Biosynthesis and Mechanism of Action of the Cell Wall Targeting Antibiotic Hypeptin

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Abstract: Hypeptin is a cyclodepsipeptide antibiotic produced by Lysobacter sp. K5869, isolated from an environmental sample by the iChip technology, dedicated to the cultivation of previously uncultured microorganisms. Hypeptin shares structural features with teixobactin and exhibits potent activity against a broad spectrum of gram-positive pathogens. Using comprehensive in vivo and in vitro analyses, we show that hypeptin blocks bacterial cell wall biosynthesis by binding to multiple undecaprenyl pyrophosphate-containing biosynthesis intermediates, forming a stoichiometric 2:1 complex. Resistance to hypeptin did not readily develop in vitro. Analysis of the hypeptin biosynthetic gene cluster (BGC) supported a model for the synthesis of the octapeptide. Within the BGC, two hydroxylases were identified and characterized, responsible for the stereoselective \( \beta \)-hydroxylation of four building blocks when bound to peptidy1 carrier proteins. In vitro hydroxylation assays corroborate the biosynthetic hypothesis and lead to the proposal of a refined structure for hypeptin.

The rapid emergence and worldwide spread of infections caused by antibiotic resistant bacteria represents a serious health threat, while the identification and development of novel antibiotic classes is scarce. Particularly, the pressing need for resistance-breaking antibiotics reinforced the focus on natural products, though increasingly becoming harder to find. Overmining of this limited resource in the 1960s ended the golden era of antibiotic discovery and, despite intensive effort, synthetic approaches were unable to replace natural products.\(^1\)

To access a greater diversity of antibiotic producing microorganisms, novel cultivation methods have been developed. The iChip (isolation chip) technology was designed for high-throughput in situ cultivation of previously “uncultured” bacteria.\(^2,3\) The iChip device enables to simultaneously cultivate and isolate about 50% of soil bacteria, compared with only 1% of strains that grow under laboratory conditions. This method facilitated the discovery of teixobactin, representing an entirely novel antibiotic class, produced by the previously uncultured \( \beta \)-proteobacterium Eleftheria terrae.\(^4\) Another extract from the same screen that led to the discovery of teixobactin showed potent activity against Staphylococcus aureus. Bioassay-guided fractionation of culture extracts, followed by MALDI-TOF analysis, identified the bioactive compound, as a peak with a \([M+H]^+\) ion at \(m/z\) 1022.489 (Figure S1). Comparison with natural product databases pointed towards the known compound hypeptin (1, Figure 1), previously isolated from Pseudomonas sp. PB-6269 by Shionogi & Co, in 1989.\(^5\) The producing strain K5869, isolated by the iChip was then cultivated in larger scale and 1 was isolated as described in the Supporting Information (SI, page 3), yielding approximately 18 mg of 1 from a 3 L culture.

NMR and other spectroscopic analyses further confirmed the identity of 1 (Figures S2–S11, Table S2), being an octadepsipeptide with a four-residue macrocycle. A comparably high proportion of amino acids, comprising half of the...
peptide backbone, are β-hydroxylated, which gave the compound the name hypeptin. Determination of the absolute stereoconfiguration of 1 had revealed three amino acids to be d-configurated and three out of four amino acids to contain R-configuration at the β-carbon (2S,3R)-3-OH-Asn4 (Has), (2R,3R)-3-OH-Asn5, (2S,3S)-3-OH-Tyr6 (Hty), and (2S,3R)-3-OH-Leu7 (Hle). The configuration of Hty had not been experimentally determined due to degradation during hydrolysis, and NMR spectra were not provided in the original publication.

