BIOSYNTHESIS OF LASALOCID A

BIOCHEMICAL ALTERATION OF POLYETHER ANTIBIOTIC PRODUCTION

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The effect of known precursors, their fluorinated analogs, and biochemical inhibitors on the production of the polyether antibiotic, lasalocid A (**1**), by resting cells of *Streptomyces lasaliensis* was determined to study the biochemistry and regulation of antibiotic biosynthesis *in vivo*.

We are interested in the biochemical parallels between the formation of fatty acids and polyketides, secondary metabolites which are made from simple fatty acids by many organisms. Polyether antibiotic formation is a polyketide pathway whose biochemistry is poorly understood, yet inherently fascinating.[1~3] We have uncovered considerable information about the biochemical mechanisms involved in the formation of lasalocid A (**1**) from the results of experiments with isotopically labeled precursors.[4] In this paper we present the results obtained from a study of the effects of known precursors, their fluorinated analogs, and various biochemical inhibitors on the formation of lasalocid metabolites by *Streptomyces lasaliensis in vivo* and discuss their implication in relation to its hypothetical biosynthetic pathway.

Results

The polyether antibiotics produced by *S. lasaliensis* include **1**, its four homologs (**2**~**5**), and isolasalocid A (**6**).[5,6] These compounds are formed by a pathway that has been postulated to involve the assembly of acetate, butyrate and propionate into a 34-carbon linear diene which is converted to **1**~**6** by oxidation with a monooxygenase and cyclization.[5,6] We studied the effect of precursor concentration, fluorinated precursor analogs, monooxygenase inhibitors, and protein biosynthesis inhibitors on the formation of the lasalocid pathway metabolites to determine three things: i) If their formation could be regulated by the concentration of the fatty acid precursor, ii) if inhibition of monooxygenase activity could reduce their formation, and iii) if new protein synthesis could be required for their formation. We also tested if the inhibition of polyether antibiotic formation could cause the accumulation of pathway intermediates since these hypothetical compounds[5,6] were apparently not accumulated by antibiotic non-producing *S. lasaliensis* mutants (H. KINASHI, unpublished results).

Lasalocid A appeared at *ca.* 70 hours after the cell growth rate had declined markedly in shake flask cultures using either a split pea - lard oil (SP/LO) or maltose - yeast extract - malt extract (MYM) media and continued to be produced up to *ca.* 168 hours (Fig. 1). Thus addition of compounds to fermentation flasks at 70 hours and thereafter was adopted to approximate a resting cell culture, which was used to determine the effect of the added compounds on polyether antibiotic production. All fermentations and polyether antibiotic assays were done in triplicate to reduce the effect of culture

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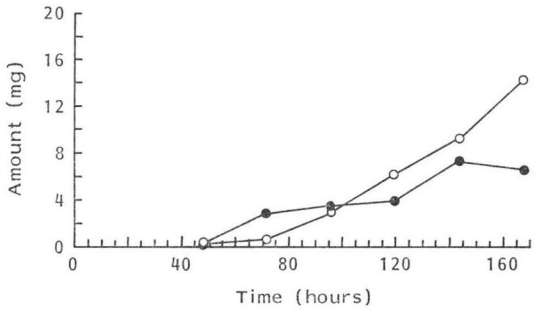
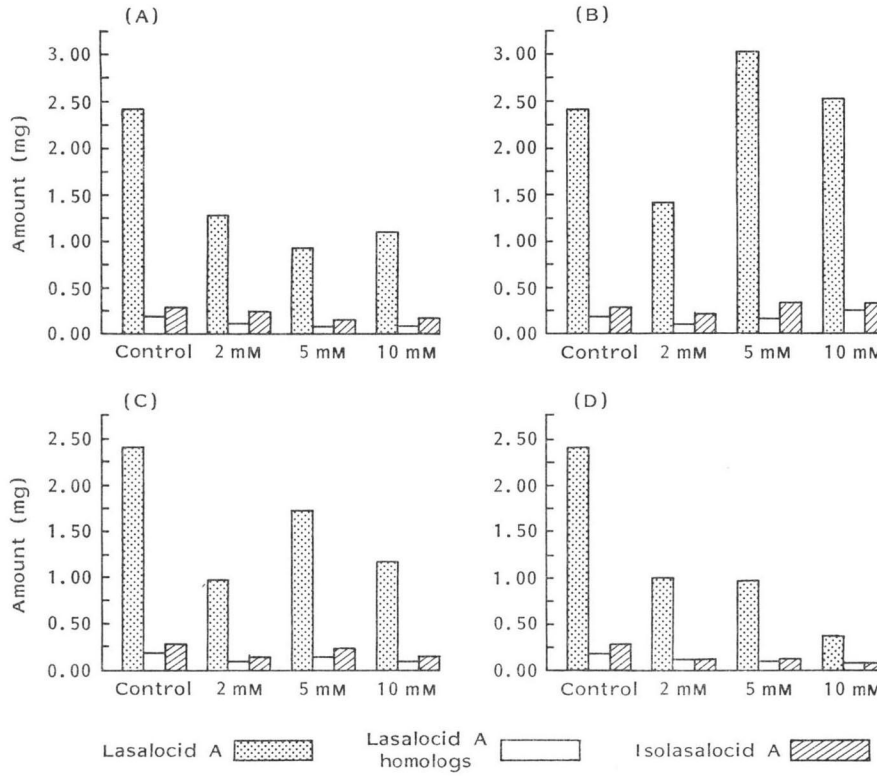
Fig. 1. Production of lasalocid A in the split pea - lard oil (SP/LO) and maltose - yeast extract - malt extract (MYM) medium.
○ SP/LO, ●MYM.



