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

David Podlesainski, Emmanuel T. Adeniyi, Yvonne Gröner, Florian Schulz, Violetta Krisilia, Nidja Rehberg, Tim Richter, Daria Sehr, Huzhuyue Xie, Viktor E. Simons, Anna-Lene Kiffe-Delf, Farnusch Kaschani, Thomas R. Ioerger, Markus Kaiser, Rainer Kalscheuer

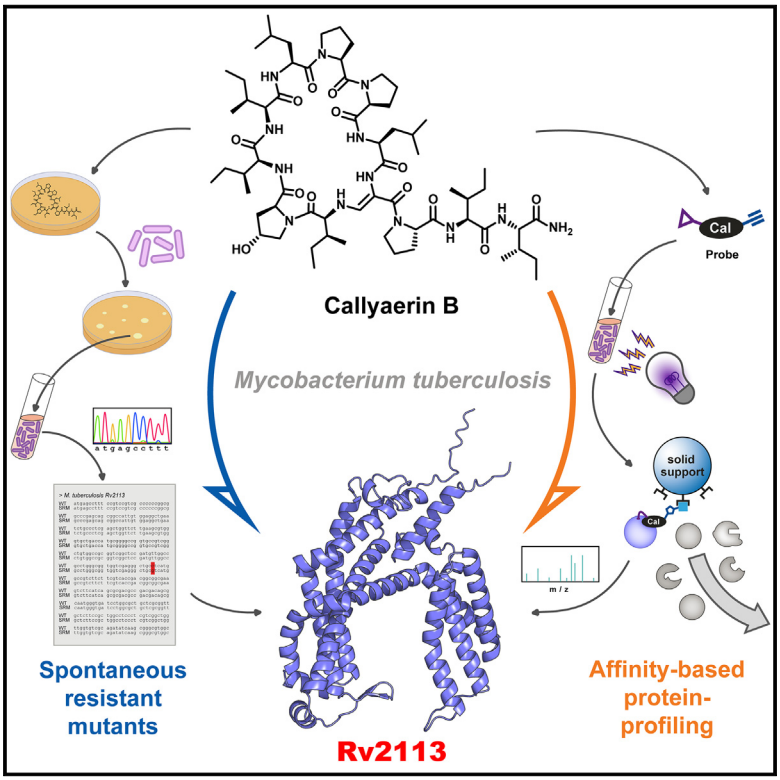
Article - Version of Record

Article

Cell Chemical Biology

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

Graphical abstract



Authors

David Podlesainski, Emmanuel T. Adeniyi, Yvonne Gröner, ..., Thomas R. Ioerger, Markus Kaiser, Rainer Kalscheuer

Correspondence

markus.kaiser@uni-due.de (M.K.), rainer.kalscheuer@hhu.de (R.K.)

In brief

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

Highlights

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

Podlesainski et al., 2024, Cell Chemical Biology *31*, 1755–1771
October 17, 2024
https://doi.org/10.1016/j.chembiol.2024.06.002

CellPress

Cell Chemical Biology

CellPress
OPEN ACCESS

Article

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

David Podlesainski,[1,4] Emmanuel T. Adeniyi,[2,4] Yvonne Gröner,[2] Florian Schulz,[1] Violetta Krisilia,[2] Nidja Rehberg,[2] Tim Richter,[1] Daria Sehr,[1] Huzhuyue Xie,[1] Viktor E. Simons,[2] Anna-Lene Kiffe-Delf,[2] Farnusch Kaschani,[1] Thomas R. Ioerger,[3] Markus Kaiser,[1,*] and Rainer Kalscheuer[2,5,*]

[1]Center of Medical Biotechnology (ZMB), Faculty of Biology, Chemical Biology, University of Duisburg-Essen, 45141 Essen, Germany
[2]Heinrich Heine University Düsseldorf, Faculty of Mathematics and Natural Sciences, Institute of Pharmaceutical Biology and Biotechnology, 40225 Düsseldorf, Germany
[3]Department of Computer Science, Texas A&M University, College Station, TX 77843, USA
[4]These authors contributed equally
[5]Lead contact
*Correspondence: markus.kaiser@uni-due.de (M.K.), rainer.kalscheuer@hhu.de (R.K.)
https://doi.org/10.1016/j.chembiol.2024.06.002

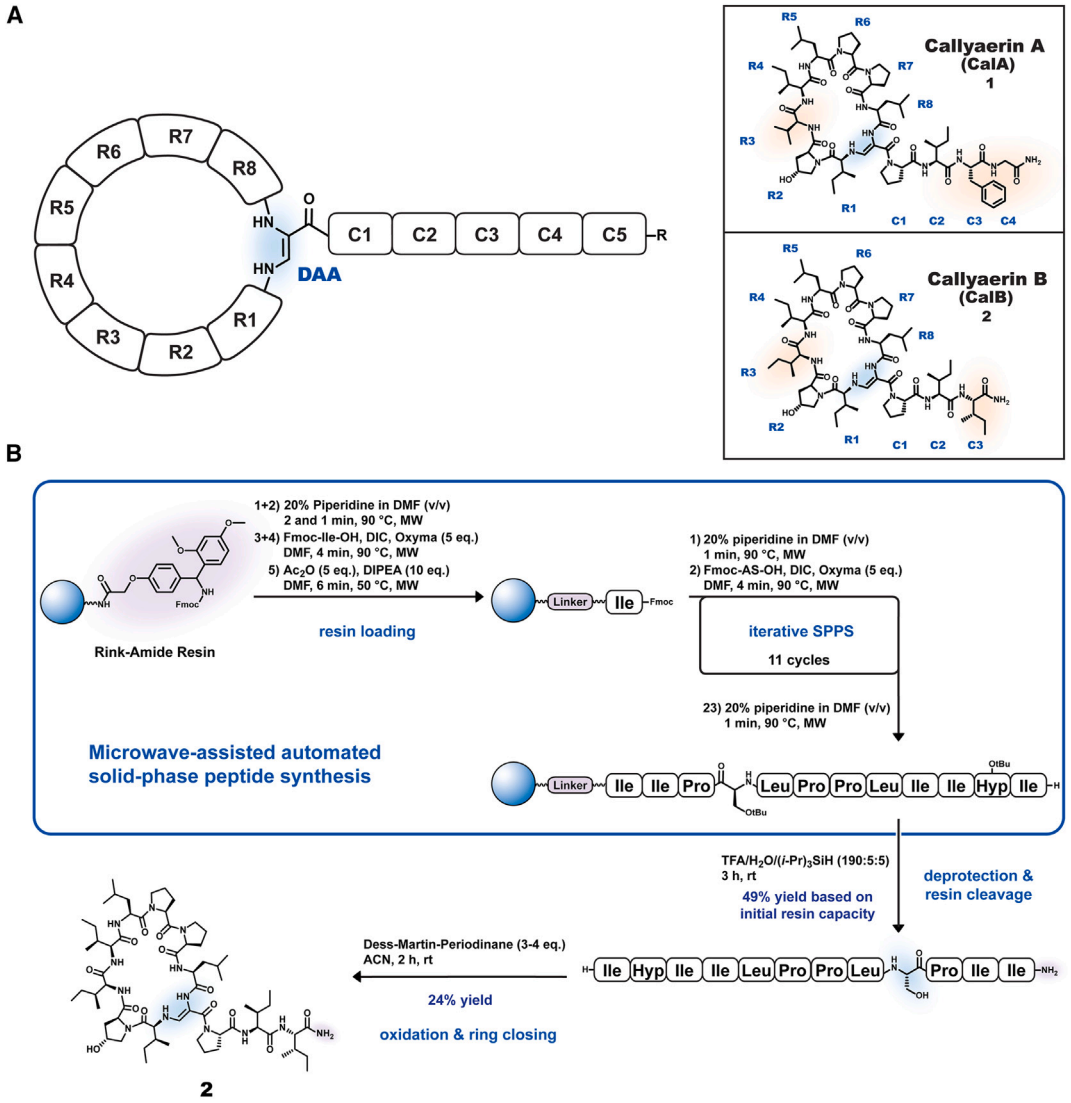
SUMMARY

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

INTRODUCTION

Tuberculosis, an ancient infectious disease caused by *Mycobacterium tuberculosis*, remains a global health threat responsible for 1.6 million deaths in 2021.[1] Despite recent advances in tuberculosis chemotherapy with addition of bedaquiline, pretomanid, and delamanid to the antimicrobial arsenal, lack of success in combating tuberculosis lingers.[2] This is partly due to the inability of drugs to accumulate in compartments harboring sequestered bacilli, as well as the length and complexity of drug regimens.[3] A standard tuberculosis treatment, for instance, requires a cocktail of antibiotics, usually starting with a 2-month combination therapy of isoniazid, rifampicin, pyrazinamide, and ethambutol, followed by a 4-month combination of rifampicin and isoniazid. This prolonged course of treatment often results in poor compliance, which consequently drives emergence of drug resistance.[4,5] As a matter of fact, in 2021, there was an estimated 3.1% global increase in the number of multidrug-resistant and rifampicin-resistant tuberculosis (MDR/RR-TB) cases compared to the previous year and about 191,000 deaths associated with MDR/RR-TB.[1] Due to the broad-spectrum activity of some of the antibiotics included in this 6-month regimen for tuberculosis, resistance development can additionally extend from *M. tuberculosis* to off-target pathogens. Especially rifampicin, which has substantial activity against gram-positive bacteria, can drive the development of such off-resistance.[6] Furthermore, long-term use of these broad-spectrum antibiotics causes major distress to the host microbiome, which can significantly impair the host's immune system as a result of dysbiosis, thereby contributing to several ill health conditions in treated patients.[7] Thus, there remains a persistent need for new therapeutic options that are active against resistant strains in the ongoing fight against tuberculosis, particularly for anti-tubercular agents with a weaker resistance development in off-target pathogens and less harm to the microbiome.

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



C

Anti-tubercular Activity of the natural Callyaerins			MIC90 [μM]
1	Callyaerin A	cyclo(Ile-Hyp-Val-Ile-Leu-Pro-Pro-Leu-) Pro-Ile-Phe-Gly-NH_2	3.13
2	Callyaerin B	cyclo(Ile-Hyp-Ile-Ile-Leu-Pro-Pro-Leu-) Pro-Ile-Ile-NH_2	1.56 - 3.13
3	Callyaerin C	cyclo(His-Hyp-Leu-Leu-Pro-Pro-Val-) Pro-Leu-Phe-Gly-NH_2	>100
4	Callyaerin D	cyclo(Ile-Hyp-Ile-Phe-Pro-Pro-Leu-) Pro-Ile-Asn-Ala-Ile-NH_2	50
5	Callyaerin E	cyclo(Leu-Pro-Phe-Phe-Pro-Pro-Val-) Pro-Ile-Ile-Gly-NH_2	50
6	Callyaerin F	cyclo(Val-Pro-Val-Phe-Pro-Pro-Leu-) Phe-Ile-NH_2	25
7	Callyaerin G	cyclo(Leu-Pro-Phe-Phe-Pro-Pro-Leu-) Pro-Pro-Phe-Gly-NH_2	50
8	Callyaerin H	cyclo(Val-Pro-Val-Phe-Pro-Pro-Leu-) Pro-Ile-NH_2	25
9	Callyaerin I	cyclo(Leu-Pro-Phe-Phe-Pro-Pro-Val-) Pro-Leu-Phe-Gly-NH_2	50
10	Callyaerin J	cyclo(Phe-Pro-Leu-Phe-Pro-Pro-Val-) Pro-Ile-Ile-Gly-NH_2	25
11	Callyaerin K*	cyclo(Phe-Pro-Phe-Gly-Leu-Pro-Pro-Phe-) Pro-Phe-Ile-Asp-OH	>100
12	Callyaerin L	cyclo(Ile-Hyp-Glu-Ile-Val-Pro-Pro-Leu-) Pro-Leu-Phe-NH_2	>100
13	Callynormine A*	cyclo(Ile-Hyp-Val-Leu-Pro-Pro-Leu-) Pro-Phe-Leu-OH	>100

*SPPS with 2-chlortritylchloride resin

(legend on next page)

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

However, an in-depth characterization including structure-activity-relationships (SAR) and mode-of-action (MOA) studies of these anti-tubercular natural products has so far not been pursued. Here, we report the development of a solid phase synthesis protocol based on a modification of a previously published solid phase synthesis of CalA[14] that enabled a straightforward and flexible synthesis of various callyaerin derivatives, some with improved anti-tubercular activities, for SAR and MOA studies. By a combination of these resources with various genetic and chemical proteomic approaches, we then identified and validated the mycobacterial-specific protein Rv2113 as the direct antimycobacterial target of callyaerins in *M. tuberculosis* H37Rv. Of particular note, Rv2113 is fully dispensable for growth and viability of *M. tuberculosis*, demonstrating that non-essential proteins can represent efficacious targets of antibacterial drugs.

RESULTS

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

CalA and CalB have previously been identified as promising anti-tubercular agents (Figure 1A).[11] However, only five of the currently fourteen known callyaerins—due to high structural similarities, we count callynormine A[15] as a further callyaerin derivative—have until now been evaluated for their antimycobacterial properties. It therefore remained unclear whether other members of the callyaerin natural product members might represent more promising starting points for further anti-tubercular drug development.

To address this current limitation and to broaden the evaluation of the anti-tubercular potential of the callyaerins, we therefore aimed to establish a straightforward and flexible synthesis route to diverse members of the callyaerin natural product family as it may also be later used to generate customized derivatives for structure-activity relationship (SAR) studies. The first solid phase peptide synthesis of the callyaerin CalA has been reported by the Brimble group.[14] This synthesis relied on the incorporation of a suitably protected, specifically synthesized α-formyl glycine building block during solid phase peptide assembly,[16] which, after deprotection, allowed the efficient installation of the DAA unit through initial imino linkage and concomitant double-bond migration. Recently, an alternative approach utilizing an acetal-protected 4-hydroxy-L-threonine building block for the introduction of α-formyl glycine was introduced by Yates et al.[16] Based on these findings, we developed a slightly modified, more straightforward callyaerin synthesis that consists of a solid-phase synthesis of a precursor peptide featuring a standard serine residue at the future DAA position, followed by a one-pot selective Dess-Martin oxidation of the hydroxyl residue of serine and concomitant cyclization (Figure 1B). This protocol allowed a flexible synthesis of diverse callyaerins typically at a 1–5 μmol scale. Notably, this approach exploits the unique chemical properties of the cyclized callyaerins that differ significantly from all other open-chain analogs (oxidized as well as non-oxidized), thereby enabling an efficient and easy purification of the final products by reversed-phase high-performance liquid chromatography (RP-HPLC).

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

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

We continued our investigations by characterizing the antibacterial and cytotoxicity profile of CalA/B-type callyaerins (Figures 2A and S2). Interestingly, we found that extensively drug-resistant (XDR) forms of *M. tuberculosis* were also susceptible to CalA and CalB with MIC_{90} ranging from 3.13–12.5 μM, indicating that these callyaerins do not share similar targets with clinical drugs to which these XDR forms of *M. tuberculosis* are resistant.

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

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

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

(C) Overview on the growth inhibition properties (measured as MIC_{90} values using resazurin dye reduction assay) of chemically synthesized callyaerins vs. *Mycobacterium tuberculosis* H37Rv. Data represent a single experiment with $n = 2–3$. A range is shown when variations in MIC_{90} values were observed between individual samples. See also Figure S1.

A

Activity spectrum of CalB	MIC_{90} [μM]
Strain	CalB
M. tuberculosis mc² 6230[a]	1.56
M. tuberculosis H37Rv[a]	1.56 - 3.13
M. tuberculosis Erdman[a]	6.25
M. tuberculosis CDC 1551[a]	12.5
M. tuberculosis KZN 06[a]	6.25
M. tuberculosis KZN 07[a]	12.5
M. tuberculosis KZN 13[a]	6.25
M. tuberculosis KZN 14[a]	3.13
M. tuberculosis KZN 15[a]	12.5
M. tuberculosis KZN 16[a]	12.5
M. tuberculosis KZN 17[a]	6.25
M. bovis AF2122/97[a]	> 50
M. bovis BCG Pasteur[a]	50
M. smegmatis mc² 155[a]	100
M. marinum ATCC 927[a]	> 50
S. aureus ATCC 700699[b]	>100
B. subtilis 168[b]	>100
E. coli ATCC BAA-2469[b]	>100
H. sapiens HEK293[c]	50*
H. sapiens THP-1[d]	50*
H. sapiens HepG2[e]	> 50*
H. sapiens MRC-5[f]	>100*

[a]Middlebrook 7H9 [b]Mueller-Hinton [c]EMEM *IC_{50} values
[d]RPMI 1640 [e]Ham`s F12 [f]DMEM

B

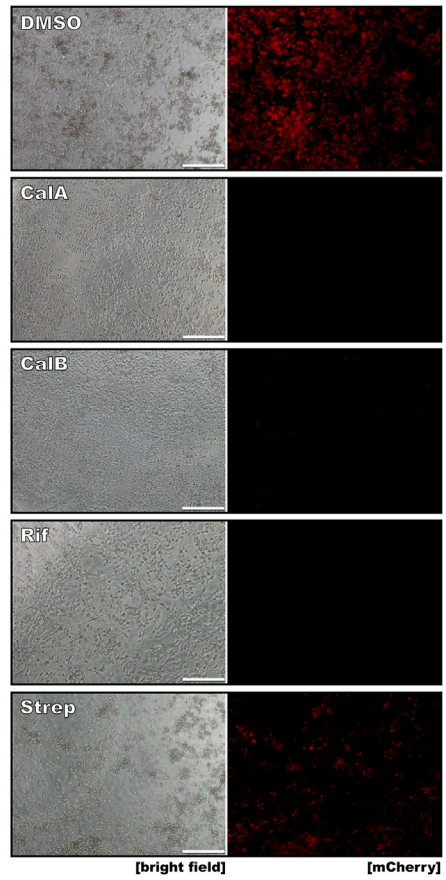


Figure 2. Callyaerins are *M. tuberculosis* selective anti-tubercular agents with intracellular growth inhibition properties

(A) MIC_{90} values (measured using resazurin dye reduction assay, $n = 2–3$) for CalB treatment of various *M. tuberculosis* strains including clinical XDR isolates and further mycobacterial, gram-positive and gram-negative strains as well as human cell lines. Media used for cultivation of the respective cells are indicated by brown superscripted letters. A range is shown when variations in MIC_{90} values were observed between samples.

(B) Fluorescence micrographs of intracellular activity of CalA and CalB in a macrophage infection assay. THP-1 cells were differentiated into macrophage-like cells using PMA. Cells were infected with a mCherry expressing reporter strain of H37Rv (multiplicity of infection MOI = 3) for 3 h and subsequently treated as indicated with 15.6 μM CalA, 1 μM rifampicin or 20 μM streptomycin (each corresponding to 5-fold MIC_{90}) or 1.95 μM CalB (corresponding to ca. 1-fold MIC_{90}). Intracellular growth was evaluated five days post infection. Scale bar represents 150 μm. See also Figures S2–S6.

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

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

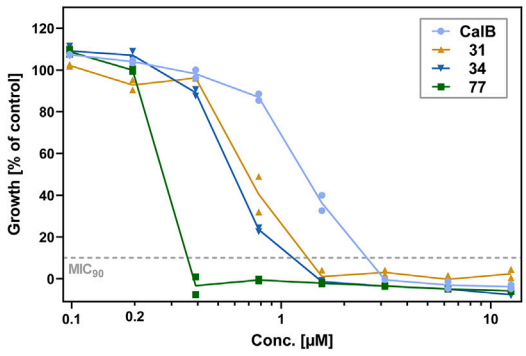
Current TB treatment relies on a combination therapy to minimize the emergence of resistance that rapidly arises during monotherapy.[3] However, the effect of a drug combination is not predictable but could potentially result in neutral, additive, synergistic, or even antagonistic interactions. We therefore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

A

Alanine scan CalA												MIC$_{90}$ [μM]
R1	R2	R3	R4	R5	R6	R7	R8	C1	C2	C3	C4	C5
14	15	16	17	18	19	20	21	22	23	24	25	26
12.5	12.5	>50	3.13	6.25	12.5	6.25	12.5	12.5	>50	>50	12.5	3.13

B

C



D

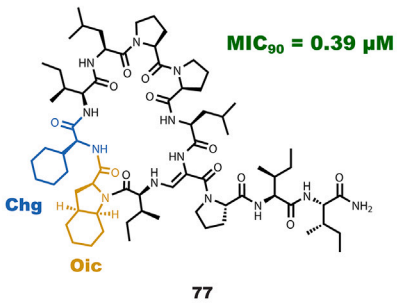


Figure 3. Overview on the structure-activity relationships of CalA/B-type callyaerins

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

(B) Overview on single amino acid substitutions performed to study structure-activity-relationships. Further evaluated derivatives are reported in Figure S7. MIC$_{90}$ values in (A) and (B) represent mean values (n = 2–3) obtained from single experiments.

(C) Concentration-dependent growth inhibition curve for CalB, the slightly more active derivatives 31 and 34 as well as of compound 77 harboring both structural modifications. Data shown as means of duplicates with SD depicted as error bars. Each data point represents one replicate (n = 2–3) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC$_{90}$ threshold corresponding to a remaining growth of 10%. All MIC$_{90}$ values and dose-response curves were determined relative to untreated DMSO control (corresponding to 100% growth) and sterile medium control (0% growth) using resazurin dye reduction assay.

(D) Chemical structure of compound 77 with a cyclohexylglycine (Chg, dark blue) modification in R3 and an octahydroindole-2-carboxylic acid (Oic, light blue) modification in R2. This rationally designed compound is ten-times more active than the parent compound CalB, displaying a MIC$_{90}$ of 0.39 μM vs. *M. tuberculosis* H37Rv. See also Figure S7.

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

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

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

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

CalA and CalB as the founding members of the CalA/B-type callyaerin class are highly hydrophobic peptides that share most of their amino acid composition. Both are characterized by a 9 amino acids-membered ring system cyclized via a DAA moiety and the conserved site-specific presence of (hydroxyl)proline residues, resulting in a unique and stable peptide fold.[14] They are structurally highly similar, differing only in their R3 and C3 position as well as by the presence of a C4 residue in CalA that is lacking in CalB (Figure 1A). However, despite these structural differences, both cyclic peptides have a similar potency against *M. tuberculosis* H37Rv (MIC_{90} = 3.13 μM, Figure 1C), indicating that at least certain positions tolerate structural modifications.

Taking advantage of our flexible chemical synthesis route, we therefore next performed a structure-activity relationship for studying their structural anti-tubercular determinants. We started with a CalA "alanine scan", i.e., we replaced successively each amino acid of CalA with alanine and determined the resulting antimycobacterial activity for these derivatives (Figure 3A). For most positions, introduction of an alanine residue resulted in a lower bioactivity, although in some cases (e.g., position R5, R7; see compound 18 and 20, respectively), these effects were only moderate, while in other positions complete loss of bioactivity was observed (e.g., position R3, C2, and C3; see compound 16, 23, and 24, respectively). A notable exception is R4 (compound 17); here, the substitution of the parent isoleucine residue by alanine was well tolerated and resulted in the same MIC_{90} values as observed for CalA and CalB. Also, an extension of CalA's exocyclic chain with a further C5 alanine residue (26) had no impact on the bioactivity, suggesting that the "end region" of the exocyclic chain may allow attachment of additional chemical tags without significant bioactivity loss.

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

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

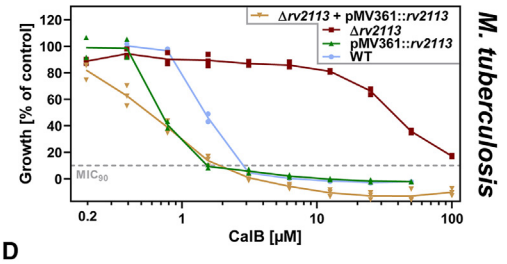
Rv2113 mediates resistance and susceptibility to CalA and CalB

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

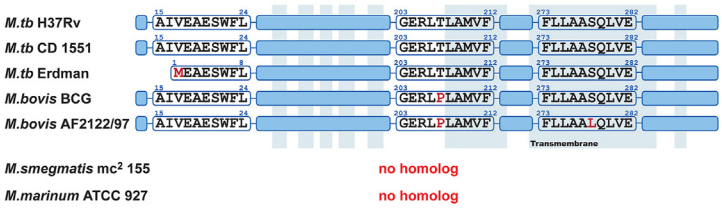
A

CalB resistant Mutants						
#	Rv2113	Rv1929c	TB15.3	atsA	ppsA	aftD
1	-c in aa 9		V128A		W1294*	
2	-c in aa 9	P200L	V128A		W1294*	
3	C58R			E514Q	W1294*	
4	-c in aa 9	P200L	V128A		W1294*	
5	+c in aa 9		V128A		W1294*	
6	-c in aa 9		V128A		W1294*	
7	-c in aa 9	P200L	V128A		W1294*	
CalA resistant Mutants						
8	T185A				H885P	
9	L28P				W1294*	
10	L341P					G882G

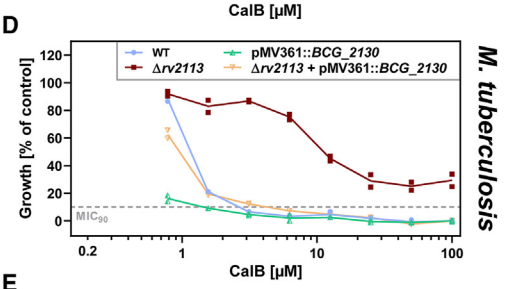
B



C



D



E

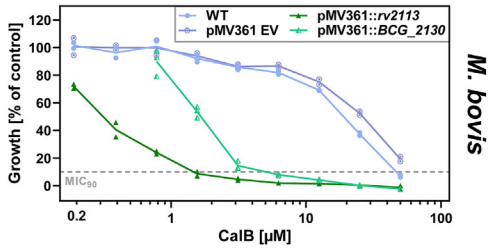


Figure 4. Rv2113 mediates resistance and susceptibility to CalA and CalB

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

(B) Dose-response curves for CalB concentration-dependent growth inhibition of H37Rv strains. The assay included the wild type, the *Δrv2113* gene deletion mutant, the complemented strain *Δrv2113* pMV361::*rv2113* and the overexpressing strain pMV361::*rv2113*.

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

(D) Dose-response curves for CalB concentration-dependent growth inhibition of H37Rv strains. The assay included the wild type, the *Δrv2113* gene deletion mutant, the pMV361::*BCG_2130* complemented mutant strain *Δrv2113* and a BCG_2130 overexpressing strain pMV361::*BCG_2130*.

(E) Dose-response curves for CalB concentration-dependent growth inhibition of *M. bovis* BCG Pasteur strains. The assay included the wild type, the empty vector control pMV361 EV and the overexpressing strains pMV361::*rv2113* and pMV361::*BCG_2130*. In case of (B), (D), and (E) growth was quantified relative to untreated DMSO control (corresponding to 100%) and sterile medium control (0%) using resazurin dye reduction assay. Each data point represents one replicate (n = 2–3) and the solid line connects the respective mean values. The horizontal gray dashed line represents the MIC_{90} threshold corresponding to a remaining growth of 10%. See also Figures S8–S16.

H37Rv were isolated after four weeks from 7H10 medium containing a 5-fold MIC of either CalA or CalB, respectively, at a frequency of 10^{-6}. In contrast to the wild type, the obtained mutants were highly resistant to CalA or CalB treatment with MIC_{90} values above 50 μM (Figures S8A and S8B) and also showed cross-resistance to the respective other callyaerin (Figure S8C), suggesting a common target for both compounds. Subsequent whole-genome sequencing of randomly selected mutants consistently revealed non-synonymous mutations in the gene *rv2113* (Figure 4A). In the majority of the analyzed clones, *rv2113* mutations were accompanied by further mutations; these mutations, however, occurred in diverse genes, strongly suggesting that mutations in *rv2113* are indeed the molecular cause for callyaerin resistance. In agreement with this, complementation of the spontaneous CalA-resistant mutant clone 8, which harbors a H885P mutation in *ppsA* in addition to a T185A mutation in *rv2113* (Figures S9A–S9C), and of the spontaneous CalB-resistant mutant clone 7, which harbors mutations in *ppsA* (W1294*), *rv1929c* (P200L), and *TB15.3* (V128A) in addition to a frameshift mutation in *rv2113* (Figures S9D–S9F), with a wild-type copy of *rv2113* constitutively expressed from a single-copy integrative plasmid (pMV361::*rv2113*), was sufficient to fully restore susceptibility toward CalB. The mutations observed in *rv2113* included non-synonymous SNPs resulting in diverse amino acid substitutions (T185A, L28P, L341P, and C58R) as well as single nucleotide +c insertions or -c deletions in a 6c homopolymer region 20 nucleotides downstream of the start of the *rv2113* gene resulting in frame shifts. The bandwidth of identified mutations suggests a loss-of-function-based resistance mechanism.

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

M. tuberculosis H37Rv, using specialized transduction (Figure S10). As expected, the generated independent clones of the *M. tuberculosis* Δ*rv2113* gene deletion mutant were found to be resistant to both CalA (Figure S11) and CalB treatment (Figure 4B), with MIC_{90} values >100 μM. Upon complementation of the deletion mutant with a wild-type copy of *rv2113* (Δ*rv2113* pMV361::*rv2113*), sensitivity to CalB was restored, thus unambiguously linking the resistance phenotype exclusively to absence of Rv2113. Finally, when the same plasmid pMV361::*rv2113* was used to generate a merodiploid strain of *M. tuberculosis* H37Rv that overexpresses Rv2113, this led to enhanced susceptibility (2-fold decrease in MIC) to CalB. Although *rv2113* is not essential for viability, these results suggest that both resistance and susceptibility of *M. tuberculosis* to CalA/B-type callyaerins are intimately linked to Rv2113.

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

To understand the basis for the selectivity of callyaerins against *M. tuberculosis*, we searched for Rv2113 homologs in the UniProtKB proteome via BLAST.[21] Notably, we found homologs (sequence identity ≥95%) of Rv2113 only in a small number of organisms including *M. tuberculosis*, *M. bovis*, *M. orygis*, *M. canettii*, and *M. shinjukuense*. Except these mycobacterial strains, the search revealed no further organisms with Rv2113 homologs with more than 70% sequence identity. Rv2113 is annotated as a "conserved probable membrane protein" that, according to sequence analysis and Alphafold prediction,[20] harbors eight transmembrane helixes. We previously tested CalA and CalB vs. various (myco)bacterial strains (Figure 2A). An analysis on the presence of Rv2113 homologs in these strains revealed that apart from the susceptible *M. tuberculosis* strains, Rv2113 homologs are only present in the virulent *M. bovis* AF122/97 (with "Mb2137" corresponding to Rv2113) and non-virulent *M. bovis* BCG Pasteur (with "BCG_2130" corresponding to Rv2113) strains (Figure 4C). In these strains, however, CalA and CalB were much less active. Although Mb2137 and BCG_2130 show high sequence identities to Rv2113, BCG_2130 harbors one (T207P) and Mb2137 two amino acid substitutions (T207P and S278L). Owing to the fact that the amino acid substitution T207P is common to both *M. bovis* strains, we reasoned that this mutation might account for the low susceptibility (MIC_{90} = 50 μM) of *M. bovis* BCG Pasteur to CalA/B-type callyaerins. We thus heterologously expressed *BCG_2130* in the *M. tuberculosis* Δ*rv2113* mutant (Δ*rv2113* pMV361::*BCG_2130*) and observed that this fully restored sensitivity to CalB (Figure 4D). Further, when *BCG_2130* or *rv2113* were overexpressed in *M. bovis* BCG Pasteur via a strong constitutive promoter from the single-copy integrative plasmids pMV361::*BCG_2130* and pMV361::*rv2113*, respectively, this strongly sensitized *M. bovis* BCG Pasteur toward CalB, with a reduction in MIC_{90} from 50 μM to 6.25 μM or 1.56 μM, respectively (Figure 4E). These findings together suggest that the basis of the poor sensitivity of *M. bovis* BCG Pasteur toward CalA/B-type callyaerins is likely tied to a low expression level of endogenous *BCG_2130*, whereas the single amino acid exchange in BCG_2130 compared to Rv2113 itself does not substantially impair interaction with CalB. Quantitative real-time PCR indeed demonstrated that the expression level of *BCG_2130* is more than four times lower in wild-type *M. bovis* BCG Pasteur than native *rv2113* expression in *M. tuberculosis* H37Rv (Figure S15A). Additionally, utilizing pMV361:*rv2113* in *M. bovis* BCG Pasteur results in at least triple the expression level of *rv2113* compared to the native variant *BCG_2130* (Figure S15B). However, while the expression level of *rv2113* or its homolog *BCG_2130* determines sensitivity toward callyaerins in *M. tuberculosis* and *M. bovis* BCG Pasteur, heterologous expression of *BCG_2130* or *rv2113* did not or only slightly increased susceptibility of *M. smegmatis* toward callyaerins (Figure S16). This suggests that the interaction of CalA/B-type callyaerins with Rv2113 is necessary, but alone not sufficient to establish an antibacterial effect; instead, additional factors that are present in *M. tuberculosis* and *M. bovis* BCG Pasteur, but absent in other bacteria, are required for anti-tubercular activity.

CalB directly targets Rv2113

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

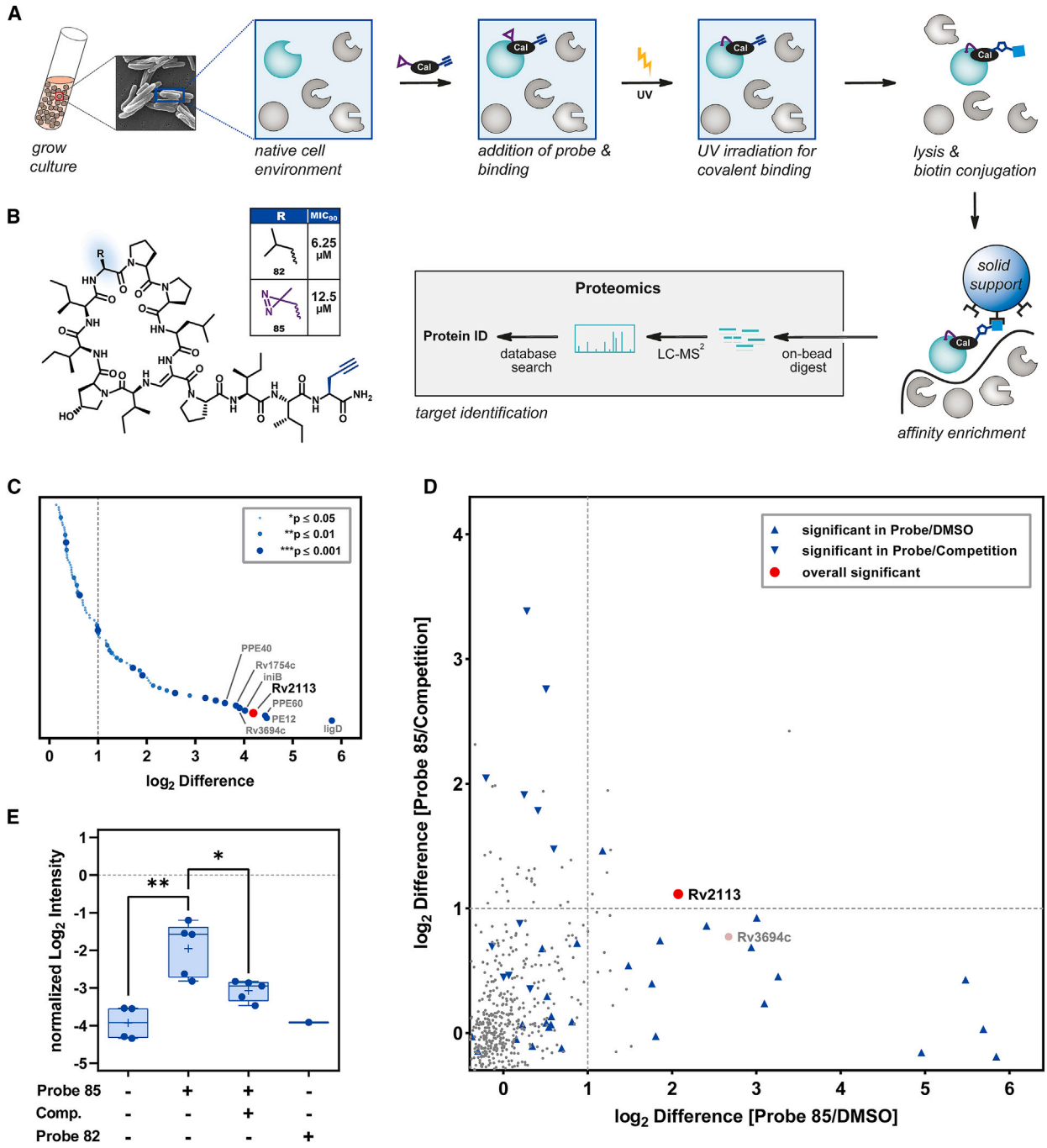


Figure 5. Photoaffinity labeling reveals Rv2113 as a direct target of CalA/B-type callyaerins

(A) Overview of the workflow for (photoaffinity) labeling of *M. tuberculosis* H37Rv cultures.

(B) Chemical structures of the probes 82 and 85 and their respective MIC_{90} values against *M. tuberculosis* H37Rv. Probe 85 is a photoaffinity probe that besides the alkyne tag for click chemistry also harbors an aziridine-based photoleucine at position R5.

(C) Significant enriched proteins ($p \leq 0.05$ after Student's t test using permutation-based FDR with 250 randomizations and FDR = 0.01) resulting from an affinity-based protein-profiling experiment with 10 µM probe 85 in *M. tuberculosis* H37Rv sorted by their $\log_2$ FC.

(D) Results from a combined photoaffinity labeling with 12.5 µM 85 ($\log_2$ FC plotted on x axis) and competitive photoaffinity labeling approach with application of 30 µM CalB prior to photoaffinity labeling ($\log_2$ FC plotted on y axis). Dashed lines indicate the $\log_2$ FC > 1 threshold. To identifying statistically significant hits from the analysis (marked in blue or red), $p \leq 0.05$ (Student's t test; Permutation-based FDR with 250 randomizations and FDR = 0.01) was applied.

(E) Boxplot representation of Rv2113 quantification data from the (photoaffinity) labeling experiments. Cells of exponentially growing cultures of *M. tuberculosis* H37Rv were pooled and aliquoted into 2-mL centrifugation tubes in a total volume of 1 mL PBS containing 0.05% tyloxapol. Samples (n = 5 replicates per condition) were subsequently treated, processed and measured independently without further pooling. Asterisks indicate significant differences between indicated samples (**$p \leq 0.01$, *$p \leq 0.05$, unpaired t test with Welch's correction). See also Figures S17–S20.

The design of the corresponding probes was deduced from the previous structure-activity relationship studies (Figure S7C), suggesting the R5 position as a suitable site for the incorporation of the photoleucine residue (Figure S17). Indeed, 82 as well as 85 both efficiently inhibited mycobacterial growth with MIC_{90} values of 6.25 and 12.5 μM, respectively, thus being only slightly less active than the parent compound CalB.

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

Accordingly, we repeated the experiment with the photoaffinity-tagged probe 85. Application of 10 μM of the probe to an *M. tuberculosis* H37Rv culture for 3 h, followed by 20 min UV radiation at 365 nm, cell lysis, click reaction with biotin azide, and finally an avidin-based affinity enrichment and LC-MS/MS analysis indeed revealed several significantly enriched targets, with log_2 fold changes (FC) > 1 as a threshold, among them Rv2113 as one of the top hits (Figure 5C). For further validation of these initial hits, a second photoaffinity labeling approach was performed at a 12.5 μM probe concentration that was combined with a second, competitive labeling approach that relied on a preincubation with 30 μM CalB (corresponding to a more than 2-fold excess of the competitor) prior to application of the photoaffinity probe 85 (Figures 5D and S19). Importantly, this combination of both experiments revealed Rv2113 as the only significantly enriched direct target with log_2 FC > 1 in both experimental setups (Figures 5D and 5E). Consistent with our previous data, probe 85 showed a significant reduction of activity against our *Δrv2113* gene deletion mutant (Figure S20A). However, labeling of the Δ*rv2113* mutant showed similar enrichment results apart from Rv2113 (Figure S20B). This non-specific labeling might be caused by the highly active carbene species generated during photoactivation of diazirine.

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

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

Our results so far point toward a central role of Rv2113 in the antibacterial mechanism of CalA/B-type callyaerins. However, whereas most anti-tubercular antibiotics typically work by inhibiting the function of an essential protein, this cannot straightforwardly explain the molecular mode of action of CalA/B-type callyaerins in *M. tuberculosis* as the Δ*rv2113* mutant is fully viable and shows no growth defects. We therefore first asked if the membrane protein Rv2113 acts as a molecular transporter of CalA and CalB, thereby mediating intracellular uptake of these compounds, which would allow the compounds to subsequently bind to the "real" targets inside the mycobacterial cell.

To investigate this potential mechanism, we synthesized fluorescent CalB derivatives by conjugating the azide-tagged fluorescent dyes Cy3 or rhodamine via a click reaction to a propargylglycine residue in the C4 position of CalB (compound 82, Figures 6A and S21). While the rhodamine derivative 88 was found to be inactive (MIC_{90} > 100 μM), the Cy3 derivative 87 (Figure 6A) was found to be more than 30-fold more potent than CalB (MIC_{90} = 0.05 μM, Figure 6B). This improvement in anti-tubercular activity was a result of the appropriate covalent modification of CalB with Cy3 as neither application of a respective Cy3 control (89) alone nor of a 1:1 molar mixture of CalB and Cy3 resulted in comparable MIC_{90} values (Figure 6B). This improved anti-tubercular activity furthermore seems to be a specific effect as analogous incorporation of a Cy3 residue at the R3 (90) or R4 (91) position of CalB again led to less active compounds (Figure S22). Importantly, the Cy3-CalB derivative 87 still inhibited the growth of *M. tuberculosis* in an Rv2113-dependent manner, since the *Δrv2113* mutant exhibited resistance (Figure 6C), demonstrating that the dye conjugate most likely relies on the same mechanism as the parent CalB. 87 also maintained its low cytotoxicity to the human cell lines THP-1 and HEK293, resulting in an impressive selectivity index (IC_{50}/MIC_{90}) of >125.

With this highly active and fluorescent derivative in hands, we next used fluorescence microscopy to measure compound uptake in *M. tuberculosis* H37Rv cells as well as in the *Δrv2113* strain (Figures 6D and 6E). Intriguingly, 87 was rapidly internalized both by cells of *M. tuberculosis* wild type and the Δ*rv2113* mutant, with intracellular staining clearly visible as early as after 30 min of incubation. Thus, CalA/B-type callyaerins likely enter *M. tuberculosis* cells independently of Rv2113, ruling out an uptake-dependent mechanism of susceptibility and resistance. As a negative control experiment, we also performed uptake studies with the inactive rhodamine-conjugated derivative (Figure S23). We were not able to detect rhodamine-mediated fluorescence in any of both strains, thus demonstrating that the observed fluorescence signal from 87 correlates with uptake and not unspecific cell surface binding.

