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Biosynthesis of the di-*meta*-hydroxyphenylglycine constituent of the vancomycin-group antibiotic chloroeremomycin

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The biosynthetic pathway to the L-di-*meta*-hydroxyphenylglycine (DHPG) constituent of chloroeremomycin (A82846B), has been investigated by a combination of feeding experiments with ^{13}C-labelled substrates, and expression and assay of HpgT from the chloroeremomycin biosynthetic cluster.

The rising incidence of resistance to vancomycin and teicoplanin[1,2]—currently the antibiotics of last resort for treating many nosocomial infections—necessitates the development of new therapeutic agents. An understanding of the mode of action of vancomycin group antibiotics[3] and the emerging resistance mechanisms[4,5] should facilitate rational design of new antibiotics capable of defeating vancomycin resistant bacteria. Some of our recent research on the vancomycin group of antibiotics has focussed on the biosynthesis of chloroeremomycin **1** (Fig. 1), also known as A82846B and LY264826, which differs from vancomycin in only its appended sugars. A gene cluster from *Amycolatopsis orientalis* A82846 for the biosynthesis of **1** has been sequenced.[6] Protein homologies have suggested roles for many of the putative enzymes encoded therein, and a number of these roles have now been confirmed experimentally.[7–11]

The heptapeptide core of chloroeremomycin contains several unusual amino acids, including four residues with D-stereochemistry at the alpha carbon, and five with non-proteinogenic side chains. Here we report two intermediates and the role of one enzyme, HpgT (*p*-hydroxyphenylglycine transaminase), in the biosynthetic pathway (Scheme 1) leading to L-di-*meta*-hydroxyphenylglycine (L-DHPG) **2**, found as the *C*-terminal residue of **1**. DHPG has previously been shown to be derived from four units of acetate,[12] but the subsequent biosynthetic steps have hitherto been uncertain.

We synthesised two possible precursors to DHPG, di-*meta*-hydroxyphenylacetic acid **3** and (±)-di-*meta*-hydroxymandelic acid **4** (Scheme 1), with ^{13}C-labelled carboxylate groups. These were characterized by NMR and mass spectroscopy; [1-^{13}C]-**3** δ_H (500 MHz, D_2O) 3.44 (2H, d, J^{13}_{CH} = 12 Hz), 6.21 (1H, s), 6.30 (2H, s): δ_C (100 MHz, D_2O, proton decoupled) 41.40 (d, $J^{13}_C{}^{13}_C$ = 54.2), 102.37, 109.56, 137.98, 157.88, 177.48 (^{13}C enriched): *m*/*z* (EI+) [M$^{+\cdot}$] = 169.0454, calculated for $^{12}C_7{}^{13}C_1{}^1H_8{}^{16}O_4$ = 169.0456; [1-^{13}C]-**4** δ_H (500 MHz, DMSO-d_6) 4.74 (1H, d, J^{13}_{CH} = 4.6 Hz), 6.02 (1H, br), 6.23 (2H, br), 9.13 (br): δ_C (125 MHz, DMSO-d_6, proton decoupled) 72.51 (d, $J^{13}_C{}^{13}_C$ = 58 Hz), 101.73, 104.84, 142.25, 158.17, 174.06 (^{13}C enriched): *m*/*z* (EI+) [M$^{+\cdot}$] = 185.0414, calculated for $^{12}C_7{}^{13}C_1{}^1H_8{}^{16}O_5$ = 185.0405. (Details of the syntheses will be published separately.) If incorporated by the producing strain, both should lead to isotopic enrichment at the carbonyl group of L-DHPG. To investigate the uptake of labelled substrates by *A. orientalis* A82846.2 (NRRL 18100), the strain was inoculated into CSM[13] (50 ml) and incubated at 30 °C/250 rpm for 3 days. A 2.5 ml aliquot of this culture was inoculated into FermB medium (50 ml) and incubated at 30 °C/250 rpm for 110 h. Labelled substrates were fed aseptically, in 4 × 5 mg portions, at either 36, 40, 44 and 48 h (for **3**) or 34, 38, 42 and 44 h (for **4**) after inoculation. Cells were removed by centrifugation, and the antibiotics purified from the filtered supernatant by affinity chromatography on sepharose-D-Ala-D-Ala (prepared from Activated CH-Sepharose 4B), desalted by reverse-phase HPLC and lyophilised. Typically, 5–10 mg of purified antibiotic was recovered. The ^{13}C NMR spectra of isolated chloroeremomycin showed enrichment of a peak at 172.1 ppm. The corresponding HMBC spectra, for **1** produced both from **3** and from **4**, showed a cross-peak to a proton at 4.42 ppm, identified as the C_α proton of DHPG, x_7 (see Fig. 1), confirming the identity of the enriched carbon as CX_7. No other cross peaks due to **1** were observed in these spectra. All spectra were run in DMSO-d_6 as **1** does not dimerise in DMSO, thus simplifying the spectra considerably. The natural abundance ^{13}C resonances were too weak to be seen in any of the spectra. Thus **3** and **4** are incorporated in the biosynthesis of DHPG.

