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PAPER

Switching a regular tryptophan *C4*-prenyltransferase to a reverse tryptophan-containing cyclic dipeptide *C3*-prenyltransferase by sequential site-directed mutagenesis

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FgaPT2 from *Aspergillus fumigatus* catalyzes a regular *C4*- and its mutant K174A a reverse *C3*-prenylation of L-tryptophan in the presence of dimethylallyl diphosphate. FgaPT2 also uses tryptophan-containing cyclic dipeptides for *C4*-prenylation, while FgaPT2_K174A showed almost no activity toward these substrates. In contrast, Arg244 mutants of FgaPT2 accept very well cyclic dipeptides for regular *C4*-prenylation. In this study, we demonstrate that FgaPT2_K174F, which catalyzes a regular *C3*-prenylation on tyrosine, can also use *cyclo*-L-Trp-L-Ala, *cyclo*-L-Trp-L-Trp, *cyclo*-L-Trp-Gly, *cyclo*-L-Trp-L-Phe, *cyclo*-L-Trp-L-Pro, and *cyclo*-L-Trp-L-Tyr as substrates, but only with low activity. Combinational mutation on Lys174 and Arg244 increases significantly the acceptance of these cyclic dipeptides. With the exception for *cyclo*-L-Trp-L-Trp, the tested dipeptides were much better accepted by FgaPT2_K174F_R244X (X=L, N, Q, Y) than FgaPT2, with an increase of two- to six-fold activity. In comparison to FgaPT2_K174F, even two- to ten-fold conversion yields were calculated for the double mutants. Isolation and structural elucidation of the enzyme products revealed stereospecific reverse *C3*-prenylation on the indole ring, resulting in the formation of *syn-cis* configured hexahydropyrroloindole derivatives. The results presented in this study highlight the convenience of site-directed mutagenesis for creating new biocatalysts.

Introduction

Cyclic dipeptides (CDPs) and derivatives are widely distributed in microorganisms and exhibit diverse biological and pharmacological activities.[1-3] In nature, these dipeptides are biosynthesized by bimodular non-ribosomal peptide synthetases or cyclodipeptide synthases.[4-6] Due to the various modification possibilities on the indole ring, tryptophan-containing CDPs are the richest precursors of natural products with pharmaceutical interest.[2,3] These dipeptides are frequently modified by methylation, hydroxylation, prenylation, dimerization, and further cyclization.[2,3,7,8] Chart 1 lists several examples of CDP derivatives such as the antitumor active rugulosuvine A from *Penicillium* species,[9] the sterol *O*-acyltransferase inhibitor amauromine from a *Nocardiopsis sp.*,[10,11] and the two mycotoxins roquefortine C and acetylaszonalenin from different fungal strains.[3,12,13] They are modification products of CDPs by tailoring enzymes including prenyltransferases (PTs).[3,14]

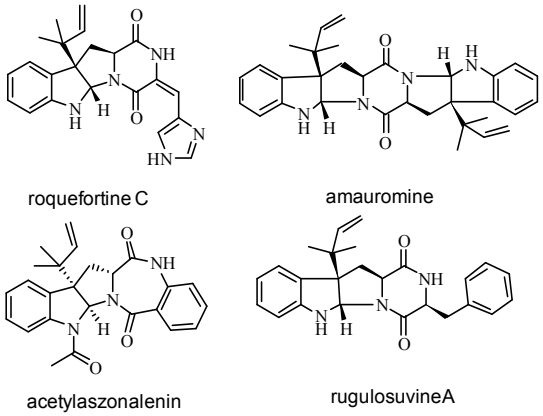

Chart 1 Examples of CDP derivatives

Prenyltransferases belong to one of the important modifying enzymes of CDPs and catalyze the transfer reactions of prenyl units (nxC_5, n=1, 2, 3, 4 *etc.*) from prenyl diphosphates to different aliphatic and aromatic acceptors.[14] Members of the most biochemically and structurally investigated PT group share meaningful sequence identities with the dimethylallyltryptophan synthase in the biosynthesis of ergot alkaloids and therefore termed DMATS enzymes.[8,14-16] Most PTs of the DMATS superfamily use dimethylallyl diphosphate (DMAPP) as donor and L-tryptophan or tryptophan-containing

