Enzyme Catalysis

An Oxidative Phenol Coupling Reaction Catalyzed by OxyB, a Cytochrome P450 from the Vancomycin-Producing Microorganism**

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During the biosynthesis of vancomycin, three oxidative phenol coupling reactions take place which lead to cross-links between aromatic amino acid side chains in the heptapeptide glycopeptide aglycone (Figure 1).[1] These cross-links constrain the peptide into a conformation that is optimal for binding to a *N*-acyl-D-Ala-D-Ala fragment, which arises during bacterial peptidoglycan biosynthesis.[2] The binding of vancomycin to *N*-acyl-D-Ala-D-Ala inhibits peptidoglycan biosynthesis, an event that is lethal for Gram-positive bacteria.

Important information about the biosynthesis of glycopeptide antibiotics has been revealed through the analysis of biosynthetic gene clusters, in particular those of chloroeremomycin[3] and balhimycin.[4] Gene knockout experiments in the balhimycin producer (balhimycin shares the same aglycone with vancomycin) identified three oxygenase genes (*oxy*A, *oxy*B, and *oxy*C) in the cluster which encode cytochrome P450-like proteins and which are responsible for the three oxidative phenol coupling reactions (Figure 1).[5] These knockout experiments indicated the order in which the three coupling reactions occur. The first coupling occurs between rings C and D and is catalyzed by OxyB, the second reaction occurs between rings D and E and is catalyzed by OxyA, and the final coupling reaction takes place between rings A and B and is catalyzed by OxyC.[6,7] So far, however, the preferred substrates of OxyA–C and hence at which step in the biosynthesis the coupling reactions occur remain unknown. Our earlier efforts to detect the turnover of the linear heptapeptides **1a** and **1b** by OxyB cloned from the vancomycin producer failed to reveal significant amounts of any product that arises from a phenol coupling reaction.[8] One reason for this failure might be that the phenol coupling occurs in vivo whilst the peptide precursor is still attached as a thioester to a peptide carrier domain (PCD) of the glycopeptide non-ribosomal peptide synthetase (NRPS). The enzyme OxyB might, therefore, be unable to catalyze the coupling of phenols on the free heptapeptide, but rather would require the peptide to be present as a thioester derivative attached to its cognate PCD (as in **2a** and **2b**). Here we report results from experiments with OxyB from the vancomycin producer *Amycolatopsis orientalis* that support this conclusion.

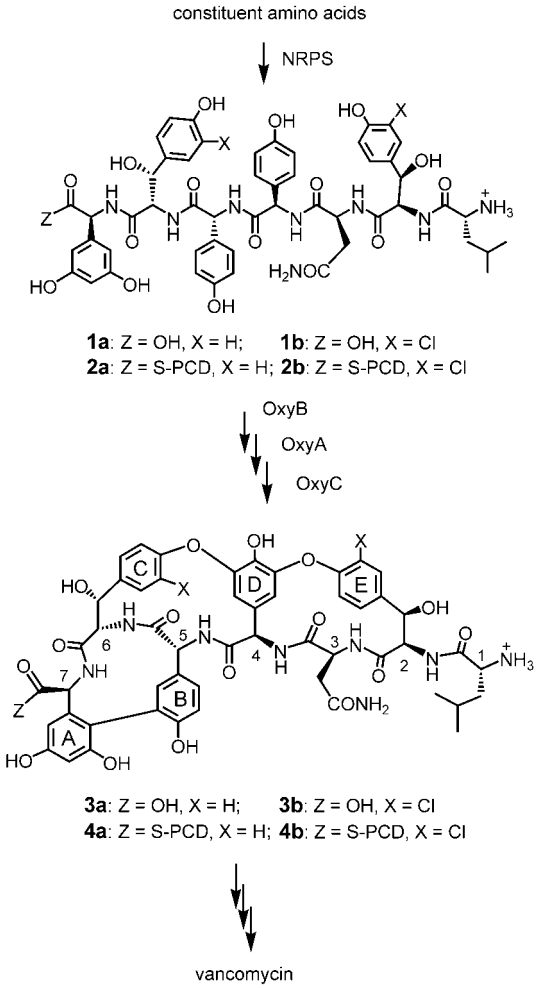

Figure 1. Outline of the biosynthesis of the glycopeptide antibiotic vancomycin. NRPS = non-ribosomal peptide synthetase, PCD = peptide carrier domain.

