Biosynthesis of the Polyether Antibiotic Monensin-A. Results from the Incorporations of Labelled Acetate and Propionate as a Probe of the Carbon Chain Assembly Processes

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The incorporation of sodium [2-$^{2}H_{2}$]- and (*S*)-[2-$^{2}H_{1}$]-propionate into the polyether antibiotic monensin-A in cultures of *Streptomyces cinnamonensis* occurs with retention of label only at C-4 and C-6, whereas during the incorporation of sodium (*R*)-[2-$^{2}H_{1}$]propionate the deuterium label is lost to the medium. These results are consistent with the formation of (*S*)-methylmalonyl-CoA from the labelled propionate by carboxylation of propionyl-CoA with loss of the 2-*pro-R* hydrogen. The (*S*)-methylmalonyl-CoA is subsequently incorporated into the antibiotic by a decarboxylative condensation occurring with overall inversion. The incorporations of sodium [1-^{13}C,2-$^{2}H_{3}$]-and [2-^{13}C,2-$^{2}H_{3}$]-acetates into monensin-A provide evidence for a pathway of metabolism leading to methylmalonyl-CoA that does not proceed *via* succinyl-CoA. Instead, acetyl-CoA may be processed *via* butyryl-CoA and isobutyryl-CoA, to afford (*S*)-methylmalonyl-CoA.

The multienzyme polyketide synthases responsible for catalysing the formation of the carbon backbones of macrolide, polyether, and related antibiotics, predominantly in *Streptomyces* species, have become the subject of intense interest recently.[1–6] The enzymes operating on these pathways are able to assemble the backbones of these complex secondary metabolites, starting typically from activated coenzyme-A derivatives of acetate, propionate, and butyrate. In general, the mode of assembly is believed to involve chemistry which is closely related to that catalysed by the fatty acid synthase complex, except that a *regular* pattern of chain elongation–reduction–elimination–reduction steps, during the biosynthesis, apparently does not take place. There exists at present, however, a paucity of structural knowledge about this family of proteins and how the carbon-chain assembly processes mediated by them are controlled, although this is vital information for an understanding of how the individual enzymes have evolved to generate specific antibiotic structures, as well as for any rational attempts to redesign, or manipulate, the biosynthetic machinery by rDNA methods.[7]

In view of the technical difficulties inherent in the isolation and characterisation of these enzymes from antibiotic-producing strains of micro-organisms, our attention has focussed initially on the application of stable isotope labelling experiments with whole cell systems in an attempt to glean information about the mechanism and stereochemistry of these carbon-chain assembly processes.[8] In particular, the biosynthesis of the polyether antibiotic monensin-A has been studied in this manner, by looking at the incorporation of deuterium labelled forms of the primary precursors acetate and propionate,[2] and full details of these experiments are presented and discussed in this paper.

Unambiguous assignments of the carbon-13 and ^{1}H n.m.r. spectra of monensin-A sodium salt have been reported already.[9,10] Experiments utilizing ^{13}C singly labelled, and ^{13}C/^{18}O doubly labelled samples of acetate, propionate, and butyrate have been described,[10,11] and have led to the identification of the biosynthetic origins of all the carbon and oxygen atoms in monensin-A. Based on these data the carbon backbone was shown to be assembled formally from five acetate, seven propionate, and one butyrate units, most plausibly by the pathway indicated in Figure 1.

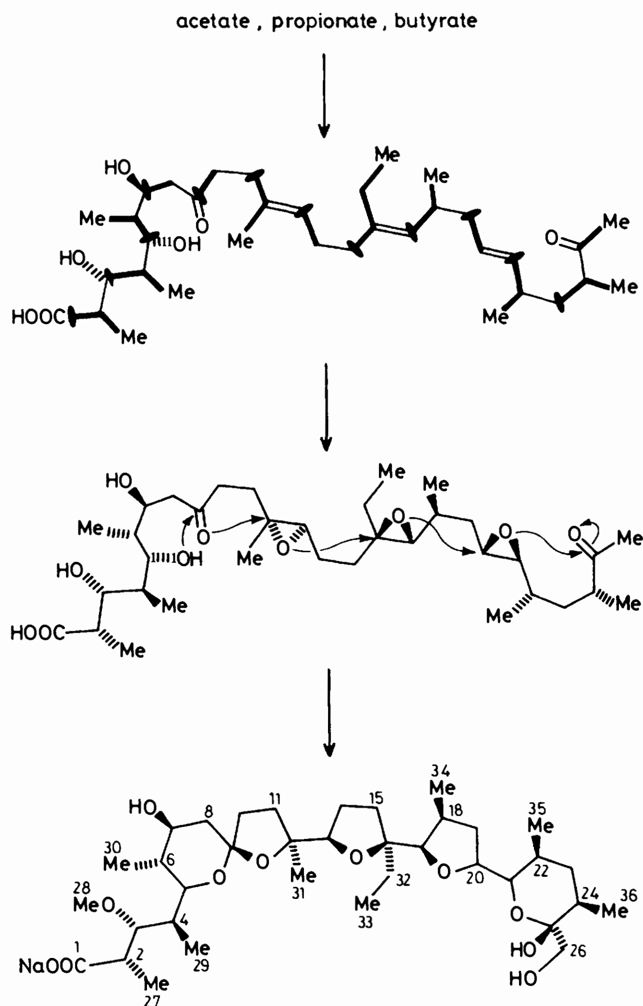


Figure 1. Proposed biosynthetic pathway to monensin-A

By following now the incorporations of these precursors specifically deuteriated at the α-positions, useful additional data can be gained concerning the mechanism and *overall* stereochemistry of reactions occurring at these deuteriated centres during the chain assembly processes. In the experiments

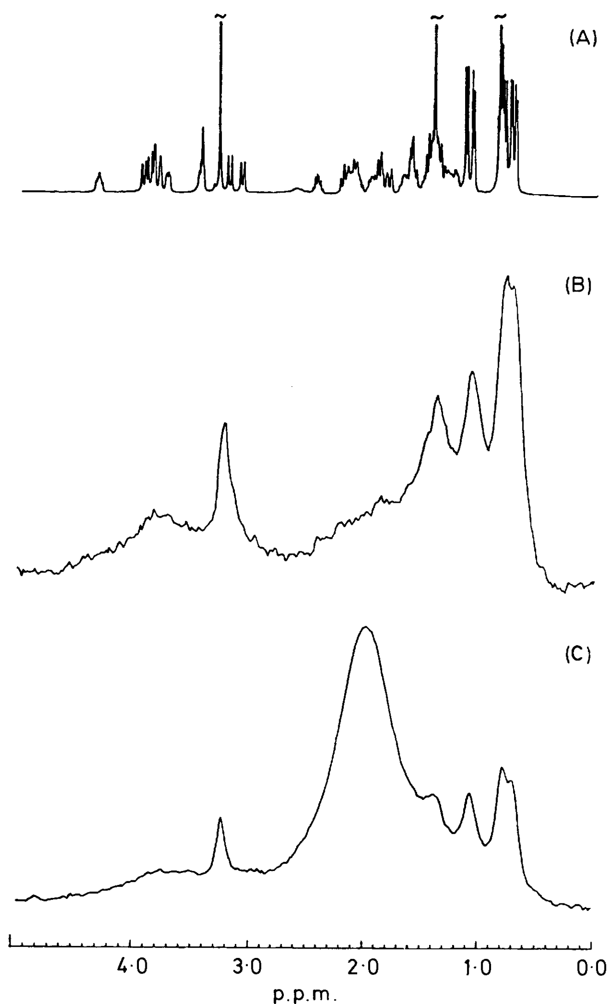


Figure 2. (A) ^{1}H N.m.r. spectrum of Na-monensin-A at 360 MHz in $CDCl_3$; (B) natural abundance $^2H\{^1H\}$ n.m.r. spectrum of Na-monensin-A at 55.3 MHz (1 g dissolved in 2 ml of $CHCl_3$, 2 K data points, acquisition time 0.655 s, 1.0 Hz line broadening with exponential multiplication); (C) $^2H\{^1H\}$ n.m.r. spectrum of Na-monensin-A biosynthesized from sodium [2-$^2H_2$]propionate run under the same conditions as in (B)

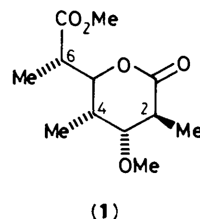
described below, this applies particularly to deuterium label retained at positions derived from C_α of propionate, since these correspond to all of the methine positions bearing methyl branch points with varying orientations in the backbone. A second useful feature arises when the deuteriated precursor is diverted into other parts of the primary metabolism of the cell prior to incorporation into the antibiotic. In such circumstances, retention of deuterium label may occur at positions that are not necessarily correlated directly with their locations in the precursor, and this can give valuable information about the intervening primary metabolic processes, and the relationship of these to the normal *in vivo* pathways generating building blocks for antibiotic production. Just such a situation arises here during the incorporation of labelled acetate into monensin-A.

