Biosynthesis of Lasalocid A: Biochemical Mechanism for Assembly of the Carbon Framework†

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ABSTRACT: Labeling experiments on the biosynthesis of the polyether antibiotic lasalocid A (**1**) using carboxylic acid precursors bearing ^{13}C, ^{2}H, and ^{3}H labels at various positions established the following: (1) ^{2}H or ^{3}H at C-2 of propionate or ^{2}H at C-2 of butyrate was partially retained at C-12 and C-14 of **1**, respectively. (2) ^{2}H at C-2 of propionate or at C-2 and C-3 of succinate did not label C-10. These and earlier data [Hutchinson, C. R., Sherman, M. M., Vederas, J. C., & Nakashima, T. T. (1981) *J. Am. Chem. Soc. 103*, 5953; Hutchinson, C. R., Sherman, M. M., McInnes, A. G., Walter, J. A., & Vederas, J. C. (1981) *J. Am. Chem. Soc. 103*, 5956] are consistent with a hypothesis for the stereochemical control of lasalocid A biosynthesis, whose main tenets are (i) that the configuration of C-12 and C-14 is determined by the stereoselectivity of the carbon chain forming condensation between acyl thio ester and 2-carboxyacyl thio ester intermediates and (ii) that the configuration of C-11 and C-15 results from the reduction of 2-keto thio ester intermediates with opposing stereospecificities.

The polyether antibiotics are branched-chain polyoxygenated carboxylic acids (Westley, 1982). These intricate structures with 10–23 chiral centers have in common the presence of five- or six-membered hydroxylated ether rings whose oxygen atoms, along with the carboxylate, account for their ionophoric properties, hence their antibiotic character.

A unifying hypothesis for the biosynthesis of the known polyether antibiotics assigns a special role to the cyclization of putative acyclic, polyepoxide intermediates (Cane et al., 1983a). To understand the nature of stereochemical control in this process, two questions must be asked: How is the configuration of the chiral centers established? What dictates the sequence by which the individual two-, three-, and four-carbon precursors are assembled into the basic carbon framework? Answers to these questions should have wide interest in view of the biochemical parallels between macrolide and polyether antibiotics (Hutchinson, 1983).

The polyether antibiotic lasalocid A (**1**) derives from three molecules of butyric acid, four of propionate, and five of acetate (Westley et al., 1974a) as shown in Figure 1. To investigate the stereochemistry of the biosynthetic process, the retention or loss of ^{2}H was determined for the incorporation of ^{13}C-, ^{14}C-, and ^{2}H-labeled precursors into **1** in whole cells of *Streptomyces lasaliensis* NRRL 3382R (Berger, 1973). Although loss of ^{2}H was to be expected through exchange reactions unrelated to lasalocid A biosynthesis, in practice enough label was retained for analysis.

Our working hypothesis, modeled on fatty acid biosynthesis, is that the configuration at C-2 of 3-keto thio ester intermediates will be carried through into many of the non-hydroxylated chiral centers of **1** (Figure 2a). The stereochemistry at such positions will be determined (a) during carboxylation of $RCH_2COSEnz$ and (b) during condensation between $RCH_2COSEnz$ and $RCH(COOH)COSEnz$. In fatty acid biosynthesis the carboxylation and condensation steps occur, respectively, with retention and inversion of the C-2 configuration (Arnstadt et al., 1975; Sedgwick & Cornforth, 1977; Sedgwick et al., 1977). The C-3 configuration of 3-hydroxy thio ester intermediates also is likely to be carried through into hydroxylated positions of **1** that are not created oxidatively after carbon skeleton assembly (Figure 2b). The chirality of these positions could result either from stereospecific reduction of the 3-keto group of the preceding intermediate, as in fatty acid biosynthesis, or from a dehydration–rehydration (Figure 2b). The latter mechanism would allow simultaneous change of the C-2 and C-3 configurations of the 3-hydroxy thio ester intermediates. Finally, since the configuration of some methine carbons of **1** is opposite to that predicted by the transformations of Figure 2a,b, epimerization by enolization at C-2 of any thio ester intermediate during carbon chain assembly may be possible (Figure 2c). Epimerization at C-3 also could occur via a redox process.

Experimental Procedures

General Methods. Streptomyces lasaliensis NRRL 3382R was obtained from the USDA, Northern Regional Resource Service, Peoria, IL. The organism was initially maintained by growth on slants at 30 °C with successive transfers and then later as frozen spore suspensions by standard methods.

The following growth medium was used for all fermentations: split pea/lard oil media (SP/LO) which contained 10 g/L lard oil, 10 g/L Stadex 60 dextrin, 4 g/L Difco yeast extract, 2 g/L K_2HPO_4, 1 L of doubly distilled H_2O, and 20 g of yellow split peas/L of medium.

Liquid cultures were grown in Erlenmeyer flasks with foam plugs and a capped side arm with plastic tubes for sampling on a New Brunswick G25 reciprocating shaker at 250 rpm and 28–30 °C without stopping the shaker during the fermentation.

Solvents and chemicals were used as commercially available, or where necessary, the solvents were redistilled from glass before use. Celite 545 was obtained from J. T. Baker. TLC plates were commercial precoated silica gel F_{254}, 0.25-mm thickness; PLC plates were made from silica gel PF_{245} at a 1.5-mm thickness. Solvents were generally evaporated either in vacuo on a rotary evaporator at 30–35 °C or under a stream of N_2 at room temperature.

Mass Spectral and Nuclear Magnetic Resonance Protocols. Most of the samples were run on an AEI MS9-DS50: 8-kV

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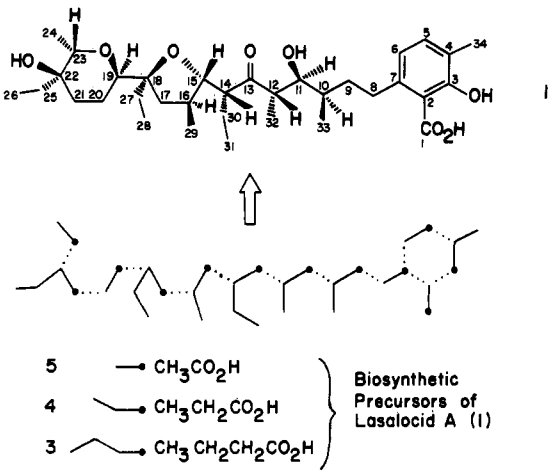


FIGURE 1: Way in which the three precursors are assembled in the biosynthesis of lasalocid A.

acceleration voltage; 70-eV ionization voltage; 130 °C source temperature. Some samples were run on a KRATOS MS25-DS55: 2-kV acceleration voltage; 70-eV ionization voltage; 150 °C source temperature; 200 °C probe temperature.

