Supporting information

Increasing cytochrome P450 enzyme diversity by identification of two distinct cyclodipeptide dimerases

Jing Liu,a Xiulan Xie,b and Shu-Ming Li*,a

a Institut für Pharmazeutische Biologie und Biotechnologie, Fachbereich Pharmazie, Philipps-Universität Marburg, Robert-Koch-Straße 4, 35037 Marburg, Germany

b Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Straße 4, Marburg 35032, Germany
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Experimental Procedures

1. Computer-assisted sequence analysis
The gene and protein sequences used in this study were obtained from NCBI databases (http://www.ncbi.nlm.nih.gov). Protein sequences were compared with each other by using BLASTP program (http://blast.ncbi.nlm.nih.gov/). The phylogenetic tree of P450s showing in Fig. S2 was created by MEGA version 7.0 (http://www.megasoftware.net). Protein sequence alignments were performed with the program ClustalW and visualized with ESPript 3.0 (http://endscript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) to identify strictly conserved amino acid residues (Fig. S1 and S3).

2. Bacterial strains, plasmids, and growth conditions
Strains and plasmids used in this study are listed in Tables S2 and Table S3, respectively. Recombinant E. coli strains were cultivated in liquid or on solid Luria-Bertani (LB) medium with 100 µg/mL ampicillin, 50 µg/mL kanamycin, 50 µg/mL apramycin or 25 µg/mL chloramphenicol, when necessary.

Saccharopolyspora antimicrobica DSM 45119 was purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). Streptomyces albus J1074¹ was kindly gifted by Prof. Luzhetskyy (Saarland University). S. albus J1074 and the generated exconjugants were maintained on MS plates (mannitol 20.0 g/L, soya flour 20.0 g/L, agar 20.0 g/L) at 28 °C for sporulation. For secondary metabolite production, S. albus J1074 transformants were cultivated in liquid modified R5 medium (sucrose 103.0 g/L, glucose 10.0 g/L, yeast extract 5.0 g/L, MgCl₂·6H₂O 10.12 g/L, K₂SO₄ 0.25 g/L, Difco casaminoacids 0.1 g/L, MOPS 21.0 g/L, trace element solution 2 mL/L, pH 7.2) at 28 °C for 7 days.

3. Genetic manipulation, PCR amplification, and gene cloning
Genetic manipulation in E. coli was performed according to the protocol by Green and Sambrook.² Isolation of genomic DNA from actinomycetes was carried out as described in the literature.³ The cdps and p450 genes were amplified by PCR from genomic DNA of S. antimicrobica DSM 45119 by using primers listed in Table S3 and Phusion® High-Fidelity DNA Polymerase from New England Biolabs (NEB). The generated PCR fragments were cloned into pGEM-T Easy vector and the sequence integrity was confirmed by sequencing. Subsequently, the fragments were released with restriction endonucleases from pGEM-T Easy and ligated into pPWW50A⁴ or pET28a (+) vector, which were digested with the same enzymes, previously. The
generated constructs (Table S3) were transformed into \textit{S. albus} J1074 or \textit{E. coli} BL21 (DE3) for gene expression.

4. Heterologous gene expression in \textit{Streptomyces albus} J1074

The constructed plasmids harbouring different genes or gene clusters were firstly transformed into the non-methylating \textit{E. coli} ET12567/pUZ8002, then conjugated with \textit{S. albus} J1074. The positive conjugants were firstly selected by the phenotype showing apramycin resistance and further confirmed by PCR. The spores of the \textit{S. albus} J1074 transformants were inoculated into 50 mL of modified R5 liquid media supplied with 50 µg/mL of apramycin in 250 mL baffled flasks and cultured at 28 °C and 200 rpm for 7 days. 1 mL of the cultures was extracted with the same volume of ethyl acetate for three times. The organic phases were combined, evaporated, and the dried residues were afterwards dissolved in 400 µL of methanol. 5 µL of such samples were subjected to LC-MS for analysis.

