INCORPORATION OF CARBON-14 IN THE BIOSYNTHESIS OF THE MACROLIDE ANTIBIOTIC, LL-F28249-α

Z. H. AHMED*, R. R. FIALA and M. W. BULLOCK

Agricultural Research Division,
American Cyanamid Company,
Princeton, New Jersey 08543-0400, U.S.A.

(Received for publication November 2, 1992)

A total of 76 mCi of ^{14}C LL-F28249-α, nemadectin (**1**), having a specific activity of 35.2 μCi/mg was isolated from a fermentation using a mixture of approximately 600 mCi of ^{14}C carboxyl labeled acetate, propionate and isobutyrate. Nemadectin was used to synthesize carbon-14 labeled moxidectin which is being developed as a highly efficient ectoparasitic anthelminth. The labeled positions were determined by ^{13}C NMR analysis of ^{13}C nemadectin which was obtained by similar incorporation of ^{13}C carboxyl labeled acetate, propionate and isobutyrate.

LL-F28249-α[1], nemadectin (**1**) is a potent antiparasitic macrolide which is chemically modified to moxidectin (**2**)[2]. Although these macrolides are closely related to other 16-membered lactones, *e.g.* avermectins[3] and milbemycins[4], there are a few important structural differences. Nemadectin bears a hydroxyl group at the 23-position as well as a unique methyl-pentenyl side-chain at C-25.

Earlier we reported the bio-origin of the carbon and oxygen atoms of nemadectin as determined by ^{13}C NMR and mass spectral fragmentation data on the enriched antibiotics obtained by feeding ^{13}C and ^{18}O labeled precursors to the fermentation broth[5].

In this paper, we wish to report the biosynthesis of ^{14}C radiolabeled nemadectin (**3**) having a specific activity of 35.2 μCi/mg. Feeding experiments in which different combinations of high levels of ^{14}C carboxyl labeled acetate, propionate and isobutyrate were added to the fermentation broth yielded the desired antibiotic which was multiply labeled with ^{14}C. The labeled positions were determined by ^{13}C NMR analyses of the corresponding ^{13}C labeled products. ^{14}C labeled nemadectin was chemically modified[6] to multiply labeled moxidectin (**2**) for use in animal metabolism studies.

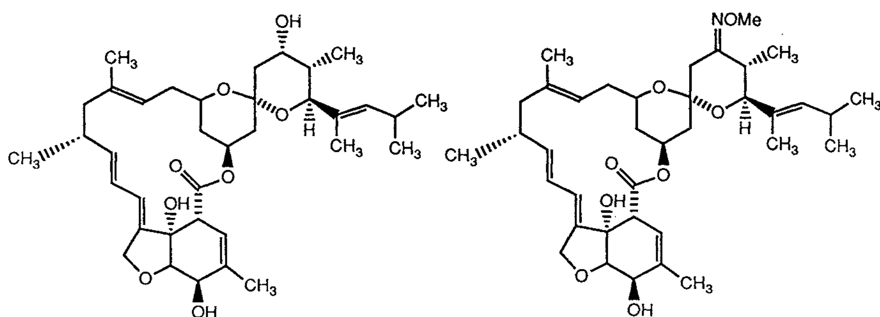
Experimental

Organism

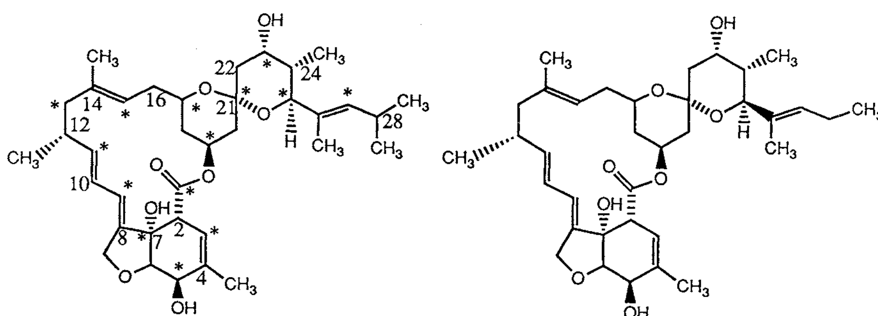
Streptomyces cyaneogriseus sp. *noncyanogenus* strain F 28249-PF3-15 was used in this study. This strain was developed by mutation and selection into a strain that yielded 4 g/liter and produced none of the ω component (an oligomycin class antibiotic) which was coproduced with the wild strain.

