BIOSYNTHESIS OF THE AVERMECTINS BY *STREPTOMYCES AVERMITILIS*

INCORPORATION OF LABELED PRECURSORS

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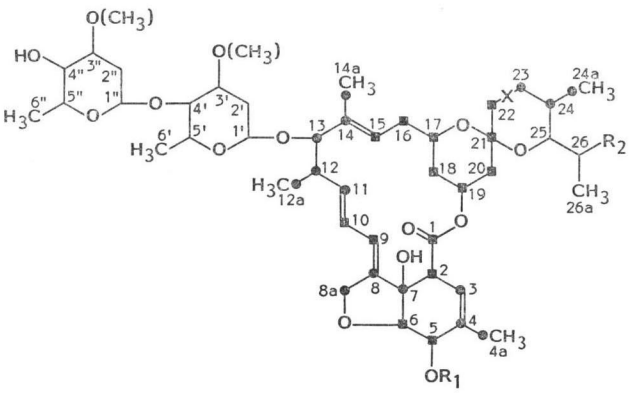
The biosynthesis of the avermectins, a group of 16 membered macrolides with potent anthelmintic and insecticidal activity produced by *Streptomyces avermitilis*, was studied by supplying cultures with ^{14}C and ^{13}C precursors. [1-^{14}C] and [2-^{14}C]acetate and propionate were poor precursors of the avermectins and were instead rapidly oxidized to $^{14}CO_2$. The *S*-methyl of methionine in contrast was incorporated extensively and equally into the three methoxyl groups of the avermectins. The carbon backbone of methionine was not a precursor of the avermectins. Feeding of [1-^{13}C]glucose yielded avermectins labeled specifically in the C1′ and C1″ of the oleandrose moiety and in the aglycone moiety in carbons known to be derived from the methyl of acetate. Feeding [*U*-^{13}C]glucose showed that the entire avermectin molecule is derived from glucose carbons.

The avermectins are oleandrose disaccharide derivatives of 16 membered pentacyclic lactones which are produced by *Streptomyces avermitilis* (Fig. 1)[1~5]. Studies employing ^{13}C-labeled acetate and propionate have shown that the aglycone moiety of the avermectins is derived from a head to tail condensation of seven acetates and five propionates[6]. The *sec*-butyl (Fig. 1, $R_2=CH_2CH_3$) or *iso*-

Fig. 1. General structure of the avermectins.

Precursors are indicated as follows: Methyl of methionine; (CH_3), acetate; ■—■, propionate; CH_3●—●—●.

Avermectin terminology is as follows: $R_1=H$; "B" components, $R_1=(CH_3)$; "A" components, X=CH=CH; "1" components, $X=CH_2CHOH$; "2" components, $R_2=CH_2CH_3$; "a" components, $R_2=CH_3$; "b" components.



propyl (Fig. 1, $R_2=CH_3$) side chains (C26~C28) were not labeled by either precursor and were instead derived respectively from isoleucine and valine (G. ALBERS-SCHÖNBERG *et al.*, unpublished observations). Use of [1-^{13}C, 1-^{18}O]propionate and [1-^{13}C, 1-^{18}O]acetate demonstrated that the oxygens at C1, C5, C7, C13, C17, C18 and C23 (for B_2a and A_2a compounds) retained their isotope content. These findings established that the avermectin "1" components which contain a double bond at C22~C23 (B_1a and A_1a) arose from the "2" components by dehydration of the hydroxyl at C23. The retention of ^{18}O at C13 further established that the avermectins are not derived from a milbemycin type intermediate *via* oxidation. Preliminary studies (G. ALBERS-SCHÖNBERG *et al.*, unpublished observations) have also revealed that the methoxy substituents at C5 of the macrolide moiety and C3′ and C3″ of the oleandrose disaccharide are derived from the methyl of methionine.

Although these studies have determined the origin of all the carbons of the avermectin aglycone and methoxyl groups of the disaccharide, they have not provided any information about the origin of the oleandrose backbone, the biosynthetic pathway of the avermectin aglycone and the metabolic origin of the avermectin precursors. To answer these questions, we have determined the metabolic fate and incorporation into avermectin of specifically labeled acetate and propionate. Using both ^{14}C and ^{13}C-labeled methionine we have shown that the *S*-methyl group and not the carbon backbone is incorporated into avermectin. The methyl is incorporated equally in the methoxyl groups at the C3′ and C3″ of the oleandrose moiety and the C5 of the aglycone. In addition we report studies utilizing glucose labeled specifically with ^{14}C or ^{13}C which showed that the oleandrose moieties are derived directly from glucose and that the entire avermectin molecule is derived from glucose carbons. The probable metabolic pathways responsible for these observations are discussed.

