Butyrate Metabolism in Streptomycetes. Characterization of an Intramolecular Vicinal Interchange Rearrangement Linking Isobutyrate and Butyrate in *Streptomyces cinnamonensis*

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The incorporations of various carbon-13 and deuterium labelled forms of isobutyrate into the polyether antibiotic monensin-A have provided evidence for the existence of a novel rearrangement in whole cells of *Streptomyces cinnamonensis*, which leads to the conversion of isobutyrate into butyrate. This rearrangement is shown to proceed in an intramolecular fashion by migration of the carboxy carbon of isobutyrate to the 2-*pro*-*S* methyl, with a concomitant back migration of a hydrogen atom from this methyl group predominantly into the 3-*pro*-*R* position in butyrate. Formally, therefore, the carboxy carbon is replaced with overall retention of configuration, in a vicinal interchange rearrangement. The significance of these observations with regard to the coenzyme requirements of the rearrangement, and its relationship to polyether and macrolide antibiotic production in Streptomycetes is discussed.

In common with many secondary metabolites produced by the genus *Streptomyces*, monensin-A and related polyether ionophores may be classified as polyketide antibiotics. Thus carbon-13 labelling experiments have shown[1] that the carbon backbone of monensin-A is assembled from five acetate, seven propionate, and one butyrate building blocks, as indicated in Figure 1. The starter unit for carbon-chain assembly should be acetyl-CoA, based on analogies to fatty acid biosynthesis, and chain elongation should occur using activated forms of the precursors, namely malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA. The fact that seven molecules of propionate are required to construct each molecule of monensin-A indicates the importance of methylmalonyl-CoA as a potential limiting precursor in the secondary metabolism.

The metabolic routes to methylmalonyl-CoA are, therefore, of great interest, and recently the roles of amino acid catabolism[2–6] (particularly valine, methionine, leucine, isoleucine, and phenylalanine) and long-chain fatty acid degradation, in addition to the Krebs cycle,[7] in furnishing building blocks for polyketide antibiotic production, have been recognized. Thus, Omura and co-workers[2–5] have described how L-valine and isobutyrate can supply both the propionate units, and the butyrate units, required for the biosynthesis of the aglycones of the 16-membered macrolide antibiotics tylosin and leucomycin. Related observations were made by Vanek and co-workers,[3] who showed that monensin-A is efficiently and specifically enriched upon incorporation of either [1-^{13}C]-butyrate or [1-^{13}C]isobutyrate; seven of these enrichments occur in propionate units formally derived from methylmalonyl-CoA, and the highest enrichment was seen from labelled isobutyrate in the single butyrate unit [*i.e.* C(15)–C(16)–C(32)–C(33)] in monensin-A (see Figure 1). Valine catabolism in other prokaryotic[8] and eukaryotic[9] organisms is known to proceed through isobutyryl-CoA, and although not all of the steps have been clearly defined at the enzymic level, the oxidation of isobutyryl-CoA to propionate is well established. On the other hand, the implied intact conversions *in vivo* of isobutyryl-CoA to butyryl-CoA, and of butyryl-CoA into methylmalonyl-CoA are without precedence in the known primary metabolism of both prokaryotes and eukaryotes. The clear implication from the labelling experiments was, however, that both isobutyrate and butyrate can afford efficiently

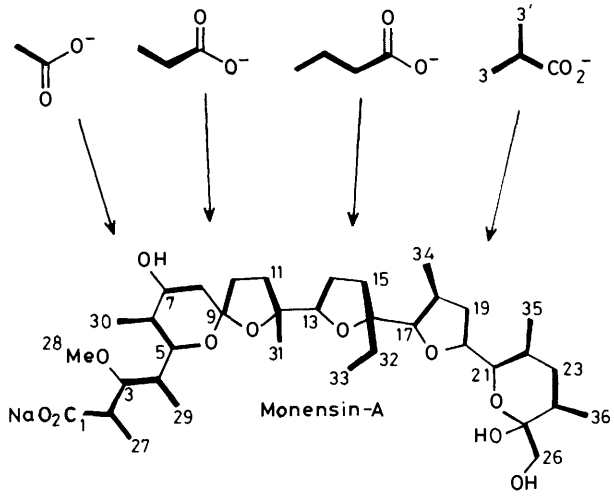


Figure 1.

methylmalonyl-CoA in Streptomycetes, and that there exists also a pathway for the conversion of isobutyrate into butyrate.

We report in this paper the full details of our experiments[10–12] on the roles of butyrate and isobutyrate in monensin biosynthesis. This includes unambiguous evidence for a direct conversion of isobutyrate into butyrate in *S. cinnamonensis*, which involves an intramolecular carbon skeleton vicinal interchange rearrangement, reminiscent of, but distinct from, the well known[13] rearrangement of succinyl-CoA into methylmalonyl-CoA. The rearrangement apparently involves the conversion of isobutyryl-CoA into butyryl-CoA, possibly catalyzed by a novel coenzyme-B_{12} dependent mutase. This constitutes an important link between straight chain and branched-chain fatty acid metabolism in these antibiotic producing organisms.

Results

Incorporation of Carbon-13 Labelled Precursors into Monensin-A.—The precursor roles of propionate, butyrate, and iso-

Table. Enrichments observed in sodium monensin-A upon incorporation of labelled precursors. Enrichment = height of enriched $^{13}C\{^1H\}$ signal ÷ average height of unenriched signal in same spectrum. All spectra are $^{13}C\{^1H\}$ at 90.5 MHz with broad band ^1H decoupling, 32 K data points, 6 s relaxation delay, 0.5 Hz line broadening with exponential multiplication before Fourier transformation. For details of feeding protocols see Experimental section. Pr = sodium propionate, Bu = sodium butyrate, and IBA = sodium isobutyrate

	[^{13}C]Enrichments in Na-monensin-A precursor administered			
Carbon	[1-^{13}C]Pr	[1-^{13}C]Bu	[1-^{13}C]IBA	[3,3′-$^{13}C_2$]IBA
1	4.0	4.1	2.5	
3	6.1	5.8	3.4	
5	5.6	5.7	2.9	
11	5.1	5.0	2.7	
15		7.5	5.9	
16				2.8 (*J* 1.8 Hz)
17	6.9	6.8	3.8	
21	6.3	6.6	3.3	
23	4.2	4.2	2.7	
27				2.1
29				2.3
30				2.2
31				2.5
33				3.8 (*J* 1.8 Hz)
34				2.1
35				2.8
36				3.4

butyrate in monensin biosynthesis[1] was tested by administering ^{13}C-labelled forms of each to shake flask cultures of *S. cinnamonensis*. Subsequently, the antibiotic was isolated and examined by high-field $^{13}C\{^1H\}$ n.m.r. spectroscopy.[14] The results of these incorporations are collated in the Table. These data show again that butyrate and isobutyrate act efficiently as precursors of all the propionate units, as well as the single butyrate unit, in monensin-A. This whole-cell assay can, therefore, be used to investigate the primary metabolism of these C_4 fatty acids. In particular, the incorporation of labelled isotopomers of isobutyrate into the single butyrate unit [C(15)–C(16)–C(32)–C(33)] in monensin-A provides data on the rearrangement of isobutyrate to butyrate.

Evidence for an Intramolecular Carbon Skeleton Rearrangement.—Two experiments with multiply carbon-13 labelled isobutyrate gave evidence for an intramolecular rearrangement *in vivo* leading from isobutyrate to butyrate. In the first, [3,3′-$^{13}C_2$]isobutyrate was administered portionwise to shake cultures until its final concentration in the broth was 15 mM. The monensin-A produced was isolated and examined by 90 MHz $^{13}C\{^1H\}$ n.m.r. spectroscopy. The enrichments, recorded in the Table, reveal that both C-16 and C-33 are labelled and the presence also of a long range coupling ($J_{1,3}$ 1.8 Hz) indicates that the isobutyrate has been incorporated intact. This was confirmed in the second experiment, upon incorporation of [1,3,3′-$^{13}C_3$]isobutyrate (each site 90 atom % enriched) diluted 1:3 with unlabelled material. Portions of the $^{13}C\{^1H\}$ n.m.r. spectrum of monensin-A generated in this way are shown in Figure 2, and include two strongly enhanced signals assigned to

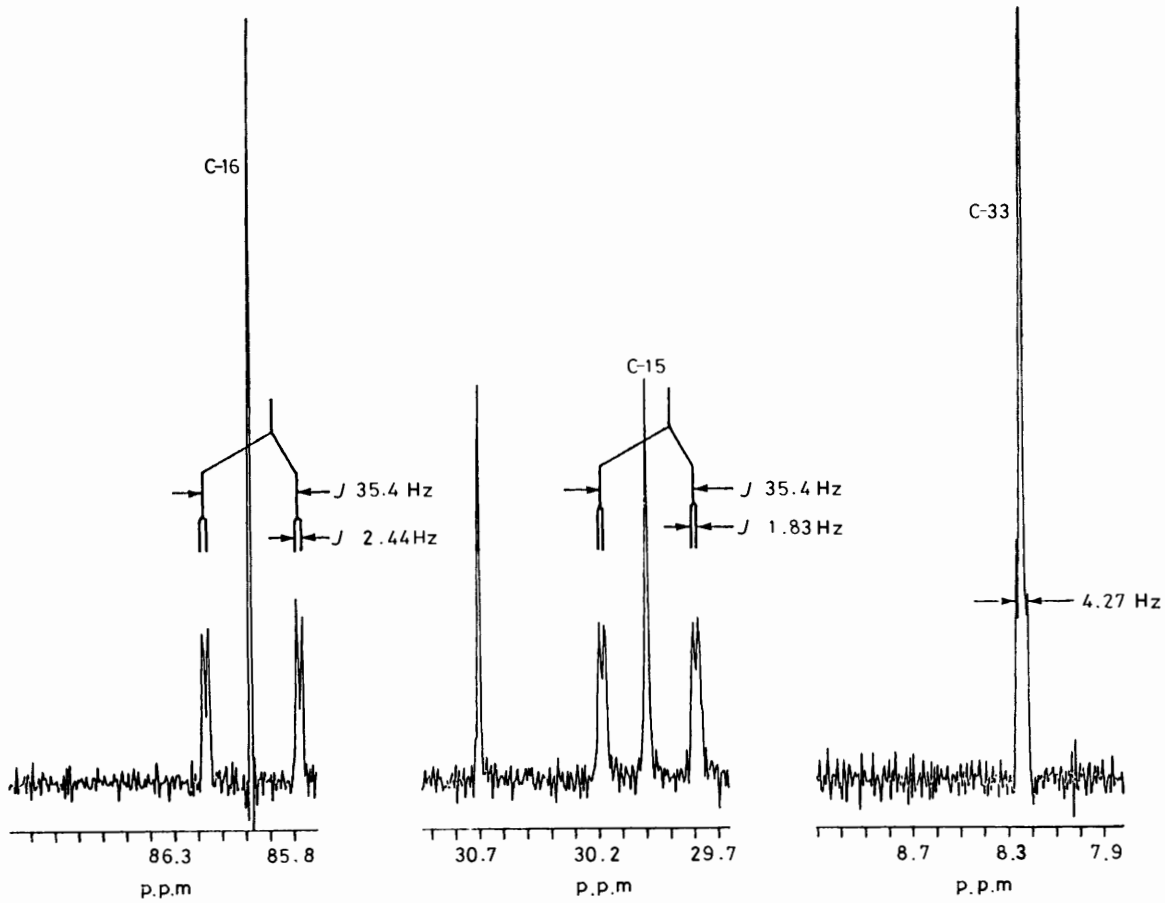


Figure 2. Portions of the 90.5 MHz $^{13}C\{^1H\}$ n.m.r. spectrum of Na-monensin-A biosynthesized from [1,3,3′-$^{13}C_3$]isobutyrate. Resonances assigned to C-16, C-15 and C-33 are highlighted

