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PAPER

Breaking the regioselectivity of indole prenyltransferases: identification of regular C3-prenylated hexahydropyrrolo[2,3-*b*]indoles as side products of the regular C2-prenyltransferase FtmPT1†

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The prenyltransferase FtmPT1 from *Aspergillus fumigatus* is involved in the biosynthesis of fumitremorgin-type alkaloids and catalysed the regular C2-prenylation of brevianamide F (*cyclo*-L-Trp-L-Pro). It has been shown that FtmPT1 also accepted a number of other tryptophan-containing cyclic dipeptides and prenylated them, in the presence of dimethylallyl diphosphate, at C-2 of the indole nucleus. Detailed analysis of the incubation mixtures of FtmPT1 with these cyclic dipeptides revealed the presence of additional product peaks in the HPLC chromatograms. Seven regularly C3-prenylated hexahydropyrrolo[2,3-*b*]indoles were isolated and identified by HR-ESI-MS and NMR analyses including HMBC, HMQC and NOESY experiments. Further experiments proved that the C2- and C3-prenylated products are both independent enzyme products. To the best of our knowledge, this is the first report on the enzymatic formation of regularly C3-prenylated indolines. A reaction mechanism for both C2- and C3-prenylated derivatives was proposed.

Introduction

Prenylated indole alkaloids are a large family of secondary metabolites with diverse biological activities.[1,2] These substances are hybrid natural products containing prenyl moieties derived from prenyl diphosphates and an indole or indoline ring from tryptophan or its precursors.[1] Prenyltransferases, especially those from the DMATS superfamily, are responsible for connection of these two structural elements.[1,3] Bioinformatic analysis indicated the presence of at least 200 members from this family in the database.[4] More than 20 of such enzymes have been characterized biochemically.[1,3,5]

These enzymes usually transfer a prenyl moiety regiospecifically to one of the seven positions of the indole nucleus. The identified enzymes, which use tryptophan as a natural or the best aromatic substrate and therefore act as dimethylallyltryptophan synthases, *e.g.* FgaPT2, 5-DMATS, IptA and 7-DMATS, catalysed regular prenylation at C-4 to C-7.[1,3,6] In contrast, indole prenyltransferases, which use tryptophan-containing cyclic dipeptides as aromatic substrates, catalysed the prenylation at N1, C-2 and C-3 of the indole ring.[1,5] For C2-prenylation, both regular (FtmPT1) and reverse prenyltransferases (NotF and BrePT) have been identified.[5,7,8] Two C3-prenyltransferases AnaPT and CdpC3PT from *Neosartorya fischeri* were found to catalyse regiospecific prenylations at C-3, resulting in the formation of hexahydropyrrolo[2,3-*b*]indoles carrying an α- and a β-configured prenyl moiety, respectively.[9–11] In both cases, the prenyl moieties are attached *via* its C-3 as reverse ones. It has also been demonstrated that CdpNPT from *Aspergillus fumigatus* was able to convert cyclic dipeptides to reversely C3-prenylated derivatives.[12,13]

Until now, no reports on enzymes were found in the literature, which catalyse a regular prenylation at C-3 of the indole ring. This could be explained by the fact that regularly C3-prenylated derivatives of cyclic dipeptides occur relatively rare in the nature and examples, *e.g.* nocardioazine B (Fig. 1), have only been recently isolated and identified.[14] In contrast, a number of reversely C3-prenylated derivatives including aszonalenin and roquefortine C (Fig. 1) have been identified as mycotoxins for decades.[1]

The aforementioned prenyltransferase FtmPT1 from *Aspergillus fumigatus* is involved in the biosynthesis of verruculogen and catalysed the prenylation of *cyclo*-L-Trp-L-Pro (**1a**, Fig. 2) at C-2, resulting in the formation of tryprostatin B (**1b**, Fig. 2).[7,15] Tryprostatin B was reported to exhibit inhibitory effects on the cell cycle progression in the G2/M phase.[16] It has also been shown that the prenyl moiety at C-2 in **1b** was essential for the cytotoxicity.[17,18] In the course of our search for cytotoxic compounds,

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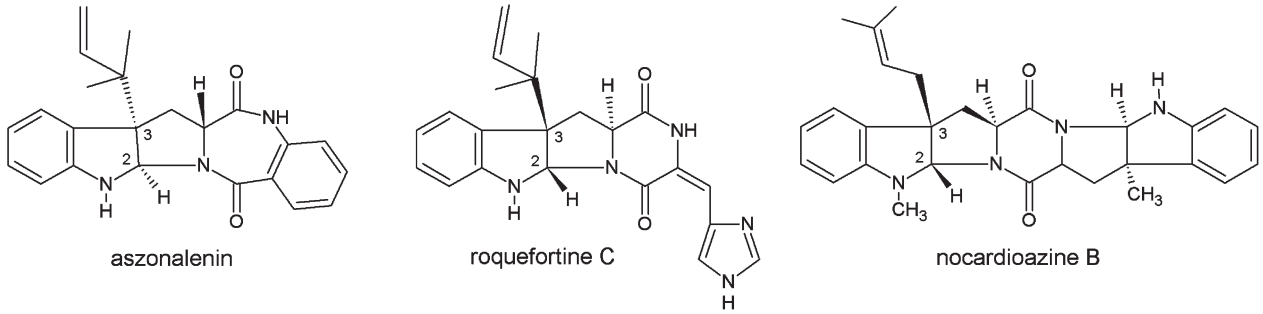


Fig. 1 Examples of naturally occurring C3-prenylated indolines.