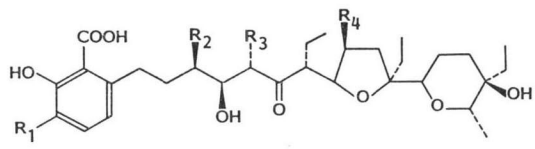
variability on the results.

We expected that the concentration of the four precursors – acetate, butyrate, propionate and succinate – in the fermentation medium would affect antibiotic yield based on the observations of DAY *et al.* with the monensin fermentation.[7] As shown in Fig. 2A ~ D, acetate, propionate and succinate, especially, inhibited polyether production at the higher concentrations used. The strong inhibitory effect of succinate was accompanied by a noticeable decrease in cell respiration as measured with an oxygen electrode; thus its effect most likely was due to inhibition of α-oxoglutarate dehydrogenase by succinylCoA[8] produced *in vivo* from the exogenous succinate. Butyrate caused an increase in polyether production at 5 mM, little change at 10 mM, and a decrease at 2 mM and greater than 10 mM (data not shown). It also increased the amount of compounds **2** ~ **5** relative to **1** at the highest concentration (Fig. 2B), which is consistent with the report of WESTLEY *et al.* that a deficiency of propionate in low-yield fermentations

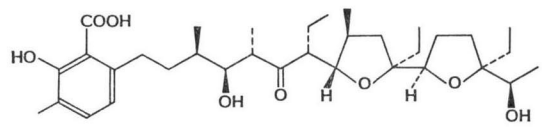
Fig. 2. The effect of varying concentrations of four known precursors on the production of lasalocid A, lasalocid A homologs and isolasalocid A.
The amounts of each compound produced in the baseline data experiment ((BDE), Experimental section) is shown on the ordinate.
(A) Acetate (BDE), (B) butyrate (BDE), (C) propionate (BDE), (D) succinate (BDE).



resulted in an increased production of the lasalocid A homologs.[5]



Lasalocid A (**1**) $R_1 = R_2 = R_3 = R_4 = CH_3$
Lasalocid B (**2**) $R_1 = C_2H_5$, $R_2 = R_3 = R_4 = CH_3$
Lasalocid C (**3**) $R_1 = R_3 = R_4 = CH_3$, $R_2 = C_2H_5$
Lasalocid D (**4**) $R_1 = R_2 = R_4 = CH_3$, $R_3 = C_2H_5$
Lasalocid E (**5**) $R_1 = R_2 = R_3 = CH_3$, $R_4 = C_2H_5$



Isolasalocid A (**6**)

Since fluorinated analogs of normal primary metabolites frequently inhibit the enzymes which process them,[9] we tested the effect of 2-fluoropropionate and 2-fluorosuccinate on the production of **1** because they could affect the biosynthesis of **1** by interfering with either the carboxylation of propionate to 2-methylmalonate,[4] the rearrangement of succinate to 2-methylmalonate,[4] or the assembly of the hypothetical 34-carbon intermediate.[5,6] These projections were based on the knowledge that 3-fluoropropionyl-CoA inhibited the carboxylation of propionyl-CoA *in vitro*,[10] that 3-chloropropionyl-CoA irreversibly inhibited mammalian fatty acid synthetase,[11] and that 2- and 3-fluoropropionate blocked the production of the macrolide antibiotic, erythromycin A (which is formed from propionate), by *Streptomyces erythraeus* when added to the fermentation medium.[12]

The production of **1** was affected in different ways when fluorinated propionate or succinate were added to the resting cell fermentation. Racemic 2-fluoropropionate increased production somewhat at 2 and 3.5 mM concentrations, but decreased it at 5~10 mM concentrations (Fig. 3A) due to the lower pH imparted to the cultures at harvest (6.6~7.0 vs. 7.3 for the control) at the higher concentrations. 2(*S*)-2-Fluoropropionate (the pH of its solution was adjusted to 7.4 before addition to the fermentation) or its ethyl ester increased lasalocid A production *ca.* 2- to 8-fold when added at 2~5 mM concentrations (Fig. 3B). Some of this increase was due to higher cell densities in the experimental cultures since the increase was only 1.3- to 1.5-fold at 108.5 hours on a mg of **1**/mg dry cell weight basis. Racemic 2-fluorosuccinate drastically decreased production when added in any concentration between 2 and 10 mM (Fig. 3C). As the final pH of these cultures at harvest was 3.2~4.1, this was the most likely reason for the low yield. The experiment was not repeated with the disodium salt of 2-fluorosuccinate due to its instability towards elimination of HF. The fluorinated succinate also caused a 50% decrease in the final cell density compared with the control, but none of the fluorinated propionates affected this value significantly at 2~10 mM levels.

