BIOSYNTHESIS OF THE ANTIBIOTIC MADURAMICIN

ORIGIN OF THE CARBON AND OXYGEN ATOMS AS WELL AS THE ^{13}C NMR ASSIGNMENTS

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The biosynthesis of maduramicin α and β in a culture of *Actinomadura yumaensis* has been studied using ^{13}C, ^{14}C and ^{18}O labeled precursors. The α component of this recently discovered polyether antibiotic, containing forty-seven carbon atoms in a seven-ring system, is derived from eight acetate, seven propionate and four methionine molecules. The β component which is missing one methoxy group incorporates three methionine methyl groups. The carbohydrate moiety was enriched by methionine, but not significantly by acetate or propionate. Studies of the incorporation of ^{13}C labeled precursors permit the ^{13}C NMR assignment of maduramicin. The origin of oxygen atoms of maduramicin has been examined by feeding [1-^{13}C, $^{18}O_2$]acetate and [1-^{13}C,$^{18}O_2$]propionate separately in the fermentation culture and the resulting doubly labeled maduramicin samples were analyzed by the isotopic shifts in the ^{13}C NMR spectra. These results are consistent with the initial formation of a triene, which is converted to maduramicin by cyclization of the triepoxide.

Among polyether antibiotics, monensin and lasalocid provide important veterinary applications in the control of coccidiosis. In this paper, we wish to report the biogenesis of maduramicin, a 20-fold more potent anticoccidial polyether antibiotic discovered independently by LABEDA[1] and LIU[2]. Coccidial infections in poultry were controlled by maduramicin at a level of 5 ppm.

The structure of maduramicin shown in Fig. 1 was determined by X-ray crystallographic analysis[2]. It shares structural similarity with other polyether ionophores, such as mutalomycin, 6061, septamycin and K-41A (Fig. 2). Based on the reported biosynthetic information of polyether ionophores[3], we speculated that the carbon skeleton of maduramicin α was derived from eight acetate, seven propionate and four methionine molecules. We were able to prove this postulation by feeding several ^{13}C and ^{14}C labeled acetates, propionates and methionine in the fermentation cultures of maduramicin. The resulting ^{13}C enriched maduramicin samples enabled us to assign the complex proton decoupled ^{13}C NMR spectra of maduramicin, which is made of forty-seven ^{13}C resonances. The accuracy of the assignments was subsequently verified when we succeeded in assigning the ^{13}C NMR spectrum of maduramicin α by double quantum NMR techniques on natural abundance maduramicin, which is the subject of another publication by us[4]. By comparison of the ^{13}C NMR spectra of maduramicin α and β, we were able to determine the structure of the minor component, maduramicin β. In addition, these incorporation con-

Fig. 1. The structure of maduramicin sodium.
Maduramicin sodium α, R=CH_3; maduramicin sodium β, R=H.

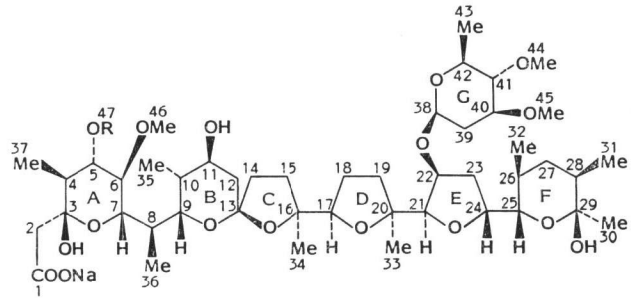
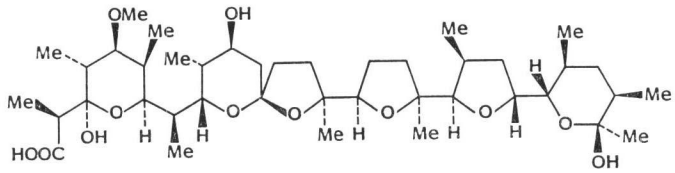
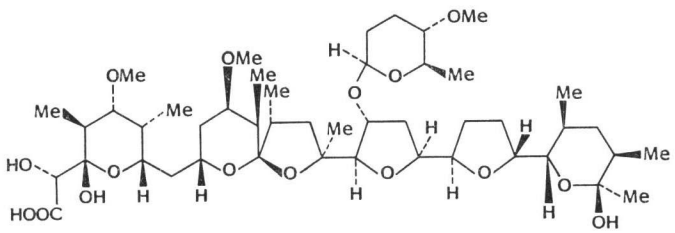


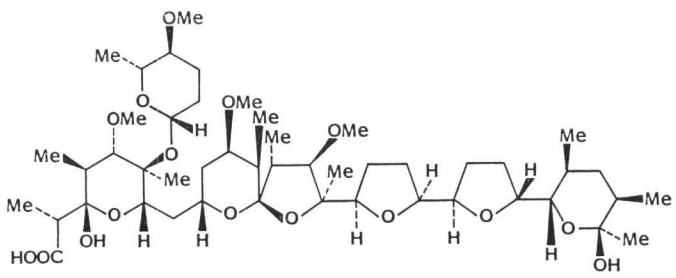
Fig. 2. The structures of mutalomycin, 6016, septamycin and K-41A.



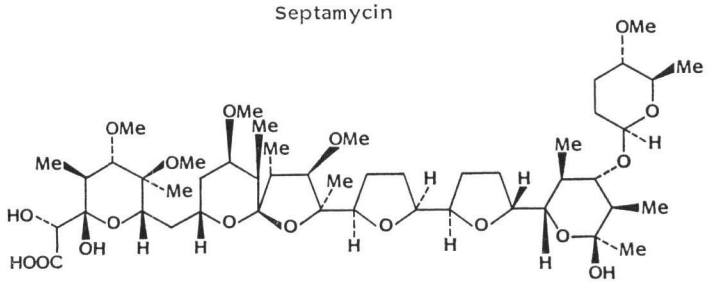
Mutalomycin



6016



Septamycin



K-41A

ditions can be used to prepare high specific activity ^{14}C and ^{3}H labeled maduramicin for tissue residue studies and radioimmunoassay of maduramicin.

