Exploring the substrate specificity of OxyB, a phenol coupling P450 enzyme involved in vancomycin biosynthesis†

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OxyB is a cytochrome P450 enzyme that catalyzes the first oxidative phenol coupling reaction during vancomycin biosynthesis. The preferred substrate is a linear peptide linked as a C-terminal thioester to a peptide carrier protein (PCP) domain of the glycopeptide antibiotic non-ribosomal peptide synthetase. Previous studies have shown that OxyB can efficiently oxidize a model hexapeptide–PCP conjugate (*R*-Leu[1]-*R*-Tyr[2]-*S*-Asn[3]-*R*-Hpg[4]-*R*-Hpg[5]-*S*-Tyr[6]-S-PCP) (Hpg = 4-hydroxyphenylglycine) into a macrocyclic product by phenolic coupling of the aromatic rings in residues-4 and -6. In this work, the substrate specificity of OxyB has been explored using a series of N-terminally truncated peptides related in sequence to this model hexapeptide–PCP conjugate. Deletion of one or three residues from the N-terminus afforded a penta- (Ac-Tyr-Asn-Hpg-Hpg-Tyr-S-PCP) and a tri- (Ac-Hpg-Hpg-Tyr-S-PCP) peptide that were also efficiently transformed into the corresponding macrocyclic cross-linked product by OxyB. The tripeptide, representing the core of the macrocycle in vancomycin created by OxyB, is thus sufficient, as a thioester with the PCP domain, for phenol coupling to occur. The related tetrapeptide–PCP thioester was not cyclized by OxyB, neither was a related model hexapeptide containing tryptophan in place of tyrosine-6, nor were tripeptides (related to the natural product K-13) with the sequence Ac-Tyr-Tyr-Tyr-S-PCP cross-linked by OxyB.

Introduction

Vancomycin, a glycopeptide produced by *Amycolatopsis orientalis*, is a clinically important antibiotic, which acts by inhibiting peptidoglycan biosynthesis in Gram-positive bacteria.[1] The vancomycin heptapeptide aglycone, which is also found in the related natural products balhimycin and chloroeremomycin (Fig. 1), contains several aromatic amino acid side chains oxidatively linked through biaryl or biaryl-ether bridges. The heptapeptide backbone itself is constructed by the actions of three non-ribosomal peptide synthetase (NRPS) subunits, whereas the oxidative cross-linking reactions are catalyzed in a defined order by three cytochrome P450 enzymes OxyA, OxyB and OxyC.[2–4] The first coupling occurs between the phenol rings in residues-4 and -6 (the C–*O*–D ring), catalyzed by OxyB, the second aryl-ether bridge is formed between side chains of residues-2 and -4 (D–*O*–E ring) by OxyA, and the last biaryl coupling between the aromatic side chains of residues-5 and -7 is carried out by OxyC (AB ring, Fig. 1). The crystal structures of OxyB (CYP165B1) and OxyC (CYP165C1) from the vancomycin producer *A. orientalis* have been reported, in substrate free form, confirming that these proteins contain a fold and heme environment typical of P450 enzymes.[5,6]

In molecular genetic studies, the inactivation of genes responsible for the biosynthesis of the seventh amino acid (3,5-dihydroxyphenylglycine, Dpg) (Δ-*dpgA*), or for its incorporation into the linear heptapeptide intermediate (Δ-*bpsC*), afforded mutants of the balhimycin producer still able to produce hexapeptides containing the C–*O*–D ring, suggesting indirectly that a linear hexapeptide may act as a substrate for OxyB.[7] Complementary *in vitro* studies, however, demonstrated that the first cross-linking reaction actually occurs when the linear peptide is attached as a thioester to a peptide carrier protein (PCP) domain in the NRPS assembly line.[8,9] Thus model linear hexa- and heptapeptides (**1** and **2**), comprising residues 1–6 and 1–7 of vancomycin aglycone but containing tyrosine at positions-2 and -6 instead of β-hydroxytyrosine derivatives, and linked as thioesters to a recombinant PCP domain from the NRPS, were transformed by OxyB into monocyclic cross-linked products (**3** and **4**) (Fig. 2). Catalytic activity with the remaining two coupling enzymes OxyA and OxyC has not yet been reported, but both are also thought to act upon peptidic intermediates attached as thioesters to the NRPS assembly line.