Results

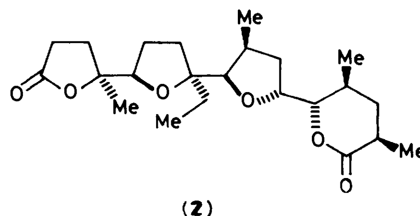
Incorporation of Labelled Propionate.—The aim of these experiments was to follow the incorporation of deuterium label from C-2 of propionate into the corresponding methine positions in monensin-A, *i.e.* C-2, C-4, C-6, C-18, C-22 and C-24. As a first step the incorporation of (*RS*)-[2-^{14}C,2-^{3}H]-propionate ($^3H/^{14}C$ = 18.0) was examined in order to assess the extent to which any label at the α-position is lost during the biosynthesis. Accordingly, after batchwise addition of the precursor to cultures of *S. cinnamonensis*,[9] [^{3}H]- and [^{14}C]-labelled monensin-A was isolated and recrystallized to constant specific activity ($^3H/^{14}C$ = 0.6). The $^3H/^{14}C$ ratio of this material indicated that a large proportion (97%) of the tritium label had been lost to the medium during the biosynthesis. In a control experiment [1-^{13}C]propionate was fed under identical conditions, and gave rise to enhanced singlets (*ca.* $\times 10$ enrichment) in the ^{13}C n.m.r. of the antibiotic at the expected positions,[10] *i.e.* C-1, C-3, C-5, C-11, C-17, C-21, and C-23.

Although most of the tritium label at C-2 in propionate is lost during the experiment, a significant proportion is retained, sufficient to make it worthwhile establishing the sites of enrichment in the antibiotic. Several experimental approaches were possible to achieve this, including the use of $^{13}C\{^1H,^2H\}$ n.m.r. spectroscopy,[12] and other edited ^{13}C n.m.r. techniques,[13] allowing detection specifically of deuteriated carbon centres. However, the inevitable low retention of deuterium label upon incorporation of [2-^{13}C,2-$^2H_2$]propionate, would be likely to cause serious difficulties in detecting weak ^{2}H–^{13}C signals. We chose, therefore, to pursue this study using ^{2}H n.m.r. spectroscopy since the natural abundance of ^{2}H is at a much lower level, and large quantities of monensin can be generated by fermentation, thereby negating problems due to the inherent low sensitivity of this n.m.r. technique.[12]

Accordingly, sodium [2-$^2H_2$]propionate (30 g), containing also a trace level of [1-^{14}C]propionate was administered batchwise to a fermentation of *S. cinnamonensis* (6 l), and this was subsequently worked up in the usual way to afford pure crystalline Na-monensin-A (3 g, m.p. 270 °C). Both the 55 MHz ^{2}H, and 360 MHz ^{1}H n.m.r. spectra of monensin-A are shown in Figure 2. The labelled monensin showed an increased background level of deuterium but, more importantly, also showed a single strongly enhanced, broad signal, centred at δ 2.0, a result which is consistent with the retention of deuterium specifically at one or more methine positions. The very broad natural abundance line widths of ^{2}H n.m.r. signals for monensin-A are to be expected for a molecule of this size. However, it could be established with reasonable confidence, from scrutiny of resolution enhanced spectra, that the deuterium enrichments were not at C-2 [δ(^{1}H) 2.53], C-22 (δ 1.36) or C-24 (δ 1.46), although the broadness of the enriched signal did not allow a distinction to be made between specific enrichments at the other methine centres, C-4 (δ 2.07), C-6 (δ 2.22), and C-18 (δ 2.26).



(**1**)



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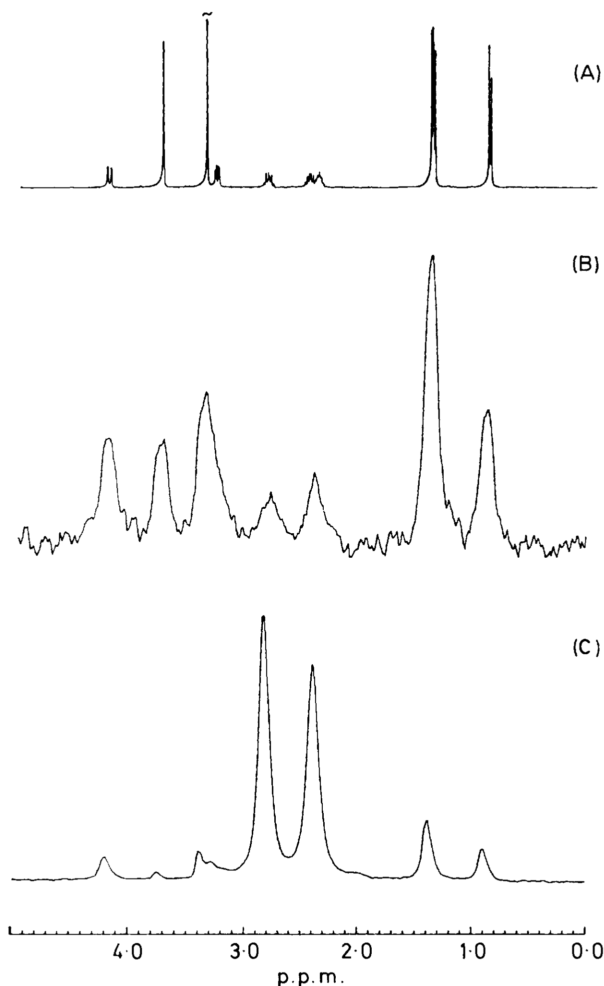
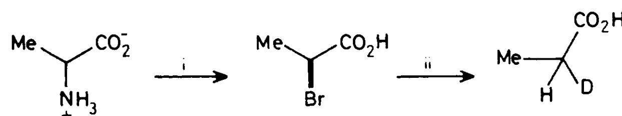


Figure 3. (A) ^{1}H N.m.r. spectrum of the lactone methyl ester (**1**) at 360 MHz in $CDCl_3$; (B) natural abundance $^{2}H\{^{1}H\}$ n.m.r. spectrum of (**1**) at 55.3 MHz (300 mg dissolved in 2 ml of $CHCl_3$. For acquisition parameters see Figure 2); (C) $^{2}H\{^{1}H\}$ n.m.r. spectrum of (**1**) derived from monensin-A biosynthesized from sodium [2-$^{2}H_2$]propionate, run under the same conditions as (B)

This ambiguity could be resolved by degrading[14] the labelled monensin-A in two steps (i, CrO_3–AcOH; ii, CH_2N_2) to afford the known[14,15] lactone methyl ester (**1**) and dilactone (**2**), each of which was purified by flash chromatography. The ^{2}H n.m.r. spectrum of the lactone (**1**) produced in this way, together with the natural abundance ^{2}H and ^{1}H n.m.r. spectra of (**1**) are shown in Figure 3. Two strongly enriched signals at δ 2.40 and δ 2.85 are evident in the biosynthetic sample, and these can be assigned *via* the ^{1}H n.m.r. spectrum to specific enrichments (30—50 fold enhancement relative to the methyl ester signal δ 3.75) at C-4 and C-6 in monensin-A. On the other hand, the ^{2}H n.m.r. spectrum of the dilactone (**2**) generated from enriched monensin-A, was virtually identical with the natural abundance spectrum, confirming that no significant retention of deuterium had occurred at C-18, C-22, or C-24, nor at any other position in monensin-A.

These results clearly show that deuterium label is retained, *specifically* at only two methine centres in monensin-A, starting from the α-position of propionate. A stereochemical correlation between the absolute configuration of these methine centres, and the position of the retained hydrogen in the precursor, could now be established by determining the fate of the enantiotopic hydrogens in propionate. This required the synthesis of stereospecifically labelled propionates.

