CdpC2PT, a reverse prenyltransferase from Neosartorya fischeri with a distinct substrate preference from known C2-prenyltransferases

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A putative prenyltransferase gene, NFIA_043650, was amplified from Neosartorya fischeri NRRL 181 and cloned into the expression vector pQE60. The deduced polypeptide consisting of 445 amino acids with a molecular mass of 51 kDa was overproduced in Escherichia coli and purified as His6-tagged protein to near homogeneity. The purified soluble protein was subsequently assayed with potential aromatic substrates in the presence of dimethylallyl diphosphate. HPLC analysis of the reaction mixtures revealed acceptance of all tested tryptophan-containing cyclic dipeptides. Isolation and structural elucidation of enzyme products of five selected substrates indicated a reverse C2-prenylation on the indole nucleus, proving the enzyme to be a cyclic dipeptide C2-prenyltransferase (CdpC2PT). Differing significantly from two known brevianamide F reverse C2-prenyltransferases NotF and BrePT which use cyclo-L-Trp-L-Pro as their preferred substrate, CdpC2PT showed a clear substrate preference for (S)-benzodiazepinedinone and cyclo-L-Trp-L-Trp with $K_m$ values of 84.1 and 165.2 μM and turnover numbers at 0.63 and 0.30 s$^{-1}$, respectively. A possible role of CdpC2PT in the biosynthesis of fellutanines is discussed.

INTRODUCTION

Prenyltransferases are a large family of enzymes found in all domains of life and are involved in the biosynthesis of primary and secondary metabolites (Heide, 2009; Li, 2010; Liang, 2009; Yazaki et al., 2009). One subgroup of the prenyltransferases from ascomycetes shares significant sequence similarities with dimethylallyltryptophan synthase (DMATS) in the biosynthesis of ergot alkaloids (Kremer & Li, 2010; Liu et al., 2013; Mundt et al., 2012; Noike et al., 2012; Pockrandt et al., 2012). Indole prenyltransferases of the DMATS superfamily catalyse attachment of prenyl moieties to different positions of the indole ring of tryptophan or derivatives thereof. The resulting prenylated indole alkaloids often exhibit biological and pharmacological activities distinct from their non-prenylated precursors (Li, 2010; Williams et al., 2000). They include important mycotoxins, such as the roquefortine-family or fumitremorgin-type alkaloids (Li, 2010; Williams et al., 2000), and important drugs, e.g. the ergot alkaloids (Lorenz & Hoseney, 1979), making prenyltransferases of interest to biologists, biochemists, medicinal chemists and biotechnologists. Bioinformatic analysis of available genome sequences revealed the presence of more than 200 putative prenyltransferases of the DMATS superfamily. More than 20 such genes have been characterized biochemically following overproduction and purification (Bonitz et al., 2011; Khaldi et al., 2010; Li, 2010; Yin et al., 2013). The characterized indole prenyltransferases have characteristic features, especially their high flexibility towards aromatic substrates and high regioselectivity regarding prenylation position at the indole ring and prenylation pattern. However, the broad substrate specificity of these prenyltransferases has certain limitations. Usually, tryptophan prenyltransferases such as FgaPT2, 5-DMATS and 7-DMATS accept cyclic dipeptides only at high protein concentrations (Kremer & Li, 2008; Steffan et al., 2007; Steffan & Li, 2009). Conversely, tryptophan is a very poor substrate for cyclic dipeptide prenyltransferases (Zou et al., 2009). Furthermore, a given cyclic dipeptide prenyltransferase has its own preference for dipeptides. For example, BrePT from Aspergillus versicolor accepted cyclo-L-Trp-L-Pro and cyclo-L-Trp-D-Pro much...
better than other cyclic dipeptides such as cyclo-1-Trp-1-Trp (Yin et al., 2013). Its orthologue NotF from Aspergillus sp. MF 297-2 exclusively used cyclo-1-Trp-1-Pro as a substrate (Ding et al., 2010). It is therefore of significant importance to identify enzymes with same function, but with different substrate preferences.

The availability of genome sequences provides a convenient strategy for the identification of candidate genes of interest. Ten putative prenyltransferase genes of the DMATS superfamily have been identified in the genome sequence of Neosartorya fischeri NRRL 181 (Khaldi et al., 2010). Eight of these were confirmed biochemically (Chooi et al., 2012; Mundt et al., 2012; Steffan et al., 2009). During the course of our search for new prenyltransferases, we identified a ninth enzyme, encoded by NFIA_043650, as a cyclic dipeptide C2-prenyltransferase (CdpC2PT). Here, we report the results of gene cloning and expression, biochemical investigation of the purified protein and its possible role in the biosynthesis of fellutanines (Kozlovsky et al., 2000, 2001).

