Biochemical investigations on bacterial and fungal prenyltransferases

Biochemische Untersuchungen an bakteriellen und pilzlichen Prenyltransferasen

Dissertation
zur
Erlangung des Doktorgrades
der Naturwissenschaften
(Dr. rer. nat.)

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Marburg/Lahn 2015
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Eingereicht am 03. Juni 2015


Hochschulkennziffer: 1180
Dedicated to
my grandparents
and parents
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Statutory Declaration

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## Share of author contributions

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<td>Site-directed mutagenesis switching a dimethylallyl tryptophan synthase to a specific tyrosine $C3$-prenylating enzyme. <em>(J. Biol. Chem.)</em></td>
<td>Fan, A.; Zocher, G.; Stec, E.; Stehle, T.; Li, S.-M.</td>
<td>60</td>
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<td>Prenylation of tyrosine and derivatives by a tryptophan $C7$-prenyltransferase. <em>(Tetrahedron Lett.)</em></td>
<td>Fan, A.; Li, S.-M.</td>
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<td>Tryptophan prenyltransferases showing higher catalytic activities for Friedel-Crafts alkylation of o- and m-tyrosine than tyrosine prenyltransferases. <em>(Org. &amp; Biomol. Chem.)</em></td>
<td>Fan, A.; Xie, X.; Li, S.-M.</td>
<td>70</td>
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<td>Creation of FgaPT2 mutants with enhanced catalytic ability and different preferences toward cyclic dipeptides by saturation mutagenesis. <em>(ChemCatChem)</em></td>
<td>Fan, A. and Li, S.-M.</td>
<td>65</td>
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Signature candidate

Signature supervisor
Oral and poster presentations

Fan, A., Zocher, G., Stec, E., Stehle, T. and Li, S.-M.

Site-directed mutagenesis switching a dimethylallyl tryptophan synthase to a specific tyrosine C3-prenylating enzyme.

Oral presentation, Annual Conference of the Association for General and Applied Microbiology (VAAM), 01. – 03. March 2015, Marburg

Fan, A., Zocher, G., Stec, E., Stehle, T. and Li, S.-M.

Partner changes of tryptophan and tyrosine prenyltransferases.

Short lecture and poster presentation, 27. Irseer Naturstofftage (Dechma), 25. – 27. February 2015, Kloster Irsee

Fan, A. and Li, S.-M.

One substrate – seven products with different prenylation positions in one-step reactions by using fungal prenyltransferases.

Abbreviations

The international system of units and units derived thereof have been used.

[M]$^+$
molecular ion

[M+H]$^+$
molecular ion plus hydrogen

[M+Na]$^+$
molecular ion plus sodium

$\times$ g
gravitational acceleration

2D
two-dimensional

2-pentenyl-PP
2-pentenyl diphosphate (also termed *trans*-pentenyl diphosphate)

5-DMATS$_{Sc}$
5-dimethylallyltryptophan synthase from *Streptomyces coelicolor*

6-DMATS$_{Sa}$
6-dimethylallyltryptophan synthase from *Streptomyces ambofaciens*

6-DMATS$_{Sv}$
6-dimethylallyltryptophan synthase from *Streptomyces violaceusniger*

*A. clavatus*
*Aspergillus clavatus*

*A. flavus*
*Aspergillus flavus*

*A. fumigatus*
*Aspergillus fumigatus*

*A. nidulans*
*Aspergillus nidulans*

*A. niger*
*Aspergillus niger*

*A. terreus*
*Aspergillus terreus*

*A. versicolor*
*Aspergillus versicolor*

Ac
acetyl

Ant
anthranilate

Ar
aromatic ring

benzyl-PP
benzyl diphosphate

BLAST
basic local alignment search tool

bp
base pair

br
broad (NMR signal)

*C. purpurea*
*Claviceps purpurea*

CD$_3$OD
deuterated methanol

CDCl$_3$
deuterated chloroform
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Comp.</td>
<td>compound</td>
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<tr>
<td>Conc.</td>
<td>concentration</td>
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<tr>
<td>$J$</td>
<td>coupling constant</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Prod</td>
<td>cyclo- L-tryptophanyl- L-prolinyl doublet</td>
</tr>
<tr>
<td>D$_2$O</td>
<td>deuterium oxide</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>dd</td>
<td>double doublet</td>
</tr>
<tr>
<td>DMA</td>
<td>dimethylallyl</td>
</tr>
<tr>
<td>DMAPP</td>
<td>dimethylallyl diphosphate</td>
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<tr>
<td>DMAT</td>
<td>dimethylallyltryptophan</td>
</tr>
<tr>
<td>DMATS</td>
<td>dimethylallyltryptophan synthase</td>
</tr>
<tr>
<td>DMS</td>
<td>dimethyl sulfide</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxid</td>
</tr>
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<td>DMSO-d$_6$</td>
<td>deuterated dimethyl sulfoxid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<td>EI-MS</td>
<td>electron impact mass spectrometry</td>
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<tr>
<td>ESI-MS</td>
<td>electrospray ionization spectrometry</td>
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<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<td>FPP</td>
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<tr>
<td>gDNA</td>
<td>genomic DNA</td>
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<tr>
<td>GGPP</td>
<td>geranylgeranyl diphosphate</td>
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<td>GPP</td>
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<td>HTS</td>
<td>high throughput screening</td>
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<tr>
<td>His$_6$</td>
<td>hexahistidine</td>
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<td>HMBC</td>
<td>heteronuclear multiple bond correlation</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<tr>
<td>HR</td>
<td>high resolution</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>Hz</td>
<td>hertz</td>
</tr>
<tr>
<td>IPP</td>
<td>isopentenyl diphosphate</td>
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<tr>
<td>IPTG</td>
<td>isopropyl β-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>$k_{\text{cat}}$</td>
<td>turnover number</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>$K_M$</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>L. maculans</td>
<td><em>Leptosphaeria maculans</em></td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani or lysogeny broth</td>
</tr>
<tr>
<td>L-Trp</td>
<td>L-tryptophan</td>
</tr>
<tr>
<td>m</td>
<td>multiplet</td>
</tr>
<tr>
<td>m/z</td>
<td>mass-to-charge ratio</td>
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<tr>
<td>MAPP</td>
<td>monomethylallyl diphosphate (also termed methylallyldiphosphate)</td>
</tr>
<tr>
<td>MHz</td>
<td>mega hertz</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MTase</td>
<td>methyltransferase</td>
</tr>
<tr>
<td>multi</td>
<td>multiplicity</td>
</tr>
<tr>
<td>n×C5</td>
<td>number of C5 units, minimum 1</td>
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<tr>
<td>N. fischeri</td>
<td><em>Neosartorya fischeri</em></td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>nickel-nitrilotriacetic acid</td>
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<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOESY</td>
<td>nuclear Overhauser effect spectroscopy</td>
</tr>
<tr>
<td>NRPS</td>
<td>non-ribosomal peptide synthetase</td>
</tr>
<tr>
<td>OD$_{600}$</td>
<td>optical density at 600 nm</td>
</tr>
<tr>
<td>P. aethiopicum</td>
<td><em>Penicillium aethiopicum</em></td>
</tr>
<tr>
<td>P. roqueforti</td>
<td><em>Penicillium roqueforti</em></td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PPi</td>
<td>inorganic pyrophosphate</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>q</td>
<td>quartet</td>
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rel. conv. relative conversion
RNA ribonucleic acid
RP reverse phase
rpm revolutions per minute
s singlet
SAR structure-activity relationship
*S. ambofaciens* *Streptomyces ambofaciens*
*S. cerevisiae* *Saccharomyces cerevisiae*
*S. cinnamonensis* *Streptomyces cinnamonensis*
*S. coelicolor* *Streptomyces coelicolor*
*S. violaceusniger* *Streptomyces violaceusniger*
SDS sodium dodecyl sulfate
SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis
sp. species (sing.)
t triplet
TB Terrific-Broth
TBA tetrabutylammonium
TRAIL TNF-related apoptosis-inducing ligand
Tris 2-amino-2-(hydroxymethyl)-propan-1,3-diol
UV ultraviolet
v/v volume per volume
w/v weight per volume
$\delta_C$ chemical shift of $^{13}$C
$\delta_H$ chemical shift of $^1$H
Summary

Prenylated natural products are widely distributed in nature and demonstrate an amazing variety of structures and promising biological activities, which are usually distinct from their non-prenylated precursors. Therefore, they are intensively studied by researchers from different disciplines. These include investigations on their biosynthesis as well as on the involved key enzymes such as prenyltransferases, which contribute significantly to the structural diversity and biological activity. Prenyltransferases catalyze the transfer reactions of prenyl moieties from different prenyl donors to various aliphatic or aromatic acceptors. This thesis focuses on the prenyltransferases from the dimethylallyltryptophan synthase (DMATS) superfamily.

A new member of this superfamily TyrPT was characterized biochemically in vitro. The responsible gene An13g01840 had been identified in the genome sequence of A. niger and cloned into pET28a. In this thesis, TyrPT was found to catalyze the O-prenylation of tyrosine as well as C7-prenylation of tryptophan. It was further compared with the known tyrosine O-prenyltransferase SirD and tryptophan C7-prenyltransferase 7-DMATS toward a series of tyrosine and tryptophan derivatives. TyrPT exhibited a broader substrate spectrum and significantly higher catalytic activity for several substrates than the other two enzymes. Kinetic parameters of TyrPT reactions with ten substrates were determined. This study not only provides a new enzyme, but also enhances the relationships between tyrosine O- and tryptophan C-prenyltransferases.

Further studies demonstrated that tryptophan prenyltransferases FgaPT2 and 7-DMATS also accepted tyrosine and its derivatives as substrates and catalyzed a unique C3- and O-prenylation, respectively. A mechanism of the FgaPT2 reaction with tyrosine was proposed based on the molecular modeling results with the available crystal structure of FgaPT2. Based on this hypothesis, sixteen mutated FgaPT2 derivatives were tested with tryptophan and tyrosine as substrates. The mutant K174F demonstrated much higher catalytic ability toward tyrosine than FgaPT2 and showed almost no activity toward tryptophan. Therefore, tryptophan C4-prenyltransferase was switched to a specific tyrosine C3-prenyltransferase by site-directed mutagenesis. This strategy was also used for enhancing the catalytic activity of FgaPT2 as a C4-prenylating enzyme of cyclic dipeptides. Thirteen mutated FgaPT2 with much higher
catalytic activities and different substrate preferences toward cyclic dipeptides were obtained as new biocatalysts.

Inspired by the results above, the L-tyrosine analogs, L-o- and L-m-tyrosine, were tested with the tryptophan prenyltransferases FgaPT2, 5-DMATS, 6-DMATS\textsubscript{Sv} and 7-DMATS as well as the tyrosine prenyltransferases SirD and TyrPT. Surprisingly, SirD hardly accepted these tyrosine analogs. In contrast, tryptophan prenyltransferases generally demonstrated higher catalytic activities toward these two substrates. Product isolation and structure elucidation proved C5-prenylated o-tyrosine as unique product of these enzymes, and both C4- and C6-prenylated derivatives were identified as the products of the FgaPT2 reaction with L-m-tyrosine. These results revealed that chemical category of the aromatic nucleus was not the essential feature for the acceptance of a substrate by tryptophan or tyrosine prenyltransferases. It strongly depends on the substitution positions of the functional groups on the aromatic nucleus, which directly interact with the enzyme active sites.

Aforementioned results exhibit fairly the broad substrate spectra of DMATS enzymes. However, the tryptophan prenyltransferases usually accepted cyclic dipeptides only at high protein concentrations. Consequently, tryptophan was a very poor substrate for cyclic dipeptide prenyltransferases. In this thesis, the substrate promiscuity of indole prenyltransferases including tryptophan and tryptophan-containing cyclic dipeptide prenyltransferases was further expanded by their acceptance of the synthesized unnatural cyclo-L-homotryptophan-D-valine with one additional C-atom between the indole and the diketopiperazine rings. This compound was well accepted by all the tested prenyltransferases including three tryptophan and five cyclic dipeptide prenyltransferases. Seven prenylated products with one prenyl moiety at each position of the indole nucleus were isolated from the enzyme assays. This was the first report on the production of seven monoprenylated products from one substrate by one-step reactions.

The results presented in this thesis demonstrate that prenyltransferases of the DMATS superfamily are a rich source of biocatalysts, which could play an important role for production of prenylated compounds.
Zusammenfassung


Weitere Untersuchungen ergaben, dass die Tryptophan-Prenyltransferasen FgaPT2 und 7-DMATS auch Tyrosin und dessen Derivate als Substrate akzeptieren und jeweils eine C3- und O-Prenylierung katalysieren. Ein Mechanismus der FgaPT2-Reaktion mit Tyrosin wurde durch molekulares modellieren basierend auf der verfügbaren Kristallstruktur postuliert. Ausgehend von dieser Hypothese wurden 16 FgaPT2-Mutanten mit Tryptophan und Tyrosine als Substrate getestet. Die Mutante K174F zeigte eine viel höhere katalytische Aktivität gegenüber Tyrosin als FgaPT2, jedoch kaum Aktivität mit Tryptophan. Somit wurde die Tryptophan-C4-Prenyltransferase durch zielgerichtete Mutagenese zu einer spezifische Tyrosin-C3-Prenyltransferase umgewandelt. Diese Strategie wurde auch eingesetzt um die
katalytische Aktivität von FgaPT2 als C4-prenylierendes Enzym von zyklischen Dipeptiden zu erhöhen. 13 FgaPT2-Mutanten mit deutlich höherer katalytischer Aktivität und unterschiedlichen Substratspräferenzen gegenüber Dipeptiden konnten als neue Biokatalysatoren erhalten werden.

Motiviert durch die zuvor genannten Ergebnisse wurden zwei Analoga von L-Tyrosin, L-\(\alpha\)- und L-\(m\)-Tyrosin, mit den Tryptophan-Prenyltransferasen FgaPT2, 5-DMATS, 6-DMATS\(_{Sv}\) und 7-DMATS sowie den Tyrosin-Prenyltransferases SirD und TyrPT getestet. Interessanterweise akzeptierte SirD die zwei Tyrosin Analoga nur gering. Im Gegensatz dazu, wiesen die Tryptophan-Prenyltransferasen eine höhere katalytische Aktivität gegenüber diesen Substraten auf. Durch Produkt-Isolierung und Strukturaufklärung konnte bezüglich TyrPT und den Tryptophan-Prenyltransferasen C5-prenyliertes \(\alpha\)-Tyrosin als einziges Produkt nachgewiesen werden. Für die FgaPT2-Reaktion wurden C4- und C6-prenyliertes \(m\)-Tyrosin identifiziert. Diese Ergebnisse lassen erkennen, dass die Grund Struktur der Substrate keine essentielle Rolle für die Akzeptanz von Tryptophan- oder Tyrosin-Prenyltransferasen spielt, sondern von der Substitutionsposition der funktionellen Gruppe am aromatischen Kern abhängt, welche in direkte Wechselwirkung mit dem aktiven Zentrum des Enyzms tritt.


Die aus dieser Dissertation hervorgegangenen Ergebnisse demonstrieren, dass die Prenyltransferasen der DMATS-Superfamilie eine wertvolle Quelle für Biokatalysatoren darstellen und damit eine wichtige Rolle in der Produktion prenylierter Verbindungen spielen könnten.
1. Introduction

1.1. Prenylated aromatic compounds in nature

Natural products and their derivatives are of high significance for drug discovery and development process (1). These include prenylated natural compounds, which contain aromatic scaffolds and one or more prenyl moieties, e.g. prenylated indole alkaloids, tyrosines, xanthones, flavonoids, hydroxynaphthalenes and so on (Figure 1-1A) (2-5). Such compounds are abundant in terrestrial and marine organisms (6-9) and demonstrate a wide range of promising biological and pharmacological activities (3, 10-12), which are often distinct from their non-prenylated precursors. The distinctive prenyl moieties play an important role in the structural diversity of these natural products, due to various prenylation positions on the aromatic nucleus and different patterns (regular or reverse) and lengths of the prenyl chain (Figure 1-1B). In addition, the prenylated compounds can be further modified by rearrangement, cyclization, oxidation and hydroxylation (Figure 1-1C) (11, 13, 14). The prenylated aromatic metabolites are classified, summarized and described below according to their aromatic nucleus.

Figure 1-1. (A) Numbering of common aromatic scaffold; (B) Examples of regular and reverse prenyl pattern and different lengths of prenyl moieties; (C) Examples of bioactive prenylated natural products.
1.1.1. Prenylated indole alkaloids

Prenylated indole alkaloids comprising of indole nucleus and prenyl moiety are widely distributed in microorganisms, especially in filamentous fungi, e.g. the genera *Penicillium* and *Aspergillus* of Ascomycota (9). They demonstrate various chemical structures and biological activities, which even have great influence in human history, e.g. ergot alkaloids (15-17). Therefore, prenylated indole alkaloids are of great interest for scientists from different disciplines and significant progress has been achieved concerning their isolation, structure elucidation, biological and pharmacological activities as well as their biosynthetic pathways. Based on the structure of aromatic core, prenylated indole alkaloids can be further classified into different subgroups, such as prenylated tryptophan and simple indole derivatives like α-cyclopiazonic acid, prenylated tryptophan-containing cyclic peptide derivatives (tryprostatins A and B), indole terpenes (paxilline) (18) and prenylated isoindolinone (aspernidines A and B) (19, 20), etc (Figure 1-2). This thesis focuses on the prenyltransferases catalyzing the formation of the former two main subgroups, which are further depicted below.

![Figure 1-2. Examples of prenylated indole alkaloids.](image-url)
1.1.1.1. Prenylated tryptophan and simple indole derivatives

The most famous representatives of prenylated tryptophan are the ergot alkaloids, which were first identified in ergot fungus *Claviceps purpurea* (*C. purpurea*) and further isolated from different fungi of the phylum Ascomycota, including *Claviceps*, *Aspergillus* and *Penicillium* as well as some plant families (17). Ergot alkaloids are featured with a tetracyclic ergoline ring, derived from 4-dimethylallyltryptophan and display numerous biological activities. Ergot alkaloids and their semi-synthetic derivatives are applied in the clinic for treatment of different diseases, e.g. ergometrine for postpartum hemorrhage, ergotamine for migraine (Figure 1-1C) and cabergoline for parkinsonism (16). More regular $C4$-prenylated indole derivatives have been identified, such as the mycotoxin alpha-cyclopiazonic acid (Figure 1-2) from *Penicillium cyclopium* (21) and rugulovasines A and B as well as 8-chloro-rugulovasines A and B from *Penicillium islandicum* (22). Furthermore, reversely $N1$-prenylated tryptophan was found in the biosynthetic pathway of the anti-inflammatory cyclomarin A and antibacterial cyclomarazine A in the actinobacterium *Salinispora arenicola* CNS-205 (23). Regularly or reversely prenylated simple indole alkaloids containing hexahydropyrrolo[2,3-b]indole (HPI) unit, such as flustramines or flustraminols are widely distributed and isolated from plant *Selaginella moellendorfii* and even animal like the marine organism *Flustra foliacea* and Australian frog *Pseudophryne coriacea* (24). 5-Dimethylallylindole-3-acetonitrile (5-DMAIAN, Figure 1-2) represents a novel tryptophan metabolite of *Streptomyces coelicolor* A3 (25). Simple indole alkaloids including 5- and 7-dimethylallylindole, 3,7-diprenylated indole and hexalobines (Figure 1-2) were also isolated from plants (26, 27). Semicochliodinols A and B (Figure 1-2) as well as isocochliodinol (C6-diprenylated indolylbenzoquinones) were produced by *Chrysosporium merdarium* as inhibitors of HIV-1 protease (28). Penipaline B (Figure 1-2) and penipaline C isolated from the deep-sea-sediment derived fungus *Penicillium paneum* SD-44 showed potent cytotoxic activities against A-549 and HCT-116 cell lines (29).

1.1.1.2. Prenylated tryptophan-containing peptide derivatives

A major group of prenylated indole alkaloids belong to prenylated cyclic dipeptides, which consist of a 2,5-diketopiperazine or benzodiazepindine unit derived from
tryptophan and tryptophan/another amino acid biosynthesized by non-ribosomal peptide synthetase (NRPS). They are ubiquitous in nature and mainly in the phylum Ascomycota, notably in *Aspergillus* and *Penicillium* sp. A plurality of prenylated cyclic dipeptides are cyclo-tryptophanyl-prolinyl (cyclo-Trp-Pro) derivatives, e.g. brevianamides, mostly with a distinctive C2-reverse prenylation, i.e. deoxybrevianamide E, which can be further modified or rearranged to more complicated natural products, e.g. brevianamide A and stephacidin A (Figure 1-2) (30, 31), brevianamide S-U (32), notoamides (30, 31, 33), mangrovamides A-C (34) and versicamides A-H (35). Another group of C2-prenylated cyclo-Trp-Pro derivatives are the bioactive tryprostatins (36), e.g. the microtube inhibitor tryprostatins A and B (Figure 1-2) and fumitremorgins as well as verruculogen (36), which usually have a regular dimethylallyl substitution at C-2 of the indole ring (Figure 1-2). Furthermore, mainly multiple prenylated cyclo-tryptophanyl-alaninyl derivatives also contribute greatly to the diversity of prenylated cyclic dipeptide, e.g. echinulins (37, 38), rubrumlines A-O (38) and variecolorins (39). The third type of prenylated cyclic dipeptides is represented by the roquefortine C (Figure 1-2) and comprises of roquefortines (40-42) and meleagrin (43), which are the reversely syn-cis C3-prenylated cyclo-tryptophanyl-histidinyl derivatives. More members of prenylated cyclic dipeptides might contain cyclo-tryptophanyl-tryptophanyl, such as gypsetin (44) or cyclo-tryptophanyl-anthranilate, like (acetyl)azonalenin (9, 45). Further examples are dipeptides of tryptophan with leucine, isoleucine, valine or serine in the core, which demonstrate activities to regulate plant growth (46-48) or anti-inflammatory (49).

In addition, prenylated linear peptides and other cyclic peptides enrich the diversity of prenylated tryptophan-containing peptide derivatives as well (50, 51).

Since the prenylated tryptophan-containing peptide derivatives display the bulk structural diversity and promising biological activities, they are substantially investigated on their occurrence, biological activity, biosynthesis (9, 50, 52, 53), total synthesis (24, 54, 55) and even structure-activity relationship (SAR) (56, 57).

### 1.1.2. Prenylated tyrosine derivatives

Tyrosine is an important proteinogenic amino acid, which is synthesized from the essential amino acid phenylalanine in human being and via the shikimate pathway in
plants, fungi, bacteria and algae (58). By the virtue of its 4-hydroxyl group, tyrosine contributes significantly to post-translational modifications, biosynthetic transformations and molecular interactions, which resulted from phosphorylation, sulfation, nitration, oxidation, halogenation, glycosylation, AMPylation or cross-linking, etc (58). Prenylated tyrosine derivatives are not as common as indole derivatives in the secondary metabolism in nature. The O- or C-prenylated tyrosyl exists mostly in peptides or cyclic peptides (59) such as aestuaramides from *Lyngbya aestuarii* (60), pullularins from *Bionectria ochroleuca* (61) as well as prenylagaramides A and B from *Oscillatoria agardhii* (NIES-205) and *Oscillatoria agardhii* (NIES-596), respectively (62). It was proven that some regularly C-prenylated products were the results of Claisen rearrangement of reversely O-prenylated precursors (Figure 1-3A) (63, 64). Several simple O- or C3-prenylated tyrosine derivatives, e.g. stachylines were also found in *Stachylidium* sp., *Pithomyces* sp. and *Streptomyces* sp. IFM 10937 (65-67). For example, stachyline A (Figure 1-3B) was identified as a O-prenylated tyrosine derivative with a unique terminal oxime group (65) and a C3-prenylated tyrosine (Figure 1-3C) was isolated during a screening program for TRAIL-resistance-overcoming activity (66). Furthermore, O-prenylated tyrosine is the precursor of the phytotoxin sirodesmin PL (Figure 1-1C) in *Leptosphaeria maculans* (*L. maculans*) (68).

**Figure 1-3.** (A) O-prenylation and Claisen rearrangement to C-prenylated derivative; (B) An example of simple prenylated tyrosine derivative; (C) C3-prenylated tyrosine; (D) Examples of prenylated xanthones; (E) Examples of prenylated flavonoids, naphthalenes and quinones.
1.1.3. Prenylated xanthones

Prenylated xanthones are a widespread and structurally diverse group of natural products frequently found in plants, fungi and lichens (69). The prenyl moieties usually attach to hydroxyl groups or the ortho-positions of hydroxyl group and contribute greatly to the bioactivity of prenylated xanthones (70, 71). For example, garcinone C (Figure 1-3D) and γ-mangostin from *Garcinia mangostana* show cytotoxic activity (72) and AChE inhibitory activity (70), respectively. Maculaxanthone B from *Maclura tinctoria* was proven to have HIV-inhibitory activity (73), cudracuspixanthone A (Figure 1-3D) from *Cudrania tricuspidata* antiproliferative activity (4) and garciaciowone A (Figure 1-3D) from *Garcinia cowa* antimicrobial activity (74). Furthermore, there are structurally more complicated prenylated xanthone dimers in nature (69) such as bigarcinenone A (Figure 1-3D) with one geranyl and four dimethylallyl groups exhibiting a strong antioxidant activity (75).

1.1.4. Prenylated flavonoids, naphthalenes and quinones

Flavonoids are a huge group of structurally diverse and bioactive secondary metabolites with a distinctive C6-C3-C6 carbon framework mainly distributed in plant kingdom. Prenylation, frequently regular C-prenylation, represents one of the most important step in structural elaboration of flavonoids. So far, about 1000 prenylated flavonoids are characterized structurally and well investigated in their occurrence, biological activities chem- and biosynthesis (76-80). Dimethylallyl and geranyl substituted flavonoids and isoflavonoids have been substantially isolated, while farnesylated flavonoids are extremely rare. They have been reported to possess various biological activities and widely utilized in pharmaceutical, food and cosmetic industries. For example, xanthohumol (Figure 1-3E) from hops exhibits multiple pharmacological activities including anticancer (81, 82), antihyperglycemia (83) and antiviral activities (84). Other biologically active prenylated flavonoids include pannokin D (Figure 1-3E) with TRAIL-resistance-overcoming activity (85), quercetin-3-O-α-L-rhamnoside with strong antioxidant activity (86) and lespecrytin E₃ with potent melanin biosynthesis inhibitory activity (87).

Prenylated naphthalenes are not as common as flavonoids in nature. Cytotoxic adenafiorins A-D and vismiones B (Figure 1-3E), E and F were isolated from *Adenaria*
floribunda and Cratoxylum sp., respectively (88, 89). Vismione B also demonstrates potent anti-malarial activity (89).

Prenylated quinones often referred as meroterpenoids from mixed polyketide and isoprenoid biosynthetic origins, have been substantially isolated from marine organisms (3, 90) and terrestrial plants and animals (91-93). They exhibit a wide variety of structural diversity and biological activities, especially for anti-inflammatory and anticancer activity (3). In addition to the versatile lapachol (92), dimeric prenylated quinone scabellone B (Figure 1-3E) from Aplidium scabellum demonstrates selective anti-malarial activity without detectable apoptosis toward human neutrophils, which could be a novel lead for the development of new drugs for treatment of malaria (94).

1.2. Biosynthesis of prenylated aromatic secondary metabolites

In recent years, investigations on the biosynthesis of secondary metabolites grew vigorously and significant progress has been achieved due to the development of various biotechnologies and the availability of genome sequences. With the help of genome mining, feeding experiment, gene deletion and overexpression as well as heterologous expression, more and more biosynthetic pathways have been identified in microorganisms and plants. Meanwhile, bioinformatic analysis (95) has greatly accelerated this process. Owing to the importance of prenylated indole alkaloids, the gene clusters responsible for their biosynthesis are intensively studied and reviewed (53, 96-98). Moreover, achievements were made in biosynthetic pathways of prenylated xanthones (99, 100) and epipolythiodioxopiperazine as well. Some prenyltransferases used in this thesis are involved in several biosynthetic pathways, which are described in the following paragraphs. Furthermore, the interests of scientists have expanded to activate numerous silent secondary metabolite gene clusters to identify the unprecedented biosynthetic pathways and to regulate the production of natural products (2, 101).

1.2.1. Biosynthetic pathway of fumigaclavine C in Aspergillus fumigatus
Fumigaclavine C belongs to the bioactive ergot alkaloids, some of which are clinically used as mentioned before (17). Its biosynthesis in *Aspergillus fumigatus* (*A. fumigatus*) (Scheme 1-1A) started with the C4-prenylation of L-tryptophan (102) followed with methylation by FgaMT (103), oxidation by FgaOx1 and dehydrogenation by FgaDH to form the key intermediate chanoclavine-1-aldehyde, which was the ancestor of festuclavine in *A. fumigatus*, pyroclavine in *Penicillium commune* or agrocavine in *C. purpurea* (17, 104, 105).

Up to chanoclavine-1-aldehyde, the biosynthesis of ergot alkaloids in these three species shares the common steps. Chanoclavine-1-aldehyde was converted to festuclavine in *A. fumigatus* by FgaOx3 and FgaFS (106), which was further hydroxylated and acetylated to produce (8S,9S)-fumigaclavine A (15, 107). Another prenyltransferase FgaPT1 catalyzed the C2-reverse prenylation of (8S,9S)-fumigaclavine A for the final step in the biosynthesis of (8S,9S)-fumigaclavine C (108).

### 1.2.2. Biosynthetic pathway of prenylated brevianamide F derivatives

Brevianamide F, i.e. cyclo-L-Trp-L-Pro, assembled by bimodular NRPS, is a common precursor in the biosynthesis of a wide range of prenylated compounds, e.g. tryprostatins, fumitremorgins, spirotryprostatins, stephacidins and notoamides (Scheme 1-1B) (9, 97, 98, 109-111). Regular C2-prenylation of brevianamide F by FtmPT1 leads to the formation of tryprostatin B, which can be converted to tryprostatins, spirotryprostatins or fumitremorgins (112) by further modifications, e.g. oxidation, methylation, prenylation and rearrangement. Alternatively, reverse C2-prenylation by BrePT or NotF leads to deoxybrevianamide E, which is the precursor of stephacidins and notoamides (Scheme 1-1B) (30, 113).

### 1.2.3. Biosynthetic pathway of acetylaszonalenin

Acetylaszonalenin is a N1-acetylated and reversely C3-prenylated derivative of *R*-benzodiazepinedione, isolated from *N. fischeri* NRRL181. Its biosynthetic pathway begins with the formation of *R*-benzodiazepinedione, which is assembled by a NRPS AnaPS and followed by prenylation with AnaPT and acetylation with AnaAT (Scheme 1-1C) (9, 114).
Scheme 1-1. (A) Biosynthetic pathway of fumigaclavine C in *A. fumigatus*; (B) Biosynthetic pathways of brevianamide F derivatives; (C) Biosynthetic pathway of acetylaszonalenin; (D) Proposed biosynthetic pathway of sirodesmin PL.

1.2.4. Biosynthetic pathway of sirodesmin PL
SIRODESMIN PL is an epidithiodioxopiperazine derived from tyrosine and serine. It was isolated from the *L. maculans* and is responsible for the blackleg disease (115, 116). The biosynthesis of sirodesmin PL has been extensively studied and its biosynthetic pathway is proposed (Scheme 1-1D) (68, 117-120). Our group reported the biochemical characterization of SirD, which catalyzed the regular O-prenylation of L-tyrosine as the first step in its biosynthesis (68). Welch et al proposed a biosynthetic pathway for sirodesmin PL as depicted in Scheme 1-1D (117).

1.3. Prenyltransferases

Prenyltransferases (PTs) are a large family of enzymes that catalyze the attachment of prenyl moieties (n × C5) derived from the terpenoid biosynthetic pathways, e.g. dimethylallyl (C5), geranyl (2 × C5), farnesyl (3 × C5) or geranylgeranyl (4 × C5), to various aliphatic or aromatic acceptors including small-molecule natural products, peptides and proteins (13) and even a few t-RNAs (121). They play an important role in both primary and secondary metabolism in nature. According to their sequences, structures, biochemical properties and functions, PTs can be classified into different subgroups, like protein PTs, cis- and trans-PTs and aromatic PTs. Our group focuses on the aromatic PTs of DMATS superfamily, therefore it will be described in details.

1.3.1. Protein prenyltransferases

Protein PTs attach a farnesyl or geranylgeranyl moiety to a conserved cysteine residue in a “CaaX box” at the C-terminus of several proteins and peptide substrates. Prenylation of protein is a ubiquitous post-translational modification found in all eukaryotic cells, which is required for the proper cellular activity of many proteins, e.g. Ras family GTPases and heterotrimeric G-proteins. Therefore, protein prenylation has been extensively investigated as drug targets for treatment of cancers, progeria, aging, parasitic diseases and bacterial as well as viral infections (122).

1.3.2. Prenyl diphosphate synthases: *trans-* and *cis*-prenyltransferases

Prenyl diphosphate synthases catalyze the transfer of prenyl moiety to a specific acceptor, isopentenyl diphosphate (IPP) and are responsible for the biosynthesis of the
carbon skeleton of over 55,000 isoprenoids, which are high value components of pharmaceutical products, cosmetics, fragrances, flavors and foods (123-125). Based on the trans- or cis-configuration of formed prenyl units, prenyl diphosphate synthases are classified into two major groups, trans-PTs, e.g. GGPP synthases (126) and cis-PTs, e.g. undecaprenyl diphosphate synthase (UPPs) (127, 128). Although these two groups of PTs utilize the same substrates and both are Mg$^{2+}$-dependent, they have different amino acid sequences and three-dimensional structures (123, 124). Trans-PTs consist of $\alpha$-helices with two conserved aspartate-rich DDxxD motifs which allowed the binding of prenyl diphosphate in complex with Mg$^{2+}$ (129-131), while cis-PTs are featured with an Asp in the conserved P-loop involved in the chelating of Mg$^{2+}$ (132-134).

1.3.3. Aromatic prenyltransferases

Aromatic PTs catalyze the formation of prenylated natural products by attaching prenyl moieties to a wide variety of aromatic substrates and contribute significantly to their structural diversity and biological activities (9, 11, 13, 14). Therefore, aromatic PTs are intensively investigated during the past decades. Based on their differences on sequences, structures and biochemical properties, such as distinctive substrate binding motifs or metal ion dependency, aromatic PTs could be classified into membrane-bounded PTs, i.e. PTs of the UbiA family and soluble PTs, e.g. CloQ/NphB group and DMATS superfamily including LtxC group (13).

1.3.3.1. Prenyltransferases of the UbiA superfamily

PTs of the UbiA superfamily are involved in the biosynthesis of ubiquinones, menaquinones (135) and membrane lipids in archaea (136) as well as in plant secondary metabolites (137). UbiA or UbiA-like enzymes catalyze mainly the C-prenylations of benzoic and naphthoic acids and flavonoids. So far, all of them are membrane-bound proteins (138) and divalent ion-dependent (139). The structure of UbiA from Aeropyrum pernix was solved recently (140). In addition to the representative C-prenylation of most UbiA enzymes, a membrane-bound PT CnqPT1 from Streptomyces sp. CNQ-509 was identified to catalyze O-prenylation of 1,6-dihydroxyphenazine (141).
1.3.3.2. Prenyltransferases of the CloQ/NphB group

PTs of the CloQ/NphB group catalyze mainly prenylations of naphthalenes, quinones as well as phenolic compounds. These soluble proteins from bacteria and fungi display a novel and distinctive structure with five repeating “αββα” barrel fold, named PT-barrel (13, 142-144). In contrast to the aforementioned UbiA enzymes, most of them have no conserved aspartate-rich motif, thus independent of divalent metal ions. Furthermore, they also demonstrate higher substrate flexibility than UbiA enzymes. The CloQ/NphB group is named after the first identified enzymes CloQ from *Streptomyces roseochromogenes* catalyzing the prenylation of 4-hydroxyphenylpyruvic acid (142) and the NphB from *Streptomyces* sp. attaching a geranyl group to a 1,3,6,8-tetrahydroxynaphthalene (143). More and more PTs are identified from this family (145-150) and used for chemoenzymatic synthesis of prenylated compounds (151).

1.3.3.3. Prenyltransferases of the DMATS superfamily

Our group focuses on the study of PTs from the DMATS superfamily, which is one of the most investigated subgroup of aromatic PTs. Until now, more than forty such enzymes from fungi and bacteria have been identified by genome mining and characterized biochemically by using recombinant proteins. Due to the conflict of numerous PTs and limited space, only PTs used in this thesis were summarized in Table 1-1. According to their major function, the DMATS enzymes could be further classified into subgroups, such as tryptophan, cyclic peptide and tyrosine PTs, etc. They exhibit common characteristics, such as solubility, divalent metal ions independency as well as broad aromatic substrate promiscuity. Furthermore, they usually display high specificity toward prenyl donor (152-154). Dimethylallyl diphosphate (DMAPP) serves as the most common prenyl donor, while GPP and FPP are only accepted by a few members of the DMATS superfamily.

