Chapter 6

Facile Synthetic Access to Glycopeptide Antibiotic Precursor Peptides for the Investigation of Cytochrome P450 Action in Glycopeptide Antibiotic Biosynthesis

Clara Brieke, Veronika Kratzig, Madeleine Peschke, and Max J. Cryle

Abstract

The glycopeptide antibiotics are an important class of complex, medically relevant peptide natural products. Given that the production of such compounds all stems from in vivo biosynthesis, understanding the mechanisms of the natural assembly system—consisting of a nonribosomal-peptide synthetase machinery (NRPS) and further modifying enzymes—is vital. In order to address the later steps of peptide biosynthesis, which are catalyzed by Cytochrome P450s that interact with the peptide-producing nonribosomal peptide synthetase, peptide substrates are required: these peptides must also be in a form that can be conjugated to carrier protein domains of the nonribosomal peptide synthetase machinery. Here, we describe a practical and effective route for the solid phase synthesis of glycopeptide antibiotic precursor peptides as their Coenzyme A (CoA) conjugates to allow enzymatic conjugation to carrier protein domains. This route utilizes Fmoc-chemistry suppressing epimerization of racemization-prone aryl glycine derivatives and affords high yields and excellent purities, requiring only a single step of simple solid phase extraction for chromatographic purification. With this, comprehensive investigations of interactions between various NRPS-bound substrates and Cytochrome P450s are enabled.

Key words Glycopeptide antibiotics, Solid phase peptide synthesis, Coenzyme A, Bio-conjugation, Nonribosomal peptide synthetase, Cytochrome P450

1 Introduction

Nonribosomal peptide synthetases (NRPSs) are large, multi-enzyme complexes acting as modular peptide assembly lines that are involved in the biosynthesis of numerous peptide natural products independent of the ribosome [1]. NRPSs are organized into functional modules, in which each module enables the incorporation of one amino acid and consists of several domains: these domains are responsible for the selection/activation of amino acids and the elongation of the growing peptide chain (*see* Fig. 1). During all steps following amino acid activation the peptide is conjugated as a thioester to peptidyl carrier protein domains (PCP- or

Bradley S. Evans (ed.), *Nonribosomal Peptide and Polyketide Biosynthesis: Methods and Protocols*, Methods in Molecular Biology, vol. 1401, DOI 10.1007/978-1-4939-3375-4_6,

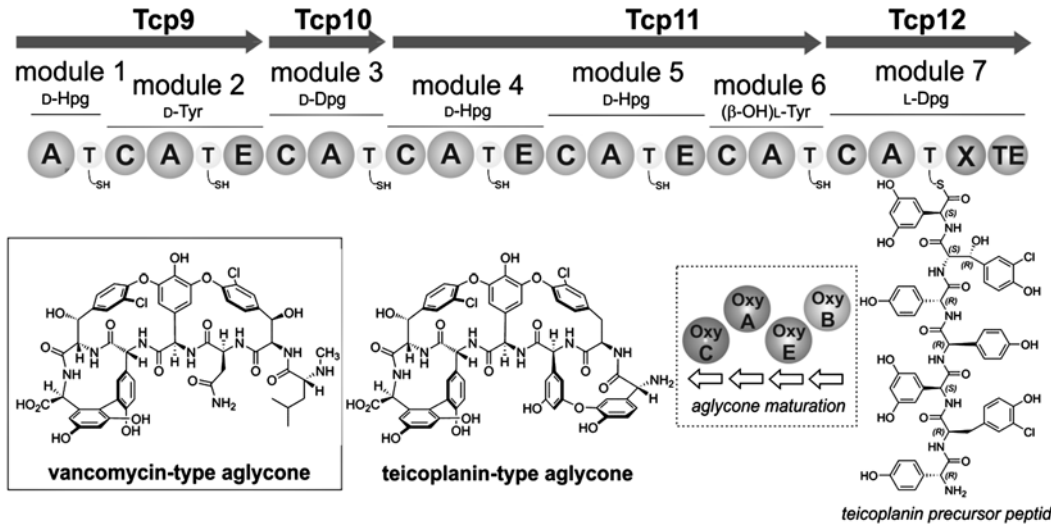

Fig. 1 Schematic pathway of teicoplanin biosynthesis by nonribosomal peptide synthesis and structures of vancomycin and teicoplanin aglycones (type I- and type IV-GPAs). A: adenylation; C: condensation; E: epimerization; T: thiolation/peptidyl carrier protein; TE: thioesterase; X: oxygenase recruitment domain

thiolation (T)-domains) via a posttranslationally introduced 4′-phosphopantetheine linker. Thus, assembly of a peptide requires multiple interactions between catalytic domains within the NRPS-machinery and is further complemented by additional interactions with enzymes acting in *trans*, which are often focused on the final peptide maturation process.

A prominent class of NRPS-metabolites that require extensive external modification of the NRPS-bound peptide is the glycopeptide antibiotics (GPAs) such as vancomycin and teicoplanin [2, 3]: a group of peptide natural products that are clinically relevant for the effective treatment of gram-positive bacterial infections that includes multidrug-resistant strains. These compounds possess a complex structure consisting of a heptapeptide backbone with a high content of non-proteinogenic amino acids that is modified by glycosylation and further decorations. Moreover, the amino acid side chains are highly cross-linked through several phenolic and aryl cross-links leading to the rigid 3D structure of GPAs that is crucial for their antibiotic activity [2]. During biosynthesis, these cross-links are introduced by several Cytochrome P450 enzymes (P450s) that act on the peptide while it remains bound to the NRPS machinery (Fig. 1).

Cytochromes P450 (P450s) are heme-dependent monooxygenases with a highly conserved overall protein fold found in prokaryotes and eukaryotes. These enzymes are capable of catalyzing a broad range of oxidative transformations, often in a stereo- as well as regioselective manner. A special group of P450s, which is involved in the biosynthesis of secondary metabolites, oxidizes their substrates that include amino acids, fatty acids or peptides

only when these are bound to a carrier protein domain from an NRPS, polyketide, or fatty acid synthase [3, 4]. The interaction between the P450 and the NRPS systems in GPA biosynthesis is transient in nature and thus challenging to investigate [5].

In the biosynthesis of GPAs, three (in the case of vancomycin, type I) or four (in the case of teicoplanin, type IV) P450s named Oxys are necessary for aglycone maturation. Several of these enzymes have been characterized and the order of oxidation and individual catalytic roles have been assigned for type I and type IV GPAs [3, 6–10]. Recently, Haslinger et al. revealed that a special NRPS-domain in the final module of the teicoplanin NRPS machinery—the so-called X-domain—is crucial for the recruitment of these Oxy enzymes to the NRPS, thus providing new insight into the mode of interaction of these enzymes in teicoplanin biosynthesis [11]. In this study it was also shown that this recruitment event is essential for in vitro activity of the both P450s catalyzing the C-*O*-D ($OxyB_{tei}$) and D-*O*-E ($OxyA_{tei}$) cyclization of a teicoplanin precursor peptide bound to a PCP-X di-domain construct. In contrast to this, the C-*O*-D ring catalyzing P450 from the vancomycin gene cluster ($OxyB_{van}$) exhibits not only in vitro oxidation activity on a precursor peptide bound to a single PCP domain in the absence of an X-domain [12, 13], but it also accepts peptide/PCP-pairs from alternate GPA gene clusters, thus showing a degree of promiscuity regarding the peptide substrate as well as the PCP domain [14].