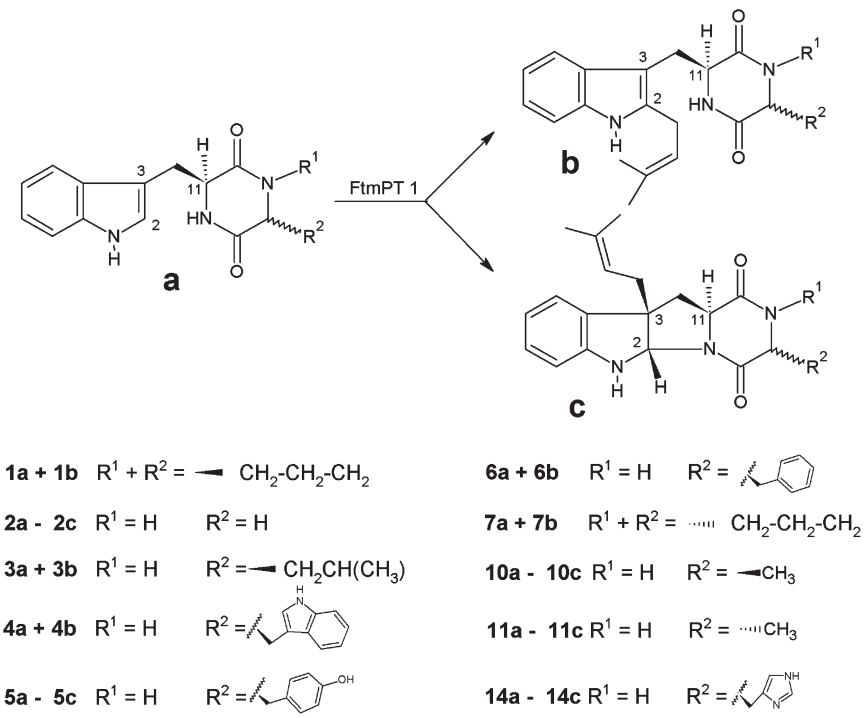


Fig. 2 Conversion of L-tryptophan-containing cyclic dipeptides by FtmPT1 to regular C2- and C3β-prenylated derivatives.

14 regularly C2-prenylated tryptophan-containing diketopiperazines were prepared by incubation of the respective cyclic dipeptides with FtmPT1 in the presence of dimethylallyl diphosphate.[19] In at least seven of these incubation mixtures, regularly C3-prenylated indolines were identified as side products of the enzymatic reactions. In this study, we describe the isolation and structure elucidation of these products and postulate a plausible reaction mechanism for the formation of both C2- and C3-prenylated derivatives.

Results and discussion

Detection of additional products in the incubation mixtures of FtmPT1

During the isolation of **1b–14b** (Fig. 2 and 3) for cytotoxic testing, the preparative HPLC chromatograms of the incubation mixtures of 14 cyclic dipeptides (**1a–14 a**) were recorded and illustrated in Fig. 4. The structures of **1b–14b** were unequivocally identified by NMR and MS analyses as regularly C2-prenylated diketopiperazines.[19] It showed clearly that the natural substrate of FtmPT1, *cyclo*-L-Trp-L-Pro (**1a**), was converted to tryprostatin B (**1b**) as the predominant product in this enzyme assay, corresponding very well to the results reported previously.[7] Due to the increased amount of the injected sample under the preparative conditions, one additional minor peak (approximately 0.7%) with a large retention time was detected. Inspection of other 13 chromatograms revealed the presence of two or more product peaks. Similar to that of **1a**, C2-prenylated diketopiperazines **3b**, **7b** and **8b** were predominant products in the incubation mixtures of *cyclo*-L-Trp-L-Leu (**3a**) and the two *cyclo*-Trp-Pro isomers **7a** and **8a** (Fig. 2 and 3). The second product peak (**c** series) in other reaction mixtures was significantly higher than those of **1a**, **3a**, **7a** and **8a**. In the cases of **5a** and **9a**, a third product (**d** series) each was detected.

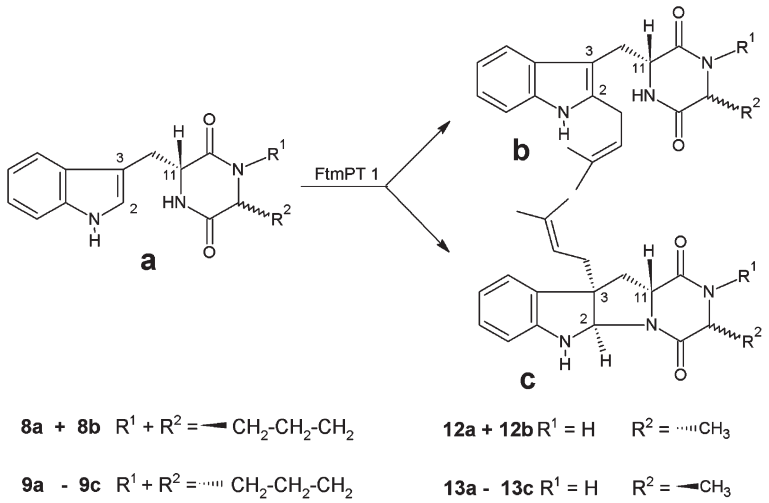


Fig. 3 Conversion of D-tryptophan-containing cyclic dipeptides by FtmPT1 to regular C2- and C3α-prenylated derivatives.

For better quantification, we carried out incubations at 37 °C in 100 µl scales with 5 µg FtmPT1 for 2 h. The yields of products **b** and **c** are summarized in Table 1. Under these conditions, yields of more than 10% were detected for the products of the **c** series in five of the used substrates, *i.e.* **2a**, **9a**, **10a**, **11a** and **13a**. The product yield of 18.6% for **2c** is about one-third of that of **2b**. The product yields of the **c** series in incubation mixtures of **9a**, **10a**, **11a** and **13a** were found to be about one-fourth of those of the **b** series. In the incubation mixture of **9a**, the product yield of an additional product **9d** reached 24% of that of **9b**.