We next sequenced the genome of the newly isolated producing strain K5869. 16S rDNA analysis revealed the organism to belong to the genus Lysobacter, γ-proteobacteria known to produce a range of secondary metabolites including compounds with antibacterial and antifungal bioactivities. The overall structure of 1 suggested a nonribosomal origin. Nonribosomal peptide synthetases (NRPS) are multimodular megaenzymes that assemble peptides in a thiotemplated manner. A minimal NRPS elongation module, known to recruit specific amino acid building blocks and extend the growing peptide chain, consists of condensation (C), adenylation (A), and thiolation (T) domains, also named peptidyl carrier proteins. Some A domains are dependent on the interaction with a small MbtH-like protein (MLP) to maintain their correct conformation and/or catalytic activity. To analyze the biosynthesis of 1, the genome sequence was searched for candidate NRPS biosynthetic gene clusters (BGC). AntiSMASH analysis revealed a BGC with two NRPS genes (Table S3), that we termed hynA (19.4 kb) and hynB (7.3 kb), encoding six and two modules, respectively (Figure 1). The number of modules and predicted A domain specificities, as well as C domain functions, were consistent with the overall structure of 1. Module 5 harbors a scarce additional C domain, that clusters together with known Cβ epimerases in phylogenetic analysis (Figure S12, Table S4). Interestingly, the active site of the C domain contains a HRxxxDR sequence, which would render the domain inactive by the bulky side chains of the arginines. The identical Cβ configuration of Has and Hs6 in the final peptide strongly supports this theory. BLAST analysis of the genes hynAB revealed an identical BGC in the genome of Lysobacter psychrotolerans ZS60 (NZ_RBS01000005.1), which helped to manually determine the borders of the BGC. Despite extensive bioinformatic searches with BLAST and BigFAM, no further related BGCs were identified in the databases. In addition to the two NRPS genes hynAB, bioinformatic analysis revealed two putative hydroxylases to be encoded in the BGC: HynC is a non-heme diiron monooxygenase (NHDM), a barely studied enzyme family, so far only described in the biosyntheses of chloramphenicol, teicoplanin, and FR900359, while HynE is annotated as an α-ketoglutarate-dependent oxygenase (αKG). Additional genes (hynDFG) likely represent transporter-related genes. The hyn BGC, comprising a 35.6 kb region, is located

![Figure 1. Gene organization of the hyn BGC and biosynthetic pathway of hypeptin (1). The NRPS HynAB assemble a linear octapeptide which is finally released and cyclized by HynB. The tailoring hydroxylases HynC and HynE (green) modify the building blocks during assembly. Has: 3-Hydroxyasparagine Hty: 3-Hydroxytyrosine Hle: 3-Hydroxyleucine.](image-url)
in the 5'-end of a giant cluster with four other BGCs. The 3'-end of this region encodes a stand-alone MLP (hynMLP) that was assumed to be involved in the biosynthesis of Hty.

The different stereoconfiguration of the β-hydroxyl groups in Hty raised questions about the hydroxylation reactions in hyепtin biosynthesis. We thus focused on the characterization of the two different hydroxylases HynC and HynE, for which the nature of substrates is unclear. β-hydroxyl moieties in amino acids can be introduced by different mechanisms, either on the free amino acid or on the aminoacylated T domain during NRPS assembly. Comparable examples of NHDM and αKG were shown to hydroxylate their substrate amino acids when covalently bound to the cognate T domain.[14,15] Interestingly, all characterized NHDM are known to hydroxylate their substrate l-amino acids in synconfiguration[16-18] whereas for αKG, hydroxylation of products in both configurations is reported.[19] Therefore, we hypothesized that the αKG HynE might hydroxylate either free or T domain-bound Asn, Asp, and Tyr prior to further modification, while the NHDM HynC would act on Leucinyl-T. We aimed at analyzing β-hydroxylation reactions in vitro to determine the substrate specificity of HynC and HynE. To this end, modules 4, 5, 6, and 7 were cloned and expressed in E. coli for in vitro reconstitution. As NRPS multidomain proteins frequently show difficulties to express heterologously in a soluble and active form,[13] we cloned different constructs of each module: AT, CAT, ATC or CA-T, the latter two designed after the recently introduced XU- and XUC-exchange modules.[17] These were developed as exchange modules for NRPS engineering, but we also found them effective to define appropriate borders for successful in vitro reconstitution. Most of the attempted constructs yielded truncated or insoluble proteins, even when co-expressed with chaperones. The enzymatic activity of the A domains within the constructs was verified with the γ18O2-ATP exchange assay.[10] Finally, we obtained the soluble and functional enzymes HynA4CaccATCdon, HynA5CAT, HynA6CAT, and HynB7AT. HynA5CAT and HynA6CAT needed to be co-expressed with HynMLP, which is located 300,000 bp downstream of the hyn genes, to exhibit adenylating activity towards their preferred substrates l-Asn and l-Tyr. HynE was efficiently expressed in E. coli BL21(DE3), whereas HynC had to be co-expressed with each of the four different NRPS modules to be soluble and stable in vitro (Figure S13).

