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## **Discovery of novel antibiotics from actinomycetes by integrated metabolomics & genomics approaches**

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## Chapter 10

### **Leucanicidin and endophenazines result from methyl-rhamnosylation by the same tailoring enzymes in *Kitasatospora* sp. MBT66**

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**ABSTRACT**

The increasing bacterial multi-drug 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<sub>1</sub> 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 candidate for the methyl-rhamnosylation of natural products, and mutation of *leuB* abolished glycosylation of both families of natural products. Thus, LeuAB mediate the post-PKS methyl-rhamnosylation of bafilomycin A<sub>1</sub> 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.

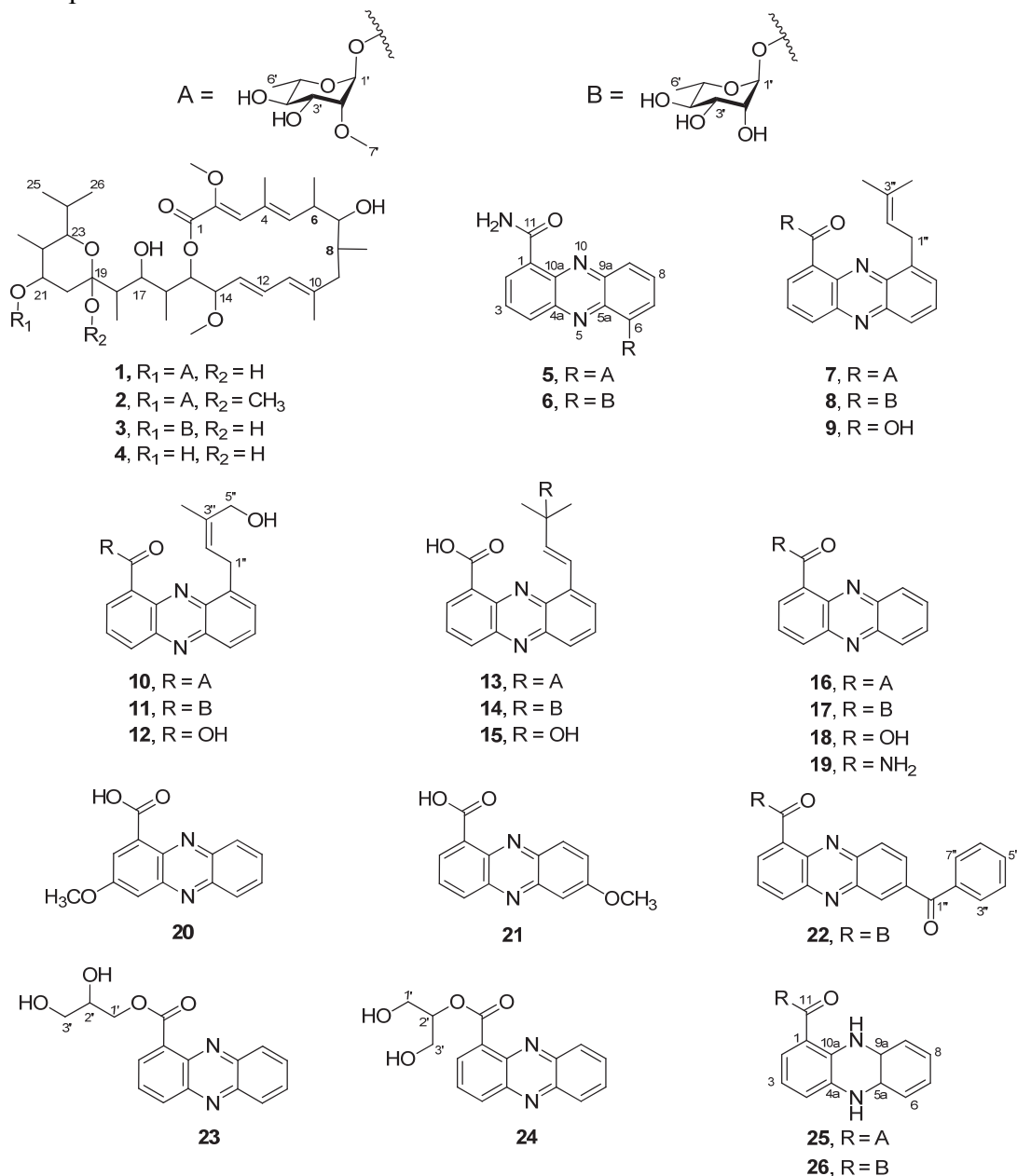
## 1. INTRODUCTION

The rapid increase in antimicrobial resistance poses one of the major threats to human health.<sup>1</sup> A particular problem with drug discovery from microbial sources is the high frequency of re-discovery of known compounds, which necessitates new approaches to replenish the antimicrobial drug pipelines.<sup>2–5</sup> 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.<sup>2,6,7</sup> Sequencing of the genomes of actinomycetes revealed that the natural products producing potential of even the best-studied model organisms has been underestimated.<sup>8–10</sup> However, many of these gene clusters are poorly expressed in the laboratory.<sup>11–14</sup> 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 is glycosylated, with structurally highly diverse aglycones containing one or more glycosyl groups.<sup>15</sup> Glycosylation can dramatically influence the pharmacological properties of the parent scaffold and directly mediate bioactivity, such as in anthracycline, aureolic acid and enediyne antibiotics.<sup>16</sup> Many microbial glycosides also find their applications in agriculture, such as the insecticide avermectin.<sup>17</sup> Antibiotic glycan alteration (so-called glycorandomization) is a potentially powerful strategy in combating emerging bacterial resistance.<sup>18–20</sup>

