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Geranylation of Cyclic Dipeptides by the Dimethylallyl Transferase AnaPT Resulting in a Shift of Prenylation Position on the Indole Ring

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The dimethylallyl transferase AnaPT from *Neosartorya fischeri* is involved in the biosynthesis of acetylaszonalenin and catalyses the regioselective and stereospecific C3α-prenylation of (*R*)-benzodiazepinedione in the presence of dimethylallyl diphosphate. This enzyme also converts several tryptophan-containing cyclic dipeptides to C3α-prenylated indolines. In this study, we demonstrate the geranylation of (*R*)-benzodiazepinedione and five other cyclic dipeptides by AnaPT in the presence of geranyl diphosphate (GPP). Interestingly, structure elucidation by NMR and MS analyses revealed that, with GPP, the geranyl moiety is attached to C-6 or C-7 rather than C-3 of the indole ring of the enzyme products. For (*R*)-benzodiazepinedione, one dominant C6-geranylated derivative was obtained, whereas the other five substrates yielded both C6- and C7-geranylated products. Neither acceptance of GPP by a dimethylallyl transferase from the dimethylallyltryptophan synthase superfamily, nor the alkylation shift from C-3 to the benzene ring of the indole nucleus has been reported previously.

Introduction

Attachment of a prenyl moiety to aromatic compounds contributes significantly to the structural diversity of secondary metabolites, including prenylated indole alkaloids.[1] Natural products of this group are widely distributed in both terrestrial and marine microorganisms.[2–4] In most cases, they are derivatives of tryptophan or tryptophan-containing cyclic dipeptides with one or more dimethylallyl moieties.[2,3] However, indole derivatives with larger prenyl moieties have also been found in nature, for example, indole sesquiterpenoid xiamycin and derivatives from *Streptomyces*,[5,6] and indole diterpenoids paxilline and aflatrem from *Penicillium* and *Aspergillus* species.[7–9] In many cases, the prenylated derivatives differ clearly from their precursors in their biological and pharmacological activities.[10–12] Prenyltransferases from plants, bacteria and fungi are responsible for the transfer reactions of the prenyl moieties, and belong to different superfamilies/groups, based on their primary amino acid sequences and biochemical properties.[1,13,14] Indole prenyltransferases of the DMATS superfamily show broad substrate specificity towards their aromatic substrates but high regioselectivity for the prenylation position on the indole ring.[14] These characteristics render members of this superfamily a valuable toolbox for the production of prenylated indole derivatives. In a sharp contrast to the flexibility for aromatic substrates, most of the enzymes accept exclusively dimethylallyl diphosphate (DMAPP, C_5 unit) as the prenyl donor.[14] Only one enzyme from the DMATS superfamily, VrtC from *Penicillium aethiopicum*, is known to use geranyl diphosphate (GPP, C_{10} unit), rather than DMAPP, as the prenyl donor.[15] Interestingly, several homologues of VrtC from *Neosartorya fischeri*, *Microsporum canis, Aspergillus fumigatus* and *Trichophyton tonsurans* catalyse similar prenylation reactions, but these use exclusively DMAPP as the prenyl donor.[15,16] This clearly demonstrates the high specificity of these enzymes for their prenyl donors. Yet, it can be speculated that the prenyl diphosphate binding sites of the different members in this superfamily are derived from the same ancestor, or evolved by successive modifications. In both cases, enzymes that accept both DMAPP and GPP should exist within the DMATS superfamily. Therefore, we tested the acceptance of GPP and farnesyl diphosphate (FPP) by several known prenyltransferases from different fungi,[14] for example, 7-DMATS, CdpC3PT, CdpNPT and AnaPT, in the presence of their natural (or best accepted) aromatic substrates, that is, tryptophan or tryptophan-containing cyclic dipeptides. HPLC analysis revealed that additional peaks were only detected in the incubation mixtures of AnaPT with GPP. AnaPT was identified in the genome sequence of *N. fischeri* NRRL181 by genome mining and catalyses the C3α reverse prenylation of (*R*)-benzodiazepinedione (**1a**) in the presence of DMAPP (Scheme 1). It was then shown that AnaPT also accepts **2a** (the enantiomer of **1a**) and tryptophan-containing cyclic dipeptides with a diketopiperazine skeleton, for example, **3a**–**6a** (Figure 1). The reactions are C3α reverse prenylations in all these cases.[17,18] Here, we report the acceptance of GPP by AnaPT and identification of C6- and C7-prenylated derivatives as the enzyme products.

