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Biochemical Investigations of Two 6-DMATS Enzymes from *Streptomyces* Reveal New Features of L-Tryptophan Prenyltransferases

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Two putative prenyltransferase genes, *SAML0654* and *Strvi8510*, were identified in *Streptomyces ambofaciens* and *Streptomyces violaceusniger*, respectively. Their deduced products share 63% sequence identity. Biochemical investigations with recombinant proteins demonstrated that L-tryptophan and derivatives, including D-tryptophan, 4-, 5-, 6- and 7-methyl-DL-tryptophan, were well accepted by both enzymes in the presence of DMAPP. Structural elucidation of the isolated products revealed regiospecific prenylation at C-6 of the indole ring and proved unequivocally the identification of two very similar 6-dimethylallyltryptophan synthases (6-DMATS). Detailed biochemical investigations with SAML0654 proved L-tryptophan to be the best substrate (K_m 18 μM, turnover 0.3 s^{-1}). Incubation with different prenyl donors showed that they also accepted GPP and catalyzed the same specific prenylation. Utilizing GPP as a prenyl donor has not been reported for tryptophan prenyltransferases previously. Both enzymes also catalyzed prenylation of some hydroxynaphthalenes; this has not previously been described for bacterial indole prenyltransferases. Interestingly, SAML0654 transferred prenyl moieties onto the unsubstituted ring of hydroxynaphthalenes.

Introduction

Natural products and their derivatives are important resources for drug discovery and development.[1] Prenylated aromatic compounds, including prenylated indole derivatives, represent a large group of secondary metabolites and are widely distributed across bacteria, fungi, and plants. Prenylation (transfer reactions of nxC5-units) contributes largely to structural diversity and often significantly increases the biological and pharmacological activity of the resulting compounds. These prenylation reactions and the responsible enzymes are of interest not only to biologists but also to scientists from other disciplines.[2–6] The transfer reactions of prenyl moieties from prenyl donors (usually dimethylallyl diphosphate, DMAPP) onto indole nuclei are catalyzed in nature by indole prenyltransferases. A large number of such enzymes of the dimethylallyltryptophan synthase (DMATS) superfamily have been identified in recent years in bacteria and fungi, and these have been studied biochemically.[7–10] They mainly accept tryptophan and tryptophan-containing cyclic dipeptides as substrates and catalyze regiospecific prenylation at the indole ring.[8] Three DMATS enzymes that use L-tryptophan and DMAPP as substrates have been identified in *Aspergillus fumigatus* and *Aspergillus clavatus*: FgaPT2, 5-DMATS, and 7-DMATS. They were shown to catalyze prenylation of L-tryptophan at C-4, C-5, and C-7, respectively.[11–13] Three bacterial DMATSs have shown prenylation at positions N1, C-5, and C-6: CymD (*Salinispora arenicola*), SCO7467 (*Streptomyces coelicolor*), and IptA (*Streptomyces* sp. SN-593), respectively.[7,9,10,14] Fungal DMATS enzymes share very little sequence similarity with those from bacteria.

Similarly to most known indole prenyltransferases, FgaPT2, 5-DMATS, IptA, and 7-DMATS exhibit high specificity for their prenyl donor DMAPP and do not accept geranyl diphosphate (GPP) as a substrate.[9,11,13,15] In contrast, they show high promiscuity for the aromatic substrate and accept a large number of tryptophan derivatives.[9,11,16,17] FgaPT2 and 7-DMATS even used hydroxynaphthalenes as prenyl acceptors;[18] these are natural substrates of prenyltransferases of the CloQ/NphB group, from both bacteria and fungi.[19,20] The acceptance of hydroxynaphthalenes by FgaPT2 and 7-DMATS encouraged us to compare the biochemical properties and substrate specificities of DMATSs from bacteria and fungi. BLAST searching with the 6-DMATS IptA from *Streptomyces* sp. SN-593 and the 5-DMATS SCO7467 from *S. coelicolor* revealed several homologues in bacterial genomes, including SAML0654 from *Streptomyces ambofaciens* ATCC2387[7,21] and Strvi8510 from *Streptomyces violaceusniger* Tü 4113. SAML0654 shares 75% amino acid sequence identity with SCO7467, 60% with IptA, and 63% with Strvi8510, thus it was speculated that SAML0654 functions as a 5-DMATS. Strvi8510 showed 60 and 64% identity with SCO7467 and IptA, respectively, so the specific reaction catalyzed by this enzyme could not be predicted from sequence analysis. Here, we report the cloning and expression of SAML0654 and Strvi8510 and their identification as 6-DMATSs.

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Biochemical characterization of the recombinant proteins revealed several new features for the tryptophan prenyltransferases in terms of substrate specificity towards both prenyl donor and acceptor as well as the unusual prenylation position of hydroxynaphthalenes.

Results

Sequence analysis

SAML0654 is a member of a small gene cluster (type B) found in several actinomycetes strains, for example, *S. coelicolor* and *Streptomyces lividans*; it contains genes for a prenyltransferase and for a flavin-dependent monooxygenase.[7,9] Heterologous expression of the prenyltransferase gene SCO7467 and the flavin-dependent monooxygenase gene SCO7468 from *S. coelicolor* A3(2) in *S. lividans* TK23 resulted in the formation of 5-dimethylallylindole-3-acetonitrile.[7] It was speculated that similar clusters in other strains including *S. ambofaciens* would also be responsible for the biosynthesis of 5-dimethylallylindole-3-acetonitrile.[7] In comparison to those in *S. coelicolor* A3(2) and *S. lividans*, the *SAML0654*-containing gene cluster in *S. ambofaciens* has an additional gene coding for a cytochrome P450, but its end product is unknown.[7] *Strvi8510* from *S. violaceusniger* and *IptA* from *Streptomyces* sp. SN-593 belong to type A gene clusters found in different actinomycetes.[7] Although no gene coding for a flavin-dependent monooxygenase is found in these clusters, there is a gene for tryptophanase. These clusters were speculated to be responsible for the formation of 6-dimethylallylindole-carbardehyde.[7]

The deduced product of *SAML0654* has 375 amino acids and a calculated mass of 39.9 kDa. The deduced product of *Strvi8510* has 380 amino acids and a calculated mass of 40.9 kDa.

Cloning and purification of overexpressed His-tagged SAML0654 and Strvi8510

To prove its function biochemically, *SAML0654* was amplified by PCR from genomic DNA of *S. ambofaciens* DSM40053. The PCR product (1133 bp) was cloned into pGEM-T Easy and then subcloned into vector pQE-60, thereby resulting in the expression construct pJW12. The C-terminal His_6-tagged protein was overproduced in *Escherichia coli* M15 (induction with 0.5 mM IPTG) at 30 °C for 16 h and purified by Ni-NTA affinity chromatography.

