(CCl_4) δ 1.1 (s, 9 H), 2.65 (s, 4 H), 3.6-4.4 (m, 3 H); MS (EI), *m/e* 261, 263 (1:1, M^+), 204, 206 (1:1, $M^+ - C_4H_9$), 182 ($M^+ - Br$). ^1H NMR integrations were used to determine the absolute yield in product mixtures.

Acknowledgment. Financial support for this work came from the National Science Foundation (Grant CHE-7810049).

Registry No. Neopentane, 463-82-1; *n*-butane, 106-97-8; cyclopentane, 287-92-3; isobutane, 75-28-5; 2,3-dimethylbutane, 79-29-8; cyclohexane, 110-83-8; benzene, 71-43-2; 1,3-butadiene, 106-99-0; bromine, 7726-95-6; chloroform, 67-66-3; *N*-bromosuccinimide, 128-08-5; 1-bromo-2-succinimidylcyclohexane, 82469-57-6; 1-bromo-4-succinimidyl-2-butene, 82469-58-7; 3-bromo-4-succinimidyl-1-butene, 82469-59-8; *N*-(2-bromo-3,3-dimethyl-1-butyl)succinimide, 72323-45-6.

Polyether Biosynthesis. 2. Origin of the Oxygen Atoms of Monensin A

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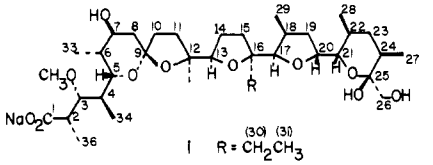
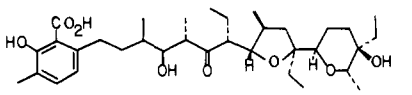
Contribution from the Department of Chemistry, Brown University, Providence, Rhode Island 02912. *Received April 26, 1982*

Abstract: Feeding of [1-^{13}C]acetate to cultures of *Streptomyces cinnamonensis* gave monensin A labeled at carbons 7, 9, 13, 19, and 25, as established by ^{13}C NMR analysis. Similarly, incorporation of [1-^{13}C]propionate resulted in enrichment of carbons 1, 3, 5, 11, 17, 21, and 23. Further incorporations of [1,2-$^{13}C_2$]acetate, [1,2-$^{13}C_2$]propionate, [2-^{13}C]propionate, and [2,3-$^{13}C_2$]succinate and analysis by ^{13}C NMR, including extensive homonuclear $^{13}C\{^{13}C\}$ decoupling, established the biosynthetic origins of all the carbon atoms of monensin, while allowing a complete assignment of the ^{13}C NMR spectrum. When [1-^{13}C,1-$^{18}O_2$]propionate was fed, isotopically shifted peaks indicating the presence of oxygen-18 at C-1, C-3, and C-5 were observed, whereas feeding of [1-^{13}C,1-$^{18}O_2$]acetate gave rise to excess oxygen-18 at C-7, C-9, and C-25. Three of the remaining ether oxygens, O(7), O(8), and O(9), were shown to be derived from molecular oxygen by growth of *S. cinnamonensis* in an atmosphere of $^{18}O_2$ and ^{13}C NMR analysis of the resulting labeled monensin A. These results are consistent with initial formation of the *all-E*-triene **7**, which can be converted to monensin by cyclization of the triepoxide **8**.

The polyether antibiotics, a group of more than 60 naturally occurring ionophores,[2] have attracted intense chemical and biochemical attention since the determination of the structure of monensin A (**1**) only 15 years ago.[3] Two of these compounds, monensin and lasalocid (**2**),[4] have found important veterinary applications in the control of coccidiosis in poultry and as agents for the improvement of feed utilization in ruminant livestock. The



1 R = CH_2CH_3
6 R = CH_3



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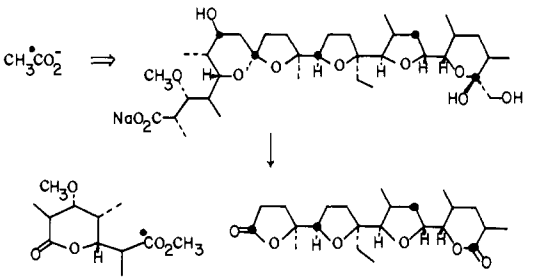
Scheme I



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vast majority of these polyoxygenated, branched-chain fatty acids are produced by species of the genus *Streptomyces*. Recent investigations in several laboratories of the biosynthesis of these substances have focussed on identification of the simple precursors acetate, propionate, and butyrate and suggested an analogy to the well-understood formation of saturated fatty acids as well as to the biosynthesis of a second major class of polyoxygenated *Streptomyces* metabolites, the macrolide antibiotics.[5] In our own work we have been interested in establishing the details of the pathways by which both macrolides[6] and polyethers are formed from their simple precursors. Our initial efforts have concentrated on determining the extent to which the implied analogy to classical fatty acid biosynthesis is in fact applicable to the formation of these functionally and stereochemically far more complex analogues. To address this question, we have recently determined the origin of the oxygen atoms of monensin, and our results are described below.[7]

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

(2) Reviews: "Polyether Antibiotics: Carboxylic Ionophores", Westley, J. W., Ed.; Marcel Dekker: New York, 1982; Vol. I (Biology), Vol. II (Chemistry). Westley, J. W. *Annu. Rep. Med. Chem.* **1975**, *10*, 246. Westley, J. W. *Adv. Appl. Microbiol.* **1977**, *22*, 177. Pressman, B. C. *Annu. Rev. Biochem.* **1976**, *45*, 501.

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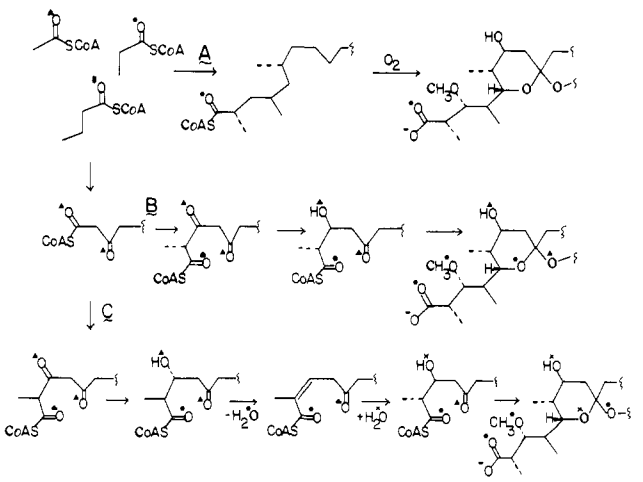

Figure 1. Hypothetical Pathways for polyether chain formation.

At the outset of our investigations, prior experiments by investigators at Eli Lilly using ^{14}C-labeled precursors followed by partial degradation of the derived monensin had already indicated that the carbon chain is assembled from five acetate, seven propionate, and one butyrate molecule, while the C-3 *O*-methyl is derived as expected from methionine.[8] For example, feeding of [1-^{14}C]acetate followed by chromic acid oxidation of the labeled monensin gave two fragments, a C-11 lactone ester (**3**) and C-23 dilactone diether (**4**) bearing 18.7% and 83.67%, respectively, of the molar ^{14}C activity of monensin, in accord with the suggested presence of one label at C-7 and a total of four at (presumably) C-9, C-13, C-19, and C-25 (Scheme I). These early results proved to be consistent with subsequently reported ^{13}C NMR biosynthetic studies of the polyethers lasalocid,[9] narasin,[10] salinomycin,[11] lysocellin,[12] lonomycin A,[13] and dianemycin.[14,15]

To date, the closest apparent model for polyether biosynthesis as well as for the biosynthesis of macrolides and related branched-chain polyacetate—propionate metabolites has been classical saturated fatty acid biosynthesis, a process that has been thoroughly investigated in a variety of organisms from the point of view of mechanism, stereochemistry, and enzymology.[16] Whether catalyzed by a single, multisite polypeptide or by a multienzyme system, the elaboration of the fatty acid chain is known to involve a simple sequence of four reactions: decarboxylative condensation of malonyl-CoA with a starter unit of acetyl-CoA or a growing fatty acyl thioester chain to generate a β-keto acyl thio ester, reduction to a D-β-hydroxy ester, syn dehydration to the corresponding enoyl thio ester derivative, and a second reduction to a saturated acyl thio ester homologated by two additional carbon atoms. This sequence is repeated several times by using additional malonyl-CoA units until the final 16-carbon chain of palmitate is released. At first glance this process would appear to provide a good analogy for the formation of the far more highly functionalized polyethers. For example, hydroxyl functions are present in monensin at C-3, C-5, and C-7, and the ketals at C-9 and C-25 are clearly derived from the corresponding ketones. Several positions no longer bear oxygen, however, such as the saturated carbons C-11, C-15, C-19, and C-23. The question arises, therefore, as to the mechanism by which the ultimate oxidation state at each site is determined. Any explanation must also account for the stereochemistry of the various secondary hydroxyl functions and of the methyl groups that are present with both D and L configurations.