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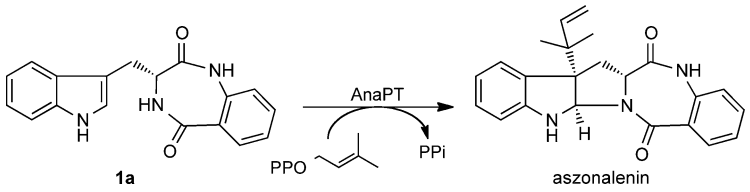


Scheme 1. C3α prenylation of **1a** by AnaPT in the presence of DMAPP.

Results

Acceptance of GPP by AnaPT

AnaPT was assayed with six tryptophan-containing cyclic dipeptides (**1a**–**6a**), including its natural substrate (*R*)-benzodiazepinedione (**1a**) in the presence of GPP or FPP. Enzymatic conversion was observed in all of the six incubation mixtures with GPP (Figure 1). Product formation was strictly dependent on the presence of active enzyme and GPP. Negative controls with heat-inactivated enzyme (boiled for 30 min) showed only substrate peaks in the HPLC chromatograms (data not shown). No conversion was detected for enzyme assays with FPP as prenyl donor (data not shown). Initial HPLC analysis of the GPP reaction mixtures of **2a**–**6a** with a reversed-phase C-18 column revealed only a single product peak for each; this was proven to be mixtures of at least two compounds after purification and NMR analysis (data not shown). Analysis of these reaction mixtures by HPLC with a normal-phase column clearly showed the presence of two or more enzyme products (Figure 1). Therefore, we used a normal-phase HPLC column for analysis and purification of the enzyme products. As shown in Figure 1, **1a** and its enantiomer **2a** were very well accepted by AnaPT in the presence of GPP. In the incubation mixture of **1a**, one dominant enzyme product (**1b**) was detected after incubation for 16 h, with a conversion yield of 88%. The reaction mixture of **2a** showed two dominant product peaks, **2b** and **2c** (ratio 1:2), with a total conversion yield of 91% (slightly higher than that for **1a**). Two major peaks were also detected in the reaction mixtures of cyclo-L-Trp-L-Leu (**3a**), cyclo-L-Trp-L-Trp (**4a**), cyclo-L-Trp-L-Tyr (**5a**) and cyclo-D-Trp-L-Tyr (**6a**). The total conversion yields were 33–72%. In the incubation mixtures of L-tryptophan-containing substrates (**2a**–**5a**), the yield of the b-series product (with a larger retention time) was clearly lower than that of the c-series product (shorter retention time). In contrast, **6a** (D-tryptophanyl moiety) produced more b-series product than c-series. In addition to **1b** (the dominant product), another product, **1c**, with an identical retention time to that of **2c** was detected at 14.2 min in the incubation mixture of **1a**.

AnaPT catalyses both C6- and C7-geranylation of tryptophan-containing cyclic dipeptides