The first isolated and characterized DMATS enzyme was the C4-tryptophan PT, DmaW, which is involved in the biosynthesis of ergot alkaloids in *Claviceps* (155, 156). As mentioned above, the orthologue FgaPT2 is involved in the biosynthesis of fumigaclavine C in *A. fumigatus* (Scheme 1-1A) (15, 102, 157). So far, N1-reverse, C4-, C5-, C6- and C7-regular prenylations of tryptophan were found to be catalyzed by
CymD, FgaPT2, 5-DMATS, 6-DMATS$_{Sa}$ or 6-DMATS$_{Sv}$ and 7-DMATS as well as their homologues DamW-Cs (158, 159), 5-DMATS$_{Sc}$ (160), IptA (161) and 7-DMATS$_{Neo}$ (162), respectively (Table 1-1). CymD, DmaW-Cs, 5-DMATS$_{Sc}$, IptA and 7-DMATS are involved in the biosynthesis of cycloamarin/cyclomarazine, ergot alkaloids, 5-DMAI-3-acetonitrile, 6-DMAI-3-carbaldehyde and astecochrome, respectively (23, 158, 160, 161, 163). CymD and IptA along with LtxC (164) and TleC (165), which catalyze the C7-geranylation of (-)-indolactam V, are bacterial origin and were cataloged to an individual LtxC group. They share common features with DMATS enzymes from fungi and are therefore integrated into DMATS superfamily.

**Table 1-1. PTs of the DMATS superfamily used in this thesis.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Organism</th>
<th>Prenylated substrate, position and pattern</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tryptophan PTs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FgaPT2</td>
<td><em>A. fumigatus</em></td>
<td>L-Trp, C-4, regular</td>
<td>(102)</td>
</tr>
<tr>
<td>5-DMATS</td>
<td><em>A. clavatus</em></td>
<td>L-Trp, C-5, regular</td>
<td>(166)</td>
</tr>
<tr>
<td>6-DMATS$_{Sa}$</td>
<td><em>S. ambofaciens</em></td>
<td>L-Trp, C-6, regular</td>
<td>(167)</td>
</tr>
<tr>
<td>6-DMATS$_{Sv}$</td>
<td><em>S. violaceusniger</em></td>
<td>L-Trp, C-6, regular</td>
<td>(167)</td>
</tr>
<tr>
<td>7-DMATS</td>
<td><em>A. fumigatus</em></td>
<td>L-Trp, C-7, regular</td>
<td>(163)</td>
</tr>
<tr>
<td><strong>Tryptophan-containing cyclic peptides PTs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FtmPT1</td>
<td><em>A. fumigatus</em></td>
<td>brevianamide F, C-2, regular</td>
<td>(168)</td>
</tr>
<tr>
<td>BrePT</td>
<td><em>A. versicolor</em></td>
<td>brevianamide F, C-2, reverse</td>
<td>(30)</td>
</tr>
<tr>
<td>CdpC3PT</td>
<td><em>N. fischeri</em></td>
<td>cyclo-L-Trp-L-Leu, syn-cis C3-reverse</td>
<td>(169)</td>
</tr>
<tr>
<td>AnaPT</td>
<td><em>N. fischeri</em></td>
<td>cyclo-D-Trp-Ant, anti-cis C3-reverse</td>
<td>(114)</td>
</tr>
<tr>
<td>CdpNPT</td>
<td><em>A. fumigatus</em></td>
<td>cyclo-L-Trp-Ant, syn/anti-cis C3-reverse</td>
<td>(170)</td>
</tr>
<tr>
<td><strong>Tyrosine and xanthone PTs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SirD</td>
<td><em>L. maculans</em></td>
<td>L-Tyr O-regular</td>
<td>(68)</td>
</tr>
<tr>
<td>XptB</td>
<td><em>A. nidulans</em></td>
<td>xanthone O-regular</td>
<td>(100)</td>
</tr>
</tbody>
</table>

Compared to the most tryptophan PTs, tryptophan-containing cyclic peptide PTs are usually responsible for the biosynthesis of structurally more complicated natural products (Table 1-1). As aforementioned, FtmPT1, FtmPT2 and FtmPT3 from A. fumigatus/N. fischeri catalyze the sequential, regular C2-, N1- and O-prenylations in the biosynthesis of fumitremorgins and verruculogen, respectively (Scheme 1-1B) (109, 168, 171), while NotF and NotC from Aspergillus species prenylate sequentially on the C-2 (reversely) and C-7 (regularly) of the indole ring in the biosynthesis of notoamides (Scheme 1-1B). The C2-prenylation was also observed with BrePT from Aspergillus versicolor (30) and CdpC2PT from N. fischeri with different substrate preferences. CdpC2PT is proposed to be involved in the biosynthesis of fellutanine (172). Two C3-PTs CdpC3PT and AnaPT are characterized from N. fischeri and found to produce syn-cis and anti-cis configured reverse C3-prenylated pyrroloindolines with high stereoselectivity (114, 169). In contrast, the third reverse C3-PT CdpNPT from A. fumigatus exhibited low stereoselectivity and catalyzed both syn-cis and anti-cis configured prenylations (170, 173). CTrpPT from Aspergillus oryzae catalyzed the simultaneous regular C7- (major) and reverse N1-prenylation (minor) of cyclo-L-Trp-L-Trp with high substrate specificity (174), while a new member of the DMATS family, CdpC7PT from Aspergillus terreus (175) demonstrated much higher substrate flexibility and converted six cyclic dipeptides to ten prenylated products, including the first O-prenylated cyclo-L-Tyr-L-Tyr (175).

PTs of other indole derivatives were also identified, such as didemethylasterriquinone D PT TdiB (176), asterriquinone D PT AstPT (177) and indole diterpene PT AtmD (178) and PaxD (179).

Compared to the numerous indole PTs, a few DMATS enzymes catalyze prenylations of non-indole derivatives, such as tyrosine O-PT SirD (68) and xanthone PT XptB as well as the distinctive reverse O-PT of glucose moiety in fusicoccin P PAPT (180).

Despite of their low to moderate similarities on the amino acid level, similar crystal structures with five repeating “αββα” (ABBA) barrel fold were found for FgaPT2 (Figure 1-4) (181), FtmPT1 (182), CdpNPT (183) and AnaPT (173), which were used as basis for understanding the prenyl transfer reactions (181, 182, 184). Their active sites are located in the center of the barrel and no metal ions were detected in the structures. The amino acid residues in the DMAPP binding sites seem to be fairly
conserved in these four structures, whereas the binding sites of the aromatic substrates differ from each other. Further experiments showed that the enzyme reactions catalyzed by the DMATS superfamily might contain three common steps: it firstly started with ionization of DMAPP to form the dimethylallyl carbocation, which was confirmed by a positional isotope exchange in isotopically-labeled DMAPP (184); then nucleophilic attack of the aromatic ring to the primary center (regular prenylation) or the tertiary center (reverse prenylation) of the dimethylallyl carbocation; at last rearomatization of the aromatic ring to produce the final product (Figure 1-4, FgaPT2 as example) (181). Most controversy over the mechanism focused on the second step: whether it went under a directly nucleophilic attack by the prenylated position to the dimethylallyl carbocation or the nucleophilic attack first happened at other position and followed by different rearrangements (cope rearrangement or aza-cope rearrangement or 1,2 shift) to obtain the final products (185). All these results illuminate the route of function study and rational modification of DMATS to improve their catalytic ability.

The distinct common structure, “ABBA” fold, of CloQ/NphB group and DMATS superfamily provides evidence for a common ancestry of these enzymes (186). Therefore, they also termed ABBA-PTs.

![Figure 1-4. Structure of FgaPT2 and proposed mechanism of its prenyl transfer reaction (modified from (181)).](image)

### 1.3.4. Potential uses of DMATS enzymes for production of prenylated compounds

As mentioned above, prenylated natural products display a wide array of biological activities (3, 9-12, 64). Thus, synthesis of such compounds is of great interest and different strategies have been developed consequently. There are two most commonly used synthetic strategies: addition of a prenyl moiety from prenyl bromide to a substrate, which was activated by strong base before; or by coupling reactions catalyzed by metal salts (55, 57, 187). Reactions of both types are usually carried out under anhydrous or
anaerobic conditions with special temperature control using dangerous and environment hazardous chemicals and organic solvent (55, 57, 187). Even so, only electron-rich positions, e.g. N-1, C-2 and C-3 of the indole ring, are preferred by chemical prenylation. Meanwhile, additional steps are usually necessary for protection and deprotection of functional groups (55, 188).

Due to the availability of an increasing number of PTs, chemoenzymatic synthesis of prenylated compounds are now considered as a promising alternative of classic chemical synthesis, because it is more efficient, environment-friendly and easier to handle than the traditional synthesis in organic solvent. The reactions catalyzed by PTs are mostly regio- and stereoselective and carried out in aqueous solutions at low temperature, e.g. 37 °C, without any involvement of protection groups (154, 189).

Before my PhD study, regularly C4- and C7-monoprenylated tryptophan and derivatives as well as C4- and C7-diprenylated tryptophan were synthesized by using the DMATS enzymes (163, 190, 191). For cyclic dipeptides, regiospecific reverse C2- and C3-prenylated derivatives were able to be produced (114, 168-170, 192). During the recent four years, characterization of DMATS enzymes as well as their substrate promiscuity developed rapidly. More than ten DMATS enzymes were newly characterized and used for the prenylations of indole, tyrosine, xanthone and flavonoid derivatives. They accept not only natural substrate analogues with similar structures, but also compounds with quite different aromatic nucleus. Thus, a large number of simple indole derivatives, cyclic dipeptides as well as tyrosine and xanthone derivatives were converted by these enzymes to their prenylated derivatives (100, 193, 194). Until now, reversely N1-, regularly C4-, C5-, C6- and C7-prenylated tryptophan and derivatives were synthesized by the DMATS enzymes (Figure 1-5A) (23, 163, 166, 167, 190, 191). Regiospecific synthesis of N1-, C2-, C3- and C7-prenylated tryptophan-containing cyclic dipeptides by cyclic dipeptide PTs were also reported (Figure 1-5B) (168-170, 172-175, 192). Remarkably, the bacterial metabolites such as prenylated hydroxynaphthalenes and indolocarbazoles as well as plant metabolites, e.g. prenylated flavonoids and acylphloroglucinols can also be produced from their non-prenylated precursors by DMATS enzymes, which are generally more convenient and efficient than membrane-bound PTs (Figure 1-5C) (195-198). Until 2012, prenyl transfer reactions mentioned above all took place on the aromatic nucleus. Chen et al reported that cyclic dipeptide PT FtmPT1 was able to catalyze the formation of the unnatural α-
prenylindolylbutenone, demonstrating the prenylation of a nonaromatic carbon by an indole PT (199). This example indicated that further investigations on the substrate promiscuity and catalytic ability of PTs could lead to production of novel unnatural products.

However, one barrier for the use of DMATS enzymes as biocatalysts is their high specificity toward prenyl donors. They usually stick to their natural donor, e.g. DMAPP, GPP or FPP and do not accept other prenyl diphosphates. During the past four years, efforts and progresses have been made to expand their acceptance for other natural prenyl donors (200, 201) as well as some unnatural alkyl, even benzyl donors, which can be used to produce novel unnatural prenylated indole or tyrosine derivatives (202-206). For instance, AstPT from *A. terreus*, catalyzed *N*1- and *C*2-prenylation of bisindolyl benzoquinone with extremely high substrate specificity, (177) could even use DMAPP, GPP and FPP for *O*-prenylation of hydroxyxanthones (Figure 1-5D) (201).

![Figure 1-5](image-url)  
*(A) – (C) Examples of prenylated compounds synthesized by DMATS enzymes; (D) and (E) Examples of unnatural alkylated or benzylated compounds synthesized by DMATS enzymes.*
Furthermore, several unnatural DMAPP analogues were synthesized and tested as prenyl donors for indole and tyrosine PTs (202-206). It has shown that the double bond at the β-position to pyrophosphate is essential for an acceptance by these enzymes. One methyl group can be deleted as in the case of monomethylallyl (MAPP) or shifted to the δ-position as in the case of 2-pentenyl-PP (2-pen-PP) (Figure 1-5E) (205). Even more space-demanding benzyl-PP can be accepted by some DMATS (Figure 1-5E) (202, 204). Additionally, the alkylation position of the products can differ from those with DMAPP, depending on the used prenyl donors. As an example, the tryptophan C4-PT FgaPT2 used these unnatural donors and the prenyl positions were partially (MAPP) or total shifted (2-pen-PP and benzyl-PP) to C-5 (Figure 1-5E) (204).

Unfortunately, biocatalyst application is often hampered by its long-term stability under process conditions and by difficulties in recovery and recycling. To overcome this problem, enzyme immobilization was proposed (207). Furthermore, coexpression of PT genes with NRPS genes in fungi resulted in high product yields of prenylated derivatives and did not need addition of expensive DMAPP (208, 209). Additionally, the whole cell biocatalyst by Autodisplay FgaPT2 on the surface of E. coli demonstrates high catalytic efficiency and stability, which can be easily separated from reaction system by centrifugation for reuse (210).

Investigation on the substrate and catalytic promiscuity of the DMATS enzymes demonstrated their potential application in the chemoenzymatic synthesis and synthetic biology. So far, more than 250 prenylated compounds have been produced by using DMATS enzymes as biocatalysts (211).

Enzyme promiscuity is latent skills of enzymes during evolution and widely considered and investigated for developing biocatalysts (212, 213). Mechanism of enzyme promiscuity might involve the variation in the mode of substrate binding interactions or conformational diversity. In a few cases, changes on other parts of the catalytic machinery occurred (214). If a natural enzyme cannot fulfill task, protein engineering can be used to improve its properties, such as regio-/stereoselectivity, desired substrate scope or stability to heat or organic solvents. Random mutagenesis/directed evolution combined with high throughput screening (HTS) as well as rational protein design methods are considered as useful tools (215-217). As refer to PTs, crystal structures of DMATS enzymes provide not only detailed insights into the reaction mechanism of the
prenyl transfer reactions, but also basic information for protein engineering to create new biocatalysts with desirable features (182). Due to the difficulty of HTS for PT mutants, rational design based on the available crystal structure is more efficient and preferred. Structure-based engineering of EpzP resulted in a novel phenazine PT with conformational changes at C-termini and increased its catalytic turnover rate (218).

In addition to biochemical investigation on a new PT, substrate promiscuity of DMATS enzymes and attempt to construct mutants with desired property are studied in this thesis.
2. Aims of this thesis

The following issues have been addressed in this thesis:

**Prenylation of unnatural cyclo-L-homotryptophan-D-valine by both tryptophan and tryptophan-containing cyclic dipeptide prenyltransferases**

Tryptophan and tryptophan-containing cyclic dipeptide PTs comprise the majority of DMATS enzymes. Usually, the former enzymes accepted cyclic dipeptides only at high protein concentrations. Likewise, tryptophan was a very poor substrate for cyclic dipeptide PTs. Furthermore, each cyclic dipeptide PT has its own preference for substrates. The aim of this project was to elucidate the behavior of these two enzyme groups toward unnatural cyclo-L-homotryptophan-D-valine, which bears one additional C-atom between the indole and diketopiperazine rings. The following experiments were planed:

- Synthesis of cyclo-L-homotryptophan-D-valine
- Overproduction and purification of cyclic dipeptide PTs BrePT, FtmPT1, CdpC3PT, CdpNPT, AnaPT as well as tryptophan PTs FgaPT2, 5-DMATS and 7-DMATS
- Enzyme assays and HPLC analysis
- Enzyme product isolation on HPLC and structure elucidation by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS)
- Determination of kinetic parameters of the enzyme reactions

**Functional proof of a putative prenyltransferase gene An13g01840 from Aspergillus niger FGSC A1180**

One putative PT CAK41583 (latter named TyrPT), deduced from the gene An13g01840 in *A. niger*, shares sequence identities of 36 and 30 % with SirD and 7-DMATS on the amino acid level, respectively. In order to characterize this enzyme biochemically, the following experiments were planed:

- Sequence analysis of neighboring genes of tyrPT from *A. niger* CBS513.88 for secondary metabolite information and mRNA isolation from *A. niger* FGSC A1180 and RT-PCR to verify the tyrPT expression
AIMS OF THIS THESIS

- Overproduction and purification of TyrPT
- Enzyme assays of TyrPT with various substances and HPLC analysis
- Enzyme product isolation on HPLC and structure elucidation by NMR and MS
- Determination of ion dependency of TyrPT reactions
- Overproduction and purification of SirD and 7-DMATS
- Comparison the enzyme activities of TyrPT with SirD and 7-DMATS toward tryptophan and tyrosine as well as their derivatives
- Determination of kinetic parameters of TyrPT and 7-DMATS reactions

Prenylation of tyrosine and its derivatives by tryptophan prenyltransferase 7-DMATS

7-DMATS from *A. fumigatus* and SirD from *L. maculans* catalyze a C7-prenylation of L-tryptophan and an O-prenylation of L-tyrosine in nature, respectively. SirD was reported also to catalyze the C7-prenylation of L-tryptophan *in vitro*. The aim of this project was to elucidate the behavior of 7-DMATS toward L-tyrosine and its derivatives. The following experiments were planed:

- Overproduction and purification of 7-DMATS
- Enzyme assays and HPLC analysis
- Enzyme product isolation on HPLC and structure elucidation by NMR and MS
- Determination of kinetic parameters of the enzyme reactions

Creation a specific tyrosine C3-prenylating enzyme from tryptophan prenyltransferase FgaPT2

The tryptophan PTs FgaPT2 and 7-DMATS from *A. fumigatus* catalyze C4- and C7-prenylations at the indole ring, respectively. 7-DMATS was found to accept L-tyrosine as substrate as well and converted it to an O-prenylated derivative. The original aim of this project was to elucidate the behavior of other available tryptophan PTs including FgaPT2, 5-DMATS and 6-DMATS$_{sa}$ toward tyrosine. Further study demonstrated a C3-prenylation of tyrosine and 4-amino-L-phenylalanine by FgaPT2. The second aim of this project was to improve the catalytic efficiency of FgaPT2 toward tyrosine. The following experiments were planed:

- Overproduction and purification of used PTs
AIMS OF THIS THESIS

- Enzyme assays of the tryptophan prenyltransferases with L-tyrosine
- Enzyme product isolation on HPLC and structure elucidation by NMR and MS.
- Determination of kinetic parameters of the enzyme reactions
- Molecular modeling-guided site-directed mutagenesis experiments to obtain FgaPT2 mutants
- Enzyme assays of mutated enzymes and FgaPT2 with L-tryptophan and L-tyrosine
- Analysis and comparison of the enzyme activities on HPLC
- Enzyme product isolation on HPLC and structure elucidation by NMR and MS

Regular C-prenylation of o-tyrosine and m-tyrosine by tryptophan and tyrosine prenyltransferases

Previous study found that tyrosine O-PTs SirD and TyrPT catalyzed the same C7-prenylation of tryptophan as 7-DMATS. Meanwhile, the tryptophan C4- and C7-PT FgaPT2 and 7-DMATS catalyzed the C3- and O-prenylations of tyrosine, respectively, which indicated the close relationship between tryptophan and tyrosine PTs. This project focuses on the behavior of these two enzyme groups toward isomers of tyrosine, i.e. o-tyrosine and m-tyrosine. The following experiments were planed:

- Overproduction and purification of used PTs
- Enzyme assays with tryptophan, tyrosine, o-tyrosine and m-tyrosine and HPLC analysis
- Enzyme product isolation on HPLC and structure elucidation by NMR and MS
- Determination of kinetic parameters of the enzyme reactions

Creation of FgaPT2 mutants for production of C4-prenylated cyclic dipeptides with high efficiency by saturation mutagenesis

Until now, N1-, C2-, C3- and C7-prenylated cyclic dipeptides can be synthesized with cyclic dipeptide PTs. Tryptophan C4-PT FgaPT2 was reported to accept a few cyclic dipeptides using high amount of protein. The aim of this project was to enhance the catalytic ability of FgaPT2 toward cyclic dipeptides by site-directed mutagenesis and to create new biocatalysts for C4-prenylation of cyclic dipeptides with high efficiency. The following experiments were planed:
• Overproduction and purification of FgaPT2 and available mutants
• Enzyme assays with cyclic dipeptides and HPLC analysis
• Enzyme product isolation on HPLC and structure elucidation by NMR and MS
• Saturation mutagenesis of R244 to obtain FgaPT2 mutants
• Attempt for optimization screening process of mutants by enzyme assays with mixtures of cyclic dipeptides and crude mutated proteins
• Overproduction and purification of selected mutants
• Characterization of the selected mutants by enzyme assays and HPLC analysis as well as isolation and structure elucidation of the enzyme products by NMR and MS
• Determination of kinetic parameters of enzyme reactions
3. Results and discussion

3.1. Prenylation of cyclo-L-homotryptophan-D-valine by both tryptophan and tryptophan-containing cyclic dipeptide prenyltransferases

The enzymes of the DMATS superfamily are known for their broad substrate spectra. However, the tryptophan PTs accepted cyclic dipeptides only at high protein concentrations. Consequently, tryptophan was a very poor substrate for cyclic dipeptide PTs. In this project, we used eight DMATS enzymes, including tryptophan and tryptophan-containing cyclic dipeptide PTs to catalyze the prenylations of an unnatural cyclic dipeptide cyclo-L-homotryptophan-D-valine.

To fulfill this aim, cyclo-L-homotryptophan-D-valine with one additional C-atom between the indole and diketopiperazine rings was synthesized. It was then tested as substrate with five cyclic dipeptide PTs including BrePT, FtmPT1, CdpC3PT, CdpNPT, and AnaPT as well as three tryptophan PTs FgaPT2, 5-DMATS and 7-DMATS. HPLC analysis of the reaction mixtures revealed that cyclo-L-homotryptophan-D-valine was well accepted by all the tested PTs. This could be explained by the increased similarity of this substance with L-tryptophan and reduced steric hindrance of the diketoperazine ring after insertion of one CH$_2$ between the indole and diketoperazine rings. Seven products with one prenyl moiety at each position of the indole nucleus and one diprenylated derivative were isolated from enzyme assays (Figure 3-1). Along with the enhanced product yields, the regioselectivity decreased in the presence of this unnatural aromatic substrate. This phenomenon was also observed for PTs with unnatural alkyl donors (205). To solve this problem, molecular modeling-guided mutagenesis experiments might be a useful tool.

To the best of our knowledge, this was the first report for production of seven monoprenylated products from one substrate by one-step reactions.
3.2. Prenylation of both tyrosine and tryptophan derivatives by TyrPT from Aspergillus niger FGSC A1180

A putative PT gene An13g01840 of the DMATS family was identified in the genome sequence of A. niger CBS513.88. The deducted protein CAK41583 shares sequence identities of 36 and 30 % with SirD and 7-DMATS on the amino acid level, respectively. Therefore, it could be a tyrosine or indole PT.

To evaluate the gene function, the entire DNA sequence of An13g01840 was PCR amplified from genomic DNA of A. niger FGSC A1180 and the intron sequence was eliminated by QuickChange Site-Directed Mutagenesis. The obtained coding sequence

For detailed information about this work, please see the publication (section 4.1)

was cloned into pET28a and resulted in the plasmid pHC16. The works described above were carried out by Huizhi Chen within the PPP program between DAAD and CSC.

In this thesis, the plasmid pHC16 was transformed into various *E. coli* strains to find a best condition for protein overproduction. The soluble His\(_6\)-tagged protein from *E. coli* SoluBL21 was purified to near homogeneity and used for enzyme assays with diverse aromatic substrates in the presence of DMAPP.

Lacking the information about its possible natural substrate, tyrosine, tryptophan and their derivatives as well as peptides were tested as potential substrates for this enzyme. HPLC analysis revealed that tyrosine and its derivatives were well accepted and product formations were detected in the incubation mixtures of L-tyrosine and five derivatives thereof. L-Tryptophan and two derivatives were also used by this enzyme as substrates. Structure elucidation of the enzyme products by NMR and MS analyses confirmed O- or N-prenylations of tyrosine and derivatives (Figure 3-2), which proved the identification of a tyrosine O-PT, named TyrPT. It was further compared with SirD toward tyrosine and its derivatives.

Similar to SirD from *L. maculans*, TyrPT also accepted L-tryptophan for a C7-prenylation (Figure 3-2). Therefore, it was further compared with SirD and 7-DMATS toward tryptophan and derivatives. The \(K_M\) values of TyrPT for L-tyrosine, L-tryptophan and DMAPP were determined to be 0.24, 0.19, and 0.71 mM, respectively. The \(k_{cat}\) of tyrosine and tryptophan reactions were determined at 0.58 and 0.0053 s\(^{-1}\), respectively.

The results presented in this study enhance the relationship of tyrosine O- and tryptophan C7-PTs and provide meanwhile a new enzyme for production of prenylated compounds. In comparison to the known tyrosine PT SirD, TyrPT exhibited broader substrate spectrum and significantly higher catalytic activity for several substrates, e.g. 4-amino-L-phenylalanine as well as 4- and 5-methyl-DL-tryptophan.

For detailed information about this work, please see the publication (section 4.2)

3.3. Prenylation of tyrosine and its derivatives by tryptophan prenyltransferase 7-DMATS

7-DMATS from *A. fumigatus* and SirD from *L. maculans* catalyze C7-prenylation of tryptophan and O-prenylation of tyrosine *in vivo*, respectively. SirD was reported to catalyze mainly C7-prenylation of tryptophan and some derivatives thereof *in vitro*. This inspired us to investigate the acceptance of tyrosine and its derivatives by 7-DMATS.

Therefore, tyrosine and nine derivatives were incubated with 7-DMATS in the presence of DMAPP. HPLC analysis of the incubation mixtures revealed clear product formation in several enzyme reactions. Product yields of 36, 70 and 97 % were calculated for tyrosine, 4-amino-L-phenylalanine and 3-iodo-L-tyrosine, respectively and the same O- or N-prenylated products as SirD were obtained and characterized by NMR and MS.
analyses (Figure 3-3). Determination of kinetic parameters revealed that high $K_M$ and low turnover number were obtained for 7-DMATS with tyrosine, whereby comparable $K_M$ value was calculated for 7-DMATS with 3-iodo-L-tyrosine to that of SirD with tyrosine or 7-DMATS with tryptophan.

These results demonstrated the similar substrate and catalytic promiscuity of SirD and 7-DMATS and indicated their close relationship in the evolution. Comparable studies on their structures and identification of their active sites could provide detailed insights into their difference and similarity, which would help us to understand these intriguing DMATS enzymes. At the same time, it could even provide information for enzyme modification to create new biocatalysts with higher substrate flexibility and regioselectivity.

Figure 3-3. Enzyme reactions catalyzed by 7-DMATS toward tyrosine and its derivatives.

For detailed information about this work, please see the publication (section 4.3)


### 3.4. Creation a specific tyrosine C3-prenylating enzyme from tryptophan prenyltransferase FgaPT2 by molecular modeling-guided site-directed mutagenesis

As mentioned above, tryptophan PT 7-DMATS catalyzes *in vitro* O-prenylation of tyrosine and its derivatives. Meanwhile, tyrosine O-PTs SirD and TyrPT can also catalyze the same C7-prenylation of tryptophan as 7-DMATS, which indicated close relationship between tryptophan and tyrosine PTs and encouraged us to investigate the
acceptance of tyrosine by other tryptophan PTs including FgaPT2, 5-DMATS and 6-DMATS<sub>Sa</sub>.

For this purpose, FgaPT2, 5-DMATS, 6-DMATS<sub>Sa</sub> and 7-DMATS were purified and incubated with tyrosine in the presence of DMAPP. A unique product peak was observed in the HPLC chromatogram of FgaPT2 reaction mixture, whose retention time was 2 min shorter than that of 7-DMATS with tyrosine. After isolation and structure elucidation, the enzyme product was proven to be a unique C3-prenylated tyrosine, which was also a natural product isolated from <i>Streptomyces</i> sp. IFM 10937 in 2008 during a screening program for TRAIL-resistance-overcoming activity. So far, there is neither report about its biosynthesis nor an enzyme for tyrosine C3-prenylation. Therefore, this was the first report on an enzymatic C-prenylation of tyrosine as free amino acid.

In order to figure out the catalytic mechanism of this reaction, molecular modeling experiment was carried out by Dr. Zocher from Universität Tübingen based on the available crystal structure of FgaPT2. According to this model,Thr-102 was proposed to interact with the hydroxyl group of tyrosine, while Arg-244, Tyr-191, Leu-81, and Ile-80 to stabilize the side chain. Tyr-413, Lys-187, Arg-100, Tyr-409, Arg-404, Arg-257, Gln-343 and Lys-259 are involved in the diphosphate binding sites. The carboxyl entity of Glu-89, which has an interaction with N-1 of tryptophan, is oriented toward the C-3 of tyrosine and therefore suitable to function as a base to abstract the proton from the σ-complex, which was carried out by Lys-174 in the FgaPT2 reaction with tryptophan. To prove the proposed mechanism, eleven FgaPT2 mutants were newly constructed by site-directed mutagenesis. Together with six available mutants, these proteins were purified and tested for their enzyme activities toward tryptophan and tyrosine. HPLC analysis of the reaction mixtures led to identify an enzyme derivative FgaPT2_K174F, which showed practically no activity toward L-tryptophan, whereas the acceptance of L-tyrosine by this enzyme was improved significantly (Figure 3-4). Therefore, we created a specific tyrosine C3-prenylating enzyme from FgaPT2 by molecular modeling-guided mutagenesis. Based on the modeling and mutagenesis results, a mechanism for FgaPT2 reaction toward tyrosine was also proposed.
RESULTS AND DISCUSSION

Figure 3-4. Switching a tryptophan prenyltransferase to a tyrosine C3-prenylating enzyme by molecular modeling-guided site-directed mutagenesis (graphical abstract of (220)).

For detailed information about this work, please see the publication (section 4.4)


3.5. Regular C-prenylation of o-tyrosine and m-tyrosine by tryptophan and tyrosine prenyltransferases

As aforementioned, tryptophan C4- and C7-PTs FgaPT2 and 7-DMATS catalyze C3- and O-prenylation of tyrosine in vitro, respectively. In return, tyrosine O-PTs TyrPT and SirD prenylate tryptophan at C-7 of the indole ring. These results demonstrated complementary substrate promiscuity of these four enzymes, indicated close relationship between tryptophan and tyrosine PTs and prompted investigation on the acceptance of tyrosine isomers, i.e. L-0-tyrosine and L-0-tyrosine by these two PT groups.

For this purpose, FgaPT2, 5-DMATS, 6-DMATS_{Sv} and 7-DMATS were purified and incubated with L-0-tyrosine and L-0-tyrosine as well as tryptophan and tyrosine under the same condition in the presence of DMAPP. HPLC chromatograms showed interesting results: both L-0-tyrosine and L-0-tyrosine are hardly accepted by the tyrosine PT SirD. Meanwhile, tryptophan PTs FgaPT2, 6-DMATS_{Sv} and 7-DMATS
RESULTS AND DISCUSSION

exhibited higher catalytic ability toward \( o \)-tyrosine than another tyrosine PT TyrPT. In addition, \( m \)-tyrosine was better converted by FgaPT2 than TyrPT. After product isolation and structure elucidation, \( C5 \)-prenylated \( L \)-\( o \)-tyrosine was identified as a unique product of FgaPT2, 5-DMATS, 6-DMATS\(_{Sv} \), 7-DMATS and TyrPT. \( C4 \)- and \( C6 \)-prenylated \( L \)-\( m \)-tyrosine were obtained from FgaPT2 reaction (Figure 3-5).

These results suggest that the acceptance of a substrate by tryptophan or tyrosine PTs would not strictly depend on the chemical category of the aromatic nucleus, but strongly rely on the positions of the functional groups on the aromatic nucleus, which perform the direct interactions with the enzyme active sites. Furthermore, these results revealed that tryptophan and tyrosine PTs possess very likely similar active sites and strengthen their close relationship in the evolution.

![Enzyme reactions catalyzed by tryptophan and tyrosine PTs toward \( L \)-\( o \)-tyrosine and \( L \)-\( m \)-tyrosine.](image)

**Figure 3-5.** Enzyme reactions catalyzed by tryptophan and tyrosine PTs toward \( L \)-\( o \)-tyrosine and \( L \)-\( m \)-tyrosine.

For detailed information about this work, please see the publication (section 4.5)

**Fan, A.,** Xie, X. and Li, S.-M. (2015). Tryptophan PTs showing higher catalytic activities for Friedel-Crafts alkylation of \( o \)- and \( m \)-tyrosine than tyrosine prenyltransferases. *Org. & Biomol. Chem.* (online published).
3.6. Creation of FgaPT2 mutants for production of $C_4$-prenylated cyclic dipeptides with high efficiency by saturation mutagenesis

Tryptophan $C_4$-PT FgaPT2 was reported to catalyze the $C_4$-prenylations of cyclic dipeptides with high concentration of protein as well. To improve the catalytic ability of FgaPT2 toward cyclic dipeptides, saturation mutagenesis experiments were carried out on the key amino acid R244, which is involved in the binding of tryptophan side chain. Fifteen additional mutants of FgaPT2 were obtained. Thirteen mutated FgaPT2 were found to demonstrate much higher catalytic efficiency toward eight tryptophan-containing cyclic dipeptides with up to 155-fold catalytic efficiency of that of FgaPT2 and catalyze the same $C_4$-prenylation as non-mutated FgaPT2. Among these mutants, R244L showed the highest activity toward most of the tested cyclic dipeptides. The highest product yield of 49.9 % toward cyclo-$\text{L}$-Trp-$\text{D}$-Pro was obtained with 0.08 $\mu$g/$\mu$L R244L and 1.5 h incubation time. In addition, cyclo-$\text{L}$-Trp-$\text{L}$-Pro, cyclo-$\text{L}$-Trp-$\text{L}$-Leu were also well accepted substrates by these mutants, with product yields of 32.8 and 37.8 %, respectively. More importantly, these mutants showed different substrate preferences toward these eight cyclic dipeptides. Product yields of 10.5 – 49.9 % were achieved for the eight cyclic dipeptides by using the respective best mutants.

These results provide not only new biocatalysts for production of $C_4$-prenylated cyclic dipeptides, but also abundant data for further investigations on the catalytic mechanism of FgaPT2 and its mutants toward cyclic dipeptides.

For detailed information about this work, please see the submitted manuscript (section 4.6)

3.7. Impacts and perspectives of prenyltransferases of the DMATS superfamily for use in biotechnology

In the past decade, significant achievement has been obtained on the PTs of the DMATS superfamily. More than forty members of this family have been characterized biochemically by genome mining and four crystal structures of them were solved, which served us not only with more biocatalysts for production of prenylated products, but also valuable information to understand these prenyl transfer reactions. Compared to their high substrate specificity on prenyl donors, DMATS enzymes are well-known for their broad substrate promiscuity on the aromatic substrates. Numerous investigations on substrate and catalytic promiscuity of these enzymes have been published and demonstrated their potential application in the chemoenzymatic synthesis and synthetic biology, which are summarized in this review.

Additionally, efforts and progresses have been achieved to expand their acceptance for other prenyl donors, e.g. GPP and FPP as well as some unnatural alkyl, even benzyl donors, which can be used to produce novel unnatural prenylated products (202-205). Furthermore, the potential usage of these enzymes was expanded by coexpression the PT genes with other genes like NRPS and by development of whole cell biocatalyst.

More than 250 prenylated compounds produced by using DMATS enzymes as biocatalysts are summarized in this review. Availability of more crystal structures of these enzymes will provide bases for creation of new desirable biocatalysts with broad substrate specificity, high catalytic efficiency and regio- as well as stereoselectivity for unnatural substrates by site-directed mutagenesis.

For detailed information about this review, please see the submitted manuscript (section 4.7)

4. Publications and manuscripts

4.1. One substrate - seven products with different prenylation positions in one-step reactions: Prenyltransferases make it possible
One Substrate – Seven Products with Different Prenylation Positions in One-Step Reactions: Prenyltransferases Make it Possible

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Abstract: Prenylated indole alkaloids derived from \( \text{L-tryptophan} \) are widely distributed in nature and show diverse biological and pharmacological activities, usually distinct from their non-prenylated precursors. Prenyltransferases catalyze the transfer reactions of prenyl moieties onto the indole nucleus and contribute largely to the structural diversity of these compounds. In this study, we demonstrate the acceptance of cyclo-\( \text{L-homotryptophan-d-valine} \), an unnatural cyclic dipeptide, by eight prenyltransferases of the dimethylallyltryptophan synthase superfamily. Seven products with one prenyl moiety at each position of the indole nucleus and one diprenylated derivative were isolated from enzyme assays of cyclo-\( \text{L-homotryptophan-d-valine} \) with dimethylallyl diphosphate. To the best of our knowledge, this is the first report for production of seven monoprenylated products from one substrate by one-step reactions.

Keywords: dimethylallyltryptophan synthase; enzyme catalysis; Friedel–Crafts alkylation; prenyltransferase

Introduction

Natural products and their derivatives contribute significantly to drug discovery and development. From 1981 to 2012, they comprised 26% of all new approved drugs, meanwhile 20% of new drugs are natural product mimics. Prenylated indole alkaloids including prenylated indole diketopiperazines constitute a natural product class that is widely distributed in both terrestrial and marine organisms. These compounds show potent biological and pharmacological activities for example, anti-cancer and anti-fungal activities, which are usually distinct from those of their non-prenylated precursors. Therefore, different approaches and agents were developed for synthesis of the prenylated indole derivatives. Prenyl bromide, isoprenyl bromide or prenyl acetate with \( \text{NaH} \), lithium salt or other metal catalysts are often used in the prenylation reactions. However, chemical prenylation usually has some limitations such as different reactivities of C-atoms on the indole nucleus, low selectivity of the prenylation for several positions, side products, toxicity and danger of the reagents and solvents for products and environment. In addition, protection and deprotection of functional groups are usually necessary in the synthetic route, which decreases the efficiency of desired products.