Stereospecifically tritiated propionic acid has been made previously,[16,17] for studies of the steric course of the propionyl-CoA carboxylase reaction. The route we employed to synthesize (*R*)- and (*S*)-[2-$^{2}H_1$]propionate is based upon chemistry described by Armarego and co-workers,[18] and is shown in the Scheme. Thus (*R*)- and (*S*)-alanine could be converted



Scheme. *Reagents: i,* $NaNO_2$, KBr H_2SO_4; *ii,* $LiEt_3BD$, THF

smoothly into (*S*)- and (*R*)-[2-$^{2}H_1$]propionates, respectively, *via* the reaction of 2-bromopropionate with superdeuteride. The enantiomeric purity of these products was expected to be high (>95%), and this was confirmed by converting each into the corresponding methyl mandelate derivative, for analysis by ^{2}H and ^{1}H n.m.r. spectroscopy, according to the method of Parker.[19] This synthetic route is thus straightforward, and amenable to the production of large amounts of chiral deuteriated propionate.

With these materials in hand two additional feeding experiments were carried out with (*R*)- and (*S*)-[2-$^{2}H_1$]propionate, each containing also a trace level of [1-^{14}C]propionate. The labelled materials (10 g) were again administered batchwise to separate fermentations of *S. cinnamonensis* (5 l), and in each case the derived Na-monensin-A contained ^{14}C label corresponding to a specific incorporation of *ca.* 16%. Importantly, however, the ^{2}H n.m.r. spectrum of Na-monensin-A biosynthesized from the *S* isomer again showed the enriched signal at δ 2.0 (as in Figure 2C), whereas the *R*-isomer led to Na-monensin-A showing no incorporation of ^{2}H label. Thus it follows that the 2-*pro-S* hydrogen in propionate is retained stereospecifically at C-4 and C-6, in monensin, whereas the 2-*pro-R* hydrogen is lost.

Incorporation of Labelled Acetate.—During the biosynthesis of monensin-A, five acetate units are required to complete the construction of the carbon backbone.[10] Primary incorporations from labelled acetate should, therefore, be expected in these five, two-carbon units (see Figure 1), and deuterium label incorporated intact will appear at C-8, C-10, C-14, C-20, and C-26 in monensin. Our first attempts to detect deuterium incorporations in this way relied upon feeding [1-^{13}C,2-$^{2}H_3$]-and [2-^{13}C,2-$^{2}H_3$]-acetates to shake cultures of *S. cinnamonensis*, so that the labelled monensins could be analysed using the well established α- and β-isotope shift techniques,[12,20] and ^{13}C n.m.r. spectroscopy.

As before, the enriched precursors (90 atom % ^{13}C, 98% deuteriated), diluted 1:1 with unlabelled material were batch fed to *S. cinnamonensis*. In the case of monensin-A biosynthesized from [1-^{13}C,2-$^{2}H_3$]acetate, no evidence could be detected in the $^{13}C\{^{1}H\}$ n.m.r. spectrum, for *intact*, deuterium retention at C-8, C-10, C-14, C-20, and C-26. Thus, within the limits of detection, all of the deuterium label is lost during the direct incorporation of acetate units into monensin-A. However, monensin-A biosynthesized in this way did contain substantial deuterium retentions in each of the methyl groups formally derived from propionate. Thus Figure 4A (see also Table 1) shows some of the ^{13}C signals assigned to these methyls, and β-shifted resonances corresponding to molecules containing ^{13}C-CH_2D, ^{13}C-CHD_2, and ^{13}C-CD_3, at these positions, are clearly identifiable. No other incorporations of

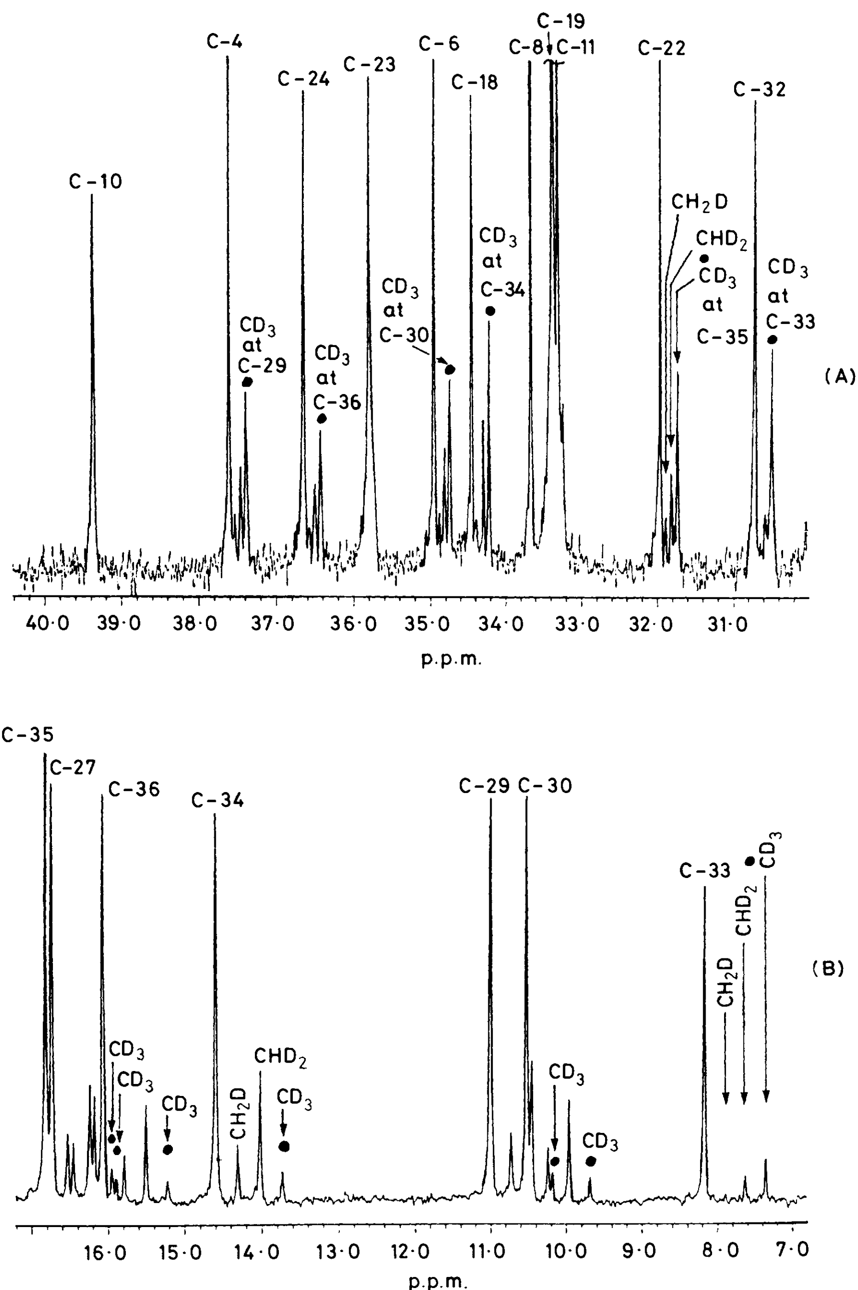


Figure 4. (A) Portion of the $^{13}C\{^1H\}$ n.m.r. spectrum of Na-monensin-A biosynthesized from sodium $[1\text{-}^{13}C,2\text{-}^2H_3]$acetate at 90.5 MHz in $CDCl_3$ (32 K data points, acquisition time 0.819 s, relaxation delay 6.0 s, with gaussian multiplication, 60° flip angle); (B) portion of the $^{13}C\{^1H,^2H\}$ n.m.r. spectrum of Na-monensin-A biosynthesized from sodium $[2\text{-}^{13}C,2\text{-}^2H_3]$acetate (64 K data points, no relaxation delay, with 1.0 Hz line broadening and exponential multiplication of FID): ● = indicates shifted resonance due to CD_3 enrichments

deuterium were detectable using this technique. Apparently, therefore, the labelled acetate can enter the primary metabolism of the cell and afford eventually methylmalonyl-CoA containing up to *three* intact deuterium atoms on the methyl group.