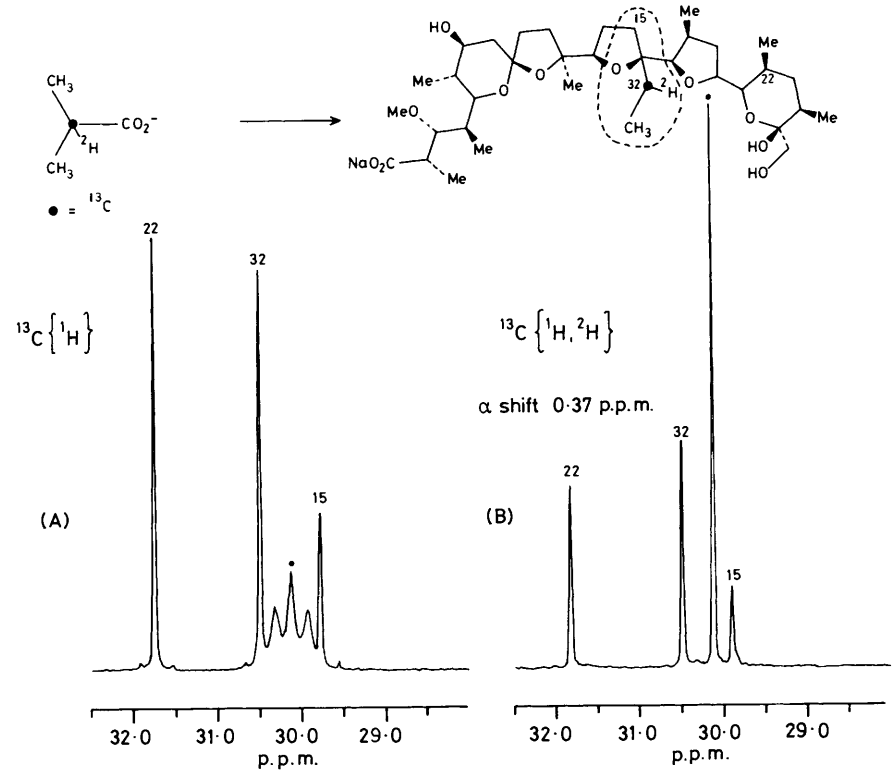


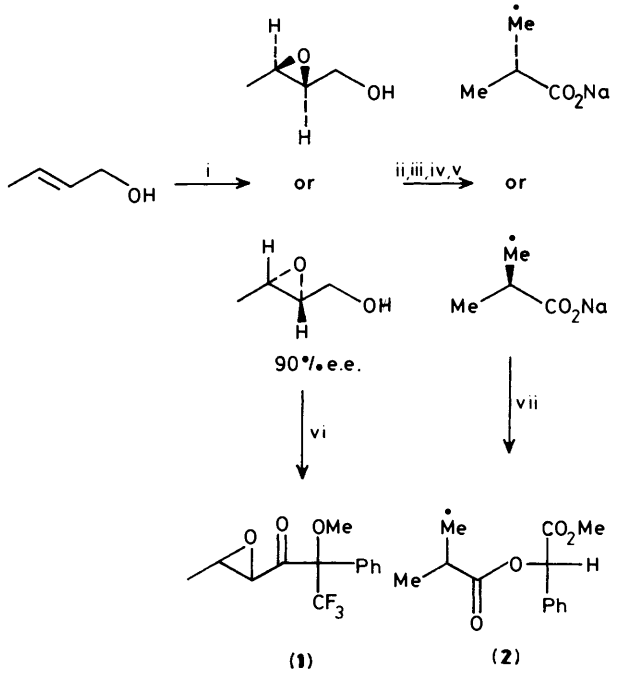
Figure 3. Portions of the 90.5 MHz ^{13}C n.m.r. spectrum of Na-monensin-A biosynthesized from [2-^{13}C,2-^{2}H]isobutyrate; (A) with ^{1}H decoupling only; (B) with ^{1}H and ^{2}H broad band decoupling

C-15 and C-16, appearing as a doublet of doublets (J 35.4 and J 1.8 Hz for C-15; J 35.4 and J 2.4 Hz for C-16). Moreover, in a resolution enhanced ^{13}C spectrum, the signal assigned to C-33 is also flanked by two satellite signals separated by *ca.* 4.3 Hz, representing the outer lines of a doublet of doublets [J 1.8 Hz for C(33)–C(15) coupling and J 2.4 Hz for C(33)–C(16) coupling]. These enriched signals can only arise from the simultaneous presence of three ^{13}C labels at C-15, C-16, and C-33, and represent typical one, two, and three bond coupling constants. It follows that isobutyrate has furnished *intact* the four carbons representing the butyrate unit, at this position in monensin-A, and hence that an intramolecular rearrangement has occurred, quite possibly involving the direct conversion of isobutyryl-CoA into butyryl-CoA.

Fate of the α-Hydrogen of Isobutyrate.—When [1-^{14}C, 2-^{3}H] isobutyrate (1.32×10^{8} d.p.m. mmol^{-1} ^{14}C, 1.13×10^{8} d.p.m. mmol^{-1} ^{3}H; $^{3}H/^{14}C$ ratio 0.86) was administered to cultures of *S. cinnamonensis*, monensin-A was biosynthesized, which after isolation and recrystallization to constant specific activity (2.67×10^{7} d.p.m. mmol^{-1} ^{14}C, 3.47×10^{7} d.p.m. mmol^{-1} ^{3}H) showed a $^{3}H/^{14}C = 1.30$. In order to establish the fate of the α-hydrogen [2-^{13}C, 2-^{2}H]isobutyrate together with unlabelled isobutyrate were incorporated in a similar fashion. The recovered antibiotic was examined by $^{13}C\{^{1}H\}$ and $^{13}C\{^{1}H, ^{2}H\}$ n.m.r. spectroscopy and this revealed a substantial *intact* incorporation of ^{2}H and ^{13}C, only at C-32. The $^{13}C\{^{1}H\}$ spectrum (see Figure 3) shows an up-field shifted ($\Delta\delta = 0.37$ p.p.m.) triplet assigned to ^{13}C-^{2}H enrichment at C-32, and in the $^{13}C\{^{1}H, ^{2}H\}$ spectrum this triplet has collapsed into an intense singlet confirming that during the rearrangement of isobutyrate to butyrate, the α-hydrogen of isobutyrate is retained intact at the migration origin.

The Stereochemical Course of the Rearrangement.—The overall stereochemical course, although not the stereospecificity, of the rearrangement of isobutyrate to butyrate could be established by examining the incorporation of stereospecifically labelled isotopomers of isobutyrate into monensin-A. Accordingly, sodium (*S*)-[3-^{13}C]-, (*S*)-[*methyl*-$^{2}H_3$]-, and (*R*)-[*methyl*-$^{2}H_3$]-isobutyrates were synthesized by the route shown in Scheme 1, which is a modification of the method developed by Aberhart.[15,16] The optical purities of the epoxy alcohols arising from the Sharpless epoxidation[17–19] of crotyl alcohol were assayed by 360 MHz ^{1}H n.m.r. spectroscopy, after conversion into the corresponding Mosher[20] derivatives (**1**) by reaction with (−)-α-methoxy-α-trifluoromethylphenylacetyl chloride. Similarly, the configurational purity of each labelled isobutyrate was monitored by ^{1}H and either ^{13}C or ^{2}H n.m.r. spectroscopy after conversion into a mandelate derivative[21] (**2**), by reaction with (+)-methyl mandelate and dicyclohexylcarbodi-imide. These acids contained an approximate 90% enantiomer excess of the designated isotopomer, and were subsequently used in feeding experiments to shake cultures of *S. cinnamonensis*.

The incorporation of (*S*)-[3-^{13}C]isobutyrate led specifically to a four-fold enrichment in monensin-A, only at C-16, the position formally derived from C-2 of butyrate. No other significant incorporations into propionate derived units were detected by ^{13}C n.m.r. spectroscopy. Taken with the labelling experiments described earlier, this result indicates that the carboxy carbon of isobutyrate migrates to the *pro-S* methyl to afford a butyrate specifically enriched at C-2. Moreover, the non-appearance of ^{13}C label in the propionate units is consistent also with the conversion of (*S*)-[3-^{13}C]isobutyrate into (*S*)-[*carboxy*-^{13}C]methylmalonyl-CoA, by oxidation of the *pro-S* methyl group; the ^{13}C label is subsequently lost as



Scheme. *Reagents:* i, (+)- or (−)-di-isopropyl tartrate, Bu^{t}OOH, Ti(OPr^i)$_4$; ii, Bu^{t}Me$_2$SiCl; iii, Me$_2$CuLi; iv, F$^-$; v, NaIO$_4$, KMnO$_4$; vi, (−)-(MeO)(CF$_3$)CPhCOCl, *N*,*N*-dimethylaminopyridine, CH$_2$Cl$_2$; vii, (+)-methyl mandelate, dicyclohexylcarbodi-imide, Et$_2$O, *N*,*N*-dimethylaminopyridine

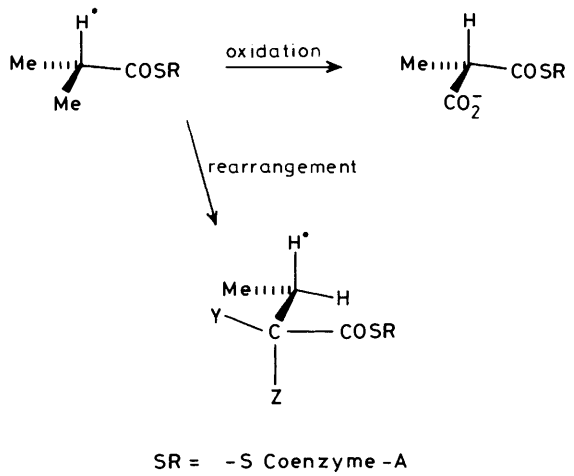
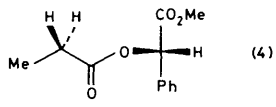


Figure 4. Two pathways of metabolism from isobutyryl-CoA: one leading to butyryl-CoA, the other to (*S*)-methylmalonyl-CoA

$^{13}CO_2$ and does not appear in the propionate units within the backbone (see Figure 4).