For the overproduction of Strvi8510, expression plasmid pJW13 was constructed by PCR amplifying the gene from genomic DNA of *S. violaceusniger* Tü 4113 and subcloning into pQE-70. After unsuccessful expression attempts with pJW13, the gene was again amplified, and the product (1155 bp) was cloned into pGEM-T Easy and subcloned into vector $pHIS_8$, thereby resulting in the expression construct pJW18. The N-terminal His_8-tagged protein was overproduced in *E. coli BL21 (DE3) pLysS* cells (induction with 0.5 mM IPTG) at 22 °C for 16 h and purified by Ni-NTA affinity chromatography.

SDS-PAGE analysis of the purified proteins showed significant bands just below the 45 kDa size marker (Figure S1 in the Supporting Information); these correspond well to the calculated masses of the His_6- and His_8-tagged proteins (41.1 and 43.3 kDa, respectively). Protein yields of 3.5 mg purified protein per liter of culture were obtained for both enzymes.

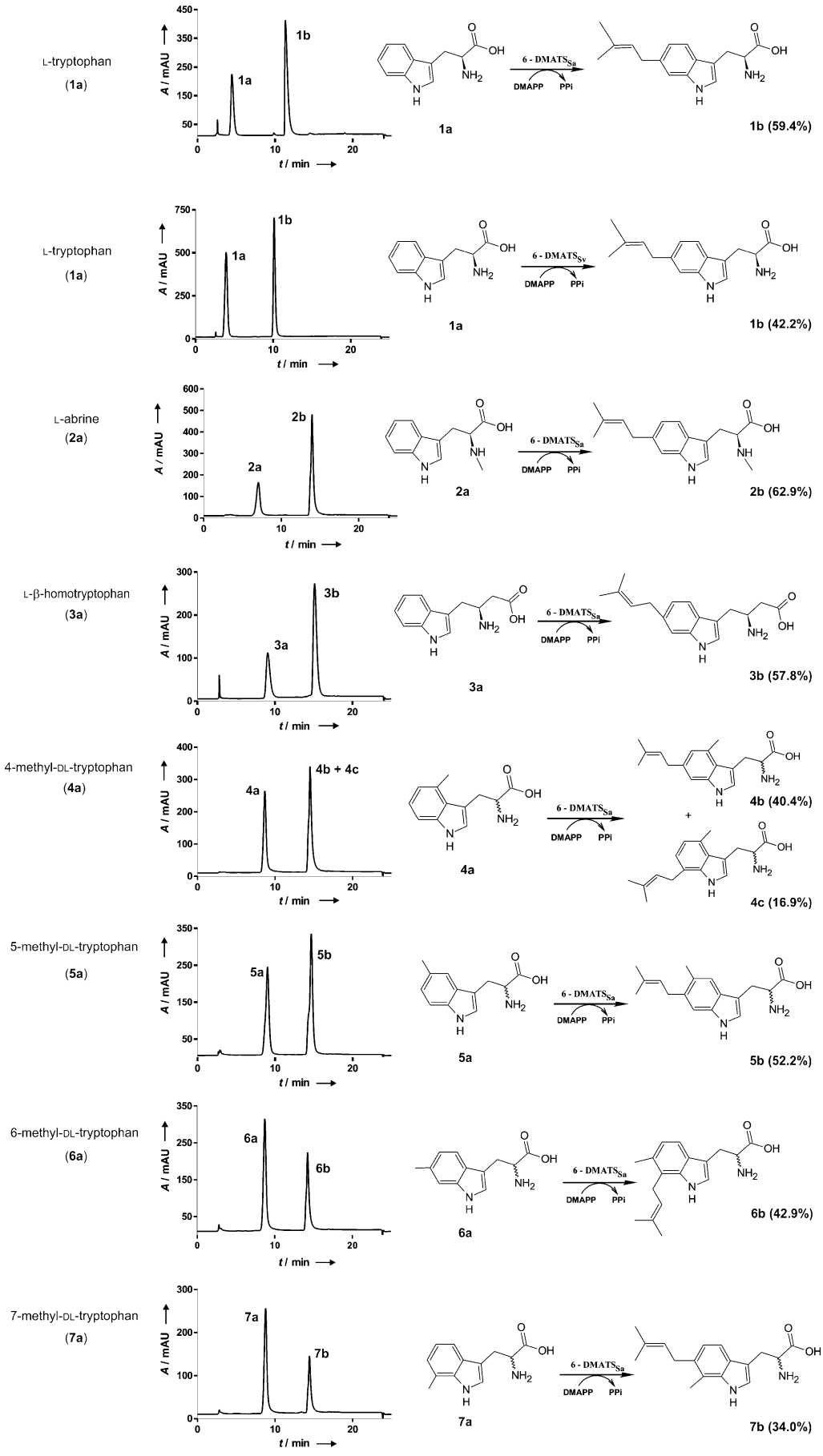
Acceptance of tryptophan and derivatives by SAML0654 and Strvi8510

Because of their high sequence similarity to tryptophan prenyltransferases, such as SCO7467 from *S. coelicolor* and IptA from *Streptomyces* sp. SN-593, SAML0654 and Strvi8150 were firstly assayed with L-tryptophan (**1a**) in the presence of DMAPP. HPLC analysis clearly revealed the presence of one significant product peak each in the incubation mixtures (Figure 1); this was dependent on the presence of prenyl donor and active protein (data not shown). Incubation of 1 mM **1a** with 1 mM DMAPP and 2.4 or 2.3 μM recombinant enzyme for 16 h at 37 °C achieved product yields of 59.4 and 42.2% for SAML0654 and Strvi8510, respectively.

D-tryptophan and 12 tryptophan derivatives (modifications on the indole ring and at the side chain) were assayed subsequently with the purified proteins in the presence of DMAPP. One product for each was detected in the incubation mixtures of the tested tryptophan derivatives (Table S1). HPLC analysis showed that D-tryptophan was well accepted by SAML0654 and Strvi8510, respectively (activities of 73.5 and 60.7% relative to those for L-tryptophan; Table S1). Such high relative activity for D-tryptophan by an L-tryptophan prenyltransferase has not been reported previously. The known DMATS enzymes FgaPT2, IptA, and 7-DMATS showed activities of 1.8, 4.3, and 15.5%, respectively, relative to those with L-tryptophan.[9,12,13]

High conversion yields were also achieved for tryptophan derivatives. In the incubation mixtures of SAML0654, conversion yields for L-abrine (**2a**) and L-β-homotryptophan (**3a**) were nearly the same as for L-tryptophan. Some of the incubation mixtures contained racemic substrates, and the conversion yields of each enantiomer could not be determined. However, the sum of their conversion yields was estimated from the total amount of substrates. Relative activities of 96.4, 87.9, 72.2, and 51.3% (relative to L-tryptophan) were calculated for 4- (**4a**), 5- (**5a**), 6- (**6a**), and 7-methyl-DL-tryptophan (**7a**), respectively (Figure 1, Table S1), thus proving high conversion of D-configured tryptophan derivatives. In comparison, low product formation was observed for 1-methyl-DL-tryptophan under the same conditions.