Culture Conditions

The seed medium consisted of (in g/liter) glucose (20), Na_2SO_4 (0.5), $MgSO_4$ (1), KH_2PO_4 (1), K_2HPO_4 (1) and corn steep liquor (15) adjusted to pH 6.5 with NaOH before autoclaving. 40 ml of the medium in a 250-ml baffled Erlenmeyer flask was seeded with 0.5 ml of a frozen spore suspension and grown in a rotary shaker at 30~32°C for 48 hours. One ml of the seed culture was used to inoculate 40 ml of production medium in a 250-ml baffled flask. The production medium consisted of (in g/liter) glucose (50), Pharmamedia (Traders Cotton Seed Flour) (25), lactose (25) and $CaCO_3$ (Missippi lime) (4). The pH was 7.2 after autoclaving at 121°C for 20 minutes. The flasks were incubated on a rotary shaker



Nemadectin (**1**) Moxidectin (**2**)



[^{14}C]Nemadectin (**3**) LL-F28249-γ (**4**)

* Indicates position of ^{14}C.

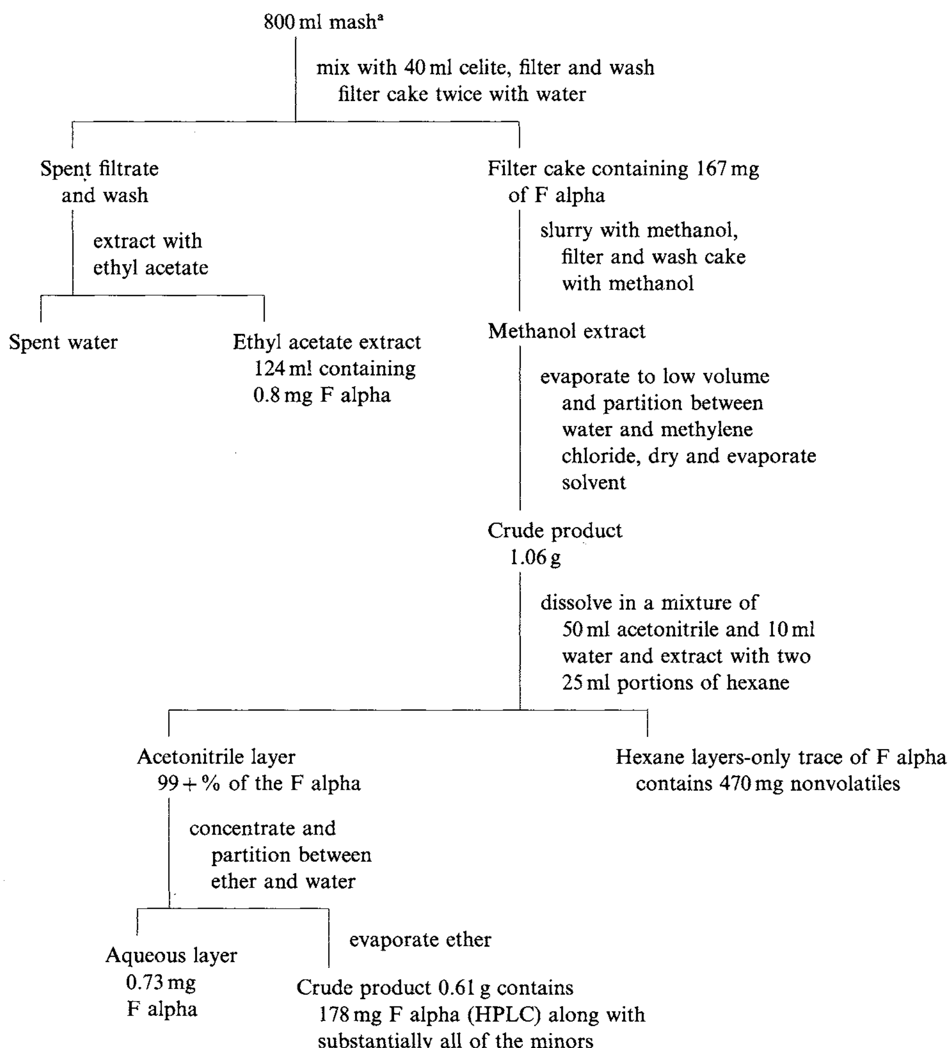
at 30~32°C until harvested (8 to 9 days). The yield of the antibiotic at different times was determined quantitatively by mixing an aliquot with two volumes of ethanol, sonicating a few minutes, centrifuging at 3,000 rpm for 15 minutes and assaying the supernatant by HPLC. The peak containing the α component was collected and counted by liquid scintillation techniques to determine the specific activity of the product.

