Appl Microbiol Biotechnol
DOI 10.1007/s00253-015-6452-1

Tyrosine *O*-prenyltransferases TyrPT and SirD displaying similar behavior toward unnatural alkyl or benzyl diphosphate as their natural prenyl donor dimethylallyl diphosphate

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Received: 16 December 2014 / Revised: 23 January 2015 / Accepted: 31 January 2015

Abstract Prenyltransferases of the dimethylallyltryptophan synthase (DMATS) superfamily are involved in the biosynthesis of secondary metabolites and contribute as modification enzymes significantly to structural diversity of natural products. They show usually broad specificity toward their aromatic substrates with regiospecific prenylations on aromatic rings. However, most members of this superfamily exhibit a high specificity toward their prenyl donors and usually accept exclusively dimethylallyl diphosphate (DMAPP). Recently, several indole prenyltransferases from this family were also demonstrated to accept unnatural DMAPP analogs such as methylallyl, 2-pentenyl and benzyl diphosphate for alkylation, or benzylation of the indole ring. Partial or complete shift of the substitution position was observed for these enzymes. In this study, we report the acceptance of these DMAPP analogs by two tyrosine *O*-prenyltransferases TyrPT from *Aspergillus niger* and SirD from *Leptosphaeria maculans* for alkylation or benzylation of tyrosine and derivatives. NMR and mass spectrometry (MS) analyses of nine isolated enzyme products confirmed the regiospecific *O*- or *N*-alkylation or benzylation at position C-4 of the aromatic ring, which is the same prenylation position of these enzymes in the presence of DMAPP.

Keywords DMATS superfamily · Unnatural alkyl donors · Tyrosine and derivatives · Enzyme catalysis

Introduction

Attachment of a prenyl moiety to an aromatic core plays an important role for creation of structural diversity of secondary metabolites (Heide 2009). Prenyltransferases catalyzing such attachments are found in many living organisms including plants, bacteria, and fungi and can be classified in different subgroups based on their amino acid sequences and biochemical properties (Heide 2009; Yazaki et al. 2009; Yu and Li 2012). The members of the dimethylallyltryptophan synthase (DMATS) superfamily catalyze the transfer reactions of prenyl moiety from prenyl donors, usually dimethylallyl diphosphate (DMAPP), to diverse aromatic substrates (Chooi et al. 2012; Yu and Li 2012). The resulted prenylated products often show interesting biological and pharmacological activities distinct from their non-prenylated precursors, which makes these enzymes to be interesting biocatalysts for pharmaceutical and medicinal research (Li 2010; Wollinsky et al. 2012). So far, more than 40 such enzymes have been characterized biochemically by using heterologously overproduced and purified proteins (Fan et al. 2014; Pockrandt et al. 2014; Wunsch et al. 2015; Yu and Li 2012). The majority of these enzymes accepts indole derivatives (Yu and Li 2012), while several members use tyrosine or nitrogen-free aromatic compounds as natural substrates (Fan et al. 2014; Kremer and Li 2010; Pockrandt et al. 2012, 2014). Until now, two L-tyrosine prenyltransferases have been identified and characterized biochemically. The first one, SirD, is involved in the biosynthesis of sirodesmin PL in *Leptosphaeria maculans* (Fig. 1) (Gardiner et al. 2004; Kremer and Li 2010). The very recently identified TyrPT from an unknown biosynthetic pathway in *Aspergillus niger* (Fig. 1) showed similar biochemical features

Electronic supplementary material The online version of this article (doi:10.1007/s00253-015-6452-1) contains supplementary material, which is available to authorized users.

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Published online: 24 February 2015

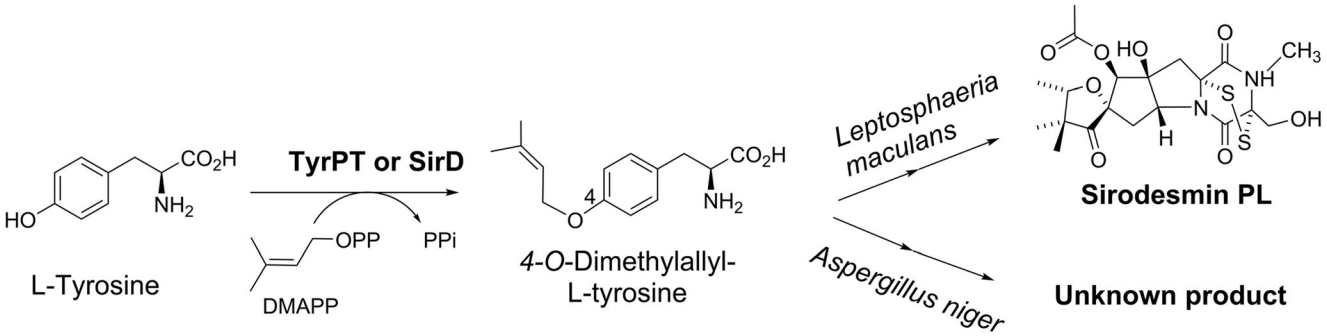


Fig. 1 Prenylations catalyzed by TyrPT and SirD and their role in nature

as SirD (Fan et al. 2014). Both enzymes catalyze the same *O*-prenylation of the hydroxyl group at the benzene ring (Fan et al. 2014; Kremer and Li 2010).

One common feature of the DMATS superfamily enzymes is their high flexibility toward aromatic substrates. In addition to tryptophan derivatives, indole prenyltransferases also accept distinct structures such as hydroxynaphthalenes (Yu et al. 2011), flavonoids (Yu and Li 2011), or xanthones (Tarcz et al. 2014) as prenyl acceptors. The tryptophan *C4*-(FgaPT2) and *C7*-prenyltransferase (7-DMATS) have also been demonstrated to accept tyrosine and derivatives as substrates (Fan et al. 2015; Fan and Li 2014). Similar to the behavior of indole prenyltransferases, the tyrosine prenyltransferases SirD and TyrPT also accept a number of tyrosine derivatives and catalyze the *O*-prenylation of the hydroxyl or *N*-prenylation of the amino group at the aromatic rings (Fan et al. 2014; Kremer and Li 2010; Zou et al. 2011). Furthermore, they also catalyze prenylation reactions of tryptophan and derivatives (Fan et al. 2014; Kremer and Li 2010; Rudolf and Poulter 2013).

