BIOSYNTHESIS OF LASALOCID. I

INCORPORATION OF ^{13}C AND ^{14}C LABELLED SUBSTRATES INTO LASALOCID A

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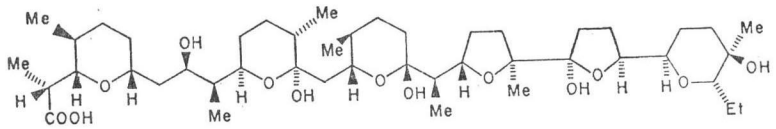
The biosynthesis of lasalocid A (formerly antibiotic X-537 A) in cultures of *Streptomyces lasaliensis* has been studied using ^{13}C- and ^{14}C-labelled precursors. The antibiotic is derived from five acetate, four propionate and three butyrate units. This study has provided the first illustration of an ethyl group arising from butyrate and 2-ethylmalonate.

The isolation of three crystalline antibiotics, X-206, X-464 and X-537 A from three different *Streptomycete* species was reported from this laboratory[1] in 1951. The structure and absolute configuration of antibiotic X-206 (**1**) was established[2] from an X-ray crystallographic analysis of its silver salt. Antibiotic X-464 (**2**) was shown to be identical[3] to nigericin[4] and polyetherin A[5]. The structure of the third antibiotic, lasalocid A, formerly called X-537 A (**3**), was determined by X-ray crystallography of the barium salt[6] and the absolute configuration was established by spectropolarimetry of a degradation product[7] containing a single asymmetric center.

Lasalocid A, produced by *Streptomyces lasaliensis*, is unique in containing three C-ethyl groups and is also the only polyether antibiotic[8] so far reported to possess an aromatic chromophore (Fig. 1). For these reasons, a study of the biosynthesis of lasalocid A was undertaken.

An early proof of the polyacetate rule[9] resulted from a study of the assembly of the carbon skeleton of 6-methylsalicylic acid (6-MSA) from four acetate units and the biosynthesis of 6-MSA is now thought to be accomplished on a multienzyme complex[10]. The similarity of the chromophores of **3** and 6-MSA suggested that the antibiotic is probably also assembled by a malonyl-coenzyme A mechanism

Fig. 1.



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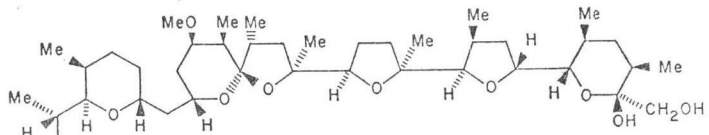

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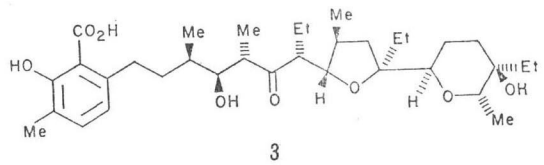

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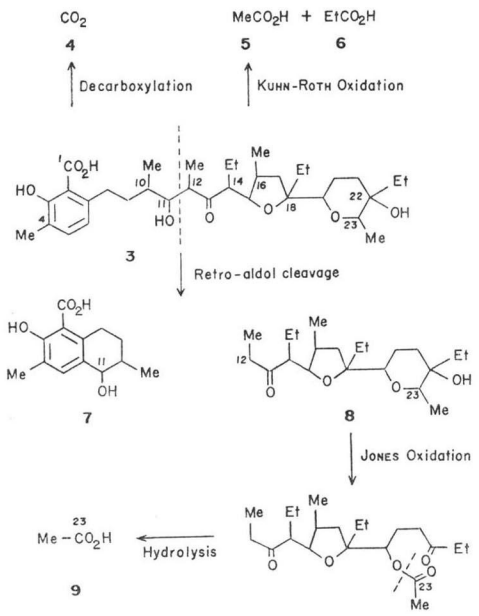
involving the initial formation of a polyketide chain on a multi-enzyme complex. The five C-methyls could arise through transmethylation[11] of the polyketide precursor or by the insertion of propionic acid units[12,13] into the polyketide skeleton. Likewise, the C-ethyl groups could be formed from successive C-methylation steps[14], by transethylation[15] or by insertion of butyrate units.

