The Biosynthesis of Monensin-A: Thymine, β-Aminoisobutyrate and Methacrylate Metabolism in *Streptomyces cinnamonensis*

DAVID O'HAGAN* and SARAH V. ROGERS

Department of Chemistry, University of Durham, Science Laboratories,
South Road, Durham, DH1 3LE, United Kingdom

GORDON R. DUFFIN

Isotope Chemistry Laboratories, Department of Preclinical Metabolism and Pharmacokinetics,
Sanofi Research Division,
Willowburn Avenue, Alnwick, Northumberland, NE66 2JH, United Kingdom

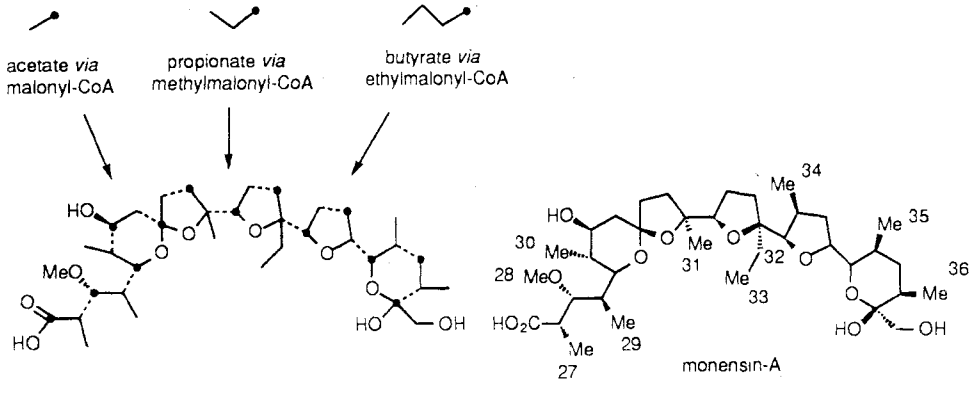
KEVIN A. REYNOLDS

Department of Biomedicinal Chemistry, School of Pharmacy,
The University of Maryland at Baltimore,
Baltimore, Maryland 21201, U.S.A.

(Received for publication May 24, 1995)

Feeding experiments with isotopically labelled samples of [^{13}C,$^{2}H_3$-*methyl*]-thymine, (R,S)-[1-^{13}C]- and (R,S)-[^{13}C-*methyl*]-β-aminoisobutyrates into monensin-A demonstrate that these metabolites can all contribute to the methylmalonyl-CoA pool in *Streptomyces cinnamonensis*, and implicate DNA catabolism as a contributory metabolic source of propionate carbon atoms for secondary metabolite biosynthesis. Further, these labelled compounds were unexpectedly incorporated into the butyrate unit of monensin-A, an observation which can be rationalised if β-aminoisobutyrate is converted to methacrylyl-CoA and then reduced to isobutyryl-CoA prior to conversion to butyryl-CoA by the action of isobutyryl-CoA mutase. Feeding experiments with [1-^{13}C]- and [^{13}C-*methyl*]- methacrylates and [3-^{13}C]-isobutyrate suggest that these metabolites partition similarly between the butyrate and propionate units of monensin-A consistent with the view that isobutyryl-CoA and methacrylyl-CoA have a close metabolic relationship.

It is widely appreciated that the aglycones of the polyether and macrolide antibiotics produced by Actinomycetes derive from C_2 acetate, C_3 propionate and C_4 butyrate units[1]. For example monensin-A is derived[2] from five acetate (malonyl-CoA), seven propionate (methylmalonyl-CoA) and a butyrate (ethylmalonyl-CoA) unit as shown. The malonyl-CoA precursors to these subunits are derived from the catabolism of primary metabolites[3] such as amino acids, fatty acids and intermediates of the citric acid cycle. For example it is well known[4] that the amino acid L-valine can contribute carbon atoms to both the propionate and butyrate subunits of monensin-A and other macrolide and polyether antibiotics. L-Valine is metabolised to iso-butyryl-CoA, a pivotal metabolite, which is converted either to methylmalonyl-SCoA and thus contributes to the



propionate units or it is acted upon by isobutyryl-CoA mutase and isomerised to butyryl-CoA and then incorporated into the butyrate unit of monensin-A[5] (see Scheme 1). In this paper we explore the role of the DNA base thymine and its catabolic product β-aminoisobutyrate in contributing to the C_3 and C_4 subunits in monensin-A biosynthesis. Some of these results have been communicated[6]. The pathway for conversion of isobutyryl-CoA to methylmalonyl-CoA implicates methacrylyl-CoA as an intermediate. We have thus studied and report the incorporation of [1-^{13}C]- and [^{13}C-*methyl*]-methacrylates into monensin-A and compare these to the incorporation of [3-^{13}C]-isobutyrate as the metabolism of these compounds should be closely linked.

Materials and Methods

Labelled Compounds

(R,S)-[3-^{13}C]-β-aminoisobutyrate (99% ^{13}C enriched) was synthesised as previously reported[6] following a modification of the route of BOHME *et al.*[7] and [$^{13}C^2H_3$-*methyl*]-thymine (99% ^{13}C, ^{2}H enriched) was prepared by a modification of the method of SHIUE *et al.*[8] using [$^{13}C^2H_3$]-methyl iodide as the source of the isotopes. Sodium [3-^{13}C]-isobutyrate (99% ^{13}C enriched) was prepared as previously described[9].

