Molecular Analysis of a 4-Dimethylallyltryptophan Synthase from Malbranchea aurantiaca**§

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Prenyltransferases are widely distributed in prokaryotes and eukaryotes and play critical roles in cell signaling, protein trafficking, and elaboration of complex molecules in secondary metabolism. Numerous prenylated natural products have been isolated from diverse microorganisms, including bacteria and fungi. These complex metabolites possess a wide range of biological activities, with some showing promise as medicinal agents. On the other hand, many prenylated secondary metabolites have been described as toxins such as ergot alkaloids that have potent psychotropic activity. We have characterized a new prenyltransferase isolated from genomic DNA of Malbranchea aurantiaca RRC1813. Enzyme specificity was investigated with a series of amino acid substrates revealing its function as a 4-dimethylallyltryptophan synthase. Polypeptide sequence alignment analysis showed that it groups with a new class of prenyltransferase enzymes that lack the typical (N/D)DXXD motif found in these polypeptides. MaPT activity was not dependent on a divalent cation cofactor, although it was reversibly inactivated by 5 mM EDTA. Analysis of kinetic parameters showed reduced enzyme efficiency upon simple modification of L-Trp. Moreover, Δ-Trp had 0.5% relative activity and functioned as a competitive inhibitor with a $K_i$ of 40.41 μM. Finally, Thr-105, Asp-179, Lys-189, and Lys-261 in MaPT were serially mutated, and the resulting lesions displayed low or complete loss of activity. This study provides a detailed characterization of a prenyltransferase in Malbranchea species, revealing two enzyme inhibitors, and through site-directed mutagenesis identified several key amino acid residues in catalysis, yielding new insights into this important yet understudied class of natural product biosynthetic enzymes.

Fungi are phylogenetically diverse microorganisms that produce thousands of low molecular weight natural products. A significant number of these metabolites are in clinical use as human therapeutics, including antibacterial penicillins, cephalosporins, immunosuppressive cyclosporine A, and cholesterol-lowering HMG-CoA reductase inhibitor "statins" (1). However, drug-resistant bacteria like methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus faecalis continue to emerge as a threat to human health (2–4). Along with medical needs to treat a spectrum of diseases, an increasing number of natural products have been isolated from fungal sources and screened for potential bioactive secondary metabolites (5). Recently, new natural products like malbraninc, dihydromalbraninc, penicillic acid, and malbrancheamide A with promising bioactivity profiles have been isolated from Malbranchea aurantiaca and other related species (6–10). Our efforts have focused broadly on the secondary metabolome of this strain for isolation of natural products and elucidation of the putative malbrancheamide and ergot alkaloid gene clusters. These efforts have led to the identification of a new prenyltransferase (PT)** whose characterization is the subject of this report.

PTs are a family of enzymes that catalyze prenyl transfer to a variety of acceptors including isoprenoids, aromatics groups, and specific amino acid residues of proteins to produce over 50,000 metabolites (11, 12). These compounds are critical in biological processes of bacteria, plants, and animals. PTs are classified into three main groups based on their catalytic function (11). The first class is comprised of isoprenyl diphosphate (IPP) synthases that uses IPP as a building block to produce different length allylic diphosphates. Enzymes in this class share relatively high identities and have two conserved (N/D)DXXD sequence motifs for binding substrate and require Mg$^{2+}$ as cofactor (13). According to the stereochemical outcome of reaction products, this enzyme class is further divided into trans- and cis-PTs (11). The second class of PT utilizes proteins as their substrates and catalyzes farnesylation at a CaaX motif located at the G-protein C terminus with Zn$^{2+}$ as cofactor. There is considerable evidence that dysfunction of this class of PTs plays a role in malignant transformation of cells.

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The last class of PTs catalyze cyclization of farnesyl diphosphate (FPP) or geranylgeranyl diphosphate in a Mg$^{2+}$-dependent manner to produce numerous isoprenoid natural products (15, 16). This class of enzymes also contains a conserved (N/D)DXXD sequence motif.

