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MACROLIDE BIOSYNTHESIS—II

ORIGIN OF THE CARBON SKELETON AND OXYGEN ATOMS OF THE ERYTHROMYCINS

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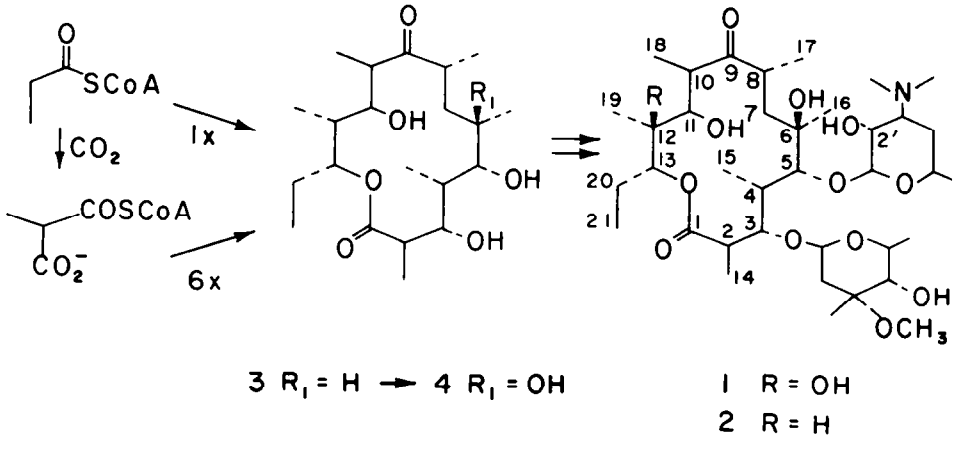
Abstract—Feeding of [1-^{13}C]propionate to cultures of *Streptomyces erythreus* gave erythromycin A and B labeled at C-1, 3, 5, 7, 9, 11, and 13, as established by ^{13}C NMR analysis of the derived 2′-benzoate esters. Incorporation of [2-^{13}C]propionate labeled C-2, 4, 6, 8, 10, 12, and 20 while feeding of ethyl [2,3-$^{13}C_2$]succinate, an *in vivo* precursor of [2,2′-$^{13}C_2$]methylmalonate, gave rise to seven pairs of enhanced and coupled doublets in the spectra of labeled erythromycin A and B benzoate. Subsequent incorporation of [1-$^{18}O_2$, 1-^{13}C]propionate and ^{13}C NMR analysis established the presence of excess ^{18}O at C-1, 3, 5, 9, 11, and 13 in both erythromycins A and B, as evidenced by the corresponding isotopically shifted $^{13}C^{18}O$ peaks. These results establish that in the biosynthesis of erythromycin all the O atoms of the initial aglycone, 6-deoxyerythronolide B (**3**) originate directly from propionate.

Although the general outlines of the biosynthesis of macrolide and polyether antibiotics are by now reasonably well understood, knowledge of the details of the key chain-building reactions leading to the formation of these stereochemically complex, polyoxygenated fatty acids is still primitive and rests largely on analogy to the far better studied biosynthesis of fatty acids themselves in both procaryotic and eucaryotic organisms.[2] For example, although the derivation of erythromycin A (**1**) from seven propionates is by now secure, and examination of a series of blocked mutants as well as enzyme-level studies has revealed the sequence of steps connecting 6-deoxyerythronolide B (**3**) to erythromycins A (**1**) and B (**2**), no intermediates preceding the parent lactone **3** have ever been observed, and published attempts to isolate the key 6-deoxyerythronolide B synthetase have been uniformly unsuccessful.[3] Analogous studies of the formation of the sixteen-membered macrolide tylosin have elucidated many of the details of the steps linking the parent aglycone protylonide[4] and tylosin, but have yet to reveal any intermediates between protylonide and the established precursors acetate, propionate, and butyrate.[5] Baltz has reported the interesting observation that among 72 tylosin-negative mutants of *Streptomyces venezuelae*, 59 appeared to be blocked in the formation of protylonide itself.[6] The observation that none of these protylonide mutants could act as secretors in cosynthesis assays suggested the absence of any diffusable intermediates. Similar observations have recently been made by Hutchinson for a series of 6-deoxyerythronolide B mutants of *S. erythreus*.[7]

To attempt to decipher the cryptic series of reactions by which the erythromycin skeleton is constructed, four years ago we initiated studies intended to establish the origin of the oxygen atoms of this macrolide. By using ^{18}O-labeled precursors we hoped to establish the connection between the chain-building reactions and the observed stereochemistry and functional group distribution in the product. The results of these studies, some of which have already been reported in preliminary form,[8] are described below.