The Labeled Substrates

Sodium [1-^{14}C]acetate, sodium [1-^{14}C]propionate, [1-^{14}C]isobutyric acid and L-[U-^{14}C]valine were purchased from commercial sources. [1-^{14}C](E)-2,4-Dimethyl-2-pentenoic acid was prepared by condensation of [1-^{14}C]2-bromopropionic acid with 2-methylpropionaldehyde by the method of BRITTELLI[7]. The labeled product was about 85% E isomer mixed with 10% Z isomer. [1-^{14}C]Propionamide and [1-^{14}C]isobutyramide were prepared from the corresponding acids by the carbodiimide method of SHEEHAN[8]. [2-^{14}C]2-Oxo-3-methylbutyric acid was prepared from carboxyl labeled isobutyric acid by conversion in two steps to the aldehyde, and then to the cyanohydrin with trimethylsilyl cyanide[9]. The cyanohydrin was then converted to the *tert*-butylamide and hydrolyzed to the 2-oxo acid by literature methods[10].

The sodium salts of the acids or water soluble amides were dissolved in water and sterile filtered prior to addition to the fermentation flasks. For screening studies, the concentrations of the substrates were made 0.4 mmol/ml and each ml was spiked with approximately 20 μCi/ml of labeled substrate. Final radioactivity concentrations were verified by counting aliquots of the solutions by liquid scintillation techniques. For the high level production run, 175 mCi of sodium acetate, 230 mCi sodium propionate and 230 mCi sodium isobutyrate, all carboxyl labeled and having specific activities of 56~57 mCi/mmol were combined and dissolved in 20 ml of water. The solution was sterile filtered and 1 ml aliquots (0.56 mmol, 31.6 mCi) added to each of 20 flasks.

Scheme 1. Recovery of LL-F28249-α (F alpha) from mash.



[a] 210 μg/ml (168 mg F alpha HPLC yield), grown in a chemostat with 25 ml antifoam used.

Isolation from Mash and Purification of the α Component

In connection with our earlier work on the biosynthesis of this anthelmintic agent[5], we developed an isolation procedure for recovering the product from the mash. This method is summarized in Scheme 1, where F alpha refers to LL-F28249-α.

The initial runs were designed to determine the addition and harvest times and the levels of useful substrates. No attempt was made to isolate the desired components in a preparative way. For the practice run and the production run for the ^{14}C preparation, the procedure outlined in Scheme 1 above was used.

In the original isolation of the milbemycins, CARTER[1] mixed the mash with a filtering aid, collected the mycelia on a filter and extracted the products from the mash cake with methanol. After removal of the methanol, the crude product was partitioned between methylene chloride and water. The methylene chloride extract was loaded onto a silica gel column. After chromatography on silica gel, the fractions were purified further by reversed phase chromatography on C-18. Although we used this isolation procedure in our earlier work on the biosynthesis[5], the procedure suffers in that the crude product

contains excessive fat-like materials. To circumvent some of the difficulties with this method we developed the isolation procedure outlined in Scheme 1. This method gave excellent recoveries from the mash and the hexane - acetonitrile partition removed fats and excess antifoam from products that had been prepared by tank fermentations. Some difficulty was experienced in recovering the product from the acetonitrile-water mixture due to foaming when the solution was concentrated. Therefore the crude product was loaded on to an Amberlite XAD-2 column directly from the acetonitrile - water mixture and the desired product was selectively eluted by increasing the concentration of acetonitrile. Two applications of Amberlite XAD-2 chromatography are outlined below:

A chromatographic column, 19 mm i.d., was slurry packed with 110 ml Amberlite XAD-2 resin (Applied Sciences purified grade) in acetonitrile. After washing the column with 100 ml of a solution of 60 : 40 acetonitrile - water, a solution of 2.0 g of 79.5% pure nemadectin in this mixture was added to the column. The product was eluted with a step gradient from 70% acetonitrile to 100% acetonitrile. The desired product eluted between 80 and 100% acetonitrile, and assayed 98.8% pure.