In a parallel incorporation experiment, $[2\text{-}^{13}C,2\text{-}^2H_3]$-acetate afforded labelled monensin-A whose $^{13}C\{^1H,^2H\}$ n.m.r. spectrum also revealed *intact* deuterium incorporations at these methyl groups (see Figure 4B, and Table 1). Now the upfield α-shifted resonances corresponding to $^{13}CH_2D$, $^{13}CHD_2$, and $^{13}CD_3$ groups can be observed, and as expected, removal of the deuterium decoupling caused the collapse of each of these resonances into a band of weak overlapping multiplets. The α-shifted peaks are notable also for the comparative weak intensity of those assigned to $^{13}CD_3$ groups. This probably reflects the loss in this case of a proton n.O.e. to the ^{13}C, rather than a drop in the relative number of molecules with $^{13}CD_3$ groups at these positions. Indeed, the corresponding resonances shown in Figure 4A, where the β-shift technique is employed, indicate that the largest population of deuteriated methyl groups actually contain *three* deuterium atoms (Table 1). As before, no other intact incorporations of deuterium were observed in this experiment.

The lack of observable deuterium retention at C-26, which is part of the presumptive acetate starter unit for carbon chain assembly, is surprising although not without precedence, and is discussed later.

Table. Isotope shifted ^{13}C resonances in sodium monensin-A upon incorporation of: (A), sodium [1-^{13}C,2-$^{2}H_3$]acetate; (B), sodium [2-^{13}C,2-$^{2}H_3$]-acetate. See Figure 4 for the spectra

(A) ^{13}C-Enrichments and β-deuterium shifts after incorporation of Na[1-^{13}C,2-$^{2}H_3$]acetate

Carbon:	C-7	C-9	C-13	C-15	C-19	C-25		
^{13}C enrichment	2.6	2.3	2.9	1.4	3.1	3.0		
Carbon:	C-2	C-4	C-6	C-12	C-18	C-22	C-24	C-32
β-shifts[a] for								
CH_2D		0.07	0.06	0.05		0.08	0.07	
CHD_2		0.15	0.13	0.09	0.16	0.15	0.15	
CD_3	0.21	0.22	0.20	0.15	0.23	0.23	0.22	0.23

(B) ^{13}C-Enrichments, α-deuterium shifted signals and their intensities, after incorporation of Na[2-^{13}C,2-$^{2}H_3$]acetate

Carbon:	C-8	C-10	C-14	C-20	C-26			
^{13}C enrichment	1.8	1.8	2.1	2.1	1.9			
Carbon:	C-27	C-29	C-30	C-31	C-33	C-34	C-35	C-36
α-shifts[a] for								
CH_2D[b]	0.28 (14)	0.27 (17)	0.28 (14)			0.29 (15)	0.29 (16)	0.28 (12)
CHD_2[b]	0.56 (26)	0.54 (36)	0.56 (33)	0.57 (29)	0.54 (7)	0.58 (33)	0.58 (27)	0.56 (24)
CD_3[b]	0.84 (6)	0.81 (8)	0.84 (6)	0.85 (6)	0.82 (13)	0.87 (8)	0.87 (8)	0.84 (5)

[a] The isotope induced shifts are given as Δ p.p.m. upfield from the natural abundance singlet. [b] The intensity of the shifted signal as a percentage of the intensity of the non-shifted signal, at the natural abundance chemical shift, is given in each case in parentheses.

Another perspective on the incorporation of deuterium label from sodium [2-$^{2}H_3$]acetate was obtained by analysing the enriched antibiotic using ^{2}H n.m.r. spectroscopy. The $^{2}H\{^{1}H\}$ n.m.r. spectrum of monensin-A biosynthesized from sodium [2-$^{2}H_3$]acetate was similar in appearance to the natural abundance spectrum, although there were clearly high levels of enrichment present. The enriched antibiotic was therefore degraded, by the method described earlier, to afford the dilactone (**2**) and the lactone methyl ester (**1**), and the ^{1}H and $^{2}H\{^{1}H\}$ n.m.r. spectra of these materials were recorded. For the ester (**1**), deuterium enrichments are seen at all positions *except* the methyl-bearing methine centres (derived from C-2, C-4, and C-6 in monensin-A). This includes each of the methyl groups, and those methine centres bearing an oxygen substituent (C-3 and C-5). The dilactone (**2**) also showed ^{2}H enrichments at many sites along the carbon backbone, although overlapping resonances in both the ^{1}H and ^{2}H n.m.r. spectra makes it difficult in many cases to assign these enrichments to specific regio- or stereo-heterotopic positions. Since it is clear from the earlier experiments that the only *intact* incorporations to have occurred (*i.e.* without C–D bond cleavage from C-2 of acetate) are those into the propionate derived methyl groups (as in Figure 4), these other indirect incorporations must arise from the *in vivo* metabolism of [2-$^{2}H_3$]acetate, including deuterium transfers *via* nicotinamide coenzymes. The full details of these intervening processes are, however, ill-defined.

Incorporation of Deuterium from the Medium.—The exchange of protons in substrates with protons in the medium, has been inferred often during the stable isotope labelling experiments described above. Direct information about this can be obtained by allowing the biosynthesis to proceed in a deuteriated medium. Thus, a shake culture of *S. cinnamonensis* was grown in 60% D_2O–40% H_2O. At higher ratios of deuterium oxide cell growth is severely inhibited. The monensin-A sodium salt generated in this way was characterized by $^{13}C\{^{1}H\}$ and $^{13}C\{^{1}H,^{2}H\}$ n.m.r. spectroscopy, and relevant parts of these spectra are shown in Figure 5. These show substantial deuterium enrichments at each of the methyl-bearing centres (*e.g.* C-2; Figure 5) and O-bearing methine centres (*e.g.* C-7), all of the methyl groups *except* C-33, and at C-26 in the starter unit. A single, major β-shifted resonance for C-33, reveals the presence largely of one deuterium atom at the adjacent methylene group (C-32). Given the substantial levels of deuterium enrichment at every other protonated carbon in this sample of monensin-A, the presence of only a single deuteron in the C(33)–C(32) unit indicates that this has not been derived from primary metabolism as acetyl-CoA. This two-carbon unit is part of the single butyrate unit [C(15)–C(16)–C(32)–C(33)] in monensin-A, which may instead originate directly from branched-chain amino acid catabolism, or conceivably from only the terminal four carbon atoms of fatty acids *via* fatty acid catabolism (*vide infra*).

Discussion

Whilst a great deal remains unknown about the mechanisms of stereo- and regio-control over polyketide antibiotic production, the results described above strengthen the often implied analogy to fatty acid biosynthesis, particularly with regard to chemical aspects of the key carbon–carbon bond forming reactions, during the biosynthesis of monensin-A. Thus the condensing enzyme of the fatty acid synthase (FAS) complex catalyses the decarboxylative-condensation of malonyl-CoA onto an acetyl starter unit, or an enzyme bound acyl thioester intermediate, with overall inversion of configuration at the nucleophilic carbon.[21–23] Analogous processes during antibiotic production would require the involvement of not only malonyl-CoA, but also methylmalonyl-CoA and ethylmalonyl-CoA. Since both epimers of methylmalonyl-CoA may, in principle, participate in chain elongation, a potentially elegant method of controlling the configuration of the newly formed chiral centre becomes possible, by selecting the appropriate stereoisomer of methylmalonyl-CoA and processing this in a stereospecific manner, by a mechanism analogous to that catalysed by the FAS condensing enzyme. This concept has been expounded previously, notably by Hutchinson and co-workers.[1,24] Thus the selection of (*R*)-methylmalonyl-CoA leads to a condensation product with a newly formed chiral centre of *S* absolute configuration, whereas the use of (*S*)-methylmalonyl-CoA leads to an *R* centre (see Figure 6).

