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A Membrane-Bound Prenyltransferase Catalyzes the O-Prenylation of 1,6-Dihydroxyphenazine in the Marine Bacterium *Streptomyces* sp. CNQ-509

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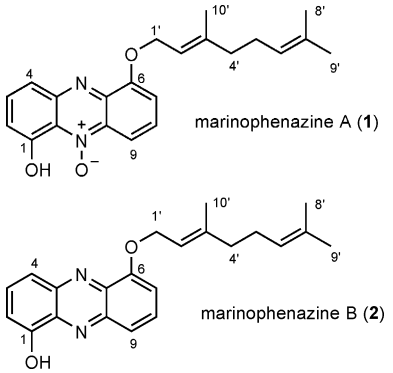
Streptomyces sp. CNQ-509 produces the rare O-prenylated phenazines marinophenazines A and B. To identify the enzyme catalyzing the O-prenyl transfer in marinophenazine biosynthesis, we sequenced the genome of *S.* sp. CNQ-509. This led to the identification of two genomic loci harboring putative phenazine biosynthesis genes. The first locus contains orthologues for all seven genes involved in phenazine-1-carboxylic acid biosynthesis in pseudomonads. The second locus contains two known phenazine biosynthesis genes and a putative prenyltransferase gene termed *cnqPT1*. *cnqPT1* codes for a membrane protein with sequence similarity to the prenyltransferase UbiA of ubiquinone biosynthesis. The enzyme CnqPT1 was identified as a 1,6-dihydroxyphenazine geranyltransferase, which catalyzes the C–O bond formation between C-1 of the geranyl moiety and O-6 of the phenazine scaffold. CnqPT1 is the first example of a prenyltransferase catalyzing O-prenyl transfer to a phenazine.

Introduction

Sediment-derived marine actinomycetes are widely recognized as an emerging source of novel and bioactive secondary metabolites.[1] They have been phylogenetically classified in 13 groups (MAR1–MAR13) within six families.[1a] The MAR4 group (family *Streptomycetaceae*) comprises 57 cultured strains[2] and is known to be a prolific source for a broad range of structurally unprecedented and pharmacologically active hybrid polyketide-terpenoid compounds, such as marinone, azamerone, and napyradiomycins.[3] One member of the MAR4 group is *Streptomyces* sp. CNQ-509, isolated from a marine sediment sample collected off La Jolla, California, USA. Analysis of this strain recently led to the isolation of the cytotoxic prenylated nitropyrroles nitropyrrolins A–E,[4] and O-prenylated phenazines (Scheme 1), termed marinophenazine A and marinophenazine B[5] (also known as phenaziterpene A).[6] Such O-prenylated phenazine ethers are rare and are produced exclusively by actinomycetes (e.g., phenaziterpenes A and B)[6] and by the archaeon *Methanosarcina mazei* Gö1 (methanophenazine).[7]

Previous studies have identified several prenyltransferases that catalyze the C-prenylation of phenazines in the secondary metabolism of *Streptomyces* strains.[8] However, no enzyme has been identified to catalyze the O-prenylation of phenazines. In order to elucidate the genetic and biochemical basis of this reaction, we sequenced the genome of *Streptomyces* sp. CNQ-509. This led to the discovery of a complete phenazine biosynthetic gene cluster as well as of an additional genetic locus containing two putative phenazine biosynthesis genes and a gene termed *cnqPT1*. The latter shows similarity to membrane-bound prenyltransferases of ubiquinone biosynthesis and was heterologously expressed in *Escherichia coli*. Biochemical investigation proved that the gene product converted 1,6-dihydroxyphenazine (1,6-DHP) and geranyl diphosphate (GPP) into marinophenazine B. CnqPT1 also accepted 1-hydroxyphenazine and the polyketide flaviolin as substrates, thus leading to the enzymatic formation of two new O-prenylated compounds, termed marinophenazine C and 2-*O*-geranylflaviolin, respectively.



Scheme 1. Structures of the prenylated phenazine ethers marinophenazine A (**1**) and B (**2**) from *Streptomyces* sp. CNQ-509.

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Results

Analysis of the *Streptomyces* sp. CNQ-509 genome sequence

In order to identify the gene cluster for marinophenazine biosynthesis, we sequenced the genomic DNA of *Streptomyces* sp. CNQ-509 and obtained a draft genome sequence. Analysis on the antiSMASH 2.0 platform[9] identified a putative phenazine gene cluster with 26 coding sequences (Figure 1 A, Table S1 in the Supporting Information). Seven genes (designated *cnq726–731* and *cnq734*) showed high similarity to the genes of the *phzABCDEFG* operon identified in several *Pseudomonas* strains.[10] In pseudomonads these genes code for enzymes that catalyze the formation of phenazine-1-carboxylic acid (PCA) from intermediates of the shikimate pathway. Near these core phenazine biosynthesis genes we also identified three genes coding for putative phenazine-modifying enzymes. *cnq735* codes for a putative flavin-dependent monooxygenase similar to PhzS from *Pseudomonas aeruginosa* PAO1,[11] and *cnq725* codes for a putative methyltransferase showing similarity to PhzM from the same *Pseudomonas* strain.[10b] Furthermore, Cnq738 showed similarity to prenyltransferases of the ABBA superfamily,[12] for example, PpzP and EpzP, the two prenyltransferases of endophenazine biosynthesis.[8a,b] Therefore, we first speculated that Cnq738 catalyzes O-prenylation in marinophenazine biosynthesis. In a postulated pathway for the biosynthesis of marinophenazine A and B (**1** and **2**; Scheme 2), **2** is a geranylated derivative of 1,6-DHP, and 1,6-DHP is likely derived from phenazine-1,6-dicarboxylic acid (PDC). PDC is a key intermediate in the biosynthesis of phenazines in some *Streptomyces* strains.[13] In analogy to the reaction catalyzed by PhzS,[11] PDC might be converted into 1,6-DHP in two hydroxylative decarboxylation steps. Subsequent transfer of a geranyl moiety (C_{10}) and oxidation of the nitrogen would lead to **1**.

