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Chapter 9

Identification of novel endophenaside antibiotics produced by *Kitasatospora* sp. MBT66

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ABSTRACT

Actinomycetes are a major source of bioactive secondary metabolites and are a focal point in the search for novel antimicrobial compounds that are needed to combat multidrug-resistant pathogens. Here we report the discovery of several novel phenazine-type antibiotics produced by *Kitasatospora* sp. MBT66. These include the novel glycosylated endophenazines A–E (**1**–**5**), together with *N*-prenylated endophenazine F1 (**6**). Compounds **1** and **3** contain a 2'-*O*-methylation of the sugar moiety, which is rare in nature and reported for the first time in connection with phenazines. The structures of the new compounds were determined on the basis of their spectral data, including NMR and HR-MS, and the gene cluster responsible for the biosynthesis of phenazines was identified. All phenazine derivatives showed antimicrobial activity against the Gram-positive *Bacillus subtilis*, while compounds **1**–**3** and **5** also inhibited growth of the Gram-negative *Escherichia coli*.

1. INTRODUCTION

Infections caused by multiple drug-resistant bacteria are a major threat to human health, both in the nosocomial environment and in the general community.[1–3] It has become increasingly difficult to find novel antibiotics with efficacy against multidrug-resistant pathogens such as MDR-TB (multidrug-resistant *Mycobacterium tuberculosis*), MRSA (methicillin-resistant *Staphylococcus aureus*), and multidrug-resistant Gram-negative pathogens.[3] Indeed, despite intensive screening programs in the pharmaceutical industry, hardly any new drugs have been identified in this millenium, which underlines the urgent need for novel approaches to find antimicrobial drugs.[4] Actinomycetes are Gram-positive mycelial bacteria with a complex multicellular life style.[5,6] They are industrially highly relevant due to their ability to produce a plethora of natural products, including anticancer, antifungal, anthelmantic and antibacterial drugs.[7] Of all known naturally occurring antibiotics, some two-third is derived from actinomycetes, the majority of which are produced by streptomycetes.[8] Sequencing of *Streptomyces* genomes established the presence of silent antibiotic biosynthetic gene clusters, suggesting that the potential of these organisms for novel drug production is much larger than originally anticipated.[9] As an example, many novel gene clusters were identified even in the extensively studied model streptomycetes *S. coelicolor*,[10] *S. griseus* [11] and *S. lividans*.[12] This has led to novel approaches to identifying specific conditions or agents that elicit the expression of antibiotics.[13–15]

In a recent study, we screened a collection of over 800 actinomycetes using 40 different growth conditions, which identified among others *Kitasatospora* sp. MBT66 that was shown to be a prolific producer of antibiotics with activity against Gram-positive and Gram-negative pathogens.[16] Preliminary analysis predicted that it produced a number of compounds exhibiting similar chemical properties (TLC and HPLC-UV detection) to 1-carbomethoxy phenazine that was described in another actinomycete *Streptomyces* sp. MBT70.[16] The phenazines are a group of heterocyclic, nitrogen-containing compounds isolated primarily from *Pseudomonas* and *Streptomyces* bacteria.[17] They differ in chemical and physical properties according to the nature and/or position of functionalities, and thus give rise to a full spectrum of colours ranging from the deep red to the light blue.[18] Phenazines are redox-active and can reduce molecular oxygen, leading to the generation of toxic reactive oxygen species and explaining their broad-spectrum antibiotic activity toward bacteria, fungi, and plant and animal tissues.[19,20] Clofazimine is an example of a phenazine compound that is successfully applied in the clinic, where it is used for the treatment of leprosy, while clofazimine also has efficacy against MDR *Mycobacterium tuberculosis*.[21] In addition, phenazines play multiple roles in the producing bacteria for survival and interaction with the environment, such as protection of plants by rhizobacteria against bacterial and fungal pathogens,[22] reducing environmental Fe^{3+} to the more soluble Fe^{2+} to aid iron acquisition,[23] and in primary energy metabolism by mediating the reoxidation of NADH and utilization of pyruvate under oxygen-limiting conditions.[24] Although more than 6000 phenazine-containing compounds have been described over the past century, fewer than 100 are of natural origin.[25]

Very few glycosylated phenazines have so far been found in nature, and these are without exception compounds with bioactivity that are derived from 6-deoxy-L-glycopyranosides.[20] In this work, we report the isolation, structure determination and antimicrobial activity of novel