HPLC analysis of the incubation mixtures of Strvi8510 showed that it, too, accepted well the tested tryptophan derivatives. The relative activities of tryptophan derivatives with Strvi8510 were in the same range as for SAML0654 (Table S1). One significant exception was **3a** (product yield 36.7%, relative to that of **1a**; Table S1). Remarkably, for a given substrate the products of Strvi8510 and SAML0654 had the same retention times in the HPLC chromatograms, thus indicating the presence of same product. On the basis of substrate specificity towards methylated tryptophan derivatives, one would conclude



prenylation at position N1 by the tested enzymes. This conflicts with the high sequence similarity of SAML0654 and Strvi8510 with the C5-prenyltransferase SCO7467 and C6-prenyltransferase IptA.

Confirmation of the prenylation position of the enzyme products

Based on the high sequence similarity with SCO7467, we speculated that 5-DMAT could be the enzyme product of SAML0654 with L-tryptophan and DMAPP. In the HPLC chromatogram of the incubation mixture with **1a**, the enzyme peak showed nearly the same retention time as C5-prenylated tryptophan produced by 5-DMATS from *A. clavatus* (data not shown). To confirm prenylation and determine the prenylation position, seven enzyme products (**1b**–**7b**) were isolated by HPLC from incubation mixtures of SAML0654 with **1a**–**7a** in the presence of DMAPP and subjected to MS and NMR analyses (Tables S3–S4.1 and Figures S3.1–S3.7). High resolution EI-MS (HR-EI-MS) revealed molecular masses 68 Da larger than those of the respective substrates, thus verifying the attachment of one dimethylallyl moiety to the substrates. The signals in the ^{1}H NMR spectra of the isolated enzyme products at $\delta_H =$ 3.37–3.56 (d, 2H-1′), 5.09–5.35 (t sept, H-2′), 1.74–1.83 (s, 3H-4′), 1.68–1.75 ppm (s, 3H-5′) confirmed the attachment of a regular dimethylallyl moiety to a C atom.[9,11,16,17] Comparison of the NMR spectra of **1b**–**7b** with those of **1a**–**7a** revealed the disappearance of signals for one aromatic proton, thus proving the attachment of the prenyl moiety at the indole ring. The singlet at $\delta_H =$ 7.08–7.15 ppm for H-2 in all the spectra indicated that prenylation did not take place at C-2.

In the ^{1}H NMR spectra of the enzyme products of **1a**–**3a**, the ABM coupling systems of three protons with coupling constants of 8.2 and 1.2–1.5 Hz indicated attachment of a prenyl moiety to C-5 or C-6. The ^{1}H NMR spectra of **1b**–**3b** clearly differed from those of the enzyme products of 5-DMATS with **1a**–**3a** (coupling pattern and chemical shifts),[11] thus indicating prenylation at C-6. Furthermore, the ^{1}H NMR spectra of **1b** and **2b** corresponded well to those of the enzyme products of IptA with **1a** and **2a**.[9] These findings unequivocally prove C6-prenylation in **1b**–**3b** (Figure 1).

The NMR spectrum of the product peak of **4a** represents a mixture of two products, **4b** and **4c** (2.4:1). Unfortunately, **4b** and **4c** could not be separated. However, it was possible to assign the prenylation positions as C-6 in **4b** and C-7 in **4c**. The presence of three singlets for aromatic protons in the spectra of **4b** and **5b** unambiguously confirmed prenylation at C-6. The two doublets at 7.4 and 6.9 ppm with a coupling constant of 8.1 Hz in **6b** and **7b** proved prenylation at C-7 and C-6, respectively. The structure of **4c** was elucidated by interpretation of the NMR spectrum and by comparison with those of C5- and C7-prenylated derivative of **4a**.[11,22]

In conclusion, SAML0654 from *S. ambofaciens* catalyzed the prenylation of L-tryptophan and derivatives at C-6 of the indole ring; it therefore functions as a 6-DMATS, (hereafter termed 6-DMATS$_{Sa}$). If this position is blocked, for example by a methyl group as in the case of **6a**, the prenylation is shifted to C-7, as observed for another 6-DMATS, IptA. Interestingly, blocking of the C-6 by the methyl group did not significantly reduce the enzyme activity of 6-DMATS$_{Sa}$. For **6a** the activity was 72.2% relative to that of **1a**. In the case of IptA, for **6a** the activity was only 9.3% relative to that of **1a**.

The enzyme product of Strvi8510 with **1a** and DMAPP was also isolated, and its structure was elucidated by NMR analysis: the product (**1b)** was the same as for the incubation mixture with SAML0654. This demonstrates clearly that Strvi8510 from *S. violaceusniger* also functions as a tryptophan C6-prenyltransferase (hereafter termed 6-DMATS$_{Sv}$). As mentioned, the product with 6-DMATS$_{Sv}$ for a given tryptophan derivative showed the same HPLC retention time as that with 6-DMATS$_{Sa}$. We concluded that it produces the same products and decided not to isolate these peaks.

GPP also serves as a prenyl donor for 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$

HPLC analysis of the DMATS$_{Sa}$ reaction with **1a** and GPP or farnesyl diphosphate (FPP) indicated product formation in the reaction mixture of GPP, but not in that of FPP (data not shown). Therefore, **1a** and six derivatives (**2a**–**7a**) with high conversion yields in the presence of DMAPP were incubated with GPP and 6-DMATS$_{Sa}$ or 6-DMATS$_{Sv}$. Product formation was detected in five cases with 6-DMATS$_{Sa}$ (Table S1). In the assays with GPP under the condition used for DMAPP, **1a**, **2a**, and **5a** were accepted by 6-DMATS$_{Sa}$ with conversion yields of 26.45, 10.8, and 21.6%, respectively (Figure 2), that is, with activities of 46.2, 18.8, and 37.8% relative to that of **1a** with DMAPP (Table S1). With 9.6 μM 6-DMATS$_{Sa}$ in the reaction mixtures of L-tryptophan and GPP, an absolute conversion yield of 40% was achieved. In comparison, GPP was a poor prenyl donor for 6-DMATS$_{Sv}$: low product formation was only detected with **1a** and **5a** (activities of 3.7 and 6.2%, respectively, relative that for DMAPP with **1a**). For structure elucidation, **1a**, **2a**, and **5a** were incubated with 6-DMATS$_{Sa}$ in large scale (10 mL) at 37 °C for 16 h. The enzyme products **1c**, **2c**, and **5c** with GPP were isolated on HPLC and subjected to MS and NMR analyses.

MS analysis showed that the molecular masses of **1c**, **2c**, and **5c** were 136 Da larger than those of **1a**, **2a**, and **5a**, respectively, thus proving attachment of a geranyl moiety. Detailed inspection of the ^{1}H NMR spectra revealed signals for a regular geranyl residue attached at a C atom.[15] The chemical

Figure 1. HPLC analysis of enzyme assays with **1a**–**7a** in the presence of DMAPP (left), and prenyl transfer reactions catalyzed by 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ (right). Reaction mixtures (100 μL) contained 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM aromatic substrate, 1 mM DMAPP, 0.15–5% (*v/v*) glycerol, 0–5% (*v/v*) DMSO, and 2.4 or 2.3 μM purified recombinant protein, and were incubated at 37 °C for 16 h. Detection was carried out with a photodiode array detector (absorption at 277 nm). Conversion yields are shown in parentheses (mean of two independent measurements).