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In our hands, it has so far proven difficult to produce **2a** or **2b** in sufficient quantities to perform assays with OxyB. Therefore, we sought to simplify the synthetic problem by synthesizing and testing the derivative **8** (Figure 2). This

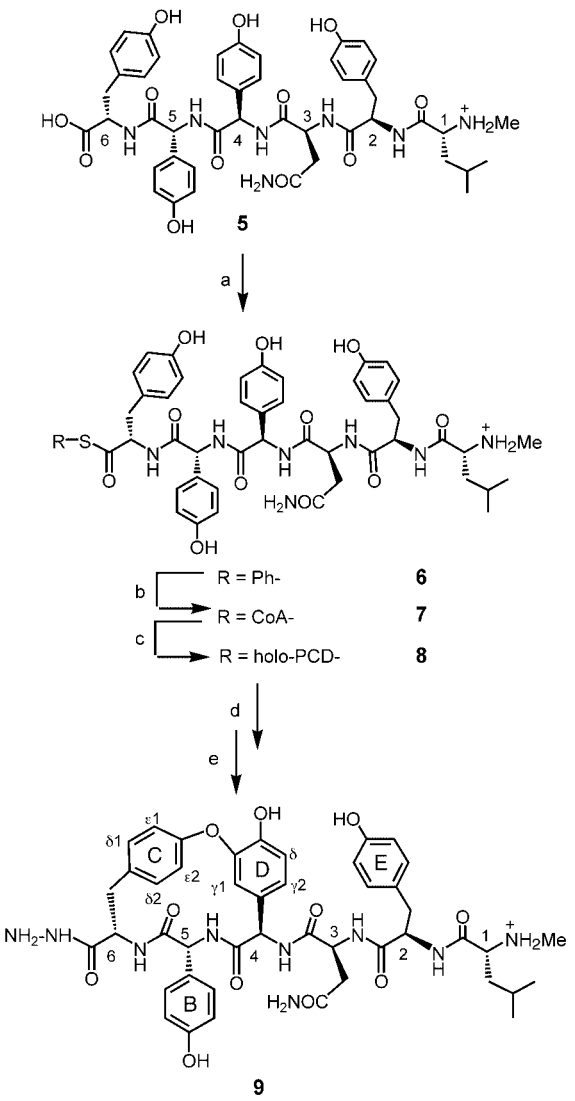
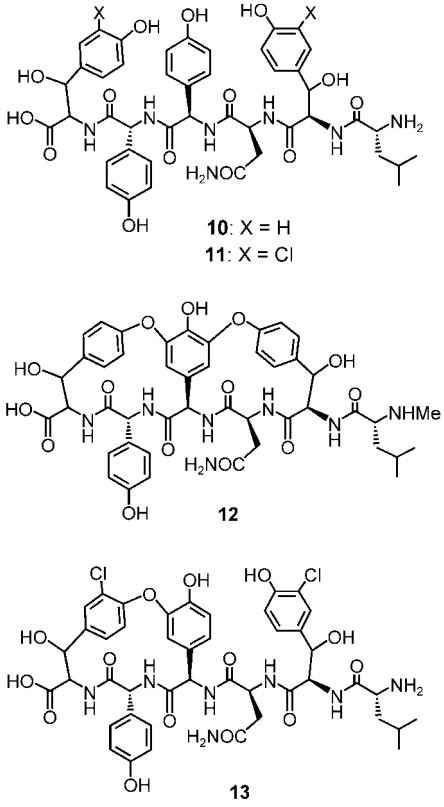


