Macrolide Biosynthesis. 3. Stereochemistry of the Chain-Elongation Steps of Erythromycin Biosynthesis

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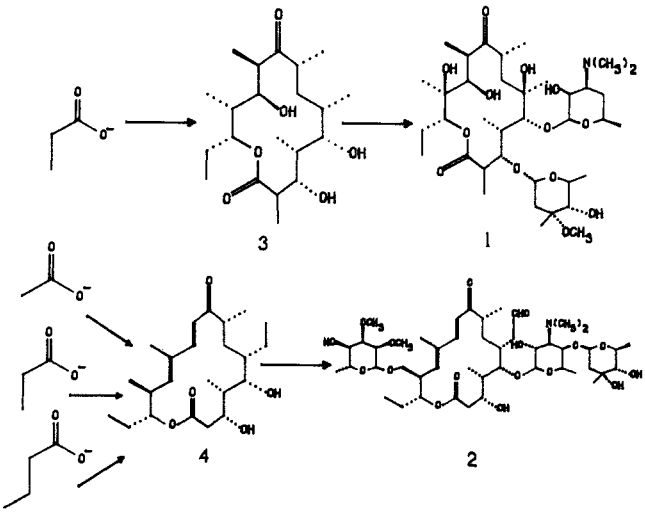
Abstract: The stereochemical course of the chain-elongation steps of the biosynthesis of the macrolide antibiotic erythromycin has been investigated. Incorporation of [2-$^2H_2$,2-^{13}C]propionate into erythromycins A and B by cultures of *Streptomyces erythreus* was followed by analysis of the corresponding 2′-benzoate esters **9** and **10** by difference $^{13}C\{^1H,^2H\}$ NMR, revealing the presence of deuterium label at C-2, C-4, and C-10, as well as C-20, of each macrolide. The three carbinyl carbons all have the same D-methyl configuration, whereas the corresponding L-methyl centers, C-8 in erythromycin A benzoate and C-8 and C-12 in erythromycin B benzoate, were devoid of deuterium. These results are consistent with stereospecific carboxylation of propionyl-CoA to give (2*S*)-[2-^2H,2-^{13}C]methylmalonyl-CoA which then undergoes condensation with the appropriate enzyme-bound acyl thio ester by a process involving decarboxylative inversion, in direct analogy to the established mechanism and stereochemistry of condensation of malonyl-CoA in fatty acid biosynthesis. Attempts to establish the origin of the L-methyl centers of the macrolides by complementary incorporations of diethyl [2-$^2H_2$,2-^{13}C]succinate were thwarted by extensive loss of deuterium due to adventitious exchange processes.

The macrolide antibiotics have been the subject of intense chemical and biological interest since the discovery of pikromycin 35 years ago. Since that time nearly 100 representatives of this class of polyoxygenated 12-, 14-, and 16-membered lactone rings have been reported.[1] By far the most thoroughly investigated from the point of view of chemical synthesis, biological origin, molecular genetics, and mode of action are the *Streptomyces* metabolites erythromycin A (**1**) and tylosin (**2**) (Scheme I). Although the basic biological precursors—acetate, propionate, and butyrate—have been identified for several macrolide antibiotics and the later stages of macrolide modification, including oxidation, methylation, and glycosylation, have been reasonably well-defined for both erythromycin[2] and tylosin[2b,3] biosynthesis, knowledge of the details of the key polyketide chain-building steps by which the polyoxygenated, branched-chain fatty acid is assembled and cyclized to the parent aglycones, 6-deoxyerythronolide B (**3**) and tylactone (protylonide) (**4**), is still at a primitive level. Our own finding that the oxygen atoms of **1** are derived from the carboxyl oxygens of the propionate precursor has suggested that the oxidation level and stereochemistry of the growing polyketide chain is adjusted as each successive methylmalonate unit is added.[4] Completely consistent findings have been reported for the biosynthesis of tylactone.[5] The fact that more than 100 12-, 14-, and 16-membered macrolides conform to the pattern of oxidation and stereochemistry summarized by the Celmer macrolide model[6,7] strongly implies that the majority of macrolide synthetases are acting in a closely related fashion, their major diversity lying in the choice of individual malonate, methylmalonate, and ethylmalonate precursors for the several condensation steps and the occasional dehydration of a β-hydroxyacyl-CoA intermediate. These conclusions would also appear to be applicable to the formation of polyether antibiotics whose structural similarities to the macrolides clearly reflect closely related biosynthetic origins.[7,8]

Scheme I



The mechanism of macrolide and polyether chain elongation presents a host of intriguing problems at the chemical, biochemical, and genetic level. The difficulty in confronting these problems lies in the fact that no intermediates of the chain-elongation process have ever been isolated nor have any of the relevant chain-building enzymes been properly characterized. To date, there have been no published descriptions of the successful isolation of a cell-free synthetase supporting formation of 6-deoxyerythronolide B (**3**).[2,9] An extremely promising approach, still in its relative infancy, is the study of the genetics and molecular biology of macrolide biosynthesis, which holds the promise of providing information on the number, location, organization, and regulation of the genes responsible for antibiotic formation.[3b,10] Very recently, Baltz and his collaborators have reported the cloning of the structural genes for erythromycin A biosynthesis.[11] The entire set of genes appears to be encoded by a 35 kbp segment of *S. erythreus* DNA, a remarkably small length in view of the large number of discrete

(1) *Macrolide Antibiotics: Chemistry, Biology, and Practice*; Omura, S., Ed.; Academic: New York, 1984.
(2) (a) Corcoran, J. W. In *Antibiotics IV. Biosynthesis*; Corocoran, J. W., Ed.; Springer-Verlag: New York, 1981; pp 132–174. (b) Omura, S.; Tanaka, Y. In *Macrolide Antibiotics: Chemistry, Biology, and Practice*; Omura, S., Ed.; Academic: New York, 1984; pp 199–299.
(3) (a) Omura, S.; Nakagawa, A. In *Antibiotics IV. Biosynthesis*; Corocoran, J. W., Ed.; Springer-Verlag: New York, 1981; pp 175–192. (b) Baltz, R. H. *Basic Life Sci.* **1982**, *19*, 431.
(4) (a) Cane, D. E.; Hasler, H.; Liang, T.-C. *J. Am. Chem. Soc.* **1981**, *103*, 5960. (b) Cane, D. E.; Hasler, H.; Taylor, P. B.; Liang, T.-C. *Tetrahedron* **1983**, *39*, 3449.
(5) O'Hagan, D.; Robinson, J. A.; Turner, D. L. *J. Chem. Soc., Chem. Commun.* **1983**, 1337.
(6) Celmer, W. D. *J. Am. Chem. Soc.* **1965**, *87*, 1801. Celmer, W. D. *Pure Appl. Chem.* **1971**, *28*, 413. Celmer, W. D. *Ann. N.Y. Acad. Sci.*, in press.
(7) Cane, D. E.; Celmer, W. D.; Westley, J. W. *J. Am. Chem. Soc.* **1983**, *105*, 3594.
(8) Hutchinson, C. R. *Acc. Chem. Res.* **1983**, *16*, 7.
(9) For reference to unpublished results involving cell-free synthesis of 6-deoxyerythronolide B, see: Hunaiati, A. A.; Kolattukudy, P. E. *Antimicrob. Agents Chemother.* **1984**, *25*, 173.
(10) (a) Hutchinson, C. R. *Nat. Prod. Rep.*, in press. (b) Hopwood, D. A.; Bibb, M. J.; Bruton, C. J.; Chater, K. F.; Feitelson, J. S.; Gil, J. A. *Trends Biotechnol.* **1983**, *1*, 42. (c) Hopwood, D. A.; Kieser, T.; Lydiate, D. J.; Bibb, M. J. In *Antibiotic-Producing Streptomyces, Volume IX, The Bacteria*; *A Treatise on Structure and Function*; Day, L. E., Queener, S. W., Eds.; Academic: New York, in press.
(11) Stanzak, R.; Matsushima, P.; Baltz, R. H.; Rao, R. N. *Bio/Technology* **1986**, *4*, 229.