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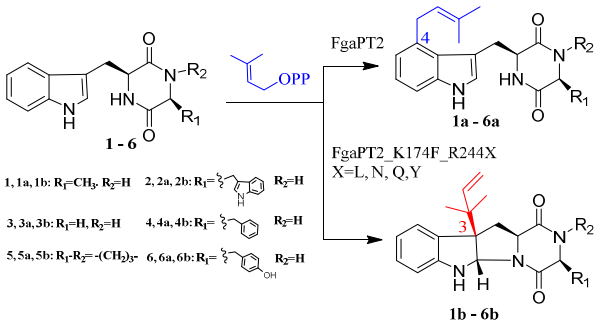
cyclic dipeptides as acceptors and are involved in the biosynthesis of diverse indole alkaloids.[8,14] The dimethylallyl moieties are attached to N-1, C-2, C-3, C-4, C-5, C-6, or C-7 of the indole ring in a regular or reverse manner. For the regular prenylation, DMAPP was proposed to form a dimethylallyl cation/pyrophosphate ion pair. The primary center of DMAPP is attacked by the electron-rich aromatic ring with a concerted displacement of pyrophosphate to form the arenium ion intermediate, which re-aromatizes by deprotonation to form the final product.[17-19] For the reverse prenylation, the nucleophilic attack takes place at the tertiary center instead of the primary center of the dimethylallyl carbocation. Reverse prenylation on the indole ring takes mainly place at C-2 and C-3 of the cyclic dipeptides, like those of *cyclo*-L-Trp-L-Pro catalyzed by NotF and BrePT,[20,21] *cyclo*-L-Trp-L-Ala by EchPT1,[22] *cyclo*-L-Trp-Ant by AnaPT,[12] and *cyclo*-L-Trp-L-Leu by CdpC3PT.[23] L-Tryptophan is usually regularly prenylated at C-4, C-5, C-6, and C-7, as demonstrated for FgaPT2,[24] 5-DMATS,[25] 6-DMATS,[26] and 7-DMATS, respectively.[27]

FgaPT2 from *Aspergillus fumigatus* was identified as a *C4*-DMATS, which initiates the biosynthesis of the ergot alkaloids fumigaclavines.[24] The crystal structure of FgaPT2 contains a rare ß/α fold (PT barrel) and serves as a basis for understanding the prenylation mechanism and for enzyme design.[18,19] Structure-based molecular modelling and site-directed mutagenesis revealed that Lys174 facilitates the entrance of substrates in the reactive center and Ile80, Leu81, Tyr191, and Arg244 anchor the polar side chain of L-tryptophan.[19]



Scheme 1 Prenylation of cyclic dipeptides by FgaPT2 and its mutants

Mutation of Lys174 to Phe switches FgaPT2 to a tyrosine *C3*-prenyltransferase FgaPT2_K174F.[28] Arg244 mutants enhance significantly the catalytic ability of FgaPT2 toward tryptophan-containing CDPs and keep the prenylation position at C-4.[29] Luk *et al*[30] demonstrated that FgaPT2_K174A can prenylate L-tryptophan at both C-3 and C-4 position and proposed the *C4*-prenylated derivative to be the result of Cope rearrangement of a *C3*-prenylated derivative. Acceptance of tryptophan-containing CDPs by FgaPT2_K174A has not been tested before, while FgaPT2 was also demonstrated to prenylate these compounds at C-4, *i.e.* the same position as for the natural substrate L-tryptophan (Scheme 1). All these results proved FgaPT2 as an excellent model for enzyme design by site-directed mutagenesis. In this study, we describe the creation of a reverse *C3*-prenyltransferase for tryptophan-containing CDPs by two step-mutations on Lys174 and Arg244.

