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Biosynthesis of the vancomycin group of antibiotics: characterisation of a type III polyketide synthase in the pathway to (*S*)-3,5-dihydroxyphenylglycine†

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3,5-dihydroxyphenylacetate, a precursor for the non-proteinogenic amino acid 3,5-dihydroxyphenylglycine occurring in glycopeptide antibiotics, is determined to be catalysed by a type III polyketide synthase using malonyl-CoA as a starter unit.

Vancomycin **1** (Fig. 1) is currently the antibiotic of last resort against methicillin-resistant *Staphylococcus aureus* (MRSA).[1] We are investigating the biosynthetic pathway of chloroeremomycin **2**, another important member of this family of glycopeptide antibiotics.

A number of unusual amino acids, such as (*S*)-4-hydroxyphenylglycine (4-HPG) and (*S*)-3,5-dihydroxyphenylglycine (3,5-DHPG) are found in glycopeptide antibiotics. Recently the pathway to 4-HPG from tyrosine has been elucidated[2–4] and the pivotal enzyme, 4-hydroxymandelic acid‡ synthase determined to be a novel dioxygenase.[2] In this paper we report the identification of the protein coded for by *orf*27 from the chloroeremomycin gene cluster, now named 3,5-dihydroxyphenylacetate synthase (DhpaS), as the first enzyme involved in the pathway to 3,5-DHPG. DhpaS catalyses the formation of 3,5-dihydroxyphenylacetate (3,5-DHPA) from four malonyl-CoA units and is only the second type III polyketide synthase fully characterised in bacteria.

Previous feeding studies using vancomycin-producing organisms and labeled acetate showed that the carbon skeleton of 3,5-DHPG was of polyketide origin.[5] Recently we have shown the involvement of 3,5-DHPA in the pathway to this amino acid by feeding experiments using the chloroeremomycin-producing strain *Amycolatopsis orientalis*. NMR studies showed that ^{13}C-labeled 3,5-DHPA was incorporated into the 3,5-DHPG moiety of the isolated antibiotic.[6] Homology searches of the open reading frames (*orfs*) from the biosynthetic gene cluster of chloroeremomycin[7] revealed that *orf*27 had homology (37% similarity, 28% identity) to a group of plant chalcone synthases which provide precursors for flavonoids.[8] This family of small discrete enzymes have been called type III polyketide synthases. They differ from other polyketide synthases by not possessing a phosphopantetheine prothetic group. Chalcone synthases typically use aromatic coenzyme A esters, such as coumaryl-CoA, as starter units and malonyl-CoA as the chain extender unit. Based on these findings we felt that ORF27 might catalyse the formation of 3,5-DHPA **4** from an acetyl-CoA starter unit and three malonyl-CoA extender units. Hydroxylation at the benzylic position of **4** would lead to 3,5-dihydroxymandelic acid **5**, oxidation to 3,5-dihydroxyphenylglyoxylic acid **6** and subsequent transamination should then furnish 3,5-dihydroxyphenylglycine **7** (Scheme 1).

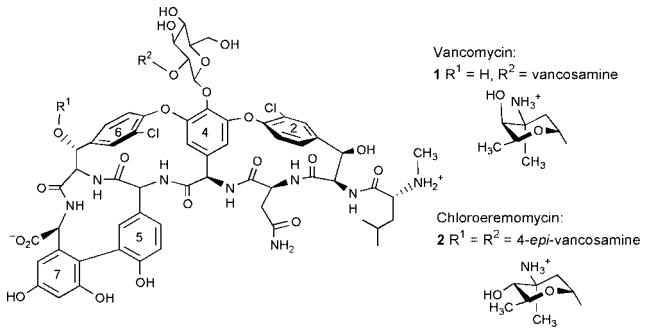
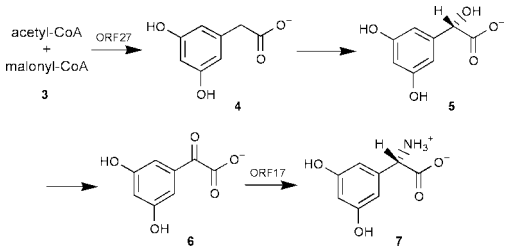


Fig. 1 Structures of vancomycin and chloroeremomycin.