glycosylated endophenazines (**1–5**) and an *N*-prenylated endophenazine (**6**) produced by *Kitasatospora* species MBT66, and present the gene cluster responsible for their biosynthesis.
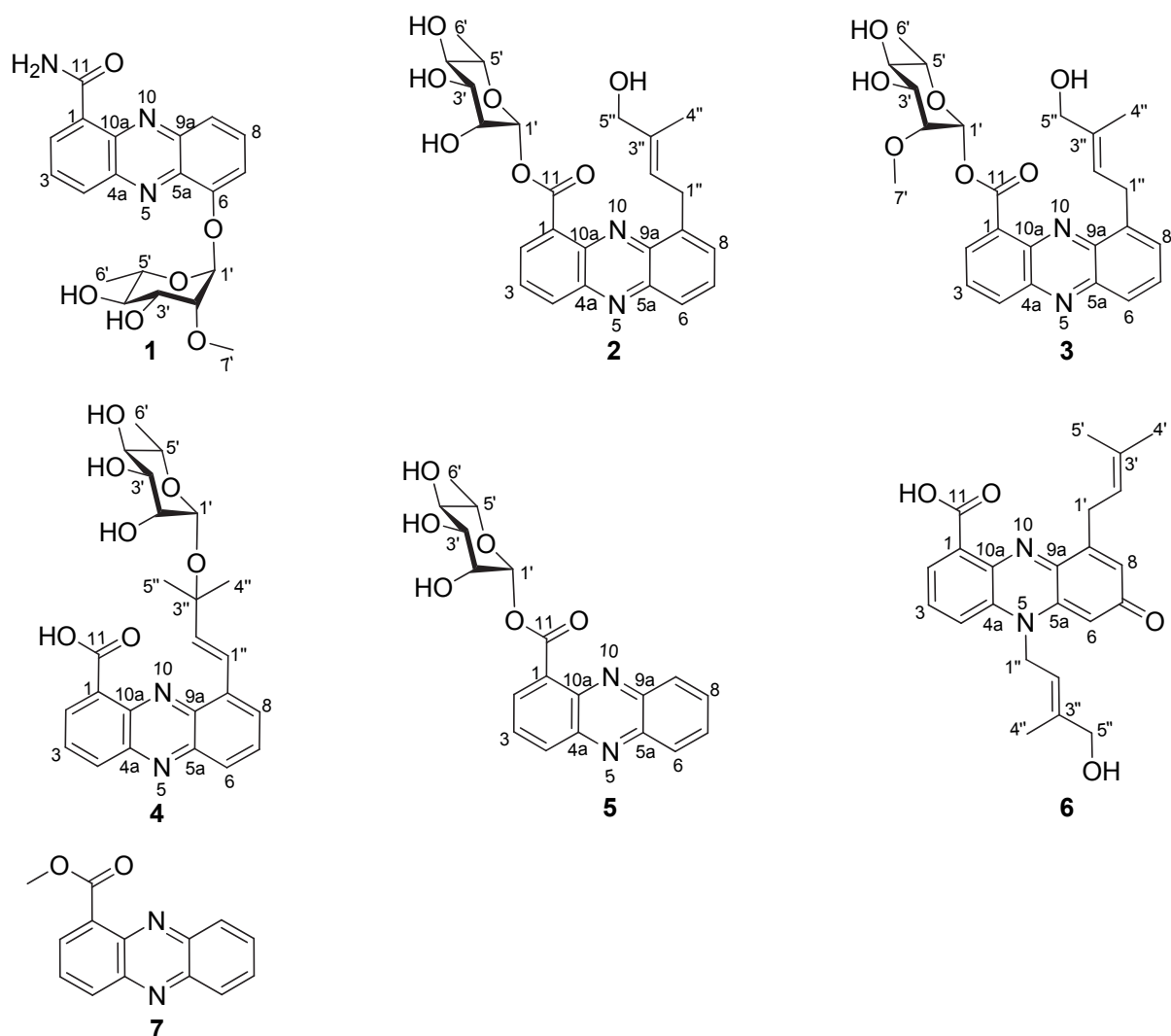

Figure 1. Phenazine derivatives from *Kitasatospora* sp. MBT66. Compounds **1–5** are glycosylated endophenazines, compound **6** is an *N*-prenylated endophenazine. Compounds **1**, and **3** were methylated at the 2'-*O* position of the rhamnose moiety.

2. RESULTS AND DISCUSSION

We previously performed a screen of a collection of some 800 actinomycetes obtained from mountain soils, so as to identify optimal laboratory conditions for antimicrobial secondary metabolites production.[16] *Kitasatospora* sp. MBT66[26] was one of our 96 most prolific antibiotic-producing identified actinomycetes,[16] which was selected for further phytochemical investigation. Preliminary TLC detection of the crude extract of compounds produced by *Kitasatospora* sp. MBT66 on MM agar plates showed several yellow UV-active spots (254 nm) and orange fluorescence at 366 nm, which turned reddish-brown by staining with anisaldehyde/sulfuric acid reagent. Repeated chromatographic separation of up-scaled extracts (2.0 g) coupled with subsequent spectroscopy data analysis resulted in the characterization of five previously undescribed rhamnosylated phenazines, endophenasides A–E (**1–5**) and one *N*-prenylated phenazine endophenazine F1 (**6**) with minor yield, together with the previously identified 1-carbomethoxy phenazine (**7**).[16] The structures of the compounds are presented in

Figure 1.