The propionate origin of erthromycin A was originally established independently by Corcoran[9] and by



Scheme 1.

Grisebach,[10] based on feedings of radioactive propionate and methylmalonate to *Streptomyces erythreus* followed by partial degradation of the labeled macrolide. Subsequently, unpublished experiments by Nourse,[11] carried out in collaboration with the Abbott group, utilized ^{13}C NMR to confirm the intact incorporation of seven units of [1-^{13}C]propionate into erythronolide B (**4**), an intermediate which accumulates in a blocked mutant of *S. erythreus*. Our initial experiments with [^{13}C]propionate were therefore designed primarily to establish optimum conditions for eventual feeding of multiply-labeled precursors. Following preliminary incorporations of varying concentrations of radioactive substrates, a protocol was developed in which 0.333 g of sodium [1-^{13}C]propionate (90 at.%), mixed with 0.667 g of unlabeled sodium propionate[12] and 9.74×10^6 dpm of sodium [1-^{14}C]propionate as internal standard in 10 mL of water was administered in three portions (2 mL after 48-hr, 3 mL after 72-hr, and 5 mL after 96-hr incubation time) to 100 mL of a fermentation culture of *S. erythreus*. After a total of 6 days, the resulting erythromycins A and B were extracted into chloroform and converted to the readily crystallized 2′-benzoate esters. Separation of the two esters was achieved by chromatography on Sephadex LH 20 (1:1 chloroform–hexane)[13] to give 27 mg of erythromycin A 2′-benzoate and 16 mg of erythromycin B 2′-benzoate as well as 11 mg of a mixture of the two esters. The 62.9 MHz ^{13}C NMR spectrum of each of the labeled macrolide esters displayed enhanced signals corresponding to the expected sites of labeling, carbons 1, 3, 5, 7, 9, 11 and 13. (Table 1, Scheme 2) The approximately 13-fold ^{13}C enrichment per labeled site was in accord with the enrichment calculated from the measured specific activity. A slightly greater enhancement of the signals corresponding to C-13 in each sample resulted from the direct utilization of the propionyl CoA starter unit.[14]

Using the same feeding and purification protocol, labeled samples of erythromycin A and B 2′-benzoates were also generated by incorporation of sodium [2-^{13}C]propionate.[16] Analysis of the derived ^{13}C NMR spectra showed the expected distribution of label based on the appearance of enhanced signals corresponding to C-2, -4, -6, -8, -10, -12 and -20. (Table 1, Scheme 2)

Three research groups have reported assignments of the ^{13}C NMR spectra of erythromycins A and B.[17] Although there was substantial agreement on the attribution of most of the individual signals, several specific assignments have remained in doubt. For example, the pair of signals corresponding to C-2 and C-8 was either reported as unresolved or was not individually assigned. A similar ambiguity characterized the C-6; C-12 pair. Several of methyl assignments are also in dispute: the assignments by Roberts[17a] for C-14, -17, -18 and -19 differ from those reported by the groups of Tori[17b] and Omura.[17c] We have now resolved the majority of these assignments by incorporation experiments with diethyl [2,3-$^{13}C_2$]succinate. The latter substrate was expected to act as an *in vivo* precursor of [2,2′-$^{13}C_2$]methylmalonyl CoA, generated by the action of methylmalonyl CoA mutase, and could therefore be considered as an *in vivo* equivalent of [2,3-$^{13}C_2$]propionate. The diethyl ester gave enrichments superior to the disodium salt, presumably due to more efficient transport across the *Streptomyces* cell wall. In the event, 0.300 g of diethyl [2,3-$^{13}C_2$]succinate, prepared from [1,2-$^{13}C_2$]dibromoethane as previously described,[16] and containing 8.27×10^6 dpm of ethyl