With all enzymes in hand, we performed hydroxylation assays for HynC and HynE, each with all four purified NRPS modules. For HynE, we could detect the mass of the hydroxylated amino acids in the assays with HynA4CaccATCdon and HynA5CAT after hydrolysis from the NRPS, but not with HynA6CAT and HynB7AT. On the other hand, the masses of Hty and Hle were detected in HynC assays with HynA4CAT and HynB7AT, respectively (Figure 2). We also detected Hle in the negative control of the assay with HynB7AT, which was probably caused by in vivo hydroxylation during co-expression. To circumvent this, we generated the inactive mutant HynC E375D. An analogous mutation in the active site of the prototype NHDM, CmlA, was reported to lack oxygen regulation and thereby impaired hydroxylation activity, without any structural changes.[10] Indeed, when using co-expressed HynC E375D as negative control in the hydroxylation assay, the signal of Hle diminished almost completely (Figure 2).

We speculated that the synthetase utilizes the two hydroxylases to obtain hydroxylated amino acids in different stereoconfiguration. Our in vitro hydroxylation assays unambiguously demonstrate, that HynE targets the T-domain bound l-Asn and l-Asp, whereas the NHDM HynC hydroxylates the T-domain bound l-Tyr and l-Leu. These results contradict the published configuration of Hty in I that was reported as (2S,3S) (anti), but, according to our in vitro data, should be (2S,3R) (syn). We were not able to verify the absolute configuration of Hty in the final peptide due to fast degradation of the amino acid during hydrolysis of I as observed previously.[19] Nevertheless, the observed high coupling constant between H3Hty and H2Hty of 7.2 Hz (Table S2) strongly indicates syn configuration in accordance with a published study on a Hty-containing peptide.[20] According to the results of our γ18O2-ATP exchange assay, the preferred substrate of the A domain is (2S)-Tyr and the module does not contain any epimerase domain. In the light of these data, we propose to reassign the absolute configuration of the Hty residue in I to (2S,3R)-OH-Tyr. Analysis of ROESY correlations supports this configuration (Figure S14).

I shows striking similarity with teixobactin, including a macrolactam ring of the same size, a comparable number of α- and l-amino acids, the presence of a guanidine amino acid, and β-hydroxy amino acids, suggesting a common mechanism of action for both compounds.[21] As observed for teixobactin, I exhibited potent antibacterial activity against gram-positive pathogens (Table 1), including drug-resistant staphylococci, such as methicillin-resistant, vancomycin intermediate-resistant and daptomycin-resistant Staphylococcus aureus (MRSA, VISA and DAPR), with minimal inhibitory concentrations (MIC) in the nmL−1 range. I further showed very good activity against mycobacteria and vancomycin-resistant enterococci (VRE) (Table 1), but was lacking activity against gram-negative species, most likely owing to the outer membrane permeability barrier, preventing penetration of the rather large compound. This is supported by the decreased MIC of E. coli strain MB5746 with a defective outer membrane.[22]

Killing kinetics of S. aureus exposed to I showed excellent bactericidal activity, even superior to vancomycin and teixobactin in killing late exponential phase cultures at 5-fold lower compound concentration (Figure 3 A, B). I had a strong lytic effect even at a concentration corresponding to 2 × MIC and showed enhanced lysis compared to vancomycin (Figure 3 C). Despite the pronounced lytic activity, I exhibits specificity for bacterial cells, indicating a favorable therapeutically window, as only moderate cytotoxic effects towards HEp-2 cells and low hemolytic activity toward red blood cells (RBCs) were observed at the highest concentration tested (128 μg mL−1) (Figure S15).

To identify the antibiotic target pathway of I, we employed pathway-selective gram-positive bioreporter strains. I specifically induced the B. subtilis Pnp-lacZ reporter strain indicative for interference with cell wall biosynthesis, while all other major biosyntheses (DNA,
RNA, protein) remained unaffected (Figure 4A). Substantiating inhibition of cell wall biosynthesis, treatment with 1 strongly inhibited incorporation of radiolabeled glucosamine, an essential precursor of cell wall biosynthetic reactions (Figure S16). Furthermore, treatment of *B. subtilis* with 1 induced severe cell-shape deformations as visualized by phase-contrast microscopy (Figure 4C). The formation of membrane bulges and blebs is characteristically induced by