Results and discussion

Acceptance of tryptophan-containing cyclic dipeptides by FgaPT2_K174F

To prove the acceptance toward tryptophan-containing CDPs, we constructed the plasmid pLZ1 for overproduction of FgaPT2_K174A (Table S1). One predominant protein band with the expected size was detected by SDS-PAGE analysis for the purified FgaPT2_K174A (Fig. S1), which was used for incubation with L-tryptophan and DMAPP. LC-MS analysis of the reaction mixture revealed the presence of two product peaks at 7.3 and 8.0 min. The peak at 8.0 min was identified as *C4*-prenylated derivative by comparison of its retention time, UV (λ_{max} 278nm), and MS data with those of the FgaPT2 product (Fig. S2). The peak at 7.3 min showed the same $[M+H]^+$ ion, but clearly different UV spectrum (λ_{max} at 240 and 294nm) as the peak at 8.0 min. These data would correspond to those of the *C3*-prenylated L-tryptophan reported for FgaPT2_K174A.[30] After proof of its functionality, the overproduced FgaPT2_K174A was incubated with six L-tryptophan-containing cyclic dipeptides, *i.e.* *cyclo*-L-Trp-L-Ala (**1**), *cyclo*-L-Trp-L-Trp (**2**), *cyclo*-L-Trp-Gly (**3**), *cyclo*-L-Trp-L-Phe (**4**), *cyclo*-L-Trp-L-Pro (**5**), and *cyclo*-L-Trp-L-Tyr (**6**) in the presence of DMAPP at 37°C for 3 h. HPLC analysis of the incubation mixtures indicated no product formation in all enzyme assays under the tested conditions (Fig. 1). LC-MS analysis with EIC detection for $[M+H]^+$ ion of monoprenylated products showed product peaks with very low intensity (Fig. S3).

In contrast, the cyclic dipeptides **1** – **6** were accepted by the wildtype FgaPT2 with product yields in the range from 6.6 to 29.5 % (Fig. 1). With the exception for **2**, one product peak each (**1a** or **3a** – **6a**) was observed. These products showed typical UV λ_{max} at 278 nm for indole system and have $[M+H]^+$ ions, which are 68 Daltons larger than the respective substrate (Fig. S3), indicating the presence of an indole chromophore and corresponding to the *C4*-prenylation of cyclic dipeptides by FgaPT2 (Scheme 1).[31] In the case of **2**, two products **2a** and **2a*** were detected, with $[M+H]^+$ ions, which are 68 and 136 Daltons larger than that of **2**, indicating a mono- and diprenylation, respectively (Fig. S3).

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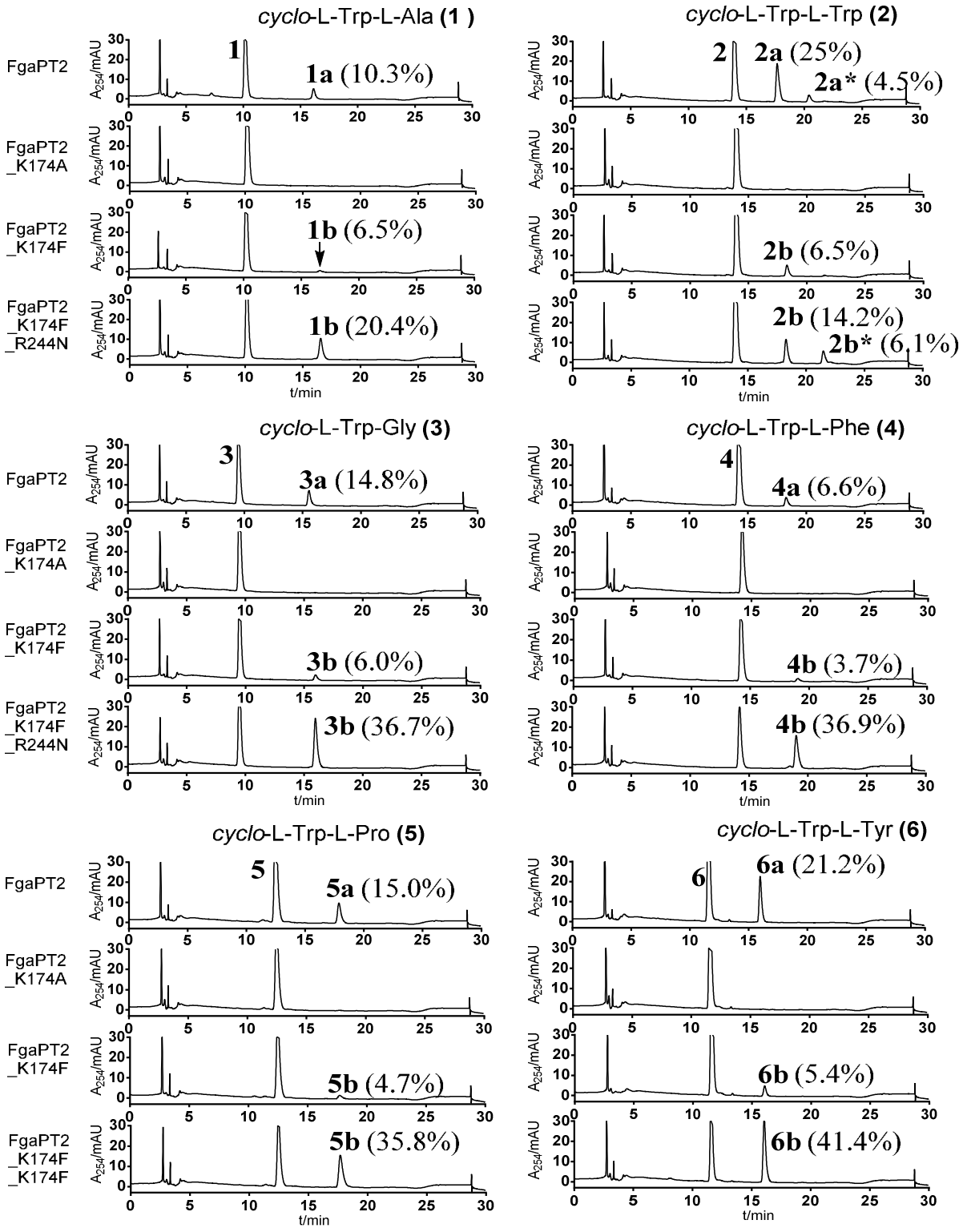

