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Oxidative Phenol Coupling Reactions Catalyzed by OxyB: A Cytochrome P450 from the Vancomycin Producing Organism. Implications for Vancomycin Biosynthesis

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Abstract: OxyB is a cytochrome P450 enzyme that catalyzes the first phenol coupling reaction during the biosynthesis of vancomycin-like glycopeptide antibiotics. The phenol coupling reaction occurs on a linear peptide intermediate linked as a C-terminal thioester to a peptide carrier protein (PCP) domain within the multidomain glycopeptide nonribosomal peptide synthetase (NRPS). Using model peptides with the sequence (*R*)(NMe)Leu-(*R*)Tyr-(*S*)Asn-(*R*)Hpg-(*R*)Hpg-(*S*)Tyr-S-PCP and (*R*)(NMe)Leu-(*R*)Tyr-(*S*)Asn-(*R*)-Hpg-(*R*)Hpg-(*S*)Tyr-(*S*)Dpg-S-PCP (where Hpg = 4-hydroxyphenylglycine, and Dpg = 3,5-dihydroxyphenylglycine), or containing (*R*)Leu instead of (*R*)(NMe)Leu, attached to recombinant PCPs derived from modules-6 and -7 in the vancomycin NRPS, we show that cross-linking of Hpg4 and Tyr6 by OxyB can occur in both hexapeptide− and heptapeptide−PCP conjugates. Thus, whereas OxyB may act preferentially on a hexapeptide still linked to the PCP-6 in NRPS subunit-2, it is possible that a linear heptapeptide intermediate linked to PCP-7 in NRPS subunit-3 may also be transformed into monocyclic product. For turnover, OxyB requires electrons, which *in vitro* can be supplied by spinach ferredoxin and *E. coli* flavodoxin reductase. Turnover is also dependent upon the presence of molecular oxygen. The model substrate (*R*)-(NMe)Leu-(*R*)Tyr-(*S*)Asn-(*R*)Hpg-(*R*)Hpg-(*S*)Tyr-S-PCP is transformed into cross-linked product by OxyB with a k_{cat} of 0.1 s^{-1} and K_m in the range 4−13 μM. Equilibrium binding of this substrate to OxyB, monitored by UV−vis, is accompanied by a typical low-to-high spin state change in the heme, characterized with a K_d of 17 ± 5 μM.

Introduction

The glycopeptide antibiotics vancomycin, balhimycin, and chloroeremomycin comprise a common heptapeptide aglycone backbone (Figure 1). The biosynthesis of this heptapeptide takes place according to the thiotemplate model of peptide assembly,[1] catalyzed by three large nonribosomal peptide synthetase (NRPS) subunits.[2,3] The first two subunits contain six modules that should generate a linear hexapeptide intermediate (**1**), still linked as a thioester to a peptide carrier protein (PCP) domain of the NRPS (Figure 2). This hexapeptide should then be transferred to the third NRPS subunit, where the seventh amino acid is added, to afford (assuming no further modification) the heptapeptide **2**.

Vancomycin aglycone also contains three cross-links between aromatic amino acid side chains.[4] Gene knock-out experiments identified the three gene products (OxyA, OxyB, and OxyC) responsible for these coupling reactions in balhimycin biosynthesis, as well as the order of the coupling steps.[5−8] The first coupling occurs between the phenol rings in residues-4 and -6, catalyzed by OxyB, the next is formed between the side chains of residues-2 and -4, catalyzed by OxyA, and the last coupling is between the phenol groups in residues-5 and -7, catalyzed by OxyC. The first coupling reaction, however, does not occur on a free linear heptapeptide.[9] Rather, *in vitro* assays only demonstrated turnover by OxyB with a linear peptide attached as a thioester to a PCP domain of the NRPS.[10] Thus, at least the first oxidative phenol-coupling reaction during vancomycin biosynthesis occurs on a peptide while it is still attached to the

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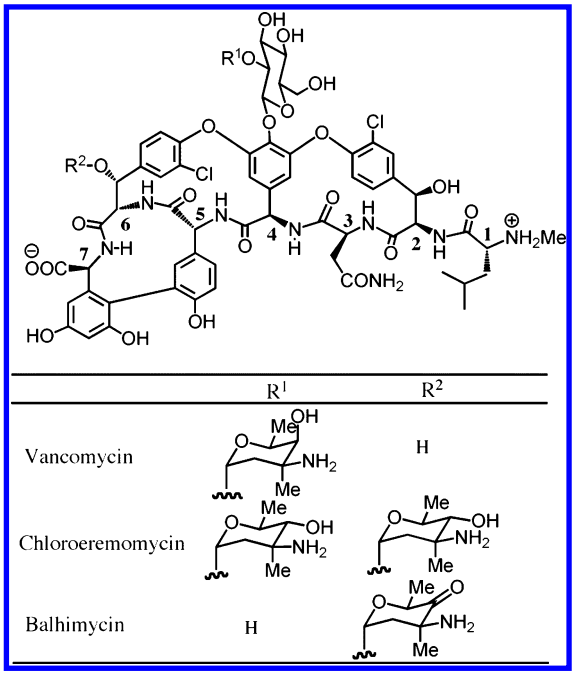

Figure 1. Structures of glycopeptides.

NRPS assembly line (Figure 2). No *in vitro* turnover has so far been reported with OxyA and OxyC, although it seems likely that the substrates for these cross-linking enzymes may also comprise NRPS-bound peptides.

A single halogenase introduces chlorine substituents in residues-2 and -6 during the process of peptide assembly.[11] Further gene knock-out studies suggest that the halogenase reactions also occur on peptidic intermediates still attached to the NRPS, although *in vitro* activity with the halogenase has so far not been reported. Conceivably, therefore, it might well be the finished vancomycin aglycone that is released from the NRPS through the action of a thioesterase (TE) domain situated at the C-terminus of NRPS subunit-3 (Figure 2). However, this remains to be proven.