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

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

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

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

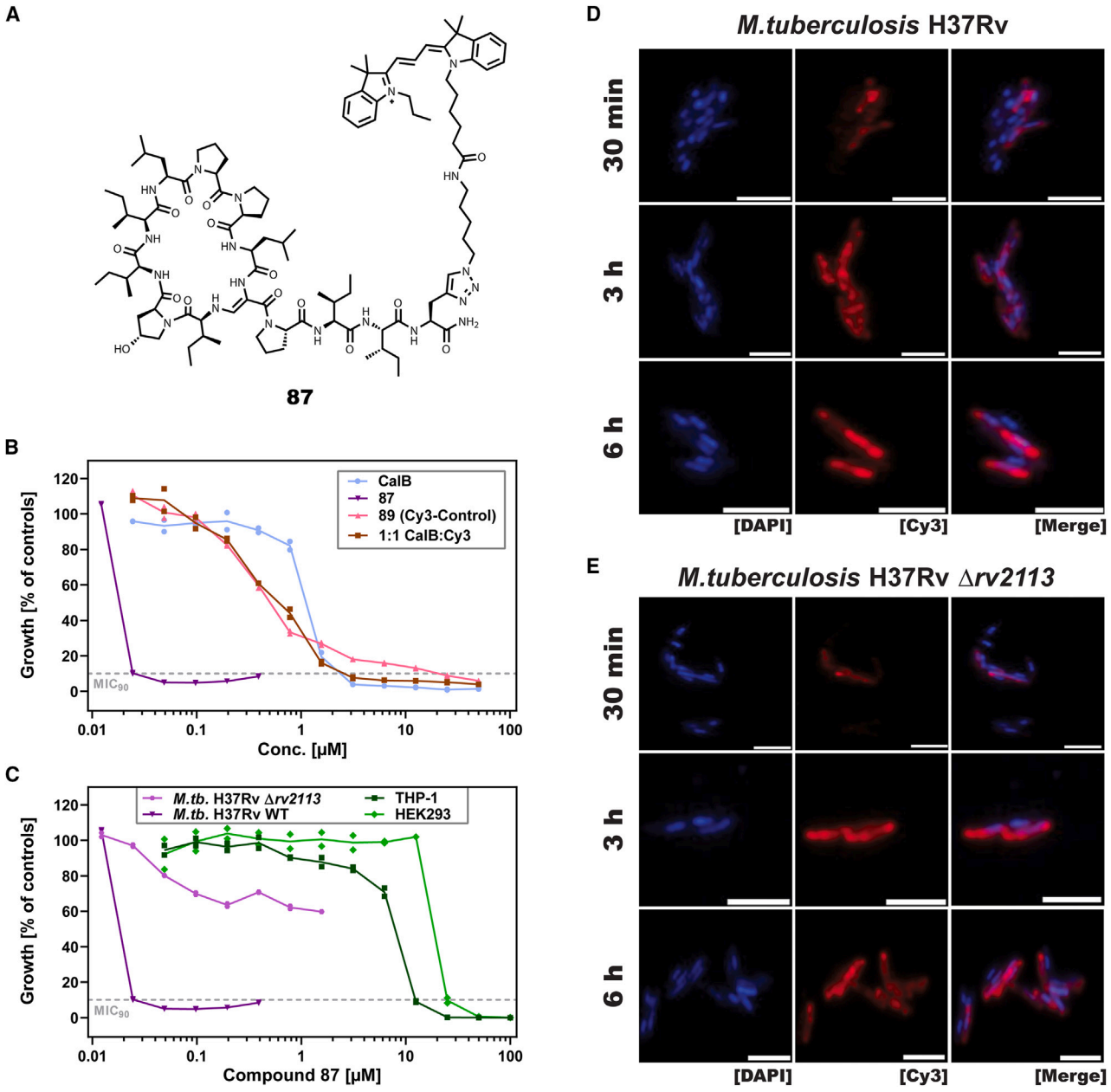


Figure 6. Rv2113 is no molecular transporter for CalB

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

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

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

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

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

We therefore concluded that CalA/B-type callyaerins act by modulating the molecular function of Rv2113, raising the question as to what is the molecular function of Rv2113 in *M. tuberculosis*. However, our bioinformatic analyses including BLAST homology search or structure-based functional predictions using homology structure search via PDBeFold[24] employing the Alphafold structure of Rv2113 or self-generated Alphafold 2-derived Rv2113-homooligomer structures failed to reveal any homolog with assigned function. Thus, the function of Rv2113 remains elusive.

To obtain insights into the cellular processes that are specifically affected by CalB treatment in an Rv2113-dependent manner, cells of *M. tuberculosis* H37Rv wild type (Figures 7D and S24A) and of the CalA and CalB-resistant Δ*rv2113* mutant (Figure S24B) were challenged with a 10-fold MIC_{90} of CalB for 48 h. Subsequently, whole protein cell lysates were prepared to perform global proteome analysis and comparison of the elicited responses. Overall, as expected, treatment of wild-type cells with CalB led to more profound dysregulations of protein expression than treatment of the resistant Δ*rv2113* mutant, which are unambiguously linked to the anti-tubercular nature of CalB, as the inactive compound 36 does not show a similar response at the proteome level (Figure S25). In wild type, the by far most predominant response was reduced abundance of diverse proteins. A GO analysis revealed that the affected processes were mainly cellular lipid metabolic processes and, to a lesser number, processes associated with DNA repair and replication. Among the underrepresented lipid biosynthesis-related proteins were, for example, Fas (Rv2524c), AccD6 (Rv2247), FadD22 (Rv2948c), FbpC (Rv0129c), FabD (Rv2243), Mas (Rv2940c), Pks15 (Rv2947c), FadD29 (Rv2950c), and Rv2949c, while the methylcitrate cycle pathway enzymes PrpC and PrpD were found to be two of the rare overrepresented proteins. However, impairment of lipid biosynthesis did not affect the integrity of the cell envelope in a way that would trigger the cell wall stress marker *iniBAC* (Figure 7C). Rv2525c was previously found to be upregulated in cells of *M. tuberculosis* that were exposed to agents (isoniazid and ethionamide) primarily inhibiting mycolic acid biosynthesis.[25] This suggests that upregulation of Rv2525c might represent an adaptive response to compounds impairing lipid biosynthesis, including callyaerins. Recently, accumulation of methylcitrate cycle intermediates has been linked to tolerance toward first- and second-line TB drugs such as isoniazid and bedaquiline.[26] Therefore, upregulation of PrpC and PrpD might represent a specific response aiming at increasing tolerance to CalB. Furthermore, the virulence-associated methyltransferase Rv1405c, among others, has been reported to be responsible for acclimatizing *M. tuberculosis* CDC1551 to acid stress in macrophages,[27] while the sigma factor SigB was reported to control a regulon involved in general stress resistance in *M. tuberculosis*.[28] This suggests that upregulation of Rv1405c and SigB may represent additional general stress adaptions in CalB-treated cells.

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

DISCUSSION

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

The binding of CalA/B-type callyaerins modulates the non-essential function of Rv2113 in a yet-elusive molecular way that elicits pleiotropic effects by simultaneous impairment of several intracellular pathways including downregulation of lipid biosynthesis, DNA repair, replication, and cell division. This mode-of-action is thus distinct from other antibiotics that typically either target single critical steps in pathways that are essential for growth or viability of bacterial cells, such as DNA replication, cell wall formation, or protein, RNA, or ATP biosynthesis, among others, or interact with membrane structures, resulting in pore formation and collapse of membrane potential. Our data however confirm that both resistance and susceptibility of *M. tuberculosis* toward CalA/B-type callyaerins are strictly linked to the non-essential membrane protein Rv2113. Rv2113 is fully dispensable for normal *in vitro* growth of *M. tuberculosis* under the tested culture conditions and, thus, has not been considered a potential drug target so far. Non-essential proteins, which are connected to the mechanism of antibacterial compounds with phenotypes similar to Rv2113 (i.e., their deletion mediates resistance, while overexpression mediates hypersensitivity), have so far been reported to be associated with only three different processes[1]: compound uptake,[2] activation of pro-drugs,[3] or deregulation and over-activation of proteases.

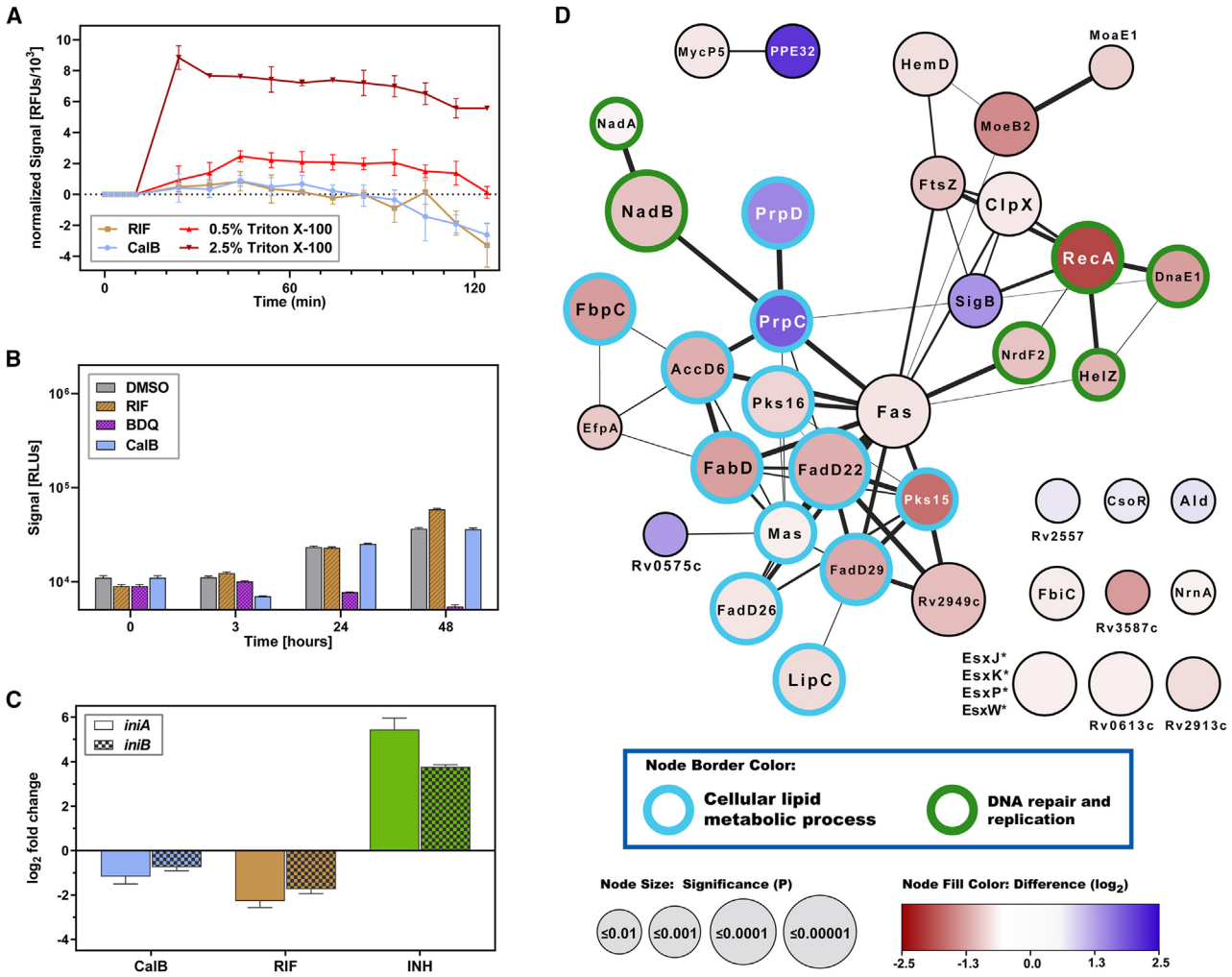


Figure 7. Rv2113-dependent proteomic dysregulation profile suggests that CalB simultaneous impairs multiple pathways

(A) Effect of CalB treatment on membrane integrity of *M. tuberculosis* as assessed by propidium iodide (PI) uptake assay. Cells of *M. tuberculosis* mc^{2}6230 were preincubated with PI to establish baseline fluorescence and then treated with 15.7 μM CalB (5-fold MIC_{90}, blue). Fluorescence intensity (excitation: 535 nm; emission: 617 nm) was measured over time. Addition of 0.5 μM rifampicin (RIF, 5-fold MIC_{90}, brown) and Triton X-100 (0.5% as light red, 2.5% as dark read) served as negative and positive controls, respectively. Data shown as means of triplicates with SD depicted as error bars.

(B) Effect of CalB on mycobacterial energy metabolism. The amount of intracellular ATP in compound-treated cells was measured as a correlate of interference with energy metabolism. Washed cell pellets of *M. tuberculosis* mc^{2}6230 treated with 31.4 μM CalB (10-fold MIC_{90}, blue) were suspended in phosphate-buffered saline and incubated with Bactiter Glo reagent. Luminescence was measured at shown intervals. DMSO (gray) and 1 μM RIF (10-fold MIC_{90}, brown) served as negative controls, while 3.9 μM bedaquiline (BDQ, 10-fold MIC_{90}, purple) served as a positive control. Data shown as means of triplicates with SD depicted as error bars.

(C) Expression of *iniA* and *iniB* of the *iniBAC* gene operon in compound-treated cells of *M. tuberculosis*. Quantification of *iniA* (plain) and *iniB* (patterned) expression by RT-qPCR in cells of *M. tuberculosis* H37Rv treated with 15.7 μM CalB (5-fold MIC_{90}) for 24 h. Log_2 fold change was determined by the ΔΔCT method, normalized to 16SrRNA with DMSO-treated cells as control (corresponding to a fold change of 0). RIF (0.5 μM, 5-fold MIC_{90}) and isoniazid (INH, 1.95 μM, 5-fold MIC_{90}) served as negative and positive controls, respectively. Data shown as means of triplicates with SD depicted as error bars.

(D) Network visualization of proteins which comply the chosen threshold (log_2 FC ≥ 0.5 or ≤ −0.5, q value ≤0.05; Student's t test; Permutation-based FDR with 250 randomizations and FDR = 0.05) from a full proteome profiling of wild type *M. tuberculosis* H37Rv cells treated for 48 h with 31.3 μM CalB (corresponding to a 10-fold MIC_{90}) vs. DMSO treatment after STRING (v11.5) analysis.[41] Node color represents the log_2-FC in protein abundance (increase showed in blue, decrease in red) and node size corresponds to significance (P). Thickness of connection lines indicated the confidence of STRING protein-protein association. Proteins assigned to the GO cluster "cellular lipid metabolic process" [GO: 0044255] or related to DNA repair and replication are shown with a turquoise or green border, respectively. Proteins marked with an asterisk could not be distinguished from each other at the MS level. See also Figures S24 and S25.

An uptake dependent role of non-essential membrane proteins is known, for example, for bacterial multi-solute transporters of the SbmA/BacA family that mediate incorporation of aminoglycoside antibiotics and antimicrobial peptides among other structurally diverse hydrophilic molecules.[35] Inactivation of these transporters cf. resistance by preventing uptake of antibacterial compounds. However, our findings with a fluorescent Cy3-CalB derivative showed that CalA/B-type callyaerins enter

M. tuberculosis cells independently of Rv2113, ruling out an uptake-related role of Rv2113.

Several antibiotics used to treat *M. tuberculosis* such as isoniazid, pyrazinamide, and ethionamide are prodrugs that require intracellular activation by enzymes including catalase/peroxidase KatG,[36] the pyrazinamidase/nicotinamidase PncA,[37] or the Baeyer-Villiger monooxygenases EthA and MymA,[38] respectively. Loss of these non-essential enzymes cf. resistance by preventing pro-drug activation. However, we have no evidence that CalA/B-type callyaerins are pro-drugs that need direct activation by Rv2113. Rv2113 is a membrane protein comprising 387 amino acids, which harbors eight transmembrane helixes according to Alphafold prediction but does not contain any known catalytic domain.

Acyldepsipeptides (ADEPs) are a class of antibacterial natural compounds that kill gram-positive bacteria by over-activation of the cytosolic ClpP protease.[39] Similarly, synthetic tripodal peptidyl compounds can inhibit bacterial growth by allosteric over-activation of the non-essential protease DegP.[40] Loss of these non-essential proteases cf. resistance by preventing disturbed proteostasis that would otherwise occur by deregulated over-activation. However, a similar mechanism is highly unlikely for CalA/B-type callyaerins for several reasons. First, according to sequence analysis, Rv2113 does not contain any known proteolytic domain nor is it predicted to possess any other catalytic activity. Second, in contrast to other gram-positive bacteria, the ClpP protease is essential in mycobacteria, and ADEPs were reported to impair growth of mycobacteria by inhibition of the essential ClpP function, not by over-activation.[32] Third, the proteomic dysregulation profile elicited by CalB treatment is distinct and differs from that of cyclic peptidic compounds that cause disturbance of proteostasis in *M. tuberculosis* by either inhibiting or deregulating ClpP function, such as cylcomarins or homoBac-PROTACs, respectively.[33]

Finally, we also found no evidence that CalA/B-type callyaerins impair structure or function of the mycobacterial membrane or cell wall that could potentially occur if binding of callyaerins to Rv2113 would lead to pore formation. While our findings do not suggest impairment of membrane potential, we acknowledge that it cannot be excluded that binding of Rv2113 by CalA/B-type callyaerins may increase the flux of ions other than protons or other metabolites that could cause a stress response.

In summary, we can rule out all known mechanisms of how a non-essential protein could mediate resistance and susceptibility toward an antibacterial compound similar to Rv2113, with deletion conferring resistance and overexpression mediating hypersensitivity. Thus, we conclude that the anti-tubercular activity of CalA/B-type callyaerins very likely relies on an unprecedented mechanism. This mechanism not only depends on presence of Rv2113 but also requires additional yet-unknown factors that are unique for *M. tuberculosis* and *M. bovis* BCG but are obviously either lacking or at least differ substantially in other mycobacteria. One could speculate that this mechanism might rely on the unspecific recruitment of essential proteins to Rv2113 following binding of CalA/B-type callyaerins, thereby impairing their function. However, further investigations are necessary to prove or disprove this and other hypotheses concerning the molecular processes that occur following binding of these natural products to Rv2113 in *M. tuberculosis*.

Altogether, all our findings indicate that Rv2113 is a highly interesting protein—it is not only specific for *M. tuberculosis*, its structure is also unique, preventing any function predictions. Our investigation on the role of Rv2113 in the anti-tubercular mechanism of the CalA/B-type callyaerins strongly suggests that compound binding modulates function of this non-essential protein in a way that derails several metabolic pathways. This mechanism is unprecedented, diametrically differing from typical antibiotics that work by inhibiting essential targets. Further investigations on the functional role of Rv2113 in *M. tuberculosis* will be pursued in the future to shed light on the underlying molecular details of this unique mechanism. Finally, further medicinal chemical optimization of CalA/B-type callyaerins and development of callyaerin conjugates might pave the way for development of urgently needed *M. tuberculosis*-specific anti-TB drugs.

Limitations of the study

This study has several limitations that warrant attention. The lack of knowledge on the exact biochemical function of Rv2113 curtails our possibilities to conduct functional studies on how CalA/CalB-type callyaerins interfere with this function or lead to a possible gain of function. While photoaffinity labeling strongly suggests interaction of CalB with Rv2113, our study does not provide direct physical evidence of binding. Further, no crystal structure of Rv2113 is available to assess where CalA/CalB-type callyaerins exactly bind to the protein. Moreover, although our investigation highlights the anti-tubercular effects of CalA/CalB-type callyaerins *in vitro*, their *in vivo* efficacy remains to be tested to reveal the clinical relevance of our findings. Additionally, as resistance-mediating mutations in a non-essential gene might not result in any fitness cost, therapeutic targeting of a non-essential protein might lead to rapid resistance development *in vivo*.

SIGNIFICANCE

The unabated spread of drug resistance in the human pathogen *Mycobacterium tuberculosis* causes the urgent need to identify new drugs employing antibacterial mechanisms and addressing cellular targets that differ from those of current antibiotics used for treatment of tuberculosis. Natural compounds continue to be a promising source of new antibiotic scaffolds but often suffer from limited supply from natural sources. Here, we describe the optimized chemical synthesis and derivatization of callyaerins, a class of natural cyclopeptides comprising an unusual (*Z*)-2,3-di-aminoacrylamide unit that has been isolated from a marine sponge. The most efficient callyaerins are bacteriostatics that potently inhibit growth of *M. tuberculosis* in liquid culture and in infected macrophages while showing negligible cytotoxicity against human cells. Antibiotics typically restrict microbial growth by inhibiting cellular targets or pathways that are essential for viability of the bacteria. In contrast, we found that the antibacterial bioactivity of callyaerins is strictly linked to a direct interaction with a non-essential target, the previously uncharacterized and *M. tuberculosis*-specific membrane protein Rv2113. While further research is needed to resolve how callyaerins exactly interact with Rv2113 and how this interaction triggers the perturbation

of several cellular processes resulting in growth inhibition of *M. tuberculosis*, this study exemplifies that non-essential proteins can represent valuable non-canonical target structures. These results can have important implications for the design of anti-tubercular drug development.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Bacterial strains and culture conditions
  - Cell lines and culture conditions
- METHOD DETAILS
  - General information about the chemical synthesis of callyaerins
  - Solid phase peptide synthesis
  - Determination of minimal inhibitory concentration (MIC)
  - Cytotoxicity determination
  - Mycobacterial killing kinetic
  - Checkerboard assay
  - Intracellular ATP measurement
  - Propidium iodide internalization assay
  - RNA extraction and real-time quantitative PCR
  - Generation of a targeted *M. tuberculosis* Δ*rv2113* gene deletion mutant
  - Heterologous gene expression in mycobacteria
  - Spontaneous resistant mutant generation
  - Whole-genome sequencing
  - Intracellular activity in infected macrophages
  - Confocal microscopy
  - Photoaffinity enrichment LC-MS/MS-based target identification
  - LC-MS/MS-based whole proteome comparison
  - Synthetic procedures and characterization of Callyaerin derivatives
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.chembiol.2024.06.002.

ACKNOWLEDGMENTS

We thank the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) (KA 2894/7-1 to M.K. and KA 2259/5-1 to R.K.) for funding. This work was further partially supported by the DFG – project number 270650915/GRK 2158 (to R.K.).

AUTHOR CONTRIBUTIONS

Conceptualization, funding acquisition, and supervision, M.K. and R.K.; investigation, D.P., E.T.A., Y.G., F.S., V.K., N.R., T.R., D.S., H.X., V.E.S., and A.K.; data analysis, D.P., E.T.A., F.K., T.R.I., M.K., and R.K.; writing – original draft, D.P., E.T.A., M.K., and R.K.; writing – review and editing, D.P., M.K., and R.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: September 1, 2023
Revised: April 19, 2024
Accepted: June 11, 2024
Published: July 8, 2024

REFERENCES

1. World Health Organization (2023). Global Tuberculosis Report 2022. https://www.who.int/publications/i/item/9789240083851.
2. Dartois, V.A., and Rubin, E.J. (2022). Anti-tuberculosis treatment strategies and drug development: challenges and priorities. Nat. Rev. Microbiol. *20*, 685–701. https://doi.org/10.1038/s41579-022-00731-y.
3. Zumla, A., Nahid, P., and Cole, S.T. (2013). Advances in the development of new tuberculosis drugs and treatment regimens. Nat. Rev. Drug Discov. *12*, 388–404. https://doi.org/10.1038/nrd4001.
4. Dean, A.S., Cox, H., and Zignol, M. (2017). Epidemiology of Drug-Resistant Tuberculosis. Adv. Exp. Med. Biol. *1019*, 209–220. https://doi.org/10.1007/978-3-319-64371-7_11.
5. Lange, C., Dheda, K., Chesov, D., Mandalakas, A.M., Udwadia, Z., and Horsburgh, C.R., Jr. (2019). Management of drug-resistant tuberculosis. Lancet *394*, 953–966. https://doi.org/10.1016/S0140-6736(19)31882-3.
6. Sacchettini, J.C., Rubin, E.J., and Freundlich, J.S. (2008). Drugs versus bugs: in pursuit of the persistent predator Mycobacterium tuberculosis. Nat. Rev. Microbiol. *6*, 41–52. https://doi.org/10.1038/nrmicro1816.
7. Shreiner, A.B., Kao, J.Y., and Young, V.B. (2015). The gut microbiome in health and in disease. Curr. Opin. Gastroenterol. *31*, 69–75. https://doi.org/10.1097/MOG.0000000000000139.
8. Newman, D.J., and Cragg, G.M. (2020). Natural Products as Sources of New Drugs over the Nearly Four Decades from 01/1981 to 09/2019. J. Nat. Prod. *83*, 770–803. https://doi.org/10.1021/acs.jnatprod.9b01285.
9. Han, J., Liu, X., Zhang, L., Quinn, R.J., and Feng, Y. (2022). Anti-mycobacterial natural products and mechanisms of action. Nat. Prod. Rep. *39*, 77–89. https://doi.org/10.1039/d1np00011j.
10. Atanasov, A.G., Zotchev, S.B., Dirsch, V.M.; International Natural Product Sciences Taskforce, and Supuran, C.T. (2021). Natural products in drug discovery: advances and opportunities. Nat. Rev. Drug Discov. *20*, 200–216. https://doi.org/10.1038/s41573-020-00114-z.
11. Daletos, G., Kalscheuer, R., Koliwer-Brandl, H., Hartmann, R., de Voogd, N.J., Wray, V., Lin, W., and Proksch, P. (2015). Callyaerins from the Marine Sponge Callyspongia aerizusa: Cyclic Peptides with Antitubercular Activity. J. Nat. Prod. *78*, 1910–1925. https://doi.org/10.1021/acs.jnatprod.5b00266.
12. Ibrahim, S.R.M., Min, C.C., Teuscher, F., Ebel, R., Kakoschke, C., Lin, W., Wray, V., Edrada-Ebel, R., and Proksch, P. (2010). Callyaerins A-F and H, new cytotoxic cyclic peptides from the Indonesian marine sponge Callyspongia aerizusa. Bioorg. Med. Chem. *18*, 4947–4956. https://doi.org/10.1016/j.bmc.2010.06.012.
13. Ibrahim, S.R.M., Edrada-Ebel, R., Mohamed, G.A., Youssef, D.T.A., Wray, V., and Proksch, P. (2008). Callyaerin G, a new cytotoxic cyclic peptide from the marine sponge Callyspongia aerizusa. Callyspongia aerizusa Arkivoc *2008* (*xii*) *2008*, 164–171.
14. Zhang, S., De Leon Rodriguez, L.M., Leung, I.K.H., Cook, G.M., Harris, P.W.R., and Brimble, M.A. (2018). Total Synthesis and Conformational Study of Callyaerin A: Anti-Tubercular Cyclic Peptide Bearing a Rare Rigidifying (Z)-2,3- Diaminoacrylamide Moiety. Angew. Chem., Int. Ed. Engl. *57*, 3631–3635. https://doi.org/10.1002/anie.201712792.
15. Berer, N., Rudi, A., Goldberg, I., Benayahu, Y., and Kashman, Y. (2004). Callynormine A, a new marine cyclic peptide of a novel class. Org. Lett. *6*, 2543–2545. https://doi.org/10.1021/ol0491787.
16. Yates, N.D.J., Warnes, M.E., Breetveld, R., Spicer, C.D., Signoret, N., and Fascione, M. (2023). Preparation and Application of an Inexpensive alpha-Formylglycine Building Block Compatible with Fmoc Solid-Phase Peptide Synthesis. Org. Lett. *25*, 2001–2005. https://doi.org/10.1021/acs.orglett.2c04059.
17. Cadena, A.M., Fortune, S.M., and Flynn, J.L. (2017). Heterogeneity in tuberculosis. Nat. Rev. Immunol. *17*, 691–702. https://doi.org/10.1038/nri.2017.69.

18. Dartois, V. (2014). The path of anti-tuberculosis drugs: from blood to lesions to mycobacterial cells. Nat. Rev. Microbiol. *12*, 159–167. https://doi.org/10.1038/nrmicro3200.

19. Rehberg, N., Akone, H.S., Ioerger, T.R., Erlenkamp, G., Daletos, G., Gohlke, H., Proksch, P., and Kalscheuer, R. (2018). Chlorflavonin Targets Acetohydroxyacid Synthase Catalytic Subunit IlvB1 for Synergistic Killing of Mycobacterium tuberculosis. ACS Infect. Dis. *4*, 123–134. https://doi.org/10.1021/acsinfecdis.7b00055.

20. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., et al. (2021). Highly accurate protein structure prediction with AlphaFold. Nature *596*, 583–589. https://doi.org/10.1038/s41586-021-03819-2.

21. Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410. https://doi.org/10.1016/s0022-2836(05)80360-2.

22. Rehberg, N., Sommer, G.A., Drießen, D., Kruppa, M., Adeniyi, E.T., Chen, S., Wang, L., Wolf, K., Tasch, B.O.A., Ioerger, T.R., et al. (2020). Nature-Inspired (di)Azine-Bridged Bisindole Alkaloids with Potent Antibacterial In Vitro and In Vivo Efficacy against Methicillin-Resistant Staphylococcus aureus. J. Med. Chem. *63*, 12623–12641. https://doi.org/10.1021/acs.jmedchem.0c00826.

23. Alland, D., Steyn, A.J., Weisbrod, T., Aldrich, K., and Jacobs, W.R., Jr. (2000). Characterization of the Mycobacterium tuberculosis iniBAC promoter, a promoter that responds to cell wall biosynthesis inhibition. J. Bacteriol. *182*, 1802–1811. https://doi.org/10.1128/JB.182.7.1802-1811.2000.

24. Krissinel, E., and Henrick, K. (2004). Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. Acta Crystallogr. D Biol. Crystallogr. *60*, 2256–2268. https://doi.org/10.1107/S0907444904026460.

25. Saint-Joanis, B., Demangel, C., Jackson, M., Brodin, P., Marsollier, L., Boshoff, H., and Cole, S.T. (2006). Inactivation of Rv2525c, a substrate of the twin arginine translocation (Tat) system of Mycobacterium tuberculosis, increases beta-lactam susceptibility and virulence. J. Bacteriol. *188*, 6669–6679. https://doi.org/10.1128/JB.00631-06.

26. Quinonez, C.G., Lee, J.J., Lim, J., Odell, M., Lawson, C.P., Anyogu, A., Raheem, S., and Eoh, H. (2022). The Role of Fatty Acid Metabolism in Drug Tolerance of Mycobacterium tuberculosis. mBio *13*, e0355921. https://doi.org/10.1128/mbio.03559-21.

27. Healy, C., Golby, P., MacHugh, D.E., and Gordon, S.V. (2016). The MarR family transcription factor Rv1404 coordinates adaptation of Mycobacterium tuberculosis to acid stress via controlled expression of Rv1405c, a virulence-associated methyltransferase. Tuberculosis *97*, 154–162. https://doi.org/10.1016/j.tube.2015.10.003.

28. Sachdeva, P., Misra, R., Tyagi, A.K., and Singh, Y. (2010). The sigma factors of Mycobacterium tuberculosis: regulation of the regulators. FEBS J. *277*, 605–626. https://doi.org/10.1111/j.1742-4658.2009.07479.x.

29. Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., Mueller, A., Schäberle, T.F., Hughes, D.E., Epstein, S., et al. (2015). A new antibiotic kills pathogens without detectable resistance. Nature *517*, 455–459. https://doi.org/10.1038/nature14098.

30. Imai, Y., Hauk, G., Quigley, J., Liang, L., Son, S., Ghiglieri, M., Gates, M.F., Morrissette, M., Shahsavari, N., Niles, S., et al. (2022). Evybactin is a DNA gyrase inhibitor that selectively kills Mycobacterium tuberculosis. Nat. Chem. Biol. *18*, 1236–1244. https://doi.org/10.1038/s41589-022-01102-7.

31. Gavrish, E., Sit, C.S., Cao, S., Kandror, O., Spoering, A., Peoples, A., Ling, L., Fetterman, A., Hughes, D., Bissell, A., et al. (2014). Lassomycin, a ribosomally synthesized cyclic peptide, kills mycobacterium tuberculosis by targeting the ATP-dependent protease ClpC1P1P2. Chem. Biol. *21*, 509–518. https://doi.org/10.1016/j.chembiol.2014.01.014.

32. Famulla, K., Sass, P., Malik, I., Akopian, T., Kandror, O., Alber, M., Hinzen, B., Ruebsamen-Schaeff, H., Kalscheuer, R., Goldberg, A.L., and Brötz-Oesterhelt, H. (2016). Acyldepsipeptide antibiotics kill mycobacteria by preventing the physiological functions of the ClpP1P2 protease. Mol. Microbiol. *101*, 194–209. https://doi.org/10.1111/mmi.13362.

33. Hoi, D.M., Junker, S., Junk, L., Schwechel, K., Fischel, K., Podlesainski, D., Hawkins, P.M.E., van Geelen, L., Kaschani, F., Leodolter, J., et al. (2023). Clp-targeting BacPROTACs impair mycobacterial proteostasis and survival. Cell *186*, 2176–2192.e22. https://doi.org/10.1016/j.cell.2023.04.009.

34. Zhang, S., Kavianinia, I., and Brimble, M.A. (2019). Naturally occurring antitubercular cyclic peptides. Tetrahedron Lett. *60*, 151339.

35. Slotboom, D.J., Ettema, T.W., Nijland, M., and Thangaratnarajah, C. (2020). Bacterial multi-solute transporters. FEBS Lett. *594*, 3898–3907. https://doi.org/10.1002/1873-3468.13912.

36. Vilcheze, C., and Jacobs, W.R., Jr. (2014). Resistance to Isoniazid and Ethionamide in Mycobacterium tuberculosis: Genes, Mutations, and Causalities. Microbiol. Spectr. *2*. MGM2-0014–2013. https://doi.org/10.1128/microbiolspec.MGM2-0014-2013.

37. Lamont, E.A., Dillon, N.A., and Baughn, A.D. (2020). The Bewildering Antitubercular Action of Pyrazinamide. Microbiol. Mol. Biol. Rev. *84*, e00070-19. https://doi.org/10.1128/MMBR.00070-19.

38. Grant, S.S., Wellington, S., Kawate, T., Desjardins, C.A., Silvis, M.R., Wivagg, C., Thompson, M., Gordon, K., Kazyanskaya, E., Nietupski, R., et al. (2016). Baeyer-Villiger Monooxygenases EthA and MymA Are Required for Activation of Replicating and Non-replicating Mycobacterium tuberculosis Inhibitors. Cell Chem. Biol. *23*, 666–677. https://doi.org/10.1016/j.chembiol.2016.05.011.

39. Brotz-Oesterhelt, H., and Vorbach, A. (2021). Reprogramming of the Caseinolytic Protease by ADEP Antibiotics: Molecular Mechanism, Cellular Consequences, Therapeutic Potential. Front. Mol. Biosci. *8*, 690902. https://doi.org/10.3389/fmolb.2021.690902.

40. Cho, H., Choi, Y., Min, K., Son, J.B., Park, H., Lee, H.H., and Kim, S. (2020). Over-activation of a nonessential bacterial protease DegP as an antibiotic strategy. Commun. Biol. *3*, 547. https://doi.org/10.1038/s42003-020-01266-9.

41. Szklarczyk, D., Kirsch, R., Koutrouli, M., Nastou, K., Mehryary, F., Hachilif, R., Gable, A.L., Fang, T., Doncheva, N.T., Pyysalo, S., et al. (2023). The STRING database in 2023: protein-protein association networks and functional enrichment analyses for any sequenced genome of interest. Nucleic Acids Res. *51*, D638–D646. https://doi.org/10.1093/nar/gkac1000.

42. Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). Int. J. Cancer *26*, 171–176. https://doi.org/10.1002/ijc.2910260208.

43. Jacobs, J.P., Jones, C.M., and Baille, J.P. (1970). Characteristics of a human diploid cell designated MRC-5. Nature *227*, 168–170. https://doi.org/10.1038/227168a0.

44. Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. *36*, 59–74. https://doi.org/10.1099/0022-1317-36-1-59.

45. Aden, D.P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B.B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature *282*, 615–616. https://doi.org/10.1038/282615a0.

46. Jain, P., Hsu, T., Arai, M., Biermann, K., Thaler, D.S., Nguyen, A., González, P.A., Tufariello, J.M., Kriakov, J., Chen, B., et al. (2014). Specialized transduction designed for precise high-throughput unmarked deletions in Mycobacterium tuberculosis. mBio *5*, e01245-14. https://doi.org/10.1128/mBio.01245-14.

47. Larsen, M.H., Biermann, K., Tandberg, S., Hsu, T., and Jacobs, W.R., Jr. (2007). Genetic Manipulation of Mycobacterium tuberculosis. Curr Protoc Microbiol *Chapter*. Curr. Protoc. Microbiol. *Chapter 10*, Unit.10A.2. https://doi.org/10.1002/9780471729259.mc10a02s6.

48. Ioerger, T.R., Feng, Y., Ganesula, K., Chen, X., Dobos, K.M., Fortune, S., Jacobs, W.R., Jr., Mizrahi, V., Parish, T., Rubin, E., et al. (2010). Variation among genome sequences of H37Rv strains of Mycobacterium tuberculosis from multiple laboratories. J. Bacteriol. *192*, 3645–3653. https://doi.org/10.1128/JB.00166-10.

49. Ninck, S., Halder, V., Krahn, J.H., Beisser, D., Resch, S., Dodds, I., Scholtysik, R., Bormann, J., Sewald, L., Gupta, M.D., et al. (2023). Chemoproteomics Reveals the Pan-HER Kinase Inhibitor Neratinib To Target an Arabidopsis Epoxide Hydrolase Related to Phytohormone Signaling. ACS Chem. Biol. *18*, 1076–1088. https://doi.org/10.1021/acschembio.2c00322.

50. Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., and Cox, J. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat. Methods *13*, 731–740. https://doi.org/10.1038/nmeth.3901.

51. Hughes, C.S., Moggridge, S., Müller, T., Sorensen, P.H., Morin, G.B., and Krijgsveld, J. (2019). Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. Nat. Protoc. *14*, 68–85. https://doi.org/10.1038/s41596-018-0082-x.

52. Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. *26*, 1367–1372. https://doi.org/10.1038/nbt.1511.

53. Cox, J., Hein, M.Y., Luber, C.A., Paron, I., Nagaraj, N., and Mann, M. (2014). Accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction, termed MaxLFQ. Mol. Cell. Proteomics *13*, 2513–2526. https://doi.org/10.1074/mcp.M113.031591.

54. Horn, D.M., Ueckert, T., Fritzemeier, K., Tham, K., Paschke, C., Berg, F., Pfaff, H., Jiang, X., Li, S., and Lopez-Ferrer, D. (2016). New Method for Label-free Quantification in the Proteome Discoverer Framework.

55. Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., and Mann, M. (2011). Andromeda: A Peptide Search Engine Integrated into the MaxQuant Environment. J. Proteome Res. *10*, 1794–1805. https://doi.org/10.1021/pr101065j.

56. Lundgren, D.H., Han, D.K., and Eng, J.K. (2005). Protein identification using TurboSEQUEST. Curr Protoc Bioinformatics *Chapter*. Curr. Protoc. Bioinformatics *Chapter 13*, Unit.13.3. https://doi.org/10.1002/0471250953.bi1303s10.

57. Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. *13*, 2498–2504. https://doi.org/10.1101/gr.1239303.

58. Martin, C.J., Booty, M.G., Rosebrock, T.R., Nunes-Alves, C., Desjardins, D.M., Keren, I., Fortune, S.M., Remold, H.G., and Behar, S.M. (2012). Efferocytosis Is an Innate Antibacterial Mechanism. Cell Host Microbe *12*, 289–300. https://doi.org/10.1016/j.chom.2012.06.010.

59. UniProt Consortium (2023). UniProt: the Universal Protein Knowledgebase in 2023. Nucleic Acids Res. *51*, D523–D531. https://doi.org/10.1093/nar/gkac1052.

60. Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F., et al. (1991). New use of BCG for recombinant vaccines. Nature *351*, 456–460. https://doi.org/10.1038/351456a0.

61. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods *9*, 671–675. https://doi.org/10.1038/nmeth.2089.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Mycobacterium tuberculosis H37Rv	Ioerger et al.,[48] gift of William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Mycobacterium tuberculosis mc^{2}6230	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Mycobacterium tuberculosis Erdman	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Mycobacterium tuberculosis KZN06, KZN07, KZN13, KZN14, KZN15, KZN16, KZN17	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Mycobacterium tuberculosis Beijing-HN878	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Mycobacterium tuberculosis CDC 1551	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Mycobacterium bovis AF2122/97	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Mycobacterium bovis BCG Pasteur	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Mycobacterium smegmatis mc^2 155	William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Mycobacterium marinum ATCC 927	DSMZ-German Collection of Microorganisms and Cell Cultures GmbH	DSM 44344
Staphylococcus aureus ATCC 700699	Doenitz Prolab	Cat#0158P
Escherichia coli ATCC BAA-2469	Doenitz Prolab	Cat#01113P
Bacillus subtilis 168	DSMZ-German Collection of Microorganisms and Cell Cultures GmbH	DSM 402
Mycobacterium tuberculosis H37Rv pBEN::mCherry (Hsp60)/GFP (Atc)	Martin et al.,[58] gift of Sarah Fortune, Harvard T.H. Chan School of Public Health	N/A
Mycobacterium tuberculosis H37Rv CalA resistant mutants	This paper	N/A
Mycobacterium tuberculosis H37Rv CalB resistant mutants	This paper	N/A
Mycobacterium tuberculosis H37Rv Δ*rv2113*	This paper	N/A
Mycobacterium tuberculosis H37Rv Δ*rv2113* pMV361::*rv2113*	This paper	N/A
Mycobacterium tuberculosis H37Rv pMV361::*rv2113*	This paper	N/A
Mycobacterium tuberculosis H37Rv pMV361::*rv2113* CalB resistant mutants	This paper	N/A
Mycobacterium tuberculosis H37Rv pMV361::BCG_2130	This paper	N/A
Mycobacterium tuberculosis H37Rv Δ*rv2113* pMV361::BCG_2130	This paper	N/A
Mycobacterium tuberculosis H37Rv CalA resistant mutant #8 pMV361::*rv2113*	This paper	N/A
Mycobacterium tuberculosis H37Rv CalB resistant mutant #7 pMV361::*rv2113*	This paper	N/A
Mycobacterium tuberculosis H37Rv CalB resistant mutant #7 pMV361 EV	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mycobacterium bovis BCG Pasteur pMV361 EV	This paper	N/A
Mycobacterium bovis BCG Pasteur pMV361::*rv2113*	This paper	N/A
Mycobacterium bovis BCG Pasteur pMV361::BCG_2130	This paper	N/A
Mycobacterium smegmatis mc^2 155 pMV361::*rv2113*	This paper	N/A
Mycobacterium smegmatis mc^2 155 pMV361 EV	This paper	N/A
Mycobacterium smegmatis mc^2 155 pMV361::BCG_2130	This paper	N/A
ΦphAE159	Jain et al.,[46] gift of William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
Chemicals, peptides, and recombinant proteins		
Tyloxapol	Sigma-Aldrich	Cat#T8761-50G; Cas: 25301-02-4
NaCl	Sigma-Aldrich	Cat#31434-1KG-M; Cas: 7647-14-5
Glycerol	Carl Roth	Cat#3783.1; Cas: 56-81-5
α-D-Glucose	Sigma-Aldrich	Cat#158968-500G; Cas: 492-62-6
Albumin (BSA) Fraction V	PanReac AppliChem ITW reagents	Cat#A1391 Cas: 9048-46-8
BD Difco™ Middlebrook 7H10 Agar	Becton Dickinson	BD 262710
BD Difco™ Middlebrook 7H9 Broth	Becton Dickinson	BD 271310
BD Difco™ Agar	Becton Dickinson	BD 214030
BD DIFCO™ Mueller Hinton Agar	Becton Dickinson	BD 225250
LB Broth (Lennox)	Carl Roth	Cat#X964.3
RPMI 1640	PAN-Biotech	Cat#P04-18500
DMEM	PAN-Biotech	Cat#P04-04510
MEM	biowest	Cat#L0416
Ham's F12	PAN-Biotech	Cat#P04-15500
Fetal Bovine Serum	gibco	Cat#A5256801
Trypsin/EDTA	PAN-Biotech	Cat#P10-0231SP
MEM Non-Essential Amino Acids (100x)	gibco	Cat#11140-035
Sodium pyruvate	PAN-Biotech	Cat#P04-43100
DPBS (10x)	PAN-Biotech	Cat#P04-53500
Hygromycin B	Carl Roth	Cat#CP12.2 Cas: 31282-04-9
Apramycin sulfate salt	Sigma-Aldrich	Cat#A2024-5G Cas: 65710-07-8
Kanamycin sulfate	Carl Roth	Cat#T832.2 Cas: 25389-94-0
Resazurin sodium salt	Sigma-Aldrich	Cat#R7017-1G Cas: 62758-13-8
ROTI® Histofix 10% Formaldehyd	Carl Roth	Cat#A146.5
Isoniazid	Supelco	Cat#I3377-5G Cas: 54-85-3
Rifampicin	Sigma-Aldrich	Cat#R3501-250MG Cas: 13292-46-1

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bedaquiline	MedChemExpress	Cat#HY-14881 Cas: 843663-66-1
Ethambutol dihydrochloride	Sigma-Aldrich	Cat#E4630-25G Cas: 1070-11-7
Delamanid	BLDpharm	Cat#BD300949 Cas: 681492-22-8
Moxifloxacin	BLDpharm	Cat#BD32396 Cas: 186826-86-8
Ethionamide	BLDpharm	Cat#BD10649 Cas#536-33-4
D-cycloserine	TCI	Cat#C1189 Cas#68-41-7
Propidium iodide	Thermo Scientific Chemicals	Cat#440300250 Cas: 25535-16-4
Streptomycin sulfate	AMRESCO	Cat#0382-EU-50G Cas: 3810-74-0
4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI)	Carl Roth	Cat#6843.1 Cas: 28718-90-3
Triton X100	PanReac AppliChem ITW reagents	Cat#A4975,0500 Cas: 9036-19-5
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich	Cat#P8139 Cas: 6561-29-8
RNAprotect Bacteria Reagent	Qiagen	Cat#76506
DNase I, RNase-free	Thermo Scientific	Cat#EN0521
Hexadecyltrimethylammonium bromide (CTAB)	Sigma-Aldrich	Cat#H5882-500G Cas: 57-09-0
Lysozyme	Thermo Scientific	Cat#89833
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D8418; Cas: 67-68-5
DMSO-d6	Sigma-Aldrich	Cat#151874; Cas: 2206-27-1
Methanol	Fisher Chemical	Cat#M/4056/17; Cas: 67-56-1
Na_2HPO_4	Sigma-Aldrich	Cat#71636; 7558-79-4
HEPES	Carl Roth	Cat#HN77; Cas: 7365-45-9
Sodium dodecyl sulfate (SDS)	Carl Roth	Cat#2326.2; CAS: 151-21-3
Fmoc-Abu-OH	Carbolution	Cat# CC05074; CAS: 135112-27-5
Fmoc-Aib-OH	Fluorochem	Cat#045381; CAS: 94744-50-0
Fmoc-Aze-OH	Bachem	Cat#4019052; CAS: 136552-06-2
Fmoc-Chg-OH	Carbolution	Cat#CC05170; CAS: 161321-36-4
Fmoc-Cpg-OH	Carbolution	Cat#CC05169; CAS: 220494-61-0
Fmoc-cPrGly-OH	Iris Biotech	Cat#FAA3410; CAS: 1212257-18-5
Fmoc-dPro-OH	Bachem	Cat#4013705; CAS: 135837-63-7
Fmoc-Hyp-OH	Carbolution	Cat#CC05041; CAS: 122996-47-8

