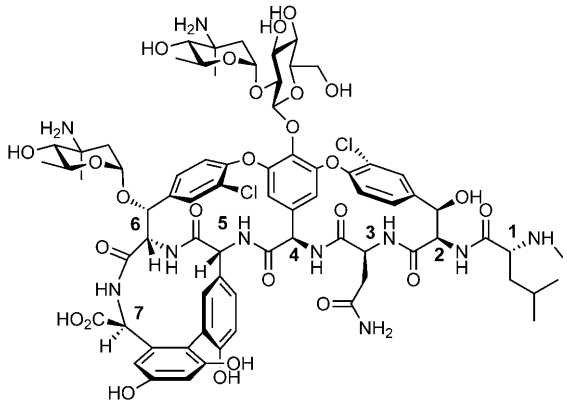
DOI: 10.1002/cbic.200800303

Investigation into the Mechanism of Phenolic Couplings during the Biosynthesis of Glycopeptide Antibiotics

Andrew N. Holding* and Jonathan B. Spencer[†][a]

Glycopeptide antibiotics such as vancomycin and teicoplanin are biosynthesised by actinomycetes[1] and have been called the drugs of "last resort" because they are often used to combat methicillin-resistant *Staphylococcus aureus* (MRSA).[2] The target site of glycopeptide antibiotics is the D-Ala-D-Ala side chain within the nascent cell wall of Gram-positive bacteria to which it binds, thereby inhibiting cell-wall biosynthesis.[3,4] Recently, vancomycin has also been shown to be more effective than metronidazole against severe infections of *Clostridium difficile*.[5] For these reasons, together with the emergence of vancomycin-resistant *S. aureus* (VRSA)[6] and community-acquired vancomycin-resistant *Enterococcus* (VRE),[7] the continued development of and research into this class of compounds are clearly of great importance.

Chloroeremomycin (Scheme 1) is a glycopeptide produced by *Amycolatopsis orientalis* that shares the same aglycone structure as vancomycin and balhimycin. It has been shown by



Scheme 1. Chloroeremomycin structure (amino acid numbering shown in bold).

heterologous complementation that the oxidative cross-linking enzymes of *A. orientalis* and the balhimycin producer *Amycolatopsis balhimycina* are functionally equivalent.[3] While chloroeremomycin is not in clinical use itself, the semisynthetic chloroeremomycin derivative oritavancin is currently approaching the end of phase III clinical trials.[8] This family of glycopeptide antibiotics shares a similar biosynthetic pathway starting with the formation of a linear peptide chain, which is produced by three nonribosomal peptide synthetase (NRPS) enzymes utilising a range of nonproteinogenic amino acids.[9] This linear chain is cross-linked between the aromatic rings in three places by a set of oxidative cytochrome P450 enzymes (OxyA, OxyB and OxyC; Scheme 5, below) to form the rigid tricyclic aglycone structure.[10] High-quality crystal structures exist for both OxyB and OxyC without substrate.[11,12]

In line with the mechanism originally suggested by Barton, it has been proposed that this family of enzymes catalyses cross-linking by the formation of two resonance-stabilised radicals.[13] The only P450 enzyme that catalyses phenolic coupling to have been successfully crystallised with its substrate bound is CYP158A2,[14] which catalyses the cross-coupling of two flaviolin molecules to form dimeric products. The diradical mechanism A (Scheme 2) seems awkward in the context of this structure, as the phenol group of the upper molecule is blocked from the haem by the lower molecule (Figure 1), an arrangement that would prevent direct abstraction of a hydrogen atom from the upper molecule by an Fe–O species. This apparent incongruity prompted us to consider other possible mechanisms for phenolic coupling that might be more consistent with this crystal structure.