The three cross-linking enzymes, OxyA−C, belong to the cytochrome P450 family of hemoproteins. The X-ray crystal structures of OxyB and OxyC from *Amycolatopsis orientalis* have been solved at 1.7 Å and 2.0 Å resolution, respectively.[9,12] Both exhibit the typical P450-fold, with a cysteine residue acting as proximal axial thiolate ligand for the heme. Heterologous complementation of Oxy-knockout mutants of the balhimycin producer *Amycolatopsis balhimycina* with the corresponding oxygenase genes (*oxy*A/B/C) from the vancomycin producer *A. orientalis* restored production of balhimycin, thus proving the functional equivalence of the vancomycin oxygenase genes used for *in vitro* assays, with those analyzed genetically in the balhimycin pathway.[13]

In vitro activity was demonstrated for OxyB using a model hexapeptide linked to a recombinant PCP domain (**3**) from module-6 in NRPS subunit-2 (Figure 2).[10] The product of this OxyB reaction (**5**) was isolated as the peptide hydrazide (**7**), after cleavage from the PCP with hydrazine (Figure 3). The hexapeptide conjugate **3** was used as a substrate in these assays, since it is synthetically easier to produce than is the hexa- or heptapeptide−PCP conjugates **1** and **2**. In this work, a more detailed study of the cross-linking reaction catalyzed by OxyB is reported. We show that OxyB can also turnover model heptapeptides linked as thioesters to a PCP, which has important implications for the timing of this cross-linking step during vancomycin biosynthesis. We report on the reduction system required to provide electrons to the P450, as well as kinetic studies with the model substrate **3**. Finally, we also show that substrate turnover by OxyB requires not only electron donors, but also molecular oxygen.

Results

Peptide Synthesis. The free peptides (**9**−**12**) were synthesized using an optimized solid-phase methodology reported earlier,[14] which makes use of temporary allyloxycarbonyl (Alloc) protection for the N(α)-amino groups, but avoids as far as possible the use of any acids or bases as well as side chain protecting groups (Figure 4). After assembly, the peptides were cleaved from 2-chlorotrityl chloride resin and purified by reverse phase HPLC (see Supporting Information).

For the synthesis of heptapeptides **11** and **12** racemic N(α)-Fmoc-3,5-dihydroxyphenylglycine (Dpg) was also used in peptide assembly, which leads in each case to a product containing a mixture of epimeric peptides, since in the course of loading onto the PCP, and also later during cleavage from the PCP with hydrazine (Figure 3), an epimerization of residue-7 could in any case not be avoided (*vide infra*). Each pair of epimers of **11** and **12** (as free C-terminal carboxylic acids) could not be resolved by analytical HPLC under the standard conditions used.

In order to couple each peptide (**9**−**12**) to a PCP, the C-terminus was activated and converted first into a *S*-phenyl thioester (Figure 5). Note, that at the stage of the C-terminal *S*-phenyl thioesters, it was possible to separate by HPLC and characterize by MS and NMR, each pair of epimers derived from **11** and **12**. At this stage the Boc group could be removed from **10** and **12** by careful treatment with TFA. Then by thioester exchange, the S-coenzyme-A (SCoA) thioesters were produced and characterized by MS and NMR (see Supporting Information). The SCoA thioesters were then used with recombinant phosphopantetheinyl transferase Sfp from *B. subtilis* to load the peptide-pantetheinyl portion onto the active site Ser residue of the *apo*-PCP domain.

PCP Domains. A PCP domain from module-6 of the vancomycin NRPS, comprising 30 residues on the N-terminal side and 52 residues on the C-terminal side of the active site Ser (i.e., 30-Ser-52 corresponding to residues 3955−4037 in VpsB), with an additional Cys-to-Ser mutation of residue 3960 near the N-terminus (called here *apo*-PCP-6S) was produced in the apo form as a His_6-tagged protein in *E. coli* as described earlier[10] (see Supporting Information).

The PCP domain from NRPS module-7 (residues 967−1043 in VpsC, or 37-Ser-39, called here *apo*-PCP-7S) was produced as a soluble N-terminal His_6-tagged fusion protein. An unpaired Cys residue near the N-terminus (residue 979) was also mutated to Ser, in order to avoid problems arising from disulfide-bridged

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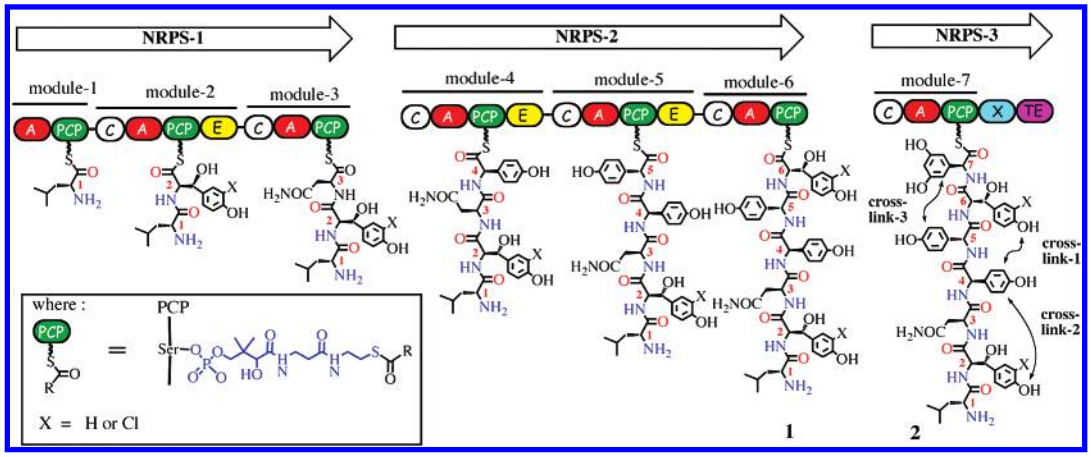

Figure 2. Schematic representation of the three subunits of the vancomycin NRPS. Illustrated are the activation (A), condensation (C), epimerization (E), peptide carrier protein (PCP), and thioesterase (TE) domains, as well as a further domain of unknown function (X). The PCPs are post-translationally modified by phosphopantetheinylation. Peptide intermediates likely to be bound to the PCPs at various stages of assembly are also shown.

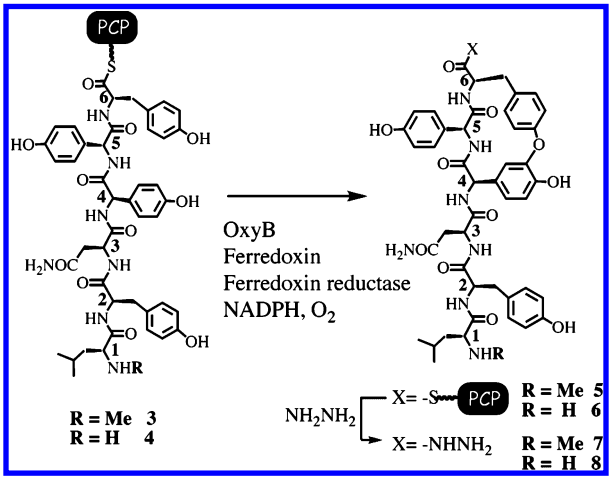

Figure 3. Conversion of a PCP-bound hexapeptide into a monocyclic product by OxyB.[10]

dimer formation. The CD spectra of *apo*-PCP-6S and *apo*-PCP-7S, as well as the peptide−PCP-7S conjugate (**3**), are shown in Figure S8 (Supporting Information).

