

# A 7-dimethylallyltryptophan synthase from *Aspergillus fumigatus*: overproduction, purification and biochemical characterization

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A putative prenyltransferase gene, *Afu3g12930*, was identified in the genome sequence of *Aspergillus fumigatus*. EAL92290, encoded by *Afu3g12930*, consists of 472 aa, with a molecular mass of about 53 kDa. The coding sequence of *Afu3g12930* was cloned in pQE60, and overexpressed in *Escherichia coli*. The soluble His<sub>6</sub>-fusion protein was purified to apparent homogeneity, and characterized biochemically. The enzyme was found to catalyse the prenylation of Trp at the C-7 position of the indole moiety, in the presence of dimethylallyl diphosphate (DMAPP); therefore, it functions as a 7-dimethylallyltryptophan synthase (7-DMATS). The structure of the enzymic product was elucidated by NMR and MS analysis.  $K_m$  values were 67  $\mu$ M for DMAPP, and 137  $\mu$ M for L-Trp. Geranyl diphosphate was not accepted as prenyl donor, while Trp-containing dipeptides were found to be aromatic substrates of 7-DMATS. 7-DMATS did not need divalent metal ions for its enzymic reaction, although Ca<sup>2+</sup> enhanced the reaction velocity slightly. The enzyme is the second dimethylallyltryptophan synthase identified in *A. fumigatus*. Interestingly, it shares a sequence identity of only 31 % at the amino acid level with another known dimethylallyltryptophan synthase, FgaPT2, from the same fungus; FgaPT2 prenylates L-Trp at the C-4 position of the indole ring. *Afu3g12930* belongs to a putative biosynthetic gene cluster consisting of eight genes. Orthologous clusters were also identified in the genome sequences of *Neosartorya fischeri* and *Aspergillus terreus*. The putative roles of the genes in the cluster are discussed.

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## INTRODUCTION

Like bacteria and plants, fungi produce pharmacologically important agents (Keller *et al.*, 2005), for example: the immunosuppressive cyclosporin A is obtained from *Tolypocladium inflatum* (Thali, 1995); statins are produced by several fungi, including *Aspergillus terreus* (Manzoni & Rollini, 2002); penicillins are produced by *Penicillium chrysogenum* and *Aspergillus nidulans* (Brakhage *et al.*, 2004); and ergot alkaloids from *Claviceps purpurea* show diverse pharmacological activities (Scharndl *et al.*, 2006). Secondary metabolites from fungi are usually active as mycotoxins and virulence factors (Bennett & Klich, 2003; Rementeria *et al.*, 2005) that could be involved in the pathogenic development of fungal infections. *Aspergillus*

**Abbreviations:** DMAPP, dimethylallyl diphosphate; DMAT, dimethylallyltryptophan; DMATS, dimethylallyltryptophan synthase; ETP, epipolythiodioxopiperazine; GPP, geranyl diphosphate; NRPS, non-ribosomal peptide synthetase.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence reported in this paper is EF539173.

*fumigatus*, a filamentous fungal saprophyte found ubiquitously in the environment, is responsible for allergenic reactions and invasive aspergillosis, which is a life-threatening disease for immunocompromised patients (Denning *et al.*, 2003). It has been discussed that the virulence of *A. fumigatus* could be based on its numerous secondary metabolites, especially gliotoxin (Latgé, 1999), which is an epipolythiodioxopiperazine (ETP) derived from Phe and Ser (Gardiner & Howlett, 2005). However, the role of these compounds remains questionable, and needs to be investigated more thoroughly (Latgé, 1999). ETPs are characterized by the presence of an internal disulphide bridge (Gardiner *et al.*, 2005). The role of gliotoxin in the pathogenic development of *A. fumigatus* has been demonstrated by deleting the non-ribosomal peptide synthetase (NRPS) gene *gliP* from the gliotoxin biosynthetic gene cluster (Cramer *et al.*, 2006a; Kupfahl *et al.*, 2006), and it was clearly demonstrated that gliotoxin production was blocked in the resulting mutants; however, the abolition of gliotoxin production had no effect on the development of invasive aspergillosis by the mutants

(Cramer *et al.*, 2006a; Kupfahl *et al.*, 2006). This proved that gliotoxin is not, or at least not alone, responsible for the pathogenic development in *A. fumigatus*. Therefore, it would be useful to identify genetic information of further secondary metabolites, which could function as virulence factors.

From the genome sequence of *A. fumigatus* Af293, at least 26 biosynthetic gene clusters for secondary metabolites have been identified (Nierman *et al.*, 2005), including the gene clusters of fumitremorgin B and fumigaclavine C, as well as that of gliotoxin (Cramer *et al.*, 2006b; Gardiner & Howlett, 2005; Grundmann & Li, 2005; Maiya *et al.*, 2006; Unsöld & Li, 2005). One putative gene cluster has been proposed for the biosynthesis of an additional unknown ETP derivative (Cramer *et al.*, 2006b). This putative gene cluster contains three genes showing significant sequence similarity to *gliC*, *gliP* and *gliM* of the gliotoxin cluster of *A. fumigatus* (Gardiner & Howlett, 2005). Homologues of these genes, i.e. *sirC*, *sirP* and *sirM*, have also been found in the gene cluster of sirodesmin, which is an ETP derivative from the phytopathogenic fungus *Leptosphaeria maculans* (Gardiner *et al.*, 2004). The gene cluster of sirodesmin contains a putative prenyltransferase gene, *sirD*, which has been proposed to be responsible for the *O*-prenylation of L-Tyr or *cyclo*-L-Tyr-L-Ser (Gardiner *et al.*, 2004, 2005). A putative prenyltransferase gene, *Afu3g12930*, has also been found in the cluster from *A. fumigatus*, and the protein that it encodes shares a sequence identity of 34% with SirD at the amino acid level; this is higher than the sequence similarity to other fungal prenyltransferases, or other entries in the databases, with the exception of its orthologues in *Neosartorya fischeri* and *A. terreus* (see Discussion). Based on this sequence similarity, it could be speculated that EAL92290, encoded by *Afu3g12930*, would catalyse the transfer reaction of a prenyl moiety to Tyr or its derivatives. To prove the function of *Afu3g12930*, we cloned and overexpressed this gene in *Escherichia coli*, and carried out biochemical investigation using the purified fusion protein. Our results showed, interestingly, that *Afu3g12930* encodes a second dimethylallyltryptophan synthase (DMATS), termed 7-DMATS, in *A. fumigatus*, and this enzyme catalyses the prenylation of Trp instead of Tyr. The first DMATS (FgaPT2) identified in *A. fumigatus* is involved in the biosynthesis of fumigaclavine C; in contrast to this, 7-DMATS catalyses the prenylation of Trp at the C-7 position, instead of at the C-4 position, of the indole ring.

