FtmPT2, an N-Prenyltransferase from Aspergillus fumigatus, Catalyses the Last Step in the Biosynthesis of Fumitremorgin B

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FtmPT2, an *N*-Prenyltransferase from *Aspergillus fumigatus*, Catalyses the Last Step in the Biosynthesis of Fumitremorgin B

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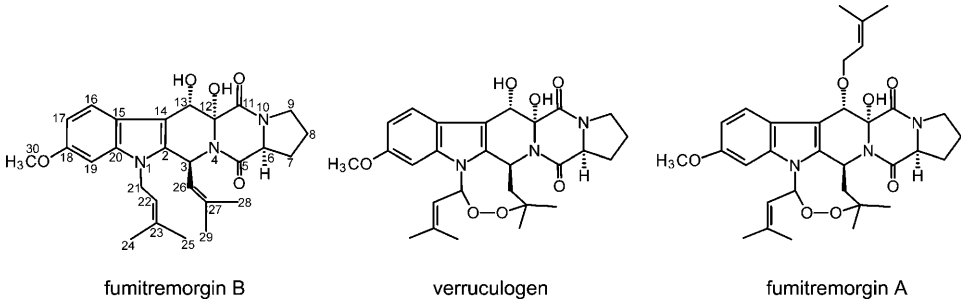
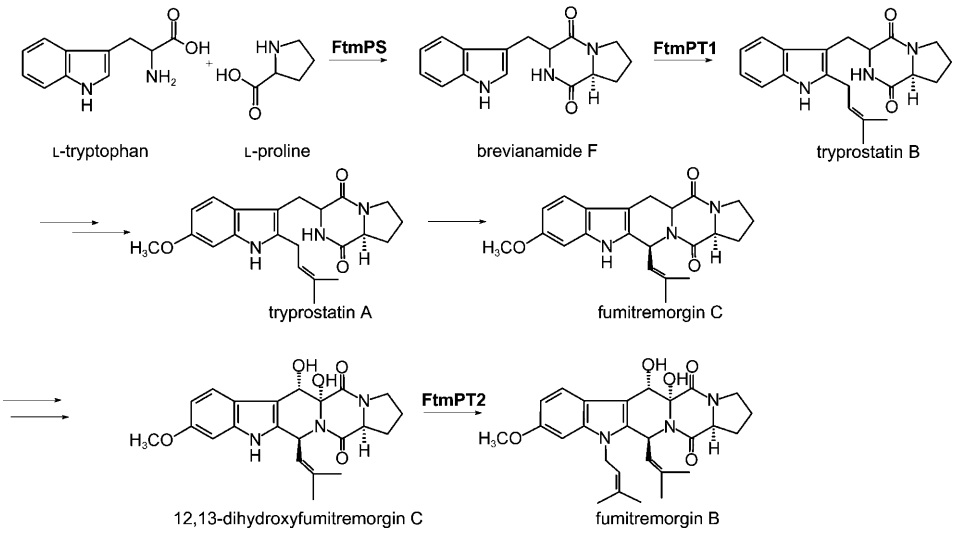
Fumitremorgin B (Scheme 1) is produced by a number of fungal strains, including *Aspergillus fumigatus*,[1–4] *Neosartorya fischeri*,[5] *Aspergillus caespitosus*,[6] *Penicillium piscarium*[7] and *Penicillium verruculosum*.[8] Some of these strains produce also related compounds, such as verruculogen or fumitremorgin A, which carries an additional *O*-prenyl moiety (Scheme 1).[1–3,6–9] Fumitremorgins and verruculogen are structurally prenylated diketopiperazine derivatives, which are derived from a cyclic dipeptide that consists of L-tryptophan and L-proline.[10] These compounds are mycotoxins and pose a problem for the food industry. However, some of their biosynthetic precursors show interesting biological and pharmacological activities. For example, tryprostatin A and B (Scheme 2) are potent inhibitors of microtubules[4] and fumitremorgin C (Scheme 2) is an inhibitor of the breast cancer resistant protein in some tumour cell lines and can reverse multidrug resistance of tumours.[11,12] Therefore, investigation of the biosynthesis of these compounds would also provide information for new strategies of drug discovery and development programs, which could be achieved by molecular biological and biochemical approaches.



Scheme 1. Chemical structures of fumitremorgins A and B and verruculogen; the proton numbering for fumitremorgin B is also indicated.



Scheme 2. Putative biosynthetic pathway of fumitremorgin B.

