

# Mutation on Gly115 and Tyr205 of the cyclic dipeptide C2-prenyltransferase FtmPT1 increases its catalytic activity toward hydroxynaphthalenes

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Received: 17 June 2016 / Revised: 28 September 2016 / Accepted: 26 October 2016 / Published online: 10 November 2016  
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**Abstract** The fungal cyclic dipeptide prenyltransferase FtmPT1 from *Aspergillus fumigatus* catalyzes a regular C2-prenylation of brevianamide F (*cyclo*-L-Trp-L-Pro) and is involved in the biosynthesis of a number of biologically active natural products including tryprostatins, spirotryprostatins, verruculogen, and fumitremorgins. FtmPT1, like other members of the dimethylallyltryptophan synthase superfamily, was shown to have high substrate promiscuity for tryptophan-containing cyclic dipeptides and a few other aromatic substrates. A previous study demonstrated the acceptance of 1-naphthol by FtmPT1, but with very low product yield. In this study, we report the significantly increased acceptance of 1-naphthol and other hydroxynaphthalenes by FtmPT1\_G115A and six FtmPT1\_Y205X single mutants as well as FtmPT1\_G115A\_Y205C. These results provided an example for creation of biocatalysts with improved catalytic activity by site-directed mutagenesis.

**Keywords** Hydroxynaphthalenes · Dimethylallyltryptophan synthase · Enzyme catalysis · Friedel–Crafts alkylation · Prenyltransferase

## Introduction

Prenyltransferases are ubiquitous in nature and catalyze the transfer reactions of prenyl moieties from different prenyl donors to various aliphatic or aromatic acceptors, including small-molecule natural products, peptides, proteins, and t-RNAs. Therefore, they play important roles in both primary and secondary metabolism (Dumelin et al. 2012; Heide 2009; Winkelblech et al. 2015). According to their amino acid sequences, structures, biochemical properties, and functions, prenyltransferases are classified into different subgroups (Bonitz et al. 2011; Heide 2009; Li 2009; Winkelblech et al. 2015). One of the most investigated subgroups is the dimethylallyltryptophan synthase (DMATS) superfamily from fungi, which share sequence homology with the dimethylallyltryptophan synthase involved in the biosynthesis of ergot alkaloids in *Claviceps sp.* (Tsai et al. 1995). Until now, more than 40 such enzymes have been identified and characterized biochemically (Winkelblech et al. 2015). The majority of the DMATS superfamily is involved in the biosynthesis of prenylated indole alkaloids and takes indole derivatives including tryptophan and tryptophan-containing cyclic dipeptides as substrates (Winkelblech et al. 2015). They contribute greatly to the structure diversity of a large number of natural products (Heide 2009; Li 2010). In comparison to their non-prenylated precursors, prenylated derivatives often show clearly distinct biological and pharmacological activities and are important sources for drug discovery and development (Botta et al.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00253-016-7966-x) contains supplementary material, which is available to authorized users.

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2005; Heide 2009; Li 2010; Wollinsky et al. 2012a; Yazaki et al. 2009), which make prenyltransferases including DMATSS valuable biocatalysts in the structural modification of small molecules (Fan et al. 2015a).

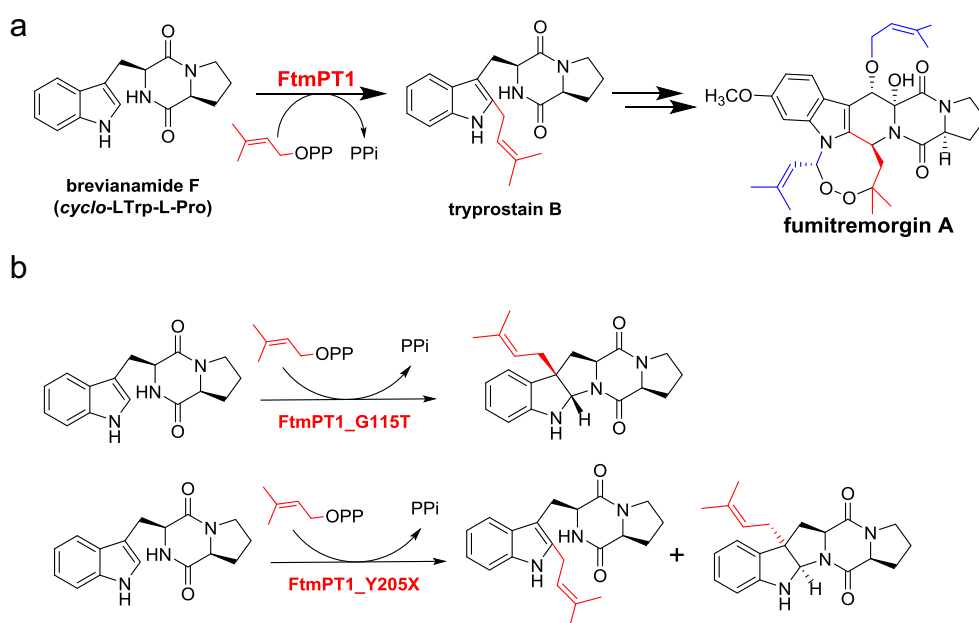
DMATSS usually demonstrate high substrate promiscuity toward aromatic substrates and catalyze mostly regioselective Friedel–Crafts alkylations on the aromatic ring system (Fan et al. 2015a; Winkelblech et al. 2015). For example, FtmPT1 from the ascomycetous fungus *Aspergillus fumigatus* catalyzes a regular C2 - prenylation on the indole ring of brevianamide F (*cyclo*-L-Trp-L-Pro) in the presence of DMAPP and is involved in the biosynthesis of several biologically active natural products including tryprostatins and fumitremorgins (Fig. 1a) (Grundmann and Li 2005; Li 2011). FtmPT1 also accepted a large number of tryptophan-containing cyclic dipeptides and other aromatic compounds like 1-naphthol for Friedel–Crafts alkylations (Chen et al. 2012; Grundmann and Li 2005; Wollinsky et al. 2012b; Yu et al. 2011). However, the product yield of 1-naphthol with FtmPT1 was significantly lower than those with other members of the DMATS superfamily such as FgaPT2, 7-DMATS, CdpNPT, and AnaPT (Yu et al. 2011). Therefore, prenylation of hydroxynaphthalenes by FtmPT1 was not further investigated in that study.