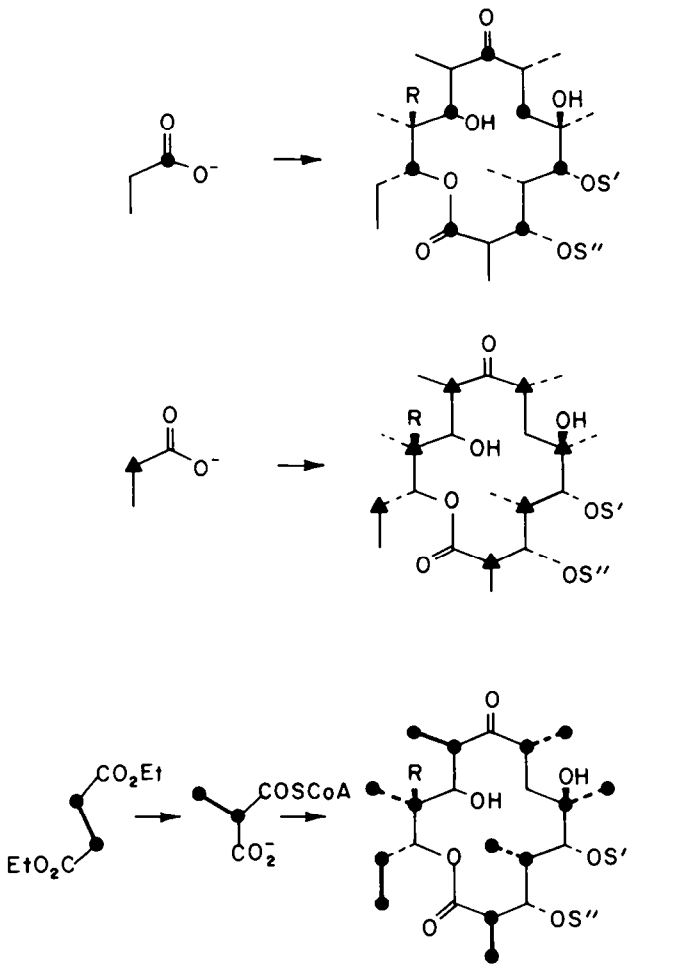
Table 1. Incorporation of [^{13}C]labeled precursors into erythromycins

	erythromycin A benzoate precursor[a]				erythromycin B benzoate precursor[a]			
chem shift[b] (m)	C	[1-^{13}C]-propionate	[2-^{13}C]-propionate	[2,3-$^{13}C_2$]-succinate J(Hz)	chem shift[b] (m)	C	[1-^{13}C]-propionate	[2-^{13}C]-propionate
175.7 (s)	1	*			176.3 (s)	1	*	
44.8 (d)	2[c]		*	35.9	44.6 (d)	2[e]		*
15.8 (q)	14[d]			34.1	15.4 (q)	14[f]		
79.8 (d)	3	*			78.0 (d)	3	*	
39.4 (d)	4		*	36.4	39.3 (d)	4		*
9.3 (q)	15			35.8	9.5 (q)	15[g]		
83.3 (d)	5	*			83.3 (d)	5	*	
75.2 (s)	6		*	38.8	75.4 (s)	6		*
27.2 (q)	16			39.1	27.6 (q)	16		
38.1 (t)	7	*			37.6 (t)	7	*	
45.2 (d)	8[c]		*	33.3	44.7 (d)	8[e]		*
18.1 (q)	17[d]			32.5	18.3 (q)	17[f]		
222.5 (s)	9	*			220.0 (s)	9	*	
37.8 (d)	10		*	34.0	39.0 (d)	10		*
11.9 (q)	18			34.0	9.0 (q)	18[g]		
68.6 (d)	11	*			69.5 (d)	11	*	
74.6 (s)	12		*	42.1	39.8 (d)	12		*
16.2 (q)	19			41.1	9.3 (q)	19[g]		
76.8 (d)	13	*			75.0 (d)	13	*	
21.1 (t)	20		*	35.8	25.5 (t)	20		*
10.6 (q)	21			35.2	10.3 (q)	21		

a. Sites of enrichment indicated by * or J_{CC} coupling constants.

b. $CDCl_3$, 62.9 MHz; spectral width 19230 Hz; 64 K data points; 0.9 Hz/pt; quadrature detection; 30° pulse; repetition rate 1.7 s.

c,d,e,f,g. Assignments within each group may be interchanged.



Scheme 2.

