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Biosynthesis of Fredericamycin A, a New Antitumor Antibiotic†

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Received June 11, 1984

ABSTRACT: Fredericamycin A (FM A), produced by a strain of *Streptomyces griseus*, represents a new structural class of antitumor antibiotics containing a spiro ring system. Studies on the producer organism showed that glucose in the fermentation medium is not utilized until late in the growth stage, just prior to synthesis of FM A. [^{14}C]Glucose tracer experiments demonstrated that glucose is incorporated into FM A by catabolism to acetate. Biosynthetic enrichment of FM A with single- and double-labeled [^{13}C]acetate showed that the entire carbon skeleton of the spiro ring system is derived from acetate. L-Methionine was shown to provide the only nonskeletal carbon in FM A, the methoxy carbon at position C-6. The direction of the polyketide chain and the position of the carbon lost during biosynthesis were established by using stable isotope experiments. A general model for FM A biosynthesis is proposed, and a possible scheme for the formation of the spiro carbon center is presented.

Fredericamycin A (FM A)[1] (NSC 305263) is an antitumor antibiotic produced by *Streptomyces griseus* (FCRC-48) (Pandey et al., 1981). In addition to possessing in vitro activity against Gram-positive bacteria and fungi, FM A was shown

† This research was supported by National Cancer Institute Contract N01-CO-23910.
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[1] Abbreviations: FM A, fredericamycin A; Me_2SO, dimethyl sulfoxide; TFA, trifluoroacetic acid; CoA, coenzyme A; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; Me_4Si, tetramethylsilane.

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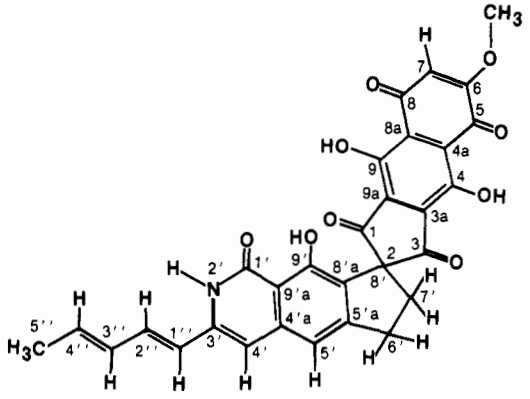

FIGURE 1: Structure of fredericamycin A.

to be cytotoxic in vitro and active in vivo against several transplantable tumors in mice (Warnick-Pickle et al., 1981).

The structure of FM A (Figure 1) was recently established by single-crystal X-ray analysis (Misra et al., 1982). Its novel spiro ring system places FM A in a new antibiotic structural class. Efforts to improve productivity of FM A by *Streptomyces griseus* (FCRC-48) led us to investigate the biosynthesis of FM A using ^{14}C- and ^{13}C-labeled precursors.

In this paper, we report the biosynthetic origin of FM A and propose a scheme for FM A biosynthesis.

Experimental Procedures

Fermentation. Streptomyces griseus strains FCRC-48 and FCRC-48, V-17 were maintained at a concentration of 3×10^9 spores/mL as frozen stocks (−70 °C) in 10% glycerol. Seed medium consisted of 1.0% soy flour (Archer Daniels Midland), 0.5% Bactopeptone (Difco), 0.5% beef extract (Gibco, dry form), 0.5% NaCl, 0.05% K_2HPO_4, and 1.5% glucose in tap water adjusted to pH 6.8. A suspension of 1×10^6 spores was used to inoculate 50 mL of seed medium in 250-mL baffled Erlenmeyer flasks. Seed flasks were incubated at 28 °C for 48 h and shaken at 250 rpm. Production of FM A was accomplished by using either 50 or 400 mL in 250- and 2000-mL baffled Erlenmeyer flasks, respectively. Production medium consisted of 1.0% dextrin (60K), 1.3% soy flour, 0.6% Bactopeptone, 0.6% beef extract, 0.6% NaCl, 0.06% K_2HPO_4, 1.5% glucose, and 0.1% Dow Corning A antifoam in distilled water adjusted to pH 7.0. Production flasks were inoculated with a 2% aliquot of 48-h seed and shaken at 250 rpm for 60–72 h at 28 °C. Addition of radioisotopes was made at 30 h unless otherwise noted. For the incorporation of stable isotopes, fermentations were carried out by using 400 mL of production medium in 3 × 3000 mL flasks. Typically, 1 g of ^{13}C-labeled precursor was added to the three flasks in two equal aliquots: the first when glucose was depleted to 1.0% and the second 5 h later. Glucose concentration was determined by using Worthington Statzyme glucose reagent, and the packed cell volume (PCV) was determined by using a DeLavel Gyro centrifuge with 10-mL conical tubes. Cerulenin (Sigma) was filter sterilized and added to the production medium at the time of inoculation.

The initial FM A biosynthetic experiments using ^{14}C-labeled precursors were carried out in shake-flask fermentations of *Streptomyces griseus* FCRC-48. Peak titers of FM A ranged from 50 to 100 μg/mL. Subsequent stable isotope experiments utilizing carbon-13 precursors were performed with a mutant, V-17, obtained by UV treatment of *S. griseus* FCRC-48. Mutant strain V-17 was not further characterized. Peak titers of FM A produced by the mutant ranged from 100 to 300 μg/mL.

Isolation of ^{14}C-Labeled Fredericamycin A. The whole broth was adjusted to pH 2.0 with 6 N HCl and extracted twice with an equal volume of ethyl acetate. The combined ethyl acetate extracts were dried over anhydrous Na_2SO_4, filtered, evaporated to dryness at 35 °C, and triturated once with ether; the residue was dissolved in chloroform–methanol–acetic acid (87:3:3) at concentrations such that 2000–10 000 cpm were present in 75 μL. The percent radioactivity in FM A was calculated by determining the radioactivity in appropriate fractions following separation on a Waters C-18 reverse-phase high-performance liquid chromatography (HPLC) column (see Chromatography). The radioactivity of each fraction was determined by liquid scintillation counting. The titer of FM A in each sample was determined by comparison of the peak height measured at 254 μm to a standard curve.