2.1. Resolution of the endophenaside structures

Compound **1** was obtained as a yellowish solid with a molecular formula of $C_{20}H_{21}N_3O_6$ established from high resolution mass $[M + H]^+$ peak at *m/z* 400.15076. The ^1H NMR (600 MHz, CD_3OD) of **1** (Table 1) showed six aromatic protons in two 1,2,3-trisubstituted aromatic spin systems, via δ_H 8.88 (dd, *J* = 7.2, 1.2, Hz, H-2), 8.07 (dd, *J* = 9.0, 7.2 Hz, H-3), and 8.54 (dd, *J* = 9.0, 1.2 Hz, H-4), along with 7.69 (dd, *J* = 7.2, 0.6 Hz, H-7), 7.97 (dd, *J* = 9.0, 7.2 Hz, H-8), and 8.15 (dd, *J* = 9.0, 0.6 Hz, H-9). Five protons in the range of δ_H 6.0–3.50, together with one methyl doublet at δ_H 1.26 (d, *J* = 6.0 Hz, H$_3$-6') in high magnetic field constituted another spin system by the confirmation of COSY experiment, which suggested the existence of a 6-deoxyhexapyranose sugar moiety (Figure 2). In addition, one distinct methoxyl δ_H 3.64 (s, H$_3$-7') was identified in the sugar region. The APT spectrum of compound **1** (Table 2) revealed 20 signals, which were assigned to carbonyl δ_C 168.7 (C-11), one oxygenated sp^2 carbon δ_C 152.8 (C-6), six sp^2 aromatic methine carbons δ_C 136.2 (C-2), 130.6 (C-3), 135.0 (C-4), 114.8 (C-7), 133.0 (C-8) and 123.5 (C-9) and five sp^2 quaternary carbons (δ_C 130.1 (C-1), 143.1 (C-4a), 137.8 (C-5a), 143.7 (C-9a), 141.7 (C-10a). Five oxygenated methine carbons δ_C 97.9 (C-1'), 81.5 (C-2'), 71.8 (C-3'), 73.7 (C-4'), and 70.9 (C-5'), together with one methyl group δ_C 17.7 (C-6'), were corresponding to the 6-deoxyhexapyranose by HSQC and APT spectra. The final structure of compound **1** was determined by analyses of the 2D NMR spectra (^1H-^1H COSY, HSQC and HMBC). The HMBC spectrum of compound **1** also suggested that it contained a 6-hydroxy-phenazine-1-carboxylic acid aglycone (H-2/C-11, C-10a, C-4; H-3/C-1; H-4/C-4a, C-2; H-7/C-9, C-6, C-5a; H-8/C-9a, C-6 and H-9/C-10a, C-7, C-5a). The aglycone was also confirmed by comparison of the spectral data with the literature.[27–29] Analysis of the COSY spectrum revealed one contiguous spin system formed by correlations of five oxygen-bearing methines and one doublet methyl signal at δ_H 1.39, confirming the deoxyhexapyranose. The connectivity of the 6-deoxysugar to the aglycone was confirmed from the HMBC long-range coupling of H-8 (δ_H 7.97) and H-1' (δ_H 6.03) to the C-6 (δ_C 152.8) of the phenazine nucleus. The additional characteristic methoxy group was attached onto C-2', based on the two key HMBC correlation H-2'/C-7' and H$_3$-7'/C-2' (Figure 2). The key HMBC correlation H-2/C-11 (δ_C 168.7) established a carbonyl substituent at C-1. To satisfy the molecular formula $C_{20}H_{21}N_3O_6$ deduced by HRMS, the substituent at C-1 had to be an amide instead of a carboxyl. Consequently, the structure of **1** was assigned as a new hydroxyphenzine-1-carboxyamide glycoside and designed as endophenaside A (Figure 1).

Compounds **2–4** and **6** possess the basic phenazine skeleton in comparison with **1**, and the structure elucidation was done by NMR and HRMS. Compound **2** gave a molecular formula of $C_{24}H_{26}N_2O_7$ established from a high-resolution mass measurement of the $[M + H]^+$ peak at *m/z* 455.18187. ^1H NMR (500 MHz, CD_3OD) comparison with compound **1** indicated the presence of a disubstituted phenazine ring, as well as the same existence of deoxyhexopyranose moiety in compound **2**. In contrast to compound **1**, the key HMBC correlation from H-1' (δ_H 6.41) to the carbonyl C-11 (δ_C 166.2) established the linkage of 6-deoxyhexopyranose moiety with phenazine ring through ester bond rather than ether bond. Moreover, an additional hydroxylated prenyl chain in **2** was established by intensive 2D NMR spectral analysis (Figure 2). NOESY experiments assigned the *trans*-position of the

hydroxymethyl group owing to coupling signals for 2″ and 5″ in combination with lacking signals for 2″ and 4″. The difference between endophenaside B (**2**) and the known compound phenazoviridin is the terminal hydroxylation in prenyl substitute.[30] The structure identification of compound **3** (endophenaside C) was effortless, since both high-resolution mass and ^{1}H NMR demonstrated an additional methyl group was added to **2**. Methylation pattern of the sugar moiety as seen for **1** was confirmed by HMBC correlation from H_3-7' to C-2' (δ_C 80.2). Endophenaside C (**3**) was a new glycosylated phenazine derivative, possessing the methylated sugar structural feature of compound **1**, as well as the hydroxylation attribute in the isoprenyl chain as in compound **2**. Compound **5** shared the same molecular formula $C_{24}H_{26}N_2O_7$ with **2**, as deduced from [M + H]$^+$ peak at *m/z* 455.18243. Though disubstituted phenazine and 6-deoxyhexopyranose feature were found in ^{1}H NMR spectrum (500 MHz, CD_3OD), difference was obviously observed as well: a *trans* double bond, δ_H 7.89 (d, *J* = 16.0 Hz, H-1″), 7.12 (d, *J* = 16.0 Hz, H-2″), and two methyls linked to saturated carbon, δ_H 1.54 (s, H_3-4″), 1.53 (s, H_3-5″). The double bond of the isoprenyl group was assumed to be rearranged from C-2″=C-3″ to C-2″=C-1″ due to the oxidation of C-3″, which was ultimately verified by the HSQC and HMBC spectral analysis (Figure 2). Remarkably, the 6-deoxyhexopyranose sugar of compound **4** (endophenaside D) was located at prenyl side chain through 3″-OH instead of 11-COOH, based on the key HMBC correlation from sugar anomeric proton H-1' (δ_H 6.43) to the oxygenated quaternary carbon C-3″ (δ_C 71.7). Compound **6** was isolated as a violet amorphous powder, whose molecular formula was deduced as $C_{23}H_{24}N_2O_4$ by experimental high resolution mass [M + H]$^+$ peak at *m/z* 393.18115. ^{1}H NMR spectrum (600 MHz, CD_3OD) of **6** indicated its attribution to phenazine type. Both ^{1}H NMR and APT spectra showed two prenyl side chains, but without the 6-deoxyhexopyranose sugar moiety. These two prenyl substitutents were respectively positioned at nitrogen atom 5 and carbon atom 9 by intensive 2D NMR analysis. The distinctive downfield carbonyl signal at δ_C 185.8 (C-7) in APT spectrum in combination with the two high field shifted narrow doublets in ^{1}H NMR spectrum, via δ_H 6.19 (d, *J* = 2.4 Hz, H-6) and 6.88 (d, *J* = 2.4 Hz, H-8), revealed oxidation pattern happening at C-7 of phenazine scaffold to form a conjugated ketone like chromophenazine C,[31] which was further reflected by additional UV/vis absorption at 518 nm and finally confirmed by the HMBC experiment. Also, the geometry of the double bond of the prenyl group was assigned as *trans* based on the NOESY correlation between H-2″ (δ_H 5.45) and H-5″ (δ_H 4.00). Notably, Heine and colleagues recently described endophenazines produced by *Kitasatospora* strain HK714, which were similar to but distinct from compound **1**.[32] Intensive spectroscopy data analysis for compounds **2–4** showed that their aglycone moieties were identical to those of previously described endophenazines,[32] except that compounds **2–4** carried an additional sugar moiety. Compound **6** (endophenazine F1) resembled endophenazine F, but contain a hydroxylated prenyl group at the *N*-5 position, with the hydroxyl group at a terminal methyl group.