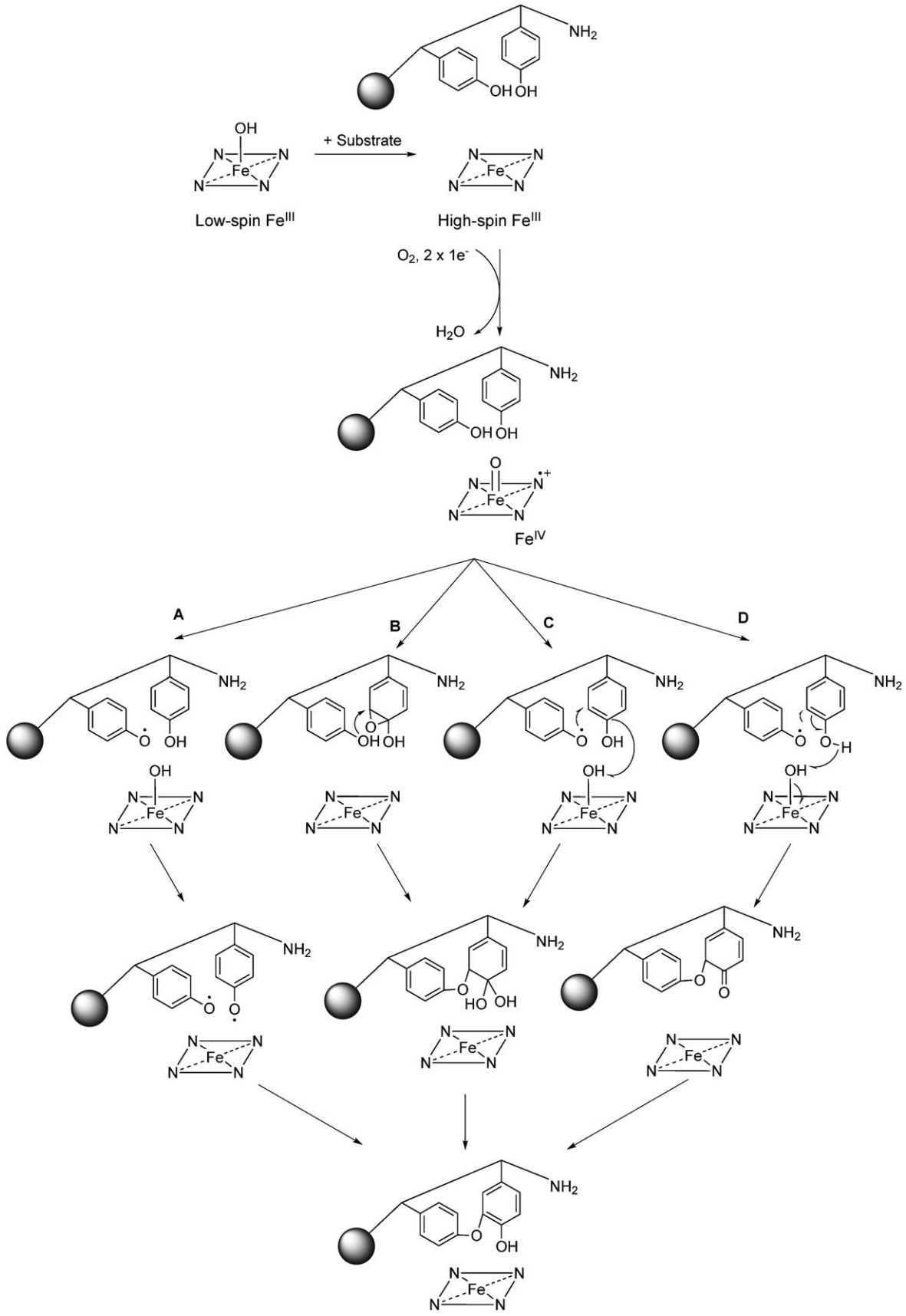
P450 enzymes catalyse a wide range of reactions, including epoxidation of aromatic compounds. If epoxidation of one of the amino acids to be cross-linked during the biosynthesis of chloroeremomycin occurred, then phenolic coupling could take place by attack of the other amino acid on this epoxide through a two-electron mechanism (B) to give a *gem*-diol intermediate (Scheme 2). This mechanism is attractive because it only requires one of the aromatic amino acids to be close to the haem, as suggested by the crystal structure of CYP158A2. This could be experimentally probed by labelling the hydroxyl group on the aromatic ring with ^{18}O. Dehydration of the *gem*-diol intermediate would lead statistically to the loss of half of this label, and provide evidence for this proposed mechanism. Recently, Robinson et al. also suggested the possibility of an epoxide intermediate. In an in vitro experiment under an atmosphere of $^{18}O_2$ with OxyB, which catalyses the first phenolic coupling of the linear hexapeptide attached to the peptidyl carrier protein (PCP),[15,16,17] they found no incorporation of label into the product; this suggests either that the epoxide was not formed or that the elimination of water from the *gem*-diol is stereoselective. In this communication, we report a complementary in vivo study using ^{18}O-labelled 4-hydroxymandelic acid. This approach has the advantage of being able to probe all three phenolic coupling steps catalysed by OxyA, OxyB and OxyC.