Given the profound biological significance of glycosylation, it is important to harness the biosynthetic machinery for the formation of glycoconjugates,<sup>21</sup> which will pave the way for the glycodiversification of NPs through genetic engineering approaches.<sup>22</sup> 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.<sup>15</sup> The plecomacrolides are a family of macrolides that typically feature a 16- or 18-membered macrolactone containing two conjugated diene units connected with a six-membered hemiacetal side chain through a C<sub>3</sub> spacer.<sup>23</sup> Endowed with biologically important intramolecular hydrogen bonding network among the lactone/C<sub>3</sub> linker/hemiacetal structural motif,<sup>24,25</sup> plecomacrolides exhibit a variety of bioactivities, including antitumor,<sup>26</sup> antifungal,<sup>27</sup> antiparasitic,<sup>28</sup> immunosuppressant,<sup>29</sup> and particularly selective vacuolar ATPase (V-ATPase) inhibitors.<sup>23,30</sup> Additional substituents (mostly on the secondary alcohol of the hemiacetal portion) and/or modification on backbone largely diversified this class of antibiotics, such as bafilomycins,<sup>31,32,33,34</sup> concanamycins,<sup>35</sup> hygrolidin,<sup>36</sup> setamycin,<sup>37</sup> micromonospolide,<sup>38</sup> and formamycin,<sup>39</sup> simultaneously affording a variation of bioactivity and toxicity.<sup>25,40</sup> The genus *Kitasatospora* has a similar life style as *Streptomyces*, and also has a rich arsenal of secondary metabolites.<sup>41</sup> Several plecomacrolide type compounds have been characterized from *Kitasatospora* species, such as bafilomycins A<sub>1</sub> and B<sub>1</sub> from *K. setae*,<sup>37</sup> bafilomycins A<sub>1</sub>–C<sub>1</sub> and respective amide derivatives from *K. cheerisanensis*.<sup>42</sup> Bafilomycins are known as specific inhibitors of vacuolar ATPases.<sup>30</sup> 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<sub>1</sub>.<sup>41</sup>

We previously characterized the endophenazines in *Kitasatospora* sp. MBT66, which

constitute a family of novel rhamnosylated phenazines.<sup>43</sup> 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**),<sup>44</sup> is not produced biosynthetically but was derived from 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 together with MS/MS based molecular networking allowed us to identify novel 2'-*O*-methylated and 2'-*O*-unmethylated rhamnosylated endophenazines **6**–**8**, **16**, **22**, **25**, and **26**, as well as an unprecedented tautomer consisting of glyceride phenazines **23** and **24**.



**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 <sup>1</sup>H NMR data assignments for these compounds are summarized in Table S3.

## 2. RESULTS & DISCUSSION

### 2.1. Biosynthetic pathway of plecomacrolide glycosides leucanicidin and NK155141

Our previous chemical investigation of *Kitasatospora* sp. MBT66 led to the discovery of five minor rhamnosylated endophenazines A–E, which were isolated from 40% methanol eluent of Macroporous resin Diaion HP-20 column chromatography.<sup>43</sup> 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),<sup>45</sup> and its methylated derivative NK155141 (**2**).<sup>44</sup> Moreover, another known plecomacrolide glycoside, bafilomycin A<sub>1</sub>-21-*O*-( $\alpha$ -L-rhamnopyranoside) (**3**),<sup>46</sup> 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,<sup>47,48</sup> because of their complex architecture and corresponding difficulty in total synthesis.<sup>49,50,51</sup>

Annotation of the genome sequence of *Kitasatospora* sp. MBT66<sup>52</sup> 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<sub>3</sub> linker/hemiacetal core (Figure S1) of bafilomycin B<sub>1</sub>.<sup>47,48</sup> However, post-PKS tailoring components for the synthesis of leucanicidin were distinct from those for bafilomycin B<sub>1</sub>. Genes required for installing the C<sub>5</sub>N moiety are absent,<sup>47,48</sup> 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<sub>1</sub>. This is a rather typical genetic configuration, as the glycosylation-associated biosynthetic genes are usually coclustered with those for aglycones in microbial genomes<sup>53</sup>. This strong linkage was the basis for the development of so-called glycogenomics, an MS<sup>n</sup>-based genome-mining method for microbial glycosylated molecules.<sup>53</sup> Therefore, we reasoned that the macrolactone core (bafilomycin A<sub>1</sub>, **4**) of leucanicidin was assembled by the classical type I PKS system. *bafAI*-*AV* encode in total 12 PKS modules, which load isobutyrate as 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<sub>1</sub><sup>54</sup> (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 NO. JODS01000000) and *Kitasatospora* sp. NRRL S-495 (GenBank accession NO. 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*.

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* non-enzymatic reaction with methyl donors during the isolation process, because cyclic hemiketal hydroxyl groups are reactive even in the presence of moderately

nucleophilic reagents.<sup>55,56</sup> To address this, we optimized leucanicidin production by MBT66. Liquid NMMP,<sup>57</sup> solid MM,<sup>57</sup> and R5,<sup>58</sup> were supplemented with different carbon sources, additional additives, and high alkalinity.<sup>59,60</sup> Varying growth conditions or the addition of chemical elicitors can be applied to activate the biosynthesis of poorly expressed natural products.<sup>59</sup> *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.<sup>61–63</sup> 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 25mM GlcNAc were dissolved in either methanol or acetonitrile without any additional catalyst. The samples were incubated at room temperature for one 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.

**Table 1.** Organization for the leucanicidin biosynthetic gene cluster of *Kitasatospora* sp. MBT66. The gene cluster architecture for the biosynthesis of bafilomycin A<sub>1</sub> is the same as in *Streptomyces griseus* DSM 2608<sup>48</sup> and *Streptomyces lohii*.<sup>47</sup> The genome sequence of *Kitasatospora* sp. MBT66 is available at GenBank with accession number JAIY000000000 and the annotation was submitted to MIBiG with accession number BGC0001232.