**Table 1:** Minimum inhibitory concentrations (MIC) of 1 against selected strains and pathogenic bacteria.

| Organism                | MIC [μg mL⁻¹] |
|-------------------------|---------------|
| *Bacillus subtilis* 168 | 0.0625        |
| *S. simulans* 22        | 0.125         |
| *S. aureus* SG 511      | 0.0625        |
| *S. aureus* SG 511 (DAP³) | 0.0625        |
| *S. aureus* LT-1334 (MRSA) | 0.25         |
| *S. aureus* 137/93C (VISA) | 0.5          |
| *Enterococcus faecium* I-11054 (VRE) | 2            |
| *Mycobacterium bovis* BGG | 0.25         |
| *E. coli* MB5746        | 4             |
| *E. coli* O-19959       | 16            |
| *Pseudomonas aeruginosa* PAO1 | >64        |

**Figure 2.** Results of in vitro assays to test NRPS and hydroxylase activities. On the left, the A domain specificity towards the substrate amino acid and the dependency of the MbtH-like protein (MLP) HynMLP was examined for each module via $^{18}$O4-ATP exchange assay. On the right, extracted LC-MS traces of the hydroxylation assays of the module construct with HynC and HynE show formation of hydroxylated amino acids in comparison with the respective negative controls. At the bottom, the formation of the hydroxylated amino acid is summarized. a) The A domain of module 4 activates l-Asn and is independent of HynMLP. HynE then hydroxylates the bound amino acid, leading to the formation of 3-hydroxyasparagine (Has) ($m/z = 147.0$). b) The A domain in module 5 activates l-Asn only in presence of HynMLP. HynE then hydroxylates the bound amino acid, leading to the formation of 3-hydroxyasparagine (Has) ($m/z = 147.0$). c) The A domain of module 6 activates l-Tyr in the presence of HynMLP. Subsequently, HynC hydroxylates the amino acid ($m/z = 196.1$). d) The A domain of module 7 activates l-Leu independently of HynMLP. HynC then hydroxylates the amino acid ($m/z = 146.1$).

**Figure 3.** 1 shows excellent bactericidal activity against *S. aureus*. Time-dependent killing of early-exponential (a) and late-exponential phase-grown (b) cells treated with 1 at 1 × MIC (open circles) and 2 × MIC (circles), with teixobactin (diamonds) and vancomycin (triangles) both at 10 × MIC. Cells left untreated are shown with squares. Data are representative of three independent experiments. c) 1-induced lysis is mediated by the major autolysin AtlA in *S. aureus*. Deletion of atlA results in markedly reduced autolysis after treatment with 1 and TEIX.
VAN-treated (1/C148 MIC) cells were used as controls. Experiments are
Photorhabdus luminescens
Angew. Chem.
many cell wall-acting antibiotics and was similarly observed
1
Figure 4. 1 targets bacterial cell wall biosynthesis. a) B. subtilis bio-
reporter strains with selected promotor-lacZ gene fusions were used to
identify interference with major biosynthesis pathways including cell wall (Pew), DNA (Pew), RNA (Pew), and protein (Pew). A blue halo at
the edge of the inhibition zone demonstrates induction of a specific
stress response by β-galactosidase expression. Antibiotics vancomycin
(VAN), ciprofloxacin, rifampicin, and clindamycin were used as pos-
itive controls. b) Treatment with 1 (1 x MIC, open circles) strongly
induced Pew as observed by expression of the lux operon from
Photobahdus luminescens in B. subtilis Pwux. VAN (triangles) and
clindamycin (CL, squares) were used as control antibiotics. c) Phase-
contrast microscopy of B. subtilis confirmed impairment of cell wall
integrity as severe cell-shape deformations and characteristic blebbing
were observed following 1 treatment. Cell wall active antibiotics
teixobactin (TEIX), VAN, plectasin (PLEC), ampicillin (AMP), and
lysozyme (LYS) were used as controls. Scale bar =2 μm. d) Intra-
cellular accumulation of the cell wall precursor UDP-MurNAc-penta-
peptide after treatment of S. aureus with 1 (5 x MIC). Untreated and
VAN-treated (5 x MIC) cells were used as controls. Experiments are
representative of 3 independent experiments each.