Both *apo*-PCP-6S and *apo*-PCP-7S could be loaded efficiently with a peptide-phosphopantetheinyl group after incubation with a peptide-SCoA thioester and the recombinant phosphopantetheinyl transferase Sfp from *B. subtilis*.[15] Each peptide-loaded PCP (**3**, **4**, **13**, or **14**) was isolated in ca. 95% purity, as analyzed by reverse phase HPLC, and each gave a molecular ion by MS consistent with the expected mass (see Experimental Section).

Reduction System. Spinach ferredoxin (sp-ferredoxin) was produced as an N-terminal His_6-tagged protein in *E. coli* and purified using metal ion affinity chromatography. The molecular weight of the protein determined by ESI-MS corresponded to the calculated mass of the His_6-tagged protein with one [2Fe-2S] cluster, and showed UV−vis absorption maxima at 328, 420, and 463 nm (see Supporting Information). The spinach ferredoxin reductase used was of commercial origin (Sigma). The *E. coli* flavodoxin (eco-Flv) and flavodoxin reductase (eco-FlvR) were also prepared as soluble N-terminal His_6-tagged proteins in *E coli* and were purified and characterized in the same way (see Supporting Information).

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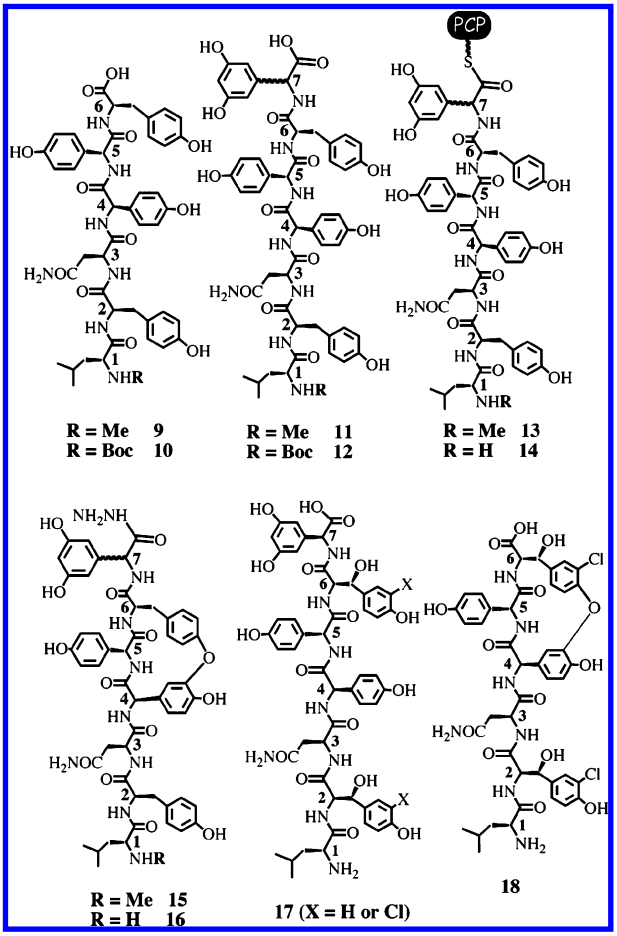

Figure 4. Model hexapeptides and heptapeptides studied in this work.

In assays with the peptide−PCP-7S construct **3**, conversion to monocyclic product (**5**) was consistently more efficient using sp-ferredoxin and spinach ferredoxin reductase than with eco-Flv/eco-FlvR. It was also observed that excellent turnover could be achieved using sp-ferredoxin and eco-FlvR, with the added advantage that both these proteins could be easily produced, purified, and characterized (see above). All assays described below, therefore, used the sp-ferredoxin/eco-FlvR pair.

Assays with OxyB. Assay conditions were first established using as substrate the hexapeptide−PCP-6S conjugate (**3**). After incubation of **3** with OxyB, sp-ferredoxin, eco-FlvR, and

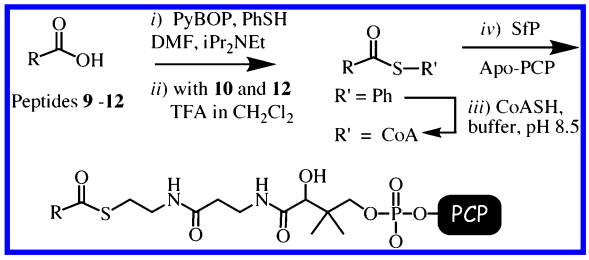

Figure 5. Activation of peptides as thioesters.

NADPH with glucose-6-phosphate and glucose-6-phosphate dehydrogenase, the peptidic products were cleaved from the PCP domain by treatment with hydrazine, and the corresponding peptide hydrazides were purified by solid-phase extraction and reverse-phase HPLC. In this way, the monocyclic product **7** could be isolated and fully characterized by MS and NMR, as described earlier.[10] In order to confirm the proposed structure, which so far had been deduced by spectroscopic methods, the monocyclic peptide (**7**) was also synthesized using established methods, as described in the Supporting Information. The synthetic material and product from the enzymatic assays were then compared by high field (600 MHz) ^{1}H NMR and ESI-MS and MS/MS analysis, which confirmed both the connectivity and the configuration of the enzymatic product.

Closer analysis of the products isolated from the enzymatic assays, however, consistently revealed in HPLC chromatograms, apart from unreacted linear peptide and the expected monocyclic product **7**, a second minor peak (ca. 10−20% of the major product) with a shorter retention time (Figure S3, Supporting Information), which by MS exhibited the same mass as the monocyclic product (**7**), and by 1D and 2D ^{1}H NMR and ESI-MS/MS fragmentation analysis the same overall connectivity. It seems most likely that this minor product is an epimer of peptide **7**. It is noteable from the NMR assignments of the major and minor products (see Supporting Information), that the chemical shifts of corresponding protons within each residue in each peptide are the same to within ±0.05 ppm, with the exception only of protons in Hpg-4 and Tyr-6, where significant differences (0.3−1.1 ppm) are seen between the major and minor forms. Hence, it seems most likely that the minor isomer arises due to an epimerization at either Tyr-6 C(α) or at Hpg-4 C(α), at some point during the assay procedure.

