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Regiospecific Benzylation of Tryptophan and Derivatives Catalyzed by a Fungal Dimethylallyl Transferase

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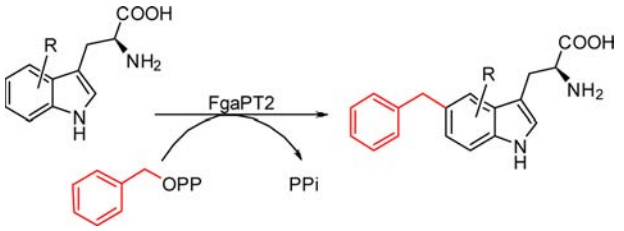
ABSTRACT



A big challenge in organic synthesis is to reach a high regioselectivity. Enzymes catalyze usually highly regiospecific reactions and can function as ideal biocatalysts for such purposes. Some secondary metabolite enzymes can even use distinctly different unnatural substrates and expand therefore their potential usage in chemoenzymatic synthesis. We report here the acceptance of benzyl diphosphate as an alkyl donor by the fungal dimethylallyl transferase FgaPT2 and the regiospecific enzymatic benzylation of tryptophan and several analogues.

Prenylated indole alkaloids such as notoamides, roquefortines, fumitremorgins, and ergot alkaloids are a large group of natural products with diverse chemical structures and strong biological activities.[1] These compounds are usually derived from L-tryptophan or derivatives thereof as backbones and prenyl moieties as modifications.[1d,e] The attachment of prenyl moieties ($n \times$ C5 units) usually increases the lipophilicity of the resulted products and strengthens their interaction with proteins and biomembranes. Prenyl moieties are often critical for the intriguing biological and pharmacological activities of the prenylated compounds.[2] Prenyl transfer reactions, i.e. prenylations, are catalyzed in nature by prenyltransferases, which can be divided into several subgroups.[3] One such subgroup comprises the members of the **dim**ethyl**a**llyl **t**ryptophan **s**ynthase (DMATS) superfamily. They are involved in the biosynthesis of secondary metabolites and mainly catalyze the transfer reactions of dimethylallyl moieties from dimethylallyl diphosphate (DMAPP) onto various positions of the indole ring of diverse indole derivatives including tryptophan, tryptophan-containing cyclic dipeptides, or complex structures.[4] For example, FgaPT2 is involved in the biosynthesis of ergot alkaloids, e.g. fumigaclavines in *Aspergillus fumigatus*, and catalyzes the prenylation of L-tryptophan at position C-4 in the presence of DMAPP (Scheme 1, a).[5] The prenylations catalyzed by the members of the DMATS superfamily are often Friedel–Crafts alkylations. Enzymes, which catalyze C-prenylations for all of the six positions (C-2 to C-7) of the indole ring, have been characterized in recent years.[4d] One common feature of these enzymes is their high flexibility toward their

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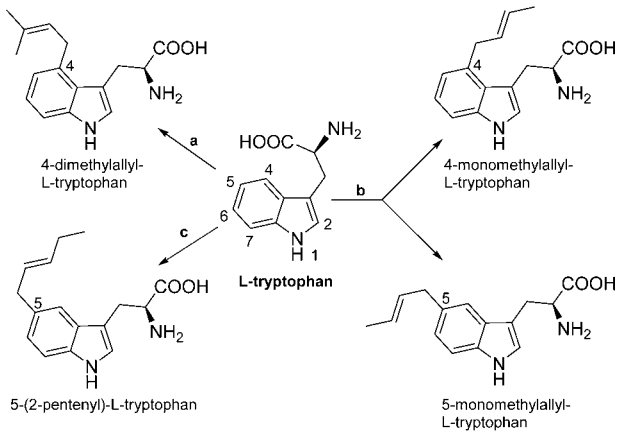
aromatic substrates. They accept not only structures similar to their natural substrates, usually L-tryptophan or tryptophan-containing cyclic dipeptides, but also significantly different structures such as flavonoids or hydroxynaphthalenes as prenyl acceptors.[4d,6] On the other hand, they display a strict substrate specificity toward their prenyl donor and usually only accept DMAPP as a substrate. One enzyme of this superfamily accepts geranyl diphosphate (GPP), but not DMAPP, as a substrate.[7] Acceptance of both DMAPP and GPP has been reported for AnaPT recently.[8] The low flexibility toward alkyl donors strongly prohibited their potential usage for chemoenzymatic synthesis and synthetic biology. In recent years, research on S-adenosyl-L-methionine (SAM) dependent methyl transferases has shown that an array of SAM analogues, with alkenyl, alkinyl, or aryl residues instead of a methyl group, were accepted as unnatural cofactors and alkylation agents.[9] We were intrigued to prove the ability of indole prenyltransferases for using unnatural alkyl donors. Therefore, we modified the DMAPP structure and tested the acceptance of several DMAPP analogues by enzymes of the DMATS superfamily.[10]