Purification of FM A Labeled with Stable Isotopes. The initial isolation of labeled antibiotic was identical with that given above for the ^{14}C-labeled FM A. Ethyl acetate extracts of whole broth yielded 100–500 mg of crude product. This was washed 4 times with 4 mL of ethyl ether and then 4 times with the lower phase of methanol–ethyl acetate–cyclohexane–water (50:5:48:30). The resulting residue was washed once with ether and dried. FM A labeled with L-[*methyl*-^{13}C]methionine was further purified by chromatography on silica gel (see Chromatography). The appropriate fractions were combined, evaporated to dryness, washed with ether, and dried under vacuum.

Chromatography. FM A titer analysis and incorporation determinations of the ^{14}C-labeled FM A were performed by using HPLC consisting of a Waters Associates Model 6000A solvent delivery system, a U6K septumless injector (Waters Associates), a Schoeffel Model SF 770 variable-wavelength UV detector set at 254 nm, and a C_{18} μBondapak column (3.9 mm × 30 cm, Waters Associates). The mobile phase consisted of methanol–water–acetic acid (70:30:1) with a flow rate of 2 mL/min. Titer and purity analysis of FM A labeled with ^{13}C were carried out as above, only using a Radial-Pak CN column in a Waters Associates RCM-100 radial compression module with methanol–2% triethylamine (60:40), pH adjusted to 2.35 with concentrated phosphoric acid, as the mobile phase at a flow rate of 2.0 mL/min. Purification of FM A labeled with L-[*methyl*-^{13}C]methionine was performed by adsorbing 30 mg of labeled FM A, purified as described above for stable isotopes, onto 1 g of silica gel, layering that on top of 10 g of silica gel (Merck, TLC grade) in $CHCl_3$, and eluting with chloroform–methanol–acetic acid (87:3:3). Fractions were monitored by using 10 × 20 cm precoated silica gel 60 F-254 thin-layer chromatography plates developed with the same solvent system. Those fractions containing essentially pure FM A were combined, evaporated to dryness under vacuum, and washed with ethyl ether; the residue was dried under high vacuum.

Radioisotopes. D-[6-^{14}C]Glucose (56.1 mCi/mmol), D-[1-^{14}C]glucose (53.0 mCi/mmol), D-[3,4-^{14}C]glucose (10.0 mCi/mmol), [2-^{14}C]glycine (47.3 mCi/mmol), [2-^{14}C]malonic acid (36.6 mCi/mmol), L-[*methyl*-^{14}C]methionine (54.0 mCi/mmol), and [G-^{14}C]shikimic acid (80.0 mCi/mmol) were purchased from New England Nuclear; [2-^{14}C]acetic acid (56.1 mCi/mmol), DL-[*carboxyl*-^{14}C]phenylalanine (7.3 mCi/mmol), and DL-[*alaninyl*-3-^{14}C]tryptophan (48.0 mCi/mmol) were from ICN; [2-^{14}C]diethyl malonate (10.0 mCi/mmol) and sodium [^{14}C]formate (58.6 mCi/mmol) were from Amersham.

Stable Isotopes. L-[*methyl*-^{13}C, 90%]Methionine, [1-^{13}C, 90%]acetic acid, [2-^{13}C, 90%]acetic acid, and [1,2-^{13}C,

Table I: Incorporation of [^{14}C]Glucose Precursors into FM A

precursor	μCi added/flask[a]	sp act. of glucose after addition of label (μCi/mmol)	FM A titer (μg/mL)	FM A sp act. (μCi/mmol)	% incorpn into FM A
[1-^{14}C]glucose	11.7	3.3	63	3.6	0.18
[3,4-^{14}C]glucose	5.0	1.4	74	0.4	0.05
[6-^{14}C]glucose	12.9	3.6	79	2.7	0.16
L-[U-^{14}C]glucose	11.7	3.3	67	2.3	0.14

[a] Additions of label were made at 24 h.

90%]acetic acid were purchased from KOR, and [2-^{13}C, 90%]malonic acid was from Merck. The acetic acid and malonic acid samples were dissolved in water, neutralized with 10% NaOH, and sterilized through 0.45-μm Millex-HA filters (Millipore) prior to addition to the fermentation flasks.

Isotope Analysis. All radioactivity measurements were carried out by using a Searle Isocap/300 liquid scintillation counter. Samples to be counted were dissolved in PCS phase combining cocktail mix (Amersham).

NMR. ^{13}C NMR spectra were obtained at 75.4 MHz on a Nicolet NT-300 wide-bore spectrometer, using either 12-mm (variable-frequency probe) or 5-mm (fixed-tune probe) spinning tubes. The central resonance of the solvent, deuteriochloroform, was used as an internal reference with shifts reported relative to Me_4Si using $\delta(Me_4Si) = \delta(CDCl_3) + 76.90$. All spectra were recorded at ambient temperature with an internal deuterium lock. Proton coupling was suppressed with 1.5 W (59 dB) of decoupler power by utilizing the MLEV-16 pulse sequence (Levitt & Freeman, 1981). Typical acquisition parameters were 25 000 scans, 1.0-s acquisition time, 0.5-s delay (1.5-s recycle time), and 40° flip angle. For off-resonance-decoupled spectra, 2.0 W of continuous wave (CW) decoupling power was used. ^{1}H NMR spectra were obtained at 300.06 MHz by using 5-mm tubes on the same spectrometer.

Results

The fermentation time courses of the two *S. griseus* FCRC-48 strains were virtually identical and are represented by that obtained for *S. griseus* FCRC-48 shown in Figure 2. Consumption of glucose did not occur until cell growth approached its maximum and just prior to the start of FM A biosynthesis. Maximum cell mass occurred at 50 h, while peak production of FM A occurred between 60 and 72 h and then sharply declined. Profiles of pH consistently showed a characteristic drop from 7.2 to 5.5 during the onset of logarithmic growth, followed by a return to alkaline values just prior to the consumption of glucose and initiation of FM A accumulation.