Furthermore, we were interested in establishing the details of the biosynthetic pathways by which maduramicin is formed from simple precursors. To address this question, we incorporated [1-^{13}C,$^{18}O_2$]-acetate and [1-^{13}C,$^{18}O_2$]propionate in the maduramicin molecule to determine the origin of the oxygen atoms of the resulting doubly labeled maduramicins by high resolution ^{13}C NMR spectroscopy.

Experimental

Organism

Actinomadura yumaensis culture NRRL 12515 was used for this work.

Culture Conditions

The ingredients of the seed culture medium used were (in g/liter): Bacto beef extract (3), Bacto Tryptone (5), glucose (10), Bacto yeast extract (5) and Bacto agar (1). The seed culture medium (65 ml) was inoculated with 1 ml of a thawed suspension of *A. yumaensis* which had been stored at −80°C, and propagated on a shaker bath (100 rpm) at 32°C for 3 days.

Two and a half ml of the inoculum were transferred to each of a series of 250-ml Erlenmeyer flasks containing 50 ml of the selected media. Three types of fermentation media were used with the following composition (g/liter): Medium A: Glucose (15), soy peptone (7.5), Mississippi lime $CaCO_3$ (0.5) and NaCl (1); Medium B: Glucose (30), soy flour (15), Mississippi lime $CaCO_3$ (1) and NaCl (2); Medium C: Glucose (75), soy flour (25), Gamaco $CaCO_3$ (2) and NaCl (2). All the fermentations were carried out on a rotary shaker at 32°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, sodium [2-^{13}C]propionate and L-[*methyl*-^{13}C]methionine of 90 atom % ^{13}C, were purchased from Merck Sharp and Dohme Isotopes (St. Louis, MO); sodium [1-^{14}C]acetate (specific radioactivity 59.0 mCi/mmol), sodium [2-^{14}C]-acetate (54.0 mCi/mmol), sodium [1-^{14}C]propionate (58.4 mCi/mmol), sodium [2-^{14}C]propionate (2.5 mCi/mmol) and L-[*methyl*-^{14}C]methionine (51.4 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Sodium [1-^{13}C,$^{18}O_2$]propionate was prepared from sodium [1-^{13}C]propionate (90 atom % ^{13}C, Merck Sharp and Dohme Isotopes, St. Louis, MO) and $H_2{}^{18}O$ (98.3 atom % ^{18}O, Prochem, London, UK) by a literature procedure[5]. The isotopic composition of the product was determined by ^{13}C NMR to be 64.4% $^{13}C^{18}O_2$, 27.2% $^{13}C^{18}O$ and 8.3% $^{13}C^{16}O_2$. Sodium [1-^{13}C,$^{18}O_2$]acetate was prepared by an analogous procedure to yield 64.2% $^{13}C^{18}O_2$, 28.4% $^{13}C^{18}O$ and 7.3% $^{13}C^{16}O_2$. Sodium [1-^{13}C, 2,2-$^{2}H_2$]propionate was synthesized by heating sodium [1-^{13}C]propionate (90 atom % ^{13}C, Merck Sharp and Dohme Isotopes) in basic $^{2}H_2O$ (99.8 atom % ^{2}H, Aldrich Chem. Co., Milwaukee, WI)[6]. The product showed 97.4 atom % ^{2}H in the C-2 position of the propionate by ^{1}H NMR spectroscopy.

Incorporation of Isotope-labeled Substrates

In order to find the optimal condition for the highest ^{13}C enrichment of maduramicin, 5 μCi of ^{14}C labeled substrate was mixed with the measured unlabeled substrate and added aseptically to each fermentation flask from 0 hour to 72 hours post-inoculation.

After seven days of fermentation at 32°C, the broth volume was adjusted to 50 ml with H_2O. A representative 25 ml of fermentation broth was withdrawn into a 250-ml centrifuge bottle, followed by addition of 25 ml of MeOH. After occasional shaking for 1 hour, 25 ml of CH_2Cl_2 was added, followed by 75 ml of H_2O. The bottle was stoppered, shaken for 2 minutes and centrifuged at 850 rpm for 15 minutes. The upper aqueous layer was siphoned and discarded. A 5-ml sample from the remaining CH_2Cl_2 solution was transferred into a flask and evaporated. The residue was dissolved in 1 ml of the solvent system, 0.1 M NH_4OAc (pH 5.0) - MeOH, 8: 92, containing di-*n*-octylphthalate as an internal standard. Then a 40-μl aliquot was injected into an HPLC equipped with a Ultrasphere-ODS column (Altex, 5 μm, 4.6 mm × 25 cm). The chromatograph was operated isocratically at a flow rate of 1.5 ml/

minute using the solvent system, 0.1 M NH_4OAc (pH 5.0) - MeOH, 6.5: 93.5. Detection was achieved by a Water's differential refractometer. The radioactivity of the collected product permitted the calculation of % ^{14}C incorporation of the substrate.

Standard solutions of authentic maduramicin were prepared containing 5 mg/ml of di-*n*-octylphthalate as internal standard. From the HPLC data of these standard solutions, a standard straight line was plotted and employed to calculate the maduramicin yield of the fermentation. From the % ^{14}C incorporation data and the fermentation yield, the ^{13}C enrichment in maduramicin was calculated for the proposed biosynthetic precursors.

