DOI: 10.1002/cbic.200500243

Evidence for the Unusual Condensation of a Diketide with a Pentulose in the Methylenomycin Biosynthetic Pathway of *Streptomyces coelicolor* A3(2)

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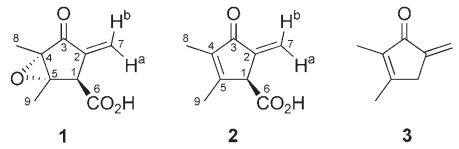
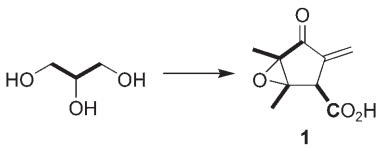
Methylenomycin A (**1**) is a cyclopentanone antibiotic that exhibits a wide spectrum of activity against Gram-positive bacteria and some Gram-negative strains, especially *Proteus* species. It was first isolated in 1974 from *Streptomyces violaceoruber* SANK95570,[1] and has since also been found to be produced by *Streptomyces coelicolor* A3(2).[2] The structurally related cyclopentanones desepoxy-4,5-didehydromethylenomycin A (here named methylenomycin C; **2**) and methylenomycin B (**3**) have also been isolated from *Streptomyces* species.[3] Incorporation experiments with radiolabelled **2** have implicated it as a biosynthetic precursor of **1**.[3]



1 **2** **3**

Both radiolabelled and stable-isotope-labelled precursors have been used to investigate the primary metabolic origins of **1**.[4] Despite feeding experiments with a wide range of labelled precursors, the origin of five of the nine carbon atoms of **1** remained unknown for more than two decades. We recently reported revised ^{1}H and ^{13}C NMR assignments for **1**, which led us to revise the published interpretation of acetate-incorporation experiments. We also reported that [U-^{13}C]glycerol is incorporated intact into C-3, C-4 and C-8 of **1**, as well as via metabolism to acetyl CoA into C-5/C-9 and C-1/C-6 (Scheme 1).[5] These experiments were carried out with *Streptomyces coelicol-*



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Scheme 1. Incorporation of [U-^{13}C]glycerol into **1**.

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or U9 in complete medium (a complex medium containing multiple carbon sources) and resulted in low incorporation levels (~0.1%). In these studies, we were unable to identify the metabolic origin of C-2 and C-7 of **1** because *S. coelicolor* U9 does not produce **1** and **2** in minimal media. Thus, we tried an alternative approach involving experiments utilising *Streptomyces lividans* 1326 transformed with a cosmid containing the gene cluster that directs methylenomycin biosynthesis in *S. coelicolor*.

Recently, the sequence of the entire giant linear plasmid SCP1 of *S. coelicolor*, which contains the *mmy* cluster that directs the biosynthesis of **1** and **2**, has been determined.[6] On the basis of a bioassay, *S. coelicolor* M145 transformed with the integrative cosmid C73 787 (containing the *mmy* cluster—from a genomic library of SCP1) has been reported to produce methylenomycins.[7] Here we present unambiguous chemical evidence that *S. lividans* 1326 transformed with cosmid C73 787 produces **1** and **2** in a supplemented minimal medium and report further incorporation experiments that reveal the unusual metabolic origin of C-2, C-3, C-4, C-7 and C-8, which has remained unclear until now.

Methylenomycins **1** and **2** were isolated from an organic extract of the acidified culture supernatant of *S. lividans* 1326 (C73 787) grown in the minimal medium, and identified by ^{1}H and ^{13}C NMR spectroscopy. The signals in the ^{1}H and ^{13}C NMR spectra of **1** have been unambiguously assigned previously.[5] The signals in the ^{1}H and ^{13}C NMR spectra of **2** were unambiguously assigned by using HMQC, HMBC and difference-NOE experiments. In the course of these experiments, the production of **3**, which could not be identified as a direct product of the fermentation, was observed when **2** was stored in acidic chloroform, presumably as a result of acid-promoted decarboxylation of **2**.