There is now great interest in exploring in more detail the substrate specificity of OxyB. In recent work, it was reported that OxyB can catalyze efficiently a phenol coupling reaction on an epimeric form of hexapeptide–PCP conjugate **1**, which contains an (*R*)-Tyr at position-6 instead of (*S*)-Tyr.[10] The product of this phenol coupling reaction, the corresponding epimeric form of monocyclic peptide **3**, had been observed in small amounts in earlier work,[9] after work-up of assays with peptide **1**; the partial epimerization of residue-6 in **1** is difficult to avoid completely and may occur during its synthesis and/or during work-up of enzyme assays (cleavage of the thioester with hydrazine), but it was a surprise to discover that the (*R*)-Tyr6-epimer is converted

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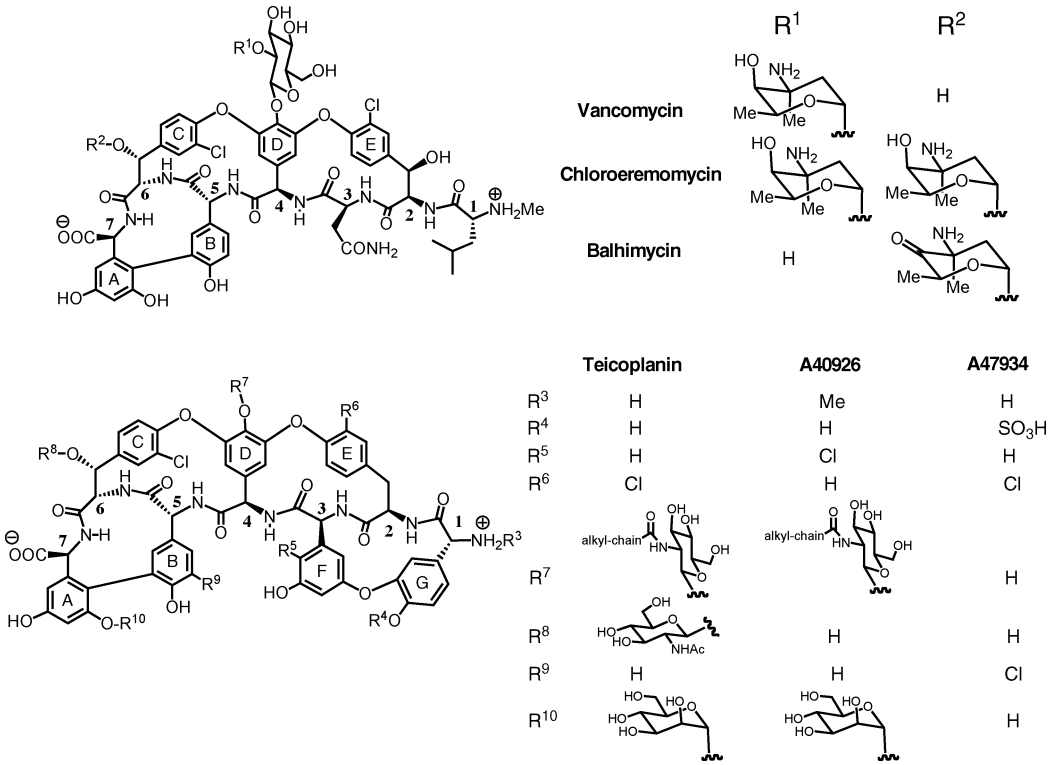

Fig. 1 Structures of vancomycin, chloroeremomycin, balhimycin, teicoplanin (alkyl chain varies), A40926 (alkyl chain varies) and A47934.

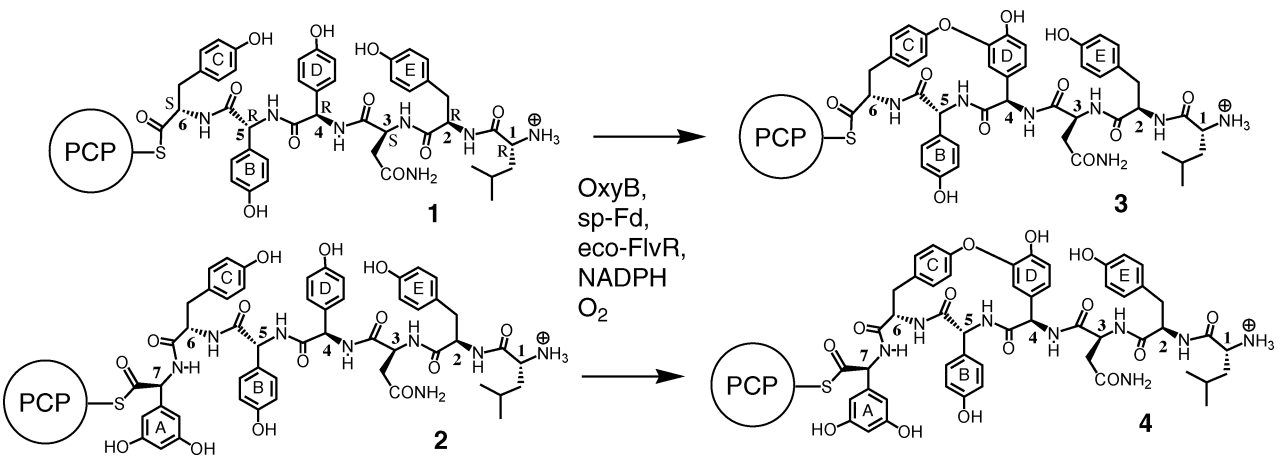

Fig. 2 Conversion of the model hexa- and heptapeptides **1** and **2** into the monocyclic products (**3** and **4**) by OxyB and ancillary electron transfer proteins.[9]

rapidly into monocyclic product by OxyB under the *in vitro* assay conditions.

