Appl Microbiol Biotechnol
DOI 10.1007/s00253-014-5509-x



# A promiscuous prenyltransferase from *Aspergillus oryzae* catalyses C-prenylations of hydroxynaphthalenes in the presence of different prenyl donors

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Received: 18 November 2013 / Revised: 23 December 2013 / Accepted: 26 December 2013


**Abstract** Prenyltransferases of the dimethylallyltryptophan synthase (DMATS) superfamily are involved in the biosynthesis of secondary metabolites and show broad substrate specificity towards their aromatic substrates with a high regioselectivity for the prenylation reactions. Most members of this superfamily accepted as prenyl donor exclusively dimethylallyl diphosphate (DMAPP). One enzyme, AnaPT from *Neosartorya fischeri*, was reported recently to use both DMAPP and geranyl diphosphate (GPP) as prenyl donors. In this study, we demonstrate the acceptance of DMAPP, GPP and farnesyl diphosphate (FPP) by a new member of this superfamily, BAE61387 from *Aspergillus oryzae* DSM1147, for C-prenylations of hydroxynaphthalenes.

**Keywords** DMATS superfamily · Prenyl donor · Hydroxynaphthalene · Enzyme catalysis

## Introduction

Prenyltransferases of the dimethylallyltryptophan synthase (DMATS) superfamily are mainly found in fungi of ascomycetes and involved in secondary metabolite biosynthesis. They catalyse transfer reactions of a prenyl moiety (n×C5 units), usually from prenyl diphosphate, e.g. dimethylallyl diphosphate (DMAPP), onto aromatic ring systems of diverse substrates (Yu and Li 2012). Prenylation usually increases the lipophilicity of the resulted products and strengthens their interaction with proteins and biomembranes and makes these enzymes interesting for pharmaceutical and medicinal research (Botta et al. 2005).

Molecular biological and biochemical investigations with genes/enzymes from this superfamily in recent years have revealed that these enzymes displayed much higher flexibility towards their aromatic substrates than our knowledge 5 years ago. At that time, substrates of known prenyltransferases from this family were exclusively indole derivatives (Steffan et al. 2009). It is known today that several enzymes from this group use tyrosine or nitrogen-free aromatic compounds such as xanthones, anthracene or naphthacene derivatives as natural substrates (Chooi et al. 2012, 2013; Kremer and Li 2010; Pockrandt et al. 2012; Sanchez et al. 2011). Biochemical investigations have also revealed that several members from this superfamily used distinctly different compounds from their natural substrates and catalysed regiospecific prenylations. These include hydroxynaphthalenes (Yu et al. 2011), flavonoids (Yu and Li 2011) and tyrosine derivatives (Zou et al. 2011).

The feature of relaxed substrate specificity of the prenyltransferases was successfully used in chemoenzymatic synthesis for the production of prenylated derivatives (Yu and Li 2012). On the other hand, most of these enzymes are restricted to the common prenyl donor DMAPP ($C_5$-unit). VrtC from *Penicillium aethiopicum* utilizes geranyl diphosphate (GPP) ($C_{10}$-unit), but not DMAPP as prenyl donor (Chooi et al. 2010). We have very recently demonstrated the acceptance of GPP by the dimethylallyltransferase AnaPT from *Neosartorya fischeri* and the regiospecific geranylation of tryptophan-containing cyclic dipeptides (Pockrandt and Li 2013). These results provided experimental evidence for the relationship of DMAPP- and GPP-using enzymes from this family. We

C. Sack and T. Kosiol contributed to this manuscript as part of their practical training course.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00253-014-5509-x) contains supplementary material, which is available to authorized users.

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Published online: 16 January 2014

speculated that some members of the DMATS superfamily could be even capable of utilizing DMAPP, GPP and farnesyl diphosphate ($C_{15}$-unit, FPP) as prenyl donors (Pockrandt and Li 2013). However, none of the known enzymes so far accepted FPP as prenyl donor. We took the new challenge of searching for prenyltransferases with broad substrate specificity towards their prenyl donors. Here, we report the biochemical characterization of such an enzyme BAE61387 from *Aspergillus oryzae* DSM1147 for prenylation of hydroxynaphthalenes.

## Materials and methods

### Computer-assisted sequence analysis

The software FGENESH (Softberry, Inc; http://www.softberry.com/berry.phtml) was used for prediction of exon and intron sequences. Alignments of amino acid sequences were carried out by using the program BLAST (http://blast.ncbi.nlm.nih.gov).

### Chemicals

DMAPP, GPP and FPP were synthesized as trisammonium salts according to the method described for GPP trisammonium (Woodside et al. 1988). Naphthalene derivatives were purchased in the highest available purity from Fluka, TCI and Acros Organics.

### Bacterial strains, plasmids and culture conditions

The expression vector pQE60 was obtained from Qiagen (Hilden, Germany). *Escherichia coli* strain XL1 Blue MRF′ (Stratagene, Amsterdam, the Netherlands) was used for cloning and gene expression. Bacteria harbouring the vectors were grown in liquid or on solid lysogeny broth (LB) media supplemented with agarose (1.5 %*w*/*v*) at 37 °C. Carbenicillin (50 μg $mL^{-1}$) was used for selection of recombinant *E. coli* strains.