Fig. 1 HPLC analysis of incubation mixtures of FgaPT2 and its mutants with cyclic dipeptides.

In a previous study, we demonstrated that Lys174 also plays an important role for aromatic substrate selection. FgaPT2_K174F accepts L-tryptophan only with a 240-fold decreased activity compared to FgaPT2, while its catalytic ability for L-tyrosine

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increased 28-fold.[28] We proved therefore its behavior toward cyclic dipeptides **1** – **6** under the same conditions used for FgaPT2_K174A. As shown in Fig. 1, all six substrates were accepted by FgaPT2_K174F with product yield of 2.5 to 6.5 %. Detailed inspection of the HPLC chromatograms revealed the presence of one product each (**1b** - **6b**) with slightly delayed retention times and clear different UV spectra than **1a** – **6a**.

The UV absorption maxima of **1b** - **6b** at 240 and 294 nm indicated the disruption of the indole and the presence of a hexahydropyrroloindole system by *C3*-prenylation. LC-ESI-HRMS analysis of the incubation mixtures of FgaPT2_K174F with **1** – **6** confirmed the monoprenylation in **1b** - **6b** by detection of $[M+H]^+$ ions, which are 68 Daltons larger than those of the respective substrate (Fig. S3). We speculated that **1** – **6** were converted to *C3*-prenylated derivatives by FgaPT2_K174F. However, the conversion yields are too low to be used as convenient biocatalyst for CDP prenylation.[10,30] In the case of **2**, a diprenylated product was also detected by LC-MS analysis (Fig. S3).

Enhancing the conversion of tryptophan-containing cyclic dipeptides by combinational mutation on Lys174 and Arg244

We demonstrated previously that Arg244 mutants, especially R244N, R244Q, R244Y, and R244L, showed strongly increased activities toward tryptophan-containing cyclic dipeptides, while L-tryptophan was less accepted by these mutants than the wildtype FgaPT2.[29] The prenylation position was remained at C-4 of the indole ring. To increase the conversion yields of cyclic dipeptides for potential *C3*-prenylation, we created double mutants by site-directed mutagenesis of Lys174 to Phe and Arg244 to Leu, Asn, Gln, and Tyr, resulting in the four double mutants FgaPT2_K174F_R244L, FgaPT2_K174F_R244N, FgaPT2_K174F_R244Q, and FgaPT2_K174F_R244Y (Table S1). The overproduced proteins with yields of 1.2 –2.3 mg per liter culture were purified to apparent homogeneity (Fig. S1) and exploited to enzyme assays with **1** – **6** under the conditions used for FgaPT2_K174F.