## METHODS

**Computer-assisted sequence analysis.** FGENESH (Softberry; [www.softberry.com/berry.phtml](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. Sequence similarities were obtained by alignments of amino acid sequences using the BLAST 2 sequences program (release 2.9.9; <http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>).

**Chemicals.** The trisammonium salt of dimethylallyl diphosphate (DMAPP) was synthesized using methods analogous to those used for

the synthesis of trisammonium geranyl diphosphate reported by Woodside *et al.* (1988). Geranyl diphosphate (GPP) was kindly provided by Wessjohann. The peptides used in this study were purchased from Bachem.

**Bacterial strains, plasmids and culture conditions.** pGEM-T and pQE60 were obtained from Promega and Qiagen, respectively. A Uni-ZAP XR premade library of *A. fumigatus* strain B5233 (ATCC 13073) was purchased from Stratagene, and used to obtain phagemids as cDNA templates for PCR amplification. *E. coli* XL1 Blue MRF' (Stratagene) was used for cloning and expression experiments, and it was grown in liquid Luria-Bertani (LB) medium, or on solid LB medium with 1.5% (w/v) agar, at 37 °C (Sambrook & Russell, 2001). Addition of 50 µg carbenicillin ml<sup>-1</sup> was used for selection of recombinant *E. coli* strains.

**DNA isolation, PCR amplification and cloning.** Standard procedures for DNA isolation and manipulation were performed, as described (Sambrook & Russell, 2001). PCR amplification was carried out on an iCycler from Bio-Rad. Using the Expand High Fidelity kit (Roche Diagnostics), a PCR fragment of 1425 bp, containing the entire coding sequence of *Afu3g12930*, was amplified from the cDNA library by using the primers 7-dmats-1 (5'-CACCATGGCCA-TCGGAGCCGAGAT-3') at the 5' end, and 7-dmats-2 (5'-TGCAGATCTGCTGTACACCCGGAG-3') at the 3' end of the gene. Bold letters represent mutations inserted to give the underlined restriction sites *NcoI*, located at the start codon in 7-dmats-1, and *BglII*, located at the predicted stop codon in 7-dmats-2. The PCR fragment was cloned into pGEM-T, resulting in plasmid pLW39, which has subsequently had its sequence confirmed (MWG-Biotech). To create the expression vector pLW40, pLW39 was digested with *NcoI* and *BglII*, and the resulting *NcoI*-*BglII* fragment of 1418 bp was ligated into pQE60, which had been digested with the same enzymes.

**Overproduction and purification of His<sub>6</sub>-7-DMATS protein.** For gene expression, *E. coli* XL1 Blue MRF' cells harbouring the plasmid pLW40 were cultivated in 300 ml Erlenmeyer flasks containing 100 ml liquid LB medium, supplemented with carbenicillin (50 µg ml<sup>-1</sup>), and grown at 37 °C to an OD<sub>600</sub> of 0.6. For induction, IPTG was added to a final concentration of 0.8 mM, and the cells were cultivated for a further 16 h at 37 °C, before harvest. The bacterial cultures were centrifuged, and the pellets were resuspended in lysis buffer (10 mM imidazole, 50 mM NaH<sub>2</sub>PO<sub>4</sub> and 300 mM NaCl, pH 8.0) at between 2 and 5 ml (g wet weight)<sup>-1</sup>. After addition of 1 mg lysozyme ml<sup>-1</sup>, and incubation on ice for 30 min, the cells were sonicated six times, for 10 s each time, at 200 W. To separate the cellular debris from the soluble proteins, the lysate was centrifuged at 14 000 g for 30 min at 4 °C. One-step purification of the recombinant His<sub>6</sub>-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 in 50 mM NaH<sub>2</sub>PO<sub>4</sub> and 300 mM NaCl, pH 8.0. In order to change the buffer, the protein fraction was passed through a NAP-5 column (GE Healthcare), which had been equilibrated with 50 mM Tris/HCl and 15% (v/v) glycerol, pH 7.5. His<sub>6</sub>-7-DMATS was eluted with the buffer described, and stored at -80 °C for enzyme assays.

**Protein analysis.** Proteins were analysed by SDS-PAGE, which was carried out according to the method of Laemmli (1970), and they were stained with Coomassie brilliant blue G-250.

