Vancomycin Biosynthesis

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Substituent Effects on the Phenol Coupling Reaction Catalyzed by the Vancomycin Biosynthetic P450 Enzyme OxyB**

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Oxidative phenol coupling reactions catalyzed by dedicated cytochrome P450 enzymes play an important role in the biosynthesis of the glycopeptide antibiotics vancomycin and balhimycin. The biosynthesis of the common heptapeptide aglycone in both antibiotics requires (2*S*,3*R*)- and (2*R*,3*R*)-β-hydroxytyrosine (Bht), two tyrosine-derived (*R*)-*p*-hydroxyphenylglycines (Hpg), a polyketide-derived (*S*)-3,5-dihydroxyphenylglycine (Dpg), and also the proteinogenic amino acids Leu and Asn (Figure 1).[1] A striking feature of the aglycone is the presence of biaryl (A-B) and biaryl ether (C-*O*-D and D-*O*-E) cross-links that create the dome-shaped structure of the molecule. Furthermore, two of the cross-linked aromatic side chains contain chlorine substituents. Molecular genetic studies of balhimycin biosynthesis have shown that the heptapeptide backbone is assembled by a non-ribosomal peptide synthetase (NRPS), whereas the cross-links are introduced in the order C-*O*-D, then D-*O*-E, and finally A-B, by the P450 enzymes OxyB, OxyA, and OxyC, respectively.[2] Two chlorination reactions are catalyzed by a single flavin-dependent halogenase.[3] Unfortunately, the exact timing of the phenol coupling and halogenation steps during vancomycin/balhimycin aglycone assembly is still not completely understood.

Earlier in vitro studies employing model hexa- and heptapeptide substrates containing Tyr residues at positions 2 and 6 showed that OxyB can catalyze a coupling reaction between the aromatic rings of Hpg4 and Tyr6 when the model peptide is linked as a C-terminal thioester to a stand-alone peptide carrier protein (PCP) domain from the vancomycin NRPS (Figure 1).[4] To generate substrates closer in structure to likely biosynthetic intermediates, we set out to replace the Tyr2/Tyr6 residues in the model peptides with Bht and/or *meta*-chloro-β-hydroxytyrosine (Cht), in the expectation that the presence of β-hydroxy and/or chlorine substituents would make the peptides better substrates for OxyB. We report herein that on the contrary, introduction of the chlorine substituents in a hexapeptide-*S*-PCP severely attenuates the rate (>20 fold lower relative to the non-chlorinated model substrate) of cross-linking by OxyB, whereas a heptapeptide-*S*-PCP with a chlorinated residue 6 is not a substrate for OxyB and is not turned over into cross-linked product. On the other hand, introducing β-hydroxy groups in the hexapeptide-*S*-PCP has only a small effect on the rate of the OxyB reaction in this in vitro system.

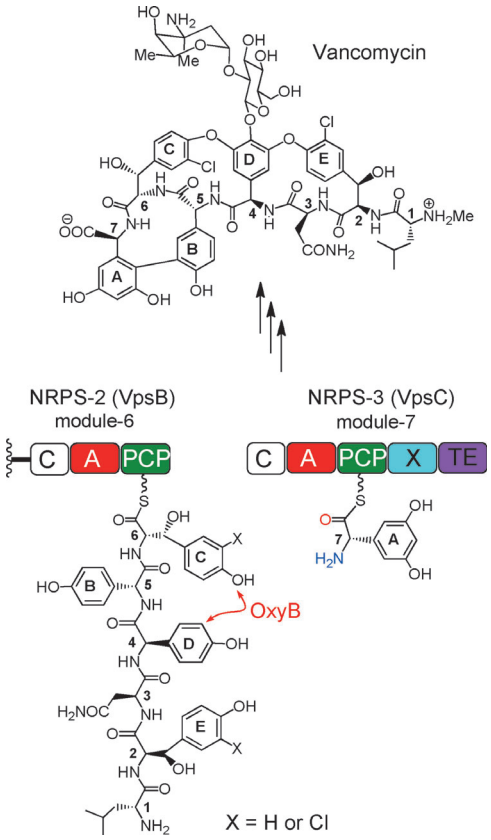

Figure 1. Key steps of backbone assembly during vancomycin and balhimycin biosynthesis (vancomycin and balhimycin share the same aglycone). Shown is the predicted domain organization of modules 6 and 7, in the vancomycin NRPS subunit 2 (VpsB) and 3 (VpsC) (C = condensation domain, A = adenylation domain, PCP = peptide carrier protein domain, TE = thioesterase domain, X = domain of unknown function). The hexapeptide-*S*-PCP thioester intermediate shown should be cross-linked by OxyB before addition of the last amino acid (Dpg7) by module 7.