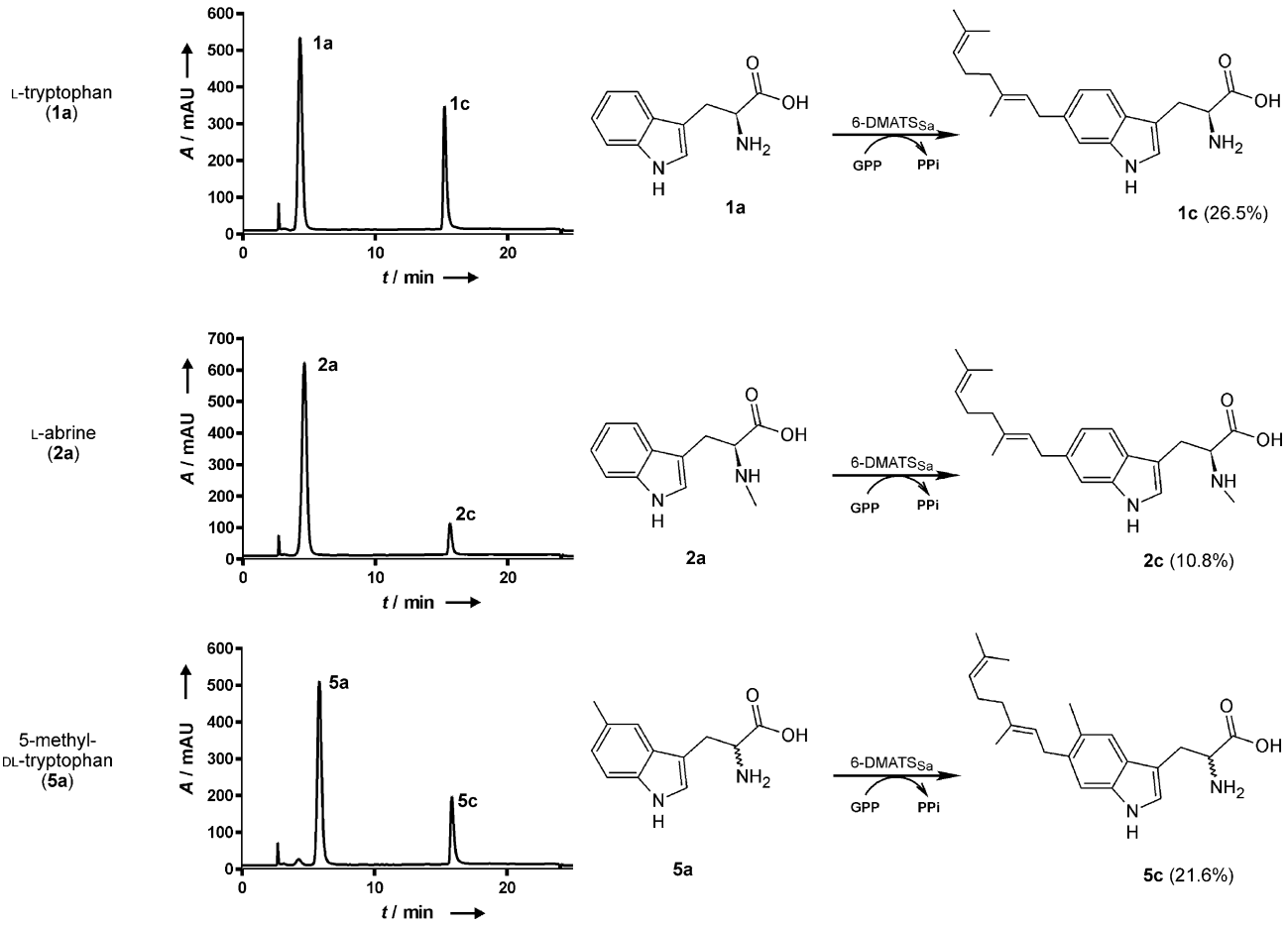


Figure 2. HPLC analysis of enzyme assays with **1a**, **2a**, and **5a** in the presence of GPP (left), and prenyl transfer reactions catalyzed by 6-DMATS$_{Sa}$ (right). Reaction mixtures (100 μL) contained 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM aromatic substrate, 1 mM GPP, 0.15–5% (*v/v*) glycerol, 0–5% (*v/v*) DMSO and 2.4 μM purified recombinant protein, and were incubated at 37 °C for 16 h. Detection was carried out with a photodiode array detector (absorption at 277 nm). Conversion yields are shown in parentheses (mean of two independent measurements).

shifts and coupling patterns for aromatic protons in the NMR spectra of **1c**, **2c**, and **5c**, respectively, were nearly early identical to those of **1b**, **2b** and **5b**, thus unequivocally proving the same prenylation position for **1c**, **2c**, and **5c** as for **1b**, **2b**, and **5b**. In other words, 6-DMATS$_{Sa}$ also catalyzed C6-prenylation of L-tryptophan and derivatives in the presence of GPP (Figure 2).

Acceptance of dihydroxynaphthalenes by 6-DMATS$_{Sa}$ and C-prenylation on the unsubstituted benzene ring

As mentioned above, hydroxynaphthalenes are natural substrates of some prenyltransferases of the CloQ/NphB group from bacteria and fungi.[19,20] These enzymes share no sequence similarity with members of the DMATS superfamily, but do share structural similarity.[23] It has been demonstrated that several enzymes of the DMATS superfamily from fungi (including FgaPT2 and 7-DMATS) also catalyze prenylation of several hydroxynaphthalenes.[18] There are no reports of acceptance of hydroxynaphthalenes by bacterial indole prenyltransferases.

For naphthalene derivatives with 9.6 μM 6-DMATS$_{Sa}$ in the presence of DMAPP, HPLC analysis revealed clear product formation in 9 of 15 incubation mixtures (Table S2). Product yields of more than 10% were achieved for five substrates after incubation at 37 °C for 16 h. For 6-DMATS$_{Sv}$, product formation was found for four hydroxynaphthalenes (Table S2). As observed for fungal indole prenyltransferases,[18] activation of the naphthalene ring was necessary for acceptance. The substrate preferences among hydroxynaphthalenes by 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ differed, however, from those of fungal indole prenyltransferases. For example, 1-naphthol was very well accepted by fungal indole prenyltransferases, but only poorly accepted by 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ (total conversion yield of 3 and $\leq$0.2%, respectively). Compounds 1,3- (**8a**), 1,7- (**9a**), and 2,3-dihydroxynaphthalene (**10a**) were very well accepted by 6-DMATS$_{Sa}$ (conversion yields 61.5, 33.7, and 43.8%, respectively; Figure 3). Compounds **8a** and **10a** were also good substrates for 6-DMATS$_{Sv}$ (conversion yields 66 and 14%, respectively; Table S2). Again, the retention times on HPLC of products of 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ for a given hydroxynaphthalene were the same, although 2,7-dihydroxynaphthalene was not accepted by DMATS$_{Sa}$ but was well accepted by DMATS$_{Sv}$ (conversion yield 42.1%). Other dihydroxynaphthalenes, such as 1,4-, 1,5-, 1,6-, and 2,6-dihydroxynaphthalene, were poor substrates for