In the last years, structures of several DMATSS including FgaPT2 (Metzger et al. 2009), FtmPT1 (Jost et al. 2010), CdpNPT (Schuller et al. 2012), and AnaPT (Yu et al. 2013) were determined and used as the basis for understanding the catalytic mechanism and for creation of new biocatalysts. Comparing these structures revealed that these enzymes share similar folds consisting of five

repeating “ $\alpha\beta\beta\alpha$ ” (ABBA) barrel folds. Their active sites are located in the center of the barrel. The amino acid residues in the DMAPP-binding sites seem to be fairly conserved, while the binding sites of the aromatic substrates differ from each other. Based on the structures of FgaPT2 and by molecular modeling-guided site-directed mutagenesis, we created several derivatives with distinct or strongly increased catalytic activity (Fan et al. 2015b; Fan and Li 2016). Recently, Mori et al. (2016) reported the alteration of the preference for prenyl chain length as well as regioselectivities and stereoselectivities of TleC and MpnD by structure-based enzyme engineering. These results demonstrated a convenient route for new enzyme design by mutagenesis.

As for FtmPT1, several amino acid residues including Gly115 and Tyr205 were proposed to be involved in the binding of brevianamide F (Jost et al. 2010). Based on the structure, a modifiable reaction chamber was identified and a mutant FtmPT1\_G115T was obtained, which still accepted brevianamide F as substrate, but catalyzed mainly a reversely *syn-cis* C3 prenylation instead of the regularly C2 - prenylation (Jost et al. 2010). In another study, several Tyr205 mutants showed comparable consumption of brevianamide F as the non-mutated FtmPT1, but with clearly increased activity for the formation of C3-prenylated derivatives (Zhou et al. 2016) (Fig. 1b). Due to the low product yields, the prenylation of hydroxynaphthalenes by FtmPT1 was not studied in detail in our previous study (Yu et al. 2011). After availability of Gly115 and Tyr205 mutants, their activities toward hydroxynaphthalenes were further investigated.

**Fig. 1** Known reactions catalyzed by FtmPT1 (a) and its Gly115 and Tyr205 mutants (b)



## Materials and methods

### Chemicals

DMAPP was synthesized according to the method described for geranyl diphosphate reported previously (Woodside et al. 1988). Naphthalene derivatives were purchased from Fluka, TCI, Acros Organics, Aldrich, and Alfa Aesar.

### Bacterial strains, plasmids, and culture conditions

*Escherichia coli* XL1-Blue MRF' (Stratagene, Heidelberg, Germany) was used for cloning and expression experiments. pAG12 containing *fmPT1* in pQE70 was constructed previously (Grundmann and Li 2005) and used as expression vector for FtmPT1 and as DNA template for site-directed mutagenesis experiments. *E. coli* cells harboring plasmids were grown in liquid Lysogeny Broth (LB) medium and on solid LB medium with 1.5% (*w/v*) agar at 37 °C. Fifty micrograms of carbenicillin per milliliter was used for selection of recombinant *E. coli* strains.

### Site-directed mutagenesis

Constructs for expression of FtmPT1 Tyr205 mutants were reported elsewhere (Zhou et al. 2016). The expression constructs pST2 and pST9–11 were described previously (Jost

et al. 2010). For creation of additional Gly115 mutants, site-directed mutagenesis was carried out as described previously (Zhou et al. 2016). Degenerated primers at bps 343–345 were used to generate mutants of FtmPT1 listed in Table 1. The Expand Long Template PCR system (Roche Diagnostics, Mannheim, Germany) was used for creation of the plasmids pKZ1–pKZ17, pKZ22, and pKZ24. pKZ3 and pKZ11 were used as templates for construction of plasmids for expression of the double mutants G115T\_Y205C, G115T\_Y205M, G115A\_Y205C, and G115T\_Y205M.

### Overproduction and purification of the recombinant proteins

Protein overproduction and purification of FtmPT1 and its mutants were carried out as described previously (Grundmann and Li 2005; Zhou et al. 2016) and analyzed on SDS-PAGE. Protein concentration was estimated by measurement of the absorption at 280 nm on a NanoDrop 2000c UV–Vis spectrophotometer and by comparison of their intensities with those of protein markers on SDS-PAGE (Fig. S1).

### Enzyme assays with purified recombinant proteins

All enzyme assays contained 50 mM Tris–HCl (pH 7.5), 5 mM CaCl<sub>2</sub>, 0.15–5% (*v/v*) glycerol, 5% (*v/v*) DMSO, and 2 mM DMAPP and were incubated at 37 °C. To determine the

**Table 1** Mutated derivatives of FtmPT1 at Gly115 and respective primers

Sequence (5'–3')	Bases at 343–345	Mutant	Plasmid	Protein yield mg/per liter of culture
fw GATTCGAATTNNGTTGATCCCGTCACGGCTGACTCTGGC	ATA	FtmPT1_G115I	pKZ1	3.7
rev GGGATCAACNNAATTCGAATCAACGCCCGAGCGACG	CAA	FtmPT1_G115Q	pKZ2	0.9
	ACC	FtmPT1_G115T	pKZ3	5.1
	GTG	FtmPT1_G115V	pKZ4	5.1
	CCT	FtmPT1_G115P	pKZ5	0.3
	AGA	FtmPT1_G115R	pKZ6	3.6
	AAC	FtmPT1_G115N	pKZ7	5.6
	CAC	FtmPT1_G115H	pKZ8	3.8
	TTT	FtmPT1_G115F	pKZ9	5.0
	AGC	FtmPT1_G115S	pKZ10	1.0
	GCA	FtmPT1_G115A	pKZ11	5.1
fw GATTCGAATTDGKTTGATCCCGTCACGGCTGACTCT	AAG	FtmPT1_G115K	pKZ12	0.6
rev GGGATCAACMHHAATTCGAATCAACGCCCGAGCGACG	TGG	FtmPT1_G115W	pKZ13	9.9
	TAT	FtmPT1_G115Y	pKZ14	4.8
	GAC	FtmPT1_G115D	pKZ15	12.2
fw GCGTATTCTGTCCGCAGCCCAAATCTGC	ACC	FtmPT1_G115T_Y205C	pKZ22	5.8
rev GGGCTGCGGACAGAAATACGCCTTGACCAG				
fw GCGTATTCAWGGCCGAGCCCAAATCTGC	ACC	FtmPT1_G115T_Y205M	pKZ24	4.9
rev GGGCTGCGGCWTGAAATACGCCTTGACCAG				
fw GCGTATTCTGTCCGCAGCCCAAATCTGC	GCA	FtmPT1_G115A_Y205C	pKZ16	7.6
rev GGGCTGCGGACAGAAATACGCCTTGACCAG				
fw GCGTATTCAWGGCCGAGCCCAAATCTGC	GCA	FtmPT1_G115A_Y205M	pKZ17	0.9
rev GGGCTGCGGCWTGAAATACGCCTTGACCAG				