Materials and Methods

Materials

[1-^{14}C]Acetate, [2-^{14}C]acetate, [1-^{14}C]propionate, [2-^{14}C]propionate, [1-^{14}C]glucose, [2-^{14}C]glucose and [6-^{14}C]glucose were from New England Nuclear; L-[*methyl*-^{14}C]methionine and L-[2-^{14}C]methionine were from Amersham. L-[*methyl*-^{13}C]Methionine, [1-^{13}C]glucose and [*U*-^{13}C]glucose were from MSD isotopes.

Culture and Incubation

S. avermitilis were grown in a modified medium B as described by BURG *et al.*[2]. For incubation with [^{14}C]acetate and [^{14}C]propionate, 0.25 ml of the radio-labeled substrate containing approximately 50 μmol (specific acitivity $\sim 1.3\times10^5$ dpm/μmol) were added to flasks in replicates of four at 24-hour intervals from 48 to 168 hours. The incubations were terminated 24 hours after each addition. The cells were harvested by centrifugation and washed with cold distilled water. The ^{14}C content was determined in samples of the whole broth, the supernatant solution and the cell paste. The quantity of avermectin in the whole broth was determined before and after addition of radioisotope. Avermectin components were isolated from the cell paste and the ^{14}C content determined. The amount of ^{14}C released as CO_2 was measured in one of the 4 replicate flasks at each time interval by passing CO_2-free sterile air through the flask and into 1 N CO_2-free NaOH. Samples of the NaOH were counted to determine the trapped $^{14}CO_2$. Additional samples of the NaOH were acidified, thoroughly flushed with air and then counted to insure that all radioactivity was due to $^{14}CO_2$. Incubations with [^{14}C]-methionine utilized the procedures described above except for the concentration of radioactive precursors. Additions of 0.02 ml of a sterile solution containing 1 μCi (specific activity 56 μCi/μmol) were made. [*methyl*-^{13}C]Methionine (5 μmol containing 90% ^{13}C in 0.1 μl) was added at 72 hours and the cells were harvested at 168 hours. The avermectins were isolated as described below.

Incubations with isotopically labeled glucoses were conducted in 2.0 ml fermentations in 25 ml

flasks. Inoculum preparation and medium composition and preparation were as described above. The sterile production medium was aseptically transferred to small flasks prior to inoculation. [^{14}C]-Glucose (5 μCi in 0.1 ml, specific activity 56 μCi/μmol) was added at 72 hours and the procedures described above for acetate and propionate were followed. For incubations with [^{13}C]glucose, the amount of glucose remaining in the medium at 120 hours was determined and an equal amount of [^{13}C]glucose was then added. Cells were harvested at 192 hours and the [^{13}C]avermectins were isolated and analyzed *via* NMR spectroscopy.

Isolation of Avermectins

Avermectins were extracted from the cell paste by vigorous shaking with methanol (~1 ml/ml packed cells) for 15 minutes on an Eberbach reciprocal shaker and the solids were removed by centrifugation. For determination of ^{14}C in avermectin, aliquots (0.02~0.05 ml) of the methanol were applied to Silica Gel 60 F_{254} precoated TLC plates (E. M. Laboratories 20×20 cm, 0.25 mm thickness). The plates were developed in methylene chloride - ethyl acetate - methanol (9: 9: 1) for 1~1.5 hours. The plates were viewed under a UV lamp to locate the avermectins and their ^{14}C content was determined with a Bioscan System 200 coupled to a Hewlet Packard 85B computer (Bioscan Inc.). For NMR analysis of [^{13}C]avermectins, the methanol was evaporated to 1 ml and then streaked onto silica gel TLC plates. The TLC plates were developed three times (1~1.5 hours each) in hexane - 2-propanol (85: 15). The UV absorbing bands were scraped from plate, extracted with methanol and purified by HPLC on a Dupont Zorbax preparative column (C-18, 21.2 mm×25 cm) using methanol - water (85: 15) as the mobile phase[4]. The avermectins were eluted from the column with a purity greater than 90%.