Three simple models,[6,31] each using fatty acid biosynthesis as a prototype, were advanced to explain the oxygenation pattern of the polyether monensin (Figure 1). (A) One hypothesis posited the initial synthesis of a branched-chain saturated or partially unsaturated fatty acid that subsequently underwent oxygenation at several sites with the appropriate stereochemistry. Each of the oxygens of the resulting polyether, save those of the carboxylate, would then be derived from molecular oxygen. (B) A second hypothesis viewed the biogenesis of the polyether skeleton as a variant of fatty acid biosynthesis in which condensation of each unit of malonyl-CoA, methylmalonyl-CoA, or ethylmalonyl-CoA, respectively, would be followed by a sequence terminating at the stage of β-keto acyl-CoA reduction, dehydration, or double-bond reduction, as appropriate, before addition of the next condensation substrate. According to this model, the stereochemistry of each ketone-group reduction would vary with its position in the growing carbon chain. Each oxygen atom at an odd-numbered polyether carbon would be derived from the corresponding carbonyl oxygens of the acetate or propionate precursors. (C) A variant of the second hypothesis envisaged 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 any adjacent secondary methyl substituent. A consequence of the latter pathway would be the derivation of certain oxygen atoms of monensin (for example, at C-5 and C-7) from water, while those at C-1 and C-3 would be derived from the precursor propionate. It was therefore recognized that determination of the origins of the individual oxygen atoms of monensin would afford considerable insight into the manner in which the completed polyether is assembled from simple two-, three-, and four-carbon precursors.[17] We chose to investigate this problem using high-resolution ^{13}C NMR spectroscopy and to establish the sites of ^{18}O enrichment by exploiting the isotope shifts in the resonances of the attached ^{13}C nuclei, a technique recently introduced independently by Van Etten[18] and Vederas.[19]

Results

Prior to our own work, the ^{13}C NMR spectrum of monensin A itself had not yet been assigned, although assignments had been reported for several other polyethers and a set of empirical rules proposed.[20] We have now established a complete set of unambiguous signal assignments based on a combination of instrumental and biosynthetic methods[21] (Table I). The oxygen-bearing

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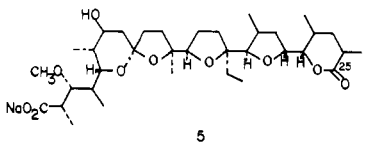
Table I. ^{13}C NMR Spectrum of Monensin A Sodium and Incorporation of ^{13}C-Labeled Precursors

chemical shift, δ (m)[a]	C	precursor[b] [1-^{13}C]-acetate	precursor[b] [1,2-$^{13}C_2$]-acetate J, Hz	C	precursor[b] [1-^{13}C]-propionate	precursor[b] [2-^{13}C]-propionate	precursor[b] [1,2-$^{13}C_2$]-propionate J, Hz	precursor[b] [2,3-$^{13}C_2$]-succinate J, Hz
181.3 (s)	1			1	*		51.6	
45.1 (d)	2			2		*	51.6	34.2[c]
16.7 (q)	36			36				34.2
83.1 (d)	3			3	*		37.8[c]	
37.5 (d)	4			4		*	37.8	36.3[c]
11.0 (q)	34			34				36.0[c]
68.4 (d)	5			5	*		37.1[c]	
34.9 (d)	6			6		*	37.0	35.2[c]
10.5 (q)	33			33				35.4[c]
70.5 (d)	7	*	37.4[c]	7				
33.6 (t)	8		d	8				
107.1 (s)	9	*	42.5	9				
39.3 (t)	10		42.1	10				
33.32 (t)	11			11	*		34.9	
85.3 (s)	12			12		*	34.9[c]	40.7
27.5 (q)	32			32				41.1
82.6 (d)	13	*	34.9[c]	13				
27.3 (t)	14		34.5	14				
29.9 (t)	15		(35.6[e])	15				
85.9 (s)	16		(35.6)[c,e]	16				
30.6 (t)	30		(34.9)[e]	30				
8.2 (q)	31		(34.9)[c,e]	31				
85.0 (d)	17			17	*		35.6[c]	
34.4 (d)	18			18		*	34.9	35.2[c]
14.6 (q)	29			29				34.9
33.26 (t)	19	*	d	19				
76.5 (d)	20		34.5[c]	20				
74.5 (d)	21			21	*		36.3[c]	
31.9 (d)	22			22		*	36.4	34.5[c]
16.8 (q)	28			28				34.5
35.8 (t)	23			23	*		*[f]	
36.6 (d)	24			24		*	*[f]	36.0[c]
16.1 (q)	27			27				36.0
98.4 (s)	25	*	45.8	25				
64.9 (t)	26		45.8	26				

[a] $CDCl_3$, 62.9 MHz; spectral width 12 000 Hz; 32K data points; quadrature detection; 30° pulse; repetition rate 1.0 s. [b] Sites of enrichment indicated by * or J_{CC} coupling constants. [c] Signal irradiated for $^{13}C\{^{13}C\}$ homonuclear decoupling. [d] Coupling constants obscured by overlap of C-8/C-19 multiplets. [e] Indirect enrichment by in vivo conversion of [1,2-$^{13}C_2$]acetate to [1,2-$^{13}C_2$]- and [3,4-$^{13}C_2$]butyrate. [f] Non-first-order ^{13}C-^{13}C coupling.

carbons of monensin A sodium were readily assigned by the application of well-precedented chemical shift parameters and decoupling techniques. The quarternary carboxylate (C-1, 181.3 ppm), ketal (C-9, 107.1), and hemiketal (C-25, 98.4) carbon signals were easily identified by their characteristic chemical shifts as well as the absence of the 98.4 ppm signal in the spectrum of the periodate oxidation product **5**.[3c,8] A distinction between the



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remaining two quarternary carbon signals corresponding to C-12 and C-16 and appearing at 85.3 and 85.9 ppm, respectively, was made on the basis of precursor incorporation and homonuclear $^{13}C\{^{13}C\}$ decoupling experiments described below. The signal at 64.9 ppm, which appeared as a triplet in the off-resonance decoupled spectrum corresponded to C-26 while the C-7 carbinyl carbon (70.5 ppm) was readily identified by its characterstic 2.8 ppm downfield shift upon acetylation.[3a,c] The six remaining oxygen-bearing carbinyl carbons were assigned by a seried of single-frequency, off-resonance decoupling experiments and analysis by the method of Birdsall.[22] The corresponding critical proton assignments were made with the aid of proton–proton decoupling of the 270-MHz ^1H NMR spectrum[23] and were in complete agreement with those made independently and reported earlier by Anteunis.[24] For example, C-3 (83.1 ppm) was correlated with H-3, which appeared as a double doublet ($J_{3,2}$ = 10.3, $J_{3,4}$ = 2.0 Hz) at δ 3.19, coupled to H-2 (δ 2.53) and H-4. Similarly, C-17 (85.0 ppm) was identified by correlation with H-17, a doublet (J = 3.5 Hz) at δ 3.94. In support of this assignment, the corresponding signal for C-17 in monensin B (**6**),[3f] which bears a methyl rather than an ethyl group at C-16, was shifted downfield to 86.5 ppm. The remaining four correlations — C-5 (68.4 ppm): H-5 (δ 4.08, dd, J = 2, 11 Hz); C-13 (82.6 ppm): H-13 (δ 3.54, dd, J = 5.4, 9.8 Hz); C-20 (76.5 ppm): H-20 (δ 4.40, ddd, J = 3.9, 6.6, 9.8 Hz); C-21 (74.5 ppm): H-21 (δ 3.80, dd, J = 3.9, 9.4 Hz) — were readily apparent from the Birdsall plots as was the assignment of the methoxyl carbon at 57.9 ppm.