enzyme activity of FtmPT1 and its mutants toward hydroxynaphthalenes, 1-naphthol (**1a**), 2-naphthol (**2a**), 1,6-dihydroxynaphthalene (**3a**), 1,7-dihydroxynaphthalene (**4a**), 2,3-dihydroxynaphthalene (**5a**), 2,6-dihydroxynaphthalene (**6a**), 2,7-dihydroxynaphthalene (**7a**), 1-hydroxy-5-aminonaphthalene (**8a**), and 1-amino-7-hydroxynaphthalene (**9a**) at a concentration of 1 mM were incubated with  $0.1 \mu\text{g } \mu\text{L}^{-1}$  of purified recombinant protein in a total volume of 100  $\mu\text{L}$ . After incubation for 2 h, the reactions were terminated by addition of 100  $\mu\text{L}$  methanol. Proteins were removed by centrifugation at 13,000 rpm for 20 min, and the supernatants were analyzed on HPLC. In order to determine the kinetic parameters of the prenyl transfer reactions, aromatic substrates at concentrations of up to 2 mM were used. Varied protein amounts and incubation times were used for assays as shown in Table 3.

For isolation of the enzyme products, reactions were carried out in large scales (15 mL) containing 1 mM aromatic substrate and 2–4 mg recombinant protein. After incubation for 16 h, the reaction mixtures were extracted with 15 mL EtOAc for four times. The extracts were concentrated on a rotating vacuum evaporator at 35 °C and then dissolved in 1 mL methanol for injection into HPLC.

#### HPLC analysis and isolation of the enzyme products

The enzyme reaction mixtures were analyzed on an HPLC (Agilent series 1200, Böblingen, Germany) by using a Multospher 120 RP-18 column (250 × 4 mm, 5  $\mu\text{m}$ , CS-Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 mL min<sup>-1</sup>. Water and methanol, both containing 0.5% trifluoroacetic acid, were used as solvents. For analysis of the enzyme products, a linear gradient of 30–100% (v/v) methanol in 20 min was used. The column was then washed with 100% (v/v) methanol for 5 min and equilibrated with 30% (v/v) methanol for 5 min. For analysis of the enzyme products as shown in Figs. S2 and S4, water and acetonitrile, both containing 0.5% trifluoroacetic acid, were used as solvents. A linear gradient of 40–100% (v/v) acetonitrile in 15 min was used for analysis. The column was then washed with 100% acetonitrile for 5 min and equilibrated with 40% acetonitrile in water for another 5 min. Detection was carried out with a photodiode array detector. For isolation of the enzyme products, the same HPLC equipment with a Multospher 120 RP-18 column (250 × 10 mm, 5  $\mu\text{m}$ , CS-Chromatographie Service, Langerwehe, Germany) was used. A linear gradient of 30–100% (v/v) of methanol in water in 40–60 min was carried out with a flow rate at 2.5 mL min<sup>-1</sup>. The column was then washed with 100% (v/v) methanol for 10 min and equilibrated with 30% (v/v) methanol for 10 min.

#### NMR and mass spectrometric analyses

For structure elucidation, the isolated enzyme products were subjected to <sup>1</sup>H NMR and LC-MS analyses. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data were obtained on a Bruker microTOF-Q III mass spectrometer. The spectrometer was equipped with an Agilent 1260 HPLC system. <sup>1</sup>H NMR spectra were recorded at room temperature with an ECX-500 spectrometer (JEOL, Tokyo, Japan) equipped with a broadband probe with z-gradient. All spectra were processed with MestReNov. 5.2.2 (Mestrelab Research, Santiago de Compostella, Spain). Chemical shifts were referenced to the solvent signal at 3.30 ppm for MeOH. For NMR spectra, please see Figs. S13, S14, S15, S16, S17, S18, S19, and S20 in Electronic Supplementary Material.

**Compound 1b:** HR-ESI-MS:  $m/z$  213.1279 ([M+H]<sup>+</sup>, calculated for C<sub>15</sub>H<sub>17</sub>O: 213.1274). <sup>1</sup>H NMR (500 Hz, CD<sub>3</sub>OD)  $\delta_H$  8.21 (ddd, 1H,  $J = 8.3, 1.4,$  and 0.6 Hz), 7.90 (br d, 1H,  $J = 8.3$  Hz), 7.45 (td, 1H,  $J = 7.5$  and 1.4 Hz), 7.40 (td, 1H,  $J = 7.5$  and 1.3 Hz), 7.09 (d, 1H,  $J = 7.7$  Hz), 6.71 (d, 1H,  $J = 7.7$  Hz), 5.33 (tsept, 1H,  $J = 7.0$  and 1.4 Hz), 3.65 (d, 2H,  $J = 7.0$  Hz), 1.80 (d, 3H,  $J = 1.0$  Hz), 1.74 (d, 3H,  $J = 1.2$  Hz). These data correspond well to those published previously (Yu et al. 2011).

**Compound 3b:** HR-ESI-MS:  $m/z$  229.1213 ([M+H]<sup>+</sup>, calculated for C<sub>15</sub>H<sub>17</sub>O<sub>2</sub>: 229.1223). <sup>1</sup>H NMR (500 Hz, CD<sub>3</sub>OD)  $\delta_H$  8.07 (d, 1H,  $J = 9.0$  Hz), 7.17 (d, 1H,  $J = 2.3$  Hz), 6.98 (m, 2H), 6.51 (d, 1H,  $J = 7.6$  Hz), 5.33 (tsept, 1H,  $J = 6.8$  and 1.4 Hz), 3.54 (d, 2H,  $J = 6.8$  Hz), 1.79 (d, 3H,  $J = 1.0$  Hz), 1.74 (d, 3H,  $J = 1.2$  Hz). These data correspond well to those published previously (Yu et al. 2011).

