

Characterization of the molecular mechanisms underlying the antibacterial activity of natural products

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Abbreviations

ABC	ATP-binding cassette
AFM	atomic force microscopy
Agr	accessory gene regulator
AMR	antimicrobial resistance
AraTs	arabinosyltransferases
ASPs	antibiotic stewardship programs
ATP	adenosine triphosphate
BCG	bacille Calmette-Guérin
BDQ	bedaquiline
BPaL	bedaquiline pretomanid linezolid
BTZ	benzothiazinone
C ₅₅ -P	undecapenyl phosphate
CGenFF	CHARMM General Force Field
CNS	central nervous system
Co-IP	co-immunoprecipitation
CP-4	quaternary ammonium-substituted pullulan
CT	computed tomography
DAA	2,3-diaminoacrylamide
D-Ala	D-Alanine
DNA	deoxyribonucleic acid
DOT	direct observed therapy
EM	electron microscopy
EMB	ethambutol
EPS	extracellular polymeric substance
HIV	Human Immunodeficiency Virus
Hlg	γ -haemolysin
INH	isoniazid
InhA	enoyl-ACP reductase
Luk	leukocidin
Lysyl-PdG	lysyl-phosphatidylglycerol
mAbs	monoclonal antibodies
MATE	multidrug and toxic compound extrusion

MBSC	Masuda borylation-Suzuki coupling
MD	molecular dynamics
MDR	multidrug resistant
MFS	major facilitator superfamily
MIC	minimum inhibitory concentration
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MS/MS	tandem mass spectrometry
MSSA	methicillin-sensitive <i>Staphylococcus aureus</i>
Mtb	<i>Mycobacterium tuberculosis</i>
MTBC	<i>Mycobacterium tuberculosis</i> complex
PBP _s	penicillin binding protein
PdG	phosphatidylglycerol
PDIM	phthiocerol dimycocerosate
PG	peptidoglycan
PMD	pretomanid
PMF	proton motive force
Ppt	phosphopantetheine
PVL	Panton-Valentine Leukocidin
PZA	pyrazinamide
RIF	rifampicin
RNA	ribonucleic acid
RND	resistance-nodulation-division
rRNA	ribosomal ribonucleic acid
SAR	structure-activity relationship
SMR	small multidrug resistance
T3SS	type III secretion system
TB	tuberculosis
tRNA	transfer ribonucleic acid
USA	United States of America
UDP-GlcNAc	uridine diphosphate <i>N</i> -Acetylglucosamine
UDP-MurNAc	uridine diphosphate <i>N</i> -acetylmuramic acid
VRE	vancomycin-resistant <i>Enterococcus</i>
WHO	World Health Organization

XDR extensively drug-resistant

Abstract

The existence and continuity of mankind is threatened by the once heralded miracle drugs losing their effectiveness amidst the growing antibiotic resistance. To alleviate this growing global health crisis, new antibacterial agents addressing novel targets are needed.

This thesis focused on the characterization of the antibacterial activities and mode(s) of action of natural products and nature-inspired compounds. In general, these compounds were screened against a broad range of bacteria, but with a focus on *Mycobacterium tuberculosis* (Mtb) and *Staphylococcus aureus*, and their molecular target(s) or mode(s) of action uncovered using a variety of techniques.

In the first part, using an optimized Masuda borylation-Suzuki coupling (MBSC) system, natural bisindole alkaloids (hyrtinadine A and alocasin A) known to exhibit diverse bioactivities but lacking antibacterial activity, were modified to generate a library of natural product-derived and synthetic (di)azine-bridged bisindole derivatives. Among these, the 5,5'-chloro front-runner compounds **4j** and **4n** displayed broad activity against Gram-positive pathogens including methicillin-resistant *S. aureus* with minimal inhibitory concentrations (MICs) in the sub-micromolar range. Both compounds, while maintaining moderate cytotoxicity against human cell lines, exhibited strong and rapid bactericidal effects and membrane-perturbing features. Their very low propensity for resistance development and potent *in vivo* efficacy in a mouse wound infection model position them as promising lead structures for antibacterial drug development.

In a follow-up study, an optimized MBSC protocol and viability assay were exploited to expand the bisindole compound library with the synthesis and screening of several undescribed *N*-tosyl bisindole derivatives against MRSA, thus broadening the understanding of the structure-activity relationship of bisindole derivatives beyond halogenation of the indole moieties. In further pursuit of the mode of action of bisindoles, mechanistic studies revealed that these molecules, renamed **5c** and **5f**, dissipate membrane potential, and permeabilize bacterial membranes by interacting with lipid II and membrane phospholipids, interfering with the lipid II biosynthetic cycle in the process.

In the third part, a previously identified anti-tubercular agent, callyaerin, was characterized. Callyaerins are cyclic peptides originally isolated from the Indonesian marine sponge *Callyspongia aerizusa*. Enabled by solid phase synthesis protocol and viability assay, structure-activity relationship studies of different callyaerins and derivatives uncovered the structural determinants of their anti-tubercular activity, with CalA and CalB being the most potent with minimal inhibitory concentrations (MICs) ranging between 1.56 and 3.13 μ M. These cyclic peptides are bacteriostatic in action and selective in activity majorly against Mtb including multidrug and extensively drug-resistant strains, with encouraging cytotoxicity profiles. A combination of mutant screens and chemical proteomics revealed Rv2113, a non-essential, integral membrane protein as the direct target of callyaerins. Further, a deep dive into the proteome of Mtb exposed to callyaerins revealed that these molecules interact with Rv2113, causing a broad downregulation of vital pathways including lipid biosynthesis, cell division, DNA repair, and replication. With excellent activity in an *ex vivo* infection model, and an unprecedented mode of action differing from all conventional antibiotics, these peptides hold promise for the development of novel tuberculosis drug.

1 Introduction

Bacteria are unicellular microorganism devoid of a nuclear membrane. They are one of the most diverse and abundant, yet relatively simple forms of life on earth, existing in various shapes and sizes. Bacteria are ubiquitous in nature and possess a remarkable ability to adapt and thrive in diverse environments. Depending on their adaptability to these environments, which vary greatly among bacterial species, and the mode of relationship established with specific hosts, these bacteria can either be pathogenic or non-pathogenic. The non-pathogenic ones are mostly beneficial to humans in various regards. For example, the intestinal microbiome provides its host with vitamins and other nutrients, thus enhancing the metabolic health of humans. Other beneficial bacteria do not inhabit the human body but offer crucial roles in ecosystem sustainability, bioremediation, food processing and production. Pathogenic bacteria, on the other hand, are associated with specific diseases. Although they constitute only a small fraction of bacterial species, they are responsible for 90% of documented hospitalized patients in developed countries. Accordingly, this underscores the importance of pathogenic bacteria in every field of medicine¹.

Toward the continuity of mankind and to contain bacterial infections, major advances in medicine have been recorded over the decades. These include the development of effective vaccines against a number of bacterial diseases, and the golden era of antibiotics ushered in by Selman Waksman, which was preceded by the accidental discovery of penicillin by Alexander Fleming². Despite this progress, bacterial infections remain a significant global health challenge, causing substantial morbidity and mortality worldwide. Two recent studies concluded in 2022 revealed that, in 2019, 34 types of bacteria were implicated in 9.1 million deaths, thus making bacterial infections the second largest cause of death globally behind cardiovascular diseases^{3,4}. Among these bacterial pathogens, *Mycobacterium tuberculosis* (Mtb) and *Staphylococcus aureus* (*S. aureus*) stand prominently as formidable adversaries, presenting intricate challenges in treatment due to their diverse virulence mechanisms, widespread prevalence, and more importantly, the emergence of antimicrobial resistance. In the next paragraphs, the pathogenicity, available treatment options and challenges, as well as antibiotic resistance development in these two pathogens will be discussed in detail.

1.1 *Staphylococcus aureus*

S. aureus is a non-motile, facultative anaerobe capable of growth at a pH range from 4.8 to 9.4. Discovered in the 1880s, it is a cocci-shaped, Gram-positive bacterium with a diameter of 0.7-1.2 μm . Cells of *S. aureus* are usually arranged in clusters, with a golden yellow appearance on growth media. Belonging to the family Staphylococcaceae, the coagulase-positive bacterium differs from other closely related staphylococci such as *S. saprophyticus* and *S. epidermidis* in its ability to ferment mannitol⁵. *S. aureus* is a commensal residing majorly in the skin and mucous membrane of humans, although its habitat also extends to the skin of sheep, poultry, cattle, cats, and dogs⁶. About 15% of the human population persistently carry *S. aureus* in their anterior nares, and it is estimated that 50% of all adults are colonized by this bacterium. However, certain populations such as health care providers, hospitalized patients, individuals with regular use of needles, immunocompromised persons, are estimated to have a higher colonization rate of about 80%⁷.

From the perspective of human health, *S. aureus* is a major human pathogen, as individuals colonized by this bacterium have increased chances of infection. *S. aureus* is responsible for several hospital- and community-acquired infections varying in their level of severity. These infections range from mild skin and soft tissue infections (boil, carbuncles, impetigo, folliculitis, furuncles, carbuncles, and cellulitis) to invasive and life-threatening diseases such as scalded skin syndrome, necrotizing pneumonia, sepsis, endocarditis, and osteomyelitis^{5,7,8}. Most of these infections, particularly bacteremia, had a fatality rate exceeding 80% before the advent of antibiotics⁹. Although the discovery of penicillin in the 1940s provided a short-lived solution, resistance was developed against it, which led to the development of methicillin. A year after the introduction of methicillin, methicillin resistant *S. aureus* (MRSA) strains surfaced^{10,11}. Several decades later, the fatality rate of *S. aureus* bacteremia is yet to improve beyond 15-50% chiefly due to resistance development, particularly with the emergence of MRSA⁹.

Resistance to methicillin and other β -lactams in *S. aureus* is mediated by penicillin binding protein 2a (PBP2a), encoded by the *mecA* gene. Penicillin binding proteins (PBPs) are transpeptidases catalyzing the cross-linking of peptidoglycan (PG) chains, a major component of the bacterial cell wall. β -Lactam antibiotics impede the function of these proteins, inevitably inhibiting cell wall synthesis. However, with the acquisition of *mecA*, MRSA is still capable of cell wall synthesis and growth even when confronted with β -lactam antibiotics due to PBP2a, which has very low affinity for these antibiotics¹². While MRSA established itself first in the

hospitals and settings of high antibiotic use, MRSA is now beyond the hospital confines, with greater fitness and altered resistance patterns in the community^{11,13}. Moreover, although MRSA infections appear to be waning in the USA and Europe due to improved hygiene and surveillance, they are still on the rise in low-income countries. In light of the increased morbidity and mortality, as well as prolonged hospital stay associated with MRSA, MRSA remains a global health threat¹⁴. To date, methicillin remains the treatment of choice for *S. aureus* infections, particularly those caused by the methicillin sensitive *S. aureus* (MSSA) strains, while vancomycin is the second-line antibiotic for MRSA infections. However, MRSA strains displaying intermediate resistance to vancomycin have emerged^{6,14}.

1.1.1 Pathogenicity and infections

Like most successful pathogens, the pathogenicity of *S. aureus* depends on several factors such as surface proteins required for adherence to host cell, as well as secreted proteins or toxins for the degradation of the host cell while evading immune cells, thereby facilitating the growth, and spread of *S. aureus* in its host⁸. These factors, termed virulence factors, range from varying pore-forming cytotoxins to exfoliative toxins and superantigens. *S. aureus* is profoundly equipped with different cytotoxins, a necessity for the establishment of infection in its host. These cytotoxins create pores in the host cell membranes or disrupt the host cell actin cytoskeleton, resulting in a breach of the endothelial cells. Among these pore-forming toxins are hemolysins, leukocidins and phenol-soluble modulins^{6,14}.

Although there are four hemolysins (α -, β -, γ -, and δ -hemolysin) encoded in *S. aureus* under the regulation of an accessory gene regulator (Agr), α -hemolysin, also referred to as α -toxin is the most characterized hemolysin and virulence factor of *S. aureus*. It is encoded by the *hla* gene. The α -toxin has affinity for mammalian cells such as epithelial cells, endothelial cells, erythrocytes, monocytes, and keratinocytes, where it causes membrane damage and apoptosis. This damage is facilitated by the presence of ADAM10, the toxin's receptor, on these cells. In principle, α -toxin binds to ADAM10 of the host cell, which triggers the stimulation of the metalloprotease activity of ADAM10, this then leads to the cleavage of the endothelial cadherin, consequently compromising the epithelial protective barrier function. This cascade of events initiates cell specific lysis and evokes cellular reactions, including transport of ions through the initiated pores, ultimately leading to target cell apoptosis. Besides these effects, during inflammation, α -toxin gives rise to tissue injury by inducing the release of cytokines and chemokines such as interleukin-1 β and TNF- α . Overall, α -hemolysin has been implicated in the

development of several severe *S. aureus* infections, including bacteremia, osteomyelitis, and pneumonia.

Another important class of pore-forming toxins contributing to the pathogenicity of *S. aureus* is bicomponent leukocidins. These toxins distort both adaptive and innate immune responses by primarily attacking white blood cells including neutrophils, macrophages, monocytes, natural killer cells, dendritic cells, and T lymphocytes^{6,15,16}. *S. aureus* has the capacity to produce different kinds of leukocidins. Five (Panton–Valentine leukocidin (PVL), γ -haemolysin AB (HlgAB), γ -haemolysin CB (HlgCB), leukocidin ED (LukED) and leukocidin AB (LukAB)) of these are associated with infections in humans while two (leukocidin MF' (LukMF') and leukocidin PQ (LukPQ)) are peculiar to zoonotic diseases¹⁶. PVL consists of two different subunits – LukS-PV and LukF-PV¹⁷. In principle, the inactive LukS first binds to the membrane of the target cell, for instance neutrophils, via the C5aR and C5L2 human complement receptors, followed by the docking of the LukF subunit. This binding cascade then leads to the multimerization of these subunits to form a pore that spans the phospholipid bilayer, which eventually leads to cell death^{16,18}. While PVL has been linked to several diseases, it is comparatively more associated with skin and soft tissue infections than invasive ones. Although the implication of PVL-positive *S. aureus* isolates in invasive infections do not often predict poor clinical manifestations, the skin and soft tissue counterparts mostly require surgical intervention¹⁹.

Besides hemolysins and leukocidins, other virulence factors also contribute to the pathogenicity of *S. aureus*. These include phenol-soluble modulins (bacteremia, skin infection), epidermal cell differentiation inhibitor (pneumonia, bacteremia, and diabetic foot ulcer), exfoliative toxins (staphylococcal scalded skin syndrome), staphylococcal enterotoxins (food poisoning), and toxic shock syndrome toxin 1 (toxic shock syndrome)⁶.

1.2 *Mycobacterium tuberculosis*

Alongside over 190 species, Mtb belongs to the genus *Mycobacterium*, a genus of non-sporulating, aerobic, non-motile, slightly curved shape Gram-positive, acid-fast bacteria with a unique cell wall rich in mycolic acids. Based on their growth rate, species in this genus are classified into fast-growing and slow-growing mycobacteria^{20,21}. Mtb is a slow-growing *Mycobacterium* with a generation time of about 20 hours. Further examples of slow-growing mycobacteria include *M. marinum*, *M. leprae* (the causative agent of leprosy), and *M. bovis*.

On the other hand, *M. smegmatis* (a widely used model organism for studying mycobacterial genetics, physiology, and drug susceptibility), *M. abscessus*, *M. chelonae*, and *M. fortuitum* exist as fast-growing mycobacteria, all of which only cause opportunistic infections in humans, particularly individuals with compromised immune systems or underlying health conditions²².

Although Robert Koch in 1882 identified Mtb as the causative agent of tuberculosis (TB), an airborne infectious disease, other tuberculosis-causing *Mycobacterium* species abound, all of which make up the *Mycobacterium tuberculosis* complex (MTBC). Members of the MTBC are closely related. They share over 99% genomic sequence similarity but differ immensely in their pathogenicity and the hosts they infect^{21,22}. Members of this complex were initially limited to Mtb (human), *M. bovis* (bovine), *M. africanum* (human), *M. canettii* (human), *M. microti* (voles), *M. pinnipedii* (seals), *M. caprae* (goats and sheep)^{20,23}, before the recent inclusion of *M. orygis* (big mammals such as oryxes, gazelles, waterbucks, and antelopes) and *M. mungi* (banded mongoose)²⁴.

1.2.1 Infection and pathogenicity

Current WHO estimates have it that about a quarter of the global population is infected with Mtb²⁵. Most Mtb infections are initiated predominantly via the respiratory routes and barely by other means such as swallowing and accidental percutaneous inoculation²⁶. Disseminated from the infected lungs of a diseased individual, to a new host in aerosol droplets via coughing or sneezing, the success of Mtb transmission is dependent among other factors, on the amount of bacteria present in the droplets²⁷. Paradoxically, unlike a few other respiratory infections caused by group A streptococcus (strep throat and scarlet fever), and *Corynebacterium diphtheriae* (diphtheria) transmitted via large aerosol droplets, the lower the number of bacteria and smaller the droplet, the higher the chances of transmission of Mtb. Typically, infection is largely and efficiently initiated in the lower lungs with small aerosol droplet nuclei containing 1 to 3 bacilli²⁷.

Unlike other feared bacterial pathogens and to the delight of bacterial pathogenesis researchers, Mtb does not seem to have classic virulence factors, as they do not produce toxins, pili, flagella, or lytic enzymes²⁸. They simply evade, and subjugate the host's immune cells, replicate massively in a privileged niche, disintegrate this niche, spread throughout the body and are ready to enter another host²⁷. Upon transmission to a new host, these bacteria are believed to first undergo phagocytosis, that is principally initiated by bacterial contact with alveolar

macrophage mannose receptors in the lungs^{29,30}. In principle, surfactant protein A, a glycoprotein located on alveolar surfaces facilitates the upregulation of mannose receptor activity, which in turn enhances the binding and uptake of the bacteria^{29,31}. The macrophage, particularly after phagosome-lysosome fusion, is a machinery sufficient for the killing of bacteria, as the phagolysosome is a compartment housing destructive enzymes and toxic reactive oxygen and nitrogen species²⁹. Unlike most bacteria, *Mtb* has devised several strategies to resist or survive killing within macrophages. For instance, *Mtb* simply prevents the fusion of the phagosome and lysosome by interfering with normal trafficking and fusion processes³². While preventing this fusion, these bacteria with the help of some secreted proteins and lipids like SapM and phthiocerol dimycocerosate (PDIM), respectively, modulate the phagosomal environment to make it less hostile while preventing its maturation. In essence, SapM dephosphorylates phosphatidylinositol 3-phosphate, a phospholipid found in the membrane crucial for phagosome maturation^{33,34}, whereas, PDIM essentially alters or modifies the phagosomal membrane in a bid to protect *Mtb* against acidic conditions as well as aid the pathogen's escape from the phagosome³⁵. Moreover, *Mtb* is also capable of resisting macrophage-killing, particularly oxidative and nitrosative stress, by neutralizing reactive oxygen and nitrogen intermediates with the expression of KatG, SodA, NrdH and AhpC²⁹. Beyond these mechanisms, *Mtb* has the capacity to escape destruction by actively evading or modulating host immune responses. *Mtb* secretes ESAT-6, an effector protein capable of disrupting host immune signaling and inducing apoptosis in immune cells³⁶. *Mtb* is also able to diminish immune response by altering cytokine (TNF- α , IL-10 and IL-12) production.

The ability of *Mtb* to evade host immune response and enter a non-replicative, dormant state mostly begets a structured collection of immune cells that ensue in an immunocompetent host's bid to contain or fight substances it cannot eliminate^{31,37}. This collection, termed granuloma, is the hallmark of TB³⁸. In this sort of event where the bacilli are well contained in the granuloma, *Mtb* is in a latent state, and the host is said to have asymptomatic TB. Neither is the host showing symptoms nor capable of transmitting the bacteria to another individual. However, in an event where the immune system of an individual is compromised or suppressed, as it is the case with TB-exposed HIV-positive individuals, the granuloma wanes, disintegrates, and there is leakage of virulent bacteria from the granuloma^{27,38}. These bacteria do not only then spread throughout the lungs, causing cavitory lesions, they also travel through the blood capillaries where they can now spread to other tissues and organs causing miliary TB. At this point, such individuals are highly contagious and said to have active or symptomatic TB^{38,39}. Nevertheless, TB is not as deadly as it sounds, as most people with TB exposure do not exhibit disease progression. For

instance, although one-third of HIV-negative individuals are currently infected with TB globally, only 3 to 5% of this number develop TB in their first year while another 3 to 5% might develop TB later in their lives²⁵. In contrast, 50% of TB-exposed HIV-positive individuals develop TB at a certain point in their lives. These patients and other immunosuppressed individuals show disease progression, thus accentuating the lethality of TB in HIV patients and individuals with suppressed immune systems. Of note, TB reactivation is not limited to HIV co-infection, other circumstances such as malnutrition, alcohol addiction, smoking, chemotherapy, diabetes mellitus, chronic renal failure are worthy triggers as well⁴⁰.

1.2.2 Tuberculosis detection and therapy

Like for other infectious or non-infectious diseases, early diagnosis and successful treatment is pivotal to the prevention of TB aggravation and death. While clinical manifestations of TB, primarily active pulmonary TB, include persistent productive cough, hemoptysis, night sweat, weight loss and low-grade fever⁴¹, depending on resource availability, several diagnostic approaches enabling TB detection in TB-exposed individuals abound. These include cultural, immunological, microscopical and radiological techniques⁴². Tuberculin skin test and interferon-gamma release assay are immunological methods for detecting TB infection⁴². The former relies on injecting a purified protein derivative into the skin and measuring the swelling after 48-72 h⁴². The latter, however, is more specific than the former, as it measures the immune response to TB antigens without cross-reactivity with BCG vaccination. Although the outcome of both techniques potentially indicates TB exposure, their con lies in their inability to differentiate between active and latent TB³⁸. Conversely, chest X-ray, a radiological tool, detects only active pulmonary TB by checking for cavities and nodules in the lungs³⁸. Beyond this, for more accurate assessment of disease process and complications, CT scanning is better suited, as this method is particularly good for characterizing lymph nodes^{43,44}. TB diagnosis can also be made microbiologically via sputum smear microscopy and culturing. Sputum smear microscopy is a quick, low-cost TB diagnostic method with limited sensitivity that examines sputum samples for acid-fast bacilli using Ziehl-Neelsen stain⁴². Culturing, on the other hand, cultivates Mtb from clinical samples such as, but not limited to, sputum, bronchoalveolar lavage, and pleural fluid. Although the results could take weeks as it is time-consuming, it is highly specific and the gold standard for TB diagnosis⁴², while CT scanning remains the gold standard for imaging primary pulmonary TB, especially in children⁴³.

The bacille Calmette-Guérin (BCG) vaccine has been the only licensed vaccine preventing miliary TB and TB meningitis in infants and children. However, unfortunately, its efficacy wanes with time, and till date, no vaccine has been found effective against this scourge in adults²⁵. 80 years after the first successful treatment of a patient suffering from TB, thanks to the discovery of the first anti-TB agent, streptomycin, in 1944, a cocktail of four antibiotics introduced in the 1970s has been the standard treatment for individuals afflicted with this dreaded disease⁴⁵⁻⁴⁷. These four antibiotics are: isoniazid (INH), rifampicin (RIF), ethambutol (EMB), and pyrazinamide (PZA)²⁵. With respect to the varying replication states of *Mtb*, specifically from actively replicating to metabolically dormant non-replicating persisters⁴⁸, this drug combination is recommended to be taken for at least 6 months in 2 phases⁴⁷. The first, referred to as the intensive phase, requires ingesting a combination of the four first-line drugs (INH, RIF, EMB, and PZA) for 2 months, followed immediately by a two-drug combination of INH and RIF for 4 months in the second phase. Although this regimen is documented to achieve about 85% cure rate⁴⁹, a recent attempt to reproduce previous meta-analyses as well as analysis of a direct observed therapy (DOT) only yielded 59% cure rate⁵⁰. This is however not entirely surprising since several side effects such as hepatotoxicity, peripheral neuropathy, ocular neuritis, central nervous system (CNS) toxicity, gastrointestinal upset and skin rash are associated with this lengthy regimen⁵¹. Consequently, there is poor compliance by many diseased individuals, which mostly then begets drug resistance.

Drug-resistance has indeed aggravated TB therapy with the emergence of multidrug resistant (MDR) and extensively drug-resistant (XDR) TB. MDR-TB is a form of TB resistant to both INH and RIF, two of the most potent first-line anti-TB drugs, whereas XDR-TB, is a subset of MDR-TB with additional resistance to any of the fluoroquinolones (moxifloxacin and levofloxacin) and either bedaquiline (BDQ) or linezolid⁵². While a number of second-line anti-TB drugs have been approved for the treatment of MDR-TB, to be administered in combination for at least 20 months, a shorter 9-month BPaL regimen consisting of BDQ, pretomanid (PMD) and linezolid has been recently recommended, albeit being expensive⁵³.

1.3 Brief history of antibiotics

Infections with rapidly growing bacteria and contagious diseases like sepsis and TB, respectively, would most likely have reduced a newborn's life expectancy to about 32 years as it was in 1900 if it was not for one of the greatest medical breakthroughs - the introduction of antibiotics into clinical use⁵⁴. Although penicillin, the first true antibiotic, rediscovered in 1928

by Alexander Fleming, ushered in this breakthrough with its introduction to the clinic in 1941, the cornerstone of modern medicine was laid as far back as 1890s by Paul Ehrlich in his search for dyes showing specific affinity toward pathogens but not human or animals^{54,55}. His idea was to find toxic agents with medically inspired discriminatory properties against infectious diseases⁵⁶. Ehrlich tested many aniline dyes and discovered that methylene blue does not only stain the parasitic protozoan *Plasmodium falciparum*, but also noted this parasite could not be detected in the blood of two patients with milder symptoms of malaria at least 8 days after methylene blue administration. Following this discovery and in search of the cure for syphilis, an incurable disease at the time, Ehrlich began an extensive screen of hundreds of atoxyl organoarsenic derivatives in syphilis-infected rabbits⁵⁷. In 1909, Compound no. 606, arsphenamine, a derivative, composed of 30% arsenic, returned as the big hit⁵⁸. Referred to as “Ehrlich 606”, and later sold as Salvarsan, the compound was described as the perfect “magic bullet” because of its ability to selectively bind *Treponema pallidum* (the causative agent of syphilis) while killing was achieved by arsenate⁵⁹. Salvarsan had serious side effects like hypersensitivity because of arsenic poisoning, nevertheless, two-thirds of syphilis-infected individuals were cured up until 1928⁵⁴. Salvarsan is widely regarded as the first modern chemotherapeutic agent.

The 1930s marked a further milestone in antibiotic history with the discovery of the sulfa drug class and its antibiotic activity by Paul Gerhard Domagk⁵⁴. Indeed, the aftermath of World War I characterized by war soldiers’ terrific injuries aggravated by bacterial infections, and the susceptibility of malnourished civilian population to infections, intensified the research and development of antibiotics⁶⁰. Inspired by Ehrlich’s success, Domagk, a German physician, who already participated in treating war wounds, was charged with the responsibility of identifying antibacterial azo dyes⁶⁰. Having failed to identify a useful antibacterial agent in earlier screening efforts of dyes due to lack of a reliable testing platform to decipher antibacterial activity, Domagk devised the mouse protection assay, a method he used to determine the survival rate of mice previously infected with a highly virulent hemolytic *Streptococcus* strain⁶¹. In 1932, using this assay, a red colored dye named sulfamidochrysoidine, was discovered to protect mice against a lethal dose of streptococci⁶¹. Although Domagk observed and proclaimed 100% efficacy *in vivo*, the substance was largely ineffective *in vitro*⁶². It was until later that it was discovered that sulfamidochrysoidine is a precursor molecule metabolized by enzymes within the body to the active sulfanilamide⁵⁴, thus making sulfamidochrysoidine a prodrug. Sulfamidochrysoidine, with the tradename prontosil, was highly potent against Gram-positive cocci by targeting the enzyme dihydropteroate synthase in the folic acid pathway that is absent

in mammalian cells⁶³. From 1935 on, deaths caused by pneumonia, meningitis, and childbed fever were drastically reduced with the sale of prontosil⁵⁴. However, today, prontosil is sparsely used because of widespread resistance that first emerged in 1942^{63,64}.

The next big milestone in antibiotic history was in 1941 with the introduction of penicillin, an antibiotic rediscovered by Alexander Fleming in 1928, into clinical use⁶⁵. The 13-year delay was chiefly because penicillin could neither be purified in significant amounts, nor its structure be elucidated⁵⁴. While working on *Staphylococcus*, Fleming observed that a mold growing on his dish of *Staphylococcus* bacterial colonies seemed to be inhibiting the growth of the bacteria around it⁶⁶⁻⁶⁸. Intrigued by his observation, he grew more cultures of mold and tested the substance produced by the mold against varying bacteria and found that the substance, which he later called penicillin, was effective against various bacteria^{67,68}. It is worth noting that, while Ernest Duchesne and many scientists before Fleming made similar observations of the antimicrobial potential of *Penicillium*⁶⁹, the antibacterial capacity of molds dates to ancient times, as witnessed by Arabian hostlers who stored their saddles in mold-supporting, dark, and moisture chamber for wound healing effect⁵⁴. Post World War II, penicillin became available and quite handy against myriads of Gram-positives including pathogens causing syphilis, gonorrhea, and puerperal infections. Fueled by the accidental discovery of the first broadly effective antibiotic, Fleming went in search of bioactive compounds from microorganisms by placing soil samples on agar plates laced with bacteria, and subsequently checked for inhibition zones⁷⁰. This cheap method, in contrast to animal disease models in terms of time and resources, would go on to revolutionize antibiotic research⁷⁰.

Despite the discovery of salvarsan, prontosil, and penicillin, most infectious diseases remained incurable in 1940. One of these incurable diseases was TB, a scourge, responsible for the death of every fourth person in USA and Europe in the 1900s⁵⁴. In the early 1940s, Selman Waksman and his students embarked on a screening campaign of soil microorganisms against targeted pathogens using Fleming's method. Their goal was to find an antibiotic that would tackle TB⁵⁴. Actinomycin and streptomycin, both produced by *Streptomyces*, were the first two promising hits from this screening campaign that inhibited the growth of Mtb^{45,71}. The former was a therapeutic liability in humans due to toxicity⁷¹, whereas the latter became the first antibiotic introduced clinically for the treatment of TB^{46,72}. In addition to finding the cure for TB, Waksman also identified the genus *Streptomyces* as a prolific producer of natural products active against bacteria, viruses, fungi, nematodes, and fungi^{46,66}. Upon Waksman's pioneering work, his template was established in various labs, and attention was focused on *Streptomyces*⁵⁴.

This resulted in hits after another between the 1940s and 1960s - the golden era of antibiotics. Arguably exhaustively explored since no new drug or novel carbon skeletons have been identified from this genus since the mid-1980s, *Streptomyces* still accounts for about two-thirds of antibiotics discovered till date^{73,74}. While attention has since shifted away from this genus, several antibiotics have been identified from other natural sources and various high throughput screens, albeit with greater difficulty and much lesser output compared to the golden era.

1.3.1 Bacterial drug targets

Some naturally occurring and synthetic antibiotics discovered so far cause direct cell death of bacteria. Others might just inhibit growth without directly affecting bacterial viability but allow elimination of the pathogenic bacteria by the host's immune defenses. Both bactericidal and bacteriostatic antibiotics target certain unique hotspots or specific cellular processes in these bacteria that include, but are not limited to, cell wall biosynthesis, DNA replication, transcription, protein synthesis, and bioenergetics.

Cell wall biosynthesis

Bacterial survival aided by the maintenance of cellular rigidity and shape, as well as protection against osmotic pressure, all provided by the cell wall, accentuates the essentiality of this structure in bacteria. This, in addition to its absence in human cells, have made its biosynthesis an attractive cellular process targeted by several antibiotics over the years. The chief component of bacterial cell wall is the PG, and its synthesis occurs in a multi-stage process. The first stage, which is cytoplasm-bound, is comprised of two key processes. The first entails synthesis of low-molecular weight precursor molecule uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) from fructose-6-phosphate (F-6-P), and subsequent conversion of UDP-GlcNAc to UDP-*N*-acetylmuramic acid (UDP-MurNAc) catalysed by an enzyme called UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA)^{75,76}. Fosfomycins simply exert their antibacterial effect on diverse bacteria by a nucleophilic attack on this enzyme⁷⁷. In the second process, a pentapeptide is added to UDP-MurNAc. Three amino acids are first added to form a tripeptide, to which D-alanyl-D-alanine dipeptide synthesized from two molecules of D-alanine by D-alanyl-D-alanine synthetase is then added⁷⁸. Alanine racemase catalyses the production of D-alanine from L-alanine. Owing to the structural similarity between cycloserine, an oral second-line anti-TB agent, and D-alanine, cycloserine outcompetes D-alanine, binds, and

inhibits both alanine racemase and D-alanyl-D-alanine synthetase, thereby abrogating cell wall synthesis^{79,80}.

The second stage is associated primarily with the membrane and catalysed by membrane-bound enzymes. At this stage, the formed UDP-MurNAc-pentapeptide is fastened to the membrane by undecaprenyl phosphate (C₅₅-P) molecule, a lipid carrier already linked to the PG precursor to form lipid I. With the addition of GlcNAc, lipid I is transformed to lipid II, which undergoes several modifications before being finally translocated across the cell membrane by flippases to the periplasm⁸¹. Bacitracin, an antibiotic peptide, interacts and forms a complex with undecaprenyl pyrophosphate, a precursor of the lipid carrier, consequently preventing the transfer of UDP-MurNAc-pentapeptide to nascent PG^{66,82}.

The third stage of PG synthesis is an extracellular phase that is comprised of polymerization and cross-linking events⁷⁸. The goal of the polymerization process is elongation of the glycan chain. In principle, repeating units of the disaccharide precursor, consisting of GlcNAc and MurNAc, both of which are part of the lipid II molecule, are linked together in a reaction catalysed by transglycosylases. This reaction leads to the formation of glycosidic bonds between one GlcNAc of one disaccharide unit and the MurNAc of another, resulting in a long glycan chain composed of GlcNAc and MurNAc residues, with the pentapeptide attached to the MurNAc^{83,84}. The cross-linking event, on the other hand, ensures the formation of covalent bonds between the peptides of adjacent glycan strands. This process is catalysed by transpeptidases, a subclass of penicillin-binding proteins (PBPs). These PBPs invariably link the terminal D-alanine of one pentapeptide to the amino group of the adjacent pentapeptide. This eventually leads to an interconnected network or mesh that gives the cell wall its mechanical strength^{83,84}. Many currently used antibiotics target both polymerization and cross-linking processes. Glycopeptides such as vancomycin kill bacteria by binding to the D-Ala-D-Ala portion of the pentapeptide, blocking both transglycosylation and transpeptidation reactions in the process⁸⁵. In contrast, beta-lactam antibiotics like penicillin exert their antibacterial effect by inhibiting transpeptidases. Consequently, cross-linking of the glycan chains is abrogated, leading to weakened cell wall and cell lysis⁸⁶. In similar manner, moenomycin impedes polymerization of glycan chains by inhibiting transglycosylase enzymes^{87,88}. Due to the difference in cell wall composition between Gram-positive and Gram-negative bacteria, particularly with the presence of an outer membrane in the latter, Gram-negatives tend to be more resistant to antibiotics.

However, additional cell wall features such as mycolic acid and arabinogalactan, unique to mycobacteria have led to the discovery of other cell wall-inhibiting antibiotics, most of which are actively relevant in TB therapy today. Both isoniazid and ethionamide represent prodrugs that are activated to active metabolites by mycobacterial enzymes, KatG and EthA, respectively^{89,90}. These active metabolites inhibit the enoyl-ACP reductase (InhA), which directly abrogate mycolic acid synthesis, leading to cell wall disruption and mycobacterial cell death⁸⁰. Other direct or indirect inhibitors of mycolic acid synthesis include pyrazinamide, delamanid and pretomanid⁸⁰. Ethambutol, on the other hand, inhibits arabinosyl transferase, an enzyme indispensable in arabinogalactan polymerization. Disruption of this process weakens the cell wall, thus making mycobacteria more susceptible to immune attack or other antibiotics^{80,91}.

DNA replication

Chromosomal DNA replication, a fundamental process for the propagation of bacteria, is dependent on the efficiency of a complex called the replisome. The bacterial replisome is a multi-protein machine composed of several key proteins and enzymes, each with a specific role in ensuring that the bacterial genome is copied error-free before cell division⁹². Among these key enzymes, DNA gyrase (topoisomerase II) and topoisomerase IV have shown to be the most successful antibacterial targets within the replisome over the years⁹³. Just ahead of the replication fork, DNA gyrase introduces negative supercoils into DNA to relieve the torsional strain generated during the unwinding of DNA by helicase, a necessary process for DNA replication and transcription. Topoisomerase IV, on the other hand, is involved in relaxation and decatenation of DNA following DNA replication⁹³. Depending on the compound and bacterial type, most fluoroquinolones such as ciprofloxacin, moxifloxacin and levofloxacin, can inhibit both enzymes with varying efficiencies. For instance, topoisomerase II is the primary target of fluoroquinolones in Gram-negatives, while topoisomerase IV is the predominant target in Gram-positives⁹⁴. Overall, fluoroquinolones exert their mode of action by first forming a complex with the topoisomerase, then stabilizing the complex in such a way that it induces double-stranded breaks in the bacterial DNA. As a result, bacterial DNA replication and transcription are prevented, resulting in not only reduced cell growth, but chromosome fragmentation that eventually leads to cell death^{95,96}.

RNA synthesis

Like the fluoroquinolones that interfere with DNA synthesis, rifamycins are a class of antibiotics that cause catastrophic effects on bacterial survival and proliferation by impairing RNA synthesis. This class of compounds specifically inhibit the bacterial RNA polymerase⁹⁷. Rifamycins bind with high affinity to the β -subunit of an actively transcribing RNA polymerase bound to DNA. This binding invariably prevents RNA synthesis from being initiated by blocking the formation of the first phosphodiester bond in the RNA chain⁹⁸. Although rifamycins were first isolated in the 1950s from *Amycolatopsis mediterranei*, mutagenesis of this bacterium has led to the emergence of more potent forms of rifamycin⁹⁹, including rifamycin SV and rifampicin that are clinically relevant till date. Rifamycins are broad-spectrum antibiotics displaying bactericidal effect against many Gram-positives, while being bacteriostatic against Gram-negative bacteria. This difference in efficacy against both classes of bacteria is not due to the difference in affinity for the β -subunit of the RNA polymerase of both Gram-positives and Gram-negatives, but the level of drug uptake between both bacteria¹⁰⁰. It is important to note that rifamycins are a first-line TB drug used in combination with other drugs to prevent resistance.

Protein synthesis

Protein synthesis is an essential process for bacterial growth and survival. This process, which entails translation of mRNA in three successive phases – initiation, elongation, and termination, is undertaken chiefly by the ribonucleoprotein machinery consisting of ribosome and varying cytoplasmic accessory factors¹⁰¹. The ribosome consists of two ribonucleoprotein subunits: the 30S and 50S subunits. Although inhibitors of protein synthesis are conventionally divided according to the subunits which they target, 30S and 50S ribosomal subunit inhibitors¹⁰², some of those targeting the same subunit still impair protein synthesis via differing mechanisms¹⁰³.

Two major antibiotic classes that bind the 30S subunit are tetracyclines and aminocyclitols¹⁰³. Both spectinomycin and aminoglycosides belong to the aminocyclitol class. While spectinomycin binds to the 30S ribosomal subunit to prevent the translocation of tRNAs and mRNA during translation without causing protein mistranslation¹⁰⁴, aminoglycosides bind to the 16S rRNA component of the 30S subunit, causing misreading of mRNA and incorporation of incorrect amino acids, giving rise to dysfunctional proteins¹⁰³. Examples of aminoglycosides are streptomycin, kanamycin, and gentamicin¹⁰³. Tetracyclines on the other hand, bind to the

same subunit and block the A site from being accessed by the aminoacyl-tRNA, thus preventing the addition of new amino acids to the growing peptide chain¹⁰⁵.

The 50S subunit inhibitors are quite broad both in terms of chemotypes and mechanisms. These are the macrolides (e.g., erythromycin and azithromycin), the lincosamides (e.g., clindamycin), oxazolidinones (e.g., linezolid), amphenicols (e.g., chloramphenicol) and streptogramins (e.g., quinupristin/dalfopristin)^{106,107}. Both lincosamides and amphenicols bind to the 50S subunit and inhibit peptide bond formation by disrupting the activity of peptidyltransferase¹⁰⁸. In contrast, macrolides interfere with protein synthesis by blocking peptide chain elongation and translocation, thereby halting the production of essential proteins^{106,108}. The streptogramins act in synergy. One component binds to the 50S subunit to inhibit peptide chain elongation, and the other prevents the extension of the peptide chain¹⁰⁷. Lastly, the oxazolidinones inhibit the initiation of protein synthesis by binding to the 50S subunit just at the interface with the 30S subunit¹⁰⁹. It is important to note that apart from the aminoglycosides that exert a bactericidal effect, all 30S and 50S ribosome inhibitors are bacteriostatic in their activity¹⁰³. Till date, aminoglycosides are included in a combination therapy as a second-line option to treat MDR-TB, while linezolid in combination with recently approved BDQ and pretomanid is used for the treatment of both MDR-TB and XDR-TB¹¹⁰.

Bioenergetics

Bacteria rely on bioenergetic processes to produce and regulate energy to power various cellular functions fundamental for their survival, growth, and replication. The key bioenergetic processes include respiration driven by the electron transport chain, ATP synthesis, and maintenance of membrane potential, all of which have recently proven to be attractive targets for antibacterial therapy¹¹¹. Among the inhibitors (BDQ, Q203, ionophores, gramicidin, phenazines, and quinones) targeting these processes¹¹¹, BDQ stands as a ground-breaking drug with a novel mode of action specifically targeting the mycobacterial ATP synthase¹¹¹. BDQ has a considerably higher affinity for the mycobacterial ATP synthase compared to its human counterpart^{112,113}. In principle, BDQ interacts with the hydrophobic region of the c-subunit of mycobacterial membrane-bound F₁F₀-ATP synthase¹¹⁴, consequently preventing the proton motive force (PMF) to produce ATP, leading to a collapse of energy storage, ATP depletion and bacterial death^{114–116}. Acting on a target distinct from those addressed by conventional TB drugs, BDQ is particularly effective against MDR-TB and XDR-TB¹¹⁰.

1.3.2 Mechanisms of antibiotic resistance

Bacteria often devise means to survive the onslaught of antibiotics in a phenomenon called antibiotic resistance. Despite the presence of drugs that should kill or inhibit their growth, these bacteria via different mechanisms would survive and multiply. Antibiotic resistance could be intrinsic or acquired. A bacterium is said to be intrinsically resistant to an antibiotic when a bacterium withstands the assault of the antibiotic due to the innate structural or functional features of the bacterium^{117,118}. An example is the intrinsic resistance of some Gram-negative bacteria to several antibacterial agents (such as vancomycin) because of the presence of an outer membrane that prevents the entry or uptake of these compounds into these bacteria¹¹⁹. By contrast, resistance to an antibiotic is said to be acquired by bacteria when resistance is developed through mutations or new genetic material acquired from other bacteria¹¹⁸.

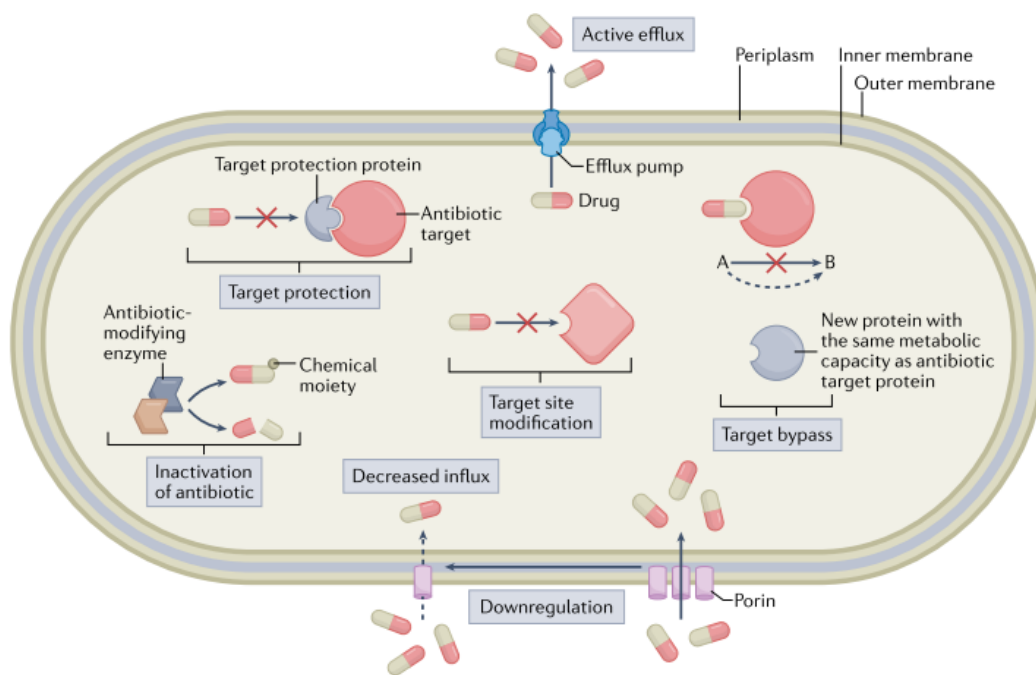


Figure 1 Mechanisms of antibiotic resistance¹²⁰.

Overall, bacteria can develop resistance to antibiotics via different mechanisms (Figure 1). These include enzymatic degradation or antibiotic modification, alteration of the target site, increased expression of efflux pumps, reduced permeability, and bypass pathways.

Antibiotic modification or inactivation

Many pathogenic bacteria survive the action of an antibiotic by degrading or chemically modifying it¹²¹. Bacteria achieve this by producing enzymes capable of either function^{120,121}. This resistance mechanism is advantageous to the bacterial cell because no mutation or alteration of the antibiotic target is involved, hence bacterial cells are less likely to suffer fitness costs¹²⁰. When the antibiotic is inactivated, its structure is damaged or degraded, thereby making the antibiotic less effective. An example of antibiotic inactivation is the hydrolysis of β -lactam antibiotics by β -lactamases¹²². β -Lactamases are enzymes that degrade β -lactam antibiotics such as penicillins, cephalosporins, by breaking the amide bond in the β -lactam ring, thereby degrading these drugs in the process¹²². Both extended-spectrum β -lactamases and carbapenemases also provide resistance to a wide range of β -lactam antibiotics in this manner^{117,120}.

The transfer of a chemical group to an antibiotic by antibiotic-modifying enzymes can also render it ineffective. Examples of antibiotics that bacteria have developed resistance against in this manner include aminoglycosides, macrolides, lincosamides, rifamycins, streptogramins, and phenicols¹²⁰. For instance, in the case of aminoglycosides, bacteria produce enzymes that chemically modify gentamicin and streptomycin, by acetylation, phosphorylation, or adenylation, leading to reduced drug binding to the bacterial ribosome¹²³. Similarly, bacteria produce ADP-ribosyltransferases, glycosyltransferases, and phosphotransferases to inactivate and modify rifamycins¹²⁴. In principle, both ADP-ribosyltransferases and glycosyltransferases modify rifamycin by glycosylating the hydroxyl position at C₂₃, while phosphotransferases basically convert rifamycin to inactive phosphorifamycin at C₂₁¹²⁵.

Target alteration and protection

Bacteria also develop resistance against antibiotics by altering the target site of the antibiotic. Once the structure of the antibiotic target is altered or protected, the antibiotic can no longer bind effectively to it, leading to resistance. A classic example is quinolone resistance. Quinolones like moxifloxacin and ciprofloxacin bind topoisomerase II and IV enzymes near their active sites to inhibit their functions. Mutations in these target proteins beget lower binding efficiency of these drugs, while the enzymes remain functional, leading to resistance. In general, altered antibiotic targets often stem from amino acid substitutions. In principle, altered target sites may arise from random point mutations that occur during bacterial growth and become

dominant when exposed to drug pressure. Alternately, mutant alleles of target genes can be frequently produced through recombination between alleles, especially if multiple copies are present in the cell. For example, mutation and recombination between homologues of the 23S rRNA gene often quickly lead to linezolid resistance in Gram-positive bacteria.

The target of an antibiotic can also be protected from the relevant antibiotic. Mechanisms of target protection include the ability of target protection proteins to bind to the antibiotic target and physically displace the antibiotic from its binding site. Additionally, the target protection proteins can bind to the antibiotic target and trigger allosteric changes, leading to the drug dissociation from its target. In some cases, these proteins can bind to the drug target and induce conformational changes that enable the target protein to continue functioning despite the presence of the drug. Target protection alone may lead to a mild increase in the minimum inhibitory concentration (MIC) of the antibiotic. However, when combined with mutations at the target site, it can result in significantly higher MICs. This is often observed with the Qnr proteins - a family of proteins encoded by plasmid-borne genes found usually in Gram-negative bacteria. These proteins protect topoisomerases from quinolone inhibition. The acquisition of a *qnr* gene leads to a mild effect on its own, but quinolone-resistant strains frequently carry a *qnr* gene alongside chromosomal mutations in *gyrA*, leading to high-level resistance.

Target bypass

To survive the effect of antibiotics, bacteria can evolve new targets or alternative metabolic pathway that perform the same biochemical functions as the original target but are not affected by the antimicrobial molecule, allowing them to completely replace or bypass the target site¹²⁶. A very good example is the development of resistance by MRSA to β -lactam antibiotics¹²⁰. β -Lactams kill bacteria by disrupting cell wall synthesis through inhibition of penicillin-binding proteins (PBPs). To survive the effect of these antibiotics, MRSA produces PBP2a, a modified PBP encoded by *mecA*, that has a low affinity for β -lactam antibiotics^{126,127}. Thus, in the presence of these β -lactam antibiotics, PBP2a in cooperation with other native PBPs, are still able to complete the peptidoglycan cross-linking and make a functional cell wall¹²⁶, allowing the bacterium to survive.

Another key example of antibiotic target bypass strategy is vancomycin resistance in bacteria¹²⁰. While glycopeptides like vancomycin inhibit bacterial cell wall synthesis by binding to the terminal D-Ala-D-Ala of peptidoglycan precursors, preventing cross-linking,

vancomycin-resistant bacteria, particularly enterococci^{120,126}, have developed mechanisms to overcome this. These bacteria acquire *van* gene clusters, which alter peptidoglycan synthesis by replacing D-Ala with D-lactate or D-serine and destroying “normal” D-Ala-D-Ala-ending precursors to block vancomycin binding¹²⁶. The D-lactate substitution reduces vancomycin's binding affinity by 1,000-fold, while the D-serine substitution affects binding less severely but still impairs antibiotic effectiveness, resulting in high-level and low-level resistance, respectively¹²⁸.

Expression of efflux pumps

Efflux pumps contribute to antibiotic tolerance and the subsequent emergence of antibiotic resistance by actively removing antibiotics from the cell, thereby lowering the concentration of the antibiotic to sub-lethal levels¹²⁹. This mechanism is one of the principal ways Gram-negative bacteria intrinsically develop resistance against drugs efficacious against Gram-positive bacteria¹¹⁷. Based on structure, molecules transported, as well as energy source required, efflux pumps are categorized into different families^{111,120}. Key families include, major facilitator superfamily (MFS), ATP-binding cassette (ABC) transporters, resistance-nodulation-division (RND) family, small multidrug resistance (SMR) family, and multidrug and toxic compound extrusion (MATE) family^{111,130}. While ABC transporters depend on ATP hydrolysis for active extrusion^{131,132}, other families of efflux pumps depend on an energized membrane and PMF¹³³. Based on molecules transported or substrate, some efflux pumps like the Tet pumps have a narrow substrate specificity while many, referred to as multidrug resistance (MDR) efflux pumps, transport a wide range of structurally and chemically unrelated molecules^{117,134}. One of such MDR efflux pumps are the RND pumps that have promoted multidrug resistance in clinical isolates¹³⁵.

Antibiotic-induced overexpression of efflux pumps is a key survival strategy used by bacteria, particularly Gram-negatives, to expel antibiotics before they accumulate to lethal levels. For example, the exposure of *E. coli* to antibiotics such as chloramphenicol, tetracycline, and puromycin usually leads to the overexpression of AcrAB-TolC and preferential extrusion of these antibiotics, and, as consequence, resistance develops¹³⁶. Besides antibiotic-induction, point mutations in the transcriptional regulators of genes encoding these pumps also promote efflux pump overexpression in clinical isolates. For example, point mutations in *mtr*, the repressor of *mtrCDE*, encoding the only RND pump in *Neisseria gonorrhoeae*, facilitates increased expression of MtrCDE efflux pump, which consequently leads to resistance against

diverse antimicrobials including penicillin, azithromycin, and cephalosporins^{137–139}. Similarly, mutations in the *rv0678* gene, a transcriptional regulator of the MmpL5-MmpS5 efflux system in *Mtb*, give rise to increased resistance to BDQ¹⁴⁰.

Efflux pumps have also been implicated in resistance to several key TB drugs, including a better part of the first-line TB drugs. For instance, efflux pump mediating resistance to isoniazid is encoded by the *efpA* gene, while efflux pumps encoded by *rv1258c* and *rv2936* conferring resistance to both rifampicin and ethambutol have also been reported in *Mtb*¹¹¹.

Reduced permeability

In addition to efflux pumps, reduced permeability is also a primary mechanism of antibiotic resistance in Gram-negative bacteria. These bacteria have a double-membrane structure that limits drug entry¹²⁰. Besides the outer membrane acting as an additional barrier that can prevent the entry of hydrophobic and large molecules not limited to antibiotics, another mechanism involves changes to the bacterial cell envelope, which decreases or restricts the entry of antibiotics reaching their intracellular targets¹²⁰. For instance, these bacteria can reduce or structurally alter the structure of porin channels, thus restricting the entry of antibiotics. For example, in *Pseudomonas aeruginosa* and *E. coli*, mutations or downregulation of the *OprD* and *OmpF* porins, respectively, reduce the uptake of β -lactam antibiotics, particularly carbapenems¹⁴¹. Gram-positive bacteria, on the other hand, do not have an outer membrane, making them inherently more permeable to many antibiotics. However, alterations in the composition of the cytoplasmic membrane that affect its fluidity have been shown to play a key role in reducing antibiotic permeability, as it is in the case of enterococci against daptomycin¹⁴². Similarly, mycobacteria produce a thick outer lipid layer and a polysaccharide capsule, which block the entry of hydrophilic molecules into the bacilli¹⁴³.

1.4 Antibiotic crisis

Antibiotic crisis, a growing global threat posed by the emergence and spread of antibiotic-resistant bacteria, is undermining the effectiveness of antibiotics. As resistance continues to spread, the capacity to cure common infections, perform surgeries, and manage immunocompromised patients is increasingly threatened¹¹⁷. An estimate has it that 25,000 deaths and €1.5 billion in costs are attributable to multidrug-resistant bacterial infections in

Europe annually¹⁴⁴. In the United states, antibiotic-resistant bacteria cause over 2 million infections, and 23,000 deaths¹⁴⁵. A key contributor to this crisis is the overuse and misuse of antibiotics. For instance, in developing countries, public hospitals are often overcrowded, and, in anticipation of high financial costs, patients frequently resort to self-medication in the hope that their ailments subside. As a result, there is a high occurrence of patients directly requesting for antibiotics, which increases antibiotic usage¹⁴⁶.

The tendency to misuse antibiotics transcends beyond patients, and extends to drug manufacturers, distributors, and healthcare providers in developing and developed nations. Owing to pharmaceutical industry promotions, competition among drug manufacturers, all of which are profit-driven, patients and healthcare providers are often swayed to use newer drugs, even when standard treatments remain effective¹⁴⁷. This, in addition to the prescription of broad-spectrum antibiotics for viral infections like common cold or flu by healthcare providers, mostly influenced by laborious or lack of diagnostic tests, potentially accelerates the development of resistance. Non-compliance with proper antibacterial therapy is another major factor contributing to antibiotic resistance¹⁴⁸. This includes the prolonged use of antibacterials beyond cure, skipping doses, and premature discontinuation of treatment¹⁴⁸. An explicit example is the treatment of TB, where patients failing to complete their full course therapy has given rise to MDR- and XDR-TB, the dangerous forms of TB.

Another significant contributory factor to the antibiotic resistance crisis is the widespread use of antimicrobials in livestock production, where over half of global antimicrobial production is used to promote growth and prevent infections in animals¹⁴⁹. This practice, involving extended or endless administration of low doses of antibiotics to animals, accelerates the emergence of antibiotic resistance. While the overall goal of the farmers is to reduce production costs, analysts believe that withholding growth-promoting antimicrobials actually boost the net value of these animals^{150,151}. This practice has been banned in the European Union since 2006¹⁵². However, in some countries like the USA and Australia, especially Australia where 78% of antimicrobials are used for animal production, antimicrobials are still permitted for livestock, with some farmers treating entire herds during disease outbreaks instead of selective treatment, further fueling antimicrobial resistance (AMR)^{148,151}. This misuse of antimicrobials in agriculture increases the transmission of resistant organisms to humans, particularly through contaminated meat and direct contact, posing serious health risks to vulnerable populations^{151,153}.

The role the environment plays in promoting antibiotic crisis, particularly as reservoir or hotspot, cannot be understated, as the excessive use of antimicrobial agents in both healthcare

and livestock production invariably lead to their accumulation in the environment¹⁵⁴. Excess antimicrobials and their metabolites from the community, healthcare and agricultural settings find their ways into the environment through poorly treated wastewater, sewage, agricultural runoff, hospital effluents and landfills¹⁵⁵. Despite adequate treatment, some of these environmental antimicrobial deposits, either in sediments or liquid, remain non-biodegradable and resistant to heat¹⁵⁶. Humans and animals get exposed to these deposits overtime, thereby fueling the emergence of antimicrobial resistance. For instance, just as a small quantity of the antibiotic neomycin found in eggs remains resistant to typical cooking temperatures¹⁵¹, ciprofloxacin and ampicillin have also been reported to be present at concentrations similar to the MIC for sensitive bacteria in hospital effluents¹⁵⁷. Paradoxically, chlorination, a standard treatment method for potable water and wastewater, reduces the overall bacterial count, while significantly increasing the levels of antimicrobial-resistant bacteria¹⁵⁸. Additionally, the discharge of pharmaceutically active compounds into the environment as effluents from antibiotic production facilities exacerbates the problem, with inadequate water treatment regulations focusing on bacterial counts but not on antimicrobial concentrations, allowing resistant microbes to thrive¹⁵¹.

Amidst several other factors contributing to antibiotic crisis including poor infection control and hygiene, global spread and travel, inadequate regulation and surveillance¹⁵⁹, of great concern is the slow development and lack of new antibiotics. Over the years, the number of antibiotics-inclined pharmaceutical companies have reduced drastically. This is due to the fact that not only are these pharmaceutical companies stiffened by regulatory barriers, the return on investment is also somewhat discouraging since antibiotics are used relatively for a short period of time before resistance is developed against them, unlike medications meant for the management of chronic ailments like diabetes and mental disorders with continuous patronage and profits¹⁶⁰. With the ongoing arms race seemingly being won by bacteria, and for mankind not to revert to the pre-antibiotic era, the search for the killers of these pathogens must be continued. As a matter of fact, if there is no action today, there might be no cure tomorrow, hence the aim of this study.

1.5 Aim of this study

The growing threat of antimicrobial resistance has intensified the need for novel therapeutic strategies to combat bacterial infections, particularly those caused by Mtb and *S. aureus*. This cumulative thesis aims to address this challenge by contributing to the identification and characterization of new antibacterial compounds with translational potential.

A key aim of this work is to identify novel, non-cytotoxic lead compounds through the synthesis and screening of two distinct chemical classes. These include naturally occurring macrocyclic callyaerins and the synthetic (di)azine-bridged bisindoles inspired by natural scaffolds. Both classes were chemically modified to enhance their potency against clinically relevant pathogens, with a particular focus on MRSA and drug-resistant Mtb. Emphasis was placed on achieving low micromolar to sub-micromolar activity while minimizing toxicity to mammalian cells, thereby aiming to establish a favorable therapeutic window.

The antibacterial activity of selected compounds was further evaluated in functional infection models. These included a murine skin infection model for *S. aureus* and an intracellular macrophage infection system for Mtb. This aimed to assess the *in vivo* and *ex vivo* efficacy of the agents under biologically relevant conditions.

Synergistic potential with clinically relevant antibiotics was also systematically investigated through combination assays. These studies aimed to determine whether the identified compounds could improve therapeutic outcomes, reduce effective doses, and potentially delay the emergence of resistance.

In parallel, this thesis aimed to uncover the molecular targets and pathways affected by these agents. These mechanistic investigations were aided by mutant screens, whole-genome sequencing, chemical proteomics, biochemical, and genetic studies.

Taken together, this thesis aims to (i) identify structurally novel, biologically active antibacterial agents, (ii) evaluate their efficacy in infection-relevant models, (iii) explore pharmacological synergy with existing drugs, and (iv) elucidate their molecular targets and resistance mechanisms. By integrating medicinal chemistry, microbiology, and *in vivo* pharmacology, this work proposes viable strategies to advance lead compounds into preclinical development and address the ongoing antibiotic resistance crisis.

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2 Nature-Inspired (di)Azine-Bridged Bisindole Alkaloids with Potent Antibacterial In Vitro and In Vivo Efficacy against Methicillin-Resistant *Staphylococcus aureus*

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Contribution to the paper:

- Drafted the second version of the manuscript.
- Demonstrated that the outer membrane restricts the activity of bisindoles in Gram-negative bacteria.
- Conducted in vitro hemolysis assays to evaluate the cytotoxicity of bisindoles.
- Generated and screened spontaneous resistant mutants of MRSA
- Extracted genomic DNA from resistant mutants for whole-genome sequencing.
- Performed microscopy analyses to assess membrane permeability in MRSA exposed to bisindoles.
- Measured propidium iodide internalization in MRSA cells exposed to bisindoles.
- Evaluated leakage of intracellular contents in MRSA following bisindole treatment.
- Assessed cell lysis in MRSA induced by bisindole exposure.

Nature-Inspired (di)Azine-Bridged Bisindole Alkaloids with Potent Antibacterial *In Vitro* and *In Vivo* Efficacy against Methicillin-Resistant *Staphylococcus aureus*

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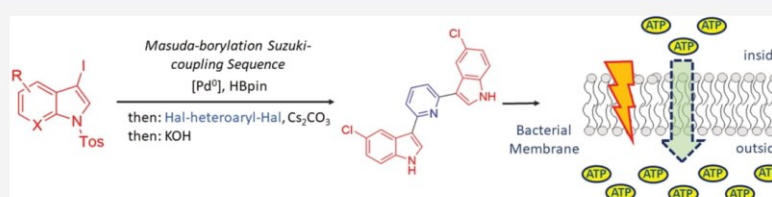
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ABSTRACT: Natural bisindole alkaloids such as Hyrtinadine A and Alocasin A, which are known to exhibit diverse bioactivities, provide promising chemical scaffolds for drug development. By optimizing the Masuda borylation–Suzuki coupling sequence, a library of various natural product-derived and non-natural (di)azine-bridged bisindoles was created. While unsubstituted bisindoles were devoid of antibacterial activity, 5,5'-chloro derivatives were highly active against methicillin-resistant *Staphylococcus aureus* (MRSA) and further Gram-positive pathogens at minimal inhibitory concentrations ranging from 0.20 to 0.78 μM . These compounds showed strong bactericidal killing effects but only moderate cytotoxicity against human cell lines. Furthermore, the two front-runner compounds **4j** and **4n** exhibited potent *in vivo* efficacy against MRSA in a mouse wound infection model. Although structurally related bisindoles were reported to specifically target pyruvate kinase in MRSA, antibacterial activity of **4j** and **4n** is independent of pyruvate kinase. Rather, these compounds lead to bacterial membrane permeabilization and cellular efflux of low-molecular-weight molecules.

HIGHLIGHTS

The synthesis and characterization of bisindoles with heterocyclic bridges are reported.
Synthetic nature-inspired bisindole derivatives were synthesized via a one-pot Masuda borylation–Suzuki coupling sequence.
Bisindoles have promising *in vitro* and *in vivo* antibacterial activity against MRSA.
Antibacterial activity of presented bisindoles is based on membrane permeabilization but not on inhibition of pyruvate kinase.

INTRODUCTION

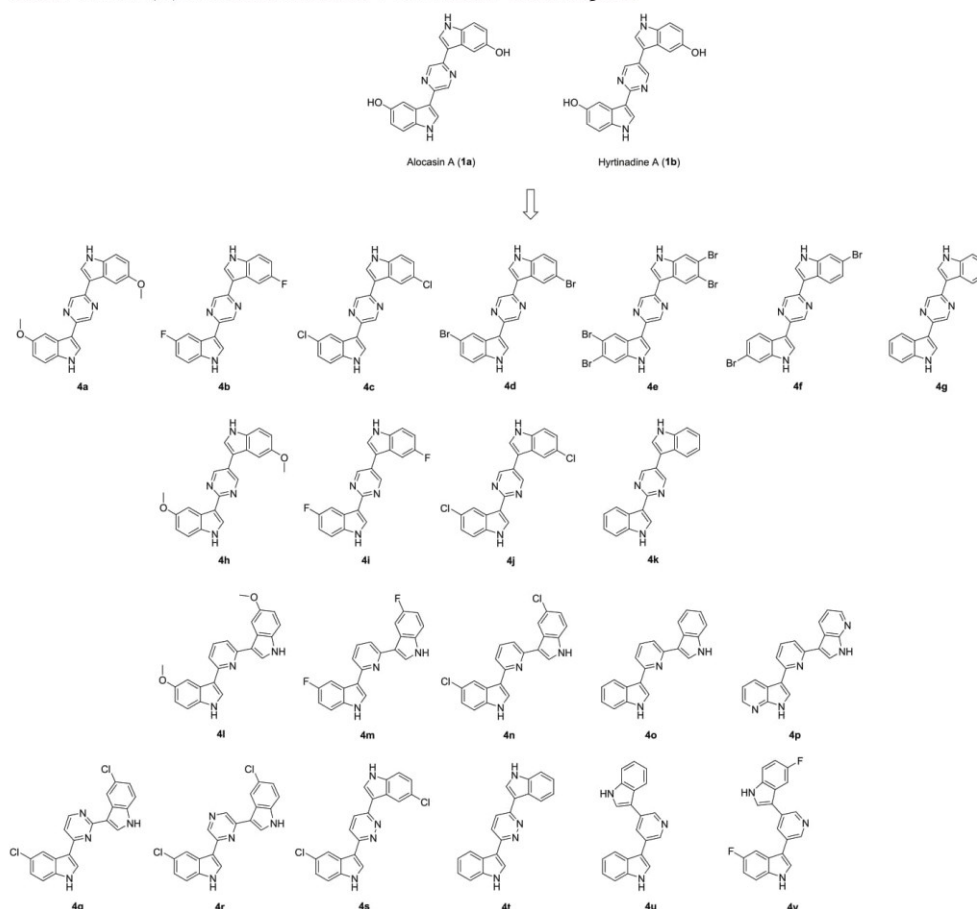
Since the introduction of penicillin, antibiotics have become one of the cornerstones of modern medicine. However, the number of multidrug-resistant bacterial pathogens steadily increased in the last years. Extensively drug-resistant strains, for which almost no antibiotic is clinically available anymore, became a major problem especially in hospitals and health care

facilities and might reemerge as a major cause of morbidity and mortality even in industrialized regions of the world in the future unless effective countermeasures are rapidly implemented.¹ Hence, new lead structures addressing novel drug targets are urgently needed for many bacterial pathogens.² *Staphylococcus aureus* is a facultative pathogenic bacterium, which can cause wound infections, pneumonia, or sepsis.³ β -Lactam antibiotics such as methicillin are the agents of choice for treatment of staphylococcus infections due to their good safety profiles. However, the widespread development of resistance against methicillin and other β -lactams as well as resistance to other antibacterial agents make treatment of *S. aureus* infections an increasing clinical challenge.⁴

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Scheme 1. Novel (di)Azine-Linked Bisindoles 4 via a Masuda–Suzuki Sequence

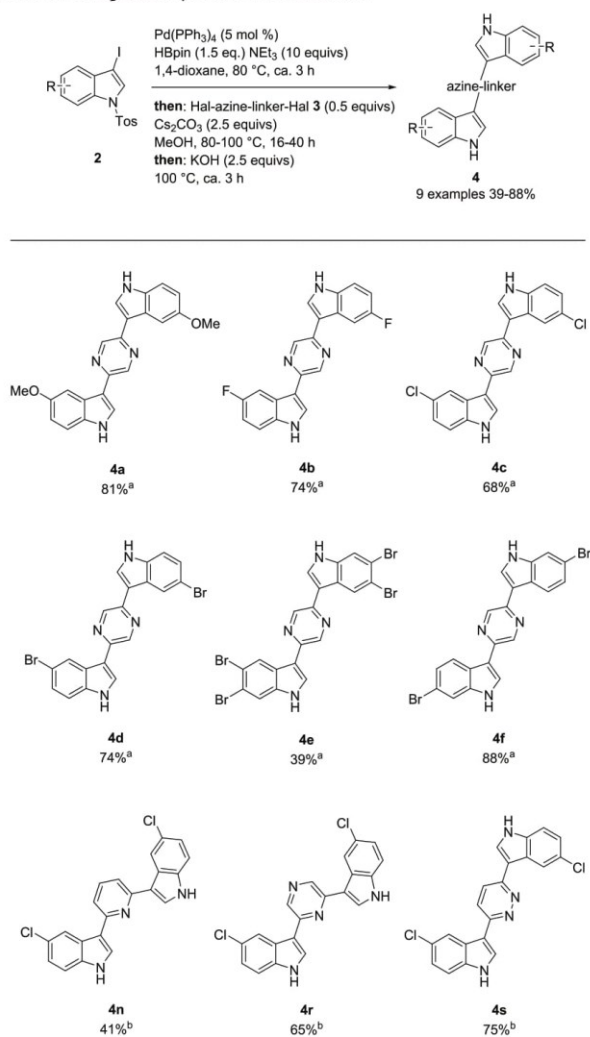


Approximately 70% of antibiotics are derived from natural compounds.⁵ In addition to soil microbes and endophytes, also, plants and marine organisms like sponges can serve as sources for identifying new natural-sourced lead structures.⁶ One promising class of antibacterial compounds is indole alkaloids, which have shown cytotoxic, antiviral, antimicrobial, antiparasitic, anti-inflammatory, and antifungal activity.^{7–9} In particular, plant- and sponge-derived indole-containing alkaloids comprise metabolites with highly diverse bioactivities such as antitumor,^{10,11} antifungal,¹² antiviral,¹³ and also antimicrobial activity.^{12,14} Two examples of natural bisindoles **1** are Alocasin A (**1a**) and Hyrtinadine A (**1b**), which consist of two indole moieties connected by a 2,5-substituted pyrazine or pyrimidine bridge. Alocasin A (**1a**) is produced by the Asian medicinal plant *Alocasia macrorrhiza* and possesses antifungal and cytostatic activity.¹⁵ In contrast, Hyrtinadine A, produced from the Red Sea sponge *Hyrtios* sp., exhibited cytotoxicity against murine leukemia and human epidermoid carcinoma

cells *in vitro*.¹⁶ However, no antibacterial activity was reported for these natural core structures. Notwithstanding, the synthetic modification of natural compounds opens possibilities to find new antibacterial lead structures, creating new pharmacophores with higher antibacterial activity than the parental molecules.

The most commonly used method for the formation of carbon–carbon bonds between two sp^2 carbon atoms is the Suzuki–Miyaura coupling.¹⁷ Starting from iodinated *N*-protected indoles **2** and suitable heterocyclic linkers **3**, the Suzuki coupling constitutes a highly reliable, versatile reaction, an ideal key step for the formation of (di)azine-linked bisindoles **4**. Furthermore, considerable functional group tolerance and applications of heteroaryls have been reported.^{18,19} Since borylated starting materials are required, a preceding borylation of an electron-rich halogenated coupling partner in the presence of pinacolyl boron (HBpin) has to be performed. As a logical combination, the Pd-

Scheme 2. MBSC Pseudo Three-Component Synthesis of Bisindoles 4



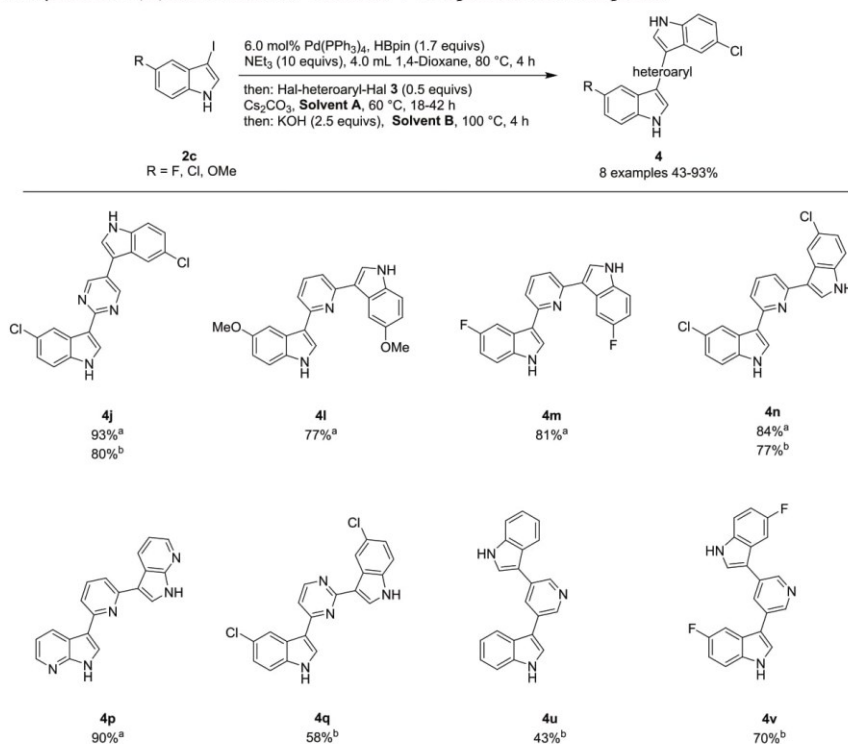
^a10 mL of 1,4-dioxane and 7.0 mL of methanol. ^b4.0 mL of 1,4-dioxane and 3.0 mL of methanol.

catalyzed Masuda borylation²⁰ and the Suzuki arylation can be consecutively employed in the same reaction vessel.^{19,21,22} For ligating two heteroaryl halides, this sequentially Pd-catalyzed one-pot reaction was developed and illustrated for the synthesis of marine alkaloids and derivatives.^{19,23–25} Here, this one-pot methodology has now been employed for the synthesis of derivatives 4 of Hyrtinadine A and Allocasin A with different substitution patterns and also various central linkers 3 to establish structure–activity relationship studies with respect to antibacterial activity (Scheme 1). Moreover, optimized

conditions for the synthesis of (di)azine-linked bisindoles 4 are reported.

RESULTS AND DISCUSSION

Synthesis of Bisindole Compounds 1 and 4. Based on our previously established protocol, nine heteroaryl bridged bisindole molecules 4 were synthesized containing a pyridazine (4s and 4t) or a pyridine bridge (4l–4p, 4u, and 4v) and eight with a pyrazine linker (4a–4g and 4r). We recently showed the successful application of the Masuda borylation–Suzuki

Scheme 3. Synthesis of (di)Azine-Linked 3,3'-Bisindoles **4** via Optimized MBSC Sequences

^aSolvent A, 7.0 mL of MeOH; solvent B, /; *t*_{SC}, 18 h. ^bSolvent A, 3.0 mL of H₂O; solvent B, 4.0 mL of MeOH; *t*_{SC}, 42 h.

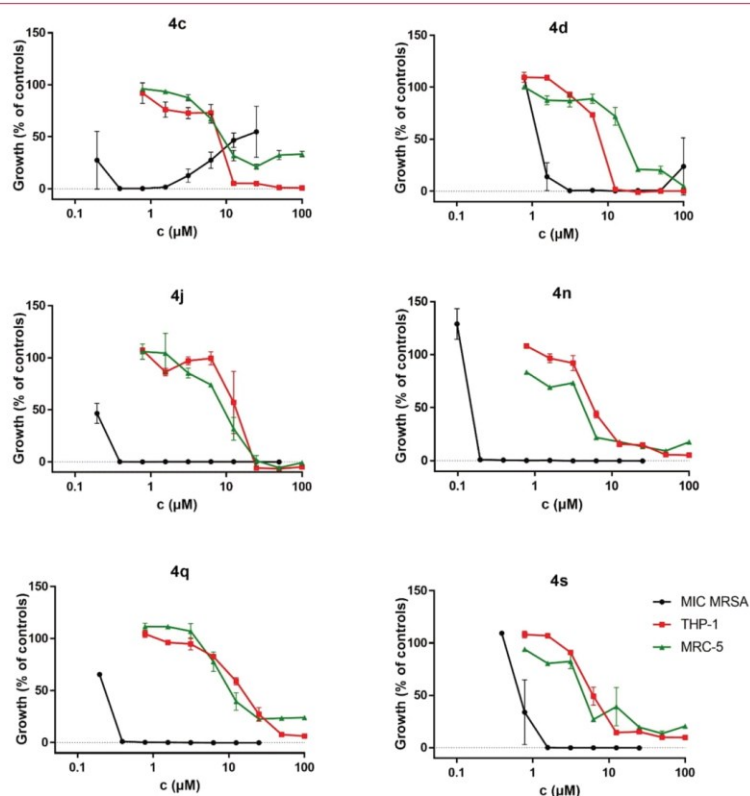
coupling (MBSC) as a one-pot reaction in the sense of a sequentially palladium-catalyzed pseudo three-component process.²⁵ In this study, Hyrtinadine A (**1b**) and several derivatives (**4h–4k**) were efficiently synthesized by this strategy, underlining the robustness of this synthetic tool for the concise preparation of bisindoles with different heterocyclic bridges (**4q**, **4s**, and **4t**) as well. A structurally related compound amenable to this synthetic protocol is the plant-derived alkaloid Alocasin A (**1a**).¹⁵ Compounds **4a–4g** and **4r** can be considered Alocasin A analogues. We synthesized a small library of derivatives **4** of these natural products with different indole moieties. Recognizing the antibacterial potency especially of chlorinated compound **4c** in this portfolio, we established new chlorinated bisindole (**4n**, **4r**, and **4s**, Scheme 2) structures by variation of linker **3**.

The indoles **2** were prepared for the sequence according to Witulski et al.²⁶ The Masuda borylation of the indoles was performed in dry 1,4-dioxane with water-free triethylamine under an argon atmosphere at 80–100 °C (monitored by TLC). After full conversion of indole halide **2**, the excess of pinacolborane was quenched by addition of methanol. The resulting mixture was also desirable for the base cesium carbonate as methanol/carbonate mixtures are preferred for Suzuki coupling reactions. The cross-coupling was successfully

performed at slightly higher temperatures. The commercially available dihaloazines **3** were employed without further purification. When using *N*-tosyl-indoles **2**, an additional cleavage step was necessary. Indeed, desulfonation is possible in a one-pot fashion by adding ground potassium hydroxide to the reaction mixture. Alocasin A (**1a**) was obtained from compound **4a** by demethylation in a mixture of aqueous hydrobromic acid (48%) and acetic acid at 120 °C. In comparison to previous studies on Masuda–Suzuki sequences,^{23–25} the more reactive iodide Suzuki coupling partners **3** (increased rate of oxidative addition, the first step in a catalytic cross-coupling cycle) are more expensive and often less stable. However, it is noteworthy mentioning that there are far more commercially available bromides (and chlorides). Therefore, the scope of this one-pot process was probed with bromo and chloro derivatives of dihaloazines **3**. For minimizing side products and impurities, a short optimization study with 5-chloro-3-iodo-1-tosyl-1*H*-indole (**2a**) and 2,6-dibromopyridine (**3b**) in the model reaction was conducted (for details see the Supporting Information). Applying H₂O as a cosolvent during Suzuki coupling finally gave high yields of the desired product, which was analytically pure after short column chromatography. Noteworthy, low temperatures, high concentration reaction mixtures, and longer reaction time gave the best

Table 1. Synthesized Substituted Compounds 1 and 4 and Their MIC against Methicillin-Resistant *S. aureus* Strain ATCC 700699 in μM and $\mu\text{g/mL}$ ^a

compound	MIC ₉₀		compound	MIC ₉₀		compound	MIC ₉₀	
	μM	$\mu\text{g/mL}$		μM	$\mu\text{g/mL}$		μM	$\mu\text{g/mL}$
1a	>100	>34.24	4f	>100	>46.82	4n	0.20	0.08
1b	>100	>34.24	4g	>100	>31.04	4o	>100	>30.94
4a	>100	>37.04	4h	50	18.52	4p	>100	>31.14
4b	>100	>34.63	4i	12.5	4.32	4q	3.13	1.19
4c	0.39	0.15	4j	0.39	0.15	4r	0.39	0.15
4d	1.56	0.73	4k	12.5	3.88	4s	0.78	0.30
4e	25	15.65	4l	12.5	4.62	4t	>100	>31.04

^aThe maximal tested concentration was 100 μM . MIC₉₀ > 100 μM is considered inactive.Figure 1. Dose–response curves of compounds 4c, 4d, 4j, 4n, 4q, and 4s against MRSA (circle) and human cell lines MRC-5 (triangle) and THP-1 (square). Data are means of triplicates $\pm$ SD. Growth was quantified using BacTiter-Glo assay for bacterial and resazurin dye reduction assay for eukaryotic growth. Resulting selectivity indices ($\text{SI} = \text{IC}_{50}/\text{MIC}_{90}$): 4d, SI = 8; 4c, SI = 32; 4j, SI = 32; 4s, SI = 8; 4q, SI = 32; 4n, SI = 32.

results. For further comparison, the best two conditions were used to synthesize different derivatives of 5,5'-chlorobisindoles 4 (Scheme 3).

While the initial conditions of the MBSC sequence were developed for a bi(hetero)aryl synthesis, an optimized process for creating an efficient pseudo three-component reaction employing bromides and chlorides now has been established.

Most critically in the Suzuki coupling, water as a less nucleophilic cosolvent and lower reaction temperatures kinetically favor cross-coupling over competing nucleophilic aromatic substitution of the heteroaryl halides 3, finally furnishing the desired bisindoles 4 in good yields. Therefore, the use of iodides is no longer necessary for obtaining pure compounds in a fast and convenient fashion. For isolating the

Table 2. Checkerboard Analysis of Compound 4j and Colistin against Gram-Negative Bacteria^a

Gram-negative bacteria	MIC (μ M)		FIC _a	FIC _b	FICI	effect
	colistin	4j				
<i>K. pneumoniae</i> ATCC 700603	1.56	>100	≤ 0.125	0.125	≤ 0.25	synergistic
<i>P. aeruginosa</i> ATCC 27853	0.39	>100	≤ 0.0625	1	≤ 1.0625	additive
<i>A. baumannii</i> ATCC BAA-1605	0.78	>100	≤ 0.0625	0.25	≤ 0.3125	synergistic
<i>E. coli</i> ATCC BAA-2469	0.78	>100	≤ 0.0313	0.064	≤ 0.0953	synergistic

^aFIC_a = MIC of the combination/MIC_a alone; FIC_b = MIC of the combination/MIC_b alone; FICI = FIC_a + FIC_b, a = 4j; b = colistin. FICI was defined as follows: synergistic effect when FICI ≤ 0.5 ; partial synergistic effect when FICI $> 0.5 \leq 0.75$; indifferent or additive effect when FICI $> 0.75 \leq 2$; antagonistic effect when FICI > 2 .

products directly from the reaction mixture, purification by fast and simple flash chromatography is sufficient. If methanol is used as a solvent for Suzuki coupling instead, further workup steps are required after chromatography.

In summary, by use of the Masuda borylation–Suzuki coupling (MBSC) sequence, a small but diverse library around the natural compounds Allocasin A and Hyrtinadine A was easily and efficiently synthesized now allowing detailed structure–activity relationship (SAR) studies regarding their antibacterial effects. Ten of these structures (4b, 4c, 4e, 4l–4n, 4p, 4q, 4r, and 4s) were not described elsewhere before.

Synthetic Bisindole Derivatives 1 and 4 Exhibit Potent Antibacterial Activity against a Panel of Pathogenic Bacterial Strains. Minimal inhibitory concentrations (MICs) of all compounds 1 and 4 were determined by screening in two-fold serial dilution at a concentration range between 100 and 0.78 μ M against Gram-positive nosocomial bacterial pathogens including methicillin-resistant *S. aureus* (MRSA), *Enterococcus faecalis*, and vancomycin-resistant *Enterococcus faecium*. None of the tested compounds 1 and 4 were active against the Gram-negative bacterium *Acinetobacter baumannii*, and they also showed no or only a weak effect against *Mycobacterium tuberculosis* (Supporting Information Table S1). However, compounds 1 and 4 showed variable inhibitory activity against the tested Gram-positive nosocomial strains. The observed antibacterial potencies highly depended on the substitution pattern at position 5' and 5'' or 6' and 6'' of the indole rings (Table 1). As already reported in the literature, the natural compounds Allocasin A (1a) and Hyrtinadine A (1b) were devoid of any inhibitory activity against the tested pathogenic bacteria. Also, 5',5''-dimethoxylated (4a and 4h) compounds did not show any (in the case of the pyrazine derivatives) or only a slight effect (in the case of the pyrimidine derivative) against *M. tuberculosis*, *S. aureus*, *E. faecalis*, and *E. faecium*. Interestingly, halogenation seemed to be essential for the antimicrobial activity. If the compound was halogenated at position 5',5'' of the indole ring, the MICs against the MRSA strain decreased (4b–4e, 4i, and 4j). Regarding the halogenated derivatives 4b and 4i as well as 4c and 4j, it could also be observed that the replacement of fluorine by chlorine led to a decrease in the MIC by a factor ≥ 32 . The replacement from chlorine to bromine increased the MIC slightly to 1.56 μ M in the case of MRSA. Furthermore, the brominated structures 4d–4f indicated that the halogenation at position 5',5'' (4d) is critical regarding antibacterial activity. The additional halogenation at position 6',6'' of the indole rings (4e) led to an increased MIC of 25–100 μ M. If only position 6',6'' was halogenated like in compound 4f, no activity could be observed in the tested concentration range.

In contrast to these relatively narrow structural requirements regarding the indole moieties, wide flexibility was tolerated

concerning the linker unit. Substitution of the linker from pyrimidine or pyrazine to pyridazine (4s) or the replacement of a 2,5-substitution to a 2,4-substituted pyrimidine ring (4q) or even the changing of the linker from pyrimidine to 3,5-disubstituted pyrazine (4r) or 2,6-disubstituted pyridine (4n) had only a slight influence on the MIC as long as the indole rings were substituted with a chlorine atom at the 5',5'' position (Table 1).

Paradoxically, a decreased inhibitory effect for compounds 4c, 4d, and 4r could be observed at concentrations higher than the MIC (Figure 1; Supporting Information Figure S1), whereas other symmetric compounds such as 4n or 4s did not show this effect. It is possible that 4c, 4d, and 4r aggregate at higher concentrations because of their axial symmetry and precipitate, thus resulting in a decreased effective concentration of the compounds in solution. Since the other symmetrical compounds 4n and 4s did not show this effect, this has to be a peculiar influence of the pyrazine linker. However, solubility of all tested derivatives (4c, 4d, 4j, 4n, and 4r) was generally very low in aqueous buffer (Supporting Information Figure S2a, b, and e; Figure S3), so that the physicochemical basis of this differential effect of compounds 4c, 4d, and 4r remains elusive.

Interestingly, the 5',5''-chloro-pyrazine derivatives 4c and 4r exhibited a very narrow specificity by being exclusively active against the MRSA strain (MIC = 0.39–0.78 μ M) but not the methicillin-sensitive parental *S. aureus* strain for yet unknown reasons. In contrast, brominated derivative 4e showed an MIC of 1.56 μ M against both *S. aureus* strains (Supporting Information Table S1). Only compounds 4j, 4n, and 4q exhibited broad antibacterial potency against all tested Gram-positive nosocomial pathogens (Supporting Information Table S1), making them the most promising candidates for further characterization.

Cytotoxicity and Therapeutic Index. To analyze the therapeutic index of these structures, compounds 4c, 4d, 4j, 4n, 4q, and 4s were tested against the human THP-1 monocytic and MRC-5 lung fibroblast cell lines. Since the compounds were found to be cytotoxic only at concentrations ≥ 12.5 μ M, the selectivity indices (IC₅₀/MIC₉₀) yielded sufficient therapeutic margins (Figure 1). 4j, 4n, and 4q were additionally tested against human kidney HEK293, human hepatocellular carcinoma HepG2, and lung carcinoma CLS-54 cell lines. The three compounds were cytotoxic only at concentrations > 12.5 μ M, confirming their rather low cytotoxic potential and resulting in sufficient antibacterial selectivity (Supporting Information Table S2). Furthermore, 4j and 4n exhibited no hemolytic activity against sheep erythrocytes even at high concentration up to 100 μ M (Supporting Information Figure S4), further adding to their low general toxicity profile.

Table 3. Checkerboard Analysis of Compound 4n and Colistin against Gram-Negative Bacteria^a

Gram-negative bacteria	MIC (μ M)		FIC _a	FIC _b	FICI	effect
	colistin	4n				
<i>K. pneumoniae</i> ATCC 700603	1.56	>100	≤ 0.125	0.125	≤ 0.25	synergistic
<i>P. aeruginosa</i> ATCC 27853	0.39	>100	≤ 0.0625	0.5	≤ 0.5625	partially synergistic
<i>A. baumannii</i> ATCC BAA-1605	0.78	>100	≤ 0.0625	0.25	≤ 0.3125	synergistic
<i>E. coli</i> ATCC BAA-2469	0.78	>100	≤ 0.0313	0.064	≤ 0.0953	synergistic

^aFIC_a = MIC of the combination/MIC_a alone; FIC_b = MIC of the combination/MIC_b alone; FICI = FIC_a + FIC_b; a = 4n; b = colistin. FICI was defined as follows: synergistic effect when FICI ≤ 0.5 ; partial synergistic effect when FICI $> 0.5 \leq 0.75$; indifferent or additive effect when FICI $> 0.75 \leq 2$; antagonistic effect when FICI > 2 .

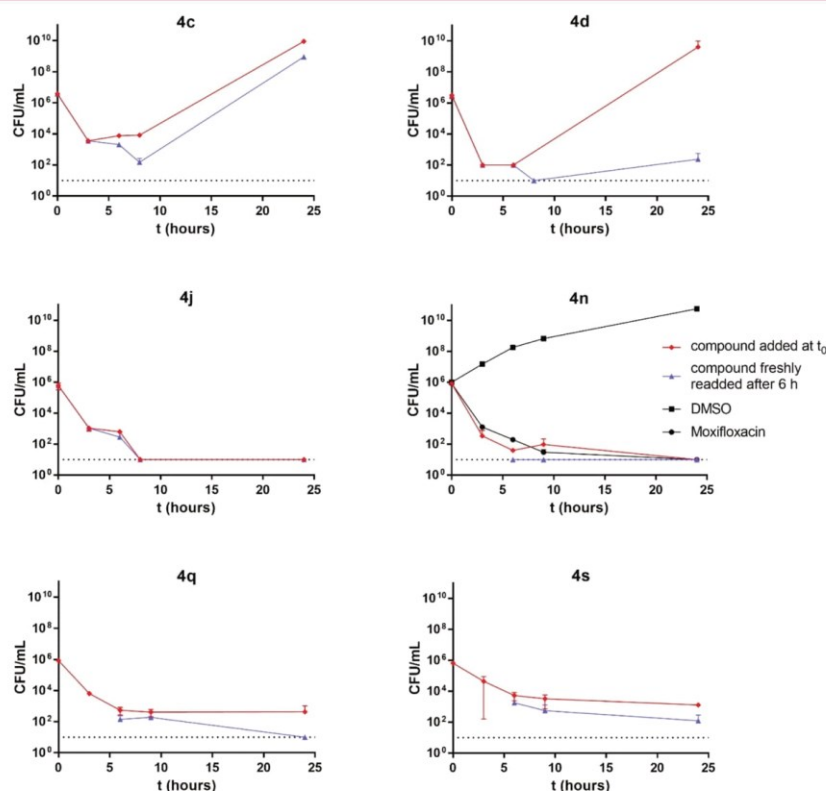


Figure 2. Time-kill curves of methicillin-resistant *S. aureus* (MRSA; ATCC 700699) for lead bisindoles 4c, 4d, 4j, 4n, 4q, and 4s. Compounds were tested at 5 $\times$ MIC (resulting in 0.02–0.08% DMSO final concentration) (diamond). Additionally, in an independent experiment, the medium was replaced, and compounds were freshly read after 6 h (triangle). Moxifloxacin at 5 $\times$ MIC (circle) and cells grown in a medium without antibiotics but only containing 0.08% DMSO (square) were included as positive and negative controls, respectively. Results are presented as the mean of duplicate experiments $\pm$ SD.

Outer Membrane Permeability Restricts Activity of Bisindole Alkaloids against Gram-Negative Bacteria.

The insusceptibility of Gram-negative bacteria to bisindoles prompted us to investigate whether the bacterial outer membrane restricts access of these compounds to their target(s). To assess this, we permeabilized the bacterial outer membrane with subinhibitory concentrations of colistin (COL)—an antibiotic whose mode of action entails disruption

of the outer membrane in a lipopolysaccharide-dependent manner in Gram-negative bacteria.²⁷ All tested Gram-negative bacteria were initially insensitive to compounds 4j and 4n. However, upon permeabilization of the outer membrane with subinhibitory concentrations of COL, there was a moderate (in the case of *Pseudomonas aeruginosa*) to strong increase (in the case of all other tested strains) in susceptibility to both compounds. As indicated in Tables 2 and 3, the presence of

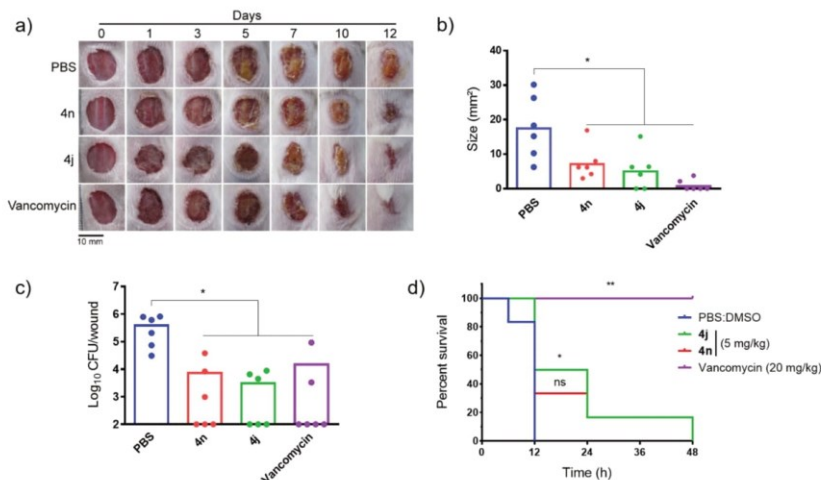


Figure 3. *In vivo* efficacy of bisindoles in mouse wound and peritonitis-sepsis infection models. (a) Pictures of skin wounds in a mouse skin wound infection model, treated with a single dose of **4j** or **4n** (each at 5 mg/kg). Vancomycin (20 mg/kg) and PBS were included as positive and negative controls, respectively. Bars represent 10 mm, $n = 6$. (b) Sizes of skin wounds and (c) bacterial counts in MRSA-infected wounds 12 days post-treatment with a single dose of **4j** or **4n** (each at 5 mg/kg). Vancomycin (20 mg/kg) and PBS were included as positive and negative controls, respectively. P -values were determined by the unpaired t test ($*p < 0.05$). $n = 6$. (d) Survival rates of mice in the mouse peritonitis-sepsis model. Six mice per group were infected via intraperitoneal injection with MRSA and treated one hour post-infection with single intraperitoneal doses of 5 mg/kg of **4j** or **4n**. Vancomycin (20 mg/kg) was used as a positive control. The percentage survival was calculated upon monitoring the mice for 48 h. P -values were determined by the logrank (Mantel-Cox) test ($*p < 0.05$; $**p < 0.01$; ns, not significant) and compared to PBS and vancomycin groups.

colistin at 0.064-fold MIC and 0.125-fold MIC robustly enhanced the susceptibility of *E. coli* (32-fold reduction in MIC) and *K. pneumoniae* (8-fold reduction in MIC) toward **4j** or **4n** resulting in FICI ≤ 0.0953 and ≤ 0.25 , respectively, denoting a rather strong synergistic effect. On the other hand, the interaction between colistin and **4j** or **4n** yielded a partially synergistic and additive effect in the case of **4n** (FICI ≤ 0.5625) and **4j** (FICI ≤ 1.0625) against *P. aeruginosa*, respectively. Overall, this indicates that the intracellular target(s) of bisindoles is(are) present in Gram-negative bacteria but that the penetration of compounds into the cells is largely restricted by the outer membrane.

In Vitro Time-Kill Studies Revealed Early Bactericidal Activity. Killing kinetics were measured to determine the potential bactericidal or bacteriostatic effect of the tested compounds on actively growing bacteria. For this, viable cell counts in treated samples were determined after different incubation times by employing colony forming unit (CFU) plating experiments. Compounds **4c**, **4d**, **4j**, **4n**, **4q**, and **4s** exhibited a strong bactericidal killing effect. After an incubation time of 6 h with compounds at five-fold MIC, bacterial viability was reduced by a factor of 10^4 – 10^5 by substances **4d**, **4j**, **4n**, and **4q**, while treatment with compounds **4c** and **4s** reduced viability only 100-fold (Figure 2). Since after 24 h, the amount of surviving bacteria remained $<10^1$ CFU/mL (representing the limit of detection in this experiment) in the presence of compounds **4j**, **4n**, **4q**, and **4s**, these highly bactericidal substances might be able to completely sterilize the culture. In contrast, some persistent bacteria remained in the cultures containing **4c** and **4d** after 6 h of treatment, giving rise to an

increasing number of cells again at prolonged treatment intervals. However, when these regrown cells were used to inoculate a fresh medium, the cultures still exhibited the same sensitivity like wild-type cells toward compounds **4c** and **4r**, indicating that they had not acquired genetically determined, inheritable resistance (data not shown). When compound **4c** was freshly added to cells after 6 h of incubation following washing and medium exchange, the viability of the culture continued to decrease to 10^2 CFU/mL initially but almost rose to the same amount as the growth control after 24 h of incubation time (Figure 2), indicating that **4c** is either spontaneously or microbially degraded or metabolized to an inactive form under the tested assay conditions. Consequently, the stability of **4c** was evaluated over time. Since compound **4c** appears stable over a period of ca. 6 h (Supporting Information Figure S5) in aqueous solution, it is unlikely that compound **4c** substantially spontaneously degrades but rather is actively metabolized or modified by the bacteria, thereby resulting in reduction of the effective concentration of **4c**. Hence, its antibacterial effect wanes after 6 h of incubation. Interestingly, regrowth of the pathogen at extended incubation times was much less pronounced when compound **4d** was freshly added to the culture after 6 h (Figure 2). Therefore, substance **4d** might be less prone to degradation or depletion than structure **4c**.

Bisindoles Exhibit Potent *In Vivo* Efficacy against MRSA in a Mouse Wound Infection Model. Due to their potent bactericidal anti-MRSA *in vitro* activity, we evaluated the potential therapeutic effect of **4j** and **4n** in a mouse wound infection model. Skin wounds were infected with MRSA strain

T144 (a multidrug-resistant clinical *S. aureus* isolate sensitive to vancomycin²⁸) and subsequently treated one hour post-infection by **4j** or **4n** (each at 5 mg/kg) employing a single-dose topical application. Treatment with both compounds promoted skin wound healing when compared to those treated with PBS (negative control) (Figure 3a and b). The effect was comparable to that of vancomycin treatment at 20 mg/kg (positive control). Likewise, both compounds significantly reduced the bacterial burden in treated wounds comparable to vancomycin treatment at a 4-fold lower dose (5 mg/kg vs. 20 mg/kg) (Figure 3c).

Next, the systemic therapeutic effect of **4j** and **4n** was evaluated in a mouse peritonitis-sepsis model. Both compounds somewhat prolonged survival of infected mice, but the effect was weaker compared to vancomycin treatment (Figure 3d). Although the survival effect was statistically significant for **4j** and both compounds could only be tested at a 4-fold lower dose compared to vancomycin (5 mg/kg vs. 20 mg/kg body weight) due to limited compound availability, the overall systemic activity of both compounds seems to be limited, presumably as a result of their poor aqueous solubility (Supporting Information Figure S2c and d). This limitation needs to be addressed in future optimization of the compounds.

Antibacterial Efficacy of Bisindole Derivatives 4 Is Independent of Pyruvate Kinase. Several marine and synthetic bisindoles have been shown to inhibit enzymatic activity of pyruvate kinase (PK) of MRSA *in vitro*.^{29–32} PK catalyzes the final reaction step in glycolysis generating pyruvate by transfer of a phosphoryl group from phosphoenolpyruvate to ADP. PK has been reported to be essential for *S. aureus* with inhibition leading to an impaired metabolism by reduced ATP production.³³ The essentiality of PK makes the enzyme a promising potential new drug target. Due to their structural similarity, it was reasonably conceivable that bisindole derivatives **4** also inhibit PK. This assumption was further supported by the fact that published bisindoles followed a similar SAR trend with halogenated derivatives inhibiting growth stronger than hydroxyl substitution at the indole rings.²⁹

To assess involvement of PK as a potential molecular target of bisindole derivatives **4** in *S. aureus*, we determined the MIC of **4c**, **4d**, **4j**, **4n**, **4q**, and **4s** against the MRSA LAC wild-type strain and against the isogenic PK knock-out strain in a minimal medium with glucose or pyruvate as a single carbon source. The PK encoding gene *pyk* is conditionally essential in *S. aureus*. A *pyk* deletion mutant is viable in a medium that lacks glucose when it is supplemented with pyruvate. Indeed, the LAC $\Delta pyk::Erm^R$ mutant was unable to grow in a medium containing glucose as a carbon source consistent with a previous report,³⁴ demonstrating PK essentiality under these conditions. Consistently, the MRSA LAC wild-type was fully susceptible to compound **4j** during growth on glucose (Figure 4). However, unexpectedly, this strain showed unaltered susceptibility to **4j** in a medium containing pyruvate as the sole carbon source, a condition where PK activity is dispensable. Furthermore, the isogenic PK-deficient LAC $\Delta pyk::Erm^R$ mutant was even slightly more sensitive toward **4j** during growth on pyruvate, while it was expected to be resistant if the compound specifically targets PK in the bacterial cell (Figure 4). Similar susceptibility patterns were also observed for compounds **4d**, **4n**, **4q**, and **4s** (Supporting Information Figure S6).

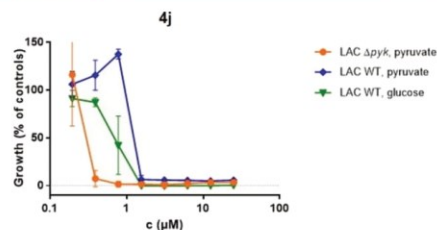


Figure 4. Antibacterial activity of **4j** against cells of MRSA LAC wild-type and LAC $\Delta pyk::Erm^R$ in a minimal M9 medium with glucose (triangle) or pyruvate (circle, diamond) as a sole carbon source. Data are means of duplicates $\pm$ SD. Growth was quantified using the resazurin reduction assay.

To directly evaluate the involvement of PK as a potential molecular target of bisindole alkaloids **4**, an N-terminally His6-tagged version of *S. aureus* PK was heterologously expressed in *E. coli* NiCo21(DE3) and purified employing nickel-NTA affinity chromatography (Figure 5a). The enzymatic activity of purified recombinant *S. aureus* PK, however, was not inhibited by compounds **4j**, **4n**, and **4q** up to concentrations of 12.5 μ M (Figure 5b), which is well above the MICs for these compounds against whole cells of *S. aureus* (Table 1). Additionally, also commercially available rabbit muscle PK, which is 97% identical to isoform M1 of human PK and 45% identical to MRSA PK, was completely insensitive to the tested compounds (Figure 5b), indicating a general lack of inhibitory properties toward PK enzymes.

In combination, these findings clearly demonstrate that the bactericidal activity of bisindole derivatives **4** on *S. aureus* cells is entirely *pyk*-independent under the tested *in vitro* culture conditions.

Resistance Studies. In order to reveal insights into the *pyk*-independent mechanism of antibacterial activity of the reported bisindole derivatives, we attempted to isolate spontaneous mutants of *S. aureus* MRSA strain ATCC 700699 resistant to **4j** on a solid medium. However, generation of single-step resistant mutants on agar supplemented with varying concentrations of bisindoles was unsuccessful. Thus, cells of *S. aureus* MRSA strain ATCC 700699 were serially passaged in a liquid medium with escalating subinhibitory concentrations of **4j**. After 38 culture passages, **4j**-resistant MRSA mutants exhibiting low-level resistance (2- to 4-fold shift in MIC) were obtained. As expected, whole genome sequencing revealed that the mutants had accumulated several mutations (Supporting Information Table S3). However, none of them pointed toward an obvious specific gene product that could be mediating resistance or directly representing a potential target. Furthermore, cross-resistance was observed among bisindoles since **4j**-resistant MRSA mutants were also resistant to **4n** with a similar 2-fold shift in MIC. In contrast, no cross-resistance was observed for antibiotics of other classes such as vancomycin, moxifloxacin, linezolid, and ramoplanin (Supporting Information Table S4), suggesting that the mechanism of resistance toward bisindoles in the studied MRSA mutants, while still elusive, is bisindole-specific.

Mode of Action Studies. One of the hallmarks of membrane-targeting antimicrobials is reduced potential for the emergence of bacterial resistance.³⁵ In like manner, difficulty in resistance generation and low-level resistance in

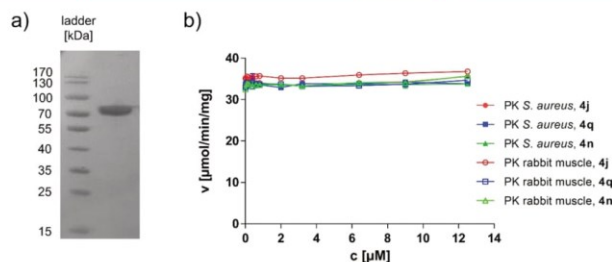


Figure 5. Bisindole derivatives **4j**, **4n**, and **4q** not inhibiting pyruvate kinase (PK) enzymatic activity *in vitro*. (a) Coomassie stained SDS-PAGE of N-terminally His6-tagged *S. aureus* PK heterologously expressed in *E. coli* NiCo21 (DE3) and purified using Ni-NTA affinity chromatography. The sample was concentrated and desalted. Purified recombinant PK (1 μg) was applied to the gel. (b) Activity of PK from *S. aureus* (filled symbols) and from rabbit muscle (empty symbols) in the presence of **4j** (red circles), **4n** (green triangles), and **4q** (blue squares). Initial enzyme velocity (v), expressed as μmol/min/mg protein, is plotted against 12 different compound concentrations ranging from 0 to 12.5 μM. Data are means of triplicates ± SEM, measured in two independent experiments.

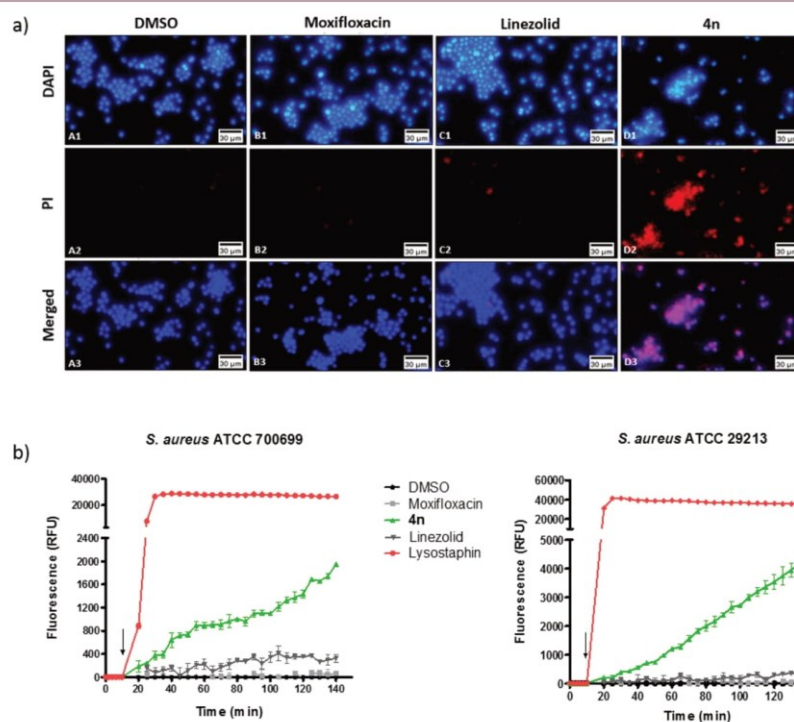


Figure 6. Membrane permeabilization of *S. aureus* cells by compound **4n**. (a) Fluorescence micrographs of *S. aureus* ATCC 29213 cells treated with 5-fold MIC of **4n** for 30 min. DMSO, moxifloxacin, and linezolid treatment served as negative controls. DAPI stains all cells irrespective of membrane integrity, whereas PI only stains cells with a permeabilized membrane. Bars represent 30 μm. (b) Propidium iodide internalization by **4n**-treated methicillin-resistant and susceptible *S. aureus* cells. PI fluorescence (excitation, 535 nm; emission, 617 nm) of cells incubated with 5-fold MIC of **4n** (green) in a black 96 well plate was measured for 2 h. Baseline fluorescence of untreated cells was established for 10 min before addition of compounds (black arrow). DMSO (0.078%), moxifloxacin, and linezolid were included as negative controls, while 10 μg/mL lysostaphin served as a positive control. Data are means of triplicates ± SEM.

conjunction with rapid bactericidal activity exhibited by bisindoles are somewhat indicators that the target of bisindoles

might not be a protein but rather the bacterial membrane or cell wall. Thus, the possibility of bisindoles interacting with the

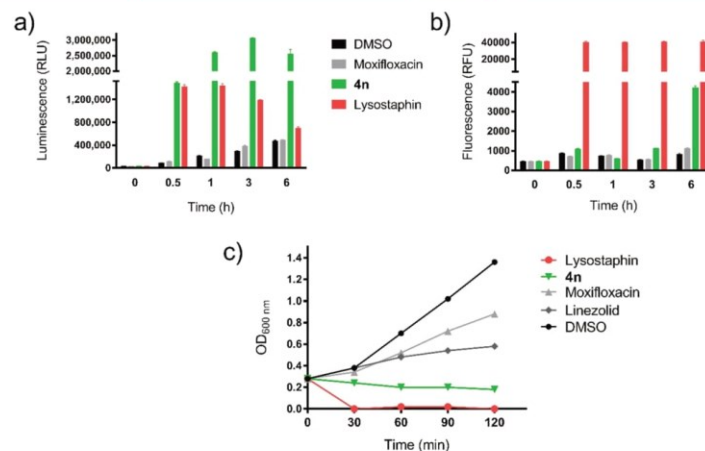


Figure 7. Leakage of intracellular contents and assessment of potential cell lysis of compound-treated *S. aureus* ATCC 700699. Efflux of (a) ATP and (b) GFP from 4n-treated recombinant *S. aureus* ATCC 700699 cells expressing GFP. Cultures were treated with 5-fold MIC of 4n. Release of ATP into cell-free culture supernatants was quantified using the bioluminescence-based BacTiter-Glo kit. The GFP level in cell-free culture supernatants at varying time points was quantified via GFP fluorescence at excitation and emission wavelengths of 475 and 509 nm, respectively. For both assays, moxifloxacin at 5-fold MIC and 0.078% DMSO served as negative controls, while 10 $\mu\text{g/mL}$ lysostaphin was used as a positive control. Data are means of triplicates $\pm$ SEM. (c) Assessment of potential lysis of *S. aureus* ATCC 700699 cells induced by treatment with 4n. Cells were incubated with 8-fold MIC of compounds. Optical density of cultures was measured at 600 nm. Decrease in turbidity would indicate lysis. Moxifloxacin and linezolid served as negative controls, 10 $\mu\text{g/mL}$ lysostaphin served as a positive control, and 0.125% DMSO was used as a solvent and growth control.

bacterial membrane and/or cell wall was investigated by fluorescence microscopy using a double staining approach. Cells of MSSA strain ATCC 29213 were treated with 4n and then costained with propidium iodide (PI) and 4',6-diamidino-2-phenylindole (DAPI). DAPI, as a membrane permeant dye, can stain the minor groove of double-stranded DNA of cells regardless of their viability or membrane integrity. However, PI only enters the cell and subsequently intercalates DNA upon loss of membrane integrity.³⁶ MSSA cells incubated with a solvent control as well as non-membrane-targeting antibiotics such as moxifloxacin and linezolid exhibited negligible PI staining. On the other hand, cells treated with 4n showed remarkable PI staining as soon as after 30 min incubation (Figure 6a), indicating substantial membrane permeabilization. Membrane permeability by 4n was further evaluated via PI internalization assay. The effects of moxifloxacin, linezolid, lysostaphin, and 4n on cells of MRSA strain ATCC 700699 and MSSA strain ATCC 29213 incubated with PI are depicted in Figure 6b. Lysostaphin, a bacteriolytic enzyme capable of punching holes in the cell wall of *Staphylococcus* species thereby causing rapid cell lysis,³⁷ was used as a positive control. As expected, lysostaphin showed a robust ability to permeabilize the bacterial membrane. In like manner, compound 4n also instigated a gradual, yet conspicuous increase in fluorescence intensity, pointing to the fact that compound 4n causes pronounced membrane permeabilization. In contrast, cells incubated with negative controls (linezolid and moxifloxacin, antibiotics impeding bacterial growth by inhibiting DNA gyrase and initiation of protein synthesis, respectively) induced very minor to no fluorescence as expected. This result is consistent with the fluorescence microscopy observations.

To corroborate the membrane-targeting features of bisindoles, cellular ATP and GFP leakage assays were conducted using the GFP-expressing recombinant *S. aureus* ATCC 700699 strain. In contrast to moxifloxacin and linezolid, 4n as well as lysostaphin induced substantial leakage of ATP from the cells as early as 0.5 h of treatment (Figure 7a), while no leakage of ATP occurs from intact cells.⁵⁸ In contrast, release of GFP was only evident after 6 h incubation for 4n-treated cells, whereas lysostaphin treatment resulted in rapid GFP efflux (Figure 7b). Next, we performed an experiment assessing potential bacterial cell lysis. 4n did not induce substantial cell lysis since there was only a very slow and minor decrease in the optical density of the bacterial liquid culture over time in contrast to lysostaphin, which caused a rapid and strong decrease in the optical density (Figure 7c). Membrane-targeting agents generally give rise to initial rapid loss of low-molecular-weight compounds such as ATP (molecular weight 0.5 kDa) from within the cell due to a membrane injury.³⁹ Considering that GFP is a macromolecule with a molecular weight of 26.9 kDa, the presented results suggest that the reported bisindoles give rise to small pores in membranes of bacteria causing efflux of low-molecular-weight molecules, which provokes rapid cell death. Only after prolonged incubation times, the membrane is likely further permeabilized to also allow efflux of macromolecules, whereas the cell wall integrity is largely maintained so that no substantial cell lysis occurs.

CONCLUSIONS

In summary, here, we report on the efficient synthesis of new derivatives 4 of the natural compounds Alocasin A (1a) and Hyrtinadine A (1b) and their strong antibacterial effect

Table 4. Experimental Details of the One-Pot Masuda–Suzuki Synthesis of the Alocasin A and Hyrtinadine A Derivatives **4a–4f**, **4n**, and **4r–4s**

entry	3-iodo indole 2 [mg] ([mmol])	t_1 [h]	heteroaryl dihalide 3 [mg] ([mmol])	t_2 [h] ([T])	yield [mg] (%)
1 ^a	427 (1.00) of 3-iodo-5-methoxy-1-tosyl-1H-indole (2a)	3	119 (0.5) of 2,5-dibromopyrazine (3a)	16 (100)	150 (81) of 4a
2 ^a	415 (1.00) of 3-iodo-5-fluoro-1-tosyl-1H-indole (2b)	3	118 (0.5) of 2,5-dibromopyrazine (3a)	16 (100)	128 (74) of 4b
3 ^a	432 (1.00) of 5-chloro-3-iodo-1-tosyl-1H-indole (2c)	3	119 (0.5) of 2,5-dibromopyrazine (3a)	16 (100)	130 (68) of 4c
4 ^a	476 (1.00) of 3-iodo-5-bromo-1-tosyl-1H-indole (2d)	3	119 (0.5) of 2,5-dibromopyrazine (3a)	16 (100)	172 (74) of 4d
5 ^a	501 (1.00) of <i>tert</i> -butyl-5,6-dibromo-3-iodo-1H-indole-1-carboxylate (2e)	3	119 (0.5) of 2,5-dibromopyrazine (3a)	16 (100)	122 (39) of 4e
6 ^a	476 (1.00) of 3-iodo-6-bromo-1-tosyl-1H-indole (2f)	3	119 (0.5) of 2,5-dibromopyrazine (3a)	16 (100)	206 (88) of 4f
7 ^b	432 (1.00) of 5-chloro-3-iodo-1-tosyl-1H-indole (2c)	3	119 (0.5) of 2,6-dibromopyridine (3b)	16 (80)	77 (41) of 4n
8 ^b	432 (1.00) of 5-chloro-3-iodo-1-tosyl-1H-indole (2c)	3	119 (0.5) of 2,6-dibromopyrazine (3c)	40 (80)	124 (65) of 4r
9 ^b	432 (1.00) of 5-chloro-3-iodo-1-tosyl-1H-indole (2c)	3	166 (0.5) of 3,6-dibromopyridazine (3d)	40 (80)	0.143 (75) of 4s

^a10 mL of 1,4-dioxane and 7.0 mL of methanol. ^b4.0 mL of 1,4-dioxane and 3.0 mL of methanol.

especially against MRSA. With respect to further preclinical development, compounds **4j** and **4n** seem to be most promising due to their broad antibacterial potency against all tested Gram-positive nosocomial pathogens, their moderate cytotoxicity toward human cells, and their *in vivo* efficacy in a mouse wound infection model upon topical application. Consequently, these compounds are interesting lead structures for further preclinical development with acceptable therapeutic margins. Future medicinal chemical optimization should focus on increasing their aqueous solubility to improve the systemic potency of the compounds. Despite structural similarity to bisindoles that were reported to inhibit PK of *S. aureus*, PK plays no role in explaining the strong bactericidal potency of compounds **4**. In contrast, we demonstrated that the reported molecules **4** likely target the bacterial membrane leading to permeabilization that causes rapid efflux of low-molecular-weight intracellular metabolites. Further studies are necessary to decipher the exact molecular target(s) and mode of interaction with the membrane of these potent bactericidal substances.

■ EXPERIMENTAL SECTION

Chemistry. The synthesis of compounds **1b**, **4g–4i**, **4k**, **4o**, and **4t** has previously been published.²⁵ Compounds **1a**, **4a–4f**, **4n**, and **4r–4s** were synthesized and isolated as described below. Compounds **4j**, **4l–4n**, **4p–4q**, **4u**, and **4v** were synthesized and isolated under optimized conditions. Purity of all compounds is >95% as determined by combustion analysis. In some cases, purity was determined by HPLC and is stated in the analytical data of the compound.

General Procedure. 3-Iodo indole **2** (1.00 mmol) and tetrakis(triphenylphosphane)palladium(0) were dissolved in dry 1,4-dioxane in a dry Schlenk tube with a magnetic stir bar under dry nitrogen (for experimental details, see Table 4). Triethylamine and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane were added dropwise, and the reaction mixture was stirred at 80 °C for the times (t_1) indicated. Thereafter, the mixture was cooled to room temperature, and methanol, cesium carbonate (0.82 g, 2.50 mmol), and heteroaryl dihalide **3** (0.5 mmol) were added to the reaction mixture. This mixture was stirred at 80–100 °C for the times (t_2) indicated. Subsequently, the mixture was cooled to room temp., potassium hydroxide (0.14 g, 2.5 mmol) was added, and the reaction was stirred again at 100 °C for 2.5 h. After cooling to room temp., the mixture was diluted with dichloromethane, adsorbed on Celite under reduced pressure, and purified by column chromatography on silica gel (100:1:1 dichloromethane/MeOH/aq. NH₃ 25%). The resulting solid was then dried for 16 h under reduced pressure (3×10^{-3} mbar). Finally, the compounds **4** were suspended in dichloromethane and subsequently in *n*-hexane for further purification.

Demethylation of Compound **3a** to Give Alocasin A (**1a**).

Compound **4a** (226 mg, 0.61 mmol) is suspended in acetic acid (5 mL) and hydrobromic acid (5 mL, 48% aq.) and heated for 16 h to 120 °C in a closed Schlenk tube with a magnetic stir bar. After cooling to room temp., the solvents were removed under reduced pressure; the residue was suspended in ca. 25% aqueous NH₃ (25 mL) and stirred at room temp. for 1 h. The mixture was extracted with ethyl acetate (2 × 25 mL), and the organic layers are separated and dried (anhydrous sodium sulfate). The product was adsorbed on Celite and purified by column chromatography on silica gel (100:1:1 DCM/MeOH/25% aq. NH₃). The resulting solid was then dried under reduced pressure (3×10^{-3} mbar) for 16 h.

Alocasin A (**1a**) (150 mg, 72%); Mp, >300 °C. ¹H NMR (600 MHz, DMSO-*d*₆): δ 6.70 (dd, J = 8.6, 2.4 Hz, 2H), 7.26 (d, J = 8.6 Hz, 2H), 7.81 (d, J = 2.4 Hz, 2H), 8.11 (d, J = 2.8 Hz, 2H), 8.82 (s, 2H), 9.00 (s, 2H), 11.34 (d, J = 2.8 Hz, 2H) (see Supporting Information Figure S7). ¹³C NMR (151 MHz, DMSO-*d*₆): δ 105.5, 111.9, 112.1, 112.1, 125.6, 125.9, 131.3, 139.6, 146.5, 151.6 (see Supporting Information Figure S8). HR-MS (ESI) m/z : calcd for [C₂₀H₁₄N₄O₂ + H]⁺, 343.1190; found, 343.1194. HPLC, 98.04%.

2,5-Bis(5-methoxy-1H-indole-3-yl)pyrazine (**4a**) (150 mg, 81%); Mp, 263 °C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 3.83 (s, 6H), 6.85 (dd, J = 8.8, 2.5 Hz, 2H), 7.37 (dd, J = 8.8, 0.6 Hz, 2H), 7.97 (d, J = 2.5 Hz, 2H), 8.20 (d, J = 2.8 Hz, 2H), 9.13 (s, 2H), 11.49 (d, J = 2.9 Hz, 2H) (see Supporting Information Figure S9). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 55.3, 103.1, 111.9, 112.4, 112.4, 125.6, 125.9, 131.9, 139.8, 146.5, 154.1 (see Supporting Information Figure S10). HR-MS (ESI) m/z : calcd for (C₂₂H₁₈N₄O₂ + H)⁺, 371.1503; found, 371.1504. Anal calcd for C₂₂H₁₈N₄O₂ (370.4): C, 71.34; H, 4.90; N, 15.13; found: C, 71.20; H, 4.93; N, 15.13.

2,5-Bis(5-fluoro-1H-indole-3-yl)pyrazine (**4b**) (128 mg, 74%); Mp, 280 °C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 7.06 (ddd, J = 9.1, 9.1, 2.7 Hz, 2H), 7.49 (dd, J = 8.9, 4.6 Hz, 2H), 8.18 (dd, J = 10.6, 2.6 Hz, 2H), 8.34 (d, J = 2.8 Hz, 2H), 9.15 (s, 2H), 11.76 (d, J = 1.9 Hz, 2H) (see Supporting Information Figure S11). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 106.1 (d, J = 24.7 Hz), 110.1 (d, J = 26.0 Hz), 112.7 (d, J = 4.7 Hz), 112.8 (d, J = 10.1 Hz), 125.4 (d, J = 10.5 Hz), 127.3, 133.5, 139.9, 146.3, 157.5 (d, J = 231.9 Hz) (see Supporting Information Figure S12). MS (ESI) m/z : calcd for (C₂₂H₁₄F₂N₄ + H)⁺, 347.4; found, 347.6. Anal calcd for C₂₂H₁₄F₂N₄ (346.3): C, 69.36; H, 3.49; N, 16.18; found: C, 69.11; H, 3.37; N, 15.93.

2,5-Bis(5-chloro-1H-indole-3-yl)pyrazine (**4c**) (130 mg, 68%); Mp, 278 °C. ¹H NMR (300 MHz, DMSO-*d*₆): δ 7.21 (dd, J = 2.1, 8.6 Hz, 2H), 7.50 (d, J = 8.6 Hz, 2H), 8.34 (d, J = 2.8 Hz, 2H), 8.51 (d, J = 2.1 Hz, 2H), 9.19 (s, 2H), 11.84 (d, J = 3 Hz, 2H) (see Supporting Information Figure S13). ¹³C NMR (75 MHz, DMSO-*d*₆): δ 112.3, 113.4, 120.7, 121.9, 124.7, 126.3, 127.1, 135.3, 140.1, 146.3 (see Supporting Information Figure S14). MS (ESI) m/z : calcd for (C₂₀H₁₂Cl₂N₄ + H)⁺, 379.2. Found, 379.3. Anal calcd for C₂₀H₁₂Cl₂N₄ (378.3): C, 63.34; H, 3.19; N, 14.77; found: C, 63.09; H, 3.17; N, 14.70.

Table 5. Experimental Details of the Optimized One-Pot Masuda–Suzuki Synthesis of the Alocasin A and Hyrtinadine A Derivatives **4** by Using Methanol as a Cosolvent

entry	(7-aza)indole 2 [mg] ([mmol])	heteroaryl dihalide 3 [mg] ([mmol])	yield [mg] (%)
1	432 (1.00) of 5-chloro-3-iodo-1-tosyl-1H-indole (2c)	142 (0.50) of 5-bromo-2-iodopyrimidine (3e)	177 (93) of 4j
2	3-iodo-5-methoxy-1-tosyl-1H-indole (2a) 427 mg/1.00 mmol	118 (0.50) of 2,6-dibromopyridine (3b) 118 mg (0.50) of 2,6-dibromopyridine (3b)	142 (77) of 4l
3	3-iodo-5-fluoro-1-tosyl-1H-indole (2b)	118 (0.50) of 2,6-dibromopyridine (3b)	140 (81) of 4m
4	432 (1.00) of 5-chloro-3-iodo-1-tosyl-1H-indole (2c)	118 (0.50) of 2,6-dibromopyridine (3b)	160 (84) of 4n
5	398 (1.00) 3-iodo-1-tosyl-7-azaindol (2g)	118 (0.50) of 2,6-dibromopyridine (3b)	120 (90) ^a of 4p

^aEven after repeated drying in vacuo at 80 °C for 42 h, dichloromethane in the crystal could not be completely removed.

2,5-Bis(5-bromo-1H-indole-3-yl)pyrazine (**4d**) (172 mg, 74%); Mp, 287 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.32 (dd, *J* = 8.6, 2.0 Hz, 2H), 7.46 (dd, *J* = 8.6, 0.6 Hz, 2H), 8.32 (d, *J* = 2.5 Hz, 2H), 8.67 (d, *J* = 1.9 Hz, 2H), 9.19 (s, 2H), 11.85 (s, 2H) (see Supporting Information Figure S15). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 112.6, 113.2, 114.2, 124.0, 124.8, 127.2, 127.3, 136.0, 140.4, 146.6 (see Supporting Information Figure S16). MS (ESI) *m/z*: calcd for (C₂₀H₁₂⁷⁹Br⁸¹BrN₄ + H)⁺, 469.2; found, 469.3. Anal calcd for C₂₀H₁₂Br₂N₄ (468.2): C, 51.31; H, 2.58; N, 11.97; found: C, 51.04; H, 2.59; N, 11.78.

2,5-Bis(5,6-dibromo-1H-indole-3-yl)pyrazine (**4e**) (122 mg, 39%); Mp, >300 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.89 (s, 2H), 8.36 (d, *J* = 2.8 Hz, 2H), 8.86 (s, 2H), 9.21 (s, 2H), 11.92 (d, *J* = 2.4 Hz, 2H) (see Supporting Information Figure S17). ¹³C NMR (126 MHz, DMSO-*d*₆) δ 113.0, 115.4, 116.9, 117.5, 126.5, 127.04, 128.7, 137.5, 141.0, 146.9 (see Supporting Information Figure S18). MS (ESI) *m/z*: calcd for (C₂₀H₁₀⁷⁹Br⁸¹Br₂N₄ + H)⁺, 627.0; found, 627.1. Anal calcd for C₂₀H₁₀Br₂N₄ (625.9): C, 38.38; H, 1.61; N, 8.95; found: C, 38.67; H, 1.66; N, 8.86.

2,5-Bis(6-bromo-1H-indole-3-yl)pyrazine (**4f**) (206 mg, 88%); Mp, 284 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.29 (dd, *J* = 8.6, 1.8 Hz, 2H), 7.67 (d, *J* = 1.5 Hz, 2H), 8.28 (s, 2H), 8.40 (d, *J* = 8.6 Hz, 2H), 9.14 (s, 2H), 11.77 (s, 2H) (see Supporting Information Figure S19). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 113.2, 114.9, 115.1, 123.5, 123.6, 124.7, 127.0, 138.3, 140.6, 146.8 (see Supporting Information Figure S20). MS (ESI) *m/z*: calcd for (C₂₀H₁₂⁷⁹Br⁸¹BrN₄ + H)⁺, 469.15; found, 469.3. Anal calcd for C₂₀H₁₂Br₂N₄ (468.2): C, 51.31; H, 2.58; N, 11.97; found: C, 50.97; H, 2.45; N, 11.65.

2,6-Bis(5-chloro-1H-indole-3-yl)pyridine (**4n**) (77 mg, 41%); Mp, 241 °C. ¹H NMR (300 MHz, DMSO-*d*₆) δ 7.19 (dd, *J* = 8.6, 2.1 Hz, 2H), 7.50 (dd, *J* = 8.6, 0.5 Hz, 2H), 7.64–7.59 (m, 2H), 7.76 (dd, *J* = 8.5, 7.1 Hz, 1H), 8.19 (d, *J* = 2.8 Hz, 2H), 8.56 (d, *J* = 2.1 Hz, 2H), 11.73 (d, *J* = 2.8 Hz, 2H) (see Supporting Information Figure S27). ¹³C NMR (75 MHz, DMSO-*d*₆) δ 113.2, 115.7, 116.1, 120.7, 121.6, 124.7, 126.3, 127.2, 135.4, 136.7, 154.0 (see Supporting Information Figure S28). MS (ESI) *m/z*: calcd for [C₂₁H₁₃Cl₂N₃ + H]⁺, 378.1; found, 378.5. Anal calcd for C₂₁H₁₃Cl₂N₃ (378.3): C, 66.68; H, 3.46; N, 11.11; found: C, 66.69; H, 3.17; N, 10.83.

2,6-Bis(5-chloro-1H-indole-3-yl)pyrazine (**4r**) (124 mg, 65%); Mp, 253 °C. ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.21 (dd, *J* = 8.6, 2.2 Hz, 2H), 7.51 (d, *J* = 8.6 Hz, 2H), 8.34 (d, *J* = 2.8 Hz, 2H), 8.52 (d, *J* = 2.1 Hz, 2H), 9.19 (s, 2H), 11.85 (d, *J* = 2.7 Hz, 2H) (see Supporting Information Figure S33). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 112.8, 113.9, 121.2, 122.4, 125.3, 126.8, 127.6, 135.9, 140.6, 146.8 (see Supporting Information Figure S34). HR-MS (ESI) calcd for (C₂₀H₁₂Cl₂N₄ + H)⁺ *m/z*, 379.0512. Found *m/z*, 379.0511. Anal calcd for C₂₀H₁₂Cl₂N₄ (379.24): C, 63.34; H, 3.19; N, 14.77; found: C, 63.15; H, 3.00; N, 14.66.

3,6-Bis(5-chloro-1H-indole-3-yl)pyridazine (**4s**) (0.143 mg, 75%); Mp, 268 °C. ¹H NMR (600 MHz, DMSO-*d*₆) δ 7.24 (dd, *J* = 8.6, 2.1 Hz, 2H), 7.53 (d, *J* = 8.6 Hz, 2H), 8.13 (s, 2H), 8.37 (d, *J* = 2.6 Hz, 2H), 8.67 (d, *J* = 2.1 Hz, 2H), 11.91–11.87 (m, 2H) (see Supporting Information Figure S35). ¹³C NMR (151 MHz, DMSO-*d*₆) δ 112.9, 113.8, 121.8, 122.5, 123.9, 125.4, 126.4, 128.8, 136.0, 154.5 (see Supporting Information Figure S36). HR-MS (ESI) *m/z*: calcd for [C₂₀H₁₂Cl₂N₄ + H]⁺, 379.0512; found, 379.0516. HPLC, 98.66%.

(7-Aza)indole (1.00 mmol) and tetrakis(triphenylphosphane)-palladium(0) (67 mg, 0.06 mmol) were placed in a dry screw-capped vessel with a magnetic stir bar (for experimental details, see Table 5). After three cycles of evacuating and refilling with dry argon, degassed dry 1,4-dioxane (4.00 mL) was added. Then, dry triethylamine (1.40 mL, 10.0 mmol) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.25 mL, 1.70 mmol) were successively added. Then, the reaction mixture was stirred in a preheated oil bath at 80 °C for 4 h. The mixture was cooled down to room temp. (water bath). Dry methanol (7.00 mL) was added, and the mixture was stirred at room temp. for 10 min. After the addition of heteroaryl dihalide **3** (0.50 mmol) and cesium carbonate (823 mg, 2.50 mmol), the mixture was stirred in a preheated oil bath at 60 °C for 18 h. After the Suzuki coupling was completed, the mixture was cooled to room temp. (water bath). Ground potassium hydroxide (140 mg, 2.50 mmol) was added, and the reaction mixture was stirred at 100 °C for 4 h. Then, after cooling to room temp. (water bath), the solvents were removed in vacuo, and the residue was adsorbed onto Celite. After purification by chromatography on silica gel (dichloromethane/methanol/aqueous ammonia, 100:1:1), the desired compounds **4** were obtained (pure according to ¹H NMR). For further purification, trituration in dichloromethane (3 × 10 mL) by ultrasonication was necessary.

3,3'-(Pyrimidine-2,5-diyl)bis(5-chloro-1H-indole) (**4j**), colorless solid (177 mg, 93%); Mp, 266 °C. ¹H NMR (DMSO-*d*₆, 600 MHz): δ = 7.21 (d, *J* = 8.6 Hz, 2H), 7.52 (dd, *J* = 8.5 Hz, 4.2 Hz, 2H), 7.97 (s, 1H), 8.01 (s, 1H), 8.30 (s, 1H), 8.62 (s, 1H), 9.13 (s, 2H), 11.80 (s, 1H), 11.86 (s, 1H) (see Supporting Information Figure S21). ¹³C NMR (DMSO-*d*₆, 150 MHz): δ = 109.8, 114.1, 115.0, 118.8, 121.5, 122.3, 122.4, 125.3, 126.4, 127.1, 130.6, 135.8, 136.0, 154.6, 160.8 (see Supporting Information Figure S22). HR-MS (ESI) *m/z*: calcd for [C₂₀H₁₂Cl₂N₄ + H]⁺, 379.0519; found, 379.0514. Anal calcd for C₂₀H₁₂Cl₂N₄ (379.2): C, 63.34; H, 3.19; N, 14.77; found: C, 63.15; H, 3.02; N, 14.51.

2,6-Bis(5-methoxy-1H-indol-3-yl)pyridine (**4l**), colorless solid (142 mg, 77%); Mp, 149 °C. ¹H NMR (DMSO-*d*₆, 300 MHz): δ 3.67 (s, 6H), 6.82 (dd, *J* = 8.8 Hz, 2.5 Hz, 2H), 7.36 (d, *J* = 8.8 Hz, 2H), 7.54 (d, *J* = 7.8 Hz, 2H), 7.72 (dd, *J* = 8.4 Hz, 1H), 8.05 (d, *J* = 2.8 Hz, 2H), 8.08 (d, *J* = 2.5 Hz, 2H), 11.36 (d, *J* = 2.9 Hz, 2H) (see Supporting Information Figure S23). ¹³C NMR (acetone-*d*₆, 75 MHz): δ 55.8, 104.9, 113.0, 113.1, 116.6, 118.2, 126.4, 127, 133.6, 137.3, 155.8, 156.2 (see Supporting Information Figure S24). MS (ESI) *m/z*: calcd for [C₂₃H₁₉N₃O₂ + H]⁺, 370.4; found, 370.3. Anal calcd for C₂₃H₁₉N₃O₂ (369.4): C, 74.78; H, 5.18; N, 11.37; found: C, 74.55; H, 5.34; N, 11.08.

2,6-Bis(5-fluoro-1H-indol-3-yl)pyridine (**4m**), colorless solid (140 mg, 81%); Mp, 227–229 °C. ¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.04 (td, *J* = 9.1 Hz, 2.6 Hz, 2H), 7.43–7.53 (m, 2H), 7.57–7.64 (m, 2H), 7.75 (dd, *J* = 8.4 Hz, 7.1 Hz, 1H), 8.19 (d, *J* = 2.7 Hz, 2H), 8.24 (dd, *J* = 10.8 Hz, 2.6 Hz, 2H), 11.60–11.73 (m, 2H) (see Supporting Information Figure S25). ¹³C NMR (DMSO-*d*₆, 75 MHz): δ 106.1 (d, *J* = 24.6 Hz), 109.8 (d, *J* = 26.1 Hz), 112.8 (d, *J* = 9.9 Hz), 115.9, 116.26 (d, *J* = 4.8 Hz), 125.6 (d, *J* = 10.8 Hz), 127.6, 133.7, 136.8, 154.2, 157.6 (d, *J* = 231.7 Hz) (see Supporting Information Figure S26). MS (ESI) *m/z*: calcd for [C₂₁H₁₃F₂N₃ + H]⁺, 346.3; found, 346.3. Anal calcd for C₂₁H₁₃F₂N₃ (345.4): C, 73.04; H, 3.79; N, 12.17; found: C, 73.06; H, 4.03; N, 11.94.

2,6-Bis(5-chloro-1*H*-indol-3-yl)pyridine (**4n**), colorless solid (160 mg, 84%). Mp, 241 °C. ¹H NMR (DMSO-*d*₆, 600 MHz): δ = 7.19 (dd, *J* = 8.6 Hz, 2.1 Hz, 2H), 7.50 (d, *J* = 8.6 Hz, 2H), 7.61 (d, *J* = 7.8 Hz, 2H), 7.76 (t, *J* = 7.8 Hz, 1H), 8.18 (d, *J* = 2.7 Hz, 2H), 8.56 (d, *J* = 2.0 Hz, 2H), 11.73 (s, 2H) (see Supporting Information Figure S27). ¹³C NMR (DMSO-*d*₆, 150 MHz): δ = 113.8, 116.7, 121.2, 122.2, 125.3, 126.8, 127.8, 135.9 (see Supporting Information Figure S28). HR-MS (ESI) *m/z*: calcd for [C₂₁H₁₃Cl₂N₃ + H]⁺, 378.0559; found, 378.0562. Anal calcd for C₂₁H₁₃Cl₂N₃ (378.3): C, 66.68; H, 3.46; N, 11.11; found: C, 66.53; H, 3.35; N, 10.84.

2,6-Bis(1*H*-pyrrolo[2,3-*b*]pyridin-3-yl)pyridine (**4p**), light yellow solid (120 mg, 90%). ¹H NMR (DMSO-*d*₆, 600 MHz): δ = 7.21 (dd, *J* = 4.6, 7.9 Hz, 2H), 7.68 (d, *J* = 7.8 Hz, 2H), 7.80 (t, *J* = 7.8 Hz, 1H), 8.27 (d, *J* = 2.6 Hz, 2H), 8.31 (dd, *J* = 4.6 Hz, 1.5 Hz, 2H), 8.77 (dd, *J* = 7.9 Hz, 1.5 Hz, 2H), 12.07 (s, 2H) (see Supporting Information Figure S29). ¹³C NMR (DMSO-*d*₆, 150 MHz): δ = 115.2, 116.8, 116.9, 118.1, 126.5, 129.9, 137.5, 143.5, 149.7, 154.4 (see Supporting Information Figure S30). Anal calcd for C₁₉H₁₃N₅ + 0.08 CH₂Cl₂ (311.3 + 6.79): C, 71.56; H, 4.15; N, 21.83; found: C, 71.16; H, 4.05; N, 21.51.

Indole **2** (1.00 mmol) and tetrakis(triphenylphosphane)-palladium(0) (67 mg, 0.06 mmol) were placed in a dry screw-capped vessel with a magnetic stir bar (for experimental details, see Table 6).

Table 6. Experimental Details of the Optimized One-Pot Masuda–Suzuki Synthesis of the Allocasin A and Hyrtinadine A Derivatives **4 by Using Water as a Cosolvent**

entry	indole 2 [mg] ([mmol])	heteroaryl dihalide 3 [mg] ([mmol])	yield [mg] (%)
1	432 (1.00) of 5-chloro-3-iodo-1-tosyl-1 <i>H</i> -indole (2c)	142 (0.50) of 5-bromo-2-iodopyrimidine (3e)	150 (80) of 4j
2	432 (1.00) of 5-chloro-3-iodo-1-tosyl-1 <i>H</i> -indole (2c)	118 (0.50) of 2,6-dibromopyridine (3b)	160 (84) of 4n
3	432 (1.00) of 5-chloro-3-iodo-1-tosyl-1 <i>H</i> -indole (2c)	75 (0.50) of 2,4-dichloropyrimidine (3f)	110 (58) of 4q
4	397 (1.00) of 5-iodo-1-tosyl-1 <i>H</i> -indole (2h)	118 (0.50) of 3,5-dibromopyridine (3g)	66 (43) of 4u
5	415 (1.00) of 3-iodo-5-fluoro-1-tosyl-1 <i>H</i> -indole (2b)	118 (0.50) of 3,5-dibromopyridine (3g)	120 (70) of 4v

After three cycles of evacuating and refilling with dry argon, degassed dry 1,4-dioxane (4.00 mL) was added. Then, dry triethylamine (1.40 mL, 10.0 mmol) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.25 mL, 1.70 mmol) were successively added. Then, the reaction mixture was stirred in a preheated oil bath at 80 °C for 4 h. The mixture was cooled down to room temp. (water bath). Distilled water (3.00 mL) was added, and the mixture was stirred at room temp. for 10 min. After the addition of heteroaryl dihalide **3** (0.50 mmol) and cesium carbonate (823 mg, 2.50 mmol), the mixture was stirred in a preheated oil bath at 60 °C for 42 h. After the Suzuki coupling was completed, the mixture was cooled to room temp. (water bath). Methanol (4.00 mL) and ground potassium hydroxide (140 mg, 2.50 mmol) were added, and the reaction was stirred at 100 °C for 5 h. Then, after cooling to room temp. (water bath), the solvents were removed in vacuo, and the residue was adsorbed onto Celite. After

purification by chromatography on silica gel (dichloromethane/methanol/aqueous ammonia, 100:1:1), the desired compounds **4** were obtained in a pure form. For removing residual solvents from chromatography, compounds were triturated in *n*-hexane under ultrasonication.