The approximate percentages of M + 1 and M + 2 ions at natural abundance were calculated according to Silverstein et al. (1974). These values were used to correct the peak heights of the M + 1 and M + 2 species for contributions from natural abundance, and the corrected values were used to determine the percentage of labeled species.

Samples were prepared for NMR spectroscopy by converting **1** either to the sodium salt or to the free acid. It was necessary to ensure that the sample was completely in one form or the other, since the free acid slowly converted to the sodium salt upon storage of solutions in glass tubes. Therefore, samples of the free acids were dissolved in 0.4–0.5 mL of $CDCl_3$ just prior to acquiring spectra. NMR spectra were recorded on several different spectrometers at four different institutions. The literature assignments for proton (Anteunis, 1976) and carbon (Seto et al., 1978) chemical shifts were checked by standard NMR methods [single-frequency decoupling, solvent-induced shifts, and the Attached Proton Test (Pratt & Schoolery, 1978)] and found to be correct for the positions of interest to this study.

Isolation and Purification of Lasalocid A (**1**). In the original method, 5 g of Celite and 50 mL of EtOAc were added to 50 mL of fermentation media, and the mixture was magnetically stirred vigorously for 1 h. The slurry was suction-filtered through Whatman No. 2 filter paper, the mycelial pad was rinsed twice with EtOAc, and the combined filtrates were extracted twice with additional EtOAc. The combined EtOAc phases were washed one with brine and dried over anhydrous Na_2SO_4, and the solvent was removed in vacuo to give a yellow oily residue.

In order to remove the bulk of the lard oil, the residue was dissolved in 95% EtOH and the solution placed in an EtOH/ice bath at about −10 °C, which caused the lard oil to solidify. This solution was filtered by suction through a medium glass frit filter funnel packed with Celite and jacketed with an EtOH/ice solution; then the filtrate was evaporated in vacuo to give a yellow residue.

This residue was applied to PLC plates developed in hexane/*i*-PrOH/AcOH (90:10:0.5). The band fluorescing bright blue under 254-nm UV was scraped and eluted with CH_2Cl_2/MeOH (4:1). After solvent removal, the residue was applied to new PLC plates and developed first in CH_2Cl_2/AcOH (98:2) and next in CH_2Cl_2/MeOH/AcOH (95:5:0.5). The blue fluorescent band was isolated as before, and the residue was crystallized from 95% EtOH.

Due to its strong ionophoric character, **1** usually was isolated as its sodium salt. To obtain the free acid, the crystals were partitioned between Et_2O and 0.1 N HCl. The Et_2O layer

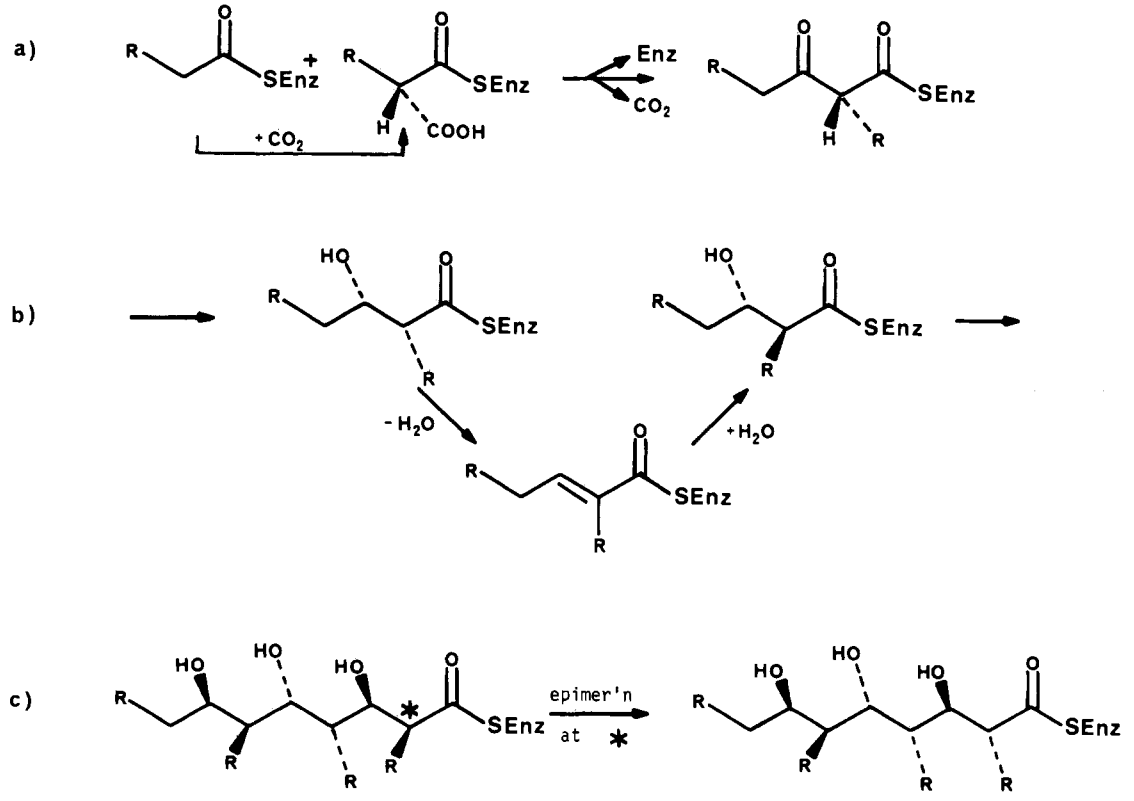


FIGURE 2: Working hypothesis for the stereochemical control of polyether antibiotic biosynthesis. All the intermediates are shown attached to an enzyme (Enz) as thio esters.

was washed twice with H_2O and then evaporated with a toluene/EtOH azeotrope, and the residue was recrystallized from 95% EtOH.