HPLC analysis of the reaction mixtures delivered enthusiastic results (Figs. 1 and 2). i) Product formation was detected in all the enzyme assays (Fig. 1, for simplifying, only the result of the best mutant for a given substrate is illustrated). ii) All double mutants exhibited increased activities toward all the tested substrates (Fig. 2). Total product yields of 20.3 to 41.4 % were calculated for the best mutant with a given substrate, *i.e.* FgaPT2_K174F_R244N for **1** – **4** and FgaPT2_K174F_R244L for **5** and **6**. This means an activity increase of two to ten-fold, in comparison to those with FgaPT2_K174F. iii) The products have same retention times and UV spectra as those of the FgaPT2_K174F for a given substrate (**1b** – **6b**). iv) The corresponding products also have almost the same $[M+H]^+$ ions by LC-MS analysis (Fig. S3). In the case of **2**, the additional peak **2b*** with a $[M+H]^+$ ion for a diprenylated derivative was detected. All these data underline successful mutational combinations for acceptance of tryptophan-containing CDPs.

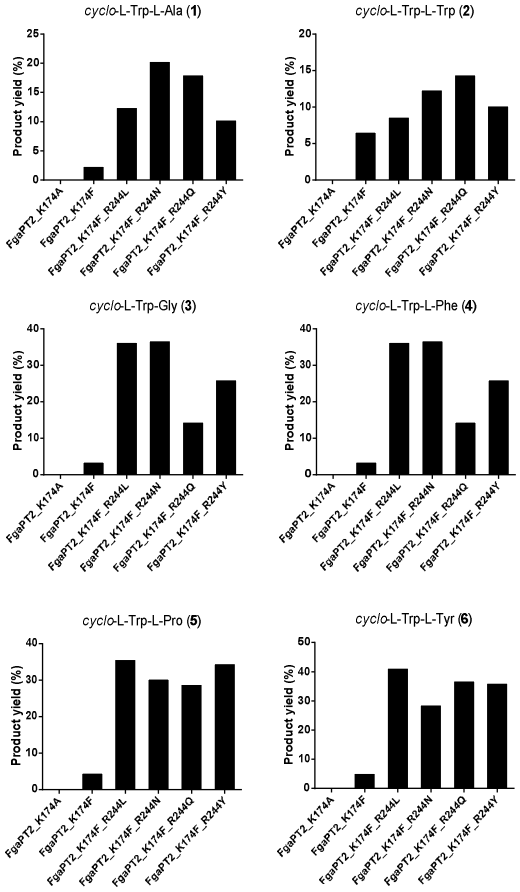

Fig. 2 Yields of **1b** - **6b** with Lys174 and Arg244 mutants. The components and incubation conditions are identical to those of Fig. 1.

Structure elucidation of the enzyme products with FgaPT2_K174F_R244X

To elucidate the structures, the enzyme products of **1** – **4** were isolated from incubation mixtures with FgaPT2_K174F_R244N and those of **5** and **6** with FgaPT2_K174F_R244L. HRMS data (Table 1) confirmed the monoprenylation in **1b** – **6b** and diprenylation in **2b***. Inspection of the ^{1}H NMR spectra of **1b** – **6b** (Figs. S4 - S9) revealed the presence of signals for a reverse prenyl moiety each at δ_h 4.97-5.13 (dd, 1H), 5.05-5.09 (dd, 1H), 5.76-5.98 (dd, 1H), 0.93-1.11 (s, 3H), and 0.79-1.01 (s, 3H) ppm (Table S2). The signals of H-2 at the original indole rings are significantly up-field shifted to 5.32 - 5.55 ppm, indicating the disruption of the indole and formation of a hexahydropyrroloindole system caused by a *C3*-prenylation.[10,23] The signals of H-11 appear as double doublets

Table 1 ESI-HRMS data of the enzyme products

Compound	Formula	$[M+H]^+$		Deviation (ppm)
		Measured	Calculated	
1b	$C_{19}H_{24}N_3O_2$	326.1858	326.1863	1.5
2b	$C_{27}H_{29}N_4O_2$	441.2292	441.2285	-1.6
2b*	$C_{32}H_{36}N_4O_2$	509.2927	509.2911	-3.1
3b	$C_{18}H_{22}N_3O_2$	312.1719	312.1707	-3.8
4b	$C_{25}H_{28}N_3O_2$	402.2187	402.2176	-2.7
5b	$C_{21}H_{26}N_3O_2$	352.2029	352.2020	-2.6
6b	$C_{25}H_{28}N_3O_3$	418.2138	418.2125	-3.1