### DNA isolation and cloning

Standard procedures for DNA isolation and cloning were performed as described elsewhere (Sambrook and Russell 2001). Genomic DNA for PCR amplification was isolated from *A. oryzae* strain DSM1147 by using phenol/chloroform extraction. The coding sequence of AO090102000322 was amplified using the primer pair BAE61387_*Nco*I (5′-GAA**CCATGG**CTCTTCGGAACG-3′) and BAE61387_*Bgl*II (5′-GA**AGATCT**AACATCAACTGTT-3′). Bold letters represent introduced restriction sites for subsequent cloning. The resulting PCR product was cloned into pGEM-T Easy (Promega) to give the plasmid pDP003, which was sequenced to confirm sequence integrity. In order to create an expression construct, the coding sequence was excised from pDP003 by digestion with *Nco*I and *Bgl*II. The *Nco*I-*Bgl*II fragment of 1,346 bp was isolated from agarose gel and ligated into pQE60 that had been previously digested with *Nco*I and *Bgl*II, yielding the expression plasmid pDP004.

### Overproduction and purification of BAE61387-$His_6$

For gene expression, *E. coli* XL1 Blue MRF′ cells harbouring plasmid pDP004 were cultivated in 2,000-mL Erlenmeyer flasks containing liquid LB media (1,000 mL) supplemented with carbenicillin (50 μg $mL^{-1}$) at 37 °C and 220 rpm to $A_{600}$=0.6. For gene induction, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. The bacteria were cultivated for a further 16 h and centrifuged to harvest the cells, which were then resuspended in lysis buffer (imidazole (10 mM), NaCl (300 mM), $NaH_2PO_4$/$Na_2HPO_4$ (50 mM, pH 8.0)) at 1.4 mL/g of pellet. After addition of the lysozyme (1 mg $mL^{-1}$) and incubation on ice for 30 min, the cells were sonicated (6× 10 s, 200 W). To separate cellular debris from soluble proteins, the lysate was centrifuged (14,000×*g*, 30 min, 4 °C). One-step purification of the recombinant BAE61387-$His_6$ by affinity chromatography with Ni-NTA agarose resin (Qiagen) was carried out according to the manufacturer's instruction. Proteins were eluted with elution buffer (imidazole (250 mM), NaCl (300 mM), $NaH_2PO_4$/$Na_2HPO_4$ (50 mM, pH 8.0)). For buffer exchange, the protein fractions were passed through PD-10 columns (GE Healthcare, Freiburg, Germany) that had been equilibrated with Tris-HCl (50 mM, pH 7.5) and glycerol (15 %*v*/*v*). The proteins were eluted with the same buffer, frozen in liquid nitrogen and stored at −80 °C until use. The protein fractions obtained from the purification process were analysed on SDS-PAGE according to the method of Laemmli (1970) and stained with Coomassie Brilliant Blue R-250. The molecular mass of the recombinant BAE61387-$His_6$ was determined on a HiLoad 16/60 Superdex 200 column (GE Healthcare, Freiburg, Germany) by using 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl as elution buffer. The column was calibrated with dextran blue 2000 (2,000 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa) (GE Healthcare).

### Enzyme assays

To determine the relative activity, enzyme assays (100 μL) contained Tris-HCl buffer (50 mM, pH 7.5), aromatic substrate (1.0 mM), DMAPP, GPP or FPP (2 mM), BAE61387 (40 μg), $CaCl_2$ (5 mM), glycerol (3 %*v*/*v*) and dimethyl sulfoxide (DMSO; 2 %*v*/*v*). The reaction mixtures were

incubated at 37 °C with mild agitation on a shaker for 16 h unless otherwise stated. After incubation, enzyme assays were extracted twice with two volumes of ethyl acetate, and the combined organic phases were evaporated to dryness in a vacuum centrifuge. The residue was dissolved in chloroform/methanol (4:1) and analysed by HPLC. For structure elucidation, enzyme assays were scaled up to 40 mL. The reaction mixtures contained Tris-HCl buffer (50 mM, pH 7.5), hydroxynaphthalenes (500 mM), DMAPP, GPP or FPP (1 mM), $CaCl_2$ (5 mM) and BAE61387 (6 mg) were incubated at 37 °C for 16 h. The assays for determination of the kinetic parameters of hydroxynaphthalenes contained DMAPP (2 mM), BAE61387 (40 μg), $CaCl_2$ (5 mM) and hydroxynaphthalenes (0.1–6.0 mM). Assays were incubated at 37 °C for 60 min. For determination of the kinetic parameters of DMAPP, GPP and FPP, the same dilution steps for the prenyl donors were used, **2a** at 1.0 mM was used as aromatic substrate.

