

C7-prenylation of tryptophanyl and O-prenylation of tyrosyl residues in dipeptides by an *Aspergillus terreus* prenyltransferase

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Abstract During our search for novel prenyltransferases, a putative gene ATEG_04218 from *Aspergillus terreus* raised our attention and was therefore amplified from strain DSM 1958 and expressed in *Escherichia coli*. Biochemical investigations with the purified recombinant protein and different aromatic substrates in the presence of dimethylallyl diphosphate revealed the acceptance of all the tested tryptophan-containing cyclic dipeptides. Structure elucidation of the main enzyme products by NMR and MS analyses confirmed the attachment of the prenyl moiety to C-7 of the indole ring, proving the identification of a cyclic dipeptide C7-prenyltransferase (CdpC7PT). For some substrates, reversely C3- or N1-prenylated derivatives were identified as minor products. In comparison to the known tryptophan-containing cyclic dipeptide C7-prenyltransferase CTrpPT from *Aspergillus oryzae*, CdpC7PT showed a much higher substrate flexibility. It also accepted *cyclo*-L-Tyr-L-Tyr as substrate and catalyzed an O-prenylation at the tyrosyl residue, providing the first example from the dimethylallyltryptophan synthase (DMATS) superfamily with an O-prenyltransferase activity

towards dipeptides. Furthermore, products with both C7-prenyl at tryptophanyl and O-prenyl at tyrosyl residue were detected in the reaction mixture of *cyclo*-L-Trp-L-Tyr. Determination of the kinetic parameters proved that (S)-benzodiazepinedione consisting of a tryptophanyl and an anthranilyl moiety was accepted as the best substrate with a K_M value of 204.1 μM and a turnover number of 0.125 s^{-1} . *Cyclo*-L-Tyr-L-Tyr was accepted with a K_M value of 1,411.3 μM and a turnover number of 0.012 s^{-1} .

Keywords *Aspergillus terreus* · Cyclic dipeptide · DMATS superfamily · Prenyltransferase · Tryptophan C7-prenylation · Tyrosine O-prenylation

Introduction

Prenyltransferases are an ubiquitous and intriguing class of enzymes involved in the biosynthesis of both primary and secondary metabolites (Heide 2009; Li 2010; Lu et al. 2009; Yazaki et al. 2009; Yu and Li 2012). One of the most investigated subgroup is the DMATS superfamily, which shares more or less sequence similarity with dimethylallyltryptophan synthase in the biosynthesis of ergot alkaloids (Tsai et al. 1995; Yu and Li 2012). Bioinformatic analysis in 2011 revealed that about 200 putative genes, mainly from genome sequencing projects, belong to this superfamily (Bonitz et al. 2011). The number of these genes has increased in the last years with the releasing of new genome sequences. So far, more than 30 such putative genes have been experimentally proven to be prenyltransferases and characterized biochemically (Pockrandt et al. 2014; Tarcz et al. 2014; Winkelblech and Li 2014; Yu and Li 2012). The majority of the DMATS superfamily uses indole derivatives including tryptophan and tryptophan-containing cyclic dipeptides as substrates and is involved in the biosynthesis of prenylated indole alkaloids.

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They catalyze transfer reactions of a prenyl moiety from prenyl diphosphate, usually dimethylallyl diphosphate (DMAPP) to different positions of the indole ring (Yu and Li 2012). In comparison to their nonprenylated precursors, prenylated indole alkaloids show distinct and in general more potent biological and pharmacological activities (Jain et al. 2008; Li 2010; Williams et al. 2000; Wollinsky et al. 2012a). The resulting products are therefore important sources of drugs or drug candidates (Li 2010; Williams et al. 2000). These characteristics open numerous multidisciplinary research opportunities for scientists from different disciplines. As a consequence, enzymes that catalyze transfer reactions of a prenyl moiety onto each position of the indole ring have been identified in the last decade and intensively studied on the molecular biological, biochemical and structural biological level. Tryptophan prenyltransferases such as FgaPT2, 5-DMATS, 6-DMATS, and 7-DMATS use logically L-tryptophan as natural substrate and catalyze prenylation reactions at C-4, C-5, C-6, and C-7 of the indole ring (Kremer et al. 2007; Unsöld and Li 2005; Winkelblech and Li 2014; Yu et al. 2012), respectively (Fig. 1). Cyclic dipeptide prenyltransferases accept in turn tryptophan-containing cyclic dipeptides as natural (BrePT, FtmPT1, and AnaPT) (Grundmann and Li 2005; Yin et al. 2009, 2013a) or best substrates (CdpNPT, CdpC2PT, and CdpC3PT) (Mundt and Li 2013; Schuller et al. 2012; Yin et al. 2010b). These enzymes mainly catalyze prenylations at C-2 or C-3 of the indole nucleus (Yu and Li 2012). The cyclic dipeptide prenyltransferase CTrpPT with its preferable substrate *cyclo*-L-Trp-L-Trp catalyzed simultaneously both *NI*- and *C7*-prenylation (Fig. 1) (Zou et al. 2010). CTrpPT is the sole cyclic dipeptide prenyltransferase, which catalyzes a prenylation at the benzene ring in the presence of its natural prenyl donor DMAPP.

In addition to indole prenyltransferases, some members of the DMATS superfamily use nonindole

compounds as substrates. For example, SirD from *Leptosphaeria maculans* catalyzes an *O*-prenylation of L-tyrosine (Kremer and Li 2010; Rudolf and Poulter 2013; Zou et al. 2011). However, an enzymatic *O*-prenylation of tyrosyl residue in a dipeptide has not been reported prior to this study.

Analysis of the genome sequence of *Aspergillus terreus* NIH2624 revealed the presence of ten putative prenyltransferase genes from the DMATS superfamily. Functions of five such genes have already been characterized molecular biologically or biochemically. We report here the cloning and expression of a putative prenyltransferase gene ATEG_04218 as well as the identification of the overproduced enzyme as a tryptophan-containing cyclic dipeptide *C7*-prenyltransferase (CdpC7PT) with an *O*-prenyltransferase activity for tyrosine-containing cyclic dipeptides.

Materials and methods

Chemicals

DMAPP was prepared according to the method described for geranyl diphosphate (Woodside et al. 1988). *Cyclo*-L-Tyr-L-Tyr was synthesized according to a protocol described previously (Jeedigunta et al. 2000). Other cyclic dipeptides were prepared as described elsewhere (Wollinsky et al. 2012a; Yu et al. 2013) or purchased from Bachem (Bubendorf, Switzerland).

Computer-assisted sequence analysis

Sequence analysis and intron prediction were performed with FGENESH-M (Softberry, Mount Kisco, NY; www.softberry.com) and geneid (<http://genome.crg.es/geneid.html>).

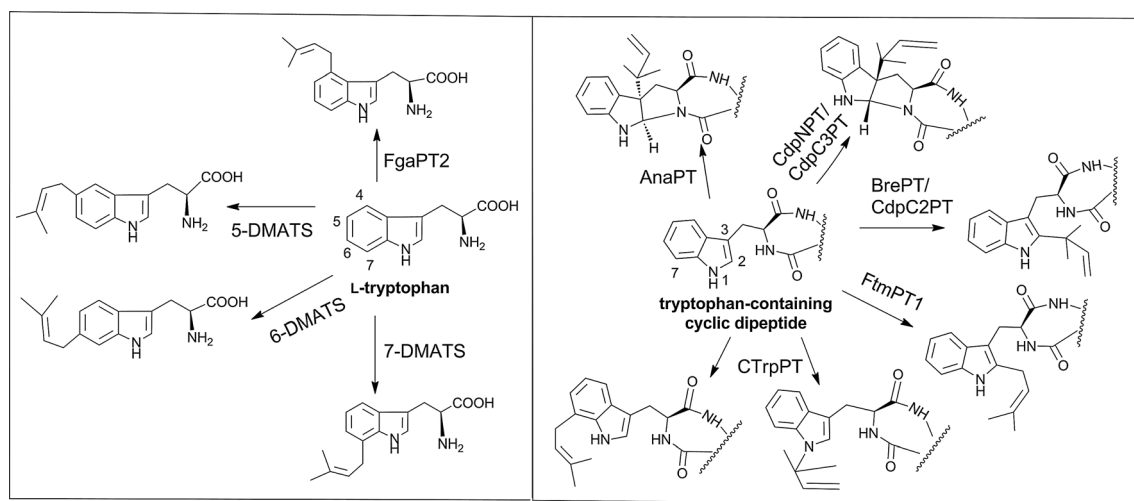


Fig. 1 Prenylation positions of tryptophan prenyltransferases (*left*) and of tryptophan-containing cyclic dipeptide prenyltransferases (*right*)

Alignments of amino acid sequences were carried out by using the programs “BLAST 2 SEQUENCES” (<http://blast.ncbi.nlm.nih.gov>) and ClustalX2 (<http://www.clustal.org/clustal2/>).

