DOI: 10.1002/cbic.200800237

Two Lysine Residues are Responsible for the Enzymatic Activities of Indole Prenyltransferases from Fungi

Edyta Stec,[a] Nicola Steffan,[a] Anika Kremer,[a] Huixi Zou,[a, b] Xiaodong Zheng,[b] and Shu-Ming Li*[a]

Recently, we identified a new family of aromatic prenyltransferases from fungi by molecular cloning and subsequent biochemical investigation. These enzymes catalyse the transfer reactions of prenyl moieties like dimethylallyl diphosphate (DMAPP) onto different positions of the indole rings of diverse secondary metabolites. For example, FgaPT1 and FgaPT2 from *Aspergillus fumigatus* are involved in the biosynthesis of fumigaclavine C and catalyse the prenylation of fumigaclavine A at position C2 and the prenylation of L-tryptophan at position C4 of the indole ring, respectively.[1,2] Therefore, FgaPT2 functions as a 4-dimethylallyltryptophan synthase. A second dimethylallyltryptophan synthase (DMATS) from the same fungus, 7-DMATS, catalyses the prenylation of L-tryptophan at position C7 of the indole ring.[3] From a biosynthetic gene cluster of fumitremorgin B in *A. fumigatus*, FtmPT1 was proven to catalyse the prenylation of cyclo-L-Trp-L-Pro (brevianamide F) at position C2 of the indole ring.[4] An additional prenyltransferase from *A. fumigatus*, CdpNPT, was found to catalyse the prenylation of tryptophan-containing cyclic dipeptides at position N1 of the indole rings.[5] From *Aspergillus nidulans*, a gene cluster for the biosynthesis of terrequinone A was identified by genomic mining,[6] and TdiB in this cluster was reported to be responsible for two prenylation steps.[7,8]

All of the indole prenyltransferases from fungi, including the previously reported dimethylallyltryptophan synthase DmaW from *Claviceps purpurea*,[9,10] show remarkable sequence similarities to each other at the amino acid level (see Table S1 in the Supporting Information). However, they do not show significant sequence similarities either to *trans*-prenyltransferases in the biosynthesis of terpenoids[11] or to membrane-bound aromatic prenyltransferases[12,13] or soluble ABBA prenyltransferases from bacteria.[14–17] The enzymatic reactions catalysed by indole prenyltransferases from fungi are independent of the presence of divalent metal ions, although calcium ions have been found to enhance the activities of some enzymes.[1,4,7,18] This behaviour is in contrast to those of *trans*-prenyltransferases and of membrane-bound aromatic prenyltransferases.[12,13,19] In these prenyltransferases, DMAPP or other prenyl diphosphates are bound by Mg^{2+} through (D/N)DXXD motifs,[12,13,19] which are absent in the fungal aromatic prenyltransferases[1–4,7,18,20] and ABBA prenyltransferases from bacteria.[17]

In contrast to the prenyltransferases that contain (D/N)DXXD motifs[11–13,19,21] and the ABBA prenyltransferases from bacteria,[17] no data are available on protein structures or substrate binding sites for the indole prenyltransferases from fungi. Based on the fact that all of the indole prenyltransferases from the above-mentioned fungi show similar biochemical properties and accept only DMAPP but not other prenyl diphosphates,[1–4,7,18] it is plausible that the binding sites for DMAPP are identical or at least similar in these prenyltransferases.

Sequence comparison of seven prenyltransferases from fungi, which were verified experimentally by molecular cloning and biochemical characterisation, revealed that these enzymes share sequence identities between 20 and 55% at the amino acid level (Table S1). By alignment of the amino acid sequences of these prenyltransferases, highly conserved regions could not be detected (data not shown). However, two motifs containing three conserved basic amino acids could be identified (Figure 1), that is, K186, R262 and K264 for DmaW-Cp from *C. purpurea*; K211, R281 and K283 for FgaPT1; K187, R257 and K259 for FgaPT2; K201, R292 and K294 for FtmPT1; K239, R311 and K313 for 7-DMATS; and K219, R284 and K286 for CdpNPT—all from *A. fumigatus*—as well as K165, R236 and K238 for TdiB from *A. nidulans*. The two conserved lysine residues in motifs I and II are separated by 66 to 92 amino acids. In motif II, the arginine and the second lysine residue are separated from each other only by one single amino acid. In addition, an aspartate residue that is eight amino acids away from the mentioned arginine was also found in motif II (Figure 1).

