Published in final edited form as:

ACS Chem Biol. 2017 September 15; 12(9): 2248–2253. doi:10.1021/acscchembio.7b00456.

In Vitro Reconstitution of OxyA Enzymatic Activity Clarifies Late Steps in Vancomycin Biosynthesis

Clarissa C. Forneris[†], Seyma Ozturk[†], Marcus I. Gibson[†], Erik J. Sorensen[†], and Mohammad R. Seyedsayamdost^{†,‡,*}

[†]Department of Chemistry, Princeton University, Princeton, New Jersey 08544, United States

[‡]Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, United States

Abstract

Studies on the biosynthesis of glycopeptide antibiotics have provided many insights into the strategies that Nature employs to build architecturally strained molecules. A key structural feature of vancomycin, the founding member of this class, is a set of three aromatic cross-links that are introduced *via* yet unknown mechanisms. Previous reports have identified three cytochrome P450 enzymes involved in this process and demonstrated enzymatic activity for OxyB, which installs the first aromatic cross-link. However, the activities of the remaining two P450 enzymes have not been recapitulated. Herein, we show that OxyA generates the second bis-aryl ether bond in vancomycin and that it exhibits strict substrate specificity toward the chlorinated, OxyB-cross-linked product. No OxyA product is detected with the unchlorinated substrate. Together with previous results, these data suggest that chlorination occurs after OxyB- but before OxyA-catalyzed cross-link formation. Our results have important implications for the chemo-enzymatic synthesis of vancomycin and its analogs.

Graphical abstract

Glycopeptide antibiotics (GPAs) have been indispensable in the fight against infectious disease. Vancomycin (**1**, Figure 1), the founding member of the GPAs, has been in clinical

*Corresponding Author: mrseyed@princeton.edu.

ORCID

Erik J. Sorensen: 0000-0002-9967-6347

Mohammad R. Seyedsayamdost: 0000-0003-2707-4854

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchem-bio.7b00456.

Materials and Methods; synthesis, purification, and characterization of substrates and substrate components **3–15**; HR-MS and HR-MS/MS data for peptide substrates **4–11** and products **16–23** (PDF)

use since 1958 and is among our 100 most essential medicines.^{1,2} It inhibits cell wall biosynthesis, and thereby bacterial growth, by sequestering the D-Ala-D-Ala terminus of the nascent peptidoglycan chain.³ Vancomycin's mode of action is enabled by its architecturally complex, cup-shaped structure, which is fixed in place by its atropisomeric nature and three aryl cross-links (Figure 1).⁴ How these cross-links are generated has been an area of immense and long-standing interest, with still many unanswered questions.^{5,6} Pioneering genetic experiments by Süssmuth and Wohlleben showed that three cytochrome P450 enzymes, OxyB, OxyA, and OxyC, in that order build two bisaryl ether bonds and a biaryl connection during the biogenesis of vancomycin-type GPAs.^{7–9} Robinson and colleagues recapitulated the enzymatic activity of OxyB *in vitro* by showing that it installs the first cross-link between rings C and D (Figure 1).^{10,11} Recent studies on the biosynthesis of teicoplanin, a GPA related to vancomycin, have afforded new insights into aryl ether bond formation as well.^{12,13} However, since the original studies by Robinson 10 years ago, cross-link formation by additional vancomycin Oxy enzymes has not been accomplished, and a number of questions, including the timing of chlorination and whether the remaining two Oxy enzymes are at all functional *in vitro*, remain unanswered. Herein, we report that OxyA catalyzes formation of the second bis-aryl ether cross-link in vancomycin. Interestingly, this enzyme shows strict specificity toward the chlorinated, singly cross-linked substrate, thereby clarifying the order of the late steps in the maturation of vancomycin.