In addition to the epoxide and the diradical mechanisms, two mechanisms that, to our knowledge, have not been pro-

[a] *A. N. Holding, Dr. J. B. Spencer*
University Chemical Laboratory, University of Cambridge
Lensfield Road, Cambridge CB2 1EW (UK)
Fax: (+44) 1223-336362
E-mail: anh25@cam.ac.uk

[†] *Deceased 6th April 2008.*

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.



Scheme 2. Summary of possible mechanistic pathways discussed in this communication.

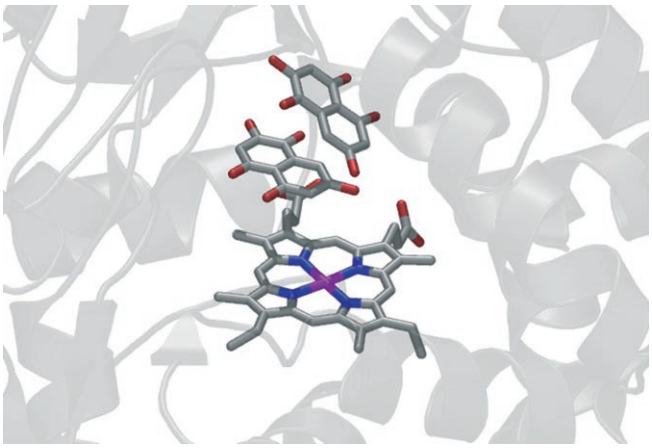
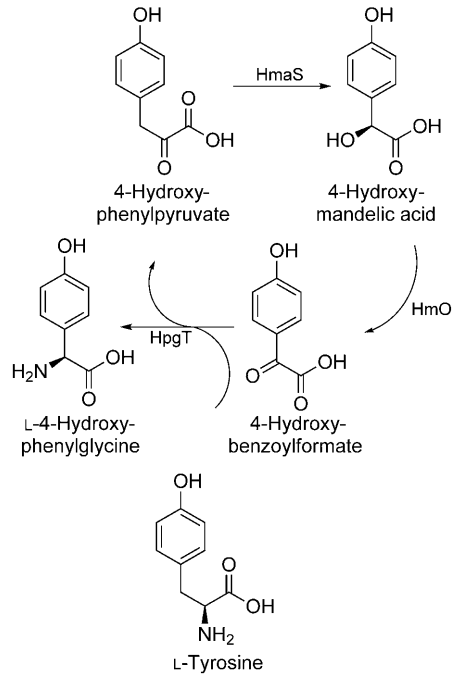


Figure 1. The binding of two flaviolin molecules in the active site of CYP158-A2 shows the difficulty of the formation of a radical on the upper flaviolin molecule due to the large distance to the haem. (Generated from PDB ID: 1T93)

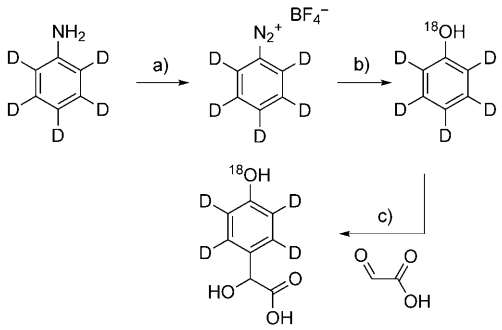
posed before, should be considered. Mechanism C shown in Scheme 2 involves initial formation of a radical on one aromatic amino acid followed by attack on the other aromatic moiety. The newly formed radical abstracts the hydroxyl group from the haem in a similar manner to the rebound mechanism proposed for the hydroxylation of many compounds by P450 enzymes.[18] As the rebound mechanism C would proceed through the same *gem*-diol intermediate as proposed in the epoxide mechanism (Scheme 2 B), these two mechanisms cannot be distinguished solely by the proposed ^{18}O labelling experiments. Both the rebound and diradical mechanisms would require the catalytic iron to be close to both aromatic rings. Although the crystal structure of CYP158A2 suggests that this is difficult, it might not truly reflect the flexibility of the active site and any changes in conformation as the reaction proceeds. Lastly, mechanism D (Scheme 2) is similar to mechanism A, except that the hydrogen atom of the phenol moiety is lost to the Fe–OH species as part of the coupling step rather than in a separate step. Like in the diradical mechanism, no exchange of label from the phenolic hydroxyl group would be predicted.