For structure elucidation, large scale incubations with **1a**–**6a** were carried out in the presence of GPP. In total, six b-series (**1b**–**6b**) and five c-series products (**2c**–**6c**) were isolated and subjected to MS and NMR analyses. HR-EI-MS data (Table 1) showed molecular ions that were 136 kDa larger than those of the respective substrates, thus indicating the presence of a geranyl moiety in the enzyme products. Inspection of the ^{1}H NMR spectra (Figures S1–S11 in the Supporting Information) revealed indeed the presence of signals for a geranyl moiety (Tables S1 and S2). The signals of H-1′ of the geranyl moiety in the range 3.3–3.6 ppm proved its attachment to the indole ring through a C–C bond. The signals of H-2 of the indole ring (range 6.5–7.2 ppm as a singlet or a doublet with a small coupling constant of approximate 2 Hz) were found in all of the spectra, thus proving geranylation not at C-2 or C-3, but on the benzene ring of the indole unit. Comparison of the spectra revealed, furthermore, that the signals of the geranylated indole ring in the b-series products (**1b**–**6b**) had a same order and similar coupling patterns. This phenomenon was also observed for the c-series products (**2c**–**6c**). The order and coupling patterns of the signals for the aromatic protons in **1b**–**6b** clearly differ from those of **2c**–**6c**. The signals of the three protons on the geranylated benzene ring in **1b**–**6b** appeared as two doublets with a coupling constant of approximate 8 Hz and one (broad) singlet (Table 2). In some cases, the doublet in the high magnetic field was further coupled with a proton (coupling constant 1.3 Hz). This coupling pattern indicated geranylation at C-5 or C-6. The order of the signals for the four protons of the indole ring of **1b**–**6b** (that is, the two singlets between the two doublets) corresponds very well to that of the known C6-, but clearly differs from that of C5-prenylated or alkylated indole derivatives.[19–21] This unequivocally proves C6-geranylation of the b-series products (**1b**–**6b**).

The signals of the three coupling protons on the geranylated benzene ring in **2c**–**6c** appeared, from low to high magnetic field, as doublet, triplet and doublet, with coupling constants of approximate 7–8 Hz (Table 3). In the case of **2c**,

Table 1. HR-EI-MS data of the isolated enzyme products.

Compound	Chemical formula	HR-MS data Calcd $[M]^+$	Measured	Deviation (ppm)
1b	$C_{28}H_{31}N_3O_2$	441.2416	441.2396	4.6
2b	$C_{28}H_{31}N_3O_2$	441.2416	441.2434	−4.1
2c	$C_{28}H_{31}N_3O_2$	441.2416	441.2423	−1.6
3b	$C_{27}H_{37}N_3O_2$	435.2885	435.2894	−2.0
3c	$C_{27}H_{37}N_3O_2$	435.2885	435.2972	−4.9
4b	$C_{32}H_{36}N_4O_2$	508.2838	508.2851	−2.6
4c	$C_{32}H_{36}N_4O_2$	508.2838	508.2844	−1.1
5b	$C_{30}H_{35}N_3O_3$	485.2678	485.2644	6.9
5c	$C_{30}H_{35}N_3O_3$	485.2678	485.2676	0.4
6b	$C_{30}H_{35}N_3O_3$	485.2678	485.2689	−2.3
6c	$C_{30}H_{35}N_3O_3$	485.2678	485.2695	−3.6

Figure 1. HPLC analysis of enzyme assays with AnaPT, GPP and cyclic dipeptides, and conversion yields of the enzyme products. Standard incubation mixtures contained 20 μg AnaPT-His_6, 1 mM aromatic substrate, 1 mM GPP, 5 mM $CaCl_2$, 50 mM Tris·HCl pH 7.5 and were incubated at 37 °C for 16 h. The illustrated chromatograms were recorded at 277 nm. Retention times are given for substrate and products.