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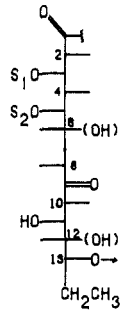
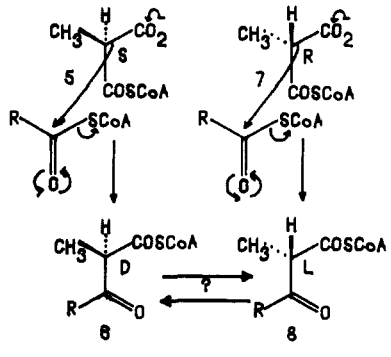


Figure 1. Fischer projection of erythromycin skeleton, showing D-methyls at C-2, C-4, and C-10 and L-methyls at C-6, C-8, and C-12.

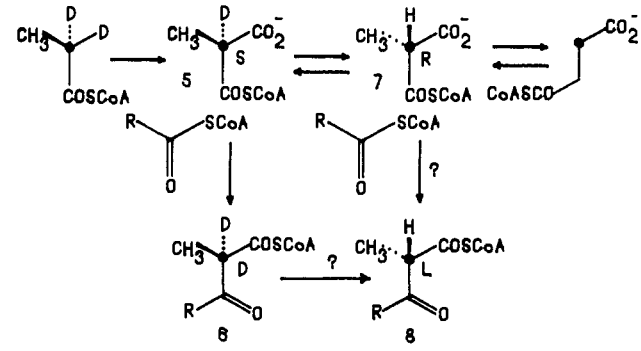
Scheme II



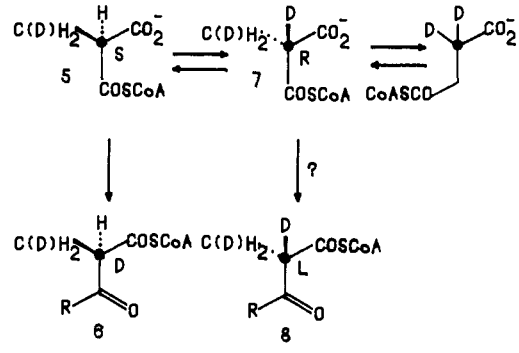
steps required for biosynthesis of the complete macrolide. Attempts have also been made to dissect the chain-building process in the biosynthesis of acetate-derived polyketides by the incorporation of specifically deuterated samples of acetate.[12] Unfortunately, detailed mechanistic interpretation of the results of the latter group of experiments has been limited by the absence of relevant stereochemical information or the lack of meaningful data for comparison.

In continuation of our own studies of erythromycin biosynthesis we have been addressing the problem of when and how the secondary methyl group stereochemistry at C-2, C-4, and C-10 (D-methyl) and C-6, C-8, and C-12 (L-methyl) is controlled (Figure 1). The configuration of the C-6 methyl is presumably the consequence of the reduction of the corresponding α-methyl-α,β-unsaturated ester intermediate. Interestingly, the same L configuration is observed at the vast majority of analogously reduced sites in macrolides and polyethers.[13] The remaining five secondary methyls in the macrolide aglycone 6-deoxyerythronolide B are introduced by the condensation of methylmalonyl-CoA with the growing polypropionate chain. In principle the eventually observed configuration may arise in one of two ways (Scheme II). (1) Utilization of either (2*R*)- or (2*S*)-methylmalonyl-CoA would result in direct formation of the corresponding D or L product. By analogy to the mechanism of malonyl-CoA condensation in fatty acid biosynthesis, known to take place by decarboxylative inversion,[14] it can be predicted that the selection of (2*S*)-methylmalonyl-CoA (**5**) will lead to the D-methyl product **6**, while condensation of the 2*R* enantiomer **7** will generate the epimeric L-methyl configuration **8**. (2) Alternatively, a single enantiomer

Scheme III



Scheme IV



of methylmalonyl-CoA may be required for all six condensation steps, with subsequent epimerization at appropriate sites most likely (but not necessarily) occurring prior to reduction of the β-keto ester.

Since methylmalonyl-CoA would not be expected to penetrate the walls of intact cells, while methylmalonate itself is achiral, we required a method for the determination of the stereochemistry of the condensation reaction based on the in vivo generation of specifically labeled (2*S*)- and (2*R*)-methylmalonyl-CoA. The approach we chose was based on the well-known pathway, illustrated in Scheme III, which links propionate to the Krebs cycle. Thus carboxylation of propionyl-CoA, with removal of the *re*-2-hydrogen atom, yields (2*S*)-methylmalonyl-CoA (**5**),[15] which is converted to its epimer by a separate epimerase. The resulting (2*R*)-methylmalonyl-CoA (**7**) serves as the substrate for coenzyme B_{12} dependent methylmalonyl-CoA mutase to generate succinyl-CoA.[16] In principle, therefore, use of [2-$^2H_2$]propionate would produce (2*S*)-[2-^{2}H]methylmalonyl-CoA, condensation of which with an acyl-CoA chain would be expected to yield the corresponding D-[α-^{2}H]-α-methyl-β-keto ester. The epimeric L-α-methyl ester would be expected to be devoid of deuterium, whether formed by epimerization of the D-[α-^{2}H]-α-methyl condensation product or by condensation of (2*R*)-methylmalonyl-CoA which necessarily has lost the deuterium label in the course of the epimerase-catalyzed reaction.[17] To distinguish the latter two

(12) See, for example: (a) Abell, C.; Staunton, J. *J. Chem. Soc., Chem. Commun.* **1981**, 856. (b) Hutchinson, C. R.; Kurobane, I.; Cane, D. E.; Hasler, H.; McInnes, A. G. *J. Am. Chem. Soc.* **1981**, *103*, 2477. (c) Moore, R. H.; Bigam, G.; Chan, J. K.; Hogg, A. M.; Nakashima, T. T.; Vederas, J. C. *J. Am. Chem. Soc.* **1985**, *107*, 3694.

(13) An exception to this generalization is the D-methyl at C-12 of the milbemycin complex of antibiotics: Mishima, H.; Kurabayashi, M.; Tamura, C.; Sato, S.; Kuwano, H.; Saito, A. *Tetrahedron Lett.* **1975**, 711. Takiguchi, Y.; Mishima, H.; Okuda, M.; Terao, M.; Aoki, A.; Fukuda, R. *J. Antibiot.* **1980**, *33*, 1120.

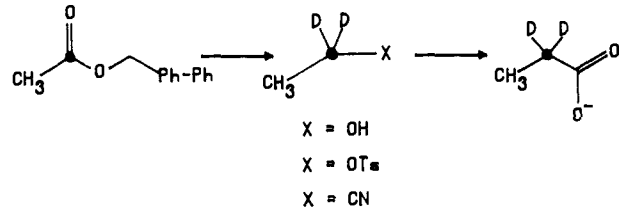
(14) Sedgwick, B.; Cornforth, J. W. *Eur. J. Biochem.* **1977**, *75*, 465. Sedgwick, B.; Cornforth, J. W.; French, S. J.; Gray, R. T.; Kelstrup, E.; Willadsen, P. *Eur. J. Biochem.* **1977**, *75*, 481. Sedgwick, B.; Morris, C.; French, S. *J. Chem. Soc., Chem. Commun.* **1978**, 93. Arnstadt, K.-I.; Schindbleck, G.; Lynen, F. *Eur. J. Biochem.* **1975**, *55*, 561.

(15) (a) Retey, J.; Lynen, F. *Biochem. Z.* **1965**, *342*, 256. Arigoni, D.; Lynen, F.; Retey, J. *Helv. Chim. Acta* **1966**, *49*, 311. (b) Kolattukudy has isolated and purified an acyl-CoA carboxylase from *S. erythreus* with a strong preference for propionyl-CoA over acetyl-CoA (relative V_{max}/K_m 20:1) and demonstrated that the product of the biotin-dependent carboxylation is (2*S*)-methylmalonyl-CoA: Hunaiti, A. R.; Kolattukudy, P. E. *Arch. Biochem. Biophys.* **1982**, *216*, 362.

(16) (a) Sprecher, M.; Clark, M. J.; Sprinson, D. B. *J. Biol. Chem.* **1966**, *241*, 872. (b) This enzyme has recently been isolated from *S. erythreus* (cf. ref 9). The same group has also isolated a (2*R*)-methylmalonyl-CoA specific decarboxylase from the same organism: Hunaiti, A. R.; Kolattukudy, P. E. *Arch. Biochem. Biophys.* **1984**, *229*, 426. The physiological role of the latter enzyme is uncertain.