The original method was later modified as follows. Celite (5 g) and EtOAc (50 mL) were added to each flask immediately at the end of the fermentation. This mixture was then worked up as described above to obtain the residue from the EtOAc extract. This residue was dissolved in 2 times 2 mL of hexane/$CHCl_3$ (2:1), loaded onto a Sep-Pak cartridge (Waters Associates, silica gel), and eluted as fraction 1. The cartridge was then rinsed with 3 times 1 mL of hexane/*i*-PrOH (9:1) as fraction 2 and then with 2 times 1 mL of $CHCl_3$/MeOH (3:1) as fraction 3. Approximately 70% of **1** was in fraction 2 and the remainder in fraction 3. The solvent in fractions 2 and 3 was evaporated under a stream of N_2, and the residue from each fraction was chromatographed on a separate PLC plate. The plates were developed first in CH_2Cl_2/AcOH (98:2), air-dried, and then developed in CH_2Cl_2/MeOH/AcOH (95:5:0.5). The blue fluorescent band (R_f ca. 0.6) was eluted with CH_2Cl_2/MeOH (4:1). After solvent evaporation, this residue was dissolved in $CHCl_3$ and filtered through a Millex-SR, 0.5-μm disposable membrane (Millipore, Bedford, MA). The $CHCl_3$ was evaporated and the residue redissolved in MeOH/$CHCl_3$ (3:1) and injected onto a semiprep high-pressure liquid chromatography (HPLC) column (Waters, μBondapak C_{18}, 7.8 mm × 30 cm). The column was eluted with MeOH/1% aqueous AcOH (75:25) at a flow rate of 1.5 mL/min, and fractions were collected at 4-min intervals. A typical column elution profile was as follows: lasalocid A, 96–124 min; lasalocid homologues, 124–144 min; isolasalocid A, 144–164 min. With this mobile phase, **1** was isolated as the free acid.

NaOMe/MeO^{2}H Exchange of **3**. The ketone **3** (0.06 mmol) was dissolved in 1 N NaOMe/MeO^{2}H (1.0 mL) under N_2 and the solution left overnight at room temperature. The solution acquired a yellow/green fluorescence that disappeared upon quenching the reaction with glacial AcOH to pH 7. Evaporation of the solvents gave a white solid that was purified by PLC on silica gel in $CHCl_3$/MeOH (95:5) to give **3**. ^{1}H NMR analysis of this material (90 MHz, $CDCl_3$) showed the disappearance of the resonances at 2.8 and 2.6 ppm for the protons at C-12 and C-14. Low-resolution mass spectrum (MS) analysis gave 68% $^2H_3$, 21% $^2H_2$, 7% $^2H_1$, 3% $^2H_0$ for the ion at m/z 354.

[2-^{2}*H*,2-^{3}*H*]*Propionate*. 2-Methylmalonate (42 mmol) was heated for 8 h at 55–60 °C with 4 mL of ^2H_2O under N_2. The ^2H_2O was evaporated, leaving a white solid that was distilled by a Kugelrohr at 135–140 °C to effect decarboxylation. The resulting liquid was filtered, titrated with NaOH to pH 8–9, and then evaporated to give [2,2-$^2H_2$]propionic acid (22 mmol), as confirmed by the absence of the resonance at 2.36 ppm corresponding to $-CH_2-$ in its ^{1}H NMR spectrum.

Sodium [2-^{2}H,2-^{3}H]propionate was generated by reacting [2,2-$^2H_2$]propionic acid (22 mmol) with ^2H_2O (9 mL), ^3H_2O (100 mCi), and clean Na (2 mmol) in a Teflon-lined stainless steel bomb at 130–140 °C for 24 h. The bomb was cooled to room temperature before opening and the solution acidified with 18 N H_2SO_4 to pH 1 and then steam-distilled. The distillate was titrated to pH 8–9 with NaOH, the solvent removed in vacuo, and the residue dried with a toluene/MeOH azeotrope giving sodium [2-^{2}H,2-^{3}H]propionate [(7.31 ± 0.2) × 10^7 dpm/mmol].

[2-^{13}*C*,2,2-^{2}*H*$_2$]*Propionate*. Sodium [2-^{13}C]propionate (5 mmol, 91.8% ^{13}C, Prochem) was placed in a Teflon-lined stainless steel bomb with NaO^{2}H generated from 0.5 mmol of Na and 2 mL of ^2H_2O under N_2. The bomb then was heated at 130–140 °C for 24 h. After the bomb was cooled to room temperature, the ^2H_2O was evaporated in vacuo and the exchange process repeated 2 more times with freshly generated NaO^{2}H each time. After the final exchange, the solution was acidified to pH 1 with 18 N H_2SO_4 and steam-distilled under N_2. The distillate was titrated to pH 8–9 with 10 N NaOH against phenolphthalein and the solvent removed in vacuo (50 °C). MS analysis of the *p*-phenylphenacyl ester of [2-^{13}C,2,2-$^2H_2$]propionate gave the following: 0.12% $^2H_0$, 10.5% $^2H_1$, and 89.3% $^2H_2$ for the M^+ ion at m/z 269.

[3-^{13}*C*,2,2-^{2}*H*$_2$]*Propionate*. Sodium [3-^{13}C]propionate (1 g, 10.3 mmol, 91.1% ^{13}C, Merck) was subjected to the deuterium exchange procedure described above. The final product was recrystallized from 95% EtOH to give 0.95 g (93%). MS analysis of its *p*-phenylphenacyl ester gave 14.2% $^{13}C_1/^2H_1$ and 83.1% $^{13}C_1/^2H_2$ for the M^+ ion at m/z 268.

[2,2,3,3-^{2}*H*$_4$]*Succinate*. The deuterium exchange was carried out as described above for propionic acid, except that the reaction was repeated 5 times to ensure complete exchange. After steam distillation and solvent evaporation, the weight of the recovered succinic acid was 1.23 g, 10.1 mmol, 79% yield. The sample was analyzed by ^{1}H NMR (200 MHz), comparing the integrals of the $-CH_2-$ resonance at 2.6 ppm (^2H_2O) between unlabeled and the deuteriated succinic acid, to show that the latter contained 11% ^{1}H and 88% ^{2}H.

