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Biosynthetic Gene Cluster of the Non-ribosomally Synthesized Cyclodepsipeptide Skyllamycin: Deciphering Unprecedented Ways of Unusual Hydroxylation Reactions

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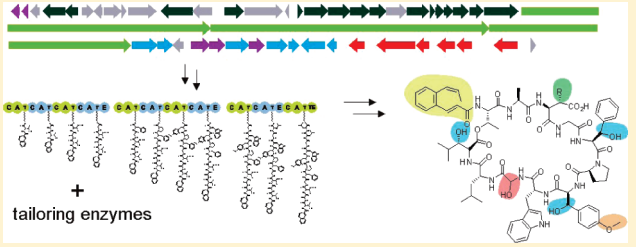
Supporting Information

ABSTRACT: The cyclic depsipeptide skyllamycin A is a potent inhibitor of the platelet-derived growth factor (PDGF) signaling pathway by inhibiting binding of homodimeric PDGF BB to the PDGF β-receptor. Its structure contains a cinnamoyl side chain and shows a high amount of β-hydroxylated amino acids as well as an unusual α-hydroxyglycine moiety as a rare structural modification. The skyllamycin biosynthetic gene cluster was cloned and sequenced from *Streptomyces* sp. Acta 2897. Its analysis revealed the presence of open reading frames encoding proteins for fatty acid precursor biosynthesis, non-ribosomal peptide synthetases, regulators, and transporters along with other modifying enzymes. Specific in-frame mutagenesis of these tailoring enzymes resulted in the production of novel skyllamycin derivatives revealing that β-hydroxy groups in skyllamycin A are introduced by a promiscuous cytochrome P450 monooxygenase, whereas a two-component flavin-dependent monooxygenase is involved in α-hydroxylation.



Cyclic depsipeptides comprise an important group of secondary metabolites produced by bacteria and fungi. Apart from their structural diversity, these natural products possess diverse biological activities and are used pharmaceutically as antibacterials, immunosuppressives, cytostatics, and antihelmintics.[1] The cyclodepsipeptide skyllamycin A (**1**) was first isolated as the antibiotic RP-1776 from *Streptomyces* sp. KY 11784[2] and reisolated from *Streptomyces* sp. Acta 2897 together with the derivative skyllamycin B (**2**).

The structure of skyllamycins (**1**, **2**, Figure 1), which has been configurationally assigned by our group,[3] consists of an 11mer macrolactone peptide with an N-terminal 2-[1-(*Z*)-propenyl]-cinnamoyl moiety attached. This residue is rarely found in peptides and has only been described for the tachykinin antagonist WS9326A[4] and the farnesyl transferase inhibitor group of pepticinnamins,[5] both isolated from *Streptomyces* species. Another remarkable feature of the skyllamycin structures is the high content of α- and β-hydroxylated amino acids. Whereas β-hydroxylated amino acids are found in a number of important antibiotics like vancomycin[6] or lysobactin,[7] a chemically stable α-hydroxy modification of a glycine residue was previously described only for the immunosuppressive peptides spergualin and 15-deoxyspergualin.[8] In addition to the unusual number of hydroxylations, the highly functionalized skyllamycin peptides A and B contain the non-proteinogenic amino acid *O*-methyltyrosine, though they differ with respect to the β-methylaspartate for skyllamycin A and to the aspartate for skyllamycin B.

Skyllamycin A has been described as a potent inhibitor of the platelet-derived growth factor (PDGF) signaling pathway.[2] PDGFs belong to a group of small homodimeric and heterodimeric proteins produced by various tissues of mammalian organisms. They act primarily as mitogens and chemotactic factors for mesenchymal tissue cells.[9] The five different forms of dimeric PDGFs known so far are active in the extracellular matrix and bind to the extracellular domains of the membrane-bound α- or β-receptor proteins. This binding results in receptor protein dimerization and induces the receptor tyrosine kinase domain that triggers intracellular signal transduction by subsequent phosphorylation of signal peptides. Deregulated or exaggerated signaling can result in pathological tissue like fibroses and arteriosclerosis, and is involved in different kinds of cancers.[9]

The synthetic drug imatinib is the first marked tyrosine kinase inhibitor used to treat cancer, e.g., chronic myelogenous leukemia.[9,10] It can also efficiently block the tyrosine kinase domain of the PDGF receptors by binding reversibly to the ATP-binding pocket. Skyllamycin A, on the other hand, selectively blocks the binding of PDGF BB homodimer to the PDGF β-receptor with an IC_{50} of 11 μM, therefore representing an alternative mode of action of PDGF signaling inhibition.[2]

The structures of skyllamycins clearly indicate their synthesis by non-ribosomal peptide synthetases (NRPS). These large multienzymes are modularly organized; that is, each successive module contains discrete catalytic domains that are responsible for building block binding and chain elongation.[11] Adenylation (A) domains activate and load the required amino acid building

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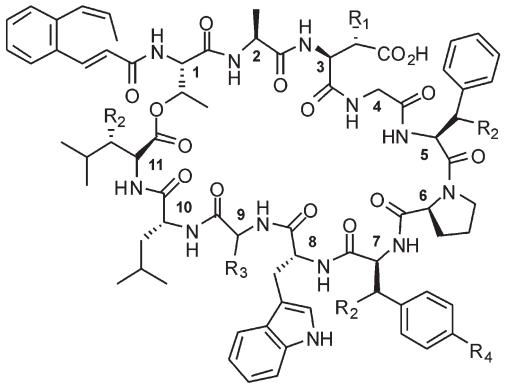

		R_1	R_2	R_3	R_4
1	skyllamycin A	CH_3	OH	OH	OCH_3
2	skyllamycin B	H	OH	OH	OCH_3
3	β-deoxy skyllamycin	CH_3	H	OH	OCH_3
4	α-deoxy skyllamycin	CH_3	OH	H	OCH_3
5	demethoxy skyllamycin	CH_3	OH	OH	H

Figure 1. Structures of skyllamycins.

blocks to the thiolation (T) domains, bound as thioesters. The condensation (C) domain performs subsequent peptide bond formation to accomplish chain elongation. An optional epimerization (E) domain directs the epimerization and incorporation of D-configured amino acid residues into the growing peptide chain. The structural diversity of secondary metabolites can be further increased by tailoring enzymes acting *in trans* to modify the building blocks or peptides.[12−14]

In the present study, we describe the sequencing and analysis of the skyllamycin biosynthetic gene cluster in *Streptomyces* sp. Acta 2897. Gene inactivation by in-frame gene deletion allowed the functional assignment of tailoring enzymes, thereby revealing new biosynthetic routes to hydroxylated amino acids in nonribosomal peptide synthesis.

MATERIALS AND METHODS

Bacterial Strains and DNA Manipulation. *Escherichia coli* DH5α was used as a host for general subcloning and manipulation of plasmid DNA following standard protocols.[15] *E. coli* BW25113/pIJ790 and *E. coli* DH5α/pIJ778 were provided by John Innes Centre (Norwich, UK) as a part of the REDIRECT technology kit.[16] *E. coli* ET12567/pUZ8002 was used as a host for conjugational transfer of plasmids into streptomycetes. *Streptomyces* sp. Acta 2897 was isolated from a sandy soil collected at a dune slack near Warkworth, UK.

Extraction of plasmids, purification of DNA, and isolation of genomic DNA were carried out using commercial kits (Fermentas, St. Leon-Rot, Germany). Restriction enzymes and molecular biological reagents were purchased from New England Biolabs (Ipswich, MA) and Fermentas (St. Leon-Rot, Germany). PCR was performed using *Taq* polymerase (Qiagen, Hilden, Germany) and Herculase II fusion DNA polymerase (Agilent Technologies, Waldbronn, Germany), and PCR products were cloned into vectors pDrive (Qiagen) or pJet1.2/blunt (Fermentas). DNA sequencing was carried out by AGOWA GmbH (Berlin, Germany). Generation of the hybridization probes was performed by PCR using DIG-labeled nucleotides (PCR DIG-labeling kit, Roche Diagnostics, Mannheim, Germany) and Southern hybridization according to the manufacturer's protocol. All primers used were ordered from biomers.net (Ulm, Germany); their respective sequences are listed in the Supporting Information.

