MILBEMYCINS, A NEW FAMILY OF MACROLIDE ANTIBIOTICS

STUDIES ON THE BIOSYNTHESIS OF MILBEMYCINS α_2, α_4 AND D USING ^{13}C LABELED PRECURSORS

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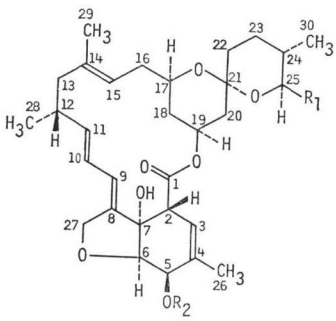
The biosynthetic origins of the carbon skeleton of milbemycins α_2, α_4 and D were studied. ^{13}C Labeled antibiotics, milbemycins α_2, α_4 and D, were isolated from the culture broth of *Streptomyces hygroscopicus* subsp. *aureolacrimosus* strain Au-3 after feeding [1-^{13}C]acetate, [1-^{13}C]-propionate, [3-^{13}C]propionate, [1-^{13}C]isobutyrate, DL-[2-^{13}C]valine and L-[*methyl*-^{13}C]methionine, and ^{13}C NMR spectra of the antibiotics thus obtained were measured. It was revealed that the carbon skeleton, except for carbon 25, of milbemycins α_2, α_4 and D are derived from seven acetate units and five propionate units. It was also shown that the methyl, ethyl and isopropyl groups at carbon 25 in milbemycins α_2, α_4 and D are derived from acetate, propionate and isobutyrate or DL-valine, respectively, and the methyl carbon of the methoxy group at carbon 5 in milbemycins α_2 and α_4 was enriched by L-[*methyl*-^{13}C]methionine.

Streptomyces hygroscopicus subsp. *aureolacrimosus* produced 20 milbemycins[1,2] with anthelmintic and insecticidal activities; all the milbemycins having a tetra- or pentacyclic ring containing the 16-membered macrocyclic lactone, the 6,6-membered spiroketal ring and the cyclohexene with/without the tetrahydrofuran ring[3,4,5]. Milbemycins α_2, α_4 and D, which were major metabolites of the strain Au-3, were chosen for the study on the biosynthetic origins of their carbon skeletons (Fig. 1). Assignments of ^{13}C NMR spectra were based on general chemical shift considerations, patterns observed in proton coupling and off-resonance proton decoupling, and selective proton noise decoupling experiments.

This paper reports on the biosynthetic origins of the carbon skeleton of milbemycins α_2, α_4 and D, determined from ^{13}C NMR data of these compounds obtained by feeding experiments using ^{13}C labeled precursors.

Fig. 1. Structure of milbemycins α_2, α_4 and D.



α_2 $R_1=\overset{31}{C}H_3$ $R_2=CH_3$

α_4 $R_1=\overset{31}{C}H_2\overset{32}{C}H_3$ $R_2=CH_3$

D $R_1=\overset{31}{C}H\langle\overset{32}{C}H_3\atop\overset{33}{C}H_3$ $R_2=H$

Materials and Methods

Culture

S. hygroscopicus subsp. *aureolacrimosus* strain Au-3, grown on a yeast extract - malt extract agar

(ISP medium 2) at 28°C for 12 days, was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of the following medium: 1% sucrose, 0.35% Polypepton and 0.05% K_2HPO_4, and cultured at 28°C for 48 hours on a rotary shaker (210 rpm, 7 cm). One milliliter of the seed culture was then transferred into a production medium (4% glucose, 1% soybean meal, 1% skim milk, 0.3% NaCl, 0.2% corn-steep liquor and 0.05% $CaCO_3$). The pH of this medium was adjusted to 7.2 before sterilization at 120°C for 20 minutes in an autoclave. Fermentation was performed in 100-ml Erlenmeyer flasks containing 20 ml of the production medium at 28°C on a rotary shaker (210 rpm, 7 cm). After 72 hours cultivation, an appropriate ^{13}C labeled precursor was added to the culture, and the cultivation was continued for an additional 48 hours. The concentrations of these precursors added were as follows: [1-^{13}C]acetate, [1-^{13}C]propionate and [3-^{13}C]propionate, 0.1% (w/v); [1-^{13}C]isobutyrate and DL-[2-^{13}C]valine, 0.01% (w/v); L-[*methyl*-^{13}C]methionine, 0.005% (w/v).