In contrast to their high substrate flexibility for acceptors, most of the DMATS enzymes show a restricted specificity toward their prenyl donors, accepting usually DMAPP as prenyl donor (Yu and Li 2012). Only a few enzymes use GPP, DMAPP and GPP, or DMAPP, GPP, and FPP as prenyl donors (Chooi et al. 2010; Pockrandt et al. 2014; Pockrandt and Li 2013; Tarcz et al. 2014). This feature prohibits their application as biocatalysts in chemoenzymatic synthesis. To broaden their specificity for alkyl donors, the acceptance of the unnatural analogs, methylallyl diphosphate (MAPP), 2-pentenyl diphosphate (2-pen-PP), and benzyl diphosphate (benzyl-PP), was tested with two tryptophan prenyltransferases FgaPT2 and 5-DMATS as well as five cyclic dipeptide prenyltransferases BrePT, FtmPT1, AnaPT, CdpNPT, and CdpC3PT in our laboratory (Liebhold et al. 2012, 2013; Liebhold and Li 2013). Our results demonstrated that the tested enzymes also used such donors for alkylation or benzylation. However, the restricted regioselectivity observed with their natural prenyl donor DMAPP has been broken. Partial or complete shifts of alkylation or benzylation were detected in these reactions (Liebhold et al. 2012, 2013; Liebhold and Li 2013). After availability of SirD and TyrPT, we decided to test the behavior of tyrosine *O*-prenyltransferases toward MAPP, 2-pen-PP, and benzyl-PP.

Materials and methods

Chemicals

MAPP, 2-pen-PP, and benzyl-PP were synthesized (Liebhold et al. 2012; Liebhold and Li 2013) following the protocol described for geranyl diphosphate (Woodside et al. 1988). Tyrosine and derivatives used for the enzyme assays were purchased from Bachem (Bubendorf, Switzerland), Sigma-Aldrich (Steinheim, Germany), Alfa Aesar (Karlsruhe, Germany), and TCI (Zwijindrecht, Belgium).

Bacterial strains, plasmids, culture conditions, overproduction, and purification of His-tagged TyrPT and SirD

pGEM-T easy used for cloning and pET28a for expression experiments were obtained from Promega (Mannheim, Germany) and Novagen (Darmstadt, Germany), respectively. *Escherichia coli* XL1 Blue MRF' (Stratagene, Amsterdam, the Netherlands) was used as host for cloning and SoluBL21 (Novagen) for expression experiments. They were grown at 37 °C in liquid Luria-Bertani (LB) or Terrific Broth (TB) medium or on solid LB medium with 1.5 % agar (*w*/*v*). For selection of recombinant *E. coli* strains, 100 μg mL^{-1} of ampicillin or 25 μg mL^{-1} of kanamycin were used.

In this study, SirD was cloned into pET28a and expressed under the control of a strong T7 promoter. Taking the former expression plasmid in pQE70 (pAK2) (Kremer and Li 2010) as template, the coding region of *sirD* was amplified with Expand High Fidelity PCR Kit (Roche Diagnostic, Mannheim, Germany) by using primers SirD_F (5-CC*ATGGCG*ATGCAGACAGCTCGTCTCTTCC-3) at the 5′-end and SirD_R (5-*GCGGCCGC*CTGTCTGTAGCGATTTGGAT-3) at the 3′-end. The underlined letters represent the restriction enzyme sites *Nco*I and *Not*I, respectively, and

italic letters indicate the inserted mutations in comparison to the original gene sequence. PCR amplification was carried out on an iCycler thermal cycler (BioRad, Munich, Germany) with annealing temperature at 56 °C. The PCR fragment of 1360 bp was cloned into pGEM-T easy vector and subsequently sequenced by Eurofins Genomics (Ebersberg, Germany) to confirm the sequence, resulting in the plasmid pHY06. The coding region was then released with *Nco*I and *Not*I and ligated into pET28a vector, which had been digested with the same enzymes previously. The resulted plasmid pHY07 was used for overproduction of SirD. pHC16 described in a previous publication (Fan et al. 2014) was used for production of TyrPT.

For gene expression, *E. coli* SoluBL21 cells harboring pHY07 or pHC16 were cultivated in a 2000-mL cylindrical flask containing 1000 mL liquid TB medium supplemented with kanamycin (25 μg mL^{-1}). After growing at 37 °C to an absorption of 0.6 at 600 nm, the gene expression was induced with isopropyl thiogalactoside at a final concentration of 0.8 mM for TyrPT and 0.5 mM for SirD at 30 °C for 16 h. The bacterial cultures were harvested by centrifugation and resuspended in lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) with 2–5 mL g^{-1} wet weight. Lysozyme from the chicken egg white was added at a final concentration of 1 mg mL^{-1}. After incubation on ice for 30 min, the cells were sonicated six times for 10 s each at 200 W. To separate the cellular debris from the soluble proteins, the lysate was centrifuged at 13,000 rpm and 4 °C for 30 min. One-step purification of the recombinant His_6-tagged fusion protein by affinity chromatography with Ni-NTA agarose resin (Macherey-Nagel, Düren, Germany) was carried out according to the manufacturer's instruction. The protein was eluted with 250 mM imidazole in 50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0. In order to remove imidazole, the protein fraction was passed through a NAP-5 column (GE Healthcare, Freiburg, Germany), which had been equilibrated with 50 mM Tris-HCl (pH 7.5) containing 15 % (*v*/*v*) glycerol. The purified TyrPT and SirD were eluted with the same buffer as for column equilibration and stored at −80 °C for enzyme assays. The protein concentration was determined on Nanodrop C 2000 (Thermo Scientific, Braunschweig, Germany) and then analyzed on 12 % (*w*/*v*) SDS-PAGE (Laemmli 1970) and stained with Coomassie Brilliant Blue G-250.