These conclusions were supported by the results from two additional feeding experiments using the (*S*)- and (*R*)-[1-^{14}C, *methyl*-$^2H_3$]isobutyrates. The specific incorporation of ^{14}C activity in each case was 8.6 and 8.3%, respectively, but in the



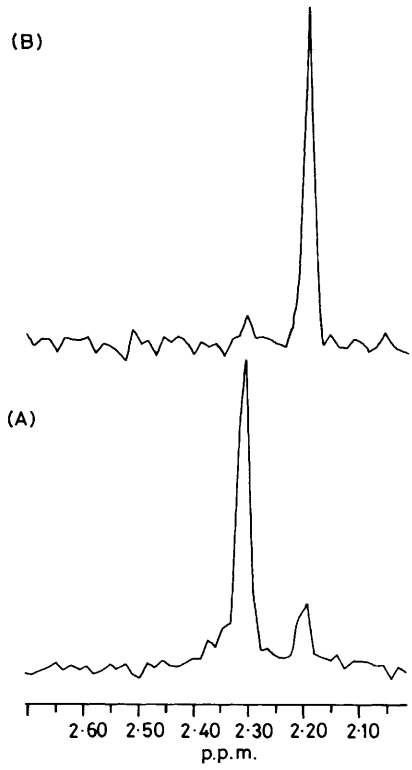


Figure 5. Portions of the 55 MHz $^2H\{^1H\}$ n.m.r. spectrum of; (A) the derivative (**4**) arising from Na-monensin-A biosynthesized from [α-^{2}H]isobutyrate; (B) the sample from (A) in admixture with (*S*)-[2-$^2H_1$]-(**4**): see text

$^2H\{^1H\}$ n.m.r. spectrum, run under identical conditions, only monensin-A biosynthesized from the *R*-isomer showed strongly enriched signals at δ 1.5, 1.25, and 0.9, which can be assigned[14] to overlapping signals due to enrichments in all of the C-methyl resonances.*

The complete stereochemical course of the isobutyrate–butyrate rearrangement includes knowledge about the final location of the α-hydrogen retained from isobutyrate. The incorporation of [α-^{2}H]isobutyrate into monensin-A in a batch fermentation of *S. cinnamonensis* afforded monensin-A whose $^2H\{^1H\}$ n.m.r. spectrum revealed a single strongly enriched broad resonance at δ 1.6, which is consistent with the location[14] of the label at C-32. This monensin-A was subsequently degraded[22] by Kuhn-Roth oxidation, and the acetic and propionic acids formed were collected by steam distillation and converted into the (+)-mandelate ester derivatives (**3**) and (**4**). After careful separation by chromatography the derivative (**4**) was characterized by ^{1}H and ^{2}H n.m.r. spectroscopy under conditions where the diastereotopic protons at C-2 are clearly resolved.[21] The ^{2}H n.m.r. spectrum at 55.3 MHz in benzene revealed two enriched signals at δ 2.35 and δ 2.23 in the ratio 85:15, corresponding to the *R*- and *S*-isomers of [2-$^2H_1$]-propionate, respectively. The correctness of these assignments was confirmed by adding a small quantity of authentic[23] (*S*)-[2-$^2H_1$]propionate derivative (**4**), synthesized from (*S*)-[2-$^2H_1$]-propionate. In this way the signal at δ 2.23 was strongly

* The sodium monensin-A derived from (*S*)-[1-^{14}C, 3-$^2H_3$]isobutyrate also showed weakly enriched ^{2}H n.m.r. signals at δ 1.5, 1.25, and 0.9, when run under identical conditions, and these are almost certainly due to the presence of *ca.* 5% of the *R* isotopomer in the synthetically derived *S* isobutyrate.

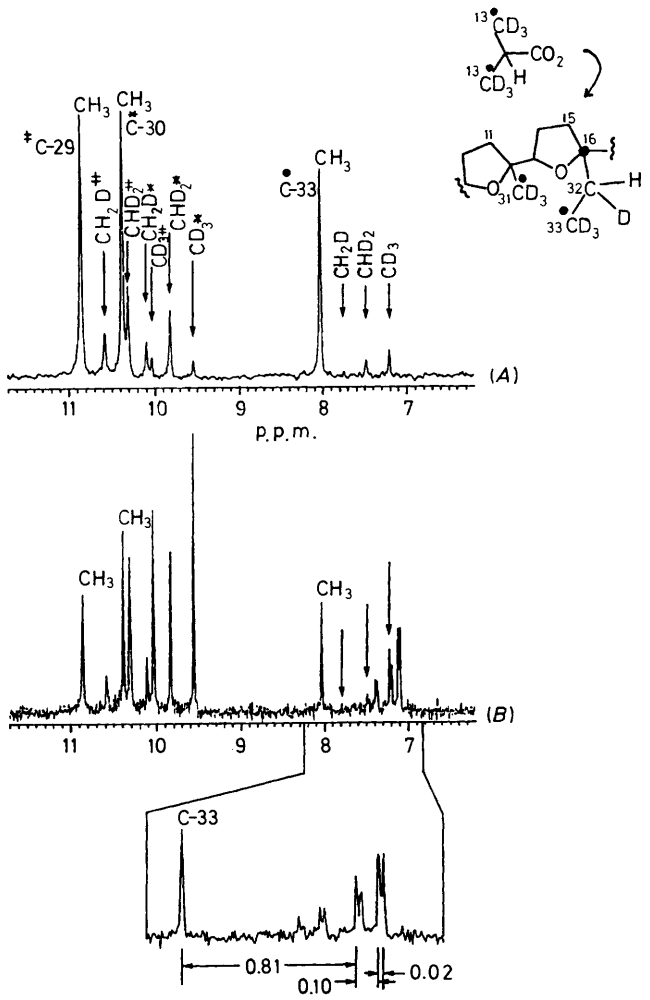


Figure 6. High-field portion of the $^{13}C\{^1H,^2H\}$ n.m.r. spectra of sodium monensin-A: (A) biosynthesized from Na[2-^{13}C, 2-$^2H_3$]acetate; (B) biosynthesized from Na[3,3′-$^{13}C_2$, *dimethyl*-$^2H_6$]isobutyrate. The arrows indicate isotopically shifted signals assigned to C-29, C-30, and C-33. Each spectrum at 90.5 MHz has 64 K data points, ^{1}H 1W and ^{2}H 3W decoupling, and was transformed after 0.4 Hz line broadening by exponential multiplication

enhanced, as shown in Figure 5. It follows that the bulk of the ^{2}H enriched propionate has the 2*R* absolute configuration, although a smaller quantity of the 2*S* isomer is also produced.

We may now integrate these results to reveal the stereochemical courses of two metabolic pathways utilizing isobutyrate, one leading to (*S*)-methylmalonyl-CoA, and the other affording a butyrate unit by a reaction (or less conceivable, by several reactions) not requiring loss of the α-hydrogen of isobutyrate, but involving the migration of the carboxy carbon, presumably activated as a coenzyme-A thiol ester, to the *pro-S* methyl group, with its replacement by a hydrogen atom occurring with overall *retention* of configuration (see Figure 4).

Evidence for a Vicinal Interchange Rearrangement.—From the results so far, the fate of the *pro-R* methyl of isobutyrate appears to be as a precursor of all the C-methyl groups in monensin-A (see Figures 1 and 4). During an attempt to show that this *pro-R* methyl is incorporated *intact*, evidence was obtained for a hydrogen migration from the *pro-S* methyl of isobutyrate to the C-3 position in butyrate, thus making the overall process a vicinal interchange rearrangement.

Thus, [3,3′-$^{13}C_2$, *dimethyl*-$^2H_6$]isobutyrate (90 atom % ^{13}C, 98% $^2H_3$, at each centre) was administered to cultures of *S. cinnamonensis*. This afforded monensin-A, and portions of the high-field $^{13}C\{^1H, ^2H\}$ n.m.r. spectrum of this material are shown in Figure 6. Included in the plot are the natural abundance signals assigned to C-29, C-30, and C-33, along with a multitude of isotopically shifted resonances.[24] For comparison the spectrum in part-A shows the same spectral region for a monensin-A sample biosynthesized from sodium [2-^{13}C, 2-$^2H_3$]acetate.[23] For each of the resonances assigned to C-29, C-30, and C-33, there appears isotopically shifted signals which can be assigned to *intact* $^{13}CH_2D$, $^{13}CHD_2$, and $^{13}CD_3$ groups. The corresponding isotope induced shifts[24] associated with C-29 and C-30 are also clearly evident in part-B (Figure 6), thus strengthening the assignments for these signals. On the other hand, the resonances for C-33 are more complex. For this carbon each of the shifted signals shows a long range ^{13}C-^{13}C coupling ($J_{1,3}$ 2 Hz), although there is no discernible signal for a $^{13}CH_2D$ group. Of more significance, there are additional signals upfield by 0.1 p.p.m. beyond the resonances assigned to *intact* $^{13}CHD_2$ and $^{13}CD_3$ groups. These may arise if there is deuterium also at C-32 in those molecules containing ^{13}C labels at C-16 and C-33; the enriched signals at C-16 and C-33 then experience an additional β-shift[25] as well as the cumulative α-shifts[24] due to directly attached deuterium. This interpretation requires that during the carbon skeleton rearrangement there is also a net intramolecular 1,2-hydrogen shift from the *pro-S* methyl of isobutyrate to the C-3 position of butyrate. Also, the *pro-R* methyl is indeed largely incorporated *intact* as $^{13}CD_3$ at each of the C-methyl groups in monensin-A.

Further support for this hydrogen migration, and its overall stereochemical course, came by following the incorporation of [*dimethyl*-$^2H_6$]isobutyrate into monensin-A. The labelled precursor was administered to a 5-l batch fermentation of *S. cinnamonensis* and the antibiotic isolated from it was degraded by Kuhn-Roth oxidation. This again afforded acetic and propionic acids, from which the derivative (**4**) could be prepared and isolated by h.p.l.c. Its ^{2}H n.m.r. spectrum in benzene now showed three major resonances at δ 2.35, 2.23, and 1.1 in the ratio 0.2:1:10 which are assigned as before to deuterium enrichments in the 2-*pro-R*, 2-*pro-S*, and 3-Me positions, respectively (see Figure 7). In a complementary fashion, [$^2H_7$]isobutyrate was incorporated into monensin-A, the monensin-A was degraded as before and the pure propionate derivative (**4**) now showed deuterium resonances at δ 2.35, 2.23, and 1.1 in the ratio 1:1:8. These experiments demonstrate again the migration of hydrogen from the *pro-S* methyl of isobutyrate, and its subsequent location largely in the *pro-S* position, at C-32 in monensin-A.

As in the experiment with [α-^{2}H]isobutyrate, a significant loss of stereochemical homogeneity is again observed during the experiment with [$^2H_6$]isobutyrate (see Figure 7). This appears not to be an artefact of the derivatisation protocol since synthetic[23] (*S*)-[2-$^2H_1$]propionate could be converted into (**4**) without any loss of optical purity. It is an open question whether this observation is a result of a partial racemization during the Kuhn-Roth oxidation, or due to *in vivo* metabolic events experienced by the precursor.