^{14}C Incorporation Experiments

Scheme 1. Chemical degradations carried out on ^{14}C-labelled lasalocid A.



To determine which of the possible pathways was being utilized in the biosynthesis of lasalocid A, a number of ^{14}C-labelled compounds were tested as substrates for the antibiotic. Preliminary results achieved with seven ^{14}C-labelled substrates were reported earlier[16] and since then a further seven have been tested as possible precursors of **3**. Of these 14 compounds, 8 were incorporated into the antibiotic (Table 1). Negative results with [^{14}C] formate, [Me-^{14}C] methionine, [Me-^{14}C] ethionine, and [2-^{14}C] mevalonate ruled out transmethylation, transethylation, or the introduction of terpenoid units as possible mechanisms leading to the branched alkyl groups in lasalocid A. The other two ^{14}C-labelled acids *not* incorporated into **3** were salicylic and shikimic acid.

The effective utilization of sodium [1-^{14}C] acetate, sodium [1-, 2-, or 3-^{14}C] propionate, and sodium [1-, 2-, or 3, 4-^{14}C] butyrate showed that the antibiotic

Table 1. Incorporation of ^{14}C-labelled substrates into lasalocid A and percentage relative molar activity[13] of fragments* from subsequent degradation reactions.

Substrate for **3** (sodium salts)	Percentage incorporation	Percentage relative molar activity of					
		4	**5**	**6**	**7**	**8**	**9**
[1-^{14}C] acetate	0.1~2	8.9	3.4	4.1	45.7	56.4	10.5
[1-^{14}C] propionate	1~4	0	0	0.8	75.3	25.1	
[2-^{14}C] propionate	0.2		11.2	0.7	47.1	50.7	
[3-^{14}C] propionate	3.7		10.5	1.0	45.3	50.8	
[1-^{14}C] butyrate	2~10	0	0.1	0.2	2.6	97.4	
[2-^{14}C] butyrate	1.6		0.3	26.6	9.5	91.2	
[3,4-^{14}C] butyrate	8.5		9.3	27.3	3.5	96.5	
[2-^{14}C] malonate	0.4				48.0	52.0	
[Me-^{14}C] 2-methylmalonate	0.5				50.4	49.5	
[2-^{14}C] 2-ethylmalonate	1.8				1.6	98.4	
[1-^{14}C] 3-hydroxybutyrate	11.1				12.6	87.4	
[1-^{14}C] stearate	0.8				40.7	57.9	

* For numbering system of compounds listed here, see Scheme 1.

was derived from these three units and incorporation of [2-^{14}C] malonate, [Me-^{14}C]-methylmalonate and [2-^{14}C] 2-ethylmalonate suggested conversion of the three monocarboxylic acids to their malonate analogs prior to incorporation into **3**. This mechanism is well established in the case of acetate[17] and propionate[12], but the involvement of butyrate and 2-ethylmalonate in the formation of C-ethyl groups has no precedent in the literature.

An alternative way in which the butyrate label could have been incorporated into the antibiotic was by β-oxidation followed by hydrolysis to *acetate* prior to incorporation into **3**. This possibility was ruled out by the degradation experiments summarized in Scheme 1 and the results in Table 1.

Chemical Degradation of ^{14}C Labelled Lasalocid A (Scheme 1)

Pyrolytic decarboxylation of lasalocid A at 220° gave radioactive CO_2 (**4**) only in the case of [1-^{14}C] acetate-derived antibiotic, showing that C-1 in **3** is derived from an acetate carboxyl. Retro-aldol cleavage of **3** followed by oxidation of **8** and hydrolysis gave acetic acid (**9**) from the C-methyl group at C-23 in **3**. This C-methyl was shown to originate as an acetate unit by obtaining radioactive **9** from [1-^{14}C] acetate-derived **3**.