METHODS

Chemicals. Dimethylallyl diphosphate (DMAPP) was prepared according to the method described for geranyl diphosphate (Woodside et al., 1988).

Computer-assisted sequence analysis. For intron prediction and sequence analysis, FGENESH (http://linux1.softberry.com) and DNASTAR (v. 2.1; Hitachi Software Engineering) were used, respectively. Sequence identities were obtained by alignments of amino acid sequences using the program BLAST 2 SEQUENCES (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Bacterial strains, plasmids and culture conditions. pGEM-T Easy and pQE60 were purchased from Promega and Qiagen, respectively. Escherichia coli DH5α (Invitrogen) and M15 (pREP4) cells (Qiagen) were used for cloning and expression experiments. Cultivation of bacteria was carried out at 30 or 37 °C in liquid or on solid Luria–Bertani medium with 1.5 % (w/v) agar (Sambrook & Russell, 2001). Kanamycin (25 μg ml⁻¹) and carbenicillin (50 μg ml⁻¹) were used for selection of recombinant E. coli strains.

Cultivation of N. fischeri NRRL 181 for DNA and RNA isolation. N. fischeri NRRL 181 was kindly provided by the Agricultural Research Service Culture Collection of the United States Department of Agriculture. For DNA isolation, the fungus was cultivated in 300 ml Erlenmeyer flasks containing 100 ml liquid potato dextrose (240.0 g l⁻¹) plus sucrose (342.3 g l⁻¹) medium at 30 °C for 5 days in darkness. For isolation of RNA, the fungus was cultivated in same medium for 3 days.

DNA propagation in E. coli, DNA and RNA isolation from fungi, and cDNA synthesis. Standard procedures for DNA isolation and manipulation in E. coli were performed as described previously (Sambrook & Russell, 2001). For isolation of genomic DNA from N. fischeri NRRL 181, the 5-day-old mycelia were collected and washed with PBS 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 Yin et al. (2013). RNA was isolated from 3-day-old mycelia by using an E.Z.N.A. fungal RNA miniprep kit (Omega Bio-Tek) according to the manufacturer’s protocol. cDNA was synthesized by using the ProtoScript M-MuLV First Strain cDNA synthesis kit (NEB).

PCR amplification and gene cloning. PCR amplification was carried out on an iCycler from Bio-Rad. For gene expression, the intron sequence was deleted by a two-round fusion PCR as described previously (Yin et al., 2009b). Two primers KM_043650-1-fw (5’-TACCATGCTATACACTCTGATGGACTGCC-3’) and KM_043650-3-rv (5’-TACGAATTCTACTGATGGACTGCC-3’) were used for amplification of the first exon and KM_043650-2-fw (5’-TGGTGATGCTACATGACCTCTTCAATCCGTAACCCA-3’) and KM_043650-4-rv (5’-AAAGATCTCAAGAACGTTTCGACAGAGC-3’) for the second exon. Bold letters in KM_043650-1-fw and KM_043650-4-rv represent mutations inserted in comparison to the original genome sequence in order to obtain the underlined restriction sites Ncol and BglII, respectively for cloning in pQE60. The underlined letters in KM_043650-2-fw and KM_043650-3-rv indicate sequences at the 5’-end of exon 2 and the 3’-end of exon 1, respectively, for fusion of the two exons.

A 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. The resulting PCR fragment of 1347 bp was cloned into pGEM-T Easy resulting in the plasmid pKM09, which was sequenced by MWG Biotech AG to confirm the sequence. pKM09 was digested with the restriction enzymes Ncol and BglII to create the expression construct pKM16. The resulting Ncol-BglII fragment of 1338 bp was isolated from agarose gel and subsequently ligated into pQE60, which had been digested with Ncol and BglII previously. To confirm the coding sequence, the program described above was used for PCR amplification with the obtained cDNA as template and KM_043650-1-fw and KM_043650-4-rv as primers. The PCR product was cloned into pGEM-T Easy vector to give pKM26, which was sequenced subsequently.

