Reconstruction of pyrrolo[2,3-b]indoles carrying an α-configured reverse C3-dimethylallyl moiety by using recombinant enzymes†

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Nine reversely C3-prenylated pyrrolo[2,3-b]indoles were successfully prepared by using two recombinant enzymes involved in the biosynthesis of acetylaszonalenin from *Neosartorya fischeri*. The prenyltransferase AnaPT catalysed the conversion of six tryptophan-containing cyclic dipeptides to reversely C3-prenylated indoline derivatives. Using cyclo-L-Trp-L-Trp as substrate, both mono- and diprenylated indolines were obtained. Two of the AnaPT products were acetylated at position N1 by the acetyltransferase AnaAT. The structures of the obtained compounds were characterised by HR-ESI-MS, ^{1}H- and ^{13}C-NMR analyses as well as by long-range ^{1}H-^{13}C connectivities in heteronuclear multiple-bond correlation (HMBC) spectra after preparative isolation. Their absolute configurations were determined by analysing the ^{1}H-^{1}H spatial correlations in rotating-frame nuclear overhauser effect spectroscopy (ROESY).

Introduction

Natural products from plants and microorganisms are among the most important sources of leading compounds for drug development.[1] Prenylated indole alkaloids are mainly found in the genera of *Penicillium* and *Aspergillus* of Ascomycota[2,3] and often possess biological activities clearly distinct from their non-prenylated aromatic precursors.[4,5] A subgroup of these compounds, C3-prenylated indoline alkaloids, usually carry a reverse prenyl moiety at position C3 of the indoline ring and a five-membered ring system between the indoline and the diketopiperazine ring. A prominent example is the mycotoxin roquefortine C, which was isolated firstly from *Penicillium roqueforti*[6] and identified later in many *Penicillium* strains.[7,8] Some of these natural products showed a wide range of biological and pharmacological activities. For example, brevicompanines A, B and C produced by *P. brevicompactum* were identified as plant growth regulators.[9,10] Fructigenines A and B (verrucofortine) (Fig. 1) from *P. fructigenum* and *P. verrucosum var. cyclopium*[11,12] were reported to show an inhibitory effect on plant growth. Rugulosuvines A and B (Fig. 1) from *P. rugulosum* and *P. piscarium* had moderate cytotoxicity against L-929, K562 and Hela cells.[13] Amauromine (Fig. 1) from *Amauroascus sp.* was reported to have a vasodilating activity.[14] Its stereoisomer epiamauromine (Fig. 1) from *Aspergillus ochraceus*, on the other hand, showed insecticidal activity.[15]

Acetylaszonalenin is a mycotoxin, which was isolated initially from an unidentified *Aspergillus* species.[16] Together with its non-acetylated form aszonalenin, it was also identified in various fungal strains including *Neosartorya fischeri*.[17,18]

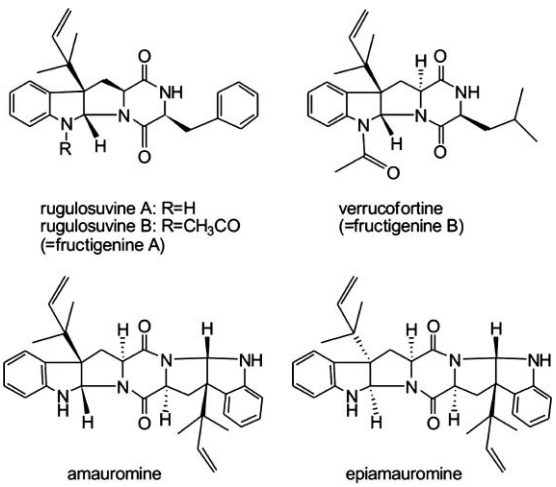

Fig. 1 Examples of C3-prenylated pyrrolo[2,3-b]indoles.

The C3-prenylated indoline derivatives mentioned above are believed to be formed biosynthetically by prenylation of tryptophan-containing cyclic dipeptides and subsequent modifications such as acetylation at position N1.[2] An experimental proof was provided recently by identification of the biosynthetic gene cluster of acetylaszonalenin from *N. fischeri*.[17] A putative prenyltransferase gene *anaPT* and a putative acetyltransferase gene *anaAT* from this cluster were cloned and overexpressed in *E. coli*, respectively. The overproduced recombinant proteins AnaPT and AnaAT were purified to homogeneity and characterised biochemically. In the biosynthesis of acetylaszonalenin, AnaPT catalysed the reverse prenylation of (*R*)-benzodiazepinedione at position C3 of the indole moiety in the presence of dimethylallyl diphosphate (DMAPP) resulting in formation of aszonalenin containing a pyrrolo[2,3-b]indole moiety. The acetyltransferase AnaAT catalysed the conversion of aszonalenin to acetylaszonalenin in the presence of acetyl-CoA.[17]

Recently, different strategies were developed for the synthesis of biologically active natural products or analogues.[19–22]

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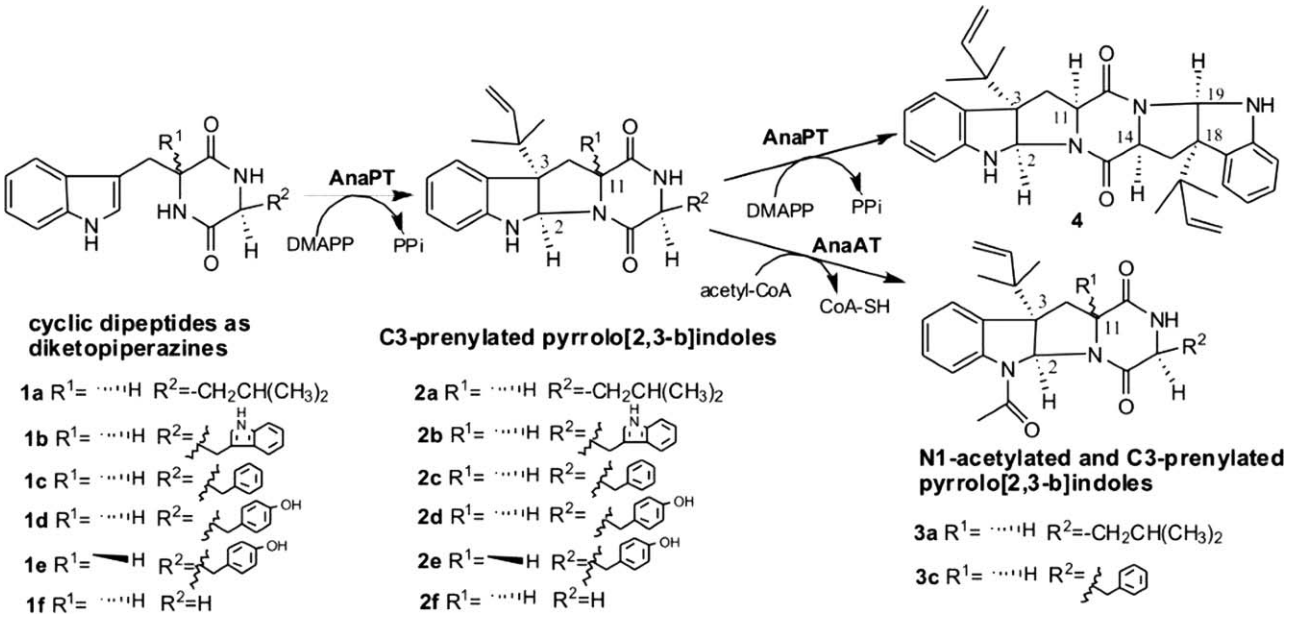


