Biosynthetic Studies of Telomycin Reveal New Lipopeptides with Enhanced Activity

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S *Supporting Information*
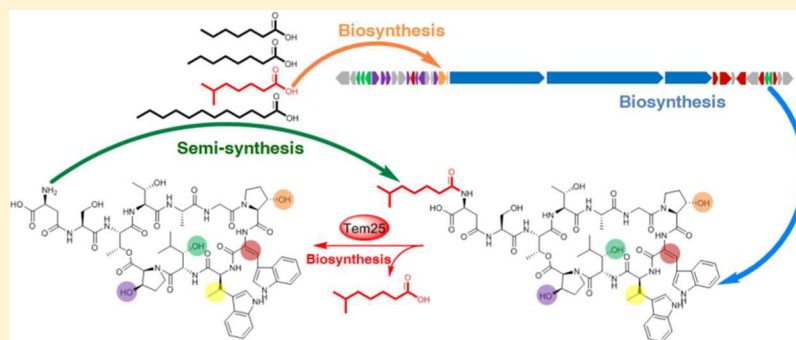
ABSTRACT: Telomycin (TEM) is a cyclic depsipeptide antibiotic active against Gram-positive bacteria. In this study, five new natural telomycin analogues produced by *Streptomyces canus* ATCC 12646 were identified. To understand the biosynthetic machinery of telomycin and to generate more analogues by pathway engineering, the TEM biosynthesis gene cluster has been characterized from *S. canus* ATCC 12646: it spans approximately 80.5 kb and consists of 34 genes encoding fatty acid ligase, nonribosomal peptide synthetases (NRPSs), regulators, transporters, and tailoring enzymes. The gene cluster was heterologously expressed in *Streptomyces albus* J1074 setting the stage for convenient biosynthetic engineering, mutasynthesis, and production optimization. Moreover, in-frame deletions of one hydroxylase and two P450 monooxygenase genes resulted in the production of novel telomycin derivatives, revealing these genes to be responsible for the specific modification by hydroxylation of three amino acids found in the TEM backbone. Surprisingly, natural lipopeptide telomycin precursors were identified when characterizing an unusual precursor deacylation mechanism during telomycin maturation. By *in vivo* gene inactivation and *in vitro* biochemical characterization of the recombinant enzyme Tem25, the maturation process was shown to involve the cleavage of previously unknown telomycin precursor-lipopeptides, to yield 6-methylheptanoic acid and telomycins. These lipopeptides were isolated from an inactivation mutant of *tem25* encoding a (de)acylase, structurally elucidated, and then shown to be deacylated by recombinant Tem25. The TEM precursor and several semisynthetic lipopeptide TEM derivatives showed rapid bactericidal killing and were active against several multidrug-resistant (MDR) Gram-positive pathogens, opening the path to future chemical optimization of telomycin for pharmaceutical application.
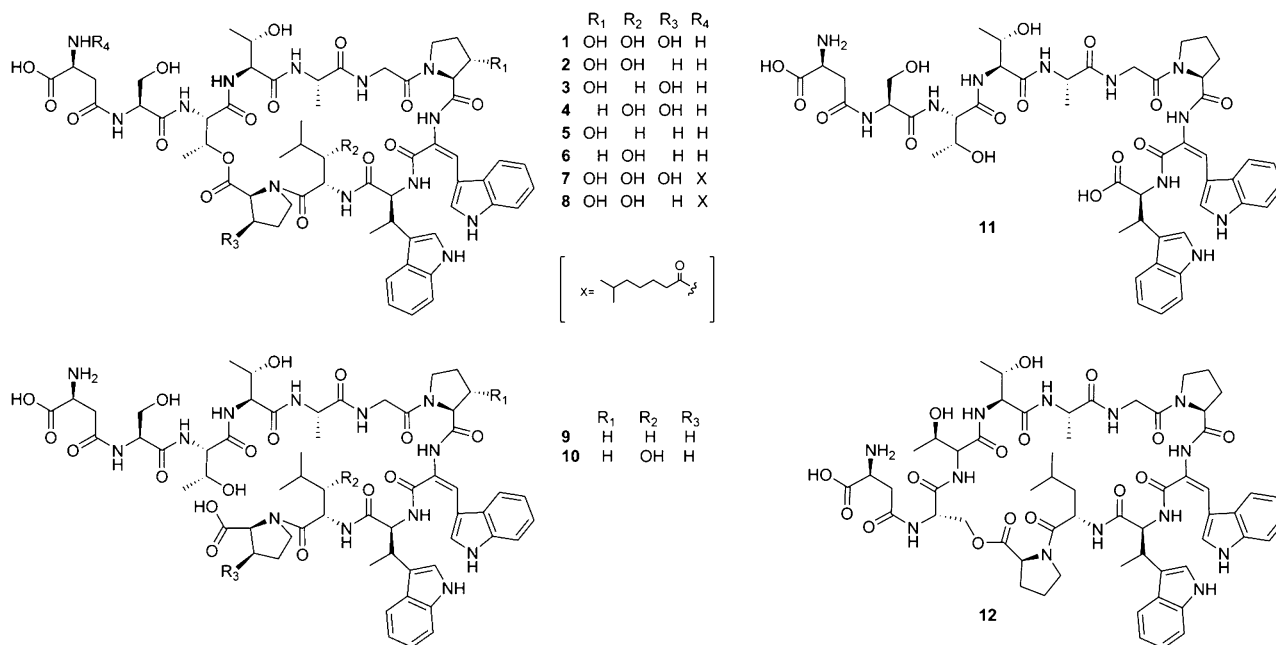
INTRODUCTION

Telomycin (**1**) is a peptide antibiotic produced by *Streptomyces canus* C159, which was described as an effective antibiotic against Gram-positive pathogens when initially isolated at the Bristol-Myers Company (New York, USA) in the 1950s.[1−3] It was demonstrated to inhibit *Staphylococcus aureus* (SA) with a minimal inhibitory concentration (MIC) of 8 μg/mL and even penicillin-resistant SA with a slightly higher MIC.[1] In the 1960s, a first partial structure of telomycin containing a permutation of

three amino acids was published.[4,5] Two nuclear magnetic resonance (NMR) based studies on telomycin in 1973 provided the correct structure of the complex molecule.[6,7] Telomycin (**1**, Chart 1) is a cyclic depsipeptide which is composed of 11 amino acids including five non-proteinogenic ones. It is composed of a nonapeptide lactone ring formed

Chart 1. Structures of Telomycins

	R$_1$	R$_2$	R$_3$	R$_4$
1	OH	OH	OH	H
2	OH	OH	H	H
3	OH	H	OH	H
4	H	OH	OH	H
5	OH	H	H	H
6	H	OH	H	H
7	OH	OH	OH	X
8	OH	OH	H	X

	R$_1$	R$_2$	R$_3$
9	H	H	H
10	H	OH	H

between the ^{4}Thr hydroxyl group and the C-terminal carboxyl group.[4−7] Only a few reports on this compound were published after the structure of telomycin was elucidated,[8−10] and to date, the mode of action (MoA) is still unclear. However, studies on a truncated analogue of telomycin lacking an aspartic acid and hydroxylation of *cis*-3-hydroxyl-proline called LL-AO341 β 1 revealed that specific inhibition of DNA, RNA, protein, lipid, or peptidoglycan synthesis by LL-AO341 β 1 could not be detected. It was postulated that LL-AO341 β 1 could cause cell lysis by membrane de-energization.[10]

The emergence of multidrug resistance (MDR) among many clinically relevant pathogens is an area of unmet medical need. Natural products play a pivotal role in antibiotic drug discovery, and scaffolds with novel MoA are urgently needed.[11,12] For several antibiotic classes, structural modifications of the natural parent molecule have been required to sufficiently optimize key parameters such as potency, efficacy, pharmacokinetics, and safety so as to identify viable development candidates, which were then successfully forwarded into clinical development.[13,14] In this study, telomycin was found to inhibit the growth of resistant pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-intermediate SA (VISA). The latter phenotype is seen with many hard-to-treat nosocomial infections. Since vancomycin is a first-line antibiotic to treat infections caused by MRSA, new antibiotics with novel MoA are needed that overcome reduced vancomycin susceptibility among clinical isolates.[14] Currently, only very limited alternatives are available including the use of naturally occurring highly potent lipodepsipeptides such as daptomycin, which is already approved for the treatment of complicated infections caused by MDR pathogens.[15]

In order to rationally optimize the fermentation yields of telomycin as prerequisite for embarking on a program of structural modifications, we investigated its biosynthesis, which, to the best of our knowledge, has not been elucidated. The depsipeptide structure of telomycin suggests that it is probably synthesized by a nonribosomal peptide synthetase (NRPS) pathway. NRPSs are multimodular enzymes in which each

module is responsible for the incorporation of a specific amino acid residue of the elongated peptide. These modules contain different catalytic domains and act in a successive way to elongate the peptide chain. A typical NRPS elongation module is composed of a condensation (C) domain, an adenylation (A) domain, and a thiolation (T) domain. The A domain selects the amino acid and activates it as aminoacyl-AMP, which is then attached to the adjacent T domain. The C domain performs the condensation step that connects the upstream T-bound peptidyl chain with the downstream T-bound aminoacyl residue through peptide bond formation.[16]

