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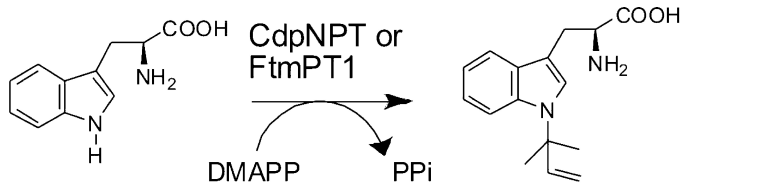

L-tryptophan

N-1-3´-(3´,3´-dimethylallyl)-L-tryptophan

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Substrate Promiscuity of the Cyclic Dipeptide Prenyltransferases from *Aspergillus fumigatus*[§]

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This study reports that a series of tryptophan derivatives with modifications on the side chain or at the indole ring were accepted by two cyclic dipeptide prenyltransferases, CdpNPT and FtmPT1, and converted to prenylated derivatives. The structures of the enzymatic products were elucidated by NMR and MS analyses. In comparison to cyclic dipeptides, which were reversely prenylated by CdpNPT at N-1 and in a regular manner by FtmPT1 at C-2, respectively, tryptophan and its simple derivatives were prenylated reversely by both enzymes at N-1. These results demonstrated the substrate promiscuity of both enzymes.

Recently, seven putative indole prenyltransferase genes were identified in the genome sequence of *Aspergillus fumigatus.*[1] Six of these prenyltransferases have been characterized biochemically and shown to catalyze transfer reactions of a prenyl moiety onto different positions of the indole rings in the biosynthesis of diverse secondary metabolites.[2−7] For example, the two dimethylallyltryptophan synthases FgaPT2 and 7-DMATS catalyze the prenylation of L-tryptophan at C-4 and C-7 of the indole ring, respectively.[3,6] CdpNPT and FtmPT1 were shown to prenylate tryptophan-containing cyclic dipeptides with a diketopiperazine ring at N-1 and C-2, respectively (Scheme 1).[2,4] All of these enzymes accepted only dimethylallyl diphosphate (DMAPP) as the prenyl donor. On the other hand, these enzymes showed broad substrate specificities toward its aromatic substrates. In previous studies, it was shown that FgaPT2 and 7-DMATS also accepted a series of simple indole derivatives, affording conversion to C-4- and C-7-prenylated derivatives,[8,9] respectively. Correspondingly, CdpNPT and FtmPT1 accepted all of the tested tryptophan-containing cyclic dipeptides and prenylated them at N-1 and C-2, respectively.[2,4,10] As expected, tryptophan was not a substrate for the cyclic dipeptide prenyltransferases CdpNPT and FtmPT1, and cyclic dipeptides were not accepted by the dimethylallyltryptophan synthase FgaPT2.[4,8,10] Interestingly, 7-DMATS accepted both tryptophan-containing linear and cyclic dipeptides as substrates.[6] The following questions had arisen from these results: Is the broad substrate specificity of 7-DMATS just an exception among the indole prenyltransferases? Were the assay conditions for CdpNPT and FtmPT1 with tryptophan not sensitive enough to see any product formation? Very recently, aminopeptidase activity of CdpNPT, FtmPT1, 7-DMATS, and FgaPT1 was detected, indicating their common evolutionary relationship.[11] Therefore, these enzymes might share many features including substrate promiscuity. These results prompted the reinvestigation of the acceptance of tryptophan and its simple derivatives by CdpNPT and FtmPT1.

Results and Discussion

Tryptophan Acceptance Was Detected at Higher Enzyme Concentration by Both Cyclic Dipeptide *N*-Prenyltransferase (CdpNPT) and Brevianamide F Prenyltransferase (FtmPT1). Under conditions described previously,[2] e.g., incubation of 1 mM tryptophan, 2 mM DMAPP, and 0.11 μM recombinant homodimeric CdpNPT in 100 μL at 37 °C for 120 min, no enzymatic product could be detected in the incubation mixture (Figure 1A). Under the same conditions, the tryptophan-containing cyclic dipeptide cyclo-D-Trp-L-Tyr was accepted, confirming previous results (Figure 1B). As expected, the conversion rate of cyclo-D-Trp-L-Tyr increased with increasing amounts of CdpNPT (Figures 1D, 1F, and 1H). By increasing protein amount, product formation was also observed with L-tryptophan. As shown in Figure 1C, one product peak at 13.9 min was detected in the reaction mixture containing 0.28 μM CdpNPT and even two product peaks at 13.9 and 16.5 min with 0.54 or 1.62 μM CdpNPT (Figures 1E and 1G). Both peaks were absent in the incubation mixture with 1.62 μM heat-inactivated CdpNPT (Figure 1I), indicating that these peaks were very likely enzymatic products. With CdpNPT at a concentration of 1.62 μM in a 100 μL assay, a conversion rate of 18.1% could be achieved for L-tryptophan after incubation at 37 °C for 2 h (Figure 1G). This result proved clearly that L-tryptophan was also accepted by CdpNPT, indicating the broad substrate specificity of this enzyme.

Similar results were also observed with the second cyclic dipeptide prenyltransferase FtmPT1. Incubation of L-tryptophan and DMAPP with FtmPT1 under similar conditions to those described in a previous study,[4] e.g., incubation with 0.11 μM monomeric FtmPT1 for 2 h, showed no enzymatic conversion (Figure 2A), while product formation at 13.5 min was clearly observed with cyclo-L-Trp-L-Pro (brevianamide F) as substrate, the natural substrate of FtmPT1[4] (Figure 2B). The product of incubation with brevianamide F was confirmed by preparative isolation and NMR analysis as tryprostatin B, which carries a regular prenyl moiety at C-2 of the indole ring (Scheme 1 and Figure 2J).[4] Brevianamide F was almost completely converted by 0.28 μM or more FtmPT1 in a 100 μL assay (Figures 2D, 2F, and 2H). Under these conditions, a product peak at 13.9 min could also be detected with L-tryptophan as substrate (Figures 2C, 2E, and 2G). Interestingly, the enzymatic product of L-tryptophan with FtmPT1 had the same retention time as the first enzymatic product of the incubation with CdpNPT, i.e., 13.9 min (Figures 1G and 2G).

With 1.62 μM CdpNPT or 1.12 μM FtmPT1 in a 100 μL enzyme assay, no conversion of tyrosine was detected in the presence of DMAPP (data not shown).