Deglycosylation of Avermectins

Avermectins B_2a and A_2a were deglycosylated as described by MROZIK *et al.*[7]. The dried methylene chloride extract was dissolved in methanol, streaked onto silica gel TLC plates and developed in methylene chloride - ethyl acetate (85: 15) for 1~1.5 hours. The UV absorbing bands were scraped from the plate, extracted with methanol and purified by HPLC on a Dupont Zorbax C-18 preparative column using methanol - water (82: 18) as the mobile phase. The avermectin aglycones eluted from the column with a purity greater than 95%.

Analysis and Determination of Avermectins

Aliquots of the fermentation broth were brought to 80% saturation with methanol, and shaken vigorously for 15 minutes on an Eberbach reciprocal shaker. The solids were removed by centrifugation and avermectin in the supernatant was determined by HPLC on a Dupont Zorbax C-18 column (4.6 mm×25 cm) at 60°C with methanol - water (85: 15) as a mobile phase[4]. The avermectins were quantitated by monitoring absorbance at 247 nm.

Other Methods

Glucose was determined colorimetrically with glucose oxidase (Sigma Chemical Co.)[8]. Radioactivity was measured in a Beckman 8100 Liquid Scintillation Spectrometer. All samples were corrected for quenching using channels ratio and an external standard. Acetate and propionate were determined by gas chromatography[9] using a Varian Aerograph Gas Chromatograph (Model 2800, Varian Instruments Inc.).

^{13}C NMR spectra were recorded in $CDCl_3$ at room temperature (~25°C) with a Varian XL-400 instrument (Varian Instrument, Inc.). ^{13}C-Enhancements of avermectins B_1a and B_2a derived from [*U*-^{13}C]glucose were determined by comparing the area of the satellite peaks to the sum of the central (unenriched) and satellite (enriched) peaks. ^{13}C-Enhancements of the methoxyl carbons were determined by comparison of the signal intensity of the CH_3O peaks in avermectins derived from 90% [*methyl*-^{13}C]methionine with those present in unlabeled avermectins.

Results

Incorporation of Specifically Labeled Acetate and Propionate into Avermectins

The incorporation of 1-^{14}C and 2-^{14}C-labeled acetate and propionate into the avermectins, cellular

Table 1. [^{14}C]Acetate and propionate feeding of *S. avermitilis*.

Precursor	^{14}C into avermectin			^{14}C in CO_2 (%)	^{14}C in cell constituents[a] (%)
	Incorporation (%)	Relative molar specific activity	Theoretical (%)		
[1-^{14}C]Acetate[b]	3.1	0.43	6.2	80~85	10~15
[1-^{14}C]Acetate[b]	3.8	0.39	5.6	65~75	20~30
[1-^{14}C]Propionate[c]	11.0	0.76	15.2	80~85	5~10
[2-^{14}C]Propionate[c]	5.5	0.37	7.2	68~75	20~30

[a] Radioactivity in the washed cells minus that present in avermectins.
[b] Specific activity varied with the amount of endogenous acetates present and was determined in each flask immediately after the addition of [^{14}C]acetate. The observed ranges were from 62,000 to 85,000 dpm/μmol. The endogenous acetate concentration decreased throughout the fermentation.
[c] Specific activity varied with the amount of endogenous propionate present and was determined in each flask immediately after the addition of [^{14}C]propionate. The observed range was 50,000 to 80,000 dpm/μmol. Endogenous propionate concentration decreased throughout the fermentation.

Table 2. [^{14}C]Methionine incorporation into avermectins.

Precursor	^{14}C in avermectins				^{14}C in cell constituents (%)
	Incorporation (%)	Specific activity of A components (dpm/μmol)	Specific activity of B components (dpm/μmol)	Ratio A/B	
[*methyl*-^{14}C]Methionine	60~63	179,155	107,521	1.67	23~27
[2-^{14}C]Methionine	0	0	0	0	16~20

Table 3. [*methyl*-^{14}C]Methionine incorporation in avermectin aglycones.