Recently, several NRPS and/or polyketide synthase (PKS) derived compounds have been shown to be processed by prodrug or precursor activation mechanisms to become the mature compound. For example, in the biosynthesis of xenocoumacin, a peptidase was found to hydrolyze the acylated D-asparagine residue from the precursor called prexenocoumacin leading to the bioactive xenocoumacin.[17] Similar mechanisms also could be found in the biosynthesis of other compounds such as zwittermicin and colibactin.[17−20] Pyoverdine is a fluorescent siderophore produced by a NRPS pathway in fluorescent pseudomonads, supposedly involved in infection in various disease models of the opportunistic pathogen *Pseudomonas aeruginosa*.[21] During pyoverdine biosynthesis, a periplasmic NTN hydrolase (PvdQ) was discovered that is responsible for the cleavage of the myristate or myristoleate moiety from the N-terminal amino acid. After knockout of *pvdQ*, larger pyoverdine derivatives harboring an ester moiety are produced.[22,23] The structure of PvdQ was characterized and the *in vitro* cleavage of a fatty acid moiety from pyoverdine precursors were shown.[24] Didemnin, a complex cyclic depsipeptide, is also thought to be synthesized by a precursor maturation mechanism. The pentaamino-alcohol zeamine I, a broad spectrum antibiotic, was discovered to be hydrolyzed from prezeamine I (with weaker activity) by a putative acylpeptide hydrolase.[25] Didemnin X and Y are acyl−glutamine ester derivatives of the bioactive didemnin B. Didemnin B is thought to be the product of a cleavage of these larger

derivatives by unknown proteins.[26] Lastly, acylated saframycin precursors were discovered by *in vitro* reconstitution experiments; however, the chemical nature of these compounds and the enzymes involved in their maturation are still elusive.[27,28]

In this study, a precursor maturation mechanism involved in telomycin biosynthesis was unveiled through both *in vivo* and *in vitro* experiments. Moreover, the cloning, sequencing, mutational and biochemical analysis, and heterologous expression of the intact telomycin biosynthetic gene cluster are described. A systematic in-frame inactivation of decorating genes was crucial for deducing the function of specific tailoring enzymes which subsequently led to the production of several novel purified and structurally elucidated telomycin derivatives. Surprisingly, the natural precursor of telomycin exhibited 2−4-fold enhanced activity against Gram-positive pathogens compared to telomycin and showed no cross-resistance to clinically used antibiotics. Based on this knowledge, a few novel semisynthetic lipopeptides were synthesized and tested for biological activities as a starting point for a future program of chemical optimization of telomycin.

■ EXPERIMENTAL SECTION

Bacterial Strains, Plasmids, and DNA Manipulation. *S. canus* ATCC 12646 was the *wild-type* telomycin producer strain used in this study. *Escherichia coli* DH10B competent cells were prepared as the host for general subcloning and plasmid manipulation. *E. coli* GB2005 cells were used as the host for homologous recombination engineering.[29,30] *E. coli* ET12567/pUZ8002 served as the host for intergeneric transfer of knock out plasmid constructs to streptomycetes by conjugation. The pIJ774 plasmid was used as the template to amplify the apramycin resistance gene *aac(3)IV* and the *oriT* cassette to replace the target gene. Two genomic libraries of *S. canus* ATCC 12646 with different size inserts were constructed (detailed procedures are described in the Supporting Information). SuperCos 1 plasmid (Agilent Technologies, USA) was used as the vector for cosmid genomic library construction while pBACe3.6 plasmid was used as the vector for bacterial artificial chromosome (BAC) genomic library construction. Expression vector pASK-IBA6 (IBA, Göttingen, Germany) was used to heterologously express Tem25 in *E. coli* BL21 (DE3).

The genomic DNA of *Streptomyces* strains was extracted and purified manually following the cetyltrimethylammonium bromide (CTAB) procedure and pulsed field gel electrophoresis (PFGE) grade chromosomal DNA preparation procedure from Streptomyces Practical Genetics.[31] *Taq* DNA polymerase (Thermo Scientific) or Phusion High-Fidelity DNA Polymerase (Thermo Scientific) was used in PCR experiments. PCR products were cloned into pCR2.1 TOPO vector (Life Technologies) or pJET1.2/blunt vector (Thermo Scientific). All standard transformations and Red/ET recombinations with *E. coli* were done by electroporation. Plasmid DNA was isolated by standard alkaline lysis method. Restriction enzymes, Shrimp Alkaline Phosphatase, T4 DNA ligase, and other common molecular biology reagents were purchased from Thermo Scientific. The end sequencing of plasmids was done by LGC genomics (Berlin, Germany). All primers (listed in Tables S1−S3 in the Supporting Information) used in this paper were synthesized by Sigma-Aldrich (Steinheim, Germany).

In-Frame Gene Inactivation. To inactivate a specific gene, the cosmid bearing the gene was engineered to exchange the gene for the *aac(3)IV* and *oriT* cassette by Red/ET recombineering.[29,30,32] The conjugation process which transferred the engineered cosmid from *E. coli* ET12567/pUZ8002 to *S. canus* ATCC 12646 was performed according to standard procedures.[33] Primers and experimental design can be found in the Supporting Information (Table S1, Figures S6−S18).

Identification, Sequencing, and Analysis of the Telomycin Biosynthetic Gene Cluster. The genomic DNA of strain ATCC

12646 was analyzed by whole-genome sequencing. In the draft genome data, a candidate NRPS gene cluster was discovered. One NRPS gene was inactivated via gene replacement. Two BAC clones P1G3 and P1N13 which harbor the telomycin biosynthetic gene cluster were also shotgun sequenced as the genome sequence was found to contain several errors. The precise restriction map of the NRPS gene region was verified by digestion of BAC clone P1B12 using *Eco*72I, *Bsp*119I, *Not*I, *Eco*47III, and *Pst*I. The restriction map of the TEM gene cluster region was verified by digesting cosmids covering the cluster by *Bam*HI and *Not*I and comparison of the predicted to real fragments. A gap region containing highly repetitive sequences was closed by a series of subcloning steps and primer walking from cosmid clone P1N6. Initial prediction and analysis of the telomycin biosynthesis gene cluster was done by using antiSMASH (http://antismash.secondarymetabolites.org/).[34] Detailed prediction of NRPS A domain specificity was performed using NRPSpredictor2 (http://nrps.informatik.uni-tuebingen.de)[35] and alignments.[36] The functional prediction of open reading frames (ORFs) encoding proteins was performed using protein blast and/or blastx program (http://blast.ncbi.nlm.nih.gov) and Pfam (http://pfam.xfam.org). Routine DNA analysis such as ORF identification, primer design, and restriction analysis was done using Geneious software (Biomatters Ltd., Auckland, New Zealand). The accession number of the sequence deposited in GenBank is KP756960.

Characterization of Telomycin Derivatives. NMR spectra were recorded on a Bruker AVANCE 500 spectrometer, a Bruker AVANCE 700 spectrometer, or a Bruker Ascend 700 spectrometer all equipped with a 5 mm TXI cryoprobe. The detailed conditions could be found in the Supporting Information. LCHRMS data was performed on a Dionex Ultimate 3000 RSLC system using a Waters BEHC18, 100 × 2.1 mm, 1.7 μm dp column. Separation of 2 μL sample was achieved by a linear gradient with (A) H_2O + 0.1% FA to (B) ACN + 0.1% FA at a flow rate of 600 μL/min and 45 °C. The gradient was initiated by a 0.5 min isocratic step at 5% B, followed by an increase to 95% B in 18 min. UV spectra were recorded by a DAD in the range from 200 to 600 nm. The LC flow was split to 75 μL/min before entering the maXis HR-ToF mass spectrometer (BrukerDaltonics, Bremen, Germany) using the standard ESI source. Mass spectra were acquired in centroid mode ranging from 150 to 2000 m/z at 2 Hz scan speed. The peptides were linearized prior to MS/MS fragmentation analysis using 50 μL of the crude extract containing the depsipeptide dissolved in methanol or 50 μg of pure depsipeptide in 50 μL of methanol. 50 μL of 1 N NaOH was added, and the sample was shaken for 4 h at 40 °C. The solvent was evaporated under vacuum and redissolved in methanol prior to MS measurements.

Heterologous Expression and Purification of Acylase Tem25. The acylase gene was first submitted to SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) for the signal peptide prediction analysis.[37] The part of the gene not encoding a possible signal peptide was amplified by PCR from the genomic DNA of *S. canus* ATCC 12646 using Phusion High-Fidelity DNA Polymerase (Thermo Scientific). The primers used in this PCR were 5′-GAATTCGCGCCCGCCCCGTCCGAC-3′ (forward primer, with *Eco*RI restriction site) and 5′-CTGCAGGGGGGGCGGTCAGGACG-ACGGT-3′ (reverse primer, with *Pst*I restriction site). The PCR product was ligated into pJET1.2/blunt vector and transformed to *E. coli* DH10B. The plasmid pJET1.2/blunt::*tem25* was extracted and digested with *Eco*RI and *Pst*I. The acylase gene fragment with correct sticky ends was inserted into pASK-IBA6 vector generating the expression plasmid pTM25. Strain *E. coli* BL21 (DE3) carrying pTM25 was grown in LB medium at 37 °C and induced by adding anhydrotetracycline to 200 ng/mL when the OD_{600} of the culture reached 0.6. The cells were harvested after 4 h of further cultivation after induction and resuspended in high sucrose concentration buffer P (100 mM Tris-HCl pH 8.0, 500 mM sucrose, 1 mM EDTA). The resuspended mixture was incubated on ice for 30 min and centrifuged for 5 min at 13,000 rpm to produce clear lysate. Further sonication was applied only if cell lysis was not sufficient. The recombinant acylase was purified using StrepTrap HP column (GE Helthcare, USA) based on Strep-Tactin affinity chromatography. The filtrated clear lysate was

loaded to the column prewashed by buffer W (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) and then washed by buffer W again after sample application. The protein was eluted by buffer E (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin).