### HPLC analysis

Enzyme assays were analysed on an Agilent Series 1200 HPLC (Agilent, Foster City, CA) with a MultoHigh 100 Si column (250×4 mm, 5 μm; CS Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 mL $min^{-1}$ with chloroform (solvent A) and methanol (solvent B) as elution solvents. The reaction mixtures were separated with a linear gradient of 0–10 % B for 30 min. After washing with 50 % B for 5 min, the column was equilibrated with 100 % A for 5 min. The substances were detected with a photodiode array detector and illustrated for absorption at 254 nm in this study. For isolation of the enzyme products, the same HPLC equipment with a semipreparative MultoHigh 100 Si column (250× 10 mm, 5 μm) at a flow rate of 2.5 mL $min^{-1}$ was used. Conversion yields of enzyme products were calculated from peak areas in HPLC chromatograms.

### NMR and mass spectrometric analyses

For structural elucidation, the isolated enzyme products were subjected to $^1H$ NMR and MS analyses. High-resolution electron impact (HR-EI) MS data were obtained on a Micromass Auto Spec spectrometer (Waters, Milford, MA) and are given in Table 2. For NMR analysis, the enzyme products were dissolved in $CD_3OD$ (650 μL). Spectra were recorded at room temperature with an ECX-500 spectrometer (JEOL, Tokyo, Japan) equipped with a broadband probe with *z*-gradient. Chemical shifts were referenced to the solvent signal at 3.30 ppm. All spectra were processed with MestReNova 5.2.2 (Mestrelab Research, Santiago de Compostella, Spain). NMR data for the isolated products are given in Table 3 and spectra in Figs. S1–S9 in electronic supplementary data.

## Results

### Sequence analysis of the putative prenyltransferase gene AO090102000322

The putative prenyltransferase gene AO090102000322 is located on supercontig 13 of *A. oryzae* RIB40 at bp 868715-870055 (Aspergillus Comparative) (Galagan et al. 2005; Machida et al. 2005) and consists of only one exon of 1,341 bp. The deduced protein BAE61387 comprises 446 amino acids with a calculated molecular mass of 50.5 kDa. BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed low sequence similarity of BAE61387 with known proteins including prenyltransferases of the DMATS superfamily. For example, BAE61387 shares a sequence identity of 28 % with VrtC, a geranyltransferase mentioned above (Chooi et al. 2012), as well as with NscD, a dimethylallyltransferase from *N. fischeri* (Chooi et al. 2013). Both VrtC and NscD use polycyclic polyketide derivatives as aromatic substrates (Chooi et al. 2012, 2013). An orthologous gene AFLA_004300 (=AFL2G_09741, coding for EED47789) was found in the genome of the close relative *Aspergillus flavus* NRRL3357. Both orthologous genes have the same length of nucleotides and amino acid chains and share a sequence identity of 99 % on the amino acid level with each other. Analysis of the neighbouring genes of AO090102000322 on supercontig 13 did not indicate the presence of a putative gene cluster for secondary metabolites (Table S1 in electronic supplementary data). No genes encoding for non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS) or other known secondary metabolite backbone genes could be identified in the vicinity of the prenyltransferase gene AO090102000322. Similar situation was also found for AFLA_004300 in *A. flavus* NRRL3357. The natural substrate of BAE61387 and EED47789 can therefore not be predicted by sequence analysis.

### Gene amplification and cloning, overproduction and purification of BAE61387-$His_6$

To prove the function of BAE61387, the coding region was amplified from genomic DNA of *A. oryzae* DSM1147 by PCR (see "Materials and methods"). The obtained PCR fragment of 1,351 bp was cloned into pGEM-T Easy and verified by sequencing. The coding region was then subcloned into the expression vector pQE60 at the *Nco*I and *Bgl*II sites to create the expression construct pDP004.

For gene expression, *E. coli* cells harbouring pDP004 were cultivated at 37 °C and induced for 16 h with isopropyl β-D-1-thiogalactopyranoside at a final concentration of 1.0 mM. The recombinant protein was purified on Ni-NTA-agarose resin, and a major protein band migrating above the 45 kDa marker band was detected on SDS-PAGE (Fig. 1). The protein size

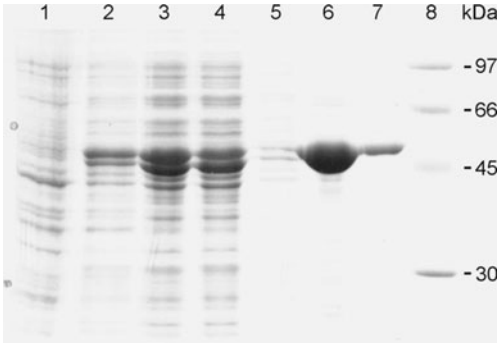



**Fig. 1** SDS-PAGE analysis of the overproduction and purification of BAE61387-$His_6$. The proteins were separated on a 12 % SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. *Lane 1*: total protein before induction, *lane 2*: total protein after induction with IPTG, *lane 3*: soluble fraction after IPTG induction, *lane 4*: flow-through, *lane 5*: wash fraction, *lane 6*: first elution fraction, *lane 7*: second elution fraction, *lane 8*: molecular mass standard

corresponds well with the calculated molecular mass of BAE61387-$His_6$ at 51.5 kDa. A protein yield of 9.0 mg purified BAE61387-$His_6$ was obtained per litre culture. The molecular mass of the native recombinant BAE61387-$His_6$ was determined by size exclusion chromatography as 93.5 kDa, indicating that it presumably acts as a homodimer.