The substrate–product relationship between glucose and FM A implied by the glucose utilization and FM A accumulation curves in Figure 2 was readily confirmed by using uniformly ^{14}C-labeled glucose as tracer. Incorporation values of [1-^{14}C]-, [3,4-^{14}C]-, and [6-^{14}C]glucose were compared to discern the pathway by which glucose was incorporated into FM A. As shown in Table I, incorporations of carbons 1 and 6 of glucose into FM A were essentially equal, while incorporation of [3,4-^{14}C]glucose was substantially less. This finding is consistent with biosynthesis through a polyketide pathway where carbons 3 and 4 of glucose are lost as CO_2 in the decarboxylation of pyruvate to acetyl-CoA. Metabolism of glucose through competing oxidative pathways resulted in some scrambling of precursor labels and accounts for the small

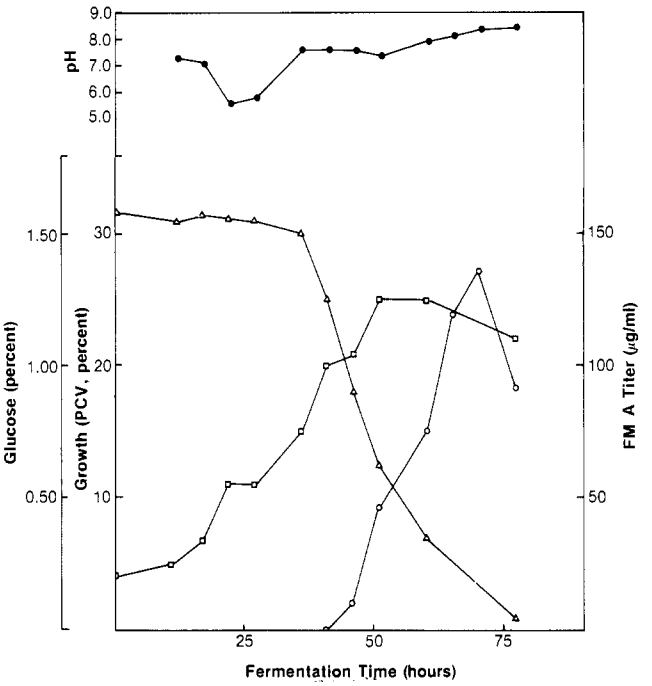

FIGURE 2: Time course of a shake-flask fermentation of FM A including growth of *S. griseus* (FCRC) determined at packed cell volume (PCV) (□), FM A titer in micrograms per milliliter (○), percent glucose present in the fermentation broth (△), and pH of the broth (●).

Table II: Incorporation of [^{14}C]Acetate and [^{14}C]Malonate into FM A

precursor	precursor sp act. (mCi/mmol)	time of addition (h)	μCi added/flask	% incorpn into FM A
[2-^{14}C]acetate	56.1	0	9.0	0.06
		48	9.0	0.40
[2-^{14}C]malonate	36.6	8	11.7	0.17
		48	11.7	2.70
[2-^{14}C]diethyl-malonate	10.0	8	5.0	0.83
		48	10.0	1.20

incorporation of [3,4-^{14}C]glucose into FM A. A polyketide biosynthetic scheme is also consistent with the finding that other potential precursors of aromatic compounds such as ^{14}C-labeled DL-phenylalanine, DL-tryptophan, and shikimic acid were not incorporated into FM A.

The polyketide precursors acetate and malonate were readily incorporated into FM A if added to the fermentation at 48 h, when antibiotic was actively being produced (Table II and Figure 2). While organisms vary in their ability to utilize exogenously added malonate as a precursor of malonyl-CoA, *Streptomyces griseus* FCRC-48 incorporated [2-^{14}C]malonate