3,3'-(Pyrimidine-2,5-diyl)bis(5-chloro-1*H*-indole) (**4j**), colorless solid (150 mg, 80%). Mp, 266 °C. ¹H NMR (DMSO-*d*₆, 600 MHz): δ = 7.21 (d, *J* = 8.6 Hz, 2H), 7.52 (dd, *J* = 8.5 Hz, 4.2 Hz, 2H), 7.97 (s, 1H), 8.01 (s, 1H), 8.30 (s, 1H), 8.62 (s, 1H), 9.13 (s, 2H), 11.80 (s, 1H), 11.86 (s, 1H) (see Supporting Information Figure S21). ¹³C NMR (DMSO-*d*₆, 150 MHz): δ = 109.8, 114.1, 115.0, 118.8, 121.5, 122.3, 122.4, 125.3, 126.4, 127.1, 130.6, 135.8, 136.0, 154.6, 160.8 (see Supporting Information Figure S22). ESI MS (*m/z* (%)): 401 (12) [M(Cl³⁵) + Na]⁺, 379 (100) [M(Cl³⁵) + H]⁺. Anal calcd for C₂₀H₁₂Cl₂N₄ (379.2): C, 63.34; H, 3.19; N, 14.17; found: C, 63.18; H, 3.47; N, 14.47.

2,6-Bis(5-chloro-1*H*-indol-3-yl)pyridine (**4n**), colorless solid (146 mg, 77%). Mp, 241 °C. ¹H NMR (DMSO-*d*₆, 600 MHz): δ = 7.19 (dd, *J* = 8.6 Hz, 2.1 Hz, 2H), 7.50 (d, *J* = 8.6 Hz, 2H), 7.61 (d, *J* = 7.8 Hz, 2H), 7.76 (t, *J* = 7.8 Hz, 1H), 8.18 (d, *J* = 2.7 Hz, 2H), 8.56 (d, *J* = 2.0 Hz, 2H), 11.73 (s, 2H) (see Supporting Information Figure S27). ¹³C NMR (DMSO-*d*₆, 150 MHz): δ = 113.8, 116.3, 116.7, 121.2, 122.2, 125.3, 126.8, 127.8, 135.9, 137.3, 154.6 (see Supporting Information Figure S28). Anal calcd for C₂₁H₁₃Cl₂N₃ (378.3): C, 66.68; H, 3.46; N, 11.11; found: C, 66.39; H, 3.43; N, 10.90.

3,3'-(Pyrimidine-2,4-diyl)bis(5-chloro-1*H*-indole) (**4q**), colorless solid (110 mg, 58%). Mp, 225 °C. ¹H NMR (DMSO-*d*₆, 600 MHz): δ = 7.22 (dd, *J* = 8.6 Hz, 2.1 Hz, 1H), 7.26 (dd, *J* = 8.6 Hz, 2.0 Hz, 1H), 7.54 (t, *J* = 8.1 Hz, 2H), 7.62 (d, *J* = 5.4 Hz, 1H), 8.31 (d, *J* = 2.7 Hz, 1H), 8.48 (d, *J* = 2.8 Hz, 1H), 8.62 (d, *J* = 2.0 Hz, 1H), 8.65 (d, *J* = 1.9 Hz, 1H), 8.67 (d, *J* = 5.4 Hz, 1H), 11.90 (s, 1H), 12.06 (s, 1H) (see Supporting Information Figure S31). ¹³C NMR (DMSO-*d*₆, 150 MHz): δ = 112.7, 113.8, 114.1, 114.2, 115.7, 121.3, 121.4, 122.4, 122.7, 125.6, 126.0, 126.7, 127.2, 130.6, 131.0, 136.0, 136.1, 156.9, 161.6, 163.4 (see Supporting Information Figure S32). MS (ESI) *m/z*: calcd for [C₂₀H₁₂Cl₂N₄ + H]⁺, 380.3; found, 380.5. Anal calcd for C₂₀H₁₂Cl₂N₄ (379.2): C, 63.34; H, 3.19; N, 14.77; found: C, 63.35; H, 3.04; N, 14.51.

3,5-Bis(1*H*-indol-3-yl)pyridine (**4u**), colorless solid (66 mg, 43%). Mp, 324 °C. ¹H NMR (DMSO-*d*₆, 600 MHz): δ = 7.15 (t, *J* = 7.1 Hz, 2H), 7.20 (t, *J* = 7.1 Hz, 2H), 7.50 (d, *J* = 8.0 Hz, 2H), 7.88–7.97 (m, 4H), 8.28 (t, *J* = 2.0 Hz, 1H), 8.79 (d, *J* = 1.9 Hz, 2H), 11.55 (s, 2H) (see Supporting Information Figure S37). ¹³C NMR (DMSO-*d*₆, 75 MHz): δ = 112.2, 112.4, 118.9, 120.1, 121.8, 124.5, 125.0, 130.7, 131.8, 137.0, 144.3 (see Supporting Information Figure S38). HR-MS (ESI) calcd for [C₂₁H₁₃N₃ + H]⁺ *m/z*, 310.1339. Found *m/z*, 310.1342.

3,5-Bis(5-fluoro-1*H*-indol-3-yl)pyridine (**4v**), light yellow solid (120 mg, 70%). Mp, 293 °C. ¹H NMR (DMSO-*d*₆, 600 MHz): δ = 7.05 (td, *J* = 9.1 Hz, 2.5 Hz, 2H), 7.50 (dd, *J* = 8.8 Hz, 4.7 Hz, 2H), 7.65 (dd, *J* = 10.3 Hz, 2.5 Hz, 2H), 8.02 (d, *J* = 2.7 Hz, 2H), 8.21 (t, *J* = 2.2 Hz, 1H), 8.78 (d, *J* = 2.1 Hz, 2H), 11.66 (s, 2H) (see Supporting Information Figure S39). ¹³C NMR (DMSO-*d*₆, 75 MHz): δ = 103.7 (d, *J* = 23.9 Hz), 109.9 (d, *J* = 25.9 Hz), 112.6 (d, *J* = 4.7 Hz), 113.2 (d, *J* = 9.9 Hz), 125.0 (d, *J* = 10.0 Hz), 126.6, 130.4, 131.3, 133.6, 144.2, 157.6 (d, *J* = 232.2 Hz) (see Supporting Information Figure S40). HR-MS (ESI) calcd for [C₂₁H₁₃F₂N₃ + H]⁺ *m/z*, 346.1150. Found *m/z*, 346.1155.

Solubility Tests. Compounds **4c**, **4d**, and **4r** as well as the *in vivo* tested compounds **4j** and **4n** have been tested for solubility in DMSO

Table 7. Absorption Coefficients ϵ for **4c, **4d**, **4r**, **4j**, and **4n** in DMSO**

coefficients	compound				
	4c	4d	4j	4n	4r
$\lambda_{\text{max,abs}}$ [nm]	375	376	339	332	365
extinction coefficient ϵ [L·mol ⁻¹ ·cm ⁻¹]	8000	7200	11,700	5800	3900
r^2	0.98658	0.9934	0.99442	0.95684	0.92262

Table 8. Absorption Coefficients ϵ for 4c, 4d, 4r, 4j, and 4n in DMSO/PBS Buffer (30:70 to 40:60, pH = 7.77)

coefficients	compound				
	4c	4d	4j	4n	4r
$\lambda_{\text{max,abs}}$ [nm]	377	377	337	328	367
extinction coefficient ϵ [L·mol ⁻¹ ·cm ⁻¹]	6000	5200	7900	4700	5300
r^2	0.99776	0.99837	0.97886	0.99785	0.99751

and PBS buffer. The longest wavelength absorption bands of the compounds were investigated at different concentrations c , and the absorption coefficients ϵ were determined (Tables 7 and 8). If the compounds are completely dissolved, then the Lambert–Beer law ($A = \epsilon_j \times c \times d$) is fulfilled.

Stability Study. To investigate the stability of compound 4c, absorption spectra of 4c were measured at different times in DMSO/PBS buffer (40:60, pH = 7.77) at a concentration of 0.19 μM . Absorption maxima of 4c were determined and plotted over time to observe potential degradation or aggregation.

Biological Evaluation. Bacterial Strains. Nosocomial bacterial reference strains were cultivated in a Mueller Hinton medium at 37 °C and included different strains of *Staphylococcus aureus* (MSSA strain ATCC 25923, MRSA/VISA strain ATCC 700699, MRSA strain LAC USA300, and pyruvate kinase-deficient MRSA strain LAC $\Delta\text{pyk}::\text{Erm}^R$ (the latter was obtained from the laboratory of Anthony R. Richardson, Department of Microbiology and Immunology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA³⁴)), *Enterococcus faecalis* (ATCC 29212, ATCC 51299 (gentamycin-resistant)), *Enterococcus faecium* (ATCC 35667, ATCC 700221 (vancomycin-resistant)), and *Acinetobacter baumannii* (ATCC BAA 1605). *Mycobacterium tuberculosis* strain H37Rv was grown aerobically in a Middlebrook 7H9 medium supplemented with 10% (v/v) ADS enrichment (5% w/v, bovine serum albumin fraction V; 2% w/v, glucose; 0.85% w/v, sodium chloride), 0.5% (v/v) glycerol, and 0.05% (v/v) tyloxapol at 37 °C.

Determination of Minimal Inhibitory Concentration (MIC). All compounds **c** were tested for their antibacterial activities against various pathogenic bacterial strains including nosocomial pathogens and *Mycobacterium tuberculosis*. For the tested nosocomial pathogens (*S. aureus*, *E. faecalis*, *E. faecium*, and *A. baumannii*), the broth microdilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) (CLSI M7-A9, 2012) was used. Briefly, bacterial cells were grown aerobically in a Mueller Hinton (MH) medium at 37 °C and 180 rpm. A preculture was grown until the log phase ($\text{OD}_{600\text{ nm}} \sim 0.5$) and then seeded at 5×10^4 CFU/well in a total volume of 100 μL in 96 well round bottom microtiter plates and incubated with serially diluted test substances **4** at a concentration range of 100–0.78 μM . Microplates were incubated aerobically at 37 °C for 24 h. MICs were determined macroscopically by identifying the minimum concentration of the compounds **4** that led to complete inhibition of visual growth of the bacteria.

The MICs of compounds **4c**, **4d**, **4j**, **4n**, **4q**, and **4s** were additionally quantified via the BacTiter-Glo assay (Promega, Madison, WI, USA). Briefly, after an incubation time of 24 h, each well was resuspended by pipetting, and 50 μL aliquots were transferred into an opaque-walled multiwell plate. Additionally, control wells containing a medium without cells to determine background luminescence were prepared. A BacTiter-Glo reagent (50 μL) was added to each well. Contents were mixed and incubated for five minutes at room temperature. Luminescence was measured using a microplate reader. Growth was calculated relative to the background luminescence (0% growth) and a DMSO solvent control (100% growth).

The influence of glucose or pyruvate as a carbon source on drug susceptibility of *S. aureus* cells was determined in a minimal medium. MIC of active compounds **4** was determined in two-fold serial dilutions ranging from 0.15 to 25 μM in a minimal medium M9 ($1 \times \text{M9 salts}$, 2 mM MgSO_4 , 0.1 mM CaCl_2 , 0.05 mM nicotinamide, and 0.125% w/v casamino acids) with either 1% glucose or pyruvate. 5×10^4 CFU/well was seeded in a total volume of 100 μL into 96 well

round bottom microtiter plates and incubated aerobically at 37 °C for 24 h. MICs were determined by using the resazurin dye reduction assay as described below for *M. tuberculosis*.

For the determination of MIC against *M. tuberculosis*, bacteria were precultured until the log phase ($\text{OD}_{600\text{ nm}} = 0.5-1$) and then seeded at 1×10^5 cells per well in a total volume of 100 μL in 96 well round bottom microtiter plates and incubated with serially diluted test compounds **4** at a concentration range of 100–0.78 μM . Microplates were incubated at 37 °C for five days. Afterward, 10 μL /well of a 100 $\mu\text{g/mL}$ resazurin solution was added and incubated at ambient temperature for further 16 h. Then, cells were fixed for 30 min after formalin addition (5% v/v, final concentration). For viability determination, fluorescence was quantified using a microplate reader (excitation, 540 nm; emission, 590 nm). Percentage of growth was calculated relative to rifampicin-treated (0% growth) and DMSO-treated (100% growth) controls.

Evaluation of Compound Interaction via Checkerboard Assay.

The fractional inhibitory concentration index (FICI) of bisindoles in combination with colistin was determined in a 96 well plate employing two-dimensional dilutions of bioactive agents. Both compounds were serially diluted in a 2-fold manner. Colistin was diluted horizontally from 3.13 to 0.025 μM , while **4j** or **4n** was diluted vertically from 100 to 0.78 μM . Respective exponential growth phase cultures of test Gram-negative bacteria were adjusted and seeded at 5×10^4 CFU/well in a total volume of 100 μL and incubated for 24 h, at 37 °C. Upon incubation, resazurin was used as a viability marker. The FICI was calculated as the sum of the quotients of the MICs of each compound when used in combination (A and B) and the MIC of the compound alone (MIC_A and MIC_B , respectively)

$$\text{FICI}(A+B) = \frac{A}{\text{MIC}_A} + \frac{B}{\text{MIC}_B}$$

Total synergism and antagonism between bisindoles and colistin were defined as $\text{FICI} \leq 0.5$ and > 2 , respectively. Partial synergism was defined by $0.5 < \text{FICI} \leq 0.75$, while an indifferent or additive effect was denoted by $0.75 < \text{FICI} \leq 2$.⁴⁰

Determination of the Cytotoxicity and Therapeutic Index. The cytotoxicity of compounds **4c**, **4d**, **4j**, **4n**, **4q**, and **4s** was determined *in vitro* using the human monocyte cell line THP-1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSM) and the human fetal lung fibroblast cell line MRC-5 (American Type Culture Collection, ATCC). Compounds **4j**, **4n**, and **4q** were additionally tested against the human kidney cell line HEK293, the human hepatocellular carcinoma cell line HepG2, and the lung carcinoma cell line CLS-54 (all obtained from CLS Cell Lines Service GmbH). THP-1 and CLS-54 cells were cultured in an RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS). MRC-5 cells were incubated in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) FBS. HEK293 cells were cultivated using an EMEM medium supplemented with 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate, and 10% (v/v) FBS. HepG2 cells were cultivated using Ham's F12 medium supplemented with 2 mM L-glutamine and 10% (v/v) FBS. All cells were cultivated at 37 °C in a humidified atmosphere of 5% CO_2 . Cells were seeded at approx. 5×10^4 cells/well in a total volume of 100 μL in 96 well flat bottom microtiter plates containing two-fold serially diluted test compounds **4** at a maximum final concentration of 100 μM . Cells treated with DMSO in a final concentration of 1% (v/v) were used as solvent controls. After an incubation time of 48 h, 10 μL of resazurin solution (100 $\mu\text{g/mL}$) was added per well and incubated for further 3 h at 37 °C in a humidified atmosphere of 5% CO_2 .

Fluorescence was quantified using a microplate reader (excitation, 540 nm; emission, 590 nm). Growth was calculated relative to non-inoculated (i.e., cell-free) (0% growth) and untreated (100% growth) controls in triplicate experiments.

For determination of the therapeutic index of the tested substances **4**, the selectivity index (SI) was determined by the quotient of the observed cytotoxic concentration reducing growth by 50% (IC₅₀) and MIC. Compounds **4** with SI ≥ 10 were characterized in further experiments.

Testing for Hemolytic Activity. Defibrinated sheep red blood cells (RBC) purchased from Thermo Fisher Scientific, Germany were washed thrice with PBS (3000 rpm, 10 min at 4 °C) and then diluted in PBS to obtain an 8% RBC suspension. 100 μ L RBC suspensions were treated with 100 μ L of compounds **4j** and **4n** at the indicated final concentrations and incubated at 37 °C. Triton X-100 (2%) and PBS served as positive and negative controls, respectively. After 1 h incubation, the mixtures were collected and centrifuged at 3000 rpm for 3 min. The supernatant (100 μ L) was transferred to a new 96 well microtiter plate, and hemoglobin release was measured in a Tecan Infinite 200pro plate reader by absorption at 540 nm.

Determination of Time-Kill Curves In Vitro. Killing kinetics were measured for compounds **4c**, **4d**, **4j**, **4n**, **4q**, and **4s** against the *S. aureus* MRSA/VISA strain ATCC 700699. An overnight culture of the strain was adjusted to a final inoculum concentration of 10⁸ CFU/mL cultivated in MH broth containing the tested compounds **4** at five-fold MIC. Cultures containing moxifloxacin at five-fold MIC (20 μ M) were used as a positive control. Furthermore, MRSA cultures cultivated in MH broth with 0.02% DMSO, correlating to the maximum amount of DMSO in the compound containing cultures, were handled as an antibiotic-free control. In a second attempt, the culture medium was removed after 6 h post-exposure to compounds **4** and replaced by a fresh compound-containing medium. This should overcome limited antibacterial activity in the case that compound **4** was degraded or consumed during the experiment. Therefore, the culture was centrifuged at 4000 rpm for 10 min. Afterward, the bacterial cell pellet was washed in PBS and suspended in a fresh medium containing the corresponding compound **4** at the initial concentration. Shaking cultures were incubated for 24 h at 37 °C at 180 rpm. After 0, 3, 6, and 24 h of incubation, viability, expressed as CFU/mL, was determined by plating 100 μ L aliquots of a serial dilution onto MH agar plates. The plates were incubated aerobically at 37 °C for 24 h before colonies were counted.

For determination of the influence of the carbon source on the killing effect, *S. aureus* LAC WT and LAC Δ pyk::Erm^R were cultivated in TSB broth without glucose with 1% sodium pyruvate and treated like described elsewhere.⁴⁴

In Vivo Efficacy Studies in a Mouse Wound Infection Model. The *in vivo* efficacy of bisindoles was investigated in a mouse skin infection model. BALB/c female mice were anesthetized with 4% chloral hydrate (0.1 mL/10 g), and 10 mm diameter wounds in the backs were achieved using surgical punches. Subsequently, a suspension containing 1 $\times 10^7$ CFU of MRSA strain T144 in PBS was inoculated onto each wound. At 1 h post-infection, PBS (vehicle), **4j** (5 mg/kg), **4n** (5 mg/kg), and vancomycin (20 mg/kg) were topically administered to the wound in a single-dose treatment. The gross and microscopic appearance of the skin surface was monitored daily for 12 days. At the end of the experiment, the wound skin was excised for homogenization to count the bacterial loads of MRSA in the skin employing CFU plating. All animal experiments were approved by the Institutional Animal Care and Use Committee of the China Agricultural University.

In Vivo Efficacy Studies in a Mouse Peritonitis-Sepsis Model. BALB/c female mice ($n = 6$ per group) were infected with 0.5 mL of an MRSA T144 suspension (5×10^8 CFU per mouse) via intraperitoneal injection. At 1 h post-infection, all mice were treated with either PBS (vehicle), **4j** (5 mg/kg), or **4n** (5 mg/kg) at single intraperitoneal doses. Vancomycin at a single dose of 20 mg/kg was used as a positive control. The survival rates of treated mice were recorded during a period of 48 h. All animal experiments were

approved by the Institutional Animal Care and Use Committee of the China Agricultural University.

Expression and Purification of Recombinant MRSA Pyruvate Kinase (PK). The *pyk* gene was amplified by PCR using genomic DNA of *S. aureus* ATCC700699 as the template and employing the oligonucleotides (restriction sites are underlined) 5'His6N_NcoI-*pyk* (5'-TTTTCCTGGATGTCATCATCATCATCATCATGATA-GAAAACTAAATTGTA-3') introducing an N-terminal His6 tag and 3'-XhoI-*pyk* (5'-TTTTCCTCGAGTTATAGTACGTTTGCA-TATCCT-3'). The resulting PCR fragment was cloned into the expression vector pET28a(+) using the restriction sites NcoI and XhoI, and the resulting plasmid pET28a(kan)::His6N-*pyk* was used to express recombinant PK in *E. coli* NiCo21(DE3). Cells were grown in a YT medium (16 g/L tryptone, 10 g/L yeast extract, and 5 g/L NaCl) at 37 °C with shaking at 180 rpm until an OD_{600 nm} of approximately 0.6 was reached, then induced by addition of 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG), and incubated at 20 °C and 50 rpm for further 24 h. Subsequently, cells were harvested by centrifugation, resuspended in lysis buffer (50 mM TRIS-HCl, (pH 7.5), 100 mM KCl, 10 mM MgCl₂, 200 mM NaCl, 10 mM imidazole, 0.5% (v/v) Triton-X100, 0.5 mM (v/v) DTT, and 10% (v/v) glycerol) containing a 1 mg/mL lysozyme and a 10 μ g/mL protease inhibitor and lysed by sonicating on ice for 1.5 min. Cell lysates were cleared by centrifuging for 30 min at 16,000g and 4 °C. A Ni-NTA agarose suspension (3 mL) was washed twice with 3 mL of lysis buffer, mixed with the cleared lysate for batch binding, and rotated gently on ice for 1 h to allow histidine-tagged proteins to bind. A Pierce disposable column with a porous polyethylene disc was washed with lysis buffer followed by adding the Ni-NTA-lysate suspension to the column. The column resin was washed with 10 column volumes of lysis buffer containing 30 mM imidazole. His-tagged proteins were then eluted with lysis buffer containing 300 mM imidazole and concentrated with a Vivaspin 20 centrifugal concentrator (MWCO 10 kDa). Finally, the protein sample was desalted by using the PD MidiTrap G-10 columns employing exchange buffer (30 mM TRIS-HCl (pH 7.5), 25 mM KCl, 5 mM MgCl₂, and 10% (v/v) glycerol). SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining were used to evaluate the protein's purity, while its concentration was determined by BCA assay.

Measuring of Pyruvate Kinase Activity. To evaluate the potential of bisindoles **4** to impair enzymatic activity of MRSA PK, activities of PKs from rabbit muscle and from MRSA were measured via a lactate-dehydrogenase (LDH)-linked assay by measuring the consumption of NADH essentially as described previously.³³ PK activity was assayed using black, flat bottom μ CLEAR 96 well plates in a total volume of 200 μ L in a reaction mixture containing 60 mM HEPES (pH 7.5), 67 mM KCl, 6.7 mM MgSO₄, 1.5 mM ADP, 1.5 mM ribose-5-phosphate, 1.5 mM phosphoenolpyruvate, 0.22 mM NADH, and 5 U/mL LDH. Bisindoles **4** were added at various concentrations as indicated. The reaction was initiated by adding 5 μ g of respective PKs, incubated at 30 °C, and quantified by measuring absorbance at 340 nm for 5 min using an Infinite M Plex Tecan reader. Inhibitor concentrations higher than 12.5 μ M were not tested as the tested compounds **4** interfered with absorbance at 340 nm under these conditions. PK activity, expressed as initial velocity in μ mol/min/mg protein, is defined as the amount of PK that catalyzes the formation of 1 μ mol of product per minute and was calculated as: v (μ mol/min/mg protein) = $[(\Delta E/\text{min})/(e \times d)/\text{mL}]/\text{mg protein}$, where ΔE = change of extinction per minute; mL = reaction volume (0.2 mL); e = extinction coefficient of NADH (6.22 mM⁻¹ cm⁻¹); d = path of light (0.64 cm). The resulting curve was fitted by nonlinear regression to the allosteric sigmoidal kinetic model of GraphPad Prism 5 software.

Fluorescence Microscopy. Fluorescence microscopy was done as described in a previous study with some modifications.⁴¹ Briefly, cells of methicillin-susceptible *S. aureus* strain ATCC 29213 grown and adjusted to a cell density of 0.3 OD_{600 nm} were incubated with 5-fold MIC of **4n** in an MH medium for 30 min at 37 °C and 150 rpm. Upon incubation, cells were pelleted (3000 g for 15 min), washed with PBS, and then incubated with PI (7.5 μ g/mL in water) in the dark for 15 min at 0–4 °C. PI-incubated cells were then washed three

times with PBS to remove excess PI and further incubated with DAPI (10 $\mu\text{g/mL}$ in water) in the dark for 15 min at 0–4 °C. After three washing steps of removing excess DAPI, cells were fixed by resuspending pellets in 4% paraformaldehyde (pH 6.9) and incubated at room temperature for 20 min. Fixed bacterial pellets were washed, suspended in PBS, and examined via the oil-immersion objective (40 $\times$) of a Nikon Eclipse TS100 fluorescence microscope.

Propidium Iodide Internalization Assay. An actively growing *S. aureus* culture was washed, harvested, and resuspended in PBS supplemented with 1% glucose. Suspended culture was adjusted to OD_{600 nm} = 0.4 and then incubated with 7.5 $\mu\text{g/mL}$ PI for 10 min at 37 °C. PI-incubated cells (100 μL) were dispensed in a black walled 96 well plate, and baseline fluorescence (excitation, 535 nm; emission, 617 nm) was established for 10 min. Cells were then treated with 100 μL of 10-fold MIC of **4n** (final concentration = 5-fold MIC). Lysostaphin was used as a positive control, while linezolid, daptomycin, moxifloxacin, and DMSO served as negative controls. Using a Tecan Infinite M Plex plate reader, fluorescence intensity of the PI dye was measured every 5 min for 2 h at 535 nm excitation wavelength and 617 nm emission wavelength.⁴²

ATP and GFP Release. To determine the leakage of adenosine triphosphate (ATP) and green fluorescent protein (GFP) at intervals upon compound treatment, cells of recombinant *S. aureus* strain ATCC 700699 expressing GFP were washed and suspended in PBS supplemented with 1% glucose and then adjusted to 0.5 OD_{600 nm}. The adjusted culture was incubated with 5-fold MIC of **4n** at 37 °C and 150 rpm. At different time points (0, 0.5, 1, 3, and 6 h), samples were collected and centrifuged at 4 °C, 10,000g for 10 min. The ATP present in the cell-free supernatants was then quantified using a BacTiter-Glo assay kit (Promega, Madison, WI, USA). For GFP, 100 μL of cell-free supernatants was dispensed in triplicates in a black walled 96 well plate, and GFP fluorescence (excitation wavelength, 475 nm; emission wavelength, 509 nm) was measured using a Tecan Infinite M Plex plate reader. Lysostaphin served as a positive control, while DMSO and moxifloxacin were negative controls for both experiments.⁴²

Assessment of Cell Lysis. Cells of *S. aureus* strain ATCC 700699 cultivated in a Mueller Hinton medium until the mid-exponential phase were adjusted to OD_{600 nm} ~ 0.3 and incubated at 37 °C with 8-fold MIC of compounds for 2 h. At different time points (0, 30, 60, 90, and 120 min), turbidity of individual cultures was monitored by measuring the optical densities of samples collected from each bacterial culture at 600 nm using a photometer. Decrease in turbidity is indicative of cell lysis.

Isolation of Genomic DNA. For isolation of the genomic DNA, individual resistant mutants were cultivated overnight. Bacterial suspensions were centrifuged at 4000 rpm for 5 min. Afterward, bacterial cell pellets were suspended in a lysostaphin-GTE solution (200 $\mu\text{g/mL}$ lysostaphin in 25 mM Tris-Cl, pH 8.0; 10 mM EDTA; 50 mM glucose) and incubated at 37 °C for 30 min. After addition of 5 μL of proteinase K (10 mg/mL), the suspensions were incubated at 65 °C for 1 h. Subsequently, an equal volume of 24:1 chloroform/isoamyl alcohol was added. The mixture was shaken vigorously and spun at 4000 rpm for 10 min. The aqueous layer was transferred in a fresh tube, and the extraction step was repeated. DNA was precipitated by addition of 60 μL of NaCl (5 M) and 1 mL of ethanol. After centrifugation and aspiration of the supernatant, the DNA pellet was air-dried for 15 min. Finally, the DNA was dissolved in TE buffer containing RNase (10 mM Tris, 0.1 mM EDTA, and pH 7.4).

Whole Genome Sequencing. Genomic DNA samples of four MRSA mutants used for genome sequencing were quantified by photometric measurement using a NanoDrop One device (Thermo Fisher Scientific, Inc.), and quality was measured by capillary electrophoresis using the Fragment Analyzer and the "High Sensitivity Genomic DNA Assay" (Agilent Technologies, Inc.). Library preparation was performed according to manufacturer's protocol using a TruePrep DNA Library Prep Kit V2 for Illumina (1 ng) (Vazyme Biotech Co., Ltd.). Libraries were normalized to 4 nM and pooled and subsequently sequenced on a MiSeq system (Illumina,

Inc.) with a read setup of 2x251 bp. Base calling was performed using Casava software, v1.8. The reads were assembled using a comparative genome assembly method and compared with the parent methicillin-resistant *S. aureus* genome (strain ATCC 700699 = Mu50; GenBank accession GCA_000009665.1). The mean depth of coverage ranged from 69 $\times$ to 114 $\times$. Sequencing was performed at the Biological and Medical Research Center, Genomics and Transcriptomics Laboratory, Heinrich Heine University Düsseldorf, Düsseldorf, Germany.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c00826>.

MICs of compounds **4** against all tested bacteria strains (Table S1); cytotoxicity data of compounds **4j**, **4n**, and **4q** against the human cell lines HEK293, HepG2, and CLS-54 (Table S2); SNPs present in MRSA resistant mutants (Table S3); MICs of compounds of different classes against MRSA resistant mutants and wild-type (Table S4); dose–response curve of compound **4r** against MRSA (Figure S1); solubility assessment of compounds **4c**, **4d**, **4j**, **4n**, and **4r** (Figure S2); absorption maxima of compounds **4c**, **4d**, **4j**, **4n**, and **4r** (Figure S3); hemolytic activity of **4j** and **4n** on sheep red blood cells (Figure S4); stability evaluation of compound **4c** (Figure S5); dose–response curves for **4d**, **4n**, **4q**, and **4s** against MRSA LAC WT and LAC $\Delta\text{pyk::Erm}^R$ in minimal medium M9 (Figure S6); studies describing optimization of reaction conditions (Scheme S1 and Table S5); analytical data of compounds **1a**, **4a–4f**, **4j**, **4l–4n**, **4p–4s**, **4u**, and **4v** including ¹H and ¹³C NMR spectra (Figures S7–S40) (PDF)

Molecular formula strings (CSV)

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Author Contributions

G.A.S. synthesized compounds **1a**, **4a–4f**, **4n**, and **4r–4s**. B.O.A.T. synthesized compounds **1a**, **4g–4i**, **4k**, **4o**, and **4t**. D.D. optimized the protocol for the pseudo three-component synthesis of heteroaryl bridged bisindoles and synthesized compounds **4j**, **4n**, **4p**, and **4q**. M.K. synthesized compounds **4l**, **4m**, **4u**, and **4v** and determined the solubility and stability of compounds. N.R. and E.T.A. conducted experiments involving MRSA and other bacteria. L.W. performed cell culture cytotoxicity profiling. E.T.A. measured hemolytic activity. K.W. purified pyruvate kinase and characterized the *in vitro* activity. S.C. and K.Z. performed mouse experiments. T.R.I. analyzed the whole genome sequencing data. R.K., T.J.J.M., and K.Z. designed experiments and analyzed data. N.R., G.A.S., D.D., E.T.A., T.J.J.M., and R.K. wrote the manuscript with contributions and edits from all authors.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

MIC, minimal inhibitory concentration; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *Staphylococcus aureus*; VISA, vancomycin intermediate-resistant *Staphylococcus aureus*; CFU, colony forming units; GFP, green fluorescent protein; OD, optical density; PK, pyruvate kinase; SAR, structure–activity relationship; Ni-NTA, nickel-nitrilotriacetic acid; MHB, Mueller Hinton broth; FBS, fetal bovine

serum; RBC, red blood cell; COL, colistin; PI, propidium iodide; DAPI, 4',6-diamidino-2-phenylindole

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Supplementary Information

Nature-inspired (Di)Azine-bridged Bisindole Alkaloids with Potent Antibacterial *In Vitro* and *In Vivo* Efficacy Against Methicillin-resistant *Staphylococcus aureus*

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Contents of Supplementary Information:

- MIC of compounds **4** against all tested bacteria strains (**Table S1**)
- Cytotoxicity data of compounds **4j**, **4n** and **4q** against the human cell lines HEK293, HepG2 and CLS-54 (**Table S2**)
- SNPs present in MRSA resistant mutants (**Table S3**)
- MICs of compounds of different classes against MRSA resistant mutants and wildtype (**Table S4**);
- Dose-response curve of compound **4r** against MRSA (**Figure S1**)
- Solubility assessment of compounds **4c**, **4d**, **4j**, **4n** and **4r** (**Figure S2**)
- Absorption maxima of compounds **4c**, **4d**, **4j**, **4n** and **4r** (**Figure S3**)
- Hemolytic activity of **4j** and **4n** on sheep red blood cells (**Figure S4**)
- Stability evaluation of compound **4c** (**Figure S5**)
- Dose-response curves for **4d**, **4n**, **4q** and **4s** against MRSA LAC WT and LAC $\Delta pyk::Erm^R$ in minimal medium M9 (**Figure S6**)
- Studies describing optimization of reaction conditions (**Scheme S1**, **Table S5**)
- Analytical data of compounds **1a**, **4a-4f**, **4j**, **4l-4n**, **4p-4s**, **4u** and **4v** including ¹H and ¹³C NMR-spectra (**Figures S7-S40**).

S1

Table S1. Minimal inhibitory concentrations (MIC) of all synthesized compounds against *Mycobacterium tuberculosis* (*M. tb.* H37Rv), *Staphylococcus aureus* (*S. aureus*, ATCC 25923) and Methicillin-resistant *S. aureus* (MRSA, ATCC 700699), *Enterococcus faecalis* (*E. faecalis*, ATCC 29212) and Vancomycin-resistant *E. faecalis* ATCC.51299), *Enterococcus faecium* (*E. faecium*, ATCC 35667) and Vancomycin-resistant *E. faecium* (VRE, ATCC 700221), and multidrug-resistant *Acinetobacter baumannii* (*A. baumannii*, ATCC BAA-1605). Maximal tested concentration was 100 μ M. MIC > 100 μ M indicates that the compound was inactive. *, 100 μ M compound **4n** reduced growth of *M. tb.* by appr. 75%.

No.	<i>M. tb.</i>	<i>S. aureus</i>		MIC (μ M) <i>E. faecalis</i>		<i>E. faecium</i>		<i>A. baumannii</i>
	H37Rv	ATCC 25923	ATCC 700699	ATCC 29212	ATCC 51299	ATCC 35667	ATCC 700221	ATCC BAA-1605
1a	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
1b	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
4a	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
4b	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
4c	> 100	> 100	0.39	> 100	> 100	> 100	> 100	> 100
4d	> 100	1.56	1.56	> 100	> 100	> 100	> 100	> 100
4e	> 100	100	25	100	> 100	50	50	> 100
4f	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
4g	> 100	> 100	> 100	12.5	25	> 100	> 100	> 100
4h	50	> 100	50	50	100	100	50	> 100
4i	> 100	100	12.5	100	100	50	50	> 100
4j	50	6.25	0.39	3.13	12.5	1.56	1.56	> 100
4k	50	100	12.5	50	100	100	100	> 100
4n	> 100*	3.13	0.20	12.5	6.25	6.25	1.56	> 100
4o	> 100	> 100	> 100	100	100	> 100	> 100	> 100
4q	> 100*	3.13	0.39	3.13	3.13	1.56	1.56	> 100
4r	> 100	> 100	0.39	> 100	> 100	> 100	> 100	> 100
4s	100	6.25	0.78	0,20	> 100	> 100	> 100	> 100
4t	> 100	> 100	> 100	100	100	> 100	> 100	> 100

Table S2. Cytotoxicity data of compounds **4j**, **4n** and **4q** against the human cell lines HEK293, HepG2 and CLS-54.

Cell line	IC ₅₀ [μM]		
	4j	4n	4q
HEK293 (CLS 300192)	12.5	12.5	25
HepG2 (CLS 300198)	25	25	25
CLS-54 (CLS 300227)	25	25	25

Table S3. Single nucleotide polymorphisms (SNPs) present in spontaneous mutants of *S. aureus* MRSA strain ATCC 700699 resistant to compound **4j** after serial enrichment with escalating subinhibitory substance concentrations. '*' means stop codon (nonsense mutation).

SNPs	Function	4j- <i>S. aureus</i> ATCC 700699 resistant mutants			
		J2	J3	J5	J9
SAV_RS00605	ATPase			+a in aa 814/1051	
SAV_RS01095	PTS glucose EIICBA component	G674D	G674D	G674D	G674D
Intergenic	-20 bp upstream of SAV_RS03295 (transcriptional regulator)	g>c	g>c	g>c	g>c
SAV_RS03635	Sensor histidine kinase	+a	+a	+a	+a
Intergenic	29 bp downstream of SAV_RS05465 (AI-2E transporter)	t>a	t>a	t>a	t>a
SAV_RS05465	AI-2E transporter	V244F	V244F	V244F	V244F
SAV_RS07330	Phosphatidylglycerol lysyltransferase	W813*	W813*	W813*	W813*
SAV_RS08525	Elongation factor 4	R6L	R6L	R6L	R6L
Intergenic	Between SAV_RS09365 (ftsK and 9370 (tRNA-binding protein)	c>a	c>a	c>a	c>a
SAV_RS13880	CHAP domain-containing protein			-38bp del (C-term.)	
SAV-10625 –					
SAV_RS10945	43053 bp deletion (63 genes)	43kb del	43kb del	43kb del	43kb del

Table S4. Minimum inhibitory concentrations (MICs) of compounds of different classes against **4j**-resistant *S. aureus* ATCC 700699 mutants and wild-type in μM .

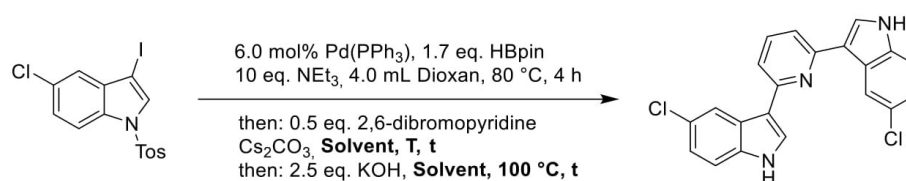
<i>S. aureus</i> ATCC700699	MIC [μM]								
	Vancomycin	Moxifloxacin	Rifampicin	Linezolid	Daptomycin	4j	4n	Bacitracin	Ramoplanin
WT	25	6.25	> 100	3.13	100	0.78	1.56	50	1.56
J2	3.13	6.25	> 100	3.13	25	1.56	3.13	50	0.39
J3	6.25	6.25	> 100	3.13 - 6.25	25	3.13	3.13	50	0.39
J5	6.25	6.25	> 100	3.13	25	1.56	3.13	50	0.39
J9	3.13	6.25	> 100	3.13 - 6.25	25	3.13	3.13	50	0.39

Synthetic derivatives

NMR spectra were recorded using Avance DRX 500, Avance III – 300 and Avance III - 600 (Bruker, Karlsruhe). Chemical shifts are reported in parts per million and the resonance of the solvent was locked as internal standard (DMSO- d_6 : ^1H δ = 2.50, ^{13}C δ = 39.52). Mass spectra were obtained using Varian MAT 311 A, Finnigan MAT 8200, Finnigan TSQ 7000, UHR-QTOF maXis 4G (Bruker Daltonics) and GCMS-QP20105 (Shimadzu). Combustion analyses were carried out on Perkin Elmer Series II Analyser 2400 and Elementarvario MICRO CUBE. The melting points were measured on a Büchi Melting Point B-540. The starting materials are commercially available or prepared according to literature syntheses.

Optimization study

For minimizing side products and impurities, a short optimization study with X and Y in the model reaction was performed (**Scheme S1**).



Scheme S1: Optimization study for 2,6-dihalopyridines.

For the Masuda borylation, we used our previously optimized conditions. Just the catalyst load was raised to 6.0 mol% as a consequence of two subsequent Suzuki couplings in the sequence (for details, see **Table S3**). For Suzuki coupling a protic solvent was crucial to scavenge the excess of pinacolyl borane. Thereafter, dihalide **Y** and cesium carbonate were added. The reaction time for Suzuki coupling were set between 18 and 42 h. Furthermore, the influence of reaction temperatures in a range between 60 and 100 °C was studied. Higher temperatures showed enhanced the oxidation of the phosphane ligand. Simultaneously, dihalide **Y** is partially consumed by nucleophilic aromatic substitution of the nucleophilic methanol/carbonate solution. If alcohol was omitted as a solvent during Suzuki coupling, it had to be added for the terminal deprotection step. If bromides instead of iodides were used the initially tested conditions failed to form the desired bisindoles (Entry 1). High temperatures during Suzuki coupling favored many side reactions like nucleophilic aromatic substitution, dehalogenation and homocoupling of the boronic acid ester. Lower temperatures and dilution of the reaction mixture reduced side reactions and gave a good yield of the desired product (Entry 3). Unfortunately, after the first Suzuki coupling, nucleophilic aromatic substitution apparently is a serious side reaction. Due to the lower reactivity of bromides this side product could still not be completely eliminated. Also milder conditions during Suzuki coupling we have previously reported for the synthesis of meriolins failed to generate good yields (Entry 4, 5). Applying H₂O as cosolvent during Suzuki coupling finally gave good yields of the desired product.

Table S5. Optimization study of MBSC sequence for the synthesis of 2,6-pyridin-linked bisindoles.

(SC: Suzuki coupling, DP: Deprotection)

Entry	Solvent SC [mL]	Temperature SC [°C]	Duration SC [h]	Solvent DP [mL]	Temperature DP [°C]	Duration DP [h]	Product [%]
1	5.0 mL MeOH	100	22	-	100	4	0 ^a
2	3.0 mL MeOH	80	42	-	100	4	0
3	7.0 mL MeOH	60	18	-	100	4	84 % ^b
4	9.0 mL DME 1.0 mL H ₂ O	100	42	-	-	-	30 % ^c
5	9.0 mL DME 1.0 mL H ₂ O	100	22	-	-	-	32 % ^d
6	3.0 mL H ₂ O	60	22	4.0 mL MeOH	100	4	48 %
7	3.0 mL H ₂ O	80	24	4.0 mL MeOH	100	4	69 %
8	3.0 mL H ₂ O	100	24	4.0 mL MeOH	100	4	58 % ^e
9	3.0 mL H₂O	60	42	4.0 mL MeOH	100	5	77 %
10	3.0 mL H ₂ O	60	42	4.0 mL MeOH	100	8	74 % ^e

^a5.0 mL 1,4-Dioxan for Masuda borylation. ^bImpured by methoxy-substituted meridianin. ^cNo deprotection in one-pot fashion. Tosylated product was isolated. ^dNo deprotection in one-pot fashion. Tosylated product was isolated. Impured by monocoupled byproduct. ^eHigh amounts of triphenylphosphane oxide detected via mass spectrometry.

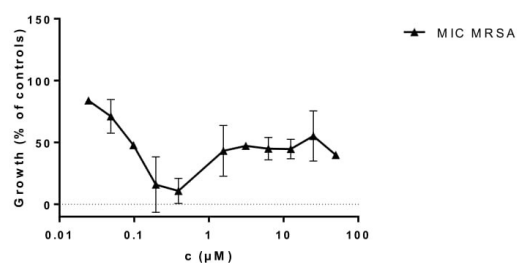
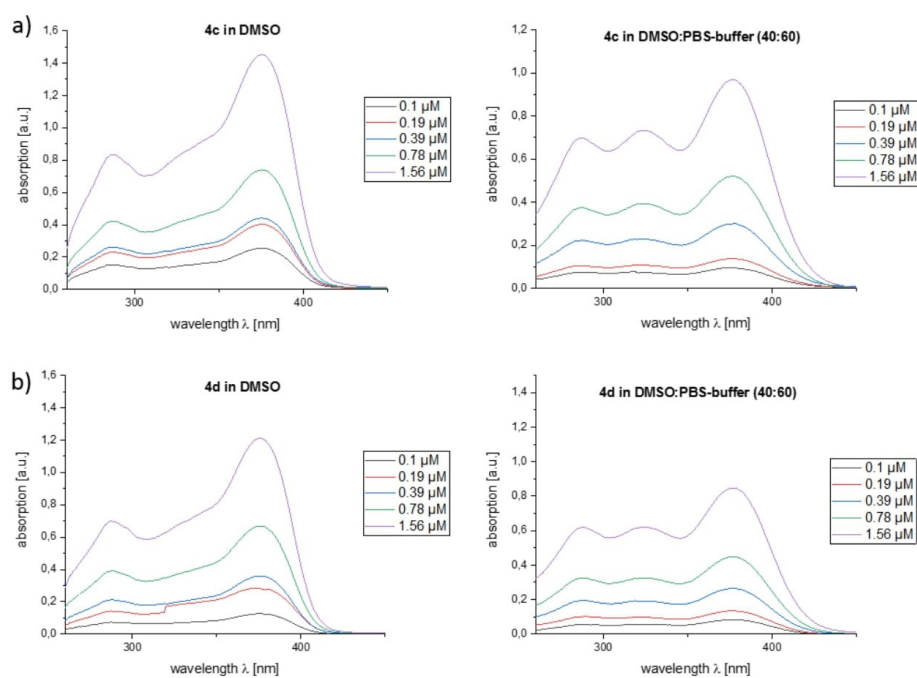


Figure S1. Dose-response curve of compound **4r** against MRSA. Data are means of duplicates $\pm$ SD. Growth was quantified using BacTiterGlo assay.



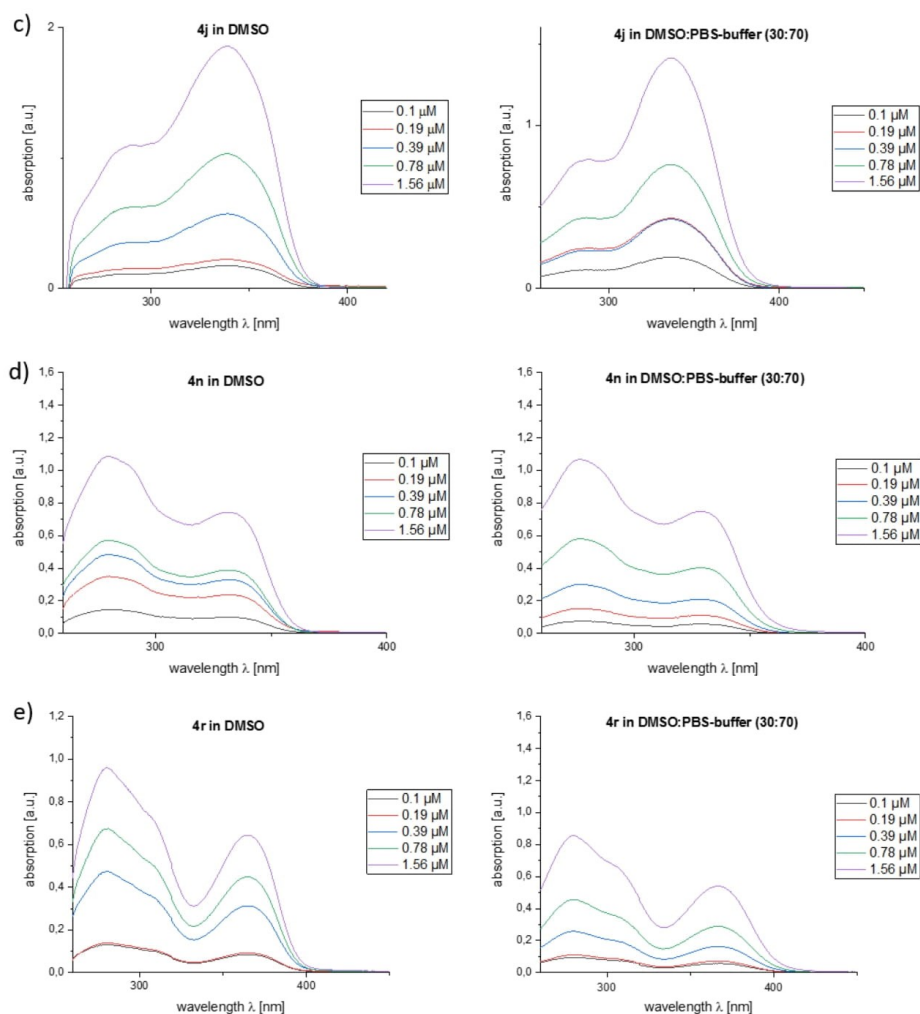


Figure S2. Solubility assessment of compounds **4c**, **4d**, **4j**, **4n** and **4r**. All tested compounds were insoluble in pure PBS-buffer (pH = 7.77) but could be solved in mixtures of DMSO and PBS-buffer. Compounds **4c** and **4d** were solved in a ratio of 40:60 DMSO:PBS-buffer (pH = 7.77) and compounds **4r**, **4j** and **4n** were solved in a ratio of 30:70 DMSO:PBS-buffer (pH = 7.77). Varying concentrations of compound (a) **4c**, (b) **4d** (c) **4j**, (d) **4n** and (e) **4r** were dissolved in DMSO and in DMSO:PBS buffer and their respective absorption spectra were monitored at 298 K.

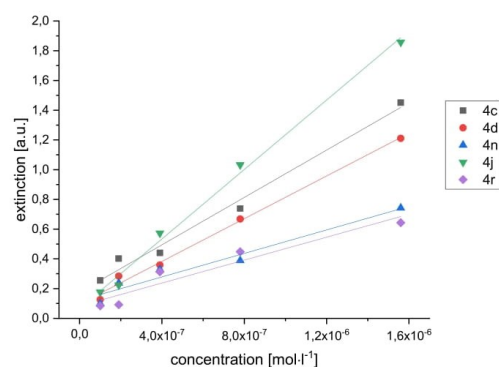


Figure S3. Absorption maxima of compounds **4c**, **4d**, **4j** **4n** and **4r** dissolved in DMSO plotted against the concentration.

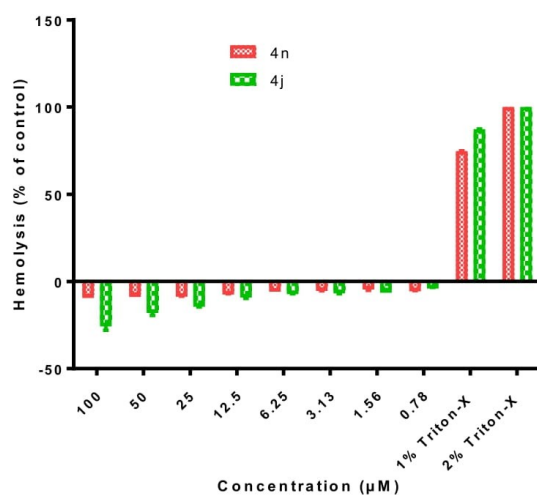


Figure S4. Lack of hemolytic activity of **4j** and **4n** on sheep red blood cells. Hemolysis was quantified by measuring hemoglobin release photospectrometrically at 540 nm. Data are means of duplicates $\pm$ SEM.

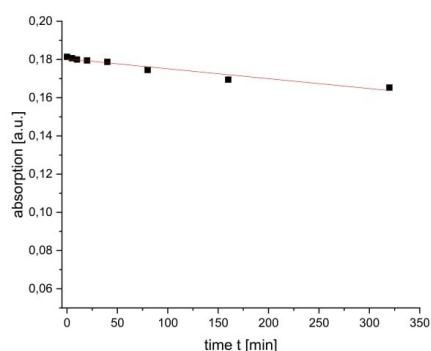


Figure S5. Stability evaluation of compound **4c**. 0.19 μM of **4c** was dissolved in DMSO:PBS (40:60) and absorption of the longest wavelength maximum was monitored over 320 min. A slight depression of the absorption of the longest wavelength maximum can be seen over a time of 320 min. This is likely caused by the formation of aggregates in solution, even though the concentration is rather low. This observation is in agreement with the solubility study of selected bisindoles.

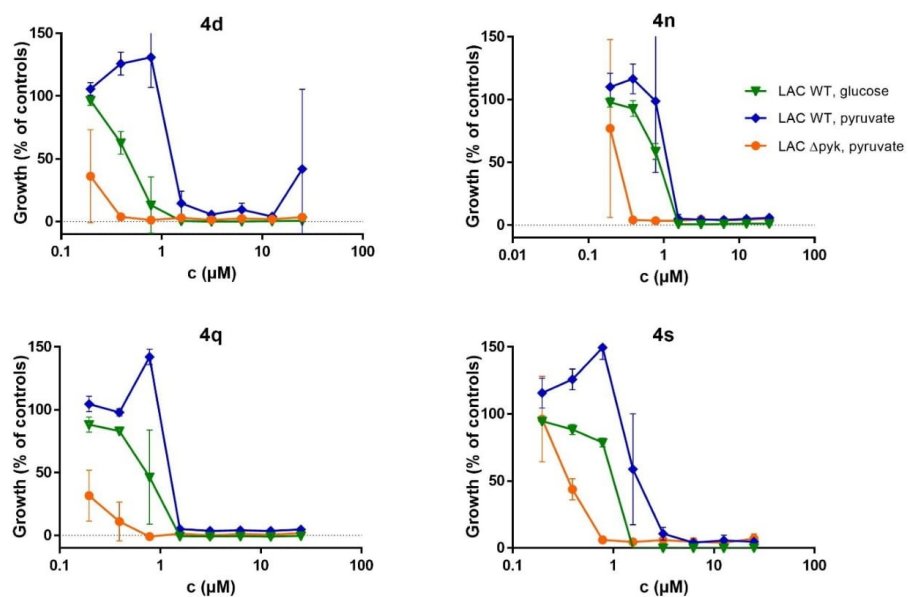


Figure S6. Antibacterial activity of **4d**, **4n**, **4q** and **4s** against MRSA LAC WT and LAC $\Delta\text{pyk}::\text{Erm}^{\text{R}}$ in minimal medium M9 with glucose ($\blacktriangledown$, $\blacklozenge$) or pyruvate ($\bullet$) as single carbon source. Data are means of duplicates $\pm$ SD. Growth was quantified using resazurin assay.

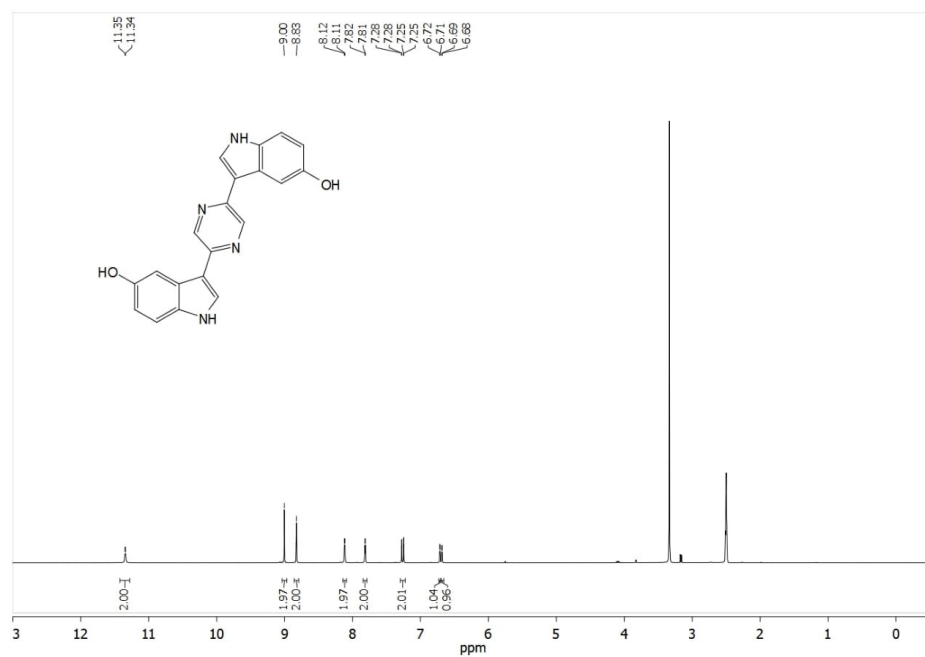


Figure S7. ¹H NMR of **1a** in DMSO-d₆ at 296 K (δ in ppm).

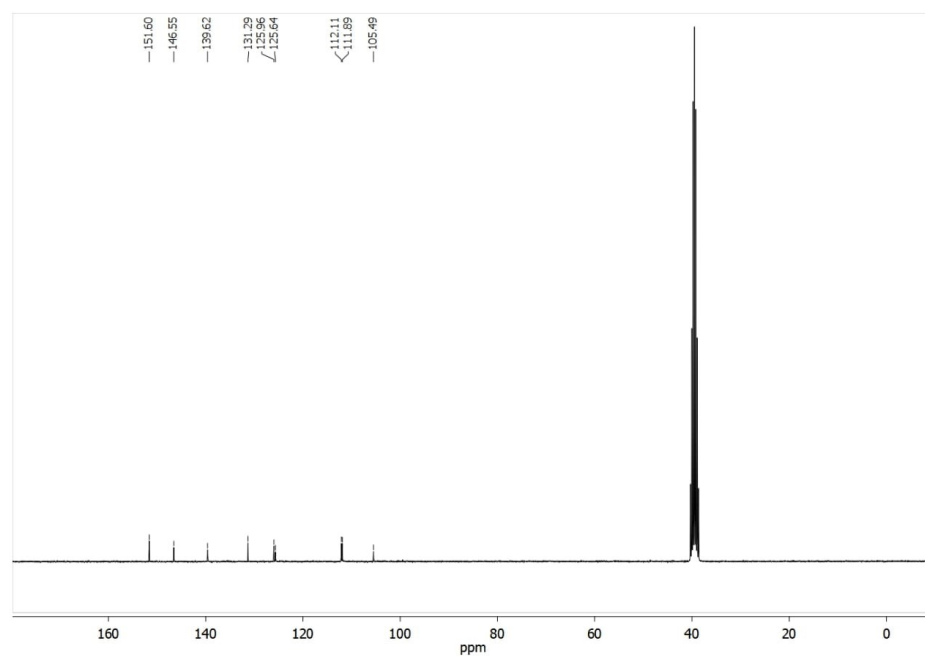


Figure S8. ¹³C NMR of **1a** in DMSO-d₆ at 295 K (δ in ppm).

S11

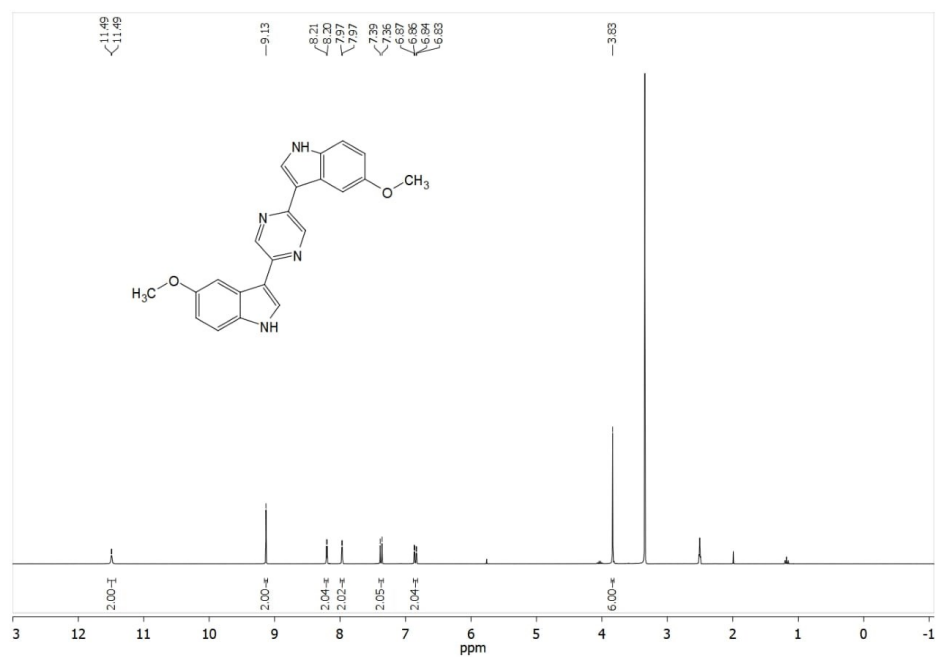


Figure S9. ¹H NMR of **4a** in DMSO-d₆ at 296 K (δ in ppm).

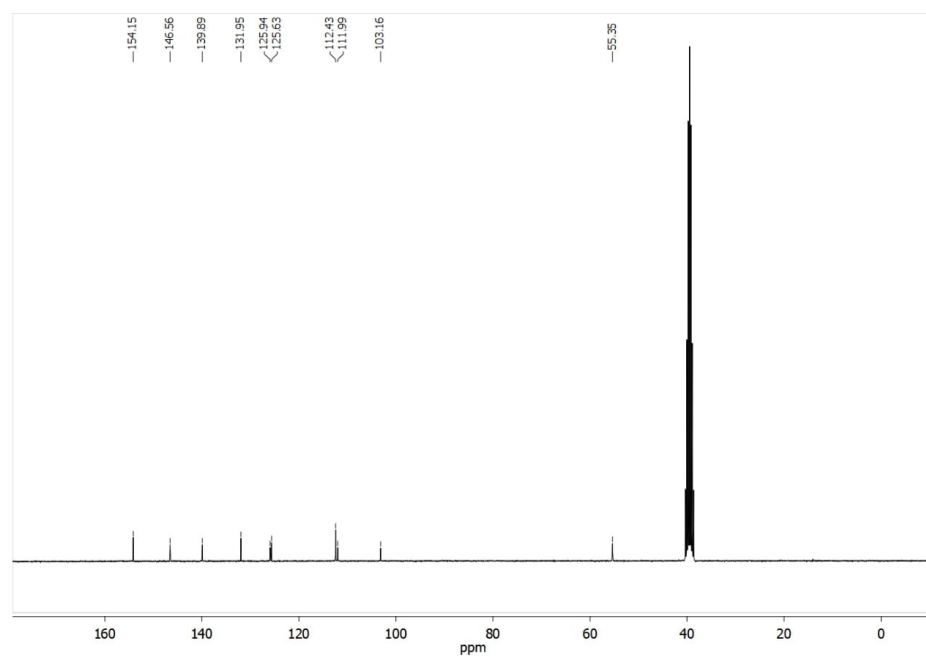


Figure S10. ¹³C NMR of **4a** in DMSO-d₆ at 295 K (δ in ppm).

S12

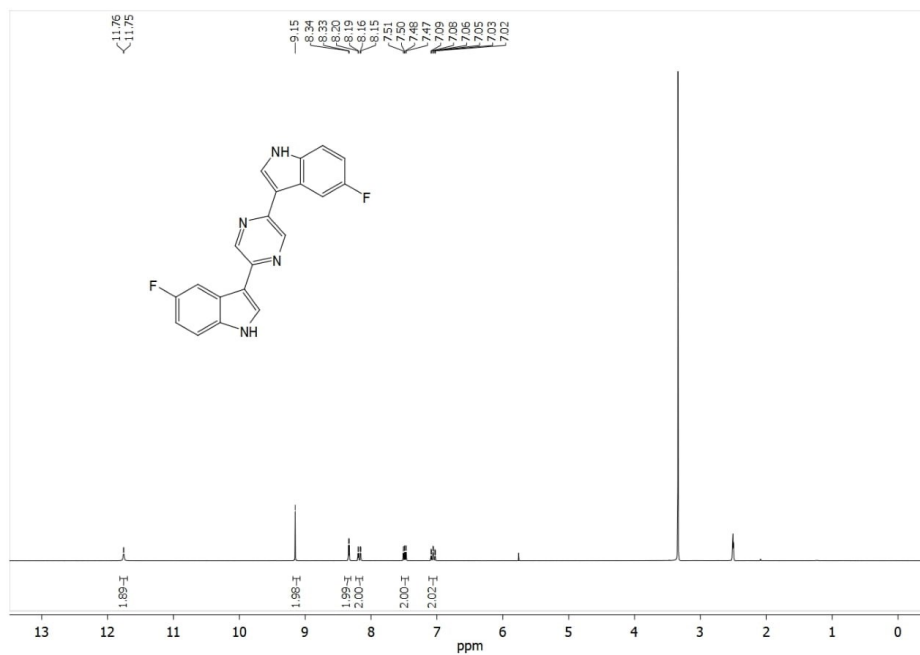


Figure S11. ¹H NMR of **4b** in DMSO-d₆ at 296 K (δ in ppm).

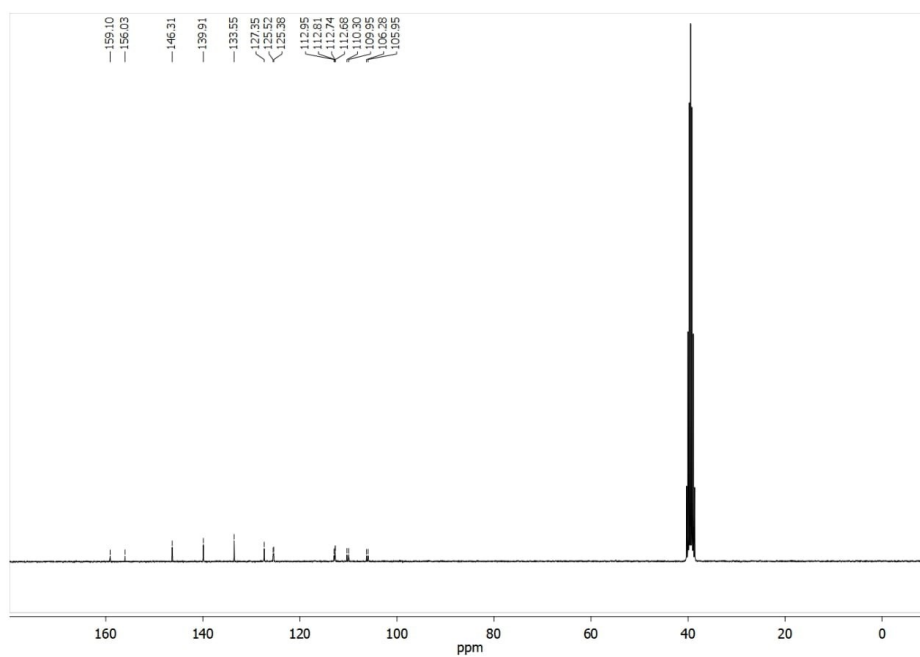


Figure S12. ¹³C NMR of **4b** in DMSO-d₆ at 295 K (δ in ppm).

S13

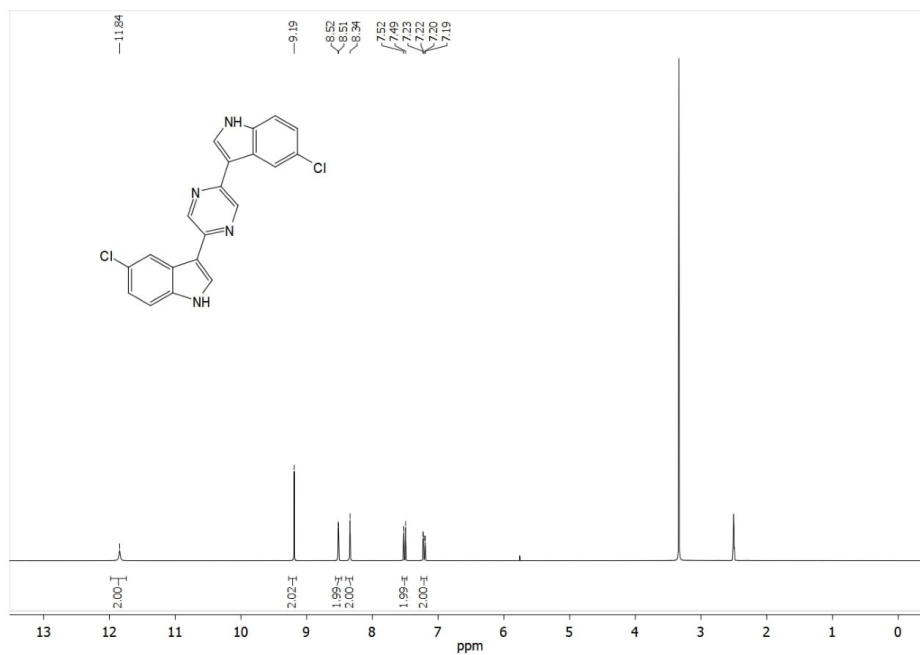


Figure S13. ¹H NMR of **4c** in DMSO-d₆ at 296 K (δ in ppm).

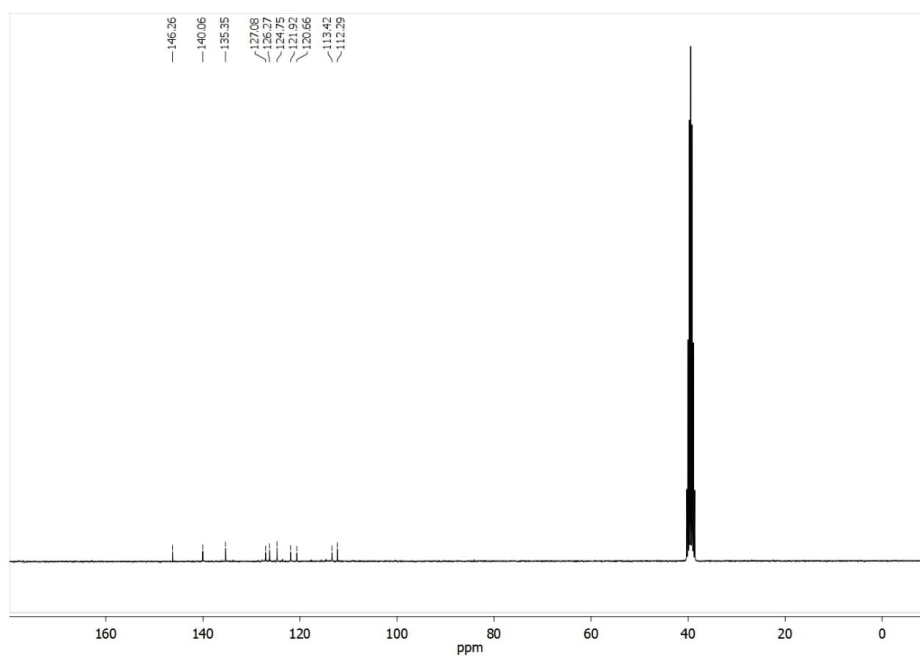


Figure S14. ¹³C NMR of **4c** in DMSO-d₆ at 295 K (δ in ppm).

S14

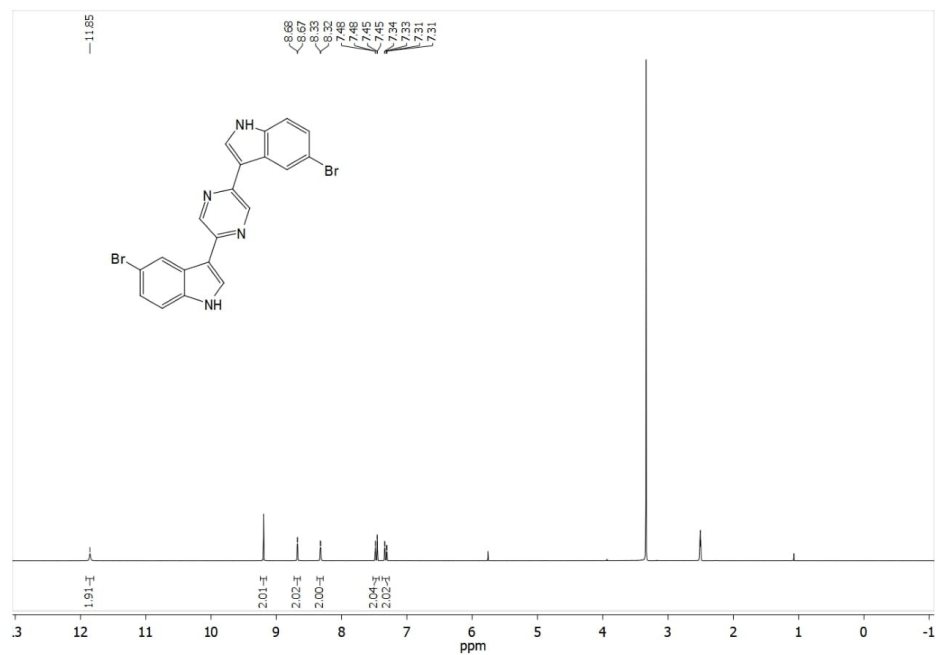


Figure S15. ^1H NMR of **4d** in DMSO-d_6 at 296 K (δ in ppm).

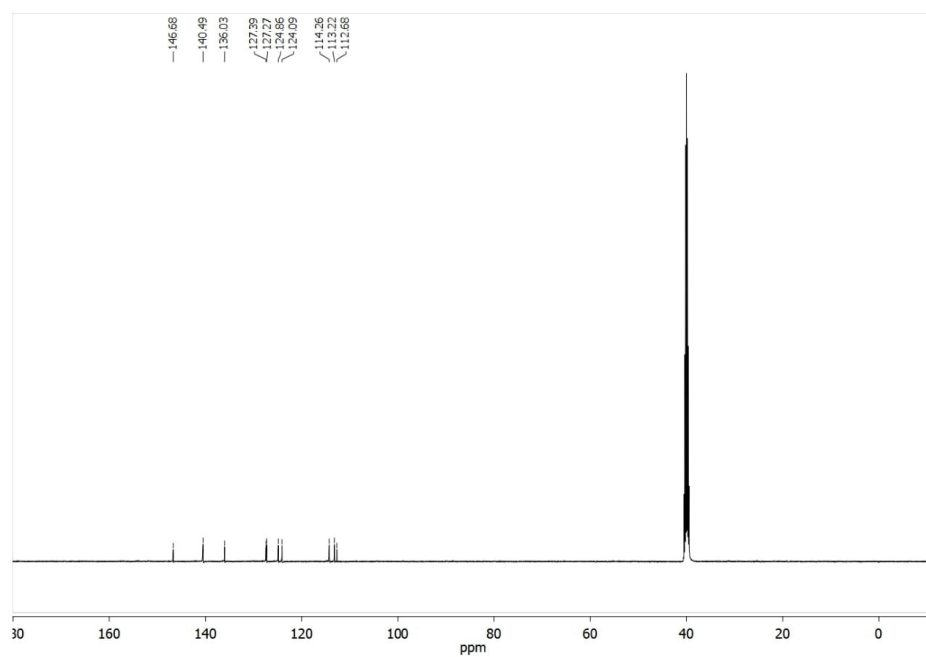


Figure S16. ^{13}C NMR of **4d** in DMSO-d_6 at 295 K (δ in ppm).

S15

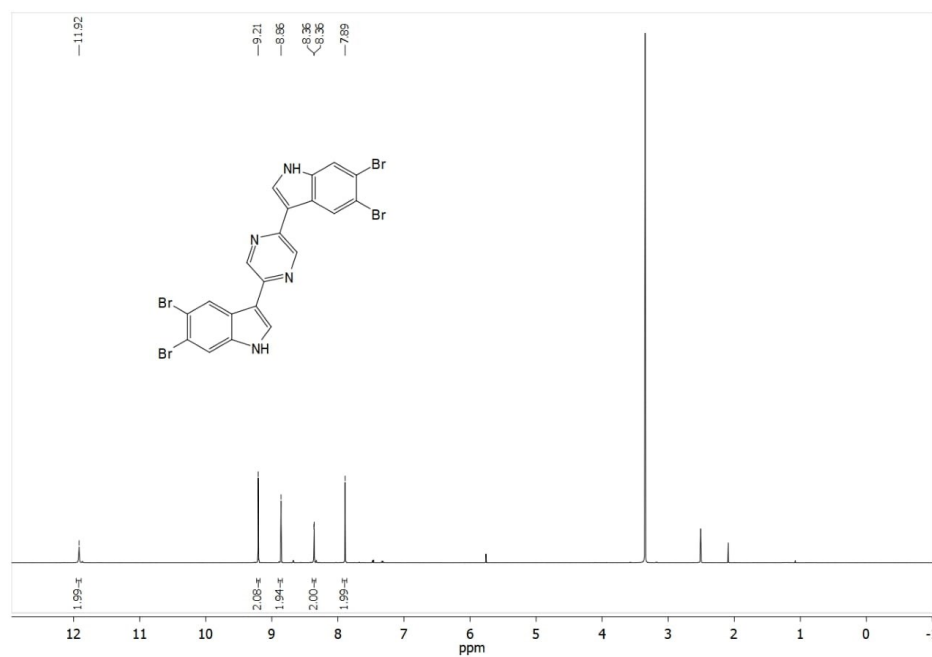


Figure S17. ¹H NMR of **4e** in DMSO-d₆ at 296 K (δ in ppm).

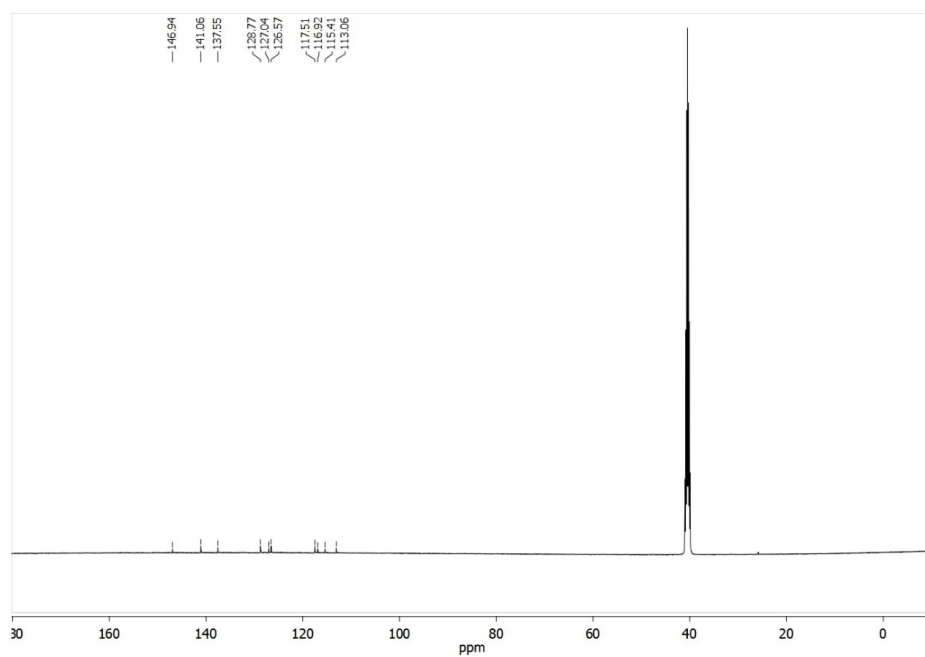


Figure S18. ¹³C NMR of **4e** in DMSO-d₆ at 295 K (δ in ppm).

S16

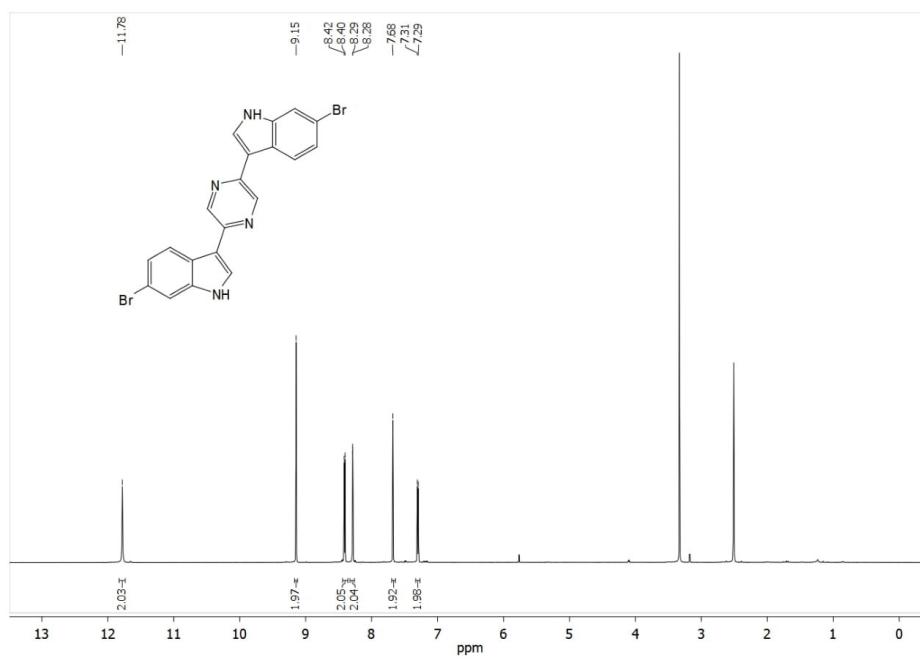


Figure S19. ¹H NMR of **4f** in DMSO-d₆ at 296 K (δ in ppm).

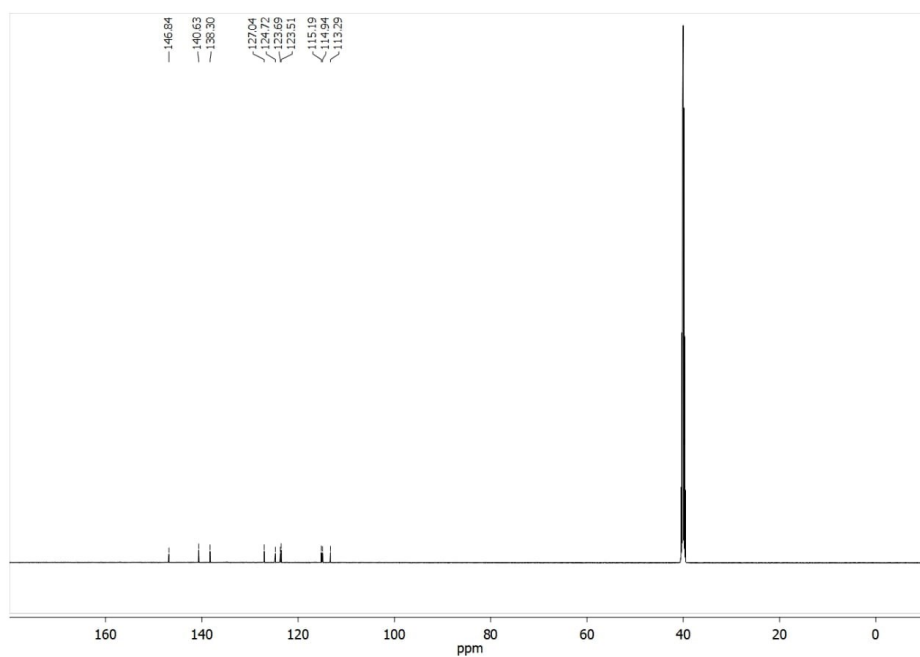


Figure S20. ¹³C NMR of **4f** in DMSO-d₆ at 295 K (δ in ppm).

S17

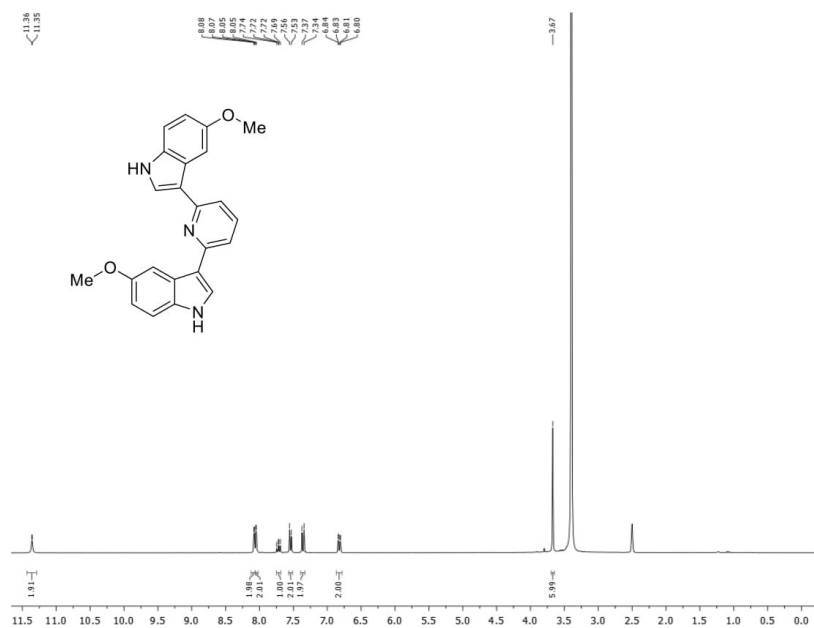


Figure S23. ¹H-NMR of **4I** in DMSO-d₆ at 293 K (δ in ppm).

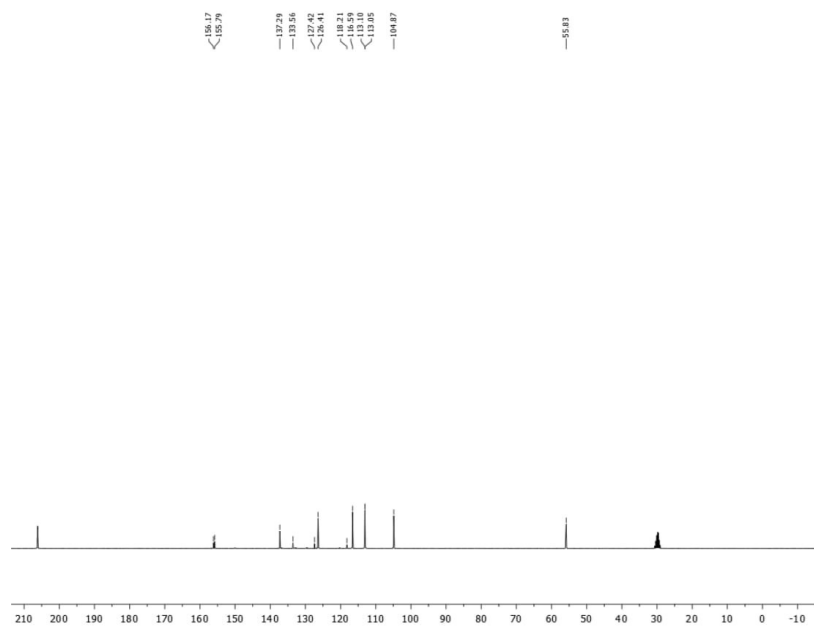


Figure S24. ¹³C-NMR of **4I** in acetone-d₆ at 293 K (δ in ppm).

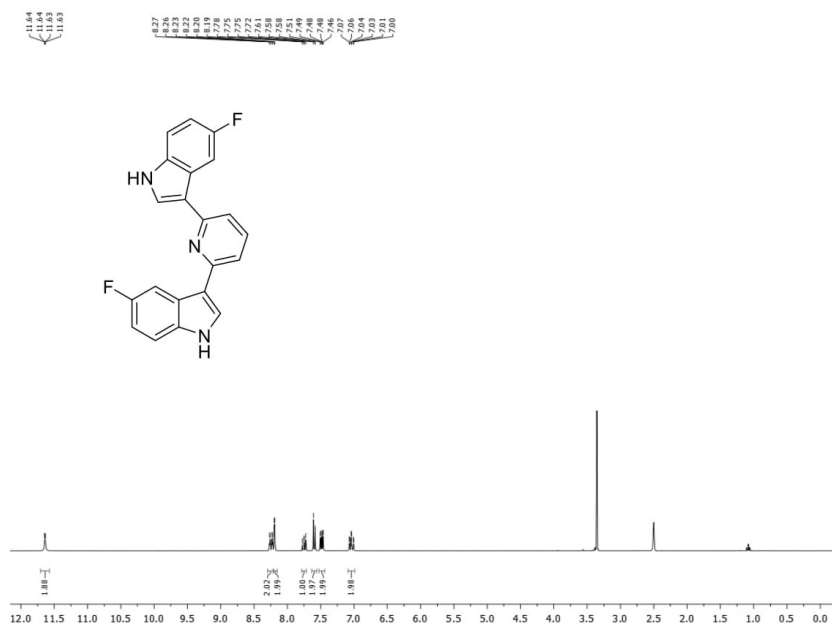


Figure S25. ¹H-NMR of **4m** in DMSO-d₆ at 293 K (δ in ppm).

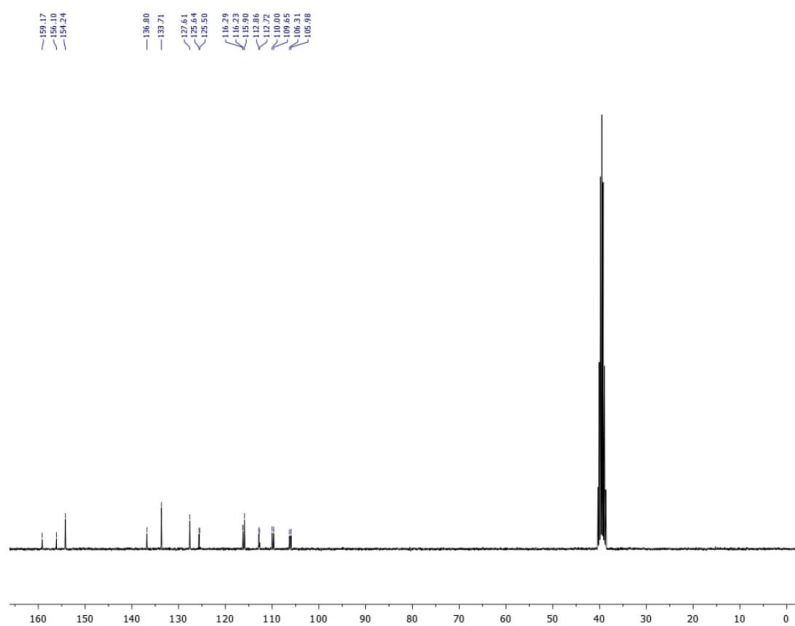


Figure S26. ¹³C-NMR of **4m** in DMSO-d₆ at 293 K (δ in ppm).

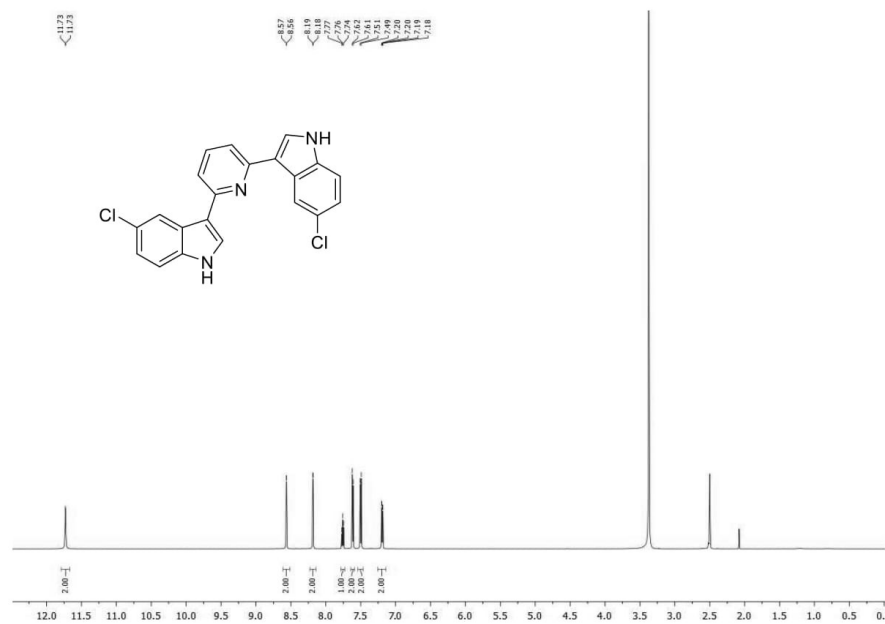


Figure S27. ¹H NMR of **4n** in DMSO-d₆ at 296 K (δ in ppm).

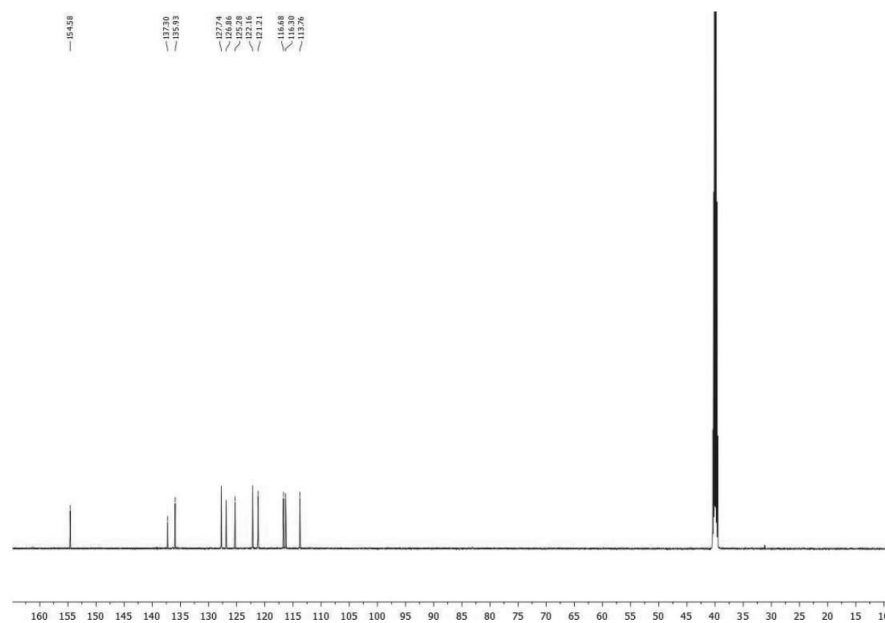


Figure S28. ¹³C NMR of **4n** in DMSO-d₆ at 295 K (δ in ppm).

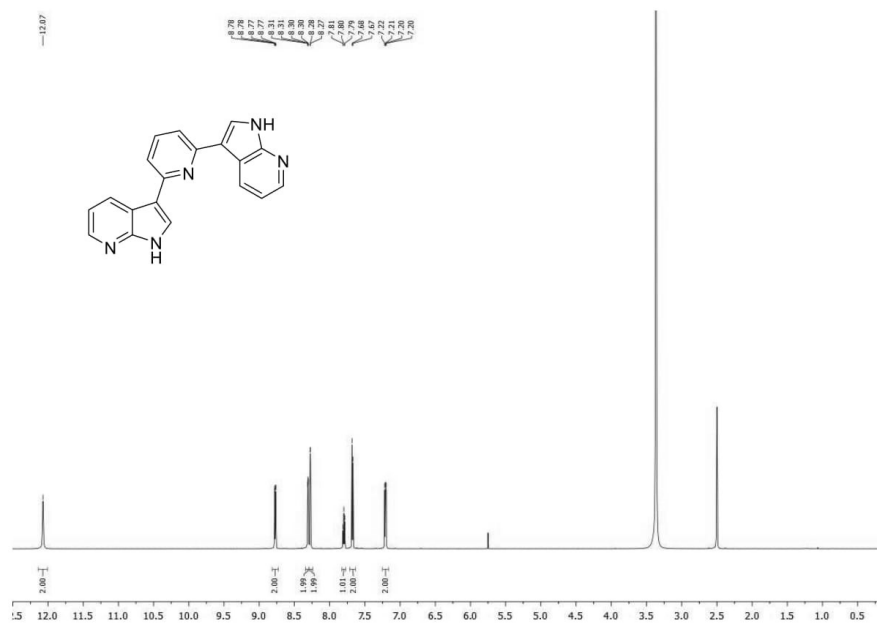


Figure S29. ¹H-NMR of **4p** in DMSO-d₆ at 296 K (δ in ppm).

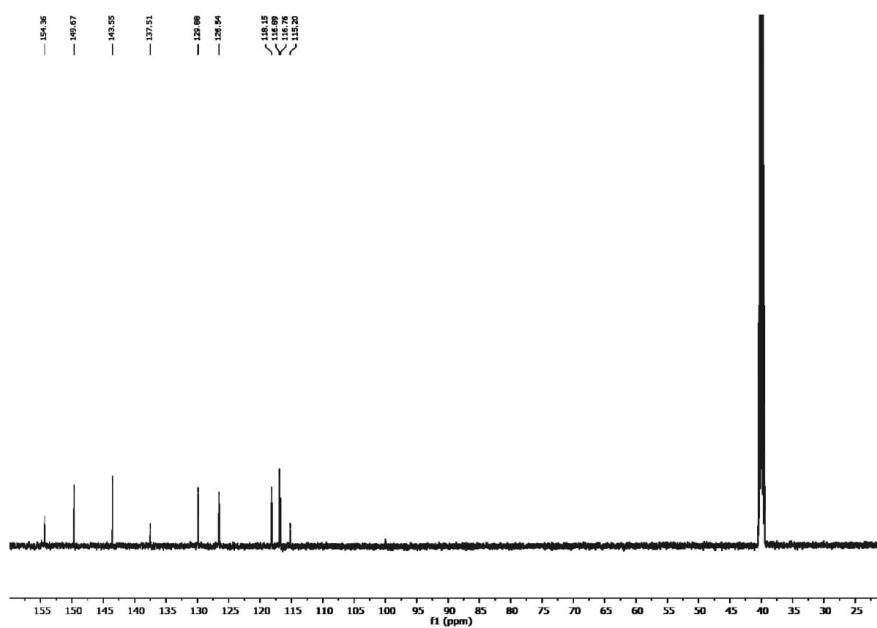


Figure S30. ¹³C-NMR of **4p** in DMSO-d₆ at 295 K (δ in ppm).

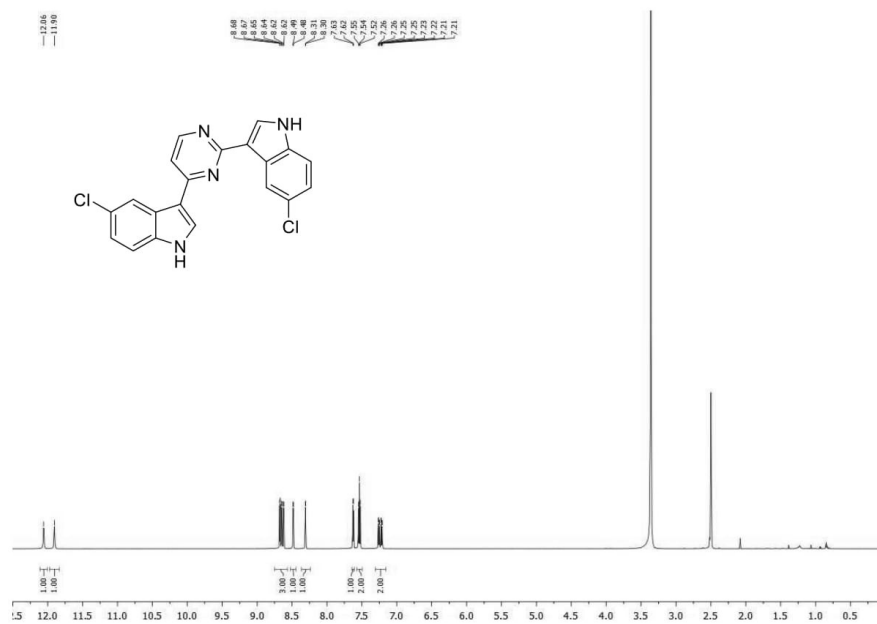


Figure S31. ¹H NMR of **4q** in DMSO-d₆ at 296 K (δ in ppm).

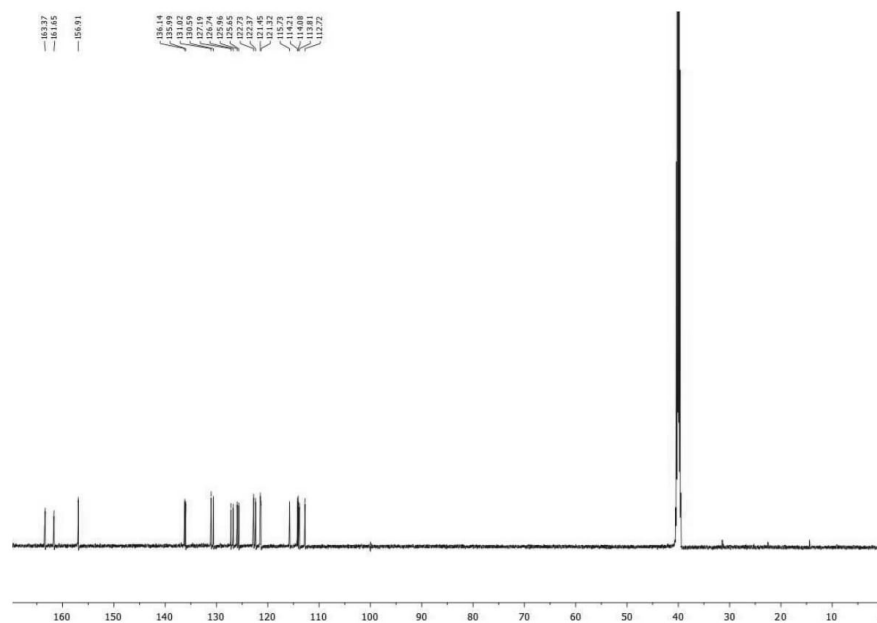


Figure S32. ¹³C NMR of **4q** in DMSO-d₆ at 295 K (δ in ppm).

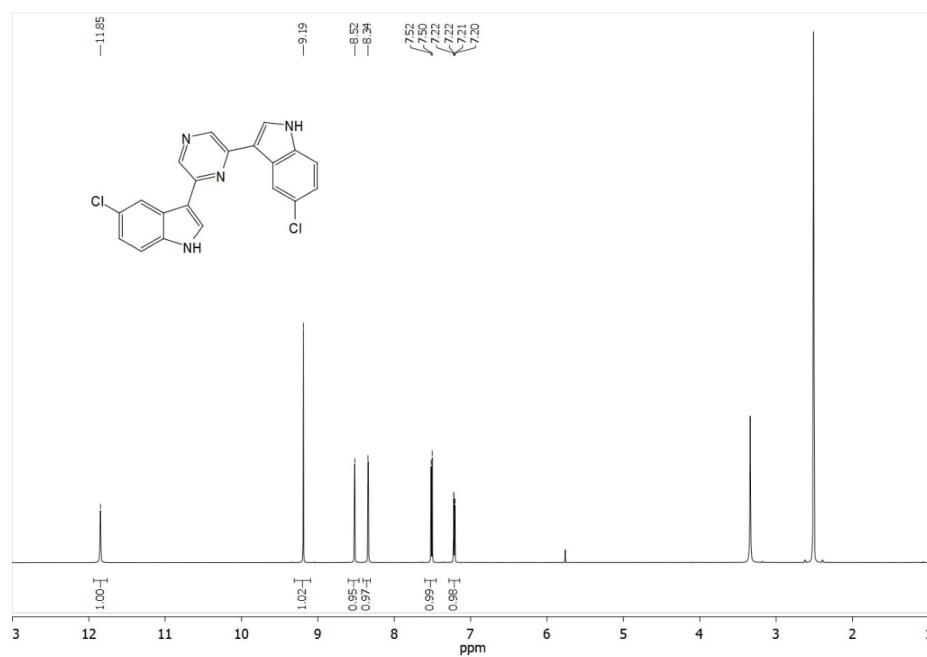


Figure S33. ¹H NMR of **4r** in DMSO-d₆ at 296 K (δ in ppm).

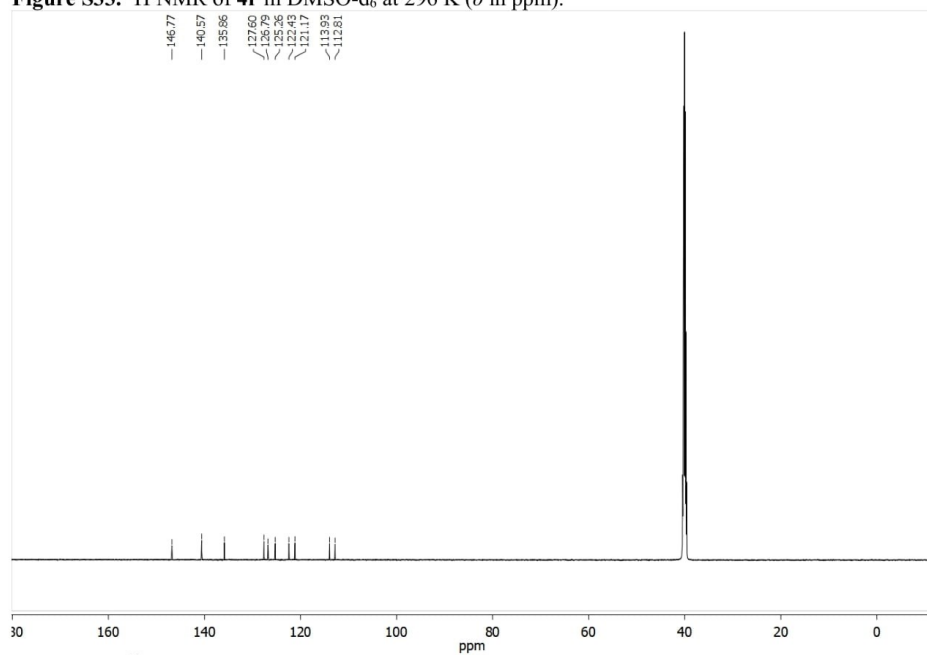


Figure S34. ¹³C NMR of **4r** in DMSO-d₆ at 295 K (δ in ppm).

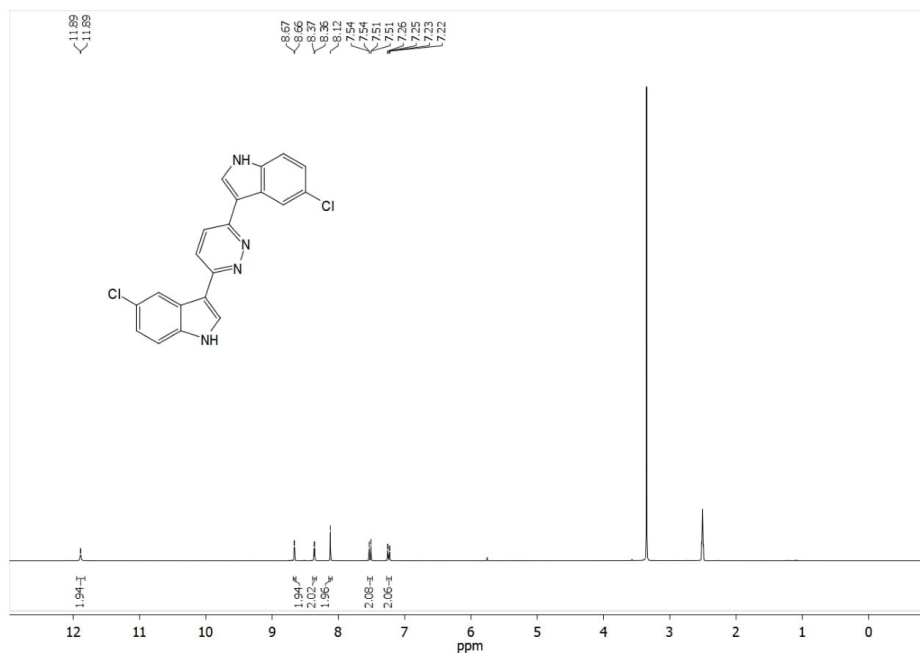


Figure S35. ¹H NMR of **4s** in DMSO-d₆ at 296 K (δ in ppm).

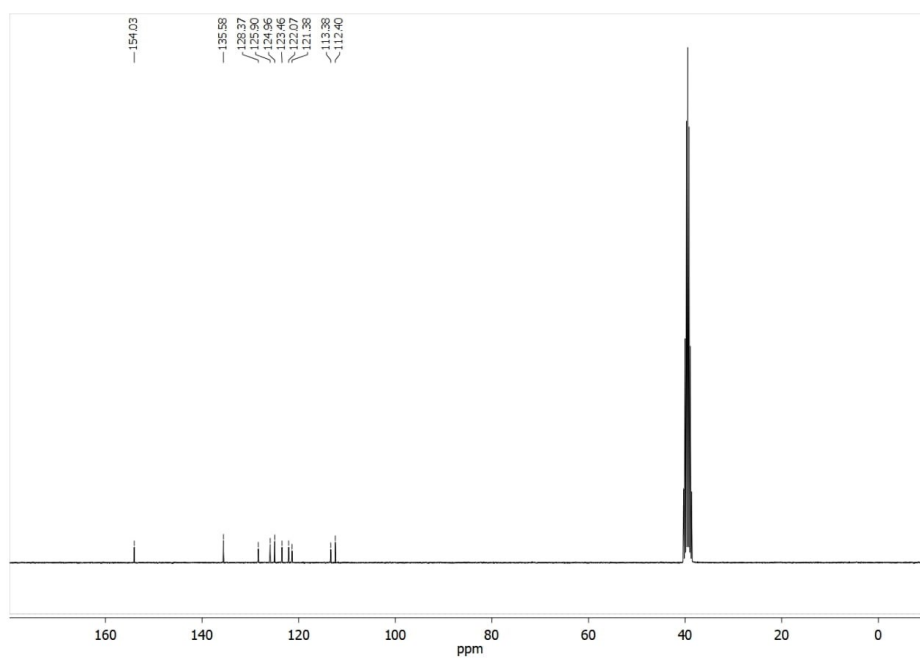
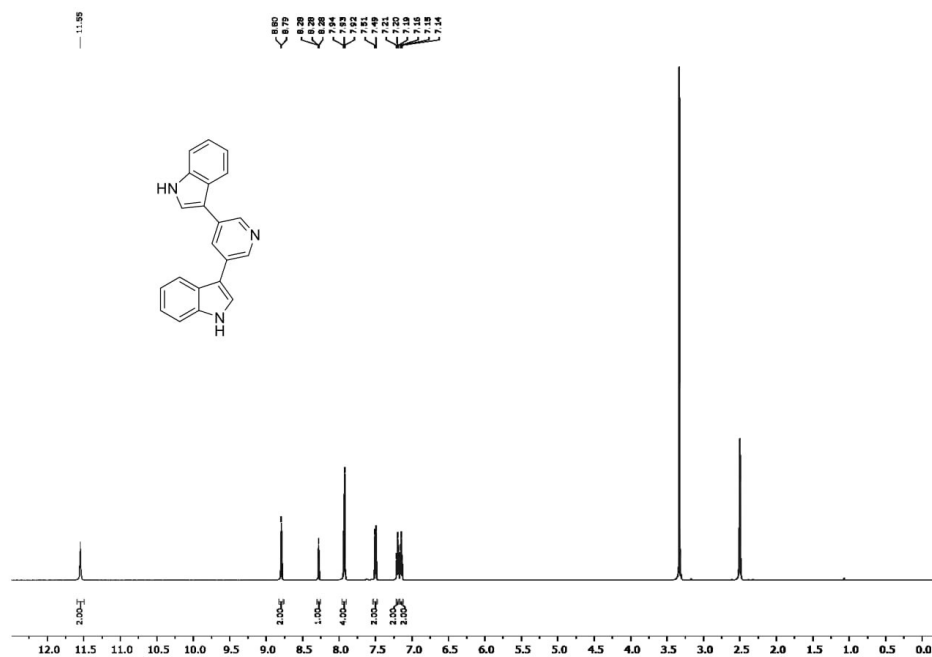


Figure S36. ¹³C NMR of **4s** in DMSO-d₆ at 295 K (δ in ppm).



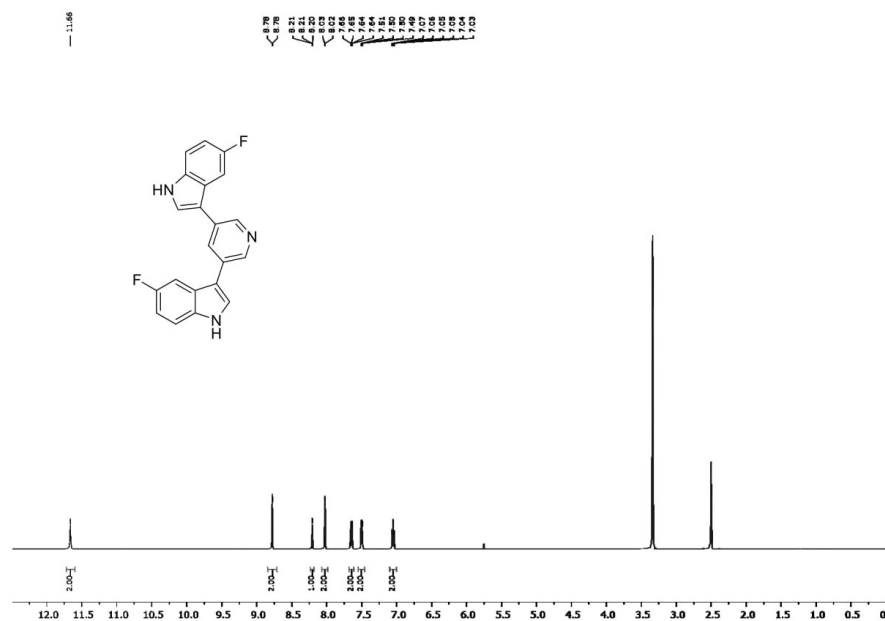


Figure S39. ¹H NMR of 4v in DMSO-d₆ at 296 K (δ in ppm).

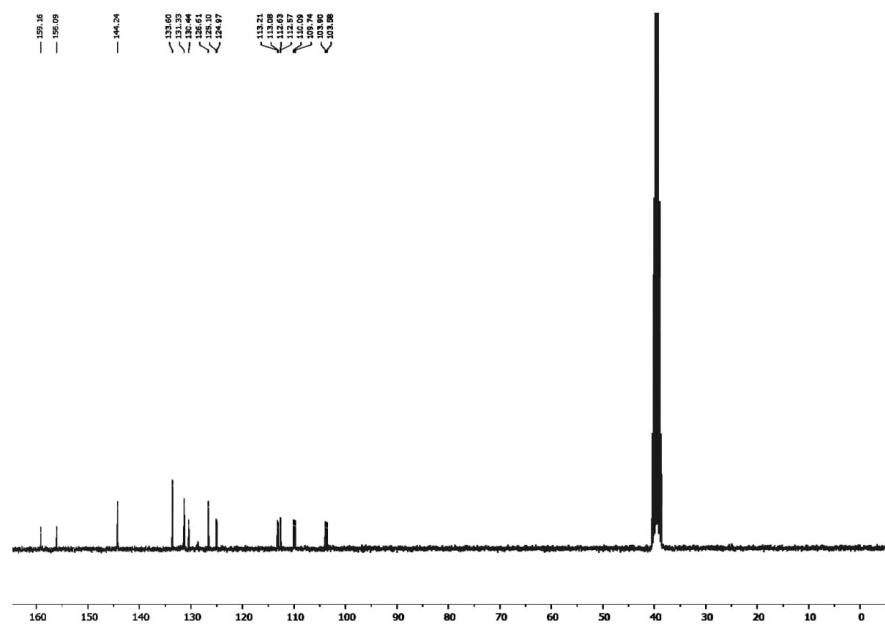


Figure S40. ¹³C NMR of 4v in DMSO-d₆ at 295 K (δ in ppm).

3 Synthesis of Bisindole Alkaloids and Their Mode of Action against Methicillin-Resistant *Staphylococcus aureus*

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Contribution to the paper:

- Wrote the manuscript.
- Determined the MICs of bisindoles against MRSA and *Bacillus subtilis*.
- Determined the MICs of bisindole derivatives for structure-activity relationship analysis.
- Investigated major cellular pathways affected by bisindoles using pathway-specific bioreporter strains.
- Assessed the impact of bisindoles on the LiaRS cell envelope stress response system.
- Measured the accumulation of UDP-MurNAc-pentapeptide in *S. aureus* upon bisindole exposure.
- Evaluated the influence of bisindoles on in vitro PBP2-catalyzed transglycosylation step of peptidoglycan synthesis.
- Investigated bisindole interference with the lipid II biosynthetic cycle.
- Evaluated the antagonistic effects of membrane lipids and phospholipids on bisindole antibacterial activity.
- Assessed bisindole-induced changes in the membrane potential of *S. aureus*.

Synthesis of Bisindole Alkaloids and Their Mode of Action against Methicillin-Resistant *Staphylococcus Aureus*

Emmanuel T. Adeniyi,^{||} Marco Kruppa,^{||} Stefania De Benedetti, Kevin C. Ludwig, Violetta Krisilia, Tobias R. Wassenberg, Melissa Both, Tanja Schneider, Thomas J. J. Müller,* and Rainer Kalscheuer**



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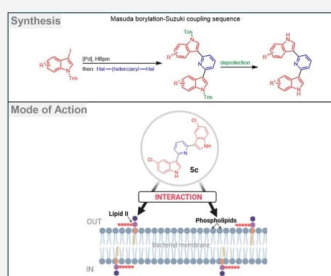
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ABSTRACT: About 100,000 deaths are attributed annually to infections with methicillin-resistant *Staphylococcus aureus* (MRSA) despite concerted efforts toward vaccine development and clinical trials involving several preclinically efficacious drug candidates. This necessitates the development of alternative therapeutic options against this drug-resistant bacterial pathogen. Using the Masuda borylation-Suzuki coupling (MBSC) sequence, we previously synthesized and modified naturally occurring bisindole alkaloids, alocasin A, hyrtinadine A and scalaradine A, resulting in derivatives showing potent *in vitro* and *in vivo* antibacterial efficacy. Here, we report on a modified one-pot MBSC protocol for the synthesis of previously reported and several undescribed *N*-tosyl-protected bisindoles with anti-MRSA activities and moderate cytotoxicity against human monocytic and kidney cell lines. In continuation of the mode of action investigation of the previously synthesized membrane-permeabilizing hit compounds, mechanistic studies reveal that bisindoles impact the cytoplasmic membrane of Gram-positive bacteria by promiscuously interacting with lipid II and membrane phospholipids while rapidly dissipating membrane potential. The bactericidal and lipid II-interacting lead compounds **5c** and **5f** might be interesting starting points for drug development in the fight against MRSA.

KEYWORDS: Methicillin-resistant *Staphylococcus aureus*, MRSA, Masuda borylation-Suzuki coupling, antibacterial, bisindole, lipid II



The unabated emergence of drug-resistant pathogens aided by antibiotic misuse and overuse has changed the narrative of most of the once-effective antibiotics to redundant chemicals against bacterial pathogens. Thus, this caused some previously simple-to-treat infections to transform into difficult-to-treat variants, resulting in increased morbidity and mortality. For instance, patients suffering from infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) are touted to be 64% more likely to die than those infected with the methicillin-sensitive strain.¹ Antibiotic resistance has since attained a crisis level and consequently has been recognized as one of the global health threats requesting dire attention. The situation is currently aggravated by the depletion of the antibiotic development pipeline and the perceived lack of access or shortage of quality therapeutics. Despite numerous reported hits resulting from joint academic and company-driven research efforts, this necessitates a continued search for novel therapeutic options.

Many antibiotics in clinical practice target the bacterial cell envelope.^{2,3} The cell envelope represents the first and major line of bacterial defense against external threats, whose integrity must be maintained at all times and at all costs. Its ability to counteract the high osmotic pressure determines the cell shape, while it also controls solute transport, interaction with other cells and others processes, making it such an

essential structure.² The bacterial cell envelope is made up of two layers: a cytoplasmic membrane and cell wall. The absence of the latter in eukaryotic cells and the crucial role it plays in bacterial survival,^{4,5} as well as its importance in the provision of structural rigidity and mechanical strength conferred by the peptidoglycan (PGN) meshwork makes the cell wall biosynthetic machinery such a prominent and successful target of antibiotics. Moreso, the highly conserved nature of the PGN precursor molecule, lipid II,⁶ and its existence in extremely low copy numbers,⁷ cements its status as a hotspot within the cell wall biosynthetic machinery and makes it an attractive target for antibiotics. With almost no tolerance for structural modification, resistance development against compounds targeting lipid II is very rare.⁸

Over the years, different classes of cell wall inhibitors have been reported to bind lipid II, consequently stalling PGN synthesis and abrogating essential cell wall functions. Most of these inhibitors are bulky peptides such as lantibiotics,

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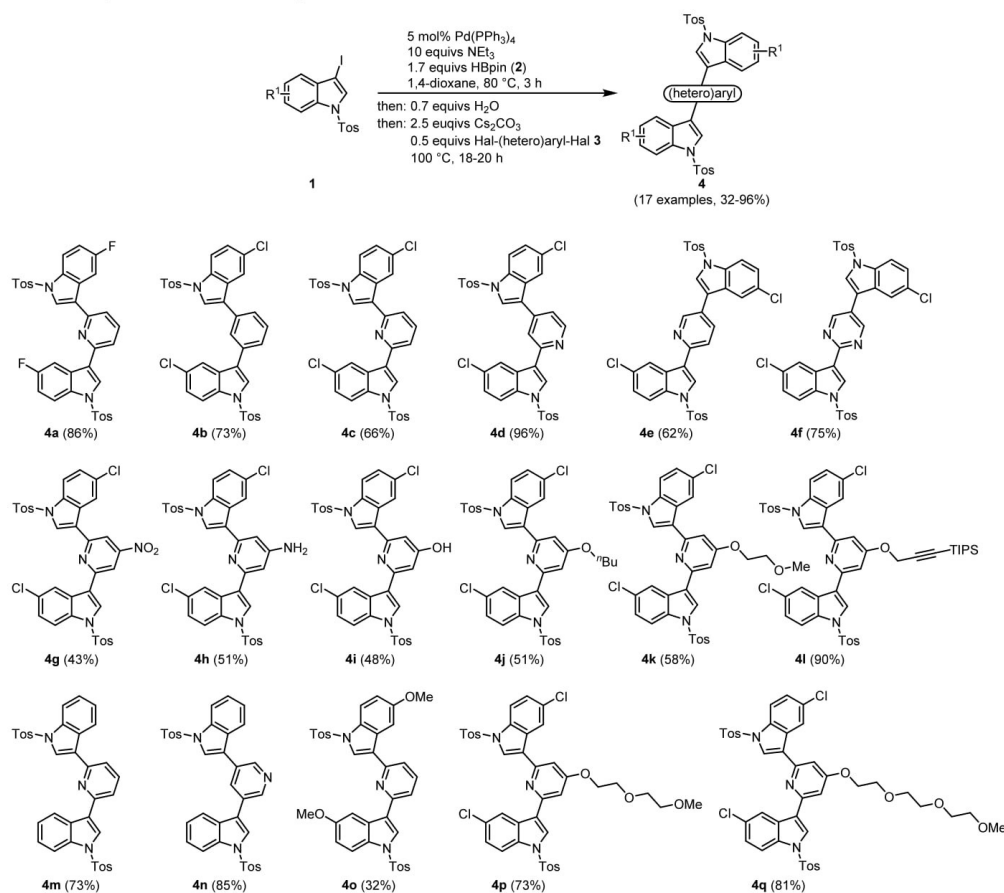


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Scheme 1. Synthesis of Novel *N*-tosyl Bisindoles 4

glycopeptides, lipopeptides, defensins and depsipeptides.^{9,10} Owing to the integration of lipid II into the cytoplasmic membrane, type-A lantibiotics such as nisin also dock onto the membrane using this high-affinity receptor molecule to form pores in the bacterial membrane. Thus, resulting in loss of membrane integrity and dissipation of membrane potential.^{7,9,11} Importantly, most of the antibacterial agents interacting with lipid II are macromolecules of natural origin,¹² with 1-amino substituted tetrahydrocarbazoles (THCz) as the only synthetic small molecules of less than 500 Da reported to target lipid II so far.⁵

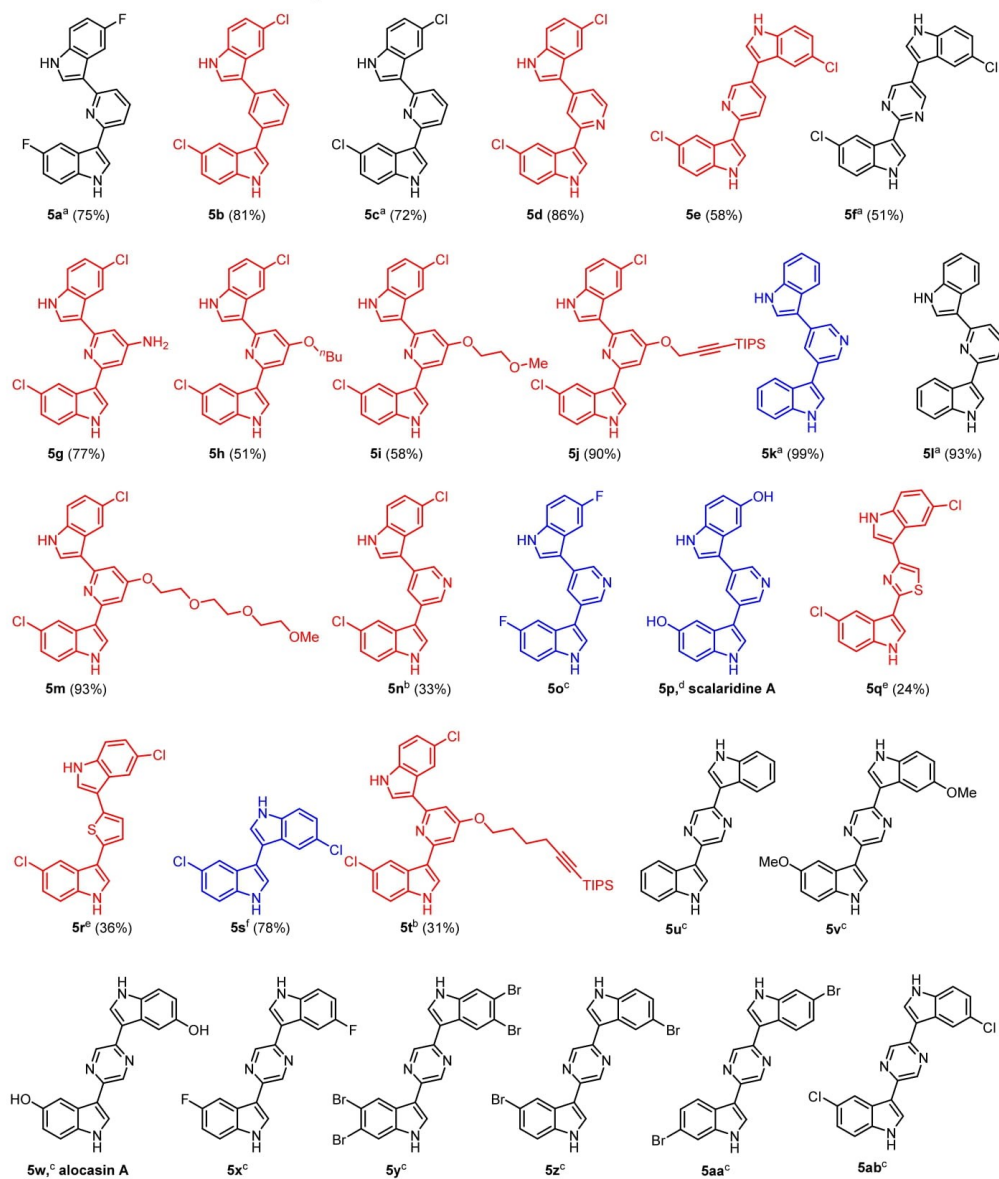
Sequel to our initial findings that nature-inspired bisindole alkaloids permeabilize the cytoplasmic membrane of *S. aureus*,¹³ leading to efflux of cellular contents, we continued to investigate the mode of action of these compounds in more detail. Further, we synthesized an expanded and diverse library of derivatives to extend our understanding of the structure–activity relationship of these molecules using our well-established and modified one-pot Masuda borylation-Suzuki coupling (MBSC) sequence.¹⁴ The MBSC sequence represents

an elegant tool, which combines a borylation reaction^{15,16} and a subsequent carbon–carbon bond formation^{17–19} in a one-pot process. This enables rapid access to highly functionalized symmetrically and unsymmetrically substituted hetero biaryls.¹⁴ Such concatenation of several elementary reaction steps in the same vessel reduces reaction time drastically, as intermediates do not require isolation and purification, resulting in less waste. Herein, we report a simplified MBSC one-pot protocol employed in the synthesis of novel *N*-tosyl-protected bisindoles. Mechanistic studies revealed that these inhibitors, in addition to permeabilizing the bacterial membrane, dissipate membrane potential by directly interacting with lipid II and membrane phospholipids and interfering with the lipid II biosynthetic cycle.

RESULTS AND DISCUSSION

Synthesis of *N*-tosyl Bisindole Compounds 4. The *N*-tosyl bisindoles **4** were synthesized with our well-established Masuda borylation-Suzuki coupling (MBSC) protocol that has been adopted for the pseudo-three-component synthesis of

Scheme 2. Bisindoles 5 via MBSC Sequence



^aResynthesis via optimized protocol; compounds have been previously described.¹³ ^bSynthesized via our previously published protocol (for experimental detail see [Supporting Information S6.6](#)). ^cThe synthesis has been described.¹⁵ ^dThe synthesis has been described.²¹ ^eSynthesized via different MBSC protocol (for experimental details see [Supporting Information S6.7](#)). ^fSynthesized via our previously published protocol (for experimental detail see [Supporting Information S6.6](#)); compound has been previously described.²² Compounds shown in red are new compounds. Compounds shown in blue have been reported previously but have not been evaluated for antibacterial activity before.

(hetero)cyclic-bridged bisindoles (Scheme 1, Scheme 2).¹³ The original MBSC protocol was varied because isolation and

purification of *N*-tosyl-protected bisindoles are easier and time and resources saved in comparison to the en route

deprotection. The reaction sequence starts with 3-iodo-1-tosyl-1*H*-indoles **1** that are readily accessible according to Witulski et al.²⁰ The iodo indoles **1** first react in the palladium-catalyzed Masuda borylation step with pinacol borane (**2**) and dry triethylamine in dry 1,4-dioxane under a nitrogen atmosphere at 80 °C (oil bath) to form the corresponding pinacol boronic acid esters. After full conversion (monitored by TLC) the excess of **2** is scavenged by the addition of stoichiometric amounts of water. After the addition of cesium carbonate and (hetero)aryl dihalides **3**, the formed boronic acid esters are reacted in a subsequent Suzuki coupling step in the same reaction vessel. Neither additional amounts of catalyst nor additional solvent or cosolvents are required. After reaction at 100 °C (oil bath) for 18 to 20 h the cross-coupling is complete (monitored by TLC) and the compounds **4** are isolated by flash chromatography on silica gel to give 17 novel *N*-tosyl bisindoles **4** in moderate to excellent yield (for experimental details, see Supporting Information S6.2 to S6.3).

Synthesis of *N*-Deprotected Bisindoles **5.** As shown in our previous study,¹³ deprotected NH bisindoles **5**, depending on their substitution pattern on the indole moiety, exhibited antibacterial activity against methicillin-resistant *Staphylococcus aureus*.¹³ To determine the potential biological activity of novel bisindole derivatives **5**, selected compounds have been detosylated and screened against MRSA. For this, *N*-tosyl bisindoles **4** were reacted with potassium hydroxide in a closed vessel in methanol at 100 °C (oil bath) for 4 h. After workup, the deprotected compounds **5a–m** were isolated by simple flash chromatography (Figure 1), resulting in 13 examples

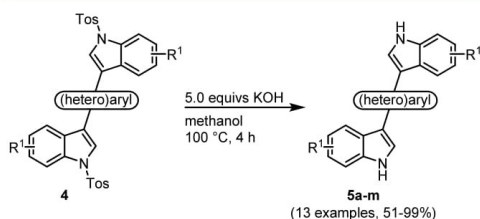


Figure 1. Synthesis of deprotected bisindoles **5a–m**.

obtained in good to excellent yield (for experimental detail, see Supporting Information S6.4 to S6.5). Compounds **5n** and **5t** have been synthesized with our previously published protocol (for experimental details, see Supporting Information S6.6) (**2**). Syntheses of **5o**, **5p** and **5u–aa** have also been reported earlier.^{13,21} For the synthesis of compounds **5q** and **5r**, see Supporting Information S6.7. Minimum inhibitory concentrations (MICs) of compounds **5** as shown in Table 1 were determined using the broth microdilution method.

Structure–Activity Relationship Study. Based on the MIC values of bisindole compounds **5**, we set out to assess the relationships between the diverse structures and their respective *in vitro* activities. In general, halide substitutions of the indole cores resulted in bioactivity. While single substitutions of both indole moieties with either bromine (**5z**) or fluorine (**5o**) resulted in good activity of MIC 1.56 and 3.13 μ M, respectively, double substitutions of each indole cores, exemplified by **5y**, led to a slightly weaker activity of 25 μ M MIC. The strongest anti-MRSA activity was interestingly exerted by **5ab** and **5n**, derivatives with chlorine substituents

Table 1. MIC and IC₅₀ Values of Bisindoles **5** and Selected *N*-tosyl Bisindoles **4** Against MRSA Strain ATCC 700699 and Human THP-1 Monocytic and HEK293 Kidney Cell Lines

Compounds	MIC [μ M]	IC ₅₀ [μ M]	
		THP-1	HEK293
5b	1.56	25	12.5
5c	0.20	6.25	12.5
5d	1.56	6.25	6.25
5e	1.56	12.5	6.25
5f	0.39	12.5	12.5
5h	50	ND	ND
5i	3.13	3.13	3.13
5k	>100	ND	ND
5m	>100	ND	ND
5n	0.78	12.5	12.5
5o	3.13	12.5	12.5
5p	>100	ND	ND
5q	0.78	12.5	12.5
5r	1.56	25	12.5
5s	6.25	50	25
5t	>100	ND ^a	ND
4b	>100	ND	ND
4c	>100	ND	ND

^aND, not determined.

with MICs 0.39 and 0.78 μ M, respectively (Figure). However, oxygen-containing groups (OH- (**5w**, **5p**), OMe- (**5v**)) and unsubstituted bisindole cores (**5u**, **5k**) led to loss of activity, with MICs > 100 μ M (Figure). This result is in tandem with our previously published results, showing that a 5,5'-dichloro substitution pattern on the indole moieties is crucial for the excellent anti-MRSA activity in the submicromolar range.

We next focused on 5,5'-dichloro-substituted bisindoles **5** and questioned the influence of the bridging heterocycle on the bioactivity of bisindoles. Thus, we synthesized and screened several azine- and diazine-bridged derivatives against MRSA. Interestingly, derivatives with electron-deficient diazines (**5ab**, **5ad**, **5ae** and **5f**) showed the most promising activity, with MICs ranging between 0.39–0.78 μ M against MRSA (Figure 2). Nonetheless, importantly, the best activity against MRSA from our bisindole library was exhibited by **5c**, a pyridine-bridged bisindole derivative with an MIC of 0.2 μ M (Table 1). Overall, compared to the indole substitution, a lot more variation is tolerated by the bridging heterocycle. Further, **5b**, a benzene-bridged bisindole showed a MIC of 1.56 μ M, a bioactivity comparable to that of pyridine-bridged bisindoles **5d** and **5e** (Table 1). However, in the case of **5s**, a bisindole derivative without a bridging unit, the activity drops significantly to 6.25 μ M, highlighting the fact that a (hetero)cyclic bridge between the indole moieties is mandatory for excellent bioactivity. Lastly, we sought to know whether protection of the indoles might improve the antibacterial activity of bisindoles. Thus, we synthesized and screened *N*-tosyl bisindoles **4b** and **4c** against MRSA. Both derivatives showed a loss of activity with MICs above 100 μ M against MRSA (Table 1), indicating that indole deprotection is required for antibacterial activity of bisindoles.

To address solubility issues as well as enable late-stage functionalization, e.g., via click chemistry, we synthesized several derivatives with diverse side chains. Regarding the effect of this side-chain functionalization on the MIC, the

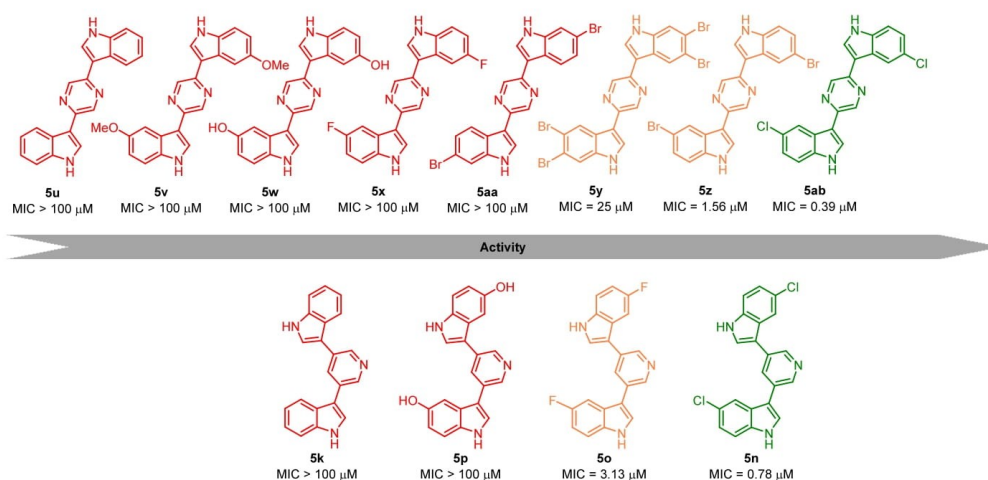


Figure 2. Influence of the indole substitution pattern on the anti-MRSA activity. The arrow indicates order of decreasing MIC. Red: MIC > 100 μM , orange: MIC = 1–100 μM , green: MIC < 1 μM .

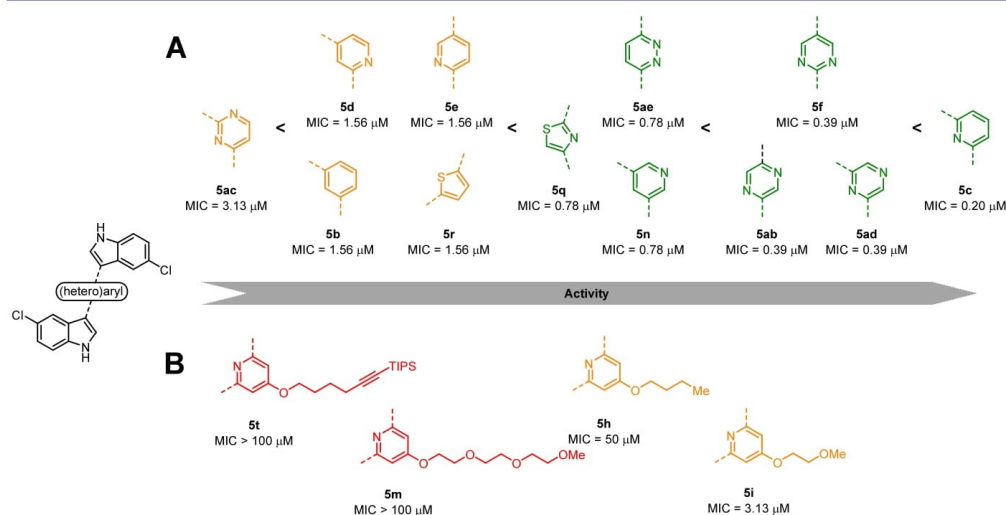


Figure 3. Structure–activity relationship of (A) (di)azine-bridged 5,5'-dichloro bisindoles 5 and (B) linker with different side chains. Compounds were screened against MRSA and the MICs are reported below each derivative. The arrow indicates order of decreasing MIC. Red: MIC > 100 μM , orange: MIC = 1–100 μM , green: MIC < 1 μM .

toleration of substituents appears to be limited. **5i**, a derivative with shorter and polar alkoxy substituents, retained activity to some extent, showing an MIC of 3.13 μM , whereas **5h**, a bisindole with an unpolar alkoxy group, led to a remarkable drop of activity with an MIC of 50 μM (Figure 3B). Meanwhile, **5m** and **5t** with longer side chains resulted in a complete loss of activity (Figure 3B). Overall, in addition to the chlorination of the indole moieties, the presence of a heterocyclic bridge and deprotection of the indole cores are

crucial for the antibacterial activity of bisindoles in the submicromolar range.

Mode of Action Studies. We have reported on the membrane-permeabilizing property of nature-inspired bisindole alkaloids in a previous study.¹³ To further characterize the mode of action of these bisindoles, we focused on our frontrunner compounds **5c** and **5f**, which are structurally identical to compounds designated as **4n** and **4j** in our previous study.¹³ We first evaluated their influence on the Lipid II-Interacting Antibiotics Response regulator and Sensor

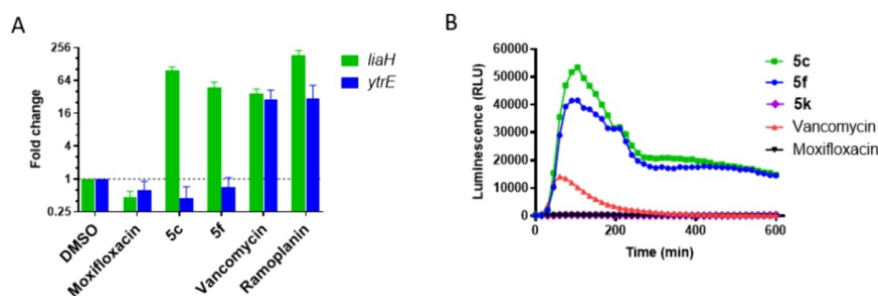


Figure 4. Influence of bisindoles on the LiaRS cell envelope stress response system. (A) Expression levels of *liaH* and *ytrE* in *B. subtilis* 168 cells exposed to bisindoles for 10 min normalized to the DMSO control. *LiaH* is a key component of the LiaRS system. *YtrE* is a membrane-bound marker protein indicative of perturbed cell wall biosynthesis.²⁶ Vancomycin and ramoplanin served as positive controls, while moxifloxacin served as a negative control. Cells were exposed to 8-fold MIC of the compounds. Data are means of triplicates. (B) Induction of the *liaI* *B. subtilis* reporter strain by active bisindoles 5c and 5f, each at 1.56 μ M, compared to the positive control 50 μ M vancomycin and 100 μ M each of the negative controls moxifloxacin and inactive bisindole 5k, indicating an interference with the lipid II biosynthetic cycle. Data shown are results of an experiment that can be repeated in two independent experiments.

