Macrolide Biosynthesis. Origin of the Oxygen Atoms in the Erythromycins

David E. Cane,*† Heinz Hasler, and Tzyy-Chyau Liang

Department of Chemistry, Brown University
Providence, Rhode Island 02912

Received June 1, 1981

Erythromycin A (**1**) is a broad spectrum antibiotic which is a potent inhibitor of protein synthesis both in vivo and in vitro.[1] The erythromycins are representative of a large class of some 50 macrolide antibiotics, substances possessing 12-, 14-, or 16-membered lactone rings and substituted with a complex array of methyl and hydroxyl functions as well as a novel group of neutral and nitrogen-containing deoxy sugars. The complex chemical structures and potent biological activity of these substances have stimulated intensive investigations of their biological origin, chemical synthesis and properties, and biochemical mode of action since the discovery of pikromycin in 1950.[2] In spite of the diversity of structures exhibited by the known macrolides, Celmer has made the intriguing observation that all these substances can be represented by a single stereochemical model.[3] While this apparent regularity has been of great utility in the structure determination of new macrolides and has important implications for the biogenesis of this class of metabolites, to date no satisfactory hypothesis has been advanced which accounts for the observed stereochemical pattern.

The general outlines of the biosynthesis of the macrolide family of antibiotics are by now reasonably well understood[4] (Scheme I). Incorporations of radioactive propionate and methylmalonate into erythromycin A (**1**) and partial degradations have confirmed the proposals of Woodward[5] and Gerzon[1] that the aglycons of these substances are formed by condensation of propionate units in a manner analogous to the synthesis of saturated fatty acids.[6] These early experiments have been placed on a more rigorous footing by recent experiments in which ^{13}C NMR spectroscopy was used to establish the positions of labeling in erythronolide B (**2**) derived from feeding [^{13}C]propionate to cultures of a blocked mutant of *Streptomyces erythreus*.[7] At the enzyme level, Lynen has investigated a propionyl-CoA carboxylase isolated from *S. erythreus*.[8] Further insight into the erythromycin biosynthetic pathway has come from the use of a series of blocked mutants.[9] The first identified intermediate after propionate and methylmalonate is

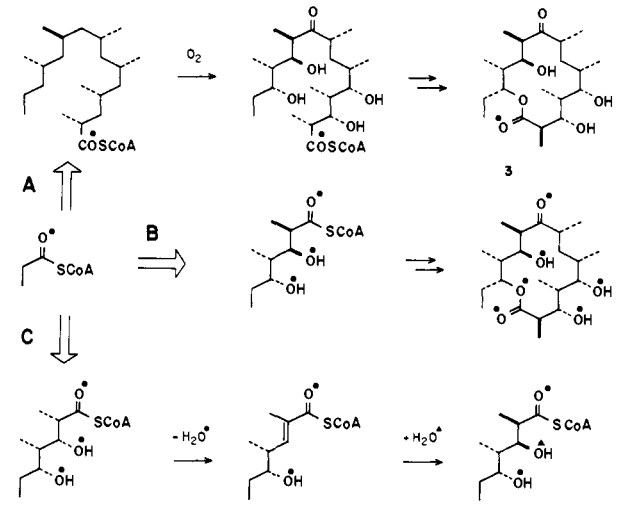
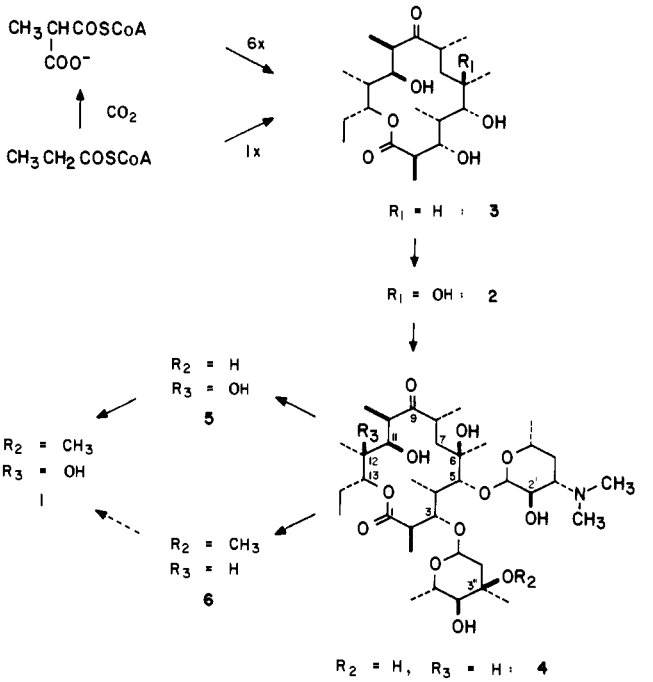


Figure 1. Hypothetical pathways of macrolide formation.