Enzyme Assays. The reaction mixtures of *in vitro* enzyme assays were prepared in 100 μL of 20 mM Tris-HCl (pH 7.4) buffer, supplemented with 0.1 mM substrate and 0.2 μM acylase. The reaction was performed at 30 °C for 4 h. The assay solution was then mixed with 300 μL of methanol and dried by evaporation. The residue was dissolved in 100 μL of methanol and centrifuged for 10 min at 21500g to remove particles before HPLC–ESI-MS analysis.

■ RESULTS AND DISCUSSION

Natural Telomycin Derivatives. From a 16 L fermentation of *S. canus* ATCC 12646, compound **2** and four additional new compounds (**9–12**) representing minor products or shunt products were isolated besides **1**. The structure of **2** was elucidated by extensive analysis of the 1D and 2D NMR data (Supporting Information). In comparison to **1**, **2** contained a methylene group instead of an oxygenated methine located in the C-terminal proline showing that it was missing the hydroxylation of [11]Pro (Chart 1). Two peaks of telomycins with the same m/z and overlapping retention time could be observed by HPLC–MS analysis (Figure 2); however, this phenomenon has been observed in other proline-containing peptides where the peaks belong to the cis–trans isomers of the same proline compound.[38–40] The structures of **9–12** were elucidated by the 1D and 2D NMR experiments (Chart 1) (Supporting Information). Compounds **9** and **10** are ring-opened telomycins with different degrees of hydroxylation; they are probably intermediates released spontaneously or by catalysis of the TE domain. Compound **11** is a shunt product representing a prematurely released intermediate while compound **12** is a macrolactone formed between [2]Ser and [11]Pro instead of lactonization between [3]Thr and [11]Pro. The implications of the identification of these shunt products with different length and hydroxylation during the biosynthesis of **1** will be discussed below. In all these telomycin analogues, the absolute stereochemistry of the β-position in the β-methyl-tryptophan (β-MeTrp) moiety could not be determined.

Antibiotic Activity. Telomycin showed good antibiotic activities against *S. aureus* as well as *Enterococcus faecium*. The natural analogues **9–12** all lost activity, revealing the nonapeptide lactone ring as critical for biological activity, whereas the loss of one hydroxylation found in **2** only led to a minor decrease of activity against *S. aureus*. Compounds **7** and **13–15** all showed comparable and by tendency improved activities compared to **1** (Table 1).

The antibacterial activity of telomycin and its semisynthetic analogues was also evaluated in a panel of resistant Gram-positive bacteria. Intriguingly, all tested derivatives were active on MRSA, VISA, and VRE in the low μg/mL range. We also assessed possible cross-resistance with structurally related daptomycin and could demonstrate that telomycins are only by a factor of 4–16 less active on an isolate that displays high-level daptomycin resistance (DRSA; >1000-fold change in MIC compared to WT). Furthermore, the MIC of daptomycin on a telomycin resistant SA strain (TRSA; >32-fold change in MIC of **15** compared to WT) did not increase when compared to SA Newman WT (Table 2).

More importantly, compound **15** showed excellent early bactericidal activity versus SA (Figure S5A in the Supporting Information) and VRE (Figure S5B in the Supporting

Table 1. MIC Values (μg/mL) of Telomycin and Analogues (see Charts 1 and 2)

telomycin	*S. aureus* Newman	*S. aureus* DSM-11822 (MRSA)	*E. faecium* DSM-20477
1	4	2	16
2	4	4	64
9	>64	>64	>64
10	>64	>64	>64
11	>64	>64	>64
12	>64	>64	>64
7	2	1	16
13	4	2	32
14	2	1	16
15	2	1	1
DAP[a]	0.5	0.25	–[b]

[a]Reference antibiotic daptomycin. [b]–, not determined.

Table 2. Cross-Resistance Evaluation of Telomycin and Selected Analogues (MIC in μg/mL; See Charts 1 and 2)

	SA N315 (MRSA)	SA Mu50 (MRSA/ VISA)	SA WT	DRSA[a]	TRSA[b]	*E. faecium* DSM-17050 (VRE)[c]
1	4	2	0.25	1	>64	16
7	1	2	0.13	2	–[d]	64
13	2	2	0.5	4	–	64
14	1	2	0.13	2	–	32
15	2	1	0.5	2	64	4
AMP[e]	>64	>64	–	–	–	–
CIP[e]	0.25	16	–	–	–	2
DAP[e]	0.13	0.25	≤0.005	5	0.5	8
ERY[e]	>64	>64	–	–	–	–
RIF[e]	0.01	>0.64	–	–	–	0.03
TET[e]	2	64	–	–	–	–
VAN[e]	1	8	–	–	–	>64

[a]*In vitro* obtained daptomycin-resistant mutant (derivative of *S. aureus* WT). [b]*In vitro* obtained telomycin analogue **15**-resistant mutant (derivative of *S. aureus* Newman). [c]Vancomycin-resistant *Enterococcus*. [d]–, not determined. [e]Reference antibiotics: AMP, ampicillin; CIP, ciprofloxacin; DAP, daptomycin; ERY, erythromycin; RIF, rifampicin; TET, tetracycline; VAN, vancomycin.

Information) in a standard time-kill assay. The bactericidal effect of **15** on SA is even slightly superior to that of daptomycin in terms of killing rate (Figure S5A in the Supporting Information).

The cytotoxicity of **1**, **7**, and **13–15** on Chinese hamster ovary (CHO-K1) cells was evaluated in standard growth inhibition experiments, and none of the tested compounds were active at concentrations up to 40 μg/mL.

Identification and Characterization of the Telomycin (TEM) Biosynthetic Gene Cluster. The structures of the telomycins suggest that their biosynthetic route is based on NRPS biochemistry. To find the corresponding biosynthetic gene cluster, the genome of *S. canus* ATCC 12646 was sequenced by Illumina sequencing technology, and one candidate NRPS gene cluster matching our expectations was found. However, the substrate specificity predictions of the A domains in this NRPS gene cluster were not consistent with all building blocks in telomycin. According to the initial genome assembly, this NRPS gene cluster exhibited only eight NRPS modules and thus seemed unlikely to be responsible for telomycin production. To test whether telomycin biosynthesis
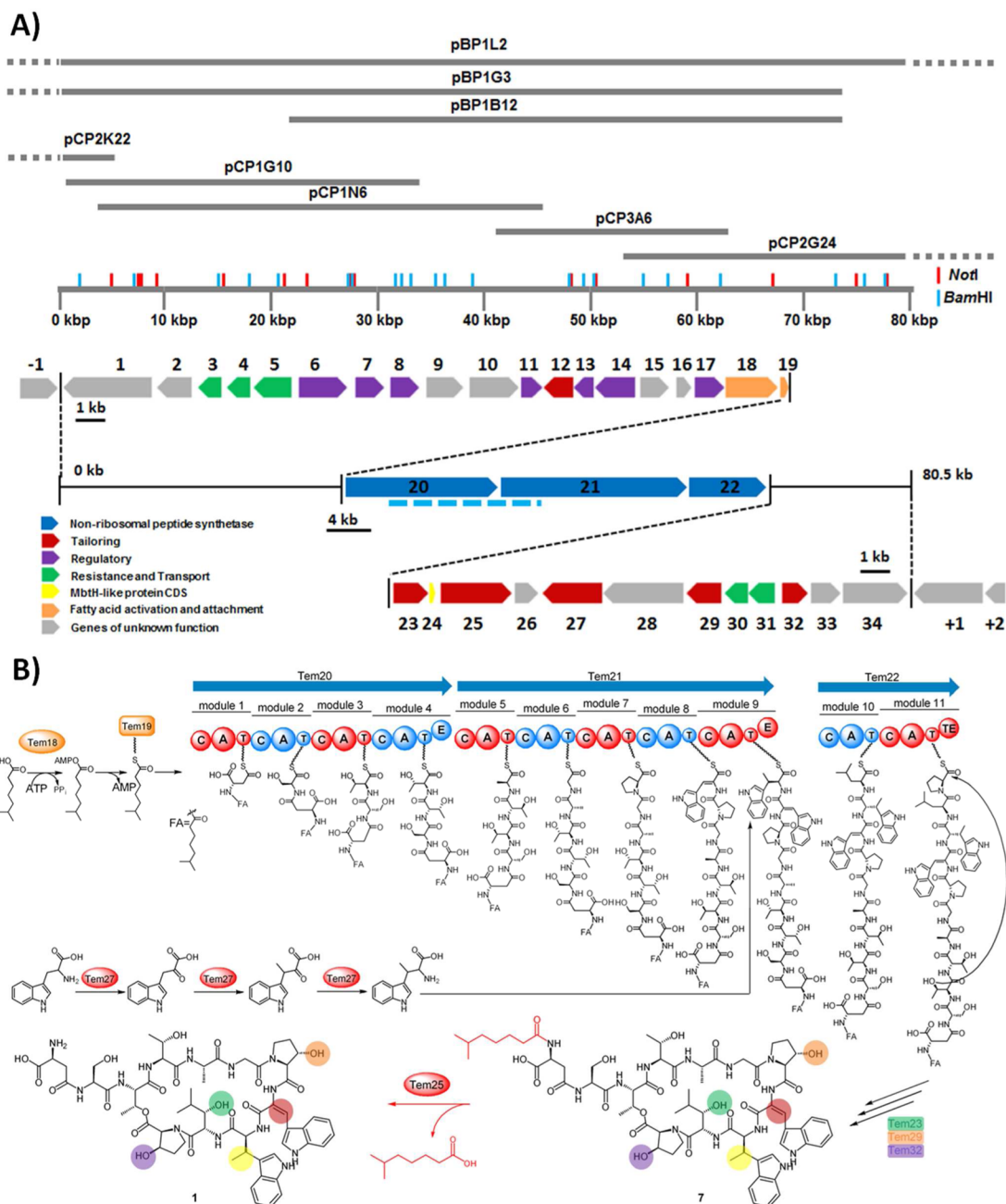
Figure 1. Gene organization and restriction map of the TEM gene cluster as well as the proposed biosynthetic pathway of telomycin. (A) Restriction map of the telomycin biosynthetic gene cluster. The dashed blue line indicates the gap containing highly repetitive DNA sequence. The restriction map of the whole DNA region covered by cosmids and BACs is found in the Supporting Information. (B) Tem18 and Tem19 initially activate a 6-methylheptanoate and transfer it to the carrier protein where Tem20, Tem21, and Tem22 build up the peptide chain. The three hydroxylation reactions performed by Tem23, Tem29, and Tem32 most likely take place post-NRPS assembly. The complex β-methylation of tryptophan is probably achieved prior to the activation by the respective A domain. After the mature lipopeptide is released and cyclized, the 6-methylheptanoyl moiety is cleaved off by Tem25 to yield telomycin.