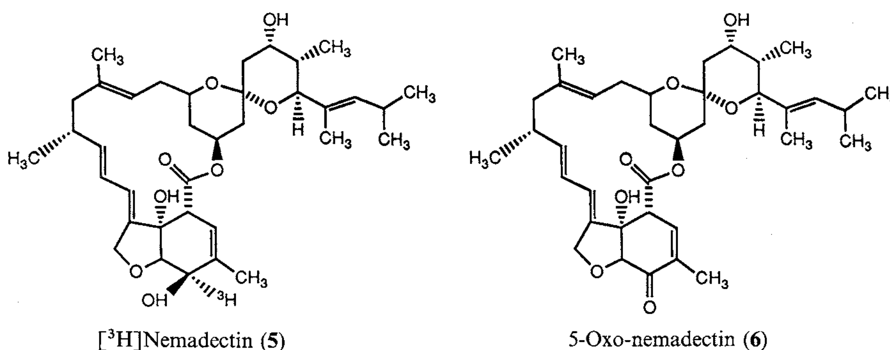
In a second run designed to evaluate the resolution of the chromatography, a 2.5 cm i.d. column was packed with 220 ml (75 g dry weight) Amberlite XAD-2 adsorbent (20~50 mesh). After equilibrating the column with 50% acetonitrile in water, a solution of 0.5 g of a crude mixture of antibiotic from a fermentation run was loaded in a 50% acetonitrile solution. The column was eluted with a step gradient from 50% to a 100% acetonitrile. The products eluted with 80 to 90% acetonitrile and there was a partial separation of the minor components from the primary product. The elution pattern appeared to be identical with that obtained with C-18 chromatography but the resolution was not as good. The products were obtained as free flowing powders when a solution of the compounds in *tert*-butanol was lypholized.

In the ^{14}C-labeled production run, we used the procedure outlined in Scheme 1 with the minor modification in that methylene chloride was substituted for ether to extract the product after the hexane-acetonitrile distribution because the methylene chloride solution was convenient to load on the silica gel column. Chromatography was by gradient elution from 90 : 10 to 80 : 20 CH_2Cl_2 - EtOAc. The γ component (**4**) was well separated from the desired α and the unwanted and more polar components remained on the column. The recovery of the purified, 95.8% pure, α component (**3**) approximated 78% of the α component of the material loaded on the column. This pure fraction which had a specific activity of 35.2 μCi/mg was used as starting material for the preparation of moxidectin (**2**)[6].

Results and Discussion

The Fermentation

In our preliminary studies, tritium labeled nemadectin (**5**) was prepared from the 5-ketone (**6**) (obtained by MnO_2 oxidation of the allylic alcohol moiety) followed by reduction of the ketone with sodium [^3H]borohydride. This product was used for conducting a preliminary metabolism study to measure blood levels in cattle.



[^3H]Nemadectin (**5**) 5-Oxo-nemadectin (**6**)

Although the tritium label would suffice to give a definitive determination of the metabolic profile of the compound, it was felt that a ^{14}C labeled material would be more desirable primarily because there could be no question about the stability of the label since multiple carbons would be labeled. In addition, there was an advantage of carbon-14 over tritium for measuring the quantities of labeled metabolites produced. Earlier we had attempted to prepare a ^{14}C labeled product by incorporation of labeled acetate and propionate which make up the major portion of the carbon skeleton[5] but with the cultures available at the time, we were not able to incorporate more than about 2% of the labeled carbon into the antibiotic.