The time-course of the OxyB reaction was followed by HPLC analysis of peptide hydrazide formation. Thus, it was consistently found that peptides loaded onto PCP-7S were significantly better substrates for OxyB than the corresponding PCP-6S conjugates; for example, under conditions where **9** loaded on PCP-6S was typically converted 50% into monocyclic product, **9** loaded on PCP-7S would be converted 90% into the same monocyclic product. Therefore, more detailed analyses were performed with peptides loaded on PCP-7S.

Using the hexapeptide−PCP-7S (**3**) as substrate, when any one of the sp-ferredoxin, eco-FlvR, NADPH and regeneration system, or OxyB was absent from the assay, no conversion of the substrate into monocyclic product was observed (see Figure S5, Supporting Information). Hence, we conclude that the OxyB reaction is strictly dependent upon the presence of all four components. We also performed assays with all these components present, but under deoxygenated conditions. Again, no monocyclic product was formed, showing that oxygen is required for turnover (see Figure S6, Supporting Information).

Substrate Specificity of OxyB. We then tested the hexapeptide−PCP-7S conjugate **4** having an unmethylated N-terminus. Under standard conditions with OxyB, complete conversion of the peptide to monocyclic products was observed (see Supporting Information for analytical data) again with the major product being **8** and a second minor cyclic product (ca. 10%) corresponding to an artefactual epimeric form, as seen during assays with **3**. We conclude that both **3** and **4** are converted into monocyclic products (**5** and **6**), with comparable rates, indicating that the presence or absence of an *N*-methyl group in (*R*)Leu-1 has no significant influence on these OxyB reactions.

We next tested the two heptapeptides **13** and **14**, each linked to PCP-7S, as substrates for OxyB. These assays were performed with a peptide comprising a mixture of two epimers at the Dpg-7 C(α) position. This was unavoidable, since during the conversion of each C-terminal *S*-phenyl thioester to the SCoA thioester (Figure 5), an epimerization in Dpg-7 occurred, and the resulting epimers could not be resolved by HPLC. After incubation with OxyB and the redox proteins and cofactors (see above), the peptidic products were again cleaved from the PCP with hydrazine and then analyzed by HPLC. Typically, starting from **13** or **14**, four peaks could be identified, corresponding to two epimeric linear peptides having the same mass by ESI-MS but differing ^{1}H NMR spectra, and two epimeric monocyclic peptides (**15** and **16**) (Figure S4, Supporting Information). These cross-linked products were purified and characterized by MS and NMR, which confirmed the formation of the macrocycle linking the aromatic rings in Hpg-4 and Tyr-6. This shows that OxyB is also able to transform heptapeptides (**13** and **14**) linked to PCP-7S into the expected cross-linked products.

The model hexapeptide (**9**) was also assayed without modification, as a free acid, as an *N*-acetylcysteamine *S*-thioester (SNAC), and as the SCoA thioester. Under standard conditions, where the hexapeptide loaded on PCP-7S (**3**) would be converted to the extent of 90% into monocyclic product within 10 min, it was possible to detect formation of small amounts of monocyclic products from these other derivatives after 60 min incubation. Thus, the SCoA thioester was converted to the extent of ca. 40−50% into the monocyclic product, the SNAC thioester to about 15−20%, and even the free carboxylic acid form **9** was transformed to the extent of 5−10% into a monocyclic product identified by HPLC and showing the expected high-resolution mass by ESI-MS (data not shown). Insufficient amounts of this last material were isolated for a full NMR analysis, but fragmentation patterns in MS/MS provide strong support for the proposed formation of a cross-link between rings in Hpg-4 and Tyr-6. These results confirm that the PCP domain in the substrate **3** contributes substantially to the efficiency of the phenol coupling reaction catalyzed by OxyB, although small amounts of cross-linked products are detectable, under these optimized conditions, with the SCoA and SNAC thioesters, and even with the peptide **9** as a C-terminal free acid.

Kinetic Studies. The steady-state kinetics of the OxyB reaction with peptide−PCP-7S **3** were investigated to determine K_m and k_{cat}. To avoid effects due to uncoupling of NADPH consumption from product formation, NADPH consumption was

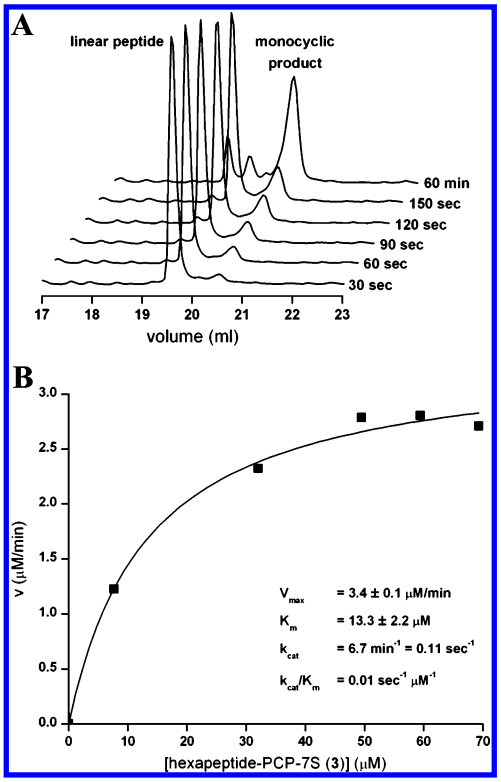

Figure 6. (A) Monitoring the rate of substrate (**3**) turnover. HPLC chromatograms obtained after 30, 60, 90, 120, 150 s and 60 min (see Experimental Section). (B) Rate vs substrate concentration with hexapeptide PCP-7S (**3**).

not used to monitor reaction rates, but rather a direct measurement of turnover rate was established based on HPLC quantitation of product formation (Figure 6A). The dependence of sp-ferredoxin and eco-FvR concentrations on the reaction rate under standard conditions were also investigated, to ensure that the concentrations of each used were high enough to saturate OxyB during the first few minutes of the reaction. In this way, an apparent K_m in the range 4−13 μM (three independent determinations) and $k_{cat} = 0.1 \pm 0.02$ s^{-1} were found (Figure 6B).