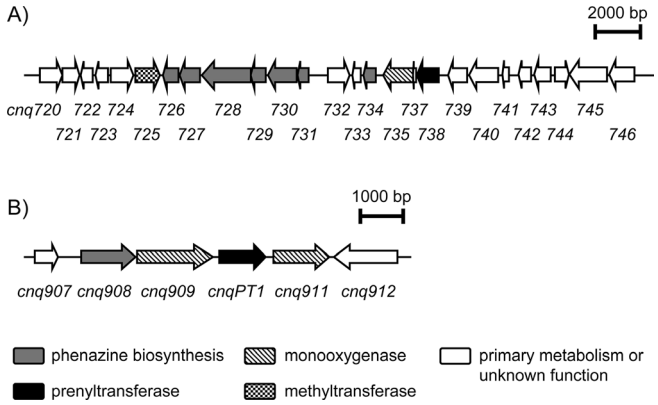
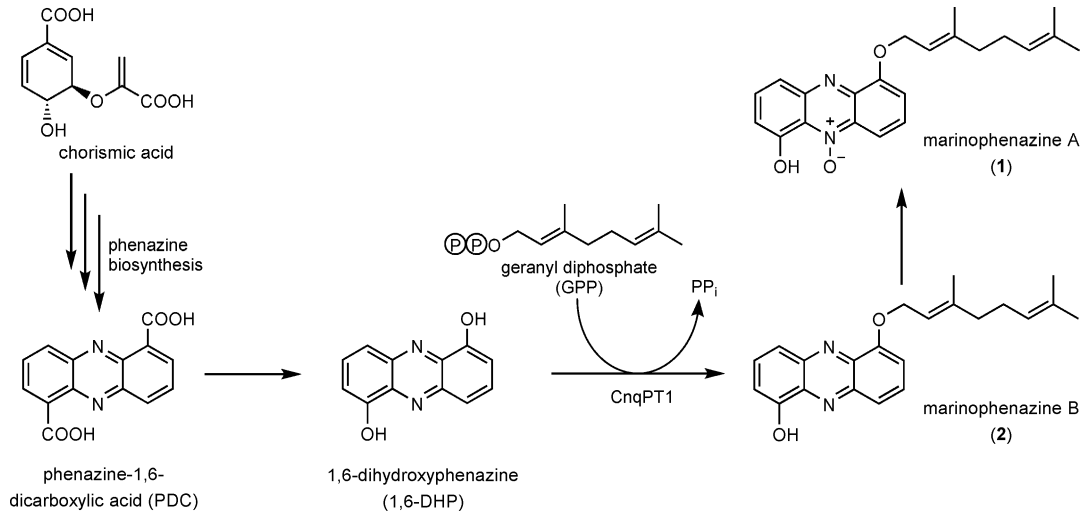


Figure 1. A) Putative phenazine biosynthetic gene cluster and B) the region around *cnqPT1* in the genome sequence of *Streptomyces* sp. CNQ-509. See Tables S1 and S2 for further information concerning these loci.

To test whether *cnq738* indeed codes for the prenyltransferase in the biosynthesis of **1** and **2**, we cloned *cnq738* into an expression vector. The corresponding His-tagged Cnq738 was overexpressed in *E. coli* and purified to apparent homogeneity. Unexpectedly, however, no formation of prenylated products was observed in incubations of Cnq738 with 1,6-DHP and GPP (data not shown). Therefore, we searched the genome of *Streptomyces* sp. CNQ-509 for further putative prenyltransferase and associated phenazine genes.

Approximately 169 kb from the phenazine gene cluster, we identified a locus that consisted of genes for a putative prenyltransferase, a putative core phenazine biosynthesis enzyme, and two putative monooxygenases (Figure 1 B, Table S2). Gene *cnq908* showed similarity to *phzF* from *Pseudomonas fluorescens* which codes for a *trans*-2,3-dihydro-3-hydroxyanthranilate isomerase, an essential enzyme in the biosynthesis of phenazines.[14] Genes *cnq909* and *cnq911* encode putative monooxygenases; Cnq909 showed similarity to a cyclohexanone monooxygenase, which catalyzes the oxidation of a cyclic ketone into a lactone.[15] The putative flavin-dependent monooxygenase Cnq911 is similar to PhzS from *Pseudomonas aeruginosa*.[11]



Scheme 2. Putative biosynthetic pathway of marinophenazine A (**1**) and B (**2**) in *Streptomyces* sp. CNQ-509.

PhzS catalyzes the oxidative decarboxylation of 5-methyl-PCA, the final step in the biosynthesis of pyocyanin from PCA. Between these two putative monooxygenase genes was a gene (designated *cnqPT1*) that codes for an enzyme with similarity to membrane-bound 4-hydroxybenzoate polyprenyltransferases of ubiquinone biosynthesis.[16] CnqPT1 comprises 334 amino acids with six transmembrane helices, as predicted by the TMHMM server (v. 2.0),[17] thus CnqPT1 was predicted to be an integral membrane protein.

