Diprenylated cyclodipeptide production by changing the prenylation sequence of the nature’s synthetic machinery

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Abstract
Ascomycetous fungi are often found in agricultural products and foods as contaminants. They produce hazardous mycotoxins for human and animals. On the other hand, the fungal metabolites including mycotoxins are important drug candidates and the enzymes involved in the biosynthesis of these compounds are valuable biocatalysts for production of designed compounds. One of the enzyme groups are members of the dimethylallyl tryptophan synthase superfamily, which mainly catalyze prenylations of tryptophan and tryptophan-containing cyclodipeptides (CDPs). Decoration of CDPs in the biosynthesis of multiple prenylated metabolites in nature is usually initiated by regiospecific C2-prenylation at the indole ring, followed by second and third ones as well as by other modifications. However, the strict substrate specificity can prohibit the further prenylation of unnatural C2-prenylated compounds. To overcome this, we firstly obtained C4-, C5-, C6-, and C7-prenylated cyclo-L-Trp-L-Pro. These products were then used as substrates for the promiscuous C2-prenyltransferase EchPT1, which normally uses the unprenylated CDPs as substrates. Four unnatural diprenylated cyclo-L-Trp-L-Pro including the unique unexpected N1,C6-diprenylated derivative with significant yields were obtained in this way. Our study provides an excellent example for increasing structural diversity by reprogramming the reaction orders of natural biosynthetic pathways. Furthermore, this is the first report that EchPT1 can also catalyze N1-prenylation at the indole ring.

Key points
• Prenyltransferases as biocatalysts for unnatural substrates.
• Chemoenzymatic synthesis of designed molecules.
• A cyclodipeptide prenyltransferase as prenylating enzyme of already prenylated products.

Keywords Cyclodipeptides · Multiple prenylation · Indole prenyltransferases · EchPT1 · Chemoenzymatic synthesis

Introduction
Microorganisms, especially ascomycetous fungi, are often found in contaminated agricultural products like food crops including corns, grain, fruits, and vegetables as well as poorly conserved foods. They produce diverse mycotoxins which are hazardous to human and animal health (Dey et al. 2022; Nan et al. 2022; Pallares et al. 2022). On the other hand, the microbial natural products including mycotoxins are important drug candidates (Newman 2021) and the enzymes involved in the biosynthesis of these compounds are valuable biocatalysts for structural modification and construction of new biosynthetic pathways (Yi et al. 2021). Among them, tailoring enzymes for modification of the backbone skeletons play key roles in increasing structural diversity and biological activities (Harken and Li 2021; Yang et al. 2022). Prenyltransferases belong to one of the important modification enzyme groups and catalyze transfer of nxC5 moieties onto different accepters (Winkelblech et al. 2015). Members of the dimethylallyl tryptophan synthase (DMATS) superfamily are most investigated prenyltransferases in the last years. They mainly catalyze prenylations of tryptophan and tryptophan-containing cyclodipeptides (CDPs) by using dimethylallyl diphosphate (DMAPP) as prenyl donor and are involved in the biosynthesis of a large number of prenylated indole alkaloids including mycotoxins (Winkelblech et al. 2015).
Prenylated indole alkaloids are widespread in bacteria, fungi, plants, and marine organisms (Klas et al. 2018; Li 2010; Yazaki et al. 2009) and exhibit clearly distinct biological activities from their nonprenylated precursors (Botta et al. 2005; Wollinsky et al. 2012). Prenylated tryptophan-containing cyclodipeptides and derivatives thereof represent an important category within the prenylated indole alkaloids. As exemplified in Fig. 1, the cytotoxic notoamides from Penicillium and Aspergillus species are derivatives of C2,C7-diprenylated and C6-hydroxylated brevianamide F (cyclo-L-Trp-L-Pro) (Klas et al. 2018). Fumitremorgins as di- and triprenylated brevianamide F products were identified as tremorgenic metabolites in Aspergillus fumigatus, Neosartorya fischeri, and other fungi (Li 2010; Mundt et al. 2012). Di- and triprenylated cyclo-L-Trp-L-Ala congeners also occur frequently in the fungal genera of Aspergillus and Eurotium with echinulin as the important representative (Chen et al. 2015; Du et al. 2017; Kamauchi et al. 2016; Li 2010; Nies and Li 2021; Wohlgemuth et al. 2017).