5. Overproduction and purification of P450s in \textit{E. coli} and \textit{Streptomyces}

For the purification of TtpB1, pJL80 was transformed into \textit{E. coli} BL21 (DE3). The recombinant \textit{E. coli} cells were cultivated for 16 h in 50 mL LB media supplied with 50 µg/mL kanamycin as preculture. 5 mL of the preculture were transferred into 500 mL LB media (with 50 µg/mL kanamycin) in 2 L-Erlenmeyer flasks and grew at 37 °C and 230 rpm to an absorption of 0.6 at 600 nm. The gene expression was induced with 0.1 mM IPTG at 16 °C for 20 h. The bacterial cultures were harvested by centrifugation (4,500 rpm, 20 min, 4 °C) and the cells were resuspended in lysis buffer (50 mM Tris-HCl, 10 mM imidazole, 300 mM NaCl, pH 8.0) with 2–5 mL/g wet weight. Lysozyme from the chicken egg white was added to the mixture at a final concentration of 1 mg/mL, which was incubated on ice for 30 min. The cells were then lysed by sonication on ice. Cell debris was removed by centrifugation at 13,000 rpm and 4 °C for 30 min. One-step purification of the recombinant His-tagged protein was performed by using Ni-NTA agarose (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instruction. The storage buffer was changed to 50 mM Tris-HCl (pH 7.5) containing 15 % (v/v) glycerol through a PD-10 column (GE Healthcare, Freiburg, Germany), which had been equilibrated with the same buffer. The obtained protein was stored frozen at -80 °C.

\textit{S. albus} J1074 harbouring pJL84 (\textit{ttpB2} in pPWW50A, Table S3) was cultivated in 50 mL tryptic soy broth (TSB) medium containing 50 µg/mL apramycin for 48 h as preculture. 5 mL of this preculture were transferred to 100 mL TSB with 50 µg/mL apramycin in 500 mL conical flasks. The cultures were further incubated at 28°C and 200 rpm for 3 days. Two litres of such cultures were harvested by centrifugation at 4 °C and 4,500 rpm for 20 min. The protein was purified as
described for *E. coli* cells.

The concentrations of TtpB1 and TtpB2 were determined on a Nanodrop C2000 (Thermo Scientific, Braunschweig, Germany) to be 0.65 mg/L and 0.05 mg/L culture, respectively. The purity of the recombinant P450s was proven by 12 % (w/v) SDS-PAGE (Fig. S29).

6. UV-Vis spectroscopic analysis of TtpB1

To measure the typical absorbance of P450 ferrous CO complex after reduction, carbon monoxide gas was bubbled into the TtpB1 solution (14 µM in 50 mM Tris-HCl containing 15% (v/v) glycerol, pH 7.5) for 2 min. After addition of 0.2 g/mL of sodium dithionite, a UV-Vis spectrum between 350 and 550 nm was taken on a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, Dreieich, Germany). UV-Vis spectra of a protein sample without any treatment and another one only bubbled with CO were taken as controls. The spectra of TtpB1 is given in Fig. S30.

7. *In vitro* assays of P450s

cWW (1, 500 µM) was first assayed with 7 µM TtpB1, 5 mM NADPH, 2 µM spinach ferredoxin (Sigma-Aldrich), 0.1 unit/mL spinach ferredoxin-NADP⁺ reductase (Sigma-Aldrich), 50 mM Tris-HCl buffer (pH 7.5) in a total volume of 50 µL at 30 °C for 30 min. Afterwards, 50 µM of 1 was incubated with 5 µM TtpB1 or 1.1 µM TtpB2 for 12 h. The reactions were quenched with 50 µL ice-cold MeOH. After centrifugation at 13,000 rpm for 5 min, 5 µL of the supernatants were subjected to LC-MS analysis. Incubations with heat-inactivated P450s, without ferredoxin, ferredoxin reductase, or NADPH were used as negative controls.

8. Determination of kinetic parameters for TtpB1

For determination of the kinetic parameters of TtpB1 towards cWW (1), the reaction mixtures (50 µL) contained 7 µM TtpB1, 5 mM NADPH, 2 µM spinach ferredoxin (Sigma-Aldrich), 0.1 unit/mL spinach ferredoxin-NADP⁺ reductase (Sigma-Aldrich), 50 mM Tris-HCl buffer (pH 7.5) and 1 at final concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.3, and 0.4 mM. The reactions were carried out at 30 °C for 30 min and terminated by addition of 50 µL ice-cold MeOH. After removal of proteins by centrifugation, 50 µL of the supernatants were subjected to HPLC analysis.