Cultivation of *Streptomyces* sp. Acta 2897 and Production of Skyllamycins and Derivatives. For the production of skyllamycins, 100 mL of KM4 medium (4 g/L glucose, 4 g/L yeast extract, 10 g/L malt extract, 2 g/L $CaCO_3$) in 500 mL baffled flasks was inoculated with 4% of a 3-days-old preculture of *Streptomyces* sp. Acta 2897 wild-type or mutant strains. Cultures were grown for 5 days at 27 °C and 120 rpm on a rotary shaker (Infors Multitron 2, Einsbach, Germany). For genomic DNA isolation, the strains were cultured in tryptic soybroth medium[17] at 27 °C and 120 rpm on a rotary shaker. To restore the production of skyllamycins in the *Streptomyces* sp. Acta 2897 Δ*orf37* mutant, 20 mL of KM4 medium supplemented with 1 mg/mL of *O*-methyltyrosine (Bachem, Bubendorf, Switzerland) was inoculated with a spore solution harvested from MS plates,[17] and the culture was incubated at 27 °C and 120 rpm on a rotary shaker. After 5 days, the supernatant was analyzed for skyllamycin production.

Isolation of Skyllamycins and High-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry (HPLC-ESI-MS). To isolate skyllamycins, the cultures were harvested by centrifugation after 5 days of growth. The supernatant was extracted twice with the same volume of ethyl acetate. After the organic phase was dried *in vacuo*, the crude extract was dissolved in CH_3CN and analyzed by HPLC-ESI-MS.

The HPLC-MS measurements were carried out on a capillary-LC system (1100 series, Agilent Technologies, Waldbronn, Germany) coupled to a QTrap2000 mass spectrometer (Applied Biosystems, Darmstadt, Germany). HPLC was performed using a 50 mm × 1 mm Luna 3μ C18(2) 100 Å column (Phenomenex, Aschaffenburg, Germany) with a flow rate of 60 μL/min using a gradient from 5% to 100% CH_3CN (0.1% HCOOH) in H_2O (0.1% HCOOH) in 10 min. Prior to tandem mass spectrometry (MS/MS) experiments, the crude extracts dissolved in CH_3CN were treated with H_2O and concentrated NH_4OH (49:49:2) for up to 4 h at room temperature to linearize the skyllamycins by hydrolysis. After the mixture was dried *in vacuo*, it was redissolved in CH_3CN and analyzed with HPLC-ESI-MS/MS under the aforementioned conditions.

Identification and Sequencing of the Skyllamycin (*sky*) Gene Cluster. A cosmid library of *Streptomyces* sp. Acta 2897 genomic DNA, comprising 2304 clones, was prepared in pOJ436[18] by Combinature Biopharm AG (Berlin, Germany). Homogenized bacterial cultures were immersed in 0.5% low-melting agarose (SeaPlaque GTG, Biozym, Germany) and incubated with 2 mg/mL lysozyme (Carl Roth, Karlsruhe, Germany) for 14 h at room temperature and with 1 mg/mL proteinase K (Merck, Darmstadt, Germany) for 24 h at 50 °C. The embedded DNA was partially digested with *Sau*3AI, extracted with gelase (Epicenter, Madison, WI), and dephosphorylated (antarctic phosphatase, New England Biolabs). Subsequently, the DNA was ligated with 750 ng of *Bam*HI-digested vector pOJ436 and then packed using Gigapack III Gold Packaging Extract (Agilent Technologies).

Cosmid clones were screened using DIG-labeled hybridization probes amplified from *Streptomyces* sp. Acta 2897 genomic DNA. For non-ribosomal peptide synthetase probes, the primer pairs oligo1/oligo5[19] and A3/A7R,[20] respectively, were employed. For screening for the large subunit of a glutamate mutase, primer pair Glm1/Glm2 (Supporting Information) was utilized. In total, the four overlapping cosmids 1J22, 1P24, 6D08, and 2G03 were fully sequenced by Agowa GmbH employing a combination of shotgun sequencing and primer walking. Cosmid 2L01 was partly sequenced by primer walking. Putative open reading frames (ORFs) were identified using the frame plots function of the Artemis sequence viewer program (http://www.sanger.ac.uk/resources/software/artemis). Likewise, the functions of encoded proteins were assigned by sequence similarity using NCBI protein−protein BLAST searches (http://www.ncbi.nlm.nih.gov/).[21] The Pfam protein family database (http://pfam.sanger.ac.uk/) was used to analyze encoded proteins for functional domains.[22] Prediction of the NRPS A

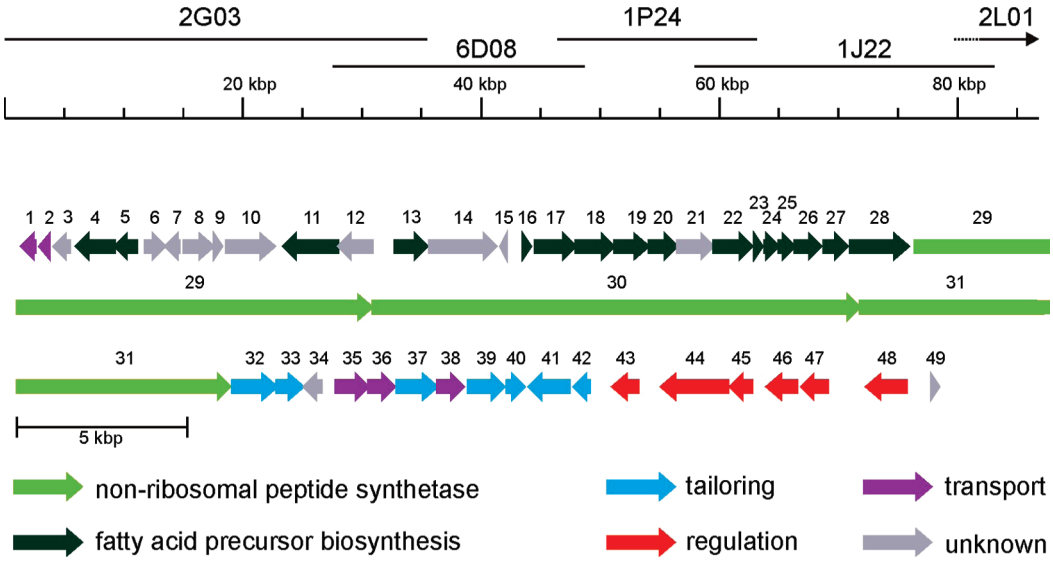

Figure 2. Organization of the skyllamycin biosynthetic gene cluster in *Streptomyces* sp. Acta 2897. Genes are color-coded according to their putative functions.

domain activation specificity was done using NRPSpredictor software (http://www-ab.informatik.uni-tuebingen.de/software/NRPSpredictor).[23] The sequence has been deposited in GenBank under accession number JF430460.

Generation of Gene Inactivation Mutants. In order to inactivate the putative NRPS gene *sky*31, an internal fragment A1 was amplified using the primers A1_fw and A1_re (Supporting Information) and cloned into vector pDrive where it was excised using *Bam*HI and *Hin*dIII and cloned into the *Bam*HI/*Hin*dIII restriction sites of pK18mob2, resulting in pK18_A1. The inactivation construct was transferred into *E. coli* ET12567/pUZ8002 and conjugated into *Streptomyces* sp. Acta 2897 spores following literature protocols.[17] Exconjugants were selected on MS plates[17] containing apramycin. The correct chromosomal integration was confirmed by Southern blot experiments on genomic DNA digested with *Blp*I using the DIG-labeled hybridization probe A1 (Supporting Information).

In order to inactivate *sky17*, which encodes a putative β-ketosynthase, an internal fragment of *sky17* was amplified using primers KS_fw and KS_re and cloned into the *Xba*I/*Hin*dIII restriction sites of pK18mob2, yielding pK18_KS. Following the transformation into *E. coli* ET12567/pUZ8002, pK18_KS was conjugated into the spores of *Streptomyces* sp. Acta 2897. The correct chromosomal integration was checked by Southern hybridization of *Pst*I-digested genomic DNA from the obtained mutant strains using the amplified internal fragment as probe.

