INTRODUCTION

Trans-prenyltransferases (Bohlmann et al., 1998; Liang et al., 2002; Sacchettini & Poulter, 1997) are involved in the biosynthesis of terpenoids from C-5 units. These enzymes usually contain one or more prenyl diphosphate binding motifs, (N/D)DXXD, in their sequences (Bohlmann et al., 1998; Liang et al., 2002; Sacchettini & Poulter, 1997). Their enzymic reaction depends strictly on the presence of metal ions such as Mg2+ or Mn2+ (Bohlmann et al., 1998; Liang et al., 2002). A special group of prenyltransferases catalyses the attachment of a prenyl moiety to an aromatic nucleus. These enzymes show similar sequence motifs and metal ion requirements to those of the trans-prenyltransferases. Examples of this group are the prenyltransferases involved in the biosynthesis of clorobiocin from Streptomyces roseochromogenes (Pojer et al., 2003) and LtxC involved in the biosynthesis of lyngbyatoxins from Lyngbya majuscula (Edwards & Gerwick 2004), have been identified. Very recently, we identified an orthologue of DMATS, FgaPT2, in the genome sequence of Claviceps majuscula (Hibino & Choshi, 2001; Tudzynski et al., 1999). Recently, two further soluble prenyltransferases with aromatic substrates, which are active in the absence of metal ions and contain no (N/D)DXXD motifs, CloQ and LtxC of lyngbyatoxins from Lyngbya majuscula (Edwards & Gerwick 2004), have been identified. Very recently, we identified an orthologue of DMATS, FgaPT2, in the genome sequence of **Aspergillus fumigatus** (Unsold & Li, 2005). Interestingly, DMATS, CloQ and LtxC share no sequence similarity, despite their similar biochemical properties. In order to obtain more insight into the mechanism and evolution of this class of prenyltransferases, we were interested to identify additional members of this class.

Prenylated indole derivatives are a large class of alkaloids containing a tryptophan moiety substituted with isoprenoid moieties, and are mostly found in fungi of the genera Aspergillus and Penicillium (Hibino & Choshi, 2001; Lounasmaa & Tolvanen, 2000; Williams et al., 2000). Most of these alkaloids contain a second amino acid, forming cyclic dipeptides with a diketopiperazine structure. L-Tryptophan and L-proline are precursors of tryprostatins, cycloprostatins, spirotroprostatins, fumitremorgins, verruculogen, brevianamides, paraherquamides and austamides, while L-tryptophan and L-alanine are precursors of the fungus Claviceps has been found to be a soluble protein and is active in a metal-free buffer containing EDTA (Cress et al., 1981; Lee et al., 1976; Tsai et al., 1995; Tudzynski et al., 1999). Recently, two further soluble prenyltransferases with aromatic substrates, which are active in the absence of metal ions and contain no (N/D)DXXD motifs, CloQ and LtxC involved in the biosynthesis of clorobiocin from Streptomyces roseochromogenes (Pojer et al., 2003) and LtxC involved in the biosynthesis of lyngbyatoxins from Lyngbya majuscula (Edwards & Gerwick 2004), have been identified. Very recently, we identified an orthologue of DMATS, FgaPT2, in the genome sequence of **Aspergillus fumigatus** (Unsold & Li, 2005). Interestingly, DMATS, CloQ and LtxC share no sequence similarity, despite their similar biochemical properties. In order to obtain more insight into the mechanism and evolution of this class of prenyltransferases, we were interested to identify additional members of this class.

**Abbreviations:** DMAPP, dimethylallyl diphosphate; DMATS, dimethylallyltryptophan synthase; EI, electron impact; FAB, fast-atom bombardment.
echinulins (Williams et al., 2000). Some prenylated indole alkaloids from fungi, such as fumitremorgin A and B, as well as verruculogen, are found to be active as tremogenic mycotoxins (Horie & Yamazaki, 1981; Schroeder et al., 1975; Steyn & Vleggaar, 1985; Yamazaki et al., 1971).