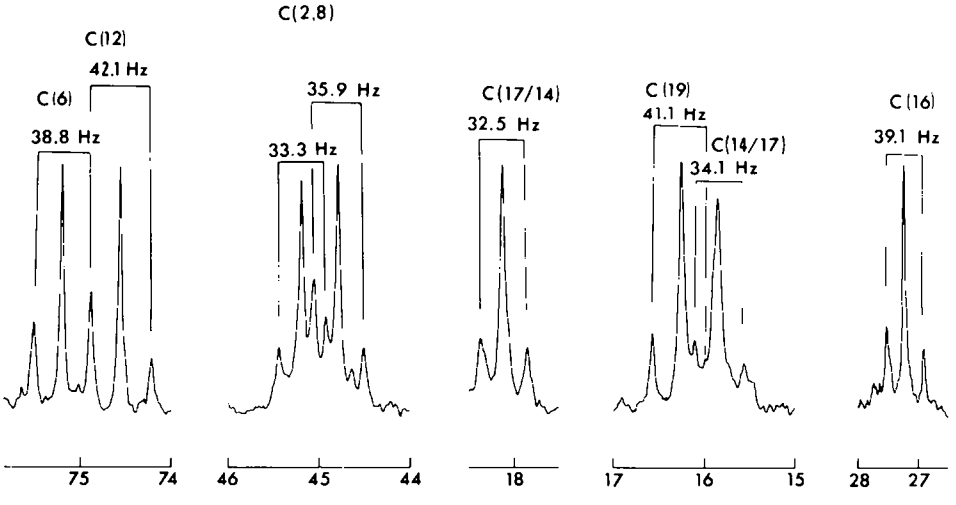


Fig. 1. Partial ^{13}C NMR spectrum of erythromycin A benzoate derived from [2,3-$^{13}C_2$]succinate and illustrating ^{13}C–^{13}C satellites.

[2-^{14}C]succinate, was administered in three portions to 100 mL of *S. erythreus* after 48 hr (60 mg), 72 hr (90 mg) and 96 hr (150 mg) and the resulting labeled erythromycins A and B 2′-benzoates were isolated and purified in the usual manner. The 62.9 MHz ^{13}C NMR spectra of erythromycin A 2′-benzoate (11.1 mg) and erythromycin B 2′-benzoate (6.3 mg) revealed the expected 7 pairs of enhanced and coupled doublets for each sample (Fig. 1, Scheme 2). Labeling of the propionate starter unit (C-20, 21) is presumably due to decarboxylation of the methylmalonyl CoA, most likely by transcarboxylase.[18] By careful measurement of coupling constants it was possible to make unequivocal assignments of several of the critical methine and methyl C signals.[19,20] For example, the signal for C-16 in erythromycin A 2′-benzoate, which appeared as a 39.1 Hz doublet centered at 27.2 ppm, was clearly coupled to the downfield resonance of the C-6; C-12 pair, which gave rise to a 38.8 Hz doublet at 75.2 ppm. The upfield doublet at 74.6 ppm (J = 42.1 Hz) was therefore assigned to C-12 and could only be coupled to the 41.1 Hz doublet at 16.2 ppm (C-19). Similar analysis of coupling constants established that the methyl signal at 18.1 ppm (J = 32.5 Hz) was coupled to the downfield methine at 45.2 ppm (J = 33.3 Hz), whereas the methyl at 15.8 ppm (J = 34.1 Hz) was coupled to the upfield methine at 44.8 ppm (J = 35.9 Hz). Although one of these groups is the C-2; C-14 pair and the other is the C-8; C-17 pair, no definitive distinction is as yet possible. Assignment of any single resonance will suffice to assign the entire set; this point is under study.

Having conclusively established the fundamental building blocks of the macrolide carbon skeleton, we then examined the origin of the O atoms of erythomycins A and B. While it had already been shown that the tertiary OH groups at C-6 and C-12, which are introduced during the conversion of 6-deoxyerythronolide to erythromycin A, are derived from molecular O_2,[3] no information was available concerning the six remaining O atoms already present in the parent macrolide **3**. In early 1980, following the report by Vederas of the use of ^{13}C NMR to determine the sites of O labeling in averufin derived from [1-$^{18}O_2$, 1-^{13}C]acetate,[21] it became clear that the use of ^{18}O-isotope shifts of high field ^{13}C NMR signals to detect sites of ^{18}O enrichment constituted a powerful, direct, and dramatically simple experimental method.[22] In order to study erythromycin biosynthesis we required a sample of [1-$^{18}O_2$, 1-^{13}C]propionate. The latter substrate was obtained in 75% overall yield by reaction of potassium [^{13}C]cyanide with ethyl iodide in methanol containing a small quantity of [^{18}O]water, followed by hydrolysis of the resulting [1-^{13}C]propionitrile with a mixture of 2.1 equiv. [^{18}O]water and 1 equiv of potassium t-butoxide in t-butyl alcohol.[16] Mass spectrometric analysis of the derived p-phenylphenacyl propionate indicated an isotopic content of 54.9% $^{18}O_2{}^{13}$C, 32.1% $^{18}O^{13}$C and 3.6% $^{16}O^{13}$C. Using the feeding protocol described above 0.333 g of sodium [1-$^{18}O_2$, 1-^{13}C]propionate, mixed with 0.667 g of unlabeled sodium propionate and 9.80×10^6 dpm of sodium [1-^{14}C] propionate, was fed to 100 mL of *S. erythreus* and the resulting labeled erythromycins A and B were isolated and purified as the derived 2′-benzoates. The sites of ^{18}O enrichment in the labeled macrolides were determined directly by 62.9 MHz ^{13}C NMR. As summarized in Table 2 and illustrated in part in Fig. 2,