Compound **5** (endophenazine E) featured a phenazine nucleus and a 6-deoxyhexopyranose sugar as shown by ^{1}H NMR. However, seven aromatic protons with overlapped and complicated coupling split patterns suggested a monosubstituted instead of a disubstituted pattern. The key HMBC correlation from sugar anomeric proton H-1' (δ_H 6.39) to C-11 (166.7) confirmed the connectivity of a sugar substituent to the phenazine core through a carbonyl group. Furthermore, endophenaside E (**5**) was prone to methanolysis to

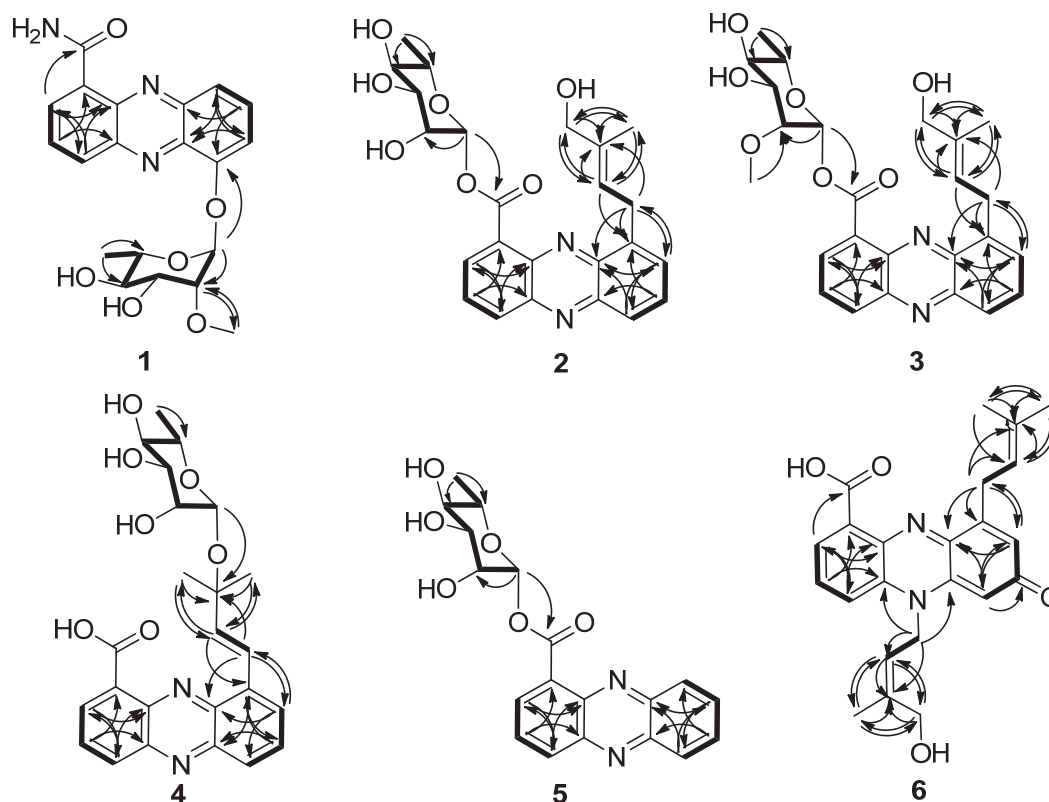generate 1-carbomethoxy phenazine (**7**) during NMR experiments (Figure S1).

Figure 2. HMBC and COSY correlations of new phenazine derivatives. Key HMBC ($\smile$) and ^{1}H-^{1}H COSY (—) NMR correlations of new phenazine derivatives are presented.

The relative stereochemistry of the 6-deoxyhexopyranose in compounds **1**–**5** was determined on the basis of NOE observations and coupling constants.[28,30] The small coupling constant ($J_{1',2'}$ 1.5 Hz) between the anomeric proton and H-2' suggested that H-1' and H-2' were both in equatorial positions and assigned the relative configuration of H-1' to be α, since the NOE was not observed either between H-1' and H-3' or between H-1' and H-5'. The NOE observed between H-3' and H-5' as well as the coupling constant between H-4' and H-5' ($J_{4',5'}$ 9.5 Hz) indicated that they were axially oriented. Based on further comparison with NMR data reported previously[28], 6-deoxyhexopyranose in compounds **1**–**5** was identified as an α-rhamnose (Figure 3). As mentioned above, few glycosylated phenazines have so far been found in nature, most of which are rhamnosides with significant bioactivity.[28,29] Endophenasides A (**1**) and C (**3**) are the first example for 2'-*O*-methylation of the rhamnose moiety in the phenazine glycosides.