**Compound 4b:** HR-ESI-MS:  $m/z$  229.1209 ([M+H]<sup>+</sup>, calculated for C<sub>15</sub>H<sub>17</sub>O<sub>2</sub>: 229.1223). <sup>1</sup>H NMR (500 Hz, CD<sub>3</sub>OD)  $\delta_H$  7.77 (d, 1H,  $J = 9.1$  Hz), 7.49 (d, 1H,  $J = 2.6$  Hz), 7.04 (dd, 1H,  $J = 9.1$  and 2.6 Hz), 6.87 (d, 1H,  $J = 7.6$  Hz), 6.64 (d, 1H,  $J = 7.6$  Hz), 5.31 (br t, 1H,  $J = 7.6$  Hz), 3.59 (d, 2H,  $J = 7.0$  Hz), 1.79 (s, 3H), 1.73 (s, 3H). These data correspond well to those published previously (Yu et al. 2011).

**Compound 7b:** HR-ESI-MS:  $m/z$  229.1203 ([M+H]<sup>+</sup>, calculated for C<sub>15</sub>H<sub>17</sub>O<sub>2</sub>: 229.1223). <sup>1</sup>H NMR (500 Hz, CD<sub>3</sub>OD)  $\delta_H$  7.56 (d, 1H,  $J = 8.8$  Hz), 7.44 (d, 1H,  $J = 8.8$  Hz), 7.12 (d, 1H,  $J = 2.4$  Hz), 6.87 (d, 1H,  $J = 8.8$  Hz), 6.83 (dd, 1H,  $J = 8.8$  and 2.4 Hz), 5.17 (tsept, 1H,  $J = 6.6$  and 1.4 Hz), 3.63 (d, 2H,  $J = 6.6$  Hz), 1.88 (d, 3H,  $J = 0.9$  Hz), 1.68 (d, 3H,  $J = 1.3$  Hz). These

data correspond well to those published previously (Yu et al. 2011).

**Compound 7c:** HR-ESI-MS:  $m/z$  229.1225 ( $[M+H]^+$ , calculated for  $C_{15}H_{17}O_2$ : 229.1223).  $^1H$  NMR (500 Hz,  $CD_3OD$ )  $\delta_H$  7.59 (dd, 2H,  $J = 8.9$  and 1.1 Hz), 7.00 (m, 2H), 6.88 (dd, 2H,  $J = 8.9$  and 2.5 Hz), 5.52 (br t, 1H,  $J = 6.6$  Hz), 4.61 (d, 1H,  $J = 6.6$  Hz), 1.81 (br s, 3H), 1.79 (br s, 3H). These data correspond well to those published previously (Yu et al. 2011).

**Compound 7d:** HR-ESI-MS:  $m/z$  297.1844 ( $[M+H]^+$ , calculated for  $C_{20}H_{25}O_2$ : 297.1849).  $^1H$  NMR (500 Hz,  $CD_3OD$ )  $\delta_H$  7.60 (d, 1H,  $J = 8.9$  Hz), 7.47 (d, 1H,  $J = 8.7$  Hz), 7.13 (d, 1H,  $J = 2.4$  Hz), 6.92 (d, 1H,  $J = 8.7$  Hz), 6.88 (dd, 1H, d,  $J = 8.9$  and 2.4 Hz), 5.52 (tsept, 1H,  $J = 6.6$  and 1.3 Hz), 5.17 (tsept, 1H,  $J = 6.7$  and 1.4 Hz), 4.62 (d, 2H,  $J = 6.5$  Hz), 3.69 (d, 2H,  $J = 6.7$  Hz), 1.90 (br s, 3H), 1.81 (br s, 3H), 1.79 (br s, 3H), 1.70 (d, 3H,  $J = 1.2$  Hz). These data correspond well to those published previously (Yu et al. 2011).

**Compound 7e:** HR-ESI-MS:  $m/z$  365.2467 ( $[M+H]^+$ , calculated for  $C_{25}H_{33}O_2$ : 365.2475).  $^1H$  NMR (500 Hz,  $CD_3OD$ )  $\delta_H$  7.39 (d, 1H,  $J = 8.7$  Hz), 7.38 (s, 1H), 7.08 (s, 1H), 6.89 (d, 1H,  $J = 8.7$  Hz), 5.54 (br t, 1H,  $J = 6.5$  Hz), 5.35 (br t, 1H,  $J = 7.4$  Hz), 5.17 (br t, 1H,  $J = 6.7$  Hz), 4.62 (d, 2H,  $J = 6.5$  Hz), 3.68 (d, 2H,  $J = 7.0$  Hz), 3.36 (d, 2H,  $J = 7.5$  Hz), 1.91 (s, 3H), 1.82 (s, 3H), 1.79 (s, 3H), 1.75 (s, 3H), 1.73 (s, 3H), 1.70 (s, 3H).

**Compound 9b:** HR-ESI-MS:  $m/z$  228.1384 ( $[M+H]^+$ , calculated for  $C_{15}H_{18}NO$ : 228.1383).  $^1H$  NMR (500 Hz,  $CD_3OD$ )  $\delta_H$  7.81 (d, 1H,  $J = 9.1$  Hz), 7.23 (d, 1H,  $J = 2.5$  Hz), 7.05 (dd, 1H,  $J = 9.1$  and 2.5 Hz), 6.89 (d, 1H,  $J = 7.5$  Hz), 6.72 (d, 1H, d,  $J = 7.5$  Hz), 5.31 (tsept, 1H,  $J = 7.0$  and 1.5 Hz), 3.60 (d, 2H,  $J = 7.2$  Hz), 1.79 (s, 3H), 1.73 (d, 3H,  $J = 1.0$  Hz).

## Results

### Acceptance of 1-naphthol by single mutants of Gly115

Four FtmPT1 mutants G115A, G115T, G115I, and G115L were created in a previous study for proof of the importance of Gly115 and showed different influence of these enzymes on the prenyl transfer reaction for brevianamide F (Jost et al. 2010). G115A did not change the prenylation position, while G115T catalyzed a reverse *C3* instead of a regular *C2* prenylation at the indole ring of brevianamide F (Jost et al.