Table 2. Incorporation of [1-$^{18}O_2$,1-^{13}C]propionate into erythromycins

	Erythromycin A 2'-Benzoate[a]			Erythromycin B 2'-Benzoate[a]		
C	^{13}C, ppm	Δδ, ppm[b]	$^{18}O:^{16}O$[c]	^{13}C, ppm	Δδ, ppm[b]	$^{18}O:^{16}O$[c]
1	175.57	0.04	50:50	176.03	0.04	50:50
3	79.85	0.02	50:50	80.24	0.03	40:60
5	83.26	0.03	45:55	83.12	0.03	50:50
7	38.25			37.81		
9	222.21	0.05	15:85	219.83	0.06	15:85
11	69.07	0.02	50:50	69.55	0.02	40:60
13	76.90	0.03	55:45	75.05	0.04	55:45

a. Bruker WM 250, 62.9 MHz; spectral width 19230 Hz, 32K time domain plus 32K zero points; quadrature detection; 90° pulse; repetition rate 1.0 s; 21000 transients; 40 mg in 2 mL $CDCl_3$, resolution enhancement was achieved by Lorentz-Gauss multiplication of the FID prior to Fourier transformation (R. R. Ernst, Adv. Magn. Res., 2, 59 (1966)), -1.0 Hz line broadening, 0.25 Gaussian multiplier; 0.009 ppm/data point.

b. $^{13}C^{18}O$ isotope shift, ± 0.01 ppm.

c. ±5; Uncorrected for contribution of natural abundance ^{13}C to $^{13}C^{16}O$ peak.

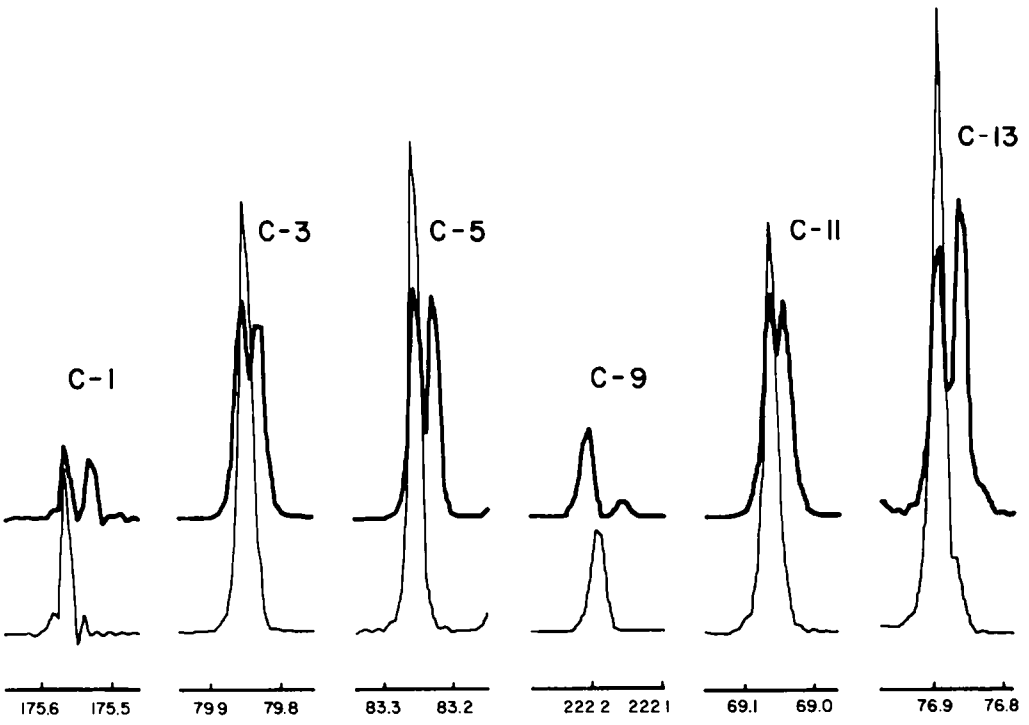
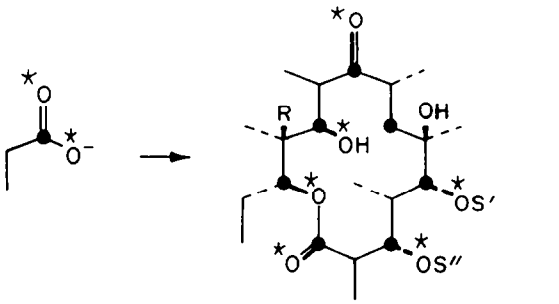


