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Leucanicidin and Endophenasides Result from Methyl-Rhamnosylation by the Same Tailoring Enzymes in *Kitasatospora* sp. MBT66

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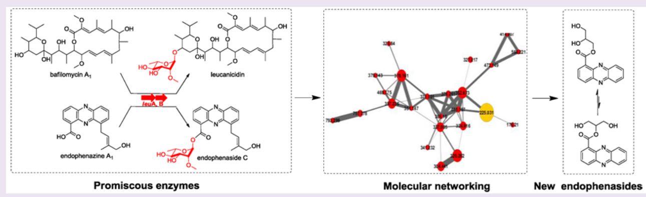
Supporting Information

ABSTRACT: The increasing bacterial multidrug resistance necessitates novel drug-discovery efforts. One way to obtain novel chemistry is glycosylation, which is prevalent in nature, with high diversity in both the sugar moieties and the targeted aglycones. *Kitasatospora sp.* MBT66 produces endophenaside antibiotics, which is a family of (methyl-)rhamnosylated phenazines. Here we show that this strain also produces the plecomacrolide leucanicidin (**1**), which is derived from bafilomycin A_1 by glycosylation with the same methyl-rhamnosyl moiety as present in the endophenasides. Immediately adjacent to the *baf* genes for bafilomycin biosynthesis lie *leuA* and *leuB*, which encode a sugar-*O*-methyltransferase and a glycosyltransferase, respectively. LeuA and LeuB are the only enzymes encoded by the genome of *Kitasatospora sp.* MBT66 that are candidates for the methyl-rhamnosylation of natural products, and mutation of *leuB* abolished glycosylation of both families of natural products. Thus, LeuA and -B mediate the post-PKS methyl-rhamnosylation of bafilomycin A_1 to leucanicidin and of phenazines to endophenasides, showing surprising promiscuity by tolerating both macrolide and phenazine skeletons as the substrates. Detailed metabolic analysis by MS/MS based molecular networking facilitated the characterization of nine novel phenazine glycosides **6–8**, **16**, and **22–26**, whereby compounds **23** and **24** represent an unprecedented tautomeric glyceride phenazine, further enriching the structural diversity of endophenasides.



The rapid increase in antimicrobial resistance poses one of the major threats to human health.[1] A particular problem with drug discovery from microbial sources is the high frequency of rediscovery of known compounds, which necessitates new approaches to replenish the antimicrobial drug pipelines.[2−5] As producers of some two-thirds of all known antibiotics and many other medically relevant natural products, actinomycetes are a major source of clinical drugs.[2,6,7] Sequencing of the genomes of actinomycetes revealed that the natural products producing potential of even the best-studied model organisms has been underestimated.[8−10] However, many of these gene clusters are poorly expressed in the laboratory.[11−14] One way of obtaining novel chemistry is by sugar-mediated tailoring, i.e., the decoration of molecules by glycosylation. Over 20% of the bacterial natural products (NPs) in the databases are glycosylated, with structurally highly diverse aglycones containing one or more glycosyl groups.[15] Glycosylation can dramatically influence the pharmacological properties of the parent scaffold and directly mediate bioactivity, such as in anthracycline, aureolic acid and enediyne antibiotics.[16] Many microbial glycosides also find their applications in agriculture, such as the insecticide avermectin.[17] Antibiotic glycan alteration (so-called glycorandomization) is a potentially powerful strategy in combating emerging bacterial resistance.[18−20]

Given the profound biological significance of glycosylation, it is important to harness the biosynthetic machinery for the formation of glycoconjugates,[21] which will pave the way for the glycodiversification of NPs through genetic engineering approaches.[22] The biosynthesis of glycosylated natural products includes (i) assembly of the aglycone, (ii) biosynthesis of an activated form of the sugar moiety, typically a nucleotide diphospho (NDP)-activated sugar, and (iii) transfer of the NDP-sugar to acceptor molecules by glycosyltransferases. Glycosylated macrolides and macrolactams represent the largest allocation in bacterial saccharidic compounds.[15] The plecomacrolides are a family of macrolides that typically feature a 16- or

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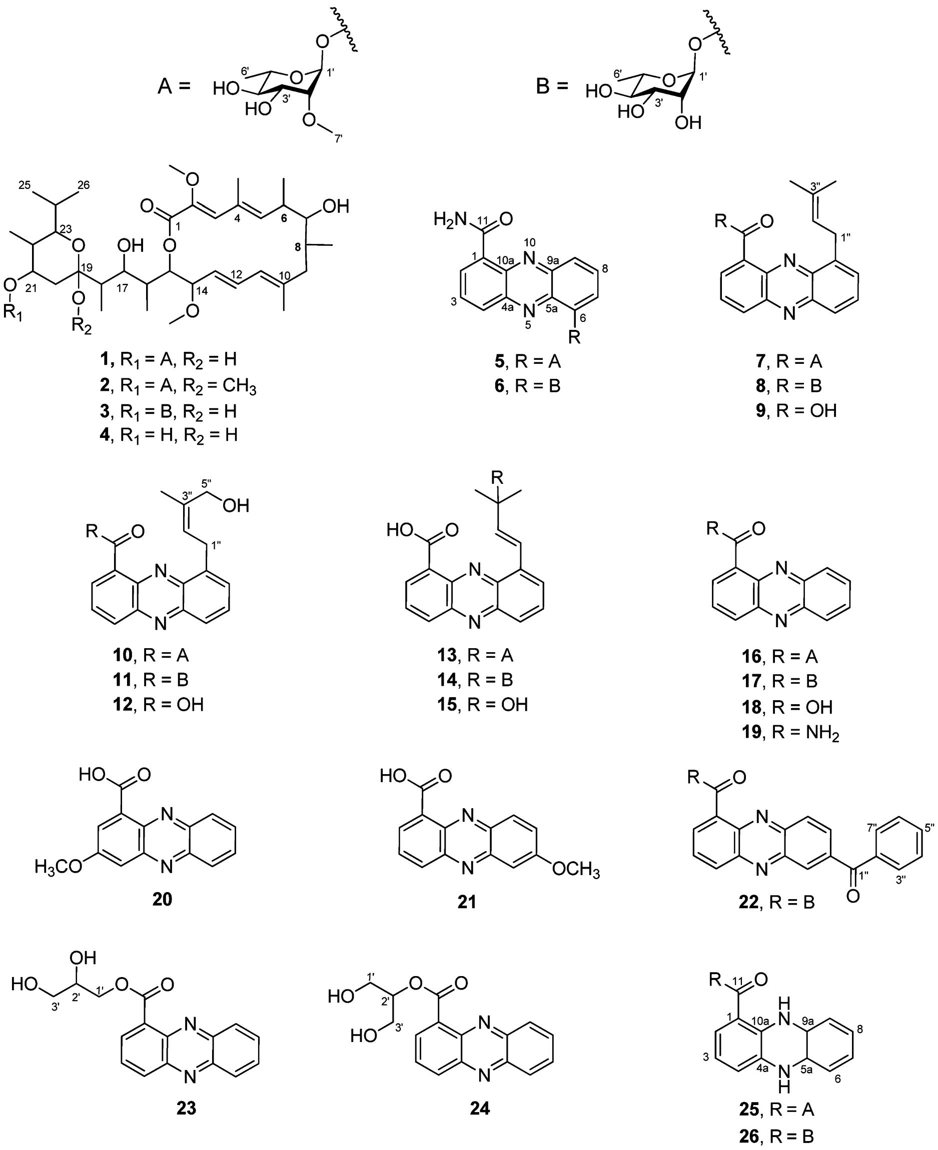

Figure 1. Glycosides from *Kitasatospora* sp. MBT66. Compounds **1**−**26** are classified into two groups according to the aglycones, namely glycosylated plecomacrolides (**1**−**4**) and phenazines (**5**−**26**), which bear the same rhamnosylation. The rhamnosyl substituents in rhamnosides are either 2′-*O*-methylated or unmethylated, except compounds **23** and **24** which are glyceride phenazine tautomers. Phenazine derivatives **6**−**8**, **16**, and **22**−**26** were previously undescribed. The ^{1}H NMR data assignments for these compounds are summarized in Table S3.

