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A New Group of Aromatic Prenyltransferases in Fungi, Catalyzing a 2,7-Dihydroxynaphthalene 3-Dimethylallyl-transferase Reaction*[S]

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Five fungal genomes from the Ascomycota (sac fungi) were found to contain a gene with sequence similarity to a recently discovered small group of bacterial prenyltransferases that catalyze the C-prenylation of aromatic substrates in secondary metabolism. The genes from *Aspergillus terreus* NIH2624, *Botryotinia fuckeliana* B05.10 and *Sclerotinia sclerotiorum* 1980 were expressed in *Escherichia coli*, and the resulting His_8-tagged proteins were purified and investigated biochemically. Their substrate specificity was found to be different from that of any other prenyltransferase investigated previously. Using 2,7-dihydroxynaphthalene (2,7-DHN) and dimethylallyl diphosphate as substrates, they catalyzed a regiospecific Friedel-Crafts alkylation of 2,7-DHN at position 3. Using the enzyme of *A. terreus*, the K_m values for 2,7-DHN and dimethylallyl diphosphate were determined as 324 ± 25 μM and 325 ± 35 μM, respectively, and k_{cat} as 0.026 ± 0.001 s^{-1}. A significantly lower level of prenylation activity was found using dihydrophenazine-1-carboxylic acid as aromatic substrate, and only traces of products were detected with aspulvinone E, flaviolin, or 4-hydroxybenzoic acid. No product was formed with L-tryptophan, L-tyrosine, or 4-hydroxyphenylpyruvate. The genes for these fungal prenyltransferases are not located within recognizable secondary metabolic gene clusters. Their physiological function is yet unknown.

Recently, a new family of prenyltransferases has been discovered in bacteria of the genus *Streptomyces* (1–4). They are involved in the biosynthesis of secondary metabolites, *e.g.* antibiotics. The members of this family are characterized by a new protein fold, termed PT barrel (1). The PT barrel consists of five repetitive $\alpha\alpha\beta\beta$ elements, with the β strands forming a central barrel. In contrast to the TIM barrel, the PT barrel consists of 10 antiparallel β strands and contains the active site of the enzyme in its spacious lumen. The members of this enzyme family catalyze Friedel-Crafts alkylations of aromatic substrates, *i.e.* the formation of carbon-carbon-bonds between C-1 or C-3 of the isoprenoid substrate and an aromatic carbon of the acceptor substrate. In contrast to the membrane-bound aromatic prenyltransferases of ubiquinone, menaquinone, and plastoquinone biosynthesis (5), the enzymes containing the PT barrel are soluble proteins and do not contain (N/D)D*xx*D motifs for binding of the isoprenoid substrate.

In fungi, the prenylation of aromatic substrates leads to a large and important class of secondary metabolites, the prenylated indole alkaloids (6). Also, the fungal indole prenyltransferases are soluble enzymes without (N/D)D*xx*D motifs (7). Very recently, we solved the first structure of a fungal indole prenyltransferase, *i.e.* of the dimethylallyltryptophan synthase. This enzyme also showed the PT barrel structure (8). For prenyltransferases characterized by the PT barrel fold, the name ABBA prenyltransferases has been suggested (3). From the currently available data, the ABBA prenyltransferases can be divided into two groups. In the dimethylallyltryptophan synthase/LtxC group, PSI-BLAST searches reveal more than a hundred entries in the database with sequence similarity to dimethylallyltryptophan synthase, mostly in fungal genomes but also some bacterial enzymes, *e.g.* LtxC (9) and CymD (10). Typically, the members of this group catalyze the prenylation of indole moieties (7). In the CloQ/NphB group, PSI-BLAST searches reveal 12 entries within the bacterial genus *Streptomyces* (and five fungal genes discussed below) that show sequence similarity to CloQ of clorobiocin biosynthesis (2) and to NphB of naphterpin biosynthesis (1) (see Fig. 1). The currently known members of this group catalyze the prenylation of phenols and phenazine derivatives (4). Sequence similarity between these two groups cannot be detected with PSI-BLAST searches but can be detected with more powerful bioinformatic techniques like HHpred (11), indicating a distant evolutionary relationship. Structure predictions (12, 13) suggest that all members of the two groups exhibit the PT barrel fold.

In the last years, genome-sequencing projects of fungi of the subphylum Pezizomycotina (the largest group of the phylum Ascomycota, *i.e.* sac fungi) revealed in five different species, including *A. terreus*, a gene with no sequence similarity to any other fungal gene but with obvious similarity to the bacterial ABBA prenyltransferases of the CloQ/NphB group (3). No data have been published on their possible function. However, 30 years ago, a soluble aromatic prenyltransferase was identified in *A. terreus* and purified to apparent homogeneity (14, 15). It was functionally identified as aspulvinone dimethylallyltransferase, catalyzing a reaction similar to that of the bacterial ABBA prenyltransferase CloQ (Fig. 1). Yet, the gene coding for aspulvi-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S5 and Figs. S1 and S2.