Scheme I



6-deoxyerythronolide B (**3**). This substance bears all the carbon atoms of the erythromycin aglycon in the proper stereochemistry, differing only by the absence of tertiary hydroxyl functions at C-6 and C-12. Oxidation of **3** at C-6 generates erythronolide B (**2**) which is further metabolized by successive introduction of mycarose and desosamine sugars at C-3 and C-5, respectively, to generate erythromycin D (**4**). At this point the pathway appears to branch. Methylation of the mycarosyl 3″-OH residue generates the corresponding cladinose derivative, erythromycin B (**6**). On the other hand, initial oxidation at C-12 produces erythromycin C (**5**) which undergoes 3″-O-methylation to produce erythromycin A (**1**). The latter steps have been studied in detail by Corcoran who has described the purification of the *S*-adenosyl-L-methionine transmethylase as well as the corresponding mixed function oxygenases.[10,11] Studies of several other macrolides, although not

† Fellow of the Alfred P. Sloan Foundation, 1978-1982; National Institutes of Health, Research Career Development Award, 1978-1983.

(1) (a) Structure: E. H. Flynn, M. V. Sigal, P. F. Wiley, and K. Gerzon, *J. Am. Chem. Soc.*, **76**, 3121 (1954); K. Gerzon, E. H. Flynn, M. V. Sigal, P. F. Wiley, R. Monahan, and U. C. Quarck, *ibid.*, **78**, 6396 (1956); P. F. Wiley, K. Gerzon, E. H. Flynn, M. V. Sigal, O. Weaver, U. C. Quarck, R. R. Chauvette, and R. Monahan, *ibid.*, **79**, 6062 (1957); P. F. Wiley, M. V. Sigal, O. Weaver, R. Monahan, and K. Gerzon, *ibid.*, **79**, 6070 (1957); D. R. Harris, S. G. McGeachin, and H. H. Mills, *Tetrahedron Lett.*, 679 (1965); (b) Mechanism of action: cf. D. Vasquez in J. W. Corcoran and F. E. Hahn, "Antibiotics III. Mechanism of Action of Antimicrobial and Antitumor Agents", Springer, New York, 1975, p 459.

(2) H. Brockmann and W. Henkel, *Naturwissenschaften*, **37**, 138 (1950).

(3) W. D. Celmer, *Pure Appl. Chem.*, **28**, 413 (1971); W. D. Celmer, *J. Am. Chem. Soc.*, **87**, 1801 (1965).

(4) S. Masamune, G. S. Bates, and J. W. Corcoran, *Angew. Chem., Int. Ed. Engl.*, **16**, 585 (1977).

(5) R. B. Woodward, *Angew. Chem.*, **69**, 50 (1957).

(6) S. M. Friedman, T. Kaneda, and J. W. Corcoran, *J. Biol. Chem.*, **239**, 2386 (1964); T. Kaneda, J. C. Butte, S. B. Taubman, and J. W. Corcoran, *ibid.*, **237**, 322 (1962); J. W. Corcoran, T. Kaneda, and J. C. Butte, *ibid.*, **235**, PC29 (1960); H. Grisebach, H. Achenbach, and W. Hofheinz, *Z. Naturforsch. B*, **15B**, 560 (1960).

(7) J. G. Nourse, Ph.D. Dissertation, California Institute of Technology, 1974.

(8) E. J. Wawszkiewicz and F. Lynen, *Biochem. Z.*, **340**, 213 (1964).

(9) J. R. Martin, T. J. Perun, and R. L. Girolami, *Biochemistry*, **5**, 2852 (1966); J. R. Martin and W. Rosenbrook, *ibid.*, **6**, 435 (1967); J. W. Corcoran and J. Majer, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34**, 589 (1975); J. R. Martin and A. W. Goldstein, *Prog. Antimicrob. Anticancer Chemother., Proc. Intl. Cong. Chemother.*, **2**, 1112 (1969).