Our results on the incorporation of labelled isotopomers of propionate at least support the operation of half of this scheme during monensin biosynthesis; that involving the use of (*S*)-

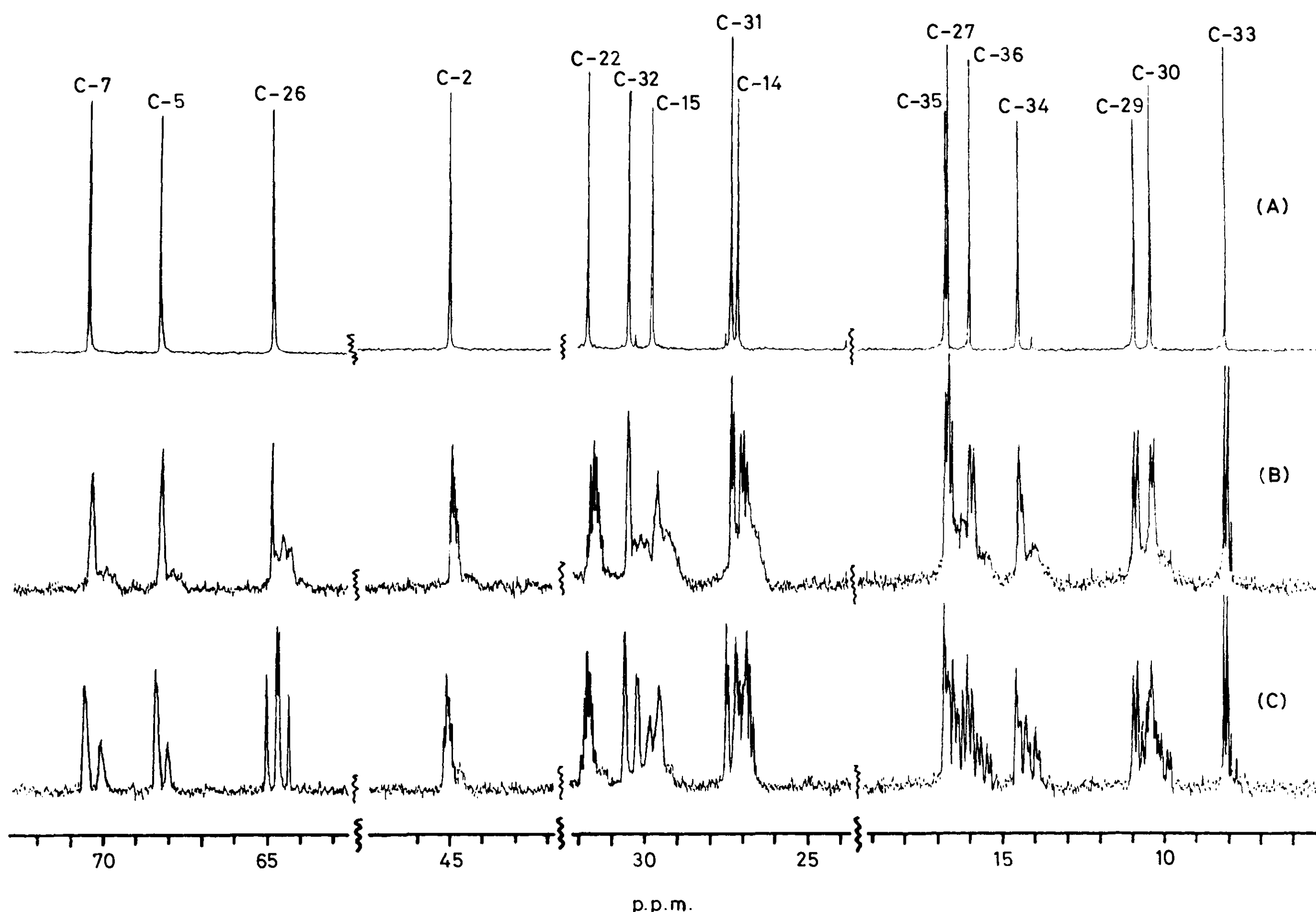


Figure 5. (A) Portions of the $^{13}C\{^1H\}$ n.m.r. spectrum of Na-monensin-A at natural abundance; (B) portions of the $^{13}C\{^1H\}$ n.m.r. spectrum of Na-monensin-A biosynthesized in a fermentation broth containing 60% vol/vol D_2O and 40% H_2O; (C) $^{13}C\{^1H,^2H\}$ n.m.r. spectrum of the same sample, 1.5W-^1H decoupling 3.0 W ^2H decoupling, 32 K data points, relaxation delay 1.0 s, 0.5 Hz line broadening

methylmalonyl-CoA. Thus the observed retention *specifically* of the 2-*pro-R* hydrogen in propionate, and its incorporation at C-4 and C-6 in monensin, strongly indicates the processing of the labelled propionyl-CoA on propionyl-CoA carboxylase to afford (*S*)-methylmalonyl-CoA, following the known stereochemical course of this enzyme isolated from *Propionibacterium shermanii.*[16,17,25] The (*S*)-methylmalonyl-CoA can then participate in antibiotic biosynthesis, by a decarboxylative-condensation, onto an existing primer, with inversion of configuration, and with deuterium retained intact at the newly formed chiral centre (*i.e.*, C-4 and C-6 in monensin-A). The C-2, C-18, C-22, and C-24 centres in monensin, on the other hand, have the opposite sense of chirality. The C-2 and C-24 centres, in particular, could arise directly through the incorporation of (*R*)-methylmalonyl-CoA, following the analogous decarboxylative-condensation route, whereas the configuration of the C-18 and C-22 centres must be influenced instead by the changes *following* carbon–carbon bond formation, including reduction–dehydration–reduction steps, in direct analogy to the corresponding steps in fatty acid biosynthesis.

On the other hand, no clear evidence exists for the action in polyether antibiotic biosynthesis of synthases that can directly utilize (*R*)-methylmalonyl-CoA. It is conceivable that the *S*-isomer alone may be employed for the insertion of all propionate units into the backbone, and alterations to the configuration of chiral methine centres, where necessary, may be catalysed by specific epimerases acting after carbon–carbon bond formation (see Figure 6). Direct evidence for the utilization of (*R*)-methylmalonyl-CoA has been sought elsewhere, by examining the incorporation of ^{13}C and ^2H labelled isotopomers of succinate into the polyether antibiotic lasalocid-A[26] (**3**), and into the macrolide antibiotic erythromycin[4] (**4**). These precursors *in vivo* may afford specifically the (*R*)-[2-^2H]methylmalonyl-CoA, through the action of methylmalonyl-CoA mutase, whose expected steric course is based on that known for the *P. shermanii* enzyme.[27–29] Unfortunately, no observable deuterium retention at methine centres derived from C-2 of methylmalonyl-CoA occurred in these systems, so the question as to whether (*R*)-methylmalonyl-CoA does participate in the carbon-chain assembly process remains open.

These data emphasize the need for a detailed knowledge of the basic biochemistry of primary metabolism in Streptomycetes, particularly as it impinges upon the secondary metabolism leading to antibiotic production. The normal *in vivo* source of methylmalonyl-CoA is an issue of particular interest in this context. Well established metabolic routes to this intermediate involve carboxylation of propionyl-CoA, and the rearrangement of succinyl-CoA. The isolation of an acyl-CoA carboxylase,[30] methylmalonyl-CoA mutase[31] and an (*R*)-methylmalonyl-CoA/malonyl-CoA decarboxylase,[32] all from the erythromycin producer *S. erythraeus*, have been reported by Kolattukudy and co-workers, although the exact relationship of these activities to erythromycin biosynthesis has not been easy to define. Other sources of methylmalonyl-CoA, however, must