Biosynthetically, the skeletons of such CDPs in fungi are usually assembled by nonribosomal peptide synthases using tryptophan and a second amino acid as substrates. The CDP core is then decorated by different tailoring enzymes including prenyltransferases from the DMATS superfamily (Li 2010; Wohlgemuth et al. 2017; Xu et al. 2014). In nature, CDPs are mostly monoprenylated at positions C-2 and C-3, and occasionally at positions C-4 to C-7. In contrast, prenylation of the free amino acid tryptophan takes place more frequently at C-4 to C-7 of the benzene ring than other positions (Winkelblech et al. 2015). As shown in Fig. 1, the biosynthesis of multiple prenylated CDPs is usually initiated by the regiospecific C2-prenylation at the indole ring, followed directly by the second prenyltransferase as in the case of echinulins or after decoration with other enzymes, e.g., in the biosynthesis of notoamides and fumitremorgins (Klas et al. 2018; Li 2011; Wohlgemuth et al. 2017). Remarkably, the members of the DMATS superfamily show high substrate flexibility toward their aromatic substrates and accept not only structurally similar, but also distinct compounds as prenyl acceptors. For example, the brevianamide F C2-prenyltransferases FtmPT1 and BrePT as well as the cyclo-L-Trp-L-Ala C2-prenyltransferase EchPT1 (Fig. 1)
accept well other CDPs for C2-prenylation (Grundmann and Li 2005; Wohlgemuth et al. 2017; Wollinsky et al. 2012; Yin et al. 2013). EchPT2 from the echinulin biosynthetic pathway (Fig. 1) converts C2-prenylated cyclo-Trp-Ala and cyclo-Trp-Pro isomers to tri- and tetruprenylated derivatives (Wohlgemuth et al. 2018). Diprenylated products, e.g., tardioxopiperazines A and B, were only detected as minor products. Furthermore, the tryptophan C6-prenyltransferase from Streptomyces ambofaciens and 7-DMATS from Aspergillus fumigatus can also prenylate CDPs at positions C-6 and C-7, respectively (Kremer et al. 2007; Liu et al. 2020; Winkelblech and Li 2014). In addition, protein engineering of the tryptophan C4-prenyltransferase from Aspergillus fumigatus led to the mutant FgaPT2_R244L with increased acceptance for CDPs (Fan and Li 2016). As a proof of concept, we decided in this study to produce unnatural C2,C4-, C2,C5-, C2,C6-, and C2,C7-diprenylated cyclo-Trp-l-Pro by combination of different prenyltransferases.

**Materials and methods**

**Chemicals**

DMAPP was chemically prepared according to the method published previously (Woodside et al. 1988). cyclo-Trp-l-Pro was synthesized as described in literature (Caballero et al. 1998).

**Bacterial strains, plasmids, and culture conditions**

*Escherichia coli* strains M15 [pREP4] (Qiagen, Hilden, Germany) and BL21 (DE3) pLysS (Invitrogen, Karlsruhe, Germany) harboring the plasmids pVW90, pALF49, pPM37, pJW12, and pLW40 were used for overproduction of the recombinant proteins EchPT1, FgaPT2_R244L, FgaPT2_Y398F, 6-DMATSSa, and 7-DMATS, respectively (Fan and Li 2016; Kremer et al. 2007; Mai et al. 2016; Winkelblech and Li 2014; Wohlgemuth et al. 2017). Terrific broth (TB) medium supplemented with 50 µg/mL carbenicillin or 25 µg/mL kanamycin was used for cultivation of recombinant *E. coli* strains.

**Protein overproduction and purification as well as enzyme assays**

Culture conditions and purification of His6-EchPT1, His6-FgaPT2_R244L, His6-FgaPT2_Y398F, 6-DMATSSa-His6, and 7-DMATS-His6 were carried out by Ni–NTA affinity chromatography (Qiagen, Hilden) as described previously (Fan and Li 2016; Kremer et al. 2007; Mai et al. 2016; Winkelblech and Li 2014; Wohlgemuth et al. 2017). SDS-PAGE analysis revealed that all the five recombinant proteins were purified to near homogeneity (Fig. 2).

To determine the enzyme activity, standard assays (50 µL) contained Tris–HCl (50 mM, pH 7.5), aromatic substrate (1 mM), DMAPP (1 mM), glycerol (0.5–5%, v/v), DMSO (2.5%, v/v), and the purified protein (4 µg). CaCl2 at a final concentration of 5 mM was added to the reaction mixtures to enhance the reaction velocity (Li 2009; Sasaki et al. 2008). After incubation at 37 °C for 16 h, the reaction mixtures were terminated by addition of 50 µL methanol. The precipitated proteins were removed by centrifugation at 13,000 × g for 20 min and analyzed on liquid chromatography coupled with mass spectrometer (LCMS) as described below.