The analysis was carried out on an Agilent HPLC 1200 series equipped with a photo diode array detector and an Eclipse XDB C18 column (5 µm, 4.6 x 150 mm). A linear gradient of 5 to 100% acetonitrile in water (0.1% formic acid) in 20 min was followed by 100% acetonitrile for 5 min and 5% acetonitrile in water for 5 min. The flow rate was set to 0.5 mL/min. Absorptions at 280 nm were illustrated in this study. The $K_M$ and $k_{cat}$ were proceeded with GraphPad Prism 8 (Fig. S32).
9. LC-MS analysis
LC-MS analysis was performed on an Agilent HPLC 1260 series system equipped with a photo diode array detector and a microTOF Qlll mass spectrometer (Bruker, Bremen, Germany) by using a Multospher 120 RP-18 column (250 x 2 mm, 5 µm, CS-Chromatographie Service GmbH). For secondary metabolite analysis, a linear gradient of 5 – 100 % acetonitrile in water, both containing 0.1 % formic acid, in 40 min and a flow rate at 0.25 mL/min were used. The column was then washed with 100 % acetonitrile containing 0.1 % formic acid for 5 min and equilibrated with 5 % acetonitrile in water for 5 min. For enzyme assay analysis, a linear gradient of 5 – 100 % acetonitrile in water in 10 min was used, and the column was then washed and equilibrated as described as the former method. The parameters of the mass spectrometer were set as following: electrospray positive ion mode for ionization, capillary voltage with 4.5 kV, collision energy with 8.0 eV.

10. Isolation of generated metabolites from S. albus J1074 transformants
For structural elucidation of the accumulated compounds, the S. albus J1074 transformants harbouring ttp(ABC)1 and ttp(AB)2 were fermented in modified R5 medium on a large scale (4 L) at 28 °C for 7 days. The cultures were extracted with equal volume of ethyl acetate for three times. The organic phases were combined and evaporated to dryness. The extracts were applied to a silica gel column and eluted with a gradient CH₂Cl₂: MeOH in ratios of 100:2, 100:3, 100:5, 100:10. The target compounds 2 and 4 were mainly found in the fractions eluted with CH₂Cl₂: MeOH of 100:5 and 3 in 100:10, respectively. These fractions were further purified on an Agilent HPLC 1260 series equipped with a photo diode array detector by using a semi-preparative Agilent ZORBAX Eclipse XDB C18 HPLC column (9.4 x 250 mm, 5 µm) with 55 % ACN in water as solvent. The flow rate was set to 2.0 mL/min.

11. Precursor feeding experiments
Precursor feeding was carried out by using 20 mM cWW in DMSO. 150 µL of this solution were added to 30 mL of 2 day-old cultures of Streptomyces transformants in modified R5 media. After cultivation at 28°C for additional 7 days, the metabolites were extracted with EtOAc and analysed on LC-MS.

12. Determination of production yields of cWW dimers in Streptomyces transformants
An Agilent HPLC 1200 series equipped with a photo diode array detector and an Agilent Eclipse XDB C18 column (5 µm, 4.6 x 150 mm) were used for quantification. A linear gradient of 10 to 100 % acetonitrile in water in 40 min was followed by 100 % acetonitrile for 5 min and then 10 %
acetonitrile in water for 5 min. The flow rate was set to 0.5 mL/min. The absorption at 280 nm was used for quantification. To ensure complete extraction of cWW dimers from mycelia, precipitants and supernatants, 1 mL whole culture of *S. albus* J1074 transformants was extracted with 1 mL ethyl acetate for three times. The organic phases were combined and evaporated to dryness. The residues were dissolved in 200 µL of methanol and 100 µL were analysed on HPLC. The isolated products were used as authentic standards for quantification.

13. NMR analysis
The NMR spectra of the purified compounds 2 and 3 were recorded on a JOEL ECA-500 MHz spectrometer (JEOL, Tokyo, Japan) in DMSO-d$_6$. The NMR spectra of compound 4 in DMSO-d$_6$ were taken at 300 K on a Bruker AVIII spectrometer (500 MHz) equipped with a 5 mm cryo BBO probe Prodigy. To obtain a better NMR signal resolution, the $^1$H NMR of 4 was also recorded in acetonitrile-d$_3$ at 273 K, 300 K, 310 K, and 320 K on a Bruker HD AVII spectrometer (500 MHz) equipped with a cryo BBO probe Prodigy. The $^{13}$C and HSQC NMR spectra of 4 were then taken at the best found temperature 310 K on the same equipment.

