BIOSYNTHESIS OF LASALOCID. II

X-RAY ANALYSIS OF A NATURALLY OCCURRING ISOMER OF LASALOCID A

J.W. WESTLEY, J.F. BLOUNT, R.H. EVANS, Jr., A. STEMPEL and J. BERGER

Chemical Research Department, Hoffmann-La Roche Inc.,
Nutley, New Jersey 07110, U.S.A.

(Received for publication April 24, 1974)

A search for precursors of lasalocid A has led to the isolation and X-ray crystallographic analysis of an isomer of the antibiotic. The isomer differs from lasalocid A in both the size and absolute configuration of the terminal cyclic ether. These differences lead to speculation on the nature of cyclization mechanisms involved in the biosynthesis of lasalocid A and the other polyether antibiotics.

The characteristic structural feature of the polyether antibiotics is the multiplicity of cyclic ether functions. Of the three antibiotics isolated in this laboratory[1], antibiotics X-206[2] and X-464[3] (nigericin[4], polyetherin A[5]) each contain six ether rings whereas lasalocid A (**1**, formerly known as antibiotic X-537A[6,7]) has only two. An isomer (*iso*-lasalocid A) of **1** has now been isolated from cultures of *Streptomyces lasaliensis*. Comparison of the structure of *iso*-lasalocid A and **1** has suggested a possible cyclization mechanism for the final step in the biosynthesis of the two antibiotics.

In earlier studies,[8,9,10] the molecule of lasalocid A(**1**) was shown to be derived from five acetate, four propionate and three butyrate molecules *via* malonyl, 2-methylmalonyl and 2-ethylmalonyl CoA respectively. In the last report,[10] the origins of the eight-branched alkyl groups in lasalocid A were assigned: the three C-ethyls are derived from butyrate; the four C-methyls at C-4, 10, 12 and 16 are propionate derived, but the one at C-23 is derived from an acetate unit. It was proposed that the carbon skeleton (**2**) of lasalocid A was formed on an enzyme complex analogous to LYNEN's hypothetical scheme[11] for 6-methylsalicyclic acid.

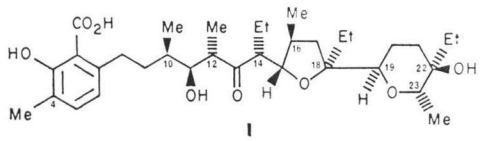
In order to determine some of the biosynthetic steps between a linear precursor such as **2** and the fully cyclized final product, **1**, a careful search was made of mother liquors from *S. lasaliensis* extracts in the hope of finding precursors to the lasalocid A molecule.

New Metabolites Isolated from *S. lasaliensis* Fermentations

The structures of the first two compounds isolated in this study were determined by proton NMR-spectroscopy with the aid of *tris* (dipivalomethanato)-europium-induced shifts.[12] Unfortunately, the two C_{17} metabolites (**3** and **4**) appeared to be totally unrelated to **1** and so the search for lasalocid A precursors was continued.

Isolation of an Isomer of Lasalocid A from *S. lasaliensis*

From the fourth crop of a crystallization of 2.66 kg lasalocid A sodium salt, 460 mg of a new crystalline sodium salt, mp 183~185°C, $[\alpha]_D^{20}$ −93.9°(*c* 1, $CHCl_3$) was isolated. Microanalysis indicated a molecular formula ($C_{34}H_{53}O_8Na$) identical to that of lasalocid A sodium salt,[7] which has a mp 168~171°C, $[\alpha]_D^{20}$ −84.6°(*c* 1, $CHCl_3$). Conversion to the acid form gave crystals ($C_{34}H_{54}O_8$), mp 203°C which were considerably less soluble than **1** (mp 100~109°C).