From the genome sequence of *A. fumigatus* Af293, a putative biosynthetic gene cluster has been identified on chromosome 8 by using a bioinformatic approach.[13] This cluster contains a putative nonribosomal peptide synthetase gene (AFUA_8G00170), two putative prenyltransferase genes (AFUA_8G00210 and AFUA_8G00250), which show significant sequence similarity to the dimethylallyltryptophan synthase DmaW from *Claviceps purpurea*,[14] and three genes that encode cytochrome P450 enzymes.[13] This gene cluster was proposed to be responsible for the biosynthesis of fumitremorgins, and the two putative prenyltransferase genes were termed *ftmPT1* (AFUA_8G00210) and *ftmPT2* (AFUA_8G00250).[13] This gene cluster is not expressed in *A. fumigatus* Af293, and, therefore, accumulation of fumitremorgin B or its precursors could not be identified in this strain.[15] However, heterologous over-expression of AFUA_8G00170 (*ftmPS* = *ftmA*) in *A. fumigatus* and *A. nidulans* resulted in formation of brevianamide F—a cyclic dipeptide of tryptophan and proline—in

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the ectopic integration mutants;[15] this demonstrates that FtmPS catalyses the condensation of the two amino acids (Scheme 2). After heterologous expression of *ftmPT1* in *E. coli*, the overproduced His_6–FtmPT1 was characterised biochemically. The FtmPT1 protein was shown to catalyse the second step in the biosynthesis of fumitremorgins, that is, the prenylation of brevianamide F at position C2 of the indole ring; this results in formation of tryprostatin B (Scheme 2).[13] The results obtained for FtmPS and FtmPT1 proved that the identified gene cluster is indeed responsible for the biosynthesis of fumitremorgins.

In this cluster, the second putative prenyltransferase gene *ftmPT2* was separated from *ftmPT1* by a DNA segment of 7 kb. Analysis by using FGENESH (see the Supporting Information) and by comparing the sequence with those of known prenyltransferases revealed that *ftmPT2* probably consists of two exons that are 1179 and 105 bp long, and are interrupted by an intron of 65 bp. The gene spans base pairs 750209 to 751557 of GenBank entry AAHF01000014.1. The deduced gene product of *ftmPT2* (EAL85141) comprises 427 amino acids and has a calculated molecular mass of 48.5 kDa; it is similar to other known indole prenyltransferases, such as FtmPT1, FgaPT1, FgaPT2, 7-DMATS and CdpNPT from *A. fumigatus*.[13,16–20] In addition, FtmPT2 also shows significant sequence similarity to these enzymes. For example, by using the program BLAST 2 SEQUENCES (Supporting Information), FtmPT2 was found to share an identity of 30% with FtmPT1,[13] 26% with FgaPT1,[17] 37% with FgaPT2,[16] 28% with CdpNPT[18,20] and 29% with 7-DMATS.[19]

The FtmPT2 protein could be involved in the conversion of 12,13-dihydroxyfumitremorgin C to fumitremorgin B, that is, prenylation at position N1 of the indole ring. However, it is also possible that this enzyme is involved in the transfer of the *O*-prenyl moiety in fumitremorgin A, or it could catalyse two different prenyl transfer reactions, that is, the N- and O-prenylation in the biosynthesis of fumitremorgin A, as observed for TdiB in the biosynthesis of terrequinone in *A. nidulans*.[21] To prove the function of FtmPT2, we decided to amplify and clone the gene from *A. fumigatus*, and overproduce and characterise the gene product biochemically.

By analogy with successful expression of other prenyltransferase genes,[13,17–19,22] we initially tried the expression of *ftmPT2* in *E. coli*. For this purpose, the coding region of *ftmPT2* was PCR amplified by using the cDNA of *A. fumigatus* strain B5233 as template, which is available in phagemid form and isolated from a cDNA library obtained from Stratagene. The PCR product was cloned—by way of using the cloning vector pGEM-T—into the expression vector pQE70 for *E. coli*, to give the construct pAG019. Sequencing of pAG019 confirmed the predicted gene structure and revealed neither a mutation nor a frame shift. However, gene expression could not be achieved under various conditions, including with different *E. coli* strains, IPTG concentrations, culture media and induction temperatures (data not shown). Therefore, we decided to carry out gene expression in *Saccharomyces cerevisiae*, which was successfully used for expression of *fgaPT2*.[16] For this purpose, *ftmPT2* was PCR amplified again by using pAG019 as template and cloned into pYES2/NTC by using pGEM-T Easy, and the expression construct pAG028 was obtained (see the Supporting Information).