Overproduction and purification of BrePT-His₅ and CdpC2PT-His₉. Overproduction and purification of His₉-BrePT was carried out as described previously (Yin et al., 2013). cdpC2PT in pKM16 was expressed in E. coli M15 pREP4 after cultivation in a 2000 ml flask with 1000 ml liquid Luria–Bertani medium supplemented with kanamycin (25 μg ml⁻¹) and carbenicillin (50 μg ml⁻¹) and grown at 37 °C to an absorption at 600 nm of 0.6. Gene expression was induced by addition of IPTG to a final concentration of 0.2 mM at 30 °C for 6 h. Protein extraction and purification were carried out as described previously (Mundt et al., 2012).

Protein analysis and determination of molecular mass of active CdpC2PT-His₉. CdpC2PT was analysed on SDS-PAGE according to the method of Laemmli (1970) and stained with Coomassie Brilliant Blue G-250. The molecular mass of the recombinant CdpC2PT-His₉ was determined on a HiLoad 16/60 Superdex 200 column (GE Healthcare) 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 (2000 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa) and RNase A (13.7 kDa) (GE Healthcare).

Assays for CdpC2PT activity. For determination of CdpC2PT activity, reaction mixtures (100 μl) containing 50 mM Tris/HCl (pH 7.5), 5 mM CaCl₂, 1 mM cyclic dipeptide, 1 mM DMAPP and 4.0 μM of purified recombinant CdpC2PT were incubated at 37 °C for 16 h. Reactions were terminated by addition of 100 μl methanol. After removal of proteins by centrifugation at 17 000 g for 20 min, enzyme products were analysed by HPLC using the method described below. Duplicate values were determined routinely for quantitative measurement of enzyme activity. The assays for
determination of the kinetic parameters of (S)-benzodiazepinedione (1a), cyclo-L-Trp-L-Trp (2a), cyclo-L-1-tert-prenyl-L-Trp-L-Trp (2b), cyclo-L-Trp-L-Leu (3a), cyclo-L-Trp-L-Phe (4a) and of cyclo-L-Trp-L-Ala (5a), contained 5 mM DMAPP, 2.0 μM CdpC2PT and aromatic substrates at final concentrations of up to 5.0 mM. For determination of the kinetic parameters of DMAPP, 2.0 μM CdpC2PT, 1 mM (S)-benzodiazepinedione, 5 mM CaCl2 and DMAPP at final concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 mM were used. Incubation was carried out at 37 °C for 60 min.

Assays for isolation of enzyme products for structural elucidation (10 ml) contained 50 mM Tris/HCl (pH 7.5), 1 mM DMAPP, 1 mM aromatic substrate, 4.0 μM of CdpC2PT, 5 mM CaCl2 and were incubated at 37 °C for 16 h.

**HPLC analysis and product isolation.** Incubation mixtures of CdpC2PT were analysed using HPLC with an Agilent series 1200 and a Multispher 120 RP-18 column (250 × 4 mm, 5 μm) and a 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 50–100 % (v/v) solvent B in A over 25 min was used. The column was then washed with 100 % solvent B for 5 min and equilibrated with 50 % solvent A for 5 min.

The enzyme products were extracted with ethyl acetate and concentrated on a rotation evaporator at 30 °C to dryness. The residues were dissolved in methanol and purified using HPLC with a Multispher 120 RP-18 column (250 × 10 mm, 5 μm) with a flow rate of 2.5 ml min⁻¹. A linear gradient of 50–100 % of solvent B in A 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. All spectra were processed using MestReNova 5.2.2 and chemical shifts were referenced to those of the solvent signals. The isolated products were also analysed with mass spectrometry on a Q-Trap Quantum (Applied Biosystems) using a high-resolution electron impact (HR-EI) ionization mode. HR-EI-MS data are given in Table S1 (available in Microbiology Online).

**RESULTS**

**Prenyltransferase genes in the genome sequence of N. fischeri**

Eight of the ten putative prenyltransferase genes of the DMATS superfamily from N. fischeri were characterized previously using recombinant proteins from N. fischeri or their orthologues from other fungi. They include three C3-prenyltransferase genes NFIA_043650, NFIA_094090 and NFIA_074280. NFIA_055300 belongs to the acetylaszone-1-enin gene cluster and its product AnaPT was shown to be responsible for the C3α-prenylation of (R)-benzodiazepinedione (Yin et al., 2009b). CdpNPT and CdpC3PT, encoded by NFIA_094090 and NFIA_074280, respectively, catalyse C3β-prenylation of tryptophan-containing cyclic dipeptides (Schuller et al., 2012; Yin et al., 2007, 2010). Three genes, NFIA_093720 coding for brevianamide F regular C2-prenyltransferase (FtmPT1), NFIA_093760 for 12,13-dihydroxyfumitremorgin C prenyltransferase (FtmPT2) and NFIA_093400 for verruculogen prenyltransferase (FtmPT3), are members of the fumitremorgin/verruculogen gene clusters and are involved in the biosynthesis of fumitremorgin A (Mundt et al., 2012). 7-DMATS encoded by NFIA_064390 and NscD by NFIA_112230 catalyse a C7-prenylation of l-tryptophan and a C-prenylation of a tetracyclic intermediate in the biosynthesis of neosartorlicin, respectively (Chooi et al., 2013; Kremer et al., 2007). Two putative genes NFIA_043650 and NFIA_062330 remained with unknown function prior to the present study.