Chemicals

Sodium [1-^{13}C]acetate, sodium [1-^{13}C]propionate, sodium [3-^{13}C]propionate, sodium [1-^{13}C]isobutyrate, DL-[2-^{13}C]valine and L-[*methyl*-^{13}C]methionine were purchased from Merck Sharp and Dohme Co., Ltd. All precursors were approximately 90 atom % of ^{13}C at the labeling sites.

Isolation of ^{13}C Labeled Milbemycins α_2, α_4 and D

Each fermentation broth (500 ml) was adjusted to pH 3.0 by 3 N H_2SO_4 and filtered with the aid of Celite. The cake was extracted with 500 ml of methanol and to the extract 500 ml of water was added. The resulting aqueous methanol solution was extracted twice with 500 ml of *n*-hexane. The *n*-hexane was evaporated under reduced pressure. The residue was chromatographed on a column of silica gel (13 g, Merck, Art. 7734). The column was eluted with a mixture of *n*-hexane and acetone (95: 5) to give fraction I (first 70 ml) and II (second 100 ml).

Fraction I, containing milbemycins α_2 and α_4, was evaporated under reduced pressure to give an oily residue. The residue was applied to a Lobar column RP-8 (Merck), which was eluted with acetonitrile - water (75: 25). The fractions were monitored by TLC and the respective fractions containing milbemycins α_2 and α_4 were collected separately. From each 500 ml of the culture broth supplemented with each ^{13}C labeled precursor, 10~50 mg of purified ^{13}C labeled milbemycins α_2 and α_4 were obtained.

Fraction II, containing milbemycins D, E and β_1, gave an oily residue on evaporation of the solvent under reduced pressure. The residue was dissolved in 1 ml of methanol, and was applied to a Lobar column RP-8, which was eluted with methanol - water (85: 15) to give fractions containing milbemycin β_1 and a mixture of milbemycins D and E. Fractions containing milbemycins D and E gave an oily residue on evaporation of the solvent under reduced pressure. The residue thus obtained was applied to a Lobar column Si 60 (Merck), which was eluted with *n*-hexane - ethyl acetate (7: 3) to give fractions containing milbemycin D. From each 500 ml of the culture broth supplemented with each ^{13}C labeled precursor, 20~50 mg of purified ^{13}C labeled milbemycin D was obtained.

NMR Spectroscopy

^{13}C NMR spectra were measured on a Jeol-JNM-FX 400 Spectrometer at 100.7 MHz. Milbemycins α_2, α_4 and D were dissolved in chloroform-*d* contained in a tube of 5 mm external diameter. Tetramethylsilane was used as an internal reference; the spectra width was 20 kHz; 16 K data points were recorded giving maximum spectra accuracy of 2.5 Hz.

Results and Discussion

Assignment of the Signals

Off-resonance proton decoupling and selective proton noise decoupling techniques were exploited for the assignments of methine and methyl signals in milbemycins α_2, α_4 and D. The signals due to carbons 4, 8 and 14 were assigned by a long range selective proton decoupling technique. The chemical shifts of each carbon atom of milbemycins α_2, α_4 and D are shown in Table 1.

Table 1. ^{13}C NMR chemical shifts.

	α_2	α_4	D		α_2	α_4	D
C- 1	173.9	173.8	173.6	18	36.6	36.7	36.7
2	45.7	45.7	45.8	19	68.7	68.7	68.7
3	118.5	118.5	118.2	20	41.1	41.3	41.4
4	137.0	137.0	137.8	21	97.6	97.4	97.5
5	77.0	77.0	67.8	22	35.7	35.7	35.8
6	77.5	77.5	79.3	23	27.7	27.9	28.1
7	80.4	80.4	80.3	24	29.3	34.2	31.6
8	139.8	139.8	139.6	25	71.3	76.0	78.4
9	119.5	119.5	120.4	26	19.9	19.9	19.9
10	123.5	123.5	123.5	27	68.3	68.3	68.5
11	142.4	142.5	142.8	28	22.3	22.3	22.3
12	35.9	35.9	36.0	29	15.5	15.5	15.5
13	48.6	48.6	48.6	30	17.9	17.8	17.4
14	135.9	135.9	136.9	31	19.4	25.7	28.4
15	120.9	120.9	121.0	32		10.1	14.2
16	34.7	34.7	34.7	33			21.0
17	67.5	67.5	67.4	5-OCH_3	57.8	57.8	