The HMBC spectrum does not allow distinguishing between 1,6- or 1,9- disubstituted phenazine.[28–30,32,33] Indeed, the prenyl- and the carboxyl-group on the phenazine core of compounds **1**–**4**, could be either 1,6- or 1,9-disubstituted. However, the presence of a 1,9-disubstituted pattern is deduced from previous analysis of the aglycone moiety[32] that is also present in compounds **2**–**4**. Moreover, close scrutiny of *Kitasatospora* sp. MBT66 by UHPLC-Q-TOF analysis showed that *Kitasatospora* sp. MBT66 could in fact also produce most of the previously described aglycons (endophenazines) produced by *Kitasatospora* sp. HK714, albeit with a low yield (Figure S2 and Table S1). Conversely, the disubstitution pattern in compound **1** was tentatively proposed as [1,6-], which was deduced from the

possible biosynthesis pathway (see below).

Table 1. ^{1}H NMR data for compounds **1–6**.

NO.	1	2	3	4	5	6
2	8.88, dd (7.2, 1.2)	8.27, dd (7.0, 1.0)	8.29, dd (7.0, 1.0)	8.30, dd (7.0, 1.0)	8.38, dd (7.0, 1.5)	7.51, dd (7.2, 1.2)
3	8.07, dd (9.0, 7.2)	8.00, dd (8.5, 7.0)	8.02, dd (8.5, 7.0)	8.02, dd (7.5, 7.0)	8.02, dd (7.5, 7.0)	7.79, dd (8.4, 7.2)
4	8.54, dd (9.0, 1.2)	8.41, dd (8.5, 1.0)	8.43, dd (8.5, 1.0)	8.42, dd (7.5, 1.0)	8.46, dd (7.5, 1.5)	7.68, dd (8.4, 1.2)
6		8.10, dd (9.0, 1.5)	8.12, dd (9.0, 1.5)	8.15, dd (7.0, 1.0)	8.01, m	6.19, d (2.4)
7	7.69, dd (7.2, 0.6)	7.91, dd (9.0, 7.0)	7.93, dd (9.0, 7.0)	7.98, dd (7.5, 7.0)	8.02, m	
8	7.97, dd (9.0, 7.2)	7.81, dd (6.5, 1.0)	7.83, dd (6.5, 1.0)	8.16, dd (7.5, 1.0)	8.28 (m, 1H)	6.88, d (2.4)
9	8.05, dd (9.0, 0.6)				8.01, m	
1'	6.03, d (1.2)	6.41, d (1.5)	6.56, d (1.5)	6.43, d (1.5)	6.39, d (1.5)	3.83, d (7.8)
2'	4.04, dd (2.4, 1.8)	4.15, dd (3.5, 1.5)	3.81, dd (3.5, 1.5)	4.27, dd (3.5, 1.5)	4.13, dd (3.5, 1.5)	5.49, m
3'	4.28, dd (9.6, 3.6)	3.75, dd (9.5, 3.5)	3.80, dd (9.5, 3.5)	3.79, dd (9.5, 3.5)	4.01, dd (9.5, 3.5)	
4'	3.52, t (9.6)	3.53, t (9.5)	3.48, t (9.5)	3.58, t (9.5)	3.60, t (9.5)	1.78, s
5'	3.81, m	3.83, m	3.80, m	3.82, m	4.21, m	1.73, s
6'	1.26, d (6.0)	1.35, d (6.5)	1.33, d (6.0)	1.33, d (6.5)	1.39, d (6.0)	
7'	3.64, s		3.63, s			
1"		4.25, dd (15.5, 7.5); 4.18, dd (15.5, 2.5)	4.27, dd (15.5, 7.5); 4.20, dd (15.5, 2.5)	7.89, d (16.0)		5.17, d (5.4)
2"		5.86, m	5.87, m	7.12, d (16.0)		5.45, m
4"		1.88, s	1.89, s	1.54, s		1.98, s
5"		4.01, s	4.02, s	1.53, s		4.00, s

Recorded in CD$_3$OD. ^{1}H NMR spectra of **2—5** were recorded at 500 MHz, **1**, **6** at 600 MHz. Proton coupling constants (J) in Hz are given in parentheses. All chemical shift assignments were done on the basis of 1D and 2D NMR techniques.

Table 2. ^{13}C NMR data for compounds **1–6**.

NO.	1	2	3	4	5	6
1	130.1	132.7	131.3	131.4	132.2	145.5
2	136.2	132.3	132.9	132.7	133.5	122.7
3	130.6	130.5	130.5	130.7	130.2	134.6
4	135.0	133.3	132.5	133.1	133.9	114.7
4a	143.1	142.9	142.1	142.1	143.5	132.1
5a	137.8	143.3	143.1	143.6	143.6	139.8
6	152.8	127.7	127.1	127.8	132.4	98.6
7	114.8	132.7	132.8	132.7	132.5	185.8
8	133.0	130.6	130.5	128.0	129.7	132.3
9	123.5	142.0	141.3	137.0	132.4	146.6
9a	143.7	144.4	143.6	142.1	144.7	146.8
10a	141.7	140.6	140.2	140.3	141.8	133.4
11	168.7	166.2	166.0	165.6	166.7	175.8
1'	97.9	96.4	93.0	96.2	96.4	29.9
2'	81.5	70.9	80.4	70.5	70.7	122.1
3'	71.8	71.7	71.2	71.3	71.5	135.7
4'	73.7	73.1	72.9	72.7	72.8	25.9
5'	70.9	72.4	72.0	72.2	72.2	18.0
6'	17.7	17.9	17.5	17.7	17.6	
7'	59.3		59.2			
1"		29.5	29.1	121.3		47.1
2"		124.1	123.7	142.7		117.2
3"		137.6	137.1	71.7		142.8
4"		13.7	13.4	29.2		14.3
5"		68.4	68.0	29.2		67.3

Table 3. Antimicrobial activity of purified phenazine derivatives **1–7**.

