Lead Structures from Nature in the Quest for New Antibacterial Drugs

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Abbreviations

µM	micromolar
A. baumannii	*Acinetobacter baumannii*
aa	amino acid
ABC	ATP-binding cassette
ACP	enoyl carrier protein
ADS	albumin dextrose sodium
AG	arabinogalactan
AHAS	acetohydroxyacid synthase
Ala	alanine
AMR	antimicrobial resistance
API	active pharmaceutical ingredients
approx.	approximately
aq.	aqueous
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
B. subtilis	*Bacillus subtilis*
BCAA	branched chain amino acid
BCG	bacillus Calmette-Guerin
BDQ	bedaquiline
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
CA	community associated
calcd.	calculated
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
Clf	clumping factor
CLSI	Clinical and Laboratory Standards Institute
CoA	coenzyme A
COL	colistin
DCM	dichloromethane
DDD	defined daily dose
Des	desaturase
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dup	duplication
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
e. g.	*exempli gratia,* for example
Eap	extracellular adhesion protein

ECDC	European Centre for Disease Prevention and Control
EDTA	ethylenediaminetetraacetic acid
Efb	extracellular fibrinogen binding protein
EMB	ethambutol
Erm	erythromycin
ESBL	extended spectrum β-lactamases
ESI	electrospray ionization
et al.	*et alii*, and others
FAME	fatty acid methyl ester
FAS-	fatty acid synthase
FBS	fetal bovine serum
FDA	Food and Drug Administration
FICI	fractional inhibitory concentration indices
Fts	filamentation temperature-sensitive
GC	gas chromatography
GISA	glycopeptide-intermediate *S. aureus*
GTE	glucose-TRIS-EDTA
GTP	guanosine triphosphate
h	hour
HAI	healthcare-associated infections
HGT	horizontal gene transfer
HIV	human immunodeficiency virus
Hpr	histidine-containing phosphorcarrier protein
Hz	Hertz
i. e.	*id est,* meaning
IC	inhibitory concertation
IFN	interferon
IGRA	interferon gamma release assay
ILV	isoleucine, leucine, valine
INH	isoniazid
ISO	isoxyl
kb	kilobase
kg	kilogram
KZN	KwaZulu-Natal
LAM	lipoarabinomannan
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LM	lipomannan
M. tb.	*Mycobacterium tuberculosis*
MA	mycolic acids
MATE	multidrug and toxic compound extrusion
MDR	multi-drug resistant
MeOH	methanol
MFS	major facilitator superfamily

mg	milligram
MGE	mobile genetic elements
MH	Müller Hinton
MIC	minimal inhibitory concentration
min	minute
mL	milliliter
Mmp	mycobacterial membrane proteins
mRNA	messenger ribonucleic acid
MRSA	Methicillin –resistant *Staphylococcus aureus*
MSSA	methicillin-sensitive *Staphylococcus aureus*
MTBC	*Mycobacterium tuberculosis* complex
NADH	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
NMR	nuclear magnetic resonance
NTM	non-tuberculous mycobacteria
OD	optical density
OSMAC	one strain many compounds
PBP	penicillin-binding proteins
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDIM	phthiocerol dimycocerosates
PEP	phosphoenolpyruvate
PG	peptidoglycan
PIM	phosphatidylinositol mannosides
PM	plasma membrane
PSM	phenol-soluble modulin
PTS	phosphotransferase system
PVL	panton-valentine-leucocidin
pyk/PK	pyruvate kinase
PZA	pyrazinamide
RIF	rifampicin
RM	resistant mutant
RNA	ribonucleic acid
RNAP	ribonucleic acid polymerase
RND	resistance-nodulation-cell-division
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal ribonucleic acid
RT-qPCR	reverse transcription quantitative polymerase chain reaction
S. aureus	*Staphylococcus aureus*
SAR	structure-activity relationship

Sas	*Staphylococcus aureus* surface
SCC	staphylococcal chromosome cassette
SD	standard derivation
SI	Selectivity index
SMR	small multidrug resistance
SNP	single nucleotide polymorphism
t	time
TB	tuberculosis
TDM	trehalose dimycolate
TLC	thin layer chromatography
TNF	tumor necrosis factor
topo	topoisomerase
TRIS	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
TSB	tryptic soy broth
TSST	toxic shock syndrome toxin
VISA	vancomycin-intermediate resistant *Staphylococcus aureus*
WHO	World Health Organization
WT	wild type
X. citri	*Xanthomonas citri*
XDR	extensively drug resistant

Abstract

To control the spread of bacterial diseases, such as tuberculosis and methicillin-resistant *Staphylococcus aureus* infections, new drugs are urgently needed.

In the first part, the thesis reviewed some of the current concepts in antibacterial drug discovery focusing on novel innovative strategies. Novel sources for antibacterial compounds, screening methods for difficult-to-treat subpopulations and alternative strategies for antimicrobial compound development are reported.

As second part, the research of this thesis focused on secondary metabolites and their derivatives isolated or derived from endophytic fungi, sponges and plants as new antibacterial lead structures. In general, compounds were screened in whole cell viability assays for their antibacterial activity against *Mycobacterium tuberculosis* and nosocomial bacteria such as *Staphylococcus aureus*. Three compound classes which each revealed a promising therapeutic range were reported here:

The flavonoid chlorflavonin was isolated from the endophytic fungus *Mucor irregularis*, which was obtained from the Cameroonian medicinal plant *Moringa stenopetala*. It exhibited potent antitubercular activity but no cytotoxicity against human cell lines. Chlorflavonin specifically inhibits the acetohydroxyacid synthase catalytic subunit IlvB1 causing auxotrophy to branched chain amino acids and pantothenic acid. Furthermore, combination treatment with the clinically used tuberculosis drugs isoniazid and particularly delamanid led to complete sterilization in liquid culture *in vitro*. Macrophage infection models revealed the intracellular activity of chlorflavonin against *Mycobacterium tuberculosis*.

Using the Masuda-Suzuki one pot synthesis, a compound library of bis-indoles derived from the natural substances hyrtinadine A and alocasin A was created. Structure-activity relationship studies revealed that 5',5''-chloro derivatives were the most active compounds against methicillin-resistant *Staphylococcus aureus* with a minimal inhibitory concentration ranging from 0.20 – 0.78 µM. The strong bactericidal killing effects with slow resistance development make these derivatives promising lead structures for antibiotics.

Gallic acid derivatives isolated from the Nigerian mistletoe *Loranthus micranthus* were screened for their antitubercular activity with the most active compounds being identified as 3-*O*-methylbutyl-, 3-*O*-methylhexyl- and 3-*O*-methyloctylgallate. While causing only a moderate reduction of bacterial viability in monotreatment, the combination with

isoniazid showed a particularly strong synergistic killing of *Mycobacterium tuberculosis* leading to complete sterilization of the culture *in vitro*. Whole genome sequencing of spontaneous resistant mutants suggests that stearoyl-desaturase DesA3 and/or the respective oxidoreductase (Rv3230c) is targeted by the gallates. This inhibition of fatty acid desaturation might not only impair regulation of cytoplasmic membrane fluidity but might also interfere with biosynthesis of long-chain fatty acid-derived cell wall lipids.

Zusammenfassung

Zur Eindämmung bakterieller Krankheiten wie Tuberkulose oder Methicillin resistente *Staphylococcus aureus* Infektionen sind neue Arzneimittel unumgänglich.

Diese Arbeit fasst im ersten Teil aktuelle Aspekte der antibakteriellen Arzneimittelforschung zusammen, wobei der Fokus auf innovative Strategien der Antibiotikaentwicklung in Bezug auf antibakterielle Verbindungen, Screeningmethoden für schwer behandelbare Populationen und alternative Herangehensweisen zur Antibiotikaentwicklung liegt.

Im zweiten Teil werden sekundäre Metabolite und ihre Derivate, welche aus Endophyten, Schwämmen und Pflanzen isoliert oder abgeleitet worden sind, als mögliche antibakterielle Leitstrukturen analysiert. Hierfür wurden Extrakte und Reinsubstanzen hinsichtlich ihrer antibakteriellen Wirkung gegen *Mycobacterium tuberculosis* und nosokomiale Erreger wie *Staphylococcus aureus* in Zell-basierten Viabilitätsstudien untersucht. Drei Substanzklassen, die eine vielversprechende therapeutische Breite aufwiesen, werden in dieser Arbeit beschrieben:

Das Flavonoid Chlorflavonin wurde aus dem Endophyten *Mucor irregularis*, welcher von der kamerunischen Heilpflanze *Moringa stenopetala* gewonnen wurde, isoliert. Es wies antituberkulöse Aktivität, jedoch keine Zytotoxizität gegen humane Zelllinien auf. Chlorflavonin inhibiert die katalytische Untereinheit IlvB1 der Acetohydroxysäure Synthase, wodurch eine Auxotrophie für verzweigte Aminosäuren und Pantothensäure verursacht wird. Außerdem führt die Kombinationstherapie mit den bekannten Tuberkulose-Antibiotika Isoniazid beziehungsweise Delamanid zu einer Sterilisation der Flüssigkultur *in vitro*. Die intrazelluläre Wirkung von Chlorflavonin gegen *Mycobacterium tuberculosis* wurde durch ein Makrophagen-Infektionsmodell bewiesen.

Mit Hilfe der Masuda-Suzuki Eintopf-Synthese wurde eine Substanzbibliothek aus Bis-Indolen erstellt, welche von den Naturstoffen Hyrtinadin A und Alocasin A abgeleitet wurde. Aufgrund von Struktur-Aktivitätsstudien erwiesen sich 5',5''-Chlorderivate als die aktivsten Verbindungen gegen Methicillin resistente *Staphylococcus aureus*, wobei sich die minimale Hemmkonzentration hierbei von 0,2 – 0,78 µM erstreckte. Aufgrund der starken bakteriziden Wirkung und der langsamen Resistenzentwicklung, stellen diese Derivate viel versprechende Leitstrukturen für Antibiotika dar.

Zudem wurden Gallussäurederivate aus dem Nigerianischen Mistelzweig *Loranthus micranthus* isoliert und auf ihre antituberkulöse Wirkung untersucht. Hierbei stellten 3-*O*-

Methylbutyl, 3-*O*-Methylhexyl- und 3-*O*-Methyloctylgallat die aktivsten Verbindungen dar. Während die Behandlung mit 3-*O*-Mehtylbutylgallat nur eine schwache Reduktion der bakteriellen Viabilität verursachte, wies besonders die Kombination mit Isoniazid eine starke synergistische Wirkung gegen *Mycobacterium tuberculosis* auf und führte zu einer Sterilisation der Flüssigkultur *in vitro*. Die Ergebnisse der Genomsequenzierung spontan resistenter Mutanten lassen vermuten, dass die Stearoyl-Desaturase DesA3 und/oder die dazugehörige Oxidoreduktase (Rv3230c) das Target der Gallate darstellt. Aufgrund der Hemmung der Fettsäuredesaturierung wird möglicherweise, neben der Regulierung der zytoplasmatischen Membranfluidität, die Synthese von Lipiden aus langkettigen Fettsäuren beeinträchtigt.

1 Introduction

1.1 Pathogenic Bacteria

Bacteria are ubiquitous, prokaryotic organisms whose deoxyribonucleic acid (DNA) is contained normally in a single chromosome located exposable in the cytoplasm. They are the most spread lifeform in the biosphere and can survive under extreme conditions like high temperatures, low pH values or high salt concentrations. Among harmless environmental bacteria, pathogenic species exist. They attack organisms by distinct strategies: competing with other organisms for space, destruction of the host's immune response or inflammation of tissue by extracellular toxins, among other mechanisms [1]. Antibiotics for treatment of bacterial infections are able to cure infested hosts. However, pathogens acquired resistance against those compounds making it difficult to struggle against the infections [2]. The next paragraphs will give a short overview about pathogens and their infectious diseases which were research object in this thesis. As second part, detailed information about distinct types of antibiotics, possible resistance mechanisms against these and about the situation of antibiotic consumption are given.

1.1.1 ESCAPE Pathogens

One group of pathogenic organisms are nosocomial bacteria which cause healthcare-associated infections (HAI). By definition, patients do not exhibit any detectable bacterial (viral, fungal or parasitic) burden at referral. It is called nosocomial if the infection can be detected 48 hours after admission to the hospital. During a hospital stay, the infections derive from different sources like (in)direct contact between patients, healthcare workers, visitors, environmental sources or contaminated objects [3]. Of these, invasive devices, like urinary catheters or ventilators, constitute a high risk for infection. The World Health Organization (WHO) reported that low- and middle-income countries are significantly more affected than high-income countries, because of additional risk factors associated with poverty such as limited resources or lack of basic hygiene. However, no country has found a solution for this problem yet [4]. In Europe alone, four million patients were affected with HAI leading to 37,000 attributable deaths every year [5].

The most severe group of nosocomial bacteria are termed as ESKAPE pathogens which "escape" biocidal action of antibiotics. However, it has to be mentioned that ESKAPE

organisms do not only occur in the healthcare sector, but also in the community. This group includes *Enterococcus faecium* (*E. faecium*), *Staphylococcus aureus* (*S. aureus*), *Klebsiella pneumoniae*, *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa*, and Enterobacter species. It was recommended to change the term from ESKAPE to ESCAPE. The new term would include "C" for *Clostridium difficile* as one of the most problematic hospital pathogen, whereas the second "E" now comprises not only *Enterobacter* species but the entire family of Enterobacteriaceae which includes the former "K" *Klebsiella pneumoniae* and also drug resistant pathogens like *Escherichia coli* and *Proteus* species [6]. However, this suggestion has not widely been implemented yet, and most researchers still use the term "ESKAPE". The main infections caused by the ESKAPE pathogens comprise bloodstream and urinary tract infections, pneumonia, and severe invasive infections. Those infections are hard to treat among ill and immunocompromised individuals due to natural or acquired resistance (see Chapter 1.2.2) [7].

1.1.2 *Staphylococcus aureus*

The yellow pigmented *S. aureus*, which is coagulase-positive, belongs to the round-shaped, non-motile, facultative anaerobe *staphylococci* genus and represents the most significant species for the clinic. As Gram-positive bacteria, *S. aureus* is characterized by a thick layer (approximately (approx.) 15-80 nm) of peptidoglycan (PG), named murein, above the cell membrane. PG is composed of long polysaccharides chains of alternating *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) monomers which are cross-linked by short peptide bridges at the *N*-acetylmuramic acid, mainly built from L-alanine (Ala) and L-lysine. The final steps of PG synthesis – transglycosylation and transpeptidation – are catalyzed by so-called penicillin-binding proteins (PBP) [8]. PBPs are localized to the extracellular surface of the cytoplasmic membrane, whereas the number of PBPs varies from species to species. In general, antibiotic sensitive species of *S. aureus* contain four PBPs, PBP 1-4, for cell wall synthesis. Of these, only PBP 2 acts as both transpeptidase and transglycosylase [9]. Methicillin-resistant *S. aureus* (MRSA) strains acquired resistance against β-lactam antibiotics by the additional PBP 2a which is not affected by β-lactam antibiotics (see chapter 1.2.2), such as methicillin [10]. The emergence of additional resistance to glycopeptides, like vancomycin, the drug of choice for treatment of multidrug resistant *S. aureus*, defines the group of glycopeptide-intermediate resistant *S. aureus*

(GISA) [11]. The first reported GISA strain (Mu-50, ATCC 700699) showed a thicker cell wall than vancomycin susceptible strains as well as an increased production of PBP 2 and PBP 2a [12].

Infection

The naturally lysozyme-resistant *S. aureus* colonizes the human microflora on the skin and mucosa and is transmitted via smear or droplet infection. The pathogen is able to cause intoxications and severe infections. Community associated (CA) *S. aureus* predominantly causes skin and soft tissue infections, whereas healthcare-associated (HA) *S. aureus* is one of the most frequent causes of lower respiratory tract, cardiovascular as well as bloodstream infection and pneumonia [13, 14]. The virulent potential varies between strains because of distinct numbers of virulence factors often located on mobile genetic elements (MGE). In general, adhesins such as clumping factor A and B (ClfA and B) or *S. aureus* surface proteins (Sas) G and X bind to human epithelial cells on which *S. aureus* colonizes in biofilm formations [15]. After entry into the bloodstream due to damage of the epithelial skin barrier, several extracellular and cell wall relevant virulence factors facilitate circumvention of the immune system leading to infection in healthy hosts: protein A (Spa) binds to immunoglobulins and thereby blocks phagocytosis of the pathogen [16]. Platelet aggregation is inhibited by extracellular fibrinogen binding protein (Efb) [17]. In addition, the recruitment of leucocytes is impaired by binding of the extracellular adhesion protein (Eap) to the respective receptor on endothelial cells [18]. For spreading and persisting in the host, the hyaluronidase lyses intercellular substances of the tissue, while the biofilm formation protects the colonies against outer impacts [19, 20]. The pathogenicity is based on several extracellular toxins. Among the enterotoxins A-E, the toxic shock syndrome toxin (TSST) and the serine proteases exfoliatin A and B, the phage-encoded panton-valentine-leucocidin (PVL) builds porins on macrophages and granulocytes and by this weakens the innate immune response [21, 22]. Interestingly, although *S. aureus* was categorized as extracellular pathogen, Kubica *et al.* showed the survival of *S. aureus* in phagocytes such as monocytes and neutrophils converting in a persistent phase [23]. After expression of phenol-soluble modulin alpha (PSM), bacteria cells escape from the phagosome into the cytosol and restart to proliferate [24].

Epidemiology

S. aureus colonizes the nasal mucosa in estimated 50 % of the human population [25]. Shortly after the introduction of penicillin in the 1940s, *S. aureus* strains developed resistance to penicillin by penicillinase production [26]. Only two years after the development of the first semi-synthetic penicillinase-resistant antibiotic methicillin in 1959, MRSA isolates were reported [27]. With the emergence of novel lineages, the first pandemic MRSA outbreak was reported in the 1980s. Nowadays resistant strains have spread worldwide with the highest incidence rates in North and South America, Asia and Malta, followed by intermediate rate in European countries, for example (lat.: *exempli gratia*, e. g.) Portugal, Greece and Italy. Annually, 171,000 cases of nosocomial MRSA infections are estimated in the EU by the European Centre for Disease Prevention and Control (ECDC) [28]. However, MRSA infections have emerged in the community and from livestock making it to an ubiquitous problem which cannot be reduced by hospital prevention alone. It is the leading cause of sepsis, endocarditis and skin infections, and the mortality rate is estimated at 20 % for severe invasive infections [15, 29]. Methicillin-resistant isolates harbor most frequently additional resistance to fluoroquinolones. Especially in Portugal and Hungary, resistance to two different antimicrobial groups occurs at a frequency of approx. 35 % among all *S. aureus* isolates [28].

1.1.3 *Mycobacterium tuberculosis*

Mycobacteria are non-sporogenic, rod-shaped bacteria growing under microaerophilic conditions. The genus *Mycobacterium*, which comprises more than 170 species, can be divided into two types: the species of the *Mycobacterium tuberculosis* complex (MTBC) comprising *M. tuberculosis* (*M. tb.*), *M. bovis*, *M. africanum*, *M. microti*, *M. canettii*, *M. orygis*, *M. caprae*, *M. pinnipedii*, *M. suricattae* and *M. mungi* cause human tuberculosis (TB) and are closely related to each other with a nucleotide sequence identity >99 % [30]. The most prominent representative of the MTBC is *M. tb* discovered 1882 by Robert Koch. It is a slow growing bacterium with a generation time of around 20 hours. The non-tuberculous mycobacteria (NTM) including *M. abscessus*, *M. avium*, *M. marinum* and *M. smegmatis* can cause diseases regarding the lung, lymph nodes or skin in immune-compromised individuals [31].

Mycobacteria can survive under harsh environmental conditions for a long period of time. One reason for that is the physically robust cell wall construction of these Gram-positive bacteria. Their cell envelope comprises a high amount of lipids which is unique for these species and provides the basis for their pathogenicity. The envelope can be divided into three distinct segments: the plasma membrane (PM), the cell wall core and the outermost layer [32]. The cell wall core consists of the PG layer which is linked covalently to arabinogalactan (AG) via phosphoryl-*N*-acetylglucosaminosylrhamnosyl linkages. Additionally, AG is esterified with long chain (C_{70}-C_{90}) α-alkyl-branched β-hydroxy fatty acids named mycolic acids (MA) forming a waxy layer. The biosynthesis of MA is catalyzed by cooperation of the multidomain fatty acid synthase I (FAS-I) and the multienzyme complex fatty acid synthase II (FAS-II). While the synthesis of the shorter α-chain consisting of 16-18 and 24-26 carbons is catalyzed by FAS-I, the longer mero chains are built by FAS-II employing elongation of FAS-I-derived C_{16}- and C_{18}-coenzyme A (CoA) chains [33].

Furthermore, the outer layer of the mycomembrane consists of a high variety of non-covalently attached glycolipids, polysaccharides, proteins and lipoglycans. The glycolipid trehalose dimycolate (TDM), also named cord factor, acts as structural component for bacterial cording and plays an important role for virulence [34]. Other cell wall-localized virulence factors like the lipoglycans phosphatidylinositol mannosides (PIM), lipomannan (LM) and lipoarabinomannan (LAM) as well as phthiocerol dimycocerosates (PDIM) interact with distinct receptors of the immune system [35]. The outermost compartment is called the capsule and contains mainly polysaccharides containing α-D-glucan, D-arabino-D-mannan and proteins, but only minor amounts of lipids [36].

Due to its lipophilic nature with a low number of porin channels, the cell envelope has a low permeability which makes the transport of substances across this structure difficult. In consequence, mycobacteria exhibit a high intrinsic resistance against chemical and physical environmental influences like many of the common antibacterial compounds, acids or extreme temperatures. Another characteristic of *M. tb*., which allows persistence for decades, is the ability to survive and proliferate in macrophages. *M. tb*. utilizes a restricted set of host derived nutrients, especially lipids such as cholesterol. Host lipids are not only used as a source of energy, but they are metabolized to acetyl- and propionyl-CoA by β-oxidation which are also used as substrates for biosynthesis of virulence factors like PDIM influencing the pathogenicity [37].

Infection

The pathogen is transmitted via aerosols by droplet infections caused by sneezing or coughing. Interestingly, respiratory secretions protect the bacilli against environmental stresses, such as dehydration or oxygen injury, leading to a half-life of *M. tb.* within aerosols of about six hours [38]. The inhalation of droplets containing as few as three to five bacilli is sufficient to establish an infection, hence primary TB mainly infects the alveoli in the lung. Receptors of alveolar macrophages including complement receptors and the mannose receptor directly interact with sugar moieties of the *M. tb.* envelope. Hence, macrophages phagocytose and engulf the TB bacilli within phagosomes and transport them into the lung tissue. Lipids, glycolipids and lipoglycans of *M. tb.* bacilli prevent the fusion of phagosomes and lysosomes, leading to survival in the intracellular niche for a prolonged period of time [39]. This process recruits and activates immune cells comprising T-cells, Langerhans cells, fibroblasts and macrophages to form an organized aggregate, the granuloma. The immune cells produce cytokines including interferon (IFN-) γ, tumor necrosis factor (TNF), inter-leukins, reactive nitrogen intermediates and oxygen, as well as nitric oxide [40-42]. Due to the nitrosative stress in the phagosomes, *M. tb.* cells become dormant. The bacteria are then able to resist eradication leading to a latent infection without any clinical symptoms, radiological abnormality or microbiological evidence [43]. If the immune system is suppressed and the immune response is dysregulated, a secondary TB can occur, and the reactivated pathogens can promote caseous necrosis. In consequence, the granuloma collapses and virulent bacteria cells enter other tissues and build more lesions. Entering the bloodstream, *M. tb*. can spread to other tissues such as the liver, lymph nodes, spleen, gut, bone marrow and urogenital tract, named as miliary TB [44]. If the destroyed granuloma is located in the lung tissue and penetrates the bronchial tract, the bacilli can be transmitted to other individuals by sputum [45]. This state is then named as active TB.

Therapy

General symptoms of pulmonary TB are relatively unspecific including coughing with purulent sputum, breathlessness, weight loss or fever with night sweats. To confirm the diagnosis, distinct assays are available ranging from microscopic evaluation and cultivation of sputum to molecular techniques like polymerase chain reaction (PCR) or IFN-γ release assay (IGRA). X-ray analysis of the patient's chest is helpful as additional investigation if sputum examinations differ from clinical suspicion [46]. After the diagnosis of TB, the

standard therapy consists of a six-month drug regimen of the four first line drugs rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazine amide (PZA) [47]. The cure rate for drug susceptible TB is 83 % [48]. However, this has been the standard therapy for more than fifty years now, leading to the consequence that tubercle cells have developed resistances against these antibiotics. The emergence of multidrug-resistant (MDR) strains which are resistant to RIF and INH are treated with combinations of second line drugs. Additional resistances against any fluoroquinolone and against the injectable drugs kanamycin, amikacin or capreomycin lead to the development of extensively drug resistant (XDR) strains which are hard to treat [49]. Since 2014, the new conditionally approved drugs bedaquiline (BDQ, Sirturo®) and delamanid (Deltyba®) are being used for treatment of MDR- and XDR-TB cases. However, the recommended treatment duration of MDR/XDR therapy takes at least 20 months accompanied with enormous side effects and costs for the health care sector of about US-$ 2,000-5,000 per person [48].

To prevent TB infection, the bacillus Calmette-Guerin (BCG), an attenuated *M. bovis* strain, is administered as vaccine. While infants and young children are protected against miliary TB and tubercular meningitis by neonatal BCG vaccination, adolescents and adults are poorly protected against pulmonary TB. Due to possible complications associated with viable, attenuated vaccines, the permanent vaccination commission has not recommended the BCG vaccination in Germany since 1998 [50].

Epidemiology

TB is the leading cause of death by an infectious disease and has replaced the human immunodeficiency virus (HIV) from first position [48]. Due to the transmission via aerosols and the unnoticed latent infection, *M. tb.* has established as a successful pathogen already early during human evolution. Approximately, one third of the world's population is infected latently. In 2016, there were 10.4 estimated new incidence cases worldwide and 1.7 million people died. Among these, 374,000 were co-infected with HIV, which triggers the development of active TB. TB is a poverty related disease with the main incidence areas located in South-East Asia and Sub-Sahara Africa (Figure 1-1 A). In addition to poverty and HIV co-infection, the neglected development of a new chemotherapy is one further reason for a TB pandemic 130 years after the discovery of the pathogen [48]. The rising number of MDR/XDR cases is getting alarming: in 2016, 4.1 % of new cases and 19 % of previously treated cases are MDR-/XDR-TB positive. Estonia, Belarus, Kazakhstan and Russia are the

countries with the highest number of such cases amounting to 47 % of all global cases and with 240,000 deaths caused by drug resistant bacilli (Figure 1-1 B) [48]. Possible explanations for regional differences between incidence and resistant rates might be the availability of anti-TB drugs but a lacking or incomplete surveillance in such countries with high resistance rates. Nevertheless, the most noteworthy outbreak of XDR strains was in the KwaZulu-Natal (KZN) region in South Africa from 2005 to 2007: 39 % of the isolates were identified as MDR and 6 % as XDR strains. The virulence of the XDR KZN strains is comparable with drug susceptible *M. tb.* strains. However, due to the high percentage of co-infection with HIV, the death rate among the co-infected individuals was 98 %. The severity and the alarming numbers of this outbreak caught worldwide attention [51].

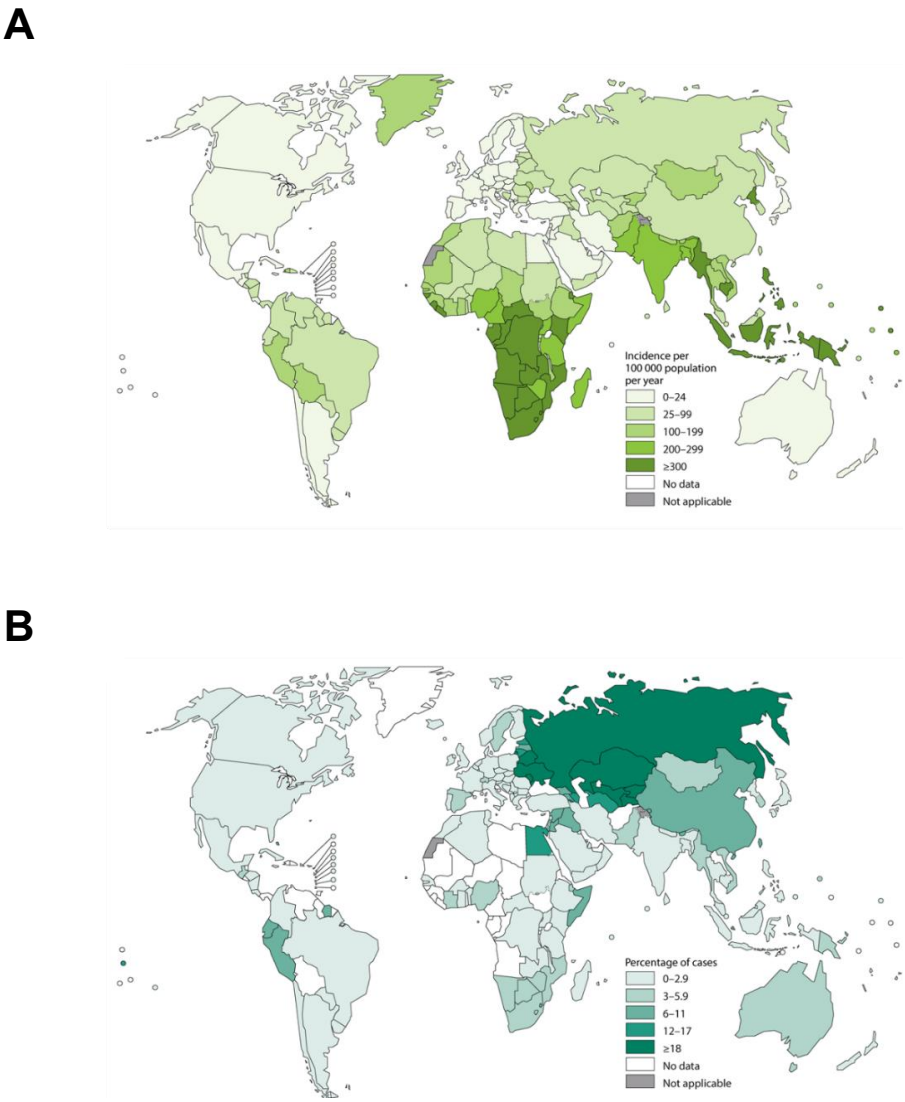

Figure 1-1 Estimated TB incidence rates, 2016 (A); percentage of new TB cases with MDR TB (B), 2016. Data source: Global Tuberculosis Report 2017, WHO, 2017; © WHO2017. All rights reserved.

1.2 Antimicrobial Chemotherapy

1.2.1 Antibacterial Drug Targets

Natural compounds as well as synthetic chemotherapeutics with antibacterial activity are termed antibiotics. Antibiotics can be differentiated by their effect into bactericidal and bacteriostatic drugs. Bactericidal drugs induce cell death, while bacteriostatic compounds inhibit cell growth and depend on the patients' immune system to break through the infection. However, the effect of an antibiotic considerably depends on its dose and its concentration at the target location, its exposure time and on the growth stage of the bacteria. Another antibiotic classification is based on their cellular component or pathway they affect. Ideally, antibiotics affect specific targets in the bacterial cell which do not have any analogs in the eukaryotic cell. By this, only the prokaryotic metabolism is impaired. In the following, the most important drug targets and their most common antibiotic classes in MRSA and TB therapy are described. Certainly, some antibiotics named below exhibit broad-spectrum efficacy so that their range of applicability is extended and not limited to MRSA and TB therapy.

Protein Synthesis

Antibiotics which target protein synthesis comprise the broadest class of drugs. They inhibit protein synthesis by interference of the translation at the bacterial ribosomes. Those drugs are classified as inhibitors of either the 50S or 30S subunit, the two ribonucleoprotein subunits of the ribosome. 50S inhibitors encompass oxazolidinones such as linezolid or cycloserine, macrolides as erythromycin (Erm), lincosamides as clindamycin, and chloramphenicol [52]. Oxazolidinones block the initiation of protein synthesis, while, for instance, macrolides and lincosamides inhibit the elongation of the protein sterically [53-55]. In their presence, peptidyl-transfer ribonucleic acid (tRNA) is hindered to bind to the ribosome and the peptidyl transferase activity is finally blocked [56].

