BIOSYNTHESIS OF LASALOCID. III ISOLATION AND STRUCTURE DETERMINATION OF FOUR HOMOLOGS OF LASALOCID A

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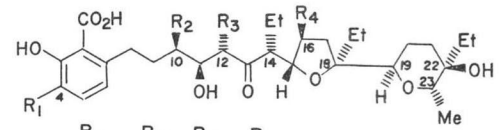
Four isomeric homologs of lasalocid A have been isolated from cultures of *Streptomyces lasaliensis*. The homologs each arise by replacement of one of the four propionate derived methyls in lasalocid A by an ethyl group, which results in each homolog molecule containing four C-ethyls.

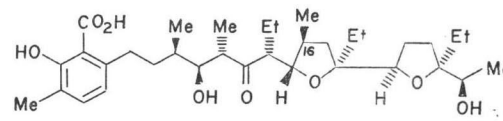
One of the unique features of the lasalocid A[1,2] molecule (**1**) is the presence of three C-ethyls as illustrated in Fig. 1. Incorporation studies with ^{13}C and ^{14}C labelled substrates revealed[3] that these three C-ethyls are each derived from a complete butyric acid molecule. In addition, four of the five C-methyls (at C-4, 10, 12 and 16) have been shown to arise from propionate. In this paper, we report the isolation and characterization of four homologs of **1** in which each propionate derived methyl is replaced in turn by an ethyl group. The structures of the homologs, lasalocids B, C, D and E (**2**, **3**, **4**, and **5**) are shown in Fig. 1.

Earlier reports from this laboratory have described the isolation,[4] structure determination,[2] biosynthesis[3,5,6] and antibacterial activity[7] of **1**. More recently,[8] the X-ray crystallographic structure determination of a naturally occurring isomer of **1**, *iso*-lasalocid A (**6**) was reported. Mass spectrometry of *iso*-lasalocid A has revealed traces of a homolog of **6**, in which the methyl at C-16 is replaced by an ethyl group. This result prompted a search for lasalocid A homologs in crude antibiotic preparations.

Fig. 1. Structure of the major components of the lasalocid antibiotic complex.



	R_1	R_2	R_3	R_4	
1	Me	Me	Me	Me	Lasalocid A
2	Et	Me	Me	Me	Lasalocid B
3	Me	Et	Me	Me	Lasalocid C
4	Me	Me	Et	Me	Lasalocid D
5	Me	Me	Me	Et	Lasalocid E



6 *iso*-Lasalocid A

Resolution of the Lasalocid Antibiotic Complex

In addition to lasalocid A, the *Streptomyces lasaliensis* fermentation has been shown to produce *iso*-lasalocid A,[8] and two C_{17} metabolites[9] structurally unrelated to the lasalocids. An additional component of the lasalocid complex was first detected by silica gel TLC using an acetic acid - 2-propanol - cyclohexane solvent system. The novel component which was sub-

sequently shown to be a mixture of four isomeric homologs of lasalocid A, had a slightly higher mobility than **1** and accounted for about 4% of the total lasalocid complex. Attempts to scale-up this separation on silica gel columns failed.

The only successful method found so far to resolve the four isomeric homologs is counter-current distribution (CCD). Using a solvent system consisting of heptane-ethyl acetate-methanol-water, CCD gave a clean separation of lasalocid A from the four homologs. Repeat CCD experiments on the homolog mixture using a heptane-ethyl acetate-ethanol-water-acetic acid system succeeded in providing sufficient quantities of each of the four homologs for their complete characterization, which was accomplished primarily by mass spectrometry as described in the next section.