## Enzyme activity and substrate specificity of BAE61387

BAE61387 was assayed in the presence of DMAPP with 34 aromatic substrates, which had been accepted by prenyltransferases of the DMATS superfamily as substrates. They included aromatic amino acids such as tryptophan and tyrosine (Yu et al. 2012; Zou et al. 2011), tryptophan-containing cyclic dipeptides (Yu and Li 2012), hydroxynaphthalenes (Yu et al. 2011), flavonoids (Yu and Li 2011) and xanthones (Pockrandt et al. 2012). The incubation mixtures were analysed on HPLC under routine conditions used in our group for detection of enzyme products. Clear product formation was detected in the incubation mixtures with 7 of 10 tested hydroxynaphthalenes. Low or no product formation was observed in the incubation mixtures with other tested substances (Table 1 and Table S2 in electronic supplementary materials). Assays of hydroxynaphthalenes with GPP and FPP led to detectable product formation in seven and five reaction mixtures, respectively. High conversion yields were achieved for 1-naphthol (**1a**), 1,7-dihydroxynaphthalene (**2a**) and 2,7-dihydroxynaphthalene (**3a**), which were also well accepted by some prenyltransferases of the DMATS superfamily in the presence of DMAPP (Yu et al. 2011). Therefore, the three substrates were chosen for further study. As shown in Fig. 2, one additional dominant peak each was detected in their enzyme assays with DMAPP, GPP and FPP. Product formation in all of the enzyme assays was strictly dependent on the presence of active enzyme and prenyl donors. Negative controls with heat-inactivated enzyme (boiled for 30 min) or without prenyl donor showed no additional peaks in HPLC chromatograms (data not shown). With an exception for the incubation mixture of **3a** and GPP, the enzymatic activity decreased with the increased chain length of the prenyl donor. For example, the conversion yields of the incubation mixtures of **1a-3a** with DMAPP were found between 20 and 72 %, followed by the incubation mixtures with GPP at 15–46 % and those with FPP at 5–18 %. In the incubation mixture of **3a** with GPP, a slightly higher conversion of 29 % was observed than in the enzyme assay with DMAPP of 20 %.

**Table 1** Acceptance of hydroxynaphthalenes by BAE61387

| Substrate | Conversion yield (%) | | |
|---|---|---|---|
| | DMAPP | GPP | FPP |
| 1-naphthol (**1a**) | 51 | 15 | 5 |
| 2-naphthol | 3 | 1 | <0.04 |
| 1,4-dihydroxynaphthalene | <0.02 | <0.02 | <0.01 |
| 1,5-dihydroxynaphthalene | <0.04 | <0.04 | <0.04 |
| 1,6-dihydroxynaphthalene | 12 | 4 | 3 |
| 1,7-dihydroxynaphthalene (**2a**) | 72 | 46 | 18 |
| 2,3-dihydroxynaphthalene | <0.07 | <0.05 | <0.04 |
| 2,6-dihydroxynaphthalene | 10 | 3 | 2 |
| 2,7-dihydroxynaphthalene (**3a**) | 20 | 29 | 10 |
| 8-amino-2-naphthoat | 5 | 2 | <0.03 |

Enzyme assays (100 μL) contained Tris-HCl buffer (50 mM, pH 7.5), aromatic substrate (1.0 mM), DMAPP, GPP or FPP (2 mM), BAE61387 (40 μg), $CaCl_2$ (5 mM), glycerol (3 %*v*/*v*) and dimethyl sulfoxide (DMSO; 2 %*v*/*v*). The reaction mixtures were incubated at 37 °C for 16 h

## BAE61387 catalyses the same regioselective C-prenylation of hydroxynaphthalenes with DMAPP, GPP and FPP

For structural elucidation, the enzyme products **1b-1d**, **2b-2d** and **3b-3d** (Fig. 2) were isolated by preparative HPLC from the incubation mixtures of **1a, 2a** and **3a,** respectively, and subjected to $^1$H NMR and MS analyses. High-resolution (HR) MS (Table 2) confirmed the presence of the respective prenyl moiety in the enzyme products. The molecular masses of **1b-3b** are 68 Da larger than those of **1a-3a**, respectively, proving the attachment of a dimethylallyl moiety. The geranylated **1c-3c** and farnesylated **1d-3d** are 136 and 204 Da larger than their respective substrates **1a-3a**.