The linearity of the EchPT1 reactions toward monoprenylated derivatives was determined up to 240 min with 4 µg protein. The assays for determination of the kinetic parameters of EchPT1 toward monoprenylated CDPs 3, 4, and 5 contained 1 mM DMAPP, 4 µg EchPT1 and the aromatic substrate at final concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mM. The reaction mixtures containing 3, 4, and 5 were incubated at 37 °C for 30, 35, and 30 min, respectively. For kinetic parameters toward DMAPP of EchPT1 in the presence of the monoprenylated compound 3, the reaction mixture contained 4 µg EchPT1, 3 (1 mM), CaCl2 (5 mM), and DMAPP at final concentrations from 0.01 to 2.0 mM, which was incubated at 37 °C for 30 min. All the assays were performed as triplicates and subsequently terminated with methanol, and analyzed on high performance liquid chromatography (HPLC) as described below. The conversion yields were calculated...
by using the isolated products as standards or by ratio of the peak areas of product and substrate in HPLC chromatograms. The data were fitted to the Michaelis–Menten equation in Prism 4.0 (GraphPad Software).

**Preparation and isolation of the enzyme products for structure elucidation**

Enzyme assays for product isolation were scaled up to a volume of 12 mL, containing Tris–HCl (50 mM, pH 7.5), DMAPP (1.5 mM), the respective aromatic substrate (1 mM), and 1–5 mg purified recombinant proteins. The reaction mixtures were incubated at 37 °C for 16 h and extracted three times with two volumes of ethyl acetate each. The resulting organic phases were combined and concentrated on a rotating vacuum evaporator at 35 °C to dryness and dissolved in 1 ml methanol for isolation.

**LCMS and HPLC conditions for analysis and isolation of the enzyme products**

LCMS analysis was performed as described previously (Zhou and Li 2021). The substances were eluted at a flow rate of 0.25 mL/min with a linear gradient from 5 to 100% ACN in 10 min. Semi-preparative HPLC was performed with an Agilent Eclipse XDB-C18 (250×9.4 mm, 5 µm) column. Water (A) and acetonitrile (B) were used as solvents at flow rate of 2 mL/min. Compounds 2, 3, 4, 5, and 6 were isolated with 55% B, 10 with 60% B and 7, 8, and 9 with 65% B.

**Nuclear magnetic resonance analysis**

NMR spectra were recorded on a JEOL ECA-500 MHz spectrometer (JEOL, Tokyo, Japan) and processed with MestReNova 6.1.0 (Metrelab). Chemical shifts were referred to those of the solvent signals. The NMR data are provided in Tables S1–S4 and spectra in Figs. S1–S25.

**Results**

**Attempts to produce diprenylated derivatives by prenylation of already C2-prenylated cyclo-L-Trp-L-Pro**

To get C2,C4-, C2,C5-, C2,C6-, and C2,C7-diprenylated cyclo-L-Trp-L-Pro, we first followed the logic of the nature’s biosynthetic strategy by using the reverse C2-prenyltransferase EchPT1 from *A. ruber* (Wohlgemuth et al. 2017) as the first biocatalyst for prenylation of cyclo-L-Trp-L-Pro (1). The obtained C2-prenylated derivative deoxybrevianamide E (2) should be then used as substrate for prenylation at C4-, C5-, C6-, and C7 of the benzene ring with other prenyltransferases. The results of the enzyme assay are shown in Fig. 3. LCMS analysis revealed that 1 was well converted to 2 having a \([M+H]^+\) ion at \(m/z\) 352.203 by EchPT1 with a conversion yield of 70.7 ± 0.5% (Fig. 3a). Isolation on HPLC and comparison of its \(^1\)H NMR data (Fig. S1) with those published previously (Schkeryantz et al. 1999) confirmed 2 to be the expected deoxybrevianamide E.