Bacterial strains, plasmids, and culture conditions

pGEM-T Easy and pQE-60 were purchased from Promega (Mannheim, Germany) and QIAGEN (Hilden, Germany) and used for cloning and expression experiments, respectively. *Escherichia coli* XL1 Blue MRF’ (Agilent Technologies, Amsterdam, the Netherlands) and M15 (pREP4) cells (QIAGEN, Hilden, Germany) were used as cloning and expression hosts. Cultivation of *E. coli* was carried out at 30 or 37 °C in liquid or on solid LB medium with 1.5 % (w/v) agar (Sambrook and Russell 2001). Kanamycin (25 µg ml⁻¹) and carbenicillin (50 µg ml⁻¹) were used for selection of recombinant strains.

Cultivation of *A. terreus* for DNA isolation

A. terreus DSM 1958 was obtained from German Collection of Microorganisms and Cell Cultures (DSMZ) and cultivated in 300-ml cylindrical flasks containing 100 ml YME medium (Yeast extract [4.0 g l⁻¹], glucose monohydrate [4.0 g l⁻¹], and Malt Extract [10.0 g l⁻¹] at 30 °C for 5 days in darkness for DNA isolation.

DNA propagation in *E. coli* and DNA isolation from fungi

Standard procedures for DNA isolation and manipulation in *E. coli* were performed as described elsewhere (Sambrook and Russell 2001).

To isolate genomic DNA from *A. terreus*, the mycelia of a 5-day-old culture were collected and washed with phosphate-buffered saline consisting of 137 mM NaCl, 2.7 mM KCl, 1 mM Na₂HPO₄, and 0.18 mM KH₂PO₄ (pH 7.3). Genomic DNA was isolated according to a method described previously (Yu and Li 2012).

PCR amplification and gene cloning

For PCR amplification, an iCycler from BioRad was used. Two primer pairs ATEG_04218_1fw (5'-**AACCATGGCCAACCGCGTGTCTAACG**-3') and ATEG_04218_3rv (5'-CGACTACTCTGAGACAAATCCACATCGGGAAATATATCTTT**CAGCATCTG**-3') were used for amplification of the first exon and ATEG_04218_2fw (5'-**CAGATGCTGAAAGATATATTTCCCGATGTGGATTG**TGTCTCAGAGTAGTCG-3') and ATEG_04218_4rv (5'-**GCAGATCTCTCTGCTAAGAATTCAGTAGAGCGCG**-3') for the second exon. Bold letters in ATEG_04218_1fw and ATEG_04218_4rv represent mutations inserted in comparison

to the original genome sequence in order to get the underlined restriction sites *NcoI* and *BgIII* for cloning in pQE-60, respectively. The underlined letters in ATEG_04218_2fw and ATEG_04218_3rv indicate sequences at 3'-end of exon 1 and 5'-end of exon 2 for fusion of the two exons. A PCR program of 35 cycles with an annealing temperature at 59 °C for 60 s and elongation at 72 °C for 120 s was used for PCR amplification. After amplification of both exons separately, the PCR products were mixed in a molar ratio of 1:1 and used for the second round PCR to get the fusion product with the expected size of 1,350 bp. The obtained PCR fragment was cloned into pGEM-T Easy vector resulting in plasmid pCaW16, which was sequenced by Eurofins Genomics GmbH (Ebersberg, Germany) to confirm the sequence. After digestion of pCaW16 with the restriction enzymes *NcoI*, *BgIII*, and *DraI*, the resulting *NcoI*-*BgIII* fragment of 1,344 bp was directly ligated into pQE-60, which had been digested with *NcoI* and *BgIII*, previously. The resulting plasmid pCaW18 was used for protein overproduction.

Overproduction and purification of CdpC7PT-His₆

For *cdpC7PT* expression with pCaW18, *E. coli* M15 [pREP] was cultivated in a 2,000-ml cylindrical flask containing 1,000 ml liquid LB medium supplemented with carbenicillin (50 µg ml⁻¹) and kanamycin (25 µg ml⁻¹). After growing at 37 °C to an optical density at 600 nm of 0.6, gene expression was induced with isopropyl thiogalactoside at a final concentration of 0.3 mM at 37 °C for 16 h. The bacterial cultures were harvested by centrifugation and resuspended in lysis buffer (10 mM imidazole, 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) at 2–5 ml g⁻¹ wet weight. After incubation for 30 min on ice with 1 mg ml⁻¹ lysozyme, the cells were sonicated six times for 10 s each at 200 W. To separate the cellular debris from the soluble proteins, the lysate was centrifuged at 14,000×g for 30 min at 4 °C. One-step purification of the recombinant His₆-tagged fusion protein by affinity chromatography with Ni-NTA agarose resin (Macherey-Nagel, Düren, Germany) was carried out according to the manufacturer's instructions. The protein was eluted with 250 mM imidazole in 50 mM NaH₂PO₄ and 300 mM NaCl, pH 8.0. In order to change the buffer, the protein fraction was passed through a NAP-5 column (GE Healthcare, Freiburg, Germany), which had been equilibrated with 50 mM Tris/HCl and 15 % (v/v) glycerol, pH 7.5. CdpC7PT-His₆ was eluted with the same buffer as for column equilibration and stored at -80 °C for enzyme assays.

Protein analysis and determination of molecular mass of active CdpC7PT-His₆

CdpC7PT was analyzed on SDS-PAGE according to the method described previously (Laemmli 1970) and stained

with Coomassie Brilliant Blue G-250. The molecular mass of the recombinant CdpC7PT-His₆ was determined on a HiLoad 16/60 Superdex 200 column (GE Healthcare, Freiburg, Germany) by using 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl as elution buffer. The column was calibrated with dextran blue 2000 (2,000 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa) (GE Healthcare, Freiburg, Germany). The molecular mass of the recombinant active CdpC7PT-His₆ was determined as 62 kDa, proving that CdpC7PT acts as a monomer.

Assays for CdpC7PT activity

For determination of CdpC7PT activity, the reaction mixtures (100 μ l) contained 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂, 1 mM cyclic dipeptide (Table 1), 2 mM DMAPP, and 50 μ g (9.0 μ M) of purified recombinant CdpC7PT and were incubated at 37 °C for 16 h. The reactions were terminated by addition of 60 μ l methanol. After removal of proteins by centrifugation at 13,000 \times g for 20 min, the enzyme products were analyzed on HPLC with the method described below. Duplicate values were determined routinely for quantitative measurement of the enzyme activity. The assays for determination of the kinetic parameters of (*S*)-benzodiazepinedione (**1a**), *cyclo*-L-Trp-L-Trp (**3a**), *cyclo*-L-Trp-L-Tyr (**4a**), *cyclo*-L-Trp-L-Phe (**5a**), *cyclo*-L-Trp-L-Leu (**6a**), *cyclo*-L-Trp-L-Ala (**7a**), and of *cyclo*-L-Tyr-L-Tyr (**18a**) contained 5 mM CaCl₂, 2 mM DMAPP, 2.0 μ M (**1a**, **3a**), or 4.0 μ M (**4a–7a**, **18a**) CdpC7PT and aromatic substrates at final concentrations of up to 5.0 mM. For determination of the kinetic parameters of DMAPP, 2.0 μ M CdpC7PT, 1 mM **1a**, 5 mM CaCl₂, and DMAPP at final concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0, 2.0, and 5.0 mM were used. Incubations were carried out at 37 °C for 60 min (DMAPP, **1a**, **3a**) or 120 min (other substrates).