Chloroeremomycin contains two 4-hydroxyphenylglycine (Hpg) residues at positions 4 and 5 that are involved in all three phenolic coupling steps. Therefore, ^{18}O-labelled Hpg should be the best probe for investigating the mechanism of OxyA, OxyB and OxyC. To obtain the appropriately labelled Hpg we took advantage of the biosynthetic pathway dedicated to this amino acid, which goes through 4-hydroxymandelic acid (Hma; Scheme 3).

The [^{18}O,$^{2}H_4$]-labelled racemic Hma was synthesised from deuterated aniline via ^{18}O-labelled phenol, as shown in Scheme 4. The deuterium label was included so that the chloroeremomycin molecules that had incorporated the labelled precursor but lost ^{18}O would be distinguished in the mass spectrum from those derived solely from unlabelled precursors (Scheme 5). Hma was produced in a yield of 20%, with an ^{18}O enrichment of 87% and deuterium enrichment in excess of 99% at the appropriate positions.

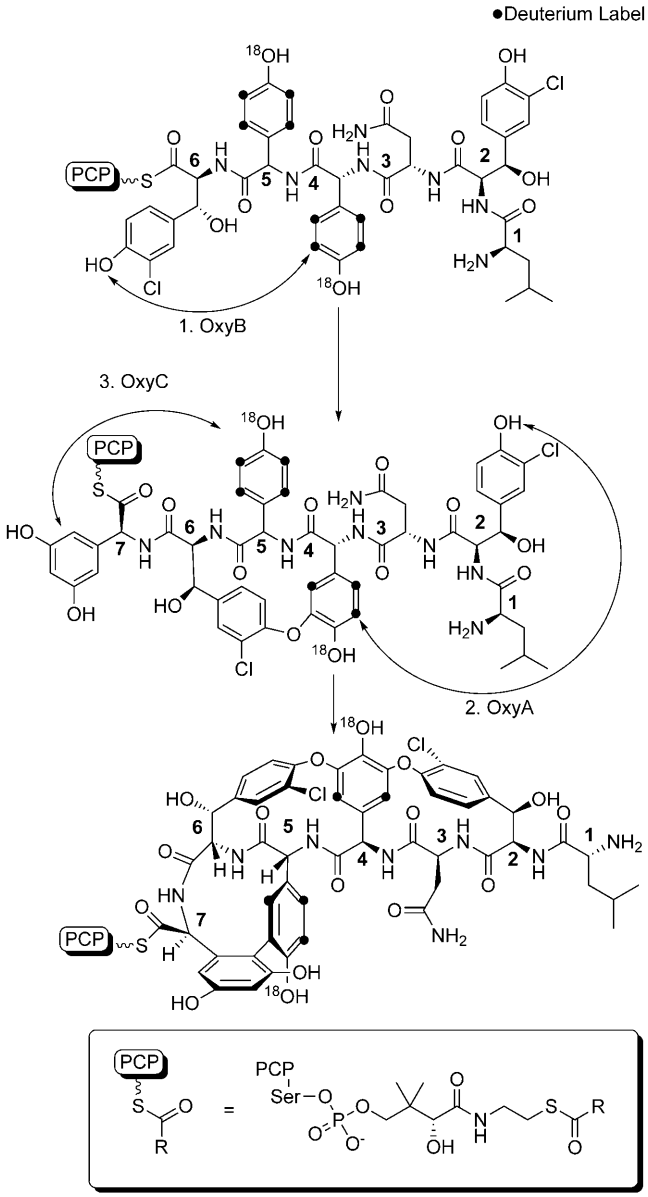


Scheme 3. L-4-Hydroxyphenylglycine biosynthetic pathway.



Scheme 4. [$^{2}H_4$,^{18}O]4-Hydroxymandelic acid synthesis. a) 0–4 °C, 30 min; 50% HBF_4, $NaNO_2$; yield: 86%. b) $H_2{}^{18}O$; conc. H_2SO_4; yield: 26%. c) NaOH; yield: 90%.

