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Prenylation of a Nonaromatic Carbon of Indolylbutenone by a Fungal Indole Prenyltransferase

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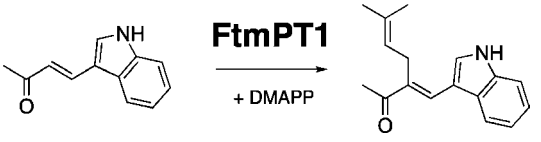
ABSTRACT



FtmPT1 from *Aspergillus fumigatus* is a fungal indole prenyltransferase (PT) that normally catalyzes the regiospecific prenylation of brevianamide F (cyclo-L-Trp-L-Pro) at the C-2 position of the indole ring with dimethylallyl diphosphate (DMAPP). Interestingly, FtmPT1 exhibited remarkable substrate tolerance and accepted (*E*)-4-(1*H*-indol-3-yl)but-3-en-2-one (1) as a substrate to produce an unnatural novel α-prenylindolylbutenone (1a). This is the first demonstration of the prenylation of a nonaromatic carbon of the acceptor substrate by a fungal indole PT.

The fungal indole prenyltransferases (PTs) catalyze the prenyl-transfer reaction of the indole core of tryptophan and its derivatives with dimethylallyl diphosphate (DMAPP) to generate the structurally divergent and biologically active prenyl indole alkaloids, such as the ergot and terrequinone alkaloids.[1,2] The enzyme reaction consists of three steps: (i) ionization of DMAPP to form the dimethylallyl carbocation, (ii) nucleophilic attack from the aromatic ring onto either the primary center (the "regular-type" prenylation) or the tertiary center (the "reverse-type" prenylation) of the dimethyl carbocation, and (iii) rearomatization of the indole to produce the final product. For example, FgaPT2 from *Aspergillus fumigatus* catalyzes the "regular-type" prenylation of L-tryptophan at the C-4 position of the indole ring to produce the biosynthetic precursor of fumigaclavine C.[1,3] On the other hand, FgaPT1 from *A. fumigatus* catalyzes the "reverse-type" prenylation of fumigaclavine A at the C-2 position of the indole ring to produce the biosynthetic precursor of fumigaclavine.[1,4] The varying substrate specificities and regiospecificities of the prenylation reactions thus contribute to the structural diversity of the prenyl indole alkaloids.

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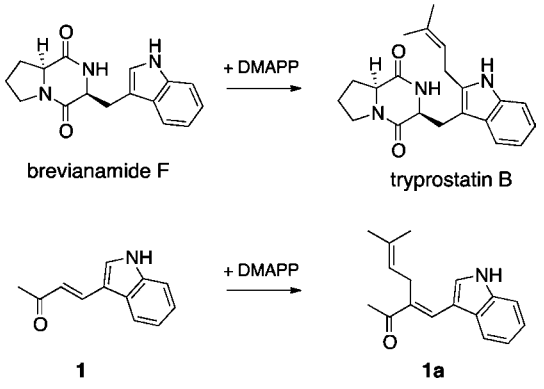
Fungal indole PTs exhibit remarkable substrate tolerance toward indole-containing substrates and efficiently catalyze prenyl-transfer reactions with different regiospecificities. They accept various simple tryptophan derivatives and tryptophan-derived cyclic dipeptides as prenyl acceptor substrates and catalyze diverse prenyl transfer reactions at different positions of the indole core.[1] Furthermore, recent studies demonstrated that the enzymes accept not only the indole-containing substrates but also other aromatic compounds such as tyrosine, hydroxynaphthalenes, and flavonoids to yield prenylated products in regiospecific manners.[5–8] The catalytic promiscuity and versatility of the enzymes thus make fungal indole PTs an excellent platform for further production of unnatural products. Here we report another example of a fungal indole PT, FtmPT1 from *A. fumigatus*, which normally catalyzes the "regular-type" prenylation of brevianamide F (cyclo-L-Trp-L-Pro), at the C-2 position of the indole ring with DMAPP, to produce tryprostatin B.[9] Interestingly, *A. fumigatus* FtmPT1 accepted (*E*)-4-(1*H*-indol-3-yl)but-3-en-2-one (**1**) as a substrate to produce an unnatural novel α-prenylindolylbutenone (**1a**) (Scheme 1). This is the first demonstration of prenylation of a nonaromatic carbon of an acceptor substrate by a fungal indole PT enzyme.