(R,S)-[1-^{13}C]-β-aminoisobutyrate (99% ^{13}C enriched) was prepared by treatment of [1-^{13}C]-α-bromopropionic acid (1.0 g, 6.49 mmol) in acetonitrile (7 ml) with sodium carbonate (0.34 g, 3.25 mmol) for 2 hours at room temperature. The sodium salt precipitated and was collected by filtration. A solution of the sodium salt (0.98 g, 5.6 mmol), NaOH (0.016 g, 0.42 mmol) and NaCN (0.27 g, 5.6 mmol) in water (5ml) was heated at 70°C for 2.5 hours. After cooling and acidification (dil HCl) the aqueous solution was extracted into diethyl ether and [1-^{13}C]-α-cyanopropionic acid recovered, after evaporation, as an oil. The oil was dissolved directly in a solution of ethanol-chloroform (10:1, 30 ml) and stirred for 18 hours with PtO_2 (100 mg) under H_2 (2.5 atm). The reaction was filtered, the solvent removed under reduced pressure and the amino acid purified by ion exchange chromatography (Dowex, H^+ form) eluting finally with ammonium hydroxide solution. Removal of the volatiles and recrystallisation from methanol afforded (R,S)-[1-^{13}C]-β-aminoisobutyrate as a white amorphous solid (54 mg, 8%); δH (D_2O) 2.96, (2H, m, CH_2), 2.54 (1H, m, CH), 1.06 (3H, dd, $^3J_{CH}$=4.6 Hz and J=7.2 Hz, CH_3); δC (D_2O) 183.4 (^{13}CO), 44.9 (CH_2), 41.5 (d, $^1J_{CC}$=51.7 Hz, CH), 17.1 (CH_3).

Sodium [1-^{13}C]-methacrylate (99% ^{13}C enriched) was prepared by addition over one hour of a solution of 2-bromopropene (2.72 g, 23 mmol) in diethyl ether (20 ml) to a suspension of magnesium turnings (6.2 g, 253 mmol) in diethyl ether (10 ml). The reaction mixture was heated at 45°C for a further hour and then the Grignard reagent was transferred to a clean flask by cannular. Labelled $^{13}CO_2$, generated by addition of concentrated H_2SO_4 (15 ml) onto $Ba^{13}CO_3$ (5 g, 25 mmol), was introduced by vacuum transfer to quench the Grignard reagent. Water was added and the solution altered to pH 3 with dilute H_2SO_4. The product was extracted into diethyl ether and concentrated to give a yellow oil, which was purified over silica gel eluting initially with dichloromethane and then ethyl acetate. Concentration of the active fractions afforded [1-^{13}C]-methacrylic acid as a colourless oil. Neutralisation to pH 7 with dilute NaOH followed by freeze drying generated sodium [1-^{13}C]-methacrylate (0.62 g, 23%) as an amorphous white solid; δH (D_2O) 5.53 ((1H, dm, $^3J_{CH}$=4.16 Hz, CH), 5.21 (1H, dm, $^3J_{CH}$=11.7 Hz, CH), 1.72 (3H, dm, $^3J_{CH}$=2.7 Hz, CH_3); δC (D_2O) 180.2 (^{13}CO), 145.1 (d, $^1J_{CC}$=61.6 Hz, C), 123.7 (d, $^2J_{CC}$=2.2 Hz, CH_2), 22.0 (d, $^2J_{CC}$=3.2 Hz, CH_3).

Sodium [^{13}C-*methyl*]-methacrylate (99% ^{13}C enriched) was prepared by stirring diethyl [3-^{13}C]-methylmalonate (3.72 g, 21.23 mmol) in an aqueous solution of formaldehyde (37%, 1.7 ml) and potassium bicarbonate (0.3 g, 3 mmol) for 2 hours at 70°C. Saturated ammonium sulphate solution (10 ml) was added and the crude product extracted into diethyl ether. Purification over silica gel, eluting with dichloromethane and then ethyl acetate, afforded diethyl [^{13}C-*methyl*]-(hydroxymethyl)methylmalonate (0.93 g, 4.53 mmol) as an oil. δH ($CDCl_3$) 4.21 (4H, q, J=7.14 Hz, CH_2), 3.86 (2H, dd, J=6.8 Hz and $^3J_{CH}$=3.2 Hz, CH_2), 3.27 (1H, t, J=6.86 Hz, OH), 1.44 (3H, d, $^1J_{CH}$=131 Hz, $^{13}CH_3$), 1.27 (6H, t, J=7.1 Hz, CH_3); δC ($CDCl_3$) 171.1 (CO), 66.1 (CH_2), 61.2 (CH_2), 55.5 (d, $^1J_{CC}$=34.7 Hz, C), 17.2 ($^{13}CH_3$), 13.6 (CH_3); Dilute hydrochloric acid (5%, 20 ml) was added directly to the oil and the reaction heated under reflux for 3 days. Extraction into diethyl ether gave a yellow oil which was purified over silica gel eluting with dichloromethane and then gradually increasing the ethyl acetate content until 100%. The active fractions were concentrated under reduced pressure, dissolved in water (10 ml) and neutralised with dilute NaOH solution. Freeze drying gave sodium [^{13}C-*methyl*]-methacrylate (0.34 g, 58%) as an amorphous white solid. mp >360°C; δH (D_2O) 5.51 (1H, dm, $^3J_{CH}$=9.56 Hz, CH), 5.2 (1H, dm, $^3J_{CH}$=11.7 Hz, CH), 1.72 (3H, dm, $^1J_{CH}$=127.4 Hz, CH_3); δC (D_2O) 180.3 (d, $^2J_{CC}$=3.32 Hz, CO), 145.1 (d, $^1J_{CC}$=43.3 Hz, C), 122.6 (CH_2), 21.9 ($^{13}CH_3$).