Incorporation of ^{13}C Labeled Precursors into Milbemycin α_2

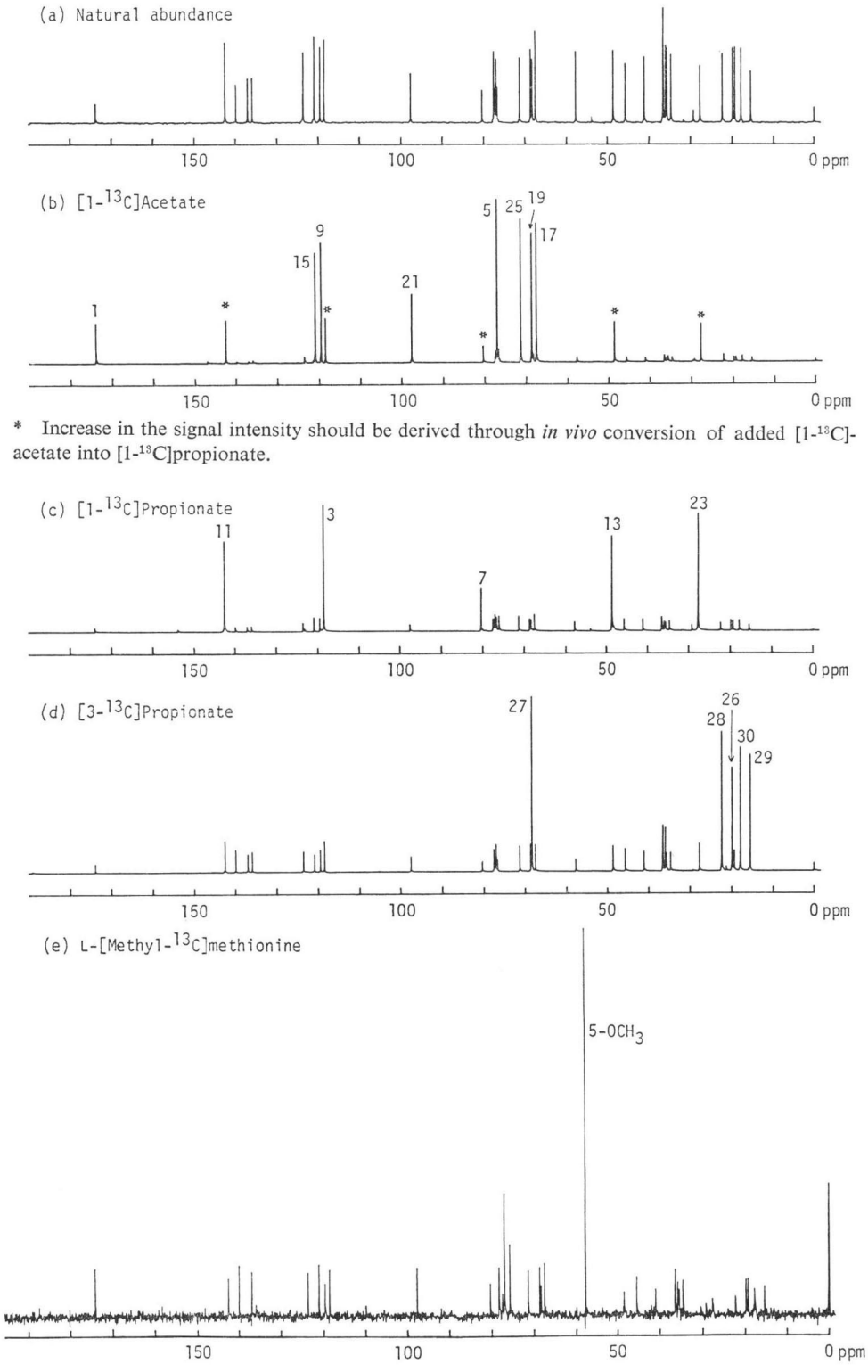
The ^{13}C NMR spectra of labeled milbemycin α_2 obtained by feeding ^{13}C labeled precursors were measured. The natural abundance of the ^{13}C NMR spectrum of milbemycin α_2 is shown in Fig. 2-a. In the sample derived from the experiments using [1-^{13}C]acetate, ^{13}C enrichment at the carbons 1, 5, 9, 15, 17, 19, 21 and 25 were observed and carbons 3, 7, 11, 13 and 23 which should biogenetically be derived from C-1 of propionate were also weakly enriched (Fig. 2-b). Peak heights on spectra of the propionate-labeled samples show that: carbons 3, 7, 11, 13 and 23 were derived from C-1 of the propionate (Fig. 2-c), and carbons 26, 27, 28, 29 and 30 were derived from C-3 of the propionate (Fig. 2-d). The results described above suggest that eight acetate units and five propionate units were incorporated into milbemycin α_2 in which carbon 25 and the methyl group attached to carbon 25 were also derived from acetate. The methyl carbon of the methoxy group at carbon 5 derived from L-[*methyl*-^{13}C]methionine (Fig. 2-e).