The reasons for these differences between highly related P450 enzymes remain to be clarified. However, in order to perform such studies the straightforward access to a variety of substrates—linear precursor peptides covalently linked to PCP domains—is crucial: such a method is discussed in this chapter.

The production of such peptide–PCP conjugates requires the initial synthesis of the linear precursor peptide and, in a second step, the conjugation of the peptide to the desired PCP domain. Tethering a cargo to *apo*-PCP domains can efficiently be performed by using the promiscuous phosphopantetheinyl transferase Sfp [15, 16]. This enzyme transfers the 4′-phosphopantetheinyl moiety of Coenzyme A (CoA) onto a conserved serine residue of PCPs while tolerating a wide range of small molecules attached to the CoA molecule via thioester bonds. The production of linear precursor peptides is practically performed using solid phase peptide synthesis methodology, which allows the assembly of peptides also containing unnatural or D-amino acids very efficiently and in high yields. For GPA precursor peptides, however, such syntheses are challenging due to the high sensitivity to epimerization of the arylglycine derivatives 4-hydroxyphenylglycine (Hpg) and 3,5-dihydroxyphenylglycine (Dpg) found in the aglycones of GPAs [17]: three such residues appear in the case of vancomycin and teicoplanin even includes five such residues. Due to this sensitivity, special care has to be taken to prepare such pep-

tides in good yields and purities. The Robinson group has developed a mild solid phase peptide synthesis (SPPS) route based on Alloc-chemistry to access vancomycin precursor peptides [18, 19]. As the respective Alloc-protected amino acid buildings blocks are not commercially available, this route required extensive effort for the synthesis and optimization of the SPPS conditions, which limits the range of accessible substrate peptides possibly suited for biochemical investigations.

Driven to overcome these obstacles our group decided to perform a careful investigation of conditions suitable for a standard SPPS strategy based on Fmoc-chemistry, which has the advantage of allowing the utilization of the broad range of commercially available reagents for such syntheses. By optimizing the conditions for amino acid coupling and for cleavage of the temporary Fmoc-protecting group on the amine functional group, racemization of arylglycine residues could be suppressed on the solid phase during synthesis [20].

This enabled us to move forward and establish a rapid route to the respective peptide-CoA conjugates (*see* Fig. 2) appropriate for the enzymatic carrier protein loading with a significant reduced purification workload during the entire synthesis. The synthesis strategy we adopted is based on the SPPS of precursor peptides using commercially available Dawson resin [21]: this resin can be activated to release the synthesized peptide directly as an activated thioester, which in a subsequent step can be converted with CoA to the respective peptide-CoA conjugate. By optimizing reaction conditions in each step, purification is reduced to a single step employing solid-phase extraction to isolate these peptide-CoA

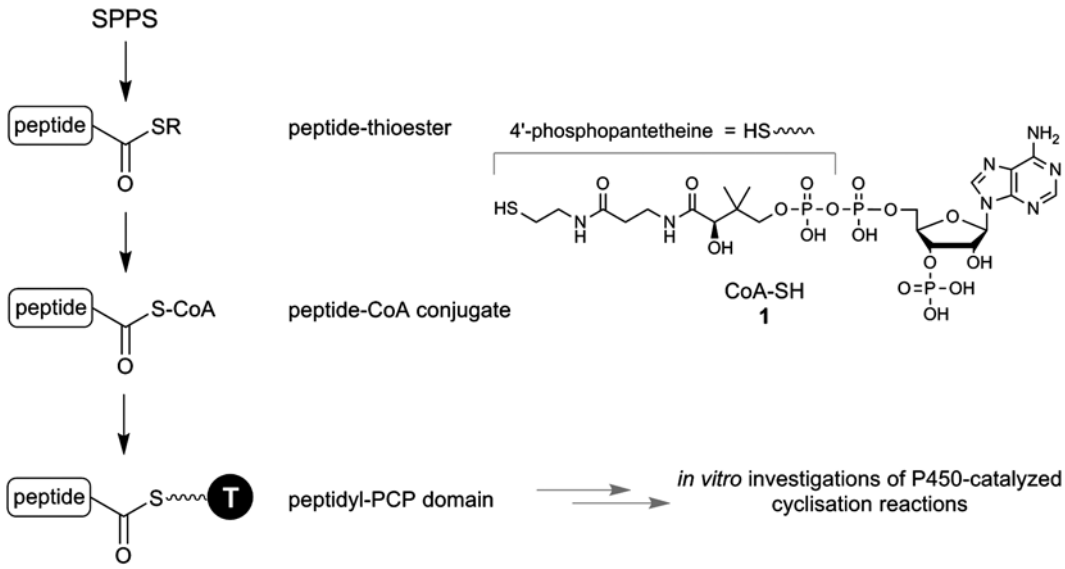

Fig. 2 Synthetic outline affording peptidyl-PCP thioester conjugates for in vitro investigations of Cytochrome P450 catalyzed cyclization reactions in the biosynthesis of GPAs

conjugates [14]. As not only GPAs but also other NRPS-derived natural products such as feglymycin [22] or β-lactam antibiotics [23] contain arylglycine derived residues, we believe that this methodology could be of general relevance for the exploration of other biosynthetic pathways.

2 Materials

2.1 Synthesis of Protected Amino Acids

1. Acetonitrile (MeCN).
2. Sodium carbonate solution: 10 % sodium carbonate (Na_2CO_3)-solution in Milli-Q water.
3. Fmoc-*N*-hydroxysuccinimide ester (Fmoc-OSu, Sigma-Aldrich).
4. (d)-4-hydroxyphenylglycine/(l)-4-hydroxyphenylglycine (Sigma-Aldrich).
5. Hydrochloric acid: 2 M aqueous hydrochloric acid.
6. Ethyl acetate.
7. Hexane.
8. Brine: saturated aqueous NaCl; prepare a solution in water by using an excess of NaCl (>360 g/L) in order to guarantee saturation of solution.
9. Sodium sulfate (Na_2SO_4).
10. 1,4-Dioxane.
11. Sodium hydroxide solution: 1 M aqueous sodium hydroxide.
12. Di-*tert*-butyl dicarbonate (Di-Boc, Sigma-Aldrich).
13. Sodium hydrogen carbonate ($NaHCO_3$).
14. Concentrated phosphoric acid (H_3PO_4, 85 %).
15. Acetone.

2.2 Solid Phase Peptide Synthesis (SPPS)

1. Commercially available amino acid building blocks: Fmoc-(l)-Tyr(tBu)-OH, Fmoc-(d)-Tyr(tBu)-OH, Fmoc-(l)-Asn(Trt)-OH, Boc-(d)-Leu-OH (Merck Novabiochem).
2. Dawson Dbz AM resin (Merck Novabiochem).
3. (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate, COMU (Merck Novabiochem).
4. Triethylamine (NEt_3, >99 %).
5. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, >99 %).
6. *N*,*N*-Dimethylformamide (DMF, >99 %).
7. Dichloromethane (DCM, reagent grade).