For structural elucidation of the enzyme products, the isolation assays (20 ml) contained 50 mM Tris-HCl (pH 7.5), 1 mM DMAPP, 1 mM aromatic substrate, 4.0–6.0 μ M of CdpC7PT, 5 mM CaCl₂ and were incubated at 37 °C for 16 h.

HPLC analysis and product isolation

Enzyme assays of CdpC7PT were analyzed on HPLC with an Agilent series 1200 (Agilent, Foster City, CA) by using a Multospher 120 RP-18 column (250 \times 4 mm, 5 μ m, CS-Chromatographie Service, Langerwehe, Germany) and flow rate of 1 ml min⁻¹. Water (solvent A) and methanol (solvent B) were used as solvents. For analysis of enzyme products, a linear gradient of 30–100 % (v/v) solvent B in A in 25 min was used. The column was then washed with 100 % solvent B for 5 min and equilibrated with 30 % solvent A for 5 min.

The assays for isolation of the enzyme products were extracted with ethyl acetate and concentrated on a rotation evaporator at 35 °C to dryness. The residues were dissolved in 800 μ l methanol and purified on an HPLC with a Multospher 120 RP-18 column (250 \times 10 mm, 5 μ m) at a flow rate of 2.5 ml min⁻¹. A linear gradient of 60–100 % of solvent B in A in 20 min was used for separation. The collected fractions were evaporated to dryness and subjected to high-resolution mass spectrometry (HR-MS) and NMR analyses.

Spectroscopic data

¹H NMR spectra were recorded on a JEOL ECA-500 spectrometer (JEOL, Akishima, Tokyo, Japan). All Spectra were processed with MestReNova.6.0.2 (Metrelab Research, Santiago de Compostella, Spain) and chemical shifts were referenced to those of the solvent signals. The NMR data are given in Table 2 and spectra as [Supplementary Material Information](#). The isolated products were also analyzed by

Table 1 Product yields of CdpC7PT and CTrpPT reactions with tested substrates

Substrate	Conversion (%)		Substrate	Conversion (%)	
	CdpC7PT	CTrpPT		CdpC7PT	CTrpPT
(<i>S</i>)-benzodiazepinedione (1a)	45.6	10.9	<i>cyclo</i> -L-Trp-D-Ala (12a)	3.4	<0.01
(<i>R</i>)-benzodiazepinedione (2a)	8.9	17.8	<i>cyclo</i> -D-Trp-D-Ala (13a)	13.0	<0.01
<i>cyclo</i> -L-Trp-L-Trp (3a)	28.1	89.4	<i>cyclo</i> -D-Trp-L-Ala (14a)	10.9	<0.01
<i>cyclo</i> -L-Trp-L-Tyr (4a)	28.2	52.5	<i>cyclo</i> -L-Trp-Gly (15a)	25.0	1.2
<i>cyclo</i> -L-Trp-L-Phe (5a)	13.6	11.4	<i>cyclo</i> -D-Trp-L-Tyr (16a)	9.7	<0.02
<i>cyclo</i> -L-Trp-L-Leu (6a)	29.2	33.6	L-tryptophan (17a)	3.2	<0.01
<i>cyclo</i> -L-Trp-L-Ala (7a)	19.5	2.2	<i>cyclo</i> -L-Tyr-L-Tyr (18a)	14.5	<0.01
<i>cyclo</i> -L-Trp-D-Pro (8a)	10.3	0.7	<i>cyclo</i> -L-Tyr-L-Ser (19a)	<0.02	<0.02
<i>cyclo</i> -D-Trp-D-Pro (9a)	6.8	2.0	<i>cyclo</i> -L-Tyr-L-Pro (20a)	<0.01	<0.01
<i>cyclo</i> -D-Trp-L-Pro (10a)	8.3	0.9	<i>cyclo</i> -L-Tyr-Gly (21a)	<0.02	<0.02
<i>cyclo</i> -L-Trp-L-Pro (11a)	21.3	6.5	L-tyrosine (22a)	<0.01	<0.01