FtmPT1 catalysed also regular C3-prenylation as side reaction

For structure elucidation, 12 products of the **c** series as well as **5d** and **9d** were isolated on HPLC from the respective incubation mixtures and subjected to spectroscopic analysis. Positive high resolution electrospray ionization mass spectrometry (HR-ESI-MS) data (Table 2) confirmed the monoprenylation in the isolated enzyme products by detection of $[M + Na]^+$ or $[M + H]^+$ ions, which are 68 daltons larger than those of the respective substrates.

UV spectra of the isolated products of the **c** series showed similar characteristic maximal absorptions at 200, 240 and 290 nm to each other and clearly differed from those of the C2-prenylated products of the **b** series with maxima at 220 and 280 nm (data not shown). The UV spectra of the products of the **c** series were very similar to those of the C3-prenylated indolines isolated from incubation mixtures of AnaPT or CdpC3PT,[9,11] indicating the presence of hexahydropyrrolo[2,3-*b*]indole systems in the structures of the products of the **c** series.

In the ^{1}H-NMR spectra of **2c**, **5c**, **9c–12c** and **14c** (Fig. S1.1–S9.4, ESI†), signals for a regular dimethylallyl moiety at δ_H 2.21–2.46 (d or dd, 2H-1′), 4.84–5.14 (br t, H-2′), 1.49–1.62 (s, 3H-4′), 1.61–1.67 (s, 3H-5′) were clearly observed (Table 3). The signals for the two protons of H-1′ of the prenyl moiety at 2.21–2.46 ppm indicted its attachment to a C- rather than to an O- or N-atom.[9,11,19–21] In comparison to those of the respective substrates, the signals for H-2 of the products of the **c** series were upfield shifted from about δ_H 7.2 to 5.18–5.41 (s, 1H), which are in a similar range to those of reversely C3-prenylated indolines.[9,11]

To determine the prenylation position, connectivities in HMBC of **2c**, **5c**, **11c** and **14c** were inspected in detail and summarized in Fig. S10 (ESI†). In these HMBC spectra, connectivities from H-1′ of the prenyl moiety at 2.21–2.46 ppm to C9 at δ_c 131.5–132.4 ppm and to C-3 at δ_c 56.1–56.5 ppm confirmed the attachment of the prenyl residue at position C-3. In HMBC spectra of **2c** and **5c** connectivities from H-10 to C-2′ were also observed.

Unambiguous proof of the β-configuration of the prenyl and H-2 in **5c**, **10c**, **11c** and **14c** was provided by NOESY experiments (Fig. S11, ESI†). In all NOESY spectra, interactions between H-10$_{anti}$ and H-1′, H-1′ and H-2 as well as H-10$_{syn}$ and H-4 were clearly detected (Table 4 and Fig. S11, ESI†). Strong NOE correlations between H-2 and the protons H-1′ of the prenyl moiety proved the *cis*-configuration between H-2 and C3-prenyl moieties of the indoline rings. For all of the analysed compounds, strong NOE correlations were observed between H-11 and H-10$_{syn}$ as well as H-1′ and H-10$_{anti}$. For **5c**, a strong correlation between H-10$_{anti}$ and H-2 was also observed. In contrast, no correlation was detected for H-11 with H-1′ and H-2′. From these results, we concluded that the regular C3-prenyl moiety in products **c** must be situated on the opposite side to H-11, *i.e.* with a β-configuration, as summarized in Fig. 2. The ^{1}H-NMR data of **10c** corresponded well to a synthetic product reported previously.[22] The ^{1}H-NMR data of **9c** are identical to its enantiomer synthesized chemically.[22] The ^{1}H-NMR data of **13c** are identical to its enantiomer **11c** reported in this study (Table 3).These results proved that hexahydropyrrolo[2,3-*b*]indoles with an α-configuration were products of the **c** series of D-tryptophan-containing cyclic dipeptides, as summarized in Fig. 3.

5d was identified as *cyclo*-N1-dimethylallyl-L-Trp-L-Tyr by comparison of the ^{1}H-NMR data with those published previously.[21] **9d** showed a nearly identical NMR spectrum as its enantiomer *cyclo*-N1-dimethylallyl-L-Trp-L-Pro.[21]

FtmPT1 products of the b and c series are independently formed during the enzyme reaction

As described above, regularly C2- and C3-prenylated products were identified in the reaction mixtures of FtmPT1. FtmPT1

