BIOSYNTHETIC ORIGIN OF THE CARBON SKELETON AND OXYGEN ATOMS OF THE LL-F28249α†, A POTENT ANTIPARASITIC MACROLIDE

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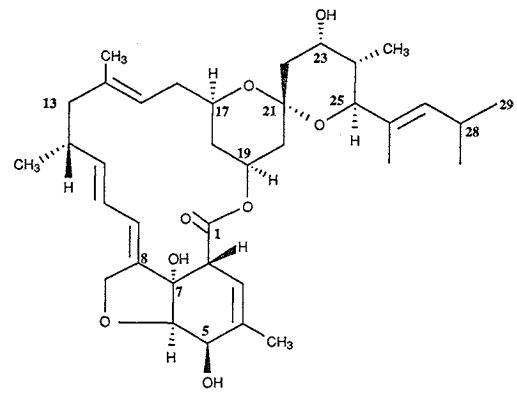
The biosynthesis of LL-F28249α in a culture of *Streptomyces cyaneogriseus* has been studied using ^{13}C, ^{14}C and ^{18}O labeled precursors. A complete ^{13}C NMR spectrum of F28249α has been assigned. Incorporation studies using ^{13}C labeled precursors indicate that the carbon skeleton of F28249α is derived from seven acetate, six propionate and one 2-methylpropionate units. The origin of the oxygen atoms of F28249α has been examined by feeding [1-^{13}C,$^{18}O_2$]acetate, [1-^{13}C,$^{18}O_2$]propionate, [2-^{13}C]acetate/$^{18}O_2$ and $^{18}O_2$ separately to the fermentation culture and analyzing the resulting labeled LL-F28249α samples by ^{13}C NMR, electron impact MS and chemical ionization MS. Out of a total of eight oxygen atoms in LL-F28249α, four oxygen atoms are derived from acetate, three from propionate and one from molecular oxygen.

Streptomyces cyaneogriseus sp. *noncyanogenus* produces the LL-F28249 antibiotics, a new group of macrolides with potent antiparasitic activity[1~5]. The structures of the LL-F28249 antibiotics[3] are related to other 16-membered lactones, *i.e.*, milbemycins and avermectins; however, significant differences exist among them. In milbemycins[6], the C-23 is not hydroxylated and the C-25 side chain is a methyl, ethyl or isopropyl group. In avermectins[7], the C-13 bears a di-(L)-oleandrose moiety, C-25 has either an isopropyl or an isobutyl side chain and in some members, a double bond exists between C-22 and C-23.

In this paper†††,[8], we wish to report the ^{13}C NMR assignments and the bio-origin of the carbon and oxygen atoms of LL-28249α (Fig. 1) as determined from ^{13}C NMR and mass spectral data of the compounds obtained by feeding experiments using ^{13}C and ^{18}O labeled precursors.

Fig. 1. Structure of LL-F28249α.



† The name nemadectin has recently been approved for LL-F28249α by the USAN council.

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††† A preliminary account of this work was previously presented ref 8.

Experimental

Organism

Streptomyces cyaneogriseus sp. *noncyanogenus* strains NRRL 15773, improved strains NS2(8), F28249-PF2 to 6 were used for this work.

Culture Conditions

The ingredients of the seed culture medium used for the incorporation of sodium [1-^{13}C]acetate, sodium [1-^{13}C]propionate and sodium [1-^{13}C]-2-methylpropionate with early strains were (in g/liter); dextrin (20), glucose (10), yeast extract (5), NZ-Amine A (5) and $CaCO_3$ (1). Culture F28249-NS2(8) was grown in two stages of inoculum in 500-ml Erlenmeyer flasks containing 100 ml of the seed culture medium.