Recently, a new group of PTs has been isolated and biochemically characterized from Streptomyces sp., Lyngbya majuscula, and the fungus Aspergillus sp. (17–27). There are several significant differences between this class and the trans-PTs/aromatic PTs that are membrane associated and contain the (N/D)DXXD motif (28). First, these enzymes (exemplified by CloQ, LtxC, FgaPT1, FgaPT2,FmtPT1, and TdiB) do not have the presumed prenyl diphosphate binding motif (N/D)DXXD (17, 18, 20, 23, 25, 26), suggesting a different substrate binding mechanism. Second, these new PTs (except Orf2) function in the absence of a divalent cation such as Mg$^{2+}$, Mn$^{2+}$, and Zn$^{2+}$ (29). Finally, Orf2 from Streptomyces sp. strain CL190 has a unique three-dimensional structure compared with previously described prenyltransferases (29). This novel PT barrel presumably represents the core structure of all other PTs in this class. From a chemical reaction point of view, these new PTs commonly catalyze C-C bond formation but some like Fnq26 and TdiB also catalyze cyclization of farnesyl diphosphate (FPP) or geranylgeranyl diphosphate in a Mg$^{2+}$-dependent manner to produce numerous isoprenoid natural products (14). The last class of PTs catalyze cyclization of farnesyl diphosphate (FPP) or geranylgeranyl diphosphate in a Mg$^{2+}$-dependent manner to produce numerous isoprenoid natural products (15, 16). This class of enzymes also contains a conserved (N/D)DXXD sequence motif.

**EXPERIMENTAL PROCEDURES**

**Materials**—DMAPP was purchased from Sigma-Aldrich or Isoprenoids. Buffer components and other reagents were purchased from Sigma-Aldrich or Thermo Fisher Scientific.

**Analytical Methods**—A Beckman Coulter HPLC, fitted with an XBridge$^{\text{TM}}$ C18 column (5 µm, 4.6 × 250 mm), coupled with a System Gold 168 Detector was routinely used for HPLC-UV analysis. The detection wavelength was set at 269 nm. A linear gradient of 20–70% acetonitrile in 0.1% trifluoroacetic acid over 20 min was used for product detection. One 10-min re-equilibration program with 20% acetonitrile in 0.1% trifluoroacetic acid followed each run. A SHIMADZU LCMS-2010EV system was used for LC-MS analysis with a linear gradient of 20–80% acetonitrile in 0.1% FA over 15 min. An XBridge$^{\text{TM}}$ C18 (3.5 µm, 2.1 × 150 mm) column was used with a flow rate of 208 µl/min. Varian Inova 500 MHz or Bruker DRX 500 MHz instruments were used to record NMR spectra. VG (Micromass) 70–250-S Magnetic Sector Mass Spectrometer in ESI$^+$ mode was used to record high resolution mass spectra.

**Bacterial Strains, Culture Conditions, and DNA Manipulation**—Escherichia coli DH5α and BL21(DE3) (Novagen) were grown in Luria-Bertani (LB) broth or on LB agar for gene cloning and protein expression, respectively. M. aurentiaca RRC1813 was grown at 28°C, 180 rpm in the dark. Standard methods for DNA isolation and manipulation were performed as described by Sambrook and Russel (30). Genomic DNA from M. aurentiaca RRC1813 was isolated with the MasterPure Yeast DNA Purification kit (Epícenter Biotechnologies) as described in the manual. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. DNA sequencing was performed by the University of Michigan DNA Sequencing Core facility using an ABI Model 3730 sequencer. The MaPT GenBank$^{\text{TM}}$ accession numbers are EU420001 for its genomic DNA and EU420002 for its putative cDNA.

**Isolation of the MaPT Gene**—Degenerate primers were designed from conserved sequences of the new class of PTs. The pair of PT-FW and PT-RV primers was used to screen putative PTs from isolated M. aurentiaca RRC1813 genomic DNA by PCR (supplemental Table S1). Fragments of about 750 bp were subcloned into the pGEM-Teasy vector (Promega) and sequenced. BLASTX in NCBI was used to identify sequenced PT fragments. TAIL-PCR was applied to amplify fragments extended from both ends of the PT gene to produce full-length genomic DNA (31). The long specific primers were designed on the known sequence, while two arbitrary primers were first used by Liu and Whittier (31). All primers used in TAIL-PCR are listed in the supplemental Table S1. BCM Gene Finder was used to predict putative introns in the full-length PT gene. A 63-bp intron was predicted and removed with primers of InterFW and InterRV along with PTFW(Ndel) and PTRV(-BamHI) to produce the MaPT cDNA (supplemental Table S1).

