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AstPT Catalyses both Reverse N1- and Regular C2 Prenylation of a Methylated Bisindolyl Benzoquinone

Sylwia Tarcz, Lena Ludwig, and Shu-Ming Li*[a]

Prenylated bisindolyl benzoquinones exhibit interesting biological activities, such as antidiabetic or anti-HIV activities. A number of these compounds, including asterriquinones, have been isolated from *Aspergillus terreus*. In this study, we identified two putative genes by genome mining, ATEG_09980 and ATEG_00702, which share high sequence similarity with the known bisindolyl benzoquinone prenyltransferase TdiB from *Aspergillus nidulans*. The coding sequences were cloned and overexpressed in *E. coli*. The overproduced recombinant proteins were purified to near homogeneity and used for enzyme assays with asterriquinone D in the presence of dimethylallyl diphosphate. HPLC analysis showed that product formation was only detected in enzyme assays with EAU29429 encoded by ATEG_09980, not in those with EAU39348 encoded by ATEG_00702. Product isolation and structure elucidation by NMR and MS analyses led to identification of N1-reversely and C2-regularly monoprenylated derivatives, as well as N1′,N1″-reversely, N1′-reversely, C2″-regularly diprenylated derivatives. This proved that EAU29429 functions as an asterriquinone prenyltransferase (AstPT) and indicated the involvement of EAU29429 rather than EAU39348 in the biosynthesis of methylated asterriquinones. Furthermore, incubation of monoprenylated enzyme products with AstPT resulted in the formation of the diprenylated derivatives.

Introduction

Bisindolyl benzoquinones are fungal secondary metabolites derived from two molecules of L-tryptophan and are therefore considered prenylated indole alkaloids.[1] They are formed through dimerisation of indolyl pyruvate catalysed by nonribosomal peptide synthetase (NRPS)-like proteins.[2,3] Most of these compounds carry prenyl moieties derived from dimethylallyl diphosphate (DMAPP).[1] Derivatisation of bisindolyl benzoquinones by prenyl residues at different positions leads to increased structural diversity as well as biological activities. A number of prenylated bisindolyl benzoquinones have been isolated from fungi[4–7] and can be divided in two main groups depending on the substitution of the benzoquinone ring. One group carries hydroxy moieties, for example, terrequinone A from *Aspergillus nidulans*[8] and *Aspergillus terreus*,[9] asterriquinone[10] and asterriquinone CT5 from *A. terreus*,[5] and cochliodinol from *Chaetomium* sp.[11] The second group bears methoxy groups instead, for example, asterriquinones A1, A3, B3, B4, C1 and F from *A. terreus* (Scheme 1).[12,13] The two unprenylated substances, didemethyl asterriquinone D (DDAQ D)[14] and asterriquinone D (AQ D; Scheme 1),[12] are also worth mentioning. Many bisindolyl benzoquinones exhibit interesting biological activities such as cytotoxic,[15–17] antifungal, or antibacterial activities.[18–20] It has been also shown that these compounds can act as insulin receptor agonists[21–23] or HIV protease inhibitors.[6]

In contrast to the large number of known structures of prenylated bisindolyl benzoquinones, biochemical investigations were carried out only for the biosynthesis of terrequinone A in *A. nidulans*.[2,3,24] A putative biosynthetic gene cluster and pathway were proposed for asterriquinones (see the Discussion).[25] In ascomycetous fungi, the prenyl moieties of the prenylated indoles are transferred from DMAPP by prenyltransferases of the dimethylallyltryptophan synthase (DMATS) superfamily.[26] Enzyme TdiB from this group is involved in the biosynthesis of terrequinone A and catalyses a reverse C2 prenylation at the indole ring and a regular prenylation at the benzoquinone ring.[2] Blasting the genome sequence of *A. terreus* NIH2624 with TdiB revealed the presence of ten putative genes, which share clear sequence similarity with known indole prenyltransferases of the DMATS superfamily.[27] Two of these, ATEG_09980 and ATEG_00702, showed significantly higher sequence similarity with TdiB than with other enzymes. Their encoded enzymes could be involved in the biosynthesis of asterriquinones. In this study, we report the cloning and overexpression of ATEG_09980 and ATEG_00702, as well as their role in the prenylation of AQ D (**1**, Scheme 1) in the presence of DMAPP.

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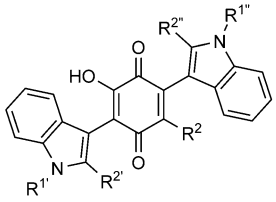
Results and Discussion

Sequence analysis and gene cloning

Sequence analyses showed that ATEG_09980 and ATEG_00702 in *A. terreus* NIH2624 have very similar exon-intron structures to *tdiB* from *A. nidulans*[24] and consist of three exons, interrupt-



derivatives with hydroxyl groups

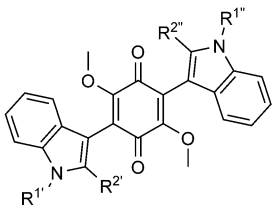
didemethyl asterriquinone D: R^2 = OH, $R^{1'}$ = $R^{1''}$ = $R^{2'}$ = $R^{2''}$ = H

asterriquinone: R^2 = OH, $R^{1'}$ = $R^{1''}$ =  $R^{2'}$ = $R^{2''}$ = H

asterriquinone CT5: R^2 = OH, $R^{1'}$ = $R^{1''}$ = H, $R^{2'}$ = $R^{2''}$ = 

terrequinone A: R^2 =  $R^{1'}$ = $R^{1''}$ = $R^{2''}$ = H

$R^{2'}$ = 



derivatives with methoxyl groups

asterriquinone D (**1**): $R^{1'}$ = $R^{1''}$ = $R^{2'}$ = $R^{2''}$ = H

asterriquinone F: $R^{1'}$ = $R^{1''}$ = H, $R^{2'}$ = $R^{2''}$ = 

asterriquinone A1: $R^{1'}$ = $R^{1''}$ =  $R^{2'}$ = $R^{2''}$ = H

asterriquinone A3: $R^{1'}$ = $R^{2''}$ =  $R^{1''}$ = $R^{2'}$ = H

asterriquinone B3: $R^{1'}$ =  $R^{1''}$ = $R^{2'}$ = $R^{2''}$ = H

asterriquinone B4: $R^{1'}$ = $R^{1''}$ = H, $R^{2'}$ = $R^{2''}$ = 

asterriquinone C1: $R^{1'}$ = $R^{1''}$ = $R^{2''}$ = H, $R^{2'}$ = 

Scheme 1. Examples of naturally occurring bisindolyl benzoquinones.

ed by two introns (Table S1). The resulting proteins EAU29429 and EAU39348 have polypeptide chains of the same length (424 amino acids) and share an amino acid sequence identity of 45 and 48%, respectively, with TdiB from *A. nidulans*.[24] The sequence identity between EAU29429 and EAU39348 was found to be 71% on the amino acid level.