For the incorporation experiments using sodium [1-^{13}C, $^{18}O_2$]acetate, sodium [1-^{13}C, $^{18}O_2$]-propionate and [2-^{13}C]acetate, [2-^{13}C]acetate/$^{18}O_2$ and $^{18}O_2$ with improved strains, the following seed culture medium was used (in g/liter); glucose (20), Na_2SO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.1), KH_2PO_4 (1), K_2HPO_4 (1), $FeSO_4 \cdot 7H_2O$ (0.05) and corn steep liquor (15) adjusted to pH 6.5. The seed culture medium (25 ml) in a 250-ml Erlenmeyer flask was inoculated with 0.4 ml of a thawed suspension of the culture and propagated on a shaker bath (120 rpm) at 27.5°C for 2 days.

One ml of the inoculum was used to inoculate the fermentation medium (100 ml/500 ml flask, 25 ml/250 ml baffled flask or 15 ml/250 ml baffled flask). Three types of fermentation media were used with the following compositions (g/liter): Medium A; molasses (20), lactose (10), Proflo (5) and $CaCO_3$ (1), medium B; glucose (60), pressed peanut meal (20), Proflo (10) and $CaCO_3$ (4), medium C; glucose (50), Proflo (25) and $CaCO_3$ (7.5). All the fermentations were carried out on a rotary shaker (235 rpm) at 27°C for 7 days unless stated otherwise.

Isotope-labeled Substrates

Sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1-^{13}C]propionate and sodium [1-^{13}C]-2-methylpropionate of 99.5 atom% ^{13}C, were purchased from Merck Sharp and Dohme Isotopes (St. Louis, MO); sodium [1-^{14}C]acetate (specific radioactivity 56.0 mCi/mmol), sodium [2-^{14}C]acetate (51.0 mCi/mmol), sodium [1-^{14}C]propionate (56.6 mCi/mmol) and sodium [1-^{14}C]-2-methylpropionate were purchased from New England Nuclear (Boston, MA). Sodium [1-^{13}C,$^{18}O_2$]acetate and sodium [1-^{13}C,$^{18}O_2$]propionate were prepared in our laboratory[9] with isotopic composition of 64.2% $^{13}C^{18}O_2$, 28.4% $^{13}C^{18}O^{16}O$, 7.3% $^{13}C^{16}O_2$ and 64.4% $^{13}C^{18}O_2$, 27.2% $^{13}C^{18}O^{16}O$ and 8.3% $^{13}C^{16}O_2$, respectively. A cylinder of $^{18}O_2$ gas (95 atom% ^{18}O) was purchased from Isotec Incorporated (Centerville, OH).

Isolation of LL-F28249α

The whole fermentation mash (425 ml) was stirred with diatomaceous earth (24 g), and filtered. The filter cake was washed with water and then extracted with MeOH. The MeOH extract was concentrated *in vacuo* to remove most of the MeOH. The antibiotic was extracted from the aqueous MeOH solution with CH_2Cl_2. The extract was dried over $MgSO_4$, filtered and evaporated to give 0.88 g of a gummy residue.

The residue along with a trace amount of indophenol dye in CH_2Cl_2 was applied to a 100-ml silica gel (Woelm TSC Activity III) column which had been slurry-packed in CH_2Cl_2. The column was eluted with CH_2Cl_2 - EtOAc (90 : 10) until the γ component[3], which eluted soon after the dye marker, was off the column and then with CH_2Cl_2 - EtOAc (80 : 20) which eluted the major α component.

The γ and α components were further purified by chromatography on a C18 reverse phase column (21.4 mm × 30 cm, Rainin Dynamax). The fractions were eluted with MeOH - H_2O gradient from 85 : 15 to 90 : 10 over 30 minutes followed by 100% MeOH for 8 minutes at a flow rate of 13.2 ml/minute. The γ and α components were eluted at 2.7 and 3.3 column volumes, respectively.

The fractions containing the desired product were combined and evaporated. The residue was dissolved in *tert*-butanol and lyophilized to yield 227.5 mg of LL-F28249α and 41.9 mg of LL-F28249γ and the ^{13}C and ^{18}O abundance were determined by ^{13}C NMR spectroscopy.