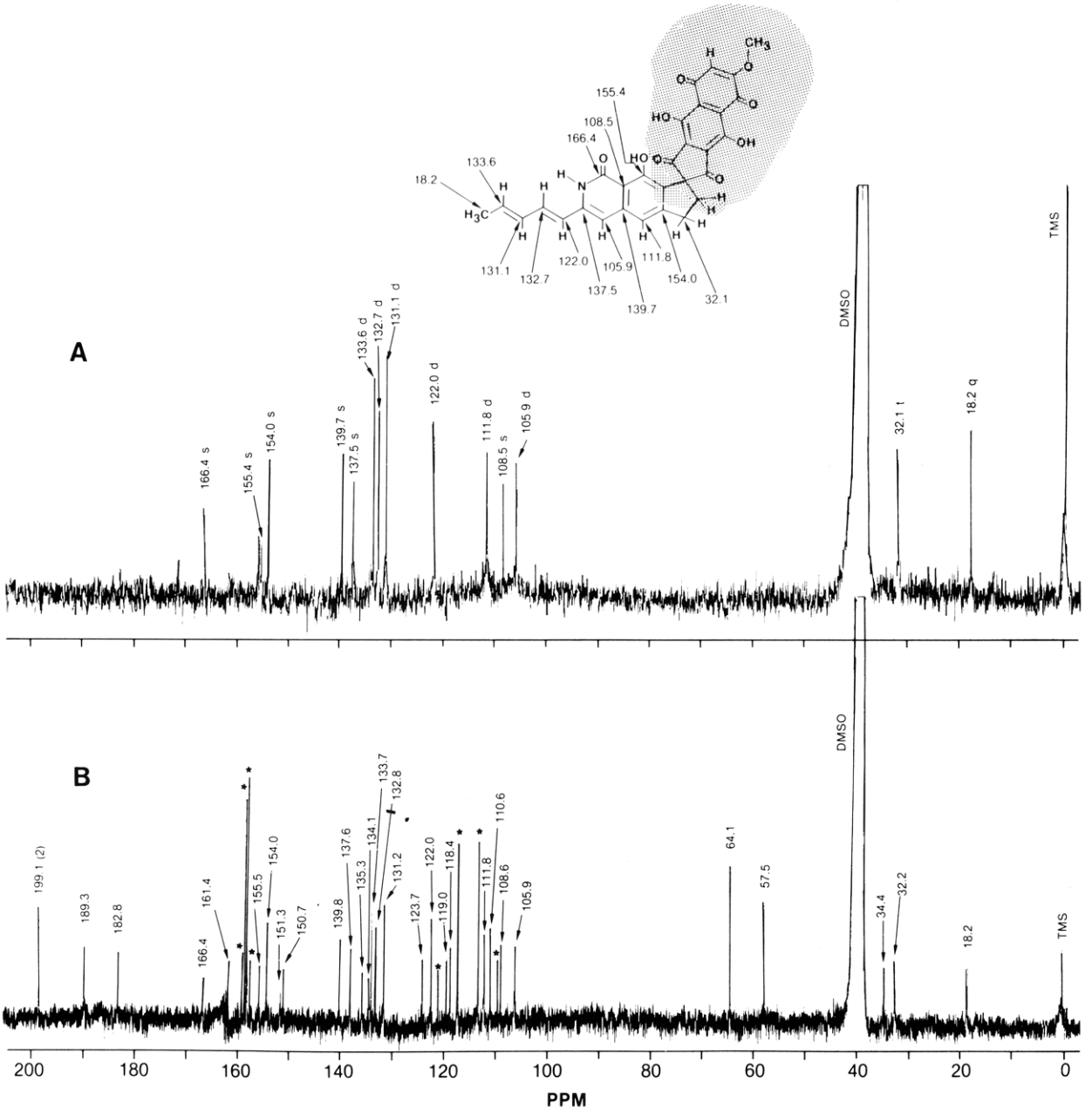

FIGURE 3: Proton noise-decoupled ^{13}C NMR spectra at 75.4 MHz of FM A in Me_2SO-d_6 (A) and in Me_2SO-d_6 containing 0.7% TFA-*d* (B). Signals due to TFA-*d* are designated with asterisks.

into FM A better than [2-^{14}C]acetate, regardless of whether the malonate was introduced as the disodium salt or the diethyl ester.

The inhibitory effect of cerulenin upon FM A production added further support for the involvement of the polyketide pathway in FM A biosynthesis. Cerulenin, an antifungal antibiotic which inhibits fatty acid biosynthesis, was recently shown to inhibit production of the polyketide-derived antibiotic daunorubicin without affecting cell growth when used at low concentrations (Omura & Takeshima, 1974; Omura, 1976; McGuire et al., 1980). Concentrations of 10 and 25 μM cerulenin were found to decrease FM A production by 62% and 92%, respectively, and completely inhibited production at 50 μM with no appreciable change in cell growth.

Confirmation of the polyketide origin and establishment of the carbon labeling pattern of FM A were obtained by feeding cultures of *S. griseus* FCRC-48 (var. 17) ^{13}C-enriched precursors. The ^{13}C NMR chemical shift assignments for each carbon in the molecule were determined by comparison to literature values of model compounds, from specific hydrogen-decoupled carbon NMR data (unpublished experiments), and from coupling constants of FM A derived from [1,2-^{13}C]acetate or a mixture of [1-^{13}C]- and [2-^{13}C]acetate.

Pure FM A is quite insoluble in most common solvents; dimethyl sulfoxide, dimethylformamide, and dimethylacetamide offer the best solubilities. However, the ^{13}C NMR spectrum of FM A in Me_2SO-d_6 exhibited only 14 resonance signals (Pandey et al., 1981) compared with the expected 30 (Figure 3A). All 14 signals arose from carbons in the isoquinoline portion of the molecule. No signals from carbons in the shaded region of Figure 3A were observed. The addition of trace quantities of trifluoroacetic acid (TFA) not only made it possible to record all of the FM A carbon signals (Figure 3B) but also led to solubilization of FM A in $CDCl_3$. Thus,

Table III: Relative Enrichments of Carbons in FM A Labeled with [1-^{13}C]Acetate and [2-^{13}C]Acetate

chemical shift[a]	carbon atom	multiplicity[b]	rel enrichment[c] [2-^{13}C]acetate	rel enrichment[c] [1-^{13}C]-acetate
18.6	5″	q	12.9	1.6
32.8	6′	t	10.2	1.1
34.7	7′	t	1.1	12.2
57.3	OCH_3	q	1.0	1.0
64.6	2	s	0.6	9.5
108.2	9′a	s	4.6	0.9
109.3	4′	d	14.3	1.7
110.9	7	d	14.2	1.6
112.9	5′	d	11.3	1.5
118.0	4a and 8a	s, s	3.7	5.9
120.6	1″	d	18.6	2.2
124.6	8′a	s	4.3	0.7
130.4	3″	d	12.8	1.3
133.0	2″	d	2.4	17.2
135.1	3a	s	0.7	13.3
135.8	4″	d	1.7	12.9
136.5	3′	s	1.3	11.4
136.9	9a	s	7.2	2.6
141.0	4′a	s	0.8	9.5
152.2[d]	9	s	1.2	6.7
152.5[d]	9′	s	0.8	14.5
153.4	4	s	7.7	0.7
155.7[d]	5′a	s	1.0	13.4
161.1	6	s	1.0	9.7
166.6	1′	s	2.0	22.5
183.3	5	s	5.7	1.4
188.6	8	s	1.2	15.0
198.8	3	s	5.0	0.7
199.0	1	s	1.0	10.5

[a] Chemical shifts (in ppm) are relative to Me_4Si. [b] The multiplicity arises from one-bond ^{13}C proton coupling. [c] Calculated by determining peak intensities relative to the intensity of the methoxy signal at 57.3 ppm and then dividing these relative intensities by the relative intensities of the same peaks (calculated in the same way) in the natural-abundance spectrum. [d] These assignments may be mixed.

Table IV: Coupling Constants of FM A Labeled with [1,2-^{13}C]Acetate and a Mixture of [1-^{13}C]- and [2-^{13}C]Acetate

carbon atom	chemical shift[a]	multiplicity[b]	acetate precursor carbon	$J_{^{13}C-^{13}C}$ (Hz) double label	$J_{^{13}C-^{13}C}$ (Hz) mixed label
5″	18.6	q	2	43.2	singlet[d]
4″	135.8	d	1	43.1	71.0
3″	130.4	d	2	56.7	71.0
2″	133.0	d	1	56.7	69.3
1″	120.6	d	2	63.2	71.0
3′	136.5	s	1	65.9	*e*
4′	109.3	d	2	55.0	71.0
4′a	141.0	s	1	56.1	56.5
5′	112.9	d	2	59.4	58.3
5′a	152.5[c]	s	1	61.3	66.1[f]
6′	32.8	t	2	33.6	40.0
7′	34.7	t	1	33.4	30.5
8′a	124.6	s	2	73.0	52.5
9′	155.7	s	1	69.5[f]	40.0 and 60.5
9′a	108.2	s	2	66.8	60.0
1′	166.6	s	1	66.9	singlet[d]
1	199.0	s	1	singlet[d]	47.0
2	64.6	s	1	45.0	31.5 and 45.0
3	198.8	s	2	44.4	50.0
3a	135.1	s	1	72.5	*e*
4	153.4	s	2	68.8[f]	*e*
4a	118.0	s	1	57.8	*e*
5	183.3	s	2	58.2	60.0
6	161.1	s	1	71.3	60.5
7	110.9	d	2	71.5	61.3
8	188.6	s	1	54.8	59.0
8a	118.0	s	2	57.8	*e*
9	152.2[c]	s	1	67.6[f]	71.5
9a	136.9	s	2	*e*	*e*
OCH_3	57.3	q		singlet[d]	singlet[d]