(LiaRS) system as a direct measure of their effect on the cell envelope, since most compounds impacting the cell envelope evoke the induction of the LiaRS system as a stress response in *B. subtilis*. The LiaRS system is a two-component and complex cell envelope stress response system, yet, a largely studied regulatory network in *B. subtilis*.^{2,23} Among members of this two-component system (TCS) is *LiaH*, a prominent marker protein predominantly upregulated in response to the impairment of cytoplasmic membrane structural integrity as well as interference with cell wall biosynthesis.^{24,25} Therefore, first, we evaluated the expression of *liaH* in *B. subtilis* cells exposed to bisindoles via RT-qPCR. Like the lipid II-binding antibiotics, vancomycin and ramoplanin, that do not exhibit membrane-disturbing function, exposure of *B. subtilis* cells to compounds 5c and 5f for 10 min led to a remarkable 50- and 100-fold upregulation of *liaH*, respectively (Figure 4A). In contrast, the negative control moxifloxacin, an antibiotic that kills bacterial cells by targeting DNA gyrase with no effect on membrane integrity or cell wall biosynthesis, led to *liaH* downregulation of about a 0.46-fold change as expected (Figure 4A).

Since the robust induction of *liaI* promoter (P_{liaI}), the driver of *liaIH* operon expression, is a direct effect of the activation of the LiaRS system in response to cell envelope-disturbing stimuli such as lipid II-targeting antibiotics,^{5,23} we further investigated the influence of bisindoles on the LiaRS stress response employing a luminescence-based reporter strain expressing P_{liaI} fused to the *lux* operon (P_{liaI} -*lux*). Consistent with the upregulation of *liaH*, active bisindoles 5c and 5f, each at 1.56 μ M, also led to a significant induction of the *liaI* reporter strain (Figure 4B). Remarkably, the *liaI* reporter-inducing capacity of active bisindoles at 1.56 μ M was superior to that of the positive control vancomycin at 50 μ M, whereas inactive bisindole 5k and negative control moxifloxacin, each at 100 μ M, did not induce the reporter strain (Figure 4B). Further, the induction of this reporter strain was also observed for both 5c and 5f at narrow concentration windows of 0.78–6.25 and 0.78–3.13 μ M, respectively (Figure S1). Taken together, with the LiaRS system known to respond to lipid II-binding antibiotics, this is the first hint that active bisindoles might be interfering with the lipid II biosynthetic cycle. This most likely explains the difficulty encountered when raising

resistant mutants against these compounds in our previous study,¹³ since lipid II is such a highly conserved molecule existing in quite a low copy number.^{6,10}

Most antimicrobials interfering with the lipid II biosynthetic cycle such as vancomycin,²⁷ ramoplanin²⁸ and teixobactin,²⁹ cause a breakdown of the cell division and membrane coordinated cell wall biosynthetic machineries.³⁰ These antibacterial agents block cell wall synthesis by binding lipid II with high affinity, thereby preventing transglycosylation and transpeptidation reactions.⁹ Since 5c or 5f did not impede the PBP2-catalyzed transglycosylation reaction step of cell wall synthesis *in vitro* at 2-fold (Figure S2) as well as 4-fold molar ratio (result not shown) of bisindoles with respect to lipid II, we next sought to determine whether bisindoles interfere with a late-stage membrane-associated PGN biosynthesis step. We checked for potential accumulation of the soluble ultimate peptidoglycan precursor UDP-N-acetyl muramic acid-pentapeptide (UDP-MurNAc-5p). Compared to vancomycin, exposure of *S. aureus* cells to 5c and 5f only led to an insignificant accumulation of the UDP-MurNAc-5p (Figure S3). Furthermore, the downregulation of *YtrE* (Figure 4A), a membrane-bound cell wall biosynthesis marker protein,²⁶ in 5c and 5f-treated *B. subtilis* cells further suggests that bisindoles do not disrupt the biosynthesis of the cell wall.

To further rule out the possibility of bisindoles interfering with the cell wall biosynthetic pathway and to get an insight into which biologically functional pathway might be impaired by these compounds, we employed pathway-specific *B. subtilis* bioreporter strains with specified promoter-*lacZ* gene fusions. Like the respective negative controls, exposure of DNA, RNA, cell wall, and protein biosynthesis pathway-specific bioreporters to 5c, 5f and 5k did not result in a blue halo around the inhibition zones of any of these strains on agar plates, including the cell wall-specific reporter strain (Figure S4). This indicates that bisindoles do not specifically interfere with protein, RNA, DNA, and cell wall biosynthetic pathways.

With our data excluding the cell wall biosynthetic machinery as the potential target of bisindoles, we turned our focus back to the membrane part of the cell envelope. Thus, we evaluated the antagonistic effects of lipid II and membrane phospholipids on the antibacterial activity of bisindoles using the *B. subtilis* *liaI* reporter. Unlike the expected selective antagonization of

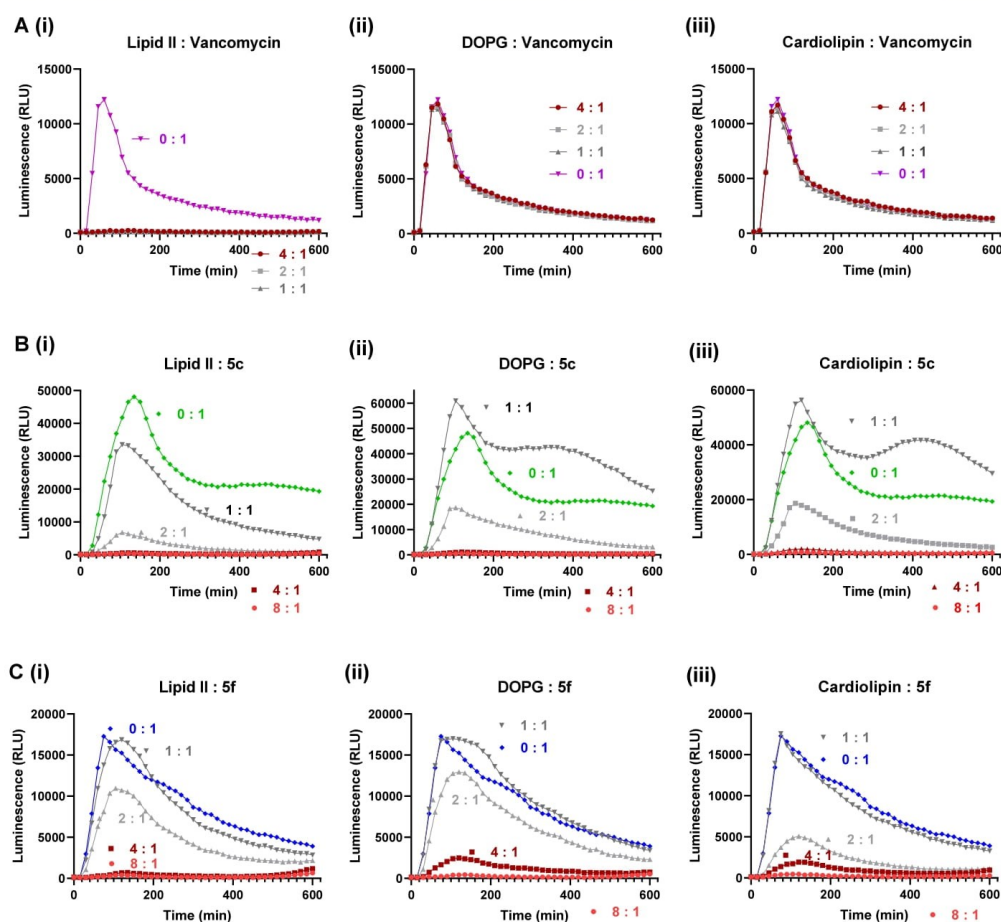


Figure 5. Antagonistic effect of membrane-bound lipid II and phospholipids on induction of the LiaRS system by bisindoles. Antagonistic effect of lipid II, DOPG and cardiolipin on the ability of (A) 6.25 μ M vancomycin, (B) 3.13 μ M 5c and (C) 3.13 μ M 5f to induce the LiaI bioreporter at different molar ratios. Data shown are results of an experiment that was repeated in an independent experiment.

vancomycin by lipid II (Figure 5Ai-iii), 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG), cardiolipin and lipid II, starting from a 2-fold molar excess, all antagonized the capability of both 5c and 5f to induce the LiaI reporter (Figure 5B and 5C). While the ability of 5c to induce the LiaI reporter seem to be antagonized by high concentrations (8-fold molar excess) of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), the undecaprenyl pyrophosphate (C_{55} -PP) part of lipid II and UDP-MurNAc-5p had no antagonizing effect on 5c and 5f (Figure 5S). Vancomycin inhibits the growth of most Gram-positive bacteria by binding to the D-alanyl-D-alanine portion of lipid II,²⁷ thereby making it unavailable for peptidoglycan synthesis, which explains the selective antagonization even at 1-fold molar excess of lipid II. On the other hand, the significant antagonization of 5c from 2-fold molar excess not only by lipid II, but also by DOPG and cardiolipin, demonstrates clearly that bisindoles interact with membrane-anchored lipid II and

negatively charged membrane phospholipids, albeit promiscuously. Thus, this suggests that bisindoles target specifically the cytoplasmic membrane, since bacterial membranes consist primarily of DOPG and cardiolipin,³¹ with lipid II having its lipid tail anchored within the membrane lipid bilayer.

Next, to corroborate the promiscuous binding perceived between bisindoles and these membrane lipids, we conducted a carboxyfluorescein (CF) release assay using lipid II-containing unilamellar vesicles. Relative to triton X-100, the addition of 5c or 5f at 5-fold MIC led to significant dye release from lipid II-containing DOPC liposomes. While 5c triggered 81% CF release like the positive control pore-forming nisin (83%) after 2 min of addition, 5f resulted in a decreased CF release of 55% (Figure 6B, 6C and 6D). This is likely due to a higher degree of affinity perceived between lipid II and 5c than with 5f, as supported by our finding that a 2-fold molar excess of lipid II almost completely antagonized the ability of 5c to induce the

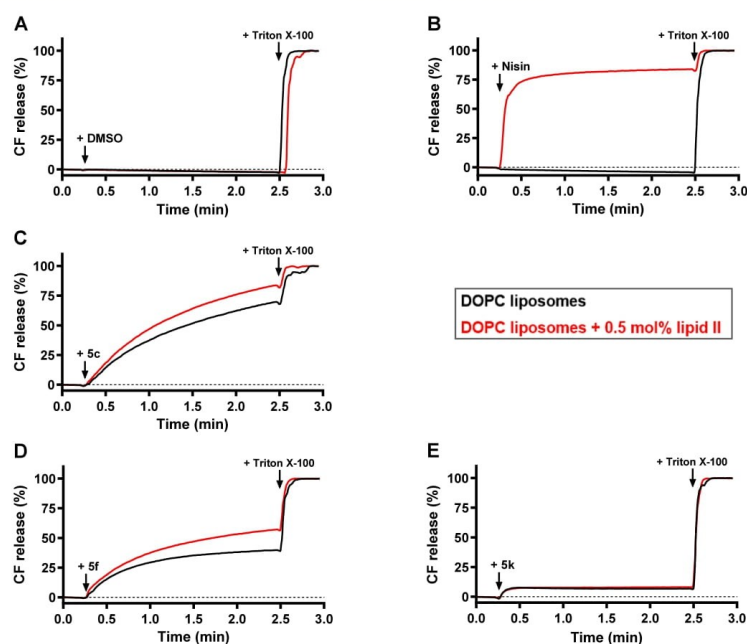


Figure 6. Leakage of CF from liposomes made of DOPC and DOPC supplemented with lipid II. Release of CF from DOPC liposomes (black) doped with 0.5 mol % lipid II (red) upon addition of DMSO (A), 1 μ M nisin (B), 1 μ M of 5c (C), 1.95 μ M 5f (D) and 4 μ M of 5k (E). Respective compounds were added to each liposome, as indicated by the black arrow. 0.1% Triton X-100 was added to achieve 100% CF leakage.

Lial reporter, but not of 5f at the same concentration (Figure 5Bi and 5Ci). Furthermore, relative to triton X-100, 5c and 5f triggered a much more reduced CF release from DOPC only liposomes than lipid II-containing DOPC liposomes, with 5c and 5f addition resulting in 67% and 39% CF release from DOPC only liposomes, respectively (Figure 6C and 6D). The decreased CF release by active bisindoles in DOPC only liposomes compared to the lipid II-containing ones is most likely due to greater affinity of bisindoles for lipid II than DOPC. This is evident in the ability of as little as a 2-fold molar excess of lipid II to antagonize the inducing effect of 5c (Figure 5Bi), compared to the antagonizing effect of DOPC obtainable only at higher concentration (8-fold molar excess) of DOPC (Figure S5 Bi). This indeed explains the moderate cytotoxicity property of bisindoles (Table 1), since most eukaryotic membranes are chiefly dominated by DOPC, which is lacking in bacteria. Interestingly, however, both lead compounds, 5c and 5f did not trigger *in vitro* hemolysis at concentrations up to 100 μ M.¹³ Of note, relative to triton X-100, compared to active bisindoles, an insignificant 8% CF release from both lipid II-containing DOPC and DOPC only liposomes was observed after 2 min of addition of 4 μ M (corresponding to 10-fold MIC of 5f) of inactive bisindole derivative 5k (Figure 6E). This indicates that the affinity between these membrane lipids and active bisindoles is profoundly higher than that with the inactive bisindole counterpart. Overall, these results clearly demonstrate that, compared to the lantibiotic nisin that damages the bacterial membrane via rapid pore formation by specifically binding to

lipid II,^{32–34} active bisindoles promiscuously interact with several membrane lipids and have only little specificity toward lipid II.

The membrane-permeabilizing feature of bisindoles are in agreement with the efflux of ATP and GFP from bisindoles-treated cells as reported in an earlier study,¹³ which is further corroborated by the CF release assay described above. We thus reasoned that these compounds might also depolarize the bacterial membrane. Hence, we investigated the effect of bisindoles on the membrane potential of *S. aureus* using the fluorescent DiOC₂(3) dye. The membrane potential sensitive probe emits a green, fluorescent signal. High membrane potential is a function of the dye molecules accumulating in the cell, and the aggregation of these dye molecules triggers a shift to red fluorescence emission. Similar to carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a protonophore that disrupts membrane potential, the addition of 5c and 5f to the bacterial cells also led to a drastic drop in red fluorescence (Figure 7A), suggesting that bisindoles rapidly dissipate membrane potential in *S. aureus*. This is in contrast to the negative controls DMSO and moxifloxacin (Figure 7A) as expected. To further investigate the membrane depolarization phenotype of bisindoles, we monitored the localization of MinD microscopically using a strain of *Bacillus subtilis* expressing GFP-MinD. MinD is a part of the cell division machinery in *B. subtilis* bound to the membrane and specifically localized at newly formed cell poles via the presence of membrane potential.³⁵ Invariably, MinD localization at the cell poles is a measure of the intact membrane

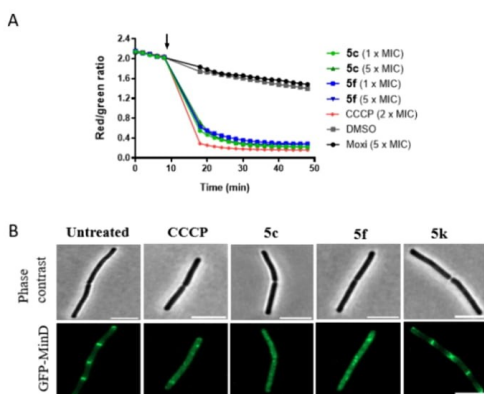


Figure 7. Influence of bisindoles on the bacterial membrane potential. (A) Effect of 5c and 5f on the membrane potential of *S. aureus* ATCC 700699. Baseline fluorescence measurements of *S. aureus* cells at $OD_{600\text{ nm}} = 0.5$ incubated with DiOC₂(3) were taken for 10 min before addition of compounds. Black arrow indicates the point at which compounds were added. Reduction in red fluorescence was measured over time as a measure of membrane potential dissipation (excitation, 488 nm; emission, 630 nm). Moxifloxacin and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) served as negative and positive controls, respectively. Data are means of triplicates. (B) Evaluation of 5c, 5f and 5k on membrane potential-driven GFP-MinD localization in *B. subtilis* 168 cells. Fluorescence micrographs of live *B. subtilis* cells expressing GFP-MinD on a thin agarose pad. Cultures were aliquoted at $OD_{600\text{ nm}} = 0.25$ before 15 min exposure to 5-fold MIC of bisindoles. Twenty-five μM CCCP was used as a positive control. Scale bar = 5 μm .

potential. Compared to the untreated control, the exposure of GFP-MinD-expressing strain of *B. subtilis* to 5c or 5f for 15 min caused a delocalization of GFP-MinD across the cell (Figure 7B). To ascertain that the membrane depolarizing feature is a phenotype specific for the biologically active bisindoles, a bisindole derivative, 5k, showing no growth inhibiting capability against MRSA (MIC > 100 μM) was included in this experiment. Like the untreated control, incubation of the cells with 5k for 15 min did not delocalize GFP-MinD (Figure 7B). Thus, consistent with the rapid dissipation of the membrane potential, active bisindoles delocalize GFP-MinD, a clear indication that active bisindoles depolarize the bacterial membrane.

CONCLUSION

In summary, here, we report a simpler and efficient MBSC sequence protocol for the synthesis of a series of previously reported and of several undescribed bisindole derivatives showing potent anti-MRSA activity, moderate cytotoxicity profile, and reasonable therapeutic margins. In addition to the chlorine substitution of the indole moieties earlier reported to be crucial for the antibacterial activity of bisindole alkaloids in the submicromolar range, synthesis of *N*-tosyl bisindoles, as well as derivatives void of (di)azine bridge, provide further insights into the basis of the antibacterial activity that the (di)azine linker between the indole moieties and indole deprotection are as well essential for anti-MRSA activity. The mechanism of action studies show that these compounds perturb the cell envelope by interfering with the lipid II

biosynthetic cycle, as demonstrated by the upregulation of *liaH* and induction of the LiaRS reporter. Furthermore, the lack of interference with any of the pathway-specific *lacZ* bioreporters by bisindoles, coupled with their interaction with membrane phospholipids as well as with the cytoplasmic membrane-anchored lipid II, suggests that these compounds specifically target the cytoplasmic membrane. This interaction subsequently leads to efflux of cellular contents and dissipation of membrane potential, perhaps as a result of bisindoles penetrating the cytoplasmic membrane and direct binding to these membrane lipids. The cytoplasmic membrane-targeting property, low resistance emergence, and promising *in vivo* activity of lead structures 5c and 5f, present both compounds as a starting point for further preclinical development in combating MRSA and *S. aureus* associated infections.

MATERIALS AND METHODS

MIC Determination. The MICs of bisindoles were determined mostly against MRSA strain ATCC 700699 using the resazurin microtiter assay. Fresh cultures of MRSA in exponential growth phase cultivated in Mueller Hinton medium were adjusted and seeded at 5×10^4 CFU per well in 96-well round-bottom microtiter plates, in a total volume of 100 μL already containing 2-fold serially diluted bisindole derivatives starting at 100 μM . Microtiter plates were incubated statically for 18–24 h at 37 °C before macroscopic evaluation and resazurin addition. Ten μL of 100 $\mu\text{g}/\text{mL}$ resazurin stock was added to each well and further incubated at 37 °C for at least 2 h. Upon color change, fluorescence intensity (excitation, 535 nm; emission, 595 nm) was measured using a microplate reader, and the MIC of each compound was determined relative to the sterile medium (0% growth) and solvent (100% growth) controls.

Cytotoxicity Determination. The cytotoxicity profiles of bisindole derivatives were determined *in vitro* using the THP-1 human monocytic cell line (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) and HEK293 human kidney cell line (CLS Cell Lines Service GmbH, Germany). THP-1 cells were cultured in RPMI 1640 medium supplemented with stable glutamine, 2.0 g/L NaHCO₃, 1 mM sodium pyruvate and 10% (v/v) fetal bovine serum (FBS), while HEK293 cells were cultured using EMEM medium supplemented with stable glutamine, 1% (v/v) nonessential amino acids, 1 mM sodium pyruvate and 10% (v/v) FBS. All cell lines were cultivated at 37 °C in a humidified atmosphere of 5% CO₂. About 5×10^4 cells per well were seeded in 96-well flat-bottom microtiter plates in a total volume of 100 μL per well, containing 2-fold serial dilutions of the selected compounds, with 100 μM as the highest concentration. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ for 48 h. Subsequently, 10 μL of a 100 $\mu\text{g}/\text{mL}$ resazurin stock was added to each well, and the plates were further incubated for 3 h at 37 °C and 5% CO₂. After incubation, fluorescence intensity was quantified using a microplate reader (excitation, 540 nm; emission, 590 nm). Cytotoxicity of compounds was determined relative to sterile medium (0% growth) and solvent (100% growth) controls in triplicates.

Membrane Depolarization. The effect of bisindoles on the membrane potential of *S. aureus* was evaluated as previously described with modifications.³⁶ An exponentially growing culture of *S. aureus* ATCC 700699 cultivated in Mueller Hinton medium was harvested, washed, and

resuspended in phosphate buffered saline supplemented with 1% glucose. Resuspended cells were adjusted to $OD_{600\text{ nm}} = 0.5$ and incubated with DiOC₃(3) (3,3'-diethyloxycarbocyanine, iodide) at 37 °C. Following dye uptake for 30 min in the dark, cells were dispensed in triplicates into a black microplate, and baseline reading was established using a Tecan plate reader. After 10 min of baseline measurement, 1-fold MIC of bisindoles were added to the cells and immediately transferred to the Tecan plate reader to resume green (excitation, 488 nm; emission, 530 nm) and red (excitation, 488 nm; emission, 630 nm) fluorescence intensity measurement in a kinetic fashion. Membrane potential was calculated as the ratio of red to green fluorescence. Moxifloxacin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) served as negative and positive controls, respectively.

Pathway-Specific β -galactosidase Reporter Assay. The impact of **5c** and **5f** on major cellular pathways (DNA, RNA, cell wall and protein biosynthesis) was evaluated as previously described.⁵ *B. subtilis* 168 *amyE::pAC6* strains containing different promoter fusions (*P_{phcA}-lacZ*, *P_{ysgS}-lacZ*, *P_{ypuA}-lacZ*, and *P_{yorB}-lacZ*; sensitive to inhibition of DNA, RNA, cell wall or protein biosynthesis, respectively) were grown in Mueller Hinton medium containing 5 μ g/mL chloramphenicol at 30 °C, 120 rpm until $OD_{600\text{ nm}} = 0.5$. Freshly prepared X-gal (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside) and cells of each strain adjusted to a density of 1×10^7 cells/mL were added to 52–55 °C Mueller Hinton melted agar supplemented with 5 μ g/mL chloramphenicol and poured aseptically. Upon drying, with reasonable distance between treatment groups, 6 μ g of **5c**, **5f** or **5k** was spotted on each plate. Plates were incubated at 30 °C for 20 h in the dark. X-gal was added to respective agar as indicated: 75 μ g/mL, cell wall; 125 μ g/mL, DNA; 250 μ g/mL, RNA and protein. While 3 μ g of clindamycin served as a positive control for the protein reporter strain and a negative control for cell wall, RNA and DNA reporter strains, 0.3 μ g of ciprofloxacin and 6 μ g of rifampicin were used as positive controls for DNA and RNA reporter strains, respectively. For positive control for the cell wall reporter strain and negative control for the protein reporter strain, 6 μ g of vancomycin was used.

Luciferase Reporter Assay. A *B. subtilis* 168 *sacA::pCHlux101* (*P_{liaI}-lux*) culture prepared by inoculating sterile chloramphenicol-supplemented Mueller Hinton medium with an overnight culture was incubated at 30 °C and 120 rpm. At $OD_{600\text{ nm}} = 0.5$, cells were added to opaque-walled microplates, in a total volume of 100 μ L containing 2-fold serially diluted compounds (**5c**, **5f** and **5k**) with a starting concentration of 100 μ M. Plates were immediately transferred to a Tecan plate reader, and luminescence was measured at 30 °C for at least 10 h. Vancomycin and moxifloxacin were used as positive and negative controls, respectively.

MinD Delocalization Assay. Cells of *B. subtilis* expressing GFP-MinD were cultivated in LB medium supplemented with 0.2% glucose at 30 °C and 120 rpm. At $OD_{600\text{ nm}} = 0.25$, with sufficient aeration, cells were exposed to 5-fold MIC of bisindole-alkaloids for 15 min. Twenty-five μ M CCCP served as a positive control. Prior to compound addition, cells were imaged to ascertain their good shape and morphology. Cells were spotted on a 1% agarose pad and visualized with a Zeiss Axio Observer Z1 fluorescence microscope (Zeiss, Jena, Germany) equipped with a HXP 120 V light source and an Axio Cam MR3 camera. Acquired images were processed by using ImageJ software.

Accumulation of UDP-MurNAc-5p. *S. aureus* SG511 cells cultivated in Mueller Hinton medium at 37 °C and 120 rpm were monitored until $OD_{600\text{ nm}} = 0.6$. Ten mL culture was further incubated for 15 min with 130 μ g/mL chloramphenicol to stop protein synthesis before challenging the cells with 2.5-, 5- or 10-fold MIC of bisindoles, with vancomycin as positive control. After 1 h incubation, cells were harvested (4500 rpm, 15 min), resuspended in 250 μ L of sterile distilled water, and boiled for 30 min at 100 °C. Supernatants were transferred to fresh eppis following centrifugation (13,000 rpm, 3 min) of cell suspensions and adjusted to pH 2 with H₃PO₄. Another centrifugation step was completed before transferring supernatants to HPLC vials for detection of the pentapeptide by HPLC.^{5,37}

Determination of Bisindole Effect on *in vitro* Transglycosylation Reaction of Cell Wall Biosynthesis. Impact of bisindoles on the *in vitro* transglycosylation reaction was determined as described elsewhere.⁵ Briefly, a reaction containing a mixture of 2 nmol of lipid II, 100 mM 2-(N-morpholino) ethanesulfonic acid (pH 5.5), 10 mM MgCl₂ and 1.25 mM CaCl₂ in a total volume of 50 μ L was initiated by the addition of PBP2. With respect to the compound to lipid II ratio, compounds were added to the lipid II mixture at 2 and 4 to 1 molar ratio. Following incubation of the resulting mixtures at 30 °C for 2 h, extraction of polyprenyl-containing product was initiated by mixing the suspension with butanol/pyridine acetate (2:1) in a 1:1 ratio, and vortexed for at least 1 min before transferring the organic (upper) phase (13,500 rpm, 3 min) to a fresh eppi. The resulting upper phase was spotted and developed on thin layer chromatography plates, followed by phosphomolybdic acid staining.

Lipid Antagonization Assay. Cells of *B. subtilis* 168 *sacA::pCHlux101* (*P_{liaI}-lux*) were cultivated in Mueller Hinton medium supplemented with 5 μ g/mL chloramphenicol at 30 °C and 120 rpm until $OD_{600\text{ nm}} = 0.5$. In triplicate, in a total volume of 100 μ L, cells were added to opaque-walled microplates containing potential antagonists: lipid II; C₅₅-PP; DOPG; DOPC; cardiolipin; UDP-MurNAc-5p and 2-fold MIC of **5c** and **5f** or 6.25 μ M of vancomycin. Plates were immediately transferred to a Tecan plate reader and luminescence was measured at 30 °C for at least 10 h.

RNA Extraction and Real-Time Quantitative PCR. Exponentially growing cells of *B. subtilis* 168 adjusted to $OD_{600\text{ nm}} = 0.6$ were incubated with 8-fold MIC of bisindoles and harvested after 10 min for RNA extraction. Harvested cells were resuspended in 1 mL of RLT buffer, transferred into Precellys tubes containing a mixture of 0.5 and 0.1 mm glass beads, and lysed by bead beating. RNA extraction was performed using the RNeasy Mini kit (Qiagen) following the manufacturer's protocol. Residual genomic DNA was removed by DNase treatment (Thermo). After an RNA integrity check using RNA 6000 Nano Chip (BMFZ, Düsseldorf), 2 μ g of RNA per sample was reverse transcribed into complementary DNA using the SuperScript III First-Strand Synthesis kit (Thermo Fisher). Gene expression was quantified by performing quantitative real-time PCR (qPCR) in an Mx3005P qPCR system (Agilent Technologies) using GoTaq qPCR Master Mix (Promega). Experiment was performed following the manufacturer's protocol while using gene-specific qPCR primers. Expression values are a product of the $\Delta\Delta C_T$ method, normalized to 16S rRNA using DMSO-treated cells as the control. All qPCR primers (Table S1) were designed by using the PrimerQuest tool.

CF Efflux Assay. Large unilamellar vesicles were prepared by the extrusion technique as described previously.³⁸ In brief, vesicles were made of DOPC (Avanti Polar Lipids) doped with 0.5 mol % lipid II (referring to the total amount of phospholipid). Vesicles loaded with CF (Sigma-Aldrich) were prepared with 50 mM CF and then diluted in 2 mL of TBS buffer (10 mM Tris-HCl, 0.85% w/v NaCl, pH 7.2) at a final concentration of 25 mM phospholipid on a phosphorus base. After the addition of compounds, the increase of fluorescence intensity was measured at 520 nm (excitation at 492 nm) on an RF-5301 spectrophotometer (Shimadzu) at room temperature. Compound-induced CF leakage was expressed relative to the total amount of CF released after vesicle solubilization by the addition of 10 μ L of 20% Triton X-100 (Carl Roth).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsinfecdis.3c00657>.

Impact of varying concentration of bisindoles on the LiaRS stress response system (Figure S1), effect of bisindoles on PBP2-catalyzed transglycosylation reaction (Figure S2), quantification of intracellular accumulation of UDP-MurNAc-5p in bisindoles-treated cells (Figure S3), influence of bisindoles on major biosynthetic pathways using pathway-specific bioreporters (Figure S4), nonantagonistic effects of DOPC, C55-PP and UDP-MurNAc-5p on the inducing capability of bisindoles (Figure S5), oligonucleotides used for RT-qPCR (Table S1), general methods for synthesis of reported compounds and spectroscopic data (PDF)

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Author Contributions

||E.T.A. and M.K. contributed equally to this paper. M.K., T.R.W. and M.B. synthesized compounds. E.T.A. and S.D.B. performed experiments regarding MRSA and *B. subtilis*. K.C.L. conducted CF release assay. V.K. determined cytotoxicity profiles. E.T.A., M.K., S.D.B., K.L., T.S., T.J.J.M. and R.K. designed experiments and analyzed data. E.T.A., M.K., T.J.J.M. and R.K. wrote the manuscript with contributions and edits from all authors.

Notes

The authors declare no competing financial interest.

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Supporting Information

Synthesis of bisindole alkaloids and their mode of action against methicillin-resistant *Staphylococcus aureus*

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Contents of Supplementary Information:

Impact of varying concentration of bisindoles on the LiaRS stress response system (**Figure S1**)

Effect of bisindoles on PBP2-catalysed transglycosylation reaction (**Figure S2**)

Quantification of intracellular accumulation of UDP-MurNAc-5p in bisindoles-treated cells (**Figure S3**)

Influence of bisindoles on major biosynthetic pathways using pathway-specific bioreporters (**Figure S4**)

Non-antagonistic effects of DOPC, C₅₅-PP and UDP-MurNAc-5p on the inducing capability of bisindoles (**Figure S5**)

Oligonucleotides used for RT-qPCR (**Table S1**)

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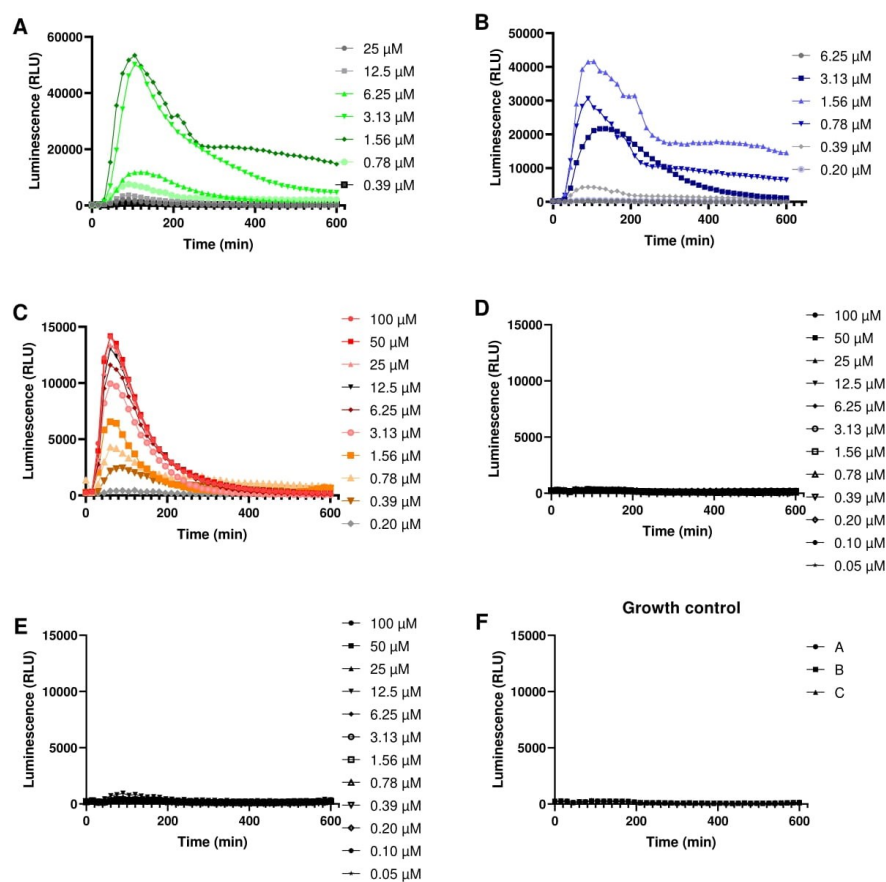


Figure S1. Impact of varying concentrations of bisindoles on the LiaRS cell envelope stress response system. Induction levels of *liaRS* *B. subtilis* reporter strain by different concentrations of (A) 5c, (B) 5f, (C) positive control, vancomycin, (D) negative control, moxifloxacin, (E) inactive bisindole, 5k and (F) growth control. Data shown are results of an experiment repeatable in two independent experiments.

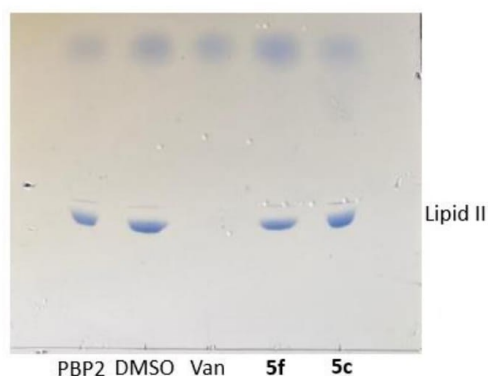


Figure S2. Effect of bisindoles on PBP2-catalysed transglycosylation reaction. Thin layer chromatography analysis of the amount of lipid II converted by PBP2-catalyzed reaction in the presence of bisindoles. Reaction was set up by addition of compounds at two-fold molar excess of lipid II. Vancomycin served as a positive control.

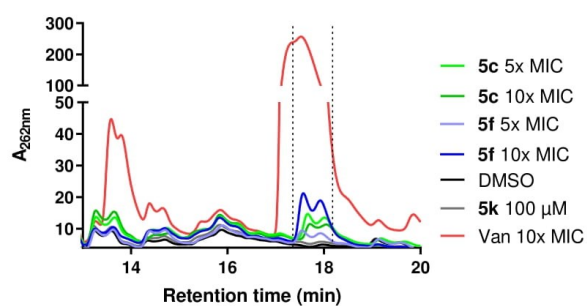


Figure S3. Intracellular accumulation of the ultimate soluble cell wall precursor UDP-MurNAc-5p in bisindole-treated *S. aureus* cells. Influence of bisindoles on the intracellular accumulation of UDP-MurNAc-5p in *S. aureus* cells analyzed by HPLC. Vancomycin served as positive control. Range of breaking lines indicates region of UDP-MurNAc-5p detection. Data shown is a result of an experiment repeatable in two independent experiments.

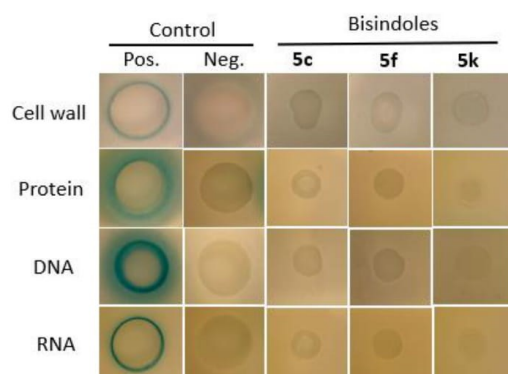


Figure S4. Influence of bisindoles on major cellular biosynthetic pathways using pathway-specific *B. subtilis* bioreporter strains. Specific promoters were fused to *lacZ* gene such that when a specific cellular pathway is interfered with by bisindole, the corresponding promoter is induced, and β -galactosidase is expressed as a green halo that appears around the inhibition zone of bisindole. The selected promoters include *P_{ypuA}* (cell wall), *P_{yheI}* (protein), *P_{yorB}* (DNA) and *P_{yvgS}* (RNA) with vancomycin, clindamycin, ciprofloxacin, and rifampicin as positive controls, respectively. Data shown are results of an experiment repeatable in three independent experiments.

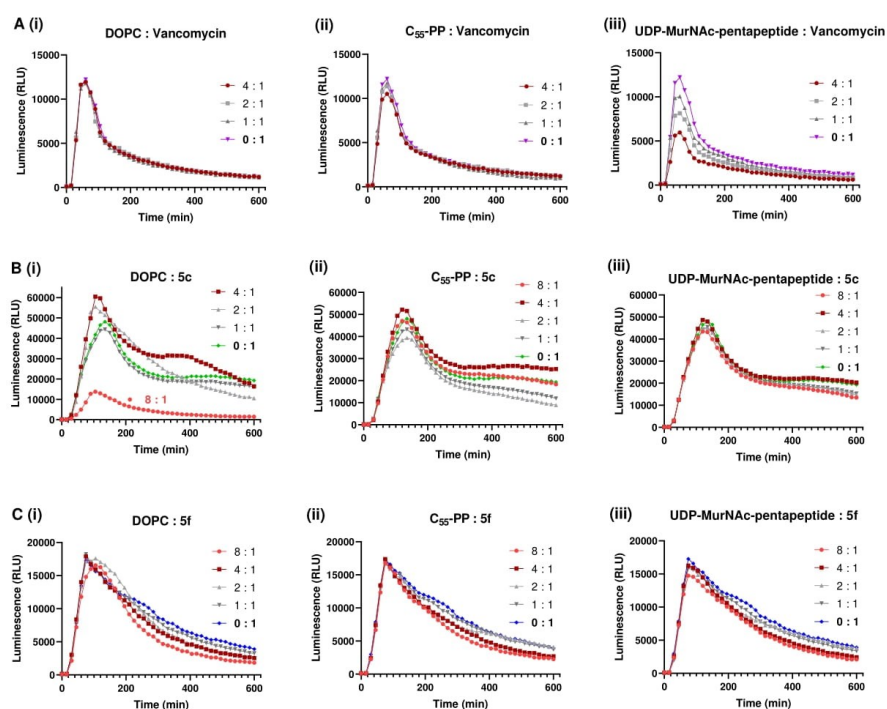


Figure S5. Influence of 1,2 dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), undecaprenyl pyrophosphate of membrane-bound lipid II (C₅₅-PP) and cell wall precursor UDP-MurNAc-5p on

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the LiaRS system inducing capability of bisindoles. Non-antagonistic effect of DOPC, C₅₅-PP and UDP-MurNAc-5p on the LiaRS bioreporter potential of (A) vancomycin, (B) **5c** and (C) **5f**.

Table S1 | Oligonucleotides used for RT-qPCR.

Gene	Forward primer	Reverse primer
<i>liaH</i>	GACGCAGGTGAAGAAGAACTG	CTTTATGCTCAGCCGCCTTC
<i>ytrE</i>	GATGAAGTCGCTTCTATCGG	AACCTCAACGGTAATTCCTC
<i>16SrRNA</i>	GTAGAGATGTGGAGGAACAC	GGGTATCTAATCCTGTTTCGC

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S6.1 General considerations

NMR spectra were recorded using Avance DRX 500, Avance III – 300 and Avance III - 600 (Bruker, Karlsruhe). Chemical shifts are reported in parts per million and the resonance of the solvent was locked as internal standard (DMSO- d_6 : ^1H δ = 2.50, ^{13}C δ = 39.52). Mass spectra were obtained using Varian MAT 311 A, Finnigan MAT 8200, Finnigan TSQ 7000, UHR-QTOF maXis 4G (Bruker Daltonics) and GCMS-QP20105 (Shimadzu). Combustion analyses were carried out on Perkin Elmer Series II Analyser 2400 and Elementarvario MICRO CUBE. The melting points were measured on a Büchi Melting Point B-540 with Kofler's methodology.[1] The starting materials are commercially available or prepared according to literature syntheses.

S6.2 General procedure I (GPI) for the synthesis of *N*-tosyl bisindoles 4

3-Iodo indole **1** (1.0 mmol) and tetrakis(triphenylphosphane)palladium(0) (57 mg, 0.05 mmol, 5 mol%) were dissolved in dry 10.0 mL 1,4-dioxane in a dry Schlenk tube with a magnetic stir bar under dry nitrogen (for experimental details, see Table S1). After the addition of dry triethylamine (1.40 mL, 10 mmol) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (**2**) (pinacolyl borane, HBpin) (0.25 mL, 1.7 mmol) the solution was stirred at 80 °C (oil bath) for 3 h. After cooling to room temp, water (0.02 mL, 0.7 mmol), cesium carbonate (814 mg, 2.5 mmol), and the corresponding heteroaryl dihalide **3** (0.5 mmol) were added to the reaction mixture. The reaction mixture was stirred at 100 °C (oil bath) for 18-20 h. After cooling to room temp, the mixture was adsorbed onto Celite® under reduced pressure and the *N*-tosyl bisindoles **4** were isolated and purified by column chromatography on silica gel (eluent: *n*-hexane/acetone 9:1). The resulting solids were dried in vacuo for 24 h. The derivatives were additionally purified by trituration in ethanol by ultrasonication.

Table S2. Indoles **1**, heteroaryl dihalides **3** and yields in the synthesis of *N*-tosyl bisindoles **4**.

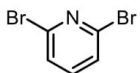
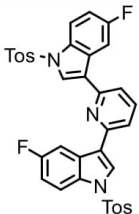
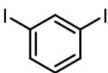
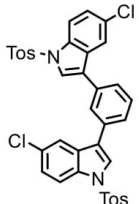
entry	indole 1 [mg] (mmol)	heteroaryl dihalide 3 [mg] (mmol)	<i>N</i> -tosyl bisindole 4	yield [mg] (%)
1	415 mg (1.0 mmol) of 5-fluoro-3-iodo-1-tosyl-1 <i>H</i> -indole (1a)	 118 mg (0.50 mmol) of 3a		282 mg (86%) of 4a
2	431 mg (1.0 mmol) of 5-chloro-3-iodo-1-tosyl-1 <i>H</i> -indole (1b)	 165 mg (0.50 mmol) of 3b		250 mg (73%) of 4b

Table S2. Indoles **1**, heteroaryl dihalides **3** and yields in the synthesis of *N*-tosyl bisindoles **4**.

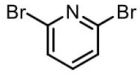
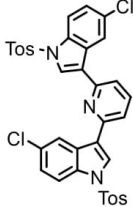
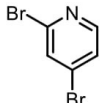
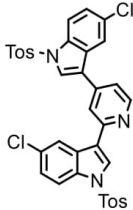
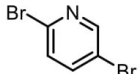
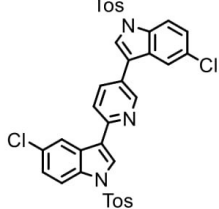
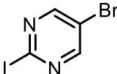
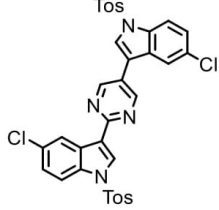
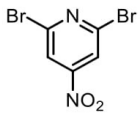
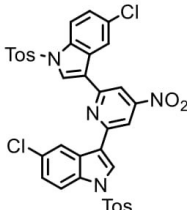
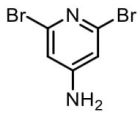
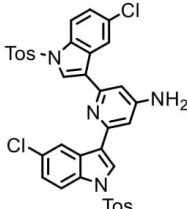
entry	indole 1 [mg] (mmol)	heteroaryl dihalide 3 [mg] (mmol)	<i>N</i> -tosyl bisindole 4	yield [mg] (%)
3	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 118 mg (0.50 mmol) of 3a		227 mg (66%) of 4c
4	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 118 mg (0.50 mmol) of 3c		330 mg (96%) of 4d
5	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 118 mg (0.50 mmol) of 3d		213 mg (62%) of 4e
6	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 142 mg (0.50 mmol) of 3e		258 mg (75%) of 4f
7	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 141 mg (0.50 mmol) of 3f		156 mg (43%) of 4g
8	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 126 mg (0.50 mmol) of 3g		179 mg (51%) of 4h

Table S2. Indoles **1**, heteroaryl dihalides **3** and yields in the synthesis of *N*-tosyl bisindoles **4**.

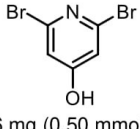
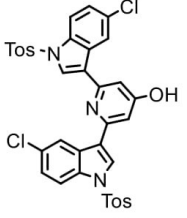
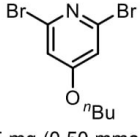
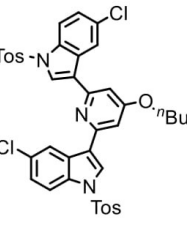
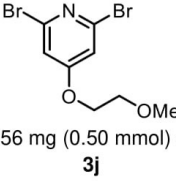
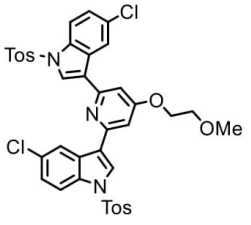
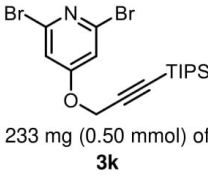
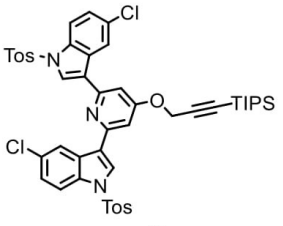
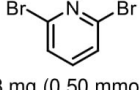
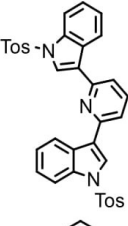
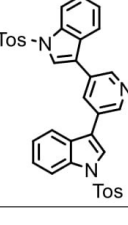
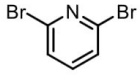
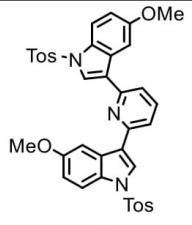
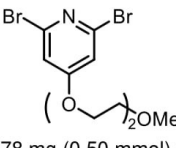
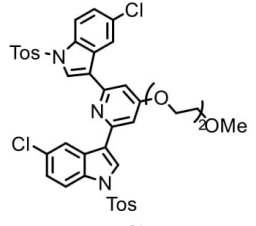
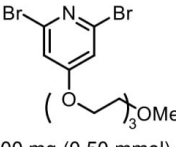
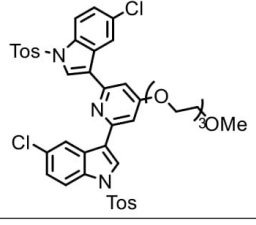
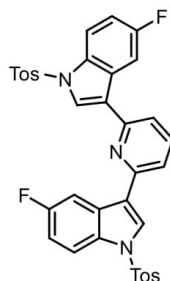
entry	indole 1 [mg] (mmol)	heteroaryl dihalide 3 [mg] (mmol)	<i>N</i> -tosyl bisindole 4	yield [mg] (%)
9	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 126 mg (0.50 mmol) of 3h		170 mg (48%) of 4i
10	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 155 mg (0.50 mmol) of 3i		193 mg (51%) of 4j
11	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 156 mg (0.50 mmol) of 3j		221 mg (58%) of 4k
12	431 mg (1.0 mmol) of 5-chloro-3- iodo-1-tosyl- 1 <i>H</i> -indole (1b)	 233 mg (0.50 mmol) of 3k		403 mg (90%) of 4l
13	397 mg (1.0 mmol) of 3-iodo-1-tosyl- 1 <i>H</i> -indole (1c)	 118 mg (0.50 mmol) of 3a		229 mg (73%) of 4m
14	397 mg (1.0 mmol) of 3-iodo-1-tosyl- 1 <i>H</i> -indole (1c)	 118 mg (0.50 mmol) of 3l		292 mg (85%) of 4n

Table S2. Indoles **1**, heteroaryl dihalides **3** and yields in the synthesis of *N*-tosyl bisindoles **4**.

entry	indole 1 [mg] (mmol)	heteroaryl dihalide 3 [mg] (mmol)	<i>N</i> -tosyl bisindole 4	yield [mg] (%)
15	427 mg (1.0 mmol) of 3-iodo-5-methoxy-1- <i>H</i> -indole (1d)	 118 mg (0.50 mmol) of 3a		108 mg (32%) of 4o
16	431 mg (1.0 mmol) of 5-chloro-3-iodo-1- <i>H</i> -indole (1b)	 178 mg (0.50 mmol) of 3m		293 mg (73%) of 4p
17	431 mg (1.0 mmol) of 5-chloro-3-iodo-1- <i>H</i> -indole (1b)	 200 mg (0.50 mmol) of 3n		344 mg (81%) of 4q

S6.3 Spectroscopic data of *N*-tosyl bisindoles 4

2,6-Bis(5-fluoro-1-tosyl-1*H*-indol-3-yl)pyridine (4a)



4a
[653.72]
 $C_{35}H_{25}F_2N_3O_4S_2$

Mp.: 207 °C.

R_f (*n*-hexane/acetone 7:3): 0.31.

¹H NMR (acetone-*d*₆, 300 MHz): δ 2.34 (s, 6 H), 7.24 (td, *J* = 9.0, 9.0, 2.7 Hz, 2 H), 7.37–7.42 (m, 4 H), 7.94–7.97 (m, 3 H), 7.97–8.03 (m, 4 H), 8.12 (dd, *J* = 9.1, 4.5 Hz, 2 H), 8.23 (dd, *J* = 9.8, 2.6 Hz, 2 H), 8.50 (s, 2 H).

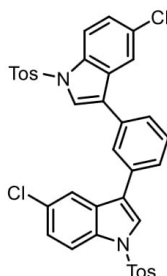
¹³C NMR (acetone-*d*₆, 75 MHz): δ 21.5 (CH₃), 109.1 (CH, *d*, *J* = 25.6 Hz), 113.9 (CH, *d*, *J* = 26.0 Hz), 115.8 (CH, *d*, *J* = 9.6 Hz), 120.6 (CH), 123.3 (C_{quat}, *d*, *J* = 4.3 Hz), 128.0 (CH), 128.42 (CH), 130.7 (C_{quat}, *d*, *J* = 10.6 Hz), 131.2 (CH), 133.0 (C_{quat}), 135.6 (C_{quat}), 138.6 (CH), 147.0 (C_{quat}), 153.14 (C_{quat}), 106.9 (C_{quat}, *d*, *J* = 238.8 Hz).

IR: $\tilde{\nu}$ [cm⁻¹]: 634 (m), 661 (s), 673 (s), 736 (w), 771 (w), 794 (m), 842 (m), 889 (m), 975 (w), 1014 (w), 1039 (w), 1087 (m), 1128 (s), 1145 (m), 1168 (s), 1193 (w), 1257 (w), 1292 (w), 1371 (w), 1442 (w), 1469 (w), 1552 (w), 1566 (w), 1589 (w).

MS (EI, *m/z* (%)): 655 ([M]⁺, 10), 654 ([M]⁺, 34), 653 (90), 499 ([C₂₈H₁₈F₂N₃O₂S]⁺, 30), 498 ([C₂₈H₁₈F₂N₃O₂S]⁺, 93), 434 (36), 345 (12), 344 ([C₂₁H₁₁F₂N₃]⁺, 39), 343 ([C₂₁H₁₁F₂N₃]⁺, 100), 342 (26), 341 (10), 324 ([C₂₁H₁₁FN₃]⁺, 19), 323 (33), 322 (17), 316 (17), 315 (19), 296 (12), 155 ([C₇H₇O₂S]⁺, 12), 92 (11), 91 ([C₇H₇]⁺, 83), 65 (26).

Anal. calcd. for C₃₅H₂₅F₂N₃O₄S₂ [653.7]: C 64.31, H 3.85, N 6.43, S 9.81; **Found:** C 64.51, H 4.35, N 5.99, S 9.35.

1,3-Bis(5-chloro-1-tosyl-1*H*-indol-3-yl)benzene (4b)



4b
[685.63]
 $C_{36}H_{26}Cl_2N_2O_4S_2$

Mp.: 220 °C

R_f (*n*-hexane/acetone 7:3): 0.47.

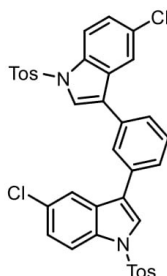
¹H NMR (acetone-*d*₆, 300 MHz): δ 2.37 (s, 6 H), 7.38–7.48 (m, 6 H), 7.66 (dd, *J* = 8.6, 6.6 Hz, 1 H), 7.72–7.80 (m, 2 H), 7.87 (d, *J* = 2.1 Hz, 2 H), 7.96–8.02 (m, 4 H), 8.02–8.06 (m, 1 H), 8.12 (d, *J* = 8.9 Hz, 2 H), 8.15 (s, 2 H).

¹³C NMR (acetone-*d*₆, 75 MHz): δ 21.7 (CH₃), 116.1 (C_{quat}), 120.8 (CH), 123.7 (C_{quat}), 125.9 (CH), 126.2 (CH), 127.9 (CH), 128.1 (CH), 128.1 (CH), 130.3 (CH), 130.6 (CH), 131.0 (CH), 131.3 (C_{quat}), 134.1 (C_{quat}), 134.7 (C_{quat}), 135.7 (C_{quat}), 146.6 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 667 (s), 690 (w), 702 (m), 756 (w), 771 (w), 790 (m), 810 (w), 869 (w), 962 (w), 1012 (w), 1035 (w), 1070 (w), 1089 (m), 1114 (m), 1139 (m), 1147 (m), 1174 (m), 1188 (w), 1228 (w), 1292 (w), 1371 (w), 1438 (w), 1597 (w), 2854 (w), 2868 (w), 2924 (w), 2953 (w). **MS (ESI, *m/z* (%)):** 704 ([³⁷Cl³⁵ClIM+NH₄]⁺, 88), 702 ([³⁵Cl³⁵ClIM+NH₄]⁺, 100), 685 ([³⁷Cl³⁵ClIM+H]⁺, 47), 687 ([³⁵Cl³⁵ClIM+H]⁺, 63).

HR-MS (ESI) (*m/z*) calcd for (C₃₆H₂₆³⁷Cl³⁵ClN₂O₄S₂+H)⁺: 678.0754; **Found:** 678.0757; **calcd for (C₃₆H₂₆³⁵Cl³⁵ClN₂O₄S₂+H)⁺:** 685.0784; **Found:** 685.0780.

1,3-Bis(5-chloro-1-tosyl-1*H*-indol-3-yl)benzene (4b)



4b
[685.63]
 $C_{36}H_{26}Cl_2N_2O_4S_2$

Mp.: 220 °C

R_f (*n*-hexane/acetone 7:3): 0.47.

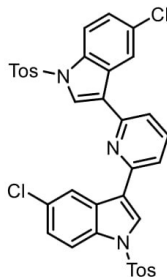
¹H NMR (acetone-*d*₆, 300 MHz): δ 2.37 (s, 6 H), 7.38–7.48 (m, 6 H), 7.66 (dd, *J* = 8.6, 6.6 Hz, 1 H), 7.72–7.80 (m, 2 H), 7.87 (d, *J* = 2.1 Hz, 2 H), 7.96–8.02 (m, 4 H), 8.02–8.06 (m, 1 H), 8.12 (d, *J* = 8.9 Hz, 2 H), 8.15 (s, 2 H).

¹³C NMR (acetone-*d*₆, 75 MHz): δ 21.7 (CH₃), 116.1 (C_{quat}), 120.8 (CH), 123.7 (C_{quat}), 125.9 (CH), 126.2 (CH), 127.9 (CH), 128.1 (CH), 128.1 (CH), 130.3 (CH), 130.6 (CH), 131.0 (CH), 131.3 (C_{quat}), 134.1 (C_{quat}), 134.7 (C_{quat}), 135.7 (C_{quat}), 146.6 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 667 (s), 690 (w), 702 (m), 756 (w), 771 (w), 790 (m), 810 (w), 869 (w), 962 (w), 1012 (w), 1035 (w), 1070 (w), 1089 (m), 1114 (m), 1139 (m), 1147 (m), 1174 (m), 1188 (w), 1228 (w), 1292 (w), 1371 (w), 1438 (w), 1597 (w), 2854 (w), 2868 (w), 2924 (w), 2953 (w). **MS (ESI, *m/z* (%)):** 704 ([³⁷Cl³⁵ClIM+NH₄]⁺, 88), 702 ([³⁵Cl³⁵ClIM+NH₄]⁺, 100), 685 ([³⁷Cl³⁵ClIM+H]⁺, 47), 687 ([³⁵Cl³⁵ClIM+H]⁺, 63).

HR-MS (ESI) (*m/z*) calcd for (C₃₆H₂₆³⁷Cl³⁵ClN₂O₄S₂+H)⁺: 678.0754; **Found:** 678.0757; **calcd for (C₃₆H₂₆³⁵Cl³⁵ClN₂O₄S₂+H)⁺:** 685.0784; **Found:** 685.0780.

2,6-Bis(5-chloro-1-tosyl-1*H*-indol-3-yl)pyridine (4c)



4c
[686.62]
 $C_{35}H_{25}Cl_2N_3O_4S_2$

Mp.: 194 °C.

R_f (*n*-hexane/acetone 7:3): 0.38.

¹H NMR (acetone-*d*₆, 600 MHz): δ 2.34 (s, 6 H), 7.39–7.41 (m, 4 H), 7.44 (dd, *J* = 8.8, 2.2 Hz, 2 H), 7.96–7.98 (m, 3 H), 8.00–8.02 (m, 4 H), 8.11 (d, *J* = 8.9 Hz, 2 H), 8.50 (s, 2 H), 8.57 (d, *J* = 2.1 Hz, 2 H).

¹³C NMR (acetone-*d*₆, 150 MHz): δ 21.5 (CH₃), 115.8 (CH), 120.7 (CH), 122.8 (C_{quat}), 123.2 (CH), 126.1 (CH), 128.0 (CH), 128.1 (CH), 130.4 (C_{quat}), 130.8 (C_{quat}), 131.2 (CH), 134.9 (C_{quat}), 135.5 (C_{quat}), 138.6 (CH), 147.1 (C_{quat}), 153.1 (C_{quat}).

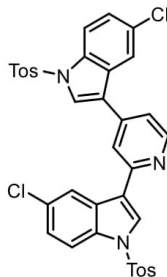
IR: $\tilde{\nu}$ [cm⁻¹]: 654 (w), 667 (s), 703 (m), 756 (w), 772 (w), 787 (m), 793 (m), 810 (w), 835 (w), 878 (w), 964 (w), 1015 (w), 1042 (w), 1070 (w), 1080 (m), 1090 (m), 1119 (m), 1148 (m), 1165 (m), 1290 (w), 1371 (w), 1435 (w), 1464 (w), 1553 (w), 1574 (w), 1595 (w), 2924 (w).

MS (EI, *m/z* (%)): 688 ([³⁷Cl³⁵ClM+H]⁺, 10), 687 ([³⁷Cl³⁵ClM]⁺, 30), 686 ([³⁵Cl³⁵ClM+H]⁺, 20), 685 ([³⁵Cl³⁵ClM]⁺, 43), 533 ([C₂₈H₁₉³⁷Cl³⁵ClN₃O₂S]⁺, 14), 532 ([C₂₈H₁₈³⁷Cl³⁵ClN₃O₂S]⁺, 38), 531 ([C₂₈H₁₉³⁵Cl³⁵ClN₃O₂S]⁺, 17), 530 ([C₂₈H₁₈³⁵Cl³⁵ClN₃O₂S]⁺, 49), 466 (11), 378 ([C₂₁H₁₂³⁷Cl³⁵ClN₃]⁺, 24), 377 ([C₂₁H₁₁³⁷Cl³⁵ClN₃]⁺, 34), 376 ([C₂₁H₁₂³⁵Cl³⁵ClN₃]⁺, 24), 375 ([C₂₁H₁₁³⁵Cl³⁵ClN₃]⁺, 50), 342 ([C₂₁H₁₁³⁷ClN₃]⁺, 12), 341 ([C₂₁H₁₂³⁵ClN₃]⁺, 16), 340 ([C₂₁H₁₁³⁵ClN₃]⁺, 35), 339 ([C₂₁H₁₀³⁵ClN₃]⁺, 20), 313 (21), 306 ([C₂₁H₁₂N₃]⁺, 56), 305 ([C₂₁H₁₁N₃]⁺, 56), 304 ([C₂₁H₁₀N₃]⁺, 56), 278 (17), 277 (14), 155 ([C₇H₇O₂S]⁺, 21), 91 ([C₇H₇]⁺, 100), 65 ([C₅H₅]⁺, 30).

HR-MS (ESI) (*m/z*) calcd for (C₃₅H₂₅³⁷Cl³⁵ClN₃O₄S₂+H)⁺: 688.0707; Found: 688.0709; **calcd for (C₃₅H₂₅³⁵Cl³⁵ClN₃O₄S₂+H)⁺:** 686.0736; Found: 686.0732.

Anal. calcd. for C₃₅H₂₅Cl₂N₃O₄S₂ [686.6]: C 61.23, H 3.67, N 6.12, S 9.32; **Found.:** C 61.47, H 3.95, N 5.84, S 9.03.

3,3'-(Pyridin-2,4-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (4d)



4d
[686.62]
 $C_{35}H_{25}Cl_2N_3O_4S_2$

Mp.: 204 °C.

R_f (*n*-hexane/acetone 7:3): 0.47.

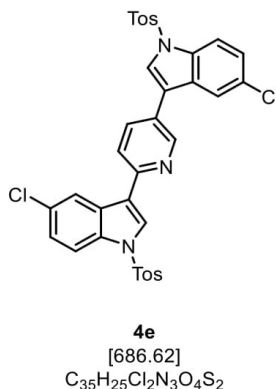
¹H NMR (acetone-*d*₆, 300 MHz): δ 2.34 (s, 3 H), 2.38 (s, 3 H), 7.36–7.52 (m, 6 H), 7.70 (dd, *J* = 5.2, 1.7 Hz, 1 H), 7.92–8.03 (m, 5 H), 8.08 (d, *J* = 8.9 Hz, 1 H), 8.13 (d, *J* = 8.9 Hz, 1 H), 8.38 (s, 1 H), 8.42–8.46 (m, 1 H), 8.65 (s, 1 H), 8.76 (d, *J* = 2.2 Hz, 1 H), 8.79 (dd, *J* = 5.1, 0.9 Hz, 1 H).

¹³C NMR (acetone-*d*₆, 75 MHz): δ 21.5 (CH₃), 21.5 (CH₃), 115.6 (CH), 116.2 (CH), 120.3 (CH), 121.0 (CH), 121.3 (C_{quat}), 121.4 (CH), 122.5 (C_{quat}), 124.0 (CH), 126.0 (CH), 126.3 (CH), 128.0 (CH), 128.1 (CH), 128.1 (C_{quat}), 128.3 (CH), 130.1 (C_{quat}), 130.5 (C_{quat}), 130.5 (C_{quat}), 131.2 (CH), 131.2 (CH), 134.8 (C_{quat}), 135.0 (C_{quat}), 135.4 (C_{quat}), 135.5 (C_{quat}), 141.7 (C_{quat}), 147.0 (C_{quat}), 147.2 (C_{quat}), 151.0 (CH), 154.4 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 667 (s), 829 (w), 966 (w), 1089 (w), 1114 (w), 1155 (m), 1371 (w), 1438 (w), 1600 (w).

HR-MS (ESI) (*m/z*) calcd for (C₃₅H₂₅³⁷Cl³⁵CIN₃O₄S₂+H)⁺: 688.0707; **Found:** 688.0707; **calcd for (C₃₅H₂₅³⁵Cl₂N₃O₄S₂+H)⁺:** 686.0736; **Found:** 686.0731.

3,3'-(Pyridin-2,5-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (4e)



Mp.: 305 °C (combustion).

R_f (*n*-hexane/acetone 7:3): 0.48.

¹H NMR (acetone-*d*₆, 300 MHz): δ 2.39 (s, 3 H), 2.40 (s, 3 H), 7.35–7.50 (m, 6 H), 7.86 (d, *J* = 2.1 Hz, 1 H), 7.91–7.99 (m, 4 H), 8.05 (d, *J* = 8.9 Hz, 1 H), 8.10 (d, *J* = 8.9 Hz, 1 H), 8.13–8.20 (m, 3 H), 8.46 (s, 1 H), 8.74 (d, *J* = 2.2 Hz, 1 H), 9.02 (dd, *J* = 2.2, 1.1 Hz, 1 H).

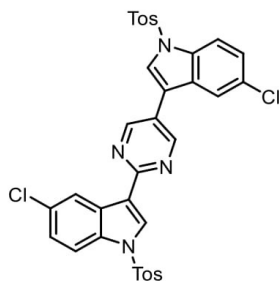
¹³C NMR (acetone-*d*₆, 75 MHz): δ 21.8 (CH₃), 105.6 (C_{quat}), 106.1 (C_{quat}), 115.4 (CH), 116.1 (CH), 120.4 (C_{quat}), 120.6 (CH), 121.8 (CH), 122.2 (C_{quat}), 124.0 (CH), 125.9 (CH), 126.1 (CH), 126.3 (CH), 127.3 (C_{quat}), 127.6 (C_{quat}), 127.9 (CH), 128.0 (CH), 130.3 (C_{quat}), 130.5 (C_{quat}), 130.9 (C_{quat}), 130.9 (C_{quat}), 131.0 (CH), 131.0 (CH), 134.4 (C_{quat}), 134.8 (C_{quat}), 135.6 (C_{quat}), 135.6 (C_{quat}), 136.1 (CH), 146.6 (C_{quat}), 148.8 (CH).

IR: $\tilde{\nu}$ [cm⁻¹]: 669 (s), 702 (w), 759 (m), 788 (s), 813 (w), 842 (w), 860 (w), 887 (w), 943 (w), 1006 (m), 1031 (m), 1053 (w), 1078 (m), 1089 (s), 1114 (m), 1155 (s), 1176 (m), 1192 (w), 1249 (w), 1273 (w), 1334 (w), 1369 (m), 1440 (w), 1581 (w), 1597 (w), 2885 (w), 2972 (w), 2985 (w).

MS (EI, *m/z* (%)): 687 ([³⁷Cl³⁵CIM]⁺, 9), 685 ([³⁵Cl³⁵CIM]⁺, 13), 533 (12), 532 ([C₂₈H₁₈³⁷Cl³⁵CIN₃O₂S]⁺, 34), 531 (18), 530 ([C₂₈H₁₈³⁵Cl³⁵CIN₃O₂S]⁺, 51), 378 (14), 377 ([C₂₁H₁₁³⁷Cl³⁵CIN₃]⁺, 29), 376 (24), 375 ([C₂₁H₁₁³⁵Cl³⁵CIN₃]⁺, 35), 350 (10), 348 (15), 340 (10), 278 (10), 227 (14), 156 (11), 155 ([C₇H₇O₂S]⁺, 47), 92 (16), 91 ([C₇H₇]⁺, 100), 65 (26).

HR-MS (ESI) (*m/z*) calcd for (C₃₅H₂₅³⁷Cl³⁵CIN₃O₄S₂+H)⁺: 688.0707; **Found:** 688.0706; **calcd for (C₃₅H₂₅³⁵Cl³⁵CIN₃O₄S₂+H)⁺:** 686.0737; **Found:** 686.0730.

3,3'-(Pyrimidin-2,5-diyl)bis(5-chloro-1-tosyl-1H-indole) (4f)



4f
[687.61]
 $C_{34}H_{24}Cl_2N_4O_4S_2$

Mp.: 310 °C.

R_f (*n*-hexane/acetone 7:3): 0.24.

¹H NMR (acetone-*d*₆+CS₂, 300 MHz): δ 2.41 (s, 6 H), 7.39–7.48 (m, 6 H), 7.92 (d, *J* = 2.0 Hz, 1 H), 7.95–8.02 (m, 4 H), 8.06 (d, *J* = 8.9 Hz, 1 H), 8.11 (d, *J* = 8.9 Hz, 1 H), 8.28 (s, 1 H), 8.58 (s, 1 H), 8.75 (d, *J* = 2.2 Hz, 1 H), 9.21 (s, 2 H).

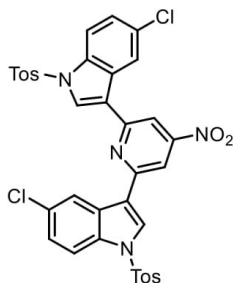
¹³C NMR (acetone-*d*₆, 150 MHz): δ 21.8 (CH₃), 115.6 (CH), 116.1 (CH), 117.4 (C_{quat}), 120.7 (CH), 121.5 (C_{quat}), 123.9 (CH), 125.1 (C_{quat}), 126.1 (CH), 126.4 (CH), 127.0 (CH), 128.1 (CH), 130.5 (C_{quat}), 130.6 (C_{quat}), 130.7 (CH), 131.0 (CH), 131.1 (CH), 131.2 (C_{quat}), 134.7 (C_{quat}), 134.9 (C_{quat}), 135.6 (C_{quat}), 135.6 (C_{quat}), 146.8 (C_{quat}), 147.0 (C_{quat}), 156.3 (C_{quat}), 161.2 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 626 (m), 669 (s), 702 (w), 759 (s), 788 (s), 810 (m), 883 (w), 954 (w), 1004 (w), 1033 (m), 1089 (s), 1109 (s), 1161 (s), 1192 (w), 1244 (w), 1278 (w), 1336 (w), 1361 (w), 1377 (w), 1425 (w), 1438 (w), 1462 (w), 1523 (w), 1550 (w), 1595 (w), 2883 (w), 2900 (w), 2922 (w), 2972 (w), 2985 (w), 3142 (w).

MS (ESI, *m/z* (%)): 689 ([³⁷C³⁵CIM+H]⁺, 90), 687 ([³⁵Cl³⁵CIM+H]⁺, 100).

Anal. calcd. for C₃₄H₂₄Cl₂N₄O₄S₂ [687.6]: C 59.39, H 3.52, N 8.15, S 9.33; **Found:** C 59.56, H 3.65, N 7.97, S 9.56.

3,3'-(4-Nitropyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (4g)



4g

[731.62]

C₃₅H₂₄Cl₂N₄O₆S₂

Mp.: 259 °C.

R_f (*n*-hexane/acetone 7:3): 0.46.

¹H NMR (acetone-*d*₆, 300 MHz): δ 2.40 (s, 6H), 7.41 (ddd, *J* = 8.6, 3.2, 1.4 Hz, 6 H), 7.97–8.03 (m, 4 H), 8.08 (d, *J* = 8.9 Hz, 2 H), 8.51 (d, *J* = 2.1 Hz, 2 H), 8.57 (s, 2 H), 8.77 (s, 2 H).

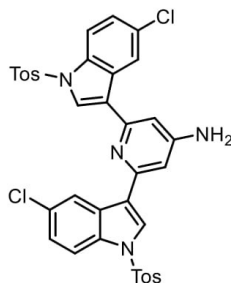
¹³C NMR (acetone-*d*₆, 75 MHz): δ 22.8 (CH₃), 113.4 (CH), 116.6 (CH), 122.3 (C_{quat}), 124.1 (CH), 127.4 (CH), 129.1 (CH), 130.7 (CH), 131.2 (C_{quat}), 131.9 (C_{quat}), 132.1 (CH), 135.7 (C_{quat}), 136.5 (C_{quat}), 147.9 (C_{quat}), 156.9 (C_{quat}), 157.28 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 669 (s), 694 (w), 706 (m), 756 (m), 790 (m), 812 (m), 835 (m), 880 (w), 895 (w), 932 (w), 955 (w), 968 (m), 1016 (w), 1030 (w), 1051 (w), 1072 (w), 1090 (m), 1109 (m), 1121 (m), 1155 (s), 1175 (m), 1211 (w), 1244 (w), 1283 (w), 1304 (w), 1327 (w), 1344 (m), 1375 (s), 1400 (w), 1439 (w), 1533 (m).

MS (EI, *m/z* (%)): 732 ([³⁷Cl³⁵ClIM], 19), 730 ([³⁵Cl³⁵ClIM]⁺, 23), 577 ([C₂₈H₁₇³⁷Cl³⁵ClN₄O₄S]⁺, 12), 575 ([C₂₈H₁₇³⁵Cl³⁵ClN₄O₄S]⁺, 17), 339 ([C₂₁H₁₀³⁵ClN]⁺, 18), 155 ([C₇H₇O₂S]⁺, 45), 91 ([C₇H₇]⁺, 18).

HR-MS (ESI) (*m/z*) calcd for (C₃₅H₂₄³⁷Cl³⁵ClN₄O₆S₂+H)⁺: 733.0558; **Found:** 733.0581; **calcd for (C₃₅H₂₄³⁵Cl³⁵ClN₄O₆S₂+H)⁺:** 731.0588; **Found:** 731.0581.

2,6-Bis(5-chloro-1-tosyl-1*H*-indol-3-yl)pyridin-4-amine (4h)



4h
[701.64]
 $C_{35}H_{26}Cl_2N_4O_4S_2$

Mp.: 188 °C.

R_f (*n*-hexane/acetone 7:3): 0.36.

¹H NMR (acetone-*d*₆, 300 MHz): δ 2.35 (s, 6 H), 5.69 (s, 2 H), 7.20 (s, 2 H), 7.41 (ddd, *J* = 8.6, 4.4, 1.5 Hz, 6 H), 7.95–8.01 (m, 4 H), 8.09 (d, *J* = 8.9 Hz, 2 H), 8.29 (s, 2 H), 8.53 (d, *J* = 2.1 Hz, 2 H).

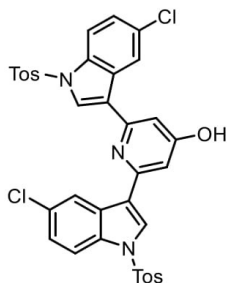
¹³C NMR (acetone-*d*₆, 75 MHz): δ 21.5 (CH₃), 106.1 (CH), 106.2 (CH), 115.8 (CH), 123.3 (CH), 123.7 (C_{quat}), 125.8 (CH), 127.2 (CH), 128.0 (CH), 130.2 (C_{quat}), 131.2 (CH), 134.9 (C_{quat}), 135.6 (C_{quat}), 147.0 (C_{quat}), 153.1 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 665 (s), 708 (m), 746 (w), 837 (w), 968 (w), 1092 (m), 1120 (m), 1148 (m), 1163 (m), 1287 (w), 1362 (w), 1435 (m), 1599 (w).

MS (EI, *m/z* (%)): 702 ([³⁷Cl³⁵ClM]⁺, 19), 701 ([³⁷Cl³⁵ClM+H]⁺, 30), 700 ([³⁵Cl³⁵ClM], 30), 699 ([³⁵Cl³⁵ClC₃₅H₂₅N₄O₄S₂]⁺, 25), 547 ([³⁷Cl³⁵ClC₂₈H₁₉N₄O₄S]⁺, 18), 546 ([³⁵Cl³⁵ClC₂₈H₂₀N₄O₄S]⁺, 16), 545 ([³⁵Cl³⁵ClC₂₈H₁₉N₄O₄S]⁺, 29), 481 (15), 394 ([³⁷Cl³⁷ClC₂₁H₁₂N₄]⁺, 10), 393 ([³⁷Cl³⁵ClC₂₁H₁₃N₄]⁺, 16), 392 ([³⁷Cl³⁵ClC₂₁H₁₂N₄]⁺, 35), 391 ([³⁵Cl³⁵ClC₂₁H₁₃N₄]⁺, 22), 390 ([³⁵Cl³⁵ClC₂₁H₁₂N₄]⁺, 43), 357 ([³⁷ClC₂₁H₁₃N₄]⁺, 14), 356 ([³⁵ClC₂₁H₁₄N₄]⁺, 17), 355 ([³⁵ClC₂₁H₁₃N₄]⁺, 55), 328 (12), 320 ([C₂₁H₁₂N₄]⁺, 36), 319 ([C₂₁H₁₁N₄]⁺, 22), 156 ([C₇H₈O₂S]⁺, 17), 155 ([C₇H₇O₂S]⁺, 16), 139 (19), 92 ([C₇H₈]⁺, 30), 91 ([C₇H₇]⁺, 100), 89 (10), 77 (12), 65 ([C₅H₅]⁺, 40), 57 (10).

Anal. calcd. for C₃₅H₂₆Cl₂N₄O₄S₂ [701.6]: C 59.91, H 3.74, N 7.99, S 9.14; **Found:** C 59.72, H 3.91, N 7.75, S 8.87.

2,6-Bis(5-chloro-1-tosyl-1*H*-indol-3-yl)pyridin-4-ol (4i)



4i
[702.62]
 $C_{35}H_{25}Cl_2N_3O_5S_2$

Mp.: 205 °C.

R_f (*n*-hexane/acetone **7:3**): 0.18.

¹H NMR (acetone-**d**₆, **600 MHz**): δ 2.32 (s, 6 H), 7.38 (d, *J* = 8.2 Hz, 4 H), 7.40–7.44 (m, 4 H), 8.00 (d, *J* = 8.5 Hz, 4 H), 8.10 (d, *J* = 8.8 Hz, 2 H), 8.42 (s, 2 H), 8.53 (d, *J* = 2.1 Hz, 2 H), 9.73 (s, 1 H).

¹³C NMR (acetone-**d**₆, **150 MHz**): δ 21.6 (CH₃), 108.5 (CH), 115.9 (CH), 123.0 (C_{quat}), 123.3 (CH), 126.1 (CH), 128.0 (CH), 128.1 (CH), 130.4 (C_{quat}), 131 (C_{quat}), 131.3 (CH), 135.0 (C_{quat}), 135.6 (C_{quat}), 147.1 (C_{quat}), 154.6 (C_{quat}), 166.0 (C_{quat}).

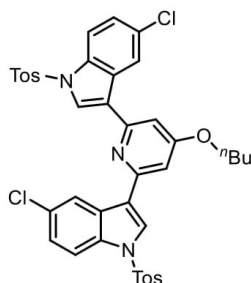
IR: $\tilde{\nu}$ [cm⁻¹]: 667 (w), 708 (m), 745 (m), 773 (w), 783 (w), 804 (m), 839 (w), 860 (w), 910 (w), 968 (m), 1003 (w), 1034 (w), 1090 (m), 1119 (m), 1150 (m), 1159 (m), 1177 (m), 1285 (w), 1327 (w), 1371 (m), 1439 (w), 1493 (w), 1557 (w), 1580 (w), 1597 (w), 2922 (w), 2951 (w).

MS (EI, m/z (%)): 703 ([³⁷Cl³⁵ClM]⁺, 19), 702 ([³⁵Cl³⁵ClM+H]⁺, 13), 701 ([³⁵Cl³⁵ClM]⁺, 38), 549 ([³⁷Cl³⁵ClC₂₈H₁₉N₃O₃S]⁺, 13), 548 ([³⁷Cl³⁵ClC₂₈H₁₈N₃O₃S]⁺, 23), 547 ([³⁵Cl³⁵ClC₂₈H₁₉N₃O₃S]⁺, 23), 546 ([³⁵Cl³⁵ClC₂₈H₁₈N₃O₃S]⁺, 32), 482 (13), 395 (10), 394 ([³⁷Cl³⁵ClC₂₁H₁₂N₃O]⁺, 21), 393 ([³⁷Cl³⁵ClC₂₁H₁₁N₃O]⁺, 30), 392 ([³⁵Cl³⁵ClC₂₁H₁₂N₃O]⁺, 33), 391 ([³⁵Cl³⁵ClC₂₁H₁₁N₃O]⁺, 30), 358 ([³⁵ClC₂₁H₁₁N₃O]⁺, 10), 357 ([³⁵ClC₂₁H₁₂N₃O]⁺, 14), 356 ([³⁵ClC₂₁H₁₁N₃O]⁺, 25), 321 ([C₂₁H₁₁N₃O]⁺, 32), 278 (11), 277 (33), 218 (10), 199 (12), 172 (20), 156 ([C₇H₈O₂S]⁺, 10), 155 ([C₇H₇O₂S]⁺, 18), 107 (15), 92 ([C₇H₈]⁺, 23), 91 ([C₇H₇]⁺, 100), 89 (11), 77 (15), 65 ([C₅H₅]⁺, 36).

HR-MS (m/z): calcd for (³⁷Cl³⁵Cl¹³CM+H)⁺: 705.0689; **found.:** 705.0685, **calcd for (³⁷Cl³⁵ClM+H)⁺:** 704.0656; **found:** 704.0660, **calcd. for (³⁵Cl³⁵Cl¹³CM+H)⁺:** 703.0719; **found:** 703.0714, **calcd for (³⁵Cl³⁵ClM+H)⁺:** 702.0685; **found:** 702.0683.

Anal. cald. for C₃₅H₂₅Cl₂N₃O₅S₂ [702.6]: C 59.83, H 3.59, N 5.98, S 9.13; **Found:** C 60.64, H 3.84, N 5.58, S 8.61.

3,3'-(4-Butoxypyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (4j)



4j
[758.73]
 $C_{39}H_{33}Cl_2N_3O_5S_2$

Mp.: 200 °C.

R_f (*n*-hexane/acetone 7:3): 0.47.

¹H NMR (acetone-*d*₆, 600 MHz): δ 1.01 (t, J = 7.4 Hz, 3 H), 1.52–1.60 (m, 2 H), 1.79–1.90 (m, 2 H), 2.34 (s, 6 H), 4.29 (t, J = 6.4 Hz, 2 H), 7.38–7.41 (m, 4 H), 7.42 (dd, J = 8.9, 2.2 Hz, 2 H), 7.55 (s, 2 H), 7.97–8.01 (m, 4 H), 8.09 (dd, J = 8.8, 0.5 Hz, 2 H), 8.52 (s, 2 H), 8.58 (dd, J = 2.2, 0.5 Hz, 2 H).

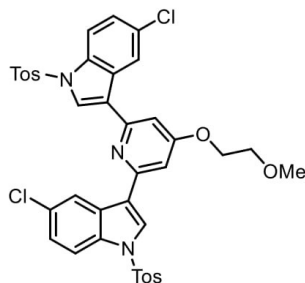
¹³C NMR (acetone-*d*₆, 150 MHz): δ 14.2 (CH), 20.0 (CH₂), 21.6 (CH), 32.0 (CH₂), 69.0 (CH₂), 107.3 (CH), 115.8 (CH), 123.0 (C_{quat}), 123.5 (CH), 126.1 (CH), 128.1 (CH), 128.2 (CH), 130.5 (C_{quat}), 131.1 (C_{quat}), 131.3 (CH), 135.0 (C_{quat}), 135.7 (C_{quat}), 147.1 (C_{quat}), 154.6 (C_{quat}), 167.6 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 665 (s), 710 (m), 795 (w), 835 (w), 968 (m), 1009 (w), 1020 (w), 1094 (m), 1117 (m), 1146 (m), 1175 (m), 1279 (w), 1304 (w), 1368 (w), 1377 (m), 1420 (w), 1433 (w), 1462 (w), 1555 (w), 1576 (w), 1597 (w), 1607 (w), 2926 (w).

MS (EI, *m/z* (%)): 760 ([³⁷Cl³⁵ClIM+H]⁺, 10), 759 ([³⁷Cl³⁵ClIM]⁺, 21), 758 [³⁵Cl³⁵ClIM+H]⁺, 24), 757 ([³⁵Cl³⁵ClIM]⁺, 34), 756 (13), 604 ([³⁷Cl³⁵ClC₃₂H₂₆N₃O₃S]⁺, 22), 603 ([³⁵Cl³⁵ClC₃₂H₂₇N₃O₃S]⁺, 16), 602 ([³⁵Cl³⁵ClC₃₂H₂₆N₃O₃S]⁺, 35), 449 ([³⁷Cl³⁵ClC₂₅H₁₉N₃O]⁺, 17), 448 ([³⁵Cl³⁵ClC₂₅H₂₀N₃O]⁺, 13), 447 ([³⁵Cl³⁵ClC₂₅H₁₉N₃O]⁺, 29), 393 ([³⁷Cl³⁵ClC₂₁H₁₁N₃O]⁺, 14), 392 ([³⁷Cl³⁵ClC₂₁H₁₀N₃O]⁺, 10), 391 ([³⁵Cl³⁵ClC₂₁H₁₁N₃O]⁺, 21), 358 ([³⁷ClC₂₁H₁₁N₃O]⁺, 13), 357 ([³⁷ClC₂₁H₁₀N₃O]⁺, 12), 356 ([³⁵ClC₂₁H₁₁N₃O]⁺, 36), 355 ([³⁵ClC₂₁H₁₀N₃O]⁺, 13), 321 ([C₂₁H₁₁N₃O]⁺, 30), 320 ([C₂₁H₁₀N₃O]⁺, 13), 155 ([C₇H₇O₂S]⁺, 22), 92 ([C₇H₈]⁺, 15), 91 ([C₇H₇]⁺, 100), 65 ([C₅H₅]⁺, 25).

Anal. calcd. for C₃₉H₃₃Cl₂N₃O₅S₂ [758.7]: C 61.74, H 4.38, N 5.54, S 8.45; **Found:** C 61.81, H 4.36, N 5.30, S 8.21.

3,3'-(4-(2-Methoxyethoxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1H-indole) (4k)



4k
[760.70]
 $C_{38}H_{31}Cl_2N_3O_6S_2$

Mp.: 183 °C.

R_f (*n*-hexane/acetone 7:3): 0.61.

¹H NMR (acetone-*d*₆, 300 MHz): δ 2.35 (s, 6 H), 3.42 (s, 3 H), 3.76–3.89 (m, 2 H), 4.38–4.49 (m, 2 H), 7.41 (ddd, J = 8.1, 6.5, 1.5 Hz, 6 H), 7.57 (s, 2 H), 7.95–8.05 (m, 4 H), 8.10 (d, J = 8.8 Hz, 2 H), 8.54 (s, 2 H), 8.58 (d, J = 2.2 Hz, 2 H).

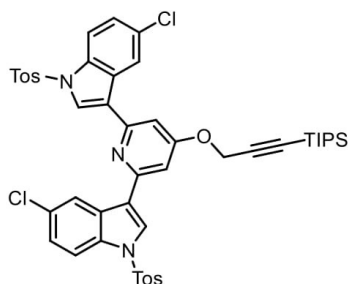
¹³C NMR (acetone-*d*₆, 150 MHz): δ 21.6 (CH₃), 59.1 (CH₃), 68.7 (CH₂), 71.6 (CH₂), 107.4 (CH), 115.8 (CH), 123.0 5 (C_{quat}), 123.5 (CH), 126.1 (CH), 128.1 (CH), 128.3 (CH), 130.5 (C_{quat}), 131.1 (C_{quat}), 131.3 (CH), 135.0 (C_{quat}), 135.7 (C_{quat}), 147.2 (C_{quat}), 154.7 (C_{quat}), 167.4 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 657 (w), 667 (s), 705 (m), 745 (w), 802 (m), 839 (w), 856 (w), 968 (m), 988 (w), 1030 (w), 1061 (w), 1092 (m), 1121 (m), 1148 (m), 1163 (m), 1285 (w), 1360 (w), 1331 (w), 1437 (w), 1595 (w).

MS (EI, *m/z* (%)): 762 ([³⁷Cl³⁵ClIM+H]⁺, 15), 761 ([³⁷Cl³⁵ClIM]⁺, 35), 760 ([³⁵Cl³⁵ClIM+H]⁺, 31), 759 ([³⁵Cl³⁵ClIM]⁺, 61), 606 ([³⁷Cl³⁵ClC₃₁H₂₄N₃O₄S]⁺, 17), 605 ([³⁵Cl³⁵ClC₃₁H₂₅N₃O₄S]⁺, 15), 604 ([³⁵Cl³⁵ClC₃₁H₂₄N₃O₄S]⁺, 24), 549 ([³⁷Cl³⁷ClC₂₈H₁₇N₃O₃S]⁺, 11), 548 ([³⁵Cl³⁷ClC₂₈H₁₈N₃O₃S]⁺, 25), 547 ([³⁵Cl³⁷ClC₂₈H₁₇N₃O₃S]⁺, 18), 546 ([³⁵Cl³⁵ClC₂₈H₁₈N₃O₃S]⁺, 37), 449 ([³⁵Cl³⁵ClC₂₄H₁₇N₃O₂]⁺, 10), 394 ([³⁷Cl³⁷ClC₂₁H₁₀N₃O]⁺, 13), 393 ([³⁷Cl³⁵ClC₂₁H₁₁N₃O]⁺, 35), 392 ([³⁷Cl³⁵ClC₂₁H₁₀N₃O]⁺, 28), 391 ([³⁵Cl³⁵ClC₂₁H₁₁N₃O]⁺, 49), 390 ([³⁵Cl³⁵ClC₂₁H₁₀N₃O]⁺, 15), 365 (13), 364 (11), 363 (11), 357 ([³⁷ClC₂₁H₁₀N₃O]⁺, 11), 356 ([³⁵ClC₂₁H₁₁N₃O]⁺, 19), 355 ([³⁵ClC₂₁H₁₀N₃O]⁺, 13), 358 (13), 188 (14), 174 (10), 155 (23), 139 (14), 92 ([C₇H₈]⁺, 26), 91 ([C₇H₇]⁺, 100), 65 ([C₅H₅]⁺, 28), 64 (16), 59 ([C₃H₇O]⁺, 10), 57 (11).

Anal. calcd. for C₃₈H₃₁Cl₂N₃O₆S₂ [760.7]: C 60.00, H 4.11, N 5.52, S 8.43; **Found:** C 59.90, H 4.25, N 5.29, S 8.23.

3,3'-(4-((3-(Triisopropylsilyl)prop-2-yn-1-yl)oxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole)
(4I)



4I
[897.01]
 $C_{47}H_{47}Cl_2N_3O_5S_2Si$

Mp.: 110 °C.

R_f (*n*-hexane/acetone 7:3): 0.50.

¹H NMR (acetone-*d*₆, 300 MHz): δ 1.01 (s, 21 H), 2.35 (s, 6 H), 5.18 (s, 2 H), 7.37–7.47 (m, 6 H), 7.64 (s, 2 H), 7.96–8.04 (m, 4 H), 8.11 (d, *J* = 8.9 Hz, 2 H), 8.52 (s, 2 H), 8.59 (d, *J* = 2.2 Hz, 2 H).

¹³C NMR (acetone-*d*₆, 75 MHz): δ 11.8 (CH), 18.8 (CH₃), 21.5 (CH₃), 57.4 (CH₂), 90.3 (C_{quat}), 102.7 (C_{quat}), 107.8 (CH), 115.7 (CH), 122.8 (C_{quat}), 123.3 (CH), 126.1 (CH), 128.0 (CH), 128.2 (CH), 130.4 (C_{quat}), 130.9 (CH), 131.2 (C_{quat}), 134.9 (C_{quat}), 135.6 (C_{quat}), 147.1 (C_{quat}), 154.5 (C_{quat}), 166.0 (C_{quat}).

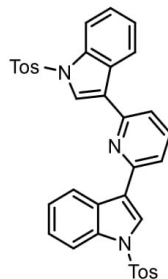
IR: $\tilde{\nu}$ [cm⁻¹]: 667 (s), 742 (w), 804 (m), 839 (w), 879 (w), 968 (w), 995 (w), 1008 (w), 1024 (w), 1055 (w), 1091 (m), 1120 (m), 1147 (m), 1163 (m), 1284 (w), 1375 (w), 1436 (w), 1560 (w), 1577 (w), 1595 (w), 2864 (w), 2941 (w).

MS (EI, *m/z* (%)): 897 ([³⁷Cl³⁵ClM]⁺, 7), 895 ([³⁵Cl³⁵ClM]⁺, 6), 854 ([C₄₄H₄₀³⁷Cl³⁵ClN₃O₅S₂Si]⁺, 4), 852 ([C₄₄H₄₀³⁵Cl³⁵ClN₃O₅S₂Si]⁺, 5) 455 (19), 454 (15), 453 (50), 325 (16), 291 (23), 279 (68), 277 (43), 199 (24), 183 (25), 155 ([C₇H₇O₂S]⁺, 70), 153 (87), 111 (39), 97 (44), 91 ([C₇H₇]⁺, 100), 83 (48), 65 (25), 59 (25).

HR-MS (ESI) (*m/z*) calcd for (C₄₇H₄₇³⁷Cl³⁵ClN₃O₅S₂Si+H)⁺: 898.2147; **Found:** 898.2151; calcd for (C₄₇H₄₇³⁵Cl³⁵ClN₃O₅S₂Si+H)⁺: 896.2176; **Found:** 896.2165.

Anal calcd for C₄₇H₄₇Cl₂N₃O₅S₂Si [897.0]: C 62.93, H 5.28, N 4.68, S 7.15; **Found:** C 63.13, H 5.50, N 4.54, S 7.02.

2,6-Bis(1-tosyl-1*H*-indol-3-yl)pyridine (4m)



4m
[617.74]
 $C_{35}H_{27}N_3O_4S_2$

Mp.: 243 °C.

R_f (*n*-hexane/acetone 7:3): 0.36.

¹H NMR (acetone-*d*₆, 300 MHz): δ 2.34 (s, 6 H), 7.24–7.51 (m, 8 H), 7.91–8.04 (m, 7 H), 8.08–8.18 (m, 2 H), 8.42 (s, 2 H), 8.52 (dt, *J* = 7.9, 1.1 Hz, 2 H).

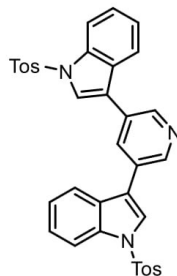
¹³C NMR (acetone-*d*₆, 75 MHz): δ 21.4 (CH₃), 114.4 (CH), 120.6 (CH), 123.5 (CH), 123.7 (C_{quat}), 124.7 (CH), 126.0 (CH), 126.7 (CH), 128.0 (CH), 129.6 (CH), 131.1 (C_{quat}), 135.8 (C_{quat}), 136.5 (C_{quat}), 138.5 (CH), 146.7 (C_{quat}), 153.5 (C_{quat}).

IR $\tilde{\nu}$ [cm⁻¹]: 611 (w), 635 (w), 650 (w), 667 (s), 700 (w), 712 (s), 748 (m), 766 (m), 777 (m), 802 (s), 829 (w), 858 (w), 924 (m), 943 (w), 962 (w), 986 (w), 1015 (m), 1088 (m), 1113 (m), 1130 (m), 1151 (m), 1163 (s), 1283 (w), 1294 (w), 1306 (w), 1344 (w), 1368 (m), 1427 (w), 1443 (w), 1557 (m), 1597 (w), 1636 (w), 2873 (w), 3002 (w), 3036 (w), 3694 (w).

MS (EI, *m/z* (%)): 618 ([MH]⁺, 12), 617 ([M]⁺, 33), 463 ([C₂₈H₂₁N₃O₂S]⁺, 28), 462 ([C₂₈H₂₀N₃O₂S]⁺, 40), 309 (30), 308 ([C₂₁H₁₄N₃]⁺, 71), 307 ([C₂₁H₁₃N₃]⁺, 100), 306 (50), 305 (18), 281 (14), 280 (34), 279 (27), 262 (14), 183 (13), 155 ([C₇H₇O₂S]⁺, 16), 103 (14), 91 (38), 65 (11).

Anal. calcd. for C₃₅H₂₇N₃O₄S₂ [617.7]: C 68.05, H 4.41, N 6.80, S 10.38 **Found:** C 67.94, H 4.32, N 6.64, S 10.26.

3,5-bis(1-tosyl-1*H*-indol-3-yl)pyridine (4n)



4n

[617.74]

C₃₅H₂₇N₃O₄S₂

Mp.: 298 °C.

R_f (*n*-hexane/acetone 7:3): 0.31.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 2.32 (s, 6 H), 7.33–7.50 (m, 8 H), 7.89–8.09 (m, 8 H), 8.38–8.48 (m, 3 H), 8.95 (d, *J* = 2.1 Hz, 2 H).

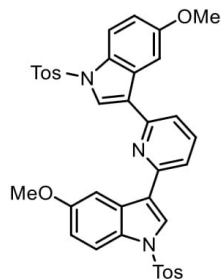
¹³C NMR (acetone-*d*₆ + CS₂, 150 MHz): δ 21.6 (CH₃), 114.8 (CH), 121.0 (CH), 121.1 (C_{quat}), 124.9 (CH), 125.5 (CH), 126.1 (CH), 128.0 (CH), 129.7 (C_{quat}), 130.1 (C_{quat}), 131.0 (CH), 134.5 (CH), 136.0 (C_{quat}), 136.4 (C_{quat}), 146.5 (C_{quat}), 148.3 (CH).

IR: $\tilde{\nu}$ [cm⁻¹]: 650 (w), 665 (s), 678 (s), 702 (m), 748 (s), 800 (w), 860 (w), 904 (w), 964 (m), 1010 (w), 1047 (w), 1089 (m), 1111 (m), 1124 (m), 1172 (s), 1228 (w), 1282 (w), 1294 (w), 1365 (m), 1446 (w), 1597 (w), 3070 (w).

MS (ESI, *m/z* (%)): 618 ([M+H]⁺, 100).

Anal calcd for C₃₅H₂₇N₃O₄S₂ [617.7]: C 68.05, H 4.41, N 6.80, S 10.38; **Found:** C 67.83, H 4.41, N 6.67, S 10.43.

2,6-Bis(5-methoxy-1-tosyl-1*H*-indol-3-yl)pyridine (4o)



4o
[677.79]
C₃₇H₃₁N₃O₆S₂

Mp.: 176 °C.

R_f (*n*-hexane/acetone 7:3): 0.41.

¹H NMR (acetone-*d*₆, 300 MHz): δ 2.35 (s, 6 H), 3.70 (s, 6 H), 7.00 (dd, *J* = 9.1, 2.6 Hz, 2 H), 7.35 (m, 4 H), 7.83–7.99 (m, 9 H), 8.04 (d, *J* = 2.6 Hz, 2 H), 8.33 (s, 2 H).

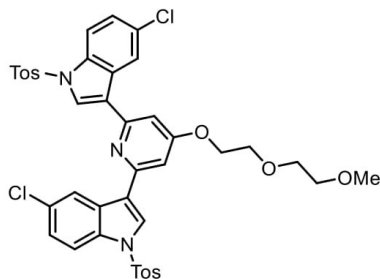
¹³C NMR (acetone-*d*₆, 75 MHz): δ 21.7 (CH₃), 55.7 (CH₃), 105.9 (CH), 115.0 (CH), 115.2 (CH), 120.1 (CH), 123.5 (C_{quat}), 127.2 (CH), 127.9 (CH), 130.6 (C_{quat}), 130.9 (CH), 131.1 (C_{quat}), 135.9 (C_{quat}), 138.2 (CH), 146.3 (C_{quat}), 153.5 (C_{quat}), 157.8 (C_{quat}).

IR $\tilde{\nu}$ [cm⁻¹]: 661 (m), 673 (s), 702 (w), 732 (w), 771 (w), 794 (m), 808 (w), 856 (w), 873 (w), 948 (w), 968 (w), 989 (w), 1018 (w), 1029 (w), 1087 (m), 1138 (s), 1111 (w), 1138 (w), 1165 (m), 1215 (w), 1259 (w), 1290 (w), 1346 (w), 1367 (m), 1400 (w), 1435 (w), 1456 (w), 1473 (w), 1552 (w), 1566 (w), 1589 (w), 1710 (w), 2833 (w), 2924 (w), 3140 (w).

MS (EI, *m/z* (%)): 678 ([¹³CM]⁺, 24), 677 ([M]⁺, 71), 523 ([¹³CM-Tos]⁺, 36), 522 ([M-Tos]⁺, 64), 368 ([¹³CM-2Tos]⁺, 44), 367 ([M-2Tos]⁺, 74), 352 ([C₂₂H₁₄N₃O₂]⁺, 100), 337 ([C₂₁H₁₁N₃O₂]⁺, 10), 324 (37), 155 ([C₇H₇O₂S]⁺, 6), 129 (14), 92 (15), 91 (65), 84 (15), 65 (26).

Anal. calcd. for C₃₇H₃₁N₃O₆S₂ [677.8]: C 65.57, H 4.61, N 6.20, S 9.46; **Found:** C 65.13, H 4.42, N 6.08, S 9.45.

3,3'-(4-(2-(2-Methoxyethoxy)ethoxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1H-indole) (4p)



4p
[804.75]
 $C_{40}H_{35}Cl_2N_3O_7S_2$

Mp.: 114 °C.

R_f (*n*-hexane/acetone 7:3): 0.28.

¹H NMR (acetone-*d*₆, 300 MHz): δ 2.34 (s, 6 H), 3.31 (s, 3 H), 3.51–3.57 (m, 2 H), 3.67–3.73 (m, 2 H), 3.89–3.94 (m, 2 H), 4.41–4.48 (m, 2 H), 7.35–7.47 (m, 6 H), 7.57 (s, 2 H), 7.97–8.03 (m, 4 H), 8.10 (d, J = 8.8 Hz, 2 H), 8.54 (s, 2 H), 8.58 (d, J = 2.2 Hz, 2 H).

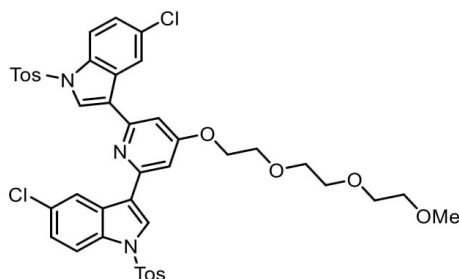
¹³C NMR (acetone-*d*₆, 75 MHz): δ 21.5 (CH₃), 58.8 (CH₃), 68.8 (CH₂), 70.1 (CH₂), 71.3 (CH₂), 72.7 (CH₂), 107.3 (CH), 115.7 (CH), 122.9 (C_{quat}), 123.4 (CH), 126.0 (CH), 128.0 (CH), 128.2 (CH), 130.4 (C_{quat}), 131.0 (C_{quat}), 131.2 (CH), 134.9 (C_{quat}), 135.6 (C_{quat}), 147.0 (C_{quat}), 154.6 (C_{quat}), 167.3 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 665 (s), 705 (m), 744 (w), 802 (m), 839 (w), 854 (w), 968 (w), 1091 (m), 1120 (m), 1147 (m), 1163 (m), 1284 (w), 1367 (w), 1436 (w), 1577 (w), 1595 (w).

MS (EI, *m/z* (%)): 805 ([³⁷Cl³⁵CIM]⁺, 8), 803 ([³⁵Cl³⁵CIM]⁺, 11), 650 ([³⁷Cl³⁵CIM-Tos]⁺, 4), 648 ([³⁵Cl³⁵CIM-Tos]⁺, 5), 548 ([C₂₈H₁₈³⁷Cl³⁵CIN₂O₃S]⁺, 11), 546 ([C₂₈H₁₈³⁵Cl³⁵CIN₂O₃S]⁺, 16), 495 ([³⁷Cl³⁵CIM-2Tos]⁺, 7), 493 ([³⁵Cl³⁵CIM-2Tos]⁺, 8), 155 ([C₇H₇O₂S]⁺, 30), 139 (10), 92 ([C₇H₈]⁺, 20), 91 ([C₇H₇]⁺, 100), 65 (30), 59 (14).

HR-MS (ESI) (*m/z*) calcd for (C₄₀H₃₅³⁷Cl³⁵CIN₃O₇S₂+H)⁺: 806.1337; **Found:** 806.1343; **calcd for (C₄₀H₃₅³⁵Cl³⁵CIN₃O₇S₂+H)⁺:** 804.1366; **Found:** 804.1362.

3,3'-(4-(2-(2-Methoxyethoxy)ethoxy)ethoxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1H-indole)
(4q)



4q
[848.80]
 $C_{42}H_{39}Cl_2N_3O_8S_2$

Mp.: 105 °C.

R_f (*n*-hexane/acetone 7:3): 0.31.

¹H NMR (Aceton-d₆, 300 MHz): δ 2.34 (s, 6 H), 3.27 (s, 3 H), 3.47 (dd, *J* = 5.8, 3.7 Hz, 2 H), 3.59 (dd, *J* = 6.0, 3.9 Hz, 2 H), 3.61–3.67 (m, 2 H), 3.68–3.74 (m, 2 H), 3.90–3.96 (m, 2 H), 4.40–4.49 (m, 2 H), 7.37–7.48 (m, 6 H), 7.57 (s, 2 H), 7.97–8.04 (m, 4 H), 8.10 (d, *J* = 8.9 Hz, 2 H), 8.54 (s, 2 H), 8.59 (d, *J* = 2.2 Hz, 2 H).

¹³C NMR (Aceton-d₆, 75 MHz): δ 21.5 (CH₃), 58.8 (CH₃), 68.8 (CH₂), 70.2 (CH₂), 71.1 (CH₂), 71.3 (CH₂), 71.5 (CH₂), 72.7 (CH₂), 107.3 (CH), 115.7 (CH), 122.9 (C_{quat}), 123.4 (CH), 126.0 (CH), 128.0 (CH), 128.2 (CH), 130.4 (C_{quat}), 131.0 (C_{quat}), 131.2 (CH), 134.9 (C_{quat}), 135.6 (C_{quat}), 147.0 (C_{quat}), 154.5 (C_{quat}), 167.3 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 667 (s), 705 (s), 744 (w), 773 (w), 802 (m), 839 (m), 854 (w), 879 (w), 968 (m), 987 (w), 1018 (w), 1035 (w), 1060 (m), 1089 (s), 1120 (s), 1147 (s), 1163 (s), 1213 (w), 1253 (w), 1284 (w), 1367 (m), 1435 (m), 1558 (w), 1573 (w), 1595 (m), 2875 (w), 3093 (w).

MS (ESI, *m/z* (%)): 703 ([C₃₅H₂₅³⁷Cl³⁵CIN₃O₅S₂]⁺, 6), 701 ([C₃₅H₂₅³⁵Cl³⁵CIN₃O₅S₂]⁺, 12), 694 ([C₃₅H₃₂³⁷Cl³⁵CIN₃O₆S]⁺, 7), 692 ([C₃₅H₃₂³⁵Cl³⁵CIN₃O₆S]⁺, 10), 393 (12), 155 ([C₇H₇O₂S]⁺, 26), 92 (26), 91 ([C₇H₇]⁺, 100), 65 (34), 59 ([C₃H₇O]⁺, 18).

HR-MS (ESI) (*m/z*) calcd for (C₄₂H₃₉³⁷Cl³⁵CIN₃O₈S₂+H)⁺: 850.1599; **Found:** 850.1607; **calcd for (C₄₂H₃₉³⁵Cl³⁵CIN₃O₈S₂+H)⁺:** 848.1629; **Found:** 848.1625.

S6.4 General procedure II (GP II) for the synthesis of *N*-deprotected bisindoles **5**

N-tosyl bisindole **4** and ground potassium hydroxide (5.0 equivs) were placed in a Schlenk tube and dissolved in 10.0 mL methanol (for experimental detail see table S2). The solution was stirred at 100 °C (oil bath) for 4 h. After cooling to room temperature, the mixture was adsorbed onto Celite® under reduced pressure and the bisindoles **5** were isolated and purified by column chromatography on silica gel (eluent: dichloromethane/methanol/ammonia aq., 25% 100:1:1). The resulting solids were additionally purified by trituration in *n*-hexane by ultrasonication and dried in vacuo for 24 h.

Table S3. *N*-tosyl bisindoles **4** and amounts of KOH in the synthesis of bisindoles **5**.

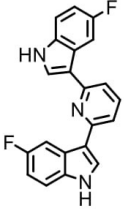
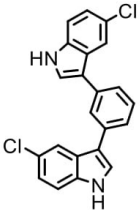
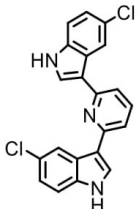
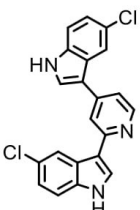
entry	<i>N</i> -tosyl bisindole 4 [mg] (mmol)	KOH [mg] (mmol)	bisindole 5	yield [mg] (%)
1	256 mg (0.39 mmol) of 4a	109 mg (1.95 mmol)		101 mg (75%) of 5a
2	130 mg (0.19 mmol) of 4b	53 mg (0.95 mmol)		57 mg (81%) of 5b
3	137 mg (0.20 mmol) of 4c	56 mg (1.0 mmol)		64 mg (85%) of 5c
4	90 mg (0.13 mmol) of 4d	36 mg (0.65 mmol)		42 mg (86%) of 5d

Table S3. *N*-tosyl bisindoles **4** and amounts of KOH in the synthesis of bisindoles **5**.

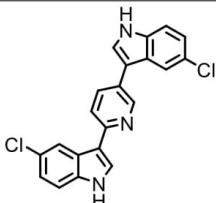
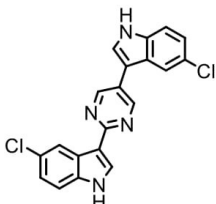
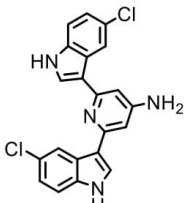
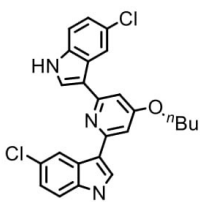
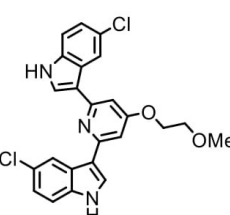
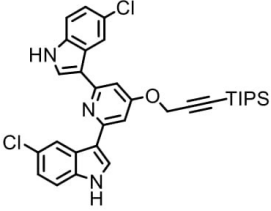
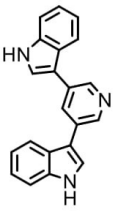
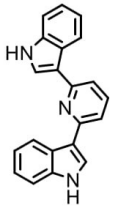
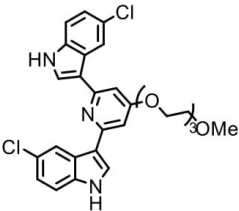
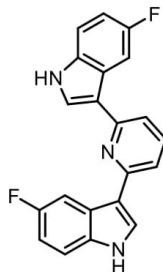
entry	<i>N</i> -tosyl bisindole 4 [mg] (mmol)	KOH [mg] (mmol)	bisindole 5	yield [mg] (%)
5	35 mg (0.05 mmol) of 4e	14 mg (0.25 mmol)		11 mg (58%) of 5e
6	154 mg (0.22 mmol) of 4f	62 mg (1.1 mmol)		43 mg (51%) of 5f
7	156 mg (0.22 mmol) of 4h	62 mg (1.1 mmol)		67 mg (77%) of 5g
8	125 mg (0.17 mmol) of 4j	48 mg (0.85 mmol)		46 mg (62%) of 5h
9	138 mg (0.18 mmol) of 4k	50 mg (0.90 mmol)		47 mg (57%) of 5i

Table S3. *N*-tosyl bisindoles **4** and amounts of KOH in the synthesis of bisindoles **5**.

entry	<i>N</i> -tosyl bisindole 4 [mg] (mmol)	KOH [mg] (mmol)	bisindole 5	yield [mg] (%)
10	158 mg (0.18 mmol) of 4l	50 mg (0.90 mmol)		94 mg (90%) of 5j
11	91 mg (0.14 mmol) of 4n	39 mg (0.70 mmol)		44 mg (99%) of 5k
12	217 mg (0.35 mmol) of 4m	101 mg (1.8 mmol)		100 mg (93%) of 5l
13	179 mg (0.21 mmol) of 4q	62 mg (1.1 mmol)		105 mg (93%) of 5m

S6.5 Spectroscopic data of 5

2,6-Bis(5-fluoro-1*H*-indol-3-yl)pyridine (5a)



5a
[345.35]
C₂₁H₁₃F₂N₃

Mp.: 228 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:1:1): 0.54.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.03 (td, J = 9.1 Hz, 9.1 Hz, 2.6 Hz, 2 H), 7.48 (dd, J = 8.8 Hz, 4.7 Hz, 2 H), 7.59 (d, J = 7.6 Hz, 2 H), 7.75 (dd, J = 8.4 Hz, 7.2 Hz, 1 H), 8.18 (d, J = 2.8 Hz, 2 H), 8.23 (dd, J = 10.8 Hz, 2.8 Hz, 2 H), 11.63 (s, 2 H).

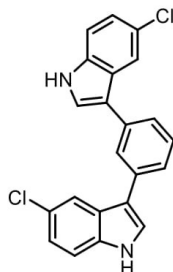
¹³C NMR (DMSO-*d*₆, 75 MHz): δ 106.2 (CH, J = 24.5 Hz), 109.9 (CH, J = 26.1 Hz), 112.9 (CH, J = 9.9 Hz), 116.0 (CH), 116.3 (C_{quat}, J = 4.8 Hz), 125.6 (C_{quat}, J = 10.8 Hz), 127.6 (CH), 133.8 (CH), 136.9 (C_{quat}), 156.2 (C_{quat}), 159.2 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 621 (m), 659 (m), 686 (w), 731 (m), 788 (s), 833 (m), 875 (m), 923 (m), 972 (w), 987 (w), 1024 (w), 1091 (m), 1118 (m), 1165 (m), 1224 (w), 1257 (w), 1296 (w), 1327 (w), 1348 (w), 1386 (w), 1417 (w), 1456 (s), 1479 (m), 1533 (m), 1591 (m), 2357 (w), 2852 (w), 2922 (w), 3053 (w), 3390 (m), 3410 (m).

MS (EI, *m/z* (%)): 345 ([M]⁺, 22), 263 (12), 212 ([C₁₃H₉FN₂]⁺, 100), 211 ([C₁₂H₈FN₂]⁺, 79), 184 ([C₁₂H₇FN]⁺, 42), 183 ([C₁₂H₆FN]⁺, 67), 85 (30), 83 (28), 71 (35), 57 (54).

Anal. calcd. for C₂₁H₁₃F₂N₃ [345.4]: C 73.04, H 3.79, N 12.17; **Found:** C 73.06, H 4.03, N 11.94.

1,3-Bis(5-chloro-1*H*-indol-3-yl)benzene (5b)



5b

$C_{22}H_{14}Cl_2N_2$
[377.26]

Mp.: 113 °C.

R_f (*n*-hexane/acetone 7:3): 0.36.

¹H NMR (acetone-*d*₆, 600 MHz): δ 7.19 (dd, *J* = 8.7, 2.0 Hz, 2 H), 7.53 (d, *J* = 8.6 Hz, 2 H), 7.55–7.58 (m, 1 H), 7.60–7.63 (m, 2 H), 7.80 (d, *J* = 2.6 Hz, 2 H), 7.93–7.98 (m, 3 H), 10.71 (s, 2 H).

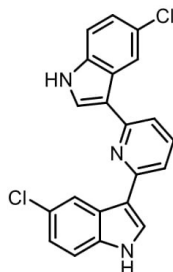
¹³C NMR (acetone-*d*₆, 150 MHz): δ 114.2 (CH), 117.9 (C_{quat}), 119.4 (CH), 122.7 (CH), 125.6 (CH), 125.6 (CH), 126.1 (C_{quat}), 126.6 (CH), 127.7 (C_{quat}), 130.3 (CH), 136.7 (C_{quat}), 136.9 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 651 (w), 673 (s), 702 (m), 750 (w), 761 (s), 783 (s), 825 (w), 869 (m), 893 (m), 958 (w), 1020 (w), 1031 (w), 1064 (w), 1105 (s), 1128 (w), 1168 (w), 1236 (w), 1280 (w), 1340 (w), 1415 (w), 1446 (w), 1463 (m), 1545 (w), 1587 (w), 1600 (w), 1645 (w), 1708 (w), 2852 (w), 3385 (m).

MS (EI, *m/z* (%)): 380 (11), 379 (17), 378 ([³⁷Cl³⁵ClM]⁺, 63), 377 (26), 376 ([³⁵Cl³⁵ClM]⁺, 100), 341 ([C₂₂H₁₄ClN₂]⁺, 11), 306 ([C₂₂H₁₄N]⁺, 11), 305 (15), 278 ([C₂₁H₁₂N]⁺, 13), 189 (12), 188 (13), 157 (12), 139 (24), 126 (18), 125 (22), 57 (14).

HR-MS (ESI) (*m/z*) calcd for (C₂₂H₁₄³⁷Cl³⁵ClN₂+H)⁺: 379.0578; **Found:** 379.0581; **calcd for (C₂₂H₁₄³⁵Cl³⁵ClN₂+H)⁺:** 377.0607; **Found** 377.0608.

2,6-Bis(5-chloro-1*H*-indol-3-yl)pyridine (5c)



5c
[378.25]
C₂₁H₁₃Cl₂N₃

Mp.: 250 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:1:1): 0.49.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.19 (dd, *J* = 8.6 Hz, 1.9 Hz, 2 H), 7.50 (d, *J* = 8.6 Hz, 2 H), 7.61 (d, *J* = 7.7 Hz, 2 H), 7.70–7.84 (m, 1 H), 8.18 (d, *J* = 1.6 Hz, 2 H), 8.57 (d, *J* = 1.6 Hz, 2 H), 11.72 (s, 2 H).

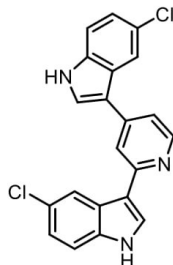
¹³C NMR (DMSO-*d*₆, 75 MHz): δ 113.2 (CH), 115.8 (C_{quat}), 116.1 (CH), 120.6 (CH), 121.6 (CH), 124.7 (C_{quat}), 126.3 (C_{quat}), 127.1 (CH), 135.4 (C_{quat}), 136.7 (CH), 154.1 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 648 (m), 680 (w), 732 (m), 788 (s), 810 (m), 831 (w), 866 (m), 893 (m), 987 (w), 1010 (m), 1028 (m), 1051 (s), 1074 (s), 1107 (m), 1128 (w), 1155 (w), 1166 (m), 1290 (w), 1321 (w), 1382 (m), 1409 (m), 1448 (m), 1533 (m), 1556 (w), 1570 (w), 1587 (w), 2362 (w), 2900 (m), 2972 (m), 2985 (m), 3369 (w).

MS (EI, *m/z* (%)): 381 ([³⁷Cl³⁷ClM]⁺, 10), 380 ([³⁷Cl³⁵ClM+H]⁺, 15), 379 ([³⁷Cl³⁵ClM]⁺, 58), 378 ([³⁵Cl³⁵ClM+H]⁺, 36), 377 ([³⁵Cl³⁵ClM]⁺, 100), 376 (26), 341 ([C₂₁H₁₂³⁵ClN₃]⁺, 12), 306 ([C₂₁H₁₁N₃]⁺, 10), 153 ([C₁₀H₅N₂]⁺, 30), 140 ([C₁₀H₆N]⁺, 26), 139 ([C₁₀H₅N]⁺, 16), 126 ([C₁₀H₆]⁺, 17).

Anal. calcd for C₂₁H₁₃Cl₂N₃ [378.3]: C 66.68, H 3.46, N 11.11; **Found:** C 66.42, H 3.65, N 10.85.

3,3'-(Pyridine-2,4-diyl)bis(5-chloro-1H-indole) (5d)



5d
[378.26]
 $C_{21}H_{13}Cl_2N_3$

Mp.: 260 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:1:1): 0.41.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.17 (dd, J = 8.6, 2.2 Hz, 1 H), 7.22 (dd, J = 8.7, 2.0 Hz, 1 H), 7.43–7.61 (m, 3 H), 8.02 (d, J = 2.0 Hz, 1 H), 8.09 (d, J = 1.7 Hz, 1 H), 8.18 (s, 1 H), 8.34 (d, J = 2.3 Hz, 1 H), 8.56–8.66 (m, 2 H), 11.72 (s, 1 H), 11.89 (s, 1 H).

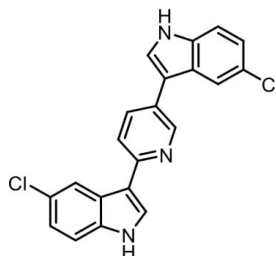
¹³C NMR (DMSO-*d*₆, 75 MHz): δ 113.2 (CH), 113.8 (CH), 115.3 (C_{quat}), 115.9 (CH), 117.3 (CH), 118.5 (C_{quat}), 121.1 (CH), 121.6 (CH), 121.9 (CH), 124.5 (C_{quat}), 125.0 (C_{quat}), 125.8 (C_{quat}), 126.6 (C_{quat}), 127.6 (CH), 135.4 (C_{quat}), 135.6 (C_{quat}), 142.7 (C_{quat}), 149.6 (CH), 155.3 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 630 (w), 667 (m), 713 (w), 758 (w), 767 (m), 800 (s), 831 (w), 852 (w), 889 (m), 933 (w), 968 (w), 997 (w), 1062 (w), 1099 (w), 1114 (w), 1228 (w), 1246 (w), 1284 (w), 1327 (w), 1346 (w), 1386 (w), 1421 (w), 1444 (m), 1454 (m), 1519 (w), 1546 (w), 1571 (w), 1597 (s), 2841 (w), 2929 (w), 3030 (w), 3122 (w), 3147 (w), 3442 (w).

MS (EI, *m/z* (%)): 380 ([³⁷Cl³⁵Cl¹³CM]⁺, 19), 379 ([³⁷Cl³⁵CM]⁺, 63), 378 ([³⁵Cl³⁵Cl¹³CM]⁺, 57), 377 ([³⁵Cl³⁵CM]⁺, 100), 376 (68), 342 ([C₂₁H₁₃ClN₃]⁺, 20), 341 (17), 306 ([C₂₁H₁₂N₃]⁺, 14), 305 (12), 278 (21), 277 (49), 199 (16), 172 (15), 171 (52), 157 (13), 153 ([C₁₁H₇N]⁺, 23), 140 (33), 139 (22), 113 (13), 112 (13).

HR-MS (ESI) (*m/z*) calcd for (C₂₁H₁₃³⁵Cl³⁵ClN₃+H)⁺: 378.0560; **Found:** 378.0565.

3,3'-(Pyridine-2,5-diyl)bis(5-chloro-1*H*-indole) (5e)



5e
[378.26]
 $C_{21}H_{13}Cl_2N_3$

Mp.: 275 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:1:1): 0.44.

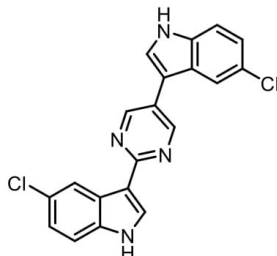
¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.17 (dd, *J* = 4.9, 2.6 Hz, 2 H), 7.20 (dd, *J* = 4.6, 2.1 Hz, 2 H), 7.47 (d, *J* = 9.3 Hz, 1 H), 7.50 (d, *J* = 9.0 Hz, 1 H), 7.87–7.98 (m, 3 H), 8.06 (dd, *J* = 8.3, 2.4 Hz, 1 H), 8.22 (s, 1 H), 8.61 (d, *J* = 2.2 Hz, 1 H), 8.96 (d, *J* = 2.4 Hz, 1 H), 11.69 (s, 2 H).

¹³C NMR (DMSO-*d*₆, 75 MHz): δ 112.5 (CH), 113.3 (CH), 113.6 (CH), 115.1 (C_{quat}), 118.2 (CH), 119.5 (CH), 121.0 (CH), 121.6 (CH), 124.5 (C_{quat}), 124.6 (C_{quat}), 125.5 (CH), 126.0 (C_{quat}), 126.4 (C_{quat}), 127.1 (C_{quat}), 127.1 (CH), 134.0 (C_{quat}), 135.4 (C_{quat}), 135.5 (C_{quat}), 146.7 (CH), 152.1 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 866 (w), 891 (w), 1014 (m), 1028 (m), 1053 (s), 1099 (w), 1228 (m), 1301 (w), 1382 (w), 1406 (m), 1479 (w), 1504 (w), 1531 (w), 1558 (w), 1687 (w), 1722 (w), 2900 (m), 2972 (s), 2989 (s), 3662 (w).

HR-MS (ESI) (*m/z*) calcd for (C₂₁H₁₃³⁵Cl³⁷ClN₃+H)⁺: 378.0560; **Found:** 378.0563; **calcd for (C₂₁H₁₃³⁷Cl³⁷ClN₃+H)⁺:** 380.0530; **Found:** 380.0535.

3,3'-(Pyrimidine-2,5-diyl)bis(5-chloro-1H-indole) (5f)



5f
[379.24]
C₂₀H₁₂Cl₂N₄

Mp.: 165 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:1:1): 0.27

¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.20 (d, *J* = 2.1 Hz, 1 H), 7.23 (d, *J* = 2.1 Hz, 1 H), 7.50 (d, *J* = 2.4 Hz, 1 H), 7.53 (d, *J* = 2.4 Hz, 1 H), 7.97 (d, *J* = 2.0 Hz, 1 H), 8.01 (s, 1 H), 8.30 (s, 1 H), 8.63 (d, *J* = 2.1 Hz, 1 H), 9.14 (s, 2 H), 11.80 (s, 1 H), 11.86 (s, 1 H).

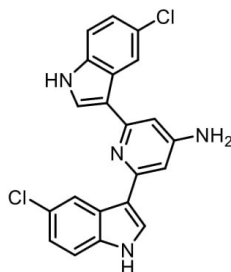
¹³C NMR (DMSO-*d*₆, 75 MHz): δ 109.3 (CH), 113.5 (CH), 113.6 (CH), 114.5 (CH), 118.4 (C_{quat}), 121.0 (CH), 121.9 (CH), 121.9 (C_{quat}), 124.7 (C_{quat}), 124.8 (C_{quat}), 125.1 (C_{quat}), 125.7 (C_{quat}), 125.9 (CH), 126.6 (CH), 130.1 (C_{quat}), 135.3 (C_{quat}), 135.6 (C_{quat}), 154.1 (CH), 160.3 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 609 (s), 655 (m), 673 (w), 715 (w), 748 (w), 786 (s), 825 (w), 840 (w), 893 (s), 927 (w), 960 (w), 995 (w), 1028 (w), 1035 (w), 1064 (w), 1072 (w), 1099 (m), 1116 (w), 1132 (s), 1157 (w), 1180 (w), 1222 (w), 1267 (w), 1286 (w), 1301 (m), 1323 (w), 1361 (w), 1379 (w), 1446 (s), 1519 (w), 1541 (s), 2900 (w), 2922 (w), 2968 (w), 2985 (w), 3113 (w), 3269 (w), 3421 (w).

MS (EI, *m/z* (%)): 381 (13), 380 ([³⁷Cl³⁵ClM]⁺, 66), 379 (22), 378 ([³⁵Cl³⁵ClM]⁺, 100), 277 (10), 189 (12), 177 ([C₉H₆ClN₂]⁺, 14), 176 (14), 175 ([C₁₀H₆ClN]⁺, 42), 140 (38), 113 (13).

HR-MS (ESI) (*m/z*) calcd for (C₂₀H₁₂³⁵Cl³⁷ClN₃+H)⁺: 379.0512; **Found:** 379.0514.

2,6-Bis(5-chloro-1*H*-indol-3-yl)pyridine-4-amine (5g)



5g
[393.27]
C₂₁H₁₄Cl₂N₄

Mp.: 205 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:5:1): 0.72.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 5.89 (s, 2 H), 6.82 (s, 2 H), 7.16 (dd, *J* = 8.6, 2.1 Hz, 2 H), 7.47 (d, *J* = 8.6 Hz, 2 H), 7.96 (d, *J* = 2.6 Hz, 2 H), 8.36–8.50 (m, 2 H), 11.58 (s, 2 H).

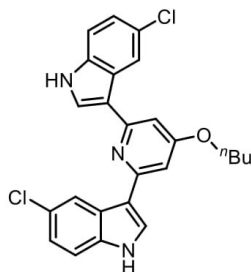
¹³C NMR (DMSO-*d*₆, 150 MHz): δ 102.1 (CH), 113.3 (CH), 116.5 (C_{quat}), 120.6 (CH), 121.4 (CH), 124.4 (C_{quat}), 126.3 (C_{quat}), 126.5 (CH), 135.4 (C_{quat}), 153.9 (C_{quat}), 155.1 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 650 (w), 685 (m), 702 (w), 752 (m), 770 (m), 797 (s), 831 (w), 878 (m), 891 (m), 910 (w), 984 (w), 1018 (w), 1103 (m), 1132 (w), 1167 (w), 1229 (w), 1281 (w), 1333 (w), 1383 (w), 1406 (w), 1449 (m), 1485 (w), 1537 (m), 1555 (m), 1598 (s), 1618 (m), 1695 (w), 3127 (w).

MS (EI, *m/z* (%)): 396 ([³⁷Cl³⁷ClIM]⁺, 66), 395 ([³⁷Cl³⁵ClIM+H]⁺, 22), 394 ([³⁷Cl³⁵ClIM]⁺, 66), 393 ([³⁵Cl³⁵ClIM+H]⁺, 64), 392 ([³⁵Cl³⁵ClIM]⁺, 100), 391 ([³⁵Cl³⁵ClC₂₁H₁₃N₄]⁺, 78), 356 ([³⁵ClC₂₁H₁₃N₄]⁺, 18), 161 (46), 147 (20), 147 (20), 113 (11), 112 (68).

HR-MS (ESI) (*m/z*) calcd for (C₂₁H₁₄³⁵Cl³⁷ClN₄+H)⁺: 395.0639; **Found:** 395.0641, **calcd for (C₂₁H₁₄³⁵Cl³⁵ClN₄+H)⁺:** 393.0669; **Found:** 393.0672.

3,3'-(4-Butoxypyridine-2,6-diyl)bis(5-chloro-1*H*-indole) (5h)



5h
[450.36]
C₂₅H₂₁Cl₂N₃O

Mp.: 194°C.

R_f (dichloromethane/methanol/aq. ammonia 100:5:1): 0.57.

¹H NMR (DMSO-*d*₆, 600 MHz): δ 0.98 (t, *J* = 7.4 Hz, 3 H), 1.50 (h, *J* = 7.5 Hz, 2 H), 1.73–1.84 (m, 2 H), 4.20 (t, *J* = 6.5 Hz, 2 H), 7.17 (dd, *J* = 8.6, 2.1 Hz, 2 H), 7.19 (s, 2 H), 7.48 (d, *J* = 8.5 Hz, 2 H), 8.22 (d, *J* = 2.7 Hz, 2 H), 8.56 (d, *J* = 2.2 Hz, 2 H), 11.71 (d, *J* = 2.7 Hz, 2 H).

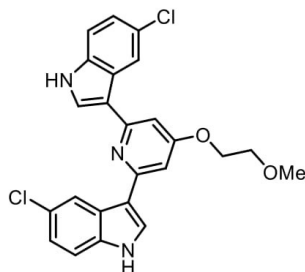
¹³C NMR (DMSO-*d*₆, 150 MHz): δ 13.8 (CH₃), 18.8 (CH₂), 30.7 (CH₂), 67.2 (CH₂), 102.8 (CH), 113.2 (CH), 116.0 (C_{quat}), 121.0 (CH), 121.7 (CH), 124.8 (C_{quat}), 126.6 (C_{quat}), 127.4 (CH), 135.4 (C_{quat}), 155.7 (C_{quat}), 165.5 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 604 (w), 683 (w), 768 (w), 799 (m), 835 (w), 851 (w), 883 (w), 891 (w), 997 (w), 1043 (w), 1099 (w), 1111 (w), 1140 (w), 1165 (w), 1217 (w), 1273 (w), 1298 (w), 1375 (w), 1431 (w), 1452 (w), 1593 (m), 2359 (w), 2855 (w), 2868 (w), 2916 (w), 2928 (w), 2951 (w), 3067 (w), 3127 (w), 3156 (w), 3296 (w), 3350 (w), 3474 (w), 3676 (w), 3711 (w), 3828 (w), 3854 (m), 3904 (w), 3964 (w), 3991 (w).

MS (EI, *m/z* (%)): 452 ([³⁷Cl³⁵ClM+H]⁺, 15), 451 ([³⁷Cl³⁵ClM]⁺, 59), 450 ([³⁵Cl³⁵ClM+H]⁺, 25), 449 ([³⁵Cl³⁵ClM]⁺, 91), 408 (12), 408 ([³⁷Cl³⁵ClC₂₂H₁₄N₃O]⁺, 12), 406 ([³⁵Cl³⁵ClC₂₂H₁₄N₃O]⁺, 25), 395 ([³⁷Cl³⁵ClC₂₁H₁₃N₃O]⁺, 14), 394 ([³⁷Cl³⁵ClC₂₁H₁₂N₃O]⁺, 16), 393 ([³⁵Cl³⁵ClC₂₁H₁₃N₃O]⁺, 14), 392 ([³⁵Cl³⁵ClC₂₁H₁₂N₃O]⁺, 14), 368 ([³⁷Cl³⁵ClC₂₀H₁₄N₃]⁺, 25), 367 ([³⁷Cl³⁵ClC₂₀H₁₃N₃]⁺, 55), 366 ([³⁵Cl³⁵ClC₂₀H₁₄N₃]⁺, 25), 365 ([³⁵Cl³⁵ClC₂₀H₁₃N₃]⁺, 100), 330 ([³⁵ClC₂₀H₁₃N₃]⁺, 14), 188 (11), 179 (10), 165 (11), 161 (41), 148 (10).

HR-MS (*m/z*): calcd. for (³⁷Cl³⁵Cl¹³CM)⁺: 455.1138; **Found:** 453.1134, calcd. for (³⁷Cl³⁵ClM)⁺: 452.1105; **Found:** 452.1107, calcd. for (³⁵Cl³⁵Cl¹³CM)⁺: 451.1168; **Found:** 451.1165, calcd. for (³⁵Cl³⁵ClM)⁺: 450.1134; **Found:** 450.1136.

3,3'-(4-(2-Methoxyethoxy)pyridine-2,6-diyl)bis(5-chloro-1*H*-indole) (5i)



5i
[452.34]
C₂₄H₁₉Cl₂N₃O₂

Mp.: 201°C.

R_f (dichloromethane/methanol/aq. ammonia 100:5:1): 0.61.

¹H NMR (DMSO-*d*₆, 600 MHz): δ 3.35 (s, 3 H), 3.71–3.77 (m, 2 H), 4.30–4.38 (m, 2 H), 7.17 (dd, *J* = 8.6, *J* = 2.1 Hz, 2 H), 7.22 (s, 2 H), 7.48 (d, *J* = 8.6 Hz, 2 H), 8.22 (d, *J* = 2.7 Hz, 2 H), 8.56 (d, *J* = 2.1 Hz, 2 H), 11.71 (s, 2 H).

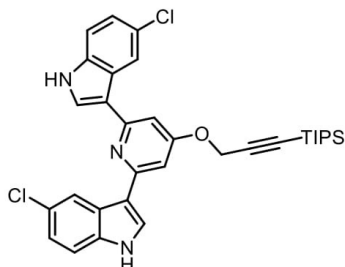
¹³C NMR (DMSO-*d*₆, 150 MHz): δ 58.4 (CH₃), 67.0 (CH₂), 70.5 (CH₂), 102.9 (CH), 113.4 (CH), 116.1 (C_{quat}), 121.1 (CH), 121.8 (CH), 124.9 (C_{quat}), 126.7 (C_{quat}), 127.6 (CH), 135.6 (C_{quat}), 155.9 (C_{quat}), 165.4 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 650 (w), 683 (w), 702 (w), 752 (w), 770 (m), 795 (s), 824 (w), 854 (w), 876 (w), 891 (m), 935 (w), 988 (w), 1032 (w), 1057 (m), 1111 (m), 1167 (w), 1198 (w), 1215 (w), 1263 (w), 1329 (w), 1362 (w), 1381 (w), 1402 (w), 1447 (m), 1533 (w), 1558 (s), 1591 (m), 2812 (w), 2876 (w), 2932 (w), 3057 (w), 3150 (w), 3335 (w), 3120 (w).

MS (EI, *m/z* (%)): 453 ([³⁷Cl³⁵ClM]⁺, 30), 452 ([³⁵Cl³⁵ClM+H]⁺, 16), 451 ([³⁵Cl³⁵ClM]⁺, 49), 395 ([³⁷Cl³⁵ClC₂₁H₁₃N₃O]⁺, 20), 394 ([³⁵Cl³⁵ClC₂₁H₁₄N₃O]⁺, 11), 393 ([³⁵Cl³⁵ClC₂₁H₁₃N₃O]⁺, 35), 368 ([³⁷ClC₂₀H₁₇N₃O₂]⁺, 14), 367 ([³⁷ClC₂₀H₁₆N₃O₂]⁺, 60), 366 ([³⁵ClC₂₀H₁₇N₃O₂]⁺, 26), 365 ([³⁵ClC₂₀H₁₆N₃O₂]⁺, 100), 330 ([C₂₀H₁₆N₃O₂]⁺, 22), 189 (11), 188 (13), 165 (15), 164 (11), 154 (11).

HR-MS (*m/z*) calcd. for (³⁷Cl³⁵Cl¹³CM)⁺: 455.0931; **Found:** 455.0936, **calcd. for (³⁷Cl³⁵ClM)⁺:** 454.0898; **Found:** 454.0911, **calcd. for (³⁵Cl³⁵Cl¹³CM)⁺:** 453.0961; **Found:** 453.0966, **calcd. for (³⁵Cl³⁵ClM)⁺:** 452.0927; **Found:** 452.0937.

3,3'-(4-((3-(Triisopropylsilyl)prop-2-yn-1-yl)oxy)pyridine-2,6-diyl)bis(5-chloro-1H-indole) (5j)



5j
[588.65]
 $C_{33}H_{35}Cl_2N_3OSi$

Mp.: 102 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:5:1): 0.65.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 0.97 (s, 21 H), 5.11 (s, 2 H), 7.18 (dd, *J* = 8.6, 2.1 Hz, 2 H), 7.27 (s, 2 H), 7.48 (d, *J* = 8.7 Hz, 2 H), 8.16 (d, *J* = 2.5 Hz, 2 H), 8.55 (d, *J* = 2.1 Hz, 2 H), 11.71 (s, 2 H).

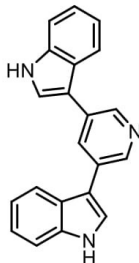
¹³C NMR (DMSO-*d*₆, 75 MHz): δ 10.5 (CH), 18.3 (CH₃), 56.1 (C_{quat}), 88.5 (C_{quat}), 102.7 (C_{quat}), 103.2 (CH), 113.2 (CH), 115.9 (C_{quat}), 120.8 (CH), 121.6 (CH), 124.8 (C_{quat}), 126.5 (C_{quat}), 127.3 (CH), 135.4 (CH), 155.6 (C_{quat}), 164.0 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹]: 678 (s), 702 (w), 752 (w), 769 (m), 796 (s), 825 (w), 881 (w), 970 (w), 989 (w), 1018 (m), 1047 (s), 1111 (m), 1132 (w), 1166 (w), 1207 (w), 1234 (w), 1274 (w), 1327 (w), 1352 (w), 1382 (w), 1444 (s), 1460 (s), 1537 (m), 1556 (m), 1570 (s), 1591 (s), 2864 (w), 2941 (w), 3398 (w), 3414 (w).

MS (ESI, *m/z* (%)): 590 ([³⁷Cl³⁵ClIM+H]⁺, 67), 588 ([³⁵Cl³⁵ClIM+H]⁺, 100).

HR-MS (*m/z*) calcd. for (C₃₃H₃₅³⁷Cl³⁵ClN₂OSi+H)⁺: 590.1970; **Found:** 590.1986; **calcd. for (C₃₃H₃₅³⁵Cl³⁵ClN₂OSi+H)⁺:** 588.2000; **Found:** 588.2009.

3,5-Di(1*H*-indol-3-yl)pyridine (5k)



5k
[309.37]
C₂₁H₁₅N₃

Mp.: 321 °C (combustion).

R_f (dichloromethane/methanol/aq. ammonia 100:1:1): 0.36.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.03–7.33 (m, 4 H), 7.43–7.62 (m, 2 H), 7.81–8.03 (m, 4 H), 8.28 (t, *J* = 2.2, 1.6 Hz, 1 H), 8.79 (d, *J* = 2.0 Hz, 2 H), 11.55 (s, 2 H).

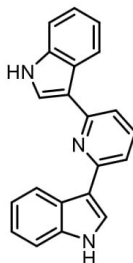
¹³C NMR (DMSO-*d*₆, 75 MHz): δ 112.2 (CH), 112.4 (C_{quat}), 118.9 (CH), 120.1 (CH), 121.8 (CH), 124.5 (CH), 125.0 (C_{quat}), 130.7 (CH), 131.8 (C_{quat}), 137.0 (C_{quat}), 144.3 (CH).

IR: $\tilde{\nu}$ [cm⁻¹]: 617 (w), 640 (w), 667 (w), 719 (s), 744 (s), 821 (w), 893 (w), 1006 (w), 1033 (w), 1056 (w), 1093 (m), 1151 (w), 1188 (w), 1242 (w), 1251 (w), 1334 (w), 1344 (w), 1361 (w), 1419 (w), 1456 (m), 1485 (w), 1531 (w), 1595 (w), 1645 (w), 1788 (w), 3030 (w), 3057 (w), 3109 (w).

MS (EI, *m/z* (%)): 310 ([*M*+*H*]⁺, 22), 309 ([*M*]⁺, 100), 155 ([C₁₀H₇N₂]⁺, 10), 154 ([C₁₁H₈N]⁺, 12), 149 (14), 141 ([C₁₀H₇N]⁺, 10), 140 (13), 127 ([C₉H₅N]⁺, 13), 113 (14), 111 (10), 99 (10), 97 (16), 85 (19), 83 (17), 73 (14), 71 (23), 69 (16), 57 (34), 55 (22).

HR-MS (ESI) (*m/z*) calcd for (¹²C₂₁¹³CH₁₅N₃+H)⁺: 311.1373; **Found:** 311.1371; **calcd for (C₂₁H₁₅N₃+H)⁺:** 310.1339; **Found:** 310.1342.

2,6-Di(1*H*-indol-3-yl)pyridine (5I)



5I
[309.37]
C₂₁H₁₅N₃

Mp.: 315 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:2:1): 0.57.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.15 (dddd, J = 17.6, 8.2, 7.0, 1.3 Hz, 4 H), 7.45–7.55 (m, 2 H), 7.55–7.67 (m, 2 H), 7.75 (dd, J = 8.5, 7.1 Hz, 1 H), 8.12 (d, J = 2.7 Hz, 2 H), 8.51 (dd, J = 7.4, 1.5 Hz, 2 H), 11.50 (d, J = 2.6 Hz, 2 H).