18-membered macrolactone containing two conjugated diene units connected with a six-membered hemiacetal side chain through a C_3 spacer.[23] Endowed with a biologically important intramolecular hydrogen bonding network among the lactone/C_3 linker/hemiacetal structural motif,[24,25] plecomacrolides exhibit a variety of bioactivities, including antitumor,[26] antifungal,[27] antiparasitic,[28] immunosuppressant,[29] and particularly selective vacuolar ATPase (V-ATPase) inhibitors.[23,30] Additional substituents (mostly on the secondary alcohol of the hemiacetal portion) and/or modification on the backbone largely diversified this class of antibiotics, such as balfilomycins,[31−34] concanamycins,[35] hygrolidin,[36] setamycin,[37] micromonospolide,[38] and formamicin,[39] simultaneously affording a variation of bioactivity and toxicity.[25,40] The genus *Kitasatospora* has a similar life style as *Streptomyces* and also has a rich arsenal of secondary metabolites.[41] Several plecomacrolide type compounds have been characterized from *Kitasatospora* species, such as bafilomycins A_1 and B_1 from *K. setae*,[37] bafilomycins A_1−C_1, and respective amide derivatives from *K. cheerisanensis*.[42] Bafilomycins are known as specific inhibitors of vacuolar ATPases.[30] So far, no detailed reports are available on the gene cluster organization for plecomacrolide scaffolds in *Kitasatospora* species, although the pioneering genome sequencing of *K. setae* predicted the plausible presence of the PKS genetic loci responsible for bafilomycin B_1.[41]

We previously characterized the endophenasides in *Kitasatospora sp.* MBT66, which constitute a family of novel rhamnosylated phenazines.[43] Here, we show that *Kitasatospora sp.* MBT66 also produces the bafilomycin-derived plecomacrolide antibiotic leucanicidin (**1**). The methylated form of leucanicidin, previously identified as NK155141 (**2**),[44] is not produced biosynthetically but was derived from the reaction with methanol. The biosynthetic gene cluster for leucanicidin was elucidated, which includes genes for a glycosyltransferase (LeuB) and a methyltransferase (LeuA); these likely modify both plecomacrolides and phenazines. The biosynthetic insights

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Table 1. Organization for the Leucanicidin Biosynthetic Gene Cluster of *Kitasatospora* sp. MBT66[a]

ORF	locus tag	protein	length	annotation	nearest homologue homology, protein, origin	GenPept accession	comments
ORF1	BI06_RS39075	BafAI	1033	PKS modules 1−4	91%, WP_018955924.1, *Streptomyces lohii*	N/A[b]	gapped sequence
ORF2	BI06_RS39070	BafAII	5019	PKS modules 5−7	90%, ADC79617.1, *Streptomyces lohii*	WP_043476519.1	
ORF3	BI06_RS39065	BafAIII	3966	PKS modules 8, 9	89%, ADC79618.1, *Streptomyces lohii*	WP_043476516.1	
ORF4	BI06_RS39060 BI06_RS32440	BafAIV	3453	PKS modules 10, 11	88%, ADC79619.1, *Streptomyces lohii*	N/A[b]	gapped sequence
ORF5	BI06_RS32435	BafAV	2158	PKS module 12+thioesterase	85%, ADC79620.1, *Streptomyces lohii*	N/A[b]	
ORF6	BI06_RS32430	BafB	296	glyceryl-ACP oxidase	93%, ADC79621.1, *Streptomyces lohii*	WP_043474332.1	
ORF7	BI06_RS32425	BafC	115	acyl carrier protein (ACP)	90%, ADC79622.1, *Streptomyces lohii*	WP_030397161.1	
ORF8	BI06_RS32420	BafD	371	acyl-CoA dehydrogenase	92%, WP_019761696.1, *Streptomyces* sp. Wigar10	WP_043474112.1	
ORF9	BI06_RS32415	BafE	377	glycerate ACP ligase	93%, WP_018568170.1, *Streptomyces* sp. PsTaAH-124	WP_051742398.1	
ORF10	BI06_RS32410	BafF	220	*O*-methyl transferase	90%, ADC79625.1, *Streptomyces lohii*	WP_043474109.1	
ORF11	BI06_RS32405	BafG	606	AfsR family transcriptional regulator	86%, ADC79626.1, *Streptomyces lohii*	WP_043474107.1	
ORF12	BI06_RS32400	BafH	253	TEII	93%, WP_019761700.1, *Streptomyces* sp. Wigar10	WP_030397166.1	
ORF13	BI06_RS32395		126	putative LuxR_C_-like protein	83%, ADC79628.1, *Streptomyces lohii*	WP_043474105.1	
ORF14			38	malonyl transferase	58%, ADC79629.1, *Streptomyces lohii*		
ORF15	BI06_RS32390	Leu A	430	sugar *O*-methyltransferase	59%, WP_005321729.1, *Streptomyces pristinaespiralis*	WP_051742397.1	
ORF16	BI06_RS32385	Leu B	394	glycosyl transferase	55%, WP_019074879.1, *Streptomyces* sp. R1-NS-10	WP_043474103.1	

[a]The gene cluster architecture for the biosynthesis of bafilomycin A_1 is the same as in *Streptomyces griseus* DSM 2608[48] and *Streptomyces lohii*.[47] The genome sequence of *Kitasatospora* sp. MBT66 is available at GenBank with accession number JAIY00000000, and the annotation was submitted to MIBiG with accession number BGC0001232. [b]N/A: Not available due to gapped sequence.

together with MS/MS based molecular networking allowed us to identify novel 2′-*O*-methylated and 2′-*O*-unmethylated rhamnosylated endophenasides **6**−**8**, **16**, **22**, **25**, and **26**, as well as an unprecedented tautomer consisting of glyceride phenazines **23** and **24**.

RESULTS AND DISCUSSION

Biosynthetic Pathway of Plecomacrolide Glycosides Leucanicidin and NK155141. Our previous chemical investigation of *Kitasatospora sp.* MBT66 led to the discovery of five minor rhamnosylated endophenasides A−E, which were isolated from 40% methanol eluent of macroporous resin Diaion HP-20 column chromatography.[43] Ongoing investigation into nonpolar fractions, via 80% methanol eluent, resulted in the purification of two additional compounds. Spectral data interpretation, including NMR, HRMS, and UV, showed that these two compounds were the plecomacrolide glycosides leucanicidin (**1**, Figure 1)[45] and its methylated derivative NK155141 (**2**).[44] Moreover, another known plecomacrolide glycoside, bafilomycin A_1-21-*O*-(α-L-rhamnopyranodise) (**3**),[46] was later identified in MBT66 crude extract by U(H)PLC-UV-TOF analysis, which was judged from comparison of the earlier retention time, the UV spectrum, and high resolution mass with those of leucanicidin. In view of the unusual structural scaffold and important biological properties, plecomacrolide polyketides and their genetics have been studied extensively,[47,48] because of their complex architecture and corresponding difficulty in total synthesis.[49−51]

Annotation of the genome sequence of *Kitasatospora* sp. MBT66[52] led to the identification of a biosynthetic gene cluster (BGC) for a PKS responsible for the biosynthesis of bafilomycins, the precursor of leucanicidin (Table 1). The domain organization of the PKS genes (ORF1−ORF13) is consistent with the assembly of macrolactone/C_3 linker/hemiacetal core (Figure S1) of bafilomycin B_1.[47,48] However, post-PKS tailoring components for the synthesis of leucanicidin were distinct from those for bafilomycin B_1. Genes required for installing the C_5N moiety are absent,[47,48] and instead two genes, encoding a putative 2′-*O* methyltransferase (ORF15) and a glycosyltransferase (ORF16), were located in the downstream region, which explain the structural differences between leucanicidin and bafilomycin B_1. This is a rather typical genetic configuration, as the glycosylation-associated biosynthetic genes are usually coclustered with those for aglycones in microbial genomes.[53] This strong linkage was the basis for the development of so-called glycogenomics, an MS^n-based genome-mining method for microbial glycosylated molecules.[53] Therefore, we reasoned that the macrolactone core (bafilomycin A_1, **4**) of leucanicidin was assembled by the classical type I PKS system. *baf*AI-AV encode in total 12 PKS modules, which load isobutyrate as a starter unit and subsequently incorporate 11 extender building blocks. The downstream genes *leuA* for a sugar-*O*-methyltransferase and *leuB* for a glycosyltransferase are likely responsible for post-PKS modification by installing a 2-*O*-methylated-rhamnosyl group at the 21-OH position of bafilomycin A_1[54] (Figure S1). The *leuA* and *leuB* genes are also present adjacent to the homologous *baf* gene clusters in the genomes of *Kitasatospora purpeofuscus* strain NRRL B-1817 (GenBank accession JODS01000000) and *Kitasatospora* sp. NRRL S-495 (GenBank accession JZWY01000000; both erroneously termed *Streptomyces* in GenBank) but are absent from all bafilomycin gene clusters in *Streptomyces* genomes. This suggests that phylogenetic linkage exists between the methyl-rhamnosylation and the genus *Kitasatospora*.