[a] Chemical shifts (in ppm) are relative to Me_4Si. [b] This multiplicity arises from one-bond ^{13}C proton coupling. [c] Assignments may be reversed. [d] These enriched carbon signals appeared as singlets. [e] These J values were indeterminable due to interfering signals. [f] These values are questionable.

it became possible to distinguish which carbons belonged to the isoquinoline or the benzindene halves of the molecule by first recording the ^{13}C NMR spectrum in Me_2SO and then in Me_2SO or $CDCl_3$ with a trace of TFA added. This approach proved extremely useful in simplifying the ^{13}C-enriched spectra, in essence permitting the total spectrum to be divided in two portions and analyzed separately.

The carbon NMR spectrum obtained after purification of natural-abundance FM A (Figure 4A) was compared with FM A enriched from [2-^{13}C]acetate (Figure 4B) and [1-^{13}C]acetate (Figure 4C). As expected, the terminal methyl (C-5″) at 18.6 ppm on the pentadienyl side chain arose from C-2 of acetate. Other biosynthetically significant carbons labeled by C-2 of acetate were the methylene (C-6′) at 32.8 ppm, the C-9′ carbon at 124.6 ppm, the C-6′ methine at 110.9 ppm, and the C-3 keto carbon at 198.8 ppm. Incorporation of [1-^{13}C]acetate into FM A was found to enrich, among others, the methylene carbon (C-7′) at 34.7 ppm, the lactam carbonyl (C-1′) at 166.6 ppm, the cyclopentyl keto carbon (C-1) at 199.0 ppm, the methoxyl-bearing carbon (C-6) at 161.1 ppm, and, most significantly, the spiro carbon (C-2) at 64.7 ppm.

Close scrutiny of the spectrum in Figure 4C reveals the presence of satellite peaks surrounding the keto carbon (C-1), the spiro carbon (C-2), and the methylene carbon (C-7′). An expansion of these three regions taken from a similar spectrum of the same sample is presented in Figure 5. The spiro carbon at C-2 is clearly coupled to both C-7′ and C-1 with J values of 31.1 and 44.7 Hz, respectively. Thus, biosynthesis of the FM A molecule leads to the direct bonding of these three carbon atoms to each other, despite the fact that each derives from the same carbon of acetate. An additional pair of satellite peaks surround the C-2 spiro carbon with a J constant of 12.8 Hz resulting from ^2J coupling with either C-3 or C-5′a.

Relative percent enrichment values for the [1-^{13}C]- and [2-^{13}C]acetate-derived FM A carbon signals were obtained by integration of the carbon signals shown in Figure 4B,C and are presented in Table III. Examination of the enrichment values reveals that the double signal at 118.0 ppm was enriched by both carbons of acetate, indicating that the two carbons contributing to the signal, C-4a and C-8a, arose from different carbons of acetate. Except for the C-1, C-2, and C-7′ array, the enrichment of the remaining carbons was as expected for a polyketide-derived compound. A total of 15 carbons arose from C-1 of acetate, while 14 were derived from C-2.

The [1-^{13}C]acetate-derived carbons in the pentadienyl side chain along with the contiguous carbons C-3′, C-4′, C-5′, and C-6′ of the isoquinoline portion of the molecule had an average relative enrichment of 13.4 compared with 6.6 for the other [1-^{13}C]acetate-derived carbons. However, labeling with [2-^{13}C]acetate did not verify a difference in relative enrichment levels between these two portions of the FM A molecule.

In addition to single ^{13}C-labeled percursors, double and mixed ^{13}C-labeled precursors were incorporated into FM A. This technique was used not only to confirm chemical shift assignments but also to aid in establishing the direction of the polyketide chains forming the FM A ring structure and to determine the one acetate cleavage site. Carbon–carbon