Figure 2. Synthetic route to the substrate **8**, and its assay with OxyB. a) PhSH, PyBOP; 95%; b) CoASH, pH 8.5; 50%; c) apo-PCD, phosphopantetheinyl transferase Sfp; 95%; d) OxyB, ferredoxin, ferredoxin–$NADP^+$ reductase, NADPH, in air; e) NH_2NH_2. PyBOP = (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate.

molecule comprises a hexapeptide **5**, which contains tyrosine in place of *m*-chloro-3-hydroxytyrosine, linked to the sixth PCD from the vancomycin NRPS in *Amycolatopsis orientalis* DSM40040. Although the exact point at which the chlorination steps take place is not yet clear,[9] the coupling reactions of the phenols can occur on non-chlorinated peptide chains.[6] Also, both non-chlorinated linear hexapeptide **10** and bicyclic *N*-methylated hexapeptide **12** have been isolated from *oxy*B and *oxy*C knockout mutants, respectively, although these hexapeptides could conceivably arise by proteolytic degradation of a heptapeptide precursor in the fermentation broth.[5,6]

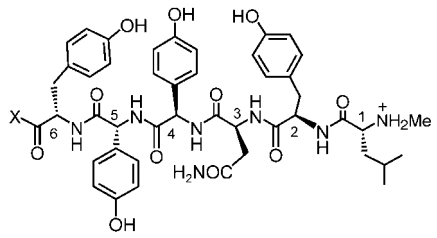


Of special interest, however, is the recent isolation of both the chlorinated linear and (in particular) monocyclic hexapeptides, **11** and **13**, respectively, from a mutant of the balhimycin producer in which a substantial portion of the *bps*C gene has been deleted.[10] The *bps*C gene is responsible for the condensation of an NRPS-bound hexapeptide with the NRPS-bound seventh amino acid 3,5-dihydroxyphenylglycine. This last result clearly suggests that the corresponding linear hexapeptide linked to the NRPS can act as a substrate for OxyB.

The PCD we chose to produce here comprises 30 residues on the N-terminal side and 52 residues on the C-terminal side of the strictly conserved active site Ser residue.[11,12] Furthermore, the Cys6 residue was modified to a Ser residue to avoid problems owing to disulfide-bridged dimer formation. This PCD, in its apo-form, was produced in *Escherichia coli* with a His_6 tag fused to the N terminus. The engineered apo-PCD was then purified to homogeneity by Ni–NTA (nickel–nitrilotriacetic acid) affinity chromatography followed by anion exchange chromatography. Electrospray ionization mass spectroscopy (ESI-MS) confirmed the expected mass of the protein (*m*/*z*: calcd: 11034; found: 11033 ± 1 ($[M+H]^+$)).

The required hexapeptide **5** was synthesized by a concise solid-phase method using Alloc ((allyloxy)carbonyl) chemistry.[13] This hexapeptide could then be converted at its C terminus into the corresponding activated phenylthioester **6** by following a previously reported method (see Figure 2).[14] For this step to work efficiently, it is important that the N terminus is *N*-methylated (as in **6**) because no further protection is then necessary: the *N*-methylamino group does

not react with the thioester under these conditions. Next, the phenylthioester **6** was converted into a coenzyme A (CoA) thioester **7**. The hexapeptide–CoA thioester was then treated with the engineered apo-PCD and the phosphopantetheinyl transferase Sfp from *Bacillus subtilis*.[15] The conversion into the hexapeptide–PCD **8** was followed by reverse-phase HPLC and found to proceed almost quantitatively. After purification by HPLC, the mass of the product **8** was confirmed by ESI-MS (m/z: calcd: 12239; found: 12239 ± 1 ($[M+H]^+$)). Upon treatment of the conjugate **8** with hydrazine, the thioester was cleaved, and the corresponding hydrazide derivative **14** was isolated and characterized by ESI-MS (m/z: calcd: 898.4; found: 898.4 ± 0.2 ($[M+H]^+$)).



