THE BIOSYNTHESIS AND PRODUCTION OF THE

POLYETHER ANTIBIOTIC LASALOCID A

A thesis submitted to the Graduate School of the University of Wisconsin-Madison in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

MARY MARTIN SHERMAN

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ACKNOWLEDGEMENTS

> So, if you think that what I say is true, then by all means agree with me; otherwise you must use all your resources of logic and arguement to refute me. Make sure I don't deceive you into sharing my own prejudices and then fly away, leaving my sting behind like a bee.
>
> PLATO, The Trial and Execution of Socrates

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LIST OF COMMON ABBREVIATIONS

1) ACP.....Acetyl Carrier Protein

2) ATP.....Adenosine Triphosphate

3) CoA.....Coenzyme A

4) C/MG.....Cpm per milligram

5) C/MM.....Cpm per millimole

6) cpm.....(Radioactivity) counts per minute

7) dpm.....Disintegrations per minute
(1 microcurie = 2.2×10^6 dpm)

8) DCW.....Dry Cell Weight

9) dd.....Double Distilled

10) FAS.....Fatty Acid Synthetase

11) HPLC.....High Performance Liquid Chromatography

12) mg.....Milligram

13) mmole.....Millimole

14) mL.....Milliliter

15) ML.....Mother Liquor

16) mp.....Melting Point

17) MS.....Mass Spectrum or Mass Spectral

18) NA.....(Data) Not Available

19) NMR.....Nuclear Magnetic Resonance

20) PCV.....Packed Cell Volume

21) PLC.....Preparative Layer Chromatography

22) S/N.....Signal-to-Noise-Ratio

22) TLC.....Thin Layer Chromatography

THE BIOSYNTHESIS AND PRODUCTION OF THE

POLYETHER ANTIBIOTIC LASALOCID A

Mary Martin Sherman

Under the supervision of Professor C. Richard Hutchinson

Lasalocid A is a polyether ionophore antibiotic produced by Streptomyces lasaliensis. It has been proposed that lasalocid A is formed on a multienzyme complex, similar to fatty acids, by the repetitive addition of simple C-2 to C-4 subunits. Unlike fatty acids, laslaocid A is a structurally complex molecule in which the biosynthesis involves the use of uncommon precursors, a specific order in which the subunits are assembled, and a precise stereochemical configuration at 10 of the carbons in the molecule. The study of lasalocid A biosynthesis was undertaken in order to better understand the complexities of this polyketide assembly pathway and its relationship to fatty acid biosynthesis; in particular, the investigations focused on the considering the origin of the C-2 to C-4 subunits and the process which controls the absolute configuration of the chiral centers.

The biosynthetic studies were approached by determining the isotopic distribution in lasalocid A by nuclear magnetic resonance and mass spectroscopic analysis of samples labelled by the incorporation of acetate, butyrate, isobutyrate, propionate and succinate in a series of different feeding experiments. The results of the precursor studies have shown that the three main subunits, acetate, propionate and butyrate, are available through

normal primary metabolic pathways and also through the interconversion of these three precursors. The interconversion between acetate and propionate, and acetate and butyrate can be explained by primary metabolic pathways, and the interconversion of propionate and butyrate has been shown to occur by the common intermediate isobutyrate. The results of the feeding experiments with the $^{13}C/^{18}O$ labelled precursors suggest that the stereocontrol exerted during the formation of the hydroxymethylene centers occurs either by stereodivergent reductases or by regiospecific epimerases during carbon chain assembly or on a free intermediate; they also support the hypothesis that lasalocid A is formed via a diene intermediate which undergoes air oxidation and subsequent cyclization to give the cyclic ethers. The studies on lasalocid A using labelled propionate, succinate and butyrate have shown that the carbon chain extension process occurs by condensation of the (2S) enantiomer of the activated CoA ester of the subunit to the growing carbon chain, and that either a racemization occurs at the time of the condensation, or a regiospecific epimerization occurs after the carbon chain has been formed.

A second series of studies was undertaken in order to investigate what factors affect the production of the polyether antibiotic laslaocid A. The following five things were examined: the effect of precursor pressure as a result of adding various levels of exogenous substrates; the effect of the fatty acid and polyketide synthetase inhibitor, cerulenin; the effect of

mono-oxygenase inhibitors, especially with regard to the putative diene biosynthetic intermediate; the effect of protein synthesis inhibitors on the antibiotic synthetase enzymes; the effect of fluorinated substrate analogues. The results of these studies were consitent with the formation of lasalocid A by a polyketide pathway in which an intermediate is oxidized by a mono-oxygenase and whose regulation is sensitive to the concentrations of normal substrates and their analogues.

INTRODUCTION

THE THESIS PROBLEM

Biosynthetic studies of secondary metabolites are often based on a comparison with the biochemistry of primary metabolites. One classical example is the comparison of polyketide biosynthesis and fatty acid biosynthesis. Polyketides are a class of secondary metabolites which are assembled from acetate subunits in a manner analogous to fatty acids; the primary difference between the two substances being the notable lack of reduction or partial reduction of the carbonyls, thus producing a wide variety of structures. The field of polyketide biosynthetic studies began in 1907 when Collie published his paper on "Derivatives of the Multiple Keten Group" in which he noted that several plant metabolites had structures that could be formed from the polymerization of the acetate unit, which he called the "Keten" group. This idea was later developed experimentally by Birch and Donovan (1953). However, it was not until 1961 when Lynen and Tada proposed a biochemical basis for the Polyacetate Rule, in which they suggested a similarity between fatty acid biosynthesis and that of several aromatic secondary metabolites. In particular, Lynen and Tada proposed a hypothetical scheme for the biosynthesis of 6-Methylsalycylic acid (6-MSA) on a multienzyme complex similar to that for fatty acid biosynthesis. Since Lynen and Tada's work, several other secondary metabolites of the polyketide class have been proposed to be synthesized similar to

fatty acids; of particular interest are the macrolide and polyether antibiotics produced by Streptomyces spp.

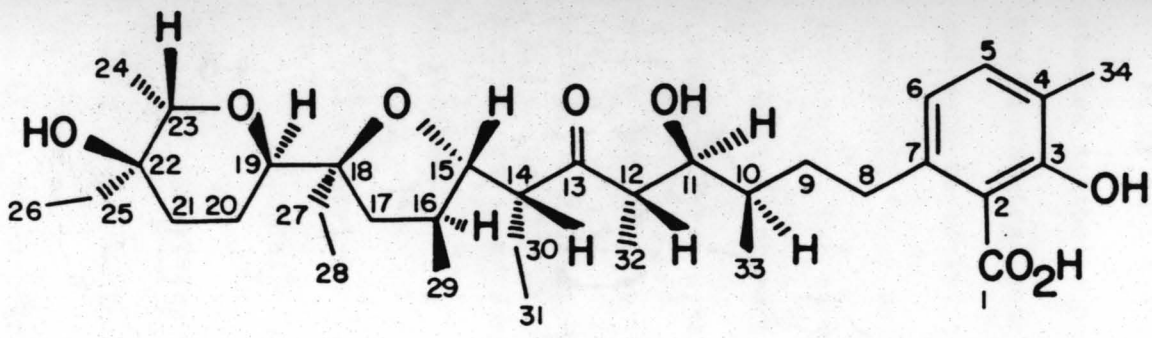
Streptomyces are gram-positive, aerobic soil bacteria belonging to the order Actinomycetes. The Actinomycetes produce the greatest number and variety of antibiotics. Lasalocid A (3-methyl-6-{7-ethyl-4-hydroxy-3,5-dimethyl-6-oxo-7-[5-ethyl-3-methyl-5-(5-ethyl-5-hydroxy-6-methyl-2-tetrahydropyranyl)-2-tetrahydrofuryl]heptyl}-salicylic acid) is a polyether ionophore antibiotic produced by Streptomyces lasaliensis. It has been proposed that lasalocid A is formed on a multienzyme complex, similar to fatty acids, by the repetitive addition of simple C-2 to C-4 acids (Westley, 1974; see Figure 1). In this work, these acid precursors or "building blocks" of the molecule, whether a fatty acid or a polyketide, will be referred to as subunits. In fatty acid biosynthesis, the repetitive addition of the same subunit (the C-2 acid: acetate or its equivalent malonyl-CoA) occurs by a stereochemically uniform mechanism. In lasalocid A biosynthesis, three different subunits are used to produce a stereochemically complex molecule with 10 asymmetric centers. The study of lasalocid A biosynthesis was undertaken in order to better understand the complexities of this polyketide assembly pathway and its relationship to fatty acid biosynthesis. The focus of the work was on three main questions.

(i) What are the origins of the C-2 to C-4 subunits?

(ii) What determines the order of assembly of the subunits?

(iii) What process controls the absolute configuration of the chiral centers?

Figure 1: Biosynthetic Precursors of Lasalocid A



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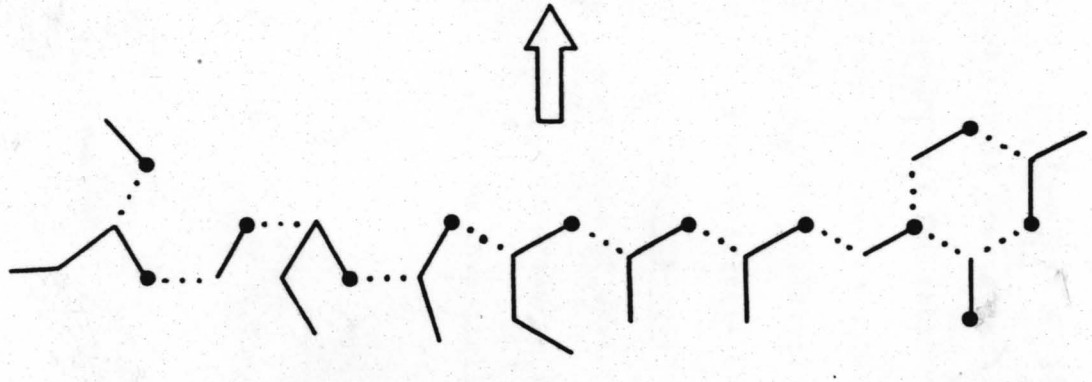


5 — CH_3CO_2H

4 — $CH_3CH_2CO_2H$

3 — $CH_3CH_2CH_2CO_2H$

Biosynthetic Precursors of Lasalocid A (I)

GENERAL BACKGROUND

Fatty Acid Biosynthesis

Fatty acid biosynthesis represents one of the most thoroughly studied metabolic pathways. It has been studied with regards to the biochemical reactions, their stereochemistry, the enzymology and more recently, the genetics. The ability to synthesize long-chain fatty acids is ubiquitous in nature. Fatty acid synthetase, the enzyme system which catalyzes the synthesis of long-chain fatty acids from simple acetate units, has been found in such diverse systems as Escherichia coli, plant chloroplasts and higher vertebrates. Regardless of what system is being studied, the chemical reactions involved in fatty acid biosynthesis have been found to be essentially the same. They are as follows.

(i) Acetyl-CoA Carboxylase

$$CH_3COCoA + HCO_3^- + ATP \xrightleftharpoons[Biotin]{} {}^-O_2CCH_2COSCoA$$

(ii) Acetyl Transacylase

$$CH_3COS\text{-}CoA + ACP\text{-}SH \rightleftharpoons CH_3COS\text{-}ACP + CoA\text{-}SH$$

(iii) Malonyl Transacylase

$$HO_2C\text{-}CH_2COS\text{-}CoA + ACP\text{-}SH \rightleftharpoons HO_2C\text{-}CH_2COS\text{-}ACP + CoA\text{-}SH$$

(iv) β-Ketoacyl-ACP Synthetase (Condensing Enzyme)

$CH_3COS\text{-}ACP + Enz\text{-}SH \rightleftharpoons CH_3COS\text{-}Enz + ACP\text{-}SH$

$CH_3COS\text{-}Enz + HO_2C\text{-}CH_2\text{-}COS\text{-}ACP \longrightarrow$

$CH_3COCH_2COS\text{-}ACP + CO_2 + Enz\text{-}SH$

(v) β-Ketoacyl-ACP Reductase

$CH_3COCH_2COS\text{-}ACP + NADPH + H^+ \rightleftharpoons$

$CH_3CHOHCH_2COS\text{-}ACP + NADP^+$

(vi) β-Hydroxyacyl-ACP Dehydratase

$CH_3CHOHCH_2COS\text{-}ACP \rightleftharpoons$ trans $CH_3CH{=}CHCOS\text{-}ACP + H_2O$

(vii) Enoyl-ACP Reductase $\longrightarrow$

$CH_3CH{=}CHCOS\text{-}ACP + NADPH + H^+$

$CH_3CH_2CH_2COS\text{-}ACP + NADP^+$

(viii) Chain Elongation

Repeat (iii) through (vii) seven times

(viii) Chain Termination

a) Animals: Thioesterase

b) Yeast: Acyltransferase

c) Bacteria: Thioesterase

The Fatty Acid Synthetase (FAS) system comprises reactions (ii) through (vii); the initial reaction catalyzed by Acetyl-CoA Carboxylase and the final chain termination reaction are separate enzymes.

The stereochemical course of these reactions has been worked out and found to be highly stereospecific throughout the entire sequence. Reaction (i), catalyzed by Acetyl-CoA carboxylase, preferentially uses the 2S enantiomer of [^{2}H,^{3}H]-acetyl-CoA and proceeds with retention of configuration (Sedgwick et al., 1977; Sedgwick and Cornforth, 1977). The reaction catalyzed by β-ketoacyl Synthetase (iv) is a concerted displacement reaction (Arnstadt et al., 1975) that occurs with inversion of configuration at C-2 of [^{2}H,^{3}H]-malonyl CoA. β-ketoacyl Reductase (reaction v) is an NADPH specific reduction in all cases, and produces the 3(R) β-hydroxy thiolester (Lynen, 1961; Wakil et al., 1962). The dehydration reaction (vi) is a syn elimination of HOH (Sedgwick et al., 1978). The Enoyl Reductase reaction is the first point of diversification in the pathway. In animals the reduction is NADPH specific; in yeast it requires in addition to NADPH, the cofactor FMN; in E. coli, there are two separate enzymes, one is NADPH specific and carries out a re-attack on the olefin, while the other enzyme is NADH specific and carries out a si-attack. In the case of yeast (Sedgwick and Morris, 1980), the Enoyl Reductase proceeds with an anti-addition of hydrogen; the H^- from NADPH is donated to the 3-proS position, while H^+ from the media is donated to the 2-proR position. In the final step, chain termination, again there is considerable diversification. In animals as in procaryotes, the final thioester is hydrolyzed to the free fatty acid by a Thioesterase, while in yeast the chain is

terminated by an Acyltransferase, giving the CoA ester of the fatty acid.

While the chemical reactions are essentially analogous in all systems, this similarity ends with the molecular organization of the Fatty Acid Synthetase enzyme system. In 1969, Brindley suggested a classification system for the two prototype Fatty Acid Synthetase enzyme systems already described in the literature. Brindley referred to Type I Synthetase as those enzymes which were multienzyme complexes, required no acetyl carrier protein (ACP), and had a molecular weight in the range of 0.5×10^6 to 2.3×10^6. The enymes from yeast, pigeon liver, rat liver and Mycobacteium phlei, among others, were classified as Type I. The Type II Synthetase enzyme systems were individual enzymes which jointly catalyzed a multi-step reaction; they were non-aggregated and required ACP. The enzymes from E. coli, Clostridium spp., Pseudomonas spp., Bacillus spp. and plant chloroplasts were included in the Type II classification. More recently (Wakil and Stoops, 1983), the classification has been further delineated to distinguish the Type IA Synthetases (those from animal tissues) from the Type IB Synthetases (those from yeast and higher bacteria).

Considerable work has been done towards elucidating the molecular and structural organization and the physiological properties of the various synthetases. Most of this information has been reviewed recently (see for example, Bloch and Vance,

1977; Wakil and Stoops, 1983; Wakil et al., 1983; Alberts and Greenspan, 1984). Some of the pertinent information is summarized here.

Among the Type II Synthetase systems, that from E. coli has been most thoroughly studied. Since this enzyme system is actually a series of non-aggregated, monofunctional enzymes, it has been most useful in studying the individual reactions. On the other hand, the Type I Synthetases have proven to be interesting with respect to their molecular and structural organization.

It was originally believed that the Type I Synthetases were simply a more organized form of the seven Type II Synthetase enzymes. The Type I enzymes were considered to be multi-enzyme complexes that could not be dissociated into their component enzymes due to tight protein-protein interactions. Brindley noted that both Type I and Type II Synthetases utilized ACP, but that Type I enzymes had the ACP molecule bound into the complex, while Type II enzymes required the ACP molecule for activation. While the animal FAS enzymes were classified together with those from yeast, it was recognized that the yeast complexes were considerably larger based on their molecular weight and that there were differences in the reactions, such as co-factor requirements and mechanisms for chain termination. Initially the higher molecular weight of the yeast enzymes was attributed to a higher degree of complexation of the subunit. Although this latter idea is basically correct, the picture that has emerged recently has

further defined the differences between the two Type I Synthetase enzyme systems and redefined the concepts of multi-functional proteins.

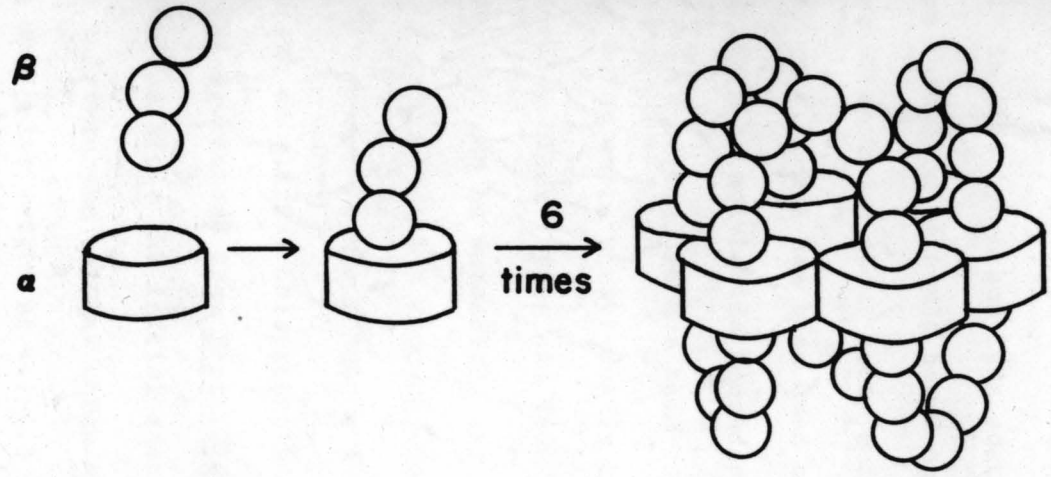
Much of the recent work on FAS enzymes has been advanced with the use of yeast FAS mutants (summarized in Schweizer et al., 1978). Two important conclusions have come out of this work. First, the isolation of fatty acid synthesis-deficient mutants in yeast led to the mapping of the fas1, fas2 and fas3 gene loci. The fas3 gene codes for acetyl-CoA carboxylase, while fas1 and fas2 code for the FAS system. It was found that the fas1 and fas2 genes are unlinked. Secondly, the isolation of a FAS mutant with lowered protease levels finally allowed the separation of the different subunits of the yeast FAS, which have been studied biochemically and by electron microscopy. It is now known that the yeast FAS system is composed of two different subunits, designated alpha and beta in the oligomeric form of $\alpha_6\beta_6$. The alpha subunit has a molecular weight of 185,000 daltons, is encoded for by the fas2 gene and contains the ACP molecules, the β-ketoacyl Synthetase and the β-ketoacyl Reductase activities. The beta subunit has a molecular weight of 180,000 daltons, is encoded for by the fas1 gene and contains the Enoyl Reductase, Dehydratase, Acetyl Transacylase and Malonyl Transacylase activities. The alpha and beta subunits differ in their structure; the alpha subunit appearing as a plate-like structure in electronmicrographs, while the beta subunit is represented by

arches on either side of the plates. The alpha subunits are arranged in reverse order so that there is a head-to-tail organization of the six sites for fatty acid synthesis (Figure 2).

The yeast FAS system has now been classified as a Type IB Synthetase. Also in this class are the synthetases consisting of oligomers of either similar subunits, such as Mycobacterium smegmatis which is an α_6-α_8 oligomer, or different subunits, as in the case of yeast. Recently, the partial purification of FAS from Streptomyces coelicolor was reported (Flatman and Packter, 1983). The initial findings for this FAS indicate that it might belong to the Type IA category. The Type IA Synthetases are from animal tissues and consist of two identical subunits, α_2. What this implies is that all of the catalytic functions involved in fatty acid biosythesis in animal tissues must be contained within one subunit, or one multi-functional protein. The fact that yeast FAS is encoded for by two unlinked gene clusters, fas1 and fas2, and the idea that animal FAS could possibly be encoded for by a single cluster gene has completely revised the understanding of the structural organization of FAS enzyme systems.

Figure 2: Proposed Model of Yeast Fatty Acid Synthetase
(Adapted from: Wakil, et. al., 1983)

YEAST FATTY ACID SYNTHETASE



α : MW 185,000

β-ketoacyl synthetase (CYS-SH)
4'-phosphopantetheine (ACP-SH)
β-ketoacyl reductase (NADPH)

fas 2

β : MW 180,000

acetyl transferase
malonyl " "
palmitoyl " "
dehydratase
enoyl reductase (NADPH, FMN)

fas 1

6-Methylsalicylic Acid Biosynthesis

6-Methylsalicylic acid (6-MSA) was first isolated from Penicillium griseofulvum, a fungus, in 1931 (Anslow and Raistrick, 1931). It has since been found in Mycobacterium spp. and in chloroplasts from dark grown barley leaves (Dain and Bentley, 1971). In 1961 Lynen and Tada postulated a hypothetical scheme for the formation of 6-MSA based on the polyketide hypothesis and what was then known about fatty acid biosynthesis. Since their initial work, 6-MSA has been used as a model for understanding the biosynthesis of polyketide secondary metabolites.

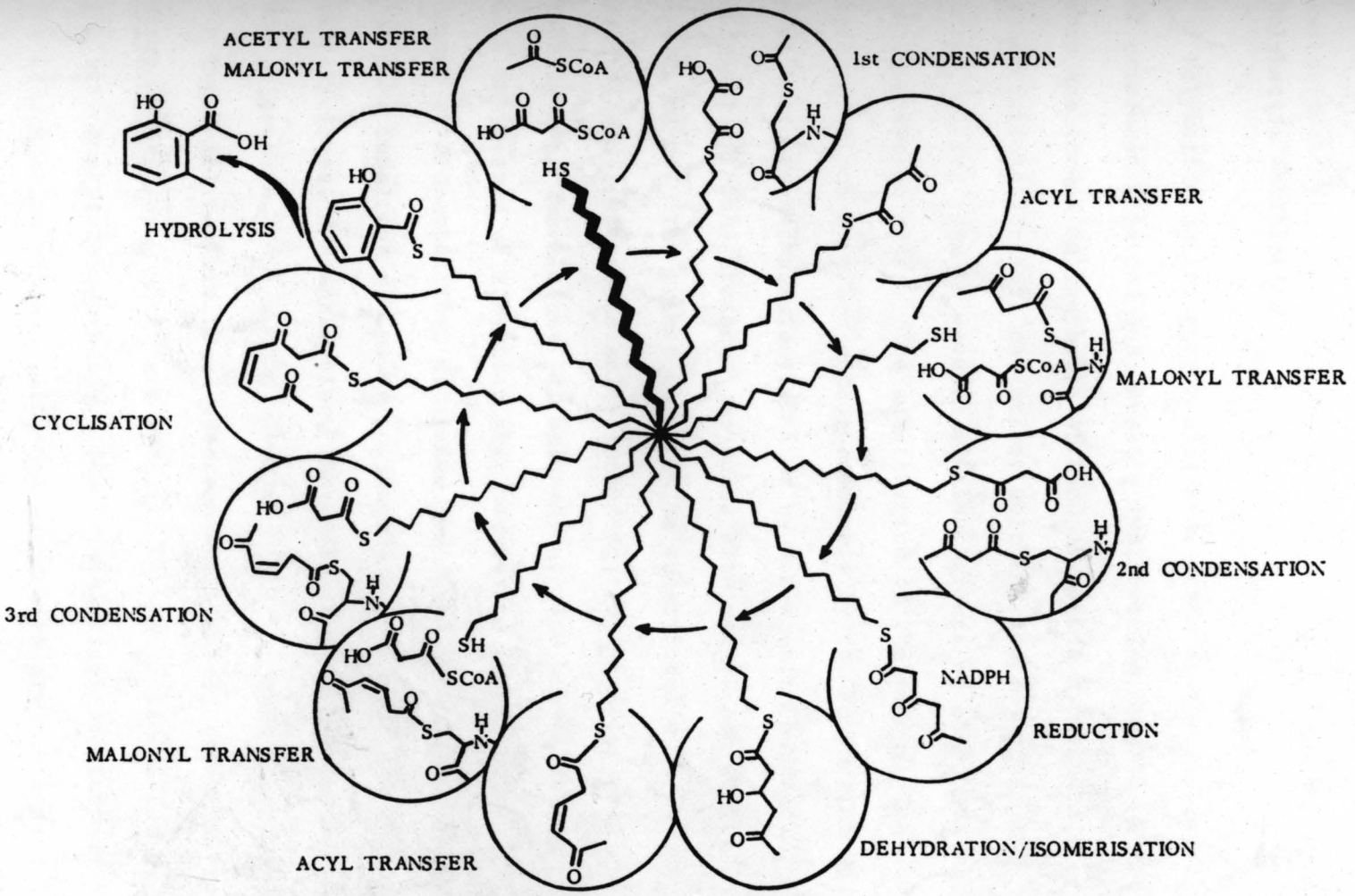
6-MSA is derived from a head-to-tail Claisen condensation of acetate units where acetyl-CoA is the primer and malonyl-CoA is the building block (Lynen and Tada, 1961; Dain and Bentley, 1971). The reaction can be summarized as:

$$CH_3CO\text{-}SCoA + 3\ HOOC\text{-}CH_2\text{-}CO\text{-}SCoA + NADPH + H^+$$
$$\longrightarrow 6\text{-MSA} + 3CO_2 + 4\ HSCoA + NADP^+ + H_2O.$$

The enzyme catalyzing this reaction is known as 6-MSA Synthetase. The 6-MSA Synthetase from Penicillium patulum can be separated from the FAS after the initial steps of purification. It is a stable multi-enzyme complex which migrates as a single particle with a molecular weight of $1.1\text{-}1.5 \times 10^6$ daltons (Dimroth et. al., 1970). It has been implicated to contain distinct central and peripheral sulfhydryl groups that form a covalent attachment to the substrates and intermediates during the synthesis of the 6-MSA molecule (Dimroth et al., 1976). However, unlike FAS, no ACP or

4'-phosphopantetheine molecules have been isolated from the 6-MSA Synthetase (Scott et al., 1974). 6-MSA Synthetase, like FAS, is susceptible to the acetylenic inhibitors 3-pentynoyl-NAC, 2-hexynoyl-NAC and iodoacetamide. 6-MSA synthesis differs from fatty acid synthesis in that only one reduction step is required for three condensation reactions; and in the dehydration step, where a cis intermediate is presumed to be formed, rather than the trans-enoyl derivative in the fatty acid pathway. 6-MSA Synthetase is specific for the priming substrate; substitution with propionyl-CoA led to the formation of 6-ethylsalicylic acid at reduced rates (Dimroth et al., 1976). In the absence of NADPH, 6-MSA Synthetase catalyzes the formation of triacetic acid lactone (TAL), a 6-carbon cyclic intermediate. The finding of TAL as a side product, led to the hypothesis that the reduction step in 6-MSA synthesis is prior to the third condensation reaction. The current model for 6-MSA Synthetase is illustrated in Figure 3.

Figure 3: Proposed Model of 6-MSA Synthetase
(From: Scott, et. al., 1974)


ACETYL TRANSFER
MALONYL TRANSFER
1st CONDENSATION
HYDROLYSIS
ACYL TRANSFER
MALONYL TRANSFER
CYCLISATION
2nd CONDENSATION
3rd CONDENSATION
NADPH
REDUCTION
MALONYL TRANSFER
ACYL TRANSFER
DEHYDRATION/ISOMERISATION

Polyketide Antibiotics

Polyketide antibiotics represent a structurally diverse class of compounds (Lancini and Parenti, 1982; Vining, 1983). While they are formed on the basis of the polyketide hypothesis, substitutions for both the primer unit and the building block occur, giving rise to several distinct groups of compounds within the polyketide class. The substitution of the malonyl-CoA building block by a C-3 unit, presumably by the conversion of propionate to methylmalonyl-CoA or by a C-4 unit (butyrate to ethylmalonyl-CoA) produces compounds with methyl and ethyl substituents. The primer unit can be substituted with any of several small activated acids including, for example, propionate, isobutyrate, isovalerate, or malonamide. The substitutions, along with the degree of reduction that occurs and consequently the degree of aromatization, determine the final structure. Some of the more important groups of polyketide antibiotics are the tetracyclines, the ansamycins, the macrolides, the polyenes and the polyethers.

Tetracyclines are broad spectrum antibiotics, isolated from Streptomyces spp., which are specific for bacteria and act by inhibiting protein synthesis. The tetracyclines are formed from the condensation of malonamide-CoA ester with eight malonate units to give methylpretetramide, which is the common precursor of tetracycline, chlortetracycline and oxytetracycline.

Methylpretatramide is fully aromatic and represents one of the simpler polyketide structures in that only the primer unit varies from the classical polyketide structure.

The ansamycins have been isolated from Norcardia spp., an actinomycete related to the Streptomyces. The ansamycins are divided into two general classes; the benzene ansamysins and the napthalene ansamysins. The benzene ansamysins, such as geldanamycin and maytansin, are generally toxic. The napthalene ansaymcins, including rifamycin, specifically inhibit RNA synthesis in bacteria by forming a complex with the bacterial RNA polymerase. The ansamycins are characterized by the ansa nucleus which is a cyclic structure consisting of the aromatic nucleus joined to a macrocycle by a lactam. The primer unit in the ansa nucleus is 3-amino-5-hydroxybenzoic acid. In the case of the napthalene ansamycins, the macrocyclic chain is formed from the condensation of two malonate units and 8 methylmalonate units. The ansamycins represent a group of polyketide antibiotics that have a composite structure in that they are both aromatic and macrocylic.

The macrolides isolated from Streptomyces spp. are typically 12- 14- or 16-membered macrocyclic lactones with deoxysugar substituents. They should be distinguished from the recently discovered avermectins, milbemycins and nargenicins, which are fused macrolides, and from the polyenes, which are large macrolide structures. The typical macrolides are antibacterial agents which

act by specifically inhibiting bacterial protein synthesis by binding to the 50S ribosomal subunit. Among this class of compounds in current use clinically are erythromycin, spiramycin, leucomycin and tylosin, the latter being used only in veterinary medicine. The aglycone portion of the molecule is formed initially, usually from acetate and propionate units; frequently a propionate serves as the primer. The condensation is assumed to follow the normal polyketide pathway; however, due to the extensive reduction of the carbonyls and the presence of methyl substituents from the propionates, no intramolecular condensation occurs to yield aromatic rings.