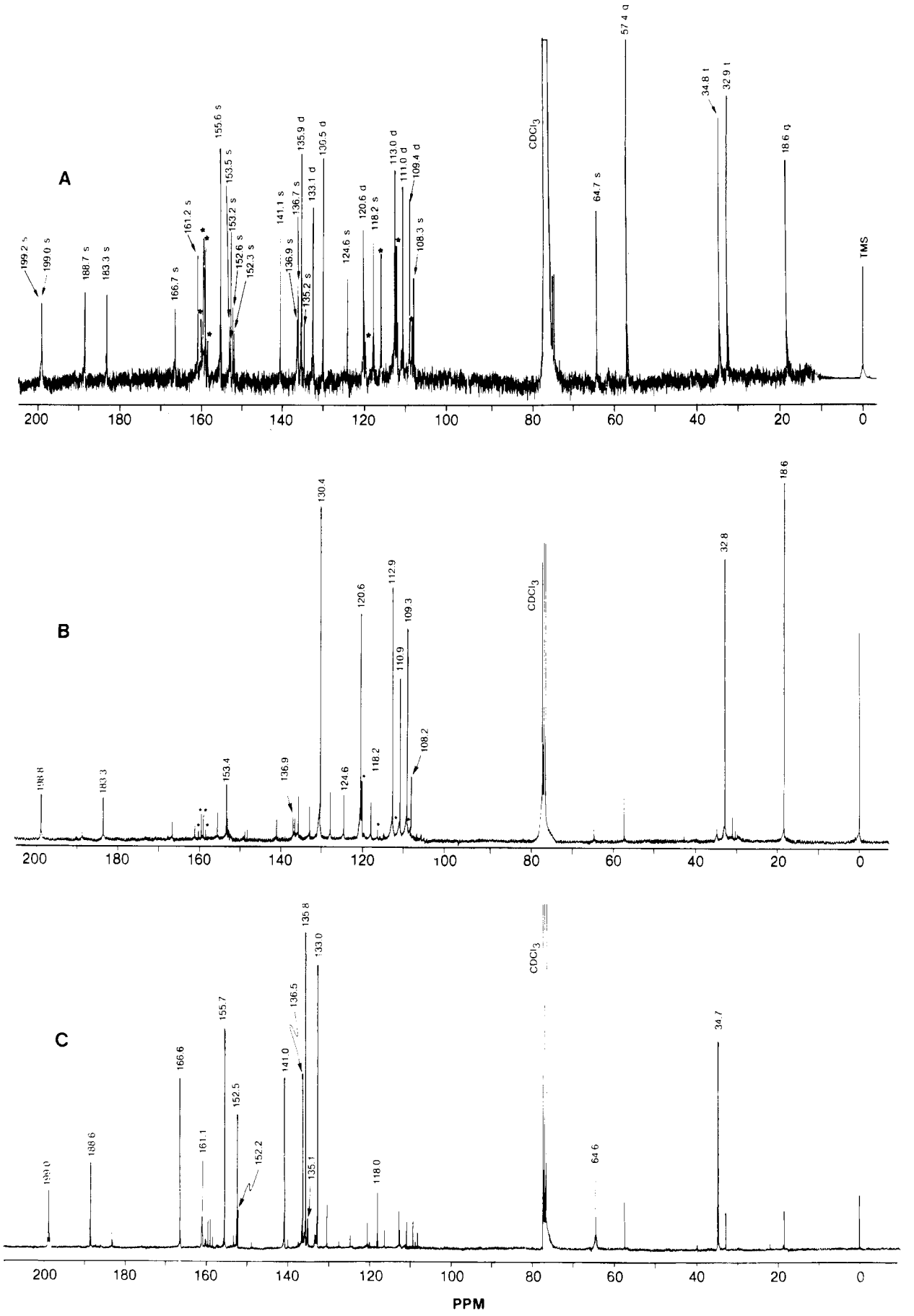

FIGURE 4: Proton noise-decoupled ^{13}C NMR spectra at 75.4 MHz of FM A. (A) Natural abundance; (B) biosynthetically enriched with [2-^{13}C]acetate; (C) biosynthetically enriched with [1-^{13}C]acetate. Enriched carbons in spectra B and C are labeled with their chemical shift value relative to Me_4Si. Signals due to TFA-*d* are designated by asterisks.

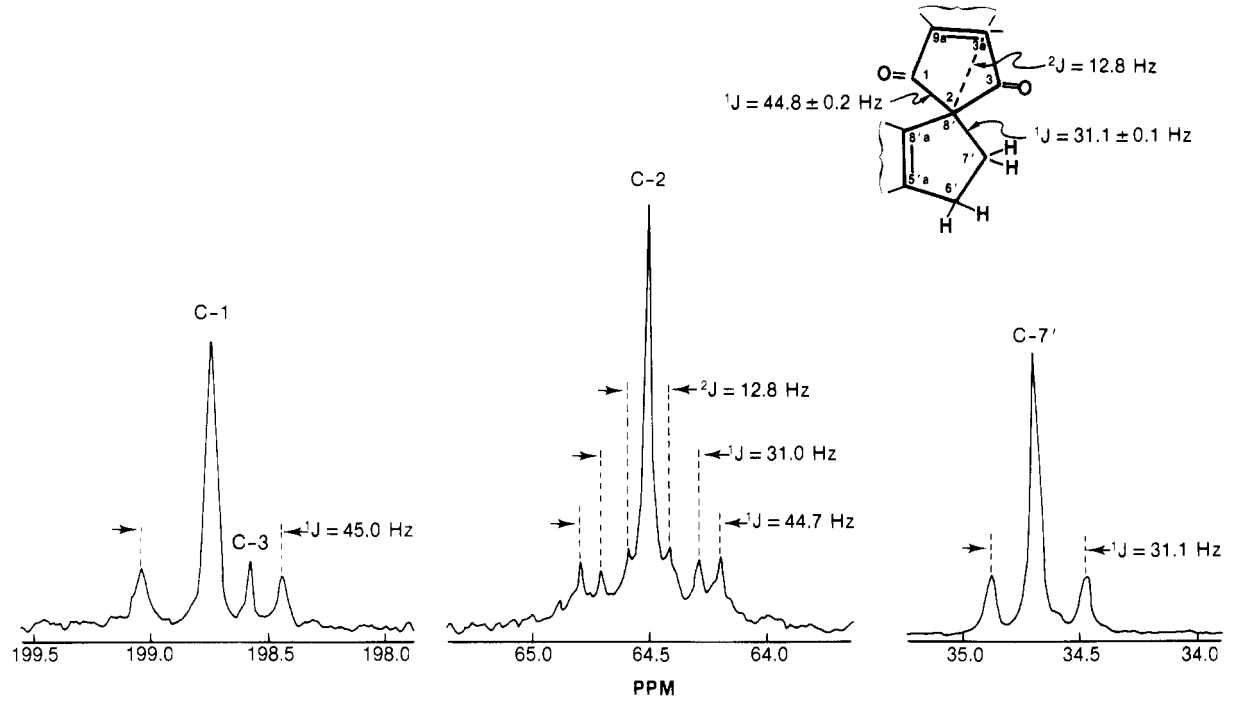

FIGURE 5: Coupling patterns of carbons in the spiro carbon region of FM A biosynthetically enriched with [1-^{13}C]acetate. The spectra were run under proton noise-decoupling conditions at 75.4 MHz. The numbering scheme for the spiro carbon region is given at top right.

coupling data obtained by using [1,2-^{13}C]acetate and a 1:1 mixture of [1-^{13}C]- and [2-^{13}C]acetate are summarized in Table IV. Coupling values were determined for most carbons, but in several instances, the complexity of the splitting pattern or low signal to noise ratios made J value calculations questionable. As expected, the methoxy carbon at 57.3 ppm showed no coupling. The C-2 acetate-derived methyl carbon at 18.6 ppm was not coupled in the spectrum of FM A derived from the mixed labeled precursor but was coupled (J = 43.1 Hz) to a C-1 acetate-derived methine carbon at 135.8 ppm (C-4″) in the spectrum of FM A derived from double-labeled acetate. This is consistent with the position of this carbon pair at C-5″ and C-4″ on the pentadienyl side chain and may represent the starter acetate unit of a polyketide chain. Similarly, the C-1 acetate-derived lactam carbonyl carbon at 166.6 ppm appeared with no satellite peaks in the spectrum of FM A derived from the mixed labeled acetates but was coupled (J = 66.8 Hz) to the C-2 acetate-derived α-carbon at 108.2 ppm in the spectrum derived from double-labeled acetate. This finding is consistent with the assignment of these resonance signals to carbons C-1′ and C-9′a and with the origin of this pair from the terminal acetate unit of a polyketide chain. Attempts to identify the polyketide starter unit(s) by feeding ^{13}C-labeled malonate were unsuccessful. Signal intensities of the ^{13}C NMR spectrum obtained of FM A enriched from [2-^{13}C]malonate were not significantly different at any resonance position from the spectrum obtained by using FM A enriched from [2-^{13}C]acetate.