Our results with monomethylallyl diphosphate (MAPP, Scheme 1, b) and 2-pentenyl diphosphate (2-pen-PP, Scheme 1, c) showed that these unnatural alkyl donors were well accepted by a number of prenyltransferases of the DMATS superfamily, resulting in the formation of different alkylated derivatives (Scheme 1).[10] These works indicated that the limited flexibility of such enzymes toward their alkyl donors can be overridden in parts and therefore encouraged us to test alkyl donors with a significantly different structure to that of DMAPP. For this purpose, we successfully synthesized benzyl diphosphate from benzyl chloride according to a method described for GPP,[11] with a total conversion of 58% and a purity of over 90%, and used it as an alkyl donor for enzyme assays of nine indole prenyltransferases with their respective natural or reported best accepted aromatic substrates. The substrates used included L-tryptophan (**1a**) for the three L-tryptophan prenyltransferases FgaPT2, 5-DMATS, and 7-DMATS and cyclic dipeptides for six cyclic dipeptide prenyltransferases.[4d] The tested dipetides were (*R*)-benzodiazepindione for AnaPT, (*S*)-benzodiazepindione for CdpNPT, *cyclo*-L-Trp-L-Pro for FtmPT1 and BrePT, *cyclo*-L-Trp-L-Leu for CdpC3PT, and *cyclo*-L-Trp-L-Trp for CTrpPT.[4d]

Scheme 1. Alkylations of L-Tryptophan Catalyzed by FgaPT2



HPLC analysis revealed that benzyl diphosphate was much better accepted by FgaPT2, with a conversion of 58.1% after incubation with 10 μg of enzyme in a 100 μL assay at 37 °C for 16 h, than other enzymes. Product formation was detected for 5-DMATS, 7-DMATS, AnaPT, and CdpNPT with conversion yields between 1% and 3% (data not shown). No product formation was observed for other enzymes under the tested conditions (data not shown). Based on the initial success with **1a**, we tested the acceptance of 25 additional tryptophan derivatives (**2a**–**26a**) with modifications on the side chain or at the indole ring by FgaPT2 in the presence of benzyl diphosphate (Table S1). HPLC analysis showed that at least 16 of these compounds were accepted by FgaPT2 with conversion yields from 0.8% for 5-methyl-DL-tryptophan (**17a**) to 54.4% for L-abrine (**2a**). D-tryptophan (**13a**) was much poorer accepted than its L-form **1a**, with a relative conversion yield of 2.6%. Racemic tryptophan was consumed to a similar conversion yield as pure L- and D-tryptophan together, indicating that D-tryptophan was consumed to a similar degree as in the enzyme assay with pure D-isomer. In the incubation mixtures with other racemic substrates, the conversion yield of each of the enantiomers could not be determined in this study. Therefore, the sum of their conversion yields were estimated from the total amount of substrates. Conversion yields of $>10\%$ were observed for **1a** and eight derivatives (**2a**–**9a**), which were thereby chosen for isolation and structure elucidation (Figure S1 and Tables 1 and S1). Total conversions of 35.5% for α-methyl-DL-tryptophan (**7a**) to 82.5% for **1a** have been even achieved in the enzyme assays for product isolation with 40 μg of protein per 100 μL assay.

HPLC chromatograms of **1a**–**9a** clearly showed one product peak each (Figure S1). **2a**, 4-methyl-DL-tryptophan (**3a**), and L-β-homotryptophan (**4a**) were consumed to a comparable degree as **1a**. Interestingly, among the five tested C5-substituted tryptophan derivatives, only 5-hydroxy-L-tryptophan (**9a**) was well accepted by FgaPT2 in the presence of benzyl diphosphate. In contrast,

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derivatives with substitutions at other positions of the indole ring, as in the cases of 4- (**3a**), 6- (**8a**), and 7-methyl-DL-tryptophan (**6a**) or 6-fluoro-DL-tryptophan (**5a**), were very well consumed. These observations could indicate C-5 of the indole ring as the favorable benzylation position by FgaPT2 in the presence of benzyl diphosphate. This hypothesis would also explain the high conversion of **3a** to **3b**. Interestingly, **5a** was much better accepted than **8a**, which could not be explained by different electron densities on the benzene ring. It could be speculated that the smaller fluorine atom would better fit in the reaction chamber than a methyl group or there are interactions between fluorine and amino acid residues in the reaction chamber.