The fact that [1-^{13}C]acetate also weakly enriched carbons 3, 7, 11, 13 and 23 which should be derived from [1-^{13}C]propionate indicated that acetate was metabolized into propionate and was incorporated into the carbon skeleton of milbemycins. Such indirect incorporation is not unprecedented as shown also in the study on the biosynthesis of rifamycin[6], leucomycin[7], tylosin[8] and lysocellin[9]. However, propionate was seemingly not metabolized into acetate, since in the experiment using [1-^{13}C]propionate no enrichment was observed at the carbons which were enriched from [1-^{13}C]acetate.

Incorporation of ^{13}C Labeled Precursors into Milbemycin α_4

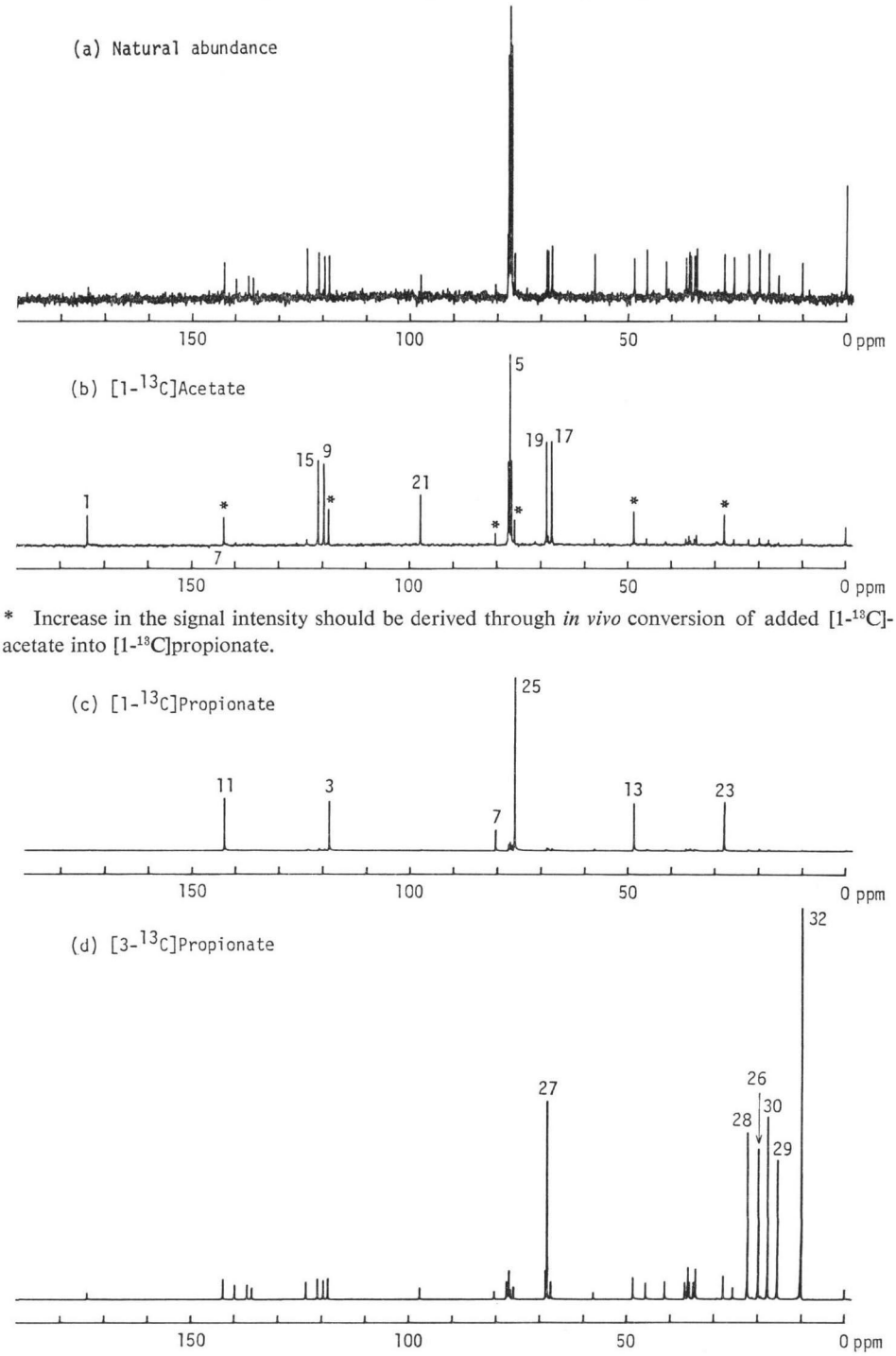
The natural abundance ^{13}C NMR spectrum of milbemycin α_4 is shown in Fig. 3-a. In the spectrum of the sample obtained from an experiment using [1-^{13}C]acetate carbons 1, 5, 9, 15, 17, 19 and 21 were shown to be enriched, indicating that seven acetate units were incorporated into milbemycin α_4 (Fig. 3-b). The experiments using ^{13}C labeled propionates showed that carbons 3, 7, 11, 13, 23 and 25 were derived from C-1 of the propionate (Fig. 3-c) and that carbons 26, 27, 28, 29, 30 and 32 were derived from C-3 of the propionate (Fig. 3-d). These results indicate that six propionate units were incorporated into

