Biosynthesis of the Vancomycin Group of Antibiotics: Involvement of an Unusual Dioxygenase in the Pathway to (*S*)-4-Hydroxyphenylglycine

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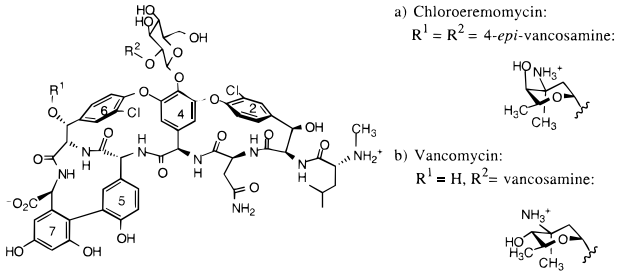
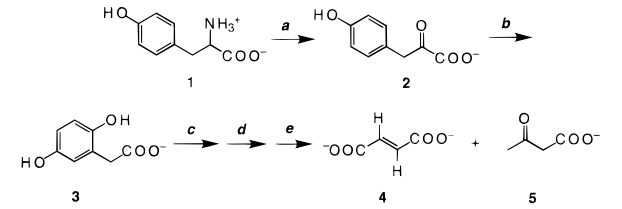
The vancomycin family of glycopeptide antibiotics are important as drugs of last resort against gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA).[1] Vancomycin was isolated some 40 years ago[2] and its structure determined by X-ray crystallography and NMR in the late 1970s.[3−5] Its complex structure has proved a formidable challenge to synthetic chemists, with the total synthesis only recently being achieved.[6−10] Bacterial strains resistant to vancomycin have been isolated in ever increasing numbers, raising the possibility that a MRSA strain may soon acquire resistance to vancomycin. An attractive approach to the synthesis of new glycopeptide antibiotics, with potentially greater potency than vancomycin, is to manipulate the biosynthetic pathway of the producing organism. To make this possible, however, each step in the biosynthetic pathway has first to be elucidated.

Recently, 72 KB of genomic DNA from *Amycolatopsis orientalis* has been sequenced and proposed to contain the biosynthetic gene cluster for chloroeremomycin, a glycopeptide antibiotic whose aglycon has the same structure as that of vancomycin (Scheme 1).[11] This proposal is strongly supported by the successful expression of a glycosyl transferase from within the gene cluster and the demonstration that it catalyzes the addition of glucose to chloroeremomycin aglycon.[12] Gene disruption experiments in the analogous balhimycin gene cluster have indicated which genes in the cluster code for the oxidative enzymes are responsible for cross-linking the aromatic residues along the peptide backbone. This has led to the isolation of the linear heptapeptide from this strain, which is presumed to be an intermediate in the biosynthesis of balhimycin.[13] In this paper we report the overexpression of the protein coded for by ORF 21 from the chloroeremomycin gene cluster, and its characterization as an unusual dioxygenase responsible for converting 4-hydroxyphenylpyruvic acid into 4-hydroxymandelic acid. Identification of 4-hydroxymandelic acid synthase leads us to propose that it is the pivotal enzyme in the biosynthesis of (*S*)-4-hydroxyphenylglycine.

Scheme 1. Structures of (a) Chloroeremomycin and (b) Vancomycin



Scheme 2: Tyrosine Degradation Pathway[a]



[a] Enzymes: (a) tyrosine transaminase, (b) 4-hydroxyphenylpyruvate dioxygenase, (c) homogentisate oxidase, (d) 4-maleylacetoacetate isomerase, and (e) 4-fumarylacetoacetate lyase.