Based on the fact that the genes of interest and *tdiB* consist of the same number of introns and exons in similar size, the coding region of ATEG_09980 was directly amplified from genomic DNA of *A. terreus* DSM1958 by two-round PCR (see the Experimental Section) and used for gene cloning and protein overproduction. The resulting PCR product without intron sequences was cloned, by using the pGEM-T Easy vector, into the expression vector pQE70, resulting in the expression construct pST24. After unsuccessful attempts to amplify the sequence of ATEG_00702 from the same strain, we used genomic DNA from the genome reference strain *A. terreus* FGSC A1156 (equivalent to *A. terreus* NIH2624) as the template for PCR amplification in a similar way. The PCR product was cloned into the expression vector pHIS8 to obtain the expression construct pST37.

Overexpression and purification

For overproduction of the recombinant protein EAU29429 (AstPT-His_6), *E. coli* XL1 Blue MRF′ cells harbouring pST24 were cultivated at 30 °C without induction by isopropyl-β-D-thiogalactopyranoside (IPTG), as addition of IPTG led only to high protein amounts of insoluble inclusion bodies (data not shown). One-step purification on Ni-NTA agarose resin resulted in a major protein band that was slightly larger than the 45 kDa size marker on SDS-PAGE (Figure S1 A). This also coincided with the calculated molecular mass of AstPT-His_6 of 48.9 kDa. A yield of 3 mg of purified protein was calculated for 1 L of bacterial culture. The molecular mass of the native recombinant protein was determined by size-exclusion chromatography as 48.9 kDa, thus indicating that AstPT-His_6 presumably acts as a monomer.

EAU39348 was overproduced by cultivating *E. coli* BL21 (DE3) pLysS cells harbouring pST37 and induction with 0.1 mM IPTG at 22 °C for 16 h. After two-step purification on Ni-NTA agarose and HisPur cobalt resin, a protein band above the 45 kDa size marker on SDS-PAGE was obtained (Figure S1 B). A yield of 0.1 mg of purified protein was obtained from 1 L of bacterial culture.

Enzyme activity and substrate specificity

AstPT was assayed with AQ D (**1**), which was synthesised from indole and 2,5-dichlorobenzoquinone in three steps, in the presence of DMAPP. After incubation at 37 °C for 1 h with 20 μg of recombinant enzyme in 100 μL assays, ethyl acetate extracts of the reaction mixtures were analysed by HPLC. Four additional peaks (**2**–**5**) were clearly observed in the HPLC chromatogram (Figure 1 A). No product formation was detected in the incubation mixtures with heat-inactivated protein or in the absence of the prenyl donor DMAPP (data not shown). The ratios of the four product peaks were obviously time-dependent (Figure 2). Peaks **2** and **3** were detected as main enzyme products in the initial phase and increased rapidly until they reached their maxima at 25 min. They subsequently decreased. In contrast, products **4** and **5** were detected somewhat later and increased slowly but continuously. After 2 h incubation, **3**, **4** and **5** reached comparable amounts. These results indicated that **2** and **3** could be intermediates for **4** and **5**. Considering the fact that mono-, di- and even triprenylated bisindolyl benzoquinones have been isolated from *A. terreus*,[12,25] it can be assumed that **2** and **3** were consumed by AstPT, leading to the formation of **4** and **5**.

To prove this hypothesis, we isolated and incubated **2** and **3** with AstPT in two parallel assays. As shown in Figure 1 B, **2** was converted to **4** and an unknown product with a slightly larger retention time than **3** after incubation at 37 °C for 16 h. In the

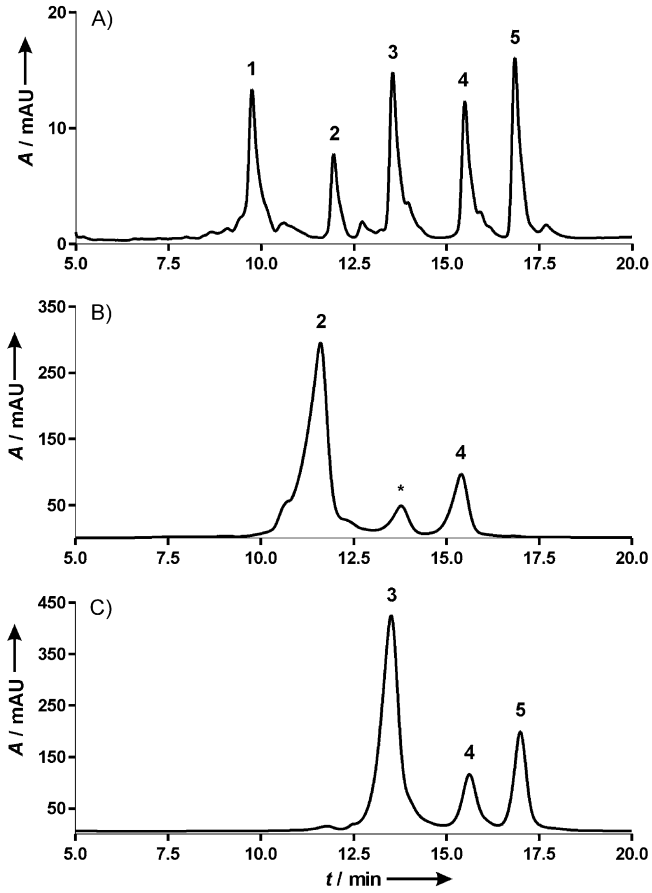


Figure 1. HPLC analysis of enzyme assays of AstPT with A) **1**, B) **2** and C) **3**. Assays with **1** were incubated for 1 h; assays with **2** and **3** were incubated for 16 h at 37 °C. Detection was carried out with a diode array detector with absorption at 277 nm. The asterisk in B) marks an unknown product that was not identified in this study due to low product quality.

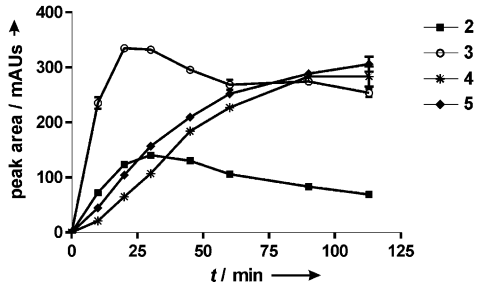


Figure 2. Dependence of product formation of AstPT reactions on incubation time. Two replicates with error bars are illustrated.

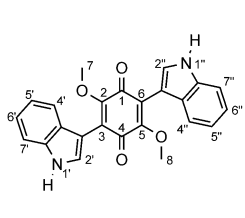
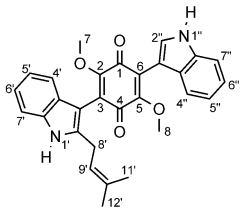
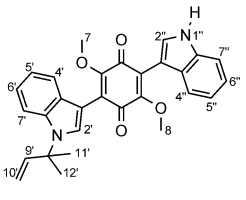
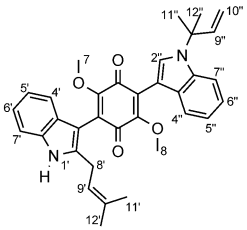
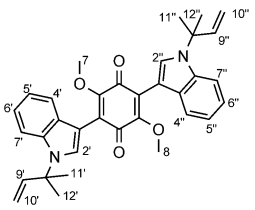
assay for **3**, compounds **4** and **5** were clearly detected (Figure 1 C).