Tetracyclines and aminoglycosides (e. g. streptomycin, kanamycin) belong to the 30S inhibitor family. The bacteriostatic tetracyclines prevent the attachment of aminoacyl-tRNA to the ribosomal 30S subunit [57]. In contrast, aminoglycosides are the only bactericidal protein synthesis inhibitors which bind to 16S ribosomal RNA (rRNA). By this, they cause reading errors at the mRNA followed by defective proteins which lose their biologic

functionality. Finally, the pathogen cannot use these nonsense proteins and dies [58]. Streptomycin is one example for an aminoglycoside used in TB therapy. Discovered in 1943, it was the first clinically used drug against TB and was isolated from *Streptomyces griseus* [59]. It is still used as second-line option in combination therapy with INH, PZA and RIF. Also other agents such as amikacin or kanamycin are second-line aminoglycosides against MDR strains of *M. tb.*.

DNA Replication

The antibacterial compound class of quinolones interferes with DNA supercoiling by targeting topoisomerase II (gyrase) or IV (topoIV), therefore they inhibit DNA replication. The binding preference to either gyrase or topoIV depends on the bacteria species. It could be shown that gyrase is the predominant target in Gram-negative bacteria such as *E. coli*, while in Gram-positive species topoIV is preferred [60, 61]. Quinolones (e. g. ciprofloxacin, levofloxacin, moxifloxacin) covalently bind to topo II/IV and form a stable drug-enzyme-DNA complex leading to double stranded DNA breaks. Although the inhibition of DNA synthesis induces the expression of DNA repair enzymes, this stress response mechanism cannot compensate the strand breaks leading to cell death [62].

RNA Synthesis

The essential RNA polymerase (RNAP) is an attractive target for drug development. So far, rifamycins are the only clinical used compounds which inhibit the RNAP [63].The first agents were isolated from *Amycolatopsis mediterranei* in 1957. A semisynthetic compound embodies RIF, a derivative from the natural compound rifamycin B isolated from *Amycolatopsis rifamycinica*. It is the most common RNA synthesis inhibitor with extremely broad efficacy spectrum. However, RIF has been mainly used in TB standard therapy since 1968 due to its ability to kill persistent bacteria [64]. Due to their lipophilic structure, rifamycins diffuse easily across the cell envelope and block the RNA synthesis [65]. They target the DNA dependent transcription by binding to a RNAP subunit located in the DNA-RNAP complex. The potent bactericidal activity is based on the impairment of initial RNA synthesis by steric inhibition of the DNA-enzyme-complex [66].

Cell Wall Synthesis

The cell wall synthesis includes a broad spectrum of targets which are affected by antibiotics leading to cell death via morphological changes and reduced mechanical strength of the prokaryotic cell. One important group of cell wall-targeting antibiotics are the β-lactams. The most common agents are the penicillins discovered by Fleming in 1928, but also carbapenems and cephalosporins are counted to that class [67-69]. All β-lactam antibiotics share – as their name implies – the β-lactam ring, whereas their activity is influenced by distinct side chains. Due to the sterical similarity to the D- alanyl-D-alanine (D-Ala-D-Ala) dipeptide of PG, β-lactams bind to the transpeptidase active site of the PBPs by acetylation, prevent the enzyme activity, and therefore block the cross-linking of the PG layer [70]. In contrast, glycopeptide antibiotics (e. g. vancomycin or teicoplanin) are able to form hydrogen bond interaction at the D-Ala-D-Ala dipeptide and block the binding of PBPs for elongation and cross-linking of the NAM and NAG polymers in Gram-positive bacteria sterically [71]. Gram-negative bacteria are not affected by glycopeptides due to low permeability through the additional outer membrane.

Additionally, some antibiotics target the wall synthesis of mycobacteria, such as INH, EMB and delamanid: the antitubercular activity of INH was first reported in 1952 and has been an inherent part of TB standard therapy since then [72]. After passive diffusion through the cell wall the prodrug INH is activated by the catalase peroxidase KatG of *M. tb*. [73]. Although the complete mechanism of action remained unclear, investigations have shown that the activated radicals inhibit the nicotinamide adenine dinucleotide (NADH)-dependent enoyl acyl carrier protein (ACP) reductase InhA [74-76]. In detail, reactive species bind to NADH resulting in dissociation of NADH from InhA. This blocks fatty acid elongation and the MA synthesis in the fatty acid synthase II system. As a result, the cell wall, which consists of MA, collapses. Additionally, it could also be shown that INH-nicotinamide adenine dinucleotide phosphate (NADP) adducts inhibit the dihydrofolate reductase which catalyzes nucleic acid synthesis [77].

In 1961, the synthetic compound EMB was found as antitubercular compound as part of a screening of randomly selected compounds [78]. Due to the pleiotropic effects of EMB on cell wall composition, the target identification has only been reported 34 years after its anti-tuberculosis activity publication [78, 79]. It has been considered that the arabinosyltransferases EmbA, EmbB and EmbC are the primary target of EMB leading to inhibition of arabinan polymerization. EmbA and EmbB are required for AG and EmbC catalyzes LAM

synthesis. It could be shown that the treatment with EMB leads to smaller species of LAM [80]. Besides that, due to the fact that most mutations in resistant isolates are located in *embB,* it is likely that EmbB and EmbC are the preferred targets of EMB [81].

The newest drug for TB treatment is delamanid. However, while it has been conditionally approved by the European Medicines Agency in 2014, the mode of action is incompletely understood. So far, it is very likely that the prodrug is activated by the deazaflavin (F_{420})-dependent nitroreductase to radical intermediates which inhibit the production of methoxymycolic and ketomycolic acids [82]. In contrast to INH, delamanid is also active against dormant bacilli [83].

ATP Synthesis

The diarylquinoline BDQ was discovered as a new TB drug with a novel target in a high throughput phenotypic screen [84]. It was approved 2012 by the American Food and Drug Administration (FDA) and has also been permitted for XDR-TB in Europe since 2014 [85]. The new target is the c-subunit of the adenosine triphosphate (ATP)-synthase of mycobacteria [86]. Due to the low degree of conservation of the gene *atpE,* which encodes the subunit c, among bacterial species, BDQ only inhibits mycobacteria but no other prokaryotic or eukaryotic species [84, 87]. As BDQ binds to the subunit c, the rotation and the proton transfer is prevented and ATP synthesis is disrupted in both replicating and dormant bacilli leading to bactericidal killing [88].

1.2.2 Resistance Mechanisms of Pathogens against Antibiotics

Natural “intrinsic” resistance means that a certain antibiotic does not have any effect on a specific pathogenic species. In contrast, the acquired resistance of an organism to an antimicrobial agent to which it was previously sensitive is called antimicrobial resistance (AMR) and displays the main focus of resistance problem. The prescribed drug is then no longer effective against the pathogen and infections can spread to other individuals. Since the development of antibiotics, bacteria have developed resistance mechanisms to escape from antibacterial effects. AMR emerges spontaneously by distinct origins. In general, the acquisition of resistance leads to a natural selection, so that AMR pathogens continue to

replicate, while drug susceptible bacteria are killed by the respective antibiotic [89]. Resistance mechanisms are acquired either by spontaneous gene mutations or by horizontal gene transfer (HGT). Gene mutations occur in an initial susceptible population of one species and cause growth inhibition of the susceptible, while resistant bacteria dominate. HGT means the acquisition of foreign DNA of MGEs such as plasmids and transposons from other species by transformation, phage transduction or conjugation. Interestingly, in *M. tb.* plasmids and genomic DNA transfer were not described, hence there is no evidence for HGT as mechanism of acquired resistance in that species [90, 91]. Instead, mycobacteria rely on *de novo* chromosomal mutations leading to a relatively slow rate of emergence of AMR with a mutation rate around 10^{-7}. In contrast, the mutation rate of *S. aureus*, whose AMR is mainly plasmid and phage mediated, is approx. 10^{-6} [90].

In general, the mechanism of resistance can affect either the structure of the antibiotic compound, its molecular target or its uptake or efflux (Figure 1-2).

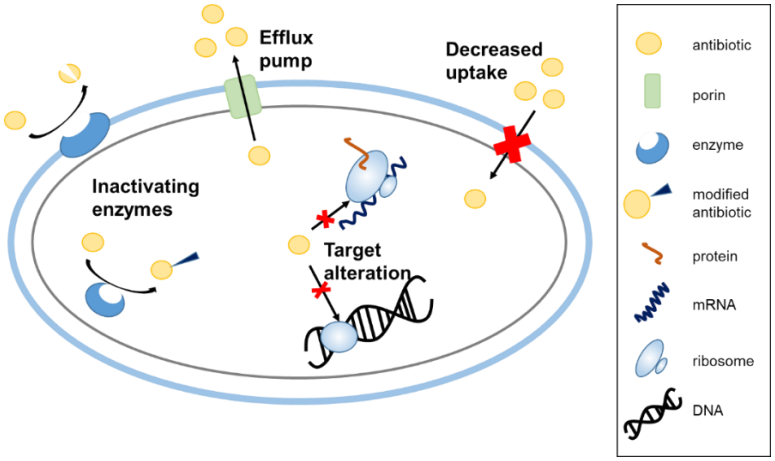

Figure 1-2 Possible mechanisms of resistance against antibiotics in bacterial cells.

Drug Modifications

The antibiotic molecule can be modified by adding moieties (e. g. acetylation, phosphorylation or methylation) or cleavage of the structure [92-94]. Additional moieties inhibit the binding of the agent sterically or via lowered binding affinity. The most prominent examples are aminoglycoside-modifying enzymes which alter the hydroxy and amino groups of the sugar rings in aminoglycosides [95]. Due to the location of the genes on gene cassettes, transposons or integrative conjugative elements, the resistance mediating genes can be incorporated by all bacteria species. Additionally, the cleavage of the amide bond in

the lactam ring by lactamases is the main resistance mechanism against β-lactam antibiotics so that the molecule cannot bind anymore to the target. Especially extended spectrum β-lactamases (ESBL) producing bacteria represent a global threat for HAI due to their broad resistance against penicillins, cephalosporines and monobactams. However, since ESBL occur mainly in Gram-negative bacteria, this AMR is not described in more detail here [96].

Not only the reactive drug, but also the prodrug can be modified or even not be activated which constitutes a common resistance mechanism for TB prodrugs: the main resistance mechanism to INH, for instance, is the reduced ability of KatG to activate the prodrug. Most commonly, amino acid mutation at position 315 from serine to threonine lead to high-level resistance. The access of INH to the active center is decreased, so that INH is not cleaved into reactive radicals [97, 98].

The same is observable for delamanid: it could be shown that resistant isolates have mutations in the gene *ddn* encoding the nitroreductase, responsible for delamanid activation, or in four other genes *(fbiA*, *fbiB*, *fbiC* or *fgd1*) which were essential for F_{420} biosynthesis in *M. tb.* [99].

Target Modifications

The target site can be avoided by replacement or overexpression. Especially, MRSA strains replace the target of β-lactams by the additional PBP 2a encoded by the gene *mecA*, which is localized on a MGE called staphylococcal chromosome cassette (SCC) [100, 101]. The protein has a low affinity to β-lactams and enables transpeptidase activity in presence of a respective antibiotic [10]. Due to the activity of PBP 2 of both transglycosylase and transpeptidase, bacteria with the additional *mecA* gene resist to penicillins, cephalosporins and carbapenems [9]. Interestingly, the resistance rate differs between strains due to the regulator Agr which influences the expression rate of *mecA*. Otherwise, the *mec* locus affects expression of *agr* [102, 103].

Another possibility of target modification are polymorphisms in the target gene leading to changes of the amino acid sequence. Due to steric changes and/or reduced binding affinity, the antibiotic could not interact anymore with its target while the enzymatic activity is not influenced by the amino acid changes. A prominent example for antibiotic resistance caused by amino acid mutation is the resistance against RIF in *M. tb*.. The point mutation in the gene *rpoB* encoding the β-subunit of RNA polymerase leads to a decrease of RIF interaction [104]. Also for the new TB drug BDQ such a resistance mechanism has already been

reported. Spontaneous point mutations in the subunit c of ATP synthase encoded by *atpE* reduce the binding affinity of BDQ to its target [105].

Decreased Uptake

To prevent the antibiotic from reaching its target in the cytoplasm or inner membrane (in case of Gram-negative bacteria), bacteria have developed mechanisms to decrease the uptake of antibacterial compounds. Hydrophilic compounds, such as β-lactams, fluoroquinolones or tetracyclines use water filled porin channels to cross the membrane barrier. By changing the number, the type or the function of porins, the influx of the antibacterial agent is reduced. These resistance mechanisms, especially a decreased number of diffusion channels, are common reasons for resistance of Gram-negative bacteria, like *A. baumannii* or *Pseudomonas aeruginosa*, against β-lactam antibiotics [106, 107]. In Gram-positive bacteria, the vancomycin intermediate resistant *S. aureus* (VISA) phenotype of *S. aureus* is based on decreased uptake of the glycopeptide vancomycin. A large number of mutations are located in stress sensing regulatory systems causing cell wall thickening. By this, vancomycin is impaired in reaching lipid II at the cell septum where PG synthesis occurs [108].

Increased Extrusion

The last presented resistance mechanism is the transfer of compounds out of the prokaryotic cell. Five different types of efflux pumps exist in bacteria which are distinguished by energy source, extruded compounds and structure. The secondary transporters energized by proton motive force are divided into the following four members: major facilitator superfamily (MFS), resistance-nodulation-cell-division family (RND), small multidrug resistance (SMR) and multidrug and toxic compound extrusion (MATE). The fifth group of efflux pumps is represented by the ATP-binding cassette (ABC) transporter family using ATP as energy source [109].

Some *tet* genes, as one example, which are located on MGE, belong to MFS and cause resistance against many tetracyclines in bacteria [57]. As another example, RNDs provide extrusion for a broad range of antibiotics. One good characterized RND system is the AcrAB-TolC protein system in *E. coli*. This efflux pump stretches from the inner membrane across the periplasmic space to the outer membrane and causes resistance against tetracyclines, chloramphenicol, β-lactams, and fluoroquinolones [110].

Mycobacteria also harbor efflux pumps. Although the genome of *M. tb*. comprises 13 different genes which encode RND proteins, it seems that RND proteins do not play any role in resistance [111, 112]. However, with the investigation of BDQ resistant isolates, it could be shown that mutations in the transcriptional regulator Rv0678 influence BDQ susceptibility. The mutations in Rv0678 lead to upregulation of the gene itself and of the neighboring genes *mmpS5* and *mmpL5* [113]. These two genes encode large and small mycobacterial membrane proteins (Mmp), with MmpL5 forming an efflux pump which is able to extrude BDQ out of the cell resulting in BDQ resistance. Interestingly, other TB drugs, such as RIF or INH are not affected by such mutations [114].

1.3 Development and Consumption of Antibiotics

1.3.1 Antibacterial Drug Discovery: from the Golden Age to Current Development

After the discovery of penicillin by Alexander Fleming in 1929 and the treatment of streptococcal infections by sulphonamides discovered by Paul Ehrlich in 1931, the starting pistol was fired for a busy period of discovery and development of antimicrobial agents [67]. On account of improved hygiene and development of antibiotics, the mortality rates caused by infectious diseases lowered. And finally, the discovery of streptomycin rang in the Golden Age of antibiotics in 1943. In the next two decades, natural sources were screened for antibacterial compounds with great success. Many of the known antibiotics were developed in this stage such as chloramphenicol, tetracycline, vancomycin, RIF and INH [115]. In the 1970s, several structures were semi-synthetically modified to achieve broader efficacy and stronger activity. In the 1980s, the occurrence of resistances increased and the development of new antibiotics derived from nature seemed to be more and more difficult due to a high rediscovery rate and the lack of finding new drug targets. After the company Bayer (Leverkusen, Germany) has introduced the fluoroquinolone ciprofloxacin to the market, the pharmaceutical industry has begun to focus on generating more potent analogues of existing drug classes by synthetic approaches [115-117]. These structural analogues, such as the third generation cephalosporins, showed better efficacy against certain diseases, where first generation antibiotics were ineffective. However, the development of new antibacterial drug classes seemed to be saturated. 80 % of the approved drugs between 1980 and 1999 belonged to the class of either β-lactams (especially cephalosporins) or fluoroquinolones [118].

The history of drug discovery showed that, while at the beginning of antibiotic drug discovery soil and fungal extracts were mainly screened for antimicrobial activity, target-based screening approaches in combination with high-throughput screens of chemical libraries became more and more important. However, hits from cell free target-based screens often showed no activity in whole cell assays *in vitro*. In addition, while chemical libraries often comprise a huge number of structures, they typically exhibit high redundancy and cover only a relatively narrow chemical space making the discovery of new drug classes difficult [119].

Companies have begun to retract from antibacterial drug development leading to a strong reduction of antibiotic approvals. The return on investment mostly is low or even negative for pharmaceutical industry. Reasons for this are the complexity of structures and synthesis of new identified compounds, the tremendous costs associated with clinical testing, and the limited time of useful clinical application due to resistance development. Furthermore, the duration and frequency of the intake of antibiotics is short in comparison to other drugs for treatment of diseases such as cancer, Alzheimer or diabetes [120]. In addition, it has to be taken into consideration that antibacterial agents with new targets are classified as reserve antibiotics which are only used if multidrug resistant species are identified. This fact drastically reduces the market potential of new antibiotics making investment for the industry more unprofitable.

However, drug development has attracted attention again from the academic sector as well as from the pharmaceutical industry in the recent years. As a result, few promising antibacterial agents have been reported in the last years and have been investigated in (pre-) clinical studies. For shortening of TB treatment, clinical trials include studies with: (i) re-purposed drugs, like clofazimine, which is used in leprosy therapy normally [121] or the gastric proton pump inhibitor lansoprazole [122]; (ii) new compounds with so far unknown targets, such as pretomanid, which is active against replicating and hypoxic non-replicating *M. tb.* cells [123]; and (iii) new chemical entities, e. g. GSK-286, which target cholesterol catabolism as novel mechanism of action against intracellular *M. tb.* [124]. Additionally, also the late-stage of clinical pipeline for MRSA harbors new classes of agents: brilacidin, debio 1450 and lefamulin for instance [125-128]. Some natural drug sources and new antibacterial compounds are also reviewed in chapter 2.1.

1.3.2 The Antibiotic Crisis

The global consumption of antibiotics rose by 65 % from the year 2000 to 2015. Nevertheless, the numbers vary strongly between different countries. The use of antibiotics has stagnated with 24.1 defined daily doses (DDD) per 1000 inhabitants per day consumed in community and hospital sector in the European Union, ranging from 20 in the Netherlands to 40 DDD per 1000 inhabitants per day in Greece. However, the consumption rate has increased by 77 % in low- and middle-income countries, for instance Turkey, Tunisia and Algeria [129, 130]. Antibiotics were not only used in human medicine but also in the veterinary and agricultural sector and impact various microbiotas in human, animals and plants [131].

In Germany, the proportion between antimicrobial consumption in human and in animals is particularly unbalanced towards the agricultural sector. However, the assessment of antimicrobial use is hard to compare. It was reported that in Germany approximately three times more milligram antibiotics per kilogram biomass were consumed in food-producing animals than in humans [132]. It is common practice giving antibiotics to the whole drove and not only to single diseased animals. Furthermore, antimicrobial drugs were also given prophylactic or even as growth enhancer in healthy animal cohorts [133].

The frequency and intensity of the use of antibiotics correlate with the frequency of resistance. The first AMR was already reported shortly after the development of penicillin as first antibiotic in 1940, and the development of new AMR is still going on [134]. This faces a worldwide problem. Due to intercontinental travel AMR pathogens spread globally. By now, MDR infections lead to an increased mortality with 50,000 deaths each year across Europe and the US. The WHO estimates that the occurrence of antibiotic resistances is one of the highest risks for human health. If the development of resistances proceeds unimpeded, ten million people would be infected by multiresistant pathogens in 2050 [135]. As a consequence, due to association with longer duration of treatment and resulting higher costs, the global economy will lose $100 trillion by AMR [136].

Reasons for the emergence of resistant populations can be diverse. The most important factor driving AMR results from a high selection pressure caused by an excessive and unilateral use of antibiotics [137]. Ideally, after identification and characterization of the pathogen(s) underlying an infection, a treatment should be initiated following the maxim: the efficacy spectrum should be as narrow as possible, but as broad as necessary. Due to time

and cost saving strategies, however, many antibiotics are prescribed on spec and without identification of the pathogen or even its antibiogram.

The number of prescriptions clearly depends on the price of the antibiotic, meaning the cheaper the antibiotic, the more frequent prescriptions are issued [138]. Evidently, in some cases antibiotics are often prescribed in cases where the chosen antibiotic is not the right one or the infection is not even caused by bacteria. In Germany, at least 30 % of prescriptions are inadequate [139]. For instance, antibiotics are given prophylactic to minimize the risk of wound infections after surgery [140].

In addition, self-medication in countries without regulation or online purchase leads to an overuse of antibiotics without any medical advice. The lacking knowledge of patients of how to consume antibiotics properly or the defiance of intake guidelines can promote the development of AMR. On the one hand, many antibiotics have to be used accumulatively, meaning that their whole effect is only achieved after a certain intake period. However, it is possible that patients quit the therapy earlier than prescribed due to faster recovery. In consequence, not all cells of the bacterial pathogen are killed, and resistant clones are able to emerge since the concentration of antibiotics is too low. On the other hand, in some cases, antibacterial agents are taken for a too long period of time. The pathogen is then already eliminated, but other bacteria may obtain resistance under high selection pressure by antibiotic treatment.

The high consumption of antibacterial agents outside the human medical sector like the veterinary and agricultural sector, is another impact on antibiotic resistance. Not only the antibiotics but also resistant bacteria arrive in the human environment by direct contact, contaminated meat or other cross-contaminated food and water [141]. The choice of antibiotic is one major problem which has to be faced: the WHO sees the use of human antibiotics in veterinary medicine critical. Especially the macrolides, cephalosporins and fluoroquinolones were classified as high priority antibiotics by the WHO meaning that those antibiotics should not be used in veterinary medicine to save their efficacy in humans [142]. Especially the reserve antibiotic colistin (COL) is detected in poultry fattening in Germany, although COL is considered to be the last option for panresistant Gram-negative pathogens [143, 144].

Among a more rational application of antibiotics, it is not realistic to quit the use of antibacterial agents completely. Facing the problem, the improvement of awareness and

surveillance of antibiotic consumption has to be combined with an ongoing search for new lead structures and new drug targets for antimicrobial therapy.

1.4 Aim of this study

To control the spread of endemic diseases such as TB and MRSA infections, new drugs are urgently needed. The history of antibacterial drug development shows that natural sources still represent a promising possibility finding new lead structures due to a greater chemical space compared to synthetic chemical libraries. However, the fast emergence and exchange of resistance mechanisms among bacterial species in a globally networked time requires that especially new drug targets have to be investigated.

In the second chapter the thesis highlights some current concepts in antibacterial drug discovery. It provides an overview of novel innovative strategies comprising novel sources for antibacterial compounds, screening methods for difficult-to-treat subpopulations and alternative strategies for antimicrobial compound development.

Searching after new antimicrobial compounds, the research of this study described in chapters 3, 4 and 5 focused on secondary metabolites and their derivatives isolated from endophytic fungi, sponges and parasitic plants. The aim was to identify novel potent antibacterial lead structures with new molecular targets and resistance mechanisms. Isolated natural compounds as well as synthetic analogs and their derivatives were screened for their antibacterial activity against *M. tb*. and nosocomial strains (*S. aureus*, *E. faecium*, *E. faecalis, A. baumannii*) in whole-cell assays. Hits with a minimal inhibitory concentration (MIC) lower than 12.5 µM were also tested for their cytotoxicity against human cell lines (THP-1 and MRC-5). If the therapeutic window is greater than 10, compounds were further characterized regarding their inhibitory effect against actively growing bacteria in liquid culture and in macrophage infection models *in vitro*. The thesis aimed to unravel the mode of action of the identified new compounds by whole genome sequencing, genetically modifications, protein isolations and enzyme activity assays.

Finally, prospective efforts are addressed which might be done for further statements about the applicability of the studied compounds as suitable lead structures from nature in the quest for new antibacterial drugs.

1.5 References

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2 (Some) Current Concepts in Antibacterial Drug Discovery

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Abstract

The rise of multidrug resistance in bacteria rendering pathogens unresponsive to many clinical drugs is widely acknowledged and considered a critical global healthcare issue. There is broad consensus that novel antibacterial chemotherapeutic options are extremely urgently needed. However, the development pipeline of new antibacterial drug lead structures is poorly filled and not commensurate with the scale of the problem since the pharmaceutical industry has shown reduced interest in antibiotic development in the past decades due to high economic risks and low profit expectations. Therefore, academic research institutions have a special responsibility in finding novel treatment options for the future. In this mini review, we want to provide a broad overview of the different approaches and concepts that are currently pursued in this research field.

3 Chlorflavonin Targets Acetohydroxyacid Synthase Catalytic Subunit IlvB1 for Synergistic Killing of *Mycobacterium tuberculosis*

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The overall contribution to the paper: 60 %.

Contribution:

- Manuscript writing (50 %)
- Cultivation of bacterial strains
- Determination of minimal inhibitory concentration against *M. tb.* via resazurin dye reduction method
- Determination of minimal inhibitory concentration against nosocomial strains
- Determination of cytotoxicity and therapeutic index
- Determination of time-kill curves *in vitro*
- Checkerboard synergy assay
- Expression and purification of catalytic subunit of AHAS
- Microplate assay of IlvB1 activity
- Intracellular activity assay via macrophage infection
- Determination of single step resistance frequency

Abstract

The flavonoid natural compound chlorflavonin was isolated from the endophytic fungus *Mucor irregularis*, which was obtained from the Cameroonian medicinal plant *Moringa stenopetala*. Chlorflavonin exhibited strong growth inhibitory activity in vitro against *Mycobacterium tuberculosis* (MIC_{90} 1.56 μM) while exhibiting no cytotoxicity toward the human cell lines MRC-5 and THP-1 up to concentrations of 100 μM. Mapping of resistance-mediating mutations employing whole-genome sequencing, chemical supplementation assays, and molecular docking studies as well as enzymatic characterization revealed that chlorflavonin specifically inhibits the acetohydroxyacid synthase catalytic subunit IlvB1, causing combined auxotrophies to branched-chain amino acids and to pantothenic acid. While exhibiting a bacteriostatic effect in monotreatment, chlorflavonin displayed synergistic effects with the first-line antibiotic isoniazid and particularly with delamanid, leading to a complete sterilization in liquid culture in combination treatment. Using a fluorescent reporter strain, intracellular activity of chlorflavonin against *Mycobacterium tuberculosis* inside infected macrophages was demonstrated and was superior to streptomycin treatment.

4 Hyrtinadine A and Alocasin A Derivatives as New Lead Structures for APIs Against Methicillin-resistant *Staphylococcus aureus* (MRSA)

Unpublished

The overall contribution to the paper: 50 %.

Contribution:

- Manuscript writing (50%)
- Cultivation of bacterial strains
- Determination of minimal inhibitory concentration
- Determination of cytotoxicity and therapeutic index
- Determination of time-kill curves *in vitro*
- Determination of single step resistance frequency
- Isolation of genomic DNA

4.1 Manuscript

Hyrtinadine A and Alocasin A Derivatives as New Lead Structures for APIs Against Methicillin-resistant *Staphylococcus aureus* (MRSA)

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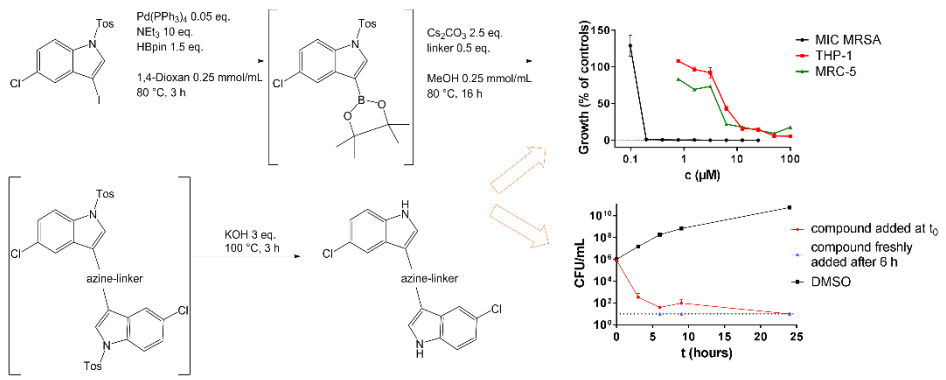
Highlights

- The synthesis and characterization of bis-indoles are reported
- Synthetic derivatives of alocasin A were synthesized via Masuda-Suzuki coupling
- Bis-indoles presented promising antibacterial effects.
- Bis indoles revealed strong bactericidal killing effect against MRSA.

Graphical Abstract



Abstract

In searching for new antibacterial lead structures, one promising class of compounds are indole alkaloids which have previously been shown to exhibit diverse bioactivities such as cytotoxic, antiviral, antimicrobial, antiparasitic, anti-inflammatory and antifungal activity. Using the Masuda-Suzuki one pot synthesis, we created a library of indoles derived from the natural products hyrtinadine A and alocasin A which was screened against *Mycobacterium tuberculosis* and several nosocomial bacterial pathogens including Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococcus faecium*. While the unsubstituted natural compounds were devoid of antibacterial activity, structure-activity relationships revealed that 5',5''-chloro derivatives were most active against MRSA with minimal inhibitory concentration ranging from 0.20 – 0.78 μM. These compounds showed strong bactericidal killing effects but only moderate cytotoxicity against human cell lines, making them promising lead structures with favorable therapeutic margins. Their structure is similar to other published bis-indoles, which have been shown to inhibit *Staphylococcus aureus* pyruvate kinase. However, albeit molecular docking studies indeed indicate alocasin A derivatives bind to pyruvate kinase, antibacterial activity was independent of pyruvate kinase in a Δ*pyk* gene deletion mutant, suggesting a yet-unknown cellular target and unrelated mechanism of action.

Keywords

Masuda-Suzuki synthesis, bis-indoles, Methicillin-resistant *Staphylococcus aureus*, bactericidal, structure-activity relationships

Introduction

Since the introduction of penicillin, antibiotics have become one of the cornerstones of modern medicine. However, the number of multidrug resistant bacterial pathogens has 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 where bacteria are confronted with a high antibiotic selection pressure. Hence, new lead structures and new drug targets are urgently needed. *S. aureus* is a facultative pathogenic bacterium which can cause wound infections, pneumonia or sepsis. β-Lactam antibiotics such as methicillin are the agents of choice for treatment of staphylococci 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.

Approximately 70 % of antibiotics are derived from natural compounds. In addition to microbes and endophytes also plants and marine organisms like sponges can serve as sources for identifying new lead structures [1]. One promising class of antibacterial compounds are indole alkaloids which have shown cytotoxic, antiviral, antimicrobial, antiparasitic, anti-inflammatory and antifungal activity [2-4]. Two examples of natural bis-indoles are alocasin A (**8**) and hyrtinadine A (**1**). Alocasin A is produced by the Asian medicinal plant *Alocasia macrorrhiza* and possesses antifungal and cytostatic activity [5]. In contrast, hyrtinadine A, produced from the Red Sea sponge *Hyrtios sp*., exhibited cytotoxicity against murine leukemia and human epidermoid carcinoma cells *in vitro* [6]. No antibacterial activity was reported for these natural compounds. However, the synthetic modification of natural compounds embodies one possibility to find new antibacterial lead structures, yielding products with higher antibacterial activity than the parental molecule.

A still very powerful tool to form carbon-carbon bonds between two sp^2 carbon atoms is the Suzuki reaction [7]. The Suzuki reaction is a palladium catalyzed cross-coupling reaction which can be performed under mild conditions and has a high functional group tolerance. In addition, this coupling is easy to perform and it is widely used for the synthesis of bi(hetero)aryls [8]. Since borylated starting materials are required, a preceding borylation of an electron rich coupling partner has to be performed using the corresponding halogenated species. As a logical combination, the palladium catalyzed Masuda borylation [9] and the Suzuki arylation can be consecutively employed in the same vessel as shown by Baudoin [10]. For ligating two heteroaryl halides this sequential palladium catalyzed one-pot reaction

was developed and illustrated for the synthesis of marine alkaloids and derivatives in the group of Müller [11-13]. In this study, this one-pot methodology has now been employed for synthesis of derivatives of hyrtinadine A and alocasin A with different substitution patterns and also various central linkers to establish structure-activity relationship (SAR) studies with respect to antibacterial activity.

Experimental Section

Synthetic derivatives. Nuclear magnetic resonance (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: ^{1}H 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.