We first established an efficient solid-phase procedure to prepare the hexapeptide precursors **1a**,**b** from the amino acid building blocks **2**–**6** (Scheme 1). These peptides contain an *N*-methyl group at the N-terminus, rather than a primary amine,

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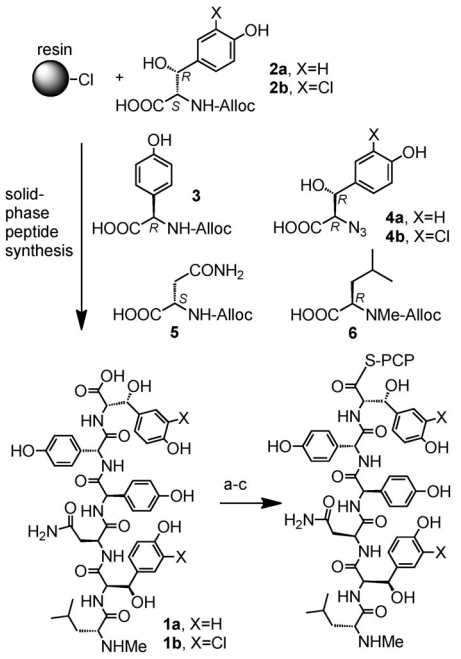


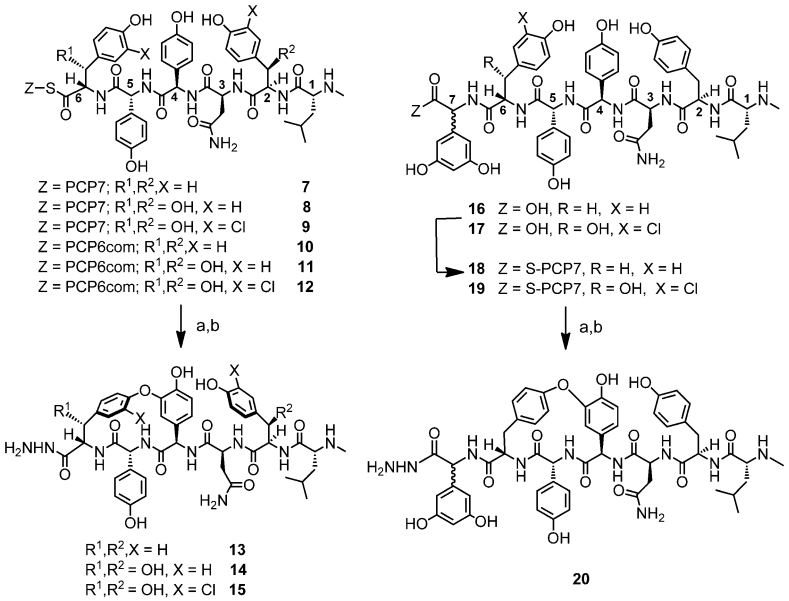
Scheme 1. Synthesis of the hexapeptide substrates. For the synthesis of amino acids **2**–**6**, and the solid-phase synthesis of **1a** and **1b**, see the Supporting Information. Reagents: a) *N*-methylmorpholine (1.2 equiv), PyBOP (1.2 equiv), PhSH (2.4 equiv), DMF, 30 min, RT; b) CoASH (4.0 equiv), phosphate buffer (50 mM, pH 7.5)-DMF (2:1), 1 h, RT; c) *apo*-PCP domain, Sfp from *B. subtilis*, 30 min, 37 °C. Alloc = allyloxycarbonyl.

as C-terminal thioester peptides with an unsubstituted N-terminus proved very difficult to prepare, whereas a secondary amine and thioester were compatible functional groups under the assay conditions used here. The N-terminus is methylated normally as the last step in aglycone biosynthesis by an *S*-adenosylmethionine-dependent methyl transferase.[5] The interpretation of in vitro assay results is then made with the proviso that a methylated N-terminus is not expected to influence significantly how the substrates interact with OxyB. The synthetic route builds upon earlier studies and largely avoids the use of acids and bases, as well as amino acid side chain protecting groups, thereby circumventing many problems that arise from the inherent reactivity of the building blocks and peptide products.[6] The peptides **1a** and **1b** were each obtained in $\geq 98\%$ purity after HPLC purification, in an overall yield of ca. 3%, and were characterized by MS and NMR methods (Supporting Information, Figure S1).