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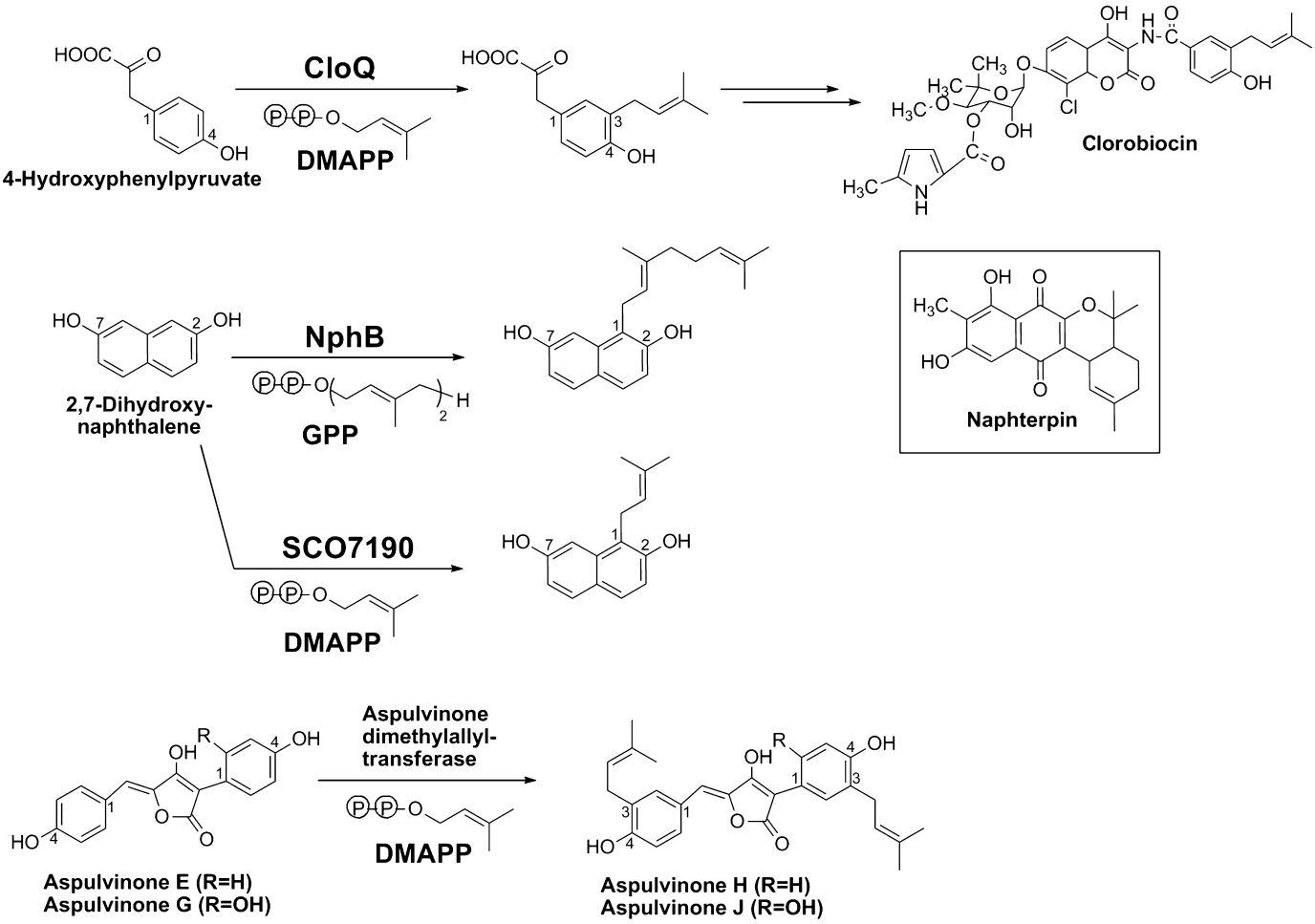


FIGURE 1. **Reactions catalyzed by the bacterial enzymes CloQ, NphB, and SCO7190 and by the fungal aspulvinone dimethylallyltransferase.** The genuine aromatic substrate of NphB in the biosynthesis of naphterpin is unknown; 2,7-dihydroxynaphthalene is one of the artificial substrates accepted *in vitro*. *GPP*, geranyl diphosphate.

none dimethylallyltransferase has not been identified. In the present study, we expressed the *A. terreus* gene with sequence similarity to CloQ as well as two orthologs from other fungal species, purified the resulting proteins, and investigated them biochemically to determine whether they indeed are aromatic prenyltransferases and whether they may represent the aspulvinone dimethylallyltransferase.

EXPERIMENTAL PROCEDURES

Chemicals—DMAPP[2] and geranyl diphosphate were synthesized according to Woodside *et al.* (16). Flaviolin was prepared as described by Gross *et al.* (17). Dihydrophenazine-1-carboxylic acid was generated as described by Saleh *et al.* (18). Aspulvinone E was synthesized according to Bernier and Brückner (19). IPTG, Tris, NaCl, glycerol, dithiothreitol, $MgCl_2$, formic acid, sodium dodecyl sulfate, polyacrylamide, and EDTA were bought at Carl Roth, Karlsruhe, Germany. 1,6-Dihydroxynaphthalene, 4-hydroxyphenyl-pyruvate, 4-hydroxybenzoic acid, TAPS, methanol, Tween 20, and imidazole were bought at Sigma Aldrich, Steinheim, Germany. 2,7-Dihydroxynaphthalene and 1,3-dihydroxynaphthalene were bought at Acros Organics. Merck delivered dipotassium hydrogen phosphate, potassium dihydrogen phosphate, β-mercaptoethanol, sodium ascorbate, methanol D_4, L-tryptophan, and L-tyrosine. Lysozyme was bought at Boehringer Ingelheim, Heidelberg, Germany.

Protein Expression and Purification—The nucleotide sequences of ptf_{Ss}, ptf_{Bf}, and the truncated ptf_{Pm}-S (lacking the coding sequence for the first 57 amino acids of XP_002143864) were optimized for expression in *Escherichia coli* with the Gene Designer Tool and synthesized commercially by DNA2.0 (Basel, Switzerland). All three genes were excised from vector pJ201 (DNA2.0) with NcoI and EcoRI and ligated into vector pHis8 (20) using the same restriction sites.

Likewise, the sequence of ptf_{At} was optimized with the Gene Designer Tool and was synthesized including the His_8 tag and the linker region as found in pHis8 (20). It was delivered in the expression vector pJExpress411 (DNA2.0) and used without further cloning. The His_8 tag and linker region were thereby identical in all four constructs.

35 ml of an overnight culture in Luria-Bertani medium (50 μg ml^{-1} kanamycin, 25 μg ml^{-1} chloramphenicol) of *E. coli*

[2] The abbreviations used are: DMAPP, dimethylallyl diphosphate; IPTG, isopropyl thiogalactoside; 2,7-DHN, 2,7-dihydroxynaphthalene; TAPS, 3-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}-1-propanesulfonic acid; HPLC-MS, high pressure liquid chromatography mass spectrometry; PSI-BLAST, position specific iterative basis local alignment search tool.

Rosetta 2 (DE3) pLysS cells harboring one of the plasmids were used to inoculate 1 liter of terrific broth (21) (50 μg ml^{-1} kanamycin, 25 μg ml^{-1} chloramphenicol). The cultures were grown at 37 °C and 250 rpm to an A_{600} of 0.6. Temperature was lowered to 20 °C, and IPTG was added to a final concentration of 0.5 mM. Synthesis of recombinant protein was allowed to proceed for 6 h. Cells were harvested by centrifugation for 20 min at 2700 × *g* at 4 °C, and the pellet was stored at −20 °C. Cells were thawed and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 20 mM imidazol, 1% Tween 20, 0.5 mg ml^{-1} lysozyme, 0.5 mM phenylmethylsulfonyl fluoride) using a ratio of 25 ml lysis buffer for 10 g of pellet. After stirring for 30 min at 4 °C, cells were ruptured with a Branson sonifier. Debris and membranes were removed by centrifugation at 38,720 × *g* for 45 min. The supernatant was applied to a nickel-nitrilotriacetic acid-agarose resin column (GE Healthcare) according to the manufacturer's instructions, using a linear gradient of 0–60% 250 mM imidazole (in 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol) in 60 min for elution. The eluate was passed over PD-10 columns (Amersham Biosciences, Freiburg, Germany) and equilibrated and eluted with 25 mM Tris-HCl, pH 8.0, 100 mM NaCl, 15% glycerol, 2 mM dithiothreitol. The proteins were stored in this buffer at −80 °C.