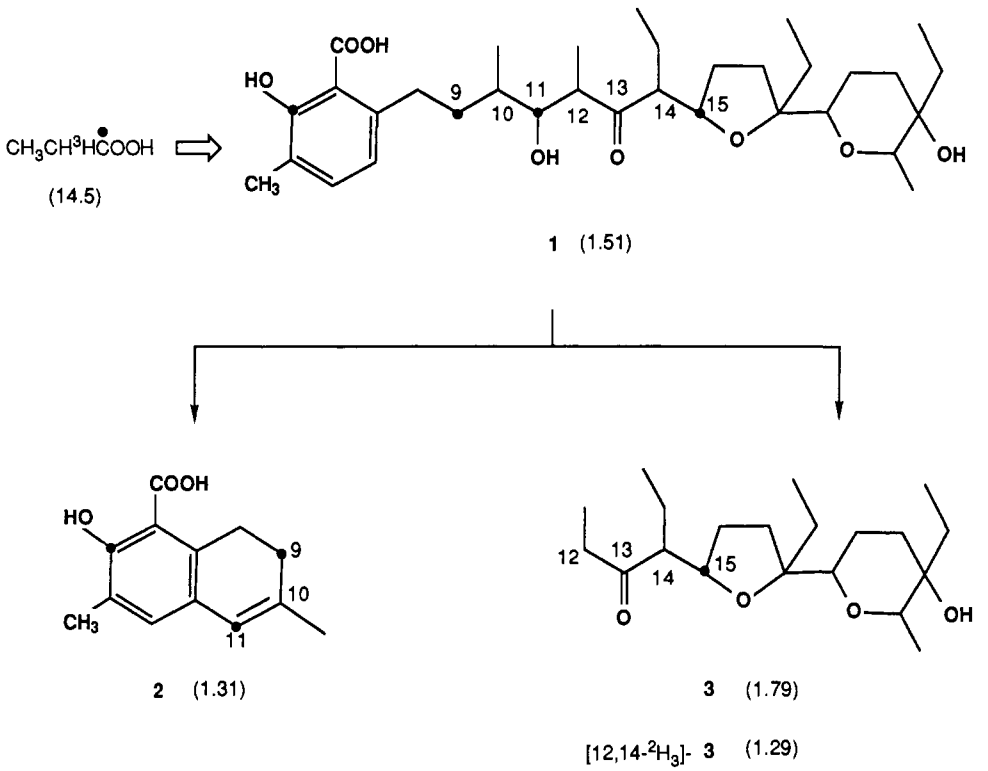
[2,3-^{13}*C*$_2$,2,2,3,3-^{2}*H*$_4$]*Succinate*. The deuterium exchange was carried out as described above for [2-^{2}H]propionate with sodium [2,3-$^{13}C_2$]succinate [2.25 mmol, prepared as reported by Cane et al. (1982)] and NaO^{2}H/^2H_2O and repeating the exchange reaction 5 times. After the final exchange, the solvent was evaporated, the pH adjusted to 1 with 18 N H_2SO_4, and the precipitated product recrystallized from EtOH. The final yield was 204 mg, 1.67 mmol, 74%. The isotopic enrichment was calculated from the low-resolution MS data of the succinic anhydride fragment: 1.8% $^{12}C_2/^2H_4$, 18.9% $^{13}C_2/^2H_2$, 12.3% $^{13}C_2/^2H_3$, and 58.8% $^{13}C_2/^2H_4$ for the ion at m/z 100. ^{13}C NMR analysis showed an intense peak at 28.05 ppm (Me_2SO), and ^{2}H NMR analysis showed a single resonance at 2.61 ppm (acetone).

[2,2-^{2}*H*$_2$]*Butyrate*. Sodium butyrate was reacted with NaO^{2}H/^2H_2O under the conditions for deuterium exchange described above for sodium [^{2}H]propionate. MS analysis of its *p*-phenylphenacyl derivative showed it to be 99.5% $^2H_2$.

General Conditions for Precursor Feedings. Unless otherwise noted, commercially available [^{14}C]propionate, [^{14}C]-succinate, or [^{14}C]butyrate was used as the reference isotopic label in admixture with the substrate with a stable isotope. The amount of ^{14}C-labeled material used was calculated to yield approximately 500 cpm/mg in the isolated **1**, and this amount was then mixed with unlabeled or stable isotope labeled precursor as an aqueous solution in a volumetric flask. Two aliquots of 0.1 mL were removed and diluted to 100 mL for radioactivity counting. The remainder of the feeding solution was filter-sterilized with a 10-mL syringe fitted with a Millipore disposable filter unit (Millex-GS, 0.22 μm), and the final volume was adjusted with additional H_2O rinses. The amount of precursor fed varied with each feeding experiment, but generally ranged from 1 to 10 mM. A 5 mM feeding was equivalent to adding 0.25 mmol of precursor per 50 mL of medium per day for example.

All feeding experiments with labeled precursors were carried out in the SP/LO medium. Seed cultures were started by innoculating 50 mL of media with ≥10^7 spores. After 72 h, fresh medium was inoculated with 2% v/v of the seed culture,

Scheme I[a]



[a] (●) Expected ^{14}C-labeling position. The numbers in parentheses are the ^{3}H:^{14}C ratios of radiochemically pure compounds.

and the cultures were usually grown for an additional 72 h before addition of the precursor. The precursor was added every 24 h for 4 days, and the cultures were harvested 24 h after the final feeding. Following the precedent of Chu and Bloomquist (1980), the [^{2}H]succinate was fed along with a 5 mM concentration of disodium malonate to suppress the conversion of succinate to fumarate by succinate dehydrogenase.

Radioactivity was measured by liquid scintillation counting in a Packard Model 3255 instrument, with either Aquasol or a toluene/EtOH cocktail [2,5-diphenyloxazole (PPO), 5 g; 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP), 0.3 g; EtOH, 300 mL; toluene, 700 mL]. Counting efficiencies were determined by using [^{3}H]hexadecane and [^{14}C]hexadecane internal standards.