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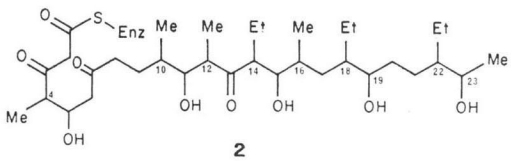

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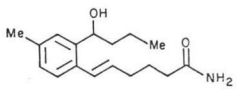

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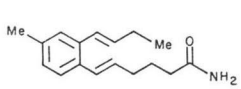

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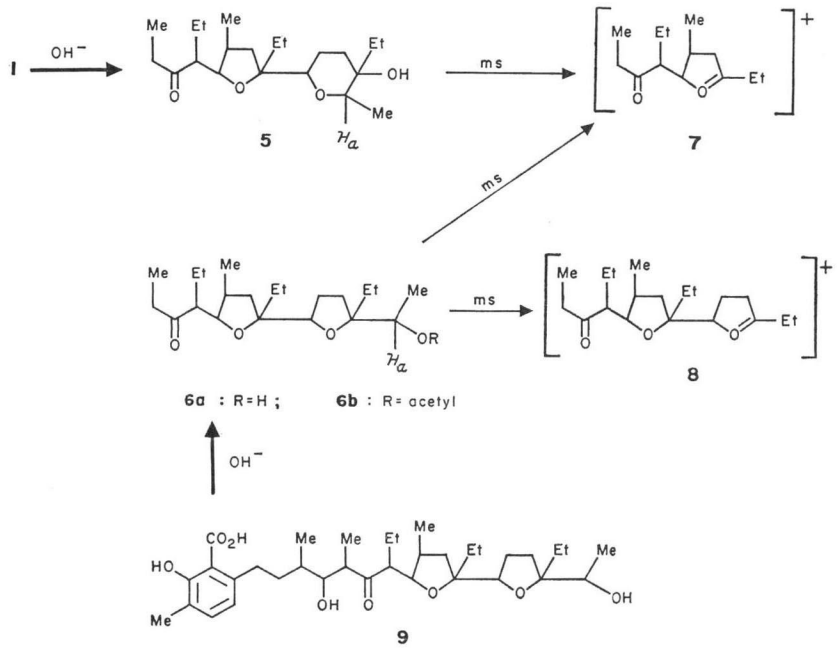
Ultraviolet and infrared spectra of this isomer of lasalocid A were virtually superimposable on the spectra of the antibiotic suggesting identical chromophores. This was confirmed by NMR ($CDCl_3$) of the sodium salt which showed the presence of an aromatic methyl singlet at δ 2.18, and two aromatic protons at δ 6.45 and 6.95 (J_{ortho} 8 Hz) consistent with the chromophore of **1**.

Base catalyzed retroaldol cleavage revealed the difference in structure of lasalocid A and the isomeric compound. Whereas, **1** gave a well-characterized ketone **5**,[13] $[\alpha]_D^{20}$ −26.5° (*c*1, $CHCl_3$), the isomer of lasalocid A gave a different ketone **6a**, $[\alpha]_D^{20}$ −10° (*c* 1, $CHCl_3$) (Scheme 1).

Comparison of the mass spectra of **5** and **6a** showed that certain ions such as the molecular ion (*m/e* 354) and **7**(*m/e* 211) were common to both whereas the ion **8** (*m/e* 309) was found only in **6a** and the acetate derivative **6b**. These results suggested the structures indicated in Scheme 1.

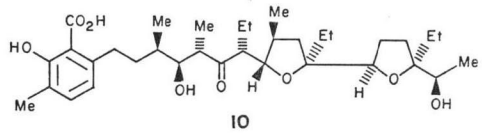
The structure assigned to **6a** was confirmed by NMR ($CDCl_3$) which gave a quartet at δ 3.75 in **5** and **6a** due to *Ha*. Acetylation caused a shift to δ 4.92 in **6b**. The ketone **5** is resistant to acetylation. These results are consistent with **9** as the structure of the lasalocid A isomer.

Scheme 1.



X-Ray Analysis of *iso*-Lasalocid A (10)

Iso-lasalocid A was converted to the *p*-bromo derivative in a manner identical to that described earlier[14] for bromo-lasalocid. The derivative crystallized in the space group $P2_12_12_1$ with four molecules in a unit cell of dimensions: $a=11.772(6)$, $b=13.013(3)$, $c=24.038(9)$ Å. A stereoview of the derivative is shown in Fig. 1.



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The structure was solved by the heavy atom method. Preliminary least squares refinement of the structure was done with isotropic temperature factors for all atoms except the bromine, which was assigned anisotropic thermal parameters. For the final refinement, hydrogen atoms were included at their calculated positions. In the case of the hydroxyl protons, they were placed at their positions on the basis of probable hydrogen bond formation; thus their positions are probable but not certain. The final refinement was carried out by full-matrix least squares with all atoms anisotropic except the hydrogens. The final discrepancy index is $R=5.5\,\%$ for 1858 observed reflections.