Structural analysis of the ABBA prenyltransferase NphB, which requires Mg^{2+} for its enzymatic activity, revealed that D62 is involved in the binding of prenyl diphosphate through Mg^{2+}.[16] Structural analysis additionally showed that basic amino acids, such as lysine and arginine, are also involved in the binding of phosphate residues in NphB,[16] as initially proposed for prenyl diphosphate binding in a farnesyl diphosphate (FPP) synthase.[22] Site-directed mutagenesis experiments indicated that two nearby arginine residues in the FPP synthase are important for catalysis.[23] In the Mg^{2+}-independent prenyltransferases CloQ and Fqn26 from the same protein family of NphB, basic amino acids are very likely directly involved in the binding of prenyl diphosphates, as suggested by modelling studies.[17] Based on these results, it can be speculated that basic amino acids in motifs I and II of the indole prenyltransferases from the mentioned-above fungi could also be involved in the direct binding of DMAPP, and, therefore, are essential for enzymatic activity.

To investigate the importance of these amino acid residues for the enzymatic activities of fungal indole prenyltransferases,

[a] E. Stec, N. Steffan, A. Kremer, H. Zou, Prof. Dr. S.-M. Li
Institut für Pharmazeutische Biologie und Biotechnologie
Heinrich-Heine-Universität Düsseldorf
Universitätsstrasse 1, 40225 Düsseldorf (Germany)
Fax: (+49) 211-81-10617
E-mail: shuming.li@uni-duesseldorf.de

[b] H. Zou, Prof. Dr. X. Zheng
Department of Food Science and Nutrition
Zhejiang University, Hangzhou 310029, Zhejiang (China)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

		Motif I			Motif II	
DmaW-Cp	185	**LK**V**Y**LY**P**HL**KS**IA**TG**V	200	252	**S**CD**LV**D**P**S**KSRIKIY**L	267
FgaPT2	186	**LK**T**Y**IY**P**AL**K**AVV**TG**K	201	247	**S**CD**L**TS**PAKSRIKIY**L	262
FgaPT1	210	**LK**S**YF**M**P**AIR**S**AI**TG**V	225	271	**S**Y**D**A**V**DAC**KARIKIY**T	286
FtmPT1	200	V**K**A**YF**Y**P**QP**KS**AV**TG**R	215	282	**ST**D**LV**E**P**G**KSR**V**K**F**Y**A	297
7-DMATS	238	T**K**A**YF**F**P**ILM**S**LK**TG**Q	253	301	**S**V**D**C**V**NEAD**SRIKIY**V	316
CdpNPT	218	A**K**E**YF**F**P**GI**K**CAA**TG**Q	233	274	CC**DLV**D**P**AHT**R**F**K**V**Y**I	289
TdiB	164	**LK**F**Y**IPSVR**K**ALA**TG**Q	179	226	CL**D**PRTHKNA**R**V**K**C**Y**L	241

Figure 1. Conserved amino acid residues in indole prenyltransferases from fungi. DmaW-Cp: 4-dimethylallyltryptophan synthase from *Claviceps purpurea* (accession no.: Q6X2E0); FgaPT2: 4-dimethylallyltryptophan synthase from *A. fumigatus* (AAX08549); FgaPT1: fumigaclavine A prenyltransferase from *A. fumigatus* (XP_756136); FtmPT1: brevianamide F prenyltransferase from *A. fumigatus* (AAX56314); 7-DMATS: 7-dimethylallyltryptophan synthase from *A. fumigatus* (ABS89001); CdpNPT: cyclic dipeptide *N*-prenyltransferase from *A. fumigatus* (ABR14712); TdiB: didemethylasterriquinone D prenyltransferase from *A. nidulans* (ABU51603).