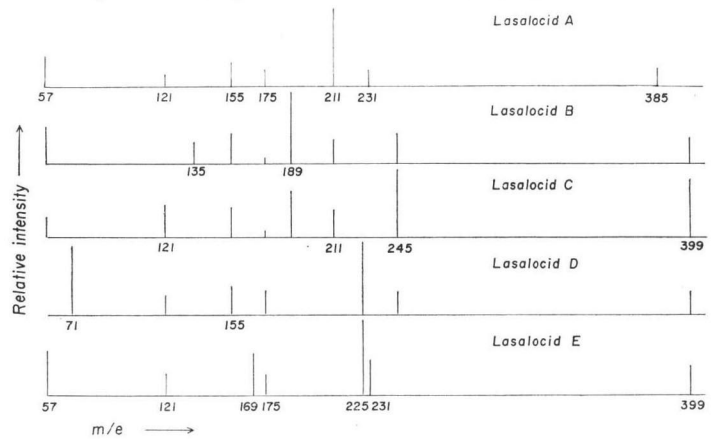
Mass Spectrometry of the Lasalocids

(1) Lasalocid A

It has been reported[10] for two other polyether antibiotics monensin and nigericin, that the alkali metal salts are sufficiently volatile to yield mass spectra which include molecular ions. The highest mass peak observed in the spectrum of lasalocid A sodium salt (mol. wt. 612, $C_{34}H_{53}O_8Na$) however is at *m/e* 528 and arises from the loss of sodium, CO_2 and H_2O.

Mass spectral fragmentation of the lasalocids (Fig. 2) proceeds primarily by two distinct routes. In the first of these, fragmentation occurs by a mechanism analogous to that described[11]

Fig. 2. Mass spectra of lasalocids A, B, C, D and E.

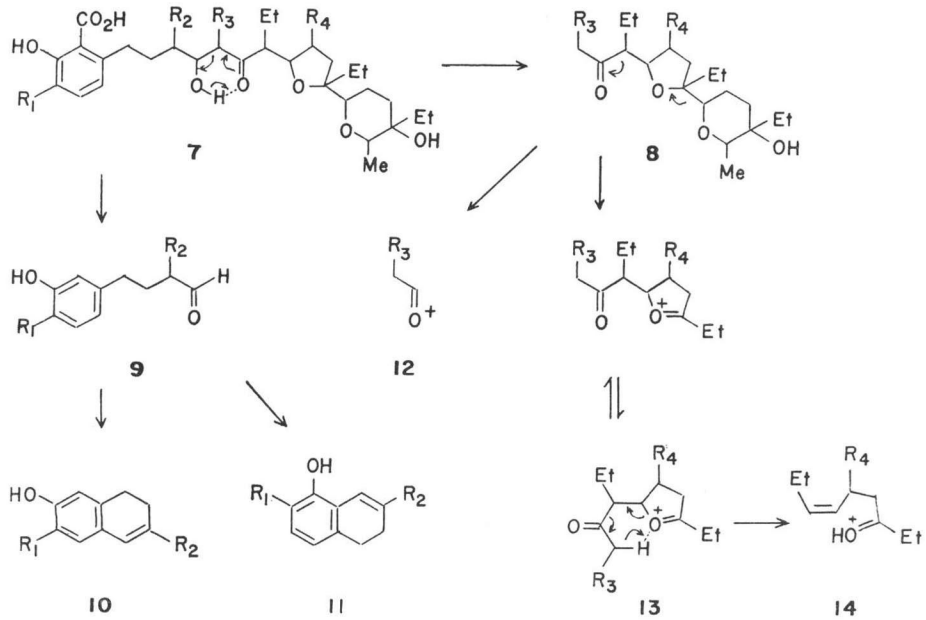


recently for the pyrolytic cleavage of lasalocid A (**1**). In the pyrolysis reaction, **1** was first cleaved to the ketone **8** and an aldehyde **9**, which spontaneously cyclized and dehydrated to a mixture of isomeric dihydronaphthols **10** and **11** (Scheme 1). Slight changes in the relative intensity of the mass spectral peaks with time indicate that pyrolysis may also occur to a limited extent in the spectrometer.

The most significant fragment which arises from the electron impact induced cleavage of lasalocid A is the ion corresponding to **8**, which is formed in the mass spectrometer by McLAFFERTY rearrangement[12] of the molecular ion corresponding to **7** as illustrated in Scheme 1. Ion **8** is the source of three of the most intense peaks in the lasalocid A mass spectrum,

including the base peak at *m/e* 211 (13, R_3=R_4=Me). Ion **13** is formed by the loss of the tetrahydropyranyl ring from **8** via α-cleavage to form a cyclic oxonium ion. This particular cleavage is known[18] to play an important role in the fragmentation of α-alkylated derivatives of tetrahydrofuran. Rearrangement of **13** (Scheme 1) involving the loss of ketene leads to a second major peak at *m/e* 155 (**14**, R_4=Me), and the third peak arising from **8** is at *m/e* 57 (**12**, R_3=Me) and is formed by α-fission at the carbonyl group.

