Tetrahedron Letters 53 (2012) 6861–6864

Contents lists available at SciVerse ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Friedel–Crafts alkylation on indolocarbazoles catalyzed by two dimethylallyltryptophan synthases from *Aspergillus*

Xia Yu [a], Aigang Yang [b], Wenhan Lin [b], Shu-Ming Li [a,c,*]

[a] *Institut für Pharmazeutische Biologie und Biotechnologie, Philipps-Universität Marburg, Marburg 35037, Germany*
[b] *State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Beijing 100191, China*
[c] *Zentrum für Synthetische Mikrobiologie, Philipps-Universität Marburg, Marburg 35032, Germany*

ARTICLE INFO

Article history:
Received 24 August 2012
Revised 5 October 2012
Accepted 9 October 2012
Available online 16 October 2012

Keywords:
Aspergillus
Enzyme catalysis
Indolocarbazoles
Prenylation

ABSTRACT

Prenylated indolocarbazoles have been reported neither from natural sources, nor by chemical synthetic approaches. In this Letter, we report a regiospecific prenylation of indolocarbazoles at the *para*-position of the indole N-atom by two recombinant enzymes from the dimethylallyltryptophan synthase (DMATS) superfamily, that is, 5-DMATS from *Aspergillus clavatus* and FgaPT2 from *Aspergillus fumigatus*.

Indolocarbazoles are a class of natural products with well known remarkable biological activities, especially their inhibitory effects against protein kinases in various organisms. Several of these compounds have already entered clinical trials for treatment of cancer and other diseases.[1,2] To overcome the activity promiscuity of naturally occurring indolocarbazoles such as staurosporine and K252d (Fig. 1) toward kinases,[3–5] numerous chemical strategies have been developed for the synthesis of their mimetics to provide specific kinase inhibitors.[6,7] Significant progress has also been achieved in the biosynthetic studies of indolocarbazoles. Identification and proof of biosynthetic genes for indolocarbazoles provided additional possibilities to create novel derivatives by combinatorial biosynthesis.[2,8] However, prenylated indolocarbazoles have been reported, neither from natural sources, nor from chemical synthetic approaches, although diverse prenylated carbazoles have been isolated from different sources.[6]

Prenylated derivatives are formed in the nature by transfer of $n \times$ C5 (n = 1, 2, 3, 4, or larger) units from their active forms, usually as diphosphate esters, to diverse acceptors. The responsible enzymes for the transfer reactions are different prenyltransferases, which are also successfully used as biocatalysts for the synthesis of prenylated compounds.[9–12] A large group of prenyltransferases belong to the dimethylallyltryptophan synthase (DMATS) superfamily. The members of this superfamily are involved in the biosynthesis of fungal secondary metabolites and catalyzed mainly the prenylation of diverse indole derivatives.[13] For example, FgaPT2 from *Aspergillus fumigatus* and 5-DMATS from *Aspergillus clavatus* catalyze the prenylation of L-tryptophan at C-4 and C-5, respectively, and therefore function as dimethylallyltryptophan synthases (Scheme 1).[14,15] It has also been demonstrated that some members of the DMATS superfamily catalyze even the prenylation of hydroxynaphthalenes and flavonoids.[16,17] These results encouraged us to test the acceptance of indolocarbazoles by members of the DMATS superfamily.

For this purpose, we synthesized four indolocarbazoles **1a**–**4a** (Scheme 2). Treatment of indole-3-acetamide with methyl indolyl-3-glyoxylate in the presence of KOBu^t afforded the intermediate arcyriarubin A,[18] which was converted to *N*-methylarcyriarubin A by treatment with methyl iodide.[19] Arcyriaflavin A (**1a**) and its N6-methylated derivative **4a** were obtained after oxidative

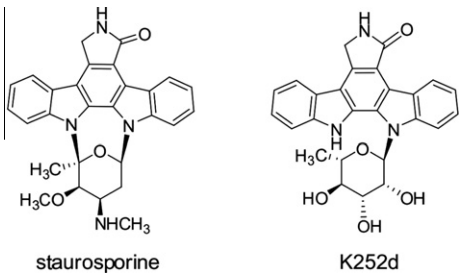

Figure 1. Structures of staurosporine and K252d.