The intra- *vs.* inter-molecular nature of the hydrogen migration is also of great interest. Our results indicate that this should be intramolecular, but do not rule out the possibility of an intermolecular shift if the mechanism of the rearrangement involves a hydrogen carrier, such as that known[26] to function during the vicinal interchange rearrangements catalyzed by coenzyme-B_{12} dependent mutases. This question is difficult to address unambiguously using the whole-cell system described here. Nevertheless, we also looked at the incorporation into monensin-A of a mixture of [2-^{13}C]- and [*dimethyl*-$^2H_6$]-

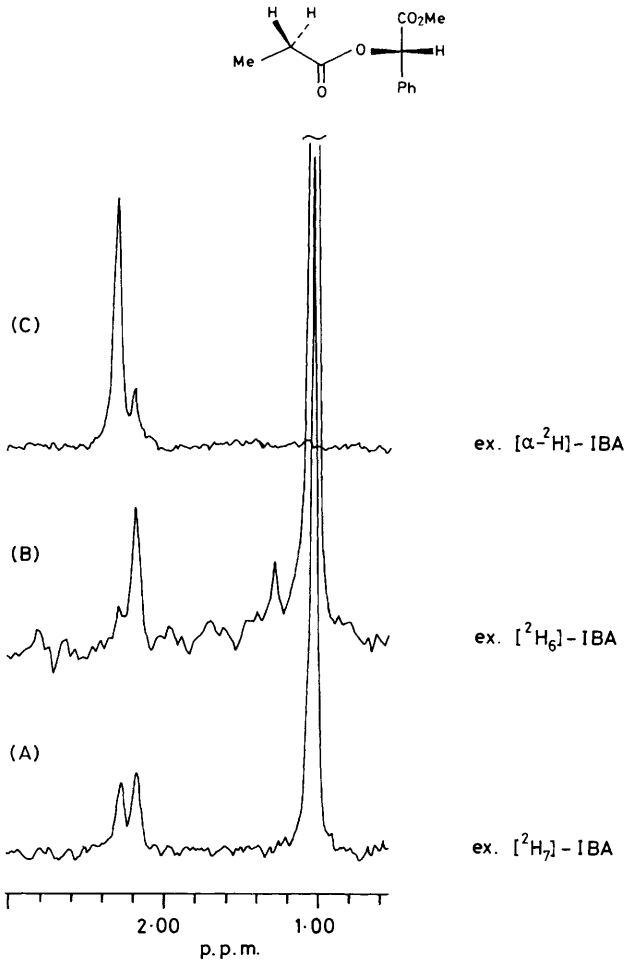


Figure 7. Portions of the 55 MHz $^2H\{^1H\}$ n.m.r. spectra of derivative (**4**) obtained from sodium monensin-A; (A) biosynthesized from [$^2H_7$]-isobutyrate; (B) biosynthesized from [*dimethyl*-$^2H_6$]isobutyrate; (C) biosynthesized from [α-^{2}H]isobutyrate

isobutyrates (in 1:3 ratio), but could see no evidence in the monensin-A produced for the direct attachment of deuterium to the ^{13}C label incorporated at C-32, which would have indicated an intermolecular shift. There are many reasons for the apparent failure of this experiment, including the operation of kinetic isotope effects, the dilution *in vivo* of labelled isobutyrate by unlabelled material, or the fact that the mechanism of the rearrangement does not allow intermolecular hydrogen transfer. This question will be addressed again, with the advent of a reliable enzyme preparation catalyzing this process.

Incorporation of Acetoacetate.—At the outset we described experiments which showed that labelled butyrate is incorporated very efficiently into the propionate units in monensin-A. This prompts the question as to how butyryl-CoA may be converted into methylmalonyl-CoA *in vivo*. The only well established primary metabolic route would lead from the linear C_4 unit, *via* β-oxidation, to acetyl-CoA and *via* the Krebs cycle intermediate succinyl-CoA, into methylmalonyl-CoA. We therefore examined the incorporation of ethyl [1,3-$^{13}C_2$]acetoacetate containing also ethyl[1,3-^{14}C]acetoacetate, and diluted 1:3 with unlabelled material, into monensin-A. This linear C_4 unit is easily prepared in doubly labelled form, and functions as a precursor *in vivo* of acetoacetyl-CoA. In the event, the labelled material was efficiently incorporated (2.7% absolute incorporation of ^{14}C) and the $^{13}C\{^1H\}$ n.m.r. spectrum of the monensin-A showed *enriched doublets* (*ca.* 10% the height of natural abundance singlets), with *J* values in the range 33—51 Hz, centred around each of the natural abundance singlets for C-1, C-2, C-3, C-4, C-5, C-6, C-11, C-12, C-17, C-18, C-21, C-22, C-23, and C-24, *i.e.* each of the positions derived from C-1 and C-2 of a propionate building block. These doublets must have arisen specifically from the *direct vicinal coupling* of adjacent ^{13}C enriched sites. This was confirmed, and the couplings were identified, from a 2D-^{13}C COSY X experiment. This surprising result requires that the acetoacetate be metabolized *without cleavage* into acetate units, by a pathway which includes a rearrangement step, highly reminiscent of the butyryl-CoA to isobutyryl-CoA rearrangement. Indeed, since carbon-skeleton rearrangements of C_4 fatty acids are rare, we propose here that [1,3-$^{13}C_2$]acetoacetyl-CoA, formed *in vivo*, is metabolized by the route shown in Figure 8, to afford [1,2-$^{13}C_2$]methylmalonyl-CoA, the precursor of the propionate units in monensin-A.

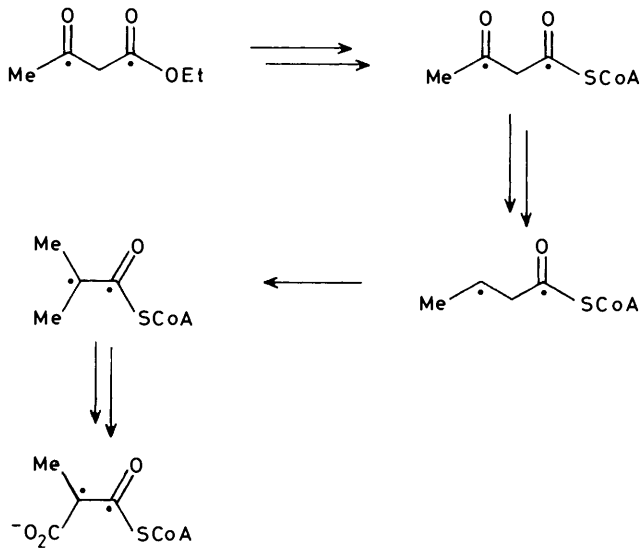


Figure 8. Proposed metabolism of acetoacetate in *S. cinnamonensis*

A closely related observation[27] has been made recently during studies of nonactin biosynthesis in *S. griseus*, where ethyl [1,2,3,4-$^{13}C_4$]acetoacetate was incorporated, again without prior β-cleavage to acetate units, into the propionate unit within this antibiotic.

Given the correctness of the interpretation, these observations also provide indirect evidence for the reversible nature of the isobutyrate to butyrate rearrangement.

Discussion

There are two principal reasons why the results described above are of special interest. Firstly, the observation of a carbon skeleton rearrangement involving a vicinal interchange of carbon and hydrogen atoms finds precedence elsewhere only in the actions of coenzyme-B_{12} dependent mutases.[26] Our labelling experiments provide unequivocal evidence for such a rearrangement in the primary metabolism of *S. cinnamonensis*, and it appears most likely that this should occur at the level of a direct interconversion of isobutyryl-CoA into butyryl-CoA, although the coenzyme requirements must be tested eventually in a cell-free preparation. Nevertheless, the rearrangement of isobutyrate to butyrate is unprecedented to date in the primary metabolism of other pro- and eu-karyotic organisms, and given

the involvement of coenzyme-B_{12}, represents a new example of this class of enzymic reaction. At the present time, an interesting comparison can be made of its overall stereochemical course, with that catalyzed by the well known[13] and widely distributed methylmalonyl-CoA mutase. As depicted in Figure 9 the latter reaction is known to involve the migration of the thioester carbon to a methyl group, with its replacement by a hydrogen atom occurring with retention of configuration,[28–31] as depicted here also for the isobutyrate–butyrate rearrangement.

The close putative similarity between the reacting substrates and coenzymes in these two processes would imply also a close structural relationship between the enzymes catalyzing these two rearrangements. The active sites of the respective enzymes need only differ in part of the substrate binding pocket, where in one case the free carboxy of (*R*)-methylmalonyl-CoA, and in the other the *pro-R* methyl of isobutyrate, are located. Indeed, it is not inconceivable that a single enzyme in *S. cinnamonensis* is responsible for catalyzing both transformations by parallel mechanisms, although distinct but closely related enzymes must be considered a more likely possibility if the carboxylate group of methylmalonyl-CoA makes an important binding interaction with residues in methylmalonyl-CoA mutase. In any event,

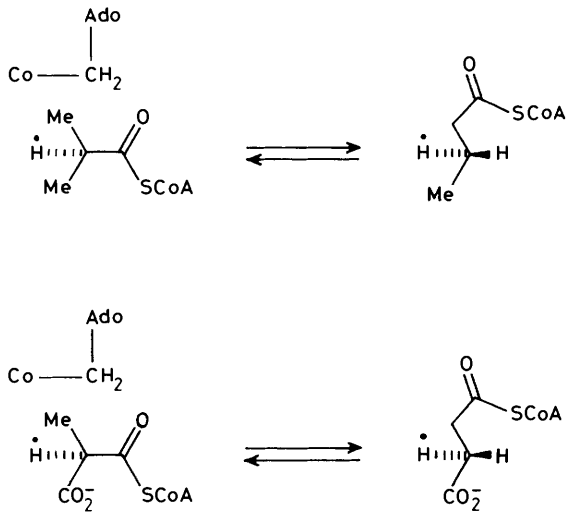
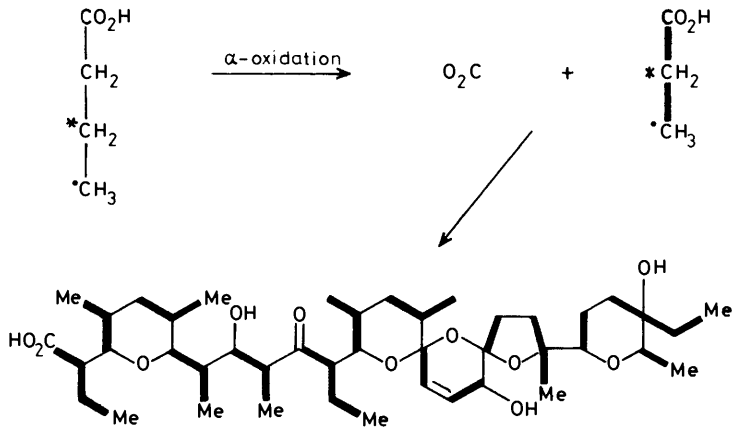


Figure 9.

these questions give an added impetus to studies of the enzymology of these rearrangements in Streptomycetes.