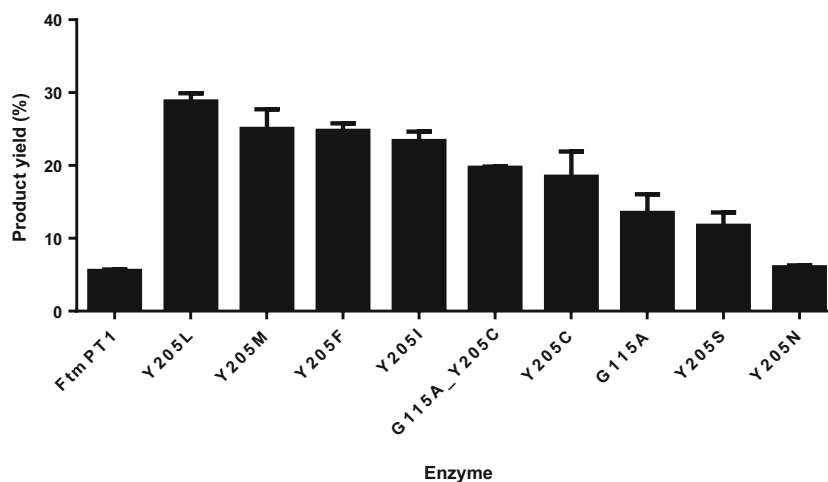
2010). Incubation of these mutants (10  $\mu$ g protein) with 1-naphthol (**1a**) at 37 °C for 2 h and analysis of their reaction mixtures on HPLC also revealed different behaviors (Fig. S2). Under this condition, FtmPT1\_G115A showed significantly higher activity toward **1a** with a product yield of  $13.5 \pm 2.5\%$  than FtmPT1 with  $5.5 \pm 0.1\%$ . G115T showed a conversion yield of  $2.1 \pm 0.2\%$ . No product formation was detected in the incubation mixtures of **1a** with mutants G115I or G115L under the tested conditions.

Our previous study showed that saturation mutagenesis could be a powerful tool for creation of enzyme derivatives (Fan and Li 2016). Following this experience, different oligonucleotides with wobbles at base pairs of 343 to 345 of *f*tmPT1 were used as degenerated primer pairs for mutagenesis (Table 1). After identification of 11 G115 mutants, the remaining mutants were obtained with more specific primers (Table 1). Constructs for additional 8 mutants were obtained in this way (Table 1). Satisfied purity and amount were achieved for nine mutants (Fig. S1). Very low protein yield and purity were obtained for G115Q, G115P, and G115K. HPLC analysis of the incubation mixtures of **1a** with these mutants revealed that G115D, G115S, G115N, and G115F showed activities, but lower than that of the non-mutated FtmPT1. No product formation was detected in the incubation mixtures of **1a** with G115H, G115Y, G115Q, G115W, G115K, G115P, G115V or G115R under the tested conditions (Fig. S2).

### Acceptance of 1-naphthol by single mutants of Tyr205

In the presence of DMAPP (2 mM), 1-naphthol (**1a**) was incubated with 10  $\mu$ g of recombinant FtmPT1 or its 19 mutants obtained from the previous study (Zhou et al. 2016) in 100  $\mu$ L enzyme assays at 37 °C for 2 h. HPLC analysis of the incubation mixtures revealed that **1a** was accepted by the tested enzymes with clearly different activities (Figs. 2 and S3). Under this condition, **1a** was converted by FtmPT1 with a product yield of  $5.5 \pm 0.1\%$ . Six mutants including Y205L, Y205M, Y205F, Y205I, Y205C, and Y205S showed higher activity toward **1a** than FtmPT1, with product yields of  $28.8 \pm 1.1$ ,  $25.0 \pm 2.7$ ,  $21.4 \pm 1.2$ ,  $23.3 \pm 1.3$ ,  $18.5 \pm 3.5$ , and  $11.7 \pm 1.8\%$ , respectively. The activities of the first five mutants are also significantly higher than that of G115A. These six Tyr205 mutants also accepted brevianamide F as well, but with activities not higher than the non-mutated FtmPT1 (Zhou et al. 2016). Six mutants Y205N, Y205V, Y205K, Y205P, Y205R, and Y205E accepted **1a** with product yields between 0.6 and 6.0%. No product peak was observed from the reaction mixtures of **1a** with Y205A, Y205T, Y205D, Y205G, or Y205W. One product peak each with the same retention time was detected for the reaction mixtures of FtmPT1 and its mutants, indicating the presence of the same product(s).

**Fig. 2** Product yields of FtmPT1 and its mutants toward 1-naphthol (**1a**). Data are presented as mean values of two independent assays. Only mutants with higher product yields than that of FtmPT1 were presented



### Acceptance of 1-naphthol by selected double mutants on Gly115 and Tyr205

The importance of Gly115 and Tyr205 for prenylation of breviramide F has been demonstrated in previous studies. That is, G115T redirected the prenylation position and several Tyr205 mutants increased the product yields of C3-prenylated derivatives (Jost et al. 2010; Zhou et al. 2016). As mentioned above, G115A, Y205L, Y205M, Y205F, Y205I, Y205C, and Y205S accepted **1a** much better than the wild type of FtmPT1. We were curious to prove the effect of mutations on both residues. Therefore, double mutants G115A\_Y205C, G115A\_Y205M, G115T\_Y205C, and G115T\_Y205M were created by site-directed mutagenesis (Table 1). HPLC analysis of the incubation mixtures of **1a** with the purified proteins under the same conditions as for the single mutants revealed that FtmPT1\_G115A\_Y205C showed higher activities toward **1a** than the wild type of FtmPT1, with a product yield of  $19.7 \pm 0.2\%$ . This activity is slightly higher than that of G115A of  $13.5 \pm 2.5\%$  and comparable with that of Y205C of  $18.5 \pm 3.5\%$ . Product yields of  $4.2 \pm 0.5$  and  $5.3 \pm 0.5\%$  were observed for G115T\_Y205C and G115T\_Y205M, respectively, which are lower than those of Y205C and Y205M. No product peak was detected in the incubation mixture of **1a** with G115A\_Y205M.