To obtain the desired diprenylated derivatives, 2 was used as substrate for the second prenylation. As mentioned in the introduction, FgaPT2_R244L, 6-DMATS, and 7-DMATS catalyze C4-, C6-, and C7-prenylation of CDPs, respectively (Fan and Li 2016; Kremer et al. 2007; Winkelblech and Li 2014). In a previous study, we have demonstrated that the mutant FgaPT2_Y398F catalyzed the C4- and C5-prenylations at the indole ring of the tripeptide derivative ardeemin FQ (Ma et al. 2016). These four enzymes were chosen for prenylation of 2 at the positions of C-4, C-5, C-6, and C-7 of the benzene ring, respectively. Unfortunately, LCMS analysis revealed that no products were detected in the incubation mixtures of FgaPT2_R244L and FgaPT2_Y398F. Formation of diprenylated products with \([M+H]^+\) ions at \(m/z\) 420.265 ± 0.005 was observed in the assays with 6-DMATS and 7-DMATS, but only detected in the extracted ion chromatograms (EICs) (Fig. 3b–e). Obviously, prenylation at C-2 of 1 prohibited its acceptance by the tested enzymes. Such low yields make almost impossible to get enough products for structural elucidation. Therefore, we changed our strategy and tested the possibility with first prenylation at the benzene ring by FgaPT2_R244L, FgaPT2_Y398F, 6-DMATS, and 7-DMATS, followed by the C2-prenylation of the monoprenylated products with EchPT1.

**Prenylation of cyclo-L-Trp-L-Pro at the benzene ring by indole prenyltransferases**

To obtain diprenylated derivatives in a different way from nature, substrate 1 was incubated with FgaPT2_R244L, FgaPT2_Y398F, 6-DMATS, and 7-DMATS in the presence of DMAPP at 37 °C for 16 h. LCMS analysis of the reaction mixtures revealed the conversion of 1 to monoprenylated products with \([M+H]^+\) ions at \(m/z\) 352.202 ± 0.005 in all the four assays. One product peak each was detected in the reaction mixtures of FgaPT2_R244L, FgaPT2_Y398F, 6-DMATS, and 7-DMATS with conversion yields of 49.6 ± 0.2, 29.9 ± 0.6, and 11.6 ± 1.4%, respectively (Fig. 4). Two peaks with a total conversion yield of 66.7 ± 0.8% were observed in the assay with FgaPT2_Y398F. Isolation and structural elucidation proved the product of FgaPT2_R244L to be C4-prenylated derivative 3 (see below for structural
elucidation). Both C4- (3) and C5-prenylated derivative 4 were isolated and identified from the reaction mixture of FgaPT2_Y398F. The sole product of 6-DMATSSa was the expected C6-prenylated cyclo-L-Trp-L-Pro (5). NMR analysis of the product peak of the enzyme assay with 7-DMATS proved the presence of both 5 and the C7-prenylated product 6 in a ratio of 0.6:1.0. Prenylation by 7-DMATS at positions C-6 and C-7 of the benzene ring was already described for cyclo-L-Homotrp-D-Val (Fan and Li 2013). Due to their similar physiochemical
properties, 5 and 6 could not be separated from each other. Nevertheless, their structures can be unequivocally elucidated by NMR analysis, because of the availability of the spectrum for 5 in high purity obtained from the enzyme assay with 6-DMATSaa.

**Confirmation of the prenylation positions of the monoprenylated products**

For structural elucidation, the enzyme products were isolated on a preparative scale and their structures were elucidated by MS and NMR analyses. ESI–MS proved that the products of 1 with FgaPT2_R244L, FgaPT2_Y398F, 6-DMATSaa, and 7-DMATS had [M+H]⁺ ions at m/z 352.202 ± 0.005, 68 Dalton larger than that of 1, indicating attachment of a dimethylallyl moiety to their structures. This hypothesis was confirmed by appearance of signals for a regular dimethylallyl residue each at δH 3.4–3.7 (d or dd, 2H-1’), 5.3–5.4 (tsept, H-2’), and 1.7–1.8 (one or two br s, 3H-4’ and 3H-5’) (Figs. S2–S4 and S9). The ¹H NMR spectra of the isolated compounds showed in the aromatic region signals for four instead of...
five protons in that of 1. The signal for H-2 was detected in all of the spectra of the isolated products 3–6, indicating the prenylation at the benzene ring. Three vicinal aromatic protons were found in the spectra of 3 and 6, suggesting for the C4- and C7-prenylation. Coupling pattern of the three aromatic protons in 4 and 5, i.e., one at meta- and two at ortho-position, is consistent with C5- and C6-prenylations. The structure of compound 5 was unequivocally determined as C6-prenylated cyclo-L-Trp-L-Pro by interpretation of its 1H, 13C, 1H-1H COSY, HSQC, and HMBC spectra. In the 13C NMR spectra of 7, 8, and 10, the signals of C-2 at the indole ring as well as those of C-1', C-2', C-3', and C-4'/'C-5' of the reverse prenyl residue were observed at δc 141.1–141.8, 39.1–39.2, 145.8–145.9, 112.8–112.9, and 27.9–28.5 ppm, respectively (Figs. S11, S16, and S25). Inspection of the 1H and 13C NMR spectra of compound 9 revealed the presence of the signals for H-2 at δH 7.11 ppm and for C-2 at δc 123.9 ppm, suggesting that the additional prenyl residue is attached to another position than C-2. Remarkably, the signal of C-1' in 9 at δH 59.1 ppm was downfield shifted for approximate 20 ppm, in comparison to those of 7, 8, and 10. This indicates its attachment to a hetero such as nitrogen than carbon atom (Fig. S20). Correspondingly, the signal of NH-1 was disappeared in the 1H NMR of 9. Together with HSQC and HMBC data (Table S4), compound 9 was proven to be N1,C6-diprenylated derivative (Fig. 5).