Table 2 ^1H NMR data of the enzymatic products of CdpC7PT

Comp.										
	δ_{C}	δ_{H} , multi., J [Hz]	δ_{H} , multi., J [Hz]		δ_{H} , multi., J [Hz]		δ_{H} , multi., J [Hz]		δ_{H} , multi., J [Hz]	
			CD ₃ OD	CDCl ₃	CD ₃ OD	CDCl ₃	CD ₃ OD	CDCl ₃	CD ₃ OD	CDCl ₃
Pos.			δ_{H} , multi., J [Hz]		δ_{H} , multi., J [Hz]		δ_{H} , multi., J [Hz]		δ_{H} , multi., J [Hz]	
NH-1	-	-	-	8.03, s	-	-	-	-	-	-
2	124.2	7.13, s	6.55, s	6.53, d, 2.2	6.57, s	6.69, s	7.05, s	-	5.52, s	
3	110.3	-	-	-	-	-	-	-	-	
4	116.0	7.26, dd, 7.7, 1.2	7.33, d, 7.8	7.41, d, 7.9	7.47, d, 8.0	7.54, 7.9	7.44, d, 7.7	7.14, d, 7.5		
5	119.6	6.88, t, 7.4	7.08, t, 7.0	7.08, t, 7.5	7.09, t, 7.4	7.11, t, 7.3	7.00, t, 7.6	6.75, t, 7.5		
6	121.1	6.85, dd, 7.1; 1.1	6.89, d, 7.0	7.02, d, 7.1	7.06, t, 7.7	7.14, t, 7.6	6.92, d, 6.9	7.10, td, 7.7, 1.1		
7	125.3 ^a	-	-	-	7.44, d, 8.0	7.48, d, 8.3	-	6.59, d, 7.9		
8	136.2	-	-	-	-	-	-	-		
9	127.7	-	-	-	-	-	-	-		
10	24.5	3.37, dd, 15.1, 5.8 3.13, dd, 15.1; 8.9	2.94, dd, 14.3, 3.9 2.36, dd, 14.3, 6.6	3.24, dd, 14.6, 3.2 2.39, dd, 14.6, 8.6	2.97, dd, 14.3, 3.9 2.25, dd, 14.3, 6.8	3.19, dd, 14.5, 3.5 2.44, dd, 14.5, 8.1	3.04, dd, 14.5, 4.1 2.92, dd, 14.5, 5.2	2.52, dd, 12.6, 6.1 2.39, dd, 12.6, 11.4		
11	53.8	4.09, dd, 9.0, 5.9	4.08, dd, 6.6, 3.9	4.19, m 5.70, s	4.07, dd, 7.3, 3.9	4.19, d, 7.7 5.70, s	4.18, t, 4.7	3.92, dd, 11.1, 6.2		
NH-12	-	-	-	-	-	-	-	-		
13	170.5	-	-	-	-	-	-	-		
14	126.5	-	4.00, dd, 7.7; 3.5	4.19, m	4.05, dd, 7.8, 4.4	4.19, d, 7.7	3.80, dd, 9.2, 3.5	4.15, dd, 10.5, 2.1		
15	137.5	-	-	-	-	-	-	-		
NH-15	-	-	-	5.73, s	-	5.74, s	-	5.55, s		
17	173.2	-	2.88, dd, 14.3, 3.5 1.91, dd, 14.3, 7.7	3.24, dd, 14.6, 3.2 2.51, dd, 14.6, 8.3	2.90, dd, 14.2; 3.9 2.09, dd, 14.2, 6.8	3.22, dd, 14.6, 3.4 2.39, dd, 14.6, 8.5	2.52, dd, 13.6, 3.7 1.24, dd, 13.6, 9.2	3.48, dd, 14.6, 3.7 2.74, dd, 14.6, 10.3		
18	121.7	7.11, d, 8.2	-	-	-	-	-	-		
19	133.7	7.52, ddd, 8.2, 7.3, 1.6	6.31, s	6.60, d, 2.2	6.39, s	6.46, s	6.59, d, 8.6	7.06, d, 8.5		
20	125.3 ^a	7.23, td, 7.6, 1.0	-	-	-	-	6.37, d, 8.4	6.80, d, 8.4		
NH-20	-	-	-	8.07, s	-	8.05, s	-	-		
21	131.1	7.77, dd, 7.9, 1.0	-	-	-	-	-	-		
22	-	-	7.29, d, 8.1	7.36, d, 8.2	7.31, d, 8.2	7.39, d, 8.0	6.37, d, 8.4	6.80, d, 8.4		
23	-	-	6.99, t, 7.3	7.22, t, 7.5	7.01, td, 7.2, 0.8	7.21, t, 7.5	6.59, d, 8.6	7.06, d, 8.5		
24	-	-	6.96, t, 7.5	7.16, t, 7.6	7.01, t, 7.6	7.14, t, 7.6	-	-		
25	-	-	7.41, d, 8.0	7.58, d, 8.0	7.62, d, 7.9	7.57, d, 8.1	-	-		
1'	30.0	3.51, d, 7.2	3.47, d, 7.0	3.52, d, 7.3	5.14, d, 10.8 5.11, d, 17.6	5.19, d, 10.8 5.16, 17.9	3.49, d, 7.3	5.13, d, 10.8 5.08, d, 17.4		
2'	122.4	5.39, t sept, 7.2, 1.4	5.30, t sept, 7.2, 1.4	5.30, t, 7.1	6.07, dd, 17.6, 10.8	6.06, d, 17.6, 10.7	5.30, t sept, 7.3, 1.4	5.95, dd, 17.3, 10.7		
3'	133.2	-	-	-	-	-	-	-		
4'	17.2 ^b	1.73, s	1.63, s	1.78, s	1.69, s	1.71, s	1.66, s	1.11, s		
5'	25.3 ^b	1.73, s	1.53, s	1.71, s	1.67, s	1.68, s	1.56, s	1.00, s		
			DMSO-d ₆	CDCl ₃						
Pos.			δ_{H} , multi., J [Hz]		δ_{H} , multi., J [Hz]		δ_{H} , multi., J [Hz]		δ_{H} , multi., J [Hz]	
NH-1	-	-	-	8.16, s	-	-	-	-	-	
2	7.03, s	-	6.97, s	6.99, s	7.06, s	7.07, s	5.54, s	6.86, d, 7.0		
3	-	-	-	-	-	-	-	6.76, d, 6.4		
4	7.58, d, 7.9	-	7.31, d, 7.8	7.48, d, 8.4	7.43, d, 8.0	7.43, d, 7.9	7.20, dd, 7.5, 0.7	-		
5	7.13, t, 7.6	-	6.92, t, 7.5	7.11, d, 7.5	6.93, t, 7.5	6.93, t, 7.5	6.72, td, 7.5, 1.0	6.76, d, 6.4		
6	7.06, t, 7.3	-	6.84, d, 6.8	7.05, d, 6.9	6.87, dd, 7.2, 0.9	6.86, dd, 7.1, 0.6	7.06, td, 7.7, 1.2	6.86, d, 7.0		
7	7.34, d, 8.1	-	-	-	-	-	6.59, d, 7.6	3.29 [*]		
8	-	-	-	-	-	-	-	3.22, dd, 11.1, 6.0		
10	3.02, dd, 14.1, 4.2 2.68, dd, 14.1,	-	2.81, dd, 14.5, 4.3 2.58, dd, 14.5, 5.4	3.27, dd, 14.6, 3.1 2.61, dd, 14.6, 8.3	3.48, dd, 14.7, 3.6 3.10, dd, 14.7, 4.6	3.44, dd, 14.7, 3.8 3.13, dd, 14.7, 4.5	2.50, dd, 12.7, 6.3 2.39, dd, 12.7, 11.3	3.84, t, 5.4		
11	4.15, t, 5.1	-	4.00, t, 4.9	4.22, m	4.27, ddd, 4.4, 3.4, 0.9	4.26, td, 4.2, 1.0	3.98, ddd, 11.3, 6.3, 2.1	3.80, t, 5.2		
14	3.92, dt, 12.5, 6.2	-	3.81, dd, 7.7, 4.6	4.09, m	3.56, ddd, 9.9, 4.1, 0.9	3.69, qd, 7.1, 1.5	4.11, qd, 6.9, 2.1	2.08, dd, 13.3, 6.3 1.98, m		
NH-12	-	-	-	5.77, s	-	-	-	-		
NH-15	-	-	-	5.71, s	-	-	-	-		
16	-	-	-	-	-	-	-	6.75, d, 6.4		
17	2.59, dd, 13.7, 3.8 1.54, dd, 13.7, 8.	-	2.35, dd, 13.5, 4.2 1.53, dd, 13.5, 5.6	3.07, dd, 13.5, 3.1 2.14, dd, 13.5, 9.1	1.13, m 0.64, ddd, 13.9, 9.7, 4.3	0.34, d, 7.0	1.38, d, 6.9	6.60, d, 6.7		
18	-	-	-	-	1.75, dd, 7.5, 1.0	-	-	-		
19	6.75, d, 8.6	-	7.12, dd, 5.0, 1.8	6.87, d, 7.0	0.59, d, 6.6	-	-	6.60, d, 6.7		
20	6.56, d, 8.6	-	6.57, dd, 6.5, 2.8	7.29 [*]	0.44, d, 6.6	-	-	6.75, d, 6.4		
21	-	-	6.48, dd, 5.5, 2.0	7.26 [*]	-	-	-	-		
22	6.56, d, 8.6	-	6.57, dd, 6.5, 2.8	7.29 [*]	-	-	-	-		
23	6.75, d, 8.6	-	7.12, 5.0, 1.8	6.87, d, 7.0	-	-	-	-		
1'	4.45, d, 6.5	-	3.41, d, 7.3	3.54, d, 6.9	3.52, d, 7.3	3.52, d, 7.2	5.14, dd, 10.9, 1.3 5.12, dd, 17.4, 1.3	4.37, d, 5.8		
2'	5.37, td, 6.8, 1.8	-	5.24, t sept, 7.3, 1.3	5.30, t, 7.2	5.43, t sept, 7.2, 1.4	5.39, t sept, 7.2, 1.4	6.06, dd, 17.4, 10.9	5.27, t, 7.0		
4'	1.69, s	-	1.58, s	1.79, s	1.76, s	1.73, s	1.13, s	1.58, s		
5'	1.67, s	-	1.49, s	1.71, s	1.75, s	1.73, s	0.99, s	1.53, s		

The spectra were taken on a JEOL ECA-500 spectrometer. Chemical shifts (δ) are given in ppm and coupling constants in Hz

* Signals overlapping with those of solvent

^a Overlapping signals

^b Interchangeable signals

mass spectrometry on a Q-Trap Quantum (Applied Biosystems, Darmstadt, Germany) by using a high-

resolution electron impact (HR-EI) ionization mode. HR-EI-MS data are given in Table S2 in the Supplementary Material.