Some of the known prenyltransferases of the DMATS superfamily were found to accept a broad spectrum of substrates, such as aromatic amino acids and cyclic dipeptides thereof, as well as flavonoids and hydroxynaphthalenes.[26] Therefore, we assayed AstPT with such aromatic substances in the presence of DMAPP. No product formation was found after incubation of AstPT with the aforementioned substances. Similarly, no product was detected in incubation mixtures of AstPT with **1** in the presence of geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), proving its high specificity for DMAPP in the presence of **1**. Incubation of **1** with purified EAU39348 in the presence of DMAPP, GPP, or FPP did not result in the formation of any enzyme product.

Structural elucidation of enzyme products

The four products, **2**–**5**, of the incubation mixtures of AstPT with **1** were isolated by preparative HPLC from combined assays of two and 16 h incubation mixtures and were subjected to spectroscopic analyses. Molecular masses in positive HR-EI-MS data confirmed the monoprenylation of **2** and **3** (68 Da larger than that of **1**) and diprenylation of **4** and **5** (136 Da larger than that of **1**; Table S2). ^{1}H NMR data clearly showed the presence of one regular prenyl moiety in each of the structures of **2** and **4** with signals at 5.39 (t sept, J = 7.3, 1.4 Hz; H-9′), 3.46 or 3.47 (m; H-8′) and 1.70 ppm (s or d, J = 1.2 Hz; H-11′ + H-12′; see the Supporting Information for spectra). The chemical shifts of H-8′ at about 3.5 ppm indicated the attachment of the prenyl moiety to a carbon atom. Signals of reverse prenyl moieties at 6.26 (dd, J = 17.5 or 17.4, 10.7 Hz; H-9′), 5.28 (dd, J = 10.7, 0.8 or 0.7 Hz; H-10′), 5.25 (dd, J = 17.5 or 17.4, 0.8 or 0.7 Hz; H-10′) and 1.86 ppm (s; H-11′ + H-12′) were observed in the spectra of **3**, **4** and **5** (Table 1). The chemical shifts of the two methyl groups indicated the attachment of the prenyl moiety to a hetero atom. ^{1}H NMR spectra of **2**–**5** showed the presence of signals for the four coupling aromatic protons of the indole ring (H-4′–H7′ as well as H4″–H7″) in the range of 7.00–7.60 ppm, indicating that prenylation did not take place at these positions. Significant differences were found between signals of NH-1′ and H-2′ and NH-1″ and H-2″. The ^{1}H NMR spectrum of **2** showed two signals for NH-1″ and NH-1″ at 10.87 (br s) and 10.32 ppm (br s), respectively, but only a signal for H-2″ at 7.68 ppm (d, J = 2.6 Hz). The appearance as a doublet with a coupling constant of 2.6 Hz proved that H-2″ still had a coupling partner at position NH-1″. Therefore, prenylation of **2** must occur at position C-2′. For **3**, only one NH signal was observed at 10.80 ppm (br s), together with two signals for H-2′ (7.74 ppm, s) and H-2″ (7.69 ppm, d, J = 2.7 Hz). The appearance of H-2′ as a singlet and H-2″ as a doublet provided a strong evidence for the attachment of the prenyl moiety to position N-1′. This compound has already been reported and named AQB3 by Arai and co-workers.[12] The ^{1}H NMR spectrum of **4** showed signals for regular and reverse prenylation. Only one NH signal (10.33 ppm, br s) and one H-2″ signal (7.73 ppm, s) were observed, indicating that regular prenylation had occurred at position C-2′ and reverse prenylation at position N-1″. This structure has already been synthesised in the context of cytotoxicity investigations.[28] Although a different solvent was used for NMR spectroscopy, good conformity was found between **4** and substance "6 b" in the published data. Because of the fact that half of the number of signals was found in the ^{1}H NMR spectrum of **5** only, we concluded that this molecule must be symmetric. No NH signals were observed, and H-2′/H-2″ appeared at 7.74 ppm as a singlet. This proved that both nitrogen atoms were reversely prenylated in **5**. A reference spec-

Table 1. ^{1}H NMR data of **1** and its enzyme products in $(CD_3)_2CO$ (500 MHz).

Compound					
proton	AQ D (**1**) δ_H, mult., *J*	**2** δ_H, mult., *J*	**3** δ_H, mult., *J*	**4** δ_H, mult., *J*	**5** δ_H, mult., *J*
7	3.81, s	3.79, s	3.83, s	3.80, s	3.82, s
8	3.81, s	3.72, s	3.81, s	3.72, s	3.82, s
1′	10.82, brs	10.32, brs	–	10.33, brs	–
2′	7.69, d, 2.7	–	7.74, s	–	7.74, s
4′	7.49, dt, 8.1, 1.1	7.31, ddd, 8.0, 1.2, 0.7	7.51, ddd, 8.2, 1.4, 0.7	7.30, ddd, 8.0, 1.2, 0.7	7.50, ddd, 7.9, 1.4, 0.7
5′	7.08, ddd, 8.1, 7.0, 1.1	7.00, ddd, 8.0, 7.1, 1.1	7.09, ddd, 8.2, 6.9, 1.2	7.00, ddd, 8.0, 6.9, 1.2	7.08, ddd, 7.9, 7.0, 1.5
6′	7.16, ddd, 8.1, 7.0, 1.1	7.09, ddd, 8.1, 7.1, 1.2	7.12, ddd, 8.3, 6.9, 1.4	7.08, ddd, 7.9, 6.9, 1.2	7.12, ddd, 8.3, 7.0, 1.4
7′	7.53, ddt, 8.1, 1.1, 0.7	7.49, ddd, 8.1, 1.1, 0.7	7.60, ddd, 8.3, 1.2, 0.7	7.49, ddd, 7.9, 1.2, 0.7	7.60, ddd, 8.3, 1.5, 0.7
8′	–	3.47, m	–	3.46, m	–
9′	–	5.39, t sept, 7.3, 1.4	6.26, dd, 17.5, 10.7	5.39, t sept, 7.3, 1.4	6.26, dd, 17.4, 10.7
10′	–	–	5.28, dd, 10.7, 0.8 5.25, dd, 17.5, 0.8	–	5.28, dd, 10.7, 0.7 5.25, dd, 17.4, 0.7
11′	–	1.70, s	1.86, s	1.70, d, 1.2	1.86, s
12′	–	1.70, s	1.86, s	1.70, d, 1.2	1.86, s
1″	10.82, brs	10.87, brs	10.80, brs	–	–
2″	7.69, d, 2.7	7.68, d, 2.6	7.69, d, 2.7	7.73, s	7.74, s
4″	7.49, dt, 8.1, 1.1	7.35, ddd, 8.1, 1.2, 0.8	7.49, dt, 8.1, 0.9	7.35, ddd, 8.1, 1.2, 0.9	7.50, ddd, 7.9, 1.4, 0.7
5″	7.08, ddd, 8.1, 7.0, 1.1	7.06, ddd, 8.1, 7.0, 1.3	7.08, ddd, 8.1, 7.0, 1.1	7.06, ddd, 8.1, 7.0, 1.1	7.08, ddd, 7.9, 7.0, 1.5
6″	7.16, ddd, 8.1, 7.0, 1.1	7.16, ddd, 8.1, 7.0, 1.2	7.16, ddd, 8.2, 7.0, 1.2	7.12, ddd, 8.3, 7.0, 1.2	7.12, ddd, 8.3, 7.0, 1.4
7″	7.53, ddt, 8.1, 1.1, 0.7	7.52, ddd, 8.1, 1.3, 0.8	7.53, ddt, 8.2, 1.1, 0.9	7.60, ddd, 8.3, 1.1, 0.9	7.60, ddd, 8.3, 1.5, 0.7
8″	–	–	–	–	–
9″	–	–	–	6.26, dd, 17.5, 10.7	6.26, dd, 17.4, 10.7
10″	–	–	–	5.28, dd, 10.7, 0.7 5.25, dd, 17.5, 0.7	5.28, dd, 10.7, 0.7 5.25, dd, 17.4, 0.7
11″	–	–	–	1.86, s	1.86, s
12″	–	–	–	1.86, s	1.86, s