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fmoc-hLeu-OH	Sigma-Aldrich	Cat#8.52327; CAS: 180414-94-2
Fmoc-Nip-OH	Fluorochem	Cat#041311; CAS: 193693-68-4
Fmoc-Oic-OH	BLDpharm	Cat#BD131378; CAS: 130309-37-4
Fmoc-Pip-OH	Carbolution	Cat#CC05038; CAS: 86069-86-5
Fmoc-Pra-OH	Carbolution	Cat#CC05047; CAS: 198561-07-8
Fmoc-β-hIle-OH	Sigma-Aldrich	Cat#03671; CAS: 193954-27-7
Fmoc-Phe-OH	Carbolution	Cat#CC05020; Cas: 35661-40-6
Fmoc-Tle-OH	TCI	Cat#F1251; Cas: 132684-60-7
Fmoc-Bpa-OH	Carbolution	Cat#CC05105; Cas: 117666-96-3
Fmoc-Aha-OH	Iris Biotech	Cat#FAA6620; Cas: 942518-20-9
Fmoc-AEEAc-OH	Carbolution	Cat#CC05196; Cas: 166108-71-0
Fmoc-Pro-OH	Biosolve	Cat#276-250-0; Cas: 71989-31-6
Fmoc-Leu-OH	Iris Biotech	Cat#FAA1120; Cas: 35661-60-0
Fmoc-Ile-OH	Sigma-Aldrich	Cat#47628; Cas: 71989-23-6
Fmoc-Val-OH	Carbolution	Cat#CC05028; Cas: 68858-20-8
Fmoc-Gly-OH	Carbolution	Cat#CC05014; Cas: 29022-11-5
Fmoc-His(Trt)-OH	Nova Biochem	Cat#8.52032; Cas: 109425-51-6
Fmoc-Asn(Trt)-OH	Carbolution	Cat#CC05007; Cas: 132388-59-1
Fmoc-Ala-OH	Nova Biochem	Cat#8.52003; Cas: 35661-89-3
Fmoc-Asp(OtBu)-OH	Carbolution	Cat#CC05008; Cas: 71989-14-5
Fmoc-Glu(OtBu)-OH	Iris Biotech	Cat#FAA1045; Cas: 71989-18-9
$CuSO_4$	Sigma-Aldrich	Cat#C1297; CAS: 7758-98-7
Azide-PEG_3-Biotin	Jena Bioscience	Cat#AZ104; CAS: 875770-34-6
Tris[(1-Benzyl-1H-1,2,3-Triazol-4-yl) methyl]amine (TBTA)	Sigma-Aldrich	Cat#678937; CAS: 510758-28-8
Tris(2-carboxyethyl)phosphine -hydrochloride (TCEP)	Sigma-Aldrich	Cat#C4706; CAS: 51805-45-9
Urea	GE Healthcare	Cat#GE17-1319-01; CAS: 17-1319-01
NH_4HCO_3 (ABC)	Sigma-Aldrich	Cat#11213; CAS: 1066-33-7

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CHROMASOLV™ Acetonitrile	Honeywell	Cat#14261; CAS: 75-05-8
CHROMASOLV™ H_2O	Honeywell	Cat#14263; CAS: 7732-18-5
Acetonitrile (ACN)	VWR	Cat#20060; Cas: 75-05-8
DL-Dithiothreitol (DTT)	Sigma-Aldrich	Cat#43815; CAS: 3483-12-3
Iodoacetamide (IAM)	Sigma-Aldrich	Cat#A3221; CAS: 144-48-9
Chloroacetamide (CAM)	Sigma-Aldrich	Cat#C0267; CAS: 79-07-2
Formic acid (FA)	Fisher Chemical	Cat#A11705AMP; CAS: 64-18-6
N,*N*′-Diisopropylcarbodiimide (DIC)	Carbolution	Cat#CC01002; CAS: 693-13-0
Ethyl cyano-hydroxyiminoacetate (Oxyma)	Carbolution	Cat#CC01024; CAS: 3849-21-6
Dimethylformamide (DMF)	Fisher Chemical	Cat#D/3841/17; CAS: 68-12-2
Piperidine	Sigma-Aldrich	Cat#104094; CAS: 110-89-4
Rink-amide resin	Iris Biotech	Cat#BR-1330
Diethyl ether	Fisher Chemical	Cat#D/2450/17; CAS: 60-29-7
N,*N*-diisopropylethylamine (DIPEA)	TCI	Cat#D1599; CAS: 7087-68-5
Dichloromethane (DCM)	Fisher Chemical	Cat#D/1852/17; CAS: 75-09-2
Acetic anhydride (Ac_2O)	Sigma-Aldrich	Cat#A6404; CAS: 108-24-7
2-chlorotrityl chloride (2-CTC) resin	Sigma-Aldrich	Cat#8.55017
Triisopropyl silane (TIS)	TCI	Cat#T1533; CAS: 6485-79-6
Trifluoroacetic acid (TFA)	Alfa Aesar	Cat#A12198; CAS: 76-05-1
Dess-Martin-Periodinane (DMP)	Carbolution	Cat#CC03024; CAS: 87413-09-0
Trypsin	Promega	Cat#V5111
Pierce™ Avidin Agarose	Thermo Scientific	Cat#20225
Callyaerin A (CalA)	This paper; Ibrahim et al.[12]	1
Callyaerin B (CalB)	This paper; Proksch et al.[12]	2
Callyaerin C	This paper	3
Callyaerin D	This paper	4
Callyaerin E	This paper	5
Callyaerin F	This paper	6
Callyaerin G	This paper	7
Callyaerin H	This paper	8
Callyaerin I	This paper	9
Callyaerin J	This paper	10
Callyaerin K	This paper	11
Callyaerin L	This paper	12
Callynormine A	This paper	13

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CalA_R1A	This paper	14
CalA_R2A	This paper	15
CalA_R3A	This paper	16
CalA_R4A	This paper	17
CalA_R5A	This paper	18
CalA_R6A	This paper	19
CalA_R7A	This paper	20
CalA_R8A	This paper	21
CalA_C1A	This paper	22
CalA_C2A	This paper	23
CalA_C3A	This paper	24
CalA_C4A	This paper	25
CalA_C5A	This paper	26
CalB_R2dPro	This paper	27
CalB_R2Aze	This paper	28
CalB_R2Pip	This paper	29
CalB_R2Nip	This paper	30
CalB_R2Oic	This paper	31
CalB_R3hLeu	This paper	32
CalB_R3Aib	This paper	33
CalB_R3Chg	This paper	34
CalB_R3F	This paper	35
CalB_R3β-hIle	This paper	36
CalB_R4β-hIle	This paper	37
CalB_R5hLeu	This paper	38
CalB_R6dPro	This paper	39
CalB_R6Aze	This paper	40
CalB_R6Pip	This paper	41
CalB_R6Nip	This paper	42
CalB_R6Oic	This paper	43
CalB_R7dPro	This paper	44
CalB_R7Aze	This paper	45
CalB_R7Pip	This paper	46
CalB_R7Nip	This paper	47
CalB_R7Oic	This paper	48
CalB_R8hLeu	This paper	49
CalB_R8Aib	This paper	50
CalB_C1dPro	This paper	51
CalB_C1Aze	This paper	52
CalB_C1Pip	This paper	53
CalB_C1Nip	This paper	54
CalB_C1Oic	This paper	55
CalB_C2β-hIle	This paper	56
CalB_C2Aib	This paper	57
CalB_C3F	This paper	58
CalB_C3Aib	This paper	59
CalB_C3cPrGly	This paper	60
CalB_C3Tle	This paper	61
CalB_C3Bpa	This paper	62
CalA_R2P	This paper	63

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CalA_R3Abu	This paper	64
CalA_R3Chg	This paper	65
CalA_R3Cpg	This paper	66
CalA_R3L	This paper	67
CalA_R3F	This paper	68
CalA_R5D	This paper	69
CalA_R8W	This paper	70
CalA_R8V	This paper	71
CalA_C3L	This paper	72
CalA_C3I	This paper	73
CalA_C3X	This paper	74
CalA_C4Aha	This paper	75
CalA_C4X	This paper	76
CalB_R2Oic_R3Chg	This paper	77
CalB_R3Pra	This paper	78
CalB_R4Pra	This paper	79
CalB_R7Pra	This paper	80
CalB_C3Pra	This paper	81
CalB_C4Pra	This paper	82
CalB_C5Pra	This paper	83
CalB_R3phLeu	This paper	84
CalB_R5phLeu	This paper	85
CalB_R8phLeu	This paper	86
CalB_C4Pra(Cy3)	This paper	87
CalB_C4Pra(Rhodamine)	This paper	88
Cy3-Control	This paper	89
2-(Acetylamino)-4-pentynamide	This paper	89a
CalB_R3Pra(Cy3)	This paper	90
CalB_R4Pra(Cy3)	This paper	91
Benzonase	EMD Milipore	Cat#71206
Critical commercial assays		
660 nm Protein Assay	Thermo Fisher Scientific	Cat#22660
Ionic Detergent Compatibility Reagent	Thermo Fisher Scientific	Cat#22663
BacTiter-Glo™ Microbial Cell Viability Assay	Promega	Cat#G8230
RNeasy Mini Kit	Qiagen	Cat#74104
SuperScript™ III First-Strand Synthesis SuperMix	Invitrogen	Cat#18080400
GoTaq® qPCR Master Mix	Promega	Cat# A6001
Deposited data		
ABPP and (competitive) A*f*BPP approach using Callyaerin probes (**82** or **85**, respectively) in *M. tuberculosis* H37Rv.	This paper	PRIDE: PXD050770
Comparative A*f*BPP experiment using a Callyaerin probe (**85**) in *M. tuberculosis* H37Rv WT and Rv2113 gene deletion mutant (Δ*rv2113*)	This paper	PRIDE: PXD050770
Callyaerin B-induced proteomic dysregulation profile in *M. tuberculosis* H37Rv WT and Rv2113 gene deletion mutant (Δ*rv2113*)	This paper	PRIDE: PXD050763

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Proteomic dysregulation profile of *M. tuberculosis* H37Rv cells treated with an inactive Callyaerin B derivative (**36**)	This paper	PRIDE: PXD050763
Figure 2B image data	This paper	https://doi.org/10.6084/m9.figshare.25569462
Figure 6D+E image data	This paper	https://doi.org/10.6084/m9.figshare.25586964
Figure S23 image data	This paper	https://doi.org/10.6084/m9.figshare.25587975
Genome sequencing data of spontaneous resistant *M. tuberculosis* mutants	This paper	https://www.ncbi.nlm.nih.gov/sra/PRJNA1098701
M. tuberculosis H37Rv reference proteome	UniProt[59]	UP000001584
STRING database	Szklarczyk et al.[41]	N/A
Experimental models: Cell lines		
Homo sapiens HEK293	CLS Cell Lines Service GmbH, Germany	Cat# 300192
Homo sapiens THP-1	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany	Cat# ACC 16
Homo sapiens HepG2	CLS Cell Lines Service GmbH, Germany	Cat# 300198
Homo sapiens MRC-5	CLS Cell Lines Service GmbH, Germany	ATCC® CCL171
Oligonucleotides		
Oligonucleotides used for RT-qPCR, see Table S1	Custom synthesis, Microsynth AG	N/A
Oligonucleotides employed for the generation of allelic exchange substrates for site-specific gene deletion of *rv2113*, see Table S2	Custom synthesis, Microsynth AG	N/A
Oligonucleotides used for cloning *rv2113* and *BCG_2130* into mycobacterial expression plasmid pMV361, see Table S3	Custom synthesis, Microsynth AG	N/A
Forward primer for qPCR validation of Rv2113/BCG_2130 expression 5′-ATCGTGCTACTGACGGCATTG-3’	Custom synthesis, Microsynth AG	N/A
Reverse primer for qPCR validation of Rv2113/BCG_2130 expression 5′-GCGATGGCGAGCAGAAAAAC-3’	Custom synthesis, Microsynth AG	N/A
Recombinant DNA		
p0004S	Jain et al.,[46] gift of William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
pMV361	Stover et al.,[60] gift of William R. Jacobs Jr., Albert Einstein College of Medicine	N/A
pMV361::*rv2113*	This paper	N/A
pMV361::*BCG_2130*	This paper	N/A
Software and algorithms		
Proteome Discoverer (v2.5)	Thermo Fisher Scientific	Cat#OPTON-31040
Perseus (v2.0.7.0)	Tyanova et al.[50]	https://maxquant.net/perseus/
MaxQuant (v2.3.0.1)	Cox & Mann[52]	https://maxquant.net/maxquant/
MaxLFQ	Cox et al.[53]	N/A
Minora LFQ	Thermo Fisher Scientific	N/A
BLAST protein alignment algorithm	Altschul et al.[21]	https://blast.ncbi.nlm.nih.gov/Blast.cgi
ChemDraw (v20.0)	PerkinElmer	N/A
MestReNova (v14.2)	Mestrelab Research	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Prism (v8.0)	Graphpad	N/A
Xcalibur (v4.1)	Thermo Fisher Scientific	N/A
Casava (v1.8)	Illumina	N/A
ImageJ	Schneider et al.[61]	https://imagej.nih.gov/ij/
Tecan i-control (v1.1)	Tecan group	N/A
MxPro – Mx3005P (v4.10)	Stratagene	N/A
NIS-Elements	Nikon	N/A
PrimerQuest	Integrated DNA Technologies	N/A
VisionWorks (v8.16090.9066)	Analytik Jena	N/A
Clone Manager 9 (v9.5.0.0)	Sci Ed Software	N/A
Cytoscape (v3.9.1)	Shannon et al.[57]	https://cytoscape.org/index.html
Other		
UV lamp	Analytik Jena	Cat#UVP 3UV™
Tough micro-organism lysing VK05 0.5mm glass beads – 2 mL	Bertin Technologies	Cat#P000913-LYSK0-A
Micro-organism lysing VK01 0.1mm glass beads – 2 mL	Bertin Technologies	Cat#P000914-LYSK0-A
0.2 μm cellulose acetate syringe filter	Starstedt	Cat#83.1826.001
GF/C Glass Fiber Filter Paper	GE Healtcare	Cat#1822-024
Empty self-pack nanoLC columns	CoAnn Technologies	Cat#ICT36007515F-50
Kinetex® XB-C18 column material	Phenomenex	Cat#4498
ReproSil-Pur C18-AQ column material	Dr. Maisch	Cat#r119.aq
Empore™ SPE Disks C18	3M	Cat#66883-U
Protein LoBind Tube 2 mL	Eppendorf	Cat#0030 108.132
Protein LoBind Tube 1.5 mL	Eppendorf	Cat#0030 108.116
Conical Tubes 15 mL	Eppendorf	Cat#0030 122.151
Sera-Mag hydrophilic SpeedBeads	Cytiva	Cat#45152105050250
Sera-Mag hydrophobic SpeedBeads	Cytiva	Cat#65152105050250
Luna 5 μm C18(2) column (21.2 × 100 mm)	Phenomenex	Cat#00D-4252-P0-AX
Nucleodur 5 μm C18 Pyramid column (4 × 250 mm)	Macherey-Nagel	Cat#760202.40
Nucleodur 5 μm C18 Pyramid column (10 × 250 mm)	Macherey-Nagel	Cat#762272.100
Nucleodur 5 μm C18 Pyramid column (32 × 250 mm)	Macherey-Nagel	Cat#762272.320
Cell culture plate, 6 well, surface: suspension, flat base	Sarstedt	Cat#83.3920.500
Micro test plate, 96 well, slip-on lid, round base, PS, transparent	Sarstedt	Cat#82.1582.001
Cell culture plate, 96 well, surface: suspension, flat base	Sarstedt	Cat#83.3924.500
Cell culture plate, 96 well, surface: standard, flat base	Sarstedt	Cat#83.3924
Nunc™ F96 MicroWell™ White Polystyrene Plate	Thermo Scientific	Cat#236105
Nunc™ F96 MicroWell™ Black Polystyrene Plate	Thermo Scientific	Cat#237105
Nalgene™ Square PETG Media Bottles with Closure: Sterile, Shrink-Wrapped Trays	Thermo Scientific	Cat#342020

RESOURCE AVAILABILITY

Lead contact

Requests for additional information on data, resources, and reagents should be directed to and will be fulfilled by the lead contact, Rainer Kalscheuer (rainer.kalscheuer@hhu.de).

Materials availability

The reagents and mutant strains generated in this study will be made available subject to a completed Materials Transfer Agreement and proof of required BSL3 and import permits.

Data and code availability

- Proteomic data have been deposited at PRIDE. Microscopy data have been deposited at (https://figshare.com). Genome sequencing data have been deposited at Sequence Read Archive (SRA). All data are publicly available as of the date of publication, and accession numbers are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Bacterial strains and culture conditions

M. tuberculosis strains including H37Rv, mc^{2}6230 (Δ*panCD* ΔRD1) and several XDR-TB clinical isolates originating from South Africa, *M. bovis* BCG Pasteur as well as *M. smegmatis* mc^{2}155 used in this study were obtained from the laboratory of William R. Jacobs Jr. (Albert Einstein College of Medicine, Bronx, NY). Cells of mycobacteria were grown at 37°C and 80 rpm in liquid Middlebrook 7H9 medium supplemented with 10% ADS enrichment (5% bovine serum albumin fraction, 2% glucose and 0.85% sodium chloride), 0.5% glycerol and 0.05% tyloxapol, or solid Middlebrook 7H10 agar supplemented with 10% ADS enrichment and 0.5% glycerol. Selective media were supplemented as required with hygromycin (50 μg/mL), apramycin (30 μg/mL) or kanamycin (20 μg/mL). Cultures of non-mycobacterial species such as *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Enterococcus faecalis*, *E. faecium*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were grown at 37°C and 180 rpm in lysogeny broth (LB) or on solid LB medium.

Cell lines and culture conditions

The human THP-1 monocytic cell line (isolated from the peripheral blood of a 1-year old male patient suffering from acute monocytic leukemia)[42] and MRC-5 lung fibroblasts (isolated from the lung tissue of a 14-week-old male fetus)[43] were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium and Dulbeccós modified Eagle medium (DMEM), respectively, both media supplemented with 10% fetal bovine serum (FBS). HEK293 kidney cells (isolated from a human female embryo)[44] were grown in Eagle's minimum essential medium (EMEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids and 10% FBS, while HepG2 cells (isolated from liver tissue of a 15-year-old Caucasian male with a hepatocellular carcinoma)[45] were cultivated in Ham's F12 medium supplemented with 2 mM L-glutamine and 10% FBS. All cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

METHOD DETAILS

General information about the chemical synthesis of callyaerins

All chemicals were purchased from *abcr*, *Acros Organics*, *Alpha Aesar*, *Bachem*, *BLD pharm*, *Carbolution*, *Fluorochem*, *Iris Biotech*, *Lumiprobe*, *Merck, TCI Chemicals*, or *Thermo scientific* and used without further purification.

Purification of peptide precursors or compounds via reversed-phase preparative high performance liquid chromatography (prep. HPLC) was performed either on a Prominence ultra fast liquid chromatography (UFLC) system from *Shimadzu* (System A) with peak detection through an UV detector at λ = 210 nm equipped with a *Phenomenex* C18 column (*Luna* 5 μm C18(2), 100 mm length, 21.2 mm ID) or a Nexera Prep HPLC system from *Shimadzu* (System B) with peak detection through a photodiode array detector at λ = 210 nm equipped with *Macherey-Nagel* C18 columns (*Nucleodur* 5 μm C18 Pyramid, 250 mm length) with either 10 mm or 32 mm ID. In both systems, the mobile phase was generated by passive mixture of solvent B (Acetonitrile (ACN) either with or without 0.1% trifluoracetic acid (TFA)) in solvent A (H_2O either with or without 0.1% TFA) in a linear gradient. Purified peptide precursors or compounds were freeze-dried using an ALPHA 2-4 LD plus lyophilizer from *Christ*.

Liquid chromatography analysis was carried out on a *ThermoFisher Scientific* Ultimate 3000 UHPLC system (UV peak detection at λ = 254 nm) equipped with a *Macherey-Nagel* C18 column (*Nucleodur* 5 μm C18 Pyramid, 250 mm length, 4 mm ID). The mobile phase was generated by passive mixture of solvent B (ACN with 0.1% formic acid (FA)) in solvent A (H_2O with 0.1% FA) in a linear gradient (hold at 10% solvent B within the first 0.5 min, rise from 10-60% in the next minute, rise to 100% in the next 4.5 min, hold for 3.2 min, decrease to 10% within 0.3 min and hold for 2.5 min). In some cases, the length of the 100% solvent B plateau

had to be increased for elution of the compounds. All compounds were analyzed from a 10 mM DMSO stock diluted 1:50 with 50% ACN in H_2O (200 μM analyte, 2% DMSO). This leads to the presence of a DMSO peak in the chromatogram of the compounds (as shown in Data S2).

High-resolution mass spectrometry (HRMS) spectra of compounds were recorded on a *ThermoFisher Scientific* Exactive Plus EMR mass spectrometer with a TriVersa NanoMate nanoESI system from *Advion* equipped with 5 μm diameter nozzle spray chips. A total sample volume of 5 μL were picked out of 96-well plates and the ESI spray was generated using 0.8 psi nitrogen backpressure combined with a positive nozzle chip voltage of 1.7 kV. The given *m/z* values represent the average of at least 200 scans.

Nuclear magnetic resonance (NMR) spectra were recorded at 25°C on a Bruker 400 MHz Avance II NMR spectrometer with a 5 mm PABBI broad band probe (400 MHz for ^{1}H and 101 MHz for ^{13}C NMR) or a 700 MHz Avance II NMR spectrometer with a 5 mm TCI cryoprobe (700 MHz for ^{1}H and 176 MHz for ^{13}C NMR) spectrometer. ^{1}H NMR spectra were reported in the following manner: chemical shifts (δ) in parts per million (ppm) refer to the residual non-deuterated solvent signals, multiplicities (s: singlet, d: doublet, t: triplet, dd: doublet of doublets, dt: doublet of triplets; td: triplet of doublets, m: multiplet), coupling constants (J) in Hertz (Hz) and number of protons (H). ^{1}H and ^{13}C NMR spectra of synthesized callyaerin derivatives are shown in Data S1.

Solid phase peptide synthesis

Peptide precursors for the synthesis of Callyaerin derivatives were synthesized using microwave-assisted automated solid phase peptide synthesis (SPPS) using functionalized polystyrene (PS) resin in a Liberty Blue HT12 Peptide Synthesizer from *CEM*. For this purpose, the respective resin was swollen in dimethylformamide (DMF). The peptide synthesizer was equipped with stock solutions of the corresponding fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids at a concentration of 0.2 M in DMF. Furthermore, stock solutions of the activator reagent *N,N′*-Diisopropylcarbodiimide (DIC, 0.5 M) and the activator base ethyl cyano-hydroxyiminoacetate (Oxyma, 1 M) in DMF as well as a 20% piperidine in DMF solution for Fmoc-deprotection were also generated. If synthesis on Rink amide resin were performed, additional stocks of *N,N*-diisopropylethylamine (DIPEA, 1 M) and acetic anhydrate (Ac_2O, 0.5 M) for capping of unreacted binding positions on the resin and optional acetylation of the peptide N-terminus were also added to the device. For the capping of 2-chlorotrityl chloride (2-CTC) resin, a DCM:MeOH:DIPEA (17:2:1) mixture was used instead.

Microwave-assisted SPPS with Rink-amide resin

Loading of Rink-amide resin with the first amino acid of the sequence was done by starting with an initial deprotection step with 20% piperidine in DMF using microwave heating to 90°C for 120 seconds. Once the reactor was drained, the deprotection was repeated again for another 65 s. After washing of the resin, the first amino acid was linked to the resin by using 5 equivalents each (compared to initial resin loading capacity) of the respective amino acid, activator and activator base at microwave heating to 90°C for 240 s. A second double-coupling step was performed, followed by a capping step by using 10 eq. of DIPEA and 5 eq. of Ac_2OH at microwave heating to 50°C for 360 s to deactivate non-reacted binding positions on the resin. After loading, the next amino acids were coupled by an iterative protocol consisting of piperidine-mediated deprotection (90°C, 65 s) and double amino acid coupling (5 eq., 90°C, 240 s) cycles. The synthesis was closed by a final Fmoc deprotection step (90°C, 65 s), thereby delivering the resin-bound precursor peptide.

Microwave-assisted SPPS with 2-CTC resin

Loading of 2-CTC resin with the first amino acid of the sequence was performed by using 10 eq. of the initial Fmoc-protected amino acid in combination with 20 eq. of DIPEA at microwave heating to 50°C for 10 minutes. Once the reactor was drained, the loading step was repeated, followed by capping of unreacted binding positions using DCM:MeOH:DIPEA (17:2:1) at microwave heating to 50°C for 10 min. After this loading procedure, an iterative protocol of deprotection with 20% piperidine in DMF (50°C, 5 min) and double amino acid coupling using 5 equivalents each of the respective amino acid, activator and activator base at microwave heating to 50°C for 10 min was used for elongation of the peptide sequence. The synthesis was closed by a final Fmoc deprotection step (50°C, 5 min), thereby delivering the resin-bound precursor peptide.

Cleavage of the SPPS-synthesized peptides from the resin

Peptides were cleaved from the resin by adding a mixture of 2.5% H_2O and 2.5% triisopropyl silane (TIS) in trifluoroacetic acid (TFA) to the peptide-loaded resin for 3 to 5 hours at room temperature. The resin was separated from the cleavage solution which was poured into ice-cold diethyl ether. The occurring peptide precipitation was supported by overnight cooling of this solution at -20°C. The peptides were separated from the solution via centrifugation at 4°C and 4200 g for 30 min, the supernatant was discarded and the peptide-pellet was air-dried for further purification via prep. HPLC.

Determination of minimal inhibitory concentration (MIC)

The MIC was determined for all bacteria using broth microdilution method. For all mycobacteria species, cultures obtained from exponentially growing cells cultured in supplemented 7H9 medium were diluted and seeded at 1 × 10^5 CFU/well in 96-well round bottom microplates, in a total volume of 100 μL of supplemented 7H9 medium containing two-fold serially diluted test compounds with a starting concentration of 100 μM. The plates were incubated at 37°C for 5 days (*M. tuberculosis* strains and *M. bovis* BCG Pasteur) or 24 h (*M. smegmatis*). 10 μL of a 100 μg/mL resazurin solution was subsequently added to each well and further incubated for 16-24 h. To fix the cells for MIC determination, 100 μL of 10% formalin was added to each well (5% final concentration) for at least

30 min. Using a microplate reader (excitation, 540 nm; emission, 590 nm), fluorescence was quantified. Percentage of growth was calculated relative to sterile medium by subtraction of background fluorescence (0% growth) and maximum growth in untreated cells in the DMSO solvent control (100% growth).

For nosocomial pathogens and *B. subtilis*, cultures obtained from exponentially growing bacteria in Mueller Hinton broth (MHB) were diluted and seeded at 5×10^4 CFU/well in 96-well round bottom microplates, in a total volume of 100 μL MHB containing two-fold serially diluted test compounds with a starting concentration of 100 μM. Microplates were incubated for 24 h at 37°C. MIC was determined macroscopically by identifying the lowest concentration that resulted in inhibition of visible bacterial growth.

Cytotoxicity determination

The cytotoxicity of callyaerins was determined using the human THP-1 monocytic, MRC-5 lung fibroblast, HepG2 liver and HEK293 kidney cell lines. Cells were diluted and seeded at 5×10^4 cells/well in tissue culture-treated 96-well flat bottom microplates, in a total volume of 100 μL of respective media containing two-fold serially diluted test compounds with a starting concentration of 100 μM. 10 μL of a 100 μg/mL resazurin dye dilution was added to each well following 48 h incubation of microplates at 37°C and 5% CO_2. After 3 h incubation with resazurin, fluorescence was quantified in a microplate reader (excitation, 540 nm; emission, 590 nm). Percentage of growth was calculated relative to sterile medium (0% growth) and DMSO solvent control (100% growth). Selectivity index of test-compounds was determined as the quotient of the cytotoxic concentration inhibiting 50% growth of human cells and the MIC resulting in 90% inhibition of mycobacterial growth (IC_{50}/MIC_{90}).

Mycobacterial killing kinetic

Killing kinetic studies were done by quantifying viable cell counts following different treatment intervals to assess bactericidal or bacteriostatic activity of compounds. For this, a mid-exponentially growing culture of *M. tuberculosis* mc^26230 was adjusted to 2×10^6 CFU/mL and challenged with 5-fold MIC of CalB alone or in combination with clinical anti-TB drugs (isoniazid, rifampicin and bedaquiline) and incubated for 5 weeks at 37°C and 80 rpm. At various time intervals, 200 μL aliquots were taken and 10-fold serially diluted. Serial dilutions were plated on Middlebrook 7H10 agar plates and incubated at 37°C for 3 weeks to count colony forming units (CFU).

Checkerboard assay

The interaction between CalB and clinical anti-TB drugs were quantified by determining the fractional inhibitory concentration indices (FICI) of CalB with these drugs in 96-well round bottom microplates. Compounds tested in combination were two-fold serially diluted in a two-dimensional fashion. Cultures of *M. tuberculosis* H37Rv and mc^26230 were diluted and added to the microplates at a density of 1×10^5 CFU/well in a total volume of 100 μL. After 5-day incubation at 37°C, 10 μL of a100 μg/mL resazurin solution was added to each well and further incubated for 16- 24 h. Mycobacterial growth was quantified as a function of fluorescence intensity measured by a microplate reader (excitation, 540 nm; emission, 590 nm). Subsequently, the FICI was calculated as the sum of the quotients of the MICs of each bioactive agent when tested in combination (A and B) and the MIC when tested alone:

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

Synergism or antagonism between CalB and clinical anti-TB drugs were represented as FICI $\leq$ 0.5 or > 2, respectively. Partial synergism was denoted by 0.5 < FICI $\leq$ 0.75, while 0.75 < FICI $\leq$ 2 indicates an additive effect.[22]

Intracellular ATP measurement

Exponentially growing culture of *M. tuberculosis* mc^26230 was adjusted to OD (optical density) 0.5 at 600 nm and challenged with 5- or 10-fold MIC of CalB and relevant controls at 37°C and 80 rpm. At various time intervals, 1 mL culture aliquots were taken, pelleted by centrifugation at 10,000 *g* for 5 min, and supernatants were discarded to remove extracellular ATP. Cell pellets were resuspended in PBS and dispensed in a white opaque 96-well microplate containing BacTiter-Glo reagent (Promega, Madison, WI, USA). The resulting 1:1 mixture was incubated for 5 min and luminescence was measured as a function of ATP content using a microplate reader.

Propidium iodide internalization assay

A culture of *M. tuberculosis* mc^26230 taken from exponential growth phase was adjusted to $OD_{600\ nm} = 0.5$, harvested by centrifugation at 4,500 *g*, 4°C for 10 min, washed and resuspended in an equal volume of PBS supplemented with 1% glucose. Cells were then incubated with 15 μg/mL propidium iodide (PI). Cells were dispensed into a black opaque 96-well microplate upon incubation of cells with PI at 37°C for 30 min to establish a baseline PI fluorescence measurement (excitation, 535 nm; emission, 617 nm) using a microplate reader. Cells were then challenged with 5-fold MIC (final concentration) of CalB or appropriate controls and immediately returned to the microplate reader to quantify uptake of PI by measuring red fluorescence in a kinetic fashion for at least 4 h.

RNA extraction and real-time quantitative PCR

Cells of an exponentially growing culture of *M. tuberculosis* were adjusted to $OD_{600\ nm} = 1.5$, incubated with 5-fold MIC of compounds and harvested after 24 h by centrifugation at 4,500 *g*, 4°C for 10 min for RNA extraction. Harvested cells were fixed overnight in 5 mL

RNA Protect reagent (Qiagen) after incubation with compounds. Fixed cells were pelleted, resuspended in 1 mL RLT buffer, and then transferred into Precellys® tubes containing a mixture of 0.5 mm and 0.1 mm silica beads. Cells were lysed by bead beating at 50 Hz, and RNA was extracted using the RNeasy Mini kit (Qiagen). Residual genomic DNA was removed by DNase treatment. After RNA integrity was checked using RNA 6000 Nano Chip, 2 μg RNA per sample was reverse transcribed into complementary DNA using the SuperScript™ III First-Strand Synthesis kit (Thermo Fisher). Gene expression was quantified by performing quantitative real-time PCR (qPCR) in a Mx3005P qPCR system (Agilent Technologies) using GoTaq® qPCR Master Mix (Promega). Analyses were performed following the manufactureŕs protocol using gene-specific qPCR primers. Expression values were determined by the $\Delta\Delta C_T$ method, normalized to *16SrRNA* using DMSO-treated cells as the control. All qPCR primers (Table S1) were designed using the PrimerQuest tool.

Generation of a targeted *M. tuberculosis* Δ*rv2113* gene deletion mutant

Site-specific gene deletion in *M. tuberculosis* H37Rv was achieved by specialized phage transduction as described elsewhere.[46] Briefly, the *rv2113* gene was replaced with a γδ-*sacB*-*hyg*-γδ cassette comprising a hygromycin resistance gene and a *sacB* counter-selectable marker flanked by *res*-sites of the γδ-resolvase. For construction of the required allelic exchange substrate, upstream and downstream flanking regions of *rv2113* were amplified by PCR using primers listed in Table S2. Subsequently, the flanking regions were digested with the indicated restriction enzymes and ligated with the *Van91*I-digested p0004S vector. The resulting allelic exchange plasmid was then linearized with *Pac*I, cloned, and packaged into the temperature-sensitive phage ΦphAE159, yielding knock-out phages that were propagated in *M. smegmatis* at 30°C. Allelic exchange in *M. tuberculosis* was achieved by specialized transduction at the non-permissive temperature of 37°C, using hygromycin for selection, resulting in gene deletion and replacement by γδres-*sacB*-*hyg*-γδres cassette. Obtained hygromycin-resistant transductants were obtained after 3 weeks of incubation and were screened for correct gene disruption by diagnostic PCR.

Heterologous gene expression in mycobacteria

For genetic complementation and overexpression purposes, the genes *rv2113* and *BCG_2130* were amplified from genomic DNA of *M. tuberculosis* H37Rv or *M. bovis* BCG Pasteur, respectively, by PCR using the primer pairs listed in Table S3, and cloned into the integrative single-copy plasmid pMV361 containing an apramycin resistance marker providing constitutive heterologous gene expression from the HSP60 promoter. The plasmids were transformed into mycobacterial cells via electroporation (1000 Ω, 25 μF, 2500 V). Transformants were selected on Middlebrook 7H10 agar plates containing apramycin (40 mg/l).

Spontaneous resistant mutant generation

Spontaneous mutants of *M. tuberculosis* H37Rv resistant to callyaerins were generated by plating 500 μL culture aliquots containing $\sim 3 \times 10^7$ CFU on 120 mm square agar plates supplemented with 5-fold MIC of CalA or CalB, respectively. After 3-4 weeks of incubation at 37°C, independent colonies were picked for further analyses. Spontaneous mutants raised in the *rv2113* merodiploid strain of *M. tuberculosis* H37Rv were generated on agar additionally containing 30 μg/mL apramycin to select for presence of the integrative plasmid carrying the merodiploid copy of *rv2113*.

Whole-genome sequencing

Genomic DNA of independent resistant mutants was extracted using the CTAB-lysozyme method as described elsewhere.[47] Genomes of resistant mutants were sequenced with an Illumina HiSeq 2500 next generation sequencer (at Texas A&M University, College Station, TX, USA & Biological and Medical Research Center, Heinrich Heine University Düsseldorf, Germany) after preparing sequencing libraries using standard paired-end genomic DNA sample prep kit from Illumina. Genome sequences were compared with that of the parent *M. tuberculosis* H37RvMA (GenBank accession GCA_000751615.1). Paired-end sequence data was collected with a read length of 106 bp. Base-calling was performed with Casava software, v1.8. The reads were assembled using a comparative genome assembly method, with *M. tuberculosis* H37RvMA as a reference sequence.[48]

Intracellular activity in infected macrophages

For differentiation into macrophage-like cells, human THP-1 cells were seeded at a density of 1×10^5 cells per well in a total volume of 100 μL RPMI 1640 medium supplemented with 10% FBS and 50 nM phorbol-12-myristate-13-acetate (PMA) in a 96-well flat-bottom microtiter plate. Cells were incubated overnight in a humidified atmosphere at 37°C and 5% CO_2. The next day, cells were washed with PBS twice, and medium was replaced with fresh RPMI 1640 medium supplemented with 10% FBS containing 3×10^5 cells per well of a precultured *M. tuberculosis* H37Rv reporter strain constitutively expressing mCherry resulting in a multiplicity of infection of three (MOI = 3). After three hours of infection, cells were washed with PBS twice, and medium was replaced with fresh RPMI 1640 medium supplemented with 10% FBS containing either 15.6 μM of CalA or 1.95 μM of CalB, respectively. DMSO and standard antibiotics (1 μM rifampicin or 20 μM streptomycin) were used as negative and positive controls, respectively. After five days of cultivation at 37°C and 5% CO_2, cells were fixed with a final concentration of 5% formalin and incubated for 30 min at room temperature. Fluorescence was quantified using a Nikon Eclipse TS100 and NIS-Elements (100 x magnification, 500 ms exposure time). Integrated density of red fluorescence was calculated using Fiji (ImageJ).

Confocal microscopy

Cells of an exponentially growing culture of *M. tuberculosis* H37Rv were adjusted to $OD_{600\ nm}$ = 0.4 in Middlebrook 7H9 medium supplemented with 10% ADS enrichment, 0.5% glycerol and 0.05% tyloxapol and were treated with 0.2 μM of either **87** or Cy3 for 0.5, 3 or 6 h. Subsequently, cells were harvested by centrifugation at 5,000 *g* for 5 min and washed once with PBS containing 0.05% tyloxapol. Next, cells were stained with 4′,6-diamidino- 2-phenylindole (DAPI) for 30 min at 4°C. Upon washing stained cells three times with PBS containing 0.05% tyloxapol, cells were fixed with 10% formaldehyde for 1 h at RT before removal from BSL-3 confinement. Fixed samples were then washed once, resuspended in PBS containing 0.05% tyloxapol and centrifuged at 800 rpm for 10 min to remove cell aggregates. Samples were imaged using Zeiss LSM710 Confocal Laser Scanning Microscope after spreading single-cell suspensions on soft agarose pad. Captured images were subsequently analyzed using ImageJ.

Photoaffinity enrichment LC-MS/MS-based target identification

Cells of an exponentially growing culture of *M. tuberculosis* H37Rv were harvested by centrifugation at 4000 *g*, 4°C for 10 min and washed thrice with PBS supplemented with 0.05% tyloxapol. The cells were resuspended in PBS (containing 0.05% tyloxapol) and divided equally into 2-mL centrifuge tubes, with each tube containing 1 mL of the suspension. In the competition approach, some samples were pretreated with 30 μM CalB for 30 minutes at 37°C, whereas the remaining samples were supplemented with 0.3% DMSO in total. Cells were subsequently treated with the corresponding probes by adding 250 μL of a 5x stock (in PBS; 0.63% DMSO in total per sample) and incubating for 180 min while shaking at 37°C. The samples were then transferred to 6-well plates and exposed to irradiation with UV light (365 nm) for 20 minutes at room temperature. The cells were transferred back to 2-mL centrifuge tubes, pelleted by centrifugation for 5 min at room temperature and 4500 *g*, and washed three times with 1.5 mL of 50 mM Na_2HPO_4 (pH 8; Sigma-Aldrich, D8418). Pellets were resuspended in 1,050 μL of 50 mM Na_2HPO_4 and cells were lysed by bead-beating using 100 μm silica zirconium beads (50 Hz; 15 min). Subsequently, 350 μL of 4% sodium dodecyl sulfate (SDS; Carl Roth, 2326.2) in 50 mM Na_2HPO_4 were added and samples were incubated at 95°C for 5 min. After cooling down the samples for 15 min while shaking continuously, lysates were cleared by centrifugation at 13,000 *g* for 5 min at room temperature. Supernatants were collected for the use as protein solutions and sterile-filtered twice through a bacteria-tight 0.2 μm cellulose acetate syringe filter to remove viable bacteria.

To apply a two-step enrichment protocol for labeled proteins, a volume corresponding to 250 μg protein (Quantification: 660 nm Protein Assay with ionic detergent compatibility reagent; Thermo Scientific, 22660 & 22663) was transferred to 15 mL centrifuge tubes and filled up with 1% SDS in 50 mM Na_2HPO_4 to a total volume of 920 μL. For SDS concentration reduction and benzonase treatment, 920 μL benzonase-containing buffer (150 u per sample; EMD Millipore, 71206) was added and the samples were incubated with shaking at 37°C for 30 min. The subsequent Copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) was performed by transferring 120 μL reagent-mixture containing 167 μM Biotin-Azide (Jena Bioscience, CLK-AZ104P4), 1.67 mM TBTA (Sigma-Aldrich, 678937) and 33.3 mM TCEP (Sigma-Aldrich, C4706) to the samples and the click reaction was started by addition of 40 μL 100 mM $CuSO_4$ (Sigma-Aldrich, C1297). The mixture was incubated under continuous rotation for 150 min at room temperature. Proteins were then precipitated by the addition of a fourfold excess of ice-cold methanol (Fisher Chemical, M/4056/17) and samples were stored overnight at -20°C. Subsequently, the proteins were sedimented by centrifugation (4°C, 3220 g, 20 min), the supernatants were discarded, and the pellets were washed with 1 mL of ice-cold methanol. After an additional centrifugation step, protein pellets were taken up in 850 μL of 2% SDS in PBS and incubated at 37°C for several minutes. The total volume was increased to a final volume of 3 mL by further addition of PBS and the remaining pellet debris were disrupted by sonication. Insoluble particles were sedimented by centrifugation (1400 g, 5 min), the supernatant was transferred to a fresh 15 mL centrifuge tube, and the SDS concentration was reduced to 0.2% by adding PBS to a total volume of 8.5 mL. For enrichment, avidin-agarose (Thermo Scientific, 20225) was equilibrated to 0.2% SDS in PBS and thereafter 100 μL of the slurry suspension was added to each sample (approx. 50 μL bead volume). The suspension was incubated under continuous rotation for 60 min at room temperature. The beads were sedimented by centrifugation (5 min, 400 g), the supernatant was discarded and the beads were washed four times with 10 mL 1% SDS in H_2O by using an iterative wash procedure consisting of incubation (5 min), centrifugation (3 min, 400 g) and supernatant removal steps. After an additional washing step with 10 mL H_2O, the beads were transferred to a 1.5 mL centrifuge tube and washed two more times with 1 mL H_2O (centrifugation 1 min, 3000 g) to remove residual SDS. For on-bead digestion, the beads were taken up in 100 μL of 0.8 M urea (GE Healthcare, GE17-1319-01) in 50 mM ammonium bicarbonate (ABC; Sigma-Aldrich, 11213) containing 10 mM dithiothreitol (DTT; Sigma-Aldrich, 43815) and incubated for 60 min with continuous shaking (1500 rpm) at room temperature. Proteins were then alkylated (1500 rpm shaking, 75 min) by adding 20 mM iodoacetamide (IAM; Sigma-Aldrich, A3221). Excess IAM was quenched by increasing the DTT concentration to 25 mM (1500 rpm, 15 min) and digestion was started by the addition of 500 ng trypsin (Promega, V5111) per sample. The digest was incubated with continuous shaking (1250 rpm) for 18 h at 37°C and stopped by the addition of 5% formic acid (FA; Fisher Chemical, A117-50). After sedimentation of the beads (5 min, 600 g), the supernatant was transferred to a fresh centrifuge tube and the beads were washed with 50 μL of 1% FA. The supernatants were combined and passed through equilibrated glass microfiber tips (pore size: 1.2 μm, thickness: 0.26 mm, two disks per tip; GE Healthcare, 1822-024) to remove any residual beads. The resulting peptide solution was desalted on home-made C18 StageTips (two discs per tip; 3M, 66883-U) as described before,[49] and dry peptides were dissolved in 16 μL 0.1% FA (15 min, 1500 rpm; Fisher Chemical, A11705AMP). For analysis, 4 μL of sample was loaded on a self-packed fused silica capillary tube with evacuated-frit tip (75 μm ID x 50 cm, 15 μm orifice; CoAnn Technologies, ICT36007515F-50) filled with either Kinetex XB-C18 (particle size 1.7 μm, Pore Size 100 Å; Phenomenex, 4498) or ReproSil-Pur 120 C18-AQ (particle size 1.9 μm, Pore Size 120 Å; Dr. Maisch, r119.aq.) material.

