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Characterisation of a hydroxymandelate oxidase involved in the biosynthesis of two unusual amino acids occurring in the vancomycin group of antibiotics†

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ORF22 from the chloroeremomycin gene cluster has been cloned, expressed and characterised as a hydroxymandelate oxidase (HmO) that is involved in the formation of both (*S*)-4-hydroxyphenylglycine and (*S*)-3,5-dihydroxyphenylglycine.

The glycopeptide antibiotics vancomycin (**1**, Fig. 1) and teicoplanin are currently the treatment of last resort against methicillin-resistant *Staphylococcus aureus*.[1] The continued rise of vancomycin-resistant bacteria has heightened the need for new therapeutic agents. Manipulation of the biosynthetic gene cluster of glycopeptide antibiotics may be a potential route to new antibiotics. To explore this approach we have set out to elucidate the function of the genes involved in chloroeremomycin biosynthesis, another important member from the vancomycin family (**2**, Fig. 1).[2] In this communication we report the overexpression of the protein coded for by *orf*22 from the chloroeremomycin gene cluster, and its characterisation as a flavin-dependent oxidase catalysing the conversion of 4-hydroxymandelic acid and 3,5-dihydroxymandelic acid to their corresponding keto acids.

(*R*)-4-Hydroxyphenylglycine, found at positions 4 and 5 of the heptapeptide backbone of chloroeremomycin, is one of a number of rare amino acids in glycopeptide antibiotics. The peptide synthetase involved in the biosynthesis of chloroeremomycin contains epimerase domains for amino acids 2, 4 and 5, which suggests that (*S*)-4-hydroxyphenylglycine is the precursor of (*R*)-4-hydroxyphenylglycyl units at positions 4 and 5. Recently we have determined that *orf*21 codes for a dioxygenase involved in the pathway to (*S*)-4-hydroxyphenylglycine.[3] This enzyme, 4-hydroxymandelic acid synthase (HmaS), catalyses the production of 4-hydroxymandelate (**4**) (4HMA) from 4-hydroxyphenylpyruvate (**3**) (4HPP) (Fig. 2). Based on this discovery, it was postulated that ORF22, which shows homology to a known glycolate oxidase[4] (50% similarity and 40% identity) might oxidise 4-hydroxymandelic acid to its keto-acid derivative (**5**) followed by transamination, possibly by ORF17, to give (*S*)-4-hydroxyphenylglycine (**6**) (Fig. 2). During the preparation of this manuscript, Hubbard *et al.* published a paper independently establishing parts of the results presented here.[5]

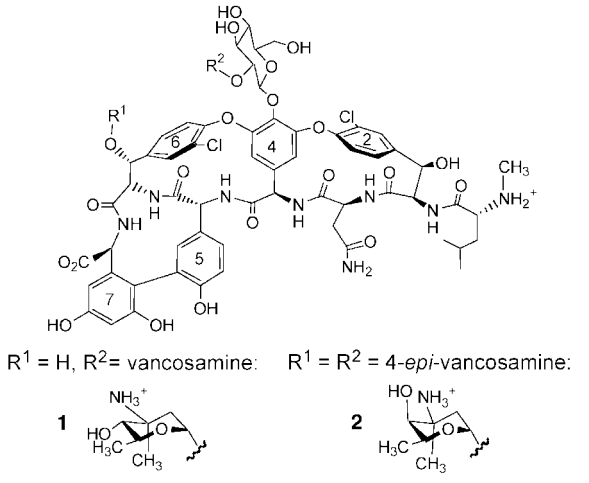

Fig. 1 Structures of vancomycin (**1**) and chloroeremomcyin (**2**).

In order to confirm the proposed pathway for the formation of 4HPG, *orf*22 was amplified by polymerase chain reaction (PCR) and cloned into the expression vector pET28a(+) (Novagen) as an *N*-terminal His_6-tagged protein. The resulting plasmid was used to transform the expression host *E. coli* BL21(DE3). Overexpression was carried out at 37 °C in 1 L 2 × YT medium with induction by isopropyl β-D-thiogalactoside (IPTG, 1 mM). The protein was recovered as an ammonium sulfate precipitate and further purified on Ni^{2+}-NTA agarose resin. The resulting protein was then transferred into 50 mM HEPES buffer, pH 7.6 using Millipore centrifugal filters. The purified protein showed a single band on a SDS-PAGE gel. The relative molecular mass was determined by ESI-MS to be 39863 ± 5 Da which was in excellent agreement with that calculated from the protein sequence (39864 Da). The enzyme contained a cofactor which was released when the protein was denatured by heat. This was determined to be FMN by HPLC analysis.