The biosynthesis of these prenylated indole alkaloids has been investigated mainly by feeding experiments with isotope-labelled precursors. Few enzymic studies have been reported, using protein extracts from producers (Williams et al., 2000). The results indicate that the cyclic dipeptide cyclo-L-Trp-L-Ala is the substrate of the prenyltransferases in the biosynthesis of echinulin (Allen, 1972a, b; Deyrup & Allen, 1975; Marchelli et al., 1975), and that the cyclic dipeptide cyclo-L-Trp-L-Pro is prenylated during the biosynthesis of brevianamides (Sanz-Cervera et al., 1993). However, the structural genes and the biochemical properties of these prenyltransferases are unknown.

We tried to identify genes of prenyltransferases involved in the biosynthesis of the indole alkaloid fumitremorgin B (Fig. 1). Fumitremorgin B is produced by A. fumigatus (Horie & Yamazaki, 1981; Yamazaki et al., 1971, 1974) and by other different fungal strains (Gallahger & Latch, 1977; Horie & Yamazaki, 1981; Schroeder et al., 1975; Vleggaar et al., 1993). It contains two prenyl moieties, attached at positions C-2 and N-1 of the indole nucleus. The biosynthesis of fumitremorgin B has been suggested to involve a prenylation of brevianamide F, yielding the intermediate tryprostatin B (Cui et al., 1996b; Williams et al., 2000). Since the genes for the biosynthesis of secondary metabolites are usually clustered in bacteria and fungi (Bhatnagar et al., 2003; Bibb, 1996; Martin & Liras, 1989; Sidhu, 2002; von Döhren & Gräfe, 1997; Yu et al., 2002), we expected that the gene for the postulated brevianamide F prenyltransferase would be located in a gene cluster containing several or all biosynthetic genes of fumitremorgin B.

The postulated brevianamide F prenyltransferase represents an interesting enzyme for the evolution of metabolic diversity, since different brevianamide F prenyltransferases probably act as branching enzymes in the biosynthetic pathways of indole alkaloids derived from cyclo-L-Trp-L-Pro (Fig. 1). Prenylation of brevianamide F by a ‘regular’ prenyltransferase at position C-2 of the indole nucleus is considered to be an initial step in the biosynthesis of tryprostatins, cyclotryprostatins, spirotryprostatins, fumitremorgins and verruculogen (Ortholand & Ganesan, 2004; Williams et al., 2000). Prenylation of brevianamide F at the same position by a ‘reverse’ prenyltransferase leads to formation of the brevianamides, the austamides and the paraherquamides (Ortholand & Ganesan, 2004; Williams et al., 2000) (Fig. 1).

‘Regular’ prenylation of brevianamide F results in the formation of the biologically active substance tryprostatin B (Fig. 1), which inhibits cell cycle progression at the G2/M phase (Cui et al., 1996a; Usui et al., 1998), while brevianamide F is inactive (Sanz-Cervera et al., 2000). Tryprostatin B and its methoxylated derivative tryprostatin A, as well as their diastereomers, have been found to exhibit cytotoxicity towards various cancer cell lines, and to be even more potent than etoposide (Zhao et al., 2002). They may therefore represent interesting candidates for the development of anticancer agents.

In the present study, we report the cloning and expression of a prenyltransferase gene, ftmPT1, as well as the biochemical characterization of the gene product, which converts brevianamide F to tryprostatin B.

**METHODS**

**Sequence analysis.** Preliminary sequence data were obtained from the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/Projects/A_fumigatus/). The cDNA sequence of ftmPT1 is available at GenBank under accession number AY861687.

FGENESH (Softberry, Inc., http://www.softberry.com/berry.phtml) and the DNASIS software package (version 2.1; Hitachi Software
Engineering) were used for intron prediction and sequence analysis, respectively. Amino acid sequence similarity searches were carried out in the GenBank database by using the BLAST program (release 2.9.9).

**Spectroscopic analysis.** 1H-NMR spectra were measured on an AMX 400 spectrometer (Bruker) using CDCl3 as solvent. Electron impact (EI) and positive-ion fast-atom bombardment (FAB) mass spectra were recorded on a TSQ70 spectrometer (Finnigan). Nitrobenzoic acid was used as matrix for measurement of FAB-MS.

**Bacterial strains, plasmids and culture conditions.** pGEM-T and pQE70 were obtained from Promega and Qiagen, respectively.

Escherichia coli XL-1 Blue MRF⁺ (Stratagene) was used for cloning experiments and grown in liquid or on solid Luria–Bertani medium with 1.5% agar at 37 °C (Sambrook & Russell, 2001).