Fig. 1. Stereoview of the bromo derivative of *iso*-lasalocid A.

The first feature distinguishing **10** from **1** emerged at an early stage of the X-ray analysis when it was found that the bromo derivative of **10** contained a single molecule per independent unit of the crystal in contrast to the bromo derivative of **1**[15], and the silver salts of **1**[16] and nitro-lasalocid A[17] all of which contained two molecules in a non-symmetrical dimeric conformation per independent unit of the crystal.

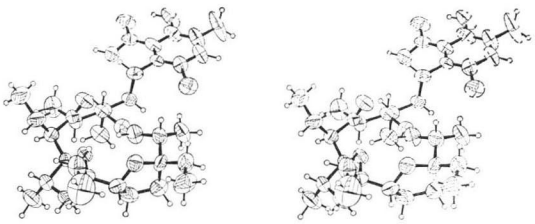
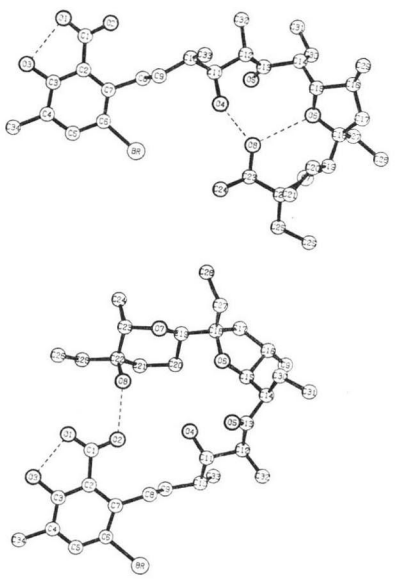


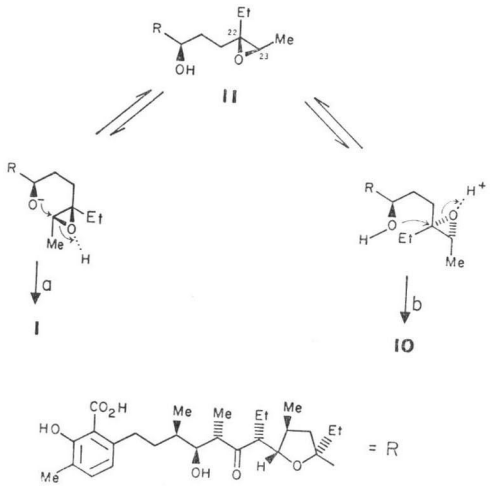
Fig. 2. Comparison of the conformation in the crystalline state of the bromo derivatives of *iso*-lasalocid A (top) and lasalocid A[15](bottom).



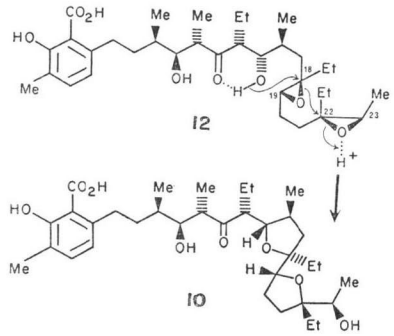
The X-ray analyses of lasalocid A derivatives were carried out in part, to determine whether there was a link between dimeric structure in the crystalline state and activity in biological systems. The X-ray result with nitro-lasalocid A,[17] a virtually inactive derivative, demonstrated poor correlation between the two parameters. Lipophilicity appears to be a more important determinant of biological activity.[14]

A second unique feature of the *iso*-lasalocid A derivative is indicated in Fig. 2, which shows that in contrast to all the other polyether antibiotics so far investigated by X-ray analysis, this compound did not exist in the characteristic cyclic conformation held together by a hydrogen bond from the

Scheme 2.



Scheme 3.



carboxyl to a terminal (tertiary) alcoholic function. This conformation was precluded by the preferred bonding of the terminal (secondary) alcohol, O-8 to the ether oxygen, O-6, and the other secondary alcohol, O-4. The different conformation assumed by the *iso*-lasalocid A derivative in the crystalline state explains the lower solubility of **10** in non-polar solvents compared to **1**. The sodium salts of **1** and **10** however are quite similar in solubility.