Despite the membrane alterations observed, 1 did not
trigger pore formation or membrane disintegration. In con-
trast to the lantibiotic nisin, no rapid pore formation was
observed (Figure S17 A). Furthermore, the membrane potential
of I-treated S. simulans 22 cells remained unaffected even
at higher concentrations (5 x MIC) (Figure S17 B), as
quantified by intra- and extracellular concentrations of
the tritium-labeled lipophilic cation TPP+. In line with these
observations, I did not impact the cellular localization of
the cell division inhibitor MinD of B. subtilis. MinD is bound to
the membrane via a C-terminal amphipathic helix and
requires the presence of the membrane potential for its
specific cellular localization pattern. In growing cells, MinD
accumulates at the newly formed cell poles to direct FtsZ to
mid-cell division site and specific FtsZ positioning to guide
division septum placement.17,21 While treatment with CCCP
resulted in a rapid delocalization and irregular dispersion of
GFP-MinD within 2 min, localization of the fusion protein
was unchanged in I-treated cells and only slightly affected
with prolonged incubation time (30 min) (Figure S17 C).

In search of the molecular target within the peptidoglycan
(PGN) biosynthesis pathway, we investigated the effect of
I on the LiaRS stress response in B. subtilis. LiaRS is a two-
component system (TCS), which is known to respond to
antibiotics that interfere with the lipid II biosynthesis cycle.25
Monitoring bioluminescence of reporter cells treated with
I over time revealed a strong induction of P_wur-lux, even
exceeding induction levels observed with the lipid II-binding
antibiotic vancomycin, indicating interference of I with
the lipid II biosynthesis cycle (Figure 4B). Mechanism of action
studies revealed that the structurally-related teixobactin
impairs cell wall biosynthesis by blocking several cell
envelope precursors containing an undecaprenyl-pyrophos-
phate linkage unit including the ultimate PGN building block
lipid II.26

PGN biosynthesis takes place in three different cellular
compartments of a bacterial cell. Synthesis starts in the
cytoplasm with the formation of the ultimate soluble pre-
cursor uridine diphosphate-N-acetylmuramic acid-penta-
tetrapeptide (UDP-MurNAc-pentapeptide), which is then transferred
to the membrane-anchor undecaprenyl phosphate (C55P) to
yield lipid I (undecaprenyl-pyrophosphoryl-MurNAc-penta-
peptide). Subsequently, the addition of N-acetylglucoasamine
(UDP-GlcNAc) yields lipid II (undecaprenyl-pyrophos-
phoryl-MurNAc-pentapeptide-GlcNAc), which can further
be species-specifically modified. Modified lipid II is trans-
located to the outer surface of the membrane and incorpo-
rated into the PGN polymer (Figure S18).

Antibiotics that interfere with late stages of PGN syn-
thesis, such as vancomycin, trigger the accumulation of UDP-
MurNAc-pentapeptide in the cytoplasm. To distinguish
whether I interferes with the early cytoplasmic or the late
membrane-associated steps of PGN synthesis, we determined
the cytoplasmic levels of UDP-MurNAc-pentapeptide of
S. aureus cells treated with I. Treatment with I led to the
intracellular accumulation of UDP-MurNAc-pentapeptide
similar to the vancomycin control (Figure 4D), suggesting
that one of the later membrane-associated or extracellular
biosynthesis steps is targeted. Taken together, results from
whole cell experiments strongly supported the hypothesis that
I and teixobactin, in accordance with their structural resem-
bance, share the same mechanism of action.

Based on this, we analyzed the impact of I on the
membrane-associated steps of PGN biosynthesis in vitro to
identify the molecular target of I. The first membrane-linked
step of PGN synthesis is catalyzed by MraY, which transfers
UDP-MurNAc-pentapeptide to the lipid carrier C55P yielding
lipid I.27 Subsequently, the glycosyltransferase MurG adds
UDP-activated GlcNAc to the muramyl moiety of lipid I,
yielding lipid II.28 Membrane preparations of M. luteus
possess the enzymatic activity of MraY and MurG to
synthesize lipid II from the substrates UDP-MurNAc-penta-
tetrapeptide, [14C]-UDP-GlcNAc and C55P.29 Testing the reactions
in the presence of increasing concentrations of I resulted in
a dose-dependent inhibition of overall lipid II synthesis. Full
inhibition was observed at a twofold molar excess of I with
regard to the substrate C_{55}P (Figure 5A). In staphylococci, lipid II is modified by the addition of five glycine residues, catalyzed by FemXAB peptidyltransferases.\(^{[30]}\) Testing the impact of 1 on the FemX-catalyzed addition of a glycine residue to lipid II revealed, that the reaction was fully blocked at a 2:1 stoichiometry (antibiotic:lipid II), indicating the formation of a complex with the substrate rather than inhibition of the enzyme (Figure 5A), as observed for teixobactin.\(^{[15]}\) In addition, 1 inhibited the YbG-catalyzed dephosphorylation of C_{55}PP to C_{55}P, although higher concentrations (10:1) were required for complete inhibition. This indicates that the pyrophosphate moiety is crucial for antibiotic interaction, but the first sugar attached to the lipid carrier contributes to high binding affinity. However, the nature of the sugar appears less important, as 1 further efficiently inhibited the synthesis of the wall teichoic acid (WTA) precursor lipid IV_{WTA} (undecaprenyl-pyrophosphoryl-GlcNAc-ManNAc) (Figure 5A, Figure S18).