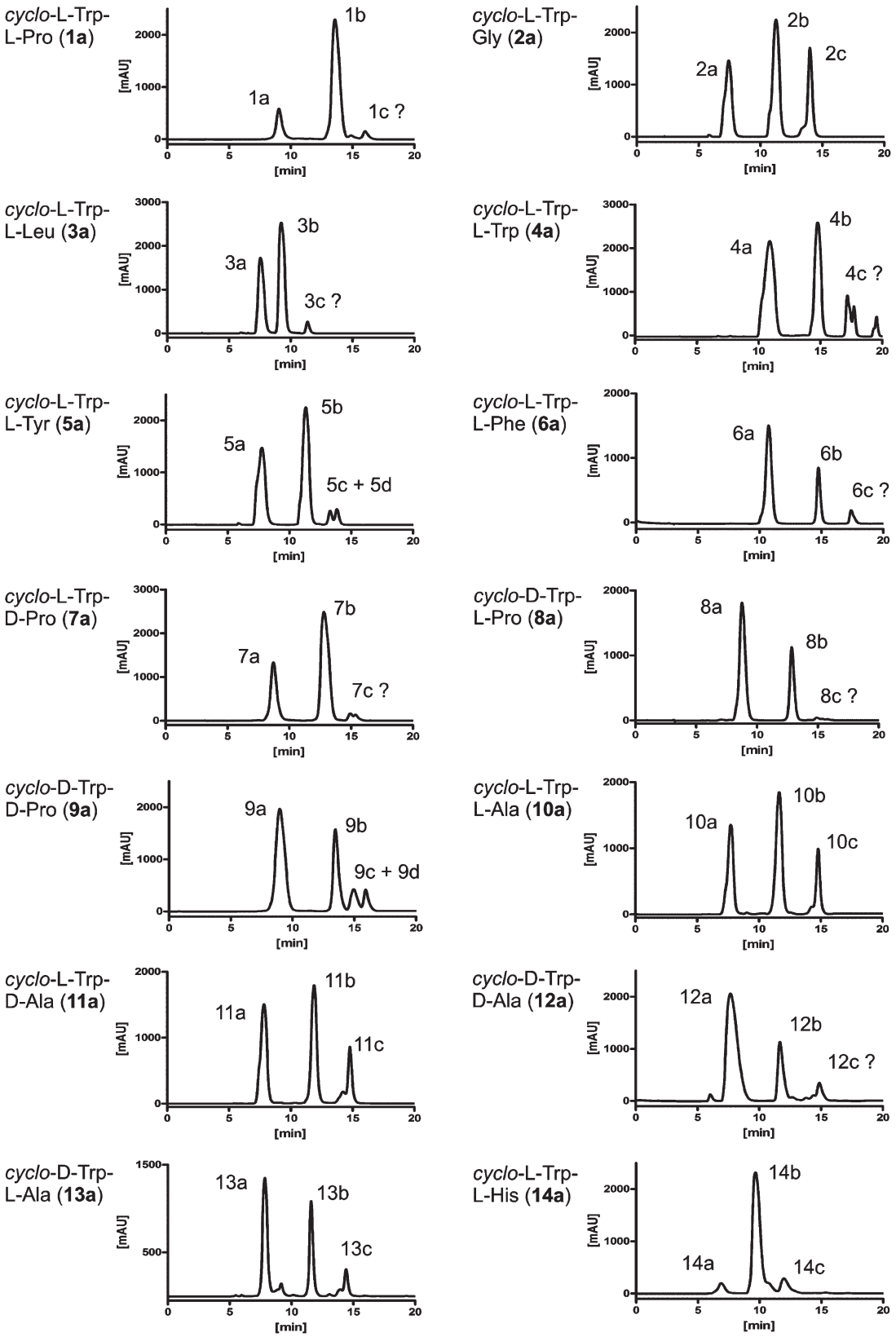


Fig. 4 HPLC chromatograms of incubation mixtures of FtmPT1 with 14 cyclic dipeptides. The reaction mixtures (50 ml) containing 1 mg FtmPT1, 10 mM $CaCl_2$, 1 mM cyclic dipeptide and 2 mM DMAPP were incubated at 37 °C for 2 h. The substances were detected with a Photo Diode Array detector and illustrated for absorption at 296 nm.

Table 1 Conversion of diketopiperazines to prenylated products by the prenyltransferase FtmPT1[a]

Substrate	Non-reacted substrate (%)	Yield of product **b** [%]	Yield of product **c** [%]
1a	7.0	82.3	0.7
2a	24.4	57.0	18.6[b]
3a	28.2	70.9	0.9[c]
4a	29.2	67.0	3.8[c]
5a	26.5	72.4	1.1[b]
6a	56.1	39.3	4.6[c]
7a	20.9	75.7	3.4
8a	70.8	27.4	1.8[c]
9a	44.8	43.4	11.8[b]
10a	33.8	53.5	12.7[b]
11a	44.0	43.9	12.1[b]
12a	86.3	8.3	5.4[c]
13a	42.0	46.4	11.6[b]
14a	25.1	68.1	6.8[b]

[a] The reaction mixtures in 100 µl scales containing 5 µg of FtmPT1, 10 mM $CaCl_2$, 2 mM DMAPP and 1 mM diketopiperazine were incubated for 2 h at 37 °C. The substances were detected at 296 nm. [b] The products were isolated on HPLC and characterized by NMR and MS analyses. [c] The products were isolated on HPLC and identified by HR-ESI-MS analysis.

Table 2 HR-ESI-MS data of the enzyme products **c** and **d** series

Comp.	Chemical formula	HR-ESI-MS data		Deviation (ppm)
		Calculated	Measured	
2c	$C_{18}H_{21}N_3O_2$	334.1531 $[M + Na]^+$	334.1559	8.2
3c	$C_{22}H_{29}N_3O_2$	390.2157 $[M + Na]^+$	390.2172	3.6
4c	$C_{27}H_{28}N_4O_2$	463.2110 $[M + Na]^+$	463.2131	4.5
5c	$C_{25}H_{27}N_3O_3$	440.1951 $[M + Na]^+$	440.1915	7.9
5d	$C_{25}H_{27}N_3O_2$	440.1950 $[M + Na]^+$	440.1999	1.9
6c	$C_{25}H_{27}N_3O_2$	424.2001 $[M + Na]^+$	424.2008	1.7
8c	$C_{21}H_{25}N_3O_2$	374.1844 $[M + Na]^+$	374.1834	2.7
9c	$C_{21}H_{25}N_3O_2$	374.1844 $[M + Na]^+$	374.1840	1.3
9d	$C_{21}H_{25}N_3O_2$	374.1844 $[M + Na]^+$	374.1844	0.1
10c	$C_{19}H_{23}N_3O_2$	348.1688 $[M + Na]^+$	348.1672	4.7
11c	$C_{19}H_{23}N_3O_2$	348.1688 $[M + Na]^+$	348.1667	6.0
12c	$C_{19}H_{23}N_3O_2$	348.1688 $[M + Na]^+$	348.1690	0.5
13c	$C_{19}H_{23}N_3O_2$	348.1688 $[M + Na]^+$	348.1671	4.8
14c	$C_{22}H_{25}N_5O_2$	392.2087 $[M + H]^+$	392.2087	0.2

catalysed the conversion of **1a** to **1b** by regular C2-prenylation, which serves as an intermediate in the biosynthesis of fumitremorgin-type alkaloids.[23] It would be not surprising that other cyclic dipeptides can also be prenylated at C-2 of the indole ring. However, a regular C3-prenylation was an unexpected event.