NFIA_043650 was proposed to be located on the genomic sequence of N. fischeri NRRL 181 between bp 30 953 and 32 173 under the accession number D5027684 (AACE03000020.1). According to the prediction given in GenBank (http://www.ncbi.nlm.nih.gov), NFIA_043650 comprises exclusively one exon of 1221 bp and the deduced polypeptide EAW25546 would have a length of 406 amino acids. By using the program FGENSEH (http://linux1.softberry.com) and by alignments with known prenyltransferases, the genomic sequence of NFIA_043650 was calculated in this study to be 170 bp larger than the predicted sequence given in GenBank, i.e. from bp 30 953 to bp 32 343. The revised sequence consists of two exons with a length of 1220 and 118 bp, respectively, interrupted by one intron of 53 bp. The deduced polypeptide comprises 445 amino acids with a calculated molecular mass of 50.5 kDa. This prediction was confirmed by PCR amplification and sequencing of a cDNA sample (see below). To distinguish the different lengths of the coding sequence, we named the polypeptide encoded by the corrected coding region of NFIA_043650 cyclic dipeptide C2-prenyltransferase. A BLAST search revealed significant sequence similarities between CdpC2PT and other known prenyltransferases of the DMATS superfamily from various fungi. For example, CdpC2PT shares an amino acid sequence identity of 40 % with the reverse C2-prenyltransferase BrePT from Aspergillus versicolor NRRL 573 (Yin et al., 2013), 42 % with NotF from Aspergillus sp. MF 297-2 (Ding et al., 2010) and 27 % with FtmPT1 from Aspergillus fumigatus (Grundmann & Li, 2005). Sequence identities of 33 % and 31 %, respectively, were found between CdpC2PT and the two C3-prenyltransferases AnaPT and CdpNPT.
from *N. fischeri* (Schuller et al., 2012; Yin et al., 2007, 2009b). Based on the relatively high sequence similarities of CdpC2PT with BrePT, NotF and other cyclic dipeptide prenyltransferases, we speculated that CdpC2PT could be responsible for a transfer reaction of a prenyl moiety to tryptophan-containing cyclic dipeptides.

**Cloning of cdpC2PT, protein overproduction and purification**

The coding region of *cdpC2PT* on two exons of 1220 and 118 bp was confirmed by sequencing of a PCR product in pGEM-T Easy vector obtained from cDNA of *N. fischeri* NRRL 181. For gene expression, the coding region was obtained from cDNA of *N. fischeri* NRRL 181 by a two-round fusion PCR and cloned into the expression vector pQE60 to create the expression construct pKM16. *E. coli* M15 (pREP4) cells harbouring the expression construct were induced at 37 °C by addition of IPTG to a final concentration of 0.2 mM. Induction of gene expression was clearly observed after cultivation in liquid Luria–Bertani medium at 30 °C for 6 h. A major protein band on SDS-PAGE with migration above the 45 kDa size marker was observed for the purified protein with Ni-NTA agarose (Fig. 1), corresponding well to the calculated mass of 51.3 kDa for CdpC2PT-His6. Protein yield was calculated to be 4 mg of purified protein per litre of culture. The molecular mass of the native recombinant CdpC2PT-His6 was determined by size-exclusion chromatography as 101 kDa, indicating that CdpC2PT presumably acts as a dimer.