Table 1. Membrane localization of CnqPT1 activity. The reaction mixtures (100 μL) contained Tris·HCl (50 mM, pH 8.8), 0.5 mM 1,6-DHP, 0.5 mM GPP, 5 mM $MgCl_2$, 20 mM NaCl, and 11.4 μL of each fraction. The mixtures were incubated for 10 min at 30 °C.

Fraction	Total protein [mg]	Product formation [nmol s^{-1}]	[%]	Specific activity [pmol s^{-1} mg^{-1}]	[%]
crude protein extract	607.8	8.9	100.0	14.6	100.0
supernatant (100 000 *g*)	516.6	0.1	1.1	0.1	0.7
membrane fraction	75.4	5.2	58.4	69.2	474.0

Enzyme activity and subcellular localization of CnqPT1

To investigate the biochemical activity of the putative prenyltransferase CnqPT1, *cnqPT1* was amplified from genomic DNA of *Streptomyces* sp. CNQ-509 and ligated into the vector pET-28a(+). The resulting plasmid pPH25 was expressed in *E. coli*, and a crude protein extract was prepared. In order to isolate the membrane fraction, the crude extract was subjected to ultracentrifugation at 100 000 *g*. The resulting pellet was resuspended and incubated with 1,6-DHP, GPP, and Mg^{2+}. HPLC analysis readily showed the formation of an enzymatic product (Figure 2 A). In a corresponding LC-MS analysis run, a molecular ion at *m/z* 349 $[M+H]^+$ was detected for the product, thus suggesting it to be a monogeranylated derivative of 1,6-DHP (incubations with a membrane fraction from cells harboring the empty vector showed no product formation; Figure 2 A). A comparative analysis of the spectral properties of the product with its reactant (1,6-DHP) as well as an independent structure elucidation employing 1D and 2D NMR spectroscopy and high-resolution MS confirmed that the enzymatic product was marinophenazine B (**2**; Scheme 1 and Figures S1–S6, Tables S3 and S4).

To verify the subcellular localization of the prenyltransferase activity we compared the crude protein extract, the supernatant after centrifugation at 100 000 *g*, and the pelleted membrane fraction (Table 1). The enzymatic activity was almost completely localized in the membrane fraction; only negligible activity was found in the supernatant. The specific activity of the membrane fraction was 4.7 times higher than that of the crude protein extract. These data proved that CnqPT1 is a membrane-bound enzyme (located in the membrane of *E. coli* after heterologous expression). All further biochemical experiments were carried out with the membrane fraction.

Biochemical properties of CnqPT1

In our standard assay for prenyltransferase activity (see the Experimental Section), product formation was linear up to 0.5 mg mL^{-1} membrane protein and up to 20 min incubation time. Maximum product formation was at pH 8.8. Addition of 20 mM NaCl slightly increased the activity (by ~6%), and therefore 20 mM NaCl was added in all subsequent assays.

Dependence of the prenyltransferase activity on divalent cations

The enzymatic activity of membrane-bound prenyltransferases of lipoquinone biosynthesis (e.g., ubiquinone biosynthesis) is

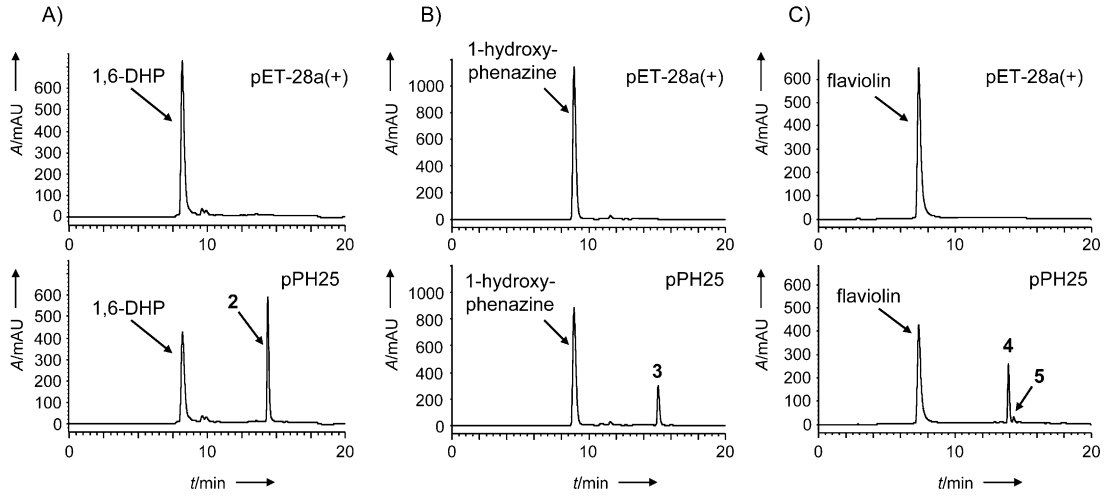


Figure 2. HPLC analysis of prenyltransferase assays. Upper chromatograms: membrane fraction from *E. coli* harboring the empty vector pET-28a(+). Lower chromatograms: membrane fraction from *E. coli* harboring the *cnqPT1* expression plasmid pPH25. Incubations contained GPP, Mg^{2+}, and aromatic substrates: A) 1,6-dihydroxyphenazine (1,6-DHP), B) 1-hydroxyphenazine, and C) flaviolin.

absolutely dependent on the presence of Mg^{2+} or similar divalent cations.[18] These enzymes contain an aspartate-rich motif (e.g., NxxxDxxxD) for binding to the prenyl diphosphate with an Mg^{2+} ion.[18–19] Likewise, the activity of CnqPT1 was also strictly dependent on the presence of Mg^{2+} ions, with the highest activity detected at 5 mM Mg^{2+}. The addition of 2 mM EDTA without divalent cations abolished product formation, thus proving the absolute requirement for divalent cations for CnqPT1 prenyltransferase activity. Replacement of Mg^{2+} by Ca^{2+} (5 mM) reduced activity by 99%. These results are consistent with the observation that CnqPT1 incorporates the aspartate-rich motif (aa 90–98: NALADREQD).