Here we report more extensive studies of the substrate specificity of OxyB, with peptide analogues having stepwise deletions of residues from the N-terminus of hexapeptide **1** (Fig. 3). We show that several of the derived penta-, tetra-, and tripeptides (all as PCP thioester conjugates) are still accepted and transformed efficiently into monocyclic products by OxyB. On the other hand, we also describe other peptide–PCP conjugates, with a substantially different backbone, either containing tryptophan, or being related to that in the natural product K-13,[11] which are not transformed by OxyB under the *in vitro* assay conditions.

Results

Peptide synthesis

The linear peptides **5A–10A** were synthesized using an optimized solid-phase methodology reported earlier.[12] This involved the use of N(α)-Alloc protection for each amino acid, but avoided as far as possible any side chain protecting groups, in order to avoid

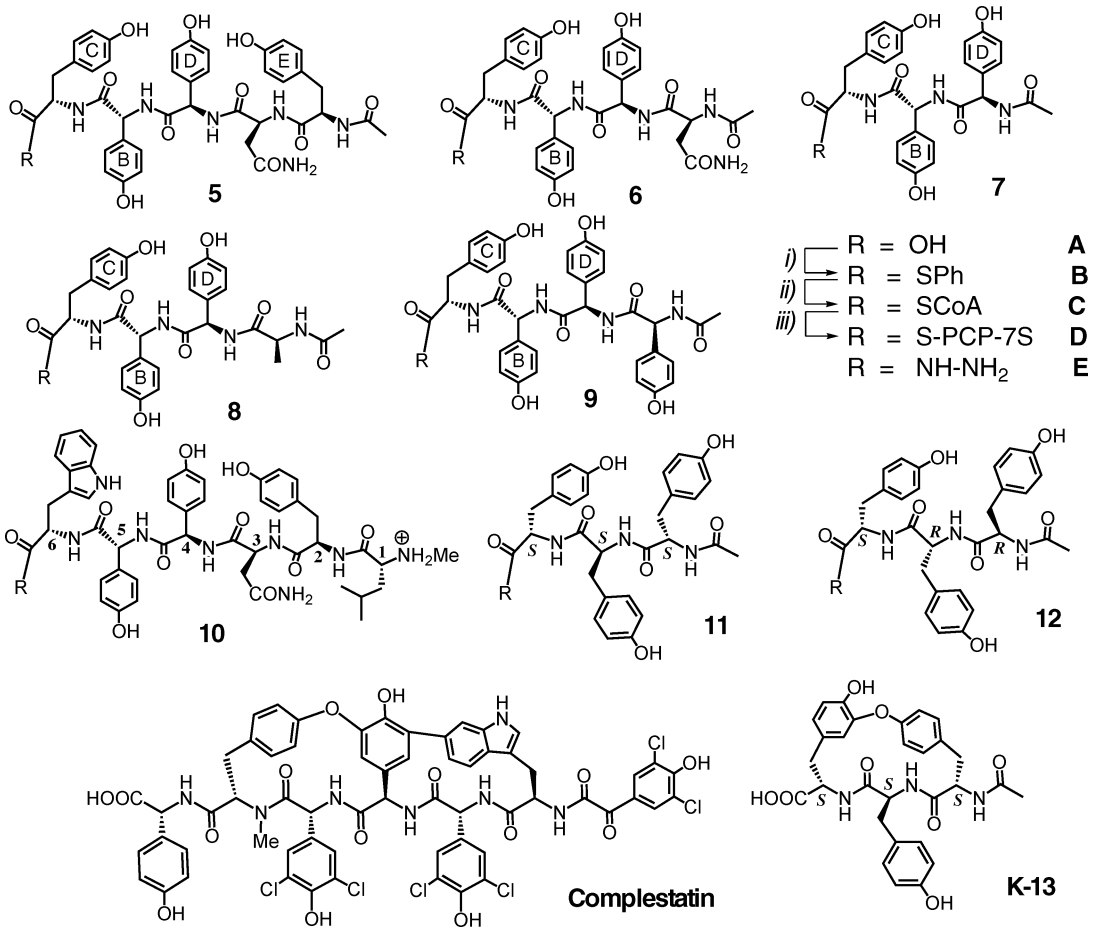

Fig. 3 Structures of complestatin and K-13 as well as the substrates (**5–12**) used in this study and their corresponding thioesters. *Reagents and conditions*: *i*) thiophenol, PyBOP, DIEA in DMF, 15 min, rt; *ii*) coenzyme-A, phosphate buffer, pH 8.5, 2 h, rt; *iii*) *apo*-PCP-7S, *B. subtilis* Sfp, $MgCl_2$, HEPES buffer, pH 7.0, 1 h, rt.