Next, conversion of the peptides **1a** and **1b** sequentially into the *S*-phenyl, the coenzyme-A (CoA-), and the *S*-PCP thioesters (Scheme 1; Supporting Information, Figure S2 and S3), required optimization of methods reported earlier.[4a,6,7] The formation of the CoA-thioesters proceeded efficiently in phosphate buffer containing DMF, although careful control of pH (7.5) is required, as the products rapidly degrade at pH 8.5. Two different stand-alone *apo*-PCP domains were prepared as recombinant His-tagged proteins in *E. coli*, one (*apo*-PCP7) derived from module-7 in subunit-3 (VpsC), and the second (*apo*-PCP6com), derived from module-6 in subunit-2 (VpsB) of the vancomycin NRPS. The latter includes a communication or COM domain and the natural C-terminus of VpsB. Similar COM domains have been found at the termini of various NRPS and polyketide synthase (PKS) subunits, and are known to play critical roles in mediating intersubunit communication.[8] Both PCPs were characterized by MS and CD (Supporting Information, Figures S4–S6). The hexapeptide-CoAs were loaded onto each *apo*-PCP domain in a reaction catalyzed by the pantetheinyl transferase Sfp to give *holo*-peptide-PCP products.

Assays with OxyB involved incubation of hexapeptide-*S*-PCP conjugates (**7**–**12**) with OxyB and a reduction system comprising spinach ferredoxin (spFd), *E. coli* flavodoxin reductase (*eco*FldR), NADPH with glucose-6-phosphate (G6P), and glucose-6-phosphate dehydrogenase (G6P-DH; Scheme 2), as described earlier.[4] Products were cleaved from the PCP domain using hydrazine, isolated by solid-phase extraction, and analyzed by analytical HPLC and mass spectrometry. Substrates **7**–**12** were first compared under identical assay conditions, based upon the extent of conversion into monocyclic product catalyzed by OxyB (Supporting Information, Figures S7 and S8).[4] In general, it was observed that substrates linked to PCP7 were converted slightly more efficiently into monocyclic product than those linked to PCP6com.

Under standard conditions, model hexapeptide-*S*-PCP7 (**7**) was converted (ca. 70% turnover) into the monocyclic



Scheme 2. In vitro conversion of linear hexapeptides (**7**–**12**) to monocyclic peptides (**13**–**15**) by OxyB, and linear heptapeptides (**16**–**19**) and only observed cross-linked product (**20**). Reagents: a) OxyB, spFd, *eco*FldR, NADPH, G6P, G6P-DH, air, 1 h, 30 °C; b) hydrazine, 30 min, 30 °C.

product **13** after 1 h (Figure 2; Supporting Information, Figure S7). Small amounts of an epimer (10–20%) as well as unreacted linear peptide were recovered from the assay. The epimeric product most likely arises owing to epimerization at Tyr^6 Cα during the assay.[4a,9] Under the same conditions, the β-hydroxylated hexapeptide-*S*-PCP7 (**8**) was converted with a similar efficiency into monocyclic product **14**. The structure of **14** was confirmed by high-resolution electrospray ionization MS/MS (HR-ESI-MS/MS) fragmentation analysis (Supporting Information, Figure S9). These results show that introducing the two β-hydroxy groups has only a minor effect on the rate of turnover by OxyB of the hexapeptides into cross-linked product with the C-*O*-D ring.

Next, β-hydroxylated and chlorinated hexapeptide-*S*-PCP7 (**9**) was assayed under the same conditions. Now, the turnover of **9** to C-*O*-D ring product (**15**) was much slower (ca. 20% turnover) than seen for **7** and **8** (Figure 2; Supporting Information, Figure S8). HR-ESI-MS/MS analysis of **15** indicated formation of the C-*O*-D ring (Supporting Information, Figure S10). Steady-state kinetics were investigated for **9** using the HPLC assay, giving an apparent K_m of 15.3 μM and a k_{cat} of 0.005 s^{-1} (Figure 2; Supporting Information, Figure S11). The kinetic parameters for the model substrate **7** were also measured and gave a K_m of 19.8 μM and a k_{cat} of 0.16 s^{-1}, which are in good agreement with values reported earlier.[4a] The results show that the rate of cross-linking by OxyB of the chlorinated substrate is severely attenuated under the conditions used ($k_{cat}/K_m \approx 25$ fold lower), with most of the effect apparent in a reduced k_{cat}.