**Assays for 7-DMATS activity.** All the enzyme assays contained 50 mM Tris/HCl, pH 7.5, 1.2–3.8% (v/v) glycerol, and 10 mM CaCl<sub>2</sub>. They differed from each other by incubation volume and time, substrate concentration, and amount of recombinant 7-DMATS. The reaction mixtures were incubated at 37 °C, and the reactions were

terminated by addition of 10  $\mu$ l TCA (1.5 M) per 100  $\mu$ l reaction volume. After removal of the protein by centrifugation (14 000 g, 10 min, 4 °C), the enzymic products were analysed on an HPLC system, as described below. The standard assays for determination of the substrate specificity (100  $\mu$ l) contained 2 mM L-Trp or another aromatic substrate, 1 mM DMAPP and 2.5  $\mu$ g purified 7-DMATS, and they were incubated for 45 min. Two independent assays were carried out for quantification. The assay for the isolation of the enzymic product for structural elucidation (5 ml) contained 400  $\mu$ g purified 7-DMATS, and was incubated for 16 h. The reaction mixture was concentrated on a rotation evaporator at 35 °C to a volume of 750  $\mu$ l, before injection of 100  $\mu$ l into HPLC for isolation of 7-dimethylallyltryptophan (7-DMAT). The assays for determination of the kinetic parameters of L-Trp contained 1 mM DMAPP, 2.5  $\mu$ g 7-DMATS, and L-Trp at a final concentration of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 or 5.0 mM, in a total volume of 100  $\mu$ l. The incubation time was 45 min. For determination of the kinetic parameters of DMAPP, 2.5  $\mu$ g 7-DMATS, 1 mM L-Trp, and DMAPP at a final concentration of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5 or 1.0 mM, were used.

**HPLC analysis and isolation of 7-DMAT.** Reaction mixtures of 7-DMATS were analysed on an Agilent HPLC Series 1100 by using an Eclipse XBD-C18 column (4.6  $\times$  150 mm, 5  $\mu$ m) at a flow rate of 1 ml min<sup>-1</sup>. Water (solvent A) and acetonitrile (solvent B), each containing 0.5% (v/v) TFA, were used as solvents. A gradient was run from 15 to 70% solvent B in 15 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 85% solvent A for 5 min. The substances were detected with a Photo Diode Array detector. 7-DMAT was isolated under the same conditions.

**Spectroscopic data.** A <sup>1</sup>H-NMR spectrum of 7-DMAT was taken on an Avance DRX 500 spectrometer (Bruker) using 0.1 M DCl in D<sub>2</sub>O as the solvent. The solvent signal at 4.81 p.p.m. was used as reference.  $\delta$  (p.p.m.): 7.53 (d, 7.6 Hz, H-4), 7.32 (s, H-2), 7.12 (t, 7.9 Hz, H-5), 7.10 (d, 8.2, H-6), 5.46 (br t, 7.0 Hz, H-2'), 4.39 (t, 6.1, H-11), 3.58 (d, 7.0 Hz, 2H-1'), 3.52 (dd, 15.5, 5.4 Hz, H-10), 3.43 (dd, 15.5, 7.0 Hz, H-10), 1.74 (s, 3H-5'), 1.72 (s, 3H-4').

Positive and negative electrospray ionization (ESI) mass spectra were obtained with a ThermoFinnigan TSQ Quantum. The mass spectrometer was coupled with an Agilent HPLC series 1100 equipped with an RP18-column (2  $\times$  250 mm, 5  $\mu$ m). For separation, the column was run with 10% solvent B (CH<sub>3</sub>OH) in solvent A (H<sub>2</sub>O) [each containing 0.1% (v/v) HCOOH] for 5 min, followed by a gradient from 10 to 100% solvent B over 30 min. After washing with 100% solvent B for 10 min, the column was equilibrated with 10% solvent B for 10 min. The flow rate was at 0.2 ml min<sup>-1</sup>.

**MS data of 7-DMAT.** Positive ESI-MS: [M + H]<sup>+</sup>:  $m/z$  273.1;  $m/z$  of [M + H]<sup>+</sup>: 272.9 (5), 256.0 (100), 240.8 (9), 214.0 (64), 212.0 (24), 199.9 (25), 156.0 (6). Negative ESI-MS: [M - H]<sup>-</sup>:  $m/z$  271.2;  $m/z$  of [M - H]<sup>-</sup>: 271.2 (100), 227.3 (40), 210.3 (65), 184.3 (94).

**MS data of the prenylated H-L-Trp-L-Gly-OH.** Positive ESI-MS: [M + H]<sup>+</sup>:  $m/z$  330.2;  $m/z$  of [M + H]<sup>+</sup>: 313.0 (25), 271.1 (34), 257.0 (41), 244.9 (16), 238.0 (47), 227.0 (7), 212.0 (100), 198.1 (25), 156.1 (11). Negative ESI-MS: [M - H]<sup>-</sup>:  $m/z$  328.4;  $m/z$  of [M - H]<sup>-</sup>: 328.2 (100), 284.3 (31), 225.3 (6), 198.2 (10), 131.1 (39).

**MS data of the prenylated cyclo-L-Trp-L-Gly.** Positive ESI-MS: [M + H]<sup>+</sup>:  $m/z$  312.2;  $m/z$  of [M + H]<sup>+</sup>: 256.0 (9), 198.1 (100). Negative ESI-MS: [M - H]<sup>-</sup>:  $m/z$  310.6;  $m/z$  of [M - H]<sup>-</sup>: 310.2 (31), 113.1 (100).