any deprotection steps requiring strong acid (as is usual in the Boc and Fmoc solid-phase methods) at the end of each synthesis. However, the tripeptides **11A** and **12A** were made using standard solid-phase Fmoc-chemistry, since these do not contain sensitive phenylglycine derivatives.

In order to couple each peptide to a PCP, the peptide was first converted into a S-phenyl thioester (**5B–12B**) and then transformed by thioester exchange into the corresponding coenzyme-A thioester (**5C–12C**), using methods reported earlier.[8,9,13] Each peptide thioester was characterized by MS and NMR spectroscopy. The peptide-SCoA thioesters were then used together with the pantetheinyl transferase Sfp from *Bacillus subtilis*,[14] and the recombinant *apo*-PCP-7S described earlier,[9] derived from module-7 of the vancomycin NRPS, to prepare the PCP conjugates **5D–12D** (Fig. 3). These peptide–PCP-7S conjugates were isolated in >95% purity, as analyzed by reverse phase HPLC, and gave the expected molecular ion by MALDI-MS.

Assays with OxyB

OxyB assays were carried out under the standard conditions reported earlier.[9] Present in the assay are spinach ferredoxin (sp-Fd), *E. coli* flavodoxin reductase (eco-FlvR) and NADPH, with glucose-6-phosphate and glucose-6-phosphate dehydrogenase as NADPH regeneration system. After reaction for 60 min at 30 °C, the peptidic products were cleaved from the PCP by hydrazine treatment and the corresponding peptide hydrazides were isolated by solid-phase extraction and purified by reverse phase HPLC.

Influence of the N-terminal residues

Under the standard assay conditions, the pentapeptide–PCP conjugate **5D**, lacking the N-terminal Leu^1 compared to the model hexapeptide (**1**), showed rapid turnover to the corresponding monocyclic peptide hydrazide (**13**) (Fig. 4). However, by HPLC analysis of the product, two epimers were detected, each with the expected mass of the monocyclic peptide (m/z = 811.3 ([M + H]$^+$)). The major product was the expected macrocyclic peptide hydrazide (**13**) and the minor product (*ca.* 10%) is most likely an epimeric form having (*R*)-Tyr in place of (*S*)-Tyr as the C-terminal amino acid, in analogy to earlier results reported for the hexapeptide **1**.[9,10] Residual unchanged linear pentapeptide hydrazide **5E** was not seen within the limits of detection, indicating essentially complete turnover of the linear peptide. The identity of the major product **13** was further confirmed by high resolution ESI-MS (exact mass calcd. for $C_{40}H_{42}N_8O_{11}Na$: 833.2871 ([M + Na]$^+$), m/z found 833.2873). The fragmentation pattern of the

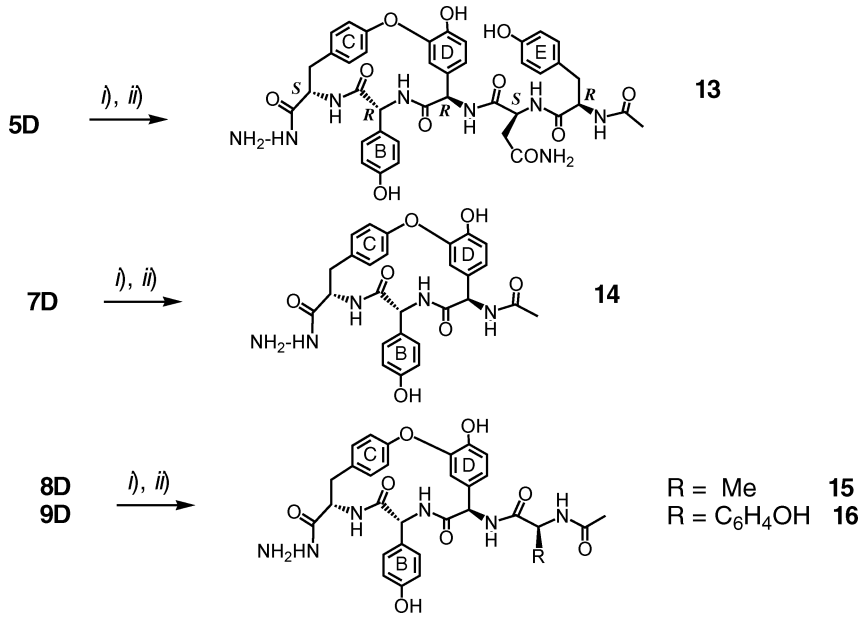

Fig. 4 Assays with OxyB. *Reagents and conditions*: *i*) OxyB, sp-Fd, eco-FlvR, NADPH, O_2, 30 °C. *ii*) NH_2-NH_2.