Substrate specificity

To test the substrate specificity of CnqPT1, the enzyme was incubated with different aromatic and isoprenoid substrates. With 1,6-DHP as the aromatic substrate, GPP was clearly the preferred isoprenoid substrate. Use of dimethylallyl diphosphate or farnesyl diphosphate resulted in only 0.5 or 1.7% activity, respectively, relative to the value observed with GPP. When CnqPT1 was incubated with 1-hydroxyphenazine and GPP, formation of **3** was detected by HPLC (Figure 2B). LC-MS analysis showed a molecular ion at *m/z* 333 $[M+H]^+$ for compound **3**, thus suggesting it to be a monoprenylated derivative of 1-hydroxyphenazine (the exact structure of **3** is described below). The reaction with 1-hydroxyphenazine and dimethylallyl diphosphate revealed 1.0% activity relative to that with GPP; with 1-hydroxyphenazine and farnesyl diphosphate it was 1.8%. No product formation was observed when CnqPT1 was incubated with the flavonoid naringenin or with 1,6-dihydroxynaphthalene. However, incubations with flaviolin (2,5,7-trihydroxy-1,4-naphthoquinone) and GPP resulted in the formation of **4** and **5** (Figure 2C). In LC-MS analyses both products showed a molecular ion at *m/z* 341 $[M-H]^-$, thus suggesting that they are monogeranylated derivatives of flaviolin (structure elucidation of the main product **4** is explained below). In comparison to the presumed genuine substrate 1,6-DHP, the enzymatic geranylation of flaviolin proceeded at least six times slower. 4-Hydroxybenzoate is the substrate of the prenylation reaction in ubiquinone biosynthesis in *E. coli*.[16] Incubations with 4-hydroxybenzoate and GPP or farnesyl diphosphate showed low product formation, but these products were also detected in incubations with membrane fractions harboring the empty vector, and therefore are most likely due to the UbiA homologue of the *E. coli* expression strain.

Steady-state kinetics of CnqPT1

The CnqPT1 reaction displayed Michaelis–Menten kinetics. For 1,6-DHP and GPP (the presumed genuine substrates) the apparent K_m values were 255 and 47 μM, respectively; the maximum velocity of this reaction was 34.0 nmol min^{-1} mg^{-1}. With 1-hydroxyphenazine as the aromatic substrate, an apparent K_m of 131 μM was determined with GPP as the isoprenoid substrate (maximum velocity 9.2 nmol min^{-1} mg^{-1}). Therefore, the catalytic activity of CnqPT1 for 1,6-DHP was nearly twice as high as for 1-hydroxyphenazine.

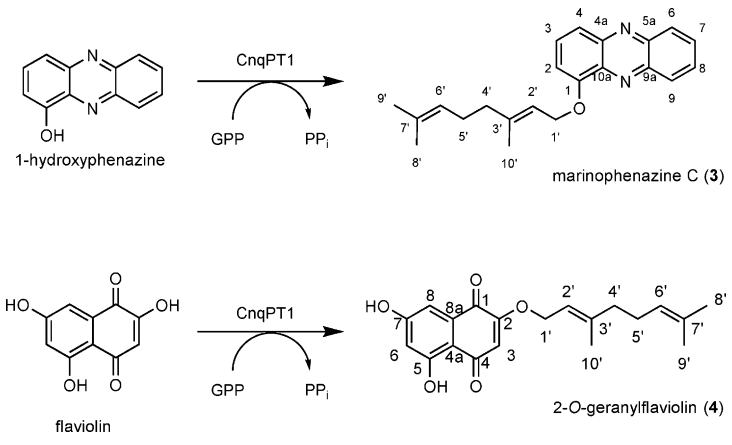
Identification of enzymatically generated marinophenazine C (3)

The structure of compound **3** was elucidated by comparison of the spectral properties of the product with that of its aromatic reactant. Product **3** was formed by the enzymatic reaction of 1-hydroxyphenazine with GPP. In comparison to 1-hydroxyphenazine, the ^{13}C NMR spectrum of **3** showed ten additional resonances (Table 2, Figure S8), attributed to a geranyl moiety by $^1H,^1H$ COSY, $^1H,^{13}C$ HSQC, and $^1H,^{13}C$ HMBC NMR experiments. Further interpretation of the NMR data revealed the regiospecificity of the prenylation reaction. In comparison with the reactant, both spin systems of the phenazine core structure remained mainly intact (Figure S7), thus suggesting that 1-hydroxyphenazine was not C-prenylated but prenylated rather at one of the heteroatoms. 2D NMR experimental results, particularly the $^1H,^{13}C$ HMBC cross correlation between CH_2-1′ and quaternary carbon C-1, confirmed that product **3** was O-prenylated (Figure S9). Product **3** was thus determined to be 1-((3,7-dimethylocta-2*E*,6-dien-1-yl)oxy)phenazine and named marinophenazine C (Scheme 3).

Identification of enzymatically generated 2-*O*-geranylflaviolin (4)

As described above, incubation of CnqPT1 with flaviolin and GPP yielded the two prenylated products **4** and **5**. Although we were able to obtain a sufficient amount of **4** for structure elucidation by a preparative-scale assay (see the Experimental Section), the low amount of **5** prevented complete elucidation of its structure.