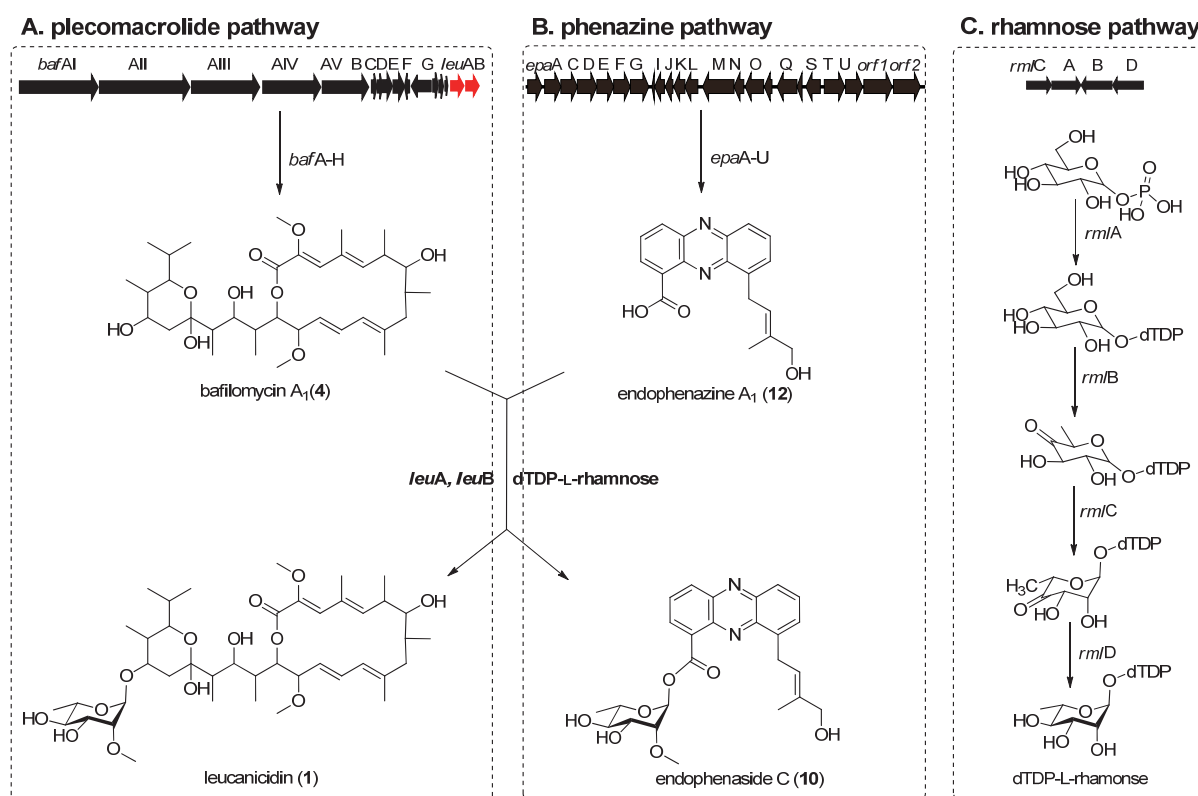
| ORF   | Locus tag                    | Protein | Length | Annotation                                    | Nearest homologue<br>Homology, Protein, origin                | GenPept<br>accession | Comments           |
|-------|------------------------------|---------|--------|---|---|----------------------|--------------------|
| ORF1  | BI06_RS39075                 | BafAI   | 1033   | PKS modules 1–4                               | 91%, WP_018955924.1,<br><i>Streptomyces lohii</i>             | N/A #                | Gapped<br>sequence |
| ORF2  | BI06_RS39070                 | BafAII  | 5019   | PKS modules 5–7                               | 90%, ADC79617.1,<br><i>Streptomyces lohii</i>                 | WP_043476519.1       |                    |
| ORF3  | BI06_RS39065                 | BafAIII | 3966   | PKS modules 8, 9                              | 89%, ADC79618.1,<br><i>Streptomyces lohii</i>                 | WP_043476516.1       |                    |
| ORF4  | BI06_RS39060<br>BI06_RS32440 | BafAIV  | 3453   | PKS modules 10, 11                            | 88%, ADC79619.1,<br><i>Streptomyces lohii</i>                 | N/A #                | Gapped<br>sequence |
| ORF5  | BI06_RS32435                 | BafAV   | 2158   | PKS module<br>12+thioesterase                 | 85%, ADC79620.1,<br><i>Streptomyces lohii</i>                 | N/A #                |                    |
| ORF6  | BI06_RS32430                 | BafB    | 296    | glyceryl-ACP oxidase                          | 93%, ADC79621.1,<br><i>Streptomyces lohii</i>                 | WP_043474332.1       |                    |
| ORF7  | BI06_RS32425                 | BafC    | 115    | acyl carrier protein<br>(ACP)                 | 90%, ADC79622.1,<br><i>Streptomyces lohii</i>                 | WP_030397161.1       |                    |
| ORF8  | BI06_RS32420                 | BafD    | 371    | acyl-CoA<br>dehydrogenase                     | 92%, WP_019761696.1,<br><i>Streptomyces</i> sp. Wigar10       | WP_043474112.1       |                    |
| ORF9  | BI06_RS32415                 | BafE    | 377    | glycerate ACP ligase                          | 93%, WP_018568170.1,<br><i>Streptomyces</i> sp. PsTaAH-124    | WP_051742398.1       |                    |
| ORF10 | BI06_RS32410                 | BafF    | 220    | <i>O</i> -methyl transferase                  | 90%, ADC79625.1,<br><i>Streptomyces lohii</i>                 | WP_043474109.1       |                    |
| ORF11 | BI06_RS32405                 | BafG    | 606    | AfsR family<br>transcriptional<br>regulator   | 86%, ADC79626.1,<br><i>Streptomyces lohii</i>                 | WP_043474107.1       |                    |
| ORF12 | BI06_RS32400                 | BafH    | 253    | TEII  | 93%, WP_019761700.1,<br><i>Streptomyces</i> sp. Wigar10       | WP_030397166.1       |                    |
| ORF13 | BI06_RS32395                 |         | 126    | Putative<br>LuxR_C <sub>2</sub> -like protein | 83%, ADC79628.1,<br><i>Streptomyces lohii</i>                 | WP_043474105.1       |                    |
| ORF14 |                              |         | 38     | malonyl transferase                           | 58%, ADC79629.1,<br><i>Streptomyces lohii</i>                 |                      |                    |
| ORF15 | BI06_RS32390                 | Leu A   | 430    | sugar<br><i>O</i> -methyltransferase          | 59%, WP_005321729.1,<br><i>Streptomyces pristinaespiralis</i> | WP_051742397.1       |                    |
| ORF16 | BI06_RS32385                 | Leu B   | 394    | glycosyl transferase                          | 55%, WP_019074879.1,<br><i>Streptomyces</i> sp. R1-NS-10      | WP_043474103.1       |                    |