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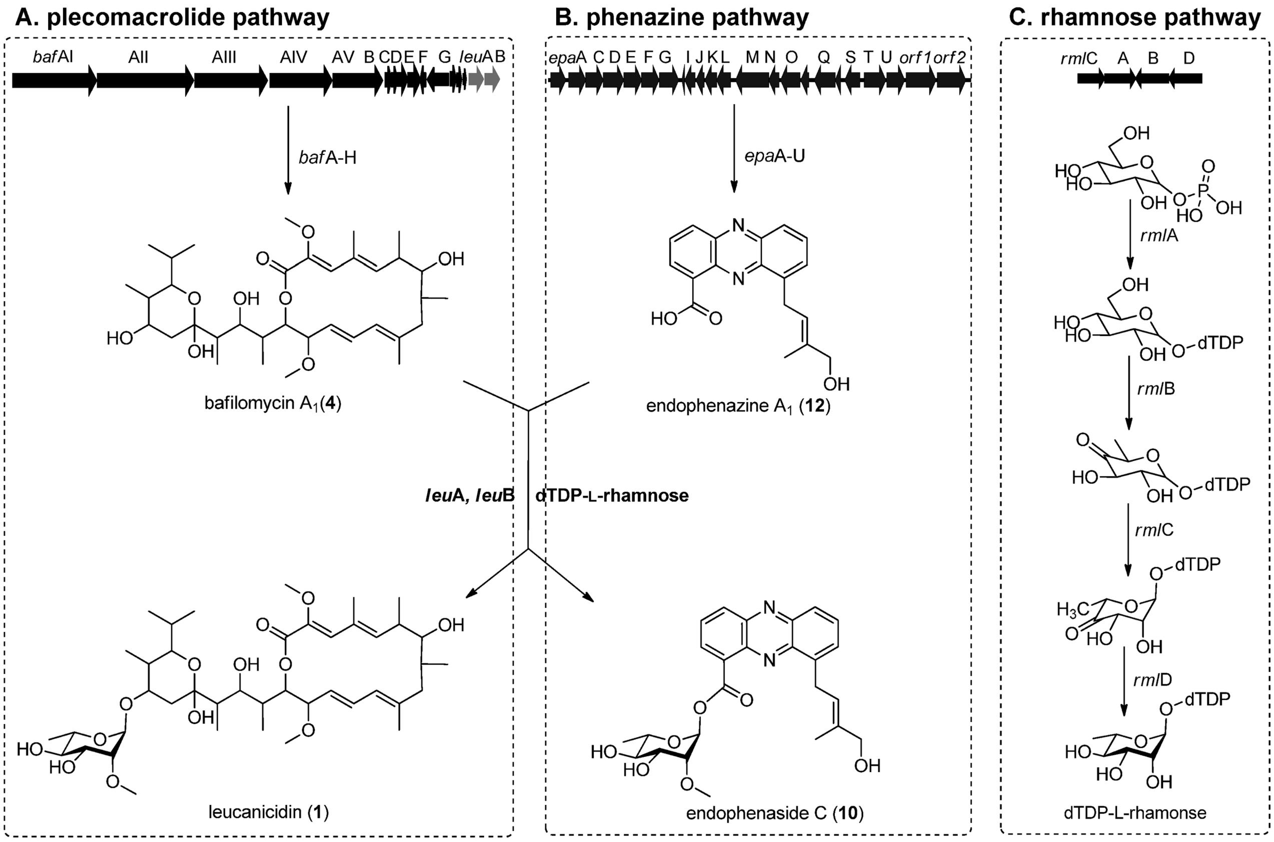

Figure 2. Promiscuous methyl-rhamnosylation of bafilomycin A_1 and endophenazine by methyltransferase LeuA and rhamnosyltransferase LeuB. Biosynthetic "crosstalk" among chromosomally distant clusters enabled *de novo* synthesis of different classes of rhamnosylated natural products. The *baf*A–H (A) and *epa*A–U (B) gene clusters are responsible for plecomacrolide and endophenazine backbone biosynthesis, respectively, while *rml*A–D (C) supply the dTDP-L-rhamnose building block for rhamnosylation. LeuA and LeuB encoded by the *leuAB* genes immediately adjacent to the *baf* cluster are the tailoring enzymes for the conversion of bafilomycin A_1 to leucanicidin and of endophenazine A_1 into endophenaside C. The type I PKS bioassembly line for the plecomacrolide backbone is detailed in Figure S1, and the shikimate pathway for endophenazine was described previously.[43]

How then is leucanicidin (**1**) methylated to generate NK155141 (**2**), and can it originate from LeuA-mediated *in vivo* enzymatic catalysis? An alternative is that **2** may have arisen from the *in vitro* nonenzymatic reaction with methyl donors during the isolation process, because cyclic hemiketal hydroxyl groups are reactive even in the presence of moderately nucleophilic reagents.[55,56] To address this, we optimized leucanicidin production by MBT66. Liquid NMMP,[57] solid MM,[57] and R5[58] were supplemented with different carbon sources, additional additives, and high alkalinity.[59,60] Varying growth conditions or the addition of chemical elicitors can be applied to activate the biosynthesis of poorly expressed natural products.[59] *N*-acetylglucosamine (GlcNAc) has previously been applied for the activation of various BGCs and acts via the metabolic inactivation of the nutrient-responsive global regulator DasR.[61−63] Indeed, growth of *Kitasatospora* sp. MBT66 on R5 agar plates with 25 mM GlcNAc effectively increased the production of leucanicidin, thus allowing ready monitoring of leucanicidin production by HPLC-UV profiling.

Crude extracts obtained from mycelia of *Kitasatospora* sp. MBT66 grown on R5 agar with 25 mM GlcNAc were dissolved in either methanol or acetonitrile without any additional catalyst. The samples were incubated at RT for 1 week and monitored by HPLC-UV. Methylated leucanicidin (NK155141) was observed exclusively in the methanol solution, whereby the leucanicidin concentration gradually decreased in favor of a time dependent increase in the level of NK155141 (Figure S2, A). However, no NK155141 was detected even after a week of incubation in acetonitrile (Figure S2, B). This *in vitro* experiment provides conclusive evidence that NK155141 is not synthesized *in vivo* but instead is an artifact resulting from the reaction of the hemiacetal hydroxyl group (19-OH) of leucanicidin with methanol.