Scheme 1. Cleavage of the β-ketol system and subsequent pyrolytic and mass spectral fragmentation of the lasalocid molecule.

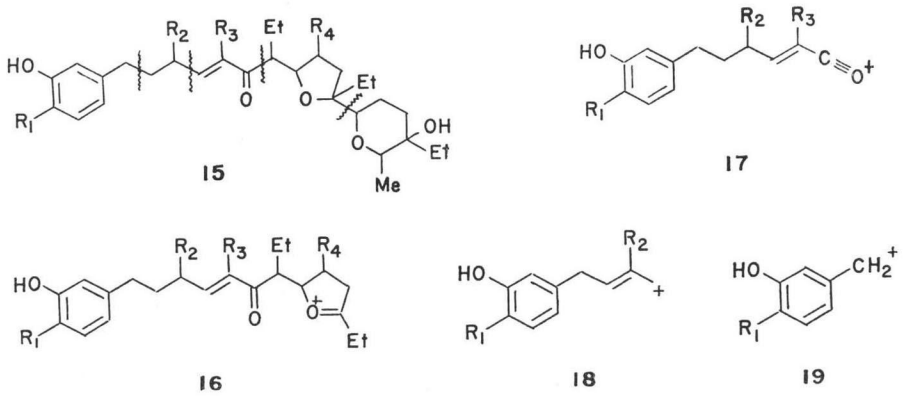


Lasalocid component	Molecular weight 7	*m/e* values for mass spectral fragments 12	13	14
1 Lasalocid A	590	57	**211**	155
2 Lasalocid B	604	57	211	155
3 Lasalocid C	604	57	211	155
4 Lasalocid D	604	71	**225**	155
5 Lasalocid E	604	57	**225**	**169**

The second major source of peaks in the mass spectra of lasalocids is the anhydro-descarboxy ion corresponding to **15** (Scheme 2). In this ion, the β-ketol system of the lasalocid molecule has been dehydrated and this precludes the McLAFFELTY rearrangement from playing a role in the further fragmentation of **15**. Cleavage does occur however at four other points in the structure of **15** as indicated in Scheme 2.

Fragment **16** arises from the loss of the tetrahydropyranyl ring[18] by **15** and is an important ion as it distinguishes lasalocid A from the four homologs, lasalocids B, C, D and E. In the antibiotic, all four R groups are methyls resulting in the appearance of **16** at *m/e* 385, but in each of the homologs one of the R groups is ethyl which leads to fragment **16** moving by

Scheme 2. Lasalocid mass spectral fragments that arise from the anhydro-descarboxy ion **15**.



Lasalocid component	*m/e* values for mass spectral fragments				
	15	**16**	**17**	**18**	**19**
1 Lasalocid A	**528**	**385**	231	175	121
2 Lasalocid B	542	399	245	**189**	**135**
3 Lasalocid C	542	399	**245**	**189**	121
4 Lasalocid D	542	399	**245**	175	121
5 Lasalocid E	542	399	231	175	121

fourteen mass units to *m/e* 399 in all four cases.

Fission of ion **15** next to the carbonyl group results in a peak at *m/e* 231 (**17**, $R_1=R_2=R_3=Me$). The other two cleavages indicated in Scheme 2 are the source of peaks at *m/e* 175 (**18**, $R_1=R_2=Me$) and 121 (**19**, $R_1=Me$) in the mass spectrum of lasalocid A (Fig. 2).

(2) Lasalocids B, C, D and E

By monitoring the *m/e* values for the seven mass spectral fragments **12**, **13**, **14** (Scheme 1), and **16**, **17**, **18** and **19** (Scheme 2) discussed above in connection with the lasalocid A spectrum it is possible to distinguish all four of the isomeric homologs. This is because each homolog has either one particular *m/e* value which is not found in the others, or a unique combination of *m/e* values for two fragments as is the case for lasalocid C, the only homolog with major peaks at both *m/e* 121 (**19**, R=Me) and 189 (**18**, $R_2=Et$).

The mass spectra of lasalocids B, D and E each contain a major peak which is unique for that particular homolog (Fig. 2). In the spectrum of lasalocid B, this diagnostic peak is at *m/e* 135 (**19**, $R_1=Et$) and the spectrum of lasalocid D is distinguished by a peak at *m/e* 71 (**12**, $R_3=Et$). The peak found only in lasalocid E is at *m/e* 169 (**14**, $R_4=Et$).