**Gene Cloning, Expression, and Protein Purification**—The purified MaPT cDNA were ligated to pET28a to produce pET28a-MaPT for transformation of E. coli DH5α competent cells. The construct was isolated and submitted for sequencing to exclude mutations introduced during PCR amplification and gene manipulation. The construct was transferred into E. coli BL21 (DE3) competent cells for protein expression. Cells harboring the construct were cultured in 4 liters of LB medium containing 25 µg/ml of kanamycin and grown at 37°C in a shaker at 200 rpm until an A$_{600}$ reached 0.6. The cultures were then induced by IPTG with a final concentration of 0.2 mM. The cultures were further grown at 16°C in a shaker at 180 rpm for 16 h. The cells were harvested by centrifugation (8000 g) and cell pellets were stored in −80°C or directly used for protein purification. Cell pellets from a 4-liter culture were suspended in 50 ml of lysis buffer (1 × PBS, 3 mM β-mercaptoethanol (BME), 10% glycerol, 20 mM imidazole, pH 7.4), which was sonicated to release soluble proteins. The supernatant after centrifugation at 35,000 × g at 4°C for 36 min was incubated with 5 ml of pre-equilibrated Ni-NTA-agarose resin (Qiagen) at 4°C for 2 h. The resin was then washed with 150 ml of lysis buffer, and recombinant His$_6$-MaPT was eluted with 10 ml of 200 mM imidazole in 1 × PBS (pH 7.4), 3 mM BME, and 10% glycerol. The protein was further purified with Superdex 200 column (Amersham Biosciences) with gel chromatography running buffer (1 × PBS, 1 mM dithiothreitol, 10% glycerol, pH 7.4). Protein absorbance at 280 nm was measured for calculating its concentration with an extinction coefficient of 88490.

The protein was then aliquoted and stored at −80°C. The final yield of His$_6$-MaPT was 2.5 mg/liter.

**Preparation of MaPT Mutants**—MaPT gene mutants were prepared following the protocols from Stratagene QuikChange$^\text{®}$ II.
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site-directed mutagenesis kit. The mutagenesis primers are shown in supplemental Table S1. The same procedures for the preparation of wild-type MaPT were followed to produce its mutants except that only Ni-NTA-agarose resin was used for protein purification.

Protein Analysis—Subunits of MaPT were analyzed by 4–12% SDS-PAGE, and protein bands were stained with Coomassie Brilliant Blue R-250. The molecular weight of MaPT with the His6 tag at its N terminus was determined by Superdex 75 column (Amersham Biosciences) with gel chromatography running buffer. The column was calibrated with bovine serum albumin (66 kDa), chicken egg ovalbumin (43 kDa), bovine trypsinogen (25 kDa), and ribonuclease A (13.7 kDa). The molecular mass of MaPT was calculated as 61.6 kDa.

Enzyme Assays—The 100-μl reaction mixture routinely contained 5 mM MgCl2, 0.25 mM L-Trp, 0.2 mM DMAPP, and 1 μl of 0.86 μg/μl MaPT in reaction buffer (1× PBS, pH 7.4, 10% glycerol, and 3 mM BME). Enzyme reactions were initiated by adding MaPT after prewarming the other components at 30 °C for 1 min and then kept at 30 °C for 30 min. Enzyme boiled at 100 °C for 10 min was used as a negative control. In the mutagenesis studies, MaPT mutants were used as the enzyme source. The reaction was stopped with 10 μl of 1.5 mM trichloroacetic acid. The mixture was agitated well and centrifuged at 13,000 × g for 5 min. The enzyme products were detected by HPLC. To investigate substrate specificity, L-Trp was substituted with a series of derivatives. IPP, GPP, or FPP also substituted DMAPP in the reactions.

Metal Dependence of MaPT—5 mM of divalent metal ions (Mg2+, Ca2+, Mn2+, Fe2+, Co2+, Ni2+, Cu2+, Zn2+, and Sn2+) were added to the routine reaction mixture. 5 mM EDTA, EGTA, EDDA, and DHPTA were added to the reaction buffer containing 0.86 μg of MaPT, and the mixture was incubated at 30 °C for 5 min. Then 0.25 mM L-Trp and 0.2 mM DMAPP were added to bring the volume to 100 μl and initiate the reaction, which was further incubated at 30 °C for 30 min before terminating with 10 μl of 1.5 mM trichloroacetic acid. The products were detected by HPLC, and experiments were performed in duplicate.

Optimal Conditions for Enzyme Reaction—For investigation of optimal temperature, reactions were conducted under 4, 15, 23, 30, 37, and 42 °C in the reaction buffer described above. For investigation of optimal reaction buffer pH value, 50 mM Tris-Cl, or sodium phosphate with various pH values (5, 6.7, 7.4, 8.9, and 10) were used in the reactions at 30 °C. The same procedure for routine enzyme assay was followed in this section.

Kinetics Analysis—To find suitable MaPT concentration linearly correlated with product generation, 0.1, 0.25, 0.5, 1, 2, 4, and 8 μl of 0.86 μg/μl enzyme was added to reaction mixtures. The reaction was incubated at 30 °C for 6 min before terminating with 10 μl of 1.5 mM trichloroacetic acid. To determine the time course of enzyme reactions, 50 μl of reaction mixture was removed and mixed with 5 μl of 1.5 mM trichloroacetic acid at 1, 3, 5, 8, 12, 16, 20, 25, 30, and 35 min. The enzyme product was detected with the same method described above.