Fig. 2. ^{13}C NMR spectra of milbemycin α_2.



* Increase in the signal intensity should be derived through *in vivo* conversion of added [1-^{13}C]-acetate into [1-^{13}C]propionate.

Fig. 3. ^{13}C NMR spectra of milbemycin α_4.



* Increase in the signal intensity should be derived through *in vivo* conversion of added [1-^{13}C]-acetate into [1-^{13}C]propionate.

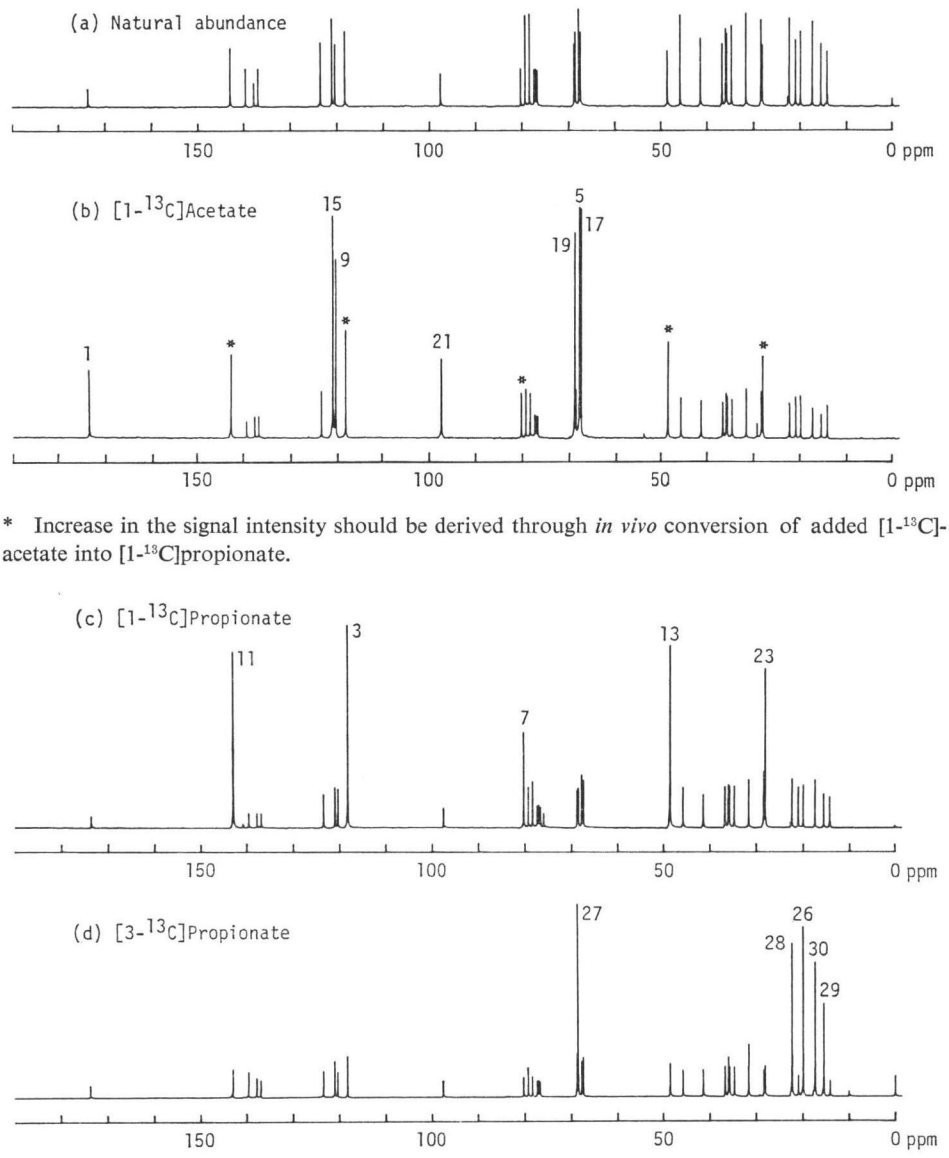
milbemycin α_4 and that carbon 25 and the ethyl group attached to carbon 25 were also derived from propionate. The methyl carbon of the methoxy group at carbon 5 in milbemycin α_4 was derived from L-[*methyl*-^{13}C]methionine as shown in the case of milbemycin α_2.