Scheme 1 Chemoenzymatic synthesis of C3-prenylated pyrrolo[2,3-b]indoles by recombinant enzymes.

Chemoenzymatic synthesis by using purified enzymes can be recognised as a potential method for this purpose. Based on this strategy, four aszonalenin stereoisomers were synthesised successfully by using two indole prenyltransferases AnaPT and CdpNPT. The conversion rates of the one-step reactions reached 85–100%. By using HPLC, only one stereoisomer each was detected from the incubation mixtures of different enzyme and substrate combinations. The enzymatic products were isolated and their structures were elucidated by NMR, MS and CD analyses.[23] These results prompted us to test the possibility for synthesis of other C3-prenylated pyrrolo[2,3-b]indoles by using AnaPT and AnaAT as catalysts and dipeptides as substrates (Scheme 1).

Results and discussion

For this purpose, six tryptophan-containing cyclic dipeptides at a final concentration of 1 mM were tested for acceptance by AnaPT in the presence of DMAPP (1 mM). After incubation with 0.54 μM AnaPT for 24 h, the reaction mixtures were analysed on HPLC. As shown in Fig. 2, all of the six substrates were accepted by AnaPT with cyclo-L-Trp-L-Leu (**1a**) as the best substrate under these conditions (Fig. 2A). One product peak each was detected for cyclo-L-Trp-L-Leu (**1a**), cyclo-L-Trp-L-Phe (**1c**), cyclo-L-Trp-L-Tyr (**1d**) and cyclo-L-Trp-Gly (**1f**) (Fig. 2A, 2E, 2G, and 2K). In the chromatograms of the incubation mixtures of cyclo-L-Trp-L-Trp (**1b**) (Fig. 2C) and cyclo-D-Trp-L-Tyr (**1e**) (Fig. 2I), two product peaks were observed. Because the unknown extinction coefficients of the enzymatic products and low amounts of the isolated compounds, the conversion rates of the AnaPT reaction could not be determined with acceptable accuracy. To determine the dependence of product formation on incubation times, we carried out incubations with all of the six mentioned cyclic dipeptides for 0, 1, 4 and 10 h and analysed the reaction mixtures with HPLC (see Figure S1, ESI†). Increasing of product formation was clearly observed for all of the substrates, although the yields for **1e** and **1f** were relative low. This indicated however that AnaPT can be in principle used for synthesis of diverse C3-prenylated indolines. For a biotechnological application, however, further experiments such as mutagenesis should be carried out to create enzyme derivatives with higher catalytic efficiency.

In a second series, the reaction mixtures of the six substrates with AnaPT were further incubated with AnaAT in the presence of acetyl-CoA. In the HPLC chromatograms of the tandem reaction mixtures, additional detectable products were only observed with cyclo-L-Trp-L-Leu (**1a**) (Fig. 2B) and cyclo-L-Trp-L-Phe (**1c**) (Fig. 2F). In the tandem assay with **1a**, the prenylated product was almost completely converted to the peak **3a**. In the case of **1c**, about two-third of the prenylated product was converted. The low concentration of the prenylation products of other cyclic dipeptides was likely responsible for their non-acceptance by AnaAT.

For structural elucidation, the products of AnaPT were isolated on a preparative scale and substances in the range of 0.2–1 mg were subjected to HR-ESI-MS and NMR analyses. HR-ESI-MS (Table 1) confirmed the presence of one dimethylallyl moiety in the structures of **2a–2f** by detection of $[M+1]^+$ or $[M+Na]^+$ ions, which are 68 daltons larger than those of the respective substrates. Comparison of the ^{1}H-NMR data of the isolated products **2a–2f** (Table 2) with those of the respective substrates (data not shown) revealed clearly the presence of signals for a reverse dimethylallyl moiety in the spectra of the isolated compounds at 5.03–5.16 (d or dd) for H-1′, 5.90–5.96 (dd) for H-2′, 0.95–0.99 ppm (s) for H-4′ and 1.05–1.14 (s) for H-5′, respectively. The signals of H-2 were strongly upfield shifted from approximately 7.0–7.2 in the spectra of the substrates to 5.4 ppm in the spectra of the enzymatic products. These shifts were similar to those observed with aszonalenins.[23] This indicated that the prenylation has very likely taken place at position C3 of the cyclic dipeptides and pyrrolo[2,3-b]indole derivatives were formed during the enzymatic reactions.