Comparison of the Structures of Lasalocid A and *iso*-Lasalocid A

The X-ray structure analysis of *iso*-lasalocid A (**10**) confirmed the presence of two tetrahydrofuran rings proposed in **9**. In addition, the analysis showed that the absolute configuration of **10** is identical to the configuration of **1** at eight asymmetric centers, but is reversed at C-22(S) and C-23(R).

These differences in ring size and configuration suggest that *iso*-lasalocid A is not a precursor of lasalocid A (**1**), but that the two compounds probably arise from a common precursor. A compound with the ability to cyclize either to **1** or **10** is the 22(R), 23(R)-epoxide (**11**) proposed in Scheme 2.

Microorganisms such as *Pseudomonas oleovorans* are known to enzymatically epoxidize alkenes[18] in the presence of NADH and molecular oxygen. The olefinic precursor of **11** could arise by dehydration of the secondary alcohol at C-23 (in **2**) at some stage during the formation of the carbon skeleton of lasalocid A by *S. lasaliensis*.

Acid catalyzed cyclization of the epoxide in Scheme 2 would be expected to follow pathway *b*. Protonation of the epoxide oxygen should lead to the selective cleavage[19] of the C-O bond to the more substituted carbon (C-22), according to the MARKOWNIKOFF rule. In this situation, bond breaking is more advanced than bond formation, giving C-22 a fractional positive charge which gains stability from the ethyl substituent.

An extrapolation of this hypothesis can be applied to the formation of the other tetrahydrofuran ring in **10**. Thus, a C-18(R),19(R),-22(R),23(R)-diepoxide (**12**) under acidic conditions could lead by a concerted mechanism (Scheme 3) to **10**.

Another, less likely mechanism leading to **10** would be the attack of hydroxyl ion at C-23 on a C-18(S),19(S)-22(S),23(S)-diepoxide. In a similar case, alkaline saponification of diepoxy

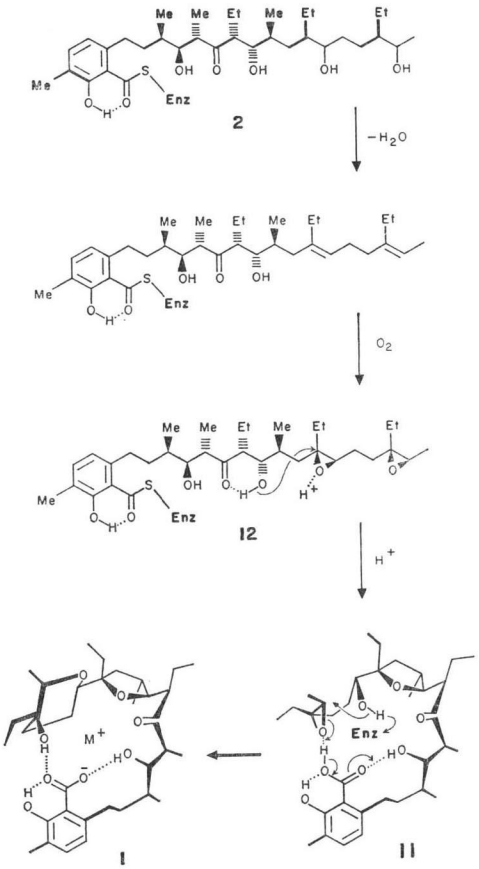
acetates from geraniol and nerol yielded mixtures of tetrahydrofurans and pyrans.[20]

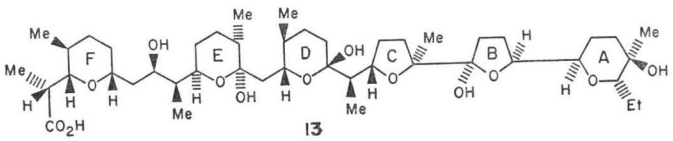
Cyclization Mechanisms Involved in the Biosynthesis of Antibiotics X-206 and X-464

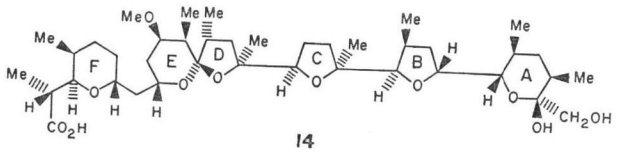
A unique feature of the antibiotic X-206 structure (**13**)[2] is the presence of three lactols (rings B, D and E) which must each be formed from the equivalent hydroxyketone. Ring A, however could clearly arise by the expoxide opening mechanism. In antibiotic X-464 (**14**)[3], ketalization is taken one step further than in **13** to form a spiro-ketal (DE) derived from a dihydroxyketone.