Generation of In-Frame Gene Deletions. In-frame gene deletions were performed following the REDIRECT protocol.[16] The template plasmid pEP, which harbors the genes of interest, was created by cloning the 20.8 kb fragment obtained after *Nsi*I and *Xba*I digestion of cosmid 1J22 into the *Pst*I, producing *Nsi*I-compatible overhangs, and *Xba*I restriction sites of pK18mob2. The streptomycin resistance gene *aadA* from pIJ778 was amplified using primers that contained additional *Spe*I restriction sites and utilized to replace target genes on vector pEP by λ-Red mediated recombination (all primers used are listed in the Supporting Information). Restriction of mutant plasmids with *Spe*I and subsequent religation led to the loss of *aadA*. The resulting pEP derivatives were transferred into *Streptomyces* sp. Acta 2897 by spore conjugation from *E. coli* ET12567/pUZ8002. Exconjugants were grown on solid apramycin containing MS medium to select for the chromosomal integration of the inactivation constructs. To achieve a loss of the target gene from the chromosome, the exconjugants were replica-plated three times onto antibiotic-free MS plates before harvesting and separating the spores of the mutants on MS plates. Single colonies were again replica-plated onto apramycin-containing MS plates and onto MS plates without antibiotic. Clones, sensitive to apramycin, were tested for target gene deletion by colony PCR. Successful in-frame gene deletion was confirmed by amplification and sequencing of the deleted target region from genomic DNA.

RESULTS

Identification and Organization of the Skyllamycin (*sky*) Cluster. A cosmid library of the *Streptomyces* sp. Acta 2897 genome was screened with probes for NRPS generated from genomic DNA of *Streptomyces* sp. Acta 2897 with degenerated primers derived from conserved core motifs of non-ribosomal peptide synthetases.[19,20] Assuming that the non-proteinogenic β-methylaspartate found in the skyllamycin A backbone is formed by a glutamate mutase, the cosmid library was screened with an additional specific probe generated with primers derived from conserved motifs of glutamate mutases.[19,24]

As a result of the screening for NRPS and glutamate mutase positive cosmids, the cosmid 1J22 was identified, fully sequenced, and used as a starting point for subsequent chromosome walking. In total, a DNA region of over 86 kbp spanning five overlapping cosmids was sequenced. Bioinformatic analysis revealed 49 ORFs whose putative functions were assigned using BLAST searches (Figure 2, Table 1).[21]

To prove the involvement of the sequenced region in skyllamycin biosynthesis, disruption mutagenesis was performed. A 640 bp fragment of the NRPS gene *sky31* was amplified from 1J22 and cloned into plasmid pK18_mob2. After conjugation of the resulting construct into *Streptomyces* sp. Acta 2897, an apramycin-resistant mutant with correct disruption of *sky31* was obtained. Analysis of the culture extractions by HPLC-ESI-MS confirmed that this mutant was unable to synthesize skyllamycin peptides (Supporting Information).

The array of genes in the skyllamycin biosynthetic gene cluster can be subdivided into three distinct regions (Figure 2). The 5′ region mainly contains genes with sequence similarity to genes

Table 1. Deduced Functions of ORFs in the Skyllamycin Biosynthetic Gene Cluster

ORF	amino acids	predicted function in skyllamycin biosynthesis	closest protein similarity	identity/ similarity [%]	reference
1	137		putative SMR-type multidrug efflux transporter *Streptomyces griseus* subsp. *griseus* NBRC 13350	89/92	YP_001824901.1
2	106		putative SMR-type multidrug efflux transporter *Streptomyces griseus* subsp. *griseus* NBRC 13350	96/99	YP_001824902.1
3	159		cytosine/adenosine deaminase *Streptosporangium roseum* DSM 43021	53/70	ZP_04473622.1
4	372	oxidoreductase	NADH:flavin oxidoreductase, *Mycobacterium smegmatis* str. MC2 155	59/69	YP_887288.1
5	218	isomerase	predicted dithiol−disulfide isomerase *Streptosporangium roseum* DSM 43021	58/70	ZP_04473623.1
6	205	N-acetyltransferase	putative acetyltransferase *Streptomyces griseus* subsp. *griseus* NBRC 13350	91/95	YP_001824903.1
7	135		hypothetical protein SGR_3392 *Streptomyces griseus* subsp. *griseus* NBRC 13350	88/92	YP_001824904.1
8	278		DNA-binding protein, *Streptomyces* sp. Mg1	59/76	ZP_05002075.1
9	78		hypothetical protein SrosN15_18654 *Streptomyces roseosporus* NRRL 15998	88/94	ZP_04694957.1
10	458		integrin-like protein, *Streptomyces roseosporus* NRRL 15998	75/83	ZP_04694954.1
11	527	carboxyltransferase	ChlJ, *Streptomyces antibioticus*	88/93	AAZ77684.1
12	347		isoprenyl diphosphate synthase *Streptomyces pristinaespiralis* ATCC 25486	44/61	ZP_05012387.1
13	305	ACP-acyltransferase	ACP-*S*-malonyltransferase, *Anoxybacillus flavithermus* WK1	38/57	YP_002316119.1
14	654		putative ABC1 family protein, *Streptomyces* sp. AA4	52/66	ZP_05478782.1
15	73	MbtH-like protein	MbtH-like protein *Saccharopolyspora erythraea* NRRL 2338	71/84	YP_001106478.1
16	84	ACP	acyl carrier protein *Streptomyces hygroscopicus* ATCC 53653	70/81	ZP_05517490.1
17	418	β-ketosynthase II	putative 3-oxoacyl-ACP synthase II *Streptomyces ghanaensis* ATCC 14672	72/84	ZP_04685000.1
18	377	β-ketosynthase II	3-oxoacyl-ACP synthase II *Streptomyces hygroscopicus* ATCC 53653	56/70	ZP_05517492.1
19	320	β-ketosynthase I	3-oxoacyl-ACP synthase I, *Streptomyces* sp. C	45/57	ZP_05511356.1
20	272	hydrolase	hydrolase, *Streptomyces avermitilis* MA-4680	46/57	NP_824839.1
21	346	thioesterase	hypothetical protein SgriT_25948, *Streptomyces griseoflavus* Tu4000	57/73	ZP_05541594.1
22	367	β-ketosynthase I	3-oxoacyl-ACP synthase I *Streptomyces avermitilis* MA-4680	45/56	NP_824834.1
23	82	ACP	acyl carrier protein *Streptomyces ghanaensis* ATCC 14672	61/75	ZP_04685010.1
24	129	dehydratase	putative dehydratase hotdog fold *Streptomyces griseus* subsp. *griseus* NBRC 13350	58/66	YP_001824760.1
25	162	dehydratase	putative 3-hydroxyacyl-ACP dehydratase *Streptomyces ghanaensis* ATCC 14672	54/70	ZP_04685012.1
26	248	reductase	SimJ2, *Streptomyces antibioticus*	59/75	AAG34189.1
27	236	isomerase	putative isomerase, *Streptomyces griseoflavus* Tu4000	60/72	ZP_05541595.1
28	555	oxidoreductase	phytoene dehydrogenase or related enzyme *Streptomyces* sp. C	61/71	ZP_05508654.1
29	4747	NRPS	PstC, *Actinoplanes friuliensis*	43/57	CAM56770
30	4728	NRPS	amino acid adenylation *Streptomyces ghanaensis* ATCC 14672	48/59	ZP_04685019.1
31	3919	NRPS	non-ribosomal peptide synthetase *Rhodococcus jostii* RHA1	44/59	YP_700135.1
32	423	cytochrome P450 monooxygenase	NovI, *Streptomyces caeruleus*	48/66	AAF67502.1
33	257	type II thioesterase	thioesterase TEII family, *Streptomyces bikiniensis*	47/61	AAS79476.1
34	173		hypothetical protein bmyco0003_44050 *Bacillus mycoides* Rock3−17	36/57	ZP_04159424.1
35	325	ABC transporter	daunorubicin resistance ABC transporter ATP-binding subunit, *Sphaerobacter thermophilus* DSM 20745	59/71	ZP_04495100.1
36	262	ABC transporter	ABC-type multidrug transport system, permease component, *Cellulomonas flavigena* DSM 20109	41/67	ZP_04365397.1
37	342	*O*-methyltransferase	QbsL, *Pseudomonas fluorescens*	28/45	AAL65279.1
38	264	ABC transporter	ABC-2 type transporter *Catenulispora acidiphila* DSM 44928	31/55	YP_003111443.1
39	356	flavin-dependent monooxygenase	MtaG, *Stigmatella aurantiaca* DW4/3−1	51/68	AAF19815.1
40	188	flavin reductase	KtzS, *Kutzneria* sp. 744	46/59	ABV56599.1
41	438	glutamate mutase subunit B	glutamate mutase subunit B, *Actinoplanes friuliensis*	59/70	CAD32908.1
42	156	glutamate mutase subunit A	glutamate mutase subunit A, *Actinoplanes friuliensis*	50/67	CAD32909.1
43	243	regulation	AreB protein, *Streptomyces clavuligerus* ATCC 27064	59/73	CAL81530.1
44	646	regulation	putative AfsR-like transcriptional regulator *Streptomyces ghanaensis* ATCC 14672	46/57	ZP_04685028.1
45	225	regulation	AreB protein, *Streptomyces* sp. SPB74	53/67	ZP_04994999.1
46	279	regulation	LuxR family transcriptional regulator *Streptomyces griseus* subsp. *griseus* NBRC 13350	56/70	YP_001824796.1
47	263	regulation	LuxR family transcriptional regulator *Streptomyces ghanaensis* ATCC 14672	48/62	ZP_04684995.1
48	381		hypothetical protein SrosN15_18619 *Streptomyces roseosporus* NRRL 15998	80/83	ZP_04694950.1
49	83	DNA polymerase	DNA polymerase III subunit beta *Streptomyces roseosporus* NRRL 15998	88/91	ZP_04694949.1