we chose FgaPT2, FtmPT1 and 7-DMATS for site-directed mutagenesis experiments (see the Supporting Information). Primers for PCR amplification are listed in Table 1 and the template plasmids were described previously.[3,4,24] The resulting plasmids pES1 to pES12 (Table 1) from mutagenesis experiments were isolated and subjected to DNA sequencing, which confirmed the desired mutations in the respective constructs. Overproduction of the recombinant proteins was carried out in *E. coli* according to the conditions that were used for the expression of wild-type proteins in our previous studies.[3,4,24]

In these experiments, lysine residues were mutated to glutamate and arginine to glycine; this resulted in the mutant proteins listed in Table 1. Based on the fact that the enzymatic reactions of the prenyltransferases are independent of divalent metal ions, it is plausible that the acidic amino acids, such as the conserved aspartate residue in motif II (Figure 1), are not directly involved in the binding of prenyl diphosphate. This aspartate residue was mutated to histidine as a negative control (Table 1).

With the exception of the negative control for FgaPT2, FgaPT2_D249H, all mutant proteins could be overproduced as His-tagged fusion proteins with yields that were comparable to those of the corresponding wild-type proteins (Table S2), which were in the range of 2 to 5 mg purified protein per litre bacterial culture. Under the conditions used in this study, expression of pES4, which was constructed for overproduction of FgaPT2_D249H, was not detected. All of the overproduced proteins could be purified to homogeneity by Ni-NTA affinity chromatography as judged by SDS-PAGE analysis (Figure 2). The molecular mass of His_8–FgaPT2 and its derivatives was found to be 57 kDa, which corresponds very well to that reported previously (Figure 2).[24] The expected molecular mass of His_6–FtmPT1[4] and His_6–7-DMATS as well as their derivatives could also be found at the expected size of 50 kDa (Figure 2).

Table 1. Primers used for site-directed mutagenesis.

Primer[a]	Sequence (5′→3′)	Mutation		Construct/protein
		DNA	AA[b]	
FgaPT2_K187fw	ATGGCCGCTTTGCACTT**G**AGACGTACATATACCCG	A559→G	K187→E	pES1/FgaPT2_K187E
FgaPT2_K187rev	CGGGTATATGTACGTCT**C**AAGTGCAAAGCGGCCAT	T821→C	K187→E	
FgaPT2_R257fw	CAGTCCTGCCAAGTCG**G**GAATCAAGATCTACCTGC	A769→G	R257→G	pES2/FgaPT2_R257G
FgaPT2_R257rev	GCAGGTAGATCTTGATTC**C**CGACTTGGCAGGACTG	T612→C	R257→G	
FgaPT2_K259fw	TGCCAAGTCGAGAATC**G**AGATCTACCTGCTGGAGC	A775→G	K259→E	pES3/FgaPT2_K259E
FgaPT2_K259rev	GCTCCAGCAGGTAGATCT**C**GATTCTCGACTTGGCA	T606→C	K259→E	
FgaPT2_D249fw	GCCTAGTGTCCTGT**C**ATCTGACCAGTCCTGCCAAG	G745→C	D249→H	pES4/FgaPT2_D249H
FgaPT2_D249rew	CTTGGCAGGACTGGTCAGAT**G**ACAGGACACTAGGC	C636→G	D249→H	
FtmPT1_K201fw	GGGACCGTCCTGGTC**G**AGGCGTATTTCTACC	A601→G	K201→E	pES5/FtmPT1_K201E
FtmPT1_K201rev	GGTAGAAATACGCCT**C**GACCAGGACGGTCCC	T795→C	K201→E	
FtmPT1_R292fw	GAACCCGGCAAATCG**G**GGGTGAAGTTCTACGC	C874→G	R292→G	pES6/FtmPT1_R292G
FtmPT1_R292rev	GCGTAGAACTTCACCC**C**CGATTTGCCGGGTTC	G522→C	R292→G	
FtmPT1_K294fw	CGGCAAATCGCGGGTG**G**AGTTCTACGCCAGCGAG	A880→G	K294→E	pES7/FtmPT1_K294E
FtmPT1_K294rev	CTCGCTGGCGTAGAACT**C**CACCCGCGATTTGCCG	T516→C	K294→E	
FtmPT1_D284fw	CATTTCCTGTCCACC**C**ACCTCGTCGAACCCGGC	G850→C	D284→H	pES8/FtmPT1_D284H
FtmPT1_D284rev	GCCGGGTTCGACGAGGT**G**GGTGGACAGGAAATG	C546→G	D284→H	
7-DMATS_K239fw	CGGGTCGTGTCACCACA**G**AGGCGTACTTTTTCCCG	A715→G	K239→E	pES9/7-DMATS_K239E
7-DMATS_K239rev	CGGGAAAAAGTACGCCT**C**TGTGGTGACACGACCCG	T705→C	K239→E	
7-DMATS_R311fw	TGAACGAGGCAGACTCT**G**GGATCAAGATATACGTG	C931→G	R311→G	pES10/7-DMATS_R311G
7-DMATS_R311rev	CACGTATATCTTGATCC**C**AGAGTCTGCCTCGTTCA	G481→C	R311→G	
7-DMATS_K313fw	GGCAGACTCTCGGATC**G**AGATATACGTGCGGATGC	A937→G	K313→E	pES11/7-DMATS_K313E
7-DMATS_K313rev	GCATCCGCACGTATATCT**C**GATCCGAGAGTCTGCC	T983→C	K313→E	
7-DMATS_D303fw	GAGATGATCAGCGTC**C**ATTGCGTGAACGAGGCAGAC	G907→C	D303→H	pES12/7-DMATS_D303H
7-DMATS_D303rev	GTCTGCCTCGTTCACGCAAT**G**GACGCTGATCATCTC	C513→G	D303→H	