Carbenicillin (50 μg ml⁻¹) was used for selection of recombinant E. coli strains.

BAC clone AfBB81 containing the putative fumitremorgin cluster of A. fumigatus was kindly provided by David Harris from the Wellcome Trust Sanger Institute (Cambridge, UK) and used as genomic DNA template for PCR amplification. A Uni-ZAP XR Premade Library of A. fumigatus strain B 5233 (=ATCC 13073) was purchased from Stratagene and used to obtain phagemids as cDNA template for PCR amplification.

**DNA isolation, manipulation and cloning.** Standard procedures for DNA isolation and manipulation were performed as described by Sambrook & Russell (2001). Isolation of BAC DNA and plasmids from E. coli was carried out with ion-exchange columns (Nucleobond AX kits, Macherey-Nagel) according to the manufacturer’s instructions.

**Gene cloning and expression, and protein purification.** For construction of the expression plasmid pAG012 (=pQE70-ftmPT1), two overlapping fragments of the coding sequences of ftmPT1 were amplified independently from each other. A genuine BamHI restriction enzyme recognition site is located in the overlapping part of both fragments. The fragment of 1269 bp at the 5’ end was amplified from genomic DNA on BAC AfBB81 by using the primers Primer-1(ftmPT11) (5’-ACGCAAATGTCCGCCGGACGA-3’) and Primer-2(ftmPT11) (5’-TAGTTCCGGATTAATCCCTG-3’). The bold type represents a mutation inserted in the original sequence to give the underlined SplI restriction site. The fragment of 277 bp at the 3’ end was amplified from cDNA by using the primers Primer-3(ftmPT12) (5’-CTGTTTCCGGATCCCCAGATGTA-3’) and Primer-4(ftmPT12) (5’-ATGGATCATTGGGGAGGACA-3’). Bold type represents mutations inserted in the original sequence to give the underlined BamHI restriction sites. Both fragments were ligated into pGEM-T to give pAG002 (=pGEMT-ftmPT11) containing the fragment at the 5’ end and pAG004 (=pGEMT-ftmPT12) containing the fragment at the 3’ end, respectively. The resulting plasmids were sequenced to identify potential errors from PCR amplification (MWG-Biotech). pAG002 was digested with SplI and BamHI and the resulting SplI–BamHI fragment of 1131 bp was ligated into pQE70, which had been digested with the same enzymes, to give pAG010 (=pQE70-ftmPT11). pAG004 was digested with BamHI and the fragment of 268 bp obtained was ligated into pAG010, which had also been digested with BamHI, to give pAG012 (=pQE70-ftmPT1). The correct orientation of the fragment of 268 bp was proven by restriction enzyme digests.

For gene expression, E. coli cells harbouring the plasmid pAG012 were cultured in a 300 ml Erlenmeyer flask containing 100 ml liquid Luria–Bertani medium with 50 μg carbenicillin ml⁻¹ grown and at 37 °C for 4 h at 170 r.p.m. (OD600=0.7). For induction, IPTG solution was added (final concentration 0.1 mM) and the bacterial culture was cultivated for a further 16 h at 30 °C before harvest. The bacterial cultures were centrifuged and the pellets were resuspended in lysis buffer (10 mM imidazole, 50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) to 2–5 ml (g wet weight)⁻¹. After addition of 1 mg lysozyme ml⁻¹ and incubation on ice for 30 min, the cells were sonicated six times for 10 s at 200 W. To separate the cellular debris from the soluble protein, the lysate was centrifuged at 10 000 g for 30 min at 4 °C. One-step purification of the recombinant His₆-tag fusion protein by affinity chromatography with Ni-NTA agarose resin (Qiagen) was carried out according to the manufacturer’s instructions. The protein was eluted with 250 mM imidazole. To exchange the diluent buffer, the protein fraction was passed through a PD-10 column (Amersham Biosciences), which had been equilibrated with 50 mM Tris/HC1 (pH 7.5).