We began our studies by expressing OxyB and OxyA from *Amycolatopsis orientalis* in an *Escherichia coli* host (Table S1), and by synthesizing the substrate for OxyB, a 7mer linear peptide that is conjugated to a peptidyl carrier protein (PCP) *via* a pendent coenzyme A (CoA) arm.^{12,14} To generate the OxyB substrate, we used a new native chemical ligation-inspired approach, as existing methods lead to facile epimerization of the C-terminal amino acid and deliver modest yields of the thioesterified product (Figure 2).¹¹ The 7mer peptide was prepared by solid-phase peptide synthesis on a 2-chlorotrityl chloride hydrazine resin. Acid-mediated cleavage from this resin delivered the acyl hydrazide form of the peptide (**4**, Figure 2B), which was then converted to the CoA-thioester according to the procedure by Zheng and co-workers *via* an acyl azide intermediate (**8**, Figure 2C, Table S2, Figures S1 and S2).¹⁵

The low pH that is maintained during this procedure prevents epimerization, and the reactive nature of the acyl azide provides good yields of the CoA-thioesterified product with an excess of CoA.

Recent work by the Cryle group has unveiled a so-called X-domain that acts as a scaffold to recruit Oxy enzymes and their PCP-bound substrates in the biosynthesis of teicoplanin.¹⁶ We therefore cloned and expressed an X-PCP didomain from the ultimate nonribosomal peptide synthetase gene from *A. orientalis* (Figure S3). The PCP domain within X-PCP was loaded with the peptide 7mer-CoA adduct using established procedures, thus completing preparation of the OxyB substrate (**12**, Figure 2D).^{12,16} Using **12**, we could reproduce results by Robinson and colleagues,¹¹ demonstrating C-O-D aromatic cross-coupling (Figure 1), as monitored by HPLC-Qtof-MS (**16**, Figure 3A,C, Tables S2–S4).

Previous studies have suggested that OxyA purifies in a catalytically incompetent form.¹⁷ In our hands, the UV–visible absorption spectrum of as-purified OxyA was very similar to that of OxyB, which is active *in vitro*. Reduction of the OxyA-heme to the Fe^{II} form and reaction with CO demonstrated formation of the well-known 450 nm feature, which is characteristic of the porphyrin–Fe^{II}–CO adduct (Figures S3–S5).¹⁸ We concluded that in our preparations, OxyA harbors the correct form of the heme cofactor, and that it could be functional if provided with its substrate under the right set of conditions. A variety of reactions were carried out in an attempt to install the second aryl ether cross-link on the 7mer peptide using OxyA. Initial attempts, in which OxyA was incubated with OxyB and its substrate, failed to deliver the doubly cross-linked product. We then conducted additional experiments by changing a number of variables, including reaction time, concentrations of the various reaction components, temperature, pH, source of reductants, dissolved O₂ levels, use of tagless OxyA in place of His₆-OxyA, provision of a discrete PCP domain, addition of X-domain in trans, and supplementation of the reaction mixture with *A. orientalis* crude extracts (to provide any potential missing components). However, all of these reactions failed to deliver the presumed OxyA product (**17**, Figure 3A,C).

The timing of chlorination at rings C and E has not been conclusively determined in the genetic experiments conducted thus far (Figure 1).⁹ It has been shown that OxyB exhibits a marked preference for the unchlorinated substrate, but the order of chlorination with respect to the transformations catalyzed by OxyA and OxyC still awaits clarification.¹⁹ We therefore hypothesized that the correct substrate was not available in our reaction mixtures above, and that OxyA may only recognize the singly cross-linked bis-chlorinated 7mer peptide. To test this idea, we synthesized the 7mer substrate, now containing 3-Cl-L-Tyr (**2**, **5**) and 3-Cl-D-Tyr (**3**, **5**) at residues 2 and 6, respectively (Figure 2A, Figures S6–S10), and subsequently performed assays initially only with OxyB. While OxyB prefers the unchlorinated substrate **12**, we could clearly see cross-linking of substrate **13** to generate product **18** (Figure 3B,D). This was verified by high-resolution (HR)-MS, which gave

$[M+H]^+_{\text{obs}} = 1086.4982$ ($[M+H]^+_{\text{calcd}} = 1086.4931$), as well as by HR-MS/MS (Table S4). The latter revealed fragmentation at each peptide bond, except for those within the macrocycle generated by the C-O-D cross-link, consistent with an aryl ether bond at this position (Figures 3E, S11).