# N/A: Not available due to gapped sequence.



## 2.2. LeuA and LeuB display broad flexibility towards their substrates

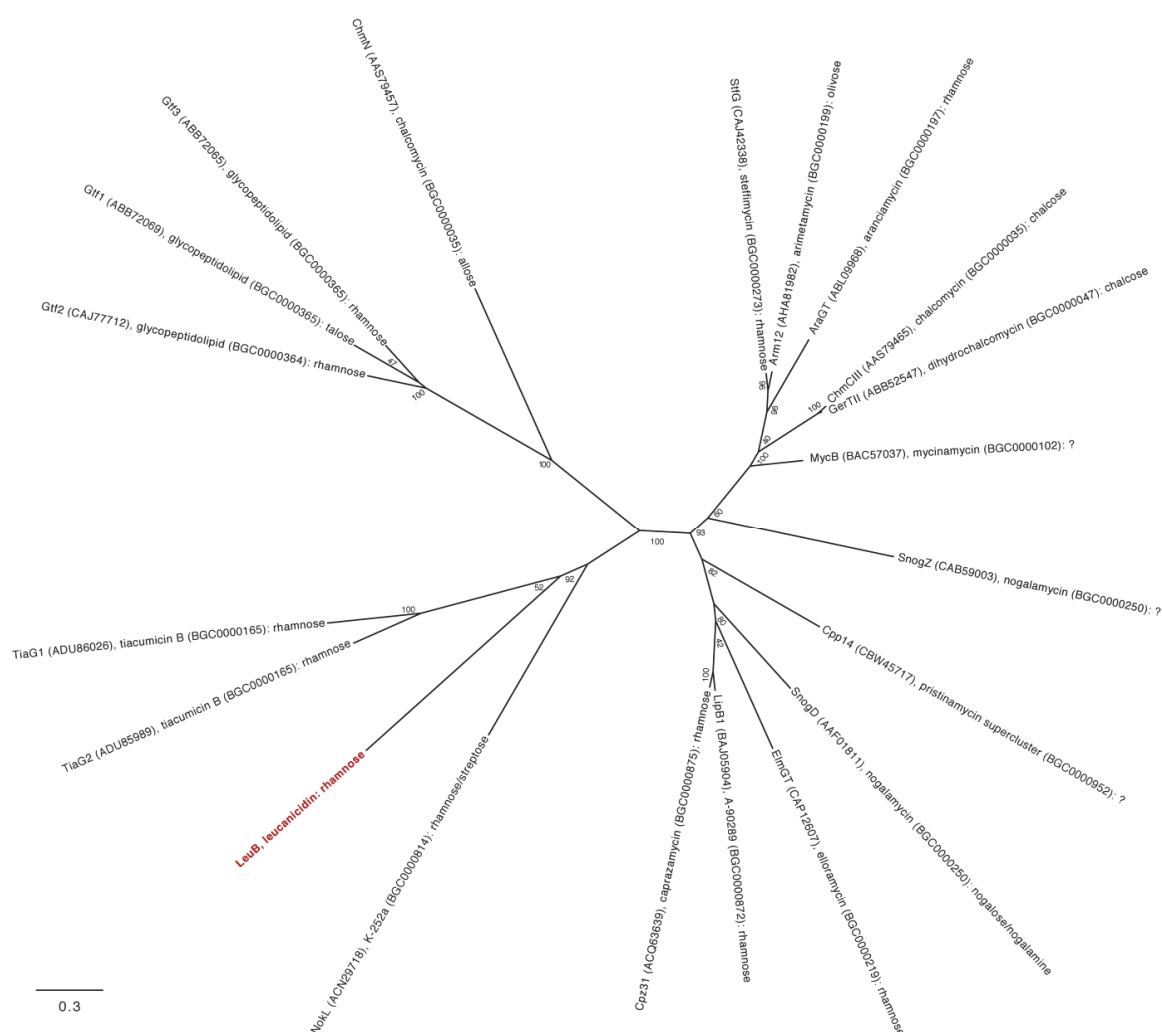
We previously reported that the *epa* BGC of *Kitasatospora* sp. MBT66 (on the genome with Genbank accession number JAIY000000000) is responsible for the assembly of the phenazine backbone in endophenazines A—E, but the essential glycosylation genes remained unresolved.<sup>43</sup> The characterization of the leucanicidin biosynthetic pathway provided more insights into the rhamnosylation of the endophenazines. Leucanicidin and the endophenazines (*i.e.* glycosylated endophenazines) contain the same  $\alpha$ -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* sub-cluster 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 decorate bafilomycin A<sub>1</sub> to leucanicidin (Figure 2, A), but also modify endophenazines<sup>54</sup> into endophenazines<sup>43</sup> (Figure 2, B).