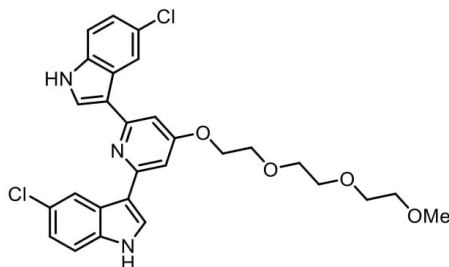
¹³C NMR (DMSO-*d*₆, 75 MHz): δ 111.7 (CH), 115.7 (CH), 116.2 (C_{quat}), 119.7 (CH), 121.2 (CH), 121.5 (CH), 125.3 (C_{quat}), 125.6 (CH), 136.5 (CH), 137.0 (C_{quat}), 154.5 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 617 (w), 640 (m), 749 (s), 800 (s), 1008 (m), 1041 (w), 1062 (m), 1093 (m), 1118 (w), 1141 (w), 1157 (w), 1168 (m), 1240 (m), 1263 (w), 1332 (w), 1417 (w), 1537 (m), 1544 (m), 1562 (w), 1593 (m), 3385 (s).

MS (EI, *m/z* (%)): 309 ([M]⁺, 20), 181 (84), 154 ([C₁₀H₆N₂]⁺, 34), 127 ([C₉H₅N]⁺, 98), 113 ([C₉H₅]⁺, 29), 105 (44), 77 ([C₅H₃N]⁺, 51), 57 (74), 55 ([C₃H₅N]⁺, 100).

HR-MS (ESI) (*m/z*) calcd for (C₂₁H₁₅N₃+H)⁺: 310.1339; **Found:** 310.1341; **calcd for (¹²C₂₀¹³CH₁₅N₃+H)⁺:** 311.1373; **Found:** 311.1370.

3,3'-(4-(2-(2-(2-Methoxyethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(5-chloro-1*H*-indole) (5m)



5m
[540.44]
 $C_{28}H_{27}Cl_2N_3O_4$

Mp.: 266 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:1:1): 0.34.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 3.22 (s, 3 H), 3.40–3.46 (m, 2 H), 3.48–3.54 (m, 2 H), 3.54–3.59 (m, 2 H), 3.63 (dt, *J* = 5.1, 2.0 Hz, 2 H), 3.75–3.90 (m, 2 H), 4.30–4.38 (m, 2 H), 7.17 (dd, *J* = 8.6, 2.1 Hz, 2 H), 7.23 (s, 2 H), 7.48 (d, *J* = 8.6 Hz, 2 H), 8.22 (d, *J* = 2.6 Hz, 2 H), 8.57 (d, *J* = 2.2 Hz, 2 H), 11.71 (d, *J* = 2.8 Hz, 2 H).

¹³C NMR (DMSO-*d*₆, 75 MHz): δ 58.0 (CH₃), 68.8 (CH₂), 69.6 (CH₂), 69.8 (CH₂), 69.8 (CH₂), 70.0 (CH₂), 71.3 (CH₂), 102.7 (CH), 113.2 (CH), 115.9 (C_{quat}), 120.9 (CH), 121.6 (CH), 124.7 (C_{quat}), 126.5 (C_{quat}), 127.4 (CH), 135.4 (C_{quat}), 155.7 (C_{quat}), 165.2 (C_{quat}).

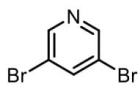
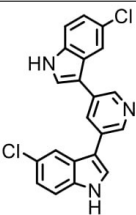
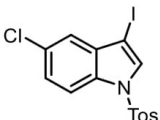
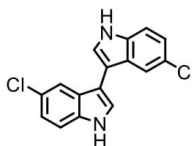
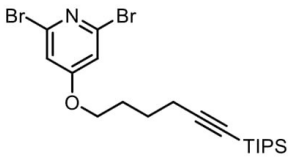
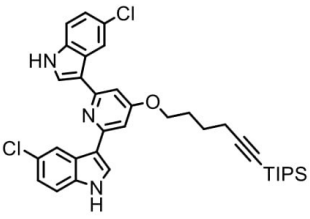
IR: $\tilde{\nu}$ [cm⁻¹] 617 (w), 640 (w), 684 (w), 702 (w), 769 (m), 785 (w), 796 (w), 850 (w), 891 (w), 948 (w), 1060 (m), 1101 (s), 1139 (m), 1170 (w), 1219 (w), 1290 (w), 1332 (w), 1346 (w), 1357 (w), 1409 (w), 1446 (m), 1552 (s), 1568 (w), 1591 (s), 2885 (w), 2895 (w), 3236 (w), 3255 (w).

HR-MS (ESI) (*m/z*) calcd for (C₂₈H₂₇³⁷Cl³⁵ClN₃O₄+H)⁺: 542.1422; **Found:** 542.1433; **calcd for (C₂₈H₂₇³⁵Cl³⁵Cl N₃O₄+H)⁺:** 540.1452; **Found:** 540.1459

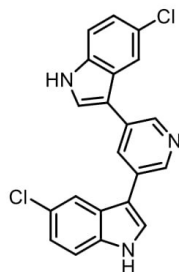
S6.6 General procedure III (GPIII) for the synthesis of *N*-deprotected bisindoles 5[2]

5-chloro-3-iodo-1-tosyl-1*H*-indole (**1b**) (431 mg, 1.00 mmol) and tetrakis(triphenylphosphane)palladium(0) (57 mg, 0.05 mmol, 5 mol%) were dissolved in 5.0 mL dry 1,4-dioxane in a dry Schlenk tube with a magnetic stir bar under dry nitrogen (for experimental detail see table S3). Triethylamine (1.4 mL, 10 mmol) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.25 mL, 1.7 mmol) (pinacolyl borane, HBpin) were added dropwise, and the reaction mixture was stirred at 80 °C for 3 h. Thereafter, the mixture was cooled to room temp, and 7.0 mL methanol, cesium carbonate (0.82 g, 2.50 mmol), and heteroaryl dihalide **3** (0.5 mmol) were added to the reaction mixture. This mixture was stirred at 60 °C for 18 h. Subsequently, the mixture was cooled to room temp, potassium hydroxide (0.14 g, 2.5 mmol) was added, and the reaction was stirred again at 100 °C for 3 h. After cooling to room temp, the mixture was diluted with dichloromethane, adsorbed onto Celite® and the bisindoles **5** were isolated and purified by column chromatography on silica gel (eluent: dichloromethane/methanol/ammonia aq., 25% 100:1:1). The resulting solids were additionally purified by trituration in *n*-hexane by ultrasonication and dried in vacuo for 24 h.

Table S4 Heteroaryl dihalides **3** and yields in the synthesis of bisindoles **5**.

entry	heteroaryl dihalide 3 [mg] (mmol)	bisindole 5	yield [mg] (%)
1	 118 mg (0.50 mmol) of 3l		62 mg (33%) of 5n
2	 431 mg (1.0 mmol) of 1b		150 mg (50%) of 5s
3	 244 mg (0.50 mmol) of 3o		97 mg (31%) of 5t

3,5-bis(5-chloro-1*H*-indol-3-yl)pyridine (5n)



5n
[378.25]
C₂₁H₁₃Cl₂N₃

Mp.: 270 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:1:1): 0.16.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.21 (dd, *J* = 8.6, 2.0 Hz, 2 H), 7.53 (d, *J* = 8.6 Hz, 2 H), 7.90 (d, *J* = 2.0 Hz, 2 H), 8.02 (d, *J* = 2.7 Hz, 2 H), 8.23 (t, *J* = 2.2 Hz, 1 H), 8.79 (d, *J* = 2.1 Hz, 2 H), 11.77 (s, 2 H).

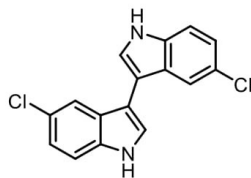
¹³C NMR (DMSO-*d*₆, 75 MHz): δ 112.2 (C_{quat}), 113.7 (CH), 118.0 (CH), 121.8 (CH), 124.7 (C_{quat}), 126.0 (C_{quat}), 126.3 (CH), 130.8 (CH), 131.1 (C_{quat}), 135.4 (C_{quat}), 144.6 (CH).

IR: $\tilde{\nu}$ [cm⁻¹] 653 (s), 678 (s), 704 (m), 746 (w), 769 (s), 785 (m), 798 (s), 817 (m), 854 (w), 879 (w), 889 (m), 960 (w), 1006 (w), 1031 (w), 1066 (w), 1114 (w), 1138 (w), 1147 (w), 1240 (w), 1284 (w), 1323 (w), 1400 (w), 1427 (w), 1452 (m), 1489 (w), 1533 (w), 1591 (m), 1618 (w), 2628 (w), 2713 (w), 2846 (w), 3010 (w), 3062 (w), 3120 (w), 3431 (w).

MS (ESI, *m/z* (%)): 380 ([³⁷Cl³⁵ClM]⁺, 65), 378 ([³⁵Cl³⁵ClM]⁺, 100).

HR-MS (ESI) (*m/z*) calcd for (C₂₁H₁₃³⁷Cl³⁵ClN₃+H)⁺: 380.0530, **Found:** 380.0534; **calcd for (C₂₁H₁₃³⁵Cl³⁵ClN₃+H)⁺:** 378.0560, **Found:** 378.0562.

5,5'-Dichloro-1*H*,1'*H*-3,3'-biindole (5s)[3]



5s
[301.17]
 $C_{16}H_{10}Cl_2N_2$

Mp.: 199 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:2:1): 0.70.

¹H NMR (DMSO-*d*₆, 300 MHz): δ 7.15 (dd, *J* = 8.6, 2.1 Hz, 2 H), 7.46 (d, *J* = 8.6 Hz, 2 H), 7.73 (d, *J* = 2.1 Hz, 2 H), 7.77 (d, *J* = 2.4 Hz, 2 H), 11.42 (d, *J* = 2.5 Hz, 2 H).

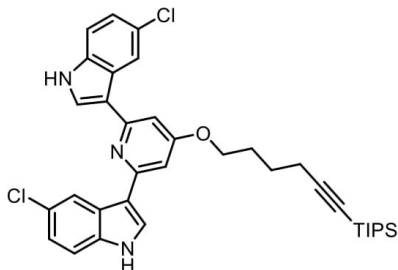
¹³C NMR (DMSO-*d*₆, 75 MHz): δ 108.8 (C_{quat}), 113.2 (CH), 118.4 (CH), 121.3 (CH), 123.7 (C_{quat}), 124.0 (CH), 127.0 (C_{quat}), 134.8 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 626 (w), 646 (w), 686 (s), 719 (w), 746 (w), 775 (s), 790 (s), 823 (w), 864 (m), 891 (m), 925 (w), 1014 (w), 1043 (w), 1062 (m), 1097 (m), 1168 (w), 1236 (w), 1251 (w), 1288 (w), 1330 (w), 1350 (w), 1402 (w), 1456 (s), 1566 (w), 1577 (w), 1612 (w), 2866 (w), 2931 (w), 2972 (w), 3417 (w).

MS (EI, *m/z* (%)): 300 ([M]⁺, 100).

HR-MS (ESI) (*m/z*) calcd for (C₁₆H₁₀³⁷Cl³⁵CIN₂)⁺: 302.0192, **Found:** 302.0185; **calcd for (C₁₆H₁₀³⁵Cl³⁵CIN₂)⁺:** 300.0221, **Found:** 300.0213.

**3,3'-(4-((6-(Triisopropylsilyl)hex-5-yn-1-yl)oxy)pyridine-2,6-diyl)bis(5-chloro-1H-indole)
(5t)**



5t
[630.73]
 $C_{36}H_{41}Cl_2N_3OSi$

Mp.: 113 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:5:1): 0.50.

¹H NMR (DMSO-*d*₆, 600 MHz): δ 1.00 (d, J = 5.7 Hz, 21 H), 1.69 (dq, J = 9.5, 7.0 Hz, 2 H), 1.88–1.95 (m, 2 H), 2.39 (t, J = 6.9 Hz, 2 H), 4.24 (t, J = 6.4 Hz, 2 H), 7.17 (dd, J = 8.6, 2.2 Hz, 2 H), 7.19 (s, 2 H), 7.44–7.52 (m, 2 H), 8.20 (d, J = 2.7 Hz, 2 H), 8.56 (d, J = 2.2 Hz, 2 H), 11.70 (d, J = 2.7 Hz, 2 H).

¹³C NMR (DMSO-*d*₆, 75 MHz): δ 10.7 (CH₂), 18.5 (CH₃), 18.9 (CH₂), 25.0 (CH₂), 27.6 (CH₂), 66.9 (CH₂), 79.9 (C_{quat}), 102.7 (CH), 109.3 (C_{quat}), 113.2 (CH), 116.0 (C_{quat}), 120.9 (CH), 121.6 (CH), 124.7 (C_{quat}), 126.6 (C_{quat}), 127.4 (CH), 135.4 (C_{quat}), 155.7 (C_{quat}), 165.3 (C_{quat}).

IR: $\tilde{\nu}$ [cm⁻¹] 657 (m), 677 (m), 702 (w), 754 (w), 769 (w), 794 (s), 823 (w), 835 (w), 871 (w), 883 (w), 893 (s), 918 (w), 947 (w), 960 (w), 987 (w), 1045 (m), 1058 (m), 1105 (w), 1143 (m), 1170 (w), 1217 (m), 1251 (w), 1278 (w), 1290 (w), 1330 (m), 1369 (w), 1411 (w), 1454 (s), 1535 (w), 1554 (s), 1570 (w), 1593 (s), 1618 (w), 1950 (w), 1961 (w), 2160 (w), 2864 (w), 2941 (w), 3111 (w), 3124 (w), 3153 (w), 3360 (w).

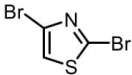
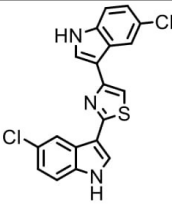
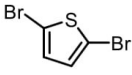
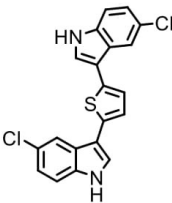
MS (ESI, *m/z* (%)): 631 ([³⁷Cl³⁵CIM]⁺, 22), 629 ([³⁵Cl³⁵CIM]⁺, 22), 588 ([C₃₃H₃₄³⁷Cl³⁵CIN₃OSi]⁺, 69), 586 ([C₃₃H₃₄³⁷Cl³⁵CIN₃OSi]⁺, 86), 508 (12), 506 (16), 450 (13), 436 (13), 192 (26), 151 (24), 149 (58), 121 (60), 93 (100), 79 (40).

Anal. calcd for C₃₆H₄₁Cl₂N₃OSi [630.7]: C 68.55, H 6.55, N 6.66; **Found:** C 68.47, H 6.51, N 6.60.

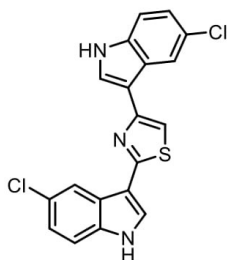
S6.7 General procedure IV (GPIV) for the synthesis of five-membered ring-bridged bisindoles 5[4]

5-chloro-3-iodo-1-tosyl-1*H*-indole (**1b**) (431 mg, 1.00 mmol) and tetrakis(triphenylphosphane)palladium(0) (57 mg, 0.05 mmol, 5 mol%) were dissolved in 5.0 mL dry 1,4-dioxane in a dry Schlenk tube with a magnetic stir bar under dry nitrogen (for experimental detail see table S4). Triethylamine (1.4 mL, 10 mmol) and 4,4,5,5-tetramethyl-1,3,2-dioxaborolane (0.25 mL, 1.7 mmol) (pinacolyl borane, HBpin) were added dropwise, and the reaction mixture was stirred at 80 °C for 3 h. Thereafter, the mixture was cooled to room temp, and 5.0 mL water, sodium carbonate (0.27 g, 2.50 mmol), triphenylphosphane (16 mg, 0.06 mmol, 6 mol%) and heteroaryl dihalide **3** (0.5 mmol) were added to the reaction mixture. This mixture was stirred at 100 °C for 18 h. Subsequently, the mixture was cooled to room temp, potassium hydroxide (0.28 g, 5.0 mmol) was added, and the reaction was stirred again at 100 °C for 3 h. After cooling to room temp, the mixture was diluted with dichloromethane, adsorbed onto Celite® and the bisindoles **5** were isolated and purified by column chromatography on silica gel (eluent: dichloromethane/methanol/ammonia aq., 25% 100:1:1). The resulting solids were dried in vacuo for 24 h.

Table S5 Heteroaryl dihalides **3** and yields in the synthesis of bisindoles **5**.

entry	heteroaryl dihalide 3 [mg] (mmol)	bisindole 5	yield [mg] (%)
1	 121 mg (0.50 mmol) of 3p		90 mg (24%) of 5q
2	 0.06 mL (0.50 mmol) of 3q		137 mg (36%) of 5r

2,4-bis(5-chloro-1*H*-indol-3-yl)thiazole (5q)



5q
[384.28]
C₁₉H₁₁Cl₂N₃S

Mp.: 211 °C.

R_f (dichloromethane/methanol/aq. ammonia 100:5:1): 0.71.

¹H NMR (acetone-*d*₆, 300 MHz): δ 7.20 (dd, *J* = 8.7 Hz, 2.1 Hz, 1 H), 7.26 (dd, *J* = 8.7 Hz, 2.1 Hz, 1 H), 7.51–7.60 (m, 3 H), 8.09 (d, *J* = 2.8 Hz, 1 H), 8.16 (d, *J* = 2.8 Hz, 1 H), 8.39 (d, *J* = 2.0 Hz, 1 H), 8.56 (d, *J* = 2.0 Hz, 1 H), 10.71 (s, 1 H), 10.99 (s, 1 H).

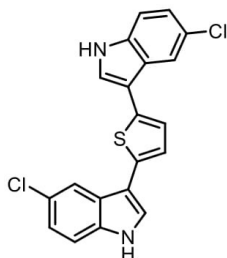
¹³C NMR (acetone-*d*₆, 75 MHz): δ 107.4 (CH), 112.5 (C_{quat}), 113.0 (C_{quat}), 114.0 (CH), 114.3 (C_{quat}), 120.9 (CH), 121.3 (CH), 122.8 (CH), 123.7 (CH), 126.2 (C_{quat}), 126.8 (CH), 126.9 (C_{quat}), 127.2 (C_{quat}), 127.4 (C_{quat}), 128.2 (CH), 136.4 (C_{quat}), 151.7 (C_{quat}), 162.8 (C_{quat}).

IR $\tilde{\nu}$ [cm⁻¹]: 633 (w), 662 (w), 698 (m), 718 (m), 750 (m), 793 (s), 829 (w), 864 (w), 876 (m), 893 (m), 928 (w), 953 (w), 978 (w), 999 (w), 1015 (w), 1053 (m), 1101 (m), 1146 (w), 1171 (w), 1215 (w), 1225 (w), 1265 (w), 1275 (w), 1339 (w), 1414 (w), 1449 (m), 1493 (w), 1549 (m), 1614 (w), 3113 (w), 3233 (w), 3433 (w).

MS (ESI, *m/z* (%)): 388 ([³⁷Cl³⁷ClC₁₉H₁₂N₃S]⁺, 14), 386 ([³⁵Cl³⁷ClC₁₉H₁₂Cl₂N₃S]⁺, 65), 384 ([³⁵Cl³⁵ClC₁₉H₁₂Cl₂N₃S]⁺, 100), 351 ([³⁷ClC₁₉H₁₂N₃S]⁺, 7), 349 ([³⁵ClC₁₉H₁₂N₃S]⁺, 12).

HR-MS (ESI) (*m/z*) calcd for (C₁₉H₁₁³⁷Cl³⁵CIN₃S+H)⁺: 386.0094, **Found**: 386.0101; calcd for (C₁₉H₁₁³⁵Cl³⁵CIN₃S+H)⁺: 384.0124, **Found**: 384.0130.

2,5-bis(5-chloro-1*H*-indol-3-yl)thiophene (5r)



5r
[383.29]
C₂₀H₁₂Cl₂N₂S

Mp.: 194 °C.

R_f (dichlormethane/methanol/aq. ammonia 100:5:1): 0.86.

¹H NMR (acetone-d₆, 300 MHz): δ 7.20 (dd, *J* = 8.7 Hz, 2.0 Hz, 2 H), 7.36 (s, 2 H), 7.52 (d, *J* = 8.6 Hz, 2 H), 7.78 (d, *J* = 1.9 Hz, 2 H), 7.98 (d, *J* = 1.8 Hz, 2 H), 10.73 (s, 2 H).

¹³C-NMR (Aceton-d₆, 150 MHz): δ 114.2 (CH), 119.6 (CH), 123.1 (CH), 123.9 (CH), 125.0 (C_{quat}), 125.2 (C_{quat}), 126.3 (CH), 127.3 (C_{quat}), 135.3 (C_{quat}), 136.4 (C_{quat}).

IR $\tilde{\nu}$ [cm⁻¹]: 692 (w), 704 (m), 723 (w), 748 (w), 785 (s), 815 (w), 866 (w), 889 (w), 934 (w), 970 (w), 997 (w), 1045 (w), 1099 (m), 1140 (w), 1171 (w), 1242 (w), 1269 (w), 1285 (w), 1341 (w), 1418 (w), 1445 (m), 1456 (m), 1562 (w), 1614 (w), 3030 (w), 3410 (w), 3426 (w).

MS (EI, *m/z* (%)): 386 ([³⁷Cl³⁷ClC₂₀H₁₂N₂S]⁺, 13), 385 ([³⁵Cl³⁷ClC₂₀H₁₃N₂S]⁺, 15), 384 ([³⁵Cl³⁷ClC₂₀H₁₂N₂S]⁺, 69), 383 ([³⁵Cl³⁵ClC₂₀H₁₃N₂S]⁺, 27), 382 ([³⁵Cl³⁵Cl C₂₀H₁₂N₂S]⁺, 100), 386 ([³⁵Cl³⁵ClC₂₀H₁₁N₂S]⁺, 11), 349 ([³⁷ClC₂₀H₁₂N₂S]⁺, 14), 347 ([³⁵ClC₂₀H₁₂N₂S]⁺, 33), 192 (14), 191 (25), 155 ([C₇H₇O₂S]⁺, 16), 142 (24).

HR-MS (ESI) (*m/z*) calcd for (C₂₀H₁₂³⁷Cl³⁵CIN₂S+H)⁺: 384.0069, **Found:** 384.0063; **calcd for (C₂₀H₁₂³⁵Cl³⁵CIN₂S+H)⁺:** 382.0098, **Found:** 382.0091.

S7 NMR spectra of *N*-tosyl bisindoles 4

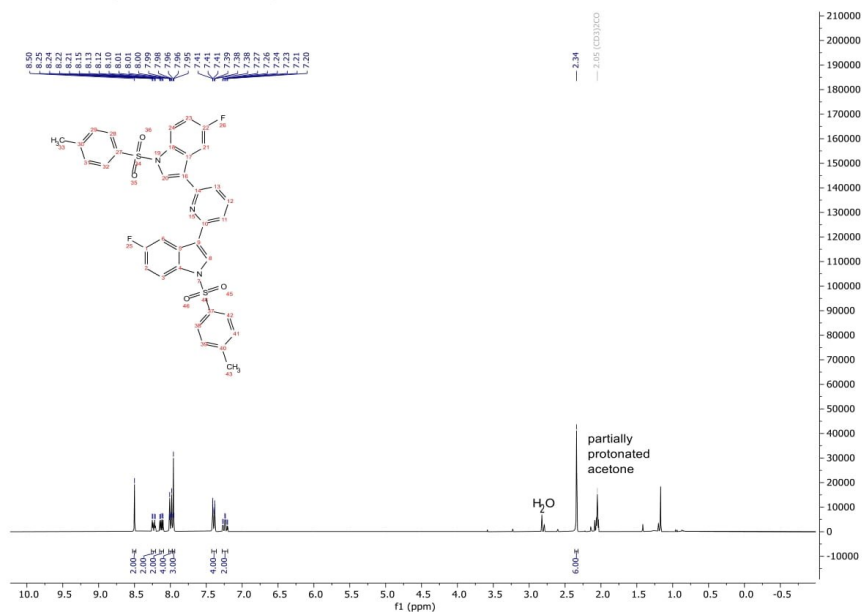


Figure 1 ¹H NMR spectrum of 2,6-bis(5-fluoro-1-tosyl-1*H*-indol-3-yl)pyridine (**4a**) (acetone-d₆, 300 MHz, 293 K).

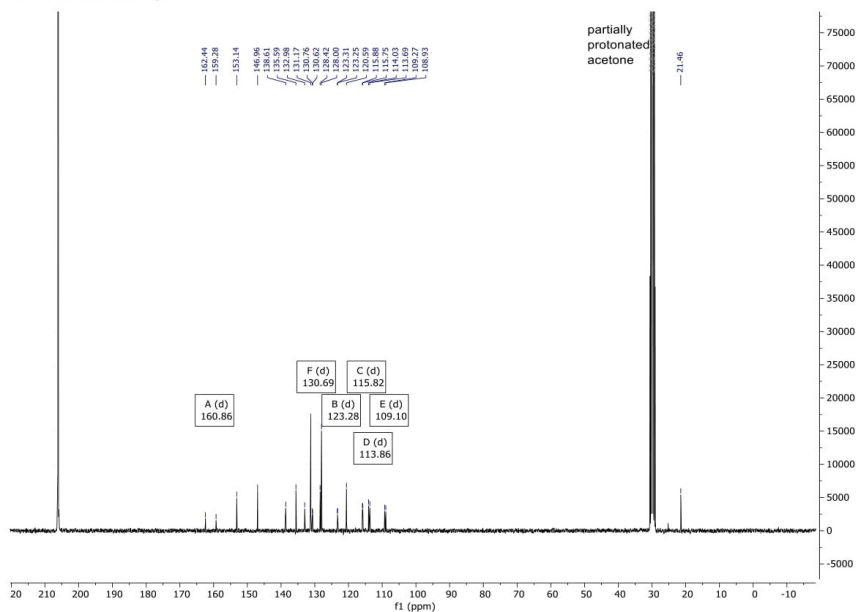


Figure 2 ¹³C NMR spectrum of 2,6-bis(5-fluoro-1-tosyl-1*H*-indol-3-yl)pyridine (**4a**) (acetone-d₆, 75 MHz, 293 K).

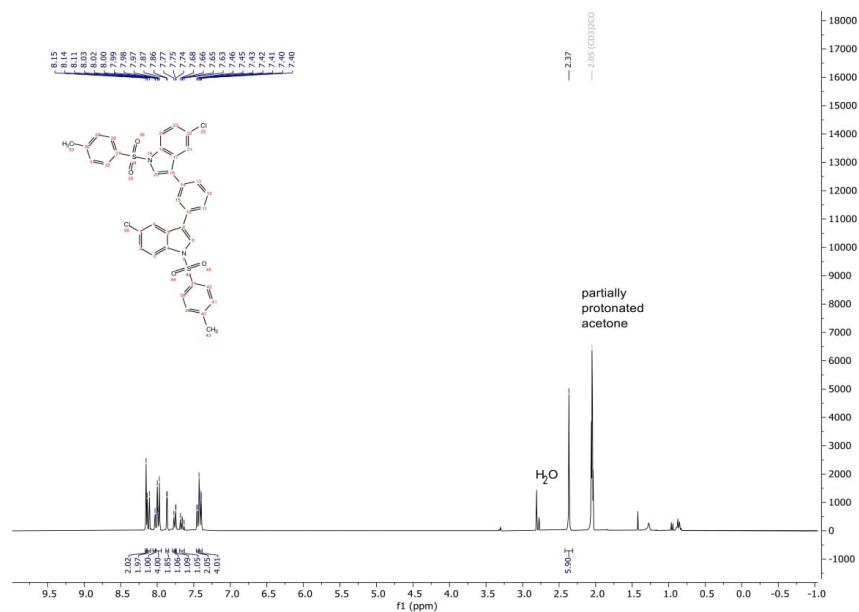


Figure 3 ¹H NMR spectrum of 1,3-bis(5-chloro-1-tosyl-1H-indol-3-yl)benzene (**4b**) (acetone-d₆, 300 MHz, 293 K).

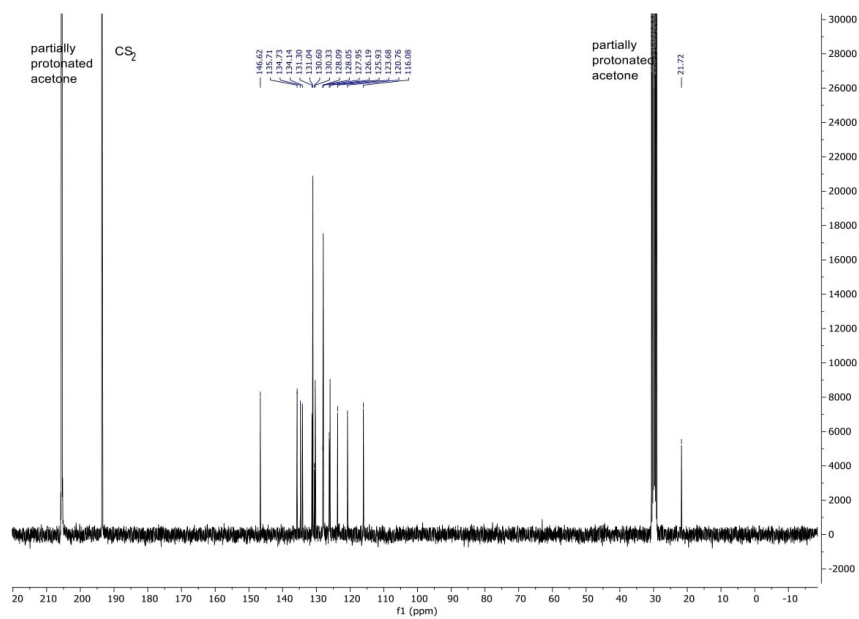


Figure 4 ¹³C NMR spectrum of 1,3-bis(5-chloro-1-tosyl-1H-indol-3-yl)benzene (**4b**) (acetone-d₆, 75 MHz, 293 K).

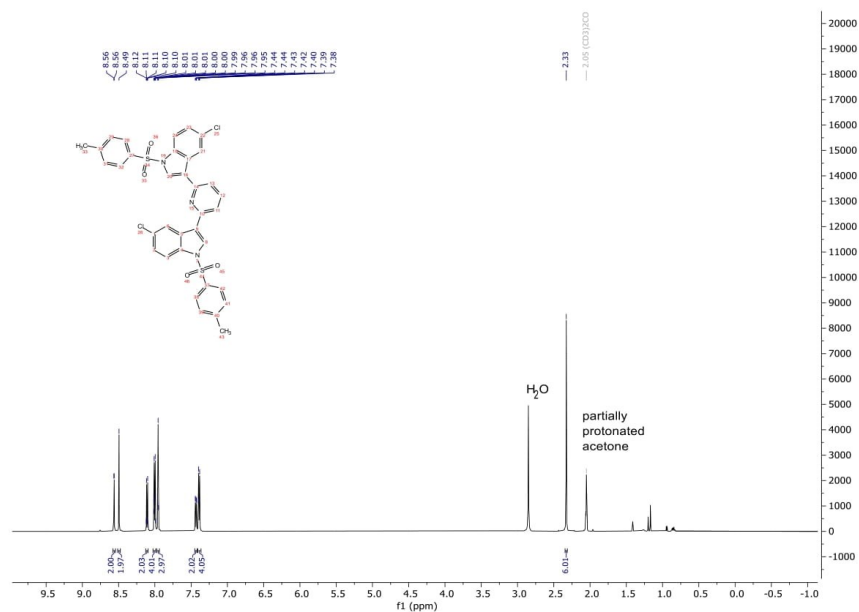


Figure 5 ¹H NMR spectrum of 2,6-bis(5-chloro-1-tosyl-1H-indol-3-yl)pyridine (**4c**) (acetone-d₆, 600 MHz, 293 K).

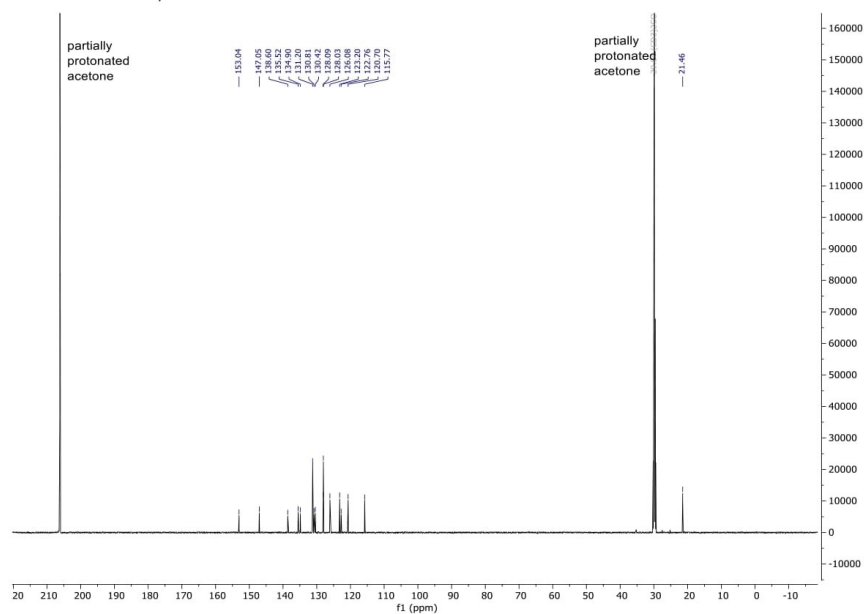
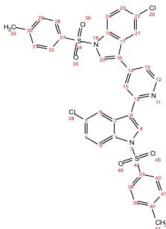


Figure 6 ¹³C NMR spectrum of 2,6-bis(5-chloro-1-tosyl-1H-indol-3-yl)pyridine (**4c**) (acetone-d₆, 150 MHz, 293 K).



154

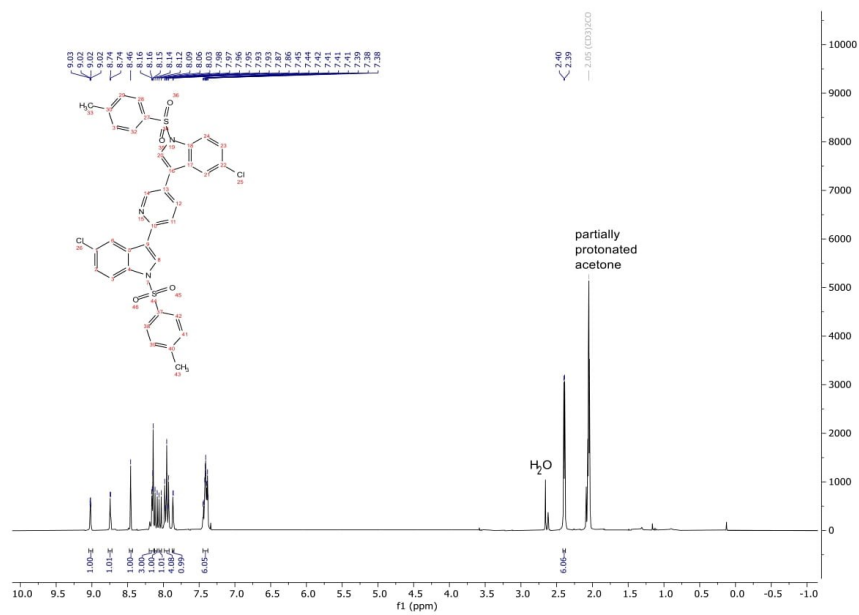


Figure 9 ¹H NMR spectrum of 3,3'-(pyridin-2,5-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4e**) (acetone-*d*₆, 300 MHz, 293 K).

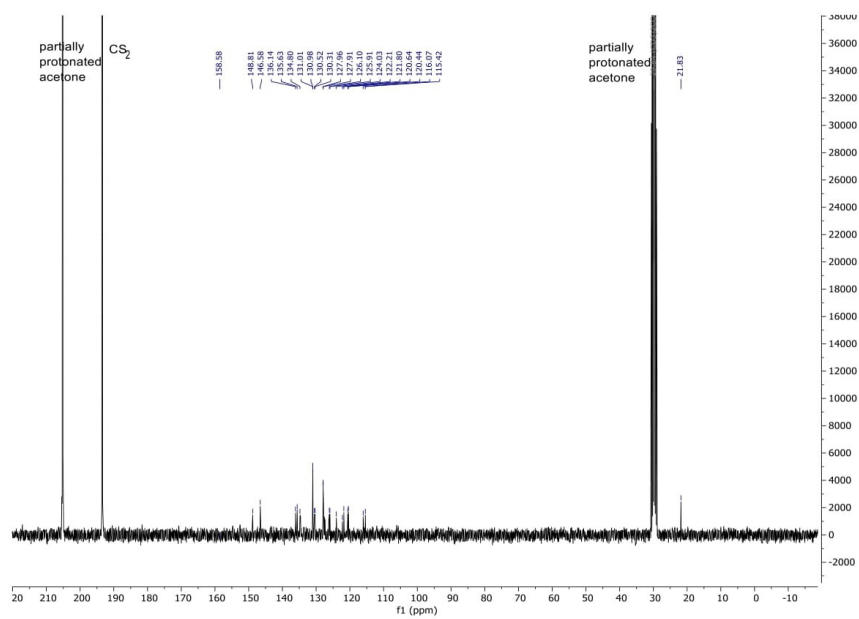


Figure 10 ¹³C NMR spectrum of 3,3'-(pyridin-2,5-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4e**) (acetone-*d*₆, 75 MHz, 293 K).

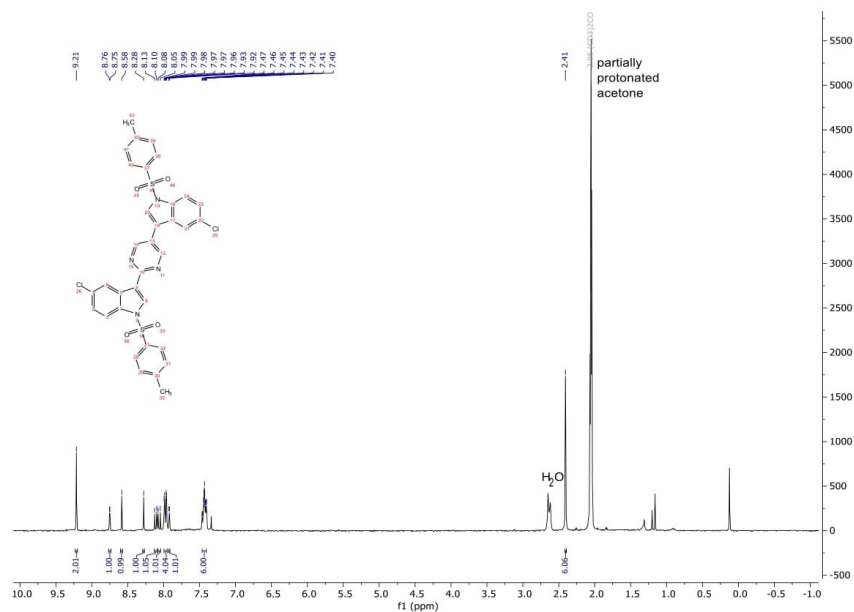


Figure 11 ¹H NMR spectrum of 3,3'-(pyrimidin-2,5-diyl)bis(5-chloro-1-tosyl-1H-indole) (**4f**) (acetone-d₆, 300 MHz, 293 K).

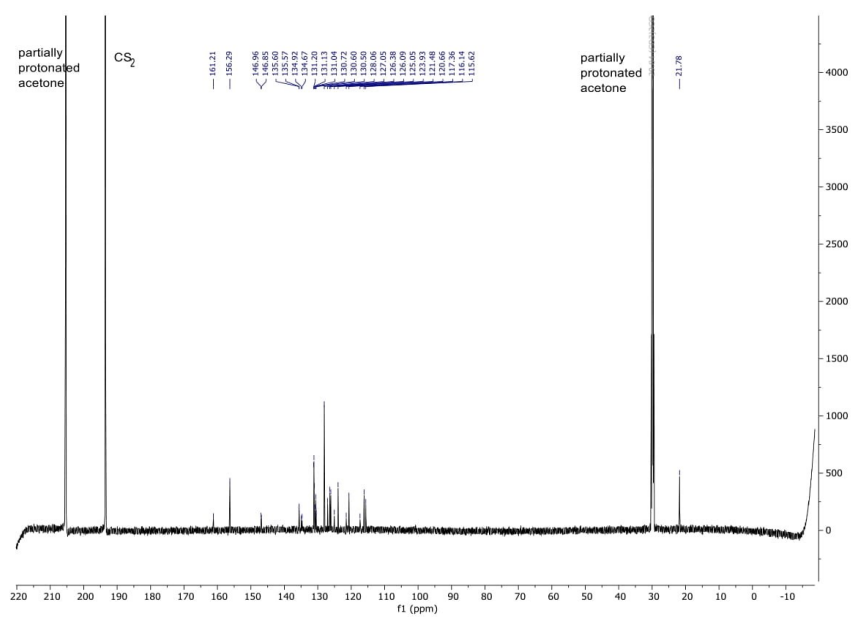


Figure 12 ¹³C NMR spectrum of 3,3'-(pyrimidin-2,5-diyl)bis(5-chloro-1-tosyl-1H-indole) (**4f**) (acetone-d₆, 150 MHz, 293 K).

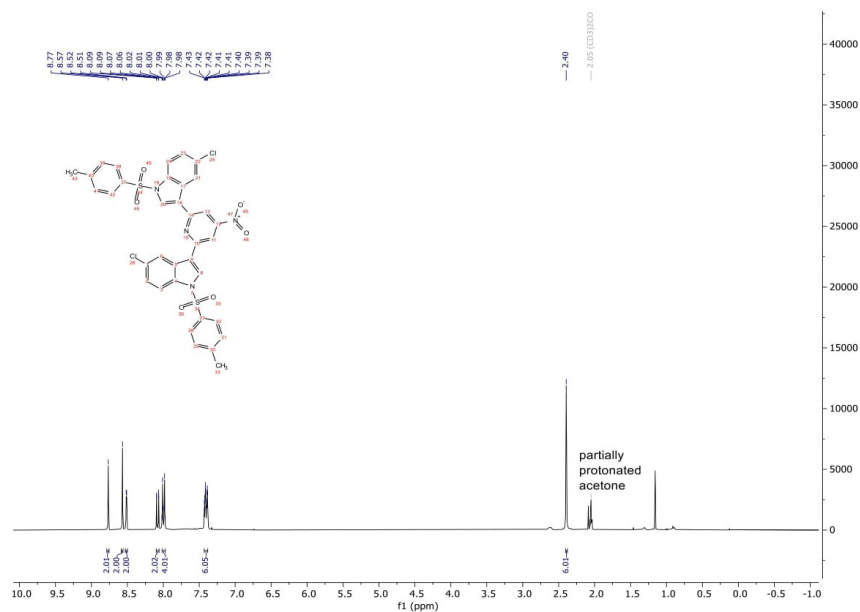


Figure 13 ¹H NMR spectrum of 3,3'-(4-nitropyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4g**) (acetone-*d*₆, 300 MHz, 293 K).

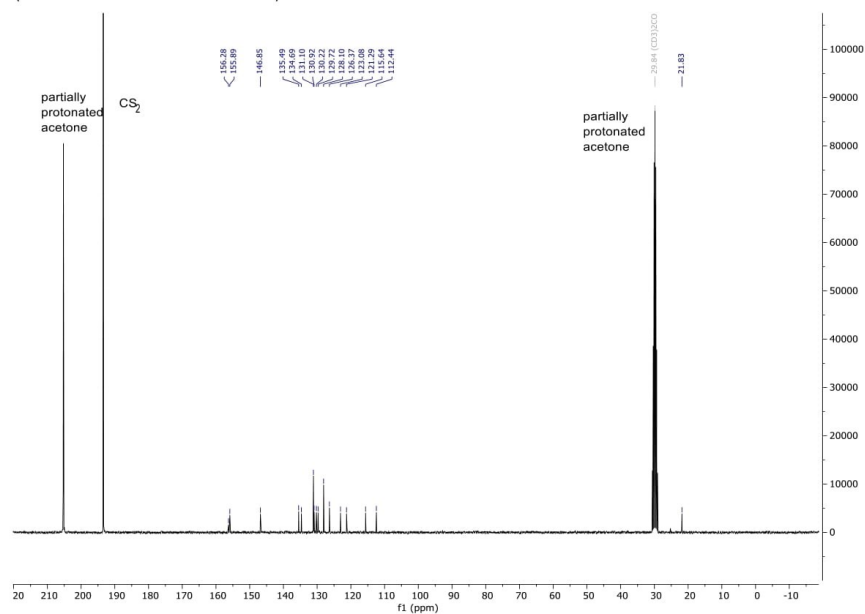


Figure 14 ¹³C NMR spectrum of 3,3'-(4-nitropyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4g**) (acetone-*d*₆, 75 MHz, 293 K).

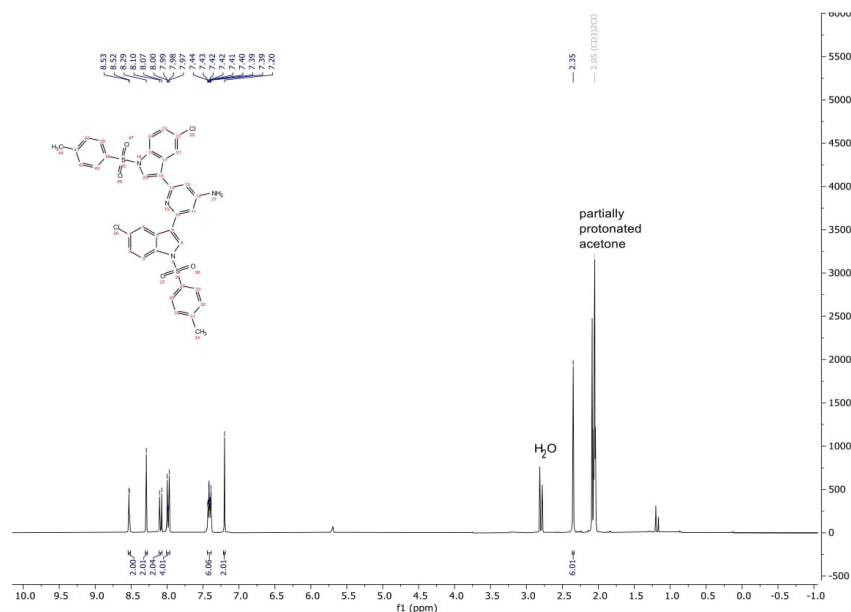


Figure 15 ¹H NMR spectrum of 2,6-bis(5-chloro-1-tosyl-1H-indol-3-yl)pyridin-4-amine (**4h**) (acetone-d₆, 300 MHz, 293 K).

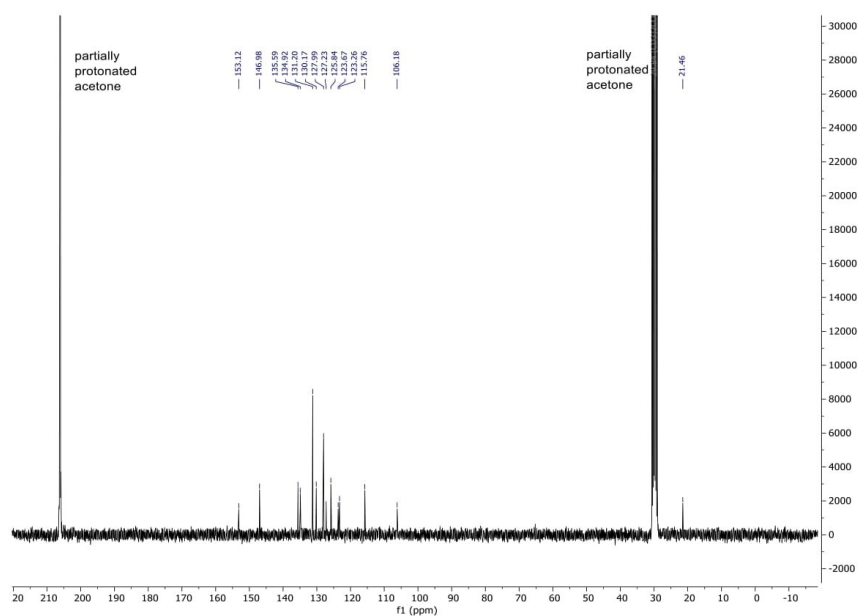


Figure 16 ¹³C NMR spectrum of 2,6-bis(5-chloro-1-tosyl-1H-indol-3-yl)pyridin-4-amine (**4h**) (acetone-d₆, 75 MHz, 293 K).

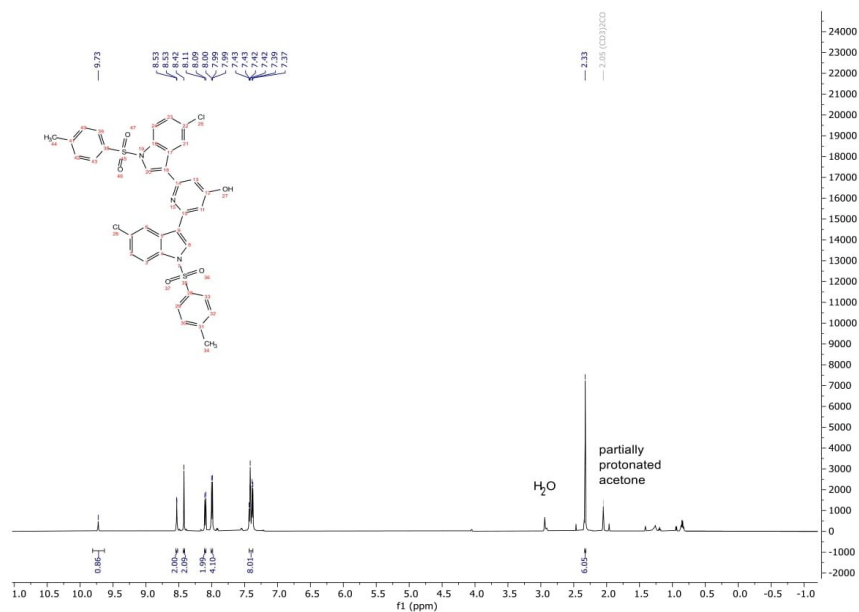


Figure 17 ^1H NMR spectrum of 2,6-bis(5-chloro-1-tosyl-1*H*-indol-3-yl)pyridin-4-ol (**4i**) (acetone- d_6 , 600 MHz, 293 K).

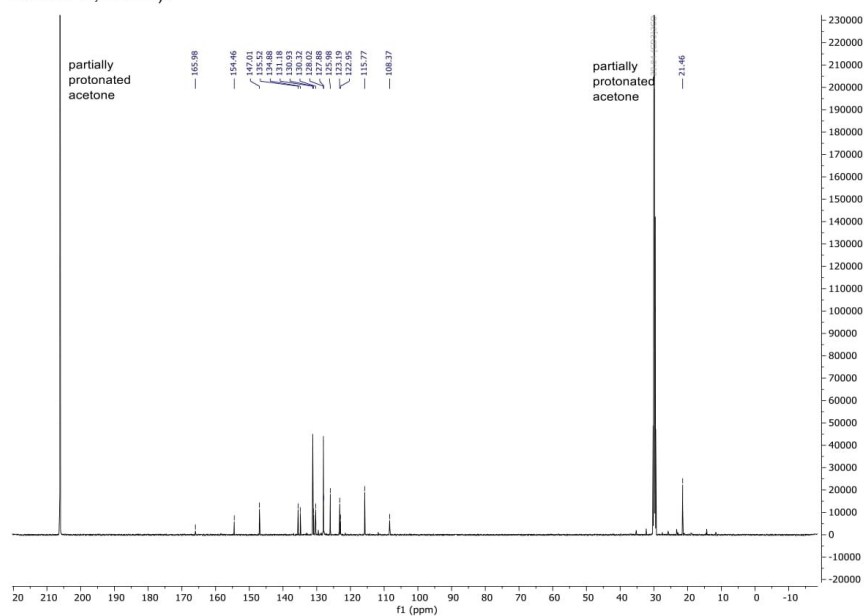


Figure 18 ^{13}C NMR spectrum of 2,6-bis(5-chloro-1-tosyl-1*H*-indol-3-yl)pyridin-4-ol (**4i**) (acetone- d_6 , 150 MHz, 293 K).

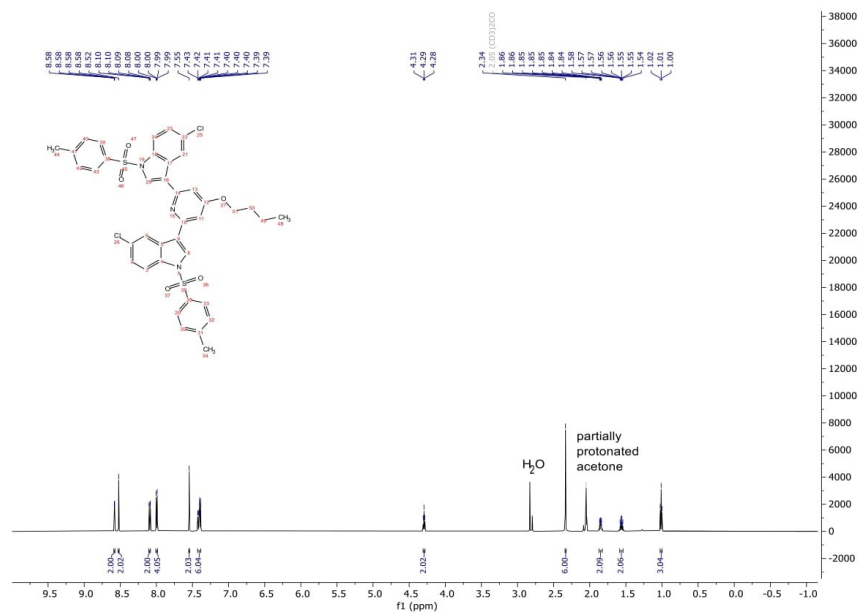


Figure 19 ¹H NMR spectrum of 3,3'-(4-butoxypyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4j**) (acetone-*d*₆, 600 MHz, 293 K).

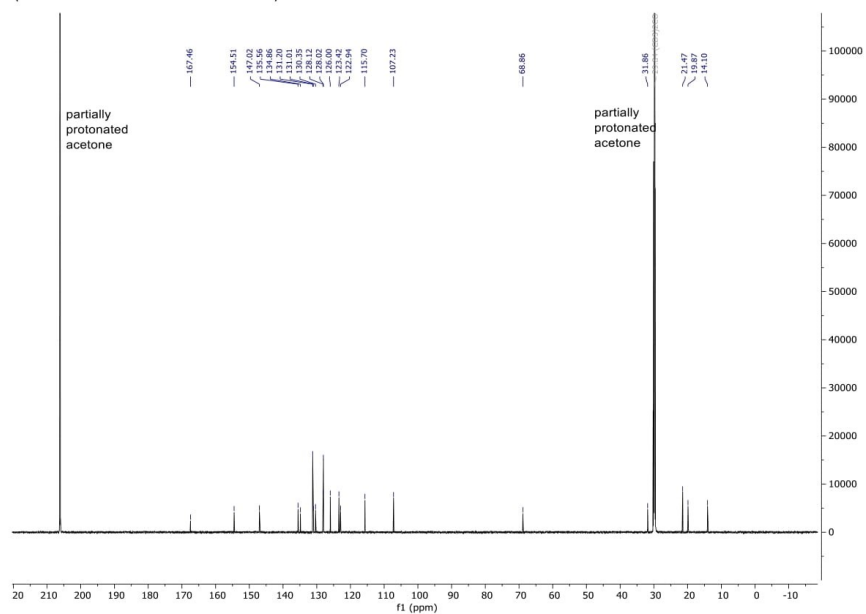


Figure 20 ¹³C NMR spectrum of 3,3'-(4-butoxypyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4j**) (acetone-*d*₆, 150 MHz, 293 K).

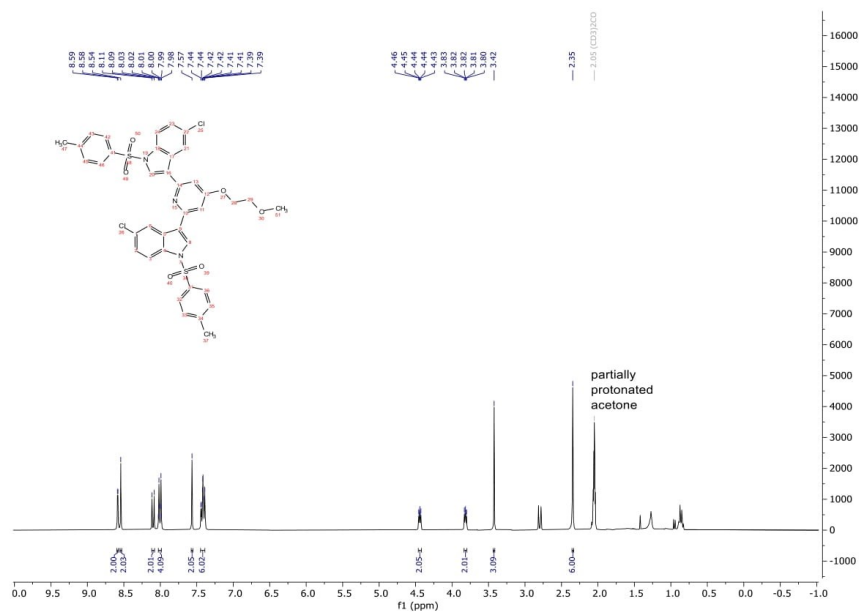


Figure 21 ^1H NMR spectrum of 3,3'-(4-(2-methoxyethoxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1H-indole) (**4k**) (acetone- d_6 , 300 MHz, 293 K).

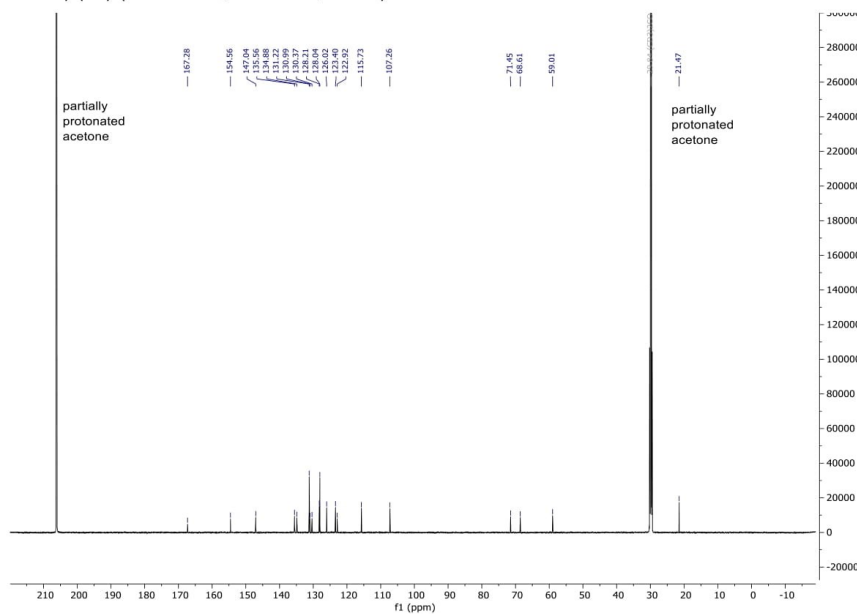


Figure 22 ^{13}C NMR spectrum of 3,3'-(4-(2-methoxyethoxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1H-indole) (**4k**) (acetone- d_6 , 150 MHz, 293 K).

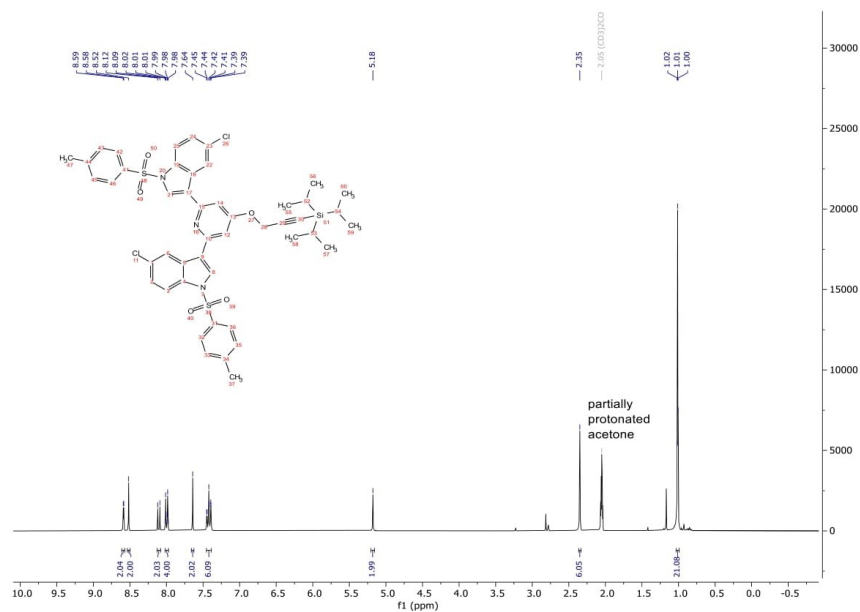


Figure 23 ¹H NMR spectrum of 3,3'-(4-((3-(triisopropylsilyl)prop-2-in-1-yl)oxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4I**) (acetone-d₆, 300 MHz, 293 K).

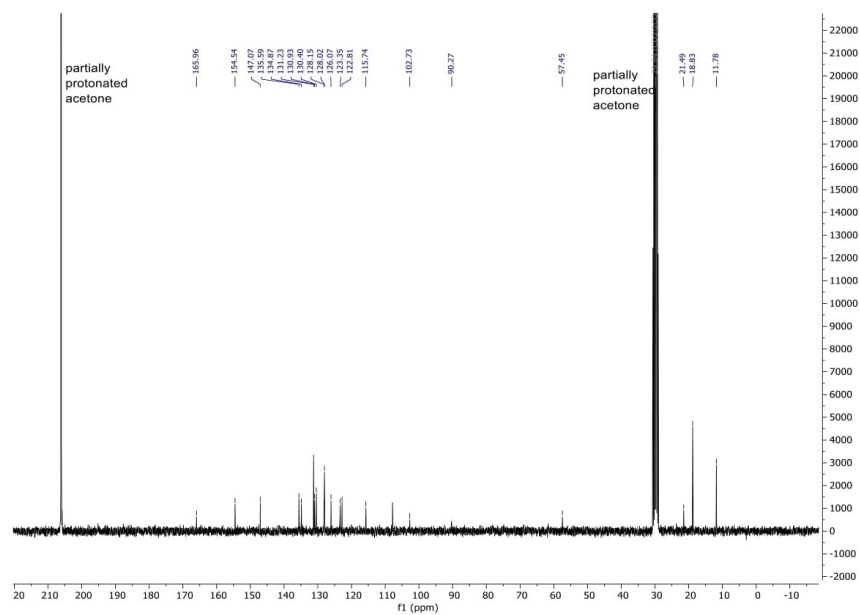


Figure 24 ¹³C NMR spectrum of 3,3'-(4-((3-(triisopropylsilyl)prop-2-in-1-yl)oxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4I**) (acetone-d₆, 75 MHz, 293 K).

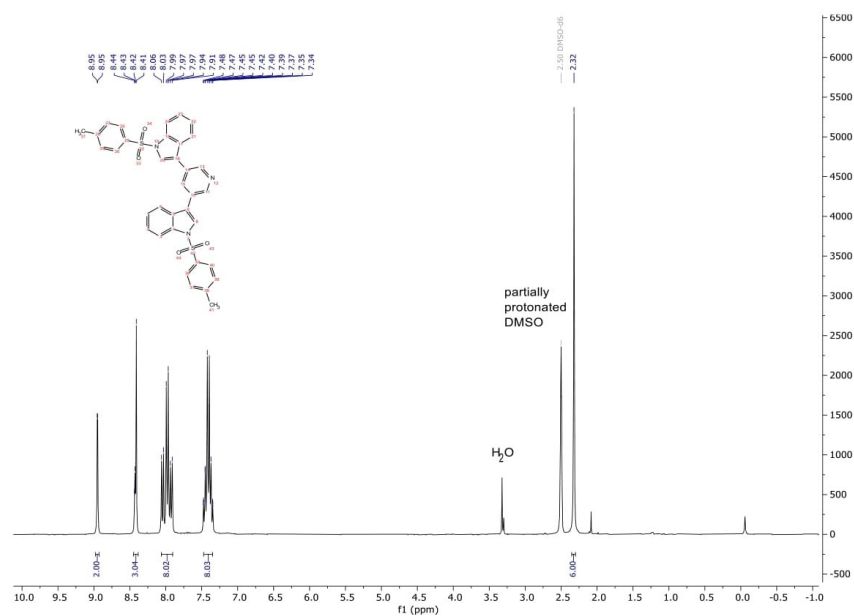


Figure 27 ¹H NMR spectrum of 3,5-bis(1-tosyl-1H-indol-3-yl)pyridine (**4n**) (DMSO-d₆, 300 MHz, 293 K).

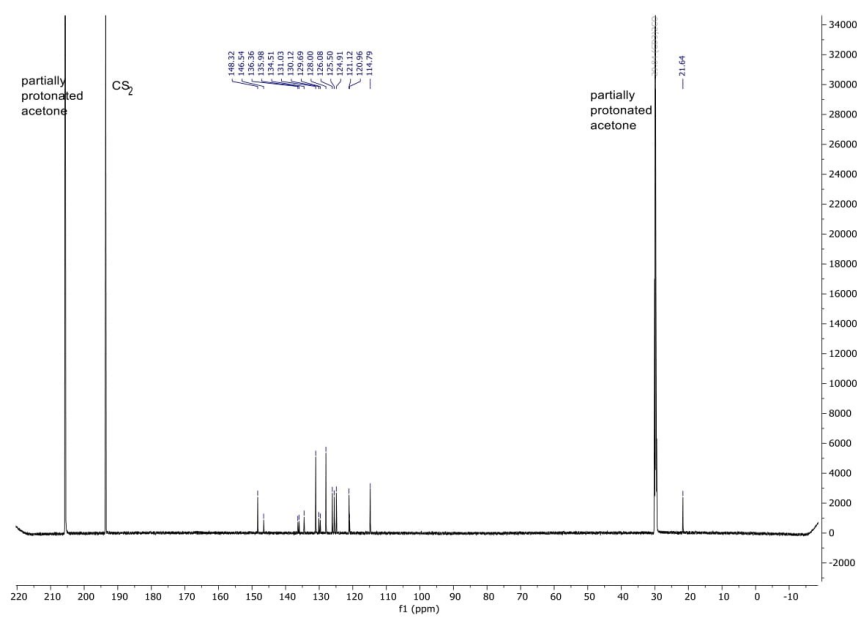


Figure 28 ¹³C NMR spectrum of 3,5-bis(1-tosyl-1H-indol-3-yl)pyridine (**4n**) (acetone-d₆, 150 MHz, 293 K).

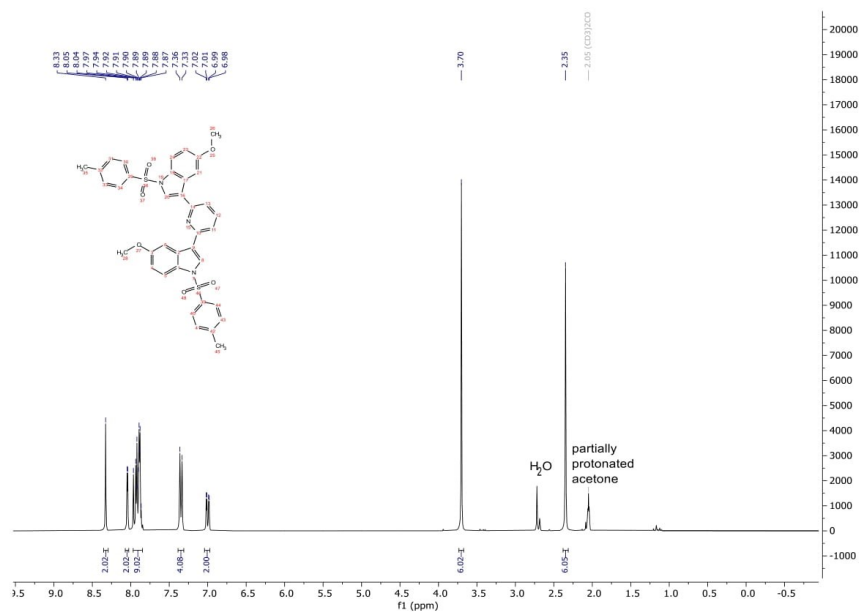


Figure 29 ¹H NMR spectrum of 2,6-bis(5-methoxy-1-tosyl-1H-indol-3-yl)pyridine (**4o**) (acetone-d₆, 300 MHz, 293 K).

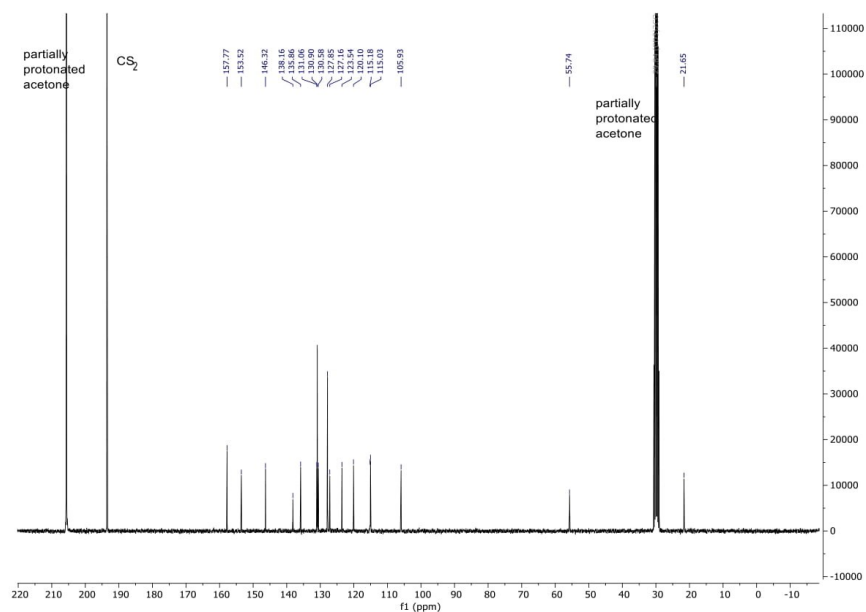


Figure 30 ¹³C NMR spectrum of 2,6-bis(5-methoxy-1-tosyl-1H-indol-3-yl)pyridine (**4o**) (acetone-d₆, 75 MHz, 293 K).

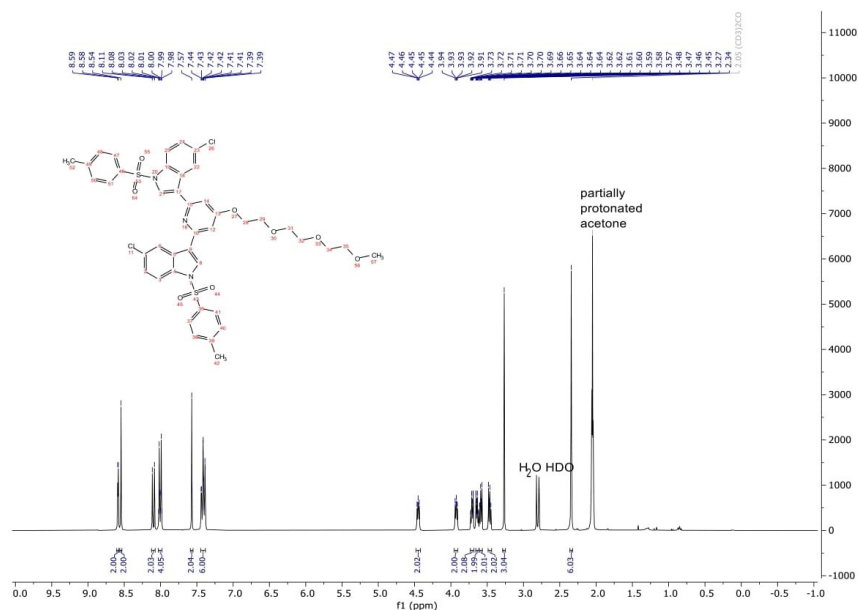


Figure 33 ¹H NMR spectrum of 3,3'-(4-(2-(2-methoxyethoxy)ethoxy)ethoxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4q**) (acetone-*d*₆, 300 MHz, 293 K).

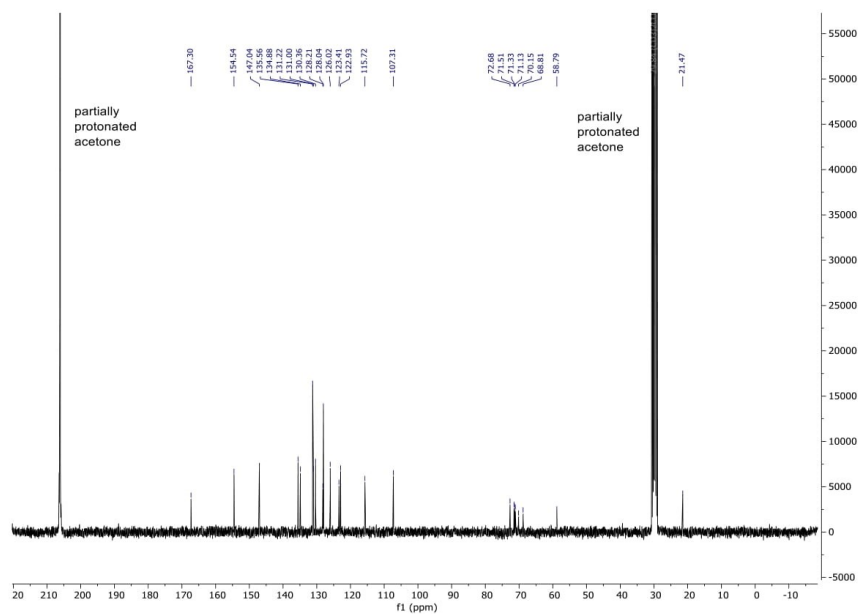


Figure 34 ¹³C NMR spectrum of 3,3'-(4-(2-(2-methoxyethoxy)ethoxy)ethoxy)pyridin-2,6-diyl)bis(5-chloro-1-tosyl-1*H*-indole) (**4q**) (acetone-*d*₆, 75 MHz, 293 K).

¹H NMR spectrum (DMSO-d₆) of 2,2'-difluoro-5,5'-bibenzimidazole.

Chemical structure: Fc1cc2c(c1)c3cc(F)cc3n2

Peak list (ppm): 8.26, 8.24, 8.22, 8.21, 8.19, 8.17, 8.15, 7.78, 7.75, 7.73, 7.72, 7.71, 7.58, 7.56, 7.55, 7.54, 7.53, 7.50, 7.49, 7.47, 7.45, 7.44, 7.43, 7.42, 7.40, 7.07, 7.05, 7.04, 7.03, 7.02, 7.00.

Integration values: 2.00H, 2.00H, 1.06H, 1.06H, 2.00H, 2.00H.

Reference peak: 2.50 ppm (DMSO-d₆).

Chemical structure diagram: The structure shows two benzimidazole rings linked at the 5,5' positions. The fluorine atoms are at the 2 and 2' positions. Protons are labeled with numbers 1 through 10.

157.71
156.17
154.29

136.87
133.77
125.70
125.55
125.35
116.29
112.82
112.80
110.87
109.72
106.19
106.03

partially
protonated
DMSO

39.52 (DMSO-d₆)

A (d)
157.71

B (d)
125.63

C (d)
116.33

D (d)
112.85

E (d)
109.89

F (d)
106.19

f1

168

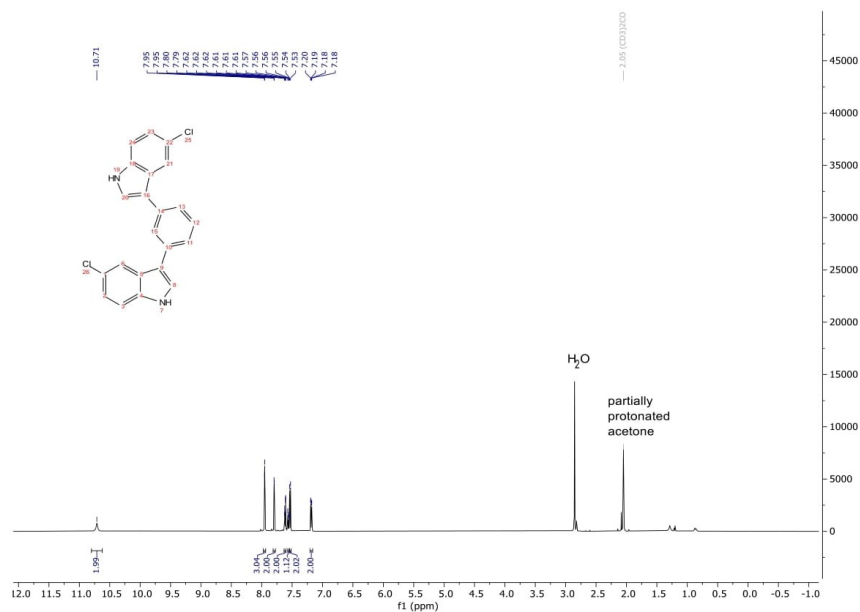


Figure 37 ¹H NMR spectrum of 1,3-bis(5-chloro-1H-indol-3-yl)benzene (**5b**) (acetone-d₆, 600 MHz, 293 K).

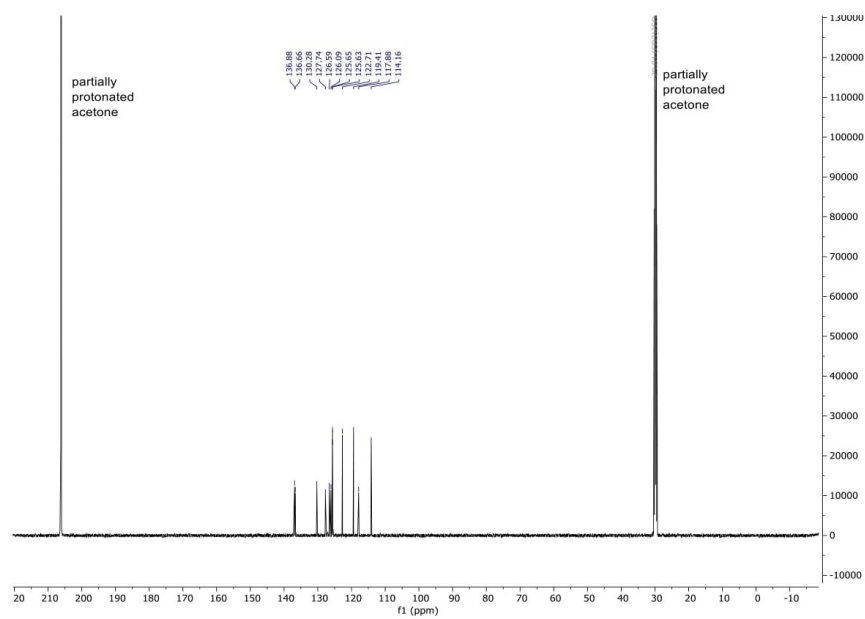


Figure 38 ¹³C NMR spectrum of 1,3-bis(5-chloro-1H-indol-3-yl)benzene (**5b**) (acetone-d₆, 150 MHz, 293 K).

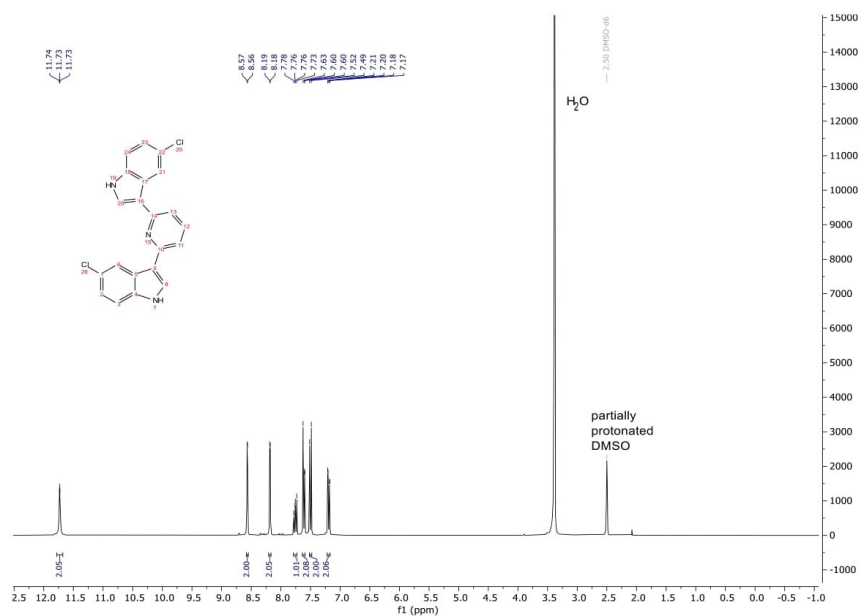


Figure 39 ¹H NMR spectrum of 2,6-bis(5-chloro-1H-indol-3-yl)pyridine (**5c**) (DMSO-d₆, 300 MHz, 293 K).

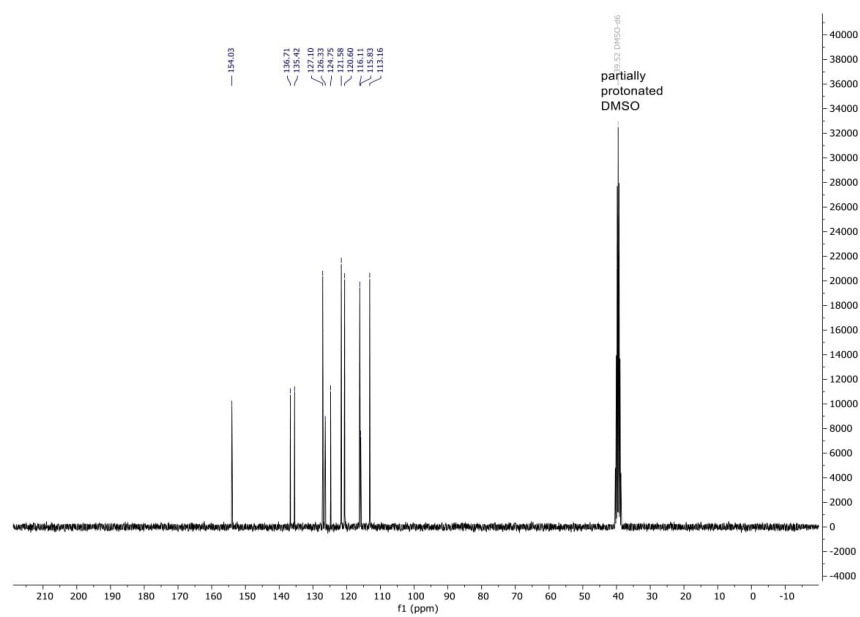


Figure 40 ¹³C NMR spectrum of 2,6-bis(5-chloro-1H-indol-3-yl)pyridine (**5c**) (DMSO-d₆, 75 MHz, 293 K).

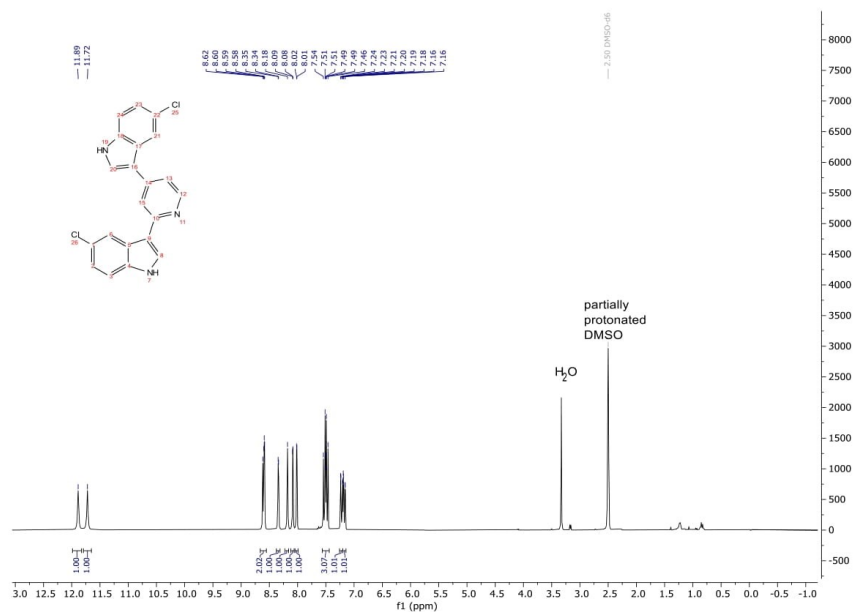


Figure 41 ^1H NMR spectrum of 3,3'-(pyridine-2,4-diyl)bis(5-chloro-1*H*-indole) (**5d**) (DMSO- d_6 , 300 MHz, 293 K).

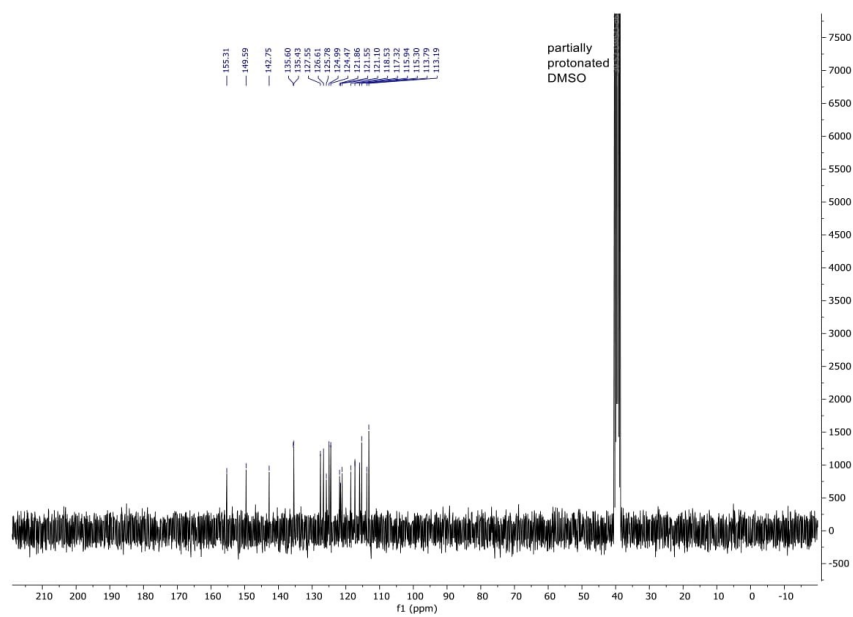


Figure 42 ^{13}C NMR spectrum of 3,3'-(pyridine-2,4-diyl)bis(5-chloro-1*H*-indole) (**5d**) (DMSO- d_6 , 75 MHz, 293 K).

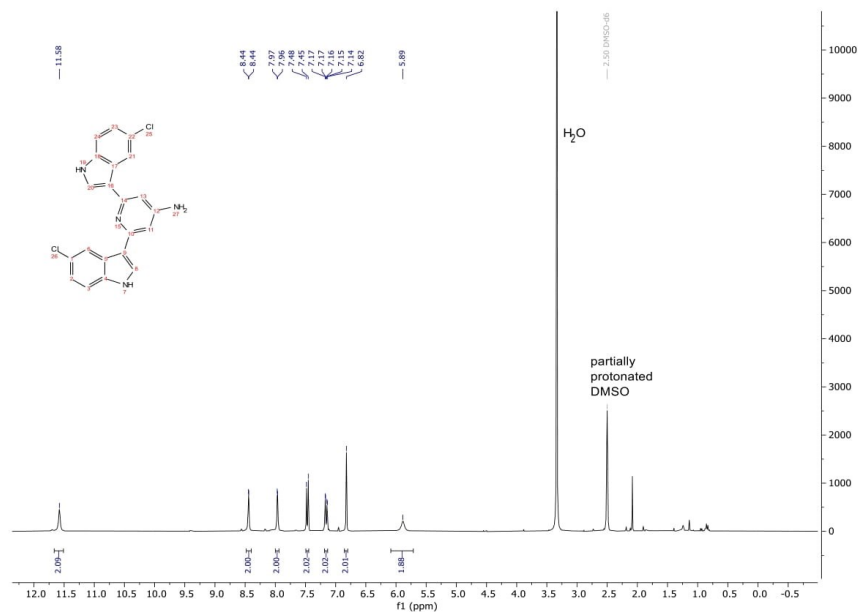


Figure 47 ¹H NMR spectrum of 2,6-bis(5-chloro-1H-indol-3-yl)pyridine-4-amine (**5g**) (DMSO-d₆, 300 MHz, 293 K).

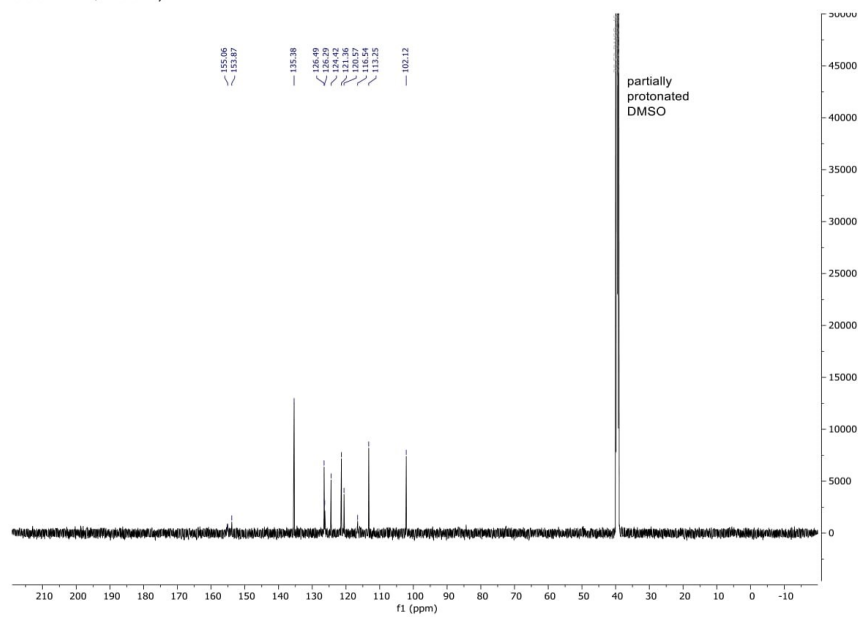


Figure 48 ¹³C NMR spectrum of 2,6-bis(5-chloro-1H-indol-3-yl)pyridine-4-amine (**5g**) (DMSO-d₆, 150 MHz, 293 K).

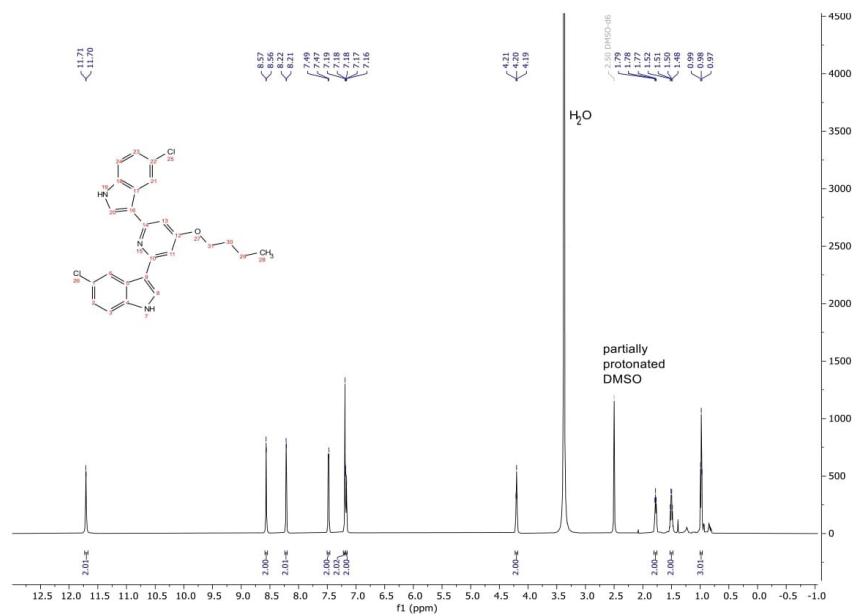


Figure 49 ^1H NMR spectrum of 3,3'-(4-butoxypyridine-2,6-diyl)bis(5-chloro-1H-indole) (**5h**) (DMSO- d_6 , 600 MHz, 293 K).

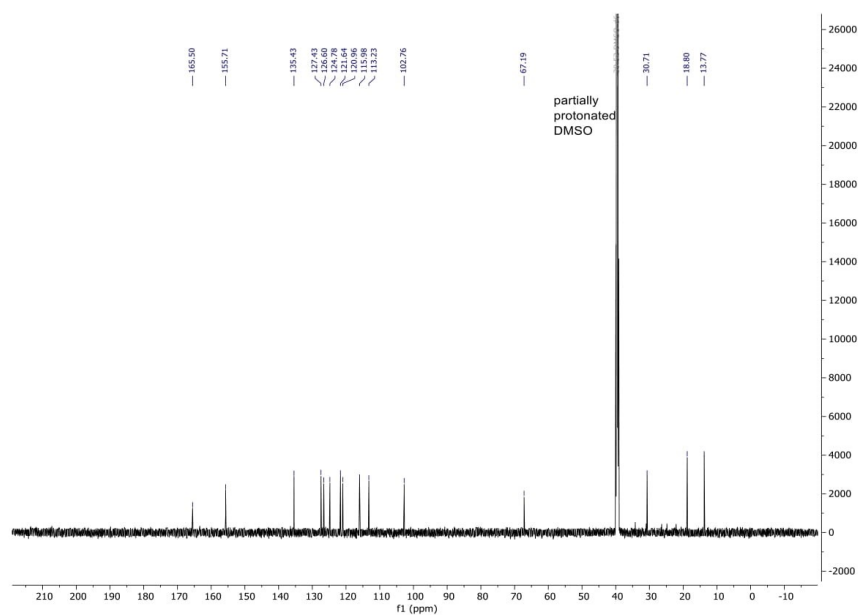
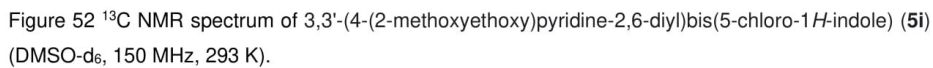
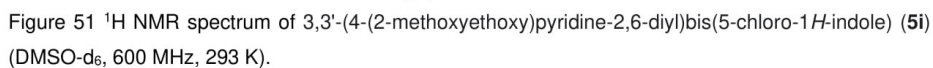


Figure 50 ^{13}C NMR spectrum of 3,3'-(4-butoxypyridine-2,6-diyl)bis(5-chloro-1H-indole) (**5h**) (DMSO- d_6 , 150 MHz, 293 K).



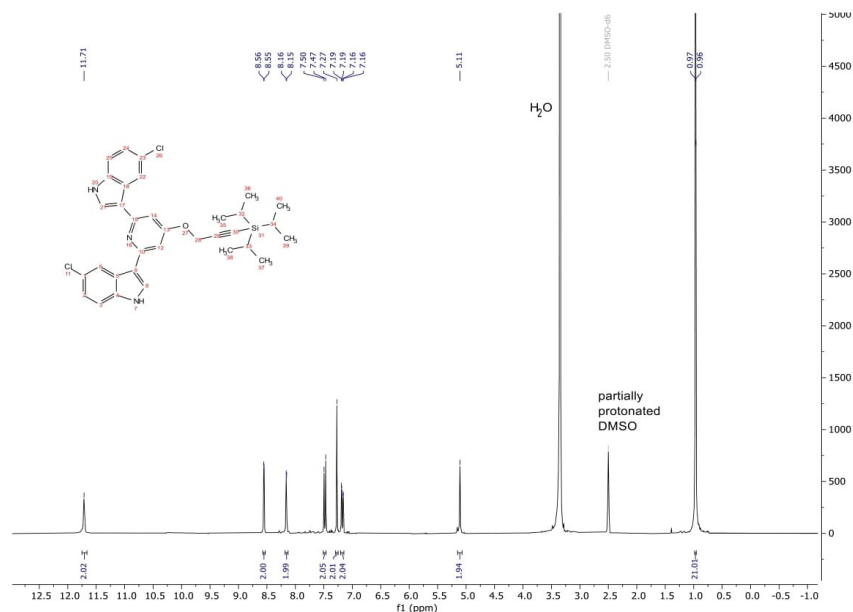


Figure 53 ^1H NMR spectrum of 3,3'-(4-((3-(triisopropylsilyl)prop-2-yn-1-yl)oxy)pyridine-2,6-diyl)bis(5-chloro-1H-indole) (**5j**) (DMSO- d_6 , 300 MHz, 293 K).

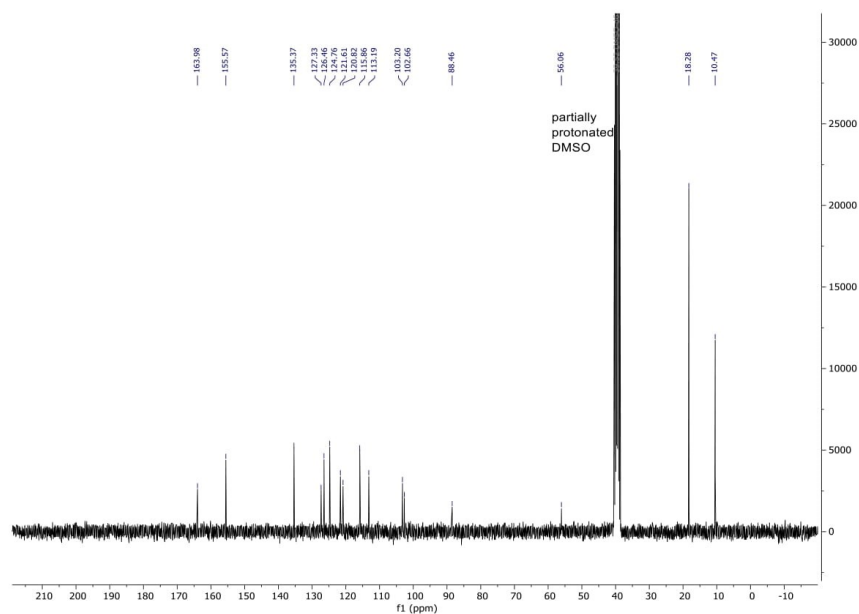
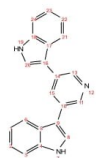


Figure 54 ^{13}C NMR spectrum of 3,3'-(4-((3-(triisopropylsilyl)prop-2-yn-1-yl)oxy)pyridine-2,6-diyl)bis(5-chloro-1H-indole) (**5j**) (DMSO- d_6 , 75 MHz, 293 K).



partially protonated DMSO

144.27
137.00
131.80
129.68
129.50
125.50
120.09
118.88
117.11
112.21

f1 (ppm)

178

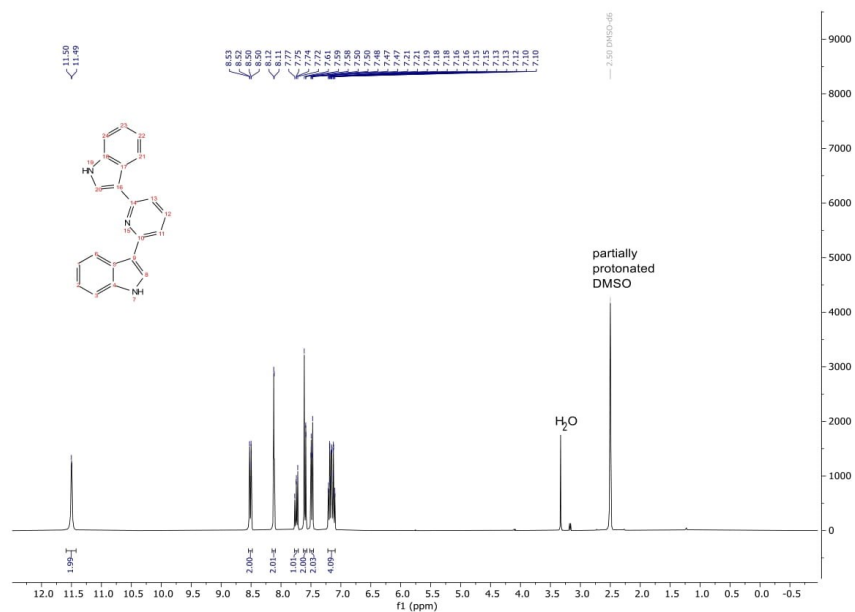


Figure 57 ¹H NMR spectrum of 2,6-di(1H-indol-3-yl)pyridine (5I) (DMSO-d₆, 300 MHz, 293 K).

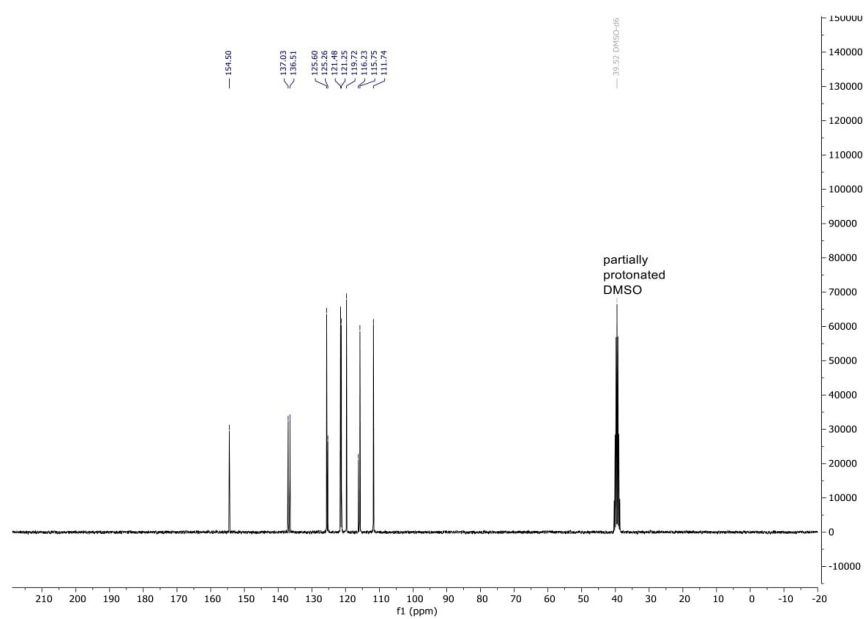


Figure 58 ¹³C NMR spectrum of 2,6-di(1H-indol-3-yl)pyridine (5I) (DMSO-d₆, 75 MHz, 293 K).

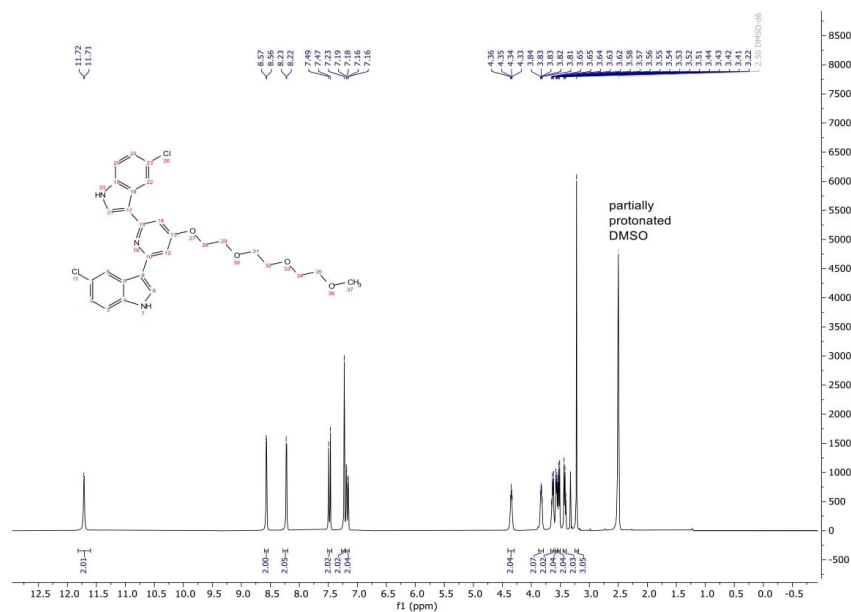


Figure 59 ¹H NMR spectrum of 3,3'-(4-(2-(2-(2-methoxyethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(5-chloro-1*H*-indole) (**5m**) (DMSO-*d*₆, 300 MHz, 293 K).

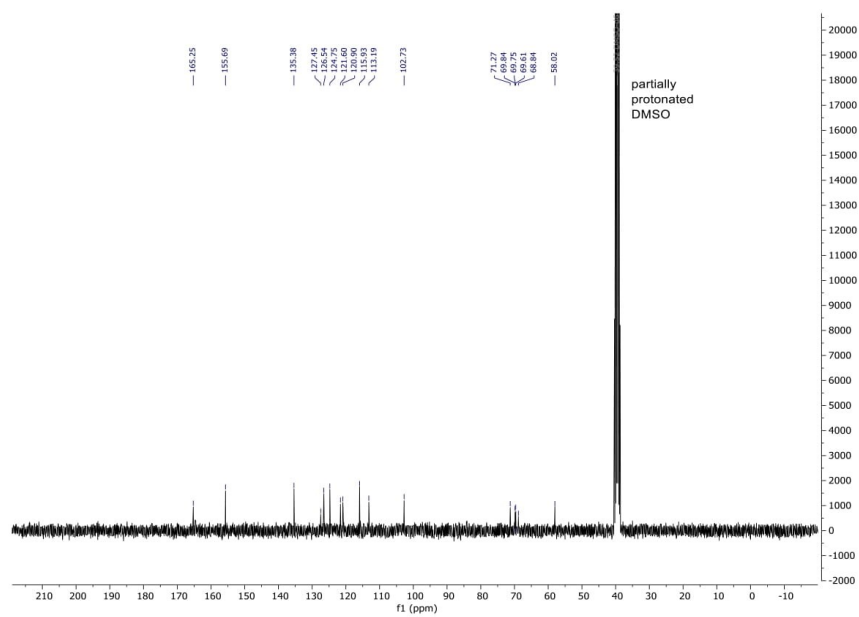


Figure 60 ^{13}C NMR spectrum of 3,3'-(4-(2-(2-methoxyethoxy)ethoxy)ethoxy)pyridine-2,6-diyl)bis(5-chloro-1*H*-indole) (**5m**) (DMSO- d_6 , 75 MHz, 293 K).

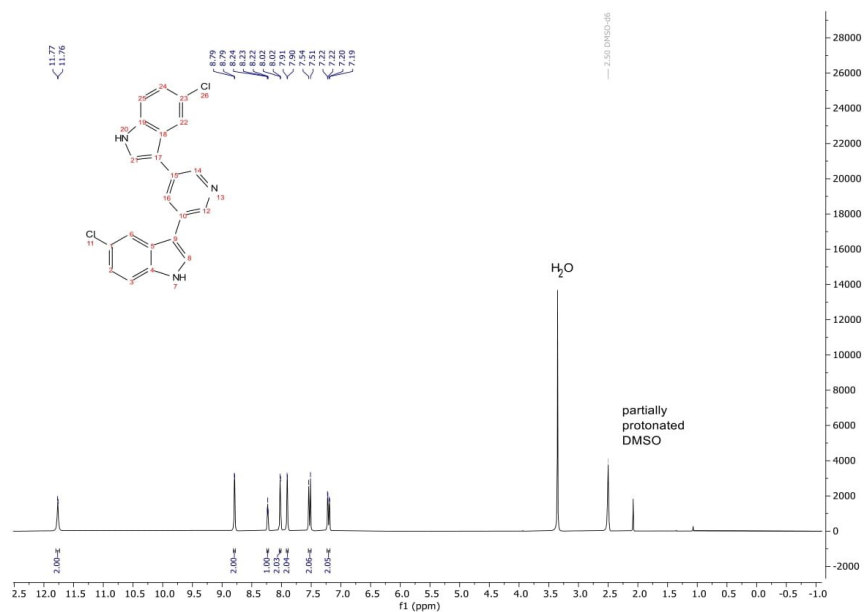


Figure 61 ¹H NMR spectrum of 3,5-bis(5-chloro-1H-indol-3-yl)pyridine (**5n**) (DMSO-d₆, 300 MHz, 293 K).

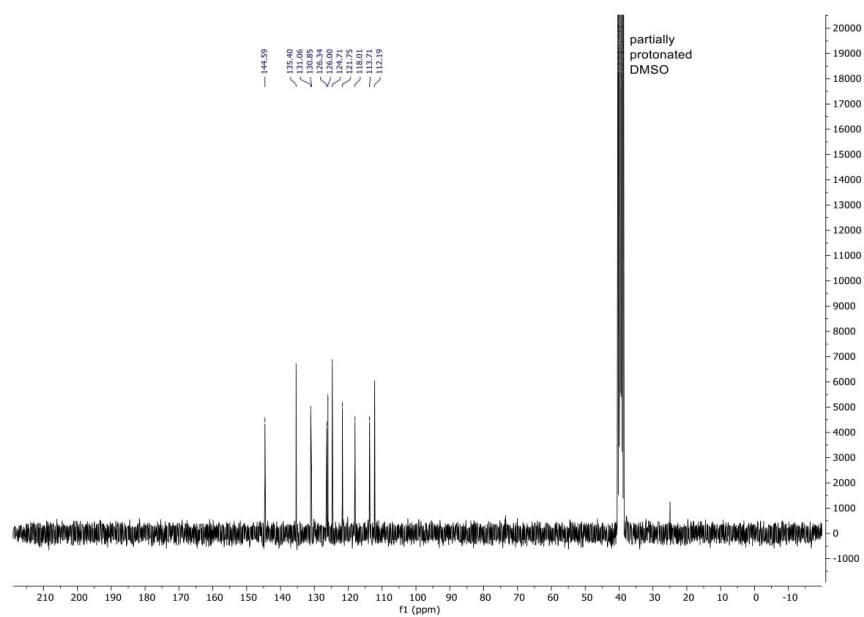


Figure 62 ¹³C NMR spectrum of 3,5-bis(5-chloro-1H-indol-3-yl)pyridine (**5n**) (DMSO-d₆, 75 MHz, 293 K).

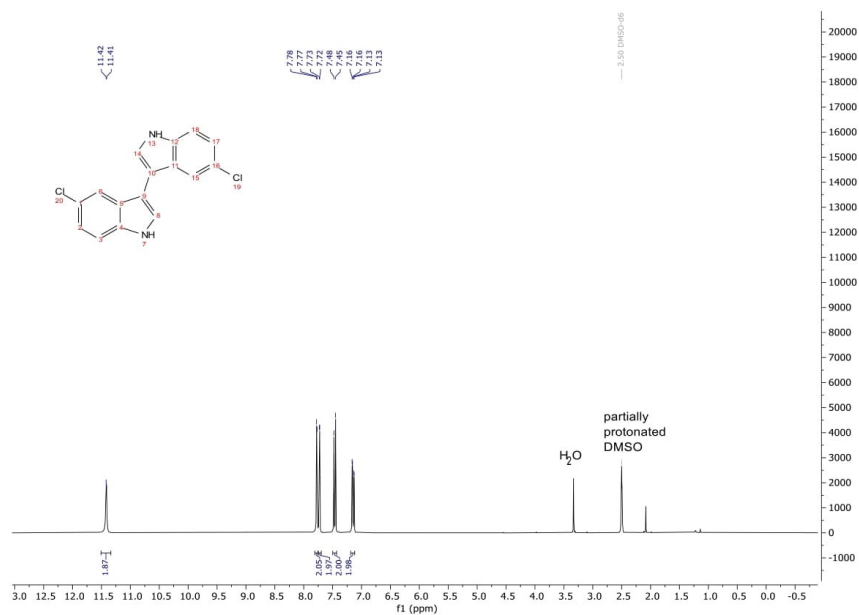


Figure 63 ¹H NMR spectrum of 5,5'-Dichloro-1*H*,1'*H*-3,3'-biindole (**5s**) (DMSO-d₆, 300 MHz, 293 K).

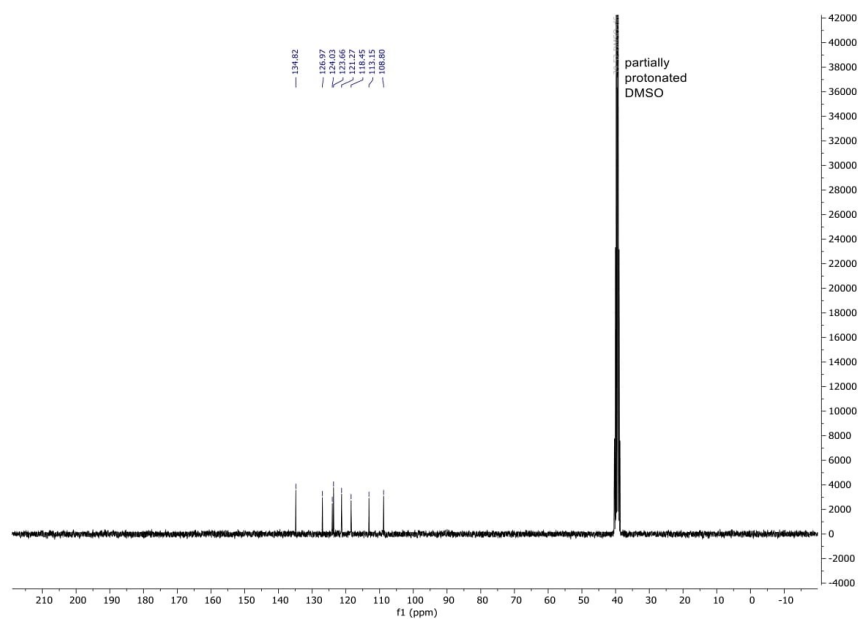


Figure 64 ¹³C NMR spectrum of 5,5'-Dichloro-1*H*,1'*H*-3,3'-biindole (**5s**) (DMSO-d₆, 75 MHz, 293 K).

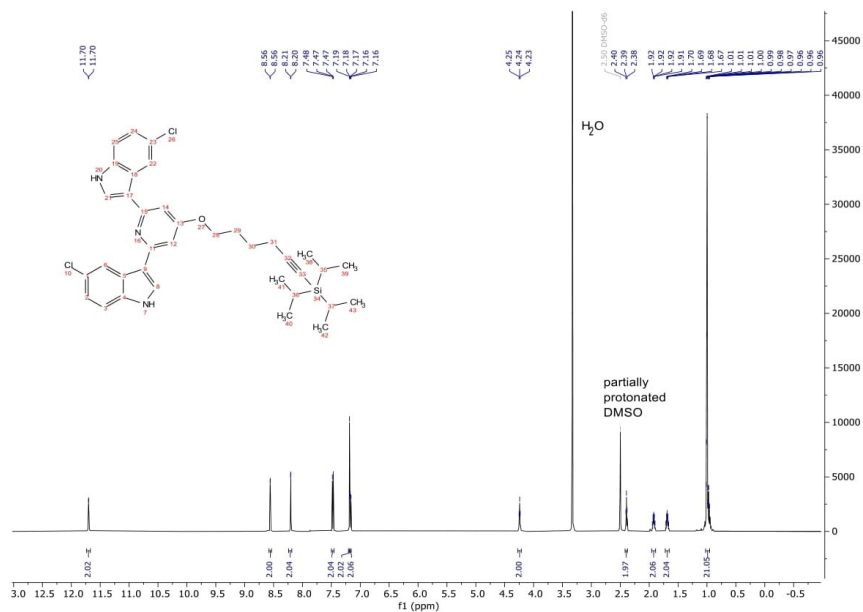


Figure 65 ¹H NMR spectrum of 3,3'-(4-((6-(Triisopropylsilyl)hex-5-yn-1-yl)oxy)pyridine-2,6-diyl)bis(5-chloro-1*H*-indole) (**5t**) (DMSO-d₆, 600 MHz, 293 K).

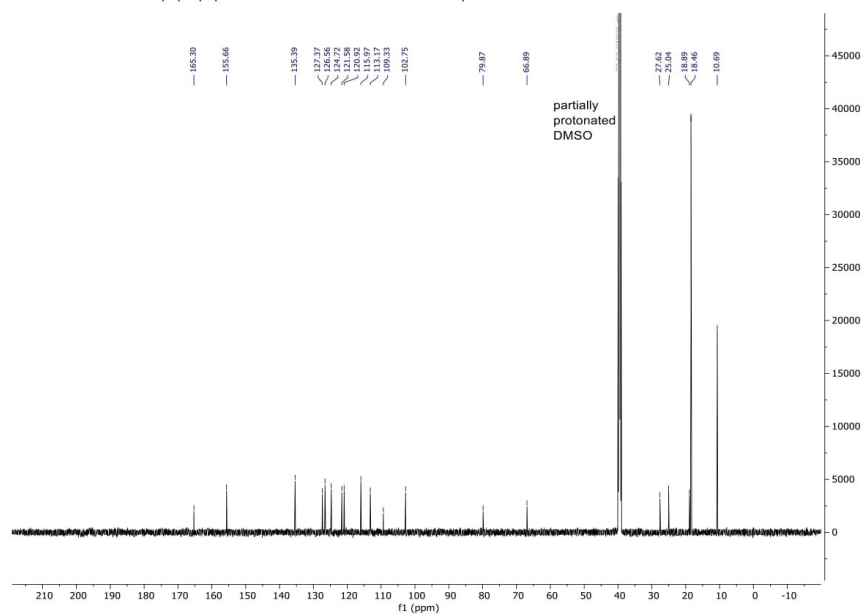


Figure 66 ¹³C NMR spectrum of 3,3'-(4-((6-(Triisopropylsilyl)hex-5-yn-1-yl)oxy)pyridine-2,6-diyl)bis(5-chloro-1*H*-indole) (**5t**) (DMSO-d₆, 150 MHz, 293 K).

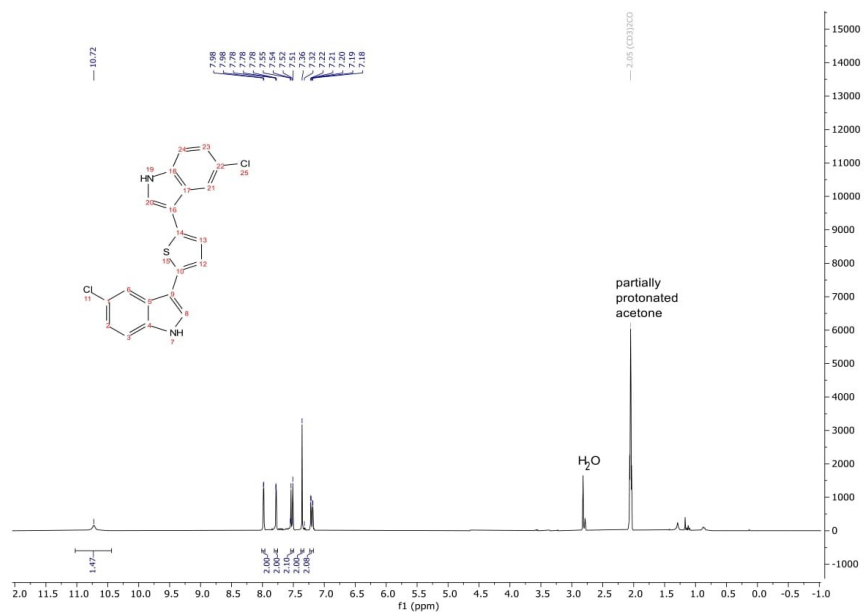


Figure 69 ¹H NMR spectrum of 2,5-bis(5-chloro-1H-indol-3-yl)thiophene (**5r**) (acetone-d₆, 300 MHz, 293 K).

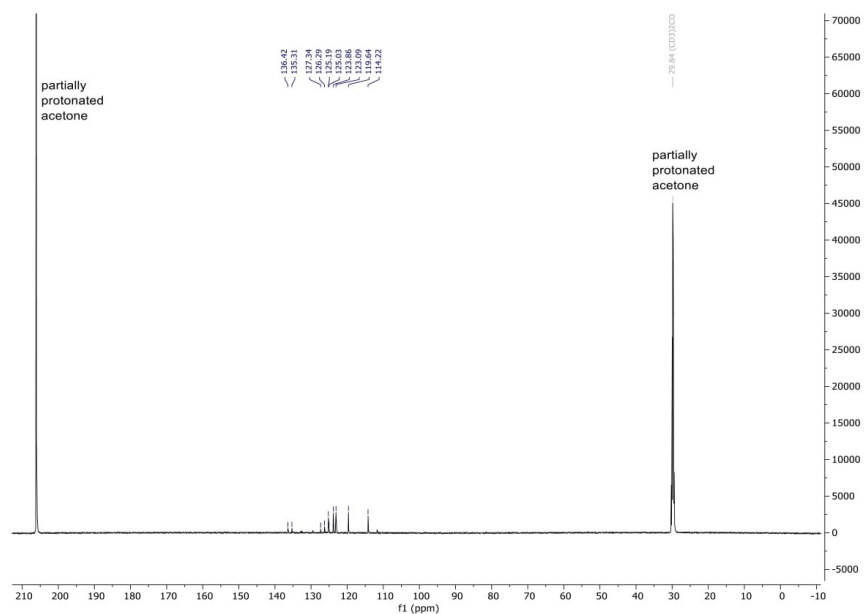


Figure 70 ¹³C NMR spectrum of 2,5-bis(5-chloro-1H-indol-3-yl)thiophene (**5r**) (acetone-d₆, 150 MHz, 293 K).

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4 The anti-tubercular callyaerins target the *Mycobacterium tuberculosis*-specific non-essential membrane protein Rv2113

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Doi: [10.1016/j.chembiol.2024.06.002](https://doi.org/10.1016/j.chembiol.2024.06.002)

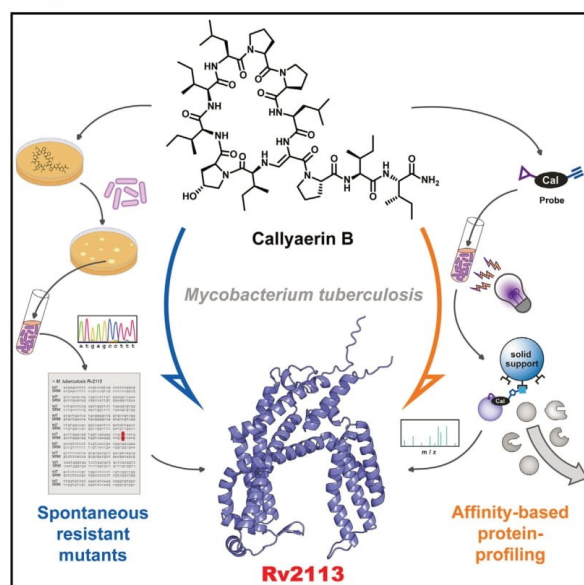
Contribution to the paper:

- Wrote the first draft of the manuscript.
- Performed MIC determination of callyaerin derivatives for structure–activity relationship analysis.
- Conducted MIC testing of derivatives against clinical XDR isolates and various *Mycobacterium* species.
- Carried out cytotoxicity profiling of derivatives against human cell lines.
- Generated and screened spontaneous CalB-resistant Mtb H37Rv mutants.
- Extracted genomic DNA from Mtb resistant mutants for whole-genome sequencing.
- Genetically manipulated Mtb and *M. bovis* BCG Pasteur strains.
- Determined in vitro time-dependent killing kinetics of CalB.
- Evaluated the interaction of CalB with selected TB drugs.
- Investigated the effect of CalB on mycobacterial energy metabolism.
- Assessed membrane integrity in Mtb exposed to CalB.
- Assessed CalB’s interference with cell wall biosynthesis.
- Evaluated the role of Rv2113 in intracellular uptake of callyaerins.
- Isolated whole-cell protein lysates of Mtb exposed to CalB for stress profiling.
- Prepared samples for photoaffinity enrichment and LC-MS/MS-based target identification.

Cell Chemical Biology

The anti-tubercular callyaerins target the *Mycobacterium tuberculosis*-specific non-essential membrane protein Rv2113

Graphical abstract



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Yvonne Gröner, ..., Thomas R. Ioerger,
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In brief

Podlesainski et al. develop natural and non-natural callyaerin derivatives that potently inhibit axenic and intracellular growth of *Mycobacterium tuberculosis* through establishing flexible synthesis of these derivatives. Chemoproteomics and mode-of-action studies identify the non-essential membrane protein Rv2113 as the target of callyaerins, which cause a broad dysregulation of the proteome.

Highlights

- Flexible synthesis of callyaerin-type natural product analogs is developed
- Callyaerins inhibit axenic and intracellular growth of *Mycobacterium tuberculosis*
- Callyaerins target the non-essential membrane protein Rv2113
- Binding of callyaerins to Rv2113 causes a broad dysregulation of the proteome



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Article

The anti-tubercular callyaerins target the *Mycobacterium tuberculosis*-specific non-essential membrane protein Rv2113

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<https://doi.org/10.1016/j.chembiol.2024.06.002>

SUMMARY

Spread of antimicrobial resistances urges a need for new drugs against *Mycobacterium tuberculosis* (Mtb) with mechanisms differing from current antibiotics. Previously, callyaerins were identified as promising anti-tubercular agents, representing a class of hydrophobic cyclopeptides with an unusual (Z)-2,3-di-amino-acrylamide unit. Here, we investigated the molecular mechanisms underlying their antimycobacterial properties. Structure-activity relationship studies enabled the identification of structural determinants relevant for antibacterial activity. Callyaerins are bacteriostatics selectively active against Mtb, including extensively drug-resistant strains, with minimal cytotoxicity against human cells and promising intracellular activity. By combining mutant screens and various chemical proteomics approaches, we showed that callyaerins target the non-essential, Mtb-specific membrane protein Rv2113, triggering a complex dysregulation of the proteome, characterized by global downregulation of lipid biosynthesis, cell division, DNA repair, and replication. Our study thus identifies Rv2113 as a previously undescribed Mtb-specific drug target and demonstrates that also non-essential proteins may represent efficacious targets for antimycobacterial drugs.

INTRODUCTION

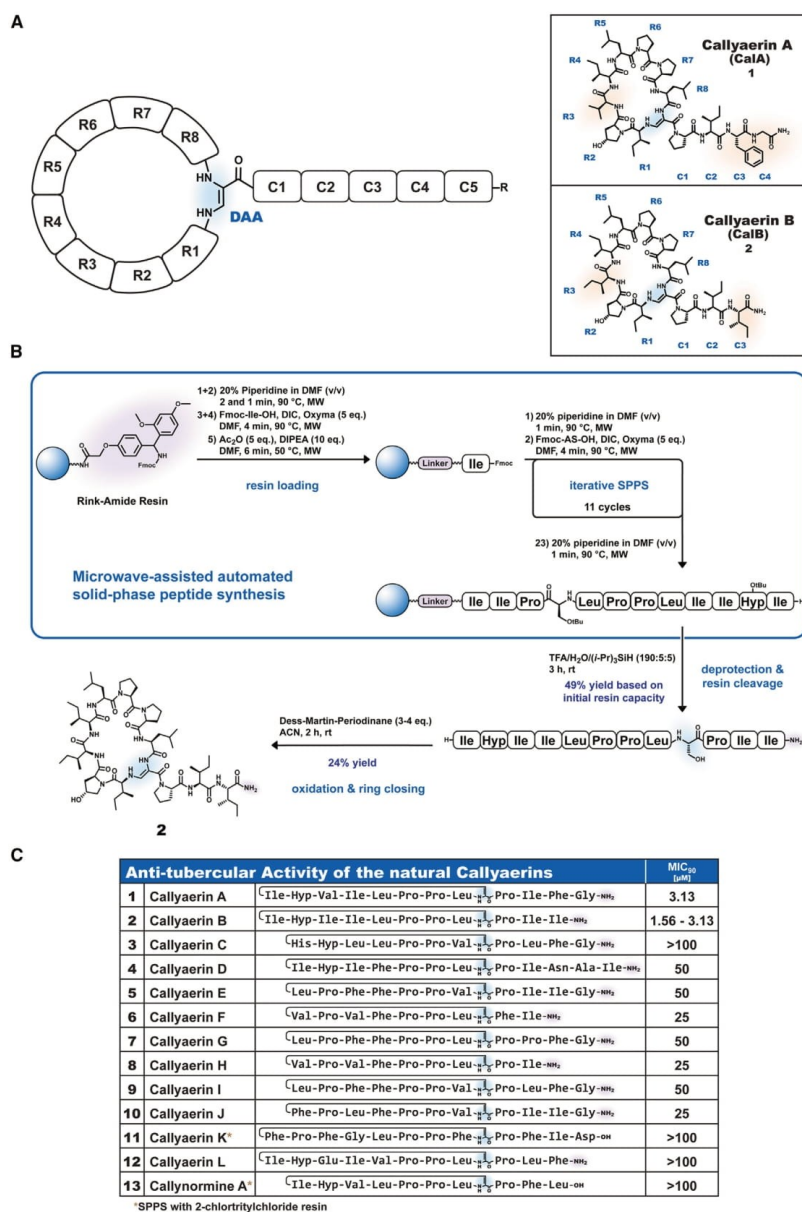
Tuberculosis, an ancient infectious disease caused by *Mycobacterium tuberculosis*, remains a global health threat responsible for 1.6 million deaths in 2021.¹ Despite recent advances in tuberculosis chemotherapy with addition of bedaquiline, pretomanid, and delamanid to the antimicrobial arsenal, lack of success in combating tuberculosis lingers.² This is partly due to the inability of drugs to accumulate in compartments harboring sequestered bacilli, as well as the length and complexity of drug regimens.³ A standard tuberculosis treatment, for instance, requires a cocktail of antibiotics, usually starting with a 2-month combination therapy of isoniazid, rifampicin, pyrazinamide, and ethambutol, followed by a 4-month combination of rifampicin and isoniazid. This prolonged course of treatment often results in poor compliance, which consequently drives emergence of drug resistance.^{4,5} As a matter of fact, in 2021, there was an estimated 3.1% global increase in the number of multidrug-resistant and rifampicin-resistant tuberculosis (MDR/RR-TB) cases compared to the previous year and about 191,000 deaths associated with

MDR/RR-TB.¹ Due to the broad-spectrum activity of some of the antibiotics included in this 6-month regimen for tuberculosis, resistance development can additionally extend from *M. tuberculosis* to off-target pathogens. Especially rifampicin, which has substantial activity against gram-positive bacteria, can drive the development of such off-resistance.⁶ Furthermore, long-term use of these broad-spectrum antibiotics causes major distress to the host microbiome, which can significantly impair the host's immune system as a result of dysbiosis, thereby contributing to several ill health conditions in treated patients.⁷ Thus, there remains a persistent need for new therapeutic options that are active against resistant strains in the ongoing fight against tuberculosis, particularly for anti-tubercular agents with a weaker resistance development in off-target pathogens and less harm to the microbiome.

Similar to many other clinically used antibiotics, many anti-TB drugs are natural product derivatives.⁸ Even today, promising starting points for anti-tubercular drug discovery can therefore be identified by screening microbial organisms for anti-tubercular natural products, particularly if less-investigated habitats



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(legend on next page)

and ecological niches such as marine environments are explored, as this lowers the risk for simple rediscovery of known antibiotics.^{9,10} Such an approach led to the discovery of the callyaerins, an anti-tubercular proline-rich cyclopeptide family from *Callyspongia aerizusa*, a marine sponge of Indonesian origin.^{11–13} Structurally, these cyclic peptides are composed of a ring system of eight to nine amino acids including an unusual (Z)-2,3-diaminoacrylamide unit (DAA, Figure 1A) that is furthermore C-terminally linked to an exocyclic side chain of two to five amino acids. Among them, particularly callyaerin A and B (CalA and CalB, Figure 1A) were found as promising anti-tubercular natural products as they possess potent antimycobacterial activity with an encouraging cytotoxic profile.¹¹

However, an in-depth characterization including structure-activity-relationships (SAR) and mode-of-action (MOA) studies of these anti-tubercular natural products has so far not been pursued. Here, we report the development of a solid phase synthesis protocol based on a modification of a previously published solid phase synthesis of CalA¹⁴ that enabled a straightforward and flexible synthesis of various callyaerin derivatives, some with improved anti-tubercular activities, for SAR and MOA studies. By a combination of these resources with various genetic and chemical proteomic approaches, we then identified and validated the mycobacterial-specific protein Rv2113 as the direct antimycobacterial target of callyaerins in *M. tuberculosis* H37Rv. Of particular note, Rv2113 is fully dispensable for growth and viability of *M. tuberculosis*, demonstrating that non-essential proteins can represent efficacious targets of anti-bacterial drugs.

RESULTS

Callyaerin A and B display the highest anti-tubercular activity within the callyaerin natural product family

CalA and CalB have previously been identified as promising anti-tubercular agents (Figure 1A).¹¹ However, only five of the currently fourteen known callyaerins—due to high structural similarities, we count callynormine A¹⁵ as a further callyaerin derivative—have until now been evaluated for their antimycobacterial properties. It therefore remained unclear whether other members of the callyaerin natural product members might represent more promising starting points for further anti-tubercular drug development.

To address this current limitation and to broaden the evaluation of the anti-tubercular potential of the callyaerins, we therefore aimed to establish a straightforward and flexible synthesis route to diverse members of the callyaerin natural product family as it may also be later used to generate customized derivatives for structure-activity relationship (SAR) studies. The first solid phase peptide synthesis of the callyaerin CalA has been reported

by the Brimble group.¹⁴ This synthesis relied on the incorporation of a suitably protected, specifically synthesized α -formyl glycine building block during solid phase peptide assembly,¹⁶ which, after deprotection, allowed the efficient installation of the DAA unit through initial imino linkage and concomitant double-bond migration. Recently, an alternative approach utilizing an acetal-protected 4-hydroxy-L-threonine building block for the introduction of α -formyl glycine was introduced by Yates et al.¹⁶ Based on these findings, we developed a slightly modified, more straightforward callyaerin synthesis that consists of a solid-phase synthesis of a precursor peptide featuring a standard serine residue at the future DAA position, followed by a one-pot selective Dess-Martin oxidation of the hydroxyl residue of serine and concomitant cyclization (Figure 1B). This protocol allowed a flexible synthesis of diverse callyaerins typically at a 1–5 μ mol scale. Notably, this approach exploits the unique chemical properties of the cyclized callyaerins that differ significantly from all other open-chain analogs (oxidized as well as non-oxidized), thereby enabling an efficient and easy purification of the final products by reversed-phase high-performance liquid chromatography (RP-HPLC).

With such a feasible synthesis route at hand, we subsequently synthesized thirteen of the so far fourteen known natural callyaerins (Figure S1, Supporting Information) and tested their *in vitro* anti-tubercular activity vs. the virulent *M. tuberculosis* strain H37Rv. Synthetic CalA and CalB were the most active callyaerins, showing potent minimum inhibitory concentrations for impairing at least 90% of growth compared to solvent controls (MIC₉₀) of 1.56–3.13 μ M, which are in agreement with previously published data for natural callyaerins (Figure 1C).¹¹ In contrast, all other tested callyaerins were significantly less active or even inactive (i.e., MIC₉₀ > 100 μ M), indicating that the macrocyclic ring size of eight amino acids plus the DAA residue in CalA and CalB, the overall fold of CalA and CalB with their distinct (hydroxyl)proline pattern as well as the presence of hydrophobic amino acids may be decisive for their anti-tubercular potential (Figure 1C). We therefore decided to focus our following investigations on the hydrophobic “CalA/B-type callyaerins” as they seem to represent the most promising anti-tubercular compounds from the callyaerin natural product family.

CalA/B-type callyaerins are *M. tuberculosis*-selective, intracellularly acting bacteriostatics

We continued our investigations by characterizing the antibacterial and cytotoxicity profile of CalA/B-type callyaerins (Figures 2A and S2). Interestingly, we found that extensively drug-resistant (XDR) forms of *M. tuberculosis* were also susceptible to CalA and CalB with MIC₉₀ ranging from 3.13–12.5 μ M, indicating that these callyaerins do not share similar targets with clinical drugs to which these XDR forms of *M. tuberculosis* are resistant.

Figure 1. Callyaerin A and B are promising anti-tubercular agents

(A) Generic structure scheme for the callyaerin natural product family (adapted from Daletos G.¹¹), (hydroxyl)proline rich hydrophobic cyclopeptides with a central (Z)-2,3-diaminoacrylic acid (DAA) unit, and chemical structure of callyaerin A (CalA) and B (CalB). Residues that are different between both compounds are indicated in orange, the DAA residue is depicted in blue.

(B) Scheme for the developed solid-phase peptide synthesis for callyaerins, here exemplarily presented for CalB.

(C) Overview on the growth inhibition properties (measured as MIC₉₀ values using resazurin dye reduction assay) of chemically synthesized callyaerins vs. *Mycobacterium tuberculosis* H37Rv. Data represent a single experiment with $n = 2–3$. A range is shown when variations in MIC₉₀ values were observed between individual samples. See also Figure S1.

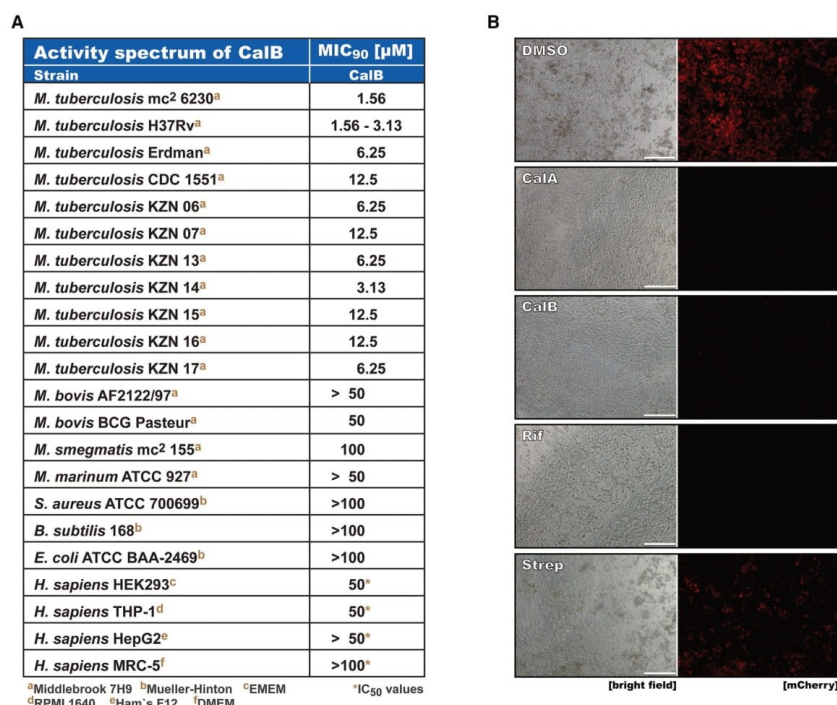


Figure 2. Callyaerins are *M. tuberculosis* selective anti-tubercular agents with intracellular growth inhibition properties

(A) MIC₉₀ values (measured using resazurin dye reduction assay, $n = 2-3$) for CalB treatment of various *M. tuberculosis* strains including clinical XDR isolates and further mycobacterial, gram-positive and gram-negative strains as well as human cell lines. Media used for cultivation of the respective cells are indicated by brown superscripted letters. A range is shown when variations in MIC₉₀ values were observed between samples. (B) Fluorescence micrographs of intracellular activity of CalA and CalB in a macrophage infection assay. THP-1 cells were differentiated into macrophage-like cells using PMA. Cells were infected with a mCherry expressing reporter strain of H37Rv (multiplicity of infection MOI = 3) for 3 h and subsequently treated as indicated with 15.6 μM CalA, 1 μM rifampicin or 20 μM streptomycin (each corresponding to 5-fold MIC₉₀) or 1.95 μM CalB (corresponding to ca. 1-fold MIC₉₀). Intracellular growth was evaluated five days post infection. Scale bar represents 150 μm. See also Figures S2–S6.

In contrast, only limited growth inhibition was found for other slow- and fast-growing mycobacteria species, including *Mycobacterium bovis* BCG Pasteur, *Mycobacterium smegmatis*, and *Mycobacterium marinum*. CalA and CalB were also inactive against other tested gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) or gram-negative (*Escherichia coli*) bacteria, demonstrating a very narrow and specific activity against *M. tuberculosis* strains. Importantly, both natural products displayed a rather low cytotoxicity with IC₅₀ values (i.e., concentrations required to inhibit 50% of growth relative to the solvent controls) ≥ 50 μM against a panel of human cell lines resulting in a promising selectivity index of 16–32.

We then performed a killing kinetic study by quantifying viable cell counts following different treatment intervals to determine how CalA/B-type callyaerins exert their growth inhibition effect on *M. tuberculosis* cells. We found that CalB displayed a bacteriostatic effect against cells of *M. tuberculosis* strain mc²6230 that was stable for two weeks (Figure S3).

Current TB treatment relies on a combination therapy to minimize the emergence of resistance that rapidly arises during monotherapy.³ However, the effect of a drug combination is not predictable but could potentially result in neutral, additive, synergistic, or even antagonistic interactions. We therefore evaluated the *in vitro* efficacy of CalB in combination with other, clinically used anti-tubercular drugs. Although addition of CalB to isoniazid and rifampicin did not enhance their bactericidal effects, the emergence of resistant mutants observed in monotherapy was effectively prevented, whereas combination with bedaquiline showed little additional effects under the tested conditions (Figure S4). We also determined fractional inhibitory concentration indices (FICI) for the interaction of CalB with either isoniazid, rifampicin, bedaquiline, delamanid, or ethambutol in the *M. tuberculosis* strains H37Rv and mc²6230 via a checkerboard assay (Figure S5). The resulting FICI values of 1–1.5 for all co-treatments suggested that CalB acts additive to these drugs, which in the case of isoniazid and rifampicin is consistent

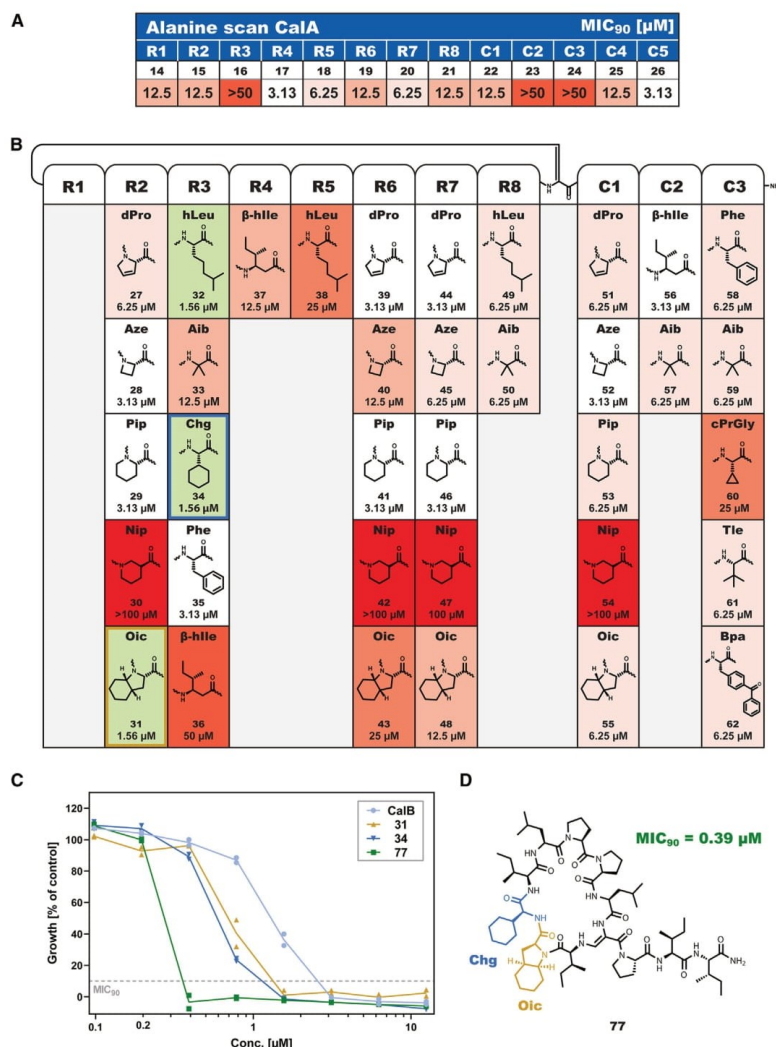


Figure 3. Overview on the structure-activity relationships of CalA/B-type callaerins

(A) An alanine scan of the different amino acids of CalA gives a first insight into structural determinants underlying bioactivity.