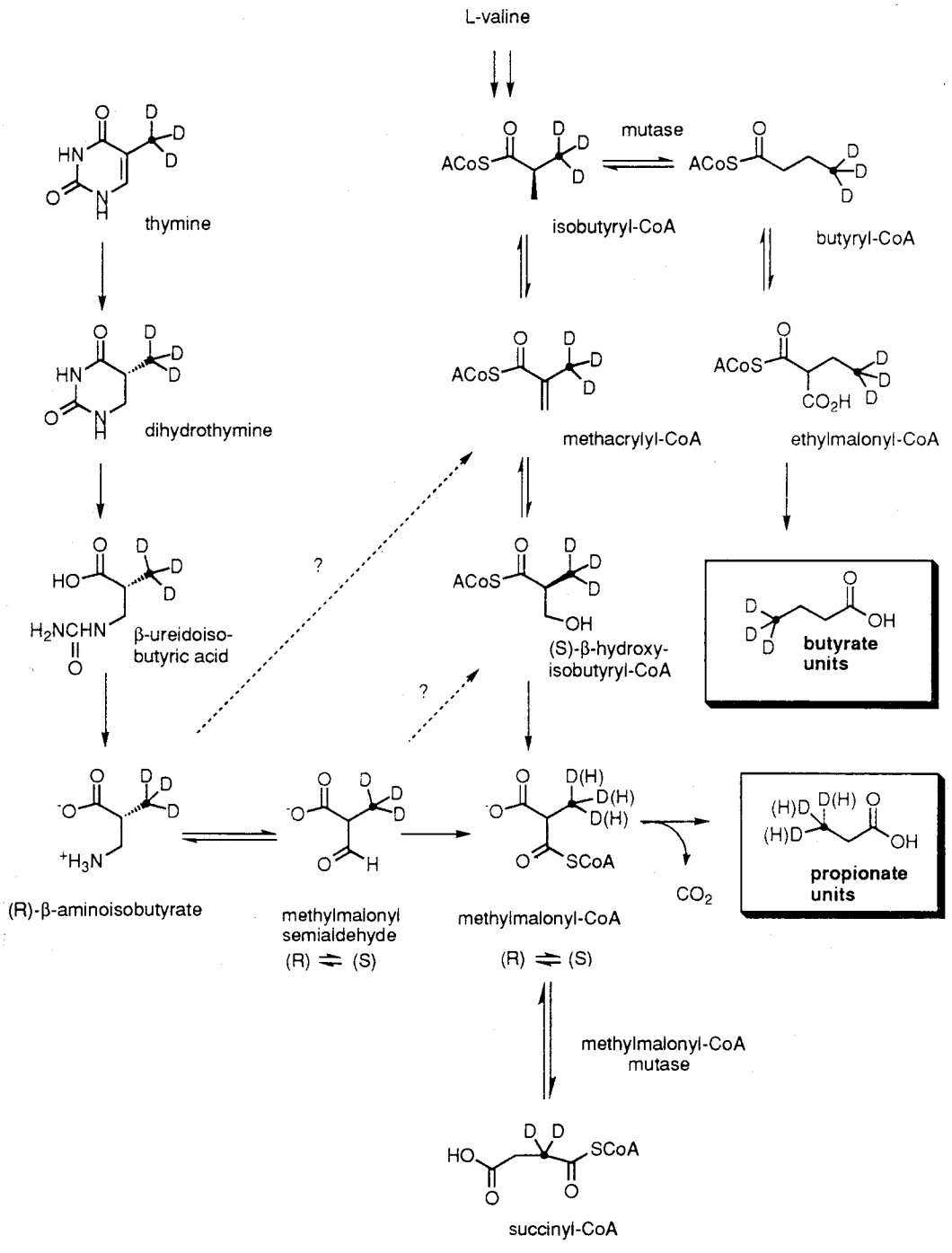
Feeding Experiments

Streptomyces cinnamonensis ATCC 15413 was grown on yeast-malt agar plates (yeast extract 2 g, malt extract 5 g, glucose 2 g, agar 7.5 g, distilled H_2O (500 ml), adjusted to pH 7.2 with dilute KOH solution) for 14 days at 30°C. Spores were used to inoculate two shake flasks each containing 70 ml of seed medium (glucose 3.36 g, soybean flour (Sigma Chem. Co.) 2.1 g, $CaCO_3$

0.42 g, $FeSO_4 \cdot 7H_2O$ 0.77 g, $MgCl_2$ 0.4 mg and distilled H_2O 140 ml) and the flasks incubated on an orbital shaker (32°C, 150 rpm) for 14 days. A 5% inoculum was used to initiate production flasks each containing 100 ml of medium (glucose 45 g, soybean flour (Sigma Chem. Co.) 13.5 g, $CaCO_3$ 2.7 g, $FeSO_4 \cdot 7H_2O$ 4.94 g, $MgCl_2$ 2.7 mg, distilled H_2O, 900 ml) which were then incubated on an orbital shaker (32°C, 200 rpm) for 10 days. Monensin-A was isolated as previously described[9] and finally purified on C_{18} reverse phase preparative chromatography plates, eluting with MeOH : H_2O (9 : 1). The samples were usually contaminated with ~10% monensin-B.

For each of the experiments the labelled compounds were pulse fed to production flasks (5 × 100 ml) of *S. cinnamonensis* at 3, 3.5 and 4 days. The final concentration was 5.0 mmol in all cases except for (R,S)-[1-^{13}C]-β-aminoisobutyrate which was fed at 2.6 mmol.

Scheme 1. The metabolic relationships between thymine and L-valine and their role in contributing to the propionate and butyrate units in monensin-A.



The scheme offers a rational for the observed labelling pattern in monensin-A after the [^{13}C, $^{2}H_3$-*methyl*]-thymine feeding experiment.