A complete set of NMR spectra revealed that **4** is an O-prenylated flaviolin derivative (Table 3, Scheme 3). The ^1H NMR spectrum exhibited three resonances, which were attributed to the methine groups of the flaviolin core, thus indicating O-prenylation of flaviolin (Figure S10). Interpretation of the 2D NMR



Scheme 3. Enzymatic products marinophenazine C (**3**) and 2-*O*-geranylflaviolin (**4**).

Table 2. NMR spectroscopic data for 1-hydroxyphenazine and marinophenazine C (**3**) in [D$_6$]acetone (δ in ppm, J in Hz).

Position	1-Hydroxyphenazine		Marinophenazine C (3)		
	δ_H[a,b]	δ_C[b,c,d]	δ_H[e]	δ_C[d,f]	HMBC[g]
1		153.7 qC		155.6 qC	
2	7.23 (dd, J=7.4, 1.2)	110.1 CH	7.23 (dd, J=7.4, 1.2)	109.1 CH	1, 4, 10a
3	7.83 (dd, J=8.9, 7.4)	132.7 CH	7.80 (dd, J=8.8, 7.4)	131.7 CH	1, 2, 4a, 10a
4	7.73 (dd, J=8.9, 1.2)	120.5 CH	7.75 (dd, J=8.8, 1.2)	121.8 CH	2, 10a
4a		144.8 qC		145.2 qC	
5a		144.9[i] qC		143.0[i] qC	
6	8.22[j] (m)	130.5[j] CH	8.19[j] (d, J=7.9)	130.2[j] CH	5a, 8
7	7.93[h] (m)	131.4[h] CH	7.91[h] (m)	131.6[h] CH	5a, 6, 9a
8	7.93[h] (m)	131.7[h] CH	7.91[h] (m)	130.9[h] CH	5a, 6, 9a
9	8.22[j] (m)	130.1[j] CH	8.26[j] (d, J=7.9)	130.8[j] CH	7, 9a
9a		142.1[i] qC		144.1[i] qC	
10a		136.0 qC		138.2 qC	
1′			4.91 (d, J=6.4)	66.8 CH_2	1, 2′, 3′
2′			5.67 (t, J=6.4)	120.6 CH	4′, 10′
3′				141.5 qC	
4′			2.14 (m)	40.2 CH_2	2′, 3′, 5′
5′			2.14 (m)	27.0 CH_2	4′, 6′, 7′
6′			5.12 (m)	124.7 CH	5′
7′				132.1 qC	
8′			1.59 (s)	17.7 CH_3	6′, 7′, 9′
9′			1.63 (s)	25.8 CH_3	6′, 7′, 8′
10′			1.82 (s)	16.8[k] CH_3	2′, 3′, 4′
OH	9.23 (br)				

[a] Recorded at 400 MHz. [b] Measured values were in good agreement with published NMR data for 1-hydroxy- or 1-methoxyphenazine.[34,36] [c] Recorded at 101 MHz. [d] Multiplicity determined by a multiplicity edited ^{1}H,^{13}C HSQC NMR experiment. [e] Recorded at 600 MHz. [f] Recorded at 151 MHz. [g] Protons showing long-range correlation with indicated carbon. [h], [i], [j] Assignments interchangeable. [k] On the basis of the ^{13}C NMR chemical shift for C-10′ (δ_C=16.8), the geometry of the $\Delta^{2',3'}$ double bond was defined as *E*.[37]

data (Figures S11–S14) further supported this assumption and revealed the regiospecificity of the prenylation reaction. Cross correlations in the ^{1}H,^{1}H ROESY NMR spectrum between CH_2-1′ and CH-3 (Figure S13), as well as the ^{1}H,^{13}C HMBC correlation between CH_2-1′ and C-2 (Figure S14), unequivocally proved that flaviolin was O-prenylated at the hydroxy group at position 2 of the flaviolin core. Thus, **4** was determined to be 2-((3,7-dimethylocta-2*E*,6-dien-1-yl)oxy)-5,7-dihydroxynaphthalene-1,4-dione (Scheme 3) and named 2-*O*-geranylflaviolin.

Discussion

Only a few O-prenylated phenazines have been isolated from microorganisms.[5–7] The unusual O-prenylation of **1** and **2** drew our attention to the corresponding prenylating enzyme. Analysis of the genome sequence identified the putative gene cluster of marinophenazines, which possess three distinctive features. 1) In contrast to most biosynthetic gene clusters of known prenylated phenazine derivatives,[8] the *cnq* genes are not physically linked but are in two separate clusters (Figure 1), here referred to as loci A and B. Such a split phenazine gene cluster has so far only been reported for the endophenazine gene cluster of *Streptomyces cinnamonensis*.[8b] 2) The genes for isoprenoid biosynthesis via the mevalonate pathway are also absent from both loci, in contrast to previously identified gene clusters for prenylated phenazine biosynthesis.[8] 3) Compared to all other biosynthetic gene clusters of prenylated phenazine derivatives, strikingly, both locus A and locus B contain a gene encoding a prenyltransferase of a different class (*cnq738* and *cnqPT1*, respectively). *cnq738* codes for a putative prenyltransferase of the ABBA superfamily,[12,20] and was initially suspected to be responsible for the prenylation reaction because of the resemblance to known gene cluster structures.[8] To our surprise, Cnq738 was unable to catalyze the expected prenylation reaction in marinophenazine biosynthesis. The physiological function, if any, of Cnq738 remains unknown. Intriguingly, *cnqPT1* is completely unrelated to ABBA prenyltransferases, and is predicted to be a membrane-bound aromatic prenyltransferase. Biochemical investigations proved that CnqPT1 transfers a geranyl moiety onto 1,6-DHP to form **2**. As **2** was also isolated from *Streptomyces* sp. CNQ-509,[5] it represents a likely inter-

Table 3. NMR spectroscopic data for 2-*O*-geranylflaviolin (**4**) in [D$_6$]acetone (δ in ppm, J in Hz).