Soluble proteins were obtained from transformants of *S. cerevisiae* that harboured pAG028 after overnight induction with raffinose (1%, *w/v*) and galactose (2%, *w/v*) at 30 °C. By using Ni–NTA agarose, His_6–FtmPT2 was purified to homogeneity as judged by SDS-PAGE (Figure 1). The observed molecular mass was 50.1 kDa and corresponded very well to the calculated mass of 49.3 kDa for His_6–FtmPT2. A protein yield of 2 mg of pure, N-terminal His_6-tagged FtmPT2 per litre culture could be obtained.

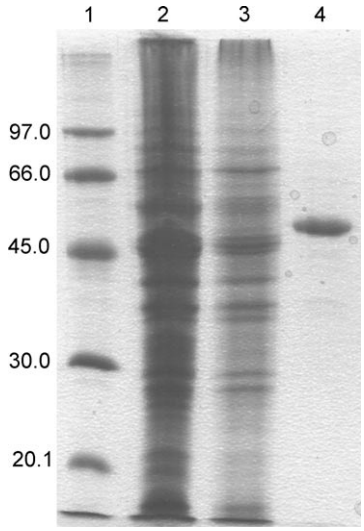

Figure 1. Purification of FtmPT2 as a His_6-tagged fusion protein after gene expression. The SDS polyacrylamide gel (12%, *w/v*) was stained with Coomassie brilliant blue R-250. Lane 1: molecular mass standard; lane 2: soluble protein before induction; lane 3: soluble protein after induction; lane 4: purified His_6–FtmPT2.

As mentioned above, FtmPT2 was expected to catalyse either the N- or O-prenylation, or both reactions in the biosynthesis of fumitremorgins. To prove this, the purified His_6–FtmPT2 was incubated with 12,13-dihydroxyfumitremorgin C in the presence of Ca^{2+} and dimethylallyl diphosphate (DMAPP). HPLC analysis showed that a product peak at 14.7 min could be detected only in the reaction mixture with active recombinant enzyme after incubation for 120 min at 37 °C, but not in the mixture with heat-denatured His_6–FtmPT2 (Figure 2). Product formation was strictly dependent on the presence of DMAPP and 12,13-dihydroxyfumitremorgin C (data not shown). Dependence of product formation on the amount of protein was found up to 50 μg per 100 μL assay with the reaction time of up to 90 min at 37 °C.

For structural elucidation, the enzymatic product was subsequently isolated on a preparative scale after incubation with 1.6 μM purified enzyme for 36 h, and subjected to NMR spectroscopy and MS analysis. Comparison of the ^1H NMR spectroscopy data of the isolated product (Table 1) with those of the substrate revealed the presence of signals for a regular di-