Chemical shifts (δ) are given in ppm and coupling constants (*J*) in Hz.

trum is given for asterriquinone A1 and, in spite of a different solvent, the signals of **5** and those of the dimethyl ether of asterriquinone prepared by Yamamoto and co-workers harmonise well.[10,12] Identification of enzyme products proved unequivocally that AstPT functions as an asterriquinone prenyltransferase and catalyses mono- and diprenylation at nitrogen and carbon atoms in the presence of DMAPP (Scheme 2).

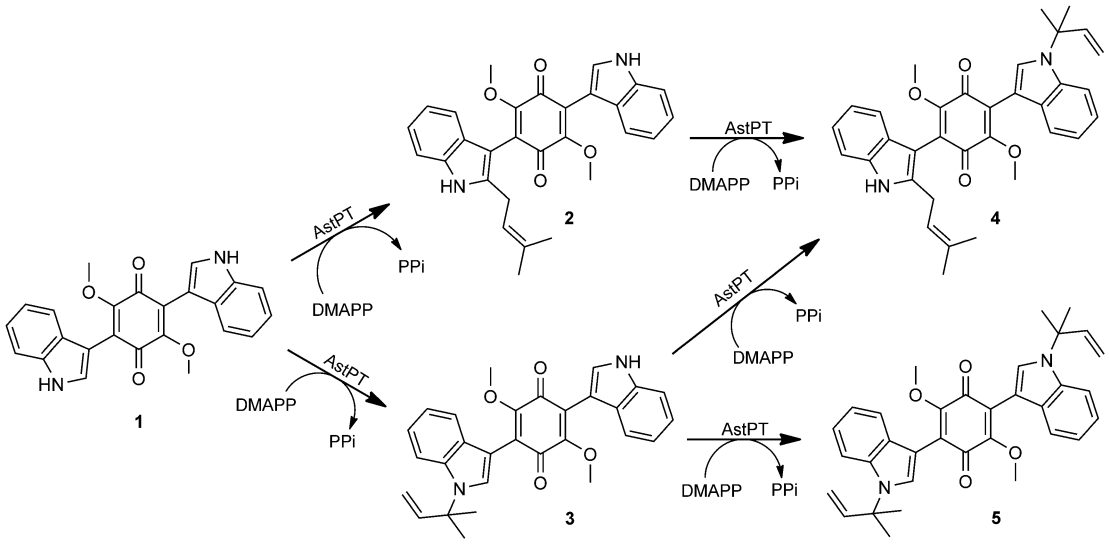
Biochemical properties and determination of kinetic parameters of AstPT

Most members of the DMATS superfamily catalyse their reactions in the absence of metal ions as well. Divalent cations such as Ca^{2+} and Mg^{2+} usually enhance their enzyme activities.[26,29] We assayed the dependence of the AstPT reaction on metal ions at final concentrations of 5 mM in the presence of 0.5 mM **1** and 2 mM DMAPP. Incubation mixtures without additives or with EDTA were used as controls. Our results showed that AstPT also catalyses its reaction in the presence of the chelating agent EDTA. However, addition of Ca^{2+} slightly increased the activity of AstPT (Figure S8).

In order to determine the affinity of AstPT towards its substrate **1**, kinetic parameters including the Michaelis–Menten constant (K_m) and turnover number (k_{cat}) were calculated from Eadie–Hofstee, Hanes–Woolf and Lineweaver–Burk plots. The reaction catalysed by AstPT apparently followed Michaelis–Menten kinetics (Figure S9). We calculated a K_m value of 463 μM with a k_{cat} of 0.16 s^{-1} for **1**. This K_m value was almost at the upper boundary of the measured concentrations. Unfortunately, due to low solubility of **1**, it was not possible to measure reaction rates at higher substrate concentrations. By using **1** at a final concentration of 0.5 mM as an aromatic substrate, K_m and k_{cat} were determined for DMAPP at 33.5 μM and 0.02 s^{-1}, respectively (Figure S10).

Cultivation of *A. terreus* and screening for methylated and unmethylated bisindolyl benzoquinones, as well as their prenylated derivatives

A. terreus DSM1958 and FGSCA1156 were cultivated as described in the Experimental Section. Culture filtrates and mycelia of both strains were analysed by LC/MS for the presence of



Scheme 2. Prenylation of **1** catalysed by AstPT.

methylated and unmethylated bisindolyl benzoquinones as well as prenylated derivatives thereof. $[M+H]^+$ ions at *m/z* 467.1966 for monoprenylated and at *m/z* 535.2588 for diprenylated derivatives of **1** were only detected in the extracts of mycelia of *A. terreus* DSM1958 (Figure S11), but not in cultures of A1156 (Figures S12 and S14). Even trace amounts of nonprenylated **1** were observed in the mycelia of DSM1958 (Figure S11 D). $[M+H]^+$ ions of a diprenylated unmethylated bisindolyl benzoquinone (DDAQ D) at *m/z* 507.2288 or 507.2281 were clearly detected in the extracts of mycelia of A1156 and the filtrate of DSM1958 (Figures S12 and S13). An $[M+H]^+$ ion of monoprenylated DDAQ D at *m/z* 439.1658 was also detected in the extracts of the culture filtrate of *A. terreus* DSM1958 (Figure S13).

Discussion

In the last decade, a large number of prenyltransferases, including the members of the DMATS superfamily, have been characterised biochemically.[26,30] Most of the known enzymes from the DMATS superfamily used tryptophan or tryptophan-containing cyclic dipeptides as natural or best substrates.[26] Despite the fact that a number of prenylated bisindolyl benzoquinones have been isolated from different fungi, only one prenyltransferase (TdiB) was characterised biochemically. In this study, we demonstrated the successful cloning, overexpression, and purification of the methylated bisindolyl benzoquinone, prenyltransferase AstPT from *A. terreus* DSM1958. By isolation and structure elucidation of the enzyme products, AstPT was shown to be responsible for the transfer of prenyl moieties from DMAPP to positions N-1 or C-2 of **1** to form mono- and diprenylated products **2**, **3**, **4** and **5**. Products **3** and **5** have been isolated from *A. terreus*.[10,12]