After the optimal condition was chosen, ^{13}C incorporation was carried out using a mixture of ^{13}C and the corresponding ^{14}C labeled substrate (3~10 μCi) for each of the ten fermentation flasks. At the end of the fermentation, a small portion of the fermentation broth was assayed by HPLC as described above and the rest was worked up to isolate maduramicin.

Isolation of Maduramicin

The fermentation broth (500 ml) was harvested and the pH was adjusted to 8.0 with sodium bicarbonate, after which it was stirred overnight with an equal volume of EtOAc. After the addition of Celite (28 g) and filtration through a Celite cake, the organic layer was separated and dried over magnesium sulfate. After filtration and evaporation, the residue was dissolved in 10 ml of $CHCl_3$ and chromatographed on a column (1.6 cm×85 cm) packed with 170 ml of Woelm silica gel. The column was eluted with the following solvent systems consecutively: (1) $CHCl_3$ - EtOAc, 7: 3, 600 ml; (2) $CHCl_3$ - EtOAc 3: 7, 400 ml and (3) EtOAc, 200 ml. Each fraction (50 ml) was checked by TLC on a silica gel plate (E. Merck) in the solvent system EtOAc - CH_2Cl_2, 7: 3. Detection was made by spraying with 5% sulfuric acid in methanol, followed by heating with a heat gun. Fractions numbered 4 to 11 were combined and the solvents were removed *in vacuo*. The residue was recrystallized from ethyl ether - *n*-hexane to yield 1.4647 g of white crystalline maduramicin sodium α, mp 195~198°C. Likewise work up of fractions numbered 14 to 19 yielded 0.1120 g of maduramicin sodium β. The % ^{13}C incorporation was determined by ^{13}C NMR spectroscopy using a Bruker CXP 300 spectrometer.

Results and Discussion

Three kinds of fermentation media were tested and their production profiles are shown in Table 1. The maduramicin production began approximately 48 hours post-inoculation and increased linearly for at least 120 hours. Among the three media, the medium containing the most glucose produced the highest yield of antibiotic.

Table 1. Maduramicin production profile in different fermentation media.

Fermentation time (hours)	Yield (g/liter)		
	Medium A[a]	Medium B[a]	Medium C[a]
48	—	0.23	—
72	—	0.89	0.40
96	1.21	1.73	1.30
120	1.49	2.02	2.48
144	1.83	2.12	2.60
168	—	—	2.76

[a] Fermentations were carried out in 250-ml Erlenmeyer flasks containing 50 ml of fermentation medium. The compositions (g/liter) of the employed fermentation media are as follows: Medium A – Glucose (15), soy peptone (7.5), Mississippi lime $CaCO_3$ (0.5) and NaCl (1). Medium B – Glucose (30), soy flour (15), Mississippi lime $CaCO_3$ (1) and NaCl (2). Medium C – Glucose (75), soy flour (25), Gamaco $CaCO_3$ (2) and NaCl (2).

Before utilizing the ^{13}C enriched substrates, a series of fermentations were performed using various amounts of ^{14}C labeled substrates in different fermentation media. The % ^{14}C incorporation and the calculated % ^{13}C enrichment based on the postulated precursors are summarized in Table 2. Whereas methionine did not stimulate the maduramicin biosynthesis, acetate at levels up to 1.15 g/liter increased the production of maduramicin. The fermentation was sensitive to early addition of propionate especially in the high glucose medium (Medium C). Even at 0.25 g/liter level, the maduramicin yield was

Table 2. Incorporation of ^{14}C labeled substrates into maduramicin sodium α.

Precursor (g/liter)		Addition time (hours)	Fermentation media[a]	Maduramicin (g/liter) at 7 days	^{14}C Incorporation (%)	Calculated ^{13}C enrichment[b] (%)
None			A	1.83[c]		
			B	2.12[c]		
			C	2.76		
[1-^{14}C]Acetate	1	0	B	2.56	4.80	2.20
	1	72	C	3.47	9.27	3.36
[2-^{14}C]Acetate	0.76	72	C	3.53	20.79	6.15
	0.93	72	C	3.15	19.76	7.91
	1.15	72	C	3.07	15.30	8.58
[1-^{14}C]Propionate	0.40	0	A	1.79	1.33	0.37
	0.40	24	A	1.78	2.65	0.75
	0.40	48	A	1.93	3.16	0.82
	0.20	0	B	1.73	16.65	2.52
	0.20	24	B	1.72	17.15	2.62
	0.20	48	B	1.96	18.30	2.45
	0.25	48	B	2.11	18.64	2.48
	0.50	48	B	1.72	12.38	4.05
	0.25	0	C	1.53	3.62	0.74
	0.50	0	C	0.87	1.39	1.00
L-[*Methyl*-^{14}C]-methionine	0.78	72	C	2.48	41.15	17.64

[a] Compositions of the fermentation media A, B and C are listed in Table 1.

[b] Percent ^{13}C enrichment was calculated on the basis that the carbon skeleton of maduramicin α was derived from eight acetate, seven propionate and four methionine molecules.

[c] Fermentation yields a 6 days.

Table 3. Summary of ^{13}C incorporation studies.

Precursor (g/liter)		Addition time (hours)	Fermentation media	Maduramicin (g/liter) at 7 days	^{14}C Incorporation[a] (%)	^{13}C Enrichment (%)
[1-^{13}C]Acetate	1	72	C	3.08	9.67	2.4
[2-^{13}C]Acetate	1	72	C	3.56	18.99	2.3
[1-^{13}C]Propionate	0.82	24~92	B	— [b]	— [b]	1.5
[1-^{13}C,2,2-$^{2}H_2$]-Propionate	0.5	0	B	1.87	10.05	4.0
[2-^{13}C]Propionate	0.5	0	B	2.05	17.43	2.8
L-[*Methyl*-^{13}C]-methionine	0.78	72	C	2.40	39.76	28.3

[a] For each ^{13}C incorporation study, 3~10 μCi of ^{14}C labeled precursor was mixed with the corresponding unlabeled precursor and added aseptically to each fermentation flask.