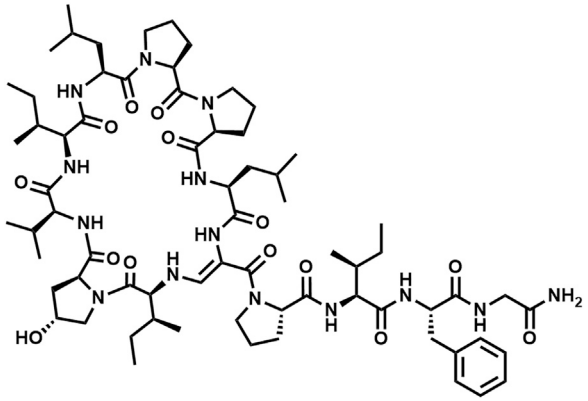
Peptides were separated using a 140 min gradient generated by an EASY-nLC 1200 liquid chromatography (Thermo Fisher Scientific) heated to 50°C by a PRSO-V1 column oven (Sonation). In case of Kinetex material, a rising proportion of 80% acetonitrile (ACN; Honeywell, 14261) in H_2O (Honeywell, 14263) with 0.2% FA (solvent B1) in 2% ACN in H_2O with 0.2% FA (solvent A1) was used for gradient preparation (3-7% B1 in A1 within the first 8 min, 7-27% in the next 84 min, 27-44% in the next 32 min, rise to 100% in the following 6 min, hold for 10 min) at a flow rate of 300 nL/min. In case of ReproSil-Pur material, a rising proportion of 80% acetonitrile (ACN) in H_2O with 0.1% FA (solvent B2) in H_2O with 0.1% FA (solvent A2) was used to prepare the gradient (6-32% B2 in A2 within the first 95 min, 32-42% in the next 20 min, rise to 100% in the following 10 min, hold for 15 min) at a flow rate of 250 nL/min. Peptides were ionized using a Nanospray Flex ion source (Thermo Fisher Scientific) with 2300-2500 V spray voltage and MS acquisition was performed in an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). MS1 data acquisition was done in a *m/z* range of 375 to 1800 at a 120000-240000 orbitrap resolution with a maximal injection time of 50 ns. Data dependent MS2 spectra were recorded using a loop cycle of 3 seconds with a dynamic exclusion duration of 20-25 seconds. For precursor isolation a 1.2 *m/z* quadrupole isolation window was used with subsequent stepped HCD fragmentation (20, 30, 40%) and data acquisition at rapid ion trap scan rate with 300% of normalized AGC target. Data processing was done with Proteome Discoverer 2.5 (Thermo Fisher Scientific) using SequestHT protein search and subsequent statistical analysis was done using Perseus 2.0.7.0.[50]

LC-MS/MS-based whole proteome comparison

Cells of exponentially growing cultures of *M. tuberculosis* H37Rv were adjusted to an $OD_{600\ nm}$ = 0.75 in a total volume of 20 mL Middlebrook 7H9 medium supplemented with 10% ADS enrichment and 0.05% tyloxapol. For treatment response analysis, cells were subjected to treatment with compound or a corresponding volume of DMSO as solvent control for 48 h at 37°C with shaking at 80 rpm. Subsequently, cells were harvested by centrifugation at 4,500 *g*, 4 °C for 10 min and washed thrice with PBS. Pellets were resuspended in 750 μL of PBS and cells were lysed by bead-beating using 100 μm silica zirconium beads (50 Hz; 15 min). After addition of 250 μL 4× lysis buffer (4% SDS, 40 mM TCEP, 160 mM chloroacetamide (CAM; Sigma-Aldrich, C0267), 200 mM HEPES (pH 8)), samples were incubated for 10 min at 95°C. Lysates were then cleared by centrifugation at 13,000 *g* and 4 °C for 5 min. Supernatants were collected to be used as protein solutions and were filter-sterilized twice through a bacteria-tight 0.2 μm cellulose acetate syringe filter to remove viable bacteria. For the digestion of proteins, a modified SP3 protocol was used.[51] For this purpose, a total volume equivalent to 15 μg protein was transferred from each sample to a fresh vessel (quantification: 660 nm protein assay with ionic detergent compatibility reagent; Thermo Scientific, 22660 & 22663) and the volume was equalized between samples by addition of 1× lysis buffer (1% SDS, 10 mM TCEP, 40 mM CAM, 50 mM HEPES). Benzonase-treatment was started by the addition of 7 u benzonase (37°C, 1200 rpm, 15-20 min) and subsequent cysteine alkylation was done using 5-10 mM IAM (37°C, 1200 rpm, 30-60 min). Hydrophilic (Cytiva, #45152105050250) and hydrophobic Sera-Mag SpeedBeads (Cytiva, #65152105050250) were mixed equally and afterwards equilibrated to water. To each sample 150 μg of particles were added and protein binding was induced by adding an equal volume of ethanol (Fisher Chemical, E/0650DF/17) to the samples (1250 rpm, 15-30 min). The particles were settled on a magnetic rack and further processed as already described.[33] For analysis, a volume equivalent to 500 ng of peptides was loaded on a self-packed fused silica capillary tube with evacuated-frit tip (75 μm ID x 50 cm, 15 μm orifice; CoAnn Technologies, ICT36007515F-50) filled with Reprosil-Pur 120 C18-AQ (particle size 1.9 μm, Pore Size 120 Å; Dr. Maisch, r119.aq.) material. Peptides were separated using a 140 min gradient generated by an EASY-nLC 1200 liquid chromatography (Thermo Fisher Scientific) heated to 50°C by a PRSO-V1 column oven (Sonation). For gradient generation, a rising proportion of 80% acetonitrile in H_2O with 0.1% FA (solvent B) in H_2O with 0.1% FA (solvent A) was used (8-33% B in A within the first 95 min, 33-42% in the next 20 min, rise to 100% in the following 10 min, hold for 15 min) at a flow rate of 250 nL/min, respectively. Peptides were ionized using a Nanospray Flex ion source (Thermo Fisher Scientific) with 2300 V spray voltage and MS acquisition was performed in an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). MS1 data acquisition was done in a *m/z* range of 375 to 1750 at a 120000-240000 orbitrap resolution with a maximal injection time of 50 ns. Data dependent MS2 spectra were recorded using a loop cycle of 3 seconds with a dynamic exclusion duration of 25-30 seconds. For precursor isolation a 1.2 *m/z* quadrupole isolation window was used with subsequent stepped HCD fragmentation (20, 30, 40%) and data acquisition at rapid ion trap scan rate with 300% of normalized AGC target. Data processing was done with MaxQuant 2.3.0.1[52] using Andromeda protein search and subsequent statistical analysis was done using Perseus 2.0.7.0.[50]

Synthetic procedures and characterization of Callyaerin derivatives

Callyaerin A (1)

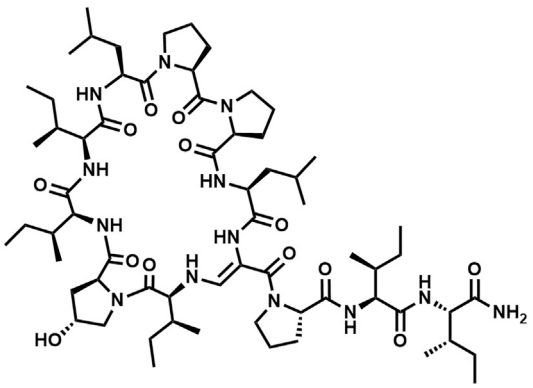


$C_{69}H_{108}N_{14}O_{14}$ M = 1357.71 g/mol

The linear precursor peptide of **1** was prepared via microwave-assisted solid-phase peptide synthesis (SPPS) using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 38 mg (27 μmol, 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of Dess-Martin-Periodinane (DMP) were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 6.1 mg (4.5 μmol, 16.4%) of **1** in a total yield of 4.5% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (700 MHz, DMSO-d_6) δ 9.02 (s, 1H), 8.81 (s, 1H), 8.28 (s, 1H), 8.15 (t, J = 6.1 Hz, 1H), 7.47 (d, J = 8.7 Hz, 1H), 7.44 (d, J = 7.0 Hz, 1H), 7.35 (d, J = 13.2 Hz, 1H), 7.26 (d, J = 8.6 Hz, 1H), 7.24 (s, 1H), 7.19-7.11 (m, 5H), 6.97 (s, 1H), 6.72 (d, J = 10.0 Hz, 1H), 5.87 (t, J = 11.7 Hz, 1H), 5.36 (d, J = 3.1 Hz, 1H), 4.61 (m, 2H), 4.40 (m, 1H), 4.30-4.22 (m, 4H), 4.09 (t, J = 10.2 Hz, 1H), 4.05 (t, J = 9.0 Hz, 1H), 3.90 (dd, J = 16.6, 7.1 Hz, 1H), 3.79 (d, J = 8.5 Hz, 2H), 3.77 (d, J = 11.6 Hz, 1H), 3.70 (dd, J = 11.5, 3.6 Hz, 1H), 3.64-3.57 (m, 2H), 3.52-3.49 (m, 2H), 3.48 (dd, J = 16.0, 4.6 Hz, 1H), 3.30 (m, 1H), 3.21 (td, J = 11.1, 5.8 Hz, 1H), 3.09 (m, 1H), 2.98 (m, 1H), 2.65 (m, 1H), 2.61 (m, 1H), 2.30 (m, 1H), 2.26 (m, 1H), 2.23 (m, 1H), 2.08 (m, 1H), 1.99 (m, 1H), 1.95-1.81 (m, 7H), 1.77 (m, 1H), 1.71 (m, 1H), 1.68-1.54 (m, 6H), 1.49 (m, 1H), 1.44-1.36 (m, 3H), 1.24 (m, 1H), 1.19-1.09 (m, 2H), 1.04 (m, 1H), 0.95 (d, J = 6.6 Hz, 3H), 0.88-0.86 (m, 6H), 0.85 (d, J = 6.6 Hz, 3H), 0.83-0.80 (m, 9H), 0.80-0.76 (m, 7H), 0.70 (t, J = 7.4 Hz, 3H), 0.42 (t, J = 7.3 Hz, 3H), 0.38 (d, J = 6.8 :Hz, 3H); ^{13}C NMR (176 MHz, DMSO-d_6) δ 173.4, 172.7, 172.4, 172.3, 172.0, 172.0, 171.5, 171.5, 171.5, 171.2, 171.1, 170.8, 167.8, 143.4, 138.1, 129.0, 129.0, 127.8, 127.8 126.1, 98.8, 68.7, 66.1, 64.4, 64.0, 62.5, 62.0, 59.8, 59.3, 58.6, 56.8, 55.7, 50.1, 49.2, 48.8, 47.0, 46.1, 42.0, 41.2, 40.6, 40.0, 37.9, 37.8, 36.2, 35.0, 29.5, 28.6, 27.2, 26.2, 25.9, 25.6, 25.1, 24.9, 24.9, 24.7, 24.6, 24.1, 23.3, 22.9, 21.9, 21.1, 19.6, 19.2, 15.2, 14.9, 14.3, 11.0, 10.6, 10.3; LC main peak retention time: 7.89 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1357.8242, found: 1357.8235 (–0.5 ppm); calculated for $[M+2H]^{2+}$: 679.4158, found: 679.4153 (–0.7 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5695 (–1.6 ppm).

Callyaerin B (2)

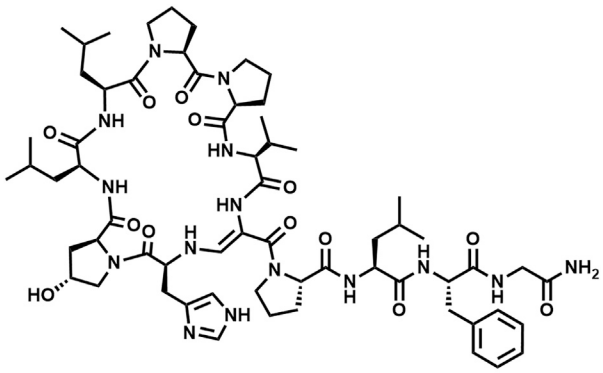


$C_{65}H_{109}N_{13}O_{13}$ M = 1280.67 g/mol

The linear precursor peptide of **2** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 63 mg (49 μmol, 49%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, 72 mg of the peptide was dissolved in 15 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 16.7 mg (13 μmol, 24%) of **2** in a total yield of 11.8% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (700 MHz, DMSO-d_6) δ 9.00 (s, 1H), 8.86 (s, 1H), 8.28 (s, 1H), 7.59 (d, *J* = 10.3 Hz, 1H), 7.37 (d, *J* = 6.9 Hz, 1H), 7.23 (d, *J* = 9.6 Hz, 1H), 7.16 (s, 1H), 7.05 (d, *J* = 13.2 Hz, 1H), 6.97 (s, 1H), 6.64 (d, *J* = 10.0 Hz, 1H), 5.71 (dd, *J* = 13.3, 10.1 Hz, 1H), 5.41 (s, 1H), 4.62 (q, *J* = 6.8 Hz, 1H), 4.55 (td, *J* = 10.4, 3.8 Hz, 1H), 4.41 (s, 1H), 4.26-4.24 (m, 2H), 4.23 (t, *J* = 4.0 Hz, 1H), 4.15 (dd, *J* = 9.6, 5.2 Hz, 1H), 4.10 (dd, *J* = 10.0, 8.6 Hz, 1H), 4.04 (t, *J* = 8.9 Hz, 1H), 3.95 (t, *J* = 10.2 Hz, 1H), 3.82-3.76 (m, 2H), 3.65 (dd, *J* = 11.2, 3.7 Hz, 1H), 3.63-3.56 (m, 2H), 3.52-3.45 (m, 2H), 3.32-3.28 (m, 1H), 3.15 (td, *J* = 9.9, 4.6 Hz, 1H), 3.07 (dd, *J* = 11.1, 6.8 Hz, 1H), 2.55-2.51 (m, 1H), 2.32-2.25 (m, 2H), 2.24-2.20 (m, 1H), 2.10-2.05 (m, 1H), 2.01-1.96 (m, 1H), 1.93-1.87 (m, 5H), 1.87-1.82 (m, 2H), 1.80-1.77 (m, 1H), 1.75 (dd, *J* = 13.6, 7.1 Hz, 1H), 1.72-1.66 (m, 2H), 1.64-1.56 (m, 3H), 1.55-1.50 (m, 1H), 1.49-1.37 (m, 7H), 1.30-1.25 (m, 1H), 1.25-1.21 (m, 1H), 1.18-1.09 (m, 2H), 1.07-1.02 (m, 1H), 1.02-0.95 (m, 2H), 0.90-0.80 (m, 30H), 0.79-0.77 (m, 6H), 0.75 (t, *J* = 7.4 Hz, 3H), 0.72 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (176 MHz, DMSO-d_6) δ 173.5, 172.8, 172.7, 172.3, 172.1, 171.5, 171.4, 171.4, 171.3, 171.2, 167.2, 142.7, 98.0, 68.8, 64.6, 64.3, 64.0, 62.5, 61.4, 60.0, 59.3, 57.9, 57.5, 56.7, 50.1, 49.2, 48.7, 47.1, 46.2, 41.2, 40.5, 37.9, 37.5, 36.4, 36.2, 36.2, 32.4, 29.7, 28.5, 26.3, 25.8, 25.6, 25.1, 24.9, 24.9, 24.8, 24.6, 23.9, 23.5, 23.1, 22.7, 22.2, 20.9, 15.8, 15.7, 15.3, 15.3, 14.4, 11.5, 11.1, 10.8, 10.3, 9.9; LC main peak retention time: 8.07 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1280.8341, found: 1280.8322 (–1.5 ppm); calculated for $[M+2H]^{2+}$: 640.9207, found: 640.9195(–1.9 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5832 (–3.6 ppm).

Callyaerin C (3)

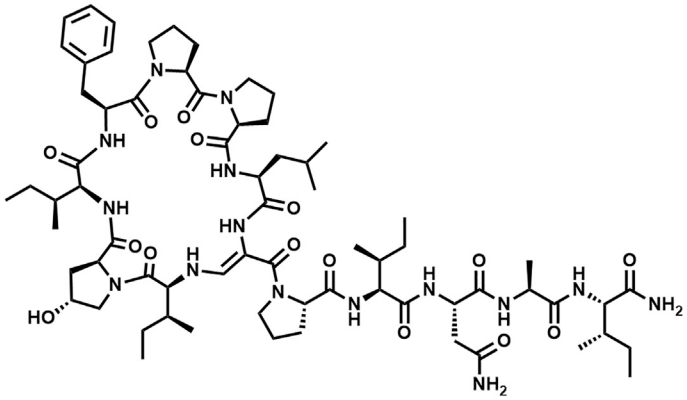


$C_{63}H_{93}N_{15}O_{13}$ M = 1268.53 g/mol

The linear precursor peptide of **3** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 8.7 mg (6.9 μmol) of **3** in a total yield of 6.9% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 12.03 (s, 1H), 9.84 (d, *J* = 7.7 Hz, 1H), 8.57 (s, 1H), 7.90 (d, *J* = 7.2 Hz, 1H), 7.80 (d, *J* = 9.3 Hz, 1H), 7.73 (dd, *J* = 7.1, 5.2 Hz, 1H), 7.65 (d, *J* = 13.8 Hz, 1H), 7.57 (d, *J* = 6.3 Hz, 1H), 7.39 (s, 1H), 7.37-7.34 (m, 2H), 7.32 (d, *J* = 10.4 Hz, 1H), 7.19 (s, 1H), 7.18-7.14 (m, 3H), 7.09 (s, 1H), 6.00 (s, 1H), 5.64 (dd, *J* = 13.8, 9.9 Hz, 1H), 5.09 (d, *J* = 3.7 Hz, 1H), 4.67 (dd, *J* = 10.2, 4.4 Hz, 1H), 4.52-4.45 (m, 1H), 4.45-4.37 (m, 2H), 4.35-4.27 (m, 2H), 4.27-4.20 (m, 1H), 4.19-4.10 (m, 2H), 4.07 (s, 1H), 3.95-3.83 (m, 2H), 3.71-3.62 (m, 1H), 3.60-3.45 (m, 6H), 3.30-3.25 (m, 1H), 3.23-3.14 (m, 1H), 2.99 (t, *J* = 12.6 Hz, 1H), 2.59-2.52 (m, 1H), 2.38-2.26 (m, 3H), 2.25-2.16 (m, 2H), 2.12-2.06 (m, 1H), 2.03-1.98 (m, 1H), 1.97-1.85 (m, 6H), 1.83-1.70 (m, 3H), 1.67-1.56 (m, 6H), 1.54-1.43 (m, 2H), 1.26-1.15 (m, 2H), 1.11 (d, *J* = 6.9 Hz, 3H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.91-0.84 (m, 12H), 0.82 (d, *J* = 6.5 Hz, 3H), 0.75 (d, *J* = 6.4 Hz, 3H); LC main peak retention time: 6.98 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1568.7150, found: 1268.7173 (1.8 ppm); calculated for $[M+2H]^{2+}$: 634.8612, found: 634.8616 (0.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 837.4618, found: 837.4627 (1.1 ppm).

Callyaerin D (4)

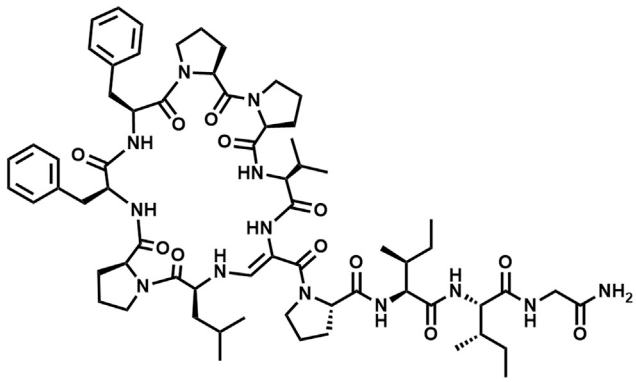


$C_{69}H_{107}N_{15}O_{15}$ M = 1386.71 g/mol

The linear precursor peptide of **4** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 5.7 mg (4.1 μmol) of **4** in a total yield of 4.1% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.42 (s, 1H), 7.88 (d, *J* = 7.5 Hz, 1H), 7.76 (d, *J* = 6.8 Hz, 1H), 7.64 (d, *J* = 5.0 Hz, 1H), 7.56 (d, *J* = 7.1 Hz, 1H), 7.53 (d, *J* = 9.3 Hz, 1H), 7.50 (d, *J* = 6.0 Hz, 1H), 7.38 (d, *J* = 3.3 Hz, 1H), 7.35 (s, 1H), 7.32-7.22 (m, 6H), 7.10 (s, 1H), 7.02 (s, 1H), 6.92 (s, 1H), 5.29 (dd, *J* = 13.8, 10.1 Hz, 1H), 5.19 (d, *J* = 3.8 Hz, 1H), 4.65-4.59 (m, 1H), 4.55 (q, *J* = 7.0 Hz, 1H), 4.51-4.44 (m, 1H), 4.44-4.38 (m, 2H), 4.36-4.30 (m, 2H), 4.21-4.11 (m, 3H), 4.05 (dd *J* = 8.9, 6.8 Hz, 1H), 4.00 (dd, *J* = 5.0, 3.4 Hz, 1H), 3.96-3.90 (m, 2H), 3.68-3.60 (m, 2H), 3.56-3.49 (m, 1H), 3.40-3.35 (m, 1H), 3.28-3.23 (m, 1H), 3.06 (dd, *J* = 13.1, 9.5 Hz, 1H), 2.80-2.73 (m, 1H), 2.73-2.66 (m, 1H), 2.39-2.30 (m, 1H), 2.27-2.12 (m, 4H), 2.10-2.04 (m, 1H), 2.02-1.88 (m, 5H), 1.86-1.68 (m, 9H), 1.65-1.56 (m, 1H), 1.52-1.30 (m, 8H), 1.27 (d, *J* = 7.2 Hz, 3H), 1.23 (s, 1H), 1.17-1.06 (m, 1H), 0.98-0.91 (m, 1H), 0.89-0.79 (m, 30H); LC main peak retention time: 6.18 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1386.8144, found: 1386.8153 (0.6 ppm); calculated for $[M+2H]^{2+}$: 693.9109, found: 693.9108 (–0.1 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 861.4869, found: 861.4870 (0.1 ppm).

Callyaerin E (5)

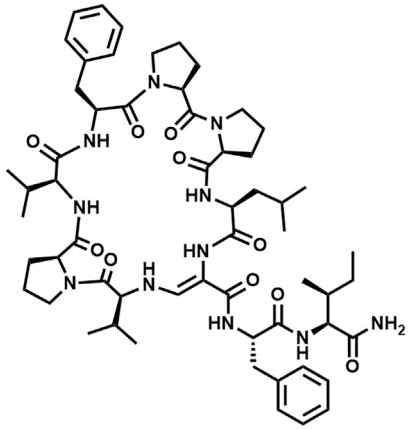


$C_{66}H_{95}N_{13}O_{12}$ M = 1262.57 g/mol

The linear precursor peptide of **5** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 130 mg (101 μmol, 44%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 60.1 mg (47.6 μmol, 46.9%) of **5** in a total yield of 20.7% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.37 (s, 1H), 7.89 (t, *J* = 6.0 Hz, 1H), 7.72 (d, *J* = 6.7 Hz, 1H), 7.63 (d, *J* = 8.0 Hz, 1H), 7.37 (d, *J* = 8.2 Hz, 1H), 7.33-7.16 (m, 12H), 7.06-7.01 (m, 2H), 6.99-6.94 (m, 1H), 5.27 (dd, *J* = 13.5, 9.7 Hz, 1H), 4.68-4.60 (m, 1H), 4.56 (dd, *J* = 10.2, 3.9 Hz, 1H), 4.45-4.32 (m, 4H), 4.16 (dd, *J* = 9.9, 7.5 Hz, 1H), 4.09 (t, *J* = 7.7 Hz, 1H), 4.04-3.96 (m, 2H), 3.96-3.90 (m, 1H), 3.71 (dd, *J* = 16.6, 6.2 Hz, 1H), 3.63-3.54 (m, 3H), 3.53-3.46 (m, 1H), 3.46-3.37 (m, 1H), 3.33-3.29 (m, 1H), 3.26-3.16 (m, 1H), 3.04-2.95 (m, 3H), 2.71 (dd, *J* = 13.4, 4.8 Hz, 1H), 2.49-2.43 (m, 1H), 2.37-2.27 (m, 1H), 2.25-2.11 (m, 4H), 2.10-2.01 (m, 1H), 1.98-1.53 (m, 14H), 1.49-1.34 (m, 3H), 1.30-1.16 (m, 3H), 1.05 (d, *J* = 6.9 Hz, 3H), 0.96 (dd, *J* = 6.7, 3.3 Hz, 6H), 0.90 (d, *J* = 6.6 Hz, 3H), 0.88-0.82 (m, 9H), 0.79 (t, *J* = 7.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.5, 172.6, 172.1, 171.7, 171.5, 171.5, 171.3, 171.0, 171.0, 169.1, 167.7, 143.1, 138.1, 137.9, 129.0, 128.4, 128.2, 128.1, 126.5, 126.4, 98.5, 63.5, 63.1, 61.0, 58.4, 57.8, 56.6, 54.0, 53.0, 48.7, 46.6, 46.4, 42.3, 42.2, 36.1, 35.5, 35.4, 35.3, 29.9, 28.8, 28.7, 27.0, 25.7, 25.5, 24.8, 24.4, 24.2, 23.8, 20.8, 18.3, 17.7, 15.7, 15.5, 11.2, 10.6; LC main peak retention time: 7.42 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1262.7296, found: 1262.7304 (0.6 ppm); calculated for $[M+2H]^{2+}$: 631.8685, found: 631.8686 (0.2 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 865.4607, found: 865.4597 (–1.2 ppm).

Callyaerin F (6)

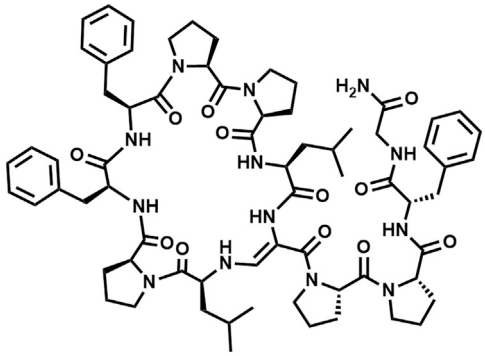


$C_{58}H_{83}N_{11}O_{10}$ M = 1094.37 g/mol

The linear precursor peptide of **6** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 116 mg (104 μmol, 45%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 14.1 mg (12.9 μmol, 12.4%) of **6** in a total yield of 5.6% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.25 (s, 1H), 7.66 (d, *J* = 9.6 Hz, 1H), 7.57-7.46 (m, 3H), 7.32-7.13 (m, 11H), 7.03 (s, 1H), 6.98 (s, 1H), 6.17 (d, *J* = 7.8 Hz, 1H), 5.14 (dd, *J* = 13.7, 10.0 Hz, 1H), 4.63 (td, *J* = 10.2, 3.9 Hz, 1H), 4.57-4.50 (m, 1H), 4.45-4.39 (m, 1H), 4.37 (d, *J* = 2.0 Hz, 1H), 4.31-4.23 (m, 2H), 4.01-3.92 (m, 2H), 3.89-3.82 (m, 2H), 3.77-3.68 (m, 1H), 3.59 (t, *J* = 8.3 Hz, 1H), 3.39-3.35 (m, 1H), 3.24 (t, *J* = 8.9 Hz, 1H), 3.01 (dd, *J* = 12.9, 9.6 Hz, 1H), 2.96-2.87 (m, 2H), 2.74 (dd, *J* = 12.9, 3.5 Hz, 1H), 2.35-2.26 (m, 2H), 2.22-2.00 (m, 5H), 1.97-1.88 (m, 2H), 1.88-1.77 (m, 4H), 1.77-1.63 (m, 4H), 1.50-1.38 (m, 2H), 1.33-1.21 (m, 1H), 1.16-1.03 (m, 1H), 0.93 (d, *J* = 4.7 Hz, 3H), 0.91 (d, *J* = 4.2 Hz, 3H), 0.88-0.84 (m, 6H), 0.82 (d, *J* = 6.3 Hz, 3H), 0.79-0.74 (m, 6H), 0.65 (d, *J* = 6.6 Hz, 3H); LC main peak retention time: 7.14 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1094.6397, found: 1094.6412 (1.4 ppm); calculated for $[M+2H]^{2+}$: 547.8235, found: 547.8242(1.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 817.4607, found: 817.4609 (0.2 ppm).

Callyaerin G (7)

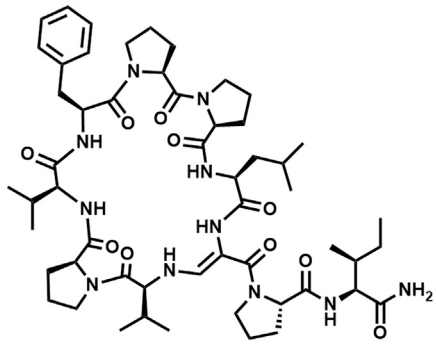


$C_{69}H_{91}N_{13}O_{12}$ M = 1294.57 g/mol

The linear precursor peptide of **7** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 83 mg (63 μmol, 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 35 mg (27 μmol, 42.9%) of **7** in a total yield of 11.7% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (s, 1H), 7.98 (dd, *J* = 7.9, 4.8 Hz, 1H), 7.75-7.66 (m, 2H), 7.52 (d, *J* = 9.7 Hz, 1H), 7.47 (d, *J* = 13.5 Hz, 1H), 7.38-7.34 (m, 2H), 7.30-7.10 (m, 16H), 5.33 (dd, *J* = 13.5, 9.9 Hz, 1H), 4.64 (q, *J* = 7.0 Hz, 1H), 4.53-4.40 (m, 2H), 4.38-4.20 (m, 5H), 4.11 (dd, *J* = 8.4, 6.6 Hz, 1H), 3.99-3.81 (m, 5H), 3.54-3.44 (m, 2H), 3.43-3.35 (m, 4H), 3.25 (dd, *J* = 13.2, 3.7 Hz, 1H), 3.04-2.94 (m, 2H), 2.93-2.84 (m, 2H), 2.79-2.72 (m, 1H), 2.68 (dd, *J* = 13.2, 6.3 Hz, 1H), 2.34-2.23 (m, 1H), 2.20-2.07 (m, 3H), 2.06-1.93 (m, 3H), 1.93-1.82 (m, 7H), 1.82-1.73 (m, 4H), 1.72-1.58 (m, 5H), 1.55-1.43 (m, 1H), 1.41-1.29 (m, 1H), 1.15-1.01 (m, 2H), 0.88 (d, *J* = 6.3 Hz, 3H), 0.84 (d, *J* = 6.2 Hz, 3H), 0.54 (d, *J* = 6.6 Hz, 3H), 0.38 (d, *J* = 6.3 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.3, 172.1, 171.9, 171.6, 171.4, 171.0, 171.0, 170.9, 170.7, 169.6, 168.1, 145.6, 138.2, 138.2, 137.7, 129.9, 129.2, 128.4, 128.2, 128.1, 128.0, 126.5, 126.3, 125.9, 98.0, 63.7, 63.0, 62.8, 61.7, 57.6, 54.6, 54.1, 52.5, 50.3, 47.7, 46.7, 46.3, 42.2, 36.9, 36.7, 35.2, 28.7, 27.9, 26.8, 25.6, 25.0, 24.4, 23.3, 22.9, 20.8, 20.2; LC main peak retention time: 6.60 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.6983, found: 1294.7013 (2.3 ppm); calculated for $[M+2H]^{2+}$: 647.8528, found: 847.8531 (0.5 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 879.4763, found: 879.4742 (–2.4 ppm).

Callyaerin H (8)



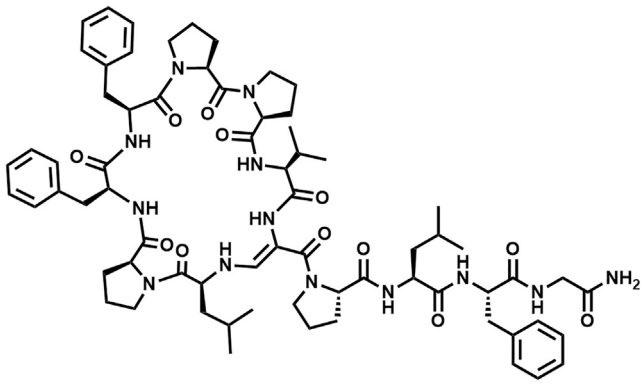
$C_{54}H_{81}N_{11}O_{10}$ M = 1044.31 g/mol

The linear precursor peptide of **8** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 98 mg (92 μmol, 40%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the

peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 4.7 mg (4.5 μmol, 4.9%) of **8** in a total yield of 2.0% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.47 (s, 1H), 7.59 0(d, *J* = 5.1 Hz, 1H), 7.55 (d, *J* = 9.6 Hz, 1H), 7.52 (s, 1H), 7.50 (d, *J* = 5.5 Hz, 1H), 7.31-7.20 (m, 6H), 6.97 (s, 1H), 6.89 (s, 1H), 5.26 (dd, *J* = 13.9, 9.9 Hz, 1H), 4.61-4.48 (m, 3H), 4.43 (d, *J* = 1.9 Hz, 1H), 4.37-4.31 (m, 2H), 4.17 (dd, *J* = 9.6, 7.7 Hz, 1H), 4.05-4.00 (m, 1H), 4.00-3.94 (m, 1H), 3.86 (t, *J* = 4.7 Hz, 1H), 3.84-3.79 (m, 1H), 3.79-3.71 (m, 1H), 3.65-3.58 (m, 1H), 3.39-3.32 (m, 1H), 3.30-3.22 (m, 2H), 3.04 (dd, *J* = 13.0, 9.5 Hz, 1H), 2.77 (dd, *J* = 13.0, 3.6 Hz, 1H), 2.40-2.30 (m, 1H), 2.28-2.15 (m, 4H), 2.14-2.07 (m, 2H), 2.07-2.02 (m, 1H), 1.99-1.89 (m, 2H), 1.89-1.81 (m, 4H), 1.80-1.65 (m, 5H), 1.60-1.36 (m, 4H), 1.24 (s, 1H), 1.22-1.13 (m, 1H), 0.93 (d, *J* = 6.9 Hz, 3H), 0.90-0.80 (m, 18H), 0.68 (d, *J* = 6.6 Hz, 3H); LC main peak retention time: 6.21 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1044.6241, found: 1044.6243 (0.2 ppm); calculated for $[M+2H]^{2+}$: 522.8157, found: 522.8137 (–3.8 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 817.4607, found: 817.4602 (–0.6 ppm).

Callyaerin I (9)

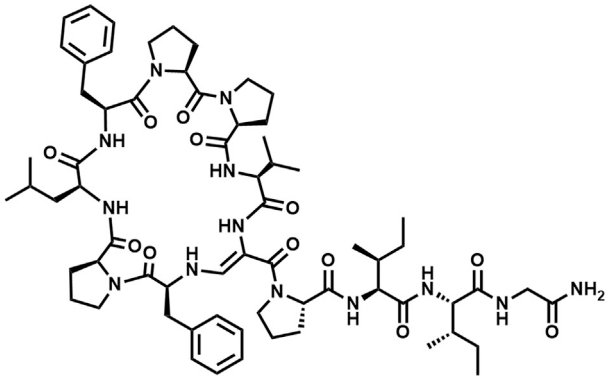


$C_{69}H_{93}N_{13}O_{12}$ M = 1296.58 g/mol

The linear precursor peptide of **9** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, 0acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 117 mg (89 μmol, 39%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 47.7 mg (36.8 μmol, 41.3%) of **9** in a total yield of 16% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.45 (s, 1H), 7.78 (d, *J* = 6.9 Hz, 1H), 7.68 (d, *J* = 9.7 Hz, 1H), 7.65 (d, *J* = 6.7 Hz, 1H), 7.53 (dd, *J* = 7.3, 5.1 Hz, 1H), 7.44 (d, *J* = 13.5 Hz, 1H), 7.32-7.22 (m, 11H), 7.21-7.18 (m, 1H), 7.16-7.11 (m, 6H), 7.03 (d, *J* = 10.1 Hz, 1H), 5.37 (dd, *J* = 13.6, 9.8 Hz, 1H), 4.68-4.59 (m, 2H), 4.47-4.35 (m, 3H), 4.30 (dd, *J* = 10.7, 7.2 Hz, 1H), 4.27-4.21 (m, 1H), 4.18 (dd, *J* = 10.0, 7.4 Hz, 1H), 4.01-3.94 (m, 2H), 3.89 (dd, *J* = 17.0, 7.4 Hz, 1H), 3.85-3.78 (m, 1H), 3.68-3.60 (m, 1H), 3.56-3.45 (m, 3H), 3.45-3.38 (m, 1H), 3.38-3.30 (m, 1H), 3.29-3.21 (m, 2H), 3.01-2.92 (m, 3H), 2.81-2.72 (m, 1H), 2.68 (dd, *J* = 13.3, 4.7 Hz, 1H), 2.48-2.43 (m, 1H), 2.34-2.27 (m, 1H), 2.26-2.19 (m, 1H), 2.18-2.10 (m, 2H), 2.07-1.99 (m, 1H), 1.96-1.82 (m, 4H), 1.81-1.68 (m, 4H), 1.67-1.49 (m, 6H), 1.48-1.36 (m, 1H), 1.26-1.22 (m, 1H), 1.20 (d, *J* = 6.9 Hz, 3H), 1.15-1.10 (m, 1H), 1.07 (d, *J* = 7.0 Hz, 3H), 1.04-0.98 (m, 1H), 0.87 (d, *J* = 6.5 Hz, 3H), 0.74 (d, *J* = 6.5 Hz, 3H), 0.44 (d, *J* = 6.4 Hz, 3H), 0.32 (d, *J* = 6.6 Hz, 3H).; ^{13}C NMR (101 MHz, DMSO-d_6) δ 174.2, 173.5, 172.1, 171.8, 171.7, 171.0, 170.7, 170.7, 169.1, 168.4, 143.5, 138.3, 138.1, 137.9, 129.8, 129.0, 128.4, 128.2, 128.1, 127.7, 126.5, 126.4, 126.0, 98.1, 63.5, 63.2, 63.0, 62.0, 57.6, 56.9, 53.8, 52.9, 48.8, 46.7, 46.4, 46.1, 42.2, 38.1, 37.7, 36.0, 35.2, 30.2, 29.4, 28.9, 28.7, 27.0, 26.0, 25.5, 24.9, 24.4, 24.2, 23.5, 23.1, 22.7, 20.8, 19.9, 18.2, 17.7; LC main peak retention time: 7.82 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1296.7139, found: 1296.7161 (1.7 ppm); calculated for $[M+2H]^{2+}$: 648.8606, found: 648.8610 (0.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 865.4607, found: 865.4597 (–1.2 ppm).

Callyaerin J (10)

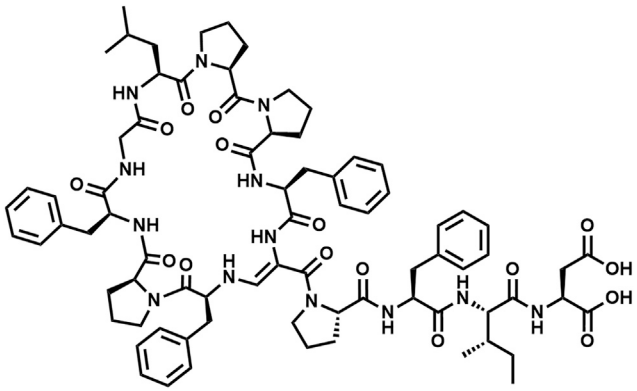


$C_{66}H_{95}N_{13}O_{12}$ **M = 1262.57 g/mol**

The linear precursor peptide of **10** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 29 mg (23 μmol) of **10** in a total yield of 23% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.21 (s, 1H), 7.84 (t, *J* = 6.0 Hz, 1H), 7.69 (d, *J* = 7.5 Hz, 1H), 7.44 (d, *J* = 6.2 Hz, 1H), 7.32 (d, *J* = 8.4 Hz, 1H), 7.29-7.16 (m, *J* = 7.2, 6.8 Hz, 10H), 7.14 (d, *J* = 6.7 Hz, 1H), 7.10 (d, *J* = 10.1 Hz, 1H), 7.07-7.01 (m, 3H), 5.44 (dd, *J* = 13.5, 9.6 Hz, 1H), 4.64-4.57 (m, 2H), 4.51 (dd, *J* = 10.2, 5.1 Hz, 1H), 4.36 (dd, *J* = 11.1, 7.7 Hz, 1H), 4.30 (t, *J* = 7.5 Hz, 1H), 4.15 (dd, *J* = 9.8, 7.5 Hz, 1H), 4.12-4.06 (m, 2H), 4.02 (dd, *J* = 10.1, 6.9 Hz, 1H), 3.98-3.92 (m, 2H), 3.89-3.80 (m, 1H), 3.70 (dd, *J* = 16.7, 6.5 Hz, 1H), 3.58-3.45 (m, 3H), 3.40-3.34 (m, 2H), 3.21-3.10 (m, 1H), 3.01 (dd, *J* = 13.5, 8.8 Hz, 1H), 2.73-2.61 (m, 2H), 2.56-2.52 (m, 1H), 2.33 (h, *J* = 6.7 Hz, 1H), 2.28-2.16 (m, 3H), 2.16-2.06 (m, 2H), 1.98-1.86 (m, 4H), 1.86-1.71 (m, 6H), 1.68-1.53 (m, 4H), 1.52-1.37 (m, 4H), 1.32-1.17 (m, 2H), 1.12-1.07 (m, 1H), 1.05 (d, *J* = 6.8 Hz, 3H), 1.01 (d, *J* = 6.8 Hz, 3H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.89-0.84 (m, 6H), 0.82 (d, *J* = 6.5 Hz, 3H), 0.69 (d, *J* = 6.9 Hz, 3H), 0.65 (t, *J* = 7.7 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.9, 172.5, 172.1, 172.0, 171.6, 171.5, 171.2, 171.0, 170.9, 169.1, 167.4, 142.3, 138.0, 136.1, 129.2, 129.0, 128.4, 128.1, 126.7, 126.4, 99.3, 63.6, 63.4, 63.0, 61.4, 60.6, 58.6, 57.2, 56.9, 52.7, 52.1, 48.6, 46.6, 42.2, 36.4, 35.5, 35.3, 29.9, 29.2, 27.0, 25.7, 25.5, 25.0, 24.4, 24.2, 22.7, 21.3, 18.9, 18.5, 15.6, 11.3, 10.9; LC main peak retention time: 7.58 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1262.7296, found: 1262.7307 (0.9 ppm); calculated for $[M+2H]^{2+}$: 631.8685, found: 631.8688 (0.5 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 865.4607, found: 865.4595 (–1.4 ppm).

Callyaerin K (11)

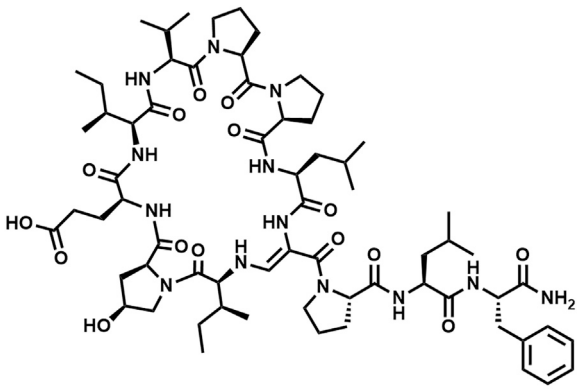


$C_{77}H_{97}N_{13}O_{16}$ **M = 1460.70 g/mol**

The linear precursor peptide of **11** was prepared via microwave-assisted SPPS using 2-CTC PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 44 mg (30 μmol, 30%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 7.6 mg (5.2 μmol, 13.5%) of **11** in a total yield of 5.2% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.26 (d, *J* = 8.4 Hz, 1H), 8.94 (s, 1H), 8.33 (d, *J* = 7.5 Hz, 1H), 7.81 (d, *J* = 9.4 Hz, 1H), 7.47 (s, 1H), 7.41-7.34 (m, 4H), 7.30-7.23 (m, 8H), 7.23-7.16 (m, 6H), 7.12-7.08 (m, 2H), 7.07-7.03 (m, 2H), 6.98 (s, 1H), 5.64-5.48 (m, 1H), 5.18-5.07 (m, 1H), 4.61 (s, 1H), 4.53-4.45 (m, 1H), 4.32 (ddd, *J* = 12.7, 9.3, 3.6 Hz, 1H), 4.22-4.12 (m, 2H), 4.10-3.97 (m, 4H), 3.96-3.86 (m, 2H), 3.66 (s, 1H), 3.56-3.44 (m, 3H), 3.29-3.21 (m, 4H), 3.21-3.14 (m, 2H), 3.10-3.04 (m, 1H), 2.99-2.91 (m, 2H), 2.90-2.82 (m, 3H), 2.73-2.63 (m, 2H), 2.58 (dd, *J* = 16.4, 6.1 Hz, 1H), 2.21-2.11 (m, 1H), 2.07-1.99 (m, 1H), 1.97-1.73 (m, 6H), 1.68-1.52 (m, 4H), 1.50-1.27 (m, 8H), 1.23 (s, 1H), 1.14-1.05 (m, 1H), 0.88 (d, *J* = 6.5 Hz, 3H), 0.86-0.81 (m, 6H), 0.70 (t, *J* = 7.3 Hz, 3H), 0.65-0.53 (m, 1H); LC main peak retention time: 6.29 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1460.7249, found: 1460.7254 (0.3 ppm); calculated for $[M+2H]^{2+}$: 730.8661, found: 730.8662 (0.1 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 970.4822, found: 940.4814 (–0.8 ppm).