To prove the relationships between the products of the **b** and **c** series, we carried out time dependence of their formation by using **2a**, **9b** and **11a** as substrates. As shown in Fig. S12 (ESI†), total yields of 60–80% were reached after incubation for 4 h. It seems that the reactions have reached their equilibrium after 4 h under these conditions. The formation of the products **b** and **c** was found however to be independent from each other in all of the cases.

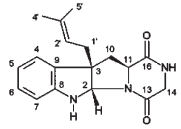
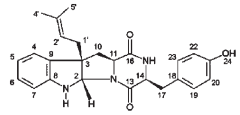
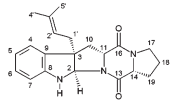
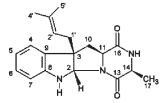
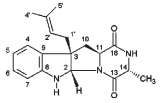
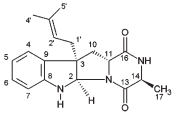
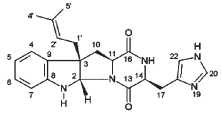
Furthermore, the isolated products **b** and **c** from **2a**, **5a**, **10a**, **11a** and **13a** were incubated at 37 °C for 2 h in the presence of DMAPP with and without FtmPT1 (Fig. S13–S17†). Incubations of the five substrates with FtmPT1 and DMAPP were used as positive controls. It has been shown that the products of the **b** and **c** series were chemically stable and no meaningful changes were detected in the incubation mixtures without FtmPT1. No additional peaks were observed in the incubation mixtures of these substances with FtmPT1, proving that no conversion had taken place between the isolated products of the **b** and **c** series (Fig. S13–S17, ESI†). It can however not be excluded that conversion between **b** and **c** takes place as enzyme-bound intermediates before their release from FtmPT1.

To compare the behavior of FtmPT1 towards different substrates and to understand the two prenylation possibilities, kinetic parameters were determined for eight substrates (Table 5, Fig. S18†). As shown in Fig. S18,† substrate inhibition at 2 mM was observed for the formation of **11b** and **13b** (Fig. S18†). In several cases, *e.g.* **9a**, **11a** and **14a**, the kinetic parameters for products of the **c** series cannot be determined, because the product formation was found still in the lineal region for substrate concentrations of up to 2 mM. From the data listed in Table 5, it is obvious that tryptophan-containing cyclic dipeptides with a smaller amino acid than proline, *e.g.* glycine in **2a** or alanine in **10a**, **11a** and **13a**, were poor substrates for FtmPT1. In the case of **14a**, electrostatic difference between proline and histidine seems to be responsible for their different affinities to FtmPT1. Furthermore, it can be interpreted that substrates with high affinity to FtmPT1 were mainly converted to products of the **b** series. As expected, FtmPT1 showed the (nearly) highest affinity and velocity towards its natural substrate **1a** with **1b** as the predominant product. The catalytic efficiency of approximately 25 312 s^{-1} M^{-1} for **1a** is much higher than other tested substrates. The two other well accepted substrates **5a** and **9a**, *i.e.* with high affinity to FtmPT1, were converted mainly to products of the **b** series with relatively high catalytic efficiencies of 6407 and 2214 s^{-1} M^{-1}, respectively. In contrast, the products of the **c** series were clearly detected in the incubation mixtures of substrates with low affinity, *e.g.* **2a** and **10a**, with K_M values of 0.77 and 0.54 mM for **2b** and **10b** as well as 0.69 and 0.48 mM for **2c** and **10c**, respectively.

Reaction mechanisms of C2- and C3-prenylation by FtmPT1

C3-prenylation by a C2-prenyltransferase sounds somewhat surprising. It can be however explained by inspection of the substrate binding pocket of the FtmPT1 structure.[24] It has been described that brevianamide F (**1a**) is placed in the PT barrel of FtmPT1 and forms a hydrogen bond with its N-1 nitrogen to the side chain carboxylate of the E102 residue of FtmPT1 (Fig. 5). The indole ring of **1a** is furthermore stabilised by tyrosine 203. As proposed previously,[24–26] a dimethylallyl cation with delocalisation of the positive charge between its C-1 and C-3 will be firstly created by elimination of the pyrophosphate group with the help of several basic amino acid residues and stabilised by cation-π interactions with the aromatic ring system, *i.e.* with its substrate **1a** from one side and the tyrosine residue Y382 from the other side (Fig. 5). Stabilisation of this cation by substrates with a smaller residue than **1a**, *e.g.* **2a**, **10a**, **11a** and **13a** or with a different electrostatic density as in the case of **14a**, can differ from that of **1a**, so that clear variation in kinetic parameters was

Table 3 ^{1}H-NMR and ^{13}C-NMR data of the enzyme products in CD_3OH (**2c**, **5c** and **10c**, **11c**, **13c**, **14c**) or $CDCl_3$ (**9c**)

	cyclo-C3β-dimethylallyl-L-Trp-Gly (**2c**)		*cyclo*-C3β-dimethylallyl-L-Trp-L-Tyr (**5c**)		*cyclo*-C3α-dimethylallyl-D-Trp-D-Pro (**9c**)		*cyclo*-C3β-dimethylallyl-L-Trp-L-Ala (**10c**)		*cyclo*-C3β-dimethylallyl-L-Trp-D-Ala (**11c**)		*cyclo*-C3α-dimethylallyl-D-Trp-L-Ala (**13c**)		*cyclo*-C3β-dimethylallyl-L-Trp-L-His (**14c**)	
														