Cultivation of *A. terreus* and analysis of secondary metabolites by LC/MS analysis

A. terreus DSM 1958 were cultivated in 300-ml cylindrical flasks containing 100 ml AMM medium (6 g/l NaNO₃; 0.52 g/l KCl, 0.52 g/l MgSO₄·7H₂O) at 30 °C and 120 rpm in darkness for 7 and 14 days. The culture filtrates were extracted twice with 100 ml ethyl acetate. The combined organic layers were dried over Na₂SO₄ and subsequently evaporated in vacuo to dryness. For LC/MS analysis, the extracts were dissolved in 1 ml of methanol. LC/MS analysis was carried out on an Agilent 1100 HPLC system (Agilent Technologies) coupled to a LTQ-FT Ultra ESI-MS instrument (Thermo Fischer Scientific) using a linear ion trap (MS) and an ICR cell (HR-MS), operated in positive ionization mode. Water (solvent A) and acetonitrile (solvent B) were used at a flow rate of 0.2 ml min⁻¹. A linear gradient of 10–100 % (v/v) solvent B in 45 min was used. The column was then washed with 100 % solvent B for 5 min and equilibrated with 10 % (v/v) solvent B for 5 min.

Accession numbers The nucleotide sequence of the genomic DNA from *A. terreus* reported in this paper has been deposited in the GenBank database under accession number CH476598.1. The coding region of *cdpC7PT* is available in GenBank under the accession number XM_0013396.1.

Results

Prenyltransferase genes in the genome of *A. terreus*

As aforementioned, five of the ten putative prenyltransferase genes of the DMATS superfamily from *A. terreus* NIH2624 had been characterized molecular biologically by gene deletion experiments or biochemically with recombinant proteins from *A. terreus* or with their orthologues from other fungi. They include one C3-prenyltransferase gene ATEG_10306 belonging to the acetylazonalenin gene cluster and its orthologue AnaPT from *Neosartorya fischeri* NRRL 181 was proven to be responsible for the C3 α -prenylation of (*R*)-benzodiazepinedione (Yin et al. 2009). 7-DMATS encoded by ATEG_08428 catalyzes the C7-prenylation of L-tryptophan and is involved in the biosynthesis of astatochrome (Kremer et al. 2007; Yin et al. 2013b). AstPT encoded by ATEG_09980 functions as a prenyltransferase of methylated asterolquinone and catalyzes both reverse *NI*- and regular C2-prenylation (Tarcz et al. 2014). Gene deletion experiments suggested the involvement of ATEG_02823 and ATEG_00702 in the biosynthesis of butyrolactones and asterolquinone CT5, respectively (Guo et al. 2013). However, no biochemical data are available for the encoded

enzymes. Other five putative genes ATEG_04999, ATEG_06111, ATEG_03092, ATEG_01730 and ATEG_04218 remained with unknown function prior to this work.

ATEG_04218 is located on the genomic sequence of *A. terreus* NIH2624 between bp 1888259 and 1889646 under the accession number CH476598.1. According to the prediction given in GenBank (<http://ncbi.nlm.nih.gov>), ATEG_04218 consists of two exons of 1,208 and 133 bp, respectively, interrupted by one intron of 47 bp. The deduced polypeptide EAU36020 (=CdpC7PT) has a length of 446 amino acids with a calculated molecular mass of 51.0 kDa. Blast search revealed clear sequence similarities between EAU36020 and several known cyclic dipeptide prenyltransferases of the DMATS superfamily from various fungi. For example, CdpC7PT shares a sequence identity of 34 % on the amino acid level with the reverse prenyltransferase CdpC2PT from *N. fischeri* (Mundt and Li 2013) and 32 % with the *cyclo*-L-Trp-L-Pro reverse C2-prenyltransferase BrePT from *Aspergillus versicolor* (Yin et al. 2013a). The sequence identity of CdpC7PT and the *cyclo*-L-Trp-L-Trp C7-prenyltransferase CTrpPT from *Aspergillus oryzae* (Zou et al. 2010) was found to be 29 %.

Analysis of the neighboring genes of ATEG_04218 on CH476598.1 did not indicate the presence of an entire putative gene cluster for secondary metabolites (Table S1 in the Supplementary Material). No genes encoding for nonribosomal peptide synthetases (NRPS), polyketide synthases (PKS), or other known secondary metabolite backbone genes have been identified in the vicinity of the prenyltransferase gene ATEG_04218. The natural substrate of ATEG_04218 can therefore not be predicted by sequence analysis. Based on its sequence similarities with cyclic dipeptide prenyltransferases, it might be speculated that these substances could be accepted by the enzyme as prenylation substrates.

Cloning of *cdpC7PT*, protein overproduction, and purification

Initial attempt to clone the coding sequence of ATEG_04218 from mRNA of *A. terreus* DSM 1958 was unsuccessful (data not shown). Its coding region consisting of two exons and was therefore amplified from genomic DNA by using two round fusion PCR and cloned into the expression vector pQE-60. Gene expression in *E. coli* and purification of the soluble protein resulted in a predominant band on SDS-PAGE with migration above the 45 kDa size marker (Fig. S1 in the Supplementary Material), corresponding well to the calculated mass of 51.8 kDa for CdpC7PT-His₆. Protein yield was calculated to be 2.4 mg of purified protein per liter of culture. The molecular mass of the native recombinant CdpC7PT-His₆ was determined by size exclusion chromatography as 62 kDa, indicating that CdpC7PT presumably acts as a monomer.

Acceptance of tryptophan-containing cyclic dipeptides by CdpC7PT as substrates

As mentioned above, CdpC7PT shares comparatively higher sequence similarities with cyclic dipeptide prenyltransferases, especially with CdpC2PT and BrePT, than with other prenyltransferases (Mundt and Li 2013; Yin et al. 2013a). Therefore, CdpC7PT was firstly incubated with 16 tryptophan-containing cyclic dipeptides (**1a–16a**, Table 1 and Fig. S2 in the Supplementary Material) and L-tryptophan (**17a**) in the presence of DMAPP as prenyl donor at 37 °C for 16 h. Enzyme assays with inactivated CdpC7PT by boiling the enzyme for 30 min were used as negative controls. HPLC analysis revealed product formation in all of the reaction mixtures with active CdpC7PT. (*S*)-benzodiazepinedione (**1a**) was found to be the best accepted substrate with a product yield of 46 %. Its enantiomer (**2a**), the natural substrate of AnaPT (Yin et al. 2009), was poorly accepted by CdpC7PT. Product yields of more than 20 % were detected for *cyclo*-L-Trp-L-Trp (**3a**), *cyclo*-L-Trp-L-Tyr (**4a**), *cyclo*-L-Trp-L-Phe (**5a**), *cyclo*-L-Trp-L-Leu (**6a**), *cyclo*-L-Trp-L-Pro (**11a**) and *cyclo*-L-Trp-Gly (**15a**) under the same conditions. As given in Table 1, cyclic dipeptides with D-configured amino acid moieties were poor substrates for CdpC7PT. Inspection of the HPLC chromatograms revealed the presence of one to three product peaks. L-Tryptophan was accepted only with a very low product yield of 3 %, excluding its role as natural substrate. Product formation was detected in the enzyme assays neither with geranyl nor farnesyl diphosphate, demonstrating its high specificity towards its prenyl donor DMAPP (Fig. S3 in the Supplementary Material).