Fig. 2. Partial ^{13}C NMR spectra of erythromycin A 2′-benzoate showing the signals of the oxygen-bearing carbon atoms of the lactone ring. Spectroscopic parameters are given in Table 2, footnote *a*. Lower trace: sample derived from [1-^{13}C]propionate. Upper trace: sample derived from [1-$^{18}O_2$,1-^{13}C]propionate.

the peaks corresponding to C-1, -3, -5, -9, -11, -13 in both erythromycin A and B benzoate each appeared as enhanced pairs of signals corresponding to ^{16}O–^{13}C and ^{18}O–^{13}C species, the latter resonance being shifted 0.02–0.05 ppm upfield, according to the type of C–O bond (Scheme 3). Variable but small amounts of oxygen exchange had occured at most sites, leading to a partial dilution of the ^{18}O label relative to ^{13}C.[23] The ketone carbonyl oxygen at C-9 was more extensively exchanged, presumably due to the basic conditiosn (pH 9) required for isolation of the macrolide. Nonetheless, a reproducible quantity (15%) ^{18}O was evident at C-9 in both erythromycin A and B.

From these results it is clear that each of the six O atoms present in the first-formed macrolide aglycone, 6-deoxyerythronolide B (**3**), was derived from the propionate precursor. Only the carbonyl O of the lactone originated from the C-1;2;14 propionate unit, The bridging ester O having been derived intact from the corresponding C-13;20;21 propionate moiety. The four secondary OH or ether functions of the macrolide ring each bore excess O isotope, irrespective of their individual *D* (C-13) or *L* (C-3, -5, -11) configuration.[26] These results strongly suggest that the oxidation level eventually observed in the parent aglycone **3** is established at each stage of the chain elongation process, thereby excluding alternative oxidation[27] or dehydration–rehydration mechanisms[8] previously considered. The potential generality of these results to the understanding of polyketide biosynthesis is emphasized by the fact that the more than 100 known macrolides can be represented by a single stereochemical model, first recognized by Celmer in 1965.[28] The results reported above are also in accord with related findings on the biosynthesis of the polyethers monensin[16,28b,29] and lasalocid,[25] as well as the non-classical macrolide avermectin.[30]



S′ = desosaminyl (benzoate)
S″ = cladinosyl
1 R = OH; 2 R = H

Scheme 3.

EXPERIMENTAL

Proton and ^{13}C NMR spectra were obtained on a Bruker WM 250 FT NMR Spectrometer at 250.0 and 62.9 MHz respectively. NMR spectra are reported as parts per million downfield of Me_4Si. (δ 0.0). Multiplicities are as follows: s = singlet, d = doublet, t = triplet, q = quartet. IR spectra were recorded on a Perkin-Elmer Model 681 Infrared Spectrophotometer. Mass spectra were obtained using the direct inlet system of a Hitachi Perkin-Elmer RMU-6D instrument at 70-eV. Mps were taken in unsealed capillary tubes in a Hoover mp apparatus and are uncorrected. Radioactivity measurements utilized a Packard 3330 liquid

scintillation counter and were carried out in 10-mL toluene solns containing 7.2 g of BuPBD and 0.45 g of PBBO/L of toluene. Fermentations were carried out in a New Brunswick G-25 gyrotory shaker.

PLC and TLC were carried out by using 20 × 20 cm Merck precoated silica gel 60 F-254 plates, 0.25 mm thickness. Compounds were visualized by UV light or by heating to 150° after spraying with arsenomolybdate soln.[31] Column chromatography employed Merck silica gel 60 (0.05–0.2 mm).

Authentic erythromycin A and B were gifts of Eli Lilly Company. Sodium [1-^{13}C]propionate (90 at.%) was purchased from Merck&Company. Sodium [2-^{13}C]propionate, [1-$^{18}O_2$, 1-^{13}C]propionate, and [2,3-$^{13}C_2$]succinate were prepared as previously described.[16] Sodium [1-^{14}C]propionate (52 mCi/mmol) was purchased from New England Nuclear. [2,3-$^{14}C_2$]Succinic acid (111.4 mCi/mmol) was obtained from Amersham.