The formation of rings C and F in the two antibiotics must involve an additional reductive step after ketalization or epoxide opening. The interesting stereochemical relationship between the two F rings has been noted earlier.[2]

Scheme 4.







Biosynthesis of Lasalocid A and *iso*-Lasalocid A

The isolation and X-ray analysis of **10** has led to speculation on the final steps of the biosynthesis of **1** (Scheme 4). As the amount of **10** compared to **1** in the fermentation is so small (about one part in five thousand), it is considered to be only a side-product of the biosynthesis of **1**. However, isolation and structure determination of **10** has resulted in three significant conclusions:

(1) Epoxides such as **11** and **12** are likely precursors in the biosynthesis of lasalocid A.

(2) The terminal, tetrahydropyran ring in **1** is the last of the antibiotic's three cyclic systems to form. According to LYNEN,[11] cyclization of 6-methylsalicylic acid follows immediately on completion of the polyketide chain and therefore, the first ring system to form in **1** is probably the aromatic chromophore.

(3) Unlike all the other polyether antibiotics, *iso*-lasalocid A lacks the cyclic conformation[2,4,6] characteristic of the class. It is therefore proposed (Scheme 4) that during the biosynthesis of lasalocid A, the precursor molecule (**11**) assumes a cyclic conformation which is the critical step in the formation of the tetrahydropyran ring. There is strong evidence supporting the intramolecular catalytic poperties of the carboxyl group (anomalous alkylation of the tertiary alcohol[14]) and in an intermediate such as **11**, epoxide opening would be more subject to conformational control than the electronic (MARKOWNIKOFF) factors which favor formation of **10** (Scheme 3).

Table 1. Relative activity of *iso*-lasalocid A (lasalocid A=100).

Organism	Percentage relative activity
Mycobacterium phlei	0.4~2
Staphylococcus aureus	3~11
Sarcina lutea	10~30
Bacillus sp. TA	40
Bacillus sp. E	40~100
Bacillus subtilis	75
Bacillus megaterium	100

Relative *in vitro* Activity of *iso*-Lasalocid A and Lasalocid A

The activity of the two antibiotics has been compared *in vitro* against a number of gram positive bacteria (Table 1).

From the results in Table 1, it is clear that the relative activity of *iso*-lasalocid A is very dependent on the test organism, varying from negligible activity *vs. M. phlei* to virtually identical efficacy to lasalocid A *vs. B. megaterium*.

Experimental

General

Melting points (mp) were determined in a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer polarimeter Model 141 using a 1 % solution at 20°C.

The nmr spectra discussed in the text were obtained with a Varian Associates HA-100 spectrophotometer and the mass spectra were taken with a CEC-2-110 mass spectrometer at 70 V.

Isolation of *iso*-lasalocid A (**10**)

Recrystallization of 2.66 kg of crude lasalocid A sodium salt gave as a fourth crop, 15.68 g of an oily, crystalline material which was shown by silica gel TLC (benzene-methanol, 9:1) to contain a compound subsequently identified as **10**.

The oily crystals were chromatographed on silica gel (Grade 62, Davison) using a gradient from methylene chloride (4 liters) to methylene chloride-ether-methanol (4 liters) in the ratio 47:47:5. Fractions containing **10** were pooled, concentrated and decolorized on a Norite A/Celite (4 g: 20 g) column to give 6.9 g of a colorless solid. The solid was dissolved in 65 ml of the solvent system: *n*-heptane-ethyl acetate-methanol-water (27:18:18:2) and chromatographed on a 200 tube (Vm=Vs=40 ml) CRAIG counter-current apparatus. After 290 transfers the following tubes were pooled:

#11 to 40: Fraction A containing **10** (K=0.14)

#55 to 90: Fraction B containing **1** (K=0.30)

Fraction A was concentrated to dryness under reduced pressure and dissolved in acetone. Addition of hexane gave 460 mg of the sodium salt of **10**, mp 183~185°C, $[\alpha]_D^{20}$ −93.9° (*c* 1, $CHCl_3$). Calcd. for $C_{34}H_{53}NaO_8$: C 66.64, H 8.72, Na 3.75; Found: C 67.01, H 8.90, Na 3.43.