In contrast, prenylation catalyzed by enzymes, i.e., prenyltransferases, provides a more selective, efficient, environment-friendly and safer alternative. The majority of the known indole prenyltransferases belongs to the dimethylallyltryptophan synthase (DMATS) superfamily. In the past 10 years, tremendous progress has been achieved on the molecular biological, biochemical and structural biological investigations on this enzyme group. They mainly catalyze transfer reactions of a dimethylallyl moiety from dimethylallyl diphosphate (DMAPP) onto different positions of the indole ring of diverse substrates. One important subgroup of the DMATS superfamily is the dimethylallyltryptophan synthases (=tryptophan prenyltransferases) such as FgaPT2, 5-DMATS and 7-DMATS which use L-tryptophan as natural substrate and catalyze prenylation reactions at C-4, C-5 and C-7 of the indole ring, respectively. The second important subgroup comprises cyclic dipeptide prenyltransferases, which accept tryptophan-containing cyclic dipeptides as natural (BrePT, FtmPT1 and AnaPT) or best substrates (CdpNPT and CdpC3PT). These enzymes
mainly catalyze prenylations at C-2 or C-3 of the indole nucleus.[13] Usually, tryptophan prenyltransferases accepted cyclic dipeptides only at high protein concentrations.[24] Conversely, tryptophan was a very poor substrate for cyclic dipeptide prenyltransferases.[25] Furthermore, each cyclic dipeptide prenyltransferase has its own preference for dipeptides. In this paper, we report the clear acceptance of cyclo-L-homotryptophan-0-valine (1) by both tryptophan and cyclic dipeptide prenyltransferases. Products 2-8 with the prenyl moiety at all positions of the indole nucleus were identified in the enzyme assays.

**Results and Discussion**

**Synthesis of Compound 1**

The synthesis of compound 1 started with lithium–halogen exchange of N-TBDMS-substituted 3-bromindole by tert-butyllithium, followed by nucleophilic attack on the ethylene oxide, the anion of which was caught by p-toluenesulfonyl chloride to generate the tosylate. Alkylation of the anion of the Schöllkopf chiral auxiliary with the tosylate produced the intermediate 10.[26] Intermediate 10 was partly hydrolyzed and deprotected to compound 1 by iodotrimethylsilane (TMSI) (Scheme 1).[27]

**Acceptance of 1 by All of the Tested Enzymes**

Compound 1 was assayed with eight overproduced and purified enzymes of the DMATS superfamily including five cyclic dipeptide prenyltransferases BrePT,[18] FtmPT1,[19] CdpC3PT,[23] CdpNPT[21] and AnaPT[20] as well as three tryptophan prenyltransferases FgaPT2,[14] 5-DMATS[16] and 7-DMATS.[17] It can be expected that 1 should be a good substrate for cyclic dipeptide prenyltransferases, because it bears just one additional C-atom between the indole and the diketopiperazine rings of naturally occurring tryptophan-containing cyclic dipeptides. HPLC analysis of the reaction mixtures revealed indeed that compound 1 was well accepted by all of the five cyclic dipeptide prenyltransferases (Figure 1A–E). Substrate consumptions (Table 1) were calculated from HPLC peak areas of products to substrate in combination with the ratio of signals in $^1$H NMR spectra and were found for BrePT, FtmPT1, CdpC3PT, CdpNPT and AnaPT to be 87, 57, 92, 73, and 55%, respectively (Table 1). Interestingly, 1 was also well accepted by the three tryptophan prenyltransferases (Figure 1F–H). The very good acceptance of 1 by three tryptophan prenyltransferases could be explained by the fact that the structure similarity of 1 with tryptophan has been increased by insertion of one CH$_2$ between the indole and the diketopiperazine rings, which reduces the steric hinderance of the diketopiperazine ring.

Inspection of the HPLC chromatograms of the reaction mixtures (Figure 1) revealed, with exceptions for 5-DMATS and 7-DMATS, the presence of two or more product peaks. One dominant peak was detected in most assays. It seems that the high regioselectivity of the prenyltransferases observed for their natural or best substrate[13] was broken in the presence of the unnatural aromatic substrate 1. This phenomenon was also observed for two tryptophan prenyltransferases with unnatural alkyl donors.[28]

**Identification of Enzyme Products with a Prenyl Moiety at Each Position of the Indole Ring**

For structure elucidation, enzyme assays in 15–25 mL scales were carried out. Enzyme products were isolated subsequently from the reaction mixtures by repeated chromatography on HPLC. One product was obtained from the reaction mixture of 5-DMATS (Figure 1G) and two from those of BrePT, FtmPT1 CdpNPT, FgaPT2 and 7-DMATS (Figure 1A, B, D, F and H). Three and five products were isolated from assays of AnaPT and CdpC3PT, respectively. $^1$H NMR and MS analyses of the isolated compounds revealed that same products have been isolated from different enzyme assays, e.g., 2 and 3 in those of BrePT and FtmPT1, and 6 and 7 in those of CdpC3PT, AnaPT and 7-DMATS (Figure 1C, E and H). In total, eight products 2, 3, 4, 5, 6, 7, 8, and 9 (Figure 2) were identified as prenylated products of 1.

MS analysis showed 2–8 are monoprenylated and 9 a diprenylated product(s) of 1. In the $^1$H NMR spectrum of 2, signals at 5.18 ppm (dd, 10.5, 1.0 Hz, 1H),
Figure 1. HPLC chromatograms of the reaction mixtures.
5.19 ppm (dd, 17.5, 1.0 Hz, 1H) and 6.12 ppm (dd, 17.5, 10.5 Hz, 1H) indicate the attachment of the 3,3-dimethylallyl moiety in a reverse pattern. Considering the chemical shift and coupling pattern of the four aromatic protons, the prenylation position is determined as C-2. This is in consistence with the reverse C-2 prenylation of BrePT for its natural substrate brevianamide F.[18] Interestingly, 2 was also identified in the enzyme assay of FtmPT1, which prenylated brevianamide F at C-2 in a regular pattern.[19]

Signals for a reverse prenyl moiety were also detected in the 1H NMR spectrum of 3 (5.06 ppm, dd, 17.5, 1.5 Hz, 1H; 5.07 ppm, dd, 11.0, 1.5 Hz, 1H; and 5.84 ppm, dd, 17.5, 11.0 Hz). The chemical shifts and coupling pattern of the four aromatic protons suggested a prenylation at C-3, which is in agreement with the presence of the signal for H-2 at 6.41 ppm. NOE effects between H-2 and H-1', H-2', H-4' and H-5' indicated that H-2 and the prenyl moiety have a cis-configuration. The NOE effect between H-2 and H-18 suggested a C-3α prenylation as shown in Figure 2. The C-3 prenylated product 3 from enzyme assays of two C-2 prenyltransferases, BrePT and FtmPT1, indicates a reduction of regioselectivity in the presence of an unnatural substrate which was also observed with FtmPT1 in the presence of some cyclic dipeptides with low conversion yields.[20] In that study, regularly C-3 prenylated products were detected.

In the 1H NMR spectra of 4, 5, 6 and 7, signals of dimethylallyl moieties at 3.44–3.73 ppm (d, 7.0 Hz, 2H), 5.35–5.40 ppm (tsept, 7.5, 1.5, 1H), 1.75–1.77 (s, 2×3H) or 1.77 (s, 3H) and 1.75 (d, 1.0, 3H) suggested regular prenylations. Interpretation and comparison of the signals for three aromatic protons each on the benzene ring with data from the literature[15,16,24,28] proved prenylation of 4, 5, 6 and 7 at C-4, C-5, C-6 and C-7, respectively. Identification of 4 and 5 in the FgaPT2 assay and 6 and 7 in the 7-DMAT assay is not surprising and indicates just a slight change of an attack position of the indole moiety. A similar phenomenon was also observed for unnatural alkyl donor.[20] However, prenylation on position C-6 and C-7 by cyclic dipeptide prenyltransferases, as in the cases of the two C-3 prenyltransferases CdpC3PT (Figure 1C) and AnaPT (Figure 1E) have not been reported before. This provides evidence of a more flexibility and higher capability of these enzymes for chemoenzymatic synthesis.

In comparison to those of 4–7 at 3.44–3.73 ppm, the signal of H-1' of 8 isolated from reaction mixtures of CdpC3PT and CdpNPT (Figure 1C and D) is strongly down-field shifted to 4.63 ppm, which is typical for regularly N-prenylated products.[21] This is also supported by the presence of signals for five aromatic protons. Compound 9 from the AnaPT assay was identified as a C-5 and C-6 diprenylated derivative (Figure 2). In summary, derivatives with prenyl moiety at N-1, C-2, C-3, C-4, C-5, C-6 and C-7 have been produced from a common substrate by one-step reactions using different prenyltransferases of the DMATS superfamily.

### Kinetic Parameters

To learn more about the behaviour of the eight prenyltransferases in the presence of 1, kinetic parameters were determined and calculated from Hanes–Woolf, Eadie–Hofstee and Lineweaver transformations (Table 1, Supporting Information). The observed

**Table 1.** The yields and kinetic parameters of enzyme reactions.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate Consumption [%]</th>
<th>Product</th>
<th>Yield [%]</th>
<th>$K_M$ [mM]</th>
<th>$k_{cat}$ [s$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrePT</td>
<td>87</td>
<td>2</td>
<td>55</td>
<td>0.25</td>
<td>0.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>24</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>7</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FtmPT1</td>
<td>57</td>
<td>2</td>
<td>14</td>
<td>0.13</td>
<td>0.0040</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CdpC3PT</td>
<td>92</td>
<td>2</td>
<td>11</td>
<td>0.23</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>2</td>
<td></td>
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<td></td>
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<td>43</td>
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<td></td>
<td>8</td>
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</tr>
<tr>
<td>CdpNPT</td>
<td>73</td>
<td>3</td>
<td>51</td>
<td>0.37</td>
<td>0.042</td>
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<tr>
<td></td>
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<td>7</td>
<td>16</td>
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<tr>
<td>AnaPT</td>
<td>55</td>
<td>6</td>
<td>23</td>
<td>0.27</td>
<td>0.009</td>
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<td></td>
<td></td>
<td>9</td>
<td>9</td>
<td></td>
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<tr>
<td>FgaPT2</td>
<td>97</td>
<td>4</td>
<td>49</td>
<td>0.54</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>5</td>
<td>49</td>
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<td></td>
</tr>
<tr>
<td>5-DMATS</td>
<td>21</td>
<td>5</td>
<td>22</td>
<td>0.34</td>
<td>0.005</td>
</tr>
<tr>
<td>7-DMATS</td>
<td>50</td>
<td>6</td>
<td>17</td>
<td>0.42</td>
<td>0.020</td>
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<tr>
<td></td>
<td></td>
<td>7</td>
<td>34</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
reactions apparently followed Michaelis–Menten kinetics. As given in Table 1, \( K_M \) values of the eight enzymes differ slightly from each other, from 0.13 for FtmPT1 to 0.54 mM for FgaPT2, clearly distinct from the significant difference of these enzymes towards their natural substrates, e.g., 0.004 mM for \( \text{L-tryptophan} \) with FgaPT2, \(^{14}\) 0.032 mM for brevianamide F with BrePT, \(^{18}\) 0.055 mM for brevianamide F with FtmPT1, \(^{19}\) or 0.23 mM for (\( \text{R} \))-benzodiazepindinone with AnaPT. \(^{20}\)

FgaPT2 showed a lower affinity, but higher turnover number of the tested eight enzymes towards 1. The \( k_{cat} \) value of 0.2 s\(^{-1} \) is approximately 54% of that of \( \text{L-tryptophan} \) with FgaPT2 at 0.37 s\(^{-1} \).\(^{14}\)

**Conclusions**

In this study, we demonstrated the prenylation of a diketopiperazine derivative 1 at each position of the indole ring by eight prenyltransferases, expanding the potential usage of the enzymes. Prenylated derivatives with prenyl moieties at each position have been produced by chemoenzymatic synthesis by using recombinant prenyltransferases.\(^{13}\) However, production of all of the mentioned products from one substrate has not been reported until now. Given the advantages of chemoenzymatic synthesis mentioned in the introduction, this work has increased significantly the substrate and catalytic promiscuity of the prenyltransferases and therefore also the structure diversity of the possible candidates for drug discovery and development.

**Experimental Section**

**General Remarks**

Indole, \( n-\text{BuLi} \), NBS, \( t-\text{BuLi} \), Schöllkopf chiral [(\( R \))-2,5-dihydro-3,6-dimethoxy-2-isopropylpyrazine], TMSI, THF were purchased from Aldrich. TBDMSCl and TsCl were from...
Acros. Ethylene oxide was obtained from Fluka. THF was dried by distillation from sodium benzophenone ketyl. Analytical TLC plates (silica gel 60 F254) were from Merck KGaA, Germany; preparative TLC, pre-coated TLC-plates SILG-200 V254 (2.0 mm silica gel with fluorescent indicator UV254, 20 × 20 cm) were purchased from Macherey-Nagel, Germany.

NMR spectra were recorded on a JEOL ECA-500 spectrometer, processed with MestRENova 5.2.2 and are supplied in the Supporting Information. Chemical shifts of protons were referenced to the signal of DMSO-d6 at 39.5 ppm. The isolated compounds were also analyzed by electron impact mass spectrometry (EI-MS) or electrospray ionization mass spectrometry (ESI-MS) on an Auto SPEC (Micromass Co. UK Ltd.).

**Synthesis of cyclo-L-Homotrp-d-Val (1)**

Compound 10 was synthesized according to the method described by Ma et al. [26] 1H NMR (CDCl3, 500 MHz): δ = 7.59 (d, J = 8.0 Hz, 1H), 7.46 (d, J = 8.0 Hz, 1H), 7.12 (m, 2H), 6.93 (s, 1H), 4.12 (m, 1H), 4.01 (t, J = 4.5 Hz, 1H), 3.74 (s, 3H), 3.71 (s, 3H), 2.72 (m, 2H), 2.27 (m, 2H), 2.11 (m, 1H), 1.07 (d, J = 8.5 Hz, 3H), 0.91 (s, 9H), 0.72 (d, J = 8.5 Hz, 3H), 0.57 (s, 6H); MS (EI): m/z = 441.281156.

To a stirred solution of compound 10 (106 mg, 0.24 mmol) in CHCl3 (2 mL), TMSI (6 equiv.) was added slowly under an N2 atmosphere at 0°C. The reaction mixture was stirred for 1 h at room temperature. The crude product was purified by preparative TLC (acetone/CH2Cl2 1:1) to afford compound 1; yield: 50 mg (0.17 mmol, 70%). [27]

**Overexpression and Purification of Recombinant Proteins**


**Enzyme Assays for Determination of Activities**

Enzyme reaction mixtures (100 μL) for determination of the activities with different proteins contained compound 1 (1 mM), CaCl2 (10 mM), DMAPP (1 mM), glycerol (1.0–9.9% v/v), dimethyl sulfoxide (DMSO, 5% v/v), 50 mM Tris-HCl (pH 7.5) and purified recombinant protein (1.5–20 μg). The reaction mixtures were incubated at 37°C for 16 h and then terminated with 100 μL MeOH. Protein was removed by centrifugation at 13000 ∗ g for 20 min.

**Enzyme Assays for Isolation and Structure Elucidation**

Enzyme Assays for Determination of Kinetic Parameters

Assays for determination of kinetic parameters contained CaCl2 (10 mM), glycerol (1.0–9.9% v/v), DMSO (5% v/v), 50 mM Tris-HCl (pH 7.5), DMAPP (1 mM) and compound 1 at final concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mM. Protein amount and incubation time varied between 2 and 20 μg, 25 and 120 min, respectively. The reactions were then terminated with 100 μL MeOH. Protein was removed by centrifugation at 13000 ∗ g for 20 min.

**HPLC Analysis and Isolation of Enzymatic Products for Structure Elucidation**

The enzyme products were routinely analyzed by HPLC on an Agilent series 1200 by using a Multispher 120 RP-18 column (250 × 4 mm, 5 μm C+5 Chromatographic Service, Langerwehe, Germany) at a flow rate of 1 mL min−1. Water (solvent A) and methanol (solvent B) were used as solvents for analysis and isolation of the enzyme products. A linear gradient of 30–100% (v/v) solvent B in 30 min was used for analysis of the enzymatic products. The column was then washed with 100% solvent B for 5 min and equilibrated with 30% solvent B for another 5 min. Detection was carried out on a photodiode array detector.

The enzyme products of compound 1 (compounds 2–9) were isolated on a Multispher 120 RP18 column (250 × 30 mm, 5 μm) with a linear gradients, 60–100% in 30 min, at 2.5 mL min−1. After each run, the column was then washed with 100% solvent B for 15 min followed by 15 min of equilibration with 60% solvent B at the beginning of each run. The mixtures of compound 6 and 7 were separated again on normal phase HPLC, MultilitHigh 100–5 Si column (250 × 4.6 mm, 5 μm) with a gradient of 0–8% MeOH (MeOH/CHCl3) in 30 min at a flow rate of 1 mL min−1. After each run, the column was washed with 50% MeOH (MeOH/CHCl3) for 5 min, followed by 5 min of equilibration with 100% CHCl3 at the beginning of each run.

**Compound 2:** 1H NMR (CDCl3, 500 MHz): δ = 7.88 (s, H-1), 7.53 (d, J = 7.5 Hz, H-4), 7.30 (d, J = 7.5 Hz, H-7), 7.14 (td, J = 7.5, 1.0 Hz, H-6), 7.09 (td, J = 7.5, 1.0 Hz, H-5), 6.12 (dd, J = 17.5, 10.5 Hz, H-2), 6.00 (s, H-13 or H-16), 5.95 (s, H-16 or H-13), 5.19 (dd, J = 17.5, 1.0 Hz, H-1′), 5.18 (dd, J = 10.5, 1.0 Hz, H-1′), 4.15 (t, J = 5.0 Hz, H-12), 3.91 (m, H-15), 2.93 (m, 2H-10), 2.45 (m, H-18), 2.25 (m, H-11), 2.14 (m, H-
One Substrate – Seven Products with Different Prenylation Positions in One-Step Reactions

Compound 1: 1H NMR (CDCl₃, 500 MHz): δ = 7.90 (d, J = 7.5 Hz, H-4), 6.91 (s, H-6, H-16), 6.30 (s, H-14 or 3H-5), 1.75 (s, 3H-4), 1.04 (d, J = 6.5 Hz, 3H-20 or 3H-19); MS (ESI): m/z = 368.24 (M+H)⁺.

Compound 2: 1H NMR (CDCl₃, 500 MHz): δ = 7.90 (s, H-5), 7.90 (br s, H-16), 6.59 (s, H-10), 2.44 (m, H-18), 2.32 (m, H-11), 1.58 (m, H-18), 1.04 (d, J = 7.0 Hz, 3H-19 or 3H-20), 1.03 (s, 3H-4 or 3H-5), 0.95 (d, J = 6.5 Hz, 3H-20 or 3H-19); MS (ESI): m/z = 368.20 (M+H)⁺.

Compound 3: 1H NMR (CDCl₃, 500 MHz): δ = 7.21 (d, J = 7.5 Hz, H-4), 7.11 (t, J = 7.5 Hz, H-7), 7.02 (br s, H-2), 6.89 (d, J = 8.0 Hz, H-5), 5.97 (s, H-13 or H-16), 5.38 (m, H-12), 4.14 (t, J = 6.0 Hz, H-12), 3.87 (t, J = 2.8 Hz, H-15), 3.75 (d, J = 6.5 Hz, 2H-1), 3.07 (t, J = 8.0 Hz, 2H-10), 2.44 (m, H-18), 2.35 (m, H-11), 2.23 (m, H-11), 1.77 (s, 3H-4 and 3H-5), 1.05 (d, J = 7.0 Hz, 3H-19 or 3H-20), 0.95 (d, J = 7.0 Hz, 3H-20 or 3H-19); MS (ESI): m/z = 390.14 (M+Na)⁺, 368.14 (M+H)⁺.

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References
One substrate – seven products with different prenylation positions in one-step reactions: Prenyltransferases make it possible

Supporting information

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Determination of kinetic parameters (Figure S1- S8)

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Figure S5. Determination of kinetic parameters of AnaPT reaction ............................................... 4
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Figure S7. Determination of kinetic parameters of 5-DMATS reaction ......................................... 5
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Figures Determination of kinetic parameters (Figure S1- S8)

**Figure S1.** Determination of kinetic parameters of BrePT reaction.

**Figure S2.** Determination of kinetic parameters of FtmPT1 reaction.
Figure S3. Determination of kinetic parameters of CdpC3PT reaction.

Figure S4. Determination of kinetic parameters of CdpNPT reaction.
Figure S5. Determination of kinetic parameters of AnaPT reaction.

Figure S6. Determination of kinetic parameters of FgaPT2 reaction.
Figure S7. Determination of kinetic parameters of 5-DMATS reaction.

Figure S8. Determination of kinetic parameters of 7-DMATS reaction.
NMR spectra

Figure S9. $^1$H NMR spectrum of compound 1 in DMSO-d$_6$ (500 MHz).

Figure S10. $^{13}$C NMR spectrum of compound 1 in DMSO-d$_6$ (125 MHz).
Figure S11. $^1$H NMR spectrum of compound 2 in CDCl$_3$ (500 MHz).

Figure S12. $^1$H NMR spectrum of compound 3 in CDCl$_3$ (500 MHz).
Figure S13. NOE spectrum of compound 3 in CDCl₃ (500 MHz).

Figure S14. ¹H NMR spectrum of compound 4 in CDCl₃ (500 MHz).
Figure S15. $^1$H NMR spectrum of compound 5 in CDCl$_3$ (500 MHz).

Figure S16. $^1$H NMR spectrum of compound 6 in CDCl$_3$ (500 MHz).
Figure S17. $^1$H NMR spectrum of compound 7 in CDCl$_3$ (500 MHz).

Figure S18. $^1$H NMR spectrum of compound 8 in CDCl$_3$ (500 MHz).
Figure S19. $^1$H NMR spectrum of compound 9 in CDCl$_3$ (500 MHz).

Figure S20. $^1$H NMR spectrum of compound 10 in CDCl$_3$ (500 MHz).
4. Publications and manuscripts

4.2. A new member of the DMATS superfamily from *Aspergillus niger* catalyzes prenylations of both tyrosine and tryptophan derivatives
A new member of the DMATS superfamily from *Aspergillus niger* catalyzes prenylations of both tyrosine and tryptophan derivatives

Aili Fan · Huizhi Chen · Rui Wu · Hui Xu · Shu-Ming Li

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**Abstract** A putative prenyltransferase gene of the dimethylallyltryptophan synthase (DMATS) family, *An13g01840*, was identified in the genome sequence of *Aspergillus niger*. The deduced polypeptide CAK41583 consists of 465 amino acids with a calculated molecular mass of 52.7 kDa. To evaluate gene function, the coding sequence was cloned into pET28a and overexpressed in *Escherichia coli*. The soluble His6-fusion protein was purified to near homogeneity on Ni-NTA agarose and used for enzyme assays with diverse aromatic substrates in the presence of dimethylallyl diphosphate. HPLC analysis revealed product formation in the incubation mixtures with L-tyrosine and five derivatives thereof. Structure elucidation of the enzyme products by NMR and MS analyses confirmed O-prenylations and proved the identification of a tyrosine O-prenyltransferase (TyrPT). As in the case of SirD from *Leptosphaeria maculans*, TyrPT also accepted 4-amino-L-phenylalanine for an N-prenylation and L-tryptophan for a C7-prenylation. The *K*~M~ values of TyrPT for L-tyrosine, L-tryptophan, and dimethylallyl diphosphate (DMAPP) were found to be 0.24, 0.19, and 0.71 mM, respectively. The *k*~cat~ of L-tyrosine and L-tryptophan reactions were determined at 0.58 and 0.0053 s~−1~, respectively. The results presented in this study enhance the relationship of tyrosine O- and tryptophan C7-prenyltransferases and provide meanwhile a new enzyme for production of prenylated derivatives. In comparison to the known tyrosine prenyltransferase SirD, TyrPT showed significantly higher catalytic activity for several substrates, e.g., 4-amino-L-phenylalanine as well as 4- and 5-methyl-DL-tryptophan.

**Keywords** *Aspergillus niger* · DMATS superfamily · DMAPP · Prenyltransferase · Enzyme catalysis

**Introduction**

Prenyltransferases catalyze transfer reactions of prenyl moieties from different prenyl donors, e.g., dimethylallyl diphosphate (DMAPP) to various aliphatic or aromatic acceptors (Heide 2009). They are involved in nature in both primary and secondary metabolism, and contribute significantly to structural diversity and bioactivities of natural products (Heide 2009). Furthermore, the biological and pharmacological activities of the prenylated products are usually distinct from their non-prenylated precursors (Botta et al. 2005; Li 2010; Wollinsky et al. 2012a), which make these enzymes to be valuable biocatalysts in the structural modification of small molecules. Based on their primary sequences, protein structures, and biochemical properties, prenyltransferases can be classified in different subgroups (Heide 2009; Li 2009). One of the most investigated subgroup is the dimethylallyltryptophan synthase (DMATS) superfamily. So far, more than 30 such prenyltransferases have been identified and characterized biochemically (Winkelblech and Li 2014; Yu and Li 2012). The majority of the DMATS superfamily uses indole derivatives including tryptophan and tryptophan-containing cyclic dipeptides as substrates. One of them is 7-
DMATS from *Aspergillus fumigatus*, which catalyzes the prenylation of L-tryptophan at position C-7 of the indole ring (Kremer et al. 2007) and is involved in the biosynthesis of astechrome (Yin et al. 2013). A few members of the DMATS superfamily are responsible for prenylation of non-indole derivatives. For example, SirD from *Leptosphaeria maculans* catalyzes O-prenylation of tyrosine (Kremer and Li 2010), the first step in the biosynthesis of sirodesmin PL (Gardiner et al. 2004). SirD also catalyzes the C7-prenylation of L-tryptophan, with a relative activity of 8.1% of that of L-tyrosine (Kremer and Li 2010). Detailed investigations on substrate specificity of SirD also demonstrated the acceptance of several methylated and halogenated tryptophan derivatives including 4-methyl-DL-tryptophan (Zou et al. 2011). Recently, Rudolf and Poulter (2013) have confirmed the C7-prenylation of four indole derivatives including L-tryptophan and 4-methyl-DL-tryptophan by SirD. In that study, reversely N1-prenylated derivatives were found as additional enzyme products (mostly as minor products). It seems that the tyrosine O-prenyltransferase SirD and the tryptophan C7-prenyltransferase 7-DMATS share similar catalytic activity toward tryptophan derivatives. An acceptance of L-tyrosine was neither reported for the mentioned 7-DMATS from *A. fumigatus* (Kremer et al. 2007) nor for the recently identified orthologue 7-DMATSNeo from a *Neosartorya* sp. (Miyamoto et al. 2014). To learn more about their relationship, it would be welcome to find additional enzymes and to compare their biochemical features including substrate specificity and kinetic parameters as well. We report here on the cloning and expression of *An13g01840*, one of the two putative prenyltransferase genes of the DMATS family from *Aspergillus niger* and identification of the recombinant protein as a tyrosine O-prenyltransferase (TyrPT).

**Materials and methods**

**Chemicals**

DMAPP was synthesized according to the method described for geranyl diphosphate (GPP) reported previously (Woodside et al. 1988). Substrates used for the enzyme assays were purchased from TCI (Zwijndrecht, Belgium), Acros Organics (Geel, Belgium), Sigma-Aldrich (Steinheim, Germany), Bachem (Bubendorf, Switzerland), and Alfa Aesar (Karlsruhe, Germany) of the highest available purity.

Bacterial and fungal strains, plasmids, and culture conditions

pGEM-T Easy and pET28a were obtained from Promega (Mannheim, Germany) and Novagen (Darmstadt, Germany), respectively. pAK2 and pLW40 were used for overproduction of SirD and 7-DMATS and propagated as described, respectively (Kremer et al. 2007; Kremer and Li 2010). *A. niger* FGSC A1180 was provided by Prof. Michael Müller (University Freiburg, Germany) and cultivated in SDA medium containing dextrose (40 g/L), peptone (10 g/L), and chloromycetin (0.2 g/L) at 30°C. The 2-day mycelia were used for isolation of genomic DNA and the 3-day mycelia for isolation messenger RNA (mRNA). mRNAs were also isolated from 3-day mycelia from cultures in YMG medium containing yeast extracts (4 g/L), malt extracts (10 g/L), and glucose (4 g/L), or PD medium containing potato infusion (4 g/L) and dextrose (20 g/L). The supernatants of the 3-day cultures in these three media were used for LC-MS analysis of accumulation of prenylated compounds.

*Escherichia coli* XL1 Blue MRF*’* (Stratagene, Amsterdam, the Netherlands) and SoluBL21 (Novagen, Darmstadt, Germany) were used for cloning and expression experiments, respectively. They were grown in liquid Lysogeny broth (LB) or Terrific broth (TB) medium and on solid LB medium with 1.5% (w/v) agar at 37°C. For selection of recombinant *E. coli* strains, 50 μg·mL<sup>−1</sup> of carbenicillin or 25 μg·mL<sup>−1</sup> of kanamycin was used.

Computer-assisted sequence analysis

Sequence identities were obtained by alignments of amino acid sequences using the “BLAST 2 sequences” (www.ncbi.nlm.nih.gov). DNASIS software package (version 2.1; Hitachi Software Engineering, Tokyo, Japan) and FGENESH-M (Softberry, Mount Kisco, NY; www.softberry.com) were used for sequence analysis and intron prediction, respectively.

DNA and RNA isolation, cDNA synthesis, PCR amplification, and gene cloning

Standard procedures for DNA manipulation in *E. coli* were performed as described (Sambrook and Russell 2001). Isolation of genomic DNA from mycelia of *A. niger* was performed as described previously (Yu and Li 2012). RNA was isolated with E.Z.N.A. Fungal RNA Kit (Omega Bio-Tek, GA, USA) and complementary DNA (cDNA) was obtained with ProtoScript First Strand cDNA synthesis Kit (Omega Bio-Tek, Norcross, GA) using oligo(dT) or specific primers AN13G01840EXP1 (5′-CACCATGGGTAGTCTT TCTCCCC-3′) and AN13G01840EXP2 (5′-GCCACAAAG ATCTTGCTGGCAGAAAT-3′).

PCR amplification was carried out on an iCycler from BioRad with annealing temperature at 55°C. The entire sequence of *An13g01840* was obtained by PCR amplification with Expand High Fidelity PCR Kit (Roche Diagnostic, Mannheim, Germany) by using genomic DNA as template. The primers were aforementioned AN13G01840EXP1 at the 5′-end and AN13G01840EXP2 at
the 3’-end. The PCR fragment of 1,471 bp was cloned into pGEM-T Easy resulting in plasmid pHC03, which was subsequently sequenced (Eurofins MWG Operon, Munich, Germany) to confirm the sequence. The intron sequence of 53 bp in An13g01840 was removed by QuickChange Site-Directed Mutagenesis PCR with pHC03 as a template, resulting in plasmid pHC09. The primers used were INTRONF (5’-CGTTAAGTATTCCACGCACGATGCTCTCCAGCAGTGGTTCTAC-3’) and INTRONR (5’-GATC GGCTG TGA AA TCTTTAACGCAGGGTTTCA GTATAGGTACGCATG-3’). Bold letters show primer-primer overlapping sequences. For construction of the expression plasmid pHC16, An13g01840 was amplified from pHC09 by PCR using primers AN13G01840EXP1 at the 5’-end and RP (5’-GATC TTGTG7CGCAAAATTTCTCGC-3’) at the 3’-end. Italic letters represent mutations inserted in comparison with the original genome sequence to give the underlined restriction site SalI in the primer RP. The PCR fragment was digested with NcoI and SalI, and ligated into pET28a, which was digested with the same enzymes previously, resulting in the expression vector pHC16.

Overproduction and purification of His-tagged recombinant proteins TyrPT, SirD, and 7-DMATS

For overproduction of TyrPT-His6, E. coli SoluBL21 cells harboring the plasmid pHC16 were cultivated in 2.5-L cylindrical flasks containing 1 L of liquid TB medium, supplemented with 25 μg·mL⁻¹ of kanamycin, and then grown at 37 °C, 220 rpm till OD 600=0.6. For induction of gene expression, isopropyl thiogalactoside (IPTG) was added to a final concentration of 0.8 mM, which had been equilibrated with 50 mM Tris-HCl (pH 7.5) containing 15 % (v/v) glycerol previously. The purified TyrPT-His6 was eluted with the same buffer and stored at −80 °C for enzyme assays.

Overproduction and purification of SirD and 7-DMATS were carried out as described previously (Kremer et al. 2007; Kremer and Li 2010).

Protein analysis

Proteins were analyzed on 12 % (w/v) SDS-PAGE (Laemmli 1970) and stained with Coomassie Brilliant Blue G-250. Protein contents were determined as described elsewhere (Bradford 1976).

The molecular mass of the native recombinant TyrPT-His6 was determined by size exclusion chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare, Freiburg, Germany). To equilibrate the column and elute proteins, 50 mM of Tris-HCl buffer (pH 7.5) containing 150 mM NaCl was used. The column was calibrated with blue dextran 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, Freiburg, Germany).

Enzyme assays

To determine the enzyme activity of TyrPT-His6, the enzyme assays contained 50 mM Tris-HCl (pH 7.5), 10 mM CaCl₂, 1 mM aromatic substrate, 1 mM DMAPP, 0.15–5 % (v/v) glycerol, 0–5 % (v/v) DMSO, and 0.054–0.11 mg/mL (1–2 μM) of purified recombinant protein in a total volume of 100 μL and were incubated at 37 °C for 16 h. The reactions were terminated by addition of 100 μL methanol. Protein was removed by centrifugation at 13,000×g for 20 min and the supernatant was analyzed on HPLC.

For determination of ion dependency of the TyrPT reaction, the enzyme assays contained 50 mM Tri-HCl (pH7.5), 1 mM L-tyrosine, 1 mM DMAPP, and 5 mM metal ion or EDTA and were incubated with 0.052 mg/mL (0.97 μM) TyrPT-His6 at 37 °C for 16 h. The enzyme activity without additive was defined as 100 %.

In order to determine the kinetic parameters of aromatic substrates, DMAPP at 1 mM and aromatic substrates with concentrations of up to 2 mM were used. Variable protein concentrations and incubation times were used for different substrates. For determination of the kinetic parameters of DMAPP, 0.091 mg/mL (1.68 μM) or 0.18 mg/mL (3.36 μM) purified recombinant protein, 1 mM L-tyrosine, or L-tryptophan with DMAPP at final concentrations of 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 mM were incubated at 37 °C for 5 or 90 min, respectively.

For isolation of the enzyme products, reactions were carried out in large scales (10 mL) containing 50 mM}

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**Mutagenesis PCR with pHC03 as a template, resulting in plasmid pHC16.**

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For isolation of the enzyme products, reactions were carried out in large scales (10 mL) containing 50 mM.
Tris-HCl (pH 7.5), 10 mM CaCl₂, 1 mM aromatic substrate, 1 mM DMAPP, and 0.081–0.16 mg/mL (1.5–3.0 μM) recombinant protein. After incubation at 37 °C for 16 h, the reactions were terminated by addition of 10 mL of methanol. After removal of the precipitated protein by centrifugation at 6,000 rpm for 30 min, the reaction mixtures were concentrated on a rotating vacuum evaporator at 35 °C to a final volume of 1 mL before injection into HPLC.

HPLC analysis and isolation of the enzyme products

The enzyme reaction mixtures were analyzed on HPLC (Agilent series 1200, Böblingen, Germany) by using a Multispher 120 RP-18 column (250×4 mm, 5 μm, C + S Chromatographic Service, Langerwehe, Germany) at a flow rate of 1 mL·min⁻¹. Water (solvent A) and methanol (solvent B) were used as solvents. For analysis of the enzyme products, a linear gradient of 30–100 % (v/v) solvent B for 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 5 min. Detection was carried out with a photodiode array detector and illustrated at 277 nm in this paper.

For isolation of the enzyme products, the same HPLC equipment with a Multispher 120 RP-18 column (250×10 mm, 5 μm, C + S Chromatographic Service, Langerwehe, Germany) was used, with a linear gradient of 50–100 % (v/v) of methanol (solvent B) in water (solvent A) in 50–80 min and a flow rate at 2.5 mL·min⁻¹. The column was then washed with 100 % (v/v) solvent B for 10 min and equilibrated with 50 % (v/v) solvent B for 10 min.

NMR and mass spectrometric analyses

For structural elucidation, the isolated enzyme products were subjected to 1H NMR and MS analyses. Electron impact mass spectrometry (EI-MS) or electrospray ionization mass spectrometry (ESI-MS) data were obtained on a Micromass Auto Spec spectrometer (Waters, Milford, MA) and are given in Table 1.

For NMR analysis, the enzyme products were dissolved in DMSO-d₆, CD₃OD, or D₂O. 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 signals at 2.50 ppm for DMSO-d₆, 3.30 ppm for CD₃OD, or 4.79 ppm for D₂O. All spectra were processed with MestReNova 5.2.2 (Metrelab Research, Santiago de Compostella, Spain). NMR data of the isolated products are given in Tables 2 and 3 and spectra as Figs. S1–S9 in Electronic supplementary materials.

Results

Sequence analysis of the putative prenyltransferase gene An13g01840

The putative prenyltransferase gene of the DMATS superfamily An13g01840, also termed as ANI_1_660114, was identified in the genome sequence of A. niger CBS 513.88 (Pel et al. 2007) by BLAST search with sequences of SirD and 7-DMATS. An13g01840 spans bp 451,850–453,300 of NT_166528 and consists of two exons of 1,274 and 124 bp, respectively, interrupted by an intron of 53 bp. Analysis of the up- and downstream genes of An13g01840 in the genome did not provide any evidence for the presence of a gene cluster for secondary metabolite biosynthesis (Table S1 in Electronic supplementary materials). The deduced product of An13g01840, CAK41583, comprises 465 amino acids and has a calculated molecular mass of 52.7 kDa. It shares sequence identities of 36 and 30 % with SirD and 7-DMATS on the amino acid level, respectively.