Examination of the region about the spiro carbon (C-2) in the spectrum of FM A derived from [1,2-^{13}C]acetate revealed that the two methylenes at 34.7 and 32.8 ppm are coupled (J = 33.5 Hz) and thus originate from one acetate unit. Coupling of the methylene at 34.7 ppm and the carbonyl at 199.0 ppm each to the spiro carbon at 64.6 ppm in the spectrum of FM A derived from mixed labeled acetates results from bond formation between these three C-1 acetate-derived carbons, in the same way as observed when [1-^{13}C]acetate was fed as a single precursor (Figure 5). The J values recorded in both experiments are in close agreement. Because of the symmetry

Table V: Incorporation of Potential Methyl Donors into FM A

precursor	μCi added/flask[a]	% incorpn into FM A	FM A sp act. (μCi/mM)
L-[*methyl*-^{14}C]methionine	5.3	9.3	51.6
[2-^{14}C]glycine	5.6	1.0	5.8
sodium [^{14}C]formate	5.3	0	0
[2-^{14}C]diethylmalonate	1.9	4.6	11.5

[a] Additions were made 48 h after inoculation of production medium.

of the benzindene portion of the molecule, except at positions C-6 and C-7, the carbon signals of this nucleus tend to occur in pairs. The carbons comprising each of these pairs arise from different acetate carbons, leading to an alternating pattern of acetate carbon origin. Such a labeling pattern strongly suggests that the benzindene ring carbons originate from one polyketide chain. The direction of the chain in this part of the molecule was determined from the [1,2-^{13}C]acetate coupling data at positions C-6 and C-7. The lack of symmetry in the ring structure at these positions facilitated the unequivocal chemical shift assignments of these carbons and, therefore, their acetate carbon origin. Coupling of the C-1 acetate-derived C-6 to the C-2 acetate-derived C-7 with a value of 71.4 Hz indicated that the benzindene ring skeleton is formed by a polyketide chain progressing in a clockwise rotation about the benzindene portion of FM A, as shown in Figure 6.

An important feature of the proposed biosynthetic schemes (Figure 6) is that one carbon must be lost at one of the carbonyl carbons (C-1 or C-3) prior to or during biogenesis of the spiro center at C-2, regardless of the chain direction around the benzindene nucleus. This conclusion was confirmed by examination of the spectrum of FM A derived from [1,2-^{13}C]acetate wherein the C-1 acetate-derived signal at 199.0 ppm appears uncoupled. This could only occur if the acetate unit from which this carbon (C-1) originates was cleaved with subsequent loss of the methyl carbon. Whether such an event occurs as two polyketide chains are fused to form the FM A

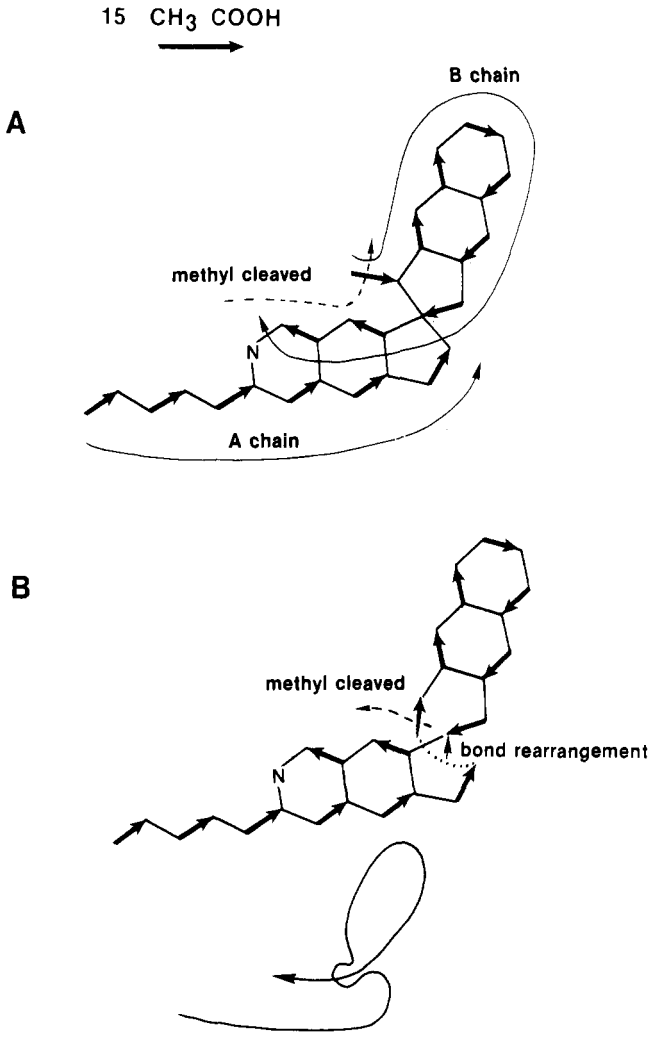

FIGURE 6: Proposed biosynthetic pathways of FM A. (A) Double-chain pathway; (B) single-chain pathway. Each arrow represents one acetate unit; the head of each arrow corresponds to C-1 of acetate.

molecule (Figure 6A) or by cleavage, methyl loss, and rejoining of a single chain (Figure 6B) remains unknown.