Scheme 1. Enzymatic Prenylation of Brevianamide F and Indolylbutenone (**1**) by *A. fumigatus* FtmPT1



The recombinant *A. fumigatus* FtmPT1 was expressed in *Escherichia coli* as a His_6-tagged protein at the C-terminus, purified by Ni-chelate affinity column chromatography, and used for the enzyme reactions. In addition to **1**, 4-hydroxybenzalacetone (**2**), benzalacetone (**3**), 3-indoleacrylic acid (**4**), *p*-coumaric acid (**5**), cinnamic acid (**6**), and 4-(1*H*-indol-3-yl)butan-2-one (**7**) were tested as prenyl acceptor substrates (Figure 1) in the presence of DMAPP as a prenyl donor substrate. Compound **1** and DMAPP

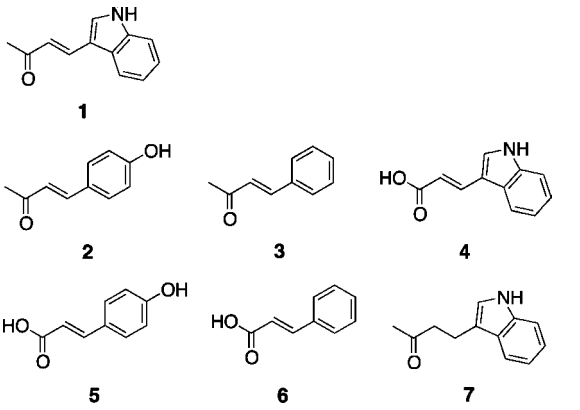


Figure 1. Structures of indolylbutenone (**1**) and related aromatic compounds (**2**–**7**) investigated in this study.

were chemically synthesized according to the published methods.[10,11]

The LC-ESIMS analyses of the enzyme reaction products revealed that *A. fumigatus* FtmPT1 accepted **1** as a substrate under the standard assay conditions and afforded the novel compound **1a** as the almost major product (46% yield), which was not detected in control experiments performed with a boiled enzyme (Figure 2). The product **1a** gave a parent ions peak $[M + H]^+$ at *m*/*z* 254, indicating the formation of a monoprenylated product. A comparison of the ^1H NMR spectrum of **1a** and **1** revealed the appearance of one olefinic proton signal at δ 4.96 (1H, brt, J = 6.0 Hz), one methylene proton signal at δ 3.25 (2H, *d*, J = 6.0 Hz), and two methyl proton signals at δ 1.74 (3H, s) and δ 1.66 (3H, s).[12] On the other hand, the olefinic signal corresponding to the α-proton of the α,β-unsaturated carbonyl group disappeared. Accordingly, a lower field β-proton showed a singlet signal at δ 7.92 (1H, s). Furthermore, the ^{13}C NMR and heteronuclear correlation spectroscopy (HMQC and HMBC) of **1a** revealed 17 carbon signals. The key correlations were from the β-proton at δ 7.92 to a methylene carbon signal at δ 26.1, from a carbonyl carbon signal at δ 198.6 to an olefinic carbon signal for the indole core at δ 111.2, and from the methylene proton signal at δ 3.25 to the carbonyl carbon signal at δ 198.6, suggesting the prenylation of **1** at the α-position of the α,β-unsaturated carbonyl group. In addition, the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum indicated that the β-olefinic proton signal at δ 7.92 correlates with an α-methyl proton signal at