After considerable strain development, the yield of the antibiotic was improved approximately ten-fold. The improved strain produced a higher percentage of the desired component at greater yields and required much less oxygen. With the improved strains, we again evaluated methods for preparing a ^{14}C labeled product. We were able to get adequate yields with 40 ml of medium in 250-ml shaker baffled flasks. The antibiotic production was underway by day 4, it increased rapidly until day seven, and then remained about constant until day 9. Changes in the amounts and compositions of the minor products were evaluated by adding different precursors in different amounts but with this strain, there was no significant increase in the relative amount of the α component for any of the potential precursors. The data from the screening runs and a representative chromatogram are included in the appendices. The incorporation of [1-^{14}C]acetate, [1-^{14}C]propionate, L-[*U*-^{14}C]valine, [2-^{14}C]2-oxo-isovaleric acid, [1-^{14}C]isobutyric acid and [1-^{14}C](*E*)-2,4-dimethyl-2-pentenoic acid were studied. It was felt that the pentenoic acid would not only label the side chain but also increase the relative yield of the α component in the fermentation mixture. This approach, wherein one feeds a "starter acid", to produce milbemycins with specific side chains has exploited by other laboratories (Merck[11], Pfizer[12] and Glaxo[13]) yielding milbemycins with unnatural side chains and enhanced biological activities. Unfortunately, in our case, none of the radioactivity appeared in the antibiotic indicating that it was not a suitable substrate. The great bulk of the radioactivity remained in the flask at the end of the fermentation which suggested that the acid was probably not metabolized. The remainder of the substrates were all incorporated into the antibiotic. L-Valine, 2-oxo-3-methylbutyric acid, which is the L-valine transamination product, and isobutyric acid all labeled the side chain of C-25 with high efficiency, but would be expected to label the lactone ring very poorly.

In order to label the "backbone" of the molecule, *i.e.* the lactone ring, one needs to incorporate acetate or propionate[5]. However, we have observed in other biosynthetic studies[14] that larger amounts of acetate and especially propionate inhibit the growth of the organism or the production of the antibiotic. In an attempt to decrease the toxicity of the acids, we prepared their amides which could be enzymatically hydrolyzed during the fermentation to supply the precursor acid at a more regulated rate, and thereby permit the feeding of the substrate at a higher rate. The results obtained with propionamide, isobutyramide and (*E*)-2,4-dimethyl-2-penten-*tert*-butylamide were negative with almost no radioactivity incorporated into the antibiotic, the bulk of the activity being found in the flask at the end of the fermentation. It would appear that the organism can not hydrolyze these amide linkages although it must be able to hydrolyze peptide bonds since proteins are useful substrates for the growth of the organism.

The efficiency of the compounds that label the side chain of C-25 are suprizingly high and large amounts can be added to yield products of high specific activity. Table 1 summarizes the efficiencies of labeling of the side chain by addition of precursors on day 4. Approximately 0.2 mmol of the α component was produced in each of the 250-ml baffled flasks containing 40 ml of production media.

Table 1. Efficiencies of incorporation of side chain precursors in the production of LL-F28249-α.

Substrate	(mmol)	Yield[a] (g/liter)	^{14}C in α component (%)	Specific activity of products[b] (μCi/mg)
Sodium [2-^{14}C]2-oxo-3-methylbutyrate	0.2	4.4	20	12.7
	0.4	4.4	16	20.3
	0.8	3.6	12	37.3
Sodium [1-^{14}C]isobutyrate	0.2	4.0	23	16.1
	0.4	4.0	18	25.2
	1.6[c]	3.2	9	63.0
L-[*U*-^{14}C]Valine	0.2	3.1	15	60.0
	0.4	3.3	12	90.0
	0.8	4.6	5	54.3

[a] Based on major α component.
[b] Calculated specific activity of products from feeding substrates of 57 mCi/mmol specific activity.
[c] All yields are an average of duplicate flasks except addition of 1.6 mmol of isobutyrate where only one flask was available.

Table 2. Efficiencies of incorporation of lactone ring precursors in the production of LL-F28249-α.

Substrate	(mmol)	Yield (g/liter)[a]	^{14}C in α component (%)	Specific activity of product[b] (μCi/mg)
Sodium [1-^{14}C]acetate	0.2	4.3	9.4	6.1
	0.4	4.3	9.0	11.7
	0.8	4.5	8.7	21.6
Sodium [1-^{14}C]propionate	0.2	4.7	13.2	7.9
	0.4	4.7	11.7	13.9
	0.8	4.3	11.1	28.9

[a] Based on the major α component.
[b] Calculated specific activity for feeding substrates at 57 mCi/mmol.