## RESULTS

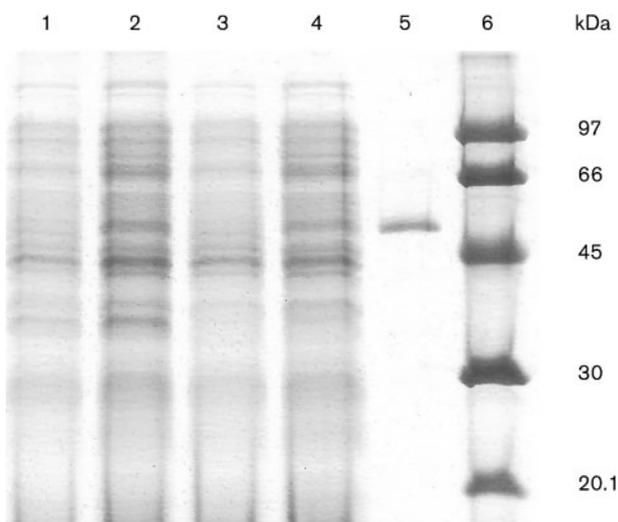
### Sequence analysis of the putative prenyltransferase gene *Afu3g12930* from *A. fumigatus*

The nucleotide sequence of the genomic DNA from *A. fumigatus* Af293 is available at GenBank under accession number AAHF01000002.1, and the genomic sequence of the putative prenyltransferase gene *Afu3g12930* spans bp 640 080–641 551, and consists of two exons of 1301 and 118 bp, respectively, interrupted by an intron of 53 bp; this was confirmed by sequencing the PCR products amplified from the cDNA of *A. fumigatus* strain B5233 (data not shown; GenBank accession no. EF539173). The predicted gene product of *Afu3g12930* is EAL92290, which comprises 472 aa, and has a calculated molecular mass of 53 kDa. EAL92290 (7-DMATS) shows significant sequence similarity to aromatic prenyltransferases from various fungi. For example, by using the BLAST 2 sequences program, we found that 7-DMATS shares a sequence identity of 34% with SirD from *L. maculans* (Gardiner & Howlett, 2005) at the amino acid level, 31% with FgaPT2 (DMATS) from *A. fumigatus* (Unsöld & Li, 2005), 28% with DMATS from *C. purpurea* and *Claviceps fusiformis* (Tsai *et al.*, 1995; Tudzynski *et al.*, 1999), and 28% with FtmPT1 (Grundmann & Li, 2005) and 26% with FgaPT1 (Unsöld & Li, 2006), which are both from *A. fumigatus*. SirD is proposed to catalyse the *O*-prenylation of L-Tyr or cyclo-L-Tyr-L-Ser in the biosynthesis of sirodesmin (Gardiner *et al.*, 2004). DMATS from different sources catalyse the prenylation of L-Trp at the C-4 position of the indole ring in the biosynthesis of ergot alkaloids (Li & Unsöld, 2006; Schardl *et al.*, 2006). FtmPT1 and FgaPT1 have been found to catalyse the prenylation reactions at the C-2 position of the indole rings of brevianamide F and fumigaclavine A (Grundmann & Li, 2005; Unsöld & Li, 2006), respectively. Based on the high sequence similarity to SirD, we speculate that 7-DMATS could be responsible for a transfer reaction of a prenyl moiety to L-Tyr or its derivatives.

### Cloning of *Afu3g12930*, and overproduction and purification of His<sub>6</sub>-7-DMATS

The coding region of *Afu3g12930* was amplified using PCR from cDNA of *A. fumigatus* strain B5233 (available in the form of phagemids isolated from a cDNA library), and cloned into the cloning vector pGEM-T. For gene expression, the coding sequence of *Afu3g12930* was released from pGEM-T, and cloned into the vector pQE60 (see Methods), resulting in the expression plasmid pLW40.

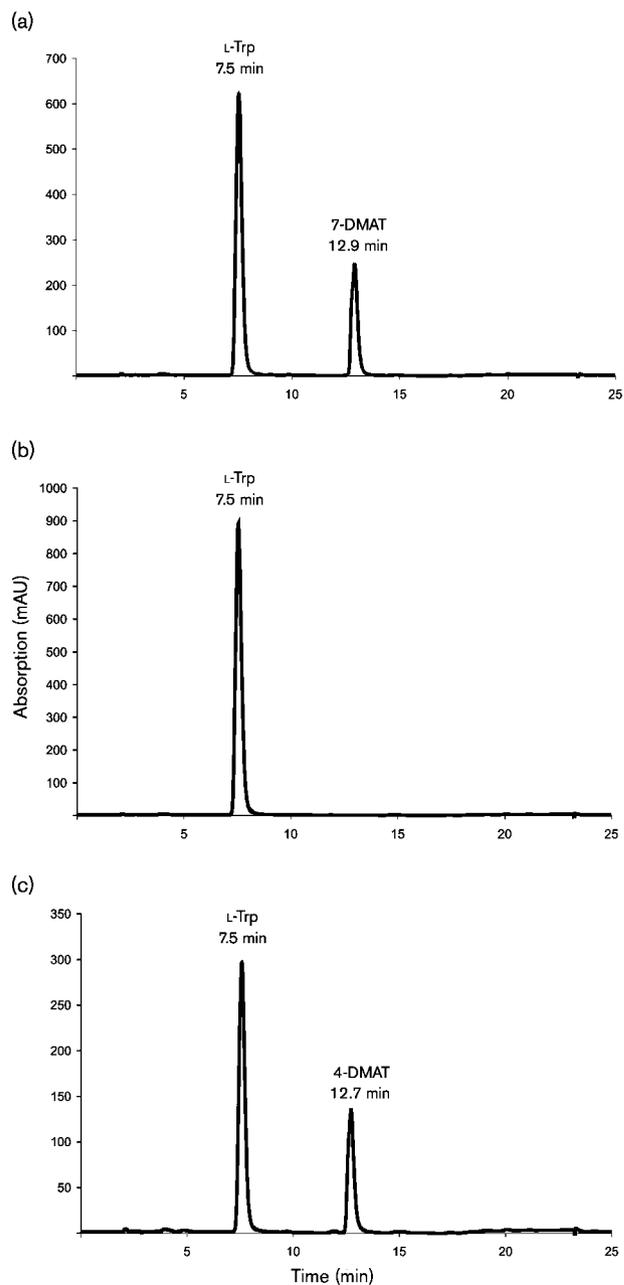
*E. coli* cells harbouring pLW40 were induced by 0.8 mM IPTG at 37 °C. His<sub>6</sub>-7-DMATS was purified with Ni-NTA agarose to apparent homogeneity, as judged by SDS-PAGE (Fig. 1), and a protein yield of 5 mg purified His<sub>6</sub>-tagged 7-DMATS per litre of culture was obtained. The observed molecular mass was 50 kDa, and this corresponded well to the calculated value of 54 kDa for His<sub>6</sub>-7-DMATS.