Following preliminary incorporations of ^{14}C-labeled substrates, cultures of *Streptomyces cinnamonensis* were fed samples of

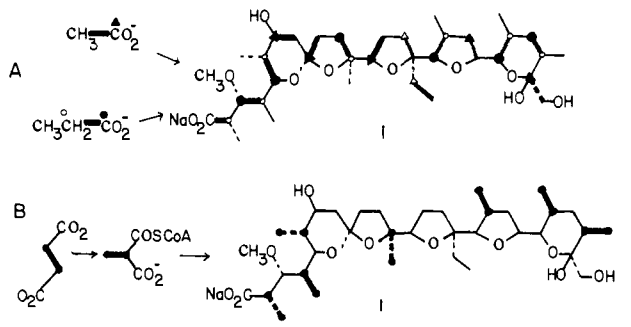
(21) After completion of the work described here, Robinson and Turner reported the assignment of the ^{13}C NMR spectrum of monensin by a combination of two-dimensional proton–carbon and carbon–carbon correlation spectroscopy. Three methylene carbons, C-8, C-11, C-19, and two methyl carbons, C-28 and C-36, were not individually assigned: Robinson, J. A.; Turner, D. L. *J. Chem. Soc., Chem. Commun.* **1982**, 148. At the same time, Dr. Michael L. Maddox of Syntex, Inc., has independently assigned the ^{13}C NMR spectrum of monensin A by extensive $^{13}C\{^1H\}$ decoupling experiments (Clark, R. D.; Hedden, G. L.; Kluge, A. F.; Maddox, M. L.; Spires, H. R.; Long, P. F. *J. Antibiot.*, in press). The results of all three groups are in complete agreement.

(22) Birdsall, B.; Birdsall, N. J. M.; Feeney, J. *J. Chem. Soc., Chem. Commun.* **1972**, 316.

(23) Carried out on a Bruker HX 270 at the Regional NMR Facility at Yale University.

(24) Anteunis, M. J. O. *Bull. Soc. Chim. Belg.* **1977**, *86*, 367.

Scheme II



sodium [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate, [1-^{13}C]propionate, and [1,2-$^{13}C_2$]propionate in separate experiments by using a standard protocol. In a typical experiment, 0.200 g of sodium [1-^{13}C]acetate (90% ^{13}C), 0.800 g of unlabeled sodium acetate,[25] and 7.34×10^6 dpm of sodium [2-^{14}C]acetate as internal standard, dissolved in 10.0 mL of water, were dispensed in lots of 2.0, 1.5, and 1.5 mL to each of two 50-mL fermentation cultures after periods of 48-, 72-, and 96-h incubation, respectively, at 32 °C. Fermentation was continued for an additional 3 days after which the resulting crude monensin was extracted into chloroform according to the literature procedure[8,26] and purified by successive silica gel column and thin-layer chromatography to give 0.022 g of monensin A sodium, 1.83×10^6 dpm/mmol. Analysis of this labeled monensin by 62.9-MHz ^{13}C NMR revealed that the signals corresponding to C-7, C-9, C-13, C-25, and C-19 had been significantly (3% excess enrichment) enhanced over natural abundance, thus providing the first direct evidence for the precursor–product relationship inferred from the original radioisotope incorporation and degradation studies (Scheme IIA, Table I). Subsequent incorporation of [1,2-$^{13}C_2$]acetate yielded a sample of labeled monensin whose ^{13}C NMR spectrum exhibited, as expected, five pairs of enhanced and coupled doublets. Analysis of the ^{13}C–^{13}C coupling constants as well as homonuclear $^{13}C\{^{13}C\}$ decoupling of four of the five pairs of doublets allowed the unambiguous assignment of the C-8, C-10, and C-14 signals and confirmed the presence of label from C-2 of acetate at C-8, C-10, C-14, C-20, and C-26. The fact that the doublet for C-20 at 76.5 ppm was coupled to the signal at 33.3 ppm (J = 34.5 Hz) was consistent with our earlier assignment of this latter resonance to C-16. In a similar set of experiments, incorporation of [1-^{13}C]propionate gave rise to 4% enrichments at C-1, C-3, C-5, C-17, C-21, and (as subsequently confirmed) C-11 and C-23. Once again, the sites of enrichment were fully consistent with those inferred on the basis of the earlier tracer studies. The ^{13}C NMR spectrum of labeled monensin B obtained from the same incorporation experiment showed an identical pattern of enrichments, with the addition of an eighth peak corresponding to C-15, resulting from the substitution of a propionate for a butyrate subunit. Subsequent incorporation of [1,2-$^{13}C_2$]propionate gave rise to a set of seven pairs of enhanced and coupled doublets, each signal of which was unambiguously assigned by extensive homonuclear $^{13}C\{^{13}C\}$ decoupling. For example, the methine carbon at 37.5 ppm was assigned to C-4 on the basis of its observed coupling (J = 37.8 Hz) to the C-3 carbinyl carbon at 83.1 ppm. Similarly, C-11 (33.3 ppm, methylene) was coupled (J = 34.9 Hz) to C-12 (85.3 ppm, quarternary). As a result, C-2 of propionate was shown to label

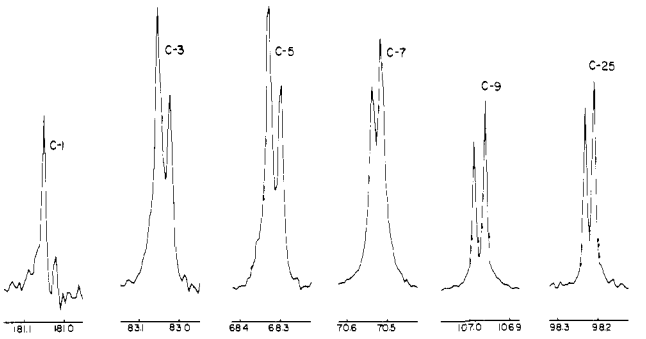
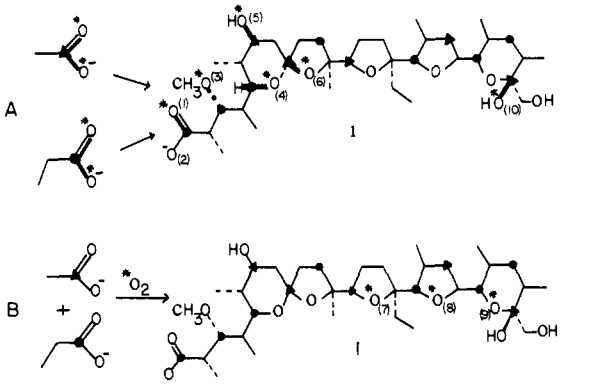


Figure 2. Partial ^{13}C NMR spectra of monensin A sodium showing the signals of carbons bearing oxygen-18. C-1, C-3, and C-5 signals are from monensin derived from [1-^{13}C,1-$^{18}O_2$]propionate. C-7, C-9, and C-25 signals are from monensin derived from [1-^{13}C,^{1}-$^{18}O_2$]acetate. (Line broadening, −1.0 Hz; Gaussian multiplier, 0.3 Hz). Acquisition parameters are given in Table II.

Scheme III



all seven methyl-bearing carbons of monensin. This result was further confirmed by an independent feeding of [2-^{13}C]propionate, which led to more than twofold enrichments at each of the seven expected sites. It was also observed that feeding of both [2-^{13}C]- and [1,2-$^{13}C_2$]propionate led to low-level enrichment of acetate-derived carbons, presumably via conversion of propionate to succinate and then to acetyl-CoA.[27] The remaining carbon signals of monensin were assigned by analysis of the ^{13}C NMR spectrum of labeled polyether obtained upon incorporation of [2,3-$^{13}C_2$]-succinate. The latter substrate acts as an in vivo precursor of methyl[2,2′-$^{13}C_2$]malonate and is therefore metabolically equivalent to [2,3-$^{13}C_2$]propionate (Scheme IIB). Homonuclear $^{13}C\{^{13}C\}$ decoupling of the resulting seven pairs of coupled doublets, combined with a knowledge of the signal assignments gained from feeding [1,2-$^{13}C_2$]propionate, resulted in the unambiguous identification of the seven propionate-derived methyl groups. In addition, the conversion of succinate to [1,2-$^{13}C_2$]acetyl-CoA, resulted in the simultaneous enrichment of each of the ten acetate-derived carbons, as evidenced by the appearance of five pairs of coupled doublets. Finally, C-15/16 and C-30/31 each appeared as coupled doublets due to the in vivo formation of labeled butyrate, a result previously obtained by feeding [1,2-$^{13}C_2$]acetate itself.

With the fundamental precursors of the carbon skeleton firmly established, we turned our attention to the origin of the oxygen atoms of monensin. The requisite [1-^{13}C,$^{18}O_2$]propionate was prepared in 75% 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 of [^{18}O]water and 1 equiv of potassium *tert*-butoxide in *tert*-butyl alcohol. Mass spectrometric analysis

(25) The ^{13}C-labeled precursors were diluted with unlabeled substrate in order to avoid excess intramolecular multiple labeling of the polyether product, which results in undesirable broadening of the resultant ^{13}C NMR resonances due to ^2J(C–C) couplings, thereby causing large variations in signal height as well as obscuring the small (0.02–0.04 ppm) isotopic shifts in subsequent experiments with ^{18}O-labeled precursors. An alternative solution to the multiple-labeling problem based on the use of a spin–echo Fourier transform (SEFT) NMR sequence has recently been described by Vederas: Hill, J. G.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1982**, *104*, 1745. Nakashima, T. T.; Vederas, J. C. *J. Chem. Soc., Chem. Commun.* **1982**, 206.