The mutations in the primer sequences are highlighted as underlined, bold letters; [a] fw: forward primer; rev: reverse primer; [b] AA: amino acid.

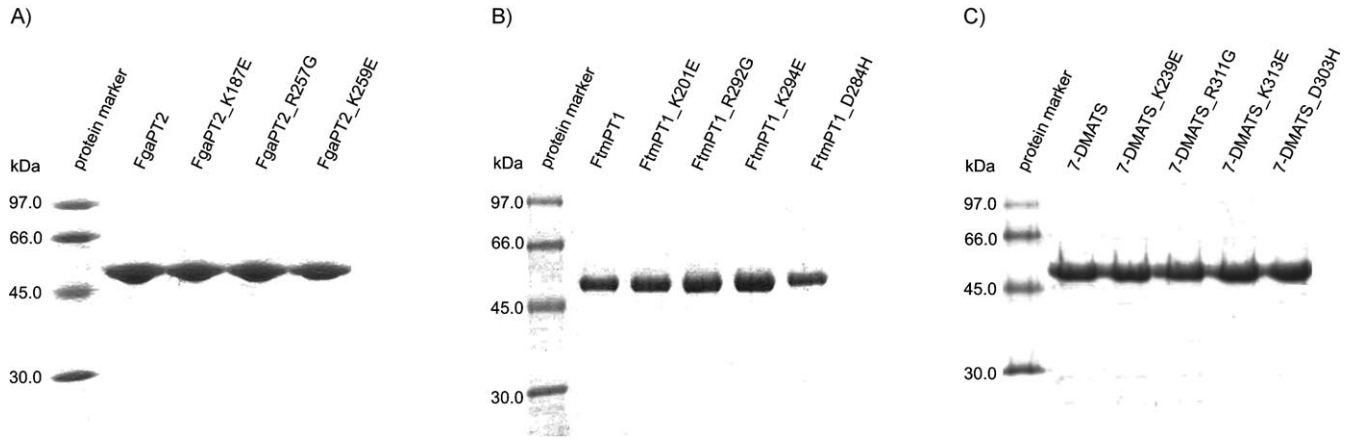

Figure 2. SDS-PAGE of the purified proteins; the gels (12%, *w/v*) were stained with Coomassie brilliant blue R-250; A) FgaPT2 and its mutant derivatives; B) FtmPT1 and its mutant derivatives; C) 7-DMATS and its mutant derivatives.

Enzymatic activities of the mutant proteins were determined by HPLC analysis after incubation with their respective substrates (see the Supporting Information). Recombinant enzymes without mutations were used as positive controls. Both FgaPT2 and 7-DMATS, as well as their mutant derivatives, were assayed by using L-tryptophan and DMAPP as substrates as described previously.[3,24] The assays for FtmPT1 and its mutant proteins were carried out by using brevianamide F and DMAPP as substrates as described previously.[4] Results of the activities of mutant and wild-type enzymes are summarised in Figure 3 and Table 2.