The enzyme (50 μg) was incubated with commercially available racemic 4HMA (20 mM) in HEPES buffer (50 mM, pH 7.6, 0.5 mL) in the presence of molecular oxygen, flavin mononucleotide (FMN, 0.5 mM) and catalase (1600 IU) at 25 °C for 16 h. The reaction was then quenched with 100 μL 6 M HCl and extracted with ethyl acetate (3 × 1 mL). The combined organic phases were dried with N_2 gas and the products converted to their methyl esters using diazomethane. The mixture was analysed by ammonia chemical ionisation GC-MS. The GC trace clearly shows that a new compound had been produced with a retention time of 16.0 min (Fig. 3, the substrate had a retention time of 16.0 min). The newly formed product

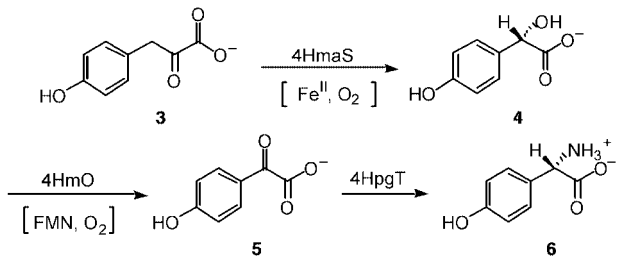

Fig. 2 Proposed biosynthetic pathway for 4-hydroxyphenylglycine (**6**): 4HmaS, 4-hydroxymandelate synthase; 4HmO, 4-hydroxymandelate oxidase; 4HpgT, 4-hydroxyphenylglyoxylate transaminase.

† Electronic supplementary information (ESI) available: SDS-PAGE analysis of HmO and molecular weight analysis of HmO protein by ESI-MS and GC/CI-MS of the enzymatic conversion of 3,5DHMA with HmO. See http://www.rsc.org/suppdata/cc/b1/b103548g/

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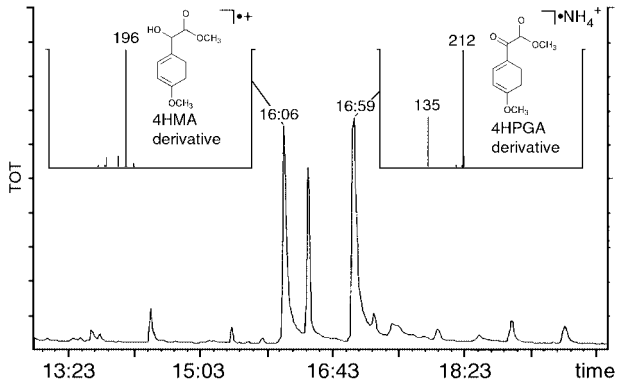

Fig. 3 GC trace and CI mass spectrum of an enzyme reaction of 4HMA with HmO after derivatisation by diazomethane showing 4-hydroxyphenylglyoxylic acid (4HPGA, product) and 4-hydroxymandelic acid (4HMA, substrate).

was identified as 4-methoxyphenylglyoxylate methyl ester (identical retention time and fragmentation pattern to standard material). This identifies *orf*22 as a hydroxymandelic acid oxidase (HmO) and strongly suggests that it is involved in the formation of (*S*)-4-hydroxyphenylglycine.