(17) Leadlay has demonstrated that the epimerization catalyzed by methylmalonyl-CoA epimerase from *Propionibacterium shermanii* takes place with essentially complete loss of the original C-2 hydrogen atom and incorporation of a proton from the solvent, consistent with a postulated two-base mechanism: Leadlay, P. F.; Fuller, J. Q. *Biochem. J.* **1983**, *213*, 635. Fuller, J. Q.; Leadlay, P. F. *Biochem. J.* **1983**, *213*, 643.

Scheme V



possibilities, it is necessary to generate the corresponding (2*R*)-[2-^{2}H]methylmalonyl-CoA. In principle, this could be accomplished by incorporation of deuterated succinate, with the expectation that the action of methylmalonyl-CoA mutase would generate the desired (2*R*)-[2-^{2}H]methylmalonyl-CoA[16] (Scheme IV). Direct condensation of the latter substrate would produce a deuteration pattern complementary to that resulting from incorporation of (2*S*)-[2-^{2}H]methylmalonyl-CoA, with label at the sites of L-methyl configuration (C-8 of erythromycin A and C-8 and C-12 of erythromycin B). Alternatively, mandatory epimerization and utilization of the undeuterated (2*S*)-methylmalonyl-CoA substrate would give a product devoid of deuterium label at all carbinyl carbons of the macrolide product.

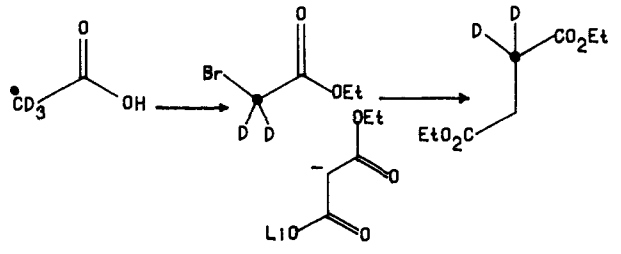
Extensive deuterium exchange has frequently complicated incorporation of deuterated acetate into polyketides and saturated fatty acids.[12,14,18] Although the exchange process itself probably has no direct *mechanistic* significance, it is possible that the condensing enzyme itself catalyzes the undesired exchange processes. The experimental situation is inherently even less favorable for the incorporation of C-2 deuterated propionate precursors, since exchange of the C-2 proton of methylmalonate is an intrinsic feature of the methylmalonyl-CoA epimerase reaction. Evidently, success in obtaining deuterated macrolides must rest in large part on the outcome of the competition between the biosynthetically relevant condensation reaction and the adventitious exchange catalyzed by the epimerase itself. Thus if the activity of the methylmalonyl-CoA epimerase significantly exceeds that of the macrolide synthetase, most or all of the deuterium label will be absent from the macrolide aglycone.

In principle, direct detection of one or more deuterium labels in erythromycin could be carried out by ^{2}H NMR spectroscopy, a technique which has gained wide acceptance over the last 10 years for the study of biosynthetic problems.[19] Unfortunately, the small chemical shift differences among the carbinyl protons H-2, H-4, H-8, H-10, and (for erythromycin B) H-12, combined with the low sensitivity of ^{2}H NMR spectroscopy, suggested that this technique would be unsatisfactory. We therefore chose to use ^{13}C{^{2}H} NMR spectroscopy for the detection of sites of deuterium enrichment based on the ^{2}H isotope shifts of ^{13}C signals corresponding to carbon atoms bearing directly attached deuterium.[19,20]

Results

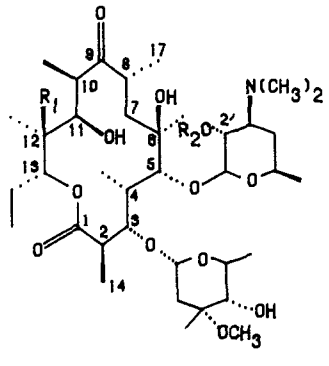
To prepare [2-^{2}H$_2$,2-^{13}C]propionate required for the intended incorporation studies, we used a variation of the synthetic sequence we had earlier developed for the preparation of [^{18}O,^{13}C]acetates and propionates,[21] a procedure which can readily be modified to deliver precursors with any desired combination of isotopes of carbon, hydrogen, and oxygen (Scheme V). Thus sodium [1-^{13}C]acetate was converted to the corresponding *p*-phenylphenacyl ester which was reduced with lithium aluminum deuteride in anhydrous diglyme to give [1-^{2}H$_2$,1-^{13}C]ethanol after being quenched with phenoxyethanol. The labeled ethanol, containing a small amount of diglyme, was removed from the product mixture in a stream of dry nitrogen and condensed in a liquid nitrogen cooled trap. Treatment with toluenesulfonyl chloride in pyridine gave the tosylate which was reacted wwith potassium cyanide in 3:1 MeOD–D_2O. The resulting [2-^{2}H$_2$,2-^{13}C]propionitrile was readily hydrolyzed by treatment with 2 equiv of KOH in refluxing D_2O. The use of deuterated solvents in the latter two reactions was necessary in order to avoid substantial washout of deuterium. Acidification of potassium propionate with phosphoric acid followed by lyophilization and titration with NaOH gave the desired sodium [2-^{2}H$_2$,2-^{13}C]propionate in overall 35% yield from [1-^{13}C]acetate.

Scheme VI



For the preparation of diethyl [2-^{2}H$_2$,2-^{13}C]succinate, sodium [2-^{2}H$_3$,2-^{13}C]acetate was first converted to [2-^{2}H$_2$,2-^{13}C]-α-bromoacetic acid by treatment with bromine in a mixture of deuterated trifluoroacetic acid–trifluoroacetic anhydride containing a small amount of thionyl chloride (Scheme VI). Esterification with ethereal diazoethane gave the corresponding ethyl [2-^{2}H$_2$,2-^{13}C]bromoacetate which was reacted with the dilithio anion of malonic acid monoethyl ester in THF–HMPA.[22] Thermolysis of the resulting alkylation product effected smooth decarboxylation to yield the desired diethyl [2-^{2}H$_2$,2-^{13}C]succinate.

Earlier work in the laboratories of Roberts,[23a] Tori,[23b] and Omura[23c] had led to the assignment of the majority of the ^{13}C NMR signals of erythromycins A and B. Subsequently, we confirmed most of these assignments and resolved several ambiguities by analysis of erythromycin A and B 2′-benzoates (**9** and **10**) derived from feedings of singly and doubly labeled propionate



9 R_1 = OH, R_2 = PhCO
10 R_1 = H, R_2 = PhCO

and doubly labeled succinate.[4b] One remaining uncertainty, however, involved the assignment of the carbinyl carbons C-2 and C-8. Although these signals are separated by no more than 0.1 ppm in the spectrum of erythromycin A, somewhat greater resolution could be achieved by analysis of the corresponding 2′-benzoate ester. Because it was observed that the precise separation

(18) (a) McInnes, A. G.; Walter, J. A.; Wright, J. L. C. *Tetrahedron Lett.* **1979**, *35*, 3245. (b) Saito, K.; Kawaguchi, A.; Nozoe, S.; Seyama, Y.; Okuda, S. *Biochem. Biophys. Res. Commun.* **1982**, *108*, 995. Saito, K.; Kawaguchi, A.; Seyama, Y.; Yamakawa, T.; Okuda, S. *J. Biochem.* (*Tokyo*) **1981**, *90*, 1697. (c) White, R. H. *Biochemistry* **1980**, *19*, 9. (d) Sherman, M. M.; Hutchinson, C. R., unpublished results.

(19) Garson, M. J.; Staunton, J. *Chem. Soc. Rev.* **1979**, 539.

(20) For an extensive compilation of recent references to detection of deuterium by ^{13}C NMR spectroscopy see ref 50 and 51 of Vederas et al., ref 12c.

(21) Cane, D. E.; Liang, T.-C.; Hasler, H. *J. Am. Chem. Soc.* **1982**, *104*, 7274.

(22) Cf.: Wierenga, W.; Skulnick, H. I. *J. Org. Chem.* **1979**, *44*, 310.