**Enzyme activity and substrate specificity of CdpC2PT**

Based on its relatively high sequence similarities to cyclic dipeptide prenyltransferases, especially to BrePT and NotF (Ding et al., 2010; Yin et al., 2013), CdpC2PT was incubated with 17 cyclic tryptophan-containing dipeptides (1a–17a; Fig. 2, Table 1) in the presence of DMAPP. Enzyme assays with CdpC2PT inactivated by boiling the enzyme for 20 min were used as negative controls and incubation mixtures with BrePT as positive controls. HPLC analysis revealed product formation in all reaction mixtures containing active CdpC2PT and BrePT. With the exception of cyclo-L-Trp-L-Trp (2a), one product peak each was detected in the enzyme assays. Interestingly, for a given substrate, the product peak in the CdpC2PT incubation mixture showed the same retention time as that of BrePT, indicating the presence of the same product in these two assays. In the enzyme assay of cyclo-L-Trp-L-Trp (2a) with CdpC2PT, two product peaks were detected, suggesting both a mono- and diprenylation of the substrate. However, the enzyme assays of CdpC2PT differed clearly from those of BrePT in terms of a preference for aromatic substrates (Fig. 2). As reported previously (Yin et al., 2013), BrePT accepted its putative natural substrate cyclo-L-Trp-L-Pro (9a) and a stereo-isomer cyclo-L-Trp-D-Pro (10a) as the best substrates and conversion yields of 78 and 72 % were observed under the conditions used in this study. By contrast, 9a and 10a were poor substrates for CdpC2PT with conversion yields of 8 and 9 %, respectively (Table 1). (S)-benzodiazepinedione (1a) and cyclo-L-Trp-L-Trp (2a) were instead the best substrates for CdpC2PT with conversion yields of 85 and 48 %, respectively. These two compounds were accepted by BrePT with only very low conversion yields of 8 % in both cases. Cyclo-L-Trp-L-Leu (3a) was accepted well by CdpC2PT and BrePT with conversion yields of 37 and 46 %, respectively. A number of tryptophan-containing dipeptides showed low conversions by both enzymes. (R)-benzodiazepinedione (17a), the enantiomer of 1a and natural substrate of AnaPT (Yin et al., 2009b), was a very poor substrate for both CdpC2PT and BrePT (Fig. 2, Table 1). No product formation was observed for the amino acids L-tryptophan and L-tyrosine or cyclic dipeptides lacking a tryptophanyl moiety, e.g. cyclo-L-Tyr-L-Pro (18a) in the presence of DMAPP (Table 1). Product formation was detected for enzyme assays with neither geranyl nor farnesyl diphosphates.

**Identification of the enzymic products**

The same retention times of the enzyme products with CdpC2PT and BrePT for all accepted substrates indicated that these enzymes catalysed the same reaction. We isolated six enzyme products (1b–5b and 2c) from incubation mixtures of selected substrates (1a–5a) using HPLC (Fig. 3) in preparative scales and determined their structures by HR-EI-MS and NMR analyses. To minimize the conversion of 2b to 2c, the reaction mixture of 2a was only incubated for 2 h. HR-EI-MS proved that the molecular masses of the isolated products 1b–5b were 68 Da larger than those of the respective substrates, indicating a monoprenylation in their structures. 2c contained an ion at *m/z* 508.2820 [M+], proving the addition of a prenyl moiety compared to 2b. Comparing the 1H NMR data of the enzyme products (Table 2; for spectra see supplementary material, available in Microbiology Online) with those of the respective substrates (data not shown) revealed clearly the presence of signals for reverse prenyl moieties at δ 6.11–6.21 (d, H-1), 5.06–5.21 (d, H-1), 5.09–5.21 (d, H-1), 1.51–1.58 (s, 3H-4') and 1.51–1.59 p.p.m. (s, 3H-5'). Inspection of the 1H NMR
spectra of the enzyme products also revealed the disappearance of the singlets for H-2 of the substrates. Signals for other aromatic protons did not change significantly. Therefore, the enzyme products 1b–5b carry a reverse prenyl moiety each at C-2 of the indole ring and CdpC2PT catalysed a reverse C2-prenylation of cyclic dipeptides. 2c is a symmetrically reversely diprenylated derivative of cyclo-L-Trp-L-Trp (Fig. 3). The 1H NMR data of 1b–5b and 2c corresponded well to those reported previously (Kozlovsky et al., 2000, 2001; Yin et al., 2009a, 2013).

### Biochemical properties and kinetic parameters of CdpC2PT

The reactions of most members of the DMATS superfamily are independent of the presence of metal ions, although addition of Ca2+ or Mg2+ increases reaction velocities (Steffan et al., 2009). To determine the ion dependency of CdpC2PT, incubations with different metal ions such as Ca2+, Mg2+, Mn2+, Co2+ or Ni2+ at final concentrations of 5 mM were carried out in the presence of (S)-benzodiazepinedione (1a) and DMAPP. Incubation mixtures with the chelating agent EDTA or without additives were used as controls. Our results (Fig. S1) showed that addition of Ca2+ and Mg2+ slightly increased the enzyme activity. Enzyme activities with Ca2+ and Mg2+ were found to be 156 and 137 % of levels for the incubation mixture without additives. The incubation mixture with EDTA showed a relative activity of 93 %. These results indicated that the enzyme reaction of CdpC2PT was independent of the presence of divalent metal ions but could be enhanced by Ca2+ or Mg2+, in agreement with previous results (Steffan et al., 2009). Incubations at different pH values and temperatures proved that the CdpC2PT reaction had a pH optimum around 8.0 and a temperature optimum at 37 °C (Figs S2 and S3).