Callyaerin L (12)

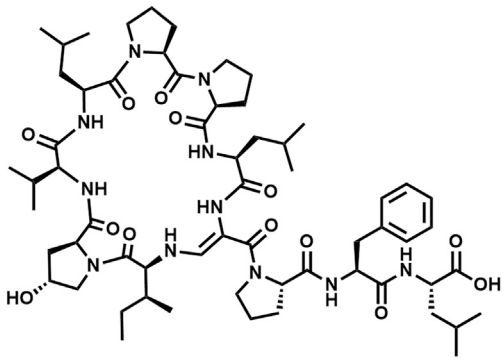


$C_{66}H_{101}N_{13}O_{15}$ M = 1316.61 g/mol

The linear precursor peptide of **12** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 43 mg (32 μmol, 32%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 8 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 3.7 mg (2.8 μmol, 8.7%) of **12** in a total yield of 2.8% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.75 (s, 1H), 8.27 (s, 1H), 7.76 (d, *J* = 7.6 Hz, 1H), 7.59-7.51 (m, 2H), 7.37 (d, *J* = 7.4 Hz, 1H), 7.32-7.26 (m, 1H), 7.25-7.18 (m, 3H), 7.14-7.04 (m, 3H), 6.91 (d, *J* = 9.9 Hz, 1H), 6.78 (s, 1H), 5.45-5.36 (m, 1H), 5.36-5.32 (m, 1H), 4.61-4.52 (m, 2H), 4.40 (s, 1H), 4.30-4.23 (m, 2H), 4.22-4.03 (m, 4H), 3.99-3.90 (m, 2H), 3.88-3.77 (m, 2H), 3.75-3.66 (m, 2H), 3.63-3.48 (m, 4H), 3.22-3.10 (m, 2H), 2.74-2.66 (m, 1H), 2.33-2.23 (m, 3H), 2.22-2.17 (m, 2H), 2.15-2.02 (m, 6H), 1.98-1.80 (m, 7H), 1.77-1.57 (m, 5H), 1.56-1.46 (m, 3H), 1.42-1.30 (m, 3H), 1.23-(s, 1H), 1.19-1.11 (m, 1H), 0.98 (d, *J* = 6.9 Hz, 3H), 0.96-0.91 (m, 6H), 0.88-0.79 (m, 9H), 0.76-0.68 (m, 9H), 0.15 (t, *J* = 7.3 Hz, 3H); LC main peak retention time: 5.46 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1316.7613, found: 1316.7621 (0.6 ppm); calculated for $[M+2H]^{2+}$: 658.8843, found: 658.884 (0.2 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 942.5295, found: 942.5301 (0.6 ppm).

Callynormine A (13)

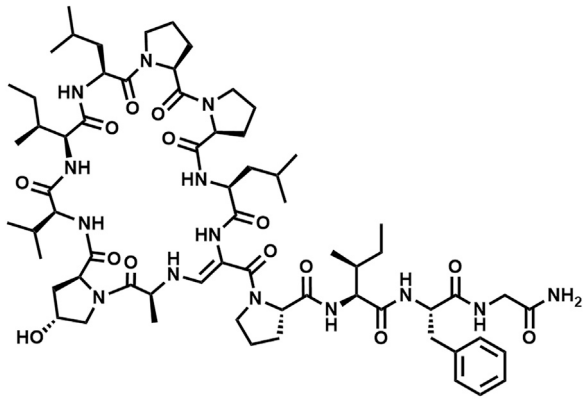


$C_{61}H_{93}N_{11}O_{13}$ M = 1188.48 g/mol

The linear precursor peptide of **13** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 55 mg (46 μmol, 46%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 17.7 mg (14.9 μmol, 32.7%) of **13** in a total yield of 14.9% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.24 (s, 1H), 7.75 (d, *J* = 9.4 Hz, 1H), 7.56 (d, *J* = 3.8 Hz, 1H), 7.54 (s, 1H), 7.37-7.26 (m, 5H), 7.20 (d, *J* = 5.9 Hz, 1H), 7.19-7.17 (m, 1H), 7.05 (d, *J* = 13.7 Hz, 1H), 5.24 (dd, *J* = 13.7, 9.4 Hz, 1H), 5.17 (d, *J* = 3.7 Hz, 1H), 4.72-4.63 (m, 1H), 4.54 (q, *J* = 6.9 Hz, 1H), 4.44-4.36 (m, 3H), 4.35-4.26 (m, 3H), 4.19-4.09 (m, 2H), 3.98-3.88 (m, 2H), 3.69-3.58 (m, 3H), 3.54-3.43 (m, 3H), 3.29-3.16 (m, 3H), 2.77 (t, *J* = 12.8 Hz, 1H), 2.35-2.26 (m, 1H), 2.23-2.10 (m, 2H), 2.09-1.95 (m, 5H), 1.94-1.79 (m, 6H), 1.74-1.57 (m, 4H), 1.56-1.43 (m, 6H), 1.41-1.33 (m, 1H), 1.19-1.08 (m, 1H), 0.96-0.75 (m, 30H); LC main peak retention time: 7.02 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1188.7027, found: 1188.7022 (–0.4 ppm); calculated for $[M+2H]^{2+}$: 594.8550, found: 594.8534 (–2.7 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 813.4870, found: 813.4849 (–2.6 ppm).

CalA_R1A (14)

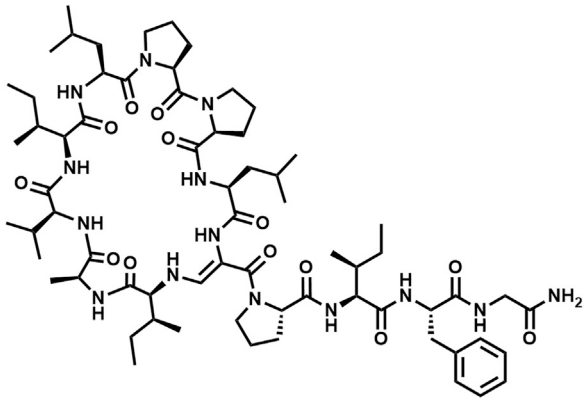


$C_{66}H_{102}N_{14}O_{14}$ M = 1315.63 g/mol

The linear precursor peptide of **14** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 102 mg (77 μmol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 8.0 mg (6.1 μmol, 7.9%) of **14** in a total yield of 2.7% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.36 (s, 1H), 8.25 (t, *J* = 6.2 Hz, 1H), 7.74 (d, *J* = 6.6 Hz, 1H), 7.59 (d, *J* = 13.6 Hz, 1H), 7.56 (d, *J* = 9.1 Hz, 1H), 7.35 (d, *J* = 7.8 Hz, 1H), 7.28 (s, 1H), 7.25-7.18 (m, 1H), 7.18-7.11 (m, 6H), 6.96 (s, 1H), 6.87 (d, *J* = 10.0 Hz, 1H), 5.77-5.67 (m, 1H), 5.33 (d, *J* = 3.3 Hz, 1H), 4.72-4.65 (m, 1H), 4.63-4.53 (m, 2H), 4.39 (s, 1H), 4.30-4.23 (m, 3H), 4.12 (dd, *J* = 10.0, 7.2 Hz, 1H), 4.06-3.95 (m, 2H), 3.94-3.84 (m, 3H), 3.67 (d, *J* = 11.3 Hz, 1H), 3.63-3.52 (m, 3H), 3.51-3.39 (m, 3H), 3.29-3.17 (m, 2H), 3.11 (dd, *J* = 14.2, 3.4 Hz, 1H), 2.70-2.61 (m, 1H), 2.30-2.21 (m, 3H), 2.17-2.10 (m, 1H), 2.06-1.91 (m, 4H), 1.90-1.80 (m, 4H), 1.76-1.66 (m, 3H), 1.64-1.42 (m, 8H), 1.39-1.30 (m, 1H), 1.20-1.12 (m, 1H), 1.09-1.01 (m, 2H), 0.99 (d, *J* = 6.5 Hz, 3H), 0.94-0.90 (m, 6H), 0.89-0.87 (m, 3H), 0.86-0.82 (m, 9H), 0.79 (d, *J* = 6.8 Hz, 3H), 0.76 (t, *J* = 7.4 Hz, 3H), 0.69 (t, *J* = 7.4 Hz, 3H), 0.50 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 6.57 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1315.7773, found: 1315.7745 (–2.1 ppm); calculated for $[M+2H]^{2+}$: 658.3923, found: 658.3919 (–0.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 884.5241, found: 884.5244 (0.3 ppm).

CalA_R2A (15)

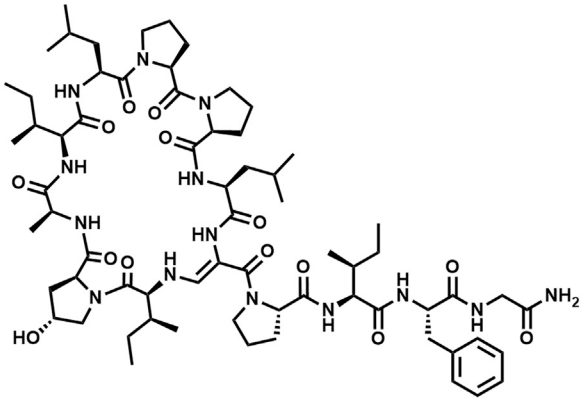


$C_{67}H_{105}N_{14}O_{13}$ **M = 1314.67 g/mol**

The linear precursor peptide of **15** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 3.2 mg (2.4 μmol) of **15** in a total yield of 2.4% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.77 (s, 1H), 8.47 (d, *J* = 6.7 Hz, 1H), 8.15 (s, 1H), 7.70 (d, *J* = 6.9 Hz, 1H), 7.64 (t, *J* = 6.2 Hz, 1H), 7.57-7.51 (m, 2H), 7.21-7.07 (m, 9H), 6.74 (d, *J* = 9.6 Hz, 1H), 5.82-5.71 (m, 1H), 4.63-4.55 (m, 1H), 4.54-4.47 (m, 1H), 4.31-4.22 (m, 3H), 3.94 (t, *J* = 8.8 Hz, 1H), 3.86-3.77 (m, 3H), 3.74 (t, *J* = 7.3 Hz, 1H), 3.63-3.55 (m, 3H), 3.54-3.45 (m, 1H), 3.43-3.38 (m, 1H), 3.30-3.22 (m, 3H), 3.01 (dd, *J* = 10.6, 6.9 Hz, 1H), 2.69-2.60 (m, 2H), 2.32-2.20 (m, 3H), 2.00-1.94 (m, 1H), 1.93-1.82 (m, 7H), 1.79-1.68 (m, 4H), 1.65-1.52 (m, 4H), 1.47-1.35 (m, 4H), 1.28 (d, *J* = 7.0 Hz, 3H), 1.23 (s, 1H), 1.16-1.12 (m, 1H), 1.11 (s, 2H), 0.95 (d, *J* = 6.4 Hz, 3H), 0.92-0.78 (m, 21H), 0.74-0.69 (m, 6H), 0.45 (d, *J* = 6.8 Hz, 3H), 0.43-0.38 (m, 3H); LC main peak retention time: 8.02 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1315.8137, found: 1315.8145 (0.6 ppm); calculated for $[M+2H]^{2+}$: 658.4105, found: 658.4105 (0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 884.5605, found: 884.5591 (–1.6 ppm).

CalA_R3A (16)

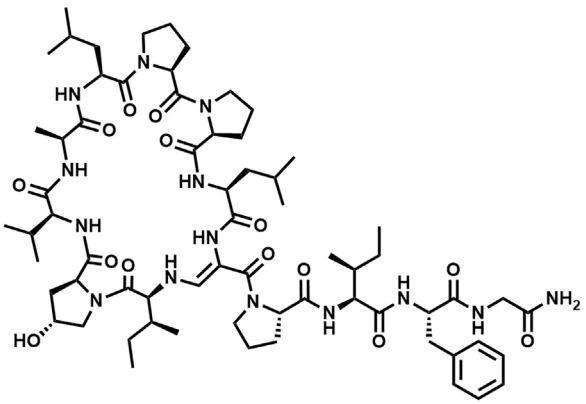


$C_{67}H_{104}N_{14}O_{14}$ M = 1329.65 g/mol

The linear precursor peptide of **16** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 8.3 mg (6.2 μmol) of **16** in a total yield of 6.2% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.01 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1329.7929, found: 1329.7947 (1.4 ppm); calculated for $[M+2H]^{2+}$: 665.4001, found: 665.4004 (0.5 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 898.5397, found: 898.5389 (–0.9 ppm).

CalA_R4A (17)



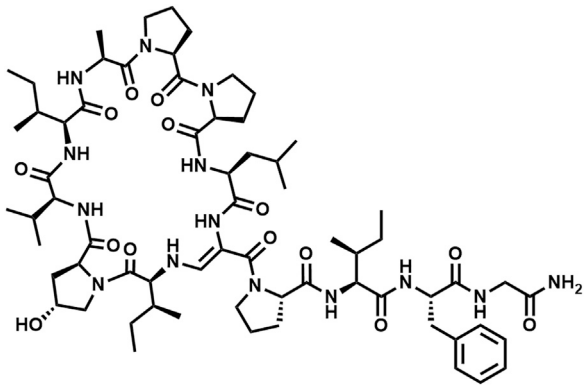
$C_{66}H_{102}N_{14}O_{14}$ M = 1315.63 g/mol

The linear precursor peptide of **17** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 21.5 mg (16.3 μmol) of **17** in a total yield of 16.3% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.91 (s, 1H), 8.68 (s, 1H), 8.30 (s, 1H), 8.20-8.12 (m, 1H), 7.50 (d, *J* = 6.9 Hz, 1H), 7.46 (d, *J* = 8.8 Hz, 1H), 7.36 (d, *J* = 13.4 Hz, 1H), 7.28-7.09 (m, 7H), 6.99-6.94 (m, 1H), 6.74 (d, *J* = 10.0 Hz, 1H), 5.84 (dd, *J* = 13.2, 10.2 Hz, 1H), 5.35 (d, *J* = 3.1 Hz, 1H), 4.65-4.57 (m, 1H), 4.57-4.52 (m, 1H), 4.41 (s, 1H), 4.31-4.21 (m, 4H), 4.12-4.02 (m, 2H), 3.97 (dd, *J* = 7.3, 5.8 Hz, 1H), 3.91 (dd, *J* = 16.5, 7.1 Hz, 1H), 3.79 (t, *J* = 8.5 Hz, 1H), 3.76-3.70 (m, 1H), 3.69-3.61 (m, 1H), 3.62-3.55 (m, 1H), 3.54-3.43 (m, 3H), 3.38-3.33 (m, 1H), 3.25-3.17 (m, 1H), 3.10 (dd, *J* = 13.9, 2.9 Hz, 1H), 3.04-2.97 (m, 1H), 2.68-2.55 (m, 2H), 2.33-2.20 (m, 3H), 2.11-2.05 (m, 1H), 2.02-1.95 (m, 1H), 1.94-1.80 (m, 7H), 1.78-1.63 (m, 5H), 1.63-1.51 (m, 4H), 1.50-1.35 (m, 4H), 1.31-1.22 (m, 1H), 1.14 (d, *J* = 7.3 Hz,

3H), 1.08-0.99 (m, 1H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.89-0.77 (m, 18H), 0.70 (t, *J* = 7.4 Hz, 3H), 0.44 (t, *J* = 7.4 Hz, 3H), 0.38 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 6.66 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1315.7773, found: 1315.7780 (0.5 ppm); calculated for $[M+2H]^{2+}$: 658.3923, found: 658.3925 (0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 884.5241, found: 884.5243 (0.2 ppm).

CalA_R5A (18)

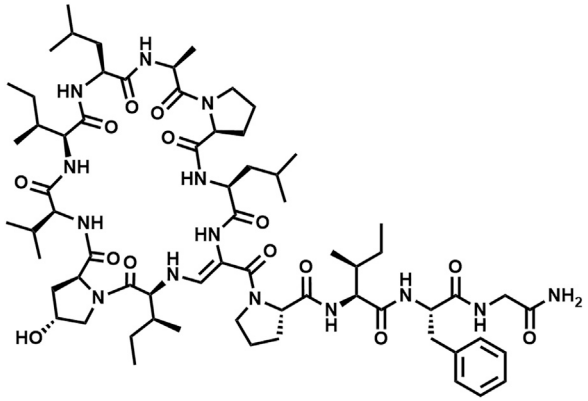


$C_{66}H_{102}N_{14}O_{14}$ M = 1315.63 g/mol

The linear precursor peptide of **18** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 34.3 mg (26.1 μmol) of **18** in a total yield of 26.1% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.02 (d, *J* = 7.0 Hz, 1H), 8.84 (s, 1H), 8.30 (s, 1H), 8.08 (t, *J* = 6.2 Hz, 1H), 7.54 (d, *J* = 8.4 Hz, 1H), 7.49 (d, *J* = 6.6 Hz, 1H), 7.32 (d, *J* = 13.4 Hz, 1H), 7.28 (d, *J* = 8.9 Hz, 1H), 7.25-7.10 (m, 6H), 7.01-6.94 (m, 2H), 5.85 (dd, *J* = 13.2, 10.2 Hz, 1H), 5.35 (d, *J* = 3.1 Hz, 1H), 4.69 (p, *J* = 6.4 Hz, 1H), 4.47-4.38 (m, 2H), 4.30-4.20 (m, 4H), 4.09-4.01 (m, 2H), 3.89 (dd, *J* = 16.5, 7.0 Hz, 1H), 3.83-3.75 (m, 1H), 3.74-3.62 (m, 4H), 3.57-3.49 (m, 2H), 3.48-3.42 (m, 2H), 3.31-3.21 (m, 1H), 3.11 (dd, *J* = 13.8, 3.0 Hz, 1H), 2.96 (dd, *J* = 10.9, 6.9 Hz, 1H), 2.69-2.57 (m, 2H), 2.31-2.16 (m, 3H), 2.13-2.05 (m, 1H), 2.04-1.98 (m, 1H), 1.96-1.81 (m, 6H), 1.80-1.59 (m, 6H), 1.54-1.42 (m, 2H), 1.42-1.30 (m, 3H), 1.28-1.19 (m, 1H), 1.16 (d, *J* = 6.2 Hz, 3H), 1.13-1.07 (m, 1H), 1.06-0.96 (m, 1H), 0.90 (d, *J* = 6.2 Hz, 3H), 0.85-0.70 (m, 23H), 0.38 (t, *J* = 7.3 Hz, 3H), 0.35 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (101 MHz, DMSO-d_6) δ 173.5, 173.0, 172.9, 172.1, 172.0, 171.8, 171.7, 171.5, 171.2, 171.2, 170.9, 167.8, 143.2, 138.1, 129.2, 127.9, 126.3, 98.4, 68.9, 64.1, 62.5, 61.9, 58.9, 56.7, 55.7, 50.7, 48.9, 47.2, 46.1, 42.1, 40.5, 38.4, 37.6, 36.2, 35.0, 29.5, 28.6, 27.2, 25.9, 25.6, 25.1, 24.2, 23.0, 20.7, 19.5, 19.2, 16.2, 15.1, 14.9, 14.3, 11.0, 10.7, 10.4; LC main peak retention time: 6.68 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1315.7773, found: 1315.7791 (1.4 ppm); calculated for $[M+2H]^{2+}$: 658.3923, found: 658.3927 (0.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 884.5241, found: 884.5246 (0.6 ppm).

CalA_R6A (19)

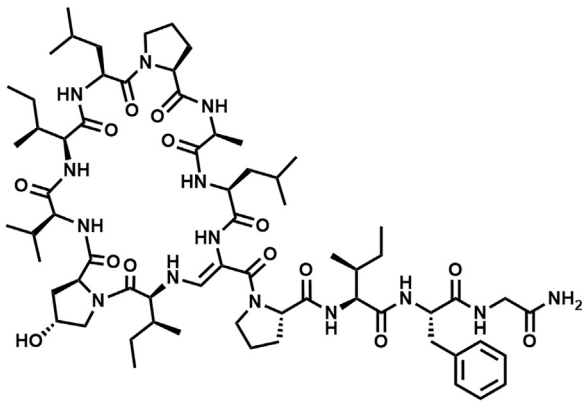


$C_{67}H_{106}N_{14}O_{14}$ M = 1331.67 g/mol

The linear precursor peptide of **19** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 12.8 mg (9.6 μmol) of **19** in a total yield of 9.6% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.43 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1331.8086, found: 1331.8096 (0.8 ppm); calculated for $[M+2H]^{2+}$: 666.4079, found: 666.4066 (–2.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 900.5554, found: 900.5546 (–0.9 ppm).

CalA_R7A (20)

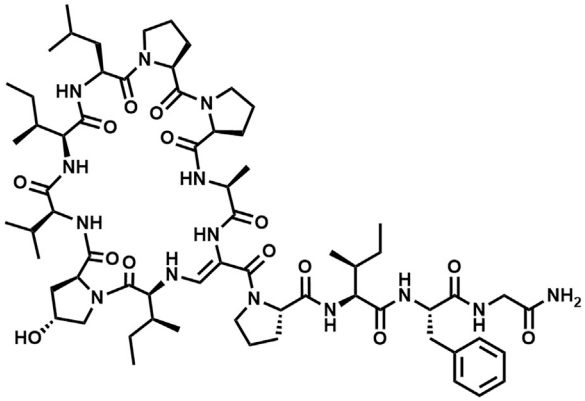


$C_{67}H_{106}N_{14}O_{14}$ M = 1331.67 g/mol

The linear precursor peptide of **20** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 8.1 mg (6.1 μmol) of **20** in a total yield of 6.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.42 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1331.8086, found: 1331.8097 (0.8 ppm); calculated for $[M+2H]^{2+}$: 666.4079, found: 666.4081 (0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 900.5554, found: 900.5558 (0.4 ppm).

CalA_R8A (21)

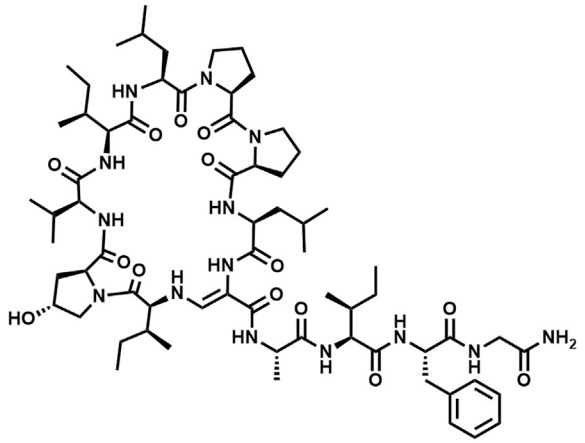


$C_{66}H_{102}N_{14}O_{14}$ M = 1315.63 g/mol

The linear precursor peptide of **21** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 11.0 mg (8.4 μmol) of **21** in a total yield of 8.4% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 (d, *J* = 6.6 Hz, 1H), 8.81 (s, 1H), 8.32 (s, 1H), 8.11 (dd, *J* = 7.0, 5.3 Hz, 1H), 7.48 (d, *J* = 8.5 Hz, 1H), 7.44 (d, *J* = 6.4 Hz, 1H), 7.35 (d, *J* = 13.4 Hz, 1H), 7.27-7.12 (m, 7H), 6.99-6.95 (m, 1H), 6.89 (d, *J* = 9.5 Hz, 1H), 5.82 (dd, *J* = 13.3, 10.1 Hz, 1H), 5.36 (s, 1H), 4.55-4.46 (m, 2H), 4.41 (s, 1H), 4.30-4.21 (m, 4H), 4.10-4.02 (m, 2H), 3.90 (dd, *J* = 16.6, 7.1 Hz, 1H), 3.79-3.68 (m, 4H), 3.65-3.57 (m, 2H), 3.55-3.46 (m, 2H), 3.30-3.21 (m, 2H), 3.10 (dd, *J* = 13.8, 3.0 Hz, 1H), 2.99 (dd, *J* = 10.8, 6.9 Hz, 1H), 2.68-2.59 (m, 2H), 2.33-2.17 (m, 3H), 2.12-2.04 (m, 1H), 2.03-1.97 (m, 1H), 1.96-1.79 (m, 7H), 1.75-1.62 (m, 3H), 1.57-1.48 (m, 3H), 1.45 (d, *J* = 7.4 Hz, 3H), 1.42-1.33 (m, 3H), 1.27-1.10 (m, 4H), 1.09-0.97 (m, 1H), 0.92 (d, *J* = 6.5 Hz, 3H), 0.89-0.85 (m, 1H), 0.84-0.75 (m, 18H), 0.72 (t, *J* = 7.4 Hz, 3H), 0.41 (t, *J* = 7.3 Hz, 3H), 0.36 (d, *J* = 6.7 Hz, 3H); LC main peak retention time: 6.83 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1315.7773, found: 1315.7780 (0.5 ppm); calculated for $[M+2H]^{2+}$: 658.3923, found: 658.3925 (0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 884.5241, found: 884.5244 (0.3 ppm).

CalA_C1A (22)



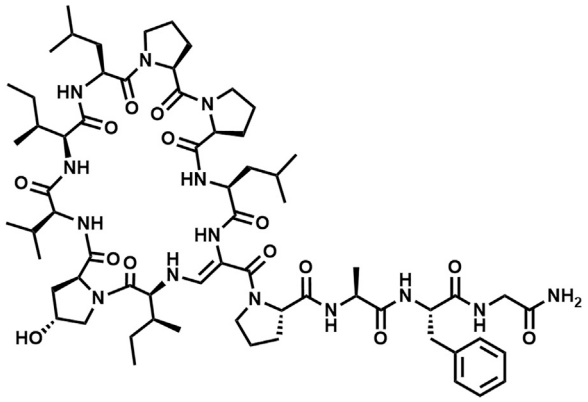
$C_{67}H_{106}N_{14}O_{14}$ M = 1331.67 g/mol

The linear precursor peptide of **22** was prepared via SPPS using Rink amide PS resin at a scale of 0.23 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 93 mg (69 μmol, 30%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the

peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 2.4 mg (1.8 μmol, 2.6%) of **22** in a total yield of 0.8% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.68 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1331.8086, found: 1331.8094 (0.6 ppm); calculated for $[M+2H]^{2+}$: 666.4079, found: 666.4071 (–1.2 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5729 (2.1 ppm).

CalA_C2A (23)

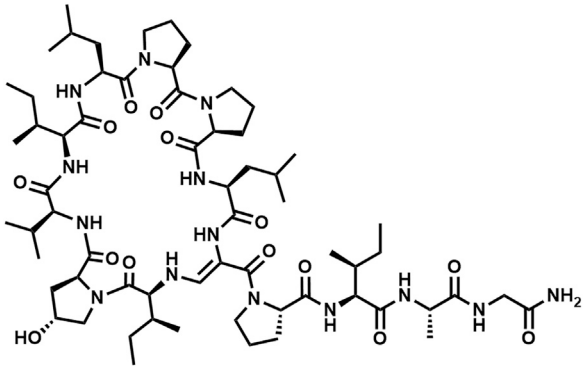


$C_{66}H_{102}N_{14}O_{14}$ M = 1315.63 g/mol

The linear precursor peptide of **23** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 3.4 mg (2.6 μmol) of **23** in a total yield of 2.6% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 (s, 1H), 8.77 (s, 1H), 8.17 (s, 1H), 7.87 (dd, *J* = 7.1, 5.2 Hz, 1H), 7.81 (d, *J* = 7.4 Hz, 1H), 7.58 (d, *J* = 9.2 Hz, 1H), 7.36 (d, *J* = 7.1 Hz, 1H), 7.27 (d, *J* = 13.3 Hz, 1H), 7.23-7.19 (m, 3H), 7.11-7.08 (m, 3H), 7.03 (s, 1H), 6.76 (d, *J* = 9.8 Hz, 1H), 5.84-5.73 (m, 1H), 4.63 (q, *J* = 6.6 Hz, 1H), 4.56 (dd, *J* = 10.0, 5.0 Hz, 1H), 4.40 (s, 1H), 4.29-4.21 (m, 4H), 4.08-4.01 (m, 1H), 3.97-3.91 (m, 2H), 3.87 (dd, *J* = 16.8, 7.2 Hz, 1H), 3.83-3.76 (m, 2H), 3.71-3.61 (m, 3H), 3.52-3.45 (m, 2H), 3.36-3.30 (m, 1H), 3.28-3.19 (m, 1H), 3.16-3.09 (m, 1H), 3.03-2.96 (m, 1H), 2.75 (t, *J* = 12.7 Hz, 1H), 2.69-2.59 (m, 1H), 2.34 – 2.18 (m, 3H), 2.13 – 2.05 (m, 1H), 2.02 – 1.96 (m, 1H), 1.95 – 1.81 (m, 8H), 1.78 – 1.67 (m, 3H), 1.66-1.52 (m, 4H), 1.50-1.43 (m, 1H), 1.42-1.31 (m, 2H), 1.30-1.25 (m, 1H), 1.23 (s, 1H), 1.17-1.10 (m, 1H), 1.08 (d, *J* = 7.4 Hz, 3H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 3H), 0.87 (d, *J* = 2.9 Hz, 3H), 0.86 (d, *J* = 3.0 Hz, 3H), 0.84-0.79 (m, 9H), 0.77 (t, *J* = 7.4 Hz, 3H), 0.74 (d, *J* = 7.2 Hz, 3H), 0.62-0.52 (m, 1H), 0.27 (t, *J* = 7.3 Hz, 3H); LC main peak retention time: 6.66 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1315.7773, found: 1315.7785 (0.9 ppm); calculated for $[M+2H]^{2+}$: 658.3923, found: 658.3925 (0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5726 (1.7 ppm).

CalA_C3A (24)

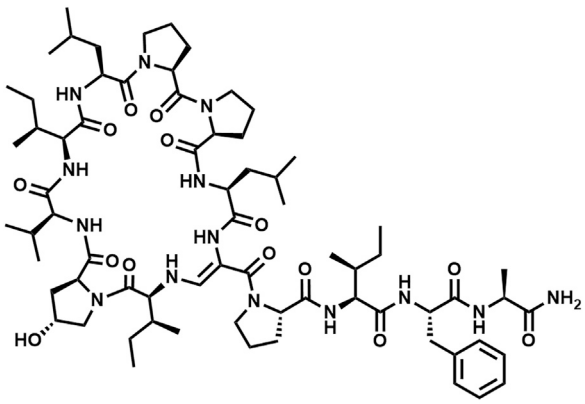


$C_{63}H_{104}N_{14}O_{14}$ M = 1281.61 g/mol

The linear precursor peptide of **24** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.4 mg (1.1 μmol) of **24** in a total yield of 1.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 6.98 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1281.7929, found: 1281.7929 (0.0 ppm).

CalA_C4A (25)



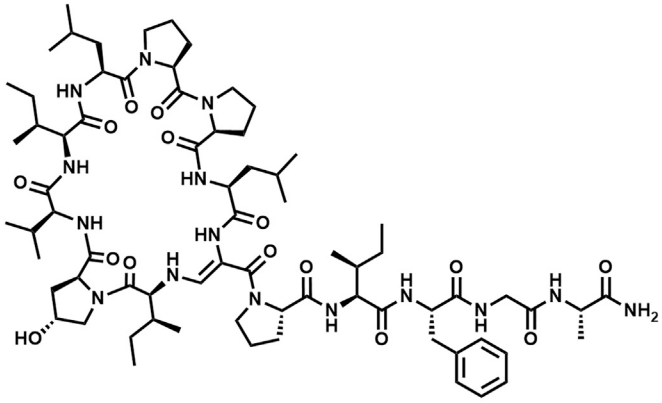
$C_{70}H_{110}N_{14}O_{14}$ M = 1371.73 g/mol

The linear precursor peptide of **25** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 2.6 mg (3.6 μmol) of **25** in a total yield of 3.6% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.98 (s, 1H), 8.77 (s, 1H), 8.13 (s, 1H), 7.72 (d, *J* = 7.3 Hz, 1H), 7.48 (d, *J* = 7.3 Hz, 1H), 7.42 (d, *J* = 8.5 Hz, 1H), 7.36 (d, *J* = 7.1 Hz, 1H), 7.26-7.20 (m, 2H), 7.18 (d, *J* = 13.3 Hz, 1H), 7.10-7.02 (m, 4H), 6.74 (d, *J* = 9.9 Hz, 1H), 6.68 (s, 1H), 5.78-5.66 (m, 1H), 4.64 (q, *J* = 6.8 Hz, 1H), 4.60-4.51 (m, 1H), 4.38 (s, 1H), 4.29-4.19 (m, 3H), 4.18-4.11 (m, 1H), 4.10-4.03 (m, 2H), 3.90-3.83 (m, 2H), 3.66-3.56 (m, 4H), 3.53-3.46 (m, 2H), 3.39-3.31 (m, 1H), 3.27-3.18 (m, 1H), 3.14-3.07 (m, 1H), 2.99 (t, *J* = 8.9 Hz, 1H), 2.81 (t, *J* = 12.7 Hz, 1H), 2.70-2.58 (m, 1H), 2.34-2.19 (m, 3H), 2.12-2.05 (m, 1H), 2.02-1.96 (m, 1H), 1.95-1.80 (m, 8H), 1.78-1.68 (m, 4H), 1.66-1.53 (m, 4H), 1.51-1.43 (m, 1H), 1.42-1.33 (m, 2H), 1.31 (d, *J* = 7.3 Hz, 3H), 1.28-1.25 (m, 1H), 1.23 (s, 1H), 1.20-1.06 (m, 3H), 0.96 (d, *J* = 6.5 Hz, 3H), 0.92 (d, *J* = 6.4 Hz, 3H), 0.88 (d, *J* = 2.2 Hz, 3H), 0.87 (d, *J* = 2.1 Hz, 3H), 0.84-0.79 (m, 9H), 0.77 (t, *J* = 7.5 Hz, 3H), 0.73 (d, *J* = 6.7 Hz, 3H), 0.70 (t, *J* = 7.4 Hz, 3H), 0.60-0.53 (m, 1H), 0.50 (d, *J* = 6.8 Hz,

3H), 0.26 (t, *J* = 7.3 Hz, 3H); LC main peak retention time: 7.82 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1371.8399, found: 1371.8411 (0.9 ppm); calculated for $[M+2H]^{2+}$: 686.4236, found: 686.4234 (–0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5709 (–0.1 ppm).

CalA_C5A (26)

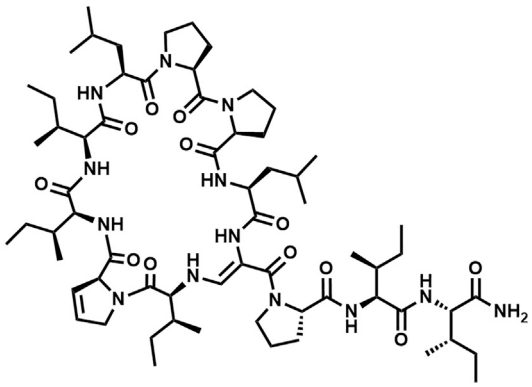


$C_{72}H_{113}N_{15}O_{15}$ M = 1428.79 g/mol

The linear precursor peptide of **26** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 3.0 mg (2.1 μmol) of **26** in a total yield of 2.1% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.04 (d, *J* = 6.9 Hz, 1H), 8.83 (s, 1H), 8.49 (t, *J* = 6.0 Hz, 1H), 8.34 (s, 1H), 8.22 (d, *J* = 7.8 Hz, 1H), 7.54 (d, *J* = 13.3 Hz, 1H), 7.47 (d, *J* = 7.2 Hz, 1H), 7.44 (d, *J* = 9.4 Hz, 1H), 7.27 (d, *J* = 8.5 Hz, 1H), 7.20-7.11 (m, 6H), 7.04 (s, 1H), 6.72 (d, *J* = 10.1 Hz, 1H), 5.96-5.87 (m, 1H), 5.36 (d, *J* = 3.2 Hz, 1H), 4.67-4.58 (m, 2H), 4.39 (s, 1H), 4.34-4.24 (m, 4H), 4.23-4.16 (m, 2H), 4.06 (t, *J* = 9.4 Hz, 1H), 4.00 (dd, *J* = 15.9, 6.5 Hz, 1H), 3.82-3.75 (m, 2H), 3.74-3.69 (m, 1H), 3.66-3.57 (m, 2H), 3.54-3.45 (m, 3H), 3.25-3.17 (m, 1H), 3.08 (d, *J* = 13.5 Hz, 1H), 3.02-2.94 (m, 1H), 2.72-2.62 (m, 1H), 2.61-2.52 (m, 1H), 2.33-2.21 (m, 3H), 2.13-2.05 (m, 1H), 2.02-1.97 (m, 1H), 1.96-1.83 (m, 7H), 1.82-1.72 (m, 3H), 1.62 (s, 6H), 1.52-1.37 (m, 6H), 1.24 (d, *J* = 4.8 Hz, 3H), 1.23-1.20 (m, 1H), 1.18-1.10 (m, 1H), 1.06-0.98 (m, 1H), 0.95 (d, *J* = 6.3 Hz, 3H), 0.88-0.76 (m, 24H), 0.73-0.67 (m, 4H), 0.45 (t, *J* = 7.2 Hz, 3H), 0.35 (d, *J* = 6.7 Hz, 3H); LC main peak retention time: 7.66 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1428.8613, found: 1428.8625 (0.8 ppm); calculated for $[M+2H]^{2+}$: 714.9343, found: 714.9346 (0.4 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5725 (1.6 ppm).

CalB_R2dPro (27)

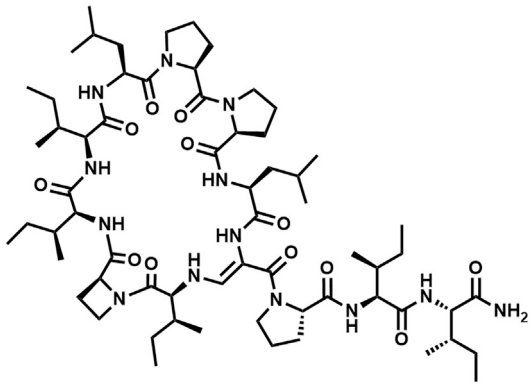


$C_{65}H_{107}N_{13}O_{12}$ M = 1262.65 g/mol

The linear precursor peptide of **27** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 69 mg (54 μmol, 54%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 0.5 mg (0.4 μmol, 0.7%) of **27** in a total yield of 0.4% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.67 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1262.8235, found: 1262.8218 (–1.3 ppm); calculated for $[M+2H]^{2+}$: 631.9154, found: 631.9143 (–1.7 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 922.5761, found: 922.5724 (–4.0 ppm).

CalB_R2Aze (28)

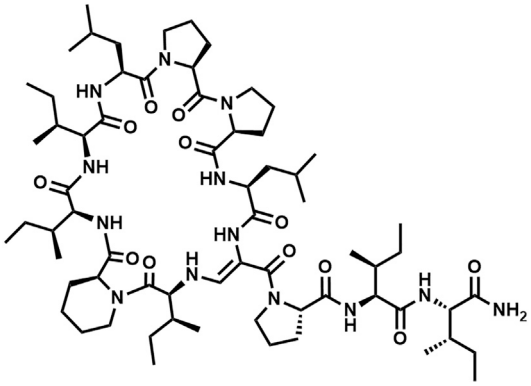


$C_{64}H_{107}N_{13}O_{12}$ M = 1250.6 g/mol

The linear precursor peptide of **28** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 55 mg (43 μmol, 43%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 8.4 mg (6.7 μmol, 15.5%) of **28** in a total yield of 6.7% (calculated in relation to initial resin capacity) as a white powder.

^1H NMR (400 MHz, DMSO-d_6) δ 9.00 (d, *J* = 7.1 Hz, 1H), 8.50 (d, *J* = 6.6 Hz, 1H), 8.22 (s, 1H), 7.59 (d, *J* = 5.8 Hz, 1H), 7.57 (d, *J* = 3.0 Hz, 1H), 7.20 (d, *J* = 9.7 Hz, 1H), 7.12 (s, 1H), 7.02 (d, *J* = 13.2 Hz, 1H), 6.91 (s, 1H), 6.73 (d, *J* = 9.8 Hz, 1H), 5.55-5.45 (m, 1H), 4.66-4.59 (m, 2H), 4.55-4.46 (m, 1H), 4.39-4.31 (m, 1H), 4.28-4.18 (m, 3H), 4.12-4.04 (m, 2H), 3.99-3.92 (m, 1H), 3.86-3.80 (m, 1H), 3.67-3.61 (m, 1H), 3.60-3.53 (m, 2H), 3.51-3.44 (m, 2H), 3.35-3.26 (m, 1H), 3.23-3.13 (m, 2H), 2.61-2.53 (m, 1H), 2.46-2.38 (m, 1H), 2.30-2.16 (m, 3H), 2.01-1.94 (m, 1H), 1.93-1.76 (m, 8H), 1.74-1.65 (m, 3H), 1.65-1.56 (m, 3H), 1.52-1.36 (m, 8H), 1.35-1.22 (m, 2H), 1.21-1.11 (m, 2H), 1.08-0.96 (m, 3H), 0.92-0.74 (m, 42H); LC main peak retention time: 8.77 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1250.8235, found: 1250.8229 (–0.5 ppm); calculated for $[M+2H]^{2+}$: 625.9154, found: 625.9148 (–1.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 910.5761, found: 910.5733 (–3.1 ppm).

CalB_R2Pip (29)

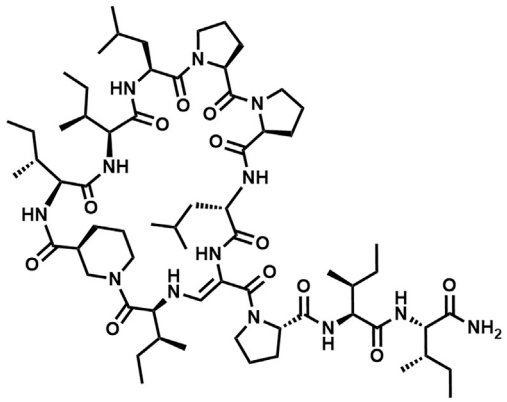


$C_{66}H_{111}N_{13}O_{12}$ M = 1278.7 g/mol

The linear precursor peptide of **29** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 38 mg (29 μmol, 29%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 8 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 2.0 mg (1.6 μmol, 5.3%) of **29** in a total yield of 1.6% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.85 (d, *J* = 7.2 Hz, 1H), 8.61 (d, *J* = 6.8 Hz, 1H), 8.24 (s, 1H), 7.57 (d, *J* = 10.2 Hz, 1H), 7.22 (d, *J* = 9.6 Hz, 1H), 7.16 (s, 1H), 7.09-7.00 (m, 2H), 6.90 (s, 1H), 6.67 (d, *J* = 10.1 Hz, 1H), 5.70 (dd, *J* = 13.2, 9.9 Hz, 1H), 4.64 (q, *J* = 6.7 Hz, 1H), 4.55 (td, *J* = 10.3, 3.6 Hz, 1H), 4.34-4.19 (m, 3H), 4.16-4.03 (m, 4H), 3.98-3.89 (m, 1H), 3.83-3.75 (m, 1H), 3.62-3.42 (m, 5H), 3.20-3.06 (m, 3H), 2.61-2.52 (m, 1H), 2.35-2.17 (m, 4H), 1.96-1.82 (m, 9H), 1.81-1.71 (m, 3H), 1.68-1.55 (m, 6H), 1.53-1.37 (m, 9H), 1.31-1.19 (m, 3H), 1.16-0.97 (m, 4H), 0.91-0.69 (m, 42H); LC main peak retention time: 10.36 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1278.8548, found: 1278.8534 (–1.1 ppm); calculated for $[M+2H]^{2+}$: 639.9311, found: 639.9300 (–1.7 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 938.6074, found: 938.6056 (–1.9 ppm).