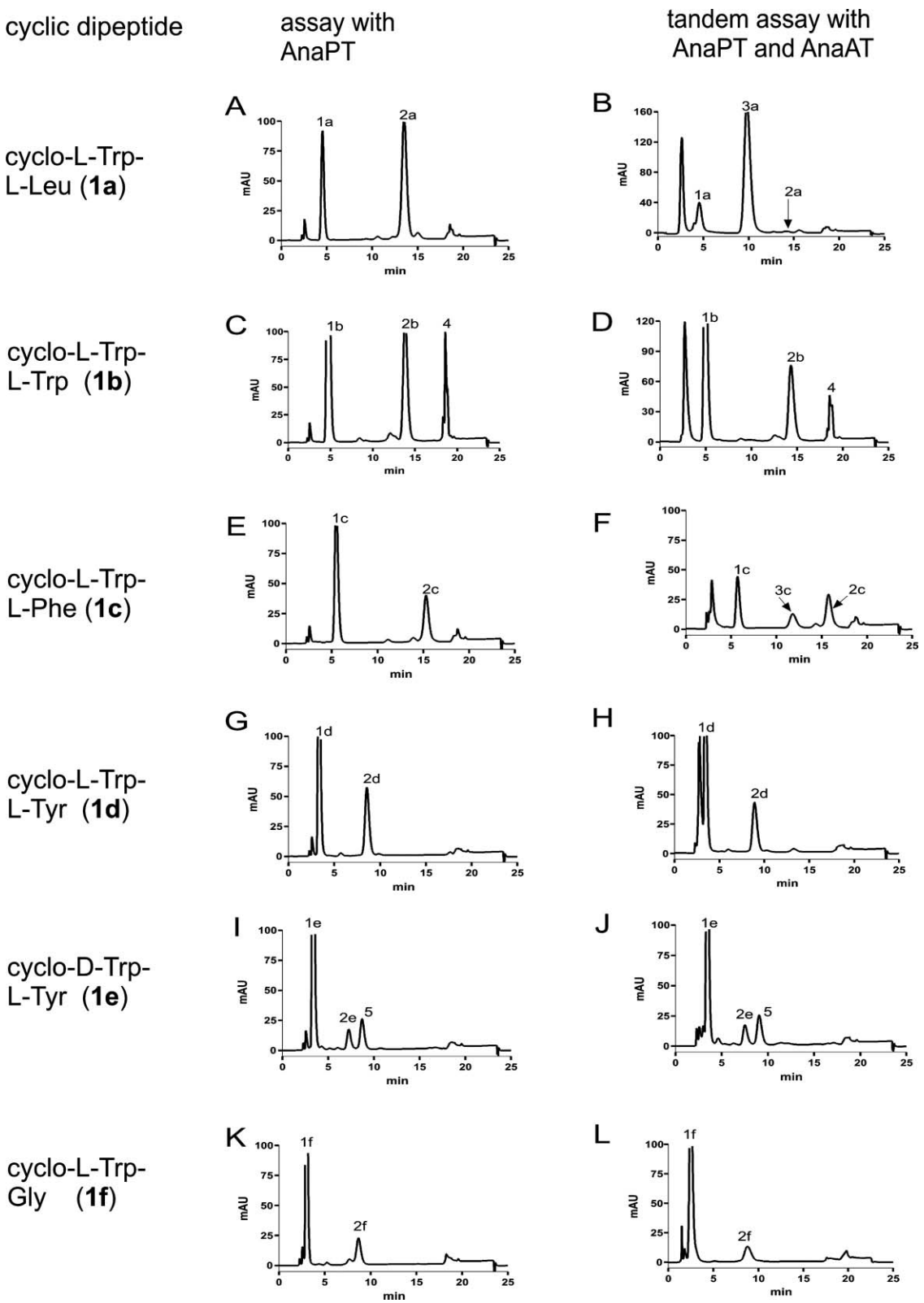


Fig. 2 HPLC chromatograms of incubation mixtures of six tryptophan-containing cyclic dipeptides with recombinant AnaPT or AnaPT and AnaAT. Detection was carried out with a photo diode array detector and illustrated for absorption at 254 nm.

Table 1 HR-ESI-MS data of the enzymatic products

Comp.	Chemical Formula	HR-ESI-MS data		
		Calculated	Measured	Deviation (ppm)
2a	$C_{22}H_{29}N_3O_2$	368.2338 $[M+1]^+$	368.2370	8.7
2b	$C_{27}H_{28}N_4O_2$	441.2291 $[M+1]^+$	441.2234	12.9
2c	$C_{25}H_{27}N_3O_2$	402.2182 $[M+1]^+$	402.2209	6.7
2d	$C_{25}H_{27}N_3O_3$	418.2131 $[M+1]^+$	418.2145	3.3
2e	$C_{25}H_{27}N_3O_3$	440.1950 $[M+Na]^+$	440.1962	2.7
2f	$C_{18}H_{21}N_3O_2$	312.1712 $[M+1]^+$	312.1732	6.4
3a	$C_{24}H_{31}N_3O_3$	410.2444 $[M+1]^+$	410.2399	11.0
3c	$C_{27}H_{29}N_3O_3$	444.2287 $[M+1]^+$	444.2309	4.9
4	$C_{32}H_{36}N_4O_2$	509.2917 $[M+1]^+$	509.2932	2.9
5	$C_{25}H_{27}N_3O_3$	418.2131 $[M+1]^+$	418.2118	3.1

The assignments listed in Table 2 were verified by analysing the ^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) spectra. Cross peaks in the HSQC spectra of **2a–2f** revealed that singlets of H-2 at 5.38–5.43 ppm correlated with the signals of C-2 at 77.5–79.5 ppm, indicating the disappearance of the double bond between C-2 and C-3 of the indole ring in the structures of **2a–2f**. HMBC spectra showed clear connectivity from H-2 to C-11 and C-13. These connectivities are conducted by the formation of a chemical bond between C-2 and N-12 of the diketopiperazine ring. These results proved that the structures of **2a–2f** are indeed C3-prenylated indolines with a fused five-membered ring between the indoline and diketopiperazine ring. This means that AnaPT catalysed, in analogy to its natural substrate (*R*)-benzodiazepinedinone, the reverse C3-prenylation of tryptophan-containing cyclic dipeptides and meanwhile the formation of a pyrrolo[2,3-b]indole structure.