A remarkable feature of AstPT is the introduction of two prenyl moieties at two distinct positions of **1** with both regular and reverse prenylations. Its homologous protein from *A. nidulans*, TdiB, is also involved in two distinct prenylation steps in the biosynthesis of terrequinone A.[2,24] On its own, it catalyses a reverse prenylation at C-2 of the indole ring[24] and, in the presence of TdiC and TdiE, prenylation at the benzoquinone ring.[2] It was proposed that bisindolyl dihydroxybenzoquinone is first reduced by TdiC to tetrahydroxybenzene, and one of the hydroxy groups is replaced by a regular prenyl moiety in the presence of TdiB and TdiE. The monoprenylated intermediate with a prenyl moiety at the benzoquinone ring is converted by TdiB to terrequinone A. In contrast to TdiB, AstPT introduces two prenyl moieties in either regular or reverse manner to two distinct sites of AQ D in the presence of DMAPP without additional enzymes. Structural analysis of enzymes of the DMATS superfamily has revealed that these enzymes share very similar structures, and only one active site was found in their PT-barrel folds.[31–33] It could be speculated that AstPT has a similar structure to other known homologues, and the substrates are placed in AstPT in such positions that the C-1′ of DMAPP is close to the C-2 of **1** and the C-3′ of DMAPP to N1 of **1**. This orientation would make a nucleophilic attack possible at both sites, N1 or C-2, and results in reverse N1 prenylation and regular C2 prenylation. A final explanation of the mechanism of the AstPT reaction will be possible when a crystal structure of this enzyme becomes available.

Incubation of the two monoprenylated products, **2** and **3**, with AstPT in the presence of DMAPP led to the formation of **4** and **5**. One uncharacterised compound was detected in the HPLC chromatogram of the incubation mixture of AstPT with **2** (marked with an asterisk in Figure 1 B). However, due to the low quality, structure elucidation was not possible in this study. It is considerable that this peak contained a substance with two regular C2 prenylations. Taking into account the relationship of structures and retention times of the enzyme products, the retention time of this substance was consistent with a presumed C2′,C2″ diprenylation, as N-prenylation led to higher hydrophobicity and therefore to longer retention times.

Analysis of the genes near to ATEG_09980 did not indicate the presence of any biosynthetic gene cluster for secondary metabolites. ATEG_09980 has only one neighbouring gene that shows 48% identity on the amino acid level to *tdiC*, which acts as NADH-dependent oxidoreductase in the biosynthesis of terrequinone A.[2] The second *tdiB* homologue, ATEG_00702 from *A. terreus*, is likely located in a similar putative biosynthetic gene cluster to that of terrequinone A, with four homologous genes. ATEG_00702 is separated from a NRPS-like *tdiA* homologue (ATEG_00700) by a *tdiC* homologue (ATEG_00701; Figure S2). Inactivation of ATEG_00700 abolished the production of asterriquinone CT5 (AQ CT5), a regularly C2′,C2′′-diprenylated bisindolyl benzoquinone.[25] Therefore, it is plausible that the putative prenyltransferase EAU39348 encoded by ATEG_00702 would catalyse the prenylation reaction in the biosynthesis of AQ CT5. However, incubation of **1** with purified EAU39348 did not result in the formation of any enzyme products. One reason might be that **1** carries two methoxy groups instead of hydroxy groups at the benzoquinone ring and therefore is a poor substrate for EAU39348. Support for this hypothesis was obtained from experiments with TdiB, which was overproduced and purified by using a successfully expressed construct as described.[24] No enzyme activity was detected with **1** and TdiB in the presence of DMAPP (data not shown). It can be assumed that EAU39348 and TdiB both show similar substrate specificities for unmethylated substances. Attempts to remove the methyl groups of **1** chemically under different conditions remained unsuccessful. The high substrate specificity of AstPT is unusual for enzymes of the DMATS superfamily, which usually show high flexibility towards their aromatic substrates. For example, the known tryptophan and tryptophan-containing cyclic dipeptide prenyltransferases also accept a large number of indole derivatives and cyclic dipeptides.[26] In contrast, incubation of AstPT with tryptophan or tryptophan-containing cyclic dipeptides did not result in the formation of any enzyme product. The high substrate specificity of AstPT cannot be explained by its sequence similarities to other known prenyltransferases. It is known that enzymes of the DMATS superfamily share low sequence similarity with each other, usually in the range of 30–35% on the amino acid level, even for those with same substrates. For example, the tryptophan C4 prenyltransferase FgaPT2[34] shows a sequence identity of only 31% to 7-DMATS,[35] which catalyses the prenylation of tryptophan at C-7. On the other hand, enzymes from this group with distinct substrates show comparable sequence similarities to each other. Examples are SirD, XptB and NscD, which catalyse prenylation reactions of tyrosine, a xanthone and an anthrachene derivative, respectively.[36–38] Therefore, it is difficult to predict the substrates of an unknown prenyltransferase or its prenyltransfer reaction, or to explain its substrate specificity by sequence comparison with other known enzymes. An explanation could be provided by analysis of the crystal structure of the enzyme.

As mentioned in the Introduction, prenylated bisindolyl benzoquinones with hydroxy and methoxy groups have been isolated from *A. terreus* (Scheme 1). LC/MS analyses in this study also revealed the presence of mono- and diprenylated derivatives of both methylated and unmethylated bisindolyl benzoquinones in cultures of *A. terreus* DSM1958 (Figures S11 and S13), although the coding sequence of EAU39349 was not amplified from genomic DNA of this strain. (Reasons for the unexpected result could be the unsuitable primers or conditions used for the PCR amplification). The two TdiB homologues AstPT and EAU39348 share a sequence identity of 71% on the amino acid level, which is extremely high for members of the DMATS superfamily. It can therefore be speculated that they accept very similar substrates and catalyse similar reactions. The results on the putative cluster of AQ CT5 reported by Guo and co-workers[25] and the fact that AstPT but not EAU39348, can convert **1** to prenylated derivatives lead to the hypothesis that AstPT is responsible for the prenylation of O-methylated benzoquinone **1** and EAU39349 for the prenylation of its demethylated form. Formation of **1** could be catalysed by an O-methyltransferase. Evidence for this hypothesis is also provided by the work of Arai and Yamamoto, who detected methylation activity using DDAQ D (Scheme 1) as the substrate for crude enzyme extracts from *A. terreus*.[39] The structure gene for this activity could be ATEG_00703, coding for EAU39349 from the AQ CT5 cluster (Figure S2),[25] as no methylation is involved in its biosynthesis. The homologue of EAU39349 from the *tdi* cluster TdiE also shows homology to methyltransferases but did not function as a methyltransferase in the biosynthesis of terrequinone A, as no methylation is necessary in that case.[2]

From the LC/MS analyses, the ratio of the total peak areas of prenylated methylated derivatives (Figure S11F and G) to those of unmethylated ones (Figure S13C and D), obtained from samples corresponding to same amounts of fungal cultures, was found to be 1:1.3. As shown in this study, AstPT has a high K_m value and low turnover number. The slow conversion of **1** by AstPT might lead to accumulation of **1**, which could inhibit the activity of the putative O-methyltransferase. As a consequence, the unmethylated bisindolyl benzoquinone would be consumed less for methylation but more for prenylation by EAU39348 so that prenylated derivatives of both substrates are produced by the strain.