Results and Discussion

Aeration had a dramatic effect on the fermentation yield of LL-F28249α. We observed that the production yield of LL-F28249α decreased considerably with increasing volume of the fermentation medium as shown in Table 1. To circumvent this problem, baffled flasks were used to allow sufficient aeration.

The bio-origin of LL-F28249α was established using ^{13}C and ^{18}O labeled substrates for incorporation into LL-F28249α. The results of the runs are summarized in Table 2. All the labeled precursors gave reasonably high ^{13}C enrichment in LL-F28249α. In particular, a 13-fold ^{13}C enrichment in LL-28249α was obtained by the incorporation of sodium [1-^{13}C]-2-methylpropionate.

The fermentation yields varied considerably in the labeling studies due to the potency of the culture, the amount of aeration and the choice of the fermentation medium. The detailed ^{13}C enrichment data and the complete ^{13}C assignments of LL-F28249α are listed in Table 3.

^{13}C NMR assignments are based on general chemical considerations and careful comparison with structurally-related macrolides, *i.e.*, milbemycins[10] and avermectins[7] and other work recently reported by us[4]. Most of the 36 ^{13}C signals were unambiguously assigned except 3 pairs of ^{13}C resonances. Among them, the two pairs of signals due to C-17, C-19, C-20 and C-22 were assigned by comparing with the corresponding signals of avermectin B_2[6]. The remaining pair of signals due to C-9 and C-15

Table 1. The effect of aeration on the fermentation yield of LL-F28249α.

Fermentation flask (250 ml)	Production yield[a] of LL-F28249α (mg/liter) in fermentation medium of		
	15 ml	25 ml	50 ml
Regular flask	300	5	0
Baffled flask	300	310	—

[a] The fermentation was carried out using seed culture F28249-PF3-2 in fermentation medium B and harvested at day 7.

Table 2. Summary of ^{13}C and ^{18}O incorporation studies.

Precursor	Addition amount (g/liter)	Addition time (hours)	LL-F28249α (mg/liter) at day 7	^{14}C incorporation[a] (%)	^{13}C incorporation[b] (%)
[1-^{13}C]Acetate[c]	0.5, 0.5	48, 72	40[d]	—	4.3
[1-^{13}C]Propionate[c]	0.5, 0.5	48, 72	30[d]	—	2.8
[1-^{13}C]-2-Methylpropionate[c]	0.25, 0.25	48, 72	19[d]	—	13.0
[2-^{13}C]Acetate[e]	1, 1	96, 120	286	2.63	3.0
[2-^{13}C]Acetate and $^{18}O_2$[e]	1, 1	96, 120, 96~168	300	3.14	3.3
[1-^{13}C, $^{18}O_2$]Acetate[e]	1, 1	96, 120	425	1.15	3.4
[1-^{13}C, $^{18}O_2$]Propionate[e]	0.67, 0.67, 0.67	72, 96, 120	228	1.77	3.0

[a] For each ^{13}C incorporation study, 5~10 μCi of ^{14}C labeled precursor was mixed with the corresponding ^{13}C labeled precursor and added aseptically to each fermentation flask.

[b] The ratio of ^{13}C enrichment to ^{13}C natural abundance.

[c] Culture F28249-NS2(8) was used. Fermentations were carried out in 500-ml Erlenmeyer flasks containing 100 ml of fermentation medium A.

[d] Fermentation yields at 6 days.

[e] An improved culture F28249-PF3-6 was developed and used in these experiments. Fermentations were carried out in 250-ml baffled flasks containing 25 ml of fermentation medium C. Only 15 ml of fermentation medium C was used in the incorporation of [2-^{13}C]acetate and $^{18}O_2$.

—: No ^{14}C labeled precursors were used.

Table 3. Incorporation of sodium [1-^{13}C,$^{18}O_2$]acetate, sodium [2-^{13}C]acetate, sodium [1-^{13}C,$^{18}O_2$]propionate and sodium [1-^{13}C]-2-methylpropionate into LL-F28249α as determined by ^{13}C NMR[a].