A vegetative medium of *Streptomyces erythreus* was prepared from 450 mg dibasic potassium phosphate (K_2HPO_4), 2.0 g saccharose ("Bacto", Difco), 1.5 g casein (acid hydrolysed, Sigma), 50 mg NaCl, 15 mg L-arginine (free base, Sigma), and 1 ml of a trace element soln containing 46.2 mg citric acid, 2 mg $FeSO_4 \cdot 7H_2O$, 1 mg $ZnSO_4 \cdot 7H_2O$, 0.8 mg $MnCl_2 \cdot 4H_2O$, 0.1 mg $CoCl_2 \cdot 6H_2O$, 50 mg $MgSO_4 \cdot 7H_2O$, and 0.12 mg ascorbic acid (Sigma), in a total of 100 mL of deionized water. The pH before sterilization was 6.9. This broth, in a 500-mL baffled DeLong flask, was innoculated with 2 mL of a culture of *Streptomyces erythreus* (Eli Lilly, E57-236, Lot 7), previously grown from a lyophilized pellet in a vegetative medium of the same composition and stored frozen in liquid N_2 in sterile "PRO-VIALS" (Cook). The flask was plugged with a cotton stopper and shaken at 250 rpm (1 in. circle stroke) at 32° for 48 hr.

Fermentation of Streptomyces erythreus. A fermentation medium consisting of 5.0 g PROFLO (cotton seed meal, Traders), 0.5 g yeast extract ("Bacto", Difco), 4.5 g dextrose ("Bacto", Difco), 0.5 mL of soybean oil (Sigma), 0.6 g $CaCO_3$, and 1 mL of the same trace element soln as above, in 100 mL tap water, was autoclaved at 120° for 20 min in a 500-mL baffled DeLong flask (pH before sterilization, 5.9). The medium was innoculated with 5.0 mL of the previously described vegetative culture by sterile syringe, the flask was covered with two layers of gauze faced cotton towels secured with rubber bands, and the culture was incubated at 250 rpm and 32°. Feeding of labeled precursors took place at intervals as described below.

After 6 days the broth from each flask (pH = 8.8–8.9) was washed with 50 mL water into centrifuge beakers and centrifuged for 30 min at 16300 g. The supernatants were poured off and the sediment was resuspended in 50 mL water. After a second centrifugation (30 min) the combined turbid brown phases were stirred for 5 min in 50 mL of 20% $ZnSO_4$ soln and the pH (5.9–6.0) was adjusted to 9.8 by the addition of 2N NaOH (*ca* 35 mL). After addition of 10 g celite 503, the ppt was filtered off by suction through filter paper and was washed with 100 mL fresh water. The resulting filtrate, which had a golden color, was extracted three times with 250 mL $CHCl_3$. The organic phases were washed with 10 mL brine and were dried over $MgSO_4$. After complete evaporation of the solvent of 40°, between 35 and 50 mg of a gold amorphous solid was obtained for every 100 mL broth. Analysis of TLC on silica ($CHCl_3$–CH_3OH–NH_4OH 8:2:0.01) showed a long spot (R_f 0.12–0.29) which corresponded to a mixture of erythromycin A and B.

Erythromycin A and B 2′-benzoate. Erythromycin was isolated conveniently as its 2′-benzoate which was prepared by stirring the crude $CHCl_3$ extract with an approximately equal weight of benzoic anhydride in 1.5 mL acetone for 12-36 hrs at room temp. The mixture was concentrated *in vacuo* and the crude benzoate esters were initially purified by column chromatography on 20 g silica, using $CHCl_3$/Me_3OH/NH_4OH 9:1:0.01 as eluant. From 100 mg crude extract as much as 75 mg crystalline material could be obtained (R_f = 0.47 in the same solvent system). Erythromycins A and B 2′-benzoates were obtained as pure crystalline materials by separation on a column of Sephadex LH 20 (1.1 × 120 cm) eluted with $CHCl_3$–hexane 1:1 (200 drops/fraction, *ca* 4 sec/drop).[13] The effluent was monitored by TLC ($CHCl_3$ Me_3OH–NH_4OH 9:1:0.01) and the purity and composition of the macrolide-containing fractions were determined by ^{13}C NMR. A typical separation gave 16.0 mg 2′-O-benzoyl-erythromycin B, (fractions 37–43), 27.0 mg 2′-O-benzoyl-erythromycin A, (fractions 46–54), and 11.0 mg of a mixture of A and B benzoates (fractions 44–45).