For further purification, Ptf_{At} was loaded on an anion exchange column (Resource Q, 30 × 6.4 mm, 15 μm; GE Healthcare) according to the manufacturer's instructions and eluted with a linear gradient of 0–50% 1 M NaCl in 25 mM Tris-HCl, pH 8.0. The eluate was again passed over PD-10 columns as described above.

Assay for Prenyltransferase Activity—The reaction mixture (100 μl) contained 100 mM sodium-TAPS pH 8.7, 2 mM aromatic substrate, 2 mM isoprenoid substrate, 10 mM $MgCl_2$, 10 mM sodium ascorbate, and either 7.2 μg Ptf_{At} or 15 μg Ptf_{Ss}, or 50 μg Ptf_{Bf}. After incubation for 10 min at 30 °C, the reaction was stopped with 100 μl of ethyl acetate/formic acid (40:1). After vortexing and centrifugation, 75 μl of the organic layer was transferred to an Eppendorf tube. The solvent was evaporated, and the residue was dissolved in 50 μl methanol and analyzed by HPLC, using an Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm, Agilent, Waldbronn, Germany) at a flow rate of 1 ml min^{-1}. Water (solvent A) and methanol (solvent B) each containing 1% (v/v) formic acid were used as eluents. A linear gradient was ran from 20–100% solvent B in 30 min. Products were detected with a photo diode array detector. Assays containing amino acids as aromatic substrates were stopped after incubation with 10 μl formic acid. The protein was removed by centrifugation at 21,460 × *g* for 10 min. The supernatant was directly analyzed by HPLC as described above.

^{1}H NMR Data of the Enzymatic Product 3-Dimethylallyl-2,7-dihydroxynaphthalene—The NMR spectrum was recorded at 250 MHz in a Bruker AC-250 spectrometer (Bruker, Rheinstetten, Germany), with CD_3OD as solvent. δ values are given in ppm. The solvent signal (3.3 ppm) was used as reference: δ, 1.73 (br s, 3*H*, H-4′), 1.76 (br s, 3*H*, H-5′), 3.37 (d, J = 7.32 Hz, 2*H*, H-1′), 5.39 (m, 1*H*, H-2′), 6.78 (dd, J = 8.67 Hz, 2.4 Hz, 1*H*, H-6), 6.84 (d, J = 2.4 Hz, 1*H*, H-8), 6.86 (s, 1*H*, H-1), 7.34 (s, 1*H*, H-4), and 7.48 (d, J = 8.85 Hz, 1*H*, H-5).

Sequence Analysis—Database searches were performed with BLASTP (http://www.ncbi.nlm.nih.gov/). Secondary structure predictions were performed with GenTHREADER (12, 13), and sequences were aligned with ClustalW2 (22) and visualized with ESPript (23).

Calculation of Kinetic Constants—K_m and k_{cat} were calculated using GraphPad Prism software, version 5.01 for Windows, GraphPad Software, Inc., La Jolla, CA.

RESULTS

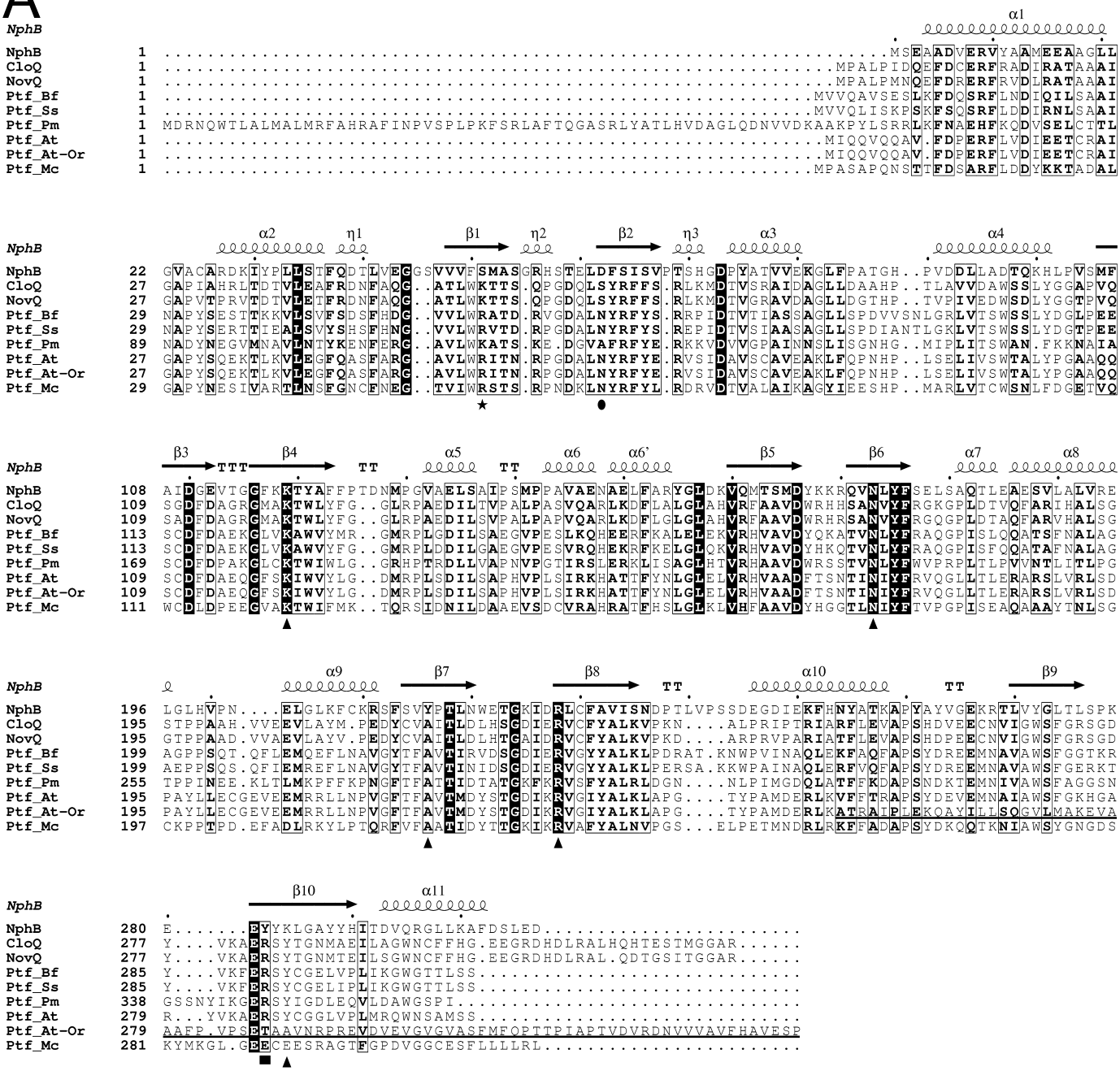
Sequence Analysis—A gene with sequence similarity to the CloQ/NphB group of ABBA prenyltransferases is found in the genomes of only five fungal species in the database: *A. terreus* NIH2624, *Botryotinia fuckeliana* B05.10, *Sclerotinia sclerotiorum* 1980, *Penicillium marneffei* ATCC18224, and *Microsporum canis* CBS113480. In this study, these genes will be designated as ptf_{At}, ptf_{Bf}, ptf_{Ss}, ptf_{Pm}, and ptf_{Mc}, respectively. In the genome sequences of the respective organisms, they have been designated as ATEG_00821, BC1G_01295, SS1G_09465, PMAA_022320, and MCYG_05060, respectively. An alignment of the amino acid sequence of their predicted gene products with the sequences of the bacterial enzymes CloQ (2), NovQ (24), and NphB (1) is shown in Fig. 2. This reveals obvious similarities between the sequences as well as conservation of several of the amino acids found to interact with the pyrophosphate moiety of the isoprenoid substrate in the x-ray structure of NphB (1).