General procedure

The 3-iodo indole (1.0 mmol) and $Pd(PPh_3)_4$ (0.05 g, 0.05 mmol) were suspended in dry 1,4-dioxane (5 mL) in a dry Schlenk tube under nitrogen (for experimental details, see Table 4-1). Triethylamine (1.4 mL, 10 mmol) and pinacolylborane (0.23 mL, 1.5 mmol) were added dropwise and the reaction mixture was stirred at 80 °C for 4 h. Thereafter the mixture was cooled to room temperature and methanol (MeOH) (5 mL), cesium carbonate (0.82 g, 2.50 mmol), and di-halo linker (0.5 mmol) were added to the reaction mixture. This mixture was stirred at 80 °C for further 16 h. Subsequently, the mixture was cooled to room temperature and sodium hydroxide (0.14 g, 2.5 mmol) was added and stirred again for 3 h at 100 °C. The mixture was allowed to cool down to room temperature diluted with dichloromethane DCM and adsorbed on Celite© under reduced pressure. The adsorbed residue was purified by column chromatography on silica gel (100:1:1/DCM:MeOH: 25 % aqueous (aq.) NH_3). The resulting solid was then dried for 16 h under reduced pressure (3×10^{-3} mbar).
The addition of sodium hydroxide is only required for desulfonating tosyl protected indoles.

Table 4-1 Experimental details of the one-pot Masuda-Suzuki synthesis of the alocasin A and hyrtinadine A derivatives.

Compound #	3-Iodo indole mg/mmol	Di-halo linker mg/mmol	Yield mg/%
5	427 mg /1 mmol of 3-iodo-5-methoxy-1-tosyl-1*H*-indole	119 mg / 0.5 mmol of 2,5-dibromopyrazine	150 mg / 81 %
6	415 mg / 1 mmol of 3-iodo-5-fluoro-1-tosyl-1*H*-indole	118 mg / 0.5 mmol of 2,5-dibromopyrazine	128 mg / 74 %
7	476 mg / 1 mmol of 3-iodo-5-bromo-1-tosyl-1*H*-indole	119 mg / 0.5 mmol of 2,5-dibromopyrazine	172 mg / 74 %
8	501 g / 1 mmol of *tert*-butyl-5,6-dibromo-3-iodo -1*H*-indole-1-carboxylate	119 mg / 0.5 mmol of 2,5-dibromopyrazine	122 mg / 39 %
9	476 mg / 1 mmol of 3-liodo-6-bromo-1-tosyl-1*H*-indole	119 mg / 0.5 mmol of 2,5-dibromopyrazine	206 mg / 88 %
10	432 mg / 1 mmol of 5-chloro-3-iodo-1-tosyl-1*H*-indole	119 mg / 0.5 mmol of 2,5-dibromopyrazine	130 mg / 78 %
12	432 mg / 1 mmol of 5-chloro-3-iodo-1-tosyl-1*H*-indole	166 mg / 0.5 mmol of 3,6-dibromopyridazine	0.143 mg / 75 %
13	432 mg / 1 mmol of 5-chloro-3-iodo-1-tosyl-1*H*-indole	119 mg / 0.5 mmol of 2,6-dibromopyrazine	124 mg / 65 %
14	432 mg / 1 mmol of 5-chloro-3-iodo-1-tosyl-1*H*-indole	75 mg / 0.5 mmol of 2,4-dichloropyrimidine	95 mg / 50 %
15	432 mg / 1 mmol of 5-chloro-3-iodo-1-tosyl-1*H*-indole	119 mg / 0.5mmol of 2,6-dibromopyridine	77 mg / 41 %
19	397 mg / 1 mmol of 3-iodo-1-tosyl-1*H*-indole	75 mg / 0.5 mmol of 2,4-dichloropyrimidine	92 mg / 59 %

In the case of alocasin A (**4**), the methoxy groups are cleaved as follows: Compound **5** (226 mg, 0.61 mmol) is suspended in acetic acid (5 mL) and hydrobromic acid (5 mL, 48 % aq.) and heated to 120 °C in a closed Schlenk tube. The solvent is removed under reduced pressure, the residue suspended in ca. 25 % aqueous NH_3 (25 mL) and stirred at room temperature for 1 h. Ethyl acetate (25 mL) is added twice, organic layers are separated and dried with anhydrous sodium sulfate. The product is then adsorbed on Celite® and purified by column chromatography on silica gel (100:1:1/DCM:MeOH: 25 % aq. NH_3). The resulting solid was then dried under reduced pressure (3×10^{-3} mbar) for 16 h.

Alocasin A (**4**) (150 mg, 72 %), Mp >300 °C. ^{1}H-NMR (600 MHz, DMSO-d_6): δ 6.70 (dd, J = 8.6, 2.4 Hz, 2 H), 7.26 (d, J = 8.6 Hz, 2 H), 7.81 (d, J = 2.4 Hz, 2 H), 8.11 (d, J = 2.8 Hz, 2 H), 8.82 (s, 2 H), 9.00 (s, 2 H), 11.34 (d, J = 2.8 Hz, 2 H) (Figure 4-4). ^{13}C 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 (Figure 4-5). HR-MS (ESI) calcd. for $[C_{20}H_{14}N_4O_2+H]^+$, m/z: 343.1190. Found m/z: 343.1194. HPLC: 98.04%.

2,5-Bis(5-methoxy-*1H*-indole-3-yl)pyrazine (**5**) (150 mg, 81 %), Mp 263 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.83 (s, 6 H), 6.85 (dd, J = 8.8, 2.5 Hz, 2 H), 7.37 (dd, J = 8.8, 0.6 Hz, 2 H), 7.97 (d, J = 2.5 Hz, 2 H), 8.20 (d, J = 2.8 Hz, 2 H), 9.13 (s, 2 H), 11.49 (d, J = 2.9 Hz, 2 H) (Figure 4-6). ^{13}C 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 (Figure 4-7). HR-MS (ESI) calcd. for $(C_{22}H_{18}N_4O_2+H)^+$ *m/z*: 371.1503. Found: *m/z*: 371.1504. Anal. calcd. for $C_{22}H_{18}N_4O_2$ (370.42): C, 71.34; H, 4.90; N, 15.13. Found: C, 71.05; H, 4.89; N, 15.08.

2,5-Bis(5-fluoro-*1H*-indole-3-yl)pyrazine (**6**) (128 mg, 74 %), Mp 280 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.06 (ddd, J = 9.1, 9.1, 2.7 Hz, 2 H), 7.49 (dd, J = 8.9, 4.6 Hz, 2 H), 8.18 (dd, J = 10.6, 2.6 Hz, 2 H), 8.34 (d, J = 2.8 Hz, 2 H), 9.15 (s, 2 H), 11.76 (d, J = 1.9 Hz, 2 H) (Figure 4-8). ^{13}C 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) (Figure 4-9). MS (ESI) calcd. for $(C_{22}H_{18}F_2N_4+H)^+$ *m/z*: 347.4. Found *m/z*: 347.6. Anal. calcd. for $C_{22}H_{18}F_2N_4$ (346.34): C, 69.36; H, 3.49; N, 16.18. Found: C, 69.11; H, 3.37; N, 15.93.

2,5-Bis(5-bromo-*1H*-indole-3-yl)pyrazine (**7**) (172 mg, 74 %), Mp 287 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.32 (dd, J = 8.6, 2.0 Hz, 2 H), 7.46 (dd, J = 8.6, 0.6 Hz, 2 H), 8.32 (d, J = 2.5 Hz, 2 H), 8.67 (d, J = 1.9 Hz, 2 H), 9.19 (s, 2 H), 11.85 (s, 2 H) (Figure 4-10). ^{13}C 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 (Figure 4-11). MS (ESI) calcd for $(C_{20}H_{12}{}^{79}Br^{81}BrN_4+H)^+$ *m/z*: 469.15. Found *m/z*: 469.3. Anal. calcd. for $C_{20}H_{12}Br_2N_4$ (468.15): 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 (**8**) (122 mg, 39 %), Mp >300 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.89 (s, 2 H), 8.36 (d, J = 2.8 Hz, 2 H), 8.86 (s, 2 H), 9.21 (s, 2 H), 11.92 (d, J = 2.4 Hz, 2 H) (Figure 4-12). ^{13}C 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 (Figure 4-13). MS (ESI) calcd. for $(C_{20}H_{10}{}^{79}Br_2{}^{81}Br_2N_4+H)^+$ *m/z*: 627.0. Found *m/z*: 627.1. Anal. calcd. for $C_{20}H_{10}Br_4N_4$ (625.94): 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 (**9**) (206 mg, 88 %), Mp 284 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.29 (dd, J = 8.6, 1.8 Hz, 2 H), 7.67 (d, J = 1.5 Hz, 2 H), 8.28 (s, 2 H), 8.40 (d, J = 8.6 Hz, 2 H), 9.14 (s, 2 H), 11.77 (s, 2 H) (Figure 4-14). ^{13}C 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 (Figure 4-15). MS (ESI) calcd. for $(C_{20}H_{12}{}^{79}Br^{81}BrN_4+H)^+$ *m/z*: 469.15. Found *m/z*: 469.3. Anal. calcd. for $C_{20}H_{12}Br_2N_4$ (468.15): C, 51.31; H, 2.58; N, 11.97; Found: C, 50.97; H, 2.45; N, 11.65.

2,5-Bis(5-chloro-*1H*-indole-3-yl)pyrazine (**10**) (130 mg, 78 %), Mp 278 °C. ^{1}H NMR (300 MHz, DMSO-d$_6$): δ 7.21 (dd, J = 2.1, 8.6 Hz, 2 H), 7.50 (d, J = 8.6 Hz, 2 H), 8.34 (d, J = 2.8 Hz, 2 H), 8.51 (d, J = 2.1 Hz, 2 H), 9.19 (s, 2 H), 11.84 (d, J = 3 Hz, 2 H) (Figure 4-16). ^{13}C 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 (Figure 4-17). MS (ESI) calcd. for $(C_{20}H_{12}Cl_2N_4+H)^+$ *m/z*: 379.2. Found *m/z*: 379.3. Anal. calcd. for $C_{20}H_{12}Cl_2N_4$ (378.26): C, 63.34; H, 3.19; N, 14.77. Found: C, 63.09; H, 3.17; N, 14.70.

3,6-Bis(5-chloro-1*H*-indole-3-yl)pyridazine (**12**) (0.143 mg, 75 %), Mp 268 °C. ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.24 (dd, J = 8.6, 2.1 Hz, 2 H), 7.53 (d, J = 8.6 Hz, 2 H), 8.13 (s, 2 H), 8.37 (d, J = 2.6 Hz, 2 H), 8.67 (d, J = 2.1 Hz, 2 H), 11.91-11.87 (m, 2 H) (Figure 4-18). ^{13}C 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 (Figure 4-19). HR-MS (ESI) calcd. for $[C_{20}H_{12}Cl_2N_4+H]^+$, *m/z*: 379.0512. Found: *m/z*: 379.0516. HPLC: 98.66 %.

2,6-Bis(5-chloro-*1H*-indole-3-yl)pyrazine (**13)** (124 mg, 65%), Mp 253 °C. ^{1}H NMR (600 MHz, DMSO-d$_6$) δ 7.21 (dd, J = 8.6, 2.2 Hz, 2 H), 7.51 (d, J = 8.6 Hz, 2 H), 8.34 (d, J = 2.8 Hz, 2 H), 8.52 (d, J = 2.1 Hz, 2 H), 9.19 (s, 2 H), 11.85 (d, J = 2.7 Hz, 2 H) (Figure 4-20). ^{13}C 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 (Figure 4-21). HR-MS (ESI) calcd. for $(C_{20}H_{12}Cl_2N_4+H)^+$ *m/z*: 379.0512. Found *m/z*: 379.0511. Anal. calcd. for $C_{20}H_{12}Cl_2N_4$ (379.24): C, 63.34; H, 3.19; N, 14.77. Found: C, 63.15; H, 3.00; N, 14.66.

3,3'-(Pyrimidine-2,4-diyl)bis(5-chloro-1*H*-indole) (**14**) (95 mg, 50 %), Mp 226 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.23 (ddd, J = 11.2, 8.6, 2.2 Hz, 2 H), 7.53 (dd, J = 8.6, 4.0 Hz, 2 H), 7.63 (d, J = 5.4 Hz, 1 H), 8.31 (d, J = 2.8 Hz, 1 H), 8.49 (d, J = 2.9 Hz, 1 H), 8.70-8.59 (m, 3 H), 11.90 (s, 1 H), 12.06 (s, 1 H) (Figure 4-22). ^{13}C NMR (75 MHz, DMSO-d_6) δ 112.2, 113.2, 113.5, 113.7, 115.2, 120.8, 120.9, 121.8, 122.2, 125.1, 125.4, 126.2, 126.6, 130.0, 130.5, 135.4, 135.6, 156.3, 161.1, 162.8 (Figure 4-23). HR-MS (ESI) calcd. for $[C_{20}H_{12}Cl_2N_4+H]^+$, m/z: 379.0512. Found m/z: 379.0510. HPLC: 98.15%.

2,6-Bis(5-chloro-1*H*-indole-3-yl)pyridine (**15**) (77 mg, 41 %), Mp 241 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.19 (dd, J = 8.6, 2.1 Hz, 2 H), 7.50 (dd, J = 8.6, 0.5 Hz, 2 H), 7.64-7.59 (m, 2 H), 7.76 (dd, J = 8.5, 7.1 Hz, 1 H), 8.19 (d, J = 2.8 Hz, 2 H), 8.56 (d, J = 2.1 Hz, 2 H), 11.73 (d, J = 2.8 Hz, 2 H) (Figure 4-24). ^{13}C 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 (Figure 4-25). MS (ESI): calcd. for $[C_{21}H_{13}Cl_2N_3+H]^+$, m/z: 378.1. Found m/z: 378.5. Anal. calcd. for $C_{21}H_{13}Cl_2N_3$ (378.26): C, 66.68; H, 3.46; N, 11.11. Found: C, 66.69; H, 3.17; N, 10.83.

3,3'-(Pyrimidine-2,4-diyl)bis(1*H*-indole) (**19**) (92 mg, 59 %), Mp 297 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.30-7.12 (m, 4 H), 7.56-7.47 (m, 2 H), 7.59 (d, J = 5.5 Hz, 1 H), 8.31 (d, J = 2.4 Hz, 1 H), 8.41 (s, 1 H), 8.68-8.57 (m, 3 H), 11.64 (s, 1 H), 11.83 (s, 1 H) (Figure 4-26). ^{13}C NMR (75 MHz, DMSO-d_6) δ 111.8, 111.9, 112.1, 113.7, 115.5, 120.1, 120.6, 121.5, 121.7, 121.9, 122.1, 125.1, 125.6, 128.8, 128.9, 137.0, 137.2, 156.1, 161.4, 163.4 (Figure 4-27). HR-MS (ESI calcd. for $[C_{20}H_{14}N_4+H]^+$, m/z: 311.1291, Found m/z: 311.1292. Anal. calcd. for $C_{20}H_{14}N_4$ (310.36): C, 77.40; H, 4.55; N, 18.05; Found: C, 77.14; H, 4.25; N, 17.76.

Bacterial strains. Nosocomial bacterial reference strains were cultivated in Müller Hinton (MH) medium at 37 °C and included different strains of *S. aureus* (MSSA strain ATCC 25923, MRSA/VISA strain ATCC 700699, MRSA strain LAC USA300, and pyruvate kinase-deficient MRSA strain LAC Δ*pyk*::Erm^{R}); *E. faecalis* (ATCC 29212, ATCC 51299 (gentamycin resistant)); *E. faecium* (ATCC 35667, ATCC 700221 (vancomycin resistant)); and *A. baumannii* (ATCC BAA 1605). *M. tb.* strain H37Rv was grown aerobically in Middlebrook 7H9 medium supplemented with 10 % (v/v) ADS enrichment (5 %, w/v,

bovine serum albumin (BSA) 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 were tested for their antibacterial activities against various pathogenic bacterial strains including nosocomial pathogens and *M. tb.*. For the tested nosocomial pathogens (*S. aureus*, *E. faecalis*, *E. faecium*, *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 MH medium at 37 °C and 180 rounds per minute (rpm). A preculture was grown until log phase (optical density $(OD)_{600\ nm} \sim 0.5$) and then seeded at 5×10^4 colony forming units (CFU) per well in a total volume of 100 µL in 96-well round bottom microtiter plates and incubated with serially diluted test substances 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 that led to complete inhibition of visual growth of the bacteria.

The MICs of compounds **7**, **10**, **11**, **12**, **14**, **15** 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 medium without cells to determine background luminescence were prepared. 50 µL BacTiter-Glo™ reagent were 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 dimethyl sulfoxide (DMSO) solvent control (100 % growth).

The influence of glucose or pyruvate as carbon source on drug susceptibility of *S. aureus* cells was determined in minimal medium. MIC of active compounds was determined in twofold serial dilutions ranging from 0.15-25 µM in minimal medium M9 (1×M9 salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.05 mM nicotinamide, 0.125 % w/v casamino acids) with either 1 % glucose or pyruvate, respectively. 5 x 10^4 CFU/well were 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. tb.*.

For *A. baumannii*, the substances were not only tested in monotherapy but additionally also in combination with sublethal concentrations of either RIF (2.5 µM) or COL (0.31 µM) in the medium, respectively. Due to the special cell wall of Gram-negative bacteria, antibiotics may not enter the cell easily. In combination with RIF, compounds can be detected which influence the membrane permeability so that RIF gets into the cell. COL causes permeability of the bacterial outer membrane so that the compounds can enter.

For the determination of MIC against *M. tb.*, bacteria were precultured until log-phase ($OD_{600\ nm}$ = 0.5-1) and then seeded at $1x10^5$ cells/well in a total volume of 100 µL in 96-well round bottom microtiter plates and incubated with serially diluted test compounds at a concentration range of 100-0.78 µM. Microplates were incubated at 37 °C for five days. Afterwards, 10 µL/well of a 100 µg/mL resazurin solution were 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 RIF treated (0% growth) and DMSO treated (100% growth) controls.

Determination of cytotoxicity and therapeutic index. The cytotoxicity of compounds **7**, **10**, **11**, **12**, **14**, **15** 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). THP-1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10 % (v/v) fetal bovine serum (FBS), while MRC-5 cells were incubated in Dulbecco's Modified Eagles Medium (DMEM) containing 10 % (v/v) FBS both 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 twofold serially diluted test compounds 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 resazurin solution (100 µg/mL) were 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 (meaning, lat.: *id est*, i.e. cell-free) (0 % growth) and untreated (100 % growth) controls in triplicate experiments, respectively.

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

Determination of time-kill curves *in vitro*. Killing kinetics were measured for compounds **7**, **10**, **11**, **12**, **14**, **15** against the *S. aureus* MRSA/VISA strain ATCC 700699. An overnight culture of the strain was adjusted to a final inoculum concentration of 10^6 CFU/mL cultivated in MH broth containing the tested compounds at five-fold MIC. Cultures containing moxifloxacin at five-fold MIC (20 µM) were used as 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 antibiotic-free control. In a second attempt, the culture medium was removed after 6 h post exposure to compounds and replaced by fresh compound-containing medium. This should overcome limited antibacterial activity in case a compound was degraded or consumed during the experiment. Therefore, the culture was centrifuged at 4000 rpm for 10 min. Afterwards, the bacterial cell pellet was washed in phosphate buffered saline (PBS) and suspended in fresh medium containing the corresponding compound 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 µL aliquots of a serial dilution onto MH agar plates. The plates were incubated aerobically at 37 °C for 24 before colonies were counted.

Additionally, the resistance rate of the cultures was determined after 6 and 24 h of incubation. For this, 20 µL of the cultures were plated on 1 mL MH agar containing the five-fold MIC of the compounds. After an incubation time of 24 h at 37 °C, the colonies were counted. The resistance rate was determined as quotient of the number of resistant colonies on compound containing agar and the total amount of viable bacteria at the corresponding time points.

For determination of the influence of the carbon source on the killing effect, *S. aureus* LAC wildtype (WT) and LAC Δ*pyk*::Erm^R were cultivated in tryptic soy broth (TSB) without glucose with 1 % sodium pyruvate and treated like described elsewhere[14].

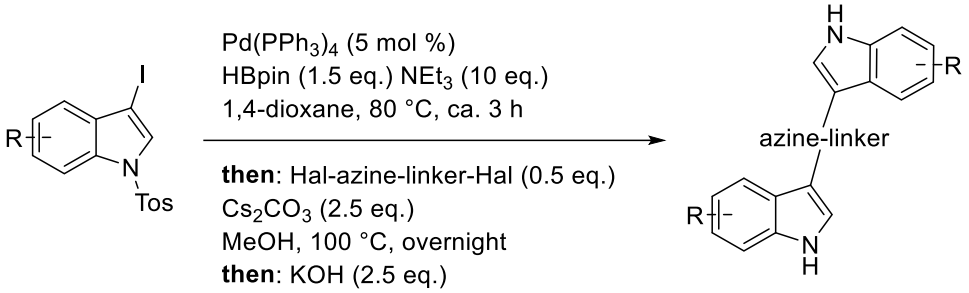
Determination of single step resistance frequency. 20 µL of bacterial cell suspensions of 1×10^6, 1×10^7 and 1×10^8 CFU/mL of the *S. aureus* MRSA strain ATCC 700699 were spread on MH agar plates containing compound **10** or **11** at 3- and 5-fold MIC. After 72 h of incubation at 37 °C, resistant colonies were quantified. To confirm the level of resistance, the MIC of the compound against the isolated clones was determined.

Isolation of genomic DNA. For isolation of the genomic DNA, individual resistant mutants (RM) were cultivated overnight. Bacteria suspensions were centrifuged at 4000 rpm for 5 min. Afterwards, bacterial cell pellets were suspended in a lysostaphin-glucose-Tris-EDTA (GTE) solution (200 µg/mL lysostaphin in 25 mM tris(hydroxymethyl)amino-methane (Tris-Cl), pH 8.0; 10 mM ethylenediaminetetraacetic acid (EDTA); 50 mM glucose) and incubated at 37 °C for 30 min. After addition of 5 µL 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 NaCl (5 M) and 1 mL ethanol. After centrifugation and aspiration of the supernatant, the DNA pellet was air-dried for 15 min. Finally, the DNA was solved in TE buffer containing RNase (10 mM Tris, 0.1 mM EDTA, pH 7.4).

Whole genome sequencing. To identify the resistance mediating mutations, genomic DNA of six independent MRSA mutants was sequenced. Libraries were prepared for sequencing using the standard paired-end genomic DNA sample prep kit from Illumina. Genomes were sequenced using an Illumina HiSeq 2500 next-generation sequencer (San Diego, CA, USA) and compared with the parent methicillin-resistant *S. aureus* genome (strain ATCC 700699 (= Mu50); GenBank accession GCA_000009665.1). Paired-end sequence data was collected with a read length of 106 bp. Base-calling was performed using Casava software, v1.8. The reads were assembled using a comparative genome assembly method. The mean depth of coverage ranged from 277× - 770×.

Results

Synthesis of bis-indole compounds. Based on our previously established synthesis scheme [15], twelve bis-indole molecules were synthesized including one with a pyridazine (**12**), and pyridine bridge (**15**), respectively, two derivatives with a pyrimidine (**14**, **19**) and eight with a pyrazine linker (**4**-**10**; **13**). Seven of these compounds represent new structures. Compound **4** embodies the natural compound alocasin A, whereas compounds **5 - 10**, and **16** are derivatives thereof. We recently showed the successful application of the Masuda borylation-Suzuki coupling as a one-pot reaction in the sense of a sequentially palladium-catalyzed process [11, 15]. In this study, hyrtinadine A **1** and several derivatives (**2**, **3** and **11**) could be synthesized with good yields using this strategy. This demonstrates that this sequence is a robust synthesis tool for bis-indoles in general allowing incorporation of different linkers (**16**, **17**, **18** and **20**) as well. Another compound amenable to this synthesis scheme is plant-derived natural compound alocasin A **4** [5]. We synthesized a small library around this natural compound with different indoles. Recognizing the antibacterial potency especially of the chlorinated compounds out of this portfolio, we started to establish new chlorinated structures by substitution of the linker (**12**, **13**, **14** and **15,** Scheme 4-1).



5 R = 5-OMe, linker = 2,5-dibromopyrazine
6 R = 5-F, linker = 2,5-dibromopyrazine
7 R = 5-Br, linker = 2,5-dibromopyrazine
8 R = 5,6-Br, linker = 2,5-dibromopyrazine with Boc-protecting group, without adding KOH
9 R = 6-Br, linker = 2,5-dibromopyrazine
10 R = 5-Cl, linker = 2,5-dibromopyrazine
12 R = 5-Cl, linker = 3,6-dibromopyridazine
13 R = 5-Cl, linker = 2,6-dibromopyrazine
14 R = 5-Cl, linker = 2,4-dichloropyrimidine
15 R = 5-Cl, linker = 2,6-dibromopyridine
19 R = H, linker = 2,6-dibromopyrimidine

Scheme 4-1 General synthesis of bis-indoles

The indoles were prepared for the sequence in the style of Witulski *et al.* [16]. The borylation of the indoles was performed in dry 1,4-dioxane with water free triethylamine under Schlenk conditions at 80 °C (checked by thin layer chromatography, TLC). The excess of pinacolborane was quenched afterwards by methanol and the resulting solvent mixture was also a better solvent system for the base cesium carbonate, which is needed for the Suzuki coupling. This was performed at a slightly higher temperature. The inserted linkers were used with no further purification and even chlorinated azines were successfully coupled (**14**). Using the tosyl-protecting group, a removing step is needed. This was also performed in a one-pot fashion by adding potassium hydroxide to the reaction mixture. Alocasin A (**4**) was synthesized out of **5** by cleaving the methyl group, heating it up to 120 °C in a mixture of aqueous hydrobromic acid (48 %) and acetic acid.

Synthetic bis-indole compounds exhibit potent antibacterial activity against a panel of pathogenic bacterial strains. MICs of all compounds were determined by screening in two-fold serial dilution at a concentration range between 100-0.78 µM against both nosocomial bacteria (including methicillin-resistant *S. aureus* (MRSA), *E. faecalis*, and vancomycin-resistant *E. faecium*) and *M. tb.*. None of the tested compounds were active against the Gram-negative bacterium *A. baumannii* and also showed no or only a weak effect against *M. tb.*

(Table 4-5). However, these compounds showed different 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 4-2). As already reported in the literature, the natural compounds hyrtinadine A (**1**) and alocasin A (**4**) did not show any inhibitory activity against the pathogenic bacteria. Also 5', 5"-dimethoxylated (**2**, **5**) 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. tb.*, *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 (**3**, **6** - **8**, **10**, **11**). Regarding the halogenated derivatives **3** and **11** as well as **6** and **10**, it could also be observed that the replacement of fluorine by chlorine led to a decrease of the MIC by a factor ≥ 32. The replacement from chlorine to bromine increased the MIC slightly to 1.56 µM in case of MRSA. Furthermore, the brominated structures **7**-**9** indicated that the halogenation at position 5',5" (**7**) is essential regarding antibacterial activity. The additional halogenation at position 6',6" of the indole rings (**8**) led to an increased MIC of 25-100 µM. If only position 6',6" was halogenated like in compound **9**, no activity could be observed in the tested concentration range.

Additionally, the comparison of the activity of the compounds **10**-**20** indicated that the substitution of the linker from pyrimidine or pyrazine both to pyridazine (**12**) and the replacement of a 2,5-substitution to a 2,4-substituted pyrimidine ring (**14**) or even the changing of the linker from pyrimidine to 3,5-disubstituted pyrazine (**13**) or 2,6-disubstituted pyridine (**15**) had only a slight influence on the MIC, respectively, as long as the indole rings were substituted with a chlorine atom at 5',5" position (Table 4-2 and 4-3).

Interestingly, the 5',5"-chlor-pyrazine derivatives **10** and **13** exhibited a very narrow specificity by inhibiting exclusively the MRSA strain (MIC = 0.39-0.78 µM), but not the methicillin-sensitive parental *S. aureus* (MSSA) strain. In contrast, the brominated derivative **7** showed a MIC of 1.56 µM against both *S. aureus* strains (Table 4-5). Only compounds **11**, **14**, and **15** exhibited broad antibacterial potency against all tested Gram-positive nosocomial pathogens. Paradoxically, a decreased inhibitory effect for compound **7**, **10** and **13** could be observed at concentrations ≥ 0.78 µM (Figure 4-1 A and B; Figure 4-28), whereas other symmetric compounds like **12** or **15** did not show this effect at concentrations higher than the MIC (Figure 4-1 D and F).

Table 4-2 Synthesized substituted compounds and their MIC against methicillin-resistant *S. aureus* ATCC 700699 in µM.

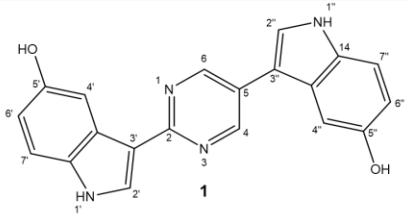
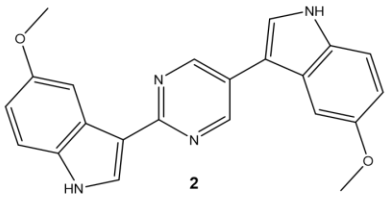
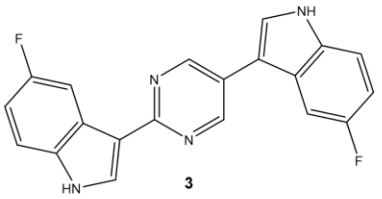
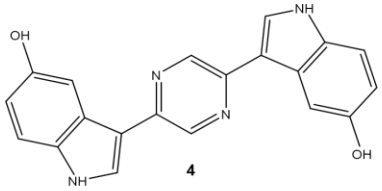
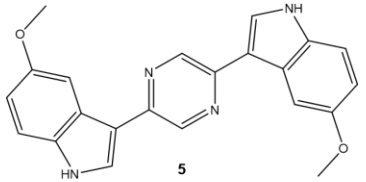
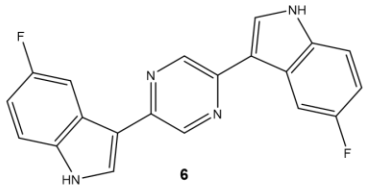
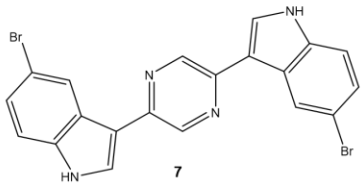
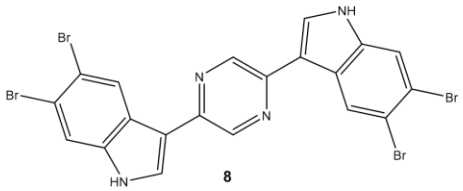
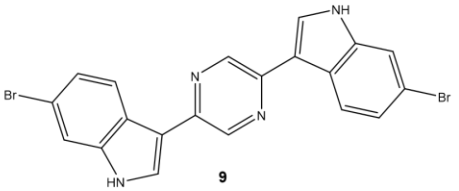
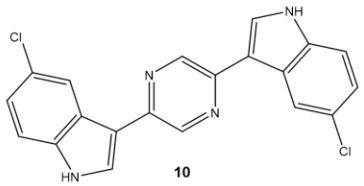
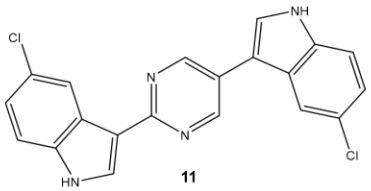
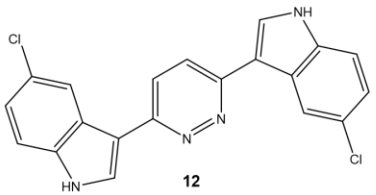
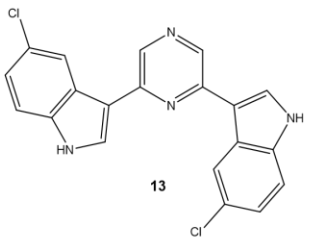
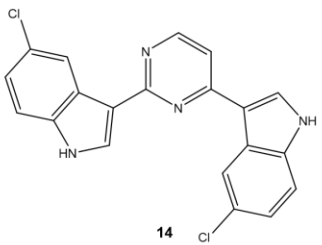
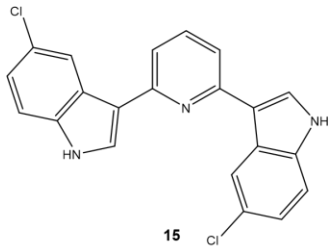
Structure	MIC_{90} [µM]	Structure	MIC_{90} [µM]	Structure	MIC_{90} [µM]
 **1**	>100	 **2**	50	 **3**	12.5
 **4**	>100	 **5**	>100	 **6**	>100
 **7**	1.56	 **8**	25	 **9**	>100
 **10**	0.39	 **11**	0.39	 **12**	0.78
 **13**	0.39	 **14**	0.39	 **15**	0.20

Table 4-3 Synthesized unsubstituted compounds and their MIC against methicillin-resistant *S. aureus* ATCC 700699 in µM.