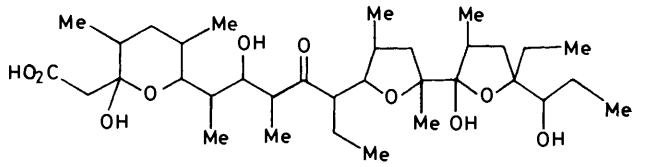
A second important point of interest concerns the biochemical and physiological role of the metabolic link established by the isobutyrate–butyrate rearrangement. This should have an important bearing on the ability of these organisms to utilize the products of both amino acid and fatty acid catabolism, in addition to components of the Krebs cycle, to furnish building blocks necessary for antibiotic production.

There is additional evidence available already that the rearrangement of isobutyrate to butyrate also operates in other antibiotic producing Streptomycetes. Indeed, its recognition and acceptance allows a considerable simplification of the interpretations accorded to earlier observations, made in other laboratories, on the incorporations of various ^{13}C labelled C_3 and C_4 fatty acids into macrolide and polyether antibiotics. For example, the incorporations of [3-^{13}C]- and [4-^{13}C]-butyrates into the polyether narasin[32] generated enrichments at positions formally derived from C-2 and C-3 of propionate, respectively, which prompted the suggestion that butyrate may undergo α-oxidation to propionate prior to incorporation (see Figure 10). In another study,[33,34] this time of lysocellin biosynthesis, the incorporation of [1-^{13}C]butyrate generated enrichments not only at the positions formally derived from C-1 of butyrate, but also at each of the positions derived from C-1 of propionate. Since α-oxidation of butyrate to propionate could not account for this result an ω-oxidation was suggested, in which butyrate is converted into succinate which, in turn, may be converted *via* succinyl-CoA into methylmalonyl-CoA (Figure 11). Similar observations[35] were made on the incorporation of [1-^{13}C]-butyrate into the macrolide antibiotics leucomycin-A_3 and tylosin. In addition, [3-^{13}C]ethylmalonic acid enriched carbons in tylosin which should arise from C-2 of propionate, and [1,3,1′-$^{13}C_3$]ethylmalonic acid gave rise to intact vicinal enrichments of each pair of carbons arising from C-1 and C-2 of propionate. This prompted the suggestion[36] that ethylmalonic acid, as its coenzyme-A thioester, might be converted into methylsuccinyl-CoA on methylmalonyl-CoA mutase, and then undergo α-oxidation to afford doubly labelled methylmalonyl-CoA (Figure 12). However, a recognition of the butyrate–isobutyrate rearrangement provides a unifying rationale for these observations, and dispenses with the need for this confusing array of alternative metabolic pathways. In each case, the labelled precursor should afford the corresponding labelled butyryl-CoA, which may isomerize to afford isobutyryl-CoA,

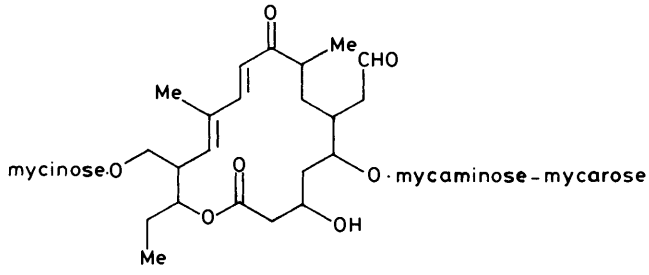


Narasin

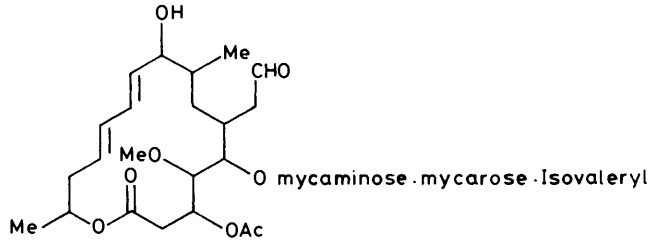
Figure 10.



Lysocellin



Tylosin



Leucomycin A_3

and then undergo oxidation to give (*S*)-methylmalonyl-CoA (Figure 13) ready for use in antibiotic production.

More direct evidence for the key rearrangement in other Streptomycetes has come from recent studies of tylosin and leucomycin-A_3 biosynthesis, where Omura and co-workers[2] have noted the incorporation of [3,3′-$^{13}C_2$]isobutyrate in the butyrate units in these macrolides. Similarly, the intact incorporation of [1,3,3′-$^{13}C_3$]- and [2,3,3′-$^{13}C_3$]-isobutyrates into the butyrate unit within the polyether lasalocid-A have been reported recently by Hutchinson and co-workers.[37]

Although the rearrangement of isobutyrate to butyrate has not to date been detected in other organisms, the oxidation of isobutyryl-CoA is a well established part of valine catabolism in both micro-organisms[8] and mammals.[9] The stereochemistry of these processes is in all cases[38–40] known to involve formation of (2*S*)-β-hydroxyisobutyryl-CoA, and this is also consistent

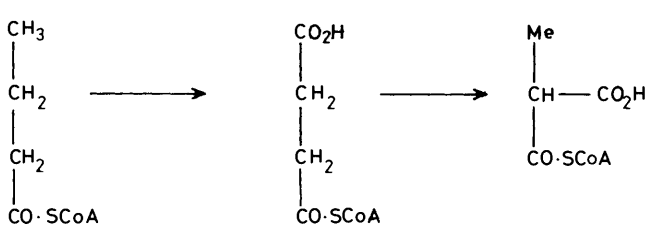


Figure 11.

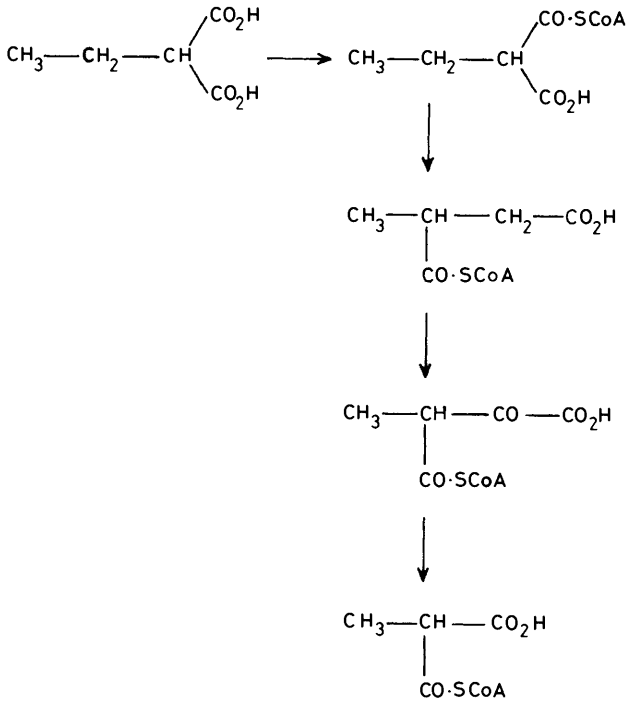


Figure 12.

with the observations made during this study (see Figure 4). However, an important difference must occur, between the subsequent course of the conversion into methylmalonyl-CoA in Streptomycetes, and that occurring in mammals,[9] and *Pseudomonas aeruginosa*[41] (the only well studied microbial system), since the labelling results described here, and elsewhere,[2,3,23] require that both [2-^{13}C]valine and [1-^{13}C]-isobutyryl-CoA should afford (*S*)-[1-^{13}C]methylmalonyl-CoA. Unfortunately, in these other organisms not all the details of the pathway have been accurately established, but the available labelling data[9] indicate that C-2 of valine is lost during catabolism in mammals as CO_2, and the end product, propionate (formally derived by decarboxylation and hydrolysis of methylmalonyl-CoA), is derived from C-3, C-4, and C-4′ of the amino acid. Much work remains to be done to clarify the details of these processes.

The labelling data establish an important role for valine catabolism in supplying the acetate, propionate, and butyrate units needed for antibiotic biosynthesis, and this view is strengthened also by physiological studies on tylosin biosynthesis showing[42,43] the inhibition of macrolide production caused by ammonium ions. This effect appears to operate through the inhibition of valine dehydrogenase, the first enzyme on the valine catabolic pathway. Isobutyryl-CoA may itself also act as a building block for antibiotic production, being incorporated as a chain starter unit in the biosynthesis of avermectins,[44] milbemycins,[45] and macrotetrolides.[46]

All these data serve to consolidate the view of valine as an important precursor of building blocks used for macrolide and polyether antibiotic production. Alongside this, the degradation of other amino acids may also lead to these same metabolic intermediates. Thus the catabolism of leucine, phenylanine, and tyrosine leads to the formation[47] of acetoacetyl-CoA. The functional role of this C_4 unit in antibiotic biosynthesis has been demonstrated during this work, where its efficient, and intact, incorporation into methylmalonyl-CoA, most likely *via* the isobutyryl-CoA mutase reaction (Figure 8) was inferred (*vide supra*). Recently direct evidence for the involvement of these

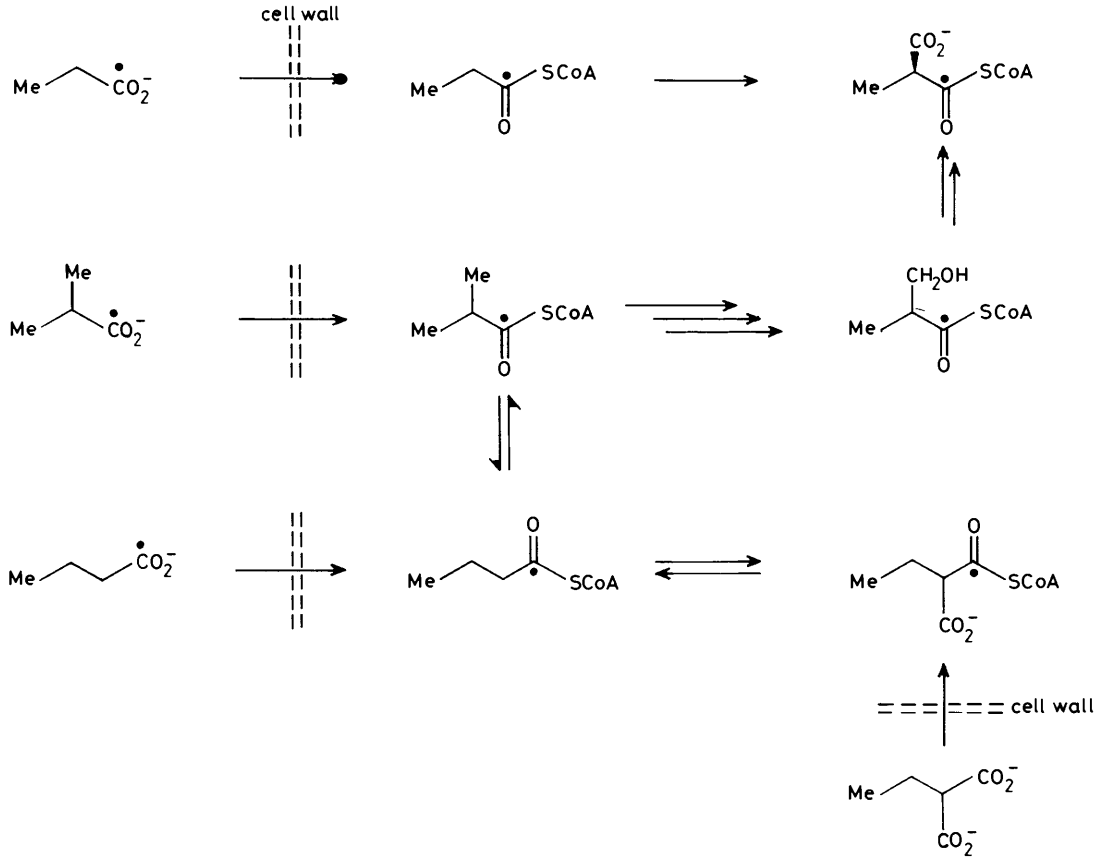
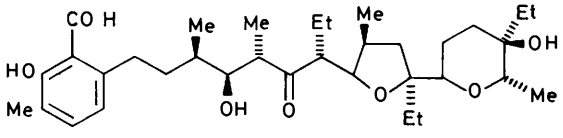