KUHN-ROTH oxidation of **3** produced acetic acid (**5**) from the eight C-methyls and propionic acid (**6**) from the three C-ethyls in the molecule. These acids were separated by silica gel chromatography as their *p*-bromophenacyl esters. If all the ^{14}C atoms in a labelled **3** preparation are situated in the C-methyl groups, the theoretical relative molar activity of **5** would be 12.5 %. Similarly, if all the ^{14}C atoms are in C-ethyl groups, the theoretical activity of **6** would be 33.3 %. When this reaction was carried out on [2- or 3-^{14}C] propionate-derived **3**, the resulting acetic acid had activity of 11.2 % and 10.5 %, respectively, whereas [1-^{14}C]-propionate-derived **3** gave inactive acetic acid. The propionic acid (**6**) produced in the oxidation was virtually inactive in all three cases. These results show clearly that the propionate units incorporated into **3** are only at C-methyl positions in the antibiotic. Retro-aldol cleavage of the three [^{14}C] propionate-derived samples of **3** demonstrated that there are four propionate units in the molecule and that one is situated at the C-11, C-12 bond, the site of this cleavage. This results in a **7** : **8** ratio of 1 : 1 (or 2 : 2) in the [2-and 3-^{14}C] propionate experiments, but 3 : 1 in the [1-^{14}C] propionate case. The four propionate-derived C-methyls must be at C-4, C-10, C-12, and C-16 as the C-23 methyl is acetate-derived.

KUHN-ROTH oxidation of the three [^{14}C] butyrate-derived samples clearly established that the butyrate units incorporated into **3** are only at the C-ethyl positions. Thus, [1-^{14}C] butyrate-derived **3** gave virtually inactive acetic and propionic acid, [2-^{14}C] butyrate-derived **3** gave inactive acetic, but active propionic acid (26.6 %, theoretical 33.3 %) and [3, 4-^{14}C] butyrate-derived **3** gave active acetic (9.3 %, theoretical 12.5 %) and active propionic acid (27.3 %). The results from retro-aldol cleavage showed that all butyrate incorporation was taking place in the ketone (**8**) part of the molecule, but could not be used to determine whether one, two, or all three of the C-ethyls were butyrate-derived. This question was subsequently answered by ^{13}C incorporation experiments described in the next section,

The suggestion that the three monocarboxylic acids were converted to their malonate analogs prior to incorporation into the antibiotic was checked by comparing relative molar activities of

the retro-aldol fragments **7** and **8** from respectively:

[1-^{14}C] acetate and [2-^{14}C] malonate derived **3**;

[2-^{14}C] propionate and [Me-^{14}C] 2-methylmalonate derived **3**;

[1-^{14}C] butyrate and [2-^{14}C] 2-ethylmalonate derived **3**.

In all three cases, the results were in good agreement with the proposed mechanism (see Table 1).

The results using [1-^{14}C] 3-hydroxybutyrate as a precursor were consistent with the known transformation[18] of malonyl CoA to butyrate, which proceeds *via* D(−)-3-hydroxybutyric acid. This particular reaction sequence is utilized by a variety of biological systems for *de novo* fatty acid synthesis and accounts for the randomization of ^{14}C label between acetate and butyrate observed in some of the results in Table 1.

Incorporation of ^{14}C from [1-^{14}C] stearate revealed that long chain fatty acids can serve as a source of acetate and butyrate in the biosynthesis of lasalocid A by *Streptomyces lasaliensis*.

^{13}C Incorporation Experiments

The use of sodium [1-^{13}C] butyrate at 0.5 g/liter in X-537 fermentations, followed by ^{13}C nmr

Table 2. Incorporation of [1-^{13}C] acetate, [1-^{13}C] propionate and [1-^{13}C] butyrate into lasalocid A (**3**) as determined by ^{13}C nmr[a]

^{13}C Shift in p.p.m.[b] in CH_2Cl_2	Carbon No. in **3**	Functional group	% Abundance[c] of ^{13}C in **3** produced from		
			$CH_3{}^{13}CO_2Na$	$CH_3CH_2{}^{13}CO_2Na$	$CH_2CH_2CH_2{}^{13}CO_2Na$
219.9	13	C=O	1	1	*4*
176.4	1	CO_2H	*2*	1	1
161.3	3	=C-OH	1	*4.5*	1
143.5	7	=C-CH_2	*2.5*	1	1
131.5	5	=CH	*3*	1	1.5
123.0	4	=C-CH_3	1	1	1
119.6	6	=CH	1	1	1
118.2	2	=C-CO_2H	1	1	1
87.6	18	C(Et)-O	1	1	1
83.1	15	CH-O	1.5	*4*	1.5
77.2	23	CH(Me)-O	*3*	1	1.5
71.5	22	C(Et)-OH	1	1	1
71.0	11	CH-OH	1.5	*4*	1.5
68.6	19	CH-O	*3*	1	1
55.9	14	CH	1	1	1
49.0	8	CH_2	1	1	1
38.1	9	CH_2	1	*4*	1
37.9	17	CH_2	1	1	*5*
29.2	21	CH_2	1	1	*4*