**Figure 2. Promiscuous methyl-rhamnosylation of bafilomycin A<sub>1</sub> 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 *bafA*—H (A) and *epaA*—U (B) gene clusters are responsible for plecomacrolide and endophenazine backbone biosynthesis, respectively, while *rmlA*—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<sub>1</sub> to leucanicidin, and of endophenazine A<sub>1</sub> into endophenaside C. The type I PKS bioassembly line for plecomacrolide backbone is detailed in Figure S1, and the shikimate pathway for endophenazine was described previously<sup>43</sup>.

We first performed a detailed computational genomic analysis of the *leuAB* genes and their homologues. A MultiGeneBlast architecture search with the *leuAB* genes as query on the

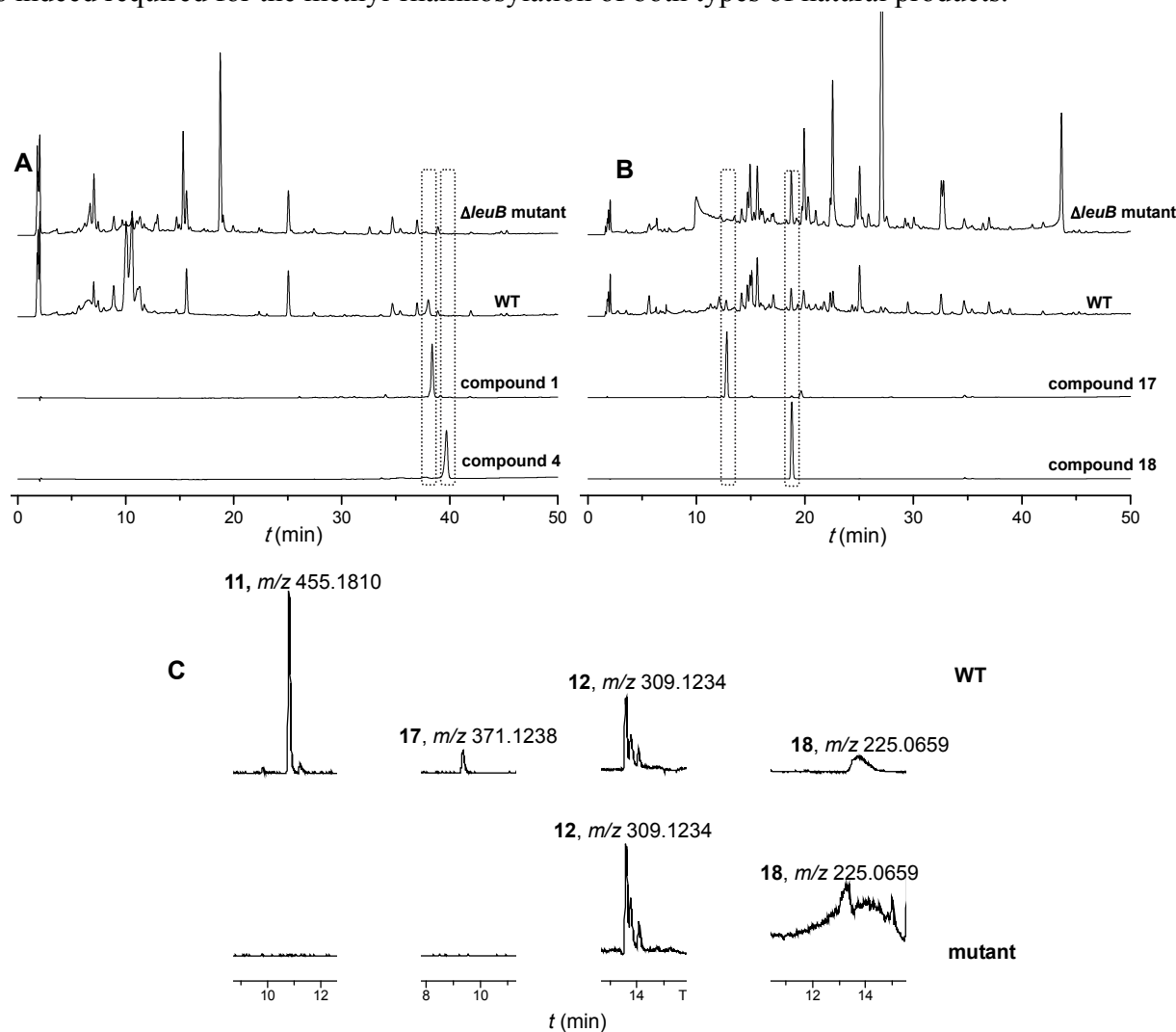
full set of 1170 BGCs from the Minimum Information about a Biosynthetic Gene cluster (MIBiG) repository<sup>64</sup> resulted in seventeen experimentally characterized BGCs that contain homologs of both *leuA* and *leuB*. All hits represented genes involved in the attachment of methylrhannose and (at 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 sub-clusters 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.



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

To create a mutation in *leuB*, we made use of the CRISPR-Cas9 system that was adapted recently for use in actinomycetes.<sup>65</sup> 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 HindIII, 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 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<sub>1</sub> (**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 4B), and this was verified by subsequent UHPLC-TOF-MS analysis (Figure 4C). 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.



**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, but without its aglycone bafilomycin A<sub>1</sub> (**4**) accumulation. **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<sub>1</sub> (**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.