The base peaks in the spectra of lasalocids B and C are at *m/e* 189 (**18**, $R_1=Et$, $R_2=Me$) and 245 (**17**, $R_1=R_3=Me$, $R_2=Et$) respectively (Fig. 2), whereas the base peaks in the spectra of lasalocids D and E at *m/e* 225 are homologous to the base peak in the lasalocid A spectrum at *m/e* 211 (**13**, $R_3=R_4=Me$).

These diagnostic peaks and others, make mass spectrometry an extremely useful tool in the identification of single components of the lasalocid complex. However, if a mixture of components is analyzed by mass spectrometry it is very difficult to determine quantitatively

the nature of the mixture because of variability in the relative intensities of the spectra. The only method devised so far to solve this problem is the pyrolysis-GLC technique described in the next section.

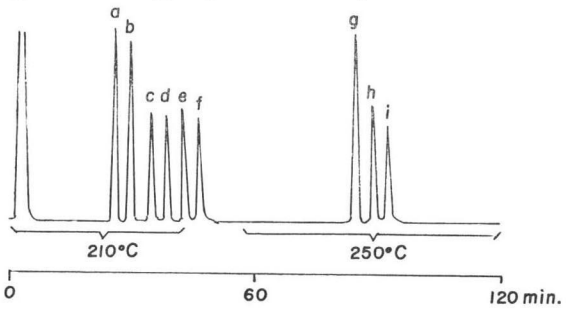
GLC Analysis of Mixtures of Lasalocid Components

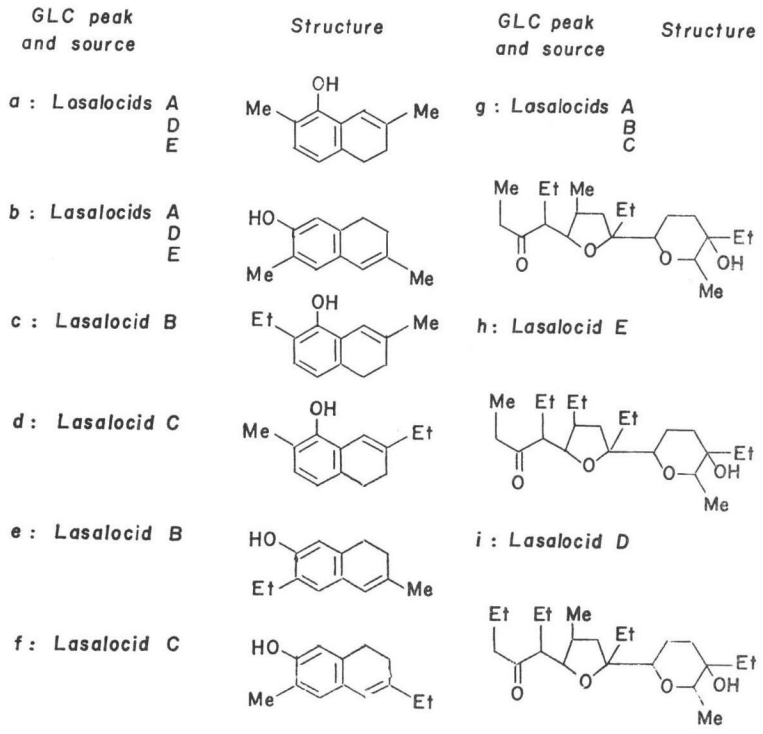
Attempts to separate the four homologs of lasalocid A by TLC have so far failed. The pyrolysis-GLC procedure[14] for the analysis of lasalocid A has therefore been adapted to the assay of the lasalocid complex as a whole. This technique is based on the quantitative retroaldol cleavage of lasalocid to the ketone **8**.

This simple technique, however, cannot be applied to the analyses of the complete lasalocid complex, because although lasalocids D and E give retroaldol ketones (**8**) of higher molecular weight, the other two homologs (**2** and **3**) give the same form of **8** ($R_3=R_4=Me$) as lasalocid A.