### Different behaviors of seven mutants of FtmPT1 toward hydroxynaphthalenes

As mentioned above, seven mutants Y205L, Y205M, Y205F, Y205C, Y205I, Y205S, and G115A\_Y205C showed high activities toward **1a**. Therefore, FtmPT1 and the seven mutants were afterwards assayed with nine hydroxynaphthalenes for 2 h at 37 °C with 10 μg proteins. These included **1a**, 2-naphthol (**2a**), 1,6-dihydroxynaphthalene (**3a**), 1,7-dihydroxynaphthalene (**4a**), 2,3-dihydroxynaphthalene (**5a**), 2,6-dihydroxynaphthalene (**6a**), 2,7-dihydroxynaphthalene

(**7a**), 1-hydroxy-5-aminonaphthalene (**8a**), and 1-amino-7-hydroxynaphthalene (**9a**). HPLC analysis of the incubation mixtures showed that the product yields of **1a** converted by FtmPT1 and its mutants were reproducible (Fig. S3). With **2a** as substrate, the conversion yield of  $0.3 \pm 0.1\%$  was observed with FtmPT1, while the tested mutants clearly accepted it with the highest product yield of  $2.4 \pm 0.3\%$  by G115A\_Y205C (Table 2, Fig. S5). **3a** and **4a** were consumed by FtmPT1 with product yields of  $2.1 \pm 0.3$  and  $5.5 \pm 0.7\%$ , respectively, while the seven mutants demonstrated clearly higher catalytic activities than FtmPT1, with the highest product yield of  $10.4 \pm 0.1\%$  for **3a** with Y205L and  $13.2 \pm 2.0\%$  for **4a** with Y205L, respectively (Table 2 and Figs. S6 and S7). This means a fivefold increase of activity for **3a** and a threefold increase for **4a**. 2,3-Dihydroxynaphthalene (**5a**) and 2,6-dihydroxynaphthalene (**6a**) were poor substrates for both FtmPT1 and its mutants with product yields of less than 7% (Table 2 and Figs. S8 and S9). Toward **7a**, the seven mutants demonstrated low activities with product yields in the range of 3.8–8.6% (Table 2 and Fig. S10). **8a** was accepted by FtmPT1 and the seven mutants with product yields of less than 1% (Table 2 and Fig. S11). **9a** was a good substrate for both FtmPT1 and its mutants. The best product yield of approximately 20% was achieved with FtmPT1\_Y205F (Table 2 and Fig. S12). Taking the results mentioned above together, some of the selected mutants Y205L, Y205M, Y205F, Y205C, Y205I, Y205S, and G115A\_Y205C were found to demonstrate clearly higher catalytic activities toward **1a**, **2a**, **3a**, **4a**, **5a**, and **9a** than the non-mutated FtmPT1 (Table 2).

### Structure elucidation of the detected enzyme products

To obtain enough enzyme products for structure elucidation, the best mutants were used for large-scale reactions (15 ml), i.e., FtmPT1\_Y205M for **1a**, **3a**, and **7a**; FtmPT1\_Y205L for **4a**; and FtmPT1\_Y205F for **9a**. The enzyme products **1b**, **3b**, **4b**, and **9b** were isolated from the

**Table 2** Product yields of hydroxynaphthalenes with FtmPT1 and its mutants

Enzyme	Product yields (%)								
	1-Naphthol (1a)	2-Naphthol (2a)	1,6-Dihydroxynaphthalene (3a)	1,7-Dihydroxynaphthalene (4a)	2,3-Dihydroxynaphthalene (5a)	2,6-Dihydroxynaphthalene (6a)	2,7-Dihydroxynaphthalene (7a)	1-Hydroxy-5-aminonaphthalene (8a)	1-Amino-7-hydroxynaphthalene (9a)
FtmPT1	5.5 ± 0.1	0.3 ± 0.1	2.1 ± 0.3	5.5 ± 0.7	0.4 ± 0.1	5.1 ± 0.1	6.2 ± 0.2	0.4 ± 0.1	12.7 ± 0.5
Y205L	28.8 ± 1.1	2.1 ± 0.1	10.4 ± 0.1	13.2 ± 2.0	6.5 ± 0.7	3.9 ± 0.1	5.5 ± 0.2	0.5 ± 0.1	15.3 ± 0.2
Y205M	25.0 ± 2.7	1.2 ± 0.1	9.2 ± 1.7	5.9 ± 0.2	4.9 ± 0.3	2.1 ± 0.2	6.1 ± 0.2	0.4 ± 0.1	15.6 ± 0.8
Y205I	23.3 ± 1.3	0.9 ± 0.2	5.2 ± 0.2	4.1 ± 0.4	4.5 ± 0.4	4.8 ± 0.4	5.5 ± 0.1	0.4 ± 0.1	21.1 ± 3.2
Y205F	21.4 ± 1.2	0.5 ± 0.3	7.6 ± 1.2	8.5 ± 1.7	4.6 ± 0.1	1.4 ± 0.1	8.6 ± 0.9	0.3 ± 0.1	16.4 ± 2.7
Y205C	18.5 ± 3.5	0.7 ± 0.2	5.8 ± 0.8	7.5 ± 0.4	3.6 ± 0.1	2.3 ± 0.1	5.7 ± 0.5	0.4 ± 0.1	16.4 ± 2.7
Y205S	9.0 ± 1.3	0.3 ± 0.1	3.7 ± 0.3	2.2 ± 1.3	3.6 ± 0.7	2.0 ± 0.1	5.7 ± 0.4	0.5 ± 0.1	16.5 ± 0.1
G115A_Y205C	21.0 ± 0.5	2.4 ± 0.3	7.6 ± 0.4	5.0 ± 0.6	3.3 ± 0.1	1.1 ± 0.1	3.8 ± 0.1	0.6 ± 0.1	15.8 ± 1.4

The enzyme assays (100  $\mu$ L) contained one of the substrates **1a–9a** (1 mM), CaCl<sub>2</sub> (5 mM), DMAPP (2 mM), glycerol (1.0–6.0% v/v), DMSO (5% v/v), Tris-HCl (50 mM, pH 7.5), and purified recombinant protein (10  $\mu$ g). The reaction mixtures were incubated at 37 °C for 2 h. Data are presented as mean values of two independent assays

reaction mixtures of **1a**, **3a**, **4a**, and **9a**, respectively. **7b**, **7c**, **7d**, and **7e** were obtained as products of **7a**. The isolated products were then subjected to MS and NMR analyses (Figs. S13, S14, S15, S16, S17, S18, S19, and S20 in Electronic Supplementary Material). The obtained spectroscopic data of **1b**, **3b**, and **4b** corresponded very well to those of the enzyme products of **1a**, **3a**, and **4a** with other DMATS enzymes published previously (Yu et al. 2011) and confirmed the *para*-prenylation of 1-hydroxynaphthalenes as given in Fig. 3.