(26) Stark, W. M.; Knox, N. G.; Westhead, J. E. *Antimicrob. Agents Chemother. (1961–70)* **1968**, 353.

(27) Labeling of propionate-derived subunits by acetate carbons is frequently observed. Cf: Omura, S.; Nakagawa, A.; Takeshima, H.; Atusmi, K.; Miyazawa, J.; Piriou, F.; Lukacs, G. *J. Am. Chem. Soc.* **1975**, *97*, 6600. White, R. J.; Martinelli, E.; Lancini, G. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3260, ref 10.

Table II. Incorporation of [1-^{13}C,1-^{18}O$_2$]Acetate and [1-^{13}C,1-^{18}O$_2$]Propionate into Monensin A

	precursor						
	[1-^{13}C,1-^{18}O$_2$]acetate				[1-^{13}C,1-^{18}O$_2$]propionate		
C	^{13}C shift, ppm[a,b,e]	Δδ, ppm[g]	^{18}O:^{16}O[h]	C	^{13}C shift, ppm[c,d,f]	Δδ, ppm[g]	^{18}O:^{16}O[h]
7	70.46	0.02	60:40	1	181.05	0.03	20:80
9	106.98	0.03	60:40	3	83.06	0.03	40:60
13	82.48	0.0	0:100	5	68.30	0.03	40:60
19	33.30			11	33.22		
25	98.25	0.02	55:45	17	84.90	0.0	0:100
				21	74.45	0.0	0:100
				23	35.71		

[a] Bruker WM 250, 62.9 MHz; spectral width 12 000 Hz; 64K points; quadrature detection; 55° pulse; repetition rate 2.7 s; 5200 transients; 0.049 g in 2.0 mL of $CDCl_3$; resolution enhancement was achieved by Lorentz–Gauss multiplication of FID prior to Fourier Transformation (Ernst, R. R. *Adv. Magn. Reson.* 1966, *2*, 59); −1.0-Hz line broadening, 0.4 Gaussian multiplier; 0.006 ppm/data point. [b] Monensin A sodium, 8.7×10^5 dpm/mmol. [c] 62.9 MHz; spectral width 12 000 Hz; 64K data points; quadrature detection; 55° pulse; repetition rate 2.7 s; 12 550 transients; 0.022 g in 2.0 mL of $CDCl_3$; −1.0-Hz line broadening, 0.3 Gaussian multiplier; 0.006 ppm/data point. [d] Monensin A sodium, 4.5×10^6 dpm/mmol. [e] Average ^{13}C enrichment, 3.1 ± 1.1%. [f] Average ^{13}C enrichment, 4.0 ± 0.2%. [g] $^{13}C^{18}O$ isotope shift, ±0.006 ppm. [h] Uncorrected for contribution of natural abundance ^{13}C to $^{13}C^{16}O$ peak; ±5.

of the derived *p*-phenylphenacyl propionate indicated an isotopic content of 54.9% $^{18}O_2{}^{13}C$, 32.17% $^{18}O^{13}C$, and 3.6% $^{16}O^{13}C$. With the feeding regimen described above, a mixture of 0.200 g of sodium [1-^{18}O$_2$,1-^{13}C]propionate, 0.800 g of unlabeled sodium propionate, and 2.2×10^7 dpm of sodium [1-^{14}C]propionate as internal standard was dispensed to each of two 50-mL fermentation cultures of *S. cinnamonensis*, and the resulting labeled monensin (0.022 g, 4.5×10^6 dpm/mmol) was analyzed by high-resolution 62.9-MHz ^{13}C NMR. The signals corresponding to C-1, C-3, and C-5 each appeared as an enhanced pair of signals corresponding to the respective $^{13}C^{16}O$ and $^{13}C^{18}O$ species (Figure 2 and Scheme IIIA). As summarized in Table II, the observed ^{18}O enrichments deviated slightly from the theoretical maximum of 78%, reflecting varying degrees of oxygen exchange at each site, the greatest amount having occurred at C-1. No $^{13}C^{18}O_2$ species were evident for the C-1 signal, consistent with the intermediacy of an acyl-CoA species bearing a single carbonyl oxygen. Interestingly, no ^{18}O was present at either C-17 or C-21, whose ^{13}C NMR signals each appeared as enhanced singlets as did those for the two non-oxygen-bearing carbons, C-11 and C-23.

Sodium [1-^{18}O$_2$,1-^{13}C]acetate (73.4% $^{18}O_2{}^{13}C$, 14.6% $^{18}O^{13}C$, 0.8% $^{16}O^{13}C$) was prepared from methyl iodide and potassium [^{13}C]cyanide by a procedure similar to that used for the preparation of the corresponding labeled propionate. A mixture of 0.200 g of sodium [1-^{18}O$_2$,1-^{13}C]acetate, 0.800 g of unlabeled sodium acetate, and 7.34×10^6 dpm of sodium [2-^{14}C]acetate was then administered by the usual feeding regimen to two 50-mL cultures of *S. cinnamonensis*. Analysis of the resultant labeled monensin A by ^{13}C NMR revealed the presence of excess ^{18}O at C-7, C-9, and C-25, as evidenced by the usual pair of signals, whereas no ^{18}O attached to C-13 was apparent. The signals corresponding to C-13 and C-19 appeared as enhanced but unsplit singlets. Since the tetrahydropyranyl oxygen [O(4)] of the C-9 spiroketal had been shown to be derived from the C-5 propionate unit, the acetate-derived oxygen at C-9 was assigned to the tetrahydrofuran moiety [O(6)]. Because a similar distinction was not possible at C-25, the labeled monensin A was oxidized with periodate to the lactone **5**,[3c,8] for which the ^{13}C NMR signals corresponding to C-25 appeared as a pair of peaks separated by 0.04 ppm, characteristic of ^{18}O in the carbonyl as distinguished from the ether oxygen.[18,19] The acetate-derived ^{18}O attached to C-25 in monensin itself was therefore assigned to the hemiketal hydroxyl oxygen [O(10)].

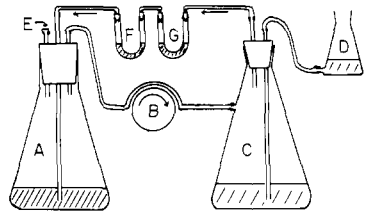


Figure 3. Schematic of apparatus for incubation of *S. cinnamonensis* with $^{18}O_2$ (not drawn to scale): (A) 500-mL Delong flask, 50 mL of culture medium; (B) peristaltic pump; (C) 1-L filter flask, $^{18}O_2$ reservoir, ca. 0.1 L of water; (D) water manostat; (E) rubber septum for introduction of labeled precursors. (F) U-tube, water; (G) U-tube, concentrated KOH.

Table III. Incorporation of [$^{18}O_2$] into Monensin A[a,b]

C	^{13}C shift, ppm	enrich, %	ppm[c]	^{18}O:^{16}O[d]
13	82.55	3.6	0.035	30:70
17	84.98	6.3	0.029	40:60
21	74.54	6.6	0.029	40:60
25	98.34	3.3	0.029	30:70

[a] Culture medium (50 mL) supplemented with 0.150 g of sodium [1-^{13}C]propionate, (90 atom % ^{13}C), 0.150 g of unlabeled sodium propionate, 0.200 g of sodium [1-^{13}C]acetate (90 atom % C), 0.100 g of unlabeled sodium acetate, and 1 μCi of sodium [1-^{14}C]propionate. Precursors added at 48 h (40%), 72 h (30%), and 96 h (30%). [b] 0.003 g in 0.4 mL of $CDCl_3$; Gaussian multiplication: LB = −1.0 Hz, GB = 0.5; 140 000 transients; acquisition parameters as in Table I. [c] $^{13}C^{18}O$ isotope shift, ±0.006 ppm. [d] ±5; uncorrected for contribution of natural abundance ^{13}C to $^{13}C^{16}O$ peak.