Simple Tryptophan Derivatives Were Also Substrates of CdpNPT and FtmPT1. After confirmation of the enzymatic products of L-tryptophan with CdpNPT and FtmPT1 as prenylated tryptophan by detection of the $[M - 1]^-$ ion at *m/z* 271.2 and $[M + 1]^+$ ion at *m/z* 273.1 with LC-MS (Supporting Information, Table S1), incubations of CdpNPT and FtmPT1 with simple indole derivatives were carried out. These substances were accepted by dimethylallyltryptophan synthases FgaPT2 and/or 7-DMATS as

[§] Dedicated to Prof. Dr. Richard D. Hutchison on the occasion of his 65th birthday.

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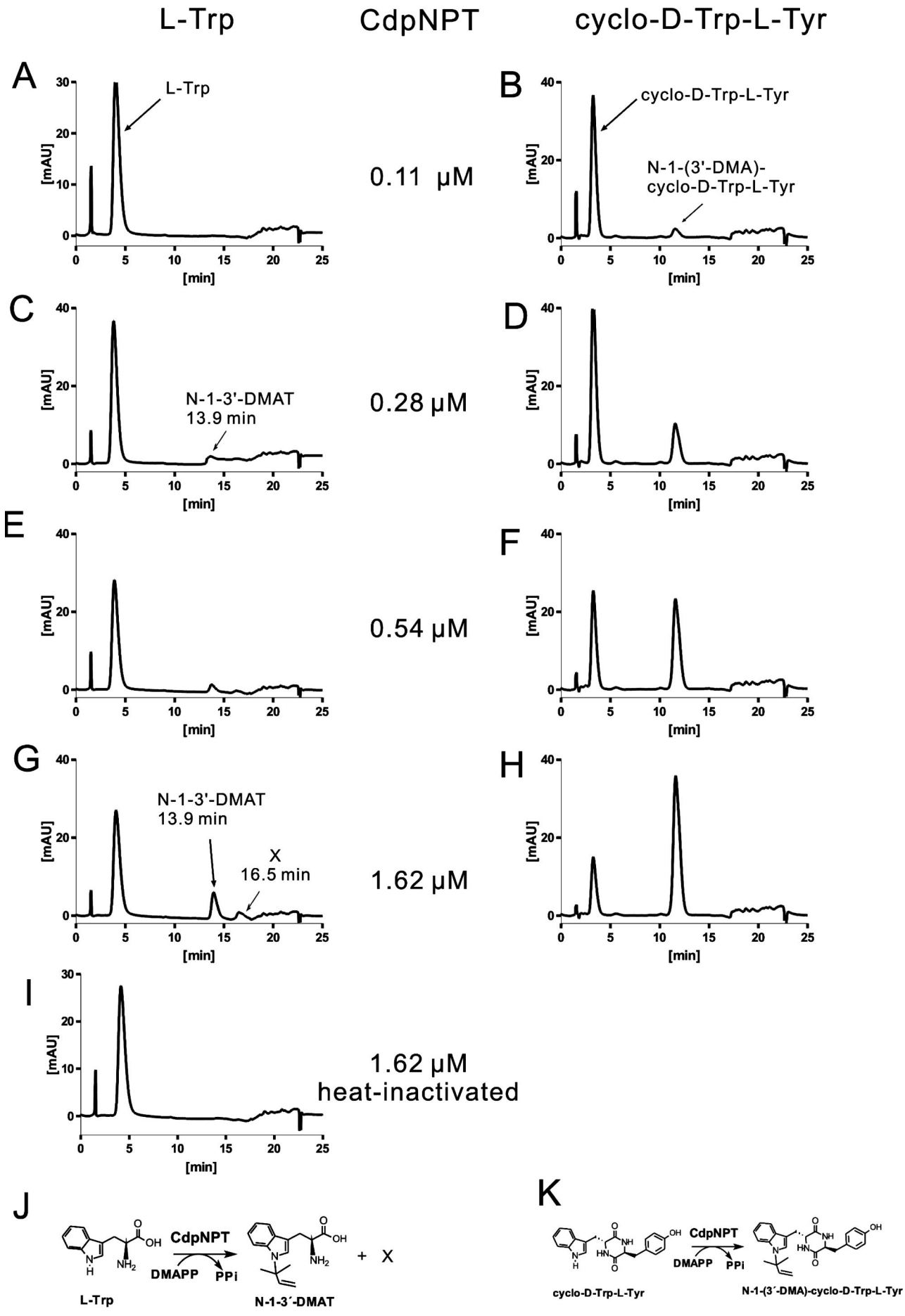


Figure 1. HPLC chromatograms of incubation mixtures of L-Trp and cyclo-D-Trp-L-Tyr with different amounts of recombinant CdpNPT.

Scheme 1. Prenyltransfer Reactions Catalyzed by Different Prenyltransferases from *Aspergillus fumigatus*: Conversion of L-Trp to 4-Dimethylallyltryptophan (4-DMAT) by FgaPT2 (A) and to 7-Dimethylallyltryptophan (7-DMAT) by 7-DMATS (B); Conversion of Brevianamide F by FtmPT1 to Tryprostatin B (C) and to N-1-3′-DMA-cyclo-L-Trp-L-Pro by CdpNPT (D)

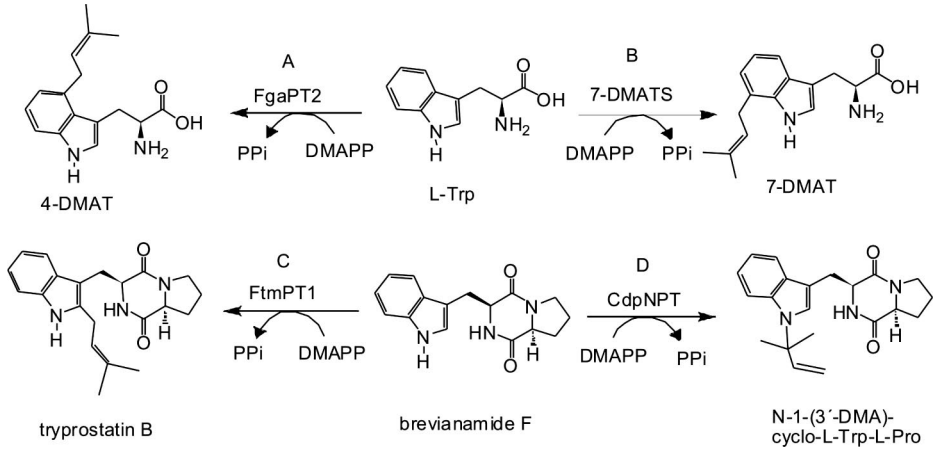
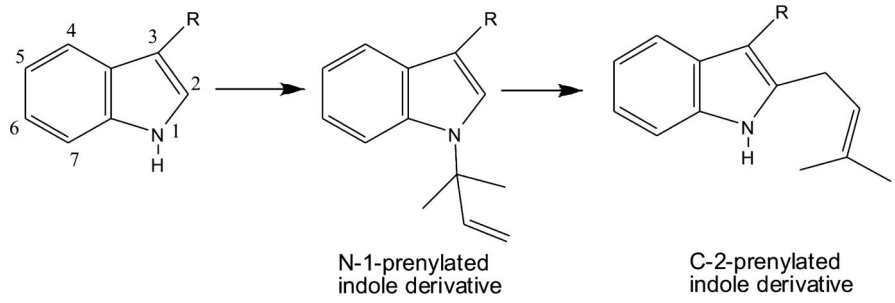