Incorporation of several one-carbon donors into FM A was first investigated by using ^{14}C-labeled precursors (Table V). L-Methionine was found to label the antibiotic at a level greater than 9%, twice the level of diethylmalonate. These data suggested that the one remaining carbon, the methoxy at position C-6, originated from L-methionine. Attempts to confirm this by using ^{13}C-labeled methionine led to the finding that substrate levels of L-methionine fed to *S. griseus* FCRC-48 (var. 17) suppress FM A production. Reductions in FM A productivity of 58% and 84% occurred when L-methionine was added to the production medium at 0.1 and 0.2 g/L, respectively. This suppressive effect may result from the repression of *S*-adenosylmethionine synthetase by methionine, as observed in *Escherichia coli* (Holloway et al., 1970). In fact, the fermentation broth appeared just as red under FM A suppressed conditions as under normal conditions. This suggests that L-methionine suppression may lead to accumulation of an intermediate late in FM A biosynthesis, after the aromatic nucleus is assembled but before final modifications, such as methylation, occur. An adequate titer of FM A was obtained when L-[*methyl*-^{13}C]methionine was added to the culture at a concentration of 0.1 g/L. The ^{13}C NMR spectrum of the purified FM A product revealed greater than a 20-fold enrichment of the methoxy signal at 57.3 ppm. Barely discernible were the methyl signal at 18.6 ppm, the two methylenes at 32.9 and 34.8 ppm, a contaminant signal at 56.8 ppm, and the spiro carbon signal at 64.5 ppm.

Discussion

The studies reported clearly establish that the biosynthesis of the FM A carbon skeleton occurs by a polyketide pathway. The acetate pool required for FM A biosynthesis is provided by glycolysis of glucose and dextrin, present in the fermentation medium. The producing organism does not utilize glucose as a primary carbon source for growth; the glucose remains unutilized until just prior to FM A biosynthesis. By adding substrate levels of labeled acetate to the production medium during the course of glucose utilization, it is possible to obtain very high levels of incorporation. The ^{13}C-labeled precursor results demonstrate that acetate provides the carbon skeleton for all of the FM A carbon atoms except the C-6 methoxy carbon which is derived from L-methionine. A total of 15 carbon atoms are derived from C-1 of acetate, while 14 carbons are derived from C-2. This necessitates cleavage of an acetate unit followed by loss of a C-2 acetate carbon during FM A biosynthesis. The ^{13}C NMR spectrum of FM A enriched from [1,2-^{13}C]acetate shows no coupling for C-1 at 199.0 ppm; thus, the C-2 carbon of this acetate unit is the carbon atom lost during biosynthesis.

Three of the most intriguing questions concerning FM A biosynthesis are the following: (1) How many chains are involved in FM A biosynthesis? (2) What is the direction of the polyketide chain(s)? (3) How is the spiro center at C-2 formed? The double and mixed ^{13}C-enriched acetate studies confirm that resonance signals corresponding to carbons C-5 and C-1′ represent the head and tail of polyketide chains but provide no information as to whether they are the head and tail of the same chain. Similarly, comparison of the ^{13}C NMR spectra of FM A derived from [2-^{13}C]malonate and [2-^{13}C]acetate shows no significant difference in signal intensity at any resonance position. Thus, for FM A, as is true for several other acetate-derived natural products (Birch et al., 1975, 1976), this technique fails to identify the position and the number of starter acetate units involved in the biosynthesis. However, the mixed- and double-labeled [^{13}C]acetate results do firmly establish the direction of the chain(s), as depicted in Figure 6.

The question of a single vs. a multiple polyketide chain biosynthesis of FM A remains unanswered. Only a small number of fungal-derived natural products are reportedly made from multiple polyketide chains wherein a carbon–carbon bond is formed between the two separate chains [i.e., citromycetin (Evans & Staunton, 1976), mollisin (Bentley, 1965), and a series of compounds related to rubropunctatin (Hadfield et al., 1967)]. Condensation of multiple polyketide chains is even more rare in *Streptomyces*, and in those instances where it is proposed to occur, i.e., aplasmomycin and boromycin (Floss & Chang, 1981), the chains do not condense through carbon–carbon bond formation.[2] Biosynthesis of FM A from one polyketide chain would require that the C-2 acetate carbon which is cleaved from C-1 of FM A during biosynthesis be bound between C-1 and C-7′ in the original polyketide chain

[2] The most notable example of a streptomycete natural product originally proposed to be formed from multiple polyketide chains is tetracycline. The interpretation of the initial ^{14}C incorporation data supporting the two-polyketide proposal was later questioned when experiments revealed that the malonyl-CoA used in polyketide biosynthesis is supplied through a number of different pathways, thus accounting for the variation in acetate incorporation values throughout the molecule (Hutchinson, 1981).

(Figure 6A). Loss of this carbon may occur during formation of the spiro center.

Formation of the spiro center remains one of the most intriguing aspects of FM A biosynthesis. A number of marine natural products are known to contain spiro carbon centers, e.g., the sesquiterpene halochamigrene derivatives spironippol (Fukuzawa et al., 1981) and spirolaurenone (Suzuki et al., 1980) and several classes of plant alkaloids, including the spiro bisindoles (Cordell & Saxton, 1981). Formation of a spiro center from a sesquiterpene (Fukuzawa et al., 1981) or a pseudopeptide (Shamma, 1971) is chemically much easier to rationalize than such a formation from one or more polyketide chains. An example of a known acetate-derived streptomycete natural product containing a spiro center is griseofulvin. In this case, however, the spiro carbon arises from a C-2 acetate-derived methylene attached to three C-1 acetate-derived carbons and one oxygen atom. The spiro carbon of FM A, however, is derived from C-1 of acetate and is bound to four carbon atoms (two derived from C-1 and two from C-2 of acetate). Since the two C-2 acetate-derived carbons are bound to the spiro carbon by virtue of the polyketide chain, the two new bonds must be formed between the three C-1 acetate-derived carbon atoms, a heretofore unreported condensation. Given the unlikely chance of these three carbons bonding directly to each other, it seems reasonable that the initial bond formation may occur between the methylene carbon (or methyl carbon, for a two-chain biosynthesis), which is eventually lost, and the C-1 acetate-derived carbon, which becomes the C-2 spiro carbon of FM A. Thus, whether FM A is derived from one or two polyketide chains, the C-2 acetate-derived carbon lost during biosynthesis may play a vital intermediary role in formation of the spiro carbon center of FM A.

Registry No. FM A, 80455-68-1; glucose, 50-99-7; acetic acid, 64-19-7; L-methionine, 63-68-3.

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