(*R*)-4-Hydroxyphenylglycine, found at positions 4 and 5 of the heptapeptide backbone of chloroeremomycin, is one of a number of rare amino acids found in glycopeptide antibiotics (Scheme 1). Since the peptide synthetase involved in chloroeremomycin biosynthesis contains an epimerase domain for amino acids **4** and **5**,[11] then (*S*)-4-hydroxyphenylglycine must be the precursor of the (*R*)-4-hydroxyphenylglycine residues. Feeding experiments have determined that it is formed from L-tyrosine.[14] However, none of the intermediates or enzymes on the pathway to (*S*)-4-hydroxyphenylglycine have been identified so far. L-Tyrosine **1** is degraded by some microorganisms by a well-characterized pathway to fumarate **4** and acetoacetate **5** (Scheme 2).[15] The second enzyme of this pathway, hydroxyphenylpyruvic acid dioxygenase (4-HPPD), catalyzes an interesting reaction: coupling the oxidative decarboxylation of the ketoacid **2** with activation of molecular oxygen which is postulated to effect the epoxidation of the aromatic ring followed by a 1,2-alkyl shift (NIH-shift) to give homogentisate **3**.[15,16] 4-HPPD bears a general sequence similarity to the large class of α-ketoglutarate-dependent dioxygenases. However, unlike α-ketoglutarate-dependent dioxygenases, the substrate acts as both the ketoacid and the substrate for hydroxylation.[17] ORF 21 showed some sequence similarity to 4-HPPD (43% similarity and 34% identity). Therefore, we hypothesized that it might be involved in the pathway to (*S*)-4-hydroxyphenylglycine, since oxidative decarboxylation of hydroxyphenylpyruvic acid would give the correct carbon skeleton for (*S*)-4-hydroxyphenylglycine.

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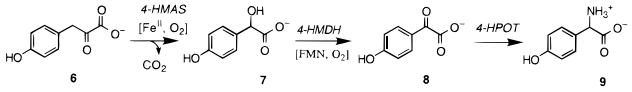
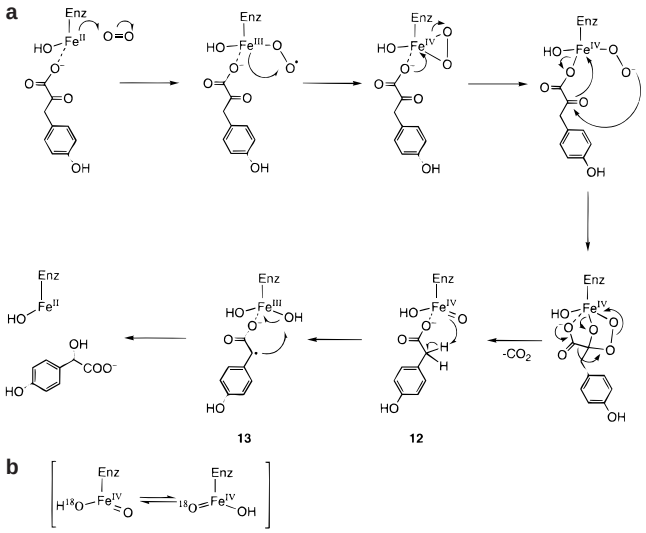
Scheme 3. Proposed Biosynthetic Pathway for 4-Hydroxyphenylglycine[a]



[a] 4-HMAS, 4-hydroxymandelate synthase; 4-HMDH, 4-hydroxymandelate dehydrogenase; 4-HPOT, 4-hydroxyphenylglyoxalate transaminase

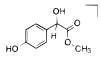
Scheme 4. (a) Proposed Mechanism and (b) "Redox Tautomerism"



To determine the function of ORF 21, the gene was amplified by polymerase chain reaction (PCR), cloned into pET28a, and overexpressed in *Escherichia coli* BL21(DE3) to give the recombinant protein His-tagged at the N-terminal end. Purification using Ni^{2+}-NTA resin gave homogeneous protein with a molecular mass of 40368 ± 17 Da as determined by electrospray-MS (calcd 40371 Da, data not shown) which runs as a homodimer on a gel filtration column. The protein was incubated with the same substrates (4-hydroxyphenylpyruvic acid, Fe^{2+}, DTT, and ascorbate) that are used by 4-HPPD to produce homogentisate.[16] The products of this reaction were extracted with diethyl ether, derivatized with diazomethane to give their methyl esters, and analyzed by GC-MS. The GC trace shows that 4-hydroxyphenylpyruvic acid is not converted to homogentisate, but rather a new compound is formed that runs 1.5 min faster. This compound was identified as the methyl ester of 4-hydroxymandelic acid **7** by comparison with an authentic sample (identical retention time and fragmentation pattern). This result establishes that the enzyme coded for by ORF 21, herewith named 4-hydroxymandelic acid synthase (4-HMAS), carries out a similar oxidative decarboxylation of hydroxyphenylpyruvic acid as 4-HPPD. However, the putative Fe^{IV}═O species[18] hydroxylates the benzylic position rather than attacking the aromatic ring (Scheme 4a).