For confirmation of the benzylation and benzylation position, the enzymatic products of the nine substrates (**1a**–**9a**) were isolated on HPLC and subjected to high resolution MS and NMR analyses. HR-EI-MS revealed that these compounds are 90 Da larger than their respective substrates and confirmed the presence of a benzyl residue in the structures of the enzymatic products. Detailed analysis of NMR spectra of the obtained enzymatic products (see Supporting Information for structure elucidation) confirmed the C5-benzylation in **1b**–**8b** from **1a**–**8a** (Table 1). From the incubation mixture of **6a**, an additional product **6c** with a C6-benzyl moiety was also identified. It seems that the methyl group at C7 of the indole ring reduced the regioselectivity of the FgaPT2 reaction. For 5-hydroxy-DL-tryptophan (**9a**), only the C6-benzylated derivative **9c** was isolated, indicating a complete shift in regioselectivity (Table 1). This proved that FgaPT2 catalyzed in most cases the transfer reaction of a benzyl moiety from benzyl diphosphate to C-5 of tryptophan and derivatives. If this position is blocked, the benzylation then took place at C-6. With its natural substrate DMAPP as the prenyl donor, shifting of the alkylation position was also observed, when the original prenylation position C-4 was blocked, as in the case of **3a**.[12]

As aforementioned, the prenyltransferase FgaPT2 catalyzes in nature the prenylation of L-tryptophan at position C-4.[5b] It has been demonstrated that, in the presence of its natural prenyl donor DMAPP, the overproduced and purified FgaPT2 accepted a large number of simple indole derivatives and tryptophan-containing cyclic dipeptides and catalyzed the regiospecific prenylation at C-4 of the indole rings.[13] In the presence of two unnatural DMAPP analogues MAPP and 2-pen-PP, FgaPT2 also catalyzed the alkylation of L-tryptophan. In the case of the larger alkyl donor 2-pen-PP, the alkylation position was shifted from C-4 to C-5 (Scheme 1). For the smaller MAPP, both C4- and C5-alkylations were observed (Scheme 1). In this study, we reported the acceptance of benzyl diphosphate by FgaPT2 and the highly regiospecific alkylation of tryptophan derivatives. As observed for 2-pen-PP,[10a] the alkylation position was shifted from C-4 to C-5. It seems

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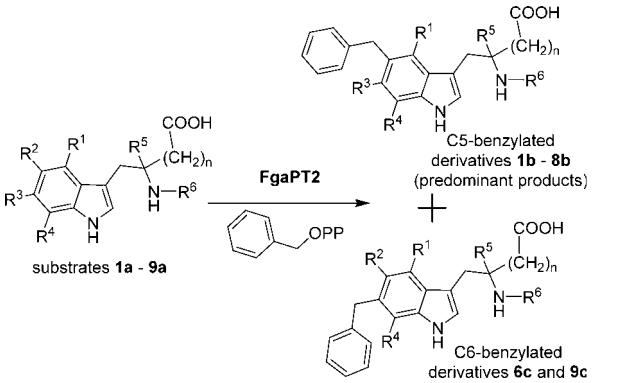
Table 1. Selected Substrates and Their Benzylated Products[a]



	yield	R^1	R^2	R^3	R^4	R^5	R^6	n	C5-benzylated	C6-benzylated
1a (L)	100.0	H	H	H	H	H	H	0	**1b**	–
2a (L)	93.6	H	H	H	H	H	**CH_3**	0	**2b**	–
3a (DL)	88.0	**CH_3**	H	H	H	H	H	0	**3b**	–
4a (L)	85.9	H	H	H	H	H	H	1	**4b**	–
5a (DL)	61.6	H	H	**F**	H	H	H	0	**5b**	–
6a (DL)	35.3	H	H	H	**CH_3**	H	H	0	**6b**	**6c**
7a (DL)	33.9	H	H	H	H	**CH_3**	H	0	**7b**	–
8a (DL)	21.3	H	H	**CH_3**	H	H	H	0	**8b**	–
9a (L)	31.2	H	**OH**	H	H	H	H	0	–	**9c**