We felt it was possible for 2(*S*)-2-fluoropropionate to be a substrate for propionyl-CoA carboxylase, which removes the 2 *pro R* hydrogen in forming (2*S*)-2-methylmalonyl-CoA[13]; if true, the 2-fluoro-2-methylmalonyl-CoA thus produced might have been incorporated into **1**. This possibility was invalidated by finding that lasalocid A produced from feeding [*U*-^{14}C]-2-(*S*)-2-fluoropropionate to *S. lasaliensis* contained neither ^{14}C nor ^{19}F as determined by radioactivity measurement and by ^{19}F NMR spectroscopy at 188 MHz.

We tested the ability of cerulenin to inhibit lasalocid A biosynthesis because cerulenin is an irreversible inhibitor of the acylthioester/α-carboxyacylthioester condensation step of fatty acid biosyn-

Fig. 3. The effect of varying concentrations of fluorinated analogs of propionate and succinate on the production of lasalocid A.

The IS No. refers to the experiment with that number in the Experimental section. The vertical arrow indicates when the fluorinated compound was added to the growth medium. The amount of lasalocid A produced by an aliquot of the culture medium is shown on the ordinate.

(A) Fluoropropionate (IS No. 5), ▼ control, ○ 2 mM, ● 3.5 mM, △ 5 mM, ▲ 6.5 mM, □ 8 mM, ■ 10 mM.

(B) Fluoropropionate (IS No. 9), ▼ control, ○ 2 mM (acid), ● 3.5 mM (acid), △ 5 mM (acid), □ 2 mM (ester), ■ 3.5 mM (ester).

(C) Fluorosuccinate (IS No. 8), ▼ control, ○ 2 mM, ● 3.5 mM, △ 5 mM, ▲ 6.5 mM, ■ 10 mM.

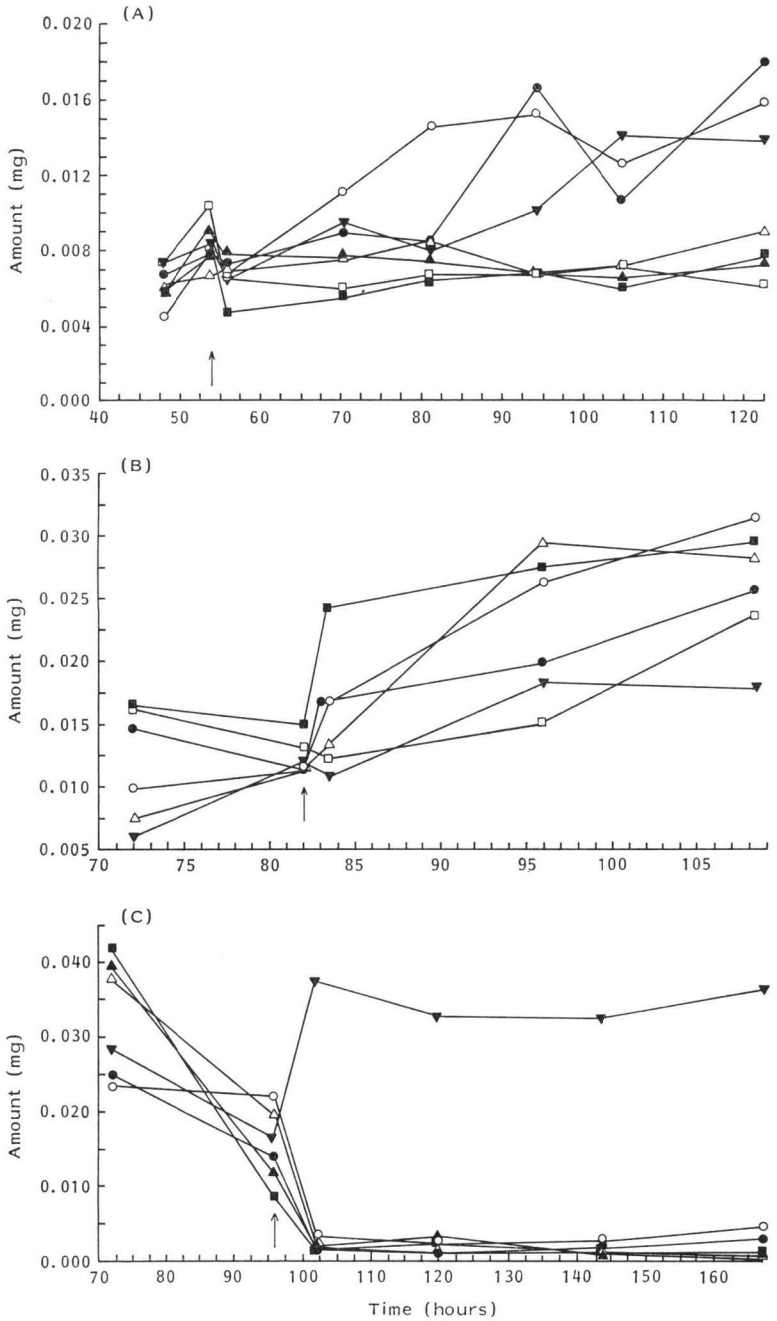


Fig. 4. The effect of varying concentrations of cerulenin on the production of lasalocid A (IS No. 8).