The promiscuity of LeuAB is not an exception, and indeed glycodiversification of bacterial secondary metabolites may arise from many glycosyltransferases with high substrate promiscuity towards 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 type of glycosidic bonding (*O*-, *S*- and *N*-glycoside).<sup>66</sup> The flexible glycosyltransferase GtfE uses variant NDP-sugars to generate glycorandomized vancomycin-analogues that rival vancomycin.<sup>18</sup>

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).<sup>67,68</sup> This dTDP-L-rhamnose then serves as a substrate for the LeuB-mediated rhamnosyl transfer to the aglycones.<sup>69,70</sup> 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 addition of rhamnose did not improve the levels of these NPs. The latter is consistent with dTDP-L-rhamnose being the substrate for glycosylation.

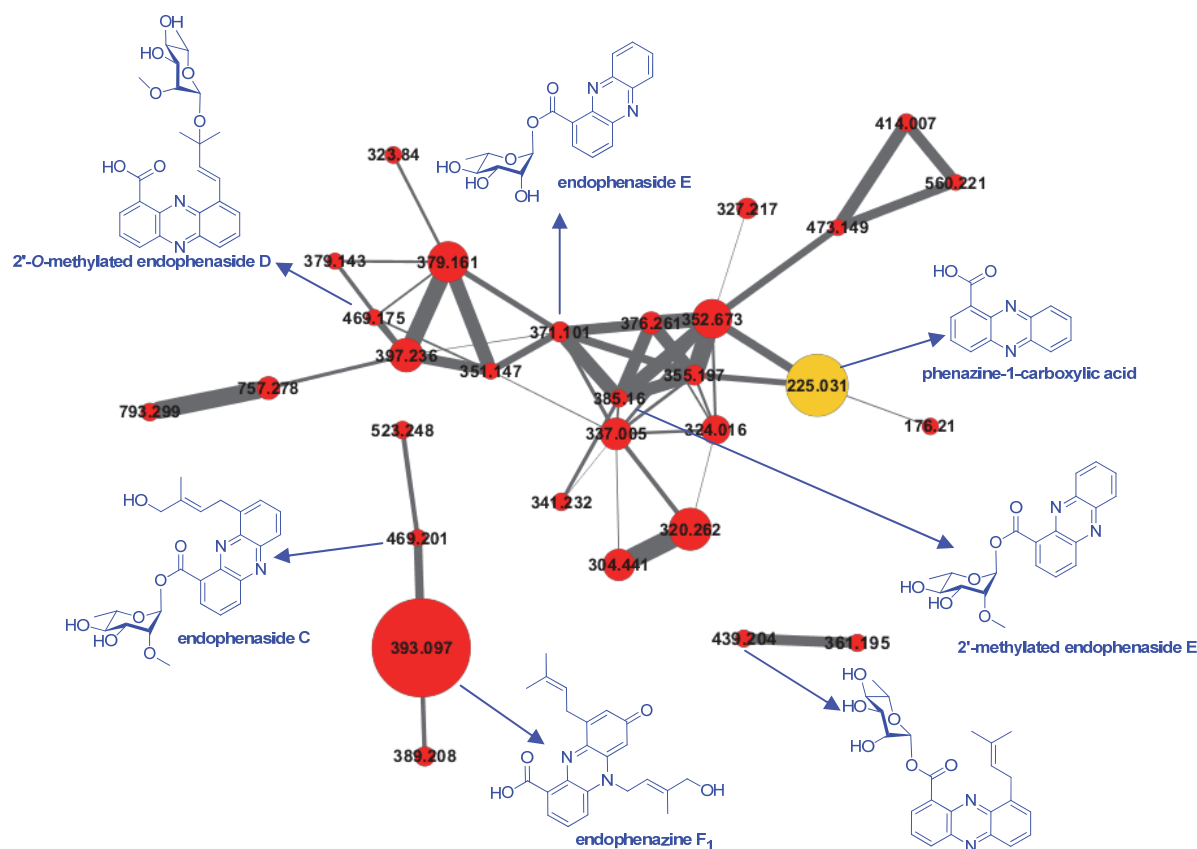
**Table 2.** Organization of the *rml* gene cluster for dTDP-L-rhamnose biosynthesis in *Kitasatospora* sp. MBT66.

| ORF  | Locus tag    | Protein | Length | Annotation                                   | Nearest homologue<br>Homology, Protein, origin             | GenPept<br>accession |
|------|--------------|---------|--------|--|--|----------------------|
| ORF1 | BI06_RS21780 | RlmC    | 202    | dTDP-4-dehydrorhamnose-3,5-epimerase         | 98%, WP_045937815.1,<br><i>Streptomyces</i> sp. NRRL S-495 | WP_030393284.1       |
| ORF2 | BI06_RS21775 | RlmA    | 291    | glucose-1-phosphate<br>thymidyltransferase   | 99%, WP_045937814.1,<br><i>Streptomyces</i> sp. NRRL S-495 | WP_030393285.1       |
| ORF3 | BI06_RS21770 | RlmB    | 321    | dTDP-glucose-4,6-dehydratase                 | 94%, WP_030232436.1,<br><i>Streptomyces</i> sp. NRRL S-350 | WP_030393286.1       |
| ORF4 | BI06_RS21765 | RlmD    | 311    | Putative dTDP-4-keto-L-rhamnose<br>reductase | 83%, WP_037899046.1,<br><i>Streptomyces</i> sp. NRRL S-350 | WP_051741708.1       |

### 2.3. 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<sub>1</sub>-21-*O*-( $\alpha$ -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 rhamnosyltransfer, 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**),<sup>43</sup> which