C7-prenylation on the indole ring as the main reaction

To prove their structures, ten enzyme products (**1b**, **3b–7b**, **3c**, **4c**, **7c**, and **4d**) were isolated on HPLC from incubation mixtures of six selected substrates (**1a**, **3a–7a**; Fig. 2) in preparative scales and subjected to MS and NMR analyses. NMR data are given in Table 2 and spectra in Figs. S4–S25 in the Supplementary Material. MS data are given in Table S2 in the Supplementary Material. HR-EI-MS data proved that the molecular masses of all the isolated products are 68 Daltons larger than those of the respective substrates, indicating a monoprenylation in their structures. The **b**-series products (**1b**, **3b–7b**), i.e., those with a smaller retention time on the RP-column than other products of a given substrate, were isolated from all the enzyme assays (**1a**, **3a–7a**). Inspection of their ¹H NMR spectra revealed clearly the presence of signals for a regular prenyl moiety each at δ_{H} 3.41–3.54 (d, H-1'), 5.24–5.43 (t, H-2'), 1.58–1.79 (s, 3H-4'), and 1.49–1.75 ppm (s, 3H-5'). The chemical shifts of H-1' proved its attachment to an aromatic C-atom (Winkelblech and Li 2014; Wollinsky et al. 2012b; Yu et al. 2012; Zou et al. 2010). This

conclusion was also supported by the disappearance of signals for one aromatic proton at H-4 or H-7, which was deduced from the number and coupling pattern of the aromatic protons. Detailed comparison of the NMR spectra of **1b** and **3b–7b** with those of their substrates indicated that the doublet for H-7 has disappeared. The chemical shifts of the three remained coupling protons on the prenylated ring clearly differed from those of C4-prenylated and corresponded well to those of C7-prenylated derivatives (Steffan and Li 2009; Zou et al. 2010). H-H COSY, HSQC and HMBC (Fig. 3 and Figs. S5–S12 in the Supplementary Material) of **1b** proved unequivocally the attachment of the prenyl moiety to C-7 of the indole ring. The NMR data of **3b** and **5b** in CDCl₃ corresponded perfectly to those of the enzyme products of CTrpPT with **3a** and **5a**, respectively, whose structures have been elucidated by NMR analysis including HSQC and HMBC in that study (Zou et al. 2010). The NMR data of **7b** are consistent well with those of terezine D (Wang et al. 1995). These compounds are therewith identified as C7-prenylated derivatives and therefore CdpC7PT functions as a cyclic dipeptide C7-prenyltransferase (Fig. 2).

Prenylations at *N1* and *C3* of the indole ring as well as *O*-prenylation on the tyrosyl residue as minor products

3c from the incubation mixture of *cyclo*-L-Trp-L-Trp (**3a**) was identified as reversely *N1*-prenylated derivative by comparison of its NMR data with those of an enzyme product of **3a** with CTrpPT published previously (Zou et al. 2010). Signals of a reverse prenyl moiety attached to C-3 of an indoline ring were detected in the spectra of **4c** and **7c**, which is characterized by the signals of the two methyl groups at high magnetic field (approx. 1 ppm) (Yin et al. 2010a, b; Yu et al. 2013). Furthermore, **4c** and **7c** were easily identified as reversely C3 β -prenylated *cyclo*-L-Trp-L-Tyr and *cyclo*-L-Trp-L-Ala with a *syn-cis* configuration by comparison of their NMR data with those published previously (Yin et al. 2010b; Yu et al. 2013). Using NMR analysis including HSQC, HMBC and NOESY, C3 β -prenylated derivatives were identified as main products in the reaction mixtures of CdpNPT and CdpC3PT with tryptophan-containing cyclic dipeptides (Yin et al. 2010b; Yu et al. 2013). Identification of these compounds as enzyme products in the incubation mixtures of CdpC7PT demonstrated the decrease of the regioselectivity due to a putative unfavorable orientation of such substrates in the active site of the enzyme, as observed previously for other enzymes (Wollinsky et al. 2012b). In comparison, only one product **1b** bearing a C7-prenyl moiety was detected with the best accepted substrate **1a** (Fig. 2).

In the spectrum of the third product **4d** from the incubation mixture of *cyclo*-L-Trp-L-Tyr (**4a**), signals for a regular prenyl moiety were observed at δ_{H} 4.45 (d, H-1'), 5.37 (td, H-2'), 1.69 (s, 3H-4'), and 1.67 ppm (s, 3H-5'). The low chemical shift of

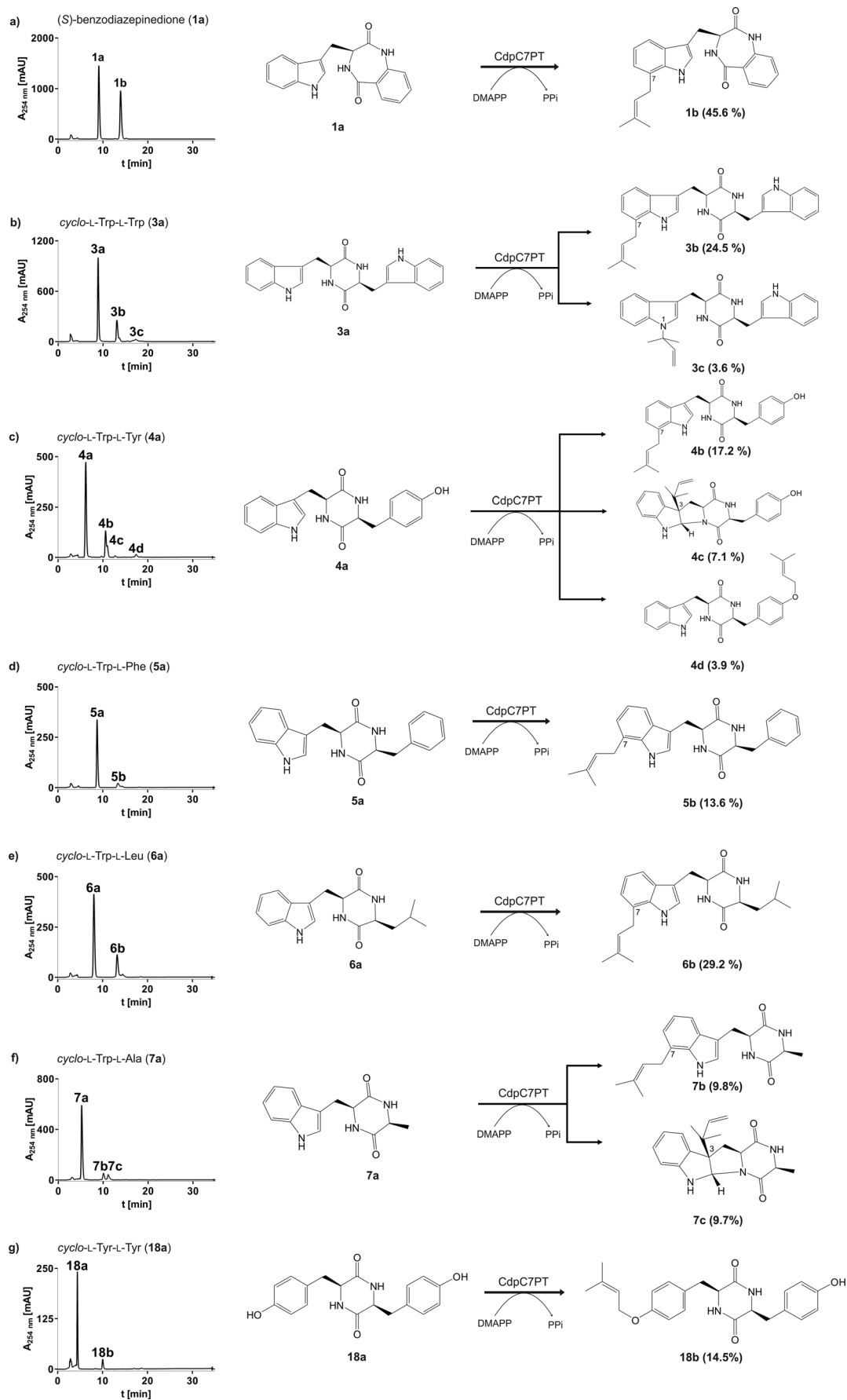


Fig. 2 HPLC analysis of the incubation mixtures of selected substrates (left) and prenylation reactions catalyzed by CdpC7PT (right). Product yields are given in parenthesis under the structures

H-1' indicates a prenylation at a hetero atom, i.e., an oxygen or nitrogen atom. This is also confirmed by the same number and coupling pattern of the aromatic protons in the spectra of **4d** and **4a**. The chemical shift of H-1' of **4d** in CD₃OD at δ_H 4.45 ppm differed clearly from that of *cyclo-NI*-dimethylallyl-L-Trp-L-Tyr at δ_H 4.68 ppm in the same solvent (Yin et al. 2007). **4d** was therefore identified as an *O*-prenylated *cyclo*-L-Trp-L-Tyr derivative. To exclude the possibility of conversions between **4b**, **4c**, and **4d** during the incubation, **4a** was incubated with CdpC7PT for different times. As shown in Fig. 4, the three products are formed independently from each other during the incubation.