The IS No. refers to the experiment with that number in the Experimental section. The vertical arrow indicates when the cerulenin was added to the growth medium. The amount of lasalocid A produced by an aliquot of the culture is shown on the ordinate.

▽ Control, ○ 5 μg/ml, ● 10 μg/ml, □ 15 μg/ml, ■ 20 μg/ml, ▲ 30 μg/ml.

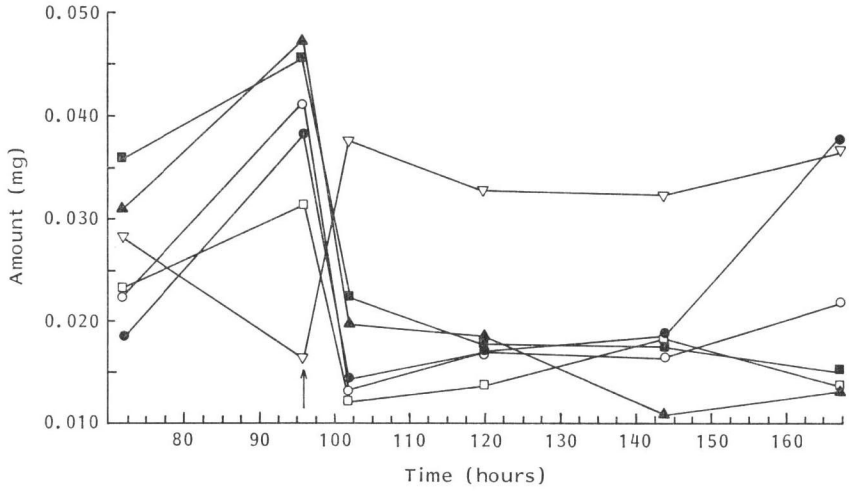
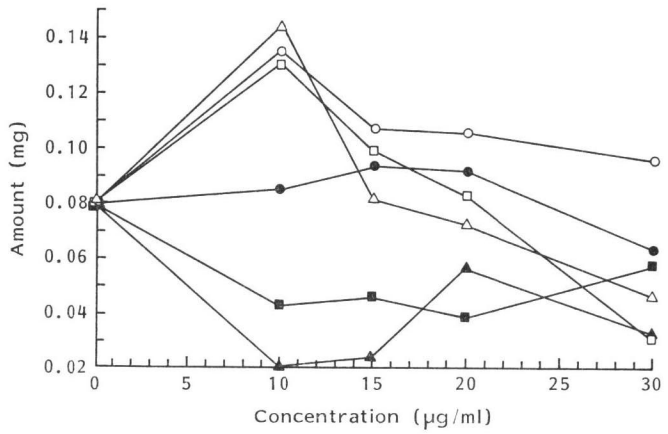


Fig. 5. The effect of varying concentrations of 2-diethyl GEB, AY 994 and SKF 525A in the growth medium on the production of lasalocid A (IS No. 7).

The IS No. refers to the experiment with that number in the Experimental section. The amount of lasalocid A produced by the culture is shown on the ordinate.

2-Diethyl GEB: □ GE, ■ GE(S)*. AY 994: ○ AY, ● AY(S)*. SKF 525A: △ SK, ▲ SK(S)*.

*(S)=Inhibitor also present in seed culture.



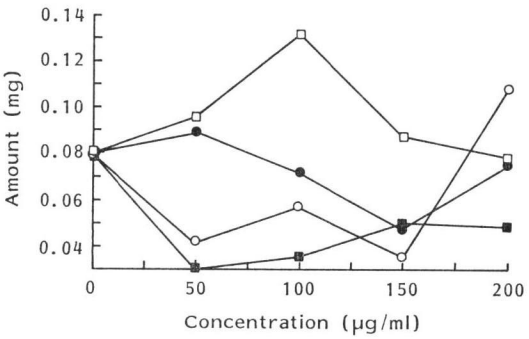
thesis and is known to inhibit the formation of several types of polyketide metabolites.[13] Polyether antibiotics had not been examined in this regard, however. When added to the resting cell fermentation at 5~30 μg/ml concentrations, cerulenin strongly inhibited the production of **1** (Fig. 4) although partial recovery from its effect was seen at the 5 and 10 μg/ml levels, presumably because the cells can slowly metabolize cerulenin.

Three known[14] inhibitors of monooxygenase enzyme activity in other bacteria – AY 994, 2-diethyl GEB and SKF 525A (see Experimental section) – were tested for their effect on the production of **1**. 2-

Fig. 6. The effect of varying concentrations of *p*-fluorophenylalanine and cycloheximide on the production of lasalocid A (IS No. 7).

The IS No. refers to the experiment with that number in the Experimental section. The amount of lasalocid A produced by the culture is shown on the ordinate.

p-Fluorophenylalanine: ○ PF, ● PF (S)*.
Cycloheximide: □ CH, ■ CH (S)*.
*(S)=Inhibitor also present in seed culture.