DNA isolation, PCR amplification, gene cloning, overproduction, and purification of TyrPT-His₆

To investigate the function of the deduced polypeptide CAK41583 in vitro, the entire DNA sequence of An13g01840 was PCR amplified from genomic DNA of A. niger FGSC A1180, and the intron sequence was eliminated by QuickChange Site-Directed Mutagenesis (see “Materials and methods”). The coding region was then cloned into pET28a, resulting in the expression vector pHC16.

After overproduction, the recombinant protein was purified on Ni-NTA-agarose resin to near homogeneity, as shown on SDS-PAGE in Fig. 1. A major protein band migrating above that of the 45 kDa protein marker corresponded well to the calculated molecular mass of TyrPT-His₆ at 53.5 kDa. A protein yield of 4.0 mg TyrPT-His₆ was calculated for 1 L of culture. The molecular mass of the native recombinant TyrPT-His₆ was determined by size exclusion chromatography to be 228 kDa, indicating that this enzyme presumably acts as a tetramer.

Acceptance of tyrosine and derivatives by TyrPT

As aforementioned, An13g01840 is not clustered with other secondary metabolite genes (Table S1 in Supplementary material) and shows moderate sequence similarities to known proteins. Consequently, the natural substrate of the encoded protein cannot be predicted by analysis of the genetic context in genome or by sequence analysis and comparison. Therefore, TyrPT was incubated with diverse aromatic substrates, which had been accepted by prenyltransferases of the DMATS superfamily. DMAPP
was used as prenyl donor for these assays. HPLC analysis of the incubation mixtures revealed that tyrosine and derivatives (1a–7a) were very well accepted by TyrPT (Fig. 2), with product yields in the range of 30 to 100 % after incubation at 37 °C for 16 h. Therefore, we tested the acceptance of these compounds by the known tyrosine O-prenyltransferase SirD under the same condition used for TyrPT and compared their conversions with each other (Fig. 2). As shown in Fig. 2, both enzymes showed comparable activities toward 1a–6a. 3-Iodo-L-tyrosine (5a) and 3,4-dihydroxy-L-phenylalanine (6a) were almost completely converted by both enzymes. Under the tested condition, TyrPT had a significantly higher conversion for 4-amino-L-phenylalanine (7a) than SirD (98 respective 23 %). Remarkably, D-tyrosine (2a) was a very good substrate for both enzymes with product yields between 30 and 33 %, demonstrating their low stereoselectivity. In contrast to the good acceptance of 3-iodo-L-tyrosine (5a), 3,5-dibromo-L-tyrosine (8a) was a poor substrate for SirD and TyrPT. It seems that the steric hindrance is responsible for the low conversions.

O-prenylation of the 4-hydroxy and N-prenylation of the 4-amino group of the selected substrates

For structural elucidation, the enzyme products 1b and 3b–7b (Fig. 2) were isolated by preparative HPLC from the incubation mixtures of 1a and 3a–7a, respectively, and subjected to 1H NMR and MS analyses. HRMS data (Table 1) proved the presence of molecular masses, which are 68 Da larger than those of their respective substrates, providing evidence for monoprenylation in all the products.

Inspection of the 1H NMR spectra of 1b and 3b–7b (Figs. S1–S6 in Supplementary material) revealed the presence of only one product each. Furthermore, the number and coupling pattern of the aromatic protons have not been changed in comparison to those of the respective substrates,

<table>
<thead>
<tr>
<th>Isolated products</th>
<th>Formula</th>
<th>MS mode</th>
<th>Calculated (M⁺)</th>
<th>Measured (M⁺)</th>
<th>Deviation (ppm)</th>
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<tr>
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<td>C₁₄H₁₉NO₃</td>
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<td>EI</td>
<td>263.1521</td>
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<td>8.4</td>
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<td>EI</td>
<td>267.1271</td>
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<td>−2.6</td>
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<tr>
<td>5b</td>
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<td>ESI</td>
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<tr>
<td>6b</td>
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<td>EI</td>
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</tr>
<tr>
<td>7b</td>
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<td>EI</td>
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**Table 2 NMR data of 1b and 3b–7b**

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<th>δ, multi, J</th>
<th>δ, multi, J</th>
<th>δ, multi, J</th>
<th>δ, multi, J</th>
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</tr>
<tr>
<td>3</td>
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<td>6.81, d, 8.5</td>
<td>6.79, d, 8.5</td>
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<td>7.03, d, 8.3</td>
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<td>–</td>
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<table>
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<tr>
<th>Solvent</th>
<th>DMSO-d₆</th>
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<th>DMSO-d₆</th>
<th>D₂O</th>
<th>D₂O</th>
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</thead>
</table>

a Chemical shifts (δ) are given in parts per million and coupling constants (J) in hertz

b Signals overlapping with those of H₂O
indicating the prenylation at O- or N-atom. The $^1$H NMR spectra of 1b in DMSO-$d_6$ and 5b–7b in D$_2$O were nearly identical to those of the enzyme products with SirD (Kremer and Li 2010; Zou et al. 2011), which proved their regular prenylation at the 4-hydroxy group (1b, 5b, and 6b) or 4-amino group (7b). The $^1$H NMR spectrum of 3b in DMSO-$d_6$ was very similar to that of 1b. They differed from each other by the signal for H-8 (3.24 ppm, t, 5.0) in 1b, which was replaced by a singlet for a methyl group at 1.23 ppm in 3b. This verified that 3b had the same prenylated position and pattern as 1b. The spectrum of 4b corresponded well to that of the O-prenylated derivatives reported previously (Zou et al. 2011). The reactions of tyrosine and derivatives catalyzed by TyrPT are illustrated in Fig. 2b.

Acceptance of L-tryptophan and derivatives by TyrPT

Previous studies have demonstrated that the tyrosine O-prenyltransferase SirD also accepted L-tryptophan and several derivatives as substrates and mainly catalyzed C7-prenylations at the indole ring (Kremer and Li 2010; Rudolf and Poulter 2013; Zou et al. 2011). Therefore, we carried out incubations of L-tryptophan (9a) and derivatives (10a–16a) with TyrPT under conditions used for tyrosine derivatives. The reaction mixtures of TyrPT were then compared with those obtained with the tyrosine O-prenyltransferase SirD and the known tryptophan C7-prenyltransferase 7-DMATS (Fig. 3). With an exception for d-tryptophan (10a), L-tryptophan (9a) and all other derivatives (11a–16a) were very well accepted by 7-DMATS, corresponding to our previously reported results (Kremer and Li 2008). L-tryptophan (9a) was accepted by SirD with a product yield of 25%. Much lower conversions were observed for SirD with 4-methyl-DL-tryptophan (11a) and L-abrine (15a). No product formation was detected in the incubation mixtures of SirD with 5-methyl-DL-tryptophan (12a), 6-methyl-DL-tryptophan

### Table 3  NMR data of 9b, 11b, 12b, and 12c

<table>
<thead>
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<th>Compound</th>
<th>Position</th>
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<th>$\delta$, multi, $^a$</th>
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<tr>
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<tr>
<td>4'</td>
<td>1.72, br s</td>
<td>1.61, s</td>
<td>1.74, s</td>
<td>1.75, s</td>
<td></td>
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<td>5'</td>
<td>1.72, br s</td>
<td>1.63, s</td>
<td>1.74, s</td>
<td>1.75, s</td>
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<tr>
<td>Solvent</td>
<td>DMSO-$d_6$</td>
<td>D$_2$O</td>
<td>CD$_2$OD</td>
<td>CD$_3$OD</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Chemical shifts ($\delta$) are given in parts per million and coupling constants (J) in hertz

$^b$ Signals overlapping with those of H$_2$O

$^c$ Signals overlapping with those of glycerol

---

![Fig. 1 SDS-PAGE analysis of the overproduction and purification of TyrPT-His$_{6\alpha}$](image-url)
(14a), and L-β-homotryptophan (16a). All these results are in good agreement with the published results (Zou et al. 2011).

In comparison to L-tyrosine and derivatives (Fig. 2), tryptophan and derivatives were poor substrates for TyrPT. TyrPT accepted L-tryptophan (9a) with a product yield of 33 %, slightly higher than SirD. Significant activity of 41 % was observed for TyrPT with 4-methyl-DL-tryptophan (11a). While at least two product peaks were detected in the incubation mixture of 11a with SirD as reported previously (Rudolf and Poulter 2013), only one product was observed in that with TyrPT. In the incubation mixture of 12a with TyrPT, a broad product peak with a product yield of 15 % was detected, which was absent in that with SirD. The other tested tryptophan derivatives were also poor substrates for TyrPT (Fig. 3).

C7-prenylation of the indole ring

For structural elucidation, the enzyme products of 9a, 11a, and 12a with TyrPT were isolated on HPLC and subjected to 1H NMR and MS analyses. The HRMS data (Table 1) confirmed the monoprenylation of the isolated products.
In the \(^{1}\text{H}\) NMR spectra of 9b and 11b, the signals of \(\text{H-2}'\) of the prenyl moiety in the region of 5.43–5.33 ppm (sept or m) verified that they are regularly prenylated products. In the spectrum of 9b, the signals of the indole moiety at \(\delta_{\text{H}}\) 7.18 ppm (1H, d, 2.4), 7.38 ppm (1H, d, 7.3), 6.92 ppm (1H, t, 7.3), and 6.85 ppm (1H, dd, 7.3,
0.7) were in agreement with those of C7-prenylated tryptophan (Rudolf and Poulter 2013). The 1H NMR data of 11b corresponded very well to the data reported previously (Rudolf and Poulter 2013). These data proved that TyrPT also catalyzed the regular C7-prenylation of L-tryptophan (9a) and 4-methyl-DL-tryptophan (11a).

Inspection of the 1H NMR spectrum of the product of 5-methyl-DL-tryptophan (12a) revealed the presence of two regularly prenylated products in a ratio of 1:2 (12b:12c) by detection of two sets of signals for H-2 at δH 5.25 ppm (br t, 7.0) and 5.41 ppm (br t, 7.2). Unfortunately, these two products could not be separated from each other in this study, even by repeated HPLC chromatography under different conditions. However, their structures can still be elucidated from the 1H NMR spectrum of the obtained mixture. The signals of the aromatic protons in 12c at δH 7.34 (1H, s), 7.15 (1H, s), and 6.75 (1H, s) corresponded very well to those of the C6-prenylated product of 6-DMATS with 12a reported previously (Winkelblech and Li 2014). The 1H NMR spectrum of 12b also showed three singlets at δH 7.47 (1H, s), 7.13 (1H, s), and 7.07 (1H, s), proving the attachment of the prenyl moiety to C7 of the indole ring. In conclusion, TyrPT catalyzes both O-prenylation of tyrosine and derivatives and C7-prenylation of tryptophan and derivatives.

Fifteen cyclic dipeptides containing tyrosine or tryptophan moieties were then tested as substrates. As observed for SirD and some tryptophan prenyltransferases (Kremer et al. 2007; Kremer and Li 2010; Steffan et al. 2007; Steffan and Li 2009; Yue et al. 2007), some tryptophan prenyltransferases (Kremer et al. 2007; Kremer and Li 2010; Steffan et al. 2007; Steffan and Li 2009; Yue et al. 2007), prenyltransferase gene. Interestingly, 11a was much better accepted by TyrPT than SirD. However, their structures can still be elucidated from the 1H NMR spectrum of the obtained mixture. The signals of the aromatic protons in 12c at δH 7.34 (1H, s), 7.15 (1H, s), and 6.75 (1H, s) corresponded very well to those of the C6-prenylated product of 6-DMATS with 12a reported previously (Winkelblech and Li 2014). The 1H NMR spectrum of 12b also showed three singlets at δH 7.47 (1H, s), 7.13 (1H, s), and 7.07 (1H, s), proving the attachment of the prenyl moiety to C7 of the indole ring. In conclusion, TyrPT catalyzes both O-prenylation of tyrosine and derivatives and C7-prenylation of tryptophan and derivatives.

Biochemical properties and kinetic parameters

Usually, the prenyltransferases of the DMATS superfamily are independent of the presence of metal ions in their reactions, although Ca2+ or Mg2+ often enhances the reaction velocities (Steffan et al. 2009). HPLC analysis showed that addition of K+, Na+, Mg2+, Mn2+, Co2+, or EDTA to the reaction mixture of TyrPT-His6 did not change the enzyme activity significantly. Zn2+ reduced slightly and Cu2+ strongly the enzyme activity (Rudolf and Poulter 2013). The 1H NMR data of 5-methyl-DL-tryptophan (9a) and 4-methyl-DL-tryptophan (11a) were determined by Lineweaver-Burk plots (Figs. S11–S20 in Supplementary material) and compared with those reported for SirD (Kremer and Li 2010; Zou et al. 2011). As given in Table 4, tyrosine was better accepted by SirD than that by TyrPT, with a threefold catalytic efficiency. The Km values and turnover numbers of TyrPT for 3a–5a differ slightly from those of SirD, so that both enzymes catalyze the O-prenylation of these substrates with comparable catalytic efficiencies. SirD and TyrPT accepted 9a with comparable Km values (0.23 and 0.19 mM), which are also in the range of Km values for 1a and derivatives 3a–7a. However, the turnover numbers for 9a at 0.06 with SirD and 0.0053 s−1 with TyrPT are significantly lower than those for 1a and derivatives. These results justified the assignment of the function of TyrPT as a tyrosine O-prenyltransferase. Interestingly, 11a was much better accepted by TyrPT than SirD.

Discussion

A putative gene An13g01840 with meaningful sequence homology to enzymes of the DMATS superfamily has been identified in the genome sequence of A. niger CBS 513.88 (Pel et al. 2007). Literature search did not result in any hits on the isolation and identification of prenylated aromatic compounds from A. niger, whose biosynthesis would involve this putative prenyltransferase gene.

In this study, the coding sequence of the putative prenyltransferase gene An13g01840 was cloned into the expression vector pET28a, and the soluble recombinant protein was purified to near homogeneity after overproduction in E. coli. Incubation with a large number of potential aromatic substrates and subsequent analysis of the reaction mixtures on

### Table 4  Comparison of kinetic parameters of TyrPT with those of SirD published previously

<table>
<thead>
<tr>
<th></th>
<th>SirD</th>
<th>TyrPT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km (mM)</td>
<td>kcat (s−1)</td>
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<tr>
<td>1a</td>
<td>0.13a</td>
<td>1.0a</td>
</tr>
<tr>
<td>3a</td>
<td>0.36b</td>
<td>0.31b</td>
</tr>
<tr>
<td>4a</td>
<td>0.24b</td>
<td>0.10b</td>
</tr>
<tr>
<td>5a</td>
<td>0.10b</td>
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</tr>
<tr>
<td>6a</td>
<td>0.21b</td>
<td>0.09b</td>
</tr>
<tr>
<td>7a</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9a</td>
<td>0.23a</td>
<td>0.06a</td>
</tr>
<tr>
<td>11a</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DMAPPP</td>
<td>0.17</td>
<td>1.0</td>
</tr>
<tr>
<td>DMAPPd</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* The data were adopted from previous publications (Kremer and Li 2010; Zou et al. 2011)

1a as aromatic substrate

9a as aromatic substrate

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 Springer
HPLC revealed acceptance of tyrosine and derivatives with excellent activities. Structure elucidation of the isolated enzyme products proved an O-prenylation at the 4-phenolic hydroxy group of tyrosine and four derivatives or N-prenylation in the case of 4-amino-L-phenylalanine. These data suggested that TyrPT probably functions as an O-prenyltransferase. Analysis and comparison of the genes down- and upstream of An13g01840 did not indicate presence of biosynthetic gene clusters for secondary metabolites. HPLC-MS analysis of the fungal cultures did not reveal the presence of prenylated tyrosine, tryptophan or their derivatives (data not shown). Therefore, the natural substrate of TyrPT and its role in the fungal strain remained unknown. To prove the tyrPT expression, the fungus was cultivated in three different media at 30 °C (see “Materials and methods”). mRNA was isolated from 3-day-old mycelia and used as a template for RT-PCR to get cDNAs. No product was detected after PCR amplification (data not shown). This indicates that tyrPT was not expressed under the tested conditions, as observed for many genes in the biosynthesis of secondary metabolites (Brakhage 2013).

Acceptance of L-tryptophan and several derivatives by TyrPT and their prenylation at C7 position just demonstrated its broad substrate specificity, as observed for its homologue SirD from L. maculans (Gardiner et al. 2004; Kremer and Li 2010). This conclusion is confirmed by determination of the kinetic parameters of SirD for L-tyrosine, L-tryptophan, and their derivatives (Table 4). As in the case of SirD, TyrPT accepted L-tryptophan with a comparable, or even smaller $K_m$ value (0.19 mM) than with L-tyrosine (0.24 mM). However, the turnover number for L-tyrosine at 0.58 s$^{-1}$ is more than 100-fold than that for L-tryptophan at 0.0053 s$^{-1}$. As given in Table 4, SirD accepted L-tyrosine (1a) and α-methyl-L-tyrosine (3a) better than TyrPT. TyrPT in contrast accepted 3,4-dihydroxy-L-phenylalanine (6a), 4-amino-L-phenylalanine (7a), 4-methyl-DL-tryptophan (11a), and 5-methyl-DL-tryptophan (12a) much better than SirD (Table 4, Figs. 2 and 3). It could be expected that these two enzymes can be used as complementary biocatalysts in the chemoenzymatic synthesis, to produce desired prenylated derivatives.

The enzymes of the DMATS superfamilly usually showed broad substrate specificity, catalyzed regiospecific prenylations, and resulted in the formation of one dominant product (Yu and Li 2012). However, this regioselectivity decreased with the reduced conversion yields of unnatural substrates and two or more products were detected in such cases (Wollinsky et al. 2012b). This phenomenon was very well demonstrated in this study. Tyrosine and derivatives (1a–7a) as well as L-tryptophan were well accepted by SirD and TyrPT, one predominant product each was found in their reaction mixtures. 4-Methyl-DL-tryptophan (11a) was accepted by SirD with a product yield of 14 %. C7- and NI-prenylated derivatives with a ratio of about 2:1 were detected as enzyme products. 11a was accepted by TyrPT with a product yield of 41 %, and only C7-prenylated derivative was detected as an enzyme product. In the case of 5-methyl-DL-tryptophan (12a) with TyrPT, a product yield of 15 % was detected and two products with prenylations at C6 and C7 with a ratio of 2:1 were found as enzyme products (Fig. 3).

Identification and biological investigation of TyrPT enhance the relationship of the substrate and catalytic promiscuity of tyrosine O- and tryptophan C7-prenyltransferases. TyrPT showed somewhat more flexible toward its aromatic substrates than SirD. Comparable studies in the future on the structure level with TyrPT, SirD, and 7-DMATS and identification of the potential active sites involved in the substrate binding could provide additional information for understanding their substrate and catalytic promiscuity. Meanwhile, it could even provide modification suggestions for creating new biocatalysts with higher substrate flexibility and regioselectivity.

Acknowledgments We thank Prof. Michael Müller (Freiburg, Germany) for providing the A. niger strain, Lena Ludwig for synthesis of DMAPP, and Nina Zitter and Stefan Newel for taking MS and NMR spectra, respectively. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (Li844/4-1 to S.-M. L.) and by a PPP program of Deutscher Akademischer Austauschdienst and China scholarship council (to S.-M. L. and H.X.). Aili Fan is a recipient of a scholarship from China scholarship council.

References


A new member of the DMATS superfamily from *Aspergillus niger* catalyzes prenylations of both tyrosine and tryptophan derivatives

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Fig. S20  Determination of kinetic parameters of TyrPT for DMAPP in the presence of L-tryptophan (9a). ................................................................. 15
**Table S1** Sequence analysis of neighboring genes of An13g01840 from Aspergillus niger CBS513.88.

ANI_1_656114: putative IQ calmodulin-binding motif gene  
ANI_1_250114: unknown gene  
ANI_1_658114: putative monoxygenase gene  
ANI_1_662114: putative amino acid transporter gene  
ANI_1_664114: unknown gene  
ANI_1_666114: putative triacylglycerol lipase  
ANI_1_668114: putative phospholipid methyltransferase

**Table S2** Enzyme activity of TyrPT towards cyclic dipeptides.

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<tr>
<th>Substance</th>
<th>Conversion yield (%)</th>
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<td>Cyclo-L-Trp-L-Tyr</td>
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<tr>
<td>Cyclo-D-Trp-L-Tyr</td>
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</tr>
<tr>
<td>Cyclo-L-Trp-L-Phe</td>
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<tr>
<td>Cyclo-L-Trp-L-Trp</td>
<td>3.1</td>
</tr>
<tr>
<td>Cyclo-L-Trp-L-Leu</td>
<td>3.0</td>
</tr>
<tr>
<td>Cyclo-L-Trp-Gly</td>
<td>2.5</td>
</tr>
<tr>
<td>Cyclo-L-Trp-L-His</td>
<td>0.5</td>
</tr>
<tr>
<td>Cyclo-L-Trp-L-Pro</td>
<td>3.4</td>
</tr>
<tr>
<td>Cyclo-L-Trp-D-Pro</td>
<td>1.3</td>
</tr>
<tr>
<td>Cyclo-D-Trp-L-Pro</td>
<td>1.2</td>
</tr>
<tr>
<td>Cyclo-D-Trp-D-Pro</td>
<td>4.2</td>
</tr>
<tr>
<td>Cyclo-L-Tyr-L-Pro</td>
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</tr>
<tr>
<td>Cyclo-L-Ser-L-Tyr</td>
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<td>(R)-Benzodiazepinedione</td>
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<tr>
<td>(S)-Benzodiazepinedione</td>
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Table S3 Enzyme activity of TyrPT towards other aromatic substances.

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<th>Conversion yield (%)</th>
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<tr>
<td>2-Aminobenzyl alcohol</td>
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</tr>
<tr>
<td>Caffeic acid</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>0.5</td>
</tr>
<tr>
<td>2,5-Di-hydroxybenzoic acid</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>DL-4-Hydroxyphenyllactic acid</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>1-Naphtol</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>2-Naphtol</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>1,4-Dihydroxynaphthalene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>2,4-Dihydroxynaphthalene</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>2,6-Dihydroxynaphthalene</td>
<td>0.5</td>
</tr>
<tr>
<td>3-Hydroxyxanthone</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>3-Hydroxyxathen-9-one</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>Esculin</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>4-Methylesceletin</td>
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<tr>
<td>4-Hydroxy-7-methoxycoumarin</td>
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<tr>
<td>Naringenin</td>
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<tr>
<td>Hesperetin</td>
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<tr>
<td>Luteolin</td>
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<tr>
<td>8-Fluoro-4-hydroxyquinoline</td>
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<tr>
<td>4-Hydroxyquinoline</td>
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</tr>
<tr>
<td>7-(Trifluoromethyl)quinoline-4-thiol</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>
Fig. S1 $^1$H NMR spectrum of 1b in DMSO-d$_6$.

Fig. S2 $^1$H NMR spectrum of 3b in DMSO-d$_6$. 
Fig. S3 $^1$H NMR spectrum of 4b in DMSO-d$_6$.

Fig. S4 $^1$H NMR spectrum of 5b in D$_2$O.
Fig. S5 $^1$H NMR spectrum of 6b in D$_2$O.

Fig. S6 $^1$H NMR spectrum of 7b in D$_2$O.
Fig. S7 $^1$H NMR spectrum of $9b$ in DMSO-d$_6$.

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Fig. S17 Determination of kinetic parameters of TyrPT for L-tryptophan (9a).

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**Fig. S19** Determination of kinetic parameters of TyrPT for DMAPP in the presence of L-tyrosine (1a).

**Fig. S20** Determination of kinetic parameters of TyrPT for DMAPP in the presence of L-tryptophan (9a).
4. Publications and manuscripts

4.3. Prenylation of tyrosine and derivatives by a tryptophan C7-prenyltransferase
Prenylation of tyrosine and derivatives by a tryptophan C7-prenyltransferase

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ABSTRACT

7-DMATS from Aspergillus fumigatus and SirD from Leptosphaeria maculans catalyse a C7-prenylation of L-tryptophan and an O-prenylation of L-tyrosine in nature, respectively. SirD was reported to catalyse the C7-prenylation of L-tryptophan and some derivatives thereof in vitro. We report here the O-prenylation of tyrosine and O- or N-prenylation of its derivatives by 7-DMATS. These results provide experimental evidence for the close relationship of tyrosine O- and tryptophan C7-prenyltransferases regarding their substrate and catalytic promiscuity.

Prenyltransferases catalyse in nature transfer reactions of prenyl moieties from different prenyl donors, for example, dimethylallyl diphosphate (DMAPP) to various aliphatic or aromatic acceptors. They are involved in both primary and secondary metabolism in living organisms, and are responsible in part for the structural diversity and bioactivities of natural products.1–3 Furthermore, the biological and pharmacological activities of the prenylated products are usually distinct from their non-prenylated precursors.4 These features make prenyltransferases interesting research topics for scientists from different disciplines. Based on their sequences and biochemical properties, prenyltransferases can be divided into different subgroups.1,5 One of the most investigated subgroups is the dimethylallyl tryptophan synthase (DMATS) superfamily. So far, more than 30 such prenyltransferases have

Scheme 1. Prenyl transfer reactions catalysed by 7-DMATS (A) and SirD (B) in nature.
been identified and characterised biochemically.\textsuperscript{3,6,7} The majority of the DMATS superfamily uses indole derivatives including tryptophan and tryptophan-containing cyclic dipeptides as substrates. One of them is 7-DMATS from \textit{Aspergillus fumigatus} (\textit{A. fumigatus}), which catalyses the prenylation of L-tryptophan at position C-7 of the indole ring\textsuperscript{8} and is involved in the biosynthesis of astechrome.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{HPLC chromatograms of the reaction mixtures of 7-DMATS with 1a–10a as substrates as well as the prenyl transfer reactions catalysed by 7-DMATS. Detection was carried out with a photo diode array detector and absorptions at 277 nm were illustrated.}
\end{figure}
An orthologue 7-DMATSNeo from a Neosartorya sp. was recently reported by Miyamoto et al. A few members of the DMATS superfamily are responsible for the prenylation of non-indole derivatives. For example, SirD from Leptosphaeria maculans catalyses an O-prenylation of tyrosine (1a), the first step in the biosynthesis of sirodesmin PL (Scheme 1B). SirD shares a sequence identity of 34% on the amino acid level with 7-DMATS and also catalyses the C7-prenylation of L-tryptophan, with a relative activity of 8.1% of that of 1a. Detailed investigations with SirD also demonstrated the acceptance of several methylated and halogenated tryptophan derivatives including 4-methyl-DL-tryptophan by SirD. Recently, Rudolf and Poulter have confirmed the C7-prenylation of three indole derivatives including L-tryptophan and 4-methyl-tryptophan by SirD. In addition, reversely N1-prenylated derivatives were found as additional enzyme products in that study (mostly as minor products). It seems that the tyrosine O-prenyltransferase SirD shares similar catalytic activity with the tryptophan C7-prenyltransferase 7-DMATS. Product formation for 1a was detected neither with 7-DMATS (after incubation with 2.5 μg recombinant protein for 45 min), nor with the recently identified 7-DMATSNeo. No data on the acceptance of 1a derivatives by 7-DMATS and 7-DMATSNeo are available in the literature. Therefore, we reinvestigated the acceptance of 1a and derivatives by 7-DMATS from A. fumigatus with higher protein concentration and longer incubation time than used in our previous studies. L-Tyrosine (1a) and nine derivatives (2a–10a, Fig. 1) were tested as substrate with 20 μg recombinant 7-DMATS in 100 μL enzyme assays at 37 °C for 16 h. HPLC analysis of the incubation mixtures showed clear product formation in several cases (Fig. 1, B–K). Detection was carried out with a photo diode array detector and illustrated for absorption at 277 nm. As shown in Figure 1B, 1a was accepted by 7-DMATS with a product yield of 36%. No product formation was detected in the reaction mixture of its enantiomer 2a (Fig. 1C). Replacement of the phenolic hydroxyl group by an amino group, as in the case of 4-amino-β-phenylalanine (3a), increased the acceptance by 7-DMATS, with a product yield of 70% (Fig. 1D). Again, its enantiomer 4a was not converted by 7-DMATS (Fig. 1E), demonstrating the high stereoselectivity of 7-DMATS towards 1a and its derivatives. Therefore, L-configured tyrosine derivatives were used for further study. Introducing an additional hydroxyl group to C3 of tyrosine (5a) resulted in a reduction of its acceptance by 7-DMATS, with a conversion yield of 12% (Fig. 1F), approximate one third of that of 1a. Interestingly, an iodine substitution to the ortho-position of the hydroxyl group (6a) increased significantly the enzyme activity, with a remarkable product yield of 97% (Fig. 1G). That is about 2.7 times of that of 1a, which might be explained by the electronic effect of this substitution on the benzene ring. The electron donating iodine at C3 position would increase the nucleophilicity of the 4-hydroxy group for dimethyallyl cation and stabilise the formed positively charged ion, which is proposed to be intermediates in the prenylation reactions (Scheme 2). In comparison, 3-nitro-L-tyrosine (7a) with a strong electron withdrawing group on the benzene ring was hardly accepted by 7-DMATS (Fig. 1H). Introducing a second iodine to 6a, as in the case of 8a, abolished the activity almost completely (Fig. 1I). Similarly, a very low activity, with a conversion yield of 2%, was observed for 3,5-dibromo-L-tyrosine (9a) (Fig. 1J). The low conversion of 8a and 9a by 7-DMATS seems to be results of steric hindrance of the two large substitutions at C3 and C5 of the benzene ring. No product peak was observed in the reaction mixture of α-methyl-L-tyrosine (10a) under this condition (Fig. 1K). In comparison, α-methyl-DL-tryptophan was accepted by 7-DMATS with a relative activity of 19.1% of that of L-tryptophan.

To elucidate their structures, the enzyme products of 1a, 3a and 6a were isolated on HPLC from 10 ml of incubation mixtures and then subjected to NMR and MS analyses. MS data supported the monoprenylation of the isolated products 1b, 3b and 6b by detection of molecular masses, which are 68 Da larger than those of the respective substrates (Table S1 in Supplementary material).

Inspection of the 1H NMR spectra (Table S2; Figs. S1–S3 in Supplementary material) of the isolated product peaks 1b, 3b and 6b revealed the presence of signals for a regular prenyl moiety at δH 3.76–4.55 (d, H-1'), 5.36–5.43 (t or tsept, H-2'), 1.73–1.79 (s or d, H-5') and 1.69–1.79 ppm (s or d, H-3'). Furthermore, signals of the aromatic protons at δH 7.15 (d, H-2,6), 6.83 (d, H-3,5) of 1b, 7.22 (d, H-2,6), 6.89 (d, H-3,5) of 3b and 7.65 (s, H-2), 6.92 (d, H-5), 7.22 (d, H-6) of 6b indicated the same number and coupling pattern as those of their respective substrates. The 1H NMR spectra of 1b in DMSO-d6 and 3b in D2O were almost identical to those of the enzyme products with SirD, which confirmed the regular prenylation at the 4-hydroxyl group (1b) or 4-amino group (3b). Comparison of the spectrum of 6b with that of 1b, the signals of H-1' (4.55, d, 6.5) and H-2' (5.43, tsept, 6.5, 1.5) of the prenyl moiety in 6b are very similar to those of 1b. These data proved unequivocally the regular prenylation of 1a and derivatives at O- or N-atom, as summarised in Figure 1A. This means that 7-DMATS catalyses both tryptophan C7- and tyrosine O-prenylation, as observed for the tyrosine O-prenyltransferase SirD.
Miyamoto et al. mentioned that the tryptophan C7-prenyltransferase 7-DMATSNeo from Neosartorya sp. did not accepted 1a as the substrate. Detailed inspection of the HPLC chromatograms in that study indicated the presence of an additional minor peak at approximate 14.7 min after incubation of 1a with 5 μg of 7-DMATSNeo for 16 h. This peak could be the prenylated product 1b identified in our present study.

To get insights into the catalytic efficiency, kinetic parameters of 7-DMATS and SirD towards L-Trp, L-Tyr (1a) and its derivatives (3a and 6a) were determined by Eadie–Hofstee, Hanes–Woolf and Lineweaver–Burk plots (Table 1; Figs. S4–S6 in Supplementary material) and compared with those of the published data for SirD and 7-DMATS. 7-DMATS showed a higher $K_M$ value of 1.7 mM and lower turnover number of 0.091 s$^{-1}$ for 1a than those of SirD with l-tryptophan. On the other hand, 6a was very well accepted by 7-DMATS with a comparable $K_M$ value (0.15 mM) to that of SirD with 1a and of 7-DMATS with l-tryptophan (Table 1). The turnover number of 7-DMATS with 6a at 0.023 s$^{-1}$ is much higher than that with 1a.

In conclusion, the results reported in this study proved unequivocally that the tryptophan C7-prenyltransferase 7-DMATS from A. fumigatus can also use tyrosine and derivatives as substrates and catalyses the same O- or N-prenylation as the tyrosine O-prenyltransferase SirD, which prenylates in turn also tryptophan and derivatives at C7 of the indole ring. These data demonstrated the similar substrate and catalytic promiscuity of the two enzymes and indicated their close relationship in the evolution. Comparable studies in the future on the structure level with 7-DMATS and SirD and identification of the active sites involved in the substrate binding could provide detailed insights into their difference and similarity. Meanwhile, it could even provide modification suggestions for creation of new biocatalysts with higher substrate flexibility and regioselectivity. Furthermore, identification of new members from the DMATS superfamily with similar substrate spectrum and/or catalytic ability would help us to understand this group of intriguing enzymes.

Acknowledgments

This work was supported in part by a Grant from DFG-Germany (Li844/4-1 to S.-M. Li). Aili Fan is a recipient of a scholarship from CSC-China. We thank Lena Ludwig for synthesis of DMAPP, Nina Zitzer and Stefan Newel, all from Philipps-Universität Marburg, for taking MS and NMR spectra, respectively.

Supplementary data

Supplementary data (experimental section, MS data, NMR data and spectra) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2014.07.080.

References and notes


Table 1
Kinetic parameters of 7-DMATS and SirD towards l-Trp, l-Tyr (1a) and its derivatives (3a and 6a) in

<table>
<thead>
<tr>
<th>Substrate</th>
<th>SirD</th>
<th>7-DMATS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_M$ (mM)</td>
<td>$k_{cat}$ (s$^{-1}$)</td>
</tr>
<tr>
<td>l-Trp</td>
<td>0.23</td>
<td>0.06</td>
</tr>
<tr>
<td>1a</td>
<td>0.19</td>
<td>0.21</td>
</tr>
<tr>
<td>3a</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6a</td>
<td>0.10</td>
<td>0.07</td>
</tr>
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</table>

$a$ The data were adopted from Refs. 8.
$b$ The data were adopted from Refs. 10.
$c$ The data were adopted from Refs. 12.
Supplementary Material

for

Prenylation of tyrosine and derivatives by a tryptophan C7-prenyltransferase

Aili Fan and Shu-Ming Li

EXPERIMENTAL

GENERAL INFORMATION

Dimethylallyl diphosphate (DMAPP) was synthesised according to the method described for geranyl diphosphate (GPP) reported by Woodside et al. Substrates used for the enzyme assays are commercially available of highest quality.

EXPERIMENTAL PROCEDURE

Overproduction and purification of 7-DMATS were carried out as described previously by Kremer et al.

ASSAYS FOR DETERMINATION OF ENZYME ACTIVITY. The enzyme assays (100 µl) contained L-tyrosine or derivatives (1 mM), CaCl₂ (10 mM), DMAPP (2 mM), glycerol [1.0 - 1.5% (v/v)], dimethyl sulfoxide [DMSO, 0 - 2.5% (v/v)], Tris-HCl (50 mM, pH 7.5) and 7-DMATS (20 µg). The reaction mixtures were incubated at 37 °C for 16 h and then terminated with 100 µl MeOH. Protein was removed by centrifugation at 13,000 × g for 20 min. Product yields were calculated by peak areas in HPLC chromatograms at 277 nm as well as by comparison of signal intensities of substrate and product in the 1H NMR spectra of the respective reaction mixture. The extinction coefficients of substrate to product were found to be 120%, 135% and 76% for 1a, 3a and 6a, respectively.

ENZYMES ASSAYS FOR DETERMINATION OF THE KINETIC PARAMETERS. The enzyme assays (100 µl) contained 1a, 3a or 6a (0.01 - 5 mM), CaCl₂ (10 mM), DMAPP (1 mM), glycerol [1.0 - 1.5% (v/v)], dimethyl sulfoxide [DMSO, 0 - 2.5% (v/v)], Tris-HCl (50 mM, pH 7.5) and 3 (6a), 7.5 (3a), or 10 µg (1a) 7-DMATS. The reaction mixtures were incubated at 37 °C for 20 (3a), 90 (6a) or 120 min (1a) and then terminated with 100 µl MeOH. Protein was removed by centrifugation at 13,000 × g for 20 min.

ENZYMES ASSAYS FOR ISOLATION OF THE ENZYME PRODUCTS. The enzyme assays (10 ml) contained 1a, 3a or 6a (1 mM), CaCl₂ (10 mM), DMAPP (1.5 mM), dimethyl sulfoxide [DMSO, 0 - 2.5% (v/v)], Tris-HCl (50 mM, pH 7.5) and 7-DMATS (2 mg). The reaction mixtures were incubated at 37 °C for 16 h and then terminated with 10 ml MeOH. Protein was removed by centrifugation at 6,000 × g for 30 min. The supernatant was concentrated on a rotary evaporator to 1 - 2 ml and used as samples for separation on HPLC.