[b] This experiment was carried out in a 2-liter chemostat. The precursor was administered continuously between 24 hours and 92 hours. Undetermined amount of maduramicin was lost due to foaming problem. Isolated yield of maduramicin was 1.03 g/liter.

reduced by almost one-half.

L-[*Methyl*-^{14}C]methionine showed a high level of incorporation, as did sodium [2-^{14}C]acetate. Interestingly, sodium [1-^{14}C]acetate was incorporated with only one-half the efficiency of [2-^{14}C]acetate. The incorporation of propionate differed markedly among different media. Both soy peptone and high glucose-containing media (Media A and C) significantly decreased the incorporation of propionate, while medium B gave reasonable incorporation. The addition time of these substrates was not critical between

Table 4. Incorporation of sodium [1-^{13}C]acetate, sodium [2-^{13}C]acetate, sodium [1-^{13}C]propionate, sodium [1-^{13}C, 2,2-$^{2}H_2$]propionate, sodium [2-^{13}C]propionate and L-[*methyl*-^{13}C]methionine into maduramicin sodium α as determined by ^{13}C NMR[a].

^{13}C Shift (ppm[b])	Carbon No. in maduramicin	% Abundance[c] of ^{13}C in maduramicin α produced from					
		[1-^{13}C]-Acetate	[2-^{13}C]-Acetate	[1-^{13}C]-Propionate	[1-^{13}C, 2,2-$^{2}H_2$]-Propionate	[2-^{13}C]-Propionate	L-[*Methyl*-^{13}C]-methionine
179.14	1	* 2.6	1.0	1.0	1.0	1.2	1.0
107.52	13	* 2.4	1.0	1.0	1.0	1.0	1.0
97.69	3	1.4	1.4	* 1.6	* 4.0	1.1	1.1
97.03	29	* 2.0	1.0	1.1	1.3	1.0	1.0
95.87	38	1.0	1.0	1.0	1.0	1.0	1.3
86.88	21	* 2.7	1.0	1.1	1.1	1.2	1.2
85.72	41	1.2	1.0	1.1	1.0	1.0	1.1
85.67	5	* 2.6	1.0	1.0	1.0	1.1	1.0
84.67	16	1.0	1.7 △	1.1	1.1	* 3.3	1.0
84.51	20	1.0	1.9 △	1.0	1.0	* 2.7	1.2
82.34	17	* 2.3	1.0	1.2	1.1	1.4	1.0
82.01	6	1.0	* 2.3	1.0	1.0	1.0	1.0
80.88	40	1.1	1.0	1.0	1.0	1.0	1.2
79.92	24	1.0	* 2.1	1.1	1.0	1.2	1.0
75.25	22	1.0	* 2.4	1.0	1.0	1.3	1.1
72.99	25	1.1	1.2	* 1.6	* 4.0	1.1	1.0
71.34	42	1.1	1.0	1.0	1.1	1.0	1.2
70.32	11	* 2.2	1.0	1.0	1.0	1.0	1.0
67.61	9	1.0	1.0	* 1.4	* 3.6	1.0	1.0
67.45	7	1.0	1.0	* 1.6	* 4.3	1.1	1.0
60.69	44	1.1	1.0	1.1	1.1	1.0	* 32.4
60.54	47	1.0	1.0	1.0	1.0	1.0	* 27.8
59.48	46	1.0	1.0	1.0	1.0	1.0	* 22.1
57.05	45	1.0	1.0	1.0	1.1	1.0	* 30.9
45.53	2	1.0	* 2.2	1.0	1.0	1.0	1.0
45.49	4	1.0	1.0	1.0	1.0	* 3.4	1.0
40.00	28	1.0	1.8 △	1.1	1.1	* 3.2	1.0
39.00	14	1.0	* 2.4	1.0	1.1	1.0	1.0
36.90	39	1.1	1.0	1.2	1.2	1.0	1.4
36.54	27	1.3	1.4	* 1.5	* 4.0	1.0	1.2
33.94	12	1.0	* 2.4	1.3	1.1	1.2	1.3
33.78	10	1.0	2.0 △	1.3	1.3	* 3.9	1.0
33.56	15	1.3	1.6	* 1.6	* 3.7	1.3	1.2
33.40	8	1.0	2.0 △	1.0	1.0	* 3.2	1.4
33.31	26	1.0	1.8 △	1.0	1.1	* 3.0	1.0
32.11	19	1.2	1.3	* 1.5	* 4.4	1.3	1.0
30.21	23	* 2.7	1.0	1.2	1.1	1.0	1.3
27.62	34	1.0	1.8 △	1.0	1.1	1.4	1.1
26.88	18	1.0	* 2.1	1.0	1.0	1.2	1.0
26.11	30	1.0	* 2.1	1.0	1.1	1.0	1.0
22.41	33	1.0	1.7 △	1.0	1.0	1.4	1.0
17.91	43	1.0	1.0	1.0	1.0	1.0	1.5
17.62	32	1.0	1.6 △	1.0	1.0	1.0	1.0
16.96	31	1.0	1.7 △	1.0	1.0	1.0	1.0
11.99	37	1.0	1.9 △	1.0	1.0	1.1	1.2
10.95	36	1.0	1.9 △	1.0	1.1	1.6	1.1
10.49	35	1.0	1.9 △	1.0	1.0	1.2	1.3

[a] The concentration of natural abundance and ^{13}C enriched maduramicin sodium was 53 mM. The broad band proton decoupled ^{13}C NMR Fourier transform spectra were recorded in 10 mm sample tubes on a Bruker CXP 300 spectrometer at 75.47 MHz using an internal deuterium lock of C^2HCl_3 at 30°C.