Avermectin component	Specific activity[a] (dpm/μmol)	Original specific activity (%)
A_1a	30,075	100
Deglycosylated A_1a	11,910	40
A_2a	45,409	100
Deglycosylated A_2a	17,787	39

[a] Determined following isolation of the pure component.

constituents and CO_2 during 24-hour intervals was measured from 48 to 168 hours. Similar results were obtained for each interval and the average values of all intervals are presented in Table 1. It is apparent from Table 1 that although acetate and propionate were incorporated into the avermectins, they were not good precursors of these compounds. Less than 4% of the added acetate was incorporated into the avermectins and the relative molar specific activity of acetate was only 6% of the theoretical value of 7. The incorporation of propionate was better than that of acetate but the relative molar specific activity was also only a fraction of the expected value of 5. The majority of ^{14}C in all these experiments was liberated as CO_2. This indicated that these compounds entered the cell and were actively metabolized.

Incorporation of Labeled Methionine into Avermectins

The incorporation of [*methyl*-^{14}C] and [2-^{14}C]methionine into the avermectins was studied over 24-hour intervals from 48 to 168 hours. Table 2 summarizes the results obtained. The methyl of methionine was incorporated extensively while the carbon backbone was not incorporated. The ratio

Table 4. Incorporation of [*methyl*-^{13}C]methionine into avermectins.

Avermectin component	Isotopic enrichment[a]		
	C3′ methoxyl	C3″ methoxyl	C5 methoxyl
$A_{2}a$	5.5	6.5	4.2
$A_{1}a$	4.3	4.2	6.2
$B_{1}a$	2.9	2.5	—

[a] The assignment of the C3′ and C3″ methoxyl carbons are not unequivocal. The C3′ methoxyl carbon is arbitrarily chosen as the high field resonance.

Table 5. Incorporation of [^{14}C]glucose into the avermectins.

Precursor	^{14}C in avermectins	
	Relative molar specific activity total avermectins[b]	Deglycosylated avermectin $A_{2}a$ % of disaccharide
[1-^{14}C]Glucose[a]	4.6～5.4[b]	53[c]
[2-^{14}C]Glucose[a]	3.8～5.0[b]	55[c]
[6-^{14}C]Glucose[a]	4.3～5.2[b]	60[c]

[a] Specific activity was dependent upon glucose concentration in the flask at the time of addition and was therefore determined for each individual flask.

[b] Range observed in 24 flasks; replicates at 72～90, 90～120, 120～144 and 144～168.

[c] $A_{2}a$ of the 6 replicates at each interval were pooled and deglycosylated. Results are the mean of all intervals.

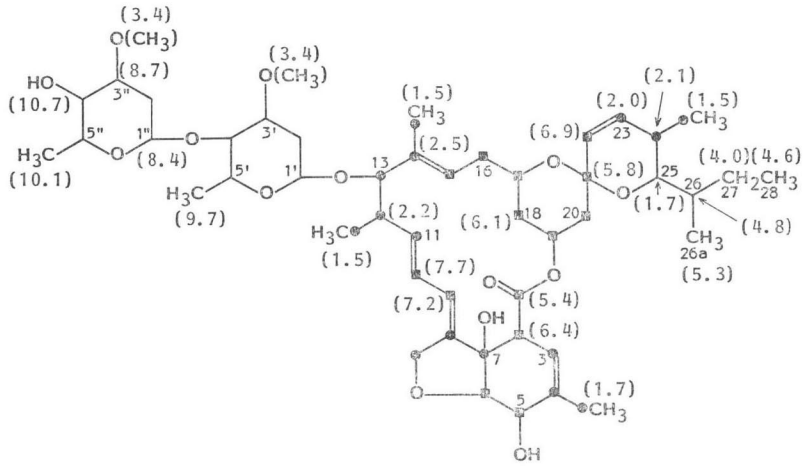
of the specific activity of the avermectin A components which possess three methoxyl groups to that of the B components which possess only two methoxyl group was close to the theoretical value of 1.5 suggesting that all three of these methoxyl groups are derived from a common pool of methionine. The avermectin A components were deglycosylated[7] and the ^{14}C in the isolated aglycones were determined (Table 3). The observed specific activities of the aglycones were close to the theoretical value of 33%.

The incorporation of the methyl of [*methyl*-^{13}C]methionine into avermectin was investigated between 72 and 168 hours and the results are shown in Table 4. Isotopic enrichment was observed at the C3′, C3″ and C5 methoxyl groups of the A components and at C3′ and C3″ methoxyl groups of the B components.