^{13}C shift (ppm)[b]	Carbon No.	Relative abundance[c] of ^{13}C in LL-F28249α produced from			
		[1-^{13}C,$^{18}O_2$]-Acetate	[2-^{13}C]-Acetate	[1-^{13}C,$^{18}O_2$]-Propionate	[1-^{13}C]-2-Methyl-propionate[d]
173.33	1	3.8*	0.8	1.5	0.8
142.21	11	1.9**	1.3	3.2*	1.4
140.35	8	1.2	1.7**	1.2	—
138.64	4	1.8	2.2**	1.1	—
137.05	27	1.0	0.9	1.0	13.0*
136.92	14	1.4	1.6**	1.6	0.9
131.74	26	1.1	1.9**	1.5	—
123.87	10	1.0	2.4*	1.0	—
120.99	15	3.0*	0.8	1.0	1.6
120.56	9	3.2*	0.8	1.0	1.4
118.06	3	1.8**	1.3	2.9*	1.0
100.15	21	2.5*	0.7	1.7	1.0
80.60	7	2.2**	0.9	4.3*	0.8
80.06	6	1.1	2.9*	1.0	—
77.33	25	1.7**	1.3	3.5*	1.3
69.15	23	1.7**	1.4	3.6*	1.2
68.98	17	3.5*	1.0	1.1	1.2
68.28	8-CH_2	0.9	2.1***	0.9	—
67.93	5	4.0*	0.9	1.0	1.5
67.67	19	3.1*	0.9	1.1	1.9
48.54	13	2.1**	1.5	2.6*	0.9
46.35	2	1.0	2.4*	1.0	—
41.59	22	1.0	3.5*	0.8	—
41.51	20	0.8	3.8*	0.8	—
36.65	24	1.0	2.4**	0.8	—
36.40	18	0.8	4.9*	0.8	0.7
36.04	12	0.9	2.1**	0.9	0.8
35.12	16	0.9	3.4*	0.8	—
27.12	28	1.1	0.8	1.0	—
23.04	29	0.8	1.0	0.8	—
22.98	28-CH_3	0.8	1.0	0.7	—
22.57	12-CH_3	0.9	2.3***	0.8	—
19.97	4-CH_3	1.0	2.2***	0.9	—
15.37	14-CH_3	0.9	2.4***	0.9	—
14.46	24-CH_3	0.9	2.6***	0.8	—
11.17	26-CH_3	0.8	2.4***	0.8	—

[a] The concentration of natural abundance and ^{13}C enriched LL-F28249α was 86 mM in C_6D_6. The broad band proton decoupled ^{13}C NMR Fourier transform spectra were recorded on a Varian XL-300 NMR spectrometer at 75.47 MHz using an internal deuterium lock of C_6D_6 at 24.6°C.

[b] Downfield from $(CH_3)_4Si$.

[c] Peak height ratio of ^{13}C enriched to natural abundance LL-F28249α.

[d] The ^{13}C NMR spectrum was recorded using $CDCl_3$ as the solvent.

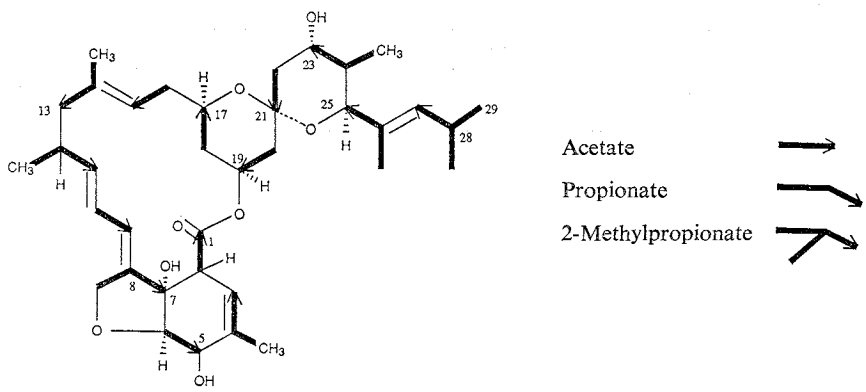
* Denotes primary ^{13}C enrichment.