To investigate conditions for the determination of kinetic parameters, dependency of product formation on incubation time was tested with 2.0 μM CdpC2PT in the presence of 1 mM (S)-benzodiazepinedione (1a) and 1 mM DMAPP. Linear dependency of up to 120 min was observed in this experiment (Fig. S4). To determine the behaviour of CdpC2PT towards DMAPP, five selected cyclic dipeptides (1a–5a) and the monoprenylated cyclo-L-Trp-L-Trp (2a), kinetic parameters including Michaelis–Menten constants (Km) and turnover numbers (kat) were calculated from Eadie–Hofstee, Hanes-Woolf and Lineweaver–Burk plots (Table 3, Figs S5–S10). The reactions catalysed by CdpC2PT apparently followed Michaelis–Menten kinetics. CdpC2PT displayed a very high affinity to its prenyl donor DMAPP with a KM value of 25.1 μM. Among the tested aromatic substrates, CdpC2PT showed the highest affinity to (S)benzodiazepinedione (1a) and cyclo-L-Trp-L-Trp (2a) with KM values of 84.1 and 165.2 μM, respectively. The reaction velocities (Vmax) for 1a and 2a were determined to be 0.74 and 0.35 μmol min−1 mg−1, i.e. kat at 0.63 and 0.30 s−1. The monoprenylated product 2b had a higher KM value of 250.6 μM than its substrate 2a, but still significantly lower than those of 3a–5a where KM exceeded 500 μM (Table 3). The turnover numbers and catalytic efficiencies of 3a–5a were much lower than those of 1a and 2a (Table 3).

### DISCUSSION

We cloned the coding sequence of the putative prenyltransferase gene NFIA_043650 from the fungus N. fischeri NRRL 181 into the expression vector pQE60 and purified the soluble recombinant protein CdpC2PT after overproduction in E. coli. Biochemical investigations revealed the acceptance of all tested tryptophan-containing cyclic dipeptides with (S)-benzodiazepinedione (1a) and cyclo-L-Trp-L-Trp (1b) as the best substrates. Structural elucidation of six enzyme products proved that CdpC2PT catalysed a reverse C2-prenylation on the indole ring of the cyclic dipeptides.

A C2-prenylated tryptophan-containing dipeptide and/or related structures derived thereof have not been reported previously for N. fischeri. Intensive searches for such compounds by cultivation of the genome reference strain N. fischeri NRRL 181 in different media under various conditions and isolation of accumulated products remained unsuccessful (B. Wollinsky & S.-M. Li, unpublished results). It seems that NFIA_043650 or related genes were not

### Table 1. Relative activities of enzyme assays with CdpC2PT and BrePT

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conversion (%)</th>
</tr>
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<tbody>
<tr>
<td>Cyclo-L-Trp-L-Trp (2a)</td>
<td>48</td>
</tr>
<tr>
<td>Cyclo-L-Trp-L-Leu (3a)</td>
<td>37</td>
</tr>
<tr>
<td>Cyclo-L-Trp-L-Phe (4a)</td>
<td>17</td>
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<tr>
<td>Cyclo-L-Trp-L-Ala (5a)</td>
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<tr>
<td>Cyclo-L-Trp-L- Ala (6a)</td>
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<tr>
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<tr>
<td>Cyclo-O-Trp-L-Ala (8a)</td>
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<tr>
<td>Cyclo-O-Trp-L-Pro (9a)</td>
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<td>Cyclo-O-Trp-L-Pro (11a)</td>
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<tr>
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<td>Cyclo-O-Trp-L-Tyr (16a)</td>
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</tr>
<tr>
<td>(S)-Benzodiazepinedione (17a)</td>
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</tr>
<tr>
<td>Cyclo-O-Trp-L-Pro (18a)</td>
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</tr>
<tr>
<td>L-Tryptophan</td>
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<tr>
<td>L-Tyrosine</td>
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See legend to Fig. 2 for conditions. –, Product not detected.
Fig. 3. HPLC analysis of incubation mixtures with CdpC2PT and selected substrates (1a–5a) for structure elucidation. Product peaks are identified as 1b–5b, and 2c). Incubation time is also given in parentheses. Detection was carried out with a diode array detector and illustrated for absorption at 277 nm.
**Table 2.** $^1$H NMR data of the enzymic products of CdpC2PT