* Corresponding author. Tel.: +49 6421 2822461; fax: +49 6421 2826678.
E-mail address: shuming.li@staff.uni-marburg.de (S.-M. Li).

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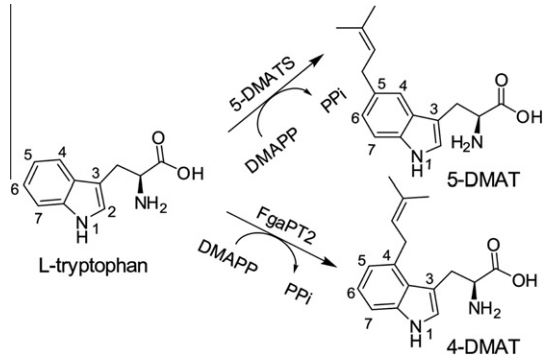
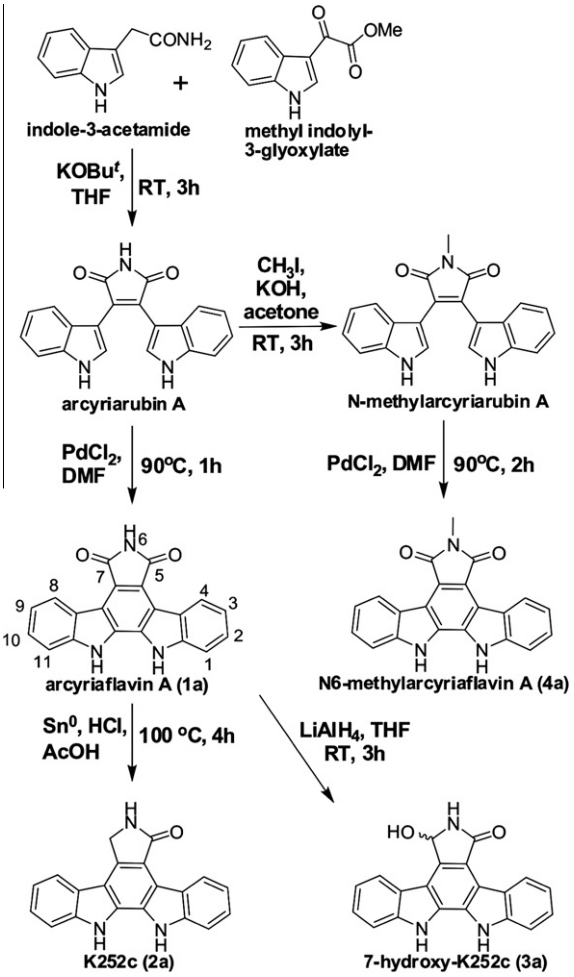
http://dx.doi.org/10.1016/j.tetlet.2012.10.039



Scheme 1. Prenyltransfer reactions of 5-DMATS and FgaPT2 for their natural substrate L-tryptophan.

cyclization of the two bisindolylmaleimides arcyriarubin A and *N*-methylarcyriarubin A, respectively.[20,21] Reduction of **1a** with tin metal in AcOH/HCl and $LiAlH_4$ in THF resulted in the formation of K252c (**2a**)[21] and 7-hydroxy-K252c (**3a**),[22] respectively. The identities of the obtained compounds were confirmed by NMR and MS analyses. Unexpectedly, two product peaks **3a** and **3a*** were observed in the HPLC chromatogram of **3a** (Fig. 2). Reanalysis of the isolated single peak **3a** on HPLC revealed still the presence of both peaks. Furthermore, the ratios of **3a*** to **3a** were found to be nearly identical in all of the incubation mixtures with **3a**. However,



Scheme 2. Synthesis of bisindolylmaleimides and indolocarbazoles as substrates.