**Fig. 1.** Analysis of the overproduction and purification of His<sub>6</sub>-7-DMATS. The proteins were separated on a 12% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue G-250. Lanes: 1, total proteins before induction; 2, total proteins after induction with 0.8 mM IPTG at 37 °C for 16 h; 3, soluble proteins before induction; 4, soluble proteins after induction; 5, purified His<sub>6</sub>-7-DMATS; 6, molecular mass standard.

### Enzymic activity and substrate specificity of 7-DMATS

Initial attempts to detect the enzymic activity were carried out by incubations of 7-DMATS with L-Tyr and *cyclo*-L-Tyr-L-Ser in the presence of DMAPP or GPP. HPLC analysis was used to monitor the formation of an enzymic product. However, no product peak could be detected under different conditions, including various substrate concentrations and amounts of purified 7-DMATS, different metal ions with various concentrations, and different pH values and buffer compositions (data not shown). After the unsuccessful attempts, we decided to test other aromatic amino acids and their derivatives. HPLC analysis of the incubation mixture of L-Trp and DMAPP showed a significant product peak with a retention time of 12.9 min; this peak was absent in the reaction mixture containing heat-denatured enzyme (Fig. 2). Dependence of the product formation on the amount of protein was found up to 4 µg per 100 µl assay, and on a reaction time of up to 50 min. Product formation was strictly dependent on the presence of His<sub>6</sub>-7-DMATS, L-Trp and DMAPP. The enzyme 7-DMATS was found to be specific for DMAPP. Product formation was observed with DMAPP only, and not with GPP. In contrast, 7-DMATS showed relative flexible substrate specificity towards its aromatic substrates. D-Trp was accepted by 7-DMATS, with a relative activity of 15.5% of its enantiomer L-Trp. Linear and cyclic dipeptides were also substrates of 7-DMATS. Product formation was clearly observed when H-L-Trp-L-Gly-OH or *cyclo*-L-Trp-L-Gly was used instead of L-Trp in



**Fig. 2.** HPLC chromatograms of enzyme assays with His<sub>6</sub>-7-DMATS (a), with heat-inactivated His<sub>6</sub>-7-DMATS by boiling of the enzyme solution for 20 min (b), and with His<sub>6</sub>-FgaPT2 (c). The reaction mixtures contained 1 mM DMAPP, L-Trp (2 mM for 7-DMATS assays, and 1 mM for the FgaPT2 assay), and 4 µg recombinant 7-DMATS or 1 µg recombinant FgaPT2, and they were incubated for 16 h. The substances were detected with a Photo Diode Array detector, and illustrated at 277 nm.

the incubation mixtures (Table 1). However, the linear tripeptide L-Trp-L-Trp-L-Trp was not accepted by 7-DMATS. No product formation was observed in incubation mixtures with L-Phe, or L-Phe-containing cyclic dipeptides (Table 1). These results demonstrated that

**Table 1.** Substrate specificity of 7-DMATS towards different aromatic substrates

For incubation conditions, see Methods.

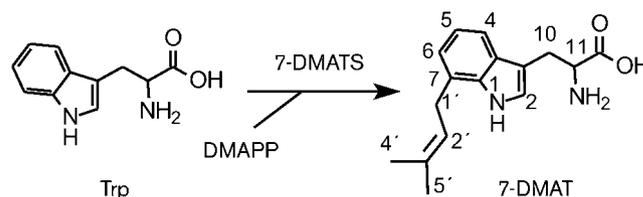
Substrate	Relative activity (%)
L-Trp	100.0 ± 1.5
D-Trp	15.5 ± 3.1
H-L-Trp-L-Gly-OH	10.9 ± 1.1
cyclo-L-Trp-L-Gly	1.8 ± 0.2
H-L-Trp-L-Trp-L-Trp-OH	<0.3
L-Tyr	<0.3
cyclo-L-Tyr-L-Pro	<0.3
cyclo-L-Tyr-L-Ser	<0.3
L-Phe	<0.3
cyclo-L-Phe-L-Phe	<0.3
cyclo-L-Phe-L-His	<0.3

L-Trp was the best substrate for 7-DMATS, under our test conditions.

### Identification of the enzymic products

For structural elucidation, the enzymic product of L-Trp was isolated on a preparative scale, and its structure was elucidated by NMR and MS analysis.