**Protein analysis.** Standard protein techniques were used as described elsewhere (Bradford, 1976; Laemmli, 1970). The subunits of FtmPT1 were analysed by SDS-PAGE, carried out according to the method of Laemmli (1970), and protein bands were stained with Coomassie brilliant blue R-250. The molecular mass of native FtmPT1 was determined by gel filtration on a HiLoad 26/60 Superdex 200 column (Amersham Biosciences) that had been equilibrated with 50 mM Tris/HC1 buffer (pH 7.5) containing 150 mM NaCl. The column was calibrated with dextran blue 2000 (2000 kDa), aldolase (158 kDa), albumin (66 kDa), ovalbumin (45 kDa) and ribonuclease A (13.7 kDa) (Amersham Biosciences). The proteins were eluted with 50 mM Tris/HC1 buffer (pH 7.5) containing 150 mM NaCl. The molecular mass of native His₆-FtmPT1 was determined as 66 kDa.

**Chemical synthesis of brevianamide F.** Brevianamide F was prepared according to the method described by Caballero et al. (1993): 1.97 g N-tetra-butyloxycarbonylproline (9-17 mmol) was stirred together with 1.78 g N'-(3-dimethylaminopropyl)-N-ethylcarbodiimid (EDC) (9-27 mmol), 2.34 g l-tryptophan methyl ester-HCl and 1.45 g triethylamine in 50 ml dry CH₂Cl₂ under argon for 24 h. The mixture was washed twice with 10 ml HCl (1 M) and 10 ml aqueous NaHCO₃ (1 M). The organic layer was dried over Na₂SO₄ and evaporated. The product was heated at 200 °C for 4 h under an argon atmosphere in a flask that had been maintained in a stream of argon for 15 min. The product was dissolved in acetone/ethyl acetate (2:1, v/v) and purified over a silica gel column, using acetone/ethyl acetate (2:1, v/v) as elution solvent. Brevianamide F (200 mg) and its (11R,14S) diastereoisomer cyclo-D-Trp-L-Pro (300 mg) were obtained.

The ¹H-NMR data of brevianamide F and its diastereoisomer in CDCl₃ are given in Table 1.

The EI-MS data of brevianamide F were as follows: m/z (intensity): 43 (17), 70 (9), 84 (18), 130 (100), 185 (6), 283 (8) [M⁺]⁻.

The EI-MS data of cyclo-(D-Trp-L-Pro) were as follows: m/z (intensity): 43 (4), 70 (4), 84 (11), 130 (100), 154 (6), 185 (2), 283 (7) [M⁺]⁻.

The structures of the products were confirmed by comparison of their spectroscopic data with the literature (Caballero et al, 2003; Steyn, 1973).

**Assay for FtmPT1 activity.** For the quantitative determination of enzyme activity, the reaction mixture (100 μl) contained 50 mM Tris/HC1 (pH 7.5), 5 mM MgCl₂, 1 mM brevianamide F or analogues, 0.5 mM dimethyllylphosphatase (DMAPP) (or isopenytenyl diposphate or geranyl diposphate) and 0.5 μg purified His₆-FtmPT1. After incubation for 10 min at 30 °C, the reaction was stopped with 10 μl TCA (1:5 M). The protein was removed by centrifugation at 13 000 g for 10 min. Enzymic products were analysed by HPLC with detection at 277 nm using a Multosphere RP 18-5...
Table 1. $^1$H-NMR data of substrates and products of FtmPT1

The spectra were taken in CDCl$_3$ and the chemical shifts (δ) are given in p.p.m. and coupling constants in Hz.

<table>
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<tr>
<th>Compound:</th>
<th>Brevianamide F</th>
<th>cyclo-D-Trp-L-Pro</th>
<th>Tryprostatin B</th>
<th>cyclo-Dimethylallyl-L-Trp-L-Gly</th>
<th>cyclo-Dimethylallyl-L-Trp-L-Leu</th>
<th>cyclo-Dimethylallyl-L-Trp-L-Trp</th>
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*†‡§Assignments for signals with the same letters are interchangeable.
column (250 × 4 mm, 5 μm, C+ S Chromatographie Service). A linear gradient of 35–100 % methanol in 1 % aqueous formic acid in 20 min was used. For quantitative measurements, duplicate values were determined routinely. The Kd value for DMAPP was determined at a brevianamide F concentration of 1 mM and for brevianamide F at a DMAPP concentration of 0.5 mM.