Calculations for the incorporation of the labeled precursors into **1** were done as follows. Specific incorporation of radioactivity was expressed as dpm/mmol of product over dpm/mmol of precursor × 100. The incorporation of ^{13}C was measured by ^{13}C NMR spectroscopy and determined by the following ratio: ^{13}C enrichment factor = (peak height of ^{13}C-enriched compound)/(peak height of natural ^{13}C abundance compound). The data and results for the precursor feeding experiments are listed in Tables I and II.[1]

Results

Oxygen-18 Labeling Experiments. The oxygens attached to C-19, C-22, and C-23 of **1**, which were not labeled by [1-^{13}C,1-^{18}O]propionate and [1-^{13}C,1-^{18}O]butyrate (Hutchinson et al., 1981a), were suggested to come from O_2 (Westley et al. 1974b). We could not verify this in subsequent work because of the lack of antibiotic production upon fermentation of *S. lasaliensis* in closed systems.

[1] The experimental details for the work reported in Hutchinson et al. (1981a,b) are available in Sherman (1986).

Table I: Precursor Feeding Experiment Data

precursor[a]	amount fed (mM, μCi, $^{3}H/^{14}C$ ratio)	lasalocid A isolated: amount (mg)	lasalocid A isolated: incorporation (%)[b]	$^{3}H/^{14}C$ ratio
[1-^{14}C,2,2-$^{2}H_2$,2-^{3}H]propionate	5, 11, 14.5	82	61 (^{14}C)	1.5
[2-^{13}C,1-^{14}C,2,2-$^{2}H_2$]propionate	5	41	*c*	*c*
[3-^{13}C,2,2-$^{2}H_2$]-propionate	5	64	*c*	*c*
[2,3-$^{13}C_2$,2,3-$^{14}C_2$,2,2,3,3-$^{2}H_4$]-succinate[d]	2.8	~30	52	*c*
[2,2,3,3-$^{2}H_4$]-succinate[d]	5	66	*c*	*c*
[2,2-$^{2}H_2$,1-^{14}C]-butyrate	5	14	*c*	*c*
[2,2-$^{2}H_2$,1-^{14}C]-butyrate	10	10	*c*	*c*

[a] All acids were fed as the sodium salt. [b] Specific incorporation. [c] Not determined. [d] Plus 5 mmol of sodium malonate.

^{3}H Labeling Experiments. In preliminary experiments with [1-^{14}C,2-^{3}H]propionate, the labeled **1** contained only 10.3% of the ^{3}H content of the precursor rather than 25%, the amount expected from retention of two the eight C-2 hydrogens of four propionates. It was degraded according to Westley et al. (1973a) to compounds **2** and **3** (Scheme I). The data shown in this scheme indicate that **2** had 71% of the ^{14}C and 61% of the ^{3}H content and **3** had 30% of the ^{14}C and 36% of the ^{3}H content of **1**. Dehydration of the tertiary carbinol precursor of **2** did not result in significant loss of ^{3}H. Exchange of the C-12 and C-14 positions of **3** with deuterium resulted in the loss of 13% of its ^{3}H label. Thus, ^{3}H was located at C-12 but not at C-10 of **1**. The location of the remaining ^{3}H label could not be easily determined because of the limitations of chemical degradations; therefore, all further work was carried out with the stable isotope, ^{2}H.