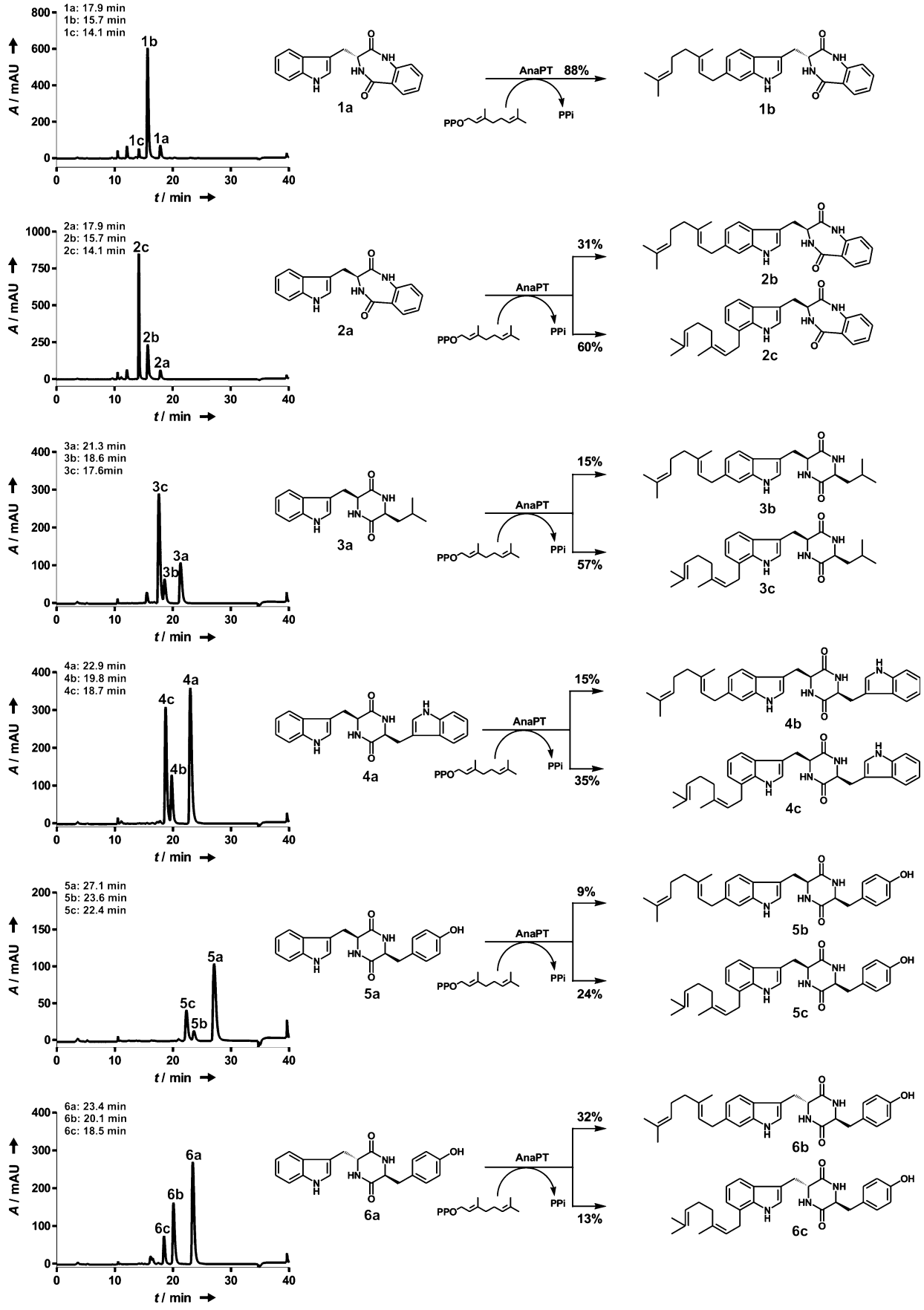
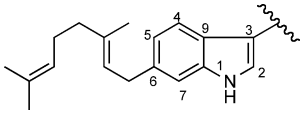
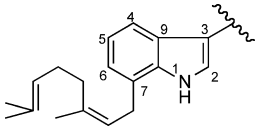

1a: 17.9 min
1b: 15.7 min
1c: 14.1 min
A / mAU
t / min
1a
1b
1c
AnaPT
88%
PPO
PPi
2a: 17.9 min
2b: 15.7 min
2c: 14.1 min
2a
2b
2c
31%
60%
3a: 21.3 min
3b: 18.6 min
3c: 17.6min
3a
3b
3c
15%
57%
4a: 22.9 min
4b: 19.8 min
4c: 18.7 min
4a
4b
4c
15%
35%
5a: 27.1 min
5b: 23.6 min
5c: 22.4 min
5a
5b
5c
9%
24%
6a: 23.4 min
6b: 20.1 min
6c: 18.5 min
6a
6b
6c
32%
13%

Table 2. Chemical shifts (ppm) of protons on the indole ring of the C6-geranylated derivatives (b-series).