HPLC ANALYSIS AND ISOLATION OF ENZYME PRODUCTS FOR STRUCTURE ELUCIDATION. The reaction mixtures were routinely analysed by HPLC on an Agilent series 1200 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. Water (solvent A) and methanol (solvent B) were used as solvents for analysis and isolation of the enzyme products. A linear gradient of 30 - 100% (v/v) solvent B in 20 min was used for analysis of the enzyme reactions. The column was then washed with 100 % solvent B for 5 min and equilibrated with 30% solvent B for another 5 min. Detection was carried out by photo diode array detector.
The products of 7-DMATS with \textbf{1a}, \textbf{3a} and \textbf{6a} were isolated on a Multospher 120 RP18 column (250 × 10 mm, 5 µm) with different gradients, \textit{e.g.} 60 – 100% in 30 min, at 2.5 ml min\(^{-1}\). After each run, the columns were then washed with 100% solvent B for 10 min followed by 8 min of equilibration with the corresponding condition at the beginning of each run.

**NMR AND MS ANALYSES.** \(^1\)H spectra were recorded on a JEOL ECA-500 MHz spectrometer. Chemical shifts were referenced to the signal of DMSO-d\(_6\) at 2.50 ppm or D\(_2\)O at 4.79 ppm. Spectra were processed with MestRENova 5.2.2. The isolated compounds were also analysed by electrospray ionization mass spectrometry (ESI-MS) on an Auto SPEC (Micromass Co. UK Ltd.).
### Table S1. ESI-MS data of the enzyme products

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<tr>
<td>1b</td>
<td>C₁₄H₁₉NO₃</td>
<td>249.14</td>
<td>250.14</td>
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<tr>
<td>3b</td>
<td>C₁₄H₁₉N₂O₂</td>
<td>248.15</td>
<td>249.12</td>
</tr>
<tr>
<td>6b</td>
<td>C₁₄H₁₈INO₃</td>
<td>375.03</td>
<td>375.96</td>
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### Table S2. ¹H NMR data of the enzyme products 1b, 3b and 6b.

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<tbody>
<tr>
<td></td>
<td></td>
<td>1b</td>
<td>3b</td>
<td>6b</td>
</tr>
<tr>
<td>2</td>
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<td>7.15, d, 8.4</td>
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<td>3</td>
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<td>6.89, d, 8.0</td>
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<td>6.89, d, 8.0</td>
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<td>8</td>
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<td>approx. 3.3</td>
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* Signals overlapping with those of H₂O
Figure S1. $^1$H NMR spectrum of 1b in DMSO-d$_6$.

Figure S2. $^1$H NMR spectrum of 3b in D$_2$O.
Figure S3. $^1$H NMR spectrum of 6b in DMSO-$d_6$. 

![NMR Spectrum](image-url)
**Figure S4.** Determination of kinetic parameters of 7-DMATS for L-tyrosine (1a).

**Figure S5.** Determination of kinetic parameters of 7-DMATS for 4-amino-L-phenylalanine (3a).
Figure S6. Determination of kinetic parameters of 7-DMATS for 3-iodo-L-tyrosine (6a).

Reference List

4. Publications and manuscripts

4.4. Site-directed mutagenesis switching a dimethylallyl tryptophan synthase to a specific tyrosine C3-prenylating enzyme
Site-directed Mutagenesis Switching a Dimethylallyl Tryptophan Synthase to a Specific Tyrosine C^3^-Prenylating Enzyme*

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The tryptophan prenyltransferases FgaPT2 and 7-DMATS (7-dimethylallyl tryptophan synthase) from Aspergillus fumigatus catalyze C^2^- and C^3^-prenylation of the indole ring, respectively. 7-DMATS was found to accept L-tyrosine as substrate as well and converted it to an O-prenylated derivative. An acceptance of L-tyrosine by FgaPT2 was also observed in this study. Interestingly, isolation and structure elucidation revealed the identification of a C^3^-prenylated L-tyrosine as enzyme product. Molecular modeling and site-directed mutagenesis led to creation of a mutant FgaPT2_K174F, which showed much higher specificity toward L-tyrosine than L-tryptophan. Its catalytic efficiency toward L-tyrosine was found to be 4.9-fold in comparison with that of non-mutated FgaPT2, whereas the activity toward L-tryptophan was less than 0.4% of that of the wild-type. To the best of our knowledge, this is the first report on an enzymatic C^3^-prenylation of L-tyrosine as free amino acid and altering the substrate preference of a prenyltransferase by mutagenesis.

According to their sequences, biochemical properties, and structures, prenyltransferases can be divided into several subgroups (1, 9, 10). One of the most investigated subgroups is the dimethylallyl tryptophan synthase (DMATS) superfamily from microorganisms. Until now, more than 40 such enzymes have been identified and characterized biochemically (8, 11–14). The majority of the DMATS superfamily is involved in the biosynthesis of prenylated indole alkaloids and takes indole derivatives including tryptophan and tryptophan-containing cyclic dipetides as substrates (3, 8). Usually, they showed significant substrate tolerance toward different aromatic substrates, but catalyzed regiospecific Friedel-Crafts alkylations of the indole ring. These features make the DMATS prenyltransferases useful tools as biocatalysts for production of prenylated products (8). For example, FgaPT2 catalyzes the first pathway-specific step in the biosynthesis of the ergot alkaloid fumigacavine C in Aspergillus fumigatus, i.e. a regular prenylation of L-tryptophan at position C-4 of the indole moiety in the presence of DMAPP (Fig. 1) (15, 16). It was later demonstrated that FgaPT2 also accepted DMAPP analogues as alkyl donors (17, 18) and accepted simple tryptophan derivatives (19), tryptophan-containing cyclic dipetides (19), or even hydroxynaphthalenes (20) as acceptors. The crystal structure of FgaPT2 was determined in 2009 and used as basis for understanding the prenylation mechanism (21, 22). Another example of this superfamily, 7-DMATS, catalyzes a C^3^-prenylation of L-tryptophan on the indole ring (23) and is involved in the biosynthesis of astechrome in A. fumigatus (24). FgaPT2 and 7-DMATS share a sequence identity of 31% on the amino acid level.

A few members of the DMATS superfamily catalyze prenylations of non-indole derivatives, and in some cases, O-prenylations (25, 26). One example of such enzymes, SirD from Lep-tosphaeria maculans, is involved in the biosynthesis of sirodesmin PL and responsible for the O-prenylation of the phenolic hydroxy group in L-tyrosine (26, 27). SirD shares a sequence identity of 34% on the amino acid level with 7-DMATS and also accepts L-tryptophan and some of its derivatives as aromatic substrates. It catalyzes mainly a C^3^-prenyla-
tion of the indole ring (26, 28, 29). C7-Prenylation of L-tryptophan by tyrosine prenyltransferases was also demonstrated recently with TyrPT from Aspergillus niger (14). Encouraged by the results obtained for SirD and TyrPT with L-tryptophan as substrate, we investigated the behavior of 7-DMATS from A. fumigatus toward L-tyrosine in the presence of DMAPP. 7-DMATS was also able to take L-tyrosine and two derivatives thereof as substrates and catalyzed the same O- or N-prenylation as the two tyrosine O-prenyltransferases SirD and TyrPT (14, 28–30). These results demonstrated the complementary substrate and catalytic promiscuity of tryptophan and tyrosine prenyltransferases and meanwhile raised the question about the acceptance of L-tyrosine and derivatives by other tryptophan prenyltransferases, such as FgaPT2, 5-DMATS, and 6-DMATSSa, which catalyze the L-tryptophan prenylation at C-4, C-5, and C-6, respectively (11, 15, 31). To gain more insights into the behavior of these enzymes toward tyrosine and derivatives, FgaPT2, 5-DMATS, and 6-DMATSSa were overproduced and purified as described previously (11, 19, 31) and incubated with L-tyrosine at 37 °C for 16 h. Incubations with L-tryptophan were used as positive controls. The incubation mixtures were then analyzed on HPLC.

**EXPERIMENTAL PROCEDURES**

Chemicals—DMAPP was synthesized according to the method described for geranyl diphosphate reported previously (32). Substrates used for the enzyme assays were purchased at the highest available purity.

Bacterial Strains, Plasmids, and Culture Conditions—Escherichia coli XL1 Blue MRF’ (Stratagene, Heidelberg, Germany) and E. coli BL21 (DE3)pLysS (Invitrogen, Karlsruhe, Germany) were used for cloning and expression experiments, respectively. pIU18 was used as construct for FgaPT2 overproduction as described previously (19) and as DNA template for site-directed mutagenesis experiments. E. coli cells harboring plasmids were grown in liquid Lysogeny Broth (LB) or Terrific Broth (TB) medium and on solid LB medium with 1.5% (w/v) agar at 37 °C. 25 μg/ml of kanamycin were used for selection of recombinant E. coli strains.

Site-directed Mutagenesis—One-step site-directed mutagenesis protocols were used to generate the mutated derivatives of FgaPT2 listed in Table 1. The QuikChange site-directed mutagenesis kit (Stratagene, Heidelberg, Germany) was used for construction of plasmids pES23 to pES26 and pES34, the Expand Long Template PCR dNTP pack (Roche Diagnostics, Mannheim, Germany) was used for plasmids pALF13, pALF15, pALF16, pALF18, and pALF22, and the Expand Long Template PCR system (Roche Diagnostic, Mannheim, Germany) was used for plasmids pALF23 to pALF28. pES26 was used as template for construction of the double mutant K174F_R244E in pES34 with the primers R244E fw and R244E rev (Table 1). The obtained plasmids were subjected to sequencing to confirm the desired mutations in the respective constructs.

Overproduction and Purification of the Recombinant Proteins—FgaPT2 and its mutated derivatives were overproduced and purified as described previously (19) and analyzed on SDS-PAGE (Fig. 2). Protein yields between 0.6 and 4.9 mg/liter of culture were obtained in this study.

**Enzyme Assays with Recombinant Purified Proteins**—To determine the enzyme activity, the incubation mixtures contained 50 mM Tris-HCl (pH 7.5), 5 mM CaCl2, 1 mM aromatic substrate, 1 or 2 mM DMAPP, 0.15–5% (v/v) glycerol, 0–5% (v/v) dimethyl sulfoxide (DMSO), and 0.2–0.4 mg/ml of purified recombinant protein in a total volume of 100 μl. After incubation at 37 °C for 6 or 16 h, the reactions were terminated by the addition of 100 μl of methanol. Protein was removed by centrifugation at 13,000 rpm for 20 min, and the supernatant was analyzed on HPLC. Data given in this study were calculated from two or three independent measurements.

To determine the kinetic parameters of aromatic substrates, DMAPP at 1 mM and aromatic substrates with concentrations of up to 2 mM were used. Variable protein concentrations and incubation times were used for different enzymes.

**FIGURE 1.** Prenyl transfer reaction catalyzed by FgaPT2 in the biosynthesis of fumigaclavine C.
For isolation of the enzyme products, reactions were carried out in large scales (10 ml) containing 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂, 1 mM aromatic substrate, 1.5 mM DMAPP, and 0.2–0.4 mg ml⁻¹ recombinant protein. After incubation at 37 °C for 16 h, the reactions were terminated by the addition of 10 ml of methanol. After removal of the precipitated protein by centrifugation at 6000 rpm for 30 min, the reaction mixtures were concentrated on a rotating vacuum evaporator at 35 °C to a final volume of 1 ml before injection into HPLC.

**HPLC Analysis and Isolation of the Enzyme Products**—The enzyme reaction mixtures were analyzed on HPLC (Agilent series 1200, Böblingen, Germany) by using a Multospher 120 RP-18 column (250 × 4 mm, 5 μm, CS-Chromatographic Service, Langerwehe, Germany) at a flow rate of 1 ml min⁻¹. Water (solvent A) and methanol (solvent B) were used as solvents. For analysis of 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 5 min. Detection was carried out with a photodiode array detector and illustrated at 277 nm in this study. For isolation of the enzyme products, the same HPLC equipment with a Multospher 120 RP-18 column (250 × 10 mm, 5 μm, CS-Chromatographic Service) was used. A linear gradient of 50–100% (v/v) of methanol (solvent B) in water (solvent A) in 50–80 min was carried out with a flow rate at 2.5 ml min⁻¹. The column was then washed with 100% (v/v) solvent B for 10 min and equilibrated with 50% (v/v) solvent B for 10 min.

**NMR and Mass Spectrometric Analyses**—For structural elucidation, the isolated enzyme products were subjected to ¹H NMR, HSQC, HMBC, and MS analyses. High resolution electron impact mass spectrometry data were obtained on a Micromass Auto Spec spectrometer (Waters, Milford, MA). The enzyme products were: 1b, m/z 249.1361 (C₁₄H₁₉NO₃, calculated, 249.1365); 3b, m/z 248.1524 (C₁₄H₂₀N₂O₂, calculated, 248.1525).

For NMR analysis, the enzyme products were dissolved in CD₃OD or D₂O. 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 for CD₃OD or 4.79 ppm for D₂O. All spectra were processed with MestReNova 5.2.2 (Mestrelab Research, Santiago de Compostela, Spain). NMR data of the isolated products are given in Table 2.

**Molecular Docking Calculation**—The structure of FgaPT2 (3I4X) in complex with tryptophan was used as a template for the docking calculation using AUTODOCK4 (33). The tryptophan and surrounding water molecules were removed, and the prenylation substrate, dimethylallyl S-thiolodiphosphate (DMSPP), was kept in the active site and assumed to be rigid. For docking calculations with L-tyrosine, Lys-174 was mutated to phenylalanine in silico. Docking calculations (genetic algorithm) were prepared with ADT (33) using a grid that covers the complete binding site. The results were visualized with PyMOL (48) and verified for chemical sense.

**RESULTS**

**Acceptance of L-Tyrosine and 4-Amino-L-phenylalanine by FgaPT2**—HPLC analysis of the incubation mixture of L-tryptophan with 0.36 μM FgaPT2 showed a conversion yield of 58 ±
1.0% (Fig. 3A). Using the same amount of protein, a minor additional peak with an approximate conversion of 1.8 ± 0.15% was detected in the incubation mixture of L-tyrosine (1a) (Fig. 3B).

By increasing the FgaPT2 concentration to 3.6 μM, a clear and unique product peak with a yield of 18 ± 1.0% was observed in the HPLC chromatogram of 1a (Fig. 3D). Under this condition, L-tryptophan was completely converted to 4-dimethylallyl tryptophan (4-DMAT) (Fig. 3C). No product formation was detected in the incubation mixtures of 1a with 3.6 μM 5-DMATS or 6-DMATSa (data not shown). Interestingly, the retention time of the enzyme product of FgaPT2 with 1a was found to be 10 min, which is 2 min shorter than that of the O-prenylated derivative obtained from the 7-DMATS assay under the same HPLC condition (data not shown) (30). This indicated the presence of different products in the reaction mixtures of 1a with both tryptophan prenyltransferases. To test the substrate specificity of FgaPT2 toward tyrosine derivatives, D-tyrosine (2a), 4-amino-L-phenylalanine (3a), α-methyl-L-tyrosine (4a), 3-fluoro-DL-tyrosine (5a), 3-iodo-L-tyrosine (6a), 3-nitro-L-tyrosine (7a), 3,5-dibromo-L-tyrosine (8a), and 3,5-diido-L-tyrosine (9a) were incubated with 3.6 μM recombinant FgaPT2 at 37 °C for 16 h. As shown in Fig. 4, FgaPT2 showed a relatively high substrate specificity toward L-tyrosine and its analog 3a. Clear product formation was only observed in the reaction mixture of 3a (data for 2a and 4a–9a not shown). With a total product yield of 70 ± 4.9%, 3a was even better accepted by FgaPT2 than 1a. Inspection of the HPLC chromatogram of the reaction mixture of 3a revealed the presence of two product peaks 3b and 3c at 9.7 and 12 min, with product yields of 67 ± 4.5 and 3 ± 0.46%, respectively (Fig. 4B).

Enzyme Product Characterization—For structure elucidation, the enzyme products 1b, 3b, and 3c were isolated from 10-ml enzyme reaction mixtures of 1a and 3a, respectively, and subjected to MS and NMR analyses. The obtained NMR data are given in Table 2. MS data indicated a monoprenylation in the isolated products 1b and 3a, respectively, and subjected to MS and NMR analyses. The obtained NMR data are given in Table 2. MS data indicated a monoprenylation in the isolated products 1b, 3b, and 3c by detection of molecular masses, which are 68 Da larger than those of the respective substrates (see "Experimental Procedures"). The 1H NMR spec-

FIGURE 3. HPLC analysis of incubation mixtures of L-tryptophan and L-tyrosine (1a) with FgaPT2 (A–D) and its mutant FgaPT2_K174F (E–H). The enzyme assays contained 0.36 or 3.6 μM of the recombinant enzymes and were incubated at 37 °C for 16 h. Detection was carried out with a photodiode array detector and illustrated for absorption at 277 nm. mAUs, milliabsorbance units.
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FIGURE 4. HPLC analysis of the reaction mixtures of 1a (A and C) and 3a (B and D) with FgaPT2 or FgaPT2_K174F as well as prenyl transfer reactions catalyzed by both enzymes (E and F). The enzyme assays contained 3.6 μM of the recombinant enzymes and were incubated at 37 °C for 16 h. Detection was carried out with a photodiode array detector and illustrated for absorption at 277 nm. mAU, milliabsorbance units.

trum of 3c corresponded perfectly to that of the regularly N-prenylated derivative of 3a, which had been identified as an enzyme product of SirD previously (28).

Inspection of the 1H NMR spectra of 1b and 3b revealed the presence of only one product each. The signal of H-2’ at 5.41 ppm (triple septettes, 7.3, 1.4 Hz for 1b) or 5.34 ppm (triple septettes, 7.2, 1.4 Hz for 3b) proved to be regularly prenylated products. Signals of three coupling aromatic protons were observed in spectra of both 1b and 3b, indicating the prenylation at an aromatic carbon atom of 1a and 3a. The coupling patterns of one doublet with a small coupling constant of 2.0 (1a) or 1.6 Hz (3b), one doublet with a large coupling constant of 8.1 Hz, and one double doublet with coupling constants of 8.1 and 2.0 Hz (1b) or 8.1 and 1.6 Hz (3b) also proved a C2- or C3-prenylation in 1b and 3b (Table 2). Given the electron donating effects of the 4-hydroxyl in 1a or 4-amino group in 3a, the Friedel-Crafts alkylation should take place at C-3 of the benzene ring. To prove this hypothesis, HSQC and HMBC spectra of 1b and 3b were then taken for structure determination.

In the HSQC spectrum of 1b, the chemical shifts of the three aromatic proton-bearing carbons are found at 130.7, 115.7, and 128.0 ppm, which were assigned to C-2, C-5, and C-6 of the C3-prenylated product, respectively (Table 2). Clear HMBC correlations between H-1’ of the prenyl moiety and C-4 of the benzene ring as well as H-2 and C-1’ proved unequivocally that 1b was the C3-prenylated product. Furthermore, the 1H NMR spectrum of 1b taken in CD3OD corresponded perfectly to that of the natural product isolated from Streptomyces sp. IFM 10937 (34). Similar correlations were observed in the HMBC spectrum of 3b, and the same conclusion can be therefore drawn for this compound. These results confirmed the C3-prenylation of 1a and 3a by FgaPT2 (Fig. 4, E and F) as the unique or main reaction (95.7%). In the case of 3a, the N-prenylation was only 4.3% of the total product formation.

Molecular Modeling and Site-directed Mutagenesis of FgaPT2—To get more insights into the catalytic mechanism of FgaPT2 toward 1a and 3a, we carried out a molecular modeling study with FgaPT2 and 1a based on the crystal ternary complex of FgaPT2 with DMSPP and l-tryptophan (21). We performed in silico docking of l-tyrosine (blue) into the binding pocket of l-tryptophan (Fig. 5). The final docking result is in good agreement with the observed catalytic activity and shows that the hydroxyl group occurs near a cavity occupied with water molecules (Fig. 5, red spheres). The C3-atom of 1a or 3a (Fig. 5, blue sphere) can easily attack the C3-atom of DMSPP. This model explained well our experimental results that 1a and 3a were also substrates for FgaPT2 and that the resulting products were C3-prenylated derivatives.

According to this model, Thr-102 was proposed to interact with the hydroxyl group of 1a or amino group of 3a through hydrogen bond. Arg-244, Tyr-191, Leu-81, and Ile-80 tend to stabilize the side chain. Tyr-413, Lys-187, Arg-100, Tyr-409, Arg-404, Arg-257, Gln-343, and Lys-259 are involved in the diphosphate binding sites. In a previous study for the FgaPT2 reaction with l-tryptophan (21), Lys-174 was proposed to act as a base for abstracting the proton at C-4 from an intermediate, which was formed after attacking of C-4 to the prenyl cation, and rebuilding the aromatic ring. Later, Luk et al. (35) reported that the FgaPT2 reaction with tryptophan might undergo a reverse prenylation at C-3 followed by a Cope rearrangement and rearomatization. In both proposed mechanisms, Lys-174 always acted as a base for regaining the aromatic ring. The function as a base is lost for K174F at this position, which is accompanied by the prenylation of l-tyrosine at its C-3 atom. In our structural model, the carboxyl entity of Glu-89, which has an interaction with N-1 of l-tryptophan (21, 35), is oriented toward the C-3 of 1a or 3a and therefore suitable to function as a base to abstract the proton from the σ-complex.

To prove the proposed mechanism, we determined the activity of I80F, E89A, K174E, and K174Q obtained in a previous study (21) toward l-tryptophan and l-tyrosine by incubation at 37 °C for 6 h. As shown in Fig. 6, relative activities of 43.2 ± 2.0, 2.3 ± 0.21, 2.0 ± 0.24, and 119.0 ± 9.3% of that of FgaPT2 were detected for I80F, E89A, K174E, and K174Q with l-tryptophan, respectively. The results for I80F, E89A, and K174E are similar to those obtained in the previous study (21). Differing from that observed by Metzger et al. (21), K174Q showed a slightly higher activity than FgaPT2. This result indicated that Gln could elim-
inactivate a proton, or that another amino acid acts as a base instead. Using L-tyrosine as prenyl acceptor, relative activities of 0.62 ± 0.05, 5.7 ± 1.0, 3.2 ± 0.26, and 9.8 ± 0.89% of that of FgaPT2 were detected for I80F, E89A, K174E, and K174Q, respectively. This indicated different behaviors of I80F and K174Q toward L-tryptophan and L-tyrosine and suggested that Lys-174 was important but not the base to abstract a proton. Meanwhile, the significant activity decrease of E89A implied that it could be the base and supported our molecular model for the FgaPT2 reaction with L-tyrosine.

For further investigation, we chose three amino acids residues, Thr-102, Lys-174, and Arg-244 in the structure of FgaPT2 as “hot spots” for site-directed mutagenesis using primers listed in Table 1. We speculated that replacement of Lys-174 by an aromatic amino acid residue would have more interaction with the benzene ring of the substrate L-tyrosine and stabilize the intermediates of the prenylation. Therefore, we prepared additional Lys-174 mutants FgaPT2_K174F, FgaPT2_K174Y, and FgaPT2_K174W.

In another previous study, we showed that the cyclic dipeptide brevianamide F prenyltransferase FtmPT1 from A. fumigatus shared a similar structure and reaction chamber with FgaPT2 (36). However, the amino acid residues involved in the substrate binding differ slightly from each other. For example, Gly-115 in FtmPT1 was proposed to be involved in the binding of brevianamide F (36). The corresponding Thr-102 in FgaPT2...
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is likely not directly involved in the binding of L-tryptophan, but located in the active site of the enzyme (21). Mutation of Gly-115 in FtmPT1 to threonine resulted in a derivative, which catalyzed a reverse C3- instead of a regular C2-prenylation of brevianamide F (36). As shown in Fig. 5, Thr-102 in FgaPT2 is suggested to be involved in the binding of L-tyrosine. Therefore, we changed this residue to five different amino acids to get FgaPT2-T102X (X = Val, Arg, Cys, Gly, or Ser). In addition, molecular modeling indicated the binding of the amino group of the L-tyrosine side chain to Arg-244. Thus, different mutants at this position, FgaPT2_R244X (X = Glu, Asn, Asp, or Gln), were constructed as described under "Experimental Procedures." In total, we designed 13 additional mutants (Table 1) on the basis of the proposed mechanism as well as physical and chemical properties of the amino acids for investigation on enzyme activities.

Identification of the FgaPT2 Mutant Carrying Tyrosine C3-Prenyltransferase but Almost No Tryptophan Prenyltransferase Activity—To evaluate the activity of the resulting mutants, the constructs obtained from site-directed mutagenesis experiments were introduced into E. coli BL21 (DE3)pLysS cells. After induction of the gene expression as described previously (19), the overproduced proteins were purified on nickel-nitrilotriacetic acid-agarose and used for enzyme assays with L-tryptophan or L-tyrosine under the same condition for FgaPT2 mentioned above (37 °C for 6 h). The obtained results for all of the 17 mutants with L-tryptophan and L-tyrosine are illustrated in Fig. 6 and compared with those of FgaPT2.

As shown in Figs. 3 and 6, clearly different activity profiles were observed for these mutants toward L-tryptophan and L-tyrosine. Similar or slightly higher, but in most cases, lower activity than that of non-mutated FgaPT2 was detected in the incubation mixtures of L-tryptophan with 0.36 μM recombinant mutants. Using L-tyrosine as substrate, most mutants showed much lower activities than FgaPT2. No significant changes on the activities were detected for T102C with L-tryptophan and L-tyrosine. Substantial increase of the enzyme activity toward L-tyrosine was detected for K174F. Approximate 3-fold activity than that of non-mutated FgaPT2 was calculated for this mutant (Fig. 3). On the other hand, no conversion was detected in the incubation mixture of l-tryptophan with 0.36 μM K174F, even after an incubation for 16 h (Fig. 3E). Increasing the protein concentration to 3.6 μM and incubation time to 16 h, a minor product peak with a yield of 0.24 ± 0.073% was detected for K174F (Fig. 3G). With this protein concentration, a product yield of 50 ± 3.5% was calculated for K174F with 1a as substrate (Fig. 3H). Isolation and structure elucidation confirmed the same enzyme product as that identified from the incubation mixture of FgaPT2 with 1a. From these results, it can be concluded that K174F functions as a tyrosine C3-prenyltransferase and not as tryptophan prenyltransferase anymore. K174F in a concentration of 3.6 μM was then incubated with eight tyrosine derivatives 2a–9a at 37 °C for 16 h. The product yield of 3a with K174F was slightly increased to 76 ± 4.4% (Fig. 4). No product formation was detected in the incubation mixtures of other substrates (data not shown).

DISCUSSION

Prenyltransferases represent key enzymes in the biosynthesis of prenylated natural products (1, 3, 4, 8, 42). Because of genome sequencing and mining of biosynthetic pathways, significant progress has been achieved on the genetics, enzymology, and structure of prenyltransferases using various aromatic substrates such as indoles including tryptophan, flavonoids, coumarins, xanthones, and naphthalenes (1, 3, 4, 8, 43). A number of studies have demonstrated the high flexibility of prenyltransferases, especially the members of the DMATS superfamily, toward their aromatic substrates (8). However, a switching of substrate preference by structure-based rational engineering has not been reported prior to this study.

Based on the fact that the L-tyrosine O-prenyltransferases SirD and TyrPT catalyzed C7-prenylation of L-tryptophan and the tryptophan C7-prenyltransferase 7-DMATS catalyzed the O-prenylation of L-tyrosine (14, 28, 30), we detected in this study the prenylation of L-tyrosine by the tryptophan C3-prenyltransferase FgaPT2. Identification of enzyme product revealed a C3-prenylation of L-tyrosine by FgaPT2, demonstrating the first enzymatic Friedel-Crafts alkylation of L-tyrosine as free amino acid. Subsequent molecular modeling-guided site-directed mutagenesis led to create an enzyme derivative K174F, which showed practically no activity toward L-tryptophan, whereas the acceptance of L-tyrosine by this enzyme was improved significantly. With 3.6 μM enzyme, a product yield of 50 ± 3.5% was detected for K174F after incubation at 37 °C for 16 h. Therefore, FgaPT2 and especially its mutant K174F represent the first series of L-tyrosine C-prenylating enzymes. Meanwhile, this study demonstrated the possibility to change
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\[ \text{FgaPT2} \rightarrow \text{C}-\text{Prenylated tyrosyl residue} \]

the substrate preference of a prenyltransferase by site-directed mutagenesis.

C-Prenylated tyrosyl residue was found in cyclic peptides, e.g. cyanobactins, from cyanobacteria (40, 44). A prenyltransferase \textit{LynF} in \textit{Lyngbya aestuarii} from the \textit{TruF} enzyme family was proven to catalyze reverse \textit{O}-prenylations of tyrosyl residues in such ribosomal cyclic peptides. C-Prenylated tyrosine-containing derivatives are then formed from the \textit{O}-prenylated tyrosyl residue by Claisen rearrangement afterward (44, 45). To exclude the possibility that the \textit{C}\textsuperscript{3}-prenylated products \textit{1b} and \textit{3b} are rearrangement rather than enzyme products during the incubation procedure, we carried out incubations with \textit{1a} and \textit{3a} for 10, 15, 30, 60, 120, and 960 min. Similar profiles of the HPLC chromatograms were observed for different incubation times (data not shown). In the incubation mixtures of \textit{1a}, only the product peak \textit{1b} was detected. In the incubation mixtures of \textit{3a}, the ratio of the peak area of \textit{3b} to that of \textit{3c} remained almost constant after different incubation times (approximately 22 ± 0.65\%). It seems that \textit{3b} and \textit{3c} are independently formed during the incubation. To provide more details on stability of \textit{3b} and \textit{3c}, \textit{3a} was incubated with FgaPT2 at 37 °C for 2 h. The reaction mixture was then heated to 80 °C and maintained for 3 h. No change was observed for the ratio of \textit{3b} and \textit{3c} in the HPLC chromatogram of the treated sample, excluding the heat-catalyzed rearrangement between \textit{3b} and \textit{3c}.

Taking the results obtained from molecular modeling and mutagenesis experiments into consideration (Figs. 5 and 6), a mechanism was proposed for FgaPT2 reaction with \textit{1a}. According to this hypothesis, the residue Thr-102 in FgaPT2 interacts with the 4-hydroxyl group of \textit{1a} in the reaction cavity and contributes to the stabilization of the intermediate. A number of basic amino acids and tyrosine residues mentioned before are involved in the binding of DMAPP and responsible for the formation of the dimethylallyl carbon cation (21, 46). A detailed course of the reaction remained to be elucidated and might start by a nucleophilic attack of electron-rich C-3 of the benzene ring onto C-1 of the dimethylallyl carbon cation, resulting in the formation of an intermediate with a non-aromatic system. A plausible deprotonation to regain aromaticity might involve Glu-89 of FgaPT2, resulting in the formation of \textit{C}\textsuperscript{3}-prenylated product \textit{1b} (Fig. 7). In comparison with that of FgaPT2, FgaPT2_E89A showed only a relative activity of 5.7 ± 1.0% toward L-tyrosine, providing strong support for this hypothesis. It is also considerable that a reverse prenylation at C-1 is followed by a Cope rearrangement as proposed for FgaPT2 with L-tryptophan (35).

Using the molecular model mentioned above, the activities of other mutants obtained in this study can also be interpreted. For the mutation at Thr-102, substitution of Thr with similar amino acids, such as Cys and Ser, (partly) retained the catalytic ability toward L-tryptophan, whereas T102C demonstrated similar catalytic ability toward \textit{1a} as FgaPT2, 1.9-fold of that of T102V, and 7.1-fold of that of T102S. This indicates that both the hydrogen donor and the methyl group are important for the activity of FgaPT2 toward \textit{1a}. Although the hydrogen bond is not crucial for the reaction (T102V), steric collisions (T102R) abolish the reaction, whereas smaller side chains avoid the correct positioning of the aromatic ring systems (T102G). Interestingly, mutant T102C slightly increases the catalytic turnover as the nucleophilic sulfur atom is more suitable to stabilize the partial positive charge of the cationic intermediate.

By substitution of Lys-174 with Glu, Tyr, or Trp, the activities toward both L-tryptophan and \textit{1a} were reduced or abolished, whereas K174F demonstrated a specific and 4.9-fold catalytic efficiency toward \textit{1a}, but almost complete loss of activity toward L-tryptophan. The rigidity and increased size of the phenyl side chain of K174F seem to support the stabilization of a smaller substrate (tyrosine) in a cavity that was designed to incorporate a larger educt (tryptophan). Besides, the introduction of the mutation K174F is likely to induce minor conformational rearrangements to its neighboring residues of the indole binding site by steric restrains. The higher activity of K174F for \textit{1a} could be interpreted by much better interaction of the modified cavity to the phenyl moiety with the benzene ring of L-tyrosine compared with the interaction with the indole ring of L-tryptophan.

All four mutated derivatives at Arg-244 showed poor conversion of L-tryptophan. Although FgaPT2 does accept the removal of the positive charge by the Arg-244 head group (R224Q and R244N), the introduction of a negative charge by R244E would abolish the prenylation of tryptophan due to charge-charge repulsion. The enzyme activities of these four
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Arg-244 mutants toward L-tyrosine were abolished completely (Fig. 6), demonstrating its essential role in the interaction of FgaPT2 with the side chain of L-tyrosine. The absolute importance of this amino acid residue was also demonstrated by the double mutant K174F_R244E.

In this study, we reported the C3-prenylation of L-tyrosine and 4-amino-L-phenylalanine by the tryptophan C3-prenyltransferase FgaPT2 from the fungus A. fumigatus and demonstrated, to the best of our knowledge, the first enzymatic Friedel-Crafts alkylation of tyrosine and derivative as free amino acids. Our results provided additional evidence for the relationships of substrate and catalytic promiscuity between tryptophan and tyrosine prenyltransferases. Furthermore, a binding site different from that of L-tryptophan was proposed for L-tyrosine and used as the basis for rational design of FgaPT2 mutants. K174F exhibited much higher catalytic efficiency toward L-tyrosine than FgaPT2, whereas its activity toward L-tryptophan was almost abolished. C3-Prenylated L-tyrosine and 4-amino-L-phenylalanine remained unique or predominant product of the mutant. The ratio of the product yields of L-tyrosine to L-tryptophan was increased from 1.31 with FgaPT2 to 208:1 with K174F. This means that K174F does not act as a L-tryptophan C3-prenyltransferase and could serve as new biocatalyst for C3-prenylation of L-tyrosine. Therefore, the result provides an exciting example for creating biocatalysts by mutation of known enzymes. It is also considerable that enzymes for specific prenylation of flavonoids or hydroxynaphthalenes could be created by mutation of some members of the DMATS superfamily because such compounds have already been accepted by some of these enzymes as prenylation substrates (20, 47).

Meanwhile, this study presents an excellent example of successful interdisciplinary cooperation and the importance of structural biology in the discovery and development of novel biocatalysts.

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REFERENCES

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4. Publications and manuscripts

4.5. Tryptophan prenyltransferases showing higher catalytic activities for Friedel-Crafts alkylation of \( o \)- and \( m \)-tyrosine than tyrosine prenyltransferases
Tryptophan prenyltransferases showing higher catalytic activities for Friedel-Crafts alkylation of \( \alpha \)- and \( m \)-tyrosine than tyrosine prenyltransferases

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Tryptophan prenyltransferases FgaPT2, 5-DMATS, 6-DMATS\textsubscript{v} and 7-DMATS catalyse regiospecific C-prenylations on the indole ring, while tyrosine prenyltransferases SirD and TyrPT catalyse the O-prenylation of the phenolic hydroxyl group. In this study, we report the Friedel-Crafts alkylation of \( \alpha \)-tyrosine by these enzymes. Surprisingly, no conversion was detected with SirD and three tryptophan prenyltransferases showed significantly higher activity than another tyrosine prenyltransferase TyrPT. \( C_3 \)-prenylated \( \alpha \)-tyrosine was identified as unique product of these enzymes. Using \( l \)-m-tyrosine as prenylation substrate, product formation was only observed with the tryptophan prenyltransferases FgaPT2 and 7-DMATS. \( C_4 \) and \( C_6 \)-prenylated derivatives were identified in the reaction mixture of FgaPT2. These results provided additional evidence for the similarities and differences of these two subgroups within the DMATS superfamily in their catalytic behaviours.