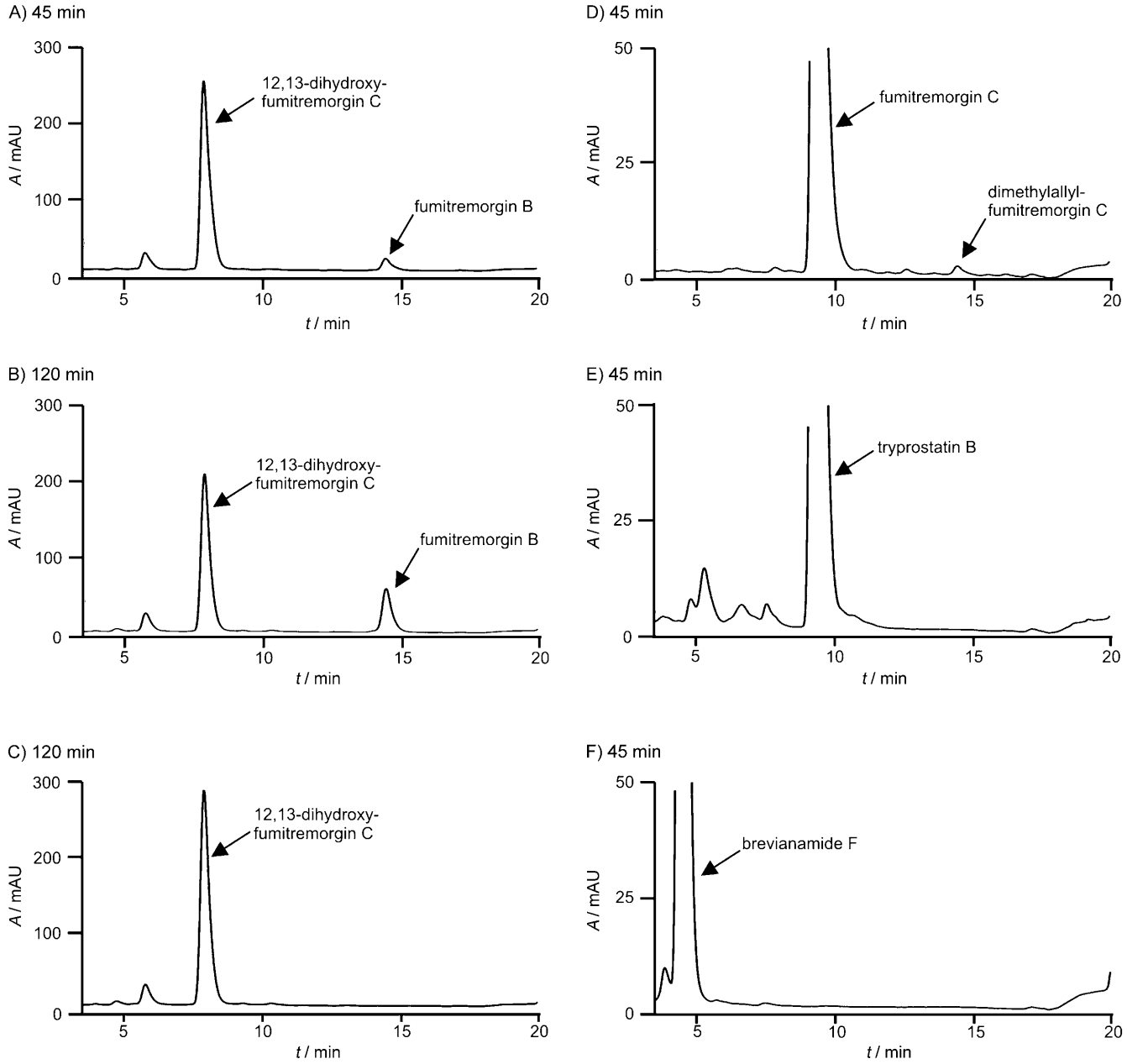

Figure 2. HPLC chromatograms of enzyme assays with recombinant His_6–FtmPT2 by using: A–C) 12,13-dihydroxyfumitremorgin C, D) fumitremorgin C, E) tryprostatin B and F) brevianamide F as substrates. The reaction mixtures were incubated at 37 °C for 45 min or 120 min. Protein inactivation for the incubation in chromatogram C) was carried out by boiling the protein at 100 °C for 20 min. Detection was carried out by using a photo diode array detector and illustrated at 277 nm.

methylallyl moiety at 4.54 (H-21), 5.04, (H-22), 1.85 (3H-24) and 1.70 ppm (3H-25); this indicates that the moiety is attached to either an N or O atom rather than to a C atom.[1,18] The NMR spectroscopy data of the isolated compound correspond well to those of fumitremorgin B described by Kodato et al.[23] Therefore, the enzymatic product of FtmPT2 could be unequivocally identified as fumitremorgin B, which was also confirmed by detection of the $[M-1]^-$ ion at *m/z* 478.4 in negative ESI-MS spectrum. These results suggest that FtmPT2 catalysed the prenylation of 12,13-dihydroxyfumitremorgin C at position N1 of the indole moiety (Figure 2). Therefore, FtmPT2 functions as an *N*-prenyltransferase, but not as an *O*-prenyltransferase, that could be involved in the biosynthesis of fumitremorgin A. The reaction catalysed by FtmPT2 represents the last step in the biosynthesis of fumitremorgin B. In the biosynthetic gene cluster of fumitremorgins from *A. fumigatus*, only two prenyltransferase genes—the previously reported brevianamide F prenyltransferase gene *ftmPT1* (Scheme 2) and *ftmPT2* described in this study—have been found by sequence analysis.[13] The results obtained for FtmPT1 and FtmPT2 indicate that fumitremorgin B or a derivative thereof, for example, verruculogen, but not fumitremorgin A, is very likely the end product of this cluster. This seems to be in contrast to the observation that fumitremorgin A is also detected in different *A. fumigatus*

Table 1. ^{1}H NMR spectroscopy data for fumitremorgin B. The spectrum was taken by using an Avance DRX 500 (Bruker) spectrometer in $CDCl_3$. Chemical shifts (δ) are given in ppm and coupling constants in Hz. Assignments of the signals were also confirmed by H,H-COSY; for numbering of the protons see Scheme 1.

