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Incorporation of [U-^{13}C]glycerol defines plausible early steps for the biosynthesis of methylenomycin A in *Streptomyces coelicolor* A3(2)†

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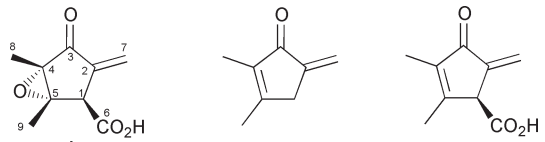
Incorporation of [U-^{13}C]glycerol into the linear plasmid-encoded secondary metabolite methylenomycin A, both intact and indirectly *via* metabolism to acetyl CoA, was observed in *Streptomyces coelicolor* suggesting plausible early biosynthetic steps for this unusual antibiotic.

Methylenomycins A and B, **1** and **2** respectively,[1] are the most well studied members of a small group of cyclopentanone antibiotics produced by various *Streptomyces* spp. Other members of this group include xanthocidin,[2] vertimycin,[3]



pentenomycin,[4] and sarkomycin,[5] which is an antitumour agent that has been used clinically in Japan. The *mmy* cluster of genes encoding methylenomycin biosynthesis and its regulation, as well as self-resistance, is localised on a large linear plasmid that is not essential for growth in *Streptomyces coelicolor* A3(2) and a large circular plasmid in *Streptomyces violaceoruber*.[5] Recently, the entire *mmy* cluster of *S. coelicolor* has been sequenced.[6] As a first step towards understanding how the enzymes encoded by this cluster assemble **1** and **2** we have reinvestigated the primary metabolic origins of these antibiotics.

Previous investigations showed that radiolabelled desepoxy-4,5-didehydro methylenomycin A **3**, which is also a metabolite of *S. coelicolor*, is incorporated into **1**.[7] Thus epoxidation of **3** appears to be the final step in the biosynthesis of **1**, whereas **2** probably derives from spontaneous decarboxylation of **3**. Additional experiments using radiolabelled precursors showed significant incorporation of acetate, pyruvate, succinate, aspartate and glycine into the carbon atoms of **1**.[8] Intact incorporation of acetate into C-4, C-8 and C-1, C-6 of **1** was deduced by ^{13}C NMR analysis of a sample isolated from a culture of *S. coelicolor* fed with [1,2-$^{13}C_2$]acetate (Scheme 1).[8]

Scheme 1

To further investigate the primary metabolic origins of the carbon atoms in **1**, the incorporation of [U-^{13}C]glycerol was examined.§ Surprisingly, the results of this experiment were inconsistent with the previously reported incorporation of [1,2-^{13}C]acetate into C-4, C-8 of **1**. The original interpretation of the acetate incorporation experiment depended on the assignment of the signal at 13.8 ppm in the ^{13}C NMR spectrum of **1** to the C-5 methyl substituent. This assignment was made on the basis of an apparent ^4J coupling between the C-5 methyl group and the hydrogen atom attached to C-1 in the ^{1}H NMR spectrum, in conjunction with heteronuclear decoupling experiments.[8] To resolve the conflict we measured the HMBC spectrum of **1**, which unambiguously showed that the signals at 13.8 and 8.2 ppm in the ^{13}C NMR spectrum are due to the C-4 and C-5 methyl groups, respectively (Fig. 1).

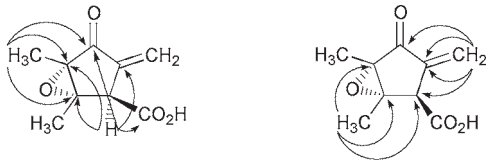
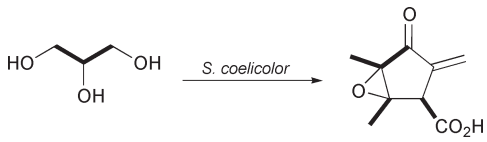


Fig. 1 Summary of HMBC correlations observed for **1**.