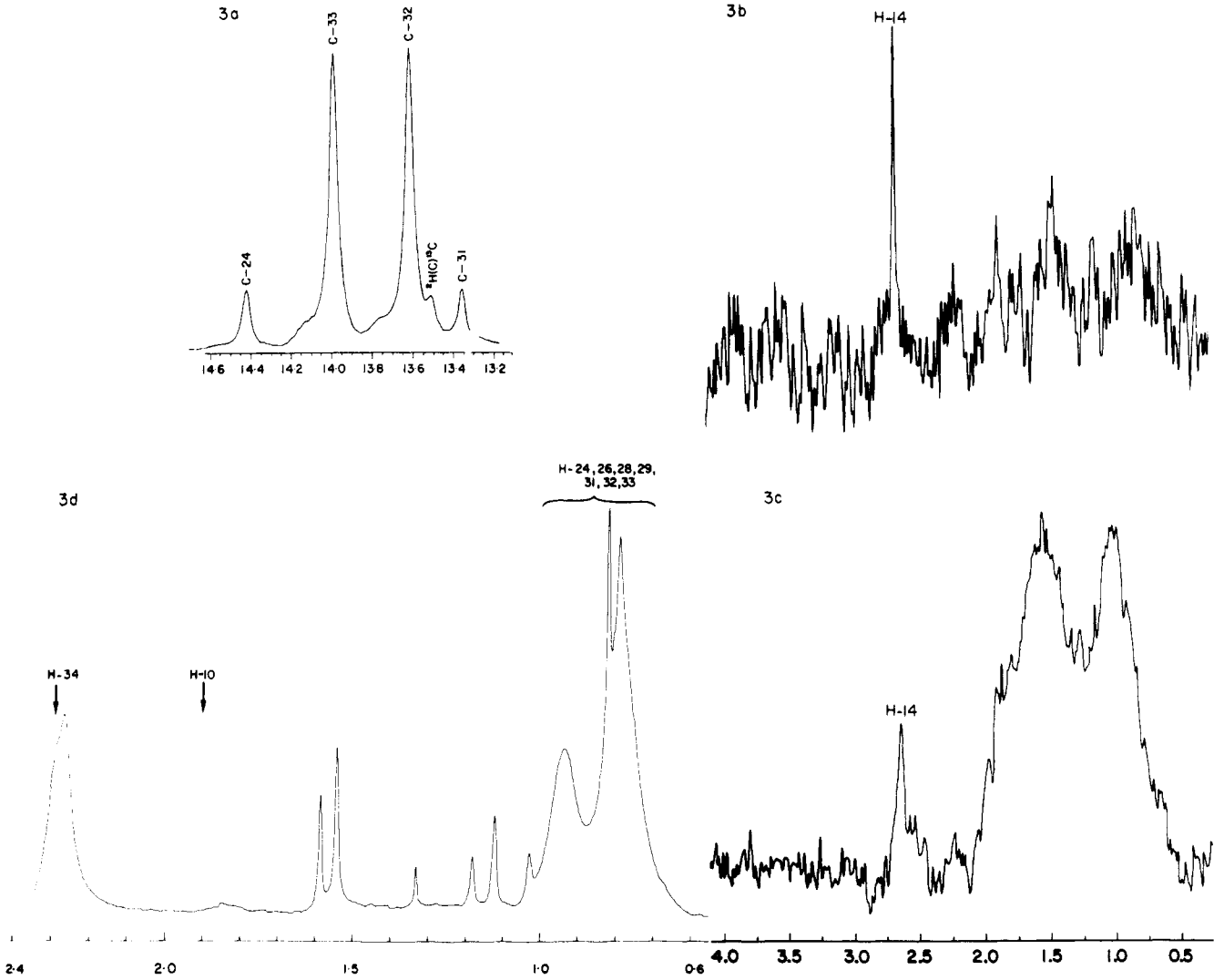


FIGURE 3: Portions of the NMR spectra of labeled lasalocid A. (a) The 13.2–14.6 ppm region of the 50-MHz $^{13}C\{^1H\}$ spectrum of **1** labeled by [3-^{13}C,2,2-$^2H_2$]propionate. (b) The 0.5–4.0 ppm region of the 30.7-MHz ^2H spectrum of **1** labeled by [2,2-$^2H_2$]butyrate fed at a 5 mM level. (c) The same region from feeding [2,2-$^2H_2$]butyrate at a 10 mM level. (d) The 0.6–2.4 ppm region of the 92.1-MHz ^2H spectrum of **1** labeled by [2,2,3,3-$^2H_4$]succinate. No signals downfield of 2.5 ppm were observed in this spectrum, and the sharper resonances between ca. 1.0 and 1.6 ppm may be spurious.

^2H Labeling Experiments. [2-^{13}C,2,2-$^2H_2$]Propionate labeled C-12 of **1** with ^{13}C and ^2H, but with a nearly 90% loss of the ^2H relative to the incorporation of ^{13}C (Hutchinson et al., 1981b). It was stated that C-10 was not also ^2H labeled, but this was an insecure result since the signals for C-8, C-10, and C-12 were not clearly resolved in the 25.1-MHz $^{13}C\{^1H,^2H\}$ NMR spectrum of **1**. This conclusion was confirmed by two new experiments. Repetition of the earlier feeding experiment with [2-^{13}C,2,2-$^2H_2$]propionate established that C-12 of **1** carried ^2H and ^{13}C with 16% retention of the ^2H relative to the ^{13}C label. High-resolution mass spectral analysis of the labeled **1** showed the presence of an isotopically labeled species containing one ^2H and one ^{13}C for the m/z 57 ion, which contains carbons 12, 13, and 32 of **1** (Westley et al., 1974c). Analysis of the 50-MHz $^{13}C\{^1H\}$ NMR spectrum of **1** labeled by [3-^{13}C,2,2-$^2H_2$]propionate showed that C-29, C-32, C-33, and C-34 were ^{13}C-enriched about 18-fold over natural abundance (Table II). The signal for C-32 at 13.607 ppm contained a small upfield resonance at 13.509 ppm (Figure 3a), representing a β ^2H isotope shifted ^2H-(C)-^{13}C species (Colli et al., 1973; Simpson & Stenzel, 1982) whose intensity corresponded to a 9.2% retention of ^2H at C-12 of **1**. Since the signal for C-33 at 13.982 ppm (Figure 3a) was well resolved from the C-32 resonance and clearly did not exhibit a β-shifted resonance, C-10 of **1** definitely was labeled with ^{13}C but not ^2H.

A plausible explanation for the selective labeling of C-12 by [2-^{13}C,2,2-$^2H_2$]- or [3-^{13}C,2,2-$^2H_2$]propionate would be for (2*S*)-2-methylmalonate and (2*R*)-2-methylmalonate to be the immediate precursors of the two three-carbon units comprising respectively C-32, C-12, and C-11 and C-33, C-10, and C-9 of **1**. The 2*S* isomer of 2-methylmalonate should be formed by the carboxylation of propionyl-CoA and the 2*R* isomer by the rearrangement of succinyl-CoA catalyzed by methylmalonyl-CoA mutase (Kaziro & Ochoa, 1964). If our hypothesis were correct, then 2,3-deuteriated succinate should label C-10 of **1**, but not C-12, provided that the racemization of (2*S*)- and (2*R*)-2-methylmalonate by epimerization (Mazumder et al., 1962) does not involve internal return of a C-2 ^2H label. This expectation has to be tempered by the fact that some loss of ^2H occurs during the rearrangement of [^2H]-succinyl-CoA to (2*R*)-2-methylmalonyl-CoA in vitro (Retey, 1982) and that 2-methylmalonyl-CoA undergoes rapid proton exchange at physiological pH in vitro (Overath et al., 1962; Mazumder et al., 1962).

Attempts to show that three different forms of deuteriated succinate labeled C-10 selectively met with qualified failure, however. Use of [2,3-$^{13}C_2$,2,2,3,3-$^2H_4$]succinate resulted in

Table II: Carbon-13 Enrichments[a] of Lasalocid A (1)

carbon	[2-^{13}C, 2,2-$^{2}H_2$]-propionate[b]	[3-^{13}C, 2,2-$^{2}H_2$]-propionate	carbon	[2-^{13}C, 2,2-$^{2}H_2$]-propionate[b]	[3-^{13}C, 2,2-$^{2}H_2$]-propionate
1	1.3	3.6	18	c	2.1
2	1.0	4.8	19	1.2	2.9
3	c	1.9	20	1.5	3.2
4	10.8	1.6	21	0.7	1.2
5	c	2.1	22	c	
6	1.0	1.3	23	0.8	2.2
7	c	2.8	24	1.7	3.4
8	c	3.3	25	0.7	0.9
9	c	1.7	26	0.7	2.6
10	12.3	1.8	27	0.6	0.9
11	c	1.6	28	0.6	3.5
12	10.6	1.8	29	0.8	20.5
13	c	1.0	30	c	1.7
14	c	2.5	31	0.8	c
15	c	1.8	32	1.2	16.9
16	12.3	3.3	33	1.3	17.8
17	c	1.3	34	0.8	16.7

