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Reinvestigation of a Cyclic Dipeptide *N*-Prenyltransferase Reveals Rearrangement of N-1 Prenylated Indole Derivatives

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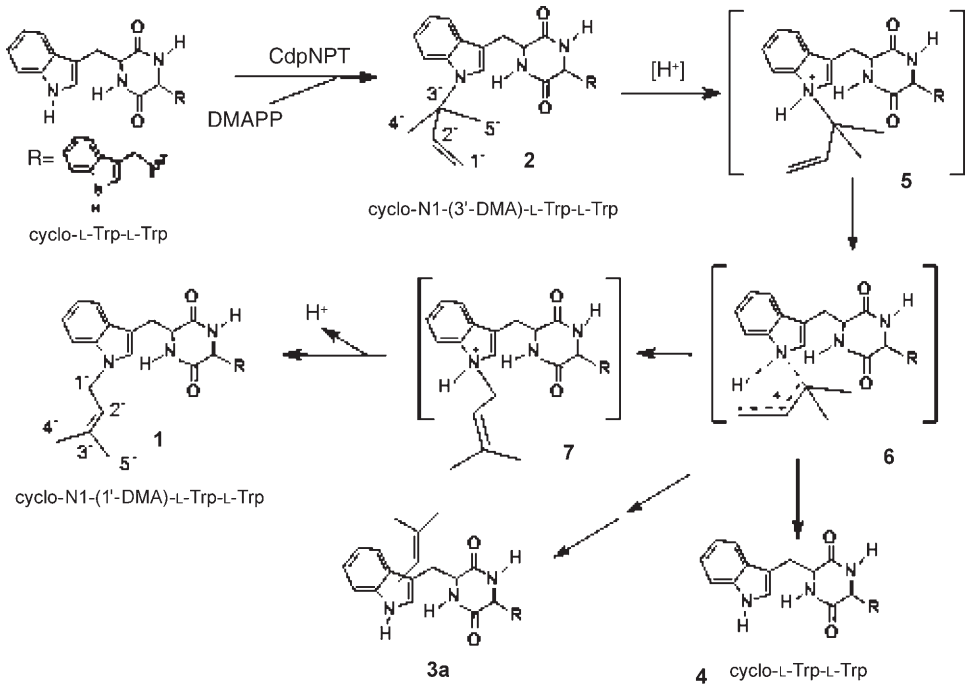
In a previous study, a **c**yclic **dip**eptide ***N*-p**renyl**t**ransferase CdpNPT from *Aspergillus fumigatus* was found to catalyse the prenylation of tryptophan-containing cyclic dipeptides at position N-1 of indole moieties. The major products were identified as derivatives that carried 1′-(3′,3′-dimethylallyl)—or 1′-DMA—moieties, for example, cyclo-N1-(1′-DMA)-L-Trp-L-Trp (**1**; Scheme 1).[1] The enzymatic reactions were terminated by addition of trichloroacetic acid (TCA) and the reaction mixtures were separated by HPLC under acidic conditions.[1] However, reinvestigation of the reaction mixture of cyclo-L-Trp-L-Trp and dimethylallyl diphosphate (DMAPP) in the presence of CdpNPT showed that the peak area of **1** decreased significantly as the incubation time of the reaction mixture after addition of TCA was shortened (Figures 1 A–C). Meanwhile, the area of the peak of **2**, which eluted before **1**, increased drastically. When the enzymatic reaction was terminated with the same volume of MeOH as that of the reaction mixture, **1** was detected only as a minor peak in the HPLC chromatogram (Figure 1 D). Instead, the peak for **2** was found to be dominant. Therefore, **1** seems to be an artefact of an enzymatic product, for example, of **2**.

To prove this hypothesis, enzymatic reactions of eight cyclic dipeptides, cyclo-L-Trp-L-Trp, cyclo-L-Trp-L-Tyr, cyclo-D-Trp-L-Tyr, cyclo-L-Trp-L-Phe, cyclo-L-Trp-L-Pro, cyclo-D-Trp-L-Pro, cyclo-L-Trp-L-Leu and cyclo-L-Trp-Gly, were terminated by addition of TCA (final concentration 136 mM) as used in a previous study.[1] The obtained mixtures had a pH value of 1 and were incubated at room temperature for 2 h. These mixtures were then neutralised to pH 7.0 by addition of NaOH and analysed by HPLC under the new conditions, which lacked acids in the elution solvents. Reaction mixtures terminated with MeOH were used as controls. With the exception of cyclo-D-Trp-L-Pro (Figure 2 K), only one dominant product peak was detected for each of the tested substrates after termination with MeOH. In contrast, two or more peaks were found for almost all of the substrates after termination with TCA (Figure 2). These results show that rearrangement of the enzymatic products of CdpNPT had in fact taken place in the presence of TCA.