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

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

* Denotes primary ^{13}C enrichment.

△ Denotes secondary ^{13}C enrichment.

Fig. 3. Natural abundance ^{13}C NMR spectrum of maduramicin sodium.
Carbonyl carbon resonance at 179.14 ppm is not shown.

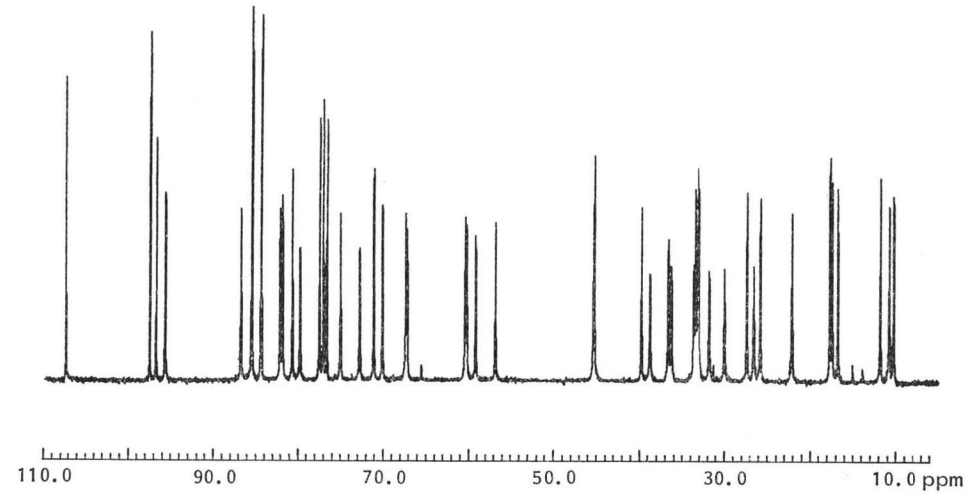
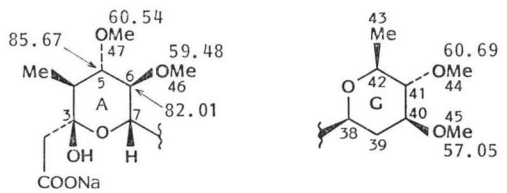
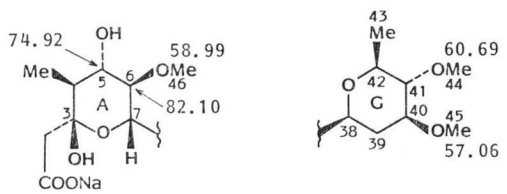


Fig. 4. ^{13}C NMR assignments of methoxy groups in maduramicin sodium α and β.



Maduramicin sodium (α)



Maduramicin sodium (β)

Table 5. Comparison of ^{13}C NMR spectra of maduramicin sodium α and β.

Carbon No.	Chemical shift (δ)		$\Delta\delta$ (ppm)
	α	β	
3	97.69	97.77	0.08
38	95.87	95.87	0
41	85.72	85.71	0.01
5	85.67	74.92	10.75
6	82.01	82.10	0.09
40	80.88	80.87	0.01
24	79.92	79.92	0
42	71.34	71.33	0.01
7	67.45	66.91	0.54
44	60.69	60.69	0
47	60.54	—	—
46	59.48	58.99	0.49
45	57.05	57.06	0.01
39	36.90	36.90	0
43	17.91	17.91	0
37	11.99	12.07	0.08

0 and 72 hours post-inoculation, although propionate added 72 hours after inoculation showed slightly better incorporation.

After the fermentation conditions for the best incorporation of substrates had been established, ^{13}C labeled substrates were utilized for incorporation into maduramicin. The results of these runs are summarized in Table 3. L-[*Methyl*-^{13}C]methionine incorporated substantially into maduramicin. [1-^{13}C]-Acetate, [2-^{13}C]acetate, [1-^{13}C]propionate and [2-^{13}C]propionate also gave reasonably high ^{13}C enrichment in maduramicin. [1-^{13}C, 2,2-$^{2}H_2$]Propionate was used to study the deuterium β-shift of the ^{13}C NMR spectrum of the corresponding labeled maduramicin and the investigation is still in progress. The broad band proton decoupled ^{13}C NMR spectra of the ^{13}C labeled maduramicin α indicated as expected that sixteen carbons were enriched by the 1 and 2 labeled acetate substrates, fourteen carbons by the 1

and 2 labeled propionate substrates and four methoxy carbons by methionine, as shown in Table 4.

A natural abundance ^{13}C NMR spectrum of maduramicin is quite complex as shown in Fig. 3. It consists of forty-seven resonances, arising from forty-two carbons in the seven-ring skeleton, a carboxyl carbon, and four methoxy carbons. Although ^{13}C assignments have been reported for structurally related polyethers, such as mutalomycin, 6016, septamycin and K-41A (Fig. 2) and also a set of empirical rules was proposed[7,8], complete ^{13}C assignments of maduramicin still presented a challenge. For example, there are two pairs of peaks (85.72, 85.67; 45.53, 45.49 ppm), each separated by only 0.05 ppm. And a set of five signals (33.31 ~ 33.94 ppm) appeared within a narrow range of 0.63 ppm. These complex peaks were unambiguously assigned by ^{13}C NMR spectra of the ^{13}C enriched maduramicin (Table 4).