It is readily apparent from the data in Table 1 that the side chain can be labeled with any of these substrates with good efficiency and that the precursors do not significantly inhibit antibiotic production. As a practical matter, the preferred substrate is [1-^{14}C]isobutyric acid since it can be obtained in good yield in a single step from [^{14}C]barium carbonate. Thus, by adding 0.4 mmol (22.4 mCi) sodium [1-^{14}C]isobutyrate having a specific activity of 56 mCi/mmol to 40 ml of production medium in 250-ml flask, one could expect to produce about 160 mg of the α component having a specific activity of 25 μCi/mg. The labeled carbon would be largely at the 27 position and based on the known metabolic fate of ivermectin[15,16] would probably be retained in all of the metabolites; however, it would be very desirable to label the lactone ring system with acetate and/or propionate precursors. The known biosynthetic pathway indicates that either of these substrates would yield a suitably labeled product. We have evaluated the efficiency of these substrates and observed that they are less efficient but still give incorporations high enough for use in the labeled synthesis since these substrates are readily available and inexpensive. The incorporation of the labeled carbons and the effect of the precursor on the fermentation yield are shown in Table 2.

It is immediately apparent that the organism can incorporate both acetate and propionate into the antibiotic and that propionate is incorporated with better efficiency. As would be expected, there is some decrease in efficiency with increasing amounts of substrate and at the same time the specific activity of

Table 3. Efficiencies of incorporation of combinations of labeled precursors in the production of LL-F28249-α.

Substrate[a] (mmol)			Yield (g/liter)	^{14}C in α component (%)	Specific activity of products (μCi/mg)[b]
Acetate	Propionate	Isobutyrate			
0.2	0.2	0.0	4.1	10.5	14.3
0.4	0.4	0.0	3.9	10.6	30.4
0.8	0.8	0.0	3.1	10.0	72.2
0.2	0.4	0.0	3.8	11.9	26.3
0.4	0.2	0.0	3.8	11.1	21.7
0.2	0.2	0.2	3.2	13.2	34.6
0.4	0.4	0.4	3.2	10.7	56.1
0.8	0.8	0.8	2.3	5.9	86.2

[a] The acids were added as sterile solutions of their sodium salts.
[b] Calculated specific activity for feeding the substrates at 57 mCi/mmol.

the product increases in a uniform and predictable way as the quantity of the labeled substrate is increased.

The incorporation of a mixture of substrates, especially acetate, propionate and isobutyrate, all labeled at the carboxyl carbon with ^{14}C would appear to be an excellent and economical way to label this molecule, provided that the incorporation of the substrates are additive. We evaluated the efficiency of incorporation of mixtures of these readily available substrates. The results are summarized in Table 3.

The data indicate that there is no significant inhibition of the use of multiple labeled substrates. From the data summarized in Tables 1, 2 and 3, we can predict the specific activity that would be obtained by the additions of individual runs. Table 1 shows that addition of 0.2 mmol of sodium isobutyrate contributed a specific activity of 16.1 μCi/mg to the product and from Table 2, it is clear that 0.2 mmol of sodium acetate contributes 6.1 μCi, and 0.2 mmol of sodium propionate contributes about 7.8 μCi/mg. The sum of the contributions of these three substrates to the total specific activity is 30 μCi/mg, which is close to the observed value to 34.6 for the mixture of the three compounds as given in Table 3. The runs with 0.4 mmol of each substrate/flask is calculated from data in Tables 1 and 2 to give a specific activity of 50 μCi/mg which compares well with the experimental value of 56.1 in Table 3.