$^1$H NMR spectra of enzyme products from the same aromatic substrate, i.e. **1b-1d**, **2b-2d** or **3b-3d** (Table 3 and Figs. S1–S9 in electronic supplementary materials), showed signals with same number and coupling pattern of aromatic protons to each other. This indicated that the prenylation position of the enzyme products of a given substrate remains the same, despite different prenyl donors (DMAPP, GPP or FPP). The chemical shifts of H-1′ of the prenyl moieties for all of the isolated enzyme products were found between 3.36–

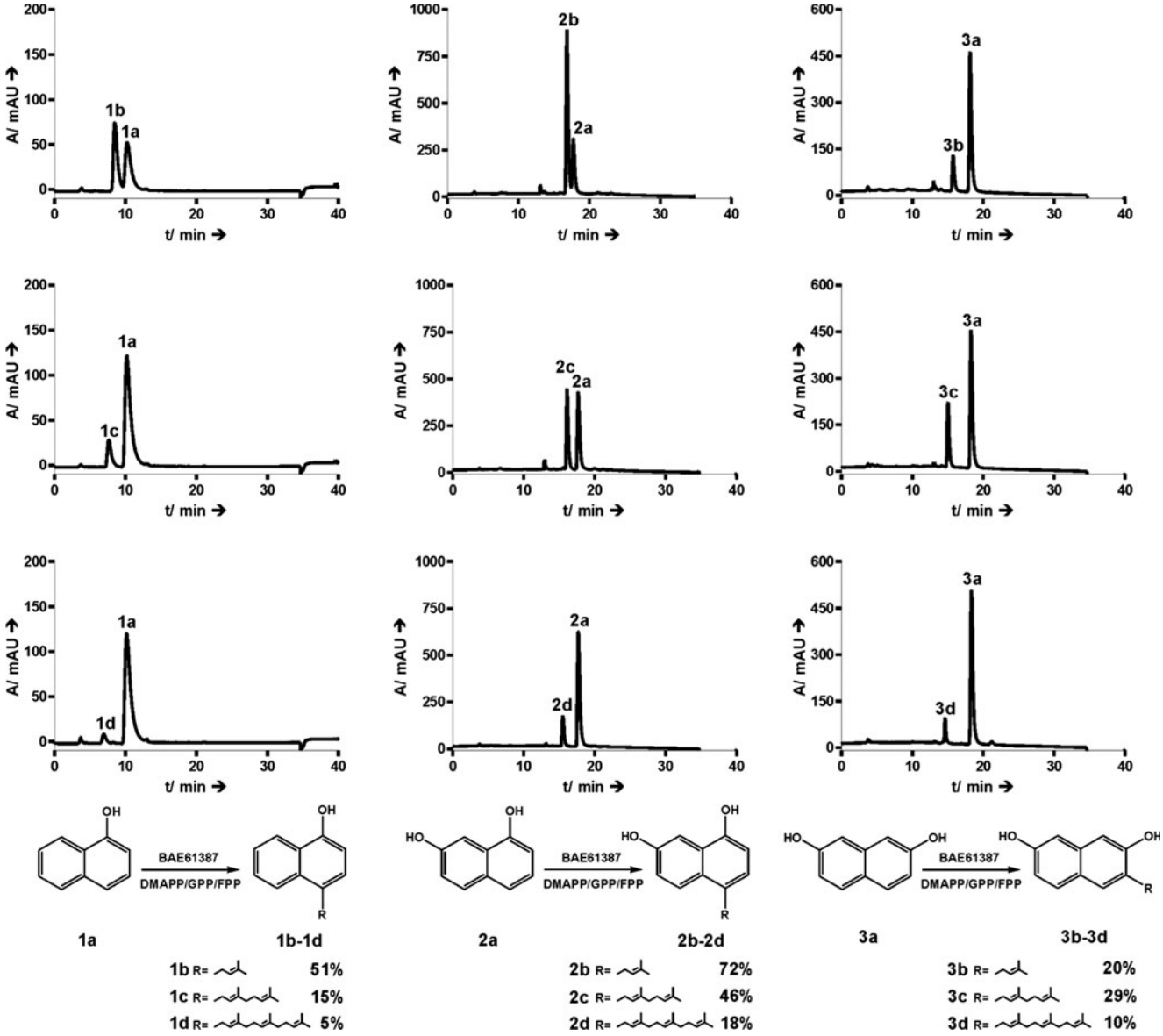



**Fig. 2** HPLC analysis of the enzyme assays of BAE61387 with hydroxynaphthalenes on a MultoHigh 100 Si column. Standard reaction mixtures contained 40 μg of BAE61387, 1.0 mM of aromatic substrate, 2.0 mM DMAPP, GPP or FPP, 5 mM $CaCl_2$ and 50 mM Tris-HCl at pH 7.5 and were incubated at 37 °C for 16 h. The illustrated chromatograms were recorded at 254 nm. Conversion yields are given under the reaction scheme

3.63 ppm, proving their attachment to a C-atom rather than to an oxygen. In the $^1$H NMR spectra of **1b-1d** and **2b-2d**, signals for two new coupling aromatic protons each with coupling constants of 7.6 or 7.7 Hz were detected, instead of three coupling aromatic protons with similar coupling constants in those of **1a** and **2a** (data not shown). This proved that **1a** and **2a** were prenylated at C-4, i.e. the *para*-position of a hydroxyl group of the same benzene ring, in the presence of DMAPP, GPP and FPP. In the $^1$H NMR spectra of **3b-3d**, two singlets each were detected, instead of three coupling aromatic protons as an ABM system in **3a** (data not shown). This led to the conclusion that **3a** was prenylated at C-3, i.e. *ortho*-position of a hydroxyl group, in the presence of DMAPP, GPP and FPP (Fig. 2). Prenylations of hydroxynaphthalenes at *para*- or *ortho*-positions of a hydroxyl group have been observed for some prenyltransferases of the DMATS superfamily in the presence of DMAPP (Yu et al. 2011). The NMR data of **1b**, **2b** and **3b** corresponded well to those reported previously (Yu et al. 2011).