Initially feeding of [$^{2}H_4$]Hma to a culture of wild-type *A. orientalis* was used to establish the level of incorporation of exogenous labelled substrate into chloroeremomycin. Three chlorination states of the glycopeptide are produced by the strain—doubly chlorinated chloroeremomycin, singly chlorinated eremomycin, and an unchlorinated product, dechloroeremomycin. For unknown reasons, dechloroeremomycin was found to have the highest incorporation of deuterium. A possible explanation of this is that the feeding of labelled 4-hydroxymandelic acid coincided with a reduction in halogenase activity. Nevertheless the higher incorporation into dechloroeremomycin simplified the analysis of results, since the unchlorinated product presents a simpler isotope pattern by mass spectrometry than the two chlorinated products. Incorporation of [4-$^{2}H_4$]4-hydroxymandelic acid resulted in three main additional isotopic species compared with natural abundance by mass spectroscopy (Table 1): a compound with an M+2 parent ion (retention of



Scheme 5. Expected labelling from cross-linking in the chloroeremomcyin aglycone assuming no oxygen label is lost. Actual timing of the action of the enzymes OxyA and OxyC is currently unknown.

Table 1. Mass spectra of dechloroeremomycin after incorporation of labelled 4-hydroxymandelic acid. The relative abundances of each peak are shown.

Mass	Unlabelled [%]	[$^2H_4$]Hma [%]	[$^2H_4$,^{18}O]Hma [%][a]	Base peak
0	100	100	100	unlabelled
+1	84	80	82	
+2	36	52	36	[$^2H_2$]
+3	11	44	12	[$^2H_3$]
+4	2	26	21	[$^2H_2$,^{18}O]
+5		63	28	[$^2H_5$] and [$^2H_3$,^{18}O]
+6		43	17	
+7		22	10	[$^2H_5$,^{18}O]
+8			8	
+9			21	[$^2H_5$,$^{18}O_2$]
+10			14	
+11			5	

[a] Combined data from two runs.

dict from the base peak of each species how much it should contribute through natural abundance to each subsequent peak. Starting with the lowest mass species, this could be repeated until all the species had been separated out, with the exception of [$^2H_5$] and [$^2H_3$,^{18}O]dechloroeremomycin, which were treated as a single species, as the ratio of the compounds would not affect the magnitude of subsequent daughter peaks. The final result is shown in Table 2 (see the Supporting Information for details of the calculation).

Table 2. Relative amounts of species.

Species	Mass increase	Relative abundance [%]
unlabelled	0	100
[$^2H_2$]	+2	0.5
[$^2H_3$]	+3	0.6
[$^2H_2$,^{18}O]	+4	18.3
[$^2H_5$] + [$^2H_3$,^{18}O]	+5	12.1
[$^2H_5$,^{18}O]	+7	5.0
[$^2H_5$,^{18}O]	+9	17.5

two deuterium atoms) from incorporation at the fourth amino acid residue; an M+3 parent ion (retention of three deuterium atoms) from incorporation at the fifth amino acid residue, and an M+5 parent ion from incorporation at both the fourth and fifth positions.

After the successful incorporation of [$^2H_4$]Hma, the experiment was repeated with [$^2H_4$,^{18}O]Hma. The resulting mass spectral data (Table 1) showed multiply labelled species due to the two different incorporation sites and the use of [$^2H_4$,^{18}O]Hma that was only 87% enriched in ^{18}O. As such, even with full retention of the oxygen label, we would expect to see the appearance of eight main species. Two of these species, [$^2H_5$] and [$^2H_3$,^{18}O]dechloroeremomcyin, would give the same mass. To dissect the contributions of all eight species, the standard isotope pattern of dechloroeremomycin was used to pre-

In principle, it should be possible to calculate the degree of retention of ^{18}O by determining the ratio of either the amount of [$^2H_2$]dechloroeremomycin to [$^2H_2$,^{18}O]dechloroeremomycin or that between [$^2H_5$,^{18}O]- and [$^2H_5$,$^{18}O_2$]dechloroeremomycin. The former comparison is likely to be less accurate, as the *M*+2 peak is dominated by the natural isotopic abundance of unlabelled dechloroeremomycin; therefore, the latter ratio was used. The resulting ratio of [$^2H_5$,$^{18}O_2$]- to [$^2H_5$,^{18}O]dechloroeremomycin over three separate experiments (3.5:1) was slightly higher than one would expect on statistical grounds if the ^{18}O enrichment remained unchanged at 87% (which would be 3.35:1), thus indicating essentially total retention of the label. A negligible amount of oxygen label was lost during the biosynthesis of the natural product; this agrees with the previous in vitro study of OxyB[15] and provides new data for both