Incorporation of Labeled Glucose into Avermectins

Experiments with [^{14}C]glucose specifically labeled in the 1, 2 or 6 positions were conducted as described in Materials and Methods and the results obtained are summarized in Table 5. All were incorporated into the avermectins equally. Deglycosylation of the avermectins showed that glucose carbons were incorporated into the aglycone as well as the oleandrose moiety of the avermectins. The aglycones contained 50～60% of the ^{14}C in the avermectin. Incubations were conducted with [1-^{13}C]-glucose and [*U*-^{13}C]glucose to determine both the site of incorporation of isotope and the amount incorporated. With [1-^{13}C]glucose, C1′ and C1″ were enriched 3.05 and 3.2-fold respectively while carbons 2, 6, 10, 16, 18, 20 and 22 which are derived from the methyl of acetate were enriched 1.2 to 1.7-fold. The remainder of the avermectin molecule was not labeled. The results obtained with [*U*-^{13}C]glucose are presented in Fig. 2. Enrichments could not be assigned to every carbon because of extensive splitting and overlap. It was, however, apparent that the entire avermectin molecule was formed from glucose carbons. The carbons of the oleandrose backbone contained the highest amounts of ^{13}C; those carbons known to be derived from acetate and the *iso*-butyl side chain (C26～C28) were also highly

Fig. 2. Incorporation of ^{13}C from [*U*-^{13}C]glucose into avermectin B_1a precursors are indicated as follows: Methyl of methionine; (CH_3), acetate; ■—■, propionate; CH_3●—●—●.
Numbers in parenthesis indicate the fold ^{13}C enrichment over background.



enriched; and those carbons known to be derived from propionate contained the lowest levels of isotope. The methoxyl of oleandrose which is derived from the methyl of methionine also contained significant amounts of ^{13}C.