Results

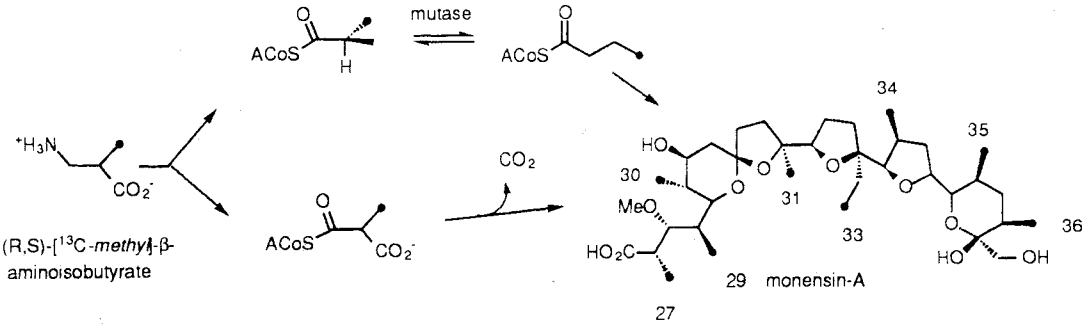
In mammals[10,11] thymine is degraded to (2R)-β-aminoisobutyrate *via* (5R)-dihydrothymine and β-ureidoisobutyric acid as illustrated in Scheme 1. (2R)-β-Aminoisobutyrate is then converted to methylmalonyl-CoA after transamination to methylmalonate semialdehyde. In Streptomycetes, if thymine can similarly contribute to the methylmalonyl-CoA pool then it will provide carbon atoms to the propionate units of the polyether and macrolide antibiotics. In an effort to establish the validity of this hypothesis we have studied the incorporations of (R,S)-[1-^{13}C]- and (R,S)-[^{13}C-*methyl*]-β-aminoisobutyrates and [$^{13}C^2H_3$ *methyl*]-thymine into the propionate units of monensin-A.

Incorporation of Isotopically Labelled (R,S)-[1-^{13}C]- and (R,S)-[^{13}C-*methyl*]-β-Aminoisobutyrates and [$^{13}C^2H_3$ *methyl*]-Thymine into Monensin-A

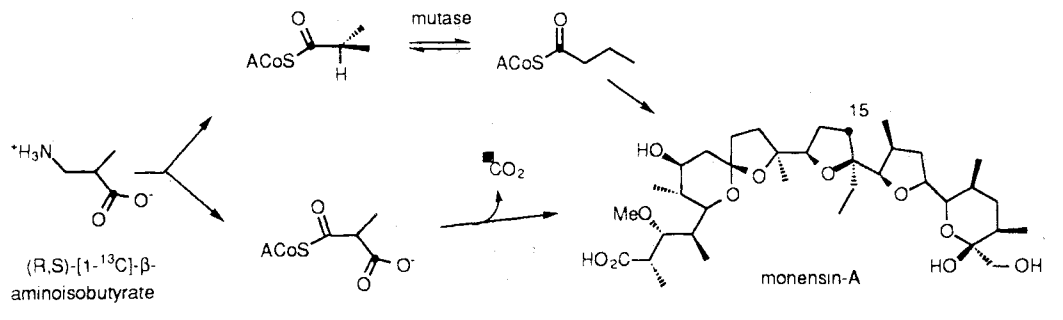
In the first instance (R,S)-[^{13}C-*methyl*]-β-aminoisobutyrate was introduced into cultures of *Streptomyces cinnamonensis* and the resultant monensin-A isolated and subjected to ^{13}C NMR analysis. All of the propionate derived methyl groups (C-27, C-29, C-30, C-31, C-34, C-35 and C-36) were significantly enriched (5~8 fold) consistent with the hypothesis that β-aminoisobutyrate is efficiently converted to methylmalonyl-CoA. In addition there was a smaller but significant (2.5 fold) incorporation into C-33, the methyl group of the butyrate derived unit in monensin-A. From this experiment therefore it would appear that β-aminoisobutyrate can also contribute to the butyrate pool most probably after its conversion to isobutyryl-CoA. These results are summarised in Scheme 2. The feeding experiment with [1-^{13}C]-β-aminoisobutyrate reinforced these conclusions. In this case only one enrichment was apparent at C-15 (2.33 fold) of monensin-A, the carbon derived from C1 of the butyrate unit. If β-aminoisobutyrate is metabolised to methylmalonyl-CoA as illustrated in Scheme 1, then the isotope at C-1 will be lost during decarboxylation and thus no isotope is incorporated into the propionate units. The single incorporation into C-15 can be rationalised as before by the conversion of β-aminoisobutyrate to isobutyryl-CoA and then incorporation into the butyrate unit after the action of isobutyryl-CoA mutase. These results are summarised in Scheme 3.

A feeding experiment with [$^{13}C^2H_3$ *methyl*]-thymine reinforced these conclusions giving a similar labelling distribution between the propionate and butyrate units in monensin-A. After incorporation of [$^{13}C^2H_3$ *methyl*]-thymine, the resultant monensin-A was analysed by subtracting the data of the ^{13}C-{^{1}H} NMR spectrum

Scheme 2. Labelling pattern in monensin-A after incorporation of (*R,S*)-[^{13}C-*methyl*]-β-aminoisobutyrate.



Scheme 3. Labelling pattern in monensin-A after incorporation of (*R,S*)-[1-^{13}C]-β-aminoisobutyrate.