Similar results were also obtained with the hexapeptide-*S*-PCP6com conjugates **10**–**12** (Scheme 2) as substrates. The hexapeptides **10** and **11** were converted into the expected cross-linked products to the extent of 45–50% and were accompanied by smaller amounts of an epimeric monocyclic product (ca. 10%), as well as unreacted linear peptide (Supporting Information, Figures S7 and S8). However, the presence of chlorine substituents in hexapeptide **12** again led to a much lower turnover (ca. 25%) into macrocyclic product **15**.

The binding of the substrates to OxyB was also monitored by UV/Vis difference spectroscopy. All substrates induced a typical type I difference spectrum upon binding to OxyB, which is caused by displacement of the axial water ligand on iron and shift in the spin-state equilibrium.[4a] The concentration dependence of the absorbance changes (ΔA) fitted well a binding equation involving a 1:1 interaction (Figure 3; Supporting Information, Figure S12). Fitting the Langmuir equation to each data set gave for **7** a K_d of (27 ± 5) μM, which is in a similar range to that reported previously.[4a] The affinity of **8** to OxyB was reduced somewhat, with a K_d of (48 ± 6) μM. However, the presence of both chlorine substituents in **9** gave a K_d of about (180 ± 30) μM. The results show that the introduction of chlorine substituents has a significant effect on the shift in spin-state equilibrium, and weakens significantly the binding affinity of the hexapeptide-*S*-PCP to OxyB. Thus, a good correlation is observed between the effect on spin-state

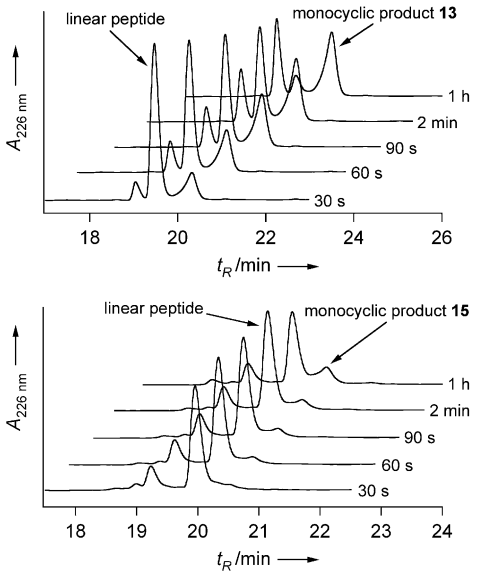


Figure 2. HPLC chromatograms showing typical time courses of product formation (**13** and **15**) from substrates **7** (top) and **9** (bottom), respectively (Supporting Information, Figure S11). The peak retention time (t_R) and peak absorbance at 226 nm are shown.

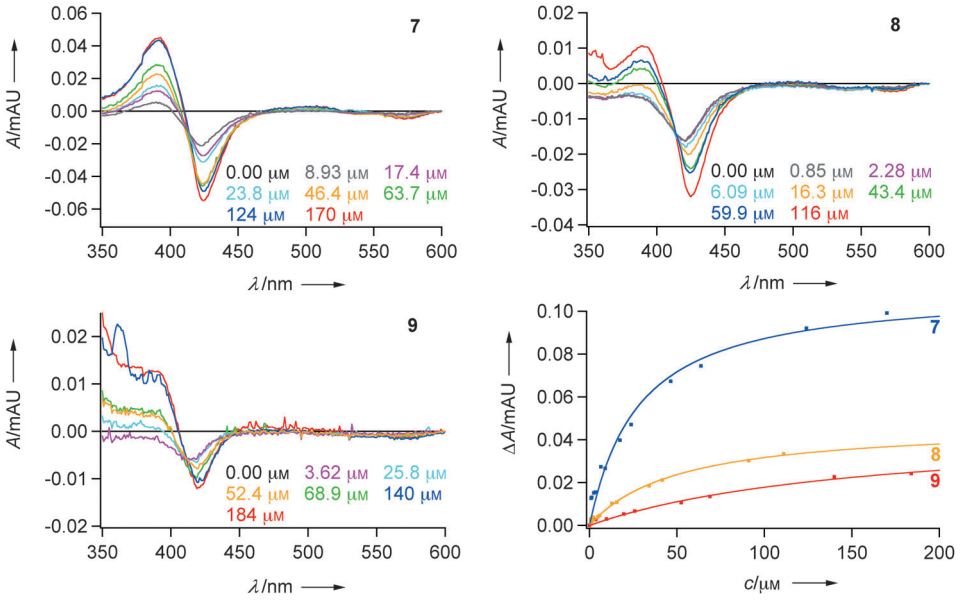


Figure 3. Difference UV/Vis spectra from binding of substrates **7**, **8**, and **9** to OxyB, and the dependence of spectral changes on the peptide concentration *c*.

equilibrium shift and catalytic activity of OxyB upon comparing these substrates.