High degrees of ^{13}C incorporation were observed at carbon 25 and 32 of milbemycin α_4 in the experiments using [1-^{13}C] and [3-^{13}C]propionates, respectively, whereas such a high degree of incorporation was not observed at carbon 25 and 31 of milbemycin α_2 in the experiment using [1-^{13}C]acetate. It is very intriguing fact that the ^{13}C labeled propionates externally added were incorporated in much higher ratios than those of the other five propionate units of milbemycin α_4, which would offer the subject of further study in connection with the role of the starter units in the biosynthesis of polyketide chains.

Incorporation of ^{13}C Labeled Precursors into Milbemycin D

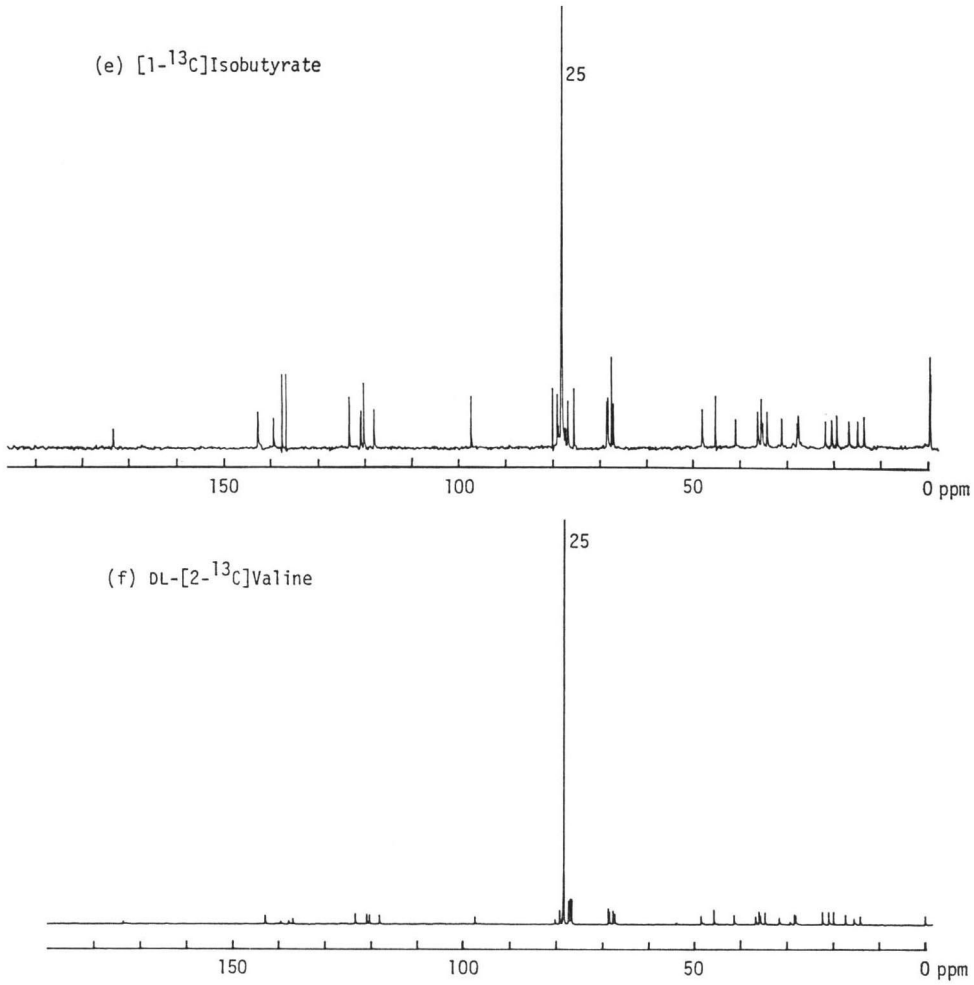
The natural abundance ^{13}C NMR spectrum of milbemycin D is shown in Fig. 4-a. In the spectrum of the sample obtained from the experiment using [1-^{13}C]acetate, ^{13}C enrichment was observed at car-