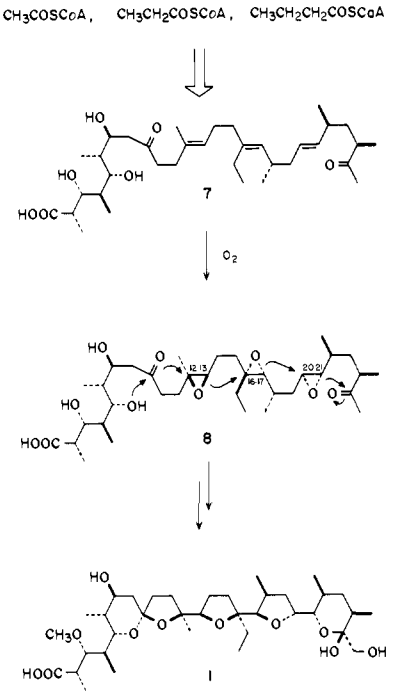
Having established that six of the oxygen atoms of monensin A are derived directly from the carboxylate oxygens of the carbon-skeleton precursors acetate and propionate, we addressed the problem of defining the origin of the remaining three ether oxygens by carrying out a growth of *S. cinnamonensis* in the presence of ^{18}O-labeled molecular oxygen. Considerable experimentation was necessary to establish suitable fermentation and labeling procedures due to the drastic reduction in monensin production resulting from the unavoidable restriction in culture aeration.[28] Eventually we settled on a protocol in which a normally grown 48-h culture was connected to a closed system utilizing a peristaltic pump to circulate the $^{18}O_2$ gas directly through the fermentation medium. A U tube with concentrated aqueous potassium hydroxide was used to trap expired carbon dioxide, and a simple manostat was used to maintain atmospheric pressure (Figure 3). To compensate for reduced monensin production, we administered a mixture of [1-^{13}C]acetate and [1-^{13}C]propionate at the start of the oxygen-18 incubation and again at 72 and 96 h in order to magnify the intensity of isotopically shifted signals. After an additional 3 days, the labeled monensin was isolated and purified and the resulting 3 mg of monensin A was analyzed by high-field ^{13}C NMR. Isotopically shifted peaks were observed for C-13, C-17, C-21, and C-25 corresponding to the presence of 30–40% ^{18}O at O(7), O(8), and O(9) (Scheme IIIB and Table III). The absence of observable $^{13}C^{18}O$ peaks corresponding to C-16 and C-20 is due to the predominant derivation of the observed natural-abundance signals from monensin biosynthesized prior to exposure to labeled molecular oxygen, after which point production was severely reduced. For the same reasons, no isotopically shifted peak could be discerned along with the unenriched natural-abundance signal for C-26.

Discussion

The above-described experiments have confirmed the previously inferred acetate and propionate origins of monensin A and have

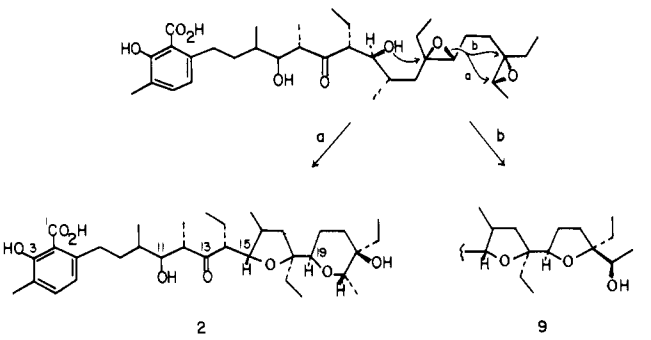
(28) For previously reported methods for incorporation of $^{18}O_2$ into microbial metabolites, see: Mabuni, C. T.; Garlaschelli, L.; Ellison, R. A.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1979**, *101*, 707. O'Sullivan, J.; Aplin, R. T.; Stevens, C. M.; Abraham, E. P. *Biochem. J.* **1979**, *179*, 47. Quigley, F. R.; Floss, H. G. *J. Org. Chem.* **1981**, *46*, 464.

Scheme IV



established for the first time the origins of 9 of the 11 oxygens of this polyether antibiotic. Analysis of these results affords considerable insight into the detailed sequence of steps by which this complex metabolite is elaborated. On the basis of the origins of six of the eleven polyether oxygen atoms from the substrates acetate and propionate, we have speculated[7] that the first-formed polyfunctional fatty acid would be the *all-E*-triene **7** (Scheme IV). The formation of this intermediate by a sequence of steps, each of which is formally analogous to the individual transformations of fatty acid synthesis, would be a completely reductive process utilizing the precursors acetyl-CoA, propionyl-CoA, and butyryl-CoA. Since the source of individual oxygen atoms is not correlated with the eventual D (C-3) or L (C-5 and C-7) configuration[29] of the various hydroxyl functions, stereospecific reduction of the corresponding β-ketoacyl-CoA intermediates in the biosynthesis of the growing polyhydroxy fatty acid can occur with either stereochemistry, strongly suggesting the operation of a distinct enzyme for each chemical step. Biosynthesis of monensin B by the same organism would presumably involve the homologous triene. Whether both trienes would be formed by a common synthetase that occasionally substitutes methylmalonyl-CoA for ethylmalonyl-CoA while leaving all other enzymatic reactions unperturbed or whether each monensin is the exclusive product of distinct but genetically related multienzyme synthetases is an intriguing question of considerable interest. Also unexplained is the biochemical mechanism by which the eventual secondary-methyl stereochemistry at C-2, C-4, C-6, and C-24 is generated. In principle, condensation might involve a single enantiomer of methylmalonyl-CoA, with subsequent epimerization of individual sites, or each enantiomer of methylmalomyl-CoA might undergo stereospecific condensation to generate one or the other epimeric α-methyl β-keto acyl ester. This point is currently under investigation. The eventual configuration of the C-18 and C-22 methyl groups, on the other hand, is presumably the consequence of the stereospecific reduction of the corresponding α-methyl enoyl thio ester intermediates. If each step in the elaboration of the carbon chain is catalyzed by an individual enzyme, the synthetase that generates the triene must consist of a minimum of 33 individual enzymes, not counting carboxylases or associated control elements. No suitable analogies are available to suggest how such a multienzyme system might be packaged or to define the control mechanisms by which each enzyme acts only on its individual substrate.

Scheme V



Once released from its synthetase, the triene **7** is postulated to undergo epoxidation by one or more oxidases to give the 12*R*,13*R*,16*R*,17*R*,20*S*,21*S*-triepoxide **8**. Attack of the C-5 hydroxyl of **8** at the C-9 carbonyl carbon would initiate a cascade of ring closures to generate all five ether rings of monensin. This hypothetical sequence, which accounts for the stereochemistry at C-12, C-13, C-16, C-17, C-20, and C-21 of monensin, is supported by the demonstrated derivation of O(7), O(8), and O(9) from molecular oxygen. The fact that nearly 20 additional pentacyclic polyethers display the identical pattern of oxidation and ether stereochemistry[30] emphasizes the potential generality of these labeling results and their biosynthetic implications.

The polyepoxide model of polyether biogenesis was first suggested by Westley in 1974 to account for the cooccurrence of lasalocid (**2**) and isolasalocid (**9**),[9b] which he proposed might be formed by alternative cyclization modes of a common diepoxide intermediate (**10**)[9b,15] (Scheme V). This suggestion has received strong experimental support from the recent demonstration by Hutchinson and Vederas that the oxygen atoms at C-1, C-3, C-11, C-13, and C-15 of lasalocid are derived from the carboxylate of their respective acetate, propionate, and butyrate precursors, the remaining oxygens at C-19 and C-22 presumably originating from molecular oxygen.[31]

Although the macrolide antibiotics represent a structurally distinct class of natural products, their branched-chain, polyhydroxy skeleton and derivation from the simple precursors acetate, propionate, and butyrate have led to frequent comparisons with the polyether ionophores. Our recent demonstration[6] that the oxygens of propionate account for all six of the carbonyl and secondary hydroxyl oxygen atoms of the macrolide aglycone of erythromycins A and B, independent of the eventual stereochemistry at each position, lends support to the implied biosynthetic analogy between polyethers and macrolides as well as further generality to our experimental findings. At the same time, a host of intriguing stereochemical, enzymatic, and genetic questions have been opened up, and these are being actively pursued.

Experimental Section

Instrumentation. ^1H and ^{13}C NMR spectra were obtained on a Bruker WM 250 FT NMR spectrometer at 250.0 and 62.9 MHz, respectively. Homonuclear $^{13}C\{^{13}C\}$ decoupling experiments were performed on a BSV-3X broad-band amplifier and with 62.85-MHz fixed-frequency booster and normal spectral acquisition parameters. NMR spectra are reported as parts per million downfield of Me_4Si or DSS (sodium 2,2-dimethyl-2-silapentane-5-sulfonate) (δ = 0.0). Multiplications are as follows: s = singlet, d = doublet, t = triplet, q = quartet. Infrared spectra were recorded on a Perkin-Elmer Model 681 infrared spectrophotometer. Mass spectra were obtained by using the direct inlet system of a Hitachi Perkin-Elmer RMU-6D instrument at 70-eV and are reported as *m/e*. Melting points were taken in unsealed capillary tubes in a Hoover melting-point apparatus and are uncorrected. Radioactivity

(29) Fisher–Klyne notation: Klyne, W. *Chem. Ind. (London)* **1951**, 1022. Eliel, E. L. "Stereochemistry of Carbon Compounds"; McGraw-Hill: New York, 1962; pp 91–92.