Table 1. Relative Substrate Specificity of CdpNPT and FtmPT1 toward Indole Derivatives[a]

	CdpNPT (1.62 μM)				FtmPT1 (1.68 μM)			
	2 h		16 h		2 h		16 h	
substrate	conversion rate [%]	rel yield [%]	conversion rate [%]	rel yield [%]	conversion rate [%]	rel yield [%]	conversion rate [%]	rel yield [%]
L-tryptophan	12.05	100	38.24	100	7.51	100	37.84	100
indole-3-acetic acid	≤0.21	≤1.7	17.33	45.3	≤0.21	≤2.8	≤0.21	≤0.6
L-β-homotryptophan	≤0.41	≤3.4	≤0.41	≤1.1	≤0.41	≤5.5	≤0.41	≤1.1
tryptamin	≤0.40	≤3.3	≤0.40	≤1.0	≤0.40	≤5.3	≤0.40	≤1.1
N-acetyl-DL-tryptophan	≤0.10	≤0.8	9.94	26.0	≤0.10	≤1.3	0.29	0.8
L-abrine (N_α-methyl-L-tryptophan)	15.14	125.6	43.4	113.5	≤0.28	≤3.7	11.12	29.4
α-methyl-DL-tryptophan	3.01	25.0	9.38	24.5	≤0.17	≤2.3	0.38	1.0
1-methyl-DL-tryptophan	≤0.13	≤1.1	≤0.13	≤0.3	≤0.13	≤1.7	≤0.13	≤0.3
4-methyl-DL-tryptophan	8.75	72.6	23.48	61.4	1.20	16.0	7.33	19.4
5-methyl-DL-tryptophan	≤0.18	≤1.5	7.71	20.2	≤0.18	≤2.4	3.89	10.3
6-methyl-DL-tryptophan	≤0.11	≤0.9	5.16	13.5	11.08	147.5	36.33	96.0
7-methyl-DL-tryptophan	6.1	50.6	14.48	37.9	5.74	76.4	24.26	64.1
5-hydroxy-L-tryptophan	≤0.10	≤0.8	≤0.10	≤0.3	≤0.10	≤1.3	≤0.10	≤0.3
5-bromo-DL-tryptophan	≤0.14	≤1.2	≤0.14	≤0.4	≤0.14	≤1.9	≤0.14	≤0.4
5-fluoro-L-tryptophan	≤0.09	≤0.7	0.72	1.9	≤0.09	≤1.2	1.64	4.3
6-fluoro-DL-tryptophan	2.13	17.7	5.29	13.8	4.14	55.1	21	55.5

[a] The reaction mixtures with 1.62 μM CdpNPT or 1.68 μM FtmPT1 were incubated for 2 and 16 h. The relative yield or relative activity was defined as the ratio of the conversion rate of a substrate to that of L-tryptophan.

Scheme 2. Proposed Relationship between N-1- and C-2-Prenylated Indole Derivatives



substrates.[8,9] In total, 16 substances (Table 1) were tested with both enzymes in the presence of DMAPP. Product formation could be detected for six and five of these substrates after incubation with CdpNPT and FtmPT1 for 2 h at 37 °C, respectively (Table 1). By expanding the incubation time to 16 h, 11 and 10 substances were accepted by CdpNPT and FtmPT1, respectively (Table 1). In summary, CdpNPT and FtmPT1 showed similar substrate specificities toward simple tryptophan derivatives, although the relative activities differed from each other. With the exception of 1-methyl-, 5-hydroxy-, and 5-bromo-DL-tryptophan, which were not accepted by any of the enzymes, tryptophan derivatives with modifications at the indole ring were in general better accepted than those with modifications at the side chain of tryptophan. Indole-3-acetic acid and *N*-acetyl-DL-tryptophan were not or almost not accepted by FtmPT1, but well accepted by CdpNPT, with a relative activity of 45% and 26% in comparison to that of L-tryptophan. L-Tryptophan and L-abrine were found to be the best simple tryptophan derivatives for CdpNPT as substrates, while L-tryptophan and 6-methyl-DL-tryptophan were the best simple tryptophan derivatives for the FtmPT1 reaction (Table 1). Interestingly, L-β-homotryptophan, which was well accepted by FgaPT2 and 7-DMATS,[8,9] was not a substrate for CdpNPT and FtmPT1. As examples shown in Figure 3, two enzymatic products were detected with all of the accepted substrates in the reaction mixtures with CdpNPT and one with