The four methoxy carbons were readily identified at 60.69, 60.54, 59.48 and 57.05 ppm by their enormous enhancement in ^{13}C NMR spectrum of L-[*methyl*-^{13}C]methionine incorporated maduramicin. Explicit assignment of these four methoxy signals relied on the difference between the ^{13}C NMR spectra of the α and β maduramicin, because the β compound has one methoxy group replaced by a hydroxyl group. Comparison of the methoxy signals of the α and β compounds clearly indicated the disappearance of one methoxy carbon signal at 60.54 ppm in the β compound, and the signal at 59.48 ppm shifted to 58.99 ppm, while the remaining pair of methoxy signals at 60.69 and 57.05 ppm were virtually unchanged (Fig. 4). In addition, resonances corresponding to C-38, C-39, C-40, C-41, C-42 and C-43 were undisturbed, while resonances from C-3, C-5, C-6, C-7 and C-37 were all shifted, especially the C-5 signal which was shifted upfield by 10.75 ppm (Table 5). Therefore, in the β compound, the methoxy group (60.54 ppm, C-47) on C-5 (in ring A) must be replaced by a hydroxyl group and the resonance of the neighboring methoxy group (C-46) on C-6 shifted to 58.99 ppm. The remaining methoxy groups (C-44 and C-45) in the G ring were then readily assigned, because C-45 was more shielded than C-44.

The complete ^{13}C assignments of maduramicin are listed in Table 4. The resonances at 79.92 and 82.01 ppm were assigned tentatively to C-24 and C-6, because both carbons were enriched by [2-^{13}C]-acetate. Also they were both methine carbons and unable to be differentiated by DEPT experiment[9], in which carbons attached to different numbers of protons can be distinguished. Furthermore, for structurally similar ionophores, C-6 in K-41A was assigned identically with C-24 in mutalomycin at 78.80 ppm[10]. This ambiguity was resolved by comparing the ^{13}C NMR spectra of maduramicin α and β, because one of these two resonances would be appreciably influenced by the replacement of a methoxy group with a hydroxyl group. In the ^{13}C NMR spectrum of maduramicin β, the resonance at 82.01 ppm was shifted by 0.09 ppm while the resonance at 79.92 ppm was virtually unchanged. Therefore resonances at 79.92 and 82.01 ppm were confidently assigned to C-24 and C-6 respectively. In addition, two signals at 10.49 and 10.95 ppm were empirically assigned to C-36 and C-35, according to the assignment of mutalomycin[10]. Later this pair of assignments was reversed based on our recent two-dimensional

Fig. 5. Bio-origin of maduramicin.

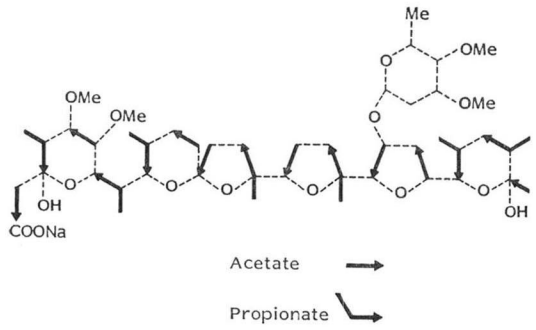
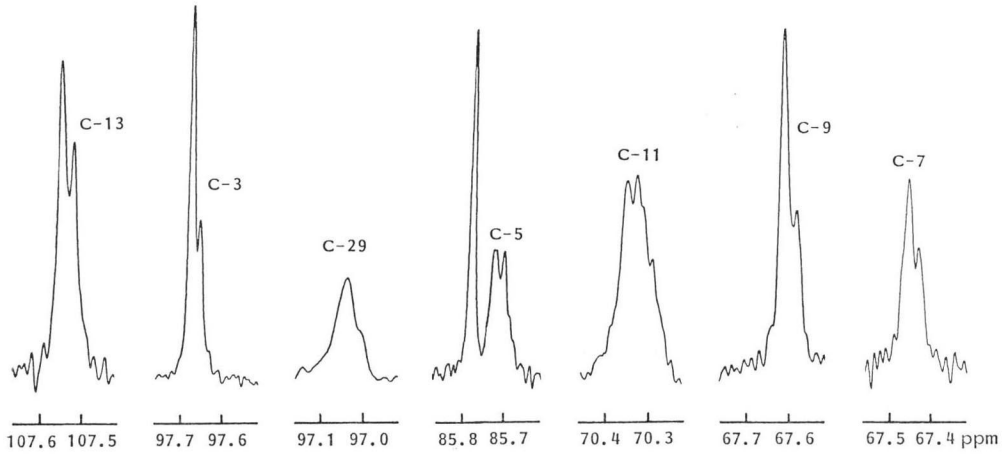
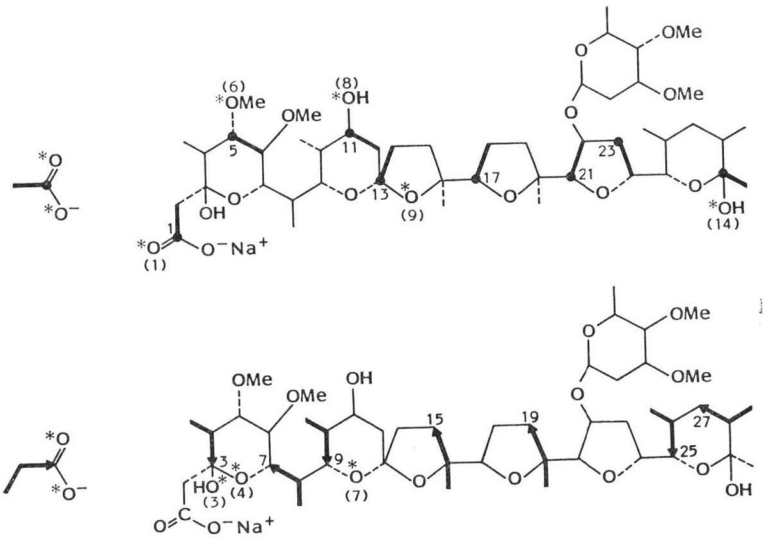


Fig. 6. Section of the 75.47 MHz broad band proton-decoupled ^{13}C NMR spectrum of ^{13}C and ^{18}O labeled maduramicin sodium α.