Enzyme assays for TyrPT and SirD

For determination of the TyrPT and SirD activity, the reaction mixtures (100 μL) contained 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 1 mM aromatic substrate (Table 1), 1 mM DMAPP or analogs, 1.5–5 % (*v*/*v*) glycerol, 0–5 % dimethyl sulfoxide (DMSO), and 20 μg of the purified recombinant protein. After incubation at 37 °C for 16 h, the reactions were terminated by addition of 100 μL methanol. Protein was removed by centrifugation at 13,000 rpm for 20 min before injection on HPLC. Two to four independent assays were carried out routinely. For determination of kinetic parameters of DMAPP analogs, 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 40 μg purified recombinant protein, 1 mM L-tyrosine or 4-amino-L-phenylalanine, and DMAPP analogs at final concentration of 0.01, 0.02, 0.05, 0.08, 0.1, 0.2, 0.5, 0.8, 1.0, 2.0, and 5.0 mM were used in enzyme assays. The reaction mixtures were incubated at 37 °C for 2 h.

Preparative synthesis of prenylated products for structural elucidation

The enzyme assays for isolation of enzymatic products contained 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$, 1 mM aromatic substrate, 1 mM DMAPP analog, and 2 mg of purified recombinant protein, in a total volume of 10 mL. The reaction mixtures were incubated in glass vials at 37 °C for 16 h, and the reactions were terminated by addition of 1 volume of methanol. After centrifugation at 6000 rpm for 30 min to precipitate protein, the supernatants were concentrated on a rotary evaporator at 35 °C to 1–2 mL and used as samples for separation on HPLC.

HPLC analysis and isolation of enzyme products for structure elucidation

The enzyme assays of TyrPT and SirD were routinely analyzed on an Agilent HPLC series 1200 (Agilent, Böblingen, Germany) by using a Multospher 120 RP-18 column (250×4 mm, 5 μm C + S Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 mL min^{-1}. Double-distilled water (solvent A) and methanol (solvent B) were used as solvents. To analyze the enzyme products, a linear gradient of 30–100 % (*v*/*v*) solvent B in 20 min was used. The column was then washed with 100 % (*v*/*v*) solvent B for 5 min and equilibrated with 30 % (*v*/*v*) solvent B for another 5 min. Detection was carried out by a Photo Diode Array detector and illustrated for absorption at 277 nm in this study.

For structure elucidation, the products were isolated on a Multospher 120 RP 18 column (250×10 mm, 5 μm) with a linear gradient of 60–100 % (*v*/*v*) of methanol (solvent B) in water (solvent A) in 60–80 min and a flow rate of 2.5 mL min^{-1}. After each run, the column was then washed with 100 % (*v*/*v*) solvent B for 10 min and equilibrated with 60 % (*v*/*v*) solvent B for 10 min. The collected fractions were evaporated to dryness and subjected to NMR and mass spectrometry (MS) analyses.

Table 1 Product yields of TyrPT and SirD reactions with tyrosine and derivatives in the presence of DMAPP and analogs

Substrate	DMAPP		MAPP		2-pen-PP		benzyl-PP	
	Conversion (%)		Conversion (%)		Conversion (%)		Conversion (%)	
	TyrPT	SirD	TyrPT	SirD	TyrPT	SirD	TyrPT	SirD
L-Tyrosine (1a)	89.1±6.20	99.3±0.06	21.2±0.72	28.0±2.7	56.6±6.21	44.3±2.90	2.2±0.27	1.3±0.24
4-Amino-L-phenylalanine (2a)	78.1±0.36	66.1±1.38	57.7±1.45	34.8±0.20	46.4±0.52	42.6±2.57	67.2±3.84	54.6±1.54
3-Fluoro-DL-tyrosine (3a)	50.1±0.15	66.5±3.24	13.1±1.15	8.9±0.66	43.9±4.69	20.5±1.23	1.5±0.40	0.4±0.05
3,4-Dihydroxy-L-phenyl-alanine (4a)	100±0.0	88.2±1.28	5.8±0.15	7.3±0.23	30.2±0.18	14.3±0.77	0.4±0.02	0.3±0.10
α-Methyl-L-tyrosine (5a)	98.3±0.03	89.4±0.44	2.9±0.06	16.0±2.11	10.3±0.44	33.5±2.23	0.1±0.02	0.5±0.01
3-Iodo-L-tyrosine (6a)	100±0.0	100±0.0	4.9±0.37	1.1±0.21	12.0±0.89	3.0±0.08	0.2±0.03	0.2±0.04
3,5-Dibromo-L-tyrosine (7a)	20.6±0.30	53.3±4.26	2.8±0.09	0.7±0.15	3.9±0.18	1.4±0.17	0.3±0.08	0.2±0.05
D-Tyrosine (8a)	59.5±0.30	57.6±4.10	0.1±0.04	0.2±0.09	1.7±0.15	0.8±0.08	0.1±0.02	0.2±0.02
3-Amino-3-(4-hydroxyphenyl)-propanoic acid (9a)	10.1±0.18	8.7±0.04	1.7±0.06	1.6±0.02	1.8±0.14	1.8±0.12	2.0±0.04	2.0±0.23
3-(3,4-Dihydroxyphenyl)-DL-serine (10a)	16.1±0.54	29.9±1.75	0.6±0.04	0.5±0.03	7.1±0.64	1.3±0.38	0.1±0.03	<0.01
DL-m-Tyrosine (11a)	1.4±0.02	0.3±0.02	0.7±0.04	0.6±0.02	0.7±0.06	0.6±0.01	0.8±0.01	0.6±0.02
3,5-Diiodo-L-tyrosine (12a)	2.2±0.14	2.2±0.01	0.1±0.03	0.1±0.01	0.1±0.04	0.2±0.07	0.1±0.01	0.1±0.01
3-Nitro-L-tyrosine (13a)	6.6±0.03	44.9±1.71	0.1±0.01	<0.01	0.1±0.02	<0.01	<0.01	<0.01

DMAPP dimethylallyl diphosphate, *MAPP* methylallyl diphosphate, *2-pen-PP* 2-pentenyl diphosphate, *benzyl-PP* benzyl diphosphate

NMR and MS analyses

The isolated enzyme products were dissolved in D_2O or CD_3OD. ^1H NMR spectra were recorded at room temperature on a JEOL ECA-500 spectometer (JEOL, Tokyo, Japan). Two-dimensional spectra HSQC and HMBC were recorded on a Bruker Avance 500 MHz spectrometer (Bruker, Karlsruhe, Germany). All of the spectra were processed with MestReNova 6.0.2 (Metrelab Research, Santiago de Compostella, Spain), and chemical shifts were referenced to the signal of D_2O at 4.79 ppm and that of CD_3OD at 3.30 ppm. The NMR data of isolated products are given in Table 2 and spectra in Figs. S1–S12 in electronic supplementary material.