(B) Overview on single amino acid substitutions performed to study structure-activity-relationships. Further evaluated derivatives are reported in Figure S7. MIC₉₀ values in (A) and (B) represent mean values ($n = 2-3$) obtained from single experiments.

(C) Concentration-dependent growth inhibition curve for CalB, the slightly more active derivatives 31 and 34 as well as of compound 77 harboring both structural modifications. Data shown as means of duplicates with SD depicted as error bars. Each data point represents one replicate ($n = 2-3$) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC₉₀ threshold corresponding to a remaining growth of 10%. All MIC₉₀ values and dose-response curves were determined relative to untreated DMSO control (corresponding to 100% growth) and sterile medium control (0% growth) using resazurin dye reduction assay.

(D) Chemical structure of compound 77 with a cyclohexylglycine (Chg, dark blue) modification in R3 and an octahydroindole-2-carboxylic acid (Oic, light blue) modification in R2. This rationally designed compound is ten-times more active than the parent compound CalB, displaying a MIC₉₀ of 0.39 μM vs. *M. tuberculosis* H37Rv. See also Figure S7.



with the killing kinetic experiment that showed no enhancement of bactericidal effects but avoidance of resistance emergence by the combination with CalB.

M. tuberculosis is an intracellular pathogen that mostly resides and replicates in phagolysosomes of macrophages.^{17,18} For evaluating the intracellular growth inhibition properties of CalA and CalB, we therefore employed a THP-1 human macrophage infection model system that relies on quantifying cell growth of a mCherry-expressing fluorescent *M. tuberculosis* reporter strain.¹⁹ To this end, macrophages were treated 3 h after infection with either DMSO, 15.6 μ M CalA, 1.95 μ M CalB, or with the clinically used anti-TB drugs rifampicin (1 μ M) or streptomycin (20 μ M). Growth inhibition efficiency was then evaluated five days post-infection using fluorescence microscopy (Figure 2B, see Figure S6 for quantification). While macrophages treated with the solvent control DMSO exhibited a heavy intracellular bacterial burden, appeared clumpy and started to detach from the surface, CalA and CalB both substantially inhibited intracellular proliferation of *M. tuberculosis* and resulted in a healthy morphology of the treated macrophages. The effects were comparable to that of the first-line anti-TB drug rifampicin. Remarkably, despite the usage of lower compound concentrations, the intracellular growth inhibition activity of CalA and CalB was superior to streptomycin, which could reduce mycobacterial growth only to about 20% of the DMSO-treated control compared to ca. 1% for CalA and CalB.

In summary, CalA/B-type callyaerins exhibit a bacteriostatic effect *in vitro*, show additive effects in combination with clinical anti-TB drugs, and can penetrate human macrophages for reaching *M. tuberculosis* cells in their phagosomal compartment, resulting in strong inhibition of intracellular growth.

Structure-anti-tubercular activity determinants of CalA/B-type callyaerins

CalA and CalB as the founding members of the CalA/B-type callyaerin class are highly hydrophobic peptides that share most of their amino acid composition. Both are characterized by a 9 amino acids-membered ring system cyclized via a DAA moiety and the conserved site-specific presence of (hydroxyl)proline residues, resulting in a unique and stable peptide fold.¹⁴ They are structurally highly similar, differing only in their R3 and C3 position as well as by the presence of a C4 residue in CalA that is lacking in CalB (Figure 1A). However, despite these structural differences, both cyclic peptides have a similar potency against *M. tuberculosis* H37Rv (MIC₉₀ = 3.13 μ M, Figure 1C), indicating that at least certain positions tolerate structural modifications.

Taking advantage of our flexible chemical synthesis route, we therefore next performed a structure-activity relationship for studying their structural anti-tubercular determinants. We started with a CalA “alanine scan”, i.e., we replaced successively each amino acid of CalA with alanine and determined the resulting antimycobacterial activity for these derivatives (Figure 3A). For most positions, introduction of an alanine residue resulted in a lower bioactivity, although in some cases (e.g., position R5, R7; see compound 18 and 20, respectively), these effects were only moderate, while in other positions complete loss of bioactivity was observed (e.g., position R3, C2, and C3; see compound 16, 23, and 24, respectively). A notable exception is R4 (compound 17); here, the substitution of the parent isoleu-

cine residue by alanine was well tolerated and resulted in the same MIC₉₀ values as observed for CalA and CalB. Also, an extension of CalA's exocyclic chain with a further C5 alanine residue (26) had no impact on the bioactivity, suggesting that the “end region” of the exocyclic chain may allow attachment of additional chemical tags without significant bioactivity loss.

We then performed single amino acid substitutions at selected positions within CalB to better test position-dependent structural determinants (Figure 3B, see Figures S7A and S7B for further CalA derivatives). We mainly focused on conservative substitutions, i.e., we replaced hydrophobic and/or cyclic amino acids with chemically similar amino acids; these substitutions also included the change of the (hydroxyl)proline residues with other cyclic amino acids. These studies revealed that the hydroxyproline residue at R2 is not essential for bioactivity and can also be replaced by a “standard” proline residue (63). Indeed, (hydroxyl)proline substitutions by other cyclic amino acids such as dehydroproline (dPro) or azetidine carboxylic acid (Aze) were often well tolerated. The incorporation of β -amino acids, in particular nipecotic acid (Nip), however, led mostly to fewer active compounds, with the exception of the incorporation of β -hIle at C2 (56) that resulted in a derivative with CalA/CalB-similar bioactivities, again highlighting the exocyclic chain end as a region amenable for modification. Most tested amino acid substitutions, however, resulted in derivatives with equal or, more frequently, lower bioactivities compared to the parent compound CalB. Only three substitutions led to moderate, 2-fold more active derivatives: (i) a substitution of hydroxyproline at R2 by octahydroindole-2-carboxylic acid (Oic, 31) and the substitution of isoleucine at R3 by either (ii) homoleucine (hLeu, 32) or (iii) cyclohexyl glycine (Chg, 34, Figures 3B and 3C). In the case of the amino acid Chg, this effect was also confirmed in a corresponding CalA derivative (65). These bioactivity improvements were found to be additive as the designed callyaerin derivative 77 containing the Oic-substitution at R2 and the Chg-substitution at R3 was roughly 10-times more active than the parent compound CalB (Figures 3C and 3D). Overall, structure-activity relationships seem particularly driven by hydrophobicity; this notion is further substantiated by the finding that an incorporation of, for example, an aspartate residue at the R5 position (69) or a tryptophane residue at the R8 position of CalA (70), completely abolishes activity (Figure S7A) and, complementarily, callyaerin K (11) and callyaerin L (12), which both belong to the CalA/B-callyaerin family but harbor acidic amino acids in their structure (Figure 1C), exhibit no activity.

Altogether, CalA/B-type callyaerins display distinct structure-activity relationships, as most substitutions, even of hydrophobic amino acids with similar chemical properties, strongly reduce bioactivity, thereby indicating that their mode-of-action relies on a defined interaction with one or more distinct target protein(s). The strong preference for hydrophobic amino acids might be a consequence of their beneficial effect for supporting passage of the compound through the membrane; in addition, it suggests that CalA/B-type callyaerins will bind to a highly hydrophobic binding site on their target protein.

Rv2113 mediates resistance and susceptibility to CalA and CalB

In order to identify the direct target(s) of the anti-tubercular callyaerins, spontaneous single-step mutants of *M. tuberculosis*

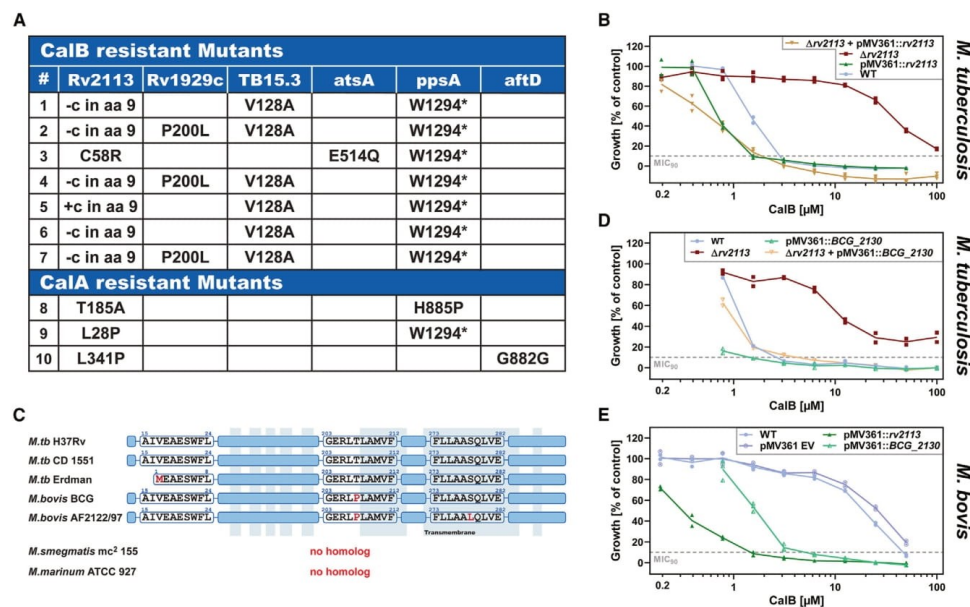


Figure 4. Rv2113 mediates resistance and susceptibility to CalA and CalB

(A) Overview on the results from screening of spontaneous CalA- and CalB-resistant *M. tuberculosis* H37Rv mutants. Spontaneous single-step resistant mutants emerged at a frequency of 10^{-6} on 7H10 agar containing concentrations corresponding to a 5-fold MIC of CalA or CalB, respectively, after 4-week incubation. The corresponding mutations found in H37Rv were mapped via whole-genome sequencing. Mutations resulting in premature termination of translation due to the introduction of a stop codon are indicated by an asterisk.

(B) Dose-response curves for CalB concentration-dependent growth inhibition of H37Rv strains. The assay included the wild type, the $\Delta rv2113$ gene deletion mutant, the complemented strain $\Delta rv2113$ pMV361::rv2113 and the overexpressing strain pMV361::rv2113.

(C) Overview on the sequence variations found in Rv2113 homologs in other mycobacterial strains tested for callyaerin susceptibility in the scope of this work.

(D) Dose-response curves for CalB concentration-dependent growth inhibition of H37Rv strains. The assay included the wild type, the $\Delta rv2113$ gene deletion mutant, the pMV361::BCG_2130 complemented mutant strain $\Delta rv2113$ and a BCG_2130 overexpressing strain pMV361::BCG_2130.

(E) Dose-response curves for CalB concentration-dependent growth inhibition of *M. bovis* BCG Pasteur strains. The assay included the wild type, the empty vector control pMV361 EV and the overexpressing strains pMV361::rv2113 and pMV361::BCG_2130. In case of (B), (D), and (E) growth was quantified relative to untreated DMSO control (corresponding to 100%) and sterile medium control (0%) using resazurin dye reduction assay. Each data point represents one replicate ($n = 2-3$) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC₉₀ threshold corresponding to a remaining growth of 10%. See also Figures S8–S16.

H37Rv were isolated after four weeks from 7H10 medium containing a 5-fold MIC of either CalA or CalB, respectively, at a frequency of 10^{-6} . In contrast to the wild type, the obtained mutants were highly resistant to CalA or CalB treatment with MIC₉₀ values above 50 μ M (Figures S8A and S8B) and also showed cross-resistance to the respective other callyaerin (Figure S8C), suggesting a common target for both compounds. Subsequent whole-genome sequencing of randomly selected mutants consistently revealed non-synonymous mutations in the gene *rv2113* (Figure 4A). In the majority of the analyzed clones, *rv2113* mutations were accompanied by further mutations; these mutations, however, occurred in diverse genes, strongly suggesting that mutations in *rv2113* are indeed the molecular cause for callyaerin resistance. In agreement with this, complementation of the spontaneous CalA-resistant mutant clone 8, which harbors a H885P mutation in *ppsA* in addition

to a T185A mutation in *rv2113* (Figures S9A–S9C), and of the spontaneous CalB-resistant mutant clone 7, which harbors mutations in *ppsA* (W1294*), *rv1929c* (P200L), and *TB15.3* (V128A) in addition to a frameshift mutation in *rv2113* (Figures S9D–S9F), with a wild-type copy of *rv2113* constitutively expressed from a single-copy integrative plasmid (pMV361::rv2113), was sufficient to fully restore susceptibility toward CalB. The mutations observed in *rv2113* included non-synonymous SNPs resulting in diverse amino acid substitutions (T185A, L28P, L341P, and C58R) as well as single nucleotide +c insertions or -c deletions in a 6c homopolymer region 20 nucleotides downstream of the start of the *rv2113* gene resulting in frame shifts. The bandwidth of identified mutations suggests a loss-of-function-based resistance mechanism.

To prove the relevance of *rv2113* for CalA and CalB resistance, we next generated a site-specific *rv2113* gene deletion mutant in



M. tuberculosis H37Rv, using specialized transduction (Figure S10). As expected, the generated independent clones of the *M. tuberculosis* $\Delta rv2113$ gene deletion mutant were found to be resistant to both CalA (Figure S11) and CalB treatment (Figure 4B), with MIC₉₀ values >100 μ M. Upon complementation of the deletion mutant with a wild-type copy of *rv2113* ($\Delta rv2113$ pMV361::*rv2113*), sensitivity to CalB was restored, thus unambiguously linking the resistance phenotype exclusively to absence of Rv2113. Finally, when the same plasmid pMV361::*rv2113* was used to generate a merodiploid strain of *M. tuberculosis* H37Rv that overexpresses Rv2113, this led to enhanced susceptibility (2-fold decrease in MIC) to CalB. Although *rv2113* is not essential for viability, these results suggest that both resistance and susceptibility of *M. tuberculosis* to CalA/B-type callyaerins are intimately linked to Rv2113.

To confirm these findings, we also raised spontaneous CalB-resistant mutants in the generated merodiploid *M. tuberculosis* H37Rv strain harboring pMV361::*rv2113*, reasoning that the presence of a second copy of *rv2113* might force the occurrence of further mutations in other genes. However, we again found relevant non-synonymous SNPs only in *rv2113* located either in the endogenous or in the merodiploid gene copy, causing A217V and L338P amino acid substitutions, respectively (Figure S12). Importantly, the spontaneous CalB-resistant mutants raised in the *Rv2113*-merodiploid strain exhibited the same MIC as the *M. tuberculosis* wild type when screened against bedaquiline and rifampicin, indicating that the mutations in *rv2113* do not cause broad-spectrum resistance but specifically only affect susceptibility toward callyaerins (Figures S13A and S13B). In agreement with this, lack of Rv2113 did not influence susceptibility of the *M. tuberculosis* H37Rv $\Delta rv2113$ gene deletion mutant toward a panel of anti-tubercular first- and second-line drugs (Figures S13C–S13H). Interestingly, four of the six mutated amino acids (T185, A217, L338, and L341) found in Rv2113 in spontaneous resistant mutants cluster within a particular region of the corresponding AlphaFold-predicted protein structure,²⁰ highlighting the potential significance of this site in mediating resistance to the callyaerins (Figure S14). These findings emphasize the crucial role of the non-essential protein Rv2113 in resistance and susceptibility of *M. tuberculosis* to CalA/B-type callyaerins, while no other relevant mutations located in other genes could be identified.

To understand the basis for the selectivity of callyaerins against *M. tuberculosis*, we searched for Rv2113 homologs in the UniProtKB proteome via BLAST.²¹ Notably, we found homologs (sequence identity $\geq 95\%$) of Rv2113 only in a small number of organisms including *M. tuberculosis*, *M. bovis*, *M. orygis*, *M. canettii*, and *M. shinjuense*. Except these mycobacterial strains, the search revealed no further organisms with Rv2113 homologs with more than 70% sequence identity. Rv2113 is annotated as a “conserved probable membrane protein” that, according to sequence analysis and AlphaFold prediction,²⁰ harbors eight transmembrane helices. We previously tested CalA and CalB vs. various (myco)bacterial strains (Figure 2A). An analysis on the presence of Rv2113 homologs in these strains revealed that apart from the susceptible *M. tuberculosis* strains, Rv2113 homologs are only present in the virulent *M. bovis* AF122/97 (with “Mb2137” corresponding to Rv2113) and non-virulent *M. bovis* BCG Pasteur (with “BCG_2130” corresponding

to Rv2113) strains (Figure 4C). In these strains, however, CalA and CalB were much less active. Although Mb2137 and BCG_2130 show high sequence identities to Rv2113, BCG_2130 harbors one (T207P) and Mb2137 two amino acid substitutions (T207P and S278L). Owing to the fact that the amino acid substitution T207P is common to both *M. bovis* strains, we reasoned that this mutation might account for the low susceptibility (MIC₉₀ = 50 μ M) of *M. bovis* BCG Pasteur to CalA/B-type callyaerins. We thus heterologously expressed BCG_2130 in the *M. tuberculosis* $\Delta rv2113$ mutant ($\Delta rv2113$ pMV361::BCG_2130) and observed that this fully restored sensitivity to CalB (Figure 4D). Further, when BCG_2130 or *rv2113* were overexpressed in *M. bovis* BCG Pasteur via a strong constitutive promoter from the single-copy integrative plasmids pMV361::BCG_2130 and pMV361::*rv2113*, respectively, this strongly sensitized *M. bovis* BCG Pasteur toward CalB, with a reduction in MIC₉₀ from 50 μ M to 6.25 μ M or 1.56 μ M, respectively (Figure 4E). These findings together suggest that the basis of the poor sensitivity of *M. bovis* BCG Pasteur toward CalA/B-type callyaerins is likely tied to a low expression level of endogenous BCG_2130, whereas the single amino acid exchange in BCG_2130 compared to Rv2113 itself does not substantially impair interaction with CalB. Quantitative real-time PCR indeed demonstrated that the expression level of BCG_2130 is more than four times lower in wild-type *M. bovis* BCG Pasteur than native *rv2113* expression in *M. tuberculosis* H37Rv (Figure S15A). Additionally, utilizing pMV361::*rv2113* in *M. bovis* BCG Pasteur results in at least triple the expression level of *rv2113* compared to the native variant BCG_2130 (Figure S15B). However, while the expression level of *rv2113* or its homolog BCG_2130 determines sensitivity toward callyaerins in *M. tuberculosis* and *M. bovis* BCG Pasteur, heterologous expression of BCG_2130 or *rv2113* did not or only slightly increased susceptibility of *M. smegmatis* toward callyaerins (Figure S16). This suggests that the interaction of CalA/B-type callyaerins with Rv2113 is necessary, but alone not sufficient to establish an antibacterial effect; instead, additional factors that are present in *M. tuberculosis* and *M. bovis* BCG Pasteur, but absent in other bacteria, are required for anti-tubercular activity.

CalB directly targets Rv2113

The genetic studies showed that Rv2113 mediates CalB resistance and susceptibility. It is however not clear if this phenotype is caused by a direct interaction with Rv2113 or is a more indirect effect. To prove direct target engagement of CalB in mycobacteria, we therefore devised a chemical proteomics strategy in which suitably modified CalB derivatives were used for direct target identification (Figure 5A). In this approach, two C4-alkyne tagged CalB derivatives, one with a native leucine residue (82, Figure 5B) and the other with an additional diaziridine-based “photoleucine” residue in R5 (85), were used in a (photo)affinity enrichment-LC-MS/MS-based target identification. To this end, a two-step labeling procedure was used in which the alkyne tag was modified with a biotin residue via click chemistry after *in situ* labeling. Accordingly, while 82 could be employed to identify proteins that are targeted by a potential covalent mechanism, e.g., via the DAA unit of the callyaerins by a so far unclear mechanism, 85 could be used to elucidate non-covalent target proteins via an additional photoaffinity labeling step (Figure 5A).

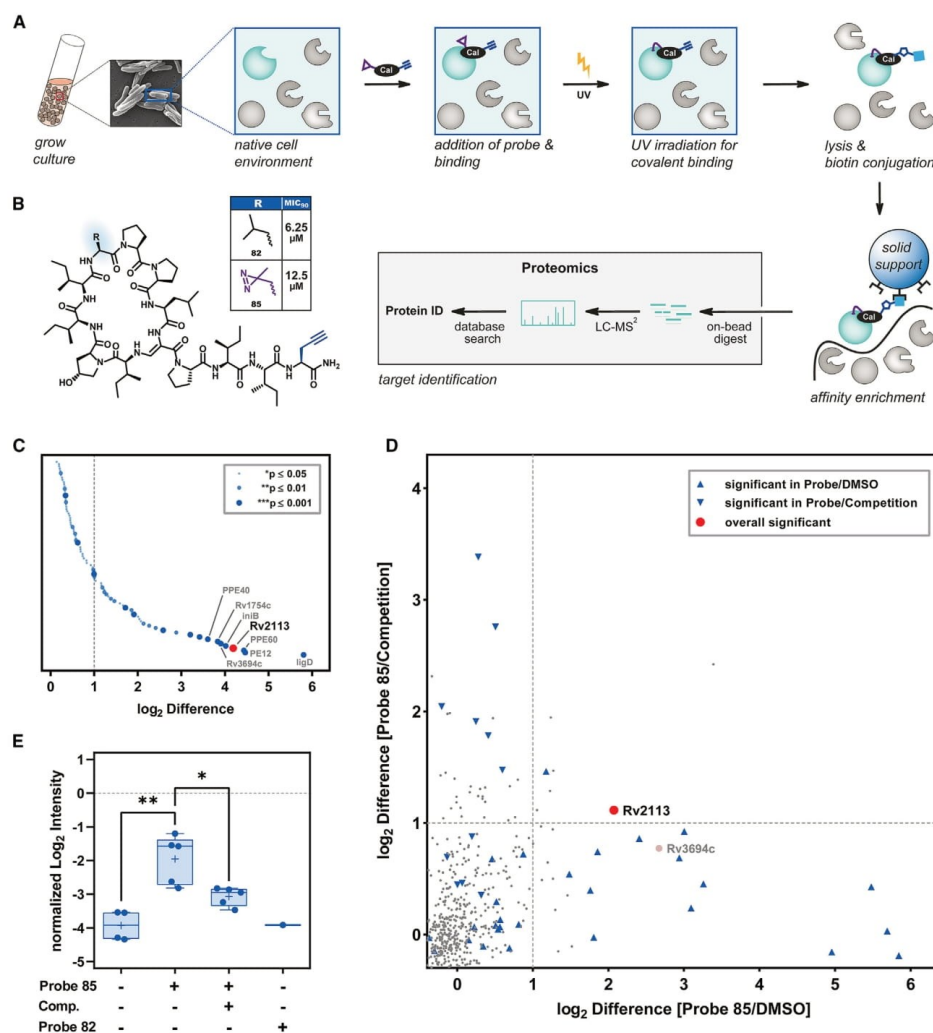


Figure 5. Photoaffinity labeling reveals Rv2113 as a direct target of CalA/B-type callyaerins

(A) Overview of the workflow for (photoaffinity) labeling of *M. tuberculosis* H37Rv cultures. (B) Chemical structures of the probes 82 and 85 and their respective MIC₅₀ values against *M. tuberculosis* H37Rv. Probe 85 is a photoaffinity probe that besides the alkyne tag for click chemistry also harbors an aziridine-based photoleucine at position R5. (C) Significant enriched proteins ($p \leq 0.05$ after Student's t test using permutation-based FDR with 250 randomizations and FDR = 0.01) resulting from an affinity-based protein-profiling experiment with 10 μ M probe 85 in *M. tuberculosis* H37Rv sorted by their log₂ FC. (D) Results from a combined photoaffinity labeling with 12.5 μ M 85 (log₂ FC plotted on x axis) and competitive photoaffinity labeling approach with application of 30 μ M CalB prior to photoaffinity labeling (log₂ FC plotted on y axis). Dashed lines indicate the log₂ FC > 1 threshold. To identifying statistically significant hits from the analysis (marked in blue or red), $p \leq 0.05$ (Student's t test; Permutation-based FDR with 250 randomizations and FDR = 0.01) was applied. (E) Boxplot representation of Rv2113 quantification data from the (photoaffinity) labeling experiments. Cells of exponentially growing cultures of *M. tuberculosis* H37Rv were pooled and aliquoted into 2-mL centrifugation tubes in a total volume of 1 mL PBS containing 0.05% tyloxapol. Samples ($n = 5$ replicates per condition) were subsequently treated, processed and measured independently without further pooling. Asterisks indicate significant differences between indicated samples (** $p \leq 0.01$, * $p \leq 0.05$, unpaired t test with Welch's correction). See also Figures S17–S20.



The design of the corresponding probes was deduced from the previous structure-activity relationship studies (Figure S7C), suggesting the R5 position as a suitable site for the incorporation of the photoleucine residue (Figure S17). Indeed, 82 as well as 85 both efficiently inhibited mycobacterial growth with MIC₉₀ values of 6.25 and 12.5 μ M, respectively, thus being only slightly less active than the parent compound CalB.

To investigate whether CalA/B-type callyaerins exert their bioactivity via a potential covalent interaction mechanism, we first performed a pulldown with 82 but were only able to identify a small number of enriched proteins with this approach (Figure S18A). The majority of these proteins are however most probable false-positive hits due to large variations within each treatment group (Figure S18B) or the generation of statistical artifacts from missing data point imputations during data processing (Figure S18C). This suggests that CalA and CalB most probably interact via a non-covalent mechanism.

Accordingly, we repeated the experiment with the photoaffinity-tagged probe 85. Application of 10 μ M of the probe to an *M. tuberculosis* H37Rv culture for 3 h, followed by 20 min UV radiation at 365 nm, cell lysis, click reaction with biotin azide, and finally an avidin-based affinity enrichment and LC-MS/MS analysis indeed revealed several significantly enriched targets, with log₂ fold changes (FC) > 1 as a threshold, among them Rv2113 as one of the top hits (Figure 5C). For further validation of these initial hits, a second photoaffinity labeling approach was performed at a 12.5 μ M probe concentration that was combined with a second, competitive labeling approach that relied on a preincubation with 30 μ M CalB (corresponding to a more than 2-fold excess of the competitor) prior to application of the photoaffinity probe 85 (Figures 5D and S19). Importantly, this combination of both experiments revealed Rv2113 as the only significantly enriched direct target with log₂ FC > 1 in both experimental setups (Figures 5D and 5E). Consistent with our previous data, probe 85 showed a significant reduction of activity against our Δ r₂₁₁₃ gene deletion mutant (Figure S20A). However, labeling of the Δ r₂₁₁₃ mutant showed similar enrichment results apart from Rv2113 (Figure S20B). This non-specific labeling might be caused by the highly active carbene species generated during photoactivation of diazirine.

Altogether, these chemical proteomics experiments provide strong evidence that Rv2113 acts as a mediator of the anti-tubercular activity of CalA/B-type callyaerins. In addition, they suggest a direct molecular interaction between this membrane protein and the compounds.

Rv2113 does not mediate intracellular uptake of CalA/B-type callyaerins

Our results so far point toward a central role of Rv2113 in the antibacterial mechanism of CalA/B-type callyaerins. However, whereas most anti-tubercular antibiotics typically work by inhibiting the function of an essential protein, this cannot straightforwardly explain the molecular mode of action of CalA/B-type callyaerins in *M. tuberculosis* as the Δ r₂₁₁₃ mutant is fully viable and shows no growth defects. We therefore first asked if the membrane protein Rv2113 acts as a molecular transporter of CalA and CalB, thereby mediating intracellular uptake of these compounds, which would allow the compounds to subsequently bind to the “real” targets inside the mycobacterial cell.

To investigate this potential mechanism, we synthesized fluorescent CalB derivatives by conjugating the azide-tagged fluorescent dyes Cy3 or rhodamine via a click reaction to a propargylglycine residue in the C4 position of CalB (compound 82, Figures 6A and S21). While the rhodamine derivative 88 was found to be inactive (MIC₉₀ > 100 μ M), the Cy3 derivative 87 (Figure 6A) was found to be more than 30-fold more potent than CalB (MIC₉₀ = 0.05 μ M, Figure 6B). This improvement in anti-tubercular activity was a result of the appropriate covalent modification of CalB with Cy3 as neither application of a respective Cy3 control (89) alone nor of a 1:1 molar mixture of CalB and Cy3 resulted in comparable MIC₉₀ values (Figure 6B). This improved anti-tubercular activity furthermore seems to be a specific effect as analogous incorporation of a Cy3 residue at the R3 (90) or R4 (91) position of CalB again led to less active compounds (Figure S22). Importantly, the Cy3-CalB derivative 87 still inhibited the growth of *M. tuberculosis* in an Rv2113-dependent manner, since the Δ r₂₁₁₃ mutant exhibited resistance (Figure 6C), demonstrating that the dye conjugate most likely relies on the same mechanism as the parent CalB. 87 also maintained its low cytotoxicity to the human cell lines THP-1 and HEK293, resulting in an impressive selectivity index (IC₅₀/MIC₉₀) of >125.

With this highly active and fluorescent derivative in hands, we next used fluorescence microscopy to measure compound uptake in *M. tuberculosis* H37Rv cells as well as in the Δ r₂₁₁₃ strain (Figures 6D and 6E). Intriguingly, 87 was rapidly internalized both by cells of *M. tuberculosis* wild type and the Δ r₂₁₁₃ mutant, with intracellular staining clearly visible as early as after 30 min of incubation. Thus, CalA/B-type callyaerins likely enter *M. tuberculosis* cells independently of Rv2113, ruling out an uptake-dependent mechanism of susceptibility and resistance. As a negative control experiment, we also performed uptake studies with the inactive rhodamine-conjugated derivative (Figure S23). We were not able to detect rhodamine-mediated fluorescence in any of both strains, thus demonstrating that the observed fluorescence signal from 87 correlates with uptake and not unspecific cell surface binding.

In summary, these experiments demonstrate that Rv2113 does not act as a membrane transporter mediating uptake of CalA or CalB, but as a functional target that is modulated upon binding of these compounds.

CalB binding to Rv2113 modulates multiple major cellular processes in *M. tuberculosis*

While all experiments so far illustrate the relevance of Rv2113 for the anti-tubercular activities of CalA/B-type callyaerins, it remains unclear how this interaction translates into a growth inhibitory effect.

Having ruled out an uptake-related role of Rv2113, our first hypothesis was that the interaction of CalA or CalB with Rv2113 might deregulate structure and function of this membrane protein, finally resulting in a loss of membrane integrity; such a mechanism is often observed for “membrane active” antibiotics that trigger an efflux of metabolites and a collapse of the membrane potential. To address this hypothesis, we performed a propidium iodide internalization experiment, which however failed to detect relevant membrane permeabilization in CalB-treated *M. tuberculosis* cells (Figure 7A). A lowering of intracellular ATP levels upon CalB treatment as a result from an impairment of

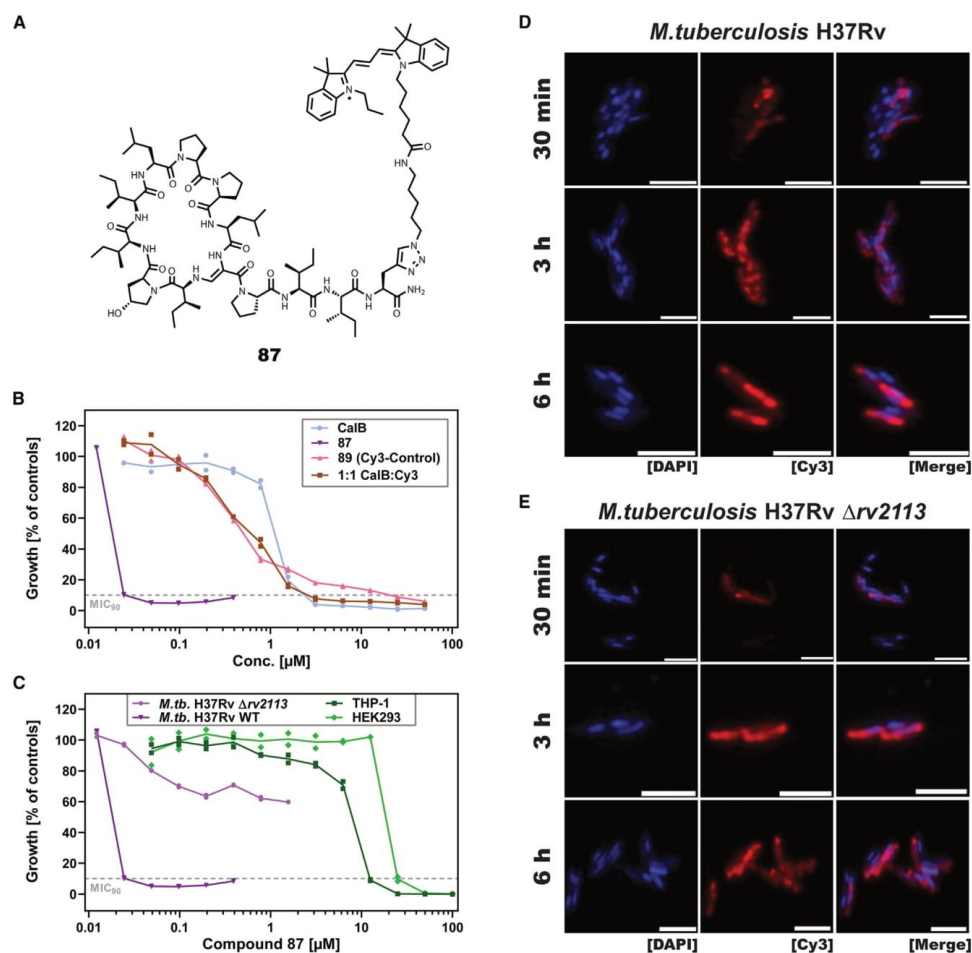


Figure 6. Rv2113 is no molecular transporter for CalB

(A) Chemical structure of the most active Cy3-CalB conjugate 87. In this derivative, the Cy3 residue has been conjugated to the C4 position via a Click reaction on a propargyl glycidyl residue.

(B) Dose-response curves for concentration-dependent growth inhibition of H37Rv strains by the Cy3-CalB derivative 87, CalB, a Cy3 control (89), and a 1:1 mixture of CalB and Cy3.

(C) Dose-response curves for the 87-mediated concentration-dependent growth inhibition of cells of *M. tuberculosis* H37Rv wild type and the $\Delta rv2113$ mutant as well as of the human cell lines THP-1 and HEK293. Growth in (B) and (C) was quantified relative to untreated DMSO control (corresponding to 100%) and sterile medium control (0%) using resazurin dye reduction assay. Each data point represents a single experiment ($n = 2$), and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC_{90} threshold corresponding to a remaining growth of 10%. This experiment has been repeated once with similar results.

(D and E) Fluorescence microscopy images of cells of *M. tuberculosis* H37Rv wild type (D) and the $\Delta rv2113$ mutant (E) treated with 0.2 μM of 87 for the indicated time intervals. After incubation, treated cells were counterstained with DAPI to label all cells. Scale bar represents 3 μm . Images are representative of one experiment. This experiment has been repeated once with similar results. See also Figures S21–S23.



energy metabolism of *M. tuberculosis* cells or a reduced ATP production due to a collapse of the membrane potential was also not observed (Figure 7B). These findings are corroborated by the fact that compounds affecting bacterial membrane integrity typically exert a strong bactericidal effect,²² while the effect of CalA and CalB on *M. tuberculosis* cells is bacteriostatic. Finally, we also measured the expression of the genes *iniA* and *iniB* from the *iniBAC* gene operon as markers for cell wall stress in mycobacteria after CalB application.²³ While these genes were found to be strongly upregulated by the positive control isoniazid, a known inhibitor of cell wall mycolic acid biosynthesis, CalB treatment led to the downregulation of both marker genes, similar to the negative control rifampicin, an RNA polymerase inhibitor with no direct effect on cell wall structure (Figure 7C). In conclusion, our experiments did not suggest that CalA/B-type callyaerins substantially impair the integrity of the cell membrane or cell wall in *M. tuberculosis*.

We therefore concluded that CalA/B-type callyaerins act by modulating the molecular function of Rv2113, raising the question as to what is the molecular function of Rv2113 in *M. tuberculosis*. However, our bioinformatic analyses including BLAST homology search or structure-based functional predictions using homology structure search via PDBFold²⁴ employing the AlphaFold structure of Rv2113 or self-generated AlphaFold 2-derived Rv2113-homooligomer structures failed to reveal any homolog with assigned function. Thus, the function of Rv2113 remains elusive.

To obtain insights into the cellular processes that are specifically affected by CalB treatment in an Rv2113-dependent manner, cells of *M. tuberculosis* H37Rv wild type (Figures 7D and S24A) and of the CalA and CalB-resistant Δ r2113 mutant (Figure S24B) were challenged with a 10-fold MIC₉₀ of CalB for 48 h. Subsequently, whole protein cell lysates were prepared to perform global proteome analysis and comparison of the elicited responses. Overall, as expected, treatment of wild-type cells with CalB led to more profound dysregulations of protein expression than treatment of the resistant Δ r2113 mutant, which are unambiguously linked to the anti-tubercular nature of CalB, as the inactive compound 36 does not show a similar response at the proteome level (Figure S25). In wild type, the by far most predominant response was reduced abundance of diverse proteins. A GO analysis revealed that the affected processes were mainly cellular lipid metabolic processes and, to a lesser number, processes associated with DNA repair and replication. Among the underrepresented lipid biosynthesis-related proteins were, for example, Fas (Rv2524c), AccD6 (Rv2247), FadD22 (Rv2948c), FbpC (Rv0129c), FabD (Rv2243), Mas (Rv2940c), Pks15 (Rv2947c), FadD29 (Rv2950c), and Rv2949c, while the methylcitrate cycle pathway enzymes PrpC and PrpD were found to be two of the rare overrepresented proteins. However, impairment of lipid biosynthesis did not affect the integrity of the cell envelope in a way that would trigger the cell wall stress marker *iniBAC* (Figure 7C). Rv2525c was previously found to be upregulated in cells of *M. tuberculosis* that were exposed to agents (isoniazid and ethionamide) primarily inhibiting mycolic acid biosynthesis.²⁵ This suggests that upregulation of Rv2525c might represent an adaptive response to compounds impairing lipid biosynthesis, including callyaerins. Recently, accumulation of methylcitrate cycle intermediates

has been linked to tolerance toward first- and second-line TB drugs such as isoniazid and bedaquiline.²⁶ Therefore, upregulation of PrpC and PrpD might represent a specific response aiming at increasing tolerance to CalB. Furthermore, the virulence-associated methyltransferase Rv1405c, among others, has been reported to be responsible for acclimatizing *M. tuberculosis* CDC1551 to acid stress in macrophages,²⁷ while the sigma factor SigB was reported to control a regulon involved in general stress resistance in *M. tuberculosis*.²⁸ This suggests that upregulation of Rv1405c and SigB may represent additional general stress adaptations in CalB-treated cells.

In summary, CalB treatment elicited a complex protein dysregulation characterized by lower abundance of proteins involved in lipid biosynthesis, DNA repair, replication, and cell division. On the other hand, the cells responded by induction of several known stress adaptations, including Rv2525c, Rv1405c, PrpC-PrpD, and SigB. This complex stress profile is unique and differs from that of other natural anti-tubercular cyclic peptides such as teixobactin,²⁹ evybactin,³⁰ lassomycin,³¹ acyldepsipeptide,³² and cyclomarin,³³ implying that callyaerins embark on a distinct mechanism of action that presumably involves simultaneous impairment of several pathways.

DISCUSSION

We have elucidated structural determinants of CalA/B-type callyaerins, a family of potent anti-tubercular natural products, and demonstrated a direct targeting of the non-essential membrane protein Rv2113. As Rv2113 is *M. tuberculosis* specific, this mode-of-action may allow the generation of bacteriostatics with less side effects to the microbiome as well as the spread of off-target pathogen antibacterial resistance. The callyaerins are therefore another promising example of the growing number of cyclo(depsi)peptide-based anti-tubercular natural products with unique mode-of-actions.³⁴

The binding of CalA/B-type callyaerins modulates the non-essential function of Rv2113 in a yet-elusive molecular way that elicits pleiotropic effects by simultaneous impairment of several intracellular pathways including downregulation of lipid biosynthesis, DNA repair, replication, and cell division. This mode-of-action is thus distinct from other antibiotics that typically either target single critical steps in pathways that are essential for growth or viability of bacterial cells, such as DNA replication, cell wall formation, or protein, RNA, or ATP biosynthesis, among others, or interact with membrane structures, resulting in pore formation and collapse of membrane potential. Our data however confirm that both resistance and susceptibility of *M. tuberculosis* toward CalA/B-type callyaerins are strictly linked to the non-essential membrane protein Rv2113. Rv2113 is fully dispensable for normal *in vitro* growth of *M. tuberculosis* under the tested culture conditions and, thus, has not been considered a potential drug target so far. Non-essential proteins, which are connected to the mechanism of antibacterial compounds with phenotypes similar to Rv2113 (i.e., their deletion mediates resistance, while overexpression mediates hypersensitivity), have so far been reported to be associated with only three different processes¹: compound uptake,² activation of pro-drugs,³ or deregulation and over-activation of proteases.

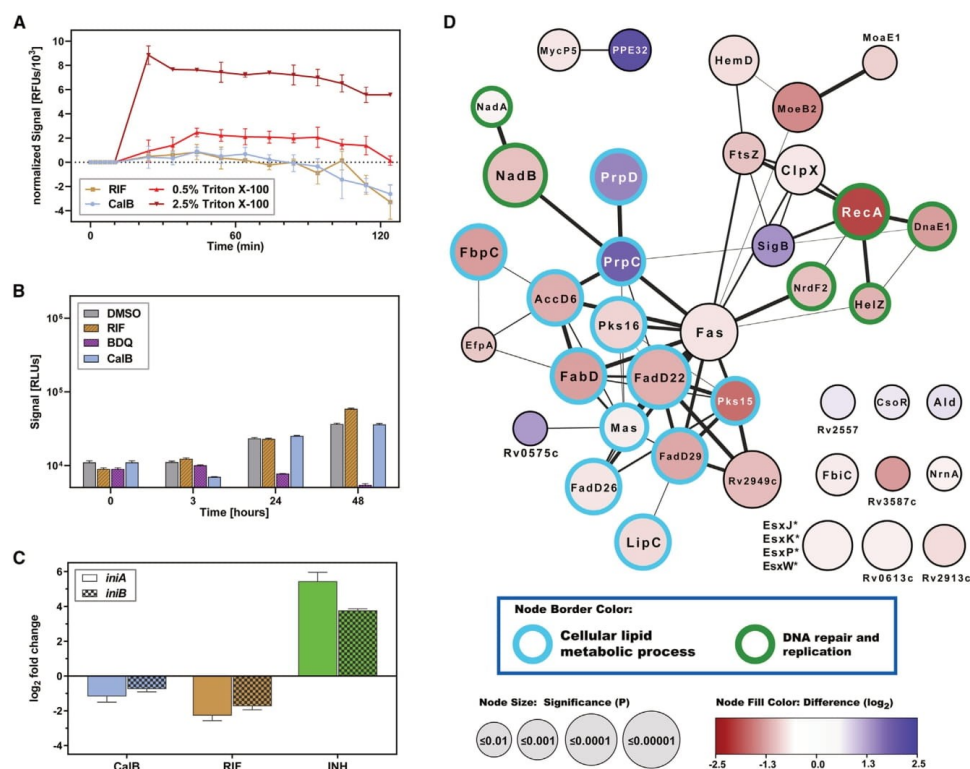


Figure 7. Rv2113-dependent proteomic dysregulation profile suggests that CalB simultaneously impairs multiple pathways

(A) Effect of CalB treatment on membrane integrity of *M. tuberculosis* as assessed by propidium iodide (PI) uptake assay. Cells of *M. tuberculosis* mc²6230 were preincubated with PI to establish baseline fluorescence and then treated with 15.7 μ M CalB (5-fold MIC₉₀, blue). Fluorescence intensity (excitation: 535 nm; emission: 617 nm) was measured over time. Addition of 0.5 μ M rifampicin (RIF, 5-fold MIC₉₀, brown) and Triton X-100 (0.5% as light red, 2.5% as dark red) served as negative and positive controls, respectively. Data shown as means of triplicates with SD depicted as error bars.

(B) Effect of CalB on mycobacterial energy metabolism. The amount of intracellular ATP in compound-treated cells was measured as a correlate of interference with energy metabolism. Washed cell pellets of *M. tuberculosis* mc²6230 treated with 31.4 μ M CalB (10-fold MIC₉₀, blue) were suspended in phosphate-buffered saline and incubated with Bactiter Glo reagent. Luminescence was measured at shown intervals. DMSO (gray) and 1 μ M RIF (10-fold MIC₉₀, brown) served as negative controls, while 3.9 μ M bedaquiline (BDQ, 10-fold MIC₉₀, purple) served as a positive control. Data shown as means of triplicates with SD depicted as error bars.

(C) Expression of *iniA* and *iniB* of the *iniBAC* gene operon in compound-treated cells of *M. tuberculosis*. Quantification of *iniA* (plain) and *iniB* (patterned) expression by RT-qPCR in cells of *M. tuberculosis* H37Rv treated with 15.7 μ M CalB (5-fold MIC₉₀) for 24 h. Log₂ fold change was determined by the $\Delta\Delta$ CT method, normalized to 16S rRNA with DMSO-treated cells as control (corresponding to a fold change of 0). RIF (0.5 μ M, 5-fold MIC₉₀) and isoniazid (INH, 1.95 μ M, 5-fold MIC₉₀) served as negative and positive controls, respectively. Data shown as means of triplicates with SD depicted as error bars.

(D) Network visualization of proteins which comply the chosen threshold (log₂ FC $\geq$ 0.5 or $\leq$ -0.5, q value $\leq$ 0.05; Student's t test; Permutation-based FDR with 250 randomizations and FDR = 0.05) from a full proteome profiling of wild type *M. tuberculosis* H37Rv cells treated for 48 h with 31.3 μ M CalB (corresponding to a 10-fold MIC₉₀) vs. DMSO treatment after STRING (v11.5) analysis.⁴¹ Node color represents the log₂-FC in protein abundance (increase showed in blue, decrease in red) and node size corresponds to significance (P). Thickness of connection lines indicated the confidence of STRING protein-protein association. Proteins assigned to the GO cluster "cellular lipid metabolic process" [GO: 0044255] or related to DNA repair and replication are shown with a turquoise or green border, respectively. Proteins marked with an asterisk could not be distinguished from each other at the MS level. See also Figures S24 and S25.

An uptake dependent role of non-essential membrane proteins is known, for example, for bacterial multi-solute transporters of the SbmA/BacA family that mediate incorporation of aminoglycoside antibiotics and antimicrobial peptides among

other structurally diverse hydrophilic molecules.³⁵ Inactivation of these transporters cf. resistance by preventing uptake of antibacterial compounds. However, our findings with a fluorescent Cy3-CalB derivative showed that CalA/B-type callyaerins enter



M. tuberculosis cells independently of Rv2113, ruling out an uptake-related role of Rv2113.

Several antibiotics used to treat *M. tuberculosis* such as isoniazid, pyrazinamide, and ethionamide are prodrugs that require intracellular activation by enzymes including catalase/peroxidase KatG,³⁶ the pyrazinamidase/nicotinamidase PncA,³⁷ or the Baeyer-Villiger monooxygenases EthA and MymA,³⁸ respectively. Loss of these non-essential enzymes *cf.* resistance by preventing pro-drug activation. However, we have no evidence that CalA/B-type callyaerins are pro-drugs that need direct activation by Rv2113. Rv2113 is a membrane protein comprising 387 amino acids, which harbors eight transmembrane helices according to AlphaFold prediction but does not contain any known catalytic domain.

Acyldepsipeptides (ADEPs) are a class of antibacterial natural compounds that kill gram-positive bacteria by over-activation of the cytosolic ClpP protease.³⁹ Similarly, synthetic tripodal peptidyl compounds can inhibit bacterial growth by allosteric over-activation of the non-essential protease DegP.⁴⁰ Loss of these non-essential proteases *cf.* resistance by preventing disturbed proteostasis that would otherwise occur by deregulated over-activation. However, a similar mechanism is highly unlikely for CalA/B-type callyaerins for several reasons. First, according to sequence analysis, Rv2113 does not contain any known proteolytic domain nor is it predicted to possess any other catalytic activity. Second, in contrast to other gram-positive bacteria, the ClpP protease is essential in mycobacteria, and ADEPs were reported to impair growth of mycobacteria by inhibition of the essential ClpP function, not by over-activation.³² Third, the proteomic dysregulation profile elicited by CalB treatment is distinct and differs from that of cyclic peptidic compounds that cause disturbance of proteostasis in *M. tuberculosis* by either inhibiting or deregulating ClpP function, such as cylcomarins or homoBac-PROTACs, respectively.³³

Finally, we also found no evidence that CalA/B-type callyaerins impair structure or function of the mycobacterial membrane or cell wall that could potentially occur if binding of callyaerins to Rv2113 would lead to pore formation. While our findings do not suggest impairment of membrane potential, we acknowledge that it cannot be excluded that binding of Rv2113 by CalA/B-type callyaerins may increase the flux of ions other than protons or other metabolites that could cause a stress response.

In summary, we can rule out all known mechanisms of how a non-essential protein could mediate resistance and susceptibility toward an antibacterial compound similar to Rv2113, with deletion conferring resistance and overexpression mediating hypersensitivity. Thus, we conclude that the anti-tubercular activity of CalA/B-type callyaerins very likely relies on an unprecedented mechanism. This mechanism not only depends on presence of Rv2113 but also requires additional yet-unknown factors that are unique for *M. tuberculosis* and *M. bovis* BCG but are obviously either lacking or at least differ substantially in other mycobacteria. One could speculate that this mechanism might rely on the unspecific recruitment of essential proteins to Rv2113 following binding of CalA/B-type callyaerins, thereby impairing their function. However, further investigations are necessary to prove or disprove this and other hypotheses concerning the molecular processes that occur following binding of these natural products to Rv2113 in *M. tuberculosis*.

Altogether, all our findings indicate that Rv2113 is a highly interesting protein—it is not only specific for *M. tuberculosis*, its structure is also unique, preventing any function predictions. Our investigation on the role of Rv2113 in the anti-tubercular mechanism of the CalA/B-type callyaerins strongly suggests that compound binding modulates function of this non-essential protein in a way that derails several metabolic pathways. This mechanism is unprecedented, diametrically differing from typical antibiotics that work by inhibiting essential targets. Further investigations on the functional role of Rv2113 in *M. tuberculosis* will be pursued in the future to shed light on the underlying molecular details of this unique mechanism. Finally, further medicinal chemical optimization of CalA/B-type callyaerins and development of callyaerin conjugates might pave the way for development of urgently needed *M. tuberculosis*-specific anti-TB drugs.

Limitations of the study

This study has several limitations that warrant attention. The lack of knowledge on the exact biochemical function of Rv2113 curtails our possibilities to conduct functional studies on how CalA/CalB-type callyaerins interfere with this function or lead to a possible gain of function. While photoaffinity labeling strongly suggests interaction of CalB with Rv2113, our study does not provide direct physical evidence of binding. Further, no crystal structure of Rv2113 is available to assess where CalA/CalB-type callyaerins exactly bind to the protein. Moreover, although our investigation highlights the anti-tubercular effects of CalA/CalB-type callyaerins *in vitro*, their *in vivo* efficacy remains to be tested to reveal the clinical relevance of our findings. Additionally, as resistance-mediating mutations in a non-essential gene might not result in any fitness cost, therapeutic targeting of a non-essential protein might lead to rapid resistance development *in vivo*.

SIGNIFICANCE

The unabated spread of drug resistance in the human pathogen *Mycobacterium tuberculosis* causes the urgent need to identify new drugs employing antibacterial mechanisms and addressing cellular targets that differ from those of current antibiotics used for treatment of tuberculosis. Natural compounds continue to be a promising source of new antibiotic scaffolds but often suffer from limited supply from natural sources. Here, we describe the optimized chemical synthesis and derivatization of callyaerins, a class of natural cyclopeptides comprising an unusual (Z)-2,3-di-aminoacrylamide unit that has been isolated from a marine sponge. The most efficient callyaerins are bacteriostatics that potently inhibit growth of *M. tuberculosis* in liquid culture and in infected macrophages while showing negligible cytotoxicity against human cells. Antibiotics typically restrict microbial growth by inhibiting cellular targets or pathways that are essential for viability of the bacteria. In contrast, we found that the antibacterial bioactivity of callyaerins is strictly linked to a direct interaction with a non-essential target, the previously uncharacterized and *M. tuberculosis*-specific membrane protein Rv2113. While further research is needed to resolve how callyaerins exactly interact with Rv2113 and how this interaction triggers the perturbation

of several cellular processes resulting in growth inhibition of *M. tuberculosis*, this study exemplifies that non-essential proteins can represent valuable non-canonical target structures. These results can have important implications for the design of anti-tubercular drug development.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.chembiol.2024.06.002>.

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AUTHOR CONTRIBUTIONS

Conceptualization, funding acquisition, and supervision, M.K. and R.K.; investigation, D.P., E.T.A., Y.G., F.S., V.K., N.R., T.R., D.S., H.X., V.E.S., and A.K.; data analysis, D.P., E.T.A., F.K., T.R.I., M.K., and R.K.; writing – original draft, D.P., E.T.A., M.K., and R.K.; writing – review and editing, D.P., M.K., and R.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>Mycobacterium tuberculosis</i> H37Rv	Ioerger et al., ⁴⁸ gift of William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
<i>Mycobacterium tuberculosis</i> mc ² 6230	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
<i>Mycobacterium tuberculosis</i> Erdman	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
<i>Mycobacterium tuberculosis</i> KZN06, KZN07, KZN13, KZN14, KZN15, KZN16, KZN17	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
<i>Mycobacterium tuberculosis</i> Beijing-HN878	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
<i>Mycobacterium tuberculosis</i> CDC 1551	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
<i>Mycobacterium bovis</i> AF2122/97	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
<i>Mycobacterium bovis</i> BCG Pasteur	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
<i>Mycobacterium smegmatis</i> mc ² 155	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
<i>Mycobacterium marinum</i> ATCC 927	DSMZ-German Collection of Microorganisms and Cell Cultures GmbH	DSM 44344
<i>Staphylococcus aureus</i> ATCC 700699	Doenitz Prolab	Cat#0158P
<i>Escherichia coli</i> ATCC BAA-2469	Doenitz Prolab	Cat#01113P
<i>Bacillus subtilis</i> 168	DSMZ-German Collection of Microorganisms and Cell Cultures GmbH	DSM 402
<i>Mycobacterium tuberculosis</i> H37Rv pBEN::mCherry (Hsp60)/GFP (Atc)	Martin et al., ⁵⁸ gift of Sarah Fortune, Harvard T.H. Chan School of Public Health	N/A
<i>Mycobacterium tuberculosis</i> H37Rv CalA resistant mutants	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv CalB resistant mutants	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv Δrv2113	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv Δrv2113 pMV361::rv2113	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv pMV361::rv2113	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv pMV361::rv2113 CalB resistant mutants	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv pMV361::BCG_2130	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv Δrv2113 pMV361::BCG_2130	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv CalA resistant mutant #8 pMV361::rv2113	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv CalB resistant mutant #7 pMV361::rv2113	This paper	N/A
<i>Mycobacterium tuberculosis</i> H37Rv CalB resistant mutant #7 pMV361 EV	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
<i>Mycobacterium bovis</i> BCG Pasteur pMV361 EV	This paper	N/A
<i>Mycobacterium bovis</i> BCG Pasteur pMV361::rv2113	This paper	N/A
<i>Mycobacterium bovis</i> BCG Pasteur pMV361::BCG_2130	This paper	N/A
<i>Mycobacterium smegmatis</i> mc ² 155 pMV361::rv2113	This paper	N/A
<i>Mycobacterium smegmatis</i> mc ² 155 pMV361 EV	This paper	N/A
<i>Mycobacterium smegmatis</i> mc ² 155 pMV361::BCG_2130	This paper	N/A
ΦphAE159	Jain et al., ⁴⁶ gift of William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Chemicals, peptides, and recombinant proteins		
Tyloxapol	Sigma-Aldrich	Cat#T8761-50G; Cas: 25301-02-4
NaCl	Sigma-Aldrich	Cat#31434-1KG-M; Cas: 7647-14-5
Glycerol	Carl Roth	Cat#3783.1; Cas: 56-81-5
α-D-Glucose	Sigma-Aldrich	Cat#158968-500G; Cas: 492-62-6
Albumin (BSA) Fraction V	PanReac AppliChem ITW reagents	Cat#A1391 Cas: 9048-46-8
BD Difco™ Middlebrook 7H10 Agar	Becton Dickinson	BD 262710
BD Difco™ Middlebrook 7H9 Broth	Becton Dickinson	BD 271310
BD Difco™ Agar	Becton Dickinson	BD 214030
BD DIFCO™ Mueller Hinton Agar	Becton Dickinson	BD 225250
LB Broth (Lennox)	Carl Roth	Cat#X964.3
RPMI 1640	PAN-Biotech	Cat#P04-18500
DMEM	PAN-Biotech	Cat#P04-04510
MEM	biowest	Cat#L0416
Ham's F12	PAN-Biotech	Cat#P04-15500
Fetal Bovine Serum	gibco	Cat#A5256801
Trypsin/EDTA	PAN-Biotech	Cat#P10-0231SP
MEM Non-Essential Amino Acids (100x)	gibco	Cat#11140-035
Sodium pyruvate	PAN-Biotech	Cat#P04-43100
DPBS (10x)	PAN-Biotech	Cat#P04-53500
Hygromycin B	Carl Roth	Cat#CP12.2 Cas: 31282-04-9
Apramycin sulfate salt	Sigma-Aldrich	Cat#A2024-5G Cas: 65710-07-8
Kanamycin sulfate	Carl Roth	Cat#T832.2 Cas: 25389-94-0
Resazurin sodium salt	Sigma-Aldrich	Cat#R7017-1G Cas: 62758-13-8
ROTI® Histofix 10% Formaldehyd	Carl Roth	Cat#A146.5
Isoniazid	Supelco	Cat#I3377-5G Cas: 54-85-3
Rifampicin	Sigma-Aldrich	Cat#R3501-250MG Cas: 13292-46-1

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bedaquiline	MedChemExpress	Cat#HY-14881 Cas: 843663-66-1
Ethambutol dihydrochloride	Sigma-Aldrich	Cat#E4630-25G Cas: 1070-11-7
Delamanid	BLDpharm	Cat#BD300949 Cas: 681492-22-8
Moxifloxacin	BLDpharm	Cat#BD32396 Cas: 186826-86-8
Ethionamide	BLDpharm	Cat#BD10649 Cas#536-33-4
D-cycloserine	TCI	Cat#C1189 Cas#68-41-7
Propidium iodide	Thermo Scientific Chemicals	Cat#440300250 Cas: 25535-16-4
Streptomycin sulfate	AMRESCO	Cat#0382-EU-50G Cas: 3810-74-0
4',6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Carl Roth	Cat#6843.1 Cas: 28718-90-3
Triton X100	PanReac AppliChem ITW reagents	Cat#A4975,0500 Cas: 9036-19-5
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich	Cat#P8139 Cas: 6561-29-8
RNAprotect Bacteria Reagent	Qiagen	Cat#76506
DNase I, RNase-free	Thermo Scientific	Cat#EN0521
Hexadecyltrimethylammonium bromide (CTAB)	Sigma-Aldrich	Cat#H5882-500G Cas: 57-09-0
Lysozyme	Thermo Scientific	Cat#89833
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D8418; Cas: 67-68-5
DMSO-d6	Sigma-Aldrich	Cat#151874; Cas: 2206-27-1
Methanol	Fisher Chemical	Cat#M/4056/17; Cas: 67-56-1
Na ₂ HPO ₄	Sigma-Aldrich	Cat#71636; 7558-79-4
HEPES	Carl Roth	Cat#HN77; Cas: 7365-45-9
Sodium dodecyl sulfate (SDS)	Carl Roth	Cat#2326.2; CAS: 151-21-3
Fmoc-Abu-OH	Carbolution	Cat# CC05074; CAS: 135112-27-5
Fmoc-Alb-OH	Fluorochem	Cat#045381; CAS: 94744-50-0
Fmoc-Aze-OH	Bachem	Cat#4019052; CAS: 136552-06-2
Fmoc-Chg-OH	Carbolution	Cat#CC05170; CAS: 161321-36-4
Fmoc-Cpg-OH	Carbolution	Cat#CC05169; CAS: 220494-61-0
Fmoc-cPrGly-OH	Iris Biotech	Cat#FAA3410; CAS: 1212257-18-5
Fmoc-dPro-OH	Bachem	Cat#4013705; CAS: 135837-63-7
Fmoc-Hyp-OH	Carbolution	Cat#CC05041; CAS: 122996-47-8

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fmoc-hLeu-OH	Sigma-Aldrich	Cat#8.52327; CAS: 180414-94-2
Fmoc-Nip-OH	Fluorochem	Cat#041311; CAS: 193693-68-4
Fmoc-Oic-OH	BLDpharm	Cat#BD131378; CAS: 130309-37-4
Fmoc-Pip-OH	Carbolution	Cat#CC05038; CAS: 86069-86-5
Fmoc-Pra-OH	Carbolution	Cat#CC05047; CAS: 198561-07-8
Fmoc-β-hlle-OH	Sigma-Aldrich	Cat#03671; CAS: 193954-27-7
Fmoc-Phe-OH	Carbolution	Cat#CC05020; Cas: 35661-40-6
Fmoc-Tle-OH	TCI	Cat#F1251; Cas: 132684-60-7
Fmoc-Bpa-OH	Carbolution	Cat#CC05105; Cas: 117666-96-3
Fmoc-Aha-OH	Iris Biotech	Cat#FAA6620; Cas: 942518-20-9
Fmoc-AEEAc-OH	Carbolution	Cat#CC05196; Cas: 166108-71-0
Fmoc-Pro-OH	Biosolve	Cat#276-250-0; Cas: 71989-31-6
Fmoc-Leu-OH	Iris Biotech	Cat#FAA1120; Cas: 35661-60-0
Fmoc-Ile-OH	Sigma-Aldrich	Cat#47628; Cas: 71989-23-6
Fmoc-Val-OH	Carbolution	Cat#CC05028; Cas: 68858-20-8
Fmoc-Gly-OH	Carbolution	Cat#CC05014; Cas: 29022-11-5
Fmoc-His(Trt)-OH	Nova Biochem	Cat#8.52032; Cas: 109425-51-6
Fmoc-Asn(Trt)-OH	Carbolution	Cat#CC05007; Cas: 132388-59-1
Fmoc-Ala-OH	Nova Biochem	Cat#8.52003; Cas: 35661-89-3
Fmoc-Asp(OtBu)-OH	Carbolution	Cat#CC05008; Cas: 71989-14-5
Fmoc-Glu(OtBu)-OH	Iris Biotech	Cat#FAA1045; Cas: 71989-18-9
CuSO ₄	Sigma-Aldrich	Cat#C1297; CAS: 7758-98-7
Azide-PEG ₃ -Biotin	Jena Bioscience	Cat#AZ104; CAS: 875770-34-6
Tris[(1-Benzyl-1H-1,2,3-Triazol-4-yl)methyl]amine (TBTA)	Sigma-Aldrich	Cat#678937; CAS: 510758-28-8
Tris(2-carboxyethyl)phosphine-hydrochloride (TCEP)	Sigma-Aldrich	Cat#C4706; CAS: 51805-45-9
Urea	GE Healthcare	Cat#GE17-1319-01; CAS: 17-1319-01
NH ₄ HCO ₃ (ABC)	Sigma-Aldrich	Cat#11213; CAS: 1066-33-7

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CHROMASOLV™ Acetonitrile	Honeywell	Cat#14261; CAS: 75-05-8
CHROMASOLV™ H ₂ O	Honeywell	Cat#14263; CAS: 7732-18-5
Acetonitrile (ACN)	VWR	Cat#20060; Cas: 75-05-8
DL-Dithiothreitol (DTT)	Sigma-Aldrich	Cat#43815; CAS: 3483-12-3
Iodoacetamide (IAM)	Sigma-Aldrich	Cat#A3221; CAS: 144-48-9
Chloroacetamide (CAM)	Sigma-Aldrich	Cat#C0267; CAS: 79-07-2
Formic acid (FA)	Fisher Chemical	Cat#A11705AMP; CAS: 64-18-6
N,N'-Diisopropylcarbodiimide (DIC)	Carbolution	Cat#CC01002; CAS: 693-13-0
Ethyl cyano-hydroxyiminoacetate (Oxyma)	Carbolution	Cat#CC01024; CAS: 3849-21-6
Dimethylformamide (DMF)	Fisher Chemical	Cat#D/3841/17; CAS: 68-12-2
Piperidine	Sigma-Aldrich	Cat#104094; CAS: 110-89-4
Rink-amide resin	Iris Biotech	Cat#BR-1330
Diethyl ether	Fisher Chemical	Cat#D/2450/17; CAS: 60-29-7
N,N-diisopropylethylamine (DIPEA)	TCI	Cat#D1599; CAS: 7087-68-5
Dichloromethane (DCM)	Fisher Chemical	Cat#D/1852/17; CAS: 75-09-2
Acetic anhydride (Ac ₂ O)	Sigma-Aldrich	Cat#A6404; CAS: 108-24-7
2-chlorotrityl chloride (2-CTC) resin	Sigma-Aldrich	Cat#8.55017
Triisopropyl silane (TIS)	TCI	Cat#T1533; CAS: 6485-79-6
Trifluoroacetic acid (TFA)	Alfa Aesar	Cat#A12198; CAS: 76-05-1
Dess-Martin-Periodinane (DMP)	Carbolution	Cat#CC03024; CAS: 87413-09-0
Trypsin	Promega	Cat#V5111
Pierce™ Avidin Agarose	Thermo Scientific	Cat#20225
Callyaerin A (CalA)	This paper; Ibrahim et al. ¹²	1
Callyaerin B (CalB)	This paper; Proksch et al. ¹²	2
Callyaerin C	This paper	3
Callyaerin D	This paper	4
Callyaerin E	This paper	5
Callyaerin F	This paper	6
Callyaerin G	This paper	7
Callyaerin H	This paper	8
Callyaerin I	This paper	9
Callyaerin J	This paper	10
Callyaerin K	This paper	11
Callyaerin L	This paper	12
Callynormine A	This paper	13

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CalA_R1A	This paper	14
CalA_R2A	This paper	15
CalA_R3A	This paper	16
CalA_R4A	This paper	17
CalA_R5A	This paper	18
CalA_R6A	This paper	19
CalA_R7A	This paper	20
CalA_R8A	This paper	21
CalA_C1A	This paper	22
CalA_C2A	This paper	23
CalA_C3A	This paper	24
CalA_C4A	This paper	25
CalA_C5A	This paper	26
CalB_R2dPro	This paper	27
CalB_R2Aze	This paper	28
CalB_R2Pip	This paper	29
CalB_R2Nip	This paper	30
CalB_R2Oic	This paper	31
CalB_R3hLeu	This paper	32
CalB_R3Aib	This paper	33
CalB_R3Chg	This paper	34
CalB_R3F	This paper	35
CalB_R3 β -hIle	This paper	36
CalB_R4 β -hIle	This paper	37
CalB_R5hLeu	This paper	38
CalB_R6dPro	This paper	39
CalB_R6Aze	This paper	40
CalB_R6Pip	This paper	41
CalB_R6Nip	This paper	42
CalB_R6Oic	This paper	43
CalB_R7dPro	This paper	44
CalB_R7Aze	This paper	45
CalB_R7Pip	This paper	46
CalB_R7Nip	This paper	47
CalB_R7Oic	This paper	48
CalB_R8hLeu	This paper	49
CalB_R8Aib	This paper	50
CalB_C1dPro	This paper	51
CalB_C1Aze	This paper	52
CalB_C1Pip	This paper	53
CalB_C1Nip	This paper	54
CalB_C1Oic	This paper	55
CalB_C2 β -hIle	This paper	56
CalB_C2Aib	This paper	57
CalB_C3F	This paper	58
CalB_C3Aib	This paper	59
CalB_C3cPrGly	This paper	60
CalB_C3Tle	This paper	61
CalB_C3Bpa	This paper	62
CalA_R2P	This paper	63

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
CalA_R3Abu	This paper	64
CalA_R3Chg	This paper	65
CalA_R3Cpg	This paper	66
CalA_R3L	This paper	67
CalA_R3F	This paper	68
CalA_R5D	This paper	69
CalA_R8W	This paper	70
CalA_R8V	This paper	71
CalA_C3L	This paper	72
CalA_C3I	This paper	73
CalA_C3X	This paper	74
CalA_C4Aha	This paper	75
CalA_C4X	This paper	76
CalB_R2Oic_R3Chg	This paper	77
CalB_R3Pra	This paper	78
CalB_R4Pra	This paper	79
CalB_R7Pra	This paper	80
CalB_C3Pra	This paper	81
CalB_C4Pra	This paper	82
CalB_C5Pra	This paper	83
CalB_R3phLeu	This paper	84
CalB_R5phLeu	This paper	85
CalB_R8phLeu	This paper	86
CalB_C4Pra(Cy3)	This paper	87
CalB_C4Pra(Rhodamine)	This paper	88
Cy3-Control	This paper	89
2-(Acetylamino)-4-pentynamide	This paper	89a
CalB_R3Pra(Cy3)	This paper	90
CalB_R4Pra(Cy3)	This paper	91
Benzonase	EMD Millipore	Cat#71206
Critical commercial assays		
660 nm Protein Assay	Thermo Fisher Scientific	Cat#22660
Ionic Detergent Compatibility Reagent	Thermo Fisher Scientific	Cat#22663
BacTiter-Glo™ Microbial Cell Viability Assay	Promega	Cat#G8230
RNeasy Mini Kit	Qiagen	Cat#74104
SuperScript™ III First-Strand Synthesis SuperMix	Invitrogen	Cat#18080400
GoTaq® qPCR Master Mix	Promega	Cat# A6001
Deposited data		
ABPP and (competitive) A/BPP approach using Callyaerin probes (82 or 85, respectively) in <i>M. tuberculosis</i> H37Rv.	This paper	PRIDE: PXD050770
Comparative A/BPP experiment using a Callyaerin probe (85) in <i>M. tuberculosis</i> H37Rv WT and Rv2113 gene deletion mutant (Δ r2113)	This paper	PRIDE: PXD050770
Callyaerin B-induced proteomic dysregulation profile in <i>M. tuberculosis</i> H37Rv WT and Rv2113 gene deletion mutant (Δ r2113)	This paper	PRIDE: PXD050763

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Proteomic dysregulation profile of <i>M. tuberculosis</i> H37Rv cells treated with an inactive Callyaerin B derivative (36)	This paper	PRIDE: PXD050763
Figure 2B image data	This paper	https://doi.org/10.6084/m9.figshare.25569462
Figure 6D+E image data	This paper	https://doi.org/10.6084/m9.figshare.25586964
Figure S23 image data	This paper	https://doi.org/10.6084/m9.figshare.25587975
Genome sequencing data of spontaneous resistant <i>M. tuberculosis</i> mutants	This paper	https://www.ncbi.nlm.nih.gov/sra/PRJNA1098701
<i>M. tuberculosis</i> H37Rv reference proteome	UniProt ⁵⁹	UP000001584
STRING database	Szklarczyk et al. ⁴¹	N/A
Experimental models: Cell lines		
<i>Homo sapiens</i> HEK293	CLS Cell Lines Service GmbH, Germany	Cat# 300192
<i>Homo sapiens</i> THP-1	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany	Cat# ACC 16
<i>Homo sapiens</i> HepG2	CLS Cell Lines Service GmbH, Germany	Cat# 300198
<i>Homo sapiens</i> MRC-5	CLS Cell Lines Service GmbH, Germany	ATCC® CCL171
Oligonucleotides		
Oligonucleotides used for RT-qPCR, see Table S1	Custom synthesis, Microsynth AG	N/A
Oligonucleotides employed for the generation of allelic exchange substrates for site-specific gene deletion of <i>rv2113</i> , see Table S2	Custom synthesis, Microsynth AG	N/A
Oligonucleotides used for cloning <i>rv2113</i> and <i>BCG_2130</i> into mycobacterial expression plasmid pMV361, see Table S3	Custom synthesis, Microsynth AG	N/A
Forward primer for qPCR validation of <i>Rv2113/BCG_2130</i> expression 5'-ATCGTGCTACTGACGGCATTG-3'	Custom synthesis, Microsynth AG	N/A
Reverse primer for qPCR validation of <i>Rv2113/BCG_2130</i> expression 5'-GCGATGGCGAGCAGAAAAAC-3'	Custom synthesis, Microsynth AG	N/A
Recombinant DNA		
p0004S	Jain et al., ⁴⁶ gift of William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
pMV361	Stover et al., ⁶⁰ gift of William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
pMV361:: <i>rv2113</i>	This paper	N/A
pMV361:: <i>BCG_2130</i>	This paper	N/A
Software and algorithms		
Proteome Discoverer (v2.5)	Thermo Fisher Scientific	Cat#OPTON-31040
Perseus (v2.0.7.0)	Tyanova et al. ⁵⁰	https://maxquant.net/perseus/
MaxQuant (v2.3.0.1)	Cox & Mann ⁵²	https://maxquant.net/maxquant/
MaxLFQ	Cox et al. ⁵³	N/A
Minora LFQ	Thermo Fisher Scientific	N/A
BLAST protein alignment algorithm	Altschul et al. ²¹	https://blast.ncbi.nlm.nih.gov/Blast.cgi
ChemDraw (v20.0)	PerkinElmer	N/A
MestReNova (v14.2)	Mestrelab Research	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Prism (v8.0)	Graphpad	N/A
Xcalibur (v4.1)	Thermo Fisher Scientific	N/A
Casava (v1.8)	Illumina	N/A
ImageJ	Schneider et al. ⁶¹	https://imagej.nih.gov/ij/
Tecan i-control (v1.1)	Tecan group	N/A
MxPro – Mx3005P (v4.10)	Stratagene	N/A
NIS-Elements	Nikon	N/A
PrimerQuest	Integrated DNA Technologies	N/A
VisionWorks (v8.16090.9066)	Analytik Jena	N/A
Clone Manager 9 (v9.5.0.0)	Sci Ed Software	N/A
Cytoscape (v3.9.1)	Shannon et al. ⁵⁷	https://cytoscape.org/index.html
Other		
UV lamp	Analytik Jena	Cat#UVP 3UV™
Tough micro-organism lysing VK05 0.5mm glass beads – 2 mL	Bertin Technologies	Cat#P000913-LYSK0-A
Micro-organism lysing VK01 0.1mm glass beads – 2 mL	Bertin Technologies	Cat#P000914-LYSK0-A
0.2 µm cellulose acetate syringe filter	Starstedt	Cat#83.1826.001
GF/C Glass Fiber Filter Paper	GE Healthcare	Cat#1822-024
Empty self-pack nanoLC columns	CoAnn Technologies	Cat#ICT36007515F-50
Kinetex® XB-C18 column material	Phenomenex	Cat#4498
ReproSil-Pur C18-AQ column material	Dr. Maisch	Cat#r119.aq
Empore™ SPE Disks C18	3M	Cat#66883-U
Protein LoBind Tube 2 mL	Eppendorf	Cat#0030 108.132
Protein LoBind Tube 1.5 mL	Eppendorf	Cat#0030 108.116
Conical Tubes 15 mL	Eppendorf	Cat#0030 122.151
Sera-Mag hydrophilic SpeedBeads	Cytiva	Cat#45152105050250
Sera-Mag hydrophobic SpeedBeads	Cytiva	Cat#65152105050250
Luna 5 µm C18(2) column (21.2 × 100 mm)	Phenomenex	Cat#00D-4252-P0-AX
Nucleodur 5 µm C18 Pyramid column (4 × 250 mm)	Macherey-Nagel	Cat#760202.40
Nucleodur 5 µm C18 Pyramid column (10 × 250 mm)	Macherey-Nagel	Cat#762272.100
Nucleodur 5 µm C18 Pyramid column (32 × 250 mm)	Macherey-Nagel	Cat#762272.320
Cell culture plate, 6 well, surface: suspension, flat base	Sarstedt	Cat#83.3920.500
Micro test plate, 96 well, slip-on lid, round base, PS, transparent	Sarstedt	Cat#82.1582.001
Cell culture plate, 96 well, surface: suspension, flat base	Sarstedt	Cat#83.3924.500
Cell culture plate, 96 well, surface: standard, flat base	Sarstedt	Cat#83.3924
Nunc™ F96 MicroWell™ White Polystyrene Plate	Thermo Scientific	Cat#236105
Nunc™ F96 MicroWell™ Black Polystyrene Plate	Thermo Scientific	Cat#237105
Nalgene™ Square PETG Media Bottles with Closure: Sterile, Shrink-Wrapped Trays	Thermo Scientific	Cat#342020

RESOURCE AVAILABILITY

Lead contact

Requests for additional information on data, resources, and reagents should be directed to and will be fulfilled by the lead contact, Rainer Kalscheuer (rainer.kalscheuer@hhu.de).

Materials availability

The reagents and mutant strains generated in this study will be made available subject to a completed Materials Transfer Agreement and proof of required BSL3 and import permits.

Data and code availability

- Proteomic data have been deposited at PRIDE. Microscopy data have been deposited at (<https://figshare.com>). Genome sequencing data have been deposited at Sequence Read Archive (SRA). All data are publicly available as of the date of publication, and accession numbers are listed in the [key resources table](#).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strains and culture conditions

M. tuberculosis strains including H37Rv, mc²6230 (Δ panCD Δ RD1) and several XDR-TB clinical isolates originating from South Africa, *M. bovis* BCG Pasteur as well as *M. smegmatis* mc²155 used in this study were obtained from the laboratory of William R. Jacobs Jr. (Albert Einstein College of Medicine, Bronx, NY). Cells of mycobacteria were grown at 37°C and 80 rpm in liquid Middlebrook 7H9 medium supplemented with 10% ADS enrichment (5% bovine serum albumin fraction, 2% glucose and 0.85% sodium chloride), 0.5% glycerol and 0.05% tyloxapol, or solid Middlebrook 7H10 agar supplemented with 10% ADS enrichment and 0.5% glycerol. Selective media were supplemented as required with hygromycin (50 μ g/mL), apramycin (30 μ g/mL) or kanamycin (20 μ g/mL). Cultures of non-mycobacterial species such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalis*, *E. faecium*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were grown at 37°C and 180 rpm in lysogeny broth (LB) or on solid LB medium.

Cell lines and culture conditions

The human THP-1 monocytic cell line (isolated from the peripheral blood of a 1-year old male patient suffering from acute monocytic leukemia)⁴² and MRC-5 lung fibroblasts (isolated from the lung tissue of a 14-week-old male fetus)⁴³ were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbeccó's modified Eagle medium (DMEM), respectively, both media supplemented with 10% fetal bovine serum (FBS). HEK293 kidney cells (isolated from a human female embryo)⁴⁴ were grown in Eagle's minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids and 10% FBS, while HepG2 cells (isolated from liver tissue of a 15-year-old Caucasian male with a hepatocellular carcinoma)⁴⁵ were cultivated in Ham's F12 medium supplemented with 2 mM L-glutamine and 10% FBS. All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂.

METHOD DETAILS

General information about the chemical synthesis of callyaerins

All chemicals were purchased from *abcr*, *Acros Organics*, *Alpha Aesar*, *Bachem*, *BLD pharm*, *Carbolution*, *Fluorochem*, *Iris Biotech*, *Lumiprobe*, *Merck*, *TCI Chemicals*, or *Thermo scientific* and used without further purification.

Purification of peptide precursors or compounds via reversed-phase preparative high performance liquid chromatography (prep. HPLC) was performed either on a Prominence ultra fast liquid chromatography (UFLC) system from *Shimadzu* (System A) with peak detection through an UV detector at λ = 210 nm equipped with a *Phenomenex* C18 column (*Luna* 5 μ m C18(2), 100 mm length, 21.2 mm ID) or a Nexera Prep HPLC system from *Shimadzu* (System B) with peak detection through a photodiode array detector at λ = 210 nm equipped with *Macherey-Nagel* C18 columns (*Nucleodur* 5 μ m C18 Pyramid, 250 mm length) with either 10 mm or 32 mm ID. In both systems, the mobile phase was generated by passive mixture of solvent B (Acetonitrile (ACN) either with or without 0.1% trifluoroacetic acid (TFA)) in solvent A (H₂O either with or without 0.1% TFA) in a linear gradient. Purified peptide precursors or compounds were freeze-dried using an ALPHA 2-4 LD plus lyophilizer from *Christ*.

Liquid chromatography analysis was carried out on a *ThermoFisher Scientific* Ultimate 3000 UHPLC system (UV peak detection at λ = 254 nm) equipped with a *Macherey-Nagel* C18 column (*Nucleodur* 5 μ m C18 Pyramid, 250 mm length, 4 mm ID). The mobile phase was generated by passive mixture of solvent B (ACN with 0.1% formic acid (FA)) in solvent A (H₂O with 0.1% FA) in a linear gradient (hold at 10% solvent B within the first 0.5 min, rise from 10-60% in the next minute, rise to 100% in the next 4.5 min, hold for 3.2 min, decrease to 10% within 0.3 min and hold for 2.5 min). In some cases, the length of the 100% solvent B plateau



had to be increased for elution of the compounds. All compounds were analyzed from a 10 mM DMSO stock diluted 1:50 with 50% ACN in H₂O (200 μ M analyte, 2% DMSO). This leads to the presence of a DMSO peak in the chromatogram of the compounds (as shown in [Data S2](#)).

High-resolution mass spectrometry (HRMS) spectra of compounds were recorded on a *ThermoFisher Scientific* Exactive Plus EMR mass spectrometer with a TriVersa NanoMate nanoESI system from *Advion* equipped with 5 μ m diameter nozzle spray chips. A total sample volume of 5 μ L were picked out of 96-well plates and the ESI spray was generated using 0.8 psi nitrogen backpressure combined with a positive nozzle chip voltage of 1.7 kV. The given *m/z* values represent the average of at least 200 scans.

Nuclear magnetic resonance (NMR) spectra were recorded at 25°C on a Bruker 400 MHz Avance II NMR spectrometer with a 5 mm PABBI broad band probe (400 MHz for ¹H and 101 MHz for ¹³C NMR) or a 700 MHz Avance II NMR spectrometer with a 5 mm TCI cryoprobe (700 MHz for ¹H and 176 MHz for ¹³C NMR) spectrometer. ¹H NMR spectra were reported in the following manner: chemical shifts (δ) in parts per million (ppm) refer to the residual non-deuterated solvent signals, multiplicities (s: singlet, d: doublet, t: triplet, dd: doublet of doublets, dt: doublet of triplets, td: triplet of doublets, m: multiplet), coupling constants (J) in Hertz (Hz) and number of protons (H). ¹H and ¹³C NMR spectra of synthesized callyaerin derivatives are shown in [Data S1](#).

Solid phase peptide synthesis

Peptide precursors for the synthesis of Callyaerin derivatives were synthesized using microwave-assisted automated solid phase peptide synthesis (SPPS) using functionalized polystyrene (PS) resin in a Liberty Blue HT12 Peptide Synthesizer from *CEM*. For this purpose, the respective resin was swollen in dimethylformamide (DMF). The peptide synthesizer was equipped with stock solutions of the corresponding fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids at a concentration of 0.2 M in DMF. Furthermore, stock solutions of the activator reagent *N,N'*-Diisopropylcarbodiimide (DIC, 0.5 M) and the activator base ethyl cyano-hydroxyiminoacetate (Oxyma, 1 M) in DMF as well as a 20% piperidine in DMF solution for Fmoc-deprotection were also generated. If synthesis on Rink amide resin were performed, additional stocks of *N,N*-diisopropylethylamine (DIPEA, 1 M) and acetic anhydride (Ac₂O, 0.5 M) for capping of unreacted binding positions on the resin and optional acetylation of the peptide N-terminus were also added to the device. For the capping of 2-chlorotrityl chloride (2-CTC) resin, a DCM:MeOH:DIPEA (17:2:1) mixture was used instead.

Microwave-assisted SPPS with Rink-amide resin

Loading of Rink-amide resin with the first amino acid of the sequence was done by starting with an initial deprotection step with 20% piperidine in DMF using microwave heating to 90°C for 120 seconds. Once the reactor was drained, the deprotection was repeated again for another 65 s. After washing of the resin, the first amino acid was linked to the resin by using 5 equivalents each (compared to initial resin loading capacity) of the respective amino acid, activator and activator base at microwave heating to 90°C for 240 s. A second double-coupling step was performed, followed by a capping step by using 10 eq. of DIPEA and 5 eq. of Ac₂O at microwave heating to 50°C for 360 s to deactivate non-reacted binding positions on the resin. After loading, the next amino acids were coupled by an iterative protocol consisting of piperidine-mediated deprotection (90°C, 65 s) and double amino acid coupling (5 eq., 90°C, 240 s) cycles. The synthesis was closed by a final Fmoc deprotection step (90°C, 65 s), thereby delivering the resin-bound precursor peptide.

Microwave-assisted SPPS with 2-CTC resin

Loading of 2-CTC resin with the first amino acid of the sequence was performed by using 10 eq. of the initial Fmoc-protected amino acid in combination with 20 eq. of DIPEA at microwave heating to 50°C for 10 minutes. Once the reactor was drained, the loading step was repeated, followed by capping of unreacted binding positions using DCM:MeOH:DIPEA (17:2:1) at microwave heating to 50°C for 10 min. After this loading procedure, an iterative protocol of deprotection with 20% piperidine in DMF (50°C, 5 min) and double amino acid coupling using 5 equivalents each of the respective amino acid, activator and activator base at microwave heating to 50°C for 10 min was used for elongation of the peptide sequence. The synthesis was closed by a final Fmoc deprotection step (50°C, 5 min), thereby delivering the resin-bound precursor peptide.

Cleavage of the SPPS-synthesized peptides from the resin

Peptides were cleaved from the resin by adding a mixture of 2.5% H₂O and 2.5% triisopropyl silane (TIS) in trifluoroacetic acid (TFA) to the peptide-loaded resin for 3 to 5 hours at room temperature. The resin was separated from the cleavage solution which was poured into ice-cold diethyl ether. The occurring peptide precipitation was supported by overnight cooling of this solution at -20°C. The peptides were separated from the solution via centrifugation at 4°C and 4200 g for 30 min, the supernatant was discarded and the peptide-pellet was air-dried for further purification via prep. HPLC.

Determination of minimal inhibitory concentration (MIC)

The MIC was determined for all bacteria using broth microdilution method. For all mycobacteria species, cultures obtained from exponentially growing cells cultured in supplemented 7H9 medium were diluted and seeded at 1×10^5 CFU/well in 96-well round bottom microplates, in a total volume of 100 μ L of supplemented 7H9 medium containing two-fold serially diluted test compounds with a starting concentration of 100 μ M. The plates were incubated at 37°C for 5 days (*M. tuberculosis* strains and *M. bovis* BCG Pasteur) or 24 h (*M. smegmatis*). 10 μ L of a 100 μ g/mL resazurin solution was subsequently added to each well and further incubated for 16–24 h. To fix the cells for MIC determination, 100 μ L of 10% formalin was added to each well (5% final concentration) for at least

30 min. Using a microplate reader (excitation, 540 nm; emission, 590 nm), fluorescence was quantified. Percentage of growth was calculated relative to sterile medium by subtraction of background fluorescence (0% growth) and maximum growth in untreated cells in the DMSO solvent control (100% growth).

For nosocomial pathogens and *B. subtilis*, cultures obtained from exponentially growing bacteria in Mueller Hinton broth (MHB) were diluted and seeded at 5×10^4 CFU/well in 96-well round bottom microplates, in a total volume of 100 μ L MHB containing two-fold serially diluted test compounds with a starting concentration of 100 μ M. Microplates were incubated for 24 h at 37°C. MIC was determined macroscopically by identifying the lowest concentration that resulted in inhibition of visible bacterial growth.

Cytotoxicity determination

The cytotoxicity of callyaerins was determined using the human THP-1 monocytic, MRC-5 lung fibroblast, HepG2 liver and HEK293 kidney cell lines. Cells were diluted and seeded at 5×10^4 cells/well in tissue culture-treated 96-well flat bottom microplates, in a total volume of 100 μ L of respective media containing two-fold serially diluted test compounds with a starting concentration of 100 μ M. 10 μ L of a 100 μ g/mL resazurin dye dilution was added to each well following 48 h incubation of microplates at 37°C and 5% CO₂. After 3 h incubation with resazurin, fluorescence was quantified in a microplate reader (excitation, 540 nm; emission, 590 nm). Percentage of growth was calculated relative to sterile medium (0% growth) and DMSO solvent control (100% growth). Selectivity index of test-compounds was determined as the quotient of the cytotoxic concentration inhibiting 50% growth of human cells and the MIC resulting in 90% inhibition of mycobacterial growth (IC₅₀/MIC₉₀).

Mycobacterial killing kinetic

Killing kinetic studies were done by quantifying viable cell counts following different treatment intervals to assess bactericidal or bacteriostatic activity of compounds. For this, a mid-exponentially growing culture of *M. tuberculosis* mc²6230 was adjusted to 2×10^6 CFU/mL and challenged with 5-fold MIC of CalB alone or in combination with clinical anti-TB drugs (isoniazid, rifampicin and bedaquiline) and incubated for 5 weeks at 37°C and 80 rpm. At various time intervals, 200 μ L aliquots were taken and 10-fold serially diluted. Serial dilutions were plated on Middlebrook 7H10 agar plates and incubated at 37°C for 3 weeks to count colony forming units (CFU).

Checkerboard assay

The interaction between CalB and clinical anti-TB drugs were quantified by determining the fractional inhibitory concentration indices (FICI) of CalB with these drugs in 96-well round bottom microplates. Compounds tested in combination were two-fold serially diluted in a two-dimensional fashion. Cultures of *M. tuberculosis* H37Rv and mc²6230 were diluted and added to the microplates at a density of 1×10^5 CFU/well in a total volume of 100 μ L. After 5-day incubation at 37°C, 10 μ L of a 100 μ g/mL resazurin solution was added to each well and further incubated for 16–24 h. Mycobacterial growth was quantified as a function of fluorescence intensity measured by a microplate reader (excitation, 540 nm; emission, 590 nm). Subsequently, the FICI was calculated as the sum of the quotients of the MICs of each bioactive agent when tested in combination (A and B) and the MIC when tested alone:

$$FICI(A+B) = \frac{A}{MIC(A)} + \frac{B}{MIC(B)}$$

Synergism or antagonism between CalB and clinical anti-TB drugs were represented as $FICI \leq 0.5$ or > 2 , respectively. Partial synergism was denoted by $0.5 < FICI \leq 0.75$, while $0.75 < FICI \leq 2$ indicates an additive effect.²²

Intracellular ATP measurement

Exponentially growing culture of *M. tuberculosis* mc²6230 was adjusted to OD (optical density) 0.5 at 600 nm and challenged with 5- or 10-fold MIC of CalB and relevant controls at 37°C and 80 rpm. At various time intervals, 1 mL culture aliquots were taken, pelleted by centrifugation at 10,000 g for 5 min, and supernatants were discarded to remove extracellular ATP. Cell pellets were resuspended in PBS and dispensed in a white opaque 96-well microplate containing BacTiter-Glo reagent (Promega, Madison, WI, USA). The resulting 1:1 mixture was incubated for 5 min and luminescence was measured as a function of ATP content using a microplate reader.

Propidium iodide internalization assay

A culture of *M. tuberculosis* mc²6230 taken from exponential growth phase was adjusted to OD_{600 nm} = 0.5, harvested by centrifugation at 4,500 g, 4°C for 10 min, washed and resuspended in an equal volume of PBS supplemented with 1% glucose. Cells were then incubated with 15 μ g/mL propidium iodide (PI). Cells were dispensed into a black opaque 96-well microplate upon incubation of cells with PI at 37°C for 30 min to establish a baseline PI fluorescence measurement (excitation, 535 nm; emission, 617 nm) using a microplate reader. Cells were then challenged with 5-fold MIC (final concentration) of CalB or appropriate controls and immediately returned to the microplate reader to quantify uptake of PI by measuring red fluorescence in a kinetic fashion for at least 4 h.

RNA extraction and real-time quantitative PCR

Cells of an exponentially growing culture of *M. tuberculosis* were adjusted to OD_{600 nm} = 1.5, incubated with 5-fold MIC of compounds and harvested after 24 h by centrifugation at 4,500 g, 4°C for 10 min for RNA extraction. Harvested cells were fixed overnight in 5 mL



RNA Protect reagent (Qiagen) after incubation with compounds. Fixed cells were pelleted, resuspended in 1 mL RLT buffer, and then transferred into Precellys® tubes containing a mixture of 0.5 mm and 0.1 mm silica beads. Cells were lysed by bead beating at 50 Hz, and RNA was extracted using the RNeasy Mini kit (Qiagen). Residual genomic DNA was removed by DNase treatment. After RNA integrity was checked using RNA 6000 Nano Chip, 2 µg RNA per sample was reverse transcribed into complementary DNA using the SuperScript™ III First-Strand Synthesis kit (Thermo Fisher). Gene expression was quantified by performing quantitative real-time PCR (qPCR) in a Mx3005P qPCR system (Agilent Technologies) using GoTaq® qPCR Master Mix (Promega). Analyses were performed following the manufacturer's protocol using gene-specific qPCR primers. Expression values were determined by the $\Delta\Delta C_T$ method, normalized to *16S rRNA* using DMSO-treated cells as the control. All qPCR primers (Table S1) were designed using the PrimerQuest tool.

Generation of a targeted *M. tuberculosis* $\Delta rv2113$ gene deletion mutant

Site-specific gene deletion in *M. tuberculosis* H37Rv was achieved by specialized phage transduction as described elsewhere.⁴⁶ Briefly, the *rv2113* gene was replaced with a $\gamma\delta$ -*sacB*-*hyg*- $\gamma\delta$ cassette comprising a hygromycin resistance gene and a *sacB* counter-selectable marker flanked by *res*-sites of the $\gamma\delta$ -resolvase. For construction of the required allelic exchange substrate, upstream and downstream flanking regions of *rv2113* were amplified by PCR using primers listed in Table S2. Subsequently, the flanking regions were digested with the indicated restriction enzymes and ligated with the *Var911*-digested p0004S vector. The resulting allelic exchange plasmid was then linearized with *PacI*, cloned, and packaged into the temperature-sensitive phage Φ phAE159, yielding knock-out phages that were propagated in *M. smegmatis* at 30°C. Allelic exchange in *M. tuberculosis* was achieved by specialized transduction at the non-permissive temperature of 37°C, using hygromycin for selection, resulting in gene deletion and replacement by $\gamma\delta$ *res*-*sacB*-*hyg*- $\gamma\delta$ *res* cassette. Obtained hygromycin-resistant transductants were obtained after 3 weeks of incubation and were screened for correct gene disruption by diagnostic PCR.

Heterologous gene expression in mycobacteria

For genetic complementation and overexpression purposes, the genes *rv2113* and *BCG_2130* were amplified from genomic DNA of *M. tuberculosis* H37Rv or *M. bovis* BCG Pasteur, respectively, by PCR using the primer pairs listed in Table S3, and cloned into the integrative single-copy plasmid pMV361 containing an apramycin resistance marker providing constitutive heterologous gene expression from the HSP60 promoter. The plasmids were transformed into mycobacterial cells via electroporation (1000 Ω , 25 μ F, 2500 V). Transformants were selected on Middlebrook 7H10 agar plates containing apramycin (40 mg/l).

Spontaneous resistant mutant generation

Spontaneous mutants of *M. tuberculosis* H37Rv resistant to callaerins were generated by plating 500 μ L culture aliquots containing $\sim 3 \times 10^7$ CFU on 120 mm square agar plates supplemented with 5-fold MIC of CalA or CalB, respectively. After 3–4 weeks of incubation at 37°C, independent colonies were picked for further analyses. Spontaneous mutants raised in the *rv2113* merodiploid strain of *M. tuberculosis* H37Rv were generated on agar additionally containing 30 μ g/mL apramycin to select for presence of the integrative plasmid carrying the merodiploid copy of *rv2113*.

Whole-genome sequencing

Genomic DNA of independent resistant mutants was extracted using the CTAB-lysozyme method as described elsewhere.⁴⁷ Genomes of resistant mutants were sequenced with an Illumina HiSeq 2500 next generation sequencer (at Texas A&M University, College Station, TX, USA & Biological and Medical Research Center, Heinrich Heine University Düsseldorf, Germany) after preparing sequencing libraries using standard paired-end genomic DNA sample prep kit from Illumina. Genome sequences were compared with that of the parent *M. tuberculosis* H37RvMA (GenBank accession GCA_000751615.1). Paired-end sequence data was collected with a read length of 106 bp. Base-calling was performed with Casava software, v1.8. The reads were assembled using a comparative genome assembly method, with *M. tuberculosis* H37RvMA as a reference sequence.⁴⁸

Intracellular activity in infected macrophages

For differentiation into macrophage-like cells, human THP-1 cells were seeded at a density of 1×10^5 cells per well in a total volume of 100 μ L RPMI 1640 medium supplemented with 10% FBS and 50 nM phorbol-12-myristate-13-acetate (PMA) in a 96-well flat-bottom microtiter plate. Cells were incubated overnight in a humidified atmosphere at 37°C and 5% CO₂. The next day, cells were washed with PBS twice, and medium was replaced with fresh RPMI 1640 medium supplemented with 10% FBS containing 3×10^5 cells per well of a precultured *M. tuberculosis* H37Rv reporter strain constitutively expressing mCherry resulting in a multiplicity of infection of three (MOI = 3). After three hours of infection, cells were washed with PBS twice, and medium was replaced with fresh RPMI 1640 medium supplemented with 10% FBS containing either 15.6 μ M of CalA or 1.95 μ M of CalB, respectively. DMSO and standard antibiotics (1 μ M rifampicin or 20 μ M streptomycin) were used as negative and positive controls, respectively. After five days of cultivation at 37°C and 5% CO₂, cells were fixed with a final concentration of 5% formalin and incubated for 30 min at room temperature. Fluorescence was quantified using a Nikon Eclipse TS100 and NIS-Elements (100 $\times$ magnification, 500 ms exposure time). Integrated density of red fluorescence was calculated using Fiji (ImageJ).

Confocal microscopy

Cells of an exponentially growing culture of *M. tuberculosis* H37Rv were adjusted to OD_{600 nm} = 0.4 in Middlebrook 7H9 medium supplemented with 10% ADS enrichment, 0.5% glycerol and 0.05% tyloxapol and were treated with 0.2 μ M of either **87** or Cy3 for 0.5, 3 or 6 h. Subsequently, cells were harvested by centrifugation at 5,000 g for 5 min and washed once with PBS containing 0.05% tyloxapol. Next, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) for 30 min at 4°C. Upon washing stained cells three times with PBS containing 0.05% tyloxapol, cells were fixed with 10% formaldehyde for 1 h at RT before removal from BSL-3 confinement. Fixed samples were then washed once, resuspended in PBS containing 0.05% tyloxapol and centrifuged at 800 rpm for 10 min to remove cell aggregates. Samples were imaged using Zeiss LSM710 Confocal Laser Scanning Microscope after spreading single-cell suspensions on soft agarose pad. Captured images were subsequently analyzed using ImageJ.

Photoaffinity enrichment LC-MS/MS-based target identification

Cells of an exponentially growing culture of *M. tuberculosis* H37Rv were harvested by centrifugation at 4000 g, 4°C for 10 min and washed thrice with PBS supplemented with 0.05% tyloxapol. The cells were resuspended in PBS (containing 0.05% tyloxapol) and divided equally into 2-mL centrifuge tubes, with each tube containing 1 mL of the suspension. In the competition approach, some samples were pretreated with 30 μ M CalB for 30 minutes at 37°C, whereas the remaining samples were supplemented with 0.3% DMSO in total. Cells were subsequently treated with the corresponding probes by adding 250 μ L of a 5x stock (in PBS; 0.63% DMSO in total per sample) and incubating for 180 min while shaking at 37°C. The samples were then transferred to 6-well plates and exposed to irradiation with UV light (365 nm) for 20 minutes at room temperature. The cells were transferred back to 2-mL centrifuge tubes, pelleted by centrifugation for 5 min at room temperature and 4500 g, and washed three times with 1.5 mL of 50 mM Na₂HPO₄ (pH 8; Sigma-Aldrich, D8418). Pellets were resuspended in 1,050 μ L of 50 mM Na₂HPO₄ and cells were lysed by bead-beating using 100 μ m silica zirconium beads (50 Hz; 15 min). Subsequently, 350 μ L of 4% sodium dodecyl sulfate (SDS; Carl Roth, 2326.2) in 50 mM Na₂HPO₄ were added and samples were incubated at 95°C for 5 min. After cooling down the samples for 15 min while shaking continuously, lysates were cleared by centrifugation at 13,000 g for 5 min at room temperature. Supernatants were collected for the use as protein solutions and sterile-filtered twice through a bacteria-tight 0.2 μ m cellulose acetate syringe filter to remove viable bacteria.

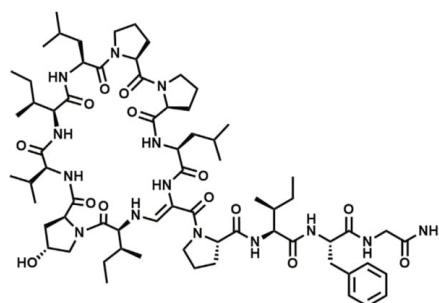
To apply a two-step enrichment protocol for labeled proteins, a volume corresponding to 250 μ g protein (Quantification: 660 nm Protein Assay with ionic detergent compatibility reagent; Thermo Scientific, 22660 & 22663) was transferred to 15 mL centrifuge tubes and filled up with 1% SDS in 50 mM Na₂HPO₄ to a total volume of 920 μ L. For SDS concentration reduction and benzonase treatment, 920 μ L benzonase-containing buffer (150 u per sample; EMD Millipore, 71206) was added and the samples were incubated with shaking at 37°C for 30 min. The subsequent Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) was performed by transferring 120 μ L reagent-mixture containing 167 μ M Biotin-Azide (Jena Bioscience, CLK-AZ104P4), 1.67 mM TBTA (Sigma-Aldrich, 678937) and 33.3 mM TCEP (Sigma-Aldrich, C4706) to the samples and the click reaction was started by addition of 40 μ L 100 mM CuSO₄ (Sigma-Aldrich, C1297). The mixture was incubated under continuous rotation for 150 min at room temperature. Proteins were then precipitated by the addition of a fourfold excess of ice-cold methanol (Fisher Chemical, M/4056/17) and samples were stored overnight at -20°C. Subsequently, the proteins were sedimented by centrifugation (4°C, 3220 g, 20 min), the supernatants were discarded, and the pellets were washed with 1 mL of ice-cold methanol. After an additional centrifugation step, protein pellets were taken up in 850 μ L of 2% SDS in PBS and incubated at 37°C for several minutes. The total volume was increased to a final volume of 3 mL by further addition of PBS and the remaining pellet debris were disrupted by sonication. Insoluble particles were sedimented by centrifugation (1400 g, 5 min), the supernatant was transferred to a fresh 15 mL centrifuge tube, and the SDS concentration was reduced to 0.2% by adding PBS to a total volume of 8.5 mL. For enrichment, avidin-agarose (Thermo Scientific, 20225) was equilibrated to 0.2% SDS in PBS and thereafter 100 μ L of the slurry suspension was added to each sample (approx. 50 μ L bead volume). The suspension was incubated under continuous rotation for 60 min at room temperature. The beads were sedimented by centrifugation (5 min, 400 g), the supernatant was discarded and the beads were washed four times with 10 mL 1% SDS in H₂O by using an iterative wash procedure consisting of incubation (5 min), centrifugation (3 min, 400 g) and supernatant removal steps. After an additional washing step with 10 mL H₂O, the beads were transferred to a 1.5 mL centrifuge tube and washed two more times with 1 mL H₂O (centrifugation 1 min, 3000 g) to remove residual SDS. For on-bead digestion, the beads were taken up in 100 μ L of 0.8 M urea (GE Healthcare, GE17-1319-01) in 50 mM ammonium bicarbonate (ABC; Sigma-Aldrich, 11213) containing 10 mM dithiothreitol (DTT; Sigma-Aldrich, 43815) and incubated for 60 min with continuous shaking (1500 rpm) at room temperature. Proteins were then alkylated (1500 rpm shaking, 75 min) by adding 20 mM iodoacetamide (IAM; Sigma-Aldrich, A3221). Excess IAM was quenched by increasing the DTT concentration to 25 mM (1500 rpm, 15 min) and digestion was started by the addition of 500 ng trypsin (Promega, V5111) per sample. The digest was incubated with continuous shaking (1250 rpm) for 18 h at 37°C and stopped by the addition of 5% formic acid (FA; Fisher Chemical, A117-50). After sedimentation of the beads (5 min, 600 g), the supernatant was transferred to a fresh centrifuge tube and the beads were washed with 50 μ L of 1% FA. The supernatants were combined and passed through equilibrated glass microfiber tips (pore size: 1.2 μ m, thickness: 0.26 mm, two disks per tip; GE Healthcare, 1822-024) to remove any residual beads. The resulting peptide solution was desalted on home-made C18 StageTips (two discs per tip; 3M, 66883-U) as described before,⁴⁹ and dry peptides were dissolved in 16 μ L 0.1% FA (15 min, 1500 rpm; Fisher Chemical, A11705AMP). For analysis, 4 μ L of sample was loaded on a self-packed fused silica capillary tube with evacuated-frit tip (75 μ m ID x 50 cm, 15 μ m orifice; CoAnn Technologies, ICT36007515F-50) filled with either Kinetex XB-C18 (particle size 1.7 μ m, Pore Size 100 Å; Phenomenex, 4498) or ReproSil-Pur 120 C18-AQ (particle size 1.9 μ m, Pore Size 120 Å; Dr. Maisch, r119.aq) material.



Peptides were separated using a 140 min gradient generated by an EASY-nLC 1200 liquid chromatography (Thermo Fisher Scientific) heated to 50°C by a PRISO-V1 column oven (Sonation). In case of Kinetex material, a rising proportion of 80% acetonitrile (ACN; Honeywell, 14261) in H₂O (Honeywell, 14263) with 0.2% FA (solvent B1) in 2% ACN in H₂O with 0.2% FA (solvent A1) was used for gradient preparation (3–7% B1 in A1 within the first 8 min, 7–27% in the next 84 min, 27–44% in the next 32 min, rise to 100% in the following 6 min, hold for 10 min) at a flow rate of 300 nL/min. In case of ReproSil-Pur material, a rising proportion of 80% acetonitrile (ACN) in H₂O with 0.1% FA (solvent B2) in H₂O with 0.1% FA (solvent A2) was used to prepare the gradient (6–32% B2 in A2 within the first 95 min, 32–42% in the next 20 min, rise to 100% in the following 10 min, hold for 15 min) at a flow rate of 250 nL/min. Peptides were ionized using a Nanospray Flex ion source (Thermo Fisher Scientific) with 2300–2500 V spray voltage and MS acquisition was performed in an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). MS1 data acquisition was done in a *m/z* range of 375 to 1800 at a 120000–240000 orbitrap resolution with a maximal injection time of 50 ns. Data dependent MS2 spectra were recorded using a loop cycle of 3 seconds with a dynamic exclusion duration of 20–25 seconds. For precursor isolation a 1.2 *m/z* quadrupole isolation window was used with subsequent stepped HCD fragmentation (20, 30, 40%) and data acquisition at rapid ion trap scan rate with 300% of normalized AGC target. Data processing was done with Proteome Discoverer 2.5 (Thermo Fisher Scientific) using SequestHT protein search and subsequent statistical analysis was done using Perseus 2.0.7.0.⁵⁰

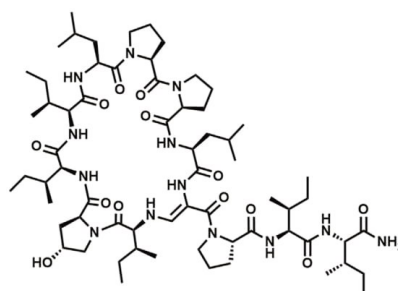
LC-MS/MS-based whole proteome comparison

Cells of exponentially growing cultures of *M. tuberculosis* H37Rv were adjusted to an OD_{600 nm} = 0.75 in a total volume of 20 mL Middlebrook 7H9 medium supplemented with 10% ADS enrichment and 0.05% tyloxapol. For treatment response analysis, cells were subjected to treatment with compound or a corresponding volume of DMSO as solvent control for 48 h at 37°C with shaking at 80 rpm. Subsequently, cells were harvested by centrifugation at 4,500 *g*, 4 °C for 10 min and washed thrice with PBS. Pellets were resuspended in 750 µL of PBS and cells were lysed by bead-beating using 100 µm silica zirconium beads (50 Hz; 15 min). After addition of 250 µL 4× lysis buffer (4% SDS, 40 mM TCEP, 160 mM chloroacetamide (CAM; Sigma-Aldrich, C0267), 200 mM HEPES (pH 8)), samples were incubated for 10 min at 95°C. Lysates were then cleared by centrifugation at 13,000 *g* and 4 °C for 5 min. Supernatants were collected to be used as protein solutions and were filter-sterilized twice through a bacteria-tight 0.2 µm cellulose acetate syringe filter to remove viable bacteria. For the digestion of proteins, a modified SP3 protocol was used.⁵¹ For this purpose, a total volume equivalent to 15 µg protein was transferred from each sample to a fresh vessel (quantification: 660 nm protein assay with ionic detergent compatibility reagent; Thermo Scientific, 22660 & 22663) and the volume was equalized between samples by addition of 1× lysis buffer (1% SDS, 10 mM TCEP, 40 mM CAM, 50 mM HEPES). Benzoylation-treatment was started by the addition of 7 U benzoylation (37°C, 1200 rpm, 15–20 min) and subsequent cysteine alkylation was done using 5–10 mM IAM (37°C, 1200 rpm, 30–60 min). Hydrophilic (Cytiva, #45152105050250) and hydrophobic Sera-Mag SpeedBeads (Cytiva, #65152105050250) were mixed equally and afterwards equilibrated to water. To each sample 150 µg of particles were added and protein binding was induced by adding an equal volume of ethanol (Fisher Chemical, E/0650DF/17) to the samples (1250 rpm, 15–30 min). The particles were settled on a magnetic rack and further processed as already described.³³ For analysis, a volume equivalent to 500 ng of peptides was loaded on a self-packed fused silica capillary tube with evacuated-frit tip (75 µm ID × 50 cm, 15 µm orifice; CoAnn Technologies, ICT36007515F-50) filled with ReproSil-Pur 120 C18-AQ (particle size 1.9 µm, Pore Size 120 Å; Dr. Maisch, r119.aq.) material. Peptides were separated using a 140 min gradient generated by an EASY-nLC 1200 liquid chromatography (Thermo Fisher Scientific) heated to 50°C by a PRISO-V1 column oven (Sonation). For gradient generation, a rising proportion of 80% acetonitrile in H₂O with 0.1% FA (solvent B) in H₂O with 0.1% FA (solvent A) was used (8–33% B in A within the first 95 min, 33–42% in the next 20 min, rise to 100% in the following 10 min, hold for 15 min) at a flow rate of 250 nL/min, respectively. Peptides were ionized using a Nanospray Flex ion source (Thermo Fisher Scientific) with 2300 V spray voltage and MS acquisition was performed in an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). MS1 data acquisition was done in a *m/z* range of 375 to 1750 at a 120000–240000 orbitrap resolution with a maximal injection time of 50 ns. Data dependent MS2 spectra were recorded using a loop cycle of 3 seconds with a dynamic exclusion duration of 25–30 seconds. For precursor isolation a 1.2 *m/z* quadrupole isolation window was used with subsequent stepped HCD fragmentation (20, 30, 40%) and data acquisition at rapid ion trap scan rate with 300% of normalized AGC target. Data processing was done with MaxQuant 2.3.0.1⁵² using Andromeda protein search and subsequent statistical analysis was done using Perseus 2.0.7.0.⁵⁰

Synthetic procedures and characterization of Callyaerin derivatives
Callyaerin A (1)

The linear precursor peptide of **1** was prepared via microwave-assisted solid-phase peptide synthesis (SPPS) using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 38 mg (27 μmol , 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of Dess-Martin-Periodinane (DMP) were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O ; solvent B: ACN) to give 6.1 mg (4.5 μmol , 16.4%) of **1** in a total yield of 4.5% (calculated in relation to initial resin capacity) as a white powder.

^1H NMR (700 MHz, $\text{DMSO}-d_6$) δ 9.02 (s, 1H), 8.81 (s, 1H), 8.28 (s, 1H), 8.15 (t, $J = 6.1$ Hz, 1H), 7.47 (d, $J = 8.7$ Hz, 1H), 7.44 (d, $J = 7.0$ Hz, 1H), 7.35 (d, $J = 13.2$ Hz, 1H), 7.26 (d, $J = 8.6$ Hz, 1H), 7.24 (s, 1H), 7.19–7.11 (m, 5H), 6.97 (s, 1H), 6.72 (d, $J = 10.0$ Hz, 1H), 5.87 (t, $J = 11.7$ Hz, 1H), 5.36 (d, $J = 3.1$ Hz, 1H), 4.61 (m, 2H), 4.40 (m, 1H), 4.30–4.22 (m, 4H), 4.09 (t, $J = 10.2$ Hz, 1H), 4.05 (t, $J = 9.0$ Hz, 1H), 3.90 (dd, $J = 16.6$, 7.1 Hz, 1H), 3.79 (d, $J = 8.5$ Hz, 2H), 3.77 (d, $J = 11.6$ Hz, 1H), 3.70 (dd, $J = 11.5$, 3.6 Hz, 1H), 3.64–3.57 (m, 2H), 3.52–3.49 (m, 2H), 3.48 (dd, $J = 16.0$, 4.6 Hz, 1H), 3.30 (m, 1H), 3.21 (td, $J = 11.1$, 5.8 Hz, 1H), 3.09 (m, 1H), 2.98 (m, 1H), 2.65 (m, 1H), 2.61 (m, 1H), 2.30 (m, 1H), 2.26 (m, 1H), 2.23 (m, 1H), 2.08 (m, 1H), 1.99 (m, 1H), 1.95–1.81 (m, 7H), 1.77 (m, 1H), 1.71 (m, 1H), 1.68–1.54 (m, 6H), 1.49 (m, 1H), 1.44–1.36 (m, 3H), 1.24 (m, 1H), 1.19–1.09 (m, 2H), 1.04 (m, 1H), 0.95 (d, $J = 6.6$ Hz, 3H), 0.88–0.86 (m, 6H), 0.85 (d, $J = 6.6$ Hz, 3H), 0.83–0.80 (m, 9H), 0.80–0.76 (m, 7H), 0.70 (t, $J = 7.4$ Hz, 3H), 0.42 (t, $J = 7.3$ Hz, 3H), 0.38 (d, $J = 6.8$ Hz, 3H); ^{13}C NMR (176 MHz, $\text{DMSO}-d_6$) δ 173.4, 172.7, 172.4, 172.3, 172.0, 172.0, 171.5, 171.5, 171.5, 171.5, 171.2, 171.1, 170.8, 167.8, 143.4, 138.1, 129.0, 129.0, 127.8, 127.8, 126.1, 98.8, 68.7, 66.1, 64.4, 64.0, 62.5, 62.0, 59.8, 59.3, 58.6, 56.8, 55.7, 50.1, 49.2, 48.8, 47.0, 46.1, 42.0, 41.2, 40.6, 40.0, 37.9, 37.8, 36.2, 35.0, 29.5, 28.6, 27.2, 26.2, 25.9, 25.6, 25.1, 24.9, 24.9, 24.7, 24.6, 24.1, 23.3, 22.9, 21.9, 21.1, 19.6, 19.2, 15.2, 14.9, 14.3, 11.0, 10.6, 10.3; LC main peak retention time: 7.89 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1357.8242, found: 1357.8235 (–0.5 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 679.4158, found: 679.4153 (–0.7 ppm); MS^2 HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 926.5710, found: 926.5695 (–1.6 ppm).

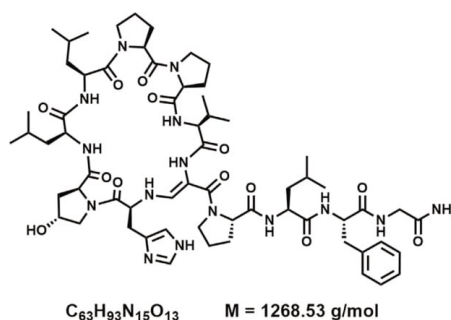
Callyaerin B (2)



The linear precursor peptide of **2** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 63 mg (49 μmol, 49%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, 72 mg of the peptide was dissolved in 15 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 16.7 mg (13 μmol, 24%) of **2** in a total yield of 11.8% (calculated in relation to initial resin capacity) as a white powder.

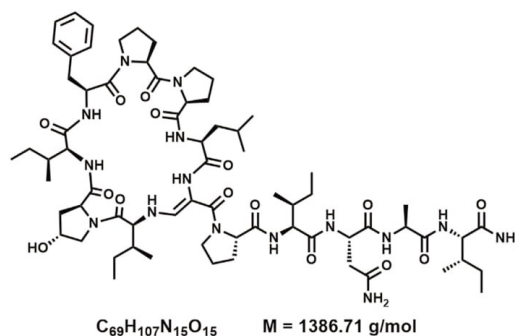
¹H NMR (700 MHz, DMSO-*d*₆) δ 9.00 (s, 1H), 8.86 (s, 1H), 8.28 (s, 1H), 7.59 (d, *J* = 10.3 Hz, 1H), 7.37 (d, *J* = 6.9 Hz, 1H), 7.23 (d, *J* = 9.6 Hz, 1H), 7.16 (s, 1H), 7.05 (d, *J* = 13.2 Hz, 1H), 6.97 (s, 1H), 6.64 (d, *J* = 10.0 Hz, 1H), 5.71 (dd, *J* = 13.3, 10.1 Hz, 1H), 5.41 (s, 1H), 4.62 (q, *J* = 6.8 Hz, 1H), 4.55 (td, *J* = 10.4, 3.8 Hz, 1H), 4.41 (s, 1H), 4.26–4.24 (m, 2H), 4.23 (t, *J* = 4.0 Hz, 1H), 4.15 (dd, *J* = 9.6, 5.2 Hz, 1H), 4.10 (dd, *J* = 10.0, 8.6 Hz, 1H), 4.04 (t, *J* = 8.9 Hz, 1H), 3.95 (t, *J* = 10.2 Hz, 1H), 3.82–3.76 (m, 2H), 3.65 (dd, *J* = 11.2, 3.7 Hz, 1H), 3.63–3.56 (m, 2H), 3.52–3.45 (m, 2H), 3.32–3.28 (m, 1H), 3.15 (td, *J* = 9.9, 4.6 Hz, 1H), 3.07 (dd, *J* = 11.1, 6.8 Hz, 1H), 2.55–2.51 (m, 1H), 2.32–2.25 (m, 2H), 2.24–2.20 (m, 1H), 2.10–2.05 (m, 1H), 2.01–1.96 (m, 1H), 1.93–1.87 (m, 5H), 1.87–1.82 (m, 2H), 1.80–1.77 (m, 1H), 1.75 (dd, *J* = 13.6, 7.1 Hz, 1H), 1.72–1.66 (m, 2H), 1.64–1.56 (m, 3H), 1.55–1.50 (m, 1H), 1.49–1.37 (m, 7H), 1.30–1.25 (m, 1H), 1.25–1.21 (m, 1H), 1.18–1.09 (m, 2H), 1.07–1.02 (m, 1H), 1.02–0.95 (m, 2H), 0.90–0.80 (m, 30H), 0.79–0.77 (m, 6H), 0.75 (t, *J* = 7.4 Hz, 3H), 0.72 (t, *J* = 7.4 Hz, 3H); ¹³C NMR (176 MHz, DMSO-*d*₆) δ 173.5, 172.8, 172.7, 172.3, 172.1, 171.5, 171.4, 171.4, 171.3, 171.2, 167.2, 142.7, 98.0, 68.8, 64.6, 64.3, 64.0, 62.5, 61.4, 60.0, 59.3, 57.9, 57.5, 56.7, 50.1, 49.2, 48.7, 47.1, 46.2, 41.2, 40.5, 37.9, 37.5, 36.4, 36.2, 36.2, 32.4, 29.7, 28.5, 26.3, 25.8, 25.6, 25.1, 24.9, 24.9, 24.8, 24.6, 23.9, 23.5, 23.1, 22.7, 22.2, 20.9, 15.8, 15.7, 15.3, 15.3, 14.4, 11.5, 11.1, 10.8, 10.3, 9.9; LC main peak retention time: 8.07 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1280.8341, found: 1280.8322 (–1.5 ppm); calculated for [M+2H]²⁺: 640.9207, found: 640.9195 (–1.9 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 940.5866, found: 940.5832 (–3.6 ppm).

Callyaerin C (**3**)



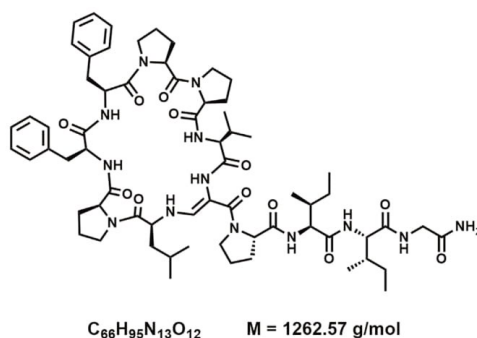
The linear precursor peptide of **3** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 8.7 mg (6.9 μmol) of **3** in a total yield of 6.9% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 12.03 (s, 1H), 9.84 (d, *J* = 7.7 Hz, 1H), 8.57 (s, 1H), 7.90 (d, *J* = 7.2 Hz, 1H), 7.80 (d, *J* = 9.3 Hz, 1H), 7.73 (dd, *J* = 7.1, 5.2 Hz, 1H), 7.65 (d, *J* = 13.8 Hz, 1H), 7.57 (d, *J* = 6.3 Hz, 1H), 7.39 (s, 1H), 7.37–7.34 (m, 2H), 7.32 (d, *J* = 10.4 Hz, 1H), 7.19 (s, 1H), 7.18–7.14 (m, 3H), 7.09 (s, 1H), 6.00 (s, 1H), 5.64 (dd, *J* = 13.8, 9.9 Hz, 1H), 5.09 (d, *J* = 3.7 Hz, 1H), 4.67 (dd, *J* = 10.2, 4.4 Hz, 1H), 4.52–4.45 (m, 1H), 4.45–4.37 (m, 2H), 4.35–4.27 (m, 2H), 4.27–4.20 (m, 1H), 4.19–4.10 (m, 2H), 4.07 (s, 1H), 3.95–3.83 (m, 2H), 3.71–3.62 (m, 1H), 3.60–3.45 (m, 6H), 3.30–3.25 (m, 1H), 3.23–3.14 (m, 1H), 2.99 (t, *J* = 12.6 Hz, 1H), 2.59–2.52 (m, 1H), 2.38–2.26 (m, 3H), 2.25–2.16 (m, 2H), 2.12–2.06 (m, 1H), 2.03–1.98 (m, 1H), 1.97–1.85 (m, 6H), 1.83–1.70 (m, 3H), 1.67–1.56 (m, 6H), 1.54–1.43 (m, 2H), 1.26–1.15 (m, 2H), 1.11 (d, *J* = 6.9 Hz, 3H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.91–0.84 (m, 12H), 0.82 (d, *J* = 6.5 Hz, 3H), 0.75 (d, *J* = 6.4 Hz, 3H); LC main peak retention time: 6.98 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1568.7150, found: 1268.7173 (1.8 ppm); calculated for [M+2H]²⁺: 634.8612, found: 634.8616 (0.6 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 837.4618, found: 837.4627 (1.1 ppm).

Callyaerin D (4)

The linear precursor peptide of **4** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O ; solvent B: ACN) to give 5.7 mg (4.1 μmol) of **4** in a total yield of 4.1% (calculated in relation to initial resin capacity) as a white powder.

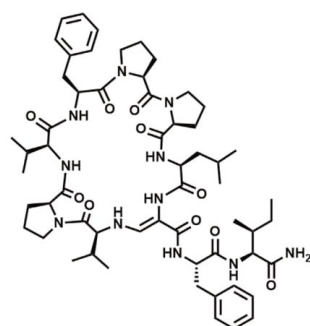
1H NMR (400 MHz, $DMSO-d_6$) δ 8.42 (s, 1H), 7.88 (d, $J = 7.5 \text{ Hz}$, 1H), 7.76 (d, $J = 6.8 \text{ Hz}$, 1H), 7.64 (d, $J = 5.0 \text{ Hz}$, 1H), 7.56 (d, $J = 7.1 \text{ Hz}$, 1H), 7.53 (d, $J = 9.3 \text{ Hz}$, 1H), 7.50 (d, $J = 6.0 \text{ Hz}$, 1H), 7.38 (d, $J = 3.3 \text{ Hz}$, 1H), 7.35 (s, 1H), 7.32–7.22 (m, 6H), 7.10 (s, 1H), 7.02 (s, 1H), 6.92 (s, 1H), 5.29 (dd, $J = 13.8, 10.1 \text{ Hz}$, 1H), 5.19 (d, $J = 3.8 \text{ Hz}$, 1H), 4.65–4.59 (m, 1H), 4.55 (q, $J = 7.0 \text{ Hz}$, 1H), 4.51–4.44 (m, 1H), 4.44–4.38 (m, 2H), 4.36–4.30 (m, 2H), 4.21–4.11 (m, 3H), 4.05 (dd, $J = 8.9, 6.8 \text{ Hz}$, 1H), 4.00 (dd, $J = 5.0, 3.4 \text{ Hz}$, 1H), 3.96–3.90 (m, 2H), 3.68–3.60 (m, 2H), 3.56–3.49 (m, 1H), 3.40–3.35 (m, 1H), 3.28–3.23 (m, 1H), 3.06 (dd, $J = 13.1, 9.5 \text{ Hz}$, 1H), 2.80–2.73 (m, 1H), 2.73–2.66 (m, 1H), 2.39–2.30 (m, 1H), 2.27–2.12 (m, 4H), 2.10–2.04 (m, 1H), 2.02–1.88 (m, 5H), 1.86–1.68 (m, 9H), 1.65–1.56 (m, 1H), 1.52–1.30 (m, 8H), 1.27 (d, $J = 7.2 \text{ Hz}$, 3H), 1.23 (s, 1H), 1.17–1.06 (m, 1H), 0.98–0.91 (m, 1H), 0.89–0.79 (m, 30H); LC main peak retention time: 6.18 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1386.8144, found: 1386.8153 (0.6 ppm); calculated for $[M+2H]^{2+}$: 693.9109, found: 693.9108 (–0.1 ppm); MS^2 HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{ring}+H]^+$: 861.4869, found: 861.4870 (0.1 ppm).

Callyaerin E (5)

The linear precursor peptide of **5** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 130 mg (101 μmol , 44%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O ; solvent B: ACN) to give 60.1 mg (47.6 μmol , 46.9%) of **5** in a total yield of 20.7% (calculated in relation to initial resin capacity) as a white powder.



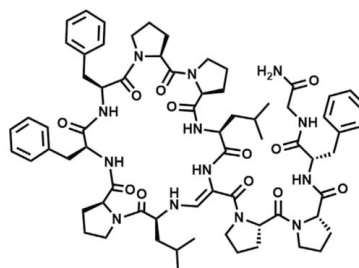
¹H NMR (400 MHz, DMSO-*d*₆) δ 8.37 (s, 1H), 7.89 (t, *J* = 6.0 Hz, 1H), 7.72 (d, *J* = 6.7 Hz, 1H), 7.63 (d, *J* = 8.0 Hz, 1H), 7.37 (d, *J* = 8.2 Hz, 1H), 7.33–7.16 (m, 12H), 7.06–7.01 (m, 2H), 6.99–6.94 (m, 1H), 5.27 (dd, *J* = 13.5, 9.7 Hz, 1H), 4.68–4.60 (m, 1H), 4.56 (dd, *J* = 10.2, 3.9 Hz, 1H), 4.45–4.32 (m, 4H), 4.16 (dd, *J* = 9.9, 7.5 Hz, 1H), 4.09 (t, *J* = 7.7 Hz, 1H), 4.04–3.96 (m, 2H), 3.96–3.90 (m, 1H), 3.71 (dd, *J* = 16.6, 6.2 Hz, 1H), 3.63–3.54 (m, 3H), 3.53–3.46 (m, 1H), 3.46–3.37 (m, 1H), 3.33–3.29 (m, 1H), 3.26–3.16 (m, 1H), 3.04–2.95 (m, 3H), 2.71 (dd, *J* = 13.4, 4.8 Hz, 1H), 2.49–2.43 (m, 1H), 2.37–2.27 (m, 1H), 2.25–2.11 (m, 4H), 2.10–2.01 (m, 1H), 1.98–1.53 (m, 14H), 1.49–1.34 (m, 3H), 1.30–1.16 (m, 3H), 1.05 (d, *J* = 6.9 Hz, 3H), 0.96 (dd, *J* = 6.7, 3.3 Hz, 6H), 0.90 (d, *J* = 6.6 Hz, 3H), 0.88–0.82 (m, 9H), 0.79 (t, *J* = 7.3 Hz, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 173.5, 172.6, 172.1, 171.7, 171.5, 171.5, 171.3, 171.0, 171.0, 169.1, 167.7, 143.1, 138.1, 137.9, 129.0, 128.4, 128.2, 128.1, 126.5, 126.4, 98.5, 63.5, 63.1, 61.0, 58.4, 57.8, 56.6, 54.0, 53.0, 48.7, 46.6, 46.4, 42.3, 42.2, 36.1, 35.5, 35.4, 35.3, 29.9, 28.8, 28.7, 27.0, 25.7, 25.5, 24.8, 24.4, 24.2, 23.8, 20.8, 18.3, 17.7, 15.7, 15.5, 11.2, 10.6; LC main peak retention time: 7.42 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1262.7296, found: 1262.7304 (0.6 ppm); calculated for [M+2H]²⁺: 631.8685, found: 631.8686 (0.2 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 865.4607, found: 865.4597 (−1.2 ppm).

Callyaerin F (6)

The linear precursor peptide of **6** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 116 mg (104 μmol, 45%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 14.1 mg (12.9 μmol, 12.4%) of **6** in a total yield of 5.6% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.25 (s, 1H), 7.66 (d, *J* = 9.6 Hz, 1H), 7.57–7.46 (m, 3H), 7.32–7.13 (m, 11H), 7.03 (s, 1H), 6.98 (s, 1H), 6.17 (d, *J* = 7.8 Hz, 1H), 5.14 (dd, *J* = 13.7, 10.0 Hz, 1H), 4.63 (td, *J* = 10.2, 3.9 Hz, 1H), 4.57–4.50 (m, 1H), 4.45–4.39 (m, 1H), 4.37 (d, *J* = 2.0 Hz, 1H), 4.31–4.23 (m, 2H), 4.01–3.92 (m, 2H), 3.89–3.82 (m, 2H), 3.77–3.68 (m, 1H), 3.59 (t, *J* = 8.3 Hz, 1H), 3.39–3.35 (m, 1H), 3.24 (t, *J* = 8.9 Hz, 1H), 3.01 (dd, *J* = 12.9, 9.6 Hz, 1H), 2.96–2.87 (m, 2H), 2.74 (dd, *J* = 12.9, 3.5 Hz, 1H), 2.35–2.26 (m, 2H), 2.22–2.00 (m, 5H), 1.97–1.88 (m, 2H), 1.88–1.77 (m, 4H), 1.77–1.63 (m, 4H), 1.50–1.38 (m, 2H), 1.33–1.21 (m, 1H), 1.16–1.03 (m, 1H), 0.93 (d, *J* = 4.7 Hz, 3H), 0.91 (d, *J* = 4.2 Hz, 3H), 0.88–0.84 (m, 6H), 0.82 (d, *J* = 6.3 Hz, 3H), 0.79–0.74 (m, 6H), 0.65 (d, *J* = 6.6 Hz, 3H); LC main peak retention time: 7.14 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1094.6397, found: 1094.6412 (1.4 ppm); calculated for [M+2H]²⁺: 547.8235, found: 547.8242 (1.3 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 817.4607, found: 817.4609 (0.2 ppm).

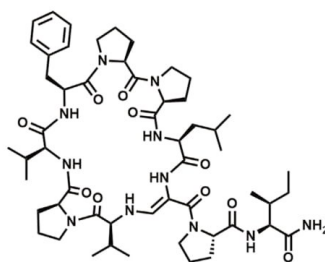
Callyaerin G (7)



The linear precursor peptide of **7** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 83 mg (63 μmol , 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O ; solvent B: ACN) to give 35 mg (27 μmol , 42.9%) of **7** in a total yield of 11.7% (calculated in relation to initial resin capacity) as a white powder.

^1H NMR (400 MHz, DMSO-d_6) δ 8.24 (s, 1H), 7.98 (dd, $J = 7.9, 4.8$ Hz, 1H), 7.75–7.66 (m, 2H), 7.52 (d, $J = 9.7$ Hz, 1H), 7.47 (d, $J = 13.5$ Hz, 1H), 7.38–7.34 (m, 2H), 7.30–7.10 (m, 16H), 5.33 (dd, $J = 13.5, 9.9$ Hz, 1H), 4.64 (q, $J = 7.0$ Hz, 1H), 4.53–4.40 (m, 2H), 4.38–4.20 (m, 5H), 4.11 (dd, $J = 8.4, 6.6$ Hz, 1H), 3.99–3.81 (m, 5H), 3.54–3.44 (m, 2H), 3.43–3.35 (m, 4H), 3.25 (dd, $J = 13.2, 3.7$ Hz, 1H), 3.04–2.94 (m, 2H), 2.93–2.84 (m, 2H), 2.79–2.72 (m, 1H), 2.68 (dd, $J = 13.2, 6.3$ Hz, 1H), 2.34–2.23 (m, 1H), 2.20–2.07 (m, 3H), 2.06–1.93 (m, 3H), 1.93–1.82 (m, 7H), 1.82–1.73 (m, 4H), 1.72–1.58 (m, 5H), 1.55–1.43 (m, 1H), 1.41–1.29 (m, 1H), 1.15–1.01 (m, 2H), 0.88 (d, $J = 6.3$ Hz, 3H), 0.84 (d, $J = 6.2$ Hz, 3H), 0.54 (d, $J = 6.6$ Hz, 3H), 0.38 (d, $J = 6.3$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.3, 172.1, 171.9, 171.6, 171.4, 171.0, 171.0, 170.9, 170.7, 169.6, 168.1, 145.6, 138.2, 138.2, 137.7, 129.9, 129.2, 128.4, 128.2, 128.1, 128.0, 126.5, 126.3, 125.9, 98.0, 63.7, 63.0, 62.8, 61.7, 57.6, 54.6, 54.1, 52.5, 50.3, 47.7, 46.7, 46.3, 42.2, 36.9, 36.7, 35.2, 28.7, 27.9, 26.8, 25.6, 25.0, 24.4, 23.3, 22.9, 20.8, 20.2; LC main peak retention time: 6.60 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1294.6983, found: 1294.7013 (2.3 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 647.8528, found: 647.8531 (0.5 ppm); MS^2 HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 879.4763, found: 879.4742 (−2.4 ppm).

Callyaerin H (8)



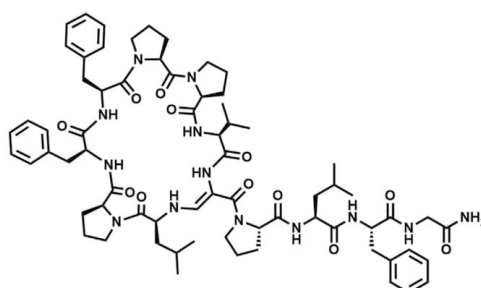
The linear precursor peptide of **8** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 98 mg (92 μmol , 40%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the



peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 4.7 mg (4.5 μ mol, 4.9%) of **8** in a total yield of 2.0% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.47 (s, 1H), 7.59 0(d, *J* = 5.1 Hz, 1H), 7.55 (d, *J* = 9.6 Hz, 1H), 7.52 (s, 1H), 7.50 (d, *J* = 5.5 Hz, 1H), 7.31–7.20 (m, 6H), 6.97 (s, 1H), 6.89 (s, 1H), 5.26 (dd, *J* = 13.9, 9.9 Hz, 1H), 4.61–4.48 (m, 3H), 4.43 (d, *J* = 1.9 Hz, 1H), 4.37–4.31 (m, 2H), 4.17 (dd, *J* = 9.6, 7.7 Hz, 1H), 4.05–4.00 (m, 1H), 4.00–3.94 (m, 1H), 3.86 (t, *J* = 4.7 Hz, 1H), 3.84–3.79 (m, 1H), 3.79–3.71 (m, 1H), 3.65–3.58 (m, 1H), 3.39–3.32 (m, 1H), 3.30–3.22 (m, 2H), 3.04 (dd, *J* = 13.0, 9.5 Hz, 1H), 2.77 (dd, *J* = 13.0, 3.6 Hz, 1H), 2.40–2.30 (m, 1H), 2.28–2.15 (m, 4H), 2.14–2.07 (m, 2H), 2.07–2.02 (m, 1H), 1.99–1.89 (m, 2H), 1.89–1.81 (m, 4H), 1.80–1.65 (m, 5H), 1.60–1.36 (m, 4H), 1.24 (s, 1H), 1.22–1.13 (m, 1H), 0.93 (d, *J* = 6.9 Hz, 3H), 0.90–0.80 (m, 18H), 0.68 (d, *J* = 6.6 Hz, 3H); LC main peak retention time: 6.21 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1044.6241, found: 1044.6243 (0.2 ppm); calculated for [M+2H]²⁺: 522.8157, found: 522.8137 (–3.8 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 817.4607, found: 817.4602 (–0.6 ppm).

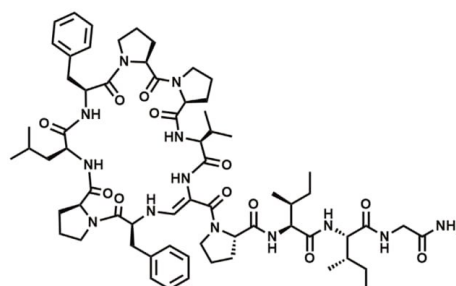
Callyaerin I (**9**)



The linear precursor peptide of **9** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 117 mg (89 μ mol, 39%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 47.7 mg (36.8 μ mol, 41.3%) of **9** in a total yield of 16% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.45 (s, 1H), 7.78 (d, *J* = 6.9 Hz, 1H), 7.68 (d, *J* = 9.7 Hz, 1H), 7.65 (d, *J* = 6.7 Hz, 1H), 7.53 (dd, *J* = 7.3, 5.1 Hz, 1H), 7.44 (d, *J* = 13.5 Hz, 1H), 7.32–7.22 (m, 11H), 7.21–7.18 (m, 1H), 7.16–7.11 (m, 6H), 7.03 (d, *J* = 10.1 Hz, 1H), 5.37 (dd, *J* = 13.6, 9.8 Hz, 1H), 4.68–4.59 (m, 2H), 4.47–4.35 (m, 3H), 4.30 (dd, *J* = 10.7, 7.2 Hz, 1H), 4.27–4.21 (m, 1H), 4.18 (dd, *J* = 10.0, 7.4 Hz, 1H), 4.01–3.94 (m, 2H), 3.89 (dd, *J* = 17.0, 7.4 Hz, 1H), 3.85–3.78 (m, 1H), 3.68–3.60 (m, 1H), 3.56–3.45 (m, 3H), 3.45–3.38 (m, 1H), 3.38–3.30 (m, 1H), 3.29–3.21 (m, 2H), 3.01–2.92 (m, 3H), 2.81–2.72 (m, 1H), 2.68 (dd, *J* = 13.3, 4.7 Hz, 1H), 2.48–2.43 (m, 1H), 2.34–2.27 (m, 1H), 2.26–2.19 (m, 1H), 2.18–2.10 (m, 2H), 2.07–1.99 (m, 1H), 1.96–1.82 (m, 4H), 1.81–1.68 (m, 4H), 1.67–1.49 (m, 6H), 1.48–1.36 (m, 1H), 1.26–1.22 (m, 1H), 1.20 (d, *J* = 6.9 Hz, 3H), 1.15–1.10 (m, 1H), 1.07 (d, *J* = 7.0 Hz, 3H), 1.04–0.98 (m, 1H), 0.87 (d, *J* = 6.5 Hz, 3H), 0.74 (d, *J* = 6.5 Hz, 3H), 0.44 (d, *J* = 6.4 Hz, 3H), 0.32 (d, *J* = 6.6 Hz, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 174.2, 173.5, 172.1, 171.8, 171.7, 171.0, 170.7, 170.7, 169.1, 168.4, 143.5, 138.3, 138.1, 137.9, 129.8, 129.0, 128.4, 128.2, 128.1, 127.7, 126.5, 126.4, 126.0, 98.1, 63.5, 63.2, 63.0, 62.0, 57.6, 56.9, 53.8, 52.9, 48.8, 46.7, 46.4, 46.1, 42.2, 38.1, 37.7, 36.0, 35.2, 30.2, 29.4, 28.9, 28.7, 27.0, 26.0, 25.5, 24.9, 24.4, 24.2, 23.5, 23.1, 22.7, 20.8, 19.9, 18.2, 17.7; LC main peak retention time: 7.82 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1296.7139, found: 1296.7161 (1.7 ppm); calculated for [M+2H]²⁺: 648.8606, found: 648.8610 (0.6 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 865.4607, found: 865.4597 (–1.2 ppm).