### Prenylation of the C4-, C5-, C6-, and C7-prenylated cyclo-L-Trp-L-Pro by EchPT1 and structural elucidation of the diprenylated products

The isolated monoprenylated samples from the enzyme assays of 1 with FgaPT2_R244L, FgaPT2_Y398F, 6-DMATS, and 7-DMATS, i.e., 3, 4, and 5 in high purity and 6 in a mixture with 5, were used for incubation with the cyclo-L-Trp-L-Ala reverse C2-prenyltransferase EchPT1 in the presence of DMAPP. LCMS analysis showed all these substrates were accepted by EchPT1 with the highest conversion of 85.7 ± 3.5% for 3 (Fig. 5). Only one product peak each was detected, even in the assay with the mixture of 5 and 6 (Fig. 5d). Isolation and structural elucidation confirmed the presence of one product each in the incubation mixtures of 3–5. As expected, the single product peak of the incubation mixture of 5 and 6 comprised two products, which can be separated from each other under optimized conditions.

The isolated products 7–10 from the enzyme assays with 3–6 were then subjected to MS and NMR analyses. ESI–MS proved that all these new products were diprenylated cyclo-L-Trp-L-Pro with [M + H]+ ions at m/z 420.265 ± 0.005. In the 1H NMR spectra of 7, 8, and 10, the signal for H-2 disappeared. Instead, additional signals for a reverse dimethylallyl moiety were detected at δH 6.12–6.14 (dd, 17, 10 Hz, H-2'), 5.17–5.20 (dd, 17, 0.6 Hz, H-3'), 5.16–5.23 (dd, 10, 0.6 Hz, H-2'), and 1.5–1.7 (two singlets, 3H-4' and 3H-5') (Figs. S10, S15, and S24). These data suggested 7, 8, and 10 to be C2,C4-, C2,C5-, and C2,C7-diprenylated cyclo-L-Trp-L-Pro by attachment of a reverse dimethylallyl moiety to position C-2 of 3, 4, and 6, respectively. This is expected for the EchPT1 reactions and also confirmed by intensive interpretation of their 13C, 1H-1H COSY, HSQC, and HMBC spectra. Together with HSQC and structural elucidation of their diprenylation.

### Determination of the kinetic parameters of EchPT1 toward monoprenylated cyclo-L-Trp-L-Pro

To better understand the behavior of EchPT1, kinetic parameters including Michaelis–Menten constant K_M and turnover number k_cat were determined for the three aromatic substrates 3–5 and DMAPP in the presence of the best accepted 3. Kinetic parameters for 6 could not be obtained due to the impurity with 5. All the reactions followed the Michaelis–Menten kinetics (Fig. 6). Similar affinities with K_M values between 0.05 and 0.08 mM were determined for 3–5, comparable to that of cyclo-L-Trp-L-Ala, the natural substrate of EchPT1 at 0.09 mM (Wohlgemuth et al. 2017). The highest k_cat value at 0.21 s⁻¹ was calculated for 3, followed by those of 4 and 5 at 0.03 and 0.007 s⁻¹, respectively. These values are much lower than that of cyclo-L-Trp-L-Ala at 6.63 s⁻¹ (Wohlgemuth et al. 2017). The catalytic efficiencies were calculated for 3, 4, and 5 to be 3500, 375, and 140 s⁻¹ mol⁻¹, respectively, being in good consistence with the observed conversion yields depicted in Fig. 5. The K_M value for DMAPP was determined in the presence of the best C4-prenylated cyclo-L-Trp-L-Pro 3 at 0.10 mM, slightly lower than in the presence of cyclo-L-Trp-L-Ala at 0.18 mM (Wohlgemuth et al. 2017) (Table 1).