Experimental Section

Chemicals: Dimethylallyl diphosphate (DMAPP), geranyl diphosphate, and farnesyl diphosphate were prepared according to the method described for geranyl diphosphate by Woodside.[40] Asterriquinone D (AQ D, **1**) was prepared through a three-step procedure. 2,5-Dichloro-3-indolyl-1,4-benzoquinone was synthesised from indole and 2,5-dichlorobenzoquinone according to the method by Pirrung et al.[41] and was used to obtain 3,6-dichloro-2,5-bis-(3-indolyl)-1,4-benzoquinone.[42] Conversion of 3,6-dichloro-2,5-bis-(3-indolyl)-1,4-benzoquinone to **1** by methoxylation was then performed according to a method described by Tanoue et al.[43] For detailed synthetic procedures, see the Supporting Information. NMR data for **1** are given in Table 1.

Computer-assisted sequence analysis: The software packages DNASIS (version 2.1; Hitachi Software Engineering, San Bruno, CA, USA) and FGENESH (Softberry, Inc; http://www.softberry.com/berry.phtml) were used for intron prediction and sequence analysis, re-

spectively. Alignments of amino acid sequences were carried out by using the program BLAST (http://blast.ncbi.nlm.nih.gov).

Bacterial strains, plasmids and culture conditions: *E. coli* XL1-Blue MRF′ (Stratagene) and *E. coli* BL21 (DE3) pLysS (AMS Biotechnology, Abingdon, UK) were used for gene cloning and expression. pGEM-T Easy and pQE70 vectors were obtained from Promega and Qiagen, respectively. pHIS8 was obtained from J. Noel.[44] Bacteria harbouring these vectors were cultivated at 37 °C in liquid or on solid lysogeny broth (LB) with agar (1.5%, *w/v*), supplemented with carbenicillin (50 μg mL^{-1}) for selection of recombinant *E. coli* strains.[45] For *E. coli* BL21 (DE3) pLysS, kanamycin (25 μg mL^{-1}) was also used.

Cultivation of *A. terreus* for DNA isolation: *A. terreus* DSM 1958 was purchased from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Braunschweig, Germany). *A. terreus* FGSCA1156 (identical to the genome reference strain NIH 2624) was kindly provided by Dr. Matthias Brock (Hans-Knöll-Institut, Jena, Germany). The strains were cultivated in 300 mL Erlenmeyer flasks containing 100 mL liquid or on plates with solid YME medium consisting of yeast extract (4.0 g L^{-1}), malt extract (10.0 g L^{-1}), glucose (4 g L^{-1}) and agar (20.0 g L^{-1}) at 30 °C in darkness.

DNA propagation, PCR amplification and gene cloning: Standard procedures for DNA isolation and manipulation in *E. coli* were performed as described previously.[45] Genomic DNA of *A. terreus* was isolated from five-day-old mycelia grown by using a stationary method on liquid YME medium according to the chloroform/isoamyl alcohol method. A MiniCycler from Bio-Rad was used for PCR amplification. Amplification of ATEG_09980 was carried out by a two-step PCR reaction with an Expand High Fidelity Kit (Roche Diagnostics) and genomic DNA of *A. terreus* DSM1958. In the first step, the three exons of ATEG_09980 were amplified in separate reactions, with primer pairs ATEG_09980_1 and ATEG_09980_2 for exon 1, ATEG_09980_3 and ATEG_09980_4 for exon 2 and ATEG_09980_5 and ATEG_09980_6 for exon 3 (see Table S3 for primer sequences). The primers ATEG_09980_2, 3, 4 and 5 contained overlapping regions with the adjacent exons, which were used for fusion of the exons in the second step of amplification. The entire coding sequence was then propagated by using primer pair ATEG_09980_1 and ATEG_09980_6 and cloned into pGEM-T Easy vector to give plasmid pST22. After confirmation of the sequence integrity of the cloned amplicon by sequencing (Eurofins MWG Operon, Eberberg, Germany), pST22 was digested with BamHI and BglII (Jena Bioscience, Jena, Germany). The resulting 1280 bp fragment was cloned into the BamHI and BglII restriction sites of pQE70 to give plasmid pST24 after confirmation of the correct orientation.

The same strategy was used for amplification of the coding sequence of ATEG_00702. PCR amplification was carried out by using genomic DNA of *A. terreus* FGSCA1156 with addition of 5% (*v/v*) DMSO, due to the high GC content of the genomic sequence. Primer pairs ATEG_00702_1_pHis and ATEG_00702_2 were used for the amplification of the first exon, ATEG_00702_3 and ATEG_00702_4 for the amplification of the second exon and ATEG_00702_5 and ATEG_00702_6_pHis for the amplification of the third exon (see Table S3 for primer sequences). The primers ATEG_00702_2, 3, 4 and 5 contained overlapping regions with the adjacent exons, which were used for fusion of the exons in the second step of amplification. The entire coding sequence was subsequently propagated by using primer pair ATEG_00702_1 and ATEG_00702_6 and cloned into pGEM-T Easy vector to give plasmid pST35. After confirmation of the sequence integrity of the cloned amplicon by sequencing (Eurofins MWG Operon), pST35 was digested with BamHI and NotI (Jena Bioscience). The resulting 1283 bp fragment was cloned into BamHI and NotI restriction sites of pHIS8 to give plasmid pST37.

Overproduction and purification of recombinant proteins: For overproduction of AstPT-His$_6$, *E. coli* XL1 blue MRF′ cells harbouring pST24 were cultivated in LB medium supplemented with carbenicillin (50 μg mL^{-1}). An overnight culture (2%, *v/v*) was used for inoculation of 1000 mL LB medium supplemented with carbenicillin (50 μg mL^{-1}) in a 2000 mL flask. After growing at 37 °C and 220 rpm for 6 h, the culture was cooled to 30 °C and subsequently incubated at 30 °C for additional 16 h. Cells were harvested by centrifugation (4000 *g*, 10 min, 4 °C) and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) at 2–5 mL per gram wet weight. After addition of lysozyme (1 mg mL^{-1}) and incubation on ice for 30 min, cells were sonicated six times for 10 s each at 200 W. To remove cellular debris from the soluble protein, the lysate was centrifuged at 20000 *g* and 4 °C for 30 min. Purification of the recombinant His$_6$-tagged fusion protein by affinity chromatography with Ni-NTA agarose resin (Macherey–Nagel, Düren, Germany) was carried out according to the manufacturer's instructions. AstPT-His$_6$ was subsequently eluted with NaH_2PO_4 (50 mM), NaCl (300 mM) and imidazole (250 mM, pH 8.0). The resulting fraction was passed through a PD-10 desalting column (GE Healthcare) that had been previously equilibrated with Tris·HCl (50 mM) and glycerol (15%, *v/v*, pH 7.5) to perform buffer exchange. AstPT-His$_6$ was eluted with the same buffer and stored at −80 °C.

For overproduction of His$_8$-EAU39348, *E. coli* BL21 (DE3) pLysS cells harbouring pST37 were cultivated in LB medium supplemented with carbenicillin (50 μg mL^{-1}) and kanamycin (25 μg L^{-1}). An overnight culture (2%, *v/v*) was used to inoculate LB (1 L) medium supplemented with carbenicillin (50 μg mL^{-1}) and kanamycin (25 μg L^{-1}) in a 2000 mL flask. Cells were grown at 37 °C to an A_{600} of 0.6. Gene expression was induced by addition of IPTG to a final concentration of 0.1 mM. The bacterial culture was cultivated for a further 16 h at 22 °C and then centrifuged to harvest the cells. Protein purification was carried out as described above, with an additional purification step on HisPur cobalt resin (Thermo Fisher Scientific) after affinity chromatography with Ni-NTA agarose resin.