In analogy to the prenylated products, the acetylated products of the tandem reactions of cyclo-L-Trp-L-Leu (**1a**) and cyclo-L-Trp-L-Phe (**1c**) with AnaPT and AnaAT were also isolated on a preparative scale and subjected to HR-ESI-MS (Table 1) and NMR analyses. HR-ESI-MS results confirmed the presence of one acetyl moiety in **3a** and **3c** by detection of $[M+1]^+$ ions, which are 42 daltons larger than those of the respective substrates, *i.e.* the enzymatic products **2a** and **2c** of AnaPT. A singlet for three protons at 2.53 ppm in the ^{1}H spectrum of **3a** and 2.56 ppm in that of **3c** were observed, respectively (Table 3). These data are in the range of signals for the N1-acetyl groups of C3-prenylated pyrrolo[2,3-b]indoles, *e.g.* 2.63 ppm for $NCOCH_3$ of fructigenine A[12] and verrucofortine.[11] Spatial correlations of H-2 and H-7 with the methyl protons of the acetyl group were clearly observed in a ROESY spectrum of **3a** (spectrum not shown). This proved that the acetyl moiety was attached to position N1 of the indoline ring and corresponded very well to the N1 acetylation of aszonalenin by AnaAT, the natural substrate of this enzyme.[17]

Until now, three C3-monoprenylated pyrrolo[2,3-b]indoles derived from the cyclic dipeptides tested in this study were isolated from various fungal cultures and identified as N1-acetylated and non-acetylated derivatives. These compounds include verrucofortine (Fig. 1),[11] termed also fructigenine B,[12] which is an N1-acetylated derivative of L-tryptophan and L-leucine. Rugulosuvine A and its N1-acetylated derivative rugulosuvine B, termed also fructigenine A,[12] (Fig. 1) are derived from L-tryptophan and L-phenylalanine.[13] All of these compounds have a configuration of 2*S* and 3*R*, *i.e.* a *cis*-configuration between C2 and C3. The absolute configuration of fructigenine A was determined by comparison of the CD spectra with those of related known compounds.[12] Taken together, both verrucofortine[11] and **3a** are N1-acetylated and C3-prenylated pyrrolo[2,3-b]indole from L-tryptophan and L-leucine. Both **3c** and fructigenine A are N1-acetylated and C3-prenylated pyrrolo[2,3-b]indole from L-tryptophan and L-phenylalanine.

To determine the stereochemistry of **3a** and **3c** at C2 and C3, we compared the ^{1}H-NMR data of **3a** with those of verrucofortine[11] and **3c** with those of fructigenine A,[12] all taken in $CDCl_3$. It turned out that the NMR data of **3a** and **3c** differed clearly from those of verrucofortine and fructigenine A, respectively, indicating that **3a** and verrucofortine as well as **3c** and fructigenine A are diastereomers to each other by differences of stereochemistry at positions C2 and C3. One of the remarkable differences of the NMR data is the chemical shift for H2, which was reported to be at 6.06 and 6.04 ppm for verrucofortine and fructigenine A, respectively. The chemical shifts of H2 of **3a** and **3c** were strongly upfield shifted to 5.76 and 5.26 ppm, respectively, being similar to the observed values for other prenylated products reported in this study. This means that both **3a** and **3c** have very likely a (2*R*,3*S*)-configuration, which is in accordance with the results obtained from the prenylation of (*R*)-and (*S*)-benzodiazepinedinone by AnaPT.[23] Because stereochemical retention was expected during an acetyl transfer reaction, the configuration of **2a** and **2c** at positions C2 and C3 must be the same as those of **3a** and **3c**, *i.e.* 2*R* and 3*S*.

CD spectra of **2a–2f** (Fig. 3) provided further evidence for their stereochemistry at C2 and C3. Fig. 3 showed clearly that all of these compounds have a positive Cotton effect at 296 nm. A positive Cotton effect at 296 nm was detected in the CD spectra of the two aszonalenins with a configuration of (2*R*,3*S*) and a negative Cotton effect at this wavelength in other two aszonalenin stereoisomers with a configuration of (2*S*,3*R*). Almost no signal was found for the both precursors of the four aszonalenins without

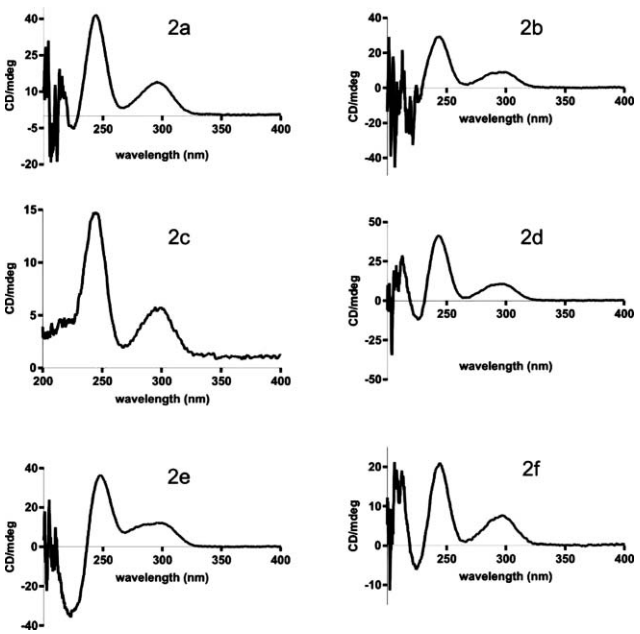


Fig. 3 CD spectra of C3-prenylated pyrrolo[2,3-b]indoles.

Table 2 ^{1}H-NMR and ^{13}C-NMR data of enzymatic products ($CDCl_3$)