ESI-MS proved that the enzymic product is a prenylated derivative of Trp by detection of  $[M+H]^+$  and  $[M-H]^-$  at  $m/z$  273 and  $m/z$  271, respectively. Comparison of the  $^1\text{H-NMR}$  spectrum of the isolated compound with that of L-Trp revealed the presence of signals for a dimethylallyl moiety at 5.46 (br t, 7.0 Hz, H-2'), 3.58 (d, 7.0 Hz, 2H-1'), 1.74 (s, 3H-5') and 1.72 p.p.m. (s, 3H-4'). Correspondingly, signals for four protons instead of five protons were found in the region of the aromatic protons of the isolated compound, i.e. signals for three vicinal protons at 7.53 (d, 7.6 Hz), 7.12 (t, 7.9 Hz) and 7.10 p.p.m. (d, 8.2), and a singlet at 7.32 p.p.m. This indicated that the prenylation had taken place at the C-4 or C-7 position of the indole moiety. The spectrum of the isolated compound differed clearly from that of 4-DMAT for all of the protons, with the exception of the proton at C-2 (Unsöld & Li, 2005). 4-DMAT, the first pathway-specific intermediate in the biosynthesis of ergot alkaloids (Schardl *et al.*, 2006; Unsöld & Li, 2005), is also formed from L-Trp by a prenyl-transfer reaction catalysed by DMATS, e.g. FgaPT2 from *A. fumigatus* (Unsöld & Li, 2005). The enzymic product of 7-DMATS showed a similar, but different, retention time to that of FgaPT2 on the HPLC chromatogram (Fig. 2). Therefore, the enzymic product of 7-DMATS was identified as 7-DMAT (Fig. 3). The enzymic products of H-L-Gly-L-Trp-OH and cyclo-L-Trp-L-Gly were unequivocally proven as prenylated derivatives by detection of  $[M+H]^+$  at  $m/z$  330.2 and 312.2, as well as  $(M-H)^-$  at  $m/z$  328.4 and 310.6, respectively.

**Fig. 3.** Enzymic reaction catalysed by 7-DMATS.

### Biochemical properties and kinetic parameters of 7-DMATS

Testing with different ions showed that metal ions, such as  $\text{Mg}^{2+}$  or  $\text{Mn}^{2+}$ , are not essential for the prenyltransferase activity of 7-DMATS. In the presence of 10 mM EDTA, a chelating agent for divalent ions, a relative activity of 82% was observed, in comparison with the incubation mixture without additives. However, an increase in enzyme activity of up to 140% of that of the incubation without additives was detected in the presence of 10 mM  $\text{Ca}^{2+}$ . This finding is similar to those of some of the soluble aromatic prenyltransferases, e.g. CloQ from *Streptomyces roseochromogenes* (Pojer *et al.*, 2003), LtxC from *Lyngbya majuscula* (Edwards & Gerwick, 2004), DMATS from *C. purpurea* (Gebler & Poulter, 1992), and FgaPT1, FgaPT2 and FtmPT1 from *A. fumigatus* (Grundmann & Li, 2005; Unsöld & Li, 2005, 2006).

The 7-DMATS reaction apparently followed Michaelis–Menten kinetics. By using Hanes–Woolf and Lineweaver–Burk plots, the  $K_m$  values were determined as 137  $\mu\text{M}$  for L-Trp, and 67  $\mu\text{M}$  for DMAPP. The maximum reaction velocity observed with 7-DMATS was 0.21  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ . These values are in the normal range of those of enzymes involved in the biosynthesis of secondary metabolites. Given that L-Trp was shown to be the best substrate in our test conditions (see above), it is very likely to be the natural substrate of 7-DMATS.

### DISCUSSION

In this study, we cloned and overexpressed the putative prenyltransferase gene *Afu3g12930* from the fungus *A. fumigatus* in a heterologous host, and proved its function by biochemical characterization of the overproduced enzyme. Investigation of substrate specificity showed that 39 of 53 compounds were accepted by 7-DMATS, with the highest conversion rate for L-Trp.  $K_m$  values of six Trp derivatives, which had modifications to the side chain and the indole ring, were determined to be in the range of 190–460  $\mu\text{M}$ , which is higher than that for L-Trp (137  $\mu\text{M}$ ).  $K_m$  values of eight linear and cyclic Trp-containing dipeptides were found to be from 180 to 500  $\mu\text{M}$  (Kremer & Li, unpublished data). This indicated that the natural substrate of 7-DMATS is very likely to be L-Trp. However, it cannot be excluded that an unidentified free or enzyme-bound

dipeptide containing a Trp moiety (see below for the discussion on the role of the NRPS) is the natural substrate of the identified enzyme. The enzymic product of 7-DMATS was unequivocally identified as 7-DMAT by NMR and MS analysis. This demonstrated clearly that 7-DMATS functions as a dimethylallyltryptophan synthase. Interestingly, 7-DMATS shares a sequence identity of only 31% at the amino acid level with another DMATS, FgaPT2 (Unsöld & Li, 2005), from the same fungus; this level of identity is slightly higher than the sequence similarities between 7-DMATS and other indole prenyltransferases from *A. fumigatus*, e.g. 7-DMATS shares a sequence identity of 28% with FtmPT1, which catalyses the prenylation of the cyclic dipeptide *cyclo*-L-Trp-L-Pro at the C-2 position of the indole moiety (Grundmann & Li, 2005), and 26% with FgaPT1, which prenylates fumigaclavine A at the C-2 position of the indole moiety (Unsöld & Li, 2006). 7-DMATS accepted Trp, but not Tyr as a substrate. However, with the exception of its orthologues in *N. fischeri* NRRL181 and *A. terreus* NIH2624, with sequence identities of 95 and 82% (Table 2, see below) at the amino acid level, respectively, 7-DMATS showed the highest sequence similarity to SirD from the biosynthetic gene cluster of sirodesmin from *L. maculans* (Gardiner *et al.*, 2004), i.e. a sequence identity of 34% at the amino acid level. SirD has been proposed to catalyse the *O*-prenylation of L-Tyr, or its cyclic dipeptide, with Ser (Gardiner *et al.*, 2005). It would be interesting to prove the function of this putative prenyltransferase biochemically.