Preparative enzymic synthesis of tryprostatin B and Analogues for structural elucidation. To a 5 ml glass vial, DMAPP (0.5 mM final concentration), brevianamide F or cyclo-L-Trp-L-Gly or cyclo-L-Trp-L-Leu or cyclo-L-Trp-L-Tyr or cyclo-L-Trp-L-Trp (1 mM), MgCl2 (5 mM), Tris·HCl (50 mM, pH 7.5) and FtmPT1 (60 μg) were added to a final volume of 2 ml. The mixtures were incubated at 30 °C for 16 h. One hundred microlitres TCA (1·5 M) was added and the protein was removed by centrifugation. The enzymic products were extracted with ethyl acetate and purified by HPLC using the method described above. The fractions containing the prenylated diketopiperazine derivatives were collected and the solvent was evaporated. The obtained products (0·2–0·5 mg) were analysed by 1H-NMR spectroscopy and by positive-ion FAB-MS.

Spectroscopic data of tryprostatin B and prenylated cyclic dipeptides. The 1H-NMR data of the enzymic products are given in Table I. Positive FAB-MS data of the isolated enzymic products are as follows.

Tryprostatin B: m/z (intensity): 198 (100), 352 (26) [M + H]+; cyclo-L-dimethylallyl-Trp-L-Trp: m/z (intensity): 198 (100), 441 (18) [M + H]+; cyclo-L-dimethylallyl-Trp-L-Leu: m/z (intensity): 198 (100), 368 (28) [M + H]+; cyclo-L-dimethylallyl-Trp-L-Gly: m/z (intensity): 198 (100), 242 (35), 312 (50) [M + H]+; cyclo-L-dimethylallyl-Trp-L-Tyr: m/z (intensity): 198 (79), 219 (100), 419 (43) [M + H]+.

RESULTS AND DISCUSSION

Analysis of the genome sequence of A. fumigatus for prenyltransferase genes

Both the postulated brevianamide F prenyltransferases and the well-examined DMATS catalyse prenylation reactions of the indole ring of (free or peptide-bound) tryptophan, so we expected that both enzymes would share some sequence similarity. Indeed, several genes with similarity to cpd1, which encodes the enzyme DMATS from Claviceps purpurea (Tudzynski et al., 1999), were found in the A. fumigatus genome. Two of them, fgaPT1 and fgaPT2, are located on BAC clones AfA32C2 and AfA31F3 and are probably situated within a putative biosynthetic gene cluster of fumigaclavine C (Unsöld & Li, 2005). FgaPT2 shows an identity of 54 % at the amino acid level to DMATS from C. purpurea and has been proven to catalyse the formation of dimethylallyltryptophan (Unsöld & Li, 2005). Two further putative orfs, named ftmPT1 and ftmPT2, were situated only 7·1 kb apart from each other in the genome, on the BAC clones AfB88B11 and AfB12D2. The proteins deduced from these orfs showed a sequence identity of 36 % (FtmPT1) and 35 % (FtmPT2) to DMATS from C. purpurea (Tudzynski et al., 1999), respectively. FtmPT1 and FtmPT2 share 36 % sequence identity with each other. Together with genes in their vicinity in the genome sequence (Fig. 2), ftmPT1 and ftmPT2 probably belong to a putative biosynthetic gene cluster for fumitremorgin B or its derivatives, because two prenyltransferases are expected to be involved in the biosynthesis of fumitremorgin B, for the prenylations at C-2 and N-1. In addition, the gene ftmPS, encoding a putative non-ribosomal peptide synthetase (NRPS), might be involved in the formation of the cyclo-L-Trp-L-Pro dipeptide structure of fumitremorgin B. The predicted protein FtmPS (243 kDa) contains two peptide synthetase modules, each comprising an adenylation domain, a peptide carrier protein (PCP) and a condensation domain. Furthermore, ORF10 and ORF12 could be responsible for the introduction of the methoxyl group onto the indole ring. ORF12 shows sequence similarity to cytochrome P450 enzymes which catalyse the hydroxylation of flavonoids (Ueyama et al., 2002), while ORF10 is a homologue of methyltransferases which catalyse the methylation of a phenolic hydroxyl group (Dickens et al., 1995). By sequence analysis, however, it is not possible to assign the prenyltransferases FtmPT1 and FtmPT2 to the corresponding reactions.