Compound	H-4	H-7	H-2	H-5
1b	7.41, d, 8.1	7.16, br s	7.11, d, 2.3	6.79, d, 8.1
2b	7.41, d, 8.1	7.17, br s	7.11, d, 2.3	6.94, dd, 8.1, 1.3
3b	7.52, d, 8.1	7.17, br s	7.04, d, 2.3	6.99, dd, 8.1, 1.3
4b	7.56, d, 8.2	7.14, s	6.51, s	6.99, d, 8.2
5b	7.34, d, 8.1	7.05, s	6.87, d, 2.0	6.78, d, 8.1
6b	7.38, d, 8.1	7.03, s	6.89, d, 2.3	6.73, dd, 8.1, 1.3

Table 3. Chemical shifts of protons on the indole ring of the C7-geranylated derivatives (c-series).



Compound	H-2	H-4	H-5	H-6
2c	7.16, d, 2.3	7.38, dd, 7.5, 0.8	7.04, t, 7.5	7.01, dd, 7.1, 1.2
3c	7.07, s	7.49, d, 7.7	7.07, t, 7.7	7.03, d, 7.1
4c	6.53, d, 2.2	7.41, d, 8.2	7.07, t, 7.2	7.03, d, 7.1
5c	6.94, d, 2.1	7.27, d, 7.7	6.87, t, 7.7	6.81, d, 7.0
6c	6.96, d, 2.3	7.32, d, 7.8	6.83, t, 7.8	6.77, d, 7.2

a long-range coupling was also observed. This indicates C4- or C7-geranylation. Comparison of the chemical shifts of the three coupling aromatic protons in **2c**–**6c** with those of C4- and C7-prenylated tryptophan-containing cyclic dipeptides[22,23] revealed the disappearance of the signals for H-7 in **2c**–**6c**, which were therefore identified as C7-geranlyated derivatives.

Determination of the kinetic parameters for AnaPT in the presence of GPP

Kinetic parameters including Michaelis–Menten constant (K_m) and turnover number (k_{cat}) were determined for GPP as well as for **1a**–**6a** (Table 4, Figure S12–S18). The AnaPT reactions apparently followed Michaelis–Menten kinetics, including when in the presence of GPP. In the presence of its natural aromatic substrate, (*R*)-benzodiazepinedione (**1a**), a K_m value of 0.16 mM was calculated, identical to that for DMAPP with GPP.[24] With GPP as the prenyl donor, a K_m value of 0.25 mM was determined for **1a**, very similar to that with DMAPP (0.23 mM).[24] However, the turnover number (0.05 s^{-1} with GPP and **1a)** was significantly lower than that with DMAPP (1.5 s^{-1}). Higher K_m values (0.35–2 mM) were determined for the other aromatic substrates. It is evident that tyrosine-containing substrates **5a** and **6a** were converted with lower reactivity than the other substrates (Figure 1 and Table 4).

Discussion

As mentioned, AnaPT belongs to prenyltransferases of the DMATS superfamily and is involved in the biosynthesis of acetylaszonalenin in *N. fischeri*. This enzyme catalyses the C3α-prenylation of (*R*)-benzodiazepinedione (**1a**), thereby resulting in formation of aszonalenin.[24] C3α-prenylation was also observed with a number of tryptophan-containing cyclic dipeptides, including **2a**–**6a,** in the presence of DMAPP.[18,25] In general, members of the DMATS superfamily use DMAPP, but not other prenyl diphosphates (e.g. GPP or FPP), as prenyl donor.[14] We have recently demonstrated that the DMAPP structure can be modified by a shift or deletion of a methyl group, but the double bond at the β-position to pyrophosphate seems to be essential as alkyl donor.[19,26] Acceptance of such unnatural alkyl donors by several prenyltransferases from this family proves that the binding sites for DMAPP in these enzymes are flexible up to a certain degree.[19,26] Identification of the geranyl transferase VrtC from *P. aethiopicum*[15] indicates that the prenyl donors of these enzymes are not limited to DMAPP in nature. Acceptance of GPP by the dimethylallyl transferase AnaPT described in this study provides experimental evidence for the relationship of DMAPP- and GPP-accepting enzymes regarding the binding sites in the evolution of this family. It is also possible that some members of the DMATS superfamily are capable of utilising DMAPP, GPP and FPP as prenyl donors; this should be investigated in the near future.