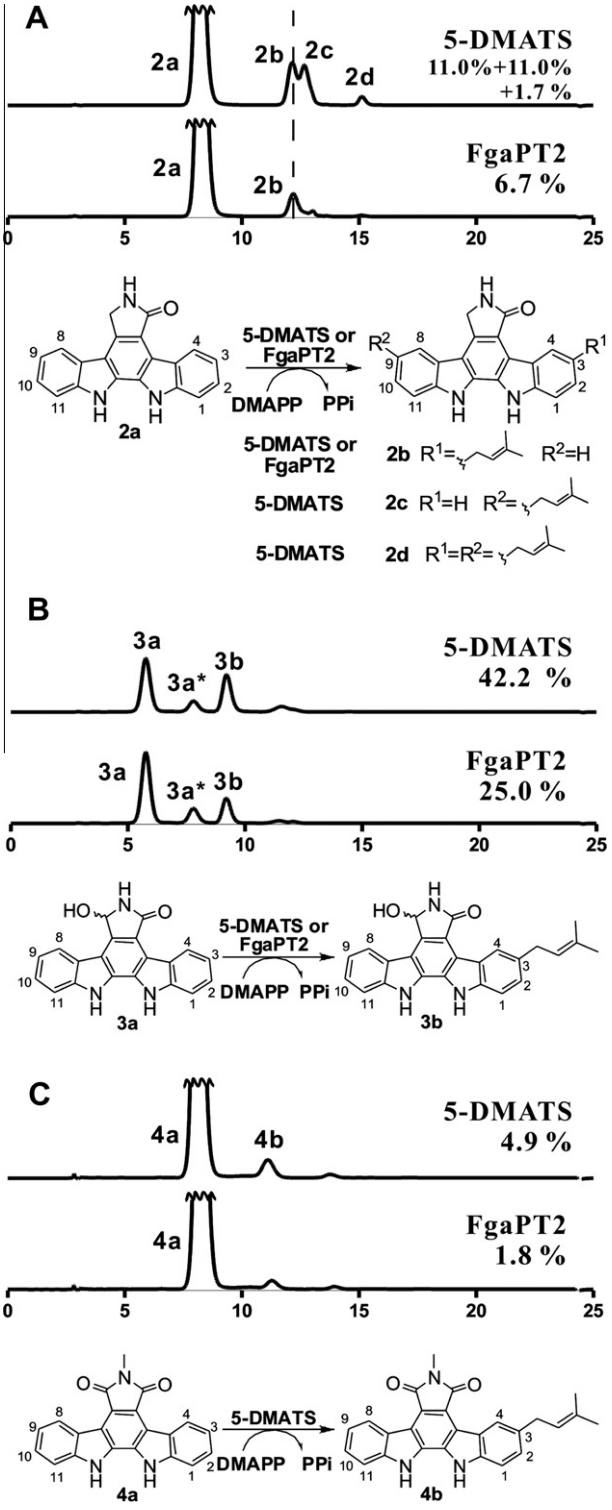


Figure 2. HPLC chromatograms and prenyl transfer reactions onto indolocarbazoles catalyzed by 5-DMATS and FgaPT2. The reaction mixtures (100 μl) containing 0.2 μg μl^{-1} of purified recombinant protein, 0.5 mM of aromatic substrate, and DMAPP in 50 mM Tris–HCl (pH 7.5) were incubated at 37 °C for 3 h. Detection was carried out on a Photodiode array detector and illustrated for absorption at 290 nm.

a ^{1}H NMR spectrum in DMSO-d_6 showed only signals for the structure of **3a**. It seems therefore that **3a*** is an isomer of **3a**, for example, a keto–enol tautomer at C-5 and N-6.