Structure	MIC_{90} [µM]	Structure	MIC_{90} [µM]	Structure	MIC_{90} [µM]
16	>100	**17**	12.5	**18**	>100
		19	>100	**20**	>100

Cytotoxicity and therapeutic index. The compounds **7**, **10**, **11**, **12**, **14**, **15** were tested against human cell lines to analyze the therapeutic index of these structures. Since the compounds are cytotoxic only at concentrations >12.5 µM, the selectivity indices (IC_{50}/MIC_{90}) yielded sufficient therapeutic margins (Figure 4-1**)**.

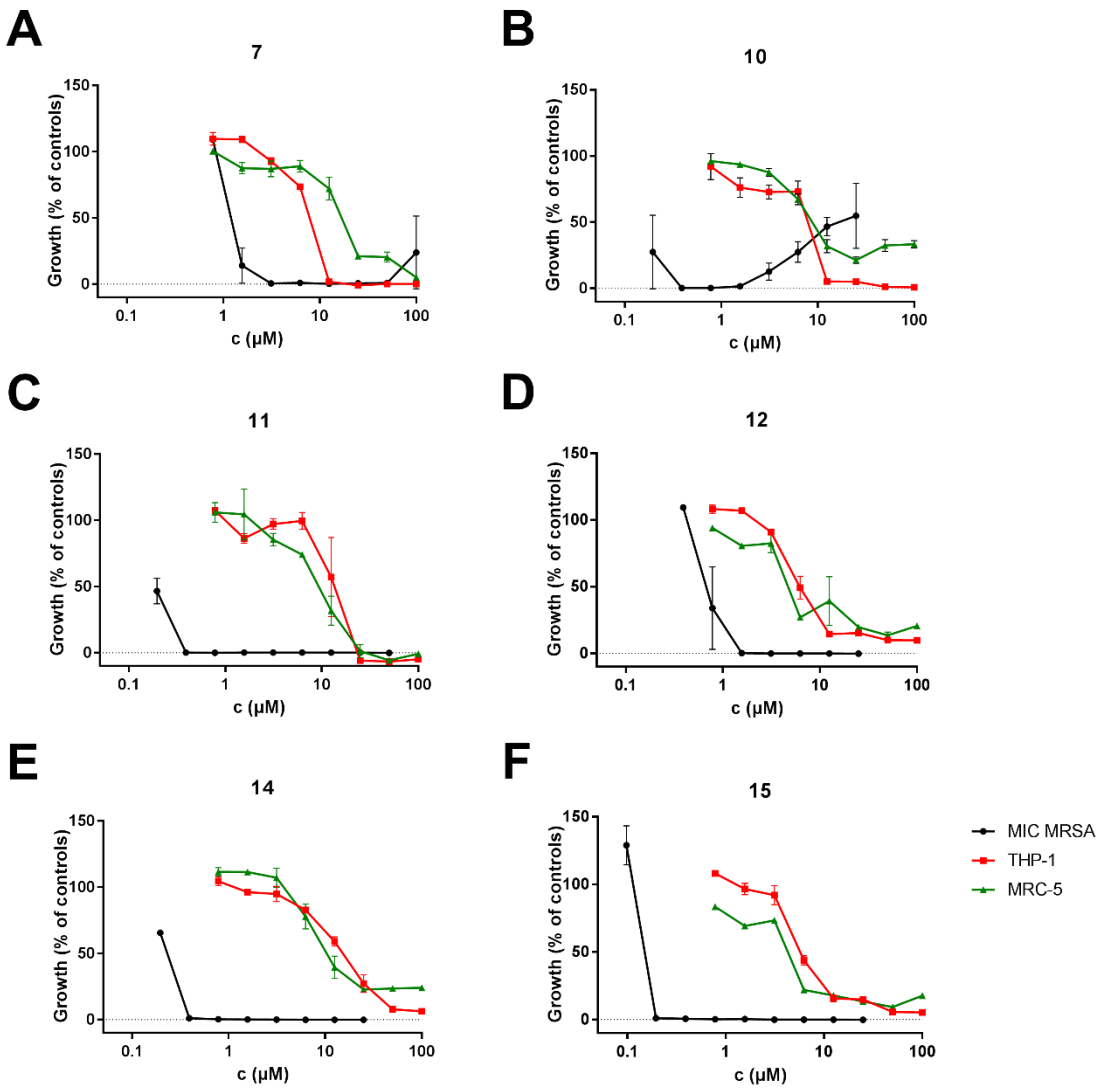


Figure 4-1 Dose-response curves of compounds **7, 10, 11, 12, 14, 15** against MRSA (●) and the human cell lines MRC-5 (▲) and THP-1 (■). Data are means of triplicates ± SD. Growth was quantified using BacTiterGlo assay for bacterial and resazurin dye reduction assay for eukaryotic growth. Resulting selectivity indices (SI = IC_{50}/MIC_{90}): **7**, SI = 8; **10**, SI = 32; **11**, SI = 32; **12**, SI= 8; **14**, SI = 32; **15**, SI = 32.

***In vitro* time kill studies revealed early bactericidal activity.** A killing kinetic was performed to determine the potential bactericidal or bacteriostatic effect of the tested compounds on actively growing bacteria. Compounds **7**, **10**, **11**, **12**, **14**, **15** exhibited a strong bactericidal killing effect. After an incubation time of 6 h with compounds at the five-fold MIC, bacterial viability was reduced by a factor of $10^4 – 10^5$ by substances **7**, **11**, **14** and **15** (Figure 4-2 A, C, E, F), while treatment with compounds **10** and **12** reduced viability only 100-fold (Figure 4-2 B and D). Since after 24 h the amount of surviving bacteria remained $<10^1$ CFU/mL (representing the limit of detection) in presence of compounds **11**, **12**, **14**, **15**, these highly bactericidal substances might be able to completely sterilize the culture. In contrast, some persistent bacteria remained in the cultures containing **7** and **10** after 6 h of treatment, giving rise to increasing number of cells again at prolonged treatment intervals. However, no significant level of resistance was detectable in these treated cultures after 6 and 24 h of incubation (Figure 4-29). When compound **10** was freshly added to washed cells after 6 h of incubation, the viability of the culture continued to decrease to 10^2 CFU/mL initially, but almost rose to the same amount than the growth control after 24 h of incubation time (Figure 4-2 B). Accordingly, compound **10** seems to be rapidly degraded or depleted in liquid culture such that the antibacterial effect wanes after more than 6 h of incubation time. Interestingly, regrowth of the pathogen at extended incubation times was much less pronounced when compound **7** was freshly added to the culture after 6 h (Figure 4-2 A). Therefore, substance **7** might be less prone to degradation or depletion than structure **10**.

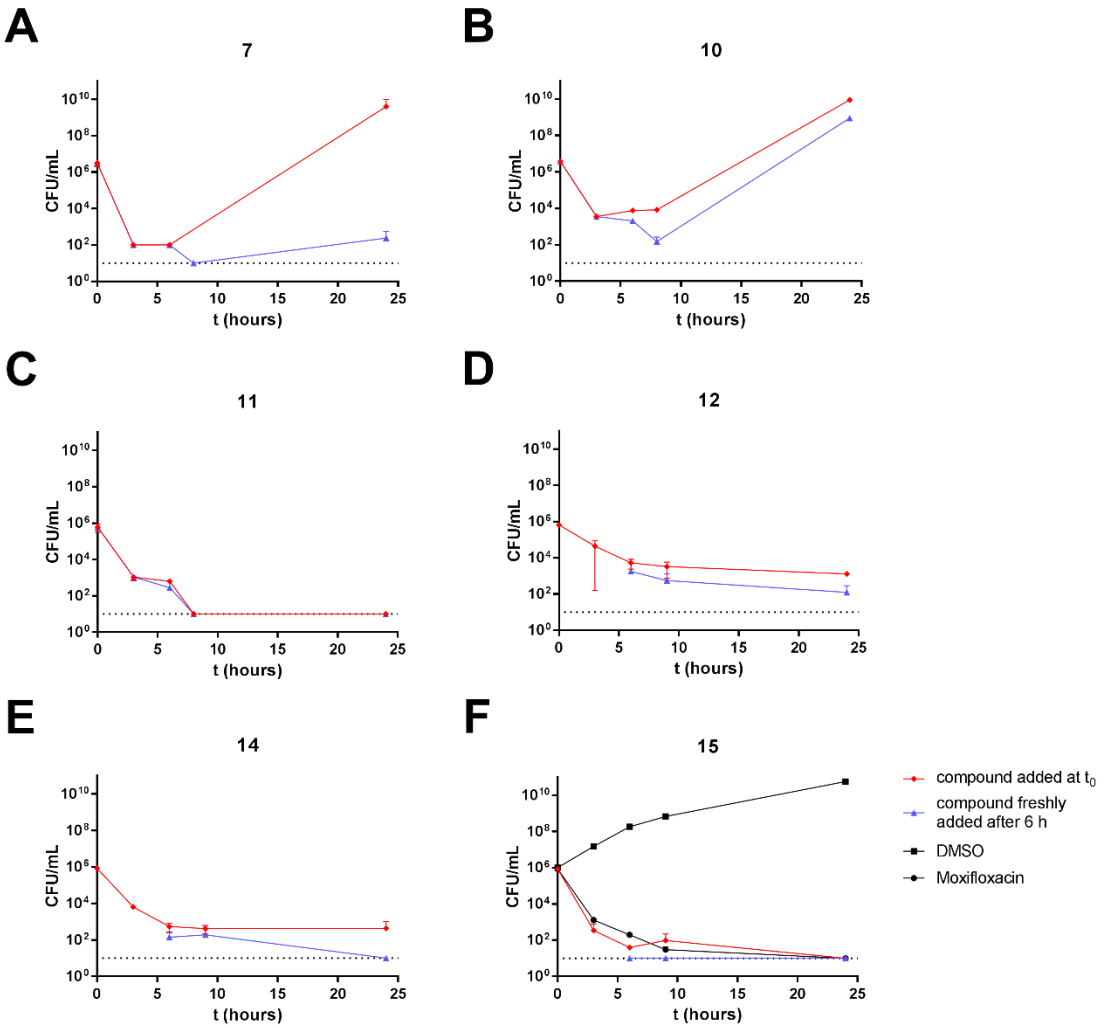


Figure 4-2 Time-kill curves of methicillin-resistant *S. aureus* (MRSA; ATCC 700699) for lead bis-indoles **7**, **10**, **11**, **12**, **14**, **15**. Compounds were tested at 5x MIC (resulting in 0.02-0.08% DMSO final concentration) (♦). Additionally, in an independent experiment, medium was replaced and compounds were freshly added after 6 h (▲). Moxifloxacin at 5x MIC (●) and cells grown in medium without antibiotic but only containing 0.08% DMSO (■) were included as positive and negative controls, respectively. Results are presented as the mean of duplicate experiments ± SD.

Antibacterial efficacy of alocasin A derivatives is independent of PK in a PK-deficient MRSA mutant. To validate involvement of PK as a potential molecular target of alocasin A derivatives in *S. aureus*, we determined the MIC of **7**, **11**, **12**, **14** and **15** against the MRSA LAC WT strain and an isogenic PK knock out strain in minimal medium with glucose or pyruvate as single carbon source. The PK encoding gene *pyk* is conditionally essential in *S. aureus*. A PK deletion mutant is viable in medium that lacks glucose when it is supplemented with pyruvate. Indeed, the LAC Δ*pyk*::Erm^R mutant was unable to grow in medium containing glucose as a carbon source consistent with a previous report [14], demonstrating PK essentiality under these conditions. Consistently, the MRSA LAC WT was fully susceptible to compound **11** during growth on glucose (Figure 4-3). However, unexpectedly the MRSA LAC WT showed unaltered susceptibility to **11** in medium containing pyruvate as the sole carbon source, a condition where PK activity is dispensable. Furthermore, the isogenic PK-deficient LAC Δ*pyk*::Erm^R mutant was even slightly more sensitive towards **11** during growth on pyruvate, while it was expected to be resistant if the compound specifically targets PK in the bacteria cell (Figure 4-3). Similar susceptibility patterns were also observed for compounds **7**, **12**, **14** and **15** (Figure 4-30). Additionally, deletion of *pyk* did not alter the bactericidal killing kinetic of the LAC Δ*pyk*::Erm^R mutant compared to the parental MRSA LAC WT strain towards compound **11** in TSB supplemented with pyruvate (data not shown). These studies showed that the bactericidal activity of alocasin A derivatives is entirely *pyk*-independent under the tested *in vitro* culture conditions.

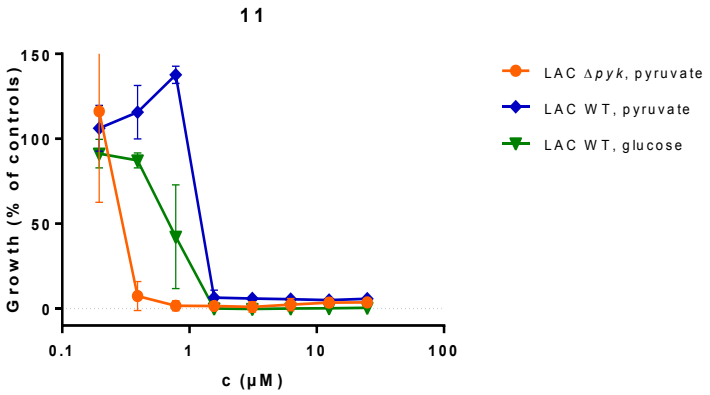


Figure 4-3 Antibacterial activity of **11** against MRSA LAC WT and LAC Δ*pyk*::Erm^R in minimal medium M9 with glucose (▼) or pyruvate (●, ♦) as single carbon source. Data are means of duplicates ± SD. Growth was quantified using resazurin assay.

Evaluation of resistant mutants with whole genome sequencing gave insights in the mode of resistance. In order to gain insight into the potential mode-of-action and the molecular target(s), spontaneous resistant MRSA mutants were isolated on solid medium containing compounds **10** or **11** at 3- or 5-fold MIC. Resistance occurred at a frequency of $5x10^{-7}$ (compound **10**) and $5x10^{-5}$ (compound **11**), respectively. The isolated individual resistant mutants were subsequently tested against compound **11** to determine their level of resistance. Table 4-4 shows that the MIC of compound **11** was 8-fold higher for the mutants than for the parent MRSA strain. Interestingly, also the two tested clones that were resistant against compound **10** showed cross-resistance against compound **11**, indicative of a common mode-of-action and target of these compounds as was expected due to their highly similar chemical structures. Whole-genome resequencing of four individual mutants resistant against compound **11** and of two mutants resistant against compound **10** was performed to reveal the resistance-mediating determinants, some of which might directly be linked to the molecular target. Several mutations were identified that were present only in one clone or that were shared only between two or three strains. Therefore, these mutations are likely not directly related to resistance. However, all six clones also harbored a single nucleotide polymorphisms (SNP) in the gene SAV_RS01095 encoding the enzyme EIICBA of the phosphoenolpyruvate-dependent glucose phosphotransferase system (PTS): mutants **10**-RM1, **10**-RM2 and **11**-RM4 carried a mutation that causes a substitution of the amino acid at position 674 from glycine to aspartic acid. Furthermore, mutants **11**-RM1, **11**-RM2, **11**-RM3 had a SNP that leads to an amino acid substitution at position 461 from valine to leucine. The common mutations in gene SAV_RS01095 in six individual mutants strongly suggests that this gene plays a direct role in resistance, and the corresponding protein might possibly even represent a direct cellular target for compounds **10** and **11**. However, the molecular consequences of the amino acid substitutions for function of the protein as well as the molecular principle underlying resistance are currently unknown.

Table 4-4 Minimal inhibitory concentration of compound **11** against spontaneous resistant mutants. Mutants were generated on agar containing 3-5-fold concentration of **10** or **11**, respectively (**10**-RM or **11**-RM). Parent MRSA strain (MRSA WT) was used as control.

***S. aureus* strain**	**MIC_{90} [µM]**
MRSA WT	0.39
10-RM1	3.13
10-RM2	6.25
11-RM1	3.13
11-RM2	3.13
11-RM3	3.13
11-RM4	3.13

Discussion and Conclusions

New compounds are urgently needed to combat multi-drug resistant bacteria. Higher plants and marine sponges may serve as a resource for new antimicrobials. In particular, plant- and sponge-derived indole containing alkaloids comprise metabolites with highly diverse bioactivities such as antitumor [18, 19], antifungal [20], antiviral [21] and also antimicrobial [20, 22] activity. Alocasin A and hyrtinadine A are two natural bis-indole alkaloids consisting of two indole moieties connected to an 2,5-substituted pyrazine or pyrimidine linker which are active against human cancer cell lines [5, 6]. However, antibacterial activity has not previously been associated with these natural core structures. Antibacterial natural products often exhibit complex structures with chiral atoms, for instance, which often can be synthesized only with poor efficiency. By use of the Masuda-Suzuki sequence, however, a small but diverse library around the natural compounds alocasin A (this study) and hyrtinadine A [15] was easily and efficiently synthesized and allowed detailed SAR studies regarding their antibacterial effects. Seven of these structures (**6**, **8**, **10**, **12**-**15**) were not described elsewhere before. SAR studies showed that halogenation is essential for the antimicrobial activity, whereas the unsubstituted natural compounds as well as their methoxylated derivatives were inactive. The halogen substitution has to be located on 5',5'' position of the indole rings, while the single or additional substitution on 6',6'' position reduced the antibacterial activity. In contrast to these relatively narrow structural requirements regarding the indole moieties, wide flexibility is tolerated concerning the linker unit. Not only hyrtinadine A or alocasin A derivatives, but also 2,4-, 2,6-, or 3,6-disubstituted pyrimidine, pyrazine, pyridine or pyridazine bis-indoles inhibit the growth of MRSA as long as they harbor chlorine atoms at 5',5'' position of the indole rings. Besides alocasin A, the use of pyrazine as linker has previously only been reported once [2]. With respect to further preclinical development, compounds **11**, **14**, and **15** seem to be most promising due to their broad antibacterial potency against all tested Gram-positive nosocomial pathogens and their moderate cytotoxicity towards human cells. Consequently, these compounds are interesting lead structures for further preclinical development with acceptable therapeutic margins.

Paradoxically, a decreased inhibitory effect for compounds **7**, **10** and **13** could be observed at higher concentrations. It is possible that **7**, **10** and **13** aggregate at higher concentrations because of their axial symmetry and precipitates, thus resulting in a decreased concentration of the compound in solution. Since the other symmetric compounds **12** and **15** did not show these effects at high concentrations, this has to be a peculiar influence of the pyrazine

linker. Regardless of the physicochemical basis of this effect, however, the limited solubility in aqueous solution makes these compound poor candidates for systemic clinical applications. Furthermore, after an initial phase of killing of bacterial cells, treatment of MRSA with **7** and **10** resulted in regrowth after 8 h. Since this regrowth could be delayed by compound replenishment after 6 h, and since no increased resistance was detected in the treated vs. naive cultures after 24 h, this likely points toward limited stability of these compounds in aqueous solution.

Several marine and synthetic bis-indoles have been shown to inhibit enzymatic activity of purified PK of MRSA *in vitro* [17, 23-25]. PK catalyzes the final reaction step in glycolysis generating pyruvate by transfer of a phosphoryl group from phosphoenolpyruvate to ADP. Zoraghi *et al.* reported that PK is an essential gene in *S. aureus* and that its inhibition leads to an impaired metabolism by reduced ATP production [26]. The essentiality of PK makes the enzyme a promising potential new drug target. Due to their structural similarity, it was conceivable that hyrtinadine A and alocasin A derivatives also inhibit PK. This assumption was further supported by the fact that published bis-indoles followed a similar SAR trend with halogenated derivatives inhibiting growth stronger than a hydroxyl substitution at the indole rings [17]. Despite the likely interaction of the studied bis-indoles with PK however, our findings clearly show that PK can be excluded as the primary target of hyrtinadine A and alocasin A derivatives in *S. aureus* cells under the tested *in vitro* conditions. *S. aureus* cells displayed unaltered susceptibility under growth conditions where PK is fully dispensable for viability. Moreover, absence of PK in the MRSA LAC Δ*pyk*::Erm^R mutant did not result in any altered susceptibility whatsoever. Thus, PK can play only a minor role, if any, in explaining the strong bactericidal potency of our compounds. In consequence, the reported molecules corrupt one or more unrelated vital processes in the cell, which independently or in combination cause the pronounced antibacterial effect. This scenario does not preclude that the studied bis-indoles actually do bind to PK intracellularly, albeit inactivation does not influence cell viability. In fact, the slightly increased sensitivity of the MRSA LAC Δ*pyk*::Erm^R mutant suggests that in WT cells binding to PK partially obscures interaction of hyrtinadine A and alocasin A derivatives with one or more other relevant target(s) in *S. aureus*.

We performed whole genome sequencing of spontaneous resistant mutants to possibly gain insights into these molecular target(s). The fact that all sequenced resistant clones harbored SNPs in one common gene (SAV_RS01095) strongly suggests that this gene plays a

direct role in resistance. The gene SAV_RS01095 encodes the enzyme EIICBA of the phosphoenolpyruvate-dependent glucose phosphotransferase system (PTS). In general, PTS catalyzes the translocation of sugar substrates through the cell membrane coupled with their concomitant intracellular phosphorylation. Enzyme I (EI) and histidine-containing phosphor-carrier protein (HPr) are located in the cytosol and are involved in the transport of all PTS sugars such as glucose, mannose or fructose [27]. As a first step, the phosphoryl group of phosphoenolpyruvate (PEP) is transferred *via* EI to HPr on a specific histidyl residue. Next, the phosphoryl group is transferred to enzyme II which is a sugar-specific permease consisting of three domains: EIIA, EIIB and EIIC. EIIA and B are located in the cytoplasm and bind the phosphoryl group at a histidine (EIIA) or cysteine (EIIB) residue. Finally, the transmembrane domain EIIC catalyzes the phosphorylation of the sugar substrate and mediates its concomitant translocation across the cytoplasm membrane [27]. While the data suggest a specific role of SAV_RS01095 in resistance, it remains unclear whether the encoded enzyme EIICBA also represents the direct antibacterial molecular target. While this EIICBA is annotated as a component of a glucose-specific PTS, this has never been verified experimentally so that the actual substrate specificity, biological function and gene essentiality remain elusive.

In summary, here we report on the efficient synthesis of new derivatives of the natural compounds alocasin A and hyrtinadine A and their strong antibacterial effect especially against MRSA. However, further studies are necessary to decode the mode-of-action and molecular target(s) of these potent bactericidal substances.

Supporting Information

Analytical data of compounds **4-10**, **12-15** and **19** including ^{1}H and ^{13}C NMR-spectra (Figure 4-4 to 4-27); MIC of compounds against all tested bacteria strains (Table 4-5); dose-response curve of compound **13** against MRSA (Figure 4-28); resistance development in MRSA during time-kill kinetics of compounds **7** and **10** (Figure 4-29); dose-response curve **7**, **12**, **14**, **15** against MRSA LAC WT and LAC Δ*pyk*::Erm^{R} in minimal medium M9 (Figure 4-30).

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Author contributions

G.A.S. synthesized compounds **4 – 10**, **12 -16**, **19**. N.R. conducted all experiments involving MRSA and other bacteria. N.P. performed molecular docking studies. T.R.I. performed and analyzed whole genome sequencing. R.K., H.G. and T.J.J.M. designed experiments and analyzed data. N.R. and R.K. wrote the manuscript with contributions and edits from all authors.

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4.2 Supporting Information

Supplementary Data

Hyrtinadine A and Alocasin A Derivatives as New Lead Structures for APIs Against Methicillin-resistant *Staphylococcus aureus* (MRSA)

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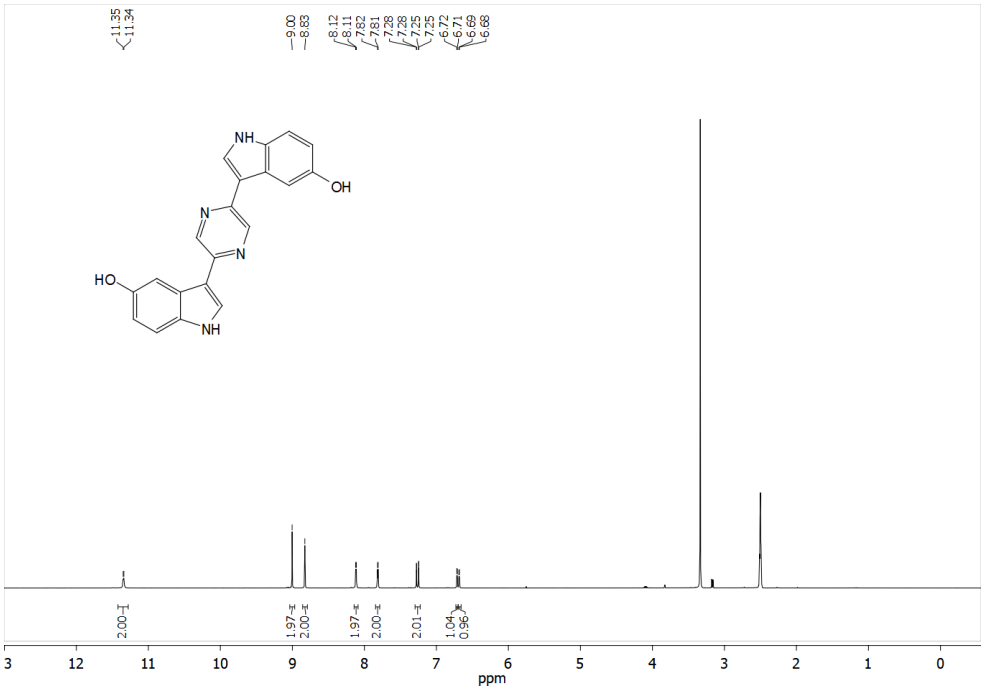

Figure 4-4 ^{1}H NMR of **4** in DMSO-d$_6$ at 296 K (δ in ppm).

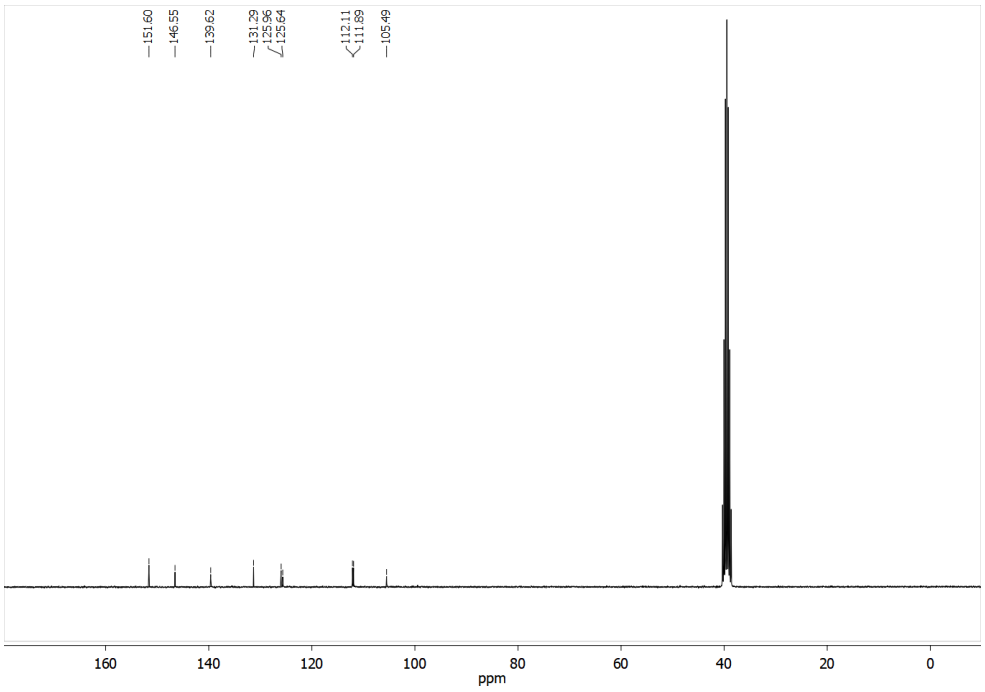

Figure 4-5 ^{13}C NMR of **4** in DMSO-d$_6$ at 295 K (δ in ppm).

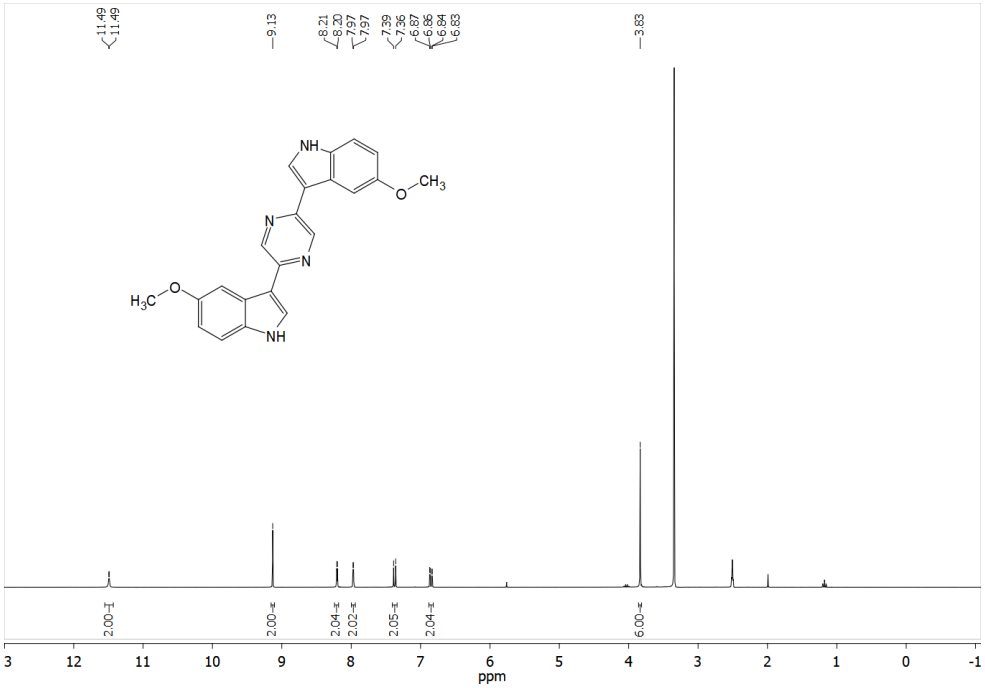

Figure 4-6 ^{1}H NMR of **5** in DMSO-d$_6$ at 296 K (δ in ppm).

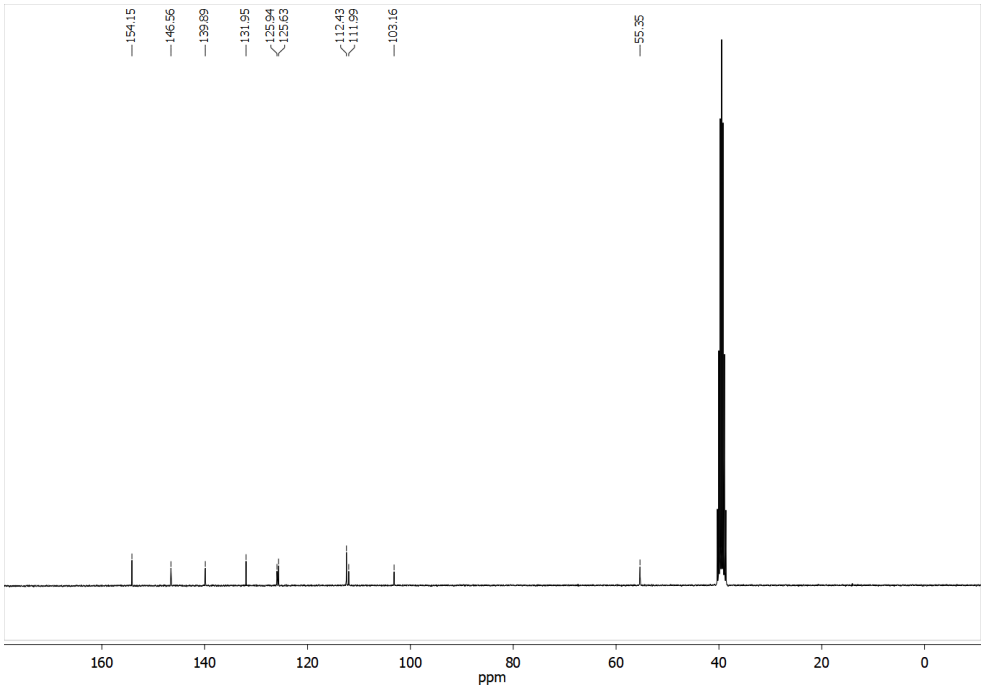

Figure 4-7 ^{13}C NMR of **5** in DMSO-d$_6$ at 295 K (δ in ppm).