Identification of the enzyme products of the six selected substrates as C6- and C7-geranylated is somewhat surprising for the C3-prenyltransferase AnaPT. However, shifts of the prenylation position and reduction of regioselectivity of prenyltransferases have also been observed with unnatural prenyl donors.[19,26] Comparison of the kinetic parameters for **1a** in the presence of GPP obtained in this study with those in the presence of DMAPP revealed similar affinity to the enzyme, thus indicating no significant change in the binding site for the aromatic substrate. Interestingly, GPP has an affinity similar to that of DMAPP. However, the AnaPT reaction was approximately 30 times faster with its natural substrate (DMAPP) than with GPP. The difference be-

Table 4. Kinetic parameters of AnaPT in the presence of GPP.

Subs.	K_m [mM]		k_{cat}[s^{-1}]		k_{cat}/K_m [s^{-1} M^{-1}]	
	b-series	c-series	b-series	c-series	b-series	c-series
1a	0.25 ± 0.03	–	0.05 ± 0.002	–	199	–
2a	0.80 ± 0.09	0.65 ± 0.04	0.10 ± 0.0008	0.17 ± 0.004	126	262
3a	2.11 ± 0.23	1.78 ± 0.17	0.07 ± 0.008	0.29 ± 0.01	33	162
4a	0.76 ± 0.06	0.83 ± 0.05	0.09 ± 0.006	0.22 ± 0.002	119	266
5a	0.38 ± 0.03	0.47 ± 0.04	0.002 ± 0.0002	0.007 ± 0.0004	5	15
6a	0.39 ± 0.02	0.35 ± 0.04	0.01 ± 0.0008	0.004 ± 0.0002	26	11
GPP[a]	0.16 ± 0.03	–	0.05 ± 0.001	–	307	–

[a] Kinetic parameters for GPP were measured with **1a** as aromatic substrate.

tween the prenylation position with GPP (this study) to that with DMAPP (previously reported) might be explained as follows. In case of DMAPP, its C-1′ is already at such a distance to C-3 of the indole moiety that the nucleophilic attack of this C-atom has to take place at C-3′ of the dimethylallyl cation after the cleavage of pyrophosphate from DMAPP. If, now, the large geranyl moiety binds in the same orientation as DMAPP, the distance of the C-1′ of GPP to C-3 of indole is even larger, and the only candidate for a nucleophilic attack seems to be the benzene ring.

Another interesting phenomenon observed in this study is the dependence of the ratios of C6- to C7-geranylated derivatives on the stereochemistry of the tryptophanyl moiety of the substrates. As mentioned above, C7-geranylated derivatives (c-series) were found as the main products in the incubation mixtures of L-tryptophan-containing substrates **2a**–**5a**, and C6-geranylated (b-series) were the main products for D-tryptophan-containing **6a** (Figure 1). This observation was confirmed by determination of the turnover number of the b- and c-series of the respective substrate (Table 4, Figures S12–S18). In the case of the D-tryptophan-containing **1a**, **1b** was detected as the predominant product. One minor peak (**1c**) with a same retention time as **2c** might indicate the presence of a C7-geranylated substance (which would be the enantiomer of **2c**) in the enzyme assay of **1a**. Because of the low quantity, this peak was not isolated and characterised. The observation of different ratios for C6- and C7-geranylated products indicates a slightly different placement of the aromatic substrates and therefore changed distance of C-6 and C-7 to the geranyl moiety. The number of the investigated substrates is of course too small to conclude the structural feature as main reason for the observed ratio of the b- and c-series products.