C-3, C-7 and C-9 signals are from maduramicin derived from [1-^{13}C,$^{18}O_2$]propionate. C-13, C-29, C-5 and C-11 signals are from maduramicin derived from [1-^{13}C,$^{18}O_2$]acetate. Acquisition parameters are given in Table 6.



Scheme 1. Incorporation of [1-^{13}C,$^{18}O_2$]acetate and [1-^{13}C,$^{18}O_2$]propionate into maduramicin sodium α. ●, ▲ Denotes ^{13}C label; * denotes ^{18}O label. Expected enrichment at O-1 was not proved due to the extensive line-broadening of C-1 signal in ^{13}C NMR spectrum.



double-quantum ^{13}C NMR study of maduramicin α, in which all of the carbon-carbon connectivities of both the aglycone and the sugar were determined[4,11]. Taken together these studies fully elucidate the ^{13}C NMR assignments of both maduramicin α and β, and designates the locations and relative concentrations of radiolabeled atoms incorporated into maduramicin from the substrates studied.

From the data given in Tables 3 and 4, it can be seen that the aglycone of maduramicin is assembled from eight acetate and seven propionate units while the four *O*-methyl carbons are derived from methionine. Neither labeled acetate nor labeled propionate produced detectable amount of enrichment

Table 6. Incorporation of [1-^{13}C,$^{18}O_2$]propionate and [1-^{13}C,$^{18}O_2$]acetate into maduramicin.

Precursor	C No.	^{13}C Shift (ppm)	Single pulse[a]		Spin-echo[b]	
			$\Delta\delta$ (ppm)	^{18}O: ^{16}O	$\Delta\delta$ (ppm)	^{18}O: ^{16}O
[1-^{13}C,$^{18}O_2$]Propionate	3	97.67	0.020	32: 68	0.021	30: 70
	7	67.45	0.021	39: 61	—	—
	9	67.61	0.028	33: 67	0.029	33: 67
	15	33.56	0	0: 100	0	0: 100
	19	32.11	0	0: 100	0	0: 100
	25	72.99	0	0: 100	0	0: 100
	27	36.54	0	0: 100	0	0: 100
[1-^{13}C,$^{18}O_2$]Acetate	1	179.14[c]	—	—	—	—
	5	85.67	0.022	47: 53	0.028	51: 49
	11	70.32	0.023	51: 49	0.020	51: 49
	13	107.52	0.024	46: 54	0.027	43: 57
	17	82.34	0	0: 100	0	0: 100
	21	86.88	0	0: 100	0	0: 100
	23	30.21	0	0: 100	0	0: 100
	29	97.03[d]	—	—	—	—

[a, b] Bruker CXP 300, 75.47 MHz; spectral width 8,772 Hz; 128 K data points; quadrature detection, acquisition time 1.87 seconds; 53 mM in $CDCl_3$. Resolution enhancement was achieved by Lorentz-Gauss multiplication of FID prior to Fourier transformation, −1.0 Hz line broadening, 0.3 Gaussian multipler; 0.134 Hz/data point. Isotopic ratio was calculated from peak height.

[a] Acquisition time 1.87 seconds; 30° flip angle; recycle delay 2 seconds; 2K-20K scans.

[b] 90-τ-180 sequence; 90° pulse, 10 μ seconds, delay 70 m seconds; recycle delay 5~15 seconds; 2K-4K scans.

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

[d] The signal was only partially resolved due to line-broadening.

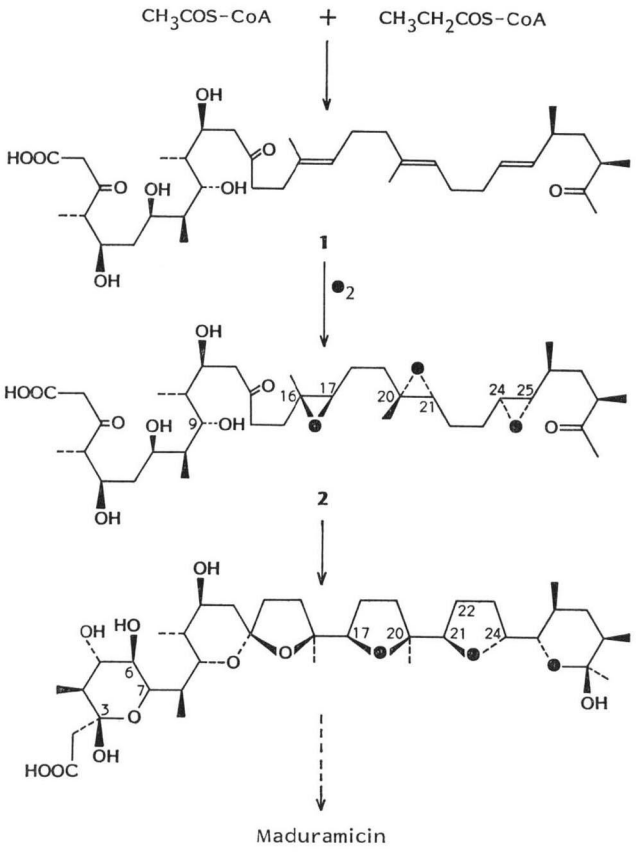
in the sugar ring (G ring) of the maduramicin (Fig. 5). It is noteworthy that incorporation of [2-^{13}C]-acetate led to secondary enrichment of [2-^{13}C]propionate and [3-^{13}C]propionate derived carbons, presumably *via* conversion of acetate to propionate during the Krebs' cycle[12~14]. This could explain the early result that [2-^{14}C]acetate was incorporated into the antibiotic twice as efficiently as [1-^{14}C]acetate.