OxyA and OxyC. The complete retention of the phenolic hydroxyl oxygen suggests that pathways B and C, which go through a *gem*-diol, are the least likely of the four proposed mechanisms. However, the elimination of water from such an intermediate could be completely stereoselective, as the catalytic iron that donated the hydroxyl group would be in a position to facilitate its removal. Of the other two mechanisms consistent with the labelling studies, the diradical mechanism A seems more awkward, as both radicals have to be generated before coupling takes place. The single radical mechanism D is more appealing, but would still require both phenolic hydroxyl groups to be positioned close to the iron centre.

In conclusion, we show that oxygen labels on both the Hpg residues of the nascent peptide chain are retained during the biosynthesis of dechloroeremomcyin. These results suggest that all three oxidative cross-linking enzymes react through a similar mechanism; this is of particular interest in the case of OxyC as it catalyses a carbon–carbon coupling rather than a carbon–oxygen coupling as catalysed by OxyA and OxyB (Scheme 4). Finally, these results suggest that the continuing study to elucidate the mechanism of all three enzymes is necessary if we are to fully understand the biosynthesis of this important class of antibiotics.

Experimental Section

[2,3,4,5,6-^{2}H]Benzenediazonium tetrafluoroborate: 98% [U-^{2}H]-aniline (0.91 mL, 9.98 mmol, Cambridge Isotopes) was dissolved in a mixture of distilled water (5 mL) and 50% aqueous fluoroboric acid (3.4 mL, Avocado Chemicals). The solution was cooled to 0 °C and a solution of sodium nitrite (0.69 g, 10 mmol) in water (1.5 mL) was added dropwise. The suspension was stirred keeping the temperature between 0 and 4 °C for 30 min and then filtered. The residue was recrystallised from acetone/diethyl ether to give the benzenediazonium salt as a white powder (1.69 g, 86%).

Synthesis of [2,3,4,5,6-^{2}H,^{18}O]phenol:[19] Concentrated sulfuric acid (125 μL) was added to a stirred paste of [$^2H_5$]benzenediazonium tetrafluoroborate (1.0 g, 5.1 mmol) in 97% [$^{18}O_2$]water (1 mL, Cambridge Isotopes). The mixture was then heated to 65 °C until evolution of nitrogen ceased. The solution was extracted with diethyl ether several times. The ethereal layer was washed with 1 M HCl and saturated brine, dried over magnesium sulfate, and concentrated under reduced pressure. The crystalline residue was purified by sublimation to give the labelled phenol as colourless crystals (134 mg, 26%). The final ^{18}O enrichment was found by GC-MS to be at least 80%.

Synthesis of [2,3,5,6-^{2}H,^{18}O]4-hydroxymandelic acid:[20] [$^2H_5$,^{18}O]Phenol (94.1 mg, 0.93 mmol) was added to a solution of sodium hydroxide (8 mg) in distilled water (0.8 mL), followed by the dropwise addition of a solution of glyoxylic acid (92 mg, 1.24 mmol) in distilled water (100 μL). The mixture was stirred for 5 h at 30 °C, then adjusted to pH 6.0 by the addition of hydrochloric acid and washed with toluene to remove unreacted phenol. The aqueous layer was then adjusted to pH 1.5, and the product was extracted into diethyl ether by the use of continuous liquid/liquid extraction overnight. Evaporation of the solvent under reduced pressure gave the labelled 4-hydroxymandelic acid (146 mg, 90%) as a white powder. The ^{18}O enrichment was found by mass spectrometry to be 87%. ^{1}H NMR (500 MHz, D_2O): $\delta = 5.2$ (s); ^{13}C NMR (500 MHz, D_2O): δ: 176.9 (C=O), 155.8 (C–OH), 130.1 (C), 128.4 (CD, t, $J_{C-D} = 24$ Hz), 115.3 (CD, t, $J_{C-D} = 24$ Hz), 72.5 (CH). HRMS (ESI) calcd for $C_8D_4H_4O_3{}^{18}ONa$: 197.0604 [M+Na]$^+$; found: 197.0608 (+2.0 ppm).

[2,3,5,6-^{2}H]4-hydroxymandelic acid was produced by the same method, except distilled water was used in place of [^{18}O]H_2O during the formation of the labelled phenol.