major product **13** by MS/MS, together with the full chemical shift assignment and analysis of NOEs by 1D and 2D ^{1}H-NMR spectroscopy confirmed that macrocycle formation had occurred between the side chains of 4-hydroxyphenylglycine-3 (Hpg^3) and tyrosine-5 (Tyr^5).

No turnover by OxyB was detected in assays with the tetrapeptide–PCP-7S conjugate (**6D**). Only the corresponding linear tetrapeptide hydrazide was detected in the product by HPLC and by ESI-MS analysis (m/z = 650.2 ([M + H]$^+$)). To confirm this assignment, the linear tetrapeptide hydrazide was isolated and fully characterized by high resolution ESI-MS (exact mass calcd. for $C_{31}H_{35}N_7O_9Na$: 672.2394 ([M + Na]$^+$), m/z found 672.2407), and by 1D and 2D ^{1}H-NMR spectroscopy. A second minor epimer (*ca.* 5%) with the same mass was also detected by HPLC.

In contrast, under standard assay conditions the tripeptide **7D** was transformed by OxyB into a macrocyclic peptide (**14**) (Fig. 4). HPLC analysis of the reaction products indicated *ca.* 80% conversion into the tripeptide hydrazide **14**, which was identified by high resolution ESI-MS (exact mass calcd. for $C_{27}H_{27}N_5O_7Na$: 556.1808 ([M + Na]$^+$), m/z found 556.1806) and ESI-MS/MS. 1D and 2D-NMR spectroscopy also confirmed the formation and the connectivity of the expected macrocycle. A second minor component (*ca.* 15%) in the product mixture was assigned to the corresponding linear tripeptide hydrazide **7E**, derived from unreacted **7D**. A further minor peak (<5%) detected by HPLC, with a mass also corresponding to macrocyclic product, was assigned again as an epimer of **14**.

Substituted tetrapeptides

Two additional tetrapeptides containing N-terminal (*S*)-Ala (**8D**) or (*S*)-Hpg (**9D**), instead of (*S*)-Asn (compare **6D**), were also tested as substrates. Under standard assay conditions, each peptide–PCP-7S conjugate (**8D** and **9D**) was converted to the extent of 10–20% into the corresponding monocyclic product (**15** and **16**). In both cases, unreacted linear peptides could be re-isolated as the corresponding peptide hydrazides. As minor products, epimeric forms of the linear tetrapeptide hydrazide and the monocyclic tetrapeptide hydrazide (**15** and **16**) were also identified. The identities of the major macrocyclic products were supported by ESI-MS (**15**: m/z = 605.4 ([M + H]$^+$), **16**: m/z = 683.4 ([M + H]$^+$)), high resolution ESI-MS (**15**: exact mass calcd. for $C_{30}H_{32}N_6O_8Na$: 627.2179 ([M + Na]$^+$), m/z found 627.2194, **16**: exact mass calcd. for $C_{35}H_{34}N_6O_9Na$: 705.2285 ([M + Na]$^+$), m/z found 705.2285) and ESI-MS/MS analysis. The poor resolution of the monocyclic and linear products in HPLC chromatograms prevented isolation of pure compounds in sufficient quantities for a full NMR analysis. However, 1D and 2D ^{1}H-NMR analyses of the product mixtures enabled a full chemical shift assignment of the linear and monocyclic tetrapeptide hydrazides **15** and **16**.

Other peptides

The hexapeptide (**10D**) containing (*S*)-Trp at position-6 was assayed under standard conditions, but afforded no monocyclic product. The corresponding linear peptide hydrazide and a putative C-terminal epimer (≈20%) were recovered, and characterized by ESI-MS (m/z = 921.6 ([M + H]$^+$)), high resolution ESI-MS (exact mass calcd. for $C_{47}H_{57}N_{10}O_{10}$: 921.4259 ([M + Na]$^+$), m/z found 921.4278), ESI-MS/MS and NMR spectroscopy. The two tripeptides related to K-13, **11D** (configuration (*S*)(*S*)(*S*) as in K-13) and **12D** (configuration (*R*)(*R*)(*S*) in analogy to **7**) were also assayed under standard conditions. Again, no monocyclic products were detected by HPLC, only unreacted linear tripeptide was recovered, isolated as the hydrazide, and confirmed by ESI-MS (**11E**: m/z = 564.4 ([M + H]$^+$), **12E**: m/z = 564.4 ([M + H]$^+$)), high resolution ESI-MS (**11E**: exact mass calcd. for $C_{29}H_{33}N_5O_7Na$: 586.2278 ([M + Na]$^+$), m/z found 586.2282, **12E**: exact mass calcd.

for $C_{29}H_{33}N_5O_7Na$: 586.2278 ([M + Na]$^+$), *m/z* found 586.2285), ESI-MS/MS and 1D and 2D ^{1}H-NMR spectroscopy.