(23) (a) Nourse, J. G.; Roberts, J. D. *J. Am. Chem. Soc.* **1975**, *97*, 4584. (b) Terui, Y.; Tori, K.; Nagashima, K.; Tsuji, N. *Tetrahedron Lett.* **1975**, 2583. (c) Omura, S.; Neszelyi, A.; Sangare, M.; Lukacs, G. *Tetrahedron Lett.* **1975**, 2939.

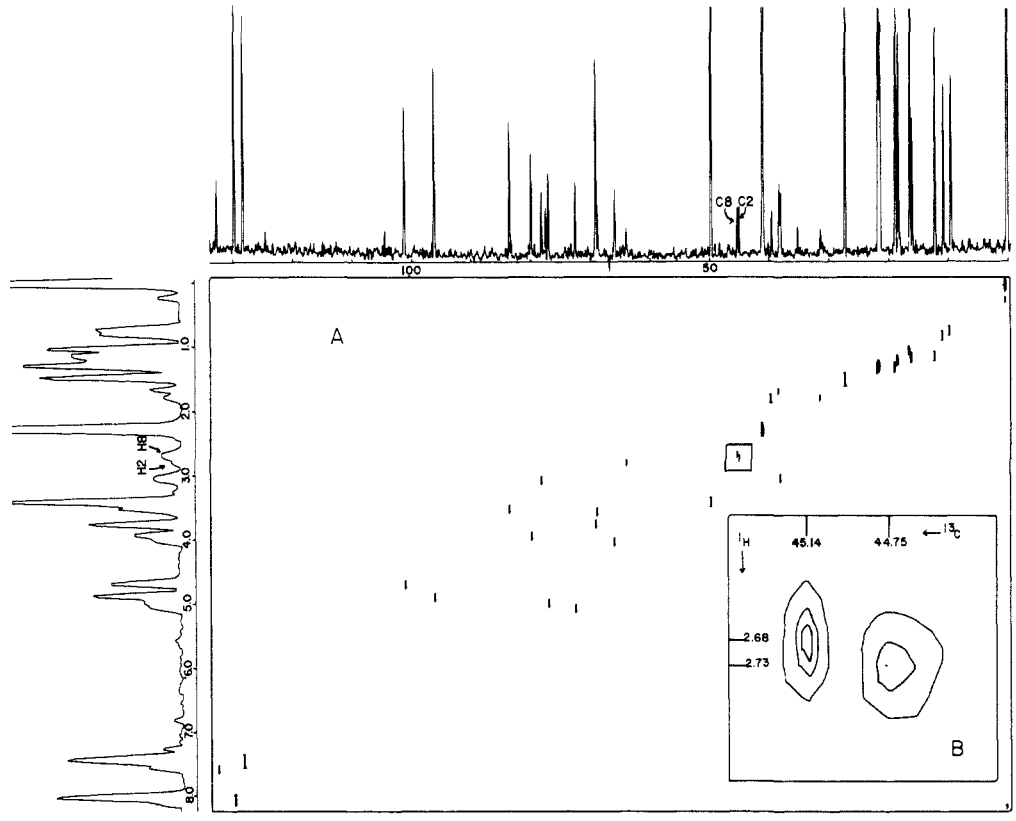


Figure 2. ^{1}H–^{13}C hetero-COSY spectrum of 0.056 M erythromycin A 2′-benzoate (**9**). Acquisition parameters are given in the Experimental Section. (A) ^{13}C (f_2) −0.7–134.0 ppm, ^{1}H (f_1) −0.3–8.2 ppm. (B) Expansion of C-2,C-8 region.

and even the order of the relevant resonances were concentration dependent, it was necessary to carry out all analyses at well-defined concentrations. In the event, it was found that for a 0.056 M solution of erythromycin A benzoate (**9**) in $CDCl_3$ the C-2/C-8 signals appeared at 44.8 and 45.2 ppm, whereas the corresponding resonances for erythromycin B benzoate (**10**) appeared at 44.6 and 44.7 ppm. The requisite assignments were in fact readily made by carrying out a combination of 2D ^{1}H COSY and ^{1}H–^{13}C hetero-COSY experiments on erythromycin A benzoate. Prior work in other laboratories[24] had left little doubt that the proton signal for H-2 (δ 2.87) appeared downfield from that corresponding to H-8 (δ 2.70) in the spectrum of erythromycin A, a result which we have confirmed by ^{1}H COSY analysis of erythromycin A. Although the corresponding protons were less well resolved in the 250-MHz ^{1}H NMR spectrum of erythromycin A benzoate, analysis of the ^{1}H COSY spectrum of **9** established that H-2 (δ 2.72), identified by its strong cross-peak with H-3 (δ 3.93), remained downfield of H-8 (δ 2.68). In the ^{1}H–^{13}C correlated spectrum, however, the H-8 proton was clearly correlated with the downfield ^{13}C NMR signal at 45.14 ppm, which was therefore assigned to C-8, while H-2 showed a clear cross-peak with the upfield ^{13}C signal at 44.78 ppm (C-2)[25] (Figure 2).

With the critical ^{13}C NMR signals firmly assigned, the individual labeled samples of sodium [2-$^2H_2$,2-^{13}C]propionate and ethyl [2-$^2H_2$,2-^{13}C]succinate were administered to actively growing cultures of *S. erythreus* according to our previously described protocols.[4b] The resulting samples of labeled erythromycins were isolated and purified after suitable incubation times, separated by reverse-phase HPLC into the components erythromycins A and B, and then subjected to further purification by conversion to the corresponding 2′-benzoate esters. Attempts at HPLC purification of the benzoates themselves led to substantial hydrolysis of the sensitive ester moiety. The latter compounds could be separated by Sephadex LH-20 chromatography, but the resulting product contained traces of impurities which interfered with subsequent ^{13}C{^{2}H} NMR analysis.

Preliminary examination of the 62.9-MHz ^{13}C NMR spectra of erythromycin A and B benzoates derived from incorporation of [2-$^2H_2$,2-^{13}C]propionate revealed the presence of deuterium in the C-20 methylene of the propionate starter unit of each macrolide, as evidenced by the observed broad multiplet which appeared upfield of the corresponding unshifted ^{13}C NMR resonance. When the same samples were analyzed by using broad-band proton and deuterium decoupling (^{13}C{^{1}H,^{2}H} NMR), the latter multiplets collapsed to a pair of signals corresponding to d_1 and d_2 species, shifted 0.4 and 0.7 ppm upfield, respectively.[19] Six additional enhanced ^{13}C NMR signals, corresponding to C-2, C-4, C-8, C-10, and C-12, were observed in the spectrum of each macrolide, as previously reported. Broad-band deuterium decoupling, however, resulted in only minor perturbations of these latter signals, indicating that extensive loss of deuterium label from the propionate precursor had occurred, presumably due to methylmalonate epimerase catalyzed exchange competing effectively with polyketide condensation. That deuterium was in fact present at C-2, C-4, and C-10 of erythromycin was eventually established by difference ^{13}C{^{1}H,^{2}H} NMR spectroscopy. Thus two blocks of spectra were acquired under essentially identical conditions with the broad-band deuterium decoupler alternately turned on and off. Subtraction of the two resulting Fourier-transformed spectra resulted in cancellation of all signals except those arising from carbon atoms with attached deuterium (Figure 3). In addition to confirming the presence of d_1 and d_2 species attached to C-20, the difference spectrum of erythromycin A benzoate exhibited three additional deuterium-shifted peaks ($\Delta\delta$ = 0.38–0.45 ppm) corresponding to C-2, C-4, and C-10 (Scheme VII and Table I). Comparison of the integrated areas of these residual peaks with those of the unshifted ^{13}C peaks in the coupled spectrum indicated that no more than 3–5% of the observed ^{13}C nuclei at C-2, C-4, and C-10 also bore deuterium.[26] Similar Fourier difference

(24) Egan, R. Ph.D. Dissertation, University of Illinois, 1971.
(25) The above experiments also led to assignments for the attached methyls, C-14 (15.8 ppm) and C-17 (18.1 ppm), based on our earlier demonstration that incorporation of diethyl [2,3-$^{13}C_2$]succinate into erythromycin A resulted in coupling between these signals and those now assigned to C-2 and C-8, respectively.[4b]

Table I. Incorporation of [2-$^2H_2$,2-^{13}C]Propionate into Erythromycins A and B[a]

	erythromycin A 2′-benzoate (**9**)			erythromycin B 2′-benzoate (**10**)		
C	$\delta(d_0)$	$\delta(d_1)$	$\Delta\delta$, ppm	$\delta(d_0)$	$\delta(d_1)$	$\Delta\delta$, ppm
2	44.807	44.443	0.364	44.685	44.322	0.363
4	39.498	38.966	0.532	39.550	39.095	0.455
10	37.874	37.522	0.352	39.122	38.721	0.401
20	21.172	20.829[b]	0.343[b]	25.548	25.199[b]	0.349[b]
		20.516[c]	0.656[c]		24.833[c]	0.715[c]

[a] Difference $^{13}C\{^1H,^2H\}$ NMR, 62.9 MHz; spectral width 19 230 Hz, 32 K data points; quadrature detection; 90° pulse; acquisition time 0.85 s; 0.056 M **9** or **10** in $CDCl_3$; broad-band ^2H decoupler 38.397 206 MHz, 3.0–3.8 W as described in Experimental Section. Chemical shifts are ± 0.019 ppm. [b] d_1 species. [c] d_2 species.