HPLC analysis (Figure 3) showed that product formation could be clearly detected in the incubation mixtures of all three wild-type enzymes, with conversion rates of 21.19% for FgaPT2, 10.62% for FtmPT1 and 9.85% for 7-DMATS (Table 1). In contrast, product was not detected in the incubation mixtures with proteins that had been mutated at the lysine residue in motif II, that is, with FgaPT2_K259E, FtmPT1_K294E or 7-DMATS_K313E. These results suggest that the lysine residue at this locus is essential for the enzymatic activity of these prenyltransferases and is probably involved in the binding of DMAPP. Product formation was not observed in the incubation mixtures of FtmPT1 and 7-DMATS derivatives that had been mutated at the conserved lysine residue in motif I, that is, FtmPT1_K201E and 7-DMATS_K239E. Only the corresponding derivative of FgaPT2, that is, FgaPT2_K187E, showed low enzymatic activity (2.8% of that of wild-type FgaPT2). This demonstrates the importance of this lysine residue, which was even essential for enzymatic activities of FtmPT1 and 7-DMATS. These results are consistent with the suggestion from the modelling studies that basic amino acids, for example, L54 in CloQ and R46 in Fqn26, could be involved in prenyl diphosphate binding by ABBA prenyltransferases from bacteria.[17] From our results, it could be speculated that these two lysine residues in fungal prenyltransferases are directly bound to the phosphate residues of DMAPP, and carbocation-mediated electrophile capture could be involved as proposed for the *trans*-prenyltransferase FPP synthase.[22] Mutation of the arginine residue in motif II had less of an effect on the enzymatic activities of FtmPT1 and 7-DMATS. Relative activities of 48.2 and 36.7% were found for FtmPT1_R292G and 7-DMATS_R311G, respectively, compared to the wild-type enzymes. This residue is unlikely to be involved in DMAPP binding by FtmPT1 and 7-DMATS. In contrast, mutation of the corresponding residue in FgaPT2 had more of an effect on the enzymatic activity. Only 4.7% of the enzymatic activity of FgaPT2 was found for FgaPT2_R257G; this indicates a possible role for the arginine during catalysis by FgaPT2. As expected, mutation at the aspartate residue had less of an effect on the enzymatic activity. The mutated proteins FtmPT1_D284H and 7-DMATS_D303H showed 33.0 and 42.5% relative activities compared to those of FtmPT1 and 7-DMATS, respectively. These results indicate that the conserved arginine and aspartate are very likely not involved in prenyl diphosphate binding by FtmPT1 and 7-DMATS. A weak binding by R257 in FgaPT2 is plausible.

In summary, we provide evidence for the first time that two lysine residues are important for enzymatic activities of the indole prenyltransferases from fungi and are very likely in-

Table 2. Prenyltransferase activities of purified proteins.

FgaPT2 and derivatives	Conversion rate [%]	Relative activity [%]	FtmPT1 and derivatives	Conversion rate [%]	Relative activity [%]	7-DMATS and derivatives	Conversion rate [%]	Relative activity [%]
FgaPT2	21.19 ± 2.18	100 ± 10.3	FtmPT1	10.62 ± 1.03	100 ± 9.7	7-DMATS	9.85 ± 1.23	100 ± 12.5
FgaPT2_K187E	0.6[a]	2.8	FtmPT1_K201E	≤ 0.05	≤ 0.6	7-DMATS_K239E	≤ 0.03	≤ 0.3
FgaPT2_R257G	1.00 ± 0.21	4.7 ± 1.0	FtmPT1_R292G	5.12 ± 0.36	48.2 ± 3.4	7-DMATS_R311G	3.61 ± 0.22	36.7 ± 2.3
FgaPT2_K259E	≤ 0.04	≤ 0.2	FtmPT1_K294E	≤ 0.05	≤ 0.6	7-DMATS_K313E	≤ 0.03	≤ 0.3
			FtmPT1_D284H	3.50 ± 0.29	33.0 ± 2.8	7-DMATS_D303H	4.19 ± 0.30	42.5 ± 3.0

For quantification two independent experiments were carried out. [a] Product formation was detected only in one of the incubation mixtures.