** Denotes secondary ^{13}C enrichment from [2-^{13}C]propionate.

*** Denotes secondary ^{13}C enrichment from [3-^{13}C]propionate.

were assigned by analogy with the milbemycins α_2, α_4 and D[10]. The ^{13}C NMR assignments of LL-F28249α have been independently determined by our colleague S. RAJAN using the "INADEQUATE" experiment, which will be published separately.

Fig. 2. Origin of the carbon atoms of LL-F28249α.

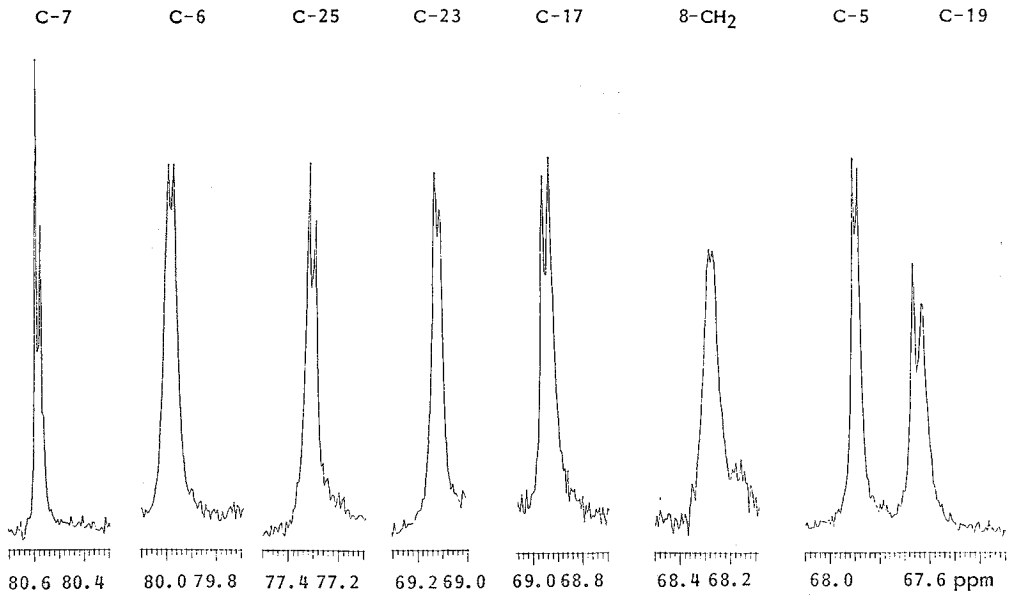


The experiments using ^{13}C labeled acetates showed that carbons 1, 5, 9, 15, 17, 19 and 21 were derived from C-1 of acetate and that carbons 2, 6, 10, 16, 18, 20 and 22 were derived from C-2 of acetate. These results indicate that seven acetate units were incorporated into LL-F28249α. In the sample derived from the experiment using [1-^{13}C]propionate, ^{13}C enrichment at carbons 3, 7, 11, 13, 23 and 25 were observed, indicating that six propionate units were incorporated into LL-F28249α. The experiment using [1-^{13}C]-2-methylpropionate showed that only carbon 27 was derived from C-1 of 2-methylpropionate. The results described above suggest that the carbon skeleton of LL-F28249α is derived from seven acetate, six propionate and one 2-methylpropionate units (Fig. 2). Excluding the side chain portion, this labeling pattern is identical to that determined for milbemycin[10] and avermectin[11].