However, in the case of the *A. terreus* protein (GenBank™ accession number XP_001210907, Ptf_{At}-Or in Fig. 2), similarity to the other enzymes stops abruptly downstream of the amino acid corresponding to position 253 in NphB (Fig. 2). Closer inspection of the nucleotide sequence showed that this position marks the beginning of the first of two predicted introns in the current annotation of the gene ptf_{At} (ATEG_00821). As the bioinformatics prediction of introns is prone to errors, we generated a translation of the genomic sequence without introns (sequence Ptf_{At} in Fig. 2), and this showed close similarity to the other fungal and bacterial enzymes. We therefore suggest that the gene ptf_{At} does not contain introns, just like ptf_{Bf}, ptf_{Ss}, and ptf_{Pm}. This suggestion was later on supported by the demonstration of enzymatic activity of the protein generated from the genomic sequence of ptf_{At} in *E. coli* (see below). The gene ptf_{Mc} is annotated to contain a small intron near its 3′ end; bioinformatic analysis does not allow to decide whether this annotation is correct.

The N terminus of the predicted gene product of ptf_{Pm} was >50 amino acids longer than that of the four other fungal proteins. For the current study, we generated a truncated version of this gene (termed ptf_{Pm}-S), lacking the coding sequence for the first 57 amino acids and thereby having a similar size as the other proteins in this study. The active site of the protein, located inside the β barrel, should not be affected by this truncation.

The sequence identity in between the five predicted fungal proteins ranges from 27 to 81% (Fig. 2*B*). As noted previously (3), they are closely related to the 4-hydroxyphenylpyruvate-3-dimethylallyltransferases CloQ and NovQ (2, 24), but only dis-

A



B Sequence identities (%)

No. of amino acids	Enzyme	NphB	NovQ	CloQ	Ptf_{Mc}	Ptf_{Pm}-S	Ptf_{Ss}	Ptf_{Bf}	Ptf_{At}
304	Ptf_{At}	15	31	31	30	27	36	38	100
309	Ptf_{Bf}	16	36	37	37	35	81	100	
309	Ptf_{Ss}	15	37	39	37	34	100		
307	Ptf_{Pm}-S	14	30	29	32	100			
314	Ptf_{Mc}	15	33	32	100				
324	CloQ	16	85	100					
323	NovQ	17	100						
307	NphB	100							

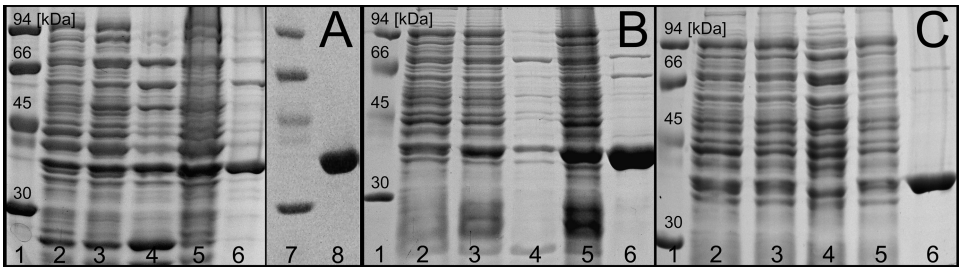


FIGURE 3. **Purification of the fungal prenyltransferases Ptf_{At} (*A*), Ptf_{Bf} (*B*), and Ptf_{Ss} (*C*) after expression in the form of His_8-tagged fusion proteins.** *Lane 1*, molecular mass standards; *lane 2*, total protein before IPTG induction; *lane 3*, total protein after IPTG induction; *lane 4*, soluble protein after IPTG induction; *lane 5*, insoluble protein after IPTG induction; *lane 6*, protein after Ni^{2+} affinity chromatography; *lane 7*, molecular mass standards; *lane 8*, protein after ion exchange chromatography. The calculated masses are 36.6 kDa for Ptf_{At}, 36.7 kDa for Ptf_{Ss}, and 36.5 kDa for Ptf_{Bf}. The 12% polyacrylamide gel was stained with Coomassie Brilliant Blue R-250.

