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# New insights into the first oxidative phenol coupling reaction during vancomycin biosynthesis

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**Abstract**—OxyB catalyzes the first oxidative phenol coupling reaction in vancomycin biosynthesis. OxyB is a P450 hemoprotein whose activity is strictly dependent upon the presence of molecular oxygen. Here, it was shown that label from $^{18}O_2$ is not incorporated into the monocyclic product during catalysis by OxyB. In addition, it was shown that OxyB can convert a model hexapeptide substrate containing (*R*)-Tyr6, instead of (*S*)-Tyr6, covalently linked as a C-terminal thioester to a peptidyl carrier protein (PCP-7S) derived from the vancomycin non-ribosomal peptide synthetase (NRPS), into the corresponding epimeric monocyclic product. The binding of this epimeric hexapeptide-PCP conjugate to the Fe(III) form of OxyB, as monitored by UV–vis spectroscopy, revealed a $K_d = 35 \pm 5$ μM. Thus, the enzyme reveals a surprising lack of stereospecificity in the binding and transformation of these epimeric substrates.


Vancomycin (Fig. 1), a glycopeptide produced by *Amycolatopsis orientalis*, is a clinically important antibiotic that acts by inhibiting cell wall biosynthesis in Gram-positive bacteria.[1] During vancomycin biosynthesis, key steps include rigidification of the heptapeptide aglycone backbone. These are accomplished by the actions of three cytochrome P450 hemoproteins OxyA, OxyB, and OxyC that catalyze three consecutive oxidative phenol coupling reactions.[2–5] The first coupling, which ultimately links the phenol rings in the side chains of Hpg4 ((*R*)-4-hydroxyphenylglycine) and Cht6 ((2*S*,3*R*)-*m*-chloro-β-hydroxytyrosine) (C-*O*-D ring), is catalyzed by OxyB. The second aryl-ether bridge is formed by OxyA and links the side chains of amino acids Cht2 ((2*R*,3*R*)-*m*-chloro-β-hydroxytyrosine) and Hpg4 (D-*O*-E ring), and the last coupling catalyzed by OxyC connects the aromatic side chains of Hpg5 and Dpg7 ((*S*)-3,5-dihydroxyphenylglycine) (AB ring).

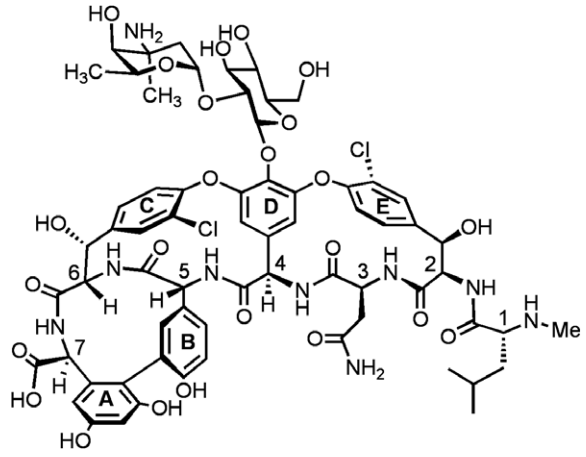

**Figure 1.** Structure of vancomycin.

We have shown previously that a model linear hexapeptide (**1**) (Fig. 2) related to the vancomycin aglycone is converted efficiently into a monocyclic product in vitro by OxyB only when the peptide is linked as a thioester to a recombinant peptide carrier protein (PCP) domain from the vancomycin non-ribosomal peptide synthetase (NRPS) (Fig. 3).[6,7] It was also demonstrated that OxyB activity is strictly dependent upon the presence of redox proteins that deliver electrons to the hemoprotein, as well as upon molecular oxygen. The model substrate, **1**-PCP-7S (Fig. 2), binds to OxyB in the absence of other ancillary proteins with a dissociation constant of 17 ± 5 μM, as determined by UV–vis difference spectroscopy.[7] Furthermore, we showed that OxyB is also able