Position	δ_H[a]	δ_C[b,c]	COSY	ROESY	HMBC[d]
1		179.9 qC			
2		160.9 qC			
3	6.15 (s)	110.8 CH		1′, 5-OH	1, 2, 4, 4a, 5, 8a
4		190.6 qC			
4a		108.8[e] qC			
5		164.3 qC			
6	6.62 (d, J=2.3)	108.8 CH	8	5-OH	4a, 5, 7, 8
7		164.6 qC			
8	7.07 (d, J=2.3)	108.6 CH	6		1, 4a, 6, 7, 8a
8a		134.1 qC			
1′	4.70 (d, J=6.6)	67.2 CH_2	2′	3, 2′,10′	2, 2′, 3′
2′	5.50 (t, J=6.6)	118.6 CH	1′	1′	1′, 4′, 10′
3′		143.6 qC			
4′	2.12 (m)	40.1 CH_2	5′		2′, 3′, 5′, 6′, 10′
5′	2.14 (m)	26.9 CH_2	4′, 6′		3′, 4′, 6′, 7′
6′	5.11 (t, J=6.3)	124.6 CH	5′		8′, 9′
7′		132.2 qC			
8′	1.59 (s)	17.7 CH_3			6′, 7′, 9′
9′	1.64 (s)	25.8 CH_3			6′, 7′, 8′
10′	1.78 (s)	16.7[f] CH_3		1′	2′, 3′, 4′
5-OH	12.50 (s)			3, 6	5, 6, 7

[a] Recorded at 500 MHz. [b] Recorded at 125 MHz. [c] Multiplicity determined by multiplicity edited ^{1}H,^{13}C HSQC and ^{13}C JMOD NMR experiments. [d] Protons showing long-range correlation with indicated carbon. [e] Extracted from the ^{1}H,^{13}C HMBC spectrum, because of overlapping resonances in the ^{13}C JMOD NMR experiment. [f] On the basis of the ^{13}C NMR chemical shift for C-10′ (δ_C=16.7), the geometry of the $\Delta^{2',3'}$ double bond was defined as *E*.[37]

mediate in the biosynthesis of **1**. The isolation of **2** indicates that the prenylation reaction occurs before N-oxidation rather than after (Scheme 2). It appears likely that the phenazine genes of locus A synthesize the phenazine precursor (probably PDC), whereas the gene products of locus B are involved in the conversion of this phenazine precursor to **1** (Scheme 2).

The biochemical results obtained for CnqPT1 strongly suggest its involvement in marinophenazine biosynthesis. However, confirmation of this hypothesis by genetic experiments was not possible. Despite repeated attempts, *Streptomyces* sp. CNQ-509 could not be manipulated genetically, thus preventing inactivation of *cnqPT1*.

In contrast to the soluble and mostly Mg^{2+}-independent ABBA prenyltransferases, CnqPT1 is membrane-bound and Mg^{2+} dependent, similar to membrane-bound aromatic prenyltransferases of primary metabolism, for example UbiA of ubiquinone biosynthesis and MenA of menaquinone biosynthesis in *E. coli*.[16,21] These membrane-bound enzymes were initially identified in microorganisms solely in primary metabolism,[18] but recently some of these enzymes were found to be involved in the biosynthesis of secondary metabolites in fungi[22] and bacteria.[8c,23] In the rare actinomycete *Actinoplanes missouriensis*, a membrane-bound prenyltransferase catalyzes O-prenylation in the biosynthesis of a polyketide.[24] Similarly to CnqPT1, this enzyme uses GPP as the prenyl donor, but with a hydroquinone instead of a phenazine as the prenyl acceptor. Enzymes involved in the O-prenylation of a phenazine scaffold have not previously been described (either from the ABBA superfamily or from membrane-bound aromatic prenyltransferases). Therefore, CnqPT1 is the first enzyme to catalyze this type of prenylation reaction. In contrast to UbiA of ubiquinone biosynthesis, the prenyl donor specificity of CnqPT1 can be judged tentatively as rather narrow (GPP as the isoprenoid substrate). In this context it is noteworthy that CnqPT1 shows a new regiospecificity regarding the tri-hydroxylated aromatic compound flaviolin. Previously described prenylated flaviolins are solely either O-prenylated at position 7 (e.g., the neuroprotective flaviogeranin)[25] or C-prenylated at position 3[26] or 6.[27] As CnqPT1 performs prenylation at position 2 of the flaviolin skeleton, this prenyltransferase represents also a useful tool to generate new bioactive prenylated aromatic compounds.

Experimental Section

Chemicals: Dimethylallyl diphosphate, GPP, and farnesyl diphosphate were synthesized according to Woodside et al.[28] Flaviolin was prepared as described by Gross et al.[29] Other chemicals and molecular biological agents were obtained from standard commercial sources.