Growth of *A. orientalis*: A 250 mL flask with spring containing common streptomyces medium (CSM; 50 mL) was inoculated with *Amycolatopsis orientalis* (A82846.2) from a frozen permanent stock and grown for three days at 30 °C with shaking at 300 rpm. An aliquot (5 mL) of this starter culture was then transferred to FermB medium (50 mL, 30 °C, 300 rpm). The culture was fed [$^2H_4$,^{18}O]4-hydroxymandelic acid (10 mg) in water (10 mL) adjusted to pH 7.0 with NaOH (0.1 M) in four portions over a period between 36–48 h of growth. The culture was harvested at 110 h and purified as described below.

FermB media: Anhydrous glucose (12.0 g), potato dextrin (24.0 g), bacteriological peptone (bacto peptone, 12.0 g), beet molasses (2.4 g) and calcium carbonate (1.2 g) were dissolved in distilled water (300 mL), and the pH was adjusted to 7 with 1 M sodium hydroxide solution. The medium was then autoclaved.

Isolation and purification of glycopeptides: The glycopeptide-containing medium was purified by the use of a D-alanine-D-alanine-based affinity column.

The column was synthesised as follows. CH-Sepharose 4B (2 g) was added to hydrochloric acid (100 mL, 1 mM) and allowed to swell before washing on a glass sinter for 10 min with hydrochloric acid (400 mL, 1 mM). The D-alanine-D-alanine (63.8 mg, 0.40 mmol) dipeptide was dissolved into coupling buffer (0.1 M $NaHCO_3$/0.5 M NaCl, 12 mL, pH 8.0) before being combined with the activated resin and rotated end over end for 90 min. The resin was extracted by filtration and washed again with coupling buffer (200 mL), then resuspended in aqueous ethanolamine hydrochloride (25 mL, 1 M, pH 8.0). After 60 min, the suspension was filtered and washed with five cycles of 0.1 M sodium acetate/0.5 M NaCl (150 mL, pH 4.0) followed by 0.1 M Tris HCl/0.5 M NaCl (150 mL, pH 8.0) alternately, and finally left to equilibrate with sodium phosphate buffer (0.02 M, pH 7.0) before use.

The production culture was pelleted by centrifugation (6470 rpm, 5000 *g*), and the cell-free supernatant was filtered through a GF/A glass fibre filter under suction. The filtrate was adjusted to pH 7.0 (where necessary) and loaded onto the D-alanine-D-alanine column by using a peristaltic pump at a flow rate of 0.5 mL min^{-1}. The column was washed sequentially with aqueous sodium phosphate (40 mL, 0.2 M, pH 7.0) and then aqueous ammonium acetate (40 mL, 0.4 M, pH 7.8), and then 10% acetonitrile in water (40 mL). The glycopeptide was eluted with 0.1 M NH_4OH/acetonitrile (1:1, 40 mL), and the eluate was lyophilised.

The column was regenerated by washing with 0.4 M aqueous sodium carbonate/30% acetonitrile (80 mL, pH 11.0), and was then re-equilibrated with sodium phosphate buffer (50 mL, 0.02 M, pH 7.0).

GC-MS analysis: GC-MS analysis of labelled phenol was performed on a Perkin–Elmer Autosystem XL GC connected to a Perkin–Elmer Turbomass Spectrometer. Helium was used as a carrier gas, and detection was carried out by electron impact ionisation. The column used was a Perkin–Elmer Elite-Pe-5MS. The method used started at

40 °C, which was held for 1 min, before heating to 230 °C at a rate of 10 °C min^{-1}. The column was then held at 230 °C for 5 min.

LC-ESI-MS analysis: Initial HPLC-ESI-MS was performed on an Agilent HP 1100 HPLC system connected to a ThermoFinnigan LCQ fitted with an electrospray ionization (ESI) source. Samples were injected onto a Phenomenex Luna C18(2) column (250×2 mm, 5 μm), eluting with a linear gradient of 0 to 60% acetonitrile (0.1% trifluoroacetic acid) in water (0.1% trifluoroacetic acid) over 30 min with a flow rate of 0.3 mL min^{-1}.

High-resolution HPLC-ESI-MS was performed on a Thermo Electron LTQ-Orbitrap. Samples were injected onto a Dionex Acclaim PepMap 100 column (C18, 3μ, 100 Å), eluting with a linear gradient of 0 to 100% acetonitrile (0.1% formic acid) in water (0.1% formic acid) over 28 min with a flow rate of 50 μL min^{-1}.