14: X = -NH-NH_2

15: X = -S-CH_2CH_2NHAc

An *N*-acetylcysteamine thioester derivative (S-NAC) **15** was also prepared by the direct coupling of *N*-acetylcysteamine to **5** because S-NAC thioesters are frequently used as simpler mimics of CoA and PCD thioesters.

To detect the turnover by OxyB, assays were performed with **8** (80 μM) or other potential substrates in the presence of His_6-tagged OxyB[8] (15 μM), an engineered spinach ferredoxin, ferredoxin–$NADP^+$ reductase (0.1 U, Sigma), an NADPH-regenerating system that comprises glucose-6-phosphate (2.5 mM) and glucose-6-phosphate dehydrogenase (1 U, Sigma), and NADPH (1 mM) in HEPES buffer (25 mM, pH 7.0) at 30 °C. When the free peptide **5** was tested in this way, no conversion into a monocyclic product could be detected by HPLC/MS which is consistent with earlier observations with putative free linear heptapeptide substrates (**1a** and **1b**).[8] Following assays with the PCD derivative **8**, the assay mixture was treated directly with excess hydrazine, and the linear open chain and the monocyclic peptides, **14** and **9**, respectively, were analyzed by HPLC/MS. Assays were also performed with **6** and **7**, but these failed to reveal any conversion into a monocyclic product. An assay with **15** revealed a small degree of conversion ($\leq 5\%$) into a new product, whose molecular mass was lower by 2 Daltons (by HPLC/MS). The extent of conversion was too low, however, to allow a full characterization of this product. Assays with the PCD derivative **8**, however, typically showed up to 80% conversion into a new product (see Figure 3) to which we assign the structure **9** (high-resolution positive-ion ESI-MS (APEX Qe FT-ICR mass spectrometer (Bruker Daltonics) equipped with a 7 tesla magnet); exact mass calcd for $C_{45}H_{54}N_9O_{11}$: 896.3943 ($[M+H]^+$); found: 896.3938). No conversion of **8** into **9** took place under the assay conditions in the absence of OxyB nor in the absence of ferredoxin and ferredoxin–$NADP^+$ reductase.

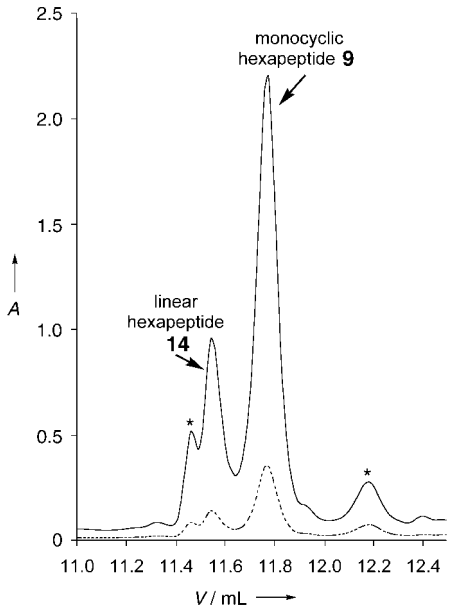


Figure 3. Analysis by HPLC (UV/Vis detection; ——= 226 nm, -----= 280 nm) of the products from the conversion of **8** with His_6–OxyB (see text for details). The peaks corresponding to linear (**14**) and monocyclic (**9**) products are shown; ⋆ = unknown. HPLC conditions: C18 Vydac column (218TP54), solvent A = water + 0.1% TFA, solvent B = MeCN + 0.1% TFA, gradient 5–40% B over 20 min, flow rate: 1 mL min^{-1}. TFA = trifluoroacetic acid, MeCN = acetonitrile.

ESI-tandem mass spectra (ESI-MS/MS) of **9** and **14** are consistent with the location of a cross-link between rings C and D in the new product **9**. Thus, **14** showed the expected fragment ions b_2, b_3, b_4, and b_5, whereas **9** showed b_2 and b_3 fragments and additional z_3, y_4/z_4, and y_5/z_5 fragments with the expected (−2) masses (Figure 4).