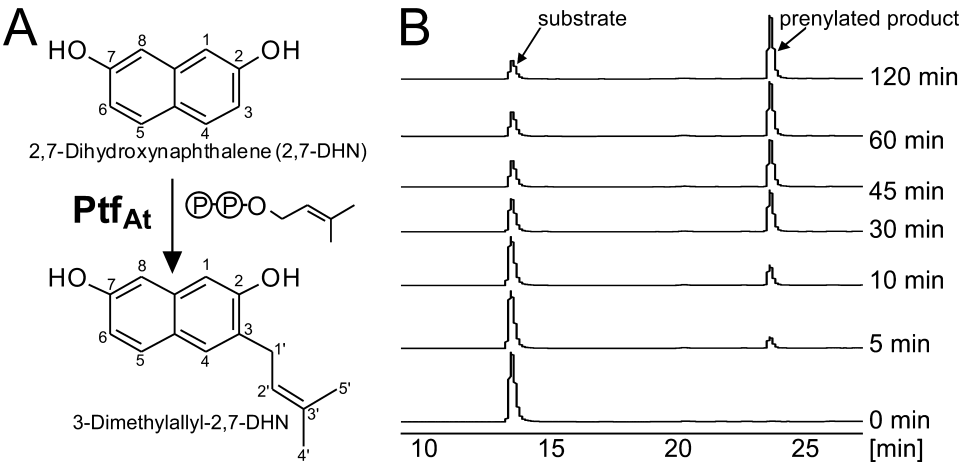


FIGURE 4. *A*, the aromatic prenylation reaction catalyzed by Ptf_{At}. *B*, HPLC analysis of prenyltransferase assays with Ptf_{At}. Detection: UV, 285 nm.

tantly to NphB (1) and the other nine bacterial ABBA prenyltransferases of the CloQ/NphB group in the database.

NphB is magnesium-dependent in its catalytic activity, but CloQ and NovQ are not. Correspondingly, the aspartate residue required for the coordination of Mg^{2+} in NphB (Asp^{62}, Fig. 2) is not conserved in CloQ and NovQ, and neither is this residue conserved in the five predicted fungal proteins. Kuzuyama *et al.* (1) suggested that the positive charge of Mg^{2+} in NphB is functionally replaced by a positively charged lysine residue in CloQ and NovQ and by an arginine residue in the related prenyltransferase SCO7190. As shown in Fig. 2, the five fungal proteins also contain arginine or lysine residues in this position, predicting that they may represent Mg^{2+}-independent prenyltransferases. This was later confirmed by biochemical investigations (see below). Structure prediction using GenTHREADER (12, 13) showed that all five fungal enzymes are likely to have the PT barrel fold.

Protein Expression and Purification—The nucleotide sequences of ptf_{At}, ptf_{Bf}, and ptf_{Ss} as well as of the truncated ptf_{Pm}-S were optimized for expression in *E. coli* and synthesized commercially (see "Experimental Procedures"). The expression plasmids were introduced into *E. coli*. After induction with IPTG, the cells were harvested, and the His_8-tagged proteins were purified by Ni^{2+} affinity chromatography (Fig. 3*A–C*). Ptf_{At} was further purified by ion exchange chromatography to apparent homogeneity (Fig. 3*A*). Although a part of the proteins remained insoluble, Ptf_{At}, Ptf_{Bf}, and Ptf_{Ss} could be obtained in yields of 21, 11, and 25 mg per liter culture, respectively. In contrast, Ptf_{Pm}-S was completely insoluble and could not be purified. The sequence of ptf_{Mc} has only been deposited in the database when the current study was already in progress. Biochemical investigations were therefore carried out initially with Ptf_{At} and later on also with Ptf_{Bf} and Ptf_{Ss}.

Demonstration of C-prenyltransferase Activity of Ptf_{At}—2,7-Dihydroxynaphthalene (2,7-DHN) is one of the aromatic substrates accepted by the bacterial ABBA prenyltransferases NphB and SCO7190 (Fig. 1) (25). When purified Ptf_{At} was incubated with DMAPP and 2,7-DHN, the enzyme- and time-dependent formation of a prenylated product was readily observed in HPLC-UV (Fig. 4*B*). HPLC-MS analysis in the positive mode showed the molecular ion of the product at m/z = 229 $[M+H]^+$, corresponding to a monoprenylated derivative of 2,7-DHN. Subsequently, the assay was repeated on a large scale, and the product was isolated by preparative HPLC and investigated by ^1H NMR and ^{13}C NMR (supplemental Figs. S1 and S2). In the ^1H NMR spectrum of the substrate 2,7-DHN, the signals of the aromatic protons can be detected at 6.91 ppm (H-1), 6.83 ppm (H-3), and 7.54 ppm (H-4). The signals of the protons at position 3 and 4 show coupling with 8.82 Hz, while the more distant protons at position 1 and 3 couple with 2.37 Hz. This results in a double doublet signal for H-3. In the symmetrical 2,7-DHN molecule, the signals of H-5, H-6, and H-8 are identical to those of H-4, H-3, and H-1, respectively.

The ^1H NMR spectrum of the enzymatic product clearly showed the signals of a dimethylallyl moiety (see

FIGURE 2. *A*, sequence comparison of the bacterial prenyltransferases NphB, CloQ, and NovQ with the fungal proteins Ptf_{At}, Ptf_{Bf}, Ptf_{Ss}, Ptf_{Pm}, and Ptf_{Mc}. For Ptf_{At}, both the current sequence in the database (Ptf_{At}-Or) and the corrected sequence (Ptf_{At}) are displayed. The amino acids of NphB involved in the binding of the pyrophosphate moiety (▲, Lys^{119}, Asn^{173}, Tyr^{216}, Arg^{228}, and Lys^{284}; over H_2O ■, Tyr^{282}) and in the coordination of the Mg^{2+} ion (●, Asp^{62}) are marked. Likewise, the position of the lysine residue of CloQ and NovQ, suggested to functionally replace the positive charge of the Mg^{2+} ion, is marked (★). The sequence was created with Clustal W2 (22) and visualized by ESPript 2.2 (23). The sequence shows the secondary structure elements of NphB: α, α helices; η, 3_{10} helices; β, β strands; *TT*, strict β turns. Strict sequence identity is shown by a *black box* with a *white character*, and similarity is shown by *bold characters in a black frame*. *B*, sequence identities (%) of the predicted gene products of the fungal genes ptf_{At}, ptf_{Bf}, ptf_{Ss}, ptf_{Pm}-S, and ptf_{Mc} with the bacterial ABBA prenyltransferases NphB, CloQ, and NovQ. For ptf_{Pm}, an N-terminally truncated version (ptf_{Pm}-S) was used for this calculation (see text).