2.3 Resin Activation and Peptide Displacement Using MPAA

1. *p*-nitrophenylchloroformate (pNPCF) solution in DCM (50 mM), immediately prepared before use.
2. *N*,*N*-diisopropylethylamine (DIPEA) solution in *N*,*N*-dimethylformamide (86 mM), immediately prepared before use.
3. 4-mercaptophenylacetic acid (MPAA), tri-*n*-butylphosphine (PBu_3, 97 %, Sigma-Aldrich), *N*,*N*-dimethylformamide (DMF, anhydrous).
4. TFA cleavage mixture: 95 % trifluoroacetic acid (TFA), 2.5 % triisopropylsilane, 2.5 % water, ice-cold diethyl ether.

2.4 Activated Peptide Thioester Displacement with CoA

1. Reaction buffer: 50 mM potassium phosphate buffer pH 8.3 water-MeCN 2:1.
2. TCEP stock solution: 500 mM TCEP in Milli-Q water pH 7 (the solution was stored in aliquots at −24 °C).
3. Coenzyme A (CoA) (Affymetrix).
4. Strata-X solid phase extraction columns (200 mg resin/3 mL tubes) (Phenomenex).
5. SPE equilibration solvent: 50 mM KPi pH 7.0.
6. SPE wash solvent: methanol, 25 % solution in Milli-Q water.
7. SPE elution solvent: 35 % MeCN solution in Milli-Q water.

2.5 PCP Loading

1. Loading buffer: 1 M Hepes buffer pH 7.0, 5 M NaCl, 1 M $MgCl_2$.
2. Peptide-CoA conjugate (*see* Subheading 3.4).
3. Expressed PCP-domain [11, 14, 24].
4. 4′-phosphopantetheinyl-transferase Sfp R4-4 mutant (Sfp) [25].
5. Centrifugal filters with a molecular weight cutoff MW = 3000 (Amicon).
6. Wash buffer: 50 mM Hepes pH 7.0, 50 mM NaCl.

2.6 OxyB-Catalyzed Turnover and Turnover Workup

1. NAD(P)H stock solution: 50 mM NADH or NADPH in Milli-Q water, make freshly every time before use.
2. Glucose oxidase stock solution: 4 mg/mL in Milli-Q water, can be stored for 1 week at 4 °C.
3. D-glucose stock solution: 20 % in Milli-Q water, stored at 4 °C.
4. Reaction buffer: 50 mM Hepes, 50 mM NaCl pH 7.0.
5. PCP loading reaction: 60 μM *peptidyl*-PCP in 50 mM Hepes, 50 mM NaCl pH 7.0 (*see* Subheading 3.5).
6. Redox system: ferredoxin HaPuX and corresponding ferredoxin reductase HaPuR (*see* **Note 28**) [26].
7. NADH-regeneration system: glucose oxidase (0.033 mg/mL) and 0.33 % *w*/*v* glucose.

8. Cytochrome P450 $OxyB_{van}$ [27].
9. SPE-columns: Strata-X 33u polymeric reversed phase solid phase extraction columns 30 mg/mL (Phenomenex).
10. SPE elution solvent: methanol including 0.1 % formic acid.
11. SPE wash solvent: 5 % MeCN in Milli-Q water, 50 % MeCN in Milli-Q water.
12. 50 % Methylhydrazine (98 %, Sigma-Aldrich) in Milli-Q water.
13. Formic acid.
14. Syringe filters, PVDV, pore size 0.45 μM.

2.7 Equipment

1. Automated solid phase peptide synthesizer (Protein Technologies).
2. SpeedVac concentrator (Eppendorf).
3. Freeze-dryer (Christ).
4. High performance liquid chromatography (HPLC) system (Shimadzu) equipped with analytical C18 columns (Waters XBridge™ BEH 300 Prep C18 column, particle size: 5 and 10 μm, 4.6 × 250 mm).
5. Mass spectrometer (Shimadzu).
6. Nuclear magnetic resonance (NMR) spectrometer (Bruker).
7. Thermomixer (Eppendorf).
8. Amicon Ultra-0.5 mL Centrifugal Filters (Merck Millipore).
9. Centrifuge (Eppendorf).

3 Methods

3.1 Synthesis of Protected Amino Acids (Fig. 3)

1. Dissolve (d)-4-hydroxyphenylglycine or (l)-4-hydroxyphenylglycine **2** (5.00 g, 29.9 mmol) in a 1:1 solution of MeCN/sodium carbonate solution (10 %, 100 mL) with stirring at room temperature. Add Fmoc-OSu (10.1 g, 29.9 mmol) in portions over 30 min to this solution (*see* **Note 1**). Leave the reaction mixture stirring overnight, and then dilute it with water (100 mL). Extract this solution two times with diethyl ether (100 mL) and separate the organic layers. Acidify the aqueous phase to pH 3 using hydrochloric acid solution (2 M) and again extract the aqueous phase with ethyl acetate (3 × 100 mL). Extract the combined ethyl acetate organic layers with brine (2 × 100 mL), separate the organic layer and dry it over Na_2SO_4. Remove the solid by filtration and concentrate the filtrate in vacuo. Recrystallize the crude product in a mixture of ethyl acetate and hexane (*see* **Note 2**). For proper crystallization, keep the flask at 4 °C overnight, then separate the