Scheme 1. Enzymatic reaction catalysed by CdpNPT and a hypothetical rearrangement mechanism of the product in the presence of acids; cyclo-L-Trp-L-Trp is used as an example.

For structural elucidation of the enzymatic products, each of the eight cyclic dipeptides (5 μmol of each) was incubated with DMAPP (5 μmol) and recombinant His_6–CdpNPT (0.4 mg) for 16 h, and the mixtures were extracted with ethyl acetate immediately after the incubation period. The conversion rates were found to be from 30 to 70% for these substrates. The enzymatic products were subsequently isolated by HPLC without acids in the elution solvents and subjected to NMR spectroscopy and MS analysis. The ^{1}H NMR spectroscopy and MS data are

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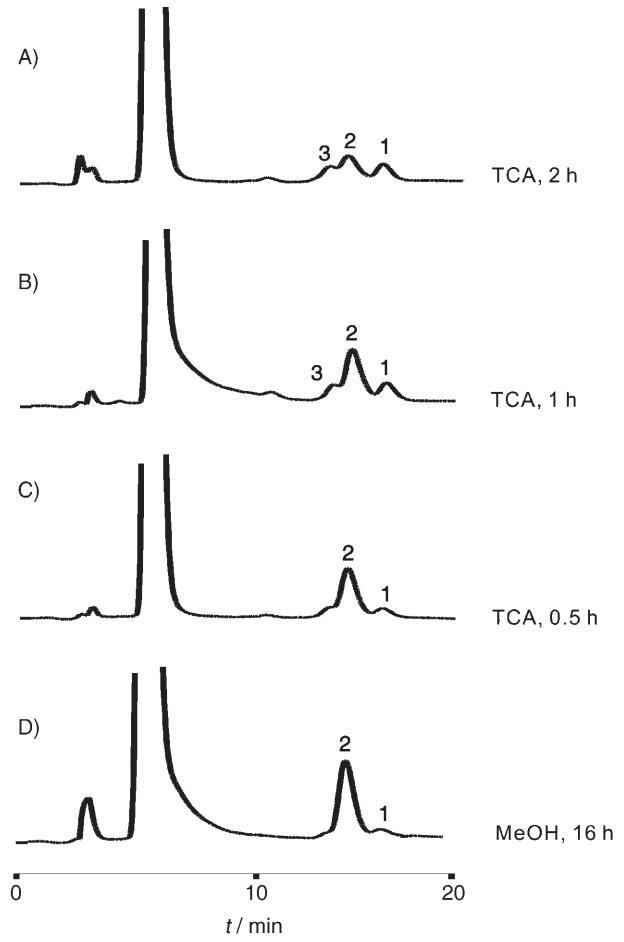

Figure 1. HPLC analysis of the reaction mixtures of cyclo-L-Trp-L-Trp after termination with MeOH and TCA (136 mM) and incubation at room temperature. The mixtures containing TCA were neutralised with NaOH (1.5 M) to pH 7.0 and analysed by HPLC.