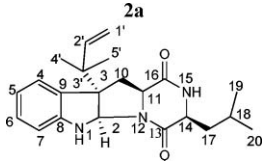
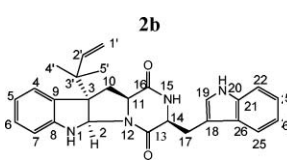
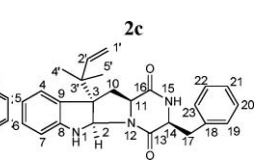
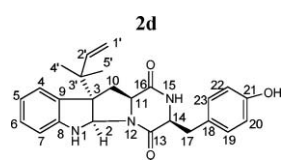
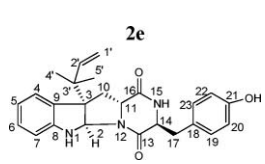
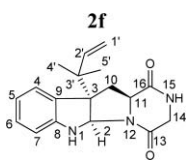
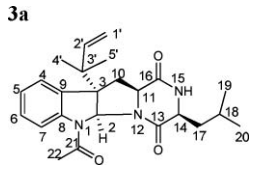
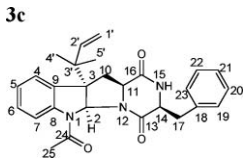
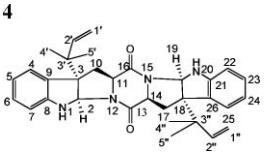
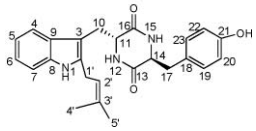
Compound	**2a** 		**2b** 		**2c** 		**2d** 		**2e** 		**2f** 	
Position	δ_C	δ_H, multi., J in Hz	δ_C	δ_H, multi., J in Hz	δ_C	δ_H, multi., J in Hz	δ_C	δ_H, multi., J in Hz	δ_C	δ_H, multi., J in Hz	δ_C	δ_H, multi., J in Hz
2	79.5	5.38, s	79.5	5.42, s	79.5	5.41, s	79.5	5.40, s	77.5	5.43, s	79.5	5.39, s
3	61.5	—	61.4	—	61.4	—	61.4	—	60.9	—	61.3	—
4	125.5	7.16, d, 7.6	125.6	7.15, dd, 7.6, 1.0	125.5	7.17, d, 7.6	125.5	7.15, d, 7.6	124.9	7.02, dd, 7.6, 0.8	125.6	7.16, ddd, 7.6, 1.2, 0.5
5	118.6	6.73, td, 7.6, 1.0	118.6	6.76, td, 7.4, 1.0	118.6	6.75, td, 7.6, 1.0	118.6	6.75, t, 7.6	118.5	6.75, td, 7.5, 1.0	118.7	6.74, td, 7.5, 1.1
6	128.1	7.07, td, 7.6, 1.3	128.2	7.11, td, 7.6, 1.3	128.2	7.11, td, 7.6, 1.0	128.3	7.12, td, 7.9, 1.0	128.6	7.18, td, 7.6, 1.2	128.3	7.08, td, 7.6, 1.3
7	108.8	6.54, d, 7.6	108.8	6.60, d, 7.6	108.8	6.60, d, 7.9	108.8	6.59, d, 7.9	108.8	6.66, d, 7.6	108.9	6.57, ddd, 7.6, 1.0, 0.5
8	148.2	—	148.3	—	148.2	—	148.2	—	150.0	—	148.1	—
9	131.1	—	131.4	—	131.2	—	131.1	—	128.5	—	131.1	—
10_{syn}	35.6	2.81, dd, 13.9, 9.2	36.2	2.79, dd, 13.8, 8.6	36.2	2.81, dd, 13.9, 8.9	36.2	2.81, dd, 13.6, 8.9	35.8	2.28, dd, 12.6, 6.0	36.3	2.84, dd, 13.9, 8.9
10_{anti}		2.55, dd, 13.9, 8.5		2.40, dd, 13.8, 9.3		2.45, dd, 13.9, 9.2		2.42, dd, 13.9, 9.2		2.20, dd, 12.4, 11.7		2.50, dd, 13.9, 8.9
11	57.4	4.12, t, 8.5	57.5	4.08, td, 9.2, 1.3	57.5	4.10, t, 9.2	57.5	4.08, t, 8.8	57.3	2.44, dd, 12.6, 7.3	56.7	4.10, td, 9.0, 1.5
13	168.3	—	167.6	—	167.4	—	167.3	—	167.5	—	165.3	—
14	53.6	3.95, dd, 9.5, 2.6	54.8	4.31, ddd, 10.7, 3.5, 1.6	56.3	4.19, ddd, 11.1, 3.8, 1.1	56.3	4.12,ddd,11.4,7.6, 3.8	58.3	4.17, dd, 8.8, 4.1	46.5	4.01, dd, 17.2, 1.8
	—	—	—	—	—	—	—	—	—	—	—	3.83, dd, 17.2, 4.2
15	—	5.54, s	—	5.57, s	—	5.45, s	—	5.45, br s	—	5.74, br s	—	5.70, br s
16	169.3	—	168.4	—	168.7	—	168.7	—	166.6	—	168.7	—
17	38.5	1.98, ddd, 14.3, 10.1, 4.0	26.9	3.71, ddd, 14.8, 3.8, 1.0	36.6	3.59, dd, 14.3, 3.6	35.6	3.48, dd, 14.7, 3.4	39.6	3.13, dd, 13.0, 3.4	—	—
17		1.51, ddd, 14.3, 9.5, 4.5		2.90, dd, 15.0, 11.1		2.69, dd, 14.5, 11.1		2.65, dd, 14.5, 10.7		2.83, dd, 13.0, 5.4		—
18	24.3	1.64, m	109.3	—	135.4	—	126.6	—	125.6	—	—	—
19	23.0	0.95, d, 6.6	123.1	7.02, d, 2.2	128.7	7.16, br d, 7.6	129.9	7.01, d, 8.2	130.7	6.88, dt, 8.5, 2.0	—	—
20	20.9	0.91, d, 6.3	—	8.04, s	129.2	7.31, t, 7.3	116.0	6.73, d, 7.6	115.6	6.43, dt, 8.5, 2.0	—	—
21	—	—	136.4	—	127.5	7.30, t, 7.6	155.2	—	155.2	—	—	—
22	—	—	111.4	7.37, d, 8.2	129.2	7.31, t, 7.3	116.0	6.73, d, 7.6	115.6	6.43, dt, 8.5, 2.0	—	—
23	—	—	122.6	7.23, td, 7.9, 1.0	128.7	7.16, br d, 7.6	129.9	7.01, d, 8.2	130.7	6.88, dt, 8.5, 2.0	—	—
24	—	—	119.8	7.13, td, 7.9, 1.0	—	—	—	—	—	—	—	—
25	—	—	118.3	7.56, d, 7.9	—	—	—	—	—	—	—	—
26	—	—	126.3	—	—	—	—	—	—	—	—	—
1′	114.4	5.15, dd, 10.7, 1.2	114.6	5.15, dd, 10.7, 1.0	114.6	5.16, dd, 10.7, 1.0	114.5	5.16, d, 10.7	114.3	5.08, dd, 10.8, 1.1	114.6	5.16, dd, 10.8, 1.1
		5.12, dd, 17.3, 1.2		5.12, dd, 17.3, 1.0		5.13, dd, 17.3, 1.0		5.13, d, 17.3		5.03, dd, 17.4, 1.1		5.11, dd, 17.4, 1.1
2′	143.4	5.96, dd, 17.3, 10.7	143.3	5.94, dd, 17.3, 10.7	143.4	5.94, dd, 17.3, 10.7	143.3	5.94, dd, 17.3, 10.7	143.2	5.90, dd, 17.4, 10.8	143.4	5.95, dd, 17.4, 10.8
3′	41.3	—	41.4	—	41.5	—	41.4	—	40.5	—	41.3	—
4′	22.3	0.98, s	22.2	0.99, s	22.2	0.99, s	22.2	0.98, s	22.6	0.95, s	22.2	0.99, s
5′	22.2	1.14, s	22.2	1.14, s	22.2	1.14, s	22.2	1.14, s	22.2	1.05, s	22.2	1.15, s