Pulse-feeding experiments with [U-^{13}C]glycerol (4×2 mM) as the main carbon source were carried out with *S. lividans* 1326 (C73 787) grown in the minimal medium. Labelled **1** and **2** were isolated and characterised by ^{13}C NMR spectroscopy. As in our previous experiments, in which we fed [U-^{13}C]glycerol to *S. coelicolor* U9 in complete medium, double doublets flanking the natural-abundance signals for C-3 and C-8 were observed in the spectra of **1** and **2** ($^{1}J_{C3-C4}=54.4$, $^{1}J_{C4-C8}=49.1$, $^{2}J_{C3-C8}=3.3$ Hz for **1**; $^{1}J_{C3-C4}=52.5$, $^{1}J_{C4-C8}=46.3$, $^{2}J_{C3-C8}=1.9$ Hz for **2**).[5] These results confirm that C-3, C-4 and C-8 in **1** and **2** can originate from an intact three-carbon precursor derived from glycerol. In addition, in the spectrum of **2**, a second, broadened doublet of doublets ($^{1}J_{C3-C4}=52.5$, $^{1}J_{C3-C2}=51.0$ Hz) overlaying and flanking the natural-abundance signal for C-3 was observed, along with a broadened doublet ($^{1}J_{C2-C7}=73.4$ Hz) flanking the natural-abundance signal of C-7, and a doublet of doublets ($^{1}J_{C4-C8}=46.3$ Hz, $^{1}J_{C3-C4}=52.5$ Hz) flanking and overlaying the natural-abundance signal of C-4. Similar additional signals were observed flanking and overlaying the natural-abundance signals of C-3, C-4 and C-7 of **1**. These results are consistent with intact incorporation of a [U-^{13}C]-labelled five-carbon precursor, derived from two molecules of [U-^{13}C]glycerol, into C-2, C-3, C-4, C-7 and C-8 of **1** and **2** (Figure 1). [U-^{13}C]glycerol was also incorporated into C-1, C-6 and C-5, C-9 of **1** and **2** by metabolism to [1,2-$^{13}C_2$]acetyl CoA, as observed previously for **1**.[5]

To explain the above observations, we reasoned that [U-^{13}C]glycerol gets metabolised to [U-^{13}C]glyceraldehyde-3-phosphate (G3P), which is incorporated by two different routes through the pentose phosphate pathway into a five-carbon precursor of C-2, C-3, C-4, C-7 and C-8 of **1** and **2**. Thus, transketolase-mediated condensation of [U-^{13}C]G3P with unlabelled fructose-6-phosphate (F6P) or seduheptulose-7-phosphate (S7P) would give xylulose-5-phosphate (Xu5P) labelled at only C-3, C-4 and C-5 with ^{13}C (and erythrose-4-phosphate (E4P) or ribose-5-phosphate (R5P), respectively). On the other hand, isomerisation of [U-^{13}C]G3P to [U-^{13}C]dihydroxyacetone phosphate (DHAP), followed by condensation with a second molecule of [U-^{13}C]G3P by gluconeogenesis would yield [U-^{13}C]F6P (Scheme 2). Given that [U-^{13}C]glycerol is fed in high concentration and is the main carbon source present in the minimal medium at the time of feeding, it seems very likely that this could occur. [U-^{13}C]F6P could then be converted to [U-^{13}C]ribulose-5-phosphate (Ru5P) by conversion to 6-phosphogluconate followed by oxidative decarboxylation. Alternatively, [U-^{13}C]F6P and another molecule of [U-^{13}C]G3P could be converted by transketolase to [U-^{13}C]Xu5P. Since Xu5P, Ru5P and R5P are rap-