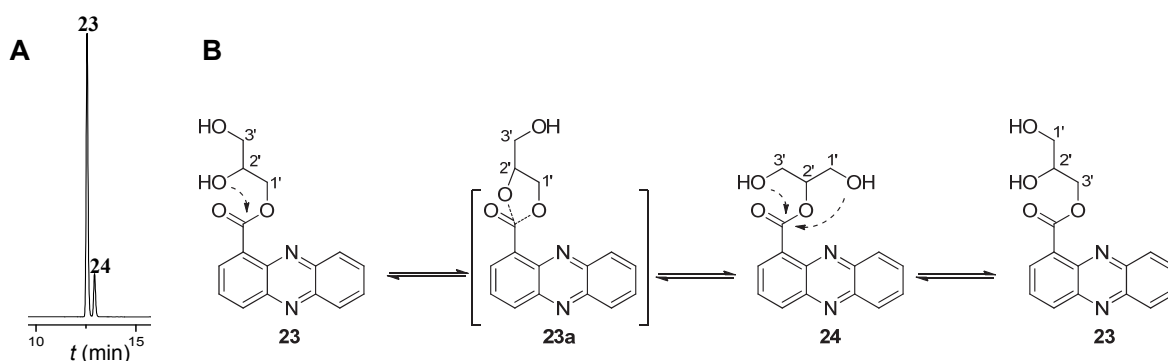
could have been missed in our prior chemical investigation due to intrinsic low yields. To test this, crude extracts of *Kitasatospora* sp. MBT66 grown on MM + 1% glucose were subjected to MS/MS-based molecular networking analysis (Table S2).<sup>71–74</sup> The fundamental principle is based on the 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<sup>54</sup> or endophenasides,<sup>43</sup> strongly suggesting that *Kitasatospora* sp. MBT66 produces many other and likely novel phenazine-type compounds.



**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.

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 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  $^1\text{H}$  NMR (Table S3) measurements unambiguously confirmed the planar structures of new compounds **6**–**8**, **16**, **25**, and **26**, by comparison with those of known endophenazines A–E<sup>43</sup> and with the endophenazines<sup>54</sup>, all of which were confirmed by high resolution mass spectrometry (for details see Supplementary data file). While the  $^1\text{H}$  NMR spectrum of **22** revealed features typical of endophenazines, the phenazine core was elucidated as 1,7-disubstituted based on the coupling of H-6 ( $\delta$  8.57, d,  $J$  = 1.2 Hz), H-8 ( $\delta$  8.34, dd,  $J$  = 9.0, 1.2 Hz), and H-9 ( $\delta$  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  $\text{C}_{26}\text{H}_{23}\text{N}_2\text{O}_7$  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  $\delta$  3.5–5.0 region. The different ester linkage was distinguished by the downfield shift of H-1' ( $\delta$  4.63 and 4.57) in **23** and H-2' ( $\delta$  5.43) in **24**, originated from the shielding effect of 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 alcoholic 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).



**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).

To get an idea of the antimicrobial activity of the newly isolated endophenazines, 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 endophenazines A–E.<sup>43</sup> 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 endophenazines A–E.<sup>43</sup> 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 endophenazines **8**, **16**, and **23–25**. For each compound, 25 µl was spotted of a 2 mg/ml solution in methanol.

| Compound NO.     | Inhibition zone (mm)     |                         |                              |                               |
|------------------|--------------------------|-------------------------|------------------------------|-------------------------------|
|                  | <i>Bacillus subtilis</i> | <i>Escherichia coli</i> | <i>Staphylococcus aureus</i> | <i>Pseudomonas aeruginosa</i> |
| <b>8</b>         | 10                       | 10                      | 0                            | 10                            |
| <b>16</b>        | 14                       | 9                       | 0                            | 9                             |
| <b>23, 24</b>    | 20                       | 10                      | 0                            | 11                            |
| <b>25</b>        | 11                       | 9                       | 0                            | 9                             |
| AMP <sup>a</sup> | 32                       | 23                      | 35                           | 0                             |
| STR              | 13                       | 18                      | 17                           | 15                            |
| NC               | 0                        | 0                       | 0                            | 0                             |

<sup>a</sup> AMP, ampicillin; STR, streptomycin; NC, negative control (methanol)

### 3. CONCLUSION

Characterization of the genetic basis for the glycosylation is of 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. However, 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<sub>1</sub> to leucanicidin. Besides plecomacrolides, *Kitasatospora* sp. MBT66 also produces a range of phenazines, including endophenazines 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 obtain 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.

## 4. EXPERIMENTAL SECTION

### 4.1. Strains and culturing conditions

*Kitasatospora* sp. MBT66 was described previously.<sup>43,52</sup> As growth media we used liquid minimal media (NMMP),<sup>57</sup> minimal media agar plates (MM),<sup>57</sup> solid R5 agar plates,<sup>58</sup> 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  $5 \times 10^7$  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  $5 \times 10^7$  spores in 250 ml flasks equipped with a spring, and grown at 30 °C with constant shaking at 220 rpm for 7 days.