Diethyl GEB and SKF 525A, inhibited antibiotic production when added to the resting cell fermentation at 20 or 30 μg/ml concentrations, and more strongly when added to the fermentation medium along with the seed culture (Fig. 5); AY 994 had a negligible effect. If this result was due to inhibition of the oxidation of a cyclic lasalocid pathway intermediates, such compounds did not appear to accumulate and be secreted into the medium, since we could not detect them by chromatographic analysis of the material in EtOAc extracts of the fermentation medium.

Since inhibitors of protein biosynthesis or protein function can distinguish between the constitutive and inducible formation of enzymes, *i.e.*, can show if new protein synthesis is need for the biosynthesis of a metabolite,[1] we tested the effect of cycloheximide and *p*-fluorophenylalanine on the production of **1**. The latter compound also has been used for the production of abnormal metabolites of a pathway, which can result from the action of abnormal enzymes produced by the incorporation of *p*-fluorophenylalanine into cellular proteins.[1] Inhibition of lasalocid A production was seen with both inhibitors, even though cycloheximide is not known to inhibit protein biosynthesis in prokaryotes (Fig. 6). Although *p*-fluorophenylalanine inhibited antibiotic formation strongly, new compounds were not detected by chromatographic analysis of EtOAc extracts of cultures grown in the presence of this inhibitor. Therefore, new enzyme synthesis is required for the formation of lasalocid A, but abnormal enzymes, if produced, did not cause the accumulation of pathway intermediates.

Discussion

Although the use of whole cells in this study prevents a clear determination of the cause of all of our observations, the following conclusions seem warranted. (1) The carbon skeleton of **1** must be assembled by enzymes which functionally resemble fatty acid synthetases since cerulenin inhibits both systems. For **1** this would correspond to inhibition of the condensation of the acylthioester and either malonyl-, 2-methylmalonyl-, or 2-ethylmalonylthioester intermediates by the putative "polyether synthase." As this reaction would be crucial to assembly of the hypothetical 34-carbon pathway intermediate,[5,6] disruption of lasalocid A formation should result as observed. (2) It is likely that epoxidation of a diene intermediate is an essential step in the lasalocid A pathway as proposed by WESTLEY and co-workers[6] since two known monooxygenase inhibitors significantly decreased the formation of **1**. The fact that this diene did not appear to accumulate in the fermentation could be due to it being enzyme bound as proposed,[6] non-diffusible into the extracellular *mileau* if free, or simply produced in an amount too low for detection by the methods used. (3) The formation of **1** and related metabolites is regulated by the amounts of its two, three, and four carbon precursors in a way that must be determined by further study. That a fluorinated analog of one of these, 2-fluoropropionate, can markedly increase lasalocid A production is striking and suggests that the levels of enzymes which make or process propionate for lasalocid A biosynthesis are increased in the presence of this compound.

Since an increasing concentration of only *n*-butyrate in the fermentation was correlated with a rise

in the amount of lasalocid A homologs produced, an excess of only one of the three α-carboxythioester substrates used for assembly of the carbon skeleton can shift the assembly pattern towards the utilization of the most abundant one and thus alter the sequence of precursor assembly. It thus seems that the 2-ethylmalonylthioester substrate can be substituted for the 2-methylmalonylthioester substrate, but not the converse. This result suggests that polyether antibiotic homologs eventually could be constructed by suitable manipulations of this enzymatic system, once it becomes available for study.

Experimental

General Procedures

Streptomyces lasaliensis NRRL 3382R was used in all of the experiments. The organism was maintained as frozen spore suspensions in 20% glycerol at −80°C.

One vial of the frozen spore suspension (*ca.* 10^9 spores/ml) was used to inoculate 50 ml of media/250 ml culture flask. SP/LO[4] or MYM [Difco malt extract 10 g, Difco yeast extract 4 g, maltose 4 g, double distilled (dd) H_2O 1 liter] media were used for the seed cultures. This seed culture was grown (28~30°C, 250 rpm) to stationary phase (usually 72 hours) and then used to inoculate the fermentation media, which was either MYM, SP/LO or 14C (dextrose 10 g, yeast extract 2 g, monosodium glutamate 0.5 g, K_2HPO_4 0.5 g, dd H_2O 1 liter) media. Once the fermentations had been started, they remained in place during the entire fermentation without stopping the shaker until ready to harvest.

Assays for antibiotic production and mycelial weight were done during the fermentation by removing a 1.0 ml aliquot from the culture through a side arm on the flask and placing it in a 14×100 mm tube. Double distilled H_2O (*ca.* 0.5 ml) was added to each tube, then the tubes were vortexed briefly and centrifuged at 3,000 rpm for 5 minutes. The aqueous layer was removed and the cells were re-extracted with *ca.* 1 ml of fresh H_2O. $CHCl_3$ (1 ml) was added to the combined aqueous layers, the mixture was vortexed well, and the $CHCl_3$ layer was separated and evaporated under a stream of N_2.