Compound #	Inhibition zone (mm)	
	Bacillus subtilis	*Escherichia coli*
1	8	7
2	8	12
3	14	9
4	8	0
5	15	7
6	8	0
7	26	0
AMP	30	21
STR	12	19
NC	0	0

\# AMP, ampicillin; STR, streptomycin; NC, negative control (methanol)
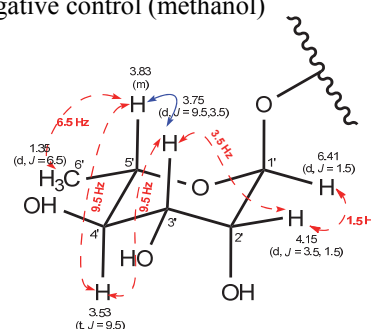

Figure 3. Selected NOE correlations ( ) and coupling constants ( ) in the sugar moiety of compound 2.

2.2. Antimicrobial activity of the endophenasides

Antimicrobial properties of compounds **1–7** were evaluated by agar diffusion assay, against the Gram-positive indicator strain *Bacillus subtilis* 168 and the Gram-negative *Escherichia coli* K12 (Table 3). Compound **5** showed highest antibiotic activity against both strains, while compounds **4** and **6** only inhibited *B. subtilis* and not *E. coli*. These antimicrobial activities are similar to the data published previously for the aglycones[32]. Remarkably, the glycosylation enhanced the activity of compounds **2** and **3** against Gram-negative *E. coli*, in contrast to what was reported for the corresponding aglycone. Taken together, our data show that the novel endophenasides described here have significant antimicrobial activity, whereby the glycosylation did not inhibit the bioactivity.

2.3. The phenazine biosynthetic gene cluster

Annotation of the genome sequence of *Kitasatospora* sp. MBT66 (Genbank accession number JAIY00000000 [26]) identified a gene cluster of around 25 kb that is likely responsible for the production of phenazine-type compounds. The gene products of the biosynthetic gene cluster (Table 4) were nearly identical to those of the endophenazine biosynthetic gene cluster of *Kitasatospora* sp. HKI 714 (*epaA-epaU* [32]; GenBank accession number: KJ207079).

Table 4. Gene organization of the phenazine biosynthetic gene cluster of *Kitasatospora* sp. MBT66 (Genbank accession number JAIY00000000). Proteins were named according to the gene cluster of *Kitasatospora* sp. HKI 714 (Genbank Accession KJ207079).[32] Literature references corresponding to the predicted functions are indicated in superscript.

ORF	Protein	Contig	Length (aa)	Annotation	Homologue in HKI 714	Homology
ORF1	EpaA	182	303	Prenyltransferase	AHW81460.1	100%
ORF2	EpaB	182	345	Mevalonate kinase	AHW81461.1	100%
ORF3	EpaC	182	353	Diphosphomevalonate decarboxylase	AHW81462.1	100%
ORF4	EpaD	182, 329	413	Phosphomevalonate kinase	AHW81463.1	98%
ORF5	EpaE	329	360	Isopentenyl-diphosphate δ-isomerase	AHW81464.1	100%
ORF6	EpaF	329	352	HMG-CoA reductase	AHW81465.1	100%
ORF7	EpaG	329	391	3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase	AHW81466.1	100%
ORF8	EpaH	329	51	Regulatory protein MerR	AHW81467.1	98%
ORF9	EpaI	329	207	Unknown function	AHW81468.1	99%
ORF10	EpaJ	329	160	Phenazine biosynthesis enzyme (PhzA/B [19])	AHW81469.1	100%
ORF11	EpaK	329	237	FMN-dependent oxidase (PhzG [19])	AHW81470.1	97%
ORF12	EpaL	329	278	*trans*-2, 3-dihydro-3-hydroxyanthranilate isomerase (PhzF [19])	AHW81471.1	99%
ORF13	EpaM	329	666	2-amino-2-desoxy-isochorismate (ADIC) synthase (PhzE [19])	AHW81472.1	99%
ORF14	EpaN	329	207	2, 3-dihydro-3-hydroxy-anthranilate (DHHA) synthase (PhzD [19])	AHW81473.1	100%
ORF15	EpaO	329	391	3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthase (PhzC [34])	AHW81474.1	99%
ORF16	EpaP	329	162	enzyme of phenazine biosynthesis (PhzA/B)	AHW81475.1	99%
ORF17	EpaQ	329	421	Rhodocoxin reductase	AHW81476.1	99%
ORF18	EpaR	329	107	Putative rhodocoxin	AHW81477.1	100%
ORF19	EpaS	329	305	Transcriptional regulator	AHW81478.1	99%
ORF20	EpaT	329	431	Cytochrome d ubiquinol oxidase subunit I	AHW81479.1	99%
ORF21	EpaU	329	360	Cytochrome d ubiquinol oxidase subunit II	AHW81480.1	99%
ORF22	*orf1*	329	598	ABC transporter ATPase	AHW81481.1	100%
ORF23	*of2*	329	569	ABC transporter	AHW81482.1	99%