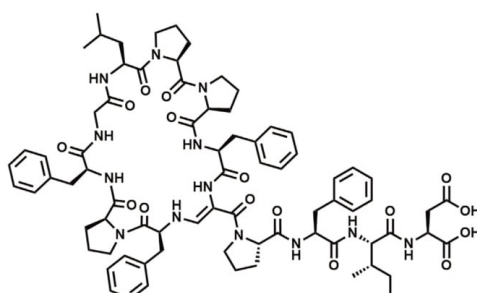
Callyaerin J (10)



The linear precursor peptide of **10** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O ; solvent B: ACN) to give 29 mg (23 μmol) of **10** in a total yield of 23% (calculated in relation to initial resin capacity) as a white powder.

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 8.21 (s, 1H), 7.84 (t, $J = 6.0$ Hz, 1H), 7.69 (d, $J = 7.5$ Hz, 1H), 7.44 (d, $J = 6.2$ Hz, 1H), 7.32 (d, $J = 8.4$ Hz, 1H), 7.29–7.16 (m, $J = 7.2, 6.8$ Hz, 10H), 7.14 (d, $J = 6.7$ Hz, 1H), 7.10 (d, $J = 10.1$ Hz, 1H), 7.07–7.01 (m, 3H), 5.44 (dd, $J = 13.5, 9.6$ Hz, 1H), 4.64–4.57 (m, 2H), 4.51 (dd, $J = 10.2, 5.1$ Hz, 1H), 4.36 (dd, $J = 11.1, 7.7$ Hz, 1H), 4.30 (t, $J = 7.5$ Hz, 1H), 4.15 (dd, $J = 9.8, 7.5$ Hz, 1H), 4.12–4.06 (m, 2H), 4.02 (dd, $J = 10.1, 6.9$ Hz, 1H), 3.98–3.92 (m, 2H), 3.89–3.80 (m, 1H), 3.70 (dd, $J = 16.7, 6.5$ Hz, 1H), 3.58–3.45 (m, 3H), 3.40–3.34 (m, 2H), 3.21–3.10 (m, 1H), 3.01 (dd, $J = 13.5, 8.8$ Hz, 1H), 2.73–2.61 (m, 2H), 2.56–2.52 (m, 1H), 2.33 (h, $J = 6.7$ Hz, 1H), 2.28–2.16 (m, 3H), 2.16–2.06 (m, 2H), 1.98–1.86 (m, 4H), 1.86–1.71 (m, 6H), 1.68–1.53 (m, 4H), 1.52–1.37 (m, 4H), 1.32–1.17 (m, 2H), 1.12–1.07 (m, 1H), 1.05 (d, $J = 6.8$ Hz, 3H), 1.01 (d, $J = 6.8$ Hz, 3H), 0.92 (d, $J = 6.5$ Hz, 3H), 0.89–0.84 (m, 6H), 0.82 (d, $J = 6.5$ Hz, 3H), 0.69 (d, $J = 6.9$ Hz, 3H), 0.65 (t, $J = 7.7$ Hz, 3H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$) δ 172.9, 172.5, 172.1, 172.0, 171.6, 171.5, 171.2, 171.0, 170.9, 169.1, 167.4, 142.3, 138.0, 136.1, 129.2, 129.0, 128.4, 128.1, 126.7, 126.4, 99.3, 63.6, 63.4, 63.0, 61.4, 60.6, 58.6, 57.2, 56.9, 52.7, 52.1, 48.6, 46.6, 42.2, 36.4, 35.5, 35.3, 29.9, 29.2, 27.0, 25.7, 25.5, 25.0, 24.4, 24.2, 22.7, 21.3, 18.9, 18.5, 15.6, 11.3, 10.9; LC main peak retention time: 7.58 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1262.7296, found: 1262.7307 (0.9 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 631.8685, found: 631.8688 (0.5 ppm); MS^2 HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 865.4607, found: 865.4595 (−1.4 ppm).

Callyaerin K (11)

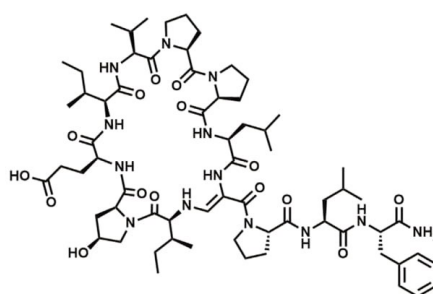




The linear precursor peptide of **11** was prepared via microwave-assisted SPPS using 2-CTC PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 44 mg (30 μ mol, 30%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 7.6 mg (5.2 μ mol, 13.5%) of **11** in a total yield of 5.2% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.26 (d, *J* = 8.4 Hz, 1H), 8.94 (s, 1H), 8.33 (d, *J* = 7.5 Hz, 1H), 7.81 (d, *J* = 9.4 Hz, 1H), 7.47 (s, 1H), 7.41–7.34 (m, 4H), 7.30–7.23 (m, 8H), 7.23–7.16 (m, 6H), 7.12–7.08 (m, 2H), 7.07–7.03 (m, 2H), 6.98 (s, 1H), 5.64–5.48 (m, 1H), 5.18–5.07 (m, 1H), 4.61 (s, 1H), 4.53–4.45 (m, 1H), 4.32 (ddd, *J* = 12.7, 9.3, 3.6 Hz, 1H), 4.22–4.12 (m, 2H), 4.10–3.97 (m, 4H), 3.96–3.86 (m, 2H), 3.66 (s, 1H), 3.56–3.44 (m, 3H), 3.29–3.21 (m, 4H), 3.21–3.14 (m, 2H), 3.10–3.04 (m, 1H), 2.99–2.91 (m, 2H), 2.90–2.82 (m, 3H), 2.73–2.63 (m, 2H), 2.58 (dd, *J* = 16.4, 6.1 Hz, 1H), 2.21–2.11 (m, 1H), 2.07–1.99 (m, 1H), 1.97–1.73 (m, 6H), 1.68–1.52 (m, 4H), 1.50–1.27 (m, 8H), 1.23 (s, 1H), 1.14–1.05 (m, 1H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.86–0.81 (m, 6H), 0.70 (t, *J* = 7.3 Hz, 3H), 0.65–0.53 (m, 1H); LC main peak retention time: 6.29 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1460.7249, found: 1460.7254 (0.3 ppm); calculated for [M+2H]²⁺: 730.8661, found: 730.8662 (0.1 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 970.4822, found: 940.4814 (–0.8 ppm).

Callyaerin L (12)



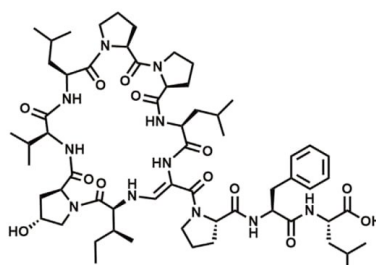
C₆₆H₁₀₁N₁₃O₁₅

M = 1316.61 g/mol

The linear precursor peptide of **12** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 43 mg (32 μ mol, 32%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 8 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 3.7 mg (2.8 μ mol, 8.7%) of **12** in a total yield of 2.8% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.75 (s, 1H), 8.27 (s, 1H), 7.76 (d, *J* = 7.6 Hz, 1H), 7.59–7.51 (m, 2H), 7.37 (d, *J* = 7.4 Hz, 1H), 7.32–7.26 (m, 1H), 7.25–7.18 (m, 3H), 7.14–7.04 (m, 3H), 6.91 (d, *J* = 9.9 Hz, 1H), 6.78 (s, 1H), 5.45–5.36 (m, 1H), 5.36–5.32 (m, 1H), 4.61–4.52 (m, 2H), 4.40 (s, 1H), 4.30–4.23 (m, 2H), 4.22–4.03 (m, 4H), 3.99–3.90 (m, 2H), 3.88–3.77 (m, 2H), 3.75–3.66 (m, 2H), 3.63–3.48 (m, 4H), 3.22–3.10 (m, 2H), 2.74–2.66 (m, 1H), 2.33–2.23 (m, 3H), 2.22–2.17 (m, 2H), 2.15–2.02 (m, 6H), 1.98–1.80 (m, 7H), 1.77–1.57 (m, 5H), 1.56–1.46 (m, 3H), 1.42–1.30 (m, 3H), 1.23–(s, 1H), 1.19–1.11 (m, 1H), 0.98 (d, *J* = 6.9 Hz, 3H), 0.96–0.91 (m, 6H), 0.88–0.79 (m, 9H), 0.76–0.68 (m, 9H), 0.15 (t, *J* = 7.3 Hz, 3H); LC main peak retention time: 5.46 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1316.7613, found: 1316.7621 (0.6 ppm); calculated for [M+2H]²⁺: 658.8843, found: 658.884 (0.2 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 942.5295, found: 942.5301 (0.6 ppm).

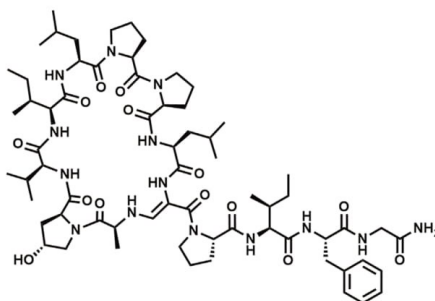
Callynormine A (13)



The linear precursor peptide of **13** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 55 mg (46 μmol , 46%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 17.7 mg (14.9 μmol , 32.7%) of **13** in a total yield of 14.9% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.24 (s, 1H), 7.75 (d, *J* = 9.4 Hz, 1H), 7.56 (d, *J* = 3.8 Hz, 1H), 7.54 (s, 1H), 7.37–7.26 (m, 5H), 7.20 (d, *J* = 5.9 Hz, 1H), 7.19–7.17 (m, 1H), 7.05 (d, *J* = 13.7 Hz, 1H), 5.24 (dd, *J* = 13.7, 9.4 Hz, 1H), 5.17 (d, *J* = 3.7 Hz, 1H), 4.72–4.63 (m, 1H), 4.54 (q, *J* = 6.9 Hz, 1H), 4.44–4.36 (m, 3H), 4.35–4.26 (m, 3H), 4.19–4.09 (m, 2H), 3.98–3.88 (m, 2H), 3.69–3.58 (m, 3H), 3.54–3.43 (m, 3H), 3.29–3.16 (m, 3H), 2.77 (t, *J* = 12.8 Hz, 1H), 2.35–2.26 (m, 1H), 2.23–2.10 (m, 2H), 2.09–1.95 (m, 5H), 1.94–1.79 (m, 6H), 1.74–1.57 (m, 4H), 1.56–1.43 (m, 6H), 1.41–1.33 (m, 1H), 1.19–1.08 (m, 1H), 0.96–0.75 (m, 30H); LC main peak retention time: 7.02 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1188.7027, found: 1188.7022 (–0.4 ppm); calculated for [M+2H]²⁺: 594.8550, found: 594.8534 (–2.7 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 813.4870, found: 813.4849 (–2.6 ppm).

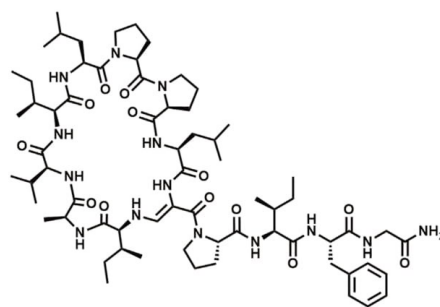
CalA_R1A (14)



The linear precursor peptide of **14** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 102 mg (77 μmol , 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 8.0 mg (6.1 μmol , 7.9%) of **14** in a total yield of 2.7% (calculated in relation to initial resin capacity) as a white powder.



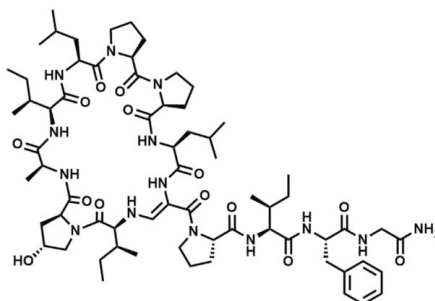
^1H NMR (400 MHz, DMSO- d_6) δ 8.36 (s, 1H), 8.25 (t, J = 6.2 Hz, 1H), 7.74 (d, J = 6.6 Hz, 1H), 7.59 (d, J = 13.6 Hz, 1H), 7.56 (d, J = 9.1 Hz, 1H), 7.35 (d, J = 7.8 Hz, 1H), 7.28 (s, 1H), 7.25–7.18 (m, 1H), 7.18–7.11 (m, 6H), 6.96 (s, 1H), 6.87 (d, J = 10.0 Hz, 1H), 5.77–5.67 (m, 1H), 5.33 (d, J = 3.3 Hz, 1H), 4.72–4.65 (m, 1H), 4.63–4.53 (m, 2H), 4.39 (s, 1H), 4.30–4.23 (m, 3H), 4.12 (dd, J = 10.0, 7.2 Hz, 1H), 4.06–3.95 (m, 2H), 3.94–3.84 (m, 3H), 3.67 (d, J = 11.3 Hz, 1H), 3.63–3.52 (m, 3H), 3.51–3.39 (m, 3H), 3.29–3.17 (m, 2H), 3.11 (dd, J = 14.2, 3.4 Hz, 1H), 2.70–2.61 (m, 1H), 2.30–2.21 (m, 3H), 2.17–2.10 (m, 1H), 2.06–1.91 (m, 4H), 1.90–1.80 (m, 4H), 1.76–1.66 (m, 3H), 1.64–1.42 (m, 8H), 1.39–1.30 (m, 1H), 1.20–1.12 (m, 1H), 1.09–1.01 (m, 2H), 0.99 (d, J = 6.5 Hz, 3H), 0.94–0.90 (m, 6H), 0.89–0.87 (m, 3H), 0.86–0.82 (m, 9H), 0.79 (d, J = 6.8 Hz, 3H), 0.76 (t, J = 7.4 Hz, 3H), 0.69 (t, J = 7.4 Hz, 3H), 0.50 (d, J = 6.8 Hz, 3H); LC main peak retention time: 6.57 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1315.7773, found: 1315.7745 (–2.1 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 658.3923, found: 658.3919 (–0.6 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 884.5241, found: 884.5244 (0.3 ppm).

CalA_R2A (15) $\text{C}_{67}\text{H}_{105}\text{N}_{14}\text{O}_{13}$ $M = 1314.67 \text{ g/mol}$

The linear precursor peptide of **15** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O ; solvent B: ACN) to give 3.2 mg (2.4 μmol) of **15** in a total yield of 2.4% (calculated in relation to initial resin capacity) as a white powder.

^1H NMR (400 MHz, DMSO- d_6) δ 8.77 (s, 1H), 8.47 (d, J = 6.7 Hz, 1H), 8.15 (s, 1H), 7.70 (d, J = 6.9 Hz, 1H), 7.64 (t, J = 6.2 Hz, 1H), 7.57–7.51 (m, 2H), 7.21–7.07 (m, 9H), 6.74 (d, J = 9.6 Hz, 1H), 5.82–5.71 (m, 1H), 4.63–4.55 (m, 1H), 4.54–4.47 (m, 1H), 4.31–4.22 (m, 3H), 3.94 (t, J = 8.8 Hz, 1H), 3.86–3.77 (m, 3H), 3.74 (t, J = 7.3 Hz, 1H), 3.63–3.55 (m, 3H), 3.54–3.45 (m, 1H), 3.43–3.38 (m, 1H), 3.30–3.22 (m, 3H), 3.01 (dd, J = 10.6, 6.9 Hz, 1H), 2.69–2.60 (m, 2H), 2.32–2.20 (m, 3H), 2.00–1.94 (m, 1H), 1.93–1.82 (m, 7H), 1.79–1.68 (m, 4H), 1.65–1.52 (m, 4H), 1.47–1.35 (m, 4H), 1.28 (d, J = 7.0 Hz, 3H), 1.23 (s, 1H), 1.16–1.12 (m, 1H), 1.11 (s, 2H), 0.95 (d, J = 6.4 Hz, 3H), 0.92–0.78 (m, 21H), 0.74–0.69 (m, 6H), 0.45 (d, J = 6.8 Hz, 3H), 0.43–0.38 (m, 3H); LC main peak retention time: 8.02 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1315.8137, found: 1315.8145 (0.6 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 658.4105, found: 658.4105 (0 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 884.5605, found: 884.5591 (–1.6 ppm).

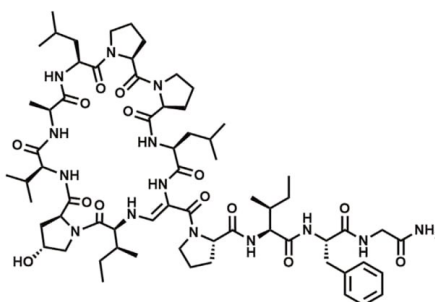
CalA_R3A (16)



The linear precursor peptide of **16** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 8.3 mg (6.2 μmol) of **16** in a total yield of 6.2% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.01 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1329.7929, found: 1329.7947 (1.4 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 665.4001, found: 665.4004 (0.5 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 898.5397, found: 898.5389 (−0.9 ppm).

CalA_R4A (17)

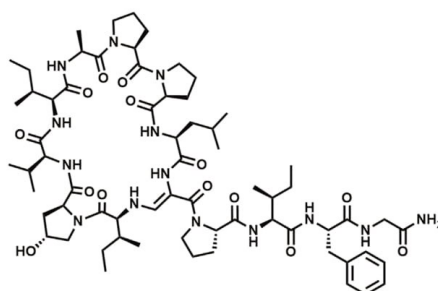


The linear precursor peptide of **17** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 21.5 mg (16.3 μmol) of **17** in a total yield of 16.3% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.91 (s, 1H), 8.68 (s, 1H), 8.30 (s, 1H), 8.20–8.12 (m, 1H), 7.50 (d, $J = 6.9$ Hz, 1H), 7.46 (d, $J = 8.8$ Hz, 1H), 7.36 (d, $J = 13.4$ Hz, 1H), 7.28–7.09 (m, 7H), 6.99–6.94 (m, 1H), 6.74 (d, $J = 10.0$ Hz, 1H), 5.84 (dd, $J = 13.2, 10.2$ Hz, 1H), 5.35 (d, $J = 3.1$ Hz, 1H), 4.65–4.57 (m, 1H), 4.57–4.52 (m, 1H), 4.41 (s, 1H), 4.31–4.21 (m, 4H), 4.12–4.02 (m, 2H), 3.97 (dd, $J = 7.3, 5.8$ Hz, 1H), 3.91 (dd, $J = 16.5, 7.1$ Hz, 1H), 3.79 (t, $J = 8.5$ Hz, 1H), 3.76–3.70 (m, 1H), 3.69–3.61 (m, 1H), 3.62–3.55 (m, 1H), 3.54–3.43 (m, 3H), 3.38–3.33 (m, 1H), 3.25–3.17 (m, 1H), 3.10 (dd, $J = 13.9, 2.9$ Hz, 1H), 3.04–2.97 (m, 1H), 2.68–2.55 (m, 2H), 2.33–2.20 (m, 3H), 2.11–2.05 (m, 1H), 2.02–1.95 (m, 1H), 1.94–1.80 (m, 7H), 1.78–1.63 (m, 5H), 1.63–1.51 (m, 4H), 1.50–1.35 (m, 4H), 1.31–1.22 (m, 1H), 1.14 (d, $J = 7.3$ Hz,



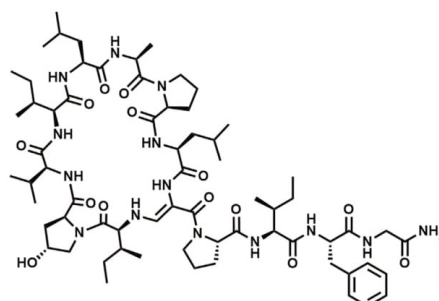
3H), 1.08–0.99 (m, 1H), 0.95 (d, $J = 6.5$ Hz, 3H), 0.89–0.77 (m, 18H), 0.70 (t, $J = 7.4$ Hz, 3H), 0.44 (t, $J = 7.4$ Hz, 3H), 0.38 (d, $J = 6.8$ Hz, 3H); LC main peak retention time: 6.66 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1315.7773, found: 1315.7780 (0.5 ppm); calculated for $[M+2H]^{2+}$: 658.3923, found: 658.3925 (0.3 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 884.5241, found: 884.5243 (0.2 ppm).

CalA_R5A (18)

The linear precursor peptide of **18** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 34.3 mg (26.1 μ mol) of **18** in a total yield of 26.1% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.02 (d, $J = 7.0$ Hz, 1H), 8.84 (s, 1H), 8.30 (s, 1H), 8.08 (t, $J = 6.2$ Hz, 1H), 7.54 (d, $J = 8.4$ Hz, 1H), 7.49 (d, $J = 6.6$ Hz, 1H), 7.32 (d, $J = 13.4$ Hz, 1H), 7.28 (d, $J = 8.9$ Hz, 1H), 7.25–7.10 (m, 6H), 7.01–6.94 (m, 2H), 5.85 (dd, $J = 13.2, 10.2$ Hz, 1H), 5.35 (d, $J = 3.1$ Hz, 1H), 4.69 (p, $J = 6.4$ Hz, 1H), 4.47–4.38 (m, 2H), 4.30–4.20 (m, 4H), 4.09–4.01 (m, 2H), 3.89 (dd, $J = 16.5, 7.0$ Hz, 1H), 3.83–3.75 (m, 1H), 3.74–3.62 (m, 4H), 3.57–3.49 (m, 2H), 3.48–3.42 (m, 2H), 3.31–3.21 (m, 1H), 3.11 (dd, $J = 13.8, 3.0$ Hz, 1H), 2.96 (dd, $J = 10.9, 6.9$ Hz, 1H), 2.69–2.57 (m, 2H), 2.31–2.16 (m, 3H), 2.13–2.05 (m, 1H), 2.04–1.98 (m, 1H), 1.96–1.81 (m, 6H), 1.80–1.59 (m, 6H), 1.54–1.42 (m, 2H), 1.42–1.30 (m, 3H), 1.28–1.19 (m, 1H), 1.16 (d, $J = 6.2$ Hz, 3H), 1.13–1.07 (m, 1H), 1.06–0.96 (m, 1H), 0.90 (d, $J = 6.2$ Hz, 3H), 0.85–0.70 (m, 23H), 0.38 (t, $J = 7.3$ Hz, 3H), 0.35 (d, $J = 6.8$ Hz, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 173.5, 173.0, 172.9, 172.1, 172.0, 171.8, 171.7, 171.5, 171.2, 171.2, 170.9, 167.8, 143.2, 138.1, 129.2, 127.9, 126.3, 98.4, 68.9, 64.1, 62.5, 61.9, 58.9, 56.7, 55.7, 50.7, 48.9, 47.2, 46.1, 42.1, 40.5, 38.4, 37.6, 36.2, 35.0, 29.5, 28.6, 27.2, 25.9, 25.6, 25.1, 24.2, 23.0, 20.7, 19.5, 19.2, 16.2, 15.1, 14.9, 14.3, 11.0, 10.7, 10.4; LC main peak retention time: 6.68 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1315.7773, found: 1315.7791 (1.4 ppm); calculated for $[M+2H]^{2+}$: 658.3923, found: 658.3927 (0.6 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 884.5241, found: 884.5246 (0.6 ppm).

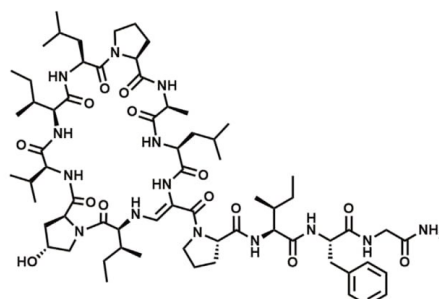
CalA_R6A (19)



The linear precursor peptide of **19** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 12.8 mg (9.6 μmol) of **19** in a total yield of 9.6% (calculated in relation to initial resin capacity) as a white powder.

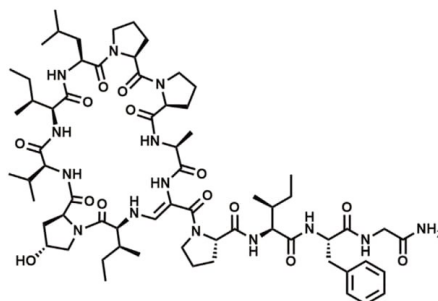
LC main peak retention time: 7.43 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1331.8086, found: 1331.8096 (0.8 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 666.4079, found: 666.4066 (−2.0 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 900.5554, found: 900.5546 (−0.9 ppm).

CalA_R7A (20)



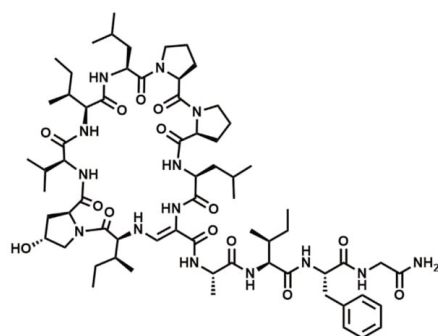
The linear precursor peptide of **20** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 8.1 mg (6.1 μmol) of **20** in a total yield of 6.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.42 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1331.8086, found: 1331.8097 (0.8 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 666.4079, found: 666.4081 (0.3 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 900.5554, found: 900.5558 (0.4 ppm).

**CalA_R8A (21)**

The linear precursor peptide of **21** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 11.0 mg (8.4 μ mol) of **21** in a total yield of 8.4% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.99 (d, *J* = 6.6 Hz, 1H), 8.81 (s, 1H), 8.32 (s, 1H), 8.11 (dd, *J* = 7.0, 5.3 Hz, 1H), 7.48 (d, *J* = 8.5 Hz, 1H), 7.44 (d, *J* = 6.4 Hz, 1H), 7.35 (d, *J* = 13.4 Hz, 1H), 7.27–7.12 (m, 7H), 6.99–6.95 (m, 1H), 6.89 (d, *J* = 9.5 Hz, 1H), 5.82 (dd, *J* = 13.3, 10.1 Hz, 1H), 5.36 (s, 1H), 4.55–4.46 (m, 2H), 4.41 (s, 1H), 4.30–4.21 (m, 4H), 4.10–4.02 (m, 2H), 3.90 (dd, *J* = 16.6, 7.1 Hz, 1H), 3.79–3.68 (m, 4H), 3.65–3.57 (m, 2H), 3.55–3.46 (m, 2H), 3.30–3.21 (m, 2H), 3.10 (dd, *J* = 13.8, 3.0 Hz, 1H), 2.99 (dd, *J* = 10.8, 6.9 Hz, 1H), 2.68–2.59 (m, 2H), 2.33–2.17 (m, 3H), 2.12–2.04 (m, 1H), 2.03–1.97 (m, 1H), 1.96–1.79 (m, 7H), 1.75–1.62 (m, 3H), 1.57–1.48 (m, 3H), 1.45 (d, *J* = 7.4 Hz, 3H), 1.42–1.33 (m, 3H), 1.27–1.10 (m, 4H), 1.09–0.97 (m, 1H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.89–0.85 (m, 1H), 0.84–0.75 (m, 18H), 0.72 (t, *J* = 7.4 Hz, 3H), 0.41 (t, *J* = 7.3 Hz, 3H), 0.36 (d, *J* = 6.7 Hz, 3H); LC main peak retention time: 6.83 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1315.7773, found: 1315.7780 (0.5 ppm); calculated for [M+2H]²⁺: 658.3923, found: 658.3925 (0.3 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 884.5241, found: 884.5244 (0.3 ppm).

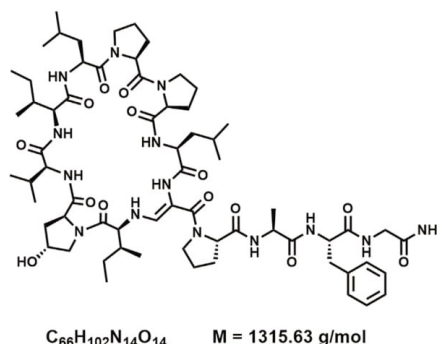
CalA_C1A (22)

The linear precursor peptide of **22** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 93 mg (69 μ mol, 30%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the

peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 2.4 mg (1.8 μ mol, 2.6%) of **22** in a total yield of 0.8% (calculated in relation to initial resin capacity) as a white powder.

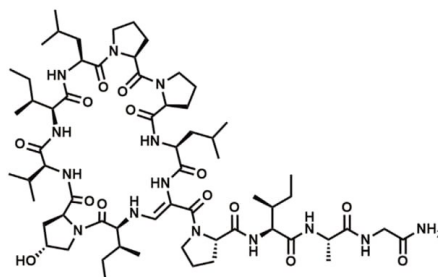
LC main peak retention time: 7.68 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1331.8086, found: 1331.8094 (0.6 ppm); calculated for $[M+2H]^{2+}$: 666.4079, found: 666.4071 (−1.2 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 926.5710, found: 926.5729 (2.1 ppm).

CalA_C2A (23)



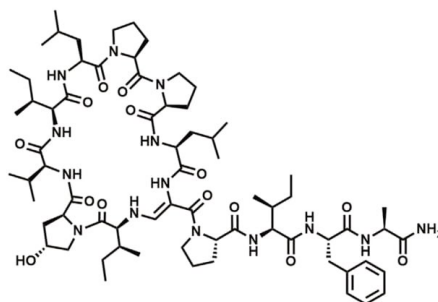
The linear precursor peptide of **23** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 3.4 mg (2.6 μ mol) of **23** in a total yield of 2.6% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.99 (s, 1H), 8.77 (s, 1H), 8.17 (s, 1H), 8.77 (dd, $J = 7.1, 5.2$ Hz, 1H), 7.81 (d, $J = 7.4$ Hz, 1H), 7.58 (d, $J = 9.2$ Hz, 1H), 7.36 (d, $J = 7.1$ Hz, 1H), 7.27 (d, $J = 13.3$ Hz, 1H), 7.23–7.19 (m, 3H), 7.11–7.08 (m, 3H), 7.03 (s, 1H), 6.76 (d, $J = 9.8$ Hz, 1H), 5.84–5.73 (m, 1H), 4.63 (q, $J = 6.6$ Hz, 1H), 4.56 (dd, $J = 10.0, 5.0$ Hz, 1H), 4.40 (s, 1H), 4.29–4.21 (m, 4H), 4.08–4.01 (m, 1H), 3.97–3.91 (m, 2H), 3.87 (dd, $J = 16.8, 7.2$ Hz, 1H), 3.83–3.76 (m, 2H), 3.71–3.61 (m, 3H), 3.52–3.45 (m, 2H), 3.36–3.30 (m, 1H), 3.28–3.19 (m, 1H), 3.16–3.09 (m, 1H), 3.03–2.96 (m, 1H), 2.75 (t, $J = 12.7$ Hz, 1H), 2.69–2.59 (m, 1H), 2.34–2.18 (m, 3H), 2.13–2.05 (m, 1H), 2.02–1.96 (m, 1H), 1.95–1.81 (m, 8H), 1.78–1.67 (m, 3H), 1.66–1.52 (m, 4H), 1.50–1.43 (m, 1H), 1.42–1.31 (m, 2H), 1.30–1.25 (m, 1H), 1.23 (s, 1H), 1.17–1.10 (m, 1H), 1.08 (d, $J = 7.4$ Hz, 3H), 0.95 (d, $J = 6.5$ Hz, 3H), 0.90 (d, $J = 6.4$ Hz, 3H), 0.87 (d, $J = 2.9$ Hz, 3H), 0.86 (d, $J = 3.0$ Hz, 3H), 0.84–0.79 (m, 9H), 0.77 (t, $J = 7.4$ Hz, 3H), 0.74 (d, $J = 7.2$ Hz, 3H), 0.62–0.52 (m, 1H), 0.27 (t, $J = 7.3$ Hz, 3H); LC main peak retention time: 6.66 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1315.7773, found: 1315.7785 (0.9 ppm); calculated for $[M+2H]^{2+}$: 658.3923, found: 658.3925 (0.3 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 926.5710, found: 926.5726 (1.7 ppm).

**CalA_C3A (24)**

The linear precursor peptide of **24** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.4 mg (1.1 μmol) of **24** in a total yield of 1.1% (calculated in relation to initial resin capacity) as a white powder.

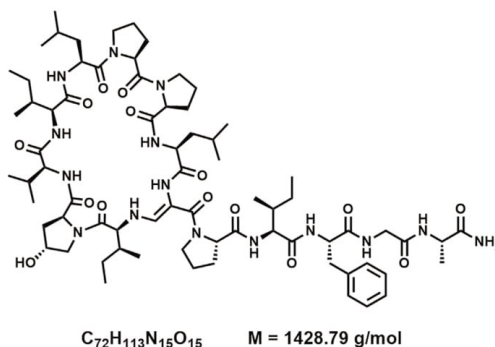
LC main peak retention time: 6.98 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1281.7929, found: 1281.7929 (0.0 ppm).

CalA_C4A (25)

The linear precursor peptide of **25** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 2.6 mg (3.6 μmol) of **25** in a total yield of 3.6% (calculated in relation to initial resin capacity) as a white powder.

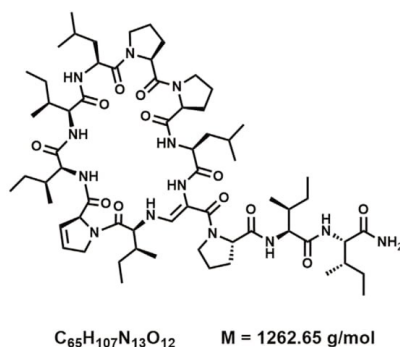
¹H NMR (400 MHz, DMSO-*d*₆) δ 8.98 (s, 1H), 8.77 (s, 1H), 8.13 (s, 1H), 7.72 (d, *J* = 7.3 Hz, 1H), 7.48 (d, *J* = 7.3 Hz, 1H), 7.42 (d, *J* = 8.5 Hz, 1H), 7.36 (d, *J* = 7.1 Hz, 1H), 7.26–7.20 (m, 2H), 7.18 (d, *J* = 13.3 Hz, 1H), 7.10–7.02 (m, 4H), 6.74 (d, *J* = 9.9 Hz, 1H), 6.68 (s, 1H), 5.78–5.66 (m, 1H), 4.64 (q, *J* = 6.8 Hz, 1H), 4.60–4.51 (m, 1H), 4.38 (s, 1H), 4.29–4.19 (m, 3H), 4.18–4.11 (m, 1H), 4.10–4.03 (m, 2H), 3.90–3.83 (m, 2H), 3.66–3.56 (m, 4H), 3.53–3.46 (m, 2H), 3.39–3.31 (m, 1H), 3.27–3.18 (m, 1H), 3.14–3.07 (m, 1H), 2.99 (t, *J* = 8.9 Hz, 1H), 2.81 (t, *J* = 12.7 Hz, 1H), 2.70–2.58 (m, 1H), 2.34–2.19 (m, 3H), 2.12–2.05 (m, 1H), 2.02–1.96 (m, 1H), 1.95–1.80 (m, 8H), 1.78–1.68 (m, 4H), 1.66–1.53 (m, 4H), 1.51–1.43 (m, 1H), 1.42–1.33 (m, 2H), 1.31 (d, *J* = 7.3 Hz, 3H), 1.28–1.25 (m, 1H), 1.23 (s, 1H), 1.20–1.06 (m, 3H), 0.96 (d, *J* = 6.5 Hz, 3H), 0.92 (d, *J* = 6.4 Hz, 3H), 0.88 (d, *J* = 2.2 Hz, 3H), 0.87 (d, *J* = 2.1 Hz, 3H), 0.84–0.79 (m, 9H), 0.77 (t, *J* = 7.5 Hz, 3H), 0.73 (d, *J* = 6.7 Hz, 3H), 0.70 (t, *J* = 7.4 Hz, 3H), 0.60–0.53 (m, 1H), 0.50 (d, *J* = 6.8 Hz,

3H), 0.26 (t, $J = 7.3$ Hz, 3H); LC main peak retention time: 7.82 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1371.8399, found: 1371.8411 (0.9 ppm); calculated for $[M+2H]^{2+}$: 686.4236, found: 686.4234 (−0.3 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 926.5710, found: 926.5709 (−0.1 ppm).

CalA_C5A (26)

The linear precursor peptide of **26** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 3.0 mg (2.1 μ mol) of **26** in a total yield of 2.1% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.04 (d, $J = 6.9$ Hz, 1H), 8.83 (s, 1H), 8.49 (t, $J = 6.0$ Hz, 1H), 8.34 (s, 1H), 8.22 (d, $J = 7.8$ Hz, 1H), 7.54 (d, $J = 13.3$ Hz, 1H), 7.47 (d, $J = 7.2$ Hz, 1H), 7.44 (d, $J = 9.4$ Hz, 1H), 7.27 (d, $J = 8.5$ Hz, 1H), 7.20–7.11 (m, 6H), 7.04 (s, 1H), 6.72 (d, $J = 10.1$ Hz, 1H), 5.96–5.87 (m, 1H), 5.36 (d, $J = 3.2$ Hz, 1H), 4.67–4.58 (m, 2H), 4.39 (s, 1H), 4.34–4.24 (m, 4H), 4.23–4.16 (m, 2H), 4.06 (t, $J = 9.4$ Hz, 1H), 4.00 (dd, $J = 15.9, 6.5$ Hz, 1H), 3.82–3.75 (m, 2H), 3.74–3.69 (m, 1H), 3.66–3.57 (m, 2H), 3.54–3.45 (m, 3H), 3.25–3.17 (m, 1H), 3.08 (d, $J = 13.5$ Hz, 1H), 3.02–2.94 (m, 1H), 2.72–2.62 (m, 1H), 2.61–2.52 (m, 1H), 2.33–2.21 (m, 3H), 2.13–2.05 (m, 1H), 2.02–1.97 (m, 1H), 1.96–1.83 (m, 7H), 1.82–1.72 (m, 3H), 1.62 (s, 6H), 1.52–1.37 (m, 6H), 1.24 (d, $J = 4.8$ Hz, 3H), 1.23–1.20 (m, 1H), 1.18–1.10 (m, 1H), 1.06–0.98 (m, 1H), 0.95 (d, $J = 6.3$ Hz, 3H), 0.88–0.76 (m, 24H), 0.73–0.67 (m, 4H), 0.45 (t, $J = 7.2$ Hz, 3H), 0.35 (d, $J = 6.7$ Hz, 3H); LC main peak retention time: 7.66 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1428.8613, found: 1428.8625 (0.8 ppm); calculated for $[M+2H]^{2+}$: 714.9343, found: 714.9346 (0.4 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 926.5710, found: 926.5725 (1.6 ppm).

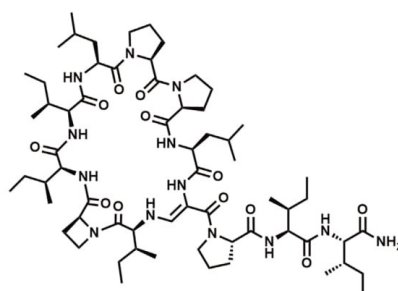
CalB_R2dPro (27)



The linear precursor peptide of **27** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 69 mg (54 μ mol, 54%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 0.5 mg (0.4 μ mol, 0.7%) of **27** in a total yield of 0.4% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.67 min; HRMS (ESI): m/z calculated for [M+H]⁺: 1262.8235, found: 1262.8218 (−1.3 ppm); calculated for [M+2H]²⁺: 631.9154, found: 631.9143 (−1.7 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment [M_{ring}+H]⁺: 922.5761, found: 922.5724 (−4.0 ppm).

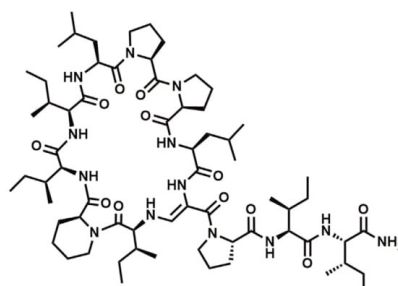
CalB_R2Aze (**28**)



The linear precursor peptide of **28** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 55 mg (43 μ mol, 43%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 8.4 mg (6.7 μ mol, 15.5%) of **28** in a total yield of 6.7% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.00 (d, $J = 7.1$ Hz, 1H), 8.50 (d, $J = 6.6$ Hz, 1H), 8.22 (s, 1H), 7.59 (d, $J = 5.8$ Hz, 1H), 7.57 (d, $J = 3.0$ Hz, 1H), 7.20 (d, $J = 9.7$ Hz, 1H), 7.12 (s, 1H), 7.02 (d, $J = 13.2$ Hz, 1H), 6.91 (s, 1H), 6.73 (d, $J = 9.8$ Hz, 1H), 5.55–5.45 (m, 1H), 4.66–4.59 (m, 2H), 4.55–4.46 (m, 1H), 4.39–4.31 (m, 1H), 4.28–4.18 (m, 3H), 4.12–4.04 (m, 2H), 3.99–3.92 (m, 1H), 3.86–3.80 (m, 1H), 3.67–3.61 (m, 1H), 3.60–3.53 (m, 2H), 3.51–3.44 (m, 2H), 3.35–3.26 (m, 1H), 3.23–3.13 (m, 2H), 2.61–2.53 (m, 1H), 2.46–2.38 (m, 1H), 2.30–2.16 (m, 3H), 2.01–1.94 (m, 1H), 1.93–1.76 (m, 8H), 1.74–1.65 (m, 3H), 1.65–1.56 (m, 3H), 1.52–1.36 (m, 8H), 1.35–1.22 (m, 2H), 1.21–1.11 (m, 2H), 1.08–0.96 (m, 3H), 0.92–0.74 (m, 42H); LC main peak retention time: 8.77 min; HRMS (ESI): m/z calculated for [M+H]⁺: 1250.8235, found: 1250.8229 (−0.5 ppm); calculated for [M+2H]²⁺: 625.9154, found: 625.9148 (−1.0 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment [M_{ring}+H]⁺: 910.5761, found: 910.5733 (−3.1 ppm).

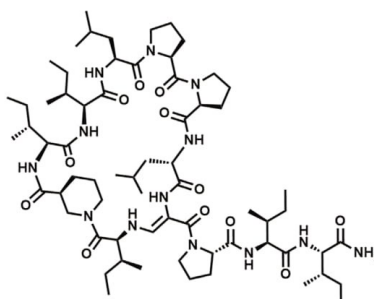
CalB_R2Pip (29)



The linear precursor peptide of **29** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 38 mg (29 μmol , 29%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 8 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 2.0 mg (1.6 μmol , 5.3%) of **29** in a total yield of 1.6% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.85 (d, *J* = 7.2 Hz, 1H), 8.61 (d, *J* = 6.8 Hz, 1H), 8.24 (s, 1H), 7.57 (d, *J* = 10.2 Hz, 1H), 7.22 (d, *J* = 9.6 Hz, 1H), 7.16 (s, 1H), 7.09–7.00 (m, 2H), 6.90 (s, 1H), 6.67 (d, *J* = 10.1 Hz, 1H), 5.70 (dd, *J* = 13.2, 9.9 Hz, 1H), 4.64 (q, *J* = 6.7 Hz, 1H), 4.55 (td, *J* = 10.3, 3.6 Hz, 1H), 4.34–4.19 (m, 3H), 4.16–4.03 (m, 4H), 3.98–3.89 (m, 1H), 3.83–3.75 (m, 1H), 3.62–3.42 (m, 5H), 3.20–3.06 (m, 3H), 2.61–2.52 (m, 1H), 2.35–2.17 (m, 4H), 1.96–1.82 (m, 9H), 1.81–1.71 (m, 3H), 1.68–1.55 (m, 6H), 1.53–1.37 (m, 9H), 1.31–1.19 (m, 3H), 1.16–0.97 (m, 4H), 0.91–0.69 (m, 42H); LC main peak retention time: 10.36 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1278.8548, found: 1278.8534 (–1.1 ppm); calculated for [M+2H]²⁺: 639.9311, found: 639.9300 (–1.7 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 938.6074, found: 938.6056 (–1.9 ppm).

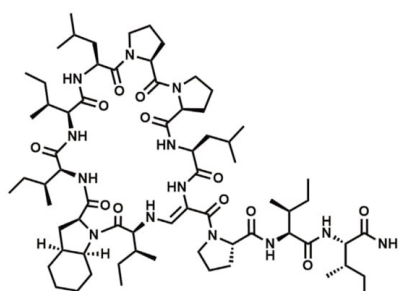
CalB_R2Nip (30)



The linear precursor peptide of **30** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 21 mg (16 μmol , 16%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.4 mg (1.1 μmol , 6.6%) of **30** in a total yield of 1.1% (calculated in relation to initial resin capacity) as a white powder.

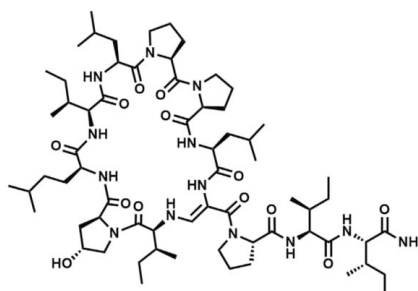


LC main peak retention time: 7.11 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1278.8548, found: 1278.8551 (0.2 ppm); calculated for $[M+2H]^{2+}$: 639.9311, found: 639.9309 (−0.3 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 938.6074, found: 938.6057 (−1.8 ppm).

CalB_R2Oic (31)

The linear precursor peptide of **31** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 61 mg (45 μ mol, 45%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 26 mg (20 μ mol, 44%) of **31** in a total yield of 19.8% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.84 (d, J = 7.0 Hz, 1H), 8.59 (d, J = 6.6 Hz, 1H), 8.27 (s, 1H), 7.60 (d, J = 3.4 Hz, 1H), 7.58 (s, 1H), 7.29 (d, J = 9.6 Hz, 1H), 7.12 (d, J = 13.4 Hz, 1H), 7.04 (s, 1H), 7.02 (s, 1H), 6.67 (d, J = 9.9 Hz, 1H), 5.62–5.53 (m, 1H), 4.65–4.57 (m, 1H), 4.57–4.48 (m, 1H), 4.31–4.20 (m, 2H), 4.20–4.14 (m, 1H), 4.14–4.01 (m, 3H), 3.95 (t, J = 8.7 Hz, 1H), 3.81 (t, J = 10.1 Hz, 1H), 3.77–3.71 (m, 1H), 3.62–3.40 (m, 4H), 3.30–3.24 (m, 1H), 3.23–3.13 (m, 1H), 3.11–3.04 (m, 1H), 2.69–2.57 (m, 1H), 2.37–2.17 (m, 3H), 2.05–1.96 (m, 2H), 1.95–1.82 (m, 8H), 1.81–1.55 (m, 10H), 1.54–1.38 (m, 8H), 1.33–0.98 (m, 10H), 0.92–0.69 (m, 44H); LC main peak retention time: 14.35 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1318.8861, found: 1318.8862 (0.1 ppm); calculated for $[M+2H]^{2+}$: 659.9467, found: 659.9461 (−0.9 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 978.6387, found: 978.6381 (−0.6 ppm).

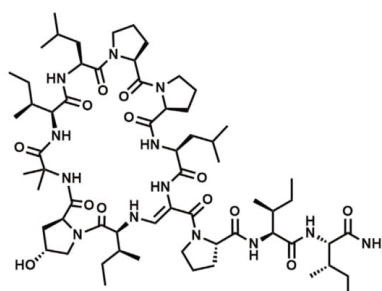
CalB_R3hLeu (32)

The linear precursor peptide of **32** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA),

25 mg (19 μ mol, 19%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 2.1 mg (1.6 μ mol, 8.5%) of **32** in a total yield of 1.6% (calculated in relation to initial resin capacity) as a white powder.

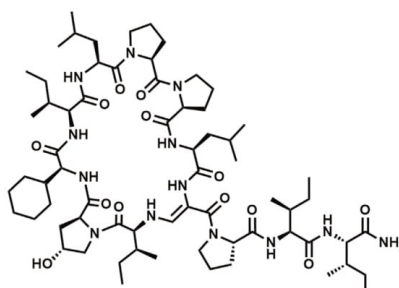
¹H NMR (400 MHz, DMSO-*d*₆) δ 8.87 (d, *J* = 6.8 Hz, 1H), 8.30 (s, 1H), 8.21 (d, *J* = 8.1 Hz, 1H), 7.60 (d, *J* = 10.0 Hz, 1H), 7.45 (d, *J* = 7.3 Hz, 1H), 7.23 (d, *J* = 9.6 Hz, 1H), 7.15 (s, 1H), 7.06 (d, *J* = 13.2 Hz, 1H), 6.97 (s, 1H), 6.67 (d, *J* = 9.9 Hz, 1H), 5.61 (dd, *J* = 13.2, 10.1 Hz, 1H), 4.66 (q, *J* = 6.8 Hz, 1H), 4.52 (td, *J* = 10.3, 3.6 Hz, 1H), 4.42 (s, 1H), 4.29–4.20 (m, 3H), 4.19–4.13 (m, 1H), 4.13–4.05 (m, 1H), 4.06–3.92 (m, 3H), 3.78–3.68 (m, 2H), 3.62–3.49 (m, 5H), 3.21–3.11 (m, 1H), 2.36–2.17 (m, 3H), 2.13–1.99 (m, 3H), 1.96–1.77 (m, 9H), 1.73–1.55 (m, 6H), 1.54–1.38 (m, 8H), 1.37–1.29 (m, 1H), 1.27–1.20 (m, 2H), 1.19–1.06 (m, 4H), 1.05–0.94 (m, 2H), 0.93–0.69 (m, 42H); LC main peak retention time: 8.00 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1294.8497, found: 1294.8481 (–1.2 ppm); calculated for [M+2H]²⁺: 647.9285, found: 647.9274 (–1.7 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 954.6023, found: 954.5990 (–3.5 ppm).

CalB_R3Aib (**33**)



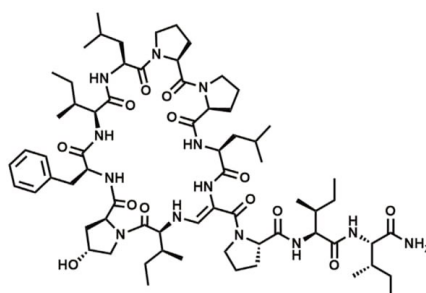
The linear precursor peptide of **33** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 42 mg (33 μ mol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 2.6 mg (2.1 μ mol, 6.3%) of **33** in a total yield of 2.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.16 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1252.8028, found: 1252.8015 (–1.0 ppm); calculated for [M+2H]²⁺: 626.9050, found: 626.9044 (–1.0 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 912.5554, found: 912.5514 (–4.4 ppm).

**CalB_R3Chg (34)**

The linear precursor peptide of **34** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 40 mg (31 μmol, 31%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 6.8 mg (5.1 μmol, 16.5%) of **34** in a total yield of 5.1% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.02 (d, *J* = 7.0 Hz, 1H), 8.86 (d, *J* = 6.6 Hz, 1H), 8.29 (s, 1H), 7.59 (d, *J* = 10.1 Hz, 1H), 7.36 (d, *J* = 7.0 Hz, 1H), 7.24 (d, *J* = 9.7 Hz, 1H), 7.17 (s, 1H), 7.05 (d, *J* = 13.3 Hz, 1H), 6.97 (s, 1H), 6.65 (d, *J* = 10.0 Hz, 1H), 5.78–5.67 (m, 1H), 5.41 (s, 1H), 4.66–4.59 (m, 1H), 4.58–4.50 (m, 1H), 4.41 (s, 1H), 4.29–4.21 (m, 3H), 4.19–4.08 (m, 3H), 4.08–4.01 (m, 1H), 4.00–3.91 (m, 1H), 3.84–3.76 (m, 2H), 3.70–3.43 (m, 6H), 3.21–3.11 (m, 1H), 3.11–3.03 (m, 1H), 2.44–2.34 (m, 1H), 2.35–2.17 (m, 3H), 2.14–2.03 (m, 1H), 2.02–1.95 (m, 1H), 1.95–1.82 (m, 8H), 1.82–1.55 (m, 10H), 1.52–1.37 (m, 8H), 1.33–1.21 (m, 3H), 1.20–1.08 (m, 4H), 1.05–0.94 (m, 3H), 0.92–0.68 (m, 36H); LC main peak retention time: 8.88 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1306.8497, found: 1306.8494 (–0.2 ppm); calculated for [M+2H]²⁺: 653.9285, found: 653.9279 (–0.9 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 966.6023, found: 966.5992 (–3.2 ppm).

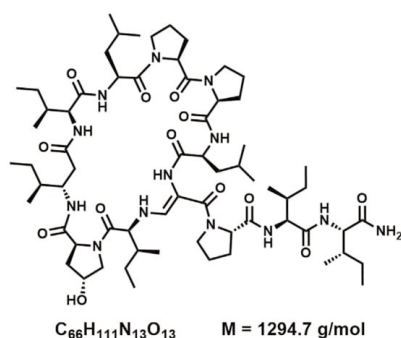
CalB_R3F (35)

The linear precursor peptide of **35** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 37 mg (28 μmol, 28%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was

shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 8.2 mg (6.2 μ mol, 22.4%) of **35** in a total yield of 6.2% (calculated in relation to initial resin capacity) as a white powder.

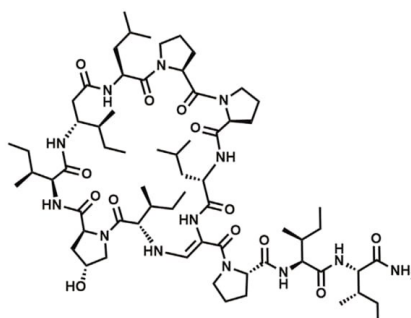
¹H NMR (400 MHz, DMSO-*d*₆) δ 8.79 (d, *J* = 6.8 Hz, 1H), 8.27 (s, 1H), 8.24 (d, *J* = 8.4 Hz, 1H), 7.58 (d, *J* = 10.1 Hz, 1H), 7.50 (d, *J* = 7.4 Hz, 1H), 7.36–7.28 (m, 2H), 7.27–7.20 (m, 2H), 7.19–7.16 (m, 1H), 7.15 (s, 1H), 7.04 (d, *J* = 13.2 Hz, 1H), 6.98 (s, 1H), 6.64 (d, *J* = 9.9 Hz, 1H), 5.67–5.56 (m, 1H), 5.33 (s, 1H), 4.68 (q, *J* = 6.8 Hz, 1H), 4.51 (td, *J* = 10.4, 3.6 Hz, 1H), 4.40 (s, 1H), 4.27–4.20 (m, 2H), 4.19–4.06 (m, 3H), 4.05–3.93 (m, 3H), 3.70–3.64 (m, 1H), 3.60–3.40 (m, 6H), 3.20–3.11 (m, 1H), 3.11–3.04 (m, 1H), 2.32–2.16 (m, 3H), 2.01–1.95 (m, 1H), 1.94–1.82 (m, 8H), 1.79–1.52 (m, 11H), 1.51–1.39 (m, 6H), 1.39–1.31 (m, 1H), 1.29–1.18 (m, 2H), 1.17–1.08 (m, 1H), 1.06–0.96 (m, 2H), 0.94–0.69 (m, 36H); LC main peak retention time: 8.00 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1314.8184, found: 1314.8184 (0.0 ppm); calculated for [M+2H]²⁺: 657.9129, found: 657.9125 (−0.6 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 974.5710, found: 974.5693 (−1.7 ppm).

CalB_R3 β -hIle (**36**)



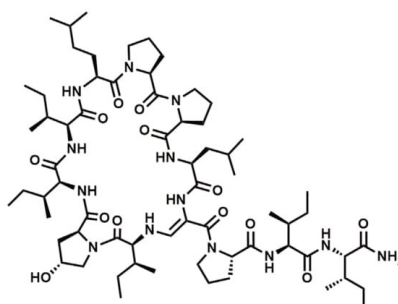
The linear precursor peptide of **36** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 51 mg (39 μ mol, 39%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 3.7 mg (2.9 μ mol, 7.4%) of **36** in a total yield of 2.9% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.14 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1294.8497, found: 1294.8488 (−0.7 ppm); calculated for [M+2H]²⁺: 647.9285, found: 647.9277 (−1.2 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 954.6023, found: 954.5994 (−3.0 ppm).

**CalB_R4β-hlle (37)**

The linear precursor peptide of **37** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 44 mg (33 μmol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 0.9 mg (0.7 μmol, 2.1%) of **37** in a total yield of 0.7% (calculated in relation to initial resin capacity) as a white powder.

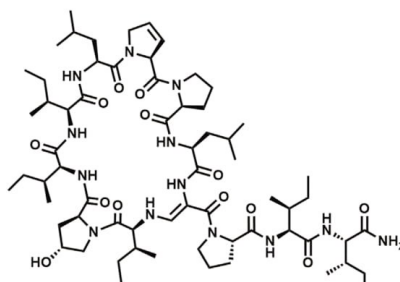
LC main peak retention time: 7.68 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1294.8497, found: 1294.8492 (−0.4 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 647.9285, found: 647.9277 (−1.2 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 954.6023, found: 954.5995 (−2.9 ppm).

CalB_R5hLeu (38)

The linear precursor peptide of **38** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 33 mg (25 μmol, 25%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 8 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 7.8 mg (6.0 μmol, 23.7%) of **38** in a total yield of 6.0% (calculated in relation to initial resin capacity) as a white powder.

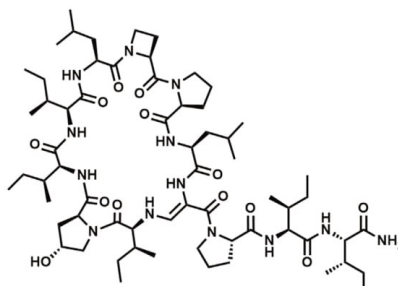
¹H NMR (400 MHz, DMSO-*d*₆) δ 8.96 (d, *J* = 6.9 Hz, 1H), 8.82 (s, 1H), 8.29 (s, 1H), 7.59 (d, *J* = 10.0 Hz, 1H), 7.33 (d, *J* = 7.0 Hz, 1H), 7.23 (d, *J* = 9.7 Hz, 1H), 7.14 (s, 1H), 7.05 (d, *J* = 13.3 Hz, 1H), 6.95 (s, 1H), 6.73 (d, *J* = 9.8 Hz, 1H), 5.68 (dd, *J* = 13.2, 10.1 Hz, 1H),

5.39 (d, $J = 3.0$ Hz, 1H), 4.59 (q, $J = 6.8$ Hz, 1H), 4.52 (td, $J = 10.2, 3.3$ Hz, 1H), 4.41 (s, 1H), 4.28–4.19 (m, 3H), 4.15–4.00 (m, 3H), 3.93 (t, $J = 10.0$ Hz, 1H), 3.82–3.75 (m, 2H), 3.69–3.63 (m, 1H), 3.61–3.48 (m, 3H), 3.47–3.38 (m, 2H), 3.21–3.12 (m, 1H), 3.12–3.03 (m, 1H), 2.33–2.17 (m, 3H), 2.11–2.04 (m, 1H), 2.03–1.94 (m, 2H), 1.94–1.75 (m, 9H), 1.73–1.56 (m, 4H), 1.55–1.37 (m, 10H), 1.28–1.10 (m, 5H), 1.06–0.94 (m, 3H), 0.91–0.69 (m, 42H); LC main peak retention time: 8.32 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1294.8497, found: 1294.8497 (0.0 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9279 (−0.9 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 954.6023, found: 954.6000 (−2.4 ppm).

CalB_R6dPro (39)

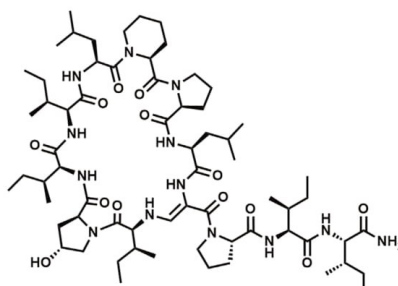
The linear precursor peptide of **39** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 23 mg (18 μmol, 18%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 5.6 mg (4.4 μmol, 25.1%) of **39** in a total yield of 4.4% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.75 (d, $J = 7.0$ Hz, 1H), 8.59 (d, $J = 6.8$ Hz, 1H), 8.32 (s, 1H), 7.63 (d, $J = 10.1$ Hz, 1H), 7.35 (d, $J = 7.2$ Hz, 1H), 7.25–7.17 (m, 2H), 7.06 (d, $J = 13.3$ Hz, 1H), 6.90 (s, 1H), 6.61 (d, $J = 9.9$ Hz, 1H), 6.14–6.09 (m, 1H), 5.85–5.80 (m, 1H), 5.68 (dd, $J = 13.2, 10.0$ Hz, 1H), 4.82–4.77 (m, 1H), 4.72 (q, $J = 6.8$ Hz, 1H), 4.55 (td, $J = 10.4, 3.7$ Hz, 1H), 4.43–4.35 (m, 2H), 4.35–4.27 (m, 1H), 4.27–4.19 (m, 2H), 4.14–4.06 (m, 3H), 3.95 (t, $J = 10.0$ Hz, 1H), 3.89 (t, $J = 7.6$ Hz, 1H), 3.77–3.72 (m, 1H), 3.69–3.63 (m, 1H), 3.57–3.49 (m, 1H), 3.29–3.21 (m, 2H), 3.20–3.12 (m, 1H), 3.11–3.05 (m, 1H), 2.48–2.41 (m, 1H), 2.31–2.19 (m, 2H), 2.09–2.01 (m, 1H), 1.93–1.81 (m, 5H), 1.80–1.59 (m, 7H), 1.56–1.38 (m, 9H), 1.38–1.30 (m, 1H), 1.23 (s, 1H), 1.19–1.08 (m, 2H), 1.06–0.95 (m, 3H), 0.94–0.70 (m, 42H); LC main peak retention time: 7.83 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1278.8184, found: 1278.8171 (−1.0 ppm); calculated for $[M+2H]^{2+}$: 639.9129, found: 639.9119 (−1.6 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 938.5710, found: 938.5702 (−0.9 ppm).

**CalB_R6Aze (40)**

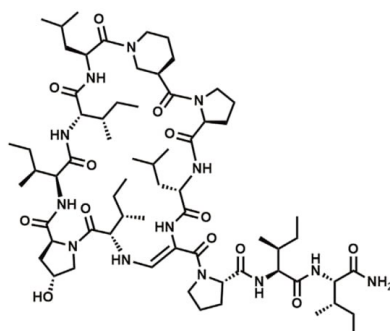
The linear precursor peptide of **40** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 30 mg (23 μmol, 23%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.1 mg (0.9 μmol, 3.7%) of **40** in a total yield of 0.9% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.96 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1266.8184, found: 1266.8150 (−2.7 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 633.9129, found: 633.9111 (−2.8 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 926.5710, found: 926.5690 (−2.2 ppm).

CalB_R6Pip (41)

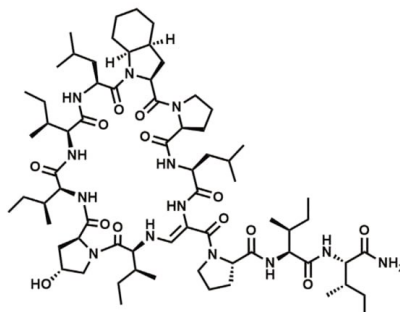
The linear precursor peptide of **41** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 57 mg (44 μmol, 44%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.1 mg (0.8 μmol, 2.0%) of **41** in a total yield of 0.8% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.84 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1294.8497, found: 1294.8492 (−0.4 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 647.9285, found: 647.9285 (−0.0 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 954.6023, found: 954.6011 (−1.3 ppm).

CalB_R6Nip (42)

The linear precursor peptide of **42** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 30 mg (23 μmol, 23%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.1 mg (0.9 μmol, 3.8%) of **42** in a total yield of 0.9% (calculated in relation to initial resin capacity) as a white powder.

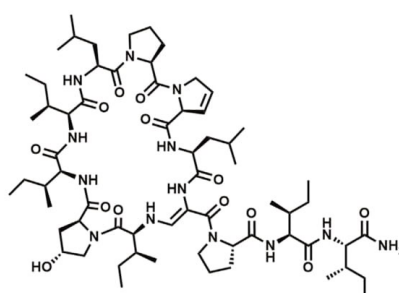
LC main peak retention time: 6.68 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1294.8497, found: 1294.8494 (−0.2 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9282 (−0.5 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 954.6023, found: 954.6000 (−2.4 ppm).

CalB_R6Oic (43)

The linear precursor peptide of **43** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 47 mg (35 μmol, 35%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.0 mg (0.7 μmol, 2.2%) of **43** in a total yield of 0.7% (calculated in relation to initial resin capacity) as a white powder.

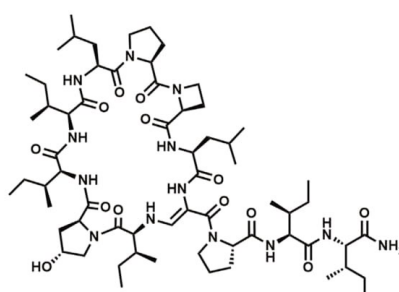


LC main peak retention time: 10.57 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1334.8810, found: 1334.8797 (−1.0 ppm); calculated for $[M+2H]^{2+}$: 667.9442, found: 667.9469 (4.0 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{ring}+H]^+$: 994.6336, found: 994.6319 (−1.7 ppm).

CalB_R7dPro (44)

The linear precursor peptide of **44** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 25 mg (19 μ mol, 19%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 3.5 mg (2.7 μ mol, 14.5%) of **44** in a total yield of 2.7% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.03 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1278.8184, found: 1278.8185 (0.1 ppm); calculated for $[M+2H]^{2+}$: 639.9129, found: 639.9124 (−0.8 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{ring}+H]^+$: 938.5710, found: 938.5672 (−4.0 ppm).

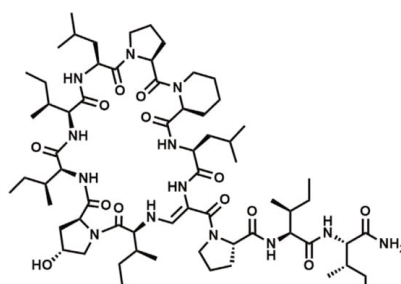
CalB_R7Aze (45)

The linear precursor peptide of **45** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 31 mg (24 μ mol, 24%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent

B: ACN) to give 2.1 mg (1.7 μ mol, 7.0%) of **45** in a total yield of 1.7% (calculated in relation to initial resin capacity) as a white powder.

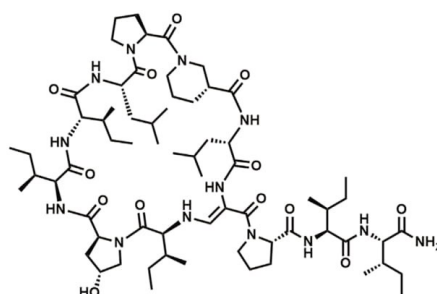
^1H NMR (400 MHz, DMSO- d_6) δ 8.98 (d, J = 7.0 Hz, 1H), 8.84 (d, J = 6.6 Hz, 1H), 8.21 (s, 1H), 7.56 (d, J = 10.1 Hz, 1H), 7.29 (d, J = 7.3 Hz, 1H), 7.20 (d, J = 9.8 Hz, 1H), 7.17 (s, 1H), 7.08 (d, J = 5.6 Hz, 1H), 7.05 (d, J = 8.4 Hz, 1H), 6.95 (s, 1H), 5.86 (dd, J = 13.2, 10.0 Hz, 1H), 5.39 (d, J = 3.1 Hz, 1H), 4.71–4.63 (m, 2H), 4.59 (dd, J = 9.3, 6.1 Hz, 1H), 4.43–4.39 (m, 1H), 4.38–4.32 (m, 1H), 4.26–4.19 (m, 2H), 4.18–4.08 (m, 2H), 4.02–3.92 (m, 2H), 3.87 (t, J = 8.4 Hz, 1H), 3.83 (d, J = 7.3 Hz, 1H), 3.78 (d, J = 11.7 Hz, 1H), 3.64 (dd, J = 11.1, 3.6 Hz, 1H), 3.56–3.40 (m, 2H), 3.06 (dd, J = 11.1, 6.9 Hz, 1H), 3.02–2.95 (m, 1H), 2.65–2.57 (m, 1H), 2.34–2.23 (m, 2H), 2.11–2.00 (m, 2H), 1.99–1.95 (m, 1H), 1.94–1.82 (m, 6H), 1.82–1.72 (m, 4H), 1.71–1.60 (m, 3H), 1.56–1.37 (m, 8H), 1.35–1.27 (m, 1H), 1.23 (s, 1H), 1.18–1.09 (m, 2H), 1.08–0.96 (m, 3H), 0.94–0.71 (m, 42H); LC main peak retention time: 7.72 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1266.8184, found: 1266.8172 (–0.9 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 633.9129, found: 633.9117 (–1.9 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 926.5710, found: 926.5674 (–3.9 ppm).

CalB_R7Pip (46)



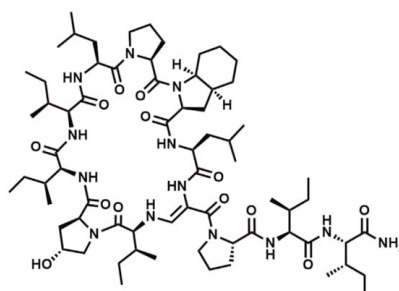
The linear precursor peptide of **46** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 35 mg (27 μ mol, 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O ; solvent B: ACN) to give 1.3 mg (1.0 μ mol, 3.8%) of **46** in a total yield of 1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.30 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1294.8497, found: 1294.8487 (–0.8 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 647.9285, found: 947.9277 (–1.2 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 954.6023, found: 954.5999 (–2.5 ppm).

**CalB_R7Nip (47)**

The linear precursor peptide of **47** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 32 mg (24 μmol, 24%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 0.8 mg (0.6 μmol, 2.6%) of **47** in a total yield of 0.6% (calculated in relation to initial resin capacity) as a white powder.

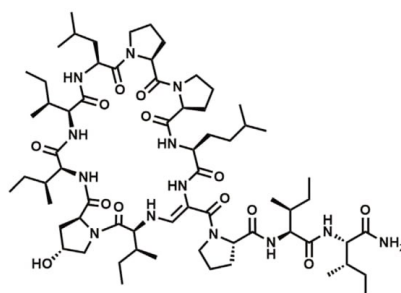
LC main peak retention time: 6.64 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1294.8497, found: 1294.8482 (−1.2 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 647.9285, found: 647.9276 (−1.4 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 954.6023, found: 954.5998 (−2.6 ppm).

CalB_R7Oic (48)

The linear precursor peptide of **48** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 47 mg (34 μmol, 34%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 4.9 mg (3.7 μmol, 10.7%) of **48** in a total yield of 3.7% (calculated in relation to initial resin capacity) as a white powder.

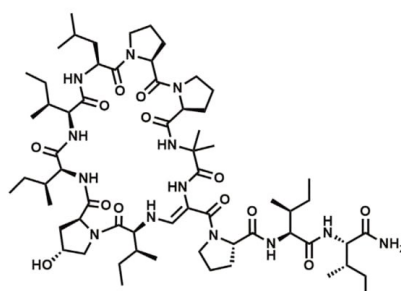
¹H NMR (400 MHz, DMSO-*d*₆) δ 9.05 (d, J = 7.0 Hz, 1H), 8.95 (d, J = 6.3 Hz, 1H), 8.26 (s, 1H), 7.56 (d, J = 10.1 Hz, 1H), 7.48 (d, J = 6.9 Hz, 1H), 7.25–7.16 (m, 2H), 7.03 (d, J = 13.2 Hz, 1H), 6.95 (s, 1H), 6.62 (d, J = 10.3 Hz, 1H), 5.92–5.81 (m, 1H), 5.41 (d, J = 2.9 Hz, 1H), 4.55 (td, J = 10.4, 3.4 Hz, 1H), 4.47–4.37 (m, 3H), 4.29–4.19 (m, 2H), 4.18–4.05 (m, 3H), 3.94 (t, J = 10.2 Hz, 1H), 3.89–3.83 (m, 1H), 3.82–3.72 (m, 2H), 3.71–3.60 (m, 2H), 3.49 (t, J = 9.1 Hz, 1H), 3.19–3.01 (m, 2H), 2.59–2.52 (m, 1H), 2.40–2.22 (m, 3H), 2.18–2.00 (m, 3H),

1.96–1.82 (m, 6H), 1.80–1.55 (m, 10H), 1.52–1.35 (m, 10H), 1.32–1.10 (m, 7H), 1.07–0.93 (m, 3H), 0.92–0.68 (m, 42H); LC main peak retention time: 12.01 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1334.8810, found: 1334.8812 (0.1 ppm); calculated for $[M+2H]^{2+}$: 667.9442, found: 667.9436 (−0.9 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 994.6336, found: 994.6326 (−1.0 ppm).

CalB_R8hLeu (49)

The linear precursor peptide of **49** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 43 mg (33 μmol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 7.5 mg (5.8 μmol, 17.8%) of **49** in a total yield of 5.8% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.00 (d, $J = 6.9 \text{ Hz}$, 1H), 8.87 (d, $J = 6.3 \text{ Hz}$, 1H), 8.28 (s, 1H), 7.58 (d, $J = 10.1 \text{ Hz}$, 1H), 7.40 (d, $J = 6.8 \text{ Hz}$, 1H), 7.24 (d, $J = 9.7 \text{ Hz}$, 1H), 7.15 (s, 1H), 7.06 (d, $J = 13.3 \text{ Hz}$, 1H), 6.97 (s, 1H), 6.64 (d, $J = 9.9 \text{ Hz}$, 1H), 5.70 (dd, $J = 13.3, 10.1 \text{ Hz}$, 1H), 5.40 (s, 1H), 4.62–4.54 (m, 2H), 4.47–4.36 (m, 2H), 4.29–4.21 (m, 3H), 4.18–4.13 (m, 1H), 4.11–4.01 (m, 2H), 3.95 (t, $J = 10.1 \text{ Hz}$, 1H), 3.81–3.72 (m, 2H), 3.69–3.55 (m, 3H), 3.54–3.45 (m, 2H), 3.29–3.22 (m, 1H), 3.20–3.10 (m, 1H), 3.10–3.03 (m, 1H), 2.34–2.19 (m, 3H), 2.12–1.96 (m, 3H), 1.97–1.82 (m, 8H), 1.82–1.55 (m, 5H), 1.53–1.34 (m, 10H), 1.30–1.21 (m, 2H), 1.20–1.10 (m, 3H), 1.07–0.94 (m, 2H), 0.92–0.68 (m, 42H); LC main peak retention time: 8.13 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1294.8497, found: 1294.8484 (−1.0 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9275 (−1.5 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 954.6023, found: 954.5991 (−3.4 ppm).

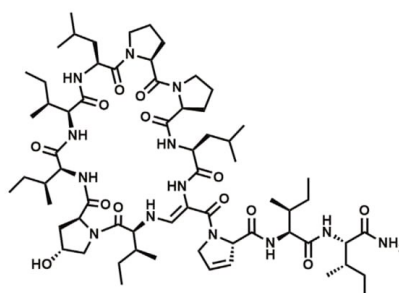
CalB_R8Aib (50)



The linear precursor peptide of **50** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 56 mg (44 μmol, 44%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 0.8 mg (0.6 μmol, 1.5%) of **50** in a total yield of 0.6% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.44 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1252.8028, found: 1252.8011 (−1.4 ppm); calculated for $[M+2H]^{2+}$: 626.9050, found: 626.9041 (−1.4 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{ring}+H]^+$: 912.5554, found: 912.5525 (−3.2 ppm).

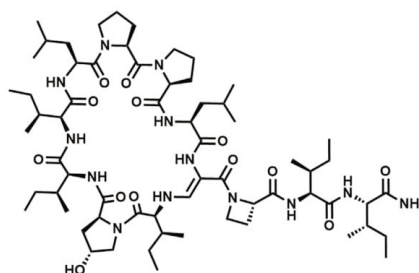
CalB_C1dPro (**51**)



The linear precursor peptide of **51** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 29 mg (22 μmol, 22%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 2.5 mg (2.0 μmol, 8.9%) of **51** in a total yield of 2.0% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.01 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1278.8184, found: 1278.8171 (−1.0 ppm); calculated for $[M+2H]^{2+}$: 639.9129, found: 639.9121 (−1.3 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5830 (−3.8 ppm).

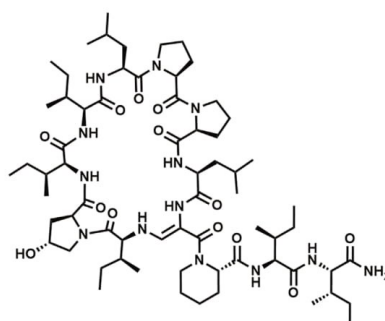
CalB_C1Aze (**52**)



The linear precursor peptide of **52** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 50 mg (39 μ mol, 39%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 3.2 mg (2.5 μ mol, 6.5%) of **52** in a total yield of 2.5% (calculated in relation to initial resin capacity) as a white powder.

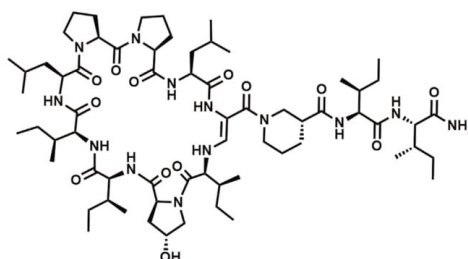
¹H NMR (400 MHz, DMSO-*d*₆) δ 8.94 (s, 1H), 8.83 (s, 1H), 7.91 (s, 1H), 7.82 (d, *J* = 9.3 Hz, 1H), 7.48 (d, *J* = 9.0 Hz, 1H), 7.38 (d, *J* = 6.9 Hz, 1H), 7.19 (d, *J* = 13.3 Hz, 1H), 7.08 (s, 1H), 6.97 (s, 1H), 6.80 (d, *J* = 10.0 Hz, 1H), 5.66–5.56 (m, 1H), 5.40 (d, *J* = 3.2 Hz, 1H), 4.65–4.46 (m, 3H), 4.40 (s, 1H), 4.27–4.15 (m, 3H), 4.11–4.05 (m, 1H), 4.05–3.98 (m, 1H), 3.98–3.90 (m, 2H), 3.88–3.73 (m, 3H), 3.72–3.65 (m, 1H), 3.64–3.53 (m, 2H), 3.52–3.42 (m, 1H), 3.27–3.19 (m, 1H), 3.13–3.05 (m, 1H), 2.44–2.32 (m, 1H), 2.31–2.17 (m, 2H), 2.13–1.68 (m, 12H), 1.66–1.33 (m, 11H), 1.32–1.21 (m, 2H), 1.20–0.93 (m, 5H), 0.92–0.71 (m, 42H); LC main peak retention time: 7.60 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1266.8184, found: 1266.8185 (0.1 ppm); calculated for [M+2H]²⁺: 633.9129, found: 633.9125 (–0.6 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 940.5866, found: 940.5855 (–1.2 ppm).

CalB_C1Pip (53)



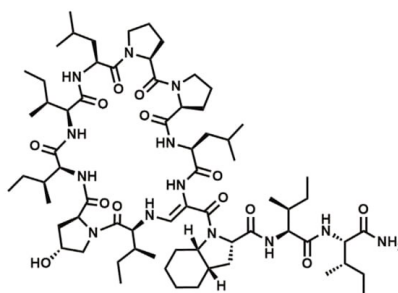
The linear precursor peptide of **53** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 59 mg (45 μ mol, 45%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 7.9 mg (6.1 μ mol, 13.7%) of **53** in a total yield of 6.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.17 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1294.8497, found: 1294.8486 (–0.8 ppm); calculated for [M+2H]²⁺: 647.9285, found: 647.9277 (–1.2 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 940.5866, found: 940.5826 (–4.3 ppm).

**CalB_C1Nip (54)**

The linear precursor peptide of **54** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 36 mg (27 μmol, 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.4 mg (1.1 μmol, 4.0%) of **54** in a total yield of 1.1% (calculated in relation to initial resin capacity) as a white powder.

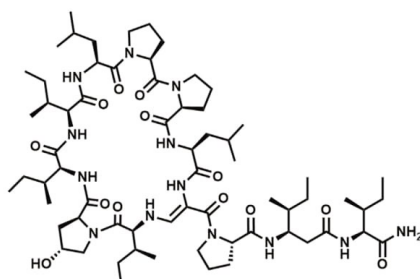
LC main peak retention time: 6.66 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1294.8497, found: 1294.8499 (0.2 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 647.9285, found: 647.9282 (−0.5 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 940.5866, found: 940.5836 (−3.2 ppm).

CalB_C1Oic (55)

The linear precursor peptide of **55** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 49 mg (36 μmol, 36%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 4.1 mg (3.1 μmol, 8.5%) of **55** in a total yield of 3.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.76 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1334.8810, found: 1334.8815 (0.4 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 667.9442, found: 667.9437 (−0.7 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 940.5866, found: 940.5851 (−1.6 ppm).

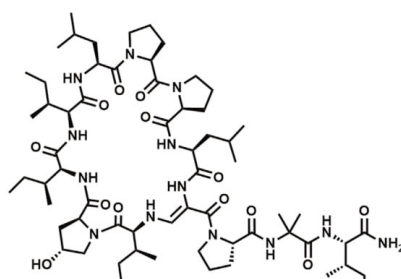
CalB_C2β-hIle (56)



The linear precursor peptide of **56** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 50 mg (38 μmol, 38%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 0.9 mg (0.7 μmol, 1.8%) of **56** in a total yield of 0.7% (calculated in relation to initial resin capacity) as a white powder.

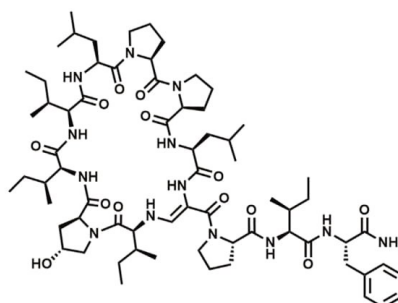
LC main peak retention time: 7.82 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1294.8497, found: 1294.8489 (−0.6 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 647.9285, found: 647.9276 (−1.4 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 940.5866, found: 940.5830 (−3.8 ppm).

CalB_C2Aib (57)



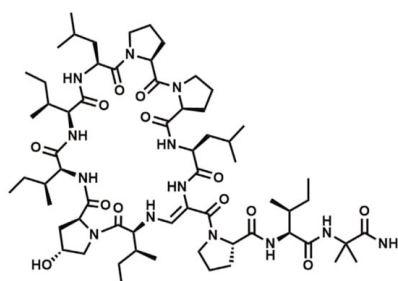
The linear precursor peptide of **57** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 60 mg (47 μmol, 47%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 6.8 mg (5.4 μmol, 11.5%) of **57** in a total yield of 5.4% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.90 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1252.8028, found: 1252.8028 (0.0 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 626.9050, found: 626.9048 (−0.3 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 940.5866, found: 940.5827 (−4.1 ppm).

**CalB_C3F (58)**

The linear precursor peptide of **58** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 81 mg (61 μmol , 61%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 13.3 mg (10.1 μmol , 16.7%) of **58** in a total yield of 10.1% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.98 (d, *J* = 7.0 Hz, 1H), 8.83 (d, *J* = 6.5 Hz, 1H), 8.27 (s, 1H), 7.49–7.42 (m, 2H), 7.39 (d, *J* = 7.0 Hz, 1H), 7.24–7.18 (m, 3H), 7.16 (s, 1H), 7.14–7.06 (m, 4H), 6.73 (d, *J* = 10.0 Hz, 1H), 5.82 (dd, *J* = 13.3, 10.0 Hz, 1H), 5.46–5.28 (m, 1H), 4.67–4.56 (m, 2H), 4.40 (s, 1H), 4.31–4.16 (m, 4H), 4.06 (t, *J* = 8.7 Hz, 1H), 3.91–3.77 (m, 4H), 3.71–3.54 (m, 3H), 3.53–3.44 (m, 2H), 3.25–3.15 (m, 2H), 3.12–3.03 (m, 1H), 2.61–2.54 (m, 1H), 2.35–2.18 (m, 3H), 2.11–2.03 (m, 1H), 2.00–1.85 (m, 8H), 1.81–1.46 (m, 8H), 1.45–1.33 (m, 4H), 1.30–1.19 (m, 1H), 1.17–0.99 (m, 2H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.91–0.72 (m, 30H), 0.67 (t, *J* = 7.4 Hz, 3H), 0.48 (t, *J* = 7.3 Hz, 3H), 0.33 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 8.19 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1314.8184, found: 1314.8175 (–0.7 ppm); calculated for [M+2H]²⁺: 657.9129, found: 657.9122 (–1.1 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 940.5866, found: 940.5837 (–3.1 ppm).

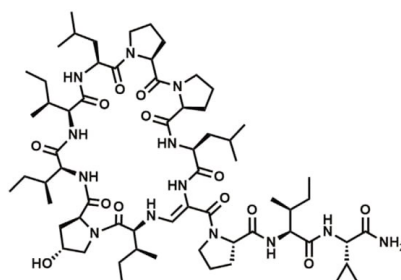
CalB_C3Aib (59)

The linear precursor peptide of **59** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 38 mg (30 μmol , 30%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was

shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 8.1 mg (6.5 μmol, 21.5%) of **59** in a total yield of 6.5% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 6.90 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1252.8028, found: 1252.8026 (−0.2 ppm); calculated for $[M+2H]^{2+}$: 626.9050, found: 626.9048 (−0.3 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5834 (−3.4 ppm).

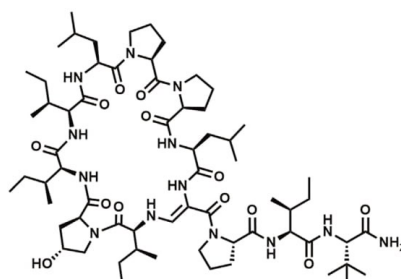
CalB_C3cPrGly (60)



The linear precursor peptide of **60** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 69 mg (54 μmol, 39%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 6.2 mg (4.9 μmol, 9.1%) of **60** in a total yield of 3.5% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (700 MHz, DMSO-*d*₆) δ 9.01 (s, 1H), 8.86 (s, 1H), 8.25 (s, 1H), 7.51 (d, $J = 10.3$ Hz, 1H), 7.48 (s, 1H), 7.38 (d, $J = 6.9$ Hz, 1H), 7.22 (s, 1H), 7.07 (d, $J = 13.3$ Hz, 1H), 6.94 (s, 1H), 6.69 (d, $J = 9.9$ Hz, 1H), 5.73 (t, $J = 10.0$ Hz, 1H), 4.63–4.58 (m, 1H), 4.55–4.50 (m, 1H), 4.41 (s, 1H), 4.27–4.21 (m, 3H), 4.14–4.09 (m, 1H), 4.04 (t, $J = 8.9$ Hz, 1H), 3.89 (t, $J = 10.2$ Hz, 1H), 3.83–3.77 (m, 2H), 3.70–3.63 (m, 1H), 3.63–3.56 (m, 2H), 3.51–3.44 (m, 1H), 3.21–3.15 (m, 1H), 2.32–2.19 (m, 3H), 2.11–2.04 (m, 1H), 2.01–1.96 (m, 1H), 1.94–1.82 (m, 8H), 1.81–1.49 (m, 12H), 1.47–1.34 (m, 8H), 1.29–1.21 (m, 2H), 1.19–1.10 (m, 2H), 1.09–0.91 (m, 5H), 0.91–0.70 (m, 36H); LC main peak retention time: 7.22 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1264.8028, found: 1264.8015 (−1.0 ppm); calculated for $[M+2H]^{2+}$: 632.9050, found: 632.9043 (−1.1 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5845 (−2.2 ppm).

CalB_C3Tle (61)

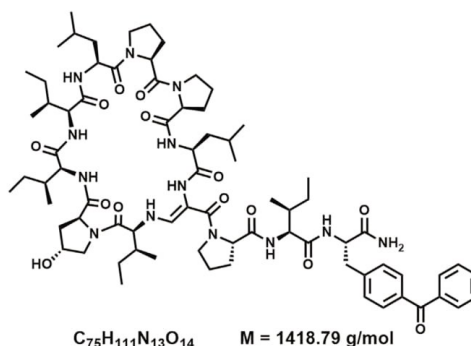




The linear precursor peptide of **61** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 51 mg (39 μmol, 28%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 6.4 mg (5.0 μmol, 12.7%) of **61** in a total yield of 3.6% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.00 (d, *J* = 6.9 Hz, 1H), 8.85 (s, 1H), 8.20 (s, 1H), 7.65 (d, *J* = 9.6 Hz, 1H), 7.37 (d, *J* = 7.1 Hz, 1H), 7.16 (d, *J* = 9.8 Hz, 1H), 6.98 (d, *J* = 14.3 Hz, 2H), 6.89 (s, 1H), 6.66 (d, *J* = 9.8 Hz, 1H), 5.63 (t, *J* = 11.6 Hz, 1H), 5.39 (s, 1H), 4.66–4.56 (m, 1H), 4.55–4.44 (m, 1H), 4.40 (s, 1H), 4.32–4.19 (m, 3H), 4.19–4.10 (m, 1H), 4.08–3.98 (m, 3H), 3.88–3.71 (m, 3H), 3.70–3.55 (m, 4H), 3.33–3.24 (m, 1H), 3.22–3.13 (m, 1H), 3.12–3.04 (m, 1H), 2.35–2.15 (m, 3H), 2.11–2.00 (m, 2H), 1.97–1.81 (m, 8H), 1.77–1.56 (m, 6H), 1.54–1.35 (m, 8H), 1.31–1.20 (m, 1H), 1.19–1.09 (m, 2H), 1.09–0.96 (m, 2H), 0.94–0.71 (m, 45H); LC main peak retention time: 8.13 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1280.8341, found: 1280.8334 (–0.5 ppm); calculated for [M+2H]²⁺: 640.9207, found: 640.9203 (–0.6 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 940.5866, found: 940.5834 (–3.4 ppm).

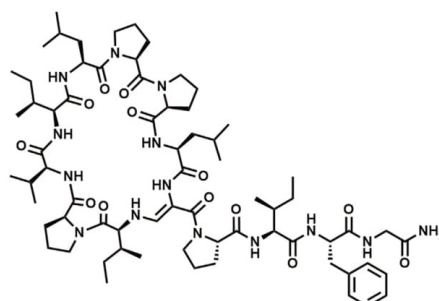
CalB_C3Bpa (**62**)



The linear precursor peptide of **62** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 65 mg (45 μmol, 32%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 3.1 mg (2.2 μmol, 4.8%) of **62** in a total yield of 1.6% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.98 (s, 1H), 8.80 (s, 1H), 8.18 (s, 1H), 7.66–7.59 (m, 4H), 7.57–7.46 (m, 5H), 7.44–7.38 (m, 2H), 7.35 (d, *J* = 7.0 Hz, 1H), 7.31 (s, 1H), 7.20 (d, *J* = 13.3 Hz, 1H), 6.93 (s, 1H), 6.67 (d, *J* = 10.0 Hz, 1H), 5.78 (t, *J* = 11.6 Hz, 1H), 5.36 (s, 1H), 4.63–4.57 (m, 1H), 4.56–4.49 (m, 1H), 4.39 (s, 1H), 4.29–4.16 (m, 5H), 4.04 (t, *J* = 8.7 Hz, 1H), 3.91–3.83 (m, 1H), 3.82–3.75 (m, 2H), 3.66–3.46 (m, 5H), 3.32–3.26 (m, 2H), 3.25–3.16 (m, 1H), 3.09–3.02 (m, 1H), 2.82 (t, *J* = 12.8 Hz, 1H), 2.35–2.17 (m, 4H), 2.07 (q, *J* = 7.0 Hz, 2H), 1.96–1.79 (m, 7H), 1.75–1.49 (m, 6H), 1.46–1.27 (m, 6H), 1.26–0.98 (m, 7H), 0.85–0.67 (m, 30H), 0.47 (d, *J* = 6.8 Hz, 3H), 0.31 (t, *J* = 7.3 Hz, 3H); LC main peak retention time: 7.81 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1418.8446, found: 1418.8438 (–0.6 ppm); calculated for [M+2H]²⁺: 709.9260, found: 709.9253 (–1.0 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 940.5866, found: 940.5846 (–2.1 ppm).

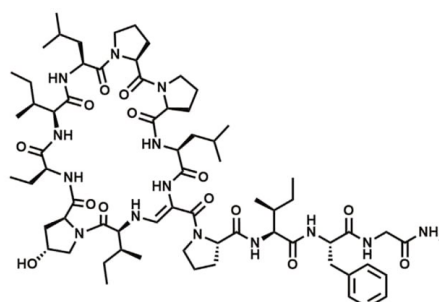
CalA_R2P (63)



The linear precursor peptide of **63** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 36.5 mg (27.2 μmol) of **63** in a total yield of 27.2% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-d₆) δ 8.94 (d, *J* = 7.0 Hz, 1H), 8.69 (d, *J* = 6.8 Hz, 1H), 8.24 (s, 1H), 8.10 (t, *J* = 6.2 Hz, 1H), 7.48 (d, *J* = 8.6 Hz, 1H), 7.44 (d, *J* = 7.0 Hz, 1H), 7.35 (d, *J* = 13.3 Hz, 1H), 7.29 (d, *J* = 8.6 Hz, 1H), 7.23–7.16 (m, 3H), 7.16–7.07 (m, 3H), 7.01 (s, 1H), 6.73 (d, *J* = 9.9 Hz, 1H), 5.84 (dd, *J* = 13.3, 10.1 Hz, 1H), 4.66–4.55 (m, 2H), 4.32–4.19 (m, 3H), 4.14–4.06 (m, 2H), 4.06–3.99 (m, 1H), 3.93–3.82 (m, 2H), 3.82–3.75 (m, 2H), 3.68–3.54 (m, 3H), 3.53–3.42 (m, 3H), 3.26–3.17 (m, 1H), 3.11 (dd, *J* = 13.8, 2.9 Hz, 1H), 3.00 (dd, *J* = 10.9, 7.0 Hz, 1H), 2.73–2.58 (m, 2H), 2.35–2.17 (m, 4H), 2.06–1.95 (m, 3H), 1.95–1.81 (m, 7H), 1.80–1.72 (m, 2H), 1.71–1.55 (m, 6H), 1.53–1.44 (m, 2H), 1.44–1.33 (m, 3H), 1.28–1.20 (m, 1H), 1.20–1.09 (m, 2H), 1.08–1.00 (m, 1H), 0.95 (d, *J* = 6.4 Hz, 3H), 0.90–0.75 (m, 24H), 0.73–0.67 (m, 4H), 0.44–0.36 (m, 6H); LC main peak retention time: 8.45 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1341.8293, found: 1341.8319 (1.9 ppm); calculated for [M+2H]²⁺: 671.4183, found: 671.4186 (0.4 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 910.5761, found: 910.5755 (–0.7 ppm).

CalA_R3Abu (64)



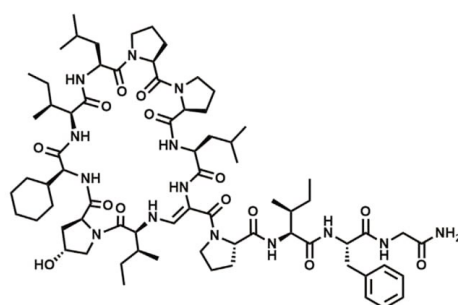
The linear precursor peptide of **64** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 90 mg (66 μmol, 47%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 20 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours



at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 21.1 mg (15.7 μ mol, 23.7%) of **64** in a total yield of 11.2% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.83 (d, *J* = 6.8 Hz, 1H), 8.31 (s, 1H), 8.17–8.08 (m, 2H), 7.55 (d, *J* = 7.3 Hz, 1H), 7.48 (d, *J* = 8.7 Hz, 1H), 7.35 (d, *J* = 13.3 Hz, 1H), 7.28–7.21 (m, 2H), 7.21–7.10 (m, 5H), 7.00–6.95 (m, 1H), 6.76 (d, *J* = 9.9 Hz, 1H), 5.80–5.70 (m, 1H), 5.37 (d, *J* = 3.0 Hz, 1H), 4.66 (q, *J* = 6.9 Hz, 1H), 4.58 (td, *J* = 10.3, 4.2 Hz, 1H), 4.41 (s, 1H), 4.32–4.20 (m, 4H), 4.12 (t, *J* = 10.2 Hz, 1H), 4.03 (t, *J* = 8.7 Hz, 1H), 3.98–3.87 (m, 2H), 3.82–3.68 (m, 3H), 3.61–3.39 (m, 6H), 3.27–3.18 (m, 1H), 3.15–3.07 (m, 1H), 2.68–2.58 (m, 1H), 2.35–2.31 (m, 1H), 2.30–2.21 (m, 2H), 2.14–2.01 (m, 3H), 1.98–1.78 (m, 9H), 1.75–1.53 (m, 9H), 1.45–1.28 (m, 5H), 1.20–0.99 (m, 3H), 0.96 (d, *J* = 6.5 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 3H), 0.86–0.75 (m, 18H), 0.71 (t, *J* = 7.4 Hz, 3H), 0.42 (t, *J* = 7.3 Hz, 3H), 0.38 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 7.43 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1343.8086, found: 1343.8087 (0.1 ppm); calculated for [M+2H]²⁺: 672.4079, found: 672.4073 (–0.9 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 912.5554, found: 912.5527 (–3.0 ppm).

CalA_R3Chg (65)



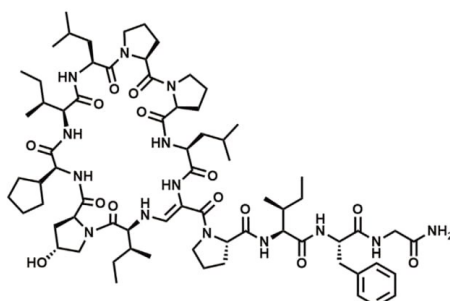
C₇₂H₁₁₂N₁₄O₁₄

M = 1397.77 g/mol

The linear precursor peptide of **65** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 40 mg (28 μ mol, 20%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 9.4 mg (6.7 μ mol, 23.8%) of **65** in a total yield of 4.8% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.01 (d, *J* = 7.0 Hz, 1H), 8.83 (d, *J* = 6.6 Hz, 1H), 8.28 (s, 1H), 8.18 (t, *J* = 6.1 Hz, 1H), 7.46 (d, *J* = 8.8 Hz, 1H), 7.42 (d, *J* = 7.0 Hz, 1H), 7.34 (d, *J* = 13.3 Hz, 1H), 7.29–7.23 (m, 2H), 7.19–7.09 (m, 5H), 6.97 (s, 1H), 6.71 (d, *J* = 10.0 Hz, 1H), 5.93–5.82 (m, 1H), 5.38 (d, *J* = 3.0 Hz, 1H), 4.64–4.55 (m, 2H), 4.40 (s, 1H), 4.30–4.20 (m, 4H), 4.10–4.00 (m, 2H), 3.93 (d, *J* = 7.2 Hz, 1H), 3.89 (d, *J* = 7.0 Hz, 1H), 3.81–3.74 (m, 3H), 3.70–3.55 (m, 4H), 3.35–3.25 (m, 1H), 3.24–3.15 (m, 1H), 3.12–3.01 (m, 2H), 2.61 (t, *J* = 12.9 Hz, 1H), 2.55–2.52 (m, 1H), 2.40–2.19 (m, 4H), 2.12–2.03 (m, 1H), 2.01–1.94 (m, 1H), 1.93–1.80 (m, 8H), 1.79–1.71 (m, 1H), 1.71–1.53 (m, 10H), 1.52–1.33 (m, 7H), 1.22 (s, 1H), 1.19–0.98 (m, 7H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.87–0.74 (m, 18H), 0.69 (t, *J* = 7.3 Hz, 3H), 0.42 (t, *J* = 7.3 Hz, 3H), 0.37 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 9.08 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1397.8555, found: 1394.8557 (0.1 ppm); calculated for [M+2H]²⁺: 699.4314, found: 699.4307 (–1.0 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 966.6023, found: 966.6001 (–2.3 ppm).

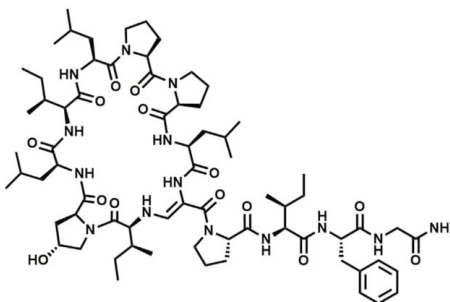
CalA_R3Cpg (66)



The linear precursor peptide of **66** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 19 mg (14 μmol, 10%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 6 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 3.5 mg (2.5 μmol, 18.5%) of **66** in a total yield of 1.0% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.08 (d, *J* = 6.9 Hz, 1H), 8.76 (d, *J* = 7.0 Hz, 1H), 8.29 (s, 1H), 8.17 (t, *J* = 6.2 Hz, 1H), 7.47 (d, *J* = 8.7 Hz, 1H), 7.42 (d, *J* = 7.0 Hz, 1H), 7.34 (d, *J* = 13.3 Hz, 1H), 7.28–7.23 (m, 2H), 7.20–7.09 (m, 5H), 6.99–6.95 (m, 1H), 6.72 (d, *J* = 10.0 Hz, 1H), 5.91–5.82 (m, 1H), 5.37 (d, *J* = 3.1 Hz, 1H), 4.65–4.57 (m, 2H), 4.40 (s, 1H), 4.31–4.21 (m, 4H), 4.12–4.01 (m, 2H), 3.96–3.87 (m, 1H), 3.85–3.74 (m, 3H), 3.72–3.66 (m, 1H), 3.63–3.55 (m, 2H), 3.53–3.44 (m, 3H), 3.25–3.16 (m, 1H), 3.13–3.05 (m, 2H), 2.97–2.86 (m, 1H), 2.62 (t, *J* = 12.9 Hz, 1H), 2.55–2.51 (m, 1H), 2.34–2.20 (m, 3H), 2.10–2.02 (m, 1H), 2.02–1.96 (m, 1H), 1.94–1.82 (m, 8H), 1.79–1.70 (m, 1H), 1.69–1.52 (m, 10H), 1.51–1.35 (m, 7H), 1.27–1.20 (m, 3H), 1.19–1.11 (m, 2H), 1.08–1.00 (m, 1H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.89–0.75 (m, 18H), 0.70 (t, *J* = 7.4 Hz, 3H), 0.42 (t, *J* = 7.3 Hz, 3H), 0.38 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 8.54 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1383.8399, found: 1383.8401 (0.1 ppm); calculated for [M+2H]²⁺: 692.4236, found: 692.4231 (−0.7 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 952.5867, found: 952.5858 (−0.9 ppm).

CalA_R3L (67)



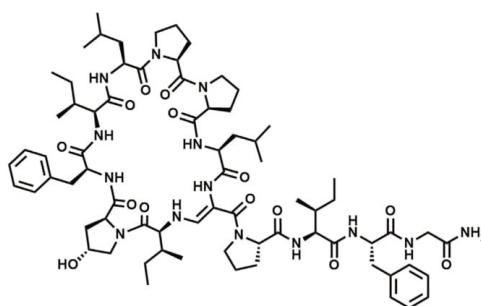
The linear precursor peptide of **67** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 26 mg (19 μmol, 13%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the



peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 6.4 mg (4.7 μ mol, 25.4%) of **67** in a total yield of 3.4% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.86 (d, *J* = 6.9 Hz, 1H), 8.31 (s, 1H), 8.19–8.12 (m, 2H), 7.54 (d, *J* = 7.3 Hz, 1H), 7.47 (d, *J* = 8.7 Hz, 1H), 7.34 (d, *J* = 13.3 Hz, 1H), 7.27–7.21 (m, 2H), 7.20–7.12 (m, 5H), 6.97 (s, 1H), 6.75 (d, *J* = 9.9 Hz, 1H), 5.82–5.72 (m, 1H), 5.39 (d, *J* = 3.1 Hz, 1H), 4.65 (q, *J* = 6.9 Hz, 1H), 4.57 (dd, *J* = 10.2, 4.1 Hz, 1H), 4.40 (s, 1H), 4.31–4.18 (m, 4H), 4.10 (t, *J* = 10.2 Hz, 1H), 4.03 (t, *J* = 8.8 Hz, 1H), 3.97–3.87 (m, 2H), 3.80–3.69 (m, 2H), 3.61–3.48 (m, 5H), 3.37–3.28 (m, 2H), 3.26–3.17 (m, 1H), 3.13–3.06 (m, 1H), 2.61 (t, *J* = 12.9 Hz, 1H), 2.54–2.51 (m, 1H), 2.35–2.30 (m, 1H), 2.29–2.20 (m, 2H), 2.13–2.03 (m, 2H), 1.97–1.81 (m, 7H), 1.73–1.36 (m, 15H), 1.34–1.25 (m, 1H), 1.22 (s, 1H), 1.20–1.12 (m, 1H), 1.11–0.99 (m, 2H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.90–0.73 (m, 24H), 0.70 (t, *J* = 7.4 Hz, 3H), 0.42 (t, *J* = 7.3 Hz, 3H), 0.37 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 8.11 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1371.8399, found: 1371.8404 (0.4 ppm); calculated for [M+2H]²⁺: 686.4236, found: 686.4233 (–0.4 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 940.5866, found: 940.5856 (–1.1 ppm).

CalA_R3F (68)

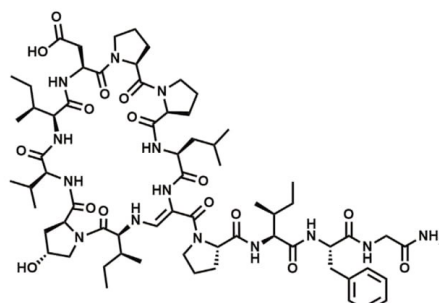


C₇₃H₁₀₈N₁₄O₁₄

M = 1405.75 g/mol

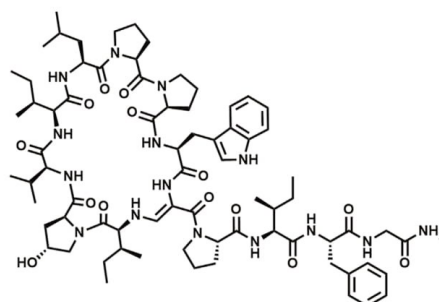
The linear precursor peptide of **68** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 7.2 mg (5.1 μ mol) of **68** in a total yield of 5.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.15 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1405.8242, found: 1405.8247 (0.4 ppm); calculated for [M+2H]²⁺: 703.4158, found: 703.4160 (0.3 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 974.5710, found: 974.5698 (–1.2 ppm).

CalA_R5D (69)

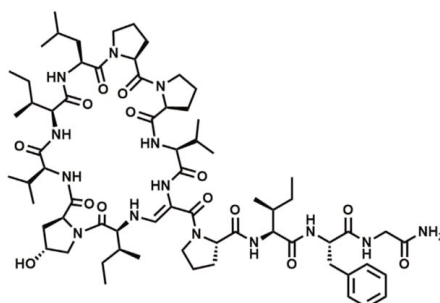
The linear precursor peptide of **69** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 4.7 mg (3.5 μ mol) of **69** in a total yield of 3.5% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 5.28 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1359.7671, found: 1359.7677 (0.4 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 680.3872, found: 680.3870 (−0.3 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 928.5138, found: 928.5136 (−0.2 ppm).

CalA_R8W (70)

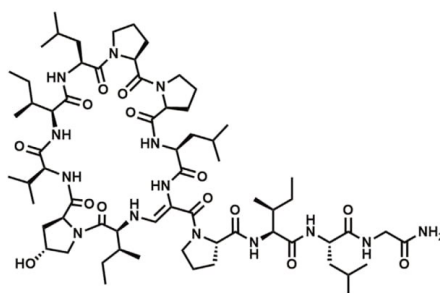
The linear precursor peptide of **70** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 6.2 mg (4.3 μ mol) of **70** in a total yield of 4.3% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 6.79 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1430.8195, found: 1430.8207 (0.8 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 715.9134, found: 715.9127 (−1.0 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 999.5662, found: 999.5626 (−3.6 ppm).

**CalA_R8V (71)**

The linear precursor peptide of **71** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 9.2 mg (6.8 μmol) of **71** in a total yield of 6.8% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.96 (s, 1H), 8.80 (s, 1H), 8.36 (s, 1H), 8.10 (t, *J* = 6.1 Hz, 1H), 7.53 (d, *J* = 8.3 Hz, 1H), 7.37 (d, *J* = 6.5 Hz, 1H), 7.31 (d, *J* = 13.3 Hz, 1H), 7.28–7.13 (m, 7H), 6.95 (s, 1H), 6.69 (d, *J* = 9.9 Hz, 1H), 5.73 (dd, *J* = 14.1, 9.9 Hz, 1H), 5.37 (d, *J* = 3.1 Hz, 1H), 4.55 (td, *J* = 10.3, 5.0 Hz, 2H), 4.40 (s, 1H), 4.30–4.20 (m, 4H), 4.07–3.97 (m, 2H), 3.90 (dd, *J* = 16.5, 7.2 Hz, 1H), 3.79–3.71 (m, 4H), 3.66–3.52 (m, 3H), 3.48 (dd, *J* = 16.7, 5.1 Hz, 1H), 3.26–3.16 (m, 2H), 3.12 (dd, *J* = 13.7, 3.1 Hz, 1H), 3.03–2.95 (m, 1H), 2.69–2.57 (m, 2H), 2.47–2.38 (m, 1H), 2.32–2.21 (m, 3H), 2.12–2.04 (m, 2H), 2.01–1.96 (m, 1H), 1.96–1.86 (m, 5H), 1.86–1.76 (m, 2H), 1.76–1.60 (m, 3H), 1.55 (td, *J* = 13.5, 6.6 Hz, 2H), 1.49–1.32 (m, 4H), 1.27–1.17 (m, 2H), 1.17–1.02 (m, 2H), 0.99 (d, *J* = 7.2 Hz, 3H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.89 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 6.5 Hz, 3H), 0.84–0.74 (m, 15H), 0.74–0.69 (m, 4H), 0.41 (t, *J* = 7.3 Hz, 3H), 0.38 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 7.69 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1343.8086, found: 1343.8092 (0.4 ppm); calculated for [M+2H]²⁺: 672.9096, found: 672.9101 (0.7 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 912.5553, found: 912.5564 (1.2 ppm).

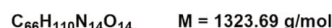
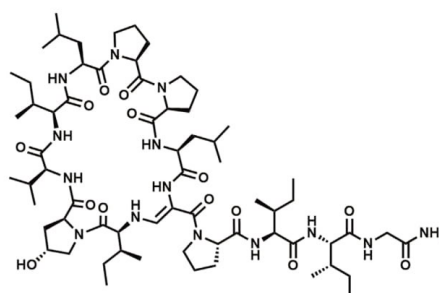
CalA_C3L (72)

The linear precursor peptide of **72** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then

dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 6.3 mg (4.8 μ mol) of **72** in a total yield of 4.8% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-d₆) δ 9.04 (d, *J* = 6.8 Hz, 1H), 8.83 (d, *J* = 5.8 Hz, 1H), 8.29 (d, *J* = 6.4 Hz, 1H), 8.28 (s, 1H), 7.46 (d, *J* = 6.9 Hz, 1H), 7.43 (d, *J* = 8.1 Hz, 1H), 7.35 (d, *J* = 6.5 Hz, 1H), 7.32 (d, *J* = 3.1 Hz, 1H), 7.19 (s, 1H), 6.91 (s, 1H), 6.63 (d, *J* = 10.1 Hz, 1H), 5.81 (dd, *J* = 12.9, 9.8 Hz, 1H), 5.36 (d, *J* = 3.0 Hz, 1H), 4.66–4.54 (m, 2H), 4.40 (s, 1H), 4.29–4.16 (m, 4H), 4.15–4.07 (m, 2H), 4.07–4.01 (m, 1H), 3.92 (dd, *J* = 16.2, 7.1 Hz, 1H), 3.79 (dd, *J* = 8.5, 6.6 Hz, 1H), 3.76–3.66 (m, 2H), 3.65–3.55 (m, 2H), 3.54–3.45 (m, 2H), 3.40 (dd, *J* = 16.2, 5.1 Hz, 1H), 3.22–3.12 (m, 1H), 3.01–2.94 (m, 1H), 2.71–2.62 (m, 1H), 2.35–2.29 (m, 1H), 2.28–2.19 (m, 2H), 2.12–2.04 (m, 2H), 2.03–1.96 (m, 1H), 1.95–1.80 (m, 7H), 1.76–1.66 (m, 3H), 1.66–1.55 (m, 3H), 1.54–1.37 (m, 8H), 1.30–1.22 (m, 2H), 1.21–1.09 (m, 1H), 1.02–0.93 (m, 1H), 0.89 (d, *J* = 5.1 Hz, 3H), 0.88–0.79 (m, 30H), 0.78 (d, *J* = 3.8 Hz, 3H), 0.76 (d, *J* = 3.8 Hz, 3H), 0.75–0.70 (m, 3H); LC main peak retention time: 7.45 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1323.8399, found: 1323.8419 (1.5 ppm); calculated for [M+2H]²⁺: 662.4236, found: 662.4237 (0.2 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 926.5710, found: 926.5701 (–1.0 ppm).

CalA_C3I (73)

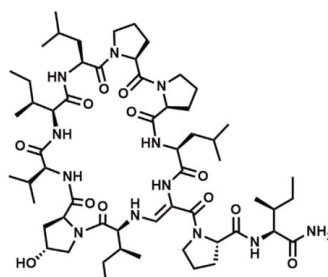


The linear precursor peptide of **73** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 2.3 mg (1.7 μ mol) of **73** in a total yield of 1.7% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.53 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1323.8399, found: 1323.8405 (0.5 ppm); calculated for [M+2H]²⁺: 662.4236, found: 662.4233 (–0.5 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 926.5710, found: 926.5721 (1.2 ppm).



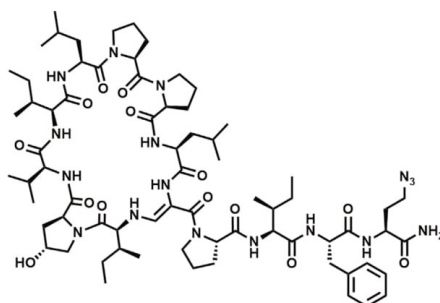
CalA_C3X (74)



The linear precursor peptide of **74** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 8.8 mg (7.6 μmol) of **74** in a total yield of 7.6% (calculated in relation to initial resin capacity) as a white powder.

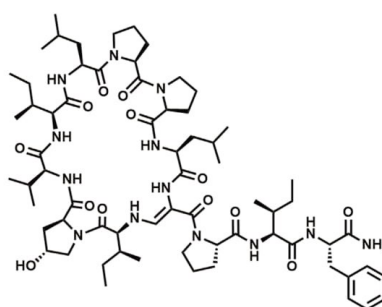
¹H NMR (400 MHz, DMSO-*d*₆) δ 8.95 (s, 1H), 8.77 (s, 1H), 8.06 (s, 1H), 7.47 (d, *J* = 9.1 Hz, 1H), 7.42 (d, *J* = 6.8 Hz, 1H), 7.15 (d, *J* = 12.9 Hz, 1H), 7.05 (s, 1H), 6.81 (s, 1H), 6.74 (d, *J* = 9.9 Hz, 1H), 5.56 (dd, *J* = 12.9, 10.2 Hz, 1H), 5.36 (d, *J* = 3.1 Hz, 1H), 4.61 (q, *J* = 6.7 Hz, 1H), 4.51–4.43 (m, 1H), 4.39 (s, 1H), 4.33 (dd, *J* = 9.4, 7.5 Hz, 1H), 4.27–4.21 (m, 1H), 4.04 (t, *J* = 9.0 Hz, 1H), 3.99 (dd, *J* = 9.1, 6.3 Hz, 1H), 3.94 (t, *J* = 9.7 Hz, 1H), 3.83 (t, *J* = 7.5 Hz, 1H), 3.78 (d, *J* = 11.4 Hz, 1H), 3.67 (dd, *J* = 10.9, 3.5 Hz, 1H), 3.64–3.54 (m, 2H), 3.54–3.43 (m, 2H), 3.29–3.23 (m, 1H), 3.21–3.13 (m, 1H), 3.07–2.97 (m, 1H), 2.69–2.58 (m, 1H), 2.32–2.15 (m, 3H), 2.12–2.04 (m, 1H), 2.02–1.98 (m, 1H), 1.97–1.84 (m, 7H), 1.80–1.69 (m, 4H), 1.66–1.58 (m, 3H), 1.56–1.37 (m, 7H), 1.36–1.27 (m, 1H), 1.22–1.10 (m, 1H), 1.08–0.94 (m, 2H), 0.92–0.89 (m, 9H), 0.86–0.79 (m, 27H); LC main peak retention time: 6.46 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1153.7343, found: 1153.7338 (−0.4 ppm); calculated for [M+2H]²⁺: 577.3708, found: 577.3702 (−1.0 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 926.5710, found: 926.5721 (1.2 ppm).

CalA_C4Aha (75)



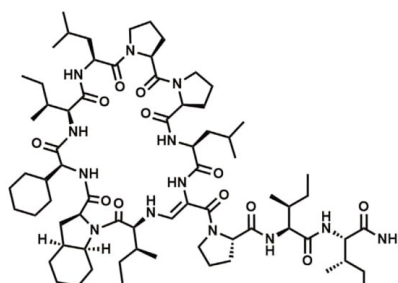
The linear precursor peptide of **75** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 8.2 mg (5.7 μmol) of **75** in a total yield of 5.7% (calculated in relation to initial resin capacity) as a white powder.

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.00 (s, 1H), 8.77 (s, 1H), 8.15 (s, 1H), 7.68 (d, $J = 7.2$ Hz, 1H), 7.45 (d, $J = 8.1$ Hz, 1H), 7.40 (d, $J = 8.4$ Hz, 1H), 7.35 (d, $J = 7.2$ Hz, 1H), 7.26–7.18 (m, 3H), 7.14–7.02 (m, 4H), 6.77 (s, 1H), 6.72 (d, $J = 9.7$ Hz, 1H), 5.77 (dd, $J = 12.9$, 10.0 Hz, 1H), 5.37 (d, $J = 3.0$ Hz, 1H), 4.64 (q, $J = 6.9$ Hz, 1H), 4.56 (dd, $J = 9.9$, 5.0 Hz, 1H), 4.39 (s, 1H), 4.31–4.24 (m, 2H), 4.22–4.12 (m, 3H), 4.10–4.01 (m, 1H), 3.85–3.70 (m, 4H), 3.64 (dd, $J = 11.1$, 3.5 Hz, 1H), 3.61–3.53 (m, 2H), 3.52–3.44 (m, 4H), 3.24 (td, $J = 11.0$, 6.0 Hz, 1H), 3.15 (dd, $J = 13.3$, 3.0 Hz, 1H), 2.99 (dd, $J = 10.9$, 6.8 Hz, 1H), 2.84–2.73 (m, 1H), 2.71–2.55 (m, 1H), 2.34–2.19 (m, 3H), 2.12–2.05 (m, 1H), 2.04–1.97 (m, 2H), 1.96–1.81 (m, 9H), 1.79–1.68 (m, 4H), 1.66–1.54 (m, 3H), 1.51–1.41 (m, 1H), 1.39–1.32 (m, 1H), 1.30–1.21 (m, 2H), 1.18–1.05 (m, 5H), 0.96 (d, $J = 6.8$ Hz, 3H), 0.91 (d, $J = 6.5$ Hz, 3H), 0.88 (d, $J = 6.6$ Hz, 6H), 0.85–0.74 (m, 15H), 0.70 (t, $J = 7.4$ Hz, 3H), 0.59–0.52 (m, 1H), 0.50 (d, $J = 6.8$ Hz, 3H), 0.26 (t, $J = 7.4$ Hz, 3H); LC main peak retention time: 8.40 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1426.8569, found: 1426.8575 (0.4 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 713.9321, found: 713.9322 (0.1 ppm); MS^2 HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 926.5710, found: 926.5727 (1.8 ppm).

CalA_C4X (76) $\text{C}_{67}\text{H}_{105}\text{N}_{13}\text{O}_{13}$ $M = 1300.66 \text{ g/mol}$

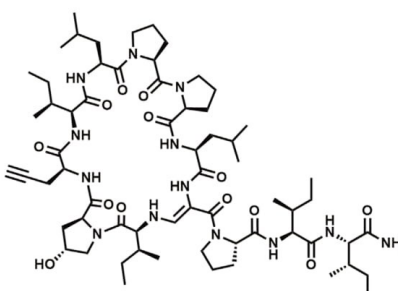
The linear precursor peptide of **76** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O ; solvent B: ACN) to give 1.9 mg (1.5 μmol) of **76** in a total yield of 1.5% (calculated in relation to initial resin capacity) as a white powder.

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 9.02 (s, 1H), 8.82 (s, 1H), 8.27 (s, 1H), 7.45 (d, $J = 9.0$ Hz, 2H), 7.39 (d, $J = 7.4$ Hz, 1H), 7.22–7.18 (m, 3H), 7.15 (d, $J = 7.8$ Hz, 1H), 7.14–7.08 (m, 4H), 6.73 (d, $J = 9.6$ Hz, 1H), 5.86–5.77 (m, 1H), 5.38 (d, $J = 3.0$ Hz, 1H), 4.66–4.55 (m, 2H), 4.41 (s, 1H), 4.30–4.17 (m, 4H), 4.06 (t, $J = 8.2$ Hz, 1H), 3.88 (t, $J = 10.5$ Hz, 1H), 3.84–3.77 (m, 3H), 3.68–3.63 (m, 1H), 3.62–3.55 (m, 2H), 3.53–3.44 (m, 2H), 3.24–3.16 (m, 2H), 3.02–2.94 (m, 1H), 2.70–2.61 (m, 1H), 2.56 (t, $J = 12.7$ Hz, 1H), 2.33–2.20 (m, 3H), 2.13–2.05 (m, 1H), 2.02–1.97 (m, 1H), 1.96–1.84 (m, 7H), 1.82–1.68 (m, 3H), 1.66–1.54 (m, 4H), 1.54–1.46 (m, 2H), 1.44–1.32 (m, 3H), 1.30–1.21 (m, 3H), 1.14–1.01 (m, 2H), 0.94 (d, $J = 6.6$ Hz, 3H), 0.90–0.77 (m, 24H), 0.74–0.70 (m, 1H), 0.67 (t, $J = 7.4$ Hz, 3H), 0.48 (t, $J = 7.2$ Hz, 3H), 0.33 (d, $J = 6.8$ Hz, 3H); LC main peak retention time: 6.98 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1300.8028, found: 1300.8048 (1.5 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 650.9050, found: 950.9052 (0.3 ppm); MS^2 HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 926.5710, found: 926.5722 (1.3 ppm).

**CalB_R2Oic_R3Chg (77)**

The linear precursor peptide of **77** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 72 mg (52 μmol, 52%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 3.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 4.1 mg (3.0 μmol, 5.8%) of **77** in a total yield of 3.0% (calculated in relation to initial resin capacity) as a white powder.

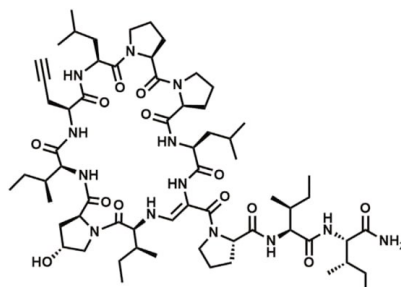
¹H NMR (700 MHz, DMSO-*d*₆) δ 8.85 (d, *J* = 7.0 Hz, 1H), 8.58 (d, *J* = 6.6 Hz, 1H), 8.26 (s, 1H), 7.62–7.55 (m, 2H), 7.30 (d, *J* = 9.6 Hz, 1H), 7.12 (d, *J* = 13.4 Hz, 1H), 7.05 (s, 1H), 7.03 (s, 1H), 6.66 (d, *J* = 10.0 Hz, 1H), 5.58 (dd, *J* = 13.4, 10.0 Hz, 1H), 4.59 (q, *J* = 6.7 Hz, 1H), 4.53 (td, *J* = 10.3, 3.8 Hz, 1H), 4.27 (dd, *J* = 10.0, 7.7 Hz, 1H), 4.23 (t, *J* = 7.9 Hz, 1H), 4.17 (dd, *J* = 9.6, 5.1 Hz, 1H), 4.13–4.06 (m, 2H), 4.04 (dt, *J* = 12.4, 6.5 Hz, 1H), 3.95 (t, *J* = 8.8 Hz, 1H), 3.81 (t, *J* = 10.2 Hz, 1H), 3.73 (dd, *J* = 9.5, 6.6 Hz, 1H), 3.60–3.51 (m, 2H), 3.51–3.45 (m, 1H), 3.45–3.41 (m, 1H), 3.28 (dt, *J* = 10.2, 7.0 Hz, 1H), 3.18 (td, *J* = 10.9, 5.8 Hz, 1H), 3.07 (dd, *J* = 11.1, 7.0 Hz, 1H), 2.49–2.44 (m, 2H), 2.34–2.30 (m, 1H), 2.29–2.25 (m, 1H), 2.25–2.19 (m, 1H), 2.03–1.96 (m, 2H), 1.93–1.83 (m, 7H), 1.82–1.78 (m, 1H), 1.77–1.72 (m, 2H), 1.71–1.64 (m, 7H), 1.63–1.55 (m, 5H), 1.54–1.40 (m, 9H), 1.31–1.18 (m, 7H), 1.17–1.10 (m, 3H), 1.06–0.99 (m, 2H), 0.94–0.90 (m, 1H), 0.90–0.86 (m, 12H), 0.86–0.85 (m, 1H), 0.84–0.77 (m, 18H), 0.75 (t, *J* = 6.4 Hz, 3H), 0.73 (t, *J* = 6.0 Hz, 3H); ¹³C NMR (176 MHz, DMSO-*d*₆) δ 173.0, 172.8, 172.8, 172.4, 171.9, 171.5, 171.4, 171.3, 171.2, 171.2, 167.2, 142.3, 98.1, 64.8, 63.9, 63.8, 62.5, 61.2, 61.1, 59.8, 58.1, 57.4, 57.3, 50.0, 49.2, 48.7, 47.1, 46.1, 41.1, 40.4, 37.5, 36.8, 36.3, 36.2, 35.8, 29.7, 29.5, 29.0, 28.5, 26.3, 25.8, 25.6, 25.2, 25.1, 25.1, 25.0, 24.9, 24.8, 24.6, 24.0, 23.6, 23.5, 23.1, 22.7, 22.2, 20.9, 19.4, 15.8, 15.7, 15.1, 11.5, 11.0, 10.8, 9.5; LC main peak retention time: 17.11 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1344.9017, found: 1344.9020 (0.2 ppm); calculated for [M+2H]²⁺: 672.9545, found: 972.9536 (–1.3 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 1004.6543, found: 1004.6513 (–3.0 ppm).

CalB_R3Pra (78)

The linear precursor peptide of **78** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 42 mg (33 μmol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 8 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 9.2 mg (7.3 μmol, 22.3%) of **78** in a total yield of 7.3% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 9.13 (d, *J* = 6.8 Hz, 1H), 8.30 (s, 1H), 7.80 (d, *J* = 9.0 Hz, 1H), 7.60 (d, *J* = 10.0 Hz, 1H), 7.49 (d, *J* = 7.6 Hz, 1H), 7.23 (d, *J* = 9.7 Hz, 1H), 7.14 (s, 1H), 7.07 (d, *J* = 13.2 Hz, 1H), 6.97 (s, 1H), 6.68 (d, *J* = 9.9 Hz, 1H), 5.62–5.50 (m, 1H), 5.40 (d, *J* = 3.1 Hz, 1H), 4.75–4.64 (m, 1H), 4.56–4.46 (m, 1H), 4.42 (s, 1H), 4.33–4.21 (m, 3H), 4.19–4.13 (m, 1H), 4.12–4.06 (m, 1H), 4.05–3.95 (m, 3H), 3.77–3.69 (m, 2H), 3.62–3.44 (m, 4H), 3.39–3.33 (m, 1H), 3.23–3.13 (m, 1H), 3.09–2.99 (m, 1H), 2.97–2.93 (m, 1H), 2.78–2.69 (m, 1H), 2.41–2.32 (m, 1H), 2.31–2.17 (m, 2H), 2.12–2.01 (m, 2H), 2.01–1.75 (m, 8H), 1.74–1.55 (m, 6H), 1.54–1.33 (m, 8H), 1.28–1.21 (m, 1H), 1.19–0.96 (m, 5H), 0.95–0.69 (m, 36H); LC main peak retention time: 7.15 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1262.7871, found: 1262.7857 (–1.1 ppm); calculated for [M+2H]²⁺: 631.8972, found: 631.8963 (–1.4 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 922.5397, found: 922.5377 (–2.2 ppm).

CalB_R4Pra (79)

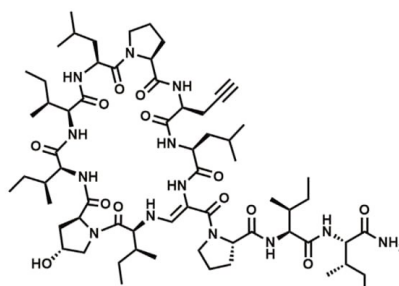


C₆₄H₁₀₃N₁₃O₁₃

M = 1262.61 g/mol

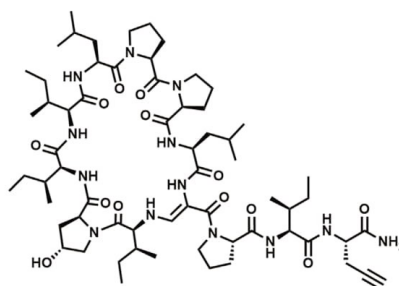
The linear precursor peptide of **79** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 29 mg (23 μmol, 23%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 2.6 mg (2.1 μmol, 9.1%) of **79** in a total yield of 2.1% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.81 (s, 1H), 8.73 (s, 1H), 8.30 (s, 1H), 7.59 (d, *J* = 9.9 Hz, 1H), 7.55 (d, *J* = 7.0 Hz, 1H), 7.23 (d, *J* = 9.6 Hz, 1H), 7.14 (s, 1H), 7.07 (d, *J* = 13.3 Hz, 1H), 6.96 (d, *J* = 2.8 Hz, 1H), 6.67 (d, *J* = 9.7 Hz, 1H), 5.70–5.60 (m, 1H), 5.45–5.33 (m, 1H), 4.63–4.56 (m, 1H), 4.56–4.48 (m, 1H), 4.42 (s, 1H), 4.28–4.18 (m, 3H), 4.17–4.10 (m, 1H), 4.10–4.01 (m, 3H), 3.98–3.89 (m, 1H), 3.78–3.67 (m, 2H), 3.61–3.48 (m, 4H), 3.34–3.28 (m, 1H), 3.23–3.13 (m, 2H), 2.98–2.94 (m, 1H), 2.60–2.53 (m, 1H), 2.33–2.20 (m, 3H), 2.11–1.97 (m, 2H), 1.95–1.84 (m, 8H), 1.73–1.56 (m, 6H), 1.51–1.39 (m, 8H), 1.24 (s, 1H), 1.17–0.95 (m, 5H), 0.90–0.76 (m, 36H); LC main peak retention time: 5.80 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1262.7871, found: 1262.7861 (–0.8 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 922.5397, found: 922.5407 (1.1 ppm).

**CalB_R7Pra (80)**

The linear precursor peptide of **80** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 40 mg (31 μ mol, 22%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.7 mg (1.3 μ mol, 4.3%) of **80** in a total yield of 0.9% (calculated in relation to initial resin capacity) as a white powder.

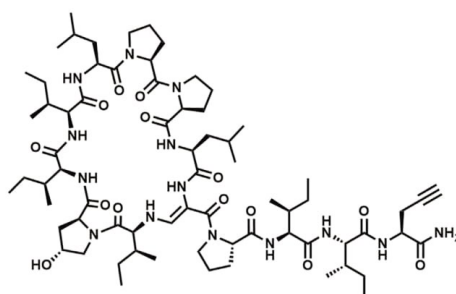
LC main peak retention time: 7.61 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1278.8184, found: 1278.8171 (−1.0 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 938.5710, found: 938.5712 (0.2 ppm).

CalB_C3Pra (81)

The linear precursor peptide of **81** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 43 mg (33 μ mol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.9 mg (1.5 μ mol, 4.5%) of **81** in a total yield of 1.5% (calculated in relation to initial resin capacity) as a white powder.

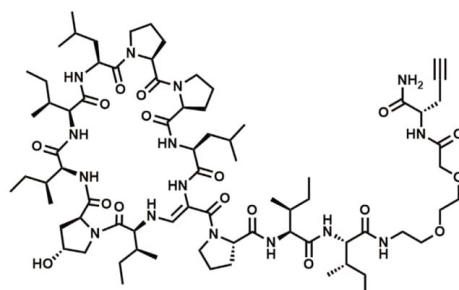
¹H NMR (400 MHz, DMSO-*d*₆) δ 8.99 (d, J = 7.0 Hz, 1H), 8.85 (d, J = 6.5 Hz, 1H), 8.35 (s, 1H), 7.78 (d, J = 8.7 Hz, 1H), 7.44–7.35 (m, 3H), 7.16 (s, 1H), 7.08 (d, J = 13.4 Hz, 1H), 6.70 (d, J = 9.8 Hz, 1H), 5.83–5.70 (m, 1H), 5.40 (d, J = 3.1 Hz, 1H), 4.64–4.49 (m, 2H), 4.41 (s, 1H), 4.28–4.19 (m, 5H), 4.18–4.12 (m, 1H), 4.06–4.00 (m, 1H), 3.91–3.84 (m, 1H), 3.82–3.75 (m, 2H), 3.70–3.56 (m, 4H), 3.52–3.45 (m, 1H), 3.23–3.13 (m, 1H), 3.12–3.03 (m, 1H), 2.73–2.68 (m, 1H), 2.68–2.59 (m, 1H), 2.31–2.18 (m, 3H), 2.14–2.04 (m, 2H), 1.99–1.77 (m, 8H), 1.71–1.48 (m, 6H), 1.47–1.32 (m, 8H), 1.31–1.21 (m, 1H), 1.19–0.99 (m, 5H), 0.92–.72 (m, 36H); LC

main peak retention time: 6.81 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1262.7871, found: 1262.7865 (−0.5 ppm); calculated for $[M+2H]^{2+}$: 631.8972, found: 631.8967 (−0.8 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 940.5866, found: 940.5854 (−1.3 ppm).

CalB_C4Pra (82)

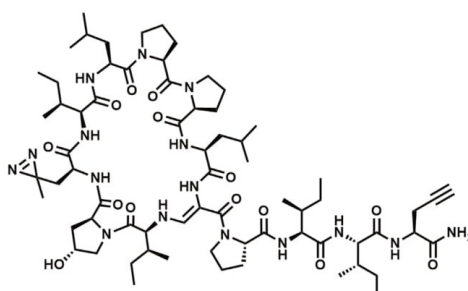
The linear precursor peptide of **82** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.2 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 130 mg (93 μ mol, 46%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 20 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 25.3 mg (18.4 μ mol, 19.7%) of **82** in a total yield of 9.0% (calculated in relation to initial resin capacity) as a white powder.

¹H NMR (700 MHz, DMSO-*d*₆) δ 9.01 (s, 1H), 8.85 (s, 1H), 8.17 (s, 1H), 8.10 (d, $J = 8.3$ Hz, 1H), 7.47 (d, $J = 8.7$ Hz, 1H), 7.36 (d, $J = 7.0$ Hz, 1H), 7.26 (d, $J = 7.7$ Hz, 1H), 7.22 (s, 1H), 6.98 (d, $J = 13.1$ Hz, 1H), 6.70 (s, 1H), 6.63 (d, $J = 9.9$ Hz, 1H), 5.71 (dd, $J = 13.3$, 10.1 Hz, 1H), 4.62 (q, $J = 6.8$ Hz, 1H), 4.52 (td, $J = 9.9$, 4.9 Hz, 1H), 4.41 (s, 1H), 4.29–4.22 (m, 4H), 4.04 (t, $J = 9.0$ Hz, 1H), 3.99 (dd, $J = 8.7$, 7.3 Hz, 1H), 3.89 (t, $J = 8.4$ Hz, 1H), 3.80 (t, $J = 7.5$ Hz, 1H), 3.68–3.66 (m, 2H), 3.66–3.56 (m, 4H), 3.32–3.28 (m, 1H), 3.23–3.18 (m, 1H), 3.09–3.05 (m, 1H), 2.85 (t, $J = 2.6$ Hz, 1H), 2.71–2.67 (m, 1H), 2.56 (ddd, $J = 16.9$, 10.0, 2.6 Hz, 1H), 2.32–2.27 (m, 1H), 2.25–2.19 (m, 2H), 2.09–2.05 (m, 1H), 2.02–1.94 (m, 2H), 1.93–1.83 (m, 6H), 1.81–1.68 (m, 4H), 1.65–1.57 (m, 4H), 1.57–1.50 (m, 3H), 1.49–1.36 (m, 6H), 1.30–1.25 (m, 1H), 1.23 (s, 1H), 1.22–1.17 (m, 1H), 1.16–1.09 (m, 2H), 1.07–1.00 (m, 1H), 0.94–0.90 (m, 1H), 0.90–0.88 (m, 9H), 0.86 (d, $J = 6.6$ Hz, 3H), 0.85–0.80 (m, 24H), 0.78 (d, $J = 6.7$ Hz, 3H), 0.71 (t, $J = 7.4$ Hz, 3H); ¹³C NMR (176 MHz, DMSO-*d*₆) δ 173.4, 172.7, 172.2, 172.2, 172.2, 171.9, 171.8, 171.5, 171.4, 171.2, 171.1, 167.3, 161.7, 141.4, 99.3, 81.7, 72.2, 68.8, 65.4, 64.0, 62.6, 61.7, 60.0, 59.4, 58.8, 58.2, 56.7, 52.4, 50.2, 49.2, 48.7, 47.0, 46.2, 41.2, 40.9, 38.1, 37.5, 36.2, 35.7, 35.4, 32.5, 29.6, 28.5, 26.3, 25.7, 25.6, 25.1, 24.9, 24.7, 24.6, 24.0, 23.2, 22.5, 22.1, 21.4, 21.3, 15.6, 15.4, 15.3, 15.3, 14.5, 14.0, 11.3, 11.2, 10.5, 10.3, 9.9; LC main peak retention time: 8.41 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1375.8712, found: 1375.8708 (−0.3 ppm); calculated for $[M+2H]^{2+}$: 688.4392, found: 688.4384 (−1.2 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 940.5866, found: 940.5852 (−1.5 ppm).

**CalB_C5Pra (83)**

The linear precursor peptide of **83** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 48 mg (31 μmol, 31%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 6.6 mg (4.3 μmol, 13.9%) of **83** in a total yield of 4.3% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.42 min; HRMS (ESI): m/z calculated for $[\text{M}+\text{H}]^+$: 1520.9451, found: 1520.9440 (−0.7 ppm); calculated for $[\text{M}+2\text{H}]^{2+}$: 760.9762, found: 760.9751 (−1.4 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}_{\text{ring}}+\text{H}]^+$: 940.5866, found: 940.5825 (−4.4 ppm).

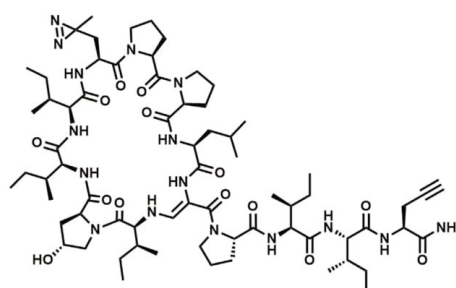
CalB_R3phLeu (84)

The linear precursor peptide of **84** was prepared via microwave-assisted SPPS up to the R2 position using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. The loading of the resin was quantified through a spectrometric Fmoc-Test and a manual coupling of photo-leucine was performed using 4 equivalents of amino acid combined with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) and hydroxybenzotriazole (HOBt) (each 4 equivalents) as well as 5 equivalents of DIPEA in 10 mL DMF by shaking the suspension over night at room temperature. The resin was then thoroughly washed with DMF and a second round of microwave-assisted SPPS was performed for completion of the peptide sequence. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 38 mg (27 μmol, 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, 36 mg of peptide was dissolved in 5 mL

of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 11.8 mg (8.5 μ mol, 33.6%) of **84** in a total yield of 8.5% (calculated in relation to initial resin capacity) as a white powder.

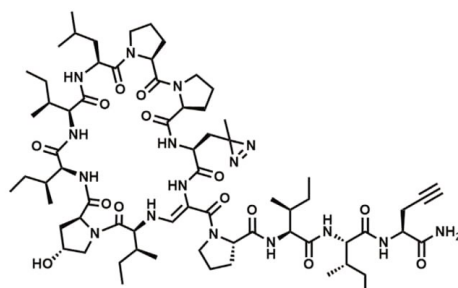
¹H NMR (400 MHz, DMSO-*d*₆) δ 9.13 (d, *J* = 6.4 Hz, 1H), 8.19 (s, 1H), 8.06 (d, *J* = 8.2 Hz, 1H), 7.88 (d, *J* = 8.6 Hz, 1H), 7.51 (d, *J* = 8.8 Hz, 1H), 7.49 (d, *J* = 7.4 Hz, 1H), 7.26 (d, *J* = 7.9 Hz, 1H), 7.21 (s, 1H), 7.00 (d, *J* = 13.1 Hz, 1H), 6.74 (s, 1H), 6.67 (d, *J* = 10.2 Hz, 1H), 5.60 (dd, *J* = 13.1, 10.2 Hz, 1H), 5.43 (d, *J* = 3.0 Hz, 1H), 4.67 (q, *J* = 7.0 Hz, 1H), 4.52–4.46 (m, 1H), 4.45 (s, 1H), 4.36–4.31 (m, 1H), 4.31–4.21 (m, 3H), 4.07–3.95 (m, 3H), 3.92 (t, *J* = 8.3 Hz, 1H), 3.79–3.73 (m, 1H), 3.72–3.64 (m, 2H), 3.63–3.51 (m, 5H), 3.50–3.44 (m, 1H), 3.27–3.18 (m, 1H), 2.85 (t, *J* = 2.6 Hz, 1H), 2.73–2.65 (m, 1H), 2.60–2.52 (m, 1H), 2.42–2.31 (m, 1H), 2.27–2.17 (m, 3H), 2.11–2.02 (m, 3H), 2.01–1.93 (m, 4H), 1.92–1.83 (m, 2H), 1.81–1.76 (m, 1H), 1.75–1.70 (m, 1H), 1.70–1.54 (m, 7H), 1.52–1.40 (m, 5H), 1.39–1.33 (m, 2H), 1.25–1.13 (m, 2H), 1.10–1.04 (m, 1H), 1.02 (s, 3H), 1.00–0.95 (m, 1H), 0.92 (d, *J* = 6.3 Hz, 3H), 0.90 (d, *J* = 6.3 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 3H), 0.86–0.79 (m, 21H), 0.79–0.77 (m, 1H), 0.76–0.70 (m, 6H); LC main peak retention time: 8.00 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1387.8460, found: 1387.8449 (–0.8 ppm); calculated for [M+2H]²⁺: 694.4267, found: 694.4256 (–1.6 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 952.5615, found: 952.5581 (–3.6 ppm).