The mycelial cells were transferred to a disposable aluminum weighing dish, dried at 90°C for 18 hours, then dried *in vacuo* at room temp for an additional 12 hours to obtain the dry cell weight (DCW). The packed cell volume (PCV) at the end of the fermentation was measured by decanting the entire contents of a flask into a 50-ml disposable conical centrifuge tube and centrifuging at 3,000 rpm for 15~20 minutes. The volume of the cell mass then was determined by the tube calibrations.

The residue from the $CHCl_3$ extract was dissolved in MeOH - $CHCl_3$ (3: 1) and used for HPLC analysis as described below to measure the amount of lasalocid A present and to determine the incorporation of ^{14}C-labeled precursors into it. The calculation for the amount of lasalocid A isolated was based on comparison of the HPLC detector responses with that obtained from a set of known antibiotic concentrations; the calculation for the radioactivity of the isolated lasalocid A was adjusted for the total amount of the fraction collected, and then divided by the amount of lasalocid A calculated to be present in the sample.

Cerulenin and *p*-fluoro-DL-phenylalanine were purchased from Sigma Chemical Co., St. Louis, MO. SKF 525A [(*N*,*N*-diethyl)-2′-aminoethyl-2,2-diphenylpentanoate hydrochloride] was a gift from Smith Kline & French Laboratiories. 2-Diethyl-GEB [3-β-(2-diethylaminoethoxy)androst-5-en-17-one hydrochloride] was a gift from the Upjohn Company. AY 994 [*trans*-1,4-*bis*(2-chlorobenzylaminomethyl)cyclohexane dihydrochloride] was a gift from the DuPont Company.

All precursors or inhibitors were prepared as aqueous solutions which were filter sterilized using a Millipore disposable filter unit (Millex-GS, 0.22 μm), except for the monooxygenase inhibitors and the protein inhibitors which were added to the seed cultures prior to sterilization following the procedure of CORCORAN and VYGANTAS.[14] All controls were fed sterile H_2O in a volume comparable to the volume of the inhibitor solution added.

Thin-layer Chromatography and Bioautography

TLC of the samples was done in duplicate on a Kieselgel 60 F254 plate by developing in EtOAc. One set of plates was visualized by an acid/vanillin/heat treatment, as described,[4] while the other set was subjected to bioautography as follows. A 2-ml spore suspension of *Bacillus subtilis* and 1 ml of 4%

aq 2,3,5-triphenyltetrazolium chloride solution were added to Difco Antibiotic Media No. 1 (200 ml) and mixed vigorously for 5 minutes. The entire mixture was poured slowly and evenly into a sterile Pyrex dish. The TLC plate was air dried and placed on the surface of the cooled agar. Once the SiO_2 layer had been H_2O saturated, the TLC plate was allowed to remain in position for 30~45 minutes before removing. The agar plate was then covered and incubated at 30°C for 8 hours. The amount of antibiotic was determined by comparison of the size of the growth inhibition zone against a standard dose-response curve prepared from lasalocid A.

Analytical and Preparative HPLC

A Waters μBondapak C_{18} column was used with a mobile phase of MeOH - 1% aq AcOH (75: 25) at a flow rate of 1.5 ml/minute. Samples were dissolved in 0.2 ml of MeOH - $CHCl_3$ (3: 1) for injection onto the column. The column effluent was monitored with a Waters Series 441 monitor at 254 nm and fractions were collected at 4-minute intervals. A normal column elution profile was: Echinomycin, 4 minutes; lasalocid A, 19 minutes; lasalocid homologs, 26 minutes; isolasalocid A, 31 minutes. The identity of all peaks was verified by standards obtained from Dr. JOHN WESTLEY, Hoffmann-La Roche Inc., Nutley, N.J., U.S.A.

Growth and Antibiotic Production

The seed cultures were used to inoculate (2%) 12 flasks of 50 ml SP/LO media/250 ml flask or 12 flasks of 50 ml MYM media/250 ml flask, respectively. Starting at 48 hours after inoculation, and repeating every 24 hours for 168 hours, 2 flasks of each media were removed from the shaker and worked-up as follows. Celite (5 g) and EtOAc (50 ml) were added to each flask, the mixture was stirred vigorously for 1~1.5 hours at room temp and filtered through Whatman No. 2 filter paper, then the filtrate was extracted with EtOAc (2×50 ml). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* on a rotary evaporator at 30°C. The resulting residue was assayed for antibiotic content by TLC and analytical HPLC as described above.

Baseline Data Experiment (BDE)

The seed culture for this experiment was grown in SP/LO media, then used to inoculate (2%) 39 flasks each containing 25 ml of SP/LO media/125 ml flask. The precursor solutions were added at 2, 5 and 10 mM levels at 72, 96, 120 and 144 hours after inoculation. Three flasks fed sterile H_2O were used as controls. At 168 hours all the flasks were harvested and worked up in triplicate groups as described above for the growth and antibiotic production. The residue from the EtOAc extracts was purified by preparative HPLC and the peak area was used to quantitate the amount of lasalocid A, lasalocid homologs and isolasalocid A present.