LeuA and LeuB Display Broad Flexibility toward Their Substrates. We previously reported that the *epa* BGC of *Kitasatospora* sp. MBT66 (on the genome with Genbank accession number JAIY00000000) is responsible for the assembly of the phenazine backbone in endophenasides A–E, but the essential glycosylation genes remained unresolved.[43] The characterization of the leucanicidin biosynthetic pathway provided more insights into the rhamnosylation of the endophenazines. Leucanicidin and the endophenasides (i.e., glycosylated endophenazines) contain the same α-L-rhamnosyl substituents and coexist in the same cultures (MM + 0.5% mannitol + 1% glycerol) of *Kitasatospora* sp. MBT66. Moreover, bioinformatic analysis showed that the *leuAB* subcluster is the only locus in the entire genome where genes for a natural product-related glycosyltransferase and methyltransferase co-occur within a 20 kb distance. Because of the absence of any other genes for candidate enzymes that may catalyze this reaction, either in the phenazine BGC or elsewhere on the genome, it is likely that LeuA and LeuB not only

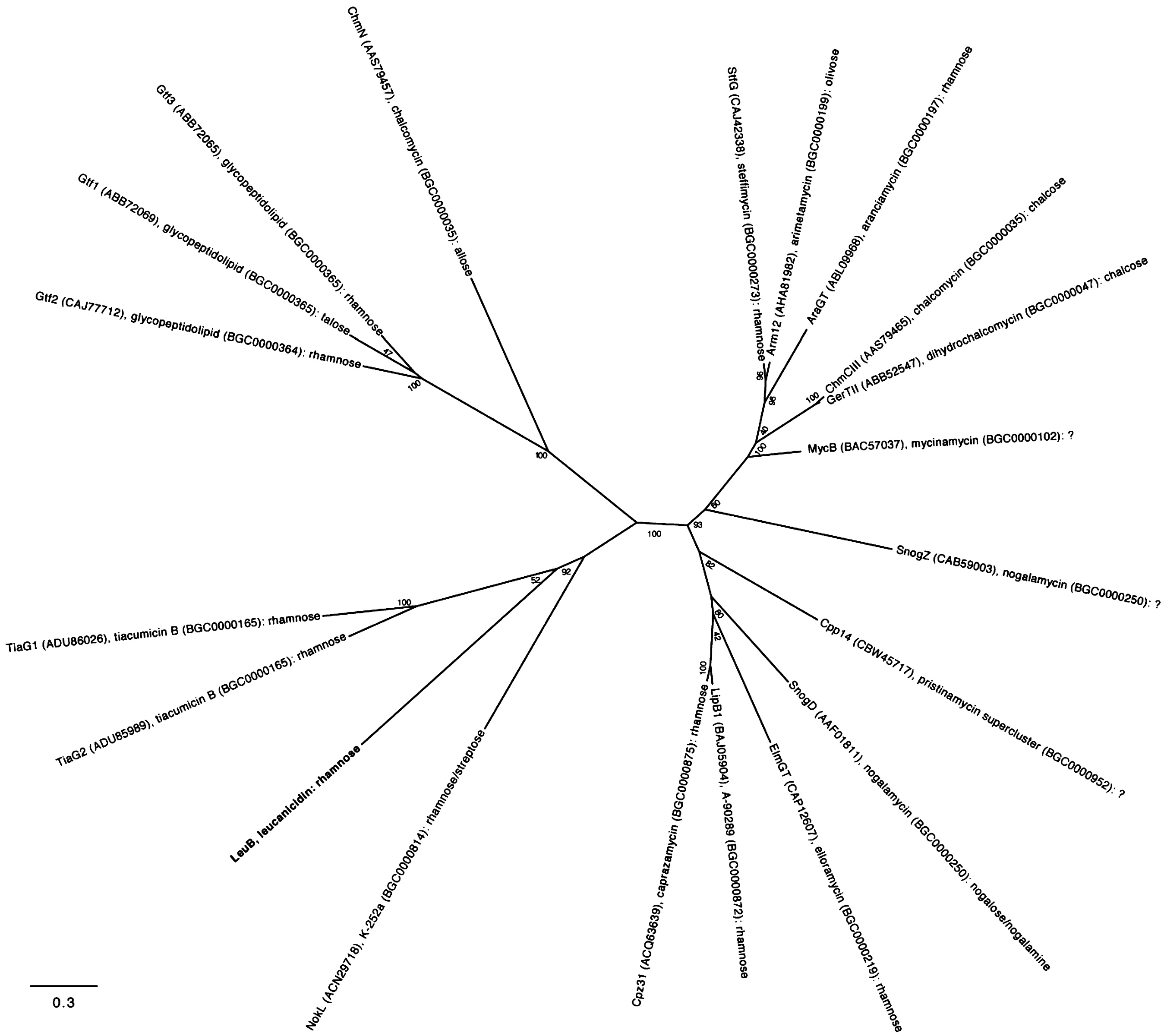

Figure 3. Unrooted maximum likelihood phylogenetic tree of glycosyltransferase LeuB and its homologues from homologous subclusters. NCBI GenPept accessions, natural product names, MIBiG BGC accessions, and substrate specificities (if known) are provided at the tips of each branch.

decorate bafilomycin A_1 to leucanicidin (Figure 2, A) but also modify endophenazines[54] into endophenasides[43] (Figure 2, B).

We first performed a detailed computational genomic analysis of the *leuAB* genes and their homologues. A MultiGeneBlast architecture search with the *leuAB* genes as a query on the full set of 1170 BGCs from the Minimum Information about a Biosynthetic Gene cluster (MIBiG) repository[64] resulted in 17 experimentally characterized BGCs that contain homologues of both *leuA* and *leuB*. All hits represented genes involved in the attachment of methylrhamnose and (at larger evolutionary distances) related deoxysugars. While a phylogenetic analysis of glycosyltransferase amino acid sequences (Figure 3) showed that the most closely related glycosyltransferases from the set (TiaG1 and TiaG2) are involved in methyl-rhamnosylation of the macrolide tiacumicin B, several other homologous *leuAB*-like subclusters are involved in the methyl-rhamnosylation of a wide range of scaffolds, including the indolocarbazole K-252a; the nucleosides A-90289 and caprazamycin; and the anthracyclines elloramycin, steffimycin, and aranciamycin. This strongly suggested that this family of glycosyltransferases (exemplified by LeuB) has great evolutionary target promiscuity.

To create a mutation in *leuB*, we made use of the CRISPR-Cas9 system that was adapted recently for use in actinomycetes.[65] For this, construct pGWS1002 was introduced into *Kitasatospora* sp. MBT66 by conjugation and ex-conjugants were selected based on their resistance to apramycin. These ex-conjugants were then propagated to select for loss of the plasmid, and the correct colonies were verified by PCR. After conjugation, single ex-conjugants were streaked onto SFM agar plates containing nalidixic acid and incubated at 30 °C for 3−5 days. Colonies were then grown in liquid TSBS for genomic isolation, followed by PCR using oligonucleotides LeuB_F-370 and LeuB_R+584 (Table S1). PCR products were digested by *Hin*dIII, and the desired frame-shift mutants were confirmed by the appearance of 401 bp and 538 bp DNA fragments (Figure S3). The obtained *leuB* frame-shift mutant and its parental strain MBT66 were grown on R5 agar plates with GlcNAc to

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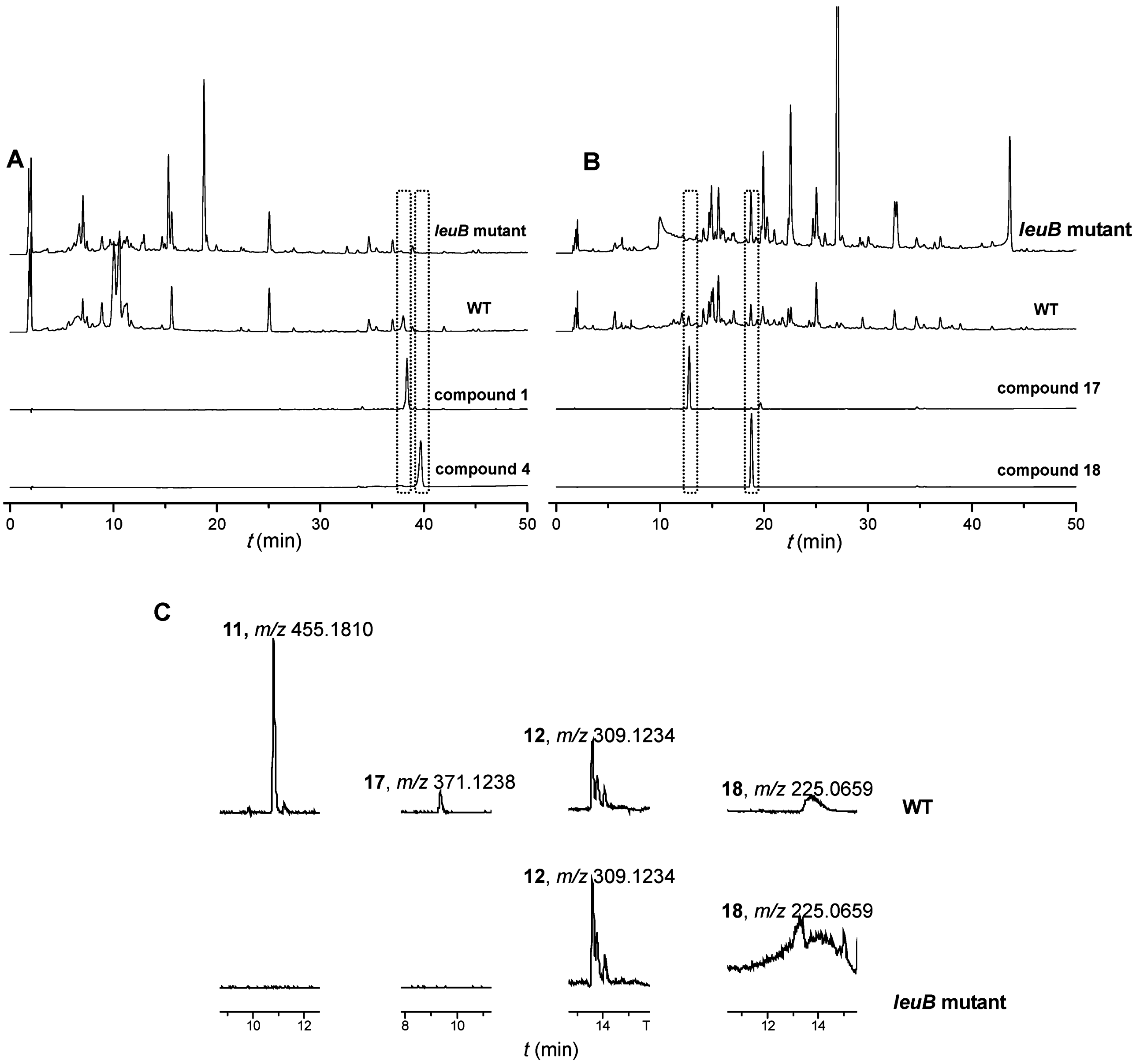