The polyenes, isolated from Streptomyces, are considered to be a subgroup of the macrolides described above. The polyenes are macrocylic lactones containing 26-38 carbon atoms and are specifically antifungal agents. The polyenes complex with the sterols in the cell membranes of eucaryotes to alter membrane function and integrity, consequently they are also toxic. Typical of this group of compounds are candicidin, nystatin and amphotericin B. For the polyene structures containing an aromatic moiety, this appears to be the primer unit and most likely originates from p-aminobenzoic acid, rather than from a polyketide pathway. The remainder of the aglycone portion is formed similar to the typical macrolides by condensation of acetate and propionate units.

Polyether Antibiotics

The polyether antibiotics are characterized by their diverse and complex structures and distinct mode-of-action (Westley, 1982; 1983); they occur naturally in at least 53 different species of Streptomyces (Westley, 1982). The discovery of this group of antibiotics dates back to 1951 when Berger et al., first reported the isolation of antibiotics X-206, X-464 (nigericin) and X-537A (lasalocid A); at the same time nigericin was isolated by a second group (Harned et al, 1951). While the structure of these compounds was not elucidated for nearly 18 years, their unusual properties were recognized immediately.

Polyether antibiotics range in molecular weight from 493 (Antibiotic X-14547A) to 1060 (K-41B). While the polyethers are characteristically acidic, they are more soluble in organic solvents than in aqueous bases, even in their salt form. In 1968, the first structure of a polyether antibiotic, monensin, was reported (Agtarap et al., 1967). This report was followed by the structure of nigericin (Steinrauf et al.,1968; Kubota et al., 1968), of lasalocid A (Westley et al., 1970; Johnson et al., 1970) and others. The structural elucidation of these compounds led to the recognition of common structural features, for example, one terminus contains a carboxylic acid, while the other terminus has a hydroxyl function. Polyethers are capable of forming hydrogen bonds between their two terminii giving a cyclic structure where the oxygen functions are concentrated towards the center. This

cyclization produces a lipohilic complex that is capable of binding and transportating ions, and thus conferring on the polyether antibiotics the property of being ionophores (Pressman et al., 1967; Westley, 1977).

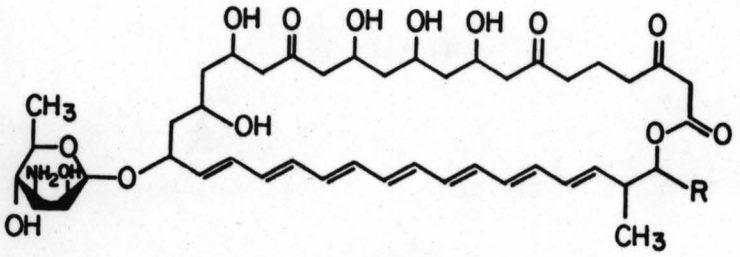
Ionophore antibiotics act by specifically increasing the permeability of membranes for certain cations. They are to be distinguished from such antibiotics as polyenes, which act as a surfactant on the membrane and generally disrupt membrane function. The polyether antibiotics are classified by their structure, and consequently by their ability to transport cations. The classes are monovalent polyethers, monovalent glycosides, divalent polyethers, divalent glycosides and divalent pyrrole ethers (Westley, 1977). Lasalocid A is classified as a divalent polyether; it is capable of existing either as a monomer or as a dimer and is therefore more flexible in its ability to complex other molecules. Studies have shown the following preference of complexation for Lasalocid A: Ca^{++}, Co^{+}> Ba^{++}> Sr^{++}> ethanolamine> Rb^{+}> Na^{+}> norepinephrine> Tm^{+++}> epinephrine> isoproterenol (Pressman and de Guzman, 1974; 1975). The polyether antibiotics, in particular lasalocid A and monensin A, have found their greatest application in veterinary medicine where they are used as poultry coccidiostats and to improve the feed efficiency in cattle and sheep (Galitzer and Oehme, 1984). Lasalocid A has also been tested for its ability to act as an antihypertensive agent (Osborne and Cohen, 1979). However, the actual mechanism by which

these antibiotics exert their effects is still not well understood.

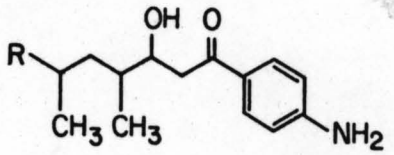
The polyether antibiotics are formed from acetate, propionate and butyrate subunits in the typical polyketide condensation pattern, yielding diverse and highly complex structures. Contributing to this diversity and complexity is the use of butyrate as a subunit which was first discovered in the polyether antibiotics; the degree of reduction of the carbonyls which varies considerably even within the individual compounds; and most notably, the stereochemical complexity of the molecules. Lasalocid has 10 asymmetric centers, monensin has 17, narasin has 19 and lonomycin has 23 asymmetric centers. These structural features make the polyether antibiotics a fascinating area in which to pursue biosynthetic studies.

Figure 4: Representative Polyketide Antibiotics:

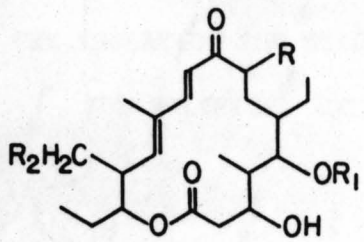
Candicidin, a polyene antibiotic
Tylosin, a macrolide antibiotic
Monensin, a polyether antibiotic



CANDICIDIN



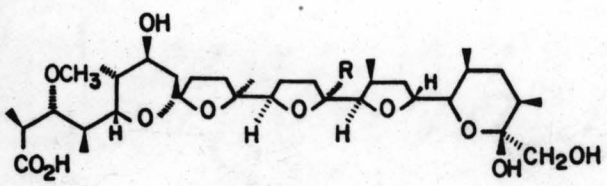
CANDICIDIN D



R = CH_3, R_1 = R_2 = H T YLACTONE

R = CHO, R_1 = – MYCAMINOSE - MYCAROSE

R = – O – MYCINOSE TYLOSIN



MONENSIN

R = CH_2CH_3 MONENSIN A

R = CH_3 MONENSIN B

CHAPTER ONE

THE ISOLATION AND STRUCTURE DETERMINATION OF THE POLYETHER ANTIBIOTIC LASALOCID A

INTRODUCTION

In 1951, Berger reported the first isolation of the polyether antibiotic Lasalocid A, initially called antibiotic X-537A. The antibiotic was isolated from a culture of Streptomyces obtained from local soil samples. Although the strucure of the antibiotic was not known for some time, its unusual physical properties were noted in Berger's report; most notably, the fact that the alkali salts of the compound were soluble in organic solvents, and insoluble in water. In 1970 the first reports on the structure of antibiotic X-537A began to appear in the literature (Westley et al., 1970; Johnson et al., 1970; Maier and Paul, 1971; Bissell and Paul, 1972; Alpha and Brady, 1973). These structural studies concentrated on X-ray analysis and chemical degradations and derivatizations. By 1974 the first NMR study on lasalocid A was reported (Schmidt et al., 1974). Also in 1974, Westley published a series of papers on the biosynthesis of lasalocid, in which various precursors were studied along with the isolation and structure determination of an isomer and four homologs of lasalocid A. This was followed in 1976 by a thorough ^{1}H-NMR study (Anteunis, 1976) and in 1978 by the complete ^{13}C-NMR assignment (Seto et al., 1978). These last two works provided much insight into the structural and ionophoric properties of the molecule.

The continual interest in the complexing abilities of lasalocid A is evidenced by the steady appearance of reports in

the literature (see for example: Richardson and Das Gupta, 1981; Grandjean and Laszlo, 1982; Hanna et al., 1983; Everett et al., 1983; Shastri and Easwaran, 1984). Lasalocid A is capable of complexing monovalent (see for example, Figure 5, p. 34), divalent and trivalent cations along with several small molecules, such as ethanolamine, norepinephrine, epinephrine and isoproterenol (Westley et al., 1977). The free acid of a lasalocid derivative is also capable of complexation; 5-bromolasalocid A forms dimeric complexes with a H_2O molecule in the center (Bissell and Paul, 1972). All of these complexes are lipid-soluble and can therefore transport the ions or molecules across membranes, thus imparting on lasolocid A, an important physiological effect.

Among its various biological applications, lasalocid A has been most widely used in veterinary medicine. It was initially used as a poultry coccidiostat (Berger, 1973), and later, also as a growth promoter in cattle and sheep (Galitzer and Oehme, 1984). The actual mechanism for these two properties is not well understood, especially in terms of the ionophore property of lasalocid A. In the case of the anticoccidial activity, it has been proposed (Wang, 1978) that there is a loss of intracellular nutrients due to the change in the membrane gradient, and that this will result in a less favorable environment for the coccidia. With regards to the growth promoter activity in cattle and sheep, it is known that lasalocid A causes a change in the rumen ecosystem resulting in overproduction of propionic acid and under

production of acetic and butyric acids. This change in rumen acid composition is believed to be the cause of the growth promotion (Ruff, 1982). A derivative of lasalocid A, 5-bromolasalocid A, has been tested for its antihypertensive activity (Osborne and Cohen, 1979), while lasalocid A and its salts have been shown to produce myocardial stimulation (Westley, 1978). It is not known, however, whether these compounds will reach the stage of being marketed for their therapeutic value in humans.

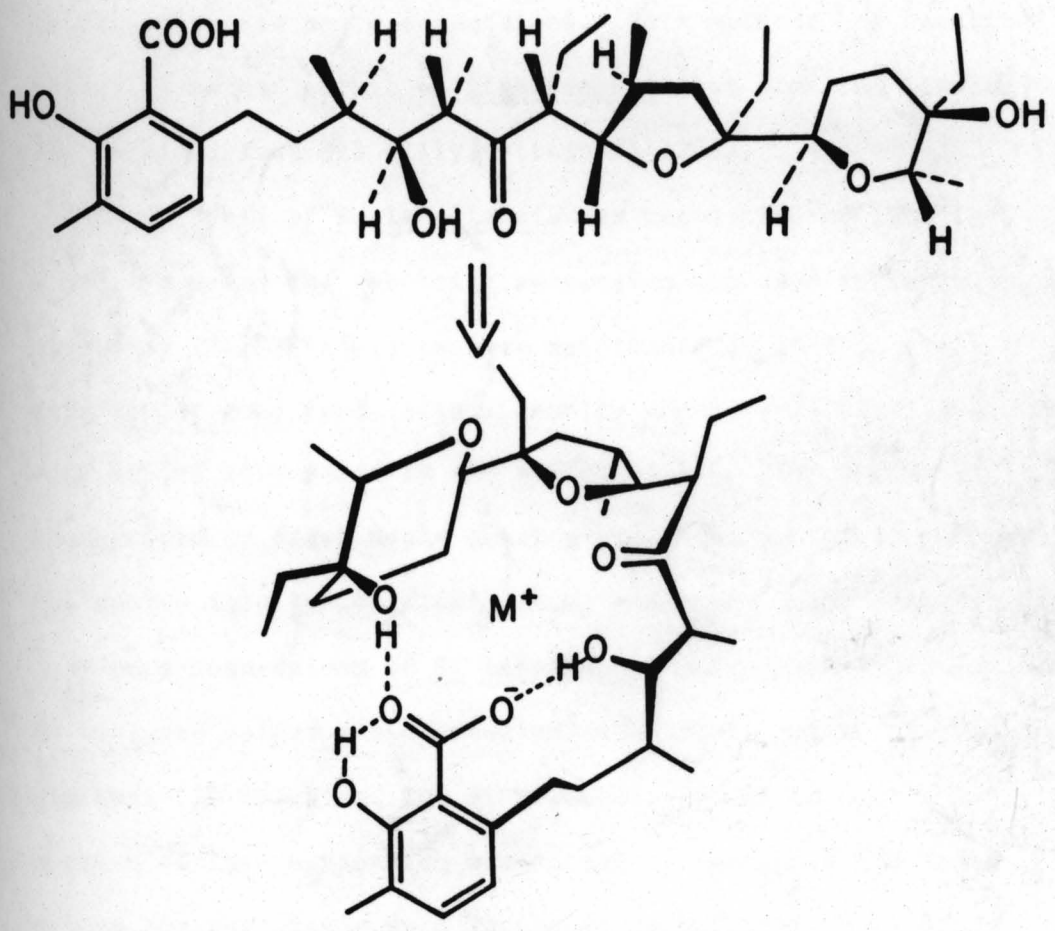
Due to both the interesting physiological properties and to a greater extent, the complex structure, two research groups have accomplished the synthesis of lasalocid A. In 1978, Kishi reported the first total synthesis of lasalocid A (Nakata et al., 1978), in which he took advantage of the fact that lasalocid A will undergo a retro-aldol cleavage in the presence of base or heat (Westley et al., 1973). Kishi approached the synthesis by carrying out the aldol reaction on the two halves of the molecule; the right half was assembled from a diene precursor, which was built up to the ketone fragment using regio- and stereo-controlled reactions, while the left-half was obtained by synthesizing a substituted benzyl salicylate, which could be ozonized to give the aldehyde fragment. Ireland presented a second total synthesis of lasalocid in 1980 (Ireland et al., 1980), in which the aldol condensation was again the means of assembling the two halves of the molecule. However, Ireland carried out the synthesis of the difficult right half of the molecule starting with natural

carbohydrate precursors. Ireland continued to develop his synthetic strategies for lasalocid A, and in 1983 reported on the total synthesis of lasalocid A from the chiral starting materials (R)-(-)-citronellene for the aldehyde and natural carbohydrate precursors for the ketone fragment (Ireland, et al., 1983). While it appears that synthetic approaches to lasalocid A will never compete with the ability of the micro-organism to produce the compound, it is still a great achievement to assemble a molecule with 10 asymmetric centers, whether synthetically or microbiologically.

In the course of investigating the production and biosynthesis of a bacterial metabolite it is necessary to establish protocols for the growth of the organism, the isolation and identification of the metabolite, and the means for analyzing any changes in the metabolite as a result of the conditions being tested. The intent of this chapter, therefore, is to present the basic methodology used for the remainder of the investigations, and to compare the methods that were developed during the course of this work with those that had been previously established in the literature. The standard methods used involved the maintenance and fermentation of the micro-organism Streptomyces lasaliensis; the means by which to assay for lasalocid A and other metabolites of interest; the isolation and purification of lasalocid A; and the analysis of lasalocid A by nuclear magnetic resonance (NMR) and mass spectral (MS) techniques.

Figure 5: Conformation of a Monovalent Metal Lasalocid A Complex

SOLUTION CONFORMATION OF LASALOCID A MONOMER



MATERIALS AND METHODS

Culture Methods: Maintenance and Fermentation Conditions

Streptomyces lasaliensis, NRRL 3382R (lyophilized 23.Jan.1976), was obtained from the USDA, Northern Regional Resource Service, Peoria, IL. The organism was initially maintained by growth on slants with successive transfers; then later, as frozen spore suspensions. Both methods are described below. A second strain of Streptomyces that produced Lasalocid A was obtained from Eli Lilly (strain #A41278).

The lyophil of S. lasaliensis was reconstituted into 1 mL sterile H_2O and the resulting suspension was used to inoculate 20 slants of TPA. The slants were maintained at 26-28°C until evidence of good sporulation, usually about 14-21 days; then they were sealed with parafilm and stored at 4°C. The spores were transferred to fresh media about every three months by scraping the spores into 1-2 mL sterile H_2O, and inoculating fresh slants.

Spore suspensions of S. lasaliensis were prepared as follows. An isolated colony was crushed and suspended in 1 mL of 20% glycerol (2G Solution: 200 mL glycerol, 800 mL dd H_2O). A 0.1 mL portion of this suspension was spread on each of 9 MYM Media plates and the plates were incubated at 30°C for 10-14 days. The spores were harvested by flooding the plates with 5 mL 20% glycerol containing 0.1% Triton X-100 and scraping the spores from the surface. The resulting spore suspension was transfered to a

screw-cap tube and vortexed vigorously. The suspension was then filtered through a spore filter tube (Hopwood, 1983) and the filtrate centrifuged at 10,000 rpm for 10 min. The supernatant was decanted and the pellet resuspended in in 20% glycerol. This last step was repeated and the pellet was resuspended in 5-10 mL 20% glycerol; this suspension was then dispensed in 1 mL aliquots and frozen at -20°C. Thirty MYM Media plates were spread with 100 uL of a 1:10 dilution of the above spore suspension. The plates were incubated at 30°C for 10-14 days, the spores were harvested as before and suspended in 20-30 mL 2G Solution, then frozen at -80°C in 1 mL aliquots.

The following solid and liquid growth media were used for culture maintenance and antibiotic fermentation.

Split Pea/Lard Oil Media (SP/LO)

10 gm/L Lard Oil

10 gm/L Stadex Dextrin #60

4 gm/L Yeast Extract

2 gm/L K_2HPO_4

1 L dd H_2O

1 gm Yellow Split Peas/50 mL media

The dd H_2O is added to the lard oil, Stadex Dextrin, yeast extract and K_2HPO_4, with heating. This mixture is then dispensed in 50 mL aliquots to the individual flasks containing the split peas, prior

to autoclaving. Care must be taken to continually mix the media while dispensing, since this is not a soluble mixture.

MYM Media

10 gm/L Malt Extract

4 gm/L Yeast Extract

4 gm/L Maltose

1 L dd H_2O

The malt extract, yeast extract and maltose are mixed with the dd H_2O with slight warming, before being dispensed to the individual flask, prior to autoclaving. The MYM agar media contains 20% Difco bactoagar in addition to the above ingredients.

14C Media

10 gm/L Dextrose

2 gm/L Yeast Extract

.5 gm/L Monosodium Glutamate

.5 gm/L K_2HPO_4

1 L dd H_2O

All ingredients are mixed with the dd H_2O, with slight warming, before being autoclaved.

Tomato Paste Agar Media (TPA)

20 gm/L Tomato Paste

10 gm/L Soyafluff 200W

10 gm/L Glucose

2 gm/L $CaCO_3$

1 gm/L Peptone

1 gm/L K_2HPO_4

20 gm/L Sucrose

20 gm/L Agar

1 L dd H_2O

The tomato paste is mixed with a small portion of the dd H_2O to aid in solubilizing the paste, then the remaining ingredients are added along with the remainder of the H_2O. The media is autoclaved prior to pouring the plates. The media is not soluble, and must be mixed continuously while dispensing.

Lilly Vegetative Media

15 gm/L Dextrose

15 gm/L Soybean Grits

10 gm/L Cornsteep Liquor

5 gm/L NaCl

2 gm/L $CaCO_3$

1 L tap H_2O

All ingredients are mixed with the H_2O, then adjust to pH 6.8 with 1 M NaOH prior to sterilization.

Lilly Fermentation Media

15 gm/L Dextrose

35 gm/L Glycerol

10 gm/L Peptone

4 gm/L NZ Amine A

5 gm/L Blackstrap Molasses

.5 gm/L $MgSO_4(7H_2O)$

2 gm/L $CaCO_3$

2 mL Czapek Mineral Stock (see below)

1 L dd H_2O

All ingredients are mixed with the H_2O and the pH is adjusted to 7.0 with 1 M NaOH prior to sterilization.

Czapek Mineral Stock

100 gm/L KCl

100 gm/L $MgSO_4.7H_2O$

2 gm/L $FeSO_4.7H_2O$ - dissolve in 2 mL conc HCl

1 L dd H_2O

"Monensin" Media

30 gm/L glucose

30 gm/L Soyafluff 200W

1 gm/L $CaCO_3$

.1 gm/L K_2HPO_4

5 gm/L Lard Oil

.1 gm/L $MnCl_2(H_2O)$ *

.6 gm/L $Fe_2(SO_4)_3$ *

.3 gm/L $ZnSO_4(7H_2O)$ *

1 L dd H_2O

All ingredients (except *) are mixed with H_2O, with heating prior to sterilization. The remainder of the ingredients (*) are filter sterilized and added after autoclaving.

Berger Patent Media

20 gm/L brown sugar

20 gm/L Soyafluff 200W

5 gm/L Cornsteep Liquor

2 gm/L Lard Oil

1 gm/L K_2HPO_4

1 L dd H_2O

All ingredients were mixed with dd H_2O with heating. The mixture is dispensed in 50 mL aliquots to the flasks prior to autoclaving. The mixture is not soluble and therfore must be mixed continually while dispensing.

Media ingredidents were obtained from the following places. Yeast extract, malt extract, Bactoagar, Bactopeptone, maltose, dextrose and Antibiotic Media #1 were from Difco Labs, Detroit, MI. The lard oil (Peacock Special Prime Burning Lard Oil) was a gift from George Pfau and Sons, Jeffersonville IN. The Stadex Dextrin #60 was received from A.E. Staley Mfg. Co., Decatur IL. The Soyfluff 200W was from Central Soya, Fort Wayne IN. NZ Amine Type A was received from Humko-Sheffield Chemical, Memphis TN. Other ingredients such as yellow split peas, soybean grits, tomato

paste, and blackstrap molasses were purchased from local grocery stores.

Stationary cultures were incubated at 30°C. Liquid cultures were grown on a continual shaker at 250 rpm at 28-30°C.

Isolation and Purification of Lasalocid A

The solvents and chemicals used were the best commercially available; where necessary, the solvents were redistilled. The Celite 545 was obtained from J.T. Baker. PLC plates were prepared from silica gel P254 and pouring to a 2 mm thickness. The TLC plates used were commercial precoated silica gel F254, 0.25 mm thickness. Solvents were generally evaporated either in vacuo on a rotary evaporator at 30-35°C, or under a stream of N_2 at room temperature.

During the course of these experiments, two procedures were developed for the isolation and purification of lasalocid A. The first was based on chromatographic separation by PLC, and then recrystallization; the second method employed the advantages of a semi-prep HPLC column.

In the first method, 5 gm Celite and 50 mL EtOAc were added to 50 mL media and the mixture stirred vigorously for 1 hour. The slurry was filtered in a Buchner funnel, using Whatman #2 filter paper to remove the mycelia. The mycelial pad was rinsed twice with EtOAc and the combined filtrates were extracted twice with additional EtOAc. The combined EtOAc phases were

washed once with brine, dried over anhydrous Na_2SO_4, then the solvent removed *in vacuo* to give a yellow oily residue.

In order to remove the bulk of the lard oil, the residue was dissolved in 95% EtOH and then placed in an EtOH/ice bath at about $-10^{o}C$, which caused the lard oil to solidify. This solution was then filtered by suction through a medium frit filter funnel packed with celite and jacketed with an EtOH/ice solution. The EtOH was evaporated from the filtrate *in vacuo* to give a yellow residue.

This residue was applied to 20 x 20 cm silica gel PF254 plates and developed in hexane:^{i}PrOH:AcOH (90:10:0.5). The blue fluorescent (short UV) band was scraped and eluted with CH_2Cl_2:MeOH (4:1). The residue was then reapplied to new plates and developed first in CH_2Cl_2:AcOH (98:2) and then in CH_2Cl_2:MeOH:AcOH (95:5:0.5). The blue fluorescent band was isolated as before, and the residue crystallized from 95% EtOH in a Craig Tube.

Due to its strong ionophoric character, lasalocid A was usually isolated as its sodium salt. To obtain the free acid, the crystals were partitioned between Et_2O and 0.1 *N* HCl. The Et_2O layer was washed twice with H_2O, then evaporated with a toluene-EtOH azeotrope, and the residue recrystallized from 95% EtOH.

The presence of lasalocid A could be checked at any time during the isolation by its characteristic bright blue

fluorescence under short UV. An appropriate TLC system was hexane:^{i}PrOH:AcOH (9:1:0.05). By spraying with 3% vanillin/0.5% conc H_2SO_4 in 95% EtOH and heating at 120°C, lasalocid A is visualized as yellow-orange. Any attempts to separate lasalocid A from its homologs or isolasalocid A by TLC were unsucessful.

The second method for the isolation of lasalocid A followed essentially the same procedure in the beginning as described above. Celite (5 gm) and EtOAc (50 mL) were added to each flask immediately at the end of the fermentation. This mixture was then worked up as described above, obtaining the EtOAc extract.

This extract was dissolved in 2 x 2 mL hexane:$CHCl_3$ (2:1), and loaded onto a Sep-Pak™ (Waters Associates, silica gel) cartridge, and eluted as "Fraction #1". The cartridge was then rinsed with 3 x 1 mL hexane:^{i}PrOH (9:1), as "Fraction #2", and then with 2 x 1 mL $CHCl_3$:MeOH (3:1), as "Fraction #3". Approximately 70% of the lasalocid A was in "Fraction #2", the remainder was in "Fraction #3" (occasionally, some would leak through into "Fraction #1", but this could be monitored by TLC). The solvent in Fractions #2 and #3 was evaporated under a stream of N_2 and the residue from each fraction was chromatographed on a separate prep plate. The plates were developed first in CH_2Cl_2:AcOH (98:2), air dried, then developed in CH_2Cl_2:MeOH:AcOH (95:5:0.5). The blue fluorescent band (Rf ca. 0.6) was scraped and eluted with CH_2Cl_2:MeOH (4:1). The material from fractions #2 and #3 were combined, the solvent removed in vacuo and the residue was tared.

This residue was dissolved in $CHCl_3$ and filtered through a Millex^R-SR, 0.5 um disposable membrane. The $CHCl_3$ was evaporated and the residue redissolved in MeOH:$CHCl_3$ (3:1) and injected onto a semi-prep HPLC column (Waters, μBONDAPAK C_{18}, 7.8 mm x 30 cm). The column was eluted with MeOH:1% Aqueous AcOH (75:25) at a flow rate of 1.5 mL/min; fractions were collected at 4 min. intervals. Typically, a column elution profile was: lasalocid A, 96-124 mins (7 fractions); homologs, 124-144 min (5 fractions); isolasalocid A, 144-164 mins (5 fractions). The total column elution time was 3-3.5 hours. With this mobile phase, lasalocid A was isolated as the free acid.

Growth Curves

Growth Curve Experiment #1

The seed cultures were started by adding 1 vial frozen spore suspension (1 mL) to each of 50 mL SP/LO media/250 mL flask and the cultures were grown for 65 hours. The cells were then spun down (10K, 10 min.), the supernatant decanted, and the cells resuspended in Na_2HPO_4 buffer (50 mM, pH 7.5). This last step was repeated, and the cells were then resuspended in 100 mL 14C media; 25 mL of this media was dispensed to 4 x 125 mL flasks. The flasks were then returned to the shaker.

Samples were removed (1.0 mL aliquots) during the course of the fermention. Two of the flasks were assayed frequently at t = 0 (time of transfer), 6, 12, 18, 24, 30, 36, 48, 54, 58.5, 72, 78,

82.5, and 96 hours. The other two flasks were assayed only at t = 0, 24, 48, 72, and 96 hours.

These samples were assayed by HPLC for antibiotic content and for dry cell weight. The 1.0 mL aliquot was spun down at 3000 rpm for 10 min; the supernatant was pipetted off and saved. The cells were washed with dd H_2O (1.0 mL) by vortexing, and recentrifugation, and the supernatant saved. The cells were resuspended in a small amount of H_2O and transferred to a tared, disposable aluminum weighing dish, dried at 90°C for 12-18 hours, then transferred to a vacuum dessicator for an additional 18-24 hours before weighing. The combined aqueous phases were extracted with $CHCl_3$ (1.0 mL), vortexed, the aqueous layer was pipetted off, and the $CHCl_3$ evaporated under N_2 at room temperature. The $CHCl_3$ extract was dissolved in 0.2 mL MeOH:$CHCl_3$ (3:1) for analysis of antibiotic content by analytical HPLC as described below.

Growth Curve Experiment #2

The seed cultures for this experiment were started by adding 1 vial frozen spore suspension (cell count: 2.8 x 10^8/0.1 mL) to 50 mL MYM media/250 mL flask and to 50 mL SP/LO media/250 mL flask. The cultures were grown at 28-30°C 250 rpm for 72 hours, and were then used to inoculate (2% v/v) 21 x 50 mL MYM media/250 mL flask and 21 x 50 mL SP/LO media/250 mL flask, respectively.

Starting 24 hours after inoculation, and repeating every 24 hours for a total of 168 hours, 3 flasks of each media were removed from the shaker and harvested. The flasks were worked-up

by measuring the pH, the packed cell volume, and then extracting with 3 x 5-10 mL $CHCl_3$. The $CHCl_3$ extracts were combined and evaporated under N_2. These extracts were assayed by TLC, by bioautography, and by injecting 1/20 of the total amount onto the analytical HPLC.

Growth Curve Experiment #3

The seed cultures for this experiment were started by adding 1 vial frozen spore suspension (cell count: 9.2 x 10^7/0.1 mL) to 50 mL SP/LO media/250 mL flask and to 50 mL MYM media/250 mL flask. The cultures were grown for 72 hours, then used to inoculate (2% v/v) 12 x 50 mL SP/LO media/250 mL flask and 12 x 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 gm) and EtOAc (50 mL) were added to each flask, and the mixture stirred vigorously for 1-1.5 hours at room temperature. The mixture was filtered through a Whatman #2 filter paper, and the filtrate was extracted with EtOAc (2 x 50 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, then evaporated *in vacuo* on a rotary evaporator at 30°C. The resulting residue was assayed by TLC and analytical HPLC as before.

Assay Methods

Thin Layer Chromatography and Bioautography

TLC of the samples was run by spotting a known quantity of the sample onto a Kieselgel 60 F-254 plate; usually 6 samples and 1 standard could fit onto one 20 x 20 cm plate. The plates were run in duplicate by developing them in 100% EtOAc. One set of plates was visualized by vanillin/Δ , as described under the Isolation and Purification section, while the other set was subjected to bioautography.

The bioautogram was prepared as follows. Antibiotic Media #1 (200 mL) was autoclaved and cooled to 55°C. Then 2 mL spore suspension of Bacillus subtilis (the original spore suspension was prepared by J.M. Weber, 1977), and 1 mL 4% aqueous 2,3,5-Triphenyl-tetrazolium chloride solution were added to the media and mixed vigorously for 5 min. The entire mixture was poured slowly and evenly into a large, sterile Pyrex baking dish. The TLC plate was air dried thoroughly, then placed on the surface of the agar; once the SiO_2 layer was H_2O saturated, the plate is allowed to remain in position for 30-45 min before removing. The agar plate was covered and incubated at 30°C for 18 hours before reading. The amount of antibiotic could be quantitated by comparison of the size of growth inhibition zone against a standard dose-response curve prepared from pure lasalocid A.

Analytical HPLC

A Waters μBONDAPAK C_{18} column was routinely used with the mobile phase being MeOH:1% aqueous AcOH (75:25) at a flow rate of 1.5 mL/min. Samples were dissolved in 0.2 mL MeOH:$CHCl_3$ (3:1) for

loading onto the column. The column effluent was monitored with a Waters Series 441 monitor set at 254 nm and fractions were collected at 4 min. intervals. A normal column elution profile was: echinomycin, 4 min; lasalocid A, 19 min; lasalocid homologs, 26 min; isolasalocid A, 31 min. All peaks were verified by standards obtained from Dr. John Westley, Hoffmann-La Roche, Nutley, N.J.