(10) A. M. Vygantas and J. W. Corcoran, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33**, 1233 (1974); J. W. Corcoran and A. M. Vygantas, *ibid.*, **36**, 663 (1975).

(11) J. Majer, J. R. Martin, R. S. Egan, and J. W. Corcoran, *J. Am. Chem. Soc.*, **99**, 1620 (1975); J. W. Corcoran, *Methods Enzymol.*, **43**, 487 (1975); T. S. McAlpine and J. W. Corcoran, *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **30**, 1168 (1971).

0002-7863/81/1503-5960$01.25/0 © 1981 American Chemical Society

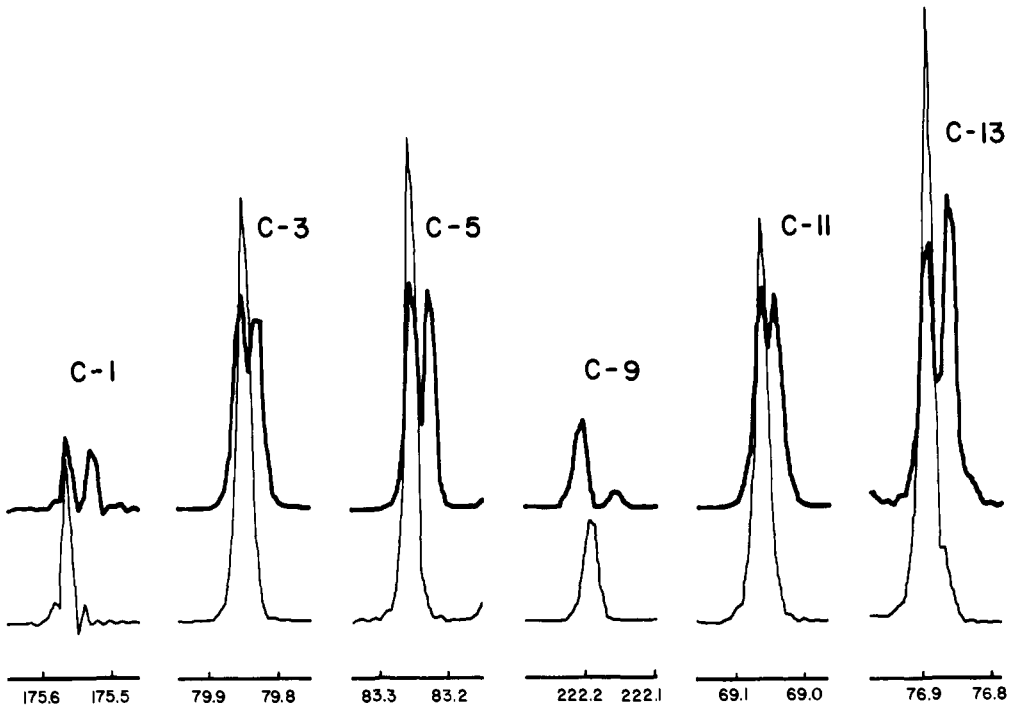


Figure 2. Partial ^{13}C NMR spectra of erythromycin A 2'-benzoate showing the signals of the oxygen-bearing carbon atoms of the lactone ring. Spectroscopic parameters are given in Table I, footnote a. Lower trace: sample derived from [I-^{13}C]propionate. Upper trace: sample derived from [1-$^{18}O_2$,1-^{13}C]propionate.

as detailed as those applied to erythromycin, are consistent with the same general picture.[4,12-15]