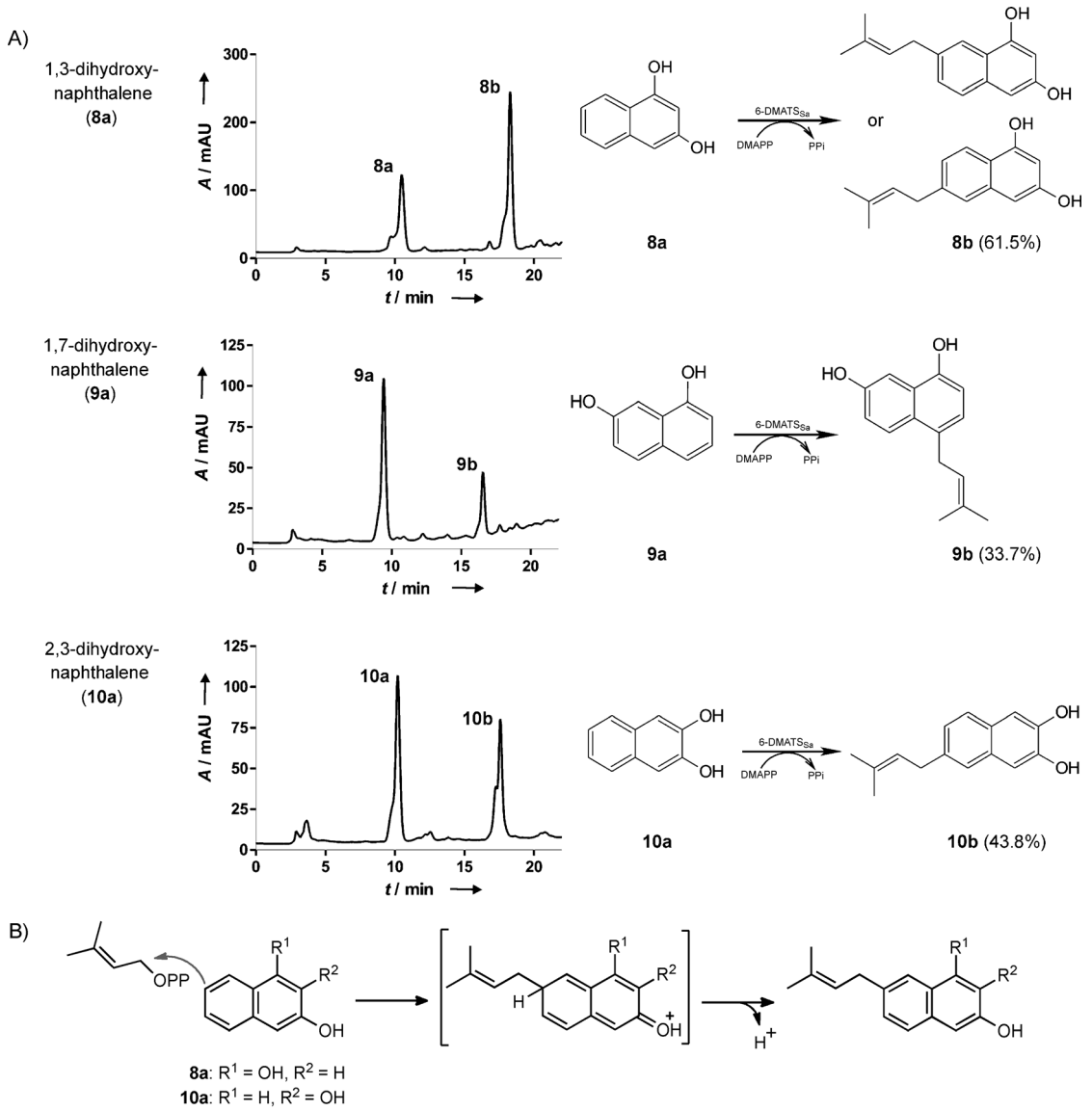


Figure 3. A) HPLC analysis of enzyme assays with **8a**–**10a** in the presence of DMAPP (left) and prenyl transfer reactions catalyzed by 6-DMATS$_{Sa}$ (right). Reaction mixtures (100 μL) contained 50 mM Tris·HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM aromatic substrate, 2 mM DMAPP, 0.15–5% (*v*/*v*) glycerol, 0–5% (*v*/*v*) DMSO and 9.6 μM purified recombinant protein, and were incubated at 37 °C for 16 h. Detection of the compounds was carried out with a photodiode array detector (absorption at 296 nm). Conversion yields are shown in parentheses. B) Proposed reaction mechanism for **8a** and **10a**.

both 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ (Table S2). For structure elucidation, **8b**–**10b** were isolated from the incubation mixtures of the three best accepted naphthalene derivatives (**8a**–**10a**) with 6-DMATS$_{Sa}$ in the presence of DMAPP (Figure 3) and subjected to MS and NMR analyses (Table S3 and Figure S3.8–S3.10). HR-EI-MS analysis confirmed a prenyl moiety in each of **8b**–**10b** by their molecular masses (68 Da larger than for the respective substrates; Table S3). The ^{1}H NMR spectra of **8b**–**10b** show clear signals for a regular dimethylallyl moiety attached to a C atom at δ_H = 3.40–3.59 (d, 2H-1′), 5.31–5.37 (t sept, H-2′), 1.76 or 1.78 (s, 3H-4′), 1.76 or 1.73 (s, 3H-5′; Table S5, Figure S3.8–S3.10). Comparison of the spectra of **9b** with those of **9a** proved prenylation at C-4 of 1,7-dihydroxynaphthalene, that is, at a *para*-position of a hydroxyl group. Compound **9b** has also been identified as an enzyme product of fungal prenyltransferases.[18]

Surprisingly, inspection of the NMR spectra of **8b** and **10b** revealed prenylation in both cases at the unsubstituted benzene ring. The structure of **10b** was easily identified as 6-dimethylallyl-2,3-dihydroxynaphthalene by the presence of one new three spin system with coupling constants at 8.4 and 1.8 Hz. The ^{1}H NMR spectrum of **8b** indicated the presence of three coupling protons at δ_H = 7.80 (br s), 7.44 (d, 8.4 Hz), and 7.15 (dd, 8.4 and 1.8 Hz) ppm, caused by prenylation at C-6 or C-7 of 1,3-dihydroxynaphthalene (this cannot be confirmed or excluded from the obtained spectrum). Even with additional spectroscopic analyses, such as HSQC, HMBC, NOE or NOESY, it would be difficult to distinguish the two possibilities. We favor