We next carried out a one-pot reaction in which OxyB and OxyA were incubated with the bis-chlorinated peptide substrate **13**, and the reaction mixture was analyzed by HPLC-Qtof-MS. The data clearly show the appearance of two peaks that are 2 and 4 Da lighter than the substrate (Figure 3A–D, Table S2). The former consists of the singly cross-linked OxyB product as determined before. We propose that the latter corresponds to the long-sought doubly cross-linked product of OxyA (**21**, Figure 3B,D). This product peak is not observed when we omit from the reaction mixture either OxyA, OxyB and OxyA, or the substrate. The product peak accumulates in a time-dependent fashion when all the necessary reaction components are present (Figure S12), and its UV–vis absorption spectrum is characteristic of phenols, all in line with our proposal that it represents the OxyA product.

To obtain further evidence for this conclusion, HR-MS and HR-MS/MS data were collected. The former was entirely consistent with a loss of four protons

($[M+H]^+_{\text{obs}}=1152.4010$, $[M+H]^+_{\text{calcd}} 1152.3995$). Further, the isotopic distribution of the product peak revealed the presence of Cl atoms, which can be identified by the relatively high abundance of the ^{37}Cl isotope. Last, HR-MS/MS data show fragmentation at all peptide bonds except for those contained within the two macrocycles created by the C-O-D and the D-O-E cross-links (Figure 3E,F and Table S4). Notably, the b2, b3, y5, and y6 ions can be observed when the substrate is reacted with OxyB alone. However, when OxyB and OxyA are present, these fragments are not observed from the -4 Da product peak, again consistent with the aryl ether bond at rings D and E (Figure 3F). Together, the data show that the one-pot incubation of the bis-chlorinated 7mer substrate **13** with OxyB and OxyA gives rise to OxyA product (Figure 3B), which contains both aryl ether cross-links in vancomycin. After a 3 h incubation, we observed 20% conversion to product **21** (relative to product **18**).

OxyA exhibits strict specificity toward the bis-chlorinated, singly cross-linked adduct. Recent results have shown that the teicoplanin OxyA, OxyA_{tei}, can accept substrates that are not chlorinated.^{16,20} In our assays, however, chlorination appears to be a prerequisite for OxyA activity. To examine whether chlorination at both or only one of the tyrosine residues was required for OxyA, we synthesized the two monochlorinated substrates, in which only the C-ring (**6**, **14**) or the E-ring (**7**, **15**) was chlorinated (Figure 1). Experiments with the latter gave negligible amounts of monochlorinated, doubly cross-linked product **23**, suggesting that chlorination at the C-ring was essential for OxyA activity, and that it must precede catalysis by OxyA (Figure S13). When only the C-ring was chlorinated, formation of the doubly cross-linked product **22** occurred at a rate of ~33% of that of the bis-chlorinated substrate (Tables S3 and S4, Figure S14). Note that the yield of the OxyB reaction is comparable with both the bis-chlorinated peptide **13** and the C-ring-chlorinated substrate **14**, but significantly lower with unchlorinated (**12**) or E-ring chlorinated (**15**) substrates.

Along with previous results, our data provide insights into the timing of chlorination in vancomycin biosynthesis (Figure 4). The linear 7mer substrate **24** is generated by three nonribosomal peptide synthetases that incorporate 3,5-dihydroxyphenylglycine, 4-hydroxyphenylglycine, and β -hydroxytyrosines, all of which are encoded in the vancomycin gene cluster. Upon formation of the linear peptide, OxyB installs the first aryl ether cross-link.⁸ OxyB can also act on the 6mer product bound to the penultimate or ultimate PCP domain, but it shows a higher $k_{\text{cat}}/K_{\text{m}}$ toward the 7mer substrate.^{11,19} Deletion of *oxyB* in *A. mediterranei* results in the accumulation of a linear, unchlorinated, β -OH-Tyr-containing substrate, indicating that β -hydroxylation occurs before OxyB-mediated cross-link formation, while β -OH-Tyr-chlorination occurs after.⁹ In accordance, *in vitro* assays of OxyB show a strict preference for substrates lacking Cl at the C-ring and tolerance toward substrates chlorinated on the E ring, which is not involved in OxyB-catalyzed cross-linking.¹⁹

We propose the next step in vancomycin biosynthesis is chlorination of the β -OH-Tyr residues catalyzed by the chlorinase VhaA, followed by the second cross-link installed by

OxyA. This proposal is consistent with prior genetic studies: knockouts in *oxyA* and *oxyC* result in accumulation of the bis-chlorinated, monocyclic product and the bis-chlorinated bicyclic intermediate, respectively.^{7–9,21} The stringent specificity of OxyA for the chlorinated, singly cross-linked substrate that we observe is in line with this proposal. Finally, OxyC-mediated biaryl bond formation followed by release of the aglycone from the PCP, glycosylation of the central 4-hydroxyphenylglycine by GtfA and GtfB, and methylation of the N-terminal Leu by MtfA complete vancomycin biogenesis.