depends on this NRPS gene cluster, the NRPS gene *tem22* was inactivated using an in-frame gene inactivation strategy (Figure S9 in the Supporting Information). The resulting mutant Δ*tem22* lost the ability to produce **1** and **2**, which proved the involvement of this NRPS gene cluster in telomycin biosynthesis (Figure S21 in the Supporting Information). Meanwhile, cosmid and BAC genomic libraries of *S. canus* ATCC 12646 were constructed. To deduce possible misassemblies causing a gap in the gene cluster, we screened these two genomic libraries with PCR probes targeting three genes located in the 5′ end,

the middle, and the 3′ end of the gene cluster (Table S2 in the Supporting Information). Through a 2-dimensional high-throughput screening strategy (Supporting Information), five cosmids (pCP2K22, pCP1G10, pCP1N6, pCP3A6, pCP2G24) spanning approximately 120 kb and three BAC plasmids (pBP1B12, pBP1G3, pBP1L2) overlapping about 104 kb were identified that covered the whole NRPS gene cluster (Figure 1). This physical genomic presentation was characterized by end sequencing and classical restriction mapping (Figure 1 and Figures S3 and S4 in the Supporting Information).

Table 3. Deduced Functions of ORFs Encoded in the Telomycin Biosynthetic Gene Cluster

gene	size (aa)[a]	predicted function in TEM biosynthesis	best match protein/organism	best match accession no. (identity/ similarity %)[a]
orf(−1)	433		hypothetical protein	CAJ87953 (69/76)
tem1	1051		hypothetic protein/Streptomyces coelicolor A3(2)	NP_624423 (93/95)
tem2	401		hydrolase/Streptomyces coelicolor A3(2)	NP_624422 (93/95)
tem3	273	ABC transporter	integral membrane permease/Streptomyces coelicolor A3(2)	NP_624421 (99/99)
tem4	280	ABC transporter	integral membrane permease/Streptomyces coelicolor A3(2)	NP_624420 (98/99)
tem5	426	ABC transporter	extracellular binding protein/Streptomyces coelicolor A3(2)	NP_624419 (96/98)
tem6	572		hypothetical protein/Streptomyces coelicolor A3(2)	NP_624418 (93/96)
tem7	297	transcriptional regulator	putative glucokinase/Streptomyces coelicolor A3(2)	NP_624417 (91/93)
tem8	347	transcriptional regulator	LacI family transcriptional regulator/Streptomyces coelicolor A3(2)	NP_624416 (96/97)
tem9	434		hypothetical protein/Streptomyces coelicolor A3(2)	NP_624415 (92/94)
tem10	567		putative hydrolase/Rhodococcus wratislaviensis NBRC 100605	GAF44958 (61/75)
tem11	267	transcriptional regulator	LuxR family transcriptional regulator/Pseudonocardia dioxanivorans CB1190	YP_004335958 (39/53)
tem12	314	tryptophan dehydrogenase	NADPH:quinone reductase/Amycolatopsis mediterranei	KDO08548 (85/93)
tem13	202		conserved hypothetical protein/Streptomyces ambofaciens ATCC 23877	CAJ89303 (58/66)
tem14	477	transcriptional regulator	transcriptional regulator, partial/Streptomyces venezuelae ATCC 10712	AAF01064 (67/75)
tem15	352		anthranilate phosphoribosyltransferase/Streptomyces toyocaensis	KES07266 (63/77)
tem16	160		secreted protein/Streptomyces sp. ML694-90F3	BAP34762 (73/84)
tem17	306	transcriptional regulator	ArsR family transcriptional regulator/Streptomyces albus J1074	YP_007746804 (51/62)
tem18	582	fatty acid CoA ligase	long chain fatty acid CoA ligase/uncultured bacterium esnapd4	AGS49393 (63/72)
tem19	89	acyl carrier protein	peptidyl carrier protein/Streptomyces albus	AEZ53971 (49/60)
tem20	4742	NRPS	NRPS/uncultured bacterium esnapd4	AGS49395 (52/63)
tem21	5824	NRPS	NRPS/uncultured bacterium esnapd2	AGS49328 (50/62)
tem22	2401	NRPS	NRPS/uncultured bacterium esnapd4	AGS49397 (54/66)
tem23	419	P450 monooxygenase	P450 monooxygenase/Streptomyces sp. Acta 2897	AEA30275 (63/78)
tem24	74	MbtH	MbtH-like protein/Streptomyces hygroscopicus	AAU34213 (79/87)
tem25	824	cyclic lipopeptide acylase	hypothetic protein/uncultured bacterium esnapd4	AGS49399 (56/68)
tem26	268		beta-ketoadipate enol-lactone hydrolase/uncultured bacterium esnapd4	AGS49400 (67/77)
tem27	715	methyltransferase	methyltransferase/Streptomyces flocculus	AFW04573 (39/54)
tem28	916		hypothetical protein/uncultured bacterium esnapd4	AGS49410 (62/75)
tem29	405	P450 monooxygenase	P450 monooxygenase/Kutzneria albida DSM 43870	AHI00608 (41/56)
tem30	272	ABC transporter	ABC transporter/Streptomyces coelicolor A3(2)	NP_627437 (41/58)
tem31	314	ABC transporter	ABC transporter/Kribbella flavida DSM 17836	YP_003381220 (71/80)
tem32	287	proline hydroxylase	chain A, L-proline-bound L-proline cis-4-hydroxylase/Mesorhizobium loti MAFF303099	PDB: 4P7W_A (31/48)
tem33	296		hypothetical protein/Streptomyces davawensis JCM 4913	YP_007592609 (29/41)
tem34	667		hypothetical protein/Amycolatopsis orientalis HCCB10007	YP_008013999 (33/45)
orf(+1)	837		ABC transporter integral membrane protein/Streptomyces davawensis JCM 4913	YP_007523698 (38/52)
orf(+2)	247		ABC transporter/Thermomonospora curvata DSM 43183	YP_003299905 (68/79)

[a] Amino acids.

By using different restriction enzymes to digest these cosmids and BAC plasmids, we found a region larger than 10 kb missing in the genome assembly (Figure S1 in the Supporting Information). To close this gap, the BAC plasmid pBP1L2, covering the whole gene cluster, was sequenced again by Illumina sequencing technology. Assembly of the resulting primary data revealed that only a 7.9 kb fragment of the gap was identified and added to the sequence, still leaving a gap approximately 5 kb in size. The sequence of this fragment was determined by a series of subcloning and primer walking steps based on cosmid pCP1N6. The completely closed gap was found to be approximately 11 kb in size, and an additional 2 kb fragment of wrong sequence assembly found immediately downstream of the gap was also revised (Figure S1 in the Supporting Information). Taking a closer look at this region, we found that it contains highly repetitive DNA segments which the employed genome assembly algorithm obviously

failed to assemble correctly, probably due to the relatively short read length of Illumina sequencing technology (Figure S2A in the Supporting Information). The first two newly identified A domains of modules 3 and 4 share 93.2% DNA identity, and more than 850 bp of their middle parts are exactly identical in sequence, probably because they are both responsible for threonine activation. As discovered before in hormaomycin biosynthesis, a recombinatorial exchange of a short DNA region approximately 420 bp led to different A domain functionality. However, the A domain of module 2 (A2) and A domain of module 5 (A5) in telomycin biosynthetic gene cluster share as high as 99.4% DNA identity except for only ten nucleotides difference. The Stachelhaus codes of A2 and A5 are exactly the same (Table S4 in the Supporting Information); seven amino acids outside the binding pocket are different (Figure S2B in the Supporting Information). However, these two A domains are expected to activate different amino acids: Ser and Ala,

respectively. These features suggest point mutations to be involved in the evolution of NRPS function as already described for the myxochromide pathway.[41] In addition, the C domains of module 3 and module 6 share as much as 93.1% DNA identity. Most likely, these repeats led to the wrong assembly of C3 and C6 and the deletion of the region in between them in the initial assemblies. The corrected gene cluster sequence was then verified by classical physical restriction mapping (Figure 1 and Figures S3 and S4 in the Supporting Information).