(30) Cf.: Cane, D. E.; Celmer, W. D.; Westley, J. W., submitted for publication.

(31) Hutchinson, C. R.; Sherman, M. M.; Vederas, J. C.; Nakashima, T. T. *J. Am. Chem. Soc.* **1981**, *103*, 5953.

measurements utilized a Packard 3330 liquid scintillation counter and were carried out in 10-mL toluene solutions containing 7.2 g of BuPBD and 0.45 g of PBBO per liter of toluene. Fermentations were carried out in a New Brunswick G-25 gyrotory shaker.

Preparative-layer chromatography (PLC) and thin-layer chromatography (TLC) were carried out by using 20 × 20 cm Merck precoated silica gel 60 F-254 plates, 0.25 mm thick. Compounds were visualized by ultraviolet light or by heating to 150 °C after spraying with arsenomolybdate solution.[32] Column chromatography employed Merck silica gel 60 (0.05–0.2 mm).

Materials. Diethylene glycol *n*-butyl ether (Aldrich) was dried by storage over calcium hydride and then distilled from lithium aluminum hydride. Ethyl iodide (Aldrich) was freshly distilled from P_2O_5 and stored over sodium metal just prior to use. Pyridine (Fisher) was distilled from CaO and then stored over 5-Å molecular sieves. Iodomethane (Aldrich) was distilled from calcium hydride and stored over molecular sieves. *tert*-Butyl alcohol (Fisher) was distilled from iodine-activated magnesium. Phenoxyethanol (Aldrich) was dried over molecular sieves. Dimethyl sulfoxide was distilled from calcium hydride. *p*-Phenylphenacyl bromide (Aldrich) was recrystallized from benzene after clarification with charcoal. *p*-Toluenesulfonyl choride (Aldrich) was purified from chloroform according to Fieser.[33] 18-Crown-6 (Aldrich) was used without further purification. Monensin sodium, a mixture of monensin A and monensin B, was a gift of Eli Lilly Co. Sodium [1-^{14}C]propionate (52 mCi/mmole and sodium [2-^{14}C]acetate (58.8 mCi/mmol) were purchased from New England Nuclear; Sodium [1-^{13}C]acetate, [$^{13}C_2$]-dibromoethane (90 atom %), and $^{18}O_2$ gas (97 atom %) were obtained from KOR Isotopes. Potassium [^{13}C]cyanide (90.7 atom %) was obtained from the Stable Isotopes Resource, Los Alamos, NM. Sodium [1-^{13}C]propionate (90 atom %) was purchased from Merck. [^{18}O]Water (95 atom %) was purchased from Mound Laboratory, Miamisburg, OH.

Streptomyces cinnamonensis A3823.5, a gift from the Eli Lilly Co., was obtained as a lyophilized pellet, which was suspended in 2.0 mL of sterile water and used to inoculate an agar slant of the following composition:[26] 10 mg/mL of bactodextrose (Difco), 10 mg/mL of Soytone (Difco), and 25 mg/mL of agar (Difco), adjusted to pH 7.3 with sodium hydroxide solution before autoclaving. After incubation for 5 days at 30 °C, the spores were harvested in 2.0 mL of nutrient broth (Difco) containing 0.1% Tween-80. This spore suspension was used to inoculate 50 mL of a sterilized medium consisting of 5 mg/mL of bactodextrose, 15 mg/mL of Nutri-soy flour (Archer-Daniels-Midland Co.), 20 mg/mL of dextrin, 2.5 mg/mL of yeast extract (Difco), and 1 mg/mL of calcium carbonate in distilled water. After incubation at 32 °C for 24 h in a 500-mL Delong flask shaken at 300 rpm, the resulting vegetative inoculum was dispensed as 0.5-mL aliquots mixed with 0.2 mL of sterile glycerol into sterile 2.0-mL plastic vials, which were sealed and stored in liquid nitrogen in an Orion ET-34 Dewar. All subsequent fermentations were initiated with inoculum from these vials.

Fermentation of *S. cinnamonensis*.[26] A vegetative inoculum of *S. cinnamonensis* A3823.5 was prepared by adding the contents of a rapidly thawed vial to 50 mL of the above-described medium and incubating at 300 rpm and 32 °C for 1 day. The fermentation medium consisting of 30 mg/mL of bactodextrose, 25 mg/mL of soy grits, 1 mg/mL of calcium carbonate, 0.1 mg/mL of KCl, 0.1 mg/mL of K_2HPO_4, 0.6 mg/mL of $MnCl_2 \cdot 4H_2O$, 0.3 mg/mL of $Fe_2(SO_4)_3 \cdot XH_2O$, 0.02 mL/mL of soybean oil (Sigma), and 0.02 mL/mL of methyl oleate (Eastman) in 50 mL of distilled water was adjusted to pH 8.0 with potassium hydroxide solution and autoclaved at 120 °C for 20 min in a 500-mL Delong flask. This medium was then inoculated with 1.0 mL of vegetative culture and incubated at 300 rpm and 32 °C for 7 days. For the isolation of monensin, the pH was adjusted to 9.0 by addition of sodium hydroxide solution, the mycelia and broth were separated by centrifugation at 16000*g*, and each was extracted with three 50-mL portions of chloroform. The combined organic extracts were filtered through a Celite pad and dried over sodium sulfate. Evaporation of the solvent gave a dark colored residue, which was chromatographed on 60 g of silica gel 60 with ethyl acetate as eluant. The crude monensin sodium thus obtained was further purified by TLC (ethyl acetate) to yield typically 20–30 mg of monensin A sodium (R_f 0.25) (monensin B sodium, R_f 0.21). Precipitation from methanol–water gave pure monensin A sodium; mp 265–268 °C (lit.[3b] mp 267–269 °C).

Precursor Incorporation. Labeled precursors containing radioactive internal standard were dissolved in distilled water and added in three portions (40%, 30%, and 30%) through a Millex disposable sterile filtration unit to 50 or 100 mL of a fermentation culture of *S. cinnamonensis* at 48, 72, and 96 h, respectively. At the conclusion of the normal 7-day fermentation period, the labeled monensin was isolated and purified in the usual manner.

Sodium [1-^{13}C,$^{18}O_2$]Acetate. A mixture of 2.26 g (16.0 mmol) of methyl iodide, 1.056 g (16.0 mmol) of potassium [^{13}C]cyanide (90.7 atom %), and 0.1 mL of [^{18}O]water (90 atom %) in 3.0 mL of anhydrous methanol was refluxed for 22 h, after which the entire mixture was distilled. To this distillate was added 0.806 g (40 mmol) of [^{18}O]water (90 atom %) and 13.0 mL (16.0 mmol) of 1.23 M potassium *tert*-butoxide in *tert*-butyl alcohol, prepared by addition of purified potassium metal[34] to anhyrous *tert*-butyl alcohol. The resulting mixture was refluxed for 48 h to hydrolyze the acetonitrile. Evaporation of the solvent and drying of the residue under high vacuum gave shiny flakes of sodium acetate, which were acidified with phosphoric acid, and the resulting solution of acetic acid was lyophilized and collected in a second flask immersed in liquid nitrogen. Titration of the lyophilizate with 12.8 mL of 1.1 N NaOH gave 1.227 g (14.1 mmol) of sodium [1-^{13}C,$^{18}O_2$]acetate (88% yield): ^{1}H NMR (D_2O, DSS) δ 1.9 (d, $^2J_{CH}$ = 6 Hz); ^{13}C NMR (D_2O, DSS, BB {^{1}H} decoupled) 26.0 (d, J = 52.3 Hz), 183.87 (s, ^{13}C-(1)$^{18}O_2$), shoulder at 183.90 (s, ^{13}C(1)^{18}O^{16}O); IR λ_{max} (KBr) 3000, 2940, 1520, 1420, 1380, 1330, 1030, 1010 cm^{-1}.

***p*-Phenylphenacyl [1-^{13}C,1-$^{18}O_2$]Acetate.** *p*-Phenylphenacyl bromide (55 mg, 0.20 mmol) was added to 17.4 mg (0.20 mmol) of sodium [1-^{13}C,$^{18}O_2$]acetate and 4 mg of 18-crown-6 in 2.0 mL of 1:1 benzene–acetonitrile and the mixture refluxed overnight. After evaporation of the solvent under a stream of nitrogen, the residue was chromatographed with benzene on 10 g of silica gel to give 47 mg (0.18 mmol) of *p*-phenylphenacyl acetate (91%): mp 110 °C (lit.[35] mp 111 °C); ^{1}H NMR ($CDCl_3$) δ 2.23 (d, $^2J_{CH}$ = 6 Hz, CH_3, 3 H), 5.32 (d, $^3J_{CH}$ = 4.5 Hz, CH_2, 2 H), 7.3–8.2 (m, ar CH, 9 H); IR λ_{max} ($CHCl_3$) 1700 (C=O), 1670 (^{13}C=^{18}O), 1605 cm^{-1} (C=C); MS, *m/e* 255–259,[36] $^{13}C^{16}O_2$ (0.8%), $^{12}C^{18}O_1$ (1.6%), $^{13}C^{18}O_1$ (14.5%), $^{12}C^{18}O_2$ (9.4%), $^{13}C^{18}O_2$ (73.4%); calcd 88.7 atom % ^{13}C, 90.9 atom % ^{18}O.