Divalent metal ions, such as Mg<sup>2+</sup> and Mn<sup>2+</sup>, are not essential for the enzymic reaction of 7-DMATS. However, like other indole prenyltransferases, such as FgaPT1, FgaPT2 and FtmPT1 mentioned above (Grundmann & Li, 2005; Unsöld & Li, 2005, 2006), as well as DMATS in the biosynthesis of ergot alkaloids from *Claviceps* (Lee *et al.*, 1976), Ca<sup>2+</sup> enhanced the enzymic reaction of 7-DMATS slightly. This finding, together with the sequence similarity, provided experimental data for the hypothesis that the indole prenyltransferases from fungi belong to the same

group of enzymes, and that they have evolved directly from a common ancestor.

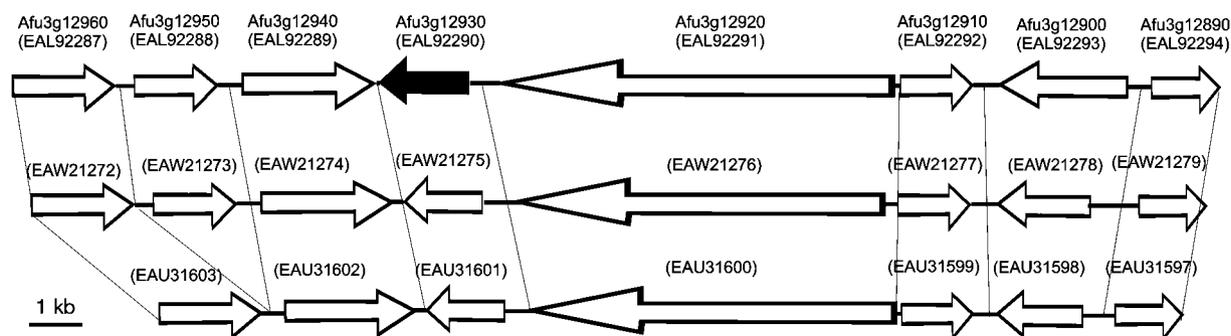
*Afu3g12930* belongs to a putative biosynthetic gene cluster consisting of probably eight genes (Fig. 4, Table 2): two putative transcription factor genes *Afu3g12940* and *Afu3g12890*, one putative transporter gene (*Afu3g12900*), one gene with unknown function (*Afu3g12950*), and four biosynthetic genes, i.e. the NRPS gene *Afu3g12920*, the prenyltransferase gene *Afu3g12930* described in this study, a gene coding for a putative cytochrome P450 enzyme (*Afu3g12960*), and a putative methyltransferase gene *Afu3g12910*. With the exception of *Afu3g12950*, orthologous genes were also found in the genome sequence of *N. fischeri* NRRL181 and *A. terreus* NIH2624, with the same orientation and same relative position of the genes (GenBank entries) (Fig. 4). The gene products of the cluster from *A. fumigatus* share sequence identities of 92–96% at the amino acid level with their orthologues from *N. fischeri*, and 73–88% with the orthologues from *A. terreus* (Table 2), respectively. The gene *Afu3g12950* in the gene cluster from *A. fumigatus* encodes a putative ubiquitin-conjugating enzyme. An orthologue of this gene was found in the gene cluster of *N. fischeri*, but not in the gene cluster of *A. terreus*. Therefore, it is questioned whether *Afu3g12950* is essential for the biosynthesis of the compound encoded by this gene cluster.

The end product of the eight-gene cluster is unknown (Cramer *et al.*, 2006b; Nierman *et al.*, 2005). Deletion of the NRPS gene *Afu3g12920* from the genome of *A. fumigatus* CEA17Δ*akuB* (Silva Ferreira *et al.*, 2006) by T. Heinekamp and A. A. Brakhage (HKI, Jena, Germany), and HPLC analysis of the cultural extracts by our group, showed that no changes of the profile of the secondary metabolite production could be observed in the obtained mutants, in comparison with that of the wild-type (data not shown). This indicated that one or more genes of this cluster were not expressed, or were expressed at very low levels, so that no product of this cluster accumulated in the

**Table 2.** ORFs of the putative cluster in *A. fumigatus* Af293

<i>A. fumigatus</i> ORF	Identity* to genes of				Putative function
	<i>N. fischeri</i>	<i>A. terreus</i>	Gliotoxin cluster	Sirodesmin cluster	
EAL92287	EAW21272 (92)	EAU31603 (85)	GliC (32)	SirC (39)	Cytochrome P450 monooxygenase
EAL92288	EAW21273 (95)	–	–	–	Ubiquitin conjugating enzyme
EAL92289	EAW21274 (96)	EAU31602 (84)	–	–	C-6 transcription factor
EAL92290 (7-DMATS)	EAW21275 (95)	EAU31601 (82)	–	SirD (34)	Prenyltransferase
EAL92291	EAW21276 (93)	EAU31600 (76)	GliP (29)	SirP (36)	NRPS containing A1-T1-C1-A2-T2-C2-T3
EAL92292	EAW21277 (96)	EAU31599 (88)	GliM (34)	SirM (38)	Methyltransferase
EAL92293	EAW21278 (96)	EAU31598 (87)	–	–	Transporter
EAL92294	EAW21279 (93)	EAU31597 (73)	GliZ (27)	SirZ (23)	C-6 transcription factor

\*Percentage similarity values of the *A. fumigatus* ORFs to those of *N. fischeri*, *A. terreus*, and the gliotoxin and sirodesmin clusters, are given in parentheses.