Sequence analysis of ftmPT1

The genomic sequence of the putative prenyltransferase gene ftmPT1 consists of two exons of 1262 and 133 bp, interrupted by one intron of 69 bp situated near the 3' end. The existence of this intron was confirmed by the sequencing of a PCR fragment (277 bp) amplified from cDNA as template (see below).

The predicted gene product of ftmPT1 comprises 464 amino acids and has a calculated molecular mass of 52·6 kDa. FtmPT1 shares sequence similarity with prenyltransferases from different fungi, such as 36 % to DMATS from C. purpurea (Tudzynski et al., 1999), 36 % to DMATS from Claviceps fusiformis (Tsai et al., 1995), 36 % to DMATS from Neotyphodium sp. (Wang et al., 2004), 36 % to FgaPT2 from A. fumigatus (Unsöld & Li, 2005) and 32 % to SirD, a putative prenyltransferase in the biosynthetic gene cluster of sirodesmin from Leptosphaeria maculans (Gardiner et al., 2004). SirD has been speculated to be involved in the prenylation of the phenolic OH group of tyrosine or of

![Fig. 2. Genomic region in the vicinity of the brevianamide F prenyltransferase gene ftmPT1 of Aspergillus fumigatus. Black bars represent introns.](http://mic.sgmjournals.org)
cyclo-L-Tyr-L-Ser (Gardiner et al., 2004). FtmPT1 shows a very low similarity to bacterial prenyltransferases with aromatic substrates, such as CloQ (Pojer et al., 2003) and LtxC (Edwards & Gerwick, 2004) (27% at the amino acid level to both enzymes). This may indicate that the ‘aromatic’ prenyltransferases from fungi and bacteria belong to different enzyme families. No sequence homology was found between FtmPT1 and trans-prenyltransferases (Liang et al., 2002). Like CloQ (Pojer et al., 2003), LtxC (Edwards & Gerwick, 2004), DMATS from C. purpurea (Tudzynski et al., 1999) and C. fusiformis (Tsai et al., 1995), and FgaPT2 from A. fumigatus (Unsöld & Li, 2005), FtmPT1 does not contain the prenyl diphosphate binding motif (N/D)DXXD, which has been found in all trans-prenyltransferases (Bohlmann et al., 1998; Liang et al., 2002; Sacchettini & Poulter, 1997). Neither the putative prenyl diphosphate binding motif DDSYN suggested by Tsai et al. (1995) for the DMATS from C. fusiformis, nor the conserved motifs I–V of the cis-prenyltransferases (Apfel et al., 1999; Asawatreratanakul et al., 2003) is present in the sequence of CloQ, DMATS, LtxC or FtmPT1. The substrate binding sites of these enzymes are unknown. An X-ray crystallographic study with crystallized FtmPT1 may provide evidence for these binding sites and the theoretical basis for site-directed mutagenesis to obtain enzymes with modified substrate specificity.

Cloning and expression of ftmPT1 and purification of FtmPT1

To eliminate the intron in the sequence of ftmPT1, a fragment of 277 bp comprising the 3′ end of the structural gene was amplified using PCR from cDNA of A. fumigatus strain B 5233, available as phagemids isolated from a cDNA library. The 5′ end of ftmPT1 was amplified as a fragment of 1270 bp from genomic DNA of A. fumigatus strain B 5233, available as BAC DNA. The two PCR fragments overlapped 138 bp in length and were ligated into a cloning vector using a genuine BamHI recognition site in the overlapping region. Sequencing of the resulting plasmids confirmed that the cloned sequence was identical to the predicted sequence.

For gene expression, the coding sequence of ftmPT1 was cloned into the expression vector pQE70. E. coli cells harbouring the expression plasmid pQE70-ftmPT1 were induced by 0·1 mM IPTG at 30 °C. Protein induction was clearly observed (Fig. 3) and a protein yield of 20 mg of purified His6-tagged FtmPT1 per litre of culture was obtained. FtmPT1 was purified on nickel-NTA agarose to apparent homogeneity as judged by SDS-PAGE (Fig. 3). The observed molecular mass was 49 kDa and corresponded reasonably to the calculated mass of 53·4 kDa for His6-FtmPT1.

Using gel-permeation chromatography, the native molecular mass of FtmPT1 was determined as 66 kDa, suggesting that the protein was active as a monomer. The enzyme was soluble and did not require the presence of detergents.