The two bisindolylmaleimides arcyriarubin A and *N*-methylarcyriarubin A, the four indolocarbazoles **1a**–**4a**, and two indolocarbazole glycosides staurosporine and K252d, which had been isolated from

Streptomyces nitrosporeus CQT14-24, were then incubated with nine prenyltransferases from the DMATS superfamily in the presence of dimethylallyl diphosphate (DMAPP). The tested enzymes included five cyclic dipeptide prenyltransferases AnaPT, BrePT, CdpC3PT, CdpNPT, and FtmPT1 with prenylation positions at C-2 or C-3 of the indole ring,[23–27] three dimethylallyltryptophan synthases FgaPT2, 5-DMATS, and 7-DMATS with prenylation positions at C-4, C-5, and C-7,[14,15,28] respectively. One tyrosine *O*-prenyltransferase SirD[29] was also tested. HPLC analysis showed that 5-DMATS from *Aspergillus clavatus* and FgaPT2 from *Aspergillus fumigatus* displayed more substrate flexibilities toward the tested substances than other enzymes (data not shown) and were studied in detail.

HPLC analysis of incubation mixtures with a 20 μg of 5-DMATS or FgaPT2 in 100 μl assay indicated that **1a** was poor substrate for both enzymes. 5-DMATS accepted **1a** only with a total conversion yield of 0.3%, while no product peak was detected in its incubation mixture with FgaPT2. Other three indolocarbazoles (**2a**–**4a**) were clearly accepted by both 5-DMATS and FgaPT2 (Fig. 2). Product formation was only detected in the incubation mixtures with active, but not in those with heat-inactivated proteins (by boiling for 20 min, data not shown). This demonstrated the importance of the oxidation grade at position C-7. Hydroxylation at this position seems better for acceptance by 5-DMATS than with a keto group. Detailed inspection of the HPLC chromatograms with **2a**–**4a** revealed that FgaPT2 showed generally a lower activity than 5-DMATS, proving again that different DMATS enzymes display different preference toward aromatic substrates.[17] Furthermore, more than one product peaks were detected in the reaction mixtures and the main product of both enzymes for a given substrate was proven to be identical (see below). For example, **2a** was converted by 5-DMATS into **2b**, **2c**, and **2d** with yields of 11, 11, and 1.7 %, respectively. **2a** was converted by FgaPT2 mainly into **2b** with a yield of 6.7 % (Fig. 2A). **3a**/**3a*** were converted into **3b** by 5-DMATS and FgaPT2 with conversion yields of 42 and 25%, respectively. The non-bridged intermediates of indolocarbazoles, that is, arcyriarubin A and *N*-methylarcyriarubin A, were not accepted by 5-DMATS and FgaPT2, indicating the importance of the presence of the indolocarbazole skeleton. Glycosides of indolocarbazoles, that is, staurosporine or K252d (Fig. 1), were also not prenylation substrates for the enzymes of the DMATS superfamily (data not shown).

For structure elucidation, three enzyme products **2b**, **2c**, and **2d** were isolated from the incubation mixture of 5-DMATS with **2a** and one each, that is, **3b** and **4b**, from those with **3a** and **4a**, respectively. **2b** and **3b** were also isolated from the reaction mixtures of FgaPT2 with **2a** and **3a**. All isolated enzyme products were subjected to MS and NMR analyses.

In the HPLC chromatograms of the incubation mixtures with **3a**, a minor product peak eluted after **3b** was also observed (Fig. 2B), which could be a prenylation product of **3a*** or formed by tautomerism of **3b**. Due to the low quantity, this minor product could not be isolated and identified. For the same reason, no enzyme product of **4a** with FgaPT2 and **1a** with 5-DMATS was isolated.

HR-EI-MS confirmed the monoprenylation in **2b**, **2c**, **3b**, and **4b** and diprenylation in **2d**, by detection of the molecular masses that are 68 and 136 Da larger than those of the respective substrate (Supplementary Table S1). The main enzyme products of FgaPT2 with **2a** and **3a** had identical ^{1}H NMR spectra as those of **2b** and **3b** from the 5-DMATS assays, respectively, proving the same structure of the enzyme products. The ^{1}H NMR signals at δ_H 3.48–3.52 (d, 2H-1′ or 2H-1″), 5.40–5.44 (t sep, H-2′ or H-2″), 1.77–1.79 (d, 3H-4′ or 3H-4″), and 1.73–1.75 ppm (d, 3H-5′ or 3H-5″) in the spectra of **2b**, **2c**, **2d, 3b**, and **4b** (Supplementary Figs. S1–S5, Table S2) revealed clearly the attachment of one regular dimethylallyl moiety to a C-atom.[17,30]