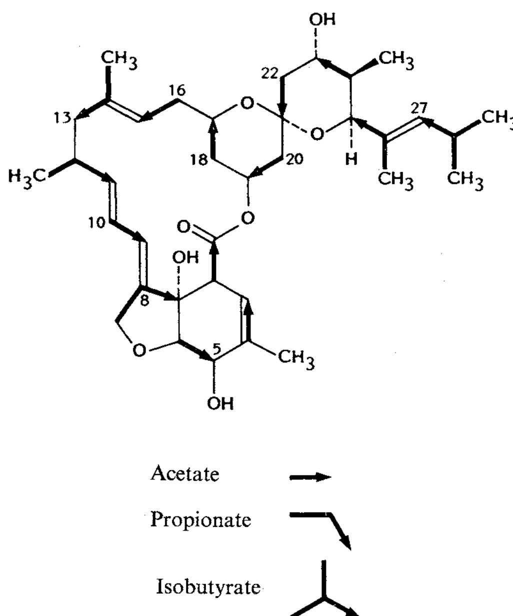
The relative efficiencies of the various substrates vary considerably with the precursors. Isobutyrate and compounds that are readily convertible to isobutyrate during the fermentation gave the best efficiency. Under the conditions that we ran the biosynthesis, each 250 ml shake flask containing 40 ml of media produced 0.2 to 0.25 mmol of the α component. When [1-^{14}C]isobutyrate was added at 0.2 mmol/flask, 23% of the label was incorporated primarily into the side chain of the product. When 0.4 mmol (200% of the amount that could theoretically be incorporated) of this precursor was added 18% was incorporated yielding a product which is primarily enriched at the C-27 carbon atom. Based on our earlier work[5)] we know that a fraction of the label will also appear in each the carbons that are derived from the acetate carboxyl. From this data, it is apparent that a significant amount of the [1-^{14}C]isobutyrate is converted to [1-^{14}C]acetate during the fermentation. The incorporation of the labeled atom from 2-oxoisovalerate and L-valine are best explained as being precursors of isobutyrate.

The lactone ring system, the backbone of the molecule, is derived from seven acetate and six propionate units, and each 0.2 mmol of product formed in the fermentation should be 1.4 mmol of acetate and 1.2 mmol of propionate. Since large amounts are required for the biosynthesis, it would

appear that acetate and propionate could be incorporated with good efficiency. Within the loading ranges that we evaluated, acetate was incorporated at about 9% efficiency and propionate at approximately 12%. The efficiency decreased by relatively small amounts with relatively large increases in the amount of substrate added. At the highest levels tested, 0.8 mmol/flask (about 1.9 g/liter of sodium propionate) the yield and incorporation data suggest that even higher levels of use would be practical. From the biosynthetic studies on the ^{13}C enriched samples[5], we can predict that the [1-^{14}C]acetate will not only label the carbons that are derived from acetate carboxyl but also that a significant amount of the labeled atoms which are primarily derived from the propionate carboxyl will be labeled. It is at once apparent that [1-^{14}C]acetate is converted to [1-^{14}C]propionate during the fermentation and this labeled propionate is incorporated into the product. However, there is no significant conversion of carboxyl labeled propionate to carboxyl labeled acetate. The results obtained from these studies indicated that LL-F28249-α can be labeled efficiently and economically in a manner suitable for metabolism studies with carboxyl labeled acetate or propionate or combinations of the two with or without carboxyl labeled isobutyrate.

The production run was carried out to prepare a large amount of labeled antibiotic for further conversion to moxidectin[6]. We employed 635 mCi of an approximately equimolar mixture of [1-^{14}C]acetate, [1-^{14}C]propionate and [1-^{14}C]isobutyrate at a specific activity of 56~57 mCi/mmol and added 0.2 mmol of each substrate/flask. The purified LL-28249-α amounted to 76 mCi, 12% of the ^{14}C added being incorporated into the antibiotic. An additional 3.3% of the labeled carbon was recovered as less pure product and other milbemycins. The insoluble solids from the methanol extracted mycelia, the insoluble cell components, contained 33 mCi, 5.2% of the radioactivity charged. Radioactivity in the mash filtrate and aqueous layers from extractions and washes which would contain unused substrates and small water soluble molecules amounted to 27 mCi, 4.2%. Material extracted from water with methylene chloride which was not eluted from the silica gel chromatographic column during the chromatography of the product amounted to 66 mCi, 10.4%. The sum of the radioactivity of these various fractions amounted to 227 mCi, 36% of the radioactivity in the substrates. The remaining 64% of the carbon-14 is best accounted for as labeled carbon dioxide which is formed during the fermentation.

Fig. 1. Origin of the carbon atoms of LL-F28249-α.



As discussed in our earlier work[5], ^{13}C NMR assignment were used to establish the position of the labels as indicated from the enrichments of individual carbons. These results are summarized in Fig. 1 which shows that carbons 1, 5, 9, 15, 17, 19 and 21 were derived from C-1 of acetate and carbons 2, 6, 10, 16, 18, 20 and 22 were derived from C-2 of acetate. When sodium [1-^{13}C]propionate was added to the fermentation, the isolated antibiotic was enriched at carbons 3, 7, 11, 13, 23 and 25 indicating that six propionate units were incorporated into LL-F28249-α. Addition of carboxyl

labeled sodium isobutyrate enriched C-27 in the methylpentenyl side-chain. Secondary enrichments were also observed with some substrates and suggested conversion of acetate to propionate during the Krebs cycle. By analogy with this distribution of multiple carbon-13 labels, the sample of ^{14}C enriched nemadectin (**3**) obtained in the production run was also similarly labeled. The product was found to have an approximate specific activity of 35.2 μCi/mg and was used for metabolic studies after chemical conversion to moxidectin (**2**).