Consistently, 1 efficiently trapped lipid intermediates containing a C_{55}PP moiety in a stable complex that prevented extraction of the lipid intermediate from the reaction mixture when added in a twofold molar excess, indicating to the formation of a 2:1 stoichiometric complex. Complex formation was not observed with C_{55}P, confirming the lipid pyrophosphate moiety to be the minimal binding motif (Figure 5B). The inability of 1 to bind to C_{55}P further shows that inhibition of the in vitro lipid II synthesis using membrane preparations (Figure 5A), relies on binding to the reaction products, lipid I and lipid II, rather than the C_{55}P substrate.

To validate that the antimicrobial activity of 1 relies on complex formation with cell wall lipid intermediates, antagonism assays with selected purified precursors were performed. In line with the in vitro analyses, the addition of C_{55}PP-containing lipid intermediates counteracted 1 from inhibiting growth of S. aureus similar to teixobactin (Table S5). However, compared to lipid I and lipid II, the addition of lipid III_{WTA} or C_{55}PP was less effective, since 4-fold higher concentrations were required to fully antagonize the antimicrobial activity of 1, pointing to differences in the binding modes, that may involve interactions with the first sugar in lipid II. As expected, C_{55}P and the anionic phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) had no antagonistic effect.

Binding of 1 to lipid II blocks PGN biosynthesis resulting in a defective cell wall ultimately leading to cell death. In contrast to PGN, WTAs are not essential per se, but blocking WTA biosynthesis can result in the lethal accumulation of toxic intermediates and indirectly effect PGN biosynthesis, as the molecular machineries of both pathways are tightly interlinked.\(^{[31]}\) In addition, WTAs anchor autolysins and thereby prevent uncontrolled hydrolysis of PGN,\(^{[32]}\) suggesting that inhibition of WTA biosynthesis by binding WTA lipid intermediates helps to liberate autolysins. In agreement, 1-induced lysis was markedly reduced in a ΔatlA mutant, compared to wildtype S. aureus cells (Figure 3C), confirming that lysis induced by 1 is dependent on the major autolysin AtlA in S. aureus. Our results further show that co-targeting of lipid II and WTA lipid intermediates by 1 cause synergistic effects by weakening the PGN structure and liberation of autolysins, which synergistically lead to cell lysis and death. In addition, multiple-targeting strongly reduced the propensity to develop resistance, as we could not generate resistant
mutations of *S. aureus* by serial passaging on incrementing concentrations of 1.

Novel antibiotics with resistance breaking mechanisms of action are urgently needed to counteract the continuing spread of drug resistant pathogens. Hypeptin (1) is a cyclodepsipeptide that shares structural similarity with teixobactin (Figure S19).

1 contains four β-hydroxylated amino acids with different stereoconfiguration. We investigated the substrate specificity and stereoselectivity of the two tailoring hydroxylases HynC and HynE in vitro, which revealed specific interactions of both hydroxylases with their cognate domains. A transient hydrophobic interaction with a cognate T domain was characterized for the skylamycin CYP450 β-hydroxylase, but the reason for specific recognition could not be determined.[33] Understanding and predicting domain interaction specificity of NRPS tailoring enzymes is a hallmark for future engineering attempts, a feature we are currently investigating. The structure revision of 1 based on bioinformatica, biochemical assays and extensive NMR analyses highlights the value of integrating these approaches for complex natural product structure elucidation.