Binding Studies by UV−vis. The binding of hexapeptide−PCP-7S conjugate (**3**) to OxyB was monitored by UV−vis spectroscopy. As reported earlier, OxyB displays a Soret band at 419 nm and β and α peaks at 537 and 571 nm, respectively, typical for those of a low spin cytochrome P450. Upon addition of hexapeptide loaded on PCP-7S (**3**) to OxyB, the Soret band is shifted to lower wavelength, as detected in a UV-difference spectrum by a positive band at 391 nm and a trough at 426 nm (Figure 7A). The concentration dependence of the peak intensities fitted well a binding equation involving a 1:1 interaction, with a K_d of 17 ± 5 μM. These data are consistent with a typical type-I ligand (substrate), which upon binding displaces the water molecule as axial Fe ligand and causes a shift in the equilibrium spin state from hexacoordinate low spin toward pentacoordinate high spin. The hexapeptide **10** loaded on PCP-7S behaved in a similar way, with a K_d of 18 ± 5 μM (data not shown).

Similar studies using hexapeptide loaded on PCP-6S (**3**) produced changes in the UV difference spectra, which were significantly different (Figure 7B). Thus the positions of the maximum (410 nm) and trough (435 nm) are shifted compared

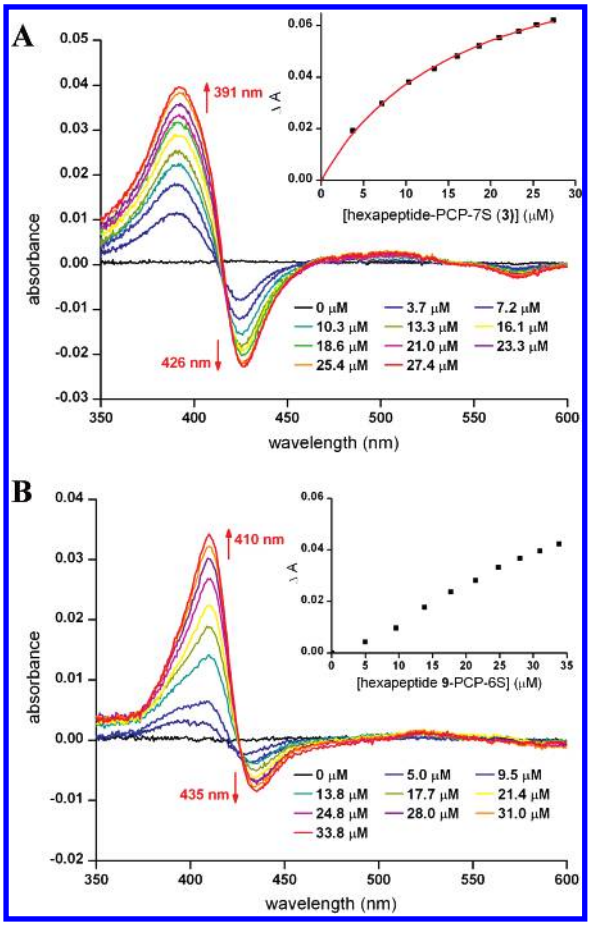

Figure 7. UV-Difference spectra of (A) hexapeptide PCP-7S (**3**) binding to OxyB, and (B) hexapeptide PCP-6S (**3**) binding to OxyB. The concentration dependence of the spectral changes and the wavelengths of the minima and maxima are shown.

to **3** on PCP-7S, the relative magnitude of the trough is much reduced, and the concentration dependence of the changes did not show saturation behavior in the range studied, suggesting that the K_d may be much higher. Similar UV/vis spectra were obtained when OxyB was titrated with the heptapeptide **14** loaded on PCP-7S (data not shown), although here the peptide comprised a mixture of two diastereomers (see above).

Discussion

The OxyB reaction belongs to one of only a handful of oxidative phenol couplings catalyzed by specific cytochrome P450 hemoproteins, which to date have been characterized biochemically. Other examples include P450s involved in plant alkaloid biosynthesis,[16−19] a *Streptomyces coelicolor* flaviolin oxidase,[20] a *Streptomyces griseus* tetrahydroxynaphthalene oxidase,[21] and an aryl−aryl coupling enzyme involved in staurosporine biosynthesis.[22]

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At the outset of our work, the preferred substrate of OxyB was unknown, although attempts to detect turnover using the free heptapeptides **17** (derived from **2**, Figure 2**)** had proven unsuccessful.[9] In more recent gene knock-out experiments, a *bps*C mutant of the balhimycin producer *A. balhimycina* (i.e., a mutated condensing domain in module-7 of NRPS subunit-3 (BpsC)) was found to produce the monocyclic hexapeptide **18**,[5] showing that the first cross-link could be introduced *in vivo* at the hexapeptide stage. Hence, attention turned to assays with hexa- and heptapeptides loaded as thioesters on PCP domains from the NRPS. For reasons of their easier synthesis, however, we chose to focus first on peptide analogues containing tyrosine at positions-2 and -6, i.e., hexapeptide−PCP conjugates **3** and **4**, and heptapeptide−PCP conjugates **13** and **14**.

The free linear peptides required for this work comprised the model hexapeptides **9** and **10**, and heptapeptides **11** and **12** (Figures 3 and 4) that could each be assayed as the corresponding peptide−PCP thioesters **3**, **4**, **13**, and **14**. The N-terminus of **3** and **9** contains (*R*)-*N*-methylleucine. N-Methylation actually occurs at a later stage in glycopeptide biosynthesis, after peptide assembly and cross-linking.[23] Our first experiments were performed with these N-methylated peptides, since it was expected that a secondary amine might be more stable in the presence of the C-terminal thioester group,[24] yet the peptides might nevertheless be acceptable substrates for OxyB. These assumptions were shown to be valid in the course of this work. Subsequently, a method for the preparation of the non-N-methylated peptides (**4** and **14**) was established, employing a temporary Boc-N-protecting group (peptides **10** and **12**), which could be removed with TFA after activation of the C-terminus as a *S*-phenyl thioester (Figure 5). Conversion into the PCP-derivatives was then achieved using the SCoA derivatives and the phosphopantetheinyl transferase Sfp from *Bacillus subtilis*.[15,24]

The PCP domains used here were derived from the vancomycin NRPS modules-6 and -7. So far no structural information is available on the vancomycin NRPS. Nevertheless, the 3D structure of a typical NRPS PCP domain, e.g., TycC3 from the tyrocidine NRPS (PDB files 2GDW/2GDX/2GDY), comprises a compact four-helix bundle,[25] with the phosphopantetheinylated active site Ser residue located in a loop connecting helix-1 and helix-2. The minimal four-helix bundle includes about 32 residues on the N-terminal side and 38 residues on the C-terminal side of the active site Ser (denoted here 32-Ser-38). We, therefore, first attempted to produce recombinant N-terminal His_6-tagged *apo*-PCP domains encompassing residues 30-Ser-40, as well as 37-Ser-40, from module-6 of the vancomycin NRPS (Figure 2). However, both were insoluble when produced in the cytoplasm of *E. coli*. On the other hand, a 30-Ser-52 construct (PCP-6S) could be produced in a soluble form and was easily purified as described earlier.[10] In addition, the PCP domain from NRPS module-7 (37-Ser-39, called PCP-7S) could be produced in the same way as a soluble N-terminal His_6-tagged fusion protein.