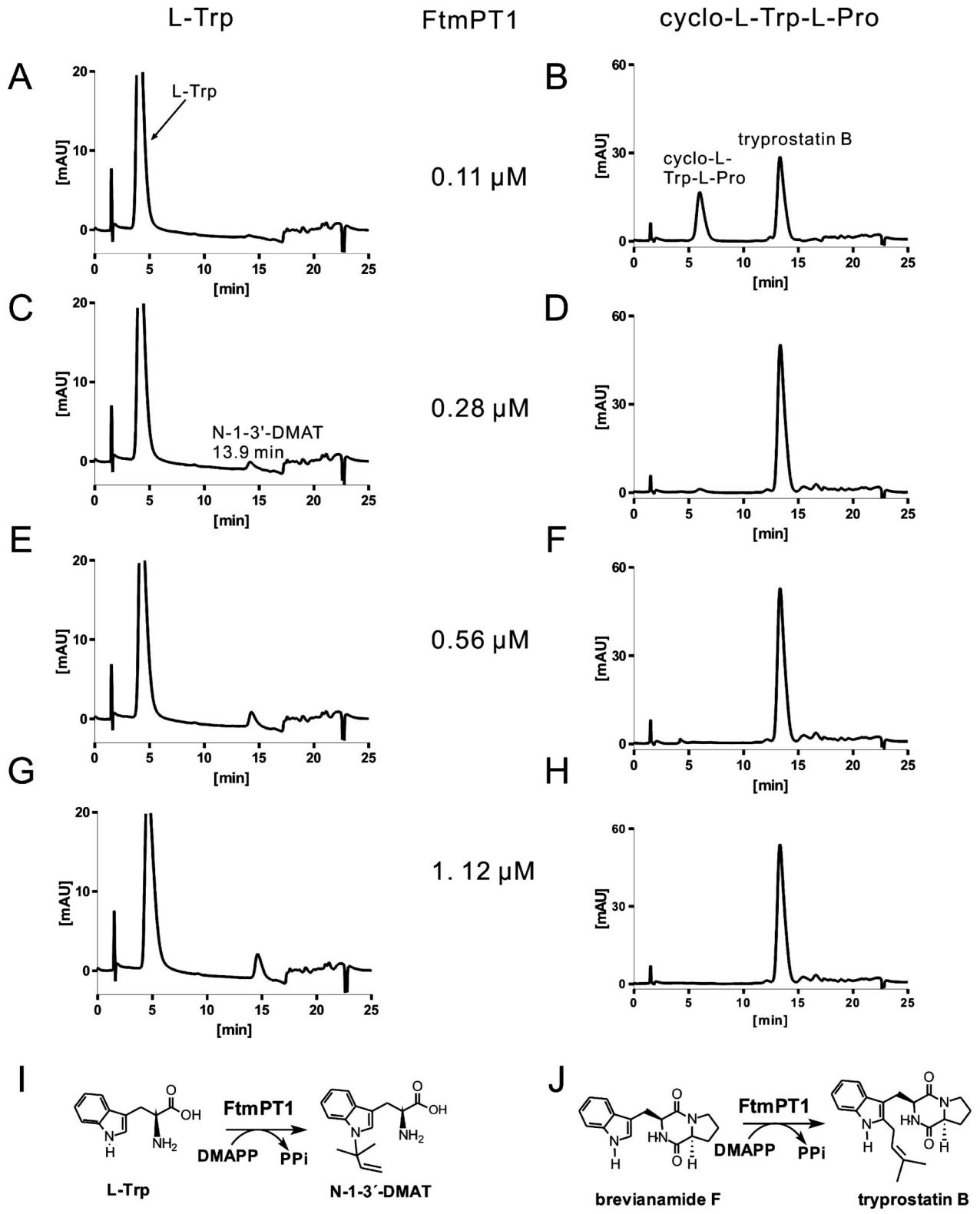


Figure 2. HPLC chromatograms of incubation mixtures of L-Trp and brevianamide F with different amounts of recombinant FtmPT1.

FtmPT1, respectively. The products in the reaction mixtures of FtmPT1 had the same retention time as those of the first product in the reaction mixtures of the respective substrate with CdpNPT (Figure 3).

***N*-1-Prenylated Derivatives Are Products of CdpNPT and FtmPT1 Reactions, When Simple Tryptophan Derivatives Were Used As Substrates.** For structural elucidation, the enzymatic products of L-tryptophan, L-abrine, and 4- and 7-methyl-DL-tryptophan were isolated from the incubation mixtures of CdpNPT on a preparative scale and subjected to NMR and MS analyses. As expected, CdpNPT catalyzes the transfer of a reverse prenyl moiety [3′-(3′,3′-dimethylallyl), 3′-DMA] onto N-1 of the tryptophan-containing cyclic dipeptides.[2] The enzymatic product peaks at 13.9, 14.9, 16.0, and 16.2 min (Figure 3) indeed carry reverse prenyl moieties at N-1 of the indole ring (Figure 4). This was unequivocally proven by detection of the prenyl signals of N-1-

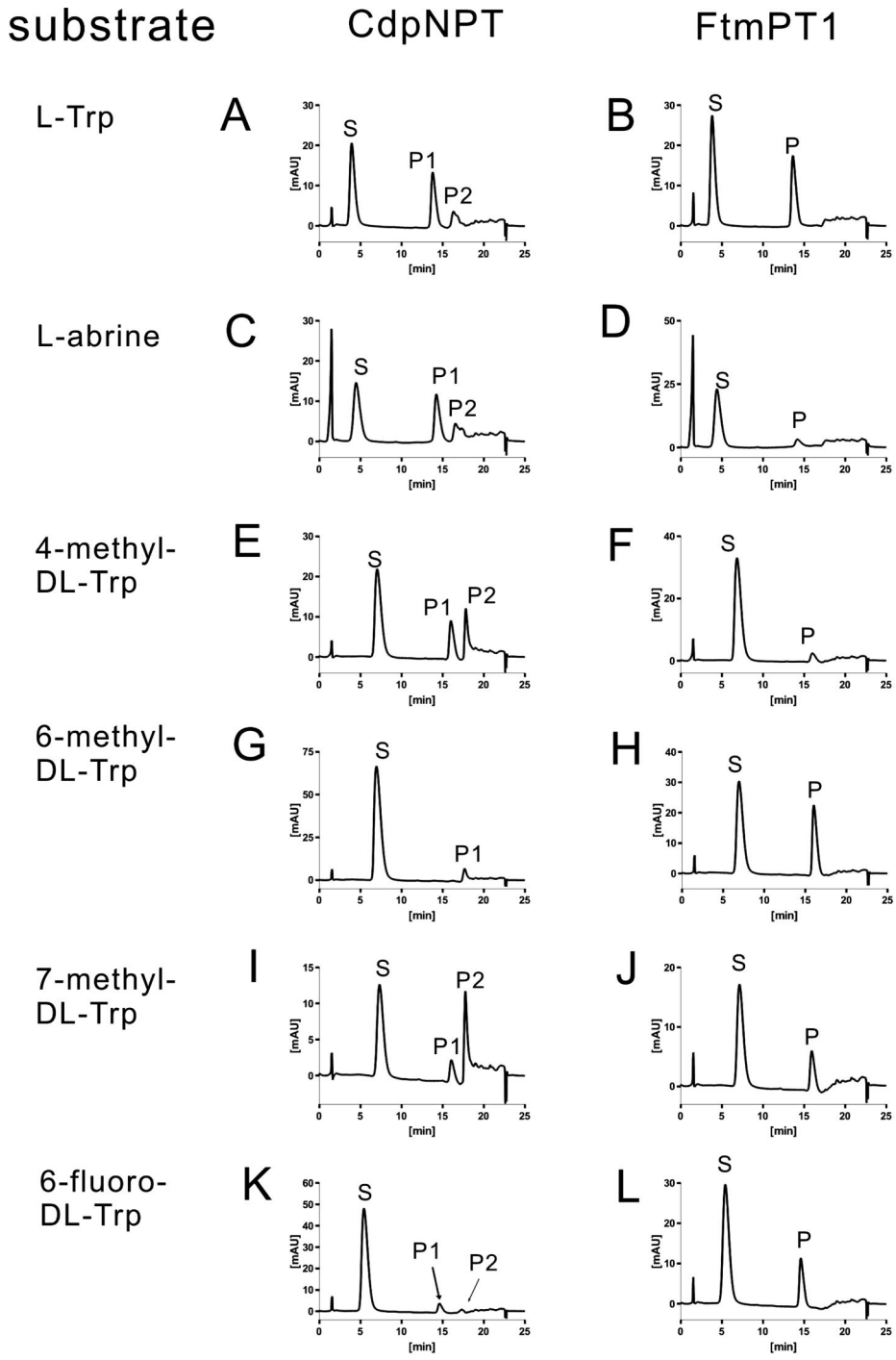
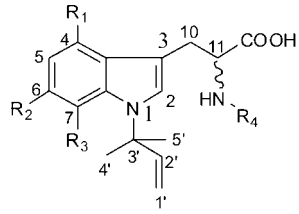