Reaction mixtures in the enzyme kinetic analysis contained 0.22 μg of MaPT, and 5 mM MgCl2. To determine kinetic parameters of L-Trp, 5-OH-L-Trp, and l-abrine, 0.2 mM DMAPP was included in the reaction and 2–600 μM L-Trp, 5–1500 μM 5-OH-L-Trp, or 5–1000 μM l-abrine was added. To determine kinetic parameters of DMAPP, 0.25 mM L-Trp, 2 mM 5-OH-L-Trp, or 1 mM l-abrine was included in the reaction while DMAPP final concentration was varied (1–100, 5–200, and 5–160 μM, respectively). The reaction mixtures without L-Trp, 5-OH-L-Trp, l-abrine, or DMAPP were prewarmed at 30 °C for 1 min. The reactions were initiated by mixing them with the single omitted component and were further incubated at 30 °C for 5 and 6 min for L-Trp and 5-OH-L-Trp or l-abrine, respectively, before terminating with 10 μl of 1.5 mM trichloroacetic acid. The mixtures were mixed well and centrifuged at 13,000 × g for 5 min. A 100-μl solution was subjected to HPLC for product detection. All experiments were performed in duplicate. The area under the product peak was measured, and the peak content amount was calculated using a dimethylallyltrypophan (DMAT) standard curve. The initial rate was calculated with equation of product (nmol)/100 μl × 110 μl/100 μl.

Characterization of Enzyme Reaction Products—2-ml reaction mixtures containing 2 mg of L-Trp, 5-OH-L-Trp, 5-Me-DL-Trp, or l-abrine and 1.2 mg of DMAPP were incubated at 30 °C overnight and stopped by adding 200 μl of 1.5 mM trichloroacetic acid. The mixtures were mixed well and centrifuged at 13,000 × g for 5 min. The products were separated by a semi-preparative XBridgeTM C18 column with the same program except using a flow rate of 3 ml/min. The isolated products were lyophilized and subjected for 1H and 13C NMR analyses and high resolution MS analysis (HRMS).

Analysis of Metal Content of MaPT—Twelve divalent metal ions (Mg2+, Ca2+, V2+, Cr2+, Mn2+, Fe2+, Co2+, Ni2+, Cu2+, Zn2+, Cd2+, Pb2+) in MaPT were detected by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Finnigan) in the Department of Geological Science, University of Michigan. The gel chromatography running buffer was used as the blank sample. All detected metal ion content measurements except Ni2+ were <0.05 mol per mol of MaPT.

RESULTS

Identification and Purification of MaPT—Several PT amino acid sequences were aligned to identify conserved regions for PT gene degenerate primer design in M. aurantiaca RRC1813 (Fig. 1A). A 750-bp fragment was isolated and predicted to be a dimethylallyltrypophan synthase (DMATS) based on sequence comparisons (data not shown). TAIL-PCR was subsequently applied to isolate the 1371-bp full-length MaPT genomic DNA (31). A 63-bp intron was predicted in the MaPT genomic DNA (BCM Gene Finder) and removed to produce the corresponding MaPT cDNA. The PT encoded from this cDNA was aligned with DMATSs from A. fumigatus (EAL94103), Balansia obtecta (Q6X1E1), Claviceps fusiformis (Q12594), C. purpurea (Q9C141), and Neothyphodium coenophialum (Q6X2E1), FgaPT1 (EAL94098), FgaPT2 (AXA05849), and
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FtmPT2 (Fig. 1A) (20). It shares two conserved regions for designing degenerate primers with other PTs and has the highest sequence identity with FgaPT2 (64%). The lack of a conserved (N/D)DXXD motif in this PT from \textit{M. aurantiaca} RRC1813 indicated that this enzyme is a member of a recently identified new class of PTs.