Introduction

Prenylated secondary metabolites are widely distributed in nature. The biological and pharmacological activities of prenylated products are usually distinct from their non-prenylated precursors and therefore contribute largely to drug discovery and development programmes.\textsuperscript{1-3} Prenyltransferases are involved in the biosynthesis of these natural products and catalyse the regiospecific, in most cases Friedel-Crafts alkylations by transferring prenyl moieties from different prenyl donors to various acceptors. The prenyl moieties with different carbon chain lengths (C5, C10, C15 or C20 units) can be attached in reverse or regular pattern and further modified by cyclization, oxidation and more. Therefore, prenyltransferases play an important role in the formation of the structural diversity of such products.\textsuperscript{4,6} These features have drawn considerable attention from scientists in different disciplines to prenyltransferases and prenylated derivatives. Based on their primary sequences, biochemical properties and structures, prenyltransferases can be divided into different subgroups including protein prenyltransferases, prenyl diphosphate synthases and aromatic prenyltransferases.\textsuperscript{4,7} In the last years, significant progress has been achieved for aromatic prenyltransferases, especially for those from the dimethyallyl tryptophan synthase (DMATS) superfamily.\textsuperscript{6,8,9} So far, more than 40 such prenyltransferases have been identified and characterised biochemically from microorganisms, especially from fungi and bacteria.\textsuperscript{6,8,9} The majority of the DMATS superfamily takes indole derivatives including tryptophan and tryptophan-containing cyclic dipeptides as substrates. In the presence of dimethyallyl diphosphate (DMAPP), for example, FgaPT2,\textsuperscript{10} 5-DMATS,\textsuperscript{11} 6-DMATS\textsubscript{v}\textsuperscript{8} and 7-DMATS\textsuperscript{12} from this family catalyse regiospecific prenylations of \( l \)-tryptophan (1a) at C-4, C-5, C-6 and C-7 of the indole ring, respectively (Fig. 1). FgaPT2 and 7-DMATS from \textit{Aspergillus fumigatus} are involved in the biosynthesis of fumigaclavine C\textsuperscript{13} and astechrome\textsuperscript{14}, respectively. 6-DMATS\textsubscript{v} is likely involved in the biosynthesis of 6-dimethylallylindole-carbardinhyde in \textit{Streptomyces violaceusniger}.\textsuperscript{8} A few members of the DMATS superfamily are responsible for the prenylation of non-indole substances. For example, SirD from \textit{Leptosphaeria maculans} catalyses an O-prenylation of tyrosine (2a) (Fig. 1),\textsuperscript{15,17} the first specific step in the biosynthesis of sirodesmin PL.\textsuperscript{16} Last year, a new tyrosine O-prenyltransferase TyrPT (Fig. 1) was identified in \textit{Aspergillus niger}, which was demonstrated to have similar functions as SirD.\textsuperscript{17} Further study showed that SirD and TyrPT also accepted \( l \)-tryptophan (1a) as substrate \textit{in vitro} and catalyse the same \( C_7 \)-prenylation as 7-DMATS (Fig. 1).\textsuperscript{15,17} Correspondingly, 7-DMATS catalyse the same O-prenylation of tyrosine as SirD and TyrPT (Fig. 1). Very recently, we demonstrated the \( C_3 \)-prenylation of \( l \)-tyrosine (2a) by the tryptophan C4-prenyltransferase FgaPT2 (Fig. 1).\textsuperscript{18} These results demonstrated the close relationship between tryptophan C-prenyltransferases and tyrosine O-prenyltransferases. The different regioselectivities of the mentioned enzymes with 2a encouraged us to test the acceptance of the 2a isomers, \( l \)-\( \alpha \)-tyrosine (3a) and \( l \)-\( \tau \)-tyrosine (4a), by tryptophan and tyrosine prenyltransferases. 3a and 4a are import amino acid analogues found in metabolic pathways of human being and have shown potentials for treatments of different diseases.\textsuperscript{19-21} Prenylation of these two compounds have not been reported yet and their prenylated derivatives could be interesting candidates for further biological and pharmacological investigations.
Fig. 1. Selected examples of prenyl transfer reactions catalysed by tryptophan and tyrosine prenyltransferases.

Results and discussion

Comparison of the enzyme activities of tryptophan and tyrosine prenyltransferases towards tryptophan (1a) and tyrosine (2a)

For better comparison of their catalytic activities, we carried out incubations of 1a and 2a with FgaPT2, 5-DMATS, 6-DMATS<sub>Sv</sub>, 7-DMATS, SirD and TyrPT under the same conditions, i.e. 2 µg enzyme for 1a and 20 µg for 2a, 1 mM 1a or 2a, 5 mM CaCl<sub>2</sub> and 1 mM DMAPP in 100 µL reaction mixtures. With the exception for 2a with 5-DMATS and 6-DMATS<sub>Sv</sub>, HPLC analysis of the reaction mixtures (Figs. 2A - 2L) revealed the formation of one product each, confirming the results published previously.\(^{17,18,22}\) Product yields of these reactions were summarized in Table 1. It is obvious that tryptophan prenyltransferases FgaPT2, 5-DMATS, 6-DMATS<sub>Sv</sub> and 7-DMATS accepted 1a much better than 2a. Over 50 % conversion yields were observed with 1a as substrate by tryptophan prenyltransferases after incubation at 37 °C for 1.5 h (Table 1; Figs. 2A - 2D). With 2a as substrate, product formation was detected only in the reaction mixtures of FgaPT2 and 7-DMATS for tryptophan prenyltransferases, with product yields of 6.2 ± 1.0 and 13.8 ± 1.3 %, respectively (Table 1). As expected, 2a was much better accepted by tyrosine than by tryptophan prenyltransferases. Almost total conversion of 2a was observed for TyrPT and SirD (Table 1; Figs. 2K and 2L). Product yields of 1a with TyrPT and SirD were found to be 5.2 ± 0.40 and 5.9 ± 1.0 % (Table 1, Fig. 2), respectively.

Acceptance of L-o-tyrosine (3a) by all the tested tryptophan prenyltransferases, but not by the tyrosine prenyltransferase SirD

In the presence of 1 mM DMAPP, L-o-tyrosine (3a) was incubated with the six prenyltransferases (20 µg in 100 µL assay) mentioned in Fig. 2 at 37 °C for 1.5 h. Subsequent HPLC analysis revealed product formation in five reaction mixtures (Figs. 2M - 2Q). Surprisingly, 3a was accepted by all the tested tryptophan prenyltransferases, but not by the tyrosine prenyltransferase SirD (Fig. 2R), although TyrPT and SirD shared nearly the same behaviour towards diverse substrates tested before.\(^{17}\) Furthermore, the tryptophan prenyltransferases FgaPT2, 6-DMATS<sub>Sv</sub> and 7-DMATS with product yields of 73.6 ± 3.6, 29.9 ± 2.5 and 40.2 ± 3.8 %, respectively, showed significantly higher activities than TyrPT with a product yield of 10.8 ± 0.7 %, which is comparable to that of 5-DMATS. The product peak detected in these assays display the same retention time, indicating the formation of an identical enzyme product by different prenyltransferases.

C5-prenylated o-tyrosine (3b) as the unique product of tyrosine and tryptophan prenyltransferases

To elucidate their structures, the enzyme products of 3a with FgaPT2, 5-DMATS, 6-DMATS<sub>Sv</sub>, 7-DMATS and TyrPT were isolated on HPLC from 5 - 10 ml of incubation mixtures and subjected to NMR (Table 2, Fig. S1 in ESI) and HR-MS analyses (Experimental Section). HR-MS data confirmed the monoprenylation of the isolated products by detection of molecular masses, which are 68 Da larger than that of 3a. \(^{1}H\)-NMR data proved that FgaPT2, 5-DMATS, 6-DMATS<sub>Sv</sub>,
Fig. 2. HPLC analysis of the reaction mixtures of 1a - 4a with tryptophan and tyrosine prenyltransferases and the enzyme reactions of 3a and 4a. The assays contained 1 mM aromatic substrate, 5 mM CaCl$_2$, 1 mM DMAPP, and 2 (for 1a) or 20 (for 2a – 4a) µg purified recombinant protein and were incubated at 37°C for 1.5 h. Results were illustrated for absorption at 277 nm.
Table 1. Prenylation positions and product yields [%] of enzyme reactions.

<table>
<thead>
<tr>
<th>1a Product</th>
<th>2a Product</th>
<th>3a Product</th>
<th>4a Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preynlated position*</td>
<td>Product yield [%]</td>
<td>Preynlated position*</td>
<td>Product yield [%]</td>
</tr>
<tr>
<td>FgaPT2</td>
<td>C-4</td>
<td>51.2 ± 4.6</td>
<td>C-3</td>
</tr>
<tr>
<td>5-DMATS</td>
<td>C-5</td>
<td>62.1 ± 2.8</td>
<td>-</td>
</tr>
<tr>
<td>6-DMATS</td>
<td>C-6</td>
<td>63.6 ± 2.9</td>
<td>-</td>
</tr>
<tr>
<td>7-DMATS</td>
<td>C-7</td>
<td>52.1 ± 1.9</td>
<td>O-4</td>
</tr>
<tr>
<td>TyrPT</td>
<td>C-7</td>
<td>5.2 ± 0.4</td>
<td>O-4</td>
</tr>
<tr>
<td>SirD</td>
<td>C-7</td>
<td>5.9 ± 1.0</td>
<td>O-4</td>
</tr>
</tbody>
</table>

*For structures see Fig. 1; “-” not determined.

7-DMATS and TyrPT converted indeed 3a to the same product 3b. Inspection of the 1H-NMR spectra (Fig. S1 in ESI) of 3b revealed the presence of signals for a regular prenyl moiety at δH 3.21 (d, H-1’), 5.25 (tsept, H-2’), 1.70 (d, H-4’) and 1.69 ppm (br s, H-5’). Furthermore, signals for three aromatic protons indicated that the prenylation took place on the aromatic ring, rather than at the hydroxyl group. The signals of the aromatic protons at δH 6.76 (d, 8.0 Hz), 6.91 (dd, 8.0, 2.0 Hz) and 6.99 (d, 2.0 Hz) of 3b proved the prenylation at C-4 or C-5 of 3a (Table 2). For the C-5-prenylated 3a, it is expected that the signal of H-3 should be found in the high-field with a coupling constant of approximate 8.0 Hz and the signal of H-6 in low-field with a coupling constant of 2.0 Hz. The obtained data corresponded very well to this prediction and therefore confirmed 3b to be the C5-prenylated product (Fig. 2Y). These results sound somewhat surprising, because O-prenylated derivative was identified in the reaction mixtures of l-tyrosine (2a) with 7-DMATS and TyrPT. However, C3-prenylated derivative was the enzyme product of FgaPT2 with 2a (Fig. 1).18

Table 2. 1H-NMR and 13C-NMR data of the enzyme products (500 MHz).

<table>
<thead>
<tr>
<th>Pos</th>
<th>δH multi, J</th>
<th>δC multi, J</th>
<th>δH</th>
<th>δC</th>
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<tr>
<td>1</td>
<td>6.69, d, 2.5</td>
<td>134.9</td>
<td>6.69, s</td>
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<tr>
<td>2</td>
<td>6.76, d, 8.0</td>
<td>135.4</td>
<td>-</td>
<td>-</td>
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<tr>
<td>3</td>
<td>6.91, dd, 8.0, 2.0</td>
<td>130.6</td>
<td>7.00, d, 7.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>7.00, d, 8.0</td>
<td>130.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>6.99, d, 2.0</td>
<td>131.2</td>
<td>6.67, d, 7.5</td>
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<tr>
<td>6</td>
<td>approx. 3.30'</td>
<td>3.42, dd, 15.0, 4.5</td>
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<tr>
<td>7</td>
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<td>2.80, dd, 15.0, 10.5</td>
<td>2.86, dd, 14.0, 9.3</td>
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</tr>
<tr>
<td>8</td>
<td>3.85, dd, 8.5, 4.0</td>
<td>3.72, dd, 10.5, 4.5</td>
<td>5.55</td>
<td>3.69, m</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>172.6</td>
<td>172.6</td>
<td></td>
</tr>
<tr>
<td>1'</td>
<td>3.21, d, 7.0</td>
<td>3.33, d, 7.0</td>
<td>30.0</td>
<td>3.24, d, 7.5</td>
</tr>
<tr>
<td>2'</td>
<td>5.25, brs, 7.5, 1.5</td>
<td>5.19, br, t, 7.0</td>
<td>123.4</td>
<td>5.28, br t, 7.5</td>
</tr>
<tr>
<td>3'</td>
<td>-</td>
<td>131.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4'</td>
<td>1.70, d, 1.0</td>
<td>1.73, s</td>
<td>16.6</td>
<td>1.70, d, 1.0</td>
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<td>5'</td>
<td>1.69, br s</td>
<td>1.72, s</td>
<td>24.4</td>
<td>1.69, s</td>
</tr>
</tbody>
</table>

Acceptance of 1-tyrosine (4a) by two tryptophan prenyltransferases and identification of C4- and C6-prenylated derivatives as products of the FgaPT2 reaction

After identification of C5-prenylated 3a from reaction mixtures of tyrosine and tryptophan prenyltransferases, we tested the behaviour of these enzymes towards 1-tyrosine (4a) under the same conditions as for 3a. As shown in Figs. 2S - 2X, product formation was only observed in the reaction mixtures of FgaPT2 and 7-DMATS, with product yields of 33.4 ± 1.4 and 4.8 ± 1.6% (Table 1), respectively. Unexpectedly, 4a was not accepted by the two tyrosine prenyltransferases SirD and TyrPT (Figs. 2W and 2X). Another surprise is the much better acceptance of 4a by FgaPT2 than by 7-DMATS (Figs. 2S and 2V), which differs clearly from the preference of 2a towards these two enzymes (Figs. 2G and 2J). It seems that the positions of the hydroxyl groups at the benzene ring of phenylalanine have critical influence on their acceptance by tryptophan and tyrosine prenyltransferases.

Inspection of the HPLC chromatograms in Figs. 2S and 2V revealed the presence of two product peaks 4b and 4c with a ratio of 1:2 in the reaction mixture of FgaPT2 at 10.3 and 11.4 min and only one in that of 7-DMATS at 11.4 min. The UV maxima of 4a at 220 and 283 nm differed slightly from those of 4c at 220 and 278 nm. For structure elucidation, 4b and 4c were isolated on HPLC from incubation mixture of 4a with FgaPT2 and subjected to NMR (Table 2; Figs. S2 - S5 in ESI) and HRMS analyses (Experimental Section). HRMS data confirmed the monoprenylation of the isolated products. Inspection of the 1H-NMR spectra (Table 2) of 4b and 4c revealed the presence of signals for a regular prenyl moiety each at δH 3.33 or 3.24 (d, H-1’), 5.19 or 5.28 (br t H-2’), 1.73 or 1.70 (s H-4’) and 1.72 or 1.69 ppm (s, H-5’). The signals of the aromatic protons at 6.69 (d, 2.5 Hz, H-2), 6.64 (dd, 8.0, 2.5 Hz, H-4), 7.00 (d, 8.0 Hz, H-5) of 4b and 6.69 (s, H-2), 7.00 (d, 7.5 Hz, H-5), 6.67 (d, 7.5 Hz, H-6) of 4c indicated the prenylation at C-4 in one case and C-6 in another case. To determine the structure of 4b and 4c, we took HSQC and HMBC spectra for 4b (Fig. 3, Figs. S3 and S4 in ESI). The important correlation between H-1’ and C-1 prove unequivocally that 4b is the C6-prenylated derivative, i.e. prenylation at the para-position to the phenolic hydroxyl group (Fig. 2Z). Consequently, 4c is the C4-prenylated product (Fig. 2Z). Due to the low quality, the structure of the enzyme product of 4a with 7-DMATS could not be elucidated in this study. However, from its same retention time and UV absorption maxima with those of 4c, it could be speculated that this substance has the same structure as 4c.
groups on the aromatic nucleus, which perform the direct interaction with the enzyme active sites. Furthermore, these results reveal that tryptophan and tyrosine prenyltransferases possess very likely similar active sites and strengthen their close relationship in the evolution.

Kinetic study of the enzymatic Friedel-Crafts reactions

To get insights into the catalytic efficiency, kinetic parameters were determined for FgaPT2, 5-DMATS, 6-DMATS, TyrPT, 7-DMATS and TyrPT towards 3a and FgaPT2 towards 4a (Table 3; Figs. S6 – S11 in ESI). As expected for unnatural substrates, $K_M$ values between 0.17 ± 0.0076 and 0.58 ± 0.081 mM were determined for 3a, much higher than for their natural substrates. $\frac{k_{cat}}{K_M}$ values between 0.29 ± 0.029 and 600 ± 58 were determined for 3a, much higher than that with 3a (Table 3). In agreement with the product yields given in Table 1, FgaPT2 showed the highest turnover number and catalytic efficiency toward 3a, followed by 7-DMATS and 6-DMATS, TyrPT and 5-DMATS demonstrated comparable kinetic parameters for their reactions with 3a (Table 3).

Table 3. Kinetic parameters of tested enzymes for 3a and 4a.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_M$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$\frac{k_{cat}}{K_M}$ (s$^{-1}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-o-tyrosine (3a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FgaPT2</td>
<td>0.47 ± 0.0024</td>
<td>0.29 ± 0.029</td>
<td>600 ± 58</td>
</tr>
<tr>
<td>5-DMATS</td>
<td>0.49 ± 0.0031</td>
<td>0.013 ± 0.00011</td>
<td>27 ± 0.40</td>
</tr>
<tr>
<td>6-DMATS</td>
<td>0.58 ± 0.081</td>
<td>0.034 ± 0.00038</td>
<td>59 ± 3.6</td>
</tr>
<tr>
<td>7-DMATS</td>
<td>0.17 ± 0.0076</td>
<td>0.045 ± 0.036</td>
<td>270 ± 8.9</td>
</tr>
<tr>
<td>TyrPT</td>
<td>0.58 ± 0.014</td>
<td>0.014 ± 0.00066</td>
<td>24 ± 1.7</td>
</tr>
<tr>
<td>L-m-tyrosine (4a)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FgaPT2</td>
<td>0.90 ± 0.013</td>
<td>0.0065</td>
<td>6.8 ± 0.093</td>
</tr>
</tbody>
</table>

Proposed reaction mechanisms of Friedel-Crafts alkylation of o- and m-tyrosine

Previous studies on structures of several prenyltransferases and mutagenesis experiments have shown that the prenyl transfer reactions catalysed by the prenyltransferases of the DMATS superfamily are initiated by the formation of a dimethylallyl carbocation. Nucleophilic attack of this ion by electron-rich aromatic ring leads to the formation of non-aromatic intermediates, which will be rearomatized by elimination of a proton and result in the formation of the prenylated derivatives. Similar mechanism could also be proposed for reactions observed in this study. As shown in Fig. 4, attack of the dimethylallyl carbocation by C-5 of 3a would result in the formation of the intermediate I, which would undergo proton elimination to form the final product 3b. For the FgaPT2 reaction with 4a, attack from two positions, C-6 and C-4, would be possible. It can be expected that two different intermediate II and III will be formed via route A and B, respectively, and then converted to 4b and 4c after proton elimination (Fig. 4).

Experimental Section

Chemicals

DMAPP was synthesized according to the method described for geranyl diphosphate (GPP) reported previously. L-o-tyrosine and L-m-tyrosine used for the enzyme assays were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Alfa Aesar (Karlsruhe, Germany), respectively.

Overexpression and purification of recombinant proteins

Protein overproduction and purification were carried out for FgaPT, 5-DMATS, 6-DMATS, 7-DMATS, TyrPT and SirD as described previously.

Assays for determination of enzyme activities

Reaction mixtures (100 µl) for determination of the enzyme activities contained aromatic substrate (1 mM), CaCl$_2$ (5 mM), DMAPP (1 mM), glycerol (1.0 – 6.0 % v/v), dimethyl sulfoxide (DMSO, 0 – 5.0 % v/v), 50 mM Tris-Cl (pH 7.5) and purified recombinant protein (2 or 20 µg). The reaction mixtures were incubated at 37 °C for 1.5 h and then terminated by addition of 100 µl MeOH. Protein was removed by centrifugation at 17,000 rpm for 20 min.

Enzyme assays for product isolation and structure elucidation

Assays for isolation of the enzyme products were carried out in large scales (5 - 10 ml) containing aromatic substrate (1 mM), DMAPP (1.5 mM), CaCl$_2$ (5 mM), glycerol (1.0 - 9.9 % v/v), DMSO (0 – 5.0 % v/v), 50 mM Tris-Cl (pH 7.5) and recombinant protein (0.2 - 0.6 mg per ml assay). After incubation for 16 h at 37 °C, the reaction mixtures were terminated by addition of 5 - 10 ml methanol. After removal of the precipitated protein by centrifugation at 6,000 rpm for 30 min, the reaction mixtures were concentrated on a rotating vacuum evaporator at 35 °C to a final volume of 1 ml before injection into HPLC.

Enzyme assays for determination of the kinetic parameters
As shown in Fig. 2, tyrosine prenyltransferases TyrPT and SirD also accepted tryptophan as substrate, but with lower activity than with tyrosine. Correspondingly, tryptophan prenyltransferases FgaPT2 and 7-DMATS also prenylated tyrosine, but with lower activity than for tryptophan. In this study, we demonstrated surprisingly that L-α-tyrosine (3a) was accepted by all of the tested tryptophan prenyltransferases for prenylations at C-4, C-5, C-6 and C-7 of the indole ring, but not by the tyrosine prenyltransferase SirD.

The unique product, C5-prenylated α-tyrosine was identified in all the reaction mixtures of FgaPT2, 5-DMATS, 6-DMATS, 7-DMATS and TyrPT, although these enzymes utilized different natural substrates and catalysed diverse regioselective reactions. 1-m-tyrosine (4a) was only converted by two tryptophan prenyltransferases, but by none of the tyrosine prenyltransferases. Structure elucidation of the enzyme products of 4a with FgaPT2 revealed a low regioselectivity. C4- and C6-prenylated m-tyrosine with a ratio of approximate 2:1 were identified as reaction products.

Acknowledgements

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Notes

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References

Electronic Supplementary Information for

Tryptophan prenyltransferases showing higher catalytic activities for Friedel-Crafts alkylation of o- and m-tyrosine than tyrosine prenyltransferases

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Fig. S2. $^1$H-NMR spectrum of 4b in MeOH-d$_4$. 
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Fig. S4. HMBC spectrum of 4b in MeOH-d₄.
Fig. S5. $^1$H-NMR spectrum of 4c in MeOH-d$_4$. 
**Fig. S6.** Determination of kinetic parameters of FgaPT2 for 3a.

- $K_M = 0.47 \pm 0.0024 \text{ mM}$
- $k_{cat} = 0.29 \pm 0.029 \text{ s}^{-1}$

**Fig. S7.** Determination of kinetic parameters of 5-DMATS for 3a.

- $K_M = 0.49 \pm 0.0031 \text{ mM}$
- $k_{cat} = 0.013 \pm 0.00011 \text{ s}^{-1}$
Fig. S8. Determination of kinetic parameters of 6-DMATSSv for 3a.

![Graph showing kinetic parameters](image)

\[ K_M = 0.58 \pm 0.081 \text{ mM} \]
\[ k_{cat} = 0.034 \pm 0.00038 \text{ s}^{-1} \]

Fig. S9. Determination of kinetic parameters of 7-DMATS for 3a.

![Graph showing kinetic parameters](image)

\[ K_M = 0.17 \pm 0.0076 \text{ mM} \]
\[ k_{cat} = 0.045 \pm 0.036 \text{ s}^{-1} \]
Fig. S10. Determination of kinetic parameters of TyrPT for 3a.

Fig. S11. Determination of kinetic parameters of FgaPT2 for 4a.
4. Publications and manuscripts

4.6. Creation of FgaPT2 mutants with enhanced catalytic ability and different preferences toward cyclic dipeptides by saturation mutagenesis
FULL PAPER

Creation of FgaPT2 mutants with enhanced catalytic ability and different preferences toward cyclic dipeptides by saturation mutagenesis

Aili Fan[a] and Shu-Ming Li*[a]

Abstract: FgaPT2 from Aspergillus fumigatus catalyzes a Friedel-Crafts alkylation at C-4 of L-tryptophan and is involved in the biosynthesis of the ergot alkaloids fumigaclavines. Several tryptophan-containing cyclic dipeptides had also been prenylated by FgaPT2 at high protein concentrations. Here, we report the generation of FgaPT2 mutants by saturation mutagenesis at amino acid residue Arg244 to improve its catalytic efficiency toward cyclic dipeptides. Thirteen mutated enzymes demonstrated of up to 155-fold higher catalytic efficiency toward eight cyclic dipeptides than FgaPT2. More importantly, the mutated enzymes exhibited different preferences toward these substrates. This study provides not only new biocatalysts for production of C4-prenylated cyclic dipeptides, but also abundant data for further investigations on the catalytic mechanism of FgaPT2 reactions toward cyclic dipeptides.

Introduction

Prenylated natural products are widespread in nature and demonstrate a wide range of promising biological activities, which contribute largely to the drug discovery and development process.[11-8] Among them, prenylated tryptophan-containing cyclic dipeptides and derivatives thereof comprise a huge group of secondary metabolites with high structural diversity and various biological activities such as the well-known cytotoxic tryprostatins and derivatives furmitermorigns.[9] Additional examples are the reversely C2-prenylated cyclo-trypophan-derivatives echinulins and isoechinulins as well as the syn-cis C3-prenylated cyclo-trypophanyl-histidinyl derivatives roquefortines.[10-14] So far, cyclic dipeptides with prenyl moieties at N-1, C-2, C-3, C-4, C-5, C-6 and C-7 of the indole ring were frequently observed in nature with anti-inflammatory, cytotoxic, anti-feeding, antifungal, antimicrobial and selective PTK inhibitory activities.[15-18] Consequently, synthesis of prenylated cyclic dipeptides has drawn lots of attention and different strategies have been developed, such as addition of prenyl moiety from prenyl bromide in the presence of strong base or coupling reactions catalyzed by metal salts.[19-23] These reactions are usually carried out under anhydrous or anaerobic conditions using environment hazardous chemicals and under special temperature control.[19-21] Nevertheless, only electron-rich positions, e.g. N-1, C-2 and C-3 of the indole ring are preferred by classic chemical synthesis. Meanwhile, additional steps are usually necessary for protection and deprotection of the functional groups.[21-22] In nature, prenyl transfer reactions on the indole ring are catalyzed by prenyltransferases, especially by those from the dimethylallyltryptophan synthase (DMATS) superfamily. DMATS enzymes are soluble prenyltransferases, mainly catalyze Friedel-Crafts alkylation by attachment of prenyl moieties to various aromatic acceptors and are involved in the biosynthesis of secondary metabolites. Until now, more than forty members of this group were characterized biochemically.[23-27] About 50 % of the DMATS enzymes use tryptophan and tryptophan-containing cyclic dipeptides as substrates. Twelve tryptophan prenyltransferases catalyze prenylations at N-1, C-4, C-5, C-6 and C-7 of the indole ring.[23,28-31] For example, FgaPT2 from Aspergillus fumigatus is involved in the biosynthesis of fumigaclavines and catalyzes a regular C4-prenylation of L-tryptophan.[32,33] To date, eleven tryptophan-containing cyclic dipeptide prenyltransferases including four C2-, three C3- and four C7-prenyltransferases have been identified and characterized.[31,34-39] The C7-prenyltransferase CTrpPT also catalyzes an N1-prenylation of cyclic dipeptides. Cyclic dipeptide C4-, C5- and C6-prenyltransferases have not been characterized biochemically. In contrast to other prenylated cyclic dipeptides, C4-prenylated cyclic dipeptides and derivatives thereof are not commonly found in nature. However, such compounds might exhibit interesting and potent biological activities.

Prenyltransferases of the DMATS superfamily usually demonstrated remarkable substrate promiscuity, especially toward their aromatic substrates. For example, with high protein amount, FgaPT2 was able to catalyze the C4-prenylation of five tryptophan-containing cyclic dipeptides[40] and C3-prenylation of tyrosine.[41] The catalytic ability of FgaPT2 toward tyrosine was enhanced by molecular modeling-guided site directed-mutation.[41] This result inspires us to improve the catalytic efficiency of FgaPT2 toward cyclic dipeptides by mutagenesis, so that biocatalysts for production of C4-prenylated diketopiperazine derivatives could be made available in this way. Fortunately, the crystal structures of FgaPT2 with and without its substrates were solved in 2009 and provided a basis for understanding the mechanism of the prenyl transfer reaction.[42] A three-step mechanism was proposed for the prenyl transfer reaction with FgaPT2 (Figure 1). Formation of a dimethylallyl cation by removal of the pyrophosphate group is considered as initiation.[43] Subsequent nucleophilic attack of this cation by the C-4 position of the indole nucleus will form the σ-complex. The resulting intermediate was then deprotonated to generate the final product.[42]
It was later proposed that the substitution might occur directly at C-3 instead of C-4, followed by a rearrangement to generate the final product. Based on the crystal structures of FgaPT2 and three cyclic dipeptide prenyltransferases FtmPT1, CdpNPT and AnaPT, it was found that the amino acids of the DMAPP binding sites seem to be conserved, while the binding sites of the aromatic substrates differ from each other.

As aforementioned, FgaPT2 also catalyzed C4-prenylation at the indole ring of tryptophan-containing cyclic dipeptides as for its natural substrate tryptophan, but with much lower efficiency. This indicated that the indolyl moiety of the cyclic dipeptides might be positioned similarly in the active site of FgaPT2 as the free amino acid tryptophan. The poor acceptance of cyclic dipeptides by FgaPT2 might result from the non-perfect fitting of the diketopiperazine ring and the second amino acid moiety in the active site. This could be overcome by changing amino acid residues involved in the binding of tryptophan side chain in the reaction cavity, so that the cyclic dipeptides can be better placed in the pocket of FgaPT2. In a previous study, fifteen FgaPT2 mutants at E89, T102, K174 and R244 were demonstrated to have different activity profiles toward L-tryptophan and L-tyrosine. T102C and K174F showed significantly higher activity for tyrosine than the non-mutated FgaPT2. Their activities toward tryptophan was almost unchanged (T102C) or reduced to only 0.4% of that of FgaPT2. Therefore, the available fifteen FgaPT2 mutants were assayed with cyclo-L-Trp-L-Tyr (1a), the best identified cyclic dipeptide substrate of FgaPT2 previously.

**Results and Discussion**

**Identification of R244 as a “hot spot” for increasing prenylation activity toward cyclo-L-Trp-L-Tyr (1a)**

In the presence of DMAPP (1 mM), cyclo-L-Trp-L-Tyr (1a) was incubated individually with 10 µg recombinant FgaPT2 and the fifteen mutants including E89A, T102V, T102C, T102S, T102R, K174E, K174Q, K174F, K174Y, R244E, R244N, R244Q, R244D and K174F-R244E in 100 µL enzyme assays at 37 °C for 16 h. HPLC analysis of the incubation mixtures demonstrated that 1a was accepted by the tested enzymes with clearly different activities (Figure 2).

Under this condition, 1a was converted by FgaPT2 with a product yield of 10.0%. Compared to the strongly reduced activity toward tyrosine and tryptophan, 180F demonstrated similar catalytic ability as FgaPT2 toward 1a, while E89A hardly accepted all these three substrates. Mutation of T102 and K174 resulted in derivatives with strongly reduced activity with 1a, which differed significantly from those with tryptophan and tyrosine as substrates observed in a previous study. In that study, T102C and K174F were found to accept tyrosine much better than FgaPT2, while T102C, T102S and K174Q showed comparable activities as FgaPT2 for tryptophan. Both T102 and K174 seem to be of essential importance for an acceptance of 1a, so that alteration of these amino acids is not allowed. Interestingly, three of the four R244 mutants, R244Q, R244N and R244D, accepted 1a much better than FgaPT2. Product yield of 44.1%, 47.4% and 22.4% were calculated for the reactions of 1a with R244Q, R244N and R244D, respectively. That is 2.2 to 4.7-fold of that of FgaPT2. In comparison, these three mutants showed almost no activity toward tyrosine and comparable or lower activity with tryptophan than FgaPT2. The results of the acceptance of tryptophan, tyrosine and the cyclic dipeptide 1a by the fifteen mutants indicated that different amino acids are involved in the binding sites of these substrates and the roles of these amino acids can be changed in the presence of different substrates. R244 was
Saturation mutagenesis resulting in mutants with enhanced catalytic ability toward eight tryptophan-containing cyclic dipeptides

The much higher catalytic ability of R244Q and R244N toward cyclic dipeptides encouraged us to get additional R244 mutants for enhancing activity toward cyclic dipeptides by different mutants. For generation of additional mutants, the saturation mutagenesis experiments were then carried out on the key amino acid R244 by site-directed mutagenesis (Tables S1 and S2). With the exception of R244C, all the other 14 remained mutants were obtained (Table S1). The plasmids obtained from this and a previous study[41] were then transformed into E. coli SoluBL21. Single colonies containing the respective mutated plasmids were inoculated into 1 mL of LB media with 25 μg mL−1 kanamycin and 0.5 mM IPTG and cultivated for 24 h. The crude protein fractions were used directly for screening of the mutants with high catalytic efficiency. For an initial selection, 7a or a mixture containing 1a, 3a, 5a, 7a and 8a or 2a, 4a, 6a, 9a and 10a was incubated with crude enzyme extract of a given mutant (Experimental section). Due to their different retention times on HPLC, the reaction mixtures containing products of different cyclic dipeptides could be separated from each other. With the exception for those of R244P, R244F and R244W, product formation was observed in enzyme assays of the fifteen mutants (data not shown).

FgaPT2 and the fifteen mutants were then overproduced and purified for further investigations. Each of the ten cyclic dipeptides 1a - 10a was incubated individually with 4 μg FgaPT2 and the fifteen mutants in 50 μL assays for 1.5 h. Under this condition 1a - 10a were converted by FgaPT2 with yields between 0.28 and 7.7 %. R244E and R244D displayed poor catalytic activity toward all the tested substrates, with product yields of lower than 5 and 15 %, respectively (Data not shown). Other thirteen mutants FgaPT2 and its mutants, with product yields of lower than 1.2 % and 5.3 %, respectively (Data not shown). Their results are therefore not presented in Figure 4. 2a and 6a were poorly accepted by FgaPT2 and its mutants, with product yields of lower than 1.2 % and 5.3 %, respectively (Data not shown). Other thirteen mutants including R244G, R244A, R244S, R244T, R244V, R244L, R244I, R244M, R244Y, R244N, R244Q, R244H and R244K utilized the eight dipeptides 1a, 3a, 4a, 5a and 7a - 10a as good substrates and significant enhancement on the catalytic ability was observed (Figure 4).

Figure 3. Product yields of R244N and R244Q reactions toward 1a – 10a (A and B) and enzyme reactions catalyzed by FgaPT2 and mutants (C). The assays (100 μL) contained 1 mM aromatic substrate, 5 mM CaCl2, 1 mM DMAPP, and 10 μg purified protein and were incubated at 37 °C for 16 h.
Figure 4. Product yields of FgaPT2 and mutated proteins reactions with 1a, 3a - 5a, 7a – 10a as substrates. The assays (50 µL) contained 1 mM aromatic substrate, 5 mM CaCl₂, 1 mM DMAPP and 4 µg purified recombinant protein and were incubated at 37 °C for 1.5 h.
From Figure 4, it is obvious that R244L accepted most of the tested cyclic dipeptides better than other mutants. Toward 1a, R244L demonstrated the highest product yield of 28.2 %, which is 28-fold of that of FgaPT2. The highest product yields of 32.8, 49.9, 10.3 and 10.1 % were obtained with R244L for 3a, 4a, 5a and 8a, respectively. For 4a, R244T, R244V, R244I and R244M showed slightly lower activities than R244L. With 5a as substrate, R244T, R244V, R244I and R244M exhibited similar catalytic activity as R244L. Furthermore, R244L displayed comparable catalytic activity toward 7a with R244S, R244T, R244Y and R244H with product yields between 35.4 – 37.8 %. 9a was better converted by R244M, R244G, R244L and R244Y with product yields of 26.0, 22.9, 22.1 and 20.8 %, respectively. R244Y showed a 16.5-fold activity of that of FgaPT2 toward 10a. This improvement might be the result of better stabilization of the phenylalanine ring in substrate by the aromatic tyrosyl residue of R244Y. Overall product yields of more than 10.5 % were achieved for the eight cyclic dipeptides by using the respective best mutants. 3a, 4a and 7a were the most well accepted substrates by these mutated proteins, with highest product yields of 32.8, 49.9 and 37.8 %, respectively.

To elucidate its structure, the enzyme product 10b was isolated from the reaction mixture of R244Y with 10a and proved to be the C4-prenylated cyclo-L-Trp-L-Phe by NMR and MS analyses. To confirm the structures of the enzyme products with different mutants, 1a, 3a, 4a, 5a and 7a were incubated with the thirteen mutated proteins listed in Figure 4 for 16 h. The ethyl acetate extracts of the reaction mixtures were subjected to 1H NMR analysis. All the signals of the enzyme products corresponded well to those obtained with R244Q and R244N, confirming the C4-prenylation by these mutants.

Different substrate preferences of FgaPT2 mutants toward cyclic dipeptides

From Figure 4, it is also obvious that these mutants showed different preferences toward these dipeptides. For better understanding the preferences of different FgaPT2 mutants toward cyclic dipeptides, dotted lines are added to indicate the product yields of different substrates with the same protein. Under the conditions used in this study, 4a and 7a with product yields of 7.7, and 7.4 %, respectively, were found to be the best substrates for FgaPT2. R244G accepted 7a and 9a better than other substrates, with comparable product yields of 22.5 and 22.9 %, respectively, while 4a and 7a are the best substrates for R244A with product yields of 30.8 and 27.1 %, respectively. For R244S, R244N and R244H, 7a was clearly better accepted than other substrates. R244T converted 4a and 7a with comparable product yields about 33 %. R244V, R244L, R244I and R244K demonstrated similar substrate preferences toward the tested substrates and accepted 4a as best substrate, followed by 7a, 2a and 9a.

The R244 mutants showing lower activity toward L-tryptophan than FgaPT2

Figure 5. Product yields of tryptophan with FgaPT2 and R244 mutants. The assays (50 μL) contained 1 mM aromatic substrate, 5 mM CaCl₂, 1 mM DMAPP and 4 μg purified protein and were incubated at 37 °C for 1.5 h.

To prove the behavior of the R244 mutants toward tryptophan, we carried out incubations with the same amount protein, incubation time and temperature as for cyclic dipeptides by using FgaPT2 as positive control. Under this condition, tryptophan was converted by FgaPT2 with a product yield of 72.7 %. As shown in Figure 5, R244H and R244K displayed slightly lower activities than FgaPT2. R244E lost almost completely its activity toward tryptophan, while other mutants showed lower, but still detectable activities. This proved that the amino acids at position 244 are in most cases not critical for the acceptance of tryptophan.