Figure 4. Leucanicidin and endophenaside production by *Kitasatospora* sp. MBT66 and its *leuB* mutant. (A) HPLC-UV profile of R5 medium (detected at 254 nm) showing that the production of leucanicidin (**1**) was abolished in the *leuB* mutant; note that also the aglycone bafilomycin A_1 (**4**) is absent. (B) HPLC-UV profile of MM medium (detected at 254 nm) showing that the production of endophenaside E (**17**) was abolished in the *leuB* mutant, accompanied by the accumulation of its aglycone phenazine-1-carboxylic acid (**18**). As references, we used the chromatographically purified compounds **1**, **17**, and **18** obtained from *Kitasatospora* sp. MBT66, while compound **4** was purchased from Sigma. (C) Ion chromatography (EIC) of UHPLC-TOF-MS analysis further confirmed that the rhamnosylation of endophenaside B (**11**, *m/z* 455.1810) and E (**17**, *m/z* 371.1238) was indeed abrogated in the *leuB* mutant, while the production of the corresponding aglycones endophenazine A_1 (**12**, *m/z* 309.1234) and phenazine-1-carboxylic acid (**18**, *m/z* 225.0659) was not affected. EIC(s) was pairwise compared between wild-type *Kitasatospora* sp. MBT66 (WT) and its *leuB* mutant.

identify bafilomycins, and on MM agar plates with mannitol and glycerol to identify phenazines. HPLC-UV analysis of R5-grown cultures demonstrated that the production of both leucanicidin (**1**) and bafilomycin A_1 (**4**) was abolished in the *leuB* mutant (Figure 4, A), which indicated that the disruption of *leuB* affected the overall gene expression of the *baf* gene cluster. Importantly, HPLC-UV analysis of cultures grown on MM agar plates showed that the rhamnosylation of the phenazines was also aborted in the *leuB* mutant (Figure 4, B), and this was verified by subsequent UHPLC-TOF-MS analysis (Figure 4, C). Taken together, analysis of the *leuB* mutant validated the bioinformatics analysis, establishing unequivocally that *leuB* is indeed required for the methyl-rhamnosylation of both types of natural products.

The promiscuity of LeuAB is not an exception, and indeed glycodiversification of bacterial secondary metabolites may arise from many glycosyltransferases with high substrate promiscuity toward either the (deoxy)sugar donors or the aglycones. For example, the versatile macrolide glycosyltransferase OleD tolerates a wide variety of aglycones including aromatics, coumarins, flavanols, and macrolides, remarkably generating three different types of glycosidic bonding (*O*-, *S*-, and *N*-glycoside).[66] The flexible glycosyltransferase GtfE uses variant

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Table 2. Organization of the *rml* Gene Cluster for dTDP-L-rhamnose Biosynthesis in *Kitasatospora* sp. MBT66

ORF	locus tag	protein	length	annotation	nearest homologue homology, protein, origin	GenPept accession
ORF1	BI06_RS21780	RlmC	202	dTDP-4-dehydrorhamnose-3,5-epimerase	98%, WP_045937815.1, *Streptomyces* sp. NRRL S-495	WP_030393284.1
ORF2	BI06_RS21775	RlmA	291	glucose-1-phosphate thymidylyltransferase	99%, WP_045937814.1, *Streptomyces* sp. NRRL S-495	WP_030393285.1
ORF3	BI06_RS21770	RlmB	321	dTDP-glucose-4,6-dehydratase	94%,WP_030232436.1, *Streptomyces sp.* NRRL S-350	WP_030393286.1
ORF4	BI06_RS21765	RlmD	311	Putative dTDP-4-keto-L-rhamnose reductase	83%, WP_037899046.1, *Streptomyces* sp. NRRL S-350	WP_051741708.1

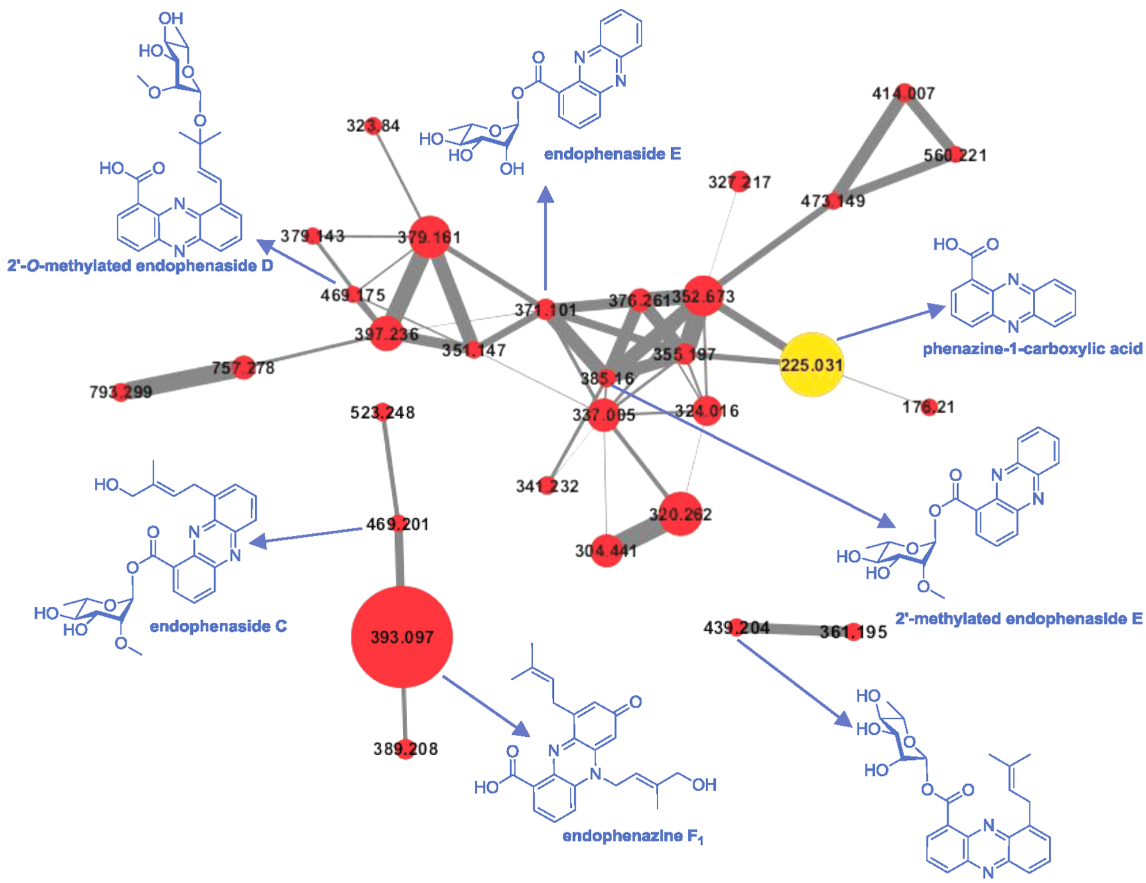

Figure 5. Molecular networking of endophenasides produced by *Kitasatospora* sp. MBT66. The size of the nodes corresponds to the signal intensities of the compounds (see Table S2), and the thickness of the edge between connecting nodes defines the degree of similarity of the MS/MS spectra. The full network of secondary metabolites produced by strain *Kitasatospora* sp. MBT66 is presented in Figure S4.