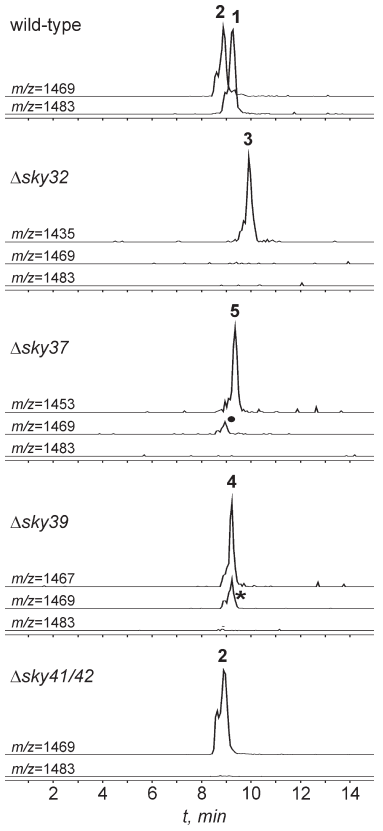

Figure 3. HPLC-ESI-MS analysis of skyllamycin production in culture extracts of the wild-type and mutant strains of *Streptomyces* sp. Acta 2897. Extracted ion chromatograms are shown for naturally occurring skyllamycin A (**1**, $[M + H]^+ = 1483$) and B (**2**, $[M + H]^+ = 1469$) and derivatives identified in each mutant strain. The peak marked with ● represents an unknown compound, whereas the peak marked with * indicates interference of the isotopic pattern of **4**.

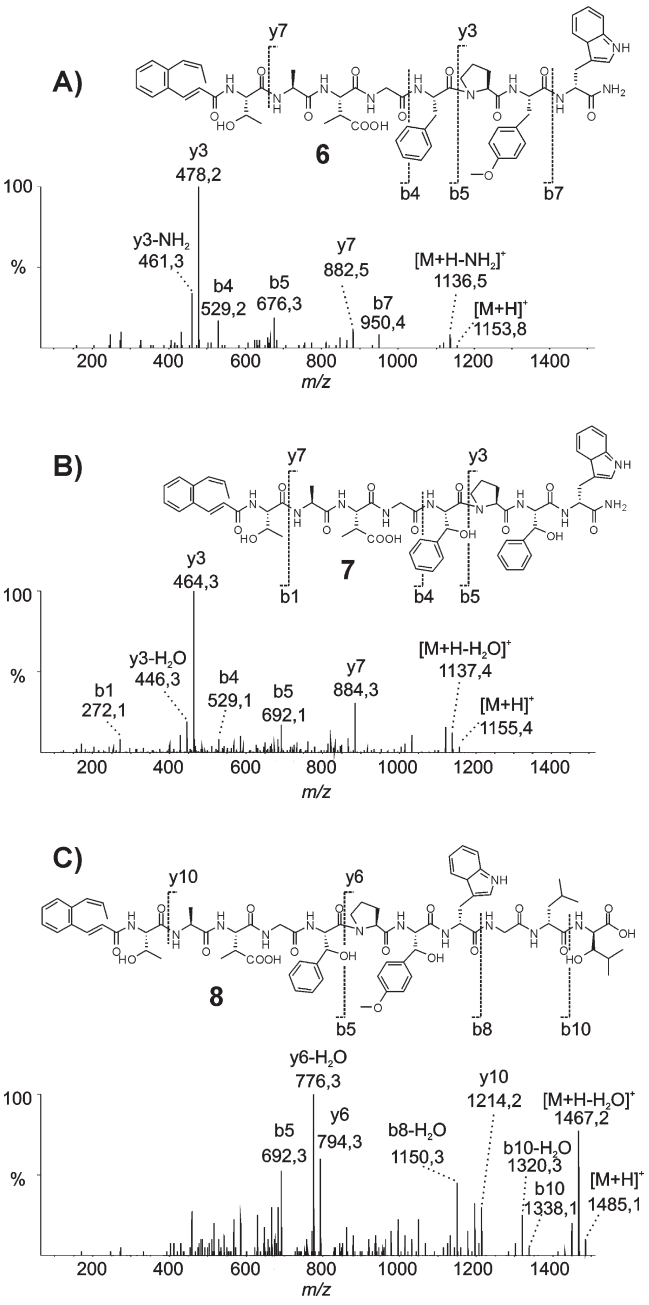

Figure 4. Structural assignment of skyllamycin derivatives. HPLC-ESI-MS/MS fragmentation of (A) **6** after hydrolysis of **3**, (B) **7** after hydrolysis of **5**, and (C) **8** after hydrolysis of **4**.

involved in bacterial fatty acid biosynthesis. The center region of the cluster is made up of three large non-ribosomal peptide synthetases (*sky29*−*sky31*) followed by genes (*sky32*−*sky48*) coding for tailoring enzymes, transporters, and regulators.

The probable downstream boundary of the *sky* cluster is marked by *orf49*, whose gene product resembles a DNA polymerase III that is most likely involved in primary metabolism. The location of the 5′-boundary is not clearly predictable, as the ORFs in this region have homologies to efflux transporters and might be involved in self-resistance of the host to skyllamycin (*sky1*, *sky2*).

Hydroxylation Reactions during the Skyllamycin Biosynthesis. Skyllamycin A and B contain three β-hydroxylated amino acids ($^5\beta$-OH-Phe, $^7\beta$-OH-*O*-Me-Tyr, $^{11}\beta$-OH-Leu) and an unusual α-hydroxylated glycyl residue ($^9\alpha$-OH-Gly). The putative gene product of *sky32* shows 48% sequence identity to the cytochrome P450 monooxygenase NovI, a tyrosine β-hydroxylating enzyme involved in the biosynthesis of the coumarin antibiotic novobiocin.[25] In order to investigate the function of Sky32, a Δ*sky32* mutant strain was constructed using in-frame gene deletion methods and was tested for skyllamycin production by subsequent HPLC-ESI-MS analysis of culture filtrates. While the wild-type peptides A (**1**) and B (**2**) were not produced in the mutant strain, a peptide **3** with a molecular mass $[M + H]^+$ at *m*/*z* of 1435 and increased hydrophobicity (t_R = 9.92 min) compared to skyllamycin A (**1**, $[M + H]^+ = 1483$, t_R = 9.23 min) was identified (Figure 3). Hydrolysis of **3** for HPLC-ESI-MS/MS analysis yielded **6**, and the fragmentation pattern (Figure 4) confirmed that the structure of the identified peptide correlated to skyllamycin A. However, the mass shift of 48 Da resulted from loss of all three β-hydroxy groups, allowing the functional assignment of Sky32 as a β-hydroxylase. A skyllamycin B derivative lacking β-hydroxylated amino acid residues was not identified in the mutant culture extracts.

The gene product of *sky39* belongs to the bacterial luciferase family of two-component flavin-dependent monooxygenases and shows 51% sequence identity to the glycine hydroxylating MonoOX domain of MtaG, an NRPS protein that is involved in

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myxothiazol biosynthesis in *Stigmatella aurantiaca* DW4/3-1.[26] In-frame deletion of *sky39* in *Streptomyces* sp. Acta 2897 led to the identification of the peptide derivative **4** ($[M + H]^+$ = 1467), with a 16 Da lower mass compared to skyllamycin A (Figure 3).