By comparison with the data in the literature (Yu et al. 2011), **7b** and **7c** were identified as *C*- and *O*-monoprenylated, respectively, while **7d** is a *C,O*-diprenylated derivative (Fig. 3). MS data indicated a triprenylation in **7e**. In the <sup>1</sup>H NMR spectrum of **7e**, signals at 5.54 (br t, 1H, 6.5 Hz), 5.35 (br t, 1H, 7.4 Hz), and 5.17 ppm (br t, 1H, 6.7 Hz) indicated the presence of three regularly attached prenyl moieties. One of them is attached to an oxygen atom due to the down-field shift of H-1 to 5.54 ppm. Considering the chemical shifts and coupling pattern of the four aromatic protons, the prenylation position is determined as *O*-2, *C*-3, and *C*-8 (Fig. 3). These results demonstrated that the products of the FtmPT1\_Y205 mutants for a given substrate are very similar or identical to those obtained with other prenyltransferases of the DMATS superfamily (Yu et al. 2011). Detection of signals for five aromatic protons in the NMR spectrum of **9b** indicated the attachment of the prenyl moiety to a *C* atom. Inspection of the NMR revealed the presence of two coupling systems. Three protons are coupled with coupling constants of 9.1 and 2.5 Hz and the other two with a coupling constant of 7.5 Hz. This proved that the prenylation had been taken place at the *ortho*-position of the amino group.

Comparing the results obtained for the pairs with different hydroxylation positions, i.e., **1a** and **2a**, **3a**, and **6a** as well as

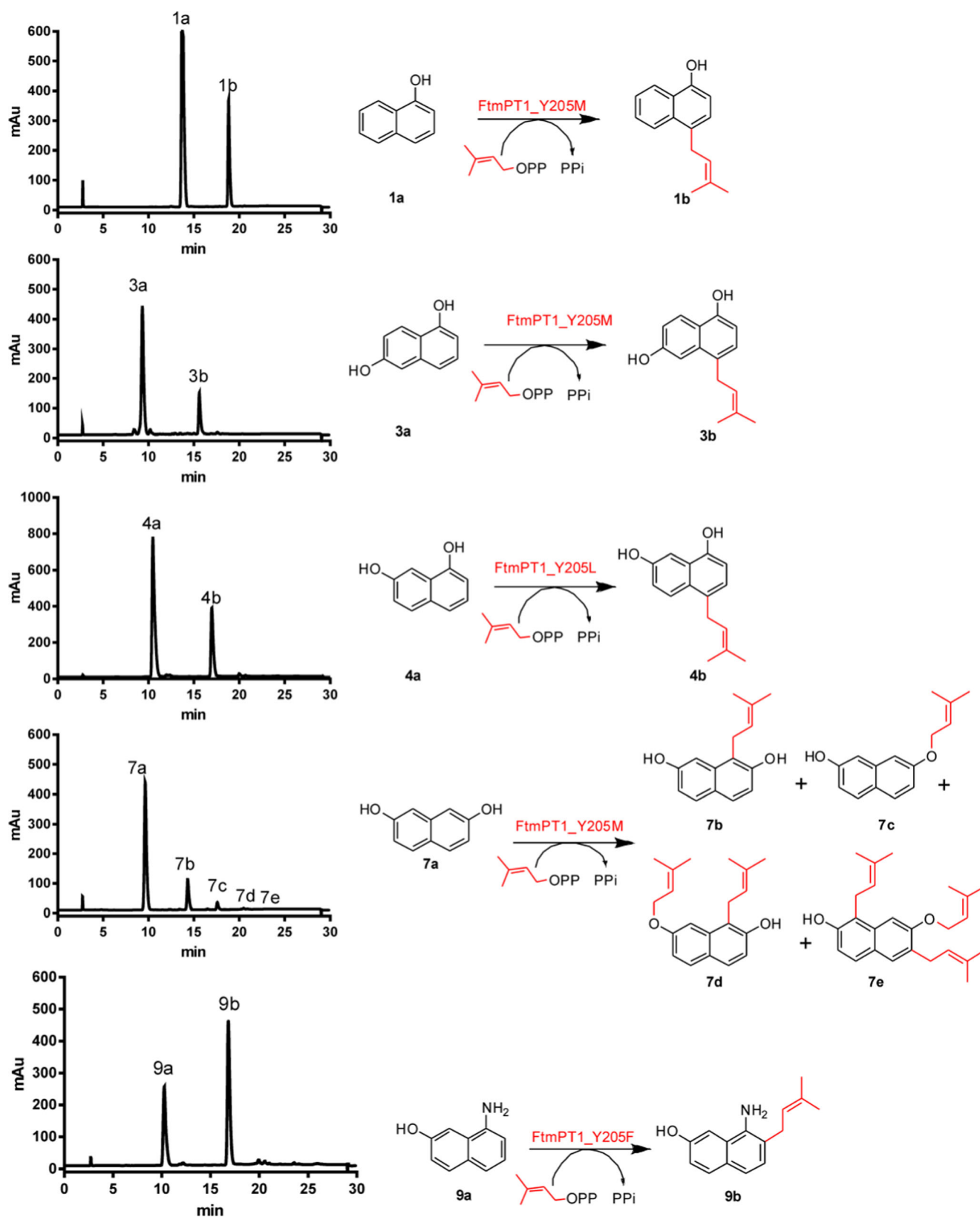
**4a** and **7a**, it seems that mutation of Tyr205 has a stronger positive influence on the acceptance of 1- than 2-hydroxynaphthalene derivatives. **1a**, **4a**, and **9a** were good substrates for FtmPT1 and its mutants.