The isolated compounds were also analyzed by high-resolution electron impact mass spectrometry (HR-EI-MS) on a Micromass Auto Spec spectrometer (Waters, Milford, MA). The obtained MS data are given in Table 3.

Results

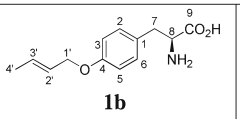
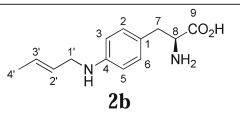
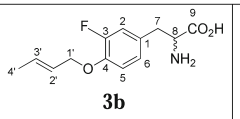
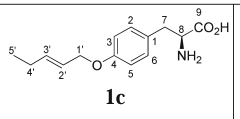
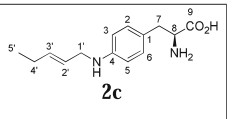
Overproduction and purification of SirD and TyrPT

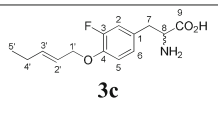
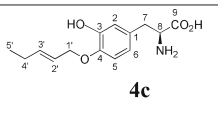
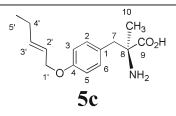
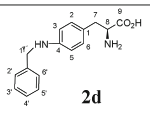
Expression of *sirD* was carried out with the newly constructed plasmid pHY07 in pET28a. Purification of the overproduced SirD led to detection of a predominant protein band on SDS-PAGE (Fig. 2a). With the help of the strong T7 promoter and cultivation in nutrient-rich TB media, the protein yield was improved from 2 to 4 mg L^{-1} bacterial culture. By incubation of L-tyrosine with DMAPP and 2-pen-PP, the activity of SirD from the new construct was proven to be nearly the same of that from the previous construct (data not shown). Therefore, SirD for enzyme assays presented in this study was obtained by using pHY07 as expression vector. TyrPT with a protein yield of 4–6 mg L^{-1} bacterial culture was overproduced and purified as described previously (Fan et al. 2014) (Fig. 2b).

Acceptance of the unnatural DMAPP analogs by tyrosine prenyltransferases

The purified SirD and TyrPT were firstly assayed with DMAPP in the presence of tyrosine (1a) and 12 derivatives (2a–13a). As shown in Table 1, the behaviors of both enzymes for these substrates were similar to those observed previously (Fan et al. 2014; Zou et al. 2011). In comparison to previous works with 3 (Zou et al. 2011) or 8.6 μg proteins per 100 μL assay (Fan et al. 2014), 20 μg were used in this study. As expected, significantly higher conversion yields were obtained for most substrates. The acceptance of 1a–13a by TyrPT and SirD was then tested in the presence of MAPP, 2-pen-PP, and benzyl-PP as alkyl or benzyl acceptors. After incubation with TyrPT and SirD under the same conditions, the reaction mixtures were analyzed on HPLC (Materials and methods). In comparison to negative controls with heat-inactivated proteins (data not shown), product formation was detected in 73 of 78 incubation mixtures (Table 1). Conversion yields of more than 10 % were calculated for 19 enzyme reactions after incubation

Table 2 NMR data of the isolated products 1b–3b, 1c–5c, and 2d

Compound	 **1b**	 **2b**	 **3b**	 **1c**	 **2c**
Position	δ_H, *multi, J*	δ_H, *multi, J*	δ_H, *multi, J*	δ_H, *multi, J*	δ_H, *multi, J*
1	-	-	-	-	-
2	7.19, dd, 8.7, 2.1	7.22, d, 8.5	7.04, dd, 12.5, 2.0	7.19, dd, 8.7, 2.0	7.22, d, 8.4
3	6.88, dd, 8.7, 2.1	6.90, d, 8.5	-	6.88, dd, 8.7, 2.1	6.90, d, 8.4
4	-	-	-	-	-
5	6.88, dd, 8.7, 2.1	6.90, d, 8.5	7.01, t, 8.0	6.88, dd, 8.7, 2.1	6.90, d, 8.4
6	7.19, dd, 8.7, 2.1	7.22, d, 8.5	6.98, dd, 8.5, 1.8	7.19, dd, 8.7, 2.0	7.22, d, 8.4
7	3.23, dd, 14.7, 4.3 2.93, dd, 14.7, 8.8	3.25, dd, 14.7, 5.1 3.09, dd,14.7, 7.9	3.19, dd, 14.7, 4.5 2.94, dd, 14.7, 8.4	3.22, dd, 14.7, 4.3 2.93, dd, 14.7, 8.8	3.21, dd, 14.6, 5.1 3.05, dd, 14.6, 7.9
8	3.71, dd, 8.8, 4.3	3.99, dd, 7.9, 5.1	3.74, dd, 8.4, 4.5	3.70, dd, 8.8, 4.3	3.93, dd, 7.9, 5.1
1’	4.44, dq, 6.0, 1.2	approx. 3.75*	4.49, d, 6.1	4.46, dd, 6.0, 1.2	3.75, d, 6.1
2’	5.68, dtq, 15.3, 6.0, 1.6	5.66, dtq, 15.4, 6.1, 1.7	5.68, dtq, 15.3, 6.1, 1.7	5.87, dtt, 15.4, 6.4, 1.3	5.89, dbrt, 15.5, 6.2
3’	5.84, dqt, 15.3, 6.5, 1.4	5.83, dqt, 15.4, 6.5, 1.4	5.85, dqt, 15.3, 6.5, 1.3	5.66, dtt, 15.4, 6.0, 1.6	5.64, dbrt, 15.5, 6.2
4’	1.72, dd, 6.5, 1.3	1.73, dd, 6.5, 1.4	1.71, dd, 6.5, 1.2	2.09, dqq, 7.2, 6.9, 1.2	2.10, dq, 7.5, 7.4
5’	-	-	-	1.01, t, 7.5	1.01, t, 7.5
Solvent	CD_3OD	D_2O	CD_3OD	CD_3OD	D_2O