Pos.	δ_H, multi, *J* in Hz	δ_C	δ_H, multi, *J* in Hz	δ_C	δ_H, multi, *J* in Hz	δ_C	δ_H, multi, *J* in Hz	δ_C	δ_H, multi, *J* in Hz	δ_C	δ_H, multi, *J* in Hz	δ_C	δ_H, multi, *J* in Hz	δ_C
NH-1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	5.34, s	80.6	5.18, s	80.3	5.41, s	80.4	5.31, s	80.4	5,37 s	80.4	5.38, s	80.4	5.27, s	80.5
3	—	56.5	—	56.5	—	*h*	—	*h*	—	56.1	—	*h*	—	56.5
4	7.10, d, 7.5	124.3	6.97, d, 7.2[b]	124.2	7.07, m[b]	124.0	7.10, br d, 7.5	124.0	7.10, d, 7.5	124.0	7.11, dd, 7.5, 1.2	124.0	7.09, br d, 7.4	124.1
5	6.70, td, 7.5, 0.8	120.0	6.65, td, 7.4, 0.8	119.6	6.75, td, 7.6, 0.8	119.8	6.71, td, 7.7, 0.9	119.8	6.70, td, 7.5, 0.7	119.6	6.72, td, 7.5, 1.0	119.6	6.70, td, 7.4, 0.9	119.8
6	7.01, td, 7.6, 1.2	129.6	6.97, td, 7.3, 1.2[b]	129.3	7.07, m[b]	129.3	7.01, td, 7.7, 1.2	129.3	7.02, td, 7.5, 1.1	129.3	7.03, td, 7.7, 1.3	129.3	7.01, td, 7.6, 1.2	129.4
7	6.58, d, 7.6	110.2	6.52, d, 7.5	109.5	6.57, d, 7.7	110.2	6.59, br d, 7.8	110.2	6.59, d, 7.6	109.9	6.60, dt, 7.8, 0.6	109.9	6.57, d, 7.7	110.0
8	—	151.0	—	151.4	—	*h*	—	*h*	—	150.8	—	150.8	—	150.8
9	—	132.4	—	131.5	—	*h*	—	*h*	—	132.0	—	132.0	—	132.0
10_{syn}[a]	2.58, dd, 12.5, 6.1	40.3	2.26, dd, 12.1, 5.5[c]	41.8	2.50, m	39.6	2.57, dd, 12.6, 6.3	39.6	2.60, dd, 12.5, 6.1	40.5	2.61, dd, 12.5, 6.1	40.5	2.57, dd, 12.6, 6.1	39.8
10_{anti}[a]	2.22, dd, 12.5, 11.5		1.19, t, 12.0		2.50, m		2.23, dd, 12.6, 11.3		2.20, dd, 12.3, 11.7		2.22, dd, 12.5, 11.5		2.09, dd, 12.5, 11.5	
11	4.00, m	59.2	3.79, ddd, 12.0, 5.4, 1.7	58.7	4.35, t, 8.7	59.6	4.03, ddd, 11.3, 6.2, 1.9	59.6	4.02, dd, 11.4, 6.0	58.4	4.02, dd, 11.5, 6.1	58.4	4.05, ddd, 12.1, 6.1, 1.8	59.6
13	—	166.2	—	166.0	—	*h*	—	*h*	—	168.7	—	168.7	—	166.4
14	4.04, dd, 17.1, 2.3	46.9	4.33, m	57.7	4.15, m	51.9	4.11, qd, 6.9, 1.8	51.9	3.90, m	53.9	3.91, qd, 7.2, 0.8	53.9	4.45, td, 6.0, 1.7	55.3
	3.73, dd, 17.0, 3.3												—	—
NH-15	8.01, s	—	7.96, s	—	—	—	8.07, br s	—	8.22, s	—	—	—	—	—
16	—	171.3	—	169.6	—	*h*	—	*h*	—	170.4	—	170.4	—	171.8
17	—	—	3.16, dd, 13.9, 4.1	38.3	3.46, m[c]	15.6	1.36, d, 6.9	15.6	1.36, d, 7.1	19.9	1.36, d, 7.1	19.9	3.19, dd, 15.5, 5.7	26.0
	—		2.92, dd, 13.9, 4.4		3.46, m[c]								3.33, dd, 4.3[g]	
18	—	—	—	126.7	1.88–2.20, m[d]	—	—	—	—	—	—	—	—	130.5
					1.88–2.20, m[d]									
19	—	—	7.01, d, 8.5[e]	132.1	1.88–2.20, m[d]	—	—	—	—	—	—	—	—	—
					1.88–2.20, m[d]									
20	—	—	6.72, dt, 8.5, 1.9[d]	116.0	—	—	—	—	—	—	—	—	8.75, s	134.8
21	—	—	—	157.6	—	—	—	—	—	—	—	—	—	—
22	—	—	6.72, 8.5, 1.9[d]	116.0	—	—	—	—	—	—	—	—	7.32, s	118.8
23	—	—	7.01, d, 8.5[e]	132.1	—	—	—	—	—	—	—	—	—	—
1′	2.45, dd, 14.5, 8.0	36.9	2.21, dd, 14.3, 8.3	37.5	2.31, m	36.6	2.39,dd, 14.0, 7.0	36.6	2.41, dd 14.6, 7.4	36.8	2.42, dd, 14.4, 7.2	36.8	2.37, d, 7.6	36.7
	2.41, dd, 14.5, 7.3		2.24, dd,[c]				2.44, dd, 14.0, 8.1		2.45, dd, 14.5, 8.1		2.46, dd, 14.3, 7.9			
2′	5.14, br t, 7.2	120.0	4.84, m[f]	119.8	5.10, t, 8.1	119.8	5.14, br t, 7.3	119.8	5.13, br t, 8.2	119.7	5.14, br t, 7.9	119.7	5.10, br t, 7.4	119.7
3′	—	136.1	—	135.3	—	*h*	—	*h*	—	135.8	—	135.8	—	136.0
4′	1.52, s	17.8	1.51, s	17.8	1.62, s	17.5	1.51, s	17.5	1.52, s	17.5	1.53, s	17.5	1.49, s	17.6
5′	1.66, s	25.9	1.61	25.6	1.67, s	25.6	1.66, s	25.6	1.66, s	25.6	1.67, s	25.6	1.65, s	25.7