The MaPT cDNA was cloned into pET28a for protein expression in \textit{E. coli} BL21(DE3). Purified polypeptide was obtained by Ni-NTA-agarose and further by gel chromatography with a Superdex 200 column to generate a product with apparent homogeneity. The observed molecular mass of the enzyme in 4–12% SDS-PAGE analysis was about 51 kDa, consistent with a theoretical weight of 52 kDa for His\textsubscript{6}-MaPT (Fig. 1B). Using gel-filtration chromatography, the native molecular mass of the enzyme was determined to be 61.6 kDa, suggesting a monomeric species (supplemental Fig. S1).
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| Substrate | Relative activity | M_0 of product | [M+1]^+ of product |
|-----------|------------------|----------------|------------------|
| L-Trp     | 100 ± 18.39      | 272.2          | 273.1            |
| d-Trp     | 0.5 ± 0.04       | 272.2          | 273.1            |
| 1-Methyl-L-Trp | 3.2 ± 0.35     | 286.2          | 287.4            |
| 5-Br-L-Trp | 0.3 ± 0.02       | 350.1          | 350.9            |
| 5-F-L-Trp | ND              | ND             | ND^a             |
| 5-Me-O-L-Trp | 16.1 ± 0.01     | 286.2          | 287.0            |
| 5-MeO-6-L-Trp | 0.7 ± 0.02      | 302.2          | 303.1            |
| 5-OH-L-Trp | 17.0 ± 0.37      | 288.2          | 289.1            |
| 6-Me-O-L-Trp | 24.9 ± 1.45     | 286.2          | 287.1            |
| 6-Me-6-L-Trp | 27.0 ± 2.66     | 286.2          | 287.1            |
| Ala-L-Trp | 0.6 ± 0.06       | 343.2          | 344.2            |
| N(2,4-Dinitrophenyl)-L-Trp | ND^a          | ND             | ND^a             |
| L-Phe | ND              | ND             | ND^a             |
| L-Tyr | ND              | ND             | ND^a             |
| L-His | ND              | ND             | ND^a             |
| Brevianamide F | 0.5 ± 0.03   | 353.2          | 354.1            |

^a ND represents no product detected.
^b Brevianamide F was labeled with two 13C atoms.

Enzyme Activity and Substrate Selectivity—L-Trp and a dipeptide, brevianamide F, were first tested as enzyme substrates. Brevianamide F was utilized by the enzyme with very poor conversion (see Table 1), whereas L-Trp was converted effectively into product, demonstrating it to be a more acceptable enzyme substrate (Table 1 and Fig. 1). The m/z of the product corresponds to the theoretical weight of DMAT, suggesting the enzyme to be a DMATS. This result further confirms that DMATS is an enzyme common in many fungal strains such as Aspergillus sp., Balanisia sp., Claviceps sp., Neurospora crassa, and the newly isolated Malbranchea sp. MaPT was specific for DMAPP as no product was detected by LC-MS when L-Trp and brevianamide F were tested with IPP, GPP, and FPP (data not shown).

Although both L-Trp and brevianamide F were accepted by MaPT, its native substrate was not predictable by comparing its sequence to other known enzymes in this class. To assess further the substrate flexibility, we interrogated 14 additional compounds as substrates with nine converted into products observed by LC-MS analysis (Table 1). The data revealed that modifications on L-Trp significantly reduced enzyme activity exemplified by 1-Me-L-Trp and the dipeptide Ala-L-Trp with relative activities of 3.2 ± 0.35 and 0.6 ± 0.06%, respectively. Additionally, amino acid chirality was critical for substrate recognition with 0.5 ± 0.04% relative activity of MaPT toward d-Trp. Finally, the enzyme is strictly specific for L-Trp and did not utilize other aromatic amino acid analogues including L-Phe, L-His, or L-Tyr.

Five substrates used in this study contained side chains with variant electronegativity at C-5 of the L-Trp indole ring. 5-OH-L-Trp was the best substrate with 17.0 ± 0.37% relative activity, followed by 5-MeO-6-L-Trp (16.1 ± 0.01%), 5-MeO-6-L-Trp (0.7 ± 0.02%), and 5-Br-L-Trp (0.3 ± 0.02%), while 5-F-L-Trp was not utilized by the enzyme. Thus, electronegativity of the side chain substituent on the L-Trp indole ring was critical for enzyme catalytic efficiency. Interestingly, FgaPT2 exhibits the same trend toward these five substrates, suggesting it is common to other DMATs (24).

Identification of MaPT Enzyme Reaction Products—Products formed by reaction of MaPT with L-Trp, L-abrine, 5-OH-L-Trp, and 5-Me-6-L-Trp in large scale reactions were purified by semi-preparative HPLC and analyzed by 1H and 13C NMR analyses and high resolution MS analysis (HRMS).