[*U*-^{14}C]-2(*S*)-2-Fluoropropionic Acid and its Ethyl Ester

These compounds were prepared according to the method of OLAH.[15] L-[*U*-^{14}C]Alanine (168.0 mCi/mmol, 100 μCi) was admixed with L-alanine (2.66 g, 29.9 mmol) to give a specific radioactivity of 3.3 μCi/mmol, and the mixture was dissolved in 75 ml of 70% polyhydrogen fluoride - pyridine contained in a teflon reaction vessel. While stirring, $NaNO_2$ (dried at 140°C for 24 hours, 3.1 g, 45 mmol) was added slowly. The reaction was run at room temp for 1 hour, quenched with ice/H_2O, then extracted 3~4 times with equal volumes of Et_2O. The combined Et_2O layers were washed with 10% aq $NaHCO_3$, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting residue was distilled by a Kugelrohr at 66~68°C, yielding [*U*-^{14}C]-2(*S*)-2-fluoropropionate (1.3 g, 12.2 mmol, 31.6% yield, 6.9×10^8 dpm/mmol). The optical rotation was not measured; for another sample of 2(*S*)-2-fluoropropionate prepared by the same procedure, the material was only 57% ee. The product was analyzed by ^{13}C NMR (50 MHz, $CDCl_3$) δ 130.5 (d, J=124 Hz, C-1), 85.2 (d, J=182.8 Hz, C-2), 18.12 (d, J=22.85 Hz, C-3); and a combined ^{1}H NMR (200 MHz) and ^{19}F NMR (188 MHz) analysis in ^2H_2O (assigned as an AMX_3 system) δ −184.56 (A, doublet of quartets, J_{AM}=48 Hz, J_{AX}=25.5 Hz), 4.95 (M, doublet of quartets, J_{AM}=48 Hz, J_{MX}=7 Hz), and 1.37 (X, J_{AX}=25.5 Hz, J_{MX}=7 Hz). The ^{19}F NMR resonances were assigned relative to $CFCl_3$ at 0.0.

Ethyl-[*U*-^{14}C]-2(*S*)-2-fluoropropionate was prepared by esterifying [*U*-^{14}C]-2(*S*)-2-propionic acid (300 mg, 3.26 mmol) with $CH_3CH_1N_2$ prepared from *N*-ethyl-*N*-nitrosourea (1 g, 8.5 mmol) to yield

ethyl-[*U*-^{14}C]-2(*S*)-2-fluoropropionate (189 mg, 1.57 mmol, 48% yield). Esterification was verified by ^{1}H NMR analysis.

(+/−)-2-Fluoropropionic Acid

The racemic fluoropropionate was prepared from ethyl 2-bromopropionate according to the procedure of ELKIK[16] followed by hydrolysis with 6 N HCl: BP 63~66°C (13 mmHg); ^{1}H NMR (90 MHz, $CDCl_3$) δ 5.05 (dq, $^{3}J_{HH}$=6 Hz, $^{3}J_{HF}$=48 Hz (CHF)), 1.60 (dd, $^{3}J_{HH}$=6 Hz, $^{3}J_{HF}$=24 Hz (CH_3CF)); MS *m/z* 92 (M^+).

Anal Calcd for $C_3H_5O_2F$: C 39.13, H 5.47, F 20.63.
Found: C 38.08, H 5.62, F 18.91.

(+/−)-2-Fluorosuccinic Acid

Racemic fluorosuccinic acid was prepared by the method of LOWE.[17] DL-Lactic acid was reacted with $SOCl_2$ - CH_3OH to give the dimethyl ester. This was reacted with Et_2NSF_3 - CH_2Cl_2 to give the 2-fluoro derivative, and then the dimethyl ester of the latter compound was hydrolyzed with H_3O^+ to give the 2-fluorosuccinic acid which was recrystallized from EtOAc (mp 145°C). The structure was verified by ^{1}H NMR (200 MHz) and ^{19}F NMR (188 MHz) of the succinic anhydride in D_2O (assigned as an AMX_2 system) δ −188.7 (A, doublet of triplets, J_{AM}=46.5 Hz, J_{AX}=25.5 Hz), 5.4 (M, doublet of triplets, J_{AM}=46.5 Hz, J_{MX}=5.0 Hz), and 3.15 (X, dd, J_{AX}=25.5 Hz, J_{MX}=5.0 Hz).

(+/−)-2-Fluoropropionic Acid (IS No. 5)

The seed cultures for this experiment were grown in 21 of 250-ml flasks each containing 50 ml of SP/LO media for 66 hours, the cells were spun down (7,500 rpm, 10 minutes), washed twice with Na_2PO_4 buffer (50 mM, pH 7.4) and resuspended in 1.0 ml of ^{14}C media, then transferred into 21 flasks each containing 50 ml of SP/LO media. At 54 hours the 2-fluoropropionic acid solutions were added to each flask at the following concentrations: 0, 2, 3.5, 6.5, 8 and 10 mM. Samples (1.0 ml aliquots) were removed from the flasks at t=0 (time of transfer), 6, 10.5, 24, 29.5, 35, 48, 54, 56, 70.5, 81.5, 94.5, 105 and 123 hours and were assayed by DCW and HPLC. After 123 hours, the flasks were harvested in triplicate groups and the final pH was recorded. The contents of the flasks in each group were combined, the PCV was measured, and the medium was extracted with EtOAc (3×100 ml). The EtOAc extract was dried over anhydrous Na_2SO_4 and evaporated, and the resulting residue was weighed, analyzed by analytical HPLC, and purified by preparative HPLC.