To determine the boundaries of the TEM cluster, a series of ORFs within the gene cluster were inactivated by gene replacement. The simultaneous inactivation of two genes *tem1* and *tem2* related to sugar metabolism dramatically impaired the production of telomycins, which suggests that these genes affect the biosynthesis of telomycin (Figures S7 and S21 in the Supporting Information). In contrast, inactivation of a hypothetical protein encoding gene *orf(−1)* did not have any influence on telomycin production, which indicates that this gene is located outside of the TEM cluster (Figures S6 and S21 in the Supporting Information). As to the 3′ end boundary, we inactivated the genes *tem33* and *tem34* exhibiting hypothetic functions, and the production of telomycins disappeared in both mutants (Figures S16, S17, and S21 in the Supporting Information). However, the inactivation of gene *orf(+1)* and *orf(+2)* simultaneously did not affect the production of telomycins (Figures S18 and S21 in the Supporting Information). In addition to the functional assignment of putative gene products within the cosmid overlapping region, these *in vivo* gene inactivation results revealed the TEM biosynthetic gene cluster to span 80,533 bp with a high GC content (73.0%) and to consist of 34 ORFs from *tem1* to *tem34*. These ORFs were initially characterized by bioinformatic analysis, and their putative functions were assigned by BLAST searches. The 34 genes can be classified in subcategories according to their functions in the biosynthesis of telomycin. First of all, in the center of the gene cluster there are three large NRPS genes (*tem20−22*). At the 5′-end of the NRPS genes, there are genes responsible for fatty acid activation and attachment (*tem18, -19*) and the remaining genes apart from several genes of unknown function are identified as encoding tailoring enzymes, transporters, and regulatory proteins (Table 3).

Heterologous Expression of the TEM Biosynthetic Gene Cluster. After the integration of BAC plasmids harboring the TEM cluster into the genome of heterologous hosts, several resulting apramycin-resistant mutants were fermented to test if any telomycin was produced. The HPLC−ESI-MS analysis revealed one telomycin derivative produced in low amount in the mutants of *Streptomyces albus* J1074 which carry the insets pBP1G3HE and pBP1L2HE, respectively (Figures S19 and S22 in the Supporting Information). This compound was identified as **2** based on the same retention time, molecular mass, and fragmentation pattern (t_R = 3.99 min, molecular mass [M + H]$^+$ at m/z of 1256.6, Figure S22 in the Supporting Information). In the mutants of *Streptomyces coelicolor* A(3)2 and *Streptomyces lividans* TK64, we did not identify any derivative of **1**. The production level of telomycins was very low, probably because of complex regulation process involved in biosynthesis of TEM, as suggested by numerous regulators located in the TEM gene cluster. Although the heterologous expression of the TEM cluster in *S. albus* did not lead to a high titer of telomycin

production, it is still a promising platform for genetic engineering. Further experiments such as promoter exchange are currently in progress to improve telomycin production in *S. albus* J1074.

Nonribosomal Peptide Synthetases. Three large genes *tem20*, *tem21*, and *tem22* are located in the center of the TEM gene cluster, and their deduced protein products are typical NRPS enzymes exhibiting a modular organization. These three giant enzymes Tem20, Tem21, and Tem22 contain 4, 5, and 2 modules, respectively, totaling 11 modules comprising C, A, and T domains (Figure 1B).

Two additional epimerization (E) domains were found in modules 4 and 9 (Figure 1B). Usually, an E domain would convert an L-amino acid to the respective D-amino acid.[16] However, the two amino acid residues incorporated by modules 4 and 9 are both L-amino acids, which still keep the L-form in the presence of E domains. The amino acid incorporated by module 4 is L-*allo*-Thr which might be formed from L-Thr since the (2*S*,3*S*) stereoisomer is rare in nature. As there is no precedent of *β*-epimerization by an E domain, there might be other enzymes involved in the configuration conversion. However, the possibility that the *β*-epimerization is catalyzed by the E domains cannot be excluded at this moment. The *β*-MeTrp residue of telomycin seems to be incorporated by module 9. However, whether it represents the (2*S*,3*S*) or (2*S*,3*R*) stereoisomer is still unclear since the absolute configuration of *β*-position of *β*-MeTrp in natural products has not been determined yet. Hence, whether the epimerization domain in module 9 is functional and what kind of function it has remain elusive.

The substrate specificity of each A domain was predicted according to the putative binding-pocket constituents (the so-called Stachelhaus-code).[36] However, the substrate predictions for A5, A8, A9 and A10 are not consistent with the amino acids present in **1** (Table S4 in the Supporting Information). All close hits predicted for the [5]Ala-activating domain A5 are Ser-activating domains found in the NRPS for CDA,[42] enterobactin,[43] nostopeptolide,[44] and belomycin[45] biosynthesis. A8 is expected to activate Trp or Z-ΔTrp, but the closest prediction for A8 is Val. Z-ΔTrp was also found in CDA, but the binding pocket code (DGWAVASVCK) of the A domain from CDA-module 11 incorporating Z-ΔTrp is quite different from the code of A8 in the TEM cluster.[42] Since module 9 of the TEM cluster may serve to incorporate *β*-MeTrp as found for the maremycin gene cluster, A9 of the TEM cluster and the A domain from the maremycin cluster were aligned.[46] Surprisingly, the A domain from the maremycin cluster (code DGYLYGSFTK) is very different from the TEM A9 code, implying that different substrates are activated by these A domains.[46]

Except for the N-terminal C domain, all other domains are found to be consistent with the expected number and order according to the chemical structure of telomycin. The C domain located at the N-terminus of module 1 of Tem20 shows similarity to enzymes which acylate the first amino acid with activated fatty acid moieties such as ACMS II,[47] DhbF,[48] and PstB.[49] Thus, the presence of the N-terminal starter C domain implies that [1]Asp might be acylated in an unknown precursor of telomycin (see below).

Natural Precursor Maturation Processed by an Acylase. Since telomycin is a cyclic peptide and not a lipopeptide, it is surprising to find a starter C domain located at the N terminus of Tem20 that cannot be functionally assigned.

Upstream of the first NRPS gene *tem20*, *tem18* is located which putatively encodes a fatty acid CoA ligase and *tem19* encodes an acyl carrier protein (ACP). According to the protein function prediction, Tem18 and Tem19 should be involved in activation of a fatty acid moiety and its loading onto the ACP. Tem18 has a characteristic domain of fatty acyl-AMP ligase (FAAL) which belongs to the class I adenylate forming enzyme family, and its homologues are assumed to be responsible for fatty acid activation in lipopeptide biosynthesis such as laspartomycin,[50] friulimicin,[51] taromycin A,[52] or glycopeptide biosynthesis, e.g., for teicoplanins.[53] Most fatty acid moieties in these lipopeptides are branched or unsaturated.

All these points suggest that the TEM biosynthetic machinery produces a typical lipopeptide. To verify our hypothesis that there might be a tailoring step involved in forming the mature peptide from a lipopeptide precursor, we turned our attention to the remaining genes in the cluster that were not functionally assigned to telomycin biosynthesis. Indeed, *tem25* encodes a putative cyclic lipopeptide acylase. The deduced product Tem25 shares a characteristic domain with a family of beta-lactam acylases which includes penicillin G acylase (PGA) and cephalosporin acylase (CA). These proteins all contain a conserved Ntn (N-terminal nucleophile) hydrolase fold and belong to the N-terminal (Ntn) hydrolase superfamily. PGA and CA share low sequence similarity with each other, but the structural similarities surrounding the active sites are very high. However, regardless of the conserved active sites, they hydrolyze the amide bonds of very different substrates.[54] Tem25 displays 63% similarity to an acylase found in *Streptomyces sp.* no. 6907 that hydrolyzes the palmitoyl moiety of the antifungal lipopeptide FR901379 produced by *Coleophoma empetri* F-11899.[55] It also shows 52% similarity with PvdQ, which is critical in pyoverdine maturation and transportation.[22,24]