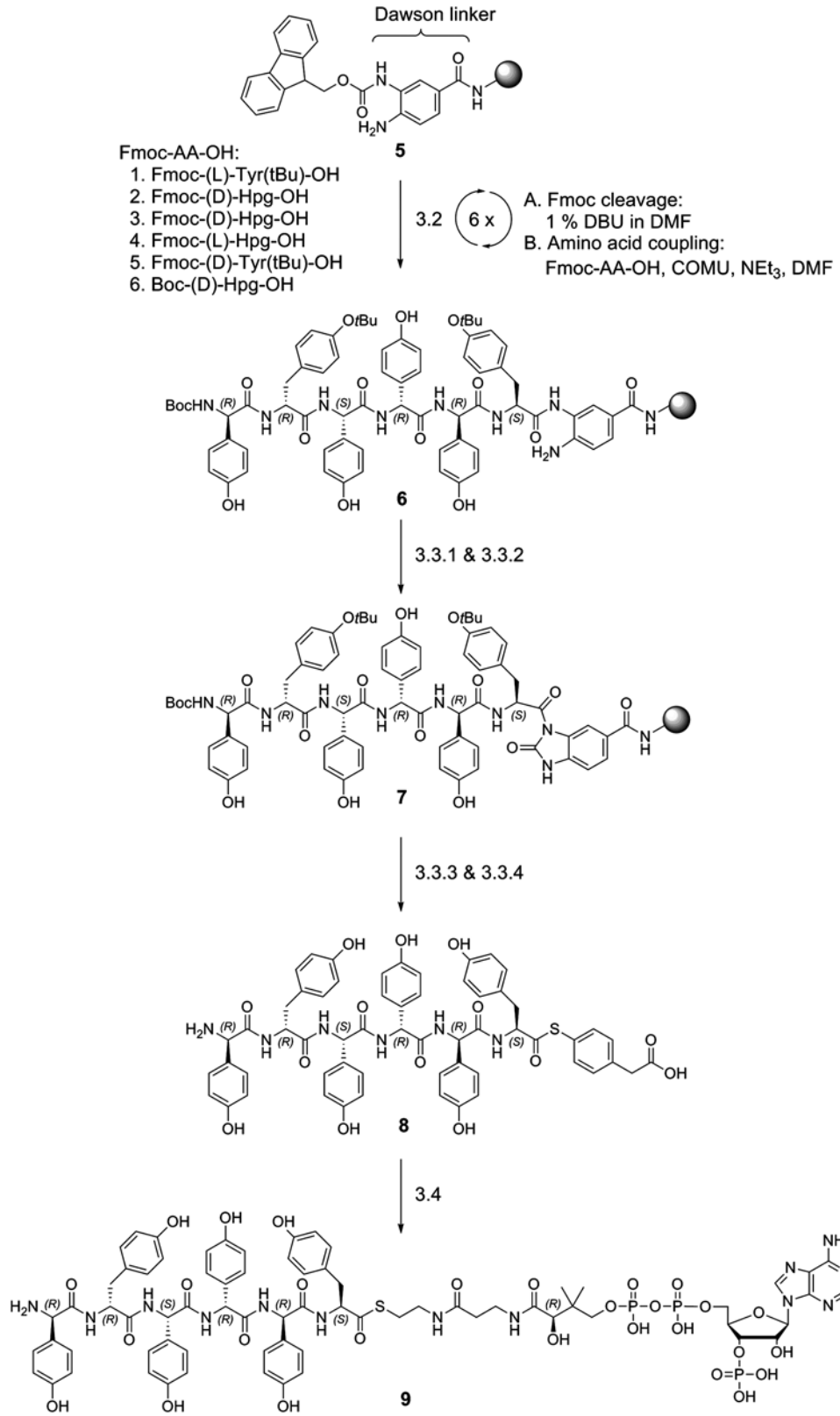

Fig. 3 Synthesis of the protected arylglycine building blocks needed for SPPS. The *numbering* refers to the corresponding paragraphs in the text

precipitated solid and dry in vacuo to obtain Fmoc-Hpg-OH **3** as a white solid (9.27 g, 23.8 mmol, 80 % yield). ^{1}H-NMR (300 MHz, DMSO-d_6): $\delta = 9.49$ (bs, 1H, COOH), 8.04 (d, 1H, NH), 7.89 (d, 2H, H4-, H5-Fmoc), 7.76 (d, 2H, H1-, H8-Fmoc), 7.44–7.38 (m, 2H, H3-, H6-Fmoc), 7.36–7.27 (m, 2H, H2-, H7-Fmoc), 7.21 (d, 2H, $^{3}J = 8.5$ Hz, Har-2,6), 6.74 (d, 2H, H_{ar}-3,5), 5.02 (d, 1H, Hα), 4.32–4.18 (m, 3H, H9-Fmoc, CH_2-Fmoc) ppm.

2. To a mixture of (d)-4-hydroxyphenylglycine **2** (2.00 g, 12.0 mmol) in 2:1 dioxane/water (30 mL), stirred on an ice bath, add sodium hydroxide solution (1 M, 10 mL) and 10 min later Di-Boc (3.9 g, 18.0 mmol) and sodium bicarbonate (1.01 g, 12.0 mmol). Leave the solution stirring overnight, allowing the mixture to warm up to room temperature. Remove the organic solvent by concentrating the solution in vacuo. Add ethyl acetate (40 mL) to the remaining mixture and acidify the organic layer to pH 3 with concentrated phosphoric acid. Separate the layers and extract the aqueous layer with ethyl acetate (2 × 40 mL). Wash the combined organic layers with brine (2 × 50 mL) and dry them over Na_2SO_4. Remove the solid by filtration and concentrate the filtrate in vacuo. Triturate the remaining oil with hexane (6 × 30 mL) until the oil becomes a paste (*see* **Note 3**). Dissolve this in acetone (100 mL) and concentrate the solution in vacuo, which affords Boc-(d)-Hpg-OH **4** (2.3 g, 8.6 mmol, 72 %) as an easily powdered white foam. ^{1}H-NMR (300 MHz, DMSO-d_6): $\delta = 7.36$ (d, 1H, NH), 7.17 (d, 2H, H_{ar}), 6.70 (d, 2H, H_{ar}), 4.95 (d, 1H, Hα), 1.38 (s, 9H, $C(CH_3)_3$) ppm.

3.2 Solid Phase Peptide Synthesis (Fig. 4)

The solid phase peptide synthesis (SPPS) of a teicoplanin precursor peptide is performed using an automated peptide synthesizer (*see* **Note 4**) on a 50 μM scale using the following conditions:

1. Resin swelling: Shake the Dawson resin for 10 min in 3 mL DMF; repeat this procedure three times (*see* **Notes 5** and **6**).
2. Fmoc deprotection (A, Fig. 4): Treat the resin with 3 mL of 1 % DBU in DMF for 30 s, filter the cleavage solution and check photometrically for the Fmoc cleavage product ($\lambda = 301$ nm). Repeat this procedure until the absorbance stays constant (*see* **Note 7**); subsequently wash the resin with DMF (five times with 3 mL).
3. Amino acid activation and coupling (B, Fig. 4): Solubilize 4 eq. of amino acid and 4 eq. of COMU activator in 2 mL of a 0.1 M Et_3N solution in DMF (4 eq.), incubate for 2 min and then add this solution to the resin. Let this resin shake for 30 min at room temperature (*see* **Note 8**). Following this, remove the soluti on and wash the resin with DMF (5 × 3 mL).
4. Repeat **steps 2** and **3** of Subheading 3.2, for each amino acid.

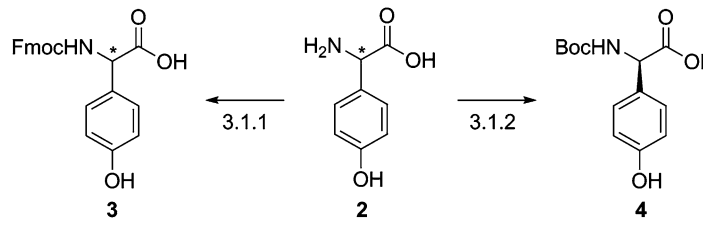

Fig. 4 Assembly of the teicoplanin-like precursor hexapeptide **6** on solid phase resin using Fmoc-chemistry followed by conversion into the activated MPAA thioester conjugate **8** and subsequently the respective CoA-conjugate **9**. *Numbering* refers to the corresponding paragraphs in the main text