Metal Dependence of MaPT—The optimal temperature and pH value for enzyme reactions were 30 °C and 7.4, respectively (supplemental Fig. S2). The product in the reaction including L-Trp was produced to be correlated linearly with up to 0.43 µg of proteins per 100-µl assay, and up to 12 min under the above optimal conditions. The enzyme activity was not dependent on any additive divalent cation while addition of 5 mM of Mg2⁺ slightly enhanced MaPT activity by about 130% (supplemental data). The products were isolated by semi-prep HPLC and subjected to 1H and 13C NMR analyses and high resolution MS analysis (HRMS).
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L-Trp concentration was varied and DMAPP concentration held constant at 0.2 mM (Table 2). The $K_m$ and $V_{max}$ values for L-Trp were 48.88 ± 1.86 μM and 3.41 ± 0.04 μM/min, respectively, giving a turnover value of 80.42 ± 0.94 min⁻¹. Interestingly, DMAPP showed self-inhibition when its concentration was varied. The $K_m$ and $K_i$ values for DMAPP were 13.97 ± 1.86 μM and 126.37 ± 27.30 μM, respectively. Its $V_{max}$ value (5.63 ± 0.45 μM/min) was higher than that of L-Trp (measured at 0.2 mM DMAPP in L-Trp kinetic analysis). Both 5-OH-L-Trp and L-abrine had higher $K_m$ values and lower $V_{max}$ values than those of L-Trp. 5-OH-L-Trp $K_m$ and $V_{max}$ values were 303.08 ± 25.73 μM and 1.43 ± 0.04 μM/min, respectively. No self-inhibition was observed for up to 200 μM DMAPP in the presence of 5-OH-L-Trp. L-Abrine was converted into product with lower catalytic efficiency and showed self-inhibition in the kinetic assays. Its $K_m$ and $K_i$ values were 105.99 ± 21.14 μM and 63.68 ± 7.08 μM, respectively. For example, $K_{n(obs)}$ and $V_{max,obs}$ values for L-Trp with 50 μM Trp was 107.79 ± 5.07 μM and 5.36 ± 0.07 μM/min, respectively, while $K_{n(obs)}$ and $V_{max,obs}$ values for DMAPP were 41.20 ± 3.48 μM and 3.10 ± 0.04 μM/min, respectively (Table 2). The $K_i$ for D-Trp was determined to be 40.41 μM after plotting $K_{in,obs}$ values and corresponding D-Trp concentrations by the equation of $K_{in,obs} = K_m [1 + [D-Trp]/K_i]$. The data revealed that D-Trp acts as a competitive inhibitor in the MaPT reaction.

Mutagenesis of MaPT—The MaPT amino acid sequence was aligned to Orf2, CloQ, and NovQ sequences by ESPript (supplemental Fig. S4). MaPT Lys-261 (corresponding to Asn-173 in Orf2) was identified as a possible key residue involved in DMAPP binding. This residue was mutated to Glu and Leu (supplemental Fig. S5). MaPT K261E was highly soluble and readily purified while its K261L form was only partially purified due to significant insolubility. No product was generated in either reaction, reflecting the potential key role of Lys-261 in the enzyme reaction followed Michaelis-Menten kinetics when

![Reversible inhibition of MaPT activity by EDTA](A) A. Inhibition of EDTA analogues to MaPT. 5 μM of each chemical was incubated with the enzyme at 30 °C for 5 min before initiating the reaction. All experiments were performed in duplicate. B. Regaining enzyme activity by dialysis. 5 mM EDTA was incubated with MaPT at 30 °C for 5 min, and then dialyzed against gel chromatography running buffer at 4 °C overnight. Line 1, reaction with 1 mM L-Trp and dialyzed MaPT treated with EDTA; line 2, reaction with 1 mM L-Trp and dialyzed MaPT untreated with EDTA. Mg²⁺ was not added to either reaction.

![Absorbance at 269 nm](B) The absorbance at 269 nm was measured for each reaction for 15 min. The absorbance was highest at the beginning of the reaction, and then decreased over time due to enzyme activity.

### Table 2

| Substrate   | $K_m$ (μM) | $V_{max}$ (μM/min) | $k_{cat}$ (μM/min) | $k_{cat}/K_m$ (μM⁻¹ min⁻¹) |
|-------------|------------|--------------------|--------------------|-----------------------------|
| L-Trp       | 48.88 ± 1.86 | 3.41 ± 0.04        | 80.42 ± 0.94       | 1.65 ± 0.04                 |
| 5-OH-L-Trp  | 303.08 ± 25.73 | 1.43 ± 0.04        | 33.73 ± 0.94       | 0.11 ± 0.09                 |
| L-Abrine    | 105.99 ± 21.14 | 2.70 ± 0.30        | 63.68 ± 7.08       | 0.60 ± 0.23                 |
| L-Trp⁺      | 107.79 ± 5.07 | 3.56 ± 0.07        | 83.96 ± 1.65       | 0.78 ± 0.05                 |
| L-Trp⁻⁵⁰    | 13.97 ± 1.86 | 5.63 ± 0.45        | 132.78 ± 10.61     | 9.50 ± 0.16                 |
| 5-OH-L-Trp⁻⁵⁰| 41.20 ± 3.48 | 1.40 ± 0.04        | 33.02 ± 0.94       | 0.80 ± 0.09                 |
| L-Abrine⁻⁵⁰ | 19.00 ± 3.34 | 1.19 ± 0.06        | 28.07 ± 1.42       | 1.48 ± 0.18                 |
| L-Trp⁻⁵⁰³⁰  | 12.14 ± 3.36 | 4.87 ± 0.70        | 114.86 ± 16.51     | 9.46 ± 0.31                 |

*K* Kinetic data were obtained in the presence of 50 μM D-Trp.