To probe whether *tem25* plays a role in telomycin biosynthesis, this gene was inactivated via replacement with the *aac(3)IV-oriT* cassette (Figure S11 in the Supporting Information). The corresponding mutant *Streptomyces canus* ATCC 12646 Δ*tem25* stopped producing **1** and **2** but accumulated larger and more hydrophobic novel products. From a 6 L fermentation of mutant strain Δ*tem25*, two new compounds were isolated and structurally elucidated as lipopeptidic telomycin derivatives **7** ($[M + H]^+$ = 1398.7, t_R = 5.11 min) and **8** ($[M + H]^+$ = 1382.7, t_R = 5.33 min), both containing a 6-methylheptanoic acid residue (MHA) (Figure 2 and Chart 1). Comparing the ^{1}H NMR data with **1**, compound **7** showed additional signals for methylene groups between 1.18 and 2.24 ppm, a methine at 1.52 ppm, and two additional methyl groups at 0.86 ppm. The sequential spin system of the 6-methylheptanoic acid residue was deduced from COSY correlations starting from the two methyl groups at δ 0.86 (Me-7 and Me-8$_{MHA}$) attached to the methine at δ 1.52 (H-6$_{MHA}$) and comprising four sequential methylene groups H-5 to H-2. Together with the previous findings, HMBC correlations from H-3MHA and H-2MHA to a carbonyl resonance at d 175.8 (C-1MHA) clearly identified MHA as a branched chain fatty acid moiety. The fatty acid was found connected to the N-terminal asparagine through a key HMBC correlation from H-2$_{Asp1}$ to the carbonyl C-1$_{MHA}$ (Supporting Information). Compound **8** showed the same pattern as **2**, identifying it as an acylated derivative missing the hydroxylation of the C-terminal proline residue (Figure S24 in the Supporting Information).
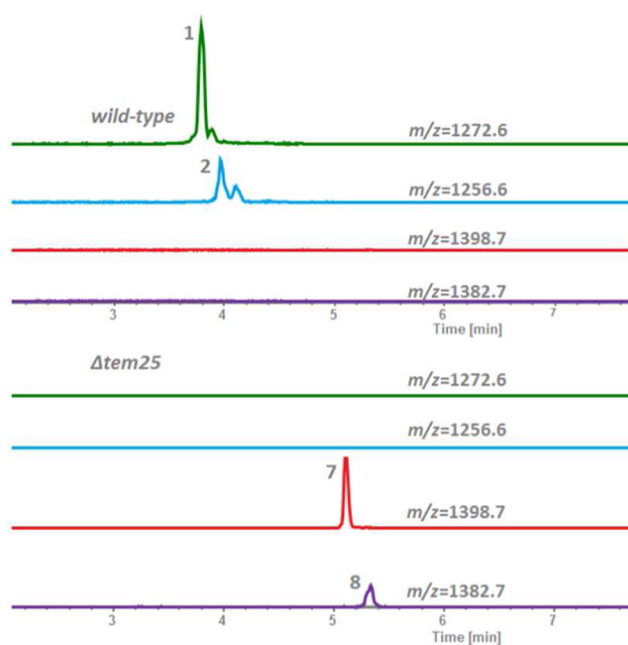
Figure 2. HPLC−MS analysis of telomycins in crude extracts (small-scale fermentation) of the *wild-type* and *tem25* gene-inactivated mutant strain of *Streptomyces canus* ATCC 12646. Extracted ion chromatograms (EIC) of telomycins (**1**, $[M + H]^+$ = 1272.6, green; **2**, $[M + H]^+$ = 1256.6, blue; **7**, $[M + H]^+$ = 1398.7, red; and **8**, $[M + H]^+$ = 1382.7, violet) were analyzed for both mutant and *wild-type* strains to test production. Compounds found in the *wild-type* and mutant strains are identified by numbers, and their structures as shown in Chart 1

Since **7** and **8** are lipopeptides containing the 6-methylheptanoyl moiety coupled to the amino group of the N-terminal Asp of **1** and **2**, respectively, the gene *tem25* was thus proposed as encoding the acylase which can hydrolyze the 6-methylheptanoyl moiety of the lipopeptidic precursor to produce the nonacylated peptide telomycins (Figure 1B).
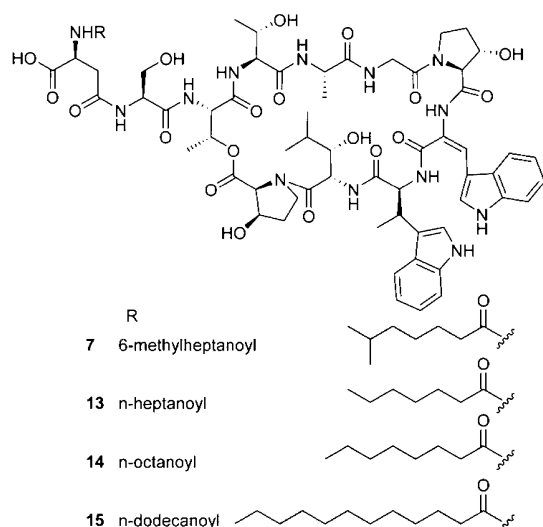
To our surprise, testing of **7** unveiled an increased activity compared to **1**. It is remarkable that the biological activities against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) were increased for the lipopeptide **7** that bears an acyl lipid side chain while the activity against *E. faecium* was not improved (Table 1).

Several semisynthetic derivatives with different acyl chains attached to the amino group of the ^{1}Asp of TEM were prepared (Scheme S1 and Supplemental Experimental Section in the Supporting Information). A selection of these tool compounds (**13**, **14**, and **15**) (Chart 2 and Supporting Information) were assessed for their antibacterial activities, which confirmed the beneficial effect of a lipid side chain on activity. The biological activity against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) was also increased for all semisynthetic lipopeptides **13**, **14**, and **15**, but activity against *E. faecium* was only increased in the case of **15**. Compound **15** displayed a 4-fold activity increase against SA and MRSA and a 16-fold activity increase against *E. faecium* (Table 1).

The question why a "more potent" precursor is hydrolyzed to give a natural product with weaker antibiotic activity currently remains elusive. One possible answer is that telomycin plays a different role in nature than acting as an antibiotic against Gram-positive bacteria.[56,57] Moreover, we considered that the deacylation step could constitute a detoxification or a so-called self-resistance mechanism. We
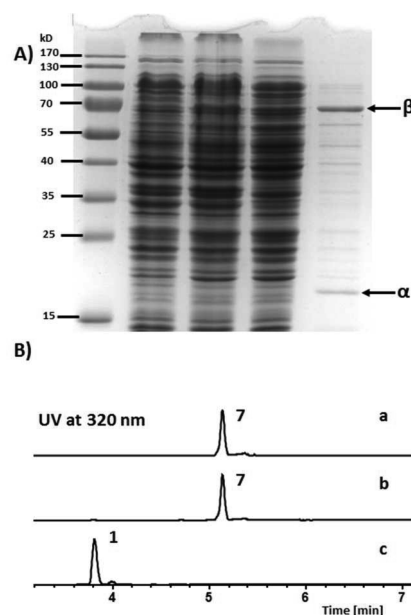
Chart 2. Chemical Structures of Semisynthetic Telomycin Analogues (7, 13−15)

|	R
7	6-methylheptanoyl
13	n-heptanoyl
14	n-octanoyl
15	n-dodecanoyl

Figure 3. SDS−PAGE analysis and *in vitro* characterization of Tem25. A standard Tem25 assay was prepared in 100 μL of 20 mM Tris-HCl (pH 7.4) buffer, supplemented with 0.1 mM substrate and 0.2 μM acylase. (A) The recombinant enzyme was purified as described in the Experimental Section. The α and β subunits are indicated by arrows. M, protein marker; lane 1, uninduced cells; lane 2, induced cells; lane 3, lysate supernatant of induced cells; lane 4, semipurified Tem25. (B) HPLC analysis of (a) 7 without adding Tem25; (b) boiled Tem25 incubated with 7; (C) Tem25 incubated with 7.

analyzed the activity of the acylase in a time course experiment of *S. canus* ATCC 12646 fermentation. The acylase activity could be detected from the third day of fermentation, which is when the telomycin production level is still low. Moreover, we never found the production of the natural precursors **7** or **8** in the fermentation of the *wild-type* telomycin producer. It thus seems that the acylase Tem25 is expressed as early as the NRPS genes. However, the concentration of telomycin in the natural environment is probably much lower than under laboratory conditions, and relatively low concentrations might already trigger the expression of Tem25.

***In Vitro* Characterization of Acylase Tem25.** To confirm the role of Tem25 in the biosynthesis of **1**, Tem25 was characterized *in vitro*. Since many PGAs and CAs from *Streptomyces* were identified as secreted proteins, the deduced product of *tem25* was analyzed for the presence of a signal peptide. Indeed, a cleavage site was predicted as APA-AP between residue 32 and 33, and the first 32 residues of Tem25 contain the typical signal peptide N, H, and C region indicative for an N-terminal signal peptide.[37,58] The part of *tem25* without the signal peptide encoding sequence was amplified from ATCC 12646 genomic DNA, and the corresponding recombinant protein was heterologously expressed in *E. coli* and purified as a Strep-tag fusion protein (Figure 3A). To test the *in vitro* hydrolytic activity of Tem25, the recombinant protein was incubated together with crude extracts from a Δ*tem25* fermentation. As expected, LC−MS analysis identified the accumulation of **1** and **2** while **7** and **8** disappeared from the extract. Furthermore, recombinant Tem25 was incubated with pure **7** *in vitro*, and the conversion from **7** to **1** was proven by LC−MS analysis (Figure 3B). These data confirmed the acylase activity of Tem25, which hydrolyzes **7** and **8** to release the 6-methylheptanoyl moiety.

To provide some insight on the substrate specificity of Tem25, the semisynthetic lipopeptides **13**, **14**, and **15** have been employed as substrates of Tem25 (Chart 2). Using HPLC−ESI-MS analysis it was found that Tem25 can hydrolyze **13** and **14** containing *n*-heptanoyl and *n*-octanoyl moieties almost completely, similar to the hydrolysis of **7** and **8**. Compound **15**, which carries the *n*-dodecanoyl moiety, could

only be partially hydrolyzed under standard conditions (Figure 4).

These assays employing different substrates including semisynthetic telomycins provided some hints as to the substrate specificity of Tem25. The enzyme shows a preference for amide bond hydrolysis preferring the connection between a fatty acid moiety and telomycin, since almost complete hydrolysis is observed for **7**, **8**, **13**, and **14**. The fatty acid chain length seems to be important, as roughly only 60% of compound **15** was hydrolyzed under standard conditions.