The sodium salt was converted to **10** by dissolving the salt in methylene chloride and washing with 1 N HCl. Crystallization was from methylene chloride-hexane to give **10**, mp 203°C, $[\alpha]_D^{20}$ −39.2°(*c* 1, $CHCl_3$). Calcd. for $C_{34}H_{54}O_8$: C 69.12, H 9.21; Found: C 69.27, H 9.11.

Retroaldol cleavage of **10** to the ketone 6a

To a solution of 500 mg **10** in 20 ml of *p*-dioxane was added 2 ml of 10 % aqueous NaOH. After 16 hours, the reaction mixture was diluted with water (50 ml) and extracted twice with ethyl acetate (2×50 ml). The extracts were pooled, dried (Na_2SO_4) and evaporated under reduced pressure to 300 mg of an oil, which was chromatographed over silica gel (18 g) using a solvent gradient of 1.5 liters of methylene chloride to 1.5 liters of methylene chloride-ether (4:1). Fractions containing **6a** were pooled and concentrated to 267 mg of an oil, which was rechromatographed over silica gel (40 g) eluting first with 200 ml methylene chloride followed by a gradient between 1 liter of methylene chloride to 1 liter methylene chloride-ether (9:1). This second column yielded 136 mg (45 %) of **6a** as a colorless oil, $[\alpha]_D^{20}$ −26.5° (*c* 1, $CHCl_3$). Calcd. for $C_{21}H_{38}O_4$: C 71.98, H 11.15; Found: C 72.22, H 11.16.

Preparation of **6b** by acetylation of retroaldol ketone **6a**

To a solution of 90 mg of **6a** in 10 ml of pyridine was added 0.5 ml acetic anhydride and the mixture stirred overnight. Water (10 ml) was added and the products extracted with chloroform (2×5 ml). The extract was washed in turn with 0.1 N HCl, saturated aqueous sodium carbonate and water and after drying (Na_2SO_4) was evaporated to an oil. The product was purified by preparative silica gel TLC (benzene-methanol, 60:1) to give as an oil, **6b**. Calcd. for $C_{23}H_{40}O_5$: C 69.66, H 10.17; Found: C 70.01, H 10.05.

Bromination of *iso*-lasalocid A

To a solution of 3.672 g (6 mmole) of the sodium salt of **10** in 500 ml of methylene chloride at 3°C, 0.323 ml (6.3 mmole) of bromine in 50 ml of methylene chloride was added over 1 hour. The solution was kept a further hour at 3°C and then allowed to warm up to 15°C at which point, 1 liter of water was added.

The solvent layer was removed, washed in turn with aqueous sodium bisulfite, aqueous sodium carbonate and water. After drying (Na_2SO_4), the solution was concentrated to an oil, which was crystallized from acetone-hexane to give 2.1 g (50.6 %) of the bromo derivative of *iso*-lasalocid A, sodium salt, mp 197~198°C, $[\alpha]_D^{20}$ −52.5° (*c* 1, $CHCl_3$). Calcd. for $C_{34}H_{52}BrNaO_8$: C 59.03, H 7.59, Br 11.55, Na 3.32; Found: C 59.22, H 7.71, Br 11.67, Na 3.46.

The second crop of crystals from acetone-hexane was dissolved in methylene chloride and washed with 1 N HCl. Evaporation of the solvent layer and crystallization from acetone-hexane gave 1.44 g (33.3 %) of the bromo derivative of *iso*-lasalocid A (see Fig. 1), which was subsequently used for X-ray analysis. The melting point of the crystals was 185~186°C, $[\alpha]_D^{20}$ −15.6° (*c* 1, $CHCl_3$). Calcd. for $C_{34}H_{53}BrO_8$: C 60.98, H 7.98, Br 11.93; Found: C 61.16, H 8.13, Br 11.96.

The crystal used for data collection on the HILGER-WATTS diffractometer was approximately 0.12×0.15×0.45 mm.

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