All spectra were processed with MestReNova 5.2.2 (Metrelab Research, S5 Santiago de Compostella, Spain). The NMR data of the identified compounds are listed as physiochemical properties and the spectra are provided in Fig. S4 – S27.

14. The physiochemical properties of the identified compounds
Tetratryptomycin A (2): 30 mg, light yellow powder; slightly soluble in modified R5 media (approx. 20 mg/L); CD (MeOH) $\lambda_{max}$ (\(\Delta\varepsilon\)) 306 (-18.4), 269 (-4.8), 249 (-29.4), 227 (+13) nm; HRMS (m/z): (ESI/[M+H$^+$]) calcd. for C$_{44}$H$_{38}$N$_8$O$_4$, 743.3089, found 743.3090. $^1$H NMR (DMSO-d$_6$, 500 MHz) $\delta$ 10.68 (s, 2H, H-20 and H-20'), 7.66 (s, 2H, H-15 and H-15'), 7.55 (d, $J$ = 7.8 Hz, 2H, H-25 and H-25'), 7.38 (d, $J$ = 8.1 Hz, 2H, H-22 and H-22'), 7.19 (d, $J$ = 7.4 Hz, 2H, H-4 and H-4'), 7.07 (s, 2H, H-19 and H-19'), 7.06 (t, $J$ = 8.1 Hz, 2H, H-23 and H-23'), 7.04 (t, $J$ = 7.8 Hz, 2H, H-6 and H-6'), 6.96 (t, $J$ = 7.8 Hz, 2H, H-24 and H-24'), 6.67 (s, 2H, H-1 and H-1'), 6.66 (t, $J$ = 7.4 Hz, 2H, H-5 and H-5'), 6.57 (d, $J$ = 7.8 Hz, 2H, H-7 and H-7'), 5.10 (s, 2H, H-2 and H-2'), 4.34 (t, $J$ = 5.0 Hz, 2H, H-14 and H-14'), 3.82 (dd, $J$ = 12.1, 5.9 Hz, 2H, H-11 and H-11'), 3.25 (dd, $J$ = 15.0, 5.7 Hz, 2H, H-17$\alpha$ and H-17$'\alpha$), 3.04 (dd, $J$ = 15.0, 5.7 Hz, 2H, H-17$\beta$ and H-17$'\beta$), 2.35 (dd, $J$ = 12.3, 5.9 Hz, 2H, H-10$\alpha$ and H-10$'\alpha$), 2.25 (t, $J$ = 11.9 Hz, 2H, H-10$\beta$ and H-10$'\beta$). $^{13}$C NMR (DMSO-d$_6$,125 MHz) $\delta$ 168.1 (C-16 and C-16'), 165.4 (C-13 and C-13'), 151.0 (C-8 and C-8'), 136.0 (C-21 and C-21'), 129.1 (C-6 and C-6'), 127.5 (C-26 and C-26'), 127.0 (C-9 and C-9'), 124.6 (C-4 and C-4'), 123.7 (C-19 and C-19'), 120.9 (C-23 and C-23'), 118.4 (C-25 and C-25'), 118.2 (C-24 and C-24'), 117.4 (C-5 and C-5'), 111.4 (C-22 and C-22'), 109.3 (C-18 and C-18'), 108.7 (C-7 and C-7'), 77.2
(C-2 and C-2'), 58.7 (C-3 and C-3'), 57.8 (C-11 and C-11'), 55.0 (C-14 and C-14'), 36.2 (C-10 and C-10'), 25.4 (C-17 and C-17').