[a] H-10_{syn} has a *cis*- and H-10_{anti} a *trans*-configuration to H-11. [b] Overlapping signals. [c] Overlapping signals. [d] Overlapping signals. [e] Overlapping signals. [f] Overlapping with water signal. [g] Overlapping with solvent signal. [h] Due to low purity, signals were not detected.

determined in this study (Table 5). In the co-structure of FtmPT1 with a non-hydrolysable substrate analogue DMASP, both C-2 and C-3 of the indole ring are within a distance of 3.8 Å to C-1 of DMASP and therefore prenylation would be possible for both positions. However, C2-prenylation of **1a** by FtmPT1 would result in the formation of a more favorable product.[24] A similar behavior can be expected for the three stereoisomers of **1a**. This hypothesis is supported by the fact that C2-prenylated derivatives were detected predominantly in the incubation mixtures of **1a**, **7a** and **8a**. Other diketopiperazines might be located, due to the given geometric and electrostatic features, in a slightly unfavourable manner in the reaction site, so that the prenylation at C-3 would be easier and indeed the yields of C3-prenylated derivatives have increased in the incubation mixtures of these compounds (Fig. 4). These observations were supported by detailed inspection of the kinetic parameters obtained for eight compounds (Table 5). As mentioned above, the three best accepted substrates **1a**, **5a** and **9a** with K_M values of 0.16, 0.27 and 0.14 mM and catalytic efficiencies (k_{cat}/K_M) of 25 312, 6407 and 2214 s^{-1} M^{-1}, respectively, were converted dominantly to products of the **b** series. In contrast, the poorly accepted substrate **2a** with K_M values of 0.77 and 0.69 mM for the products of the **b** and **c** series was converted to both products with a ratio of 3 : 1. A similar phenomenon was also observed for **14a** (Fig. S18†).

It can be proposed that both C2- and C3-prenylations begin simultaneously with the attack of the electron-rich indole ring at C2 and C-3 to C-1′ of the dimethylallyl cation (Fig. 5). Their ratio would be dependent on the position of a given aromatic substrate and accordingly the distance of C-2 to C-1′ as well as C-3 to C-1′. Attacking at C-2 and C-3 would result in the formation of the intermediate **I** *via* pathway A and **II** *via* pathway B, respectively. For formation of the C2-prenylated product **b**, just one deprotonating step to restore the indole system is necessary. The fate of **II** would be attacking the cation at position C-2 of the indole ring by the electron-rich N12 nitrogen, as proposed for reversely C3-prenylated indolines,[9] resulting in the formation of protonated form **III** of the end product **c**. Releasing of one proton leads then to regularly C3-prenylated derivative **c** (Fig. 5).

Table 4 NOE results of **10c** as an example for regular C3-prenylated diketopiperazines

Protons	Strength
H-11–H-10$_{syn}$	Strong
H-1′–H-10$_{anti}$	Medium
H-1′–H-2	Strong
H-4–H-10$_{syn}$	Medium
H-1′–H-11	Not observed
H-2′–H-11	Not observed

Table 5 Kinetic parameters for the **b** and **c**-series

	K_M [mM]		k_{cat} [s^{-1}]		k_{cat}/K_M [s^{-1} M^{-1}]	
Sub	**b** series	**c** series	**b** series	**c** series	**b** series	**c** series
1a	0.16	—	4.05	—	25 312	—
2a	0.77	0.69	3.14	2.55	4078	3696
5a	0.27	0.37	1.73	0.09	6407	246
9a	0.14	—	0.31	—	2214	—
10a	0.54	0.48	1.47	0.13	2722	271
11a	0.41	—	0.71	—	1732	—
13a	0.39	0.14	0.24	0.06	615	429
14a	0.45	—	2.14	—	4756	—

Conclusions

In this study, we proved that the prenyltransferase FtmPT1[7,27] converted tryptophan-containing diketopiperazines to their prenylated derivatives. For three of the four *cyclo*-Trp-Pro isomers and *cyclo*-L-Trp-L-Leu, regularly C2-prenylated derivatives were found as predominant products. For seven diketopiperazines, regularly C3-prenylated products were detected in the enzyme