The hydrolytic ring-opening reaction performed prior to MS/MS experiments provided an initial indication that this mass shift occurs because of the loss of α-hydroxylation at the glycyl residue. Under basic conditions, skyllamycins A and B undergo two ring cleavages at ^{9}α-OH-Gly and the lactone bond (^{1}Thr-^{11}β-OH-Leu), while the peptide **4** produced by the Δ*sky39* mutant could only be linearized at the lactone bridge (**8**, Figure 4). HPLC-ESI-MS/MS experiments clearly showed the presence of Gly instead of α-hydroxyglycine in **4**, thus revealing Sky39 as the α-hydroxylase. The reaction mechanism catalyzed by Sky39 and the configuration of the introduced stereocenter are still under investigation. Located next to *sky39*, *sky40* encodes a putative flavin mononucleotide reductase (46% identity with KtzS in *Kutzneria* sp. 744)[27] that presumably regenerates the FMN cofactor needed for the α-hydroxylation reaction.

Biosynthesis of *β*-Methylaspartate. The gene products of *sky41* and *sky42* show significant homology to the glutamate mutase subunits VinI and VinH, respectively, of *Streptomyces halstedii*[24] and to GlmB and GlmA, respectively, of *Actinoplanes friuliensis* glutamate mutase.[19] First described for the glutamate fermentation pathway in *Clostridium* spp., these adenosylcobalamin-dependent enzymes catalyze the reversible conversion of (2*S*)-glutamate to (2*S*,3*S*)-3-methylaspartate.[28] Sky41 resembles the large subunit, while Sky42 aligns with the small, cobalamin-binding subunit of the heteromeric glutamate mutase.

Skyllamycins A and B, which are produced in similar amounts by *Streptomyces* sp. Acta 2897, differ structurally solely in that a β-methylaspartate is replaced by an aspartate in skyllamycin B. To study the role of the glutamate mutase in skyllamycin biosynthesis, both genes *sky41* and *sky42* were deleted together in-frame. Culture extracts of the resulting mutant strains were analyzed by HPLC-ESI-MS analysis, revealing a loss of skyllamycin A production, while the production of skyllamycin B remained nearly unaffected (Figure 3).

Biosynthesis of *O*-Methyltyrosine. The C-terminal part of the *sky37* gene product possesses 45% similarity to the *S*-adenosyl-methionine (SAM)-dependent *O*-methyltransferase domain of QbsL from *Pseudomonas fluorescens* ATCC 17400.[29] This enzyme catalyzes the *O*-methylation of xanthurenic acid during quinolobactin biosynthesis, therefore supporting the proposition that Sky37 plays a role in *O*-methyltyrosine formation.

To verify the proposed function of Sky37, the gene was deleted in-frame, resulting in the mutant *Streptomyces* sp. Acta 2897 Δ*orf37*. No production of skyllamycins was observed in this mutant strain. Instead, a molecular mass with an $[M + H]^+$ at *m*/*z* 1453 (Δ*m* = 30 Da to **1**) was detected in the mutant culture extracts, indicating the production of peptide derivative **5** lacking a methoxy group (Figure 3).

Analysis of the fragmentation pattern of **7**, obtained by hydrolysis of **5**, confirmed the absence of ^{7}β-hydroxy-*O*-methyltyrosine, suggesting the presence of β-hydroxyphenylalanine in the skyllamycin A derivative peptide **5** (Figure 4). This finding is supported by the investigation of the non-ribosomal code of the binding pocket conferring amino acid specificity of module 7 A domain.[23,30,31] The conserved motif DAWNIAAVCK indicates a substrate specificity for phenylalanine rather than for tyrosine.

To check whether the production of skyllamycins can be restored, the Δ*sky37* mutant strain was supplemented with *O*-methyltyrosine. Extracts analyzed after 5 days of culture growth contained both skyllamycin A and B, leading to the conclusion that tyrosine is *O*-methylated as the free amino acid prior to its activation by the A domain of module 7 in Sky30. Interestingly, the deletion of *sky37* seems not to interfere with the β-hydroxylation catalyzed by Sky32, since β-hydroxyphenylalanine was found in the hydrolysis product of **5**, as demonstrated by MS/MS analysis (**7**, Figure 4).

Synthesis of the N-Terminal Cinnamoyl Residue. The cinnamoyl residue attached to Thr of the skyllamycin backbone is composed of an 1,2-dialkylated aromatic ring that lacks, in contrast to aromatic polyketides, oxygen moieties at or near the aromatic ring.[32] Hence, the C_{12}-polyene **17** (Figure 5) is the postulated precursor synthesized by those enzymes of the *sky* cluster showing homology to the bacterial type II fatty acid synthesis pathway.

Malonyl-ACP **11** as a building block is likely to be generated by carboxylation of acetyl-CoA **9** to malonyl-CoA **10** by the putative carboxyltransferase Sky11 and the subsequent transacylation to malonyl-ACP **11** by the ACP-acyltransferase homologue Sky13 utilizing either acyl carrier protein (ACP) homologue Sky16 or Sky23.

The first step in fatty acid synthesis leads to formation of acetoacetyl-ACP **13** by the condensation of malonyl-ACP with acetyl-CoA by a β-ketoacyl-ACP synthase III. Since this enzyme is missing in the sequenced *sky* cluster, it could be acquired from primary metabolism. Alternatively, it could stem from the initiation reaction that proceeds via decarboxylation of malonyl-ACP **11** to acetyl-ACP **12** with subsequent condensation of **11** and **12** to acetoacetyl-ACP **13** (Figure 5). This reaction is catalyzed by β-ketosynthases encountered in the biosynthesis of most type II polyketides and as an alternative route in the initiation of fatty acid biosynthesis.[33,34]

All ketosynthases present in the *sky* cluster, Sky17, Sky18, Sky19, and Sky22, show poor sequence homology to β-ketoacyl-ACP synthases from fatty acid metabolism or to type II polyketide synthases. The closest known homologues of Sky17 and Sky19 are AuaC (38% sequence identity) and AuaD (28% sequence identity), which are type II PKS ketosynthases from the aurachin producer *Stigmatella aurantiaca* Sg a15.[35] Sequence analysis revealed that only the N-terminal regions of Sky19 and Sky22 show homology to β-ketoacyl-ACP synthases I, suggesting that they might be inactive. The involvement of the putative β-ketoacyl-ACP synthase II Sky17 in skyllamycin biosynthesis was demonstrated by gene inactivation. *Streptomyces* sp. Acta 2897 mutant strain with disrupted *sky17* was not able to produce skyllamycin peptides (Supporting Information).

In contrast to most type II PKS systems, the β-ketoacyl-ACP intermediates **13** and **16** (Figure 5) are likely to be reduced by the β-ketoacyl-ACP reductase homologue Sky26 to **14** and afterward by β-ketoacyl-ACP dehydratase homologues Sky24 and Sky25 to a putative enoyl-ACP intermediate **15** in skyllamycin biosynthesis. Further reduction of enoyl-ACP during fatty acid biosynthesis is catalyzed by an enoyl-ACP reductase[36,37] that is, however, not present in the *sky* cluster. We assume that enoyl-ACP **15** could be used as substrate for the β-ketoacyl-ACP synthases for subsequent condensation with malonyl-ACP **11** to build up the C_{12}-polyene intermediate **17**.