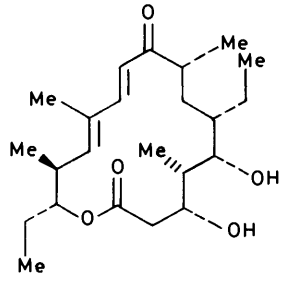
Figure 13. Metabolism of butyrate to methylmalonyl-CoA in Streptomycetes

and other amino acids in the biosynthesis of tylactone was demonstrated by Dotzlaf and co-workers.[6] It is, therefore, not valine catabolism alone that may provide C_3 and C_4 building blocks for antibiotic production, these may arise also from the catabolism of most of the common amino acids.

Other potentially major sources of carbon fragments are the fatty acids constituted as lipids, and other storage materials such as poly-β-hydroxybutyrate.[48] The catabolism of fatty acids



Lasalocid–A



Tylactone

via β-oxidation can readily afford acetyl-CoA and butyryl-CoA and the latter, in turn, can be diverted through the isobutyryl-CoA mutase reaction into the branched chain, propionate units, needed for antibiotic biosynthesis (Figure 8). The stimulatory effects of long-chain fatty acids on the production levels of polyether antibiotics in batch fermentations are well documented,[49] and the importance of this route is indicated by the efficiency with which ^{13}C labelled butyrate is incorporated into the macrolides and polyethers in shake cultures.

It seems well established that antibiotic production in Streptomycetes takes place predominantly after the initial period of rapid cell growth, when the activity of the Krebs cycle and fatty acid biosynthesis became lower. It is during this later period that the major pathways of catabolism, described above, become most active and antibiotic production reaches its highest levels. It is within this context that the isobutyrate–butyrate rearrangement may be seen as an important link between straight-chain and branched-chain fatty acid metabolism, especially for the production of branched chain macrolide and polyether antibiotics. Whether this also has a bearing on the regulatory mechanisms controlling the key metabolic switch from early to late stage growth remains to be elucidated.

Experimental

^1H, ^2H and ^{13}C N.m.r. spectra were recorded on a Bruker AM 360 spectrometer in chloroform unless stated otherwise. $^{13}C\{^1H, ^2H\}$ spectra were run unlocked with broad band ^1H (1W) and ^2H (3W) decoupling, and are referenced using the residual solvent signal (δ ^{13}C, $CDCl_3$ = 77.10, ^1H = 7.27;

C_6D_6, ^1H = 7.30), ^{13}C-labelled precursors were 90 atom % enriched unless stated otherwise. Mass spectra were run on a Kratos MS30 instrument at 70 eV. Column chromatography was by the method of Still and co-workers[50] using Kieselgel 60, 230—400 mesh, t.l.c. used pre-coated silica plates (Merck), and h.p.l.c. used a Dupont 8800 instrument with Zorbax silica or ODS-C_{18} reverse phase columns (analytical 4.6 mm × 25 cm; preparative 21.5 mm × 25 cm), and on-line UV (254 nm) detection. Conditions for the growth of *S. cinnamonensis* A 3 823.5 in shake flask cultures[14] and in batch fermentation[23] have been described previously.

(i) *Sodium* [1-^{13}C]*Isobutyrate*.—[^{13}C]Carbon dioxide, generated by dropwise addition of concentrated H_2SO_4 onto barium [^{13}C]carbonate (5 g), was passed into a solution of propan-2-ylmagnesium, prepared by addition of magnesium turnings (0.35 g) to 2-bromopropane (1.85 g) in tetrahydrofuran (20 ml) at 0 °C. After 2 h water was added followed by 50% H_2SO_4, and the product was extracted into diethyl ether. The extract was evaporated and the residue was acidified (H_2SO_4) and lyophilized. The lyophilisate was adjusted to pH 9 (1M NaOH) and freeze dried to afford sodium [1-^{13}C]isobutyrate (0.76 g). For characterization, a portion of this product was treated with 2-bromo-4′-phenylacetophenone in aqueous EtOH at 50 °C. The resulting *p*-phenylphenacyl derivative of [1-^{13}C]isobutyric acid crystallized on cooling, m.p. 88—89 °C; *m/z* 283 (M^+, 12%), 181 (100), and 152 (89).

(ii) *Sodium* [2-^{13}C]*Isobutyrate*.—Diethyl [2-^{13}C]malonate (250 mg) with sodium hydride (83 mg) in tetrahydrofuran (10 ml), was stirred with methyl iodide (500 mg) for 45 min at room temperature, and for 90 min at reflux. The mixture was then diluted with water and extracted with diethyl ether and the extract dried (Na_2SO_4) and evaporated. The resulting oil was stirred in ethanol (10 ml) with NaOH (2M; 2 ml) for 1 h and then acidified (H_2SO_4) and extracted with ethyl acetate; the extract was dried (Na_2SO_4) and evaporated. The residue in water (10 ml) was heated overnight at 160 °C, after which the mixture was cooled, adjusted to pH 8.5 (dilute NaOH), and freeze dried to afford sodium [2-^{13}C]isobutyrate (100 mg). A portion was converted into the *p*-phenylphenacyl derivative, by the method described in (i) above, showing m.p. 88—89 °C, δ(^{13}C) single enrichment at 33.92; *m/z* 283 (M^+; 12%), 181 (100), and 152 (89).

(iii) *Sodium* [3,3′-$^{13}C_2$]*Isobutyrate*.—The reaction of diethyl malonate with [^{13}C]iodomethane according to the procedure described in (ii), followed by hydrolysis and decarboxylation, gave sodium [3,3′-$^{13}C_2$]isobutyrate. This material was characterized as a (*S*)-(+)-methyl mandelate derivative, prepared as follows. The labelled isobutyrate (30 mg) in water (10 ml) was acidified (H_2SO_4) and the mixture then extracted with diethyl ether. The ether extract was dried ($MgSO_4$) and reduced in volume, and the residue was treated with (*S*)-(+)-methyl mandelate (60 mg), dimethylaminopyridine (43 mg), and dicyclohexylcarbodi-imide (73 mg) in dichloromethane (5 ml) at room temperature overnight. The solution was then washed sequentially with water, aqueous sodium hydrogen carbonate, and dilute HCl, and then dried ($MgSO_4$) and evaporated. Chromatography of the residue on silica gel (CH_2Cl_2–light petroleum, 1:1) afforded the product as a clear oil, δ(^{13}C) enriched signals at 18.7 and 18.9; *m/z* 238 (M^+, 54), and 73 (100).

(iv) *Sodium* [2-^{2}H]*Isobutyrate*.—Dimethylmalonic acid (10 g) in deuterium oxide (99.8 atom %; 50 ml) was freeze dried, redissolved in deuterium oxide (50 ml), freeze dried again, and finally decarboxylated by heating at 160 °C in deuterium oxide (30 ml) overnight. After cooling, the pH was adjusted to 8.5 (10M NaOH), and the solution was freeze dried to afford sodium [2-^{2}H]isobutyrate (8.3 g); δ(^{2}H, H_2O) 2.68 (s). A portion of the labelled isobutyrate, as its *p*-phenylphenacyl derivative, prepared as in (i) above, showed m.p. 88—89 °C, >95% monodeuteriation by ^{1}H n.m.r.; *m/z* 283 (0.8), 181 (100), and 153 (19).

(v) *Sodium* [dimethyl-$^2H_6$]*Isobutyrate*.—Diethyl malonate (12.0 g) was bis-alkylated with [*methyl*-$^2H_3$]iodomethane (24 g; 99 atom % ^{2}H) hydrolysed with ethanolic sodium hydroxide, and decarboxylated in water, following the method in (ii) above, to afford sodium [*dimethyl*-$^2H_6$]isobutyrate (3.1 g). A portion of this material was converted into the *p*-phenylphenacyl derivative, as in (ii) above; m.p. 88—89 °C, δ(^{2}H, $CHCl_3$), 1.22 (s); *m/z* 288 (M^+, 4.9%), 181 (100), and 152 (26.6).

(vi) *Sodium* [$^2H_7$]*Isobutyrate*.—[*dimethyl*-$^2H_6$]Dimethylmalonic acid (5.0 g) was prepared, following the method in (v) above. This material was dissolved in deuterium oxide (50 ml), and freeze dried. This process was repeated, and the acid was then decarboxylated in deuterium oxide (10 ml, 99.8 atom %) at 160 °C, as described in (iv) above. A portion of the sodium [$^2H_7$]isobutyrate was converted into the *p*-phenylphenacyl derivative, as in (i), m.p. 88—89 °C; >95% [$^2H_7$]isobutyrate by ^{1}H n.m.r.; δ(^{2}H, $CHCl_3$), 1.22 (6 H, s) and 2.62 (1 H, s); *m/z* 289 (M^+).

(vii) *Sodium* [1,3,3′-$^{13}C_3$]*Isobutyrate*.—Diethyl [1,3-$^{13}C_2$]-malonate (0.5 g, each site 90 atom % ^{13}C) was bis-alkylated with [^{13}C]iodomethane (1.0 g, 90 atom % ^{13}C), hydrolysed and decarboxylated in water, by the method given in (ii) to afford sodium [1,3,3′-$^{13}C_3$]isobutyrate (210 mg). A portion of this material was converted into the *p*-phenylphenacyl derivative, which showed m.p. 88—89 °C; δ(^{13}C) enriched signals at 19.2 and 176.9; *m/z* 285 (M^+, 2.7), 181 (100), and 152 (13).