Fig. 4. ^{13}C NMR spectra of milbemycin D.



* Increase in the signal intensity should be derived through *in vivo* conversion of added [1-^{13}C]-acetate into [1-^{13}C]propionate.

Fig. 4. (continued).



bons 1, 5, 9, 15, 17, 19 and 21 (Fig. 4-b). Enrichment at these carbons is completely consistent with the labeling experiment of milbemycin α_4 with [1-^{13}C]acetate. In the experiments using ^{13}C labeled propionates, enriched carbons were the same as those of milbemycin α_2 (Fig. 4-c and d). It was shown that neither acetate nor propionate was incorporated into carbon 25 and the isopropyl group attached to carbon 25 in milbemycin D. In the spectra of the samples obtained from the experiments using [1-^{13}C]-isobutyrate or DL-[2-^{13}C]valine, carbon 25 in milbemycin D was specifically enriched (Fig. 4-e and f) and no ^{13}C enrichment was observed at other carbons. This fact suggests that DL-valine is metabolized into isobutyryl-CoA, which is directly incorporated into carbon 25 and its isopropyl group. In the experiments using [1-^{13}C]isobutyrate and DL-[2-^{13}C]valine, no ^{13}C enrichment was observed in milbemycins α_2 and α_4.

The rate of increase of ^{13}C involvement was estimated from mass spectra of natural and enriched milbemycin D. In the experiments using [1-^{13}C]isobutyrate or DL-[2-^{13}C]valine, the rate of increase of ^{13}C involvement was calculated to be about 25% and 30%, respectively, based on the peak abundance at

Scheme 1. Mass fragments of milbemycin D.

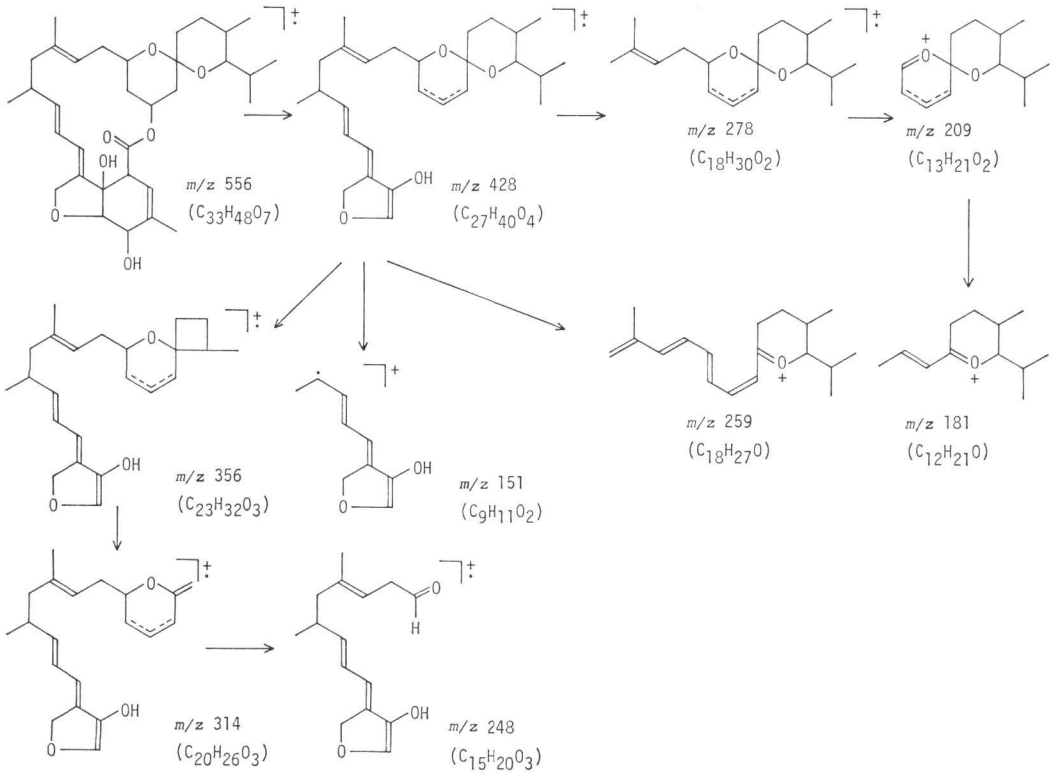


Fig. 5. Biosynthesis of milbemycins α_2, α_4 and D.