Figure 3. HPLC chromatograms of incubation mixtures of selected tryptophan derivatives with recombinant CdpNPT or FtmPT1. The reaction mixtures contained 1.62 μM CdpNPT or 1.68 μM FtmPT1 and were incubated at 37 °C for 16 h. S: substrate. P, P1, and P2: products.

3′-DMAT, 4-methyl-N-1-3′-DMAT, and 7-methyl-N-1-3′-DMAT in D_2O at δ 5.99−6.00 (H-2′), 5.10−5.17 (H-1′), 5.09−5.12 (H-1′), 1.08−1.10 (3H-4′), and 0.99−1.00 (3H-5′) ppm or of N-1-3′-DMA-L-abrine in DCl at 6.19 (H-2′), 5.34 (H-1′), 5.27 (H-1′), 1.24 (3H-4′), and 1.18 (3H-5′) ppm, respectively (Table 2). The upfield shifting of the signal for H-2 to 5.2−5.5 ppm (Table 2) confirmed the prenylation at N-1.[2,12] The structures of these products were also confirmed by MS analysis (Table S1). Due to low solubility in D_2O and low stability in DCl, no interpretable NMR spectra could be obtained for the second peak of the reaction mixtures. Therefore, the structures of these compounds could not be elucidated. However, from NMR spectra of the products isolated from the incubation mixtures of 4- and 7-methyl-DL-tryptophan, it seems that these peaks contain prenylated derivatives (data not shown).

By analogy to CdpNPT, the enzymatic products of L-tryptophan, L-abrine, and 4- and 7-methyl-DL-tryptophan with retention times at 13.9, 14.9, 16.0, and 16.2 min, respectively, were also isolated from the reaction mixtures of FtmPT1. In addition, the enzymatic product of 6-methyl-DL-tryptophan was also isolated from the incubation mixture of FtmPT1. The isolated products were subjected to NMR and MS analyses. Inspection of the ^{1}H NMR spectra surprisingly revealed that the enzymatic products isolated from incubation mixtures of FtmPT1 are identical to those identified from



N-1-3′-DMAT: R_1=H, R_2=H, R_3=H, R_4=H
4-methyl-N-1-3′-DMAT: R_1=CH_3, R_2=H, R_3=H, R_4=H
6-methyl-N-1-3′-DMAT: R_1=H, R_2=CH_3, R_3=H, R_4=H
7-methyl-N-1-3′-DMAT: R_1=H, R_2=H, R_3=CH_3, R_4=H
N-1-3′-DMA-L-abrine: R_1=H, R_2=H, R_3=H, R_4=CH_3

Figure 4. Enzymatic products of tryptophan derivatives after incubation with CdpNPT or FtmPT1.

CdpNPT assays, i.e., reversely N-1-prenylated tryptophan and derivatives (Figures 1J and 2I). This is in contrast to the regular prenylation at C-2 (Scheme 1 and Figure 2J), when tryptophan-containing cyclic dipeptides were used as substrates for FtmPT1 reactions.[4]

Two questions arise from the results obtained from incubation mixtures of tryptophan and its simple derivatives with CdpNPT and FtmPT1. Are the FtmPT1 products identified in this study probable precursors of C-2-prenylated derivatives? Furthermore, what is the relationship of the two product peaks in the incubation mixtures with CdpNPT?

4-Methyl-N-1-3′-DMAT Was Stable in the FtmPT1 Assay. It could be speculated that FtmPT1 catalyzes the prenylation of L-tryptophan and its simple derivatives as well as of tryptophan-containing cyclic dipeptides in the same manner, i.e., prenylation at N-1 followed by immigration of the prenyl moiety from N-1 to C-2 (Scheme 2), as reported for 3-alkyl-1-allylindoles.[13] In the case of cyclic dipeptides, the products of the first reaction would be immediately converted, so that only C-2-prenylated derivatives could be detected. In the case of tryptophan or its simple derivatives, the second reaction would be much slower than the first one, so that N-1-3′-DMAT or its derivatives were detected as described above. Incubation mixtures of L-tryptophan and its simple derivatives with FtmPT1 for 16 h showed only one product peak each (Figure 3). To exclude the influence of the first reaction on the second one, the behavior of N-1-3′-DMAT and simple derivatives in the absence of the substrate was investigated. For this purpose, 4-methyl-DL-tryptophan was incubated with CdpNPT in the presence of DMAPP (Figure 5A = Figure 3E). The two product peaks, i.e., 4-methyl-N-1-3′-DMAT and the unidentified products of the second peak with retention time of 16.5 min, were then isolated on HPLC and incubated again with FtmPT1 or CdpNPT in the presence or absence of DMAPP at pH 7.5 for 16 h (Figure 5). As shown in Figures 5B−D, no additional peaks were detected after the incubation with FtmPT1, demonstrating that 4-methyl-N-1-3′-DMAT was chemically stable and not converted by FtmPT1 under the tested condition. This is also true for the second product peak (Figures 5G−I). This proved that the final product of FtmPT1 reaction of 4-methyl-DL-tryptophan was indeed 4-methyl-N-1-3′-DMAT, i.e., reverse prenylation at N-1 (Figure 2I) rather than regular prenylation at C-2. Similar results are expected for tryptophan and other simple derivatives.