Proton	Kodato et al.[23]	Enzymatic product of FtmPT2
3	5.97 (d-like, $J=10.1$, 1H)	5.97 (dd, $J_1=10.0$, $J_2=0.8$)
6	4.46 (dd, $J_1=7.0$, $J_2=9.5$, 1H)	4.45 (dd, $J_1=10.0$, $J_2=7.0$)
7	2.46 (m, 1H)	2.48 (m)
	1.8–2.2 (m, 3H)	2.10 (m)
8	1.8–2.2 (m, 3H)	2.10 (m)
		1.98 (m)
9	3.64 (dd, $J_1=4.9$, $J_2=8.5$, 2H)	3.64 (dd, $J_1=9.0$, $J_2=4.5$)
OH-12	4.0 (s, exchangeable)	3.99 (s)
OH-13	4.70 (d, $J=2.7$, exchangeable)	4.70 (d, $J=2.6$)
13	5.77 (d, $J=2.7$, 1H)	5.77 (dd, $J_1=2.6$, $J_2=0.8$)
16	7.85 (d, $J=8.5$, 1H)	7.85 (d, $J=8.7$)
17	6.80 (dd, $J_1=2.4$, $J_2=8.5$, 1H)	6.80 (dd, $J_1=8.7$, $J_2=2.3$)
19	6.70 (d, $J=2.4$, 1H)	6.70 (d, $J=2.3$)
21	4.54 (d-like, $J=5.8$, 2H)	4.54 (m)
22	5.04 (t-like)	5.04 (td, $J_1=6.4$, $J_2=1.0$)
24	1.85 (s, 3H)	1.85 (d, $J=1.0$)
25	1.70 (s, 3H)	1.70 (d, $J=1.0$)
26	4.70 (d-like, $J=10.1$, 1H)	4.71 (dm, $J=10.0$)
28	1.99 (s, 3H)	1.99 (d, $J=1.2$)
29	1.63 (s, 3H)	1.63 (d, $J=1.2$)
30	3.85 (s)	3.84 (s)

strains.[1,24] However, it cannot be excluded that additional genes that encode a prenyltransferase and modification enzymes for the conversion of fumitremorgin B to fumitremorgin A, are located elsewhere in the genome.

The FtmPT2 protein was found to be specific for DMAPP as prenyl donor. Product formation was only observed with DMAPP, but not with geranyl diphosphate under the conditions that we used. FtmPT2 also showed a relatively high specificity towards its aromatic substrate. Fumitremorgin C was accepted by FtmPT2 with a relative activity of 10% that of 12,13-dihydroxyfumitremorgin C. The prenylation product of fumitremorgin C was identified by positive ESI-MS. Other biosynthetic precursors, such as brevianamide F, tryprostatin A or B, were not accepted by FtmPT2. The enzymatic reaction catalysed by FtmPT2 appears to be extremely regiospecific since a second product was not detected with 12,13-dihydroxyfumitremorgin C in the presence of DMAPP even under extended incubation conditions (data not shown).

An important parameter for prenyltransferases is the dependence of their reactions on metal ions, especially on the presence of Mg^{2+} or Ca^{2+}.[13,16,18,19,25–27] Therefore, the enzymatic activity of FtmPT2 was assayed with 12,13-dihydroxyfumitremorgin C and DMAPP in the presence of different metal ions at a final concentration of 5 mM. An incubation mixture without additives was used as control. Our results showed that product formation is independent of the presence of metal ions. Even in the presence of EDTA—a chelating agent for divalent metal ions—loss of enzyme activity was not detected. These results are in contrast to all of the known *trans*-prenyltransferases[26] and membrane-bound aromatic prenyltransferases.[27–29] After addition of Ca^{2+}, no significant enhancement of enzyme activity could be observed with FtmPT2 in comparison to the sample without additives. This feature differs from those of FgaPT2,[16] FtmPT1,[13] CdpNPT[18,20] and 7-DMATS, but is similar to that of FgaPT1.[17] This indicates that enhancement of enzyme activity by Ca^{2+} is not conserved among the indole prenyltransferases.

The FtmPT2 enzyme is active as a homodimer and the reaction apparently followed Michaelis–Menten kinetics. The K_m values were determined from Lineweaver–Burk plots to be 96 μM for 12,13-dihydroxyfumitremorgin C and 186 μM for DMAPP; the turnover number was determined to be 0.03 s^{-1}. These values are comparable to those of other indole prenyltransferases, for example, FtmPT1[13] and 7-DMATS.[19]

In summary, the present study showed that FtmPT2 catalyses the regiospecific prenylation of 12,13-dihydroxyfumitremorgin C at position N1 of the indole ring in the presence of DMAPP, and therefore represents an enzyme involved in the last reaction step in the biosynthesis of fumitremorgin B in *A. fumigatus*.

Acknowledgements

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Keywords: *Aspergillus fumigatus* · biosynthesis · gene expression · indole derivatives · *N*-prenyltransferases

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