Elucidation of the mechanism of action revealed, that 1 inhibits cell wall biosynthesis by binding to C55PP-containing lipid intermediates within PGN, WTA, and capsule biosynthesis. Binding to multiple of these non-protein target structures within different biosynthesis pathways explains the potent activity towards a broad range of gram-positive pathogens, including drug resistant and difficult to treat strains, suggesting that the concomitant targeting of these precursors confers “intrinsic synergy”. Besides the mere blocking of cell wall biosynthesis, binding to WTA precursors further triggered deregulation of autolysis resulting in rapid and uncontrolled lysis and impressive bactericidal activity.

The exact knowledge of the mode of action and molecular target, together with a deeper understanding of the structure–activity relationships (SAR) will support rational design of synthetic analogs of 1. Synthetically generated teixobactin variants with modified N-terminus, by either replacing the linear chain by a lipophilic moiety[34] or the attachment of hydrophobic residues to N-Phe1,[35] have been reported to exhibit potent anti-microbial activity. Likewise, the semisynthetic attachment of hydrophobic moieties to the N-terminal d-Ala3 may increase membrane interaction and target binding of 1. Notably, 1 was most refractory to resistance development in vitro, suggesting that the combined cellular activities, triggered by targeting different cell wall precursors, account for the reduced propensity to develop resistance, making this antibiotic class a favorable scaffold for development.

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**Conflict of interest**

The authors declare no conflict of interest.

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[1] K. Lewis, *Nature* 2012, 485, 439–440.
[2] D. Nichols, N. Cahoon, E. M. Trakhtenberg, L. Pham, A. Mehta, A. Belanger, T. Kanigan, K. Lewis, S. S. Epstein, *Appl. Environ. Microbiol.* 2010, 76, 2445 – 2450.
[3] L. L. Ling, T. Schneider, A. J. Peoples, A. L. Spoering, I. Engels, B. P. Conlon, A. Mueller, T. F. Schäbler, D. E. Hughes, S. Epstein, et al., *Nature* 2015, 517, 455–459.
[4] J. Shoji, H. Hinoo, T. Hattori, K. Hirooka, Y. Kimura, T. Yoshida, J. *Antibiot.* 1989, 42, 1460–1464.
[5] Y. Xie, S. Wright, Y. Shen, L. Du, *Nat. Prod. Rep.* 2012, 29, 1277–1284.
[6] R. D. Süssmuth, A. Mainz, *Angew. Chem. Int. Ed.* 2017, 56, 3770 – 3821; *Angew. Chem. 2017*, 129, 3824–3878.
[7] B. R. Miller, E. J. Drake, C. Shi, C. C. Aldrich, A. M. Gulick, J. *Biol. Chem.* 2016, 291, 22559–22571.
[8] K. Blin, S. Shaw, K. Steinke, R. Villebro, N. Ziemert, S. Y. Lee, M. H. Medema, T. Weber, *Nucleic Acids Res.* 2019, 47, W81–W87.
[9] K. Graupner, K. Scherlach, T. Bretschneider, G. Lackner, M. Roth, H. Gross, C. Hertweck, *Angew. Chem. Int. Ed.* 2012, 51, 13173 – 13177; *Angew. Chem. 2012*, 124, 13350–13354.
[10] J. Houl, L. Robbel, M. A. Marahiel, *Chem. Biol.* 2011, 18, 655–664.
[11] S. A. Kautsar, K. Blin, S. Shaw, T. Weber, M. H. Medema, *Nucleic Acids Res.* 2021, 49, D490–D497.
[12] C. Herms, R. Richarz, D. A. Wirtz, J. Patt, W. Hanke, S. Kehraus, J. H. Voß, J. Küppers, T. Ohiyayashi, V. Namastivayam, et al., *Nat. Commun.* 2021, 12, 144.
[13] M. Kaniusaitė, R. J. A. Goode, R. B. Schittenhelm, T. M. Makris, M. J. Czylce, *ACS Chem. Biol.* 2019, 14, 2932–2941.
[14] T. M. Makris, M. Chakrabarti, E. M. Nolan, C. T. Walsh, M. A. Marahiel, *J. Biol. Chem.* 2010, 107, 15391–15396.
[15] G. M. Singh, P. D. Fortin, A. Koglin, C. T. Walsh, *Biochemistry 2008*, 47, 11310–11320.
[16] a) Z. L. Reitz, C. D. Hardy, J. Suk, J. Bouvet, A. Butler, *Proc. Natl. Acad. Sci. USA* 2019, 116, 19805–19814; b) M. Stricker, E. M. Nolan, C. T. Walsh, M. A. Marahiel, *J. Am. Chem. Soc.* 2009, 131, 15233–15330.
[17] K. A. J. Bożyniuk, F. Fleischhacker, A. Linck, F. Wesc, A. Tietze, C.-P. Niesert, H. B. Bode, *Nat. Chem.* 2018, 10, 275 – 281.
[18] V. V. Phelan, Y. Du, J. A. McLean, B. O. Bachmann, *Chem. Biol.* 2009, 16, 473–478.
[19] A. J. Jasniewski, C. J. Knoot, J. D. Lipscomb, L. Que, *Biochemistry 2016*, 55, 5818–5831.
[20] Z. Lin, J. O. Falkingham, K. A. Tawfik, P. Jeffs, B. Bray, G. Dubay, J. E. Cox, E. W. Schmidt, *J. Nat. Prod.* 2012, 75, 1518–1523.
[21] F. von Nussbaum, R. D. Süssmuth, *Angew. Chem. Int. Ed.* 2015, 54, 6684–6686; *Angew. Chem. 2015*, 127, 6784–6786.
[22] S. Kodali, A. Galgoci, K. Young, R. Painter, L. L. Silver, K. B. Herath, S. B. Singh, D. Cully, J. F. Barrett, D. Schmatz, et al., *J. Biol. Chem.* 2008, 283, 1669–1677.
[23] T. Schneider, T. Kruse, R. Wimmer, I. Wiedemann, V. Sass, U. Pag, A. Jansen, A. K. Nielsen, P. H. Mygind, D. S. Raventós, et al., *Science* **2010**, *328*, 1168–1172.