CdpNPT Catalyzes Prenylations at Different Positions of Tryptophan and Its Simple Derivatives. Enzymatic products of cyclic dipeptides with CdpNPT were found to be unstable under acidic conditions and underwent rearrangement to different products, e.g., to regular N-1-prenylated derivatives.[2] Even during the enzymatic incubation at pH 7.5 and 37 °C, two or more products could be detected when cyclo-L-Trp-L-Pro and cyclo-D-Trp-L-Pro were used as substrates.[2] This could also be true for the CdpNPT products of tryptophan and its derivatives. It could be speculated that rearrangement of N-1-3′-DMAT derivatives had already taken place during the enzymatic incubation to the unknown products of the second peak (Figure 3). In comparison to those of CdpNPT, the incubation mixtures of L-tryptophan, L-abrine, and 4- and 7-methyl-DL-tryptophan as well as 6-fluoro-DL-tryptophan with FtmPT1 showed only one product peak each. Therefore, the second peak in the incubation mixtures of CdpNPT was an enzymatic product rather than a result of a chemical rearrangement of N-1-3′-DMAT derivatives, which would also take place during incubation with FtmPT1, because the assay conditions, e.g., pH value, incubation temperature, and time, and substrate and buffer components were identical for both enzyme reactions. As shown in Figure 1, both

Table 2. ^{1}H NMR Data of Isolated Enzymatic Products[a]

proton	N-1-3′-DMAT in D_2O	4-methyl-N-1-3′-DMAT in D_2O	6-methyl-N-1-3′-DMAT in D_2O	7-methyl-N-1-3′-DMAT in D_2O	N-1-(3′-DMA)-L-abrine in DCl (0.1 M)
H-2	5.46, s	5.24, s	5.24, s	5.46, s	5.66, s
H-4	7.39, d, 7.6	CH_3: 2.44, s	7.24, d, 7.9	7.25, d, 7.6	7.48, d, 7.6
H-5	6.95, td, 7.6, 1.0	6.76, d, 7.3	6.75, d, 7.6	6.91, t, 7.6	7.03, t, 7.6
H-6	7.24, td, 7.7, 1.2	7.15, t, 7.7	CH_3: 2.28, s	7.25, d, 7.1	7.39, t, 7.6
H-7	6.80, d, 7.6	6.62, d, 7.6	6.62, s	CH_3: 2.17, s	6.97, d, 7.9
H-10	2.60, dd, 13.1, 6.6	2.91. dd, 13.4, 6.3	2.42, dd, 13.0, 6.0	2.58, dd, 13.4, 6.5	2.84, m
	2.46, dd, 13.1, 11.4	2.52, dd, 13.4, 8.2	2.28, m	2.46, t, 11.5	2.67, t, 10.7
H-11	3.68, dd, 11.7, 6.3	3.68, t, 8.2	3.68, m	3.63, dd, 11.5, 6.5	3.77, m
H-12					
					N-CH_3: 2.73, s
H-1′	5.17, dd, 10.7, 1.3	5.10, d, 11.2	5.13, d, 10.7	5.16, dd, 10.8, 1.3	5.34, d, 10.4
	5.12, dd, 17.5, 1.3	5.09, d, 17.2	5.08, d, 17.6	5.12, dd, 17.3, 1.3	5.27, d, 16.7
H-2′	6.00, dd, 17.5, 10.7	5.99, dd, 17.2, 11.2	6.01, dd, 17.6, 10.7	5.99, dd, 17.3, 10.8	6.19, dd, 16.7, 10.4
H-4′	CH_3: 1.10, s	CH_3: 1.08, s	CH_3: 1.07, s	CH_3: 1.10, s	CH_3: 1.24, s
H-5′	CH_3: 0.99, s	CH_3: 1.00, s	CH_3: 0.99, s	CH_3: 0.99, s	CH_3: 1.18, s

[a] The chemical shifts (δ) are given in ppm and coupling constants in Hz.

Table 3. K_m Values and Turnover Numbers of CdpNPT and FtmPT1

	CdpNPT				FtmPT1			
substrate	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [mM^{-1} s^{-1}]	ratio k_{cat}/K_m	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [mM^{-1} s^{-1}]	ratio k_{cat}/K_m
L-tryptophan	0.83	0.005	0.006	1	0.82	0.12	0.15	1
cyclo-L-Trp-L-Pro					0.055	5.57	101.3	675
cyclo-D-Trp-L-Tyr	0.128	0.46	3.59	598				