supplemental Fig. S1). The chemical shift of the signal of H-1′ of the dimethylallyl moiety (3.37 ppm) showed that it is attached to an aromatic carbon, not to an oxygen atom. The NMR signals of the aromatic protons H-5, H-6, and H-8 were essentially unchanged in comparison with the substrate. However, the signal of H-3 had disappeared, and correspondingly, the signals of H-1 and H-4 now appeared as singlets rather than doublets. This proved that substitution had occurred at C-3 of the aromatic nucleus (Fig. 4*A*). Therefore, Ptf_{At} catalyzes a C-prenylation of the aromatic substrate, *i.e.* a Friedel-Crafts alkylation, similar as reported for other ABBA prenyltransferases (3, 4). The regiospecificity of the prenylation by Ptf_{At} (Fig. 4*A*) is in contrast to the prenylation of 2,7-DHN by the bacterial prenyltransferases NphB and SCO7190 (Fig. 1), both of which lead to substitution at C-1 of 2,7-DHN (25).

Testing of Aspulvinone E as Substrate of Ptf_{At}—Previous biochemical investigations of aspulvinone dimethylallyltransferase from *A. terreus* showed that aspulvinone E (Fig. 1) is readily accepted as substrate of this enzyme, leading predominantly to a diprenylated product (14, 15). For the present study, aspulvinone E was synthesized according to a published procedure (19), and the identity of the compound was confirmed by ^{1}H and ^{13}C NMR. When aspulvinone E was incubated with Ptf_{At} and DMAPP, no product formation could be observed in HPLC-UV analysis. The more sensitive HPLC-MS analysis, however, showed a low formation of a monoprenylated product (data not shown). The reaction velocity of the prenylation of aspulvinone E by Ptf_{At} was at least 60 times lower than the prenylation of 2,7-DHN, and much lower than described previously for the aspulvinone dimethylallyltransferase purified from cell-free extracts of *A. terreus* (14, 15). Therefore, aspulvinone E is not the preferred substrate of Ptf_{At}.

Biochemical Properties of Ptf_{At}—The biochemical properties of the enzyme were investigated using 2,7-DHN and DMAPP as substrates. In the assay described in the "Experimental Procedures," the formation of the prenylated product showed linear dependence on the amount of Ptf_{At} (up to 10 μg) and on reaction time (up to 30 min). During prolonged incubation, a precipitation of protein was sometimes observed. Inclusion of NaCl into the incubation mixture was not useful to resolve this problem because it reduced the reaction velocity of the prenylation (50% reduction by 500 mM NaCl). Therefore, NaCl was not included into the assays, but incubation time was reduced to 10 min. In sharp contrast to the bacterial ABBA prenyltransferase NphB (1), to the membrane-bound aromatic prenyltransferase UbiA (26), and to the transprenyltransferases such as FPP synthase (27), Ptf_{At} showed catalytic activity in the complete absence of Mg^{2+} or any other divalent cation. Addition of 10 mM EDTA did not influence the reaction velocity. Therefore, binding of the isoprenoid substrate does not occur in form of a magnesium complex, as shown for FPP synthase (27). Nevertheless, addition of $MgCl_2$ to the incubation mixture increased the reaction velocity. The optimal concentration of $MgCl_2$ was found to be 10 mM, leading to a 2.4-fold increase of product formation. Similar observations have been reported both for fungal and for bacterial aromatic prenyltransferases of the ABBA family, *e.g.* for dimethylallyltryptophan synthase (28) and for CloQ (2). The pH optimum of Ptf_{At} was determined as

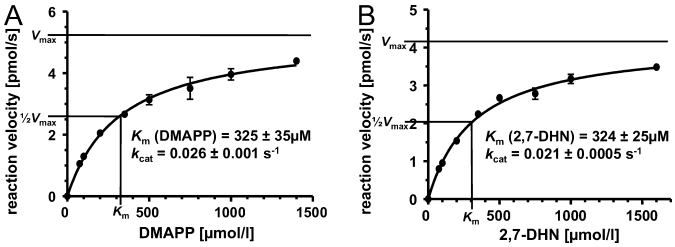


FIGURE 5. **Determination of K_m values of Ptf_{At} for DMAPP and 2,7-dihydroxynaphthalene.** In *A*, 2,7-diydroxynaphthalene was kept constant at 2 mM. In *B*, DMAPP was kept constant at 2 mM. K_m and k_{cat} values were determined by nonlinear regression, using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

8.7, with half-maximal reaction velocities at pH 8.1 and 9.4. The enzyme could be stored at −80 °C, with <5% loss of activity within two months.

Using a constant concentration of 2,7-DHN (2 mM) and varying concentrations of DMAPP, a typical hyperbolic curve of product formation over substrate concentration was obtained, indicating that the reaction followed Michaelis-Menten kinetics (Fig. 5*A*). Nonlinear regression analysis resulted in a K_m value for DMAPP of 325 ± 35 μM and a k_{cat} of 0.026 ± 0.001 s^{-1}. Using different concentrations of 2,7-DHN (up to 1.6 mM) in the presence of 2 mM DMAPP, likewise a hyperbolic curve was observed, resulting in a K_m value for 2,7-DHN of 324 ± 25 μM (Fig. 5*B*). Using these values the catalytic efficiency (k_{cat}/K_m) of the conversion of the non-genuine substrate 2,7-DHN by Ptf_{At} was calculated as 80 M^{-1} s^{-1}. This is >50 times higher than the value of 1.4 M^{-1} s^{-1} reported for the conversion of 2,7-DHN by NphB (25) but significantly lower than the values of 5280, 12400 or 46250 M^{-1} s^{-1} calculated for the prenylation of the genuine substrates of the ABBA prenyltransferases CloQ (2), PpzP (18), or dimethylallyltryptophan synthase (8). When 2,7-DHN was used in concentrations higher than 2 mM, substrate inhibition was observed. 20 mM 2,7-DHN inhibited product formation almost completely. It was not possible to calculate a K_i value, as no typical substrate inhibition curve was obtained. This may indicate multiple interactions of the phenolic substrate 2,7-DHN with the protein at high concentrations.