Since the discovery of the vancomycin biosynthetic gene cluster, one of the long-held goals has been the chemo-enzymatic preparation of vancomycin derivatives using the endogenous biosynthetic machinery. However, difficulties associated with the synthesis of the peptide substrate, its attachment to PCP and X-PCP domains, and the timing of additional modifications have made this a challenging proposition. Here, we take an important step toward this goal by showing that OxyA activity can be recapitulated *in vitro* and that a one-pot reaction containing OxyB and OxyA delivers product **21**, which is only one cross-link away from the vancomycin aglycone. Glycosylation of the aglycone has previously been demonstrated.²² Thus, further studies with OxyC could complete the chemo-enzymatic synthesis of vancomycin and possibly enable creation of new, perhaps improved, derivatives in the future.^{23,24}

METHODS

A description of procedures used to clone *oxyA*, *oxyB*, and various other proteins; reconstitute the heme cofactor in OxyA/OxyB; synthesize and characterize compounds **3–15**; and analyze enzymatic activity assays *via* HR-MS and HR-MS/MS is given in the SI.

General Procedures

UV–vis absorption spectra were acquired on a Cary 60 UV–visible spectrometer (Agilent). HPLC separations were carried out on an Agilent 1260 Infinity Series analytical or preparative HPLC system equipped with a photodiode array detector and an automated fraction collector. Low resolution HPLC-MS analysis was performed on an Agilent instrument consisting of a liquid autosampler, a 1260 Infinity Series HPLC system coupled to a photodiode array detector, and a 6120 Series ESI mass spectrometer. A Phenomenex Luna C18 column (3 μ m, 4.6 $\times$ 100 mm) was used with a flow rate of 0.5 mL/min and a gradient of 5% MeCN in H₂O to 55% MeCN over 17 min. Both MeCN and H₂O contained 0.1% (v/v) formic acid (FA). HR-HPLC-MS and HR-tandem HPLC-MS were carried out on an Agilent UHD Accurate Mass Q-tof LC-MS system, equipped with a 1260 Infinity Series HPLC, an automated liquid sampler, a photodiode array detector, a JetStream ESI source, and the 6540 Series Q-tof. Samples were separated on a Phenomenex Luna C18 column (5 μ m, 4.6 $\times$ 100 mm), operating at 0.4 mL/min with a gradient of 5% MeCN in H₂O to 44% MeCN over 13 min. Both MeCN and H₂O contained 0.1% (v/v) FA. NMR spectra were acquired at the Princeton University Department of Chemistry Facilities. ¹H NMR spectra were recorded on a Bruker 500 spectrometer (500 MHz) and are referenced relative to the residual CHCl₃ (in CDCl₃) proton signal at δ 7.26 ppm.