Conclusions

In this study, we demonstrated acceptance of GPP as substrate by a dimethylallyl transferase. To the best of our knowledge, acceptance of both DMAPP and GPP by an enzyme of the dimethylallyltryptophan synthase superfamily has not been reported before. In addition, the previously observed regioselectivity of the investigated enzyme was reduced by changing the prenyl donor, and two main products were detected in five of the six enzyme reactions. Furthermore, with GPP the prenylation position by AnaPT shifted from C-3 with DMAPP to C-6 or C-7 on the indole ring.

Experimental Section

Chemicals: Geranyl diphosphate was synthesised according to the method described by Woodside et al.[27] (*R*)-benzodiazepinedione (**1a**) and (*S*)-benzodiazepinedione (**2a**) were synthesised by condensation of D- or L-tryptophan with isatoic anhydride in the presence of triethylamine according to the method of Barrow and Sun.[28] Other cyclic dipeptides (**3a**–**6a**) were obtained from Bachem (Bubendorf, Switzerland).

Overexpression and purification of AnaPT-His$_6$ as well as enzyme assays: AnaPT-His$_6$ was overexpressed and purified as described previously.[24] In order to test the acceptance of GPP by AnaPT, enzyme assays (100 μL) containing aromatic substrate (1 mM), GPP (1 mM), $CaCl_2$ (5 mM), glycerol (2%, *v/v*), AnaPT-His$_6$ (20 μg) and Tris·HCl (50 mM, pH 7.5) were incubated at 37 °C for 16 h with mild agitation on a shaker. After incubation, the assay mixture was extracted with ethyl acetate (2×200 μL), and the combined organic phases were evaporated to dryness in a vacuum centrifuge. The residues were dissolved in $CHCl_3$ (150 μL) and analysed by HPLC as described below. For structure elucidation, enzyme assays were scaled up to 40 mL and the reaction mixtures contained aromatic substrate (1 mM), GPP (2 mM), $CaCl_2$ (5 mM), glycerol (2%, *v/v*), AnaPT-His$_6$ (6 mg) and Tris·HCl (50 mM, pH 7.5), and were incubated at 37 °C for 16 h. For determination of kinetic parameters of aromatic substrates, enzyme assays contained AnaPT-His$_6$ (20 μg), $CaCl_2$ (5 mM), GPP (1 mM), glycerol (2%, *v/v*) and aromatic substrate (0.025–4 mM), and were incubated at 37 °C for 120 min with mild agitation. For determination of the GPP dependency, the same dilution steps were used for GPP as for the aromatic substrates, with a fixed concentration of **1a** (1 mM). Conversion yields (%) were calculated from peak areas of products and substrate in HPLC chromatograms.

HPLC analysis: Reaction mixtures were analysed in an Agilent Series 1200 HPLC (Agilent, Foster City, CA) with a MultoHigh 100 Si column (250×4 mm, 5 μm; CS Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 mL min^{-1} with chloroform (solvent A) and methanol (solvent B). The reaction mixtures were separated with a linear gradient of 0–10% B for 30 min. After washing with 50% B for 5 min, the column was equilibrated with 100% A for 5 min. The substances were detected with a photodiode array detector and illustrated for absorption at 277 nm. The same HPLC equipment and solvent gradient were used for isolation of the enzyme products.

NMR and mass spectrometric analyses: For structural elucidation, the isolated enzyme products were subjected to ^{1}H NMR spectroscopic and MS analyses. For NMR analysis, the enzyme products were dissolved in $CDCl_3$ (150 μL; **1b**–**4b** and **2c**–**4c**) or [D_6]DMSO (150 μL; **5b**, **5c**, **6b** and **6c**). Spectra were recorded at room temperature with an ECX-500 spectrometer (JEOL, Tokyo, Japan) equipped with a broadband probe with z-gradient. Chemical shifts were referenced to $CHCl_3$ (7.26 ppm) or DMSO (2.50 ppm). All spectra were processed with MestReNova 5.2.2 (Metrelab Research, Santiago de Compostella, Spain). NMR data for the isolated products are given in Tables S1 and S2. The enzyme products were also analysed by HR-EI-MS. MS data are given in Table 1.

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Keywords: cyclic dipeptides · enzyme catalysis · geranylation · prenyltransferases · reaction mechanisms

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