NDP-sugars to generate glycorandomized vancomycin analogues that rival vancomycin.[18]

The next question to answer was, how is the rhamnosyl substrate for LeuB synthesized? Neither the *baf* nor the *epa* BGC contained components for biosynthesis of NDP-activated rhamnose. Scanning the *Kitasatospora* sp. MBT66 genome identified *rmlABCD* as the likely biosynthetic genes for the activated rhamnose moiety (Table 2), which are required for *de novo* biosynthesis of dTDP-L-rhamnose from D-glucose-1-phosphate (Figure 2, C).[67,68] This dTDP-L-rhamnose then serves as a substrate for the LeuB-mediated rhamnosyl transfer to the aglycones.[69,70] When *Kitasatospora* sp. MBT66 was grown in MM supplemented with different carbon sources, glucose was the best carbon source for simultaneous production of endophenasides as well as of leucanicidin, but the addition of rhamnose did not improve the levels of these NPs. The latter is consistent with dTDP-L-rhamnose being the substrate for glycosylation.

Molecular Networking-Driven Discovery of New Endophenasides. The simultaneous occurrence of both 2′-*O*-methylated and 2′-*O*-unmethylated variants, such as leucanicidin (**1**) and bafilomycin A_1-21-*O*-(α-L-rhamnopyranodise) (**3**), together with endophenaside C (**10**) and endophenaside B (**11**), suggested that either LeuB has a relatively broad substrate specificity by accepting both unmethylated and 2′-*O*-methylated dTDP-L-rhamnose as sugar donors or that LeuA regioselectively methylates the 2′-OH group after rhamnosyl transfer, regardless of the precise chemical topology of the aglycones. Based on this, we hypothesize that *Kitasatospora* sp. MBT66 may therefore have the potential to produce the corresponding counterparts of endophenasides A (**5**), D (**14**), and E (**17**),[43] which could have been missed in our prior chemical investigation due to intrinsic low yields.

To test this, crude extracts of *Kitasatospora* sp. MBT66 grown on MM + 1% glucose were subjected to MS/MS-based

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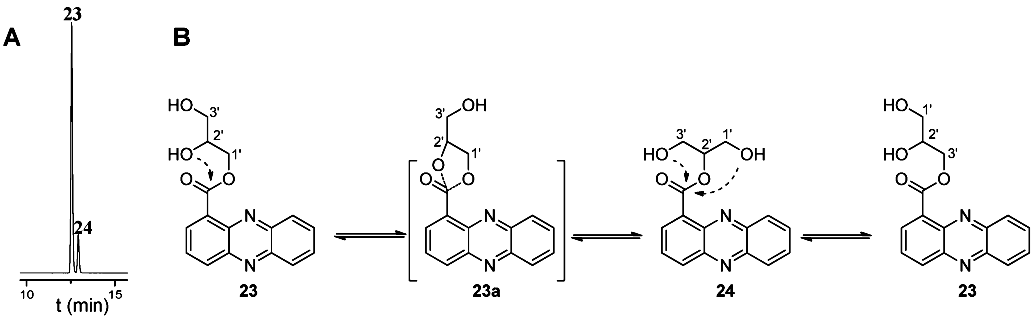

Figure 6. Diagram for the tautomerism between glyceride phenazines **23** and **24**. HPLC analysis revealed the spontaneous interconversion of **23** and **24**. When the equilibrium was reached, compound **23** was 7.5 times **24** (A). An explanatory mechanism for this phenomenon is that all the three hydroxyl groups can form ester bonds with phenazine-1-carboxylic acid. The plausible transition intermediate **23a** with a relatively stable five-member ring system probably mediated intramolecular exchange of glycerol esterification (B).

molecular networking analysis (Table S2).[71−74] The fundamental principle is based on the fact that structurally related natural products are typically characterized by similar MS/MS fragmentation patterns. The MS/MS structural relatedness among molecules can be detected in an automated manner and can subsequently generate a molecular network wherein analogues cluster together. As a result, a network of secondary metabolites produced by *Kitasatospora* sp. MBT66 was created (Figure S4), which contains subnetworks for phenazine-type molecules (Figure 5). This among others identified endophenaside E (**17**) at m/z 371 and its 2′-*O*-methylated congener (**16**) at m/z 385. Two peaks at m/z 469 corresponded to endophenaside C (**10**) and 2′-*O*-methylated-endophenaside D (**13**). Moreover, the networking analysis presented many molecular features that could not be assigned to any of the previously identified endophenazines[54] or endophenasides,[43] strongly suggesting that *Kitasatospora* sp. MBT66 produces many other and likely novel phenazine-type compounds.

To characterize these putative new phenazine derivatives, another round of up-scale fermentation of *Kitasatospora* sp. MBT66 followed by compound purification and identification was performed. As previous studies showed that endophenaside E (**17**) was prone to methanolysis, the use of methanol as a solvent during isolation was avoided. UV- and/or MS-guided fractionation indeed resulted in the elucidation of nine new phenazine glycosides **6**−**8**, **16**, and **22**−**26**, as well as their corresponding known endophenazine aglycones **9**, **12**, **15**, **18**, and **19**−**21**. Further purification of **13** was not feasible due to its extremely low abundance. The follow-up ^{1}H NMR (Table S3) measurements unambiguously confirmed the planar structures of new compounds **6**−**8**, **16**, **25**, and **26**, by comparison with those of known endophenasides A−E[43] and with the endophenazines,[54] all of which were confirmed by high resolution mass spectrometry (for details, see Supporting Information data file). While the ^{1}H NMR spectrum of **22** revealed features typical of endophenasides, the phenazine core was elucidated as 1,7-disubsitituted based on the coupling of H-6 (δ 8.57, d, J = 1.2 Hz), H-8 (δ 8.34, dd, J = 9.0, 1.2 Hz), and H-9 (δ 8.60, d, J = 9.0 Hz). In addition, the substituent at C-7 was further identified as a benzoyl group and confirmed by HRMS at m/z 475.1482 (calculated for $C_{26}H_{23}N_2O_7$ 475.1500). Exceptionally, the NMR spectrum of a mixture of **23** and **24** presented two sets of characteristic glycerol signals instead of the usual rhamnose in the δ 3.5−5.0 region. The different ester linkage was distinguished by the downfield shift of H-1′ (δ 4.63 and 4.57) in **23** and H-2′ (δ 5.43) in **24**, originated from the shielding effect of the carbonyl group (C-11). Though these isomers were chromatographically separable (Figure 6, A), they were spontaneously interconverted into one another, with a fast asymmetric equilibrium of **23** around 7.5 times that of **24**. The tautomerization likely originated from the spatially vicinal −OH group on the glycerol side chain, which could serve as an alcoholytic reagent for ester bond, while the phenazine-1-carboxyl performed as an anchor (or carrier) for self-refresh esterification of three hydroxyls (Figure 6, B).

To get an idea of the antimicrobial activity of the newly isolated endophenasides, antimicrobial assays were performed using compounds **8**, **16**, and **23**−**25** that represented substitute variation of rhamnosylated, glycerolated, and prenylated phenazines, respectively (Table 3). The assays were done according to the method that was also used for endophenasides A−E.[43] All glycosylated phenazines that have been tested inhibited growth of the Gram-negative bacteria *Escherichia coli* K12 and *Pseudomonas aeruginosa* PAO1, which was in agreement with the data obtained for endophenasides A−E.[43] The compounds, and in particular phenazines **23** and **24**, also inhibited growth of *Bacillus subtilis* 168, while conversely, the compounds had a negligible efficacy against the Gram-positive bacterium *Staphylococcus aureus* CECT976.