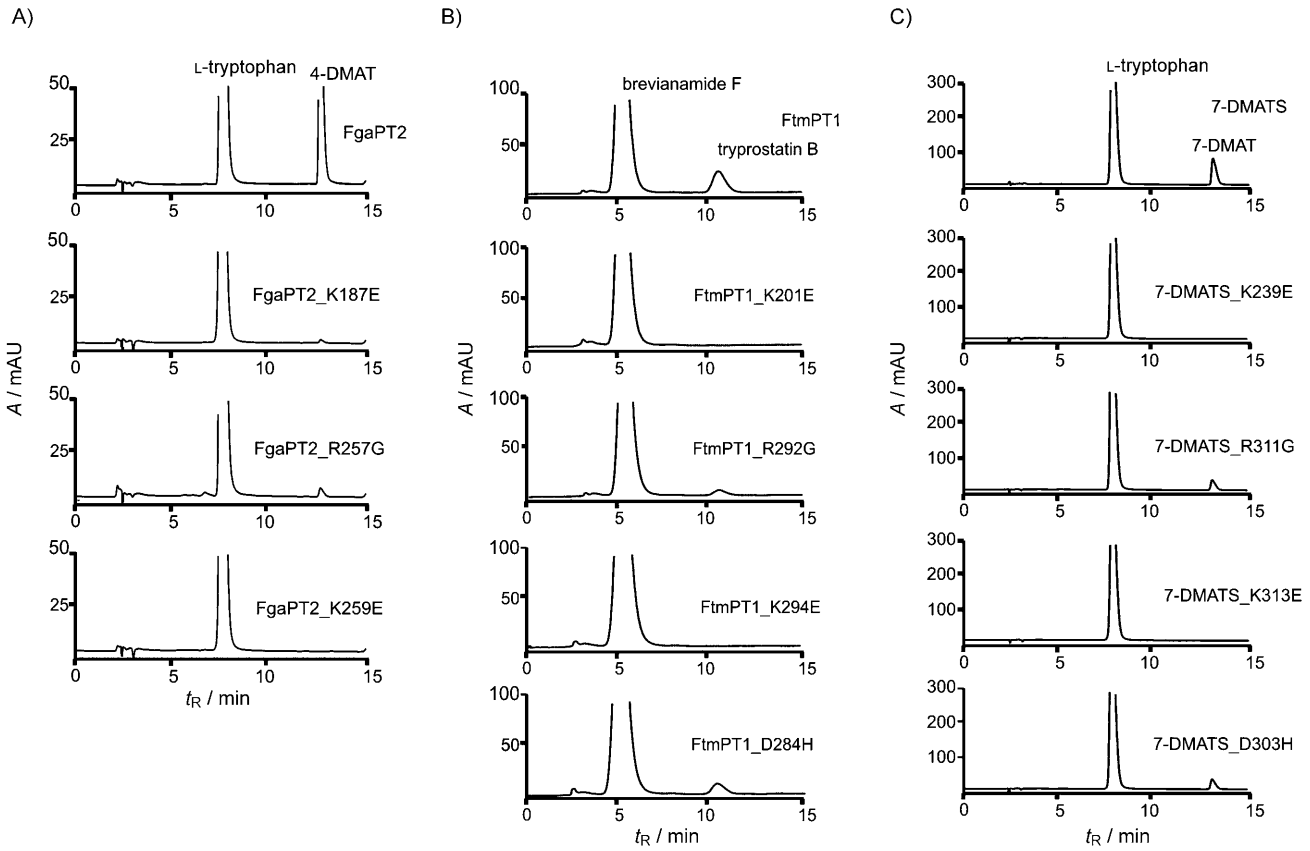

Figure 3. HPLC analysis of reaction mixtures with A) FgaPT2 and its mutant derivatives after incubation at 30 °C for 60 min, B) FtmPT1 and its mutant derivatives after incubation at 37 °C for 45 min and C) 7-DMATS and its mutant derivatives after incubation at 37 °C for 45 min; detection was carried out at 277 nm.

volved, either directly or indirectly, in prenyl diphosphate binding. Mutation of the conserved residues, arginine and aspartate, resulted in only a slight decrease in enzymatic activities; this indicates that they are not directly involved in catalysis. Structural analysis, for example, by crystallisation experiments, could provide support for our results and additional information on substrate binding sites of the indole prenyltransferases from fungi.