#### Kinetic parameters of the prenyl transfer reactions with three of the identified best Tyr205 mutants

To compare the catalytic activity of FtmPT1 and its mutated proteins toward hydroxynaphthalenes, kinetic parameters were determined for **1a** and **3a** with FtmPT1 and Y205M, **4a** and **7a** with FtmPT1 and Y205L, and **9a** with FtmPT1 and Y205F by Eadie-Hofstee, Hanes-Woolf, and Lineweaver-Burk plots (Figs. S21, S22, S23, S24, S25, S26, S27, S28, S29, and S30 in Electronic Supplementary Material) as described under the “Materials and methods” section and summarized in Table 3. The *K<sub>M</sub>* values of the mutated proteins toward **1a**, **3a**, **4a**, **7a**, and **9a** are found in the range of 0.19–0.52 mM, significantly lower than those with FtmPT1 in the range of 0.51–1.24 mM, indicating the increased affinity of the mutated proteins to these substrates. The turnover numbers of the reactions with the mutated proteins are about two to eight folds of those with FtmPT1. The kinetic parameters of **7a** are comparable with FtmPT1 and Y205L. These results correspond well to the observed product yields (Table 2).

#### Discussion

Prenyltransferases of the DMATS superfamily are capable of prenylation of various substrates, including indole, tyrosine, xanthone, flavonoid, and naphthalene derivatives. However, they show in many cases low activity toward their non-natural



**Fig. 3** HPLC analysis of the reaction mixtures of **1a**, **3a**, **4a**, **7a**, and **9a** with selected mutants as well as the prenyl transfer reactions

substrates. Several studies in the last years have demonstrated that protein engineering can also be considered as a powerful

tool for the creation of prenyltransferases with altered features (Fan et al. 2015a; Winkelblech et al. 2015).



**Table 3** Kinetic parameters of FtmPT1, Y205L, Y205F, and Y205M reactions

Substrate	FtmPT1				Mutants				
	Protein amount and incubation time	$K_M$ (mM)	$k_{cat}$ ( $s^{-1}$ )	$k_{cat}/K_M$ ( $s^{-1}/mM$ )	Protein amount and incubation time	$K_M$ (mM)	$k_{cat}$ ( $s^{-1}$ )	$k_{cat}/K_M$ ( $s^{-1}/mM$ )	
<b>1a</b>	10 $\mu$ g, 80 min	1.24	0.072	58.1	Y205M	5 $\mu$ g, 40 min	0.20	0.14	700.0
<b>3a</b>	30 $\mu$ g, 70 min	1.20	0.021	17.5	Y205M	10 $\mu$ g, 100 min	0.45	0.038	84.4
<b>4a</b>	30 $\mu$ g, 60 min	0.52	0.018	34.6	Y205L	5 $\mu$ g, 30 min	0.19	0.038	200.0
<b>7a</b>	30 $\mu$ g, 120 min	0.51	0.011	21.6	Y205L	30 $\mu$ g, 120 min	0.52	0.014	26.9
<b>9a</b>	10 $\mu$ g, 45 min	0.79	0.034	43.1	Y205F	10 $\mu$ g, 15 min	0.34	0.28	823.5

In a previous study, mutation of Gly115 to Thr reduced the formation of a C2-prenylated product tryprostatin B from its natural substrate brevianamide F. Instead, a reversely C3-prenylated derivative was identified as the main product. In contrast to G115T, G115A did not change the prenylation position (Jost et al. 2010). In another study, all 19 Tyr205 mutants were created by saturation mutagenesis (Zhou et al. 2016). Seven mutants, Y205L, Y205M, Y205F, Y205C, Y205I, Y205S, and Y205N, showed comparable or slightly lower activity than the non-mutated FtmPT1 toward its natural substrate brevianamide F. However, different products were identified in the reaction mixtures. Like FtmPT1, Y205M and Y205F converted brevianamide F mainly to a regularly C2-prenylated derivative, while Y205L, Y205C, Y205I, Y205S, and Y205N catalyzed both regular C2 and C3 prenylations of brevianamide F. The ratios of C3- and C2-prenylated derivatives varied between 1:1.5 and 2.3:1 (Zhou et al. 2016).

In this study, additional Gly115 mutants were created by saturation mutagenesis. HPLC analysis of the incubation mixtures of 1-naphthol with the obtained mutants revealed that G115A had a higher enzyme activity than the non-mutated FtmPT1 (Table 2 and Fig. S2). HPLC analysis of the reaction mixtures of **1a** with Tyr205 mutants demonstrated that six of these mutants Y205L, Y205M, Y205F, Y205C, Y205I, and Y205S were found to have clearly higher catalytic activities. Then, we mutated the two positions and created four double mutants. HPLC analysis showed that G115A\_Y205C had higher activity toward **1a** than the wild type, but lower than several Tyr205 mutants. Other three tested double mutants showed no or lower activity than FtmPT1. This indicated that combination of both positions is not the best way for increasing the enzyme activity toward **1a**. Afterwards, seven mutants, Y205L, Y205M, Y205F, Y205C, Y205I, Y205S, and G115A\_Y205C, were assayed with nine hydroxynaphthalenes **1a–9a**. Our results demonstrated that several of these mutants showed higher activity toward **1a**, **2a**, **3a**, **4a**, **5a**, and **9a** than the non-mutated FtmPT1 (Table 2). Isolation and structure elucidation revealed that for a given substrate, the products are very similar or identical to those obtained with other prenyltransferases of the DMATS superfamily (Yu et al.

2011). Since **3a–7a** are dihydroxylated isomers, their acceptance by different mutants could be of significant importance for understanding the interactions between the hydroxynaphthalenes and enzymes as well as for further optimization of the enzyme activity toward those substrates.

Prenylated naphthalenes are not as common as prenylated flavonoids or indole derivatives in nature. Therefore, only a few hydroxynaphthalene prenyltransferases have been reported (Haug-Schifferdecker et al. 2010; Kumano et al. 2008), which share no amino acid sequence homology with the members of the DMATS superfamily. Acceptance of hydroxynaphthalenes by DMATS enzymes and increasing their catalytic activity by mutagenesis expand the possibility for production of prenylated hydroxynaphthalenes by chemoenzymatic synthesis or synthetic biology. This could play an important role in the drug discovery and development process.

**Acknowledgements** We thank Lena Ludwig for synthesis of DMAPP, Rixa Kraut and Stefan Newel for taking MS and NMR spectra, respectively.

#### Compliance with ethical standards

**Funding** The Bruker microTOF-Q III mass spectrometer was funded by the Deutsche Forschungsgemeinschaft (INST 160/620-1 to S.-M. L.). Kang Zhou is a recipient of a scholarship from the China Scholarship Council (201308440282).

**Conflict of interest** The authors declare that they have no conflict of interest.

**Human and animal rights** This article does not contain any studies with human participants or animals performed by any of the authors.

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