Interestingly, the CD spectrum of the *apo*-PCP-7S showed minima at 208 and 222 nm, with a shape suggesting that the protein was folded with significant helical content, consistent with the expected four-helix bundle fold (Figure S8, Supporting Information).[26] The CD spectrum of *apo*-PCP-6S, however, showed a much more intense minimum at 202 nm, suggesting either that a significant proportion of the protein may be unfolded, or that the global fold includes a much larger proportion of disordered regions.[27,28] Certainly, the CD signatures suggest a significant difference between the structures of *apo*-PCP-6S and *apo*-PCP-7S, which is of interest here in the light of binding studies with the peptide-loaded PCPs and OxyB (see later).

Bacterial class-I P450s typically require the presence of a ferredoxin-like [Fe−S]-protein and a flavin-containing ferredoxin reductase for substrate turnover, which receive reducing equivalents from NADPH. The class-II P450s, on the other hand, usually receive electrons from NADPH through a flavin-containing reductase.[29−31] Within the published glycopeptide biosynthetic gene clusters no ferredoxin or ferredoxin reductase has been identified,[3,32−36] with the exception of a single ferredoxin in the complestatin cluster.[32] So the natural redox partners for the biosynthetic cross-linking enzymes are mostly unknown. In assays with the peptide **9** loaded on PCP-7S (**3**), the sp-ferredoxin/eco-FlvR pair was used as electron-transfer proteins.

The conversion of hexapeptide−PCP-6S (**3**) into monocyclic product (**5**), isolated as the peptide hydrazide **7**, was reported earlier.[10] We could show here for the first time that the same hexapeptide loaded on PCP-7S is converted at a significantly faster rate into the monocyclic product **5**. This observation is interesting, on the one hand, because in UV−vis binding assays we could also show that the interaction of hexapeptide−PCP-6S (**3**) with OxyB is substantially weaker than that of hexapeptide−PCP-7S (**3**) with OxyB (Figure 7). The binding data suggest a dissociation constant of hexapeptide−PCP-7S (**3**) with OxyB of about 17 μM. On the other hand, we have also seen that PCP-6S and PCP-7S have different CD signatures (Figure S8), with that of PCP-7S resembling the spectrum expected of a four-helix bundle PCP or acyl carrier protein (ACP),[26] and that of PCP-6S resembling more closely those of ACP domains

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in a partially unfolded state.[27,37] These differences between PCP-6S and PCP-7S suggest the importance of having a correctly folded PCP domain for optimum substrate recognition by OxyB. This conclusion is reinforced by the large decrease in turnover seen when the hexapeptide-SCoA and -SNAC thioesters, and even more pronounced, the hexapeptide free carboxylic acid, were tested as substrates. However, it is also relevant to note that during the biosynthesis of vancomycin *in vivo* a hexapeptide intermediate should only ever be bound to PCP-6 and not to PCP-7. At this time it is unclear which residues in the PCP are important for recognition by OxyB.

The phenol coupling reaction catalyzed by OxyB was shown here to depend upon the presence of the complete reduction system (ferredoxin, ferredoxin reductase (or equivalent), and NADPH) and molecular oxygen. When any one component was omitted, no significant turnover of substrate was observed.

OxyB assays (turnover and UV-binding) were also carried out with model heptapeptides bound to PCP-7S (**13** and **14**). These assays were complicated by the unavoidable use of heptapeptides that comprised a mixture of two diastereomers, epimeric at the Dpg-7 C(α) position. The results, however, clearly show that OxyB can catalyze a phenol coupling reaction on heptapeptide−PCP as well as on hexapeptide−PCP conjugates. The use of epimeric heptapeptides in these assays prevented more detailed kinetic studies, but the rates of conversion for hexa- and hepta-peptides appeared not to be vastly different.

These results have important implications in defining the timing of the phenol coupling reaction during vancomycin biosynthesis. Thus, whereas OxyB may function on a hexapeptide still linked to the PCP-6 in NRPS subunit-2, it seems possible that a linear heptapeptide intermediate linked to PCP-7 in NRPS subunit-3 may also be transformed into monocyclic product. Additional studies, in particular with the correct β-hydroxytyrosine-containing hexa- and heptapeptides, perhaps linked to larger, natively folded fragments of NRPS subunits-2 and -3, will be required to confirm or refine these conclusions. Peptides containing β-hydroxytyrosine residues will also be of interest, since in at least two other P450-catalyzed reactions,[38,39] hyroxyl groups in the substrate have been show to interact with and participate in the breakdown of iron-bound dioxygen during the P450 catalytic cycle.

Finally, with hexapeptide−PCP-7S (**3**) the steady-state kinetic parameters k_{cat} and K_m were measured, with the assumption that OxyB exhibits Michaelis−Menten kinetics under the conditions used. The k_{cat} of 0.1 s^{-1} found is comparable to turnover numbers determined for other cytochrome P450 enzymes,[29] and the K_m value (in the range 4−13 μM) is close to the K_d (17 μM) of hexapeptide−PCP-7S (**3**) binding to resting OxyB determined here by UV−vis spectroscopy (Figure 7A). These data should provide a useful benchmark for more detailed studies in the future with other substrate analogues.