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(12) Product **1a**: LC-ESIMS *m*/*z* 254 $[M + H]^+$; UV λ_{max} 221, 275, 346 nm; ^1H NMR (500 MHz, DMSO-d_6) δ 11.81 (1H, s), 7.92 (1H, s), 7.90 (1H, d, J = 8.0 Hz), 7.61 (1H, d, J = 3.0 Hz), 7.47 (1H, d, J = 8.0 Hz), 7.21 (1H, t, J = 8.0 Hz), 7.16 (1H, t, J = 8.0 Hz), 4.96 (1H, brt, J = 6.0 Hz), 3.25 (2H, d, J = 6.0 Hz), 2.46 (3H, s), 1.74 (3H, s), 1.66 (3H, s); ^{13}C NMR (125 MHz, DMSO-d_6) δ 198.6, 136.2, 135.3, 132.8, 132.3, 128.0, 127.9, 122.9, 122.3, 120.7, 118.9, 112.5, 111.2, 26.2, 26.1, 26.0, 18.6; HRMS (ESITOF) found for $[C_{17}H_{19}NO]^+$ 254.1543, calcd 254.1539.

δ 2.46 (3H, s), and a proton signal of the indole moiety at δ 7.61 (1H, d, J = 3.0 Hz) correlates with the methylene proton signal at δ 3.25. The structure of **1a** was thus unambiguously determined to be a novel prenylindolylbutenone, (*E*)-3-((1*H*-indol-3-yl)methylene)-6-methylhept-5-en-2-one (Scheme 1).[12] Interestingly, the enzyme reaction did not afford any products resulting from the prenylation of the indole moiety. The enzyme activity for the prenylation of **1** was maximal at pH 7.0, within the range of pH 5.5−8.5. The steady-state enzyme kinetics analysis revealed K_M = 8.8 × 10^{-1} mM and k_{cat} = 4.0 × 10^{-1} s^{-1} for **1**, with respect to the formation of **1a**. Thus, *A. fumigatus* FtmPT1 exhibited a 3.6-fold increase and 192-fold decrease in the k_{cat}/K_M values for the prenylation of L-tryptophan and the brevianamide F activities, respectively.[9,13]

In contrast, *A. fumigatus* FtmPT1 did not accept **2**−**6** as prenyl acceptors, and no products were detected in those enzyme reaction mixtures. These observations suggest that the presence of both the α,β-unsaturated ketone and the indole ring is important, but a carboxyl group is inhibitory for substrate binding at the active-site of the enzyme. In the case of L-tryptophan, substrate binding would be facilitated by the presence of the α-amino group, which leads to the "reverse-type" prenylation at the N-1 position of the indole ring, although the product yield was significantly decrease compared with that of brevianamide F.[13] As discussed below, in a conjugated system of the α,β-unsaturated ketone and the indole ring, the indole amine may relay the pair of electrons all the way to the carbonyl group to form an enol conformer for the formation of the unnatural novel α-prenylindolylbutenone. On the other hand, FtmPT1 accepted the α,β-saturated analogue **7** as a substrate to catalyze the "reverse-type" prenylation at the C-2 position of the indole ring.[14]