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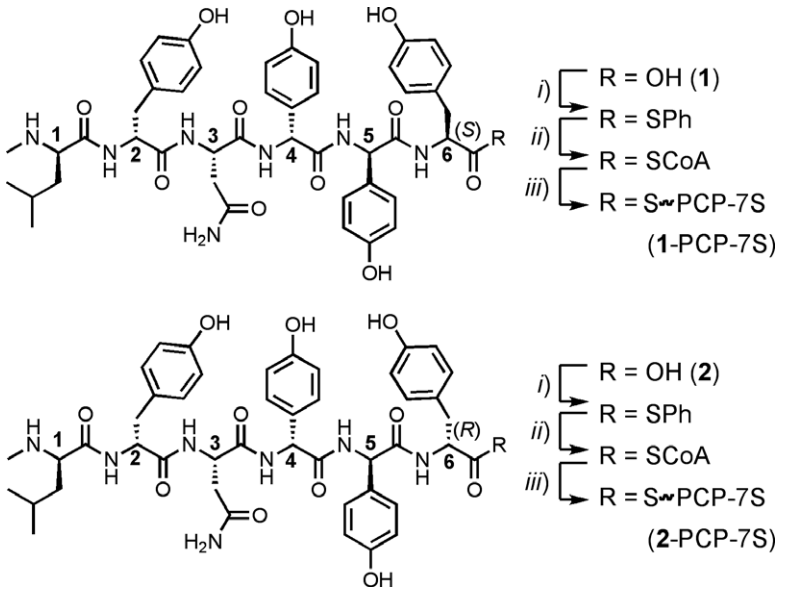


**Figure 2.** Activation of hexapeptides **1** and **2** as thioesters. Reagents and conditions: (i) peptide (1 equiv), PyBOP (1.2 equiv), *i*-$Pr_2EtN$ (1.2 equiv), PhSH (2.4 equiv), DMF, rt, 15 min; (ii) coenzyme A (4 equiv), phosphate buffer, pH 8.5, rt, 2 h; (iii) *apo*-PCP-7S, *B. subtilis* Sfp, $MgCl_2$, Tris–HCl buffer, pH 7.5, 37 °C, 30 min.

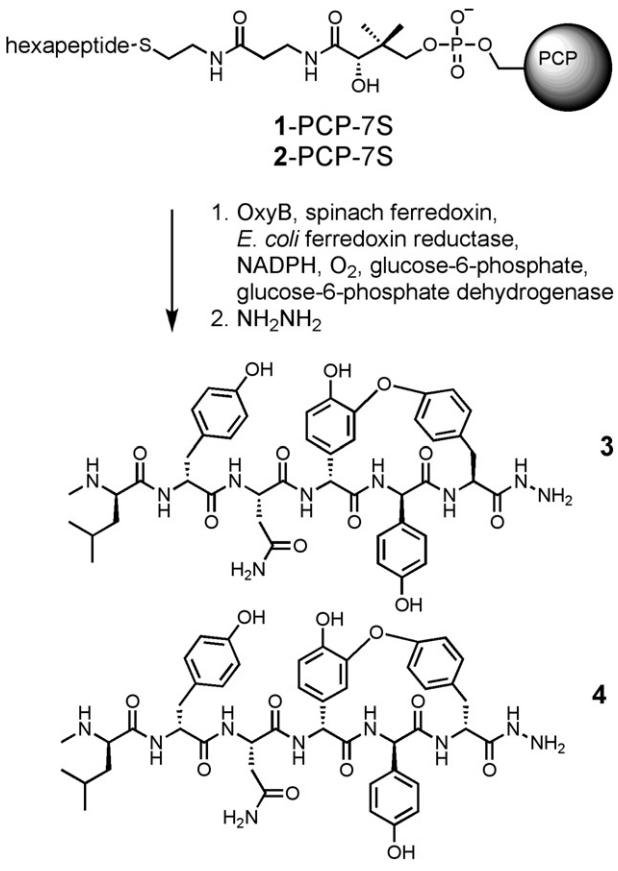


**Figure 3.** OxyB catalyzed conversion of the PCP-bound hexapeptides **1** and **2** into monocyclic products **3** and **4**.

to catalyze a comparable phenol coupling reaction on a model heptapeptide-PCP-7S conjugate, thus raising the possibility that OxyB may act upon both linear hexa- and heptapeptide intermediates whilst they are still attached to the NRPS assembly line.[7]

In this work, we report labeling studies using $^{18}O_2$ as the oxygen source for OxyB, in order to probe the mechanism of this oxidative coupling reaction. Furthermore, we describe the transformation by OxyB of an epimeric model hexapeptide (**2**) conjugated to PCP-7S, where (*S*)-tyrosine at position 6 has been replaced by (*R*)-tyrosine (Fig. 2).