Binding studies by UV-vis

The binding of the pentapeptide **5D**, the tetrapeptide **6D** and tripeptide **7D** to OxyB was monitored by UV-vis difference spectroscopy. Upon titration of **5D** with OxyB, the expected type-I binding spectrum was obtained (Fig. 5) with a peak at 393 nm, a trough at 426 nm and an isosbestic point at 413 nm. The concentration dependence of the absorbance fitted well a binding equation involving a 1 : 1 interaction, with a K_d of 28 ± 4 μM for the interaction of **5D** with OxyB.

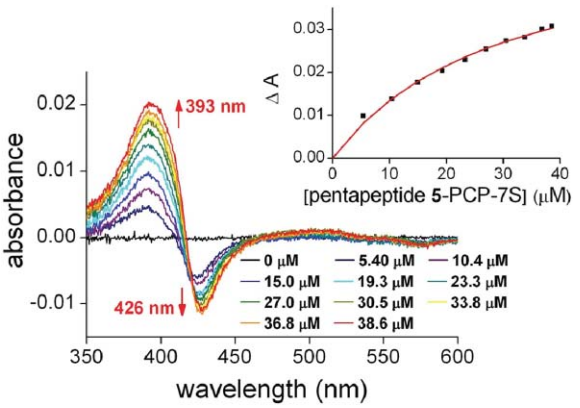

Fig. 5 UV-vis difference spectra of the pentapeptide **5**–PCP-7S binding to OxyB. The concentration dependence of the spectral changes and the wavelengths of the minima and maxima are shown.

Binding studies using the tetrapeptide **6D** and tripeptide **7D** produced significantly different changes in UV-vis difference spectra, with a shift of the positions of the maximum (to 405–414 nm) and the trough (to 435–441 nm). Additionally, the relative magnitude of the trough was much reduced and the concentration dependence of the changes was approximately linear in the range that could be tested (not shown). A similar behaviour was observed in earlier work for the interaction of OxyB with a heptapeptide–PCP-7S conjugate, and a hexapeptide–PCP-6S conjugate.[9] The results indicate that the binding affinity of **7D** to OxyB is much lower (K_d >100 μM) than that measured here for **5D**, and for **1** in earlier work (K_d 17 ± 5 μM).[9]

Discussion

In earlier work,[8,9] model hexa- and heptapeptide–PCP conjugates (**1** and **2**), related to putative intermediates in vancomycin aglycone assembly were tested *in vitro* as substrates for OxyB. These model substrates contain tyrosine at positions-2 and -6.

Glycopeptide antibiotics of the vancomycin/balhimycin/chloroeremomycin family, however, all contain *m*-chloro-β-hydroxytyrosine (Cht) at positions-2 and -6. Gene knock-out studies have indicated that β-hydroxytyrosine (β-HT), but not tyrosine nor Cht, are recognized and incorporated into the peptide backbone at positions-2 and -6 by the NRPS during balhimycin biosynthesis.[15] The timing of chlorination of both aromatic rings is not yet known, but gene knock-out studies again suggest that each chlorination may occur directly after incorporation of the respective β-HT residue into the growing peptide chain by the NRPS, catalyzed by the single halogenase encoded within the biosynthetic gene cluster.[15] This implies that OxyB should never encounter an NRPS-bound peptide substrate containing tyrosine at positions-2 and -6, only β-HT or Cht. The β-HT itself is thought to be formed by a pathway similar to that demonstrated for novobiocin biosynthesis,[16] in which a cytochrome P450 is used to hydroxylate a PCP-thioester bound form of tyrosine, which is then released as free β-HT by hydrolysis. Interestingly, the biosynthesis of glycopeptide antibiotics of the teicoplanin/A40926/A47934 family, which possess an additional side chain cross-link between amino acids 1 and 3 (an F–*O*–G link) and a Cht only at position-6, appears to proceed differently. Gene knock-out studies in the A40926 producer suggest that tyrosine, rather than β-HT, is recognized and incorporated by the NRPS at positions-2 and -6, and that subsequently only residue-6 is β-hydroxylated (exact timing uncertain), but now catalyzed by a non-heme iron oxygenase (Dbv28).[17] However, a *dbv*28 knock-out mutant does produce small amounts of a glycopeptide related to A40926, lacking the β-hydroxy group in residue-6. The timing of chlorination in the biosynthesis of this family of glycopeptides is presently also unknown. One other remarkable observation relating to the specificity of vancomycin OxyB, is that this vanOxyB was able to cross-complement a *staH* mutant of the A47934 producer.[18] StaH catalyzes the analogous cross-coupling of the C–*O*–D rings in A47934 biosynthesis, but apparently this function can also be fulfilled by vanOxyB, although the peptide backbone of A47934 is different to that of vancomycin (Fig. 1).