Mass Spectral (MS) Protocol

Mass spectral samples were prepared by dissolving the lasalocid A in CH_2Cl_2, transferring 20-30 ugm to a small capillary, then drying in vacuo for 2 hours. Most (ca. 95%) of the samples were run on an AEI MS9-DS50: 8KV acceleration voltage; 70eV ionization voltage; 130°C source temperature. The more recent samples were run on a KRATOS MS25-DS55: 2KV acceleration voltage; 70eV ionization voltage; 150°C source temperature, 200°C probe temperature.

Mass spectral analysis was used regularly to determine isotopic enrichment factors. The approximate percentages of (M+1) and (M+2) were calculated from the following formulas (Silverstein et al., 1974):

$$\%(M+1) = 100((M+1))/M = 1.1 \times \#\ C\ atoms$$

$$\%(M+2) = 100((M+2))/M$$

$$= (1.1 \times \#\ C\ atoms)^2/200 + 0.2 \times \#\ O\ atoms$$

(no corrections were made for N atoms, since none were present in any of the compounds used). These values were used to correct the peak heights of the M+1 and M+2 species for contributions from natural abundance, and the corrected values were used to determine the percentage of labelled species. Since it is impossible to distinguish enrichment due to ^{2}H from that of ^{13}C by low resolution MS methods, the enrichment values are given simply as: L0 (unlabelled), L1 (single label), L2 (double label), and so on.

Nuclear Magnetic Resonance (NMR) Protocol

Samples of lasalocid A were prepared for NMR spectroscopy by first precipitating it either to the sodium salt or to the free acid, depending on which signals were of interest. It is important, however, to ensure that the sample is completely in one form or the other, and to note that the free acid slowly converts to the sodium salt upon storage in solution in glass tubes. Samples were usually dissolved in 0.4-0.5 mL $CDCl_3$ (Aldrich Gold Label, 99.8%) just prior to acquiring spectra. Lasalocid A will decompose in $CHCl_3$ if exposed to light and/or stored at room temperature for longer than 48 hours.

During the course of these experiments, NMR spectra were acquired on different spectrometers at several different institutions. The specifics of each instrument and the parameters used are listed with each spectra or experiment.

RESULTS AND DISCUSSION

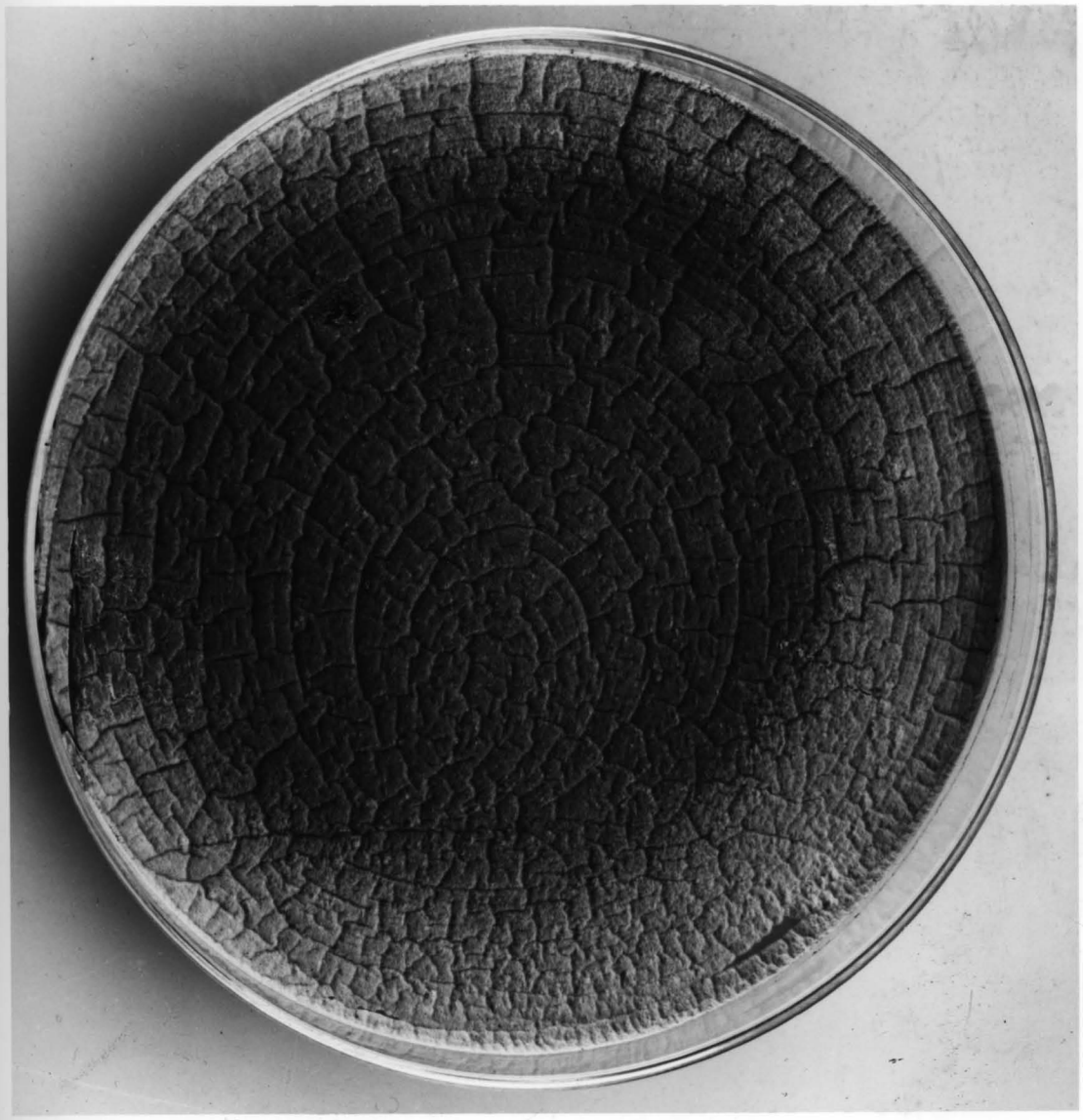
Organism and Compound

The order, Actinomycetales, consists of the filamentous, branching bacteria. Within this order the family Streptomycetaceae, genus Streptomyces, is the largest producer of the polyether antibiotics. Streptomyces lasaliensis NRRL 3382R (Stempel and Westley, 1973) was the primary organism used for the production of lasalocid A throughout these experiments. The NRRL strain (Figure 6) has been characterized taxonomically as having gray spiny spores that are formed in spiral chains (Prosser and Palleroni, 1982). A second organism that produces lasalocid A was obtained from Eli Lilly (Lilly #A41278); however it was found that this organism produces lasalocid A both later in the fermentation and in lower quantity than the NRRL strain, and consequently was not used.

The conditions under which the organism is maintained are critical. The initial method of successive slant transfer was inadequate; the organism reverted to non-producer after several transfers. The method of isolating spores from a single colony, and then storing the spores as a frozen suspension was a considerable improvement. However, what is not yet known is how long these spore suspensions remain viable, and to what degree the

Figure 6: Sporulated Plate of *Streptomyces lasaliensis* (NRRL 3382R)

The plate was prepared by suspending an isolated colony in 1 mL 2G solution, and spreading 0.1 mL onto a MYM plate. The plates were incubated for 14 days at 30°C. The dark area constitute the spores.



inoculum size is a critical factor in determining production. Experiments in which the spore suspension was at least a year old, and in which the cell count was not known, resulted in low production of lasalocid. The experiments that were carried out in the last four months using recently prepared spore suspensions of known cell count (noted in the experimental) showed high levels of antibiotic production. By following the production of lasalocid over time, with a known spore inoculum size, the viability of the spores and whether or not the cell count is a critical factor can be determined.

During the course of these experiments, various medias and growth conditions were tested in order to both improve yields and establish reproducible antibiotic production conditions. Tomato paste and MYM media were used for solid cultures used in sporulation. The following medias were tested for liquid culture conditions: SP/LO media, 14C media, "Monensin" media, Berger's Patent media, Lilly Fermentation media, and more recently, MYM media. The SP/LO media was also tested with various oils, including : lard oil, methyl oleate, soyabean oil, Pristane and Prochem 51. In addition, the size of flask, the flask angle (using 15^{o} "ErlAngle" clamps), and other shaker conditions were tested.

It was found that both tomato paste media and MYM media were good for sporulation; however, the MYM media is the preferred media, since it is considerably easier to handle, both in

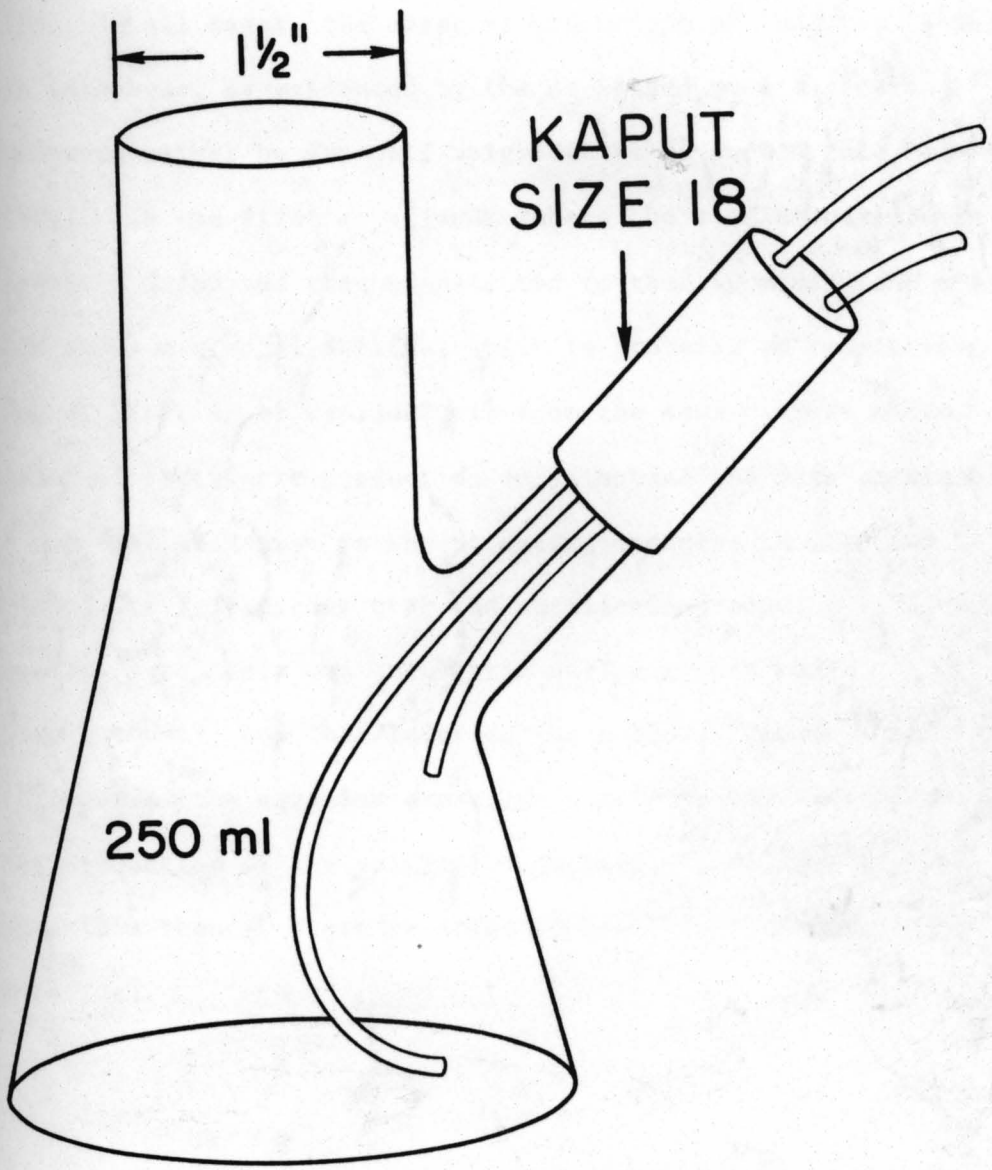
preparation and in any analytical work. Only three of the liquid culture media tested proved to be of any use, SP/LO, MYM, and to a lesser degree, 14C (see results of the growth curve experiments). All other medias tested resulted in low or no production of the antibiotic. The oil in SP/LO media is critical; it aids in the extraction of lasalocid from the aqueous media into the oil phase, presumably due to the lipophilicity of lasalocid. Omission of the oil completely from this media resulted in no production of the antibiotic, while substitution of other oils gave a decreased production. It is possible for the lard oil to go rancid, again affecting production, so it is best to store the lard oil at 4°C, which also makes it easier to handle. The 14C media is adequate and was used initially in experiments that required analysis of the metabolites during the course of the fermentation due to its relatively uncomplex composition. Surprisingly, the simple MYM media proved to be not only a good sporulation media, but also a good production media. It is also interesting to note that none of the media that are suitable for the production of monensin, a polyether antibiotic from Streptomyces cinnamonensis, will support the production of lasalocid.

The physical conditions for the growth of the organism also proved to be crucial. The growth temperature, shaker speed and aeration of the cultures all exerted an affect on the production of the antibiotic. For liquid cultures, the temperature should be maintained at 28-30°C and protected from any fluctuations due to

differences in ventilation within the shaker-incubator. The optimal shaker speed is 250 rpm; differences in actual shaker speed due to position on the shaker platform were noticeable in terms of the amount of antibiotic produced. The most critical factor in the culture conditions was the aeration. The optimal conditions are 25 mL media/125 mL erlenmeyer flask or 50 mL media/250 mL erlenmeyer flask, grown at 250 rpm on a continual shaker. Attempts to scale up the fermentation, even to 100 mL media/500 mL flask resulted in a decrease in antibiotic; likewise, scaling the fermentation down to 5 mL media/tube gave non-reproducible results. The use of the "ErlAngle" clamp had no noticeable affect on production in SP/LO media, probably due to the fact that the media is quite viscous, and consequently increasing aeration in this manner is not as effective. No comparisons were done using the simpler medias, such as 14C or MYM, however it was noticed that aeration increased when the angle clamps were used for these medias. The use of continual shaking during the course of the entire fermentation was a decisive factor in obtaining reproducible antibiotic production. Removing a flask during the fermentation, even for 1-2 minutes, could result in little or no antibiotic being produced. Consequently, a flask was designed which would allow for the feeding and sampling of the shaking culture via tubes (Figure 7). In this manner the cultures could be grown without interruption and reproducibly.

Figure 7. 250 mL Erlenmeyer Feeding Flask

The tygon tubing allows for feeding and sampling the cultures during the fermentation.


1½"
KAPUT
SIZE 18
250 ml

The growth curve experiments represent the standard conditions used for the fermentation of S. lasaliensis and the production of lasalocid during the course of these experiments (Figures 8, 9 and 10). In all cases, the onset of production of lasalocid occurred in idiophase, as evidenced by the decreased growth rate as measured either by dry cell weight (DCW) or packed cell volume (PCV). In the first experiment, where the seed cultures were grown in SP/LO and then transferred to the 14C media, the measured DCW shows a gradual decline, which is probably an indication of the utilization of residual oil from the seed culture media. The onset of antibiotic production in idiophase has been attributed to a survival mechanism in the producing organism (Martin and Demain, 1980). It is believed that the antibiotic-producing strains are sensitive to their own antibiotic during growth phase (trophophase), and that later during a slowed growth phase (idiophase) the organism expresses a self-resistance mechanism and the production of the antibiotic begins. While this is a plausible theory, there is still no conclusive evidence to support this idea.

Figure 8: Growth Curve Experiment #1

a) Tables of:
Amount (AMT) of Lasalocid A and
DCW (WT) of each aliquot sampled. p. 60

b) Plot of Amount of Lasalocid A vs
Time during the fermentation. p. 61

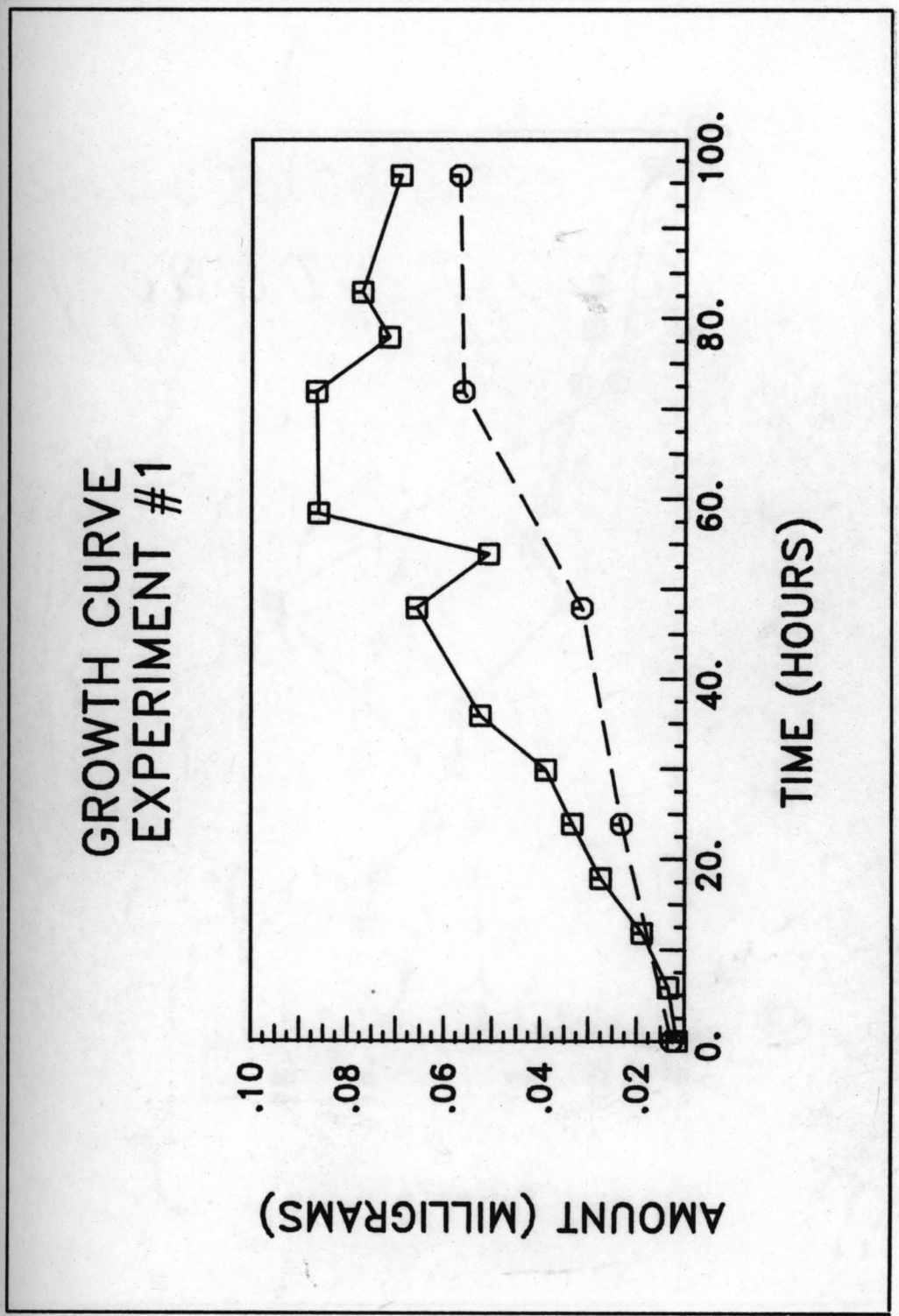
c) Plot of Weight of each aliquot
sampled vs Time during the
fermentation. p. 62

GROWTH CURVE EXPERIMENT #1

GCE1 CODE	AMT(1)	AMT(2)	AVG AMT
0 HRS	.0148	.0083	.01155
6 HRS	.0126	.0142	.0134
12 HRS	.0236	.0141	.01885
18 HRS	.0301	.0248	.02745
24 HRS	.0392	.0276	.0334
30 HRS	.0576	.0201	.03885
36 HRS	.0613	.0416	.05145
48 HRS	.0771	.0542	.06565
54 HRS	.0543	.0473	.0508
58.5 HRS	.0946	.0776	.0861
72 HRS	.0936	.0796	.0866
78 HRS	.0928	.0501	.07145
83 HRS	.0718	.0823	.07705
96 HRS	.0586	.0795	.06905
0 HRS	.013	.0123	.01265
24 HRS	.0216	.0248	.0232
48 HRS	.0291	.0336	.03135
72 HRS	.0523	.0598	.05605
96 HRS	.0677	.0461	.0569

GROWTH CURVE EXPERIMENT #1

GCE1 CODE	WT(1)	WT(2)	AVG WT
0 HRS	17.54	13.57	15.55
6 HRS	16.64	12.94	14.79
12 HRS	17.36	14.65	16.00
18 HRS	16.78	14.34	15.56
24 HRS	15.83	12.71	14.27
30 HRS	14.95	12.32	13.63
36 HRS	14.45	12.57	13.51
48 HRS	11.10	10.46	10.78
54 HRS	13.03	10.38	11.71
58.5 HRS	12.13	10.46	11.30
72 HRS	10.92	9.90	10.41
78 HRS	11.07	9.08	10.07
83 HRS	10.91	8.92	9.91
96 HRS	9.32	7.72	8.52
0 HRS	12.76	13.04	12.90
24 HRS	13.27	13.06	13.16
48 HRS	21.68	14.81	18.25
72 HRS	9.15	9.86	9.50
96 HRS	8.35	8.79	8.57


GROWTH CURVE
EXPERIMENT #1
AMOUNT (MILLIGRAMS)
.10
.08
.06
.04
.02
0.
20.
40.
60.
80.
100.
TIME (HOURS)

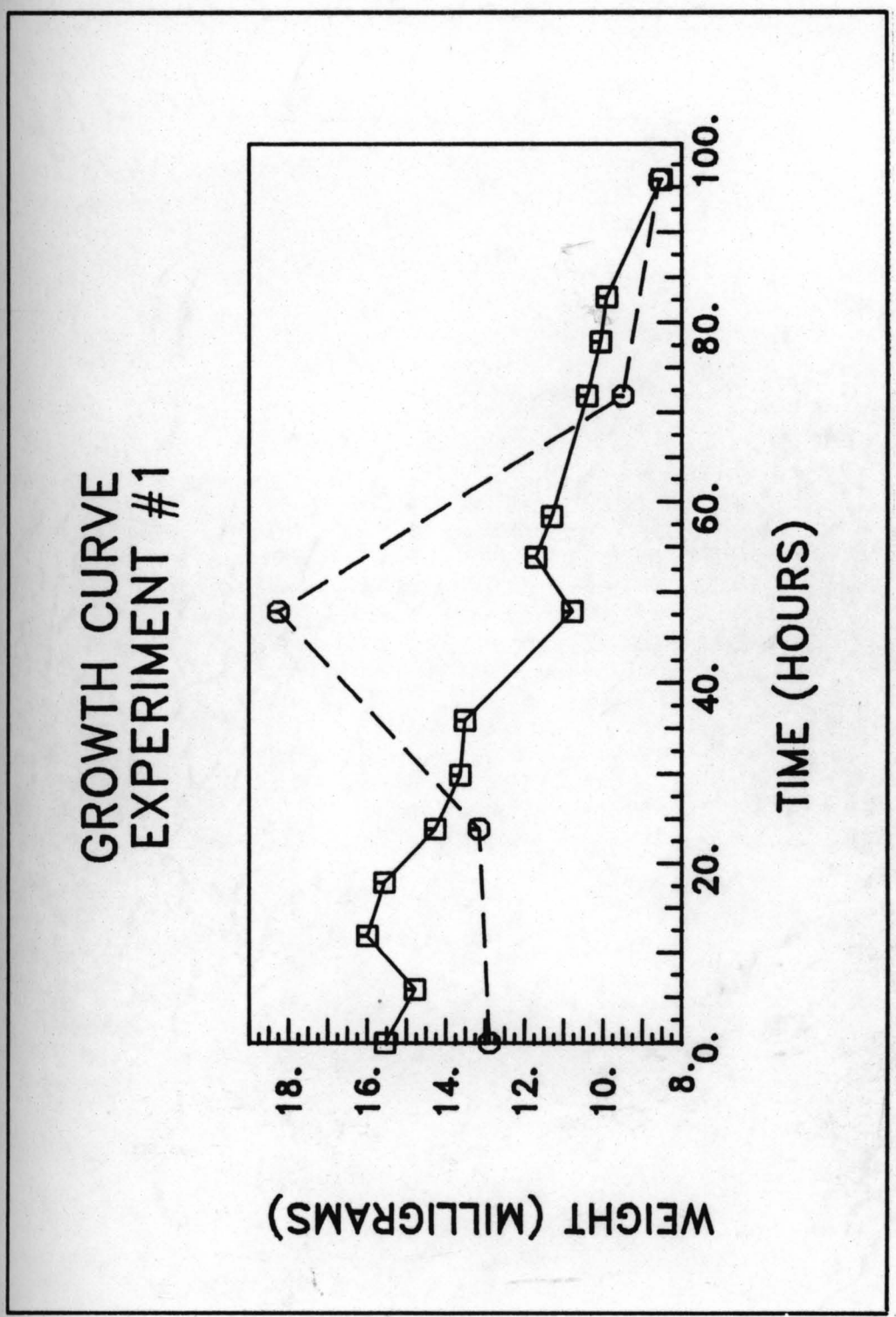

GROWTH CURVE
EXPERIMENT #1
WEIGHT (MILLIGRAMS)
18.
16.
14.
12.
10.
8.
0.
20.
40.
60.
80.
100.
TIME (HOURS)

Figure 9: Growth Curve Experiment #2

a) Table of Amount (AMT)
of Lasalocid A p. 64

b) Tables of:
pH and PCV of each flask
at end of fermentaion p. 65

c) Plot of Amount of Lasalocid A
vs Time during fermentation
SP/LO Media
MYM Media p. 66

GROWTH CURVE EXPERIMENT #2

GCE2 CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
SPLO-48	.01474	.01853	.01966	.01764	.00246
SPLO-72	NA	.00668	.02674	.01671	.01003
SPLO-96	.05308	.08552	.03853	.05904	.02349
SPLO-120	.17064	.09122	.15688	.13958	.03971
SPLO-144	.20367	.25478	.07077	.17641	.09201
SPLO-168	.19168	.14567	.30826	.21520	.08129
MYM-48	.00393	.01278	.01062	.00911	.00442
MYM-72	.17890	.10321	.18401	.15537	.04040
MYM-96	.16789	.26147	.04954	.15963	.10596
MYM-120	.35426	.31995	.32457	.33293	.01715
MYM-144	.43988	.18086	.68847	.43640	.25380
MYM-168	.27523	1.18103	.96429	.80685	.45290

GROWTH CURVE EXPERIMENT #2

GCE2 CODE	pH (1)	pH (2)	pH (3)	AVG pH
SPLO-24	6.38	6.29	6.27	6.31
SPLO-48	6.38	6.42	6.47	6.42
SPLO-72	6.74	6.69	6.72	6.72
SPLO-96	6.75	6.89	6.84	6.83
SPLO-120	6.93	6.89	6.96	6.93
SPLO-144	6.95	7.23	6.98	7.05
SPLO-168	7.32	7.09	7.34	7.25
MYM-24	5.36	5.42	5.31	5.36
MYM-48	5.88	5.88	5.88	5.88
MYM-72	5.80	5.88	5.90	5.86
MYM-96	5.94	5.69	5.96	5.86
MYM-120	6.35	5.79	5.84	5.99
MYM-144	6.39	6.22	6.52	6.38
MYM-168	7.03	6.99	7.19	7.07

	PACKED CELL VOLUME (cc)			
GCE2 CODE	(1)	(2)	(3)	AVG
SPLO-24	10	10	10	10
SPLO-48	15	15	15	15
SPLO-72	20	20	20	20
SPLO-96	20	20	22	21
SPLO-120	20	20	22	21
SPLO-144	20	18	15	18
SPLO-168	15	20	18	18
MYM-24	3	3	3	3
MYM-48	5	5	6	5
MYM-72	7	7	7.5	7
MYM-96	10	10	7	9
MYM-120	7	7.5	7.5	7
MYM-144	7	7.5	5	6
MYM-168	7	5	7	6

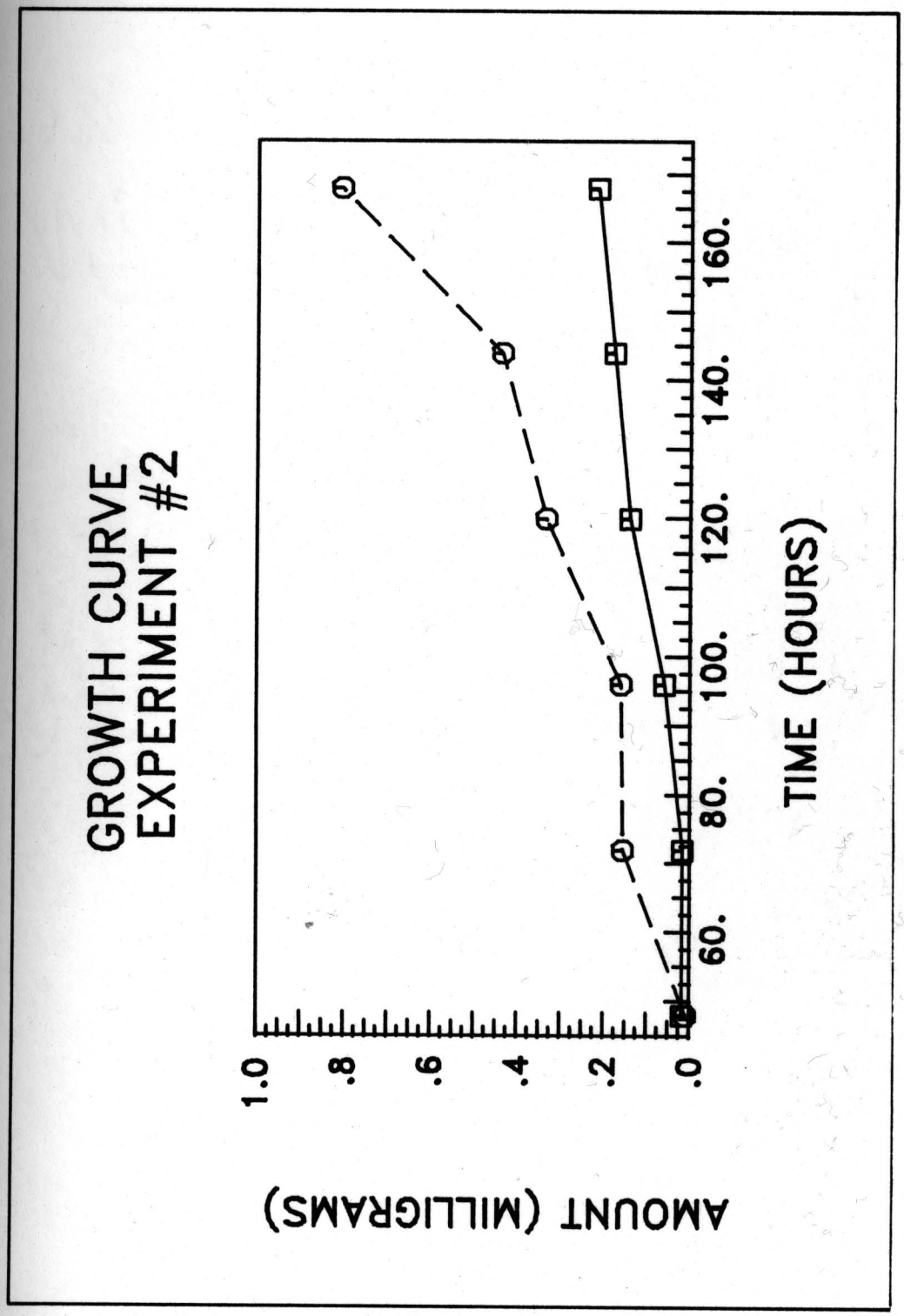

GROWTH CURVE
EXPERIMENT #2
AMOUNT (MILLIGRAMS)
1.0
.8
.6
.4
.2
.0
60.
80.
100.
120.
140.
160.
TIME (HOURS)