3.3 Resin Activation and Peptide Displacement Using MPAA (Fig. 4)

1. Wash the resin thoroughly with DCM (4 × 3 mL) before adding *p*-nitrophenylchloroformate (50 mg, 0.25 mmol, 5 eq.) dissolved in 5 mL of DCM. Agitate the resin with this solution for 40 min, then remove the solution and wash the resin with DCM (four times) and DMF (three times).
2. Add a solution of DIPEA (44 μL, 5 eq.) in 3 mL of DMF to the resin and agitate the reaction for 15 min. Wash the resin with DMF (three times) and DCM (three times) and dry it under a stream of nitrogen (*see* **Notes 9** and **10**).
3. Transfer the resin into a 50 mL falcon tube, add 3 mL of dry DMF and agitate 15 min for swelling. Saturate the mixture with an inert gas (argon or nitrogen) by bubbling it through the solution for 10 min. Add 4-mercaptophenylacetic acid (84 mg, 0.5 mmol, 10 eq.) and PBu_3 (200 μL, 1.2 mmol) to the resin (*see* **Note 11**) and leave this mixture agitating in a closed tube for 24 h at room temperature. Separate the DMF solution from the resin; wash the resin beads with DMF (2 mL) and remove the solvent in vacuo (*see* **Note 12**).
4. Suspend the remaining residue in 5 mL of freshly prepared TFA cleavage mixture and incubate it under gentle agitation for 90 min (*see* **Note 13**). Concentrate the solution under a stream of nitrogen to ~1 mL and precipitate the peptide-MPAA thioester **8** via addition of ice cold diethyl ether (~10 mL). To ensure complete precipitation, store the mixture at −24 °C overnight. After collecting the peptide-MPAA thioester via centrifugation and decanting of the ether layer, dissolve the residue in 50 % acetonitrile–50 % water, analyze the raw mixture using analytical HPLC together (*see* **Note 14**) with mass spectrometry and lyophilize the raw peptide-MPAA thioester (*see* Fig. 5a).

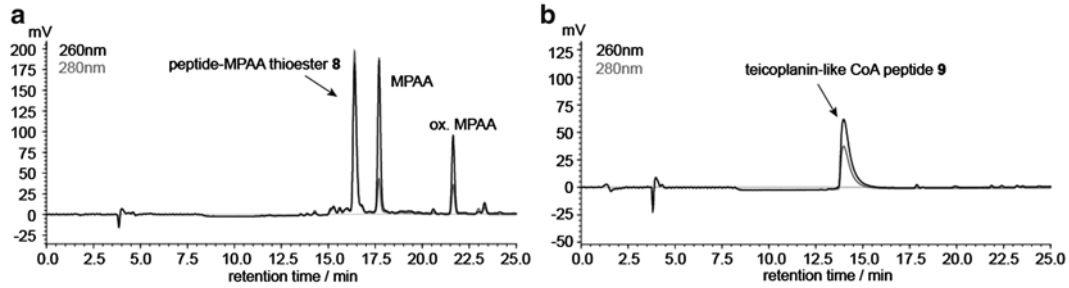

Fig. 5 Analytical reversed-phase HPLC trace of crude peptide-MPAA thioester **8** (**a**) and SPE-purified teicoplanin-like precursor CoA peptide **9** (**b**)

3.4 Activated Peptide Thioester Displacement with CoA (Fig. 4)

1. Dissolve the initial peptide-MPAA thioester **8** from 50 μmol scale synthesis in reaction buffer (~6 mL) (*see* **Note 15**) and 20 mM TCEP (*see* **Note 16**). Add CoA (35 mg, 0.875 equivalents) and let the reaction gently agitate at room temperature for 1–2 h, until—according to HPLC reaction monitoring—the peptide-MPAA thioester **8** is consumed (*see* **Note 17**).
2. In the meantime whilst the displacement reaction is underway prepare the SPE columns (four for one peptide-CoA synthesis 50 μmol scale) for purification: (1) Add methanol (5 mL) to each column for activation and let this run through the column under gravity, (2) Equilibrate the columns with SPE equilibration solvent (pH 7.0, 3 mL).
3. Dilute the reaction mixture with SPE equilibration solvent until the MeCN concentration is 5 % (*see* **Note 18**) and load it onto the equilibrated SPE columns (*see* **Note 19**). After complete loading wash the columns with SPE equilibration solvent (pH 7.0, 3 mL) and in a second wash step with SPE wash solvent in water (3 mL). To elute the peptide-CoA conjugate from the SPE columns add SPE elution solvent (pH 5–6, 3 mL) (*see* **Note 20**). Collect the elution fraction and lyophilize the isolated CoA peptide **9**. The peptides are sufficiently pure to allow for PCP loading (*see* **Note 21** and Figs. 5b and 6).

3.5 PCP-Loading (Fig. 5)

In order to study Cytochrome P450-catalyzed oxidative cross-linking reactions, PCP-domains from module 7 of several GPAs NRPS systems (balhimycin (type I), teicoplanin (type IV), complestatin (type V)) can be exploited (*see* **Note 23**).

1. Prior to the loading reaction, dilute the lyophilized peptide-CoA conjugates in Milli-Q water to a concentration of 1.5 mM (*see* **Note 24**).

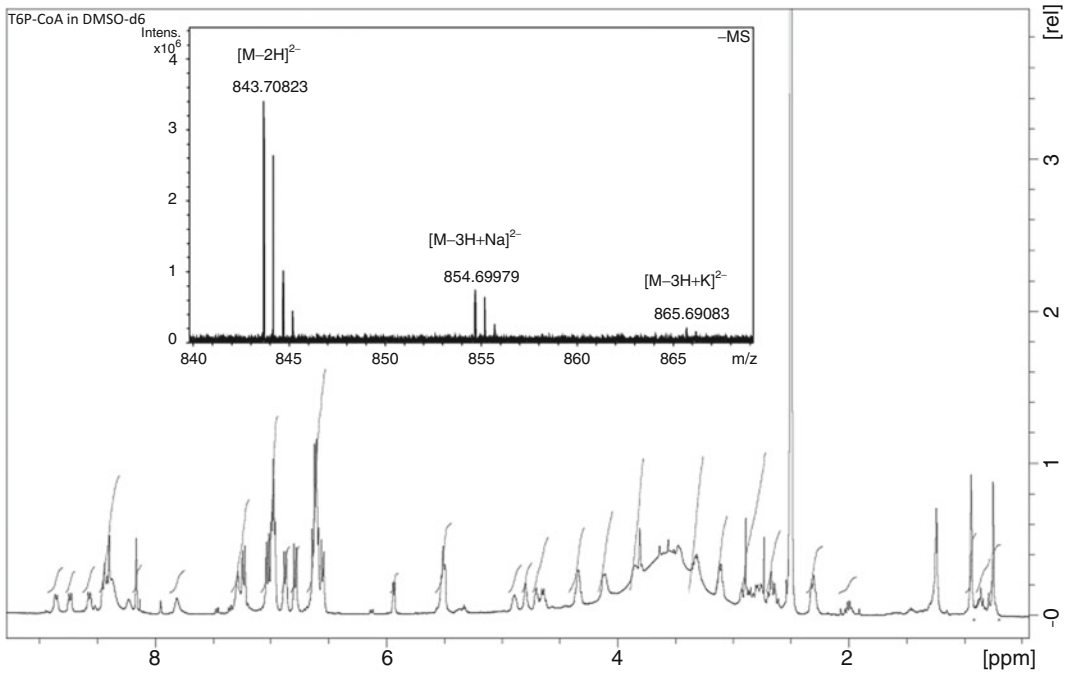

Fig. 6 ^{1}H-NMR in DMSO-d_6 (400 MHz) and HRMS spectra of HPLC-purified of teicoplanin-like CoA peptide **9** (ions are doubly charged and correspond to the protonated, sodiated, and potassiated species, *see* **Note 22**)

2. In an Eppendorf tube (*see* **Note 25**), prepare loading buffer (50 mM Hepes buffer, pH 7.0, 50 mM NaCl, 10 mM $MgCl_2$) (*see* **Note 26**) and add the expressed PCP7-construct to a concentration of 60 μM together with a fourfold excess of peptide-CoA conjugate [9].
3. Start the loading reaction by adjusting the Sfp concentration in the reaction to 6 μM (PCP:Sfp ratio 10:1). Gently mix the reaction mixture and incubate at 30 °C for 1 h (*see* **Note 27**).
4. Remove the excess of unloaded peptide by centrifugation at 4 °C using centrifugal filters with a molecular weight cutoff that allows the unloaded peptide-CoA conjugate pass through (MW 3000). Concentrate the loading reactions by centrifugation to approximately 100 μL, then add wash buffer (in a dilution of 1:5). Repeat this step three additional times. After concentrating the reaction for the fifth time, add wash buffer to reach the volume of the initial loading reaction (here: 175 μL). Keep the samples on ice until further use.