It was proposed that EpaA catalyzes the prenylation at C-9 and/or *N*-5 positions of phenazine-1-carboxylic acid, similar to the function of enzyme PpzP in *Streptomyces anulatus*,[35]

which generated the [1,9-] substitution mode on the phenazine nucleus. From the perspective of biosynthesis, the formation of phenazine-1-carboxylic acid not only depends on the enzymes encoded by the gene cluster, but also involves spontaneous reaction without enzymatic catalysis.[19,36] The carboxyl group derived from chorismate is likely cleaved by molecular oxygen through oxidative decarboxylation, which would be alternatively replaced by a hydroxyl group in the aqueous environment (Figure 4). This may explain why 6-hydroxyphenazine-1-carboxylic acid derivatives were frequently identified,[28,29,37–39] while 9-hydroxyphenazine-1-carboxylic acid [40,41] was less often found.
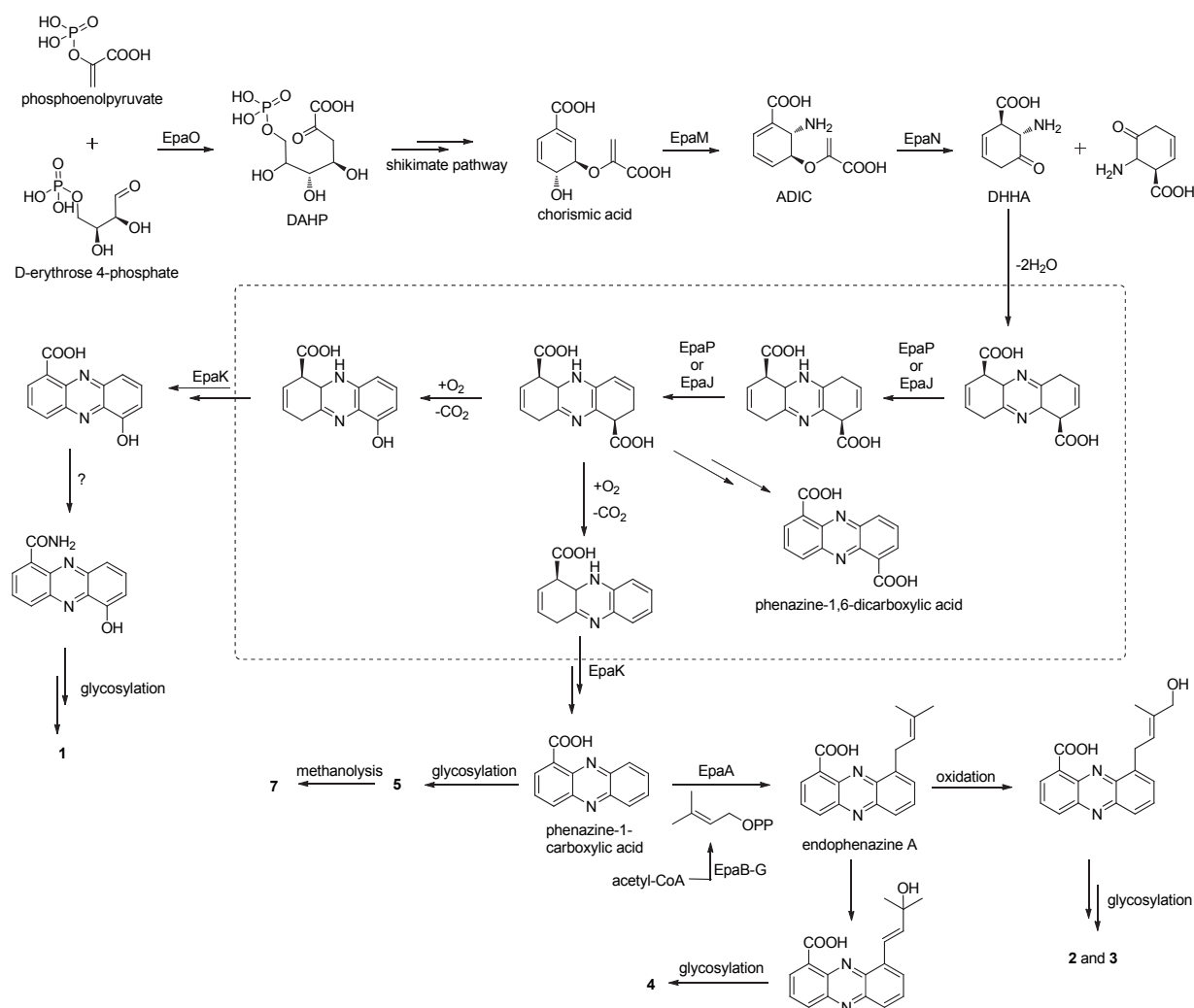

Figure 4. Proposed biogenesis of phenazine derivatives. Enzymes catalyzing the reaction correspond to Table 4. This biosynthesis pathway of phenazines was summarized according to references[19,20,34,36]. Reactions leading to the formation of the phenazine core are highlighted by a dashed box, which includes spontaneous reaction without enzymatic catalysis.[19,36] A yet uncharacterized glycosyltransferase and methyltransferase then give rise to the new glycosylated endophenazines (**1**–**5**) described in this study.

Besides the glycosylated endophenasides, we were able to show that *Kitasatospora* sp. MBT66 also produces small amounts of the same endophenazines as produced by *Kitasatospora* sp. HKI 714 (Figure S2 and Table S1). We expect that the two strains are closely related kitasatosporae, a genus closely related to *Streptomyces* in the family of *Streptomycetaceae*.[42] This should be resolved by comparison of the genomes and phenotypic characteristics of the two strains. It is yet unclear which genes are responsible for the

glycosylation of the endophenazine aglycones, as no such genes were found within or in close proximity of the *epa* gene cluster (Table 4). Therefore, the genes responsible for the transfer and methylation of the rhamnose moiety to either the carboxyl or the hydroxyl group of the phenazines should be located elsewhere on the chromosome. This is reminiscent of the biosynthesis of 7-prenylisatin in *Streptomyces* sp. MBT28, whereby the indispensable oxygenases responsible for the oxidation of 7-prenylindole into the7-prenylisatin were not found within or in close proximity of the *isa* gene cluster (Chapter 4). Research to identify the genes and enzymes responsible for the glycosylation of the endophenazines in *Kitasatospora* sp. MBT66 is described in Chapter 10.