Aromatic ring formation in such a polyene precursor could proceed analogously to the biomimetic synthesis of pseudorubrenoic acid A by 6π-electrocyclization.[32] Hence, the ring closure can only occur if the C_{12}-polyene shows a specific

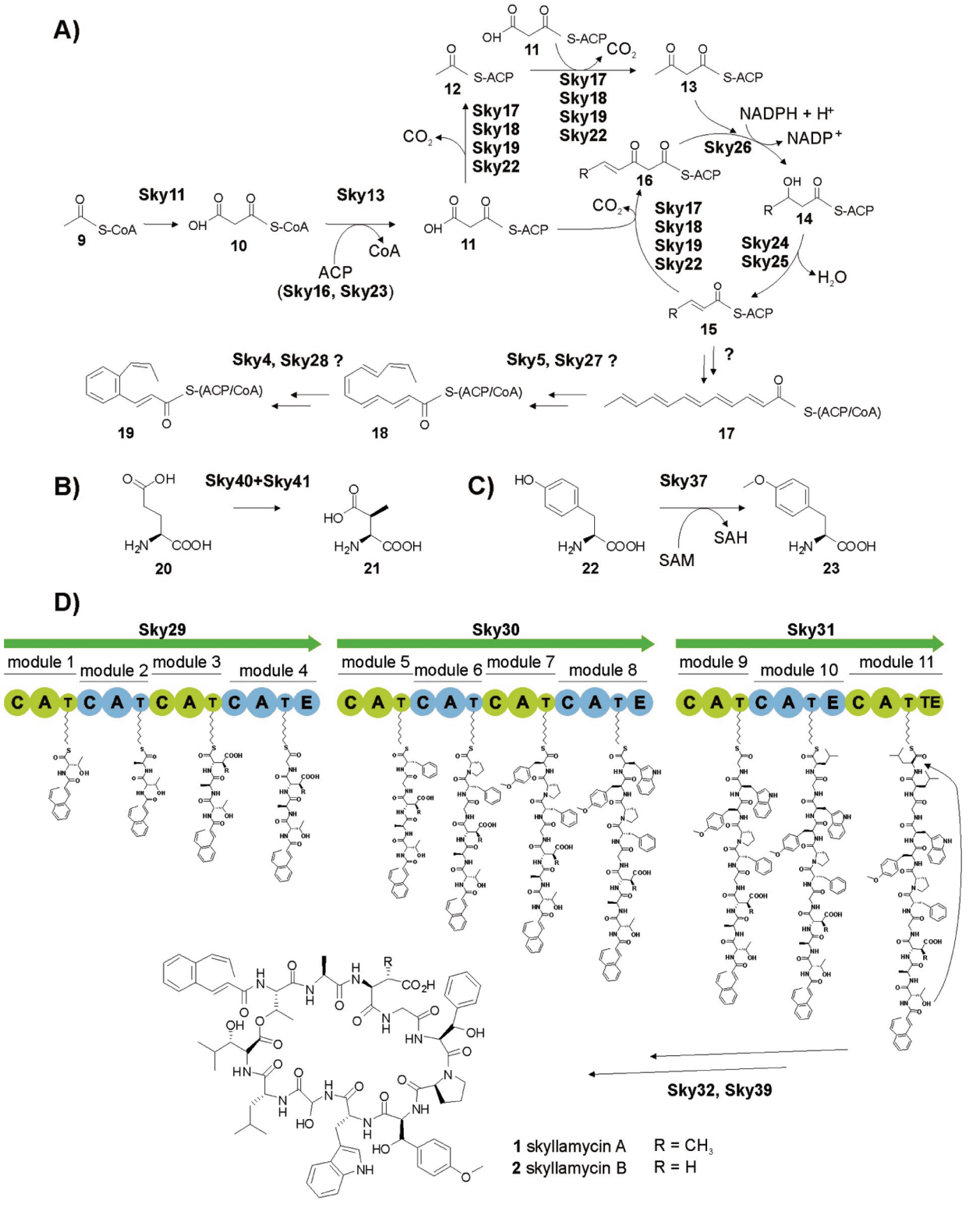

Figure 5. Predicted pathway of skyllamycin biosynthesis. (A) The cinnamoyl moiety is putatively formed via the C_{12}-polyene **17** as intermediate that then undergoes an aromatic cyclization reaction. Methylated building blocks are synthesized by (B) the two component glutamate mutase (Sky41/Sky42) that forms β-methylaspartate from Glu and (C) the methyltransferase Sky37 that forms *O*-methyltyrosine from Tyr. (D) The skyllamycin peptide chain is built up of the three non-ribosomal peptide synthetases Sky29, Sky30, and Sky31. Further modification by the α- and β-hydroxylases Sky39 and Sky32, respectively, are most likely to occur during chain elongation prior to the release of matured peptide.

(4*E*,6*Z*,8*E*)-configuration, bringing the carbon atoms within the required distance for cyclization. This prerequisite configuration, together with the 10*Z*-configuration, could possibly be introduced in **17** by the putative gene products of *sky5* and *sky27*.

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These gene products show homology to glutathione-dependent enzymes of the 2-hydroxychromene-2-carboxylate isomerase protein family encountered in the naphtalene degradation pathway in *Pseudomonas* sp. TA-2.[38]

Formation of the benzene ring, involving ring closure between the carbon atoms 4 and 9 in **18** followed by a dehydrogenation to obtain the aromatic system, could be catalyzed by either the putative oxidoreductase Sky4 or phytoene dehydrogenase-like Sky28. Alternatively, both enzymes could be involved.

It remains unclear whether the putative C_{12}-polyene precursor **17** is hydrolyzed from the ACP after its synthesis or processed in an ACP-bound state. Sky20, resembling a putative hydrolase, or Sky21, a protein not showing homology to known enzymes but possessing a hot-dog fold structure common to authentic thioesterases, might play a role in cleavage of the acyl-ACP.

Synthesis of the Skyllamycin Peptide Backbone. The three NRPSs Sky29, Sky30, and Sky31 have been assigned to the synthesis of the peptide core of skyllamycin A and B. Together they contain 11 modules with each module consisting of at least the minimal set of NRPS domains, i.e., the condensation (C), adenylation (A), and thiolation (T) domains. Prediction of amino acid activation specificity of A domains using the nonribosomal code[23,30,31] revealed that the peptide synthesis starts with Thr at module 1 of Sky29 and terminates with Leu at module 11 of Sky31 (Figure 5).

The initiation module contains an N-terminal C domain showing sequence identity to the N-terminal C domains of the NRPSs ACMSII (43%) of *Streptomyces chrysomallus*,[39] MxcG (41%) of *Stigmatella aurantiaca* Sg a15,[40] and DhbF (40%) of *Bacillus subtilis*[41] and is suggested to be involved in the coupling of the cinnamoyl moiety to module 1-bound ^{1}Thr.

Additional E domains can be found in all three NRPSs of the *sky* cluster, downstream of the C-A-T domain motifs of modules 4, 8, and 10. The E domains of modules 8 and 10 are responsible for the incorporation of the (*R*)-configured amino acids Trp and Leu, respectively. Interestingly, module 4 incorporates the achiral amino acid Gly into the peptide chain and does not require an epimerization domain. However, the E domain of module 4 is ~100 amino acids shorter in length, and its core motifs E2, E4, E5, and E7[42] are not well conserved. Most likely, this E domain is inactive due to a mutation of the catalytically important His in the second core motif HHXXXDGVSW to glycine (HGXXXDAESL).[42,43]

Structural elucidation and configurational assignment showed that β-hydroxyleucine, incorporated by module 11, also possesses an (*R*)-configuration although the corresponding module lacks an E domain.[3] A comparison between the A domains of modules 10 and 11 revealed that the amino acid residues responsible for A domain substrate specificity are identical for both domains (Supporting Information). Other enzymes that might be involved in amino acid racemization were not detected in the sequenced region.

Type II Thioesterase. The gene product of *sky33* shows homologies to type II thioesterases that are commonly found in NRPS and PKS gene clusters. This class of enzymes was assumed to have a proofreading function since they are able to hydrolyze misprimed ACP and T domains, thereby assuring the correct product formation.[44] An in-frame gene deletion of *sky33* led to a *Streptomyces* sp. Acta 2897 Δ*sky33* strain with only slightly decreased levels of skyllamycin A and B production, leaving the actual role of *sky33* in skyllamycin biosynthesis undefined.

Transporter and Regulators. The two genes *sky1* and *sky2* located at the 5′ end of the *sky* cluster encode putative multidrug efflux transporters. Until now, it is still elusive if they are involved in the skyllamycin biosynthetic machinery. Other genes coding for the putative ABC transporters Sky35, Sky36, and Sky38 have been investigated by simultaneous in-frame gene deletion of *sky35* and *sky36*. *Streptomyces* sp. Acta 2897 Δ*orf35/36* mutants showed only a weak production of skyllamycin peptides proving their involvement in skyllamycin biosynthesis. To check whether skyllamycins are accumulated in the cytoplasm, cells of the *Streptomyces* sp. Acta 2897 Δ*sky35/36* mutants were extracted with methanol and screened for both peptides. No accumulation of skyllamycin A or B could be detected, possibly due to a down-regulation of production.