Purification of OxyA and OxyB

Cloning and expression of OxyA and OxyB is described in detail in the SI. Purification of these enzymes was carried out at 4 °C. The lysis buffer consisted of 50 mM Tris, 50 mM NaCl, 5 mM imidazole, 5% glycerol, pH 7.8, and 1 mM β -mercaptoethanol. The cell pellet was resuspended in lysis buffer (5 mL/g) in a 250 mL beaker. The suspension was supplemented with protease inhibitor cocktail (0.1% v/v), PMSF (0.25 mM), lysozyme (1 mg/mL), and DNase I (10 U/mL). Subsequently, the suspension was stirred for 30 min and sonicated on ice for 2 min in 15 s on/15 s off cycles at 30% power. The sonication cycle was repeated after the suspension rested on ice for 5 min, and then cell debris was pelleted *via* centrifugation (32 000g, 1 h, 4 °C). PMSF (0.25 mM final concentration) was added to the crude extract, which was then loaded onto a nickel metal affinity column (12 mL) that had been equilibrated with lysis buffer. The column was washed with 10 CV of lysis buffer and 4 CV of wash buffer (50 mM Tris, 50 mM NaCl, 30 mM imidazole, 5% glycerol, pH 7.8, 1 mM β -mercaptoethanol and 0.25 mM PMSF). Finally, the protein was eluted with 4 CV of elution buffer (50 mM Tris, 50 mM NaCl, 300 mM imidazole, 5% glycerol, pH 7.8, 1 mM β -mercaptoethanol, and 0.25 mM PMSF). His₆-OxyA and His₆-OxyB were buffer-exchanged on a Sephadex G-25 column (~35 mL, $d = 1.25$ cm, $l = 25$ cm) that had been equilibrated with G-25 buffer (50 mM Tris, 100 mM KCl, 5% glycerol, pH 7.8). The desired protein fractions, identified by the bright red color, were pooled, analyzed by SDS-PAGE and UV-vis spectroscopy, flash frozen in liquid N₂, and stored at -80 °C (see Supporting Information Figure 3). A typical yield was 40 mg of protein per liter of culture.

Activity Assays of OxyA and OxyB

Assay conditions were based on previously described protocols.^{11,12} Reactions were typically carried out on a 100 μ L scale. Reaction buffer (50 mM HEPES, 20 mM KCl, 10 mM MgCl₂, pH 7.0) was added to an Eppendorf tube containing 20 nmol of lyophilized peptide-CoA adduct, to a final concentration of 400 μ M. Subsequently, final concentrations of 100 μ M PCP₇-X and 10 μ M of Sfp R4-4—a K28E/T44E/C77Y triple mutant of *B. subtilis* phosphopantetheinyl transferase with increased catalytic efficiency and expanded substrate scope^{12,25}—were added to the reaction mixture, which was placed in a 30 °C incubator for 1 h. In standard reactions, final concentrations of the following reagents were added to the reaction mixture, in this order: 2 mM glucose-6-phosphate, 4 units of glucose-6-phosphate dehydrogenase, 14 μ M spinach ferredoxin, 6 μ M *E. coli* flavodoxin reductase, 10 μ M OxyB, and 5 μ M OxyA. Finally, the oxidative cross-linking reaction was initiated by the addition of 2 mM NADPH. Typical assays were carried out at RT for 12 h in the dark. In order to remove the peptide from the carrier domain, 20 000 equiv of propylamine were added and the reaction mixture incubated for 15 min. Proteins were precipitated by adding 15 μ L of formic acid and 50 μ L of MeCN (+ 0.1% FA). Denatured proteins were pelleted, and the supernatant was analyzed by HR-HPLC-MS and MS/MS (see Supporting Information Figures 11, 13, and 14 and Tables 2, 3, and 4).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the National Institutes of Health (grants GM065483 to E.J.S. and DP2-AI-124786 to M.R.S.) and the Princeton Environmental Institute Innovative Research Award (to M.R.S.) for support of this work. C.C.F. was supported by an Edward C. Taylor 3rd Year Graduate Fellowship in Chemistry.