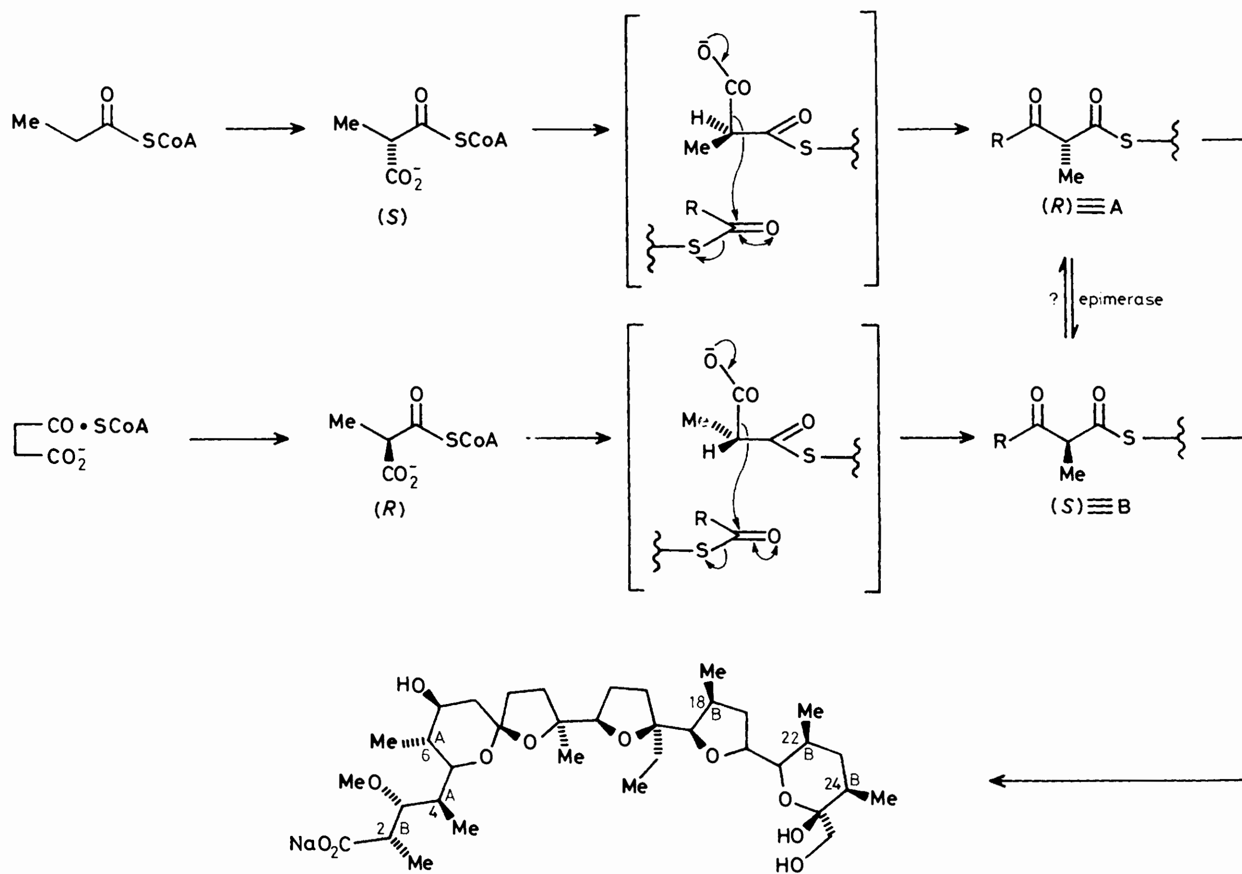
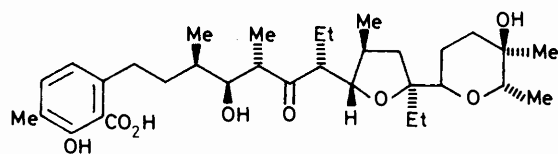
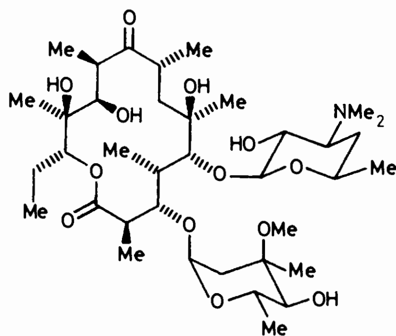


Figure 6.





also be considered, and in this context the results of our incorporations of labelled acetate into monensin, together with the results described in the following paper, point to an important role also in antibiotic production for C_4 fatty acids derived from the breakdown of long-chain fatty acids, and the branched chain amino acids.

The *direct* incorporation of [1-^{13}C,2-^{2}H$_3$]- and [2-^{13}C,2-^{2}H$_3$]-acetate into the backbone of monensin-A has been shown here to occur with complete loss of the deuterium label. This includes a complete wash-out of deuterium from the starter unit at C(25)–C(26). This is not without precedence in Streptomycetes, similar observations having been obtained for lasalocid (**3**) biosynthesis by Hutchinson and co-workers,[26] and for the polyether ICI 139603 (**5**) by Staunton and co-workers.[3] In contrast, however, the incorporation of [2-^{13}C,2-^{2}H$_3$]acetate into fatty acids in *E. coli* occurs predominantly with retention of three deuterium[33] atoms in the starter unit, and deuterium is known to be retained also at the starter unit methyl group during the biosynthesis of several other microbial metabolites.[12] One suggestion[26] is that in Streptomycetes the starter unit arises by decarboxylation of malonyl-CoA, thereby accounting for the facile wash-out of label.

The observed *indirect* but intact incorporation of methyl groups bearing one, two, or three deuterium atoms, at the propionate derived methyl groups, upon feeding either [1-^{13}C,2-^{2}H$_3$]- or [2-^{13}C,2-^{2}H$_3$]-acetate, is of interest and requires further comment. In similar experiments, conducted with both lasalocid-A[26] and ICI 139603[3] producing strains of *Streptomyces*, the incorporations of labelled acetate into either lasalocid-A or ICI 139603 were also observed, but in each case a *maximum of two* deuterons were retained at the propionate derived methyl groups, upon incorporation of [2-^{13}C,2-^{2}H$_3$]-acetate. On the other hand, in this same experiment, the methyl carbon of the butyrate unit in lasalocid-A did retain *three*, two, or one deuteron(s). These results can be interpreted very reasonably.[3,26] Firstly, butyryl-CoA should be generated *in vivo* from

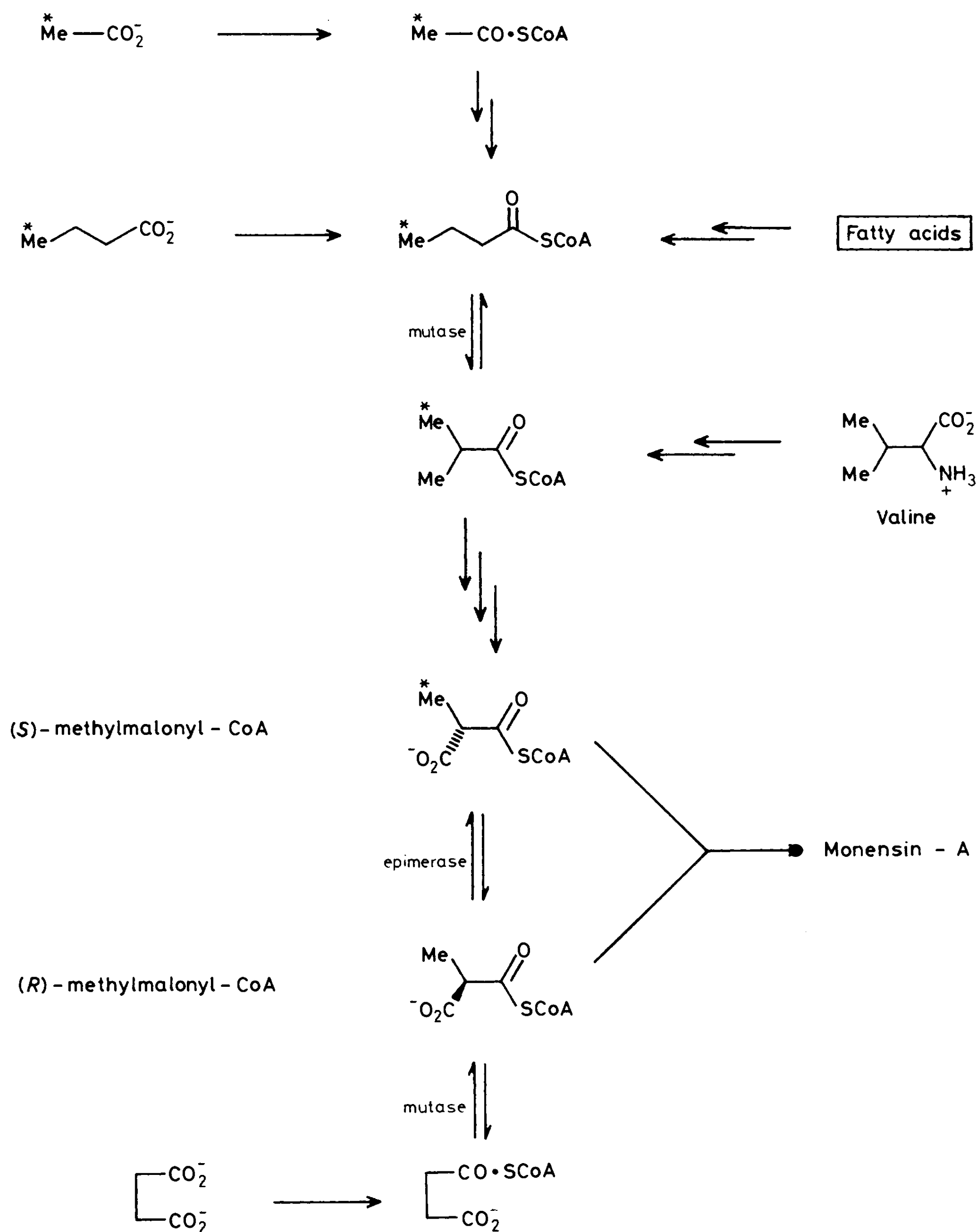
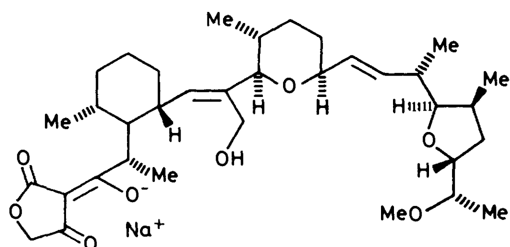