General experimental procedures: HPLC analysis was performed on a 1100 series system (Agilent Technologies) coupled to a photodiode array detector or a variable wavelength detector. ESI-LC/MS analysis was carried out with a 1200 series system (Agilent Technologies) coupled to an ESI spectrometer (LC/MSD Ultra Trap System XCT 6330). FT-IR spectra were recorded on an FT/IR-4200 spectrometer (Jasco) interfaced to a MIRacle ATR (ZnSe) system (PIKE Technologies, Madison, WI). UV spectra were obtained on a Lambda25 spectrometer (PerkinElmer). 1D and 2D NMR spectra were recorded on Avance III 400, 500, and 600 and DRX-250 spectrometers (Bruker). Spectra were referenced to residual solvent signals ([D_6]acetone or [D]chloroform; $\delta_{H/C}$ 2.04/29.8 and 7.27/77.0, respectively). High-resolution mass spectra were acquired on an HR-ESI-TOF-MS maXis 4G mass spectrometer (Bruker) and on an APEX II FT-ICR-MS instrument (Bruker).

Bacterial strains and culture conditions: *Streptomyces* sp. CNQ-509 was kindly provided by William Fenical and Paul R. Jensen (Scripps Institution of Oceanography, University of California, San Diego) and cultured as described previously.[4] *Brevibacterium iodinum* DSM 433 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany) and cultivated as recommended by the DSMZ. *E. coli* XL1 Blue MRF′ (Stratagene/Agilent Technologies) was used for cloning and grown on solid or in liquid LB medium at 37 °C. Recombinant strains were selected with kanamycin (50 μg mL^{-1}).

Genetic procedures: DNA isolation and manipulation were carried out by standard methods (Sambrook and Russell[30] and Kieser et al.).[31] Genomic-tip 100/G columns (Qiagen) were used to purify genomic DNA.

Genome sequencing and bioinformatic sequence analysis: An 8k paired-end and a whole-genome shotgun library were prepared according to standard protocols (Roche Applied Science). For sequencing of *Streptomyces* sp. CNQ-509 genomic DNA, a Genome Sequencer FLX System and Titanium chemistry (Roche Applied Science) were used. Assembly of the sequence reads was performed with GS Assembler Software (version 2.5.3; Roche). BLAST[32] was used for database searches, and the Artemis[33] program was used for sequence analysis.

Preparation of 1,6-DHP: *B. iodinum* was precultured for four days at 30 °C and 200 rpm in DSMZ medium 1 (50 mL; peptone (0.5 %) and meat extract (0.3 %); pH 7.0 before sterilization). The production medium (1.25 L; yeast extract (1 %), glucose (1 %), L-valine (0.1 %), and Diaion HP-20 (1 %; Mitsubishi Chemical); pH 7.0 before sterilization) was inoculated with preculture (20 mL) and cultured for eight days at 26 °C and 200 rpm. Subsequently, the culture broth was extracted with CH_2Cl_2 (1.25 L). The extract was passed through a filter paper, dried over Na_2SO_4, and evaporated, in order to obtain an iodinin extract (250 mg). Iodinin (1,6-dihydroxyphenazine-5,10-dioxide) was reduced to 1,6-DHP according to the method of Breitmaier and Hollstein.[34] The crude reaction product 1,6-DHP (220 mg) was purified on a silica gel 60 column (mobile phase: toluene/MeOH (975:25)) to yield 1,6-DHP (31.6 mg).

Protein expression and preparation of enzyme extracts: Primers cnqpt1_FW2 (5′-CGATCCCATGGGACGACGAGAG-3′) and cnqpt1_RV2 (5′-GTGGGAATTCGCCGGGTGGTC-3′) were used to amplify the putative prenyltransferase gene *cnqPT1* from *Streptomyces* sp. CNQ-509 genomic DNA (NcoI and EcoRI restriction sites underlined). The PCR product was cloned into pET-28a(+) (Novagen/Merck Millipore) to produce the expression construct pPH25, which was verified by restriction mapping and sequencing.

An overnight culture (17.5 mL) of *E. coli* Rosetta 2(DE3)pLysS (Novagen) harboring pPH25 in LB medium with kanamycin (50 μg mL^{-1}) and chloramphenicol (25 μg mL^{-1}) was used to inoculate Terrific Broth medium[30] (500 mL) supplemented with kanamycin (50 μg mL^{-1}) and chloramphenicol (25 μg mL^{-1}). The cells were cultured at 37 °C and 250 rpm to OD_{600} = 0.6–0.7. After lowering the temperature to 20 °C, isopropyl β-D-1-thiogalactopyranoside (IPTG)

was added (final concentration, 0.5 mM). The cells were grown overnight (15–16 h) at 20 °C and 250 rpm then harvested by centrifugation (6080 *g*, 10 min). The resulting pellet was washed with Tris·HCl (50 mM, pH 7.5) and centrifuged again. Cells were resuspended in lysis buffer (Tris·HCl (50 mM, pH 7.5), lysozyme (0.5 mg mL^{-1}), 1,4-dithiothreitol (10 mM); 2 mL per gram cell wet weight) and ruptured by sonication (W-250D Sonifier; Branson, Danbury, CT). The lysate was centrifuged (5000 *g*, 15 min, 4 °C) to remove cell debris. The obtained extract was passed through PD-10 desalting columns (GE Healthcare) and eluted with Tris·HCl (50 mM, pH 7.5). This desalted extract was used as crude protein extract in measurements of enzyme activity. For isolation of the membrane fraction the extract was centrifuged (100 000 *g*, 75 min, 4 °C), and the resulting pellet was resuspended in Tris·HCl (50 mM, pH 7.5) and centrifuged again (100 000 *g*, 75 min, 4 °C). The pellet was resuspended in Tris·HCl (50 mM, pH 7.5) and used to determine activity in the membrane fraction. Enzyme activity in the supernatant fraction was determined by using the supernatant after the first centrifugation (100 000 *g*). Protein concentration was quantified by a Bradford assay.[35]