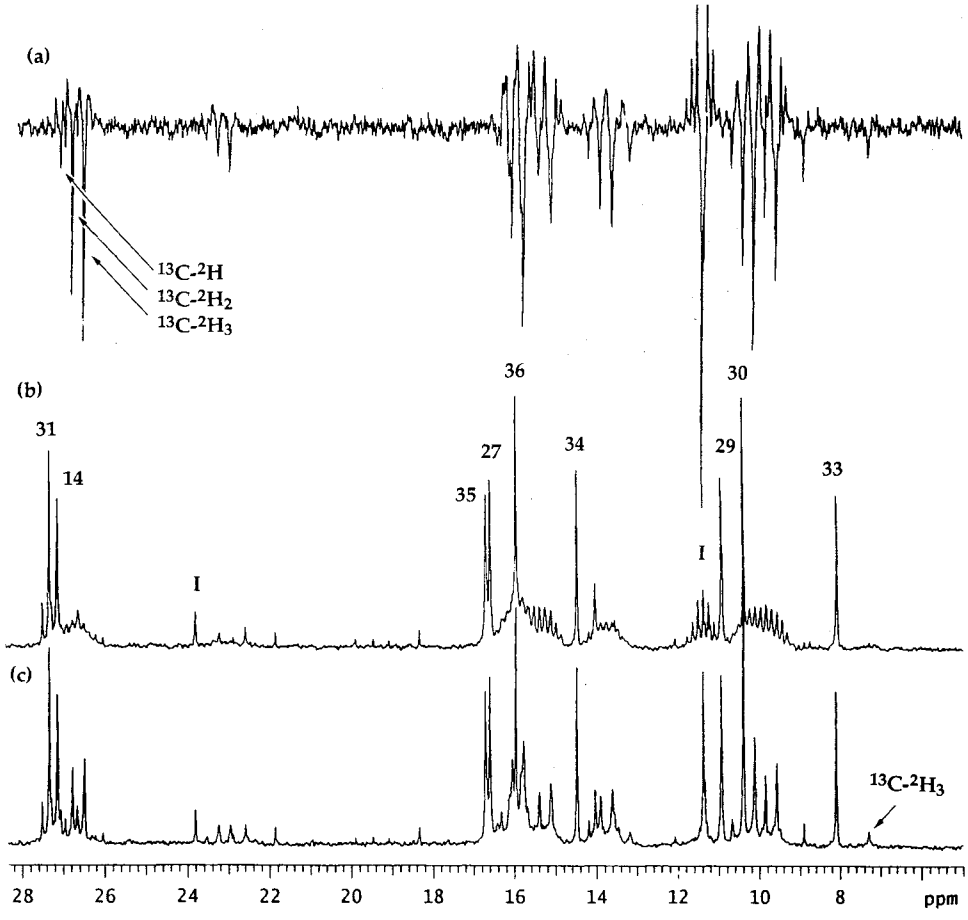
from that of the $^{13}C\{^{1}H,^{2}H\}$ spectrum. The resultant difference spectrum is shown in Figure 1. The incorporation levels were lower than that from the (R,S)-β-aminoisobutyrate experiment. From the additive deuterium induced α-shifts in the ^{13}C-NMR spectrum[12], associated with one, two and three deuterium atoms directly bonded to carbon-13, we were able to establish that there were incorporations into all seven of the propionate derived methyl groups and that the ratio of the $^{13}CD_3$, $^{13}CD_2H$ and $^{13}CH_2D$ components averaged 3:2:1 respectively. In addition there was a lower level incorporation again into C-33, the methyl group derived from butyrate. In this case there appeared to be no wash out of the deuterium atoms from the labelled methyl group and all of the enriched signal at C-33 was associated with a CD_3 component. This can be rationalised by a divergence in the pathways from β-aminoisobutyrate to methylmalonyl-CoA and isobutyryl-CoA (see Discussion).

Incorporation of [1-^{13}C]- and [^{13}C-*methyl*]-Methacrylates and [3-^{13}C]-Isobutyrate into Monensin-A

Methacrylyl-CoA is an assumed intermediate on the pathway between isobutyryl-CoA and methylmalonyl-CoA, however we are unaware of any feeding experiments involving methacrylate in Streptomyces metabolism. Methacrylyl-CoA is also implicated in the metabolism of isobutyrate in *Pseudomonas putida*[13] and methacrylate has been successfully incorporated, in a stereospecific manner into β-hydroxyisobutyrate in this bacterium. In an effort to reinforce the intermediacy of methacrylyl-CoA in isobutyrate metabolism in *S. cinnamonensis*, [1-^{13}C]- and [^{13}C-*methyl*]-methacrylates were prepared and their incorporation into monensin-A was studied. Our route to [1-^{13}C]-methacrylate was straightforward and involved the generation of a Grignard reagent from 2-bromopropene. The Grignard reagent was then quenched with [^{13}C]-carbon dioxide, generated from

Fig. 1. ^{13}C-NMR spectra of monensin-A after feeding [^{13}C-*methyl*]-thymine to *S. cinnamonensis*.

(a) Difference spectrum of data from (b) subtracted from (c). (b), $^{13}C\{^{1}H\}$ NMR spectrum of the recovered monensin-A, (c), $^{13}C\{^{1}H, ^{2}H\}$ NMR spectrum of the recovered monensin-A.



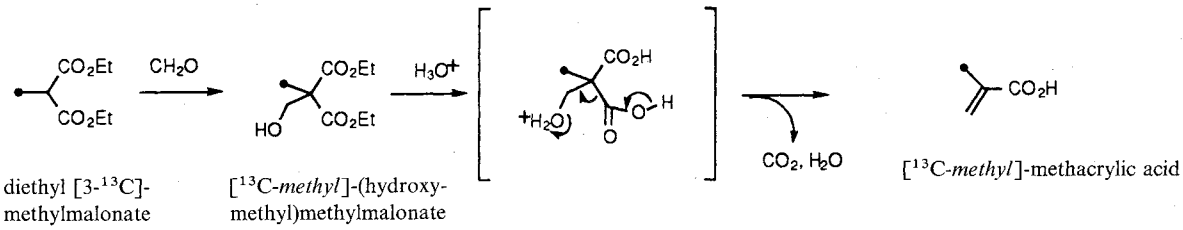
It is apparant from the additive α-shifts due to successive deuterium substitution that all of the propionate derived methyl groups contain either three, two or one deuterium atoms in a ratio ~3:2:1. Only a $^{13}C^{2}H_3$ component exists for C-33, the methyl group derived from butyrate. The peaks designated as (I) are labelled impurities, both of which could be removed after further chromatography.