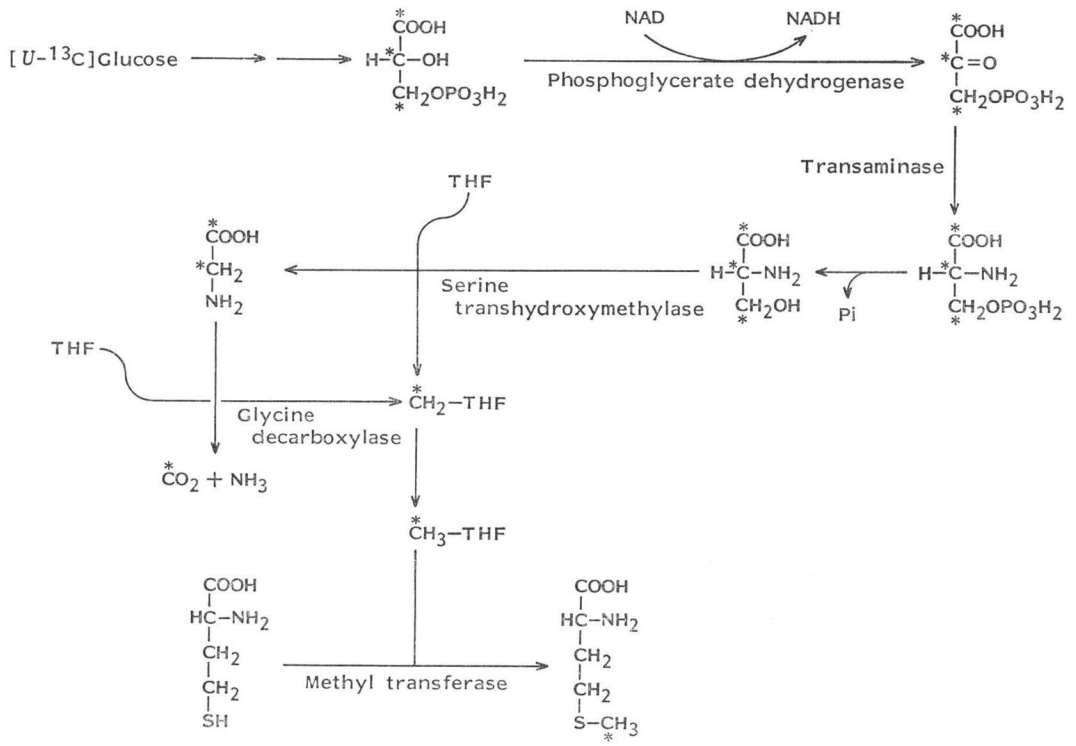
Discussion

The avermectins have been shown to contain seven acetate and five propionate moieties[6]. The evidence presented in the paper shows that acetate and propionate *per se* were poor precursors of the avermectins. The observed high level of ^{14}C liberated as CO_2 demonstrated that the poor incorporation of acetate and propionate into avermectin was not due to low permeability of these acids, to an inability of the cells to metabolize these compounds or to the presence of a large internal unlabeled pools of these compounds since these phenomena would also have limited formation of $^{14}CO_2$. The oxidation of acetate and propionate to CO_2 is assumed to have proceeded *via* activation to their respective CoA esters and subsequent oxidation *via* the tricarboxylic acid cycle. We have demonstrated the presence of acetate kinase and phosphotransacetylase in cell-free extracts of *S. avermitilis* and are investigating the enzymes responsible for propionate activation. Since these same CoA esters are also presumed to be the biosynthetic intermediates which condense to form avermectin, their disproportion in favor of CO_2 must result from either separate metabolic pools of the CoA esters or a vey high rate of oxidation compared to avermectin biosynthesis. The latter appears most probable given the high O_2 requirement reported for fermentations producing secondary metabolites[10~12]. The factors regulating the flow of intermediates between avermectin synthesis and energy metabolism await investigation.

[*methyl*-^{14}C]Methionine and [*methyl*-^{13}C]methionine in contrast to acetate and propionate were extensively incorporated into avermectin. Isotope was incorporated equally and exclusively into all three methoxyl groups. The results suggested that these methoxyl groups might arise *via* an *S*-adenosylmethionine (SAM) dependent methyltransferase as has been found with tylosin[13] and erythromycin[14]. We have consequently searched for this type of enzyme and have discovered an avermectin B-*O*-methyltransferase which transfers the S-CH_3 of SAM to C-hydroxyl of the avermectin[15].

The results obtained with labeled glucose were quite interesting. The incorporations of [1-^{14}C], [2-^{14}C] and [6-^{14}C]glucose into both the avermectin disaccharide and the avermectin aglycone were equal. Equal incorporation of isotope into the aglycone which is derived from isoleucine, acetate and propionate suggested that glucose was cleaved symmetrically (*via* the Embden-Myerhoff pathway) to yield the necessary biosynthetic units. The results with [1-^{13}C]glucose confirmed this hypothesis. [1-^{13}C]Glucose yielded avermectin enriched in those carbons of the macrolide moiety derived from the

Fig. 3. Proposed pathway for formation of [*methyl*-^{13}C]methionine from glucose.
* ^{13}C.