## Biochemical properties and determination of the kinetic parameters of BAE61387 with DMAPP, GPP and FPP

To test the dependency of the BAE61387 reaction on metal ions, we assayed **2a** with DMAPP in the presence of diverse metal ions at final concentrations of 5 mM. Incubation

**Table 2** HR-EI-MS data of the isolated enzyme products

| Compd. | Chemical formula | HR-EI-MS data | | Deviation (ppm) |
|---|---|---|---|---|
| | | calculated | measured | |
| **1b** | $C_{15}H_{16}O$ | 212.1201 | 212.1208 | 3.3 |
| **1c** | $C_{20}H_{24}O$ | 280.1827 | 280.1840 | 4.6 |
| **1d** | $C_{25}H_{32}O$ | 348.2453 | 348.2446 | −2.0 |
| **2b** | $C_{15}H_{16}O_2$ | 228.1150 | 228.1131 | −8.3 |
| **2c** | $C_{20}H_{24}O_2$ | 296.1776 | 296.1792 | 5.4 |
| **2d** | $C_{25}H_{32}O_2$ | 364.2402 | 364.2394 | −2.2 |
| **3b** | $C_{15}H_{16}O_2$ | 228.1150 | 228.1154 | 1.8 |
| **3c** | $C_{20}H_{24}O_2$ | 296.1776 | 296.1786 | 3.4 |
| **3d** | $C_{25}H_{32}O_2$ | 364.2402 | 364.2398 | −1.1 |

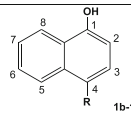
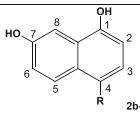
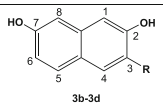
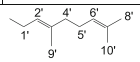
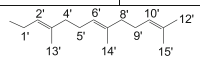
mixtures without additives or with EDTA were used as controls. Our results showed that BAE61387 catalyses its reaction also in the presence of the chelating agent EDTA. However, addition of $Ca^{2+}$ increased the enzyme activity about 40 % (Fig. S10). These results corresponded well with those observed for other members of the DMATS superfamily (Li 2009; Yu and Li 2012).

Kinetic parameters including Michaelis-Menten constant ($K_M$) and turnover number ($k_{cat}$) were determined for **1a-3a** and DMAPP, GPP and FPP (Table 4 and Figs. S11–S22 in electronic supplementary data). The enzyme reaction of BAE61387 with hydroxynaphthalenes and DMAPP, GPP and FPP apparently followed Michaelis-Menten kinetics. For the tested three substrates, their affinities to the enzyme decreased with the increased chain length of prenyl donors. In the presence of DMAPP, **2a** was accepted as the best substrate with a $K_M$ value at 0.56 mM, followed by **1a** at 1.01 mM and **3a** at 1.85 mM. These values are somewhat higher than those observed for other prenyltransferases with their natural substrates (Yu and Li 2012). In the presence of GPP, $K_M$ values for **1a**, **2a** and **3a** were determined at 1.55, 1.42 and 1.94 mM, respectively. In the presence of FPP, $K_M$ values for **1a**, **2a** and **3a** were found to be 2.08, 1.73 and 2.81 mM. The affinity of different prenyl donors to the enzyme was found to be comparable. In the presence of **2a**, FPP with a $K_M$ value of 1.03 mM showed only slightly higher affinity than DMAPP with a $K_M$ value of 1.28 mM. The velocity of the BAE61387 reaction in the presence of hydroxynaphthalenes was relative low. Turnover numbers of approximately 0.03 $s^{-1}$ were calculated for **1a-3a** in the presence of the best accepted prenyl donor DMAPP, indicating these substances are not its natural substrates.

## Discussion

In this study, we cloned and expressed a putative prenyltransferase gene AO090102000322 from *A. oryzae*, which shares meaningful sequence similarity with known members of the DMATS superfamily (Yu and Li 2012). Incubation of the recombinant enzyme BAE61387 with diverse aromatic substrates showed its ability to prenylate hydroxynaphthalenes in the presence of different prenyl donors such as DMAPP, GPP, or FPP.