References

1. Butler MS, Hansford KA, Blaskovich MAT, Halai R, Cooper MA. Glycopeptide antibiotics: Back to the future. *J Antibiot.* 2014; 67:631–644. [PubMed: 25118105]
2. WHO. Model List of Essential Medicines, 19th List. Apr. 2015
3. Kahne D, Leimkuhler C, Lu W, Walsh CT. Glycopeptide and lipoglycopeptide antibiotics. *Chem Rev.* 2005; 105:425–448. [PubMed: 15700951]
4. Nicolaou KC, Boddy CNC, Bräse S, Winssinger N. Chemistry, biology, and medicine of the glycopeptide antibiotics. *Angew Chem, Int Ed.* 1999; 38:2096–2152.
5. Hubbard BK, Walsh CT. Vancomycin assembly: Nature's way. *Angew Chem, Int Ed.* 2003; 42:730–765.
6. Yim G, Thaker MN, Koteva K, Wright G. Glycopeptide antibiotic biosynthesis. *J Antibiot.* 2014; 67:31–41. [PubMed: 24220108]
7. Bischoff D, Pelzer S, Hölzel A, Nicholson GJ, Stockert S, Wohlleben W, Jung G, Süßmuth RD. The biosynthesis of vancomycin-type glycopeptide antibiotics – new insights into the cyclization steps. *Angew Chem, Int Ed.* 2001; 40:1693–1696.
8. Bischoff D, Pelzer S, Bister B, Nicholson GJ, Stockert S, Schirle M, Wohlleben W, Jung G, Süßmuth RD. The biosynthesis of vancomycin-type antibiotics – the order of the cyclization steps. *Angew Chem, Int Ed.* 2001; 40:4688–4691.
9. Stegmann E, Pelzer S, Bischoff D, Puk O, Stockert S, Butz D, Zerbe K, Robinson J, Süßmuth RD, Wohlleben W. Genetic analysis of the balhimycin (vancomycin-type) oxygenase genes. *J Biotechnol.* 2006; 124:640–653. [PubMed: 16730832]
10. Zerbe K, Woithe K, Li DB, Vitali F, Bigler L, Robinson JA. An oxidative phenol coupling reaction catalyzed by OxyB, a cytochrome P450 from the vancomycin-producing microorganism. *Angew Chem, Int Ed.* 2004; 43:6709–6713.
11. Woithe K, Geib N, Zerbe K, Li DB, Heck M, Fournier-Rousset S, Meyer O, Vitali F, Matoba N, Abou-Hadeed K, Robinson JA. Oxidative phenol coupling reactions catalyzed by OxyB: A cytochrome P450 from the vancomycin producing organism. Implications for vancomycin biosynthesis. *J Am Chem Soc.* 2007; 129:6887–6895. [PubMed: 17477533]
12. Haslinger K, Maximowitsch E, Brieke C, Koch A, Cryle MJ. Cytochrome P450 OxyB_{tej} catalyzes the first phenolic coupling step in teicoplanin biosynthesis. *ChemBioChem.* 2014; 15:2719–2728. [PubMed: 25358800]
13. Peschke M, Brieke C, Cryle MJ. F-O-G ring formation in glycopeptide antibiotic biosynthesis is catalysed by OxyE. *Sci Rep.* 2016; 6:35584. [PubMed: 27752135]
14. Brieke C, Cryle MJ. A facile Fmoc solid phase synthesis strategy to access epimerization-prone biosynthetic intermediates of glycopeptide antibiotics. *Org Lett.* 2014; 16:2454–2457. [PubMed: 24730694]
15. Zheng JS, Tang S, Qi YK, Wang ZP, Liu L. Chemical synthesis of proteins using peptide hydrazides as thioester surrogates. *Nat Protoc.* 2013; 8:2483–2495. [PubMed: 24232250]
16. Haslinger K, Peschke M, Brieke C, Maximowitsch E, Cryle MJ. X-domain of peptide synthetases recruits oxygenases crucial for glycopeptide biosynthesis. *Nature.* 2015; 521:105–109. [PubMed: 25686610]
17. Brieke C, Peschke M, Haslinger K, Cryle MJ. Sequential in vitro cyclization by cytochrome P450 enzymes of glycopeptide antibiotic precursors bearing the X-domain from nonribosomal peptide biosynthesis. *Angew Chem.* 2015; 127:15941–15945.
18. Ortiz de Montellano, PR. Cytochrome P450. Structure, Mechanism and Biochemistry. 3. Kluwer Academic/Plenum Publishers; New York: 2005.