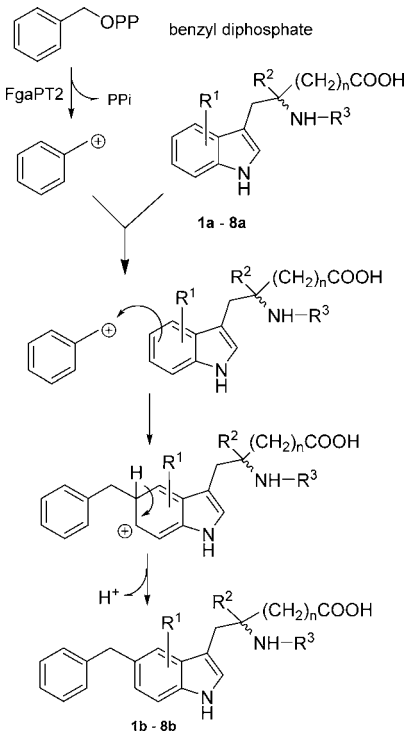
[a] Relative yields are given in %; A total conversion yield of 58% was dermined for **1a** and defined as 100%. 100 μL assays contained 1 mM aromatic substrate, 2 mM benzyl diphosphate, 10 mM $CaCl_2$, 10 μg of FgaPT2, and 50 mM Tris-HCl (pH 7.5) and were incubated at 37 °C for 16 h. Configurations of the substrates are given in parentheses. – = Not detected.

that C-5 can be reached easier by the benzyl residue than C-4. Substances with substitution at this position were very poor substrates for FgaPT2 in the presence of benzyl diphosphate. One exception was 5-hydroxy-L-tryptophan, which was converted to a C6-benzylated derivative.

Our results reported in this study provide experimental evidence that some prenyltransferases, at least FgaPT2 and several other enzymes including 5-DMATS, 7-DMATS, AnaPT, and CdpNPT, can also utilize alkyl donors with strongly modified structures from its natural substrate DMAPP for Friedel–Crafts alkylation. To the best of our knowledge, neither an acceptance of benzyl diphosphate by a prenyltransferase as an alkyl donor nor a one-step regioselective benzylation of tryptophan and derivatives by secondary metabolite enzymes has been reported previously.

Based on the results obtained from crystal structures of three indole prenyltransferases and site-directed mutagenesis experiments,[14] it was proposed that the prenyl transfer reactions catalyzed by prenyltransferases in the presence of DMAPP begin with the removal of pyrophosphate from enzyme-bound DMAPP and the formation of a positively charged dimethylallyl ion. The involvement of a cation during the prenylation was confirmed by a positional

Scheme 2. Proposed Reaction Mechanism for Benzylation of L-Tryptophan and Derivatives (see Table 1 for detailed structures)



isotope exchange in isotopically labeled DMAPP.[15] The electron-rich aromatic ring would attack the C-1 or C-3 of this cation, resulting in the formation of different intermediates, which undergo various fates to enzyme-specific products.

In analogy to prenylations, a reaction mechanism can be postulated for the benzylation of tryptophan and derivatives by FgaPT2 in the presence of benzyl diphosphate (Scheme 2). It is plausible that the pyrophosphate of benzyl diphosphate is first removed by interaction with several basic amino acids.[14a] The resulted benzyl cation will be stabilized by interaction with tyrosine residues in the active center and by its mesomeric system. It seems that benzyl diphosphate and tryptophan derivatives are placed in positions relative to each other so that an attack of the indole residue with its C-5 becomes much easier than with its C-4 as in the case of DMAPP. Removal of a proton from C-5 rearomatizes the indole ring and results in the formation of C5-benzylated products. In the case of 5-hydroxy-L-tryptophan (**9a**), the C5 is blocked and C-6 is strongly activated by the hydroxyl group so that the attack took place from C-6 of the indole ring.

Comparison of the biochemical properties of the three dimethylallyltryptophan synthases FgaPT2, 5-DMATS, and 7-DMATS revealed different behaviors toward their substrates. 7-DMATS showed a much higher flexibility than FgaPT2 and 5-DMATS toward aromatic substrates. This enzyme accepted indole derivatives, hydroxynaphthalenes, and flavonoids as substrates.[6,16] On the other hand, the two DMAPP analogues MAPP and 2-pen-PP were very well accepted by FgaPT2 and 5-DMATS, but hardly by 7-DMATS.[10a] In this study, we showed that FgaPT2 can utilize benzyl diphosphate much better than 5- and 7-DMATS. These distinctive features increase their importance as biocatalysts in chemoenzymatic synthesis and expand their potential usage for Friedel–Crafts alkylations.

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Supporting Information Available. Experimental procedures, HPLC chromatograms, kinetic parameters, HR-EI-MS and NMR data. This material is available free of charge via Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.