Acknowledgments

We wish to thank Dr. G. Asato for support and Dr. J. Growich of Lederle Laboratories for a generous supply of frozen spore suspensions and specific instructions for growing the culture in shaker flasks.

References

1) Carter, G. T.; J. A. Nietsche & D. B. Borders: Structure determination of LL-28249 α, β, γ and λ, potent antiparasitic macrolides from *Streptomyces cyaneogriseus* ssp. *noncyanogenus*. J. Chem. Soc. Chem. Commun. 1987: 402~404, 1987
2) Asato, G. & D. J. France (American Cyanamid Company): U.S. 4,916,154, Apr. 10, 1990
3) 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
4) Takiguchi, Y.; H. Mishima, M. Okuda, M. Terao, A. Aoki & R. Fukuda: Milbemycins, a new family of macrolide antibiotics: Fermentation, isolation and physico-chemical properties. J. Antibiotics 33: 1120~1127, 1980
5) Tsou, H.-R.; Z. H. Ahmed, R. R. Fiala, M. W. Bullock, G. T. Carter, J. J. Goodman & D. B. Borders: Biosynthetic origin of the carbon skeleton and oxygen atoms of the LL-F28249 α, a potent antiparasitic macrolide. J. Antibiotics 42: 398~406, 1989
6) Ahmed, Z.; M. Bullock & D. J. France: Synthesis of moxidectin labeled with ^{14}C, ^{2}H and ^{3}H. 4th International Isotope Society Symposium, Toronto, Sept. 3, 1991
7) Brittelli, D. R.: Phosphite-mediated in situ carboxyvinylation: A new general acrylic acid synthesis. J. Org. Chem. 46: 2514~2520, 1981
8) Sheehan, J. C. & G. P. Hess: A new method of forming peptide bonds. J. Am. Chem. Soc. 77: 1067~1068, 1955
9) Gassman, P. G. & J. J. Talley: Cyanohydrins—A general synthesis. Tetrahedron Lett. 1978: 3773~3776, 1978
10) Anatol, J. & A. Medete: A new general procedure for the preparation of α-oxocarboxylic acids. Synthesis 1971: 538~539, 1971
11) Chen, T. S.; E. S. Inamine, O. D. Hensens, D. Zink & D. A. Ostlind: Directed biosynthesis of avermectins. Arch. of Biochem. Biophys. 269: 544~547, 1989
12) Dutton, C. J.; S. P. Gibson, A. C. Goudie, K. S. Holdom, M. S. Pacey, J. C. Ruddock, J. D. Bu'Lock & M. K. Richards: Novel avermectins produced by mutational biosynthesis. J. Antibiotics 44: 357~365, 1991
13) Todd, M.; M. A. Haxell & G. C. Lawrence (Glaxo Group Ltd.): Eur. Pat. Appl. 241 147, Oct. 14, 1987
14) Tsou, H.-R.; S. Rajan, R. Fiala, P. C. Mowery, M. W. Bullock, D. B. Borders, J. C. James, J. H. Martin & G. O. Morton: Biosynthesis of the antibiotic maduramicin. Origin of the carbon and oxygen atoms as well as the ^{13}C NMR assignments. J. Antibiotics 37: 1651~1663, 1984
15) Chiu, S.-H.; E. Sestokas, R. Taub, J. L. Smith, B. Arison & A. Y. H. Lu: The metabolism of avermectin H-2B-1B by pig liver microsomes. Drug Metab. Dispos. 12: 464~469, 1984
16) Chiu, S.-H.; E. Sestokas, R. Taub, R. P. Buhs, M. Green, R. Sestokas, W. J. A. Vandenheuvel, B. H. Arison & T. A. Jacob: Metabolic disposition of ivermectin in tissues of cattle, sheep and rats. Drug Metab. Dispos. 14: 590~600, 1986