CalB_R2Nip (30)

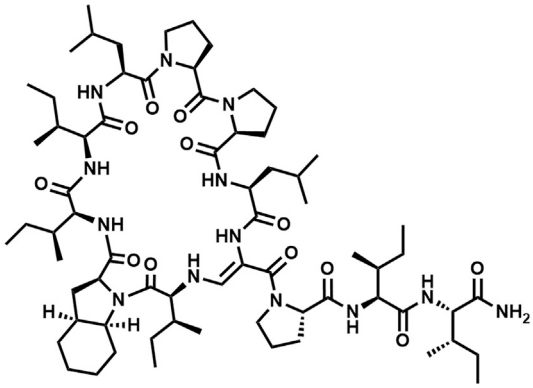


$C_{66}H_{111}N_{13}O_{12}$ M = 1278.7 g/mol

The linear precursor peptide of **30** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 21 mg (16 μmol, 16%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.4 mg (1.1 μmol, 6.6%) of **30** in a total yield of 1.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.11 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1278.8548, found: 1278.8551 (0.2 ppm); calculated for $[M+2H]^{2+}$: 639.9311, found: 639.9309 (–0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 938.6074, found: 938.6057 (–1.8 ppm).

CalB_R2Oic (31)

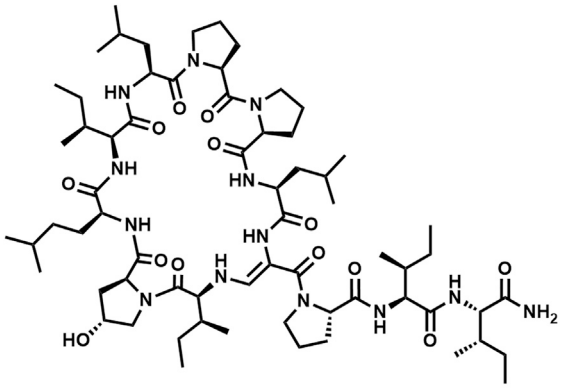


$C_{69}H_{115}N_{13}O_{12}$ M = 1318.8 g/mol

The linear precursor peptide of **31** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 61 mg (45 μmol, 45%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 26 mg (20 μmol, 44%) of **31** in a total yield of 19.8% (calculated in relation to initial resin capacity) as a white powder.

^1H NMR (400 MHz, DMSO-d_6) δ 8.84 (d, *J* = 7.0 Hz, 1H), 8.59 (d, *J* = 6.6 Hz, 1H), 8.27 (s, 1H), 7.60 (d, *J* = 3.4 Hz, 1H), 7.58 (s, 1H), 7.29 (d, *J* = 9.6 Hz, 1H), 7.12 (d, *J* = 13.4 Hz, 1H), 7.04 (s, 1H), 7.02 (s, 1H), 6.67 (d, *J* = 9.9 Hz, 1H), 5.62-5.53 (m, 1H), 4.65-4.57 (m, 1H), 4.57-4.48 (m, 1H), 4.31-4.20 (m, 2H), 4.20-4.14 (m, 1H), 4.14-4.01 (m, 3H), 3.95 (t, *J* = 8.7 Hz, 1H), 3.81 (t, *J* = 10.1 Hz, 1H), 3.77-3.71 (m, 1H), 3.62-3.40 (m, 4H), 3.30-3.24 (m, 1H), 3.23-3.13 (m, 1H), 3.11-3.04 (m, 1H), 2.69-2.57 (m, 1H), 2.37-2.17 (m, 3H), 2.05-1.96 (m, 2H), 1.95-1.82 (m, 8H), 1.81-1.55 (m, 10H), 1.54-1.38 (m, 8H), 1.33-0.98 (m, 10H), 0.92-0.69 (m, 44H); LC main peak retention time: 14.35 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1318.8861, found: 1318.8862 (0.1 ppm); calculated for $[M+2H]^{2+}$: 659.9467, found: 659.9461 (–0.9 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 978.6387, found: 978.6381 (–0.6 ppm).

CalB_R3hLeu (32)



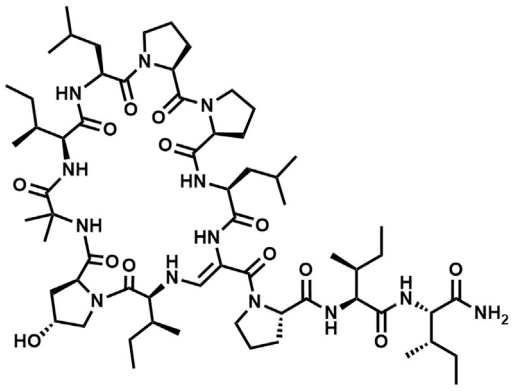
$C_{66}H_{111}N_{13}O_{13}$ M = 1294.7 g/mol

The linear precursor peptide of **32** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA),

25 mg (19 μmol, 19%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 2.1 mg (1.6 μmol, 8.5%) of **32** in a total yield of 1.6% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.87 (d, *J* = 6.8 Hz, 1H), 8.30 (s, 1H), 8.21 (d, *J* = 8.1 Hz, 1H), 7.60 (d, *J* = 10.0 Hz, 1H), 7.45 (d, *J* = 7.3 Hz, 1H), 7.23 (d, *J* = 9.6 Hz, 1H), 7.15 (s, 1H), 7.06 (d, *J* = 13.2 Hz, 1H), 6.97 (s, 1H), 6.67 (d, *J* = 9.9 Hz, 1H), 5.61 (dd, *J* = 13.2, 10.1 Hz, 1H), 4.66 (q, *J* = 6.8 Hz, 1H), 4.52 (td, *J* = 10.3, 3.6 Hz, 1H), 4.42 (s, 1H), 4.29-4.20 (m, 3H), 4.19-4.13 (m, 1H), 4.13-4.05 (m, 1H), 4.06-3.92 (m, 3H), 3.78-3.68 (m, 2H), 3.62-3.49 (m, 5H), 3.21-3.11 (m, 1H), 2.36-2.17 (m, 3H), 2.13-1.99 (m, 3H), 1.96-1.77 (m, 9H), 1.73-1.55 (m, 6H), 1.54-1.38 (m, 8H), 1.37-1.29 (m, 1H), 1.27-1.20 (m, 2H), 1.19-1.06 (m, 4H), 1.05-0.94 (m, 2H), 0.93-0.69 (m, 42H); LC main peak retention time: 8.00 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8481 (–1.2 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9274 (–1.7 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 954.6023, found: 954.5990 (–3.5 ppm).

CalB_R3Aib (33)

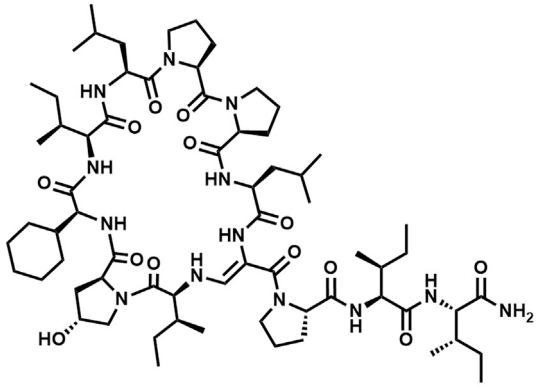


$C_{63}H_{105}N_{13}O_{13}$ M = 1252.6 g/mol

The linear precursor peptide of **33** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 42 mg (33 μmol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 2.6 mg (2.1 μmol, 6.3%) of **33** in a total yield of 2.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.16 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1252.8028, found: 1252.8015 (–1.0 ppm); calculated for $[M+2H]^{2+}$: 626.9050, found: 626.9044 (–1.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 912.5554, found: 912.5514 (–4.4 ppm).

CalB_R3Chg (34)

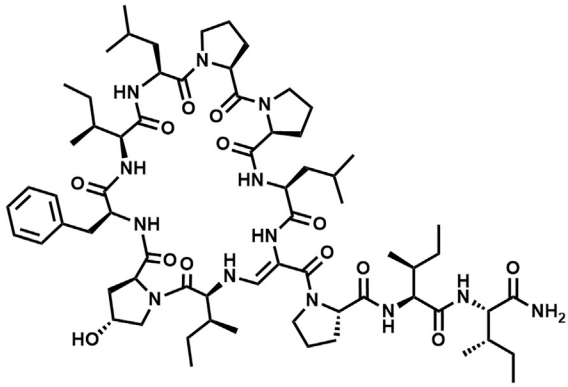


$C_{67}H_{111}N_{13}O_{13}$ M = 1306.7 g/mol

The linear precursor peptide of **34** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 40 mg (31 μmol, 31%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 6.8 mg (5.1 μmol, 16.5%) of **34** in a total yield of 5.1% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.02 (d, *J* = 7.0 Hz, 1H), 8.86 (d, *J* = 6.6 Hz, 1H), 8.29 (s, 1H), 7.59 (d, *J* = 10.1 Hz, 1H), 7.36 (d, *J* = 7.0 Hz, 1H), 7.24 (d, *J* = 9.7 Hz, 1H), 7.17 (s, 1H), 7.05 (d, *J* = 13.3 Hz, 1H), 6.97 (s, 1H), 6.65 (d, *J* = 10.0 Hz, 1H), 5.78-5.67 (m, 1H), 5.41 (s, 1H), 4.66-4.59 (m, 1H), 4.58-4.50 (m, 1H), 4.41 (s, 1H), 4.29-4.21 (m, 3H), 4.19-4.08 (m, 3H), 4.08-4.01 (m, 1H), 4.00-3.91 (m, 1H), 3.84-3.76 (m, 2H), 3.70-3.43 (m, 6H), 3.21-3.11 (m, 1H), 3.11-3.03 (m, 1H), 2.44-2.34 (m, 1H), 2.35-2.17 (m, 3H), 2.14-2.03 (m, 1H), 2.02-1.95 (m, 1H), 1.95-1.82 (m, 8H), 1.82-1.55 (m, 10H), 1.52-1.37 (m, 8H), 1.33-1.21 (m, 3H), 1.20-1.08 (m, 4H), 1.05-0.94 (m, 3H), 0.92-0.68 (m, 36H); LC main peak retention time: 8.88 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1306.8497, found: 1306.8494 (–0.2 ppm); calculated for $[M+2H]^{2+}$: 653.9285, found: 653.9279 (–0.9 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 966.6023, found: 966.5992 (–3.2 ppm).

CalB_R3F (35)



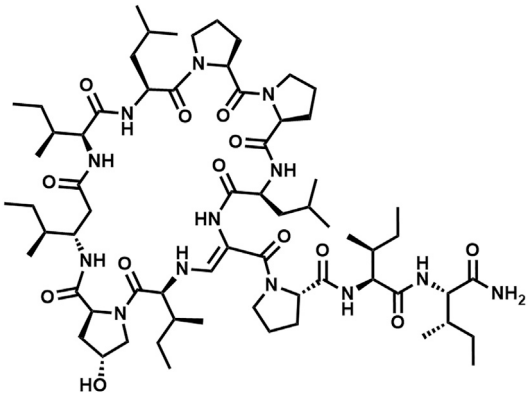
$C_{68}H_{107}N_{13}O_{13}$ M = 1314.7 g/mol

The linear precursor peptide of **35** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 37 mg (28 μmol, 28%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was

shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 8.2 mg (6.2 μmol, 22.4%) of **35** in a total yield of 6.2% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.79 (d, *J* = 6.8 Hz, 1H), 8.27 (s, 1H), 8.24 (d, *J* = 8.4 Hz, 1H), 7.58 (d, *J* = 10.1 Hz, 1H), 7.50 (d, *J* = 7.4 Hz, 1H), 7.36-7.28 (m, 2H), 7.27-7.20 (m, 2H), 7.19-7.16 (m, 1H), 7.15 (s, 1H), 7.04 (d, *J* = 13.2 Hz, 1H), 6.98 (s, 1H), 6.64 (d, *J* = 9.9 Hz, 1H), 5.67-5.56 (m, 1H), 5.33 (s, 1H), 4.68 (q, *J* = 6.8 Hz, 1H), 4.51 (td, *J* = 10.4, 3.6 Hz, 1H), 4.40 (s, 1H), 4.27-4.20 (m, 2H), 4.19-4.06 (m, 3H), 4.05-3.93 (m, 3H), 3.70-3.64 (m, 1H), 3.60-3.40 (m, 6H), 3.20-3.11 (m, 1H), 3.11-3.04 (m, 1H), 2.32-2.16 (m, 3H), 2.01-1.95 (m, 1H), 1.94-1.82 (m, 8H), 1.79-1.52 (m, 11H), 1.51-1.39 (m, 6H), 1.39-1.31 (m, 1H), 1.29-1.18 (m, 2H), 1.17-1.08 (m, 1H), 1.06-0.96 (m, 2H), 0.94-0.69 (m, 36H); LC main peak retention time: 8.00 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1314.8184, found: 1314.8184 (0.0 ppm); calculated for $[M+2H]^{2+}$: 657.9129, found: 657.9125 (–0.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 974.5710, found: 974.5693 (–1.7 ppm).

CalB_R3β-hIle (36)

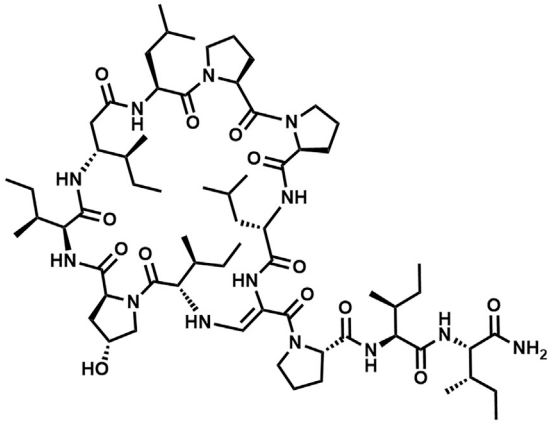


$C_{66}H_{111}N_{13}O_{13}$ M = 1294.7 g/mol

The linear precursor peptide of **36** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 51 mg (39 μmol, 39%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 3.7 mg (2.9 μmol, 7.4%) of **36** in a total yield of 2.9% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.14 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8488 (–0.7 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9277 (–1.2 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 954.6023, found: 954.5994 (–3.0 ppm).

CalB_R4β-hIle (37)

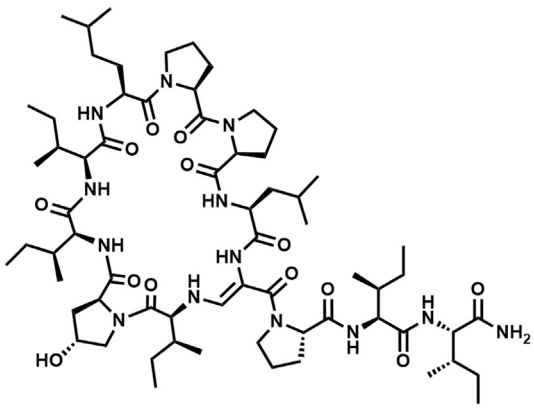


$C_{66}H_{111}N_{13}O_{13}$ M = 1294.7 g/mol

The linear precursor peptide of **37** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 44 mg (33 μmol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 0.9 mg (0.7 μmol, 2.1%) of **37** in a total yield of 0.7% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.68 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8492 (–0.4 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9277 (–1.2 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 954.6023, found: 954.5995 (–2.9 ppm).

CalB_R5hLeu (38)



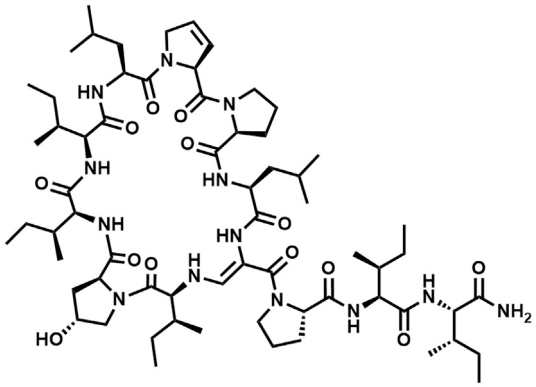
$C_{66}H_{111}N_{13}O_{13}$ M = 1294.7 g/mol

The linear precursor peptide of **38** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 33 mg (25 μmol, 25%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 8 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 7.8 mg (6.0 μmol, 23.7%) of **38** in a total yield of 6.0% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (d, *J* = 6.9 Hz, 1H), 8.82 (s, 1H), 8.29 (s, 1H), 7.59 (d, *J* = 10.0 Hz, 1H), 7.33 (d, *J* = 7.0 Hz, 1H), 7.23 (d, *J* = 9.7 Hz, 1H), 7.14 (s, 1H), 7.05 (d, *J* = 13.3 Hz, 1H), 6.95 (s, 1H), 6.73 (d, *J* = 9.8 Hz, 1H), 5.68 (dd, *J* = 13.2, 10.1 Hz, 1H),

5.39 (d, *J* = 3.0 Hz, 1H), 4.59 (q, *J* = 6.8 Hz, 1H), 4.52 (td, *J* = 10.2, 3.3 Hz, 1H), 4.41 (s, 1H), 4.28-4.19 (m, 3H), 4.15-4.00 (m, 3H), 3.93 (t, *J* = 10.0 Hz, 1H), 3.82-3.75 (m, 2H), 3.69-3.63 (m, 1H), 3.61-3.48 (m, 3H), 3.47-3.38 (m, 2H), 3.21-3.12 (m, 1H), 3.12-3.03 (m, 1H), 2.33-2.17 (m, 3H), 2.11-2.04 (m, 1H), 2.03-1.94 (m, 2H), 1.94-1.75 (m, 9H), 1.73-1.56 (m, 4H), 1.55-1.37 (m, 10H), 1.28-1.10 (m, 5H), 1.06-0.94 (m, 3H), 0.91-0.69 (m, 42H); LC main peak retention time: 8.32 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8497 (0.0 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9279 (–0.9 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 954.6023, found: 954.6000 (–2.4 ppm).

CalB_R6dPro (39)

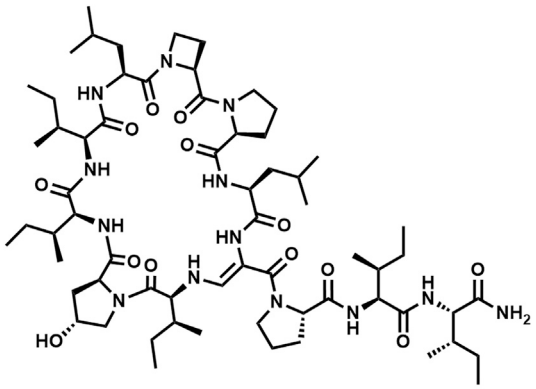


$C_{65}H_{107}N_{13}O_{13}$ M = 1278.65 g/mol

The linear precursor peptide of **39** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 23 mg (18 μmol, 18%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 5.6 mg (4.4 μmol, 25.1%) of **39** in a total yield of 4.4% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.75 (d, *J* = 7.0 Hz, 1H), 8.59 (d, *J* = 6.8 Hz, 1H), 8.32 (s, 1H), 7.63 (d, *J* = 10.1 Hz, 1H), 7.35 (d, *J* = 7.2 Hz, 1H), 7.25-7.17 (m, 2H), 7.06 (d, *J* = 13.3 Hz, 1H), 6.90 (s, 1H), 6.61 (d, *J* = 9.9 Hz, 1H), 6.14-6.09 (m, 1H), 5.85-5.80 (m, 1H), 5.68 (dd, *J* = 13.2, 10.0 Hz, 1H), 4.82-4.77 (m, 1H), 4.72 (q, *J* = 6.8 Hz, 1H), 4.55 (td, *J* = 10.4, 3.7 Hz, 1H), 4.43-4.35 (m, 2H), 4.35-4.27 (m, 1H), 4.27-4.19 (m, 2H), 4.14-4.06 (m, 3H), 3.95 (t, *J* = 10.0 Hz, 1H), 3.89 (t, *J* = 7.6 Hz, 1H), 3.77-3.72 (m, 1H), 3.69-3.63 (m, 1H), 3.57-3.49 (m, 1H), 3.29-3.21 (m, 2H), 3.20-3.12 (m, 1H), 3.11-3.05 (m, 1H), 2.48-2.41 (m, 1H), 2.31-2.19 (m, 2H), 2.09-2.01 (m, 1H), 1.93-1.81 (m, 5H), 1.80-1.59 (m, 7H), 1.56-1.38 (m, 9H), 1.38-1.30 (m, 1H), 1.23 (s, 1H), 1.19-1.08 (m, 2H), 1.06-0.95 (m, 3H), 0.94-0.70 (m, 42H); LC main peak retention time: 7.83 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1278.8184, found: 1278.8171 (–1.0 ppm); calculated for $[M+2H]^{2+}$: 639.9129, found: 639.9119 (–1.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 938.5710, found: 938.5702 (–0.9 ppm).

CalB_R6Aze (40)

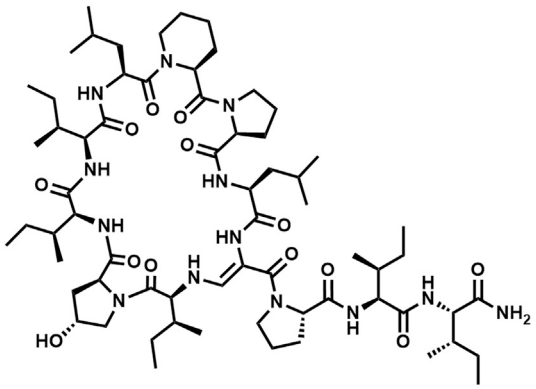


$C_{64}H_{107}N_{13}O_{13}$ M = 1266.64 g/mol

The linear precursor peptide of **40** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 30 mg (23 μmol, 23%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.1 mg (0.9 μmol, 3.7%) of **40** in a total yield of 0.9% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.96 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1266.8184, found: 1266.8150 (–2.7 ppm); calculated for $[M+2H]^{2+}$: 633.9129, found: 633.9111 (–2.8 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5690 (–2.2 ppm).

CalB_R6Pip (41)

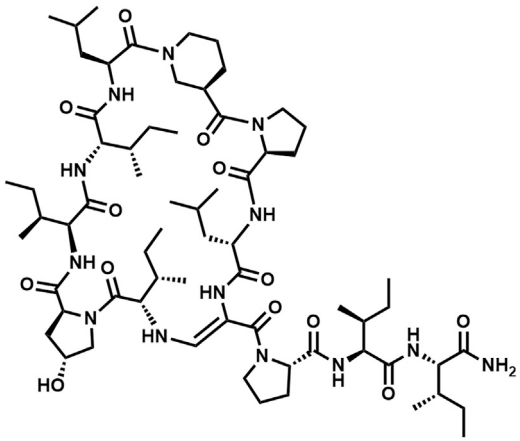


$C_{66}H_{111}N_{13}O_{13}$ M = 1294.69 g/mol

The linear precursor peptide of **41** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 57 mg (44 μmol, 44%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.1 mg (0.8 μmol, 2.0%) of **41** in a total yield of 0.8% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.84 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8492 (–0.4 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 947.9285 (–0.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 954.6023, found: 954.6011 (–1.3 ppm).

CalB_R6Nip (42)

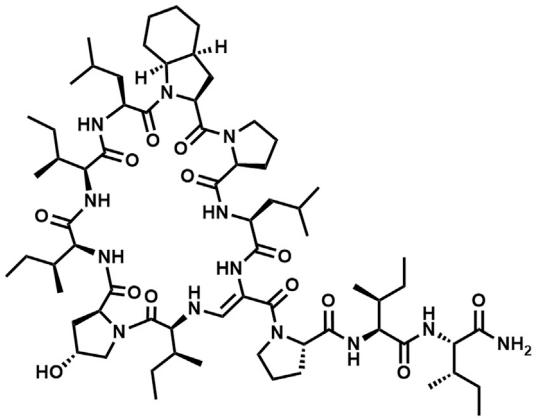


$C_{66}H_{111}N_{13}O_{13}$ M = 1294.69 g/mol

The linear precursor peptide of **42** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 30 mg (23 μmol, 23%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.1 mg (0.9 μmol, 3.8%) of **42** in a total yield of 0.9% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 6.68 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8494 (–0.2 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9282 (–0.5 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 954.6023, found: 954.6000 (–2.4 ppm).

CalB_R6Oic (43)

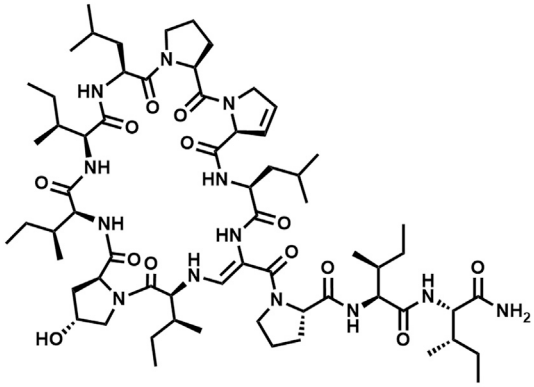


$C_{69}H_{115}N_{13}O_{13}$ M = 1334.76 g/mol

The linear precursor peptide of **43** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 47 mg (35 μmol, 35%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.0 mg (0.7 μmol, 2.2%) of **43** in a total yield of 0.7% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 10.57 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1334.8810, found: 1334.8797 (–1.0 ppm); calculated for $[M+2H]^{2+}$: 667.9442, found: 667.9469 (4.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 994.6336, found: 994.6319 (–1.7 ppm).

CalB_R7dPro (44)

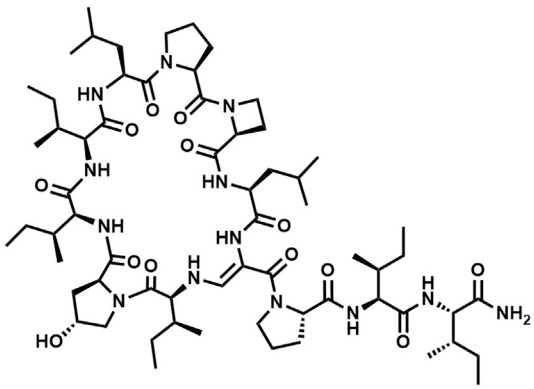


$C_{65}H_{107}N_{13}O_{13}$ M = 1278.65 g/mol

The linear precursor peptide of **44** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 25 mg (19 μmol, 19%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 3.5 mg (2.7 μmol, 14.5%) of **44** in a total yield of 2.7% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.03 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1278.8184, found: 1278.8185 (0.1 ppm); calculated for $[M+2H]^{2+}$: 639.9129, found: 639.9124 (–0.8 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 938.5710, found: 938.5672 (–4.0 ppm).

CalB_R7Aze (45)



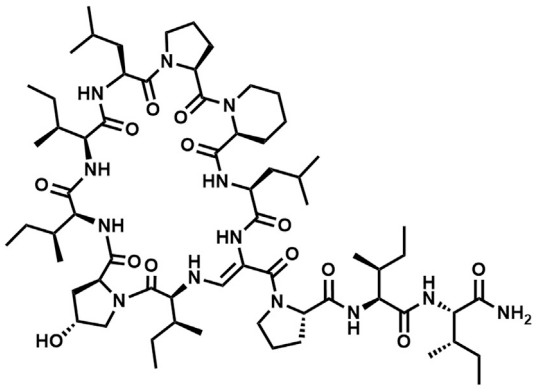
$C_{64}H_{107}N_{13}O_{13}$ M = 1266.64 g/mol

The linear precursor peptide of **45** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 31 mg (24 μmol, 24%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent

B: ACN) to give 2.1 mg (1.7 μmol, 7.0%) of **45** in a total yield of 1.7% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.98 (d, *J* = 7.0 Hz, 1H), 8.84 (d, *J* = 6.6 Hz, 1H), 8.21 (s, 1H), 7.56 (d, *J* = 10.1 Hz, 1H), 7.29 (d, *J* = 7.3 Hz, 1H), 7.20 (d, *J* = 9.8 Hz, 1H), 7.17 (s, 1H), 7.08 (d, *J* = 5.6 Hz, 1H), 7.05 (d, *J* = 8.4 Hz, 1H), 6.95 (s, 1H), 5.86 (dd, *J* = 13.2, 10.0 Hz, 1H), 5.39 (d, *J* = 3.1 Hz, 1H), 4.71-4.63 (m, 2H), 4.59 (dd, *J* = 9.3, 6.1 Hz, 1H), 4.43-4.39 (m, 1H), 4.38-4.32 (m, 1H), 4.26-4.19 (m, 2H), 4.18-4.08 (m, 2H), 4.02-3.92 (m, 2H), 3.87 (t, *J* = 8.4 Hz, 1H), 3.83 (d, *J* = 7.3 Hz, 1H), 3.78 (d, *J* = 11.7 Hz, 1H), 3.64 (dd, *J* = 11.1, 3.6 Hz, 1H), 3.56-3.40 (m, 2H), 3.06 (dd, *J* = 11.1, 6.9 Hz, 1H), 3.02-2.95 (m, 1H), 2.65-2.57 (m, 1H), 2.34-2.23 (m, 2H), 2.11-2.00 (m, 2H), 1.99-1.95 (m, 1H), 1.94-1.82 (m, 6H), 1.82-1.72 (m, 4H), 1.71-1.60 (m, 3H), 1.56-1.37 (m, 8H), 1.35-1.27 (m, 1H), 1.23 (s, 1H), 1.18-1.09 (m, 2H), 1.08-0.96 (m, 3H), 0.94-0.71 (m, 42H); LC main peak retention time: 7.72 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1266.8184, found: 1266.8172 (–0.9 ppm); calculated for $[M+2H]^{2+}$: 633.9129, found: 633.9117 (–1.9 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5674 (–3.9 ppm).

CalB_R7Pip (46)

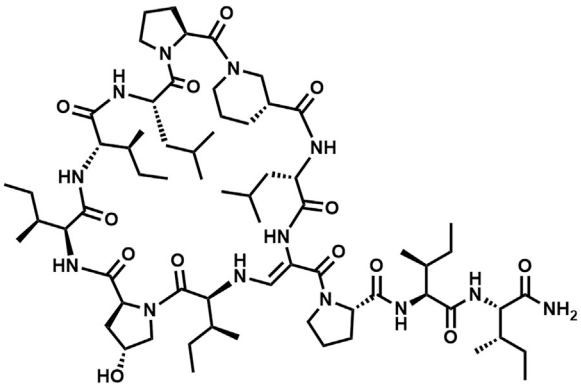


$C_{66}H_{111}N_{13}O_{13}$ M = 1294.69 g/mol

The linear precursor peptide of **46** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 35 mg (27 μmol, 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.3 mg (1.0 μmol, 3.8%) of **46** in a total yield of 1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.30 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8487 (–0.8 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 947.9277 (–1.2 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 954.6023, found: 954.5999 (–2.5 ppm).

CalB_R7Nip (47)

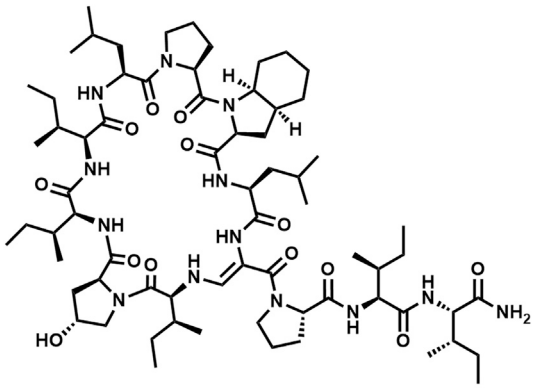


$C_{66}H_{111}N_{13}O_{13}$ M = 1294.69 g/mol

The linear precursor peptide of **47** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 32 mg (24 μmol, 24%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 0.8 mg (0.6 μmol, 2.6%) of **47** in a total yield of 0.6% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 6.64 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8482 (–1.2 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9276 (–1.4 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 954.6023, found: 954.5998 (–2.6 ppm).

CalB_R7Oic (48)



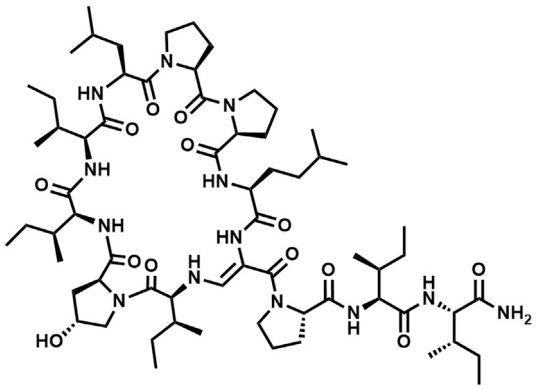
$C_{69}H_{115}N_{13}O_{13}$ M = 1334.76 g/mol

The linear precursor peptide of **48** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 47 mg (34 μmol, 34%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 4.9 mg (3.7 μmol, 10.7%) of **48** in a total yield of 3.7% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.05 (d, *J* = 7.0 Hz, 1H), 8.95 (d, *J* = 6.3 Hz, 1H), 8.26 (s, 1H), 7.56 (d, *J* = 10.1 Hz, 1H), 7.48 (d, *J* = 6.9 Hz, 1H), 7.25-7.16 (m, 2H), 7.03 (d, *J* = 13.2 Hz, 1H), 6.95 (s, 1H), 6.62 (d, *J* = 10.3 Hz, 1H), 5.92-5.81 (m, 1H), 5.41 (d, *J* = 2.9 Hz, 1H), 4.55 (td, *J* = 10.4, 3.4 Hz, 1H), 4.47-4.37 (m, 3H), 4.29-4.19 (m, 2H), 4.18-4.05 (m, 3H), 3.94 (t, *J* = 10.2 Hz, 1H), 3.89-3.83 (m, 1H), 3.82-3.72 (m, 2H), 3.71-3.60 (m, 2H), 3.49 (t, *J* = 9.1 Hz, 1H), 3.19-3.01 (m, 2H), 2.59-2.52 (m, 1H), 2.40-2.22 (m, 3H), 2.18-2.00 (m, 3H),

1.96-1.82 (m, 6H), 1.80-1.55 (m, 10H), 1.52-1.35 (m, 10H), 1.32-1.10 (m, 7H), 1.07-0.93 (m, 3H), 0.92-0.68 (m, 42H); LC main peak retention time: 12.01 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1334.8810, found: 1334.8812 (0.1 ppm); calculated for $[M+2H]^{2+}$: 667.9442, found: 667.9436 (–0.9 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 994.6336, found: 994.6326 (–1.0 ppm).

CalB_R8hLeu (49)

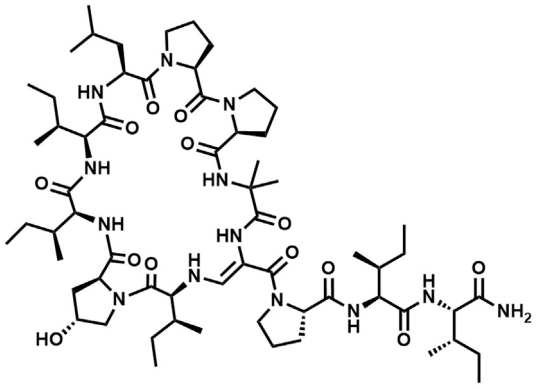


$C_{66}H_{111}N_{13}O_{13}$ M = 1294.69 g/mol

The linear precursor peptide of **49** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 43 mg (33 μmol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 7.5 mg (5.8 μmol, 17.8%) of **49** in a total yield of 5.8% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.00 (d, *J* = 6.9 Hz, 1H), 8.87 (d, *J* = 6.3 Hz, 1H), 8.28 (s, 1H), 7.58 (d, *J* = 10.1 Hz, 1H), 7.40 (d, *J* = 6.8 Hz, 1H), 7.24 (d, *J* = 9.7 Hz, 1H), 7.15 (s, 1H), 7.06 (d, *J* = 13.3 Hz, 1H), 6.97 (s, 1H), 6.64 (d, *J* = 9.9 Hz, 1H), 5.70 (dd, *J* = 13.3, 10.1 Hz, 1H), 5.40 (s, 1H), 4.62 – 4.54 (m, 2H), 4.47 – 4.36 (m, 2H), 4.29 – 4.21 (m, 3H), 4.18 – 4.13 (m, 1H), 4.11 – 4.01 (m, 2H), 3.95 (t, *J* = 10.1 Hz, 1H), 3.81-3.72 (m, 2H), 3.69-3.55 (m, 3H), 3.54-3.45 (m, 2H), 3.29-3.22 (m, 1H), 3.20-3.10 (m, 1H), 3.10-3.03 (m, 1H), 2.34-2.19 (m, 3H), 2.12-1.96 (m, 3H), 1.97-1.82 (m, 8H), 1.82-1.55 (m, 5H), 1.53-1.34 (m, 10H), 1.30-1.21 (m, 2H), 1.20-1.10 (m, 3H), 1.07-0.94 (m, 2H), 0.92-0.68 (m, 42H); LC main peak retention time: 8.13 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8484 (–1.0 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9275 (–1.5 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 954.6023, found: 954.5991 (–3.4 ppm).

CalB_R8Aib (50)

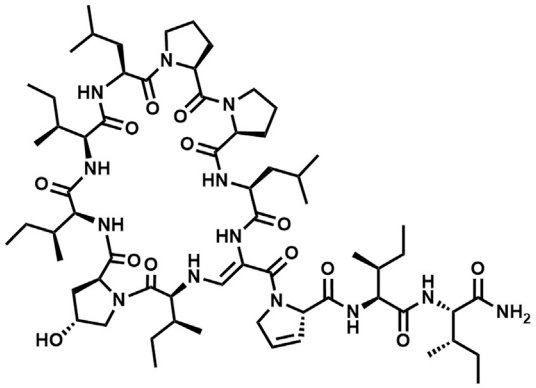


$C_{63}H_{105}N_{13}O_{13}$ M = 1252.61 g/mol

The linear precursor peptide of **50** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 56 mg (44 μmol, 44%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 0.8 mg (0.6 μmol, 1.5%) of **50** in a total yield of 0.6% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.44 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1252.8028, found: 1252.8011 (–1.4 ppm); calculated for $[M+2H]^{2+}$: 626.9050, found: 626.9041 (–1.4 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 912.5554, found: 912.5525 (–3.2 ppm).

CalB_C1dPro (51)

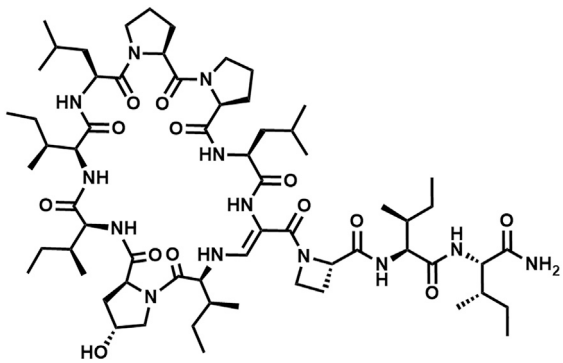


$C_{65}H_{107}N_{13}O_{13}$ M = 1278.65 g/mol

The linear precursor peptide of **51** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 29 mg (22 μmol, 22%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 2.5 mg (2.0 μmol, 8.9%) of **51** in a total yield of 2.0% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.01 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1278.8184, found: 1278.8171 (–1.0 ppm); calculated for $[M+2H]^{2+}$: 639.9129, found: 639.9121 (–1.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5830 (–3.8 ppm).

CalB_C1Aze (52)

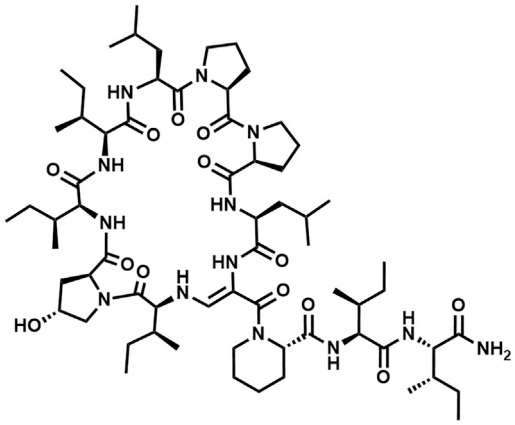


$C_{64}H_{107}N_{13}O_{13}$ M = 1266.64 g/mol

The linear precursor peptide of **52** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 50 mg (39 μmol, 39%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 3.2 mg (2.5 μmol, 6.5%) of **52** in a total yield of 2.5% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.94 (s, 1H), 8.83 (s, 1H), 7.91 (s, 1H), 7.82 (d, *J* = 9.3 Hz, 1H), 7.48 (d, *J* = 9.0 Hz, 1H), 7.38 (d, *J* = 6.9 Hz, 1H), 7.19 (d, *J* = 13.3 Hz, 1H), 7.08 (s, 1H), 6.97 (s, 1H), 6.80 (d, *J* = 10.0 Hz, 1H), 5.66-5.56 (m, 1H), 5.40 (d, *J* = 3.2 Hz, 1H), 4.65-4.46 (m, 3H), 4.40 (s, 1H), 4.27-4.15 (m, 3H), 4.11-4.05 (m, 1H), 4.05-3.98 (m, 1H), 3.98-3.90 (m, 2H), 3.88-3.73 (m, 3H), 3.72-3.65 (m, 1H), 3.64-3.53 (m, 2H), 3.52-3.42 (m, 1H), 3.27-3.19 (m, 1H), 3.13-3.05 (m, 1H), 2.44-2.32 (m, 1H), 2.31-2.17 (m, 2H), 2.13-1.68 (m, 12H), 1.66-1.33 (m, 11H), 1.32-1.21 (m, 2H), 1.20-0.93 (m, 5H), 0.92-0.71 (m, 42H); LC main peak retention time: 7.60 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1266.8184, found: 1266.8185 (0.1 ppm); calculated for $[M+2H]^{2+}$: 633.9129, found: 633.9125 (–0.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5855 (–1.2 ppm).

CalB_C1Pip (53)

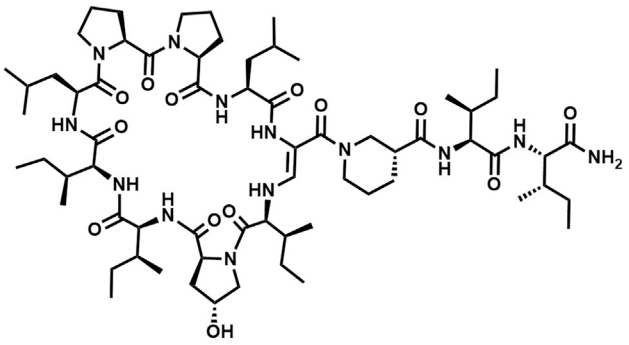


$C_{66}H_{111}N_{13}O_{13}$ **M = 1294.69 g/mol**

The linear precursor peptide of **53** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 59 mg (45 μmol, 45%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 7.9 mg (6.1 μmol, 13.7%) of **53** in a total yield of 6.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.17 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8486 (–0.8 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 947.9277 (–1.2 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5826 (–4.3 ppm).

CalB_C1Nip (54)

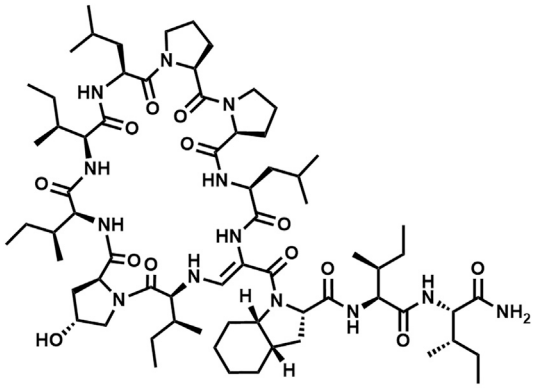


$C_{66}H_{111}N_{13}O_{13}$ M = 1294.69 g/mol

The linear precursor peptide of **54** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 36 mg (27 μmol, 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.4 mg (1.1 μmol, 4.0%) of **54** in a total yield of 1.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 6.66 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8499 (0.2 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9282 (–0.5 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5836 (–3.2 ppm).

CalB_C1Oic (55)

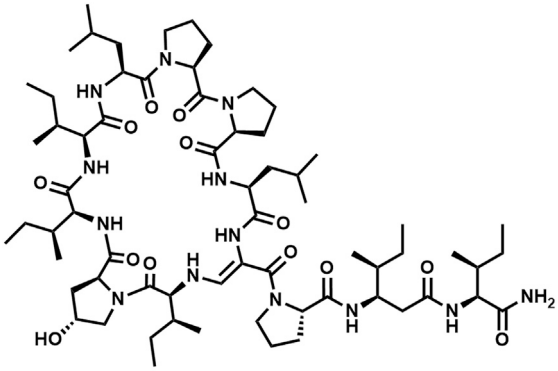


$C_{69}H_{115}N_{13}O_{13}$ M = 1334.76 g/mol

The linear precursor peptide of **55** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 49 mg (36 μmol, 36%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 4.1 mg (3.1 μmol, 8.5%) of **55** in a total yield of 3.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.76 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1334.8810, found: 1334.8815 (0.4 ppm); calculated for $[M+2H]^{2+}$: 667.9442, found: 667.9437 (–0.7 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5851 (–1.6 ppm).