given in Tables S1 and S2 in the Supporting Information, respectively. Comparison of the ^{1}H NMR data of the isolated products of cyclo-L-Trp-L-Trp, cyclo-L-Trp-L-Tyr, cyclo-D-Trp-L-Tyr, cyclo-L-Trp-L-Phe, cyclo-L-Trp-L-Leu and cyclo-L-Trp-Gly with those of the corresponding substrates revealed the presence of signals for a 3′,3′-dimethylallyl (DMA) moiety in the spectra of the isolated compounds at 5.7–6.1 (dd, 17 Hz; 11 Hz, H-2′), 5.1 (d, 11 Hz, H-1′) and 5.1 (d, 17 Hz, H-1′) as well as 1.1 and 0.9–1.0 (s, 3H-5′, 3H-4′) ppm, respectively; this indicates an attachment via its C-3′ (reverse prenylation). Signals for the four protons at positions 4, 5, 6 and 7 of the indole ring were clearly observed (Table S1), and revealed that the prenyl moiety is connected to N-1 or C-2 of the indole ring. The signals of the methyl groups in the reverse prenyl moieties attached to C-2 of the indole rings of some diketopiperazine derivatives, for example, in echnulin- and isoechinulin-type alkaloids,[2–4] in deoxybrevianamide E[5] or okaramines,[6,7] were usually found in the range of 1.40 to 1.55 ppm. In comparison, the signals of the methyl groups in the prenyl moieties of the enzymatic products of CdpNPT are significantly up-field shifted to 0.9 to 1.1 ppm. This means that the prenyl moieties of CdpNPT products are probably not attached to C-2, but to N-1 of the indole rings. Indeed, only one singlet for NH of an indole ring was detected at 8.14 ppm for the product of cyclo-L-Trp-L-Trp, but there was none for products of other cyclic dipeptides; this confirms the attachment of the prenyl moieties to position N-1. The signals of H-2 of the isolated products were strongly up-field shifted from about 7.2 to 5.1–5.7 ppm (Table S1), in comparison to those of the respective substrates. This effect is caused by the fielding of the double bond of the introduced prenyl moiety at position N-1 of the indole ring, which was also observed for other indole derivatives with an N1-(3′-DMA) moiety.[8] Therefore, the NMR data proved unequivocally that the enzymatic products of cyclo-L-Trp-L-Trp, cyclo-L-Trp-L-Tyr, cyclo-D-Trp-L-Tyr, cyclo-L-Trp-L-Phe, cyclo-L-Trp-L-Leu and cyclo-L-Trp-Gly are prenylated at position N-1 of the indole rings, and the prenyl residues are connected via its C-3′ as reverse moieties (Table S1). From the above results, it could be speculated that the products that carried N1-(1′-DMA) moieties identified in a previous study[1] were artefacts that were formed by rearrangement after termination of the enzymatic reaction with TCA and during HPLC analysis in the presence of triflouroacetic acid (see below).

Analogous to the products of the six cyclic dipeptides mentioned above, the two peaks of the enzymatic reaction of cyclo-D-Trp-L-Pro were also isolated (Figure 2K). The first peak with a smaller retention time was identified unequivocally as cyclo-N1-(3′-DMA)-D-Trp-L-Pro by ^{1}H NMR spectroscopy and MS analysis (Tables S1 and S2). The second peak was a mixture of two compounds with a ratio of 2:1 and contained the previously identified cyclo-N1-(1′-DMA)-D-Trp-L-Pro[1] as the major component. It seems that cyclo-N1-(3′-DMA)-D-Trp-L-Pro is more unstable than the enzymatic products of the other cyclic dipeptides mentioned above, and significant rearrangement had already taken place during the enzymatic incubation at pH 7.5 and 37 °C. The HPLC chromatogram of the incubation mixture of cyclo-L-Trp-L-Pro showed only one additional peak (Figure 2M) in comparison to that of the substrate. Surprisingly, after ^{1}H NMR spectroscopy it turned out that this peak was a mixture of three compounds in a ratio of 100:60:6. With the help of H,H COSY, the major component was identified to be cyclo-N1-(3′-DMA)-L-Trp-L-Pro and the minor one to be cyclo-N1-(1′-DMA)-L-Trp-L-Pro, which was identified previously.[1] These results provided additional support that the enzymatic products of cyclic dipeptides that consist of tryptophan and proline are more unstable than those of other cyclic dipeptides tested in this study.

To confirm that the previously identified derivatives with N1-(1′-DMA) moieties were rearrangement artefacts of the enzymatic products with N1-(3′-DMA) moieties, we decided to isolate the rearrangement products. For this purpose, the enzymatic product cyclo-N1-(3′-DMA)-L-Trp-L-Trp (**2**) was incubated with TCA (136 mM) for 16 h. The incubation mixture was neutralised with NaOH and analysed by HPLC. As observed with the incubation mixture mentioned above (Figure 1), enzymatic product **2** was converted to **1** and **3**. Furthermore, an additional peak (**4**) was also detected (Figure S1B in the Supporting Information). The rearrangement products (**1**, **3** and **4**) were subsequently isolated by HPLC and subjected to NMR spectrosco-