The stereochemistry of the substrate was elucidated using a racemic mixture of 4HMA and determining the optical rotation of the enantiomer remaining at the end of the incubation. Thus, the 4-hydroxymandelic acid isolated from the reaction had a negative optical rotation ($[\alpha]_D^{25}$ −5.1°, c = 2.5, ethanol) which corresponds to the same sign as synthetic (*R*)-4HMA ($[\alpha]_D^{25}$ −10.2°, c = 0.98, H_2O).[6] The enzyme was also found to oxidise commercially available (*S*)-mandelic acid but not (*R*)-mandelic acid. These combined results lead to the conclusion that (*S*)-4HMA is the natural substrate for the enzyme. Previous studies had determined that (*S*)-3,5-dihydroxyphenylglycine, another rare amino acid found in glycopeptide antibiotics, was formed from acetate *via* a polyketide pathway.[7,8] Analysis of the gene cluster reveals *orf*27 to be homologous to a number of chalcone synthases (polyketide synthases found mainly in plants). Recent work suggests that the pathway to (*S*)-3,5-dihydroxyphenylglycine involves the formation of 3,5-dihydroxyphenylacetic acid by ORF27[9a] followed by hydroxylation at the benzylic position, oxidation to the keto-acid and transamination to (*S*)-3,5-dihydroxyphenylglycine. The last two steps in this pathway are the same as those for (*S*)-4-hydroxyphenylglycine. Therefore it is tempting to suggest that ORF22 might also participate in this pathway and catalyse the oxidation 3,5-dihydroxyphenylmandelate to its keto-acid derivative. To test this, racemic 3,5-dihydroxymandelic acid[9b] was incubated with HmO as described previously (a shorter incubation time was used in this case) and the products analysed by GC-MS. The results show that 3,5-dihydroxymandelic acid is oxidised by the enzyme to give 3,5-dihydroxyphenylglyoxylic acid. This provides strong evidence that HmO is involved in the formation of both (*S*)-4-hydroxyphenylglycine and (*S*)-3,5-dihydroxyphenylglycine. The reaction catalysed by HmO can therefore be summarised as shown in Fig. 4.

Flavin-dependent enzymes are normally divided into two classes, oxidases and dehydrogenases.[10] Oxidases use molecular oxygen to oxidise the flavin after each reaction cycle with the formation of hydrogen peroxide, whereas dehydrogenases re-oxidised the flavin through electron transfer from proteins, such as ubiquinine or cytochrome b_5. To determine whether HmO is an oxidase or dehydrogenase the incubation mixtures were tested for the presence of hydrogen peroxide using a coupled assay of horseradish peroxidase (HRP) and *o*-dianisidine.[11,12] HRP generates *o*-dianisidine radicals which combine with 4HMA to give a product with an absorbance maximum at 550 nm (Fig. 5, trace a). When HmO was incubated with HRP and *o*-dianisidine an increase in absorbance at 550 nm was observed (Fig. 5, trace b). This determines that HmO is formally a flavin dependent oxidase.

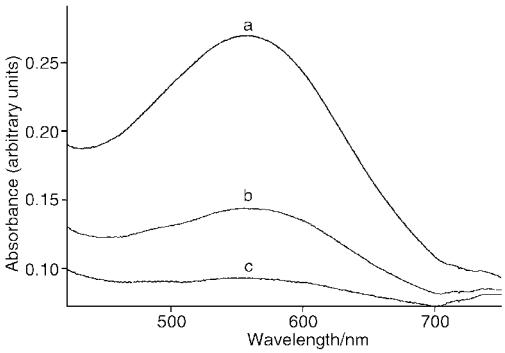

Fig. 5 Visible spectra probing the generation of H_2O_2 during HmO catalysis. a: enzymic reaction of horseradish peroxidase plus H_2O_2, *o*-dianisidine and 4HMA, b: enzymic reaction of HmO plus *o*-dianisidine and horseradish peroxidase, c: blank reaction omitting HmO.

In summary, we have identified the enzyme coded for by *orf*22, as a hydroxymandelate oxidase (HmO) which carries out the oxidation of both 4-hydroxymandelic acid and 3,5-hydroxymandelic acid. This provides strong evidence that it is involved in the pathway to both (*S*)-4-hydroxyphenylglycine and (*S*)-3,5-dihydroxyphenylglycine and sheds new light on how these important antibiotics are biosynthesised.

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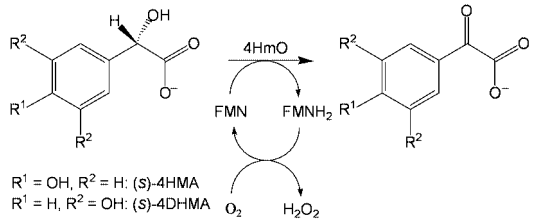

Fig. 4 Oxidation of (*S*)-4-hydroxymandelate ((*S*)-4HMA) and (*S*)-3,5-dihydroxymandelate ((*S*)-4DHMA) to 4-hydroxyphenylglyoxylate by 4HmO in the presence of FMN and molecular oxygen.

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