Precursor incorporation. Labeled propionate precursors containing radioactive internal standard were dissolved in distilled water and added in three portions (20, 30 and 50%) through a Millex disposable sterile filtration unit to 100 mL of a fermentation culture of *S. erythreus* at 48 hr, 72 hr, and 96 hr, respectively. At the conclusion of the normal 6-day fermentation period, the labeled erythromycins were isolated and purified in the usual manner.

Kuhn–Roth oxidation of erythromycin benzoate. Erythromycin benzoate (24 mg, 6.56×10^4 dpm, 2.29×10^6 dpm/mmol), obtained from incorporation of 1.05×10^7 dpm of sodium [1-^{14}C]propionate, was stirred with 10 mL of 40% chromium(VI)–oxide for 6 hr at room temp. After addition of [1-^{14}C]propionate, was stirred with 10 mL of 40% chromium(VI)–oxide for 6 hr at room temp. After addition of 3 mL orthophosphoric acid, the mixture was placed in a Wiesenberger apparatus[15] and a total of 75 mL distillate was collected. Neutralization with 0.1 N KOH required 2.2 mL base, corresponding to 76% of theoretical, based on 1 equiv propionic acid and 9 equiv AcOH. After concentration to dryness at aspirator pressure, the solid residue (9.14×10^3 dpm) was dissolved in 2 mL water and treated with 0.2 mL 85% phosphoric acid before lyophilization. Titration of the lyophilizate (0.1 N KOH, phenolphthalein) yielded a soln containing 0.195 mmol of K-salts (67%), which were collected by drying overnight under high vacuum (8.14×10^3 dpm). Treatment of the isolated K-salts with 60 mg (0.22 mmol) *p*-phenylphenacyl bromide in 3 mL acetonitrile containing 5 mg (0.019 mmol) of 18-crown-6 for 2 hr followed by addition of brine and extraction with $CHCl_3$ gave, after concentration of the dried extracts, a crude mixture of esters. Purification by PLC gave 3 mg *p*-phenylphenacyl propionate which was crystallized twice from hexane, m.p. 100.5–101° (lit.[32] m.p. 102°), 4.88×10^5 dpm/mmol (21.3% of erythromycin). An additional 41 mg *p*-phenylphenacyl acetate were also isolated, which after two crystallizations from benzene–hexane gave 38 mg of needles, m.p. 110° (lit.[32] m.p. 111°), 1.00×10^3 dpm/mol (0.04% of erythromycin).

Diethyl [2,3-$^{13}C_2$]succinate. [2,3-$^{13}C_2$]Succinic acid (0.361 g, 3.06 mmol), prepared as previously described[16] was mixed with 8.27×10^6 dpm [2-^{14}C]succinic acid in 2.3 mL EtOH. The soln was transferred to a 2-neck, 10-mL round bottom flask equipped with a condenser and serum cap. The flask was placed on a water bath at room temp. and HCl gas was bubbled into the stirring soln through the serum cap for 2 hr. $CHCl_3$ (100 mL) and 100 mL water were added to the soln and the two layers were separated. The aqueous layer was washed twice with 100 mL $CHCl_3$ and the layers were separated. The aqueous layer was washed twice with 100 mL $CHCl_3$ and the combined $CHCl_3$ extracts were washed with 100 mL each of sat $NaHCO_3$ aq and brine. The $CHCl_3$ extract was then dried with $MgSO_4$ and the solvent removed on a rotary evaporator. Further drying at high vacuum gave 0.311 g (59%) diethyl [2,3-$^{13}C_2$]succinate as a colorless liquid. ^{1}H NMR (60 MHz, $CDCl_3$) δ 4.15 (q, J = 7 Hz, 2H, CH_3CH_2–), 2.60 (d, J = 125 Hz, 2H, EtO_2C–$^{13}CH_2$, 1.27 (t, J = 7 Hz, 3H, CH_3), IR λ_{max} (neat) 1735 cm^{-1} (C–O).

Incorporation of diethyl [2,3-$^{13}C_2$]*succinate.* Diethyl (2,3-$^{13}C_2$]succinate was fed without dilution to 100 mL of an actively growing culture of *Streptomyces erythreus* in three portions of 60 mg (48 hr), 90 mg (72 hr), and 150 mg (96 hr) respectively. ^{13}C NMR analysis of the derived erythromycin A benzoate (11.1 mg) showed an enrichment of *ca* 4% at each labeled site.

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