### Discussion

In nature, cyclodipeptides are assembled by nonribosomal peptide synthases, mostly in fungi, or by cyclodipeptide synthases, mainly in bacteria (Canu et al. 2020; Mishra et al. 2017). They are then modified by different tailoring enzymes...
such as prenyltransferases, methyltransferases, cytochrome P450 enzymes and 2-oxoglutarate-dependent monooxygenases (Harken and Li 2021; Winkelblech et al. 2015). Cyclodipeptide prenylation is usually initiated at position C-2 by reverse or regular prenyltransferases (Fig. 1) (Winkelblech et al. 2015). Further prenylations take place thereafter at the benzene ring, as demonstrated in the biosynthesis of the triprenylated cyclo-L-Trp-L-Ala derivative echinulin (Wohlgemuth et al. 2017).

In this study, we first followed the nature’s biosynthetic strategy for production of diprenylated cyclo-L-Trp-L-Pro, i.e., at C2,C4-, C2,C5-, C2,C6-, and C2,C7-diprenylated derivatives. We first prepared deoxybrevianamide E by prenylation of cyclo-L-Trp-L-Pro at position C-2 with EchPT1. Unfortunately, the four prenyltransferases FgaPT2_R244L, FgaPT2_Y398F, 6-DMATSSa, and 7-DMATS used in this study showed strict substrate specificity and did not accept deoxybrevianamide E as substrate for further prenylation (Fig. 3). We therefore changed our strategy by first prenylation of cyclo-L-Trp-L-Pro at the benzene ring with the four enzymes FgaPT2_R244L, FgaPT2_Y398F, 6-DMATSSa, and 7-DMATS. As expected, three different monoprenylated derivatives, i.e., cyclo-L-Trp-L-Pro with dimethylally moieties at the positions C-4, C-5, and C-6, were isolated from the incubation mixture of 6 with 5. Absorptions at 280 nm are illustrated. All the assays were performed in duplicates. The conversion yields are given as mean values

Fig. 5 LCMS analysis of acceptance of monoprenylated derivatives (3, 4, 5, and 6) by EchPT1. Products 9 and 10 with a total conversion yield of 23.6 ± 1.4% were isolated from the incubation mixture of 6
C-7, were then successfully converted by EchPT1 to the designed diprenylated products (Fig. 5). However, to our surprise, EchPT1 catalyzed the unique $N_1$-reverse prenylation of C6-prenylated cyclo-$\text{L}_1$-Trp-$\text{L}_1$-Pro and produced $N_1\text{-}C_6$-diprenylated product. This is the first example for an $N_1$-prenylation catalyzed by EchPT1. This study expands therefore significantly our knowledge on prenyltransferases of the DMATS superfamily. It is obvious that their catalytic potential is far away from exhausted, even a large number of biochemical works have been carried out and published in the last decade (Fan et al. 2015; Mori 2020). Furthermore, our results provide a new strategy for production of designed products and increasing structural diversity by changing the normal reaction sequences.

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Author contribution S.-M. L, W. L., and C. L. conceived and planned the experiments. W. L. and C. L. carried out the experiments. J. Z. contributed to structural elucidation. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Table 1  Kinetic parameters of EchPT1 toward 3–5 and DMAPP

| Substrates          | $K_M$ (mM)  | $k_{cat}$ (s$^{-1}$)  | $k_{cat}/K_M$ (s$^{-1}$ M$^{-1}$) |
|---------------------|-------------|-----------------------|----------------------------------|
| 3                   | 0.06±0.01   | 0.21±0.02             | 3500                             |
| 4                   | 0.08±0.02   | 0.03±0.004            | 375                              |
| 5                   | 0.05±0.007  | 0.007±0.0004          | 140                              |
| DMAPP with 3        | 0.10±0.01   | 0.25±0.02             | 2500                             |

Three independent experiments were carried out and standard deviations are given as ± values.

Data availability All data generated during this study are included in this published article and its supplementary information file.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.
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