In the ^{1}H NMR spectra of the substrates **2a**–**4a** (Supplementary Figs. S6–S8), the aromatic signals appeared as two identical (**4a**) or different sets of four vicinal coupling protons (**2a** and **3a**). Each set contains two doublets and two triplets with coupling constants in the range of 7–9 Hz. In comparison, one set of signals in the ^{1}H NMR spectra of their enzyme products **2b**, **2c**, **3b**, and **4b** represent merely three protons. Two of these protons couple with each other with coupling constants of 7–9 Hz and the third one appears as a singlet or doublet with a small coupling constant of less than 2 Hz. These changes indicated that the prenylation had taken place at position C-2, C-3, C-9, or C-10. Similar phenomenon was also observed in both sets of aromatic signals in the ^{1}H NMR spectrum of **2d**, suggesting that one prenylation took place at C-2 or C-3 and the other at C-9 or C-10. Comparing the signals in the spectra of **2b**, **3b**, and **4b** with those of the respective substrate (Supplementary Figs. S6–S8) revealed that the H-4 was changed from a doublet with coupling constants of 7-9 Hz to another doublet with coupling constants small than 2 Hz, which was found in the low field in ^{1}H NMR spectra at approximately δ_H 9 ppm due to a characteristic deshielding effect from the lactam carbonyl,[31] confirming the prenylation at C-3 of **2b**, **3b**, and **4b**. In the ^{1}H NMR spectrum of **2c**, the signal of H-8 rather than that of H-4 was changed from a doublet to a singlet (Figure S6),[31] confirming that the prenylation had taken place at C-9. The structure of **2d** was assigned to a product with two prenyl moieties at C-3 and C-9, since both H-4 and H-8 were altered from doublets to singlets (Supplementary Fig. S6). This proved that both DMATS enzymes catalyzed the regiospecific C-prenyltion on the indolocarbazole system, that is, the *para*-position to the indole N-atom (C3, C9, or both) and function therefore as catalysts for Friedel–Crafts alkylations. A Friedel–Crafts alkylation catalyzed by strong Lewis acids would involve an allyl cation. This is also the case for the enzyme-catalyzed Friedel–Crafts alkylation described in this study. The formation of a dimethylallyl cation in an enzyme-catalyzed prenyl transfer reaction would be facilitated by interactions of several basic amino acid residues of the enzyme with pyrophosphate group of DMAPP.[32,33]

To elucidate the behavior of 5-DMATS and FgaPT2 toward indolocarbazoles, kinetic parameters were determined for the best accepted substrate **3a** with both enzymes by Hanes–Woolf and Eadia–Hofstee plots. Michaelis–Menten constants (K_M) were calculated to be at 87 and 136 μM for 5-DMATS and FgaPT2, respectively, while turnover numbers (k_{cat}) were found at 6.8 and 7.3 min^{-1}. The catalytic efficiency (k_{cat}/K_M) of 5-DMATS toward **3a** was 1302 s^{-1} M^{-1}, that is, 5.0 % of that of its best substrate L-tryptophan.[14] Similarly, A k_{cat}/K_M value of 891 s^{-1} M^{-1} was calculated for FgaPT2 toward **3a**, which is 3.0 % of that of L-tryptophan.[15] These data provided evidence that dimethylallyltryptophan synthases could also be used for the production of C-prenylated indolocarbazoles.

In conclusion, the present work demonstrated the acceptance of indolocarbazoles by fungal dimethylallyltryptophan synthases of the DMATS superfamily, which expands the potential usage of these enzymes in the structural modifications. To the best of our knowledge, this is the first report on the (chemoenzymatic) synthesis of prenylated indolocarbazoles.

Acknowledgments

This work was supported within the LOEWE program of the State of Hessen (SynMikro to S.-M. Li). Xia Yu is a recipient of a fellowship from China Scholarship Council. We thank Dr. Ortmann and Laufenberg for taking NMR and mass spectra.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2012.10.039.

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