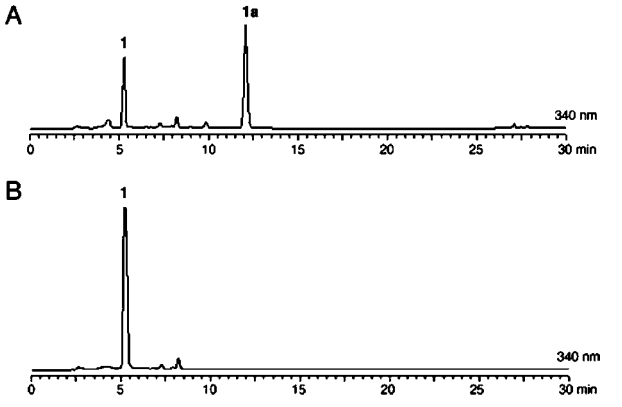
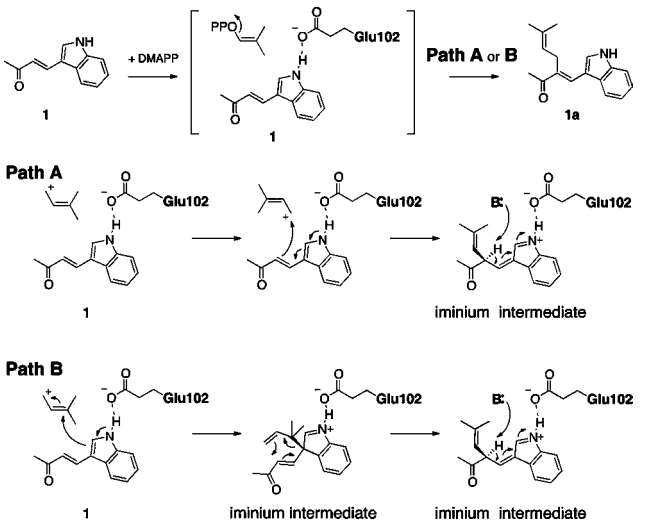


Figure 2. HPLC elution profiles of the enzyme reaction products from indolylbutenone (**1**) and DMAPP by (A) *A. fumigatus* FtmPT1, and (B) boiled enzyme.

For the enzymatic prenylation of brevianamide F (the normal substrate) by *A. fumigatus* FtmPT1 (Scheme 1), two distinct catalytic mechanisms have been proposed. First, Jost et al. proposed that FtmPT1 catalyzes the "regular-type" prenylation (attack at the primary center of the dimethylallyl carbocation), at the C-2 position of the indole ring of brevianamide F, to produce tryprostatin B.[15] On the other hand, Luk et al. recently proposed that FtmPT1 initially catalyzes the "reverse-type" prenylation (attack at the tertiary center of the dimethylallyl carbocation) at the C-3 position of the indole ring, followed by subsequent rearrangement and rearomatization to lead to the production of tryprostatin B.[16]

Scheme 2. Proposed Mechanisms for the Formation of the Prenylindolylbutenone (**1a**) by *A. fumigatus* FtmPT1



In a similar manner, we propose that two distinct pathways are possible for the formation of the unnatural novel α-prenylindolylbutenone by *A. fumigatus* FtmPT1 (Scheme 2). One possibility is the direct "regular-type" prenylation at the α-position of the α,β-unsaturated carbonyl group of **1** to generate an iminium ion intermediate. In this case, deprotonation of the indole amine may relay the pair of electrons all the way to the carbonyl group to form an enol conformer; the deprotonation of the enol by an adjacent base would then lead to the formation of the α-prenyl iminium intermediate. Finally, deprotonation by an unidentified base restores the aromaticity to yield **1a**. The second pathway involves a two-step prenylation via an initial "reverse-type" prenylation at the C-3 position of the indole ring, and a subsequent rearrangement to generate

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(14) 4-(2-(2-Methylbut-3-en-2-yl)-1*H*-indol-3-yl)butan-2-one: LC-ESIMS *m*/*z* 256 $[M + H]^+$; UV λ_{max} 226, 283 nm; ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.42 (1H, s), 7.38 (1H, d, J = 8.0 Hz), 7.29 (1H, d, J = 8.0 Hz), 7.00 (1H, t, J = 8.0 Hz), 6.93 (1H, t, J = 8.0 Hz), 6.13 (1H, dd, J = 17.3, 10.8 Hz), 5.04 (1H, d, J = 10.8 Hz), 5.02 (1H, d, J = 17.3 Hz), 2.88 (2H, t, J = 7.4 Hz), 2.63 (2H, t, J = 7.4 Hz), 2.09 (3H, s), 1.46 (6H, s); HRMS (ESITOF) found for $[C_{17}H_{21}NO]^+$ 256.1692, calcd 256.1696.