Deletion of halogenase genes in the balhimycin producer (*bha*A) abolished halogenation, and afforded the corresponding dechloro-balhimycin.[19] This shows that OxyB can catalyze cross-linking in the absence of chlorine substituents, in agreement with the *in vitro* studies. In related work, feeding various isomers of fluoro-β-hydroxytyrosine to a Δ-*bhp* mutant provided novel fluorobalhimycins,[20] and growth of the balhimycin producer on media containing bromide (but no chloride) afforded bromobalhimycins.[21] Remarkably, a Δ-*dpgA* mutant of the balhimycin producer was able to yield very small amounts of a tricyclic glycopeptide aglycone with the usual 3,5-dihydroxyphenylglycine (Dpg) replaced by 4-hydroxyphenylglycine (Hpg),[7] although in related work it was reported that feeding Hpg to this mutant led to no (detectable) glycopeptide production.[22] Similarly, feeding the *dpgA* mutant with Dpg analogues afforded tricyclic aglycones containing methoxy groups in amino acid residue-7.[22] These knock-out studies do not directly inform about the substrate specificity of OxyB, not least due to uncertainties in the timing of several key steps in the biosynthetic pathway. It should also be borne in mind that fermentations of gene knock-outs may lead to the isolation of intermediates that have been processed in ways that do not reflect the preferred pathway in the wild-type strain. Moreover, some intermediates might be modified slowly by enzymes unrelated to glycopeptide biosynthesis (*e.g.* by proteolysis) and accumulate only during the course of long fermentations. It is, therefore, important to complement such *in vivo* studies with direct enzymic studies *in vitro*.

The work described here extends our earlier *in vitro* studies using peptide **1**, with the aim of exploring further the substrate tolerance of OxyB using firstly the series of peptides **5–7**, which

represent stepwise deletions of residues from the N-terminus of **1**. The pentapeptide **5D** lacking the N-terminal Leu was converted into the monocyclic hydrazide **13** almost quantitatively under the assay conditions. Indeed, the dissociation constant ($K_d \approx 28$ μM) for this interaction, determined by UV-vis difference spectroscopy (Fig. 5), is only slightly lower than that observed for the model hexapeptide **1** ($K_d \approx 17$ μM). Hence, the Leu[1] residue does not contribute significantly to substrate recognition by OxyB. Kinetic studies of this enzymic reaction, using methods described earlier,[9] were prevented by the fact that the linear and cyclic peptide products could not be sufficiently well resolved by HPLC.

When peptide **6D** was tested, however, no turnover to cross-linked tetrapeptide product was detected under the standard assay conditions. This result would not be so surprising if it were not for the fact that the tripeptide **7D** was converted, quite efficiently (80% conversion) under the standard assay conditions, into cross-linked product **14**. The turnover of **7D** was slower than for pentapeptide **5D**, and this was also reflected in binding studies to OxyB by UV-vis-difference spectroscopy, which now suggested a much weaker interaction of **7D** with the enzyme ($K_d > 100$ μM). It was technically not possible to determine kinetic parameters (k_{cat}, K_m) for this reaction, due to the high concentrations of substrate required. We conclude, therefore, that the tripeptide **7D**, representing the core of the macrocycle created by OxyB is sufficient for phenol coupling to occur. These observations also explain why vanOxyB is able to cross-complement a *staH* mutant of the A47934 producer, as discussed above.

The reasons for the failure to detect cross-linking with the tetrapeptide **6D** are unclear. At the end of the assay, the tetrapeptide could be re-isolated as the hydrazide in good yield. To explore this further, two additional tetrapeptides **8D** and **9D** were prepared, and shown to be converted into macrocyclic products **15** and **16**, respectively, albeit with significantly lower overall turnover (10–20%) under the standard assay conditions. With these substrates at least some cross-linking occurs, but again not as efficiently as with the tri-, penta- and hexa-peptides. We speculate that the reason for the lack of turnover seen with **6D**, and the poor turnover seen with **8D** and **9D**, might be linked to the adoption of non-productive binding conformations in the corresponding enzyme–substrate complexes, but this remains to be proven.