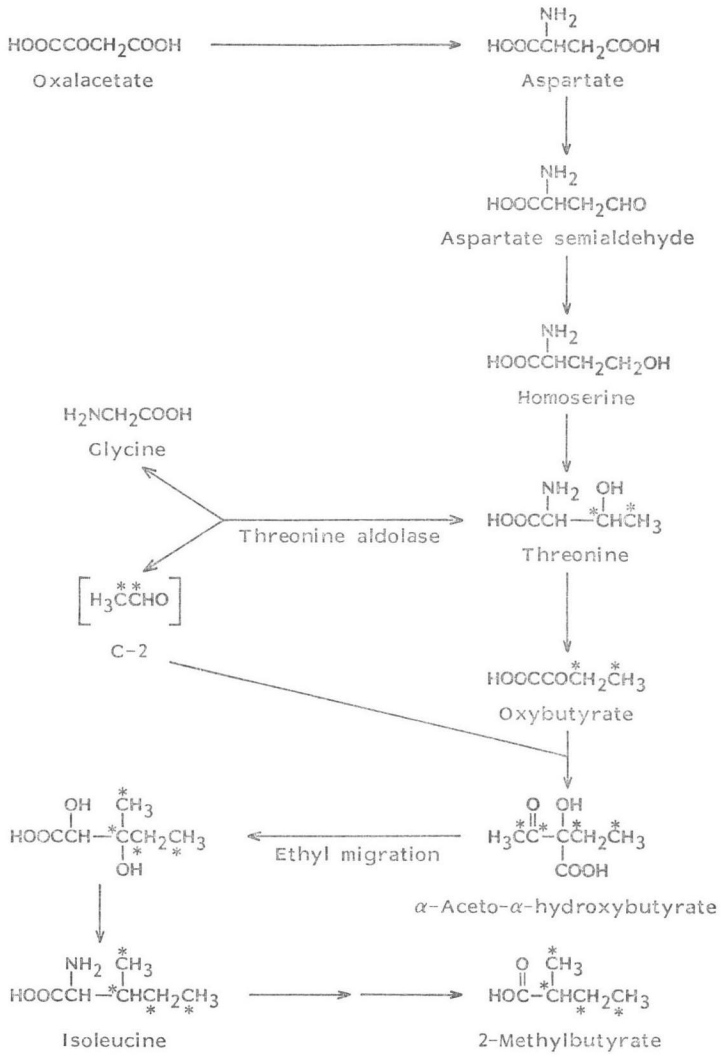


methyl of acetate, as expected from an Embden-Myerhoff cleavage. Surprisingly, there was no detectable enrichment of the carbons derived from the methyl and methylene of propionate which should have arisen from acetate *via* the tricarboxylic acid cycle. Since the ^{13}C incorporation in this experiment was low, we thought that the absence of detectable isotope in the propionate carbons might have been due to dilution and therefore did an experiment with [*U*-^{13}C]glucose in order to use carbon-carbon splitting to verify the presence or absence of ^{13}C at specific sites. The results with [*U*-^{13}C]glucose (Fig. 2) clearly showed that carbons derived from both acetate and propionate were enriched with ^{13}C. The carbons of propionate origin had approximately 3.3-fold lower enrichment than those of acetate origin. This is compatible with the hypothesis that glucose was cleaved symmetrically *via* the Embden-Myerhoff pathway to yield acetate which could either be directly incorporated into avermectin or could enter the tricarboxylic acid cycle and subsequently give rise to propionate (*via* succinate) which could then be incorporated into avermectin. Passage through the tricarboxylic acid cycle could account for the observed isotope dilution.

The observations that the oleandrose moieties were only enriched in C1′ and C1″ by [1-^{13}C]glucose and contained the highest level of isotope in both [^{13}C]glucose feedings indicate that the carbon skeletons of these sugars were derived directly from glucose. The enzymatic steps for formation of 2,6-deoxyhexoses *via* sugar nucleotide intermediates have been described[16]. The methoxyls of the oleandrose moieties (C3′ and C3″) were also enriched by [*U*-^{13}C]glucose. These are derived from the methyl of methionine. Methyl-labeled methionine can be synthesized from glucose *via* a well known microbial pathway which involves phosphoglycerate, serine and serine transhydroxymethylase[17,18] (Fig. 3).

The asymmetric labeling of the 2-methylbutyrate derived side chain (C25 ~ C28) by [*U*-^{13}C]glucose merits discussion because all but the carboxyl carbon (C25) had 4 ~ 6-fold enrichment (similar to acetate derived carbons) while the carboxyl carbon had a 1.7-fold enrichment (similar to propionate derived carbons). The labeling pattern can be explained by the biosynthetic pathway shown in Fig. 4[17,18]. Glu-

Fig. 4. Proposed pathway for formation of [^{13}C]-2-methylbutyrate.
* Acetate carbons. → Enzymatic reaction.



cose is metabolized to acetate which enters the tricarboxylic acid cycle and gives rise to oxalacetate with a ^{13}C enrichment similar to propionate carbons. The oxalacetate is then converted to threonine which in the presence of threonine aldolase[17,18] becomes labeled at the acetate level in carbons 3 and 4. Subsequent conversion of threonine to isoleucine followed by oxidative deamination would yield 2-methylbutyrate containing ^{13}C enrichments equivalent to acetate in all carbons but that of the carboxyl which would have an enrichment equivalent to that of propionate carbons.