[^{13}C]barium carbonate. For the synthesis of [^{13}C-*methyl*]-methacrylate we have developed the route shown in Scheme 4.

Treatment of diethyl [3-^{13}C]-methymalonate with aqueous formaldehyde and potassium bicarbonate generated diethyl [^{13}C-*methyl*]-(hydroxymethyl)methylmalonate. The key step in the synthesis is a decarboxylative dehydration (see Scheme 4) of (hydroxymethyl)methylmalonic acid to generate [^{13}C-*methyl*]-methacrylic acid. This reaction proceeds efficiently under aqueous acidic conditions and provides an excellent route to the appropriately labelled material. In each case the methacrylates were neutralised with dilute NaOH and fed as their sodium salts.

Monensin-A isolated after a feeding experiment with sodium [1-^{13}C]-methacrylate was labelled at C-15 (4.30 fold), the carbon derived from C1 of butyrate. Enrichments, at a level just above natural abundance, were apparant in some of the C1 propionate derived carbons indicative of low incorporation into these sites (see Table 1). In a complementary manner sodium [^{13}C-*methyl*]-methacrylate labelled C-33 of monensin-A (4.36 fold), the butyrate derived methyl group. [^{13}C-*methyl*]-Methacrylyl-CoA is thus reduced in a stereospecific manner to (R)-[3-^{13}C]-isobutyryl-CoA and is then isomerised to [4-^{13}C]-butyryl-CoA and incorporated into the butyrate unit. The carbon atoms derived from the methyl group of propionate were also labelled, although the incorporation into these sites was low and again just above the observable threshold (see Table 1). These results are entirely consistent with the pathway outlined in Scheme 1. The interconversion of isobutyryl-CoA and methacrylyl-CoA is reversible and the results suggest that under the fermentation conditions, the metabolic flux on this pathway favours butyrate over propionate. It is of course possible that the methylmalonyl-CoA pool is committed to several metabolic pathways resulting in a lower than expected incorpora-

Scheme 4. Synthesis of [^{13}C-*methyl*]-methacrylate.



diethyl [3-^{13}C]-methylmalonate

[^{13}C-*methyl*]-(hydroxymethyl)methylmalonate

[^{13}C-*methyl*]-methacrylic acid

Table 1. ^{13}C enrichments from labelled precursors into the carbons of monensin-A.

(a) Normalised relative to C10 = 1, (b) normalised relative to C28 = 1, (c) 125 MHz, (d) 100 MHz.

Carbon number in monensin-A	^{13}C NMR/ppm	[^{13}C-*methyl*]-β-aminoisobutyrate (a, c)	[1-^{13}C]-β-aminoisobutyrate (a, d)	[^{13}C-*methyl*]-methacrylate (b,c)	[1-^{13}C]-methacrylate (a, c)	[3-^{13}C]-isobutyrate (a, c)
C1	181.26	—	0.94	—	1.14	—
C3	82.93	—	0.99	—	0.93	—
C5	68.32	—	1.04	—	1.02	—
C6	34.83	0.93*	0.87*	0.91*	0.88*	0.70*
C9	106.94	0.71*	0.87*	1.20*	0.93*	0.54*
C11	33.16	—	1.08	—	1.26	—
C15	29.81	—	2.33	—	4.3	—
C16	85.79	—	—	—	—	9.09
C17	84.97	0.84*	1.03	0.9*	0.88	0.38*
C19	33.24	1.07*	1.03*	1.32*	1.11*	0.49
C21	74.46	—	0.96	—	1.16	—
C23	35.60	—	1.10	—	1.35	—
C27	16.69	6.55	—	1.4	—	2.31
C29	11.07	7.11	—	1.74	—	2.67
C30	10.50	7	—	1.24	—	2.41
C31	27.44	5.58	—	1.57	—	2.66
C33	8.23	1.58	—	4.36	—	9.86
C34	14.57	7.08	—	1.43	—	2.78
C35	16.82	6.14	—	1.21	—	2.45
C36	16.07	6.85	—	1.19	—	2.71

* Unenriched C as standard.

tion into the propionate units in these experiments. In an effort to gain more information on the metabolic flux we then fed (R,S)-[3-^{13}C]-isobutyrate to *S. cinnamonensis* cultures. The resultant ^{13}C NMR spectrum of monensin-A isolated after introduction of (R,S)-[3-^{13}C]-isobutyrate indicated two large enrichments (9～10 fold) at C-16 and C-33, corresponding C2 and C4 of the butyrate unit. This is entirely consistent with previous studies[14] on the stereochemical course of this mutase where the (S) enantiomer labels C2 of the butyrate and the (R) enantiomer labels C4. All of the propionate derived methyl groups were also significantly enriched (2～3) consistent with processing of (S)-[3-^{13}C]-isobutyrate to [^{13}C-*methyl*]-methylmalonyl-CoA. In the case of the (R) enantiomer the label is lost during decarboxylation of [^{13}C-*carboxyl*]-methylmalonyl-CoA. It is interesting to note that in this experiment the butyrate-propionate incorporation ratio is also 4:1, the ratio observed in the methacrylate feeding experiments.