Hydroxylation of Leucine. The deduced product of *tem23* is a characteristic cytochrome P450 dependent enzyme. According to Blast searches, the putative gene product of *tem23* shows 78% similarity with Sky32, a cytochrome P450 monooxygenase from *Streptomyces sp.* Acta2897. The latter enzyme is responsible for three β-hydroxylations to form β-OH-Phe, β-OH-*O*-Me-Tyr, and β-OH-Leu in skyllamycin biosynthesis.[59,60] A *tem23* inactivation mutant was created, and the crude extract from the fermentation broth was analyzed by HPLC−ESI-MS (Figures S10 and S20 in the Supporting Information). We found that **1** and **2** were not produced by Δ*tem23*. However, two new peptides, **3** and **5**, were discovered in the culture broth (Chart 1 and Figure S20 in the Supporting Information). Linearization of these peptides for MS/MS analysis yielded the linearized analogues **16** and **17**, respectively. The fragmentation pattern of **16** proved that the hydroxylation of the leucine residue was missing in **3**, and the fragmentation pattern of **17** showed that the hydroxyl groups of β-OH-Leu and *cis*-3-OH-Pro were both lost in **5** (Figure 5A). Peptide **3** and **5** thus lack the β-hydroxyl group of β-OH-Leu compared to **1** and **2**, respectively, revealing that Tem23 is the
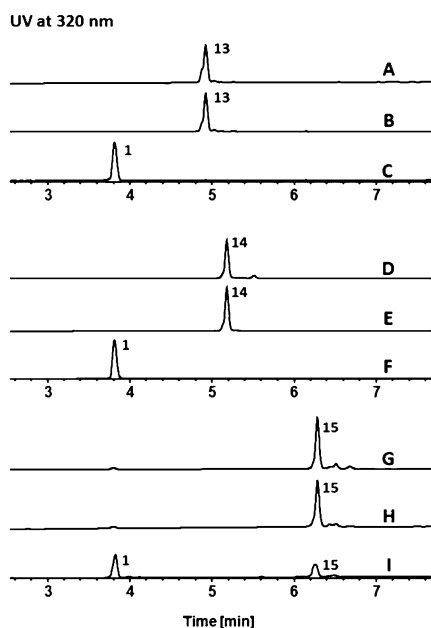
Figure 4. HPLC analysis (UV absorption at 320 nm) of various substrates incubated with recombinant Tem25. A standard Tem25 assay was prepared in 100 μL of 20 mM Tris-HCl (pH 7.4) buffer, supplemented with 0.1 mM substrate and 0.2 μM acylase. (A) **13** without Tem25; (B) **13** with inactivated Tem25; (C) **13** with Tem25; (D) **14** without Tem25; (E) **14** with inactivated Tem25; (F) **14** with Tem25; (G) **15** with Tem25; (H) **15** with inactivated Tem25; (I) **15** with Tem25.

P450 monooxygenase responsible for β-hydroxylation of leucine as found in telomycin.

Interestingly, Sky32 was shown to interact with PCP-bound amino acids in skyllamycin biosynthesis by *in vivo* and *in vitro* experiments.[59,60] The specificity of Sky32 was demonstrated to be mediated by a carrier protein loaded with the respective amino acid, which is thought to be the reason why this P450 hydroxylated specific amino acids. A skyllamycin derivative without any β-hydroxylated amino acid residues was discovered from the *sky32* inactivation mutant.[59,60] No natural dehydroxy-skyllamycin derivatives could be isolated from the *wild-type* producer strain.[59] However, natural telomycins **9** and **12** exhibiting Leu instead of β-OH-Leu moieties could be isolated from the *wild-type* telomycin producer in low yields. Furthermore, the natural telomycin derivative **10** with only the β-OH-Leu but without the other two hydroxylated amino acids has also been discovered. Because of these natural dehydroxy-telomycins and the accumulation of **3** and **5** in the $\Delta tem23$ mutant, it is likely that the β-hydroxylation of leucine happened after the NRPS assembly. However, there is another possibility which implies that the A domain and its downstream C domain have relaxed substrate specificity. Hence, these telomycins exhibiting different hydroxylation patterns might also be formed during NRPS assembly.

Hydroxylation of Two Proline Residues by a P450 Monooxygenase and a Hydroxylase. Apart from β-OH-Leu, telomycin contains two more hydroxylated amino acids (*trans*-3-OH-[7]Pro and *cis*-3-OH-[11]Pro). Gene *tem29* was annotated as encoding a cytochrome P450 dependent monooxygenase. The putative product of *tem29* shows 58% similarity to a P450 monooxygenase of unknown function from *Streptomyces eurythermus* involved in angolamycin biosyn-

thesis.[61] It also exhibits 55% similarity to a P450 mono-oxygenase which might be responsible for the hydroxylation of the macrolide ring of tylactone and tylosin produced by *Streptomyces fradiae*.[62] To probe the function of *tem29*, the gene was inactivated, and the resulting mutant $\Delta tem29$ lost the ability to produce **1** and **2** and accumulated new products **4** and **6** (Chart 1; Figures S13 and S20 in the Supporting Information). Compound **4** showed almost the same retention time and m/z as **2** and **3**. However, the hydrolysis of **4** produced **18**, MS/MS analysis of which proved that **4** differs from **2**. The *trans*-3-OH-[7]Pro residue was replaced by Pro in **4**, whereas the *cis*-3-OH-[11]Pro was substituted for Pro in **2**. Compound **6** was also hydrolyzed to give **19**. This served to prove that both *trans*-3-OH-[7]Pro and *cis*-3-OH-Pro were substituted with Pro in **6**. The *trans*-3-OH-[7]Pro moiety found in compounds **1** and **2** was substituted with Pro to result in structures **4** and **6**, respectively (Figure 5B). These data revealed that Tem29 is the P450 monooxygenase responsible for the *trans*-3-hydroxylation of [7]Pro.

Tem29 showed low similarity to other P450 monooxygenases. Since most of these homologues were assumed to be involved in polyketide biosynthesis,[61,62] it is hard to predict the timing of Tem29 activity by a comparison with homologues. Due to the presence of telomycin derivatives **4** and **6** in mutant $\Delta tem29$ and due to the natural intermediates **9**−**12** (Chart 1), in which the *trans*-3-OH-[7]Pro was substituted by Pro, the hydroxylation probably occurred post NRPS assembly. In addition, the Tem29 homologue TpdJ2 was reported to be involved in the biosynthesis of the thiopeptide GE2270. One hypothetic function of TpdJ2 is the β-hydroxylation of phenylalanine. TpdJ2 would thus use the ribosomally synthesized thiopeptide core as substrate, which further implied that Tem29 could perform the hydroxylation after the telomycin peptide intermediate was released.[63] As discussed before, the hydroxylation might also take place earlier due to relaxed substrate specificity of the NRPS enzymes.

The deduced product of *tem32* has a characteristic domain of aspartyl/asparaginyl beta-hydroxylases including proline hydroxylases, which are iron(II)/2-oxoglutarate (2-OG)-dependent oxygenases. Tem32 shows 48% similarity with a L-proline *cis*-4-hydroxylase from *Mesorhizobium loti* MAFF303099[64] and 47% similarity with a proline 3-hydroxylase (2-oxoglutarate dependent) from *Streptomyces sp.* strain TH1.[65] To verify the hypothesis that Tem32 plays a similar role in the synthesis of *cis*-3-OH-[11]Pro in **1**, a *tem32* mutant $\Delta tem32$ was constructed and probed for telomycin production (Figure S15 in the Supporting Information). The HPLC−ESI-MS analysis revealed that the production of **1** was lost while **2** was still produced (Chart 1 and Figure S20 in the Supporting Information). Compound **2** was subjected to base hydrolysis to yield the corresponding linearized peptide **20**. The MS/MS fragmentation pattern analysis of **20** revealed that, in **2**, the *cis*-3-OH-[11]Pro was replaced by Pro (Figure 5C). Therefore, Tem32 is assumed to be a proline 3-hydroxylase responsible for the formation of *cis*-3-OH-[11]Pro.