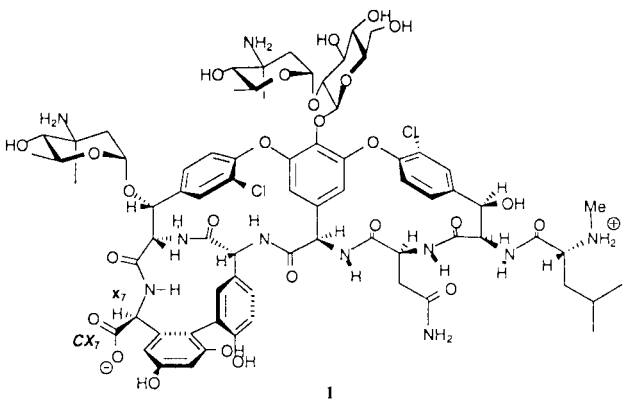
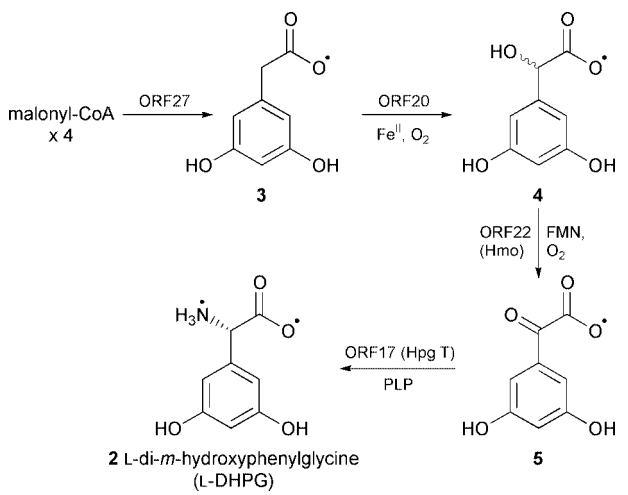


Fig. 1 The structure of chloroeremomycin **1**, showing the carbonyl position of DHPG, CX_7, and the adjacent C_α proton, x_7.



Scheme 1 Proposed biosynthetic pathway to L-DHPG.

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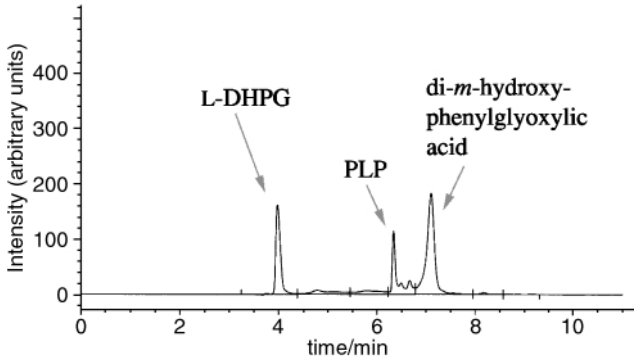

Fig. 2 HPLC trace (monitored at 254 nm) showing the HpgT-catalysed formation of di-*meta*-hydroxyphenylglyoxylic acid **5** from L-DHPG **2**.

To investigate the role of HpgT in the formation of DHPG, *hpgT* from the chloroeremomycin gene cluster was PCR amplified and cloned into the expression vector pET28a(+) (Novagen) as an *N*-terminal His_6-tagged protein. The resulting recombinant plasmid was used to transform the expression host *E. coli* BL21(DE3) (Novagen), and the cells grown for 16 h at 16 °C in 2 × YT medium, following induction by isopropyl β-D-thiogalactoside (IPTG, 1 mM). HpgT was purified using Novagen His-Bind Quick 900 cartridges, and then transferred to Tris-HCl (50 mM, pH 7.5) using Millipore™ centrifugal filters. The purified protein was visualised as a single band by SDS-PAGE, and the relative molecular mass determined by electrospray ionisation (ESI) mass spectrometry to be 49.80 kDa (predicted = 49.808 kDa).

The HpgT catalysed interconversion of **5** (Scheme 1) and L-DHPG was assayed as follows: HpgT (100 μg) was incubated with L-DHPG (1 mM), α-ketoglutarate (2 mM) and pyridoxal phosphate (PLP, 100 μM) in Tris-HCl (300 μl) for 1 h at 30 °C, after which time HPLC analysis showed a new, strongly absorbing peak (Fig. 2). ESI mass spectrometric analysis identified this peak as di-*meta*-hydroxyphenylglyoxylic acid ($[M + H]^+$ = 183.0, calculated for $C_8H_7O_5^+$ = 183.14). These results indicate that, in addition to its previously documented role in *p*-hydroxyphenylglycine biosynthesis,[11] HpgT is responsible for the transamination from **5** to L-DHPG, **2**.

Since another enzyme from the cluster, Hmo, has been shown to oxidise **4** to **5**,[14] the results given here identify the biosynthetic steps and intermediates from di-*meta*-hydroxyphenylacetate to L-DHPG in *A. orientalis* (Scheme 1) except the required benzylic hydroxylation (**3** to **4**). We have shown that ORF27 (a chalcone synthase homologue) produces di-*meta*-hydroxyphenylacetate from malonyl-CoA[15] and propose ORF20 (which shows homology to the P450 family of heme-binding proteins) from the chloroeremomycin biosynthetic cluster as a candidate for the benzylic hydroxylation; experiments are underway to investigate these proposals.

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