Tetratryptomycin B (3): 40 mg, light yellow powder; slightly soluble in modified R5 media (approx. 20 mg/L); CD (MeOH) \( \lambda_{\text{max}} (\Delta \varepsilon) \) 300 (+11.4), 268 (-5.34), 245 (+4.8), 240 (+7.0), 229 (+47.0), 214 (-66.5) nm; HRMS (m/z): (ESI/[M+H]^+) calcd. for C_{44}H_{36}N_{8}O_{4}, 743.3089, found 743.3119. ^1H NMR (DMSO-\( d_6 \), 500 MHz) \( \delta \) 10.95 (s, 1H, H-20'), 10.86 (s, 1H, H-20), 8.10 (s, 1H, H-15'), 7.94 (s, 1H, H-15), 7.62 (d, \( J = 1.9 \) Hz, 1H, H-12'), 7.58 (d, \( J = 7.5 \) Hz, 1H, H-25), 7.54 (d, \( J = 7.7 \) Hz, 1H, H-25'), 7.38 (d, \( J = 8.0 \) Hz, 1H, H-22'), 7.34 (d, \( J = 8.0 \) Hz, 1H, H-22), 7.28 (s, 1H, H-19), 7.24 (d, \( J = 7.8 \) Hz, 1H, H-4'), 7.19 (d, \( J = 3.6 \) Hz, 1H, H-1), 7.15 - 7.06 (m, 4H, H-6, H-23, H-23' and H-24'), 6.99 (t, \( J = 7.5 \) Hz, H-24), 6.95 (t, \( J = 7.8 \) Hz, 1H, H-5'), 6.88 (t, \( J = 7.8 \) Hz, 1H, H-6'), 6.88 (s, 1H, H-19'), 6.73 (d, \( J = 7.5 \) Hz, 1H, H-4), 6.70 (d, \( J = 7.8 \) Hz, 1H, H-7), 6.54 (d, \( J = 7.8 \) Hz, 1H, H-7'), 6.53 (t, \( J = 7.5 \) Hz, 1H, H-5), 6.47 (s, 1H, H-2'), 5.82 (d, \( J = 3.6 \) Hz, 1H, H-2), 4.67 (dd, \( J = 13.9 \), 5.9 Hz, 1H, H-11), 4.39 (t, \( J = 5.1 \) Hz, 1H, H-14), 4.04 (m, 1H, H-14'), 3.71 (m, 1H, H-11'), 3.48 (dd, \( J = 14.6 \), 5.9 Hz, 1H, H-10\( \alpha \)), 3.39 (dd, \( J = 15.4 \), 5.1 Hz, 1H, H-17\( \alpha \)), 3.08 - 2.98 (m, 2H, H-17\( \beta \) and H-17'\( \beta \)), 2.85 (dd, \( J = 14.1 \), 4.4 Hz, 1H, H-17'\( \alpha \)), 2.64 (dd, \( J = 14.0 \), 3.3 Hz, 1H, H-10'\( \alpha \)), 2.23 (t, \( J = 14.2 \) Hz, 1H, H-10\( \beta \)), 1.27 (dd, \( J = 14.0 \), 9.6 Hz, 1H, H-10'\( \beta \)). ^13C NMR (DMSO-\( d_6 \),125 MHz) \( \delta \) 169.2 (C-16), 167.8 (C-13), 166.8 (C-16'), 166.6 (C-13'), 148.0 (C-8), 136.0 (C-21), 136.0 (C-21'), 135.0 (C-8'), 129.6 (C-6), 129.2 (C-9'), 128.6 (C-9), 127.7 (C-26'), 127.3 (C-26), 124.8 (C-19'), 124.7 (C-2'), 124.1 (C-19), 122.2 (C-4), 121.0 (C-6'), 120.9 (C-23'), 120.8 (C-23), 119.2 (C-5'), 119.0 (C-25'), 118.6 (C-4'), 118.5 (C-24'), 118.4 (C-25), 118.3 (C-24), 118.1 (C-5), 111.8 (C-7'), 111.5 (C-22'), 111.3 (C-22), 110.0 (C-7), 109.5 (C-18), 109.1 (C-3'), 108.6 (C-18?), 81.4 (C-2), 73.4 (C-3), 57.4 (C-11), 55.4 (C-14'), 55.2 (C-14), 54.5 (C-11'), 39.1 (C-10), 30.2 (C-10'), 29.8 (C-17'), 24.4 (C-17).