Kinetic parameters of the prenyl transfer reactions of 1a, 3a, 5a, 7a – 10a with the best identified R244 mutants

To compare the catalytic efficiency of FgaPT2 and mutated proteins toward cyclic dipeptides, kinetic parameters were determined for the eight cyclic dipeptides with the best identified mutants as described under "Experimental section" and summarized in Table 1 (see also Supporting information Figure S1 - S8). The $K_{M}$ values of the mutated proteins toward cyclic dipeptides are found in the range of 0.17 – 0.74 mM, comparable to those with FgaPT2[47]. However, the turnover numbers of the reactions with the mutated proteins in the range of 0.83 to 6.4 min⁻¹ are 8.3 to 76.5-fold of those with FgaPT2. The largest $k_{cat}$ was calculated for R244L toward 4a, which also exhibited highest product yield of 49.9 %. In the case of 3a, the mutant R244L demonstrated a substantial 155-fold catalytic efficiency of that of FgaPT2. With 8a as substrate, mutant R244L displayed the relatively low catalytic efficiency of 1.6 min⁻¹ mM⁻¹ among all the tested mutants. Even though, it is still 10.7-fold of that of FgaPT2 toward 8a. The higher catalytic efficiencies of mutated enzymes toward cyclic dipeptides than FgaPT2 are corresponded to their higher product yields. Therefore, FgaPT2 mutants with enhanced catalytic ability toward cyclic dipeptides are generated by saturation mutagenesis.
Conclusions

Prenyltransferases of the DMATS superfamily are capable of prenylating a wide range of substrates with different skeletons including indole, tyrosine, xanthone, flavonoid and naphthalene derivatives. However, for some of the unnatural substrates, these enzymes could not be directly used as biocatalysts, due to low catalytic efficiency or regioselectivity. Therefore, protein engineering of known enzymes are needed to improve such features. In the present study, we enhanced significantly the catalytic efficiency of FgaPT2 toward different cyclic dipeptides by saturation mutagenesis on the key amino acid R244. Thirteen R244 mutants displayed differently increased enzyme activities for tryptophan-containing cyclic dipeptides, with up to 155-fold catalytic efficiency of that of FgaPT2. More importantly, different preferences for cyclic dipeptides were observed for these mutants. These results provide not only new biocatalysts for production of C4-prenylated cyclic dipeptides, but also abundant data for further investigations on the catalytic mechanism of FgaPT2 and its mutants toward cyclic dipeptides.

Experimental Section

Chemicals

DMAPP was synthesized according to the method described for geranyl diphosphate reported previously. Substrates used for the enzyme assays were purchased from Bachem (Bubendorf, Switzerland) or synthesized as described previously.

Bacterial strains, plasmids and culture conditions

Escherichia coli XL1 Blue MRF’ (Stratagene, Heidelberg, Germany) was used as host strain for site-directed mutagenesis experiments. E. coli BL21 (DE3)pLysS (Invitrogen, Karlsruhe, Germany) and SoluBL21 (Novagen, Darmstadt, Germany) were used for expression experiments. pIU18 was used as construct for FgaPT2 overproduction as described previously and as DNA template for site-directed mutagenesis experiments.

Expression constructs for overproduction of T102 and K174 mutants as well as I80F, R244E, R244N, R244Q and R244D are described previously.

E. coli cells harboring plasmids were grown in liquid Lysogeny-Broth (LB) or Terrific-Broth (TB) medium and on solid LB medium with 1.5 % (w/v) agar at 37 °C. 25 µg mL⁻¹ of kanamycin were used for selection of recombinant E. coli strains.

Site-directed mutagenesis

Expand Long Template PCR system (Roche Diagnostic, Mannheim, Germany) was used for construction of plasmids pALF39 – pALF53 (Table S1). The obtained plasmids were sequenced to confirm the desired mutations in the respective constructs.

Overproduction and purification of the recombinant proteins

FgaPT2 and its mutated derivatives were overproduced and purified as described previously and protein yields between 0.6 and 6.6 mg per liter culture were obtained in this study.

Enzyme assays with recombinant purified proteins

To determine the enzyme activity toward 1a, the incubation mixtures contained 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂, 1 mM 1a, 1 mM DMAPP, 0.15 - 5 % (v/v) glycerol, 0 - 5 % (v/v) DMSO and 0.1 µg µL⁻¹ of purified recombinant protein in a total volume of 100 µL. 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 and the supernatant was analyzed on HPLC.

To determine the enzyme activity of R244N and R244Q toward 1a - 10a, the incubation mixtures contained 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂, 1 mM aromatic substrate, 1 mM DMAPP, 0.15 - 5 % (v/v) glycerol, 0 - 5 % (v/v) DMSO and 0.1 µg µL⁻¹ of purified recombinant protein in a total volume of 100 µL. After incubation at 37 °C for 16 h, the reactions were terminated by addition of 100 µL methanol. Protein was removed by

### Table 1. Comparison of kinetic parameters of mutated proteins with those of FgaPT2 published previously.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>$K_{m}$ (mM)</th>
<th>$V_{max}$ (µmol min⁻¹ mg⁻¹)</th>
<th>$K_{cat}$ / $K_{m}$ (min⁻¹ mM⁻¹)</th>
<th>Ratio of $K_{cat}$ / $K_{m}$ mutant/FgaPT2</th>
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<tbody>
<tr>
<td>1a</td>
<td>R244L</td>
<td>0.40</td>
<td>0.83</td>
<td>2.1</td>
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<tr>
<td>3a</td>
<td>R244L</td>
<td>0.28</td>
<td>5.2</td>
<td>18.6</td>
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</tr>
<tr>
<td>4a</td>
<td>R244L</td>
<td>0.46</td>
<td>6.4</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>5a</td>
<td>R244L</td>
<td>0.65</td>
<td>0.95</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>7a</td>
<td>R244Y</td>
<td>0.42</td>
<td>5.6</td>
<td>13.3</td>
<td></td>
</tr>
<tr>
<td>8a</td>
<td>R244L</td>
<td>0.74</td>
<td>1.2</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>9a</td>
<td>R244M</td>
<td>0.22</td>
<td>1.9</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>10a</td>
<td>R244Y</td>
<td>0.17</td>
<td>1.9</td>
<td>11.2</td>
<td></td>
</tr>
</tbody>
</table>

[a] The data are adopted from [47]
centrifugation at 13,000 rpm for 20 min and the supernatant was analyzed on HPLC.

To determine the enzyme activities of FgaPT2 and its mutants with crude protein extracts, single colonies of E. coli harboring the respective plasmids were inoculated into 1 mL of TB media with 25 μg mL⁻¹ of kanamycin and 0.5 mM IPTG and cultivated at 37 °C with shaking at 220 rpm for 24 h. The cultures were harvested by centrifugation at 4500 rpm for 10 min. 100 μL Tris-Cl (50 mM, pH 7.5) were added to the pellets. The suspensions were frozen at -30 °C for 45 min and then thaw on ice with vortex. Repeat the freeze-thaw procedure for twice and the lysates were centrifuged at 13,000 rpm for 10 min and the supernatants were used directly for enzyme assays. Each assay contains 60 μL supernatant, 5 mM CaCl₂, 1 mM aromatic substrate, 1 mM DMAPP, 0 - 5 % (v/v) DMSO in a total volume of 100 μL. After incubation at 37 °C for 16 h, the reactions were terminated by addition of 100 μL methanol. Due to the numbers of cyclic dipeptides and different substrate preferences of known mutants, two cyclic dipeptide mixtures for the first trial of desired variants were used as aromatic substrates. The mixture contained 1a, 3a, 5a, 7a and 8a or 2a, 4a, 6a, 9a and 10a, with different retention times on HPLC. Due to the high column was then washed with 100 % (v/v) methanol for 10 min and equilibrated with 60 % (v/v) methanol for 10 min.

NMR and mass spectrometric analyses

For structural elucidation, the isolated enzyme products were subjected to 1H NMR and MS analyses. Mass spectrometry was carried out on a Q-Trap Quantum (Applied Biosystems, Darmstadt, Germany). 1H NMR 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 7.26 ppm for CDCl₃ or 2.49 ppm for DMSO-d₆. All spectra were processed with MestReNova 5.2.2 (Mestrelab Research, Santiago de Compostela, Spain).

### Compound 1b: 1H NMR (500 MHz, CDCl₃-d₆): δ=10.86 (d, 2.0 Hz, 1H, NH), 7.92 (d, 2.5 Hz, 1H, NH), 7.49 (d, 2.7 Hz, 1H, NH), 7.15 (d, 7.6, 1.0 Hz, 1H, CH), 6.93 (t, 7.6 Hz, 1H, CH), 6.84 (d, 8.5 Hz, 2H, CH), 6.82 (d, 2.5 Hz, 1H, CH), 6.69 (dd, 7.6, 1.0 Hz, 1H, CH), 6.68 (d, 8.5 Hz, 2H, CH), 5.25 (tsept, 7.0, 1.5 Hz, 1H, CH), 3.98 (dd, 7.0, 4.5 Hz, 1H, CH), 3.79 (dt, 8.5, 3.0 Hz, 1H, CH), 3.62 (d, 7.0 Hz, 2H, CHa), 3.08 (dd, 14.6, 3.5 Hz, 1H, CHa), 2.67 (dd, 13.5, 4.5 Hz, 1H, CHa), 2.57 (dd, 13.5, 5.5 Hz, 1H, CHa), 1.96 (dd, 14.6, 9.0 Hz, 1H, CHa), 1.70 (br s, 3H, CH₃), 1.69 (s, 3H, CH₃).

MS (ESI): m/z=440.17 [M+Na]+.

### Compound 3b: 1H NMR (500 MHz, CDCl₃-d₆): δ=8.18 (br s, 1H, NH), 7.25 (dd, 8.5, 1.0 Hz, 1H, CH), 7.15 (t, 8.0 Hz, 1H, CH), 7.07 (d, 2.5 Hz, 1H, CH), 6.93 (d, 7.5, 1.0 Hz, 1H, CH), 5.82 (br s, 1H, NH), 5.32 (sept, 6.8, 1.5 Hz, 1H, CH), 4.29 (dd, 11.5, 1.8 Hz, 1H, CH), 4.09 (t, 8.0 Hz, 1H, CH), 3.97 (ddd, 15.4, 3.5, 1.0 Hz, 1H, CHa), 3.75 (dd, 16.5, 6.8 Hz, 1H, CHa), 3.72 (dd, 16.5, 8.6 Hz, 1H, CHa), 3.66 (m, 1H, CHa), 3.60 (m, 1H, CHa), 2.98 (dd, 15.4, 11.5 Hz, 1H, CHa), 2.35 (m, 1H, CHa), 2.11 (m, 1H, CHa), 2.05 (m, 1H, CHa), 1.93 (m, 1H, CHa), 1.75 (s, 3H, CH₃), 1.74 (d, 1.0 Hz, 3H, CH₃).

MS (ESI): m/z=374.17 [M+Na]+.

### Compound 4b: 1H NMR (500 MHz, CDCl₃-d₆): δ=8.22 (br s, 1H, NH), 7.23 (dd, 7.5, 1.0 Hz, 1H, CH), 7.13 (t, 7.5 Hz, 1H, CH), 7.03 (d, 2.5 Hz, 1H, CH), 6.92 (dd, 7.5, 1.0 Hz, 1H, CH), 5.93 (d, 2.5 Hz, 1H, NH), 5.31 (sept, 6.8, 1.5 Hz, 1H, CH), 4.15 (dt, 9.0, 4.0 Hz, 1H, CH), 3.77 (br t, 5.5 Hz, 2H, CHa), 3.67 (t, 8.8 Hz, 1H, CH), 3.65 (ddd, 14.8, 3.8, 1.0 Hz, 1H, CHa), 3.63 (m, 1H, CHa), 3.44 (ddd, 12.0, 9.3, 2.6 Hz, 1H, CHa), 3.19 (dd, 14.8, 9.3 Hz, 1H, CHa), 2.31 (ddd, 12.0, 7.0, 1.5 Hz, 1H, CHa), 1.98 (m, 1H, CHa), 1.89 (m, 1H, CHa), 1.78 (d, 1.0 Hz, 3CH₃), 1.74 (d, 1.0 Hz, 3CH₃), 1.73 (m, 1H, CH₃).

MS (ESI): m/z=374.17 [M+Na]+.

### Compound 5b: 1H NMR (500 MHz, CDCl₃-d₆): δ=8.29 (br s, 1H, NH), 7.23 (d, 7.0 Hz, 1H, CH), 7.12 (t, 7.0 Hz, 1H, CH), 7.03 (d, 2.0 Hz, 1H, CH), 6.92 (d, 7.0 Hz, 1H, CH), 5.91 (br s, 1H, NH), 5.33 (sept, 7.0, 1.5 Hz, 1H, CH), 4.15 (m, 1H, CH), 3.77 (approx. m, 2H, CHa), 3.67 (approx. m, 1H, CHa), 3.64 (m, 1H, CHa), 3.63 (m, 1H, CHa), 3.44 (m, 1H, CHa), 3.18 (approx. m, 1H, CHa), 2.31 (m, 1H, CHa), 1.97 (m, 1H, CHa), 1.87 (m, 1H, CHa), 1.78 (br s, 3H, CHa), 1.74 (br s, 3H, CHa), 1.73 (approx. m, 1H, CHa), MS (ESI): m/z=374.17 [M+Na]+.

### Compound 7b: 1H NMR (500 MHz, CDCl₃-d₆): δ=8.20 (br s, 1H, NH), 7.24 (d, 7.5 Hz, 1H, CH), 7.15 (t, 7.5 Hz, 1H, CH), 7.08 (d, 2.0 Hz, 1H, CH), 6.94 (d, 7.5 Hz, 1H, CH), 6.00 (br s, 1H, NH), 5.87 (br s, 1H, NH), 5.33 (sept, 6.6, 1.3 Hz, 1H, CH), 4.22 (br d, 10.5 Hz, 1H, CH), 3.98 (br d, 9.5 Hz, 1H, CH), 3.87 (dd, 15.0, 3.0 Hz, 1H, CH), 3.79 (dd, 15.5, 6.5 Hz, 1H, CH), 3.72 (dd, 15.5, 6.5 Hz, 1H, CHa), 3.04 (dd, 15.0, 11.0 Hz, 1H, CH), 1.84 (dd, 13.8, 9.5, 3.5 Hz, 1H, CHa), 1.75 (s, 3H, CH₃), 1.74 (m, 1H, CHa), 1.51 (add, 13.8, 10.0, 4.5 Hz, 1H, CH₃), 0.98 (d, 6.5 Hz, 3H, CH₃), 0.94 (d, 6.5 Hz, 3H, CH₃). MS (ESI): m/z=390.24 [M+Na]+.
Acknowledgements

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Keywords: biocatalysis • cyclic dipeptide • transferase • dimethylallyltryptophan synthase • saturation mutagenesis

[34] A. Grundmann, S.-M. Li, Microbiology 2005, 151, 2199-2207.


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FULL PAPER

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Aili Fan, Shu-Ming Li*

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Creation of FgaPT2 mutants with enhanced catalytic ability and different substrate preferences toward cyclic dipeptides by saturation mutagenesis
Supporting information for

Creation of FgaPT2 mutants with enhanced catalytic ability and different preferences toward cyclic dipeptides by saturation mutagenesis

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**Table S1.** Mutated derivatives of FgaPT2 and respective primers used for site-directed mutagenesis.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Designed mutation</th>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
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**Table S2.** PCR protocol for construction of FgaPT2 mutants *

<table>
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<th>Step</th>
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<td>1</td>
</tr>
<tr>
<td>denaturation</td>
<td>60 s</td>
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<td></td>
</tr>
<tr>
<td>annealing</td>
<td>60 s</td>
<td>55 - 65 °C</td>
<td>20 - 30</td>
</tr>
<tr>
<td>elongation</td>
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<td>68 °C</td>
<td></td>
</tr>
<tr>
<td>final elongation</td>
<td>7 min</td>
<td>68 °C</td>
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</tr>
<tr>
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<td></td>
<td>4 °C</td>
<td>1</td>
</tr>
</tbody>
</table>

* Expanded long template PCR system (Roche Diagnostics, Mannheim, Germany) was used.
Figure S1. Determination of kinetic parameters of R244L for 1a.

\[ K_M = 0.40 \text{ mM} \]
\[ k_{cat} = 0.83 \text{ min}^{-1} \]

Figure S2. Determination of kinetic parameters of R244L for 3a.

\[ K_M = 0.28 \text{ mM} \]
\[ k_{cat} = 5.2 \text{ min}^{-1} \]
Figure S3. Determination of kinetic parameters of R244L for 4a.

Figure S4. Determination of kinetic parameters of R244L for 5a.
Figure S5. Determination of kinetic parameters of R244Y for 7a.

\[ K_M = 0.42 \text{mM} \]
\[ k_{cat} = 5.6 \text{ min}^{-1} \]

Figure S6. Determination of kinetic parameters of R244L for 8a.

\[ K_M = 0.74 \text{mM} \]
\[ k_{cat} = 1.2 \text{ min}^{-1} \]
Figure S7. Determination of kinetic parameters of R244M for 9a.

Figure S8. Determination of kinetic parameters of R244Y for 10a.
4. Publications and manuscripts

4.7. Impacts and perspectives of prenyltransferases of the DMATS superfamily for use in biotechnology
Impacts and perspectives of prenyltransferases of the DMATS superfamily for use in biotechnology

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Abstract

Prenylated natural products are ubiquitously found in nature and demonstrate interesting biological and pharmacological activities. Prenyltransferases catalyze the attachment of prenyl moieties from different prenyl donors to various acceptors and contribute significantly to the structural and biological diversity of natural products. In the last decade, significant progress has been achieved for the prenyltransferases of the dimethylallyltryptophan synthase (DMATS) superfamily. More than forty members of these soluble enzymes are identified in microorganisms and characterized biochemically. These enzymes were also successfully used for production of a large number of prenylated derivatives. C4-, C5-, C6- and C7-prenylated tryptophan and N1-, C2-, C3-, C4- and C7-prenylated tryptophan-containing peptides were obtained by using DMATS enzymes as biocatalysts. Tyrosine and xanthone prenyltransferases were used for prenylation of O-prenylated derivatives of their analogs. More interestingly, the members of the DMATS superfamily demonstrated intriguing substrate and catalytic promiscuity and also used structurally quite different compounds as prenyl acceptors. Prenylated hydroxynaphthalenes, flavonoids, indolocarbazoles and acylphloroglucinols, which are typical bacterial or plant metabolites, were produced by using several fungal DMATS enzymes. Furthermore, the potential usage of these enzymes was further expanded by using natural or unnatural DMAPP analogs as well as by coexpression with other genes like NRPS and by development of whole cell biocatalyst.

Keywords

Prenyltransferase; Dimethylallyltryptophan synthase; Biocatalyst; Chemoenzymatic synthesis; Prenylated compound.
Introduction

Prenylated natural products including prenylated indole derivatives are widely distributed in terrestrial and marine organisms and exhibit a wide range of biological activities (Li 2010; Liu et al. 2013; Oya et al. 2015; Raju et al. 2011; Sunassee and Davies-Coleman 2012). Therefore, different strategies have been developed for synthesis of such compounds. The most commonly used synthetic strategies are either by addition of a prenyl moiety from prenyl bromide to a substrate, which was activated by strong base before, or by coupling reactions catalyzed by metal salts (Schkeryantz et al. 1999; Yamakawa et al. 2010; Zhao et al. 2002). Both types of reactions are usually carried out under extreme conditions, e.g. anhydrous or anaerobic condition with special temperature control (Schkeryantz et al. 1999; Yamakawa et al. 2010; Zhao et al. 2002). Even so, some positions, e.g. C-4 to C-7 of the indole ring, are not preferred by chemical prenylation. Meanwhile, additional steps are usually needed for protection and deprotection of the functional group in the reactants (Yamakawa et al. 2010; Zhao et al. 2012).

Chemoenzymatic synthesis by using enzymes as biocatalysts has drawn attention from scientists of different disciplines and now widely considered as a practical alternative in the chemical synthesis, because it is more efficient, environment-friendly and easier to handle than the traditional organic synthesis (Alcantara et al. 2014; Gröger and Hummel 2014; Okamoto et al. 2014). The reactions catalyzed by enzymes are mostly regio- and stereoselective and occur under mild conditions, e.g. in aqueous solution at low temperature, e.g. 37 °C, without involvement of any protection groups (Alcantara et al. 2014; Gröger and Hummel 2014; Okamoto et al. 2014). This strategy was also successfully used for synthesis of prenylated aromatic compounds (Li 2009a; Yu and Li 2012).
In nature, the prenyl (nxC₅) moieties are transferred from activated prenyl donors, usually prenyl diphosphates, by prenyltransferases (PTs) to aliphatic or aromatic acceptors (Winkelblech et al. 2015a). During the past decade, PTs have received a substantial amount of attention, among which the dimethylallyltryptophan synthase (DMATS) superfamily involved in the biosynthesis of diverse secondary metabolites is one of the most investigated subgroup. Since the first reviews on the DMATS enzymes in 2009 (Li 2009a; Li 2009b; Steffan et al. 2009), significant progress has been achieved for the enzymes of this group. Until now, more than forty members of this superfamily were identified in bacteria and fungi and characterized biochemically.

The members of the DMATS superfamily usually demonstrate significant substrate flexibility toward aromatic substrates. They accept not only natural substrate analogs with similar structures, but also compounds with quite different skeletons. For example, tryptophan, tryptophan-containing cyclic dipeptides, tyrosine and xanthones are natural substrates of a number of DMATS enzymes. Therefore, a large number of simple indole derivatives, cyclic dipeptides, tyrosine and xanthone derivatives can be converted by these enzymes to their prenylated derivatives (Fan et al. 2014; Pockrandt et al. 2012; Zou et al. 2011). So far, regularly C4-, C5-, C6- and C7-monoprenylated tryptophan as well as C4- and C7-diprenylated tryptophan were synthesized by the DMATSs (Kremer et al. 2007; Ruan et al. 2009; Unsöld and Li 2005; Winkelblech and Li 2014; Yu et al. 2012b). Regiospecific production of N1-, C2-, C3-, C4- and C7-prenylated tryptophan containing cyclic dipeptides with different stereoselectivity were also reported (Grundmann and Li 2005; Mundt and Li 2013; Wunsch et al. 2015b; Yu et al. 2013; Zou et al. 2010). Interestingly, the bacterial metabolites such as prenylated hydroxynaphthalenes and indolocarbazoles as well as plant metabolites like prenylated flavonoids and acylphloroglucinols can also be produced from their non-prenylated...
precursors by using DMATS enzymes (Yu et al. 2012a; Yu et al. 2011; Yu and Li 2011; Zhou et al. 2015).

Compared to their aromatic substrates, one bottleneck for the use of DMATS enzymes in the biotechnology is their high specificity toward prenyl donors. Dimethylallyl diphosphate (DMAPP) serves as the most common prenyl donor of the DMATS enzymes and other prenyl diphosphates like geranyl or farnesyl diphosphate (GPP or FPP) are only accepted by few enzymes. Efforts and progresses have been made recently to expand their acceptance for other prenyl donors like GPP or FPP as well as some unnatural alkyl, even benzyl donors, which can be used to produce novel unnatural prenylated products (Liebhold et al. 2012; Liebhold et al. 2013; Liebhold and Li 2013; Winkelblech et al. 2015b).

To understand these prenyl transfer reactions, crystal structures of DMATS enzymes provide not only detailed insights into the reaction mechanism, but also basic information for protein engineering to create new biocatalysts with desirable features (Fan et al. 2015; Jost et al. 2010). Coexpression of PT genes with non-ribosomal peptide synthases (NRPS) for production of prenylated cyclic dipeptides in vivo and development of whole cell biocatalyst with PTs are new potential application of these intriguing enzymes.

**Chemoenzymatic synthesis of prenylated simple indole derivatives**

Until now, twelve tryptophan PTs have been identified in bacteria and fungi and characterized biochemically (Winkelblech et al. 2015a). These enzymes showed remarkable flexibility toward tryptophan analogs with modifications at both the indole
ring and the side chain and several of them were used for production of prenylated tryptophan analogs (Table 1). In this way, C4-, C5-, C6- and C7-prenylated simple indoles were obtained by regiospecific transfer reactions catalyzed by FgaPT2 (Steffan et al. 2007), 5-DMATS (Yu et al. 2012b), 6-DMATSsA (Winkelblech and Li 2014) and 7-DMATS (Kremer et al. 2007), respectively. C4- and C7-diprenylated derivatives were obtained by tandem incubation with FgaPT2 and 7-DMATS (Ruan et al. 2009). In addition, the two tyrosine PTs SirD and TyrPT were also found to catalyze the same C7-prenylation of L-tryptophan and derivatives as 7-DMATS (Fan et al. 2014; Rudolf and Poulter 2013). More than one product with prenylations at different positions of the indole ring were obtained by these two enzymes in most cases (Fan et al. 2014; Rudolf and Poulter 2013). These results demonstrated that more prenylated products could be obtained by using DMATS enzymes, which provided targets for protein engineering in the future for creation of enzymes with regiospecific prenylation of other substrates or at other positions than for their natural substrates.

**Chemoenzymatic synthesis of prenylated cyclic dipeptides**

Cyclic dipeptide PTs from fungi take dipeptides with a diketopiperazine or benzodiazepinedinone system as substrates, while indolactams are prenylated by cyclic dipeptide PTs from bacteria (Edwards and Gerwick 2004; Winkelblech et al. 2015a). The first group of cyclic dipeptide PTs were also utilized for production of prenylated cyclic dipeptides (Table 2). For example, FtmPT1 from *Aspergillus fumigatus* (A. fumigatus) catalyzes in nature a regular C2-prenylation of brevianamide F (cyclo-L-Trp-L-Pro) in the biosynthesis of fumitremorgins (Grundmann and Li 2005). Further study showed that it also accepted other cyclic dipeptides as substrates and produced
mainly C2-prenylated derivatives and regularly C3-prenylated pyrrolo[2,3]indole diketopiperazines as byproducts (Table 2) (Grundmann and Li 2005; Wollinsky et al. 2012). The two reverse C2-PTs BrePT from Aspergillus versicolor and CdpC2PT from Neosartorya fischeri also prenylate a series of cyclic dipeptides at C-2, but with reverse pattern and different substrate preference (Mundt and Li 2013; Yin et al. 2013). In total, thirty regularly or reversely C2-prenylated compounds were synthesized by these three enzymes (Table 2).

CTrpPT from Aspergillus oryzae catalyzed the simultaneous regular C7- (major) and reverse N1-prenylation (minor) of cyclo-L-Trp-L-Trp with high substrate specificity (Zou et al. 2010), which could not be used for production of other prenylated dipeptides. Recently, regularly C7-prenylated cyclic dipeptides were successfully obtained by using a new member of the DMATS family, CdpC7PT from Aspergillus terreus (Wunsch et al. 2015b). CdpC7PT demonstrated much higher substrate flexibility and converted six cyclic dipeptides to 10 prenylated products (Table 2). It also accepted cyclo-L-Tyr-L-Tyr as substrate and catalyzed an O-prenylation at the tyrosyl residue, which was the first example of DMATS enzymes with an O-prenyltransferase activity toward such dipeptides (Wunsch et al. 2015b). Five regularly C4-prenylated cyclic dipeptides were obtained by using the tryptophan PT FgaPT2 (Steffan and Li 2009). In comparison to those with cyclic dipeptide PTs, product yields with FgaPT2 between 22 - 35 % are relatively low (Table 2).

The cyclic dipeptide C3-PTs, AnaPT, CdpC3PT and CdpNPT all accepted a number of cyclic dipeptides as substrates and catalyzed stereoselective prenylation, resulting in the formation of different stereoisomers (Table 3). The stereoselectivities of AnaPT and CdpC3PT toward cyclo-Trp-Ala and cyclo-Trp-Pro mainly depended on the configuration of the tryptophanyl moiety in the substrates. They produced anti-cis and
*syn-cis* configured prenylated pyrroloindolines, respectively. CdpNPT catalyzed both *syn-cis* and *anti-cis* configured prenylations (Table 3) (Yu et al. 2013).

As mentioned before, tryptophan PTs accept cyclic dipeptides as well, but with very low activity (Steffan et al. 2007; Steffan and Li 2009). In return, tryptophan is a poor substrate for cyclic dipeptide PTs (Zou et al. 2009). However, one unnatural cyclic dipeptide, *cyclo*-L-homotryptophan-D-valine with one additional C-atom between the indole and the diketopiperazine rings was demonstrated to be well accepted by five cyclic dipeptide PTs (BrePT, FtmPT1, AnaPT, CdpNPT and CdpC3PT) and three tryptophan PTs (FgaPT2, 5-DMATS and 7-DMATS). Seven enzymatic products with one prenyl moiety at each position of the indole nucleus (N-1, C-2 to C-7) were produced (Fan and Li 2013). This was the first report on production of seven monoprenylated products from one substrate by one-step reactions.

The prenylation reaction mentioned above all took place on the aromatic nucleus. The first exception reported in 2012 by Chen et al showed that cyclic dipeptide C2-PT FtmPT1 also accepted (E)-4-((1H-indol-3-yl)but-3-en-2-one as substrate and catalyzed the formation of the unnatural α-prenylindolylbutenone, demonstrating the prenylation of a nonaromatic carbon by a indole PT (Chen et al. 2012). These examples indicated that further investigations on the substrate promiscuity and catalytic ability of PTs could lead to production of novel unnatural products.

**Chemoenzymatic synthesis of prenylated phenylalanine/tyrosine derivatives**

The tyrosine PTs SirD and TyrPT catalyze the *O-, N-* or *S-* prenylations of tyrosine and derivatives thereof (Table 4) (Fan et al. 2014; Rudolf and Poulter 2013; Zou et al.
Two tryptophan PTs FgaPT2 and 7-DMATS take L-tyrosine and 4-amino-L-phenylalanine as substrates as well and produce the unique C3- and O-prenylated tyrosine or 4-amino-L-phenylalanine, respectively (Table 4) (Fan et al. 2015; Fan and Li 2014). Together with the results of C7-prenylation of L-tryptophan by SirD and TyrPT, these four PTs demonstrated complementary substrate and catalytic promiscuity and share very likely similar reaction cavities in their structures.

**Chemoenzymatic synthesis of prenylated xanthones**

In the presence of DMAPP, the xanthone PT XptB from *Aspergillus nidulans* (*A. nidulans*) involved in the biosynthesis of shamixanthone converted four of the ten tested xanthones to O7-prenylated derivatives (Table 5) (Pockrandt et al. 2012). One special feature of XptB was that Mn$^{2+}$ and Co$^{2+}$ strongly enhanced its activity (up to eightfold), which has not been reported for the DMATS before. Recently, an N1- and C2-PT AstPT from *Aspergillus terreus* was proven to be highly specific for its substrate bisindolyl benzoquinone. Tryptophan and derivatives as well as tryptophan-containing cyclic dipeptides were not accepted by this enzyme (Tarcz et al. 2014a). However, AstPT used DMAPP, GPP and FPP for O-prenylation of hydroxyxanthones (Table 5) (Tarcz et al. 2014b). AstPT and XptB showed different substrate preference and regioselectivity toward xanthones.

**Chemoenzymatic synthesis of prenylated naphthalenes**
The structure of FgaPT2 unexpectedly revealed the common architecture with another PT NphB from the CloQ/NphB subgroup, which utilizes hydroxynaphthalenes as aromatic substrates (Kuzuyama et al. 2005). This result indicated a possibility for production of prenylated hydroxynaphthalenes by DMATS enzymes. Four indole PTs, 7-DMATS, AnaPT, CdpNPT and CdpC3PT (Kremer et al. 2007; Schuller et al. 2012; Yin et al. 2009b; Yin et al. 2010b), were found indeed to accept diverse hydroxynaphthalenes as substrates and catalyzed mainly a regular C-prenylation at para- or ortho-position to the hydroxyl group of 1-naphthol or 2-naphthol derivatives. 11 prenylated hydroxynaphthalenes were isolated and identified as main products (Table 6) (Yu et al. 2011). Additionally, the recently characterized 6-DMATS$_{S_s}$ and 6-DMATS$_{S_v}$ from Streptomyces species were also found to catalyze prenylation of three hydroxynaphthalenes. Interestingly, different from the products with 7-DMATS, AnaPT, CdpNPT and CdpC3PT mentioned above, the prenyl moieties were transferred onto the unsubstituted ring of naphthalenes (Table 6) (Winkelblech and Li 2014). Therefore, these four enzymes could be considered as complementary biocatalysts for production of prenylated hydroxynaphthalenes.

**Chemoenzymatic synthesis of prenylated flavonoids**

Prenylated flavonoids and isoflavonoids are widely distributed in nature, predominantly in plants (Botta et al. 2005; Botta et al. 2009). Prenylations of flavonoids in plants are catalyzed by membrane-bound proteins, which are generally more difficult to be overproduced and purified than soluble DMATS enzymes. In addition, they usually exhibit very high substrate specificity and relative low catalytic ability (Chen et al. 2013;
Sasaki et al. 2011). These features prohibit their potential use in biotechnology. Tryptophan C7-PT 7-DMATS (Table 1) was reported to convert a number of flavonoids to their prenylated derivatives, especially C6-prenylated flavanones and isoflavonoids (Figure 1) (Yu and Li 2011). Thus, 7-DMATS could be a valuable alternative biocatalyst for chemoenzymatic synthesis or in vivo-production of prenylated flavonoids.

**Chemoenzymatic synthesis of prenylated indolocarbazoles**

Indolocarbazoles are a class of natural products with remarkable biological activities. Several of these compounds have already entered clinical trials for treatment of cancer and other diseases (Salas and Mendez 2009; Sánchez et al. 2006). FgaPT2 and 5-DMATS were reported to catalyze regiospecific prenylations of indolocarbazoles at the para-position of the indole N-atom and four prenylated products were obtained. These results expanded not only the potential usage of these enzymes in the structural modification, but also provided leading compounds for further biological research (Figure 2) (Yu et al. 2012a).

**Chemoenzymatic synthesis of prenylated acylphloroglucinol**

Naturally occurring prenylated acylphloroglucinol derivatives are plant metabolites with diverse biological activities and membrane-bound PTs are responsible for the prenylation steps (Li et al. 2015; Tsurumaru et al. 2012). Recently, Zhou et al
demonstrated the prenylation of such compounds by the soluble cyclic dipeptide C3-PT AnaPT and nine prenylated acylphloroglucinols were then acquired (Table 7). Compared to a microsomal fraction containing an overproduced PT from the plant hop, AnaPT is more efficient for the chemoenzymatic synthesis (Zhou et al. 2015).

Expanding the usage of the DMATS enzymes by altering the prenyl donors

In the last years, the potential usage of DMATS enzymes as biocatalysts was expanded significantly by their acceptance of different prenyl donors. In addition to their remarkable high flexibility toward aromatic substrates, recent studies manifested that several members of this family accepted not only DMAPP, but also other alkyl donors such as GPP and FPP. Before 2012, the acceptance of these donors was only reported for several bacterial PTs of the CloQ/NphB subgroup such as NphB (Kumano et al. 2008; Kuzuyama et al. 2005), Fnq26 (Haagen et al. 2007) and Fur7 (Kumano et al. 2010). Meanwhile, we demonstrated that the unnatural DMAPP analogs like monomethylallyl (MAPP), 2-pentenyl (2-pentenyl-PP) and benzyl diphosphate (benzyl-PP) can also be used for alkylation or benzylation of tryptophan and tryptophan-containing cyclic dipeptides by a number of DMATS enzymes (Liebhold et al. 2012; Liebhold et al. 2013; Liebhold and Li 2013; Winkelblech et al. 2015b).

Acceptance of GPP and FPP by the DMATS enzymes

Identification of the DMATS enzyme VrtC from Penicillium aethiopicum as a geranyltransferase in the biosynthesis of viridicatumtoxin (Chooi et al. 2010)
demonstrated that the prenyl donors of these enzymes are not limited to DMAPP in nature. This led to intensive investigations on known DMATS enzymes regarding their acceptance of GPP as prenyl donor. Indeed, GPP can be used by AnaPT from *N. fischeri* for geranylation of (R)-benzodiazepinedione (*cyclo*-L-Trp-Ant) and five other cyclic dipeptides. *C6*- and *C7*-geranylated derivatives were identified as enzyme products (Table 8) (Pockrandt and Li 2013). Obviously, the regioselectivity of the prenyl transfer reaction was reduced in the presence of the long chain donor.

Moreover, GPP also serves as a prenyl donor for the recently identified 6-DMATS<sub>Sa</sub> and 6-DMATS<sub>Sv</sub>, but with retained prenylation position at C-6 of the indole ring. Thus, these bacterial enzymes represent the first examples of tryptophan PTs that accept both DMAPP and GPP as prenyl donors and produce 6-dimethylallyl or 6-geranyl tryptophan and derivatives thereof (Winkelblech and Li 2014).

AstPT demonstrated extraordinarily high substrate specificity toward both prenyl donor DMAPP and acceptor bisindolyl benzoquinone (AQ D) (Tarcz et al. 2014a). In the presence of GPP or FPP, none of the tested indole derivatives including tryptophan and cyclic dipeptides, hydroxynaphthalenes and flavonoids was accepted by this enzyme. Remarkably, AstPT accepted a number of hydroxyxanthones in the presence of not only DMAPP, but also GPP and FPP and catalyzed *O*-prenylations at the same position of the benzene ring (Table 9) (Tarcz et al. 2014b).