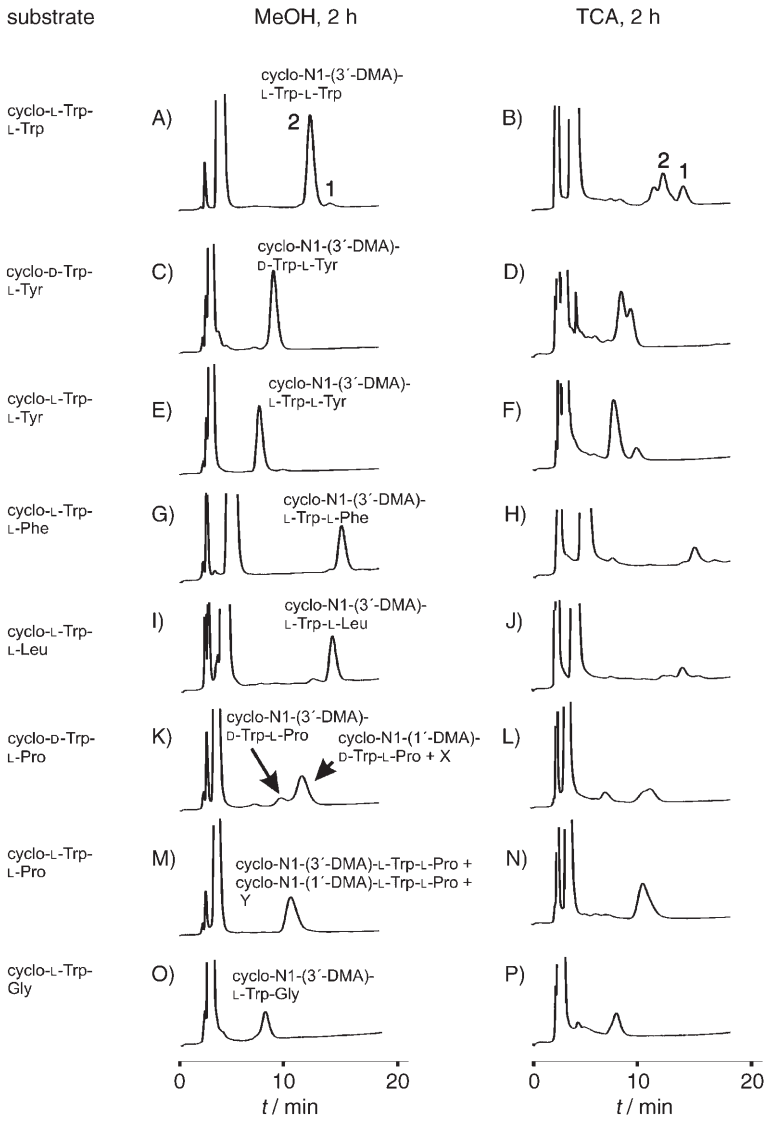

Figure 2. Stabilities of the enzymatic products of CdpNPT in the presence of MeOH and TCA. For determination of stability in MeOH, the reaction mixtures were terminated with MeOH in a ratio of 1:1 (*v/v*), incubated at room temperature for 2 h and analysed directly by HPLC. For determination of the stability in TCA, the reaction mixtures were terminated with TCA (final concentration 136 mM) and incubated at room temperature for 2 h. The mixtures were neutralised with NaOH (1.5 M) to pH 7.0 and analysed subsequently by HPLC. X: unknown compound; Y: unknown compound with a similar ^{1}H NMR spectrum to that of cyclo-N1-(3′-DMA)-L-Trp-L-Pro.

py and MS analysis. As expected, the peak for **1** was unequivocally identified to be cyclo-N1-(1′-DMA)-L-Trp-L-Trp;[1] this confirmed that it was indeed an artefact of the enzymatic product. The peak for **3** was a mixture that consisted of at least two compounds (**3a** and **3b**); **3a** bears a 1′-(3′,3′-dimethylallyl) moiety attached to a C atom; this was deduced by the presence of the signals at 5.37 (1H, t, 6.6 Hz), 3.43 (2H, 6.6 Hz), 1.74 (3H, s) and 1.72 (3H, s), respectively. This indicated that a rearrangement of the prenyl moiety from N-1 to a C atom also took place. From the ^{1}H NMR spectrum, it could be excluded that cyclo-2-(1′-DMA)-L-Trp-L-Trp[9] was in the mixture. The peak for **4** was unequivocally identified to be cyclo-L-Trp-L-Trp by comparison of its ^{1}H NMR and MS spectra with those of an authentic compound. Based on the fact that the isolated enzymatic product **2** was free from its substrate before incubation with TCA (Figure S1 A), it can be concluded that this part of cyclo-L-Trp-L-Trp was formed by hydrolysis of **2** or its rearrangement product. The rearrangement observed for cyclo-N1-(3′-DMA)-L-Trp-L-Trp could also be true for enzymatic products of other tryptophan-containing cyclic dipeptides of CdpNPT. However, it can be expected that the degrees of the rearrangement differ from each other.