***p*-Phenylphenacyl [1-^{13}C]Acetate.** Sodium [1-^{13}C]acetate (90 atom %) (2.07 g, 25.0 mmol) was reacted as above with 6.95 g (25.0 mmol) of *p*-phenylphenacyl bromide to give, after purification, 6.0 g (23.5 mmol) of *p*-phenylphenacyl [1-^{13}C]acetate (94%): mp 109–110 °C; ^{1}H NMR ($CDCl_3$) δ 2.2 (d, $^2J_{CH}$ = 6.5 Hz, CH_3, 3 H), 5.3 (d, $^3J_{CH}$ = 4.5 Hz, CH_2, 2 H), 7.2–8.1 (m, ar CH, 9 H); IR λ_{max} ($CHCl_3$) 3015, 2940, 1700 C=O, ^{13}C=O), 1605 cm^{-1} (C=C).

[1-^{13}C]Ethanol. A 100-mL, two-neck flask was charged with 6.0 g (25.3 mmol) of *p*-phenylphenacyl [1-^{13}C]acetate in 50 mL of anhydrous diglyme and cooled with a dry ice–carbon tetrachloride bath. Lithium aluminum hydride (1.40 g, 36.8 mmol) was added in portions to this mixture with rapid stirring over a period of 20 min, after which the reaction was stirred for 4 h at room temperature. After quenching of the reaction by addition of 25 mL of phenoxyethanol, the mixture was heated to 50 °C and the product [1-^{13}C]ethanol was swept in a slow stream of nitrogen into a trap immersed in liquid nitrogen over a period of 8 h. The ethanol thus obtained was converted to the tosylate without further purification. [1-^{13}C]ethanol: ^{1}H NMR (H_5-pyridine, 50% solution, H-2 (Py) = 8.60 ppm) δ 1.20 (t, J = 7.5 Hz, d, $^2J_{CH}$ = 4.5 Hz, CH_3, 3 H), 3.85 (q, J = 7.5 Hz, d, $^1J_{CH}$ = 138 Hz, $^{13}CH_2$, 2 H), 5.70 (br s, OH, 1 H).

[1-^{13}C]Ethyl Tosylate. Crude [1-^{13}C]ethanol obtained in the above reaction was mixed with 5.0 mL of dry pyridine and cooled in a dry ice–carbon tetrachloride bath with magnetic stirring. Toluenesulfonyl chloride (3.8 g, 20 mmol) was slowly added, the mixture stirred for 2 h, and then quenched by addition of ice-cold concentrated hydrochloric acid. Ethyl tosylate was precipitated by cooling in an ice bath, and the crystals were collected by filtration and dried in a dessicator at 0 °C to give 2.50 g (12.5 mmol) of [1-^{13}C]ethyl tosylate (53% based on *p*-phenylphenacyl acetate) (If ethyl tosylate fails to crystallize, the oily precipitate can be extracted into ether followed by evaporation of the solvent and drying of the residue.): ^{1}H NMR ($CDCl_3$) δ 1.29 (t, J = 7.2 Hz, d, $^2J_{CH}$ = 4.4 Hz, CH_3, 3 H), 2.45 (s, CH_3, 3 H), 4.10 (q, J = 7.2 Hz, d, $^1J_{CH}$ = 149 Hz, $^{13}CH_2$, 2 H), 7.34 (d, J = 8 Hz, CH, 2 H), 7.80 (d, J = 8 Hz, CH, 2 H); IR λ_{max} (neat) 1365, 1350, 1185, 1175, 1095 cm^{-1}.

[1,2-$^{13}C_2$]Propionitrile. Potassium [^{13}C]cyanide (90.7 atom %) (0.815 g, 12.3 mmol) was added to 2.50 g (12.5 mmol) of [1-^{13}C]ethyl tosylate dissolved in a mixture of 9.0 mL of 2:1 methanol–water. After 2 h at reflux, the ethyl tosylate had been consumed, as determined by TLC analysis, and the reaction mixture was distilled into a 25-mL round-bottom flask and used directly for the next reaction.

Sodium [1,2-$^{13}C_2$]Propionate. To the above-described distillate was added 2.0 g of potassium hydroxide in 2.0 mL of water and the resulting

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mixture refluxed for 3 days. Acidification with phosphoric acid, lyophilization, titration of the distillate with 1.1 N sodium hydroxide solution, and removal of the solvent by a second lyophilization gave 0.6 (6.12 mmol) of sodium [1,2-$^{13}C_2$]propionate (50% yield): ^{1}H NMR (D_2O, DSS) 1.04 (t J = 7.4 Hz, d, $^2J_{CH}$ = 4.4 Hz, d, $^3J_{CH}$ = 4.9 Hz, CH_3, 3 H), 2.16 (q, J = 7.4 Hz, d, $^1J_{CH}$ = 126.8 Hz, d, $^2J_{CH}$ = 6.0 Hz, $^{13}CH_2$, 2 H); ^{13}C NMR (D_2O, DSS) 12.8 (m, C-3), 33.4 (d, J = 51.6 Hz, C-2) 187.5 (d, J = 51.6 Hz, C-1); ^{13}C enrichment (^{13}C NMR) C-1 (90%), C-2 (90%).

Sodium [2-^{13}C]Propionate. Sodium [2-^{13}C]propionate was prepared from [1-^{13}C]acetate and unlabeled potassium cyanide by procedures analogous to those described above.

Sodium [1-^{13}C,1-$^{18}O_2$]Propionate. Iodoethane (4.0 g, 25.1 mmol) was reacted at 70 °C for 12 h with 1.625 g (25 mmol) of potassium [^{13}C]-cyanide (90.7 atom %) in 5.05 g of anhydrous methanol containing 0.24 mL of [^{18}O]water (^{18}O, 94.92 atom %; ^{17}O, 2.00 atom %; ^{16}O, 3.08 atom %). After an additional 33 h at 80 °C, the entire reaction mixture was distilled and the distillate treated with 1.06 g (53.0 mmol) of [^{18}O]water and 29.5 mL (25.1 mmol) of 0.85 M potassium *tert*-butoxide in *tert*-butyl alcohol, prepared as described above. The resulting solution was refluxed for 48 h and then concentrated under vacuum to leave 3.0 g of solid. The residue was taken up in water, treated with 42.5 mequiv of Dowex 50W-X8 (H^+), filtered, and titrated with 1.1 N sodium hydroxide. Evaporation of the solvent gave 1.89 g (75%) of sodium [1-^{13}C,$^{18}O_2$]propionate: ^{1}H NMR (D_2O, DSS) δ 1.04 (t, J = 7.7 Hz, d, $^3J_{CH}$ = 5.0 Hz, CH_3, 3 H), 2.16 (q, J = 7.7 Hz, d, $^2J_{CH}$ = 7.6 Hz, CH_2, 2 H); ^{13}C NMR (D_2O, DSS) δ 12.8 (C-3), 33.4 (d, J = 55 Hz, C-2), 187.45 (s, $^{13}C(1)^{18}O_2$), shoulder at 187.48 (s, $^{13}C(1)^{18}O^{16}O$); IR λ_{max} (KBr) 2980, 2940, 1510, 1280, 1075, 1000 cm^{-1}.