Monooxygenase reactions catalyzed by P450 hemoproteins, such as the well-known camphor hydroxylase P450cam, not only require molecular oxygen they also usually incorporate one oxygen atom from molecular oxygen into the product.[8] Since, the incorporation of one oxygen atom from $O_2$ into the product cannot be ruled out a priori during the OxyB reaction, we set out to test this using O-18 labeled oxygen. We again used the model hexapeptide-PCP conjugate (**1**-PCP-7S) as substrate and a reduction system consisting of recombinant spinach ferredoxin, *Escherichia coli* flavodoxin reductase, and NADPH.[7] Additionally, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were used for NADPH regeneration. After incubation of these redox proteins and OxyB with the substrate and subsequent cleavage of the peptide products from the PCP with hydrazine (Fig. 3), the resulting peptide hydrazides were purified and analyzed by analytical HPLC. As reported earlier,[7] under the standard assay conditions, the substrate **1**-PCP-7S is converted almost quantitatively into the expected monocyclic product-hydrazide **3** as well as a minor monocyclic product-hydrazide, assigned as the Tyr6 epimer **4** (vide infra). For the $^{18}O$ labeling studies, the assay solutions were degassed by freeze-thaw cycles under vacuum, and the assay was performed in an anaerobic glove box. Under these conditions no

significant product formation was observed. Upon introduction of $^{18}O_2$ (95% $^{18}O_2$, Cambridge Isotope Laboratories Inc.), the catalytic activity of OxyB was restored. However, electrospray MS analysis of the isolated major monocyclic hexapeptide hydrazide **3** gave *m/z* 896 $[M+H]^+$, indicating that within the limits of detection no label from $^{18}O_2$ had been incorporated into the product.

The lack of $^{18}O$ incorporation during the phenol coupling reaction catalyzed by OxyB is of interest when considering possible mechanisms for this transformation. We have described evidence that binding of substrate to OxyB induces a spin-state shift toward the high spin Fe(III) form.[7] Since both electrons and molecular oxygen are required for catalysis by OxyB, then in analogy to the P450cam catalytic cycle, adding electrons and oxygen to the heme Fe in OxyB should lead to the so-called compound-0 intermediate (Fig. 4). Although there is no evidence so far that this intermediate is indeed formed on OxyB, it seems reasonable to suggest this. From here onwards, the mechanism of the oxidative coupling becomes unclear. Even in the case of the very well-studied P450cam, there is still no broad consensus on the reaction mechanism following the formation of compound-0.[9,10] However, should the OxyB mechanism involve oxygen atom transfer to one aromatic ring, to form an arene epoxide (Fig. 4), a type of process that is thought to occur (but has not been proven) during oxidation of aromatic hydrocarbons by P450 enzymes (e.g. in the liver),[8] followed by rearrangement to the macrocyclic product, then this must occur without retention of the oxygen atom derived from molecular oxygen. Clearly other pathways cannot presently be ruled out.

As mentioned above, the standard assay for OxyB affords mainly the expected monocyclic product, and also around 10–20% of a minor monocyclic product that we had earlier assigned as the Tyr6 epimer.[7] In order to

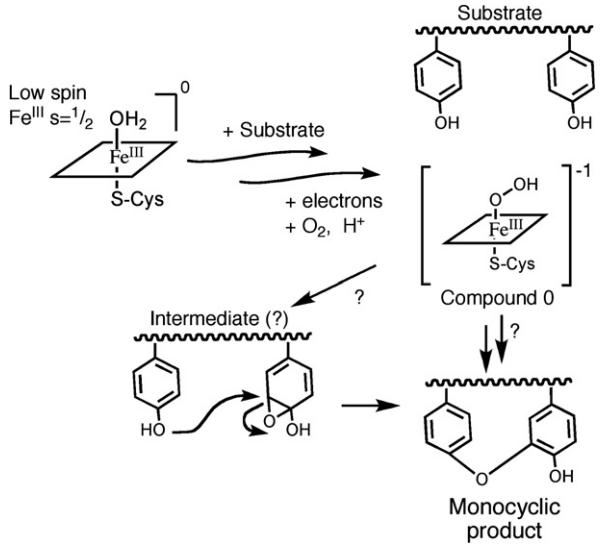


**Figure 4.** The OxyB catalytic cycle should proceed through a compound-0 like intermediate. The subsequent steps, however, remain to be established (see text).

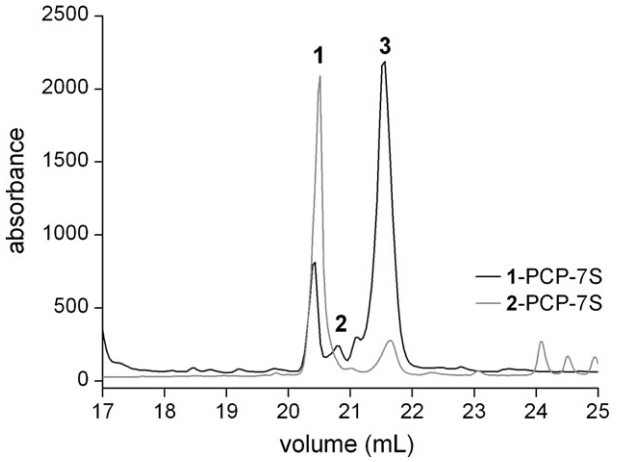


**Figure 5.** HPLC chromatograms showing peptide hydrazide products of OxyB reaction following hydrazine cleavage using **1**-PCP-7S (black) and **2**-PCP-7S (gray) as substrates. Peak 1, monocyclic product (**4**); peak 2, unreacted linear peptide; peak 3, monocyclic product (**3**) (see Fig. 3).