Although a great deal is known about the stereochemistry and detailed enzymology of fatty acid biosynthesis,[16] the relationship to the enzymes responsible for the assembly of the macrolide aglycons remains obscure, and nothing is known about the details of the steps by which the simple carboxylic acid precursors are converted to the corresponding branched chain polyhydroxy lactones. Several explanations have been advanced for the oxygenation pattern of the macrolides (Figure 1). (A) According to one hypothesis, a branched chain saturated fatty acid is first assembled and subsequently undergoes oxygenation at the several sites with the appropriate stereochemistry.[17] All the oxygen atoms of the aglycon, save the lactone carbonyl, would therefore be derived from molecular oxygen. (B) An alternative hypothesis views the biogenesis of the macrolide carbon skeleton as a variant of fatty acid biosynthesis in which condensation of each methylmalonyl-CoA can be followed by a sequence terminating with β-ketoacyl-CoA reduction, dehydration, or double-bond reduction, respectively, before addition of the next unit of methylmalonyl-CoA. Furthermore, the stereochemical course of each ketone group reduction would depend on the position in the growing carbon chain.[4,6,17] In the latter case each oxygen atom at an odd-numbered center of the macrolide would be derived from the precursor propionyl-CoA. (C) A third alternative envisages reduction of each intermediate β-keto ester with a common stereochemistry, followed by inversion of specific hydroxyl groups by a dehydration–rehydration sequence with concomitant epimerization of the adjacent secondary methyl substituent. A consequence of the latter pathway would be the derivation of certain hydroxyl oxygen atoms (for example, at C-3, -5 and -11) of the macrolide from water with the remainder originating directly from the carbonyl oxygens of propionate. We report below the results of [^{18}O]propionate incorporations which establish unambiguously that in the biosynthesis of erythromycin all the oxygen atoms of the initial macrolide aglycon **3** are retained from the precursor.

Table I. 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 shift, ppm	Δδ, ppm[b]	$^{18}O/^{16}O$[c]	^{13}C shift, 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

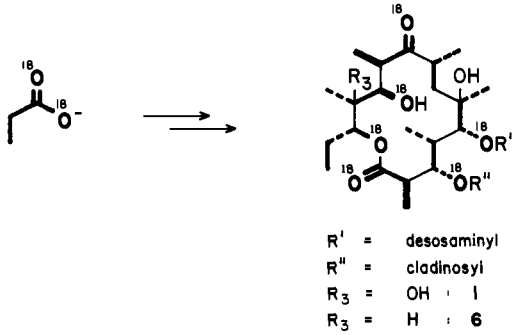
[a] Bruker WM 250, 62.9 MHz; spectral width 19230 Hz, 32K time domain plus 32K zero points; quadrature detection; 90° pulse; repetition rate 1.0 s; 21 000 transients; 40 mg in 2 mL of $CDCl_3$, δ(Me_4Si) 0; resolution enhancement was achieved by Lorentz–Gauss multiplication of the FID prior to Fourier transformation [R. R. Ernst, *Adv. Magn. Reson.*, 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.

To a solution of 0.333 g, 90 atom %), mixed with 0.667 g of unlabeled sodium propionate[18] and 9.74×10^6 dpm of sodium [1-^{14}C]propionate as internal standard in 10 mL of

(12) A. J. Birch, C. Djerassi, J. D. Dutcher, J. Majer, D. Perlman, E. Pride, R. W. Rickards, and P. J. Thomson, *J. Chem. Soc.*, 5274 (1964).
(13) H. Achenbach and H. Grisebach, *Z. Naturforsch. B*, **19B**, 561 (1964); H. Grisebach and C. A. Weber-Schilling, *ibid.*, **23B**, 655 (1968); D. Srinivasan and P. R. Srinivasan, *Biochemistry*, **6**, 3111 (1967).
(14) S. Omura, A. Nakagawa, H. Takeshima, K. Atusmi, J. Miyazawa, F. Piriou, and G. Lukacs, *J. Am. Chem. Soc.*, **97**, 6600, 6765 (1975).
(15) S. Omura, A. Nakagawa, H. Takeshima, J. Miyazawa, C. Kitao, F. Piriou, and G. Lukacs, *Tetrahedron Lett.*, 4503 (1976); S. Omura, H. Takeshima, A. Nakagawa, N. Kanemoto, and G. Lukacs, *Bioorg. Chem.*, **5**, 451 (1976).
(16) F. Lynen, *Biochem. J.*, **102**, 381 (1967); K. Bloch and D. Vance, *Annu. Rev. Biochem.*, **46**, 263 (1977); B. Sedgwick and J. W. Cornforth, *Eur. J. Biochem.*, **75**, 465 (1977); B. Sedgwick, J. W. Cornforth, S. J. French, R. T. Gray, E. Kelstrup, and P. Willadsen, *ibid.*, **75**, 481 (1977); B. Sedgwick, C. Morris, and S. J. French, *J. Chem. Soc., Chem. Commun.*, 193 (1978).
(17) Cf. H. Grisebach, "Biosynthetic Patterns in Microorganisms and Plants", Wiley, New York, 1967, pp 32–65. The biosynthesis of branched chain fatty acids has been studied: J. S. Buckner, P. E. Kolattuckady, and L. Rogers, *Arch. Biochem. Biophys.*, **186**, 152 (1978).