Substrate Specificity of Ptf_{At}—A range of aromatic compounds, previously found to be accepted by various bacterial or fungal ABBA prenyltransferases, were tested as substrates of Ptf_{At}, each in a concentration of 2 mM. Of these, only 5,10-dihydrophenazine 1-carboxylic acid yielded a product formation that was detectable by HPLC-UV analysis. HPLC-MS analysis showed that it was a monoprenylated product with the same properties as the product formed by PpzP (18) in a positive control assay. Yet, reaction velocity with this substrate was nearly 50 times lower than with 2,7-DHN (Table 1). Incubation of Ptf_{At} with flaviolin (2,5,7-trihydroxy-1,4-naphthoquinone) yielded a very low enzymatic formation of a monoprenylated product, detectable only in HPLC-MS. For other aromatic substances, including L-tryptophan, L-tyrosine, 4-hydroxyphenylpyruvate, 4-hydroxybenzoic acid, 1,3-dihydroxynaphthalene, and 1,6-dihydroxy-naphthalene, neither HPLC-UV nor HPLC-MS analysis showed any enzymatic product formation, whereas positive control assays with dimethylallyltryptophan synthase (28), CloQ (2), Fnq26 (29), and NphB (1) readily

TABLE 1

Formation of prenylated products by the fungal prenyltransferases Ptf_{At}, Ptf_{Ss}, and Ptf_{Bf} from different aromatic substrates and DMAPP. n.d., no product detectable in HPLC-UV and HPLC-MS analysis. See text for further explanations.

Aromatic substrate	Ptf_{At}		Ptf_{Ss}		Ptf_{Bf}	
	pmol product × s^{-1} × mg $protein^{-1}$	%	pmol product × s^{-1} × mg $protein^{-1}$	%	pmol product × s^{-1} × mg $protein^{-1}$	%
2,7-Dihydroxy-naphthalene	570	100	280	100	76	100
5,10-Dihydro-phenazine-1-carboxylic acid	12	2.1	7.5	2.7	7.7	10.1
Flaviolin	<10	<1.7	<5	<1.8	<1.5	<2
Aspulvinon E	<10	<1.7	<5	<1.8	n.d.	n.d.
p-Hydroxy-benzoic acid	n.d.	n.d.	<9	<3.2	11	14.5

showed the formation of prenylated products from these aromatic substrates. This proves that the specificity of Ptf_{At} for its aromatic substrate is different from that of any other prenyltransferase investigated so far.

Ptf_{At} was found to be specific for DMAPP. When DMAPP was replaced by geranyl diphosphate, no formation of a prenylated product from 2,7-DHN was observed. As expected, also isopentenyl diphosphate gave no product formation.

Biochemical Investigation of Ptf_{Ss} and Ptf_{Bf}—The enzymes from *S. sclerotiorum* and *B. fuckeliana*, Ptf_{Ss} and Ptf_{Bf}, showed very similar biochemical properties as Ptf_{At} from *A. terreus*. Upon incubation with 2,7-DHN and DMAPP, both enzymes gave a single product that was identical to that formed by Ptf_{At} in HPLC and mass spectrometry. Both enzymes were active in the absence of Mg^{2+}, but addition of 10 mM $MgCl_2$ increased reaction velocity by a factor of 2.6 for Ptf_{Ss} and 2.2 for Ptf_{Bf}.

The K_m values for 2,7-DHN were determined as 590 ± 136 μM for Ptf_{Ss} and 996 ± 177 μM for Ptf_{Bf}. For DMAPP, the respective values were 1232 ± 75 μM and 1010 ± 125 μM. The catalytic efficiency (k_{cat}/K_m) for the prenylation of 2,7-DHN resulted as 22 and 5 M^{-1} s^{-1}, still higher than the value of 1.4 M^{-1} s^{-1} reported for the prenylation of 2,7-DHN by NphB (25) but somewhat lower than the value observed of Ptf_{At}.

Like Ptf_{At}, the enzymes from *S. sclerotiorum* and *B. fuckeliana* were specific for DMAPP and did not accept geranyl diphosphate or isopentenyl diphosphate. Also their specificity for aromatic substrates resembled that of Ptf_{At} (Table 1); other than 2,7-DHN, only 5,10-dihydrophenazine-1-carboxylic acid gave an enzymatic product formation that was detectable in HPLC-UV analysis. With flaviolin, product formation was detectable only in HPLC-MS. Product formation with aspulvinone E could be detected in HPLC-MS for Ptf_{Ss} but remained below detection limit for Ptf_{Bf}. In all cases, the prenylation products formed by Ptf_{Ss} and Ptf_{Bf} were identical to those formed by Ptf_{At} in their chromatographic and mass spectrometric properties.

In contrast to Ptf_{At}, both Ptf_{Ss} and Ptf_{Bf} gave a monoprenylated product with 4-hydroxybenzoic acid, but due to the low amount of product, its structure could not be solved. Just as for Ptf_{At}, no product formation was observed when Ptf_{Ss} or Ptf_{Bf} were incubated with L-tryptophan, L-tyrosine, 4-hydroxyphenylpyruvate, 1,3-dihydroxynaphthalene, or 1,6-dihydroxynaphthalene as aromatic substrates. Therefore, the properties of Ptf_{Ss} and Ptf_{Bf} are similar to those of Ptf_{At} but at variance to those reported for any other previously investigated prenyltransferase.

DISCUSSION

The present study proves that the *ptf* genes of *A. terreus*, *S. sclerotiorum*, and *B. fuckeliana* code for functional prenyltransferases that attach isoprenoid moieties to carbon atoms of aromatic substrates in an enzyme-catalyzed Friedel-Crafts reaction. Their substrate specificity is clearly different from that of any other known prenyltransferase. In the prenylation of the artificial substrate 2,7-DHN they show a different regiospecificity than the previously examined bacterial enzymes NphB and SCO7190 (1, 25). Therefore, these fungal enzymes represent an interesting addition to the presently available aromatic prenyltransferases, which can be used for the chemoenzymatic synthesis of bioactive molecules (25, 30–32).

The amino acid sequences of Ptf_{At}, Ptf_{Ss}, and Ptf_{Bf}, as well as those of the gene products of ptf_{Pm} and ptf_{Mc}, are closely related to the bacterial enzymes NovQ and CloQ, which catalyze the 3-prenylation of 4-hydroxyphenylpyruvate in the biosynthesis of clorobiocin and novobiocin (2, 24, 33). The protein encoded by ptf_{Mc} has therefore been annotated as "NovQ" (GenBank™ accession number EEQ32241). However, in clear contrast to NovQ and CloQ, none of the three fungal proteins investigated in this study accepted 4-hydroxyphenylpyruvate as substrate.