pursue this observation, we decided here to test directly as substrate the epimeric hexapeptide **2**, possessing (*R*)-Tyr instead of (*S*)-Tyr at position 6, linked as a thioester to PCP-7S. This hexapeptide was synthesized and converted into the corresponding PCP-7S conjugate by a standard procedure (Fig. 2).[7] OxyB assays using this substrate resulted in almost complete conversion into two products, one major and one minor, identified by HPLC analysis (Fig. 5). Interestingly, the two observed peaks exhibited identical retention times but inverse intensities to those observed when using the epimeric model hexapeptide **1**-PCP-7S as substrate. A full characterization by MS and NMR of the major and minor products obtained from transformation of peptide **2**-PCP-7S revealed that the expected C-*O*-D macrocycle had been formed in both. Moreover, $^1H$ 2D COSY, TOCSY, and NOESY spectra of these major and minor products (Fig. 5, peaks 1 and 3, respectively) proved that the minor monocyclic product (peak 3) of this conversion was identical to the major monocyclic hexapeptide hydrazide (**3**) derived from assays using **1**-PCP-7S. Additionally, the major monocyclic product (peak 1, Fig. 5) was identical to the minor monocyclic product (**4**) derived from **1**-PCP-7S.

The binding of the hexapeptide **2**-PCP-7S conjugate to OxyB was also monitored by UV–vis difference spectroscopy. Upon titration of OxyB with hexapeptide **2**-PCP-7S, the expected type I binding spectrum (Fig. 6) showing a peak at 392 nm, a trough at 427 nm and an isosbestic point at 413 nm, was observed. The concentration dependence of the changes in absorbance fitted well a binding equation describing a 1:1 interaction, with a $K_d$ of 35 ± 5 μM, indicating a slightly weaker interaction with OxyB compared to the standard hexapeptide conjugate (**1**-PCP-7S: $K_d$ = 17 ± 5 μM).[7] This binding behavior mirrors nicely the results from the OxyB activity assays, in which both substrates show excellent turnover into monocyclic products under otherwise comparable assay conditions.

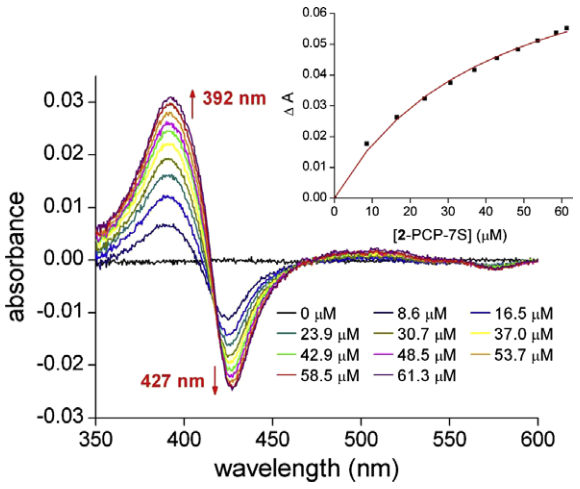


**Figure 6.** Difference UV–vis spectra of hexapeptide **2**-PCP-7S binding to OxyB. The concentration dependence of the spectral changes and the wavelengths of the minima and maxima are shown.

These complementary and interlocking results obtained with peptides **1** and **2** serve first to confirm the proposed structure of the minor product obtained from assays with the normal model hexapeptide **1**-PCP-7S, described earlier.[7] They also demonstrate, perhaps surprisingly, that the enzyme OxyB can transform efficiently the epimeric hexapeptide **2**-PCP-7S into a monocyclic product, apparently without the change in sense of chirality in residue-6 having a substantial effect on either binding, the shift in heme spin-state equilibrium, or turnover, compared to **1**-PCP-7S. Presently, it seems most likely that the minor epimers seen to arise in these assays are formed during the preparation of the peptide-PCP conjugates, and/or during peptide cleavage from the PCP using hydrazine. Finally, these results indicate that a more extensive study of the mechanism and substrate specificity of OxyB is warranted, not least since this catalyst might prove useful in the preparation of other related macrocyclic peptides.

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