3.6 OxyB-Catalyzed Turnover and Turnover Workup (Fig. 7)

1. Prepare sufficient volumes of the NADH/NADPH, glucose oxidase and glucose stock solutions (*see* Subheading 2.6, **Note 28**).
2. Set up the turnover reaction in a total volume of 210 μL in reaction buffer. Thus, mix peptidyl-PCP from the PCP loading

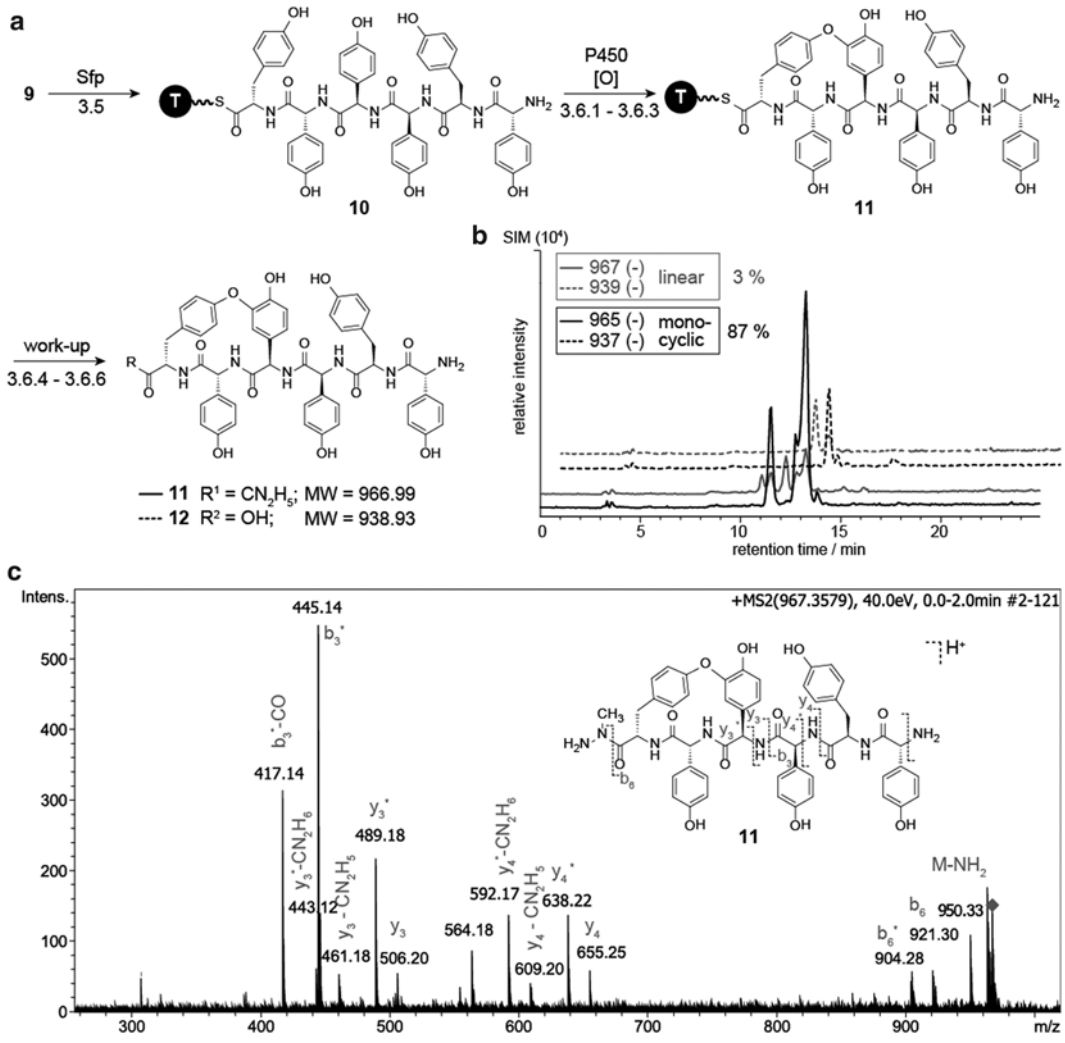

Fig. 7 (**a**) Enzymatic loading of teicoplanin-like CoA peptide **9** onto a PCP-domain (T) using the promiscuous transferase Sfp and oxidation of *peptidyl*-PCP **10** by the Cytochrome P450 $OxyB_{van}$ followed by workup. (**b**) HPLC-MS analysis of turnover reaction using single ion monitoring in negative mode; the masses correspond to the mono-cross-linked methylhydrazine (**11**, 965) and hydrolysis (**12**, 937) products and to the linear methylhydrazine (967) and hydrolysis (939) peptides. (**c**) ESI-MS/MS analysis of the C-*O*-D cross-linked product **11** from $OxyB_{van}$-catalyzed turnover. *Numbering* refers to the corresponding paragraphs in the main text

reaction (*see* Subheading 3.5, **Note 29**), ferredoxin, ferredoxin reductase (*see* **Note 28**) and OxyB in a micromolar ratio of 50:5:1:2. Add the NADH-regeneration system to the reaction. Start the reaction by adding 2 mM NADH/NADPH and incubate the mixture for 1 h at 30 °C with gentle shaking (*see* **Note 30**).

3. In the meantime prepare the solid phase extraction cartridge for workup of the turnover reaction: wash the cartridge with 1.5 mL of methanol followed by an equilibration step with water (1.5 mL). Do not allow the cartridge to dry.

4. Stop the turnover reaction by adding 30 μL of the methylhydrazine solution and incubate the mixture for 15 min at room temperature.
5. After dilution and neutralization of the turnover mixture using 11 μL of formic acid (*see* **Note 31**), load the solution onto the equilibrated SPE-column and allow the solution enter the column bed by gravity flow. Wash the column with a 5 % methanol solution (1 mL) and elute the peptides from the cartridge with 500 μL methanol (incl. 0.1 % formic acid). Concentrate the elution fraction in vacuo (*see* **Note 32**).
6. For HPLC-MS analysis, dilute the elution fraction to a final volume of 100 μL using 50 % MeCN in water and filter the sample. Analyze the samples by analytical reversed-phase HPLC-MS using single ion monitoring (*see* **Note 33**).