Chloroeremomcyin, eremomycin and dechloroeremomycin were all identified by high-resolution accurate mass to within 3 ppm of the predicted mass.

Acknowledgements

This work was supported by a project grant from the Biotechnology and Biological Sciences Research Council (BBSRC) to J.B.S; A.N.H. would like to thank the BBSRC for his studentship and F. J. Leeper for his help with proofreading this work.

Keywords: biosynthesis · enzymes · glycopeptides

[1] R. D. Süssmuth, W. Wohlleben, *Appl. Microbiol.* **2004**, *63*, 344–350.
[2] M. Khare, D. Keady, *Expert Opin. Pharmacother.* **2003**, *4*, 165–177.
[3] H. R. Perkins, *Biochem. J.* **1969**, *111*, 195.
[4] J. C. J. Barna and D. H. Williams, *Annu. Rev. Microbiol.* **1984**, *38*, 339–357.
[5] R. C. Read, *J. Infect.* **2007**, *55*, 483.
[6] P. C. Appelbaum, *Clin. Microbiol. Infect.* **2006**, *12 S1*, 16–23.
[7] K. B. Stevenson, K. Searle, G. J. Stoddard, M. H. Samore, *Emerging Infect. Dis.* **2005**, *11*, 895–903.
[8] G. Poulakou, H. Giamarellou, *Expert Opin. Invest. Drugs* **2008**, *17*, 225–243.
[9] A. M. A. van Wageningen, P. N. Kirkpatrick, D. H. Williams, B. R. Harris, J. K. Kershaw, N. J. Lennard, M. Jones, S. J. M. Jones, P. J. Solenberg, *Chem. Biol.* **1998**, *5*, 155–162.
[10] E. Stegmann, S. Pelzer, D. Bischoff, O. Puk, S. Stockert, D. Butz, K. Zerbe, J. Robinson, R. D. Süssmuth, W. Wohlleben, *J. Biotechnol.* **2006**, *124*, 640–653.
[11] K. Zerbe, O. Pylypenko, F. Vitali, W. Zhang, S. Rouset, M. Heck, J. W. Vrijbloed, D. Bischoff, B. Bister, R. D. Süssmuth, Stefan Pelzer, W. Wohlleben, J. A. Robinson, I. Schlichting, *J. Biol. Chem.* **2002**, *277*, 47476–47485.
[12] O. Pylypenko, F. Vitali, K. Zerbe, J. A. Robinson, I. Schlichting, *J. Biol. Chem.* **2003**, *278*, 46727–46733.
[13] D. H. R. Barton, R. B. Boar, D. A. Widdowson, *J. Chem. Soc.* **1970**, 1208–1213.
[14] B. Zhao, F. P. Guengerich, A. Bellamine, D. C. Lamb, M. Izumikawa, L. Lei, L. M. Podust, M. Sundaramoorthy, J. A. Kalaitzis, L. Manmohan Reddy, S. L. Kelly, B. S. Moore, D. Stec, M. Voehler, J. R. Falck, T. Shimada, M. R. Waterman, *J. Biol. Chem.* **2005**, *280*, 11599–11607.
[15] K. Zerbe, K. Woithe, D. B. Li, F. Vitali, L. Bigler, J. A. Robinson, *Angew. Chem.* **2004**, *116*, 6877–6881; *Angew. Chem. Int. Ed.* **2004**, *43*, 6709–6713.
[16] K. Woithe, N. Geib, D. B. Li, M. Heck, S. Fournier-Rousset, O. Meyer, F. Vitali, N. Matoba, K. Abou-Hadeed, J. A. Robinson, *J. Am. Chem. Soc.* **2007**, *129*, 6887–6895.
[17] N. Geib, K. Woithe, K. Zerbe, D. B. Li, J. A. Robinson, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 3081–3084.
[18] P. Hlavica, *Eur. J. Biochem.* **2004**, *271*, 4335–4360.
[19] L. Kupczyk-Subotkowska, W. H. Saunders, Jr., H. J. Shine, *J. Am. Chem. Soc.* **1988**, *110*, 7153–7159.
[20] N. P. Powar, S. B. Chandalia, *J. Chem. Technol. Biotechnol.* **1989**, *46*, 219–232.

Received: May 1, 2008
Published online on August 1, 2008