Compound	 **3c**	 **4c**		 **5c**	 **2d**
Position	δ_H, *multi, J*	δ_H, *multi, J*	δ_C	δ_H, *multi, J*	δ_H, *multi, J*
1	-	-	128.9	-	-
2	7.03, dd, 12.4, 2.0	6.86, brs	116.6	7.14, d, 8.6	7.18, d, 8.4
3	-	-	145.8	6.83, d, 8.7	6.90, d, 8.5
4	-	-	145.4	-	-
5	7.00, t, 8.0	7.06, d, 8.3	115.3	6.83, d, 8.7	6.90, d, 8.5
6	6.97, dd,8.4, 1.9	6.82, d, 8.3	121.3	7.14, d, 8.6	7.18, d, 8.4
7	3.13, dd, 14.6, 4.7 2.88, dd, 14.6, 7.9	3.19, dd, 14.6, 5.0 3.01, dd, 14.6, 7.9	35.8	3.16, d, 14.2 2.77, d, 14.2	3.22, dd, 14.7, 5.1 3.04, dd, 14.7, 8.0
8	3.63, dd, 7.9, 4.7	3.93, dd, 7.6, 5.1	55.9	-	3.95, dd, 8.0, 5.1
9	-	-	174.5	-	-
10	-	-	-	1.42, s	-
1’	4.49, dd, 6.1, 1.1	4.61, d, 6.3	70.3	4.42, d, 5.9	-
2’	5.87, dtt, 15.4, 6.4, 1.3	6.00, d brt, 15.4, 6.2	139.2	5.84, d brt, 15.4, 6.2	7.49, d, 7.4
3’	5.65, dtt, 15.4, 6.1, 1.6	5.73, d brt, 15.4, 6.2	123.1	5.63, d brt, 15,4, 6.0	7.46, t, 7.2
4’	2.07, dq, 7.6, 7.5	2.11, dq, 7.2, 7.0	24.6	2.05, dq, 7.5, 7.4	7.38, t, 7.2
5’	0.99, t, 7.5	1.01, t, 7.5	12.2	0.98, t, 7.5	7.46, t, 7.2
6’	-	-	-	-	7.49, d, 7.4
7’	-	-	-	-	4.43, s
Solvent	CD_3OD	D_2O	D_2O	CD_3OD	D_2O

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

brt broad triplet

* Signals overlapping with ethanol (impurity)

with 20 μg protein at 37 °C for 16 h. Inspection of the HPLC chromatograms of the reaction mixtures revealed the presence of one product peak each. HPLC chromatograms of nine selected enzyme assays are illustrated in Fig. 3.

As given in Table 1, TyrPT and SirD displayed similar preference for tyrosine and derivatives in the presence of all three DMAPP analogs. Higher conversion yields were detected for incubations of TyrPT and SirD in the presence of 2-pen-PP than MAPP, corresponding to those observed with indole prenyltransferases (Liebhold et al. 2012, 2013). 1a and 2a were well accepted by TyrPT and SirD in the presence of MAPP with conversion yields of more than 20 %, while 1a–5a were good acceptors for 2-pen-PP. Conversion yields of more than 20 % were calculated for incubations of 1a–4a with TyrPT and 1a–3a and 5a with SirD. In comparison to MAPP and 2-pen-PP, benzyl-PP was a poor donor for both enzymes. Conversion yields of 67.2 and 54.6 % were detected for 2a with TyrPT and SirD, respectively. Much lower conversion yields of less than 3 % were found for other substrates. As given in Table 1, 2a was accepted as the best substrate by TyrPT and SirD in the presence of MAPP and benzyl-PP. Comparable acceptance was observed for 1a and 2a by both enzymes in the presence of 2-pen-PP. Overall, 2a was the best or one of the best substrates for both TyrPT and SirD in the presence DMAPP analogs, which differs clearly from their preference toward aromatic substrates in the presence of DMAPP. Using DMAPP as prenyl donor, TyrPT showed higher activities toward 4a–6a than 2a and SirD accepted 6a

Table 3 HR-EI-MS data of the isolated enzyme products

Product	Formula	Calculated (M_r)	Measured $[M]^+$	Deviation (ppm)
1b	$C_{13}H_{17}NO_3$	235.1208	235.1215	−3.0
2b	$C_{13}H_{18}N_2O_2$	234.1368	234.1379	−4.5
3b	$C_{13}H_{16}FNO_3$	253.1114	253.1109	2.0
1c	$C_{14}H_{19}NO_3$	249.1365	249.1378	−5.2
2c	$C_{14}H_{20}N_2O_2$	248.1525	248.1530	−2.0
3c	$C_{14}H_{18}FNO_3$	267.1271	267.1285	−5.2
4c	$C_{14}H_{19}NO_4$	265.1314	265.1323	−3.4
5c	$C_{15}H_{21}NO_3$	263.1521	263.1533	−4.6
2d	$C_{16}H_{18}N_2O_2$	270.1368	270.1384	−5.9

much better than 2a (Table 1). It seems that the 4-amino group of 2a had a better interaction with the enzymes in the presence of the unnatural donors, so that the cation intermediates (Liebhold et al. 2012) are more stable than in the cases of other aromatic substrates.