The results presented here show that OxyB has spectroscopic properties and substrate requirements (electron-transfer proteins, molecular oxygen) that mirror those seen in many other P450 enzymes. The crystal structure of substrate-free OxyB revealed a typical P450-fold, with the L-helix containing the Cys^{347} residue that provides the axial thiolate ligand of the heme iron. Moreover, the F and G helices are rotated out of the active site compared to other closely related P450s, such as P450nor, creating a much more open active site, most likely for binding the large peptide−PCP substrate.[9]

The UV−vis changes observed upon titrating the protein with hexapeptide−PCP-7S (**3**), in particular the shift of the Soret band to 391 nm (Figure 7), are consistent with the displacement of the axial water ligand and a low- to high-spin conversion, as seen after substrate binding to other P450s such as P450cam.[29] Following substrate binding to OxyB, the reaction mechanism most likely involves first electron transfer from the ferredoxin to the heme-Fe(III), and subsequent binding of molecular oxygen (Figure 8). After a second electron transfer from the ferredoxin, cleavage of the Fe-bound dioxygen should occur with loss of a water molecule and formation of compound-I, a Fe(IV)═O porphyrin-π-radical cation.[29]

In the catalytic cycle of a typical P450 hydroxylase like P450cam, this intermediate oxidizes an aliphatic substrate, first by hydrogen atom abstraction and then by hydroxyl radical rebound from the heme to the substrate.[40] However, it now seems clear with several P450 hydroxylases, including P450cam, that a competing intramolecular electron transfer can occur, from tyrosine and/or tryptophan residues to the transient Fe(IV)═O porphyrin-π-radical cation, to form protein-based tyrosyl or tryptophan radicals[41−43] and a new Fe═OH species, called compound-II. Conceivably, a similar electron transfer might occur during the OxyB reaction, except now the electron is transferred from a phenol group provided by the bound peptide−PCP substrate, and the process is not a competing side reaction but part of the normal catalytic cycle of the enzyme. This would leave the second electron-transfer step, from the second phenol group in the peptide substrate to compound-II, to afford a substrate biradical, which could collapse by radical coupling and deprotonation to give the product. A similar process is thought to occur during peroxidase-mediated phenol coupling reactions.[44] Presently, however, alternative mechanisms cannot be excluded. One possibility is that compound-I might oxidize a phenol group in the substrate to form an arene oxide, a process that is thought to occur in P450s that catalyze the hydroxylation of aromatic groups.[45] Rather than a rearrangement of the arene oxide to an aromatic system, a nucleophilic attack by the second phenol ring in the substrate followed by elimination of water could lead to the desired product (Figure 8). Clearly, more experiments will be required to distinguish between these and other mechanisms.

Experimental Section

General Method for the Synthesis of Peptide *S*-Phenyl Thioesters. The peptide (**9**−**12**) (2 mg) in freshly distilled DMF (1 mL) was stirred with PyBOP (1.2 equiv), iPr_2EtN (1.2 equiv), and thiophenol (2.4 equiv)

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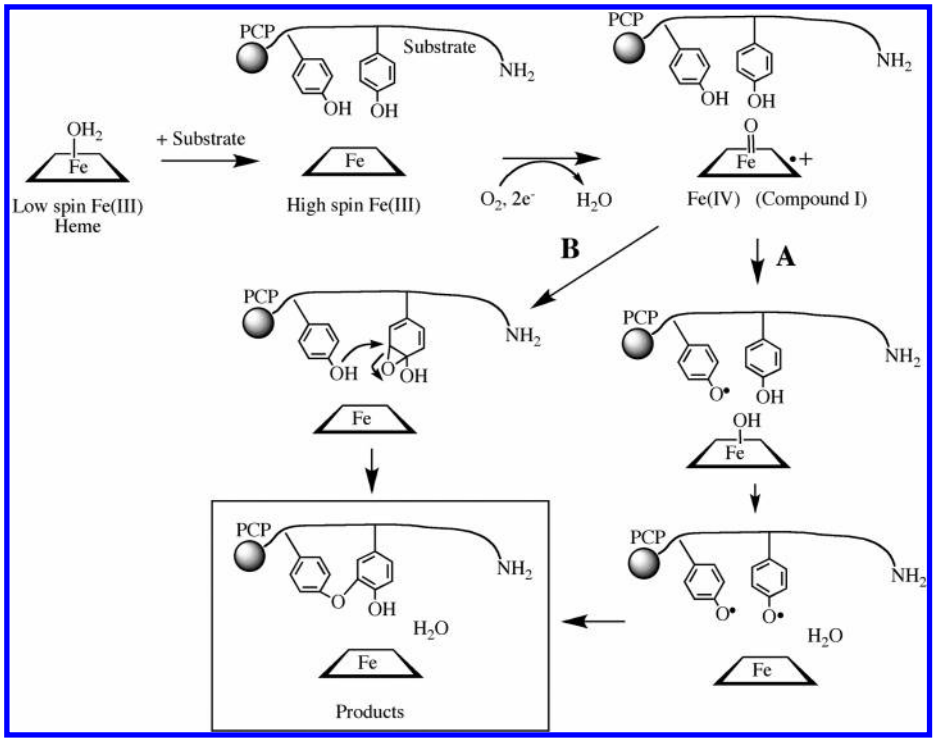

Figure 8. Putative mechanisms for the OxyB reaction (see text). After formation of compound-I, path-A leads via single electron transfers to a phenolic biradical, whereas path-B involves formation of an arene-oxide intermediate.

under N_2. The reaction was monitored by LC/MS and was typically complete after 5−15 min. After lyophilization, the product was purified by semipreparative reverse-phase HPLC (C_{18} Vydac 218TP1010, 250 × 10 mm, pore diameter 300 Å, particle size 5 μm) with a gradient of 5−100% MeCN/0.1% TFA in water/0.1% TFA in 5 column volumes at a flow rate of 5 mL/min. The corresponding peptide *S*-phenyl thioester was obtained in >90% yield. When the reaction was started from **10** and **12**, the Boc group was removed by stirring the peptide *S*-phenyl thioster (2 mg) in CH_2Cl_2/TFA (10:1, 2 mL) at rt for 2 h. After being dried in vacuo, the product was isolated by HPLC as above, in near quantitative yield. For all analytical data see Supporting Information.

General Method for the Synthesis of Peptide-SCoA Thioesters. The corresponding peptide-*S*-phenyl thioester (2 mg) and coenzyme A (4 equiv) were stirred in phosphate buffer (4 mL, 50 mM, pH 8.5). The reaction was complete after 15−120 min, as monitored by LC/MS, and the product was purified by semipreparative HPLC on a reverse-phase C_{18} column (Vydac 218TP1010, 250 × 10 mm, pore diameter 300 Å, particle size 5 μm) using a gradient of 45−100% MeCN in 20 mM NH_4AcO in 5 column volumes, with a flow rate of 5 mL/min, desalting: 25−40% MeCN/0.1% TFA in water/0.1% TFA in 5 column volumes at 5 mL/min. The corresponding peptide-SCoA thioesters were recovered in 40−60% yield. For characterization see Supporting Information.