The ^{1}H NMR and 2D COSY, TOCSY, NOESY, and ROESY spectra of **9** allowed a full assignment of the ^{1}H NMR spectrum (see Table 1) and strongly support the proposed connectivity. From 2D spectra, the connectivity of the peptide backbone was established by using standard methods.[16] Also,

Table 1: ^{1}H NMR chemical shift assignments (ppm, 500 MHz) of monocyclic peptide **9** measured in [D_6]DMSO at 300 K.

Residue	NH	C(α)−H	C(β)−H	Others
Leu1	8.60(br)	3.58	1.49, 1.37	CH(γ) = 1.46, CH_3(δ) = 0.80, 0.73, NMe = 1.96
Tyr2	8.81	4.78	2.96, 2.57	C(δ)H = 7.04, C(ε)H = 6.62, OH = 9.17
Asn3	8.51	4.73	2.56, 2.38	N(δ)H(*E*) = 7.33, N(δ)H(*Z*) = 6.94
Hpg4	7.96	5.28	–	C(γ1)H = 5.95, C(γ2)H = 6.62, C(δ)H = 6.76, OH = 9.40
Hpg5	8.89	5.21	–	C(γ)H = 7.08, C(δ)H = 6.67, OH = 9.40
Tyr6	7.30	4.62	2.90	C(δ1)H = 7.21, C(δ2)H = 7.27, C(ε1)H = 6.90, C(ε2)H = 7.09
$CONH.NH_2$	–	–	–	7.19, 7.08, 6.98