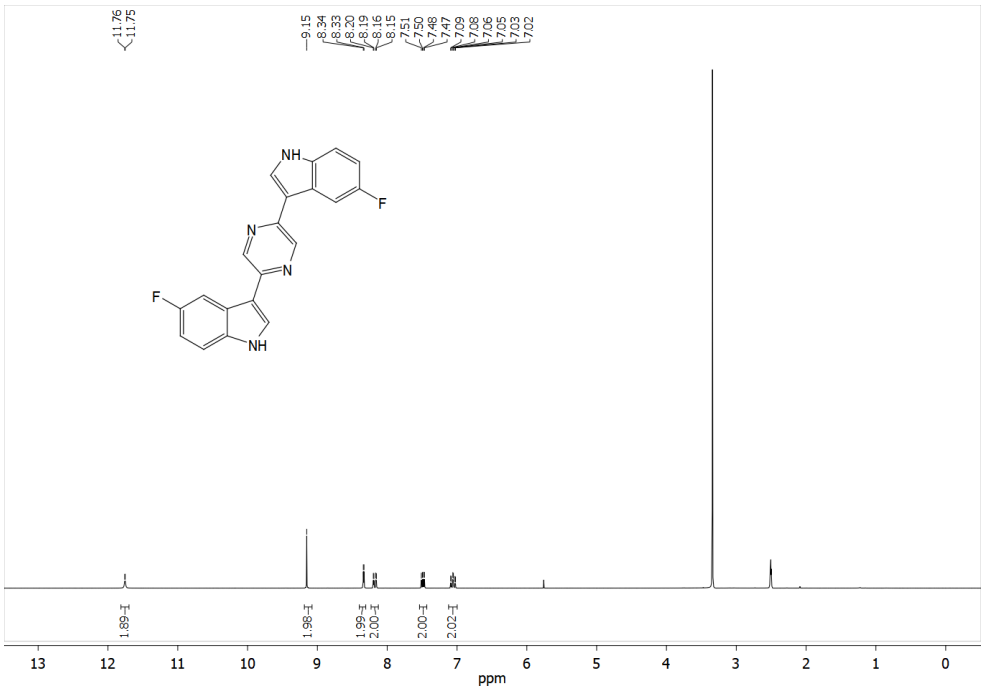

Figure 4-8 ^{1}H NMR of **6** in DMSO-d_6 at 296 K (δ in ppm).

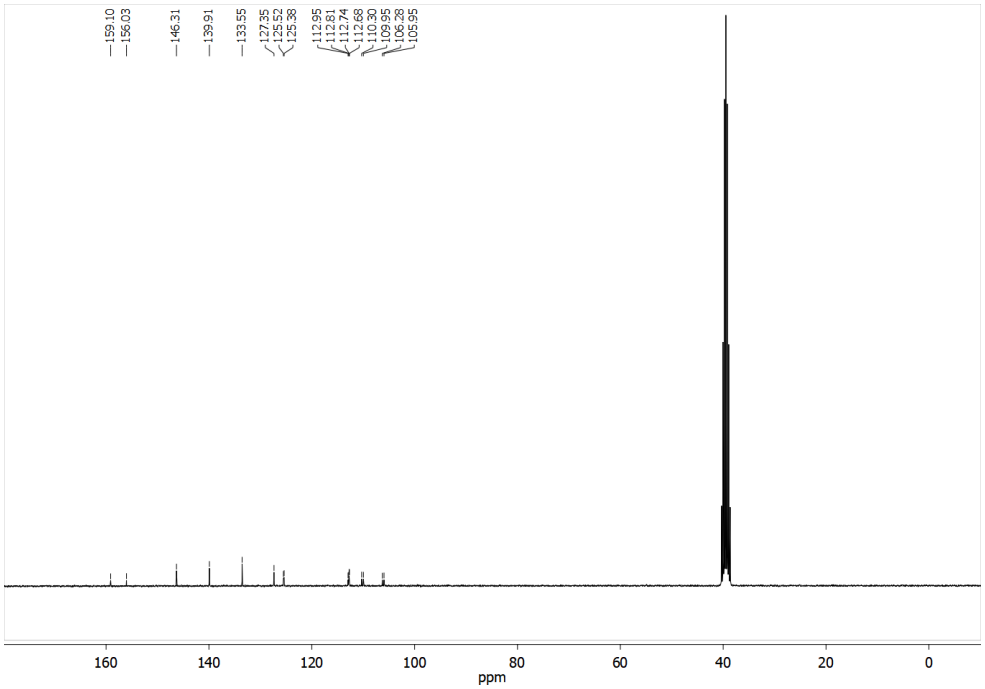

Figure 4-9 ^{13}C NMR of **6** in DMSO-d_6 at 295 K (δ in ppm).

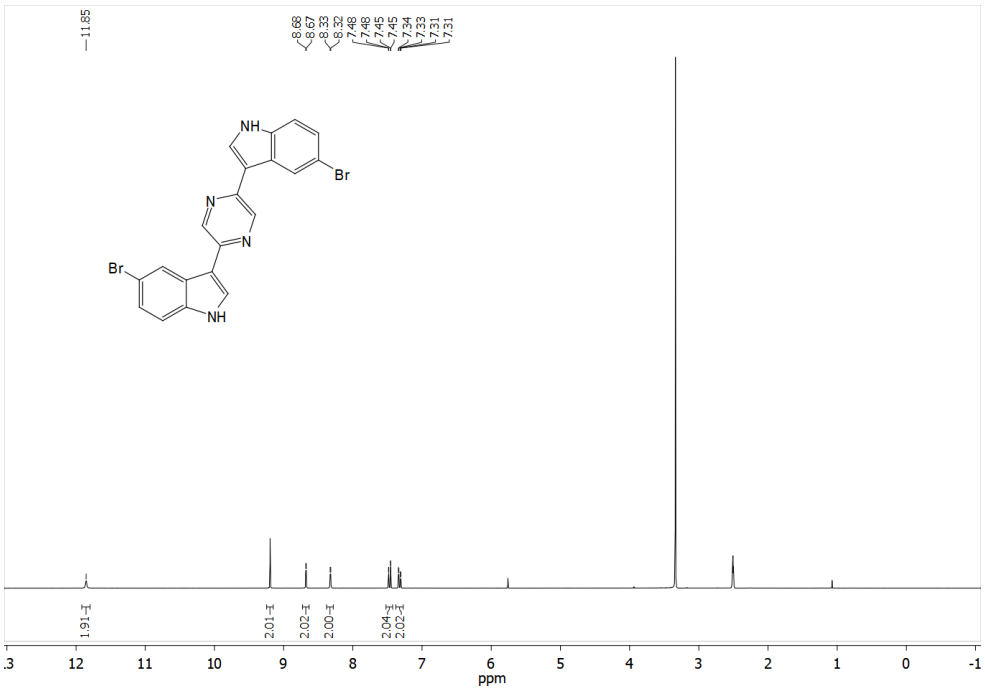

Figure 4-10 ^{1}H NMR of **7** in DMSO-d_6 at 296 K (δ in ppm).

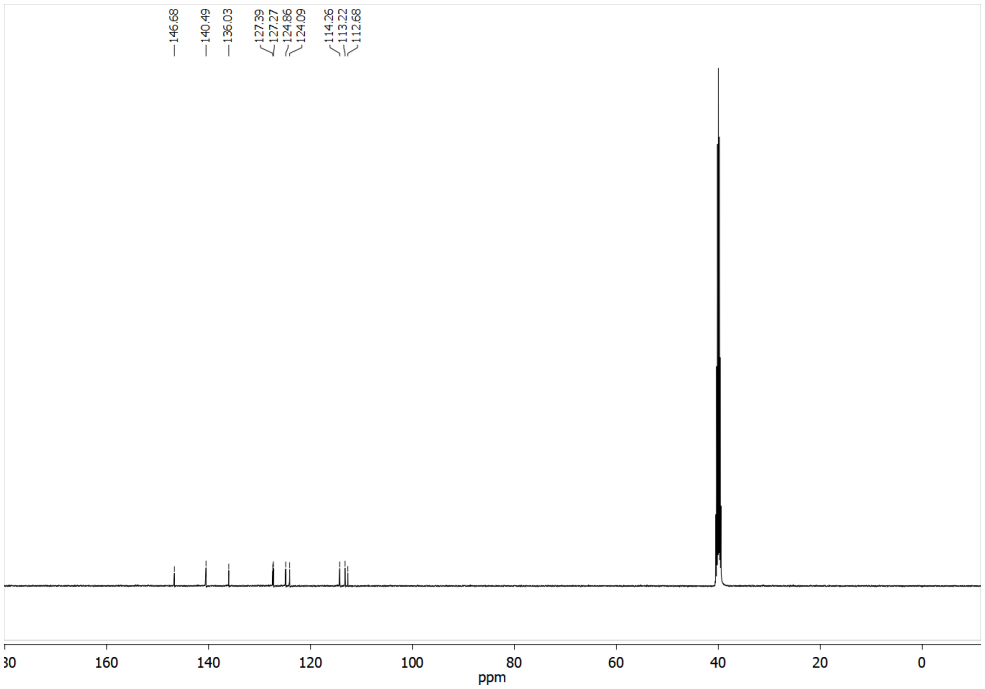

Figure 4-11 ^{13}C NMR of **7** in DMSO-d_6 at 295 K (δ in ppm).

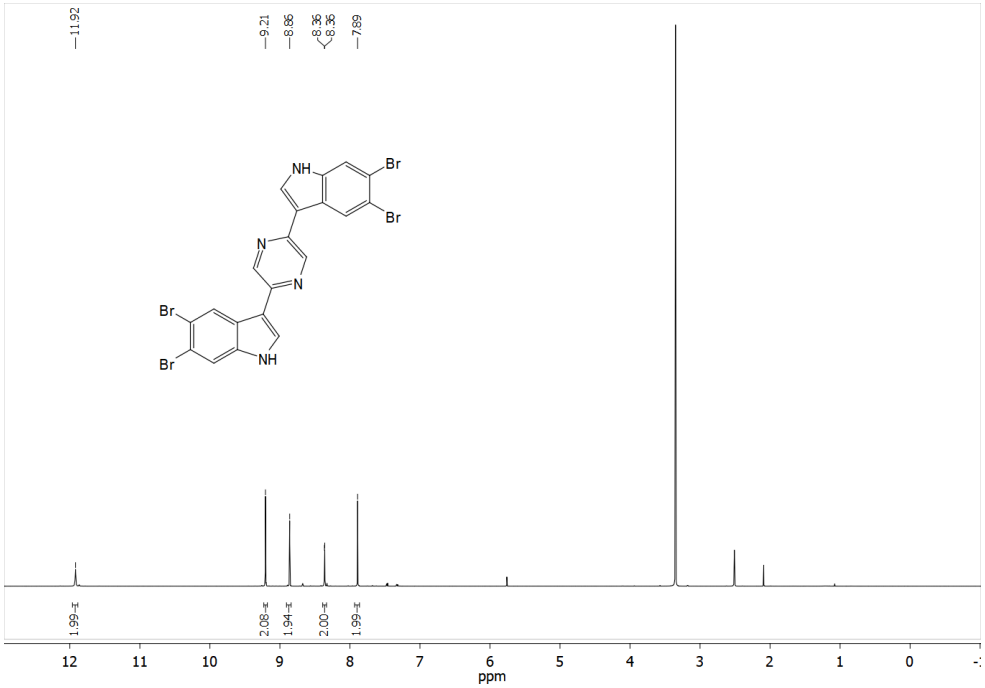

Figure 4-12 ^{1}H NMR of **8** in DMSO-d_6 at 296 K (δ in ppm).

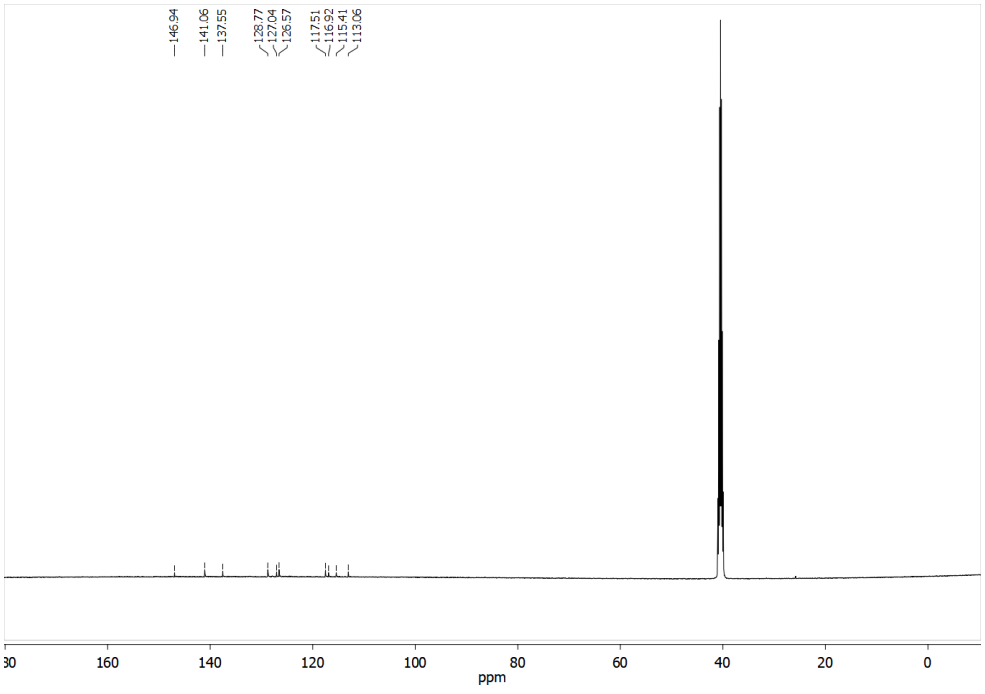

Figure 4-13 ^{13}C NMR of **8** in DMSO-d_6 at 295 K (δ in ppm).

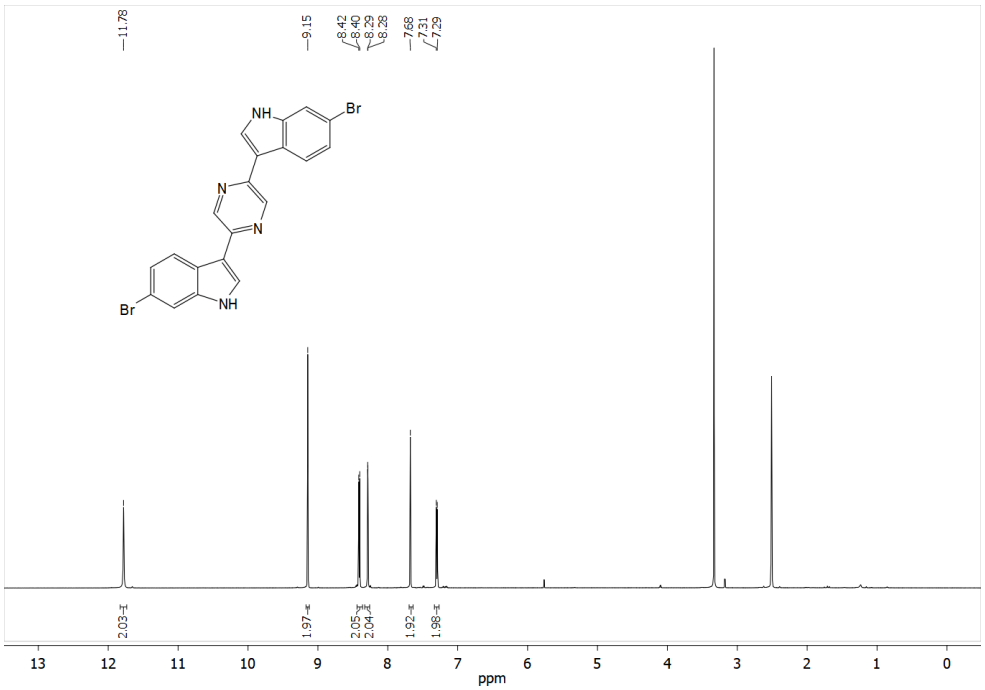

Figure 4-14 ^{1}H NMR of **9** in DMSO-d_6 at 296 K (δ in ppm).

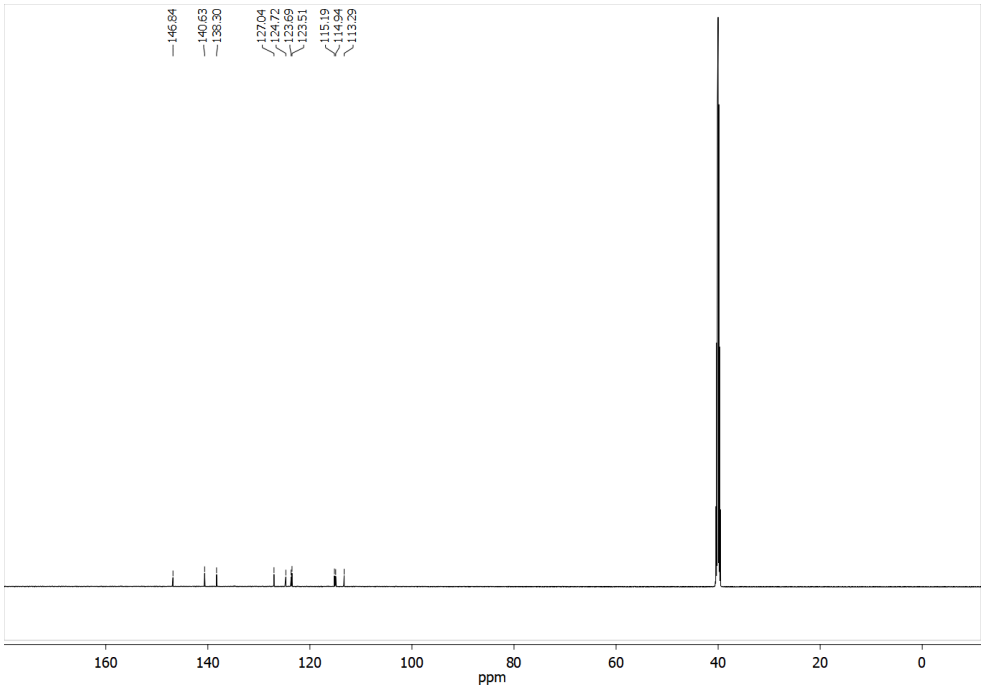

Figure 4-15 ^{13}C NMR of **9** in DMSO-d_6 at 295 K (δ in ppm).

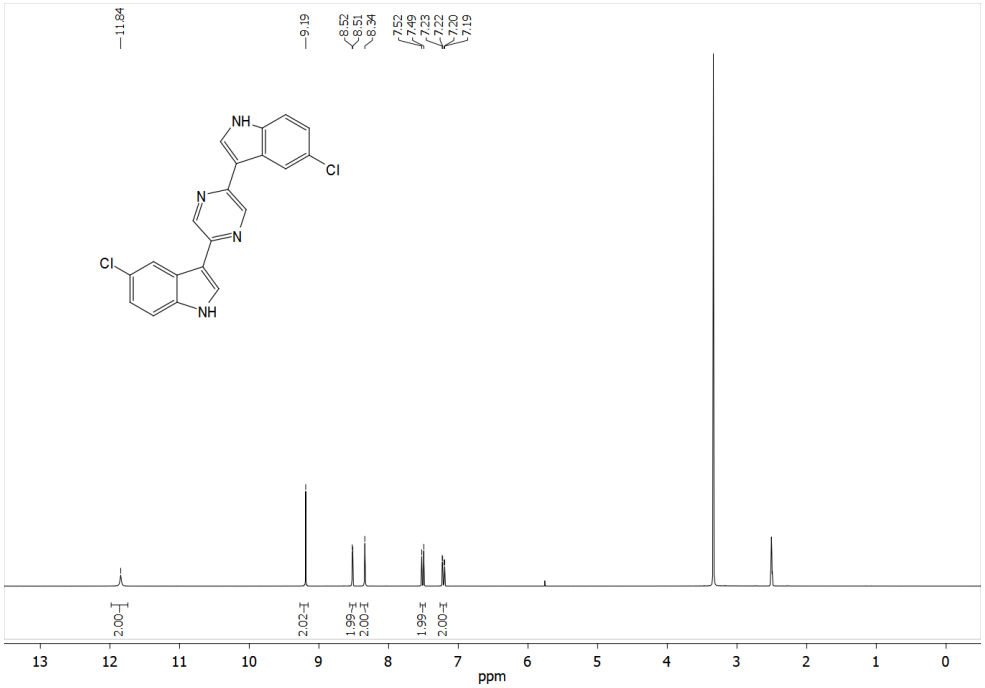

Figure 4-16 ^{1}H NMR of **10** in DMSO-d_6 at 296 K (δ in ppm).

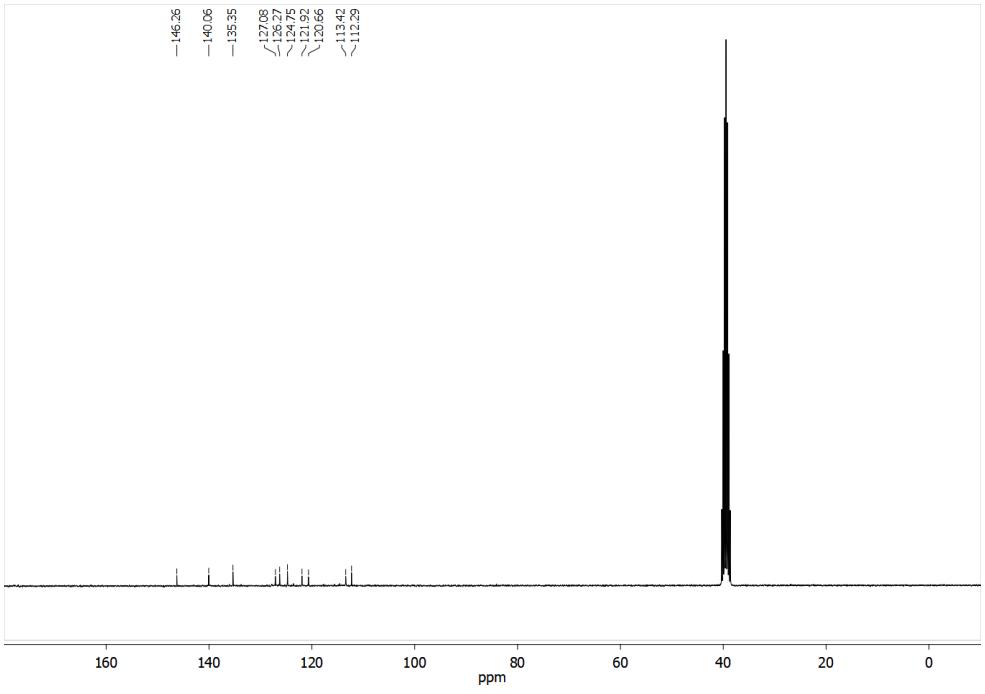

Figure 4-17 ^{13}C NMR of **10** in DMSO-d_6 at 295 K (δ in ppm).

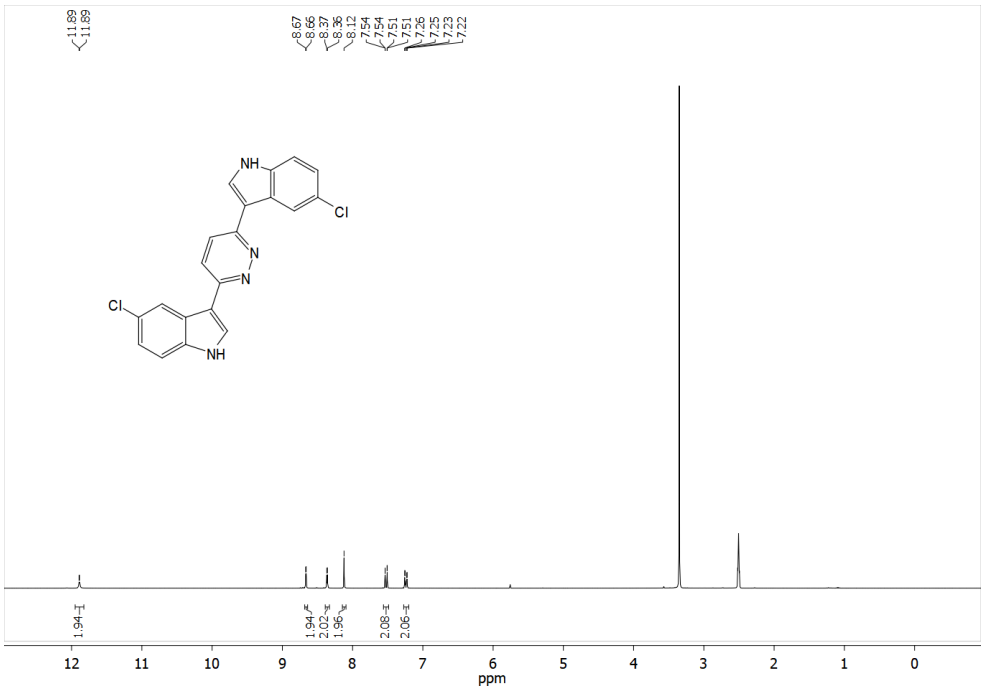

Figure 4-18 ^{1}H NMR of **12** in DMSO-d_6 at 296 K (δ in ppm).

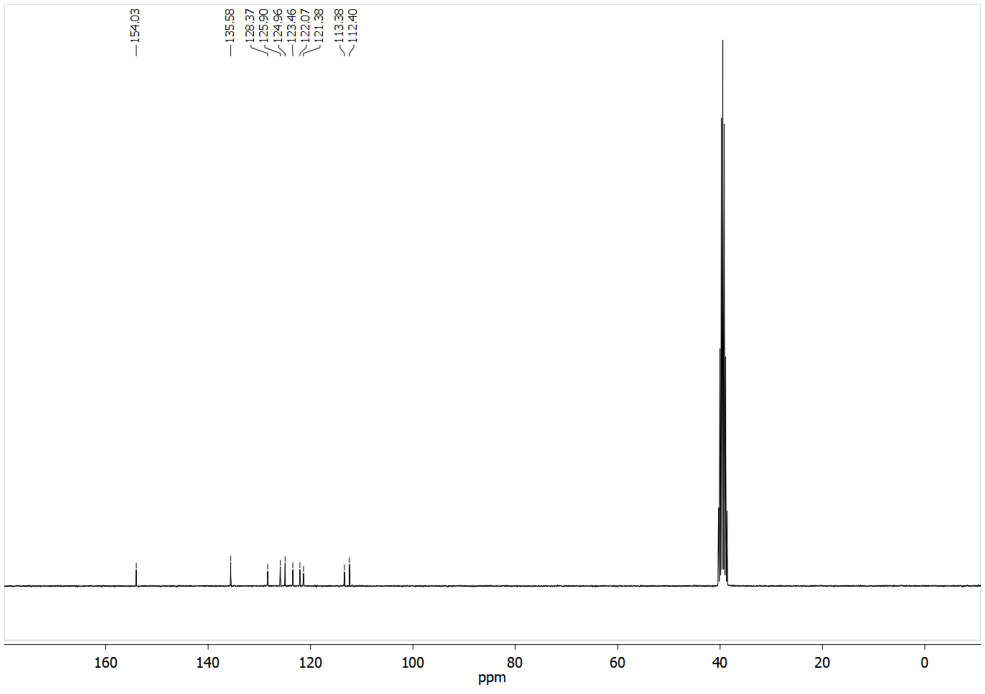

Figure 4-19 ^{13}C NMR of **12** in DMSO-d_6 at 295 K (δ in ppm).

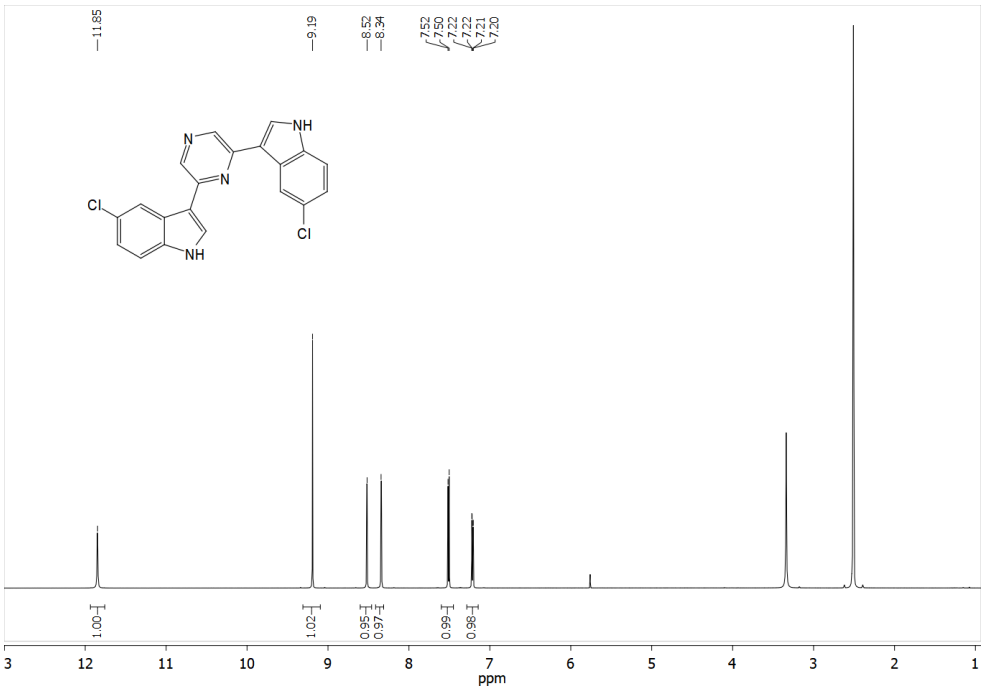

Figure 4-20 ^{1}H NMR of **13** in DMSO-d_6 at 296 K (δ in ppm).

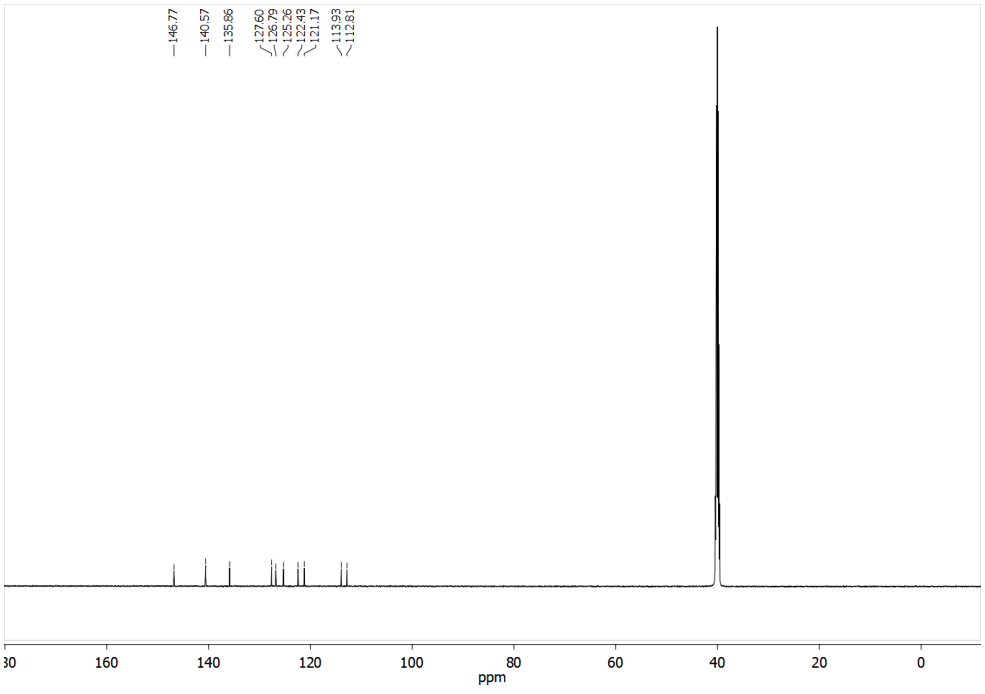

Figure 4-21 ^{13}C NMR of **13** in DMSO-d_6 at 295 K (δ in ppm).