Furthermore, the acceptance of DMAPP, GPP and FPP was reported for *C*-prenylations of hydroxynaphthalenes by BAE61387, a member of the DMATS superfamily from *Aspergillus oryzae* (Pockrandt et al. 2014). BAE61387 catalyzed the prenyl transfer reactions to the same position with all the used donors and produced nine dimethylallyl-, geranyl- and farnesylhydroxynaphthalenes with product yields of up to 72 % (Figure 3).
Acceptance of unnatural DMAPP analogs by the DMATS enzymes

In 2012, several unnatural DMAPP analogs were synthesized and tested as alkyl donors for L-tryptophan PTs (Liebhold et al. 2012). It has shown that the double bond at the β-position to pyrophosphate is essential for an acceptance by these enzymes. One methyl group can be deleted as in the case of MAPP or shifted to the δ-position as in the case of 2-pentenyl-PP (Liebhold et al. 2012). Furthermore, the alklylation position of the products can differ from those with DMAPP, depending on the used prenyl donors. In the presence of 2-pentenyl-PP, C5- and C6-alkylated tryptophan was obtained with the C4-PT FgaPT2 and C5-PT 5-DMATS, respectively (Table 10). In the presence of MAPP, a mixture of C4- and C5-alkylated tryptophan was observed for FgaPT2 reaction, and C5- and C6-alkylated derivatives for 5-DMATS reaction (Table 10). Latter, it has been shown that FgaPT2 even used the more space-demanding substrate benzyl-PP as donor and produced regiospecifically C5-benzylated tryptophan (Table 10) and eight derivatives thereof (Liebhold and Li 2013).

The chemoenzymatic synthesis of unnatural alkylated or benzylated products was expanded by further investigations with tryptophan C5-, C6- and C7-prenylating enzymes. The behaviors of two C5-PTs 5-DMATS from Aspergillus clavatus (Yu et al. 2012b) and 5-DMATSSc from Streptomyces coelicolor (Subramanian et al. 2012), two C6-PTs 6-DMATSSa and 6-DMATSSv from Streptomyces species (Winkelblech and Li 2014) as well as TyrPT with a tryptophan C7-prenylation activity (Fan et al. 2014), were investigated in the presence of MAPP, 2-pentenyl-PP and benzyl-PP (Table 10). All the tested enzymes accepted the three DMAPP analogs with different relative activities. Enzyme products with the allyl or benzyl moiety attached to different
positions were identified, whereby C6-alkylated or benzylated L-tryptophan was found as sole or one of the main products in all of these assays (Table 10) (Winkelblech et al. 2015b). This demonstrated a clear preference of the five PTs toward C-6 of the indole ring in the presence of unnatural DMAPP analogs. Molecular dynamics simulation experiments with a homologous model of 5-DMATS from A. clavatus leading to a distance-based explanation of their reactions with MAPP and 2-pentenyl-PP (Winkelblech et al. 2015b). Site-directed mutagenesis based on the molecular modeling experiments could lead to enzymes with increased regioselectivity for DMAPP analogs.

The acceptance of the unnatural DMAPP analogs was also demonstrated with three cyclic dipeptide reverse C3-PTs, AnaPT, CdpNPT and CdpC3P (Schuller et al. 2012; Yin et al. 2009b; Yu et al. 2013), as well as with two C2-PTs FtmPT1 and BrePT (Grundmann and Li 2005; Yin et al. 2013). All of these enzymes catalyzed the Friedel-Crafts alkylation of cyclic dipeptides. Remarkably, C2- and C3-reverse monoalkylated derivatives were identified as enzyme products in all the reaction mixtures, although different ratios were observed depending on the used donor and enzyme (Table 11) (Liebhold et al. 2013).

Moreover, we reported very recently the alkylation or benzylation of tyrosine and its derivatives with these DMAPP analogs by the two tyrosine O-PTs TyrPT and SirD (Table 12). In the presence of MAPP, 2-pentenyl-PP and benzyl-PP, TyrPT and SirD produced the same O- or N-alkylated or benzylated products, which displayed a similar behavior as for their natural prenyl donor DMAPP (Yu et al. 2015).
Molecular modeling guided site-directed mutagenesis as tools for creation of enzymes with new features

The crystal structures of FgaPT2 (Metzger et al. 2009), FtmPT1 (Jost et al. 2010), CdpNPT (Schuller et al. 2012) and AnaPT (Yu et al. 2013) have been determined during the past six years and were used as a basis for understanding the prenyl transfer reactions (Jost et al. 2010; Luk and Tanner 2009; Metzger et al. 2009). The structure of FtmPT1 allowed us to identify a reaction chamber with modifiable specificity. FtmPT1_G115T obtained by mutation of G115 to threonine still accepted brevianamide F as substrate, but yielded mainly a reversely syn-cis C3-prenylated derivative instead of the regularly C2-prenylated (Jost et al. 2010).

Another example of the usage of structure information for mutant design is shown with FgaPT2 (Table 1). Initial investigation demonstrated that FgaPT2 can convert L-tyrosine to 3-dimethylallyl-L-tyrosine (Table 4), but with a very low activity. Molecular modeling-guided site-directed mutagenesis of FgaPT2 resulted in a mutant, FgaPT2_K174F, which showed much higher specificity toward L-tyrosine than L-tryptophan. The catalytic efficiency of this mutant toward L-tyrosine was found to be 4.9-fold of that of wild-type, while its activity toward L-tryptophan was less than 0.4 % of that of FgaPT2 (Fan et al. 2015). Therefore, we created the first specific tyrosine C3-prenylating enzyme and altered the substrate preference of a PT by molecular modeling-guided site-directed mutagenesis.
Whole cell biocatalyst for the efficient prenylation of indole derivatives by Autodisplay of FgaPT2

The tryptophan C4-PT FgaPT2 was displayed on the surface of *Escherichia coli* cells by using Autodisplay technique for chemoenzymatic synthesis (Figure 4) (Kranen et al. 2011). Indole-3-propionic acid and L-β-homotryptophan were tested with the FgaPT2 whole cell biocatalyst and conversion yields of 30 and 13 % were obtained, respectively. Its catalytic efficiency was much higher than that of the purified FgaPT2 in 24 h assay toward indole-3-propionic acid, which could result from the enormously improved enzyme stability by immobilizing in the outer membrane of *E. coli* (Kranen et al. 2011). The whole cell biocatalyst endured a storage period of one month at 8 °C without any detectable loss in activity. Reusability was confirmed by recycling the biocatalyst by centrifugation. After three cycles of consecutive use, the whole cell biocatalyst retained a conversion rate of 46 % of indole-3-propionic acid and 23 % of L-β-homotryptophan (Kranen et al. 2011). Compared to the conventional whole cell catalysts, whose enzyme of interest within the cell cytoplasm, the Autodisplay strategy demonstrated many advantages. First of all, transport of substrates or products across membranes is not necessary; secondly, substrates and products can be easily separated from catalyst and catalyst can be recovered by centrifugation. At last, it is possible to avoid the formation of byproducts or metabolism of the substrate or product inside the cell (Kranen et al. 2011).

**Targeted production of prenylated compounds by coexpression of non-ribosomal peptide synthase and prenyltransferase genes in *Aspergillus***
The flexibility of the PTs toward aromatic substrates and high regio- and stereoselectivity regarding prenylation position at the indole ring as well as different prenylation patterns also make these enzymes interesting tools for production of natural or unnatural prenylated compounds by synthetic biological approach. As a proof of principle, the NRPS gene \textit{ftmPS} was expressed alone or together with the PT gene \textit{ftmPT1}, both from the biosynthetic gene cluster of verruculogen in \textit{A. fumigatus}. Expression of \textit{ftmPS} in \textit{A. fumigatus} and \textit{A. nidulans} resulted in the formation of brevianamide F (Figure 5) with product yields of 32 and 45 mg per liter culture, respectively (Maiya et al. 2006). Coexpression of \textit{ftmPS} and \textit{ftmPT1} as one construct in \textit{A. nidulans} resulted in the formation of tryprostatin B (Figure 5) with product yield of up to 260 mg per liter culture (Maiya et al. 2009). That is an up to 650-fold increase in comparison to that of \textit{A. fumigatus} BM939 (Cui et al. 1995). These results led to test the potential for production of other prenylated compounds by combination of genes from different clusters. For this purpose, the orthologous NRPS gene \textit{ftmPS} (NFIA\_093690) from \textit{N. fischeri} encoding a cyclic dipeptide-forming enzyme was cloned in one construct and three characterized PT genes in other constructs. These included one reverse cyclic dipeptide C2-PT gene \textit{cdpC2PT} from \textit{N. fischeri} and two reverse C3-prenyltransfersae genes, i.e., \textit{cdpC3PT} from \textit{N. fischeri} and \textit{cdpNPT} from \textit{A. fumigatus} (Mundt and Li 2013; Yin et al. 2010a; Yu et al. 2013). The genes are expressed under the control of the constitutive \textit{gpdA} promoter and \textit{trpC} terminator. Expression of \textit{ftmPS} from \textit{N. fischeri} alone in \textit{A. nidulans} resulted in the formation of the expected cyclic dipeptide brevianamide F (Figure 5) with a yield of up to 37 mg per liter culture. Introducing \textit{cdpC2PT} and \textit{cdpNPT} into a \textit{ftmPS} overproduction strain led to accumulation of reversely C2- and C3-prenylated derivatives, respectively. Coexpression of \textit{ftmPS} with the reverse C3-PT gene \textit{cdpC3PT} resulted in the formation
of N1-regularly, C2- and C3-reversely prenylated derivatives (Figure 5) (Wunsch et al. 2015a). The prenyl transfer reactions catalyzed by CdpC2PT, CdpNPT and CdpC3PT in vivo correspond well to those detected with purified proteins (Mundt and Li 2013; Yin et al. 2010b; Yu et al. 2013). This proved that the data obtained from enzyme assays with PTs can be transferred directly for in vivo production of such compounds by synthetic biology.

**Conclusion and outlook**

In the past ten years, significant progress has been achieved on the PTs of the DMATS superfamily. More than forty members of this family have been characterized biochemically and four structures of them were solved, which provided us not only with more biocatalysts for production of prenylated products, but also useful information to understand these biocatalysts. Investigation on the substrate and catalytic promiscuity of the DMATS enzymes demonstrated their potential application in the chemoenzymatic synthesis and synthetic biology. More than 250 prenylated compounds have been produced by using PTs as biocatalysts and are summarized in this review. Availability of more structures will provide basis for creation of new desirable biocatalysts by site-directed mutagenesis, especially such enzymes with broad substrate specificity, high catalytic efficiency and high regio and stereoselectivity for unnatural substrates.

**Acknowledgement**
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Yin W-B, Grundmann A, Cheng J, Li S-M (2009b) Acetylaszonalenin biosynthesis in *Neosartorya fischeri*: Identification of the biosynthetic gene cluster by genomic...


Legends to Figures

**Figure 1.** Chemoenzymatic synthesis of prenylated flavonoids.

**Figure 2.** Chemoenzymatic synthesis of prenylated indolocarbazoles.

**Figure 3.** Prenylation of hydroxynaphthalenes catalyzed by BAE61387 in the presence of DMAPP, GPP and FPP.

**Figure 4.** The Autodisplay procedure of FgaPT2 on the surface of *E. coli.* (modified according to (Kranen et al. 2011))

**Figure 5.** Coexpression of NRPS and PTs genes in *Aspergillus* to produce prenylated cyclic dipeptides.
Table 1 Product yields of prenylated simple indoles by tryptophan PTs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>FgaPT2 (C-4)</th>
<th>5-DMATS (C-5)</th>
<th>6-DMATS$_{\text{Sa}}$ (C-6)</th>
<th>7-DMATS (C-7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Trp</td>
<td>100</td>
<td>100</td>
<td>59.4</td>
<td>41.3</td>
</tr>
<tr>
<td>D-Trp</td>
<td>9.7</td>
<td>n.t.</td>
<td>43.7</td>
<td>4.9</td>
</tr>
<tr>
<td>1-methyl-L-Trp</td>
<td>0.4</td>
<td>46.9</td>
<td>1.3</td>
<td>14.8</td>
</tr>
<tr>
<td>4-methyl-DL-Trp</td>
<td>n.d.</td>
<td>56.6</td>
<td>57.3</td>
<td>36.9</td>
</tr>
<tr>
<td>5-methyl-DL-Trp</td>
<td>8.1</td>
<td>1.7</td>
<td>52.2</td>
<td>30.9</td>
</tr>
<tr>
<td>5-hydroxy-L-Trp</td>
<td>21.0</td>
<td>n.t.</td>
<td>n.t.</td>
<td>11.0</td>
</tr>
<tr>
<td>5-bromo-DL-Trp</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.t.</td>
<td>3.0</td>
</tr>
<tr>
<td>6-methyl-DL-Trp</td>
<td>15.2</td>
<td>53.2</td>
<td>42.9</td>
<td>8.2</td>
</tr>
<tr>
<td>6-fluoro-DL-Trp</td>
<td>n.d.</td>
<td>69.5</td>
<td>4.9</td>
<td>13.9</td>
</tr>
<tr>
<td>7-methyl-DL-Trp</td>
<td>16.3</td>
<td>57.8</td>
<td>30.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>indole-3-propionic acid</td>
<td>R$_3$=(-\text{COOH})</td>
<td>32.2</td>
<td>70.7</td>
<td>18.5</td>
</tr>
<tr>
<td>L-(\beta)-homo-Trp</td>
<td>R$_3$=(-\text{COOH})</td>
<td>51.5</td>
<td>48.8</td>
<td>57.7</td>
</tr>
<tr>
<td>N-acetyl-DL-Trp</td>
<td>R$_3$=(-\text{COOH})</td>
<td>n.d.</td>
<td>54.4</td>
<td>16.0</td>
</tr>
<tr>
<td>L-abrine</td>
<td>R$_3$=(-\text{COOH})</td>
<td>58.0</td>
<td>90.9</td>
<td>63.0</td>
</tr>
<tr>
<td>11-methyl-DL-Trp</td>
<td>R$_3$=(-\text{COOH})</td>
<td>1.6</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>DL-indole-3-lactic acid</td>
<td>R$_3$=(-\text{COOH})</td>
<td>10.5</td>
<td>67.3</td>
<td>16.0</td>
</tr>
<tr>
<td>(\text{trans})-indole-3-acrylic acid</td>
<td>R$_3$=(-\text{COOH})</td>
<td>n.t.</td>
<td>38.0</td>
<td>20.5</td>
</tr>
</tbody>
</table>

The data are adopted from publications for FgaPT2 after incubation with 0.18 µM protein for 1 h (Steffan et al. 2007); 5-DMATS (1 µM, 7 h) (Yu et al. 2012b); 6-DMATS$_{\text{Sa}}$ (2.3 µM, 16 h) (Winkelblech and Li 2014) and 7-DMATS (0.6 µM, 1 h) (Kremer and Li 2008). Prenylation positions are given in parenthesis under the enzyme names. n.d.: not detected; n.t.: not tested.
Table 2 Product yields of prenylated cyclic dipeptides by DMATS enzymes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>FtmPT1 C-2 regular</th>
<th>BrePT C-2 regular</th>
<th>CdpC2PT C-3 regular</th>
<th>CdpC7PT C-2 reverse</th>
<th>FgaPT2 C-7 regular</th>
<th>C-4 regular</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclo-L-Trp-L-Pro</td>
<td>82.3</td>
<td>n.i.</td>
<td>syn-cis</td>
<td>78</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-L-Trp-D-Pro</td>
<td>75.7</td>
<td>n.i.</td>
<td>syn-cis</td>
<td>72</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-D-Trp-L-Pro</td>
<td>27.4</td>
<td>n.i.</td>
<td>syn-cis</td>
<td>13</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-D-Trp-D-Pro</td>
<td>43.4</td>
<td>11.8</td>
<td>syn-cis</td>
<td>25</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Tyr</td>
<td>74.2</td>
<td>1.1</td>
<td>syn-cis</td>
<td>11</td>
<td>n.i.</td>
<td>28.2</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Trp</td>
<td>67.0</td>
<td>n.i.</td>
<td>syn-cis</td>
<td>8</td>
<td>48</td>
<td>28.1</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Leu</td>
<td>70.9</td>
<td>n.i.</td>
<td>syn-cis</td>
<td>46</td>
<td>37</td>
<td>29.2</td>
</tr>
<tr>
<td>cyclo-L-Trp-Gly</td>
<td>5.07</td>
<td>18.6</td>
<td>syn-cis</td>
<td>39</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Phe</td>
<td>39.3</td>
<td>n.i.</td>
<td>syn-cis</td>
<td>31</td>
<td>17</td>
<td>13.6</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-His</td>
<td>68.1</td>
<td>6.8</td>
<td>syn-cis</td>
<td>22</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Ala</td>
<td>53.5</td>
<td>12.7</td>
<td>syn-cis</td>
<td>35</td>
<td>8</td>
<td>19.5</td>
</tr>
<tr>
<td>cyclo-L-Trp-D-Ala</td>
<td>43.9</td>
<td>12.1</td>
<td>syn-cis</td>
<td>29</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-D-Trp-L-Ala</td>
<td>46.4</td>
<td>11.6</td>
<td>syn-cis</td>
<td>22</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-D-Trp-D-Ala</td>
<td>8.3</td>
<td>n.i.</td>
<td>syn-cis</td>
<td>13</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-L-Trp-Ant</td>
<td>n.t.</td>
<td>6</td>
<td>syn-cis</td>
<td>n.t.</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>cyclo-D-Trp-Ant</td>
<td>n.t.</td>
<td>8</td>
<td>syn-cis</td>
<td>85</td>
<td>45.6</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

The data are adopted from publications for FmPT1 (0.94 µM, 2 h) (Wollinsky et al. 2012); BrePT (3.0 µM, 16 h) (Mundt and Li 2013); CdpC2PT (4.0 µM, 16 h) (Mundt and Li 2013); CdpC7PT (9 µM, 16 h) (Wunsch et al. 2015b); FgaPT2 (1.8 µM, 16 h) (Steffan and Li 2009). n.i.: not isolated; n.t.: not tested.
Table 3 Product yields of prenylated cyclic dipeptides by DMATS enzymes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CdpC3PT C3-IIA/C&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AnaPT C3-IIB/C/D&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CdpNPT C3-IIA/B/C/D&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclo-L-Trp-L-Pro</td>
<td>58.3 (A)</td>
<td>15.2 (D)</td>
<td>4.8 (D)/75.1 (A)</td>
</tr>
<tr>
<td>cyclo-L-Trp-D-Pro</td>
<td>69.6 (A)</td>
<td>n.i.</td>
<td>62.5 (A)</td>
</tr>
<tr>
<td>cyclo-D-Trp-L-Pro</td>
<td>3.7 (C)</td>
<td>n.i.</td>
<td>n.i./n.d.</td>
</tr>
<tr>
<td>cyclo-D-Trp-D-Pro</td>
<td>2.7 (C)</td>
<td>3.2 (B)</td>
<td>94.1 (B)/n.i.</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Tyr</td>
<td>30 (A)</td>
<td>25.7 (D)</td>
<td>n.t.</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Trp</td>
<td>12 (A)</td>
<td>37.0 (D)</td>
<td>n.t.</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Leu</td>
<td>31 (A)</td>
<td>63.6 (D)</td>
<td>n.t.</td>
</tr>
<tr>
<td>cyclo-L-Trp-Gly</td>
<td>37 (A)</td>
<td>12.3 (D)</td>
<td>n.t.</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Phe</td>
<td>6 (A)</td>
<td>31.4 (D)</td>
<td>n.t.</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-His</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Ala</td>
<td>39.5 (A)</td>
<td>39.7 (D)</td>
<td>38.4 (D)/22.0 (A)</td>
</tr>
<tr>
<td>cyclo-L-Trp-D-Ala</td>
<td>32.0 (A)</td>
<td>29.1 (D)</td>
<td>34.3 (D)/51.9 (A)</td>
</tr>
<tr>
<td>cyclo-D-Trp-L-Ala</td>
<td>17.8 (C)</td>
<td>21.7 (B)</td>
<td>26.1 (B)/36.2 (C)</td>
</tr>
<tr>
<td>cyclo-D-Trp-D-Ala</td>
<td>11.0 (C)</td>
<td>10.8 (B)</td>
<td>72.7 (B)/n.i.</td>
</tr>
<tr>
<td>cyclo-L-Trp-Ant</td>
<td>n.t.</td>
<td>100 (C)</td>
<td>77.1 (B)</td>
</tr>
<tr>
<td></td>
<td>n.t.</td>
<td>80.8 (D)</td>
<td>(two byproducts)</td>
</tr>
<tr>
<td></td>
<td>99.4 (A)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data are adopted from publications for CdpC3PT (1.3 µM, 24 h) (Yin et al. 2010b); (8 µM, 3 h) (Yu et al. 2013); AnaPT (0.54 µM, 24 h) (Yin et al. 2010a); (8 µM, 3 h) (Yu et al. 2013); CdpNPT (0.38 µM, 24 h) (Yin et al. 2009a); (8 µM, 3 h) (Yu et al. 2013).

The codes for the prenylated products are given in parenthesis after product yields.

<sup>a</sup>C3-IIA/C refers to C3-IIA or C3-IIC, C3-IIB/C/D for C3-IIB, C3-IIC or C3-IID, and C3-IIA/B/C/D for C3-IIA, C3-IIB, C3-IIC or C3-IID. n.d.: not detected; n.i.: not isolated; n.t.: not tested.
Table 4 Product yields of prenylated tyrosine derivatives by DMATS enzymes.

<table>
<thead>
<tr>
<th>substrate</th>
<th>SirD</th>
<th>TyrPT</th>
<th>7-DMATS</th>
<th>FgaPT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tyr</td>
<td>63.4</td>
<td>64</td>
<td>36</td>
<td>18</td>
</tr>
<tr>
<td>D-Tyr</td>
<td>n.i.</td>
<td>n.i.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3-fluoro-DL-Tyr</td>
<td>34.0</td>
<td>50</td>
<td>n.t.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3-iodo-L-Tyr</td>
<td>17.3</td>
<td>100</td>
<td>97</td>
<td>n.d.</td>
</tr>
<tr>
<td>3-amino-L-Tyr</td>
<td>68.5</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
<tr>
<td>4-amino-L-Phe</td>
<td>6.7</td>
<td>98</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>3,4-dihydroxy-L-Phe</td>
<td>12.0</td>
<td>100</td>
<td>n.i.</td>
<td>n.t.</td>
</tr>
<tr>
<td>α-methyl-L-Tyr</td>
<td>63.0</td>
<td>64</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>3-(3,4-dihydroxy-phenyl)-DL-Ser</td>
<td>11.4</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
</tr>
</tbody>
</table>

The data are adopted from publications for SirD (0.58 µM, 16 h) (Zou et al. 2011), TyrPT (1.6 µM, 16 h) (Fan et al. 2014), 7-DMATS (0.58 µM, 16 h) (Fan and Li 2014) and FgaPT2 (3.6 µM, 16 h) (Fan et al. 2015). n.d.: not detected; n.i.: not isolated; n.t.: not tested.
Table 5 Product yields of prenylated xanthones by XptB and AstPT.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>XptB</th>
<th>AstPT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>prenylated position</td>
<td>conversion yield [%]</td>
</tr>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>7-O</td>
<td>23</td>
</tr>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>7-O</td>
<td>25</td>
</tr>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>7-O</td>
<td>31</td>
</tr>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>7-O</td>
<td>76</td>
</tr>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>n.d.</td>
<td>3-O and 6-O</td>
</tr>
<tr>
<td><img src="image" alt="Chemical Structure" /></td>
<td>n.d.</td>
<td>3-O and 7-O</td>
</tr>
</tbody>
</table>

The data are adopted from publication for XptB (3.8 µM, 16 h) (Pockrandt et al. 2012) and for AstPT (4.1 µM, 16 h) (Tarcz et al. 2014b). n.d.: not detected.
Table 6 Product yields of prenylated hydroxynaphthalenes by DMATS enzymes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>AnaPT</th>
<th>CdpNPT</th>
<th>CdpC3PT</th>
<th>7-DMATS</th>
<th>6-DMATS&lt;sub&gt;sa&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-4</td>
<td>91.5</td>
<td>C-4</td>
<td>93.3</td>
<td>C-4</td>
<td>14.1</td>
</tr>
<tr>
<td>C-4</td>
<td></td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>C-4</td>
<td>n.i.</td>
<td>C-4</td>
<td></td>
<td>C-4</td>
<td>92.6</td>
</tr>
<tr>
<td>C-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-6/C-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>61.5</td>
</tr>
<tr>
<td>C-4</td>
<td>88.7</td>
<td>C-4</td>
<td>46.7</td>
<td>C-4</td>
<td>9.8</td>
</tr>
<tr>
<td>C-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-4</td>
<td>(25.7)</td>
<td>n.d.</td>
<td>n.d.</td>
<td>C-4</td>
<td>59.0</td>
</tr>
<tr>
<td>C-4</td>
<td></td>
<td>n.i.</td>
<td>n.i.</td>
<td>n.i.</td>
<td></td>
</tr>
<tr>
<td>C-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>43.8</td>
</tr>
<tr>
<td>C-1</td>
<td>42.7</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>C-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-1 (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-3 (+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The data are adopted from publications for AnaPT, CdpNPT, CdpC3PT and 7-DMATS (20 µg, 7 h) (Yu et al. 2011); 6-DMATS<sub>sa</sub> (9.6 µM, 16 h) (Winkelblech and Li 2014). +: major product; n.i.: not isolated; n.d.: not detected; n.t.: not tested.
Table 7 Product yields of prenylated acylfloroglucinols by AnaPT.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Prenylated Position</th>
<th>Product Yield [%]</th>
<th>Substrate</th>
<th>Prenylated Position</th>
<th>Product Yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-2</td>
<td>C-6</td>
<td>4.6</td>
<td>C-2</td>
<td>C-6</td>
<td>4.6</td>
</tr>
<tr>
<td>C-2 and C-4</td>
<td>8.4</td>
<td></td>
<td>C-4</td>
<td>C-4</td>
<td>9.3</td>
</tr>
<tr>
<td>C-6</td>
<td>C-6</td>
<td>32.1</td>
<td>C-4</td>
<td>C-4</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td>32.3</td>
<td></td>
<td></td>
<td>32.2</td>
<td></td>
</tr>
</tbody>
</table>

The data are adopted from publication for AnaPT (10.1 µM, 6 h) (Zhou et al. 2015).

Table 8 Product yields of geranylated cyclic dipeptides by AnaPT

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Prenylated Position</th>
<th>Product Yields [%]</th>
<th>Substrate</th>
<th>Prenylated Position</th>
<th>Product Yields [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>cyclo-L-Trp-Ant</td>
<td>C-6</td>
<td>88</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>cyclo-D-Trp-Ant</td>
<td>C-6</td>
<td>31</td>
<td>C-7</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Leu</td>
<td>C-6</td>
<td>15</td>
<td>C-7</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Trp</td>
<td>C-6</td>
<td>15</td>
<td>C-7</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Tyr</td>
<td>C-6</td>
<td>9</td>
<td>C-7</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>cyclo-D-Trp-L-Tyr</td>
<td>C-6</td>
<td>32</td>
<td>C-7</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

The data are adopted from publication for AnaPT (4.0 µM, 16 h) (Pockrandt and Li 2013). n.d.: not detected.
Table 9 Product yields of prenylated xanthones by AstPT.

<table>
<thead>
<tr>
<th>substrate</th>
<th>prenylated position</th>
<th>dimethylallyl (n=1) product yield [%]</th>
<th>geranyl (n=2) product yield [%]</th>
<th>farnesyl (n=3) product yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[structural formula]</td>
<td>7-0</td>
<td>23.1</td>
<td>26.1</td>
<td>18.8</td>
</tr>
<tr>
<td>[structural formula]</td>
<td>3-0</td>
<td>2.1</td>
<td>9.1</td>
<td>5.3</td>
</tr>
<tr>
<td>[structural formula]</td>
<td>6-0</td>
<td>8.9</td>
<td>2.1</td>
<td>0.87</td>
</tr>
<tr>
<td>[structural formula]</td>
<td>3-0</td>
<td>n.d.</td>
<td>8.4</td>
<td>14.0</td>
</tr>
</tbody>
</table>

The data are adopted from publication for AstPT (4.1 µM, 16 h) (Tarcz et al. 2014b). n.d.: not detected.

Table 10 Product yields of prenylated L-tryptophan by DMATS enzymes with unnatural alkyl/benzyl donors.

<table>
<thead>
<tr>
<th>DMATS enzyme</th>
<th>prenylated position and product yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAPP</td>
</tr>
<tr>
<td>FgaPT2</td>
<td>C-4 (19)*</td>
</tr>
<tr>
<td>5-DMATS</td>
<td>C-5 (13.6), C-6 (45.3)</td>
</tr>
<tr>
<td>5-DMATS</td>
<td>C-5 (2.3), C-6 (9.3), C-7 (9.3), C-6 (27.9)</td>
</tr>
<tr>
<td>6-DMATS</td>
<td>C-6 (51.1)</td>
</tr>
<tr>
<td>6-DMATS</td>
<td>C-6 (37.6), C-6 (89.3)</td>
</tr>
<tr>
<td>TyrPT</td>
<td>C-6 (5.9), C-7 (8.9), byproducts</td>
</tr>
</tbody>
</table>

* The data are adopted from publication for FgaPT2 (0.89 µM, 16 h) (Liebhold et al. 2012); b for FgaPT2 (1.8 µM, 16 h) (Liebhold and Li 2013); For the rest data: enzyme (7.5µM, 16 h) was adopted here (Winkelblech et al. 2015b).
**Table 11** Product yields of prenylated cyclic dipeptides by DMATS enzymes with unnatural alkyl donors.

<table>
<thead>
<tr>
<th>Substrate with enzyme</th>
<th>donor</th>
<th>prenylated position and product yields [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C2-alkylated</td>
</tr>
<tr>
<td>cyclo-L-Trp-Ant + AnaPT</td>
<td>MAPP</td>
<td>65.2</td>
</tr>
<tr>
<td></td>
<td>2-pen-PP</td>
<td>26.8</td>
</tr>
<tr>
<td>cyclo-D-Trp-Ant + CdpNPT</td>
<td>MAPP</td>
<td>6.2 (3:1)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2-pen-PP</td>
<td>14.2 (5:1)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Leu + CdpC3PT</td>
<td>MAPP</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>2-pen-PP</td>
<td>15.3</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Pro + FtmPT1</td>
<td>MAPP</td>
<td>74.7 (1:4:1)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2-pen-PP</td>
<td>52.5 (1:3:4)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>cyclo-L-Trp-L-Pro + BrePT</td>
<td>MAPP</td>
<td>22.9 (1:1:1)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2-pen-PP</td>
<td>59.3 (5:1)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The data are adopted from publication for AnaPT (4.0 µM), CdpNPT (9.8 µM), CdpC3PT (10.5 µM), FtmPT1 (3.7 µM), BrePT (10.0 µM) and incubation time 16 h (Liebhold et al. 2013).

<sup>a</sup> the ratio of two isomers.
Table 12 Product yields of prenylated tyrosine by tyrosine PTs with unnatural alkyl/benzyl donors.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DMAPP yield [%]</th>
<th>MAPP yield [%]</th>
<th>2-pen-PP yield [%]</th>
<th>benzyl-PP yield [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tyr</td>
<td>89.1</td>
<td>99.3</td>
<td>21.2</td>
<td>28.0</td>
</tr>
<tr>
<td>4-amino-L-Phe</td>
<td>78.1</td>
<td>66.1</td>
<td>57.7</td>
<td>34.8</td>
</tr>
<tr>
<td>3-fluoro-DL-Tyr</td>
<td>50.1</td>
<td>66.5</td>
<td>13.1</td>
<td>8.9</td>
</tr>
<tr>
<td>3,4-dihydroxy-L-Phe</td>
<td>100</td>
<td>88.2</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
<tr>
<td>8-methyl-L-Tyr</td>
<td>98.3</td>
<td>89.4</td>
<td>n.i.</td>
<td>n.i.</td>
</tr>
</tbody>
</table>

The data are adopted from publication for TyrPT and SirD (3.7 or 3.9 µM, 16 h) (Yu et al. 2015). n.i.: not isolated
Figure 1

**Isoflavone**

![Chemical structure of isoflavone](image)

**Flavanone**

![Chemical structure of flavanone](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>H</td>
<td>H</td>
<td>12.3%</td>
</tr>
<tr>
<td>b</td>
<td>H</td>
<td>H</td>
<td>46.2%</td>
</tr>
<tr>
<td>c</td>
<td>H</td>
<td>H</td>
<td>24.1%</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Figure 2

![Chemical structure of figure 2](image)

Figure 3

![Chemical structure of figure 3](image)

<table>
<thead>
<tr>
<th>Prenylation Position</th>
<th>Dimethylallyl (n=1)</th>
<th>Geranyl (n=2)</th>
<th>Farnesyl (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1=R2=R3=H</td>
<td>51%</td>
<td>15%</td>
<td>5%</td>
</tr>
<tr>
<td>R1=R3=OH, R2=H</td>
<td>72%</td>
<td>46%</td>
<td>18%</td>
</tr>
<tr>
<td>R1=H, R2=R3=OH</td>
<td>60%</td>
<td>29%</td>
<td>10%</td>
</tr>
</tbody>
</table>

37
Figure 4

![Diagram of FgaPT2 and its association with the outer membrane, periplasm, inner membrane, host ribosome, and cytoplasm.]

Figure 5

![Diagram showing the synthesis of brevianamide F and tryprostatin B from FtmPS from A. fumigatus or N. fischeri, CdpC3PT from N. fischeri, and CdpC5PT from A. fumigatus.]

- Reversely C2-prenylated derivative
- Regularly N1-prenylated derivative
- Reversely C1-prenylated syn-cis configured derivative
5. Conclusions and future prospects

In this thesis, a new member of DMATS superfamily was characterized biochemically and the close relationship and complementary substrate promiscuity of tyrosine and tryptophan PTs were reported. Meanwhile, the potential usage of DMATS enzymes was further expand for chemoenzymatic synthesis of prenylated tryptophan, tyrosine and cyclic dipeptide derivatives, in some cases with enhanced catalytic activity by molecular modeling-guided mutagenesis.

The results of this thesis brought the close relationship and complementary substrate promiscuity of tyrosine and tryptophan PTs into light (Figure 5-1). This topic started with the characterization of a new member of DMATS superfamily TyrPT from Aspergillus niger. TyrPT was found to catalyze the O-prenylation of tyrosine as well as C7-prenylation of tryptophan. It was further compared with the known tyrosine O-PT SirD and tryptophan C7-PT 7-DMATS toward tyrosine and tryptophan derivatives. TyrPT exhibited broader substrate spectrum and significantly higher catalytic activity for several substrates. This work provides a new enzyme for production of prenylated derivatives and enhances the relationship of tyrosine O- and tryptophan C7-PTs.

Further study revealed that tryptophan C4- and C7-PTs FgaPT2 and 7-DMATS also accepted tyrosine and its derivatives as substrates and catalyzed a unique C3- and O-prenylation, respectively. A mechanism of FgaPT2 reaction with tyrosine was proposed based on the molecular modeling results with the available crystal structure of FgaPT2. The catalytic efficiency of FgaPT2 toward tyrosine was then improved by molecular modeling-guided site-directed mutagenesis. One mutant FgaPT2_K174F showed practically no activity toward L-tryptophan, whereas the acceptance of L-tyrosine was improved significantly.

Additionally, a tyrosine analog, L-o-tyrosine was also accepted by the tryptophan PTs FgaPT2, 5-DMATS, 6-DMATSs, and 7-DMATS as well as by the tyrosine PT TyrPT, but not by SirD. Another tyrosine analog L-m-tyrosine was only converted by FgaPT2 and 7-DMATS. For both substrates, tryptophan PTs demonstrated higher activities than tyrosine PTs. C5-prenylated o-tyrosine as well as C4- and C6-prenylated m-tyrosine were identified as products of these enzymes.
CONCLUSIONS AND FUTURE PROSPECTS

Figure 5-1. Complementary substrate promiscuity of tryptophan and tyrosine PTs.

Previous reports demonstrated that the tryptophan PTs accepted cyclic dipeptides only at high protein concentrations. Correspondingly, tryptophan was a very poor substrate for cyclic dipeptide PTs. In this thesis, an unnatural cyclic dipeptide cyclo-L-homotryptophan-D-valine with one additional C-atom between the indole and diketopiperazine rings was synthesized and found to be well accepted by three tryptophan PTs FgaPT2, 5-DMATS and 7-DMATS as well as by five cyclic dipeptide PTs BrePT, FtmPT1, CdpC3PT, CdpNPT and AnaPT. Seven prenylated products with one prenyl moiety at each position of the indole nucleus and one diprenylated derivative were isolated from enzyme assays.

Instead of modification of substrate, the acceptance of cyclic dipeptides by tryptophan C4-prenyltransferase FgaPT2 was significantly improved by saturation mutagenesis experiments on the key amino acid R244. Thirteen R244 mutants demonstrated differently increased enzyme activities for tryptophan-containing cyclic dipeptides, with up to 155-fold catalytic efficiency of that of FgaPT2. Furthermore, different preferences for cyclic dipeptides were observed for these mutants. Therefore, thirteen new biocatalysts were created from FgaPT2 for efficient production of C4-prenylated cyclic dipeptides.
CONCLUSIONS AND FUTURE PROSPECTS

In the future, the following works should be continued:

- Further study on single or double mutations of FgaPT2, especially for the key amino acid Y191, L81 and I80, involved in the binding of tryptophan side chain, to expand its aromatic substrate spectrum.
- Mutation study on E298 of 7-DMATS, which is corresponding to R244 of FgaPT2, to testify whether this amino acid residue is important for the acceptance of cyclic dipeptides.
- Collect all available data of mutated DMATS enzymes and try to rational design PTs which could catalyze additional Friedel-Crafts reactions for production of desired products.
6. References


REFERENCES


Ich, Aili Fan, versichere, dass ich meine Dissertation:

„Biochemische Untersuchungen an bakteriellen und pilzlichen Prenyltransferasen“

selbständig ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen bedient habe. Alle vollständig oder sinngemäß übernommenen Zitate sind als solche gekennzeichnet.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den.................................

.......................................................

Aili Fan
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