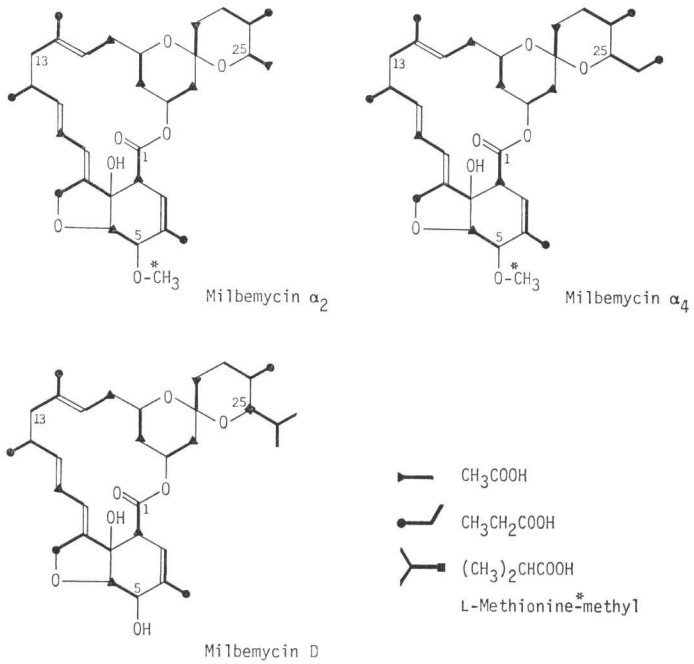


Table 2. The rate of increase of ^{13}C involvement estimated from mass spectra of milbemycin D, both natural and enriched by DL-[2-^{13}C]valine and [1-^{13}C]isobutyrate.

m/z	Relative intensity (%)			Rate of increase (%)	
	Enriched by DL-[2-^{13}C]-valine	Enriched by [1-^{13}C]-isobutyrate	Natural	DL-[2-^{13}C]-valine	[1-^{13}C]-isobutyrate
556	15.6	28.3	28.3		
557	11.4	19.9	10.6	25.9	23.9
428	33.9	35.5	48.7		
429	24.6	22.6	15.7	30.5	23.8
410	11.3	13.4	13.9		
411	7.8	8.6	4.4	28.6	24.8
356	14.0	10.0	8.7		
357	3.7	3.2	2.3	−0.2	4.4
314	14.0	14.0	13.9		
315	3.8	4.3	3.7	0.6	3.4
278	19.0	21.0	21.0		
279	12.1	12.4	5.2	31.3	27.6
259	45.5	32.2	40.0		
260	29.4	18.8	10.9	29.4	24.4
248	25.0	23.7	25.6		
249	6.3	6.4	5.4	3.6	4.8
209	48.0	43.0	52.4		
210	26.1	19.4	8.2	33.6	25.5
181	32.0	34.4	44.0		
182	17.6	15.6	6.9	34.2	25.7
151	100	100	100		
152	15.6	15.0	11.7	3.5	3.0

The rate of increase was estimated according to the equation $(b-a/a+100)\times 100$, in where a is the ratio of a nominal height of fragment ion to that of fragment ion +1 in the mass spectrum of natural milbemycin D and b is the ratio of a nominal height of fragment ion to that of fragment ion +1 in the mass spectra of milbemycin D enriched by DL-[2-^{13}C]valine or [1-^{13}C]isobutyrate.

m/z 557, 429, 411, 279, 260, 210 and 182 (Table 2). The structures of these mass fragment ions are shown in Scheme 1.

As shown in Fig. 5, it is proposed that the carbon skeleton except for carbon 25 of milbemycins α_2, α_4 and D are biosynthetically composed of seven acetate units and five propionate units, and the methyl, ethyl and isopropyl groups at carbon 25 originate from acetate, propionate and isobutyrate or DL-valine, respectively. The methyl carbon of the methoxy group at carbon 5 in milbemycins α_2 and α_4 was derived from methionine.

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