CalB_C2β-hIle (56)

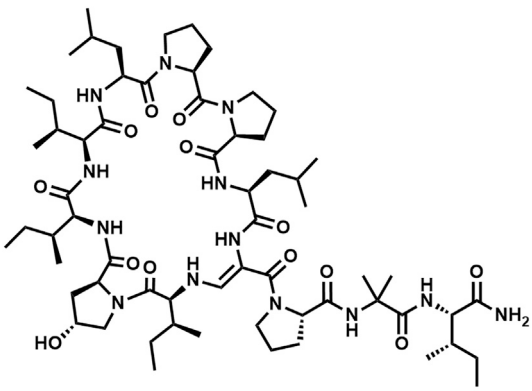


$C_{65}H_{109}N_{13}O_{13}$ M = 1280.67 g/mol

The linear precursor peptide of **56** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 50 mg (38 μmol, 38%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 0.9 mg (0.7 μmol, 1.8%) of **56** in a total yield of 0.7% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.82 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1294.8497, found: 1294.8489 (–0.6 ppm); calculated for $[M+2H]^{2+}$: 647.9285, found: 647.9276 (–1.4 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5830 (–3.8 ppm).

CalB_C2Aib (57)

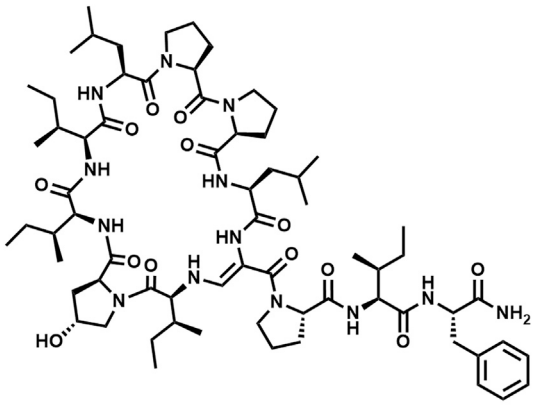


$C_{63}H_{105}N_{13}O_{13}$ M = 1252.61 g/mol

The linear precursor peptide of **57** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 60 mg (47 μmol, 47%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 6.8 mg (5.4 μmol, 11.5%) of **57** in a total yield of 5.4% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.90 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1252.8028, found: 1252.8028 (0.0 ppm); calculated for $[M+2H]^{2+}$: 626.9050, found: 626.9048 (–0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5827 (–4.1 ppm).

CalB_C3F (58)

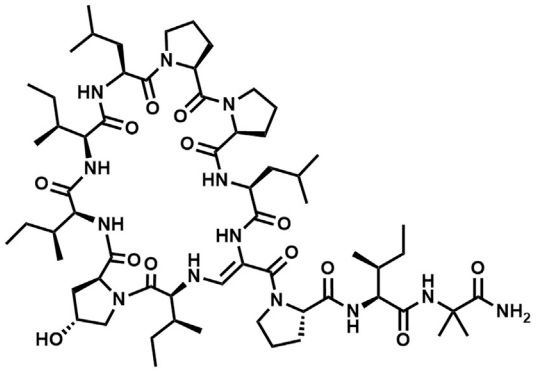


$C_{68}H_{107}N_{13}O_{13}$ M = 1314.68 g/mol

The linear precursor peptide of **58** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 81 mg (61 μmol, 61%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 13.3 mg (10.1 μmol, 16.7%) of **58** in a total yield of 10.1% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.98 (d, *J* = 7.0 Hz, 1H), 8.83 (d, *J* = 6.5 Hz, 1H), 8.27 (s, 1H), 7.49-7.42 (m, 2H), 7.39 (d, *J* = 7.0 Hz, 1H), 7.24-7.18 (m, 3H), 7.16 (s, 1H), 7.14-7.06 (m, 4H), 6.73 (d, *J* = 10.0 Hz, 1H), 5.82 (dd, *J* = 13.3, 10.0 Hz, 1H), 5.46-5.28 (m, 1H), 4.67-4.56 (m, 2H), 4.40 (s, 1H), 4.31-4.16 (m, 4H), 4.06 (t, *J* = 8.7 Hz, 1H), 3.91-3.77 (m, 4H), 3.71-3.54 (m, 3H), 3.53-3.44 (m, 2H), 3.25-3.15 (m, 2H), 3.12-3.03 (m, 1H), 2.61-2.54 (m, 1H), 2.35-2.18 (m, 3H), 2.11-2.03 (m, 1H), 2.00-1.85 (m, 8H), 1.81-1.46 (m, 8H), 1.45-1.33 (m, 4H), 1.30-1.19 (m, 1H), 1.17-0.99 (m, 2H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.91-0.72 (m, 30H), 0.67 (t, *J* = 7.4 Hz, 3H), 0.48 (t, *J* = 7.3 Hz, 3H), 0.33 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 8.19 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1314.8184, found: 1314.8175 (–0.7 ppm); calculated for $[M+2H]^{2+}$: 657.9129, found: 657.9122 (–1.1 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5837 (–3.1 ppm).

CalB_C3Aib (59)



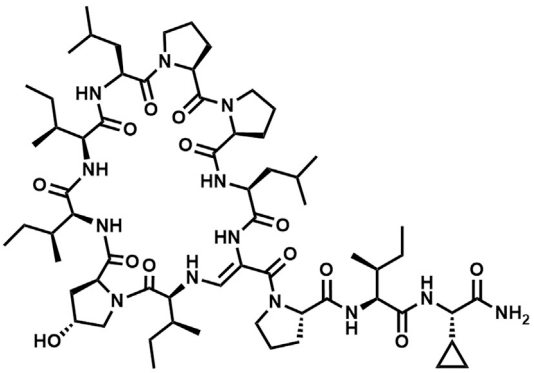
$C_{63}H_{105}N_{13}O_{13}$ M = 1252.61 g/mol

The linear precursor peptide of **59** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 38 mg (30 μmol, 30%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was

shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 8.1 mg (6.5 μmol, 21.5%) of **59** in a total yield of 6.5% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 6.90 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1252.8028, found: 1252.8026 (–0.2 ppm); calculated for $[M+2H]^{2+}$: 626.9050, found: 626.9048 (–0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5834 (–3.4 ppm).

CalB_C3cPrGly (60)

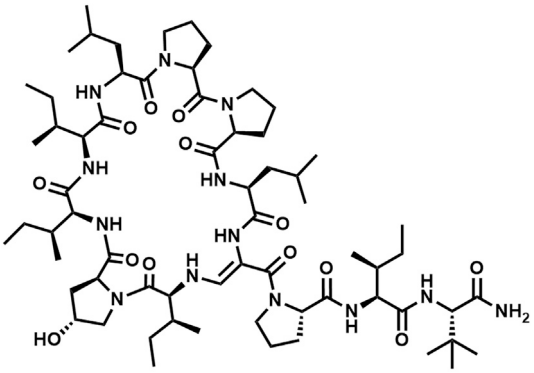


$C_{64}H_{105}N_{13}O_{13}$ M = 1264.62 g/mol

The linear precursor peptide of **60** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 69 mg (54 μmol, 39%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 6.2 mg (4.9 μmol, 9.1%) of **60** in a total yield of 3.5% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (700 MHz, DMSO-d_6) δ 9.01 (s, 1H), 8.86 (s, 1H), 8.25 (s, 1H), 7.51 (d, *J* = 10.3 Hz, 1H), 7.48 (s, 1H), 7.38 (d, *J* = 6.9 Hz, 1H), 7.22 (s, 1H), 7.07 (d, *J* = 13.3 Hz, 1H), 6.94 (s, 1H), 6.69 (d, *J* = 9.9 Hz, 1H), 5.73 (t, *J* = 10.0 Hz, 1H), 4.63-4.58 (m, 1H), 4.55-4.50 (m, 1H), 4.41 (s, 1H), 4.27-4.21 (m, 3H), 4.14-4.09 (m, 1H), 4.04 (t, *J* = 8.9 Hz, 1H), 3.89 (t, *J* = 10.2 Hz, 1H), 3.83-3.77 (m, 2H), 3.70-3.63 (m, 1H), 3.63-3.56 (m, 2H), 3.51-3.44 (m, 1H), 3.21-3.15 (m, 1H), 2.32-2.19 (m, 3H), 2.11-2.04 (m, 1H), 2.01-1.96 (m, 1H), 1.94-1.82 (m, 8H), 1.81-1.49 (m, 12H), 1.47-1.34 (m, 8H), 1.29-1.21 (m, 2H), 1.19-1.10 (m, 2H), 1.09-0.91 (m, 5H), 0.91-0.70 (m, 36H); LC main peak retention time: 7.22 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1264.8028, found: 1264.8015 (–1.0 ppm); calculated for $[M+2H]^{2+}$: 632.9050, found: 632.9043 (–1.1 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5845 (–2.2 ppm).

CalB_C3Tle (61)

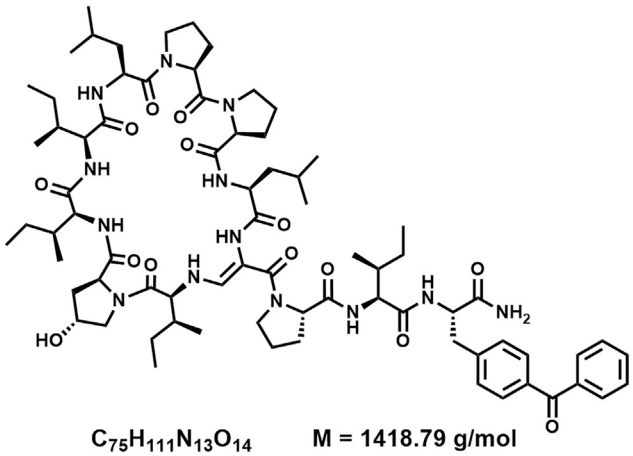


$C_{65}H_{109}N_{13}O_{13}$ M = 1280.67 g/mol

The linear precursor peptide of **61** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 51 mg (39 μmol, 28%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 6.4 mg (5.0 μmol, 12.7%) of **61** in a total yield of 3.6% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.00 (d, *J* = 6.9 Hz, 1H), 8.85 (s, 1H), 8.20 (s, 1H), 7.65 (d, *J* = 9.6 Hz, 1H), 7.37 (d, *J* = 7.1 Hz, 1H), 7.16 (d, *J* = 9.8 Hz, 1H), 6.98 (d, *J* = 14.3 Hz, 2H), 6.89 (s, 1H), 6.66 (d, *J* = 9.8 Hz, 1H), 5.63 (t, *J* = 11.6 Hz, 1H), 5.39 (s, 1H), 4.66-4.56 (m, 1H), 4.55-4.44 (m, 1H), 4.40 (s, 1H), 4.32-4.19 (m, 3H), 4.19-4.10 (m, 1H), 4.08-3.98 (m, 3H), 3.88-3.71 (m, 3H), 3.70-3.55 (m, 4H), 3.33-3.24 (m, 1H), 3.22-3.13 (m, 1H), 3.12-3.04 (m, 1H), 2.35-2.15 (m, 3H), 2.11-2.00 (m, 2H), 1.97-1.81 (m, 8H), 1.77-1.56 (m, 6H), 1.54-1.35 (m, 8H), 1.31-1.20 (m, 1H), 1.19-1.09 (m, 2H), 1.09-0.96 (m, 2H), 0.94-0.71 (m, 45H); LC main peak retention time: 8.13 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1280.8341, found: 1280.8334 (–0.5 ppm); calculated for $[M+2H]^{2+}$: 640.9207, found: 640.9203(–0.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5834 (–3.4 ppm).

CalB_C3Bpa (62)

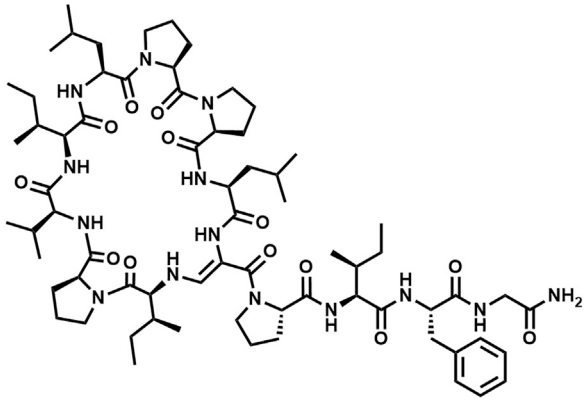


$C_{75}H_{111}N_{13}O_{14}$ M = 1418.79 g/mol

The linear precursor peptide of **62** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 65 mg (45 μmol, 32%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 3.1 mg (2.2 μmol, 4.8%) of **62** in a total yield of 1.6% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.98 (s, 1H), 8.80 (s, 1H), 8.18 (s, 1H), 7.66-7.59 (m, 4H), 7.57-7.46 (m, 5H), 7.44-7.38 (m, 2H), 7.35 (d, *J* = 7.0 Hz, 1H), 7.31 (s, 1H), 7.20 (d, *J* = 13.3 Hz, 1H), 6.93 (s, 1H), 6.67 (d, *J* = 10.0 Hz, 1H), 5.78 (t, *J* = 11.6 Hz, 1H), 5.36 (s, 1H), 4.63-4.57 (m, 1H), 4.56-4.49 (m, 1H), 4.39 (s, 1H), 4.29-4.16 (m, 5H), 4.04 (t, *J* = 8.7 Hz, 1H), 3.91-3.83 (m, 1H), 3.82 – 3.75 (m, 2H), 3.66 – 3.46 (m, 5H), 3.32 – 3.26 (m, 2H), 3.25 – 3.16 (m, 1H), 3.09 – 3.02 (m, 1H), 2.82 (t, *J* = 12.8 Hz, 1H), 2.35-2.17 (m, 4H), 2.07 (q, *J* = 7.0 Hz, 2H), 1.96-1.79 (m, 7H), 1.75-1.49 (m, 6H), 1.46-1.27 (m, 6H), 1.26-0.98 (m, 7H), 0.85-0.67 (m, 30H), 0.47 (d, *J* = 6.8 Hz, 3H), 0.31 (t, *J* = 7.3 Hz, 3H); LC main peak retention time: 7.81 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1418.8446, found: 1418.8438 (–0.6 ppm); calculated for $[M+2H]^{2+}$: 709.9260, found: 709.9253 (–1.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5846 (–2.1 ppm).

CalA_R2P (63)

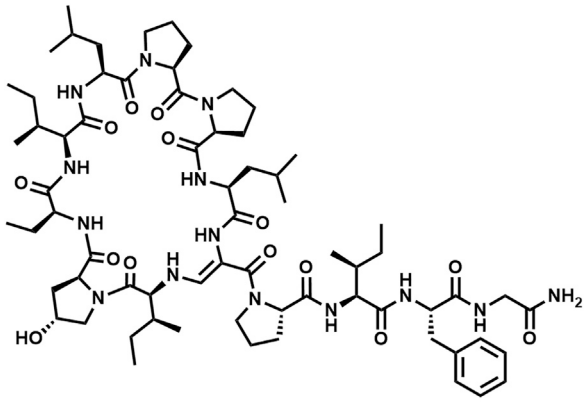


$C_{69}H_{108}N_{14}O_{13}$ M = 1341.71 g/mol

The linear precursor peptide of **63** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 36.5 mg (27.2 μmol) of **63** in a total yield of 27.2% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.94 (d, *J* = 7.0 Hz, 1H), 8.69 (d, *J* = 6.8 Hz, 1H), 8.24 (s, 1H), 8.10 (t, *J* = 6.2 Hz, 1H), 7.48 (d, *J* = 8.6 Hz, 1H), 7.44 (d, *J* = 7.0 Hz, 1H), 7.35 (d, *J* = 13.3 Hz, 1H), 7.29 (d, *J* = 8.6 Hz, 1H), 7.23-7.16 (m, 3H), 7.16-7.07 (m, 3H), 7.01 (s, 1H), 6.73 (d, *J* = 9.9 Hz, 1H), 5.84 (dd, *J* = 13.3, 10.1 Hz, 1H), 4.66-4.55 (m, 2H), 4.32-4.19 (m, 3H), 4.14-4.06 (m, 2H), 4.06-3.99 (m, 1H), 3.93-3.82 (m, 2H), 3.82-3.75 (m, 2H), 3.68-3.54 (m, 3H), 3.53-3.42 (m, 3H), 3.26-3.17 (m, 1H), 3.11 (dd, *J* = 13.8, 2.9 Hz, 1H), 3.00 (dd, *J* = 10.9, 7.0 Hz, 1H), 2.73-2.58 (m, 2H), 2.35-2.17 (m, 4H), 2.06-1.95 (m, 3H), 1.95-1.81 (m, 7H), 1.80-1.72 (m, 2H), 1.71-1.55 (m, 6H), 1.53-1.44 (m, 2H), 1.44-1.33 (m, 3H), 1.28-1.20 (m, 1H), 1.20-1.09 (m, 2H), 1.08-1.00 (m, 1H), 0.95 (d, *J* = 6.4 Hz, 3H), 0.90-0.75 (m, 24H), 0.73-0.67 (m, 4H), 0.44-0.36 (m, 6H); LC main peak retention time: 8.45 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1341.8293, found: 1341.8319 (1.9 ppm); calculated for $[M+2H]^{2+}$: 671.4183, found: 671.4186 (0.4 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 910.5761, found:910.5755 (–0.7 ppm).

CalA_R3Abu (64)



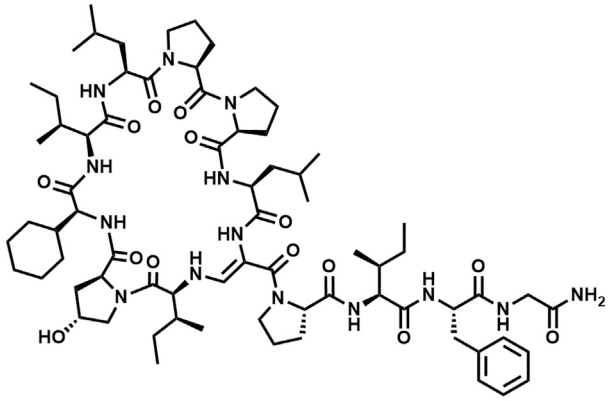
$C_{68}H_{106}N_{14}O_{14}$ M = 1343.68 g/mol

The linear precursor peptide of **64** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 90 mg (66 μmol, 47%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 20 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours

at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 21.1 mg (15.7 μmol, 23.7%) of **64** in a total yield of 11.2% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.83 (d, *J* = 6.8 Hz, 1H), 8.31 (s, 1H), 8.17-8.08 (m, 2H), 7.55 (d, *J* = 7.3 Hz, 1H), 7.48 (d, *J* = 8.7 Hz, 1H), 7.35 (d, *J* = 13.3 Hz, 1H), 7.28-7.21 (m, 2H), 7.21-7.10 (m, 5H), 7.00-6.95 (m, 1H), 6.76 (d, *J* = 9.9 Hz, 1H), 5.80-5.70 (m, 1H), 5.37 (d, *J* = 3.0 Hz, 1H), 4.66 (q, *J* = 6.9 Hz, 1H), 4.58 (td, *J* = 10.3, 4.2 Hz, 1H), 4.41 (s, 1H), 4.32-4.20 (m, 4H), 4.12 (t, *J* = 10.2 Hz, 1H), 4.03 (t, *J* = 8.7 Hz, 1H), 3.98-3.87 (m, 2H), 3.82-3.68 (m, 3H), 3.61-3.39 (m, 6H), 3.27-3.18 (m, 1H), 3.15-3.07 (m, 1H), 2.68-2.58 (m, 1H), 2.35-2.31 (m, 1H), 2.30-2.21 (m, 2H), 2.14-2.01 (m, 3H), 1.98-1.78 (m, 9H), 1.75-1.53 (m, 9H), 1.45-1.28 (m, 5H), 1.20-0.99 (m, 3H), 0.96 (d, *J* = 6.5 Hz, 3H), 0.90 (d, *J* = 6.4 Hz, 3H), 0.86-0.75 (m, 18H), 0.71 (t, *J* = 7.4 Hz, 3H), 0.42 (t, *J* = 7.3 Hz, 3H), 0.38 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 7.43 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1343.8086, found: 1343.8087 (0.1 ppm); calculated for $[M+2H]^{2+}$: 672.4079, found: 672.4073 (–0.9 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 912.5554, found: 912.5527 (–3.0 ppm).

CalA_R3Chg (65)

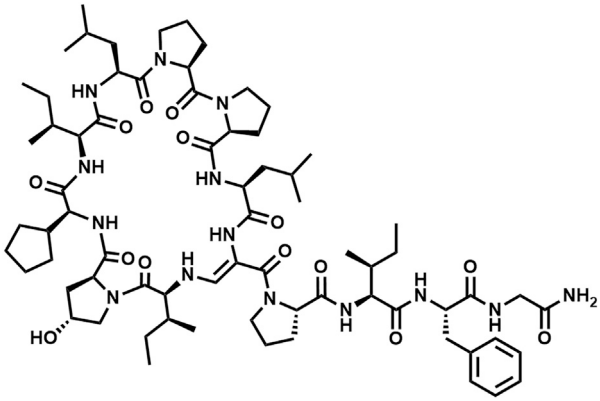


$C_{72}H_{112}N_{14}O_{14}$ **M = 1397.77 g/mol**

The linear precursor peptide of **65** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 40 mg (28 μmol, 20%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 9.4 mg (6.7 μmol, 23.8%) of **65** in a total yield of 4.8% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.01 (d, *J* = 7.0 Hz, 1H), 8.83 (d, *J* = 6.6 Hz, 1H), 8.28 (s, 1H), 8.18 (t, *J* = 6.1 Hz, 1H), 7.46 (d, *J* = 8.8 Hz, 1H), 7.42 (d, *J* = 7.0 Hz, 1H), 7.34 (d, *J* = 13.3 Hz, 1H), 7.29-7.23 (m, 2H), 7.19-7.09 (m, 5H), 6.97 (s, 1H), 6.71 (d, *J* = 10.0 Hz, 1H), 5.93-5.82 (m, 1H), 5.38 (d, *J* = 3.0 Hz, 1H), 4.64-4.55 (m, 2H), 4.40 (s, 1H), 4.30-4.20 (m, 4H), 4.10-4.00 (m, 2H), 3.93 (d, *J* = 7.2 Hz, 1H), 3.89 (d, *J* = 7.0 Hz, 1H), 3.81-3.74 (m, 3H), 3.70-3.55 (m, 4H), 3.35-3.25 (m, 1H), 3.24-3.15 (m, 1H), 3.12-3.01 (m, 2H), 2.61 (t, *J* = 12.9 Hz, 1H), 2.55-2.52 (m, 1H), 2.40-2.19 (m, 4H), 2.12-2.03 (m, 1H), 2.01-1.94 (m, 1H), 1.93-1.80 (m, 8H), 1.79-1.71 (m, 1H), 1.71-1.53 (m, 10H), 1.52-1.33 (m, 7H), 1.22 (s, 1H), 1.19-0.98 (m, 7H), 0.94 (d, *J* = 6.5 Hz, 3H), 0.87-0.74 (m, 18H), 0.69 (t, *J* = 7.3 Hz, 3H), 0.42 (t, *J* = 7.3 Hz, 3H), 0.37 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 9.08 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1397.8555, found: 1394.8557 (0.1 ppm); calculated for $[M+2H]^{2+}$: 699.4314, found: 699.4307 (–1.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 966.6023, found: 966.6001 (–2.3 ppm).

CalA_R3Cpg (66)

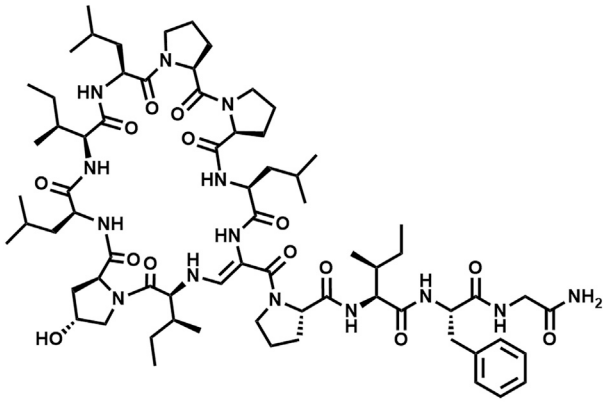


$C_{71}H_{110}N_{14}O_{14}$ M = 1383.75 g/mol

The linear precursor peptide of **66** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 19 mg (14 μmol, 10%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 6 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 3.5 mg (2.5 μmol, 18.5%) of **66** in a total yield of 1.0% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.08 (d, *J* = 6.9 Hz, 1H), 8.76 (d, *J* = 7.0 Hz, 1H), 8.29 (s, 1H), 8.17 (t, *J* = 6.2 Hz, 1H), 7.47 (d, *J* = 8.7 Hz, 1H), 7.42 (d, *J* = 7.0 Hz, 1H), 7.34 (d, *J* = 13.3 Hz, 1H), 7.28-7.23 (m, 2H), 7.20-7.09 (m, 5H), 6.99-6.95 (m, 1H), 6.72 (d, *J* = 10.0 Hz, 1H), 5.91-5.82 (m, 1H), 5.37 (d, *J* = 3.1 Hz, 1H), 4.65-4.57 (m, 2H), 4.40 (s, 1H), 4.31-4.21 (m, 4H), 4.12-4.01 (m, 2H), 3.96-3.87 (m, 1H), 3.85-3.74 (m, 3H), 3.72-3.66 (m, 1H), 3.63-3.55 (m, 2H), 3.53-3.44 (m, 3H), 3.25-3.16 (m, 1H), 3.13-3.05 (m, 2H), 2.97-2.86 (m, 1H), 2.62 (t, *J* = 12.9 Hz, 1H), 2.55-2.51 (m, 1H), 2.34-2.20 (m, 3H), 2.10-2.02 (m, 1H), 2.02-1.96 (m, 1H), 1.94-1.82 (m, 8H), 1.79-1.70 (m, 1H), 1.69-1.52 (m, 10H), 1.51-1.35 (m, 7H), 1.27-1.20 (m, 3H), 1.19-1.11 (m, 2H), 1.08-1.00 (m, 1H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.89-0.75 (m, 18H), 0.70 (t, *J* = 7.4 Hz, 3H), 0.42 (t, *J* = 7.3 Hz, 3H), 0.38 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 8.54 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1383.8399, found: 1383.8401 (0.1 ppm); calculated for $[M+2H]^{2+}$: 692.4236, found: 692.4231 (–0.7 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 952.5867, found: 952.5858 (–0.9 ppm).

CalA_R3L (67)



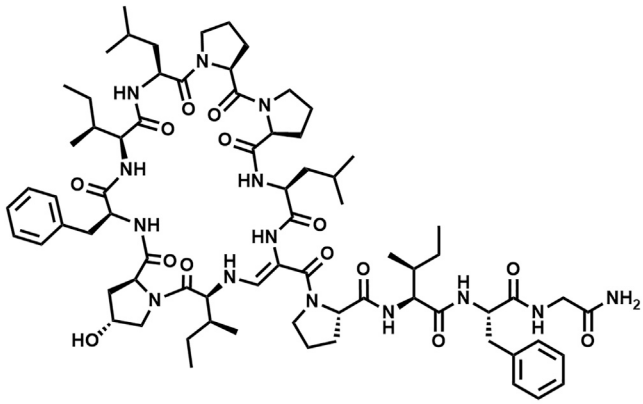
$C_{70}H_{110}N_{14}O_{14}$ M = 1371.73 g/mol

The linear precursor peptide of **67** was prepared via SPPS using Rink amide PS resin at a scale of 0.14 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 26 mg (19 μmol, 13%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the

peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 6.4 mg (4.7 μmol, 25.4%) of **67** in a total yield of 3.4% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.86 (d, *J* = 6.9 Hz, 1H), 8.31 (s, 1H), 8.19-8.12 (m, 2H), 7.54 (d, *J* = 7.3 Hz, 1H), 7.47 (d, *J* = 8.7 Hz, 1H), 7.34 (d, *J* = 13.3 Hz, 1H), 7.27-7.21 (m, 2H), 7.20-7.12 (m, 5H), 6.97 (s, 1H), 6.75 (d, *J* = 9.9 Hz, 1H), 5.82-5.72 (m, 1H), 5.39 (d, *J* = 3.1 Hz, 1H), 4.65 (q, *J* = 6.9 Hz, 1H), 4.57 (dd, *J* = 10.2, 4.1 Hz, 1H), 4.40 (s, 1H), 4.31-4.18 (m, 4H), 4.10 (t, *J* = 10.2 Hz, 1H), 4.03 (t, *J* = 8.8 Hz, 1H), 3.97-3.87 (m, 2H), 3.80-3.69 (m, 2H), 3.61-3.48 (m, 5H), 3.37-3.28 (m, 2H), 3.26-3.17 (m, 1H), 3.13-3.06 (m, 1H), 2.61 (t, *J* = 12.9 Hz, 1H), 2.54-2.51 (m, 1H), 2.35-2.30 (m, 1H), 2.29-2.20 (m, 2H), 2.13-2.03 (m, 2H), 1.97-1.81 (m, 7H), 1.73-1.36 (m, 15H), 1.34-1.25 (m, 1H), 1.22 (s, 1H), 1.20-1.12 (m, 1H), 1.11-0.99 (m, 2H), 0.95 (d, *J* = 6.5 Hz, 3H), 0.90-0.73 (m, 24H), 0.70 (t, *J* = 7.4 Hz, 3H), 0.42 (t, *J* = 7.3 Hz, 3H), 0.37 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 8.11 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1371.8399, found: 1371.8404 (0.4 ppm); calculated for $[M+2H]^{2+}$: 686.4236, found: 686.4233 (–0.4 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5856 (–1.1 ppm).

CalA_R3F (68)

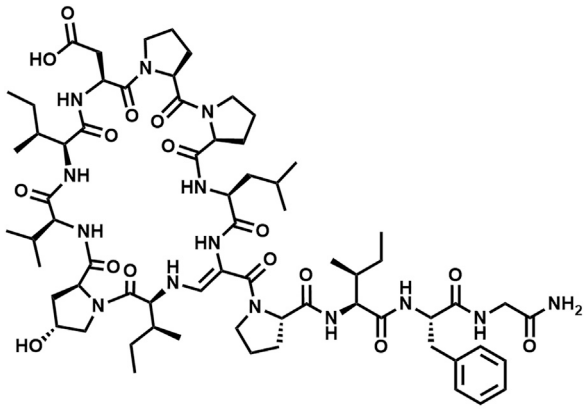


$C_{73}H_{108}N_{14}O_{14}$ M = 1405.75 g/mol

The linear precursor peptide of **68** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 7.2 mg (5.1 μmol) of **68** in a total yield of 5.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.15 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1405.8242, found: 1405.8247 (0.4 ppm); calculated for $[M+2H]^{2+}$: 703.4158, found: 703.4160 (0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 974.5710, found: 974.5698 (–1.2 ppm).

CalA_R5D (69)

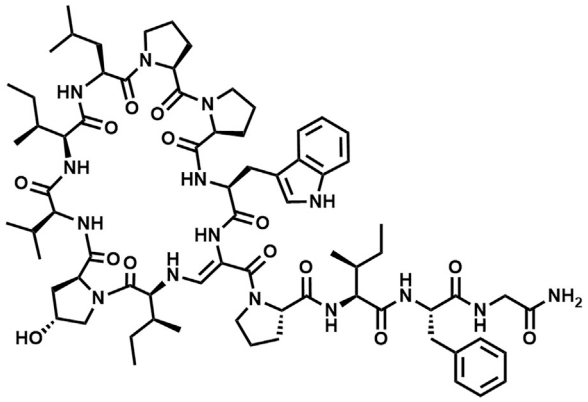


$C_{67}H_{102}N_{14}O_{16}$ M = 1359.64 g/mol

The linear precursor peptide of **69** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 4.7 mg (3.5 μmol) of **69** in a total yield of 3.5% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 5.28 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1359.7671, found: 1359.7677 (0.4 ppm); calculated for $[M+2H]^{2+}$: 680.3872, found: 680.3870 (–0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 928.5138, found: 928.5136 (–0.2 ppm).

CalA_R8W (70)

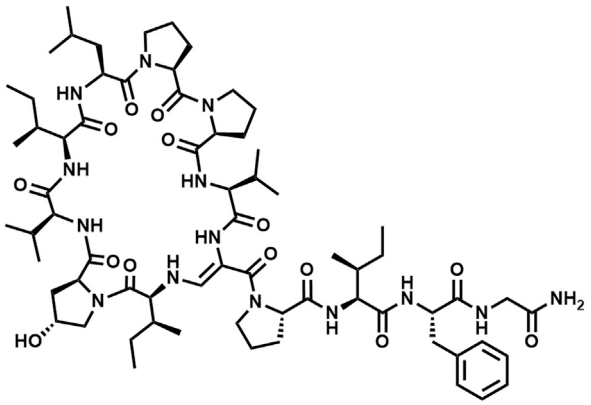


$C_{74}H_{107}N_{15}O_{14}$ M = 1430.76 g/mol

The linear precursor peptide of **70** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 6.2 mg (4.3 μmol) of **70** in a total yield of 4.3% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 6.79 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1430.8195, found: 1430.8207 (0.8 ppm); calculated for $[M+2H]^{2+}$: 715.9134, found: 715.9127 (–1.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 999.5662, found: 999.5626 (–3.6 ppm).

CalA_R8V (71)

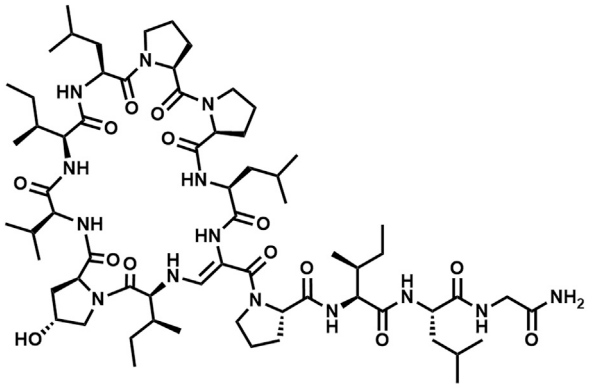


$C_{68}H_{106}N_{14}O_{14}$ M = 1343.68 g/mol

The linear precursor peptide of **71** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 9.2 mg (6.8 μmol) of **71** in a total yield of 6.8% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H), 8.80 (s, 1H), 8.36 (s, 1H), 8.10 (t, *J* = 6.1 Hz, 1H), 7.53 (d, *J* = 8.3 Hz, 1H), 7.37 (d, *J* = 6.5 Hz, 1H), 7.31 (d, *J* = 13.3 Hz, 1H), 7.28-7.13 (m, 7H), 6.95 (s, 1H), 6.69 (d, *J* = 9.9 Hz, 1H), 5.73 (dd, *J* = 14.1, 9.9 Hz, 1H), 5.37 (d, *J* = 3.1 Hz, 1H), 4.55 (td, *J* = 10.3, 5.0 Hz, 2H), 4.40 (s, 1H), 4.30-4.20 (m, 4H), 4.07-3.97 (m, 2H), 3.90 (dd, *J* = 16.5, 7.2 Hz, 1H), 3.79-3.71 (m, 4H), 3.66-3.52 (m, 3H), 3.48 (dd, *J* = 16.7, 5.1 Hz, 1H), 3.26-3.16 (m, 2H), 3.12 (dd, *J* = 13.7, 3.1 Hz, 1H), 3.03-2.95 (m, 1H), 2.69-2.57 (m, 2H), 2.47-2.38 (m, 1H), 2.32-2.21 (m, 3H), 2.12-2.04 (m, 2H), 2.01-1.96 (m, 1H), 1.96-1.86 (m, 5H), 1.86-1.76 (m, 2H), 1.76-1.60 (m, 3H), 1.55 (td, *J* = 13.5, 6.6 Hz, 2H), 1.49-1.32 (m, 4H), 1.27-1.17 (m, 2H), 1.17-1.02 (m, 2H), 0.99 (d, *J* = 7.2 Hz, 3H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.89 (d, *J* = 6.6 Hz, 3H), 0.87 (d, *J* = 6.5 Hz, 3H), 0.84-0.74 (m, 15H), 0.74-0.69 (m, 4H), 0.41 (t, *J* = 7.3 Hz, 3H), 0.38 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 7.69 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1343.8086, found: 1343.8092 (0.4 ppm); calculated for $[M+2H]^{2+}$: 672.9096, found: 672.9101 (0.7 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 912.5553, found: 912.5564 (1.2 ppm).

CalA_C3L (72)



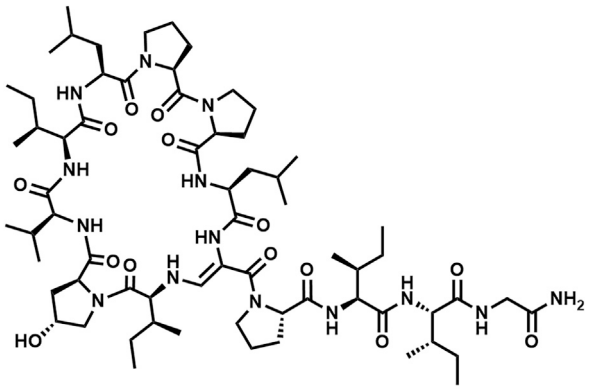
$C_{66}H_{110}N_{14}O_{14}$ M = 1323.69 g/mol

The linear precursor peptide of **72** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then

dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 6.3 mg (4.8 μmol) of **72** in a total yield of 4.8% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.04 (d, *J* = 6.8 Hz, 1H), 8.83 (d, *J* = 5.8 Hz, 1H), 8.29 (d, *J* = 6.4 Hz, 1H), 8.28 (s, 1H), 7.46 (d, *J* = 6.9 Hz, 1H), 7.43 (d, *J* = 8.1 Hz, 1H), 7.35 (d, *J* = 6.5 Hz, 1H), 7.32 (d, *J* = 3.1 Hz, 1H), 7.19 (s, 1H), 6.91 (s, 1H), 6.63 (d, *J* = 10.1 Hz, 1H), 5.81 (dd, *J* = 12.9, 9.8 Hz, 1H), 5.36 (d, *J* = 3.0 Hz, 1H), 4.66-4.54 (m, 2H), 4.40 (s, 1H), 4.29-4.16 (m, 4H), 4.15-4.07 (m, 2H), 4.07-4.01 (m, 1H), 3.92 (dd, *J* = 16.2, 7.1 Hz, 1H), 3.79 (dd, *J* = 8.5, 6.6 Hz, 1H), 3.76-3.66 (m, 2H), 3.65-3.55 (m, 2H), 3.54-3.45 (m, 2H), 3.40 (dd, *J* = 16.2, 5.1 Hz, 1H), 3.22-3.12 (m, 1H), 3.01-2.94 (m, 1H), 2.71-2.62 (m, 1H), 2.35-2.29 (m, 1H), 2.28-2.19 (m, 2H), 2.12-2.04 (m, 2H), 2.03-1.96 (m, 1H), 1.95-1.80 (m, 7H), 1.76-1.66 (m, 3H), 1.66-1.55 (m, 3H), 1.54-1.37 (m, 8H), 1.30-1.22 (m, 2H), 1.21-1.09 (m, 1H), 1.02-0.93 (m, 1H), 0.89 (d, *J* = 5.1 Hz, 3H), 0.88-0.79 (m, 30H), 0.78 (d, *J* = 3.8 Hz, 3H), 0.76 (d, *J* = 3.8 Hz, 3H), 0.75-0.70 (m, 3H); LC main peak retention time: 7.45 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1323.8399, found: 1323.8419 (1.5 ppm); calculated for $[M+2H]^{2+}$: 662.4236, found: 662.4237 (0.2 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5701 (–1.0 ppm).

CalA_C3I (73)

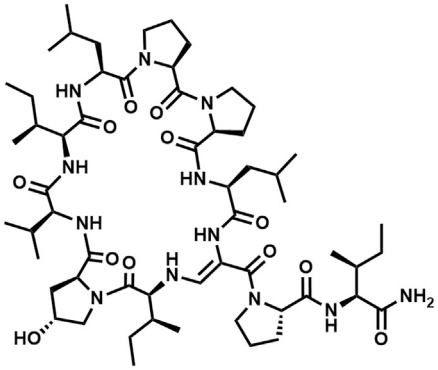


$C_{66}H_{110}N_{14}O_{14}$ M = 1323.69 g/mol

The linear precursor peptide of **73** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 2.3 mg (1.7 μmol) of **73** in a total yield of 1.7% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.53 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1323.8399, found: 1323.8405 (0.5 ppm); calculated for $[M+2H]^{2+}$: 662.4236, found: 662.4233 (–0.5 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5721 (1.2 ppm).

CalA_C3X (74)

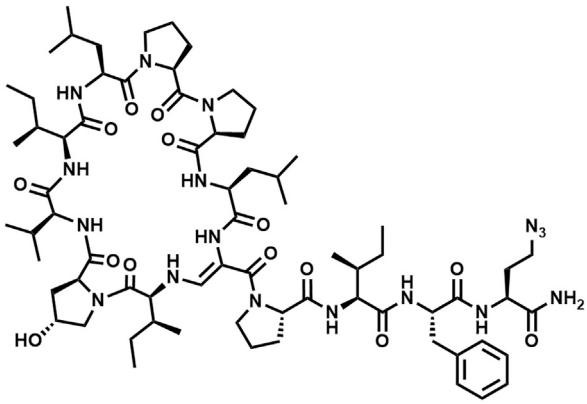


$C_{58}H_{96}N_{12}O_{12}$ **M = 1153.48 g/mol**

The linear precursor peptide of **74** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 8.8 mg (7.6 μmol) of **74** in a total yield of 7.6% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.95 (s, 1H), 8.77 (s, 1H), 8.06 (s, 1H), 7.47 (d, *J* = 9.1 Hz, 1H), 7.42 (d, *J* = 6.8 Hz, 1H), 7.15 (d, *J* = 12.9 Hz, 1H), 7.05 (s, 1H), 6.81 (s, 1H), 6.74 (d, *J* = 9.9 Hz, 1H), 5.56 (dd, *J* = 12.9, 10.2 Hz, 1H), 5.36 (d, *J* = 3.1 Hz, 1H), 4.61 (q, *J* = 6.7 Hz, 1H), 4.51-4.43 (m, 1H), 4.39 (s, 1H), 4.33 (dd, *J* = 9.4, 7.5 Hz, 1H), 4.27-4.21 (m, 1H), 4.04 (t, *J* = 9.0 Hz, 1H), 3.99 (dd, *J* = 9.1, 6.3 Hz, 1H), 3.94 (t, *J* = 9.7 Hz, 1H), 3.83 (t, *J* = 7.5 Hz, 1H), 3.78 (d, *J* = 11.4 Hz, 1H), 3.67 (dd, *J* = 10.9, 3.5 Hz, 1H), 3.64-3.54 (m, 2H), 3.54-3.43 (m, 2H), 3.29-3.23 (m, 1H), 3.21-3.13 (m, 1H), 3.07-2.97 (m, 1H), 2.69-2.58 (m, 1H), 2.32-2.15 (m, 3H), 2.12-2.04 (m, 1H), 2.02-1.98 (m, 1H), 1.97-1.84 (m, 7H), 1.80-1.69 (m, 4H), 1.66-1.58 (m, 3H), 1.56-1.37 (m, 7H), 1.36-1.27 (m, 1H), 1.22-1.10 (m, 1H), 1.08-0.94 (m, 2H), 0.92-0.89 (m, 9H), 0.86-0.79 (m, 27H); LC main peak retention time: 6.46 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1153.7343, found: 1153.7338 (–0.4 ppm); calculated for $[M+2H]^{2+}$: 577.3708, found: 577.3702 (–1.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5721 (1.2 ppm).

CalA_C4Aha (75)

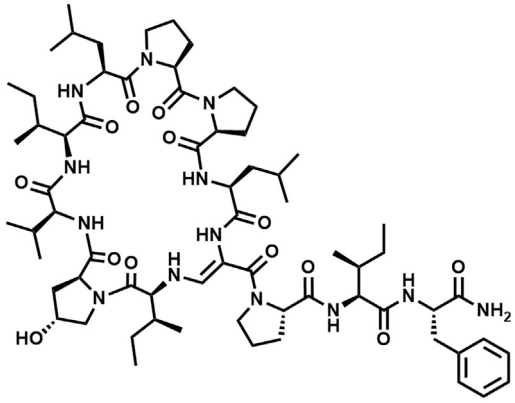


$C_{71}H_{111}N_{17}O_{14}$ **M = 1426.77 g/mol**

The linear precursor peptide of **75** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 8.2 mg (5.7 μmol) of **75** in a total yield of 5.7% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.00 (s, 1H), 8.77 (s, 1H), 8.15 (s, 1H), 7.68 (d, *J* = 7.2 Hz, 1H), 7.45 (d, *J* = 8.1 Hz, 1H), 7.40 (d, *J* = 8.4 Hz, 1H), 7.35 (d, *J* = 7.2 Hz, 1H), 7.26-7.18 (m, 3H), 7.14-7.02 (m, 4H), 6.77 (s, 1H), 6.72 (d, *J* = 9.7 Hz, 1H), 5.77 (dd, *J* = 12.9, 10.0 Hz, 1H), 5.37 (d, *J* = 3.0 Hz, 1H), 4.64 (q, *J* = 6.9 Hz, 1H), 4.56 (dd, *J* = 9.9, 5.0 Hz, 1H), 4.39 (s, 1H), 4.31-4.24 (m, 2H), 4.22-4.12 (m, 3H), 4.10-4.01 (m, 1H), 3.85-3.70 (m, 4H), 3.64 (dd, *J* = 11.1, 3.5 Hz, 1H), 3.61-3.53 (m, 2H), 3.52-3.44 (m, 4H), 3.24 (td, *J* = 11.0, 6.0 Hz, 1H), 3.15 (dd, *J* = 13.3, 3.0 Hz, 1H), 2.99 (dd, *J* = 10.9, 6.8 Hz, 1H), 2.84-2.73 (m, 1H), 2.71-2.55 (m, 1H), 2.34-2.19 (m, 3H), 2.12-2.05 (m, 1H), 2.04-1.97 (m, 2H), 1.96-1.81 (m, 9H), 1.79-1.68 (m, 4H), 1.66-1.54 (m, 3H), 1.51-1.41 (m, 1H), 1.39-1.32 (m, 1H), 1.30-1.21 (m, 2H), 1.18-1.05 (m, 5H), 0.96 (d, *J* = 6.8 Hz, 3H), 0.91 (d, *J* = 6.5 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 6H), 0.85-0.74 (m, 15H), 0.70 (t, *J* = 7.4 Hz, 3H), 0.59-0.52 (m, 1H), 0.50 (d, *J* = 6.8 Hz, 3H), 0.26 (t, *J* = 7.4 Hz, 3H); LC main peak retention time: 8.40 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1426.8569, found: 1426.8575 (0.4 ppm); calculated for $[M+2H]^{2+}$: 713.9321, found: 713.9322 (0.1 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5727 (1.8 ppm).