General Method for the Synthesis of Peptide−PCP Conjugates. The reaction mixture contained the following components at the concentrations given: *apo*-PCP (120 μM), peptide-SCoA thioester (100 μM), *B. subtilis* Sfp (5 μM), and $MgCl_2$ (50 mM) in Tris/HCl buffer (50 mM, pH 7.5) and was incubated at 37 °C for 30 min. The reaction was monitored by analytical HPLC (Vydac C18 218TP54 column, 250 × 4.6 mm, pore diameter 300 Å, particle size 5 μm) using a gradient of 5−100% MeCN/0.1% TFA in water/0.1% TFA over 4 column volumes at 1 mL/min. The conversion proceeded quantitatively. Each peptide−PCP conjugate was collected from the HPLC column and analyzed by MALDI-MS; **3**−PCP-6S, *m*/*z* 11373 ± 2 $[M + H]^+$, calcd mass 11373; **3**−PCP-7S, *m*/*z* 11564 ± 2 $[M + H]^+$, calcd mass 11563; **4**−PCP-7S, *m*/*z* 11551 ± 2 $[M + H]^+$, calcd mass 11549; **13**−PCP-7S, *m*/*z* 11730 ± 2 $[M + H]^+$, calcd mass 11728; **14**−PCP-7S, *m*/*z* 11715 ± 2 $[M + H]^+$, calcd mass 11714.

Upon complete reaction, the mixture was dialyzed against HEPES buffer (25 mM, pH 7.0, 50 mM NaCl), and the resulting solution containing peptide−PCP conjugate could be used in assays without further purification.

For kinetic and UV binding studies, the peptide−PCP conjugate was first purified by anion exchange chromatography (MonoQ HR10/10, 20 mM diethanolamine, pH 8.5, using a gradient of 0 to 0.8 M KCl over 10 column volumes). The eluate was dialyzed against Tris-HCl buffer (50 mM, pH 7.5) and concentrated to 100−150 μM using an Amicon ultrafiltration cell containing a YM-3 membrane. The concentration of peptide−PCP was determined by UV using the following molar extinction coefficients: **3**−PCP-6S ϵ_{280} = 12810 cm^{-1} M^{-1}; **3**−PCP-7S ϵ_{280} = 7120 cm^{-1} M^{-1}; **13**−PCP-7S ϵ_{275} = 8868 cm^{-1} M^{-1}.

General Assay Procedure with OxyB. The assay contained the following components at the concentrations given: peptide−PCP-conjugate (80 μM), OxyB (8 μM), spinach ferredoxin (20 μM), *E.coli* flavodoxin reductase (10 μM), NADPH (1 mM), glucose-6-phosphate (1 mM), glucose-6-phosphate-dehydrogenase (0.5 U, *Sigma*) in HEPES buffer (25 mM, pH 7.0, 50 mM NaCl), incubated at 30 °C for 60 min. Addition of 1/10 volume of a 25% v/v aqueous hydrazine and incubation at 30 °C for 30 min yielded the peptide hydrazides, which were separated from proteins by solid-phase extraction (Waters OASIS HLB cartridges). The peptidic fraction was analyzed by analytical HPLC on a reverse-phase C_{18} Zorbax Eclipse XDB column (Agilent, 250 × 4.6 mm, pore diameter 80 Å, particle size 5 μm) under the following conditions: 5−40% acetonitrile/0.1% TFA in water/0.1% TFA in 8 column volumes with a flow rate of 1 mL/min.

Anaerobic assays were performed in a glovebox with oxygen levels below 2 ppm. All solutions were degassed in four freeze−thaw cycles. The His_6-OxyB activity assay was initiated by addition of NADPH, incubated at room temperature and quenched by addition of trichloroacetic acid (TCA) prior to removal from the glovebox.

Kinetic Studies. Assays containing peptide−PCP-conjugate (5−100 μM), spinach ferredoxin (20 μM), *E. coli* flavodoxin reductase (10 μM), glucose-6-phosphate (1 mM), and glucose-6-phosphate-dehydrogenase

(0.5 U, Sigma) in Tris-HCl buffer (1.2 mL, 50 mM, pH 7.5) were incubated at 30 °C. The reaction was initiated by addition of a mixture of His_6-OxyB (0.5 μM) and NADPH (1 mM). Samples (200 μL) were withdrawn at time intervals (t = 15, 30, 75, 60, 75, 90 s) and added to TCA (20 μL, 6 M). The pellet was washed with 10% (v/v) TCA in Tris-HCl (50 mM, pH 7.5), and resuspended in 2.5% (v/v) hydrazine in Tris-HCl (50 mM, pH 8.0). After 30 min at 30 °C, protein was precipitated with TCA. The supernatant containing the peptide hydrazides was analyzed by analytical reverse-phase HPLC (Agilent C_{18} Zorbax Eclipse XDB column, 250 × 4.6 mm, pore diameter 80 Å, particle size 5 μm) using a gradient of 5−40% MeCN/0.1% TFA in water/0.1% TFA in 8 column volumes, flow rate 1 mL/min. The peaks corresponding to the linear and monocyclic peptide hydrazides were integrated to give initial velocities, which were used with the Michaelis−Menten equation to derive the steady-state kinetic parameters K_m and V_{max}.

Binding Studies by UV. Absorbance spectra were recorded using a double beam Varian Cary300 spectrophotometer. OxyB (1.6 μM) in Tris-HCl (2.4 mL, 50 mM, pH 7.5) was divided between reference and sample cuvette. After thermal equilibration at 30 °C, a baseline was recorded between 600 and 350 nm, followed by sequential additions (25 μL) of concentrated substrate solution (200 μM substrate in 50 mM Tris-HCl pH 7.5, containing 1.6 μM OxyB to maintain a constant OxyB concentration) to the sample cuvette, with stirring, resulting in a final substrate concentration in the range 0−60 μM. UV-difference spectra were recorded after each addition. A maximal overall absorption change (A_{obs}) was taken from the absolute difference betwen the positive and negative peaks in each difference spectrum. This value is related to the total substrate concentration (S), the total enzyme concentration (E_t), and the K_d through the following eq 1 describing a 1:1 binding interaction:[46]

$$A_{obs} = \frac{A_{max}}{2[E_t]}[(S + E_t + K_d) - \sqrt{(S + E_t + K_d)^2 - (4SE_t)}] \quad (1)$$

A_{max} and K_d were obtained by nonlinear least-squares curve fitting of data using eq 1 and Origin software (Microcal).

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Supporting Information Available: Complete ref 20, synthesis and analytical data of the linear and cyclic peptides and all thioesters, production and characterization of all recombinant proteins, total synthesis of the monocyclic peptide **7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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