(a) Concentration of natural abundance and ^{13}C-enriched lasalocid A was 0.76 M. The ^{1}H decoupled ^{13}C nmr FOURIER transform spectra were recorded in 10 mm spinning sample tubes on a Bruker HFX-90/6 spectrometer at 22·63 MHz, using an internal ^{19}F lock of C_6F_6 at 84·66 MHz. A Fabritek FT-1083 Computer was used for accumulation of free induction decays and FOURIER transformation.

(b) Downfield from internal Me_4Si.

(c) Corrected to nearest 0.5 %.

Fig. 2. ^{13}C nmr spectra of 0.76 M lasalocid A in CH_2Cl_2 (A) natural abundance, and (B) [1-^{13}C] butyrate-derived.

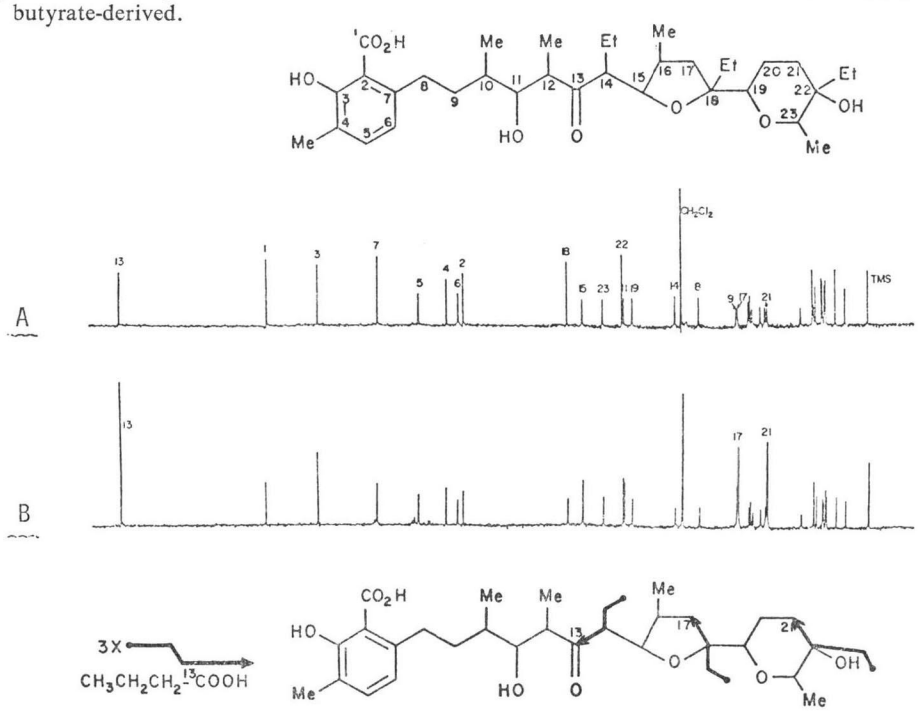


Fig. 3. ^{13}C nmr spectra of 0.76 M lasalocid A derived (A) from [1-^{13}C] propionate, and (B) from [1-^{13}C] acetate.

spectroscopy of the isolated antibiotic clearly demonstrated[19] that *all three* ethyl groups in **3** are derived from butyric acid (Fig. 2).

The proton-decoupled natural abundance ^{13}C spectrum of the antibiotic in chloroform exhibited thirty three singlets of which twenty were assigned. Methylene chloride has now been found preferable to chloroform as the interference between the signal due to C-23 and the solvent was removed and all thirty-four singlets were resolved accounting for all the carbon atoms in **3** (Table 2). Enrichment was calculated from the ratio of peak areas in ^{13}C enriched and the natural abundance spectra. The peaks not included in Table 2 were at 6.7, 9.5, 12.4, 12.6, 13.3, 13.6, 15.3, 16.0 16.2, 19.5, 29.8, 31.1, 33.4, 34.1 and 34.5 p.p.m.