CalB_R5phLeu (85)



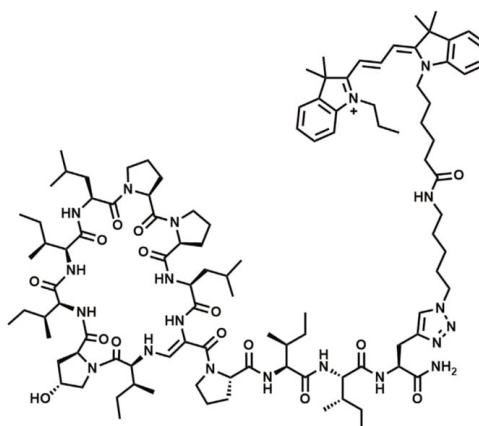
The linear precursor peptide of **85** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 42 mg (30 μ mol, 30%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 11.2 mg (8.1 μ mol, 26.8%) of **85** in a total yield of 8.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.32 min; HRMS (ESI): *m/z* calculated for [M+H]⁺: 1387.8460, found: 1387.8451 (–0.6 ppm); calculated for [M+2H]²⁺: 694.4267, found: 694.4267 (–2.7 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 952.5615, found: 952.5582 (–3.5 ppm).

**CalB_R8phLeu (86)**

The linear precursor peptide of **86** was prepared via microwave-assisted SPPS up to the R2 position using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. The loading of the resin was quantified through a spectrometric Fmoc-Test and a manual coupling of photo-leucine was performed using 4 equivalents of amino acid combined with HBTU and HOBt (each 4 equivalents) as well as 5 equivalents of DIPEA in 10 mL DMF by shaking the suspension over night at room temperature. The resin was then thoroughly washed with DMF and a second round of microwave-assisted SPPS was performed for completion of the peptide sequence. After purification by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 19 mg (13 μ mol, 13%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 6.5 hours at RT and immediately purified by HPLC (solvent A: H₂O; solvent B: ACN) to give 1.4 mg (1.0 μ mol, 7.6%) of **86** in a total yield of 1.0% (calculated in relation to initial resin capacity) as a white powder.

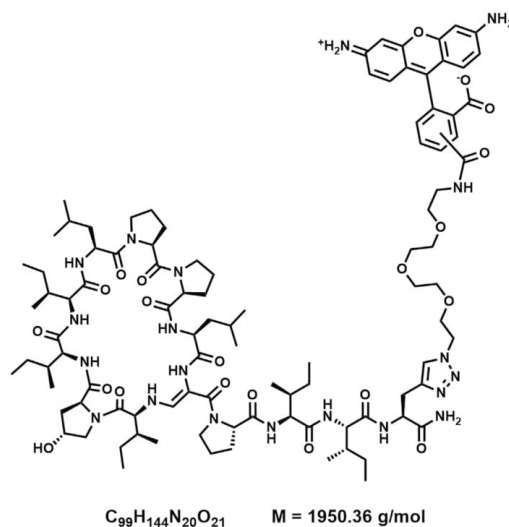
LC main peak retention time: 8.13 min; HRMS (ESI): m/z calculated for $[M+H]^+$: 1387.8460, found: 1387.8451 (−0.6 ppm); calculated for $[M+2H]^{2+}$: 694.4267, found: 694.4256 (−1.6 ppm); MS² HRMS (ESI, HCD): m/z calculated for ring fragment $[M_{\text{ring}}+H]^+$: 952.5615, found: 952.5597 (−1.9 ppm).

CalB_C4Pra(Cy3) (87)

For the copper-catalyzed azide-alkyne cycloaddition (CuAAC), 12.2 mg of **82** were dissolved in 1 mL of ACN and 1.8 equivalents of Cy3-azide, 0.4 eq. Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amin (TBTA) as well as 4 eq. of DIPEA were added. To start the reaction, 0.6 eq. of CuI were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 26 hours at room temperature. Another 0.1 equivalents of CuI were added and shaking was continued overnight. The crude product was purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 13.3 mg (6.4 μmol) of **87** in a total yield of 72% as a deep purple powder.

LC main peak retention time: 6.04 min; HRMS (ESI): *m/z* calculated for M⁺: 1971.2792, found: 1971.2798 (0.3 ppm); calculated for [M⁺+H]²⁺: 986.1432, found: 986.1426 (−0.6 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M⁺_{ring}+H]⁺: 940.5866, found: 940.5839 (−2.9 ppm).

CalB_C4Pra(Rhodamine) (**88**)

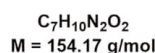
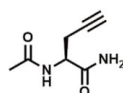
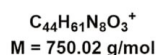
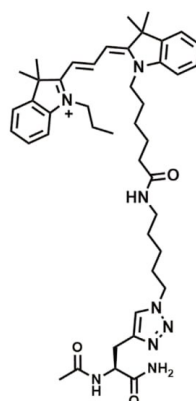


For CuAAC, 1.0 mg of 5/6-Carboxy-rhodamine-azide (CLK-AZ105, Jena Bioscience) was dissolved in 300 μL ACN and 1.6 eq. of **82**, 0.4 eq. TBTA as well as 4 eq. of DIPEA were added. To start the reaction, 0.6 eq. of CuI were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 24 hours at room temperature. The crude product was purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 1.4 mg (0.7 μmol) of **88** in a total yield of 25% as a brown powder.

LC main peak retention time: 4.64 min; HRMS (ESI): *m/z* calculated for M⁺: 1951.0922, found: 1951.0919 (−0.2 ppm); calculated for [M+H]²⁺: 976.0497, found: 976.0497 (−0.5 ppm); MS² HRMS (ESI, HCD): *m/z* calculated for ring fragment [M_{ring}+H]⁺: 940.5866, found: 940.5829 (−3.9 ppm).



Cy3-Control (89)

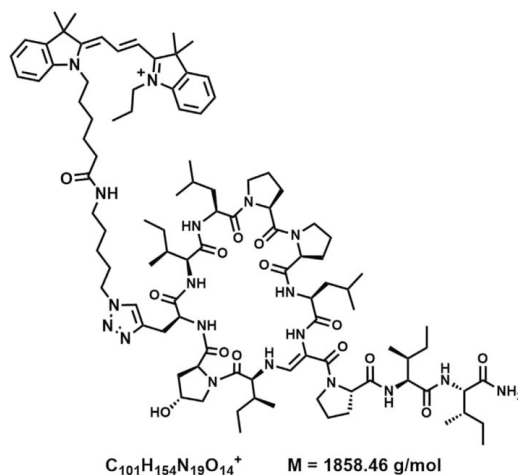


N-Fmoc-L-propargylglycine (CC05047, Carbolution) was amidated and acetylated via an automated microwave-assisted protocol in a Liberty Blue peptide synthesizer (CEM) using Rink-Amide PS resin and the obtained amino acid building block **89a** was purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.04 (d, *J* = 8.2 Hz, 1H), 7.42 (s, 1H), 7.12 (s, 1H), 4.32 (td, *J* = 8.0, 5.6 Hz, 1H), 2.82 (t, *J* = 2.7 Hz, 1H), 2.58-2.52 (m, 1H), 2.46-2.36 (m, 1H), 1.86 (s, 3H); ¹³C NMR (101 MHz, DMSO) δ 171.82, 169.20, 80.88, 72.69, 51.25, 22.53, 21.76; HRMS (ESI): *m/z* calculated for [M+H]⁺: 155.0815, found: 155.0813 (−1.3 ppm);

For CuAAC, 5.0 mg of Cy3-Azide (dissolved in DMSO) was mixed with 1.5 eq. of **89a** and 0.2 eq. TBTA as well as 4 eq. of DIPEA were added. To start the reaction, 0.6 eq. of CuI were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 24 hours at room temperature. The crude product was purified by HPLC (solvent A: H₂O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 6.1 mg (7.1 μmol) of **89** in a total yield of 84% as a deep purple powder.

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.36 (t, *J* = 13.4 Hz, 1H), 8.01 (d, *J* = 8.4 Hz, 1H), 7.76-7.70 (m, 2H), 7.64 (dd, *J* = 7.5, 4.0 Hz, 2H), 7.52-7.40 (m, 4H), 7.38 (s, 1H), 7.33-7.26 (m, 2H), 7.04 (s, 1H), 6.52 (dd, *J* = 13.4, 8.7 Hz, 2H), 4.43 (td, *J* = 8.7, 5.1 Hz, 1H), 4.27 (t, *J* = 7.0 Hz, 2H), 4.11 (t, *J* = 7.3 Hz, 4H), 3.04 (dd, *J* = 14.7, 5.1 Hz, 1H), 3.00-2.92 (m, 2H), 2.82 (dd, *J* = 14.7, 9.0 Hz, 1H), 2.05 (t, *J* = 7.3 Hz, 2H), 1.81-1.79 (m, 3H), 1.79-1.71 (m, 6H), 1.70 (s, 6H), 1.69 (s, 6H), 1.55 (p, *J* = 7.4 Hz, 2H), 1.41-1.29 (m, 4H), 1.19-1.10 (m, 2H), 0.98 (t, *J* = 7.4 Hz, 3H); LC main peak retention time: 5.85 min; HRMS (ESI): *m/z* calculated for M⁺: 749.4861, found: 749.4857 (−0.5 ppm); calculated for [M+H]²⁺: 375.2467, found: 375.2463 (−1.1 ppm).

CalB_R3Pra(Cy3) (90)

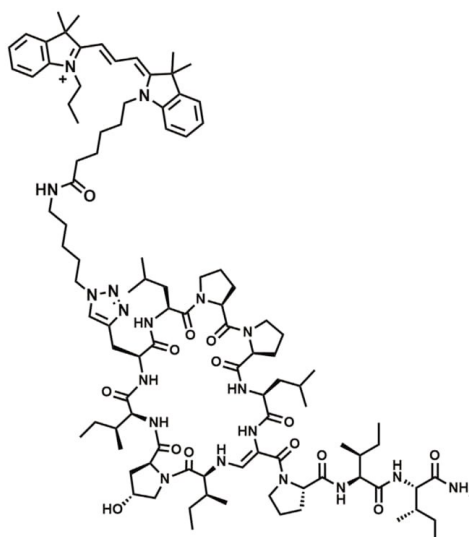


For CuAAC, 4.3 mg of **78** were dissolved in 100 μL of ACN and 1.2 equivalents of Cy3-azide (500 mM DMSO stock), 0.3 eq. TBTA (5 mM stock) as well as 4 eq. of DIPEA were added. To start the reaction, 0.5 eq. of CuI (10 mM ACN stock) were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 4 hours at room temperature. Another 0.1 eq. of CuI were added and shaking was continued overnight. The crude product was purified by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 0.6 mg (0.3 μmol) of **90** in a total yield of 9% as a deep purple powder.

LC main peak retention time: 5.36 min; HRMS (ESI): m/z calculated for M^+ : 1858.1951, found: 1858.1942 (−0.5 ppm); calculated for $[\text{M}^+ + \text{H}]^{2+}$: 929.6012, found: 929.6005 (−0.8 ppm); MS^2 HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}^+_{\text{ring}} + \text{H}]^{2+}$: 758.9758, found 758.9727 (−4.1 ppm).



CalB_R4Pra(Cy3) (91)



For CuAAC, 1.2 mg of **79** were dissolved in 100 μL of ACN and 1.3 equivalents of Cy3-azide (500 mM DMSO stock), 0.5 eq. TBTA (5 mM stock) as well as 4 eq. of DIPEA were added. To start the reaction, 0.5 eq. of CuI (10 mM ACN stock) were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 4 hours at room temperature. Another 0.25 eq. of Cy3-azide as well as 0.2 eq. of CuI were added and shaking was continued overnight. The crude product was purified by HPLC (solvent A: H_2O , acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 0.9 mg (0.5 μmol) of **91** in a total yield of 49% as a deep purple powder.

LC main peak retention time: 4.98 min; HRMS (ESI): m/z calculated for M^+ : 1858.1951, found: 1858.1937 (−0.8 ppm); calculated for $[\text{M}^+ + \text{H}]^{2+}$: 929.6012, found: 929.5993 (−2.0 ppm); MS^2 HRMS (ESI, HCD): m/z calculated for ring fragment $[\text{M}^+_{\text{ring}} + \text{H}]^{2+}$: 758.9758, found 758.9746 (−1.6 ppm).

QUANTIFICATION AND STATISTICAL ANALYSIS

All figure legends provide details on the quantification type and statistical analysis, including the statistical tests used, the exact value of n or the definition of center and precision measures for each experiment.

For the quantification of proteomic data, the MaxLFQ⁵³ (whole proteome comparison) or Minora⁵⁴ (ABPP & A/BPP) label-free quantification algorithms were used. For this purpose, either the software MaxQuant 2.3.0.1. (whole proteome comparison) employing the Andromeda search engine⁵⁵ or Proteome Discoverer 2.5 (Thermo Fisher Scientific) employing the Sequest-HT algorithm⁵⁶ were used for protein search. Statistical analysis was performed using the software Perseus 2.0.7.0.⁵⁰ LFQ quantification data was transformed to a $\log_2$ -scale and filtered for consistently identified proteins. Missing LFQ data points were replaced with values from the lower bound of the data's normal distribution. Student's T-test with permutation-based FDR was used to identify changes, and protein groups combining a $\log_2$ difference of ≥ 1 or ≤ -1 with a P value equal to or less than 0.05 were generally considered to be significantly changed. For the generation of a protein-protein interaction network, protein groups combining a $\log_2$ difference of ≥ 0.5 or ≤ -0.5 with a q value equal to or less than 0.05 were used as input for the STRING database (v.11.5)⁴¹ and the resulting network was further processed using the Cytoscape 3.9.1 software.⁵⁷

Supplemental information

**The anti-tubercular callyaerins target
the *Mycobacterium tuberculosis*-specific
non-essential membrane protein Rv2113**

David Podlesainski, Emmanuel T. Adeniyi, Yvonne Gröner, Florian Schulz, Violetta Krisilia, Nidja Rehberg, Tim Richter, Daria Sehr, Huzhuyue Xie, Viktor E. Simons, Anna-Lene Kiffe-Delf, Farnusch Kaschani, Thomas R. Ioerger, Markus Kaiser, and Rainer Kalscheuer

Supplementary Figures

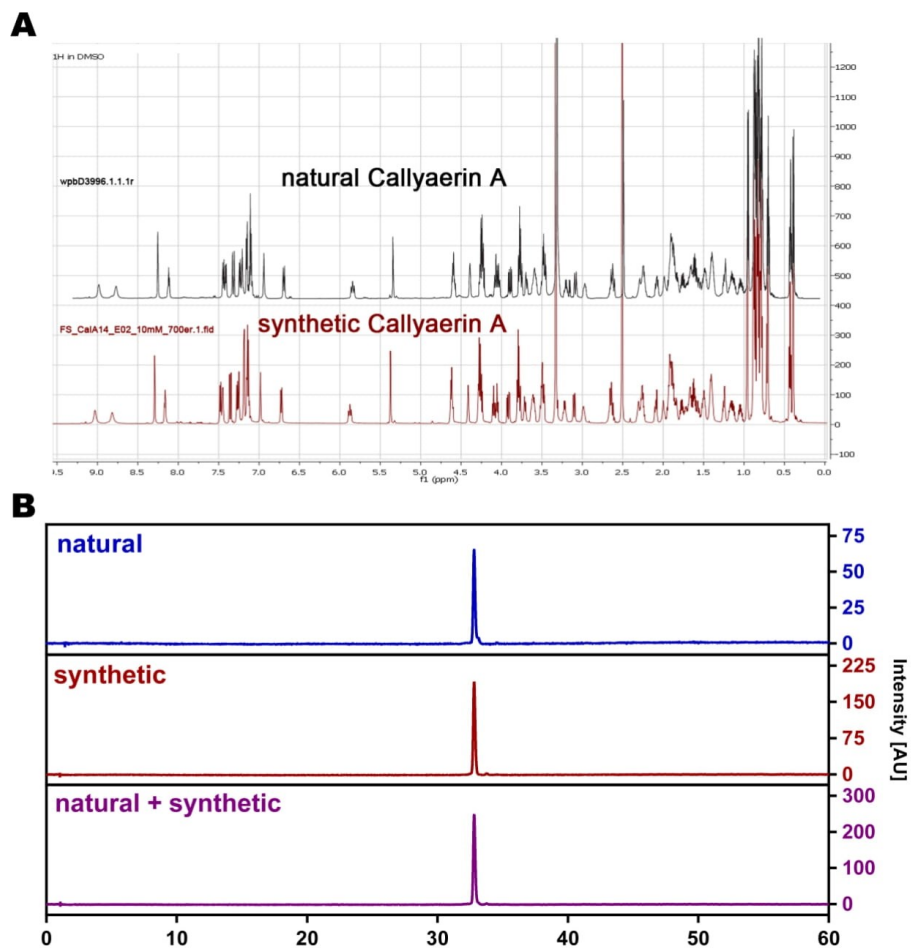


Figure S1: Comparing analysis of natural and synthetic Callyaerin A (CaA), related to Fig. 1. Superposition of ^1H NMR data (A) and LC-MS co-elution (B) of the isolated natural Callyaerin A (CaA) and our synthetic variant show structural similarity, thereby confirming the established synthetic approach.

Activity spectrum of CalA	MIC ₉₀ [μM]
Strain	CalA
<i>M. tuberculosis</i> mc ² 6230 ^a	1.56
<i>M. tuberculosis</i> H37Rv ^a	3.13
<i>M. tuberculosis</i> CDC 1551 ^a	3.13
<i>M. tuberculosis</i> KZN 06 ^a	12.5
<i>M. tuberculosis</i> KZN 13 ^a	12.5
<i>M. tuberculosis</i> KZN 14 ^a	12.5
<i>M. tuberculosis</i> KZN 16 ^a	6.25
<i>M. tuberculosis</i> Beijing-HN878 ^a	25
<i>M. bovis</i> AF2122/97 ^a	> 50
<i>M. bovis</i> BCG Pasteur ^a	> 50
<i>M. smegmatis</i> mc ² 155 ^a	>100
<i>M. marinum</i> ATCC 927 ^a	> 50
<i>S. aureus</i> ATCC 700699 ^b	>100
<i>H. sapiens</i> HEK293 ^c	50*
<i>H. sapiens</i> THP-1 ^d	50*
<i>H. sapiens</i> HepG2 ^e	50*

^aMiddlebrook 7H9 ^bMueller-Hinton ^cEMEM ^{*}IC₅₀ values
^dRPMI 1640 ^eHam's F12

Figure S2: Activity spectrum of CalA, related to Fig. 2. MIC₉₀ values of CalA for a variety of fast- and slow-growing mycobacterial strains including clinical isolates and for the nosocomial pathogen *S. aureus* as well as IC₅₀ values of human cell lines determined by resazurin dye reduction assay (single experiment with *n* = 2-3). Media used for cultivation of the respective cells are indicated by brown superscripted letters.

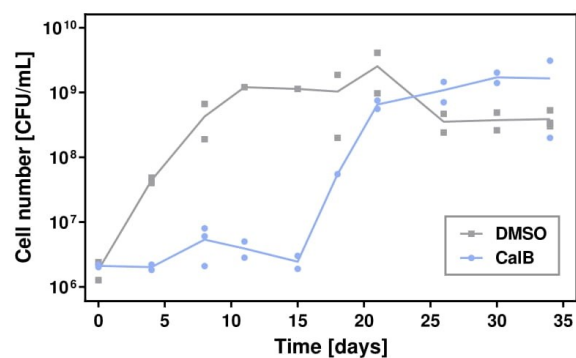


Figure S3: CalB shows bacteriostatic properties against *M. tuberculosis* mc²6230, related to Fig. 2. Overview on the killing kinetic of *M. tuberculosis* mc²6230 cells in presence of 15.7 μ M CalB (5-fold MIC₉₀, blue) or DMSO (grey) over a period of 35 days. Viable cell number was determined by plating serially diluted culture aliquots on Middlebrook 7H10 agar plates to quantify colony forming units (CFU) after incubation at 37 °C for three weeks. Each data point represents one replicate ($n = 2-3$) and the solid line connects the respective mean values.

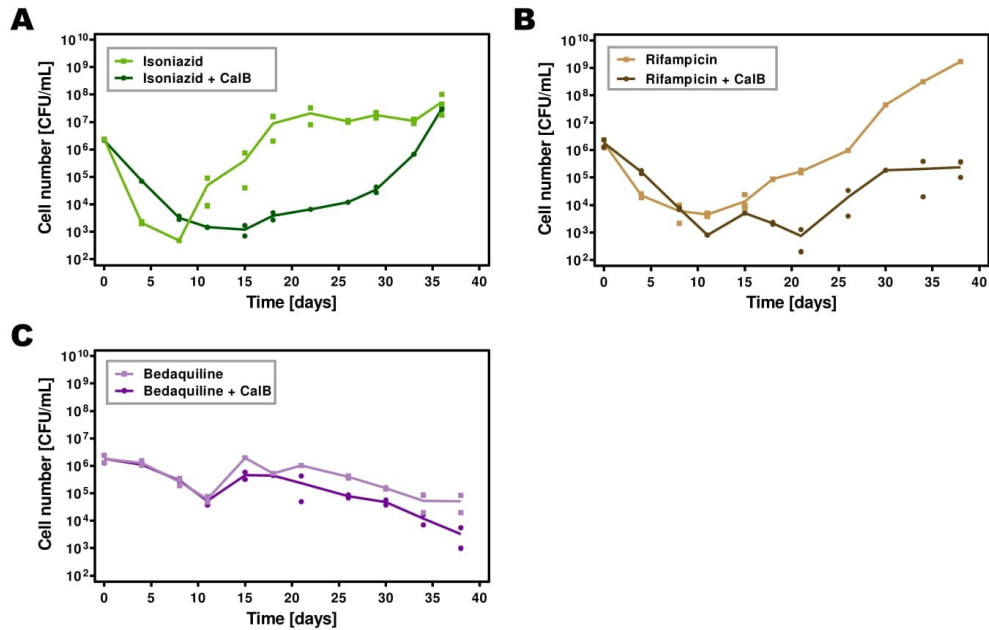


Figure S4: CalB shows additive effects in combination with clinically used anti-tubercular drugs and slows the emergence of resistant mutants to these agents, related to Fig. 2. Killing kinetic of *M. tuberculosis* mc²6230 treated with **A**) isoniazid (1.95 μ M, 5-fold MIC₉₀, green), **B**) rifampicin (0.5 μ M, 5-fold MIC₉₀, brown) or **C**) bedaquiline (1.95 μ M, 5-fold MIC₉₀, purple) in monotherapy (lighter color) or in combination with CalB (15.65 μ M, darker color) over a time course of 36 to 38 days. Viable cell number was determined by plating serially diluted culture aliquots on Middlebrook 7H10 agar plates to quantify colony forming units (CFU) after incubation at 37 °C for 3 weeks. Each data point represents one replicate ($n = 2$) and the solid line connects the respective mean values.

Combinatorial Effects				<i>M. tuberculosis</i> H37Rv			
anti-TB drug	MIC ₉₀ [μM]			FIC _A	FIC _B	FICI	Effect
	Drug	Drug + CalB (A)	CalB + Drug (B)				
CalB	3.13						
BDQ	0.39	0.200	3.13	0.5	1.0	1.5	additive
RIF	0.10	0.050	3.13	0.5	1.0	1.5	additive
INH	0.39	0.200	3.13	0.5	1.0	1.5	additive
EMB	3.13	1.560	3.13	0.5	1.0	1.5	additive
DEL	0.05	0.025	3.13	0.5	1.0	1.5	additive
Combinatorial Effects				<i>M. tuberculosis</i> mc ² 6230			
CalB	1.56						
BDQ	0.39	0.200	0.78	0.5	0.5	1.0	additive
RIF	0.05	0.025	1.56	0.5	1.0	1.5	additive
INH	0.20	0.100	1.56	0.5	1.0	1.5	additive
EMB	1.56	0.780	1.56	0.5	1.0	1.5	additive
DEL	0.05	0.025	1.56	0.5	1.0	1.5	additive

Figure S5: Determination of combinatorial effects of CalB with known anti-tubercular drugs, related to Fig. 2. Fractional inhibitory concentrations were determined via checkerboard assay ($n = 3$) through combination of a twofold serial dilution of an anti-tubercular drug (bedaquiline: BDQ, rifampicin: RIF, isoniazid: INH, ethambutol: EMB, delamanid: DEL) with different concentrations of CalB (A) or combination of a twofold serial dilution of CalB with different concentrations of the indicated anti-tubercular drugs (B) in *M. tuberculosis* H37Rv or mc²6230. All values are given in μM. Calculation of FIC: $FIC = MIC_{90} \text{ of drug} / \text{combinatorial } MIC_{90}$. Calculation of fractional inhibitory concentration indices (FICI) by addition of the respective FIC_A and FIC_B. FICI was defined as follows: synergistic effect when $FICI \leq 0.5$; partial synergistic effect when $0.5 < FICI \leq 0.75$; additive effect when $0.75 < FICI \leq 2$; antagonistic effect when $FICI > 2$.

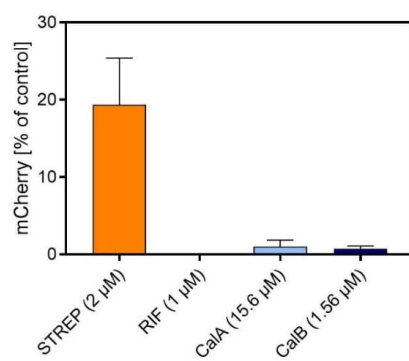


Figure S6: Quantification of integrated fluorescence intensity in a macrophage infection assay, related to Fig. 2. The integrated density of red fluorescence (mCherry) from the obtained photomicrographs (shown as mean of three fields per view $\pm$ SEM) of cells treated with streptomycin (STREP, 2 μ M), rifampicin (RIF, 1 μ M), CalA (15.6 μ M) and CalB (1.95 μ M) was normalized to the signal of the DMSO-treated control (corresponding to 100% growth).

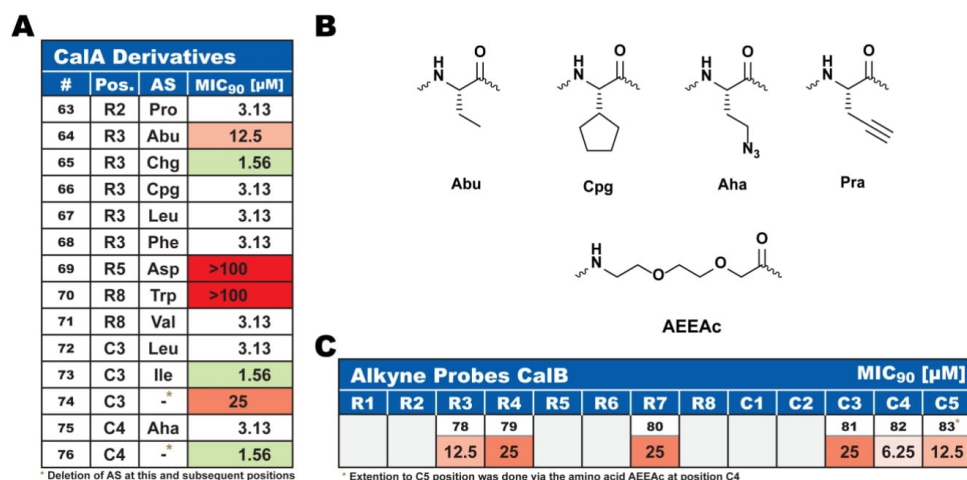


Figure S7: Structure-activity relationships for further CalA and CalB derivatives, related to Fig. 3. **A)** Additionally synthesized CalA derivatives and their corresponding MIC₉₀ values against *M. tuberculosis* H37Rv determined by resazurin dye reduction assay ($n = 2-3$). **B)** Chemical structures corresponding to the abbreviations used in A or B. **C)** Overview on the generated alkyne-modified CalB derivatives and their corresponding MIC₉₀ values (determined by resazurin dye reduction assay, $n = 2$) in *M. tuberculosis* H37Rv. The alkyne modification was incorporated into the compound structure via the amino acid propargyl glycine (Pra).

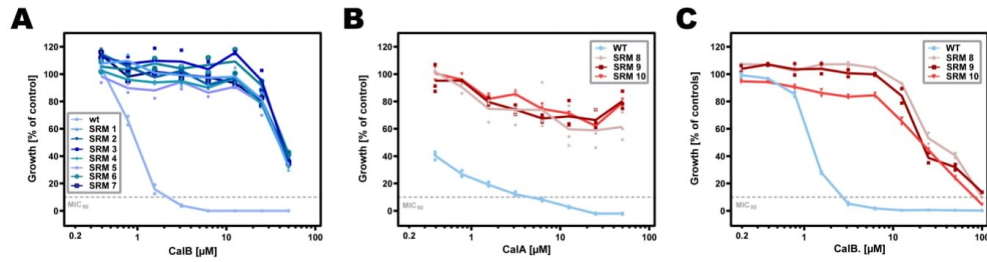


Figure S8: Dose-response curves for isolated spontaneous resistant mutants (SRM) of *M. tuberculosis* H37Rv, related to Fig. 4. **A)** Dose-response curves reporting CalB-dependent growth inhibition of SRM clones 1-7 isolated from CalB containing media plates compared to wild type (WT). **B)** Dose-response curves reporting CalA-dependent growth inhibition of SRM clones 8-10 isolated from CalA containing media plates compared to wild type (WT). **C)** Dose-response curves reporting CalB-dependent growth inhibition of SRM clones 8-10 isolated from CalA containing media plates compared to wild type (WT). This experiment validates cross-resistance of isolated SRM. Growth in A-C was quantified relative to untreated DMSO control (corresponding to 100% growth) and sterile medium control (0% growth) using resazurin dye reduction assay. Each data point in A-C represents one replicate ($n = 2-3$) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC_{90} threshold corresponding to a remaining growth of 10%.

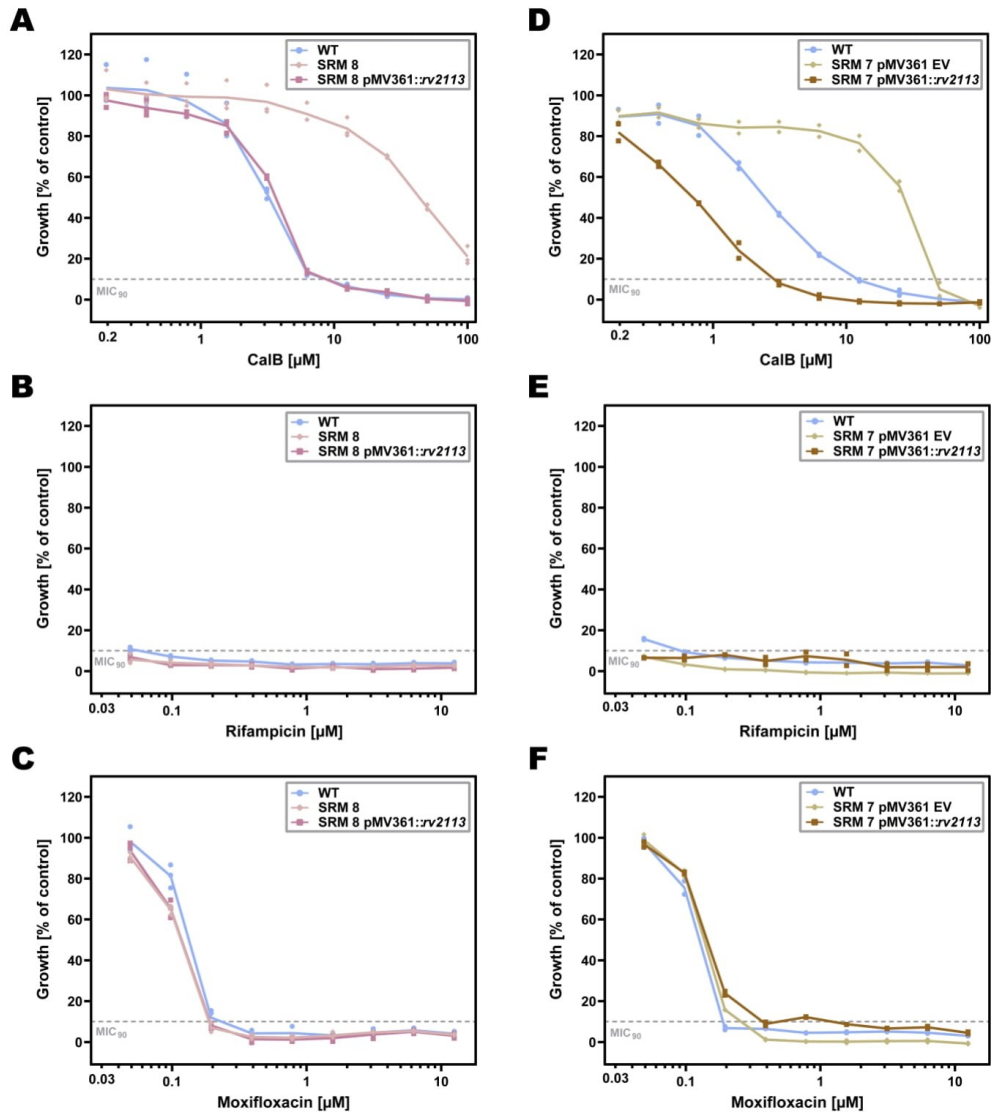


Figure S9: Resistance of isolated spontaneous resistant mutants (SRM) to CalB specifically rely on mutation of *rv2113*, related to Fig. 4. Dose-response curves of CalB (A), rifampicin (B), and moxifloxacin (C) against *M. tuberculosis* H37Rv wild type, the CalA-resistant SRM clone 8 and the complemented SRM clone 8 expressing *rv2113* (SRM 8 pMV361::rv2113). Dose-response curves of CalB (D), rifampicin (E), and moxifloxacin (F) against *M. tuberculosis* H37Rv wild type, the CalB-resistant SRM clone 7 pMV361 EV control and the complemented SRM clone 7 expressing *rv2113* (SRM 7 pMV361::rv2113). Growth in A-F was quantified relative to untreated DMSO control (corresponding to 100% growth) and sterile medium control (0% growth) using resazurin dye reduction assay. Each data point in A-F represents one replicate ($n = 2-3$) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC₉₀ threshold corresponding to a remaining growth of 10%.

9

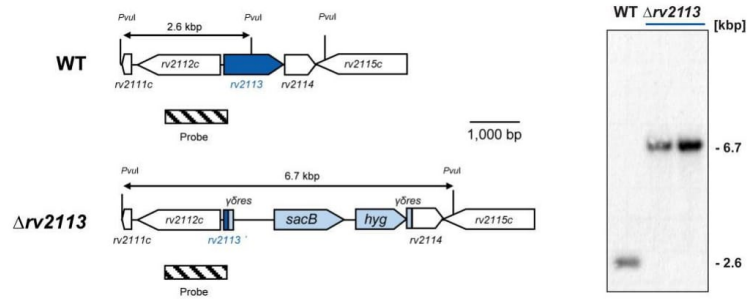


Figure S10: Overview on the cloning strategy enabling the generation of the site-specific $\Delta rv2113$ *M. tuberculosis* H37Rv gene deletion mutant, related to Fig. 4. A) Organization of the *rv2113* locus in *M. tuberculosis* wild type (WT) as well as in the $\Delta rv2113$ gene deletion mutant. The sizes of relevant fragments as well as the location of the probe used for Southern analysis are indicated. *γδ-res*: *res*-sites of the *γδ*-resolvase; *hyg*: hygromycin resistance gene; *sacB*: levansucrase gene from *Bacillus subtilis*. **B)** Southern analyses of *PvuI*-digested genomic DNA from cells of *M. tuberculosis* wild type as well as the $\Delta rv2113$ gene deletion mutant using a probe hybridizing to the position indicated in A, showing *rv2113* gene deletion.

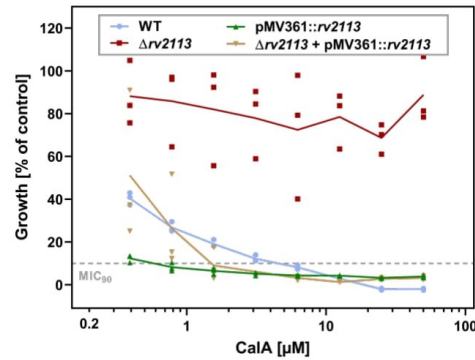


Figure S11: Dose-response curves for CalA concentration-dependent growth inhibition of *M. tuberculosis* H37Rv strains, related to Fig. 4. The assay included the wild type, the $\Delta rv2113$ gene deletion mutant, the complemented mutant strain $\Delta rv2113$ pMV361::rv2113 and the merodiploid strain overexpressing Rv2113 from plasmid pMV361::rv2113. Growth relative to untreated DMSO control (corresponding to 100%) and sterile medium control (0%) was quantified using resazurin dye reduction assay. Each data point represents one replicate ($n = 2-3$) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC₉₀ threshold corresponding to a remaining growth of 10%.

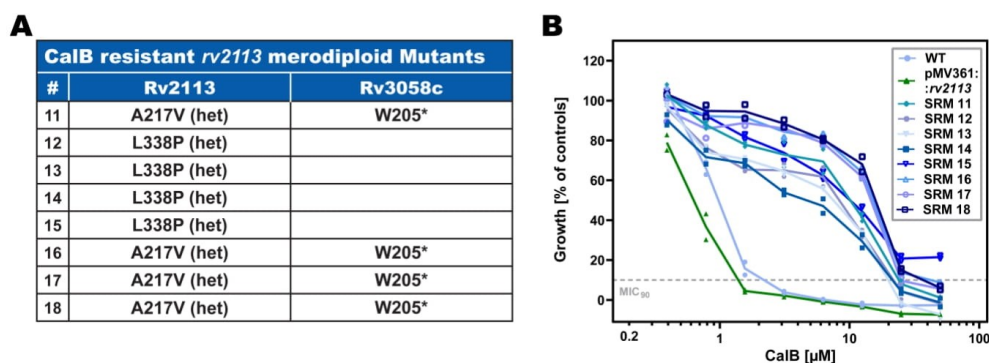


Figure S12: Overview on the results from the spontaneous CalB-resistant mutant screening raised in the merodiploid strain *M. tuberculosis* H37Rv pMV361::*rv2113* harboring two copies of the *rv2113* gene, related to Fig. 4. **A)** The identified spontaneous resistant mutants (SRMs) emerged at a frequency of $\sim 10^{-6}$ on 7H10 agar containing 15.7 μ M CalB (5-fold MIC₉₀) and 30 μ g/mL apramycin after 4-week incubation. The corresponding mutations were mapped via whole-genome sequencing. Heterogeneous results (het) were obtained for *rv2113*, meaning that the relevant SNP was found in ca. 50% of reads. This indicates that the mutation was only manifested either in the endogenous gene or in the merodiploid locus, which could not be distinguished. **B)** Dose-response curves of CalB against *M. tuberculosis* H37Rv wild type (WT), the merodiploid strain *M. tuberculosis* H37Rv pMV361::*rv2113* and isolated SRMs raised in the merodiploid strain *M. tuberculosis* H37Rv pMV361::*rv2113* as described in A. Growth relative to untreated DMSO control (100%) and sterile medium control (0%) was quantified using resazurin dye reduction assay. Each data point represents one replicate ($n = 2$) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC₉₀ threshold corresponding to a remaining growth of 10%.

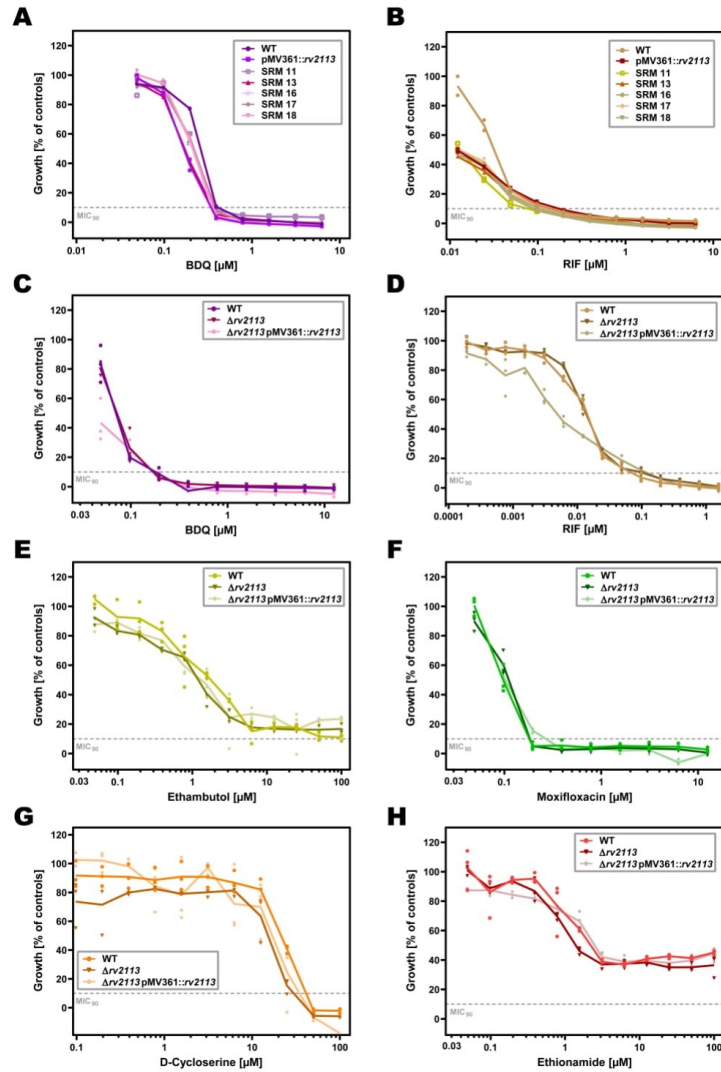


Figure S13: Susceptibility profiles to antitubercular drugs of *M. tuberculosis* H37Rv *rv2113* mutant strains, related to Fig. 4. Dose-response curves for **A)** bedaquiline (BDQ, purple) or **B)** rifampicin (RIF, brown) against wild type (WT) *M. tuberculosis* H37Rv, the merodiploid strain *M. tuberculosis* H37Rv pMV361::*rv2113* and the spontaneous CalB-resistant mutants (SRMs) raised in the merodiploid strain *M. tuberculosis* H37Rv pMV361::*rv2113* as reported in Figure S12. **C-H)** Dose-response curves for BDQ (**C**), RIF (**D**) ethambutol (**E**), moxifloxacin (**F**), D-cycloserine (**G**), or ethionamide (**H**) against wild type *M. tuberculosis* H37Rv, the *M. tuberculosis* H37Rv Δ *rv2113* gene deletion mutant, and the complemented mutant *M. tuberculosis* H37Rv Δ *rv2113* pMV361::*rv2113*. Growth relative to untreated DMSO control (corresponding to 100%) and sterile medium control (0%) was quantified using resazurin dye reduction assay. Each data point in **A-H** represents one replicate ($n = 2-3$) and the solid line connects the respective mean values. The horizontal gray dashed lines in **A-H** represent the MIC₉₀ threshold corresponding to a remaining growth of 10%.

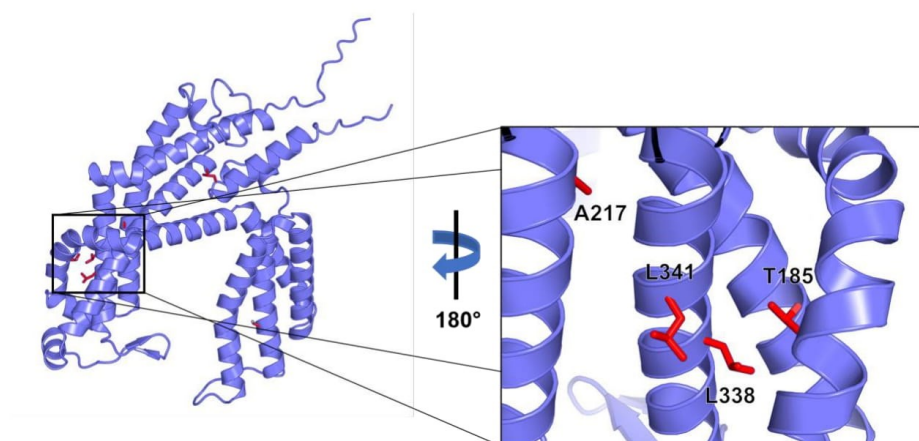


Figure S14: A significant number of identified mutation sites in Rv2113 cluster within a specific region of the protein structure predicted by AlphaFold, related to Fig. 4. Rv2113 model structure (AF-033248-F1) shown in cartoon illustration with amino acids altered in callyaerin resistant mutants of *M. tuberculosis* H37Rv WT or merodiploid pMV361::*rv2113* strain highlighted in red.

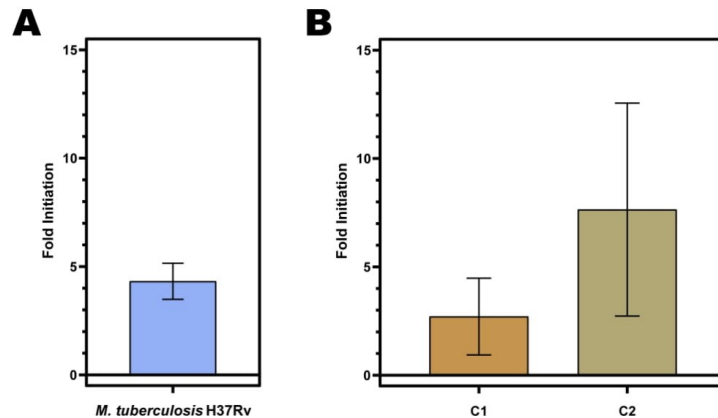


Figure S15, Quantitative analysis of *rv2113* and *BCG_2130* expression using RT-qPCR, related to Fig. 4. RT-qPCR was performed using complementary DNA synthesized from RNA samples of *M. bovis* BCG Pasteur WT, the recombinant *M. bovis* BCG Pasteur pMV361::*rv2113* strain, the recombinant empty vector control strain *M. bovis* BCG Pasteur pMV361::EV and *M. tuberculosis* H37Rv WT. 16sRNA was used as reference gene. **A)** Expression of *rv2113* in *M. tuberculosis* H37Rv WT was calculated with reference to *BCG_2130* expression in *M. bovis* BCG Pasteur WT. **B)** Combined expression level of *rv2113* + *BCG_2130* in a *M. bovis* BCG Pasteur strain heterologously expressing *rv2113* were normalized to expression of *BCG_2130* in the empty vector control strain *M. bovis* BCG Pasteur pMV361::EV. Because of identical sequences of the respective binding sites, the used primer pair could not discriminate between expression of *rv2113* and *BCG_2130*. Analysis of data was performed using the Livak method (Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25: 402-408). Data represents $n = 2 \pm SD$, obtained in two independent experiments.

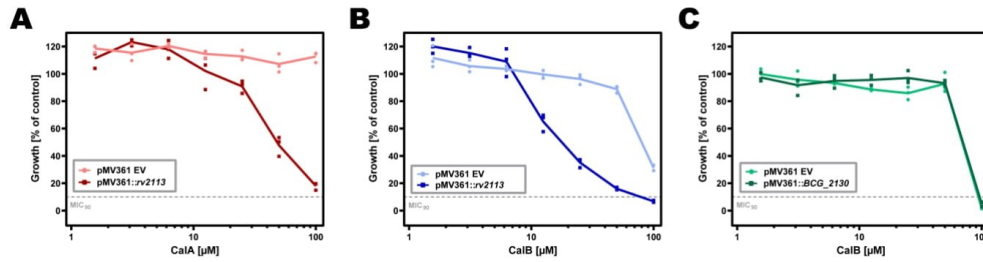


Figure S16: Expression of Rv2113 in *M. smegmatis* mc² 155 does not increase the bacterial sensitivity against Callyaerins significantly, related to Fig. 4. Dose-response curves of CalA (red, **A**) and CalB (blue, **B**) against *M. smegmatis* mc² 155 pMV361::rv2113 cell line (dark color) compared to the pMV361 empty vector control (EV, light color). **C:** Dose-response curves of CalB against *M. smegmatis* mc² 155 pMV361::BCG_2130 expressing the *M. bovis* BCG Pasteur's rv2113 homologue BCG_2130 (dark green) compared to the pMV361 empty vector control (EV, light green). Growth relative to untreated DMSO control (corresponding to 100%) and sterile medium control (0%) was quantified using resazurin dye reduction assay. Each data point represents one replicate ($n = 3$) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC_{90} threshold corresponding to a remaining growth of 10%.

CalB Diazirine Probes								MIC ₉₀ [μM]			
R1	R2	R3	R4	R5	R6	R7	R8	C1	C2	C3	C4
		84		85			86				Pra
		50		12.5			50				

Figure S17: Probe design by single amino acid substitutions with photoleucine, related to Fig. 5. Overview on synthesized photoactive CalB probes for the use in an affinity-based protein-profiling approach and corresponding MIC₉₀ values in *M. tuberculosis* H37Rv (determined by resazurin dye reduction assay, $n = 2$). The corresponding photoleucine has been introduced at different positions within the CalB core structure, while an alkyne ligation handle was introduced at C4 position via incorporation of propargylglycine (Pra).

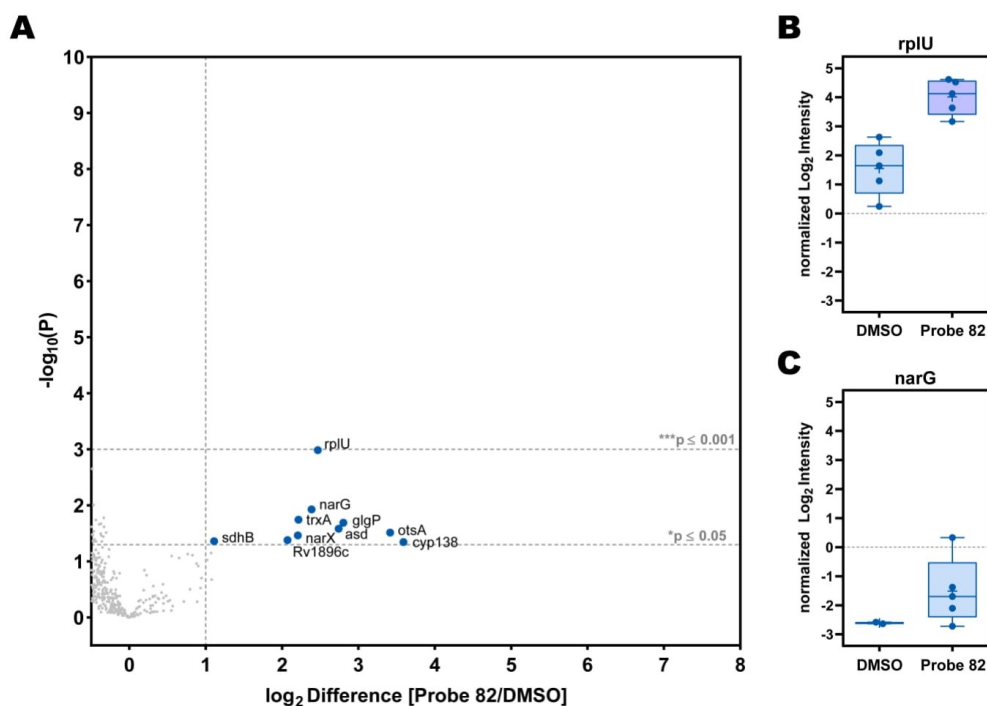


Figure S18: Activity based protein profiling with a CalB-based alkyne probe (82) shows no covalent interaction in *M. tuberculosis* H37Rv, related to Fig. 5. A) Proteomic data of *M. tuberculosis* H37Rv cells treated with 12.5 μ M probe **82** for 3 hours compared to a DMSO control. To identify statistically significant hits from the analysis, $P \leq 0.05$ (Student's T-test; permutation-based FDR with 250 randomizations and FDR = 0.01) was applied. Proteins complying with the chosen threshold of significance and showing a $\log_2$ -fold change ≥ 1 are marked in blue. Quantification via label free quantification (LFQ) algorithm of five replicates per sample group. Boxplot presentation (Min to Max, with all data points shown and respective mean value marked as cross) of quantification data from ABPP approach described in A for exemplary proteins B) *rplU* and C) *narG*.

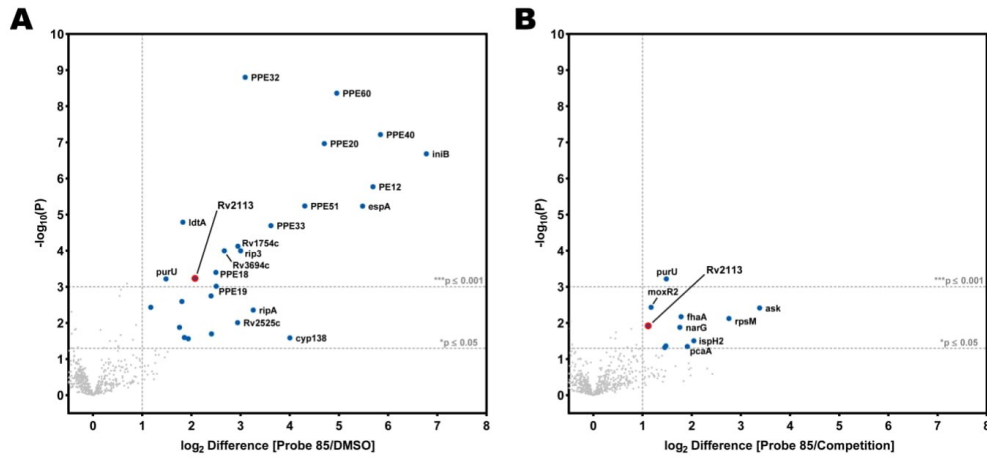


Figure S19: Affinity-based protein-profiling with a CalB-based photoaffinity-tagged probe (85) in combination with competitive labeling in *M. tuberculosis* H37Rv, related to Fig. 5. Proteomic data of *M. tuberculosis* H37Rv cells treated with 12.5 μ M probe **85** for 3 hours compared to a DMSO control (**A**) or a competitive labeling approach with preincubation with 30 μ M CalB (**B**). To identify statistically significant hits from the analysis, $P \leq 0.05$ (Student's T-test; permutation-based FDR with 250 randomizations and $FDR = 0.01$) was applied. Proteins complying with the chosen threshold of significance and showing a $\log_2$ -fold change ≥ 1 are marked in blue. Quantification was done via label free quantification (LFQ) algorithm of five replicates per sample group.

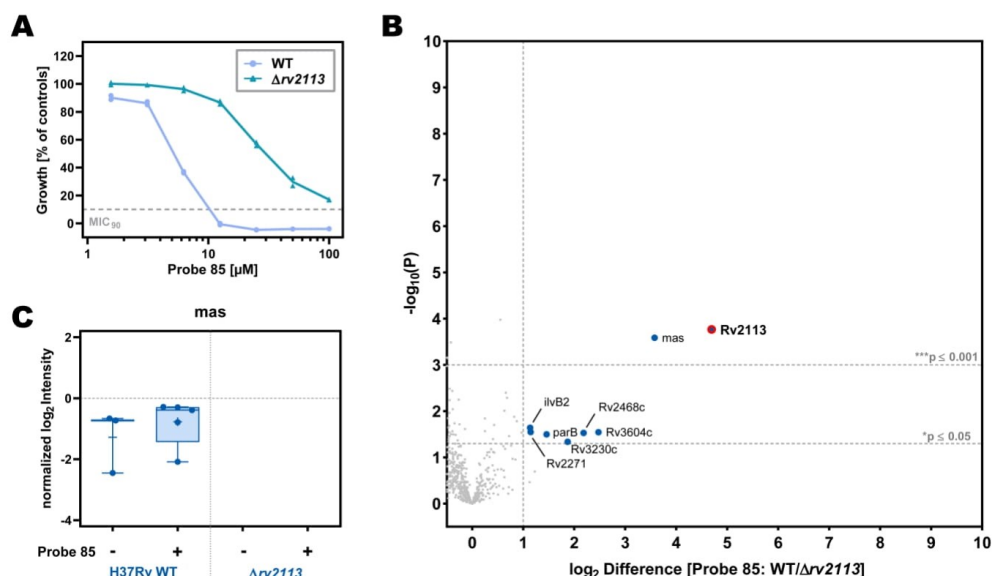


Figure S20: Photoaffinity labeling of WT in comparison to the $\Delta rv2113$ gene deletion mutant, related to Fig. 5. **A)** Dose-response curve for CalB-based photoaffinity-tagged probe (**85**) in *M. tuberculosis* H37Rv WT or $\Delta rv2113$ gene deletion mutant. Growth relative to untreated DMSO control (corresponding to 100%) and sterile medium control (0%) was quantified using resazurin dye reduction assay. Each data point represents one replicate ($n = 3$) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC₉₀ threshold corresponding to a remaining growth of 10%. **B)** Comparative affinity-based protein-profiling with **85** in *M. tuberculosis* H37Rv WT and $\Delta rv2113$ gene deletion mutant. Proteomic data of cells treated with 12.5 μ M probe **85** for 3 hours compared to a DMSO control. To identify statistically significant hits from the analysis, $P \leq 0.05$ (Student's T-test; permutation-based FDR with 250 randomizations and FDR = 0.01) was applied. Proteins complying with the chosen threshold of significance and showing a log₂-fold change ≥ 1 are marked in blue. Quantification was done via label free quantification (LFQ) algorithm of five replicates per sample group. **C)** Boxplot representation (Min to Max, with all data points shown and respective mean value marked as cross) of mas quantification data from the (photoaffinity) labelling experiments ($n = 5$ technical replicates).

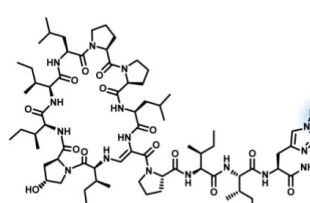
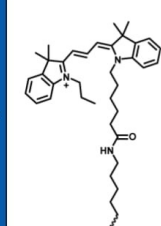
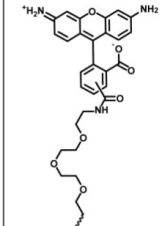
		
	87	88
	MIC ₉₀ [μM]	
	0.05	>100

Figure S21: A Cy3-tagged CalB derivative shows high activity against *M. tuberculosis* H37Rv, related to Fig. 6. Chemical structures and MIC₉₀ values against *M. tuberculosis* H37Rv (determined by resazurin dye reduction assay, $n = 2$) of fluorescently-tagged C4-modified CalB derivatives. Compound **87** correspond to a Cy3 tag and **88** represents a rhodamine tag.

CalB-Cy3 Conjugates											MIC ₉₀ [μM]
R1	R2	R3	R4	R5	R6	R7	R8	C1	C2	C3	C4
		90	91								87
		>1.56	>1.56								0.05

Figure S22: The beneficial effect of Cy3-conjugation relies on the location at the C-terminal tail of CalB, related to Fig. 6. Overview on synthesized Cy3-tagged CalB derivatives and corresponding MIC₉₀ values in *M. tuberculosis* H37Rv (determined by resazurin dye reduction assay, *n* = 2). The corresponding Cy3 tag has been introduced at different positions within the CalB core structure. Only incorporation at C4 leads to a highly active derivative.

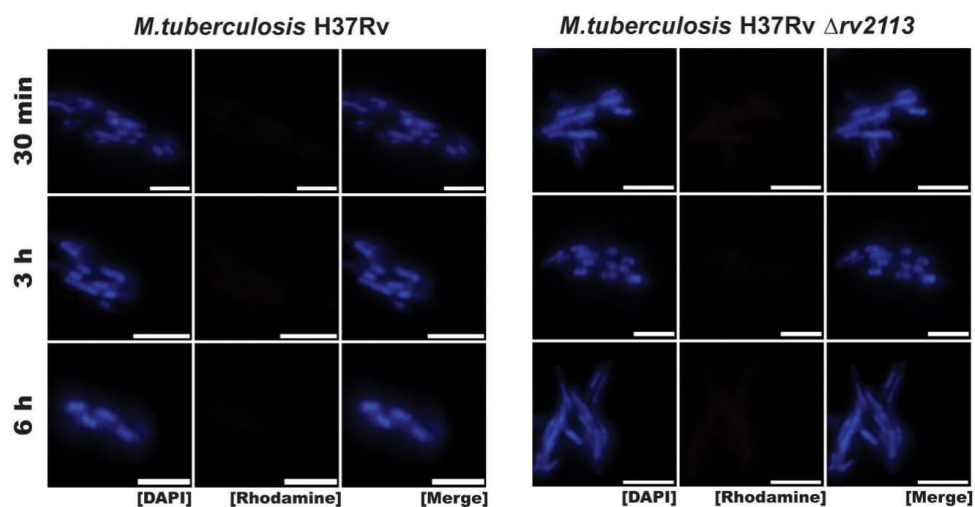


Figure S23: The inactive rhodamine-tagged CalB derivative 88 shows no intracellular fluorescence signal, related to Fig. 6. Fluorescence microscopy images of cells of *M. tuberculosis* H37Rv treated with 0.2 μ M of the rhodamine-tagged derivative **88** for the indicated time intervals. After incubation, treated cells were counterstained with DAPI to label all cells. Scale bar represents 3 μ m. Images are representative of a single experiment.

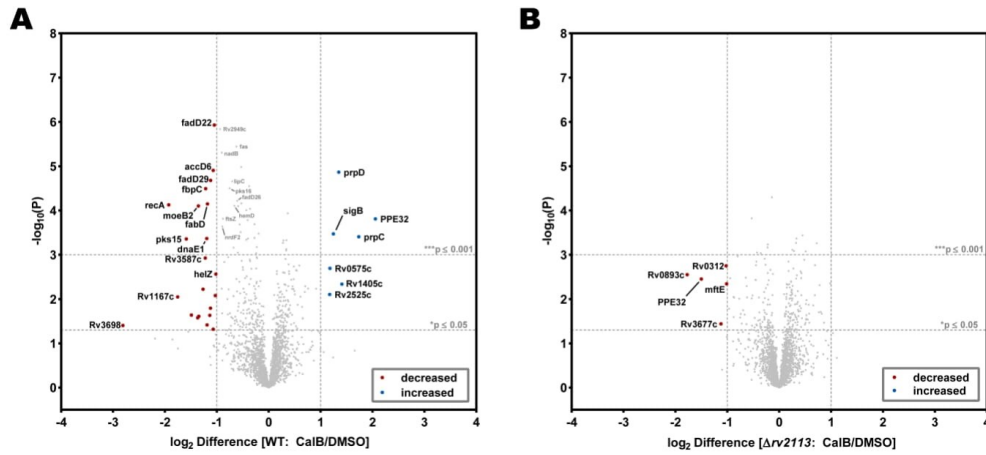


Figure S24: LC-MS/MS-based proteomic stress profiling of CalB-treated *M. tuberculosis* cells, related to Fig. 7. Whole protein analysis of cells of *M. tuberculosis* H37Rv wild type (WT, **A**) or the $\Delta rv2113$ gene deletion mutant (**B**) treated with 31.3 μ M CalB (10-fold MIC_{90}) for 48 hours compared to the corresponding DMSO control. To identify statistically significant hits from the analysis, $P \leq 0.05$ (Student's T-test; permutation-based FDR with 250 randomizations and FDR = 0.01) was applied. Proteins complying with the chosen threshold of significance and showing a $\log_2$ -fold change ≥ 1 or ≤ -1 are marked in blue or red, respectively. Quantification was done via label free quantification (LFQ) of five replicates per sample group.

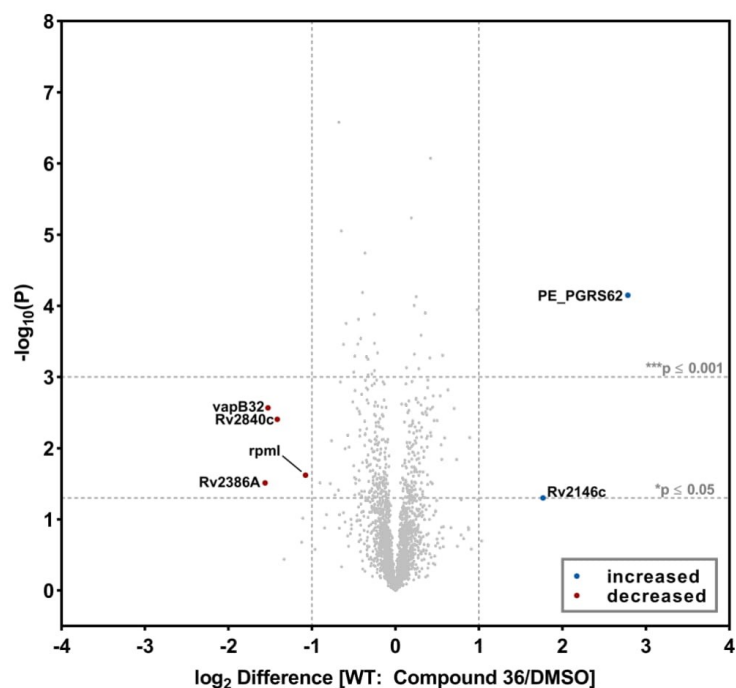


Figure S25: LC-MS/MS-based proteomic stress profiling of inactive derivative 36 against *M. tuberculosis* shows no distinct response on proteome level, related to Fig. 7. Whole protein analysis of *M. tuberculosis* H37Rv cells treated with 31.3 μ M derivative 36 for 48 hours were compared to a DMSO control. To identify statistically significant hits from the analysis, $P \leq 0.05$ (Student's T-test; permutation-based FDR with 250 randomizations and FDR = 0.01) was applied. Proteins complying with the chosen threshold of significance and showing a log₂-fold change ≥ 1 or ≤ -1 are marked in blue or red, respectively. Quantification was done via label free quantification (LFQ) algorithm of five replicates per sample group.

Supplementary Tables

Table S1. Oligonucleotides used for RT-qPCR, related to Fig. 7.

Gene	Forward primer	Reverse primer
<i>iniA</i>	CAAAGAGCTC AACGAAGAGT CC	TAGTTCGGAA CCTAGAGACA CC
<i>iniB</i>	GGGCATCGCT AGCCAGATCG	TAACGGGCCC GACAGATGAG G
<i>16SrRNA</i>	GGAATTACTG GGCGTAAAGA GC	CTCCTGATAT CTGCGCATTC C

Table S2. Oligonucleotides employed for the generation of allelic exchange substrates for site-specific gene deletion of *rv2113*, related to Fig. 4. The restriction sites used for cloning of allelic exchange substrates are underlined.

	5'-end primer	3'-end primer	Restr. site
Upstream flanking sequence	TTTTTCCATA <u>AATTGGACCT</u> GCCGGGATAC CAGAAAGGG	TTTTTCCATT <u>TCTGGCGAG</u> GGCAGACCAC GCTTCAAGAA C	Van91I
Downstream flanking sequence	TTTTTCCATA <u>GATTGGGCCG</u> GTCACGTCGA TGA TAGGTT C	TTTTTCCATC <u>TTTGGTCGC</u> TGGTAAGCAC GCGGGTATG	Van91I

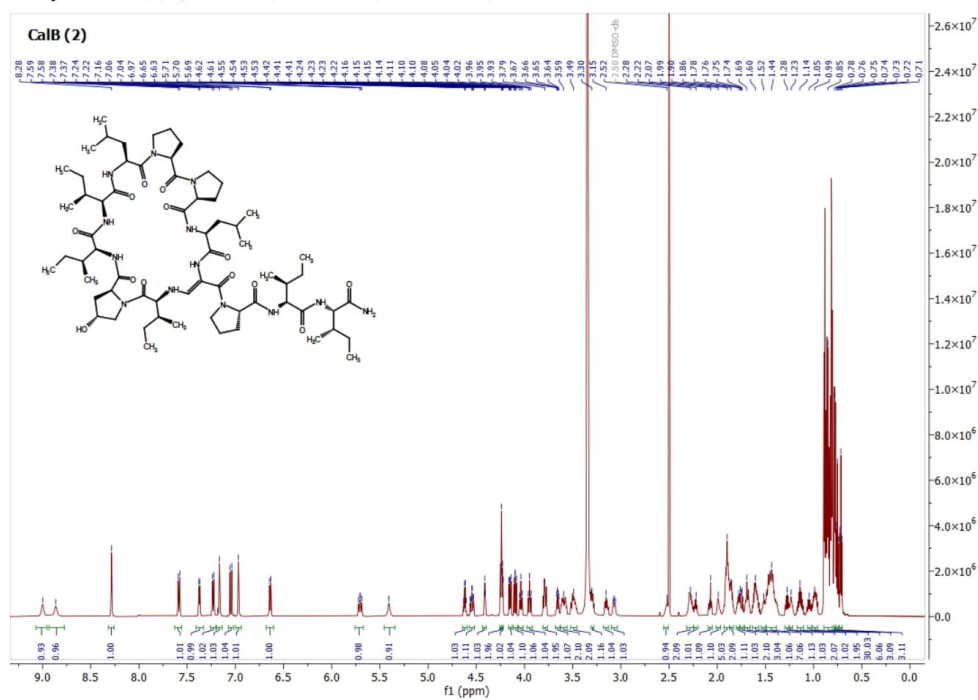
Table S3. Oligonucleotides used for cloning into mycobacterial expression plasmid pMV361, related to Fig. 4. Relevant restriction sites are underlined.

Gene	Forward primer	Restr. site	Reverse primer	Restr. site
<i>rv2113</i>	TTTTTTTAAT <u>TAATGAGCCT</u> TTCCGTCCGT CGC	<i>PacI</i>	TTTTTAAGCT <u>TCTAGTCATC</u> GACGTGACCG GCG	<i>HindIII</i>
<i>BCG_2130</i>	GGCCGCTTAA <u>TTAAATGAGC</u> CTTCCGTCC GTC	<i>PacI</i>	GAGCCGGAAG <u>CTTCTAGTCA</u> TCGACGTGAC C	<i>HindIII</i>

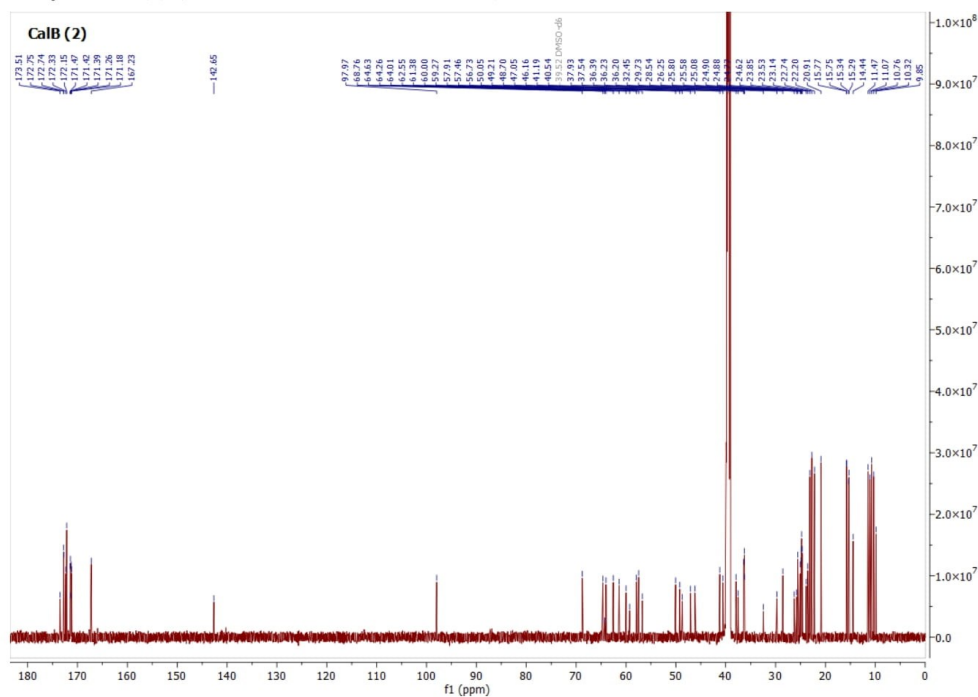
Data S1: ^1H and ^{13}C spectra of synthesized callyaerin derivatives, related to STAR Methods.

[illegible]

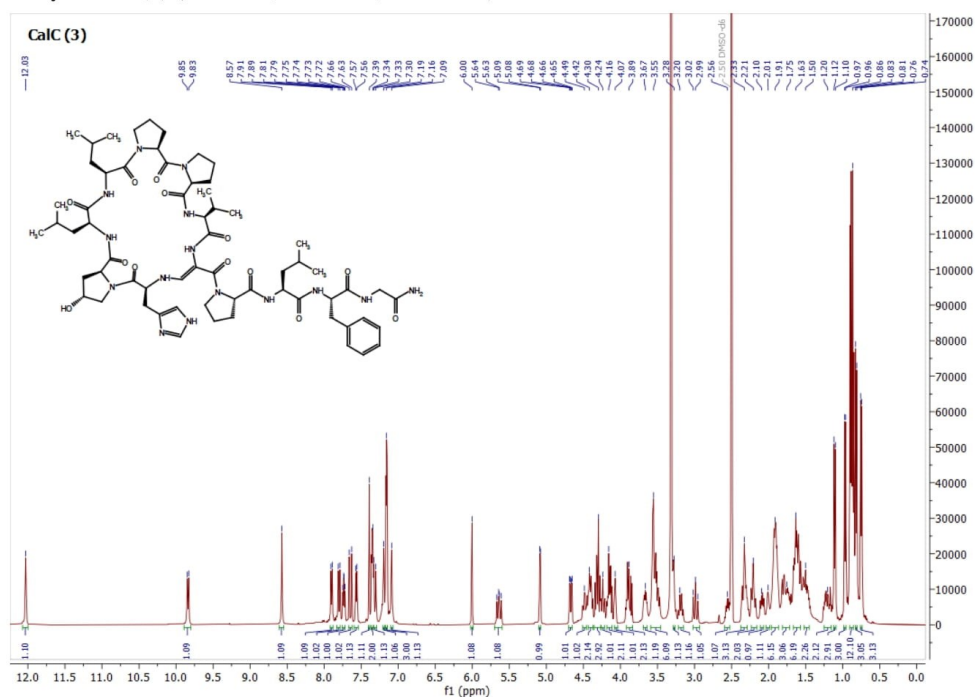
Callyaerin B (**2**) (^1H NMR; 700 MHz, DMSO-d_6)



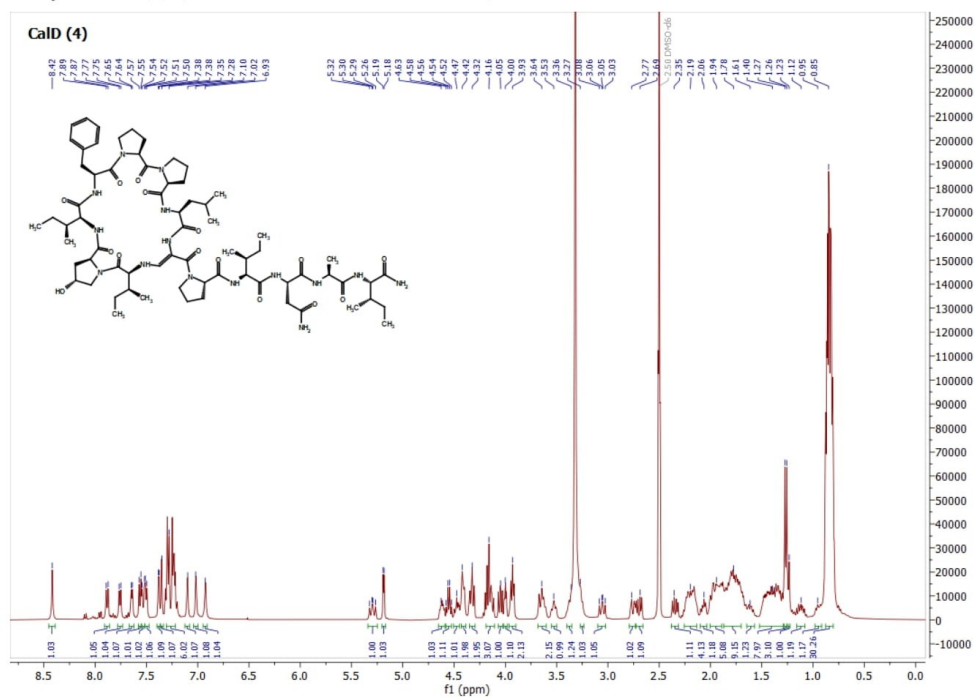
Callyaerin B (**2**) (^{13}C NMR; 176 MHz, DMSO-d_6)



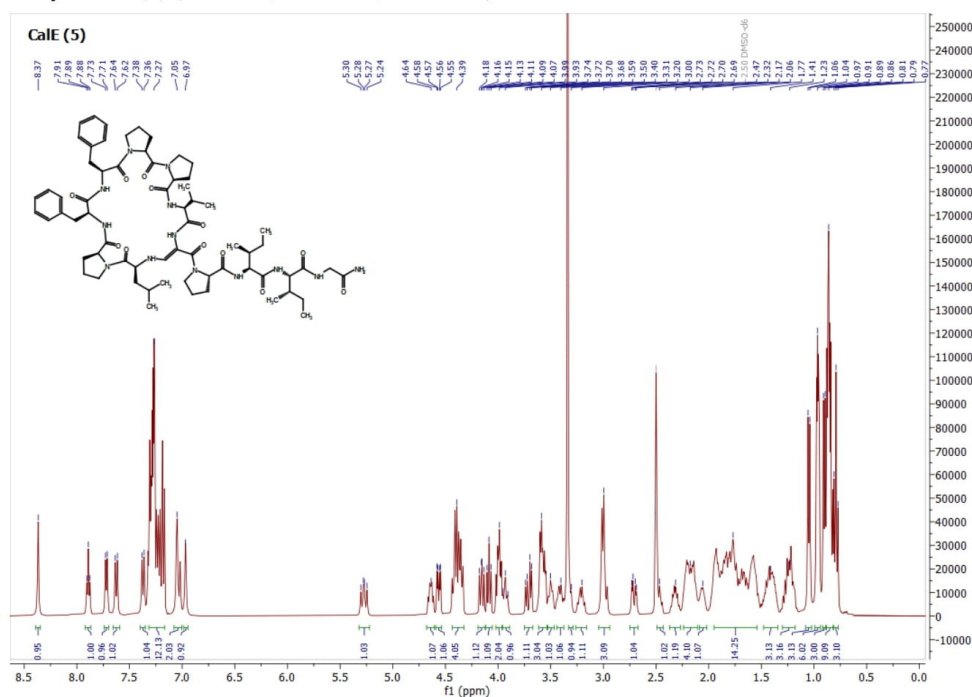
Callyaerin C (**3**) (^1H NMR; 400 MHz, DMSO-d_6)



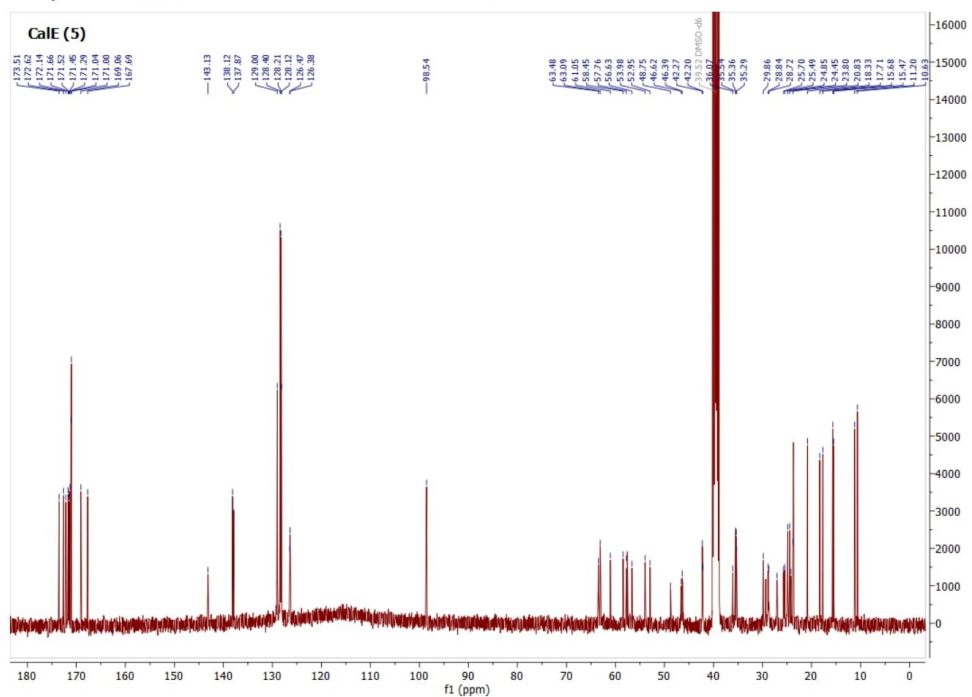
Callyaerin D (**4**) (^1H NMR; 400 MHz, DMSO-d_6)



Callyaerin E (**5**) (^1H NMR; 400 MHz, DMSO- d_6)



Callyaerin E (**5**) (^{13}C NMR; 101 MHz, DMSO- d_6)



[illegible]

CalG (7)

Chemical structure of compound 7 is shown in the top left corner.

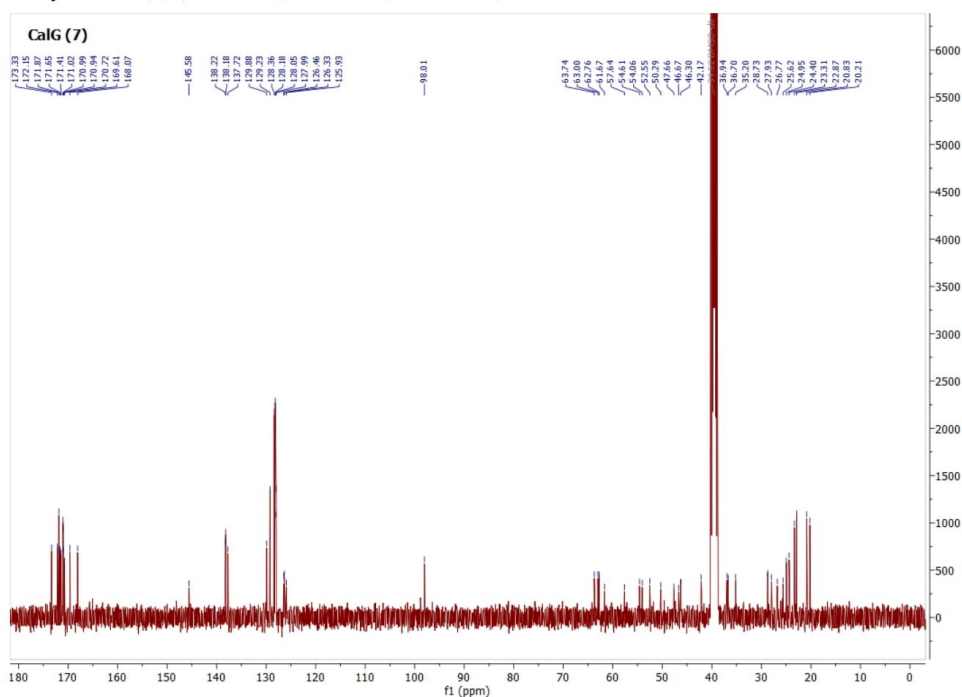
¹H NMR spectrum (CDCl₃) of compound 7. The x-axis represents the chemical shift in ppm (f1 (ppm)), ranging from 0.0 to 8.5. The y-axis represents the intensity in arbitrary units, ranging from 0 to 260,000.

Integration values (from left to right): 0.99, 1.00, 2.10, 1.00, 1.00, 1.10, 1.00, 16.07, 1.00, 1.00, 5.15, 1.00, 5.10, 2.67, 3.99, 1.18, 2.67, 1.07, 1.07, 1.14, 1.14, 3.63, 3.63, 7.99, 4.67, 5.08, 5.08, 1.22, 1.22, 2.65, 3.12, 3.12, 3.11, 3.99.

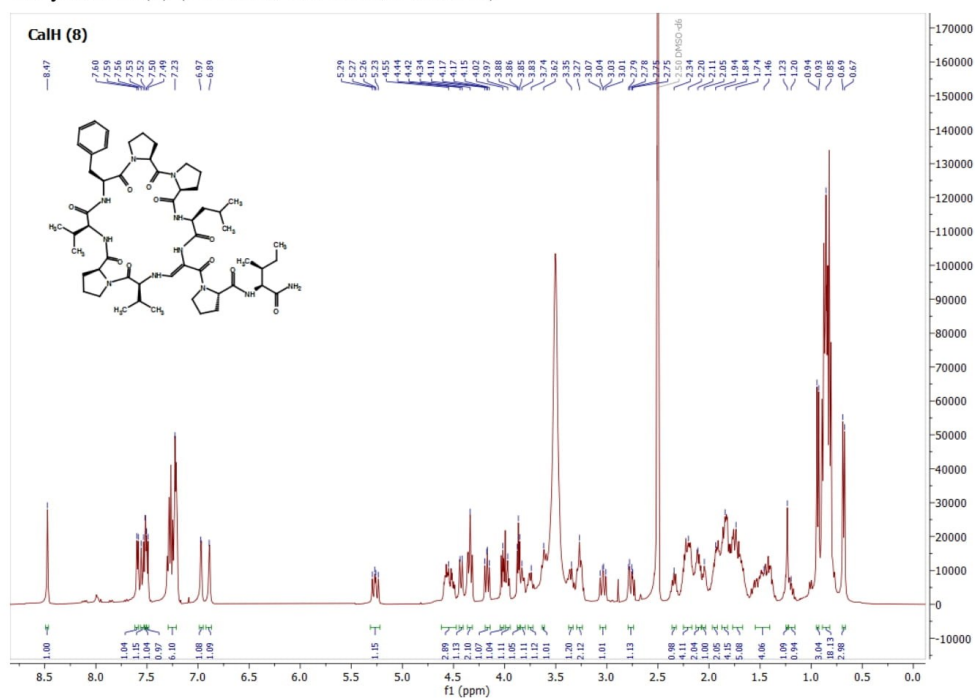
Peak lists (from left to right):

- 8.23, 7.99, 7.99, 7.97, 7.97, 7.95, 7.52, 7.50, 7.48, 7.46, 7.35, 7.19
- 5.36, 5.33, 5.33, 5.30, 5.30
- 4.66, 4.65, 4.63, 4.63, 4.61, 4.45, 4.45, 4.27, 4.27, 4.11, 4.11, 3.99, 3.99, 3.47, 3.40, 3.40, 3.25, 3.23, 3.23, 3.22, 2.88, 2.75, 2.75, 2.68, 2.66, 2.66, 2.65 (TMSO-d6)
- 2.28, 2.12, 1.98, 1.98, 1.77, 1.68, 1.68, 1.55, 1.34, 1.07, 0.88, 0.88, 0.84, 0.84, 0.54, 0.54, 0.39, 0.39

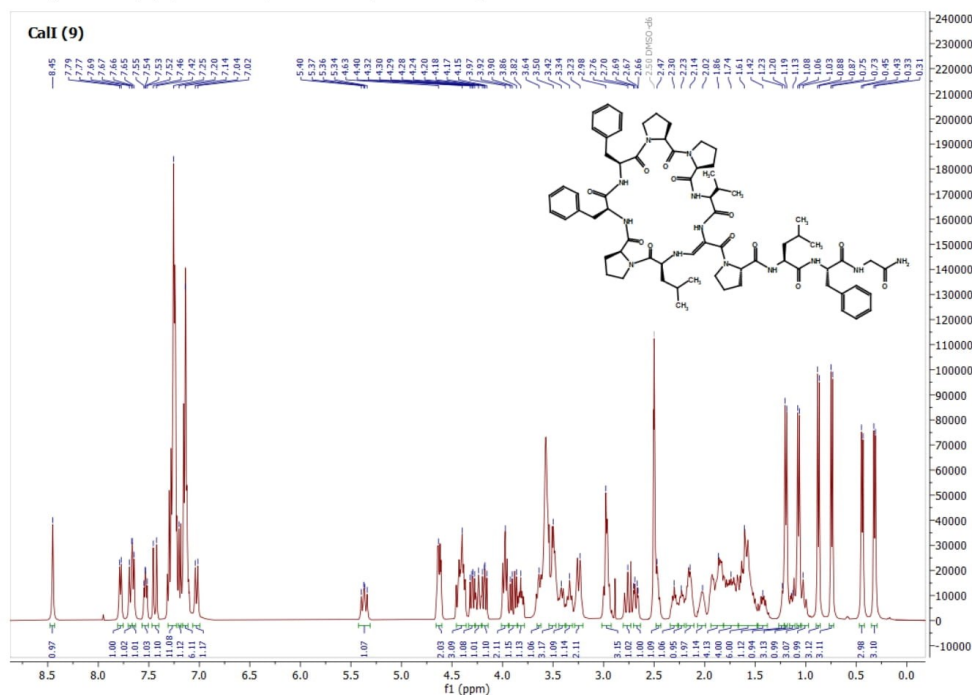
Callyaerin G (**6**) (^{13}C NMR; 101 MHz, DMSO- d_6)



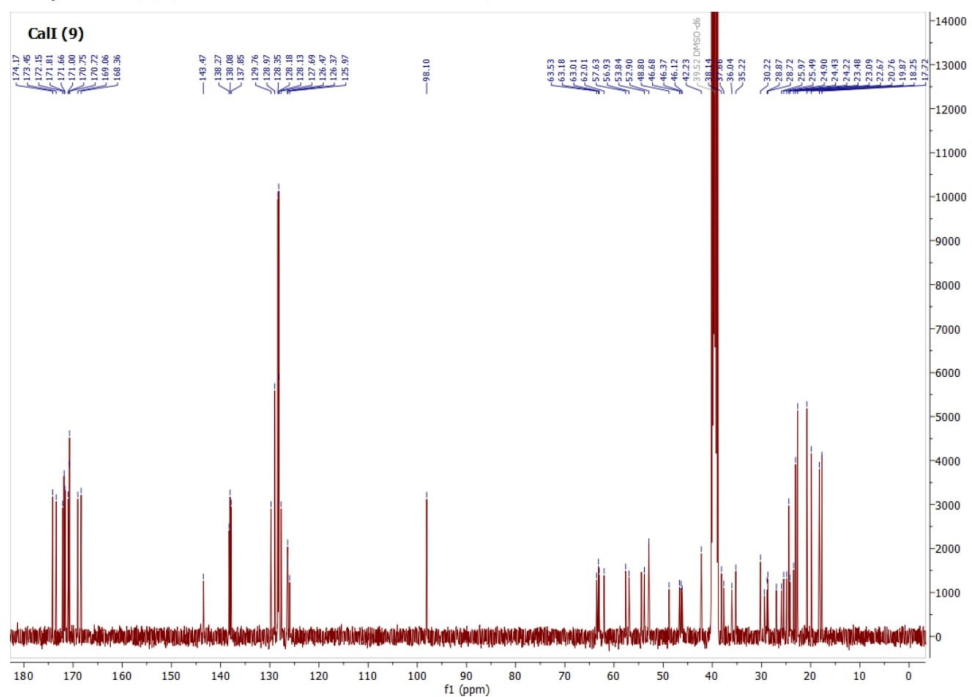
Callyaerin H (**8**) (^1H NMR; 400 MHz, DMSO- d_6)



Callyaerin I (**9**) (^1H NMR; 400 MHz, DMSO- d_6)



Callyaerin I (**9**) (^{13}C NMR; 101 MHz, DMSO- d_6)



CalD (10)

Chemical structure of compound 10 is shown above the spectrum. The structure is a complex molecule with multiple amide and ester groups, and several chiral centers.

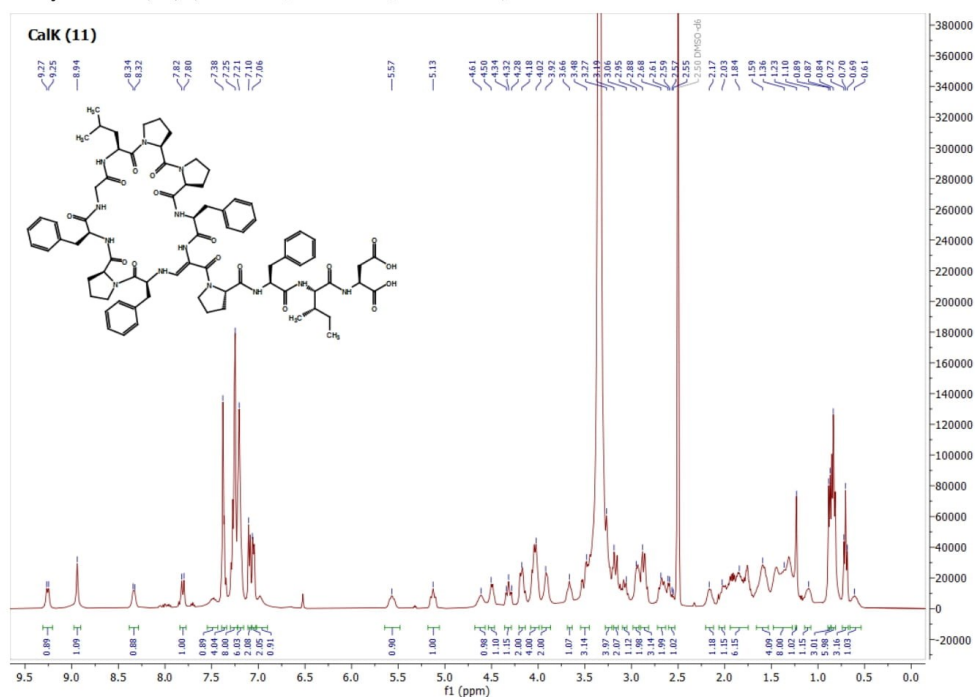
¹H NMR spectrum (CDCl₃) of compound 10. The x-axis represents the chemical shift (δ) in ppm, ranging from 0.0 to 8.2. The y-axis represents the intensity in arbitrary units, ranging from -10,000 to 230,000. The spectrum shows several peaks, with integration values provided below the baseline. The chemical shifts (δ) are listed on the right side of the spectrum.

Chemical shifts (δ) listed on the right side of the spectrum (ppm): 8.21, 7.86, 7.84, 7.82, 7.70, 7.68, 7.46, 7.45, 7.33, 7.31, 7.21, 7.17, 7.14, 7.13, 7.12, 7.10, 7.08, 7.04, 5.47, 5.46, 5.43, 5.41, 4.61, 4.60, 4.51, 4.50, 4.38, 4.36, 4.35, 4.33, 4.32, 4.30, 4.28, 4.17, 4.16, 4.13, 4.09, 4.04, 4.02, 4.00, 3.95, 3.86, 3.77, 3.72, 3.69, 3.52, 3.36, 3.16, 3.02, 3.00, 2.99, 2.67, 2.52, 2.51, 2.27, 2.22, 2.11, 2.02, 1.92, 1.88, 1.58, 1.47, 1.06, 1.04, 1.00, 0.93, 0.86, 0.83, 0.81, 0.67, 0.68, 0.63.

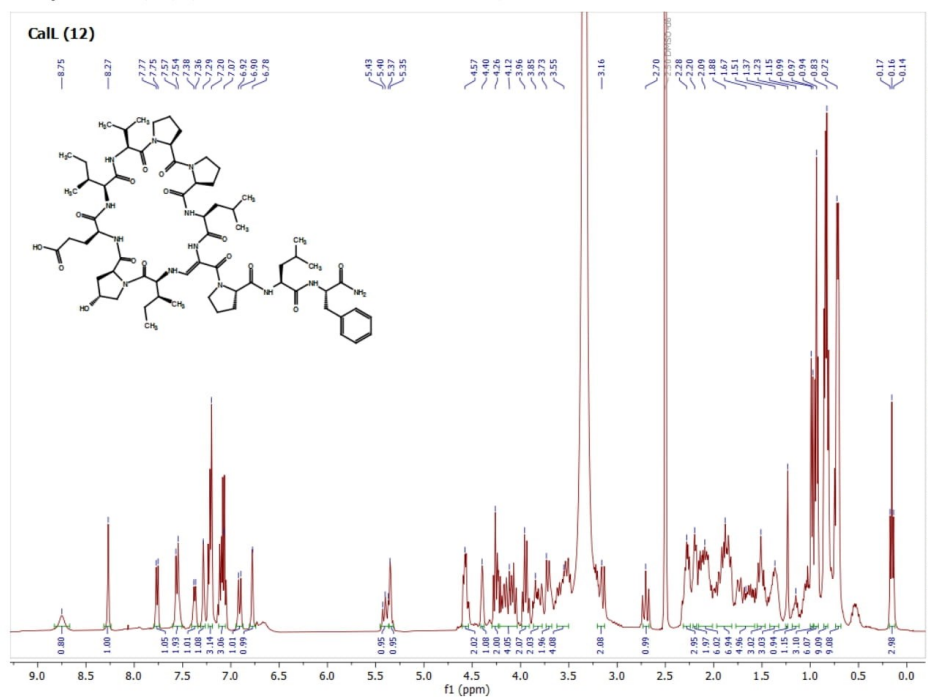
Integration values (from left to right): 1.00, 1.02, 1.00, 1.07, 10.02, 2.99, 1.03, 2.09, 0.99, 1.01, 1.01, 1.01, 2.14, 1.09, 1.95, 1.07, 3.06, 2.01, 1.08, 1.00, 2.09, 1.04, 1.05, 2.14, 4.13, 5.97, 4.03, 2.08, 3.00, 2.97, 3.10, 3.01, 3.05, 3.02.

[illegible]

Callyaerin K (**11**) (^1H NMR; 400 MHz, DMSO- d_6)



Callyaerin L (**12**) (^1H NMR; 400 MHz, DMSO- d_6)



Calynormine A (13)

Chemical structure of Calynormine A (13) is shown above the spectrum.

¹H NMR spectrum (DMSO-d₆) of Calynormine A (13). The x-axis represents the chemical shift in ppm (f1), ranging from 0.0 to 8.5. The y-axis represents the intensity, ranging from 0 to 500,000.

Chemical shifts (ppm) labeled above the spectrum:

- 8.24
- 7.77, 7.74, 7.55, 7.54, 7.21, 7.20, 7.19, 7.07, 7.07
- 5.27, 5.25, 5.21, 5.17, 5.16
- 4.68, 4.55, 4.31, 4.13, 3.94
- 3.64, 3.47
- 3.24
- 2.80, 2.77, 2.74
- 2.58 (DMSO-d₆), 2.31, 2.00, 1.88, 1.77, 1.52, 1.37
- 1.14, 0.91

Integration values (labeled below the baseline):

- 0.98
- 1.00, 1.02, 1.00, 0.99, 1.04, 1.01
- 1.03, 0.98
- 1.06, 1.07, 1.00, 1.06, 2.00, 2.04
- 3.00, 2.94
- 3.02
- 1.06
- 1.11, 1.00, 2.00, 6.05, 4.06, 0.07, 1.07
- 0.99
- 30.30

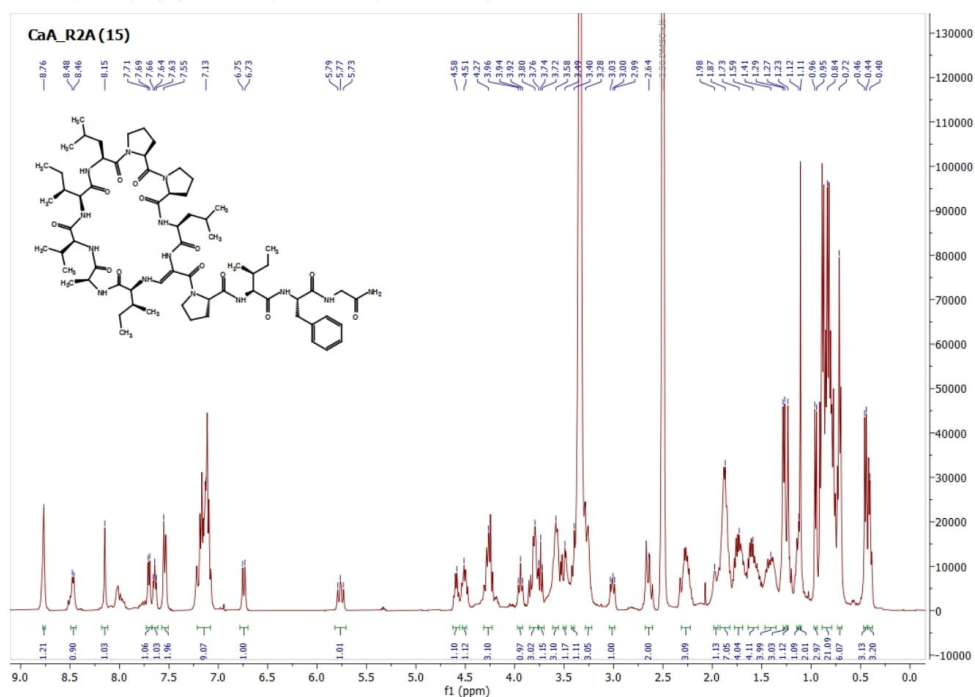
CalA_R1A (14)

Chemical structure of compound 14 is shown above the spectrum.

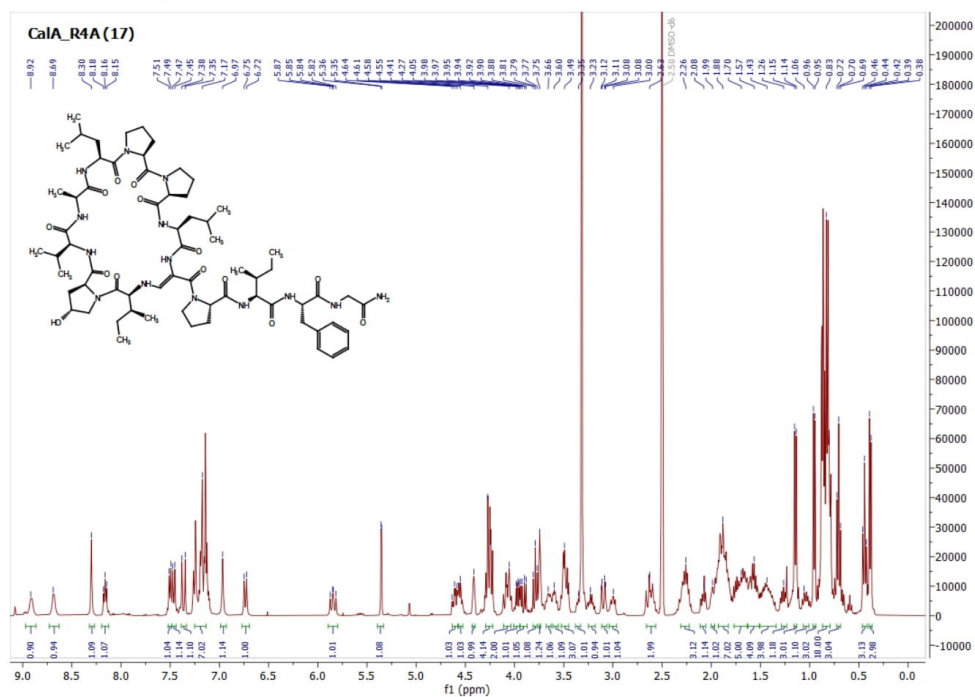
¹H NMR spectrum (CDCl₃) showing peaks from 0.0 to 8.5 ppm. The spectrum includes integration values below the peaks and a list of peak positions (ppm) on the right side.

Peak positions (ppm): 8.36, 8.27, 8.25, 8.24, 7.75, 7.71, 7.58, 7.55, 7.55, 7.36, 7.34, 7.14, 6.96, 6.85, 5.72, 5.54, 5.34, 4.70, 4.68, 4.67, 4.65, 4.58, 4.39, 4.27, 4.25, 4.12, 4.10, 3.89, 3.69, 3.66, 3.66, 3.45, 3.25, 3.12, 3.09, 3.08, 2.86, 2.66, 2.53, 2.52, 2.26, 2.15, 1.98, 1.96, 1.86, 1.84, 1.54, 1.05, 0.98, 0.98, 0.92, 0.89, 0.89, 0.84, 0.84, 0.77, 0.76, 0.74, 0.69, 0.67, 0.50.

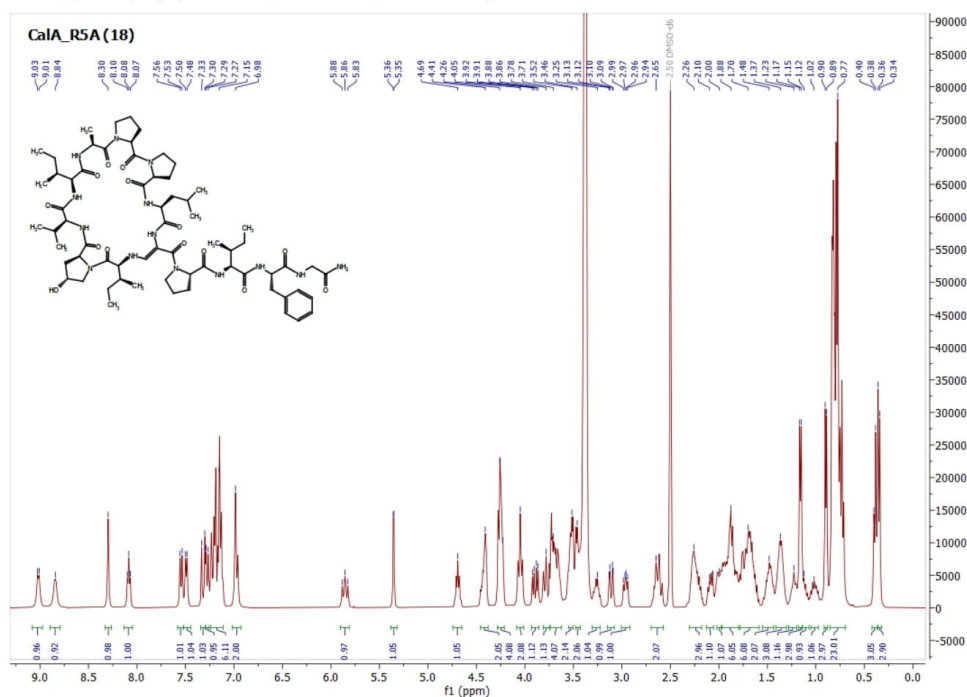
CalA_R2A (15) (^1H NMR; 400 MHz, DMSO- d_6)



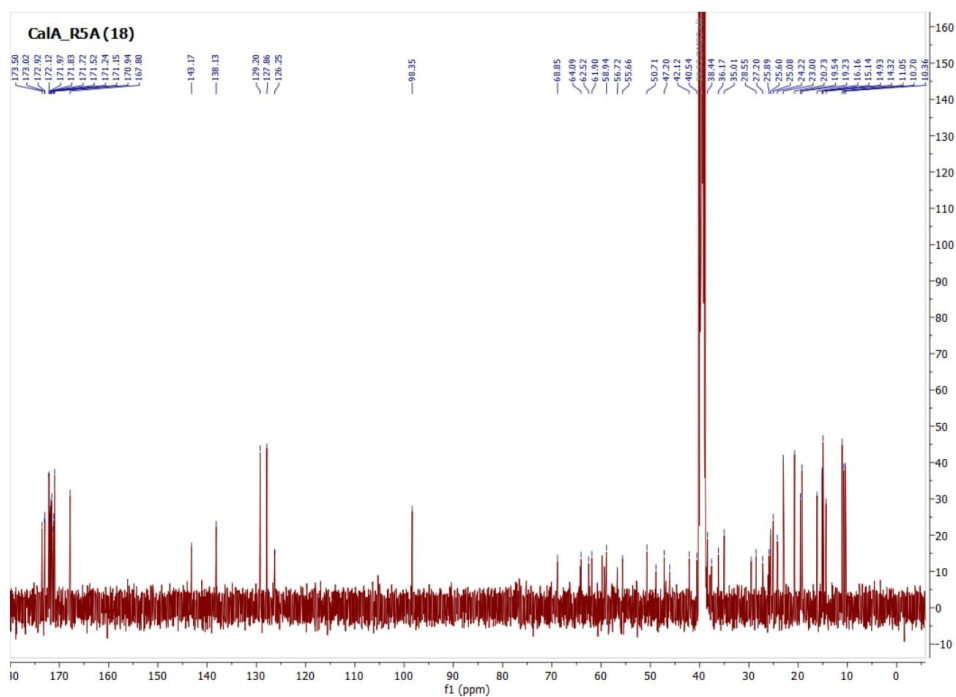
CalA_R4A (17) (^1H NMR; 400 MHz, DMSO- d_6)



CalA_R5A (18) (^1H NMR; 400 MHz, DMSO- d_6)



CalA_R5A (18) (^{13}C NMR; 101 MHz, DMSO- d_6)



CaA_R8A (21)

Chemical structure of **CaA_R8A (21)** is shown above the spectrum.

Integration values (bottom):

- 9.05, 0.96, 0.95, 1.00, 0.95, 1.06, 7.13, 1.06, 0.96, 0.99, 0.85, 2.62, 1.61, 1.61, 2.10, 1.69, 4.10, 2.61, 2.10, 1.62, 0.95, 1.94, 3.64, 1.68, 7.64, 2.92, 3.68, 2.86, 1.63, 2.94, 18.11, 2.81, 2.96

Peak list (right):

- 4.52, 4.41, 4.21, 4.05, 3.93, 3.89, 3.87, 3.74, 3.64, 3.50, 3.26, 3.12, 3.09, 3.08, 3.00, 2.99, 2.98, 2.97, 2.63, 2.25, 2.07, 2.00, 1.68, 1.51, 1.49, 1.44, 1.39, 1.19, 0.93, 0.91, 0.74, 0.72, 0.70, 0.41, 0.40, 0.38

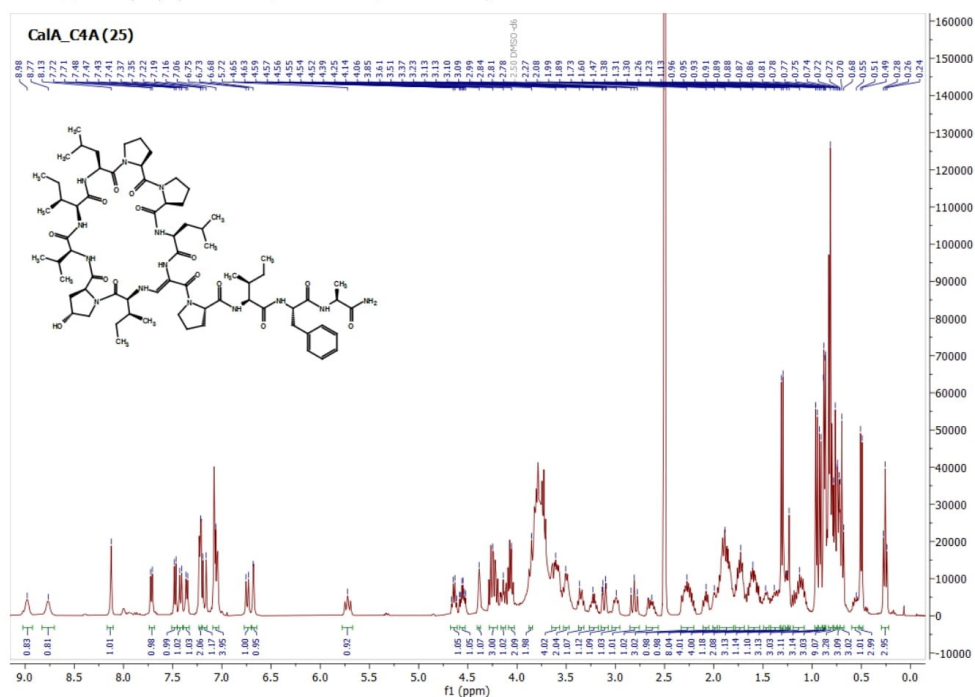
The figure displays the chemical structure of compound 23 and its corresponding ¹H NMR spectrum recorded at 600 MHz in DMSO-d₆. The chemical structure is a complex molecule featuring multiple amide bonds, a hydroxyl group, and various alkyl side chains.

The ¹H NMR spectrum shows peaks from 9.0 ppm down to 0.25 ppm. Key features include:

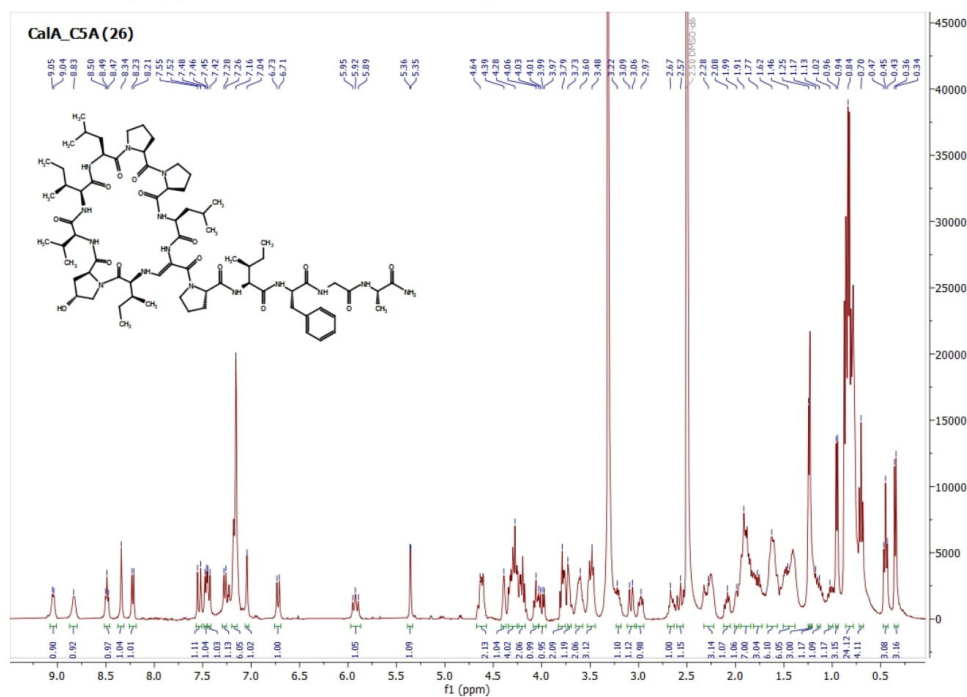
- Aromatic protons between 7.0 and 8.0 ppm.
- A broad peak around 6.8 ppm, likely due to the exchangeable NH proton.
- Aliphatic protons in the range of 3.0 to 5.5 ppm.
- A sharp singlet at approximately 2.5 ppm, possibly representing a solvent or water peak.
- Multiplets and doublets in the aliphatic region from 0.5 to 2.0 ppm.

Integration values are provided below the baseline of the spectrum, indicating the relative areas under the peaks.

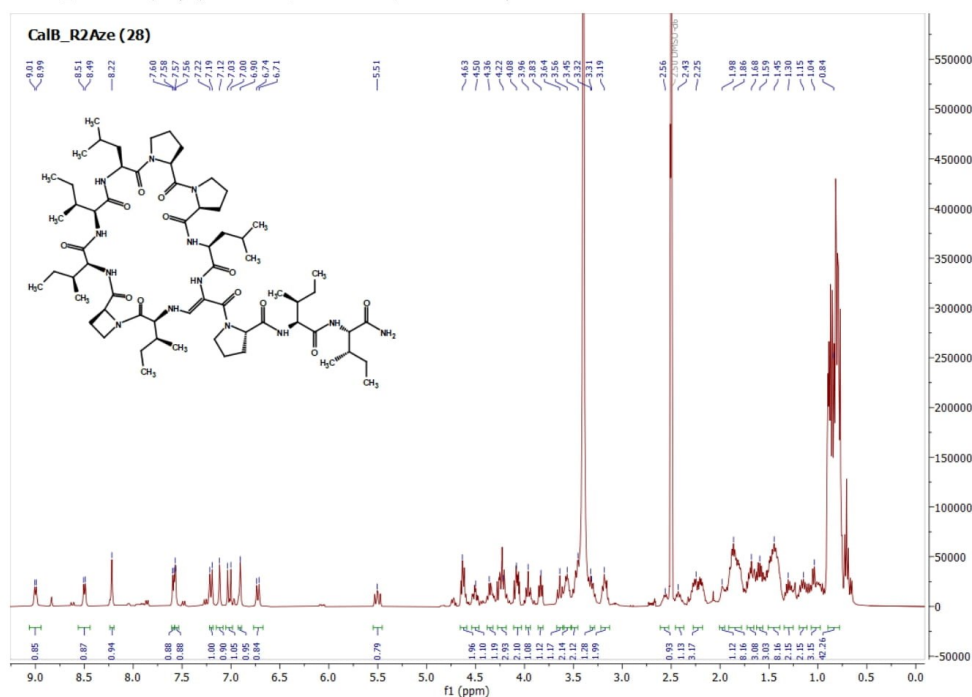
CalA_C4A (25) (^1H NMR; 400 MHz, DMSO- d_6)



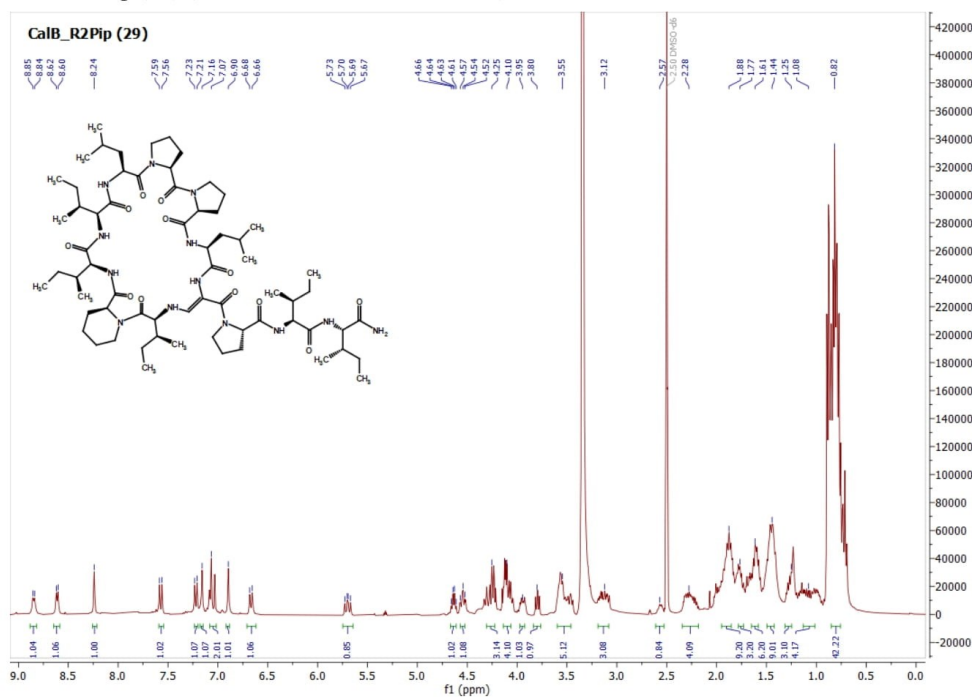
CalA_C5A (26) (^1H NMR; 400 MHz, DMSO- d_6)



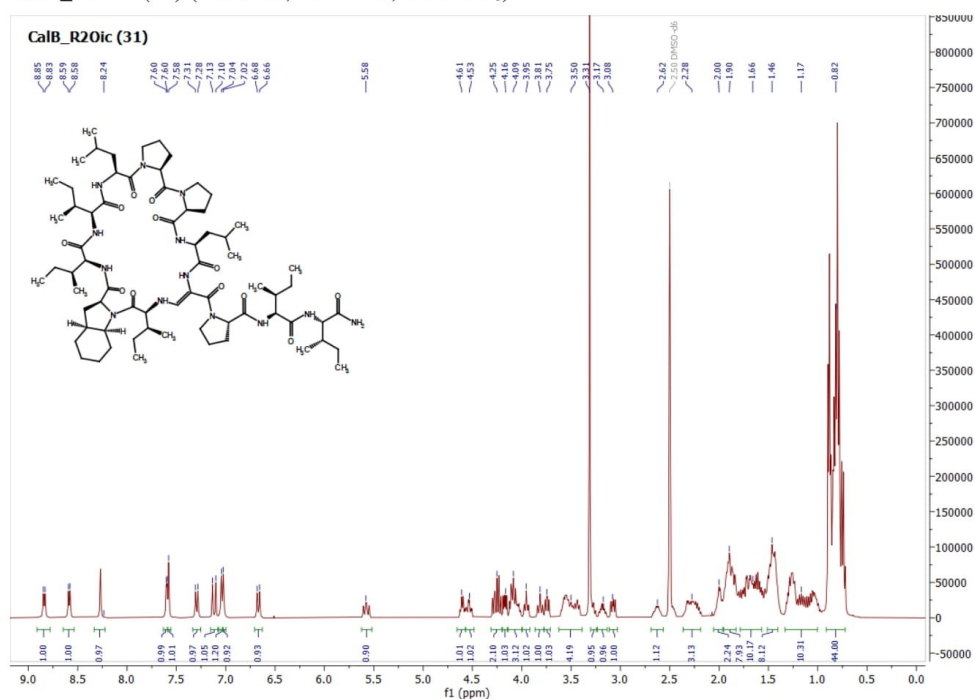
CalB_R2Aze (**28**) (^1H NMR; 400 MHz, DMSO- d_6)



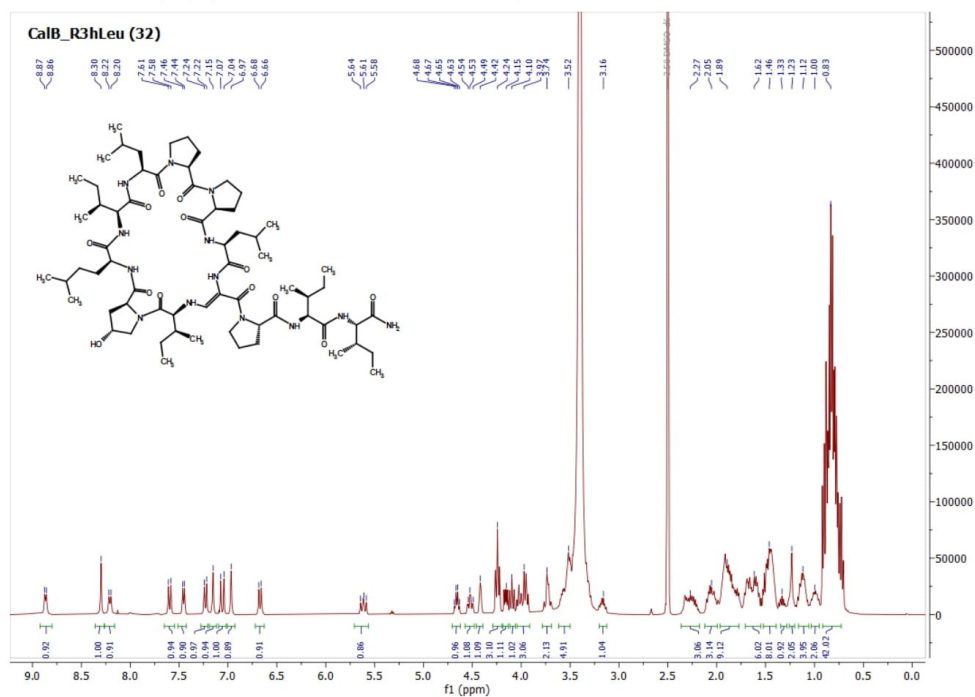
CalB_R2Pip (**29**) (^1H NMR; 400 MHz, DMSO- d_6)



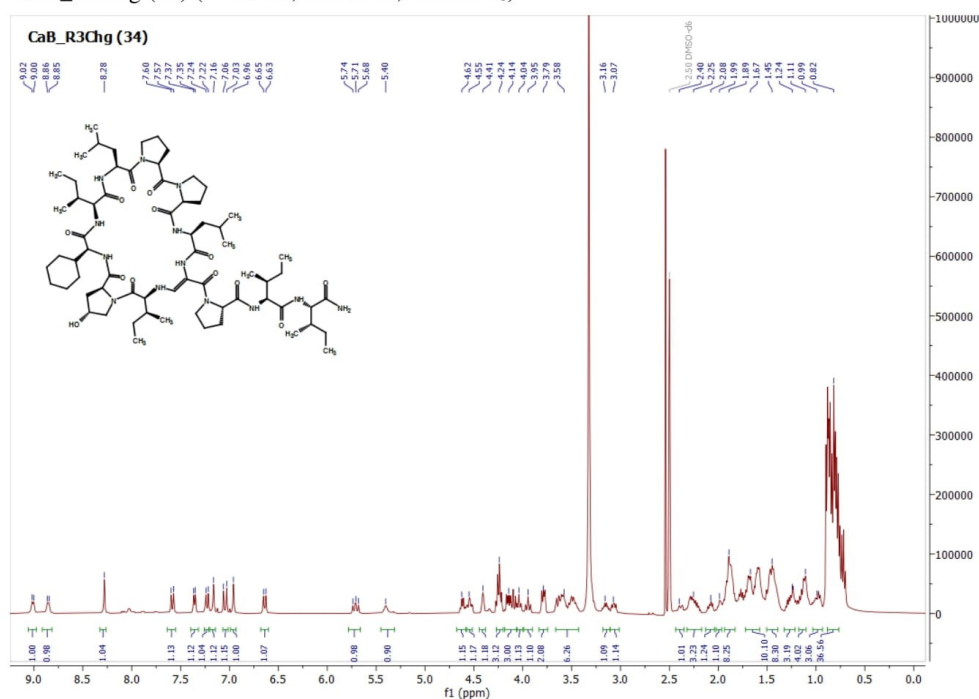
CalB_R2Oic (**31**) (^1H NMR; 400 MHz, DMSO- d_6)



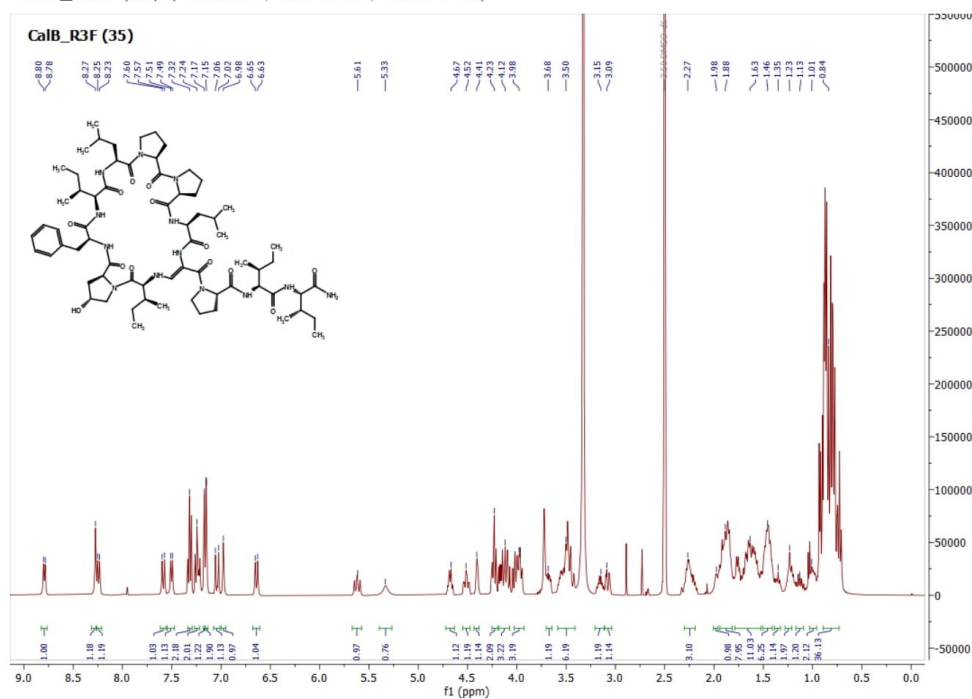
CalB_R3hLeu (**32**) (^1H NMR; 400 MHz, DMSO- d_6)



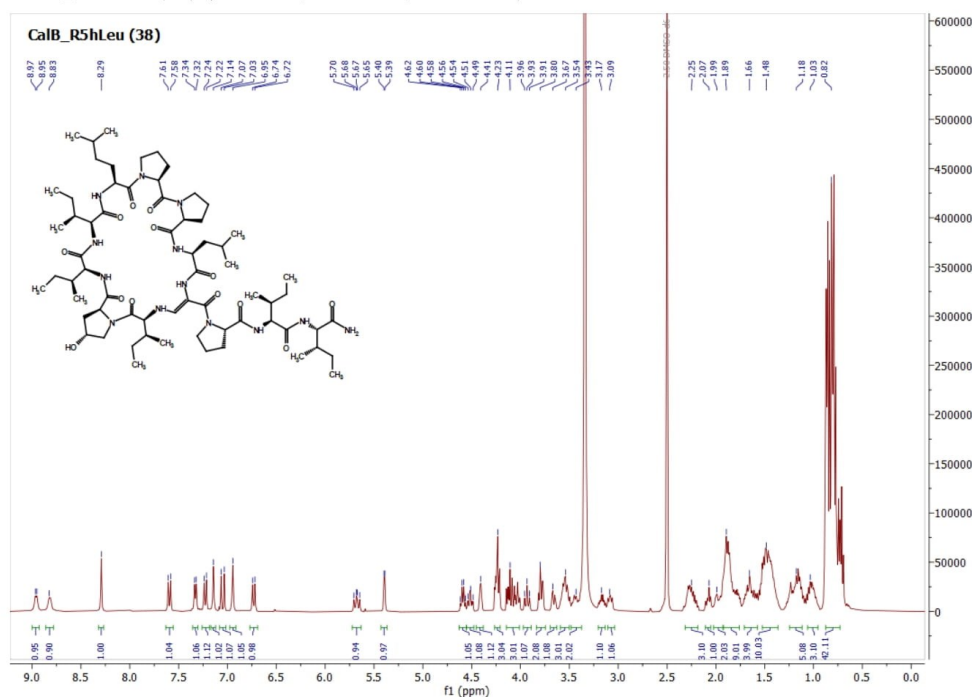
CalB_R3Chg (**34**) (^1H NMR; 400 MHz, DMSO- d_6)



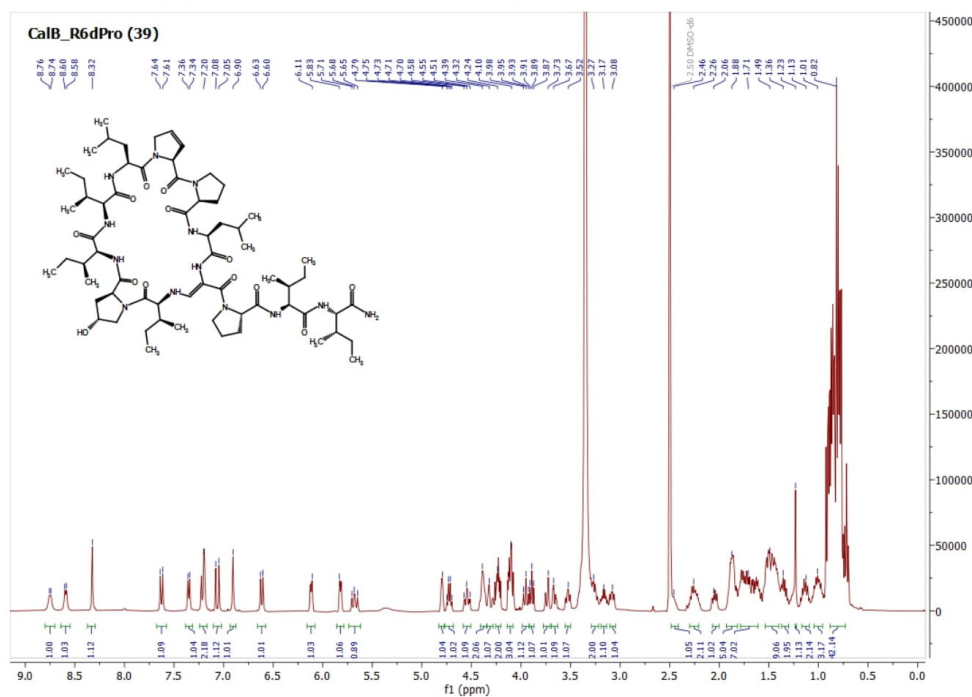
CalB_R3F (**35**) (^1H NMR; 400 MHz, DMSO- d_6)



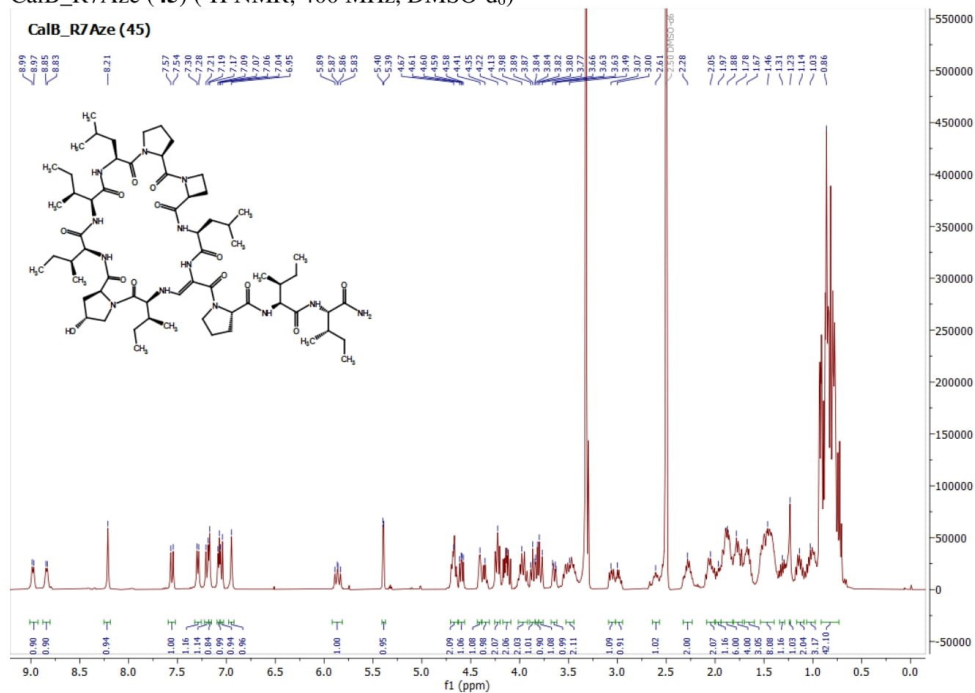
CalB_R5hLeu (38) (^1H NMR; 400 MHz, DMSO- d_6)



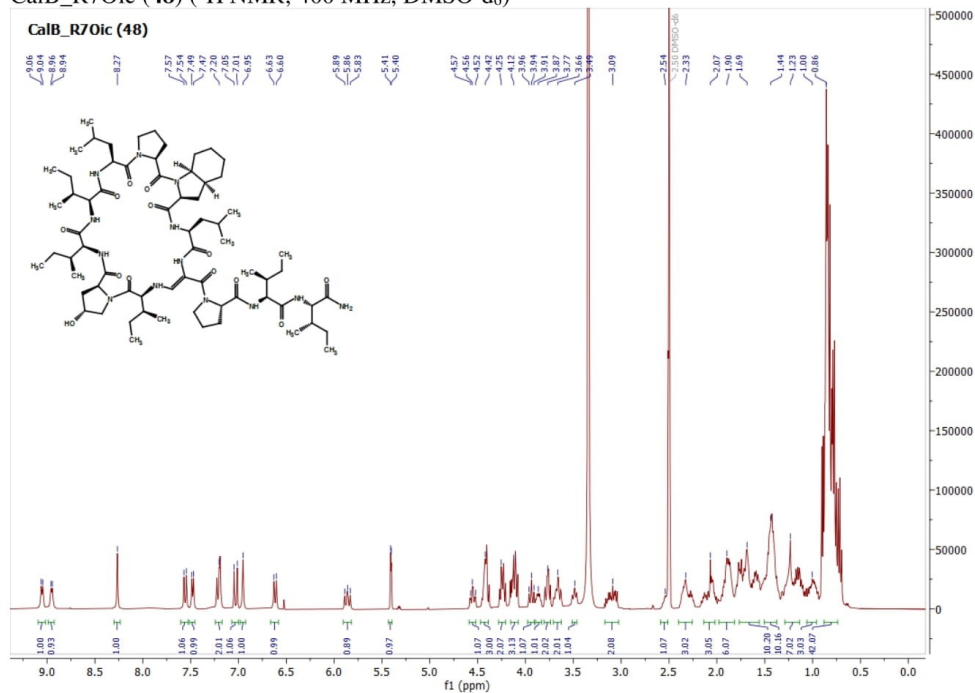
CalB_R6dPro (39) (^1H NMR; 400 MHz, DMSO- d_6)



CalB_R7Aze (**45**) (^1H NMR; 400 MHz, DMSO- d_6)

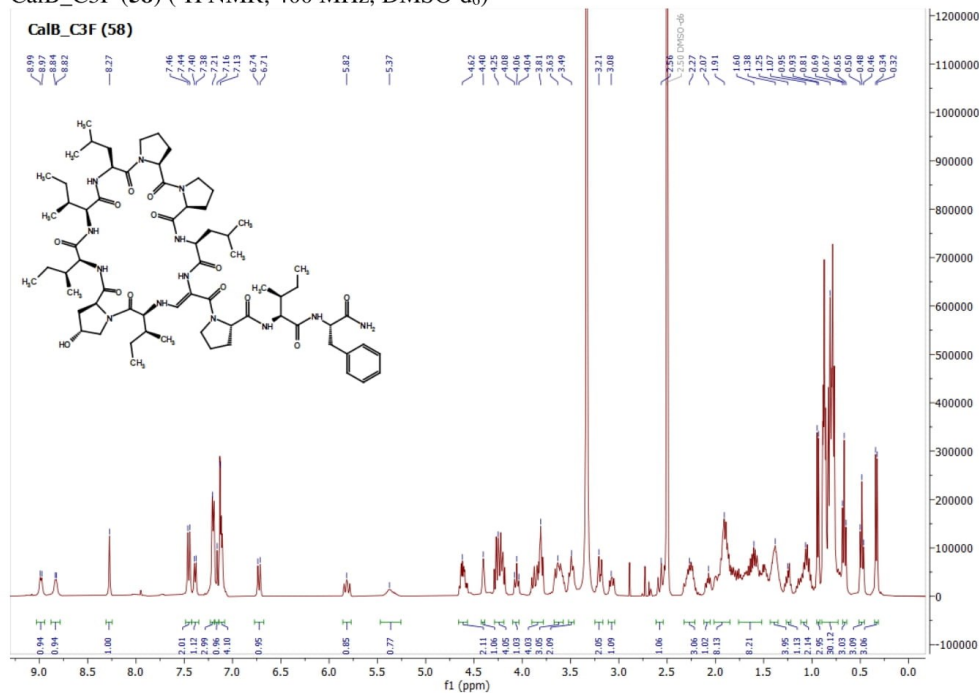


CalB_R7Oic (**48**) (^1H NMR; 400 MHz, DMSO- d_6)

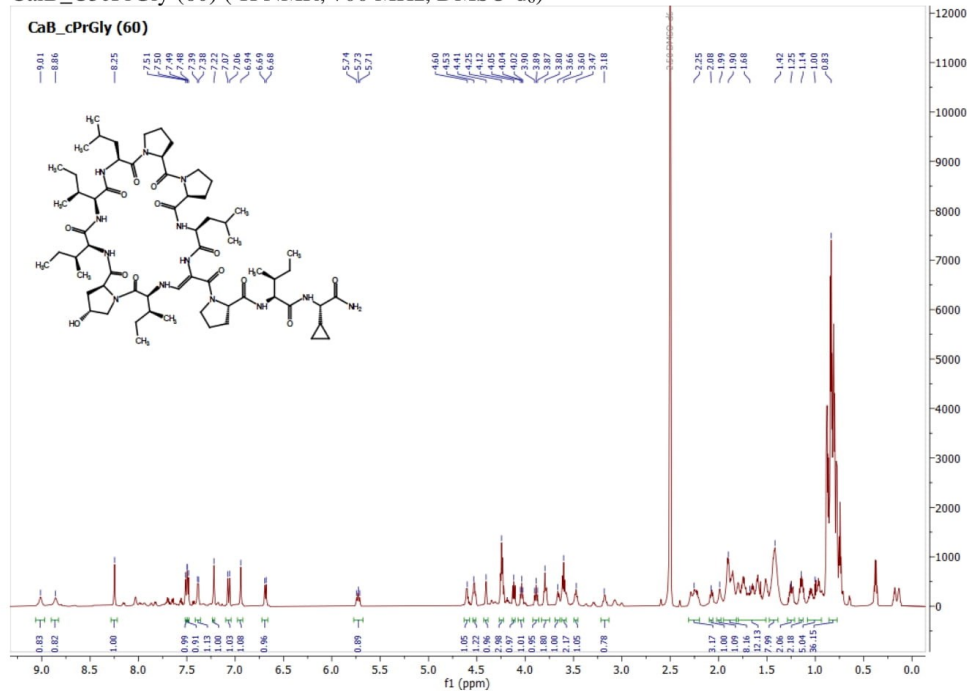


[illegible][illegible]

CalB_C3F (**58**) (^1H NMR; 400 MHz, DMSO- d_6)



CalB_C3cPrGly (**60**) (^1H NMR; 700 MHz, DMSO- d_6)



CaB_C3Tle (61)

Chemical structure of CaB_C3Tle (61) is shown, a complex cyclic peptide derivative. The structure features a large macrocyclic core with several side chains, including a long aliphatic chain with a terminal amide, a branched aliphatic chain, and a side chain with a terminal amide and a methyl group. The structure is labeled with 'CaB_C3Tle (61)'.

¹H NMR spectrum (400 MHz, DMSO-d₆) of CaB_C3Tle (61). The x-axis represents the chemical shift in ppm (f1), ranging from 0.0 to 9.0. The y-axis represents the intensity, ranging from 0 to 250,000. The spectrum shows several sharp peaks, with the most prominent ones around 1.0 ppm and 2.5 ppm. Integration values are provided below the baseline for various peak regions.

Integration values (from left to right):

- 9.01, 8.99, 8.85
- 0.20
- 7.67, 7.64, 7.39, 7.37, 7.17, 7.15, 7.15, 6.96, 6.89, 6.87, 6.85
- 5.66, 5.64, 5.61, 5.40
- 4.42, 4.50, 4.39, 4.35, 4.35, 4.02, 3.80, 3.60
- 3.31, 3.18, 3.07
- 2.25, 2.05, 1.95, 1.89
- 1.43, 1.43, 1.14, 1.02, 0.83

Integration values (from left to right):

- 0.96, 0.90
- 0.88
- 1.01
- 1.00, 1.00, 2.00, 1.05
- 0.87
- 0.76, 0.89
- 1.01, 1.20, 1.15, 0.99, 0.99, 2.99, 2.95, 4.16
- 1.06, 1.06, 1.02
- 3.14, 3.05, 6.31, 7.99, 2.11, 2.81, 6.20

CalB_C3Bpa (62)

Chemical structure of **CalB_C3Bpa (62)** is shown above the spectrum.