Tetratryptomycin C (4): 10 mg, light yellow powder; slightly soluble in modified R5 media (approx. 20 mg/L); CD (MeOH) \( \lambda_{\text{max}} (\Delta \varepsilon) \) 267 (-75.4), 216 (-2.7), 242 (+14.3), 224 (-1.4), 219 (+2.2) nm; HRMS (m/z): (ESI/[M+H]^+) calcd. for C_{44}H_{38}N_{8}O_{4}, 743.3089, found 743.3113. ^1H NMR (DMSO-\( d_6 \), 500 MHz) \( \delta \) 10.81 (br s, 1H, H-20), 10.73 (br s, 1H, H-20'), 7.95 (br s, 1H, H-15'), 7.63 (d, \( J = 7.6 \) Hz 1H, H-25'), 7.60 (s, 1H, H-15), 7.58 (d, \( J = 7.8 \) Hz 1H, H-25), 7.33 (d, \( J = 7.9 \) Hz, 1H, H-22), 7.28 (d, \( J = 7.5 \) Hz, 1H, H-22'), 7.19 (s, 1H, H-19), 7.15 (s, 1H, H-19'), 7.08 (t, \( J = 7.9 \) Hz, 1H, H-23), 7.02 – 6.93 (m, 5H, H-6, H-6', H-23', H-24 and H-24'), 6.45 (m, 6H, H-4, H-4', H-5, H-5', H-7 and H-7'), 5.37 (br s, 1H, H-2), 5.13 (br s, 1H, H-2'), 4.35 (m, 1H, H-14'), 4.29 (m, 1H, H-14), 4.07 (m, 1H, H-11), 3.77 (m, 1H, H-11'), 3.36 (m, 1H, H-17\( \alpha \)), 3.24 - 3.16 (m, 2H, H-17'\( \alpha \) and H-17'\( \beta \)), 2.96 (dd, \( J = 14.4 \), 6.7 Hz, 1H, H-17'\( \beta \)), 2.37 (m, 1H, H-10'\( \alpha \)), 2.27 (dd, \( J = 13.0 \), 6.3 Hz, 1H,
1H NMR (acetonitrile-d₃, 500 MHz, 310 K) $\delta$ 9.68 (br s, 1H, H-20), 9.19 (br s, 1H, H-20′), 7.65 (d, $J = 8.0$ Hz, 1H, H-25′), 7.63 (d, $J = 8.0$ Hz, 1H, H-25), 7.41 (d, $J = 8.0$ Hz, 1H, H-22′), 7.39 (d, $J = 7.9$ Hz, 1H, H-22), 7.18 − 7.14 (m, 3H, H-19, H-23′ and H-23), 7.11 − 7.04 (m, 5H, H-6, H-6′, H-19′, H-24′ and H-24), 6.58 − 6.51 (m, 6H, H-4, H-4′, H-5, H-5′, H-7 and H-7′), 6.45 (s, 1H, H-15′), 6.03 (s, 1H, H-15), 5.65 (br s, 1H, H-1), 5.47 (s, 1H, H-2), 5.23 (br s, 1H, H-1′), 5.12 (s, 1H, H-2′), 4.39 (t, $J = 4.6$ Hz, 1H, H-14′), 4.36 (dd, $J = 8.0$, 4.9 Hz, 1H, H-14), 4.18 (t, $J = 8.2$ Hz, 1H, H-11), 3.78 (dd, $J = 12.0$, 5.4 Hz, 1H, H-11′), 3.47 (dd, $J = 15.0$, 4.5 Hz, 1H, H-17α), 3.36 (dd, $J = 14.8$, 5.0 Hz, 1H, H-17′α), 3.49 (dd, $J = 15.0$, 4.2 Hz, 1H, H-17′β), 3.11 (dd, $J = 15.2$, 7.6 Hz, 1H, H-17β), 2.42 − 2.38 (m, 2H, H-10′α and H-10′β), 1.73 (t, $J = 12.0$ Hz, 1H, H-10′β), 1.31 (m, 1H, H-10β). $^{13}$C NMR (acetonitrile-d₃, 125 MHz, 310 K) $\delta$ 169.3 (C-16), 168.8 (C-13), 166.6 (C-16′), 164.8 (C-13′), 151.1 (C-8), 148.8 (C-8′), 136.6 (C-21), 136.4 (C-21′), 130.3 (C-9), 129.2 (C-6), 128.8 (C-6′), 127.9 (C-9′), 127.8 (C-26′), 127.3 (C-26), 124.5 (C-4), 124.2 (C-4′), 124.0 (C-19), 123.9 (C-19′), 121.7 (C-23), 121.6 (C-23′), 119.1 (C-24), 119.1 (C-24′), 118.9 (C-25′), 118.7 (C-5), 118.5 (C-5′), 118.4 (C-25), 111.7 (C-22′), 111.5 (C-22), 109.3 (C-18), 109.3 (C-18′), 109.3 (C-7), 108.8 (C-7′), 78.9 (C-2′), 76.5 (C-2), 60.1 (C-3), 60.0 (C-3′), 57.4 (C-11′), 57.3 (C-11), 56.5 (C-14′), 55.4 (C-14), 39.0 (C-10′), 35.0 (C-10), 27.9 (C-17′), 25.2 (C-17).
**Supplementary Tables**