The physiological function of these enzymes in the respective fungal organism remains unknown. Our study disproves the hypothesis that they may be responsible for the prenylation reaction in the biosynthesis of aspulvinone H and J (Fig. 1) (14, 15). An inspection of the genes located in the vicinity of the *ptf* genes in the genomes of the fungi *A. terreus*, *B. fuckeliana*, *S. sclerotiorum*, *P. marneffei*, and *M. canis* gives no indication of a secondary metabolic gene cluster to which the *ptf* genes may belong (see supplemental Tables S1–S5). This fact, *i.e.* that the *ptf* genes are not part of a recognizable secondary metabolic gene cluster, is at variance with the previously investigated fungal genes for indole prenyltransferases of the dimethylallyltryptophan synthase/LtxC group (7), but reminiscent of the bacterial genes SCO7190 and SCO7467 in *Streptomyces coelicolor* A3(2), the best examined bacterium of the genus *Streptomyces* (34). The predicted gene products of both genes show obvious similarity to aromatic prenyltransferases with a PT barrel fold, and the enzymatic activity of the protein encoded by SCO7190 has already been demonstrated *in vitro* (1, 25). Yet, a physiological function is not known for either of these two bacterial genes, and no primary or secondary metabolite resulting from an aromatic prenylation reaction possibly catalyzed by the enzymes encoded by these genes has been identified in *S. coelicolor*. Therefore, the present study clearly proved that Ptf_{At},

Ptf_{Ss}, and Ptf_{Bf} exhibit a specific, new prenyltransferase activity, but it remains unknown whether these enzymes have a physiological function in their respective fungal organisms.

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REFERENCES

1. Kuzuyama, T., Noel, J. P., and Richard, S. B. (2005) *Nature* **435,** 983–987
2. Pojer, F., Wemakor, E., Kammerer, B., Chen, H., Walsh, C. T., Li, S. M., and Heide, L. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 2316–2321
3. Tello, M., Kuzuyama, T., Heide, L., Noel, J. P., and Richard, S. B. (2008) *Cell. Mol. Life Sci.* **65,** 1459–1463
4. Saleh, O., Haagen, Y., Seeger, K., and Heide, L. (2009) *Phytochemistry* **70,** 1728–1738
5. Heide, L. (2009) *Curr. Opin. Chem. Biol.* **13,** 171–179
6. Williams, R. M., Stocking, E. M., and Sanz-Cervera, J. F. (2000) *Top. Curr. Chem.* **209,** 97–173
7. Steffan, N., Grundmann, A., Yin, W. B., Kremer, A., and Li, S. M. (2009) *Curr. Med. Chem.* **16,** 218–231
8. Metzger, U., Schall, C., Zocher, G., Unsöld, I., Stec, E., Li, S. M., Heide, L., and Stehle, T. (2009) *Proc. Natl. Acad. Sci. U.S.A.* **106,** 14309–14314
9. Edwards, D. J., and Gerwick, W. H. (2004) *J. Am. Chem. Soc.* **126,** 11432–11433
10. Schultz, A. W., Lewis, C. A., Luzung, M. R., Baran, P. S., and Moore, B. S. (2010) *J. Nat. Prod.* **73,** 373–377
11. Söding, J., Biegert, A., and Lupas, A. N. (2005) *Nucleic Acids Res.* **33,** W244–248
12. McGuffin, L. J., and Jones, D. T. (2003) *Bioinformatics* **19,** 874–881
13. Jones, D. T. (1999) *J. Mol. Biol.* **287,** 797–815
14. Takahashi, I., Ojima, N., Ogura, K., and Seto, S. (1978) *Biochemistry* **17,** 2696–2702
15. Sagami, I., Ojima, N., Ogura, K., and Seto, S. (1985) *Methods Enzymol.* **110,** 320–326
16. Woodside, A. B., Huang, Z., and Poulter, C. D. (1988) *Organic Syntheses* **66,** 211–215
17. Gross, F., Luniak, N., Perlova, O., Gaitatzis, N., Jenke-Kodama, H., Gerth, K., Gottschalk, D., Dittmann, E., and Müller, R. (2006) *Arch. Microbiol.* **185,** 28–38
18. Saleh, O., Gust, B., Boll, B., Fiedler, H. P., and Heide, L. (2009) *J. Biol. Chem.* **284,** 14439–14447
19. Bernier, D., and Brückner, R. (2007) *Synthesis* **15,** 2249–2272
20. Jez, J. M., Ferrer, J. L., Bowman, M. E., Dixon, R. A., and Noel, J. P. (2000) *Biochemistry* **39,** 890–902
21. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York
22. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) *Bioinformatics* **23,** 2947–2948
23. Gouet, P., Courcelle, E., Stuart, D. I., and Métoz, F. (1999) *Bioinformatics* **15,** 305–308
24. Ozaki, T., Mishima, S., Nishiyama, M., and Kuzuyama, T. (2009) *J. Antibiot.* **62,** 385–392
25. Kumano, T., Richard, S. B., Noel, J. P., Nishiyama, M., and Kuzuyama, T. (2008) *Bioorg. Med. Chem.* **16,** 8117–8126
26. Melzer, M., and Heide, L. (1994) *Biochim. Biophys. Acta* **1212,** 93–102
27. Poulter, C. D. (2006) *Phytochem. Rev.* **5,** 17–26
28. Unsöld, I. A., and Li, S. M. (2005) *Microbiology* **151,** 1499–1505
29. Haagen, Y., Unsöld, I., Westrich, L., Gust, B., Richard, S. B., Noel, J. P., and Heide, L. (2007) *FEBS Lett.* **581,** 2889–2893
30. Botta, B., Delle, Monache, G., Menendez, P., and Boffi, A. (2005) *Trends Pharmacol. Sci.* **26,** 606–608
31. Koehl, P. (2005) *Nat. Chem. Biol.* **1,** 71–72
32. Macone, A., Lendaro, E., Comandini, A., Rovardi, I., Matarese, R. M., Carraturo, A., and Bonamore, A. (2009) *Bioorg. Med. Chem.* **17,** 6003–6007
33. Steffensky, M., Li, S. M., Vogler, B., and Heide, L. (1998) *FEMS Microbiol. Lett.* **161,** 69–74
34. Bentley, S. D., Chater, K. F., Cerdeño-Tárraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., Harris, D. E., Quail, M. A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C. W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C. H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M. A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B. G., Parkhill, J., and Hopwood, D. A. (2002) *Nature* **417,** 141–147