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Cyclo-1-2-tert-prenyl-Trp (2b, CDCl$_3$)</th>
<th>Cyclo-bis-(1-2-tert-prenyl-Trp) (2c, CDCl$_3$)</th>
<th>Cyclo-1-2-tert-prenyl-Trp-Leu (3b, CD$_3$OD)</th>
<th>Cyclo-1-2-tert-prenyl-Trp-Phe (4b, CD$_3$OD)</th>
<th>Cyclo-1-2-tert-prenyl-Trp-Ala (5b, CD$_3$OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos.</td>
<td>$\delta$, multi., $J$</td>
<td>$\delta$, multi., $J$</td>
<td>$\delta$, multi., $J$</td>
<td>$\delta$, multi., $J$</td>
<td>$\delta$, multi., $J$</td>
</tr>
<tr>
<td>NH-1</td>
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<td>8.01, br s</td>
<td>8.08, br s</td>
<td>–</td>
<td>–</td>
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<td>3.51, ddd, 14.7, 8.5</td>
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<td>10b</td>
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<td>3.01, dd, 14.4, 9.4</td>
<td>3.26, dd, 14.6, 11.8</td>
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<td>22</td>
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<td>7.66, d, 7.9</td>
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<td>1'</td>
<td>5.21, d, 17.5</td>
<td>5.20, d, 10.6</td>
<td>5.21, d, 17.5</td>
<td>5.16, ddd, 17.5, 1.2</td>
<td>5.06, d, 10.4</td>
</tr>
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<td>2'</td>
<td>5.19, d, 10.6</td>
<td>5.16, d, 17.3</td>
<td>5.21, d, 10.4</td>
<td>5.11, ddd, 10.6, 1.2</td>
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<td>4'</td>
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<td>6.11, dd, 17.3, 10.6</td>
<td>6.21, dd, 17.6, 10.4</td>
<td>6.21, dd, 17.5, 10.6</td>
<td>6.21, dd, 17.5, 10.6</td>
</tr>
<tr>
<td>5'</td>
<td>1.55, s</td>
<td>1.52, s</td>
<td>1.58, s</td>
<td>1.56, s</td>
<td>1.51, s</td>
</tr>
</tbody>
</table>

Chemical shifts ($\delta$) are given in p.p.m. and coupling constants ($J$) in Hz. *Signals overlapping with those of solvent.
expressed or expressed only at very low levels under the tested conditions, as observed for many genes in the biosynthesis of secondary metabolites (Brakhage, 2013).

A number of reverse C2-prenylated cyclic dipeptides and derivatives are known in fungi, e.g. stephacidin, notoamides and brevianamides derived from L-tryptophan and L-proline, echinulins from L-tryptophan and L-alanine, as well as fellutanines from two tryptophan molecules (Ding et al., 2010; Li, 2010, 2011; Wang et al., 2007; Williams et al., 2000). A C2-prenylated (S)-benzodiazepinedione (1b) or derivatives have not been reported previously. Its enantiomer (R)-benzodiazepinedione serves as a substrate for the C3-prenyltransferase AnaPT in the biosynthesis of acetylaszonalenin, which was isolated from different fungal strains including N. fischeri NRRL 181 (Yin et al., 2009b).

In the biosynthesis of stephacidin, notoamides and brevianamides, cyclo-L-Trp-L-Pro (brevianamide F, 9a) is reverse prenylated at the C-2 position of the indole ring (Ding et al., 2010; Li, 2011). A gene cluster for the biosynthesis of these compounds and two brevianamide F prenyltransferases (NotF and BrePT) has been reported recently (Ding et al., 2010; Yin et al., 2013). As shown in Table 1 and Fig. 2, brevianamide F was a very poor substrate for CdpC2PT, which thus is unlikely to be responsible for its prenylation in nature.

Echinulin and its analogues build a large group of fungal metabolites derived from cyclo-L-Trp-L-Ala (5a) and are mono-, di- or triprenylated at different positions of the indole ring. However, one reverse prenyl moiety exists almost at C-2 of the indole ring and therefore a reverse C2-prenylation of cyclo-L-Trp-L-Ala (5a) is necessary.