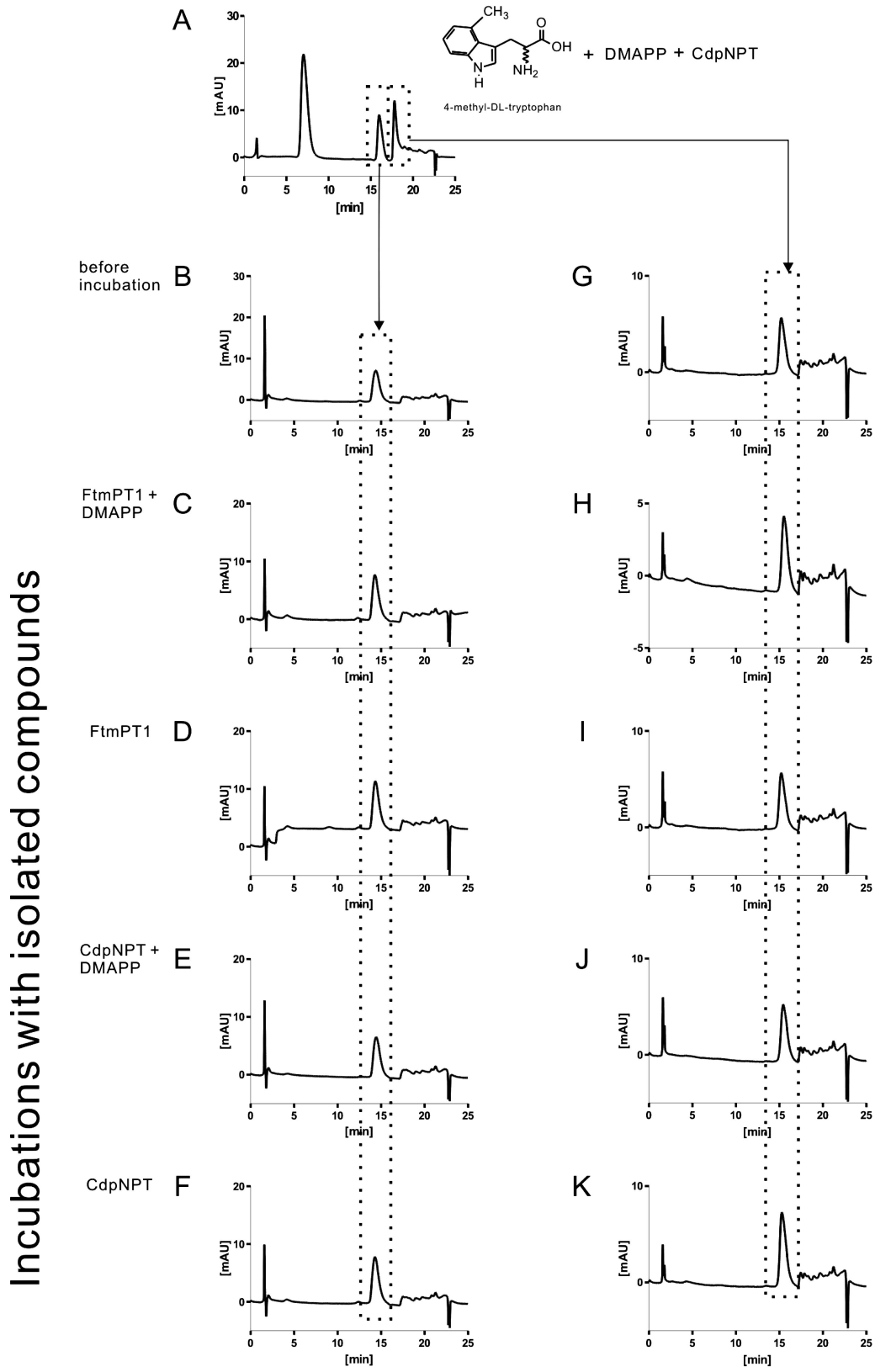


Figure 5. HPLC chromatograms of incubation mixtures of isolated products of 4-methyl-DL-tryptophan with CdpNPT or FtmPT1. The reaction mixtures contained 1.62 μM CdpNPT or 1.68 μM FtmPT1 and were incubated at 37 °C for 16 h.

product peaks in the incubation mixtures of tryptophan were detected only in the presence of active, but not of heat-inactivated, CdpNPT.

By comparison of the results obtained from CdpNPT and FtmPT1, it could be speculated that the unidentified compounds of the second peak were enzymatic products of CdpNPT with L-tryptophan or derivatives. Three possibilities could be envisaged: (a) Both products were formed directly from L-tryptophan or its derivatives; (b) N-1-3′-DMAT derivatives were formed from L-tryptophan or its derivatives and converted to the products of the second peaks; (c) N-1-3′-DMAT derivatives were formed from the products of the second peak.

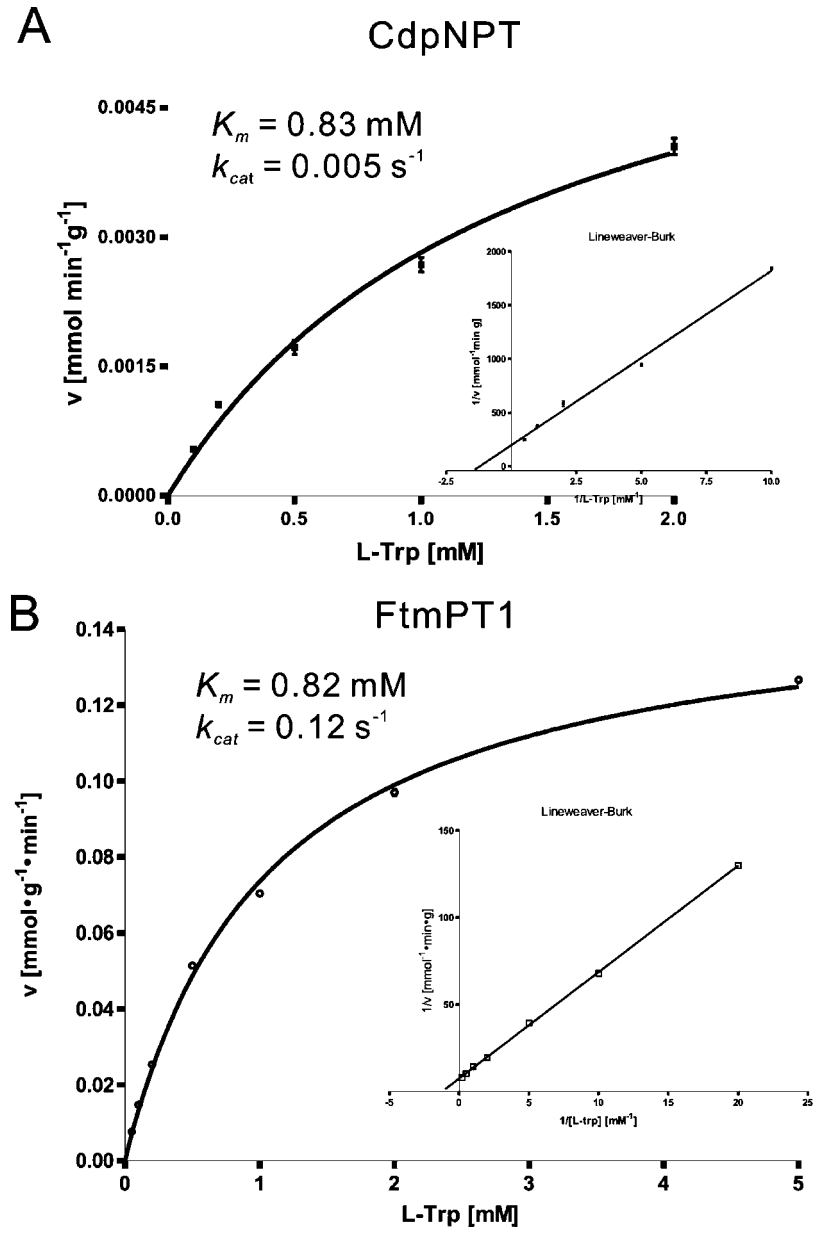


Figure 6. Determination of kinetic parameters of L-tryptophan for CdpNPT (A) and FtmPT1 (B).