In a previous study, TyrPT and SirD were demonstrated to be low stereoselective in the presence of DMAPP as prenyl donor (Fan et al. 2014). D-Tyrosine (8a) was accepted by TyrPT and SirD with relative activities of 50 and 30 % of those of 1a, respectively, which was also confirmed in this study (Table 1). As given in Table 1, 8a was a very poor substrate for both enzymes in the presence of the three DMAPP analogs, and conversion yields of less than 2 % were detected in this study. It can be speculated that in the presence of the unnatural alkyl or benzyl donors, both enzymes had changed

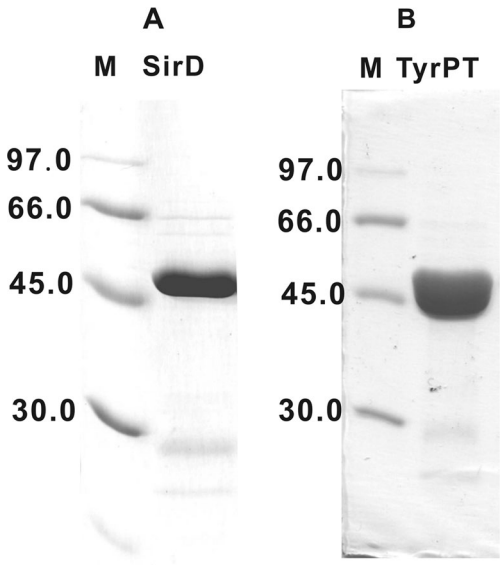


Fig. 2 SDS-PAGE analysis of purified TyrPT-His_6 and SirD-His_6. The proteins were separated on a 12 % SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. **a** Purified SirD and **b** purified TyrPT; *M* molecular mass standard

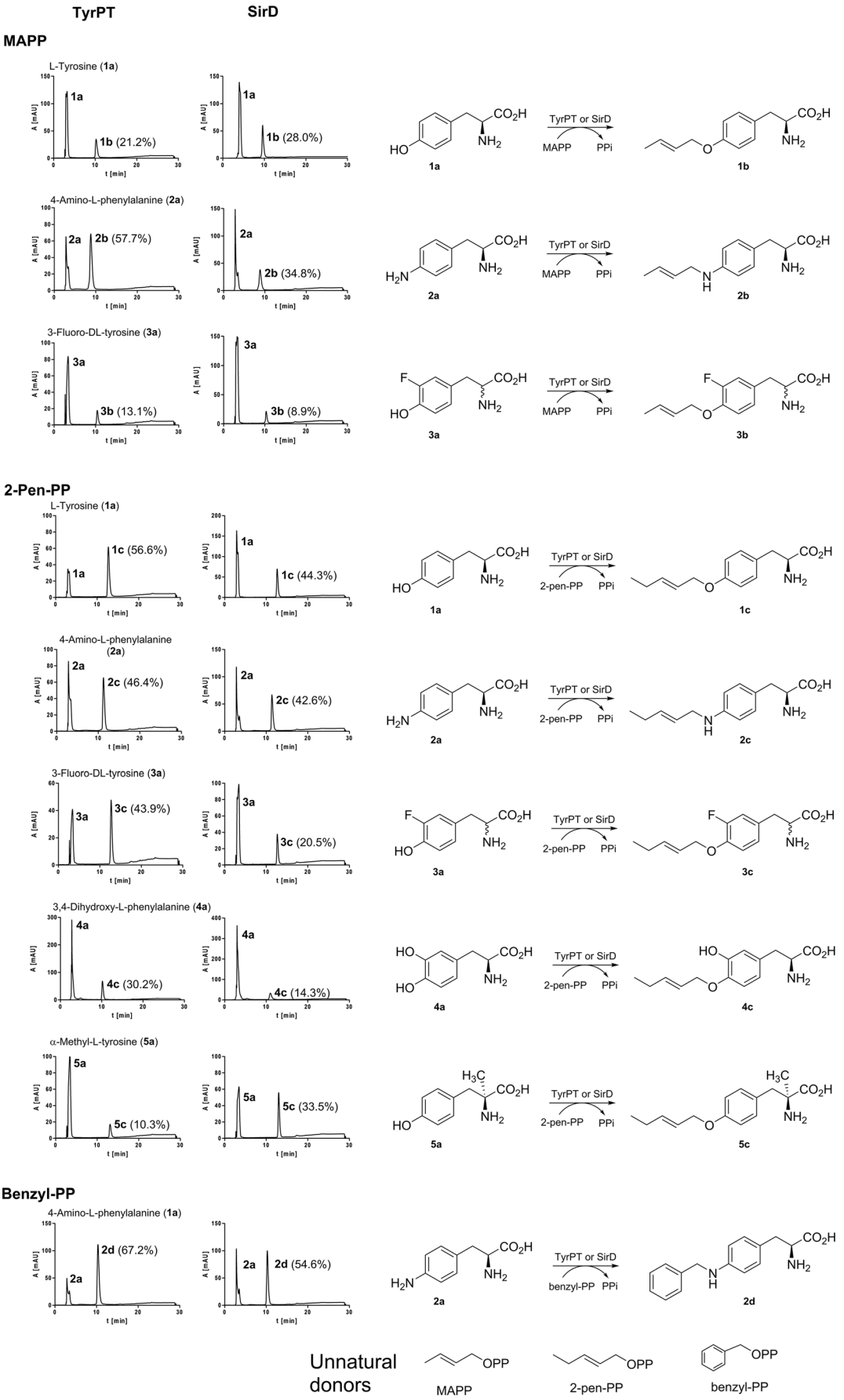
Fig. 3 HPLC analysis of the incubation mixtures of selected substrates with TyrPT (*left column*) and SirD (*middle column*) as well as the transfer reactions catalyzed by TyrPT and SirD (*right column*). The reaction mixtures contained 20 μg TyrPT or SirD, 1.0 mM of aromatic substrate, 1.0 mM DMAPP analog, 10 mM $CaCl_2$, and 50 mM Tris-HCl (pH 7.5) and were incubated at 37 °C for 16 h. The illustrated chromatograms were recorded at 277 nm. Product yields are given in *parentheses* after the product numbers

their conformations, so that 8a was much poorly fixed into the reaction cavities of both enzymes than 1a.