The GLC analysis had to be extended therefore (by temperature programming) to include

Fig. 3. Schematic diagram of the pyrolytic-GLC analysis of the lasalocid complex.





the α- and β-dihydronaphthols (**10, 11**) which are also produced in the pyrolytic cleavage reaction (Scheme 1). The nine peaks resulting from injection of the complete lasalocid complex (**1**~**5**) into a gas chromatograph are shown schematically in Fig. 3. The peaks (*a-i*) were assigned by gas chromatography-mass spectrometry[14] (GC-MS) and comparison with authentic samples. Each peak area (A_a, A_b, *etc.*) is determined by electronic integration and the percentage composition of the lasalocid complex can be calculated in the following manner:

$$\% \text{ lasalocid B} = \frac{100 \cdot \sum_A c, e}{\sum_A a, b, c, d, e, f},$$

$$\% \text{ lasalocid C} = \frac{100 \cdot \sum_A d, f}{\sum_A a, b, c, d, e, f},$$

$$\% \text{ lasalocid D} = \frac{100Ai}{\sum_A g, h, i},$$

$$\% \text{ lasalocid E} = \frac{100Ah}{\sum_A g, h, i},$$

$$\% \text{ lasalocid A} = 100 - \% \text{ lasalocids B, C, D and E.}$$

In a typical experiment, a crude fermentation extract was converted to both lasalocid and lasalocid sodium salt and each of the two forms was analyzed four times by the pyrolysis-GLC technique. The results (Table 1) indicate that the level of total homologs in the particular sample was approximately 11 %.

Table 1. Results of repetitive pyrolysis-GLC analyses of a crude, crystalline lasalocid preparation in both the sodium salt and free acid forms

Form of the crude lasalocid analyzed	% Level of the four homologs:				% Antibiotic
	lasalocid B **2**	C **3**	D **4**	E **5**	lasalocid A **1**
Sodium salt	2.8	2.7	2.5	2.0	90.0
	2.8	2.2	2.3	2.2	90.5
	3.4	2.1	2.4	2.1	90.0
	4.4	1.9	2.6	2.0	89.1
Free acid	3.6	2.3	2.3	2.7	89.1
	4.6	2.1	2.4	2.6	88.3
	4.6	2.4	2.5	2.8	87.3
	4.2	2.1	2.4	2.6	88.7
Mean	3.8	2.2	2.4	2.4	89.2
Range	±1.0	±0.5	±0.2	±0.4	± 1.5

Biosynthesis of the Lasalocid Homologs

The homologs of lasalocid A arise most probably by replacement of each propionate in the antibiotic precursor molecule by a fourth butyrate unit. This replacement would in turn result in the formation of the four distinct isomeric homologs of lasalocid A, each of which contains four C-ethyl groups (Fig. 1). As the total homolog content in lasalocid fermentation varies from 4 % up to 16 %, it appears that the replacement of each propionate by butyrate can occur from one to four times per hundred molecules of lasalocid generated by *S. lasaliensis*.

We found no evidence of an ethyl homolog at C-23, which supports the thesis[3] that this particular C-methyl arises from acetate in contrast to the other four propionate derived methyls at C-4, 10, 12 and 16.

In low yielding fermentations, the percentage of total homologs increases, suggesting insufficient propionate precursors in the fermentation medium. Potential sources of propionate or 2-methylmalonate have been discussed earlier[3] and are presently being tested for incorporation into the lasalocid molecule.

Relative *in vitro* Activity of the Natural Analogs of Lasalocid A

The activities of lasalocids B, C, D and E have been compared with those of lasalocid A and *iso*-lasalocid A *in vitro* vs *Bacillus* sp. TA (ATCC-27860) in a microbiological agar diffusion assay. The dose-response curves were parallel in all six cases and assuming the antibiotics had equal diffusion coefficient, their relative activities were determined. The results (based on lasalocid A=100) are summarized in Table 2.

Table 2. Relative *in vitro* activities of the six major lasalocids vs. *Bacillus* sp. TA (cup-plate agar diffusion assay, lasalocid A=100)

	Compound	% Relative *in vitro* activity corrected to the nearest 10%
1	Lasalocid A	100
2	Lasolocid B	90
3	Lasalocid C	180
4	Lasalocid D	160
5	Lasalocid E	170
6	*iso*-Lasalocid A	40

A qualitative correlation between the partition coefficient (in octanol-water) and *in vitro* activity vs. *Bacillus* sp. TA has been established[7] for a series of synthetic analogs of lasalocid A. This effect probably also contributes to the difference in relative activities of the natural analogs of the antibiotic. The homologs for instance are slightly more solvent soluble than lasalocid A; and three of them (lasalocids C, D and E) exhibit considerably higher *in vitro* activity than the major antibiotic. Conversely, *iso*-lasalocid A is both less soluble[8] and less active than lasalocid A against *Bacillus* sp. TA (ATCC-27860).