Figure 7. Metabolic pathways leading to methylmalonyl-CoA; from succinyl-CoA, catalysed by methylmalonyl-CoA mutase; from valine and fatty acid catabolism *via* oxidation of isobutyryl-CoA

acetyl-CoA with complete retention of three deuterons in the methyl group, following the coupling of two C_2 units in the usual way. The acetyl-CoA may also enter the Krebs cycle, and give rise to labelled succinyl-CoA and succinate. In this way [2-^{13}C,^{2}H$_3$]acetyl-CoA would afford both [2-^{13}C,2-^{2}H$_2$]- and [3-^{13}C,3-^{2}H$_2$]-succinyl-CoAs. The former of these two isotopomers may then furnish (*R*)-[3-^{13}C,3-^{2}H$_2$-*methyl*]methylmalonyl-CoA through the action of methylmalonyl-CoA mutase. This pathway, therefore, explains how up to *two* deuterons can be incorporated from acetate, but cannot account for the formation of a significant population of [3-^{13}C,3-^{2}H$_3$-*methyl*]methylmalonyl-CoA molecules from [2-^{13}C,2-^{2}H$_3$]-acetate.

A likely explanation arises, however, through consideration of the role of butyrate and isobutyrate as precursors of the polyether carbon-chain building blocks. This is discussed in detail in the following paper,[34] where convincing evidence is presented for the occurrence of a novel metabolic reaction in *S. cinnamonensis*, involving the reversible rearrangement of isobutyrate into butyrate. As shown in Figure 7, this allows the conversion of [2-^{13}C,2-^{2}H$_3$]acetyl-CoA firstly into butyryl-CoA, then into isobutyryl-CoA, with oxidation of the latter affording methylmalonyl-CoA containing three deuterons on the key methyl group. The implied link between straight-chain and branched-chain fatty acid metabolism appears to be an important one in terms of the provision of carbon building blocks for polyketide antibiotic production in this organism. As to why during the incorporations of [2-^{13}C,2-^{2}H$_3$]acetate into lasalocid-A[26] and ICI 139603 (**5**)[3] this pathway is apparently not as dominant, it is possible that this merely reflects variations

in the culture conditions for the different organisms since this will certainly influence the expression of genes encoding the relevant primary metabolic pathways.



ICI 139603 (**5**)

In conclusion, the labelling experiments reported above have shown some features of the complex primary and secondary metabolism operative during polyether antibiotic production in *S. cinnamonensis*. This information is almost certainly relevant also when considering the pathways to other closely related families of antibiotics, including the macrolides, polyenes, and avermectins/milbemycins. With techniques for gene cloning in *Streptomyces* now well advanced,[35] new opportunities for studying these pathways at the level of individual enzymes and genes are emerging. This may well help in the clarification of the unanswered questions posed by this work, and may lead also to many other novel findings in this area over the next few years.

Experimental

^1H, ^2H, and ^{13}C N.m.r. spectra were measured on a Bruker AM360 spectrometer. $^{13}C\{^1H,^2H\}$ Spectra were recorded with continuous ^1H (1W) and ^2H (3W) decoupling, and were run unlocked, using the drift compensation circuit on the spectrometer. Sodium [2-^{13}C,2-$^2H_3$]acetate, and sodium [1-^{13}C,2-$^2H_3$]acetate (90 atom % ^{13}C, 98 atom % ^2H) were supplied by B.O.C. Prochem. All other routine analytical methods, including conditions for the growth of the micro-organism were carried out as described previously.[9]

Sodium [2-$^2H_2$]*Propionate Feeding Experiment.*—Sodium [2-$^2H_2$]propionate was prepared by decarboxylating methylmalonic acid at 150 °C in deuterium oxide. The deuterium content at C-2 was >95 atom %, based on ^1H n.m.r. analysis. Five 60-ml vegetative cultures of *S. cinnamonensis* were prepared as described previously.[9] These were added to a fermentation vessel containing soybean grits (150 g), D-glucose (180 g), $CaCO_3$ (6 g), $MnCl_2 \cdot 2H_2O$ (1.2 g), $FeSO_4 \cdot H_2O$ (1.8 g), KCl (600 mg), soybean oil (90 g), methyl oleate (120 g; technical grade) and lard oil (30 g; Sigma), dispersed in distilled water (6 l), and previously sterilized at 121 °C for 45 min. The fermentation was continued at 32 °C with vigorous stirring (1 000 r.p.m.) and aeration (1 vol/vol/min of air). Sodium [2-$^2H_2$]propionate (15 g) in water (75 ml) was sterilized at 121 °C for 15 min, and then added in equal portions after 24, 32, and 38 h into the fermentation. The fermentation was continued for a total of 74 h after which it was stopped and adjusted to pH 8 with aqueous NaOH; the cells were then collected by centrifugation (5 000 r.p.m., Beckman JA-10 rotor, 20 min). The supernatant was extracted twice with chloroform, whereas the cells were washed three times with methanol. The methanol extract was diluted with an equal volume of water and extracted with chloroform. The combined chloroform extracts were evaporated and the residue was chromatographed on a silica column eluting first with methylene dichloride and then with ethyl acetate. Fractions containing monensin-A were combined and evaporated. The residue was chromatographed again under these conditions, and the monensin-A recovered was crystallized twice from methanol–water to afford white crystals (3 g), m.p. 270 °C (lit.,[36] 267—269 °C). The $^2H\{^1H\}$ n.m.r. spectrum of this material is shown in Figure 2C. The ^1H n.m.r. spectrum was identical with that shown in Figure 2A.

Degradation of Monensin-A.—Monensin-A (5.0 g; from two feeding experiments with sodium [2-$^2H_2$]propionate) in acetic acid (75 ml) was added with cooling to CrO_3 (6.8 g) in acetic acid (60 ml) and water (15 ml). The mixture was stirred overnight at room temperature and then saturated aqueous NaCl (1 l) was added; the mixture was then extracted ($\times 4$) with $CHCl_3$. The organic extract was washed (saturated aqueous NaCl), dried (Na_2SO_4), and evaporated and the residue redissolved in diethyl ether. The solution was then filtered to remove insoluble inorganic salts, washed ($\times 3$) with saturated aqueous $NaHCO_3$, dried, and evaporated to leave a yellow oil (1.6 g; neutral fraction). The aqueous layers were acidified to (pH 1, dilute HCl), extracted ($\times 3$ in Et_2O). The organic extracts were then dried and evaporated to yield a yellow oil (1.8 g; acidic fraction). This was redissolved in diethyl ether and the solution treated with an excess of diazomethane and evaporated. The resulting oil was chromatographed on silica, eluting with light petroleum–diethyl ether (4 l), and fractions containing the lactone (**1**) were combined and evaporated. This crystallized from hexane as white needles (0.4 g), m.p. 100—101 °C (lit.,[14] 102—104 °C). The $^2H\{^1H\}$ n.m.r. spectrum is shown in Figure 3; $\delta_H(CDCl_3)$, 4.23 (1 H, dd, 5-H), 3.78 (3 H, s, Me-ester), 3.40 (3 H, s, OMe), 3.30 (1 H, dd, 3-H), 2.86 (1 H, m, 6-H), 2.50 (1 H, m, 2-H), 2.41 (1 H, m, 4-H), 1.43 (3 H, d, 6-Me), 1.40 (3 H, d, 2-Me), and 0.91 (3 H, d, 4-Me); δ_C 4.59, 14.85, 15.40, 31.24, 38.74, 42.25, 52.00, 56.64, 80.20, 82.67, 173.02, and 174.07.