It is noteworthy that incorporation of [1-^{13}C]acetate led to secondary enrichment of carbons derived from [1-^{13}C]propionate, and incorporation of [2-^{13}C]acetate led to secondary enrichment of carbons derived from [2-^{13}C]propionate and [3-^{13}C]propionate. Such secondary enrichments were also shown in the biosynthesis of maduramicin[9], milbemycin[10], rifamycin[12], leucomycin[13], tylosin[14], lysocellin[15] and oligomycin A[16]. Presumably acetate is converted to propionate during the Kreb's cycle[9]. On the other hand, the incorporation experiment using [1-^{13}C]propionate did not result in the enrichment of carbons originating from [1-^{13}C]acetate or [2-^{13}C]acetate.

With the fundamental precursors of the carbon skeleton firmly established, we directed our effort to the determination of the origin of the oxygen atoms of LL-F28249α. Sodium [1-^{13}C,$^{18}O_2$]acetate (1 g/liter) was added at 96 hours and at 120 hours to each fermentation flask. After an additional 48 hours at 27°C (235 rpm), the resulting doubly labeled LL-F28249α was isolated and analyzed by 75.47 MHz ^{13}C NMR (Fig. 3). The signals for C-5, C-17 and C-19 each appeared as an enhanced pair of resonances corresponding to the respective $^{13}C^{16}O$ and $^{13}C^{18}O$ species (Table 4). The signal corresponding to C-1 was too broad to observe the expected pair of signals. Therefore (O)-2, (O)-3, (O)-6 and perhaps (O)-1 are ^{18}O enriched and derived from acetate (Fig. 4). Similarly, sodium [1-^{13}C,$^{18}O_2$]propionate (0.67 g/liter) was administered at 72, 96 and 120 hours, respectively to each fermentation flask. After an additional 48 hours at 27°C (235 rpm), LL-F28249α was isolated and analyzed by ^{13}C NMR. The ^{13}C NMR revealed the presence of excess ^{18}O at C-7, C-23 and C-25, as evidenced by the enhanced pair of signals (Fig. 3). Therefore (O)-4, (O)-8 and (O)-7 are ^{18}O enriched and derived from propionate (Fig. 4). The presence of ^{18}O label at (O)-4 suggests that the

Fig. 3. Section of the 75.47 MHz broad band proton-decoupled ^{13}C NMR spectrum of ^{13}C and ^{18}O labeled LL-F28249α.



C-5, C-17 and C-19 signals are from LL-F28249α derived from [1-^{13}C,$^{18}O_2$]acetate. C-7, C-23 and C-25 signals are from LL-F28249α derived from [1-^{13}C,$^{18}O_2$]propionate. C-6 and 8-CH_2 signals are from LL-F28249α derived from [2-^{13}C]acetate and $^{18}O_2$ gas.

Table 4. Incorporation of [1-^{13}C,$^{18}O_2$]acetate, [1-^{13}C,$^{18}O_2$]propionate and [2-^{13}C]acetate-$^{18}O_2$ gas into LL-F28249α.

Precursor	Carbon No.	^{13}C shift (ppm)	$\Delta\delta$ (ppm)	$^{18}O:^{16}O$
[1-^{13}C,$^{18}O_2$]Acetate	1	173.33[a]	—	—
	21	100.15	0	0:100
	17	68.98	0.027	51:49
	5	67.93	0.027	51:49
	19	67.67	0.046	48:52
[1-^{13}C,$^{18}O_2$]Propionate	7	80.60	0.022	40:60
	25	77.33	0.028	46:54
	23	69.15	0.033	47:53
[2-^{13}C]Acetate and $^{18}O_2$ gas	6	80.06	0.033	—
	8-CH_2	68.28	0.021	—

[a] The peak was too broad to observe the expected doublet.

formation of the cyclohexene ring may arise through aldol condensation rather than electrophilic cyclization of polyolefinic intermediates. Only a singlet peak corresponding to the spiroketal carbon C-21 was observed in the doubly labeled samples incorporated by either sodium [1-^{13}C,$^{18}O_2$]acetate or sodium [1-^{13}C,$^{18}O_2$]propionate. This suggests that the hydroxyl groups (O)-6 and (O)-7 from C-17 and C-25 react with keto group of C-21 to generate the spiroketal. This biosynthetic pathway is supported by the work of CANE *et al.*[11] on avermectin.