C7-prenylated 1,3-dihydroxynaphthalene as a plausible structure for **8b**, because the prenylation position is at the *para*-position of a hydroxyl group, although they are located on two benzene rings. Previously, prenylation of hydroxynaphthalenes at the unsubstituted benzene ring (as in the case of **8b** and **10b**) has not been reported for either indole prenyltransferases of the DMATS superfamily, or enzymes of the NphB/CloQ group. The bacterial prenyltransferase Fnq26 of NphB/CloQ group catalyzed O-prenylation of **8a**.[24]

Biochemical characterization and kinetic parameters of 6-DMATS$_{Sa}$

The dependency of the 6-DMATS$_{Sa}$ reaction on metal ions was examined by incubation of DMAPP with **1a** and different metal ions (5 mM). Reaction mixtures without additives (or with the chelating agent EDTA) served as controls (Figure S2). Addition of Mg^{2+}, Ca^{2+}, or EDTA did not have any influence on the enzyme activity, thus indicating that the 6-DMATS$_{Sa}$ reaction is likely independent of the presence of divalent ions. $CuCl_2$, $ZnCl_2$, and $NiSO_4$ strongly inhibited enzyme activity.

To better understand the 6-DMATS$_{Sa}$ reaction, kinetic parameters (Michaelis–Menten constant K_m, turnover number k_{cat}) were determined for tryptophan (**1a**), six derivatives (**2a**–**7a**), three hydroxynaphthalenes (**8a**–**10a**), DMAPP, and GPP. The reactions with all tested substrates were consistent with Michaelis–Menten kinetics. For calculation of the kinetic parameters (Table 1), data processing was carried out by constructing

Table 1. Kinetic parameters of 6-DMATS$_{Sa}$ for selected substrates.

Substrate	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]	Relative catalytic efficiency [%]
L-tryptophan (**1a**)	0.018	0.30	16.67	100
L-abrine (**2a**)	0.023	0.27	11.74	69
L-β-homotryptophan (**3a**)	0.092	0.23	2.50	15
4-methyl-DL-tryptophan (**4a**)	0.063	0.19	3.02	18
5-methyl-DL-tryptophan (**5a**)	0.074	0.43	5.81	35
6-methyl-DL-tryptophan (**6a**)	0.030	0.028	0.93	5.6
7-methyl-DL-tryptophan (**7a**)	0.072	0.013	0.18	1.1
1,3-dihydroxynaphthalene (**8a**)	0.29	0.43	1.47	8.8
1,7-dihydroxynaphthalene (**9a**)	0.059	0.088	1.49	8.9
2,3-dihydroxynaphthalene (**10a**)	0.28	0.13	0.46	2.7
DMAPP[a]	0.095	0.63	6.63	40
GPP[a]	0.070	0.043	0.61	3.7

[a] L-tryptophan (**1a**) as prenyl acceptor.

Hanes–Woolf, Lineweaver–Burk, and Eadie–Hofstee plots (Figure S4.1–S4.7). High affinities (K_m 0.018 to 0.092 mM) were determined for **1a**–**7a**, with **1a** as the best aromatic substrate (K_m 0.018 mM, k_{cat} 0.3 s^{-1}, thus catalytic efficiency 16.7 s^{-1} mM^{-1}). 1,7-Dihydroxynaphthalene (**9a**, K_m 0.059 mM) showed affinity similar to those of the indole derivatives. The other two hydroxynaphthalenes showed much lower affinity. Consistent with the high conversion yields for **2a**–**5a** (Figure 1), high turnover numbers (0.19–0.43 s^{-1}) were determined for these substrates. Consequently, 6-DMATS$_{Sa}$ had higher catalytic efficiency with **1a**–**5a** (Table 1). The kinetic parameter of 6-DMATS$_{Sa}$ towards L-tryptophan and derivatives are comparable with those of the C6-prenyltransferase IptA (Takahashi et al).[9] GPP (K_m 0.070 mM) showed a slightly higher affinity to 6-DMATS$_{Sa}$ than did DMAPP (K_m 0.095 mM). However, GPP turnover (k_{cat} 0.043 s^{-1}) was much slower than for DMAPP (k_{cat} 0.63 s^{-1}).

Discussion

Indole prenyltransferases catalyze the transfer of prenyl moieties onto different positions of indole rings; they have been identified mainly in fungi (ascomycetes)[8] and occasionally in bacteria (actinomycetes).[7,9,10] These enzymes belong to the DMATS superfamily and accept tryptophan, tryptophan-containing cyclic dipeptides, or more-complex indole derivatives as prenyl acceptors. The important features of these enzymes are high promiscuity with the aromatic substrate, high regioselectivity of the prenylation position, and high substrate specificity towards the prenyl donor. The known tryptophan prenyltransferases use solely DMAPP as the prenyl donor.[7–10,14]

In this study, we successfully identified two putative prenyltransferases, SAML0654 from *S. ambofaciens* and Strvi8510 from *S. violaceusniger*, as indole prenyltransferases with broad substrate specificity. Determination of the kinetic parameters with diverse aromatic substrates showed that L-tryptophan was best accepted as a substrate, and that the enzymes function as a 6-DMATS. Detailed biochemical characterization of the enzymes (especially of 6-DMATS$_{Sa}$) revealed a number of new features of these indole prenyltransferases:

1) The identification of SAML0654 as a 6-DMATS was somewhat surprising, because amino acid sequence alignment revealed much higher similarity to the 5-DMATS SCO7467 (75%) from *S. coelicolor*[10] than to the 6-DMATS IptA (60%) from *Streptomyces* sp. SN-593.[9] Our results highlight that the functions of enzymes should be proven experimentally, not just inferred from sequence comparison with known proteins.
2) 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ accepted well a number of indole derivatives, including D-tryptophan and 6-methyl-DL-tryptophan (**6a**; Figure 1 and Table S1). Conversion yields of 73.5 and 60.7% of that of L-tryptophan were calculated for D-tryptophan. The conversion yields for 6-methyl-DL-tryptophan were 72.2 and 82%. These values are unusually high for L-tryptophan C6-prenyltransferases. Other known DMATSs, such as IptA (*Streptomyces* sp. SN-593), FgaPT2, and 7-DMATS (both from *A. fumigatus*), accepted D-tryptophan with activities of only 4.3, 1.8, and 15.5%, respectively, relative to that for L-tryptophan.[9,12,13] IptA also accepted 6-methyl-DL-tryptophan as a substrate, but with activity of only 9.33% relative of that of L-tryptophan. As in the case of IptA, highly regiospecific C7-prenylation of 6-methyl-DL-tryptophan (**6a**) was observed with 6-DMATS from *S. ambofaciens*.