Chemical Shifts (ppm):

- 8.98, 8.79, 8.18, 7.62, 7.50, 7.41, 7.35, 7.31, 7.22, 7.18, 6.93, 6.68, 6.66, 5.78, 5.36, 4.57, 4.52, 4.23, 4.06, 4.04, 3.81, 3.58, 3.28, 3.21, 3.06, 2.95, 2.82, 2.78, 2.27, 2.07, 1.87, 1.63, 1.37, 1.12, 0.76, 0.48, 0.46, 0.33, 0.30

Integration values:

- 0.97, 1.04, 1.00, 4.25, 4.95, 7.00, 1.04, 1.35, 0.96, 1.05, 0.95, 1.04, 1.00, 1.01, 5.01, 1.15, 1.95, 5.14, 2.69, 1.18, 1.09, 1.09, 4.12, 1.95, 7.04, 6.27, 6.08, 7.02, 30.28, 3.01, 3.00

CalA_R2P (63)

Chemical structure of CalA_R2P (63) is shown above the spectrum. The structure is a complex polypeptide derivative with various side chains, including aromatic, aliphatic, and functional groups like amides and esters.

¹H NMR spectrum (400 MHz, DMSO-d₆) of CalA_R2P (63) is displayed below the structure. The x-axis represents the chemical shift in ppm (f1), ranging from 0.0 to 9.0. The y-axis represents the intensity in arbitrary units, ranging from 0 to 120,000. The spectrum shows several sharp peaks, with the most intense peak at approximately 2.5 ppm. Integration values are provided below the baseline for each major peak group.

Chemical shift data (ppm) and integration values are listed below the spectrum:

Chemical Shift (ppm)	Integration
8.95, 8.93, 8.70, 8.24, 8.12, 8.10, 7.76, 7.33, 7.28, 7.19, 7.01, 6.74, 6.72	0.98, 0.99, 0.99, 1.02, 1.00, 1.00, 1.00, 1.00
5.87, 5.84, 5.81	1.00
4.51, 4.26, 4.10, 4.03, 3.78, 3.60, 3.40, 3.22, 3.13, 3.12, 3.09, 3.02, 2.99, 2.86	2.08, 3.10, 2.60, 2.60, 2.07, 2.10, 2.10, 2.03, 1.10, 1.12, 1.09, 2.62
2.50 (DMSO-d ₆), 2.27, 2.00, 1.88, 1.76, 1.68, 1.48, 1.38, 1.24, 1.04, 0.86, 0.84, 0.70, 0.40	4.10, 3.05, 2.01, 6.06, 2.99, 1.01, 2.01, 2.97, 24.02, 4.03, 6.01

CalA_R3Abu (64)

Chemical structure of compound 64 is shown above the spectrum. The spectrum displays peaks corresponding to the structure, with integration values provided below the baseline and peak lists above the spectrum.

Peak list (ppm):

- 8.84, 8.83
- 8.31, 8.13
- 7.55, 7.50, 7.49, 7.47, 7.37, 7.34, 7.24, 7.14, 6.77, 6.75
- 5.28, 5.26, 5.22, 5.20, 4.88, 4.67, 4.65, 4.58, 4.56, 4.40, 4.31, 4.28, 4.14, 4.09, 4.05, 4.01, 3.95, 3.78, 3.55
- 3.24, 3.09
- 2.63, 2.50 (DMSO-d6), 2.26, 2.07, 1.88, 1.86, 1.39, 1.12, 1.06, 1.06, 0.91, 0.91, 0.95, 0.90, 0.71, 0.69, 0.64, 0.41, 0.39, 0.37

Integration values (from left to right):

- 0.96
- 1.00
- 2.07
- 1.07, 1.09, 1.10, 1.09, 5.10, 1.64, 1.01
- 0.85
- 0.99
- 1.02, 1.06, 0.88, 4.08, 1.02, 1.02, 2.07, 3.04, 6.13, 1.12, 1.01
- 1.11
- 1.14, 2.08, 3.12, 9.05, 9.01
- 5.09
- 3.05, 3.05, 3.02, 18.03, 3.05
- 3.05, 3.05

Cal: R3Chg (65)

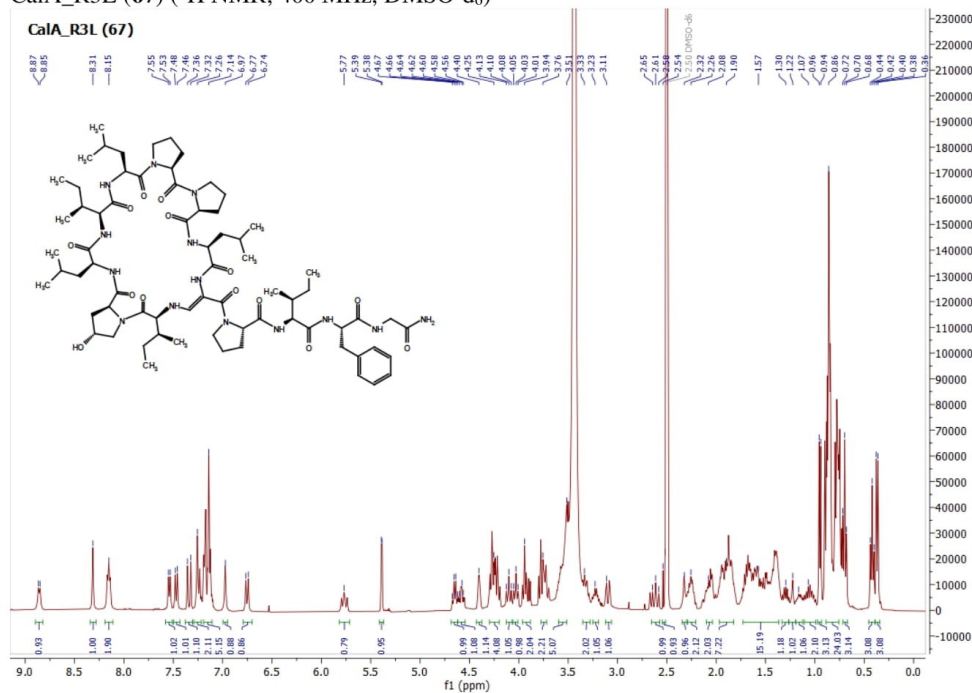
Chemical structure of compound 65 is shown above the spectrum. The spectrum displays peaks corresponding to the structure, with integration values and chemical shifts (ppm) labeled below the baseline.

Chemical shift (ppm) labels (from left to right): 9.02, 9.00, 8.83, 8.28, 8.19, 8.18, 8.16, 7.47, 7.45, 7.45, 7.41, 7.35, 7.35, 7.26, 7.13, 7.13, 6.72, 6.70, 5.91, 5.88, 5.85, 5.59, 5.58, 5.38, 4.65, 4.40, 4.27, 4.06, 3.95, 3.92, 3.92, 3.90, 3.80, 3.78, 3.60, 3.31, 3.31, 3.31, 3.07, 2.64, 2.54, 2.58, 2.27, 2.07, 1.99, 1.99, 1.76, 1.59, 1.59, 1.22, 1.09, 0.95, 0.95, 0.85, 0.71, 0.67, 0.43, 0.42, 0.38, 0.36.

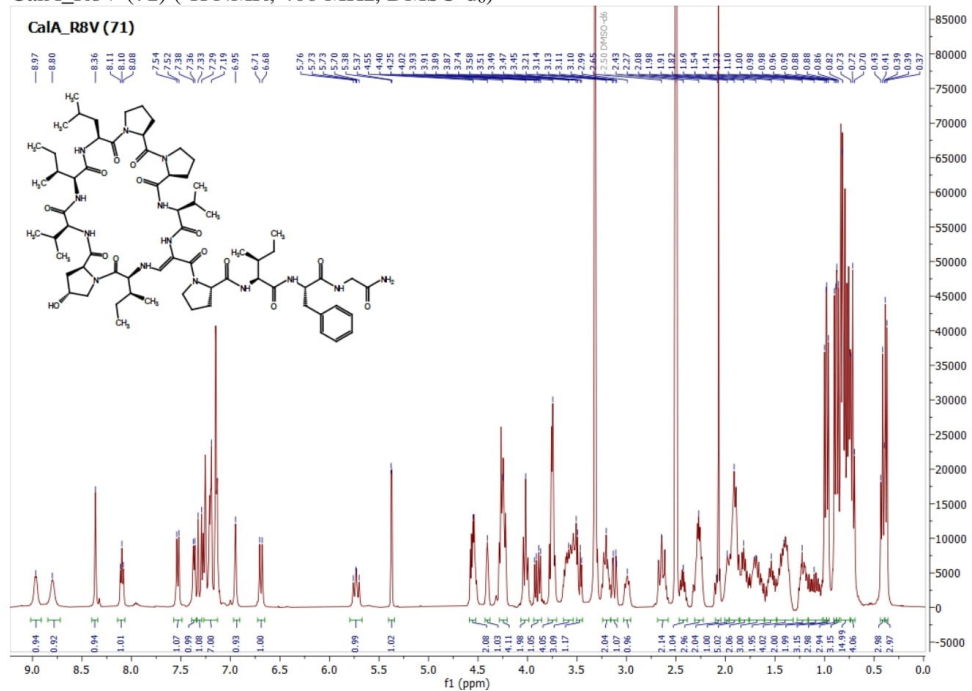
Integration values (from left to right): 1.03, 0.86, 1.00, 1.04, 1.14, 1.15, 2.69, 5.03, 0.99, 0.86, 0.88, 1.06, 2.15, 1.11, 0.95, 3.05, 0.63, 1.08, 1.08, 1.11, 1.11, 1.10, 1.13, 1.13, 2.09, 1.10, 1.12, 4.13, 1.73, 1.73, 8.62, 1.08, 1.08, 1.18, 1.18, 2.15, 18.13, 3.17, 3.64, 3.63.

[illegible]

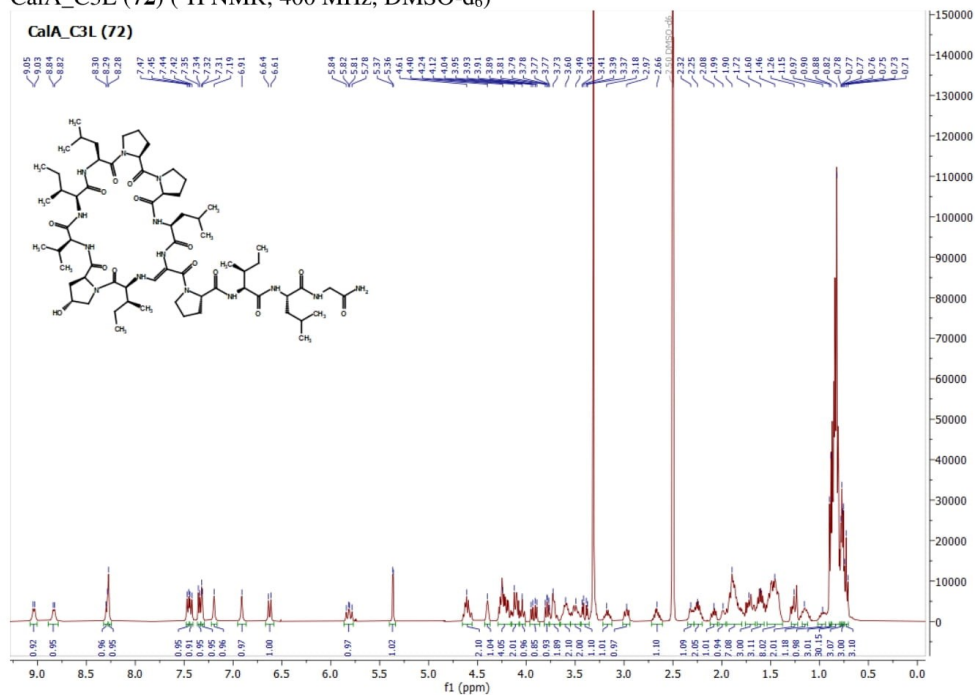
CalA_R3L (67) (^1H NMR; 400 MHz, DMSO- d_6)



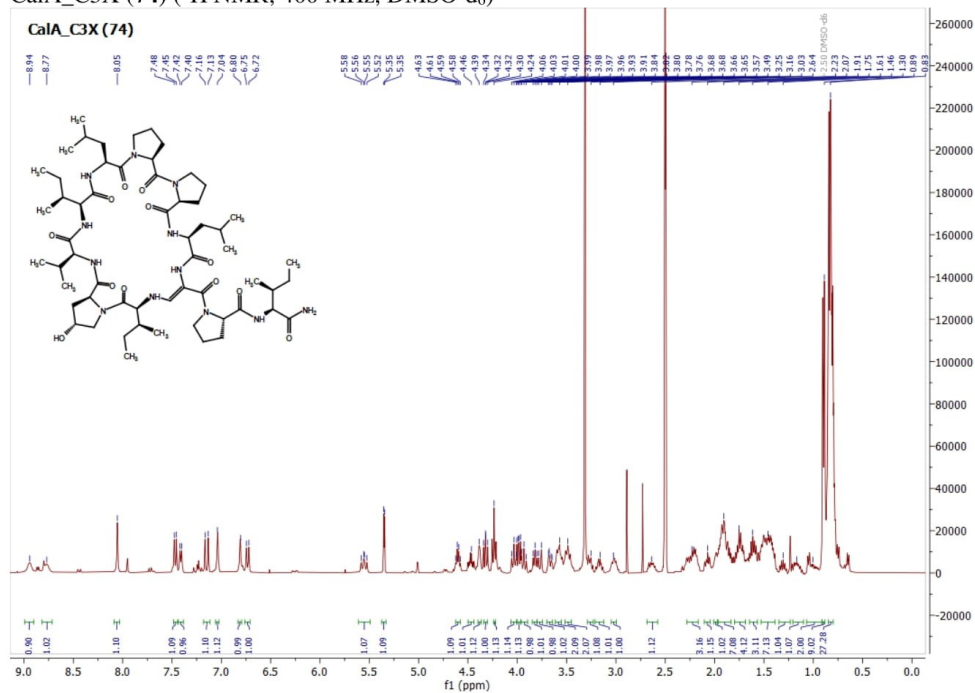
CalA_R8V (71) (^1H NMR; 400 MHz, DMSO- d_6)



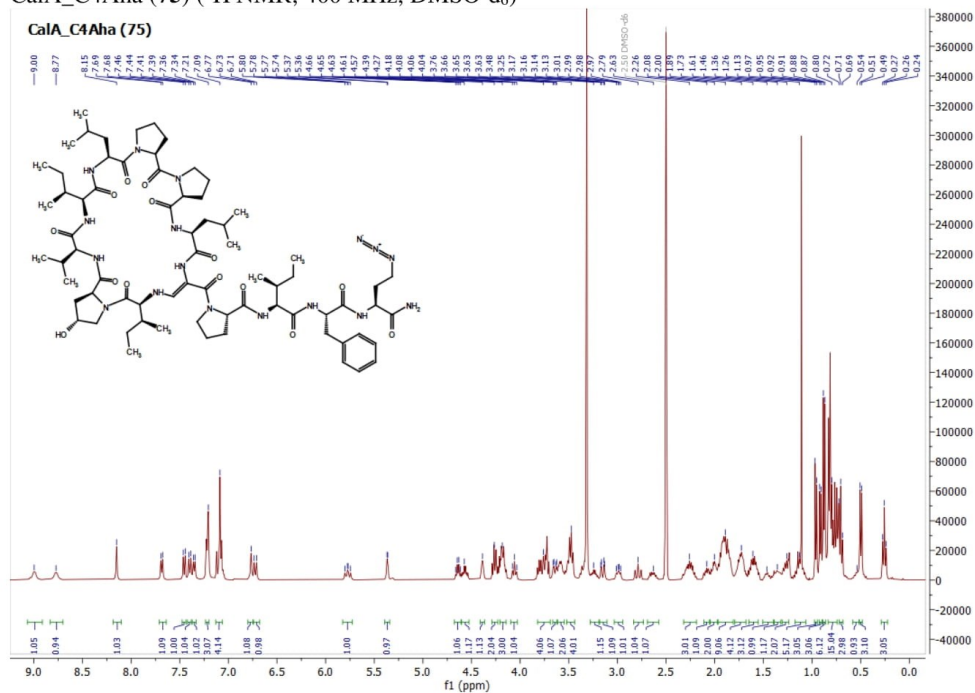
CalA_C3L (72) (^1H NMR; 400 MHz, DMSO- d_6)



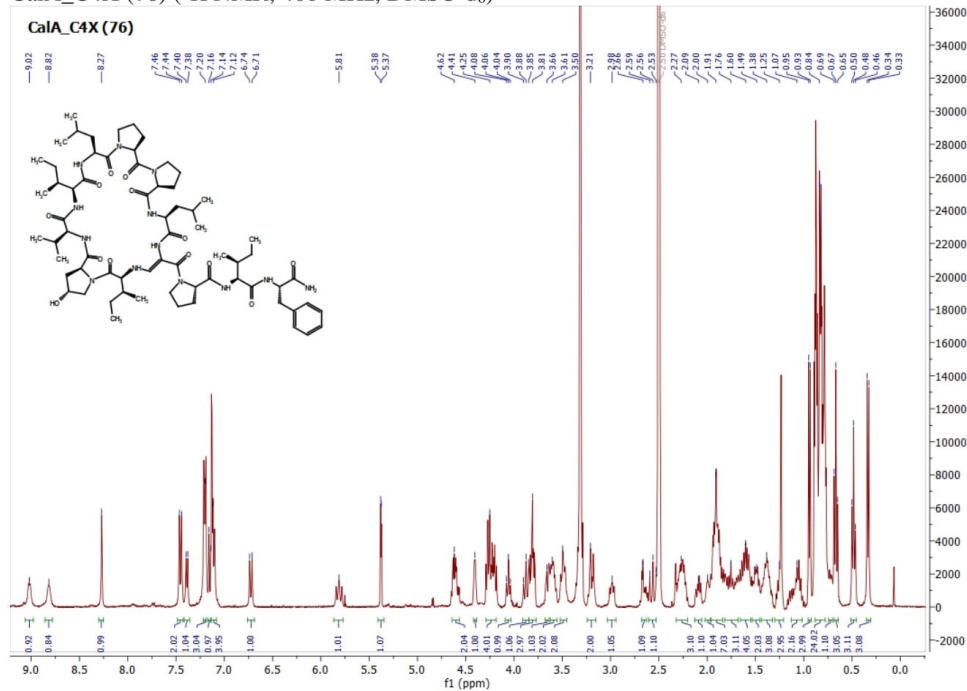
CalA_C3X (74) (^1H NMR; 400 MHz, DMSO- d_6)



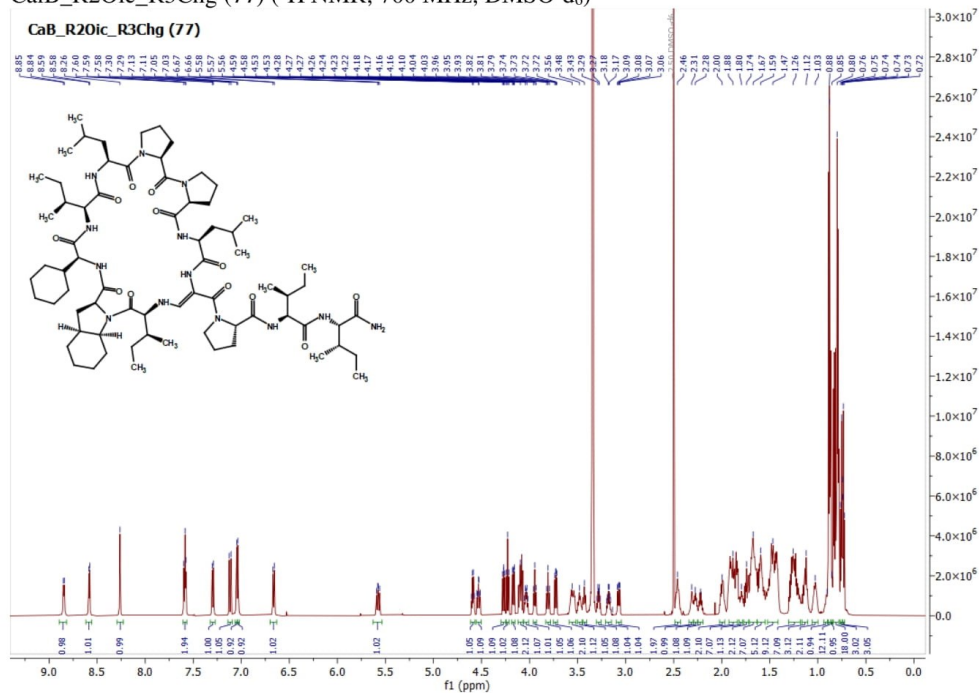
CalA_C4Aha (75) (^1H NMR; 400 MHz, DMSO- d_6)



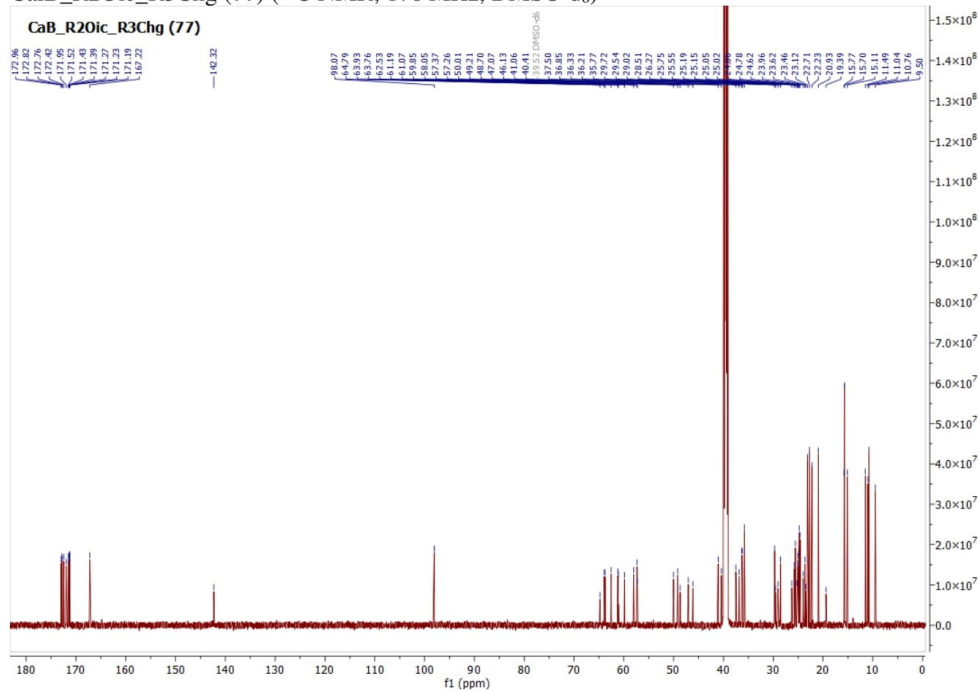
CalA_C4X (76) (^1H NMR; 400 MHz, DMSO- d_6)



CalB_R2Oic_R3Chg (77) (^1H NMR; 700 MHz, DMSO- d_6)



CalB_R2Oic_R3Chg (77) (^{13}C NMR; 176 MHz, DMSO- d_6)



CalB_R3Pra (78)

Chemical structure of CalB_R3Pra (78) is shown above the spectrum. The structure is a complex molecule with multiple amide and ester groups, and a terminal hydroxyl group.

Peak List (ppm):

- 9.14, 9.13
- 8.30
- 7.81, 7.79, 7.78, 7.59, 7.50, 7.48, 7.24, 7.22, 7.14, 7.14, 7.05, 6.97, 6.87, 6.87
- 5.57, 5.41, 5.40
- 4.70, 4.50, 4.50, 4.37, 4.27, 4.16, 4.10, 4.10, 4.01
- 3.74, 3.74, 3.55, 3.55, 3.38, 3.38, 3.17, 3.04, 3.04, 2.75, 2.75
- 2.50 (DMSO-d6)
- 2.36, 2.36, 2.07, 2.07, 1.91
- 1.61, 1.61, 1.46, 1.23, 1.10
- 0.82

Integration Values:

- 0.95, 1.00, 0.92, 1.00, 0.99, 1.02, 0.97, 1.06, 0.92, 0.95
- 0.87, 0.96
- 0.96, 1.05, 1.05, 3.00, 3.00, 1.05, 1.05, 3.02
- 2.15, 1.60, 1.60, 1.06, 0.96, 1.05, 0.96
- 1.00, 1.00, 2.07, 8.09
- 6.09, 8.14, 1.05, 5.01
- 36.02

CalB_R4Pra (79)

Chemical structure of compound 79 is shown above the spectrum. The spectrum displays peaks corresponding to the structure, with integration values indicated below the baseline. The x-axis represents the chemical shift in ppm (f1), ranging from 0.0 to 8.5. The y-axis represents the intensity, ranging from 0 to 650,000. The spectrum includes a list of peak chemical shifts on the right side.

Peak chemical shifts (ppm): 8.21, 8.14, 8.30, 7.60, 7.58, 7.56, 7.54, 7.24, 7.22, 7.20, 7.18, 7.05, 6.86, 6.66, 5.64, 5.39, 4.65, 4.63, 4.42, 4.34, 4.32, 4.07, 3.94, 3.92, 3.58, 3.32, 3.19, 2.86, 2.55, 2.27, 2.25, 1.91, 1.67, 1.46, 1.23, 1.06, 0.82.

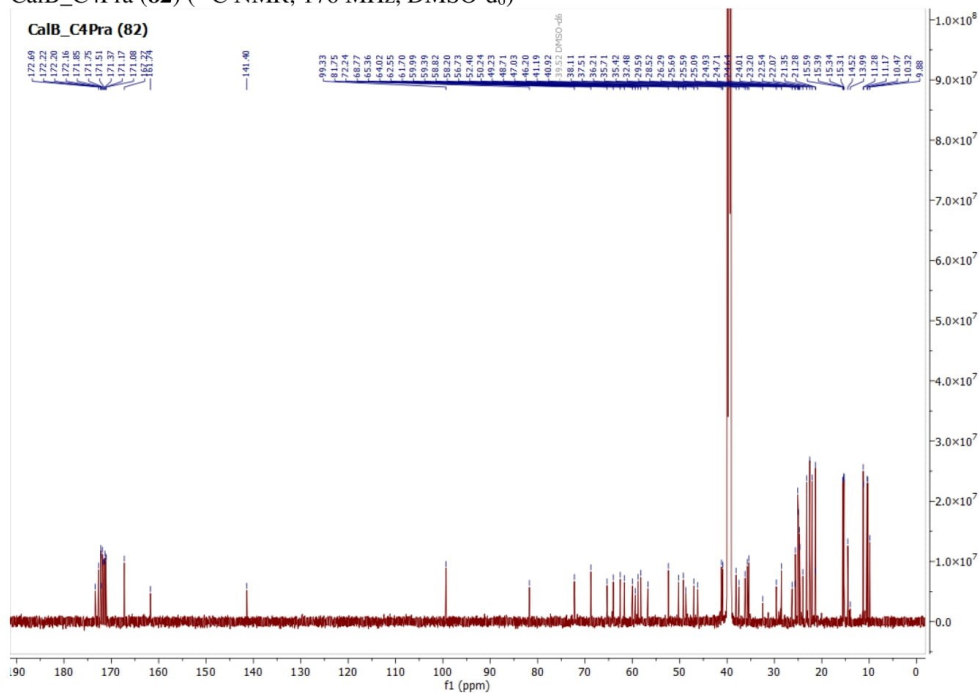
CalB_C3Pra (81)

Chemical structure of CalB_C3Pra (81) is shown above the spectrum. The spectrum displays peaks from 0.0 to 9.0 ppm. Key peaks include a broad peak at ~8.3 ppm (NH), a sharp peak at ~7.2 ppm (OH), a large peak at ~3.2 ppm (CH₂), and a large peak at ~0.8 ppm (CH₃). Integration values are provided below the baseline.

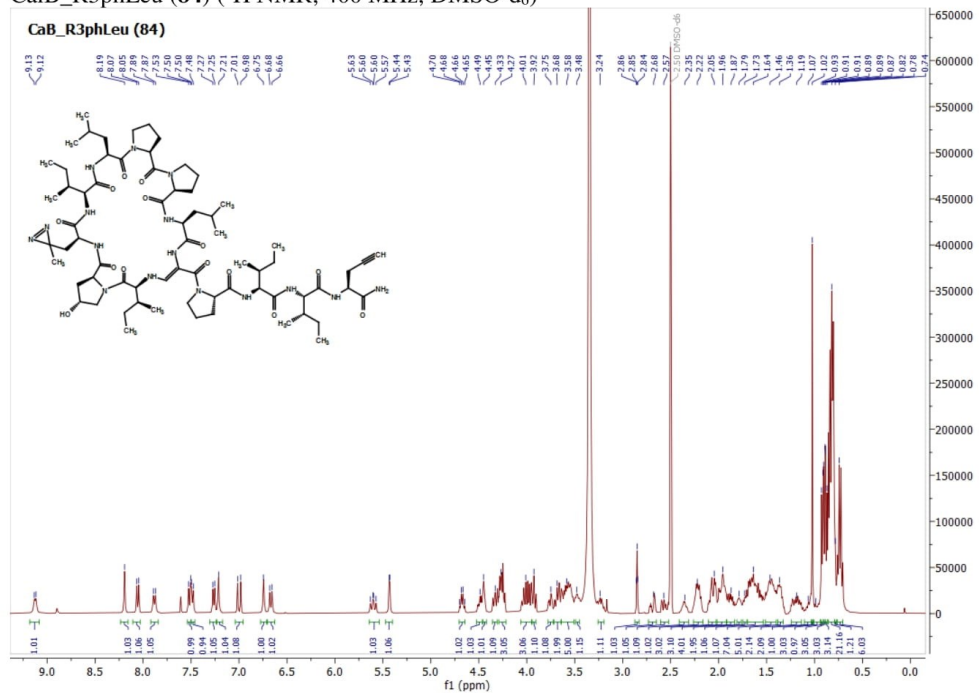
Chemical Shift (ppm)	Integration
~8.35	1.00
~7.27	0.96
~7.27	1.00
~7.10	1.05
~7.07	3.14
~7.07	0.96
~6.99	1.08
~6.99	0.97
~5.77	0.90
~5.40	0.96
~5.39	0.96
~4.55	2.09
~4.41	1.14
~4.23	5.07
~4.15	1.95
~4.05	1.95
~3.87	1.08
~3.80	2.09
~3.74	1.08
~3.69	1.13
~3.20	1.12
~3.08	1.11
~2.70	1.12
~2.67	1.64
~2.51	2.11
~2.46	2.11
~2.11	8.14
~1.90	6.08
~1.61	0.99
~1.42	0.99
~1.28	5.10
~1.12	36.11
~0.82	36.11

Chemical structure of compound 82 is shown in the top left. The ¹H NMR spectrum (DMSO-d₆) is displayed below the structure, showing peaks from 0.0 to 9.0 ppm. The x-axis is labeled 'f1 (ppm)' and the y-axis is labeled 'CalB_C4Pra (82)'. The spectrum shows a complex pattern of peaks, with a large peak at approximately 3.5 ppm and a smaller peak at approximately 1.0 ppm. The chemical structure of compound 82 is a complex molecule with multiple amide and ester groups, and a long alkyl chain.

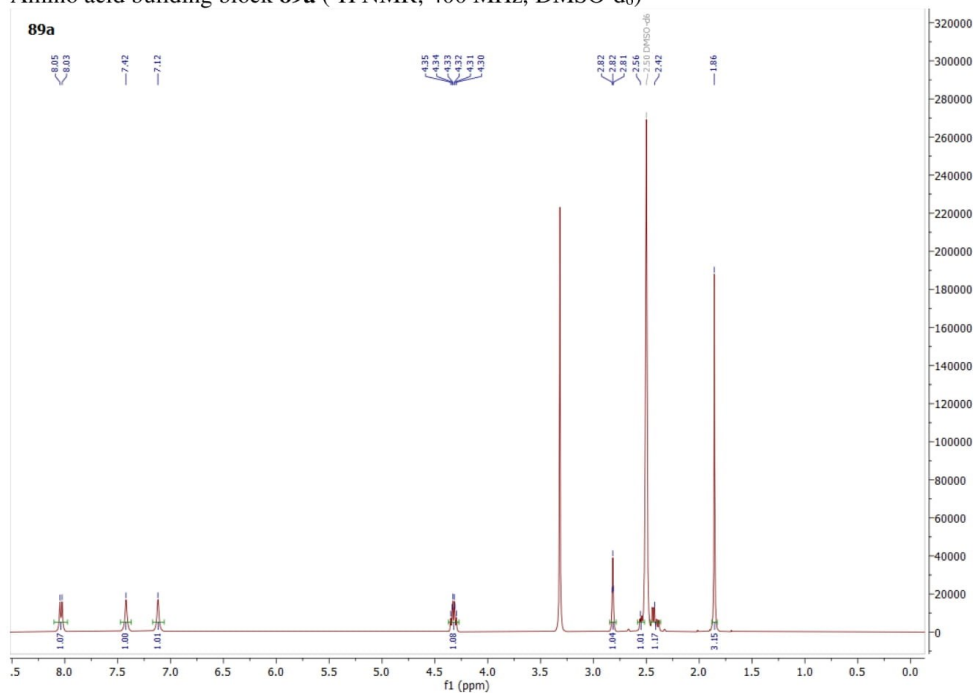
CalB_C4Pra (82) (^{13}C NMR; 176 MHz, DMSO- d_6)



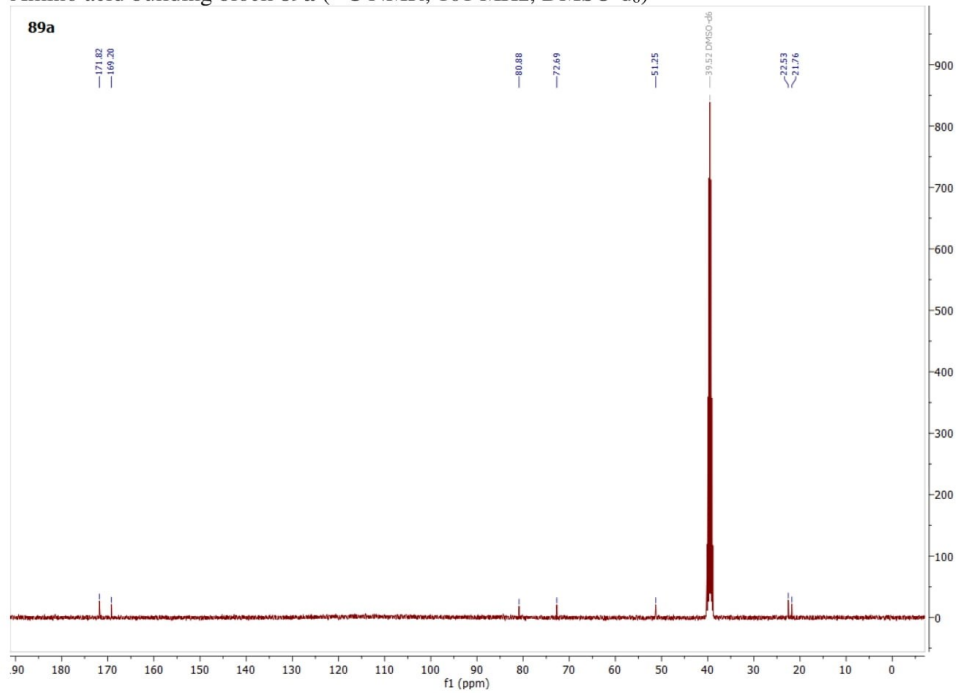
CalB_R3phLeu (84) (^1H NMR; 400 MHz, DMSO- d_6)



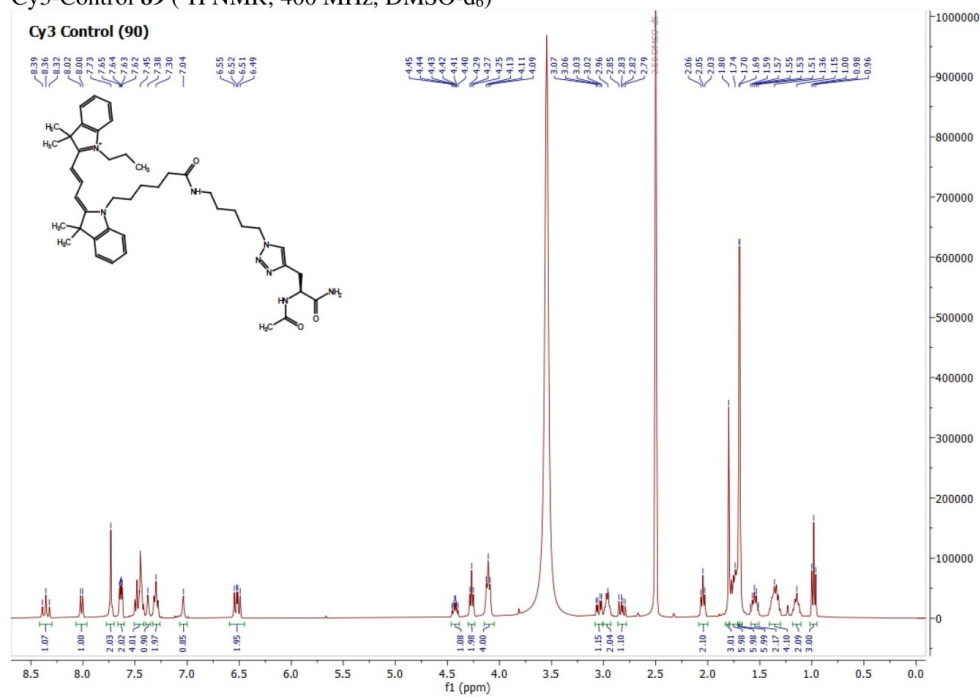
Amino acid building block **89a** (^1H NMR; 400 MHz, DMSO- d_6)



Amino acid building block **89a** (^{13}C NMR; 101 MHz, DMSO- d_6)

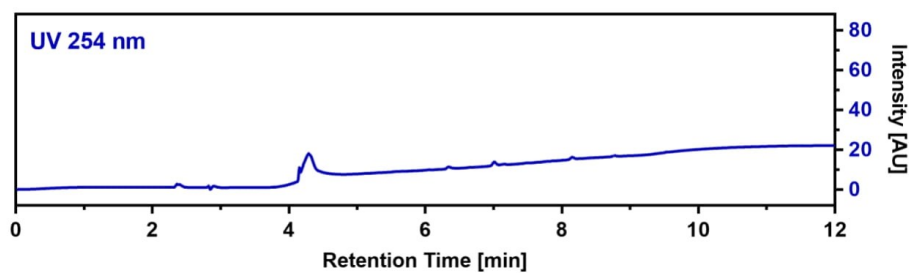


Cy3-Control **89** (^1H NMR; 400 MHz, DMSO- d_6)

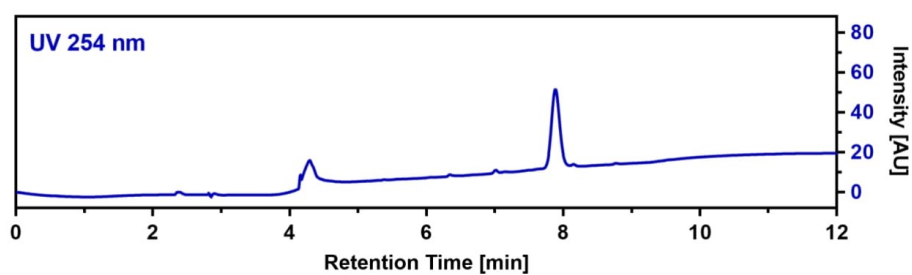


Data S2: UV traces of synthesized callyaerin derivatives, related to STAR
Methods.

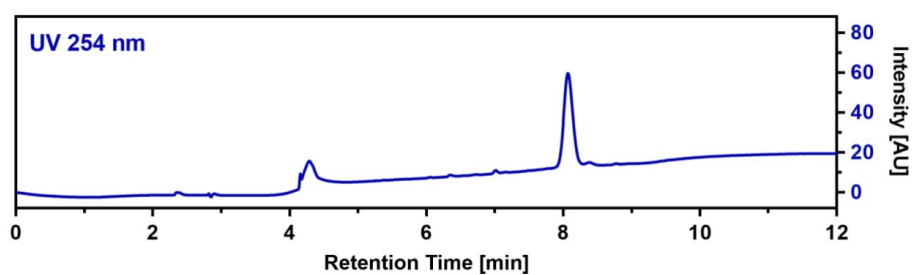
Solvent control (2% DMSO)



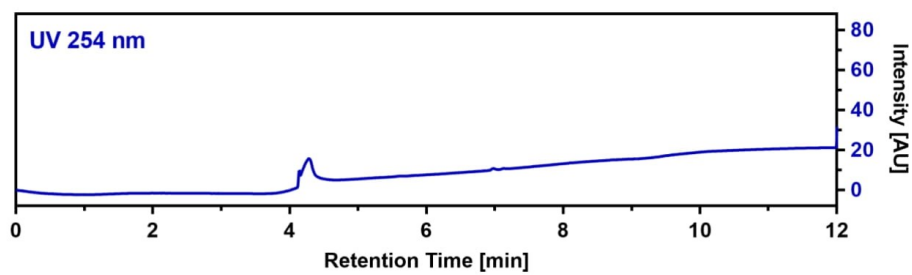
Callyaerin A (1)



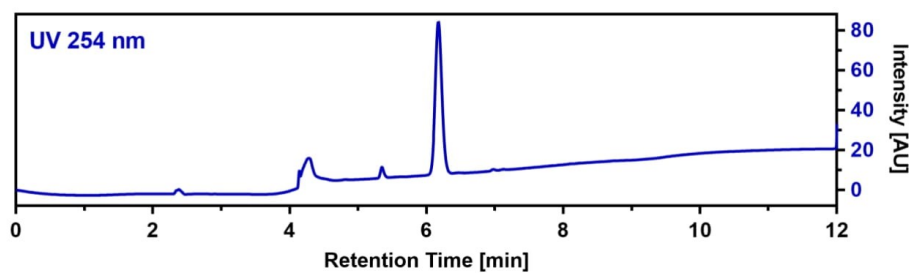
Callyaerin B (2)



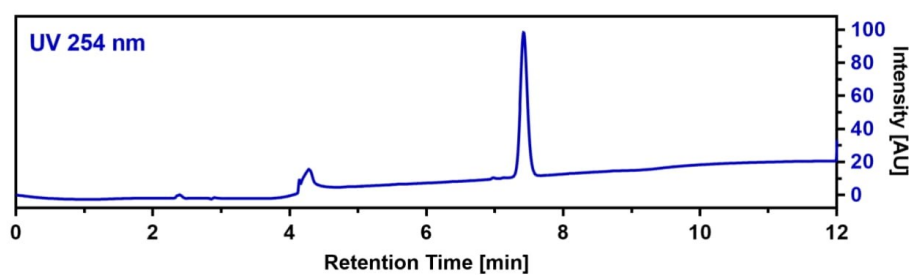
Callyaerin C (3)



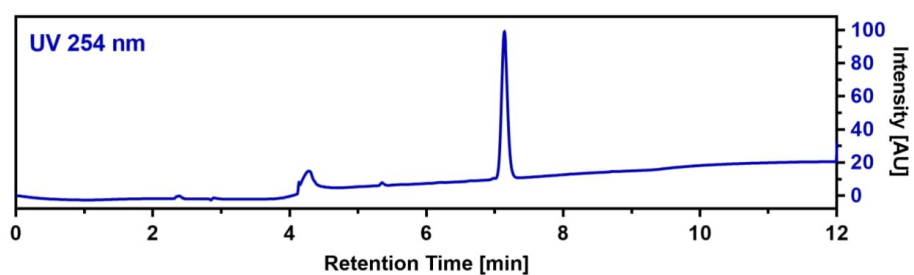
Callyaerin D (4)



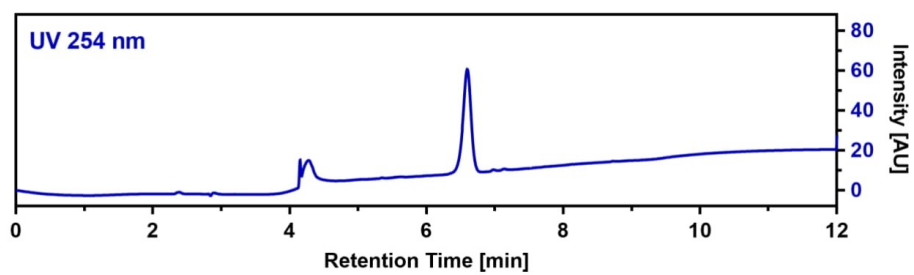
Callyaerin E (5)



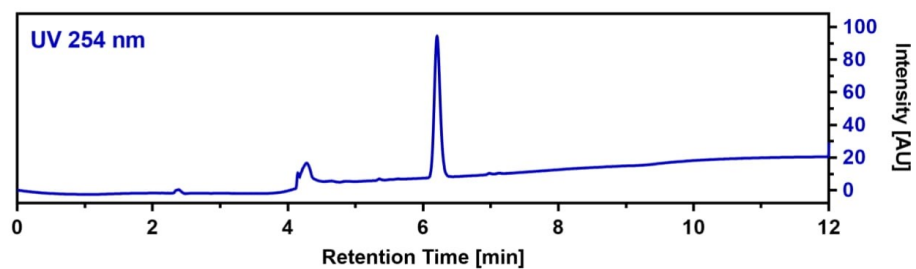
Callyaerin F (6)



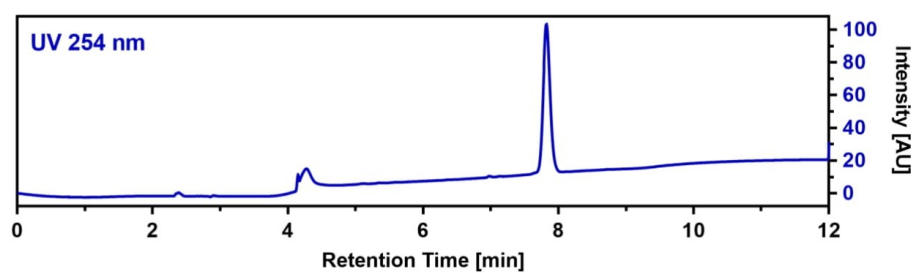
Callyaerin G (7)



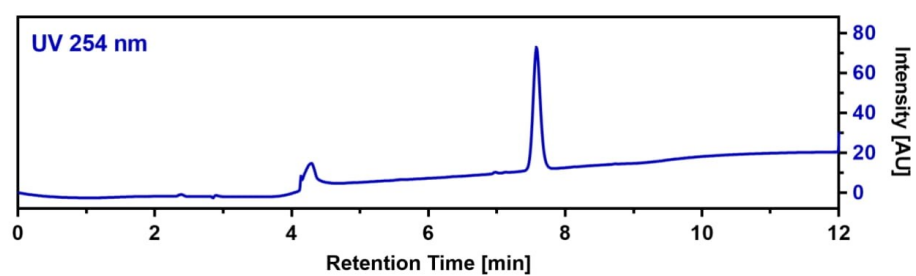
Callyaerin H (8)



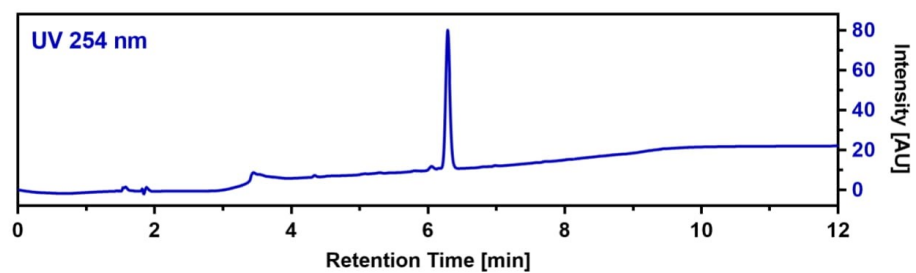
Callyaerin I (9)



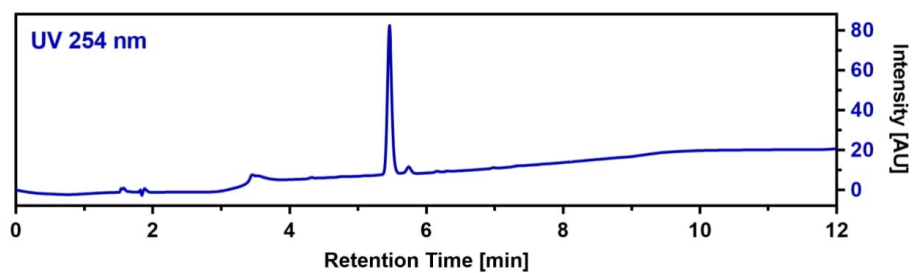
Callyaerin J (10)



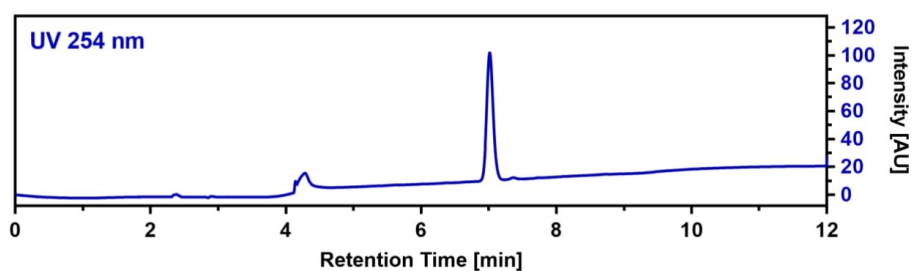
Callyaerin K (11)



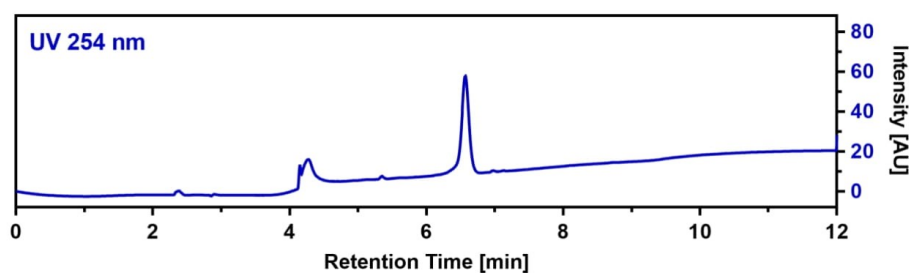
Callyaerin L (12)



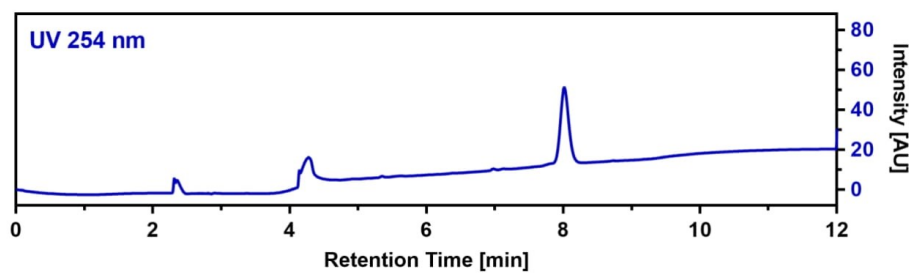
Callynormine A (13)



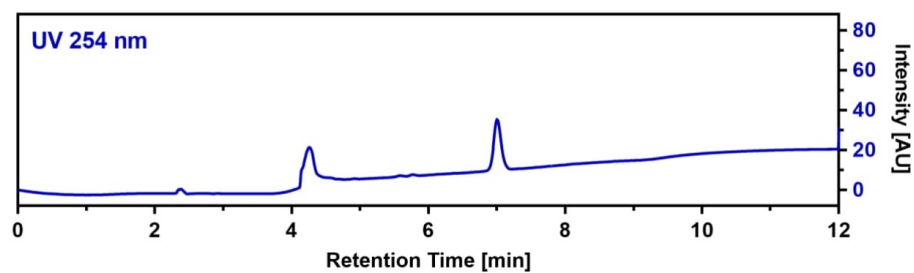
CalA_R1A (14)



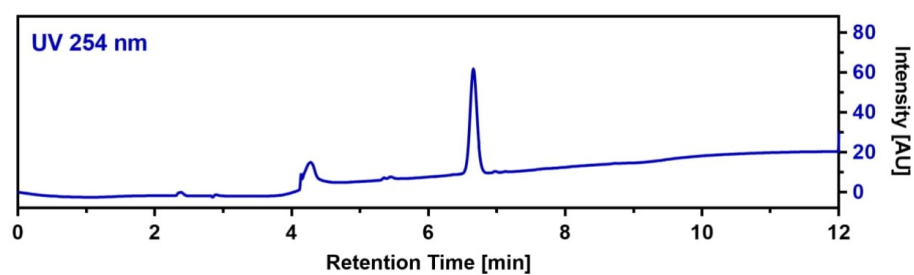
CalA_R2A (15)



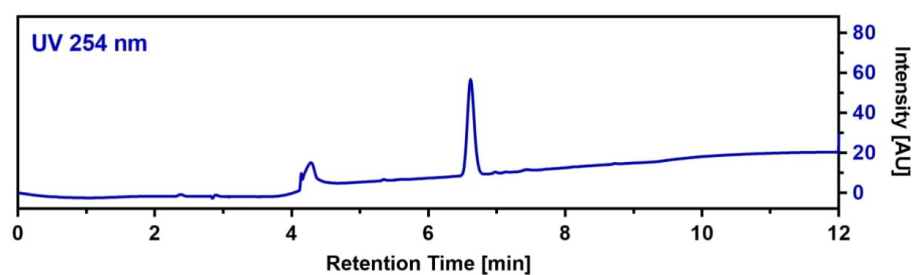
CalA_R3A (16)



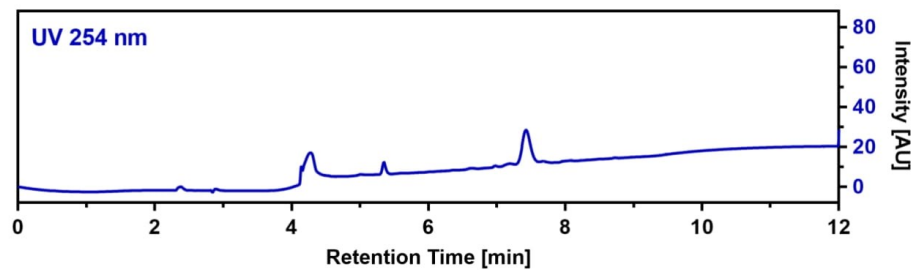
CalA_R4A (17)



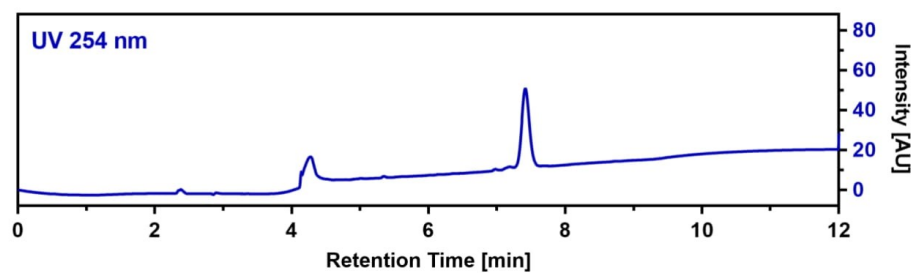
CalA_R5A (18)



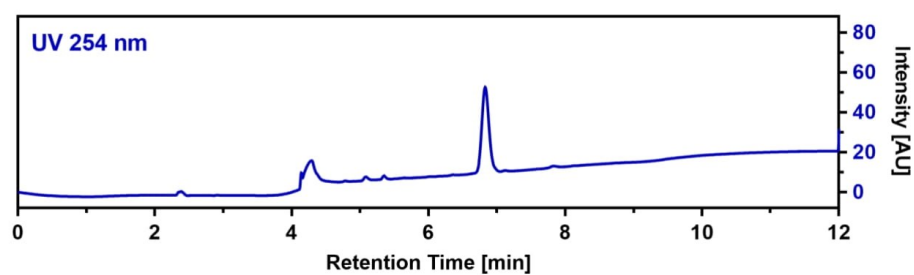
CalA_R6A (19)



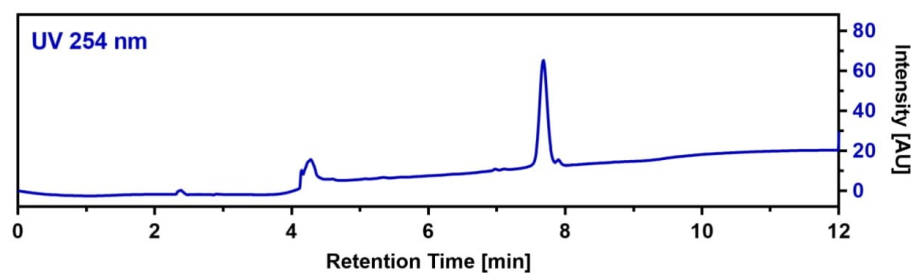
CalA_R7A (20)



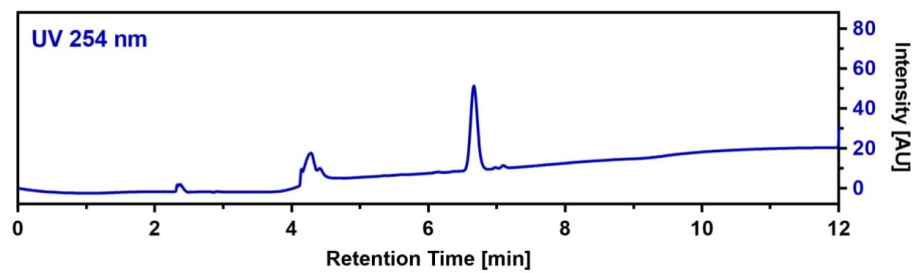
CalA_R8A (21)



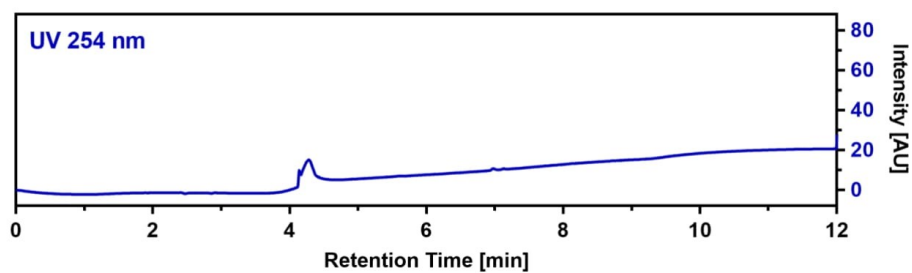
CalA_C1A (22)



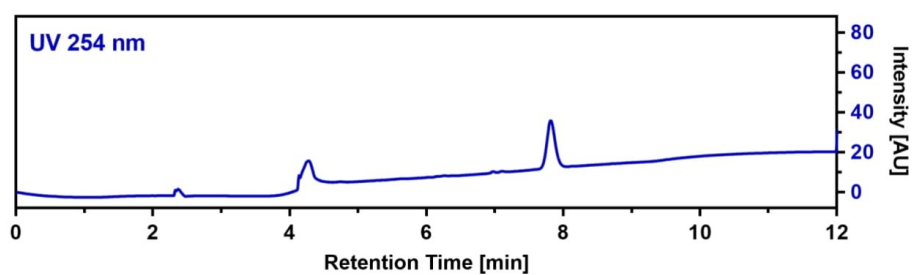
CalA_C2A (23)



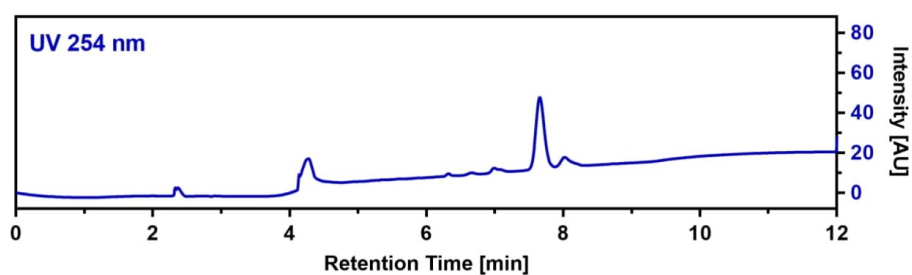
CalA_C3A (24)



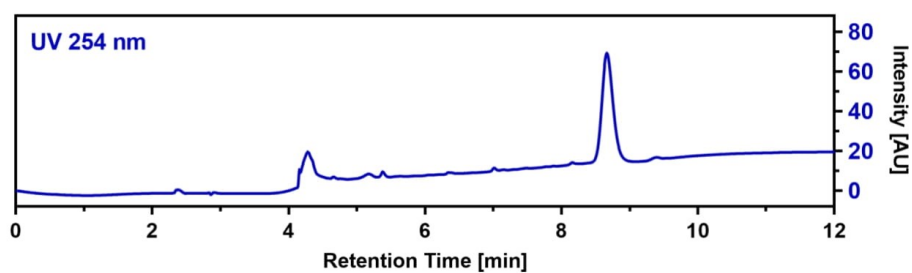
CalA_C4A (25)



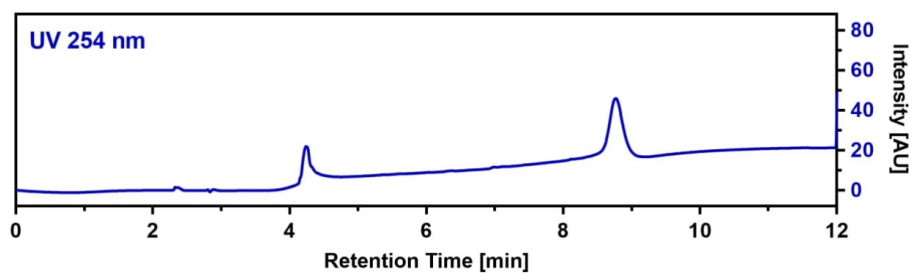
CalA_C5A (26)



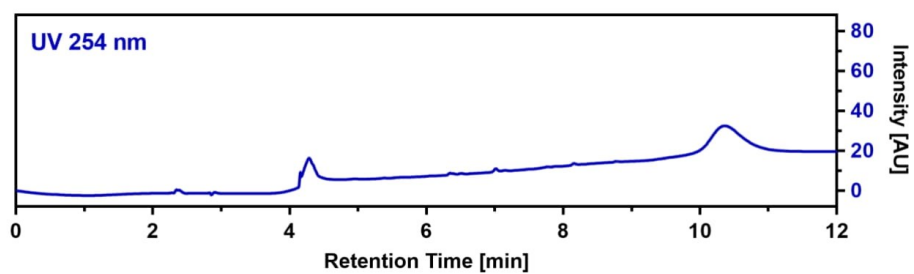
CalB_R2dPro (27)



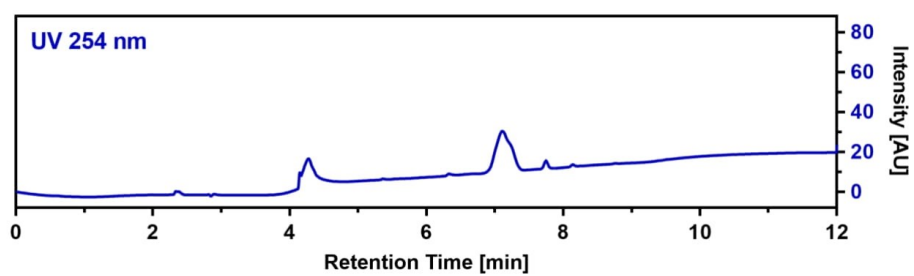
CalB_R2Aze (28)



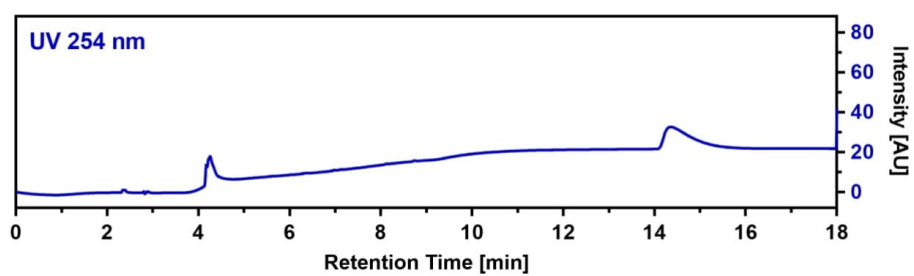
CalB_R2Pip (29)



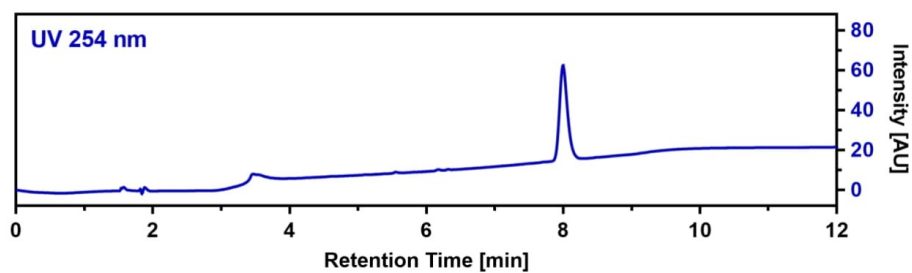
CalB_R2Nip (30)



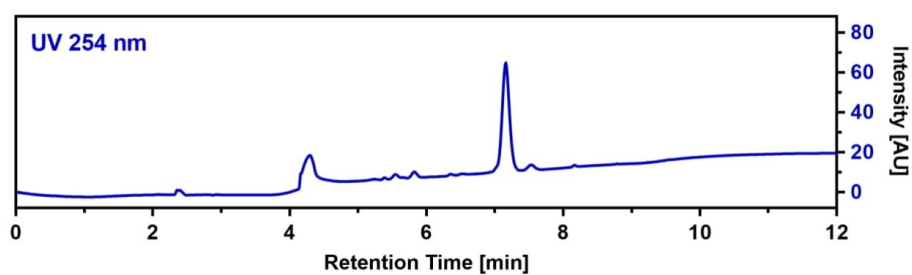
CalB_R2Oic (31)



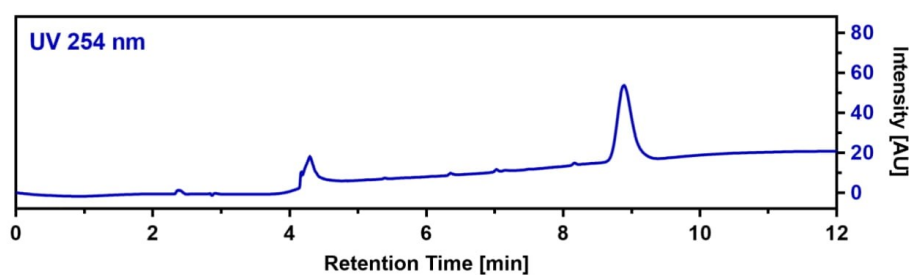
CalB_R3hLeu (32)



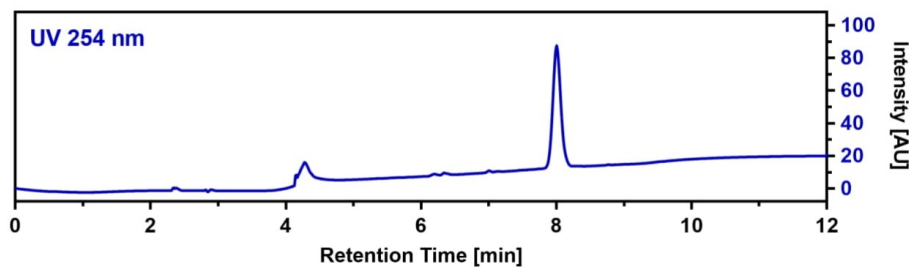
CalB_R3Aib (33)



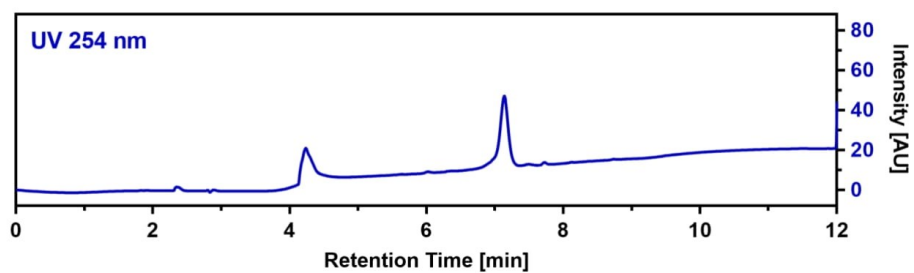
CalB_R3Chg (34)



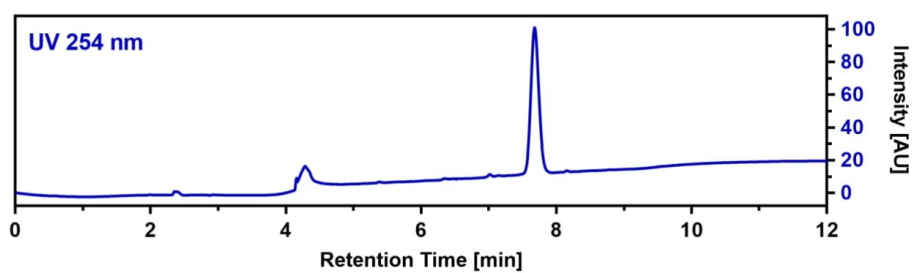
CalB_R3F (35)



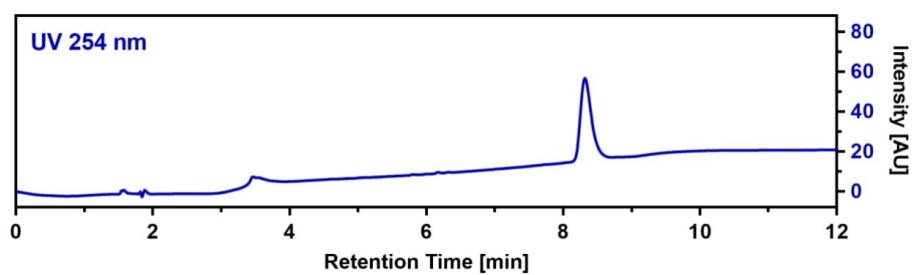
CalB_R3 β -hIle (36)



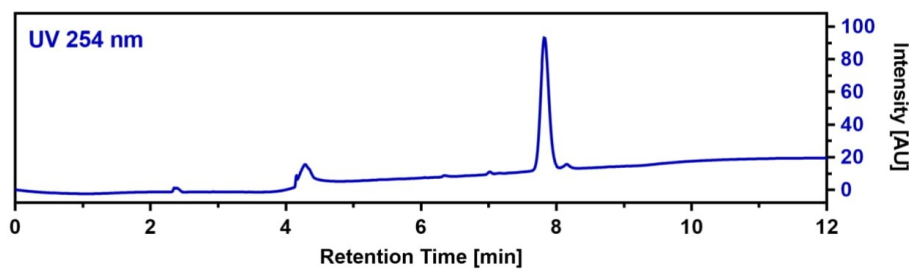
CalB_R4 β -hIle (37)



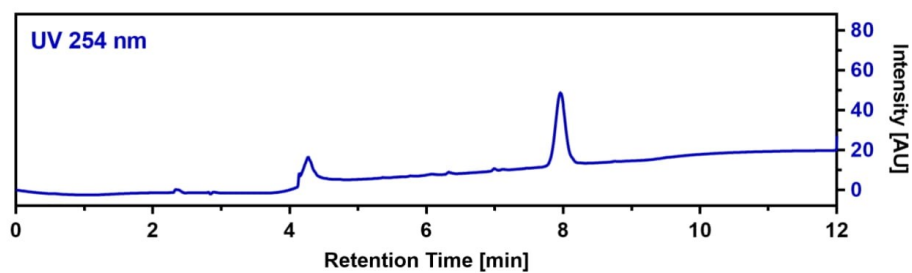
CalB_R5hLeu (38)



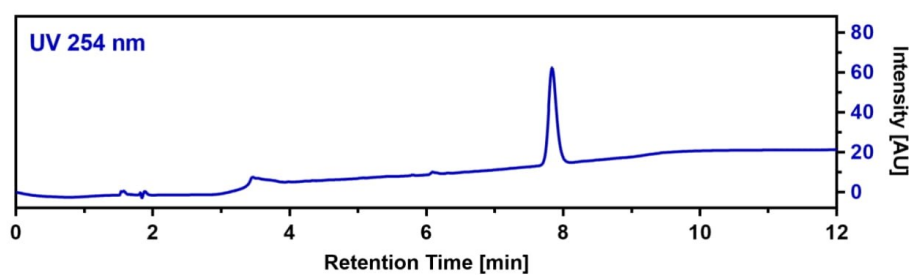
CalB_R6dPro (39)



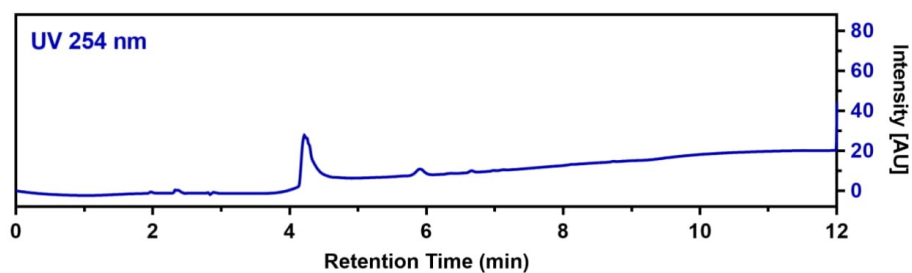
CalB_R6Aze (40)



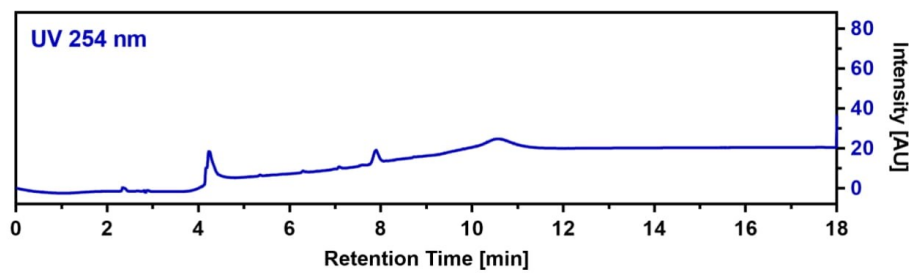
CalB_R6Pip (41)



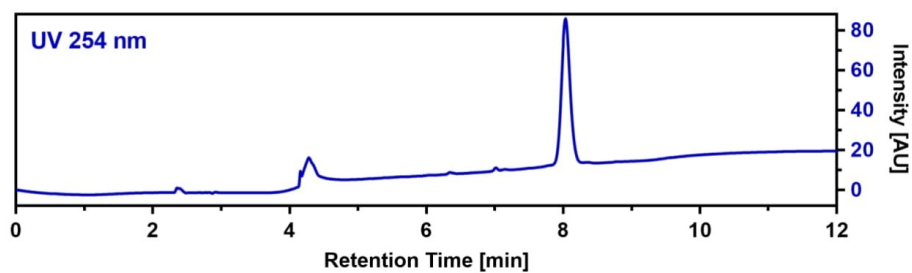
CalB_R6Nip (42)



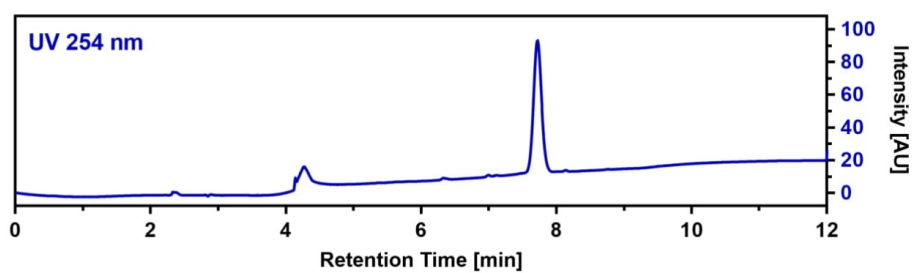
CalB_R6Oic (43)



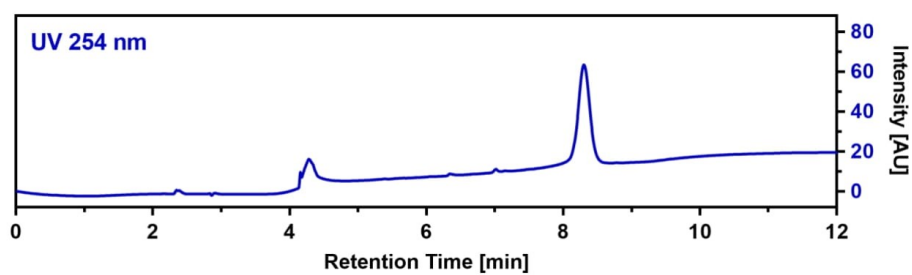
CalB_R7dPro (44)



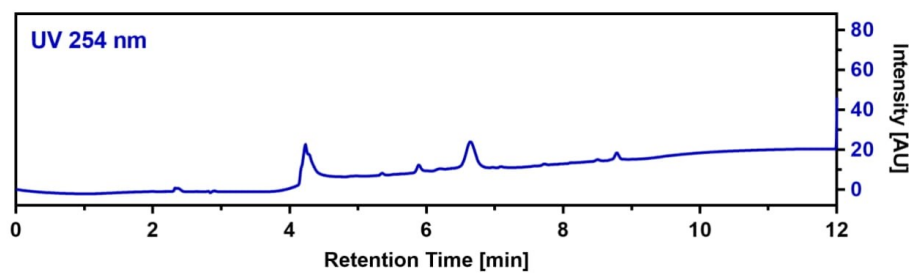
CalB_R7Aze (45)



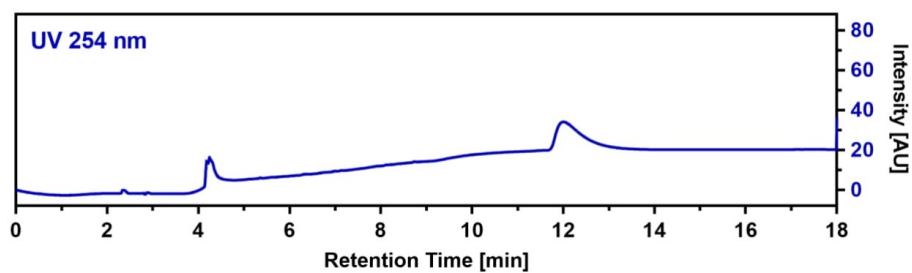
CalB_R7Pip (46)



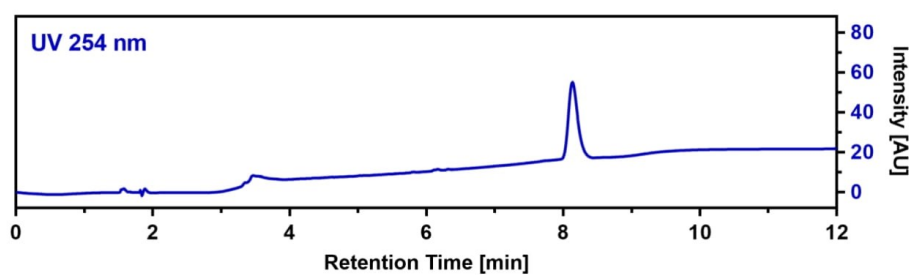
CalB_R7Nip (47)



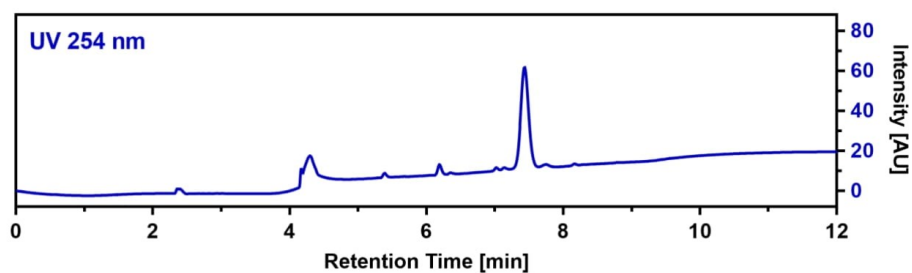
CalB_R7Oic (48)



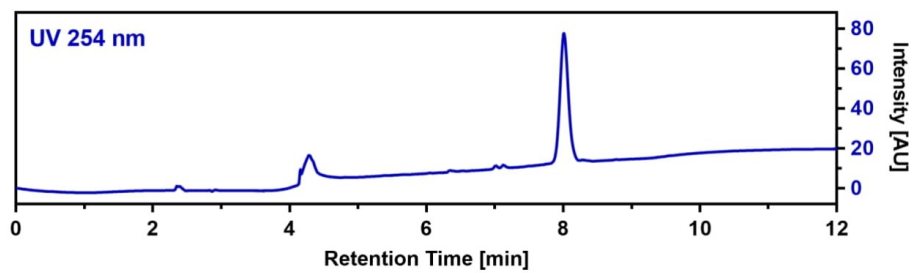
CalB_R8hLeu (49)



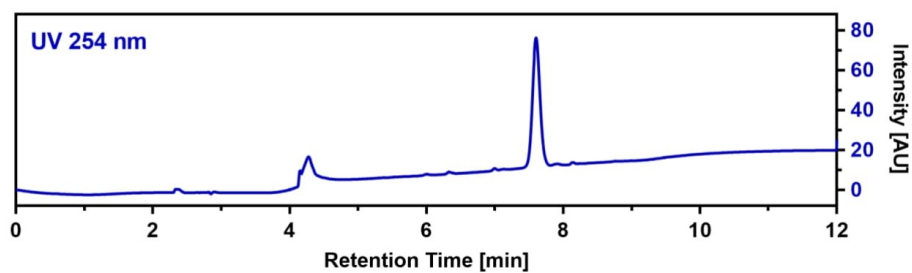
CalB_R8Aib (50)



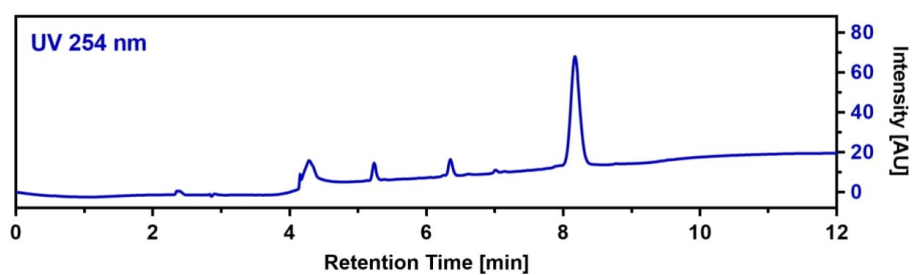
CalB_C1dPro (51)



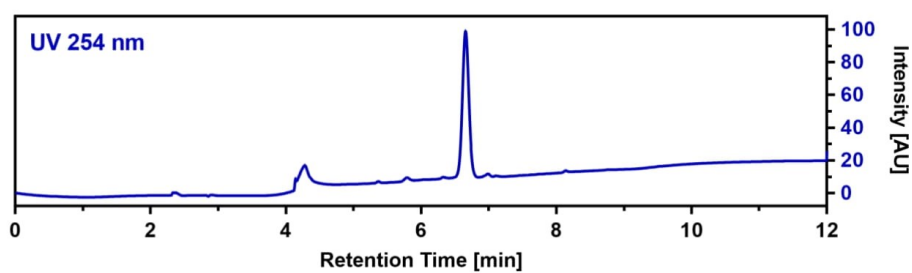
CalB_C1Aze (52)



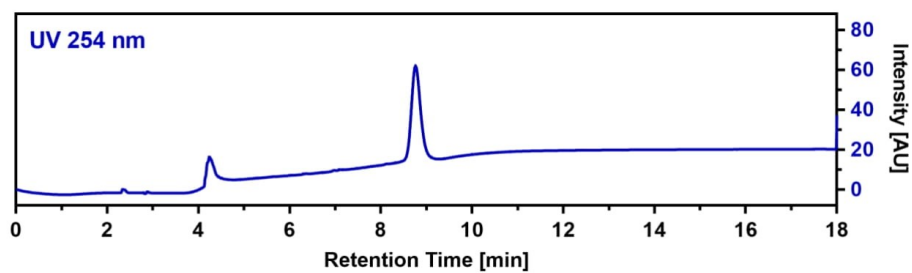
CalB_C1Pip (53)



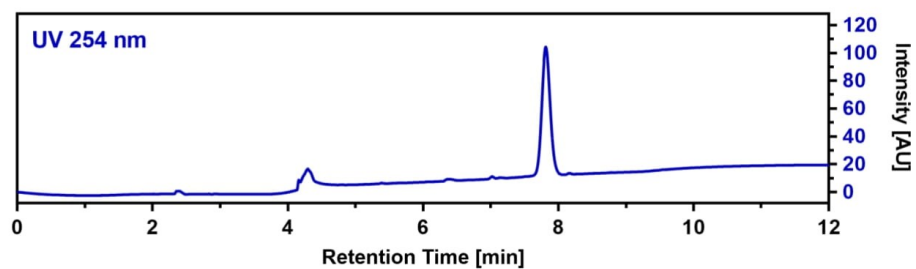
CalB_C1Nip (54)



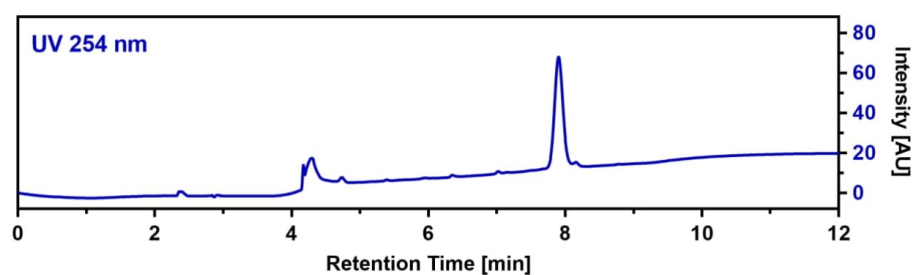
CalB_C1Oic (55)



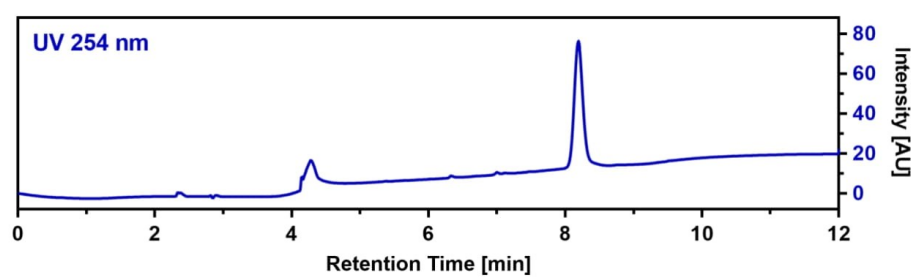
CalB_C2 β -hIle (**56**)



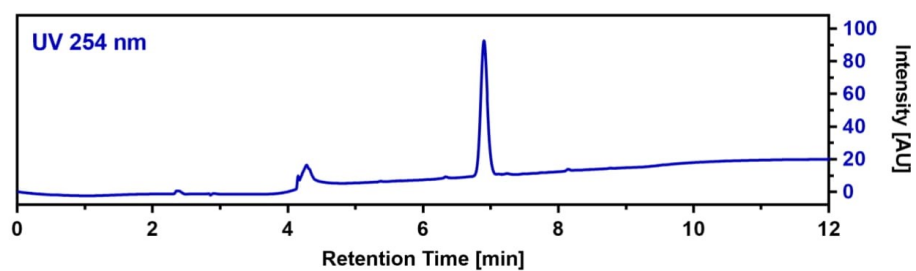
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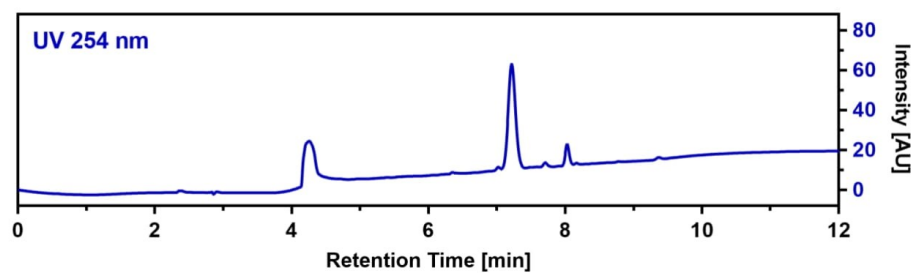
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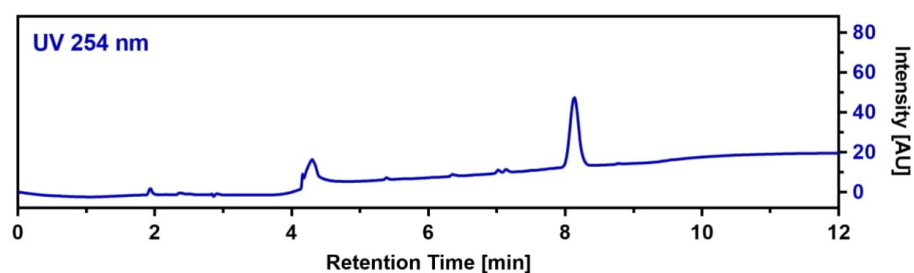
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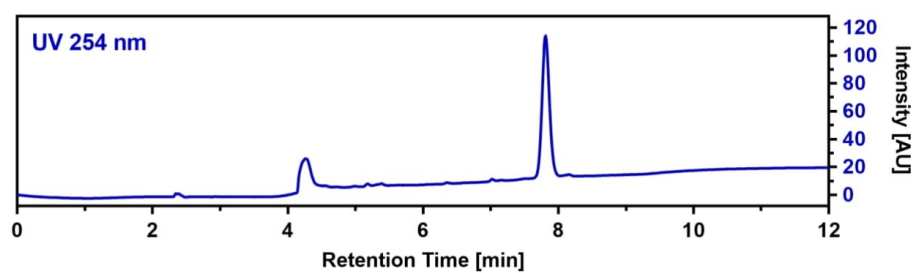
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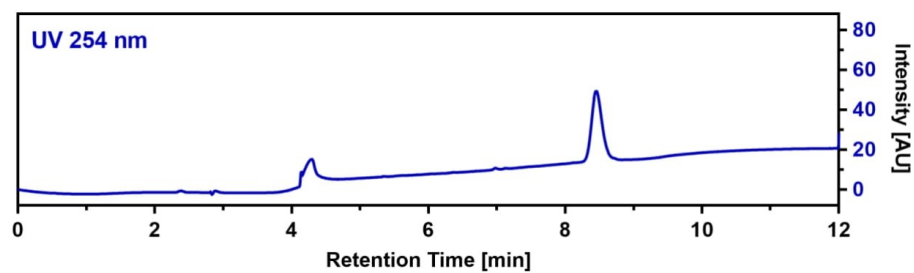
CalB_C3Tle (**61**)



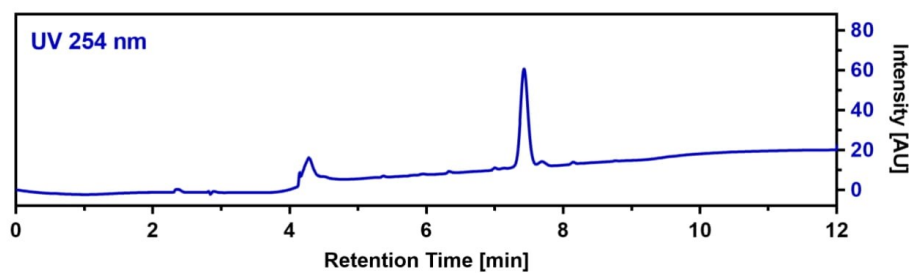
CalB_C3Bpa (**62**)



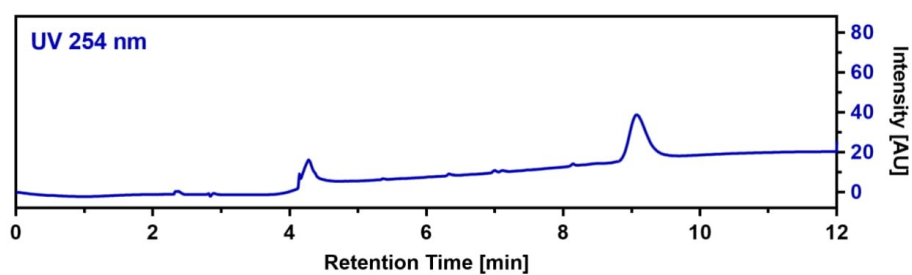
CalA_R2P (**63**)



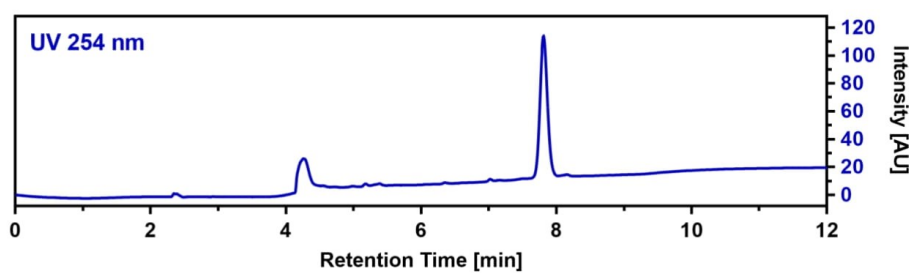
CalA_R3Abu (64)



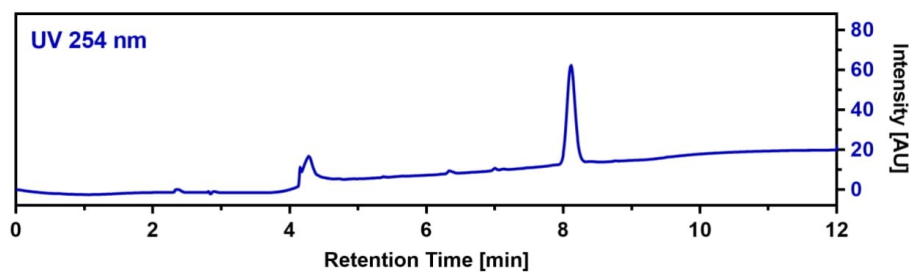
CalA_R3Chg (65)



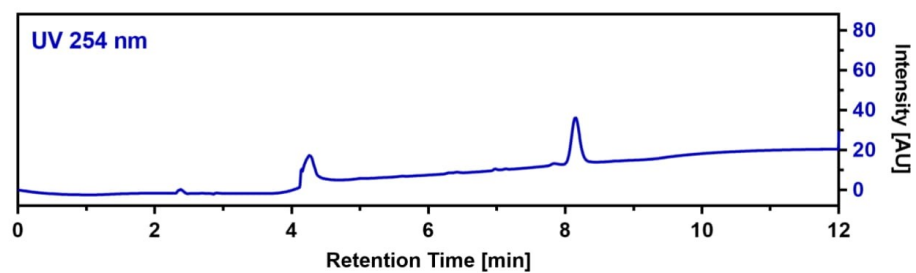
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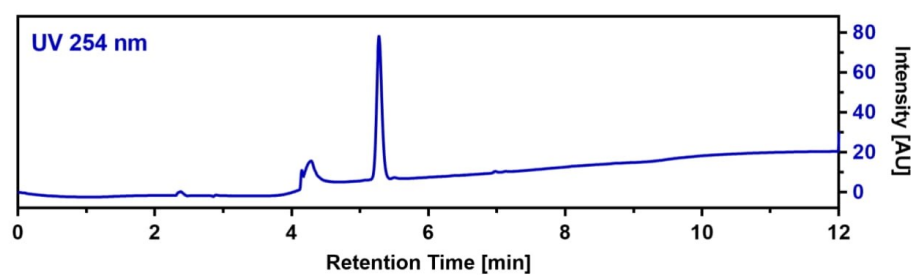
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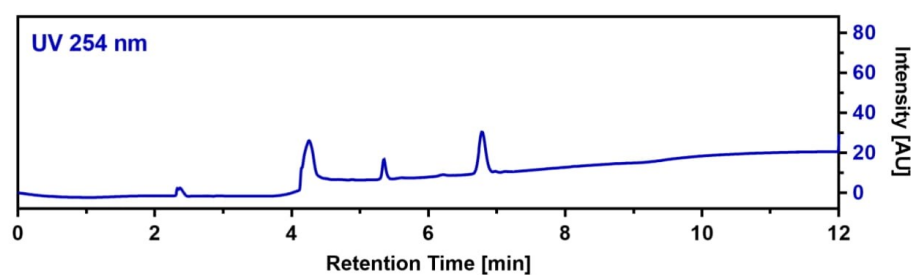
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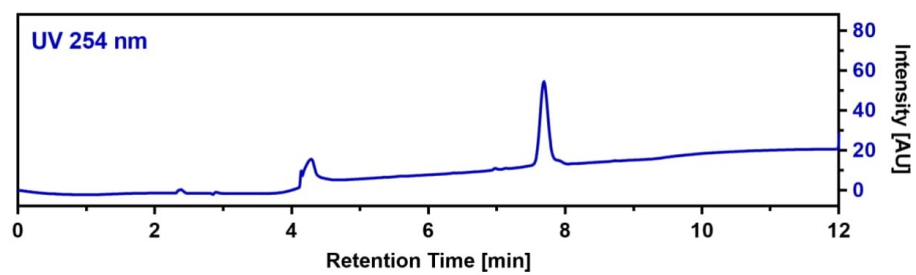
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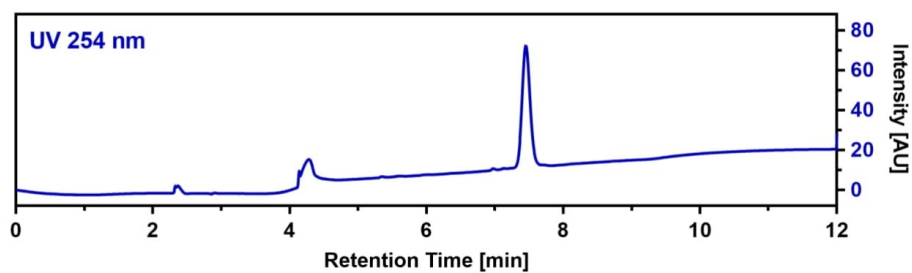
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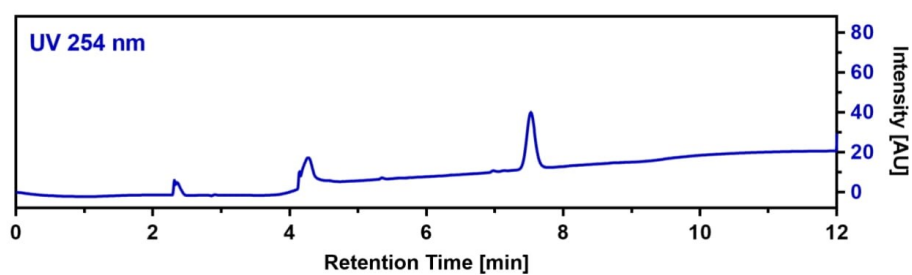
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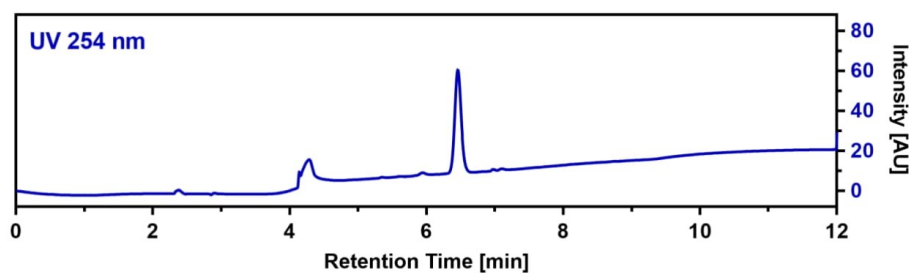
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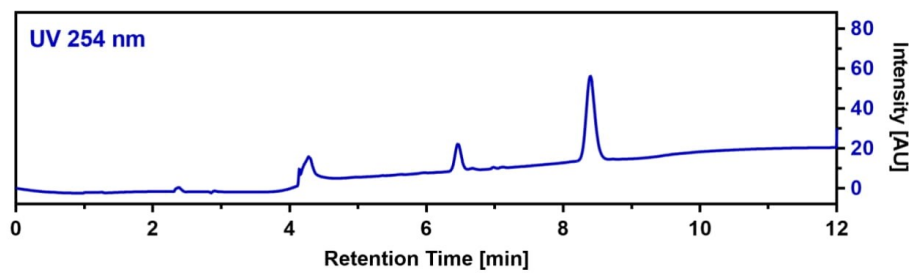
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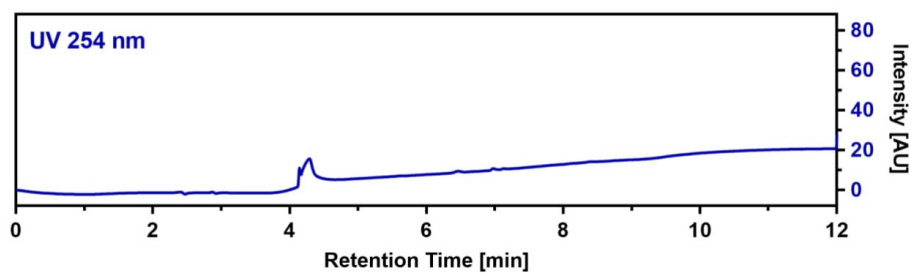
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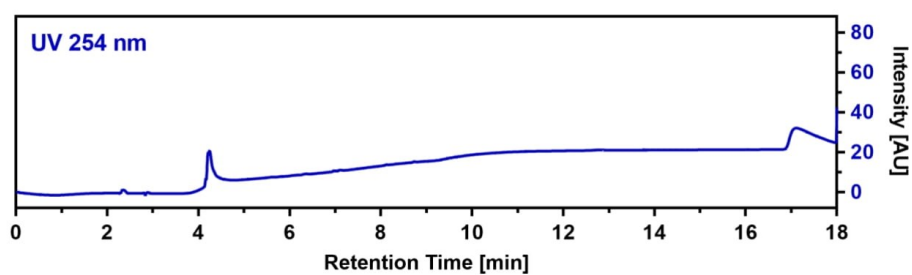
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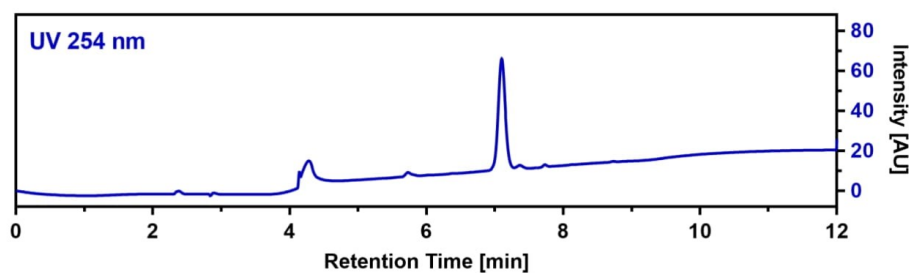
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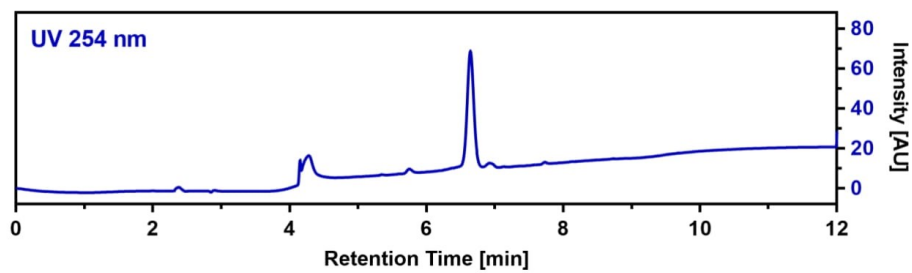
CalB_R2Oic_R3Chg (77)



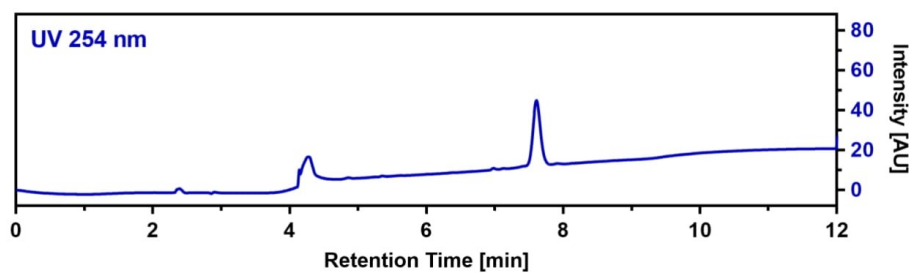
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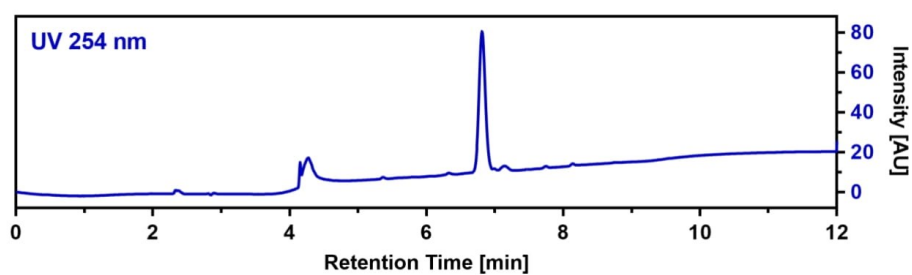
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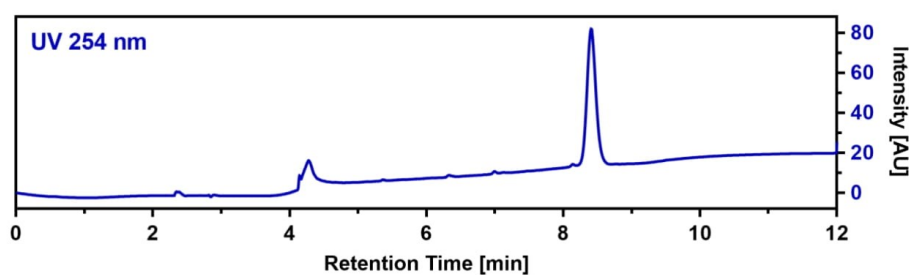
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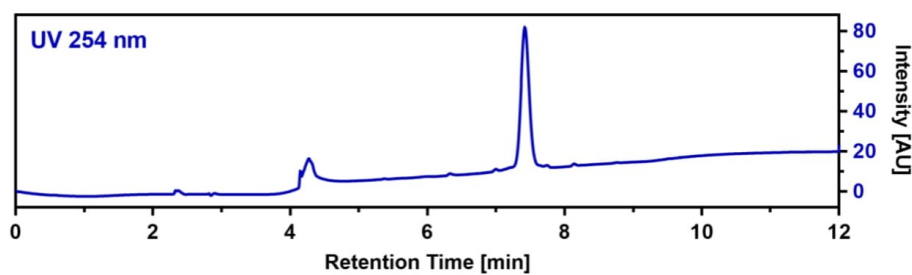
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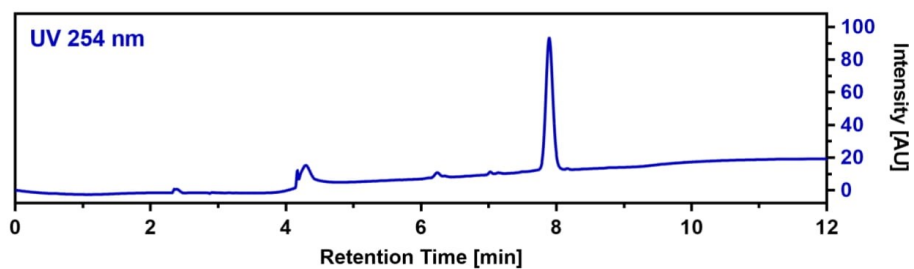
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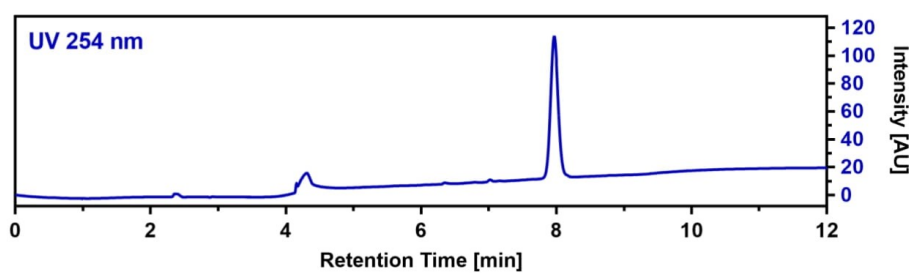
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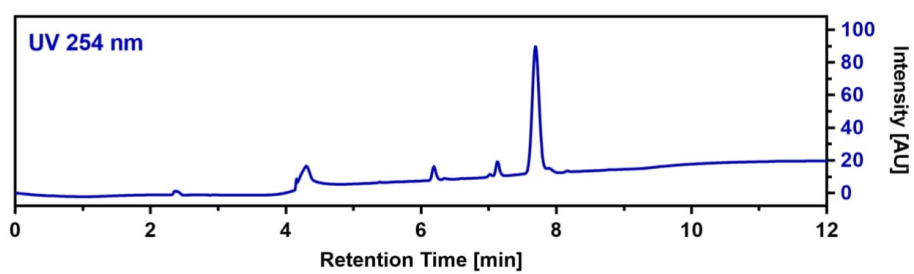
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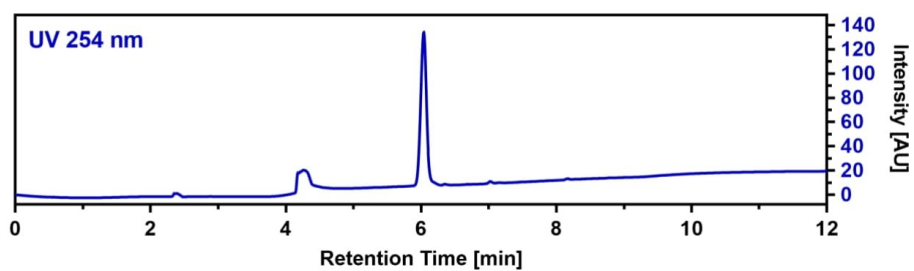
CalB_R5phLeu (85)



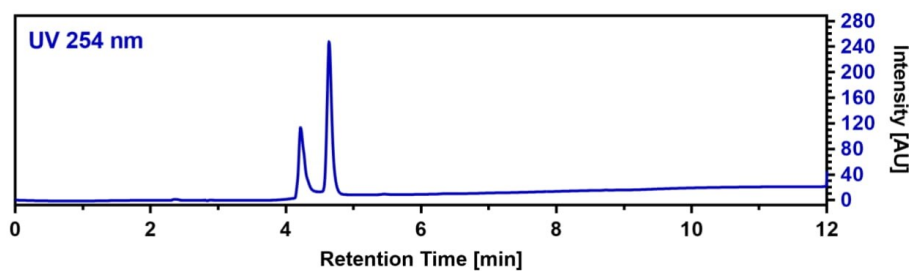
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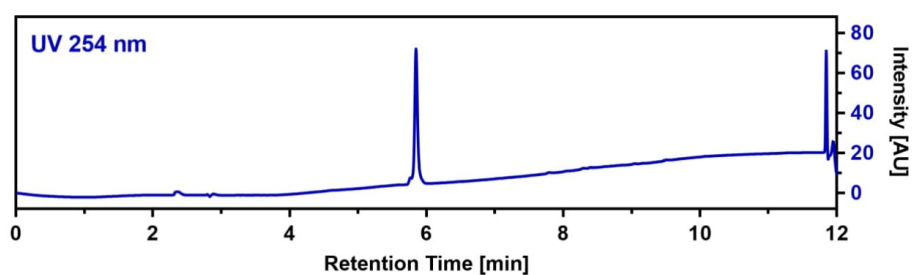
CalB_C4Pra(Cy3) (87)



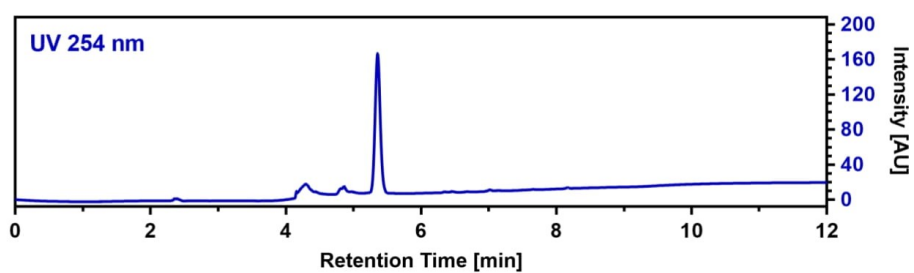
CalB_C4Pra(Rhodamine) (88)



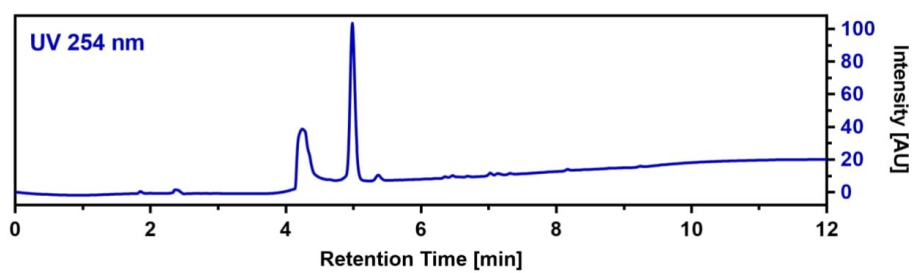
Cy3-Control (89)



CalB_R3Pra(Cy3) (90)



CalB_R4Pra(Cy3) (91)



5 Discussion

These findings represent substantial advances in drug discovery. However, they also highlight limitations and possibilities for future research, which are explored in detail in this section.

5.1 Nature-Inspired (di)Azine-Bridged Bisindole Alkaloids with Potent Antibacterial In Vitro and In Vivo Efficacy against Methicillin-Resistant *Staphylococcus aureus*

We demonstrated that (di)azine-bridged bisindole alkaloid derivatives, inspired by natural products, show promise for further development as antibacterial agents. Indole alkaloids, particularly those derived from plants and sponges, are a class of compounds with diverse bioactivities, including antitumor, antifungal, antiviral, antimicrobial effects. Alocasin A and Hyrtinadine A isolated from *Alocasia macrorrhiza* and *Hyrtios* sp., respectively, are classic natural examples of this compound class. Although both natural products reportedly lacked antibacterial effect, as confirmed in this study, synthetic modifications of these compounds, achieved via an optimized Masuda borylation-Suzuki coupling (MBSC) sequence, birthed a library of 21 natural product-derived and non-natural (di)azine-bridged bisindole derivatives that enabled structure-activity relationship studies.

While unsubstituted bisindoles displayed no antibacterial activity, halogenation of the indole moieties proved crucial for the antibacterial activity of the derivatives, with 5,5'-chloro derivatives- **4j** and **4n** (front-runner compounds), exhibiting the most impressive activity against MRSA and other Gram-positive pathogens with MICs ranging from 0.20 to 0.78 μ M. However, none of these compounds were active against Gram-negative bacteria chiefly due to the presence of an outer membrane (absent in Gram-positives) that makes these bacteria intrinsically resistant to several antibiotics. With **4j** and **4n** displaying moderate cytotoxicity against human cells, administering them to mouse wounds infected with *S. aureus* promoted wound healing. Moreover, the *in vitro* bactericidal effect of both compounds was also replicated *in vivo* by drastically reducing the bacterial burden in these wounds. Contrary to reports mapping the target of bisindole-alkaloids to bacterial pyruvate kinase and despite the structural similarity to these reported bisindoles, the antibacterial activity of **4j** and **4n** is independent of pyruvate kinase. Instead, these compounds target the bacterial membrane, causing membrane permeabilization leading to cellular efflux of low-molecular-weight molecules.

Although significant progress was made during this investigation, several important gaps abound that require further research. These include the cytotoxicity and solubility issues of the compounds, identifying additional structural components critical for antibacterial activity, unraveling the exact molecular target(s), and understanding how these molecules interact with the membrane. Most of these gaps were addressed in a follow-up study.

5.2 Synthesis of Bisindole Alkaloids and Their Mode of Action against Methicillin-Resistant *Staphylococcus aureus*

We further showed in a subsequent study described in chapter 3 that bisindole compounds are promising candidates for future drug development against drug-resistant *S. aureus* strains. By exploring a modified one-pot MBSC protocol, several undescribed *N*-tosyl-protected bisindoles, derivatives without a bridging unit, as well as derivatives with novel substitutions were synthesized in addition to the previously reported compounds. Increasing the library size in this manner pointed out clearly that not only halogenation, especially chlorination of the indole moieties, but heterocyclic bridge presence and deprotection of the indole cores are also essential for the antibacterial activity of these molecules in the sub-micromolar range. It must however be stated that despite varying different structural components of bisindoles, the current optimization campaign did not result in a better derivative in terms of antibacterial potency than the front-runner molecules **4j** and **4n** in chapter 2, renamed **5c** and **5f** in chapter 3. To extend the *in vivo* studies of these compounds beyond the excellent efficacy demonstrated in the mouse wound infection model, additional infection models such as pneumonia or sepsis should be explored. The sepsis model previously failed, presumably due to the poor solubility of the compounds in aqueous buffers. Therefore, an elaborate optimization campaign should be embarked upon.

The goal of the campaign should not only be to chemically modify bisindole compounds to enhance their antibacterial potency, but also to improve their solubility and reduce their cytotoxicity significantly. Moreover, these modifications would be a pedestal to uncover additional structural features pivotal for their antibacterial activity. Besides this, investigating the level of interaction of bisindole alkaloids with clinically relevant antibiotics might reveal synergistic effects of these compounds on currently used antibiotics. Potentially, this translates to *in vivo* clearance of MRSA and other Gram-positive pathogens with lower doses of these compounds, thus, resulting in minimal side effects and negligible likelihood of resistance

emergence of both bisindoles and the synergizing antibiotics. For instance, Swift *et al.* demonstrated that exebacase (CF-301), a novel antistaphylococcal lysin, synergized with daptomycin *in vitro*, resulting in 64-fold reduction in the MICs of daptomycin against both MSSA and MRSA¹. This synergy was replicated in a murine model of staphylococcal pneumonia, where the combination of exebacase and daptomycin resulted in 70% survival compared to 50% and 0% survival in exebacase- and daptomycin-alone mice, respectively¹. Likewise, in a recent study, laurocapram, a widely used transdermal enhancer, potentiated the antibacterial activity of cephalosporins against MRSA². Compared to laurocapram- and cefixime-alone treatment groups, the combination treatment not only remarkably reduced the degree of pulmonary edema in pneumonia-laden mice, but also drastically reduced the bacterial burden in the mice lung tissues by 4 log magnitude².

Resilience of infection is a common place in the management of MRSA infections. One of the contributory factors to this is drug resistance endowed by mutations³. The difficulty encountered in raising resistant mutants to bisindoles despite several attempts and methods, means that bisindoles overcame this first factor to an extent. However, pathogen survival strategies like biofilm formation, is another contributory factor. This factor, which is deployed by MRSA, increasingly poses apprehension in the medical field. These biofilms routinely possess extracellular polymeric substance (EPS) matrix that acts as a physical protective barrier, limiting antibiotic penetration and shielding from immune defenses or surveillance³. Considering that most membrane-active antistaphylococals often display antibiofilm property⁴⁻⁸, it will be worthwhile to screen these compounds for such potential. For this, biofilm-forming *S. aureus* strains, preferably strong biofilm formers such as NCIM 5021 are required. To assay for the antibiofilm property of bisindoles, a cell density of 1×10^6 CFU/mL of the strain should be employed to initiate biofilm formation in the presence and absence of these compounds in 96, 48 or 24-well plates. After 24 hours, the planktonic cells should be removed, the formed biofilms washed with PBS and stained with crystal violet for 15 minutes before quantifying the mass of the biofilms by measuring absorbance at 590 nm. In addition, the compounds should also be investigated for their capacity to disrupt preformed biofilms. To visualize the direct effect of bisindoles on formed biofilms and perhaps determine live and dead populations within such biofilms, samples should be stained using the BacLight live/dead viability kit and subsequently imaged under a confocal microscope.

Upon uncovering the membrane-permeabilizing properties of bisindole alkaloids, we further demonstrated that these compounds interfere with the lipid II biosynthetic cycle, interact

promiscuously with lipid II and membrane phospholipids while also depolarizing bacterial membrane. However, detailed mechanistic insights into how exactly these compounds interact with membrane lipids or bacterial membrane remains to be determined. One advanced technique that would provide deeper understanding of these interactions is molecular dynamics (MD) simulation^{9,10}. To achieve this, first, accurate models of both the compound and the bacterial membrane should be prepared using appropriate tools like CHARMM-GUI^{11,12}. For accurate molecular representation, based on documented experimental findings^{13,14}, the membrane composition must reflect the lipid characteristics of staphylococcal membrane, such as the combination of 250 lipids, each being a phosphatidylglycerol (PdG), lysyl-phosphatidylglycerol (Lysyl-PdG) or cardiolipin¹⁵. Subsequently, the compound modelled using the CHARMM General Force Field (CGenFF)¹⁶ should be integrated into the membrane model, and simulation parameters such as temperature, pressure, varying compound concentrations and boundary conditions should be defined. Upon integration and parameter definition, simulation should be run using NAMD¹⁷. By this, the mode(s) of action of a couple of bacterial membrane-active agents have been resolved in detail^{15,18,19}. This includes a study by Kumari *et al.* where this technique revealed how a strong interaction between a quaternary ammonium-substituted pullulan (CP-4) and DOPG lipids in *S. aureus* membranes is driven by electrostatic forces, leading to lipid clustering and the formation of PdG-rich domains that alter membrane properties¹⁸. These interactions increase membrane fluidity and permeability, with CP-4 primarily engaging with polar lipid head groups rather than penetrating deeply into the membrane core¹⁸.

With particular attention to the binding and influence of bisindoles on membrane properties, simulations should be run, and trajectories analyzed to observe compound-membrane interactions over time. Building on our published data, key analyses should include investigating potential electrostatic interactions, as well as hydrogen bonding between bisindoles and the lipids. Additional analyses should cover how these compounds potentially induce lipid reorganization or randomness, and influence on membrane permeability and integrity.

To buttress the membrane-perturbing mechanism of action of bisindoles, obtaining direct information or physical evidence of the alterations caused by bisindoles on the membrane would be ideal. An ideal methodology to this effect is atomic force microscopy (AFM)^{20,21}. For this, *S. aureus* cells should be exposed to varying sub-lethal and lethal concentrations of bisindoles for certain periods: 0.5, 1, 5 and 24 h, after which the cells should be prepared for

AFM. Compared to cells not exposed to bisindoles, changes on the bacterial surface not limited to vesiculation, membrane carpeting, surface roughness, thickness reduction, and loss of membrane structure are not unexpected. By employing this technique, Barbosa *et al.* reported the significant alterations in the surface roughness and morphology of *S. aureus* caused by the lantibiotic lichenicidin, leading to bacterial cell damage and lysis over time²². Alternatively, electron microscopy (EM) may be employed, but AFM should be preferred since AFM requires minimal sample preparation in near-native conditions, resulting in a three-dimensional output rich in a sample's surface details, texture, and height.

5.3 The anti-tubercular callyaerins target the *Mycobacterium tuberculosis*-specific non-essential membrane protein Rv2113

We demonstrated that callyaerins are a promising antitubercular agent differing from all currently used TB drugs with an unprecedented molecular target. Characterized by their unique cyclic peptide structures and an unusual (*Z*)-2,3-diaminoacrylamide (DAA) unit, callyaerins were originally isolated from *Callyspongia aerizusa*, a marine sponge of Indonesian origin. So far, a total of 14 callyaerin types have been identified, but only five have been reported to possess antitubercular activity. To know whether the other nine types possess activity against *Mtb* and perhaps represent a better starting point for TB drug development, coupled with the limited supply of resources, time- and cost-intensive, and laborious purification processes often accompanying natural products, we developed and employed a straightforward synthesis protocol. This protocol, a slight modification of those reported by the Brimble group²³ and Yates *et al*²⁴, facilitated not only the synthesis of 13 of the 14 callyaerin types, but also ensured, together with alanine scan, a seamless structure-activity relationship (SAR) investigation that was pivotal in uncovering the structural determinants essential for their antitubercular activity. These revealed that the macrocyclic ring size of 8 amino acids, the presence of the unusual DAA unit, and hydrophobicity of the amino acids are all crucial for activity. In agreement with Daletos *et al*²⁵, CalA and CalB are the most potent of the callyaerin types. Importantly, modifications enabled by this protocol yielded more potent derivatives than the parent CalB. Notably, callyaerins display bacteriostatic effect, effectively inhibiting the growth of *Mtb*, including extensively drug-resistant (XDR) strains, while demonstrating an encouraging low cytotoxicity profile against human cell lines.

Through a combination of mutant screens and chemical proteomics approaches, we identified Rv2113, a non-essential, Mtb-specific integral membrane protein, as the direct molecular target of callyaerins. However, the exact molecular mechanism by which callyaerins bind and inhibit this protein remains unclear. Detailed structural studies employing techniques such as X-ray crystallography and, or cryo-EM of Rv2113 alone and in complex with callyaerins may be critical in providing insights into the interactions, as well as the binding mechanism between callyaerins and Rv2113. Moreover, these insights may later prove crucial in guiding the rational design of more efficacious callyaerin derivatives. By employing these techniques, interactions and binding mode of several antibiotics to their molecular targets have been investigated. For instance, by exploiting X-ray crystallography alone, Batt *et al.* identified that while benzothiazinone (BTZ), an Mtb inhibitor in clinical trials, forms a covalent bond with Cys387, the conserved residue in the active site of the mycobacterial membrane-bound DprE1, some BTZ derivatives also form a stable non-covalent complex with DprE1, highlighting the trifluoromethyl group of these inhibitors as a key determinant for binding²⁶. Similarly, using cryo-EM, Zhang *et al.* revealed that ethambutol, a clinically relevant TB drug, targets and binds the active sites of the membrane-embedded EmbB and EmbC proteins, inhibiting their cell-free arabinosyltransferases (AraTs) in the process²⁷.

While the aforementioned techniques should be employed to demystify the biochemical function and interaction partners of Rv2113, as well as the binding mechanism between Rv2113 and callyaerins, challenges associated with characterizing such a protein may not be unexpected since it is a protein embedded in quite a unique, lipid-rich mycobacterial cell envelope. Such proteins require a stabilizing agent outside a lipid bilayer, and detergents are widely used for this purpose²⁸. This, however, does not come without a cost. They tend to disrupt hydrophobic interactions, potentially destabilizing protein structure²⁸. In cryo-EMs for instance, the use of detergents often results in density artefacts, disruption of native-like interactions, ultimately leading to inaccurate or less precise representation of protein structure and lower resolution. These issues, particularly density artefacts were witnessed by Zhang *et al* while resolving the interaction and binding between ethambutol and the membrane-embedded Emb proteins²⁷. To mitigate the challenges posed by detergents, the use of nanodiscs or peptidiscs is highly recommended. Like nanodiscs, peptidiscs are amphipathic peptides with the advantages of preserving a membrane protein in its native lipid environment, long-term stability, and a uniform environment, facilitating high resolution for studies like cryo-EM²⁸.

Through proteomics studies, we further demonstrated that the binding of callyaerins to Rv2113 triggers a complex dysregulation of the Mtb proteome. An interaction of callyaerins with Rv2113 leads to a global downregulation of pathways involved in lipid biosynthesis, cell division, DNA repair, and replication. While this widespread disruption hampers Mtb's ability to proliferate, it begs two salient questions. The first is: what is the exact biochemical function of Rv2113? So far, all that is known about this protein is that it is a membrane protein consisting of 387 amino acids and, according to Alphafold prediction, harbors eight transmembrane helices without any known catalytic domain. To understand the biochemical role of Rv2113, structural databases like DALI server²⁹ should be utilized to conduct a structural similarity search with the 3D structure of Rv2113 obtained via X-ray crystallography or cryo-EM. By comparing the structure to known protein structures, hits are generated that would ultimately reveal potential active sites (if Rv2113 is an enzyme- which is less probable following the revelation of absence of catalytic domains by Alphafold prediction), substrate- or ligand-binding sites, and function. By conducting structural similarity search, Mosior *et al.* confirmed PptH encoded by *rv2795c*, to most closely resemble a metallophosphoesterase, particularly in the conserved metal-binding active site, likely utilizing a metal-associated solvent as the catalytic nucleophile³⁰. These predictions were later confirmed experimentally and the biochemical role of PptH was demonstrated in removing the phosphopantetheine (Ppt) group from holo-acyl carrier proteins, which is essential for fatty acid and mycolic acid biosynthesis³¹. Accordingly, based on the prospective results of the Rv2113 structural similarity search, experiments should be designed and performed to unravel the biochemical function of Rv2113.

The second key question is: what are the interaction partners of Rv2113? One of the techniques to address this is co-immunoprecipitation (co-IP), a method that has the capacity of capturing a protein's interaction partners *in vivo*, followed by protein identification using tandem mass spectrometry (MS/MS). Although a model organism like *M. smegmatis* would have been ideal for this experiment because of its non-pathogenic nature and faster doubling time compared to Mtb, the lack of a Rv2113 homolog in *M. smegmatis* necessitates that this work would be done using the non-virulent *M. bovis* or in Mtb most preferably. To do this, Rv2113 should be cloned encoding a FLAG tag for detection and isolation. Lysates from large cultures of the FLAG-tagged Mtb should then be subjected to co-IP using anti-FLAG beads to isolate Rv2113-FLAG along with any interacting proteins. With Mtb cultures expressing only a FLAG vector as a control, MS/MS analysis should then be used to analyze the protein complexes captured to identify the peptides corresponding to potential Rv2113 interaction partners. Considering that pull-down assay was attempted at some point in this study to achieve similar goal, however

without any concrete outcome, it is quite important to pay attention to the result of the Rv2113 3D structure similarity search. Based on this, the cultures for the co-IP experiments can be stimulated accordingly so that the pathway toward which Rv2113 and its interacting partners are functioning are better expressed for significant co-IP capture. By this, de Miranda *et al.* identified IrtB, MmpL5, MmpS4, MmpS5, and EccE3 as partners of FecB interacting to play a role in apo-siderophore export and ferric-siderophore import³².

Without doubt, providing experimentally validated answers to these two key questions is vital to understanding how callyaerins interfere with the function of Rv2113. Since characterizing a protein of unknown function can sometimes be likened to searching for a needle in a haystack, additional approaches are necessary. Considering Rv2113 is a membrane protein, and the membrane hosts approximately a third of the proteome's proteins and serves as the site for vital processes, including nutrient and waste transport, respiration, proton motive force generation, ATP synthesis, and cell-to-cell communication within biofilms^{33,34}, it may be worthwhile to investigate the role of Rv2113 in these processes. Having demonstrated that Rv2113 does not mediate the uptake of callyaerins, the possibility of transporting vital molecules cannot be entirely ruled out. To investigate the role of Rv2113 in molecule transport, both Mtb wildtype and *rv2113* gene deletion mutant strains should be subjected to membrane permeability assay. If the gene deletion mutant shows a reduced membrane permeability phenotype compared to the wildtype, then Rv2113 is most likely involved in molecule transport. In like manner, both strains should be used to investigate the role of Rv2113 in respiration, proton motive force generation, ATP synthesis, and cell-to-cell communication within biofilms. This approach will eventually be helpful in narrowing down the search for the exact biochemical function of Rv2113.

The identification of Rv2113, a non-essential protein, as a drug target in this study is an unprecedented feat that challenges the conventional dogma of drug discovery that focuses mainly on essential proteins as drug targets. While targeting non-essential yet functionally significant proteins like Rv2113 by callyaerins offers a novel strategy to combat Mtb, the likelihood of quicker resistance emergence compared to targeting essential proteins is a limitation that should not be overlooked. To address this, callyaerins should be combined with other drugs to delay or prevent resistance emergence. Interestingly, callyaerins showed no antagonistic interaction, but an additive effect with bedaquiline, rifampicin, isoniazid, ethambutol, and delamanid. While this interaction is beneficial in terms of the prospect of administering callyaerins in a combination therapy, a synergistic interaction would be more

beneficial. Not only will this delay resistance emergence, but there might also be a chance of achieving sterility from Mtb. Thus, in search of synergistic interaction, in a checkerboard assay, callyaerins should be tested in combination with other clinical TB drugs (such as linezolid, fluoroquinolones, pretomanid, pyrazinamide, ethionamide, cycloserine, streptomycin, and amikacin) and agents currently in clinical trials. With the tolerability of position C4 of CalB that yielded compound 87, the most potent derivative with an MIC₉₀ of 25 nM in mind, it might be possible to conjugate callyaerin and the TB drug to which it synergizes with, in one molecule. When such conjugate is synthesized, the cytotoxicity profile should be ascertained against a host of human cell lines. If not cytotoxic, this may pave way for a dual targeting anti-TB agent with a low likelihood of resistance emergence.

Finally, while valuable insights into the efficacy and molecular target of callyaerins have been gained through *in vitro* findings, the absence of *in vivo* data currently limits their translational relevance. To address this, preclinical studies in animal models should be conducted to evaluate the pharmacokinetics, pharmacodynamics, efficacy and safety profiles of callyaerins. Based on the outcome, the chemical structure of callyaerins should be optimized with the flexible synthesis protocol accordingly. Not only is extending this study to clinical trials critical for bolstering TB treatment options, but it may also pave way for innovative approaches to fight other infectious diseases.

5.4 Strategies to mitigate the antibiotic crisis

The search for new antibacterial agents remains a means to fight bacterial infectious diseases, but there exists a compounding problem in antibiotic resistance. The rate at which drug-resistant strains emerge and spread currently outpaces global antibacterial drug discovery efforts. In fact, recently, an in-depth analysis of antimicrobial resistance estimated that over 39 million people are bound to die from antibiotic-resistant infections between now and 2050^{35,36}. In the face of this gloomy future painted by Naddaf, the same analysis also forecasted that about 92 million lives could be saved between 2025 and 2050³⁶, however, not until certain strategies are in place to mitigate antibiotic resistance crisis. These strategies include, but are not limited to, implementing proper infection control measures and antibiotic stewardship programs, improved diagnostics, and the development of novel and alternative therapeutics.

Alleviating the current antibiotic crisis should not be limited to the development and usage of new antibiotics but rather be initiated by ensuring proper infection control measures are in

place. Hand hygiene is a critical strategy to curb antibiotic resistance, as it prevents microbial transmission during patient care and reduces healthcare-associated infections, thus limiting antibiotic use. As far back as 2009, the World Health Organization (WHO) released the guidelines for the implementation of Multimodal Hand Hygiene Improvement Strategies which borders around 5 moments before and after contact with patient, aseptic operations, and patient's environment^{37,38}. If implemented, hand hygiene can prevent 50% of avoidable infections including drug-resistant ones that may occur during healthcare³⁹. Despite the need and obvious benefit, implementing hand hygiene remains challenging. Although significant improvement in hand hygiene adherence was observed among healthcare workers during the first wave of the COVID-19 pandemic^{40,41}, adherence has since waned⁴². To improve hand hygiene compliance among healthcare workers, monitoring and providing feedback on its implementation must be ensured.

Sanitation and vaccines are other infection control measures that can play crucial roles in addressing the root causes of infectious diseases from different angles. Proper sanitation, including clean water and hygienic waste disposal, reduces the spread of infectious diseases, whereas vaccines prevent infections that would otherwise require antibiotic treatment by providing immunity. With stronger hand hygiene adherence, improving sanitation and expanding vaccine coverage, the spread of infections can be reduced, and the over-reliance of antibiotics can be curbed.

Indeed, the global issue of antibiotic resistance has placed us in a critical situation that demands us to effectively reduce antibiotic consumption. Some of the activities leading to this dire situation include unregulated over-the-counter sales of drugs resulting in self-medication, patient's failure to complete prescribed antibiotic regimen as is often the case with TB patients, overuse of antibiotics in agriculture for disease prevention and growth promotion. To restrict this inordinate use of antibiotics, strict implementation of antibiotic stewardship programs (ASPs) is necessary in healthcare settings. With ASPs, not only are healthcare providers educated on ensuring that antibiotics are prescribed and used only when necessary and appropriate, but also antibiotic prescription, dosage, duration, and choice are monitored⁴³.

Moreover, in general, implementing a framework that addresses the interconnected factors among humans, animals, and the environment contributing to resistance is key. Such a framework exists in the One Health approach⁴⁴. The contributions of this initiative include developing risk analysis guidelines for foodborne antibiotic resistance; implementing integrated surveillance across human, animal, environmental sectors to address sources of resistance

through pollution control; and monitoring antibiotic use across various sectors⁴⁴. It also fosters global collaboration among health science professionals across related disciplines and institutions to achieve optimal health for humans, domestic animals, wildlife, plants, and the environment⁴⁵. By enforcing ASPs and the One Health initiative, judicious use of antibiotics is guaranteed to an extent, thus slowing down the fast pace of antibiotic resistance evolution.

Pivotal to the implementation of ASPs and mitigation of antibiotic crisis at large are improved diagnostics. In many low- and middle-income countries, the lack of appropriate diagnostic and clinical tools leads to high rates of inappropriate antibiotic use. Basic diagnostic assays, such as blood cultures, urinalysis, and radiographs, are crucial for medical decision-making but are often unavailable or too costly in these nations. Without these diagnostic assays, and sometimes due to time constraints, clinicians tend to prescribe antibiotics as a safeguard against severe infections, even when not necessary, consequently leading to antibiotic misuse and overuse. Studies have shown that about 50% of current antibiotic use could be prevented without adverse effects to patients if proper diagnostic tools were available⁴⁶. Nonetheless, with advanced diagnostic tools that enable rapid differentiation between bacterial and non-bacterial infections, as well as identification of specific pathogens with their antibiotic susceptibility profiles, precision of antibiotic use could be enhanced, and the effectiveness of currently used antibiotics could be preserved.

Another strategy to preserve the effectiveness of the once heralded miracle drugs is the widespread adoption of alternative therapeutics. With this, there will be less consumption pressure on antibiotics. Examples of alternative therapeutics are bacteriophages, bacteriocins, and monoclonal antibodies (mAbs). Bacteriophages, also called phages, are viruses that infect bacteria and are not inherently toxic to human hosts. Two forms of phages exist- temperate and lytic phages. The latter is preferred in phage therapy primarily because of its strict lytic cycle resulting in immediate host (bacteria) cell death. Historically, phage therapy has been around since the early 1900s with the first reported use as a therapeutic in 1921^{47,48}. However, with the increasing threat posed by MDR amidst dwindling antibiotics, phage therapy has made a return to the forefront as a potential treatment option to alleviate the antibiotic crisis. While no single naturally-occurring phage has all the features required to be an ideal therapeutic agent, targeted genome engineering of phage isolates with the desired criteria have been instrumental in modeling phages as therapeutics for MDRs⁴⁹. In comparison to antibiotics, phage therapy offers specificity that spares beneficial microflora, auto-dosing at infection sites, minimal side effects, and compatibility with antibiotics for enhanced treatment⁵⁰. Just recently, Braunstein *et al.*

employed phage-antibiotic therapy to successfully treat an MDR *Pseudomonas aeruginosa* implant-associated infection in a Siamese cat⁵¹. Moreso, several phage therapy candidates have reached phase III trials and may be in the market within the next five years⁵². However, challenges like bacterial resistance to phages, complex pharmacokinetics, regulatory hurdles, potential immune responses may hinder its widespread adoption⁵⁰. Despite these obstacles, phage therapy holds tremendous potential for addressing the antibiotic crisis.

Another alternative to conventional antibiotics that is gaining interest is bacteriocin. These are ribosomally synthesized antimicrobial peptides that are secreted by bacteria to inhibit closely related species by disrupting their plasma membranes via pore formation. Found in about 99% of bacteria, they exhibit significant diversity. This diversity could be exploited for therapeutic purposes⁵³. A prominent example is nisin A produced by lactic acid bacteria, widely used as food preservative and adaptable for medical applications⁵⁴. For instance, bacteriocins have shown efficacy against 29 strains of vancomycin-resistant *Enterococcus* (VRE), while sparing other pathogens⁵⁵, illustrating their very little to no propensity for off-target effect and anti-dysbiosis nature. However, challenges include the potential for resistance development under prolonged exposure, degradation of larger bacteriocins by environmental stresses, and their narrow spectrum, which limits their range of targets⁵⁶. While bacteriocins are promising alternatives to antibiotics, careful implementation is required to address these limitations for broader therapeutic use.

mAbs are another viable option that could reduce the pressure on antibiotics. mAbs are highly specific proteins designed to target antigens on pathogens, offering an alternative to antibiotics by neutralizing pathogens or provoking immune responses without inducing resistance⁵⁷. They bypass traditional antibiotic mechanisms, offering specificity, reduced risk of resistance by targeting non-essential virulence factors, longer half-lives, safety, and efficacy, while preserving the microbiota⁵⁷. While three antibacterial mAbs neutralizing the endotoxins of *Bacillus anthracis* (Raxibacumab and Obiltoxaximab) and *Clostridium difficile* (Bezlotoxumab) have received FDA approval so far, giant stride is also being made to target other important pathogens^{58,59}. Just recently, Simonis *et al.* discovered a highly neutralizing mAb with broad antibiotic-like *in vivo* activity against MDR strains of *P. aeruginosa*, targeting the type III secretion system (T3SS) needle-tip protein PcrV⁶⁰. However, mAbs are not without limitations. These include high production costs, challenges in accessing target antigens due to biofilms or capsules, and a narrow spectrum of activity⁵⁷. Despite these challenges, the future

looks bright for mAbs to emerge as a worthy alternative to traditional antibiotics, to mitigate the current antibiotic crisis.

It is quite important to note that antibiotics cannot be entirely replaced, and that in addition to the aforementioned strategies, tremendous drug discovery efforts will constantly be required to curb the current antibiotic crisis. Thus, in a local drug discovery effort, this thesis successfully characterized experimental drugs against two notorious bacterial pathogens, one against MRSA, and the other against Mtb, eventually increasing the number of potential TB drug targets combined with the identification of a novel mode-of-action.

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Further Publications

Reiners, J., Lagedroste, M., Gottstein, V., **Adeniyi, E. T.**, Kalscheuer, R., Poschmann, G., Stühler, K., Smits, S. H. J. & Schmitt, L. Insights in the antimicrobial potential of the natural nisin variant Nisin H. *Front. Microbiol.* **11**, 573614 (2020). <https://doi.org/10.3389/fmicb.2020.573614>

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Statutory Declaration

I declare under oath that I have compiled my dissertation independently and without any undue assistance by third parties under consideration of the “Principles for the Safeguarding of Good Scientific Practice at Heinrich Heine University Düsseldorf”.

I declare that I have not used sources or means without declaration in the text. All the passages taken from other works in the wording or in the meaning have been clearly indicated with sources. This thesis has not been used in the same or similar version to achieve an academic grading or is being published elsewhere.

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