These results are consistent with our earlier conclusion[7] that lipophilicity is a more important property than pKa, or the tendency to form dimeric complexes, in determining the *in vitro* activity of lasalocid analogs.

Unlike *iso*-lasalocid A,[8] the homologs were active anticoccidial agents in chicks when administered at feed levels found effective in earlier experiments[15] with lasalocid A.

EXPERIMENTAL

Melting points (mp) were determined on a Thomas-Hoover capillary apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer polarimeter Model 141 using 1 % solutions in chloroform at 25°C. Mass spectra were taken with a CEC-21-110 mass spectrometer at 70 eV (Fig. 2) and GC-MS results were obtained using a Finnigan Quadrupole MS 1015 connected to a Becker 409 gas chromatograph.

All UV and NMR spectral data were compatible with the assigned structures.

(1) Silica Gel TLC of the Lasalocids

A number of different solvent systems have been tested in an attempt to completely resolve the lasalocid complex by silica gel TLC. Earlier, lasalocid A and *iso*-lasalocid A were easily resolved using benzene-methanol (19:1), but this solvent system failed completely to resolve the homologs from lasalocid A.

Although at this time we cannot separate the four isomeric homologs from each other, a number of systems, listed in Table 3 have been found that resolve lasalocid A from the mixed homologs.

(2) GLC Analysis of the Lasalocid Complex

Apparatus

The gas chromatograph used for quantitative homolog determinations was a Becker 409

Table 3. R_f values determined for lasalocid A and the homolog mixture on silica gel TLC*

TLC solvent system		R_f values for:	
Solvents	Proportions	Lasalocid A	Homologs
I: 2-propanol-acetic acid-cyclohexane	5:9:86	0.52	0.60
II: methanol-octanoic acid-*n*-hexane	5:6:190	0.40	0.48
III: methanol-octanoic acid-*n*-hexane	5:4·111	0.35	0.43
IV: 2-propanol-acetic acid-*n*-hexane	3:3:19	0.45	0.54

* E. Merck Pre-coated TLC plates: Silica Gel 60 F-254. Detection was achieved by either scanning with a short wave UV lamp for absorbing spots or spraying with a 1:1 solution of conc. sulfuric acid in methanol followed by charring of the compounds to form black spots at 100°C.
Improved resolution is achieved with all four systems by employment of the multiple development technique.

fitted with a 3 meter×3 mm i.d. coiled glass column.

The extended portion of the column which fitted snugly into the injection port was packed with 120~140 mesh silanized glass beads to form a reactor whose temperature could be varied by the injector port controller.

The optimal conditions using this equipment are as follows:

Packing: 10 percent by weight of phenyl methyl silicone (OV-17) on 100~120 mesh Gas Chrom Q (Applied Science Labs).
Carrier gas: Nitrogen (oxygen-free) at 30 ml min^{-1}.
Detector: Flame-ionization.
Reactor temperature: 300°C.
Column temperature: Isothermal at 210°C for 40 minutes.
Programmed from 210°C to 250°C at 2.5° min^{-1}.
Isothermal at 250°C for 40 minutes.

Procedure

Weigh approximately 50 mg of crude lasalocid complex into a 1-ml volumetric flask, dissolve and make up to 1 ml with chloroform. Aliquots (2 μl) of this solution are injected directly into the gas chromatograph.

The results of injecting replicate samples of a particular crude lasalocid sample first as the sodium salt and secondly as the free acid are summarized in Table 1. The results were calculated using the formulae discussed in the text.