(viii) *Sodium* [3,3′-$^{13}C_2$,3,3,3,3′,3′,3′-$^2H_6$]*Isobutyrate*.—Diethyl malonate (1.0 g) was bis-alkylated with [^{13}C,$^2H_3$]iodomethane (2.0 g; 90 atom % ^{13}C, 98 atom % ^{2}H), saponified, decarboxylated in water, according to the method in (ii) above, and purified to afford sodium [3,3′-$^{13}C_2$,3,3,3,3′,3′,3′-$^2H_6$]-isobutyrate (482 mg). A portion of this material was converted into the *p*-phenylphenacyl derivative, m.p. 87—88°; δ(^{13}C) enriched multiplet 19.1—17.7: *m/z* 290 (M^+, 2.1), 181 (100), and 152 (14).

(ix) *Sodium* [2-^{13}C, 2-^{2}H]*Isobutyrate*.—Diethyl [2-^{13}C]-malonate (250 mg; 90 atom % ^{13}C) was bis-alkylated with iodomethane (500 mg) and saponified, in the manner described in (ii) above, to afford [2-^{13}C]dimethylmalonic acid. This was dissolved in deuterium oxide (10 ml; 99.8 atom % ^{2}H) and freeze dried; this procedure was then repeated. The residue was redissolved in deuterium oxide (10 ml) and decarboxylated by heating to 160 °C. Neutralization (NaOH) and freeze drying, gave sodium [2-^{13}C, 2-^{2}H]isobutyrate (130 mg). A portion of this material was converted into the *p*-phenylphenacyl derivative, as in (i) above, m.p. 88—89 °C; δ(^{13}C) enriched triplet 33.6; >98 atom % ^{2}H from ^{1}H n.m.r.; *m/z* 284 (M^+, 5%) and 181 (100).

(x) *Ethyl* [1,3-$^{13}C_2$, 1,3-^{14}C]*Acetoacetate*.—Sodium [1-^{13}C]acetate (2.0 g; 99 atom % ^{13}C) and sodium [1-^{14}C]acetate (200 μCi, 50 mg) were heated with triethyl phosphate (2.5 ml) at 230 °C for 2 h. Distillation at atmospheric pressure afforded ethyl [1-^{13}C, 1-^{14}C]acetate (1.2 g), b.p. 76—77 °C. This was refluxed in diethyl ether (20 ml) with sodium hydride (0.39 g) for 18 h, and then treated with dilute HCl. The aqueous phase was extracted with ether, and the ether extracts were dried (Na_2SO_4) and evaporated. The residue was distilled to afford ethyl [1,3-$^{13}C_2$, 1,3-^{14}C]acetoacetate (180 mg), b.p. 180—182 °C (lit.,[52]

181 °C); $\nu_{max.}$ 3 000, 1 750, and 1 720 cm^{-1}; δ(^{1}H), 1.28 (3 H, t), 2.25 (3 H, d), 3.43 (2 H, t), 4.2 (2 H, dq), 1.95 (0.3 H, d), and 4.98 (0.1 H, m); δ(^{13}C) enriched signals at 200.4 and 167.2; *m/z* 132 (M^+, 5%), 87 (15), and 44 (100). In order to determine the specific activity of this material a crystalline derivative was prepared. Ethyl [1,3-$^{13}C_2$, 1,3-^{14}C]acetoacetate (5.15 mg) and unlabelled ethyl acetoacetate (96.68 mg) in methanol (1.0 ml) was combined with semicarbazide hydrochloride (102 mg) and sodium acetate (75 mg) in water (5 ml). After the mixture had been warmed to 50 °C for 5 min and then cooled, the white semicarbazide crystallized out and was collected, m.p. 128—130 °C (from aqueous EtOH) (lit.,[52] 133 °C) specific activity of diluted material 4.2 × 10^5 d.p.m. $mmol^{-1}$, and of undiluted material 8.3 × 10^6 d.p.m. $mmol^{-1}$.

(xi) (2R,3S)-2,3-*Epoxybutan*-1-*ol*.—To a solution of titanium(IV) isopropoxide (98 g, 0.34 mol) in dichloromethane (3 l) was added sequentially (+)-di-isopropyl tartrate (81.2 g, 0.34 mol), crotyl alcohol (25 g, 0.34 mol), and a solution of t-butyl hydroperoxide in dichloromethane (4.16M; 175 ml). The mixture was left at −20 °C for 24 h after which time diethyl ether (3 l) was added followed by saturated aqueous Na_2SO_4 (50 ml); the mixture was then stirred vigorously for 3 h. A precipitate formed and this was removed by centrifugation. The solvent was removed at atmospheric pressure and the product was distilled at reduced pressure; yield 8.1 g (27%), b.p. 64 °C at 15 mmHg with traces of Bu^{t}OOH; $[\alpha]_D^{20}$ −33.6° (*c* 0.1, CH_2Cl_2); δ 1.3 (3 H, d), 2.9 (1 H, m), 3.1 (1 H, m), and 3.7 (3 H, m). The Mosher[20] derivative was formed as follows. (2*R*,3*S*)-2,3-Epoxybutan-1-ol (41 mg), (−)-α-methoxytrifluoromethylphenylacetyl chloride (150 mg), and dimethylaminopyridine (73 mg) in dichloromethane (1 ml) and pyridine (1 ml) was stirred at 18 °C for 3 h. The solution was diluted (CH_2Cl_2), washed (dilute HCl), dried, and evaporated to afford an oil which after chromatography (silica, 100% benzene) gave the product (100 mg) as a clear oil, $[\alpha]_D^{20}$ −27.6° (*c* 0.1, CH_2Cl_2); $\nu_{max.}$ 2 950, 1 755, and 1 150 cm^{-1}; δ 1.32 (3 H, d), 2.97 (2 H, m), 3.59 (3 H, s), 4.23 (1 H, dd), 4.55 (1 H, dd), 7.42 (3 H, m) and 7.55 (2 H, m); *m/z* 304 (M^+, 85), 290 (29), and 189 (100).

(xii) (2S,3R)-2,3-*Epoxybutan*-1-*ol*.—This was prepared by the method described in (xi), using (−)-di-isopropyl tartrate; $[\alpha]_D^{20}$ +29.1° (*c* 0.06, CH_2Cl_2); δ1.3 (3 H, d), 2.9 (1 H, m, CH), 3.1 (1 H, m, CH), and 3.7 (3 H, m). The (−)-α-methoxytrifluoromethylphenylacetyl derivative showed $[\alpha]_D^{20}$ +33° (*c* 0.16, CH_2Cl_2); δ 1.32 (3 H, d), 2.95 (1 H, m), 3.00 (1 H, m), 3.59 (3 H, s), 4.21 (1 H, dd), 4.61 (1 H, dd), 7.42 (3 H, m), and 7.55 (2 H, m). A comparison of this ^{1}H n.m.r. spectrum with that from (xi) above, showed each epoxide to have 90% e.e.

(xiii) (2R,3S)-1-*Dimethyl-t-butylsiloxy*-2,3-*epoxybutane*.—Imidazole (6.38 g), dimethyl-t-butylsilyl chloride (5.07 g), and (2*R*,3*S*)-2,3-epoxybutan-1-ol were stirred at 18 °C for 12 h. The mixture was diluted with water and the product extracted into diethyl ether: the extract was dried ($MgSO_4$) and evaporated to leave an oil, which after chromatography (methylene dichloride–light petroleum, 1:1) gave the title compound (4.9 g, 83%); $[\alpha]_D^{20}$ −9.9° (*c* 0.15, CH_2Cl_2); $\nu_{max.}$ 2 900, 1 100, and 850 cm^{-1}; δ 0.1 (6 H, s), 0.9 (9 H, s), 1.3 (3 H, d), 2.9 (2 H, m), and 3.8 (2 H, m).

(xiv) (2S,3R)-1-*Dimethyl-t-butylsiloxy*-2,3-*epoxybutane*.—This was prepared from (2*S*,3*R*)-2,3-epoxybutan-1-ol by the method described in (xiii); $[\alpha]_D^{20}$ +10.8° (*c* 0.1, CH_2Cl_2); δ 0.1 (6 H, s), 0.9 (9 H, s), 1.3 (3 H, d), 2.9 (2 H, m), and 3.8 (2 H, m).

(xv) [^{13}C]- *and* [$^2H_3$]-*Methyl-lithium*.—[^{13}C]- or [$^2H_3$]-iodomethane (8.0 g, 56 mmol) was added to butyl-lithium (1.6 M in hexane; 35 ml, 56 mmol) at 0 °C under argon. After 20 min at 18 °C the mixture was centrifuged (3 000 r.p.m., 20 min) and the supernatant discarded. The pellet was washed twice in hexane, and then resuspended in diethyl ether (25 ml). The methyl-lithium did not dissolve completely and was used as a suspension. An aliquot was carefully added to water and titrated against acid (1M HCl). Typically this indicated 1.4M methyl-lithium in ether.

(xvi) *Sodium* (2S)-[3-^{13}C]*Isobutyrate*.—The [^{13}C]methyl-lithium (1.4M; 35 ml, 50 mmol), prepared as described above, was added to a slurry of copper(I) iodide (4.76 g, 25 mmol) in diethyl ether (20 ml) at −60 °C and stirred for 2 h. (2*R*,3*S*)-1-Dimethyl-t-butylsiloxy-2,3-epoxybutane (2.61 g, 12.9 mmol) was added, and the mixture stirred at −60 °C for 2 h, and at 18 °C for 12 h. Saturated aqueous ammonium sulphate (5 ml) was added, followed by water, and the product was extracted into diethyl ether; the extract was dried (Na_2SO_4) and evaporated and the residue chromatographed (Si, CH_2Cl_2) to afford (2*S*,3*R*)-[1-^{13}C]-3-methyl-1-dimethyl-t-butylsiloxybutan-2-ol (1.69 g, 60%) as an oil. To this, in tetrahydrofuran (10 ml) was added tetrabutylammonium fluoride in THF (1.0M; 15 ml). The mixture was stirred at 18 °C for 4 h after which it was added to sodium periodate (8 g) and potassium permanganate (237 mg) in water (50 ml) at 0 °C with vigorous stirring for 12 h. Dropwise addition of hydrogen peroxide (30%) to precipitate manganese dioxide, and filtration, gave a clear solution. This was adjusted to pH 14 (NaOH) and reduced to 10 ml under reduced pressure. The solution at pH 3.9 (dilute H_2SO_4) was lyophilized, and the lyophilisate at pH 9 (dilute NaOH) was freeze dried, to afford the title compound as a white powder (0.47 g, 50%), contaminated with sodium formate (20% by ^{1}H n.m.r.); δ(^{1}H, D_2O), 1.05 (6 H, $J_{H,H}$ 7 Hz, $J_{C,H}$ 126 Hz), 2.38 (1 H, m), and 8.5 (s, formate); δ(^{13}C), 21.95 (enriched, q), 39.37 (d), and 190.0 (s). A portion of the salt (30 mg) was extracted from water at pH 1 into diethyl ether. After evaporation of the ether, the labelled isobutyric acid in methylene dichloride (5 ml) with (*S*)-(+)-methyl mandelate (60 mg), dimethylaminopyridine (43 mg), and dicyclohexylcarbodiimide (73 mg) was stirred at 18 °C overnight. The solution was washed sequentially with water, dilute HCl, and aqueous sodium hydrogen carbonate, dried ($MgSO_4$), and evaporated. After chromatography (silica, CH_2Cl_2–light petroleum, 1:1) the (*S*)-(+)-methyl mandelate derivative of the title compound was obtained as a colourless oil (50 mg, 59%); $\nu_{max.}$ 3 000 and 1 750 cm^{-1}; δ(^{1}H), 1.25 (3 H, dd, $J_{H,H}$ 7 Hz, $J_{C,H}$ 130 Hz), 1.30 (3 H, dd, $J_{H,H}$ 7 Hz, $J_{C,C,H}$ 5 Hz), 2.74 (1 H, m), 3.73 (3 H, s), 5.95 (1 H, s), and 7.45 (5 H, m); δ(^{13}C), 18.78 (enriched, 0.93 C, q), 18.98 (enriched, 0.07 C, q), 33.92 (d), 52.48 (q), 74.35 (d), 127.69 (d), 128.88 (d), 129.25 (d), 134.28 (s), 169.43 (s), and 176.29 (s); integration of ^{13}C resonances shows the enrichment to be 93% (2*S*)- and 7% (2*R*)-[3-^{13}C]isobutyrate; *m/z* 237 (M^+, 4%), 72 (61), and 44 (100).