***p*-Phenylphenacyl [1-^{13}C,1-$^{18}O_2$]Propionate.** Sodium [1-^{13}C,$^{18}O_2$]-propionate (10 mg, 0.10 mmol) was reacted at 95 °C for 2 h with 65 mg (0.24 mmol) of *p*-phenylphenacyl bromide and 4 mg of 18-crown-6 in 2.0 mL of 1:1 benzene–acetonitrile. After removal of the solvent with a stream of nitrogen, the oily residue was taken up in a mixture of 2 mL of water and 2 mL of chloroform, the phases separated, and the aqueous layer extracted with two further 3.0-mL portions of chloroform. The combined extracts were washed with water, shaken with brine, and dried over sodium sulfate. Evaporation of the solvent gave 57 mg of residue, which was purified by column chromatography (benzene) on 4.0 g of silica gel to give 10 mg of crude *p*-phenylphenacyl propionate (TLC (benzene) R_f 0.1). Recrystallization from ethanol–water gave 6.0 mg of ester: mp 103 °C (lit.[35] mp 102 °C); ^{1}H NMR ($CDCl_3$) δ 1.25 (t, J = 7.5 Hz, d, $^3J_{CH}$ = 5.5 Hz, CH_3, 3 H), 2.55 (q, J = 7.5 Hz, d, $^2J_{CH}$ = 7.5 Hz, CH_2, 2 H), 5.40 (d, $^3J_{CH}$ = 4.5 Hz, CH_2, 2 H), 7.35–8.15 (m, Ar CH, 9 H); IR λ_{max} ($CHCl_3$) 1700 (C=O), 1665 (^{13}C=^{18}O), 1603 cm^{-1} (C=C); isotopic composition calculated from m/e 268 to 273 and corrected for ^{17}O content of labeled water: $^{12}C^{16}O_2$, 0.1%; $^{13}C^{16}O_2$, 3.1%; $^{12}C^{16}O^{17}O$, 0.5; $^{13}C\ ^{16}O^{17}O$, 0.5%; $^{12}C\ ^{16}O^{18}O$, 2.8%; $^{13}C^{16}O^{18}O$, 30.1%; $^{12}C^{17}O^{18}O$, 0.2%; $^{13}C^{17}O^{18}O$, 2.1%; $^{12}C^{18}O_2$, 5.6%; $^{13}C^{18}O_2$, 54.9%; ^{13}C, 90.7 atom %; ^{18}O, 78.1 atom %.

[2,3-$^{13}C_2$]Succinonitrile.[37,38] Sodium cyanide (0.636 g, 13.0 mmol) was added to 1.00 g (5.33 mmol) of [1,2-$^{13}C_2$]dibromoethane in 30 mL of anhydrous dimethyl sulfoxide under nitrogen. After 5 h at 90 °C, the reaction mixture was poured into 100 mL of saturated aqueous ammonium chloride and the aqueous phase extracted with three 100-mL portions of ethyl acetate. The combined ethyl acetate fractions were washed with three 100-mL volumes of water to ensure removal of dimethyl sulfoxide, dried over magnesium sulfate, and concentrated under reduced pressure. The residue was bulb-to-bulb distilled (95 °C, 1.00 mm) to give 0.347 (4.34 mmol, 81%) of [2,3-$^{13}C_2$]succinonitrile: mp 46–49 °C (lit.[39] mp 56.6 °C); ^{1}H NMR (60 MHz, $CDCl_3$) δ 2.8 (d, $^1J_{CH}$ = 135 Hz, superimposed on m); IR λ_{max} (neat) 2250 cm^{-1} (C≡N).

[2,3-$^{13}C_2$]Succinic Acid.[37,40] [2,3-$^{13}C_2$]Succinonitrile (0.347 g, 4.34 mmol) was dissolved in 15.0 mL of 30% aqueous potassium hydroxide in a two-neck, 25-mL round-bottom flask, and 1.72 mL (20.0 mmol) of 30% hydrogen peroxide was added with stirring. A stream of nitrogen was passed through the solution, which was first heated for 1 h at 40 °C and then refluxed until the exit gas was free of ammonia (ca. 3 h), as determined by passage over litmus paper and exposure to the vapors of concentrated HCl. Excess peroxide was destroyed by treatment with 50 mL of saturated sodium metabisulfite, the pH was adjusted to 2.0 with concentrated HCl, and the solution was continuously extracted for 36 h with ether. Evaporation of the solvent and recrystallization of the residue from ethanol gave 0.339 g (combined first and second crop) (2.87 mmol, 66%) of [2,3-$^{13}C_2$]succinic acid: mp 187–190 °C (lit.[41] mp 189–198 °C); ^{1}H NMR (60 MHz, Me_2SO-d_6) δ 2.4 (br d, $^1J_{CH}$ = 126 Hz); IR λ_{max} (KBr) 3700–2800 (OH), 1700 cm^{-1} (C=O). For incorporation experiments, the succinic acid was converted to the disodium salt by titration with 2 equiv of 1.0 N sodium hydroxide.

Incorporation of Sodium [2,3-$^{13}C_2$]Succinate. Sodium [2,3-$^{13}C_2$]-succinate (0.200 g) was mixed with 0.600 g of sodium succinate and 5.5 × 10^6 dpm of sodium [2,3-^{14}C]succinate and fed in three portions to 50 mL of *S. cinnamonensis* according to the usual protocol. ^{13}C NMR analysis of the derived monensin A (0.040 g) indicated an average ^{13}C enrichment at each labeled site of ca. 2%.

Incubation of *S. cinnamonensis* with $^{18}O_2$. A 500-mL Delong flask containing 50 mL of the usual fermentation medium was inoculated with 1.0 mL of a vegetative culture of *S. cinnamonensis* and incubated at 32 °C and 300 rpm for 48 h. The flask was then fitted with a three-hole rubber stopper, flushed with nitrogen, and connected to a reservoir containing 1.0 L of $^{18}O_2$ (97 atom %). A peristaltic pump was used to circulate the labeled air directly through the culture medium at ca. 15 mL/min, and the carbon dioxide evolved was removed by passage through a U tube containing concentrated aqueous potassium hydroxide (Figure 3). A simple manostat served to maintain constant pressure throughout the incubation period. The medium was supplemented with portions of a mixture of 0.300 g of sodium [1-^{13}C]propionate (45 atom %), 0.300 g of sodium [1-^{13}C]acetate (60 atom %), and 2.2 × 10^6 dpm of sodium [1-^{14}C]propionate at intervals of 48 h (40%), 72 h (30%), and 96 h (30%). After a total of 7 days, the labeled monensin was isolated and purified in the usual fashion.

Oxidation of [^{18}O]Monensin A to 5.[3c,8] [^{13}C,^{18}O]Monensin A sodium (0.040 g, 0.057 mmol), obtained from incorporation of [1-^{13}C,$^{18}O_2$]-acetate, was dissolved in a mixture of 2.0 mL of *tert*-butyl alcohol and 0.5 mL of methanol and treated at room temperature with 0.037 g (0.17 mmol) of sodium metaperiodate in 1.0 mL of water. The reaction was monitored by TLC (30:1 EtOAc/MeOH, R_f(**1**) 0.3, R_f(**5**) 0.27). After 24 h, 5 mL of water was added and the reaction mixture extracted with four 10-mL portions of ether. The combined organic extracts were washed with water (10 mL) and brine (10 mL) before drying over sodium sulfate. Evaporation of the solvent gave 0.038 g of oily residue, which was chromatographed on 10.0 g of silica gel (30:1 EtOAc/MeOH) to yield 0.035 g of lactone **5** (87%): ^{13}C NMR ($CDCl_3$) (enhanced signals) δ 35.24 (C-19), 71.54 (with $^{13}C^{18}O$ shoulder)(C-7), 83.18 (C-13), 107.56/107.53 (C-9), 174.32/174.28 (C-25).

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Registry No. 1 free acid, 17090-79-8; **5** free acid, 83587-69-3; **6** free acid, 30485-16-6; **7**, 83587-70-6; **8**, 83587-71-7; O_2, 7782-44-7; [7,9,13,19,25-^{13}C]monensin A, 83587-66-0; [1,3,5,11,17,21,23-^{13}C]-monensin A, 83587-67-1; [7,9,13,19,25-^{13}C,7,9,25-^{18}O]monensin A, 83603-91-2; [1,3,5,11,17,21,23-^{13}C,1,3,5-^{18}O]monensin A, 83587-68-2; acetic acid, 64-19-7; propionic acid, 79-09-4; succinic acid, 110-15-6; sodium [1-^{13}C,$^{18}O_2$]acetate, 83587-72-8; *p*-phenylphenacyl [1-^{13}C,1-$^{18}O_2$]acetate, 83603-92-3; *p*-phenylphenacyl [1-^{13}C]acetate, 73377-65-8; [1-^{13}C]ethanol, 14742-23-5; [1-^{13}C]ethyl tosylate, 83587-73-9; [1,2-$^{13}C_2$]propionitrile, 83587-74-0; sodium [1,2-$^{13}C_2$]propionate, 83587-75-1; sodium [2-^{13}C]propionate, 83587-76-2; sodium [1-^{13}C,1-$^{18}O_2$]propionate, 83587-77-3; *p*-phenylphenacyl [1-^{13}C,1-$^{18}O_2$]propionate, 83587-78-4; [2,3-$^{13}C_2$]succinonitrile, 83587-79-5; [2,3-$^{13}C_2$]succinic acid, 61128-08-3; sodium [2,3-$^{13}C_2$]succinate, 83587-80-8.

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