TyrPT and SirD catalyze the same *O*-/*N*-alkylation/benzylation of tyrosine and derivatives

For structure elucidation, nine enzyme products were isolated on preparative HPLC from the incubation mixtures of TyrPT and SirD in different combinations of tyrosine and derivatives with the three DMAPP analogs. 1b–3b were obtained from the incubation mixtures of 1a–3a with MAPP, respectively, 1c–5c from those of 1a–5a with 2-pen-PP, and 2d from that of 2a with benzyl-PP. The obtained products were subjected to MS and NMR analyses. Identical ^{1}H NMR spectra of the enzyme products were obtained from incubation mixtures of TyrPT and SirD with a given substrate illustrated in Fig. 3 (data not shown), proving the same function of both enzymes in the presence of unnatural DMAPP analogs.

Molecular formula deduced from high-resolution electron impact mass spectrometry (Table 3) proved the nine isolated products to be monoprenylated or benzylated derivatives of the respective substrate. Inspection of the ^{1}H NMR spectra (Figs. S1–S12 in the Supplementary Material) of 1b–3b, 1c–5c, and 2d revealed the presence of only one product each. The coupling pattern of the signals for the methylallyl and 2-pentenyl residues are comparable to those of previously reported compounds with a regular alkyl moiety (Liebhold et al. 2012), proving their attachment in a regular pattern. In comparison to those of the respective substrate, the chemical shifts, number, and coupling pattern of the signals for protons at the aromatic ring and the side chain of the isolated products were nearly unchanged and are similar to those of the prenylated derivatives with DMAPP (Fan et al. 2014; Kremer and Li 2010; Zou et al. 2011). All these observations suggested the alkylation at the hydroxyl or amino group of the aromatic ring. The chemical shifts of H-1' of the alkyl moieties for products 1b, 1c, 3b, 3c, and 5c between 4.44 and 4.49 ppm in CD_3OD and for 4c at 4.61 ppm in D_2O proved unequivocally their attachment to *O*-atoms (Fan et al. 2014; Kremer and Li 2010; Zou et al. 2011). In the case of 3,4-dihydroxy-L-phenylalanine (4a), *O*-alkylation at position C-3 or C-4 would be possible. The correlations found between H-1' and C-4 in the HMBC spectra of 4c (Fig. 4 and Figs. S9 and S10 in the Supplementary Material) revealed that the 2-pentenyl moiety was attached to *O*-atom at position C-4


TyrPT
SirD
MAPP
L-Tyrosine (1a)
1a
1b (21.2%)
1b (28.0%)
4-Amino-L-phenylalanine (2a)
2a
2b (57.7%)
2b (34.8%)
3-Fluoro-DL-tyrosine (3a)
3a
3b (13.1%)
3b (8.9%)
TyrPT or SirD
MAPP
PPi
2-Pen-PP
1c (56.6%)
1c (44.3%)
2c (46.4%)
2c (42.6%)
3c (43.9%)
3c (20.5%)
3,4-Dihydroxy-L-phenylalanine (4a)
4a
4c (30.2%)
4c (14.3%)
α-Methyl-L-tyrosine (5a)
5a
5c (10.3%)
5c (33.5%)
2-pen-PP
Benzyl-PP
2d (67.2%)
2d (54.6%)
benzyl-PP
Unnatural donors
MAPP
2-pen-PP
benzyl-PP

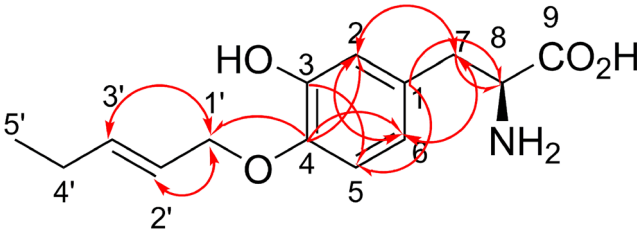


Fig. 4 Selected HMBC correlations observed in 4c

of the aromatic ring. The chemical shifts of H-1' of the alkyl moiety at 3.75 ppm were observed in the spectra of the products 2b and 2c from the incubation mixtures of 2a with MAPP and 2-pen-PP, proving an *N*-alkylation in their structures (Fan et al. 2014; Zou et al. 2011). In the spectrum of 2d from the incubation mixture of 2a with benzyl-PP, the signals for both benzene rings have been nearly unchanged and the signal of the methylene group was detected at 4.43 ppm. These data proved unequivocally the benzylation of the amino group at the aromatic ring. In conclusion, both TyrPT and SirD accepted unnatural alkyl/benzyl donors and catalyzed the *O*-/*N*-alkylation/benzylation of tyrosine and derivatives at the same position as in the presence of their natural prenyl donor DMAPP.

Kinetic parameters of TyrPT and SirD toward the unnatural DMAPP analogs

To compare the catalytic efficiencies of TyrPT and SirD in the presence of unnatural alkyl or benzyl donors with those of their natural prenyl donor DMAPP, kinetic parameters including Michaelis-Menten constant (K_M) and turnover number (k_{cat}) were determined for MAPP and 2-pen-PP by using 1a as substrate (Table 4 and Figs. S13–S16 in the Supplementary Material). Due to the low conversions of benzyl-PP with 1a, its kinetic parameters were obtained from incubation mixtures with 2a (Table 4 and Figs. S17 and S18 in the Supplementary Material). MAPP was accepted by both enzymes with very small K_M values, lower than those of 2-pen-PP, benzyl-PP, and DMAPP. All three unnatural donors showed much lower turnover numbers than those of DMAPP.