Table 3 ^{1}H-NMR and ^{13}C-NMR data of enzymatic products

Compound	**3a** 		**3c** 		**4** 	**5** 
Position	δ_C	δ_H, multi., J in Hz in $CDCl_3$	δ_H, multi., J in Hz in $CDCl_3$		δ_H, multi., J in Hz in $CDCl_3$	δ_H, multi., J in Hz in DMSO-d_6
1	—	—	—		7.52, s	10.65, s
2	80.8	5.76,br s	5.26, s		5.27, s	—
3	60.8	—	—		—	—
4	125.5	7.30, d, 7.5	7.24, d, 7.1		7.12, d, 7.3	7.40, d, 8.1
5	124.0	7.10, td, 7.6, 1.1	7.11, td, 7.5, 1.0		6.70, td, 7.5, 1.0	6.91, td, 10.3, 2.4
6	128.6	7.25, td, 7.6, 1.3	7.22, t, 7.6		7.01, td, 7.8, 1.2	7.18, t, 7.9
7	118.6	7.91, br s	7.08, d, 7.0		6.30, d, 7.8	7.06, d, 8.1
8	141.1	—	—		—	—
9	132.9	—	—		—	—
10_{syn}[a]	30.1	2.93, m	3.46, dd, 13.5, 4.9		2.71, dd, 13.8, 9.3	3.08, dd, 14.3, 4.4
10_{anti}[a]		2.66, dd, 13.9, 10.3	2.65, m		2.71, dd, 13.8, 9.3	2.87, m
11	57.6	4.16, dd, 10.2, 4.5	4.12, dd, 11.6, 3.3		4.08, br t, 8.8	4.42, dd, 11.2, 3.6
13	168.0	—	—		—	—
14	53.8	3.85, dd, 9.2, 3.9	4.09, dd, 10.6, 3.9		4.08, br t, 8.8	4.30, dd, 10.6, 5.6
15	—	5.45, s	—		5.57, s	5.45, s
16	168.9	—	—		—	—
17	37.7	1.89, ddd, 14.0, 9.6, 3.9	2.67, dd, 10.2, 5.8		2.55, dd, 13.8, 7.3	2.84, m
	—	1.42, ddd, 14.5, 9.2, 5.3	2.63, dd, 10.2, 6.6		2.55, dd, 13.8, 7.3	2.59, m
18	24.3	1.56, m	—		—	—
19	22.8	0.88, d, 6.6	7.23, d, 7.6		5.27, s	6.60, t, 8.5
20	21.0	0.86, d, 6.6	7.28, t, 7.6		7.52, s	6.87, t, 8.5
21	169.9	—	7.25, m		—	9.17, s
22	23.7	2.53, s	7.28, t, 7.6		6.30, d, 7.8	6.87, t, 8.5
23	—	—	7.23, d, 7.6		7.01, td, 7.8, 1.2	6.60, t, 8.5
24	—	—	—		6.70, td, 7.5, 1.0	—
25	—	—	2.56, br s		7.12, d, 7.3	—
1′ (1″)	114.5	5.14, d, 10.8	5.15, d, 10.8		5.12, dd, 10.8, 0.9	3.27, m
	—	5.11, d, 17.3	5.12, d, 17.4		5.08, dd, 17.4, 1.1	—
2′(2″)	143.3	5.83, dd, 17.3, 10.8	5.83, dd, 17.4, 10.8		5.92, dd, 17.4, 10.8	5.32, t, 6.1
3′(3″)	41.4	—	—		—	—
4′(4″)	22.2	0.92, s	0.93, s		0.99, s	1.69, s
5′(5″)	22.2	1.14, s	1.14, s		1.14, s	1.70, s

[a] not determined for **5**.

chiral atoms at these positions.[23] Therefore, the Cotton effect at 296 nm is very likely caused by the chiral centres at positions C2 and C3. This proved that the prenylated products described in this study should have the same configuration at C2 and C3 as those of (2*R*,3*S*,11*R*)- and (2*R*,3*S*,11*S*)-aszonalenin as well as of **3a** and **3c**, *i.e.* 2*R* and 3*S*. This corresponded also to the observation that AnaPT introduced a α-dimethylallyl group and catalysed the formation of an fused ring between the indoline and the diketopiperazine rings.[23]

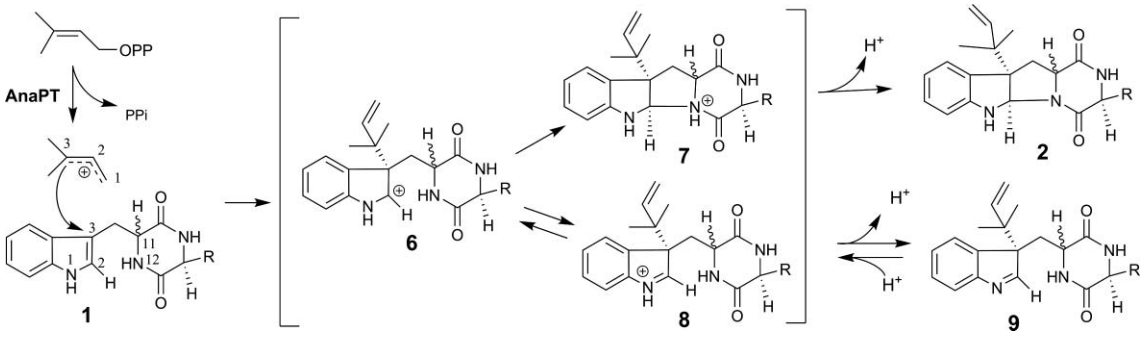
Unambiguous proof of the stereochemistry was provided by ROESY experiments for **2a–2f** and **3a** (Table 4). Strong NOE correlations between H-2 and H-2′, H-4′and H-5′of the dimethylallyl moiety proved the *cis*-configuration between C-2 and C3 of the indoline rings.