**Fig. 4.** Putative unknown gene cluster from different *Aspergillus* strains. Top, *A. fumigatus* Af293; middle, *N. fischeri* (*Aspergillus fischerianus*) NRRL181; bottom, *A. terreus* NIH2624. Introns are not shown. The deduced protein names are given in parentheses.

wild-type, and, therefore, no effect on secondary metabolite production could be observed after deletion of a gene from the cluster. This finding is in accordance with the results reported by Cramer *et al.* (2006b) that *Afu3g12920* is not expressed, or is expressed at very low levels, under the conditions tested in their study. However, it can not be excluded that this cluster could be expressed *in situ* after inhalation of the fungus, and the secondary metabolite may then play a role as virulence factor.

Comparison with database entries revealed that the four biosynthesis enzymes EAL92287, EAL92290 (7-DMATS), EAL92291 and EAL92292 share sequence identities of 39, 34, 36 and 38% with SirC, SirD, SirP and SirM, respectively (Table 2), which have been identified in the gene cluster of sirodesmin, which is an ETP from *L. maculans* (Gardiner *et al.*, 2004). Sequence identities of 32, 29 and 34% were also observed between EAL92287 and GliC, EAL92291 and GliP, as well as EAL92293 and GliM, respectively (Table 2). GliP, GliC and GliM are members of the gene cluster of the ETP gliotoxin from *A. fumigatus* (Gardiner & Howlett, 2005). The sequence identities of EAL92294 to SirZ and GliZ were found to be 23 and 27%, respectively (Table 2). Therefore, the cluster discussed here is proposed to function in the biosynthesis of an ETP derivative (Cramer *et al.*, 2006b; Nierman *et al.*, 2005).

However, database and literature searches revealed that none of the known ETP derivatives contain a prenylated Trp moiety (Gardiner *et al.*, 2005). Also, and more importantly, a homologue of the putative thioredoxin reductase, GliT/SirT, in the gene cluster of gliotoxin/sirodesmin, which is proposed to be responsible for the formation of the disulphide bond of the ETP derivatives (Gardiner *et al.*, 2005; Gardiner & Howlett, 2005), is absent in the cluster. Therefore, it could be speculated that the cluster discussed here is not intact, or that the product of the gene cluster has a structure other than an ETP or a derivative thereof. Considering the fact that the cluster is present in the three fungi mentioned above, it seems that the latter is the most plausible explanation. The product of the gene cluster could

be a derivative of a cyclic dipeptide containing 7-DMAT. It is plausible that L-Trp is converted to 7-DMAT, and that this is catalysed by the prenyltransferase 7-DMATS characterized in this study. 7-DMAT would then undergo the condensation with a second amino acid, which could not be predicted by the sequence analysis, to a prenylated (cyclic) dipeptide, under the catalysis of the putative NRPS EAL92291. Like GliP, EAL92291 has a trimodular architecture (A<sub>1</sub>-T<sub>1</sub>-C<sub>1</sub>-A<sub>2</sub>-T<sub>2</sub>-C<sub>2</sub>-T<sub>3</sub>) (Balibar & Walsh, 2006). This is in contrast to that of SirP of the sirodesmin cluster, and FtmPS for the formation of *cyclo*-L-Trp-L-Pro from the fumitremorgin cluster, as these do not contain the third thiolation domain T<sub>3</sub> (Gardiner *et al.*, 2004; Maiya *et al.*, 2006). It has been proven by biochemical investigation that the A<sub>1</sub> and A<sub>2</sub> domains in GliP are responsible for the activation of L-Phe and L-Ser, respectively, which are then loaded onto the T<sub>1</sub> and T<sub>2</sub> domains of GliP, respectively. The linear enzyme-bound peptide L-Phe-L-Ser-T<sub>2</sub> was formed under the catalysis of the condensation domain C<sub>1</sub> (Balibar & Walsh, 2006). The roles of the second condensation domain C<sub>2</sub>, and the third thiolation domain T<sub>3</sub>, are unclear, although the results of mutational experiments have suggested that they are involved in the same process (Balibar & Walsh, 2006). It has been speculated that the subsequent reactions to gliotoxin, e.g. methylation or sulphur insertion, may occur while the linear dipeptide is still covalently bound to the NRPS GliP (Balibar & Walsh, 2006). The release of an intermediate in the biosynthesis could occur at some later step. This could also be the case for EAL92291. This means that an unmodified cyclic dipeptide with the prenylated tryptophan is probably not involved in the biosynthesis of the unknown compound discussed here. The putative methyltransferase EAL92290 is probably responsible for the N-methylation of an enzyme-bound linear dipeptide or the diketopiperazine ring, similar to the proposed function of GliM and SirM in the biosynthesis of gliotoxin and sirodesmin (Gardiner & Howlett, 2005). The putative cytochrome P450 EAL92287 could be involved in the modification of the enzyme-bound dipeptide or a derivative thereof. The two regulators EAL92289 and EAL92294 are

probably involved in the regulation, and the putative transporter EAL92293 could be responsible for the efflux, of the product of the gene cluster from the fungus. Deletion of the genes from the genome of *N. fischeri* or *A. terreus*, in which this gene cluster is expressed, would explore the natural role of the gene cluster.

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