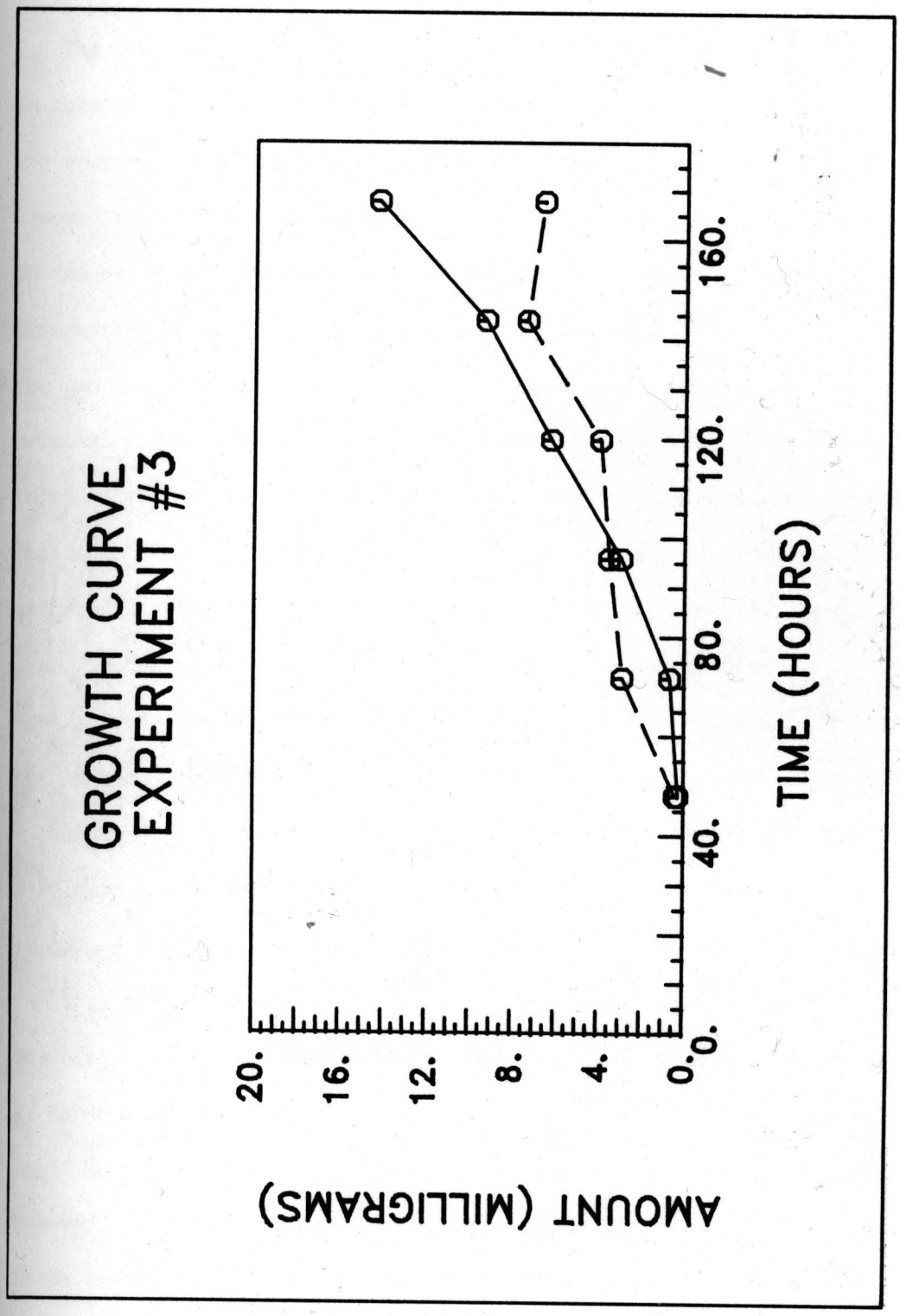
Figure 10. Growth Curve Experiment #3

a) Table of Amount (AMT)
of Lasalocid A p. 68

b) Plot of Amount of Lasalocid A
vs Time during fermentation
SP/LO Media
MYM Media p. 69

GROWTH CURVE EXPERIMENT #3

GCE3 CODE	AMT(1)	AMT(2)	AVG AMT
SPLO-48	.1966	.3539	.2752
SPLO-72	1.1304	.2202	.6753
SPLO-96	3.3342	2.5557	2.9449
SPLO-120	4.7674	7.7379	6.2526
SPLO-144	8.5570	10.0557	9.3064
SPLO-168	17.9882	10.6081	14.2981
MYM-48	.1769	.6605	.4187
MYM-72	3.8532	1.9345	2.8938
MYM-96	1.8165	5.2372	3.5269
MYM-120	4.0380	3.8807	3.9594
MYM-144	8.5871	6.1946	7.3909
MYM-168	6.1435	7.0773	6.6104


GROWTH CURVE
EXPERIMENT #3
AMOUNT (MILLIGRAMS)
20.
16.
12.
8.
4.
0.
0.
40.
80.
120.
160.
TIME (HOURS)

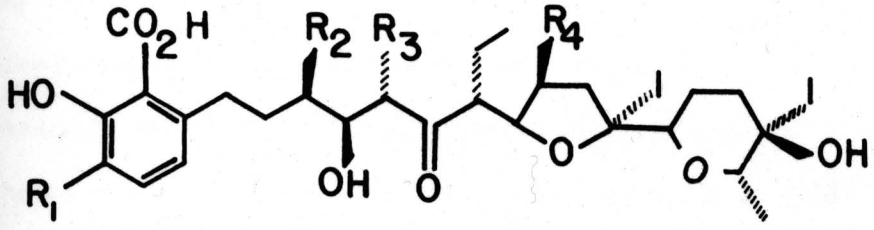
The 14C media and MYM media can be reproducibly extracted with $CHCl_3$ in tubes which was the method used for assaying aliquots removed during the course of the fermentation. The SP/LO media, due to its complex composition, can not be readily assayed by simple extraction procedures. In comparing the results of the second and third experiments, it is interesting to note the increased level of isolatable compound. While the two experiments used different solvents for the extraction, the critical difference is more likely the actual procedure. That is, in the third experiment, a full extraction using Celite to bind the mycelia and stirring the mixture for 1-1.5 hours resulted in a large increase of isolated lasalocid. Lasalocid, due to its lipophilic nature, readily binds to the mycelia which is primarily lipid in composition. Consequently, a complete extraction has to involve a means for extracting the lasalocid from the mycelia as well as from the pure aqueous phase.

Of the two methods developed for the extraction and purification of lasalocid, the second method utilizing HPLC purification, is by far superior. In 1974, Westley reported the isolation and structure of four homologs and an isomer of lasalocid A, (Westley et al., 1974b; 1974c). The homologs are referred to as lasalocid B, C, D and E, while the isomer is known as isolasalocid A (Figure 11). Westley stated that lasalocid could be isolated from its homologs and isomer by various TLC systems; however, the TLC separation never proved successful during the course of these investigations. Consequently, the HPLC separation was developed, based on a previous report in the literature (Hagel, 1978), and is the preferred method for the purification of lasalocid.

Figure 11. Structure of the Lasalocids

Lasalocid A - major metabolite
Lasalocids B, C, D and E - homologs *
Isolasalocid A - isomer

* Collectively referred to as the "Homologs"



Lasalocid

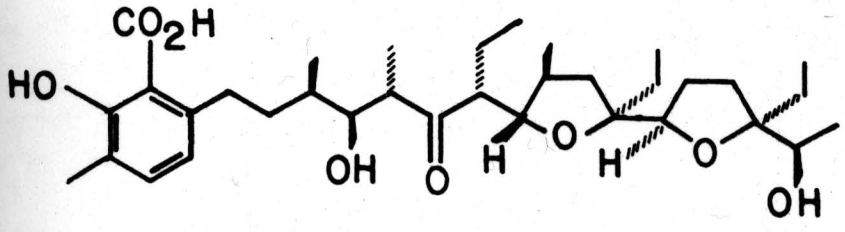
$R_1 = R_2 = R_3 = R_4 = CH_3$ Lasalocid A

$R_1 = CH_2CH_3$, $R_2 = R_3 = R_4 = CH_3$ Lasalocid B

$R_2 = CH_2CH_3$, $R_1 = R_3 = R_4 = CH_3$ Lasalocid C

$R_3 = CH_2CH_3$, $R_1 = R_2 = R_4 = CH_3$ Lasalocid D

$R_4 = CH_2CH_3$, $R_1 = R_2 = R_3 = CH_3$ Lasalocid E



Isolasalocid A

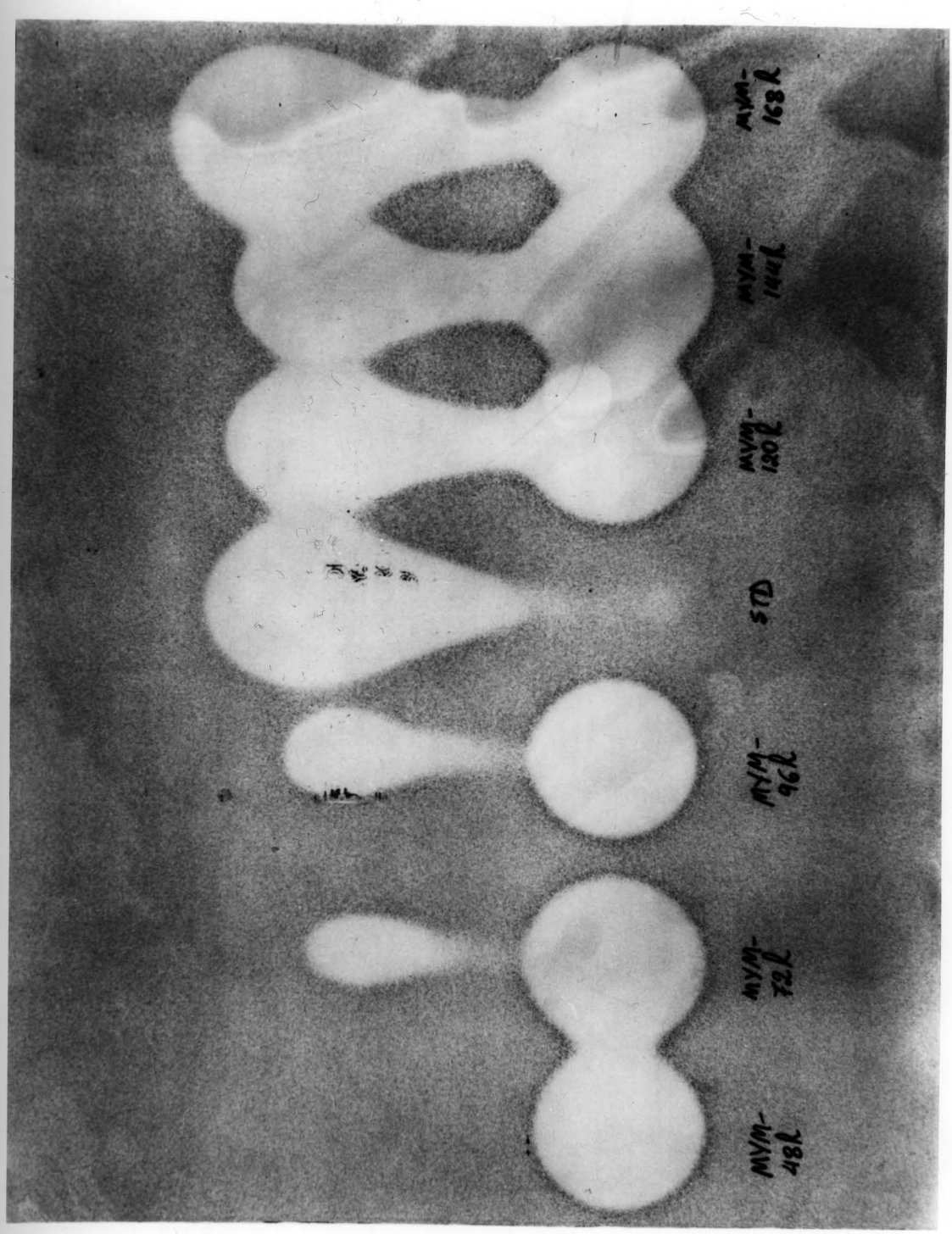
Two different means of assaying lasalocid were developed and used during these experiments. The first method is a bioassay which uses the combined results of TLC and bioautography (Figure 12). The simple disc bioassay (Kavanagh, 1975) for antibiotics will not work in this case due to the co-production of a second antibiotic, echinomycin (Figure 13), by S. lasaliensis; the bioautography allows for the separation of echinomycin from the lasalocids. When developing the TLC plates for use in bioautography, it is important to use EtOAc as the solvent system, rather than the hexane:^{i}PrOH:AcOH mixture, since any residual acid on the plates will interfere with the tetrazolium chloride dye reaction and inhibit the growth of the test organism. Later in these experiments the analytical HPLC assay was employed and was found to be superior to the bioautography both in levels of detection and in separation of lasalocid A from its homologs and its isomer (Figure 14). In a typical growth curve experiment, lasalocid can be detected at 48 hours after inoculation by HPLC, whereas by bioautography lasalocid is not readily detectable until 72 hours after inoculation (see Figures 9 and 12 for comparison).

Figure 12. Bioautograms of Growth Curve Experiment #2

a) MYM Media at 48, 72, 96, 120 144 and 168 hours p. 75

b) SP/LO Media at the same time intervals p. 76

In both cases, the lower R_f zone of inhibition is due to echinomycin. In the case of the SP/LO media, the results of the assay are erratic due to the complex media.


MVM-48h
MVM-72h
MVM-96h
STD
MVM-120h
MVM-144h
MVM-168h

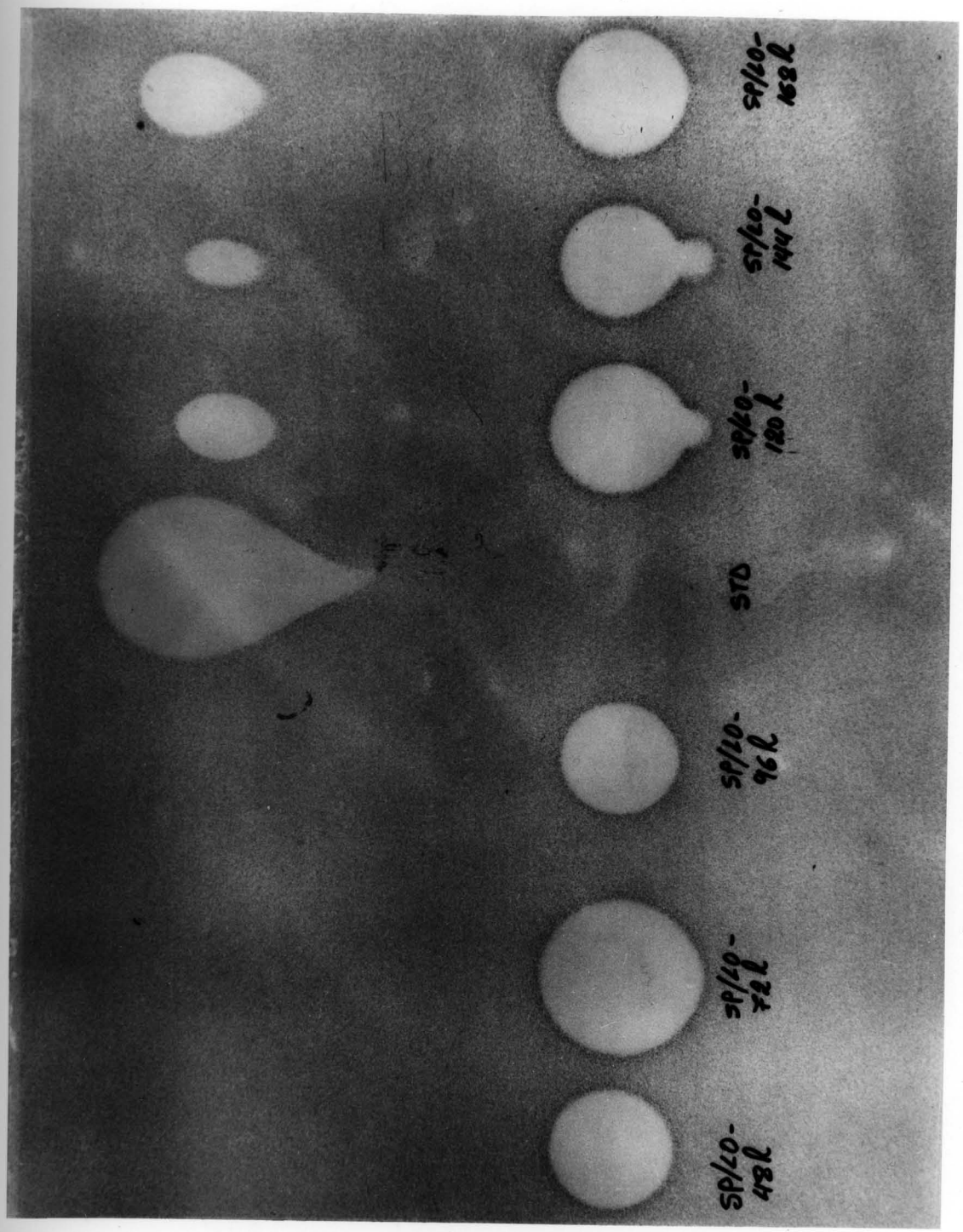
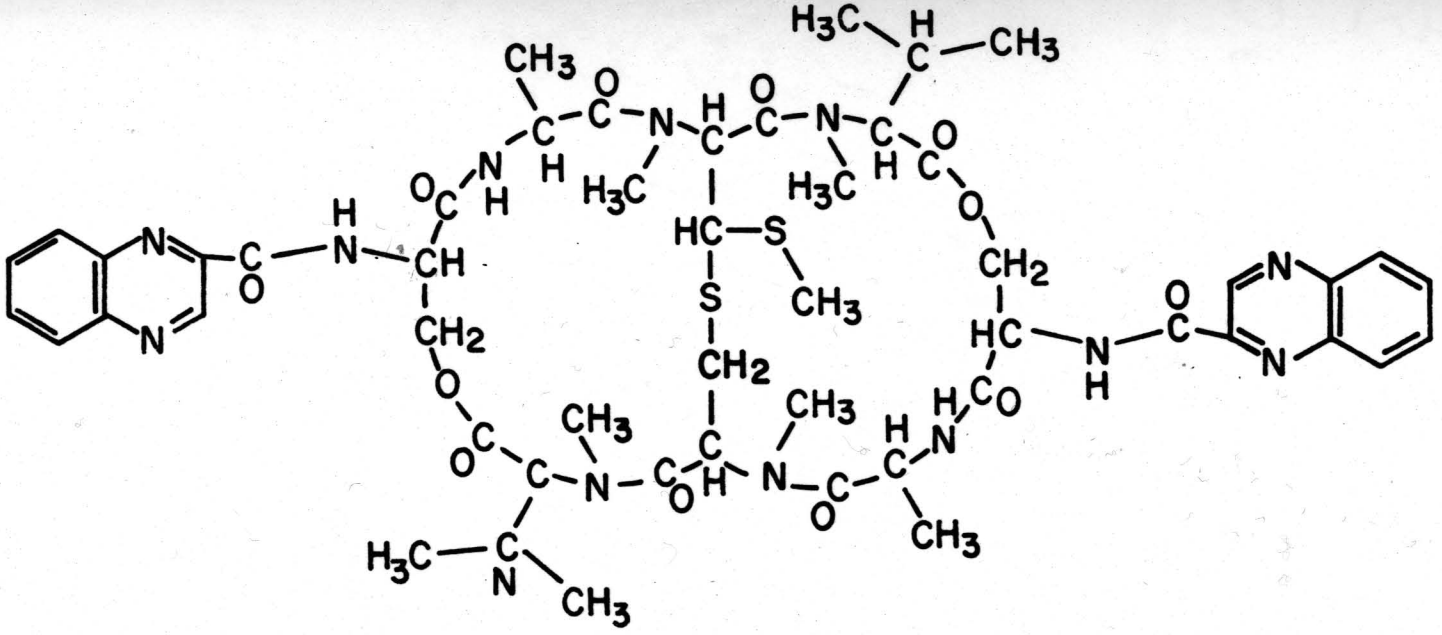

SP/40-48L
SP/40-72L
SP/40-96L
STD
SP/40-120L
SP/40-144L
SP/40-168L

Figure 13. Structure of Echinomycin,
a Quinoxaline Oligopeptide Antibiotic

Echinomycin is co-produced by
Streptomyces lasaliensis

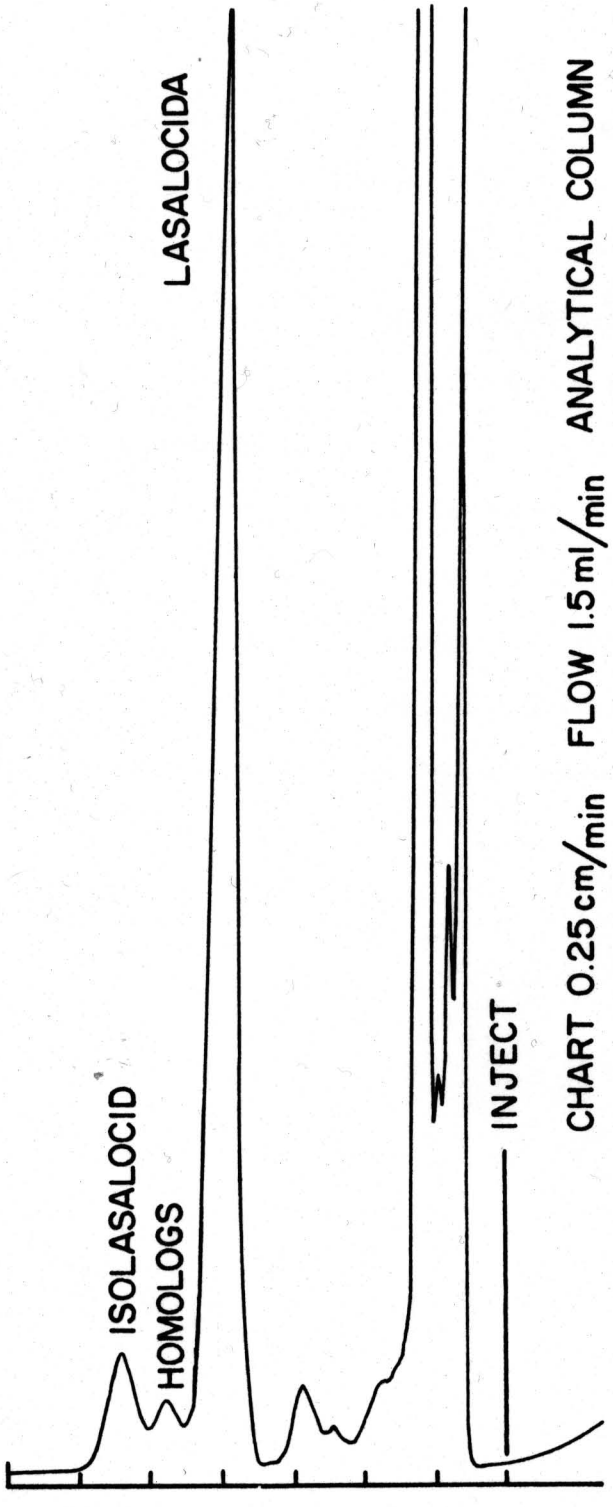


ECHINOMYCIN

Figure 14. High Performance Liquid Chromatogram of the Lasalocids

Waters uBondapak C_{18} Analytical Column
Mobile Phase: MeOH:1% Aq. AcOH (75:25)
Flow Rate: 1.5 mL/min
Monitored at 254 nm
Chart Speed: .25 cm/min

The initial peaks are solvent and echinomycin.


ISOLASALOCID
HOMOLOGS
LASALOCIDA
INJECT
CHART 0.25 cm/min FLOW 1.5 ml/min ANALYTICAL COLUMN

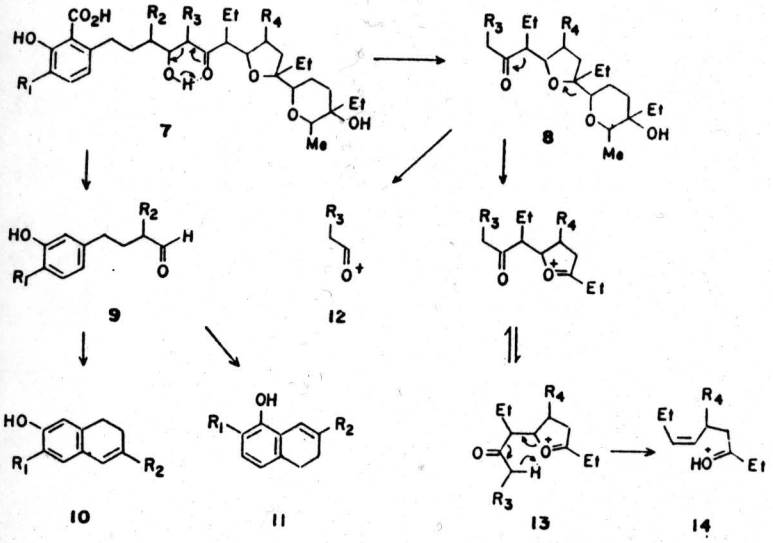
MS and NMR Analysis

The mass spectral analysis of the lasalocids was reported by Westley et al. (1974c) in their structural determination of the four homologs and isomer of lasalocid A (Figure 15). These assignments were found to be consistent with both the standards obtained from Westley and from the isolated fermentation product, and were therefore used throughout these investigations. The predominant pathway is the cleavage of the β-ketol system (Scheme 1, Figure 15), resulting in the peaks at m/e 211, m/e 155 and m/e 174 which served as the basis for analysis of enrichment. The fragments arising from the anhydro-descarboxy ion (Scheme 2, Figure 15) were always a minor contribution, and could readily be distinguished from any enriched peaks in the primary pathway by high-resolution MS.

Figure 15. Mass Spectral Fragmentation Pattern of the Lasalocids

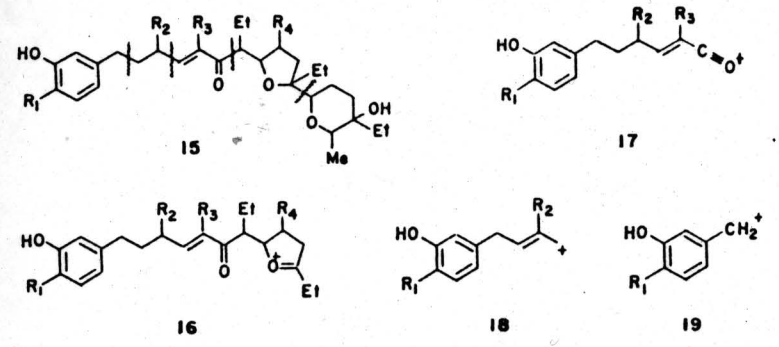
Tables are from Westley, et al., 1974c

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

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

The molecular weights of each fragment were calculated using the following values (Silverstein, 1974).

$^{12}C = 12.0000$ $^{13}C = 13.00336$

$^{1}H = 1.00783$ $^{2}H = 2.01410$

$^{16}O = 15.9949$ $^{18}O = 17.9992$

Thus, for the fragments of interest the calculated molecular weights for the unenriched peaks are:

$\underline{m/e}$ 211 = $C_{13}H_{23}O_2$ = 211.16989

$\underline{m/e}$ 155 = $C_{10}H_{19}O_1$ = 155.14367

$\underline{m/e}$ 174 = $C_{12}H_{14}O_1$ = 174.10452

In the case of distinguishing a peak at m/e 174 that is enriched from that of the minor pathway at m/e 175, the following values would be expected:

$\underline{m/e}$ 175 = $C_{12}H_{15}O_1$ = 175.11235

$\underline{m/e}$ 175 = $^{12}C_{11}{}^{13}C_1H_{14}O_1$ = 175.1079

$\underline{m/e}$ 175 = $C_{12}{}^{2}H_1{}^{1}H_{13}O_1$ = 175.11079

Under optimum conditions, these differences in molecular weights could be determined by high-resolution MS on the AEI MS9.

The nuclear magnetic resonance (NMR) spectroscopy of the polyether antibiotics has presented a considerable challenge due to the highly complex nature of this class of compounds; lasalocid A is one of the simpler polyethers in that it only contains 34 carbons and 54 protons. Two groups have published results of the systematic study of the NMR spectra of the polyethers; Anteunis _et al._ have pursued the ^{1}H-NMR assignments, while Seto _et al._ have undertaken the ^{13}C-NMR studies. In

both cases, the investigations did not begin until the early 1970's when higher-field nmr spectrometers became available.

Anteunis published his ^{1}H-NMR study of lasalocid A in 1976, using a 300 MHz instrument. By combining the use of double resonance and homo-INDOR studies on different forms of lasalocid A, the majority of the proton assignments could be made. Lasalocid A will change conformations between the sodium salt and the free acid forms, and it will undergo aromatic solvent induced shifts (ASIS). Thus, sodium lasalocid A in $CDCl_3$ is in the closed complex form (see Figure 5), while lasalocid A free acid in C_6D_6 is an open chain form. Despite his rigorous study, several ambiguities remained in the proton assignments.

At the beginning of these investigations in an attempt to resolve some of these uncertainties, the 400 MHz ^{1}H-NMR of sodium lasalocid and the free acid form were run by J.C. Vederas, University of Alberta (Figure 16). Vederas used single-frequency-off-resonance-decoupling (SFORD) techniques to make the assignments. In most cases, but not all, the assignments made by Vederas and those made by Anteunis coincide (Figure 16).

Most notably, the 9 line multiplet at 2.14 ppm (sodium lasalocid A in $CDCl_3$) was assigned to H-10 by Anteunis and to H-16 by Vederas. The assignment made by Vederas was based on irradiating H-29, H-15 and H-17; of these only H-15 is clearly resolved from any other resonance. Anteunis, on the other hand, noted that there was a large inverse ASIS for H-10 vs H-16 in $CDCl_3$, and he could therefore not rely on a simple comparison between solvents in order to make the assignments.