**Table S1.** Comparison of CDPSs and P450s from the two clusters in *Saccharopolyspora antimicrobica* DSM 45119

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<th>Protein</th>
<th>Accession No.</th>
<th>Length (aa)</th>
<th>Protein</th>
<th>Accession No.</th>
<th>Length (aa)</th>
<th>Sequence identity (%)</th>
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<td>31 to TtpB1 39 to TtpB2</td>
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**Table S2. Bacterial strains used in this study**

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<th>Strain</th>
<th>Source</th>
<th>Cultivation media</th>
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<td><em>E. coli</em> DH5α</td>
<td>Invitrogen</td>
<td>LB</td>
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<tr>
<td><em>E. coli</em> BL21(DE3)</td>
<td>Novagen</td>
<td>LB</td>
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<td><em>E. coli</em> ET12567/pUZ8002</td>
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<td>LB</td>
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<tr>
<td><em>Streptomyces albus</em> J1074</td>
<td>1</td>
<td>MS</td>
</tr>
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<td><em>Saccharopolyspora antimicrobica</em> DSM 45119</td>
<td>DSMZ</td>
<td>modified R5</td>
</tr>
</tbody>
</table>

DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen

LB medium: tryptone 10.0 g/L, yeast extract 5.0 g/L, NaCl 10.0 g/L.

Modified R5 medium: sucrose 103.0 g/L, glucose 10.0 g/L, yeast extract 5.0 g/L, MgCl₂.6H₂O 10.12 g/L, K₂SO₄ 0.25 g/L, Difco casaminoacids 0.1 g/L, MOPS 21.0 g/L, trace element solution 2 mL/L, pH 7.2.

MS medium: mannitol 20.0 g/L, soya flour 20.0 g/L, agar 20.0 g/L.
### Table S3. Cloning and expression constructs used in this study

<table>
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<tr>
<th>Gene</th>
<th>Primer sequences (5’-3’)</th>
<th>Cloning constructs</th>
<th>Expression vector</th>
<th>Cloning sites</th>
<th>Expression constructs</th>
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</table>

Restriction sites for cloning are underlined in the primer sequences. Cloning constructs are based on pGEM T Easy vector.
Supplementary Scheme