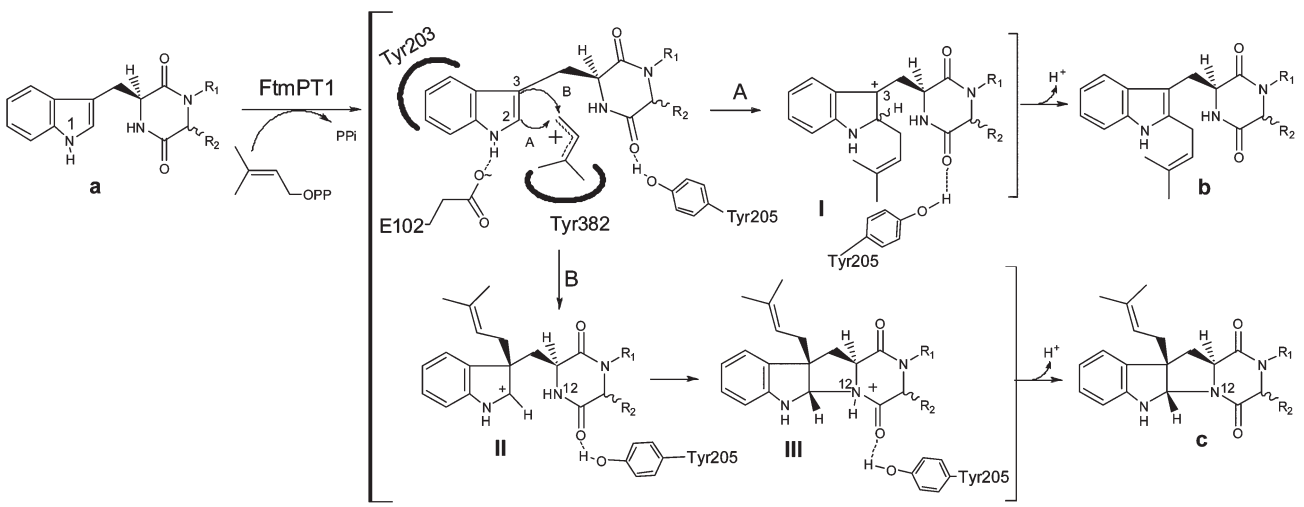


Fig. 5 Proposed reaction mechanism of the formation of C2- and C3-β-prenylated products catalysed by FtmPT1 with L-tryptophan-containing cyclic dipeptides as examples. In analogy, attacking of the prenyl moiety from the opposite side of H-11 of the D-tryptophan-containing cyclic dipeptides would result in formation of C3-α-prenylated derivatives. For R^1 and R^2 moieties see Fig. 2.

assays with total yields of up to 19%. Their structures were elucidated unequivocally by NMR and MS analyses. This indicated that the previously observed regioselectivity of many of the identified prenyltransferases can be interrupted, at least in parts, by using non-natural substrates. This reduces the convenience for product isolation, increases however the structure diversity obtained by a chemoenzymatic synthesis approach.

Experimental section

Chemicals and protein purification

Synthesis and availability of dimethylallyl diphosphate (DMAPP) and cyclic dipeptides were described previously.[19]

To get recombinant FtmPT1 for enzyme assays, *Escherichia coli* M15 cells harbouring the plasmid pAG012[7] were cultivated in 2 l Erlenmeyer flasks containing 1 l liquid Luria–Bertani medium supplemented with carbenicillin (50 μg ml^{-1}) and kanamycin (25 μg ml^{-1}). The cultures were grown at 37 °C for 4 h at 220 rpm until an absorption of 0.7 at 600 nm was reached. For induction, IPTG was added to the cultures to a final concentration of 0.1 mM and the bacteria were cultivated for a further 16 h at 30 °C before harvest. Purification of the recombinant FtmPT1 was carried out according to the QIAexpressionist™ protocol from Qiagen.

Enzyme assay and HPLC analysis

Standard enzyme assays (100 μl) containing 50 mM Tris-HCl, pH 7.5, 2 mM DMAPP, 1 mM cyclic dipeptide, 10 mM $CaCl_2$ and 5 μg FtmPT1 were incubated at 37 °C for 2 h. K_M values were determined in 100 μl assays containing 0.4 μg FtmPT1, 2 mM DMAPP and cyclic dipeptides at concentrations of up to 2 mM. The assays were incubated at 37 °C for 30 min. The reactions were terminated by addition of 100 μl methanol.

After removal of the protein by centrifugation (15000*g*, 10 min, 4 °C), the enzyme products were analyzed on an Agilent HPLC-Series 1200 by using a Multospher 120 RP-18-5 column (250 × 4 mm, 5 μm, Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 ml min^{-1}. Water (solvent A) and methanol (solvent B) were used as solvents. For analysis of the assay with *cyclo*-L-Trp-L-His, both solvents contained 0.5% (v/v) trifluoroacetic acid. The assays were analyzed with a gradient from 30% to 100% B over 30 min and a washing phase with 100% solvent B for 5 min was used. The column was equilibrated with 30% solvent B for 5 min. The substances were detected with a Photo Diode Array detector and illustrated at 296 nm in this study.

Isolation of the enzyme products

The preparative enzymatic synthesis for the structural elucidation was carried out as follows. To a 50 ml reaction tube, DMAPP (2 mM), cyclic dipeptide (1 mM), $CaCl_2$ (10 mM), Tris-HCL (50 mM, pH = 7, 5) and FtmPT1 (1 mg) were added to a final volume of 50 ml. The reaction mixtures were incubated at 37 °C for 2 h and subsequently extracted twice with equal volume of ethyl acetate and evaporated *in vacuo*. The products were purified on HPLC (Agilent series 1200) with detection at 296 nm using a Multospher 120 RP-18-5 column (250 × 10 mm, 5 μm, C+S Chromatographie Service) and a flow rate of 2.5 ml min^{-1}. Water (solvent A) and methanol (solvent B) were used as solvents. For isolation of **14c**, 0.5% trifluoroacetic acid was added to solvents A and B. For analysis of enzyme products, a linear gradient of 65 to 100% (v/v) solvent B in 15 min was used. The column was then washed with 100% solvent B for 5 min and equilibrated with 65% (v/v) solvent B for 5 min. The fractions containing the prenylated diketopiperazines were collected. After evaporation of the solvents, the obtained products were analysed spectroscopically.

ESI-MS and NMR analysis of the enzyme products

The obtained products were analyzed by NMR (in CD_3OH, CD_3OD or $CDCl_3$) and ESI-MS analyses. NMR spectra were recorded at room temperature on a Bruker Avance 600 MHz or a JEOL ECA-500 spectrometer. The heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra were recorded with standard methods.[28] Gradient-selection for nuclear Overhauser effect spectroscopy (NOESY) experiment was performed in phase-sensitive mode[29] and processed with a Bruker TOPSPIN 2.1 or a MestReNova.5.2.2. The positive electrospray ionization mass spectrometry (HR-ESI-MS) was carried out on an AutoSpec instrument (Micromass Co. UK Ltd).

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