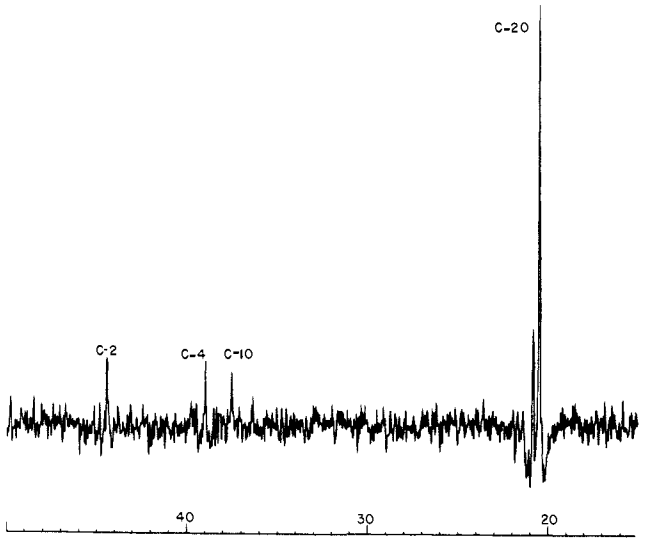
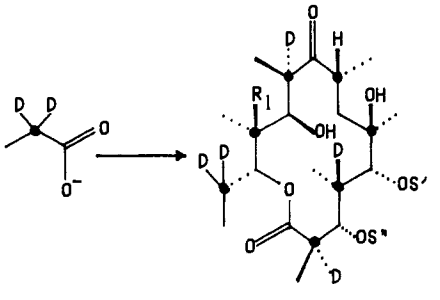


Figure 3. Difference $^{13}C\{^1H,^2H\}$ NMR spectrum of 0.056 M erythromycin A 2′-benzoate (**9**), obtained by incorporation of [2-$^2H_2$,2-^{13}C]-propionate.

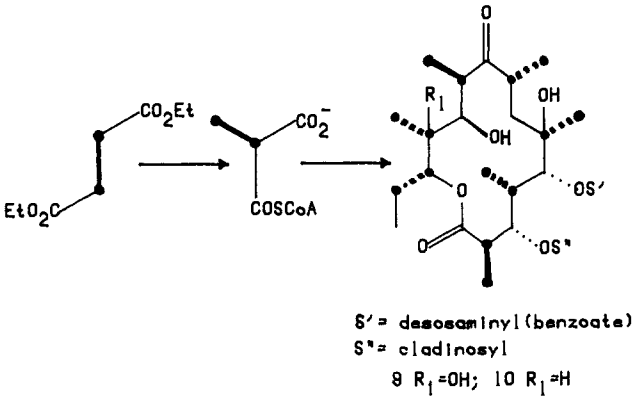
Scheme VII



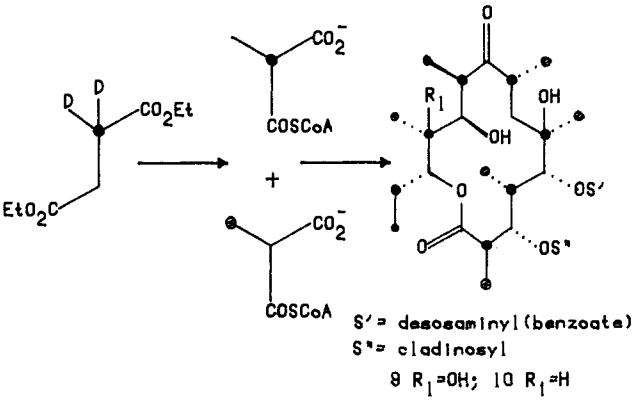
$^{13}C\{^1H,^2H\}$ analysis of erythromycin B benzoate confirmed the presence of comparable quantities of deuterium label at the analogous secondary methyl sites, C-2, C-4, and C-10. No deuterium was detected at C-8 of either macrolide or at C-12 of erythromycin B. The significance of these results is discussed below.

Incorporation of labeled succinate into erythromycin is complicated by the fact that metabolism of succinate by reactions of the Krebs cycle can lead to partial or even complete randomization of the carbon skeleton and washout of attached deuterium labels.

Scheme VIII



Scheme IX

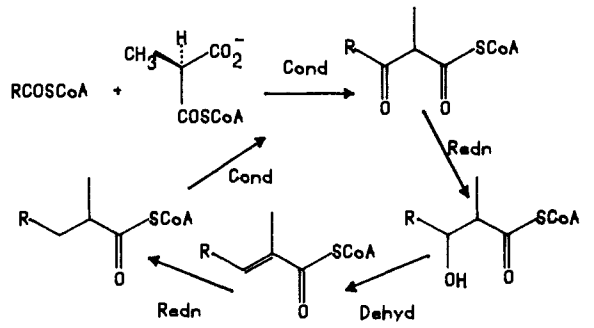


Nonetheless, we had previously demonstrated that incubation of ethyl [2,3-$^{13}C_2$]succinate with *S. erythreus* gave erythromycins A and B whose derived ^{13}C NMR spectra exhibited seven pairs of enhanced and coupled doublets, indicating incorporation of at least a portion of the succinate by rearrangement to methylmalonyl-CoA[4b] (Scheme VIII). Labeling of the propionate starter unit was presumably due to epimerization to (2*S*)-[2,2′-$^{13}C_2$]-methylmalonyl-CoA and conversion to [2,3-$^{13}C_2$]propionyl]-CoA by transcarboxylase[27] or by decarboxylation of (2*R*)-methylmalonyl-CoA by malonyl decarboxylase.[16b] On the other hand, the enrichments observed in the latter incorporation experiments were more than a factor of 10 lower than the enrichments achieved from propionate itself, in spite of the use of the diethyl ester in place of free succinate. Initial attempts to extend these results by the incorporation of [2,3-$^2H_4$,2,3-$^{13}C_2$]succinate produced labeled erythromycin samples whose ^{13}C NMR spectra showed no evidence for the presence of deuterium attached to the macrolide ring. Interpretation of the latter spectra was further complicated by the extra C–C couplings and the possibility of both β- and α-deuterium isotope shifts. In the hope of obtaining more easily interpretable data, ethyl [2-$^2H_2$,2-^{13}C]succinate, prepared as described above, was administered to cultures of *S. erythreus*. Since utilization of this substrate would lead to generation of equal

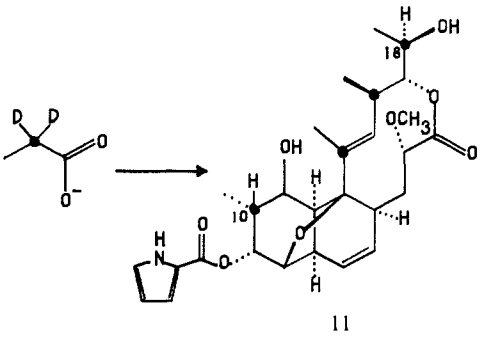
(26) The use of difference $^{13}C\{^1H,^2H\}$ NMR spectroscopy is considerably more complicated than the detection of deuterium using β-isotope-induced shifts. The latter method is often attractive because the shifted ^{13}C NMR signals ($\Delta\delta$ = 0.03–0.05 ppm/β-deuterium) appear as singlets due to the absence of observable two-bond coupling to the I = 1 deuterium nucleus.[20] On the other hand, when less than 10% of the ^{13}C nuclei have neighboring deuterium atoms, the resultant signals are obscured by the base of the downfield, unshifted ^{13}C resonance. In the latter case, difference decoupling methods extend the useful range of detection by an additional factor of 3–5.