To test the stability of the rearrangement products as well as their relationship to the enzymatic product and to each other, the rearrangement peaks for **1** and **3** were incubated with TCA (136 mM) at room temperature for 16 h. Incubations in MeOH instead of TCA were used as controls. Both peaks were relatively stable in MeOH for at least 16 h at room temperature (Figure S1). With the exception of a TCA peak at the beginning, there was almost no difference between the chromatograms of **1** after incubations with TCA (Figure S1 D) and that with MeOH (Figure S1 C); this indicates that **1** was relatively stable in the presence of 136 mM TCA. Similar results were also observed for **3** (Figures S1 E and F). By comparing the HPLC chromatograms (compare Figure S1 B with Figures S1 D and F), it could be deduced that **4** was very probably derived directly from the enzymatic product **2** or other rearrangement products rather than from **1** or **3**. The rearrangement results of **2** are summarised in Scheme 1. Different ions, such as **5**, **6** and **7**, could be involved in the rearrangement.

In conclusion, this study demonstrated that the enzymatic products of CdpNPT are derivatives of tryptophan-containing cyclic dipeptides with 3′-DMA moieties, known as "reverse" prenyl residues, attached to N-1 of the indole rings (Table S1); therefore, CdpNPT functions as a reverse *N*-prenyltransferase (Scheme 1). Most of the cyclic dipeptides with an N1-(3′-DMA) moiety tested in this study were relatively stable at pH 7.5 and 37 °C, but underwent rearrangement in the presence of TCA even at room temperature; this resulted in the formation of derivatives that carried N1-(1′-DMA) moieties identified in a previous study.[1] Cyclo-N1-(3′-DMA)-L-Trp-L-Pro and cyclo-N1-(3′-DMA)-D-Trp-L-Pro were more unstable and underwent rearrangements already during the enzymatic incubation at pH 7.5 and 37 °C.

Strong acids such as TCA are often used for protein precipitation and termination of enzymatic reactions.[10] Our results show that such additives could catalyse further reactions of the enzymatic products, and can result in misinterpretation of the enzymatic reaction. Therefore, it is worth to consider other reagents, such as MeOH, for this purpose. Addition of MeOH to the reaction mixture in a volume ratio of 1:1 was efficient, and undesired effects were not observed for immediate HPLC analysis, as shown in this study.

Experimental Section

Overproduction and purification of His_6–CdpNPT as well as conditions of the enzymatic reactions were described in a previous study.[1] HPLC analysis was carried out with the same equipment as described previously.[1] For separation, a linear gradient of MeOH (10–50%, *v*/*v*) in H_2O over 15 min was used. The column was then washed with MeOH (100%) for 5 min and equilibrated with MeOH (10%, *v*/*v*) for 5 min. Detection was carried out by using a photodiode array detector; the results at 254 nm are illustrated in the figures in this paper.

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[1] W.-B. Yin, H.-L. Ruan, L. Westrich, A. Grundmann, S.-M. Li, *ChemBioChem* **2007**, *8*, 1154–1161.
[2] T. Itabashi, N. Matsuishi, T. Hosoe, N. Toyazaki, S. Udagawa, T. Imai, M. Adachi, K. Kawai, *Chem. Pharm. Bull.* **2006**, *54*, 1639–1641.
[3] W. L. Wang, Z. Y. Lu, H. W. Tao, T. J. Zhu, Y. C. Fang, Q. Q. Gu, W. M. Zhu, *J. Nat. Prod.* **2007**, *70*, 1558–1564.
[4] Y. Li, X. Li, S. K. Kim, J. S. Kang, H. D. Choi, J. R. Rho, B. W. Son, *Chem. Pharm. Bull.* **2004**, *52*, 375–376.
[5] J. M. Schkeryantz, J. C. G. Woo, P. Siliphaivanh, K. M. Depew, S. J. Danishefsky, *J. Am. Chem. Soc.* **1999**, *121*, 11964–11975.
[6] Y. Shiono, K. Akiyama, H. Hayashi, *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1910–1920.
[7] P. R. Hewitt, E. Cleator, S. V. Ley, *Org. Biomol. Chem.* **2004**, *2*, 2415–2417.
[8] P. S. Baran, C. A. Guerrero, E. J. Corey, *J. Am. Chem. Soc.* **2003**, *125*, 5628–5629.
[9] A. Grundmann, S.-M. Li, *Microbiology* **2005**, *151*, 2199–2207.
[10] E. F. Rossomando in *Methods of Biochemical Analysis Vol. 38: HPLC in Enzymatic Analysis* (Ed.: E. F. Rossomando), Wiley, New York, **1998**, p. 12.

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