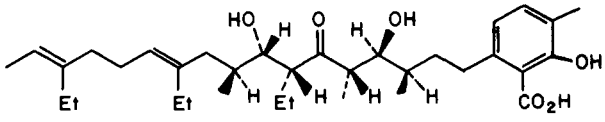
[a] Calculated as described under Experimental Procedures. [b] Calculated as described in Hutchinson et al. (1981b). [c] Negligable ^{13}C enrichment was observed.

a complex ^{13}C {^{1}H,^{2}H} NMR spectrum due to the one-bond ^{13}C–^{13}C couplings. Spectral simplification by subtraction of the ^{13}C {^{1}H} NMR spectrum from the ^{13}C {^{1}H,^{2}H} NMR spectrum (Cane et al., 1986) did not reveal the presence of an upfield multiplet due to a ^{13}C–^{2}H species for the C-10 resonance although the signal to noise ratio was good. Diethyl [2-^{13}C,2,2-$^{2}H_2$]succinate, prepared as described by Cane et al. (1986) and anticipated to give a less complex ^{13}C NMR spectrum, surprisingly was not incorporated into **1**. Consequently, [2,2,3,3-$^{2}H_4$]succinate was used as the precursor, and the ^{2}H-labeled **1** was analyzed by ^{2}H NMR spectroscopy at 92.1 MHz. Although several positions clearly were deuterium labeled, due to the catabolism of the precursor to [^{2}H]-propionate and the general ^{2}H labeling of intermediary metabolites by exchange of label from the precursor via enolization, no signal was detected for ^{2}H at C-10 or any other positions downfield of 2.5 ppm (Figure 3d). Calculations based on the observed signal to noise ratio and deuterium enrichment of **1** determined by mass spectrometry indicated that a signal could have been detected if only 11% of the molecules had been ^{2}H labeled at C-10.

[2,2-$^{2}H_2$]Butyrate labeled **1** at C-14 (Figure 3b), the only position corresponding to the 2-position of butyrate and still retaining a hydrogen atom. This precursor also labeled other methine and methylene positions of **1** to an increasing extent as the concentration of [2,2-$^{2}H_2$]butyrate in the fermentation was raised from 5 mM (Figure 3b) to 10 mM (Figure 3c). This most likely resulted from ^{2}H exchange and catabolism of the precursor to [2-^{2}H]acetate (White, 1980) or its conversion to [3-^{2}H]propionate (Sherman et al., 1986).

Discussion

The results of the labeling experiments described earlier (Hutchinson et al., 1981a,b) and above provide some insight about the stereochemical control of the biosynthesis of **4**, the



4

hypothetical 34-carbon precursor of lasalocid A (Westley et al., 1974b).

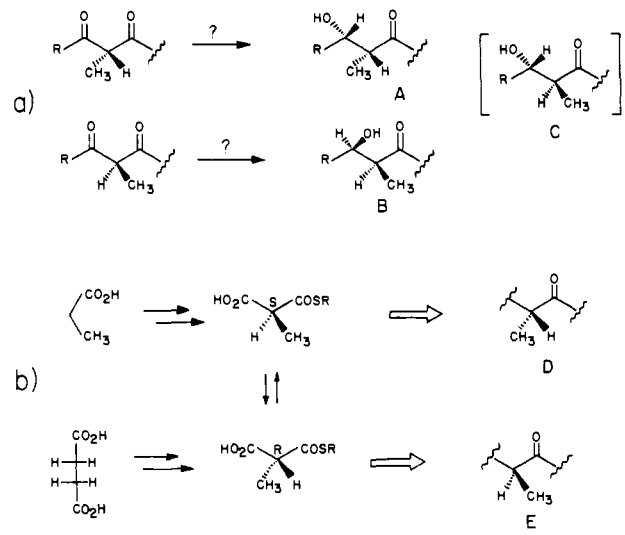


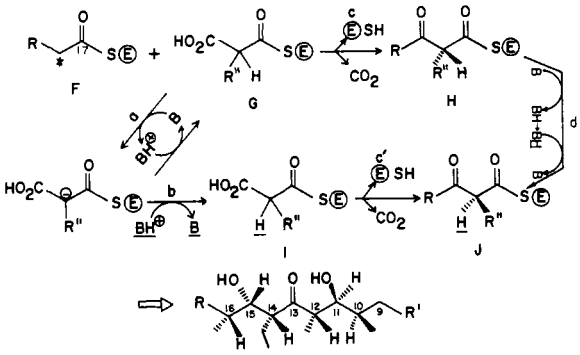
FIGURE 4: Hypothetical relationships between the absolute configuration of precursors and structural subsets of polyether biosynthetic intermediates.

Since the ^{18}O labeling experiments showed that the oxygens at C-1, C-3, C-11, and C-15 of **1** came directly from the carboxyl oxygens of the precursors (Hutchinson et al., 1981a), the configuration of position 11 or 15 of **4** is not established by a dehydration–rehydration mechanism (Figure 2b). This conclusion would be invalid, however, if the oxygen removed by dehydration were added back to the same molecule in the rehydration step. On the basis of the monensin precedent (Cane et al., 1982), the oxygens at C-19/C-23 and C-22 of **1** presumably come from molecular oxygen; therefore, **4** is not assembled by oxidation of some fully reduced intermediate but is subsequently oxidized to a diepoxide that in turn is cyclized to **1**, the concept originally suggested by Westley et al. (1974b) and further extended by Cane et al. (1983a).

The stereochemistry of positions 11 and 15 of **1** and **4**, which have the opposite configuration, could result from reduction of carbonyl groups present in the 2-position of the most recently added C_2–C_4 unit during assembly of the carbon framework. This could be achieved by two stereospecific oxidoreductases or by the same enzyme if the carbonyl were to bind in two heterotopic forms, perhaps as dictated by some conformational influence of the portion of **4** that already had been assembled. When this idea is viewed as shown in Figure 4a, it appears that the stereochemistry of the α-carbon of the hypothetical acyl thio ester intermediate might be directing the stereoselectivity of carbonyl reduction since the substituents at the α and β positions of A and B have a cis relationship. While this could be an important principle of stereochemical control in this case, it cannot be general since there are portions of avermectin (Cane et al., 1983b) and tylactone (O'Hagan et al., 1983) whose hydroxyl oxygens are believed to originate in the same way but have a trans relationship with the substituents on the adjacent position, as in C of Figure 4a.