Discussion

At the outset of this study we anticipated isotopically labelled incorporations from thymine and β-aminoisobutyrate, into the propionate subunits of monensin-A as these metabolites were expected to be metabolised to methylmalonyl-CoA. This emerged to be the case and there is a particularly efficient conversion of β-aminoisobutyrate into methylmalonyl-CoA in *S. cinnamonensis* as judged by the high incorporations into the propionate derived carbons in monensin-A. Regiochemical information was obtained. In the (R,S)-[3-^{13}C]-β-aminoisobutyrate feeding experiment, there was only one enrichment into C-33, the methyl group derived from butyrate. There was no incorporation into the propionate derived methyl groups. Thus it is the amino-methyl carbon of (R)-β-aminoisobutyrate which becomes the thioester carbonyl of methylmalonyl-CoA, and the original carboxylate carbon of the amino acid which becomes the carboxylate carbon in methylmalonyl-CoA and is lost as carbon dioxide. These observations are consistent with transamination and then the action of an aldehyde dehydrogenase acting on methylmalonyl semialdehyde.

The concomitant incorporations of thymine and β-aminoisobutyrate into the the butyrate unit of monensin-A were unexpected and require that the branched carbon skeleton of β-aminoisobutyrate is isomerised to a linear butyrate skeleton. The most reasonable rationale in our view is the conversion of (R)-β-aminoisobutyrate to isobutyryl-CoA. Isobutyryl-CoA is then acted on by isobutyryl-CoA mutase and is directly converted to butyryl-CoA. Methylmalonyl-CoA mutase, which interconverts methylmalonyl-CoA and succinate would also generate a linear from a branched framework, however such a rearrangement is inconsistent with the intact incorporation of the [^{13}C,$^{2}H_3$]-methyl group of thymine into C-33, the methyl group derived from butyrate. Also there is no evidence that Streptomycetes can reduce succinate to butyrate. Supporting evidence for a divergence in pathways from β-aminoisobutyrate to the propionate and butyrate units comes from the differential extent of deuterium washout from the methyl group during the [^{13}C$^{2}H_3$-*methyl*]-thymine feeding experiment. The hydrogen atoms of the propionate derived methyl groups were partially exchanged whereas those of the butyrate derived methyl group were not. This exchange could reasonably occur by the reversible action of methylmalonyl-CoA mutase acting on the methylmalonyl-CoA pool prior to polyketide assembly as indicated in Scheme 1. The lack of exchange at C-33, the butyrate derived methyl group in monensin-A, suggests that methylmalonyl-CoA is not an intermediate between (R)-β-aminoisobutyrate and isobutyryl-CoA, thus it would appear that there is a more direct pathway between these metabolites. A direct deamination of (R)-β-aminoisobutyrate to generate methacrylate as shown in Scheme 1 would account for this. Deamination is attractive as it is a non redox process and would generate methacrylate directly, however such a β-aminoisobutyrate deaminase has not been reported. [1-^{13}C]- and [3-^{13}C]-β-aminoisobutyrates were fed as their racemates however we can deduce from the [^{13}C$^{2}H_3$-*methyl*]-thymine feeding experiment that the putative deaminase will act on the (R) enantiomer of β-aminoisobutyrate, the enantiomer generated from thymine degradation in mammalian systems. An alternative, but less direct pathway would involve the reduction of methylmalonyl semialdehyde to generate β-hydroxyisobutyrate, followed by further processing to isobutyryl-CoA (Scheme 1). The delineation and stereochemical aspects of the pathways between β-aminoisobutyrate and the butyrate unit is currently under investigation in our laboratory.

This study also reinforces methacrylyl-CoA as a *bona fida* intermediate between isobutyryl-CoA and methylmalonyl-CoA. The labelling patterns in the resultant monensin-A are consistent with the exogenously added methacrylate contributing directly to the methacrylyl-CoA pool and being processed in a stereospecific manner to isobutyryl-CoA. [^{13}C-*methyl*]-Methacrylate labelled only C-33 and not C-16 of monensin, therefore it can be deduced that the reduction of the labelled methacrylate gave only (R)-[3-^{13}C]-isobutyryl-CoA, as expected[9,14] and was acted on by the mutase to generate [4-^{13}C]-butyryl-CoA. Also the partitioning ratio of 4:1 in favour of the butyrate over the propionate units was common to both the methacrylate and isobutyrate feeding experiments suggesting that they have intercepted the same pathways.

This study further establishes a link between primary and secondary metabolism and it would appear that methylmalonyl-CoA and possibly methacrylyl-CoA are intermediates common to both the L-valine and thymine

catabolic pathways. Both of these intermediates are involved in pathways delivering carbon atoms for the production of secondary metabolites. A key role for L-valine catabolism in the biosynthesis of the macrolide antibiotics tylosin and spiramycin has been demonstrated[15] by inactivation of the valine dehydrogenase gene in the producing strains. The mutants, which were presumably defficient in their capacity to utilise L-valine, produced six fold and four fold less of the antibiotics respectively. It is relevant to this study that antibiotic production was not completely inhibited and clearly the organisms can obtain biosynthetic intermediates from other catabolic pathways. In a recent study[16] deletion of α-keto acid dehydrogenase activity in a *Streptomyces avermitilis* strain resulted in complete loss of avermectin production, which could be regenerated by supplementation of the medium with methylbutyrate or isobutyrate. A key role for branched chain intermediates from L-valine or L-isoleucine is implied here.

Thus branched chain fatty acid metabolism is an important controlling pathway in the biosynthesis of the polyether and macrolide antibiotics in Streptomyces. Our study does reveal however a novel pathway linking primary and secondary metabolism and is the first study to implicate the DNA bases as a metabolic source for antibiotic production in Streptomyces. It is perhaps noteworthy in this context that the production of methylenomycin[17] from *Streptomyces coelicolor* and candicidin[18] from *Streptomyces griseus* are reported to be maximal during DNA degredation in the idiophase.