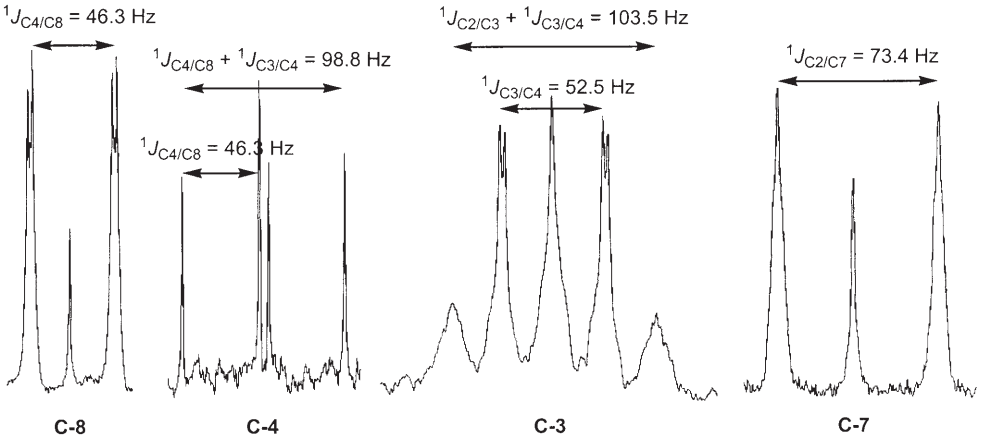
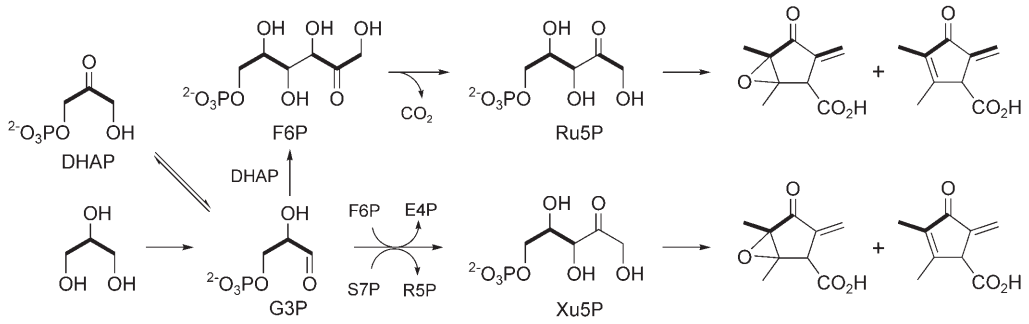


Figure 1. Selected signals from the ^{13}C NMR spectrum of **2** isolated after feeding [U-^{13}C]glycerol to *S. lividans* C73 787.