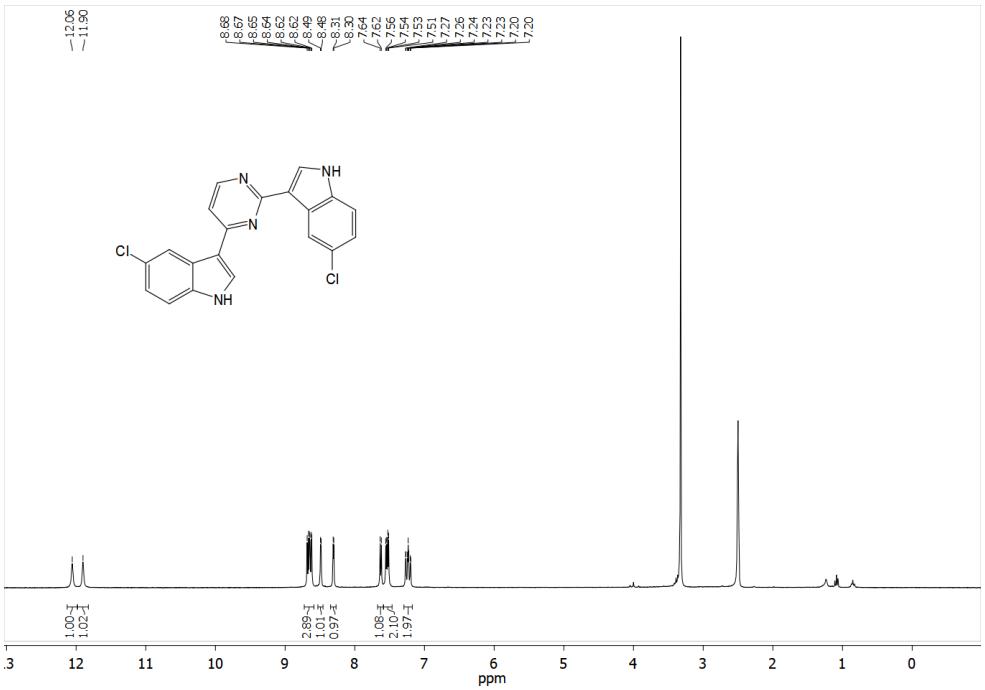

Figure 4-22 ^{1}H NMR of **14** in DMSO-d_6 at 296 K (δ in ppm).

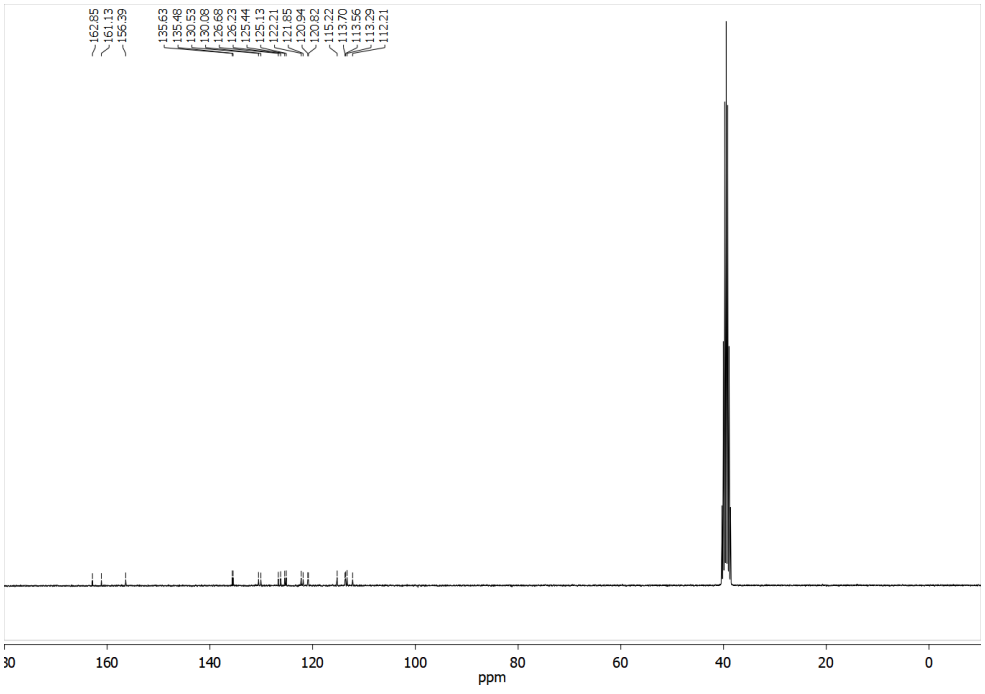

Figure 4-23 ^{13}C NMR of **14** in DMSO-d_6 at 295 K (δ in ppm).

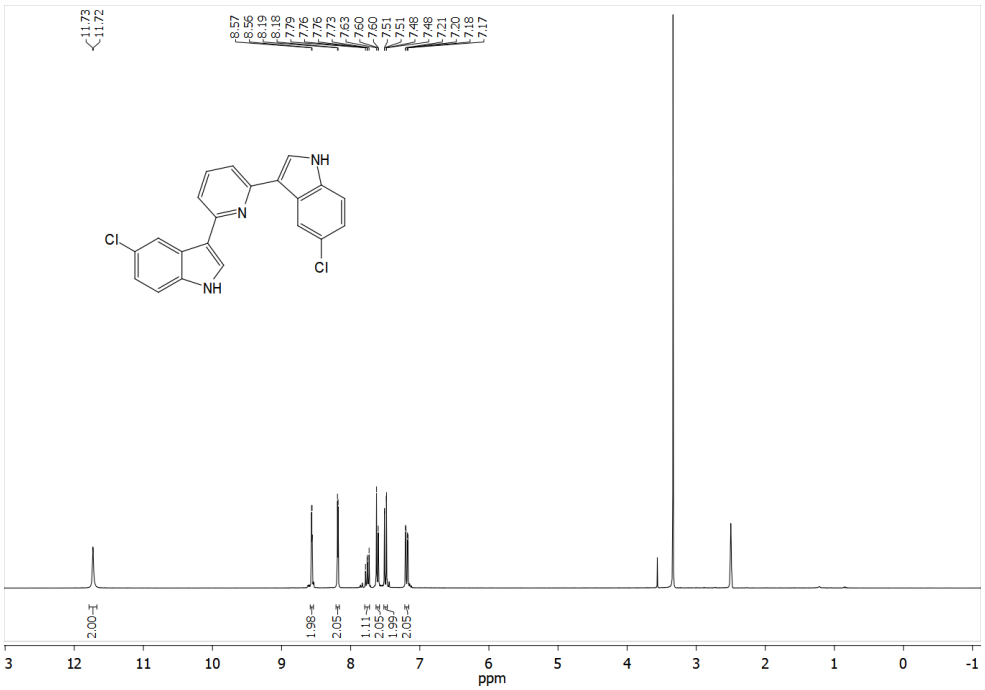

Figure 4-24 ^{1}H NMR of **15** in DMSO-d_6 at 296 K (δ in ppm).

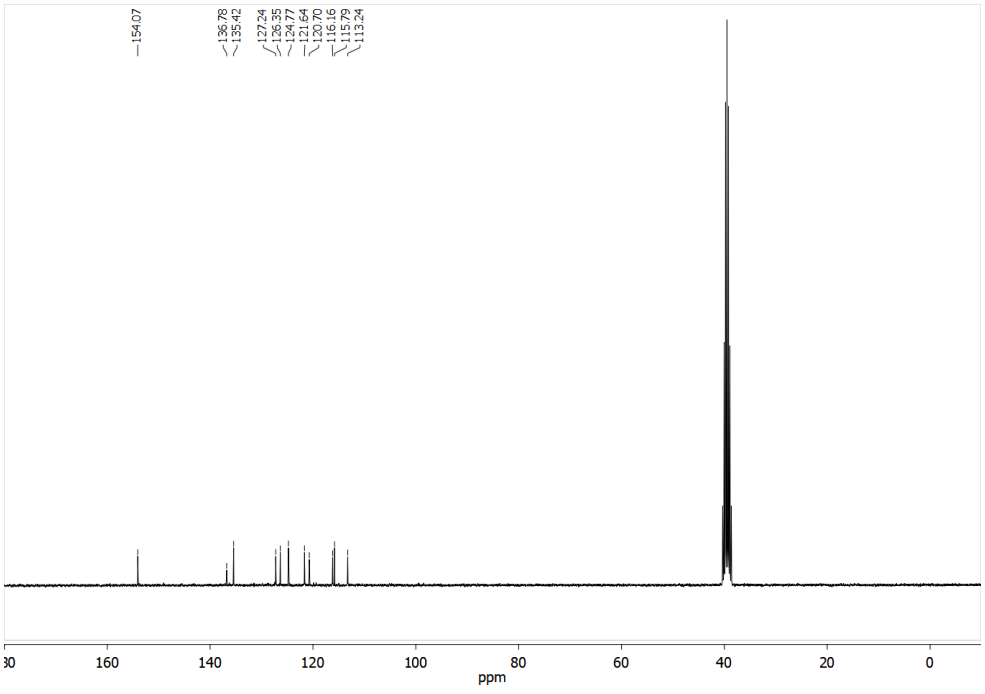

Figure 4-25 ^{13}C NMR of **15** in DMSO-d_6 at 295 K (δ in ppm).

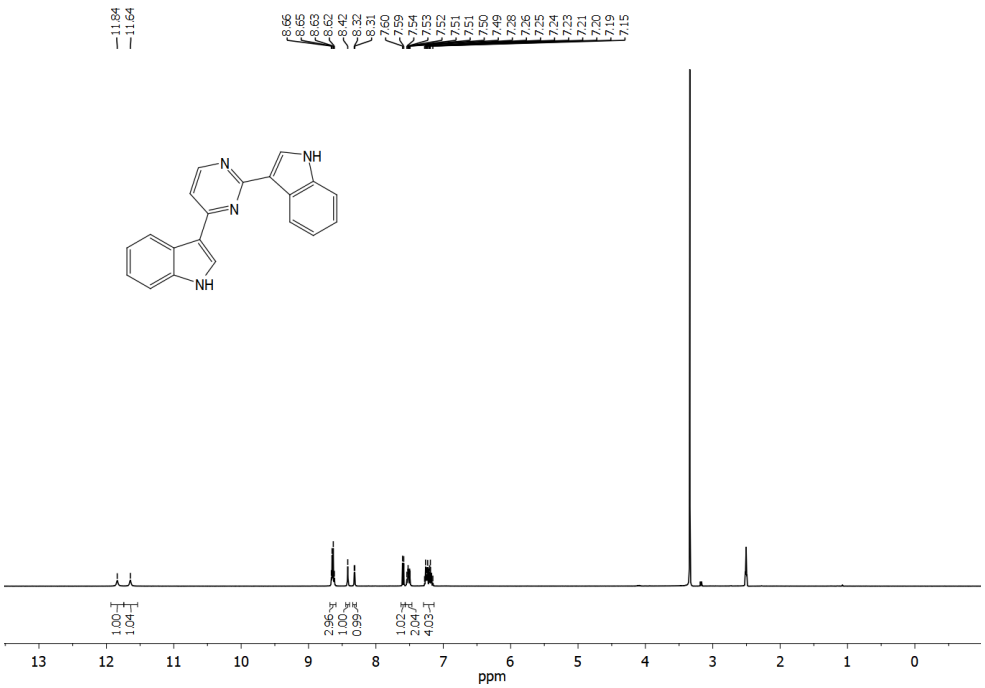

Figure 4-26 ^{1}H NMR of **19** in DMSO-d_6 at 296 K (δ in ppm).

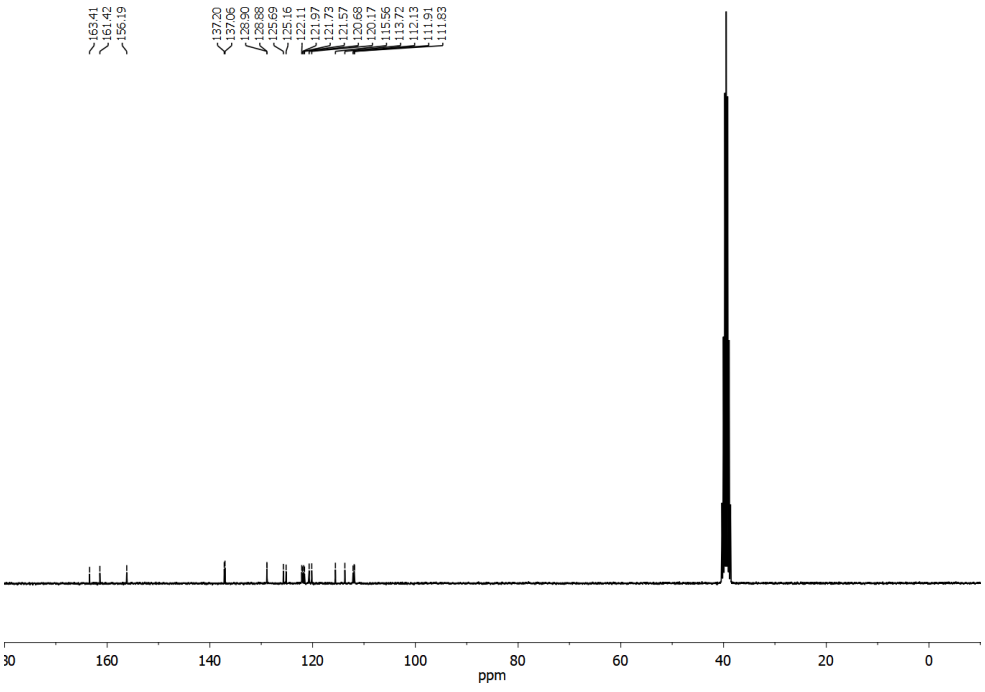

Figure 4-27 ^{13}C NMR of **19** in DMSO-d_6 at 295 K (δ in ppm).

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Table 4-5 Minimal inhibitory concentration (MIC) of all synthesized compounds against *Mycobacterium tuberculosis* (*M. tb*., H37Rv*), Staphylococcus aureus* (*S. aureus*, ATCC 25923), Methicillin-resistant *S. aureus* (MRSA, ATCC 700699), *Enterococcus faecalis* (*E. faecalis*, ATCC 29212 and 51299), *Enterococcus faecium* (*E. faecium*, ATCC 35667) and Vancomycin-*resistant E. faecium* (VRE, ATCC 700221), *Acinetobacter baumannii* (*A. baumannii*, ATCC BAA-1605). Compounds were tested against *A. baumannii* in single and combination treatment with sub lethal doses of colistin (COL, 0.31 µM) or rifampicin (RIF, 2.5 µM)

	MIC (µM)									
	M. tb.	*S. aureus*		*E. faecalis*		*E. faecium*		*A. baumannii*		
No.	H37Rv	ATCC 25923	ATCC 700699	ATCC 29212	ATCC 51299	ATCC 35667	ATCC 700221	ATCC BAA-1605	ATCC BAA-1605, COL	ATCC BAA-1605, RIF
1	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
2	50	> 100	50	50	100	100	50	> 100	> 100	> 100
3	> 100	100	12.5	100	100	50	50	> 100	> 100	> 100
4	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
5	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
6	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
7	> 100	1.56	1.56	> 100	> 100	> 100	> 100	> 100	> 100	> 100
8	> 100	100	25	100	> 100	50	50	> 100	> 100	> 100
9	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
10	> 100	> 100	0.39	> 100	> 100	> 100	> 100	> 100	> 100	> 100
11	50	6.25	0.39	3.13	12.5	1.56	1.56	> 100	> 100	> 100
12	100	6.25	0.78	0.20	> 100	> 100	> 100	> 100	> 100	> 100
13	> 100	> 100	0.39	> 100	> 100	> 100	> 100	> 100	> 100	> 100
14	> 100*	3.13	0.39	3.13	3.13	1.56	1.56	> 100	> 100	> 100
15	> 100*	3.13	0.20	12.5	6.25	6.25	1.56	> 100	> 100	> 100
16	> 100	> 100	> 100	12.5	25	> 100	> 100	> 100	> 100	> 100
17	50	100	12.5	50	100	100	100	> 100	> 100	> 100
18	> 100	> 100	> 100	100	100	> 100	> 100	> 100	> 100	> 100
19	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
20	> 100	> 100	> 100	100	100	> 100	> 100	> 100	> 100	> 100

* 100 µM compound inhibited growth of *M. tb*. down to appr. 25

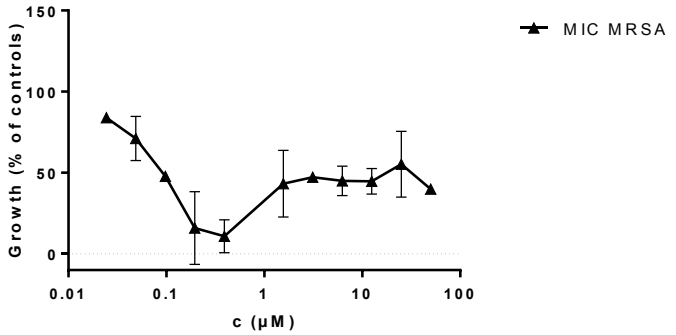

Figure 4-28 Dose-response curve of compound **13** against MRSA. Data are means of duplicates ± SD. Growth was quantified using BacTiterGlo assay.

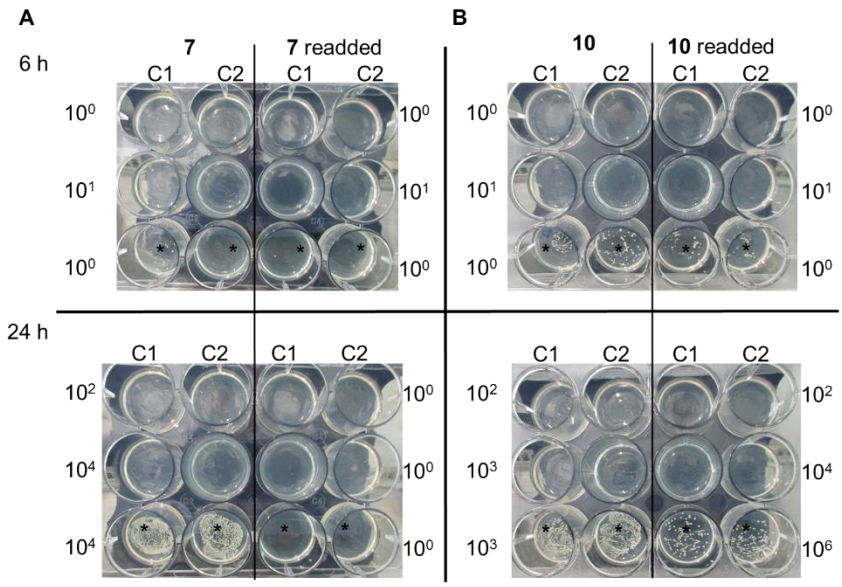

Figure 4-29 Resistance development in Methicillin-resistant *S. aureus* (MRSA; ATCC 700699) during time-kill kinetics of compounds **7** (**A**) and **10** (**B**). Two independent cultures (C1 and C2) were tested for each attempt. Compounds were tested at 5x MIC (resulting in 0.02-0.08% DMSO final concentration). In an independent experiment, medium was replaced and compounds were freshly added after 6 h ("**7** readded" or "**10** readded"; see also Figure 2). 20 µL of different dilution of the cultures after either 6 or 24 h of incubation were plated out on MH agar containing either no antibiotics (*) or compounds **7** (**A**) and **10** (**B**) at 5x MIC, respectively.

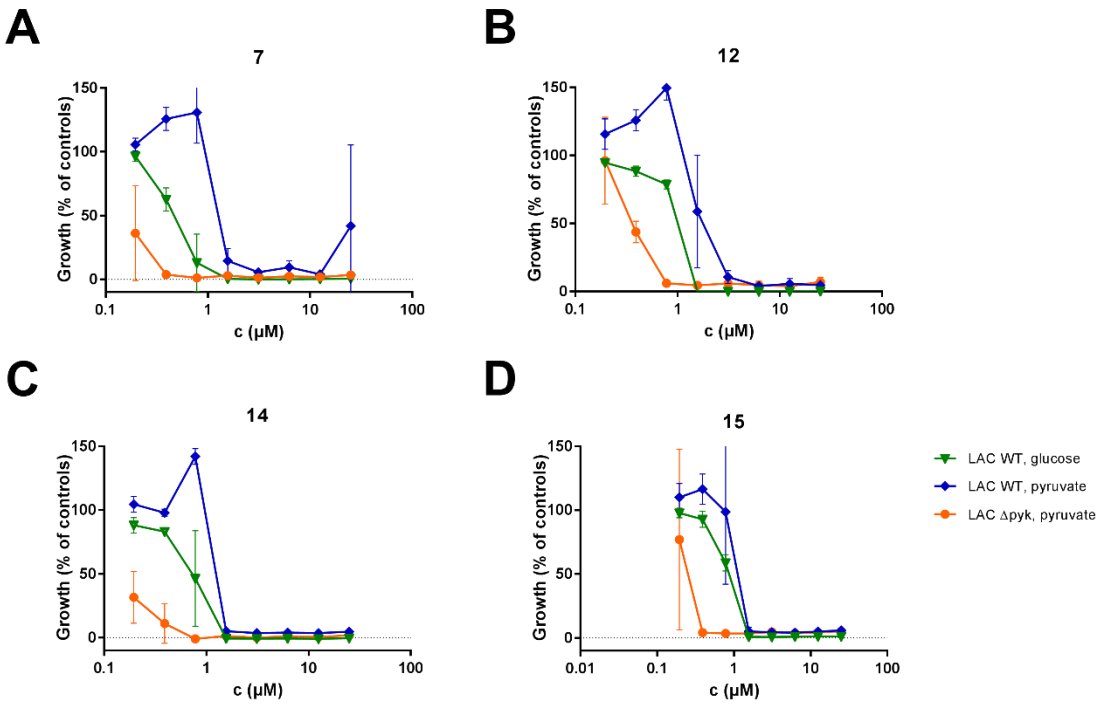

Figure 4-30 Antibacterial activity of **7**, **12**, **14**, **15** against MRSA LAC WT and LAC Δ*pyk*::Erm^R in minimal medium M9 with glucose (▼) or pyruvate (●, ♦) as single carbon source. Data are means of duplicates ± SD. Growth was quantified using resazurin assay.

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5 3-*O*-Methylalkylgallates Inhibit Fatty Acid Desaturation in *Mycobacterium tuberculosis*

Unpublished

The overall contribution to the paper: 50 %.

Contribution:

- Cultivation of bacterial strains
- Determination of minimal inhibitory concentration against *M. tb.* via resazurin dye reduction method
- Determination of minimal inhibitory concentration against nosocomial strains
- Determination of cytotoxicity and therapeutic index
- Determination of time-kill curves *in vitro*
- Checkerboard synergy assay
- Determination of single step resistance frequency

5.1 Manuscript

3-*O*-Methylalkylgallates Inhibit Fatty Acid Desaturation in *Mycobacterium tuberculosis*

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Abstract

In the quest for new antibacterial lead structures, activity screening against *Mycobacterium tuberculosis* identified antitubercular effects of gallic acid derivatives isolated from the Nigerian mistletoe *Loranthus micranthus*. Structure activity relationship studies indicated that 3-*O*-methylalkylgallates comprising aliphatic ester chains with four to eight carbon atoms result in strongest growth inhibition *in vitro* against *M. tuberculosis* with a minimal inhibitory concentration of 6.25 μM. Furthermore, the most active compounds (3-*O*-methylbutyl-, 3-*O*-methylhexyl-, 3-*O*-methyloctylgallate) were devoid of cytotoxicity against the human cell lines MRC-5 and THP-1. Whole genome sequencing of spontaneous resistant mutants indicated that the compounds target the stearoyl-CoA delta-9 desaturase DesA3 and thereby inhibit oleic acid synthesis. Supplementation assays demonstrated that oleic acid addition to the culture medium antagonizes the inhibitory properties of the gallic acid derivatives, while sodium salts of saturated palmitic and stearic acid did not show compensatory effects. The moderate bactericidal effect of 3-*O*-methylbutylgallate in monotreatment was synergistically enhanced in combination treatment with isoniazid leading to sterilization in liquid culture.

Introduction

Tuberculosis (TB), caused by *M. tb.*, is the leading cause of death by an infectious disease and even outcompetes the human immune deficiency virus (HIV) in this respect [1]. 10.4 million people were newly infected by *M. tb.* in 2016, and worldwide 1.7 million patients died of TB. The main endemic areas are located in Sub-Sahara Africa and Southeast Asia. Frequent HIV co-infection, poverty and a drug development which has been neglected for decades complicate the containment of the disease. For more than fifty years now, the standard therapy of TB consists of a six-month drug regimen of the four drugs isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA). Although combination treatment reduces the likelihood of resistance development, multi- and even pan-resistant strains have evolved over the years and are seriously complicating control of the TB pandemic [2]. Multidrug resistant (MDR) strains, which are resistant to RIF and INH, and extensively drug resistant (XDR) strains, which are additionally resistant to any fluoroquinolone and to one of the three injectable drugs capreomycin, amikacin or kanamycin, are a great burden for the healthcare sector due to a prolonged therapy duration for 10-24 months, thereby tremendously rising costs. In recent years, two new TB drugs, bedaquiline (BDQ) and delamanid, were conditionally approved for treatment of MDR TB infections, but still undergo phase III clinical evaluations [3, 4]. Nevertheless, new lead structures are still urgently needed since resistances against BDQ and delamanid have already been reported [5, 6]. Since the discovery of penicillin [7], antibacterial compounds have been searched for from natural sources such as plants and marine or soil-dwelling microorganisms, among many others. Until today, approx. 70 % of all available antibiotics are derived from nature [8]. The variety of secondary metabolites protecting plants from microbial enemies seems to be innumerous. The semi parasitic Nigerian mistletoe *Loranthus micranthus* is used in African traditional medicine for various indications [9-11]. Also, antibacterial activity of extracts from this plant has been reported [12-15]. Therefore, in this study, we investigated the antitubercular properties of compounds isolated from *Loranthus micranthus* and characterized the anti-TB effect of identified natural alkylgallates and synthetic derivatives thereof.

Results and Discussion

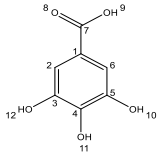
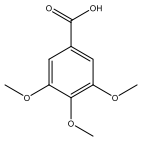
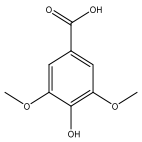
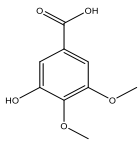
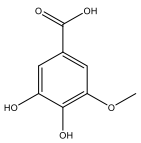
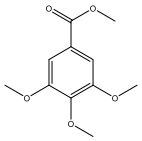
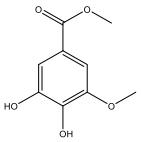
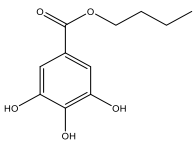
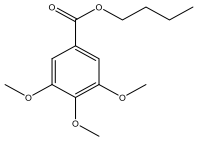
Antitubercular activity and structure-activity relationships of gallic acid derivatives. In our screening efforts, the gallic acid derivatives **1**-**5**, **8** and **10** isolated from the *n*-butanol extract of *Loranthus micranthus* (detailed isolation described elsewhere) were identified to exhibit antibacterial whole-cell activity against *M. tb.*. Based on commercially available gallic acid derivatives as precursors, a variety of structural variants (compound **6**, **7**, **9**, **11**-**19**) were synthesized and tested for antibacterial activity against *M. tb.* and nosocomial pathogens including *S. aureus*, *E. faecalis*, *E. faecium.* and *A. baumannii*. The antibacterial activity screening revealed that compounds **10**, **11** and **12** were most active against *M. tb.* with a MIC of 6.25 µM. SAR showed that compounds with a free carboxyl moiety (**1**-**5**) did not or only slightly inhibit the growth *M. tb.* (Table 5-1). Esterification of the carboxyl group was crucial for antitubercular activity. Increasing of apolarity by elongation of the aliphatic side chain from methyl- (**7**) to propyl- (**14**) to butyl- (**10**) to octylgallate (**12**) resulted in augmented anti-TB potency. The butylgallate derivatives **8**, **9** and **10** further demonstrated that methoxylation of the hydroxyl group at position 3 further substantially increased anti-tubercular potency, while additional methoxylation of the hydroxyl groups at position 4 and 5 was detrimental for antibacterial activity. Derivatives with sterically enlarged side chains such as 3-*O*-Methyl-cyclohexyl- (**13**), 3-*O*-Methylisopropyl- (**15**) or 3-*O*-Methylisobutyl-gallate (**16**) did not show any effect in the tested concentration range, so that a linear aliphatic chain seems to be essential for activity.

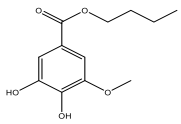
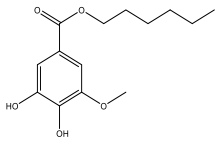
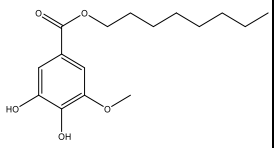
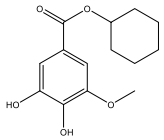
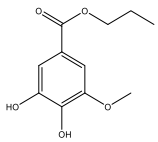
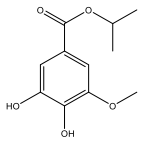
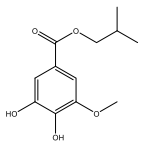
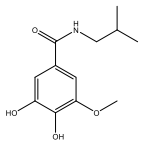
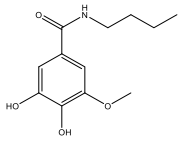
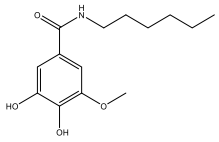
Replacement of ester-linked alkyl chains with amide derivatives (**17**, **18**, **19**) decreased the MIC, suggesting that the enlarged delocalized π-electron system in the amide derivatives reduced interaction with the target. The common characteristics of the most active compounds comprise a methoxy group in *meta* position and an ester-linked aliphatic alkyl side chain consisting of four to eight carbon atoms.

Because previous studies already reported on the antibacterial activity of some other alkylgallates against *Bacillus subtilis* (*B. subtilis*), *S. aureus* and the Gram-negative bacterium *Xanthomonas citri* (*X. citri*) [16-19], we also checked the antibacterial effect of our compounds against several nosocomial strains. Our observations are in agreement with the literature in that the optimal length of the alkyl side chain ranges from ca. four to eight carbons. However, interestingly, only compound **12** inhibited the growth of sensitive and resistant species of *S. aureus* and *E. faecium*, although the inhibitory effect was weak with MIC ranging from 25 to 100 µM (Table 5-3). Therefore, 3-*O*-methylalkylgallates display

relative specific antitubercular activity. In addition, *in vitro* cytotoxicity studies revealed that gallic acid derivatives were not or only moderate cytotoxic towards the human cell lines THP-1 and MRC-5, yielding a selectivity index (IC_{50}/MIC_{90}) of ≥16 for compound **10** (Table 5-1).

Table 5-1 Structure of gallic acid derivatives, their antibacterial activity against *M. tuberculosis* (*M. tb.*, H37Rv) and their cytotoxicity against the human cell lines THP-1 and MRC-5. Antibacterial activity is shown as the concentration reducing bacterial growth by 90 % relative to controls (MIC_{90}). Cytotoxicity is displayed as the concentrations reducing growth of human cells by 50 % or 90 %, respectively, relative to controls (IC_{50}, IC_{90}).