With an exception for **2e**, NOE correlation with a medium intensity has also been observed between H-2 and H-11. In the case of **2e**, only weak NOE was detected for H-2 and H-11. More importantly, strong NOEs were found between H-11 and H-10_{syn}, which was not observed in **2e**. The same proton (H-10_{syn}) showed medium or strong NOE correlations with H-2′, H-4′and H-5′of the dimethylallyl moiety. For **2e**, a correlation between H-11 and

Table 4 NOE results of C3-prenylated pyrrolo[2,3-b]indoles (with exception for **3c** and **4**)

Protons	Strength
H-2 to H-1′	Weak
H-2 to H-2′	Strong
H-2 to H-4′	Strong
H-2 to H-5′	Strong
H-2 to H-11	Medium[a]
H-10_{syn} to H-1′	Weak
H-10_{syn} to H-2′	Medium
H-10_{syn} to H-4′	Strong
H-10_{syn} to H-5′	Strong
H-10_{syn} to H-11	Strong[b]
H-10_{anti} to H-4	Strong
H-11 to H-2′	Very weak[c]
H-11 to H-4′	Weak[c]
H-11 to H-5′	Weak[c]

[a] Only weak correlation was observed in **2e**. [b] Not observed in **2e**. For **2e**, a correlation between H-10_{anti} and H-11 was observed instead. [c] Not observed in **2e**.



Scheme 2 Possible mechanism of AnaPT reaction.

H-10$_{anti}$ was detected instead. These results proved that, with an exception of **2e**, the dimethylallyl moiety was attached from the same side of H-11. This means that H-2, C3-prenyl and H-11 are on the same side of the ring systems as illustrated in Scheme 1 and Tables 2 and 3. In the case of **2e**, H-11 is located at the opposite side of H-2 and C3-dimethylallyl moiety. This conclusion was also supported by the weak NOEs between H-11 and H-2′, H-4′or H-5′of the prenyl moiety in **2a–2d**, **2f** and **3a**. No NOE correlations for these protons were detected in **2e**.

These results and those from our previous study[17] showed clearly that the reaction catalysed by AnaPT includes at least three steps, *i.e.* attachment of a reverse prenyl moiety to C3 of the indole ring, breaking of the double bond between C2 and C3 and the formation of a C–N bond between C2 and N12. The detailed mechanism of AnaPT reaction is unknown. We speculate that generating of a dimethylallyl cation would initiate the enzymatic reaction (Scheme 2), as in the case of other prenyltransferases, *e.g.* FgaPT2.[24] Attacking of this intermediate *via* its C3 to C3 of the indole ring of the cyclic dipeptides would result in formation of the intermediate **6** with a positive charge at C2. Two possible fates could be expected for **6**. Formation of a C–N bond between C2 and N12 leads to **7**, which is then converted to the enzymatic products **2** by releasing of a proton. The second possibility would be the formation of a double bond between N1 and C2 results in cation **8**, which would be then converted to **9** by releasing of a proton. Using cyclo-L-Trp-L-Leu (**1a**) and DMAPP as substrates and AnaPT as catalyst, we obtained indeed a product, which could be interpreted by ^{1}H-NMR analysis as a structure like **9**. However, the NMR data were insufficient for an unequivocal structure elucidation. Furthermore, the ^{1}H-NMR spectrum of the obtained compound in $CDCl_3$ was changed to that of **2a**, when the sample was analyzed again after storage at room temperature for four days. We have tried to isolate enough substance for an unambiguous structure elucidation by spectroscopic methods including ^{13}C-NMR and HMBC. Chemical shift of C2 in **9** should clearly differ from that of **2**. Unfortunately, the initial observed results could not be reproduced. Instead, **2a** was isolated directly from the reaction mixture. Plausible explanation for this phenomenon would be the low stability of **9**, which was rapidly converted to **2a** during the incubation or isolation. We are now looking for suitable conditions to isolate this compound for evaluation of the reaction mechanism.

The second product peak **4** of cyclo-L-Trp-L-Trp (**1b**) (Fig. 2C) was also isolated after repeated chromatography and subjected to MS analysis. The positive HR-ESI-MS of **4** showed an ion at *m/z* 509.2932 (Table 1), which can be interpreted as $[M+1]^+$ of a diprenylated derivative with a molecular mass 136 daltons larger than that of the substrate. The ^{1}H-NMR spectrum of **4** showed signals of two identical tryptophanyl moieties (Table 3), which are reversely prenylated at position C3 of the indoline rings. C3-diprenylated derivatives of cyclo-L-Trp-L-Trp containing two pyrrolo[2,3-b]indole systems have been reported, *e.g.* amauromine (Fig. 1) from *Amauroascus sp*[14] and epiamauromine (Fig. 1) from *Aspergillus ochraceus*.[15] However, the ^{1}H-NMR data of **4** differed clearly from those reported for amauromine[14] or epiamauromine.[15] Thus, **4** has different configurations at C2 and C3 of the indoline ring as those in amauromine or epiamauromine. The absolute configuration of **4** was not determined in this study. However, it can be expected that the symmetrical **4** was formed by a second prenylation and cyclisation of **2b** under the catalysis of AnaPT. Therefore, both tryptophanyl moieties must have the same configuration as in **2b**, *i.e.* 2*R* and 3*S* as well as 19*R* and 18*S* (Scheme 1 and Table 3). Identification of **4** as a diprenylated product of AnaPT indicated that the two prenyl moieties of some natural products derived from cyclo-Trp-Trp, *e.g.* amauromine and epiamauromine mentioned above, could also be introduced by one prenyltransferase.

From the reaction mixture of **1e** (Fig. 2I), the second product peak **5** was also isolated and subjected to ^{1}H-NMR analysis. ^{1}H-NMR indicated the presence of a regular prenyl moiety with signals at 3.27 (m) for H-1′, 5.32 (t) for H-2′, 1.70 (s) for H-5′ and 1.69 ppm (s) for H-4′, respectively (Table 3). The signal of proton H-2 disappeared in comparison to that of the substrate (data not shown). Therefore, this compound was identified as a regular C2-prenylated cyclo-D-Trp-L-Tyr (Table 3). Assignments of NMR signals (Table 3) for this compound were also confirmed by H–H-COSY (data not shown). The structure of **5** was confirmed by detection of the $[M+1]^+$ ion at *m/z* 418.2118 in HR-ESI-MS.