[24] H. Strahl, L. W. Hamoen, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 12281–12286.

[25] a) J. Radeck, S. Gebhard, P. S. Orchard, M. Kirchner, S. Bauer, T. Mascher, G. Fritz, *Mol. Microbiol.* **2016**, *100*, 607–620; b) T. Mascher, S. L. Zimmer, T.-A. Smith, J. D. Helmann, *Antimicrob. Agents Chemother.* **2004**, *48*, 2888–2896.

[26] A. Müller, A. Klöckner, T. Schneider, *Nat. Prod. Rep.* **2017**, *34*, 909–932.

[27] M. Ikeda, M. Wachi, H. K. Jung, F. Ishino, M. Matsuhashi, *J. Bacteriol.* **1991**, *173*, 1021–1026.

[28] S. Ha, B. Gross, S. Walker, *Curr. Drug Targets Infect. Disord.* **2001**, *1*, 201–213.

[29] Y. van Heijenoort, M. Derrien, J. van Heijenoort, *FEBS Lett.* **1978**, *89*, 141–144.

[30] a) T. Schneider, M. M. Senn, B. Berger-Büchi, A. Tossi, H.-G. Sahl, I. Wiedemann, *Mol. Microbiol.* **2004**, *53*, 675–685; b) J. M. Monteiro, G. Covas, D. Rausch, S. R. Filipe, T. Schneider, H.-G. Sahl, M. G. Pinho, *Sci. Rep.* **2019**, *9*, 5010.

[31] T. Roemer, T. Schneider, M. G. Pinho, *Curr. Opin. Microbiol.* **2013**, *16*, 538–548.

[32] R. Biswas, R. E. Martinez, N. Göhring, M. Schlag, M. Josten, G. Xia, F. Hegler, C. Gekeler, A.-K. Gleske, F. Götz, et al., *PLoS One* **2012**, *7*, e41415.

[33] K. Haslinger, C. Bricke, S. Uhmann, L. Sieverling, R. D. Süssmuth, M. J. Cryle, *Angew. Chem. Int. Ed.* **2014**, *53*, 8518–8522; *Angew. Chem.* **2014**, *126*, 8658–8662.

[34] H. Yang, K. H. Chen, J. S. Nowick, *ACS Chem. Biol.* **2016**, *11*, 1823–1826.

[35] Y. Zong, F. Fang, K. J. Meyer, L. Wang, Z. Ni, H. Gao, K. Lewis, J. Zhang, Y. Rao, *Nat. Commun.* **2019**, *10*, 3268.

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