Compound			c (µM)				
			M. tb.	THP-1		MRC-5	
			MIC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
1	Gallic acid		100	100	>100	100	>100
2	Eudesmic acid		>100	>100	>100	>100	>100
3	3,5-*O*-Dimethyl-gallic acid		>100	100	>100	>100	>100
4	3,4-*O*-Dimethyl-gallic acid		>100	>100	>100	>100	>100
5	3-*O*-Methyl-gallic acid		100	50	100	>100	>100
6	Trimethyl-methyl-gallate		>100	>100	>100	>100	>100
7	3-*O*-Methyl-methyl-gallate		>100	100	>100	>100	>100
8	Butyl-gallate		25	50	>100	>100	>100
9	Trimethyl-butylgallate		>100	>100	>100	>100	>100

10	3-*O*-Methyl-butylgallate		6.25	100	100	>100	>100
11	3-*O*-Methyl-hexylgallate		6.25	50	100	>100	>100
12	3-*O*-Methyl-octylgallate		6.25	50	100	>100	>100
13	3-*O*-Methyl-cyclohexyl-gallate		>100	100	>100	>100	>100
14	3-*O*-Methyl-propylgallate		50	100	>100	>100	>100
15	3-*O*-Methyl-isopropyl-gallate		>100	>100	>100	>100	>100
16	3-*O*-Methyl-isobutyl-gallate		50	25	>100	100	>100
17	*N*-2-methyl-propyl-3,4-dihydroxy-5-methoxy-benzamide		100	100	>100	>100	>100
18	*N*-butyl-3,4-dihydroxy-5-methoxy-benzamide		>100	25	>100	100	>100
19	*N*-hexyl-3,4-dihydroxy-5-methoxy-benzamide		50	6.25	50	50	100

In vitro killing kinetics. In order to further characterize the antitubercular effect of 3-*O*-methylbutylgallate (**10**), a killing kinetic was performed. 3-*O*-methylbutylgallate in monotherapy showed a moderate bactericidal effect with ~ 99% reduction of viability after 10 days (Figure 5-1 A). After this time point, however, bacterial growth resumed. Individual clones isolated after three weeks of incubation were still fully sensitive towards the compound. This particularly contrasts with the tested first line drug INH which exhibited strong bactericidal killing effect after four days but resulted in outgrowth of resistant mutants after three weeks (Figure 5-1 E). This indicated that regrowth in the 3-*O*-methylbutylgallate treated culture was rather caused by compound depletion or degradation and not by development of resistances. Because TB chemotherapy relies on a combination treatment including several drugs, the effect of combining 3-*O*-methylbutylgallate with the first line drugs EMB, RIF and INH or the new conditionally approved second-line agents BDQ and delamanid were investigated (Figure 5-1 B-F). In combination with EMB or BDQ, 3-*O*-methylbutylgallate caused an additive effect and delayed or prevented the outgrowth of mutants that was observable in monotherapy with EMB or BDQ, respectively (Figure 5-1 B and D). The combination of RIF, INH or delamanid with 3-*O*-methylbutylgallate, respectively, caused a synergistic killing effect (Figure 5-1 C, E, F). In case of delamanid, the combination first reduced the number of bacteria down to the detection limit of 10^1 CFU/mL. However, after three weeks bacterial regrowth was observable again (Figure 5-1 F). Interestingly, these clones exhibited resistance only against delamanid but not 3-*O*-methylbutylgallate, indicating that repeated addition of 3-*O*-methylbutylgallate to the culture might be able to prolong and pronounce this synergistic killing effect.

INH combined with 3-*O*-methylbutylgallate revealed a strong synergistic killing effect leading to complete sterilization of the culture after one week (Figure 5-1 E). This interaction was also investigated in a checkerboard assay by determination of the fractional inhibitory concentration indices (FICI) for combination of 3-*O*-methylbutylgallate with RIF, delamanid or INH, respectively. The presence of RIF at 0.25×MIC (0.078 µM) revealed a partial synergistic effect (FICI = 0.562), resulting in increased sensitivity towards 3-*O*-methylbutylgallate (16-fold reduction in MIC). In contrast, combination with delamanid (0.146 µM) or INH (0.156 µM) each at 0.25×MIC, respectively, resulted in synergism (FICI < 0.5) and strongly increased sensitivity towards 3-*O*-methylbutylgallate (Table 5-4).

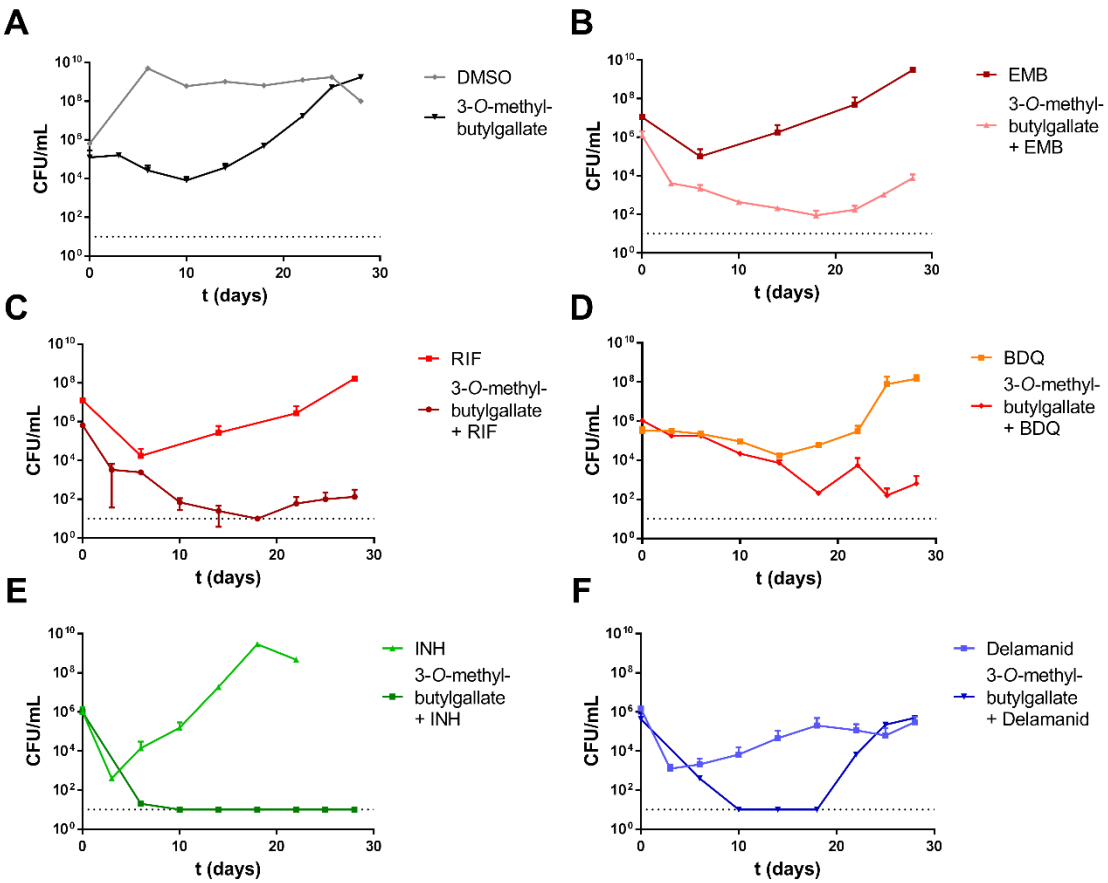


Figure 5-1 Time-killing curves of *M. tuberculosis* H37Rv in 3-*O*-methylbutylgallate- drug combination treatments. (**A**) 31.25 µM 3-*O*-methylbutylgallate (▼) and solvent control 3.1% DMSO (●); (**B**) 10 µM ethambutol (EMB) (■) and combination of 10 µM EMB and 31.25 µM 3-*O*-methylbutylgallate (▲); (**C**) 1 µM rifampicin (RIF) (■) and combination of 1 µM RIF and 31.25 µM 3-*O*-methylbutylgallate (▼); (**D**) 0.5 µM bedaquiline (BDQ) (■) and combination of 0.5 µM BDQ and 31.25 µM 3-*O*-methylbutylgallate (♦); (**E**) 10 µM isoniazid (INH) (▲) and combination of 10 µM INH and 31.25 µM 3-*O*-methylbutylgallate (■); (**F**) 0.5 µM delamanid (■) and combination of 0.5 µM delamanid and 31.25 µM 3-*O*-methylbutylgallate (▼). Limit of detection (indicated by the dotted line) was 10 colony forming units (CFU)/mL in all experiments. Data are means of duplicate measurements. Experiments have been repeated once with similar results.

Mode of action and resistance mechanism. To get insights into the mode of action, the molecular target(s) as well as possible mechanisms of resistance of 3-*O*-methylalkylgallates, spontaneous resistant mutants of *M. tb.* were isolated on solid medium containing 3-*O*-methylbutylgallate at 5×MIC, which occurred at a frequency of 3.3×10^{7}. Five independent mutants were randomly selected for further characterization. All clones exhibited a 8- to 16-fold shift in MIC for 3-*O*-methylbutylgallate and were also cross-resistant against 3-*O*-methylhexyl- and 3-*O*-methyloctylgallate (Figure 5-2 and 5-4). Subsequently, the resistance-mediating mutations were mapped employing whole genome sequencing. Three of the mutants harbored duplications of a common region spanning the genes Rv3208 to Rv3324, while two clones exhibited a single nucleotide polymorphism (SNP) in the putative promotor region of the gene Rv3230c (Table 5-2). Three of these mutants harbored additional SNPs in other loci such as genes involved in PDIM biosynthesis (*ppsC* or *mas*). Mutations in PDIM biosynthesis are known to occur frequently during *in vitro* culture and were thus estimated to be unrelated to resistance (Table 5-2).

Taken the results of genome sequencing together, one emerging possible resistance mechanism might be overexpression of the gene Rv3230c and/or the downstream gene Rv3229c which forms a two-gene operon with Rv3230c. This overexpression might have occurred either by gene duplication or by a SNP in the promotor region upstream of Rv3230c. Rv3229c (*desA3*) encodes one of the three aerobic desaturases (DesA1, DesA2 and DesA3) present in the genome of *M. tb.* H37Rv [20]. The integral membrane stearoyl-CoA desaturase DesA3 and the corresponding oxidoreductase Rv3230c convert saturated stearic acid to unsaturated oleic acid using molecular oxygen and NADPH for synthesis [20, 21]. The two homologs *desA1* and *desA2* are predicted to have a specific role in mycolic acid biosynthesis as they encode putative acyl-ACP desaturases which might introduce double bonds into the mero chain of mycolic acids after those have been formed by the FAS-II enzyme complex [22, 23]. Oleic acid plays a crucial role in membrane composition for maintenance of physiological functions. Incorporation of unsaturated fatty acid into membrane lipids regulates membrane fluidity [24]. In addition, also mycolic acid synthesis is believed to require oleic acid as a precursor for introduction of double bounds into certain mycolic acid structures. This implicates that the genes Rv3229c and Rv3230c might be essentially involved in mycobacterial lipid metabolism for both cell membrane and cell wall formation in *M. tb.*.

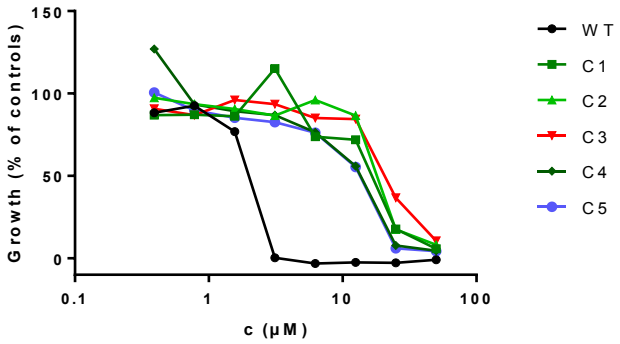


Figure 5-2 Dose-response curves of 3-*O*-methylbutylgallate against spontaneously resistant mutants of *M. tuberculosis* H37Rv (C1-C5) in comparison to the sensitive WT. Strains were grown in absence or presence of exogenously added fatty acids as indicated. Growth was quantified using the resazurin dye reduction assay and calculated relative to controls. Data represent single measurements.

Table 5-2 Mutations in 3-*O*-methylbutylgallate resistant *M. tuberculosis* H37Rv mutants identified by whole-genome sequencing.

3-*O*-Methylbutylgallate resistant mutant	Mutation(s) gene: SNP or length of duplication
C1	306 kb dup (Rv3208-Rv3473c)
C2	146 kb dup (Rv3188-Rv3324); g>a 154 bp upstream of *mas*
C3	t>g 61 bp upstream of Rv2576c; *ppsC*:+c in aa 897; g>t 23 bp upstream of Rv3230c
C4	t>g 61 bp upstream of Rv2576c; *ppsC*:+c in aa 897; g>t 23 bp upstream of Rv3230c; c>t 10 bp upstream of Rv2652c
C5	146 kb dup (Rv3188-Rv3324)

To investigate a possible role of altered fatty acid desaturation in resistance, the influence of various fatty acids on the sensitivity of *M. tb.* to 3-*O*-methylalkylgallates during supplementation to the medium was tested. The supplementation with the unsaturated fatty acids palmitoleic acid and oleic acid virtually completely compensated the inhibitory activity of the tested 3-*O*-methylalkylgallates in the tested concentration range. In contrast, supplementation with the corresponding saturated derivatives palmitate and stearate did not or only slightly decreased sensitivity of the cells, corroborating that 3-*O*-methylalkylgallates very likely target the fatty acid desaturation pathway (Figure 5-3 and 5-5 A and B).

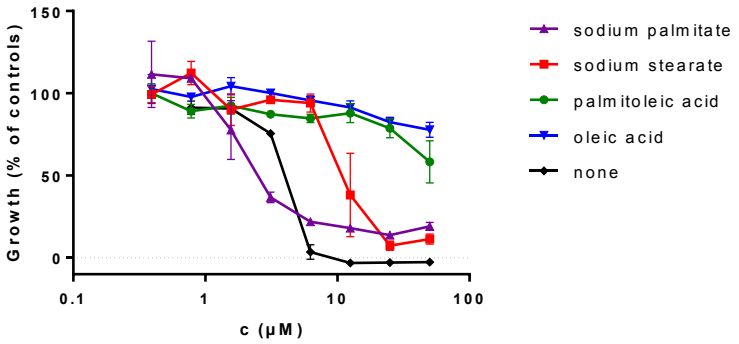


Figure 5-3 Dose-response curves of 3-*O*-methylbutylgallate during supplementation with sodium palmitate (▲), sodium stearate (■), palmitoleic acid (●) oleic acid (▼) and none supplements (♦). Growth was quantified using the resazurin dye reduction assay. Data are means of triplicates ± SD. Growth was quantified using the resazurin dye reduction assay.

A previous study has suggested that the antibiotic isoxyl (ISO), which has been clinically used in the treatment of TB in the past, also inhibits DesA3 activity [24]. Supporting this assumption, similar to our observations, addition of oleic acid to the medium could partially restore growth of ISO-treated *M. tb.* cells on solid medium [24]. However, direct physically interaction of ISO with purified DesA3 has not been demonstrated. In contrast, a more recent study suggests that, rather than DesA3 inhibition, inhibition of the (3*R*)-hydroxyacyl-acyl carrier protein dehydratases encoded by *hadABC* is more relevant for antitubercular activity of ISO, thereby targeting FAS-II-mediated mycolic acid rather than fatty acid desaturation [25]. Supporting the latter hypothesis, our own investigations confirmed that supplementation with fatty acids did not antagonize the inhibitory effect of ISO against *M. tb.* H37Rv in liquid culture *in vitro* (Figure 5-5 C). Additionally, the lack of cross-resistance of spontaneous 3-*O*-methylbutylgallate resistant mutants against ISO indicate that similarities between the putative targets of gallic acid derivatives and ISO are unlikely (data not shown).

In general, inhibition of stearoyl desaturases is known from other drugs against *Toxoplasma gondii* or cancer progression, for instance [26, 27]. Since oleic acid is the most abundant unsaturated fatty acid in mycobacteria and plays a crucial role for membrane lipid and mycolic acid synthesis, DesA3 displays a lethal target for drug therapy. Due to the strong compensation of the inhibitory effect of 3-*O*-methylalkylgallates by oleic acid supplementation, specific targeting of DesA3 is very likely.

Non-methylated alkylgallates were already described in the literature as antibacterial compounds which inhibit growth of *B. subtilis, S. aureus,* and of the Gram-negative plant

pathogen *X. citri* [16, 18, 19]. Also, the inhibitory effect of ethyl gallate against *M. tb.* has already previously been described [28]. However, the reported activities of these non-methylated derivatives were rather low. In fact, a study about 3-*O*-acetylated alkylgallates were published recently demonstrating that acetylation increases the potency against *X. citri* [29]. The published data indicate that the filamentation temperature-sensitive protein Z (FtsZ) as well as permeabilization of bacterial membranes represent potential drug targets of alkylgallates in these bacteria. FtsZ is a self-activating GTPase that polymerizes at the future site of division forming a ring and is essential for cell division in bacteria. In *X. citri*, it was postulated that the effect of alkylgallates on FtsZ might be indirect by disturbing membrane integrity due to the surfactant structure of the gallic acid derivatives, thereby dislocating FtsZ from the membrane [17, 30]. In addition to membrane permeabilization, also a direct interaction of alkylgallates with FtsZ was demonstrated for *B. subtilis* [19]. However, 3-*O*-methylalkylgallates have a rather specific effect on *M. tb.* but not on other bacteria. Also, targeting of FtsZ in *M. tb.* would not easily explain the compensatory effect of oleic acid on antitubercular activity of 3-*O*-methylalkylgallates. Therefore, the possible involvement of FtsZ in explaining the antibacterial effect of 3-*O*-methylalkylgallates on *M. tb.* has to be assessed in further studies.

Unfortunately, several tested *M. tb.* XDR strains were resistant against 3-*O*-methylbutylgallate (data not shown), indicating that this compound might have limited clinical application only for treatment of infections with drug-sensitive strains. Nevertheless, the observed pronounced synergistic killing effects with RIF and INH suggest a promising alternative for TB standard therapy which might shorten treatment duration for drug susceptible *M. tb.* strains. Further studies are required to evaluate the *in vivo* efficacy of 3-*O*-methylalkylgallates and their clinical applicability and usefulness.

Material and Methods

Bacterial strains and growth conditions. Cells of *M. tb.* H37Rv, and of several XDR-TB clinical isolates from South Africa [31] were grown aerobically in Middlebrook 7H9 medium supplemented with 10 % (v/v) ADS enrichment (5 %, w/v, BSA fraction V; 2 %, w/v, glucose; 0.85 %, w/v, sodium chloride), 0.5 % (v/v) glycerol, 0.05 % (v/v) tyloxapol at 37°C. XDR-TB clinical strains originating from South-Africa were obtained from William. R. Jacobs Jr (Albert Einstein College of Medicine, Bronx, USA) and exhibited the following resistances: 1 mg/L INH, 1 mg/L RIF, 10 mg/L EMB, 2 mg/L streptomycin, 100 mg/L pyrazinamide, 5 mg/L ethionamide, 5 mg/L kanamycin, 4 mg/L amikacin, 10 mg/L capreomycin, 2 mg/L ofloxacin.

Nosocomial bacterial strains were cultivated in MH medium at 37°C and included *S. aureus*: MSSA strain ATCC 25923, MRSA/VISA ATCC 700699; *E. faecalis*: ATCC 29212, ATCC 51299 (vancomycin resistant); *E. faecium*: ATCC 35667, ATCC 700221 (vancomycin resistant); and *A. baumannii*: ATCC BAA 1605 (multi-drug resistant).

Determination of minimal inhibitory concentration against *M. tb.* via resazurin dye reduction method. For the determination of MIC against *M. tb.*, bacteria were precultured until log-phase ($OD_{600\ nm}$ = 0.5-1) and then seeded at $1x10^5$ cells/well in a total volume of 100 µL in 96-well round bottom microtiter plates and incubated with twofold serially diluted compounds at a concentration range of 100-0.78 µM. Microplates were incubated at 37 °C for five days. Afterwards, 10 µL/well of a 100 µg/mL resazurin solution were 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 RIF treated (0 % growth) and DMSO treated (100 % growth) controls.

Determination of MIC against nosocomial strains. MIC of gallates for various typical nosocomial bacterial pathogens (*S. aureus*, *E. faecalis*, *E. faecium*, *A. baumannii*) were determined by the broth microdilution method according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [32]. For preparation of the inoculum, the growth method was used. Bacterial cells were grown aerobically in MH medium at 37 °C

and 180 rpm. A preculture was grown until log phase ($OD_{600\,nm}$ ~ 0.5) and then seeded at 5×10^4 bacteria/well in a total volume of 100 µL in 96-well round bottom microtiter plates and incubated with twofold serially diluted compound at a concentration range of 100-0.78 µM. Microplates were incubated aerobically at 37 °C for 24 h. MIC was determined by identifying the minimum concentration of the compound that led to complete inhibition of visual growth of the bacteria.

Determination of cytotoxicity and therapeutic index. The cytotoxicity of the compounds was determined *in vitro* using the human monocyte cell line THP-1 (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and the human fetal lung fibroblast cell line MRC-5 (American Type Culture Collection). THP-1 cells were cultured in RPMI 1640 medium containing 10 % (v/v) FBS, while MRC-5 cells were incubated in DMEM containing 10 % (v/v) FBS both 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 twofold serially diluted compound at a maximum final concentration of 100 µM. Cells treated with DMSO in a final concentration of 1 % (v/v) served as solvent controls. After an incubation time of 48 h, 10 µL resazurin solution (100 µg/mL) were 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, respectively. For determination of the therapeutic index of the substance, the SI was determined by the quotient of cytotoxic concentration and MIC.

Determination of time-kill curves *in vitro.* Bacteria cells were grown aerobically at 37 °C in 10 ml Middlebrook 7H9 liquid media supplemented with 0.5 % (v/v) glycerol, 0.05 % (v/v) tyloxapol and 10 % (v/v) ADS enrichment as shaking cultures. Exponentially growing cultures were diluted to a titer of ca. 1×10^6 CFU/ml as estimated from optical density measurement based on the calculation that an $OD_{600\,nm}$ of 1 translates into 3×10^8 CFU/ml. **10** was added at a concentration of 31.25 µM ($5\times MIC_{100}$) either alone or in individual combination with clinical drugs (1 µM RIF, 10 µM INH, 10 µM EMB, 0.5 µM BDQ, 0.5 µM delamanid). Culture aliquots were taken at different time points, and tenfold serial dilutions were plated on Middlebrook 7H10 agar plates to count viable cells after 3 weeks

of incubation at 37 °C. To check the outgrowth of spontaneous resistant mutants after three weeks, aliquots were plated out on compound containing agar.

Checkerboard synergy assay. The FICI of **10** with INH, delamanid or RIF, respectively, was determined in a 96-well plate format employing 2-dimensional dilutions of compounds. *M. tb.* H37Rv strain was seeded at 10^5 CFU/well in a total volume of 100 µL and incubated at 37 °C for five days. For quantification, the resazurin assay was used as described above. The FICI was calculated as sum of the quotients of the lowest inhibitory concentration in a row (A and B, respectively) and the MIC of the compound (MIC_A and MIC_B, respectively):

$$FICI = \frac{A}{MIC_A} + \frac{B}{MIC_B}$$

Total synergism (FICI ≤ 0.5), partial synergism (0.5 < FICI ≤ 0.75), no effect (0.75 < FICI ≤ 2) or antagonism (FICI > 2) between chlorflavonin and the tested antibiotics was deduced from the observed FICI values[33].

Determination of single step resistance frequency. Spontaneous resistant mutants were isolated by plating approximately 1×10^8 CFU on agar (1 mL per well in a 6-well microtiter plate) containing **10** at 5×MIC. Spontaneous resistant colonies were obtained at a frequency of ca. 1×10^{-7} after 3 weeks of incubation at 37 °C. Five independent clones were selected, which all exhibited moderate resistance against **10**, **11**, **12** in liquid culture.

Whole genome sequencing. To identify the resistance mediating mutations, genomic DNA of five independent mutants was isolated as described previously [34]. Libraries were prepared for sequencing using the standard paired-end genomic DNA sample prep kit from Illumina. Genomes were sequenced using an Illumina HiSeq 2500 next-generation sequencer (San Diego, CA, USA) and compared with the parent *M. tb.* H37RvMA genome (GenBank accession GCA_000751615.1). Paired-end sequence data was collected with a read length of 106 bp. Base-calling was performed using Casava software, v1.8. The reads were assembled using a comparative genome assembly method, using *M. tb.* H37RvMA as a reference sequence [35]. The mean depth of coverage ranged from 277× - 770×.

5.2 Supporting information

Supplementary Data

3-*O*-Methylalkylgallates Inhibit Fatty Acid Desaturation in *Mycobacterium tuberculosis*

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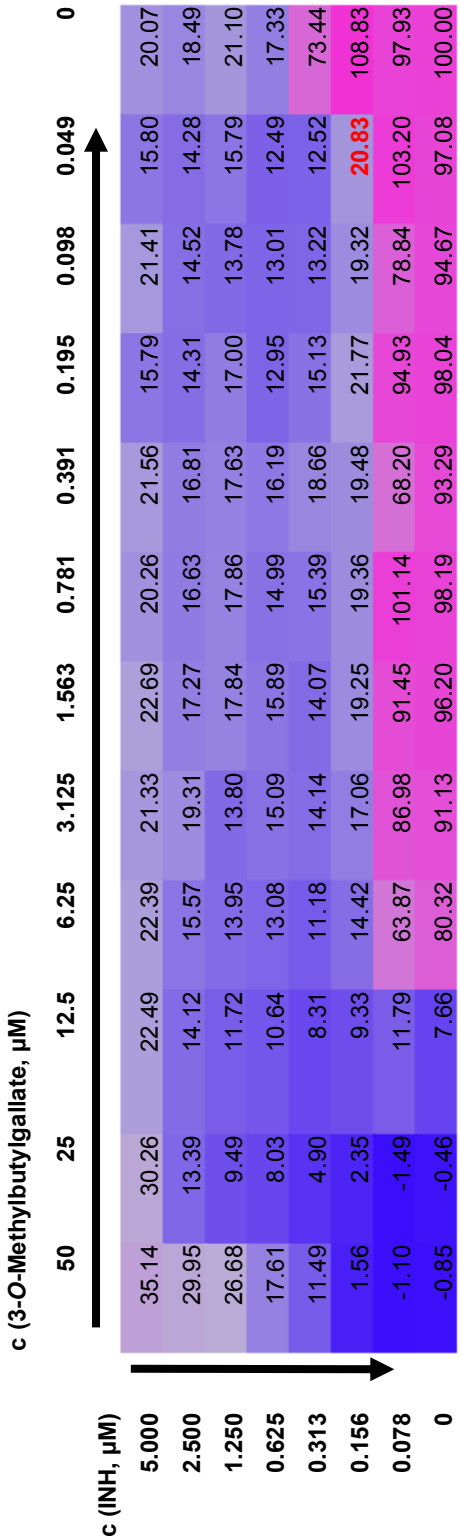
Table 5-3 Minimal inhibitory concentration (MIC) of all compounds against *Staphylococcus aureus* (*S. aureus*, ATCC 25923), Methicillin-resistant *S. aureus* (MRSA, ATCC 700699), *Enterococcus faecalis* (*E. faecalis*, ATCC 29212 and 51299), *Enterococcus faecium* (*E. faecium*, ATCC 35667) and Vancomycin-resistant *E. faecium* (VRE, ATCC 700221), *Acinetobacter baumannii* (*A. baumannii*, ATCC BAA-1605). Compounds were tested against *A. baumannii* in single and in combination treatments with sublethal doses of colistin (COL, 0.31 µM) or rifampicin (RIF, 2.5 µM)

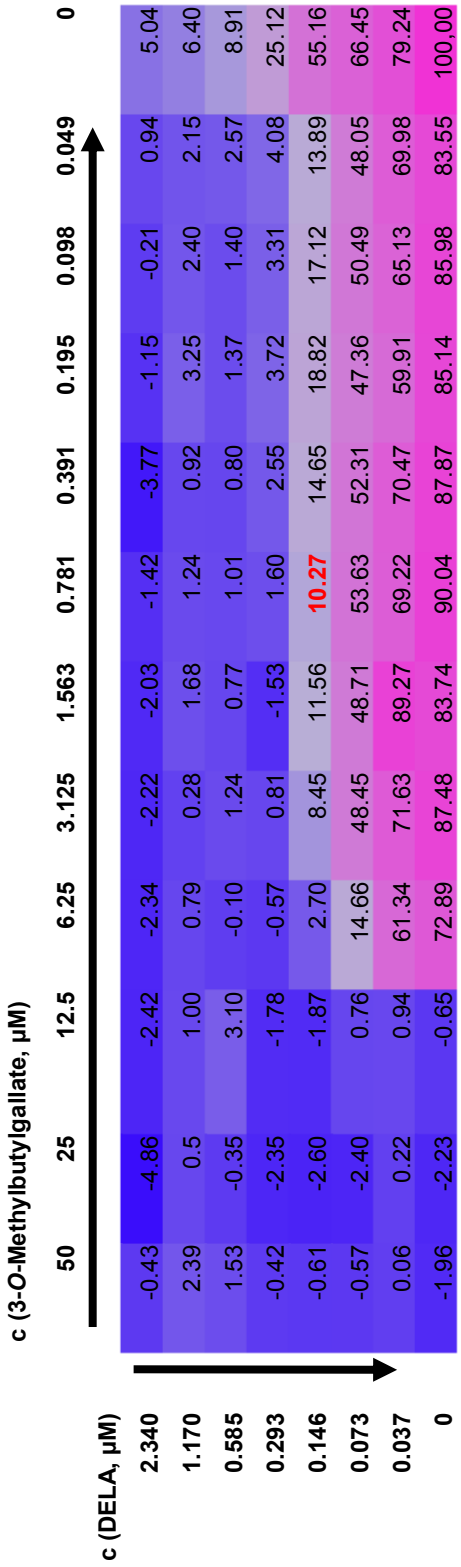
	S. aureus		*E. faecalis*		*E. faecium*		*A. baumannii*		
	ATCC 25923	ATCC 700699	ATCC 29212	ATCC 51299	ATCC 35667	ATCC 700221	ATCC BAA-1605	ATCC BAA-1605, COL	ATCC BAA-1605, RIF
1	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
2	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
3	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
4	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
5	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
6	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
7	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
8	> 100	50	> 100	> 100	> 100	> 100	> 100	> 100	> 100
9	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
10	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
11	> 100	25	> 100	> 100	> 100	> 100	> 100	> 100	> 100
12	25	25	> 100	> 100	25	100	> 100	> 100	> 100
13	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
14	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
15	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
16	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
17	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
18	> 100	100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
19	100	> 100	> 100	> 100	> 100	100	> 100	> 100	> 100

Table 5-4 Checkerboard synergy assay**.** The fractional inhibitory concentration of 3-*O*-methylalkylgallate was determined in combination with RIF, INH or delamanid. *M. tuberculosis* H37Rv was inoculated at a concentration of 10^6 CFU/mL. Growth of bacteria was determined after five days via resazurin dye reduction assay. Table shows growth of bacteria as percent of controls. Growth below 10% relative to controls is marked in blue, growth greater than 10% is marked in purple. No-growth wells relevant for FICI calculation are highlighted in red.

c (RIF, µM) \ c (3-*O*-Methylbutylgallate, µM)	50	25	12.5	6.25	3.125	1.563	0.781	0.391	0.195	0.098	0.049	0
1.250	-6.41	-2.97	-4.10	-3.10	-4.16	-4.50	-3.39	-2.59	-2.44	-2.38	-2.47	2.19
0.625	-2.96	-7.35	-8.81	-8.02	-7.48	-5.82	-7.39	-6.72	-7.81	-7.22	-7.24	-0.82
0.313	-1.21	-6.97	-6.92	-6.27	-4.77	-7.45	-4.65	-4.04	-4.29	-4.63	-3.75	-0.94
0.156	-0.70	-5.69	-5.37	-4.96	-4.48	-4.12	-2.71	-2.60	-2.56	-3.18	-3.05	27.69
0.078	0.31	-6.57	-5.69	-1.18	5.60	6.07	8.37	11.62	13.01	13.86	16.26	104.23
0.039	1.23	-5.17	-3.97	19.93	63.88	62.61	61.94	72.48	73.73	73.77	71.27	124.33
0.020	1.52	-3.89	-1.01	79.33	107.65	110.90	116.87	121.26	116.72	116.82	116.37	77.79
0	1.80	-2.47	10.83	128.04	163.76	169.69	163.33	172.47	176.96	161.40	149.05	100.00



The values for high INH concentrations in the checkerboard assay seem to enhance the growth of bacteria cells. However, macroscopical investigation confirmed bacteria pellets only in wells with calculated growth >36 % (stained in purple).


c (3-O-Methylbutylgallate, µM)
50 25 12.5 6.25 3.125 1.563 0.781 0.391 0.195 0.098 0.049 0
c (DELA, µM)
2.340 -0.43 -4.86 -2.42 -2.34 -2.22 -2.03 -1.42 -3.77 -1.15 -0.21 0.94 5.04
1.170 2.39 0.5 1.00 0.79 0.28 1.68 1.24 0.92 3.25 2.40 2.15 6.40
0.585 1.53 -0.35 3.10 -0.10 1.24 0.77 1.01 0.80 1.37 1.40 2.57 8.91
0.293 -0.42 -2.35 -1.78 -0.57 0.81 -1.53 1.60 2.55 3.72 3.31 4.08 25.12
0.146 -0.61 -2.60 -1.87 2.70 8.45 11.56 10.27 14.65 18.82 17.12 13.89 55.16
0.073 -0.57 -2.40 0.76 14.66 48.45 48.71 53.63 52.31 47.36 50.49 48.05 66.45
0.037 0.06 0.22 0.94 61.34 71.63 89.27 69.22 70.47 59.91 65.13 69.98 79.24
0 -1.96 -2.23 -0.65 72.89 87.48 83.74 90.04 87.87 85.14 85.98 83.55 100.00

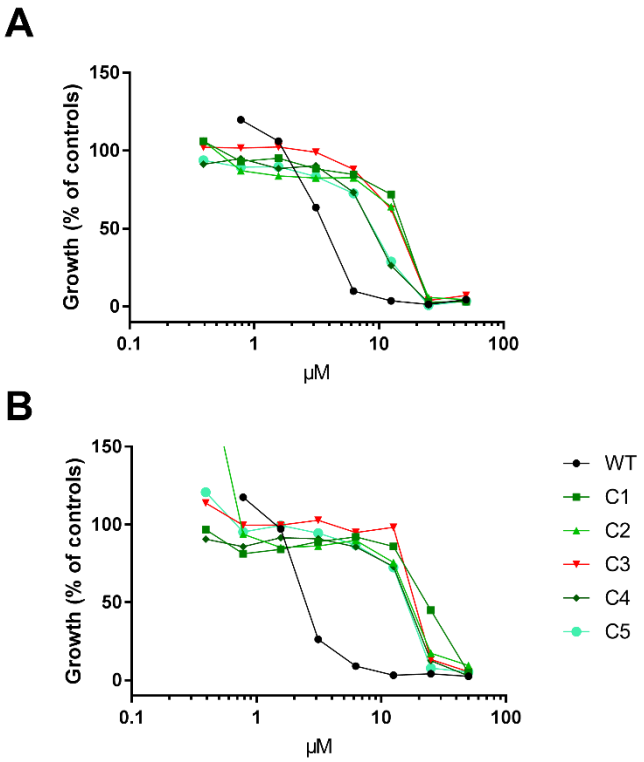


Figure 5-4 Cross resistance of spontaneous resistant mutants (C1-C5) generated with 3-*O*-methylbutylgallate against 3-*O*-methylhexylgallate (**A**) and 3-*O*-methyloctylgallate (**B**) in comparison to *M. tuberculosis* H37Rv WT. Growth was quantified using the resazurin dye reduction assay. Data represent single measurements.