### Table 3. Kinetic parameters of CdpC2PT with selected substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(K_M) (µM)</th>
<th>(V_{max}) (µmol min(^{-1}) mg(^{-1}))</th>
<th>(k_{cat}) (s(^{-1}))</th>
<th>(k_{cat}/K_M) (s(^{-1}) M(^{-1}))</th>
<th>(k_{cat}/K_M) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMAPP</td>
<td>25.1</td>
<td>0.25</td>
<td>0.22</td>
<td>8765</td>
<td>117.0</td>
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<tr>
<td>(S)-Benzodiazepinedione (1a)</td>
<td>84.1</td>
<td>0.74</td>
<td>0.63</td>
<td>7491</td>
<td>100.0</td>
</tr>
<tr>
<td>Cyclo-L-Trp-L-Trp (2a)</td>
<td>165.2</td>
<td>0.35</td>
<td>0.30</td>
<td>1815</td>
<td>24.2</td>
</tr>
<tr>
<td>Cyclo-L-2-tert-prenyl-Trp-L-Trp (2b)</td>
<td>250.6</td>
<td>0.12</td>
<td>0.10</td>
<td>399</td>
<td>5.3</td>
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<tr>
<td>Cyclo-L-Trp-L-Leu (3a)</td>
<td>510.3</td>
<td>0.05</td>
<td>0.05</td>
<td>98</td>
<td>1.3</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Phe (4a)</td>
<td>565.0</td>
<td>0.02</td>
<td>0.02</td>
<td>35</td>
<td>0.5</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Ala (5a)</td>
<td>833.7</td>
<td>0.03</td>
<td>0.03</td>
<td>36</td>
<td>0.5</td>
</tr>
</tbody>
</table>

(a) Structure of fellutanine D. (b) A putative gene cluster in Neosartorya fischeri NRRL 181. The prenyltransferase CdpC2PT reported in this study is encoded by NFIA_043650. NFIA_043660 codes for a putative amine oxidase and NFIA_043670 for a putative bimodular non-ribosomal peptide synthetase.
(Harrison & Quinn, 1983; Li, 2010; Wang et al., 2007; Williams et al., 2000). CdpC2PT accepted 5a with a $K_m$ value of 833.7 μM and a $k_{cat}$ of 0.03 s$^{-1}$ (Table 3). It is therefore not plausible to assign this enzyme to the prenylation of 5a in the biosynthesis of echinulins in nature.

In addition to (S)-benzodiadepinedione (1a), CdpC2PT showed excellent affinity to cyclo-L-Trp-L-Trp (2a) with a $K_m$ value of 165.2 μM and $k_{cat}$ of 0.30 s$^{-1}$ (Table 3). The kinetic data of CdpC2PT towards the monoprenylated product of cyclo-L-Trp-L-Trp (2b) are also in the normal range for many secondary metabolite enzymes. This could justify its role in the biosynthesis of fellutanines, which have been isolated from the fungus Penicillium fellutanum (Kozlovsky et al., 2000). For the biosynthesis of the end product fellutain D with its revised stereochemistry (Kozlovsky et al., 2001) as shown in Fig. 4a, at least three enzymes are necessary; an enzyme for the formation of the cyclic dipeptide, enzyme(s) for the two prenylations at C-2 of both indole rings and enzyme(s) for the fusion of the indole with the diketopiperazine rings. CdpC2PT could be responsible for the two prenylation steps. Formation of the diketopiperazine ring could be catalysed by a non-ribosomal peptide synthetase (Ames & Walsh, 2010; Maiya et al., 2006; Yin et al., 2009b). Inspection of the genome sequence of N. fischeri revealed the presence of a bimodular non-ribosomal peptide synthetase gene NFIA_043670 close to NFIA_043650 (Fig. 4, Table 4). An in silico prediction approach (Prieto et al., 2012; Röttig et al., 2011) suggested a possible specificity of this non-ribosomal peptide synthetase for proline and tyrosine, which differs clearly from the observed acceptance of tryptophan-containing cyclic dipeptides by CdpC2PT. Proline- and tyrosine-containing dipeptides were poor substrates for CdpC2PT. This discrepancy could be explained by the fact that the prediction-based data are mainly obtained from bacteria. One putative gene NFIA_043660 with sequence homology to amino oxidases is located between these two genes and could be responsible for the fusion of the indole and diketopiperazine ring systems. It can therefore be speculated that these three genes belong to a gene cluster for the biosynthesis of fellutanines. Experimental proof for this hypothesis is under investigation.

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biosynthetic gene cluster.

fungus

glucose moiety of fusicoccin A, a diterpene glucoside produced by the

synthesis.

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