3. CONCLUSION

We isolated a group of novel glycosylated endophenazines A–E (**1**–**5**), together with *N*-prenylated endophenazine F1 (**6**) from *Kitasatospora* sp. MBT66. The new compounds featured unusual structural properties including a 2'-*O*-methylation of the rhamnosyl moiety, which enriches the structural diversity of phenazine-class antibiotics. All phenazine derivatives showed antimicrobial activity against the Gram-positive *Bacillus subtilis*, while compounds **1**–**3** and **5** also inhibited growth of the Gram-negative *Escherichia coli*. The gene cluster (*epa*) responsible for the biosynthesis of phenazine core was identified in *Kitasatospora* sp. MBT66, which shows high homology to that of *Kitasatospora* sp. HKI 714. However, the genes responsible for the transfer and methylation of the rhamnose moiety to the phenazines are missing in *epa*, which must be located elsewhere on the chromosome (Chapter 10). We tentatively conclude that a non-clustered or subclustered genome arrangement for secondary metabolites biosynthesis in actinomycetes is more common than previously suggested. Though synthetic biology approaches will be a major trend for future antibiotics discovery (Chapter 1),[43] it may be less effective to handle this type of intertwined metabolic pathways, which could be instead overcome by the traditional systematic isolation methodology.

4. EXPERIMENTAL SECTION
4.1. General Experimental Procedures
FT-IR was measured on Perkin-Elmer FT-IR Spectrometer Paragon 1000. UV measurements were performed using a Shimadzu UV mini-1240. NMR spectra were recorded in methanol-d_4 on a Bruker DMX 500 MHz NMR and 600 MHz calibrated to a residual methanol-d_4 (3.30 ppm).

High resolution mass data (HRESIMS) were collected on an Agilent 1200 series HPLC conne cted to a LTQ-Orbitrap spectrometer. Semi-preparative HPLC (pHPLC) was performed with a Shimadzu HPLC system and a 5 mL Rheodyne manual injection loop, equipped with a reversed-phase C18 coloumn (Phenomenex Luna C18 (2) 100 A 5 micron 250 × 10 mm). All the pHPLC experiments used 2 mL/min flow rate and fraction collection based on detected peak. TLC was performed with silica gel 60 (Merck, Darmstadt, Germany) plates using CHCl$_3$/MeOH 10:1 and visualized with anisaldehyde/sulfuric acid reagent. All solvents and chemicals were of analytical and HPLC grade.

4.2. Microorganisms and Culture Conditions

Kitasatospora sp. MBT66 was isolated from Himalayan mountain soil as described previously.[16] The strain was cultivated on minimal media agar plates (MM)[44] with glycerol and mannitol (1% w/v) as the carbon sources. Square agar plates (12 cm × 12 cm) were inoculated with 5 x 10^7 spores from a fresh spore suspension. In total 125 plates were used, and incubated at 30 °C for 6 days. *Escherichia coli* K12 and *Bacillus subtilis* 168 were used for screening of antimicrobial activity.

4.3. Extraction and Isolation

Kitasatospora sp. MBT66 was fermented by stationary cultivation with a total volume of 5 liter agar medium. After fermentation for 6 days, mycelia were combined and extracted with ethyl acetate by soaking in solvent overnight at room temperature. The extraction was evaporated under reduced pressure to obtain 2.0 g of a crude residue. This extract adsorbed by silica gel was first chromatographed on a Macroporous resin Diaion HP-20 from Supelco (Bellefonte, PA, USA) by eluting stepwise from H_2O to MeOH. For monitoring, 20 μL of each fraction was subjected to TLC on premade silica gel plates (Merck, Darmstadt, Germany) using developing solvent system of chloroform and methanol (10:1). The 40% (v/v) methanol eluent showing interesting bands on TLC and moderate antibiotic activity in disc paper agar diffusion test with bacterium *Bacillus subtilis*, was subsequently 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 four subfractions (sfr.1–sfr.4). Sfr.2 was rechromatographed on Sephadex LH-20 coloumn, to purify the compound **2** (2.5 mg) and compound **6** (0.2 mg). Semi-preparative reversed-phase HPLC (Phenomenex Luna C18 (2) 100 A 5 micron 250 × 10 mm) purification of sfr.1, eluting with a gradient of MeOH in H_2O from 80% to 100% in 30 min, gave the pure compound **3** (t_R = 30.25 min, 0.3 mg) and **4** (t_R = 29.10 min, 0.2 mg). Separation of sfr.3 by HPLC using the gradient of MeOH in H_2O from 50% to 80% in 30min resulted in the purification of **5** (t_R = 21.25 min, 1.3 mg), and **7** (t_R = 28.50 min, 0.2 mg). Sfr.4 was similarly subjected to pHPLC employing the mobile phase MeOH in H_2O from 50% to 80% in 30 min and led to the separation of **1** (t_R = 17.7 min, 0.35 mg).

4.4. Antimicrobial activity assays

Antimicrobial activity was determined basically following the methods described in reference.[32] The respective compound (**1**–**7**) was dissolved in methnal (2 mg/ml), and 25 μl of the solution was applied on a paper disk (d = 6 mm). The disks were then placed onto an agar plate containing a softagar overlay with either *Bacillus subtilis* or *Escherichia coli*. 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.

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