Discussion

As aforementioned, TyrPT and SirD belong to the prenyltransferases of the DMATS superfamily. Similar to other members of this family, TyrPT and SirD showed high substrate flexibility toward tyrosine and derivatives and catalyzed regioselective transfer reactions, i.e., the 4-*O*- or 4-*N*-prenylation (Fan et al. 2014; Kremer and Li 2010; Zou et al. 2011). Both enzymes also accepted tryptophan and derivatives as substrates and mainly catalyzed the *C7*-prenylation at the indole ring (Fan et al. 2014; Kremer and Li 2010; Rudolf and Poulter 2013). In comparison, the tryptophan *C4*-prenyltransferase FgaPT2 and *C7*-prenyltransferase 7-DMATS also accepted L-tyrosine and a few number of its derivatives as prenylation substrates, demonstrating the complementary substrate promiscuity of tryptophan and tyrosine prenyltransferases. However, differing from the two tyrosine *O*-prenyltransferases TyrPT and SirD with the same *C7*-prenylated product from L-tryptophan, FgaPT2 catalyzed a *C3*- and 7-DMATS an *O*-prenylation of tyrosine, respectively (Fan et al. 2015; Fan and Li 2014). Similar to most of the DMATS enzymes, these two enzymes showed strict substrate specificity toward DMAPP and did not accepted geranyl diphosphate as prenyl donor (Kremer and Li 2010) (data for TyrPT not shown). Acceptance of MAPP, 2-pen-PP, and benzyl-PP by TyrPT and SirD presented in this study provided experimental evidence on their substrate promiscuity for prenyl donor and expanded their potential usage for increasing the structural diversity of alkylated compounds in the chemoenzymatic synthesis.

In previous studies, we have demonstrated that several indole prenyltransferases including tryptophan and tryptophan-containing cyclic dipeptide prenyltransferases used MAPP and 2-pen-PP as alkyl donors and the tryptophan *C4*-

Table 4 Comparison of kinetic parameters of TyrPT and SirD toward alkyl/benzyl donors

Donor	TyrPT			SirD		
	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} mM^{-1})
DMAPP[a]	0.71	41.4	58.3	0.17	60	352.9
MAPP[b]	0.029±0.0036	0.12±0.013	4.14	0.13±0.025	0.16±0.03	1.23
2-pen-PP[b]	0.23±0.052	0.20±0.022	0.87	0.22±0.053	0.13±0.004	0.59
benzyl-PP[c]	0.52±0.022	0.96±0.003	1.85	0.75±0.0021	1.68±0.01	2.24

DMAPP dimethylallyl diphosphate, *MAPP* methylallyl diphosphate, *2-pen-PP* 2-pentenyl diphosphate, *benzyl-PP* benzyl diphosphate

[a] Data for DMAPP were adopted from previous studies (Fan et al. 2014; Kremer and Li 2010)

[b] Data were obtained from four independent assays by using 1a as aromatic substrate

[c] Data were obtained from two independent assays by using 2a as aromatic substrate

prenyltransferase FgaPT2 even used benzyl-PP for benzylation (Liebhold et al. 2012, 2013; Liebhold and Li 2013). These enzymes catalyzed regiospecific and stereospecific prenyl transfer reactions from DMAPP onto indole rings (Grundmann and Li 2005; Unsöld and Li 2005; Yin et al. 2009, 2010, 2013; Yu and Li 2012). In the presence of the unnatural DMAPP analogs, the alkylation/benzylation positions were partially or completely shifted to the neighboring position, e.g., from exclusively at C-2 with *C2*-prenyltransferases BrePT and FtmPT1 to both C-2 and C-3 (Liebhold et al. 2013). *C2*- and *C3*-alkylations with MAPP and 2-pen-PP were also observed for the three *C3*-prenyltransferases AnaPT, CdpNPT, and CdpC3PT (Liebhold et al. 2013). The alkylation positions of the two tryptophan prenyltransferases FgaPT2 and 5-DMATS were partially (in the presence of MAPP) or completely (in the presence of 2-pen-PP) shifted (Liebhold et al. 2012). The tryptophan *C4*-prenyltransferase FgaPT2 catalyzed the benzylation at C-5 of the indole ring (Liebhold and Li 2013). As shown in Fig. 3, only one enzyme product each was detected in the incubation mixtures of selected well accepted substrates and identified as *4-O*- or *4-N*-alkylated or benzylated derivative of the respective substrate. This means that the TyrPT and SirD products with the unnatural DMAPP analogs carry the alkyl/benzyl moieties at the same position as their products with the natural donor DMAPP, although alkylation at other positions of tyrosine and derivatives such as C-3 would be possible. Together with N-acetyl-3-prenyl-L-tyrosine, 3-dimethylallyl-L-tyrosine was isolated from *Streptomyces* sp. IFM 10937 (Ahmed et al. 2008). Members of the DMATS superfamily are also able to catalyze a *C3*-prenylation of tyrosine or derivatives, as demonstrated very recently with FgaPT2 and especially with its mutant FgaPT2_K174F by using L-tyrosine (1a) and 4-amino-L-phenylalanine (2a) as substrates (Fan et al. 2015). This high regioselectivity of TyrPT and SirD toward tyrosine and derivatives with different alkyl donors would be of essential importance for controlled structure modification.

As mentioned in the "Introduction" section, SirD is involved in the biosynthesis of sirodesmin (Fig. 1) (Gardiner et al. 2004; Kremer and Li 2010) and the genetic context of TyrPT in *A. niger* is unknown (Fig. 1) (Fan et al. 2014). As shown in this and in the previous studies (Fan et al. 2014; Kremer and Li 2010; Zou et al. 2011), both enzymes display similar substrate specificities toward donors and acceptors. It can be speculated that the structures of these two enzymes are highly conserved. Furthermore, both DMAPP and the tested DMAPP analogs are placed in their reaction chambers in similar positions and undergo similar reactions.

Determination of the kinetic parameters revealed that TyrPT and SirD showed high affinity to MAPP and 2-pen-PP (Table 4). However, the turnover numbers of the TyrPT and SirD reactions with the three unnatural DMAPP analogs were found between 0.1 and 1.0 min^{-1}, comparable to those of the cyclic dipeptide prenyltransferases (Liebhold et al. 2013) and lower than those of tryptophan prenyltransferases with these DMAPP analogs (Liebhold et al. 2012). The low catalytic efficiency of TyrPT and SirD toward MAPP, 2-pen-PP, and benzyl-PP should be improved in the future by site-directed mutagenesis experiments after availability of the structures of TyrPT or/and SirD.

Acknowledgments We thank Nina Zitzer and Stefan Newel for taking MS and NMR spectra, respectively. This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (Li844/4-1 to S.-M L.). Huili Yu is a recipient of a scholarship from the China Scholarship Council.

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