Due to the discovery of natural telomycin derivatives with unhydroxylated [11]Pro (such as **9**, **10**, and **12**) in addition to **2** and the abolishment of the production of **1** after the inactivation of *tem32*, it is possible that Tem32 catalyzes the hydroxylation after the NRPS assembly. However, the homologue of Tem32 found in streptomycetes acts as proline 3-hydroxylase (with 2-oxoglutarate-dependent dioxygenase properties), hydroxylating free L-proline to form *cis*-3-OH-L-
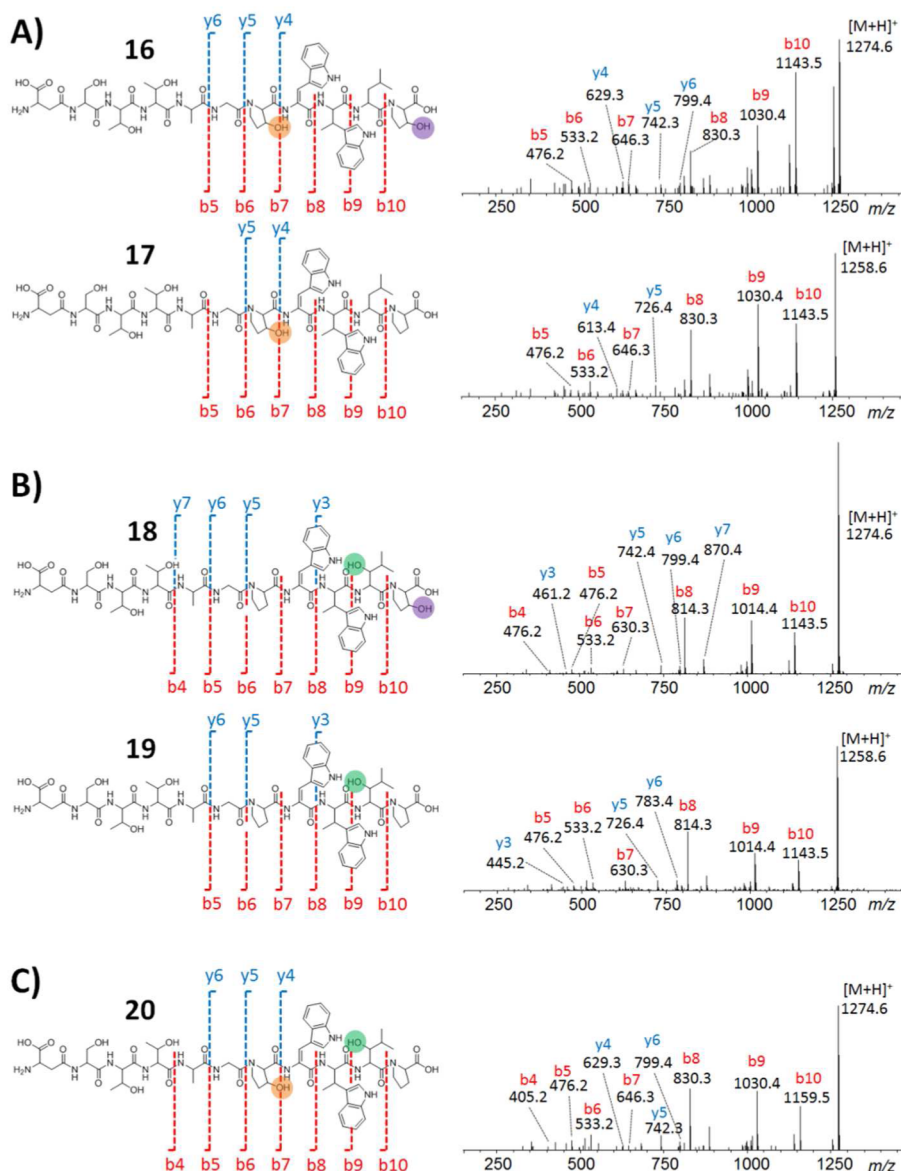
Figure 5. Characterization of telomycin derivatives. HPLC−MS/MS fragmentation analysis of (A) **16** and **17** formed by hydrolysis of **3** and **5**, respectively, (B) **18** and **19** formed by hydrolysis of **4** and **6**, respectively, and (C) **20** formed by hydrolysis of **2**.

proline.[65] Hence, the *cis*-3-hydroxylation in telomycin biosynthesis might occur on free proline or after the NRPS assembly.

Genes Putatively Involved in Resistance, Transport, Regulation, and Substrate Biosynthesis of Unknown Function. The three genes *tem3*, *tem4*, and *tem5* located at the 5′ end of the TEM cluster putatively encode ABC transporter subunits and might be involved in the telomycin export (self-resistance) and biosynthesis. Genes *tem30* and *tem31* also encode putative ABC transporters and were therefore inactivated simultaneously resulting in the mutant strain *Streptomyces canus* ATCC 12646 Δ*tem30*−*31* (Figure S14 in the Supporting Information). In several repeats of fermentation of the mutant strain *Streptomyces canus* ATCC 12646 Δ*tem30*−*31*, we never detected any telomycin in the supernatant of the culture broth (Figure S21 in the Supporting Information). However, we detected trace amounts of telomycins in the cells of the fermentation twice but not every time. We hypothesize that this unsteady and low amount of production in the cells is caused by transcription adaption (suppressor mutation) since

the initially synthesized amount of telomycin cannot be exported and thus leads to cell death.

Interestingly, various genes encoding transcription regulators were discovered in the 5′ region of the TEM cluster, which suggested a sophisticated regulation system for telomycin production. The gene *tem7*, which putatively encodes a sugar kinase, belongs to the ROK family of transcription regulators. The deduced product of *tem8* belongs to the LacI transcriptional regulator family. Since *tem6* is located close to *tem7*, and since its putative product is a mannose-6-phosphate isomerase-like protein involved in sugar metabolism, we assume that Tem6 is possibly functionally connected to Tem7. Although the function of the gene product of *tem9* is still elusive, this protein structurally belongs to the 6 hairpin glycosidase superfamily, which suggests that Tem9 might be functional in conjunction with Tem8. The gene *tem11* encodes a putative LuxR transcriptional regulator, which is a DNA-binding protein. Interestingly, the putative product of *tem13* seems to contain a characteristic AmiR and NasR transcription antitermination

regulator (ANTAR) domain representing a RNA-binding domain found in bacterial transcription antitermination regulatory proteins.[66] The gene *tem14* was annotated to encode a GntR family transcriptional regulator, which has a similar N-terminal DNA binding domain but a heterogeneous C-terminal effector-binding domain. In addition *tem17* encodes a protein containing a characteristic domain of the ArsR subfamily of helix−turn−helix bacterial transcriptional regulators.

The gene *tem27* encodes a bifunctional enzyme in which the N-terminal part is an aminotransferase and the C-terminal part is a methyltransferase. This gene might be involved in the β-methylation of tryptophan according to the reported case in biosynthesis of maremycins.[46] However, the inactivation of *tem27* led to the abolishment of all telomycins (Figures S12 and S21 in the Supporting Information). Whether this β-MeTrp is formed by Tem27 or other enzymes could not be assigned yet. There is another Trp residue in telomycin which represents a Z-2,3-dehydrotryptophan (Z-ΔTrp). *tem12* containing a characteristic domain belonging to the medium chain reductase/dehydrogenase (MDR)/zinc-dependent alcohol dehydrogenase-like family was inactivated. However, telomycin production was abolished after deletion of *tem12* (Figures S8 and S21 in the Supporting Information). Thus, whether *tem12* is involved in the formation of Z-ΔTrp remains an open question. A detailed discussion of the function of these two genes can be found in Supporting Information.

The gene *tem15* encodes an anthranilate phosphoribosyltransferase (TrpD) which is involved in tryptophan biosynthesis, and therefore is speculated to be involved in the production of tryptophan used in telomycin biosynthesis.

The deduced product of *tem24* shows homology to the MbtH family of proteins which are prevalent in NRPS biosynthetic pathways. MbtH proteins are found to be required for the efficient biosynthesis of many secondary metabolites depending on NRPSs.[67−69]

There are additional genes found in the TEM cluster which cannot be assigned to any function in telomycin biosynthesis. For instance, the gene *tem10* encoding a YtcJ-like metal dependent amidohydrolase, *tem16* encoding a prokaryotic phospholipase A2, and *tem28* encoding a representative of a family of bacterial ferritin-like proteins.

CONCLUSIONS

Novel lipopeptides were identified as natural precursors in the telomycin biosynthesis. Surprisingly, the precursor of telomycin displayed enhanced activity against Gram-positive pathogens versus telomycin itself. Telomycins are particularly important antibiotics as they most likely exhibit a novel mode of action as demonstrated by a lack of cross-resistance with several clinically important antibiotics. We could also demonstrate that telomycin **15** displays favorable early bactericidal activity on methicillin-sensitive *S. aureus* (MSSA) and VRE.

The deacylase TEM25 was characterized by *in vivo* and *in vitro* experiments, explaining the biochemistry behind the unusual maturation process of telomycins: a novel telomycin lipopeptide precursor was purified from a *tem25* mutant, structurally elucidated, and the enzyme Tem25 was found to deacylate this precursor to eventually form telomycin. However, the natural function of this transformation currently remains unclear, and it will be interesting to study why the more active lipopeptide is formed initially and then deacylated.

The biosynthetic gene cluster for the biosynthesis of the nonribosomal peptide telomycin was cloned, sequenced, and heterologously expressed. A series of *tem* genes were inactivated in the wild-type producer strain to identify the cluster boundaries and the precursor maturation related genes as well as to elucidate the function of enzymes involved in non-proteinogenic amino acid biosynthesis, including the hydroxylation of three amino acid residues, the double-bond formation of the first tryptophan residue, and the methylation of the second tryptophan residue. From these mutants, seven new telomycin biosynthetic intermediates were identified, which not only helped to elucidate the respective enzyme's functions but also expanded the chemical diversity of the telomycins. Additionally, five new telomycin intermediates isolated from the natural producer *S. canus* ATCC 12646 also provided hints to the timing of non-proteinogenic amino acid formation and contributed to preliminary structure activity relationships. This study, describing the synthesis of lipidated derivatives of telomycin displaying enhanced antibacterial activity, the identification of the biosynthetic gene cluster with an unusual maturation process, the identification of tailoring genes, and the establishment of a heterologous expression system, now forms the basis for the further pharmaceutical development of the antibiotic class of telomycins.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplemental experimental section, primer sequences, design and verification of mutants, and NMR spectra and assignment. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b01794.

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Notes

The authors declare no competing financial interest.

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