*DMAPP, kinetic parameters.*
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Although Asp-62 is not a conserved residue, Asp-110 is seemingly conserved among these PTs, casting some doubt on a role limited to metal binding. In this study, MaPT Thr-105 was mutated to Asp and Val while Asp-179 (corresponding to Asp-110 in Orf2) was mutated to Asn, Glu, and Val (supplemental Fig. S5). MaPT T105V retained about 11% of wild-type enzyme activity while the T105D mutant form was almost completely inactive (1.4% of wild-type MaPT) (Fig. 5). Considering Ser-51 in Orf2 also points toward the GPP-binding pocket (5–7 Å distance), this result suggests that Thr-105 along with other hydrophobic residues possibly defines a binding pocket for DMAPP. MaPT D179N, D179E, and D179V retained 40, 25, and 7% of wild-type enzyme activity, respectively.

**DISCUSSION**

_Malbranchea_ sp. and other fungi are a rich source of bioactive natural products (6–10). Some of these products (e.g. lovastatin) have been clinically approved for disease treatment (37). On the other hand, fungal mycotoxins cause serious health problems and enormous economic losses (38). Ergot alkaloids, commonly produced by _Claviceps_ sp., _Aspergillus_ sp., and _Penicillium_ sp., are related to a human disease called ergotism, or St. Anthony’s Fire (1). This group of mycotoxins has not been reported previously in _Malbranchea_ sp. Herein, we reported the identification of MaPT from _M. aurantiaca_ RRC1813, which may be involved in ergot alkaloid biosynthesis. MaPT catalyzes a prenylation step to C-4 in L-Trp indole, which is the first committed step in the biosynthesis of ergot alkaloids from DMAT. Further studies will be required to identify these secondary metabolites generated by _M. aurantiaca_ RRC1813. MaPT also represents the first enzyme identified and characterized from this fungal species.

MaPT consists of 435 amino acids that share high sequence similarity to FagPT2 and DMATSs from _Aspergillus_ sp., _Balanisia_ sp., Claviceps_ sp., and _Neotyphodium_ sp. (Fig. 1). These enzymes belong to a new class of PTs including CloQ, involved in the biosynthesis of chlorobiocin (23). MaPT does not contain the (N/D)DXXD motif, the characteristic feature of this class of PTs. The enzyme efficiently converted L-Trp into DMAT without addition of any divalent metal ion, although its activity was enhanced by adding 5 mM Mg\(^{2+}\) or Ca\(^{2+}\). However, this conversion was totally eliminated after incubating 17 μM enzyme with ≈4 mM EDTA, which was reversible upon dialysis. The mechanism of this type of EDTA inhibition remains unclear. In a H101N variant of human liver arginase, EDTA exhibited a predominant competitive inhibition, and its carboxylic acid group was presumably important for this effect (34). EDTA was also shown to be a competitive inhibitor of horseradish peroxidase by acting as an electron donor to compete with substrate iodine (33). Moreover, Chan and Anthony (39) found that EDTA bound to lysyl or arginyl residues near the cytochrome-binding domain of methanol dehydrogenase. X-ray crystal studies of MaPT will likely provide details about EDTA inhibition of this enzyme.

Our studies have demonstrated that the MaPT catalytic activity is affected by several possible factors relating to substrate structure. The first factor is the specific chirality of Trp. MaPT exhibited 0.5% relative activity toward D-Trp compared...
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with l-Trp, and the unnatural amino acid acted as an inhibitor, the first identified competitive inhibitor for this new class of PTs (Table 1 and Fig. 4). This observation suggests that the reported kinetic parameters of FgaPT2 toward substrates containing both D- and L-configuration might require further refinement (24). Second, Gebler et al. (40) found that substrates with increasing electron-withdrawing property at the C-7 position of the indole ring decreased the turnover of DMATS from C. purpurea. In the current study, the electronegativity of substituents has a similar affect on MaPT catalytic ability. 5-OH-l-Trp was the best substrate with the highest relative enzyme activity, while no product was detected by LC-MS in the reaction with 5-F-dl-Trp as substrate. Third, the regiochemistry of methylation on the indole ring was also important for enzyme activity. 6-Me-dl-Trp was a better MaPT substrate than 5-Me-dl-Trp, while 1-Me-l-Trp served most poorly. Fourth, the modifications on amino or carboxylic acid group of l-Trp also affected enzyme activity, exemplified with relative activities of l-abrine, Ala-Trp, brevianamide F, and N-(2,4-dinitrophenyl)-l-Trp. Finally, an intact indole ring is important for enzyme catalysis. In the competitive analysis, l-Phe presumably did not bind to the same binding pocket of l-Trp because it had no effect on l-Trp kinetics compared with D-Trp. MaPT did not accept either l-His or l-Tyr as substrates.