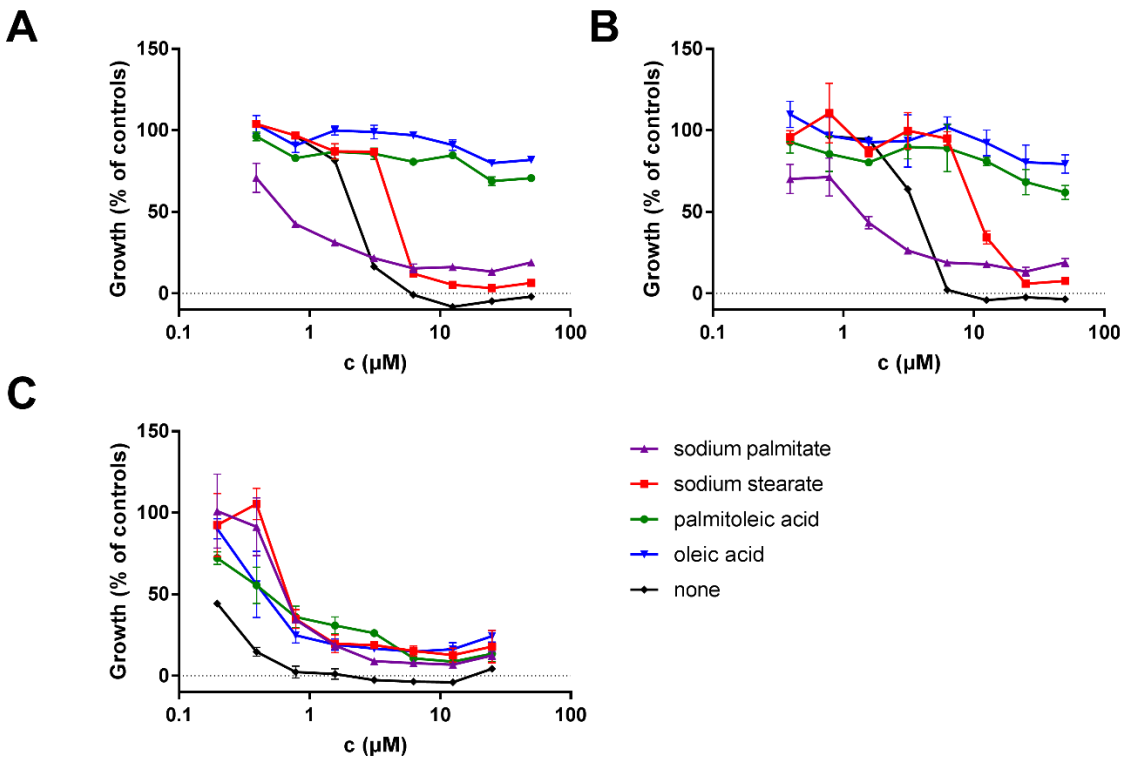


Figure 5-5 MIC assay of 3-*O*-methylhexylgallate (**A**), 3-*O*-methyloctylgallate (**B**) and isoxyl (**C**) with supplementation with sodium palmitate (▲), sodium stearate (■), palmitoleic acid (●), oleic acid (▼) and none supplements (♦). Growth was quantified using the resazurin dye reduction assay. Data are means of triplicates ± SD.

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6 Discussion and Prospects

6.1 Chlorflavonin, an Acetohydroxyacid Synthase Inhibitor as Promising Lead Structure against *Mycobacterium tuberculosis*

We demonstrated that chlorflavonin isolated from the endophyte *Mucor irregularis* is a promising new lead structure for TB therapy due to its strong growth inhibition against *M. tb*. cells. Chlorflavonin belongs to the class of flavones; secondary metabolites produced by plants and fungi. After phenylalanine biosynthesis via the Shikimate pathway, the amino acid is metabolized to cinnamate acid which is hydroxylated to p-coumaric acid. Subsequently, it condensates with three malonyl-CoA units to chalcone which cyclizes to a flavanone. In general, flavonoids are known for their biological activity as antioxidants or as antifungal and antiviral agents [1]. In 1969, Bird and Marshal reported on the structure of chlorflavonin as the only known naturally occurring flavonoid with a chlorine atom isolated from the fungus *Aspergillus candidus* [2]. In 2001, Watanabe *et al.* isolated chlorflavonin from the fungus *Acanthostigmella sp*. and confirmed its antifungal activity against *Candida albicans* and *Aspergillus fumigatus* [3].

It targets the catalytic subunit IlvB1 of acetohydroxyacid synthase (AHAS) in *M. tb.*, causing combined branched chain amino acid (BCAA) and pantothenic acid auxotrophy. Since the target is not affected by resistance mechanisms of XDR strains, chlorflavonin also impairs growth of XDR strains likewise. It has already been reported that chlorflavonin is not cytotoxic against HeLa cells [3]. We could confirm this with other human cell lines as well. Mammals are not able to synthesize branched chain amino acids and pantothenic acids on their own but rely on their dietary uptake, which explains the lack of cytotoxicity of chlorflavonin against human cell lines.

For further investigations about chlorflavonin, the chemical synthesis and/or the biotechnological production of the compounds have to be established. In our study chlorflavonin was isolated from the fungus *Mucor irregularis*. However, after several passages of subcultivation, the fungus stopped the production of chlorflavonin. Our efforts to obtain chlorflavonin in larger quantities from the known alternative producer *Aspergillus candidus*, CMI 16046 [4] have failed for the same reasons. Recently, Kjærbølling *et al.* identified the putative gene cluster for chlorflavonin production in *Aspergillus campestris*. However, the identified cluster lacks a candidate for the essential chlorinating enzyme, which is located somewhere else in the genome, but the cluster is currently the best candidate for trying to

establish recombinant chlorflavonin production. In addition, a homologous cluster pattern was also found in the chlorflavonin-producing strain *Aspergillus candidus*, but not in *Aspergillus taichungensis* which is unable to produce chlorflavonin, further supporting a role of the identified gene cluster in the proposed pathway. Unfortunately, the gene cluster is too large to be transferred to, and functionally expressed in, a heterologous host by available method [5]. However, as reviewed in chapter 2, there are plenty possibilities for yielding higher amounts of chlorflavonin in the natural producers *Mucor irregularis* or *Aspergillus candidus* by fermentation. As example, chlorflavonin production might be enhanced by employing OSMAC, co-cultivation or epigenetic modifications to cultivation. As a consequence, in prospective attempts the cultivation conditions should be varied: for instance, one possible reason for reduced chlorflavonin production is an insufficient provision of nutrients. Higher sodium chloride concentration in the medium might enhance the chlorflavonin production if the chlorination is the limiting step. As another possibility, chlorflavonin production might be triggered by effector molecules of the hosts or of pro- or eukaryotic competitors occupying the same ecological niche so that co-cultivation might influence the yield of the compound positively.

The chemical synthesis, already reported in 1981, embodies another possibility to gain chlorflavonin in larger quantity to allow further preclinical evaluation and development [6]. In addition, analogs of the antitubercular compound were also synthesized in the past [7, 8]. Nevertheless, almost 40 years after the first synthesis, the synthesis scheme still does not seem to be optimized and suffers from insufficient yield particularly of the last reaction step. Without optimization of the chemical synthesis protocol, chlorflavonin cannot be provided in sufficient quantity for further pharmacokinetic, pharmacodynamics and *in vivo* efficacy studies.

As soon as chlorflavonin production is optimized, further analogs should be synthesized for SAR. To get hints which types of derivatives may increase the binding affinity to the target, *in silico* modelling studies should be performed as virtual screening of derivatives [9]. The best computed analogs are expected to be synthesized and evaluated in activity assays. Our investigations have already shown that dechlorflavonin is not active against *M. tb.* and that the chlorine atom is essential for its drug activity but not for the uptake into the bacteria cells. These observations fit with the publication of Watanabe *et al.* in which the antifungal activity of chlorflavonin was stronger than of dechlorflavonin [3]. In addition, they described a bromine derivative of chlorflavonin which was slightly less active against

tested fungi [3]. SAR regarding the antitubercular activity of chlorflavonin derivatives will give some indication how the drug might be optimized. Referring to antifungal activity, it is feasible that the substitution from chlorine to bromine will also reduce the activity against *M. tb.*. Another option is that a fluorine atom or a trifluoromethyl group will lead to lower MICs. It should also be proven whether the position of the chlorine atom influences the interaction of the agent with the AHAS. Besides this, previous studies demonstrated that acetylation of free hydroxy group enhance bioactivity which should be checked for synthetic chlorflavonin derivatives as well [10].

M. tb. cells seem to be unable to use BCAA and pantothenic acid from their host in macrophage infection models which is supported by the IlvB1-dependent activity of chlorflavonin in human THP-1 macrophages. It is known that three isoforms (IlvB2, IlvG and IlvX) exist in *M. tb.* which might be able to compensate the lack of IlvB1 in mice experiments leading only to a slight attenuation of *ilvB1*-deficient *M. tb.* strains [11]. Singh *et al.* have already shown that recombinant IlvG possess AHAS activity, while IlvX does not, and IlvB2 was only active in crude extracts but not as purified protein [11]. For analyzing potency of chlorflavonin, the isoenzymes should be purified and the inhibitory concentration of chlorflavonin against their enzyme activity should be proven. Basic local alignment search tool for proteins (BLASTP) query revealed that homology of IlvB1 to IlvB2 and IlvG is at 87 % and 78 %, respectively, making activity of chlorflavonin against these isoforms likely. Regarding the results of Singh *et al.* with IlvX not showing AHAS activity, the binding affinity between the purified enzymes and chlorflavonin could be analyzed via isothermal calorimetry, for instance. From this, it is not possible to draw conclusions about the inhibition of IlvX by chlorflavonin, but the comparison of the binding affinities with IlvB1 allows prediction whether chlorflavonin interacts with all homologs [11, 12]. Even if chlorflavonin will not interact with the isoenzymes *in vitro*, it is still possible that IlvB1 homologues are not expressed in human host, since we demonstrated that chlorflavonin is active in human macrophage infection model.

Furthermore, *in vivo* experiments will give some prediction about the effect of chlorflavonin in humans. Keeping the costs as low as possible, relevant and predictive animal infection models will have to be chosen wisely. In case of TB investigations, *in vivo* experiments have to be conducted under biosafety level III conditions, and drug regimens often must be given over several weeks. These aspects implicate huge efforts concerning animal care and drug administration. Most obviously, the mouse model seems to be the infection

model of choice because it is the most cost-effective model generating data which are most reproducible in clinical studies [13]. In case of chlorflavonin, it might be possible, that chlorflavonin might only delay growth of *M. tb.* in mouse infection models if the compound specifically inhibits IlvB1 but not the other isoforms. Further investigations are then necessary to dissect whether this phenotype is mouse specific or transferable to humans. However, comparison of our observations in human THP-1 cells with the reported low degree of attenuation of the *M. tb. ilvB1* mutant in murine macrophages strongly suggests that chlorflavonin targets all AHAS isoforms that are expressed during intracellular growth conditions. In any case, other parameters such as tolerated drug dose, bioavailability and tissue distribution can be analyzed to assess chlorflavonin's drug-like properties. Due to the fact that TB therapy will always remain a combination therapy, the combination with other drugs, especially INH and delamanid, should be tested. A synergistic killing of chlorflavonin in combination with INH or delamanid was demonstrated in our *in vitro* studies, indicating that these combinations have persistence-breaking properties and are able to completely eliminate *M. tb.*, thereby drastically reducing the risk of relapse of the infection by persisting bacterial cells.

For better understanding of how the antibacterial effect of chlorflavonin can be further enhanced, intrinsic resistance mediating genes can be identified during drug therapy. By targeting the intrinsic resistance mechanisms, the susceptibility of *M. tb.* to chlorflavonin might be increased. Former studies by Xu *et al.* demonstrated that transposon insertion sequencing display a useful tool for identification of conditionally required genes in stress conditions, such as sub-lethal treatment with chlorflavonin [14]. Sequencing of transposon mutants will reveal a chemical genetic interaction profile specific for chlorflavonin. Additionally, explanation for the synergy of chlorflavonin with INH or delamanid might be possible if gene clusters, which are targeted by these antibiotics, are identified by transposon sequencing.

6.2 Synthetic Derivatives of Hyrtinadine A and Alocasin A as New Lead Structures against Methicillin-resistant *Staphylococcus aureus*

Our studies showed that synthetic hyrtinadine A and alocasine A derivatives, especially those with chlorine instead of hydroxy groups and distinct six membered nitrogen heterocycles, represent interesting lead structures for MRSA therapy with MICs in sub-micromolar range.

Due to the analysis of 20 derivatives, we have already performed meaningful SAR about bis-indoles with a six-membered linker. Due to the easy and reliable performance of the Masuda-Suzuki sequence for the synthesis of heteroaryls, other linkers such as five membered should be analyzed next to possible identify even more potent candidates or candidates with equal potency but otherwise improved drug-like properties. The unsubstituted bis-indoles connected by a thiazole linker inhibited growth of MRSA (data not shown here). If the unsubstituted thiazole derivative already shows activity, it is likely that halogen substituted derivatives linked by thiazole have MICs against MRSA in the nanomolar range.

The pyruvate kinase is discussed as possible target in chapter 4.1 due to structural similarities with known bis-indoles which were reported as PK inhibitors [15]. *In silico* docking studies have already confirmed that halogenated hyrtinadine A derivatives bind to PK of MRSA (personal communication Nicola Porta and Holger Gohlke, Institute of Medicinal and Pharmaceutical Chemistry, Heinrich Heine University Düsseldorf). However, experiments with a PK-deficient strain showed that MIC and bactericidal killing effect of the compounds are not affected by PK depletion meaning bis-indoles might have another mode of action. Conceivably, hyrtinadine A derivatives bind to purified PK although PK is not the relevant target in prokaryotic cells. The isolation of recombinant (His-tagged) PK and subsequently enzyme activity assay will give predictions if hyrtinadine A derivatives show binding affinity to PK like calculated in docking studies [16].

To get hints for another mode of action and resistance, a transcriptome and proteome profile might give information which metabolic pathways are affected by treatment with hyrtinadine A derivatives. The comparison of the proteome profile with profiles of antibiotics with known mode of action might enable elucidation of the affected pathway due to similar protein patterns. However, even in case the proteome pattern is incomparable with known antibiotics, the analysis of marker proteins and characteristic protein modifications permits to hypothesize about the mode of action. By this, the new antibiotic target ClpP (proteolytic core of major protein degradation) inhibited by acyldepsipeptides was identified

by Brötz-Oesterhelt *et al.* [17]. Additionally, affinity chromatography is a helpful tool for identifying cytosolic proteins that interact with hyrtinadine A derivatives. For instance, one possibility is the conjugation of the bioactive compounds with biotin: after incubation with cell lysates, all enzymes, bound to biotin-tagged compounds, will be enriched via biotin-avidin affinity purification and subsequently identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) [18]. However, it has to be checked in advance if the conjugation with biotin significantly impairs bioactivity of the bis-indoles.

Further descriptive assays might give additional hints about the mechanisms of action: Bacterial cells treated with sublethal concentration of the compounds can be analyzed microscopically. On the one hand, the shape of the cells might reveal direct morphological impacts of antibacterial agents on the bacterial envelope, such as damage of membrane integrity using electron microscopy [19]. On the other hand, the property of hyrtinadine A derivatives to induce fluorescence in protic solvents might be helpful for localization of the compounds within prokaryotic cells by fluorescence microscopy [20].

To investigate the activity of the hyrtinadine A derivatives against MRSA *in vivo*, infection models have to be conducted. For this, standard infection models include pneumonia or wound infection models and are available through commercial service providers. Due to the cytotoxicity of the bis-indoles against human cell lines, the topical and/or systemic tolerated drug dose should be determined in infection models. As one option, the bactericidal effect of the compounds might be determined on surgical site infections in murine *in vivo* wound infection models [21]. However, murine or also porcine *in vivo* wound infections harbor the disadvantages of poor representation of human skin, high costs, and ethical issues. As an alternative for animal testing, human skin equivalents can be used which are physiologically comparable to the human skin. This model is already highly developed, so that distinct parameters such as cell viability, immunological and antibacterial responses are able to be evaluated without any ethical issues [22, 23].

6.3 3-*O*-Methylalkylgallates as Possible Inhibitors of Stearoyl Desaturase of *Mycobacterium tuberculosis*

In Chapter 5, the antitubercular activity of 3-*O*-methylalkylgallates is reported. At the present stage, the investigations are at the very beginning making consolidated predictions about the mode of action against *M. tb.* impossible. Nevertheless, the whole genome sequencing of resistant mutants gave a hint that the overexpression of Rv3229c and/or Rv3230c mediates resistance against the compounds. Likewise, due to strong antagonism of oleic acid towards the inhibitory effect of the analyzed compounds, the crucial role of oleic acid pathway is assumable [24]. Since oleic acid is the most abundant unsaturated fatty acid in mycobacteria and is a crucial component of the prokaryotic membrane, its pathway represents an possible drug target [25].

Comprehensive studies need to be conducted for confirming the assumption. The overexpression of the genes Rv3229c (*desA3*) and Rv3230c in *M. tb.* via integral and episomal expression plasmid will give indications on the influence of the overexpressed desaturase and oxidoreductase on the MIC of 3-*O*-methylakylgallates. If the antitubercular compounds target the desaturase step of oleic acid synthesis, the overexpression of DesA3 and Rv3230c might increase the MIC at least comparable to MIC of resistant mutants. If no changing of the MIC is observable in this assay, one possible reason is that protein transcription is suppressed by any regulator. Hence, additional verifications on messenger RNA (mRNA) and protein level should be performed. To detect enhanced translation of mRNA of respective genes, the transcribed complementary DNA (cDNA) can be quantified via reverse transcription quantitative PCR (RT-qPCR). For this, RNA will be extracted from resistant mutants and H37Rv WT strain, reverse transcribed into cDNA and afterwards quantified in a real-time PCR system. Threshold cycles are normalized to those for housekeeping genes [26].

If the enhanced translation of *desA3* and/or Rv3230c is confirmed, enzyme activity assays with the purified enzymes are inevitable. A method for the purification of His-tagged *M. tb.* DesA3 has already been published. The respective enzyme activity assay works with radiolabeled ^{14}C stearoyl-CoA as substrate which is incubated with purified enzyme in a defined reaction buffer. After methylation, the fatty acid methyl esters (FAME) are separated by thin layer chromatography (TLC), visualized by argentation and quantified by Bioscan system [27]. If 3-*O*-methykalkylgallates inhibit the activity of DesA3, the visualized band of oleic acid have to be of lower intensity than the respective band of untreated control.

Certainly, radiolabeled analysis is not practicable in every lab, hence the effect of gallic acid derivatives on lipid composition should be characterized. FAME of treated and untreated cell extracts can be quantified and identified via gas chromatography (GC) coupled with MS.

Since FtsZ was named as target for alkylgallates in literature, this hypothesis should be tracked in further studies [28]. To check whether 3-*O*-methylalkylgallates might target FtsZ in *M. tb*., treated cells can be analyzed microscopically. If FtsZ activity is inhibited, cells display elongation and filamentation caused by delayed or defective cell division [29]. After affirmation of the hypothesis, further experiments regarding FtsZ inhibition will be inevitable. The binding of recombinant FtsZ to alkylgallates can determined indirectly over fluorescence emission shift of alkylgallates by fluorescence spectroscopy [28]. In addition, membrane integrity should be measured because previously described FtsZ inhibitors also affect cell membranes [30]. Nevertheless, the strong antagonism of oleic acid on antitubercular activity of 3-*O*-methylalkylgallates indicates that FtsZ inhibition might just be an additional target.

6.4 Further Strategies to Minimize Antibacterial Drug Crisis

As elucidated in this thesis, intensifying antibiotic research is crucial to address the problem of multidrug resistance, but the problem of resistance development cannot be solved by new antibiotics alone. The resistance crisis is not confined to the healthcare sector but also concerns the community, the individual or the agricultural sector. All distinct groups including prescribers, consumers/patients, industry, farmers, politicians and researchers, are obligated to accept responsibility for minimizing resistance development. Due to complexity of the resistance crisis, the following will focus on further research fields while responsibility of the other groups is summarized in table 6-1, and already reported elsewhere [31, 32].

Table 6-1 Responsibilities of distinct groups for reduction of antibiotic resistance.

Patients/ Consumer
• Following prescriptions while antibiotic therapy • No self-prescription without medical advice • Appropriate hygiene, but no overuse of disinfectant cleaner and sanitary products to avoid selection pressure • Changing in consumer behavior regarding meat from factory farming
Prescribers
• Avoiding too fast prescription of broad-spectrum antibiotcs outside the hospital • Diagnosis of pathogen with antibiogram (if patient's status allows delayed therapy)
Politics
• Ongoing surveillance of antibacterial consumption • Educational programs for behavior shaping and changing • New provision for use of new approved antibiotcs
Agricultural/ animal breeding sector
• Stepwise reorganization of animal fattening for making preventive antibiotic therapy redundant

For deceleration of resistance development, it would be appreciable if especially the consumption of broad-spectrum antibiotics will be reduced. However, in the health-care sector, physicians have to act quickly due to severe afflictions of patients. Doctors often prescribe antibiotics without analyzing the pathogen due to reasons regarding time- and cost-saving motives and patients' expectations. In the hospital, another important aspect has to be taken into account: identification of pathogens in blood culture can take several days, a period of time in which the clinical symptoms of the patient might increase in severity. As a consequence, the doctors prescribe broad-spectrum antibiotics to enhance the possibility to

treat the right pathogen. The analysis of the pathogen species and antibiogram have to be as quick and as cheap as possible to reduce the consumption of broad-spectrum antibiotics. Hence, research should target improved diagnostic methods for pathogen identification regarding time and cost efficiency. Recently, a novel multiplex real-time PCR assay was published by which species and resistance markers of the most relevant pathogenic bacteria including *S. aureus* are targeted [33]. This approach enables results within three hours so that physicians can choose an appropriate narrow-spectrum antibiotic almost immediately after patients' check-up.

Also in case of TB, the diagnosis has to be improved. In general, the diagnosis is based on microscopy, but this technique relies on human skills, training and experience regarding interpretation of results which make the analysis uncertain. Therefore, diagnostic methods have to become cheaper and easier to handle especially in geographical regions with poor lab infrastructure, so that decisions about the choice of antibiotics can be made within a narrow time frame [34-37].

Among antibacterial drugs, alternative therapy approaches can be combined with antimicrobial drug therapy as additional strategy to achieve most efficient results against drug resistance bacteria. On the one hand, phage therapy is one known alternative to antibacterial drug therapy: phages are not able to reproduce outside their hosts. They bind to specific receptors of the bacterial cell surface and inject their genetic material into the host. At this, bacteriophages infect bacteria specifically leading to lysis of bacteria cell or to cell death caused by expressed lethal genes [38]. It could already be shown in several studies that the combination of phages and antibacterial drug therapy leads to synergistic killing effect against Gram-negative pathogens but also *S. aureus* biofilms [39-41]. However, the application of phages for therapeutic purposes is technically difficult and will most often be limited to topical applications such as treatment of wound infections but not for deeper tissues.

Another problem which has to be keep in mind regards the pharmacokinetics of compounds. Especially in case of TB treatment, drugs have to overcome biological barriers since *M. tb* largely resides in infected macrophages (see chapter 1.1.3). Some antimicrobial agents are highly active against *M. tb. in vitro*, but they are inappropriate as drug candidates due to insufficient solubility, penetration or bio-distribution. Nano drug delivery systems can enhance the efficiency of antibiotics to penetrate cell membranes [42]. As one example, liposomes loaded with an antibacterial compound can be used as drug delivery system. Due

to the lipid bilayer of the liposome, the nanoparticle fuses with infectious prokaryotic cells and carries the compound within the microorganism. The surface of the liposome can be modified leading to increased blood circulation or binding affinity to specific tissue, such as infected lung tissue [43, 44]. Evaluations showed that encapsulated RIF exhibits more efficiency, low cytotoxicity and a controlled delivery with high retention time [42, 45, 46].

Summarizing, the rising number of drug resistant microbes is getting alarming. And although drug resistant bacteria have comparable virulence as the respective drug susceptible strains, multidrug resistance develops to one major problem in health care sector globally due to lacking therapeutic options. The successful development of new antibiotics depends on a robust and sustainable pipeline of antibacterial drug candidates. In consequence, this thesis makes a contribution to the necessary discovery of new antibacterial agents and, especially, new drug targets.

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mycobacterial infection: a quantitative proteomic study, Drug design, development and therapy, 9 (2015) 4441-4470. Doi 10.2147/DDDT.S79369
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Research contributions

Journal publications

Parts of the work presented here were published in peer reviewed scientific journals:

- **Rehberg N.**[*], Akone H. S.[*], Iorger T. R., Erlenkamp G., Daletos G., Gohlke H., Proksch P., Kalscheuer R., Chlorflavonin targets acetohydroxyacid synthase catalytic subunit IlvB1 for synergistic killing of *Mycobacterium tuberculosis*, ACS Infect. Dis., 6. Nov. 2017, Doi: 10.1021/acsinfecdis.7b00055.
  * authors contributed equally
- van Geelen L.[*], Meier D.[*], **Rehberg N.**[*], Kalscheuer R., (Some) current concepts in antibacterial drug discovery. Appl. Microbiol. Biotechnol., 2018 Feb 17.
  Doi: 10.1007/s00253-018-8843-6.
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In preparation for submission:

- **Rehberg N.**[*], Sommer G. A.[*], Porta N., Iorger T. R., Gohlke H., Müller T. J. J., Kalscheuer R., Hyrtinadine A and Alocasin A derivatives as new lead structures for APIs against Methicillin-resistant *Staphylococcus aureus* (MRSA), (2018)
  * authors contributed equally

Patent

- Kalscheuer R., Müller T. J. J., **Rehberg N.**, Sommer G. A., Tasch B. O. A., Bis-indole derivatives for use in the treatment of bacterial infections, (2016),
  EP 3 301 096 A1

Poster Presentation

- **Rehberg N.**, Akone H. S., Proksch P., Kalscheuer R., Chlorflavonin inhibits growth of *Mycobacterium tuberculosis* by targeting branched-chain amino acid biosynthesis. Poster on Gordon Research Conference "New antibacterial discovery and development", Lucca, IT, March 2016

Further Publications

Additionally, further contributions to other works not presented here have been published in peer reviewed scientific journals:

- Kamdem R. S. T., Pascal W., **Rehberg N.**, van Geelen L., Höfert S. P., Knedel T. O., Janiak C., Sureechatchaiyan P., Kassack M. U., Lin W., Kalscheuer R., Liu Z., Proksch P., Metabolites from the endophytic fungus *Cylindrocarpon sp.* isolated from tropical plant *Sapium ellipticum*. Fitoterapia. 128, 175-179, (2018).
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- Marmouzi I., Faouzi M. E., Saidi N., Cherrah Y., **Rehberg N.**, Ebada S. S., Ebrahim W., Kalscheuer R., Proksch P., Bioactive secondary metabolites from *Chaetomium globosum*, an endophyte from the Moroccan plant *Avena sativa*. Chem Nat Compd 53, 1208-1211, (2017).
  Doi: 10.1007/s10600-017-2242-6
- Mokhlesi A., Stuhldreier F., Wex K. W., Berscheid A., Hartmann R., **Rehberg N.**, Sureechatchaiyan P., Chaidir C., Kassack M. U., Kalscheuer R., Brötz-Oesterhelt H., Wesselborg S., Stork B., Daletos G., Proksch P., Cyclic cystine-bridged peptides from the marine sponge *Clathria basilana* induce apoptosis in tumor cells and depolarize the bacterial cytoplasmic membrane.
  J Nat Prod 80, 2941-2952, (2017).
  Doi: 10.1021/acs.jnatprod.7b00477
- Ancheeva E., Küppers L., Akone S. H., Ebrahim W., Liu Z., Mándi A., Kurtán T., Lin W., Orfali R., **Rehberg N.**, Kalscheuer R., Daletos G., Proksch P., Expanding the metabolic profile of the fungus *Chaetomium sp*. through co-culture with auto-claved *Pseudomonas aeruginosa*. Eur J Org Chem 2017, 3256–3264, (2017). Doi:10.1002/ejoc.201700288
- Nkwouano V., Witkowski S., **Rehberg N.**, Kalscheuer R., Nausch N., Mayatepek E., Jacobsen M., A novel mycobacterial in vitro infection assay identifies differences of induced macrophage apoptosis between CD4+ and CD8+ T cells. PLoS One 12, e0171817, (2017).
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- Ebrahim W., El-Neketi M., Lewald L. I., Orfali R. S., Lin W., **Rehberg N.**, Kalscheuer R., Daletos G., Proksch P., Metabolites from the fungal endophyte *Aspergillus austroafricanus* in axenic culture and in fungal-bacterial mixed cultures. J Nat Prod 79, 914-922, (2016).
  Doi: 10.1021/acs.jnatprod.5b00975

Curriculum vitae

Personal data

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since 07/2014	**Doctorate** (Dr. rer. nat.) at the Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, Düsseldorf • Thesis: "New lead structures from nature in the quest for antibacterial therapy"
05/2013 – 04/2014	**Second state examination:** certified food chemist
10/2010 – 09/2012	**Master program** (M. Sc.): Food chemistry, Westfälische Wilhelms University Münster • Master thesis: "Preparation and characterization vesicle-associated virulence factors of enterohaemorrhagic *Escherichia coli*"
10/2007 – 09/2010	**Bachelor program** (B. Sc.): Food chemistry, Westfälische Wilhelms University Münster • Bachelor thesis: "HPTLC-MS – principle and possible applications in food chemistry"
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Eidesstattliche Erklärung

Ich, Frau M. Sc. Nidja Rehberg, versichere an Eides Statt, dass die vorliegende Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Düsseldorf, den _____

Nidja Rehberg