Identification of the enzymic product

HPLC analysis of the enzymic mixture showed a product peak with a retention time of 18 min (Fig. 4B), which was absent in the reaction mixture containing heat-denatured enzyme (Fig. 4A). This peak had the same retention time as that of an authentic tryprostatin B (Fig. 4C). Product formation showed a linear dependence on the amount of protein (up to 1·5 µg per 100 µl assay) and on the incubation time (up to 30 min). The reaction was strictly dependent on the presence of both brevianamide F and DMAPP.

The enzymic product was subsequently isolated on a preparative scale using HPLC, and subjected to NMR and MS analysis. Comparison of the 1H-NMR spectrum of the isolated product with that of brevianamide F (Table 1) revealed that in the spectrum of the isolated compound the doublet at 7·1 p.p.m. for H-2 of brevianamide F had disappeared. Instead, additional signals for a dimethylallyl moiety were observed at 3·46 (br t, 6·9 Hz, H-1′), 5·29 (br t, 7·0 Hz, H-2′), 1·74 (s, 3H-4′) and 1·77 (s, 3H-5′) p.p.m., respectively. The positive FAB-mass spectrum showed an ion at m/z 352 ([M + H]+), in accordance with the expected mass of tryprostatin B. The NMR and MS data correspond well to those of tryprostatin B reported by Cui et al. (1996b). This proved unequivocally that FtmPT1 catalyses the prenylation of brevianamide F at the C-2 position of the indole nucleus to give tryprostatin B. Furthermore, our results suggest that FtmPT1 is an enzyme in the biosynthesis of fumitremorgin B, and that the genes located in the vicinity of ftmPT1 in the genome of A. fumigatus represent probably a biosynthetic gene cluster for the biosynthesis of fumitremorgin B or its derivatives.

This is the first report on the cloning and biochemical investigation of an enzyme involved in the biosynthesis of a prenylated indole alkaloid with a dipeptide structure, a large

**Fig. 3.** Analysis of overproduction and purification of FtmPT1.

The proteins were separated on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lanes: 1, protein marker; 2, soluble fraction before induction; 3, soluble fraction after induction with 0·1 mM IPTG at 30 °C for 16 h; 4, purified His6-FtmPT1.
and a diverse class of secondary metabolites (Williams et al., 2000).

**Biochemical properties and kinetic parameters of FtmPT1**

**Dependence on metal ions.** Testing with different ions showed that metal ions are not essential for the prenyltransferase activity. Even in the presence of 5 mM EDTA, full total activity was observed. Mn$^{2+}$ and Zn$^{2+}$ at 5 mM, and Mg$^{2+}$ at higher concentrations (≥10 mM), reduced the product formation. Mg$^{2+}$ at 2–5 mM and Ca$^{2+}$ at 5–20 mM slightly enhanced the prenyltransferase activity (up to 140% of the activity without additive). This finding is in sharp contrast to the absolute requirement for divalent cations reported for most prenyltransferases (Collakova & DellaPenna, 2001; Liang et al., 2002; Schledz et al., 2001; Turunen et al., 2004; Yazaki et al., 2002), but is similar to the data reported for the prenyltransferase DMATS from the fungus C. purpurea (Gebler & Poulter, 1992) and FgaPT2 from A. fumigatus (Unsold & Li, 2005), catalysing the transfer of a dimethylallyl moiety to the C-4 position of tryptophan; CloQ from the soil bacterium Streptomyces roseochromogenes (Pojer et al., 2003), catalysing the transfer of a dimethylallyl moiety to the C-3 position of 4-hydroxyphenylpyruvate; and LtxC from the marine cyanobacterium Lyngbya majuscula (Edwards & Gerwick, 2004), catalysing the transfer of a geranyl moiety to the C-8 position of the indole ring of (−)-indolactam V. All four enzymes are soluble enzymes and active in the absence of divalent metal ions.

**Kinetic parameters and substrate specificity.** The FtmPT1 reaction apparently followed Michaelis–Menten kinetics. The $K_m$ values were determined from Hanes–Woolf plots as 55 μM for brevianamide F and 74 μM for DMAPP, respectively. The maximum reaction velocity observed with FtmPT1 was 104 nmol s$^{-1}$ mg$^{-1}$, corresponding to a turnover number of 5.57 s$^{-1}$.