Scheme S1 Known biosynthetic pathways of CDP dimers in *Aspergillus flavus* (A) and *Streptomyces* species (B and C)
Fig. S1 Alignments of CDPSs from bacteria. Amir_4627, NozA, NcdA, WP_016576960, EPD89497_1, CWWS1_D46488, CWWS2_D46488, CWWS1_NF5123, AspA, NasA, GutA3589, GutA24309, and CWLS1_NF5053 have been characterised as tryptophan-containing CDP synthases.
Fig. S2 Phylogenetic analysis of P450s investigated in this study (in bold red) and functionally characterised P450s from bacteria. GutD_{2774}, GutD_{5414}, GutD_{2430}, GutD_{3589}, P450_{5737}, CYP121, NasB, NascB, AspB, CYP134A1, and BcmD are members of the CDPS-related pathways. Htm and Clp are involved in the biosynthesis of himastatin and chloptosin, respectively. Other enzymes are structurally characterised natural product P450s mentioned in the review by Podust et al. The protein sequences were downloaded from NCBI database.
Fig. S3 Alignments of CDP dimerization P450s from bacteria. The origins of the enzymes were mentioned in the legend to Fig. S2.
Fig. S4 $^1$H NMR spectrum of tetratryptomycin A (2) in DMSO-$d_6$ at 300 K (500 MHz).
Fig. S5 $^{13}\text{C}$ NMR spectrum of tetratryptomycin A (2) in DMSO-$d_6$ at 300 K (125 MHz).
Fig. S6 $^1$H-$^1$H COSY spectrum of tetratryptomycin A (2) in DMSO-$d_6$ at 300 K (500 MHz).
Fig. S7 HSQC spectrum of tetratryptomycin A (2) in DMSO-$d_6$ at 300 K (500 MHz, 125 MHz).
Fig. S8 HMBC spectrum of tetratryptomycin A (2) in DMSO-\textit{d}_6 at 300 K (500 MHz, 125 MHz).
Fig. S9 NOESY spectrum of tetratryptomycin A (2) in DMSO-$d_6$ at 300 K (500 MHz).
Fig. S10 $^1$H NMR spectrum of tetratryptomycin B (3) in DMSO-$d_6$ at 300 K (500 MHz).
Fig. S11 $^{13}$C NMR spectrum of tetratryptomycin B (3) in DMSO-$d_6$ at 300 K (125 MHz).
Fig. S12 $^1$H-$^1$H COSY spectrum of tetratryptomycin B (3) in DMSO-$d_6$ at 300 K (500 MHz).
Fig. S13 HSQC spectrum of tetratryptomycin B (3) in DMSO-$d_6$ at 300 K (500 MHz, 125 MHz).
Fig. S14 HMBC spectrum of tetratryptomycin B (3) in DMSO-$d_6$ at 300 K (500 MHz, 125 MHz).
Fig. S15 NOESY spectrum of tetratryptomycin B (3) in DMSO-$d_6$ at 300 K (500 MHz).
Fig. S16 $^1$H NMR spectrum of tetratryptomycin C (4) in DMSO-$d_6$ at 300 K (500 MHz).
Fig. S17 $^{13}$C NMR spectrum of tetratryptomycin C (4) in DMSO-$d_6$ at 300 K (125 MHz).
Fig. S18 $^1$H-$^1$H COSY spectrum of tetratryptomycin C (4) in DMSO-$d_6$ at 300 K (500 MHz).
Fig. S19 HSQC spectrum of tetratryptomycin C (4) in DMSO-$d_6$ at 300 K (500 MHz, 125 MHz).
Fig. S20 HMBC spectrum of tetratryptomycin C (4) in DMSO-$d_6$ at 300 K (500 MHz, 125 MHz).
Fig. S21 NOSEY spectrum of tetratryptomycin C (4) in DMSO-\textit{d}_6 at 300 K (500 MHz).
Fig. S22 $^1$H NMR spectrum of tetratryptomycin C (4) in acetonitrile-$d_3$ at 273 K (500 MHz).
Fig. S23 $^1$H NMR spectrum of tetratryptomycin C (4) in acetonitrile-$d_3$ at 300 K (500 MHz).
**Fig. S24** $^1$H NMR spectrum of tetratryptomycin C (4) in acetonitrile-$d_3$ at 310 K (500 MHz).
Fig. S25 $^1$H NMR spectrum of tetratryptomycin C (4) in acetonitrile-$d_3$ at 320 K (500 MHz).
Fig. S26 $^{13}$C NMR spectrum of tetratryptomycin C (4) in acetonitrile-$d_3$ at 310 K (125 MHz).
Fig. S27 HSQC spectrum of tetratryptomycin C (4) in acetonitrile-$d_3$ at 310 K (500 MHz, 125 MHz).
Fig. S28 CD spectra of tetratryptomycins.
Fig. S29 SDS-PAGE analysis of the purified P450s.
**Fig. S30** UV-Vis spectroscopic analysis of TtpB1.
Fig. S31 HPLC analysis of the enzyme assays with TtpB1 (A) and TtpB2 (B).
Fig. S32 Determination of kinetic parameters of TtpB1 for cWW (1).
**Fig. S33** UV-Vis spectroscopic analysis of TtpB1 with its substrate cWW (A) and other CDPs (B).
Fig. S34 LC-MS analysis for tetratryptomycin production in *S. antimicrobica* DSM 45119.
References