4 Notes

1. Slow dissolution of the amino acid as well as Fmoc-OSu can occur, in which case more MeCN can be added (up to 50 mL). Overnight, the reaction mixture becomes a clear solution.
2. Dissolve the crude product in as little as possible volume of ethyl acetate (~50 mL) and add hexane drop wise until a solid precipitates and does not dissolve anymore with gentle shaking.
3. Add hexane to the remaining oil and sonicate or mix this mixture (~5 min) for dissolving impurities into the hexane layer. After separation of the phases, remove the hexane layer by careful decanting or pipetting.
4. The solid phase peptide synthesis was performed on a Tribute UV peptide synthesizer from Protein Technologies, but it can also be performed manually.
5. Do not use a magnetic stir bar to mix the resin as this destroys the beads.
6. It is mandatory that the resin is swollen well to guarantee efficient solvent access to the resin.
7. The Tribute UV peptide synthesizer features feedback monitoring allowing to measure the amount of Fmoc cleaved after DBU treatment and to decide automatically if further cleavage cycles are necessary for complete deprotection (typically three to four cycles). Best results are obtained by incubating the resin more often with a fresh DBU solution than incubating it over a longer period.
8. Effective mixing of the resin is important to ensure proper coupling.

9. Resin activation can also be performed on an automated peptide synthesizer.
10. The dried resin can be stored in a closed tube at –24 °C until further conversion.
11. PBu_3 is oxidized by oxygen and is therefore unstable at air; use syringes for transfer.
12. This can be done by filtering the resin through a glass pipette equipped with a piece of cotton wool. The filtrate can be collected in 2 mL tubes and concentrated in vacuo using a SpeedVac concentrator.
13. By adding the TFA cleavage mixture a white solid can be formed due to oxidation of MPAA. Its formation is reduced by proper saturating the reaction mixture with inert gas and by adding PBu_3 (*see* Subheading 3.3, **step 3**). This solid should be kept in the following reaction steps.
14. For analytical HPLC a Waters XBridge BEH300 Prep C18 column (10 μm, 4.6 × 250 mm) was used. The solvents used were water + 0.1 % formic acid (solvent A) and HPLC-grade acetonitrile + 0.1 % formic acid (solvent B); gradient: 0–2 min 95 % solvent A, 2–25 min up to 25 % solvent B, flow rate 1 mL/min.
15. Check the pH of the solution and adjust it carefully to pH 8.3 using 1 M NaOH if necessary.
16. TCEP is added to reduce oxidation of excess MPAA in the reaction mixture. Oxidized MPAA has proven to be hard to separate during SPE purification and to have a negative influence the reaction yield due to solubility problems.
17. The pH can drop during the reaction slowing down the reaction and therefore needs to be checked. If necessary, readjust it to pH 8.3. For HPLC *see* **Note 14**.
18. MeCN can interfere with the SPE purification: at excessively high MeCN concentrations the product may not bind to the solid phase, which results in reduced yields.
19. For proper product adsorption to the solid phase use gravity flow in the loading step. For washing and elution steps some pressure can be generated by using a pipette bulb.
20. The amount of MeCN varies depending on the polarity of the peptide: for vancomycin-like precursor peptides 25 % MeCN has proven to be sufficient for peptide elution, for teicoplanin-like precursor peptides a higher content of acetonitrile is needed.
21. After SPE purification no other CoA species, which could interfere with PCP loading, can be detected in the samples according to HPLC-MS analysis (*see* **Note 14**).

22. ^{1}H-NMR (400 MHz, 1H-1H-COSY, DMSO-d_6): $\delta = 8.86$ (d, 1H, $^{3}J = 7.8$ Hz, Tyr_6^{NH}), 8.74 (d, 1H, $^{3}J = 8.3$ Hz, Hpg^{NH}), 8.57 (d, 1H, $^{3}J = 7.5$ Hz, Hpg^{NH}), 8.47–8.32 (m, 5H, {8.44} Tyr_2^{NH}, {8.40} H8-CoA, Hpg^{NH}, NH_2), 8.23 (m, 1H, N***H***CH_2CH_2S-CoA), 8.17 (s, 1H, H2-CoA), 7.81 (m, 1H, N***H***CH_2CH_2CO-CoA), 7.34–7.20 (m, 4H, H_{ar}), 7.07–6.94 (m, 7H, H_{ar}), 6.87 (m, 2H, H_{ar}), 6.79 (m, 2H, H_{ar}), 6.66–6.53 (m, 9H, H_{ar}), 5.94 (d, 1H, $^{3}J = 5.3$ Hz, H1′-CoA), 5.56–5.48 (m, 3H, 3 × Hpg^{α}), 4.89 (m, 1H, H3′-CoA), 4.70 (m, 1H, H2′-CoA), 4.65 (m, Tyr_2^{α}), 4.37–4.29 (m, 2H, H4′-CoA, Tyr_6^{α}), 4.11 (m, 2H, H5′-CoA), 3.87–3.78 (m, under water peak, {3.85} OC***H***$_2^{a}$C$(CH_3)_2$-CoA, Hpg_1^{α}), 3.59 (m, under water peak, C***H***(OH)CO-CoA), 3.46 (m, under water peak, OC***H***$_2^{b}$C$(CH_3)_2$-CoA), 3.35–3.28 (m, under water peak, NHC***H***$_2$$CH_2$CO-CoA), 3.15–3.07 (m, 2H, NHC***H***$_2$$CH_2$S-CoA), 2.94–2.62 (m, 6H, $NHCH_2C$***H***$_2$S-CoA, $Tyr_6\beta$, $Tyr_2\beta$), 2.34–2.26 (m, 2H, $NHCH_2C$***H***$_2$CO-CoA), 0.94, 0.75 (s, 2 × 3H, 2 × gem-CH_3-CoA) ppm.
Numbering of Coenzyme A accords to [28].
23. To increase the yield and in vitro-stability of isolated PCP7-domains it is recommended to express them as fusion proteins with thioredoxin or GB1 (IgG binding B1 domain) [14, 24, 29].
24. Always dilute the peptide-CoA conjugate freshly prior to each experiment due to instability of the peptide-CoA conjugate dissolved in water.
25. The total volume of a loading reaction for one turnover reaction (*see* Subheading 3.6) is 175 μL. For scaling up increase the total volume of the reaction, not the concentration of the components.
26. The peptide loading reaction is best carried out at pH 7.0 as higher pH leads to a faster hydrolysis of peptide-CoA minimizing the yield of the loading reaction.
27. The reaction conditions for the loading reaction described here might not be optimal for every system and can be changed if necessary (e.g., incubation temperature, incubation time).
28. The correct coenzyme (NADH or NADPH) needs to be selected depending on the redox system used. It has been shown that several redox systems such as HaPuR/HaPuX [26], PuR/PuxB [30] (NADH-dependent) or spinach ferredoxin together with *E. coli* flavodoxin reductase [12] (NADPH-dependent) are compatible with $OxyB_{van}$.
29. Always use the *peptidyl*-PCP directly after the PCP loading reaction due to the hydrolysis of the peptide from the PCP.