A set of various regulators is found in the 3′-region of the *sky* cluster. The genes *sky43* and *sky45* putatively belong to the IclR family and *sky46* and *sky47* to the LuxR family of transcriptional regulators, whereas the gene product of *sky44* shows homology to the AfsR transcriptional regulator found in *Streptomyces coelicolor* A3(2).[45] To be active, AfsR is phosphorylated by a serine/threonine kinase, a function that could be carried out by the putative protein kinase Sky14. Furthermore, the putative DNA-binding proteins Sky8 and Sky48 show homologies to transcriptional regulators. These DNA-binding proteins as well as the 78 aa hypothetical protein Sky9 might be involved in skyllamycin biosynthesis. Sky9 belongs to a family of proteins containing a domain of yet unknown function (PF04149), but some of its members are suggested to take part in transcriptional regulation as well.[46]

Genes of Unknown Function. No functions in skyllamycin biosynthesis could be assigned to the hypothetical proteins ORF7, ORF9, and ORF34, to ORF3, which resembles a nucleoside deaminase, to the integrin-like protein ORF10, and to the putative prenyltransferase ORF12. The sequence of the *sky15* gene product resembles a MbtH-like protein abundant in many bacterial NRPS gene clusters.[47,48] Even though they seem to play a significant role in non-ribosomal peptide synthesis, their exact function remained unknown for a long time.[49,50] New insights into the mode of action of MbtH-like proteins were recently given by Felnagl et al. and Zhang et al., who showed that these proteins interact with the A domains of NRPSs, thereby enabling amino acid activation.[51,52] However, this observation does not apply to all A domains of an NRPS assembly line.

■ DISCUSSION

The biosynthetic pathway leading to skyllamycin A and B was investigated by cloning and sequencing the *sky* cluster from *Streptomyces* sp. Acta 2897. One precursor required for peptide synthesis is the N-terminal 2-[1-(*Z*)-propenyl]-cinnamoyl moiety bearing similarity to pseudorubrenoic acids, isolated from *Pseudomonas fluorescens*,[32] and serpentene, isolated from *Streptomyces* sp. Tü-3851.[53] Although the biosynthetic pathway of these natural products is unknown, a biosynthesis via fatty acid or polyketide pathways has been predicted for serpentene formation, based on feeding experiments with ^{13}C-labeled acetate.[53]

Aromatic substructures of precursors in secondary metabolites are often produced by type II polyketide synthases.[33] In these biosyntheses, malonyl-ACP building blocks are condensed to

polyketo-ACP intermediates, which may undergo subsequent cyclization by aldol reactions catalyzed by cyclases and aromatases. This cyclization and aromatization results in phenolic hydroxyl groups, which would have to be removed in order to yield the cinnamoyl-like structure **19**. Polyketide reduction prior to cyclization would electronically and stereoelectronically disfavor aldol condensation.[32] We therefore consider it more likely that the cinnamoyl residue of skyllamycins is formed via 6π-electrocyclization of a polyene precursor, as shown in the biomimetic synthesis of pseudorubrenoic acid by Rickards et al.[32]

The described aromatization pathway would require a C_{12}-polyene which, as there are no type I PKS enzymes present in the *sky* cluster, could be synthesized following the pattern given in Figure 5. In this model, β-ketoacyl-ACP building blocks are formed and reduced to enoyl-ACP intermediates that are subsequently elongated to yield the polyene **17** containing a conjugated double bond system.

During fatty acid biosynthesis, unsaturation can be introduced by the β-ketoacyl-ACP dehydratase FabA that is not only able to perform the dehydration reaction, but can also isomerize *E*-2-decenoyl-ACP to *Z*-3-decenoyl-ACP. A subsequent condensation with malonyl-ACP, catalyzed by the β-ketosynthase I FabB, prevents further reduction.[36] This mechanism of desaturation and isomerization can be almost excluded for the cinnamoyl residue formation since it is most efficient on C_{10} substrates[36] and would lead to an unconjugated double bond system. Therefore, the isomerization of double bonds in the cinnamoyl precursor is suggested to be performed by the putative isomerases Sky5 or Sky27.

The ketosynthases Sky17, Sky18, Sky19, and Sky22 do not share high sequence similarity to characterized bacterial fatty acid or to type II polyketide ketosynthases. The nearest known homologues of Sky17 and Sky19 are AuaC and AuaD, respectively, from *Stigmatella aurantiaca* Sg a15. These homologues represent type II ketosynthases belonging to a new group of PKSs suggested to be an evolutionary link between type II PKS and type III PKS or the fatty acid biosynthesis.[35] Sequence analysis further revealed that both β-ketosynthases II Sky17 and Sky18 possess the conserved catalytic cysteine residue. Sky19 and Sky22 not only lack the conserved C-terminal part of β-ketosynthases but also possess a mutation of the conserved glutamine requisite for the catalytic activity as chain length factor (CLF) ketosynthase in type II PKS biosynthesis.[54] Consequently, Sky19 and Sky22 might be catalytically inactive but could contribute to protein/protein interaction as already suggested for AuaD, which also lacks the conserved active site glutamine residue.[35] The mechanisms of polyene formation and aromatization in the production of the cinnamoyl precursor in skyllamycin biosynthesis are still under investigation.

After its formation, the cinnamoyl precursor must be coupled to the N-terminus of the T-domain-bound Thr at module 1 of the NRPS Sky29. Since there are two ACPs (Sky16, Sky23) present in the *sky* cluster as well as a hydrolase-like protein (Sky20), a putative thioesterase-like protein (Sky21), and a putative acetyltransferase (Sky6), it remains unclear whether the cinnamoyl transfer occurs through an acyl-CoA or an acyl-ACP intermediate assisted by one of these enzymes.

The N-terminal C domain of module 1 of Sky29 might also be involved in the attachment of the aromatic fatty acid moiety to ^{1}Thr due to its homology to the N-terminal C domain of NRPSs ACMSII, MxcG, and DhbF.[39−41] At these peptide synthetases, the aromatic building blocks 4-methyl-3-hydroxyanthranilic acid for ACMSII and 3-dihydroxybenzoic acid for MxcG and DhbF, respectively, are transferred to the amino group of the T-domain-bound amino acid residues. The involvement of starter C domains in fatty acid transfer was recently demonstrated for the biosynthesis of the lipopeptide surfactin from *Bacillus subtilis*.[11,55] In a first step, aliphatic 3-hydroxytetradecanoic acid activated as CoA thioester is transferred to the T-domain-bound glutamate on the first module of SfrAA, catalyzed by the initial condensation domain. Similar reactions are hypothesized for the biosynthesis of other lipopeptides, e.g., the calcium-dependent antibiotic (CDA) from *Streptomyces coelicolor*,[48] and therefore might also occur during skyllamycin biosynthesis.

Skyllamycin A and B contain the three (*R*)-configured amino acids ^{8}Trp, ^{10}Leu, and $^{11}\beta$-OH-Leu, but only for Trp and Leu the corresponding modules contain E domains. The identity of the binding pocket active site residues of the A domains of modules 10 and 11 suggests that (*S*)-configured Leu is the substrate for both modules, even though the active site residues are only poorly conserved among known leucine binding motifs. Epimerization to (*R*)-Leu at module 10 is carried out by the E domain, but the required enzymatic mechanism remains unknown for module 11. The incorporation of (*R*)-configured Leu by an NRPS module lacking an E domain was shown to occur in the biosynthetic pathways of type I glycopeptides like vancomycin and balhimycin.[56,57] There, the corresponding A domains accept both (*S*)- and (*R*)-configured Leu with a preference for the (*S*)-epimer, suggesting that an *in-trans* working epimerase or racemase could be responsible for epimerization of Leu.[57] In the sequenced *sky* cluster, no genes with homologies to such amino acid racemases have been found, but it might be possible that these genes are encoded elsewhere in the genome. Another possible route for (2*R*)-β-OH-Leu incorporation could be the direct activation of (*R*)-Leu by the A domain of module 11, as demonstrated for the activation of (*R*)-configured alanine in the first step of leinamycin biosynthesis.[58] Balibar et al. described that (*R*)-amino acids can also be generated at the NRPS by dual-functional condensation/epimerization domains in the upstream module.[59] However, as β-OH-Leu is incorporated at the termination module 11, an epimerization reaction by such dual-functional domains is excluded for skyllamycin biosynthesis.