Confirmation that ^{2}H or ^{3}H at C-2 of propionate labels C-12 of **1**, and the newer finding that ^{2}H at C-2 of butyrate labels C-14, supports the original suggestion that the stereochemistry of these two centers is determined by the stereoselectivity of the reaction between an acyl thio ester and (2*S*)-2-methylmalonate or its 2-ethyl analogue (Hutchinson et al., 1981b). This reaction must take place with inversion of the C-2 configuration of the substituted malonates (cf. fatty acid biosynthesis), as illustrated in Figure 4b for the methylmalonate case, if the carboxylation of propionate and butyrate gives their 2*S* analogues. The data of Sood et al. (1984) are consistent

Scheme II



with propionate being carboxylated to (2*S*)-2-methylmalonate in the biosynthesis of monensin A. Furthermore, an acyl-CoA carboxylase from *Streptomyces erythreus* that acts on propionyl-CoA and butyryl-CoA also has this same stereoselectivity (Huniati & Kolattukudy, 1982).

Incorporation of (2*R*)-2-methylmalonate into carbons 9, 10, and 33 was initially favored as the way in which the C-10 configuration (E in Figure 4b) of **1** and **4** was created (Hutchinson et al.., 1981b). Yet we recognized that this could not be distinguished from the alternative possibility that (2*S*)-2-methylmalonate gives rise to both configurations D and E (Figure 4b) with the data available at that time. Sood et al. (1984) have proposed the latter explanation for the biosynthesis of monensin A: that chain-extending condensation with (2*S*)-2-methylmalonate is followed by epimerization to create the asymmetric centers having a configuration opposite to those centers retaining the C-2 ^2H label of propionate. Again, this was done without conclusive data. The present results from the labeling experiment with [2,2,3,3-$^2H_4$]-succinate, if a negative result is valid evidence, indicate that the latter hypothesis apparently is correct.

The underlying biochemistry is more complex than either of these ideas suggest. Scheme II shows how the C-10, C-12, and C-14 stereochemistry of carbons 9–16 of **4** could be set during its biosynthesis. If F represents the portion that has been assembled from the C_2 starter unit through carbon 17, then there are at least four possible mechanisms for extension of F by a three- or four-carbon unit. (i) G, coming from (2*S*)-2-methylmalonate, could condense with F by path c giving H (R″ = CH_3) with configurational inversion. (ii) G, again from (2*S*)-2-methylmalonate, could be epimerized to I by paths a and b but without internal return of the proton removed in step a. Then I would condense with F by path c′ giving J, again with configurational inversion. (iii) I could come directly from (2*R*)-2-methylmalonate and form J as before. There is the possibility that step c sometimes could take place with retention rather than inversion of the C-2 configuration in G to produce J, but this possibility is discounted on the basis of the existing precedents [Sood et al. (1984) and references cited therein]. (iv) H could be epimerized to J without internal return of the proton removed in step d.

The configuration drawn for C-12, C-14, and C-16 of **4** could result by path c and that for C-10 by paths a, b and c′ or paths c and d in Scheme II. [The configuration of C-16 is drawn like C-12 for consistency in the argument; it has the opposite stereochemistry in **1**, which was proposed to be the outcome of an enoyl thio ester intermediate: Hutchinson et al. (1981).] [2-^2H]Propionate or -butyrate can label **4** by path c and probably lose some of their ^2H label by path a. Operation of paths a, b, or d would result in loss of this label, but if path c′ used I formed from [^2H]succinate, then J should be ^2H labeled unless the ^2H were lost during the condensation reaction. Since loss of hydrogen isotope does not take place during the analogous condensation in fatty acid biosynthesis (Arnstadt et al., 1975), the results of the labeling experiments described here and elsewhere (Hutchinson et al., 1981a,b; Sood et al., 1984) lead to the proposal that mechanism iii is excluded for the biosynthesis of **1**, that mechanism i is operating, and that mechanism ii or iv may be operating to establish the C-10 stereochemistry. This matter will only be clarified when the mechanistic questions can be examined with purified enzymes.

It was surprising to find that [2-^{13}C,2,2,2-$^2H_3$]acetate did not label C-24 of **1** with ^2H since carbons 23 and 24 are believed to represent the starter unit for assembly of the carbon framework. [^2H is known to be retained at the starter unit methyl group in the biosynthesis of several other microbial metabolites (Hutchinson, 1983).] The same outcome has been reported in biosynthetic studies of two other polyether antibiotics (Doddrell et al., 1984; H. Seto, personal communication). One explanation for these observations is formation of the starter unit by decarboxylation of malonate, which, being more acidic than acetate, could more easily lose its ^2H label. Malonyl-CoA decarboxylase activity has been observed with enzymes that catalyze the formation of unusual branched-chain fatty acids from acetyl-CoA and 2-methylmalonyl-CoA (Kim & Kolattukudy, 1978; Rainwater & Kolattukudy, 1985) or odd-numbered fatty acids from acetyl-CoA, malonyl-CoA, and propionyl-CoA (Arai et al., 1982), and in the microorganism that makes the macrolide antibiotic erythromycin A (Hunaiti & Kolattukudy, 1984). In three of these cases this enzyme activity is believed to remove malonate so that only 2-methylmalonate is available for chain extension. If this biochemical strategy were part of the "polyether synthase" system, it would be a way for the bacteria to distinguish the starter units of fatty acid and polyether biosynthesis, thereby posttranslationally regulating the operation of these two pathways.

The information discussed above partly validates our working hypothesis, which predicts a close similarity between the biosynthesis of **1** and fatty acids. The mechanism of stereochemical control in lasalocid A biosynthesis should hold in principal for all polyether antibiotics, but due to the limitations of in vivo experimentation, several mechanistic questions remain unanswered. The answers to these and other questions will have to come from studies of more complex biosynthetic intermediates and the enzymology and genetics of the polyether antibiotic formation.

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