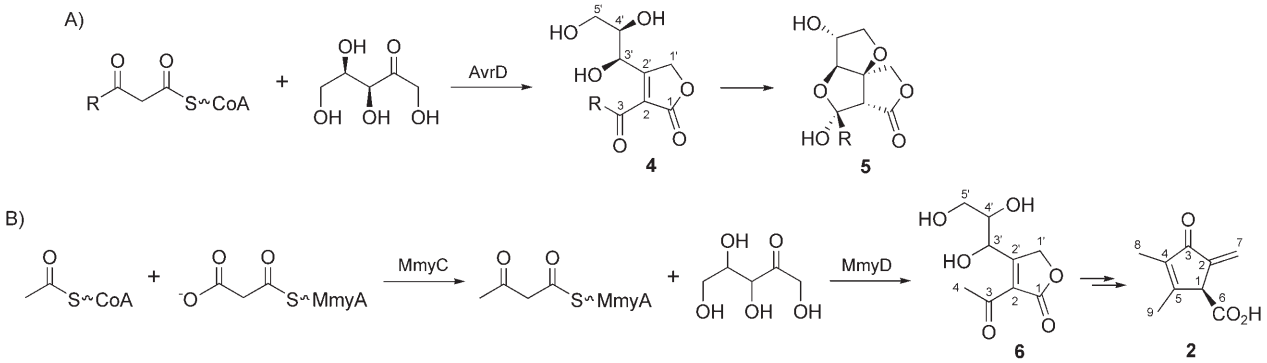


Scheme 2. Pathways for incorporation of [U-^{13}C]glycerol into C-2, C-3, C-4, C-7 and C-8 of **1** and **2**. [U-^{13}C]glycerol was also incorporated into C-5/C-9 and C-1/C-6 of **1** and **2** by metabolism of glyceraldehyde-3-phosphate to acetyl CoA. This is not shown in the scheme for reasons of clarity.

idly equilibrated, any one of these three intermediates of the pentose phosphate pathway could be the precursor of C-2, C-3, C-4, C-7 and C-8 of **1** and **2**. This model is fully consistent with the observed incorporation pattern of [U-^{13}C]glycerol into **1** and **2** in the minimal medium. It also explains why labelling of **1** at C-3, C-4 and C-8 but not C-2 or C-7 occurred when [U-^{13}C]glycerol was fed to *S. coelicolor* U9 in complete medium, because no significant gluconeogenesis (required for incorporation of [U-^{13}C]glycerol into C-2 and C-7 of **1**) would occur in such a complex medium, rich in diverse carbon sources.

To further examine the role of a pent(ul)ose as a direct precursor for C-2, C-3, C-4, C-7 and C-8 of **1** and **2**, [U-^{13}C]-D-ribose (15 mg) was fed 53 hours after inoculation to a culture of *S. lividans* 1326 (C73 787) growing in minimal medium. Labelled **1** and **2** were isolated and analysed by ^{13}C NMR spectroscopy. Broadened doublets flanking the natural-abundance signals for C-8 ($^1J_{C4-C8}$ = 46.3 Hz) and C-7 ($^1J_{C2-C7}$ = 73.4 Hz), and a broadened double doublet flanking and overlaying the signal for C-3 ($^1J_{C3-C4}$ + $^1J_{C2-C3}$ = 103.5 Hz) were observed in the spectrum of **2**. A similar incorporation pattern was observed for **1**. The coupling constants are fully consistent with those observed for incorporation of a [U-^{13}C]pent(ul)ose into **1** and **2** in the [U-^{13}C]glycerol incorporation experiment. In addition, C-8, C-3 and C-7 in **2** were all enriched with ^{13}C to a similar level of ~4%. There was also no evidence that an isolated, intact $^{13}C_3$ unit had been incorporated into C-3, C-4 and C-8 of **1** or **2**. Taken together, these data are clearly consistent with intact incorporation of a five-carbon precursor originating from [U-^{13}C]-D-ribose into **1** and **2**. These results are also entirely consistent with related feeding studies demonstrating that an intact pentose is incorporated into the carbon skeleton of vitamins B6 and B1.[9] It is also interesting to note that the onset of methylenomycin biosynthesis has previously been correlated with high pentose phosphate-pathway activity in *Streptomyces coelicolor,*[8] presumably because high pentose phosphate-pathway activity is required to make appropriate quantities of a pent(ul)ose available for methylenomycin assembly.

The complete sequence of the *mmy* cluster of genes that directs methylenomycin biosynthesis in *Streptomyces coelicolor* A3(2) has recently become available.[6] Based on sequence analysis of the proteins encoded by genes in this cluster, our previous feeding experiments[5] and the results reported herein, we propose plausible early steps for methylenomycin biosynthesis. Our previous experiments and those reported here both suggest that acetoacetyl ACP is likely to be an intermediate in the biosynthesis of **1** and **2.**[5] This proposal is consistent with the presence of *mmyA* and *mmyC*, which code for an acyl carrier protein and a ketoacyl synthase III (KASIII) enzyme, respectively, in the *mmy* cluster. MmyC could catalyse the decarboxylative condensation of malonyl-MmyA with acetyl CoA to form acetoacetyl-MmyA (Scheme 3). This hypothesis suggests that MmyC is highly selective for acetyl CoA over isobutyryl or isovaleryl CoA—unlike primary metabolic KASIII enzymes in *Streptomyces* spp., which are usually capable of incorporating all three of these starter units into fatty acids.[12] Syringolides **5** (R = nC_6H_{13} or nC_8H_{17}) are elicitors isolated from