Assays for prenyltransferase activity: Standard enzyme assays (100 μL) were performed in Tris·HCl (50 mM, pH 8.8) containing 1,6-DHP (0.5 mM), GPP (0.5 mM), $MgCl_2$ (5 mM), NaCl (20 mM), and membrane protein (0.25 mg mL^{-1}) for 10 min at 30 °C. The reaction mixture was extracted with ethyl acetate/formic acid (100 μL; 975:25). The organic layer was evaporated to dryness, and the dried residue was dissolved in MeOH for HPLC and LC-MS analysis. HPLC analysis was performed with an Eclipse XDB-C18 column (4.6×150 mm, 5 μm; flow rate 1 mL min^{-1}; Agilent Technologies). A linear gradient (40–100% solvent B over 12 min) with an additional 5 min of 100% solvent B was used (solvent A: H_2O/formic acid (999:1), solvent B: MeOH/formic acid (999:1)). UV detection was carried out at 270 nm (1-hydroxyphenazine), 275 nm (1,6-DHP), 306 nm (flaviolin), or 370 nm (1,6-DHP and 1-hydroxyphenazine). LC-MS analysis was performed with a Nucleosil 100 C18 column (100×2 mm, 3 μm; flow rate 0.4 mL min^{-1}; Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) at 40 °C. A linear gradient (40–100% solvent B over 12 min) was used with an additional 5 min of 100% solvent B (solvent A: H_2O (0.1% formic acid), solvent B: MeOH (0.06% formic acid)). UV detection was carried out at 260, 275, 305, and 370 nm. Electrospray ionization (negative and positive) was performed in ultrascan mode (capillary voltage 3.5 kV, 350 °C).

Enzymatic production and isolation of marinophenazine B (2), marinophenazine C (3), and 2-*O*-geranylflaviolin (4): Incubations (25–175 mL scale) were carried out in Tris·HCl (50 mM, pH 8.8) containing the respective aromatic substrate (1 mM), GPP (1 mM), NaCl (20 mM), $MgCl_2$ (5 mM), and membrane protein (2 mg mL^{-1} (**2**) or 1 mg mL^{-1} (**3**, **4**)). The reaction mixtures were incubated for 4 h at 30 °C and extracted with ethyl acetate/formic acid (975:25). Ethyl acetate layers were evaporated, and the residue was dissolved in MeOH. The products were purified on a Multospher 120 RP 18HP column (8×250 mm, 5 μm; Ziemer Chromatographie, Langerwehe, Germany). Isocratic elution was performed with 85% (**2**, **4**) or 90% (**3**) solvent B for 20 min (**2**, **3**) or 25 min (**4**) (solvent A: H_2O/formic acid (999:1), solvent B: MeOH/formic acid (999:1)) at a flow rate of 2.5 mL min^{-1}. The purification yielded 2.5 mg (**2**), 5.7 mg (**3**), and 4.0 mg (**4**). Compound **4** was further purified by isocratic elution with acetonitrile (85% in H_2O) for 15 min using a Kinetex PFP column (4.6×250 mm, 5 μm; Phenomenex, Aschaffenburg, Germany) at a flow rate of 1 mL min^{-1} to yield 2.4 mg of **4**.

Marinophenazine B, 6-((3,7-dimethylocta-2*E*,6-dien-1-yl)oxy)-1-hydroxyphenazine, 2: Yellow powder; UV(MeOH): λ_{max} (log ε) = 272 (4.73), 371 nm (3.41); IR (ATR): $\tilde{\nu}$ = 2964, 2924, 2856, 1530, 1485, 1358, 1125, 802, 738 cm^{-1}; ^{1}H and ^{13}C NMR spectroscopic data measured in [D]chloroform (Table S4) and [D_6]acetone (Table S3); HR-ESI-MS: *m/z* 371.1733 $[M+Na]^+$ (calcd for $[C_{22}H_{24}N_2O_2Na]^+$: 371.1730, Δ = +0.8 ppm).

Marinophenazine C, 1-((3,7-dimethylocta-2*E*,6-dien-1-yl)oxy)phenazine, 3: Yellow powder; UV(MeOH): λ_{max} (log ε) = 261 (4.61), 365 nm (3.80); IR (ATR): $\tilde{\nu}$ = 2965, 2922, 2854, 1520, 1479, 1087, 761, 741 cm^{-1}; ^{1}H and ^{13}C NMR spectroscopic data in Table 2; HR-ESI-TOF-MS: *m/z* 333.1963 $[M+H]^+$ (calcd for $[C_{22}H_{25}N_2O]^+$: 333.1961, Δ = +0.6 ppm).

2-*O*-Geranylflaviolin, 2-((3,7-dimethylocta-2*E*,6-dien-1-yl)oxy)-5,7-dihydroxynaphthalene-1,4-dione, 4: Orange powder; UV-(MeOH): λ_{max} (log ε) = 216 (4.17), 263 (3.81), 303 nm (3.60); IR (ATR): $\tilde{\nu}$ = 2926, 2361, 1629, 1237, 794 cm^{-1}; ^{1}H and ^{13}C NMR spectroscopic data in Table 3; HR-ESI-MS: *m/z* 341.1385 $[M-H]^-$ (calcd for $[C_{20}H_{21}O_5]^-$: 341.1384, Δ = +0.3 ppm).

Nucleotide sequence accession numbers: The nucleotide sequences of the two genomic loci reported in this study are available in the GenBank database under accession numbers KJ451627 and KJ451628.

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