(27) Cf.: Walsh, C. *Enzymatic Reaction Mechanisms*; Freeman: San Francisco, 1979; pp 725–727.

Scheme X



Scheme XI



11

quantities of [2-^{13}C]- and [2′-^{13}C]methylmalonyl-CoA, ignoring possible deuterium isotope effects on the methylmalonyl-CoA mutase reaction, the maximum enrichments at each site of the resulting labeled erythromycin would now be expected to be half of those achieved with the [2,3-$^{13}C_2$] precursor. In the event, the samples of erythromycin A and B benzoates which were obtained showed only marginal enrichments above natural abundance ^{13}C (Scheme IX). Futhermore, analysis of difference $^{13}C\{^1H,^2H\}$ NMR failed to reveal deuterium at any of the ring carbons of the macrolide. In fact, only traces of deuterium were evident in the ring methyl groups, indicating extensive washout of deuterium from the succinate precursor *prior* to isomerization to methylmalonyl-CoA, most likely due to the action of Krebs cycle enzymes.[28]

Discussion

Over the last 10 years, an increasing body of evidence has accumulated indicating that the apparent structural similarities between saturated fatty acids and oxygenated, branched-chain polyketide natural products such as macrolide and polyether antibiotics reflect close parallels in biogenetic mechanisms, enzymology, and molecular biology. Thus not only are both classes of compounds derived from simple short-chain organic acid precursors, primarily acetate, propionate, and butyrate, but the demonstrated origins of the oxygen atoms of the macrolides and polyethers are fully consistent with a postulated mechanism for polyketide chain elongation closely analogous to fatty acid biosynthesis in which each successive condensation with malonyl-CoA or methylmalonyl-CoA is followed by an appropriate sequence terminating with β-(ketoacyl)-CoA reduction, dehydration, or double bond reduction (Scheme X). Further circumstantial evidence for the close similarity between the two chain-elongation mechanisms has been based on the observation that cerulenin, a known inhibitor of the β-ketoacyl synthetase step of fatty acid biosynthesis,[29] also functions as an effective in vitro inhibitor of the biosynthesis of the polyketide portions of macrolide, polyether, and aromatic polyketide antibiotics.[30] Recently, Leadlay has noted that cerulenin inhibits erythromycin production when added to resting cells of *S. erythreus* and has reported that [^{3}H]tetrahydrocerulenin binds irreversibly to a 35–37-kDa polypeptide present only in extracts of *S. erythreus* at the antibiotic-producing stage, suggesting that this polypeptide may be related to the condensing enzyme activity of 6-deoxyerythronolide B synthetase.[31] Although the enzymology of macrolide and polyether chain assembly remains essentially unexplored, mechanistic and enzymological studies of 6-methylsalicylate synthetase, an enzyme of MW 1.1–1.5 × 10^6, isolated from *Penicillium patulum* and purified to apparent homogeneity, have established the close similarity betwween this tetraketide-synthesizing enzyme and yeast fatty acid synthetase.[32] More recent supporting evidence has come from a variety of genetic experiments. Thus mutants of *S. erythreus* and *S. fradiae*, blocked in the formation of the parent macrolide aglycones, 6-deoxyerythronolide B[33] and tylactone,[34] respectively, do not act as secretors in cosynthesis experiments, consistent with the absence of freely diffusible intermediates of the chain-elongation process. Genetic recombination experiments in these organisms have indicated that the requisite biosynthetic genes are closely clustered, a result analogous to the demonstrated segregation of yeast fatty acid synthetase genes at two (unlinked) genetic loci, *fas 1* and *fas 2*, coding for two mulifunctional polypeptide subunits, β (MW 203 000) and α (MW 213 000), respectively.[35] The recent cloning of the erythromycin biosynthetic genes within a 35 kbp segment of DNA should soon allow more extensive comparisons of the macrolide and fatty acid synthetase systems.[11]

In spite of the apparent similarities between the polyketide and fatty acid biosynthetic pathways, the far greater complexity of the myriad of polyketide natural products has raised a host of difficult and intriguing questions. Nothing is known about the mechanisms by which a given enzyme mediates the formation of a single macrolide or polyether product, requiring complete fidelity in the sequential utilization of the various malonyl-CoA, methylmalonyl-CoA, and ethylmalonyl-CoA precursors, stereospecific formation of both D- and L-β-hydroxyacyl thio esters, and adjustment of the oxidation level subsequent to each condensation step. The presence of both D and L secondary methyl groups in the macrolide and polyether chains adds a further degree of complexity to the problem, raising the question as to whether the polyketide synthetase makes appropriate choices between both enantiomers of methylmalonyl-CoA, both of which are readily available in vivo, or whether the synthetase utilizes a single enantiomer of methylmalonyl-CoA and carries out selective epimerizations on the α-methyl-β-ketoacyl thio ester product of the condensation. The work described above has provided a partial answer to this question.

The observed incorporation of deuterium label from [2-$^2H_2$,2-^{13}C]propionate into erythromycins A and B at C-2, C-4, and C-10 implicates (2*S*)-methylmalonyl-CoA as the direct

(28) By contrast, Kolattukudy has reported little apparent loss of tritium label during incorporation of [2,3-^{3}H,1,4-^{14}C]succinate into erythromycin A.[9]

(29) (a) Omura, S. *Methods. Enzymol.* **1981**, *72*, 520. (b) D'Agnolo, G.; Rosenfeld, I. S.; Awaya, J.; Omura, S.; Vagelos, P. R. *Biochim. Biophys. Acta* **1973**, *326*, 155. (c) Kawaguchi, A.; Tomoda, H.; Nozoe, S.; Omura, S.; Okuda, S. *J. Biochem.* (*Tokyo*) **1982**, *92*, 7.

(30) (a) Omura, S.; Takeshima, H. *J. Biochem.* (*Tokyo*) **1974**, *75*, 193. (b) Takeshima, H.; Kitao, C.; Omura, S. *J. Biochem.* (*Tokyo*) **1977**, *81*, 1127. (c) Martin, J. F.; McDaniel, L. E. *Biochim. Biophys. Acta* **1975**, *411*, 186. (d) Ohno, H.; Ohno, T.; Awaya, T.; Omura, S. *J. Biochem.* (*Tokyo*) **1975**, *78*, 1149. (e) Sherman, M. M.; Hutchinson, C. R., unpublished results.

(31) Roberts, G.; Leadlay, P. F. *Biochem. Soc. Trans.* **1984**, *12*, 642. Roberts, G.; Leadlay, P. F. *FEBS Lett.* **1983**, *159*, 13.

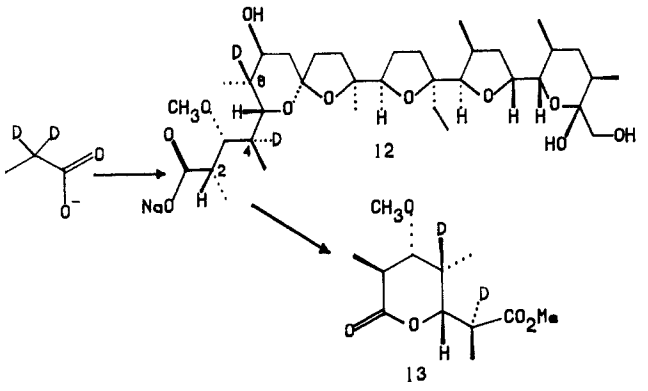
(32) (a) Dimroth, P.; Ringelmann, E.; Lynen, F. *Eur. J. Biochem.* **1976**, *68*, 591. (b) Dimroth, P.; Walter, H.; Lynen, F. *Eur. J. Biochem.* **1970**, *13*, 98. (c) Scott, A. I.; Phillips, G. T.; Kircheis, U. *Bioorg. Chem.* **1971**, *1*, 380.