These assignments forced us to conclude that [1,2-^{13}C]acetate is incorporated intact into C-5, C-9 and C-1, C-6, rather than C-4, C-8 and C-1, C-6 as previously reported.[8] Consistent with this reinterpretation, the ^{13}C NMR spectrum of **1** isolated from the glycerol feeding experiment showed indirect incorporation of [U-^{13}C]glycerol into C-5, C-9 and C-1, C-6 presumably by catabolism *via* pyruvate to [1,2-^{13}C]acetyl CoA. In addition, double doublets centred on the signals for C-3 and C-8 were also observed in the spectrum, demonstrating that these carbons are coupled ($^2J_{C-C}$ = 3.4 Hz; Fig. 2), which is consistent with intact incorporation of [U-^{13}C]glycerol into C-3, C-4 and C-8. Such intact incorporation of glycerol is relatively unusual and has only been reported for four other microbial secondary metabolites.[9] The results of the glycerol incorporation experiment are summarised in Scheme 2.



Scheme 2

On the basis of these results, we propose a pathway for the biosynthesis of **1** in *S. coelicolor* involving nucleophilic addition of C-2 of lactate (X = H, Y = OH) or of a glycolytic intermediate such as glyceraldehyde-3-phosphate (X = OPO_3^{2-}, Y = H) to C-3 of acetoacetyl ACP. This pathway, which is mechanistically distinct from those proposed for the incorporation of glycerol into other metabolites,[9] is consistent with the presence of the *mmyA* and *mmyC* genes in the methylenomycin cluster, which encode homologues of an ACP and ketoacyl synthase III (KAS III), respectively.[6] However, reduction of acetoacetyl ACP to 3-hydroxybutyryl ACP followed by nucleophilic displacement of the hydroxy group with C-2 of lactate or glyceraldehyde-3-phosphate cannot be

† Electronic supplementary information (ESI) available: HMBC, ^{1}H and ^{13}C NMR spectra of methylenomycin A. See http://www.rsc.org/suppdata/cc/b1/b101615f/

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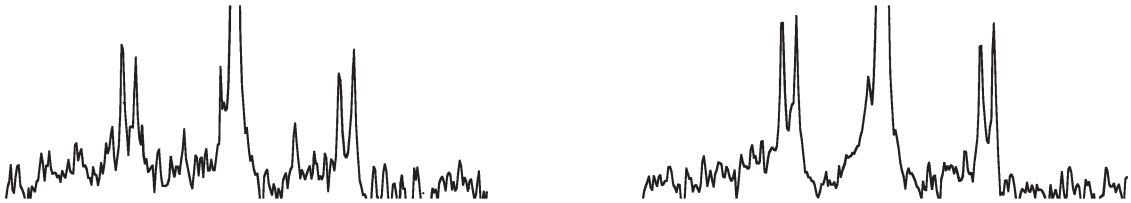
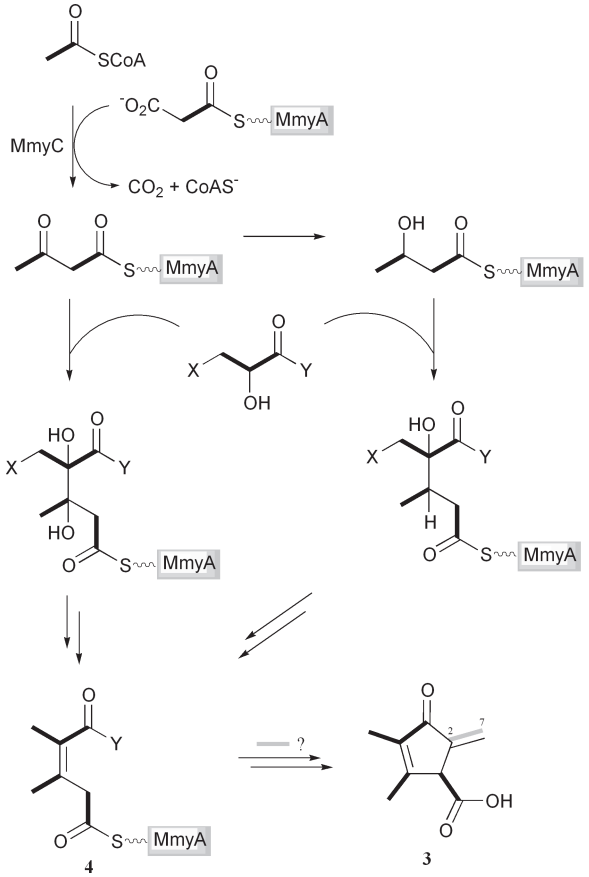