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of doublets with coupling constants of approximate 11, 6.0, and 2.0 Hz, proving the *3ß*-prenylation of L-configured tryptophanyl moiety, *i.e. syn-cis* configuration (3R, 2S, 11S).[10] Literature search confirmed the structures **1b** – **6b** (Scheme 1) as reported previously.[10,23] They were obtained as enzyme products of the prenyltransferase CdpC3PT from *Neosartorya fischeri*.[10,23] In the case of **2b***, the structure cannot be confirmed by NMR analysis, due to the low quantity. We speculated here *C3*-prenylation at the both indole rings, as observed for other *C3*-prenyltransferases with *cyclo*-L-Trp-L-Trp.[14]

To get detailed insights into the reaction mechanism, we carried out molecular docking by using the program AutoDock Vina.[32] The models of FgaPT2_K174A, FgaPT2_K174F, and FgaPT2_K174F_R244L were created from FgaPT2 structure (PDB code: 3I4X) by using SWISS-MODEL. The tested cyclic dipeptides and DMAPP were docked in silico into the models of FagaPT2 and its mutants. Unfortunately, the observed enzyme activities and prenylation positions can be explained neither by the distances of C-1/C-3 of DMAPP to C-3/C-4 of the indole ring of the cyclic dipeptides, nor by $\pi - \pi$ or Van der Waals force interaction (data not shown). Therefore, crystal structures of the mutants are needed for understanding the observed results presented in this study, which should be explorated in the near future.

Determination of kinetic parameters

Kinetic parameters including Michaelis Menten constants (K_M) and turnover numbers (k_{cat}) were determined at pH 7.5 for the two best mutants in the presence of DMAPP, *i.e.* FgaPT2_K174F_R244N with **1** – **4** as well as FgaPT2_K174F_R244L with **5** and **6**. The reactions catalyzed by both mutants apparently followed the Michaelis Menten kinetics (Figs. S10 - S15). With the exception for **3**, K_M values in the range of 0.15 to 0.79 mM were determined. The high catalytic efficiencies of FgaPT2_K174F_R244N for **4** and FgaPT2_K174F_R244L for **5** and **6** confirmed their high conversions illustrated in Fig. 1.

Table 2 Kinetic parameters of FgaPT2_K174F_R244N and FgaPT2_K174F_R244L

S	FgaPT2_K174F_R244N			FgaPT2_K174F_R244L		
	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}M^{-1}$]	K_M [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M [$s^{-1}M^{-1}$]
1	0.36	0.012	33.3	-	-	-
2	0.79	0.13	164.6	-	-	-
3	3.10	0.19	61.3	-	-	-
4	0.15	0.03	200.0	-	-	-
5	-	-	-	0.41	0.15	365.9
6	-	-	-	0.62	0.25	403.2

S: substrate

Experimental

Chemicals

DMAPP was synthesized according to the method described for geranyl diphosphate reported previously.[33] Substrates used for the enzyme assays were purchased from Bachem (Bubendorf, Switzerland) or synthesized as described previously.[34]

Bacterial strains, plasmids, and culture conditions

E. coli BL21 (DE3) pLysS (Invitrogen, Karlsruhe, Germany) and SoluBL21 (Novagen, Darmstadt, Germany) were used for expression experiments. pIU18 was used as DNA template for creation of FgaPT2_K174A. pES26 containing the mutation for FgaPT2_K174F [28] was used as template for construction of the double mutants FgaPT2_K174F_R244L, FgaPT2_K174F_R244N, FgaPT2_K174F_R244Q, and FgaPT2_K174F_R244Y. *E. coli* cells harboring plasmids were grown in liquid Lysogeny Broth (LB) or Terrific Broth (TB) medium and on solid LB medium with 2 % (w/v) agar at 37 °C. Kanamycin (50 μg mL^{-1}) was used for selection of recombinant *E. coli* strains.