Incubations with substrate analogues showed that FtmPT1 is specific for its substrate DMAPP. No product formation was observed when isopentenyl diphosphate or geranyl diphosphate was used instead of DMAPP. On the other hand, FtmPT1 accepted different L-tryptophan-containing cyclic dipeptides (Table 2): the stereoisomer of brevianamide F, cyclo-(D-Trp-L-Pro), was accepted with a relative activity of 33% compared to brevianamide F.

**Table 2. Substrate specificities of FtmPT1 towards different aromatic substrates**

Assays were carried out as described in Methods. Values are averages of two replicates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
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<tr>
<td>Brevianamide F</td>
<td>100.0 ± 8.9</td>
</tr>
<tr>
<td>cyclo-(D-Trp-L-Pro)</td>
<td>32.6 ± 3.9</td>
</tr>
<tr>
<td>cyclo-(L-Trp-L-Leu)</td>
<td>21.9 ± 1.2</td>
</tr>
<tr>
<td>cyclo-(L-Trp-L-Trp)</td>
<td>6.4 ± 1.2</td>
</tr>
<tr>
<td>cyclo-(L-Trp-L-Tyr)</td>
<td>5.0 ± 0.5</td>
</tr>
<tr>
<td>cyclo-(L-Trp-L-Gly)</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>cyclo-(L-Trp-L-Phe)</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>cyclo-(L-Pro-L-Tyr)</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>cyclo-(L-Pro-L-Phe)</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>HO-L-Trp-L-Pro-H</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>(−)-Indolactam V</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>N-Ac-Trp</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>L-Trp</td>
<td>&lt;0.3</td>
</tr>
</tbody>
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**Fig. 4.** HPLC analysis of the enzymic product. (A) Enzyme assay with the heat-inactivated FtmPT1; (B) enzyme assay with the active FtmPT1; (C) authentic tryprostatin B from Alexis (Grüningen, Germany). AU, absorbance units.
cyclo-L-Trp-L-Leu showed a relative activity of 22%. Towards the other cyclic dipeptides tested, such as cyclo-L-Trp-L-Trp, cyclo-L-Trp-L-Tyr, cyclo-L-Trp-L-Phe and cyclo-L-Trp-L-Gly, FmPTP1 showed a lower, but significant, enzymic activity (3–6% compared to brevianamide F). In addition to MS analysis (except for cyclo-L-dimethylallyl-Trp-L-Phe), the identities of the enzymic products of the cyclic peptides (except for cyclo-L-dimethylallyl-Trp-L-Tyr and cyclo-L-dimethylallyl-Trp-L-Phe) were confirmed by 1H-NMR analysis (Table 1) and comparison with 1H-NMR spectra of the non-prenylated cyclic dipeptides (Caballero et al., 1998; Hodge et al., 1988; Maes et al., 1986; Santamaria et al., 1999). In the 1H-NMR spectra of the enzymic products, signals for a dimethylallyl moiety were found at 3.38–3.46 (2H-1’), 5.29 (1H-2’), 1.74–1.76 (3H-4’) and 1.77–1.80 (3H-5’) p.p.m. The 1H-NMR spectra of the prenylated cyclic dipeptides proved that the prenylation had taken place at position C-2 of the indole nucleus, since the signal for H-2 at 7.1 p.p.m. had disappeared. With cyclo-L-Trp-L-Trp, only the monoprenylated product was detected under our reaction conditions. No detectable product formation was observed with cyclo-L-Pro-L-Tyr, cyclo-L-Pro-L-Phe, (+)-indolactam V and (-)-indolactam V (the substrate of LtxC), L-tryptophan, N-acetyl-L-tryptophan or linear dipeptides of L-tryptophan and L-proline as substrates (Table 2). This indicated that the diketopiperazine ring of the cyclic dipeptides is essential for the enzyme activity. The tolerance of FmPTP1 for different tryptophan-containing cyclic dipeptides opens the possibility of generating different prenylated diketopiperazine derivatives by chemoenzymic synthesis in vitro (Xu et al., 2004). Such substances may show interesting biological activities, such as the inhibition of cancer cell lines (Zhao et al., 2002).

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