Acceptance and *O*-prenylation of *cyclo*-L-Tyr-L-Tyr by CdpC7PT

Identification of **4d** with an *O*-prenylated tyrosyl residue as an enzyme product of *cyclo*-L-Trp-L-Tyr (**4a**) prompted us to test the acceptance of tyrosine-containing cyclic dipeptides lacking a tryptophanyl moiety such as *cyclo*-L-Tyr-L-Tyr (**18a**), *cyclo*-L-Tyr-L-Ser (**19a**), *cyclo*-L-Tyr-L-Pro (**20a**) and *cyclo*-L-Tyr-L-Gly (**21a**) as well as L-tyrosine (**22a**). Under the same conditions used for tryptophan-containing cyclic dipeptides, **18a** was accepted by CdpC7PT with a product yield of 14.5 %. That is approximately 31.8 % of that of the best accepted **1a** and 51.2 % of that of **4a**. An acceptance of *cyclo*-L-Tyr-L-Tyr by a prenyltransferase has not been reported previously. No product formation was observed in the incubation mixtures of **19a–22a** (Table 1). The sole product **18b** of the reaction mixture with **18a** was isolated on HPLC and its structure was elucidated by NMR and MS analyses (Fig. S25 and Table S2 in the Supplementary Material). As in the case of

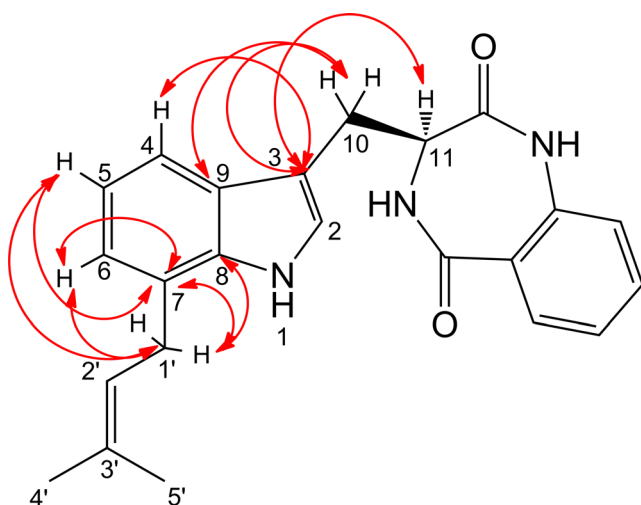


Fig. 3 Selected HMBC correlations observed in **1b**

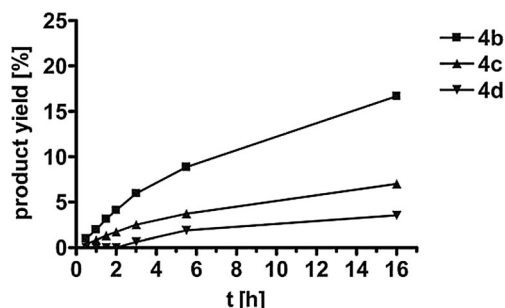


Fig. 4 Time dependency of product formation in the incubation mixture of CdpC7PT with *cyclo*-L-Trp-L-Tyr (**4a**)

4d, no significant changes were observed for the number and coupling pattern of the aromatic protons in **18b**, in comparison to those of **18a**. The chemical shift of H-1' also proved the regular *O*-prenylation in **18b** (Fig. 2).

Comparison of the substrate specificity of CdpC7PT with that of CTrpPT

As aforementioned, CTrpPT from *A. oryzae* catalyzes simultaneous *C7*- and *NI*-prenylation of cyclic dipeptides with *cyclo*-L-Trp-L-Trp as the preference substrate (Zou et al. 2010). To compare their behavior towards cyclic dipeptides as well as L-tryptophan and L-tyrosine, the same amount of CTrpPT (9.0 μ M) was incubated with the substrates tested for CdpC7PT (Table 1) and reaction mixtures were analyzed under the same HPLC conditions. As shown in Fig. S2 in the Supplementary Material, *cyclo*-L-Trp-L-Trp was confirmed to be the best substrate for CTrpPT with a product yield of about 89 %, followed by *cyclo*-L-Trp-L-Tyr, *cyclo*-L-Trp-L-Leu and *cyclo*-L-Trp-L-Phe with 53, 34, and 11 %, respectively. These substrates were also well accepted by CdpC7PT. In comparison to the results obtained with CdpC7PT, CTrpPT had a clear preference for these substrates. A number of cyclic dipeptides, including *cyclo*-L-Tyr-L-Tyr and other tyrosine-containing cyclic dipeptides, were not converted by CTrpPT (Table 1). **1a**, the best substrate of CdpC7PT, was only accepted by CTrpPT with a relative activity of 11 % of that of **3a**. For a given substrate, the main product peak in both incubation mixtures with CdpC7PT and CTrpPT showed the same retention time, indicating the same main products of the two enzymes. In comparison to CTrpPT, CdpC7PT showed somewhat lower reaction velocity, but much higher flexibility towards tryptophan-containing cyclic dipeptides. For example, *cyclo*-L-Trp-L-Pro (**11a**) and *cyclo*-L-Trp-L-Gly (**15a**) were well accepted by CdpC7PT, but only poorly by CTrpPT.

Biochemical properties and kinetic parameters of CdpC7PT

Usually, the members of the DMATS superfamily are independent of the presence of metal ions in their reactions,

Table 3 Kinetic parameters of CdpC7PT with selected substrates

Substrate	K_M (μM)	V_{\max} ($\mu\text{mol min}^{-1}\text{mg}^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_M ($\text{s}^{-1}\text{M}^{-1}$)	k_{cat}/K_M (%)
DMAPP	78.5	0.077	0.067	847.5	138.3
(<i>S</i>)-benzodiazepinedione (1a)	204.1	0.145	0.125	612.8	100.0
<i>cyclo</i> -L-Trp-L-Trp (3a)	254.0	0.041	0.035	139.3	22.7
<i>cyclo</i> -L-Trp-L-Tyr (4a)	602.2	0.049	0.042	70.0	11.4
<i>cyclo</i> -L-Trp-L-Phe (5a)	239.3	0.035	0.030	126.6	20.7
<i>cyclo</i> -L-Trp-L-Leu (6a)	746.9	0.056	0.049	65.3	10.6
<i>cyclo</i> -L-Trp-L-Ala (7a)	744.7	0.011	0.009	12.3	2.0
<i>cyclo</i> -L-Tyr-L-Tyr (18a)	1411.3	0.014	0.012	8.3	1.3

although Ca^{2+} or Mg^{2+} enhances the reaction velocities (Steffan et al. 2009). For determination of the ion dependency of CdpC7PT, incubation mixtures with different divalent metal ions such as Ca^{2+} , Mg^{2+} , Mn^{2+} , Cu^{2+} , Zn^{2+} , Fe^{2+} , and also monovalent ions such as K^+ and Na^+ at final concentrations of 5 mM were carried out in the presence of **1a** and DMAPP (Fig. S26 in the Supplementary Material). Incubations with the chelating agent EDTA or without additives were used as controls. Our results showed that addition of Ca^{2+} , Mn^{2+} , and Mg^{2+} resulted in a slight increase of the enzyme activity to 158, 131, and 120 % of that of the incubation mixture without additives. Addition of EDTA to the incubation mixture did not change the enzyme activity significantly. These results indicated that, similar to those of most the DMATS enzymes, the enzyme reaction of CdpC7PT was independent of metal ions, but can be enhanced by Ca^{2+} , Mn^{2+} , and Mg^{2+} .