Surprisingly, the glycine-activating module 4 contains an epimerization domain that is hypothesized to be inactive because of its mutated catalytic core motifs. A similar E domain can be found in the NRPS protein LgrA that takes part in linear gramicidin biosynthesis.[60] Studies with the gramicidin S synthetase of *Bacillus brevis* imply that E domains not only act in the epimerization of peptidyl residues but also fulfill important architectural bridging functions between neighboring modules on different protein chains.[11,61] Therefore, even if the module 4 E domain is catalytically inactive, it might be necessary for correct peptide assembly.

In skyllamycin biosynthesis, the two methylated non-proteinogenic amino acids β-methylaspartate **21** and *O*-methyltyrosine **23** are assumed to be synthesized prior to peptide assembly (Figure 5). Interestingly, according to our *in vivo* results from gene inactivation mutants, the corresponding A domains of modules 3 and 7 show relaxed substrate specificity. The ability of a β-methylaspartate-activating NRPS to also process Asp has already been described in friulimycin biosynthesis.[19] In contrast, friulimycins lacking β-methylaspartate were only produced to a small extent, while module 3 of Sky29 accepts both aspartate and β-methylaspartate, leading to similar yields of skyllamycin A and

B when synthesized by *Streptomyces* sp. Acta 2897. In the culture extracts of Δ*sky32* and Δ*sky37* mutant strains, skyllamycin peptide derivatives lacking β-methylaspartate were not identified, presumably due to a decreased turnover rate of peptide synthesis. This gives the glutamate mutase Sky41/Sky42 enough time to provide sufficient amounts of the methylated precursor **21**. Toki et al. described the purification only of skyllamycin A from *Streptomyces* sp. KY11784 as well as its mode of action.[2] Further work is needed to establish whether skyllamycin B is also produced by this strain and to define its role in PDGF signal inhibition.

Skyllamycin peptides are highly hydroxylated by the action of two different types of monooxygenases encoded by the biosynthetic gene cluster. One enzyme is the β-hydroxylating cytochrome P450 monooxygenase Sky32, whose homologues NovI and OxyD are involved in the biosyntheses of novobiocin[25] and type I glycopeptides like vancomycin and balhimycin,[62] respectively. In these pathways, tyrosine is the target of β-hydroxylation after it has been loaded on an A-T-containing didomain NRPS.[63] In type I glycopeptide biosynthesis, β-hydroxytyrosine is cleaved off by a thioesterase and subsequently loaded on the corresponding module of the peptide-forming NRPS.[25,62,63] Deletion of any enzyme participating in β-hydroxytyrosine biosynthesis abolishes balhimycin production, which can be bypassed upon feeding β-hydroxytyrosine.[62] In contrast, no stand-alone didomain NRPS is present in the *sky* gene cluster, and inactivation of P450 monooxygenase Sky32 led to production of skyllamycin derivative **3** with a complete absence of β-hydroxylated amino acid residues. This observation is reminiscent of the biosynthesis of A40926, a type IV glycopeptide produced by *Nonomuraea* sp. ATCC39727.[64] In this biosynthetic pathway, tyrosine is activated by the peptide-forming NRPS and suggested to be β-hydroxylated during peptide assembly.[65] Deletion of the corresponding monooxygenase ORF28 of the A40926 gene cluster led to the production of a peptide derivative that contained Tyr instead of β-hydroxytyrosine, and feeding of β-hydroxytyrosine did not restore wild-type production.[65] Interestingly, the monooxygenase ORF28, involved in A40926 biosynthesis, belongs to the group of Fe(II)/α-ketoglutarate-dependent non-heme dioxygenases,[65] which are involved in direct β-hydroxylation of soluble asparagine in lipopeptide CDA biosynthesis.[66]

Because of the absence of dehydroxy-skyllamycin derivatives in the wild-type cultures of *Streptomyces* sp. Acta 2897 and the formation of **3** in the Δ*sky32* mutants, it is most likely that Sky32 catalyzes β-hydroxylation at a T-domain-bound peptidyl chain directly at the peptide-forming NRPSs (Sky30, Sky31), as suggested for type IV glycopeptide antibiotic biosynthesis.[65] The mechanisms controlling substrate specificity of Sky32 remain unknown. It is also noteworthy that stereochemical aspects of skyllamycin hydroxylation differ significantly from previously described biosyntheses. Whereas NovI and OxyD homologues catalyze the formation of a (3*R*)-configuration of the newly formed β-hydroxy stereocenter,[25] Sky32 favors formation of the (3*S*)-configuration[3] similar to Fe(II)/α-ketoglutarate-dependent non-heme dioxygenases mentioned above.[25,62,66]

Finally, the structural feature of α-hydroxylation is highly unusual and rarely found in natural products since its hemiaminal character directs reactivity toward a facilitated hydrolysis and cleavage, releasing the peptides to an amide and a α-keto carboxylic acid, as seen for the maturation of various neuropeptide amides from insects and mammals.[67] There, the ascorbate- and Cu(II)-dependent bimodular monooxygenase PAM, consisting of the hydroxylating domain PHM and lyase domain PAL, α-hydroxylates peptides at a terminal glycine residue and catalyzes its cleavage to form the peptide amide.[67] In secondary metabolite biosyntheses, the myxothiazol precursor undergoes glycine hydroxylation and subsequent cleavage at the hydroxylation site to release the mature myxothiazol terminal amide.[26] The α-hydroxylation of an alanyl residue, putatively carried out by a cytochrome P450 monooxygenase, and a resulting cyclization to a hemiketal marks the last step in ergotamine biosynthesis.[68,69] Whereas linear peptides appear prone to hydrolytic cleavage and maturation, the α-hydroxylation of a phenylalanyl residue in thaxtomin biosynthesis,[70] catalyzed by the cytochrome P450 monooxygenase TxtC, is supposedly stabilized by ring formation.

Arguing in favor of a stabilizing effect of peptide ring formation, the α-hydroxyl group in skyllamycins is not cleaved under physiological conditions and remains stable in the mature peptide. Biosynthetically, it is introduced by Sky39, a two-component flavin-dependent monooxygenase that belongs to the group of bacterial luciferases, which differ significantly from the eukaryotic PAM enzyme. Similar hydroxylation reactions of two-component flavin-dependent monooxygenase have been previously described for the degradation of the chelating agents nitrilotriacetate and EDTA by different bacteria.[71,72] It remains unknown whether the introduction of the α-hydroxyl group occurs during peptide assembly or if it is a post-NRPS process. Because of the chemical instability of a free α-hydroxylated glycine, it is probable that the hydroxylation occurs during chain growth at the non-ribosomal peptide synthetase or after the cyclization and release of the peptide from the NRPS.

Hydroxylations during natural product biosynthesis often account for biological activity or product maturation and can be used as an attachment point for further modifications, e.g., glycosylations. The identification of a promiscuous β-hydroxylating enzyme as well as a monooxygenase introducing a stable glycyl-α-hydroxylation in skyllamycin biosynthesis increases our knowledge of the mechanism of non-ribosomal peptide hydroxylation and will give the opportunity to study these important reactions in more detail.

■ CONCLUSIONS

We have identified and sequenced the biosynthetic gene cluster responsible for the biosynthesis of the non-ribosomal peptides skyllamycin A and B. Essential enzyme functions contributing to the assembly of non-proteinogenic amino acid biosynthesis and the skyllamycin backbone were identified. Furthermore, a model of the skyllamycin biosynthesis has been proposed. Synthesis of the unusual N-terminal cinnamoyl moiety of skyllamycin most likely follows a mechanism similar to fatty acid biosynthesis, comprising an alternative pathway for aromatic ring formation. Other remarkable aspects of the skyllamycin biosynthesis include a high degree of tailoring of the peptide core, primarily involving hydroxylation reactions. The β-hydroxylations in skyllamycins are introduced by a single promiscuous P450 monooxygenase, while the α-hydroxylating monooxygenase catalyzing the stable α-hydroxylation of a glycine residue is one of the first enzymes of this type to be identified in peptide secondary metabolism. Various gene deletion mutant strains of these tailoring enzymes are found to synthesize new skyllamycin derivatives, opening the way for future mutasynthesis and combinatorial biosynthesis experiments.

■ ASSOCIATED CONTENT

Supporting Information. Primer sequences, additional experimental procedures, and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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