To test how chlorination of a heptapeptide-*S*-PCP substrate influences turnover by OxyB, a heptapeptide-*S*-PCP7 substrate **18** and the analogue **19** (Scheme 2) containing (2*S*,3*R*)-β-hydroxy-*m*-chlorotyrosine (Cht) at position 6 were synthesized (see the Supporting Information). The PCP7 thioester conjugates (**18** and **19**) were assayed with OxyB under standard conditions. As reported earlier,[4a] model heptapeptide **18** was converted (60–70%) into the C-*O*-D macrocyclic product **20**. However, similar assays with **19** repeatedly failed to reveal the formation of any C-*O*-D ring product; only the corresponding linear peptide hydrazide was recovered (ca. 90%; Supporting Information, Figure S13). This dramatic effect observed in vitro upon introducing the chlorine substituent suggests that a linear heptapeptide-*S*-PCP chlorinated in residue 6 is not a viable intermediate in vancomycin assembly, as it would not be a substrate for OxyB.

Two key observations reported herein provide important new insights into vancomycin/balhimycin biosynthesis. First, the conclusion that a chlorinated linear heptapeptide-*S*-PCP is not a viable intermediate in vancomycin assembly rests on the proviso that in vitro studies with the unnatural substrates reveal properties of the biosynthetic reactions as they occur naturally within the cell. Although difficult to prove unambiguously, this seems a reasonable conclusion, as OxyB catalyzes efficient cross-linking in vitro on hexapeptide-*S*-PCP substrates. On the other hand, fully chlorinated linear (that is, non-cross-linked) hexa- and heptapeptides have been isolated from different null mutants of the balhimycin producer.[2a,10] It is possible, however, that these chlorinated linear peptides might be shunt products produced in minor amounts over long fermentation times. One very interesting shunt metabolite isolated from one of the mutants (a *dpgA* mutant) was a cross-linked heptapeptide containing Hpg7 in place of Dpg7.[10]

The second point concerns the timing of the halogenation steps. The halogenase is unusual in that it must recognize two rather different sites to chlorinate residues 2 and 6 in the peptide backbone. Free Cht is not a naturally occurring precursor and is not used for glycopeptide biosynthesis,[11] so chlorination most likely occurs on an NRPS-bound intermediate. Chlorination might take place directly after incorporation of each Bht residue on the NRPS. The in vitro experiments described above, however, show that a chlorine substituent in residue 6 of the hexapeptide-*S*-PCP slows dramatically the cross-linking reaction catalyzed by OxyB. This raises the question as to whether, during the biosynthesis, cross-linking by OxyB might occur before chlorination. Although earlier molecular genetic studies revealed chlorinated hexa- and heptapeptides from balhimycin null mutants,[2a,10] later in-frame deletions of the oxygenase genes, designed to avoid polar effects on downstream genes (including the halogenase), gave *oxyA* or *oxyC* mutants that produced only singly or doubly cross-linked but fully chlorinated products, whereas an *oxyB* mutant gave only non-chlorinated linear hexa- and heptapeptide products, suggesting that the action of the halogenase might depend in some way on the presence of OxyB as well as the NRPS.[12] A final interesting observation is that feeding 3-fluoro-β-hydroxytyrosine to a *bhp* deletion mutant (Bhp is required for Bht biosynthesis) allowed isolation of a balhimycin derivative fluorinated in residues 2 and 6 (fluorobalhimycin), showing that OxyB can turn over in vivo, at least slowly, a fluorinated linear peptide precursor.[13] This result also illustrates the potential of the mutabiosynthesis approach for generating novel glycopeptide antibiotic analogues. Unfortunately, there have been no reports so far of in vitro activity with the halogenase, so its substrate preferences are unknown. Presently, this makes it difficult to define the preferred order of the chlorination and cross-linking steps. We anticipate, however, that the synthetic methods described herein may facilitate in vitro studies on this key halogenase.

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