(33) Weber, J. M.; Wierman, C. K.; Hutchinson, C. R.; Wang, Y.-G.; Davies, J. E. In *Trends is Antibiotic Research. Genetics, Biosyntheses, Actions, and New Substances*; Umezawa, H., Demain, A. L., Hata, T., Hutchinson, C. R., Eds.; Japan Antibiotics Research Association: Tokyo, 1982; pp 55–64. Weber, J. M.; Wiermann, C. K.; Hutchinson, C. R. *J. Bacteriol.* **1985**, *164*, 425.

(34) Baltz, R. H.; Seno, E. T.; Stonesifer, J.; Matsushima, P.; Wild, G. M. In *Trends is Antibiotic Research. Genetics, Biosyntheses, Actions, and New Substances*; Umezawa, H., Demain, A. L., Hata, T., Hutchinson, C. R., Eds.; Japan Antibiotics Research Association: Tokyo, 1982; pp 65–72. Baltz, R. H.; Seno, E. T. *Antimicrob. Agents Chemother.* **1981**, *20*, 214. Baltz, R. H.; Seno, E. T.; Stonesifer, J.; Matsushima, P.; Wild, G. M. In *Microbiology*; Schlessinger, D., Ed.; American Society of Microbiology: Washington, DC, 1981; pp 371–375.

(35) Schweizer, E. In *Multifunctional Proteins*; Bisswanger, H., Schmincke-Ott, E., Eds.; Wiley: New York, 1980; pp 197–215.

Scheme XII



precursor of these three sites in the macrolide (Scheme VII). The exclusive incorporation of deuterium label at carbinyl carbons with D-methyl groups further implies that the mechanism of polyketide chain elongation involves decarboxylative inversion of the methylmalonyl-CoA substrate, in direct analogy to the reaction of malonyl-CoA in the β-ketoacyl-CoA synthetase reaction of fatty acid biosynthesis (Scheme III).

In attempts to extend the same methodology to the study of additional macrolides and polyethers, we have also carried out incorporations of [2-$^2H_2$,2-^{13}C]propionate into the macrolide antibiotic nargenicin A_1 (**11**).[36] Although, in keeping with our earlier results,[37] efficient incorporations of ^{13}C were achieved, no deuterium could be detected at the expected sites, C-10 and C-18, apparently due to complete exchange of deuterium by methylmalonyl-CoA epimerase (Scheme XI). Similar attempts to label the polyether monensin A (**12**) were only slightly more successful.[38] In one series of experiments, Fourier difference ^{13}C{^{1}H,^{2}H} NMR analysis of monensin A derived from incubation of [2-$^2H_2$,2-^{13}C]propionate with cultures of *S. cinnamonensis* suggested the presence of minor amounts of deuterium label at the two sites bearing D-methyl groups, C-4 and C-6. Interpretation of the data was complicated, however, by the presence of several additional peaks due to apparent impurities, while attempts to repeat the incorporations failed to give reproducible results. In the meantime, Robinson has independently established the incorporation of deuterium from C-2 of propionate into H-4 and H-6 of monensin in a thoroughly convincing manner[39] (Scheme XII). Large quantities of [2-$^2H_2$]propionate were administered to *S. cinnamonensis*, and the sites of labeling in the resulting labeled monensin were determined by 55-MHz ^{2}H NMR analysis of the lactonic ester **13** obtained by chemical degradation. These workers also demonstrated that carboxylation of the propionate substrate involves removal of the *re* H-2 proton, in agreement with the previously established behavior of propionyl-CoA carboxylase.[15a] Completely consistent results for the labeling of the C-12 D-methyl center of the polyether lasalocid A by [2-$^2H_2$,2-^{13}C]propionate were first reported 4 years ago by Hutchinson.[40] Full details of this earlier work, along with a report of the successful labeling of C-14 bearing a D-ethyl group by [2-$^2H_2$]butyrate, can be found in an upcoming paper.[18d]

The results from three independent laboratories involving a variety of macrolides and polyethers are therefore all consistent with the formation of secondary D-methyl centers during polyketide chain elongation by condensation of (2*S*)-methylmalonyl-CoA. Unfortunately, attempts to confirm or rule out the intermediacy of the enantiomeric substrate, (2*R*)-methylmalonyl-CoA, in the generation of the corresponding L-methyl centers of macrolides and polyethers have been thwarted by inefficient incorporations of succinate, used as an in vivo precursor of (2*R*)-methylmalonyl-CoA, and by extensive loss of deuterium from the substrate due to adventitious metabolic processes. Although it is conceivable that, sooner or later, a macrolide or polyether-producing organism might be found in which the chain-elongation reactions of polyketide biosynthesis compete more favorably with endogenous exchange processes, determination of the complete stereochemistry of the polyketide chain-building process must probably await the preparation of viable cell-free extracts supporting the formation of a macrolide aglycone.

Experimental Section

Instrumentation. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker WM 250 FT NMR spectrometer at 250.0 and 62.9 MHz, respectively. ^{1}H COSY spectra of erythromycin A benzoate were obtained on 0.056 M solutions in $CDCl_3$, using 512 × 1024 data points in the f_1 and f_2 dimensions, respectively, with symmetrization of the resulting data set and a total acquisition time of 10 h. Two-dimensional ^{1}H–^{13}C correlated spectroscopy (hetero-COSY) was carried out on a 0.056 M solution of erythromycin A benzoate in $CDCl_3$, using a time domain of 128 in the f_1 (^{1}H) dimension (SW = 2092 Hz, 256 data points) and a time domain of 2K in the f_2 (^{13}C) dimension (SW = 8474 Hz, 4K data points). The total acquisition time was 15 h. Broad-band deuterium and proton-decoupled ^{13}C NMR spectra (^{13}C{^{1}H,^{2}H}) were acquired in unlocked mode by using a PTS-160 frequency generator set at 38.397 206 MHz (ca. 3.5 ppm in the deuterium spectrum) and a BSV-3X amplifier operated in the broad-band mode at 3.0–3.8 W, being alternately switched on and off every four scans under software control. Both blocks of spectra, obtained by using broad-band proton decoupling, were separately accumulated and subjected to Fourier transformation. After inspection of the corresponding frequency domain spectra, the coupled spectrum (decoupler off) was subtracted from the fully decoupled spectrum (decoupler on). No attempt was made to eliminate NOE effects since quantitative deuterium enrichment data were not required. All other routine analytical methods (IR, mass spectra, liquid scintillation spectrometry) as well as standard chromatographic techniques were performed as previously reported.[4b]

Materials. Sodium [1-^{13}C]acetate (90 and 99 atom %) was from KOR Isotopes. Lithium aluminum deuteride was obtained from Merck Sharp & Dohme. Authentic erythromycins A and B were gifts of Eli Lilly Company. All other reagents and solvents were of reagent grade, with any additional purification as previously described.[21]

Fermentation and Precursor Incorporation. Strains of *S. erythreus* were a gift of Dr. L. E. Day of the Eli Lilly Co. Maintenance, preparation of vegetative inocula, and fermentation methods have been previously described.[4b] The administration of labeled precursors was carried out in accord with previously developed protocols.[4b]

Erythromycin A and B 2′-Benzoates. Crude extracts of labeled erythromycins A and B could be converted to the corresponding 2′-benzoate esters and purified by sequential silica gel column chromatography and Sephadex LH-20 chromatography, as previously described.[4b] Attempted further purification by HPLC was accompanied by substantial hydrolysis of the sensitive benzoate ester. An alternative isolation and purification procedure was therefore developed. The crude chloroform extract from 100 mL of *S. erythreus* fermentation broth (35–50 mg) was subjected to preliminary purification by preparative-layer chromatography on silica gel (chloroform/methanol/concentrated ammonium hydroxide, 8:2:0.01), and the band corresponding to erythromycins A and B (R_f 0.12–0.29) was eluted, resulting in the recovery of 30–40 mg of an approximately 2:1 mixture of erythromycins A and B. Separation of the two components could be effected by HPLC on a Waters 5-μm C-18 Radial-PAK liquid chromatography cartridge (8-mm i.d.) using a mobile phase consisting of acetonitrile/methanol/0.2 M ammonium acetate/water (45:10:10:25), pH adjusted to 6.5 with HCl or NaOH, at a flow rate of 2.0 mL/min (1300 psi).[41] Erythromycins A and B had retention times of 6.6 and 10.8 min, respectively. Preparative separations were carried out by using 5-μL samples at a concentration of 2 mg/μL. In this manner 12–14 mg of erythromycin A and 10–12 mg of erythromycin B were obtained. The individual labeled macrolides were converted to the corresponding benzoate esters and purified by silica gel chromatography as previously described.[4b]