Site-directed mutagenesis, overproduction and purification of the recombinant proteins

Expand™ Long Template PCR system (Roche Diagnostic, Mannheim, Germany) was used for construction of plasmids listed in Table S1. The primers are given in Table S1. The obtained plasmids were sequenced by Seqlab Sequence Laboratories (Göttingen, Germany) to confirm the desired mutations in the respective constructs. FgaPT2 and its mutated derivatives were overproduced and purified as described previously.[28,35]

Enzyme assays with purified recombinant proteins

To test the enzyme activity, the reaction mixtures (50 μL) containing 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 0.5 mM cyclic dipeptide, 1 mM DMAPP, and 10 μg of the purified recombinant protein were incubated at 37 °C for 3 h. The reactions were terminated by addition of 50 µL methanol. After removal of the precipitated protein by centrifugation at 13,000 × *g* for 30 min, 20 μL supernatant was subjected to HPLC or LC-HRMS analysis.

Enzyme assays for determination of kinetic parameters (50 μL) contained 1 mM DMAPP, 5 – 10 µg FgaPT2_K174F_R244N or FgaPT2_K174F_R244L and the aromatic substrates at final concentrations from 0.025 to 5 mM. The incubations were carried out at 37 °C for 30 min. After addition of MeOH and centrifugation, the supernatants were analyzed on HPLC.

HPLC analysis of enzyme assays

Enzyme assays were analyzed on an Agilent HPLC series 1200 (Agilent Technologies) equipped with an Agilent Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm). A linear gradient from 10 to 90 % CH_3CN in H_2O in 20 min was used. The column was then washed with 100 % CH_3CN for 5 min and equilibrated with 10 % CH_3CN in H_2O for another 5 min. Detection was carried out with a photodiode array detector and absorptions at 254 nm were illustrated in this study.

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Isolation of the enzyme products

For isolation of the enzyme products, the assays (10 mL) contained 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, 1 mM cyclic dipeptide, 1 mM DMAPP, and 0.2 – 0.5 mg mL^{-1} recombinant protein. After incubation at 37 °C for 24 h, the reaction mixtures were extracted with equal volume of EtOAc for three times. The extracts were concentrated on a rotating vacuum evaporator at 30 °C and the residues are dissolved in 1 mL MeOH for isolation on HPLC. A semipreparative Multospher 120 RP-18 column (5 μm, 10 × 250 mm) was used for isolation of the enzyme products on the same HPLC system mentioned above with the same solvents at a flow rate of 2.5 mL/min. Separation was done by isocratic elution with 40 – 70% CH_3CN for 10-20 min. The fractions of interest were collected, combined, and concentrated to dryness on a rotating vacuum evaporator at 30 °C. The isolated enzyme products were then subjected to NMR and MS analyses.

LC-MS and MS analysis

Enzyme products were also analyzed on an Agilent HPLC 1260 series system equipped with a Bruker microTOF QIII mass spectrometer by using the Agilent Eclipse XDB C18 column (5 μm, 4.6 × 150 mm). Separation was performed at a flow rate of 0.5 mL/min with a 10 min linear gradient from 5 to 100 % CH_3CN in H_2O, both containing 0.1 % (v/v) formic acid. The column was then washed with 100 % CH_3CN for 5 min and equilibrated for 5 min. The parameters of the spectrometer were set as the following: electrospray positive ion mode for ionization, capillary voltage with 4.5 kV, collision energy with 8.0 eV.

NMR analysis

NMR spectra of the isolated enzyme products were recorded at room temperature on a JEOL ECA-500 (JEOL, Akishima, Tokyo, Japan). The samples were dissolved in DMSO-*d*6 or $CDCl_3$. All spectra were processed with MestReNov.6.1.0 (Mestrelab Research, Santiago de Compostella, Spain).

Conclusions

In summary, we demonstrated that FgaPT2_K174F catalyzes a reverse *C3*-prenylation of cyclic dipeptides, instead of *C4*-prenylation by the non-mutated enzyme, but only with low activity. Significant increase of the reaction velocity was achieved by additional mutation on Arg244. Our results provide an excellent example for protein engineering by site-directed mutagenesis on the basis of structural information.

Conflicts of interest

The authors declare no competing financial interest.

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