Conclusions

In this study, we reported the successful synthesis of nine reversely C3-prenylated pyrrolo[2,3-b]indoles by chemoenzymatic strategy using recombinant and purified enzymes of secondary metabolism. The structures of these compounds were characterized by HR-ESI-MS and NMR analyses. NOE results (Table 4) confirmed the absolute configuration of the enzymatic products. Similar structures have been identified in different fungal strains.[17,25] The approach described in this study could therefore also find usage for synthesis of natural products. Conversion of cyclo-L-Trp-L-Trp to the diprenylated derivative **4** provided

evidence that only one prenyltransferase is necessary for introducing of two prenyl moieties. It would be interesting to find out the substrate binding sites of the enzyme and to clarify the two prenylation steps. Although the efficiency of the enzymatic conversion is still low for a biotechnological application, such approaches offer however the potential to significantly increase the structural diversity in drug discovery programmes. The low catalytic efficiency could be improved by using enzyme derivatives from mutagenesis experiments.

Experimental section

Chemicals

DMAPP was prepared according to the method described for geranyl diphosphate by Woodside.[26] Acetylcoenzyme A (Trilithium Salt) was obtained from Calbiochem (Darmstadt, Germany). Cyclo-L-Trp-L-Leu, cyclo-L-Trp-L-Trp, cyclo-L-Trp-L-Phe, cyclo-L-Trp-L-Tyr, cyclo-D-Trp-L-Tyr and cyclo-L-Trp-Gly were obtained from Bachem (Bubendorf, Switzerland).

Bacterial strains, plasmids and cultural conditions

Plasmids pWY22 and pWY23[17] were used for overproduction of AnaPT and AnaAT, respectively.

Escherichia coli XL1 Blue MRF′ (Stratagene) was used for overexpression experiments and grown in liquid or on solid Luria-Bertani medium with 1.5% (w/v) agar at 37 °C. Carbenicillin (50 μg mL^{-1}) was used for selection of recombinant *E. coli* strains.

Preparation of AnaPT and AnaAT

For preparation of AnaPT, *E. coli XL1* Blue MRF′ cells harbouring pWY22 were induced by 0.5 mM of IPTG at 37 °C. His_6-AnaPT was purified with Ni-NTA agarose to homogeneity as judged by SDS-PAGE and a protein yield of 25 mg of purified His_6-tagged AnaPT per litre of culture was obtained.[17]

For preparation of AnaAT, *E. coli XL1* Blue MRF′ cells harbouring pWY23 were induced by 0.7 mM of IPTG at 37 °C. His_6-AnaAT was purified with Ni-NTA agarose to homogeneity as judged by SDS-PAGE and a protein yield of 5 mg of purified His_6-tagged AnaAT per litre of culture was obtained.[27]

HPLC conditions for analysis and isolation of enzymatic products

The enzymatic products of the incubation mixtures of AnaPT and tandem incubation of AnaPT and AnaAT were analysed by HPLC on an Agilent series 1200 by using a LiChrospher RP 18-5 column (125 × 4 mm, 5 μm, Agilent) at a flow rate of 1 mL min^{-1}. 50% methanol in water (solvent A) and pure methanol (solvent B) were used as solvents. For quantitative analysis of enzymatic products, a linear gradient of 10–50% (v/v) solvent B in solvent A in 15 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. Detection was carried out by a Photo Diode Array detector.

For isolation, the same HPLC equipment with a Multospher 120 RP-18 column (250 × 10 mm, 5 μm, C+S Chromatographie Service, Langenfeld, Germany) was used. A linear gradient of 10–50% (v/v) solvent B in A in 15 min at a flow rate of 2.5 mL min^{-1} 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.

Circular dichroism spectra

The samples were dissolved in ethanol. CD spectra were recorded on a JASCO J-810 CD spectropolarimeter by using Spectra Manager Software (JASCO Inc). Spectral data were collected from 400 to 200 nm with a data pitch of 0.1 nm. A band width of 1 nm was used with a detector response time of 0.5 s and scanning speed of 200 nm min^{-1}. Each spectrum or data point was acquired 5 times, and mean values were used.

Enzymatic assays and syntheses of reversely C3-prenylated pyrrolo[2,3-b]indoles

Enzyme assays (100 μL) contained substrates **1a** to **1f** (1 mM), DMAPP (1 mM), $CaCl_2$ (5 mM), Tris-HCl (50 mM, pH 7.5), glycerol 1.5% (v/v) and AnaPT (0.54 μM) were incubated at 37 °C. For tandem reaction, the reaction mixtures of AnaPT were incubated with acetylcoenzyme A (1 mM) and AnaAT (0.88 μM) at 37 °C for further 24 h. The reaction was then terminated with the same volume of methanol. After removal of the protein by centrifugation (15,000 x *g*, 10 min, 4 °C), the enzymatic products were analysed on a HPLC system described above. For synthesis of C3-prenylated pyrrolo[2,3-b]indoles, **1a** to **1f** (1 mM), DMAPP (2 mM), $CaCl_2$ (5 mM), Tris-HCl (50 mM, pH 7.5), glycerol 1.5% (v/v) and AnaPT (0.54 μM) in total volumes of 10 mL were incubated at 37 °C for 24 h. For synthesis of acetylated indole alkaloids, the reaction mixture of AnaPT with **1a** or **1c** was incubated with acetylcoenzyme A (2 mM) and AnaAT (0.88 μM) at 37 °C for further 24 h. The reaction mixtures were extracted with ethyl acetate subsequently. After evaporation of the solvent, the residues were dissolved in methanol and purified on HPLC under conditions described above.

NMR experiments

Small amount (less than 1 mg) of each sample was dissolved in 0.2 mL of $CDCl_3$. Samples were filled into Wilmad 3 mm tubes from Rototec Spintec. Spectra were recorded at room temperature on a Bruker Avance 600 MHz spectrometer equipped with an inverse probe with z-gradient. The HSQC and HMBC spectra were recorded with standard methods.[28] ROESY experiment[29] was performed in phase-sensitive mode using State-TPPI technique.[30] For all two-dimensional spectra, 32 to 64 transients were used. For ROESY spectra, a mixing time of 300 ms and a relaxation delay of 3.0 s. ^1H spectra were acquired with 65 536 data points, while 2D spectra were collected using 4096 points in the F_2 dimension and 512 increments in the F_1 dimension. Typical experiment time for the HMBC and ROESY measurements was about 12 h. Chemical shifts were referenced to $CDCl_3$. All spectra were processed with Bruker TOPSPIN 2.1.

HR-ESI-MS

The isolated products were analysed by high resolution (HR) electrospray ionization (ESI) mass spectrometry (MS) with a Q-Trap Quantum (Applied Biosystems). Positive HR-ESI-MS data of the enzymatic products are in Table 1.

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