(xvii) *Sodium* (2S)-[3,3,3-$^2H_3$]*Isobutyrate*.—The title compound was prepared from [$^2H_3$]methyl-lithium and (2R,3*S*)-1-dimethyl-t-butylsiloxy-2,3-epoxybutane, by the procedure described in (xvi) above, and showed δ(D_2O), 1.05 (3 H, d, *J* 7 Hz), 2.38 (1 H, m), and 8.5 (s, formate). A portion of this product was converted into the (*S*)-(+)-methyl mandelate derivative, which showed: $\nu_{max.}$ 3 000 and 1 750 cm^{-1}; δ(^{1}H, C_6D_6), 1.20 (0.18 H, d), 1.30 (2.82 H, d), 2.62 (1 H, q), 3.30 (3 H, s), 6.22 (1 H, s), and 7.2—7.6 (5 H, m); δ(^{2}H, C_6H_6) 1.20 (s, 94%) and 1.27 (s, 6%); *m/z* 239 (M^+, 0.7%), 74 (100), and 46 (73). Both ^{1}H and ^{2}H n.m.r. resonances indicate 94% (2*S*) and 6% (2*R*)-[3,3,3-$^2H_3$]isobutyrate.

(xviii) *Sodium* (2R)-[3,3,3-$^2H_3$]*Isobutyrate*.—The title com-

pound was prepared from [$^2H_3$]methyl-lithium and (2*S*,3*R*)-1-dimethyl-t-butylsiloxy-2,3-epoxybutane, by the procedure described in (xvi) above, and showed δ(D_2O) 1.05 (3 H, d, *J* 7 Hz), 2.38 (1 H, m), and 8.5 (s, formate). A portion was again converted into the (*S*)-(+)-methyl mandelate derivative, as described in (xvi), which showed; $\nu_{max.}$ 3 000 and 1 750 cm^{-1}; δ(^1H, C_6D_6), 1.2 (2.85 H, d, *J* 7 Hz), 1.30 (0.15 H, d, *J* 7 Hz), 2.62 (1 H, q), 3.30 (3 H, s), 6.22 (1 H, s), and 7.2—7.6 (5 H, m); δ(^2H, C_6H_6) 1.20 (s, 5%) and 1.27 (s, 95%); *m/z* 239 (M^+, 0.7), 74 (100), and 46 (65); both ^1H and ^2H n.m.r. indicates 95% (2*R*)- and 5% (2*S*)-[3,3,3-$^2H_3$]isobutyrate.

(xix) *Growth of* Streptomyces cinnamonensis.—*S. cinnamonensis* strain A 3 823.5 was kindly provided by Eli Lilly and Co., and was stored with glycerol (20% v/v) in liquid nitrogen. A two-stage fermentation cycle was used. A vegetative culture comprising: D-glucose (0.3 g), soybean flour (0.9 g), potato flour (1.2 g), yeast extract (0.15 g), calcium carbonate (0.06 g), and distilled water (60 ml), in a wide neck conical flask (500 ml), loosely closed with cotton wool, was sterilized (121 °C, 20 min), inoculated with *S. cinnamonensis* mycelial fragments, and incubated on a rotary shaker at 32 °C for 18 h. This corresponded to the end of the log growth phase. The second stage culture comprised: soybean grits (2.5%), D-glucose (3.0%), calcium carbonate (0.1%), manganous chloride hydrate (0.02%), ferric sulphate hydrate (0.03%), potassium chloride (0.01%), soybean oil (1.5%), methyl oleate (2.0%; technical grade), and lard oil (0.5%), dissolved in distilled water. Second-stage fermentations were either carried out in 60 ml shake flask cultures in a wide neck conical flask (500 ml), on a rotary shaker at 32 °C, or as 5.0- or 6.0-l batch fermentations in an LH 2 000 series fermenter, at 32 °C with aeration (1 vol vol^{-1} min^{-1} air). A 5% inoculum of vegetative culture was used. The antibiotic was extracted and purified by the standard method, described earlier.[13,51] Briefly, the whole broth was homogenized with methanol, filtered, and extracted with chloroform. The chloroform extracts were dried (Na_2SO_4) and evaporated to leave a dark brown oil. This was chromatographed (silica) eluting successively with methylene dichloride, methylene dichloride–ethyl acetate (1:1), and with ethyl acetate. Fractions containing monensins-A and -B were pooled and evaporated. The cultures typically produced monensins-A and -B as their sodium salts, in a 10:1 ratio. The isomers are readily separated by flash chromatography[50] on silica, or by reversed phase h.p.l.c. (Zorbax ODS column, eluting MeOH–H_2O, 97:3, analytical column 25 cm × 4.6 mm, 1 ml/min, with refractive index detector). Recrystallization from MeOH–H_2O gave Na-monensin-A, m.p. 269—270 °C (lit.,[51] m.p. 267—269 °C). The full assignments of ^1H and ^{13}C n.m.r. spectra are reported elsewhere.[14]

(xx) *Feeding Experiments.*—All labelled compounds were fed batchwise in equal portions after 48, 60, and 72 h during the fermentation, as sterile solutions in distilled water at pH 7.0. The quantities administered were: sodium [1-^{13}C]-, [2-^{13}C]-, and [3,3′-$^{13}C_2$]-isobutyrates (100 mg per culture) each to two shake flask cultures; sodium [1,3,3′-$^{13}C_3$] + unlabelled isobutyrate (75 + 225 mg, respectively) distributed (100 mg per culture) to three shake flask cultures; sodium (2*S*)-[3-^{13}C]isobutyrate (60 mg per culture) to two shake flask cultures; sodium (2*S*)-[3,3,3-$^2H_3$]- and (2*R*)-[3,3,3-$^2H_3$]-(47 mg) each with sodium [1-^{14}C]isobutyrate (3 mg; 2.16 × 10^8 d.p.m./mmol), distributed (25 mg per culture) in two shake flask cultures; sodium [2-^3H][1-^{14}C]isobutyrate (1.32 × 10^8 d.p.m./mmol ^{14}C, 1.13 × 10^8 d.p.m./mmol ^3H; $^3H/^{14}C$ = 0.86) (100 mg per culture) to two shake flask cultures; sodium [3,3′-$^{13}C_2$, *dimethyl*-$^2H_6$]isobutyrate (100 mg per culture) to three shake flask cultures; sodium [2-^{13}C, 2-^2H]isobutyrate (75 mg) + unlabelled isobutyrate (225 mg) distributed (100 mg per culture) to three shake flask cultures; sodium [2-^{13}C]isobutyrate (80 mg) + [*dimethyl*-$^2H_6$]isobutyrate (240 mg) distributed (100 mg per culture) to three shake flask cultures; ethyl [1,3-$^{13}C_2$, 1,3-^{14}C]acetoacetate (60 mg) + unlabelled ethyl acetoacetate (240 mg) distributed (150 mg per culture) to two shake flask cultures; sodium [2-^2H]isobutyrate (6 g) to a 6.0 l fermentation; sodium [*dimethyl*-$^2H_6$]isobutyrate (3.5 g), and [$^2H_7$]isobutyrate (3.5 g), each to a 5.0 l batch fermentation. At the end of the fermentation (day 7) monensin titres were typically 1.0—1.5 mg/ml.

(xxi) *Degradation of Monensin-A.*—Sodium monensin-A (2.1 g) was added with stirring to a solution of potassium dichromate (5.3 g) in water (200 ml) containing concentrated H_2SO_4 (75 ml), and the mixture was refluxed for 30 min. Acetic and propionic acids were then recovered by steam distillation. The distillate was adjusted to pH 11 (10M NaOH) and freeze dried. The residue in water (1.0 ml) at pH 1 (concentrated H_2SO_4) was mixed with anhydrous sodium sulphate (40 g). Soxhlet extraction of the solid with diethyl ether for 18 h, followed by evaporation, gave acetic and propionic acids. These were added with stirring to 4-dimethylaminopyridine (120 mg), (*S*)-(+)-methyl mandelate (1.83 g) and dicyclohexylcarbodiimide (2.27 g) in methylene dichloride (30 ml) at −20 °C. The solution was then filtered, washed with aqueous $NaHCO_3$ and dilute HCl, dried (Na_2SO_4), and evaporated to yield a yellow oil. The (*S*)-methyl mandelate derivatives of acetate and propionate were obtained after flash chromatography (silica) eluting with light petroleum–diethyl ether (4:1). Where necessary, further purification could be effected by h.p.l.c. (silica Zorbax column, 25 cm × 2.1 cm, eluting with dichloromethane–hexane, 9:1, 25 ml/min, with u.v. on-line detector at 254 nm), to give the mandelate ester of propionate (56 mg) as a colourless oil (Found: C, 64.8; H, 6.4; $C_{12}H_{14}O_4$ requires C, 64.9; H, 6.3); δ(^1H, C_6D_6), 1.10 (3 H, t), 2.23 (1 H, m, 2 H_S), 2.35 (1 H, m, 2 H_R), 3.30 (3 H, s), 6.22 (1 H, s), and 7.2—7.6 (5 H, m); *m/z* 220 (M^+, 0.7%), 163 (1.4), 105 (15), 77 (10), and 57 (100); as well as the mandelate ester of acetate (320 mg) as a colourless oil (Found: C, 63.2; H, 5.8; $C_{11}H_{12}O_4$ requires C, 63.5; H, 5.8); δ(^1H, C_6D_6) 1.87 (3 H, s), 3.31 (3 H, s), 6.18 (1 H, s), 7.2—7.6 (5 H, m), chemical shifts relative to residual C_6H_6 at δ 7.30; *m/z* 208 (M^+, 0.9%), 149 (14), 121 (13), 107 (48), 77 (25), and 43 (100).

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