Fig. 2 Sections of the ^{13}C NMR spectrum of **1** isolated from cultures of *S. coelicolor* fed with [U-^{13}C]glycerol showing double doublets flanking the C-3 (left; 196.4 ppm) and C-8 (right; 8.2 ppm) resonances. While the larger splitting in each is due to ^{1}J coupling between C-3 or C-8 and C-4, the smaller splitting of 3.4 Hz in both is due to ^{2}J coupling between C-3 and C-8, consistent with intact incorporation of [U-^{13}C]glycerol into C-3, C-4 and C-8 of **1**.



Scheme 3

ruled out.[10] In either case, several alternating dehydrations and enoyl reductions of the product would yield the common intermediate **4** (Scheme 3).

Conversion of **4** to the known biosynthetic intermediate **3** would require condensation with unknown precursors of C-2 and C-7 and hydrolysis of the thioester linkage to the ACP MmyA. Previous experiments eliminated the possibility that C-2 and C-7 are derived either from actetate as an intact two-carbon unit or from two *S*-adenosylmethionine methyl groups.[8] However, radioactivity from [2-^{14}C]glycine, has been shown to incorporate into **1** and this amino acid could therefore act as either an intact two-carbon precursor of C-2, C-7 or as a one-carbon donor for C-2 and C-7 *via* metabolism to methylene tetrahydrofolate.[8] To test this hypothesis we examined the incorporation of [1,2-$^{13}C_2$]glycine into **1**. To our surprise, there was no incorporation of ^{13}C into C-2 or C-7, instead approximately equal labelling of individual molecules at either C-5 and C-9, or C-1 and C-6 was observed. This result can be explained by transfer of ^{13}C-2 from one molecule of [1,2-$^{13}C_2$]glycine to another by serine hydroxymethyltransferase to give [U-^{13}C]serine, which is then metabolised to [1,2-$^{13}C_2$]acetyl CoA *via* pyruvate and incorporated into **1**. Thus, we have ruled out the derivation of C-2 and C-7 from intact glycine or from two one-carbon units donated by methylene tetrahydrofolate. Further experiments will be required to identify the cryptic two-carbon precursor of C-2 and C-7.

In conclusion, intact and indirect incorporation of [U-^{13}C]glycerol has elucidated the metabolic origins of the majority of carbon atoms in **1** and has defined plausible early steps for the biosynthesis of this metabolite in *S. coelicolor* A3(2).

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Notes and references

§ A solution of 50 mg of labelled glycerol in water was added to a 3-day-old 500 ml culture of *S. coelicolor* U9 grown in complete medium at 28 °C from spores as in ref. 8. After 24 h further incubation, the culture was filtered, and the filtrate was adjusted to pH 2 with 6 M HCl and extracted with an equal volume of ethyl acetate. **1** was purified from the organic extract by flash column chromatography (silica, AcOH–PhMe 1:9) and analysed by ^{13}C NMR spectroscopy at 100 MHz.

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10 Similarly the dehydration of 3-hydroxybutyryl ACP to give crotonyl ACP followed by conjugate nucleophilic addition of C-2 of lactate or glyceraldehyde-3-phosphate can not be ruled out, although it gives the same product.