Consequently, he used consequtive INDOR and double resonance experiments irradiating H-8 (A and B) and H-9 (A and B) to assign H-10; and H-30 (A and B), H-14 and H-15 to assign H-16. Again, while these methods are rigorous the resonances for these protons are not clearly distinguishable at 300 MHz. Interestingly though, the two assignments for H-10 and H-16 of lasalocid A free acid in C_6D_6 coincide; that is, H-10 is assigned to 1.87 ppm while H-16 is assigned to 1.65 ppm. For the purposes of these experiments, the ^{1}H-NMR of lasalocid A was run using the free acid in C_6D_6 in order to distinguish these two resonances.

In one case the ^{1}H-NMR of sodium lasalocid A was obtained on the 600 MHz instrument at Carnegie-Mellon. While the higher field clearly resolved several overlapping multiplets (see Figure 16), no rigorous decoupling experiments were carried out, and consequently very few of the ambiguities in the spectra were resolved. As a result, the ^{1}H-NMR of lasalocid A is still not absolutely assigned; however, the available data were adequate for the purposes of these investigations. With the increase in availability of high-field spectrometers (400-500 MHz), and the use of 2D spectroscopy, especially ^{13}C-^{1}H correlated spectroscopy, it should now be considerably easier to make the ^{1}H assignments.

Figure 16: ^{1}H-NMR Assignments of Lasalocid A

a) The different numbering systems used in the literature for lasalocid A. The currently accepted numbering system is that proposed by Westley, which is base on a common polyether nucleus (Westley, et al., 1979) For simplification, all assignments are given using Westley's numbering. p. 88

b) Tables of ^{1}H-NMR Assignments p. 89
p. 90
p. 91

All chemical shifts are in ppm relative to TMS.

The abbreviations A and B refer to the two protons of the CH_2 group; the abbreviations a and e refer to the axial and equatorially oriented protons for the given position.

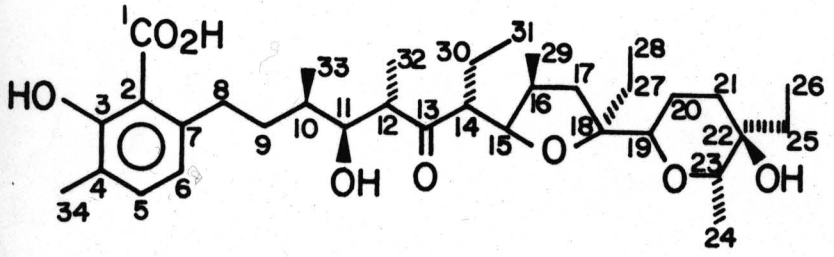
c) ^{1}H-NMR Spectra Obtained at 400 MHz

Sodium Lasalocid A in $CDCl_3$ p. 92
Expansion p. 93

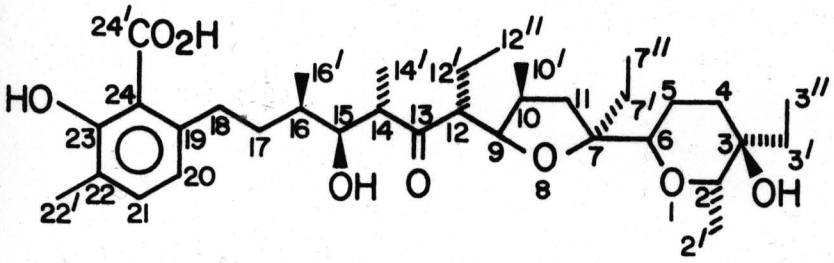
Lasalocid A Free Acid in C_6D_6 p. 94
Expansion p. 95

NUMBERING SYSTEM FOR LASALOCID A

WESTLEY



ANTEUNIS



^{1}H-NMR ASSIGNMENTS OF SODIUM LASALOCID A IN $CDCl_3$

Anteunis		Vederas		
δ	Position	δ	Multiplicity J(Hz)	Position
6.95	5	6.95	d,8	5
6.45	6	6.45	d,8	6
4.80	OH	4.89	s	OH
4.52	11	4.57	d,10	11
3.98	8A	4.00	m	8,23
3.98	23			
3.7	15	3.69	d,10	15
3.45	19	3.45	d,12	19
2.82	12	2.84	d q,7,10	12
2.53	14	2.57	d,10	14
2.23	8B	2.22	br s	34,8
2.20	34			
2.14	10	2.14	9 line m	16
1.93	30A	1.92	d of q	30
1.84	20a	1.85	m	20a
1.79	9A	1.78	m	17,9
1.60	16	1.62	d of d	25
1.48	25A	1.55		9
1.43	21e			
1.3	9B			
1.3	30B			
1.19	20e			
1.07	24			
0.99	33			
0.95	26			
0.82	29			
0.8	32			
0.76	28			
0.57	31			

^{1}H-NMR ASSIGNMENTS OF SODIUM LASALOCID A IN C_6D_6

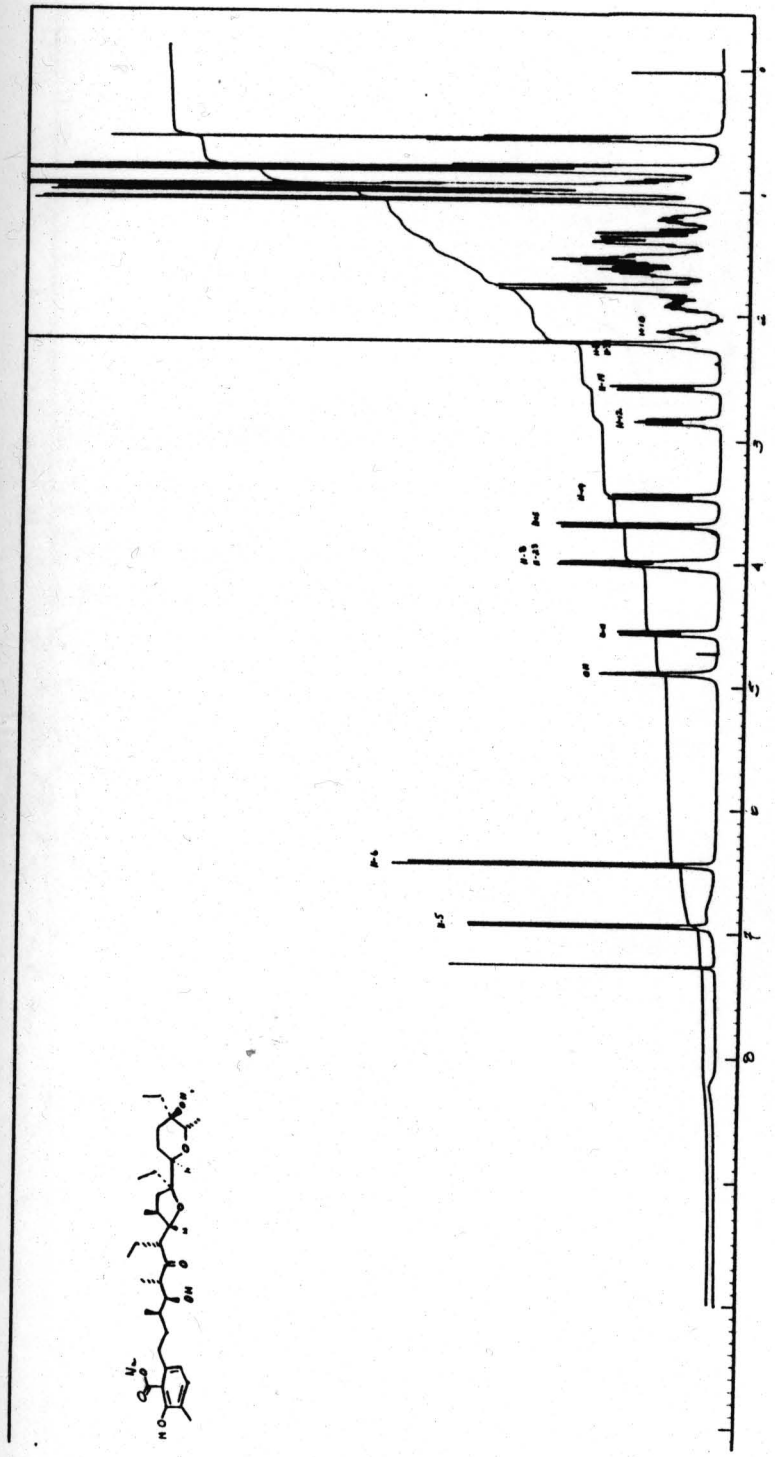
δ	Position
7.08	5
6.67	6
4.68	11
4.1	23
4.09	15
2.80	12
2.47	34
2.38	14
2.09	10
1.72	16
0.94	24
0.89	33
0.80	32
0.82	28

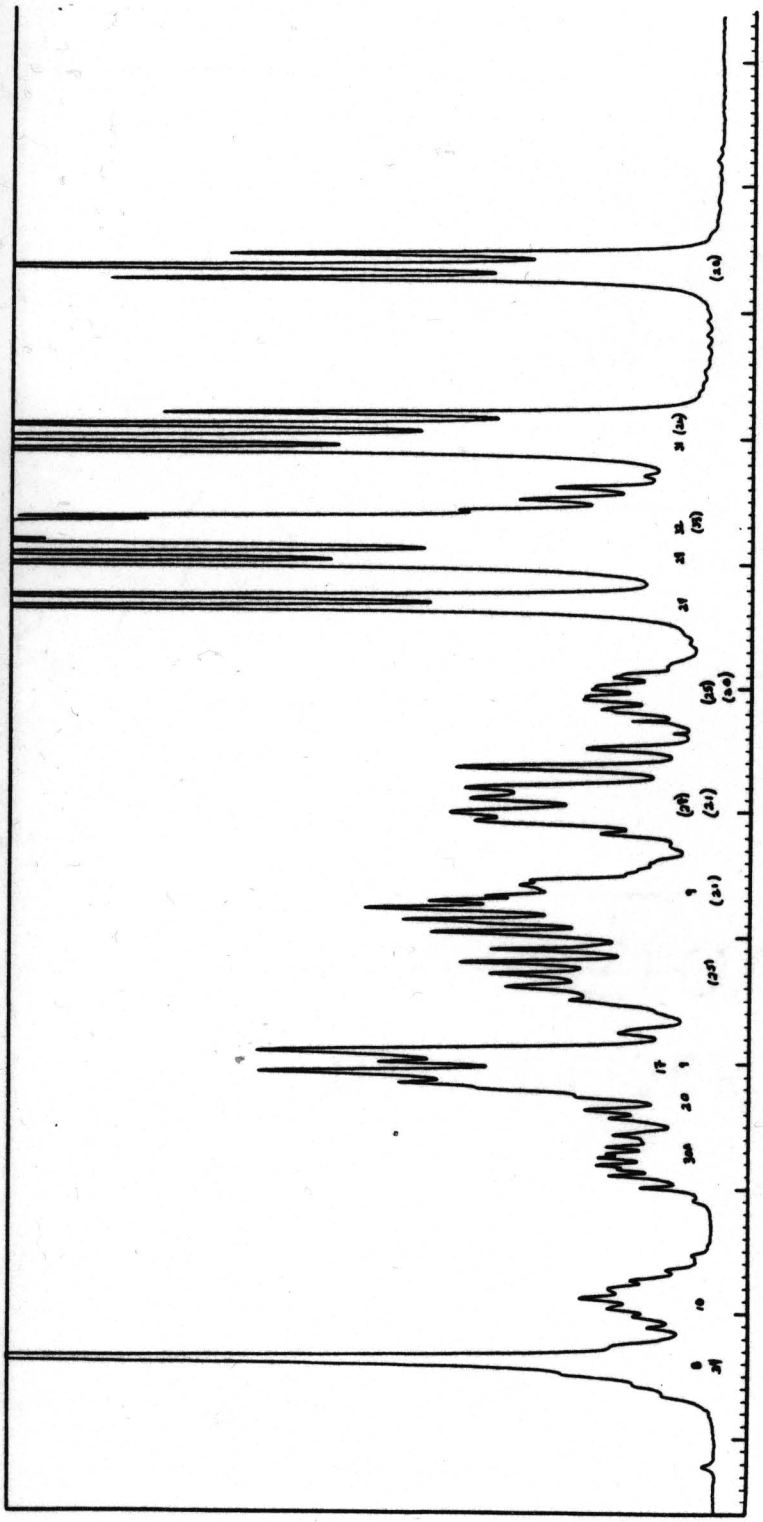
^{1}H-NMR ASSIGNMENTS OF SODIUM LASALOCID A (600 MHz) in $CDCl_3$

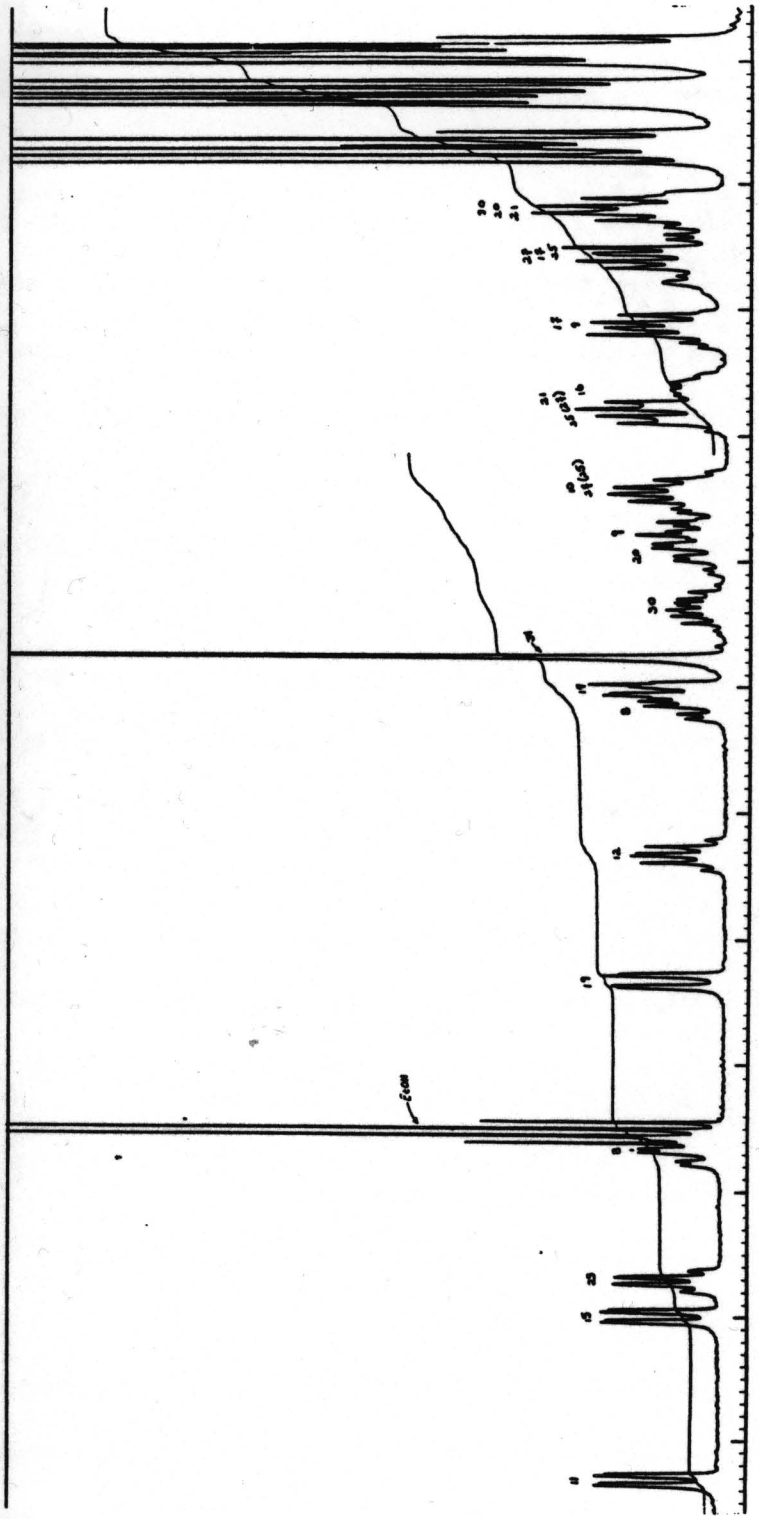
δ	Multiplicity J(Hz)	Position
1.069	d,7	24
0.998	d,6.6	33
0.994	t,7.4	26
0.936	d,7	29
0.817	d,6.5	32
0.776	t,7.4	28
0.57	t	31

^{1}H-NMR ASSIGNMENTS OF LASALOCID A FREE ACID IN C_6D_6

Anteunis		Vederas		
δ	Position	δ	Multiplicity J(Hz)	Position
7.01	5	7.03	d	5
6.56	6	6.58	d	6
6.1	OH			
4.35	11	4.38	d d,8,2	11
3.96	15	3.97	d d,8,2	15
3.86	23	3.88	q,6	23
3.52	8A	3.55	t d,10,3	8A
3.13	19	3.12	d d,8	19
2.80	12	2.80	d q,6,8	12
2.39	8B	2.42	t d,10,3	8B
2.39	14	2.40	d,8	14
2.29	34	2.31	s	34
2.13	30A	2.18	d q,10,6	30A
2.04	20a	2.04	d q,3,10	20a
1.97	9A	2.00	t t	9A
1.88	27A			
1.87	10	1.90	m	10
1.68	25A,21e	1.70		25A
1.65	16	1.64		16
1.49	9B	1.48		9B
1.45	17			
1.28	27B			
1.20	17,25			
1.17	30B	1.19		30B
1.13	20e			
1.08	21a			
1.00	26	1.00		26,33
0.90	29	0.91		29
0.87	24,31	0.89		31
		0.86		24
0.81	33			
0.80	32	0.80		32
0.76	28	0.76		28







The ^{13}C-NMR spectrum of the polyether antibiotics can be as complex as the ^{1}H-NMR spectrum due to overlapping resonances. Nevertheless, the ^{13}C-NMR assignment of lasalocid A was done by Seto et al. in 1978 at 22.5 MHz. The assignments were made based on various model compounds representing fragments of lasalocid A, as well as with biosynthetically enriched samples of lasalocid A. The two drawbacks to this study are: first, the use of a relatively low-field spectrometer; and secondly, the use of biosynthetically enriched samples to make assignments. At the time that this study was done a thorough biosynthetic study of laslocid A had not been completed, so that to use biosynthetically enriched samples as a means to make assignments becomes a circular argument.

Despite the complexity of the ^{13}C-NMR spectrum, there are three strategies that can be used to clarify the spectra. First, the ^{13}C-NMR spectrum, like the ^{1}H-NMR spectrum, shows characteristic shifts between the sodium salt form and the free acid of lasalocid A (both in $CDCl_3$), and resonances that overlap in one form often are separated in the other. Secondly, the use of higher-field nmr spectrometers which will resolve the resonances. At the beginning of these investigations the ^{13}C-NMR spectra of both the free acid and sodium salt of lasalocid were obtained at 100 MHz by J.C. Vederas (Figure 17). Finally, the use of some of the recent NMR pulse programs which will distinguish CH_3- from $-CH_2-$ and $-CH_1-$, in particular, the Attached Proton Test (APT) developed by Patt and Schoolery (1981), has proven to be a useful method for distinguishing the resonances of lasalocid A at 50 MHz (Figure 17) which until very recently was the maximum field strength available on this campus.

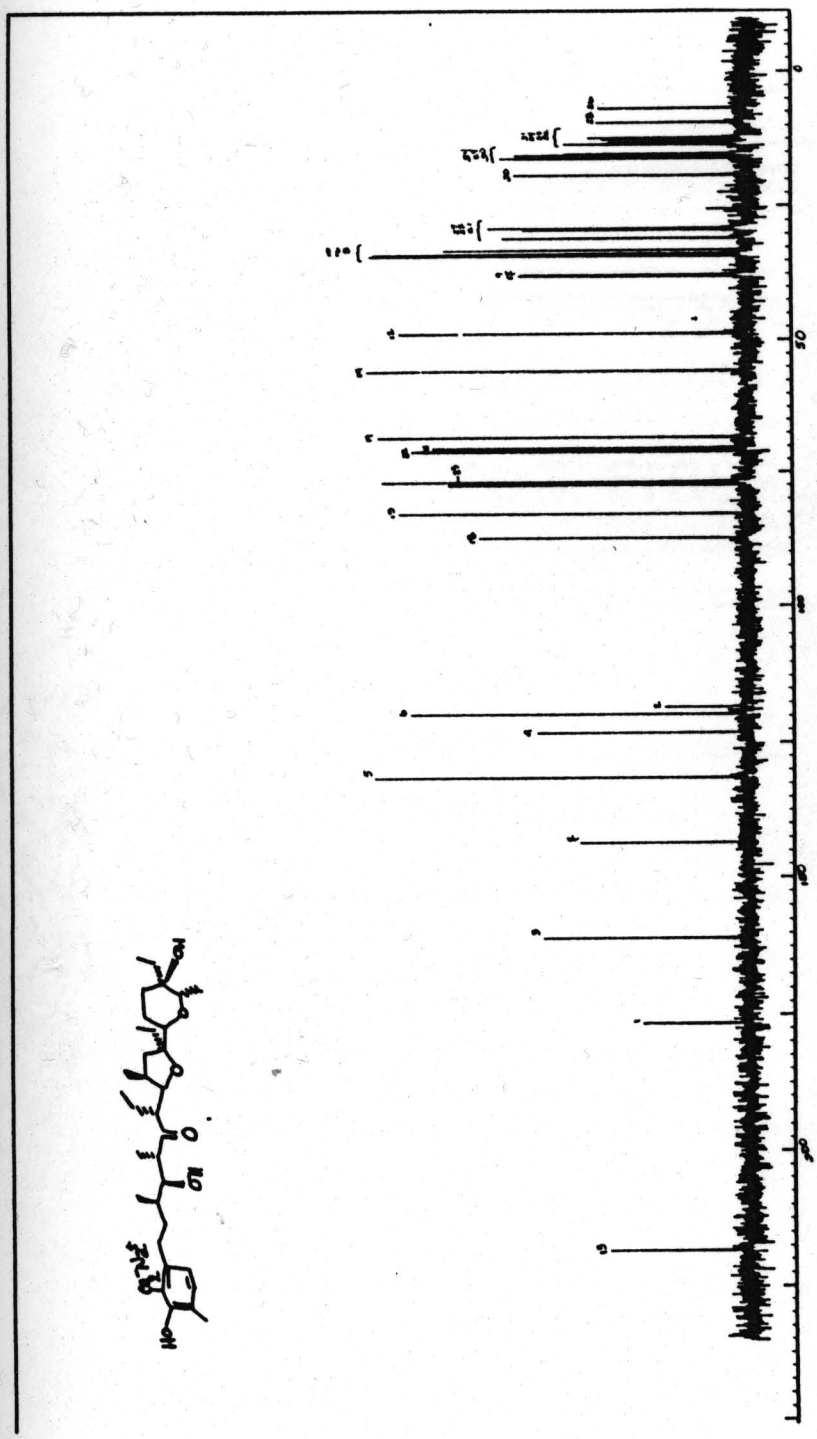
The APT experiment is a double spin-echo pulse sequence which converts the J coupling into amplitude modulation, resulting in a ^{13}C-NMR spectra which distinguishes carbons of different multiplicity. The APT experiment is an alternative to SFORD, and in larger molecules is often more accurate than SFORD techniques. In the case of lasalocid A, the pulse sequence: $90^{\circ}-\tau-180^{\circ}-\tau-\Delta-180^{\circ}-\Delta-$, was used, with τ set to 8 msec and Δ to 1 msec. This sequence gave a spectra in which the methyl and methine carbons are negative signals, the methlyene carbons are positive signals and the quartenary carbons are essentially unmodulated. In the APT spectra of lasalocid A, it was possible to distinguish several signals which were overlapping in the broadband decoupled ^{13}C-NMR spectra. In particular, C-23 which normally lies under or very close to the $CHCl_3$ triplet was resolved as a negative signal, while the solvent peaks remained positive; C-11 and C-22 which coincide at 50 MHz at ca. 72 ppm were distinguised as a positive signal for C-22 and a negative signal for C-11 that switch relative positions in going from the sodium salt to the free acid form of lasalocid A; the signals for C-10, C-16 and C-8 at ca. 34 ppm differ by only 0.1-0.3 ppm, however in the APT spectra C-8 could be resolved as a negative peak, while C-10 and C-16 were positive. The data from the Vederas and APT studies gave assignments of lasalocid A corresponding to those made by Seto et al. The absolute chemical shifts (in ppm, relative to $CDCl_3$ assigned to 77.0 ppm) will differ by a few tenths of a ppm from those given by Seto et al.; however, the relative sequence of the peaks remains the same.

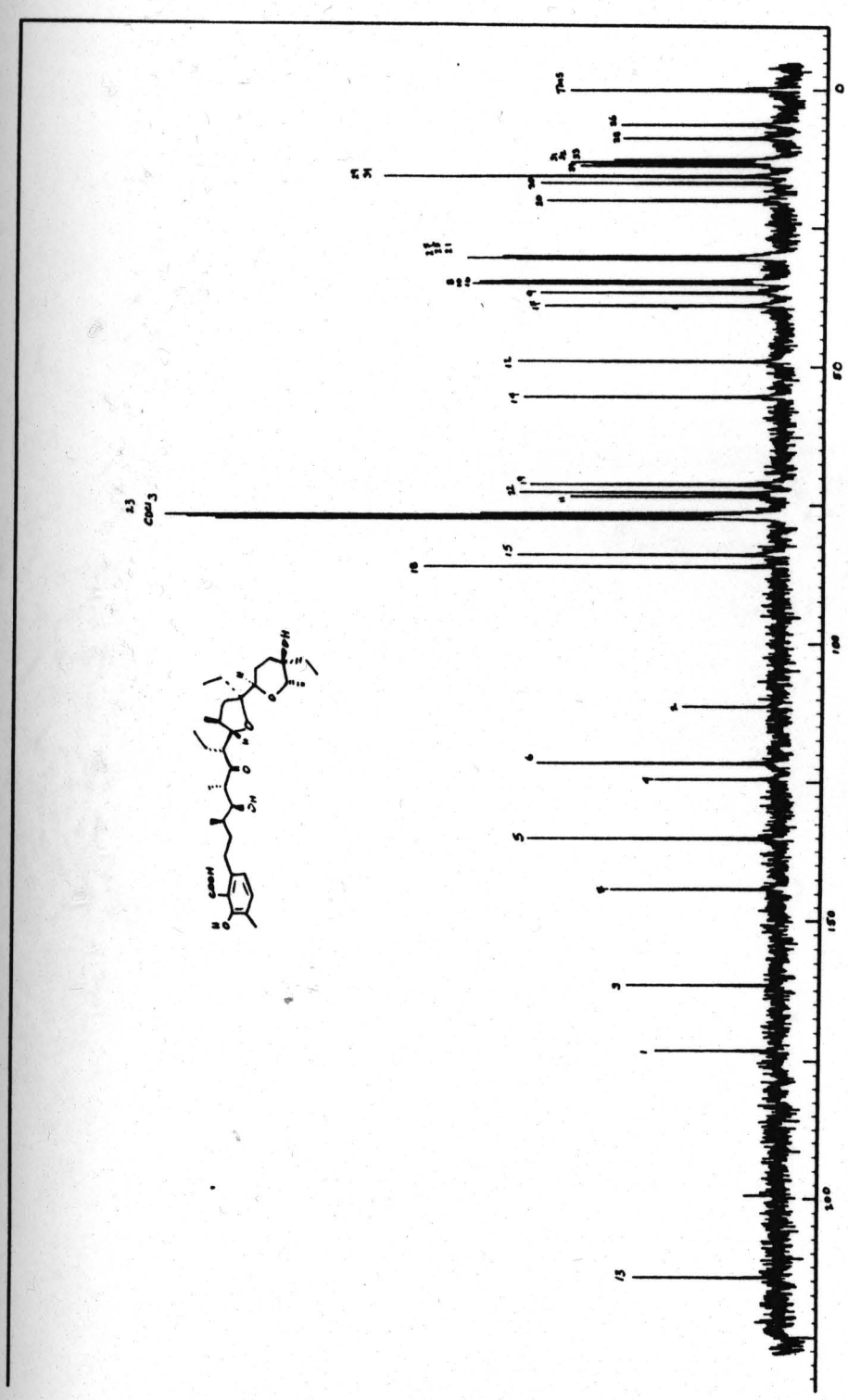
The combined analytical techniques of HPLC, MS and NMR, used during these investigations, assures that lasalocid A can be readily identified, and separated from any interfering compounds, such as its homologs or its isomer; and, that accurate assignments can be made in order to follow the biosynthetic pathways.