(18) The ^{13}C-labeled precursor was diluted with unlabeled substrate in order to avoid excess intramolecular multiple labeling of the macrolide product which results in undesirable broadening of the resultant ^{13}C NMR resonances due to ^2J(C–C) couplings.

Scheme II



water was administered in portions (2 mL after 48-h, 3 mL after 72-h, and 5 mL after 96-h incubation time) to 100 mL of a growing fermentation of *Streptomyces erythreus*[19] in a complex cotton seed medium. After a total of 6 days, the resulting mixture of antibiotics was extracted with chloroform and the mixture of erythromycins A and B isolated as the readily crystallized 2'-benzoate esters. Separation of the two esters was achieved by chromatography on Sephadex LH 20 (1:1 chloroform-hexane)[11] 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. Analysis of the 62.9-MHz ^{13}C NMR spectrum of each of the labeled macrolide esters established that carbons 1, 3, 5, 7, 9, 11, and 13 were labeled as expected.[7,20] The observed signal enhancements (ca. 13% ^{13}C per labeled site) were in accord with the enrichment calculated from the measured specific activity (2.59×10^6 dpm/mmol). A starter effect[6] was apparent in the slightly greater enhancement of the signals corresponding to C-13.[21]

Suitable conditions for incorporation of labeled propionate having been established, 100 mL of *S. erythreus* were fed a mixture of sodium [1-$^{18}O_2$,1-^{13}C]propionate[23] (0.333 g; 54.9% $^{18}O_2{}^{13}C$, 32.1% $^{18}O^{13}C$, 3.6% $^{16}O^{13}C$), 0.667 g unlabeled sodium propionate, and a trace of sodium [1-^{14}C]propionate and the resulting labeled erythromycins A and B were isolated and purified as the derived 2'-benzoates in the manner described above. The sites of ^{18}O enrichment were determined directly by ^{13}C NMR spectroscopy by taking advantage of the isotope shifts on the resonances of the attached ^{13}C nuclei, a technique recently introduced independently by Van Etten[24] and Vederas.[25] As summarized in Table I and illustrated in part in Figure 2, the peaks corresponding to C-1, -3, -5, -9, -11, and -13 in both erythromycin A and B benzoate each appeared as enhanced pairs of signals corresponding to ^{13}C-^{16}O and ^{13}C-^{18}O species, the latter resonances being shifted 0.02-0.05 ppm upfield, according to the type of C-O bond. From these results it is clear that each oxygen atom present in the first-formed macrolide aglycon, 6-deoxyerythronolide B (**3**), must have been derived largely from the precursor propionate (Scheme II). In particular, the four secondary hydroxyl sites (including C-13) all bore excess oxygen isotope, irrespective of their individual configurations. Interestingly although the carbonyl group at C-9 appeared to have undergone considerable oxygen exchange, a small amount of ^{18}O (ca 15%) was still evident. Variable but much smaller amounts of exchange had occurred at each of the remaining sites.

Since the tertiary hydroxyl groups at C-6 and C-12 of erythromycin A and at C-6 of erythromycin B have already been shown to originate from molecular oxygen,[10,11] the origin of all the macrolide aglycon oxygens has now been established. The above results clearly exclude both the oxidation pathway A and the dehydration-rehydration pathway C described earlier. Whether the stereochemical arrangement of the secondary methyl groups at C-2, -4, -8, -10, and -12 is determined by the choice of 2(*R*) or 2(*S*)-methylmalonyl-CoA as condensation substrate or whether condensation of a single enantiomer of methylmalonyl-CoA is followed in some instances by epimerization of the α-methyl-β-ketoacyl-CoA intermediate remains to be established. Nonetheless the stereochemical homology among the various known macrolides embodied by the Celmer Model emphasizes the potential generality of our results which are also completely in accord with related findings on the biosynthesis of the polyethers monensin[26] and lasalocid[27] described in the accompanying papers.