CalA_C4X (76)

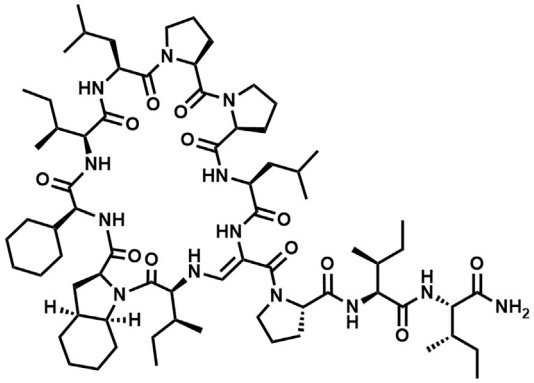


$C_{67}H_{105}N_{13}O_{13}$ M = 1300.66 g/mol

The linear precursor peptide of **76** was prepared via SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity and purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). The obtained peptide was then dissolved in 10 mL of ACN and 4 equivalents DMP were added. The resulting suspension was shaken for one hour at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.9 mg (1.5 μmol) of **76** in a total yield of 1.5% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.02 (s, 1H), 8.82 (s, 1H), 8.27 (s, 1H), 7.45 (d, *J* = 9.0 Hz, 2H), 7.39 (d, *J* = 7.4 Hz, 1H), 7.22-7.18 (m, 3H), 7.15 (d, *J* = 7.8 Hz, 1H), 7.14-7.08 (m, 4H), 6.73 (d, *J* = 9.6 Hz, 1H), 5.86-5.77 (m, 1H), 5.38 (d, *J* = 3.0 Hz, 1H), 4.66-4.55 (m, 2H), 4.41 (s, 1H), 4.30-4.17 (m, 4H), 4.06 (t, *J* = 8.2 Hz, 1H), 3.88 (t, *J* = 10.5 Hz, 1H), 3.84-3.77 (m, 3H), 3.68-3.63 (m, 1H), 3.62-3.55 (m, 2H), 3.53-3.44 (m, 2H), 3.24-3.16 (m, 2H), 3.02-2.94 (m, 1H), 2.70-2.61 (m, 1H), 2.56 (t, *J* = 12.7 Hz, 1H), 2.33-2.20 (m, 3H), 2.13-2.05 (m, 1H), 2.02-1.97 (m, 1H), 1.96-1.84 (m, 7H), 1.82-1.68 (m, 3H), 1.66-1.54 (m, 4H), 1.54-1.46 (m, 2H), 1.44-1.32 (m, 3H), 1.30-1.21 (m, 3H), 1.14-1.01 (m, 2H), 0.94 (d, *J* = 6.6 Hz, 3H), 0.90-0.77 (m, 24H), 0.74-0.70 (m, 1H), 0.67 (t, *J* = 7.4 Hz, 3H), 0.48 (t, *J* = 7.2 Hz, 3H), 0.33 (d, *J* = 6.8 Hz, 3H); LC main peak retention time: 6.98 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1300.8028, found: 1300.8048 (1.5 ppm); calculated for $[M+2H]^{2+}$: 650.9050, found: 950.9052 (0.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 926.5710, found: 926.5722 (1.3 ppm).

CalB_R2Oic_R3Chg (77)

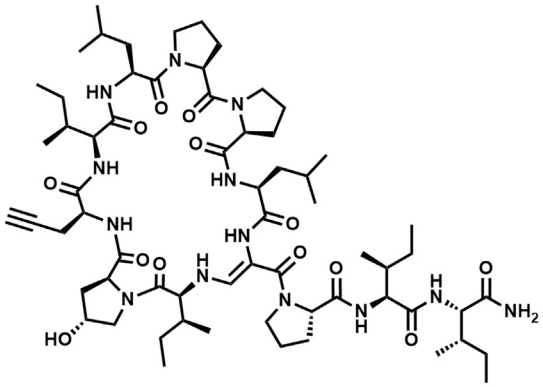


$C_{71}H_{117}N_{13}O_{12}$ M = 1344.80 g/mol

The linear precursor peptide of **77** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 72 mg (52 μmol, 52%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 15 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 3.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 4.1 mg (3.0 μmol, 5.8%) of **77** in a total yield of 3.0% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (700 MHz, DMSO-d_6) δ 8.85 (d, *J* = 7.0 Hz, 1H), 8.58 (d, *J* = 6.6 Hz, 1H), 8.26 (s, 1H), 7.62 – 7.55 (m, 2H), 7.30 (d, *J* = 9.6 Hz, 1H), 7.12 (d, *J* = 13.4 Hz, 1H), 7.05 (s, 1H), 7.03 (s, 1H), 6.66 (d, *J* = 10.0 Hz, 1H), 5.58 (dd, *J* = 13.4, 10.0 Hz, 1H), 4.59 (q, *J* = 6.7 Hz, 1H), 4.53 (td, *J* = 10.3, 3.8 Hz, 1H), 4.27 (dd, *J* = 10.0, 7.7 Hz, 1H), 4.23 (t, *J* = 7.9 Hz, 1H), 4.17 (dd, *J* = 9.6, 5.1 Hz, 1H), 4.13-4.06 (m, 2H), 4.04 (dt, *J* = 12.4, 6.5 Hz, 1H), 3.95 (t, *J* = 8.8 Hz, 1H), 3.81 (t, *J* = 10.2 Hz, 1H), 3.73 (dd, *J* = 9.5, 6.6 Hz, 1H), 3.60-3.51 (m, 2H), 3.51-3.45 (m, 1H), 3.45-3.41 (m, 1H), 3.28 (dt, *J* = 10.2, 7.0 Hz, 1H), 3.18 (td, *J* = 10.9, 5.8 Hz, 1H), 3.07 (dd, *J* = 11.1, 7.0 Hz, 1H), 2.49-2.44 (m, 2H), 2.34-2.30 (m, 1H), 2.29-2.25 (m, 1H), 2.25-2.19 (m, 1H), 2.03-1.96 (m, 2H), 1.93-1.83 (m, 7H), 1.82-1.78 (m, 1H), 1.77-1.72 (m, 2H), 1.71-1.64 (m, 7H), 1.63-1.55 (m, 5H), 1.54-1.40 (m, 9H), 1.31-1.18 (m, 7H), 1.17-1.10 (m, 3H), 1.06-0.99 (m, 2H), 0.94-0.90 (m, 1H), 0.90-0.86 (m, 12H), 0.86-0.85 (m, 1H), 0.84-0.77 (m, 18H), 0.75 (t, *J* = 6.4 Hz, 3H), 0.73 (t, *J* = 6.0 Hz, 3H); ^{13}C NMR (176 MHz, DMSO-d_6) δ 173.0, 172.8, 172.8, 172.4, 171.9, 171.5, 171.4, 171.4, 171.3, 171.2, 171.2, 167.2, 142.3, 98.1, 64.8, 63.9, 63.8, 62.5, 61.2, 61.1, 59.8, 58.1, 57.4, 57.3, 50.0, 49.2, 48.7, 47.1, 46.1, 41.1, 40.4, 37.5, 36.8, 36.3, 36.2, 35.8, 29.7, 29.5, 29.0, 28.5, 26.3, 25.8, 25.6, 25.2, 25.1, 25.1, 25.0, 24.9, 24.8, 24.6, 24.0, 23.6, 23.5, 23.1, 22.7, 22.2, 20.9, 19.4, 15.8, 15.7, 15.1, 11.5, 11.0, 10.8, 9.5; LC main peak retention time: 17.11 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1344.9017, found: 1344.9020 (0.2 ppm); calculated for $[M+2H]^{2+}$: 672.9545, found: 972.9536 (–1.3 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 1004.6543, found: 1004.6513 (–3.0 ppm).

CalB_R3Pra (78)

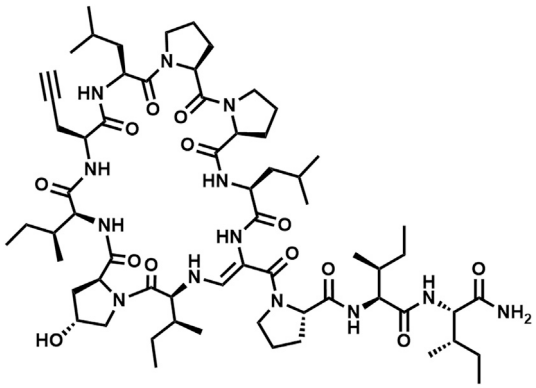


$C_{64}H_{103}N_{13}O_{13}$ M = 1262.61 g/mol

The linear precursor peptide of **78** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 42 mg (33 μmol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 8 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 3 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 9.2 mg (7.3 μmol, 22.3%) of **78** in a total yield of 7.3% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.13 (d, *J* = 6.8 Hz, 1H), 8.30 (s, 1H), 7.80 (d, *J* = 9.0 Hz, 1H), 7.60 (d, *J* = 10.0 Hz, 1H), 7.49 (d, *J* = 7.6 Hz, 1H), 7.23 (d, *J* = 9.7 Hz, 1H), 7.14 (s, 1H), 7.07 (d, *J* = 13.2 Hz, 1H), 6.97 (s, 1H), 6.68 (d, *J* = 9.9 Hz, 1H), 5.62-5.50 (m, 1H), 5.40 (d, *J* = 3.1 Hz, 1H), 4.75-4.64 (m, 1H), 4.56-4.46 (m, 1H), 4.42 (s, 1H), 4.33-4.21 (m, 3H), 4.19-4.13 (m, 1H), 4.12-4.06 (m, 1H), 4.05-3.95 (m, 3H), 3.77-3.69 (m, 2H), 3.62-3.44 (m, 4H), 3.39-3.33 (m, 1H), 3.23-3.13 (m, 1H), 3.09-2.99 (m, 1H), 2.97-2.93 (m, 1H), 2.78-2.69 (m, 1H), 2.41-2.32 (m, 1H), 2.31-2.17 (m, 2H), 2.12-2.01 (m, 2H), 2.01-1.75 (m, 8H), 1.74-1.55 (m, 6H), 1.54-1.33 (m, 8H), 1.28-1.21 (m, 1H), 1.19-0.96 (m, 5H), 0.95-0.69 (m, 36H); LC main peak retention time: 7.15 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1262.7871, found: 1262.7857 (–1.1 ppm); calculated for $[M+2H]^{2+}$: 631.8972, found: 631.8963 (–1.4 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 922.5397, found: 922.5377 (–2.2 ppm).

CalB_R4Pra (79)

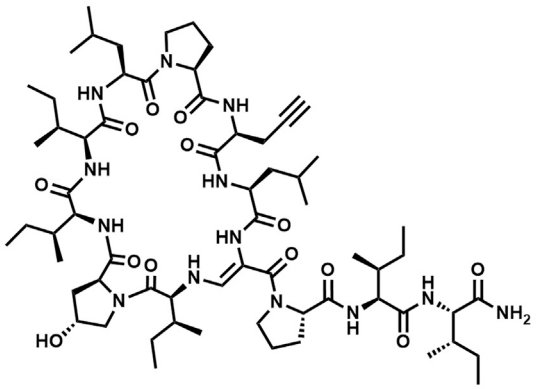


$C_{64}H_{103}N_{13}O_{13}$ **M = 1262.61 g/mol**

The linear precursor peptide of **79** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 29 mg (23 μmol, 23%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 2.6 mg (2.1 μmol, 9.1%) of **79** in a total yield of 2.1% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.81 (s, 1H), 8.73 (s, 1H), 8.30 (s, 1H), 7.59 (d, *J* = 9.9 Hz, 1H), 7.55 (d, *J* = 7.0 Hz, 1H), 7.23 (d, *J* = 9.6 Hz, 1H), 7.14 (s, 1H), 7.07 (d, *J* = 13.3 Hz, 1H), 6.96 (d, *J* = 2.8 Hz, 1H), 6.67 (d, *J* = 9.7 Hz, 1H), 5.70-5.60 (m, 1H), 5.45-5.33 (m, 1H), 4.63-4.56 (m, 1H), 4.56-4.48 (m, 1H), 4.42 (s, 1H), 4.28-4.18 (m, 3H), 4.17-4.10 (m, 1H), 4.10-4.01 (m, 3H), 3.98-3.89 (m, 1H), 3.78-3.67 (m, 2H), 3.61-3.48 (m, 4H), 3.34-3.28 (m, 1H), 3.23-3.13 (m, 2H), 2.98-2.94 (m, 1H), 2.60-2.53 (m, 1H), 2.33-2.20 (m, 3H), 2.11-1.97 (m, 2H), 1.95-1.84 (m, 8H), 1.73-1.56 (m, 6H), 1.51-1.39 (m, 8H), 1.24 (s, 1H), 1.17-0.95 (m, 5H), 0.90-0.76 (m, 36H); LC main peak retention time: 5.80 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1262.7871, found: 1262.7861 (–0.8 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 922.5397, found: 922.5407 (1.1 ppm).

CalB_R7Pra (80)

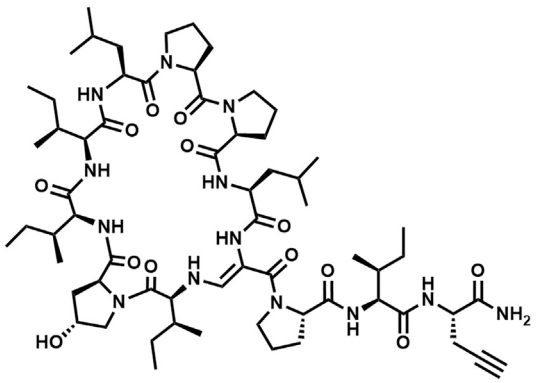


$C_{65}H_{107}N_{13}O_{13}$ M = 1278.65 g/mol

The linear precursor peptide of **80** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 40 mg (31 μmol, 22%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.7 mg (1.3 μmol, 4.3%) of **80** in a total yield of 0.9% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.61 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1278.8184, found: 1278.8171 (–1.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 938.5710, found: 938.5712 (0.2 ppm).

CalB_C3Pra (81)



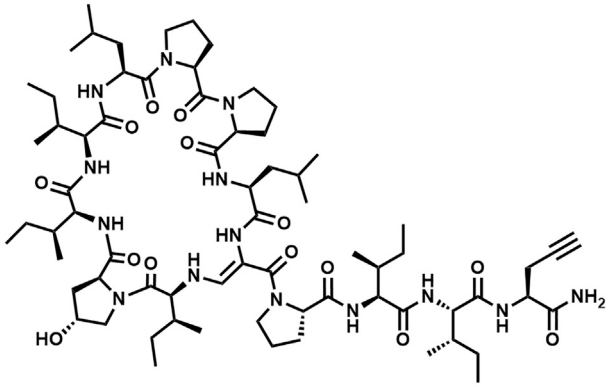
$C_{64}H_{103}N_{13}O_{13}$ M = 1262.61 g/mol

The linear precursor peptide of **81** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 43 mg (33 μmol, 33%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.9 mg (1.5 μmol, 4.5%) of **81** in a total yield of 1.5% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 (d, *J* = 7.0 Hz, 1H), 8.85 (d, *J* = 6.5 Hz, 1H), 8.35 (s, 1H), 7.78 (d, *J* = 8.7 Hz, 1H), 7.44-7.35 (m, 3H), 7.16 (s, 1H), 7.08 (d, *J* = 13.4 Hz, 1H), 6.70 (d, *J* = 9.8 Hz, 1H), 5.83-5.70 (m, 1H), 5.40 (d, *J* = 3.1 Hz, 1H), 4.64-4.49 (m, 2H), 4.41 (s, 1H), 4.28-4.19 (m, 5H), 4.18-4.12 (m, 1H), 4.06-4.00 (m, 1H), 3.91-3.84 (m, 1H), 3.82-3.75 (m, 2H), 3.70-3.56 (m, 4H), 3.52-3.45 (m, 1H), 3.23-3.13 (m, 1H), 3.12-3.03 (m, 1H), 2.73-2.68 (m, 1H), 2.68-2.59 (m, 1H), 2.31-2.18 (m, 3H), 2.14-2.04 (m, 2H), 1.99-1.77 (m, 8H), 1.71-1.48 (m, 6H), 1.47-1.32 (m, 8H), 1.31-1.21 (m, 1H), 1.19-0.99 (m, 5H), 0.92-.72 (m, 36H); LC

main peak retention time: 6.81 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1262.7871, found: 1262.7865 (–0.5 ppm); calculated for $[M+2H]^{2+}$: 631.8972, found: 631.8967 (–0.8 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5854 (–1.3 ppm).

CalB_C4Pra (82)

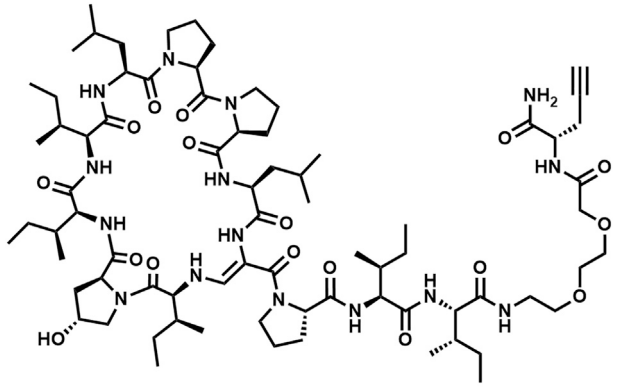


$C_{70}H_{114}N_{14}O_{14}$ **M = 1375.77 g/mol**

The linear precursor peptide of **82** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.2 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 130 mg (93 μmol, 46%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 20 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 25.3 mg (18.4 μmol, 19.7%) of **82** in a total yield of 9.0% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (700 MHz, DMSO-d_6) δ 9.01 (s, 1H), 8.85 (s, 1H), 8.17 (s, 1H), 8.10 (d, *J* = 8.3 Hz, 1H), 7.47 (d, *J* = 8.7 Hz, 1H), 7.36 (d, *J* = 7.0 Hz, 1H), 7.26 (d, *J* = 7.7 Hz, 1H), 7.22 (s, 1H), 6.98 (d, *J* = 13.1 Hz, 1H), 6.70 (s, 1H), 6.63 (d, *J* = 9.9 Hz, 1H), 5.71 (dd, *J* = 13.3, 10.1 Hz, 1H), 4.62 (q, *J* = 6.8 Hz, 1H), 4.52 (td, *J* = 9.9, 4.9 Hz, 1H), 4.41 (s, 1H), 4.29-4.22 (m, 4H), 4.04 (t, *J* = 9.0 Hz, 1H), 3.99 (dd, *J* = 8.7, 7.3 Hz, 1H), 3.89 (t, *J* = 8.4 Hz, 1H), 3.80 (t, *J* = 7.5 Hz, 1H), 3.68-3.66 (m, 2H), 3.66-3.56 (m, 4H), 3.32-3.28 (m, 1H), 3.23-3.18 (m, 1H), 3.09-3.05 (m, 1H), 2.85 (t, *J* = 2.6 Hz, 1H), 2.71-2.67 (m, 1H), 2.56 (ddd, *J* = 16.9, 10.0, 2.6 Hz, 1H), 2.32-2.27 (m, 1H), 2.25-2.19 (m, 2H), 2.09-2.05 (m, 1H), 2.02-1.94 (m, 2H), 1.93-1.83 (m, 6H), 1.81-1.68 (m, 4H), 1.65-1.57 (m, 4H), 1.57-1.50 (m, 3H), 1.49-1.36 (m, 6H), 1.30-1.25 (m, 1H), 1.23 (s, 1H), 1.22-1.17 (m, 1H), 1.16-1.09 (m, 2H), 1.07-1.00 (m, 1H), 0.94-0.90 (m, 1H), 0.90-0.88 (m, 9H), 0.86 (d, *J* = 6.6 Hz, 3H), 0.85-0.80 (m, 24H), 0.78 (d, *J* = 6.7 Hz, 3H), 0.71 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (176 MHz, DMSO-d_6) δ 173.4, 172.7, 172.2, 172.2, 172.2, 171.9, 171.8, 171.5, 171.4, 171.2, 171.1, 167.3, 161.7, 141.4, 99.3, 81.7, 72.2, 68.8, 65.4, 64.0, 62.6, 61.7, 60.0, 59.4, 58.8, 58.2, 56.7, 52.4, 50.2, 49.2, 48.7, 47.0, 46.2, 41.2, 40.9, 38.1, 37.5, 36.2, 35.7, 35.4, 32.5, 29.6, 28.5, 26.3, 25.7, 25.6, 25.1, 24.9, 24.7, 24.6, 24.0, 23.2, 22.5, 22.1, 21.4, 21.3, 15.6, 15.4, 15.3, 15.3, 14.5, 14.0, 11.3, 11.2, 10.5, 10.3, 9.9; LC main peak retention time: 8.41 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1375.8712, found: 1375.8708 (–0.3 ppm); calculated for $[M+2H]^{2+}$: 688.4392, found: 688.4384 (–1.2 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5852 (–1.5 ppm).

CalB_C5Pra (83)

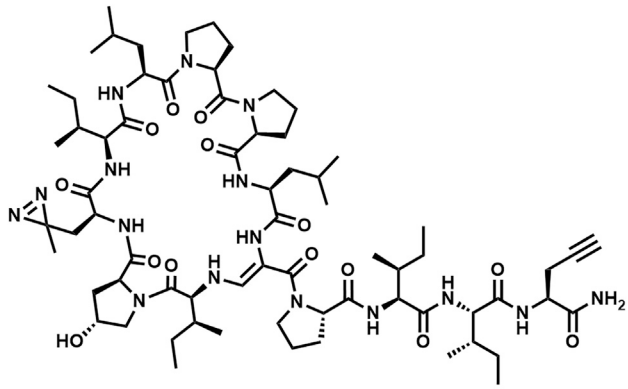


$C_{76}H_{125}N_{15}O_{17}$ M = 1520.92 g/mol

The linear precursor peptide of **83** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 48 mg (31 μmol, 31%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 10 mL of ACN and 3 equivalents of DMP were added. The resulting suspension was shaken for 2.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 6.6 mg (4.3 μmol, 13.9%) of **83** in a total yield of 4.3% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 7.42 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1520.9451, found: 1520.9440 (–0.7 ppm); calculated for $[M+2H]^{2+}$: 760.9762, found: 760.9751 (–1.4 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 940.5866, found: 940.5825 (–4.4 ppm).

CalB_R3phLeu (84)



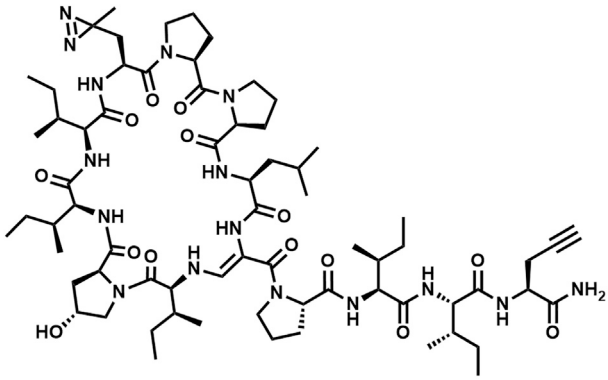
$C_{69}H_{110}N_{16}O_{14}$ M = 1387.74 g/mol

The linear precursor peptide of **84** was prepared via microwave-assisted SPPS up to the R2 position using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. The loading of the resin was quantified through a spectrometric Fmoc-Test and a manual coupling of photo-leucine was performed using 4 equivalents of amino acid combined with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) and hydroxybenzotriazole (HOBt) (each 4 equivalents) as well as 5 equivalents of DIPEA in 10 mL DMF by shaking the suspension over night at room temperature. The resin was then thoroughly washed with DMF and a second round of microwave-assisted SPPS was performed for completion of the peptide sequence. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 38 mg (27 μmol, 27%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, 36 mg of peptide was dissolved in 5 mL

of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 4.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 11.8 mg (8.5 μmol, 33.6%) of **84** in a total yield of 8.5% (calculated in relation to initial resin capacity) as a white powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 9.13 (d, *J* = 6.4 Hz, 1H), 8.19 (s, 1H), 8.06 (d, *J* = 8.2 Hz, 1H), 7.88 (d, *J* = 8.6 Hz, 1H), 7.51 (d, *J* = 8.8 Hz, 1H), 7.49 (d, *J* = 7.4 Hz, 1H), 7.26 (d, *J* = 7.9 Hz, 1H), 7.21 (s, 1H), 7.00 (d, *J* = 13.1 Hz, 1H), 6.74 (s, 1H), 6.67 (d, *J* = 10.2 Hz, 1H), 5.60 (dd, *J* = 13.1, 10.2 Hz, 1H), 5.43 (d, *J* = 3.0 Hz, 1H), 4.67 (q, *J* = 7.0 Hz, 1H), 4.52-4.46 (m, 1H), 4.45 (s, 1H), 4.36-4.31 (m, 1H), 4.31-4.21 (m, 3H), 4.07-3.95 (m, 3H), 3.92 (t, *J* = 8.3 Hz, 1H), 3.79-3.73 (m, 1H), 3.72-3.64 (m, 2H), 3.63-3.51 (m, 5H), 3.50-3.44 (m, 1H), 3.27-3.18 (m, 1H), 2.85 (t, *J* = 2.6 Hz, 1H), 2.73-2.65 (m, 1H), 2.60-2.52 (m, 1H), 2.42-2.31 (m, 1H), 2.27-2.17 (m, 3H), 2.11-2.02 (m, 3H), 2.01-1.93 (m, 4H), 1.92-1.83 (m, 2H), 1.81-1.76 (m, 1H), 1.75-1.70 (m, 1H), 1.70-1.54 (m, 7H), 1.52-1.40 (m, 5H), 1.39-1.33 (m, 2H), 1.25-1.13 (m, 2H), 1.10-1.04 (m, 1H), 1.02 (s, 3H), 1.00-0.95 (m, 1H), 0.92 (d, *J* = 6.3 Hz, 3H), 0.90 (d, *J* = 6.3 Hz, 3H), 0.88 (d, *J* = 6.6 Hz, 3H), 0.86-0.79 (m, 21H), 0.79-0.77 (m, 1H), 0.76-0.70 (m, 6H); LC main peak retention time: 8.00 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1387.8460, found: 1387.8449 (–0.8 ppm); calculated for $[M+2H]^{2+}$: 694.4267, found: 694.4256 (–1.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 952.5615, found: 952.5581 (–3.6 ppm).

CalB_R5phLeu (85)

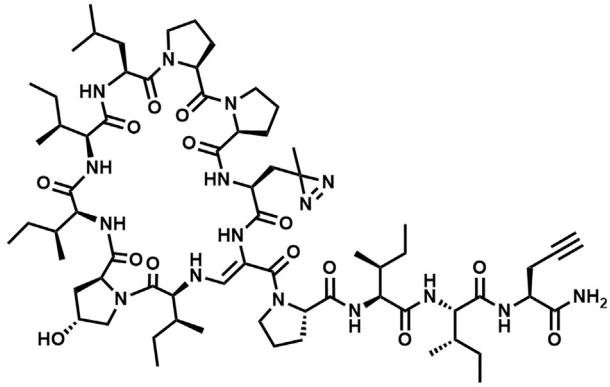


$C_{69}H_{110}N_{16}O_{14}$ M = 1387.74 g/mol

The linear precursor peptide of **85** was prepared via microwave-assisted SPPS using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 42 mg (30 μmol, 30%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 2 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 11.2 mg (8.1 μmol, 26.8%) of **85** in a total yield of 8.1% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.32 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1387.8460, found: 1387.8451 (–0.6 ppm); calculated for $[M+2H]^{2+}$: 694.4267, found: 694.4267 (–2.7 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 952.5615, found: 952.5582 (–3.5 ppm).

CalB_R8phLeu (86)

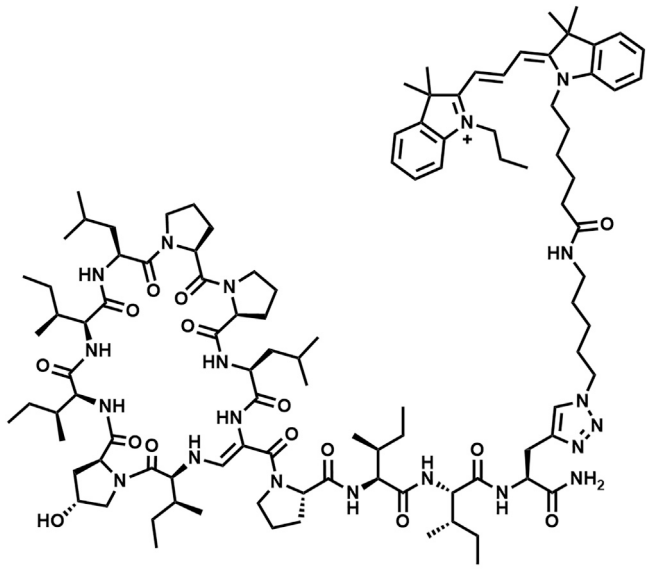


$C_{69}H_{110}N_{16}O_{14}$ M = 1387.74 g/mol

The linear precursor peptide of **86** was prepared via microwave-assisted SPPS up to the R2 position using Rink amide PS resin at a scale of 0.1 mmol initial resin capacity. The loading of the resin was quantified through a spectrometric Fmoc-Test and a manual coupling of photo-leucine was performed using 4 equivalents of amino acid combined with HBTU and HOBt (each 4 equivalents) as well as 5 equivalents of DIPEA in 10 mL DMF by shaking the suspension over night at room temperature. The resin was then thoroughly washed with DMF and a second round of microwave-assisted SPPS was performed for completion of the peptide sequence. After purification by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA), 19 mg (13 μmol, 13%) of the peptide precursor was obtained as a white powder. For the oxidation of the serine residue and concomitant cyclization, the peptide was dissolved in 5 mL of ACN and 4 equivalents of DMP were added. The resulting suspension was shaken for 6.5 hours at RT and immediately purified by HPLC (solvent A: H_2O; solvent B: ACN) to give 1.4 mg (1.0 μmol, 7.6%) of **86** in a total yield of 1.0% (calculated in relation to initial resin capacity) as a white powder.

LC main peak retention time: 8.13 min; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 1387.8460, found: 1387.8451 (–0.6 ppm); calculated for $[M+2H]^{2+}$: 694.4267, found: 694.4256 (–1.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}+H]^+$: 952.5615, found: 952.5597 (–1.9 ppm).

CalB_C4Pra(Cy3) (87)

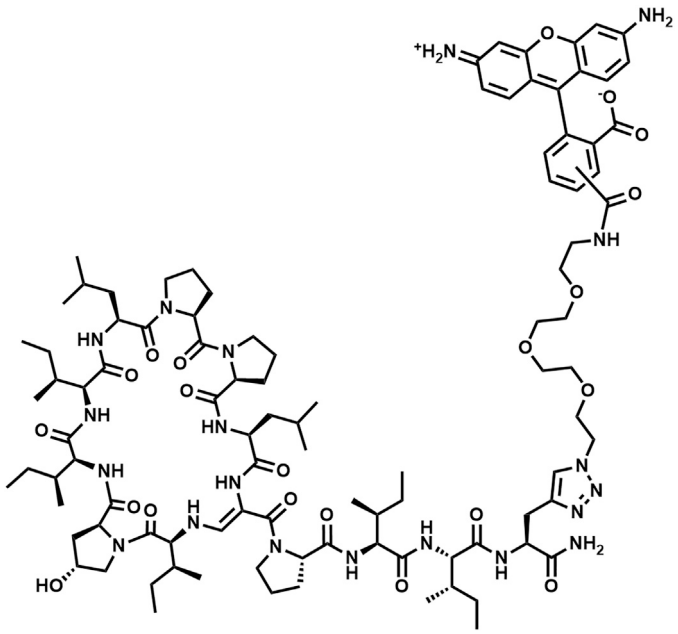


$C_{107}H_{165}N_{20}O_{15}^+$ M = 1971.62 g/mol

For the copper-catalyzed azide-alkyne cycloaddition (CuAAC), 12.2 mg of **82** were dissolved in 1 mL of ACN and 1.8 equivalents of Cy3-azide, 0.4 eq. Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amin) (TBTA) as well as 4 eq. of DIPEA were added. To start the reaction, 0.6 eq. of CuI were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 26 hours at room temperature. Another 0.1 equivalents of CuI were added and shaking was continued overnight. The crude product was purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 13.3 mg (6.4 μmol) of **87** in a total yield of 72% as a deep purple powder.

LC main peak retention time: 6.04 min; HRMS (ESI): *m/z* calculated for M^+: 1971.2792, found: 1971.2798 (0.3 ppm); calculated for $[M^+{+}H]^{2+}$: 986.1432, found: 986.1426 (–0.6 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M^+_{ring}{+}H]^+$: 940.5866, found: 940.5839 (–2.9 ppm).

CalB_C4Pra(Rhodamine) (88)

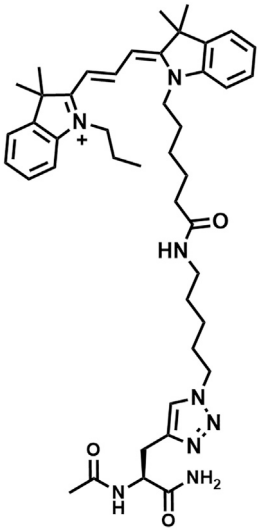


$C_{99}H_{144}N_{20}O_{21}$ M = 1950.36 g/mol

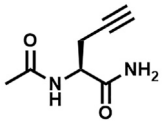
For CuAAC, 1.0 mg of 5/6-Carboxy-rhodamine-azide (CLK-AZ105, Jena Bioscience) was dissolved in 300 μL ACN and 1.6 eq. of **82**, 0.4 eq. TBTA as well as 4 eq. of DIPEA were added. To start the reaction, 0.6 eq. of CuI were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 24 hours at room temperature. The crude product was purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 1.4 mg (0.7 μmol) of **88** in a total yield of 25% as a brown powder.

LC main peak retention time: 4.64 min; HRMS (ESI): *m/z* calculated for M^+: 1951.0922, found: 1951.0919 (–0.2 ppm); calculated for $[M{+}H]^{2+}$: 976.0497, found: 976.0497 (–0.5 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M_{ring}{+}H]^+$: 940.5866, found: 940.5829 (–3.9 ppm).

Cy3-Control (89)



$C_{44}H_{61}N_8O_3^+$
M = 750.02 g/mol



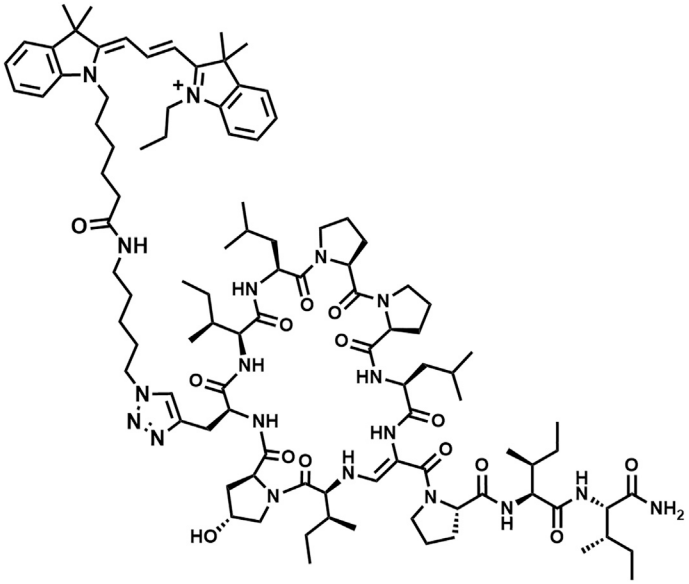
$C_7H_{10}N_2O_2$
M = 154.17 g/mol

N-Fmoc-L-propargylglycin (CC05047, Carbolution) was amidated and acetylated via an automated microwave-assisted protocol in a Liberty Blue peptide synthesizer (CEM) using Rink-Amide PS resin and the obtained amino acid building block **89a** was purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (d, *J* = 8.2 Hz, 1H), 7.42 (s, 1H), 7.12 (s, 1H), 4.32 (td, *J* = 8.0, 5.6 Hz, 1H), 2.82 (t, *J* = 2.7 Hz, 1H), 2.58-2.52 (m, 1H), 2.46-2.36 (m, 1H), 1.86 (s, 3H); ^{13}C NMR (101 MHz, DMSO) δ 171.82, 169.20, 80.88, 72.69, 51.25, 22.53, 21.76; HRMS (ESI): *m/z* calculated for $[M+H]^+$: 155.0815, found: 155.0813 (–1.3 ppm);

For CuAAC, 5.0 mg of Cy3-Azide (dissolved in DMSO) was mixed with 1.5 eq. of **89a** and 0.2 eq. TBTA as well as 4 eq. of DIPEA were added. To start the reaction, 0.6 eq. of CuI were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 24 hours at room temperature. The crude product was purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 6.1 mg (7.1 μmol) of **89** in a total yield of 84% as a deep purple powder.

^{1}H NMR (400 MHz, DMSO-d_6) δ 8.36 (t, *J* = 13.4 Hz, 1H), 8.01 (d, *J* = 8.4 Hz, 1H), 7.76-7.70 (m, 2H), 7.64 (dd, *J* = 7.5, 4.0 Hz, 2H), 7.52-7.40 (m, 4H), 7.38 (s, 1H), 7.33-7.26 (m, 2H), 7.04 (s, 1H), 6.52 (dd, *J* = 13.4, 8.7 Hz, 2H), 4.43 (td, *J* = 8.7, 5.1 Hz, 1H), 4.27 (t, *J* = 7.0 Hz, 2H), 4.11 (t, *J* = 7.3 Hz, 4H), 3.04 (dd, *J* = 14.7, 5.1 Hz, 1H), 3.00-2.92 (m, 2H), 2.82 (dd, *J* = 14.7, 9.0 Hz, 1H), 2.05 (t, *J* = 7.3 Hz, 2H), 1.81-1.79 (m, 3H), 1.79-1.71 (m, 6H), 1.70 (s, 6H), 1.69 (s, 6H), 1.55 (p, *J* = 7.4 Hz, 2H), 1.41-1.29 (m, 4H), 1.19-1.10 (m, 2H), 0.98 (t, *J* = 7.4 Hz, 3H); LC main peak retention time: 5.85 min; HRMS (ESI): *m/z* calculated for M^+: 749.4861, found: 749.4857 (–0.5 ppm); calculated for $[M+H]^{2+}$: 375.2467, found: 375.2463 (–1.1 ppm).

CalB_R3Pra(Cy3) (90)

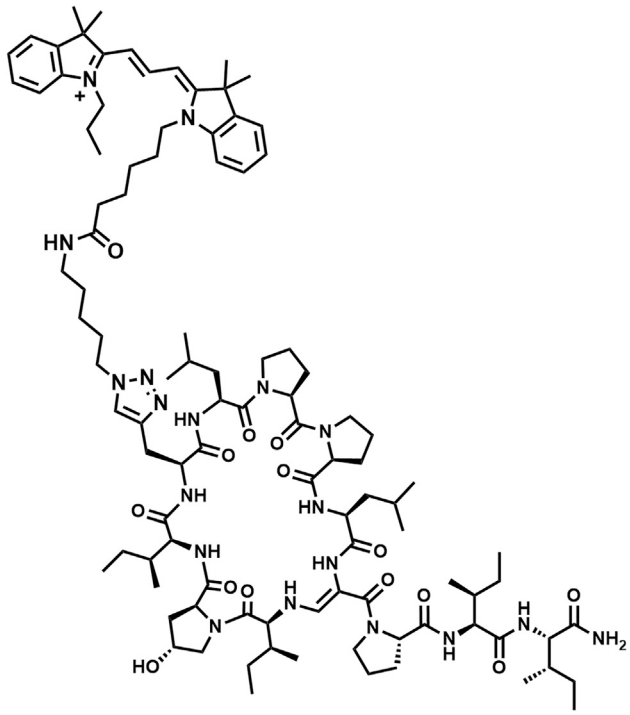


$C_{101}H_{154}N_{19}O_{14}^{+}$ **M = 1858.46 g/mol**

For CuAAC, 4.3 mg of **78** were dissolved in 100 μL of ACN and 1.2 equivalents of Cy3-azide (500 mM DMSO stock), 0.3 eq. TBTA (5 mM stock) as well as 4 eq. of DIPEA were added. To start the reaction, 0.5 eq. of CuI (10 mM ACN stock) were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 4 hours at room temperature. Another 0.1 eq. of CuI were added and shaking was continued overnight. The crude product was purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 0.6 mg (0.3 μmol) of **90** in a total yield of 9% as a deep purple powder.

LC main peak retention time: 5.36 min; HRMS (ESI): *m/z* calculated for M^+: 1858.1951, found: 1858.1942 (–0.5 ppm); calculated for $[M^+{+}H]^{2+}$: 929.6012, found: 929.6005 (–0.8 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M^+_{ring}{+}H]^{2+}$: 758.9758, found 758.9727 (–4.1 ppm).

CalB_R4Pra(Cy3) (91)



$C_{101}H_{154}N_{19}O_{14}^{+}$ M = 1858.46 g/mol

For CuAAC, 1.2 mg of **79** were dissolved in 100 μL of ACN and 1.3 equivalents of Cy3-azide (500 mM DMSO stock), 0.5 eq. TBTA (5 mM stock) as well as 4 eq. of DIPEA were added. To start the reaction, 0.5 eq. of CuI (10 mM ACN stock) were added. Immediately, the micro reaction vessel was flooded with argon and the mixture was shaken for 4 hours at room temperature. Another 0.25 eq. of Cy3-azide as well as 0.2 eq. of CuI were added and shaking was continued overnight. The crude product was purified by HPLC (solvent A: H_2O, acidified with 0.1% TFA; solvent B: ACN, acidified with 0.1% TFA) to give 0.9 mg (0.5 μmol) of **91** in a total yield of 49% as a deep purple powder.

LC main peak retention time: 4.98 min; HRMS (ESI): *m/z* calculated for M^+: 1858.1951, found: 1858.1937 (–0.8 ppm); calculated for $[M^+{+}H]^{2+}$: 929.6012, found: 929.5993 (–2.0 ppm); MS^2 HRMS (ESI, HCD): *m/z* calculated for ring fragment $[M^+_{ring}{+}H]^{2+}$: 758.9758, found 758.9746 (–1.6 ppm).

QUANTIFICATION AND STATISTICAL ANALYSIS

All figure legends provide details on the quantification type and statistical analysis, including the statistical tests used, the exact value of n or the definition of center and precision measures for each experiment.

For the quantification of proteomic data, the MaxLFQ[53] (whole proteome comparison) or Minora[54] (ABPP & A*f*BPP) label-free quantification algorithms were used. For this purpose, either the software MaxQuant 2.3.0.1. (whole proteome comparison) employing the Andromeda search engine[55] or Proteome Discoverer 2.5 (Thermo Fisher Scientific) employing the Sequest-HT algorithm[56] were used for protein search. Statistical analysis was performed using the software Perseus 2.0.7.0.[50] LFQ quantification data was transformed to a $\log_2$-scale and filtered for consistently identified proteins. Missing LFQ data points were replaced with values from the lower bound of the data's normal distribution. Student's T-test with permutation-based FDR was used to identify changes, and protein groups combining a $\log_2$ difference of $\geq$ 1 or $\leq$ -1 with a P value equal to or less than 0.05 were generally considered to be significantly changed. For the generation of a protein-protein interaction network, protein groups combining a $\log_2$ difference of $\geq$ 0.5 or $\leq$ -0.5 with a q value equal to or less than 0.05 were used as input for the STRING database (v.11.5) [41] and the resulting network was further processed using the Cytoscape 3.9.1 software.[57]