The bio-origin of all the oxygen atoms was definitively established except for (O)-1 which was ambiguous and (O)-5, which we speculated was derived from molecular oxygen. To test this hy-

Fig. 4. Origin of the oxygen atoms of LL-F28249α.

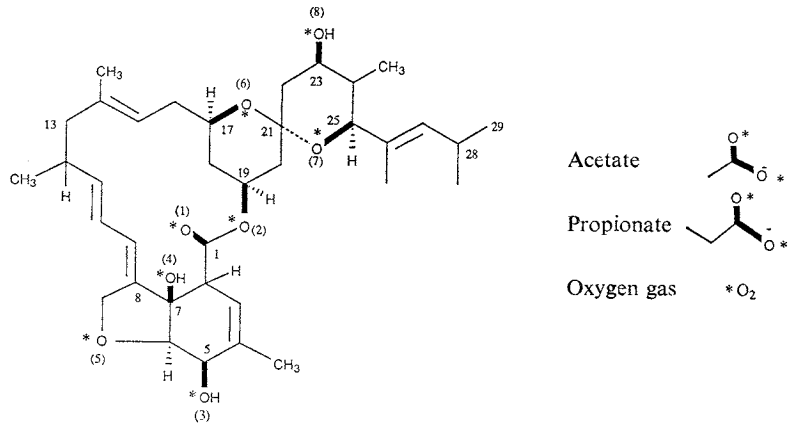
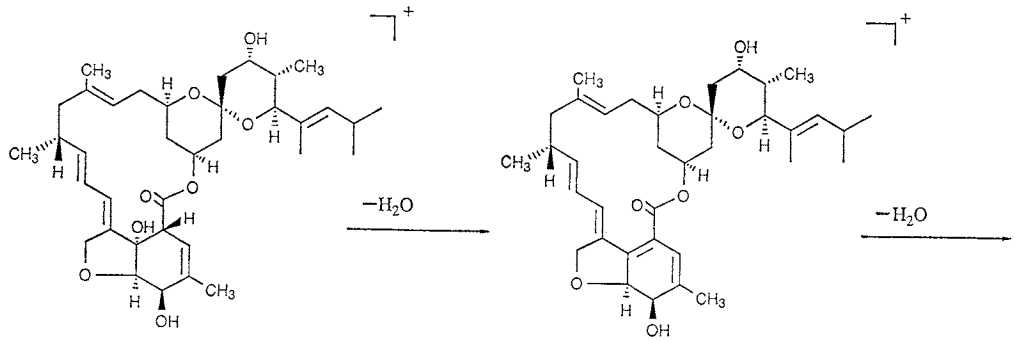
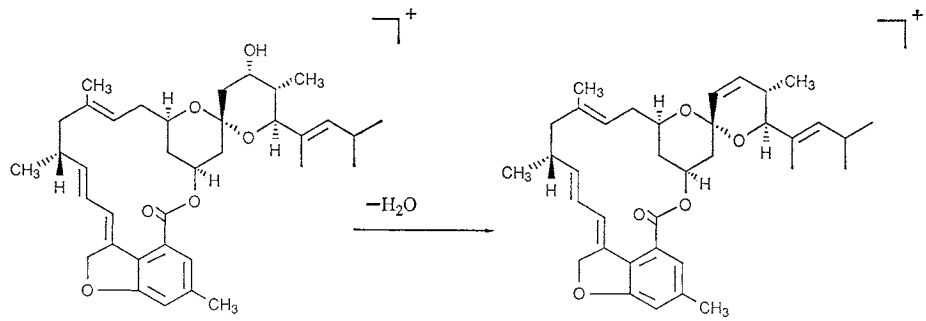


Fig. 5. CI-MS fragmentation of LL-F28249α.