Table 3. Antimicrobial Activity of Representative New Endophenasides 8, 16, and 23−25[a]

	inhibition zone (mm)			
compound no.	*Bacillus subtilis*	*Escherichia coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*
8	10	10	0	10
16	14	9	0	9
23, 24	20	10	0	11
25	11	9	0	9
AMP[b]	32	23	35	0
STR	13	18	17	15
NC	0	0	0	0

[a]For each compound, 25 μL was spotted of a 2 mg/mL solution in methanol. [b]AMP, ampicillin; STR, streptomycin; NC, negative control (methanol).

Conclusion. Characterization of the genetic basis for the glycosylation is of the utmost significance, because it can expedite the downstream biochemical investigation to refresh the chemistry of natural products and accordingly optimize their pharmaceutical properties. In this study, we characterized a type I PKS gene cluster that encodes the biosynthesis of the rhamnosylated plecomacrolide antibiotic leucanicidin. How-

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ever, the previously described NK155141 (19-methyl-leucanicidin) was shown not to be produced *in vivo* but an artifact from the reaction of leucanicidin with the solvent methanol. The gene cluster includes the *leuAB* genes for the methyl-rhamnosylation of bafilomycin A_1 to leucanicidin. Besides plecomacrolides, *Kitasatospora* sp. MBT66 also produces a range of phenazines, including endophenasides that are phenazines decorated with the same methyl-rhamnosyl group as leucanicidin. MS/MS-based molecular networking guided the further identification of nine new phenazine-type antibiotics, including a pair of interconverting glyceride phenazines. Since LeuA and LeuB are the only enzymes for the methyl-rhamnosylation of natural products encoded by the genome of *Kitasatospora* sp. MBT66, it is likely that these enzymes use both plecomacrolides and phenazines as the substrate. Such promiscuity is surprising but at the same time not unprecedented. In view of the urgent need for new antimicrobials and the challenge of discovering molecules with a novel chemical scaffold, modification of known structures is an attractive alternative to obtaining molecules with novel bioactivities and pharmacokinetic properties. The inherent flexibility of the LeuA and LeuB enzymes described in this work may be applicable for the glycorandomization of a broad range of natural products, and the same may well be true for other glycosyltransferases. The application of such promiscuous natural product tailoring for drug discovery is currently under investigation in our laboratory.

■ EXPERIMENTAL SECTION

Strains and Culturing Conditions. *Kitasatospora* sp. MBT66 was described previously.[43,52] As growth media, we used liquid minimal media (NMMP),[57] minimal media agar plates (MM),[57] solid R5 agar plates,[58] and soy flower medium (SFM) agar plates. Culture media were supplemented with different carbon sources (glycerol, mannitol, glucose, rhamnose, *N*-acetylglucosamine), additional additives (yeast extract, potato extract, peptone, starch, soy flower), or high alkalinity (pH 10). Agar plates (12 cm × 12 cm Petri dishes) were inoculated with 5×10^7 spores from a fresh spore suspension and incubated at 30 °C for 7 days. For liquid-grown cultures, 50 mL of NMMP media with additives were inoculated with 5×10^7 spores in 250 mL flasks equipped with a spring and grown at 30 °C with constant shaking at 220 rpm for 7 days.

Extraction and Isolation of Metabolites. The extraction of metabolites produced by *Kitasatospora* sp. MBT66 basically followed our method published previously.[43] Briefly, after 7 days of incubation, agar plates were cut into pieces and soaked in ethyl acetate (EtOAc) overnight at RT. The EtOAc was removed under a vacuum at 40 °C, and the residue was dissolved in methanol (MeOH) or acetonitrile (ACN) for HPLC-UV and/or UHPLC-TOF-MS analysis.

The first round of isolation was done to enable chemical investigation of *Kitasatospora* sp. MBT66.[43] After 6 days of incubation of MBT66 in 5 L of MM supplemented with 1% glycerol and 0.5% mannitol (w/v), the EtOAc-soluble component (2.0 g) was fractionated on a Macroporous resin Diaion HP-20 from Supelco (Bellefonte, PA, USA) by eluting stepwise from H_2O to MeOH, to give 20%, 40%, 60%, and 80% MeOH fractions. Previous investigation of the 40% (v/v) MeOH fraction identified the novel endophenasides A−E.[43] Here, the 80% (v/v) MeOH fraction was further separated by silica gel (pore size 60 Å, 70−230 mesh, St. Louis, MO, USA) column chromatography employing gradient elution from $CHCl_3$ to MeOH, to give 14 subfractions (sfr.1−sfr.14). Sfr.11 was purified by semipreparative reversed-phase HPLC (Phenomenex Luna C18 (2) 100 Å 5 μm 250 × 10 mm) on a Shimadzu HPLC system and a 5 mL Rheodyne manual injection loop, eluting with a gradient of MeOH in H_2O from 80% to 100%, to isolate compound **1** (t_R = 25.75 min, 0.65 mg) and **2** (t_R = 28.10 min, 0.50 mg). The 60% (v/v) MeOH fraction was defatted with *n*-hexane, which was further separated by semipreparative reversed-phase HPLC (Phenomenex Luna C18 (2) 100 Å 5 μm 250 × 10 mm) on an Agilent 1200 series HPLC apparatus (Agilent technologies Inc., Santa Clara, CA, USA), eluting with a gradient of ACN in H_2O from 20% to 100% at a flow rate of 2 mL/min in 40 min. The peaks detected in the HPLC chromatogram at 254 nm were manually collected, which gave semipure **7** (t_R = 30.12 min, 0.58 mg), **8** (t_R = 25.48 min, 1.19 mg), semipure **9** (t_R = 38.16 min, 0.46 mg), **10** (t_R = 18.90 min, 0.82 mg), a mixture of **12** and **15** (t_R = 20.48 min, 0.77 mg), **14** (t_R = 17.24 min, 0.70 mg), **19** (t_R = 19.61 min, 1.50 mg), and impure **22** (t_R = 22.22 min, 0.20 mg).

The second round of isolation was performed on 1 L of *Kitasatospora* sp. MBT66 culture grown under the same conditions as above, but using different fractionation methods. Specifically, after EtOAc extraction, 0.5 g of extract was first separated using silica gel, employing a gradient elution by acetone/*n*-hexane as the solvent. All fractions were pooled into seven fractions based on TLC (Silica gel 60 F_{254}, Merck, Darmstadt, Germany) detection under UV light 254 nm. These fractions (Fr.1−Fr7) dissolved in ACN were analyzed in parallel by TLC, HPLC-UV, and UHPLC-TOF-MS (see below). Fr.3 and Fr.4 that contained compounds that displayed a UV spectrum and mass typical of endophenazines or endophenasides[43,54] were purified further. Fr.3 was separated by semipreparative reversed-phase HPLC (Phenomenex Luna 5 μm C18 (2) 100 Å column, 250 × 10 mm) on an Agilent 1200 series HPLC (Agilent technologies Inc., Santa Clara, CA, USA), eluting with a gradient of ACN in H_2O adjusted with 0.1% TFA from 5% to 36%. HPLC peaks were manually collected, resulting in the isolation of compound **6** (semipure; t_R = 28.55 min, 0.25 mg), **21** (semipure; t_R = 30.49 min, 0.79 mg), **26** (t_R = 32.48 min, 0.32 mg), **20** (semipure; t_R = 33.74 min, 0.82 mg), **19** (semipure; t_R = 35.44 min, 0.73 mg), **25** (t_R = 37.97 min, 0.63 mg), and **18** (t_R = 43.40 min, 2.08 mg). Fr.4 was further subjected to preparative TLC (PLC Silica gel 60 F_{254}, 1 mm, Merck, Darmstadt, Germany), migrated with solvent system *n*-hexane/acetone (1:1) and detected under UV light of 254 nm. Two closely migrating dark bands were scraped off and rinsed with acetone and identified as semipure compound **17** (1.62 mg) and coded as Fr.4−1, respectively. Subsequently, Fr.4−1 was separated using reversed-phase chromatography on an Agilent 1200 HPLC, which was separated into **23** (t_R = 18.89 min, 2.25 mg; convertible into **24**), **24** (t_R = 19.68 min, 0.35 mg; convertible into **23**), **16** (t_R = 21.30 min, 0.82 mg), and **18** (semipure; t_R = 30.31 min, 0.20 mg), eluting with a gradient of ACN in H_2O from 30% to 100%.