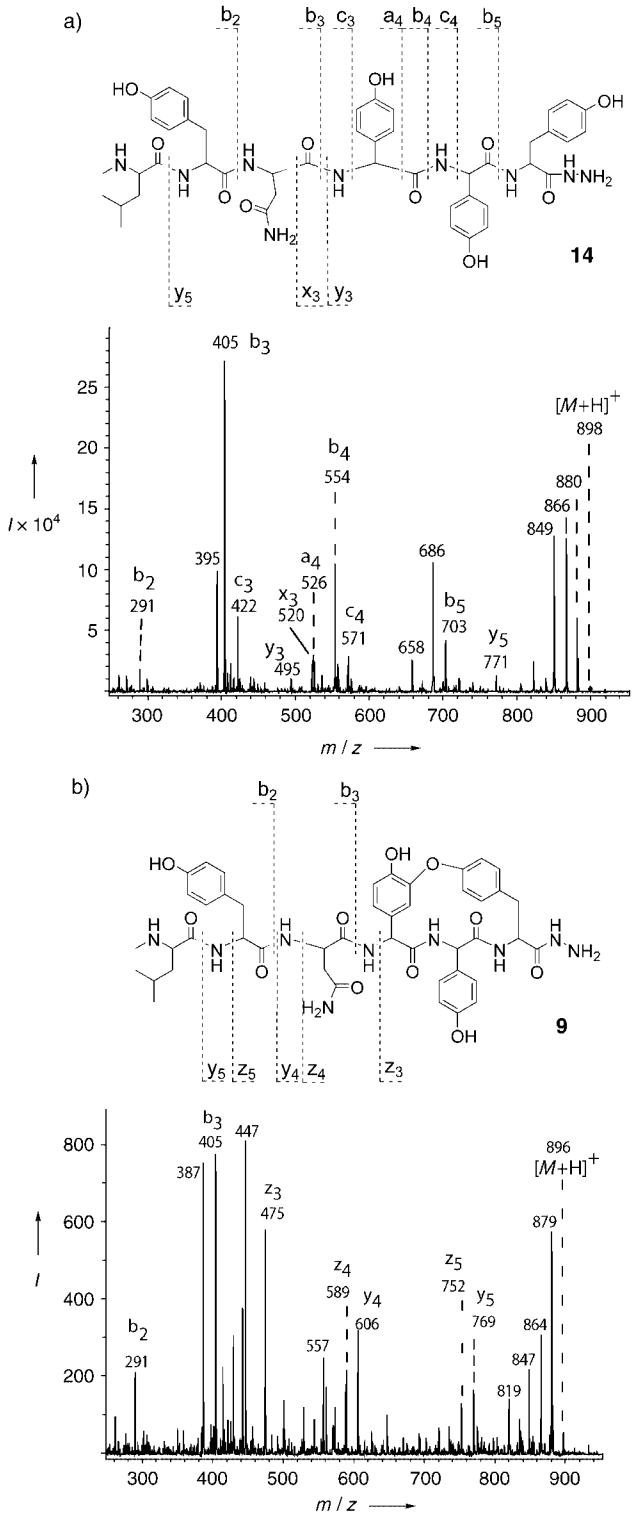


Figure 4. ESI-MS/MS of a) the linear peptide **14** and b) the monocyclic product **9.** The spectra were run on a Bruker ESQUIRE-LC quadrupole ion-trap mass spectrometer. Samples as solutions in MeOH/H_2O (1:2) with 0.05% TFA were introduced through the ES interface at 5 μL min^{-1}. The protonated quasi-molecular ions $[M+H]^+$ were selected and subjected to helium gas collision (fragmentation amplitude of 0.9 in the smart fragmentation mode).

the intact connectivity of the side chains of residues 1, 2, 3, and 5 was apparent in the 2D spectra. However, the side chains of residues 4 and 6 in this product had clearly been altered relative to the starting hexapeptide **5** (see Figure 2 for labeling of the rings, residues, and carbon atoms). The proton at C(α) of residue 4 showed NOE connectivities to a singlet peak at $\delta = 5.95$ ppm, which we assign to the hydrogen atom at C(γ1) in the aromatic side chain. This aromatic proton is shifted upfield as expected from its close proximity to the face of the aromatic ring C in residue 6 in **9** (see Figure 2). The proton of the N-H group of residue 4 shows an NOE interaction with the hydrogen atom at C(γ2) in ring D, with the latter hydrogen atom coupling to that at C(δ) to give rise to a doublet-of-doublets. In the case of residue 6, the protons of the aromatic ring C appear as two sets of doublet-of-doublets, which indicates hindered rotation of the aromatic ring and thus renders the two sides diastereotopic as would be expected for **9**. A weak NOE interaction is also observed between the hydrogen atoms at C(γ1) in ring D and C(ε2) in ring C, respectively. At a later stage, we hope to prepare **9** synthetically to prove the identity of this enzymic product by an alternative method, but at this point, the data from MS and NMR spectroscopy experiments strongly support the proposed structure of **9**.

These results provide the first direct evidence that OxyB can catalyze a phenol coupling reaction not on a free peptide but rather on a peptide attached as a thioester to a PCD. A simpler S-NAC thioester mimic, such as **15**, is not sufficient to render the peptide a viable substrate for OxyB. This further suggests that at least the OxyB-catalyzed coupling reaction, and perhaps all of the oxidative phenol couplings during glycopeptide antibiotic biosynthesis, occur whilst the peptide intermediates are attached to cognate PCDs within the NRPS as suggested indirectly by the results from other studies.[10] However, many unanswered questions remain. A key uncertainty is whether the enzyme can also transform heptapeptides such as **2a** and **2b** (and whether this is more efficient), or whether the first phenol coupling reaction occurs preferentially on a hexapeptide (that is, before the last amino acid is added to the chain by the NRPS). Renewed efforts should now be made to prepare and perform assays with heptapeptide–PCD conjugates such as **2a** and **2b**. Evidence from gene knockout experiments suggest already that the biosynthetic enzymes may not have a strict substrate specificity.[10] Clearly, a 3-hydroxy group in residue 6 is not necessary for the coupling reaction that is catalyzed by OxyB to occur. However, the influences of a β-hydroxy group and the chlorine atom in the *m*-chloro-3-hydroxytyrosine residue (compare **2a** and **2b** with **8**) on the rate of the reaction remain to be defined.

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