**Table 3** $^1$H-NMR data of the enzyme products in $CD_3OD$

| Compd. |  1b-1d |  2b-2d |  3b-3d | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| |  DMA: |  Geranyl: |  Farnesyl: | | | | | | |
| pos. | **1b R=DMA** | **1c R=Geranyl** | **1d R=Farnesyl** | **2b R=DMA** | **2c R=Geranyl** | **2d R=Farnesyl** | **3b R=DMA** | **3c R=Geranyl** | **3d R=Farnesyl** |
| | - | - | - | - | - | - | 6.85, s | 6.85, s | 6.85, s |
| 2 | 6.69, d, 7.7 | 6.70, d, 7.7 | 6.69, d, 7.7 | 6.62, d, 7.6 | 6.62, d, 7.6 | 6.61, d, 7.6 | - | - | - |
| 3 | 7.07, d, 7.7 | 7.08, d, 7.7 | 7.08, d, 7.7 | 6.85, d, 7.6 | 6.87, d, 7.6 | 6.87, d, 7.6 | - | - | - |
| 4 | - | - | - | - | - | - | 7.33, s | 7.34, s | 7.34, s |
| 5 | 7.88, d, 7.5 | 7.88, d, 7.7 | 7.88, d, 8.2 | 7.75, d, 9.1 | 7.75, d, 9.1 | 7.75, d, 9.1 | 7.47, d, 8.6 | 7.46, d, 8.7 | 7.45, d, 8.7 |
| 6 | 7.44, ddd, 8.4, 6.8, 1.5 | 7.44, ddd, 8.4, 6.8, 1.5 | 7.43, ddd, 8.3, 6.9, 1.4 | 7.02, dd, 9.1, 2.6 | 7.02, dd, 9.1, 2.6 | 7.02, dd, 9.1, 2.6 | 6.77, dd, 8.7, 2.4 | 6.77, dd, 8.7, 2.4 | 6.77, dd, 8.7, 2.4 |
| 7 | 7.38, ddd, 8.1, 6.8, 1.2 | 7.38, ddd, 8.1, 6.8, 1.2 | 7.38, ddd, 8.0, 6.9, 1.1 | - | - | - | - | - | - |
| 8 | 8.19, dd, 8.3, 0.9 | 8.19, dd, 8.3, 0.9 | 8.19, dd, 8.4, 0.9 | 7.47, d, 2.6 | 7.47, d, 2.6 | 7.47, d, 2.6 | 6.83, d, 2.3 | 6.83, d, 2.4 | 6.83, d, 2.5 |
| 1' | 3.63, d, 7.0 | 3.64, d, 7.0 | 3.64, d, 6.8 | 3.58, d, 7.0 | 3.58, d, 7.0 | 3.59, d, 6.9 | 3.36, d, 7.2 | 3.37, d, 7.2 | 3.37, d, 7.3 |
| 2' | 5.31, br. t, 7.0 | 5.32, br. t, 7.0 | 5.32, t, 6.8 | 5.29, t, 7.0 | 5.29, t, 7.0 | 5.30, t, 6.9 | 5.39, t, 7.2 | 5.40, t, 7.2 | 5.40, t, 7.3 |
| 4' | 1.78, s | 2.10, m | 2.11, m | 1.77, s | 2.09, m | 2.10, m | 1.75, s | 2.12, m | 2.14, m |
| 5' | 1.72, s | 2.04, m | 2.05, m | 1.71, s | 2.03, m | 2.04, m | 1.72, s | 2.06, m | 2.08, m |
| 6' | - | 5.06, br. t, 6.9 | 5.07, br. t, 6.9 | - | 5.06, t, 6.8 | 5.08, t, 6.8 | - | 5.12, t, 7.6 | 5.14, t, 6.9 |
| 8' | - | 1.77, s | 1.98, m | - | 1.75, s | 1.98, m | - | 1.71, s | 2.00, m |
| 9' | - | 1.60, s | 1.89, m | - | 1.60, s | 1.90, m | - | 1.64, s | 1.93, m |
| 10' | - | 1.55, s | 5.03, br. t, 7.1 | - | 1.55, s | 5.03, t, 6.9 | - | 1.58, s | 5.04, t, 7.1 |
| 12' | - | - | 1.54, s | - | - | 1.55, s | - | - | 1.53, s |
| 13' | - | - | 1.78, s | - | - | 1.76, s | - | - | 1.71, s |
| 14' | - | - | 1.62, s | - | - | 1.62, s | - | - | 1.62, s |
| 15' | - | - | 1.54, s | - | - | 1.55, s | - | - | 1.58, s |

Chemical shifts are given in parts per million and coupling constants in hertz

**Table 4** Kinetic parameters of BAE61387

| Compd. | $K_M$ [mM] | | | $k_{cat}$ [$s^{-1}$] | | | $k_{cat}/K_M$ [$s^{-1}M^{-1}$] | | |
|---|---|---|---|---|---|---|---|---|---|
| | DMAPP | GPP | FPP | DMAPP | GPP | FPP | DMAPP | GPP | FPP |
| **1a** | 1.01±0.15 | 1.55±0.17 | 2.08±0.38 | 0.03±0.005 | 0.01±0.001 | 0.006±0.0003 | 30 | 6.5 | 3 |
| **2a** | 0.56±0.06 | 1.42±0.12 | 1.73±0.20 | 0.03±0.001 | 0.03±0.003 | 0.007±0.001 | 54 | 21 | 4 |
| **3a** | 1.85±0.15 | 1.94±0.11 | 2.81±0.14 | 0.03±0.003 | 0.02±0.001 | 0.005±0.0003 | 16 | 10 | 2 |
| DMAPP[a] | 1.28±0.30 | – | – | 0.025±0.004 | – | – | 20 | – | – |
| GPP[a] | – | 1.77±0.08 | – | – | 0.022±0.0001 | – | – | 12 | – |
| FPP[a] | – | – | 1.03±0.06 | – | – | 0.01±0.0004 | – | – | 10 |