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SPP 1152; to S.-M.L.). H.Z. was a recipient of a fellowship from China Scholarship Council.

Keywords: *Aspergillus fumigatus* · biosynthesis · indole prenyltransferases · natural products · site-directed mutagenesis

[1] I. A. Unsöld, S.-M. Li, *Microbiology* **2005**, *151*, 1499–1505.
[2] I. A. Unsöld, S.-M. Li, *ChemBioChem* **2006**, *7*, 158–164.
[3] A. Kremer, L. Westrich, S.-M. Li, *Microbiology* **2007**, *153*, 3409–3416.
[4] A. Grundmann, S.-M. Li, *Microbiology* **2005**, *151*, 2199–2207.
[5] H.-L. Ruan, W.-B. Yin, J.-Z. Wu, S.-M. Li, *ChemBioChem* **2008**, *9*, 1044–1047.
[6] J. W. Bok, D. Hoffmeister, L. A. Maggio-Hall, R. Murillo, J. D. Glasner, N. P. Keller, *Chem. Biol.* **2006**, *13*, 31–37.
[7] P. Schneider, M. Weber, D. Hoffmeister, *Fungal Genet. Biol.* **2008**, *45*, 302–309.
[8] C. J. Balibar, A. R. Howard-Jones, C. T. Walsh, *Nat. Chem. Biol.* **2007**, *3*, 584–592.
[9] P. Tudzynski, K. Holter, T. Correia, C. Arntz, N. Grammel, U. Keller, *Mol. Gen. Genet.* **1999**, *261*, 133–141.
[10] H. F. Tsai, H. Wang, J. C. Gebler, C. D. Poulter, C. L. Schardl, *Biochem. Biophys. Res. Commun.* **1995**, *216*, 119–125.
[11] P. H. Liang, T. P. Ko, A. H. Wang, *Eur. J. Biochem.* **2002**, *269*, 3339–3354.
[12] K. Yazaki, M. Kunihisa, T. Fujisaki, F. Sato, *J. Biol. Chem.* **2002**, *277*, 6240–6246.
[13] K. Ohara, K. Yamamoto, M. Hamamoto, K. Sasaki, K. Yazaki, *Plant Cell Physiol.* **2006**, *47*, 581–590.
[14] F. Pojer, E. Wemakor, B. Kammerer, H. Chen, C. T. Walsh, S.-M. Li, L. Heide, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 2316–2321.
[15] D. J. Edwards, W. H. Gerwick, *J. Am. Chem. Soc.* **2004**, *126*, 11432–11433.
[16] T. Kuzuyama, J. P. Noel, S. B. Richard, *Nature* **2005**, *435*, 983–987.
[17] M. Tello, T. Kuzuyama, L. Heide, J. P. Noel, S. B. Richard, *Cell Mol. Life Sci.* **2008**, *65*, 1459–1463.
[18] W.-B. Yin, H.-L. Ruan, L. Westrich, A. Grundmann, S.-M. Li, *ChemBioChem* **2007**, *8*, 1154–1161.
[19] K. Sasaki, K. Mito, K. Ohara, H. Yamamoto, K. Yazaki, *Plant Physiol.* **2008**, *146*, 1075–1084.
[20] A. Markert, N. Steffan, K. Ploss, S. Hellwig, U. Steiner, C. Drewke, S.-M. Li, W. Boland, E. Leistner, *Plant Physiol.* **2008**, *147*, 296–305.
[21] L. Song, C. D. Poulter, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3044–3048.
[22] L. C. Tarshis, M. Yan, C. D. Poulter, J. C. Sacchettini, *Biochemistry* **1994**, *33*, 10871–10877.
[23] L. Song, C. D. Poulter, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 3044–3048.
[24] N. Steffan, I. A. Unsöld, S.-M. Li, *ChemBioChem* **2007**, *8*, 1298–1307.

Received: April 9, 2008
Published online on August 1, 2008