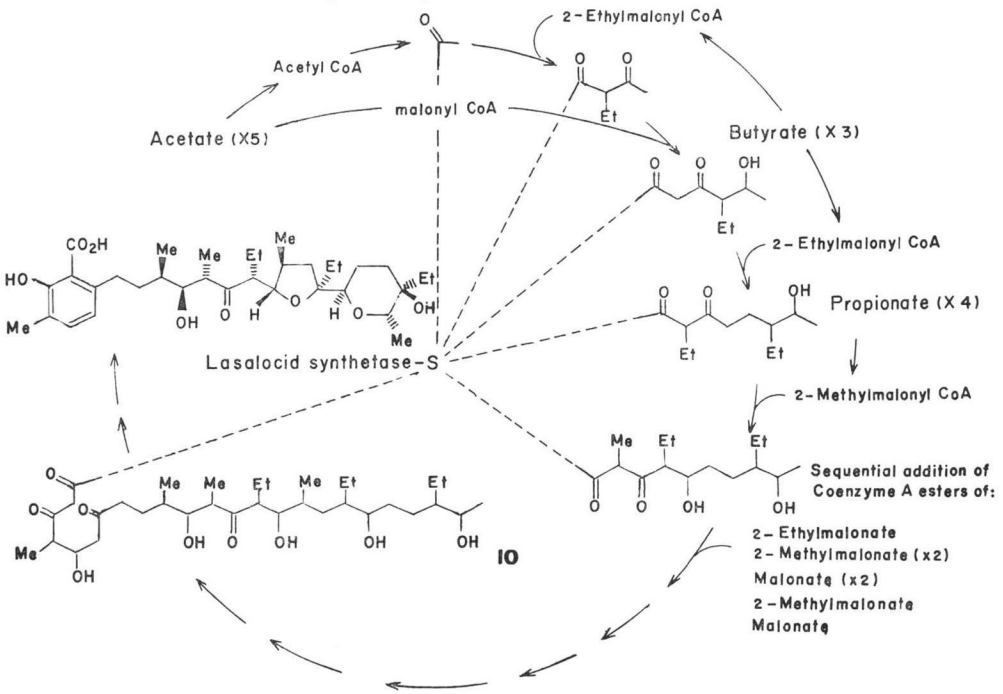
The enrichments considered most significant were those which resulted in at least a doubling of the natural abundance signal as determined by ^{13}C nmr. In the case of [1-^{13}C] butyrate-derived **3**, three carbons were enriched consistent with the C-ethyls at C-14, 18 and 22 being butyrate-derived. The results with [1-^{13}C] propionate-derived **3** were consistent with the conclusions from the earlier ^{14}C experiments that the four C-methyl groups at C-4, 10, 12 and 16 were propionate-derived (Fig. 3).

Sodium [1-^{13}C] acetate at 0.5 g/liter in X-537 fermentations gave antibiotic in which five carbons were enriched (Fig. 3). The enrichments at C-1 and C-23 confirmed our earlier results with ^{14}C acetate-derived **3** and the other three enrichments at C-5, 7 and 19 accounted for the biosynthetic origin of the remaining six carbon atoms in the molecule.

Conclusions

The carbon skeleton of lasalocid A is derived from five acetate, four propionate and three

Scheme 2. Hypothetical biosynthetic scheme outlining the formation of the carbon skeleton of lasalocid A on a multi-enzyme complex (after LYNEN[10]).



butyrate units. Prior to incorporation into the polyketide precursor of the antibiotic, the three acids are converted into malonate, 2-methylmalonate and 2-ethylmalonate respectively. Using LYNEN's scheme for 6-methylsalicylic acid biosynthesis[10] as a model, a speculative partial pathway for lasalocid A biosynthesis on a multienzyme complex is proposed (Scheme 2). According to this scheme, biosynthesis starts and terminates with acetate units which appear in lasalocid A as the C-methyl at carbon 23 and the carboxyl group of the salicylic acid chromophore respectively. The remaining four methyl groups are derived from propionate.