[a] Kinetic parameters were measured with **2a** as aromatic substrate

Literature search did not reveal any report on prenylated hydroxynaphthalenes or derivatives thereof from *A. oryzae*. Analysis of the genes up- and downstream of AO090102000322 indicate the presence of neither polyketide synthase genes for the formation of a naphthalene backbone (Chooi and Tang 2012; Fujii et al. 2000) nor non-ribosomal peptide synthetase for a peptide backbone (Condurso and Bruner 2012) or other biosynthetic genes of secondary metabolites (Table S1). Prenylated hydroxynaphthalenes are a group of secondary metabolites mainly isolated from plants (Hussein et al. 2003, 2004) and bacteria (Kumano et al. 2010; Shin-Ya et al. 1990). In bacteria, prenyltransferases of the CloQ/NphB group are responsible for the prenylation of hydroxynaphthalenes. Examples are NphB (also called Orf2) in the biosynthesis of naphterpin in *Streptomyces* sp. strain CL190 (Kumano et al. 2008; Kuzuyama et al. 2005), Fnq26 in the biosynthesis of furanonaphthoquinone I in *Streptomyces cinnamonensis* DSM 1042 (Haagen et al. 2007) and Fur7 in the biosynthesis of furaquinocin in *Streptomyces* sp. strain KO-3988 (Kumano et al. 2010). All the mentioned enzymes used GPP as prenyl donor. Several homologous genes from different fungi like *Aspergillus terreus*, *Botryotinia fuckeliana* and *Sclerotinia sclerotiorum* were found to catalyse the prenylation of 2,7-dihydroxynaphthalene. These fungal enzymes share conserved amino acid residues of the members of CloQ/NphB group and used DMAPP as prenyl donor (Haug-Schifferdecker et al. 2010). BAE61387 shares sequence similarity with hydroxynaphthalene prenyltransferases from neither bacteria nor fungi. Therefore, a hydroxynaphthalene is unlikely the natural substrate of BAE61387. The acceptance of hydroxynaphthalenes by BAE61387 demonstrated just its high flexibility towards aromatic substrates, as observed for several members of the DMATS superfamily (Yu et al. 2011). The low affinity and turnover numbers of BAE61387 for hydroxynaphthalenes (Table 4) provided strong supports for this hypothesis. The natural substrate of BAE61387 remains unknown.

Remarkably, BAE61387 used DMAPP, GPP and FPP as prenyl donors, which had not been reported for an aromatic prenyltransferase prior to this study. The promiscuous bacterial geranyltransferase Fur7 used also DMAPP as prenyl donor for its prenylation of hydroxynaphthalenes (Kumano et al. 2010), and the fungal indole dimethylallyltransferase AnaPT accepted both DMAPP and GPP as prenyl donors (Pockrandt and Li 2013). However, there are no reports on an acceptance of FPP by these enzymes. Acceptance of DMAPP, GPP and FPP by BAE61387 could indicate a flexible biding site for prenyl donors in the reaction chamber of this enzyme.

Attachment of $C_5$-, $C_{10}$- and $C_{15}$- units to naphthalene backbone increases the structural diversity of prenylated compounds, and regiospecific prenylation would be welcome for structure modification. BAE61387 catalysed the monoprenylation of hydroxynaphthalenes at *para*- or *ortho*-position of a hydroxyl group, but not at both positions. Therefore, one predominant product each was obtained from incubation mixtures of **1a**-**3a** with DMAPP, GPP and FPP. In comparison, products with geranylations at *para*- and *ortho*-positions have been isolated from incubation mixtures of NphB with a given hydroxynaphthalene (Kumano et al. 2008).

We have recently demonstrated that the prenylation positions of indole derivatives were often shifted to other positions of the indole ring by dimethylallyltransferases of the DMATS superfamily, when DMAPP analogues, GPP or benzyl diphosphate instead of DMAPP itself were used as alkyl donors (Liebhold et al. 2012, 2013; Liebhold and Li 2013; Pockrandt and Li 2013). BAE61387 catalysed the prenyl transfer reactions to the same position, i.e. *para*- or *ortho*-position to a hydroxyl group despite different chain length of the prenyl donors. It seems that one of the activated positions at the naphthalene ring is the most preferable prenylation position of BAE61387 reaction.

**Acknowledgments** We thank Dr. Edyta Stec for synthesis of GPP and FPP, Lena Ludwig for synthesis of DMAPP, Nina Zitzer and Stefan Newel, all from Philipps-Universität Marburg, for taking MS and NMR spectra, respectively. This work was supported in part by the Deutsche Forschungsgemeinschaft (grant Li844/4-1 to S.-M.Li).

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