ORF 22, which immediately follows *HmaS* (previously named ORF21*)*, has sequence similarity to glycolate oxidase. Therefore, it is tempting to suggest that 4-hydroxymandelic acid is oxidized to its ketoacid derivative **8** and then transaminated, possibly by ORF 17, to give (*S*)-4-hydroxyphenylglycine **9** (Scheme 3).[19] The gene cluster does not contain any ORF homologous to L-tyrosine transaminase. However, it is likely that the 4-hydroxyphenylpyruvate formed as a result of the normal degradation pathway for L-tyrosine would be sufficient for the synthesis of 4-hydroxymandelic acid.

Table 1. Incorporation of ^{18}O into 4-Hydroxymandelic Acid

			% abundance			
				enzymic incubation in the presence of		
ion	*m/z*	^{18}O atoms	standard	$^{16}O_2$/ $H_2{}^{16}O$	$^{18}O_2$/ $H_2{}^{16}O$	$^{16}O_2$/ $H_2{}^{18}O$
molecular ion:						
 **10**	182	0	100	100	18	11
	184	1	2	3	47	48[a]
	186	2	0	0	35	41[a]
first fragment ion:						
 **11**	123	0	100	100	53	60
	125	1	2	2	47	40

[a] See ref 20.

To determine the origin of the oxygen atoms in 4-hydroxymandelic acid **7**, the reaction was carried out under an atmosphere of $^{18}O_2$. The methyl ester **10** of 4-hydroxymandelic acid gives a useful fragmentation pattern in the mass spectrometer (EI) with cleavage of the ester group to give fragment **11** (Table 1). This allows accurate determination of the incorporation of ^{18}O into the carboxyl group and the benzylic hydroxyl group. The results show that one oxygen atom from molecular oxygen is incorporated into the carboxyl group and one into the benzylic hydroxyl group, thus supporting the mechanism shown in Scheme 4a. The incorporation of ^{18}O into the hydroxyl group shows considerable dilution by ^{16}O (approximately 55%), whereas very little dilution has occurred in the incorporation into the carboxyl group. To test if the dilution of ^{18}O arose from exchange with water, the reaction was carried out in $H_2{}^{18}O$. The results (Table 1) show approximately 50% incorporation into the hydroxyl group.[20] Previous work with model systems has found that a water (or hydroxyl) ligand on the pressumed Fe^{IV}═O species, such as intermediate **12** (Scheme 4a), can be deprotonated and undergo "Redox Tautomerism" to give 50% incorporation of label from the water (Scheme 4b).[21] Exchange with water is not commonly observed with most hydroxylases, such as those containing cytochrome P_{450}, but has been reported to take place with some other non-heme Fe-containing oxygenases.[22]

In summary, we have demonstrated that *HmaS*, previously referred to as ORF 21, codes for an α-ketoacid dioxygenase that converts 4-hydroxyphenylpyruvate to 4-hydroxymandelic acid. The enzyme is closely related to 4-HPPD, which is involved in the degradation of L-tyrosine, suggesting that 4-HMAS may have evolved from this enzyme. This study implies that the pathway to (*S*)-4-hydroxyphenylglycine from L-tyrosine involves the intermediacy of 4-hydroxymandelic acid and, therefore, sheds new light on how the biosynthesis of these important antibiotics takes place.

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Supporting Information Available: Experimental procedures, including the subcloning and expression of 4-HMAS, reaction conditions for the formation of 4-hydroxymandelic acid from 4-hydroxyphenylpyruvate by 4-HMAS, and GC-MS data for 4-hydroxymandelic acid (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(19) We have subcloned and expressed ORF 17 and preliminary results show that it catalyzes the formation of (*S*)-4-hydroxyphenylglycine from 4-hydroxyphenylglyoxalate in the presence of pyridoxal phosphate as a cofactor.

(20) The incorporation of ^{18}O into the carboxyl group of hydroxymandelic acid occurs because the keto group of 4-hydroxyphenylpyruvic acid rapidly exchanges with $H_2{}^{18}O$ [see also: Lindblad, B.; Lindstedt, G.; Lindstedt, S. *J. Am. Chem. Soc.* **1970**, 92, 7446−7449].

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