To test this hypothesis *orf*27 from the chloroeremomycin gene cluster was amplified by polymerase chain reaction using the cosmid pCZA361. Ligation into pET28 (Novagen) and transformation into *E. coli* BL21(DE3) yielded the *N*-terminal His_6-tagged protein which was purified using Ni^{2+}-NTA agarose. Electrospray mass spectrometry of the purified protein confirmed the right molecular weight for the protein without a phosphopantheine arm, therefore being consistent with a type III polyketide synthase. The enzyme was incubated in Hepes buffer (50 mM, pH 7.2) containing DTT (1,4-sulfanylbutane-2,3-diol,1.0 mM), acetyl-CoA (3.3 mM) and malonyl-CoA (10 mM) for 2 h. The enzymic mixture was acidified and extracted with ethyl acetate, silylated and analyzed by GC-CI-MS (NH_3 as reaction gas). The GC trace revealed a new peak when compared to a control reaction (Fig. 2). This compound was identified as the tris(trimethylsilyl) derivative of 3,5-dihydroxyphenylacetate by comparison with an authentic sample (identical retention time and fragmentation pattern) (Fig. 3). This proves that ORF27 is a type III polyketide synthase that catalyses the first step in the pathway to 3,5-DHPG.

The mechanism in which the polyketide chain cyclises to form 3,5-DHPA is interesting since it predicts that, if acetyl-CoA is the starter unit, an anion must be formed from a methyl ketone **8** (Scheme 2, A). In the vast majority of polyketides the folding pattern arises from an anion generated at a more acidic methylene position. It has recently been reported that malonyl-CoA serves as the starter unit for the formation of the polyketide 1,3,6,8-tetrahydroxynaphthalene.[9] If malonyl-CoA was used as the starter unit for 3,5-DHPA then the anion of the methyl ketone could be generated by decarboxylation of **9**,[10] which would be energetically more favorable than deprotonation of the



Scheme 1 Proposed pathway for 3,5-dihydroxyphenylglycine formation.

† Electronic supplementary information (ESI) available: electrospray mass spectrum of DhpaS. See http://www.rsc.org/suppdata/cc/b1/b106638b/

 DOI: 10.1039/b106638b

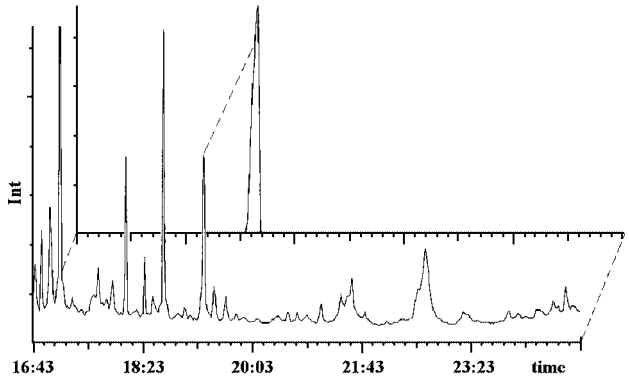


Fig. 2 GC trace on an enzymic reaction of malonyl-CoA in the presence of ORF27 (standard of 1-[^{13}C]-3,5-DHPA in the background).

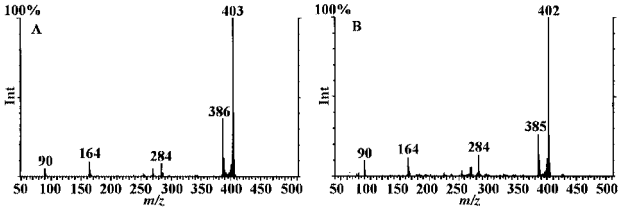
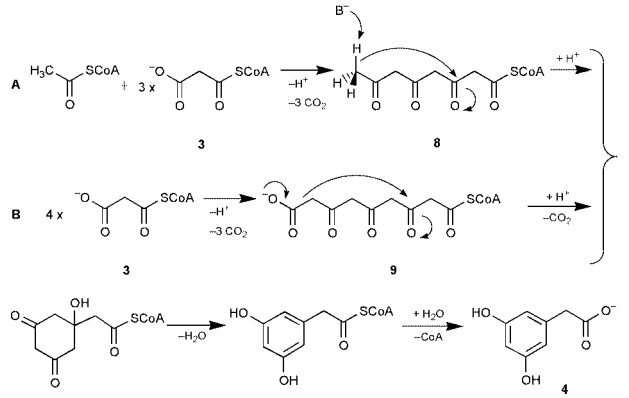
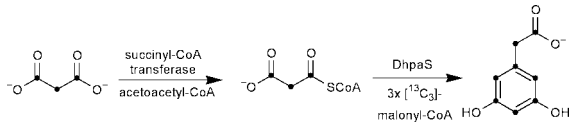