#### 4.2. Extraction and isolation of metabolites

The extraction of metabolites produced by *Kitasatospora* sp. MBT66 basically followed our method published previously.<sup>43</sup> Briefly, after 7 days of incubation, agar plates were cut into pieces and soaked in ethyl acetate (EtOAc) overnight at room temperature. The EtOAc was removed under 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.<sup>43</sup> After 6 days of incubation of MBT66 in 5 liter 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<sub>2</sub>O to MeOH, to give 20%, 40%, 60%, and 80% MeOH fractions. Previous investigation of the 40% (v/v) MeOH fraction identified the novel endophenazines A—E.<sup>43</sup> 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<sub>3</sub> to MeOH, to give 14 subfractions (sfr.1–sfr.14). Sfr.11 was purified by semi-preparative reversed-phase HPLC (Phenomenex Luna C18 (2) 100 Å 5 micron 250 × 10 mm) on a Shimadzu HPLC system and a 5 ml Rheodyne manual injection loop, eluting with a gradient of MeOH in H<sub>2</sub>O 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 semi-preparative reversed-phase HPLC (Phenomenex Luna C18 (2) 100 Å 5 micron 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<sub>2</sub>O from 20% to 100% at flow rate 2 ml/min in 40 min. The peaks detected in the HPLC chromatogram at 254 nm, were manually collected, which gave semi-pure **7** ( $t_R$  = 30.12 min, 0.58 mg), **8** ( $t_R$  = 25.48 min, 1.19 mg), semi-pure **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 liter 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<sub>254</sub>, Merck, Darmstadt, Germany) detection under UV light 254 nm. These fractions (Fr.1—Fr.7) 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 endophenazines<sup>43,54</sup> were purified further. Fr.3 was separated by semi-preparative reversed-phase HPLC (Phenomenex Luna C18 (2) 100 Å 5 micron 250 × 10 mm) on an Agilent 1200 series HPLC (Agilent technologies Inc, Santa Clara, CA, USA), eluting with a



gradient of ACN in H<sub>2</sub>O adjusted with 0.1% TFA from 5% to 36%. HPLC peaks were manually collected, resulting in the isolation of compound **6** (semi-pure;  $t_R$  = 28.55 min, 0.25 mg), **21** (semi-pure;  $t_R$  = 30.49 min, 0.79 mg), **26** ( $t_R$  = 32.48 min, 0.32 mg), **20** (semi-pure;  $t_R$  = 33.74 min, 0.82 mg), **19** (semi-pure;  $t_R$  = 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<sub>254</sub>, 1 mm, Merck, Darmstadt, Germany), migrated with solvent system *n*-hexane/acetone (1:1) and detected under UV light 254 nm. Two closely migrating dark bands were scraped off and rinsed with acetone, and identified as semi-pure 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** (semi-pure;  $t_R$  = 30.31 min, 0.20 mg), eluting with a gradient of ACN in H<sub>2</sub>O from 30% to 100%.

### 4.3. Antimicrobial activity assays

Antimicrobial activity of purified new compounds was determined according to a disc diffusion method as described.<sup>43</sup> 25  $\mu$ l of the novel endophenaside compounds **8**, **16**, and **23–25** (2 mg/ml 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.

### 4.4. NMR measurements

NMR sample preparation and measurements were performed according to a protocol that was published previously.<sup>75,76</sup> Briefly, 500  $\mu$ l of methanol-*d*<sub>4</sub> were added to freeze-dried samples, and the resultant mixtures were vortexed for 10 sec and sonicated for 20 min at 42 kHz using an Ultrasonicator 5510E-MT (Branson, Danbury, CT, USA), followed by centrifugation at 16,000x *g* at room temperature for 5 min. The supernatant (300  $\mu$ l) was transferred to a 3 mm micro NMR tube and analyzed. The <sup>1</sup>H 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 <sup>1</sup>H NMR spectrum consisted of 128 scans using the following parameters: 0.16 Hz/point, pulse width (PW) = 30 (11.3 s) 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*<sub>4</sub> at 3.30 ppm, using MestReNova 8.1.

### 4.5. HPLC-UV analysis

HPLC analysis was performed with an Agilent 1200 series HPLC apparatus (Agilent technologies Inc, Santa Clara, CA, USA), using a 150  $\times$  4.6 mm Luna 5 micron C18 (2) 100 Å column equipped with a guard column containing C18 4  $\times$  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 minutes at a flow rate of 1.0 ml/min. Chromatograms were recorded at 210 nm, 254 nm, and 280 nm. The injection volume was 10 µl.

#### 4.6. UHPLC-ToF-MS analysis

Mass analysis was performed on an Agilent 1290 UHPLC coupled to a Bruker Daltonics microToF-QII equipped with standard electrospray source. The instrument was fitted with a Kinetex C18 column (50cm x 2.1 mm, 100 Å). A linear gradient analysis from 5% B to 100% B was performed over 25 minutes with mobile phase A (H<sub>2</sub>O 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 5 MS/MS scans following each precursor MS<sup>1</sup> scan.

#### 4.7. MS/MS-based molecular networking

Mass spectral networks were assembled as described in reference.<sup>77</sup> Tandem MS spectra were clustered using MS-Clustering<sup>78</sup> that builds consensus spectra for repeatedly observed ions (this was performed using the natural product analysis infrastructure at <http://gnps.ucsd.edu>). The MS<sup>2</sup> 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<sup>2</sup> 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 is available as MSV000079139 and MSV000079279. at <http://gnps.ucsd.edu>.

#### 4.8. Bioinformatics

Genome sequencing and annotation of *Kitasatospora* sp. MBT66 was described previously,<sup>52</sup> 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 dataset 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* sub-cluster against MIBiG BGCs<sup>64</sup> were performed with a 15% sequence identity threshold and otherwise default parameters. Multiple sequence alignment of glycosyltransferases was performed using Muscle v3.8.31,<sup>79</sup> using default parameters. Phylogenetic trees were

calculated in MEGA 6.06,<sup>80</sup> using the maximum likelihood method, with 100 bootstrap replicates.

#### 4.9. 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,<sup>81</sup> the generated *leuB* spacer insert was cloned into plasmid pCRISPomyces-2,<sup>65</sup> 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.<sup>82</sup> Fragments were then digested with XbaI and HindIII, 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 HindIII 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 HM125 by conjugation as described.<sup>57</sup> *E. coli* ET12567/pUZ8002 containing pGWS1002 were incubated to OD<sub>600</sub> of 0.4-0.6, and mixed with 10<sup>8</sup> 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 HindIII, whereby the correct mutants were identified by bands of 401bp and 538bp, followed by DNA sequencing.

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