Scheme 3. A) Proposed pathway for syringolide biosynthesis in *P. syringae* pathovars. B) Hypothetical early steps for methylenomycin biosynthesis in *S. coelicolor.*

Pseudomonas syringae pathovars.[10] Expression of the *Pseudomonas syringae* gene *avrD* in *Escherichia coli* causes their production.[13] It has been suggested that the syringolides are assembled by condensation of β-ketohexanoyl CoA or β-ketooctanoyl CoA with xylulose-5-phosphate (Xu5P) to form a butenolide intermediate **4** (R = nC_6H_{13} or nC_8H_{17}), which undergoes subsequent cycloketalisation and intramolecular cyclisation through conjugate addition of the C-5′ hydroxyl group to C-2′ to form **5** (Scheme 3).[10] While there is no direct evidence (e.g. from feeding experiments) that syringolides derive from condensation of a β-ketothioester with a pentulose, the chemical feasibility of this proposal has been demonstrated by biomimetic synthesis studies.[11] The *mmyD* gene encodes a protein with 47% similarity over 333 amino acids to AvrD. It is therefore tempting to speculate that MmyD catalyses condensation of acetoacetyl-MmyA with a pentulose to form a butenolide intermediate **6** similar to the proposed intermediate in syringolide biosynthesis **4** (Scheme 3). The presumably facile conversion of intermediate **6** to a syringolide-like structure could be prevented by rapid transformation of **6** into an intermediate unable to undergo the cyclisation reactions proposed to lead to syringolides, for example, by reduction of the C=C double bond or phosphorylation of the C-5′ hydroxyl group and oxidation of the C-3′ hydroxyl group to a keto group (this carbon bears a keto group at C-3 in **1** and **2**). Completion of the assembly of the carbon skeleton of **1** and **2** requires formation of a carbon–carbon bond between C-3 and C-4′ in **6**, which could be accomplished by oxidation of the C-3′ hydroxyl group to a keto group, followed by an aldol condensation between C-4′ and C-3. The elaboration of **6** to **2** also requires hydrolysis of the lactone, several reductions and the elimination of several molecules of water. Experiments are in progress to examine whether **6** is an intermediate in methylenomycin biosynthesis and to determine the nature and order of the reactions catalysed by enzymes encoded within the *mmy* cluster involved in the assembly of **1** and **2.**

In conclusion, we have defined the metabolic origins of all of the carbon atoms of the methylenomycins. This has facilitated assignment of plausible hypothetical roles to some of the enzymes encoded within the *mmy* cluster and has led us to propose that condensation of acetoacetyl ACP with a pentulose to form a butenolide intermediate is a key step in methylenomycin biosynthesis.

Experimental Section

Isolation of 1 and 2 from *S. lividans* 1326 (C73 787): A culture of *S. lividans* 1326 (C73 787; 1 L) was grown in supplemented minimal medium (SMM) from spores. SMM consists of $MgSO_4 \cdot 7H_2O$ (5 mM final), TES buffer pH 7.2 (25 mM final), NaH_2PO_4 + K_2HPO_4 (0.5 mM of each final), trace-element solution (0.1 mL for 100 mL), casamino acids (0.2% *w/v* final), glucose (0.2% *w/v* final). Trace-element solution consists of: $ZnSO_4 \cdot 7H_2O$ (0.1 g L^{-1}), $FeSO_4 \cdot 7H_2O$ (0.1 g L^{-1}), $MnCl_2 \cdot 4H_2O$ (0.1 g L^{-1}), $CaCl_2 \cdot 6H_2O$ (0.1 g L^{-1}), NaCl (0.1 g L^{-1}).