Fig. 3 (A) CI-MS spectrum of [1-^{13}C]-3,5-DHPA standard silylated (note that the standard has one ^{13}C atom and therefore gives a molecular ion one mass higher than the enzymic sample). (B) CI-MS spectrum of the peak at 19.2 minutes from the enzymic reaction (Fig. 2).



Scheme 2 Formation of 3,5-dihydroxyphenylacetic acid using either acetyl-CoA and malonyl-CoA (A) or malonyl-CoA only (B).

methyl ketone (Scheme 2, B). To determine the origin of the starter unit and investigate these two possible reaction mechanisms, labeled malonyl-CoA (formed *in situ* by transfer of CoA from acetoacetyl-CoA to [1,2,3-$^{13}C_3$]malonic acid using succinyl-CoA transferase[11]) was incubated with DhpaS in the presence of unlabeled acetyl-CoA (Scheme 3). The mass spectrum of the isolated 3,5-DHPA gives only an $[M + 8]^+$ peak, clearly showing that all the carbon atoms come from malonyl-CoA (Fig. 4).



Scheme 3 *In situ* formation of [1,2,3-$^{13}C_3$]malonyl-CoA and its utilisation by DhpaS to form labeled 3,5-DHPA (^{13}C represented as •).

The family of chalcone synthases are relatively small proteins that appear to have only one active site.[12] This is in contrast to type I polyketide synthases which are typically very large multifunctional proteins with a separate active site for each

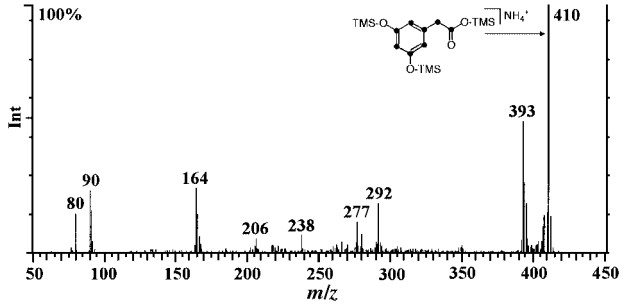


Fig. 4 Mass spectrum of labeled 3,5-DHPA formed by enzymic reaction of dihydroxyphenylacetate synthase with [1,2,3-$^{13}C_3$]malonyl-CoA in the presence of unlabeled acetyl-CoA.

reaction that they catalyse. It is therefore tempting to propose that the cyclisation to form 3,5-DHPA might be catalyzed by the same amino acids that carry out the Claisen condensation of malonyl-CoA with the growing polyketone chain since the two reactions are very similar.

In summary, we have shown that ORF27 from the chloroeremomycin gene cluster is an unusual type III polyketide synthase catalyzing the formation of 3,5-dihydroxyphenylacetic acid. Labeling experiments prove that malonyl-CoA acts not only as the chain-extender unit but also as the starter unit. This leads us to propose that cyclisation to form 3,5-dihydroxyphenylacetic acid is initiated by decarboxylation of the polyketone chain **9**.[13] This study determines that the biosynthesis of 3,5-dihydroxyphenylglycine begins with the formation of 3,5-dihydroxyphenylacetic acid or its CoA-ester, and therefore affords a new insight into how the biosynthesis of these important antibiotics occurs.

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Notes and references

‡ The IUPAC name for mandelic acid is phenylhydroxyacetic acid.

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10 Alternatively, decarboxylation could occur after cyclisation which would also be a more attractive pathway than using acetyl-CoA as the starter unit.
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13 The labeling experiments would also be consistent with the first malonyl unit loaded onto the enzyme being decarboxylated to give an acetyl starter unit. However, this reaction pathway seems unlikely since acetyl-CoA is not accepted at all by the enzyme.

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