3) 6-DMATS$_{Sa}$ showed relatively high promiscuity towards the prenyl donor. In the presence of L-tryptophan and several tryptophan derivatives, GPP was accepted by 6-DMATS$_{Sa}$ as the prenyl donor (activities of up to 46.2% relative of that for DMAPP); 6-DMATS$_{Sv}$ also accepted GPP, but with lower conversion yields. In contrast, no known DMATS (including *A. fumigatus* FgaPT2, *A. clavatus* 5-DMATS, *Streptomyces* sp. SN-593 IptA, and *A. fumigatus* 7-DMATS) have been reported to use GPP or other prenyl donors.[9,11–13] Therefore, 6-DMATS$_{Sa}$ from *S. ambofaciens* and 6-DMATS$_{Sv}$ from *S. violaceusniger* represent the first examples of tryptophan prenyltransferases that accept both DMAPP and GPP as prenyl donors.
4) Isolation and structure elucidation of the enzyme products from L-tryptophan (**1a**) and two derivatives (**2a** and **5a**) with GPP led to the identification of C6-geranylated derivatives, that is, the same prenylation position as for the products from DMAPP. This differs from the situation for some fungal indole prenyltransferases in the presence of DMAPP analogues, GPP, or benzyl diphosphate. In those cases, complete or partial shifts of the prenylation position were observed.[25–27]
5) 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ also accepted several hydroxynaphthalenes as prenyl acceptors; these are natural substrates of prenyltransferases of the bacterial and fungal CloQ/NphB group.[19,20,24] It has been demonstrated that some members of the fungal DMATS superfamily can also use these compounds as substrates.[18] However, there is no report on the behavior of bacterial indole prenyltransferase like IptA or SCO7467 towards hydroxynaphthalenes.[7,9,10] Interestingly, all fungal indole prenyltransferases accept 1-naphthol as one of the best substrates. In contrast, 1-naphthol was not accepted by 6-DMATS$_{Sv}$, and it was barely accepted by 6-DMATS$_{Sa}$ (<3%, Table S2). In contrast 1,3- and 2,3-dihydroxynaphthalenes were better accepted by 6-DMATS$_{Sa}$ than by fungal indole prenyltransferases. 1,7-Dihydroxynaphthalene was accepted by both bacterial and fungal enzymes.
6) Structure elucidation of the enzyme products of 1,3- (**8a**) and 2,3-dihydroxynaphthalenes (**10a**) identified the prenylation position at the unsubstituted benzene ring. This clearly differs from fungal indole prenyltransferases and prenyltransferases of the NphB/CloQ group; these attached prenyl moieties to *para*- or *ortho*-position of a hydroxyl group at the same benzene ring, or onto a hydroxyl group.[18–20,28] The structure of **10b** was unequivocally proven to be 6-dimethylallyl-2,3-dihydroxynaphthalene. In the case of **8b**, we favor 7-dimethylallyl-1,3-dihydroxynaphthalene as the more likely of the two possible structures. This means that prenyl moieties in both structures are *para* to a hydroxy group on the second benzene ring. It could be speculated that activation by a hydroxyl group (although at another benzene ring) is important for the stabilization of an intermediate cation (Figure 3B).

Conclusion

In summary, the identified and characterized 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$ showed several new intriguing biochemical features, and greatly expand our knowledge of bacterial indole prenyltransferases. These enzymes are very good candidates for further investigations, such as protein crystallization and structural analysis of the active sites, to understand their broad substrate specificity towards prenyl donor and acceptor, as well as their reaction mechanisms.

Experimental Section

Computer-assisted sequence analysis: Sequence identities were obtained by alignments of amino acid sequences with the program BLASTP (http://www.ncbi.nlm.nih.gov).

Chemicals: DMAPP, GPP, and FPP were prepared according to the method described for GPP by Woodside et al.[29] Indole and hydroxynaphthalene derivatives of the highest available purity were purchased from TCI, Acros Organics, Sigma–Aldrich, Bachem, and Alfa Aesar.

Bacterial strains, plasmids, and culture conditions: pGEM-T Easy and pQE-60/70 were purchased from Promega and Qiagen, respectively. pHIS$_8$[30] was a kind gift from Prof John Noel (Salk Institute for Biological Studies). *E. coli* strains XL1 Blue MRF′ (Stratagene/Agilent Technologies), M15 [pREP4] (Qiagen), and BL21 (DE3) pLysS (AMS Biotechnology, Abingdon, UK) were used for cloning and expression. They were grown in liquid lysogeny broth (LB) or on solid LB medium (with agar (1.5% *w/v*)) at 37 or 30 °C. Carbenicillin (50 μg mL^{-1}) or kanamycin (25 μg mL^{-1}) were used for selection of recombinant *E. coli* XL1 Blue MRF′ strains containing pQE-60 or pHIS$_8$ constructs, respectively. Kanamycin (25 μg mL^{-1}) with carbenicillin (50 μg mL^{-1}) or chloramphenicol (12.5 μg mL^{-1}) was used for selection of recombinant *E. coli* M15 [pREP4] or BL21 (DE3), respectively. *S. ambofaciens* DSM40053 (ATCC 23877) was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *S. violaceusniger* Tü 4113 was obtained from Prof. Wolfgang Wohlleben (Tübingen University). For genomic DNA isolation, these were cultivated in a 300 mL cylindrical flask containing liquid YMG medium (50 mL; yeast extract (0.4% *w/v*), malt extract (1% *w/v*), glucose (0.4% *w/v*)) at 28 °C on a rotary shaker (160 rpm) for 3 days.

DNA manipulation and PCR amplification: DNA propagation in *E. coli* was carried out by standard methods.[31] DNA isolation from *Streptomyces* strains was carried out by phenol-chloroform extraction.[32] PCR amplification was performed on an iCycler (Bio-Rad). PCR products containing the coding sequence of SAML0654 and Strvi8510 were obtained by using Expand High Fidelity kit (Roche Diagnostic) with genomic DNA as template. The primers for SAML0654 were SAML0654_fw (5′-CCATGG CCACCG TACGGA CCGGCG CGG-3′) and SAML0654_r (5′-GGATCC GCGCAC CGCCAC CGGGCG G-3′), and for Strvi8510 they were Strvi_bam_fw (5′-GGATCC ATGAAC GGTTTC CATTCG GGTG-3′) and Strvi_hind3_r (5′-AAGCTT TCACAG CCCTGC CCGCAC C-3′). Underlined letters represent the restriction sites for NcoI, BamHI, and HindIII, located at the predicted start and stop codon in the primer sequences. The PCR products for SAML0654 (1133 bp) and Strvi8510 (1155 bp) were cloned into pGEM-T Easy to create plasmids pJW09 and pJW16, and then subcloned into pQE-60 and pHIS$_8$ to produce the expres-

sion plasmids pJW12 (SAML0654) and pJW18 (Strvi8510), respectively.