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an iminium ion intermediate. Molecular modeling studies predicted that a prenylation at the C-3 position of the indole ring would allow the terminal olefinic carbon of the dimethylallyl moiety to reside within a reasonable distance from the α-carbon of the α,β-unsaturated carbonyl group, allowing for the rearrangement reaction. However, as described above, the enzyme reaction did not afford any products resulting from the prenylation of the indole moiety of **1**. On the other hand, interestingly, when the α,β-saturated analogue **7** was tested as a substrate, *A. fumigatus* FtmPT1 catalyzed the "reverse-type" prenylation at the C-2 position of the indole ring. This may suggest another possibility of a rearrangement reaction, leading to the formation of α-prenylindolylbutenone.

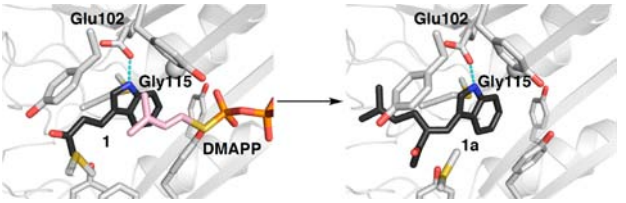

Figure 3. Three-dimensional models of indolylbutenone (**1**) and α-prenylindolylbutenone (**1a**) docked into the active-site cavity of *A. fumigatus* FtmPT1.

The model also suggested that, during the enzyme reaction, the catalytic residue Glu102[15] of *A. fumigatus* FtmPT1 plays important roles in facilitating the nucleophilic attack at the dimethyl carbocation, by either deprotonation or stabilization of the resulting iminium intermediate (Figure 3, Scheme 2). Indeed, it was reported that substitution of Glu102 with Gln resulted in the loss of enzyme activity in *A. fumigatus* FtmPT1.[15] Furthermore, Gly115 was shown to be important for controlling the regiospecificities of the prenylation reactions; the FtmPT1 G115T mutant catalyzed the "reverse-type" prenylation at the C-3 position, instead of the "regular-type" prenylation at the C-2 position of the indole ring of brevianamide F.[15] Therefore, to further evaluate the importance of Gly115, we constructed a set of site-directed mutants (G115T, G115S and G115A) and investigated the effects of the mutagenesis on the enzyme activity. Consistent with the previous report,[15] all the mutants retained prenylation activity with brevianamide F as a substrate; interestingly, however, the mutants no longer accepted **1** as a substrate, and completely lost activity. Presumably, the mutants could not correctly fold the small, nonphysiological analogues within the active-site, and thus the essential interactions with the catalytic Glu102 could not be formed, which led to the loss of the enzyme activity.

The nonphysiological substrate **1** has been chemically synthesized[17] and also isolated from the marine sponge *Tedania ignis* as an artifact derived from indole-3-acetaldehyde via the aldol condensation with acetone during extraction.[18] In the later study, moderate brine shrimp cytotoxicity of **1** was reported. Generally, the prenylation of a compound often significantly affects its biological activity. Therefore, the above obtained **1a** was tested for its antibacterial activity against methicillin-susceptible *Staphylococcus aureus* (MSSA), *E. coli*, and *Bacillus subtilis* and for its cytotoxic activity against murine leukemia P388 cells, according to the methods as described previously.[19] Although **1a** was not active against any bacteria, it exhibited significantly higher cytotoxic activity (IC_{50} = 13.1 μg/mL) against murine leukemia P388 cells than **1** (IC_{50} = 29.9 μg/mL). The bioassay thus revealed that the prenylation of **1** increased its biological activity. Further investigations to elucidate the detailed mechanism of the cytotoxicity are now in progress.

In summary, the present work describes the enzymatic formation of an unnatural novel α-prenylindolylbutenone by *A. fumigatus* FtmPT1. This is the first demonstration of the prenylation of a nonaromatic carbon of the acceptor substrate by a fungal indole PT enzyme. These results suggest that further exploitation of the substrate promiscuity and catalytic potential of fungal indole PT enzymes could lead to the production of unnatural novel prenyl alkaloids.

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Supporting Information Available. Experimental details and a complete set of NMR data and charts. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.