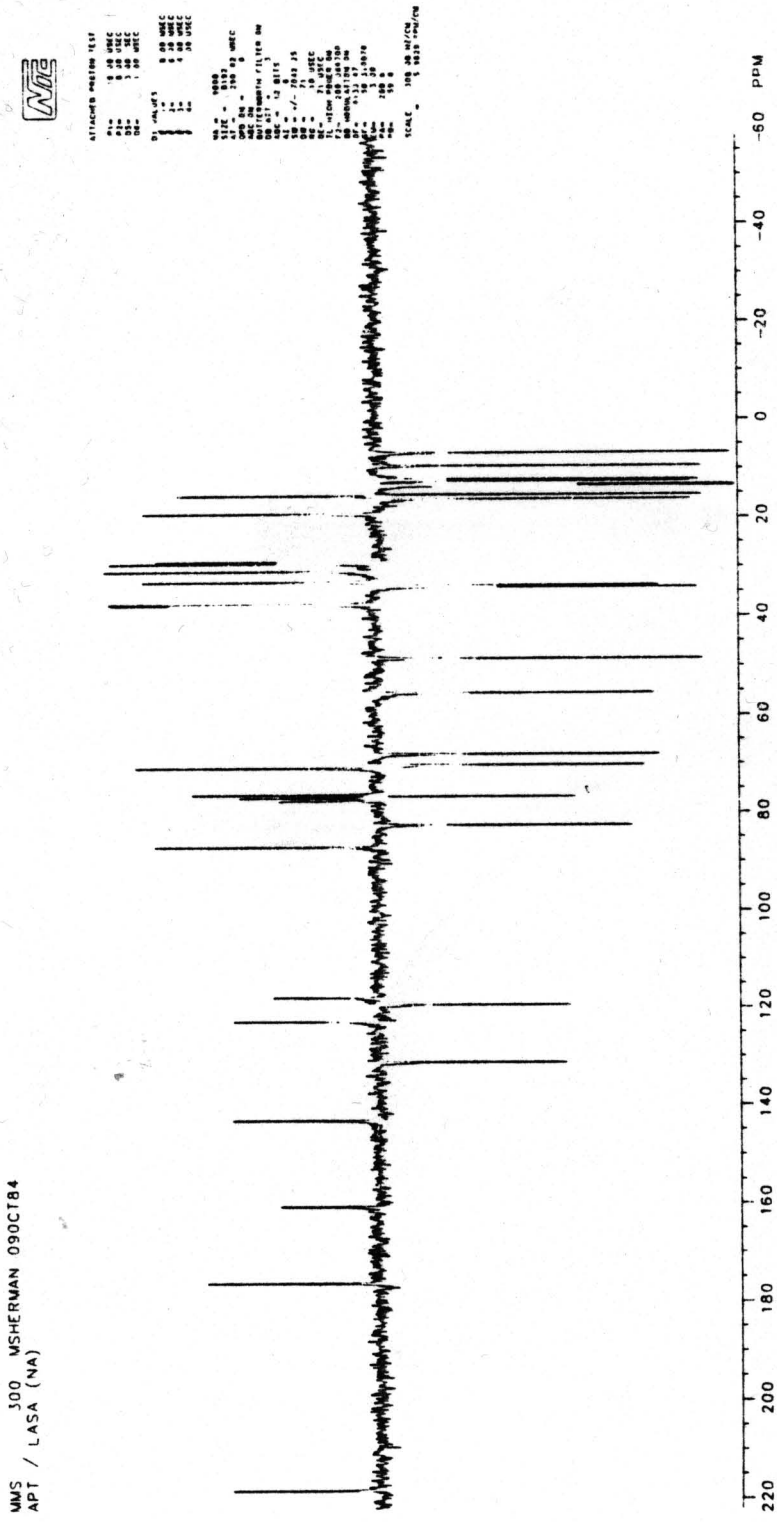
Figure 17. ^{13}C-NMR Assignments of Lasalocid A

^{13}C-NMR ASSIGNMENTS OF LASALOCID A IN CD_2Cl_2

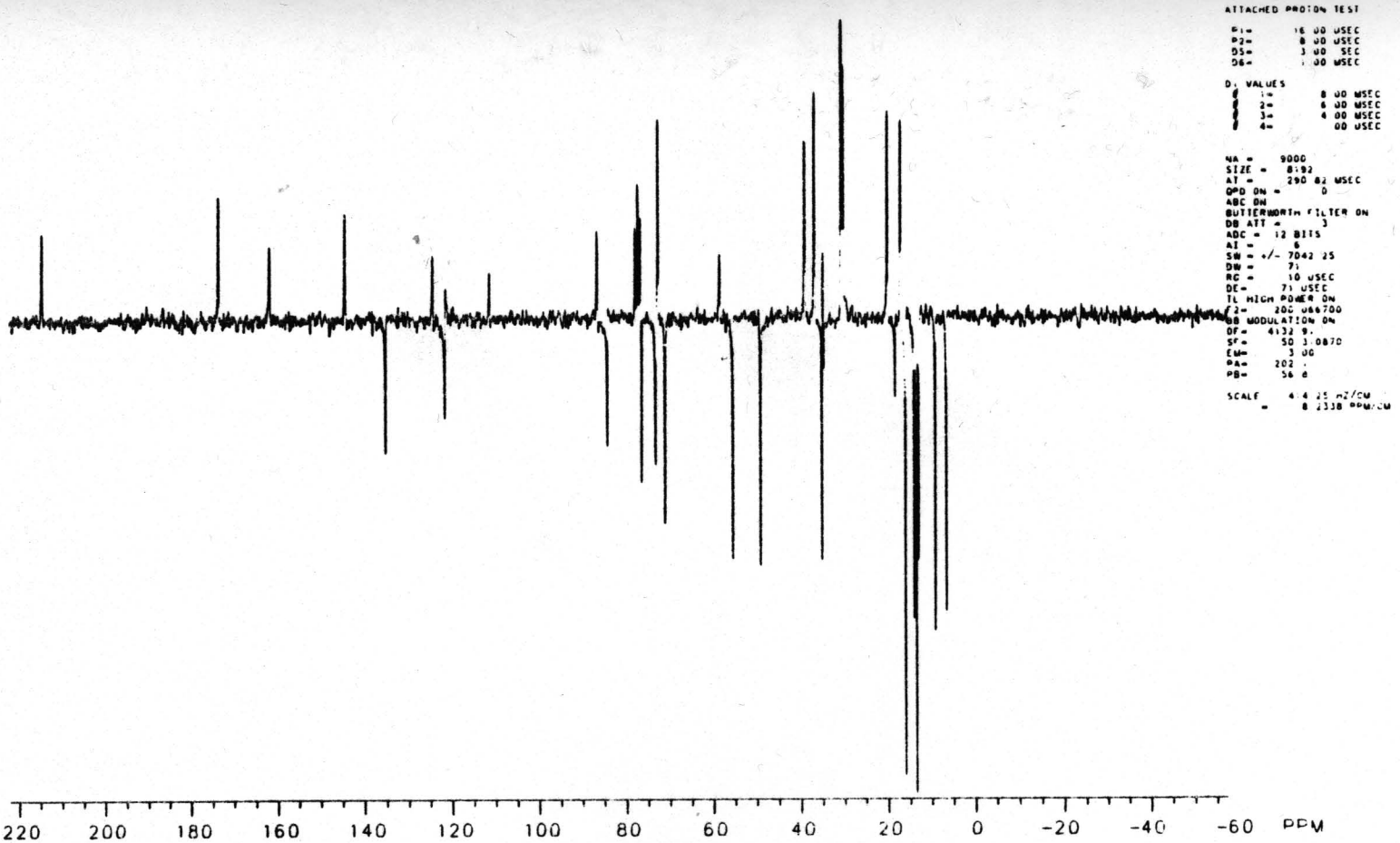
Position	Free Acid	Sodium Salt
1	173.6	176.4
2	111.2	118.2
3	161.6	161.3
4	124.1	123.0
5	135.1	131.5
6	124.1	123.0
7	144.4	143.5
8	34.8	33.4
9	37.0	38.1
10	34.9	34.5
11	72.8	71.0
12	48.9	49.0
13	214.1	219.9
14	55.4	55.9
15	84.2	83.1
16	34.8	34.1
17	38.7	37.9
18	86.7	87.6
19	70.9	68.6
20	20.0	19.5
21	30.2	29.2
22	72.4	71.5
23	76.6	77.2
24	14.0	13.6
25	30.7	31.1
26	6.6	6.7
27	30.6	29.8
28	9.2	9.5
29	15.9	16.0
30	16.7	16.2
31	12.9	12.4
32	13.2	12.6
33	13.4	13.3
34	15.7	15.3






WWS 300 WSHERMAN 090CT84
APT / LASA (NA)
PPM
-60
-40
-20
0
20
40
60
80
100
120
140
160
180
200
220

MMS 310 MSHERMAN 100CT84
APT / LASA (ACID)



CHAPTER TWO

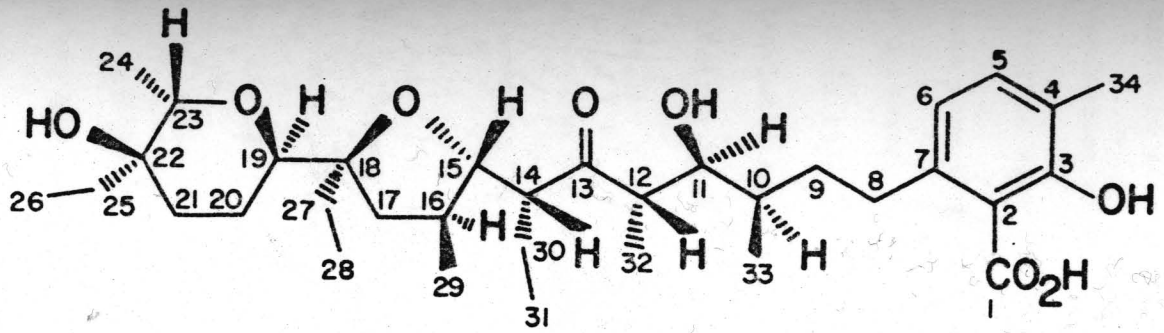
BIOSYNTHESIS OF THE POLYETHER ANTIBIOTIC LASALOCID A: STABLE ISOTOPE STUDIES

INTRODUCTION

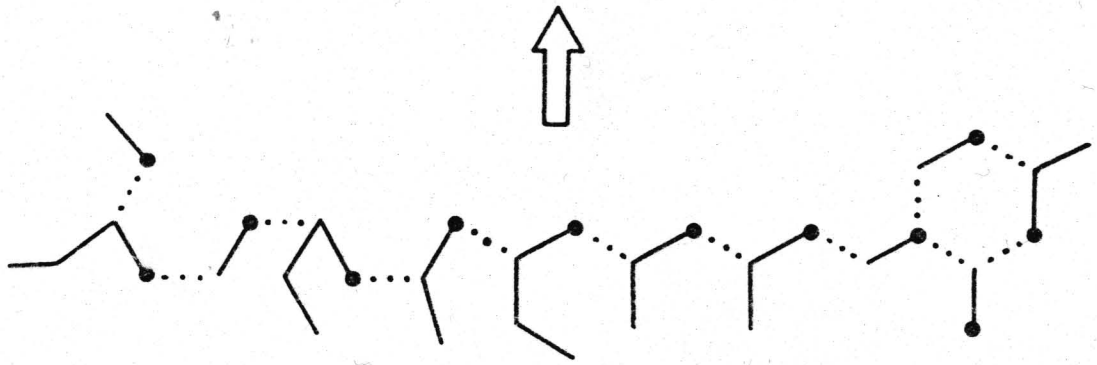
In 1970, Westley et al. reported the results of the initial biosynthetic studies on Antibiotic X-537A (lasalocid A) in which they investigated the incorporation of several small ^{14}C labelled precursors. These investigators were interested particularly in the origin of the methyl and ethyl groups of lasalocid A. Their study showed that the primary precursors for lasalocid A were acetate, propionate and butyrate; and that formate, methionine, ethionine and mevalonate were not incorporated. Based on these results it was concluded that the methyl and ethyl groups in lasalocid A arise from the propionate and butyrate precursors, and not by transmethylation, transethylation or introduction of terpenoid units (Figure 18).

This report was followed by a second study in which the same group (Westley et al., 1972) looked at the incorporation of [1-^{13}C]-propionate and [1-^{13}C]-butyrate into lasalocid A by ^{13}C-NMR. The results of this study showed that [1-^{13}C]-propionate enriched the carbons at C-3, C-9, C-11 and C-15 about four times above natural abundance; while [1-^{13}C]-butyrate enriched the carbons at C-13, C-17 and C-21 about four times above natural abundance, and those at C-5, C-11, C-15 and C-23 about 1.5 times natural abundance. From these data it was proposed that all methyl groups, except the one at C-23, arise from a propionate precursor; while all three ethyl groups arise from a butyrate

Figure 18. Biosynthetic Precursors of Lasalocid A



I



5 — CH_3CO_2H

4 — $CH_3CH_2CO_2H$

3 — $CH_3CH_2CH_2CO_2H$

Biosynthetic Precursors of Lasalocid A (I)

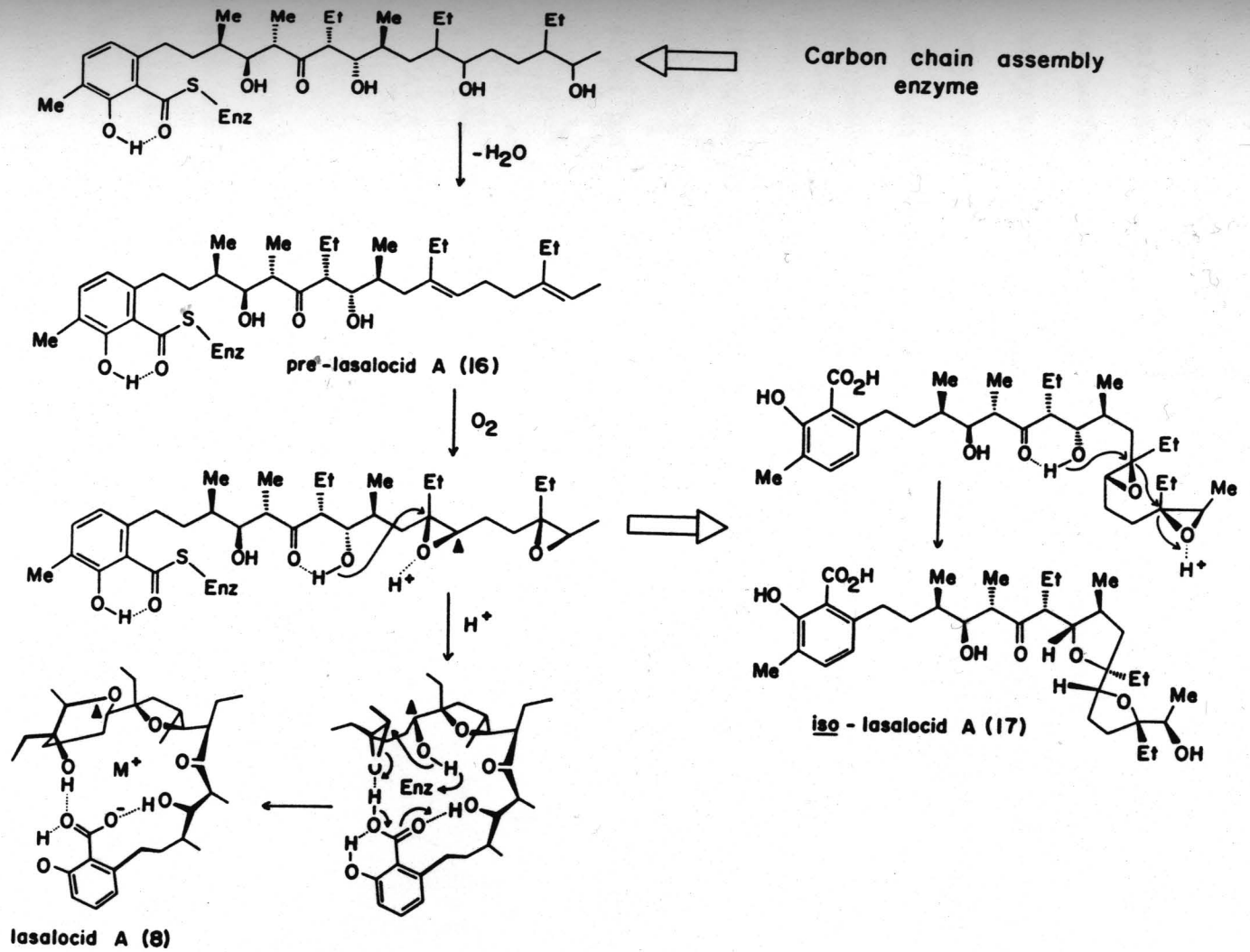
precursor. The authors noted that there is an interconversion of acetate and butyrate, probably via condensation of two acetate units to acetoacetate and thence butyrate, or conversely, by β-oxidation of butyrate to acetate, based on the labelling pattern observed with [1-^{13}C]-butyrate and [1-^{14}C]-acetate. While their data show that there is an apparent conversion of butyrate to propionate, no mention was made of this in the report.

In 1974, a series of papers appeared on the biosynthesis of lasalocid A (Westley et al., 1974a; 1974b; 1974c) in which the investigators looked not only at the incorporation of ^{13}C and ^{14}C labelled precursors into lasalocid A, but also reported the isolation and structural elucidation of four homologs and an isomer of lasalocid A (Figure 11, p. 72). From these studies, the investigators concluded that lasalocid A was formed from 5 acetate, 4 propionate and 3 butyrate subunits; that acetate served as the starting unit at C-23 and C-24; and that the ethyl groups in lasalocid A arose from a butyrate precursor via ethylmalonate, the first such case to be reported. It was also suggested that the lasalocid homologs arise as a result of propionate deficiences in the fermentation media (Westley et al., 1974c). Westley et al. (1974a; 1974b) then advanced a biosynthetic pathway for lasalocid, in which the assembly of the lasalocid subunits occurs on a multienzyme complex similar to fatty acids and 6-MSA. It was proposed that the formation of lasalocid proceeds through a diene

intermediate, which after oxidation to the diepoxide, can rearrange to either lasalocid A or isolasalocid A (Figure 19).

Figure 19. Hypothetical Scheme for the Biosynthesis of Lasalocid A

Adapted from Westley, et al., 1974b


Carbon chain assembly enzyme
-H2O
pre-lasalocid A (16)
O2
H+
lasalocid A (8)
iso - lasalocid A (17)

The model proposed by Westley et al. serves as a good starting point for additional investigations into the assembly of the polyether antibiotic lasalocid A. While Westley's investigations concentrated on tracing out the different subunits involved in the biosynthesis of lasalocid, they did not delve further into the actual assembly process. Lasalocid A is a structurally complex molecule in which the biosynthesis involves the use of uncommon precursors, a specific order in which the subunits are assembled, and a precise stereochemical configuration at 10 of the carbons in the molecule. The focus of my investigations, and in particular, the work presented in this chapter, was therefore on the following three questions.

(i) What are the origins of the C-2 to C-4 subunits?

(ii) What determines the order of assembly of the subunits?

(iii) What process controls the absolute configuration of the chiral centers?

The ground work laid out by Westley's investigations suggested that the C-2 to C-4 subunits of lasalocid are derived from intact acetate, propionate and butyrate via their respective malonylCoA esters. However, these conclusions were reached by looking at the [1-^{13}C] labelled acids, or at variously ^{14}C labelled acids. In the case of the ^{13}C labelled acids, since only the C-1 position contained a label, it could not be concluded for certain that the subunits were incorporated intact. The investigations with the

^{14}C labelled acids were more thorough in that different positions of the acids contained the radioactive label. However, in order to trace the biochemical metabolism of a radioactively labelled carbon, chemical degradations must be carried out to isolate the carbon of interest. This is not feasible for every carbon of lasalocid A (Westley et al., 1970). While the published results rule out the possibility of carbons arising from transmethylation or transethylation processes, they do not consider the possibility of interconversion of the various C-2 to C-4 subunits, nor the possibility that these subunits might arise from unusual or unexpected pathways. By attempting to trace the source of all the carbons in the molecule, it was hoped to gain further insight into the metabolic pathways that give rise to the subunits necessary for the assembly of lasalocid A.

The second and third questions pertaining to the order of assembly of the subunits and the determination of the absolute configuration of the chiral centers are more difficult to pursue by using whole-cell studies. In the former case, it is realistically only possible to postulate a model based on what is known about other systems. However, in the latter case, by looking at the origin of the oxygen and hydrogen atoms, it is possible to consider various points of stereocontrol in the assembly process.

Consequently, what is presented in this chapter is an attempt to trace the biochemical origin of most of the atoms involved in

the assembly of the polyether antibiotic, laslaocid A, and from these studies to postulate a model which will consider the three questions pertaining to the origin of the subunits, the order of their assembly, and the determination of absolute configuration of this antibiotic.

MATERIALS AND METHODS

Degradations/Derivatizations

Esterification of RCO_2Na for MS and NMR Analysis

All of the short chain mono-carboxylic acid precursors used in the feeding experiments were derivatized to give their respective crystalline, p-phenylphenacyl esters.

The derivatization reaction was carried out by adding 0.22 mmole p-phenylphenacyl bromide (recrystallized from EtOH) to 0.2 mmoles RCO_2H in DMF (4 mL, distilled and dried over 4A molecular sieves), then stirring the mixture overnight at room temperature under N_2. The DMF was then evaporated *in vacuo* at 40°C and the residue dried *in vacuo* for 2-3 hours. The dried residue was purified by PLC on silica gel in hexane:EtOAc (8:2), developing the plates twice. The lower R_f band corresponded to the ester, and after eluting, was recrystallized from EtOH to give colorless crystals having the expected melting point.

1,3-bis-OMe-Lasalocid A (2)

Lasalocid A free acid (0.5 mmole) was added to 5 mL DMF before adding freshly prepared and dried Ag_2O (2.6 mmole) and CH_3I (4 mL). The mixture was stirred under N_2 overnight at room temperature. The slurry was then filtered through a medium frit filter packed with Celite using DMF as rinses. The filtrates were evaporated *in vacuo* and the residue was partitioned between CH_2Cl_2

and H_2O. Upon evaporation the CH_2Cl_2 fraction gave a yellow gum which was purified by PLC on silica gel in $CHCl_3$:MeOH (95:5), leaving a yellow oil upon elution and evaporation. The ^1H-NMR and MS data were consistent with the reported values (Westley et al., 1973a).

1,3-bis-OMe-11,22-bis-Trifluoroacetyl-Lasalocid A (3)

1,3-bis-OMe-Lasalocid A (30 mg, 0.05 mmole) in dry pyridine (1 mL) was cooled to 0°C, then TFAA (0.1 mL, 150 mg, 0.7 mmole) was added dropwise with stirring. After warming to room temperature, the reaction was stirred under N_2 for 2 hours, then quenched with ice and extracted with EtOAc. The EtOAc extracts were washed with 1 N HCl, then 0.3 M Na_2CO_3 followed by brine, dried over anhydrous Na_2SO_4, and the EtOAc was evaporated, giving a white solid (37.5 mg, 0.046 mmole, 92.5% yield).

A sample of this material was analyzed by high-resolution MS and high-field ^{13}C-NMR. MS analysis gave: m/e (100%): 571.29 ($C_{30}H_{42}O_7F_{30}$); m/e (11.95%): 539.26 ($C_{29}H_{38}O_6F_3$); m/e (11.53%):457.3 ($C_{21}H_{45}O_{10}$); m/e (18.23%): 303.16 ($C_{18}H_{23}O_4$); m/e (18.5%): 275.16 ($C_{17}H_{23}O_3$); m/e (29%): 175.08 ($C_{11}H_{11}O_2$). The ^{13}C-NMR spectrum showed a downfield shift for C-11 (located at about 81 ppm vs. 73 ppm in the free acid form) and C-22 (91 ppm vs 72 ppm), and an upfield shift for C-23 (72 ppm vs 77 ppm).

This derivatization reaction was used for the samples of lasalocid A isolated from the [1-^{13}C, 1-^{18}O]-propionate feeding and the [1-^{13}C, 1-^{18}O]-butyrate feeding.

Retro-Aldol Cleavage of Lasalocid A

Lasalocid A free acid (0.8 mmole) was reacted with 8% aq. NaOH (2 mL) and dioxane (5 mL) at 25°C, overnight. Evaporation of an EtOAc extract of this mixture yielded **3**. The H_2O solubles were acidified (solid at pH 2) and extracted with Et_2O. The residue obtained from the evaporation of the Et_2O extract was heated at 0.05-0.1 Torr, 190-200°C for 10 mins, yielding a yellow sublimate. ^{1}H-NMR (90 MHz) analysis of the sublimate supported structure **8**. Low-resolution MS confirmed this, m/e, 218 (M^+ 29); 200 (100); and 172 (28). The results were consistent with the reported values (Westley et al., 1973a).

Pyrolytic Degradation of Lasalocid A

The pyrolysis of lasalocid A was carried out in the manner described by Westley et al. (1973b). The reaction yielded the ketone 4 and the rearranged aldehyde fragments, **9a** and **9b**. ^{1}H-NMR of the ketone showed the α-carbonyl proton to appear at 2.4 and 2.6 ppm based on ^{1}H:^{1}H double irradiation experiments; for the aldehyde, assignments were made for -C<u>H</u>O (9.61 ppm), -C<u>H</u>$_3$ (1.12 ppm, J = 6.9 Hz); aromatic -C<u>H</u>$_3$ (2.26 ppm); $CO_2C\underline{H}_3$ (3.77 ppm); -OC<u>H</u>$_3$ (3.92 ppm); aromatic H's (6.88 ppm, 7.15 ppm, J = 7.8 Hz).

Dehydration of **7**

The acid fragment, **7** was dehydrated to **8** by dissolving **7** in MeOH, acidifying with **3** N HCl and evaporating under N_2 and heat. This process was repeated once. The residue was then

recrystallized from CH_2Cl_2-Skelly B. The mp MS and NMR data were consistent with the reported values (Westley et al., 1973b).

NaOMe/MeO^{2}H Exchange of **4**

The ketone fragment **4** (0.06 mmole) was dissolved 1 N NaOMe/MeO^{2}H (1.0 mL) under N_2 and the solution left overnight at room temperature. Upon addition of the base the solution acquired a yellow/green fluorescence. This color disappeared upon quenching the reaction with glacial AcOH to pH 7. Evaporation of the solvent gave a white solid, which was purified by PLC on silica gel in $CHCl_3$:MeOH (95:5) to give **4**. ^{1}H-NMR analysis of this material showed the disappearance of the resonances at 2.8 ppm and 2.6 ppm for the protons at C-12 and C-14. Its mass spectrum showed it to be 68% d_3, 21% d_2, 7% d_1, 3% d_0 (m/e 354).

This reaction was subsequently carried out on **4** from the degradation of lasalocid A from the [2-^{3}H, 2-^{2}H, 1-^{14}C]-propionate feeding and the corresponding butyrate feeding experiments. The resulting residue was purified by PLC in $CHCl_3$:MeOH (95:5), and then distilled at 205°C, 0.1 mm, for 30 mins, before counting.

Precursor Synthesis

[2-^{2}H, 2-^{3}H]-Propionate

2-Methylmalonate (42 mmole) was heated for eight hours at 55-60°C with 4 mL ^2H_2O (Aldrich Gold Label 99.5%) under N_2. The ^2H_2O was evaporated leaving a white solid which was distilled by a Kugelrohr at 135-140°C to effect decarboxylation. The resulting

liquid was filtered, titrated with NaOH to pH 8-9, then evaporated to give [2-$^{2}H_2$]-propionic acid (22 mmmole), as confirmed by the absence of the resonance at 2.36 ppm corresponding to -C$\underline{H}_2$-in the ^{1}H-NMR spectrum.

Sodium [2-^{2}H, 2-^{3}H]-propionate was generated by reacting [2-$^{2}H_2$]-propionic acid (22 mmole) with $^{2}H_2O$ (9 mL), $^{3}H_2O$ (100 mCi) and Na (2 mmole) in a teflon-lined stainless steel bomb (Parr) at 130-140^{o}C for 24 hours. The bomb was cooled to room temperature before opening and the solution was acidified with 18 $\underline{N}$ H_2SO_4 to pH 1, then steam distilled. The distillate was titrated to pH 8-9 with NaOH, the solvent was removed $\underline{in\ vacuo}$ and the residue dried with a toluene/MeOH azeotrope. Counts of the sodium [2-^{2}H, 2-^{3}H]-propionate gave 7.31 +/- 0.2 x 10^{7} dpm/mmole.

[1-^{13}C, 1-^{18}O]-$\underline{\text{Propionate}}$

2 mmoles sodium [1-^{13}C]-propionate (KOR, 90% ^{13}C) was placed in a glass tube with 1 mL $H_2{}^{18}O$ (Monsanto, 90% ^{18}O, 6% ^{16}O; 49.5 mmole ^{18}O, 3.3 mmole ^{16}O), and a strip of ColorpHast^{TM} pH paper. Dry HCl (from NaCl and H_2SO_4) was bubbled into the tube until the pH was 1. The pH strip was removed, the tube was sealed and placed in a furnace at 105^{o}C for 24 hours. After removing the tube and cooling it on ice, it was opened and the solution titrated to pH 8-9 with NaOH. The solvent was evaporated $\underline{in\ vacuo}$ (50^{o}C), followed by toluene-MeOH azeotrope, yielding $CH_3CH_2{}^{13}C{}^{18}O_2$ Na/NaCl.

An aliquot of the sodium [1-^{13}C, 1-^{18}O]-propionate was derivatized to its p-phenylphenacyl ester for mass spectral analysis using the procedure described previously. Low resolution mass spectral analysis of the ester showed $^{13}C:^{18}O$ = 90:72 (62 mole% $^{13}C_1 + {}^{18}O_2$; 20 mole% $^{13}C_1 + {}^{18}O_1 + {}^{16}O_1$) for the M^+ ion at m/e 269.

[2-^{13}C, 2-^{2}H]-Propionate

Sodium [2-^{13}C]-propionate (5 mmoles, Prochem, 91.8% ^{13}C) was placed in a teflon-lined stainless steel bomb (Parr) with NaO^2H. (The NaO^2H was generated from 0.5 mmole Na and 2 mL ^2H_2O (Aldrich Gold Label, 99.5%) under N_2). The bomb was then heated at 130-140°C for 24 hours. After cooling the bomb to room temperature, the ^2H_2O was evaporated in vacuo. The process was repeated two more times, using freshly generated NaO^2H each time. After the final exchange, the solution was acidified to pH 1 with 18 N H_2SO_4, then steam distilled under N_2. The distillate was titrated to pH 8-9 with 10 N NaOH using phenolpthalein as indicator and the solvent was removed in vacuo (50°C). The p-phenylphenacyl ester of [2-^{3}C, 2-$^{2}H_2$]-propionate was generated in the same manner as above. The derivative was analyzed by low resolution MS: to give (uncorrected): 0.12% d_0; 10.5% d_1; 89.3% d_2 for the M^+ ion at m/e 269.

[1-^{13}C, 2-$^{2}H_2$]-Propionate (Repeat)

Sodium [1-^{13}C]-propionate (5 mmoles, Amersham, 90% ^{13}C) was reacted as described above. The reaction was worked up after the final exchange by evaporating the solvent, redissolving the residue in H_2O, adjusting to pH 1 with 18 N H_2SO_4, and evaporation and recrystallization from warm 95% EtOH. This yielded 3.25 mmoles (63%) of Na-propionate. The product was analyzed by ^{1}H-NMR which showed the absence of a resonance at 2.36 ppm for $-C\underline{H}_2-$.

[2-^{13}C, 2-$^2H_2$]-Propionate (Repeat)

This sample of labelled propionate was prepared by Dr. Akira Hori using a Grignard reaction between [1-^{13}C]-ethyl iodide (Los Alamos, 91% ^{13}C) and CO_2 to prepare the sodium [2-^{13}C]-propionate. The sodium [2-^{13}C, 2-$^2H_2$]-propionate was prepared in the usual manner by reacting [2-^{13}C]-propionate with $NaO^2H/^2H_2O$ three times. The p-phenylphenacyl ester was made and analyzed by low-resolution MS: 79% 2-^{13}C/2-$^2H_2$; 8% 2-^{12}C/2-$^2H_2$; 8% 2-^{13}C/2-$^2H_1$; 1% 2-^{12}C/2-$^2H_1$; 4% 2-^{13}C/2-$^2H_0$ for M^+ at m/e 269.

[3-^{13}C, 2-$^2H_2$]-Propionate

Sodium [3-^{13}C]-propionate (1 gm, 10.3 mmole; MSD Isotopes, 91.1% ^{13}C) was subjected to the usual procedure for deuterium exchange. The final product was recrystallized from 95% EtOH to give 0.95 gm (93%). MS analysis of the p-phenylphenacyl ester gave: 14.2% $^{13}C_1/^2H_1$; 83.1% $^{13}C_1/^2H_2$ for M^+ at m/e at 268.

[2,3-$^2H_4$]-Succinate

The deuterium exchange was carried out as described for propionic acid, except that the reaction was repeated 5 times to ensure complete exchange. After steam distillation, and solvent evaporation, the weight of the recovered succinic acid was 1.23 gm, 10.1 mmoles, 79% yield. All attempts at derivatizing the succinate to a mono- or diester were unsucessful. The sample was analyzed by ^{1}H-NMR (200 MHz) comparing the integrals of the $-CH_2-$ resonance at 2.6 ppm ($^{2}H_2O$) between an unlabelled sample of succinic acid and the deuterated succinic acid. After correcting for differences in weight between the two samples, it was found that the labelled succinic acid contained 11.4% ^{1}H, and 88.6% ^{2}H.