$C_{36}H_{52}O_7{}^{18}O$ m/z 615 (M+1) $C_{36}H_{50}O_6{}^{18}O$ m/z 597 (M+1$-H_2O$)

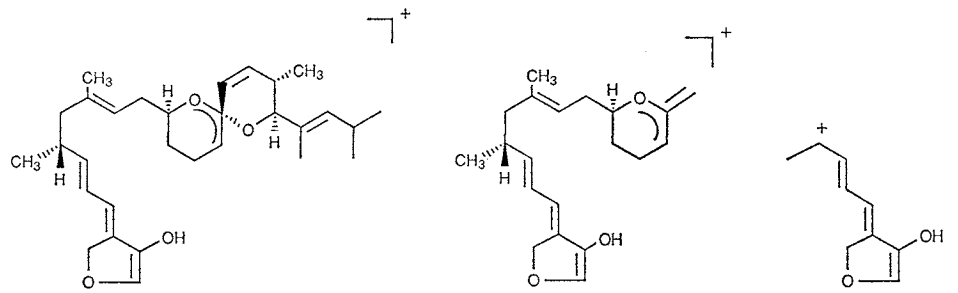


$C_{36}H_{48}O_5{}^{18}O$ m/z 579 (M+1$-2H_2O$) $C_{36}H_{46}O_4{}^{18}O$ m/z 561 (M+1$-3H_2O$)

pothesis, sodium [2-^{13}C]acetate (1 g/liter) was added to a specially-designed fermentation flask[17] at 96 hours and again at 120 hours. Furthermore, $^{18}O_2$ was administered to the same fermentation flask from 96 to 168 hours. At the end of 168 hours, the ^{13}C NMR analysis of the doubly labeled LL-F28249α indicated the presence of excess ^{18}O at (O)-5 as evidenced by the enhanced pair of signals at C-6 and a pair of signals at the 8-methylene carbon.

The bio-origin of (O)-5 was independently supported by mass spectrometry of LL-F28249α produced in the presence of $^{18}O_2$. The chemical ionization mass spectrum of the ^{18}O labeled sample

Fig. 6. EI-MS fragmentation of LL-F28249α.



$C_{30}H_{42}O_3{}^{18}O$ m/z 468 $C_{20}H_{26}O_2{}^{18}O$ m/z 316 $C_9H_{11}O^{18}O$ m/z 153

showed that 84% of the molecule contained one ^{18}O and that 15% had none. Signals were observed at m/z 615, 597, 579 and 561 corresponding to $(M+H)^+$, $(M+H-H_2O)^+$, $(M+H-2H_2O)^+$ and $(M+H-3H_2O)^+$ ions (Fig. 5). Corresponding positive ions were also observed for the unlabeled LL-F28249α. Presumably these losses of water involve the hydroxyl groups and therefore these data suggests that none of the hydroxyl groups are derived from molecular oxygen. The electron impact (EI) ionization fragmentation pattern gave intense ions at m/z 153, 316, 427, 450 and 468 (Fig. 6). Each of these ions had one heavy oxygen atom. Less intense signals seen at m/z 219, 237, 247 and 265 have one to three oxygen atoms but no ^{18}O label. An inspection of the structures shows that only fragments containing the furan ring have the labeled oxygen atom. The simple 3-hydroxy-5,6-dihydrofuran fragment m/z 153 has only two oxygen atoms and since the hydroxyl oxygen was lost as $H_2{}^{16}O$ from the chemical ionization (CI)-MS spectrum, the labeled oxygen atom must be the oxygen in the furan ring connecting carbon 6 and 8-methylene carbon. Furthermore, the absence of other ^{18}O labeled fragments indicates that the (O)-1 is not derived from oxygen gas and therefore must be brought in with the acetate molecule.

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