(+/−)-2-Fluorosuccinic Acid (IS No. 8)

The seed cultures for this experiment were grown in 50 ml of MYM media, then used to inoculate (2%) 18 flasks each containing 25 ml of MYM media/125 ml flask. The 2-fluorosuccinic acid was added at 96 hours at the following concentrations: 0, 2, 3.5, 5, 6.5 and 10 mM. The pH of the fluorosuccinate solution was 1.5. Samples (1.0 ml aliquots) were removed from the flasks at t=72 (after inoculation), 96, 102, 120, 144 and 167.5 hours and were assayed in triplicate by DCW and HPLC. After 167.5 hours, all the flasks were harvested and the pH and PCV was measured for each flask. The contents were extracted with 15 ml $CHCl_3$/25 ml media and the $CHCl_3$ extracts were analyzed by HPLC.

[*U*-^{14}C]-2(*S*)-2-Fluoropropionic Acid and Ethyl-[*U*-^{14}C]-2(*S*)-2-fluoropropionate (IS No. 9)

The seed cultures for this experiment were grown in 50 ml of MYM media and used to inoculate (2%) 22 flasks each containing 50 ml of MYM media/250 ml flask. The fluorinated propionate solutions were added at 82 hours as follows: 2 mM 2-fluoropropionic acid (4 flasks); 3.5 mM 2-fluoropropionic acid (4 flasks); 5 mM 2-fluoropropionic acid (4 flasks); 2 mM ethyl 2-fluoropropionate (3 flasks); 3.5 mM ethyl 2-fluoropropionate (3 flasks); sterile H_2O (4 flasks). Before addition, the pH of the fluorinated propionate solutions was adjusted to 7.4. Samples (1.0 ml aliquots) were removed from the flasks at t=72 (after inoculation), 82, 83.5, 96 and 108.5 hours and were assayed by DCW and HPLC. The flasks all were harvested at 124 hours and the pH and PCV of each flask was measured. The contents were extracted with 25 ml $CHCl_3$/50 ml media and the $CHCl_3$ extracts were analyzed by HPLC.

Cerulenin (IS No. 8)

The seed cultures for this experiment were grown in 50 ml of MYM medium and then used to inoculate (2%) 18 flasks each containing 25 ml of MYM media/125 ml flask. Cerulenin was added at 96 hours at the following concentrations: 5, 10, 15, 20 and 30 μg/ml per flask. Samples were removed (1.0 ml aliquots) at t=72 (time of inoculation), 96, 102, 120, 144 and 167.5 hours and analyzed for DCW and by HPLC. At 167.5 hours, all the flasks were harvested in triplicate groups by measuring the pH and PCV, then extracting the media with 15 ml $CHCl_3$/25 ml media. The $CHCl_3$ extracts were evaporated and 1/20 the total from each residue was analyzed by HPLC.

Protein Inhibitors and Mono-oxygenase Inhibitors (IS No. 7)

The seed cultures for this experiment were grown in 6 of 250-ml flasks containing 50 ml of MYM media. The following inhibitors were added to five of the seed culture flasks prior to sterilizing the medium: (i) 15 μg/ml 2-diethyl GEB, (ii) 15 μg/ml AY 994, (iii) 15 μg/ml SKF 525A, (iv) 100 μg/ml *p*-fluorophenylalanine, and (v) 100 μg/ml cycloheximide. The sixth flask contained no inhibitors. The seed cultures were grown for 72 hours, then 126 loosely plastic capped tubes (16×150 mm) with 5 ml MYM media/tube were inoculated (2%) with seed culture as follows: 12 tubes were inoculated with seed culture (i), 12 with seed culture (ii), 12 with seed culture (iii), 12 with seed culture (iv), 12 with seed culture (v), and 66 tubes with seed culture (vi). The tubes were then placed at *ca.* a 45° angle in wire test tube racks in the shaker at 28~30°C, 250 rpm.

To another set of tubes, the inhibitors were added at 80 hours after inoculation with the seed culture as follows: 2-diethyl GEB (at concentrations of 10, 15, 20 and 30 μg/ml) to 12 tubes containing seed culture (i) and 12 tubes containing seed culture (vi); AY 994 (at the same concentrations) to 12 tubes of seed culture (ii) and 12 tubes of seed culture (vi); SKF 525A (at the same concentrations as the *p*-fluorophenylalanine) to 12 tubes of seed culture (iii) and 12 tubes of seed culture (vi); *p*-fluorophenylalanine (at concentrations of 50, 100, 150 and 200 μg/ml) to 12 tubes of seed culture (iv) and 12 tubes of seed culture (vi); cycloheximide (at the same concentrations) to 12 tubes of seed culture (v) and 12 tubes of seed culture (vi). The six control tubes, inoculated with seed culture (vi), had H_2O added. At 120 hours after inoculation, all 126 tubes were harvested and worked up using the procedure given for the 1.0 ml aliquot assays. The DCW was measured and the entire $CHCl_3$ extract was analyzed by HPLC.

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