Acknowledgment. This work was supported by NIH Grant GM 22172. Strains of *S. erythreus* were kindly provided by Dr. L. E. Day of the Eli Lilly Co. who also furnished helpful information on fermentation conditions. A sample of erythromycin A was a generous gift from Eli Lilly. The [^{13}C]cyanide was obtained from the National Stable Isotopes Resource, Los Alamos Scientific Laboratory, supported by the U.S. DOE and the NIH (RR-00962). The Bruker WM 250 NMR spectrometer used in this work was purchased with funds provided by the NSF and the Montedison Group of Milan. We would like to thank Professor Richard Hutchinson of the University of Wisconsin for frequent mutual exchanges of information and for generously offering to delay publication of his own work[27] in order to allow simultaneous submission of our manuscripts.

(19) *S. erythreus*, Eli Lilly strain E57-236.

(20) The ^{13}C NMR spectra of erythromycins A and **B** have been completely assigned: J. G. Nourse and J. D. Roberts, *J. Am. Chem. Soc.*, **97**, 4584 (1975); Y. Terui, K. Tori, K. Nagashima, and N. Tsuji, *Tetrahedron Lett.*, 2583 (1975); S. Omura, A. Neszmelyi, M. Sangare, and G. Lukacs, *ibid.*, 2939 (1975).

(21) In agreement with these observations, Kuhn-Roth oxidation[22] of a sample of labeled erythromycin benzoate obtained from a separate feeding of 1.0 g of [1-^{14}C]propionate gave propionic acid whose *p*-phenylphenacyl ester bore 21.3% of the specific activity of the intact macrolide. The lack of activity in acetic acid isolated from the same Kuhn-Roth degradation established that no significant randomization of label had occurred.

(22) E. Wiesenberger, *Mikrochim. Acta*, **33**, 51 (1948).

(23) [1-^{13}C]Propionitrile was obtained by reacting 25.0 mmol of potassium [^{13}C]cyanide (90 atom%) with 25.1 mmol of ethyl iodide in 5.0 g of absolute methanol to which 0.24 mL of [^{18}O]water (95 atom %) had been added. (70 °C/12 h and then 80 °C/38 h). The entire mixture was distilled, mixed with 53 mmol of [^{18}O]water (95 atom %) and 29.5 mL of 0.85 M potassium *tert*-butoxide in *tert*-butyl alcohol, and refluxed for 48 h. The residue obtained upon evaporation of the solvent was redissolved in distilled water, acidified with phosphoric acid, and lyophilized. Titration of the lyophilizate with sodium hydroxide gave sodium [1-$^{18}O_2$,1-^{13}C]propionate (75% yield), a portion of which was converted to the *p*-phenylphenacyl ester for mass spectrometric analysis.

(24) J. M. Risley and R. L. Van Etten, *J. Am. Chem. Soc.*, **101**, 252 (1979); *ibid.*, **102**, 4609, 6699 (1980).

(25) J. C. Vederas, *J. Am. Chem. Soc.*, **102**, 374 (1980); J. C. Vederas and T. T. Nakashima, *J. Chem. Soc., Chem. Commun*, 183 (1980).

(26) D. E. Cane, T. C. Liang, and H. Hasler, *J. Am. Chem. Soc.*, following paper in this issue.

(27) C. R. Hutchinson, M. R. Sherman, J. C. Vederas, and T. T. Nakashima, *J. Am. Chem. Soc.*, first paper in this series; C. R. Huchinson, M. M. Sherman, J. C. Vederas, A. G. McInnes, and J. A. Walters, *ibid.*, preceding paper in this issue.

Polyether Biosynthesis. Origin of the Oxygen Atoms of Monensin A

David E. Cane,*† Tzyy-Chyau Liang, and Heinz Hasler

Department of Chemistry, Brown University
Providence, Rhode Island 02912

Received June 1, 1981

The polyether antibiotic monensin A (**1**), an important agent in the control of coccidiosis in poultry,[1] is one of a large class of naturally occurring ionophores which have attracted increasing

† Fellow of the Alfred P. Sloan Foundation, 1978-1982; National Institutes of Health, Research Career Development Award, 1978-1983.

0002-7863/81/1503-5962$01.25/0 © 1981 American Chemical Society