30. To minimize peptide hydrolysis from the PCP prior to the turnover reaction the reaction can be prepared during the PCP loading reaction. Therefore mix the required volumes of reaction buffer and 0.33 % glucose in the bottom of a 1.5 mL Eppendorf tube. Add the remaining components of the reaction except *peptidyl*-PCP separated from each other to the wall of the Eppendorf tube. Start the reaction after addition of *peptidyl*-PCP through centrifugation.
31. Depending on the peptide-PCP combination used neutralization of the solution can cause protein precipitation, which leads to a reduction of the observed peptide signal during HPLC-MS analysis. In this case another acid or a different neutralization procedure can be used.
32. Do not remove the complete solvent during peptide concentration as it can be difficult to properly redissolve the peptide fraction.
33. Waters XBridge BEH 300 Prep C18 column (particle size: 5 μm, 4.6×250 mm); gradient: 0–4 min 95 % solvent A, 4–4.5 min up to 15 % solvent B, 4.5–25 min up to 50 % solvent B; flow rate 1 mL/min (*see* **Note 14**).

References

1. Hur GH, Vickery CR, Burkart MD (2012) Explorations of catalytic domains in non-ribosomal peptide synthetase enzymology. Nat Prod Rep 29:1074–1098
2. Yim G, Thaker MN, Koteva K et al (2014) Glycopeptide antibiotic biosynthesis. J Antibiot 67:31–41
3. Cryle MJ, Brieke C, Haslinger K (2014) Oxidative transformations of amino acids and peptides catalysed by Cytochromes P450. In: Farkas E, Ryadnov M (eds) Amino acids, peptides and proteins, vol 38. Royal Society of Chemistry, Cambridge, pp 1–36
4. Cryle MJ, Schlichting I (2008) Structural insights from a P450 carrier protein complex reveal how specificity is achieved in the $P450_{BioI}$-ACP complex. Proc Natl Acad Sci U S A 105:15696–15701
5. Haslinger K, Brieke C, Uhlmann S et al (2014) The structure of a transient complex of a non-ribosomal peptide synthetase and a cytochrome P450 monooxygenase. Angew Chem Int Ed 53:8518–8522
6. Süssmuth RD, Pelzer S, Nicholson G et al (1999) New advances in the biosynthesis of glycopeptide antibiotics of the vancomycin type from *Amycolatopsis mediterranei*. Angew Chem Int Ed 38:1976–1979
7. Bischoff D, Pelzer S, Holtzel A et al (2001) The biosynthesis of vancomycin-type glycopeptide antibiotics—new insights into the cyclization steps. Angew Chem Int Ed 40:1693–1696
8. Bischoff D, Pelzer S, Bister B et al (2001) The biosynthesis of vancomycin-type glycopeptide antibiotics—the order of the cyclization steps. Angew Chem Int Ed 40:4688–4691
9. Hadatsch B, Butz D, Schmiederer T et al (2007) The biosynthesis of teicoplanin-type glycopeptide antibiotics: assignment of P450 mono-oxygenases to side chain cyclizations of glycopeptide A47934. Chem Biol 14:1078–1089
10. Stegmann E, Pelzer S, Bischoff D et al (2006) Genetic analysis of the balhimycin (vancomycin-type) oxygenase genes. J Biotechnol 124:640–653
11. Haslinger K, Peschke M, Brieke C et al (2015) X-domain of peptide synthetases recruits oxygenases crucial for glycopeptide biosynthesis. Nature. 521:105–109
12. Woithe K, Geib N, Zerbe K et al (2007) Oxidative phenol coupling reactions catalyzed by OxyB: a cytochrome P450 from the vancomycin producing organism. Implications for vancomycin biosynthesis. J Am Chem Soc 129:6887–6895

13. Schmartz PC, Wölfel K, Zerbe K et al (2012) Substituent effects on the phenol coupling reaction catalyzed by the vancomycin biosynthetic P450 enzyme OxyB. Angew Chem Int Ed 51:11468–11472
14. Brieke C, Kratzig V, Haslinger K et al (2015) Rapid access to glycopeptide antibiotic precursor peptides coupled with cytochrome P450-mediated catalysis: towards a biomimetic synthesis of glycopeptide antibiotics. Org Biomol Chem 13:2012–2021
15. Quadri LEN, Weinreb PH, Lei M et al (1998) Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases. Biochemistry 37:1585–1595
16. Vitali F, Zerbe K, Robinson JA (2003) Production of vancomycin aglycone conjugated to a peptide carrier domain derived from a biosynthetic non-ribosomal peptide synthetase. Chem Commun 21:2718–2719
17. Nicolaou KC, Boddy CNC, Bräse S et al (1999) Chemistry, biology, and medicine of the glycopeptide antibiotics. Angew Chem Int Ed 38:2096–2152
18. Freund E, Robinson JA (1999) Solid-phase synthesis of a putative heptapeptide intermediate in vancomycin biosynthesis. Chem Commun 24:2509–2510
19. Bo Li D, Robinson JA (2005) An improved solid-phase methodology for the synthesis of putative hexa- and heptapeptide intermediates in vancomycin biosynthesis. Org Biomol Chem 3:1233–1239
20. Brieke C, Cryle MJ (2014) A facile Fmoc solid phase synthesis strategy to access epimerization-prone biosynthetic intermediates of glycopeptide antibiotics. Org Lett 16:2454–2457
21. Blanco-Canosa JB, Dawson PE (2008) An efficient Fmoc-SPPS approach for the generation of thioester peptide precursors for use in native chemical ligation. Angew Chem Int Ed 47: 6851–6855
22. Dettner F, Hänchen A, Schols D et al (2009) Total synthesis of the antiviral peptide antibiotic feglymycin. Angew Chem Int Ed 48: 1856–1861
23. Davidsen JM, Bartley DM, Townsend CA (2013) Non-ribosomal propeptide precursor in nocardicin A biosynthesis predicted from adenylation domain specificity dependent on the MbtH family protein NocI. J Am Chem Soc 135:1749–1759
24. Haslinger K, Maximowitsch E, Brieke C et al (2014) Cytochrome P450 $OxyB_{tei}$ catalyzes the first phenolic coupling step in teicoplanin biosynthesis. ChemBioChem 15:2719–2728
25. Sunbul M, Marshall NJ, Zou Y et al (2009) Catalytic turnover-based phage selection for engineering the substrate specificity of Sfp phosphopantetheinyl transferase. J Mol Biol 387:883–898
26. Bell SG, Tan ABH, Johnson EOD et al (2010) Selective oxidative demethylation of veratric acid to vanillic acid by CYP199A4 from *Rhodopseudomonas palustris* HaA2. Mol Biosyst 6:206–214
27. Zerbe K, Pylypenko O, Vitali F et al (2002) Crystal structure of OxyB, a cytochrome P450 implicated in an oxidative phenol coupling reaction during vancomycin biosynthesis. J Mol Biol 277:47476–47485
28. Dordine RL, Paneth P, Anderson VE (1995) 13C NMR and 1H-1H NOEs of coenzyme-A: conformation of the pantoic acid moiety. Bioorg Chem 23:169–181
29. Bogomolovas J, Simon B, Sattler M et al (2009) Screening of fusion partners for high yield expression and purification of bioactive viscotoxins. Protein Expr Purif 64:16–23
30. Bell SG, Xu F, Johnson EOD et al (2010) Protein recognition in ferredoxin-P450 electron transfer in the class I CYP199A2 system from *Rhodopseudomonas palustris*. J Biol Inorg Chem 15:315–328