To find out suitable conditions for determination of kinetic parameters, dependency of product formation on incubation time was proven with 4.0 μM CdpC7PT in the presence of 1 mM **1a** and 2 mM DMAPP. Linear dependency of up to 120 min was observed in this experiment. For seven selected cyclic dipeptides (**1a**, **3a–7a**, and **18a**), kinetic parameters including Michaelis-Menten constants (K_M) and turnover numbers (k_{cat}) were determined and calculated from Eadie-Hofstee, Hanes-Woolf, and Lineweaver-Burk plots (Table 3; Figs. S27–S34 in the Supplementary Material). The reactions catalyzed by CdpC7PT apparently followed Michaelis-Menten kinetics. CdpC7PT showed a high affinity to its prenyl donor DMAPP with a K_M value of 78.5 μM and a k_{cat} of 0.067 s^{-1} . In the cases of the tested aromatic substrates, CdpC7PT showed the highest affinity to **1a** with a K_M value of 204.1 μM and a turnover number k_{cat} of 0.125 s^{-1} . For the other tryptophan-containing cyclic dipeptides (**3a–7a**), K_M values between 239.3 and 746.9 μM and turnover numbers in the range of 0.009 and 0.049 s^{-1} were determined (Table 3). A significantly higher K_M value of 1,411.3 μM was calculated for *cyclo*-L-Tyr-L-Tyr with a relative catalytic efficiency of 1.3 % of that of **1a**.

Cultivation of *A. terreus* and screening for C7-prenylated cyclic dipeptides

For analysis of secondary metabolite accumulation, *A. terreus* DSM 1958 were cultivated as described in “Materials and methods” section. Culture filtrate extracts were analyzed by LC/MS for the presence of prenylated dipeptides by using enzyme assays of (*S*)-benzodiazepinedione with CdpC7PT and AnaPT as positive controls (Fig. S35 in the Supplementary Material). Initial analysis with extract from 14-day-old AMM culture revealed clearly the presence of $[\text{M}+\text{H}]^+$ ion at $m/z=374.1863$ ($\text{C}_{23}\text{H}_{24}\text{N}_3\text{O}_2$) for aszonalenin, the AnaPT product with (*R*)-benzodiazepinedione, and the absence of the product of CdpC7PT with (*S*)-benzodiazepinedione (Fig. S36 in the Supplementary Material). Detailed analysis of extracts from 7- to 14-day-old cultures confirmed the presence of aszonalenin and prenylated *cyclo*-L-Trp-L-Ala. Ions from prenylated *cyclo*-L-Trp-L-Trp, *cyclo*-L-Trp-L-Phe, *cyclo*-L-Trp-L-Leu and *cyclo*-L-Trp-L-Tyr were not detected (Figs. S37 and S38 in the Supplementary Material).

Discussions

In this study, we focused on a putative prenyltransferase from the ascomycete *A. terreus*. The coding sequence of the putative prenyltransferase gene ATEG_04218 was cloned into the expression vector pQE-60 and the soluble recombinant protein CdpC7PT was purified to near homogeneity after overproduction in *E. coli*. Biochemical investigations revealed the acceptance of all the 16 tested tryptophan-containing cyclic dipeptides. Structure elucidation of the isolated enzyme products from six selected substrates indicated regularly C7-prenylated derivatives as main products and proved that CdpC7PT functions as a cyclic dipeptide C7-prenyltransferase. In addition, *N*1- and *C*3-prenylated derivatives were also identified as minor enzyme products.

(*S*)-benzodiazepinedione (**1a**) was accepted by CdpC7PT as the best substrate. Interestingly, this compound was also found to be the best substrate of the reverse *C2*-prenyltransferase CdpC2PT from *N. fischeri* (Mundt and Li 2013). However, neither a *C7*- nor a *C2*-prenylated (*S*)-benzodiazepinedione or a derivative thereof was isolated from nature. Its enantiomer (*R*)-benzodiazepinedione (**2a**) is involved in the biosynthesis of acetylaszonalenin, which has been intensively studied in the last years (Ames and Walsh 2010; Yin et al. 2009). AnaPT encoded by ATEG_10306 is responsible for **2a** prenylation (Yin et al. 2009).

As aforementioned, the natural substrate of CdpC7PT cannot be predicted by sequence analysis of the genetic context in the genome because this gene is not clustered with necessary backbone genes of secondary metabolism (Table S1 in the Supplementary Material). HPLC-MS analysis of the fungal cultures reveal the presence of aszonalenin, the product of AnaPT, but not *C7*-prenylated benzodiazepinedione, *cyclo*-L-Trp-L-Trp, *cyclo*-L-Trp-L-Tyr, *cyclo*-L-Trp-L-Phe or *cyclo*-L-Trp-L-Leu (Figs. S35–S38 in the Supplementary Material). This demonstrated that *cdpC7PT* was not expressed under our tested conditions or the expression level was very low, so that the accumulated product could not be detected by LC-MS analysis. This might be also the reason for unsuccessful PCR amplification of this gene from mRNA mentioned above.

A few fungal natural products including astechrome, terezine D, and echinulins (Arai et al. 1981; Birch et al. 1961; Wang et al. 1995, 2007) are derived from *C7*-prenylated *cyclo*-L-Trp-L-Ala. The biosynthetic gene cluster of astechrome containing a NRPS gene has been identified in *Aspergillus fumigatus*, *N. fischeri*, and *A. terreus* (Kremer et al. 2007; Yin et al. 2013b). Astechrome biosynthesis in *A. fumigatus* begins with the *C7*-prenylation of L-tryptophan catalyzed by 7-DMATS and terezine D was detected as a shunt product of the astechrome pathway (Yin et al. 2013b). Similar reaction steps are also expected for the astechrome biosynthesis in *A. terreus* and the product of ATEG_08428 would catalyze the prenylation of tryptophan. The detected prenylated *cyclo*-L-Trp-L-Ala by LC-MS could be terezine D. For the biosynthesis of echinulins, a NRPS would be necessary for the formation of the cyclic dipeptide *cyclo*-L-Trp-L-Ala. As shown in Table S1 in the Supplementary Material, no such gene is located in the vicinity of *cdpC7PT* in the genome of *A. terreus*. Furthermore, *cyclo*-L-Trp-L-Ala was poorly accepted by CdpC7PT with a K_M value of 744.7 μM and a turnover number of 0.009 s^{-1} (Fig. 2). Therefore, *cyclo*-L-Trp-L-Ala seems not to be the natural substrate for CdpC7PT and consequently, CdpC7PT is not involved in the biosynthesis of echinulins. Other *C7*-prenylated cyclic dipeptides or derivatives thereof are not isolated from nature. Therefore, the natural substrate of CdpC7PT and its role in the fungal strain remained unknown.

In comparison to the known *C7*- and *NI*-prenyltransferase CTrpPT from *A. oryzae* (Zou et al. 2010), CdpC7PT showed somewhat lower reaction velocity, but much higher flexibility towards tryptophan-containing cyclic dipeptides. Furthermore, we demonstrated in this study that CdpC7PT also accepted *cyclo*-L-Tyr-L-Tyr as substrate and catalyzes an *O*-prenylation on the phenolic hydroxyl group (Fig. 2). An *O*-prenylated derivative was also isolated as minor product from reaction mixture of CdpC7PT with *cyclo*-L-Trp-L-Tyr. To the best of our knowledge, this is the first report on an enzymatic *O*-prenylation of tyrosine-containing cyclic dipeptides. The known *O*-prenyltransferase SirD catalyzes *O*-prenylation of L-tyrosine, but not cyclic dipeptides (Kremer and Li 2010). SirD also prenylates L-tryptophan and several derivatives at C-7 of the indole ring, assuming the relationship of *C7*- and *O*-prenyltransferases in the evolution (Kremer and Li 2010; Rudolf and Poulter 2013; Zou et al. 2011). *O*-prenylation of tyrosyl moiety in *cyclo*-L-Trp-L-Tyr and *cyclo*-L-Tyr-L-Tyr by a tryptophan-containing cyclic dipeptide *C7*-prenyltransferase demonstrated similar behavior of cyclic dipeptide prenyltransferases and supported this hypothesis.

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