Acknowledgements

We thank Drs. JOHN PARKINSON and IAN SADLER of the University of Edinburgh for $^{13}C\{^{1}H,^{2}H\}$ NMR spectra. We also thank, Dr. JOHN A. ROBINSON of the University of Zurich for providing a suitable strain of *S. cinnamonensis*. We acknowledge financial support in the form of an EPSRC CASE studentship (SVR) and a NATO Cooperative grant.

References

1) O'HAGAN, D.: The Polyketide Metabolites. Ellis Horwood Ltd., Chichester, 1991
2) DAY, L. E.; J. W. CHAMBERLIN, E. Z. GORDEE, S. CHEN, M. GORMAN, R. L. HAMILL, T. NESS, R. E. WEEKS & R. STROSHANE: Biosynthesis of monensin. Antimicrob. Agents. Chemother. 4: 410～414, 1973
3) DOTZLAF, J. E.; L. S. METZGER & M. A. FOYLESONG: Incorporation of amino acid derived carbons into tylactone by *Streptomyces fradiae* GS14. Antimicrob. Agents. Chemother. 25: 216～220, 1984
4) ŌMURA, S.; Y. TANAKA, H. MAMADA & R. MASUMA: Bioconversion and biosynthesis of 16-membered macrolide antibiotics XXIX. Effect of ammonium ion, inorganic phosphate and amino acids on the biosynthesis of protylonolide, a precursor of the tylosin aglycone. J. Antibiotics 37: 494～502, 1984
5) ŌMURA, S.; K. TSUZUKI, Y. TANAKA, H. SAKAKIBARA, M. AIZAWA & G. LUKACS: Valine as a precursor of the n-butyrate unit in the biosynthesis of macrolide aglycone. J. Antibiotics 36: 614～616, 1983
6) O'HAGAN, D.; S. V. ROGERS, K. A. REYNOLDS & G. R. DUFFIN: The incorporation of thymine and β-aminoisobutyrate into the polyether antibiotic, monensin-A. J. Chem. Soc., Chem. Commun. 1577～1578, 1994
7) BOHME, H.; R. BROESE & F. EIDEN: Amidomethylierungen CH-acider verbindungen mit diacylimido-chlormethanen. Chem. Ber. 92: 1258～1266, 1959
8) SHIUE, C.-Y.; A. P. WOLF & D. N. SLATKIN: Synthesis of 5-deuteriomethyl-2′-deoxyuridine (thymine-α,α,α,-d_3) and related compounds. J. Labelled Cpds., Radiopharm. 17: 177～184, 1980
9) REYNOLDS, K. A.; D. O'HAGAN, D. GANI & J. A. ROBINSON: Butyrate metabolism in Streptomycetes. Characterisation of an intramolecular vicinal interchange rearrangement linking isobutyrate and butyrate in *Streptomyces cinnamonensis*. J. Chem. Soc., Perkin Trans. I: 3195～3207, 1988
10) GANI, D.; P. B. HITCHCOCK & D. W. YOUNG: Stereochemistry of catabolism of the DNA base thymine and of the anti-cancer drug 5-fluorouracil. J. Chem. Soc., Perkin Trans. I. 1363～1372, 1985
11) GRIFFITH, O. W.: β-Amino acids: Mammalian metabolism and utility as α-amino acid analogues. Ann. Rev. Biochem. 55: 855～878, 1986
12) GARSON, M. J. & J. STAUNTON: Some new NMR methods for tracing the fate of hydrogen in biosynthesis. Chem. Soc. Rev. 8: 539～561, 1979
13) ABERHART, D. J. & T. CHOU-HONG: Stereochemistry of the conversion of methacrylate to β-hydroxyisobutyrate in *Pseudomonas putida*. J. Chem. Soc., Perkin Trans 1. 939～942, 1979
14) GANI, D.; D. O'HAGAN, K. REYNOLDS & J. A. ROBINSON: Biosynthesis of the polyether antibiotic monensin-A: Stereochemical aspects of the incorporation and metabolism of isobutyrate. J. Chem. Soc. Chem. Commun. 1002～1004, 1985
15) TANG, L.; Y. X. ZHANG & C. R. HUTCHINSON: Amino acid catabolism and antibiotic synthesis. Valine is a source of precursors for macrolide biosynthesis in *Streptomyces ambofaciens* and *Streptomyces fradiae*. J. Bacteriology 176: 6107 ～ 6119, 1994
16) DENOYA, C. D.; R. W. FEDECHKO, E. W. HAFNER, H. A. I. MCARTHUR, M. R. MORGENSTERN, D. D. SKINNER, K. STUTZMAN-ENGWALL, R. G. WAX & W. C. WERNAU: A second branched chain α-keto acid dehydrogenase gene cluster (*bkd*FGH) from *Streptomyces avermitilis*. Its relationship to avermectin biosynthesis and the construction of a *bkd*F mutant suitable for the production of novel antiparasitic avermectins. J. Bacteriol. 177: 3504～3511, 1995
17) HOBBS, G.; A. I. C. OBANYE, J. PETTY, J. C. MASON, E. BARRAT, D. C. J. GARDNER, F. FLETT, C. P. SMITH, P. BRODA & S. G. OLIVER: An integrated approach to studying regulation of production of the antibiotic methylenomycin by *Streptomyces coelicolor*. J. Bacteriol. 174: 1487～1494, 1992
18) MARTIN, J. F. & L. E. MCDANIEL: Kinetics of biosynthesis of polyene macrolide antibiotics in batch cultures: Cell maturation time. Biotechnol. Bioeng. 17: 925～938, 1975