[2,3-$^{13}C_2$, 2,3-$^{2}H_4$]-Succinate

[2,3-$^{13}C_2$] Succinonitrile

Potassium cyanide (850 mg, 13 mmole) was added to [1,2-$^{13}C_2$]-dibromoethane (1 gm, 5.3 mmole; MSD Isotopes, 91.3% ^{13}C) in 25 mL of anhydrous DMSO under N_2, using an additional 3 x 1 mL of DMSO to rinse the vial containing the dibromoethane. The reaction was stirred for 5 hours, 90-100°C, under N_2. The reaction mixture was poured into 100 mL saturated NH_4Cl solution, extracted with 3 x 100 mL EtOAc, then 2 x 50 mL EtOAc, washed 2 times with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The yield of crude product was 373 mg. The residue was not purified further.

[2,3-$^{13}C_2$]-Succinic Acid

All of the [2,3-^{13}C]-succinonitrile (373 mg crude extract) was dissolved in 30% KOH (15 mL), then 1.75 mL 30% H_2O_2 (20 mmole) was added. The reaction was heated for 1 hour at 40°C under N_2, then refluxed for ca. 3 hours until the exit gas was free of ammonia. The reaction was worked up by adding 50 mL sat. $NaHSO_3$ to destroy excess peroxide, acidifing to pH 2 with conc. HCl, then continuously Et_2O extracting for 60 hours. The Et_2O extract was dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The resulting residue was recrystallized from acetone/hexane to give 270 mgs (2.25 mmoles, 42.7% yield; mp 187-189°C). The mass spectrum showed *m/e* 102, corresponding to [2,3-$^{13}C_2$]-succinic anhydride; *m/e* 119 and *m/e* 121 corresponding to the M-1 and M+1 peaks, respectively (all three peaks are characteristic of small dicarboxylic acids). The ^{1}H-NMR spectrum showed satellite resonances for 2.58 ppm, $J(^{13}C^1H)$ = 125 Hz (acetone, d_6). The sample was then titrated with 2 *N* NaOH and dried *in vacuo* to give the disodium salt.

[2,3-$^{13}C_2$, 2,3-$^2H_4$]-Succinate

The deuterium exchange was carried out as described before, using all of the sodium [2,3-$^{13}C_2$]-succinate and $NaO^2H/^2H_2O$, and repeating the exchange reaction 5 times. After the final exchange, the solvent was evaporated, the pH adjusted to 1 with 18 *N* H_2SO_4, and the product recrystallized from EtOH. The final yield was 204 mg, 1.67 mmoles, 74% yield. The product was analyzed by MS, ^{13}C-NMR and ^{2}H-NMR spectroscopy. The enrichment

was calculated from the MS data of the succinic anhydride fragment (m/e 100); found: 1.8% $^{12}C_2/^2H_4$, 18.9% $^{13}C_2/^2H_2$, 12.3% $^{13}C_2/^2H_3$, 58.8% $^{13}C_2/^2H_4$. The ^{13}C-NMR analysis showed an intense peak at 28.05 ppm (DMSO, d_6). The ^{2}H-NMR analysis showed a resonance at 2.61 ppm (acetone).

[1,4-$^{13}C_2$, 2,3-$^2H_4$]-Succinate

[1,4-$^{13}C_2$]-Succinate was prepared following the same procedure as outlined above for [2,3-$^{13}C_2$]-succinate. The starting materials were cold dibromoethane (1.5 gm, 0.75 mL, 8 mmole) and [^{13}C]-KCN (MSD Isotopes, 90% ^{13}C; 1.43 gm. 21.7 mmole). The residue recovered from the continuous Et_2O extraction was recrystallized from EtOH. The final yield was 680 mgs, 5.67 mmoles, 70.8% yield, mp 188-189°C (sharp). ^{1}H-NMR analysis showed a resonance at 2.58 ppm, d, J = 3 Hz (acetone, d_6).

The deuterium exchange was carried out as before. After the final exchange, the solvent was evaporated, the pH adjusted to 1 with 4 N HCl and the product recovered and dried in vacuo, then recrystallized from EtOH. After conversion to its Na-salt, the final yield of sodium [1,4-$^{13}C_2$, 2,3-$^2H_4$]-succinate was 622 mg, 3.7 mmole, 46.3%. The low-resolution MS analysis gave 13.0% $^{13}C_1/^2H_4$; 6.61% $^{13}C_2/^2H_3$; 80.31% $^{13}C_2/^2H_4$ for the ion at m/e 100. The ^{1}H-NMR analysis showed the absence of the resonance at 2.58 ppm (acetone, d_6). The ^{13}C-NMR analysis showed an intense peak at 173 ppm (acetone, d_6).

The second [1,4-$^{13}C_2$, 2,3-$^{2}H_4$] feeding was done with a sample of the labelled succinate prepared by C.Q. Sun using the same procedure as outlined here.

[2-^{13}C, 2-$^{2}H_2$]-Diethyl Succinate

This was prepared by a procedure developed by D.E. Cane and co-workers. [2-^{13}C]-Acetic acid (MSD Isotopes, 92.9% ^{13}C; 1 gm, 16.4 mmoles) was subjected to the normal deuterium exchange conditions, repeating the reaction 5 times to ensure complete deuterium exchange. After the final exchange, the solvent was evaporated, the product was rinsed 3 times with H_2O, the H_2O removed azeotropically with toluene/EtOH, then the residue dried in vacuo for several hours,. The ^{13}C-NMR spectrum of the recovered product showed a heptet at 22.7 ppm ($^{1}H_2O$/p-dioxane, d_8) for the $^{13}C^{2}H_3$ group.

Trifluoroacetic anhydride (3.4 mL, 5.25 gm, 25 mmole) and $^{2}H_2O$ (0.5 mL, 0.5 gm, 25 mmole) were mixed at -15°C, then rotoary evaporated briefly at 0°C, to remove excess TFAA. The labelled, dry $^{13}C_2{}^{2}H_3CO_2Na$ (1.376 gms, 16 mmole), and the $CF_3CO_2{}^{2}H$ (2.65 gm, 23 mmole) were cooled to -15°C, and TFAA (3.3 mL, 5 gm, 24 mmole) was added dropwise with stirring. The reaction was warmed to room temperature and stirred for 1 hour. Then $SOCl_2$ (distilled, 4-5 drops) was added, followed by dropwise addition of Br_2 (1.6 mL, 2.6 gm, 16 mmole). The reaction was stirred at room temperature for 2 hours, and then refluxed at 66-68°C for 3-4 hours. The reaction was cooled to room temperature, then quenched with $^{2}H_2O$

(0.55 mL, 0.6 gm, 30 mmole). The $CF_3CO_2H(D)$ was removed by a rotary evaporator at 0°C, then the $Br^{13}C^2H_2COH$ was distilled out of the mixture by a Kugelrohr at 170°C.

N-ethyl-N-nitrosourea (1 gm) which was prepared according to the method of Arndt (1943) was dissolved in Et_2O (6 mL) at 0°C; 4 mL 40% aqueous NaOH was added and the mixture was stirred vigorously for 30 mins. The ethereal EtN_2 was co-distilled into an ice cold solution of the $Br^{13}C^2H_2CO_2H$ in Et_2O (10 mL). The distillate was dried over anhydrous Na_2SO_4, then distilled by Kugelrohr to give $Br^{13}C^2H_2CO_2Et$ (1.05 gm, 6.3 mmole, 38%). The ^1H-NMR analysis of the product showed only the two resonances for the ethylester.

n-BuLi (1.55M, 9.3 mL, 1 eq.; Aldrich) was added slowly to $^iPr_2NH$ (1.98 mL, 1.05 eq.; Aldrich, 99%) in THF (5.5 mL, distilled) under N_2 at -78°C. The reaction was stirred for 30 min. Then monoethylmalonate (871 mg, 6.6 mmole; freshly prepared by hydrolysis of diethylmalonate with 1 eq. KOH) in THF (5.5 mL) was added dropwise to the LDA and the reaction was immediately warmed to 0°C and stirred for 15 min. Then dry HMPA (2.2 mL) was added, followed by $Br^{13}C^2H_2CO_2Et$ in THF (4.4 mL) all at once. The reaction was stirred for 2 hours at room temperature, then cooled to 0°C, quenched with 1 N HCl (5 mL) and extracted with Et_2O (3 x 50 mL). The combined Et_2O extracts were washed with 1 N HCl, H_2O, brine and dried over anhydrous Na_2SO_4. The Et_2O was evaporated off slowly under N_2 at room temperature to give a residue that was

heated in a small flask fitted with a reflux condensor attached to an oil bubbler by raising the temperature of the bath surrounding the flask slowly to 170°C, then holding the temperature at 150°C for 1 hour. The resulting residue was distilled by Kugelrohr at 210°C, recovering 437 mg, 2.5 mmole (15% overall yield) of [2-^{13}C, 2-$^{2}H_2$]-diethyl succinic acid. The MS analysis gave 94.8% $^{13}C/^{2}H_2$; 10.5% $^{13}C/^{2}H_1$ for the ion at m/e 174. The ^{1}H-NMR analysis showed a resonance at 2.58 ppm, (acetone, d_6) along with the two resonances for the ethyl ester at 1.2 ppm and 4.2 ppm. The integrals were not checked.

[2-^{3}H, 2-^{2}H]-Butyrate

Sodium [2-^{3}H, 2^{2}H]-butyrate was prepared in the same manner as described above for [2-$^{2}H_2$, 2-^{13}C]-propionate, except the $^{2}H_2O$ exchange was carried out only twice. The third exchange was done using sodium [2-$^{2}H_2$]-butyrate (10 mmole), Na (1 mmole), $^{2}H_2O$ (Aldrich Gold Label, 5 mL), and 45 mCi $^{3}H_2O$. The reaction and work-up were carried out the same as for the $^{2}H_2O$ exchanges. Counts of the resulting sodium $^{2}H,^{3}H$-butyrate gave 3.01 +/- 0.02 x 10^7 dpm ^{3}H/mmole. The p-phenylphenacyl ester was prepared from 0.18 mmole of the [2-^{2}H, 2-^{3}H]-butyrate for mass spectral analysis. The uncorrected isotopic enrichment data were: 0% unlabelled, 2.3% d_1, 86.6% d_2.

[1-^{13}C, 1-^{18}O]-Butyrate

Sodium [1-^{13}C]-butyrate (5 mmole; Prochem, 92.6% ^{13}C) was reacted with 1 mL $H_2{}^{18}O$ (95% ^{18}O) by the same process as described above for [1-^{13}C, 1-^{18}O] propionate. The p-phenylphenacyl ester was prepared for mass spectral analysis using 0.13 mmole [1-^{3}C, 1-^{18}O]-butyrate. The low resolution MS gave $^{13}C:{}^{18}O$ = 92.6:69.3 (55.9 mole% $^{13}C_1 + {}^{18}O_2$; 26.8 mole% $^{13}C_1 + {}^{18}O_1$) for the M^+ ion at m/e 282.

[2-$^{2}H_2$]-Butyrate

Cold Na-butyrate was reacted with $NaO^2H/{}^2H_2O$ using the conditions for deuterium exchange described for sodium propionate. After the final exchange, the pH was adjusted to 1 with 18 N H_2SO_4, and the [2-$^{2}H_2$]-butyric acid steam distilled. After adjusting the pH to 8, the solvent was evaporated in vacuo, and the residue dried in vacuo after rinsing first with H_2O, then toluene/EtOH. The sodium butyrate was recovered in 100% yield.

The sodium [2-$^{2}H_2$]-butyrate (0.2 mmole) was derivatized to its p-phenylphenacyl ester for MS analysis, which showed it to be equivalent to 99.5% butyrate-d_2.

[1-^{13}C, 2-$^{2}H_2$]-Butyrate

Sodium [1-^{13}C]-butyrate (Prochem, 92.6% ^{13}; 1.0 gm, 9.1 mmole) was subjected to the conditions for deuterium exchange described above; yield 80%.

This sodium [1-^{13}C, 2-$^{2}H_2$]-butyrate (0.24 mmole) was derivatized to its p-phenylphenacyl ester. MS analysis gave: 2.1% $^{13}C_1/^{2}H_2$; 97.7% $^{13}C_1/^{2}H_2$ for the M^+ ion at me 282.

A second sample of sodium [1-^{13}C, 2-$^{2}H_2$]-butyrate was prepared following the same procedures. The starting material, sodium [1-^{13}C]-butyrate, was also from Prochem (92.6% ^{13}C), but from a different batch. MS analysis of the p-phenylphenacyl ester gave: 14.9% $^{13}C_1$; 30.4% $^{13}C_1/^{2}H_1$; 45.4% $^{13}C_1/^{2}H_2$ for the M^+ ion at m/e 282.

The third sample of sodium [1-^{13}C, 2-$^{2}H_2$]-butyrate was prepared from 2.0 gms of sodium [1-^{13}C]-butyrate (MSD Isotopes; 90% ^{13}C) by the same procedures; yield 95%. MS analysis of the p-phenylphenacyl ester gave: 1.12% $^{13}C_1$; 2.98% $^{13}C_1/^{2}H_1$; 85.9% $^{13}C_1/^{2}H_2$ for the M^+ ion at m/e 282.

[2,3,3'-$^{14}C_3$] Isobutyrate

Cold disodium malonate (2.96 gm, 20 mmole) and disodium [2-^{14}C]-malonate (0.1 mCi; Amersham, 19.9 mCi/mmole) were admixed, the pH adjusted to 2 with 6 N HCl, the solvent evaporated, and the residue dried in vacuo. The [2-^{14}C]-malonic acid-NaCl mixture was dissolved in EtOH (15 mL, dried over Mg) with heating, HCl gas was bubbled in, and the reaction mixture was refluxed for 3.5 hours. The reaction was cooled to room temperature, the material was rinsed out of the flask with 100 mL H_2O and extracted 3 times with 100 mL Et_2O. The Et_2O layers were combined and dried over anhydrous Na_2SO_4 and evaporated in vacuo at room temperature. The

recovered material (2.2 gm, 13.8 mmole, 68.8% yield), was checked by ^{1}H-NMR ($CDCl_3$) which showed two resonances corresponding to the ethyl ester at 1.25 ppm and 4.2 ppm and a resonance at 3.35 ppm, corresponding to the methylene protons; and its radioactivity was counted (7.3 μCi/mmole).

Clean sodium (700 mg, 30.4 mmole) was placed in a dry, N_2 flushed 25 mL flask fitted with reflux condenser attached to an oil bubbler so that N_2 flow could be maintained throughout the reaction. EtOH (3 mL, dried over Mg) was added dropwise to the Na with stirring at room temperature. The reaction was heated until all the Na had dissolved; additional EtOH (4.5 mL) was then added dropwise and the solution refluxed for 1 hour more. The reaction was cooled until reflux had subsided, then all of the [2-^{14}C]-diethyl malonate prepared above was added dropwise. The reaction mixture was stirred vigorously with mild heating until all of the precipitate had dissolved, and then these conditions were maintained for an additional 1.5 hours. [Methyl-^{14}C]-iodide (Amersham, 58 mCi/mmole; diluted with cold CH_3I to give 8.47 μCi/mmole; used 131 μCi, 15.46 mmole) was added slowly to the reaction mixture, without heating. After addition was complete, the reaction was refluxed for 5 hours then cooled to room temperature. The reaction mixture was washed with H_2O, then evaporated partially to remove excess EtOH, then extracted several times with Et_2O (50 mL). The Et_2O layers were dried over

anhydrous Na_2SO_4 and evaporated to give 1.0 gm (5.32 mmole, 38.9% yield) of 2,2-dimethylmalonic acid diethyl ester.

A cold solution of 5% aqueous NaOH (2.2 eq.) was added to the crude ester and the reaction stirred at room temperature for 20 hours. After cooling, the reaction mixture was acidified to pH 1 with 6 N HCl, and extracted 3 times with Et_2O (50 mL). The Et_2O layers were combined, dried over anhydrous Na_2SO_4, evaporated *in vacuo* to give 690 mg (5.23 mmoles; 98.3% yield) of orange crystals of 2,2-dimethyl malonic acid which were recrystallized from hexane.

The crystals were placed in a dry 25 mL flask, fitted with a reflux condenser attached to an oil bubbler and stir bar. The temperature of the bath surrounding the flask was then raised slowly to 170°C, and then held at 150°C for 1 hour. The flask was cooled to room temperature to give a residue weighing 307 mg (3.53 mmole, 67.5% yield). The ^{1}H-NMR analysis of this material showed resonances at 1.22 ppm, d, J = 7.5 Hz and 2.58 ppm, heptet, J = 7.5 Hz for the protons at C-3,3' and C-2, respectively, of isobutyric acid. An aliquot was counted to give 4.2 μCi/mmole.

[2,3,3'-$^{13}C_3$]-Isobutyrate

The ^{13}C isobutyrate was prepared following the same procedure as outlined above for ^{14}C isobutyrate. The starting material consisted of [2-^{13}C]-malonic acid (Amersham, 91% ^{13}C; 1.25 gms, 11.9 mmole) which was esterified as before, yielding 1.3 gms, 8.1 mmole, 68% yield of its diethyl ester. This was admixed with

[2-^{13}C]-diethly malonate (MSD Isotopes, 90% ^{13}C; 1.0 gm, 6.2 mmole). The ^{13}C-NMR spectrum showed an intense resonance at 41.0 ppm ($CDCl_3$) corresponding to the methylene carbon of the malonic acid and two other resonances at 60.5 ppm and 13.0 ppm corresponding to the carbons of the ethyl ester.

[Methyl-^{13}C]-iodide (MSD Isotopes, 90% ^{13}C; 4 x 1.0 gm, 27.6 mmoles) was used directly as received. After decarboxylation, the final residue was 57 mg, 0.63 mmole, 4.3% overall yield of isobutyric acid as compared with 17.6% overall yield in the previous synthesis. The ^{13}C-NMR analysis (in $CDCl_3$) of this material showed resonances at 40.65 ppm, t, $\underline{J}$ = 25 Hz; 20.44 ppm).

MS analysis was done on a sample of the admixture of ^{14}C and ^{13}C labelled sodium isobutyrate that remained after the feeding experiment. The mass spectrum showed a peak at $\underline{m/e}$ 91, corresponding to [$^{13}C_3$] isobutyrate, which after correcting for dilution, was consistent with only 5.87% ^{13}C enrichment; a second peak was seen at $\underline{m/e}$ 61 corresponding to [2-^{13}C]-acetic acid which indicates that the alkylation reaction had not gone to completion for an unknown reason.

Feeding Experiments

General: Precursor Solutions/Counting Procedures

Unless otherwise noted, commercially available ^{14}C-acetate, -propionate, -succinate or -butyrate was used as the reference isotopic label. The feeding solutions were thus admixtures of the substrate labelled with the stable isotope and the appropriate ^{14}C labelled substrate. The amount of ^{14}C labelled material used was calculated to yield approximately 500 cpm/mg in the isolated lasalocid A, and this amount was then mixed with the stable isotope labelled precursor in an aqueous solution in a volumetric flask. Two aliquots of 0.1 mL were removed, and diluted to 100 mL for radioactivity counting. The remainder of the feeding solution was filter sterilized using a 10 cc "Luer-Lok" syringe fitted with a Millipore disposable filter unit (Millex^{R}-GS, 0.22 μm) and the final volume was adjusted with additional H_2O rinses. The amount of precursor fed varied with each feeding experiment, but generally ranged from 1mM to 10mM. A 5mM feeding is equivalent to 0.25 mmoles precursor/50 mL media/day. A "cold" precursor refers to any precursor that is unlabelled, either with radioactive or with stable isotopes.

All feeding experiments with labelled precursors were carried out using the split pea/lard oil (SP/LO) media. Cultures were grown at 28-30^{o}C, 250 rpm, continual shake (see Chapter 1, Results and Discussion). Seed cultures were started (Day 0) by

innoculating 50 mL media with spores from a slant (initial experiments) or from a frozen spore suspension (latter experiments). 72 Hours later (Day 3), fresh media was innoculated with 2% v/v of the seed culture. Unless otherwise noted, the cultures were grown for an additional 72 hours before addition of the precursor. The precursor was then added every 24 hours for four days (Days 6,7,8,9). The cultures were harvested 24 hours after the final feeding (Day 10). (Further details on the fermentation, isolation and purification procedures are given in Chapter One).

Radioactivity was measured by liquid scintillation counting in a Packard model #3255 instrument, using either Aquasol^{R} (NEN) or a toluene/EtOH cocktail (PPO 5 gm; POPOP 0.3 gm; EtOH 300mL; toluene 700 mL). Counting efficiences were determined by using ^{3}H Hexadecane and ^{14}C Hexadecane internal standards (Amersham).

Calculations for the incorporation of the labelled precursors into lasalocid A were done as follows. Total incorporation is a measure of the total radioactivity present in the isolated product; it is expressed as a percentage of the total radioactivity fed in the precursor. Specific incorporation is a measure of the molar radioactivity in the product as it relates to the precursor; that is:

$$\text{Specific Incorporation (Sp. Inc.)} = \frac{\text{dpm/mmole product}}{\text{dpm/mmole precursor.}}$$

It usually is expressed as a percentage also. The $^{3}H/^{14}C$ ratio is obtained by dividing the dpm ^{3}H/mmole by the dpm ^{14}C/mmole. This ratio is used in double-label experiments when it is important to distinguish how much of the ^{3}H label is lost due to exchange processes. The incorporation of ^{13}C labels is measured in ^{13}C-NMR spectroscopy and determined by the following ratio:

$$^{13}C \text{ Enrichment Factor} = \frac{\text{peak height of } ^{13}C \text{ enriched compound}}{\text{peak height of natural } ^{13}C \text{ abundance compound}}$$

In this case, the two spectra are run under identical conditions, or the peak heights are normalized to correct for any factors which might alter the intensity of the resonance. The enrichment of other stable isotopes, such as ^{2}H or ^{18}O, was determined by various spectroscopic analysis, both MS and NMR, as described in the Results and Discussion section of this chapter.

[2-$^{2}H_3$, 1-^{14}C]-Acetate Feeding

Using $C^{2}H_3CO_2Na$ (Aldrich Gold Label) and [1-^{14}C]-acetate of 0.96 +/- 0.1 x 10^{8} dpm ^{14}C/mmole, the admixture was fed (5mM acetate/feeding) to each of 3 flasks containing 100 mL media. One flask was harvested after 2 feedings (Day 8), one flask after 3 feedings (Day 9), and one flask after 4 feedings (Day 10). From the Day 8 flask (fed a total of 1.0 mmole $C^{2}H_3{}^{14}CO_2Na$), lasalocid A was isolated (32 mgs/100 mL media, 5.15 x 10^{7} dpm ^{14}C/mmole) to give 10.7% specific incorporation of ^{14}C. The Day 9 flask (fed

1.5 mmole $C^2H_3 14CO_2Na$) yielded lasalocid A (38 mg/100 mL media, 7.36×10^7 dpm ^{14}C, 15.3% specific incorporation). The Day 10 flask received a total of 2.0 mmole of $C^2H_3{}^{14}CO_2Na$ and yielded lasalocid A (35 mg/100 mL media, 7.69×10^7 dpm ^{14}C, 16.07% specific incorporation).

After counting, the sample from Day 8 was stored, and the samples from Day 9 and 10 were combined ($7.53 \pm 0.3 \times 10^7$ dpm ^{14}C/mmole). The crystals were then combined with the ML's, partitioned between Et_2O and 0.1 N H_2SO_4, and recrystallized. The resulting crystals were then sent to Alberta for spectral analysis.

[2-$^2H_3$, ^{13}C, 1-^{14}C]-Acetate Feeding

[2-$^2H_3$, 2-^{13}C]-Acetate (Prochem) and [1-^{14}C]-acetate (Amersham-Searle, 58 mCi/mmole) were mixed to give a solution of $7.32 \pm 0.8 \times 10^6$ dpm ^{14}C/mmole acetate. The cultures were fed (5mM), harvested and worked-up as usual, yielding 84 mgs lasalocid A from 200 mL media. The product was recrystallized, from which the bulk of the material was submitted for spectral analysis, and the remainder was recrystallized before counting. The lasalocid A was successively counted twice, giving $6.72 \pm 0.23 \times 10^6$ dpm ^{14}C/mmole lasalocid A or 18.4% specific incorporation of ^{14}C. Spectral analysis of this sample was carried out at Alberta and Halifax.

[2-$^3H_3$, 1-^{14}C]-Acetate Feeding

A solution of cold Na-acetate, sodium [2-$^{3}H_{3}$]-acetate (NEN, 2.73 x 10^{4} mCi/mmole) and sodium [1-^{14}C]-acetate (Amersham-Searle, 58 mCi/mmole) was prepared, giving 2.24 +/- 0.2 x 10^{7} dpm ^{3}H/mmole; 7.96 +/- 0.7 x 10^{6} dpm ^{14}C/mmole; or ^{3}H/^{4}C ratio = 2.8. After feeding (5mM) and working-up as usual, 89 mgs of lasalocid A were isolated from 200 mL media. The product was recrystallized and counted once, giving 1.07 x 10^{7} dpm ^{3}H/mmole lasalocid A; 3.32 x 10^{6} dpm ^{14}C/mmole lasalocid A; or ^{3}H/^{14}C = 3.2. This corresponds to 8.3% specific incorporation of ^{14}C and 9.5% specific incorporation of ^{3}H.

[2-$^{3}H_{3}$, 1-^{14}C]-Acetate Feeding (Repeat)

Cold Na-acetate, [2-$^{3}H_{3}$]-acetate (NEN, 2.73 x 10^{4} mCi/mmole) and [1-^{14}C]-acetate were admixed to give a solution of 7.75 +/- 0.05 x 10^{6} dpm ^{14}C/mmole; 1.35 +/- 0.02 x 10^{8} dpm ^{3}H/mmole; ^{3}H/^{14}C ratio = 17.35 +/- 0.05.

The aliquot for counting was then diluted with cold acetate (12 fold) and derivatized to give the p-phenylphenacyl ester, which was recrystallized to a constant specific activity of 7.3 +/- 0.4 x 10^{6} dpm ^{14}C/mmole; 1.17 +/- 0.4 x 10^{8} dpm ^{3}H/mmole; ^{3}H/^{14}C ratio = 16.15 +/- 1.55.

The feeding experiment was carried out as usual (5mM) yielding 80 mgs lasalocid A/300 mL media, which was recrystallized and counted to constant specific activity: 4.51 +/- 0.2 x 10^{6} dpm ^{14}C/mmole lasalocid A; 6.57 +/- 0.13 x 10^{7} dpm ^{3}H/mmole lasalocid A; ^{3}H/^{14}C ratio = 14.6 +/- 0.9. This corresponded to 61.8%

specific incorporation of ^{14}C; 56% specific incorporation of ^{3}H; 90.4% retention of ^{3}H label.

[2-$^{2}H_2$, 1-^{14}C]-Propionate Feeding

[2-$^{2}H_2$]-Propionate and [1-^{14}C]-propionate (NEN, 48 mCi.mmole), were admixed to give 5.7 x 10^7 dpm ^{14}C/mmole. The feeding was carried out the same as for [2-$^{2}H_3$, 1-^{14}C]- acetate. The Day 8 harvest yielded 30 mgs lasalocid A at 7.14 x 10^7 dpm ^{14}C/mmole (31.4% specific incorporation); Day 9 yielded 53 mgs lasalocid A at 6.8 x 10^7 dpm ^{14}C/mmole (29.8% specific incorporation); Day 10 yielded 66 mgslasalocid A at 7.65 x 10^7 dpm ^{14}C/mmole (33.5% specific incorporation).

As before the sample from Day 8 was stored after counting, while the samples from Days 9 and 10 were combined. The combined sample was recrystallized and counted twice to give: 7.21 +/- .09 x 10^7 dpm ^{14}C/mmole lasalocid A. A ^{13}C-NMR spectrum was run, but was inconlusive due to low S/N. The ML from above was partitioned between Et_2O and 0.2 N H_2SO_4, the Et_2O soution evaporated, and the residue was submitted to Alberta for ^{2}H-NMR analysis. Then all samples of ML and crystals were combined, repartitioned between Et_2O and 0.1 N HCl to give lasalocid A free acid, which was then methylated to give the 1,3-bis-OMe-lasalocid A (**2**). The sample was then resubmitted to Alberta for ^{2}H-NMR spectral analysis.

[2-^{2}H, 2-^{3}H, 1-^{14}C]-Propionate Feedings

[2-^{2}H, 2-^{3}H]-Propionate and [1-^{14}C]-propionate were admixed to give a solution of [2-^{2}H, 2-^{3}H, 1-^{14}C]-propionate with 6.92 x 10^{6} dpm ^{3}H/mmole; 4.96 +/- x 10^{6} dpm ^{14}C/mmole; ^{3}H/^{14}C ratio = 14.5 +/- 0.3. The counting aliquots of the admixture were diluted with cold Na-propionate to a final dilution of 42 fold; this was then derivatized to give the p-phenylphenacyl ester of propionic acid, which was recrystallized and counted three times, giving: 1.65 +/- 0.1 x 10^{5} dpm ^{3}H/mmole; 1.14 +/- 0.02 x 10^{5} dpm ^{14}C/mmole; ^{3}H/^{14}C ratio = 14.5 +/- 0.3.

This feeding experiment was carried out using 3 different concentrations of lard oil in the media: 5%, 10% and 20% w/v. The 5% lard oil yielded 86 mgs lasalocid A/200 mL media, with 5.70 x 10^{6} dpm ^{3}H/mmole; 4.17 x 10^{6} dpm ^{14}C/mmole; ^{3}H/^{14}C ratio = 1.37. The 10% lard oil yielded 48 mgs lasalocid A/200 mL media, with 4.29 x 10^{6} dpm ^{3}H/mmole; 3.43 x 10^{6} dpm ^{14}C/mmole; ^{3}H/^{14}C ratio = 1.25. The 20% lard oil yielded 34 mgs lasalocid A/200 mL media with 3.35 x 10^{6} dpm ^{3}H/mmole; 2.78 x 10^{6} dpm ^{14}C/mmole; ^{3}H/^{14}C ratio = 1.21.

The 5% lard oil lasalocid A sample was recrystallized and counted: 8.11 x 10^{6} dpm ^{3}H/mmole; 5.12 x 10^{6} dpm ^{14}C/mmole; ^{3}H/^{14}C ratio = 1.58. This represented (averaging the two counts) to 10.4% retention of ^{3}H or 3% specific incorporation of ^{3}H/C-3 unit. The crystals were recombined with ML, partitioned between Et_2O and 0.1 N H_2SO_4, and the Et_2O solubles evaporated to yield 68 mgs of sample. This sample was sent to Alberta for spectral

analysis prior to converting it to its 1,3-bis-OMe-lasalocid A derivative and resubmitting this for spectral analysis.

The lasalocid A isolated from the 10% and 20% lard oil fractions was combined, diluted 3 fold with cold lasalocid A, and recrystallized and counted three times: 1.72 +/- 0.03 x 10^6 dpm ^3H/mmole lasalocid A; 1.14 +/- 0.02 x 10^6dpm ^{14}C/mmole lasalocid A; $^3H/^{14}C$ ratio = 1.51 +/- 0.01. The diluted lasalocid A was then subjected to the following degradations and derivatizations.