Sodium [2-$^2H_2$,2-^{13}C]Propionate. Sodium [2-$^2H_2$,2-^{13}C]propionate was prepared by the method previously described for the preparation of sodium [2-^{13}C]propionate,[21] substituting lithium aluminum deuteride for lithium aluminum hydride in the formation of [1-$^2H_2$,1-^{13}C]ethanol, using 3:1 MeOD–D_2O as solvent for the preparation of [2-$^2H_2$,2-^{13}C]propionitrile, and substituting D_2O for H_2O in the hydrolysis of the nitrile to

(36) Cane, D. E.; Yang, C.-C., unpublished results.
(37) Cane, D. E.; Yang, C.-C. *J. Antibiot.* **1985**, *38*, 423. Cane, D. E.; Yang, C.-C. *J. Am. Chem. Soc.* **1984**, *106*, 784.
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(39) Sood, G. R.; Robinson, J. A.; Ajaz, A. A. *J. Chem. Soc., Chem. Commun.* **1984**, 1421.
(40) Hutchinson, C. R.; Sherman, M. M.; McInnes, A. G.; Walter, J. A.; Vederas, J. C. *J. Am. Chem. Soc.* **1981**, *103*, 5956.
(41) Tsuji, K.; Goetz, J. *J. Chromatogr.* **1978**, *147*, 359.

[2-$^2H_2$,2-^{13}C]propionate. Analysis of the derived *p*-phenylphenacyl propionate, prepared as previously described,[21] indicated that the ^{13}C-labeled species consisted of 75.3% $^{13}CD_2$, 13.6% ^{13}CDH, and 1.6% $^{13}CH_2$.

Ethyl [2-$^2H_2$,2-^{13}C]Bromoacetate. Deuterated trifluoroacetic acid (2.32 mL, 3.42 g, 30 mmol), prepared by reaction of trifluoroacetic anhydride with 0.9 equiv of D_2O (99.8 atom %), was added with stirring to 0.820 g (10 mmol) of sodium [2-$^2H_3$,2-^{13}C]acetate (99 atom % ^2H, 99 atom % ^{13}C) at −15 °C followed by 2.82 mL (4.2 g, 20 mmol) of trifluoroacetic anhydride. After 1 h at room temperature, 3 drops of thionyl chloride and 1.1 mL (3.20 g, 10 mmol) of bromine were added, and the mixture was stirred for a further 2 h at room temperature and then refluxed at 66–68 °C for 3–4 h. The reaction was accompanied by the formation of considerable precipitate. After the mixture cooled to room temperature and excess anhydride was decomposed with 0.36 mL (0.36 g, 20 mmol) of D_2O, followed by distillation of the resulting trifluoroacetic acid, the residual product was distilled to give a 55% yield of [2-$^2H_2$,2-^{13}C]bromoacetic acid, mp 47–49 °C. Ethereal diazoethane was generated by the reaction of 1 g of *N*-(nitroso)ethylurea with 4 mL of 40% aqueous NaOH, and the product was distilled directly into an ethereal solution of [2-$^2H_2$,2-^{13}C]bromoacetic acid until the faint yellow color of the reagent persisted. Drying over anhydrous sodium sulfate and evaporation of the solvent gave an essentially quantitative yield of ethyl [2-$^2H_2$,2-^{13}C]bromoacetate: ^1H NMR ($CDCl_3$) δ 1.30 (t, J = 7.1 Hz, CH_3, 3 H), 4.23 (q, J = 7.1 Hz, CH_2O), no signal at 3.88 ($^{13}C^2H_2Br$); ^{13}C NMR ($CDCl_3$) δ 25.24 (quintuplet, 1:3:5:3:1, $J(^2H-^{13}C)$ = 23 Hz, CD_2Br) indicating essentially exclusive D_2 species (unlabeled ethyl bromoacetate, ^{13}C NMR δ 13.6 (CH_3), 25.6 (CH_2Br), 61.8 (CH_2O), 166.9 (C=O)).

Diethyl [2-$^2H_2$,2-^{13}C]Succinate. A solution of lithium diisopropylamide, generated by the reaction of 0.91 mL (0.66 g, 6.51 mmol) of diisopropylamine with 3.87 mL (6.58 mmol) of 1.7 M *n*-butyllithium in 2.5 mL of tetrahydrofuran at −78 °C under argon, was reacted with 0.39 g (3.0 mmol) of malonic acid monoethyl ester in 2.5 mL of THF which was added dropwise at −78 °C.[22] The reaction mixture was immediately warmed to 0 °C and stirred for 15 min before the addition of 1 mL of HMPA followed by 0.50 g (3.0 mmol) of ethyl [2-$^2H_2$,2-^{13}C]bromoacetate in 2 mL of THF in a single portion. After stirring for 2 h at room temperature, the mixture was cooled to 0 °C, and the reaction was quenched with 1 N HCl. (Quenching with water resulted in significant scrambling of the deuterium label.) Extraction with two portions of ether was followed by back-extracting with 1 N HCl, water, and saturated brine, drying over sodium sulfate, and evaporating the solvent. The residue was heated to 160 °C overnight to effect decarboxylation. Distillation in a Kugelrohr apparatus resulted in a 52% yield of diethyl[2-$^2H_2$,2-^{13}C]succinate: ^1H NMR ($CDCl_3$) δ 1.26 (t, J = 7.1 Hz, CH_3, 6 H), 2.61 (d, $J(^{13}C-^1H)$ = 4.0 Hz, CH_2 and CHD, 1.8 H), 4.15 (q, J = 7.1 Hz, CH_2O, 4 H); ^{13}C NMR ($CDCl_3$) δ 13.8 (CH_3), 60.2 (CH_2O), 171.8–172.6 (m, C=O), 28.3 (m, CD_2). Expansion of the 28.3 ppm multiplet showed a 1:1:1 triplet, J ($^2H-^{13}C$) = 19.95 Hz, 28.52 ppm (CDH), superimposed on a 1:3:5:3:1 quintuplet, J ($^2H-^{13}C$) = 19.92 Hz, 28.25 ppm (CD_2), as well as small β-shifted peaks. The corresponding CH_2 peak is observed at 28.93 ppm in the spectrum of unlabeled diethyl succinate. Analysis by $^{13}C\{^1H,^2H\}$ NMR collapsed the multiplets at 28 ppm to a set of four signals at 28.50, 28.41, 28.24, and 28.16 ppm corresponding to ca. 15% $^{13}CHDCH_2$, 5% $^{13}CCHDCHD$, 69% $^{13}CD_2CH_2$, and 11% $^{13}CD_2CHD$, respectively. The percentages calculated underestimate CD_2 species since no attempt was made to suppress NOE effects. The presence of deuterium at the vicinal carbon is presumably due to exchange during the alkylation of the malonic acid ester dianion.

Acknowledgment. This work was supported by NIH Grant GM 22172 and by an NIH Research Career Development Award to D.E.C. (1978–1983). The Bruker WM 250 NMR spectrometer used in this study was purchased with funds provided by the NSF and the Montedison Group of Milan.

Note Added in Proof. Everett and Tyler (Everett, J. R.; Tyler, J. W. *J. Chem. Soc., Perkin Trans. 1* **1985**, 2599) have recently assigned the ^1H and ^{13}C NMR spectra of erythromycin A by using ^2D NMR methods. Their assignments for C-2 (45.02 ppm) and C-8 (44.93 ppm) are opposite to ours for C-2 and C-8 of erythromycin 2′-benzoate. The difference is probably due to either a concentration effect or the fact that different compounds were analyzed by each group.

Registry No. 9, 22372-28-7; **10**, 88640-92-0; $Me^{13}CD_2CO_2Na$, 102940-43-2; $Br^{13}CD_2CO_2Et$, 102940-44-3; $EtO_2CCH_2{}^{13}CD_2CO_2Et$, 102940-45-4; erythromycin A, 114-07-8; erythromycin B, 527-75-3; propionate, 79-09-4; succinate, 110-15-6.