(3) Countercurrent Distribution of Crude Lasalocid Mother Liquors and Crystals

Resolution of a mixture of lasalocids A(**1**), D(**4**), E(**5**) and *iso*-lasalocid A(**6**)

Part of the final mother liquor (containing 22 g solids) from the large scale crystallization of 10 kg of the sodium salt of **1**, was distributed in a 200-tube (40 ml of upper and 40 ml of lower phase) CCD instrument using the solvent system, heptane-ethyl acetate-methanol-water (27:18:18:2). The solution containing the crude mixture was placed in the first two tubes and the machine operated using the single withdrawal technique.[16]

After 380 transfers, the tubes were analyzed by UV, silica gel TLC and pyrolysis/GLC. Based on the analytical results, three pooled fractions were obtained as indicated in Table 4.

Fraction **1a** was evaporated and the residue distributed in a 500-tube (10 ml upper and 10 ml lower phase) CCD instrument using the solvent system, heptane-ethyl acetate-ethanol-water-glacial acetic acid (10:5:9:3:1). After 3,800 transfers (top phase recycle), the tubes were analyzed (pyrolysis/GLC) and five fractions were pooled as indicated in Table 5.

Fraction **2a** was crystallized from aqueous 2-propanol to give crystalline lasalocid E(**5**), mp 90°C, $[\alpha]_D$ −42.1°. Calcd. for $C_{35}H_{56}O_8$: C 69.50, H 9.35; Found: C 69.34, H 9.36. Part

Table 4. Fractions from the first CCD experiment

Fraction	Tubes[a] combined	Partition[b] ratio, C	TLC[c] R_f value	Lasalocid components	Weight in grams
1a	135~185	0.72	0.60	D, E	5.5
1b	110~134	0.45	0.52	A	4.5
1c	60~ 90	0.25	0.50	*iso*-lasalocid A	1.0

a) Tubes numbered from the initial tube on the CCD instrument.
b) $C=r^*\div(n-r^*)$, where r^*=peak tube and n=number of transfers.
c) Solvent system I (Table 3).

of **5** (200 mg) was dissolved in methylene chloride and treated with saturated aqueous Na_2CO_3. The solvent was separated and allowed to evaporate with addition of hexane to give 104 mg of the crystalline sodium salt of **5**, mp 181~182°C, $[\alpha]_D$ −79.5°. Calcd. for $C_{35}H_{55}O_8Na$: C 67.07, H 8.85, Na 3.66; Found: C 67.40, H 9.06, Na 3.51.

Fraction **2e** was recrystallized from aqueous 2-propanol to give lasalocid D(**4**) containing 2-propanol as solvent of crystallization, mp 102~104°C, $[\alpha]_D$ −63.6°. Calcd. for $C_{35}H_{56}O_8\cdot C_3H_8O$: C 68.64, H 9.70; Found C 68.68, H 9.85.

Table 5. Fractions from the second CCD experiment

Fraction number	Tubes combined	Weight (mg)	Pyrolysis-GLC analysis	
			% Lasalocid D	% Lasalocid E
2a	110~160	270	0	100
2b	161~180	320	10	40
2c	181~210	568	20	80
2d	211~240	438	50	50
2e	241~300	210	100	0

Resolution of a lasalocid mixture consisting of lasalocids A(**1**), B(**2**) and C(**3**)

A crude crystalline preparation of lasalocid A (44 g) was distributed in the 200-tube CCD instrument using the solvent system heptane-ethyl acetate-ethanol-water-acetic acid (10:5:9:3:1). From tubes 86~130, 4.58 g of a mixture enriched in **2** and **3** was isolated and rechromatographed in the 500-tube CCD instrument using the same solvent system.

After 2,000 transfers were completed and the tubes analyzed as described earlier, tubes 200~400 containing lasalocid A were decanted off and fresh solvent added. After an additional 2,000 transfers, the major peak (K=1.0) was divided into thirteen fractions. One fraction (tubes 450~490) was concentrated to dryness *in vacuo* and crystallized from aqueous 2-propanol to yield 230 mg of lasalocid B(**2**), mp 86~87°C, $[\alpha]_D$ −36.3°. Calcd. for $C_{35}H_{56}O_8$: C 69.50, H 9.33; Found: C 69.51, H 9.67.

Another fraction (tubes 71~80) was concentrated to dryness *in vacuo* and crystallized from aqueous 2-propanol to yield 360 mg of lasalocid C(**3**) containing 2-propanol as solvent of crystallization, mp 97~100°C, $[\alpha]_D$ −52.7°. Calcd. for $C_{35}H_{56}O_8\cdot C_3H_8O$: C 68.64, H 9.70; Found: C 68.55, H 9.68.

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