Antimicrobial Activity Assays. Antimicrobial activity of purified new compounds was determined according to a disc diffusion method as previously described.[43] A total of 25 μL of the novel endophenaside compounds **8**, **16**, and **23**−**25** (2 mg mL^{-1} in methanol) was spotted onto paper discs (6 mm diameter) placed on agar plates containing a soft agar overlay with indicator bacteria. Indicator bacteria were *Bacillus subtilis* 168, *Escherichia coli* K12, *Staphylococcus aureus* CECT976, or *Pseudomonas aeruginosa* PAO1. Ampicillin and streptomycin were used as positive controls, and the solvent methanol as the negative control. After incubation at 37 °C for 18 h, growth inhibition zones (in mm) were recorded as antimicrobial activity.

NMR Measurements. NMR sample preparation and measurements were performed according to a protocol that was published previously.[75,76] Briefly, 500 μL of methanol-d_4 were added to freeze-dried samples, and the resultant mixtures were vortexed for 10 s and sonicated for 20 min at 42 kHz using an Ultrasonicator 5510E-MT (Branson, Danbury, CT, USA), followed by centrifugation at 16 000*g* at RT for 5 min. The supernatant (300 μL) was transferred to a 3 mm micro-NMR tube and analyzed. The ^1H NMR spectra were recorded at 25 °C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz. Deuterated methanol was used as the internal lock. Each ^1H NMR spectrum consisted of 128 scans using the following parameters: 0.16 Hz/point, pulse width (PW) = 30 (11.3 μs), and relaxation delay (RD) = 1.5 s. Free induction decays (FIDs) were Fourier transformed with a line broadening (LB) = 0.3 Hz. The resulting spectra were manually phased and baseline corrected and calibrated to residual methanol-d_4 at 3.30 ppm, using MestReNova 8.1.

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HPLC-UV Analysis. HPLC analysis was performed with an Agilent 1200 series HPLC apparatus (Agilent technologies Inc., Santa Clara, CA, USA), using a 150 × 4.6 mm Luna 5 μm C18 (2) 100 Å column equipped with a guard column containing C18 4 × 3 mm cartridges (Phenomenex Inc., Torrance, CA, USA). The mobile phase consisted of water (A) and acetonitrile (B, HPLC grade) in a linear gradient program from 10% B to 100% B in 50 min at a flow rate of 1.0 mL/min. Chromatograms were recorded at 210, 254, and 280 nm. The injection volume was 10 μL.

UHPLC-TOF-MS Analysis. Mass analysis was performed on an Agilent 1290 UHPLC coupled to a Bruker Daltonics microTOF-QII equipped with a standard electrospray source. The instrument was fitted with a Kinetex C18 column (50 cm × 2.1 mm, 100 Å). A linear gradient analysis from 5% B to 100% B was performed over 25 min with mobile phase A (H_2O with 0.1% formic acid) and mobile phase B (acetonitrile with 0.1% formic acid). For each sample, 10 μL was injected onto the column at a flow rate of 0.5 mL/min. The mass spectrometer was programmed to acquire MS/MS in a data-dependent manner, acquiring five MS/MS scans following each precursor MS^1 scan.

MS/MS-based Molecular Networking. Mass spectral networks were assembled as described in ref 77. Tandem MS spectra were clustered using MS-Clustering[78] that builds consensus spectra for repeatedly observed ions (this was performed using the natural product analysis infrastructure at http://gnps.ucsd.edu). The MS^2 spectra were scored based on their similarity; a cosine score of 1 indicates identical spectra, while a cosine score of 0 indicates no similarity. The cosine score threshold to make a match was set to 0.7 and the minimum matched peak was 6. The algorithm assumed a parent peak mass tolerance of 2.0 Da and an MS^2 peak tolerance of 0.5 Da. The networks were visualized with Cytoscape software, whereby consensus spectra are represented as nodes connected by edges to aligning nodes. The thickness of the edge indicates the level of similarity between the nodes. The FM3 layout was used to organize and align nodes within the network. The data are available as MSV000079139 and MSV000079279. at http://gnps.ucsd.edu.

Bioinformatics. Genome sequencing and annotation of *Kitasatospora* sp. MBT66 was described previously,[52] and is available under Genbank accession number JAIY00000000. MultiGeneBlast architecture searches to exhaustively scan the *Kitasatospora* sp. MBT66 genome for loci with glycosyltransferases and methyltransferases were performed using default parameters, using a data set of glycosyltransferases (accessions AAN65238, AAN65243, AAG29794, AAG29803, AAK83176, AAL06683, ABY66020, ABY66027, AAM77991, AAM77984, CAC93718, ABC02795, ABC02796, BAC55213, BAC55218, CCD33145, BAA84598, AAG23269, AAG23270, AAG23272, AAG23280, ABI22137, ABI22145, AAS79443, and AAU93810) and methyltransferases (accessions AAN65229, AAG29785, AAC01731, CBA11567, AAK83182, AAK83192, AAK83193, AAM77992, CAC93713, BAC55209, CAA76551, CAA76552, CAA76553, CCD33143, CCD33144, AAG23268, and AAZ94402) known to be involved in natural product methylglycosylation. MultiGeneBlast searches of the *leuAB* subcluster against MIBiG BGCs[64] were performed with a 15% sequence identity threshold and otherwise default parameters. Multiple sequence alignment of glycosyltransferases was performed using Muscle v3.8.31,[79] using default parameters. Phylogenetic trees were calculated in MEGA 6.06,[80] using the maximum likelihood method, with 100 bootstrap replicates.

Construction of a *leuB* Frame-Shift Mutant Using the CRISPR/Cas9 System. All oligonucleotides used in this work are listed in Table S1. The *leuB* spacer insert was generated by annealing oligonucleotides LeuB_spacer_For and LeuB_spacer_Rev. By using Golden Gate assembly,[81] the generated *leuB* spacer insert was cloned into plasmid pCRISPomyces-2,[65] which was obtained from Addgene (Plasmid #61737), to generate construct pGWS1001. The approximately 1 kb left and right flanking regions of the *leuB* editing template were amplified by PCR from the genomic DNA of *Kitasatospora* sp. MBT66 using primer pairs LeuB_LF-976_EX + LeuB_LR+33_H and LeuB_FS_RF+47_H + LeuB_RR+1032_EX, respectively. PCRs were done as described.[82] Fragments were then digested with XbaI and *Hin*dIII and ligated into pGWS1001 to generate construct pGWS1002. In the *leuB* editing template, the +34/+46 part of *leuB* (whereby +1 refers to the first nt of the translational start codon) was erased and replaced by a *Hin*dIII site, so as to introduce a frame-shift in *leuB*. The correct construct assembly was confirmed by DNA sequencing (BaseClear B.V., Leiden, The Netherlands).

Construct pGWS1002 was then introduced into the parental strain *Kitasatospora sp.* MBT66 by conjugation as described.[57] *E. coli* ET12567/pUZ8002 containing pGWS1002 were incubated to an OD_{600} of 0.4−0.6 and mixed with 10^8 spores. Ex-conjugants were selected by overlaying each plate with water containing apramycin (1 mg) and nalidixic acid (500 μg). After conjugation, single ex-conjugants were streaked onto SFM agar plates containing nalidixic acid and incubated at 30 °C for 3−5 days. Colonies were then grown in liquid TSBS for genomic isolation, followed by PCR using oligonucleotides LeuB_F-370 and LeuB_R+584. PCR products were digested by *Hin*dIII, whereby the correct mutants were identified by bands of 401bp and 538bp, followed by DNA sequencing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00801.

Figures S1−S4, Tables S1−S3, supporting data (PDF)

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Notes

The authors declare no competing financial interest.

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