The neutral fraction containing the dilactone (**2**), was chromatographed on a silica column eluting with light petroleum (b.p. 40—60 °C)—ethyl acetate (2:1). Fractions containing the dilactone were evaporated to leave a colourless oil (0.61 g); $\delta_H(CDCl_3)$, 4.31 (1 H, m, 12-H), 4.15 (1 H, dd, 13-H), 4.03 (1 H, d, 9-H), 3.92 (1 H, dd, 5-H), 2.90 (1 H, q), 2.73 (1 H, dt), 2.45 (2 H, m), 2.30 (1 H, m), 2.15 (1 H, m), 2.07 (1 H, m), 1.92 (1 H, m), 1.83 (1 H, m), 1.8—1.35 (8 H, overlapping multiplets), 1.30 (3 H, s, Me), 1.25 (3 H, d, Me), 1.07 (3 H, d, Me), 0.94 (3 H, d, Me), and 0.81 (3 H t, CH_2CH_3); δ_C 7.48 (q), 15.16 (q), 16.78 (q), 17.75 (q), 23.47 (q), 27.65 (t), 28.75 (t), 29.59 (t), 29.78 (t), 29.96 (t), 31.02 (d), 34.28 (t), 34.54 (d), 35.65 (d), 37.03 (t), 76.83 (d), 82.51 (d), 86.41 (d), 87.07 (s), 87.63 (d), 87.77 (s), 173.39 (s), and 177.25 (s).

Sodium (R)-[2-$^2H_1$]*Propionate.*—L-Alanine (10 g, 112 mmol) in dilute sulphuric acid (1M; 800 ml) containing KBr (80 g, 6 equiv.) was treated portionwise with $NaNO_2$ (20.9 g, 2.7 equiv.) at 0 °C, and stirred for 1 h. The solution was then extracted with diethyl ether and the extract dried (Na_2SO_4) and evaporated to leave an oil. This reaction was repeated twice, and the oils obtained from three experiments were combined and distilled to afford the (*S*)-2-bromopropionic acid (30 g, 58%), b.p. 50 °C at 0.1 mmHg, $[\alpha]_D$ −24.7° (neat). The optical purity of this material was assessed after forming a derivative with (*R*)-(−)-methyl mandelate, as follows. The (*S*)-2-bromopropionic acid (306 mg, 2 mmol) was stirred at −10 °C with 4-dimethylaminopyridine (5 mg), dicyclohexylcarbodi-imide (412 mg), and (*R*)-methyl mandelate (332 mg) in dry CH_2Cl_2 (10 ml). After 3 h the solution was filtered, evaporated under reduced pressure, and the residue purified by column chromatography on silica, eluting with hexane–ethyl acetate; $\delta_H(CDCl_3)$, 1.78 (3 H, d), 3.60 (3 H, s), 4.38 (1 H, q), 5.87 (1 H, s), and 7.35 (5 H, m). Double

irradiation at δ 1.78 caused collapse of the quartet at δ 4.38 to a sharp singlet, indicating 94% e.e. From racemic 2-bromopropionic acid as starting material a derivative showing two singlets at δ 4.43 and δ 4.38 was obtained.

(*S*)-2-Bromopropionic acid (10 g) was added to 1M lithium triethyldeuterioborate (182 ml; Aldrich) in tetrahydrofuran, and the mixture refluxed under N_2 for 2 h; it was then left overnight at room temperature. To this was added water (30 ml) followed by H_2O_2–NaOH (1M) (1:1; 120 ml). The resulting solution was washed with diethyl ether, acidified to pH 2 with dilute H_2SO_4, saturated with Na_2SO_4, and continuously extracted with diethyl ether overnight. The ether extract was extracted with 1M NaOH (3 × 25 ml) and the aqueous phase was freeze dried. The resulting solid was redissolved in water, acidified (pH 1, H_2SO_4), and then lyophilized. The lyophilisate was adjusted to pH 9 (NaOH) and freeze dried to afford sodium (*R*)-[2-$^2H_1$]-propionate as a white solid (5.8 g). The optical purity of this salt was assessed as follows. The salt (240 mg) was dissolved in dilute HCl, saturated with NaCl, and extracted continuously overnight with diethyl ether. The ether extract was dried (Na_2SO_4) and most of the ether was distilled off at atmospheric pressure. The residual acid was redissolved in CH_2Cl_2 (10 ml) at −15 °C and 4-dimethylaminopyridine (5 mg), (*R*)-methyl mandelate (332 mg), and dicyclohexylcarbodi-imide (412 mg) were added. After being stirred for 3 h the solution was filtered, evaporated, and the product purified by flash chromatography (silica) eluting first with hexane, and then with hexane–ethyl acetate (9:1). The mandelate derivative showed δ(C_6D_6) 1.03 (3 H, d), 2.30 (1 H, m), 3.30 (3 H, s), 6.15 (1 H, s), and 7.1—7.6 (5 H, m); $\delta_{^2H}$(C_6H_6), 2.20 (1 H, s). The optical purity, based upon spectral comparison with the corresponding derivative of [2-$^2H_2$]propionate, was 96% (±2%).

Sodium (S)-[2-$^2H_1$]*Propionate*.—This was prepared from D-alanine, by the procedure described above for sodium (*R*)-[2-$^2H_1$]propionate. Again the product was obtained in high optical purity [>95% (*S*)] based on ^1H n.m.r. analysis of its derivatives with (*R*)-methyl mandelate.

Feeding Experiment with Sodium [2-^{14}C,2-^3H]*Propionate*.—Sodium [2-^{14}C,2-^3H]propionate was prepared by mixing ^3H and ^{14}C labelled materials, and a portion was converted into the *p*-bromophenacyl derivative to determine the specific activity {3.93 × 10^7 d.p.m./mmol [^3H]; 2.18 × 10^6 d.p.m./mmol [^{14}C], $^3H/^{14}C$ = 18.0}. Three fermentation cultures of *S. cinnamonensis* A3823.5 (60 ml each) were inoculated in the usual way, and grown on an orbital shaker at 32 °C. The sodium [2-^{14}C,2-^3H]propionate (115 mg) was added in three equal portions, as a sterile solution in water, after 24, 32, and 50 h of growth. The cultures were harvested to recover Na-monensin-A after 7 days, and the antibiotic was recrystallized to constant specific activity: 4.47 × 10^5 d.p.m./mmol [^3H], 7.15 × 10^5 d.p.m./mmol [^{14}C], [^3H]/[^{14}C] = 0.63. Specific incorporation of ^{14}C label was 4.7%, assuming the incorporation of 7 mol of propionate per mol of monensin-A.

Feeding Experiment with Sodium (S)-[1-^{14}C,2-$^2H_1$]*Propionate*.—To sodium (*S*)-[2-$^2H_1$]propionate (10 g) was added *ca.* 500 μCi of sodium [1-^{14}C]propionate (50 mCi/mmol). A small portion (50 mg) of the resulting sodium propionate was converted into the *p*-bromophenacyl derivative and recrystallized to constant specific activity (8.49 × 10^6 d.p.m./mmol). The remaining sample in water (75 ml), was adjusted to pH 7.5 (dilute HCl) and sterilized at 121 °C for 15 min. This was subsequently added to a 5 l fermentation of *S. cinnamonensis* (prepared as described earlier), in three equal portions, 24, 30, and 40 h after inoculation. The fermentation was worked up after 4 days to give recovery of pure sodium monensin-A (2.0 g), m.p. 270 °C (1.11 × 10^7 d.p.m./mmol; specific incorporation of ^{14}C 18.7%).

Feeding Experiment with Sodium (R)-[1-^{14}C,2-$^2H_1$]*Propionate*.—Sodium (*R*)-[1-^{14}C,2-$^2H_1$]propionate (10 g; 8.20 × 10^6 d.p.m./mmol ^{14}C), prepared in an analogous fashion, was added to a 5 l fermentation of *S. cinnamonensis*, following the same protocol, as for the experiment with (*S*)-[1-^{14}C,2-$^2H_1$]propionate. Sodium monensin-A (3.0 g) m.p. 272 °C was recovered (9.26 × 10^6 d.p.m./mmol ^{14}C; specific incorporation of ^{14}C 16.1%).

Feeding Experiments with Labelled Acetates.—The labelled substrate (sodium [1-^{13}C, 2-$^2H_3$]acetate or sodium [2-^{13}C,2-$^2H_3$]acetate) (600 mg) was diluted with an equal weight of unlabelled sodium acetate. The mixture was sterilized in water at 121 °C, 15 min, and a total of 300 mg was added batchwise, in three equal portions, to a fermentation culture (60 ml; prepared as described earlier[9]) at 24, 45, and 54 h into the fermentation. After a total of 6 days growth at 32 °C on an orbital shaker, the monensin-A was isolated and purified,[9] for analysis by $^{13}C\{^1H\}$ and $^{13}C\{^1H,^2H\}$ n.m.r.

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