19. Schmartz PC, Wölfel K, Zerbe K, Gad E, El Tamany ES, Ibrahim HK, Abou-Hadeed K, Robinson JA. Substituent effects on the phenol coupling reactions catalyzed by the vancomycin biosynthetic P450 enzyme OxyB. *Angew Chem, Int Ed.* 2012; 51:11468–11472.
20. Peschke M, Brieke C, Goode RJA, Schittenhelm RB, Cryle MJ. Chlorinated glycopeptide antibiotic peptide precursors improve cytochrome P450-catalyzed cyclization cascade efficiency. *Biochemistry.* 2017; 56:1239–1247. [PubMed: 28218515]
21. Schmartz PC, Zerbe K, Abou-Hadeed K, Robinson JA. Bis-chlorination of a hexapeptide-PCP conjugate by the halogenase involved in vancomycin biosynthesis. *Org Biomol Chem.* 2014; 12:5574–5577. [PubMed: 24756572]
22. Nakayama A, Okano A, Feng Y, Collins JC, Collins KC, Walsh CT, Boger DL. Enzymatic glycosylation of vancomycin aglycone: Completion of a total synthesis of vancomycin and N- and C-terminus substituent effects of the aglycone substrate. *Org Lett.* 2014; 16:3572–3575. [PubMed: 24954524]
23. Okano A, Nakayama A, Schammel AW, Boger DL. Total synthesis of [Ψ [C(=NH)NH]Tpg⁴]vancomycin and its (4-chlorobiphenyl)methyl derivative: Impact of peripheral modifications on vancomycin analogues redesigned for Dual D-Ala-D-Lac binding. *J Am Chem Soc.* 2014; 136:13522–13525. [PubMed: 25211770]
24. Okano A, Nakayama A, Wu K, Lindsey EA, Schammel AW, Feng Y, Collins KC, Boger DL. Total syntheses and initial evaluation of [Ψ [C(=S)NH]Tpg⁴]vancomycin, [Ψ [C(=NH)NH]Tpg⁴]vancomycin, [Ψ [CH₂NH]Tpg⁴]vancomycin, and their (4-chlorobiphenyl)methyl derivatives: Synergistic binding pocket and peripheral modifications for glycopeptide antibiotics. *J Am Chem Soc.* 2015; 137:3693–3704. [PubMed: 25750995]
25. Sunbul M, Marshall NJ, Zou Y, Zhang K, Yin J. Catalytic turnover-based phage selection for engineering the substrate specificity of Sfp phosphopantetheinyl transferase. *J Mol Biol.* 2009; 387:883–898. [PubMed: 19340948]

Figure 1. Structure of vancomycin. Aromatic cross-links catalyzed by OxyB, OxyA, and OxyC are highlighted.

Figure 2.

Preparation of substrates for OxyB by a native chemical ligation-type approach. (A) Synthesis of *N*-Fmoc-3-Cl-L-Tyr-OH and *N*-Fmoc-3-Cl-D-Tyr-OH (Figures S6 and S7). (B) Schematic for the synthesis of the 7mer peptide hydrazide using SPPS. Use of variants **2** and **3** in this sequence allowed synthesis of mono- and dichloro-derivatives **5–7**. (C) Conversion of the 7mer peptide-hydrazide to the thioesterified CoA derivatives **8–11** via an acyl azide intermediate. (D) Attachment of 7mer-pantetheinyl group to the X-PCP domain using the enzyme Sfp R4-4 to give substrates **12–15**.

Figure 3.

Enzymatic assays with non-, mono-, and dichlorinated peptide substrates. (A) Scheme describing results with substrate **12**. Only the OxyB product was observed. (B) Scheme describing results with chlorinated substrates **13–15**. Both products of OxyB and OxyA are observed. % Conversions of the linear substrate to the OxyB-product and of the OxyB-product to the OxyA-product, determined by HPLC-Qtof-MS, are shown for **12–15**. (C, D) HPLC-Qtof-MS data for the reaction of substrate **12** (C) or **13** (D) with OxyB/OxyA. In both plots, the traces from top to bottom correspond to base peak chromatogram (BPC, black trace), extracted ion chromatogram (EIC) for the OxyB product by HR-MS (blue trace), EIC for the OxyB y5 ion by HR-MS/MS (blue trace), EIC for the OxyA product by HR-MS (red trace), and EIC for the OxyA b7 ion by HR-MS/MS. (E, F) Observed HR-MS/MS fragments for the product of OxyB (E) and OxyA (F) using bis-chlorinated substrate **13**.

Figure 4.

Updated biosynthetic scheme for vancomycin. A partial gene cluster from *A. orientalis* with the relevant genes is shown. Our data suggest that upon cross-link formation by *OxyB*, chlorination by *VhaA* at the β -OH-Tyr residues precedes aryl ether bond formation by *OxyA*. The final C–C bond, installed by *OxyC*, followed by release from the PCP domain as well as by glycosylation (by *GtfA* and *GtfB*) and *N*-methylation at the first, residue (by *MtfA*) complete the biosynthesis of **1** according to previous results.^{3–5,9}