With the fundamental precursors of the carbon skeleton firmly established, we directed our effort to determining the origin of the oxygen atoms of maduramicin. For monensin, CANE and coworkers[12,15] have recently established the origin of oxygen atoms by incorporation of [1-^{13}C,$^{18}O_2$]acetate, [1-^{13}C,$^{18}O_2$]-propionate and $^{18}O_2$ gas respectively into monensin. A similar study of incorporation of molecular oxygen in monensin was reported by AJAZ and ROBINSON[16].

Sodium [1-^{13}C,$^{18}O_2$]propionate was added to the fermentation culture and the resulting doubly labeled maduramicin was analyzed by high-resolution, 75.47 MHz ^{13}C NMR using both single pulse and spin-echo techniques[17] (Fig. 6). The signals corresponding to C-3, C-7 and C-9 each appeared as an enhanced pair of signals corresponding to the respective $^{13}C^{16}O$ and $^{13}C^{18}O$ species (Scheme 1). As summarized in Table 6, the observed ^{18}O enrichments deviated slightly from the theoretical maximum of 70%, reflecting varying degrees of oxygen exchange at each site. No ^{18}O was present at C-25, whose ^{13}C NMR signal appeared as an enhanced singlet as did those for the three non-oxygen-bearing carbons, C-15, C-19 and C-27. Therefore O-3, O-4 and O-7 are ^{18}O enriched. It is also clear that the tetrahydropyranyl O-4 and the hemiketal hydroxyl O-3 were derived from C-7 and C-3 propionate units respectively.

Similarly labeled maduramicin was prepared by feeding [1-^{13}C,$^{18}O_2$]acetate. The ^{13}C NMR revealed the presence of excess ^{18}O at C-5, C-11 and C-13, as evidenced by the enhanced pair of signals. Since

Scheme 2. Postulated mechanism for the biosynthesis of maduramicin.



the tetrahydropyranyl oxygen, O-7 of the C-13 spiroketal had been shown to be derived from the C-9 propionate unit, the acetate-derived oxygen at ^{13}C was assigned to the tetrahydrofuran moiety, O-9. A similar distinction was not possible at C-29. However based on the assignment of monensin[12], the acetate-derived ^{18}O attached to C-29 in maduramicin appeared to be the hemiketal hydroxyl oxygen. The signal corresponding to C-1 was too broad to observe the expected doublet, whereas signals for C-17, C-21 and C-23 each appeared as enhanced singlets. In general, spin-echo experiments produced better resolution of the paired signals from $^{13}C^{18}O$ and $^{13}C^{16}O$ species than did the single pulse experiments. This is due to the suppression of overlap due to ^{13}C-^{13}C couplings.

Having established that at least seven, probably eight oxygen atoms of maduramicin are derived from the carboxylate oxygens of the carbon-skeleton precursors acetate and propionate (Scheme 1), we turned our attention to speculate the biosynthetic pathways which would allow us to explain all of our established results.

Recently, CANE *et al.*[18] proposed a unified stereochemical model for more than 30 different polyether antibiotics. According to this model, maduramicin is clearly a new member of the APPA class of polyethers. In the APPA class, the first four biogenetic units giving rise to this ring in all cases are acetate, propionate, propionate and acetate. Recent investigations of the biosynthesis of one member of the APPA class of polyethers, monensin, have provided some experimental evidence for the bio-

chemical model[12,15]. It was postulated that epoxidation of the initially formed triene, followed by a cascade of cyclization of the triepoxide to generate all five ether rings of monensin. This mechanistic postulation is consistent with WESTLEY's early suggestion that the various tetrahydrofuran and tetrahydropyran rings of the polyethers are formed by sequential opening of a polyepoxide precursor[19].

Analysis of our incorporation results suggests that maduramicin biosynthesis shares a similar biochemical mechanism. The first-formed polyfunctional fatty acid would be the all-*E*-triene **1** (Scheme 2). The triene **1** presumably undergoes epoxidation by one or more oxidases to give 16*R*, 17*R*, 20*R*, 21*R*, 24*S*, 25*S*-triepoxide **2**. Then ring A was formed by the attack of C-7 hydroxyl on the C-3 carbonyl oxygen. The remaining five ether rings might be generated through a cascade of ring closures initiated by the attack of C-9 hydroxyl of **2**. This hypothetical sequence would account for the stereochemistry at C-6, C-7, C-16, C-17, C-20, C-21, C-24 and C-25 of maduramicin. Therefore it is likely that three out of a total of seventeen oxygens originate from molecular oxygen and C-6 hydroxyl from water. Hydration at C-22 is thought to be catalyzed by a hydrolase. It is not clear when the glycoside linkage is formed.

Although no sugar-deficient maduramicin was observed in the fermentation broth, the glycoside formation may well be the last step as indicated in the biosynthesis of erythromycin[20]. However the possibility of glycoside formation before the simultaneous ring closures may not be ruled out. Work is continuing to clarify these complex biochemical mechanisms.

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