To prove the possibility (b), 4-methyl-N-1-3′-DMAT was incubated with CdpNPT in the absence or presence of DMAPP for 16 h. No additional peak was detected after incubation (Figures 5B, 5E, and 5F), proving that possibility (b) could be excluded. Similar results were obtained after incubation of the isolated compounds of the second peak with CdpNPT in the absence or presence of DMAPP [possibility (c)] (Figures 5G, 5J, and 5K). This indicated that the two product peaks of CdpNPT reactions are very probably independently formed from tryptophan and its simple derivatives [possibility (a)]. However, it cannot be excluded that the conversion between the two compounds occurs only during the enzymatic reactions, but not after releasing of the products from the enzyme.

Kinetic Parameters of Selected Compounds. For comparison of the behavior of CdpNPT and FtmPT1 toward tryptophan and cyclic dipeptides, kinetic parameters of CdpNPT were determined for L-tryptophan and cyclo-D-Trp-L-Tyr. Kinetic parameters of FtmPT1 were also obtained for L-tryptophan and compared with those of cyclo-L-Trp-L-Pro (brevianamide F), the natural substrate of FtmPT1.[4] The reactions of CdpNPT and FtmPT1 apparently followed Michaelis−Menten kinetics by using these substrates in the presence of DMAPP. Michaelis constant K_m, turnover number k_{cat}, and enzymatic rate constant k_{cat} and K_m^{-1} were determined by Lineweaver−Burk and Hanes−Woolf analyses[14] and are given in Table 3. K_m values of CdpNPT and FtmPT1 were determined for L-tryptophan at 0.83 and 0.82, respectively, and are much higher than those for cyclic dipeptides (Table 3). The ratio of enzymatic rate constants k_{cat} and K_m^{-1} of CdpNPT for L-tryptophan and cyclo-D-Trp-L-Tyr was found to be 1:598. In the case of FtmPT1, it was found to be 1:675 for L-tryptophan and cyclo-L-Trp-L-Pro (Table 3) (Figure 6). These data proved the high efficiency of the prenyltransferases CdpNPT and FtmPT1 toward cyclic dipeptides. Regarding the low enzymatic rate constants of CdpNPT and FtmPT1 toward tryptophan, no plausible biological relevance could be predicted for their substrate promiscuity *in vivo*.

In this study, evidence that the two cyclic dipeptide prenyltransferases CdpNPT and FtmPT1 from *Aspergillus fumigatus* accepted also tryptophan and its simple derivatives was provided and, therefore, demonstrated that the broad substrate specificity observed for 7-DMATS[9] is also applicable for other indole prenyltransferases, at least for the enzymes described in this study. This feature makes this new enzyme group especially useful as tools for production of

prenylated compounds by chemoenzymatic synthesis.[8,9] Interestingly, the FtmPT1 products of tryptophan and simple derivatives differ clearly from those of tryptophan-containing cyclic dipeptides by different prenylation positions and prenylation patterns, i.e., reverse N-1-prenylation for the former and regular C-2-prenylation for the last case (Figures 2I and 2J). These results demonstrated the substrate promiscuity of this enzyme group, in addition to their catalytic promiscuity.[11]

Experimental Section

Chemicals. Trisammonium salt of DMAPP was prepared by analogy with the synthesis of trisammonium geranyl diphosphate reported by Woodside.[15]

Overproduction and Purification of His_6-CdpNPT and His_6-FtmPT1. Overproduction and purification of His_6-CdpNPT and His_6-FtmPT1 were described previously.[4,10]

Enzyme Assays. The enzyme assays contained 50 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$, differing from each other by incubation volumes, amounts of FtmPT1 or CdpNPT, and incubation times. The reaction mixtures of the standard assay for determination of the substrate specificity (100 μL) contained 1 mM tryptophan or derivatives, 2 mM DMAPP, and 1.68 μM purified His_6-FtmPT1 or 1.62 μM His_6-CdpNPT. After incubation for 2 h at 37 °C, the reaction was stopped by addition of 100 μL of MeOH. After removal of the protein by centrifugation (15000*g*, 10 min, 4 °C), the enzymatic products were analyzed on an HPLC system described below. For quantification, two independent assays were carried out routinely. For determination of the kinetic parameters, DMAPP at a final concentration of 2 mM and L-tryptophan of up to 5 mM were used. The assays for isolation of the enzymatic products for structural elucidation (5 mL) used 1 mM tryptophan or derivatives, 2 mM DMAPP, and 1.68 μM purified His_6-FtmPT1 or 1.62 μM His_6-CdpNPT and were incubated for 16 h. The reaction mixtures were concentrated on a rotation evaporator at 30 °C to a volume of 500 μL before injection.

HPLC Analysis and Determination of the Conversion Rate. Reaction mixtures of CdpNPT and FtmPT1 were analyzed on an Agilent HPLC Series 1100 by using a LiChrospher RP 18-5 column (125 × 4 mm, 5 μm, Agilent) at a flow rate of 1 mL min^{-1}. The substances were detected with a photodiode array detector. H_2O (solvent A) and MeOH (solvent B) were used as solvents. For separation of L-tyrptophan and its simple derivatives, the samples were eluated with 30% B for 2 min, followed by a gradient from 30% to 70% B in 13 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 30% solvent B for 5 min. For cyclic dipeptides, a gradient 50% to 80% was used instead of 30% to 70%. To determine the stability of the enzymatic products (Figures 5Band 5I), a gradient of 40% to 75% was used instead.

Spectroscopic Analysis. The isolated products (50−200 μg) were analyzed by ^{1}H NMR spectroscopy, ^{1}H−^{1}H-COSY, and positive and negative electrospray ionization (ESI) mass spectrometry with a ThermoFinnigan TSQ Quantum. The mass spectrometer was coupled with an Agilent HPLC series 1100 equipped with a RP18-column (10 × 250 mm, 5 μm). For separation, the column was run with 50% solvent B (MeOH) in solvent A (H_2O), each containing 0.1% HCO_2H for 5 min, followed by a gradient from 10% to 100% B over 30 min. After washing with 100% B, the column was equilibrated with 10% B for 10 min. The flow rate was 0.2 mL min^{-1}.

NMR spectra were recorded on an Avance DRX 500 spectrometer (Bruker). The solvent signal of D_2O at 4.81 ppm was used as reference. NMR and MS data are given in Tables 2 and S1, respectively.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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