Next, we were encouraged to test linear hexapeptide **10**, which contains tryptophan in place of Tyr[6], since a side chain cross-linking reaction involving Trp must occur during complestatin biosynthesis, catalyzed by a cytochrome P450-like enzyme related to OxyB.[23] It was of interest to test whether or not OxyB from the vancomycin producer could oxidize a Trp residue within the otherwise familiar scaffold of peptide **1**. The results show conclusively that this is not possible.

Two P450-like enzymes have been implicated in cross-coupling reactions during complestatin biosynthesis, ComI and ComJ, which share 47% and 54% identity, respectively, with OxyB. OxyB normally catalyzes a C–O–C coupling and not a C–C-biaryl coupling reaction. The structural and mechanistic basis for selectivity between aryl ether and biaryl cross coupling in this class of enzymes is not yet understood. However, assays were also performed here with **10D** and OxyC, which does catalyze a C–C coupling (identity with ComI and ComJ: 46% and 42% respectively), and OxyA (identity with ComI and ComJ: 45% and 52% respectively) again without success (data not shown). Clearly, more detailed studies of these cross-linking enzymes are warranted to understand the requirements for Trp oxidation.

Peptides with structural similarities to vancomycin aglycone, in particular with similar aryl-ether bridged macrocycles, are found in many natural products, including K-13, an inhibitor of angiotensin I converting enzyme (Fig. 3).[11] This, together with the observation made here that OxyB can cross-link the tripeptide **7D**, suggested it might be interesting to examine the two tripeptides **11D** and **12D** as potential substrates for OxyB. The absolute configurations of **11** and **12** are related to those in K-13 and **7**, respectively. Each tripeptide was assayed as a thioester conjugate to PCP-7S from the vancomycin NRPS (**11D** and **12D**), however, under standard conditions no conversion to a macrocyclic product could be detected. Hence, we conclude that the configuration and identity of the building blocks in the minimal tripeptide core, in particular Hpg, are important for recognition and turnover by OxyB.

The results described here, with those reported earlier, help to define more clearly the substrate specificity of OxyB. One remaining aspect of this specificity that should now be addressed in more detail, is the influence of the β-hydroxylation of tyrosine and the chlorination of the aromatic rings on these oxidative phenol coupling reactions.

Experimental section

Reverse phase HPLC was performed using a Pharmacia ÄKTA Purifier system with an Agilent Zorbax Eclipse XDB (RP C18, 250 × 4.6 mm, 5 μm particle size, 80 Å pore size) for analysis of assay products. One dimensional and two dimensional ^{1}H-NMR spectra were recorded at 500 MHz on a Bruker AV2-500 or at 600/700 MHz on a Bruker DRX-600 or AV-600 or AV-700 typically at a peptide concentration of *ca.* 1–10 mg ml^{-1}. The solvent was DMSO-d_6 (Cambridge Isotope Laboratories). For complete spin system assignments DQF-COSY, TOCSY, ROESY and NOESY experiments were carried out. Chemical shift values are given in ppm relative to the solvent resonances or to the internal standard tetramethylsilane. Electrospray ionisation (ESI) mass spectrometry was performed on a Finnegan TSQ-700, MALDI-TOF spectra were recorded on a Autoflex I MALDI-TOF with sinapinic acid as matrix (Bruker). UV-vis (difference) absorbance spectra were recorded using a double beam Varian Cary300Bio spectrophotometer.

Peptide synthesis

The peptides were synthesized by the methods described in earlier work.[9] Relevant analytical data for each new compound are given in the supplementary material.†

Synthesis of peptide–PCP-7S conjugates

The synthesis of peptide–PCP-7S conjugates used in this study was accomplished according to the general method described earlier (Fig. 3).[9] Thioester products were analyzed by MS and 1D/2D ^{1}H-NMR spectroscopy. NMR assignments were made by standard methods, using 2D DQF-COSY, TOCSY and NOESY spectra. Relevant analytical data for the thioester intermediates is given in the supplementary material.†

OxyB activity assays

The general OxyB assay was carried out according to the procedure described earlier.[9] The peptide hydrazide products were purified by analytical HPLC and analyzed by MS and 1D and 2D ^{1}H-NMR spectroscopy. NMR assignments were made by standard methods, using 2D DQF-COSY, TOCSY and NOESY spectra, and are given in the supplementary material.†

UV-vis binding studies

To monitor substrate binding to OxyB by UV-vis difference spectroscopy, the peptide–PCP-7S conjugate was purified by anion exchange chromatography and concentrated as described earlier.[9] The concentration of peptide–PCP-7S conjugates was determined by UV using the following extinction coefficients: **5D** $\varepsilon_{280} = 7120\ \text{cm}^{-1}\ \text{M}^{-1}$; **6D** $\varepsilon_{280} = 5630\ \text{cm}^{-1}\ \text{M}^{-1}$; **7D** $\varepsilon_{280} = 4517\ \text{cm}^{-1}\ \text{M}^{-1}$.

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