References

1) Albers-Schönberg, G.; B. H. Arison, J. C. Chabala, A. W. Douglas, P. Eskola, M. H. Fisher, A. Lusi, H. Mrozik, J. L. Smith & R. L. Tolman: Avermectins. Structure determination. J. Am. Chem. Soc. 103: 4216~4221, 1981

2) Burg, R. W.; B. M. Miller, E. E. Baker, J. Birnbaum, S. A. Currie, R. Hartman, Y.-L. Kong, R. L. Monaghan, G. Olson, I. Putter, J. B. Tunac, H. Wallick, E. O. Stapley, R. Ōiwa & S. Ōmura: Aver-

mectins, new family of potent anthelmintic agents: Producing organism and fermentation. Antimicrob. Agents Chemother. 15: 361～367, 1979

3) EGERTON, J. R.; D. A. OSTLIND, L. S. BLAIR, C. H. EARY, D. SUHAYDA, S. CIFELLI, R. F. RIEK & W. C. CAMPBELL: Avermectins, new family of potent anthelmintic agents: Efficacy of the B_{1a} component. Antimicrob. Agents Chemother. 15: 372～378, 1979
4) MILLER, T. W.; L. CHAIET, D. J. COLE, L. J. COLE, J. E. FLOR, R. T. GOEGELMAN, V. P. GULLO, H. JOSHUA, A. J. KEMPF, W. R. KRELLWITZ, R. L. MONAGHAN, R. E. ORMOND, K. E. WILSON, G. ALBERS-SCHÖNBERG & I. PUTTER: Avermectins, new family of potent anthelmintic agents: Isolation and chromatographic properties. Antimicrob. Agents Chemother. 15: 368～371, 1979
5) SPRINGER, J. P.; B. H. ARISON, J. M. HIRSHFIELD & K. HOOGSTEEN: The absolute stereochemistry and conformation of avermectin B_{2a} aglycon and avermectin B_{1a}. J. Am. Chem. Soc. 103: 4221～4224, 1981
6) CANE, D. E.; T.-C. LIANG, L. KAPLAN, M. K. NALLIN, M. D. SCHULMAN, O. D. HENSENS, A. W. DOUGLAS & G. ALBERS-SCHÖNBERG: Biosynthetic origin of the carbon skeleton and oxygen atoms of the avermectins. J. Am. Chem. Soc. 105: 4110～4112, 1983
7) MROZIK, H.; P. ESKOLA, B. H. ARISON, G. ALBERS-SCHÖNBERG & M. H. FISHER: Avermecin aglycones. J. Org. Chem. 47: 489～492, 1982
8) SOLOMON, L. L. & J. E. JOHNSON: Enzymatic microdetermination of glucose in blood and urine. Anal. Chem. 31: 453～456, 1959
9) SCHULMAN, M. D. & D. VALENTINO: Studies on the mechanism of action of 4-amino-6-trichloroethenyl-1,3-benzenedisulfonamide against *F. hepatica*: effect on glycolysis *in vitro*. Exp. Parasitol. 49: 206～215, 1980
10) GRAY, P. P. & S. BHUWAPATHANAPAN: Production of the macrolide antibiotic tylosin in batch and chemostat cultures. Biotechnol. Bioeng. 22: 1785～1804, 1980
11) NASH, C. H., III: Effect of carbon dioxide on synthesis of erythromycin. Antimicrob. Agents Chemother. 5: 544～545, 1974
12) PACA, J.; P. EHLER & V. GREGR: Oxygen transfer rate in media used for erythromycin biosynthesis. J. Ferment. Technol. 56: 144～151, 1978
13) SENO, E. & R. H. BALTZ: Properties of *S*-adenosyl-L-methionine: Macrocin *O*-methyltransferase in extracts of *Streptomyces fradiae* strains which produce normal or elevated levels of tylosin and in mutants blocked in specific *O*-methylations. Antimicrob. Agents Chemother. 20: 370～377, 1981
14) CORCORAN, J. W.: *S*-Adenosylmethionine: Erythromycin C *O*-methyltransferase. Methods Enzymol. 43: 487～498, 1975
15) SCHULMAN, M. D.; D. VALENTINO & C. RUBY: Avermectin B *O*-methyltransferase of *Streptomyces avermitilis*. Fed. Proc. 44: 931, 1985
16) GLASER, L. & H. ZARKOWSKY: Dehydration in nucleotide-linked deoxysugar synthesis. *In* The Enzymes. *Ed.*, P. D. BOYER, Vol. 3, pp. 465～480, Academic Press, New York, 1971
17) FLAVIN, M.: Methionine biosynthesis. *In* Metabolic Pathways. *Ed.*, D. M. GREENBERG, Vol. 5, pp. 457～503, Academic Press, New York, 1975
18) UMBARGER, H. E.: Amino acid biosynthesis and its regulation. Ann. Rev. Biochem. 47: 533～606, 1978