After a total of 7 days of incubation at 28 °C and 200 rpm, the cultures were filtered, and the filtrate was adjusted to pH 2 with HCl (2 M) and extracted with an equal volume of ethyl acetate. The resulting organic extract was dried over $MgSO_4$, concentrated in vacuo and separated by flash column chromatography (silica, AcOH/PhMe 1:9) to yield methylenomycins A and C (5 mg each). Methylenomycin A: $R_f = 0.35$ (silica, AcOH/PhMe 1:9); ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 6.28$ (d, $J = 1.5$ Hz, 1H, H-7b), 5.67 (d, $J =$ 1.5 Hz, 1H, H-7a), 3.85 (s, 1H, H-1), 1.62 (s, 3H, H-9), 1.52 (s, 3H, H-8); ^{13}C NMR (125 MHz, $CDCl_3$) $\delta = 196.4$ (C-3), 174.0 (C-6), 140.2 (C-2), 123.3 (C-7), 66.3 (C-5), 65.2 (C-4), 51.3 (C-1), 13.8 (C-9), 8.2 (C-8). Methylenomycin C: $R_f = 0.22$ (silica, AcOH/PhMe 1:9); ^{1}H NMR (500 MHz, $CDCl_3$) $\delta = 6.21$ (d, $J = 2.0$ Hz, 1H, H-7b), 5.65 (d, $J =$ 2.0 Hz, 1H, H-7a), 4.10 (s, 1H, H-1), 2.13 (s, 3H, H-9), 1.85 (s, 3H, H-8); ^{13}C NMR (125 MHz, $CDCl_3$) $\delta = 194.3$ (C-3), 174.5 (C-6), 160.0 (C-5), 141.2 (C-4), 140.1 (C-2), 117.8 (C-7), 65.2 (C-4), 52.4 (C-1), 15.6 (C-9), 8.7 (C-8). Assignments of the signals in the ^{1}H and ^{13}C NMR spectra of **1** have been determined previously.[5] Assignments of the signals in the ^{1}H and ^{13}C NMR spectra of **2** were confirmed by HMQC, HMBC and difference NOE experiments.

Incorporation of [U-^{13}C]-glycerol into 1 and 2: [U-^{13}C]-glycerol was purchased from Cambridge Isotope Laboratories, Inc. (Andover, USA). Four solutions each containing [U-^{13}C]-glycerol (19 mg) in water (ca. 2 mL) were filter sterilised and added after 48 h, 72 h, 96 h and 120 h to a culture of *S. lividans* 1326 (C73 787; 100 mL) growing in SMM at 28 °C and at 200 rpm. After 7 days of incubation, **1** and **2** were isolated as described above, and the incorporation patterns were analysed by ^{13}C NMR spectroscopy.

Incorporation of [U-^{13}C]-D-ribose into 1 and 2: [U-^{13}C]-D-ribose was purchased from Cambridge Isotope Laboratories, Inc. (Andover, USA). A solution containing [U-^{13}C]-D-ribose (15 mg) in water (ca. 2 mL) was filter sterilised and added after 53 h to a culture of *S. lividans* 1326 (C73 787; 100 mL) growing in SMM at 28 °C and at 200 rpm. After 7 days of incubation, **1** and **2** were isolated as described above, and the incorporation patterns were analysed by ^{13}C NMR spectroscopy.

Acknowledgements

We thank Dr. Adam J. Clarke for help with the NMR analyses, Dr. Bertolt Gust for S. lividans *1326 (C73 787) and the EU (Integrated Project: Actinogen) for financial support.*

Keywords: antibiotics · biosynthesis · cyclopentanone · methylenomycin · pentose · *Streptomyces*

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Received: June 7, 2005
Published online on November 7, 2005