The size of fungal enzymes in the new class of PTs such as FgaPT2 and MaPT are noticeably larger (>11 kDa) than those from bacteria such as Orf2, CloQ, NovQ, and Fnq26. This change may have occurred during enzyme evolution, possibly suggesting a different mode of substrate binding in fungal PTs. However, only substrate binding in bacterial PT Orf2 has been investigated by x-ray crystallography, and a fungal PT crystal structure remains unavailable (29). Herein, site-directed mutagenesis indirectly provided evidence that a different substrate binding mode is present in MaPT. In PT Orf2, the β-phosphate of GSPP was tethered in the network formed by Lys-119, Asn-173, Arg-228, and Tyr-282 while its α-phosphate interacted with Tyr-216, Lys-284, and cofactor Mg2+. A group of similar amino acid residues were found in CloQ, NovQ, and Fnq26 for β-phosphate binding (supplemental Fig. S4). A positive charged residue such as Lys and Arg in these enzymes, corresponding to Ser-51 of Orf2 (supplemental Fig. S4), was suggested to act as surrogate for Mg2+ in α-phosphate binding (21, 29). In MaPT, Lys-189, Lys-261, Tyr-325, and Tyr-394 were in the positions corresponding to those of Orf2 for β-phosphate binding (supplemental Fig. S4). Mutating both Lys-189 and Lys-261 to Glu and Leu confirmed the critical roles of these residues in enzyme catalysis (Fig. 5). The role of Lys-189 might be partially compensated by other proximal (basic) residues in the mutants, which made them partially active. However, Thr-105 instead of a basic residue in MaPT was in the same position as Ser-51 in PT Orf2, suggesting a different mode for α-phosphate binding (Fig. S4). Moreover, this residue is conserved in many fungal PTs (data not shown). Although Thr-105 may not play the same role as other positive charged residues or Mg2+, it is still an important residue for enzyme catalysis (Fig. 5). The nearly complete loss of activity of MaPT T105D reflected the importance of a hydrophobic side chain in this position. Thr-105 in MaPT may be responsible for binding of hydrophobic DMAPP because Ser-51 is also close to the GSPP-binding pocket in the Orf2 crystal structure. One Asp residue is conserved among bacterial PTs and MaPT in amino acid sequence alignment analysis (supplemental Fig. S4). Although Asp-110 in Orf2 is involved in metal binding, the role of this acidic residue is not established in other PTs that are metal ion cofactor-independent. In the mutagenesis study, side chain hydrophilicity of Asp-179 was important for enzyme activity since D179V contained the lowest enzyme catalysis among the three mutants. The side chain length was also found to be important by comparing activities of wild-type enzyme and its D179E mutant. Thus, mutagenesis demonstrated that MaPT has a different binding mode of isoprene compared with bacterial PTs and revealed two important residues (Thr-105 and Asp-179) for enzyme activity.

The new class of PTs is comprised of several members including CloQ and TdiB (17, 23). Most of these enzymes have been identified by searching putative PT genes in databases containing microbial genome sequences. The studies herein provide an alternative strategy to identify new members of this class of PTs. Through designing degenerate primers and performing TAIL-PCR, the MaPT gene was isolated from genomic DNA of the recently isolated fungus, M. aurantiaca RRC1813. This enzyme encoded by the MaPT CDNA was characterized in detail to reveal substrate selectivity, kinetic parameters and optimal reaction conditions. In addition, two inhibitors (D-Trp and EDTA) were identified toward MaPT, representing two small molecules capable of affecting the activity of enzymes in this new class. Mutagenesis studies of four key residues in MaPT confirmed the putative role of Lys-189 and Asn-261 in substrate binding and assigned additional functions to Thr-105 and Asp-179 in enzyme catalysis, providing additional insights into this class of enzymes. Also, the high catalytic efficiency of MaPT suggests that it might become a useful tool in synthetic biology, and chemoenzymatic assembly of prenylated indole derivatives, which may be precursors for a number of complex biologically active molecules.

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