Protein analysis and determination of the molecular mass of active AstPT-His$_6$: Purity of AstPT-His$_6$ and His$_8$-EAU39348 was analysed by SDS-PAGE according to the method of Laemmli[46] with 12% polyacrylamide gels by using Coomassie Brilliant Blue G-250 for staining. Protein quantification was carried out according to the method of Bradford.[47] The molecular mass of the recombinant AstPT-His$_6$ was determined by size-exclusion chromatography on a HiLoad 16/60 Superdex 200 column (GE Healthcare, Freiburg, Germany) that had been equilibrated with Tris·HCl buffer (50 mM, pH 7.5) containing NaCl (150 mM). The column was calibrated with Blue Dextran 2000 (2000 kDa), ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa; GE Healthcare). Separation was performed on an ÄKTA Purifier FPLC system (GE Healthcare) at a flow rate of 1 mL min^{-1}.

Enzyme assays with AstPT: The enzyme reaction mixtures (100 μL) contained Tris·HCl (50 mM, pH 7.5), $CaCl_2$ (5 mM), **1** (0.5 mM), DMAPP (2 mM), 0.8% (*v/v*) glycerol, and purified recombinant AstPT-His$_6$ (20 μg, 4.1 μM). The reaction mixtures were incubated at 37 °C for 1 or 16 h. For determination of the kinetic parameters of **1**, the assays contained DMAPP (2 mM), **1** (0.02, 0.05, 0.08, 0.1, 0.2,

0.3, 0.4 and 0.5 mM), and purified recombinant AstPT-His_6 (10 μg). The kinetic parameters of DMAPP were determined with **1** (0.5 mM) and DMAPP (0.02, 0.05, 0.1, 0.3, 0.5, 1.0, 1.5 and 2.0 mM). Incubation mixtures for determination of the kinetic parameters were incubated for 20 min. The enzymatic reaction was stopped by three extractions with EtOAc (200 μL). After evaporation of the combined organic phases to dryness, the residue was dissolved in DMSO (20 μL), diluted with CH_3CN (100 μL), and analysed by HPLC as described below. Two independent incubations were carried out routinely. Kinetic parameters were calculated as average values from Eadie–Hofstee, Hanes–Woolf, and Lineweaver–Burk plots. Conversion yields and velocities were calculated from the amount of consumed substrate by measuring the decrease in the peak area of **1** in the HPLC chromatogram.

For isolation and structure elucidation of enzyme products, incubations were carried out at 37 °C for 2 or 16 h. The incubation mixtures (20 mL) contained Tris·HCl (50 mM, pH 7.5), $CaCl_2$ (5 mM), **1** (0.5 mM), DMAPP (2 mM) and purified recombinant protein (4.8 mg).

Enzyme assays with EAU39349: The enzyme reaction mixtures (100 μL) contained Tris·HCl (50 mM, pH 7.5), $CaCl_2$ (5 mM), **1** (0.5 mM), DMAPP (2 mM), 4.5% (*v/v*) glycerol and purified recombinant His_8-EAU39349 (6.3 μg). The reaction mixtures were incubated at 37 °C for 16 h, extracted three times with EtOAc (200 μL), and analysed by HPLC, as described below.

HPLC conditions for analysis and isolation of enzyme products: The enzyme products of the incubation mixtures were analysed on an Agilent series 1200 HPLC system (Agilent Technologies) by using a Multospher 120 RP 18-5 column (250×4 mm, 5 μm, CS-Chromatographie Service, Langerwehe, Germany) at a flow rate of 1 mL min^{-1}. Water ("A") and CH_3CN ("B") were used as solvents. For analysis of enzyme products, a linear gradient of 50–100% (*v/v*) solvent B in 20 min was used. The column was then washed with 100% solvent B for 5 min and equilibrated with 50% (*v/v*) solvent B for 5 min. Detection was carried out on a photodiode array detector, and absorption at 277 nm was illustrated in this study.

For isolation of the enzyme products, the same HPLC equipment with a Multospher 120 RP18 HP column (250×10 mm, 5 μm, Chromatographie Service) was used. Separation of the enzyme products was initiated with 70% (*v/v*) solvent B for 2 min at a flow rate of 2.5 mL min^{-1} and continued over a linear gradient of 70–100% (*v/v*) solvent B. The column was then washed with 100% solvent B for 5 min and equilibrated with 70% (*v/v*) solvent for 5 min.

Spectroscopic analysis of the enzyme products: The isolated enzyme products were dissolved in MeOH and analysed by high-resolution electron impact mass spectrometry (HR-EI-MS) on an AutoSpec instrument (Micromass, Manchester, UK). Positive HR-EI-MS data of the enzyme products are given in Table S2. For NMR analysis, the isolated products were dissolved in 0.75 mL of $(CD_3)_2CO$. Spectra were recorded at room temperature on an ECA-500 spectrometer (JEOL). Spectra were processed with MestReNova 6.0.2 (Metrelab Research, Santiago de Compostela, Spain), and chemical shifts were referenced to the signal of $(CD_3)_2CO$ at 2.05 ppm. NMR data are given in Table 1.

Cultivation of *A. terreus* and analysis of secondary metabolites by LC/MS analysis: *A. terreus* DSM1958 and FGSCA1156 were cultivated in 300 mL Erlenmeyer flasks containing YME medium (100 mL) at 30 °C and 120 rpm in darkness for 10 days. Mycelia and culture filtrates were separated by filtration and each extracted with EtOAc (3×100 μL). The combined organic layers were dried over Na_2SO_4 and subsequently evaporated in vacuo to dryness. For LC/MS analyses, the extracts were dissolved in CH_3CN. LC/MS analysis was carried out on a 1100 HPLC system (Agilent Technologies) coupled to an LTQ-FT Ultra ESI-MS instrument (Thermo Fischer Scientific) by using a linear ion trap (MS) and an ICR cell (HR-MS), operated in positive ionisation mode. Water (solvent A) and CH_3CN (solvent B) were used at a flow rate of 1 mL min^{-1}. A linear gradient of 10–100% (*v/v*) solvent B in 20 min was used. The column was then washed with 100% solvent B for 5 min and equilibrated with 10% (*v/v*) solvent B for 5 min.