The most significant outcome of this study was the clear demonstration of the involvement of butyrate and 2-ethylmalonate in the formation of the three C-ethyl groups. Although there have been many demonstrations[10~13] of the involvement of acetate and propionate in the biosynthesis of polyketide-type natural products, lasalocid A (**3**) has provided the *first* example of a C-ethyl group arising from a complete butyrate unit. A second example was reported recently[20] for monensin. Incorporation of butyric acid into lasalocid A could be considered as a special case of mycolic condensation as demonstrated[21] in the biosynthesis of corynemycolic acid by *Corynebacterium diphtheriae* from two molecules of palmitic acid.

The major source of acetate in the fermentation medium is probably carbohydrate, although β-oxidation of long chain fatty acids such as stearic acid has been shown to provide acetate and hence butyrate available for antibiotic synthesis. The conversion of acetate to butyrate apparently proceeds *via* 3-hydroxybutyrate in a sequence of reactions very similar to that demonstrated[18] for fatty acid biosynthesis.

There are several potential sources of propionate or 2-methylmalonate in fermentation broths. For *S. venezuelae*, which utilizes acetate and propionate in the biosynthesis of methymycin[22], it was proposed that the propionate units arise from the carboxylation of pyruvate to oxalacetate, followed by reduction to succinate and isomerization to 2-methylmalonate. Studies of erythromycin biosynthesis by *S. erythreus* suggest amino acids such as alanine[23], valine[23,24] and leucine[24] as possible sources of propionate and hence erythromycin.

These potential pathways to the four propionate units in lasalocid A will be tested in future incorporation studies of the *S. lasaliensis* fermentation. Another interesting aspect of lasalocid A biosynthesis involves the cyclization reactions employed in the formation of the tetrahydropyran and tetrahydrofuran rings from a linear precursor such as **10** (Scheme 2). Possible mechanisms involved in the formation of the cyclic ether functions will be the subject of the next report in this series.

Experimental

Fermentation Methods to Produce Labelled Antibiotic

All fermentations were run at 28°C on a rotary shaker in 500-ml Erlenmeyer flasks containing 100 ml of medium. Inoculum was grown from a frozen spore suspension of *Streptomyces lasaliensis* NRRL-3382.

(**1**) Experiments with ^{14}C-labelled substrates

Two ml of the spore suspension were added to a medium containing (in g/liter): dextrose, 10; yeast extract, 2; monosodium glutamate, 0.5; and K_2HPO_4, 0.5. After 3 days of incubation, 2 ml of the resulting culture were used to inoculate a second flask of the same medium. After 2 days of incubation, ^{14}C-labelled substrate was added to second flask and incubation was continued for 5 days. The yield of lasalocid A averaged about 100 mg per liter.

(**2**) Experiments with ^{13}C-labelled substrates

Two ml of the spore suspension were added to a medium containing (in g/liter): Yellow split peas, 20; lard oil, 20; cornstarch, 10; yeast extract, 2; and K_2HPO_4, 1. After 3 days of incubation, 2 ml of the resulting culture were used to inoculate a second flask of the same medium. ^{13}C-Labelled substrate was added to the second flask as the sodium salt in sterile aqueous solution on days 3, 4, 5 and 6. The yield of lasalocid A averaged about 1 g per liter on the seventh day of fermentation.

Isolation of ^{14}C-labelled Lasalocid A Sodium Salt from a Fermentation Grown in Medium Enriched with [1-^{14}C] Sodium Butyrate

To 100 ml of a 7 day old fermentation mash grown on a medium containing 24 μC of 1-^{14}C sodium butyrate was added 25 g of Celite and 100 ml of ethyl acetate. The mixture was stirred for 30 minutes, filtered and the pad washed with additional ethyl acetate. The solvent was separated and concentrated to dryness *in vacuo*. The resulting oil was chromatographed on a thick layer plate using the system methylene chloride-ethyl acetate (1 : 1). The antibiotic zone was eluted from the silica gel with methanol and concentrated *in vacuo* to an oil (1.423 μC, 5.9 % incorporation of the ^{14}C label). To the oil in methylene chloride-hexane were added 5 g of cold lasalocid A, sodium salt and the antibiotic recrystallized until constant activity was attained (569 dpm/mg).