Gene expression and purification of recombinant 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$: For expression of *SAML0654*, an overnight culture of *E. coli* M15 [pREP4] harboring pJW12 was inoculated into LB medium (1 L) supplemented with carbenicillin (50 μg mL^{-1}) and kanamycin (25 μg mL^{-1}), and cultivated at 37 °C and 220 rpm to A_{600} = 0.6. For induction of gene expression, isopropyl thiogalactosid (IPTG, 0.5 mM) was added. After further cultivation for 16 h at 30 °C, cells were harvested by centrifugation and the pellet was resuspended in lysis buffer (NaH_2PO_4 (50 mM, pH 8.0), imidazole (10 mM), NaCl (300 mM)) at 2–5 mL per gram wet weight. After addition of lysozyme (1 mg mL^{-1}), the cell suspension was incubated on ice for 30 min then sonicated (×6, 10 s, 200 W). Cellular debris was separated from the soluble proteins by centrifugation (20000 *g*, 30 min, 4 °C). Recombinant His$_6$-tagged fusion protein was isolated by affinity chromatography with Ni-NTA agarose resin (Qiagen) according to the manufacturer's instructions. The protein was eluted with imidazole (250 mM) in NaH_2PO_4 (50 mM, pH 8.0) with NaCl (300 mM). In order to change the buffer, the protein fraction was passed through a PD-10 column (GE Healthcare), previously equilibrated with Tris·HCl (50 mM, pH 7.5) containing glycerol (15% *v/v*). Purified 6-DMATS-His$_6$ was used immediately for enzyme assays or stored at −80 °C. The proteins were analyzed by 12% (*w/v*) SDS-PAGE gels according to the method of Laemmli[33] and stained with Coomassie Brilliant Blue G-250.

For overproduction of His$_8$-Strvi8510, *E. coli* BL21 (DE3) pLysS cells harboring pJW18 were cultivated in LB medium (1 L) supplemented with chloramphenicol (12.5 μg mL^{-1}) and kanamycin (25 μg mL^{-1}) at 37 °C to A_{600} = 0.6. Gene expression was induced by addition of IPTG (0.5 mM). Cells were cultivated for a further 16 h at 22 °C and then centrifuged to harvest the cells. Protein purification was carried out as described above.

Enzyme assays for 6-DMATS$_{Sa}$ and 6-DMATS$_{Sv}$: To determine the relative activities of 6-DMATS$_{Sa}$ for different substrates, enzyme assay mixtures (100 μL) contained Tris·HCl (50 mM, pH 7.5), $MgCl_2$ (5 mM), aromatic substrate (1 mM), DMAPP or GPP (1 or 2 mM), glycerol (0.15–5% *v/v*), DMSO (0–5% *v/v*), and purified recombinant protein (10 or 40 μg; 2.4 or 9.6 μM). Mixtures were incubated at 37 °C for 16 h, before reaction termination by addition of methanol (100 μL). For determination of the kinetic parameters of L-tryptophan and derivatives, DMAPP (1 mM) and aromatic substrate (up to 2 mM) were used. Protein concentrations: 61 nM (for **1a** and **2a**), 122 nM (**3a**, **4a**, and **5a**), 244 nM (**6a**), 732 nM (**7a**). Incubation times were 15 min (**1a**–**5a**), 30 min (**6a**), or 60 min (**7a**). For determination of the kinetic parameters of DMAPP, **1a** (1 mM) was used as prenyl acceptor. DMAPP (up to 2 mM) and protein (122 nM) were incubated for 15 min. For determination of the kinetic parameters of GPP, the reaction mixtures contained GPP (up to 1 mM) and protein (732 nM) and were incubated for 30 min. For determination of the kinetic parameters of dihydroxynaphthalenes, the assays contained aromatic substrate (up to 5 mM), DMAPP (1 mM), and protein (0.5 μM for **8a** and **10a**, 0.73 μM for **9a**) and were incubated at 37 °C for 30 min (**8a**, **10a**) or 45 min (**9a**).

For determination of 6-DMATS$_{Sv}$ substrate specificity, the enzyme assays were carried out as for 6-DMATS$_{Sa}$. The reaction mixtures of simple indole derivatives and naphthalene derivatives contained DMAPP (1 mM) and recombinant protein (10 μg (2.3 μM) or 20 μg (4.6 μM), respectively).

Preparation and isolation of enzyme products for structure elucidation: For isolation of the enzyme products, large-scale enzyme reactions (10 mL) were carried out. The reaction mixtures contained substrate **1a**–**10a** (1 mM), DMAPP (2 mM), $MgCl_2$ (5 mM), Tris·HCl (50 mM, pH 7.5) and 6-DMATS$_{Sa}$ (2.4 μM for **1a**–**7a**, 4.8 μM for **8a**–**10a**). The reaction mixture with 6-DMATS$_{Sv}$ and **1a** was carried out as described above for 6-DMATS$_{Sa}$ (2.3 μM). For isolation of geranylated products, **1a**, **2a**, and **5a** (1 mM) were also incubated with GPP (2 mM), $MgCl_2$ (5 mM), Tris·HCl (50 mM, pH 7.5), and 6-DMATS (2.4–10 μM) at 37 °C for 16 h. For **1a**–**7a** reactions were terminated by addition of methanol (10 mL). The precipitated protein was removed by centrifugation, and the obtained supernatants were concentrated on a rotating vacuum evaporator at 30 °C to 0.5–1 mL for HPLC purification. For **8a**–**10a** the mixtures were extracted three times with ethyl acetate immediately after incubation. The ethyl acetate phases were concentrated on a rotating vacuum evaporator at 30 °C to dryness and dissolved in methanol (0.5–1 mL) for HPLC purification.

HPLC conditions for analysis and isolation of enzyme products: The enzyme products of the incubation mixtures were analyzed on an series 1200 HPLC device (Agilent Technologies) with a Multospher 120 RP-18 column (250×4 mm, 5 μm; C+S Chromatography Service, Langenfeld, Germany) at a flow rate of 1 mL min^{-1}. Water (solvent A) and methanol (solvent B) were used with trifluoroacetic acid (0.5% *v/v*) added if necessary. For analysis of enzyme products of tryptophan and simple indole derivatives, a linear gradient of 20–100% or 40–100% solvent B over 15 min was used. For analysis of products from hydroxynaphthalene derivatives, a linear gradient of 40–100% solvent B over 15 or 20 min was used. The column was then washed with solvent B for 5 min and equilibrated with 20 or 40% solvent B for 5 min. Detection was carried out with a photodiode array detector.

The same HPLC equipment and a larger column (250×10 mm) were used to isolate enzyme products. The flow rate was 2.5 mL min^{-1}. Water (solvent A) and methanol (solvent B) without acid were used as solvents: 40–100 or 65–100% solvent B over different times. The column was then washed with solvent B for 5 min and equilibrated with 40 or 65% solvent B for 5 min.

NMR spectroscopic analysis and high-resolution electrospray ionization mass spectra (HR-EI-MS): The isolated products were analyzed by HR-EI-MS with a AutoSpec (Micromass). Positive EI-MS data are given in Table S3. ^{1}H NMR spectra were recorded on an ECA-500 spectrometer (JEOL). Chemical shifts were referenced to CD_3OD at 3.31 ppm. All spectra were processed with MestReNova 5.2.2 software (see the Supporting Information). The NMR data are given in Tables S4 and S5.

Nucleotide sequence accession numbers: The protein sequences of SAML0654 and Strvi8510 are available at GenBank (accession numbers CAJ89640 and YP_004818099.1).

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