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[1] S.-M. Li, *Nat. Prod. Rep.* **2010**, *27*, 57–78.
[2] C. J. Balibar, A. R. Howard-Jones, C. T. Walsh, *Nat. Chem. Biol.* **2007**, *3*, 584–592.
[3] P. Schneider, M. Weber, K. Rosenberger, D. Hoffmeister, *Chem. Biol.* **2007**, *14*, 635–644.
[4] S. Sekita, *Chem. Pharm. Bull.* **1983**, *31*, 2998–3001.
[5] U. Mocek, L. Schultz, T. Buchan, C. Baek, L. Fretto, J. Nzerem, L. Sehl, U. Sinha, *J. Antibiot.* **1996**, *49*, 854–859.
[6] A. Fredenhagen, F. Petersen, M. Tintelnot-Blomley, J. Rosel, H. Mett, P. Hug, *J. Antibiot.* **1997**, *50*, 395–401.
[7] K. A. Alvi, H. Pu, M. Luche, A. Rice, H. App, G. McMahon, H. Dare, B. Margolis, *J. Antibiot.* **1999**, *52*, 215–223.
[8] J. W. Bok, D. Hoffmeister, L. A. Maggio-Hall, R. Murillo, J. D. Glasner, N. P. Keller, *Chem. Biol.* **2006**, *13*, 31–37.
[9] J. He, E. M. K. Wijeratne, B. P. Bashyal, J. Zhan, C. J. Seliga, M. X. Liu, E. E. Pierson, L. S. Pierson, H. D. VanEtten, A. A. L. Gunatilaka, *J. Nat. Prod.* **2004**, *67*, 1985–1991.
[10] Y. Yamamoto, K. Nishimura, N. Kiriyama, *Chem. Pharm. Bull.* **1976**, *24*, 1853–1859.
[11] D. Brewer, W. A. Jerram, A. Taylor, *Can. J. Microbiol.* **1968**, *14*, 861–866.
[12] K. Arai, K. Masuda, N. Kiriyama, K. Nitta, Y. Yamamoto, S. Shimizu, *Chem. Pharm. Bull.* **1981**, *29*, 961–969.
[13] K. Arai, *PhD Thesis*, Kyoto University (Japan), **1984**.
[14] K. Arai, S. Shimizu, Y. Taguchi, Y. Yamamoto, *Chem. Pharm. Bull.* **1981**, *29*, 991–999.
[15] Y. Yamamoto, N. Kiriyama, S. Shimizu, S. Koshimura, *Gann* **1976**, *67*, 623–624.
[16] A. Kaji, T. Iwata, N. Kiriyama, M. Nomura, K. Miyamoto, *J. Antibiot.* **1998**, *51*, 235–238.
[17] S. Shimizu, Y. Yamamoto, S. Koshimura, *Chem. Pharm. Bull.* **1982**, *30*, 1896–1899.
[18] D. Brewer, W. A. Jerram, D. Meiler, A. Taylor, *Can. J. Microbiol.* **1970**, *16*, 433–440.
[19] D. Meiler, A. Taylor, *Can. J. Microbiol.* **1971**, *17*, 83–86.
[20] D. Brewer, W. C. Jen, G. A. Jones, A. Taylor, *Can. J. Microbiol.* **1984**, *30*, 1068–1072.
[21] B. Zhang, G. Salituro, D. Szalkowski, Z. Li, Y. Zhang, I. Royo, D. Vilella, M. T. Díez, F. Pelaez, C. Ruby, R. L. Kendall, X. Mao, P. Griffin, J. Calaycay, J. R. Zierath, J. V. Heck, R. G. Smith, D. E. Moller, *Science* **1999**, *284*, 974–977.
[22] S. A. Qureshi, V. Ding, Z. Li, D. Szalkowski, D. E. Biazzo-Ashnault, D. Xie, R. Saperstein, E. Brady, S. Huskey, X. Shen, K. Liu, L. Xu, G. M. Salituro,

J. V. Heck, D. E. Moller, A. B. Jones, B. B. Zhang, *J. Biol. Chem.* **2000**, *275*, 36590 – 36595.
[23] K. Liu, L. Xu, D. Szalkowski, Z. Li, V. Ding, G. Kwei, S. Huskey, D. E. Moller, J. V. Heck, B. B. Zhang, A. B. Jones, *J. Med. Chem.* **2000**, *43*, 3487 – 3494.
[24] P. Schneider, M. Weber, D. Hoffmeister, *Fungal Genet. Biol.* **2008**, *45*, 302 – 309.
[25] C. J. Guo, B. P. Knox, J. F. Sanchez, Y. M. Chiang, K. S. Bruno, C. C. C. Wang, *Org. Lett.* **2013**, *15*, 3562 – 3565.
[26] X. Yu, S.-M. Li, *Methods Enzymol.* **2012**, *516*, 259 – 278.
[27] N. Khaldi, F. T. Seifuddin, G. Turner, D. Haft, W. C. Nierman, K. H. Wolfe, N. D. Fedorova, *Fungal Genet. Biol.* **2010**, *47*, 736 – 741.
[28] A. Kaji, K. Kimura, M. Teranishi, N. Kiriyama, M. Nomura, K. Miyamoto, *Chem. Pharm. Bull.* **1998**, *46*, 1325 – 1329.
[29] S.-M. Li, *Appl. Microbiol. Biotechnol.* **2009**, *84*, 631 – 639.
[30] L. Heide, *Curr. Opin. Chem. Biol.* **2009**, *13*, 171 – 179.
[31] U. Metzger, C. Schall, G. Zocher, I. Unsöld, E. Stec, S.-M. Li, L. Heide, T. Stehle, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 14309 – 14314.
[32] M. Jost, G. Zocher, S. Tarcz, M. Matuschek, X. Xie, S.-M. Li, T. Stehle, *J. Am. Chem. Soc.* **2010**, *132*, 17849 – 17858.
[33] J. M. Schuller, G. Zocher, M. Liebhold, X. Xie, M. Stahl, S.-M. Li, T. Stehle, *J. Mol. Biol.* **2012**, *422*, 87 – 99.
[34] I. A. Unsöld, S.-M. Li, *Microbiology* **2005**, *151*, 1499 – 1505.
[35] A. Kremer, L. Westrich, S.-M. Li, *Microbiology* **2007**, *153*, 3409 – 3416.
[36] A. Kremer, S.-M. Li, *Microbiology* **2010**, *156*, 278 – 286.
[37] D. Pockrandt, L. Ludwig, A. Fan, G. M. Konig, S. M. Li, *ChemBioChem* **2012**, *13*, 2764 – 2771.
[38] Y. H. Chooi, J. Fang, H. Liu, S. G. Filler, P. Wang, Y. Tang, *Org. Lett.* **2013**, *15*, 780 – 783.
[39] K. Arai, Y. Yamamoto, *Chem. Pharm. Bull.* **1990**, *38*, 2929 – 2932.
[40] A. B. Woodside, Z. Huang, C. D. Poulter, *Org. Synth.* **1988**, *66*, 211 – 215.
[41] M. C. Pirrung, L. Deng, Z. Li, K. Park, *J. Org. Chem.* **2002**, *67*, 8374 – 8388.
[42] M. C. Pirrung, Y. Liu, L. Deng, D. K. Halstead, Z. Li, J. F. May, M. Wedel, D. A. Austin, N. J. G. Webster, *J. Am. Chem. Soc.* **2005**, *127*, 4609 – 4624.
[43] Y. Tanoue, T. Motoi, Y. Masuda, N. Kai, K. Sakata, M. Hashimoto, T. Nagai, *J. Heterocycl. Chem.* **2008**, *45*, 1509 – 1511.
[44] J. M. Jez, J. L. Ferrer, M. E. Bowman, R. A. Dixon, J. P. Noel, *Biochemistry* **2000**, *39*, 890 – 902.
[45] J. Sambrook, D. W. Russell in *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, New York, **2001**.
[46] U. K. Laemmli, *Nature* **1970**, *227*, 680 – 685.
[47] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248 – 254.

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