Degradation Reactions of [^{14}C]-Labelled Lasalocid A (**3**)

(**1**) Pyrolytic decarboxylation

In a pyrex glass tube fitted with a gas trap 590 mg (1 mmole) of [1-^{14}C] acetate-derived lasalocid A (11,800 dpm/mmole) was heated at 230°C for one hour under a stream of nitrogen. The evolved gas was trapped in 4.8 ml of a 2 : 1 solution of ethanolamine-ethanol. The residue (467 mg) contained 10,171 dpm or 86 % of the radioactivity, while the gas trap contained 1,055 dpm or 8.9 %.

(**2**) KUHN-ROTH oxidation

To 1.224 g (2 mmole) of 3,4-^{14}C-butyrate-labelled **3** (26,764 dpm/mmole) was added 250 ml of KUHN-ROTH oxidizing solution[25] and the mixture refluxed for 1.5 hours and steam distilled. The pH of the distillate was adjusted to 11 with 2 N LiOH and the water reduced to a small volume *in vacuo*. The pH was adjusted to 6.3 with N HCl and 2.5 g of α, *p*-dibromoacetophenone in ethanol was added and the mixture refluxed for 2 hours. The reaction was cooled, diluted with water and extracted with $CHCl_3$. The solvent was separated and concentrated to a small volume and chromatographed on a 375 g silica gel column prepared in hexane. The column was eluted with a gradient between 6-liter hexane-methylene chloride (3 : 1) and 6-liter methylene chloride. Two major fractions were collected: (A) #21~59 and (B) #81~180. From fraction A after crystallization 300 mg of the *p*-bromophenacyl ester of propionic acid was recovered with a radioactivity of 7,310 dpm/mmole, while from fraction B, 863 mg of the crystalline *p*-bromophenacyl ester of acetic acid was recovered with a radioactivity of 2,478 dpm/mmole.

(**3**) Retrograde aldol reaction

To 1 g of **3** (labelled with 2-^{14}C butyrate, 20,304 dpm/mmole) in 10 ml of *p*-dioxane was added 4 ml of aqueous 10 % NaOH. After 24 hours the reaction was diluted with water and extracted with ethyl acetate. The solvent was separated and concentrated *in vacuo* to 667 mg of clear oil **8** (18,519 dpm/mmole, 91.2 %). The water layer was acidified to pH 2 and extracted with ethyl ether. Concentration of the ether with addition of hexane afforded 125 mg of **7** (1,932 dpm/mmole, 9 %).

The above reactions were repeated on **3** that had been isolated from fermentations grown on media enriched with [1-^{14}C] acetate, [2-^{14}C] propionate, and [3-^{14}C] butyrate.

(**4**) Oxidation and hydrolysis of the retro-aldol ketone **8**

The experimental details of these reactions have been described elsewhere[26].

Isolation of ^{13}C Labelled Lasalocid A

To 1 liter of fermentation mash derived from a fermentation medium enriched with 500 mg of [1-^{13}C] sodium butyrate was added an equal volume of ethyl acetate and 10 % (w/v) of Celite as a filter aid. The mixture was stirred vigorously for 1 hour and then filtered, the filter pad being washed with additional solvent. The aqueous layer was extracted a second time and the solvent extracts combined and concentrated *in vacuo* to an oil (2.64 g) containing ~740 mg of **3** by bioassay. The oil was dissolved in methylene chloride and chromatographed on a 100 g Florisil column. The column was eluted with 200 ml of methylene chloride and then a gradient between 1.2 liter of methylene chloride to 1.2 liter of 7 % methanol in methylene chloride. Fractions (15 ml each) #47~73 were pooled and the solvent removed *in vacuo* to give 809 mg of oil which was crystallized from methylene chloride/hexane. Recrystallization from ether gave 296 mg of **3**.

From 1 liter of fermentation mash, which was grown on a fermentation medium enriched with 500 mg of [1-^{13}C] sodium butyrate, 540 mg of **3** were isolated by the same procedure.

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