A base catalyzed retro-aldol cleavage was carried out. The base soluble fraction (acid fragment, **7**) was counted, re-precipitated and recounted to give: 9.17 +/- 0.02 x 10^5 dpm ^3H/mmole; 7.94 +/- 0.36 x 10^5 dpm ^{14}C/mmole; $^3H/^{14}C$ ratio = 1.16 +/- 0.05. The acid fragment was subsequently dehydrated and recrystallized to constant specific activity: 1.05 +/- 0.02 x 10^6 dpm ^3H/mmole; 8.12 +/- 0.36 x 10^5 dpm ^{14}C/mmole; $^3H/^{14}C$ ratio = 1.30 +/- 0.09. (The ^3H value = 61.0% of the radioactivity of the starting material, and the ^{14}C value = 71.2%). The EtOAc soluble fraction (ketone fragment, 4) was distilled to give a colorless oil, which was counted, redistilled and re-counted to give: 4.83 +/- 1.60 x 10^5 dpm ^3H/mmole; 2.61 +/- 0.85 x 10^5 dpm ^{14}C/mmole; $^3H/^{14}C$ ratio = 1.84 +/- 0.03. The ketone fragment was then subjected to base exchange with NaOMe/MeO^{2}H to verify the presence of ^3H at the α-carbonyl positions. The resulting glass was distilled and counted three times giving: 3.92 +/- 0.16 x 10^5 dpm ^3H/mmole; 3.04 +/- 0.03 x 10^5 dpm ^{14}C/mmole; $^3H/^{14}C$ ratio = 1.29

+/- 0.03. (The ^{3}H value = 22.8% of the radioactivity of the starting material; the ^{14}C value = 26.7%). The base exchanged ketone fragment was submitted for both ^{1}H-NMR (270 MHz) and MS analysis, both of which confirmed d_3 as the major species.

The lasalocid A-EtOH crystalline solvate (0.1 mmole) was pyrolyzed, giving 50 mg of distillate which upon purification by PLC (as described previously) and then distillation of the eluted residue in a Kugelrohr at 207°C, 0.1 mm Hg, yielded 33 mgs of colorless liquid. The distillate was counted and redistilled 3x to give: 6.17 +/- .18 x 10^5 dpm ^{3}H/mmole; 3.54 +/- 0.18 x 10^5 dpm ^{14}C/mmole; $^{3}H/^{14}C$ ratio = 1.75 +/- 0.04. (The ^{3}H value = 36% of the radioactivity of the starting material, and the ^{14}C value = 30%).

[1-^{13}C, 1-^{18}O, 1-^{14}C]-Propionate Feeding

[1-^{13}C, 1-^{18}O]-Propionate and [1-^{14}C]-propionate (NEN, 48 mCi/mmole) were admixed to give 2.69 +/- 0.19 x 10^6 dpm ^{14}C/mmole. The feeding experiment (5mM) was done in the usual manner, yielding 42 mg lasalocid A from 100 mL broth; about 3/4 of this was sent to Alberta for spectral analysis, while the remainder was recrystallized and counted twice: 2.63 +/- .03 x 10^6 dpm ^{14}C/mmole lasalocid A, or 24.4% specific incorporation of ^{14}C. Subsequently, all crystals and ML's were combined and methylated to give the 1,3-bis-OMe-lasalocid A (**2**), which was sent to Alberta for NMR analysis. The 1,3-bis-OMe-lasalocid A was then derivatized to give the 1,3-bis-OMe-11,22-bis-TFA-lasalocid A (**3**),

which was analyzed by high-resolution MS and sent to Alberta for NMR analysis.

[2-^{13}C, 2-$^{2}H_2$, 1-^{14}C]-Propionate Feeding

The solution of [2-^{13}C, 2-$^{2}H_2$, 1-^{14}C]-propionate was prepared from [2-^{13}C, 2-$^{2}H_2$]-propionate and [1-^{14}C]- propionate (NEN 48 mCi/mmole) to give 3.63 +/- 0.5 x 10^6 dpm ^{14}C/mmole. The feeding experiment was carried out as usual (5mM), yielding 74 mgs of lasalocid A from 200 mL broth. After the product was recrystallized, the majority was reserved for spectral analysis at Alberta and Halifax, while the remainder was counted and recrystallized three times: 3.59 +/- 0.3 x 10^6 dpm ^{14}C/mmole lasalocid A or 24.8% specific incorporation of ^{14}C.

[2-^{13}C, 2-2H_2, 2-^{14}C]-Propionate Feeding (Repeat)

[2-^{13}C, 2-$^{2}H_2$]-Propionate and [2-^{14}C]-propionate were admixed to give a solution of 2.39 x 10^6 dpm ^{14}C/mmole. The propionate was fed at 5mM levels to 8 x 50 mL media/flask over the normal four day period. The flasks were worked-up with EtOAc extraction, Sep-Pak^{TM} separation, PLC and HPLC. The final yield was 41 mg lasalocid A/400 mL media. The final product was not counted, but was submitted to the following spectral analyses: MS, ^{13}C-NMR, ^{2}H-NMR, and ^{13}C{^{2}H, ^{1}H}-NMR.

[1-^{13}C, 2-$^{2}H_2$, 1-^{14}C]-Propionate Feeding

[1-^{13}C, 2-$^{2}H_2$]-Propionate and [1-^{14}C]-propionate were admixed to give a solution of 1.65 x 10^6 dpm ^{14}C/mmole. The propionate

was fed at 1mM levels to 4 x 50 mL media/flask and at 2.5 mM levels to 4 x 50 mL media/flask over the normal four day period. Flasks were assayed during the course of the fermentation by TLC and, in some cases by HPLC. Flasks were harvested and worked-up with EtOAc extraction, Sep-Pak™ separation, PLC and finally HPLC. The final yields were 5 mg lasalocid A/200 mL media for the 1mM feeding and 11 mg lasalocid A/150 mL media for the 2.5 mM feeding. The final products were not counted. The sample isolated from the 2.5 mM level feeding was converted to the free acid, and submitted to Yale for high-field ^{13}C-NMR.

[3-^{13}C, 2-$^{2}H_2$]-Propionate Feeding

The [3-^{13}C, 2-$^{2}H_2$]-propionate was not admixed with a ^{14}C substrate. The propionate was fed at 5mM levels to 10 x 50 mL media/flask over the normal four day period. The flasks were harvested in the usual manner with EtOAc, Sep-Pak™, PLC and HPLC. The final yield was 64 mg lasalocid A/500 mL media. The product was analyzed by MS and ^{13}C-NMR spectroscopy.

[2,3-$^{2}H_4$, 1,4-$^{14}C_2$]-Succinate Feeding

[2,3-$^{2}H_4$]-Succinate and [1,4-$^{14}C_2$]-succinate (Amersham 118 mCi/mmole) were admixed to give a solution of 4.41 x 10^5 dpm ^{14}C/mmole. The succinate was fed at 5mM levels to 3 x 50 mL media/250 mL flask and 2 x 100 mL media/500 mL flask.

The flasks were assayed by TLC/bioautograms during the course of the fermentation. After the feeding was completed the flasks

were worked-up by EtOAc extraction and PLC. The final product was recrystallized and counted to a constant specific activity of 3.69 x 10^4 dpm ^{14}C/mmole lasalocid A, which is equivalent to a specific incorporation of 16.72%. (Note: in the case of [1,4-$^{14}C_2$] succinate, the specific incorporation was calculated using the assumption that 1/2 of the ^{14}C label is lost in the decarboxylation of succinate to propionate. This assumption was verified in the experiments using the appropriate ^{13}C labels). The recovered weights after the counts were: 33 mg lasalocid A/150 mL media (250 mL flasks); 28 mg lasalocid A/100 mL media (500 mL flask); 0 mg lasalocid A/100 mL media (500 mL flask). The final product was analyzed by MS, ^{13}C-NMR (22.5 MHz, 50 MHz), ^{1}H-NMR (600 MHz) and ^{2}H-NMR (30.6 MHz, 92.2 MHz).

[1,4-$^{14}C_2$]-Succinate / "Malonate" Feeding

Cold disodium succinate was admixed with [1,4-$^{14}C_2$]-succinate (Amersham 118 mCi/mmole) to give a solution of 5.33 x 10^5 dpm ^{14}C/mmole. The other solutions prepared were: disodium malonate (cold, aqueous), malonic acid (cold, aqueous), diethyl malonate (commercial grade, distilled). All solutions were filter sterilized as usual.

The feeding experiment was carried out by feeding 5mM succinate to each of twelve flasks (50 mL media/flask), and co-feeding one of the "malonates" as follows: 5mM Na-malonate (2 flasks); 50mM Na-malonate (1 flask); 500mM Na-malonate (1 flask); 5mM malonic acid (2 flask); 50mM malonic acid (1 flask); 500mM

malonic acid (1 flask); 5mM diethyl malonate (2 flask); 50mM diethyl malonate (1 flask); and 500mM diethyl malonate (1 flask). The flasks were assayed by TLC/bioautogram during the course of the fermentation, then harvested and worked-up separately through the EtOAc extraction. The work-up was continued only for the 5mM level "malonates", since the general appearance and weights of the residues from the EtOAc extractions appeared unaffected by the addition of "malonate".

The lasalocid A was isolated, weighed, then recrystallized and counted to constant specific activity. 5mM Succinate/5mM Na-malonate yielded 20 mgs lasalocid A/100 mL media, with a specific incorporation of 27%. 5mM Succinate/5mM malonic acid yielded 34 mgs lasalocid A/100 mL media; specific incorporation of ^{14}C = 16%. 5mM Succinate/5mM diethyl malonate yielded 60 mgs lasalocid A with a specific incorporation of ^{14}C = 11%.

[1,4-$^{14}C_2$]-Succinate / Na-Malonate Feedings

[1,4-$^{14}C_2$]-Succinate was admixed with cold disodium succinate to give a solution of 5.38 x 10^6 dpm ^{14}C/mmole. An aqueous solution of disodium malonate was prepared to a known concentration and filter sterilized.

The feeding experiment was carried out by feeding 5mM succinate to 8 x 50 mL media/flask, and co-feeding the disodium malonate as follows: sterile H_2O (2 flasks), 2.5mM (2 flasks); 5mM (2 flasks); 10 mM (2 flasks). Each flask was worked-up separately through EtOAc extraction, Sep-Pak™ separation, and PLC. The

isolated lasalocid A from the duplicate flasks was combined after weighing and prior to recrystallizing and counting to constant specific activity. The 5mM succinate/sterile H_2O feeding yielded a combined weight of 6 mg lasalocid A/100 mL media with a specific incorporation of 5.9%. The 5mM succinate/2.5mM malonate sample yielded 9 mg Lasalocid A with specific incorporation of ^{14}C = 13.6%. 5mM Succinate/5mM malonate yielded 4 mg lasalocid A, specific incorporation of ^{14}C = 9.4%. The 5mM succinate/10mM malonate feeding yielded 14 mg lasalocid A with 20% specific incorporation of ^{14}C.

[2-^{14}C]-Malonate / Na-Succinate Feedings

[2-^{14}C]-Malonate (Amersham 55.8 mCi/mmole) was admixed with cold disodium malonate to give a solution of 4.91×10^6 dpm ^{14}C/mmole. The disodium succinate was dissolved in H_2O to give a solution of known concentration and then filter sterilized.

The feeding experiment was carried out by feeding 5mM [2-^{14}C]-malonate to each of four flasks (50 mL media/flask), and then co-feeding the following: sterile H_2O (2 flasks); 2.5mM cold succinate (2 flasks). The flasks were assayed by TLC/bioautograms during the course of the fermentation, then harvested. Each flask was worked-up separately by EtOAc extraction and PLC, then the lasalocid A was recrystallized and counted to constant specific activity. The isolated lasalocid A was weighed prior to recrystallization and averaged 45 mg/50 mL media for all four flasks. In the case of co-feeding sterile H_2O, the specific

incorporation of ^{14}C was 13.3% +/- 0.6; in the case of co-feeding succinate, the specific incorporation of ^{14}C was 13.5% +/- 0.85.

[1,4-$^{14}C_2$]-Succinate / [2,3-$^{14}C_2$]-Succinate

Malonate and Malate or Fumarate

[1,4-$^{14}C_2$]-Succinate (Amersham 118 mCi/mmole) was admixed with cold disodium succinate to give a solution of 5.83 x 10^6 dpm ^{14}C/mmole. [2,3-$^{14}C_2$]-Succinate (NEN 68.1 mCi/mmole) was admixed with cold disodium succinate to give a solution of 2.4 x 10^6 dpm ^{14}C/mmole. Na-Malonate, Na-malate and Na-fumarate were prepared as aqueous solutions of known concentration, then filter sterilized.

The experiment was carried out by feeding [2,3-$^{14}C_2$]-succinate at 2.5mM levels to 8 x 50 mL media/flask. These same flasks were then co-fed the following: sterile H_2O (2 flasks); 5mM malonate (2 flasks); 5mM malonate and 5mM malate; 5mM malonate and 5mM fumarate. The same series of feedings was carried out using the [1,4-$^{14}C_2$]-succinate solution at 2.5mM levels. The flasks were assayed by TLC/bioautogram during the course of the fermentation.

At the end of the fermentation, each flask was worked-up separately by EtOAc extraction, Sep-Pak^{Tm} separation, PLC, then the lasalocid A was recrystallized and counted to constant specific activity. The ^{14}C incorporations into lasalocid A were:
[2,3-$^{14}C_2$]-succinate/H_2O, 5.24 x 10^4 dpm/mmole;
[2,3-$^{14}C_2$]-succinate/malonate, 5.05 x 10^5 dpm/mmole;

[2,3-$^{14}C_2$]-succinate/malonate/fumarate, 2.27 x 10^5 dpm/mmole;

[2,3-$^{14}C_2$]-succinate/malonate/malate, 5.38 x 10^4 dpm/mmole;

[1,4-$^{14}C_2$]-succinate/H_2O, 7.46 x 10^4 dpm/mmole;

[1,4-$^{14}C_2$]-succinate/malonate, 1.99 x 10^5 dpm/mmole;

[1,4-$^{14}C_2$]-succinate/malonate/fumarate, 5.77 x 10^4 dpm/mmole;

[1,4-$^{14}C_2$]-succinate/malonate/malate, 5.38 x 10^4 dpm/mmole.

[1,4-$^{14}C_2$]-Succinate / [2,3-$^{14}C_2$]-Succinate Malonate and Malate or Fumarate (Repeat)

The feeding solutions were prepared the same as in the above experiment. The [1,4-$^{14}C_2$]-succinate solution was 1.43 x 10^7 dpm/mmole. The [2,3-$^{14}C_2$]-succinate solution was 2.5 x 10^6 dpm/mmole.

This experiment was carried out similar to the above experiment. The [2,3-$^{14}C_2$]-succinate solution was fed at 2.5mM levels to 8 x 50 mL media/flask. These flasks were co-fed as follows: sterile H_2O (2x flasks; 5mM malonate (2 flasks); 5mM malonate and 5mM fumarate (2 flasks); 5mM malonate and 5mM malate (2 flasks). The [1,4-$^{14}C_2$]-succinate solution was fed at 2.5mM levels to 6 x 50 mL media/flask, and co-fed as follows: sterile H_2O (2 flasks); 5mM malonate (2 flasks); 5mM malonate and 5mM fumarate (1 flasks); 5mM malonate and 5mM malate (1 flask). The [1,4-$^{14}C_2$]-succinate solution was also fed twice/day (every 12 hours) to 4 x 50 mL media/flask, and co-fed as follows: sterile H_2O (2 flasks); 5mM malonate (2 flasks). The flasks were fed over

the normal four day period during which the antibiotic production was followed by TLC.

At the end of the fermentation the flasks were worked-up individually by EtOAc extraction, Sep-Pak™ separation, PLC, then the lasalocid A was recrystallized and counted to constant specific activity. The ^{14}C incorporations into Lasalocid A were as follows: [2,3-$^{14}C_2$]-succinate/sterile H_2O, 3.67 x 10^5 dpm/mmole; [2,3-$^{14}C_2$]-succinate/malonate, 8.34 x 10^4 dpm/mmole; [2,3-$^{14}C_2$]-succinate/malonate/fumarate, 4.05 x 10^5 dpm/mmole; [2,3-$^{14}C_2$]-succinate/malonate/malate, 1.49 x 10^5 dpm/mmole; [1,4-$^{14}C_2$]-succinate/sterile H_2O, 3.05 x 10^5 dpm/mmole; [1,4-$^{14}C_2$]-succinate/malonate, 7.96 x 10^4 dpm/mmole; [1,4-$^{14}C_2$]-succinate/malonate/fumarate, 2.33 x 10^5 dpm/mmole. For the four flasks that were fed every 12 hours the ^{14}C incorporations are: [1,4-$^{14}C_2$]-succinate/sterile H_2O, 7.96 x 10^5 dpm/mmole; [1,4-$^{14}C_2$]-succinate/malonate, 5.12 x 10^5 dpm/mmole.

[2,3-$^{13}C_2$, 2,3-$^{2}H_4$, 2,3-$^{14}C_2$]-Succinate Feeding

[2,3-$^{13}C_2$, 2,3-$^{2}H_4$]-Succinate was admixed with [2,3-$^{14}C_2$]-succinate (NEN 68.1 mCi/mmole) to give a solution of 8.93 x 10^6 dpm ^{14}C/mmole.

The experiment was carried out by feeding the succinate solution at 2.75mM levels to 3 x 50 mL media/flask. Control flasks were fed: sterile H_2O; [2,3-$^{14}C_2$]-succinate (2.75mM); [1,4-$^{14}C_2$]-succinate (2.75mM). All flasks that were fed succinate solutions were also co-fed cold disodium malonate (5mM). Only the

control flasks were assayed by TLC/bioautogram during the course of the fermentation.

At the end of the fermentation, the flasks were worked-up individually by EtOAc extraction and PLC. The H_2O controls were then stored, and the lasalocid A from the other flasks was recrystallized and counted to constant specific activity. The average weight of lasalocid A recovered prior to recrystallization was: H_2O (7 mg/50 mL media); succinate solutions (10 mg/50 mL media). The specific incorporation of ^{14}C into lasalocid A was: [2,3-$^{14}C_2$]-succinate (52%); [1,4-$^{14}C_2$]-succinate (14.3%). The sample of lasalocid A labelled with [2,3-$^{13}C_2$, 2,3-$^{2}H_4$]-succinate was then analyzed by MS (high-res), ^{13}C-NMR (high-field, broad band {^{1}H}; Triple Resonance; INADEQUATE) spectroscopy.

[1,4-$^{13}C_2$, 2,3-$^{2}H_4$, 1,4-$^{14}C_2$]-Succinate Feeding

[1,4-$^{13}C_2$, 2,3-$^{2}H_4$]-Succinate and [1,4-$^{14}C_2$]- succinate (Amersham 118 mCi/mmole) were admixed to give a solution of 7.01 x 10^6 dpm/mmole.

The experiment was carried out by feeding the succinate solution at 2.5mM levels to 6 x 50 mL media/flask over the normal four day period. Each of the six flasks was also co-fed 5mM cold malonate. Control flasks were fed sterile H_2O, and were assayed for lasalocid A production during the course of the fermentation.

On Day 10, the flasks were harvested and worked-up separately by EtOAc extraction and PLC. In all cases the recovered weight of lasalocid A prior to recrystallization was high due to oil

contamination. The lasalocid A isolated from the six flasks fed the succinate solution was combined after the first set of radioactivity counts, and recrystallized and counted to constant specific activity of ^{14}C = 8.01%. This sample was then analyzed by MS (low-res); ^{13}C-NMR (50 MHz, 118 MHz).

[1,4-$^{13}C_2$, 2,3-$^{2}H_4$, 1,4-$^{14}C_2$]-Succinate Feeding (Repeat)

[1,4-$^{13}C_2$, 2,3-$^{2}H_4$]-Succinate was admixed with [1,4-$^{14}C_2$]-succinate (Amersham, 118 mCi/mmole) to give a solution of 7.11×10^6 dpm ^{14}C/mmole.

The experiment was carried out by feeding the succinate solution every 12 hours at 2mM levels to 4 x 50 mL media/flask over the normal four day period. Each of the four flasks was also co-fed 5mM cold malonate. The cultures were assayed by TLC only and after the fermentation and the flasks were worked-up as usual with EtOAc extraction and PLC. The combined weight of the recovered lasalocid A was 55 mg/200 mL media. The sample was later purified by HPLC, where only 3 mg of pure lasalocid A was recovered. The sample was analyzed by ^{13}C-NMR spectroscopy both before and after HPLC purification.

[2-^{13}C, 2-$^{2}H_2$]-Diethyl Succinate Feeding

[2-^{13}C, 2-$^{2}H_2$]-Diethyl succinate was fed at 2.75mM levels to 4 x 50 mL media/flask. The flasks were also co-fed 5mM cold malonate. The flasks were fed over the normal four day period, but were not assayed during the course of the fermentation.

After feeding, the flasks were worked-up by EtOAc extraction, Sep-Pak™ separation, PLC and HPLC purification. A total of 14.3 mg lasalocid A was recovered. The sample was analyzed by ^{13}C-NMR spectroscopy.

[2,3-$^{2}H_4$]-Succinate Feeding

[2,3-$^{2}H_4$]-Succinate was fed at 5mM levels to 10 x 50 mL media/flask over the normal four day period. The flasks were co-fed 5mM cold malonate, but the flasks were not assayed for antibiotic production during fermentation.

After the fermentation, the flasks were worked-up as usual by EtOAc extraction, Sep-Pak™ separation, PLC and HPLC. A total of 66 mg lasalocid A was recovered after purification. The sample was analyzed by MS (low-res), and ^{2}H-NMR (30 MHz) before being submitted to Carnegie-Mellon for high field ^{2}H-NMR and ^{1}H-NMR spectroscopic analysis.

[2-^{3}H, 2-$^{2}H_2$, 1-^{14}C]-Butyrate Feeding

[2-$^{2}H_2$, 2-^{3}H]-Butyrate and [1-^{14}C]-butyrate (Amersham-Searle 58 mCi/mmole) were admixed to give a solution of 3.45 +/- 6.0 x 10^7 dpm ^{3}H/mmole; 1.05 +/- 0.3 x 10^6 dpm ^{14}C/mmole; $^{3}H/^{14}C$ ratio = 32.8. The aliquot for counting was then diluted with cold butyrate (2.7 fold), and derivatized to give the p-phenylphenacyl ester which was recrystallized to a constant specific activity of 4.54 +/- 0.8 x 10^7 dpm ^{3}H/mmole; 1.27 +/- 0.2 x 10^6 dpm ^{14}C/mmole; $^{3}H/^{14}C$ ratio = 35.7.

The feeding experiment was carried out as usual (5mM), yielding 73 mgs laslaocid A/200 mL media which was recrystallized and counted once: 7.84 x 10^6 dpm ^3H/mmole; 2.78 x 10^6 dpm ^{14}C/mmole; $^3H/^{14}C$ ratio = 2.82. The bulk of the material was saved, while the remainder was diluted with cold lasalocid A (2.9) fold, recrystallized to a constant specific activity of 5.81 +/- 0.3 x 10^6 dpm ^3H/mmole lasalocid A; 2.05 +/- 0.1 x 10^6 dpm ^{14}C/mmole; $^3H/^{14}C$ = 2.82 +/- 0.15. This corresponded to: 53.8% specific incorporation of ^{14}C; 4.3% specific incorporation of ^3H; 7.9% retention of ^3H label.

After spectral analysis, the sample was diluted (4.73 fold) with cold lasalocid A, and recrystallized and counted to constant specific activity prior to degradation: 2.7 +/- 0.05 x 10^6 dpm ^{14}C/mmole; 1.04 +/- 0.05 x 10^7 dpm ^3H/mmole; $^3H/^{14}C$ ratio = 3.85 +/- 0.12. The sample was then pyrolyzed (200°C, 11 μ, 45 min), anad the ketone fragment was collected, distilled and counted two times: 3.68 +/- 0.02 x 10^5 dpm ^{14}C/mmole; 9.57 +/- 0.31 x 10^5 dpm ^{3}/mmole; $^3H/^{14}C$ ratio = 2.60 +/- 0.07. The ketone was then subjected to base exchange using 0.5 N NaOMe/MeOD. The deuterated ketone was isolated, distilled and counted three times: 5.11 +/-0.73 x 10^5 dpm ^{14}C/mmole; 1.33 +/- 0.12 x 10^6 dpm ^{3}/mmole; $^3H/^{14}C$ ratio = 2.61 +/- 0.14. Another sample of the diluted lasalocid A was then subjected to base-catalyzed retro-aldol cleavage. The ketone fragment was then isolated, distilled and counted: 3.65 x 10^5 dpm ^{14}C/mmole; 8.99 x 10^5 dpm ^3H/mmole; $^3H/^{14}C$

ratio = 2.46. This sample was then subjected to base exchange (0.5 N NaOMe/MeOD); and the deuterated ketone fragment was isolated, distilled and counted three times: 3.60 +/- 0.33 x 10^5 dpm ^{14}C/mmole; 8.81 +/- 0.24 x 10^5 dpm ^{3}H/mmole; $^{3}H/^{14}C$ ratio = 2.46 +/- 0.27.

[2-^{3}H, 2-$^{2}H_2$, 1-^{14}C]-Butyrate Feeding (Repeat)

[2-^{3}H, 2-$^{2}H_2$]-Butyrate and [1-^{14}C]-butyrate (Amersham-Searle 58 mCi/mmole) were admixed to give a solution of 1.52 +/- 0.03 x 10^6 dpm ^{14}C/mmole; 4.84 +/- 0.02 x 10^7 dpm ^{3}H/mmole; $^{3}H/^{14}C$ ratio = 31.6. The aliquot for counting was then diluted with cold butyrate (2.65 fold) and derivatized to give the p-phenylphenacyl ester, which was recrystallized to a constant specific activity of: 1.34 x 10^6 dpm ^{14}C/mmole; 4.66 x 10^7 dpm ^{3}H/mmole; $^{3}H/^{14}C$ ratio = 34.8.

The experiment was carried out by feeding the butyrate at 5mM levels to 2 x 100 mL media/flask over the normal four day period. After the fermentation, the flasks were worked-up by EtOAc extraction and PLC yielding 25 mg lasalocid A/200 mL media. The final product was counted and recrystallized to constant specific activity: 1.95 +/- 0.03 x 10^6 dpm ^{14}C/mmole; 6.41 +/- 0.07 x 10^6 dpm ^{3}H/mmole; $^{3}H/^{14}C$ ratio = 3.3 +/- 0.03. This corresponds to a specific incorporation of 119.6% ^{14}C (or 39.9% ^{14}C/C-4 unit) and 11.5% retention of ^{3}H.

The sample was diluted, recrystallized and counted prior to degradation. Pyrolytic cleavage was done at 200°C, 10μ, 45 min.

The distillate was worked-up as usual and the ketone fragment recovered, distilled and counted three times: 1.89 +/- 1.0 x 10^5 dpm ^{14}C/mmole; 5.98 +/- 2.91 x 10^5 dpm ^{3}H/mmole; $^{3}H/^{14}C$ ratio = 3.19 +/- 0.1.

[1-^{13}C, 1-^{18}O, 1-^{14}C]-Butyrate Feeding and

[2-$^{2}H_2$, 2-^{3}H]-Propionate Feeding

[1-^{13}C, 1-^{18}O]-Butyrate, [1-^{14}C]-butyrate (Amersham-Searle 58 mCi/mmole) and [2-$^{2}H_2$, 2-^{3}H]-propionate were admixed to give a solution of: 3.40 +/- 0.03 x 10^5 dpm ^{14}C/mmole butyrate and 8.23 +/- 0.08 x 10^7 dpm ^{3}H/mmole propionate (the propionate was used at tracer levels). The feeding was carried out as usual (5mM), and the isolated lasalocid A counted twice: 5.1 +/- 0.19 x 10^5 dpm ^{14}C/mmole lasalocid A or 50.0% specific incorporation of butyrate, and 1.15 +/- 0.08 x 10^6 dpm ^{3}H/mmole lasalocid A or 0.85% specific incorporation of propionate. The product was subsequently converted to its 1,3-bis-OMe-lasalocid A (2), and sent to Alberta for spectral analysis. This sample was later derivatized to give 1,3-bis-OMe-11,22-bis-TFA-lasalocid A (**3**) which was used for high-resolution MS analysis and further NMR analysis at Alberta.

[2-$^{2}H_2$, 1-^{14}C]-Butyrate Feeding

[2-$^{2}H_2$]-Butyrate was admixed with [1-^{14}C]-butyrate (Amerhsam-Searle, 58 mCi/mmole). An aliquot was removed, diluted, derivatized and counted to constant specific activity: 1.61 x 10^6 dpm ^{14}C/mmole.

The experiment was carried out using four different medias and the seed cultures were started with the corresponding media. In each case, 2 x 100 mL media/flask was fed as follows: SP/LO media (5mM); SP/LO media (10mM); Berger patent media (10mM); "Monensin" media with lard oil (10mM); "Monensin" media without lard oil (10mM). The flasks were fed over the normal four day period, then harvested and worked-up as usual by EtOAc extraction and PLC.

The "Monensin" media without lard oil yielded an EtOAc extract which could not be verified as lasalocid A. The "Monensin" media with lard oil yielded a residue (13 mgs) which corresponded to lasalocid A, but which could not be recrystallized, and therefore was not counted. Similarly, the Berger patent media yielded a residue (16 mgs) which would not recrystallize. Finally, the SP/LO medias yielded lasalocid A which was recrystallized and counted: 10mM feeding (52 mgs), 6.62 +/- 0.23 x 10^6 dpm ^{14}C/mmole, 4.1% specific incorporation of ^{14}C; 5mM feeding (12 mgs), 2.97 +/- 0.02 x 10^6 dpm ^{14}C/mmole, 1.85% specific incorporation of ^{14}C.

[2-$^2H_2$, 1-^{14}C]-Butyrate Feeding (Repeat)

[2-$^2H_2$]-Butyrate was admixed with [1-^{14}C]-butyrate (Amersham-Searle, 58 mCi/mmole). An aliquot was removed, diluted, derivatized to the p-phenylphenacyl ester, and counted to constant specific activity: 1.7 x 10^6 dpm ^{14}C/mmole.

The experiment was carried out using SP/LO media (100 mL media/flask) with a total of 16 flasks and feeding as follows: Day 6 - feed 8 flasks at 5mM levels, 8 flasks at 10mM; Day 7 - feed 6

flasks at 5mM, 6 flasks at 10mM, harvest 2 flasks at 5mM, 2 flasks at 10mM; Day 8 - feed 4 flasks at 5mM, 4 flasks at 10mM, harvest 2 flasks of each concentration; Day 9 - feed 2 flasks at 5mM, 2 flasks at 10mM, harvest 2 flasks of each concentration; Day 10 - harvest remaining 2 flasks. The flasks were worked up as usual by EtOAc extraction and PLC.

The recovered lasalocid A was then recyrstallized and counted three times: Day 7/5mM (18 mgs), 1.65 +/- 0.14 x 10^6 dpm ^{14}C/mmole, 96.8% specific incorporation of ^{14}C; Day 7/10mM (12 mgs), 3.86 +/- 0.27 x 10^6 dpm ^{14}C/mmole, 226.5% specific incorporation of ^{14}C; Day 8/5mM (28 mgs) 2.64 +/- 0.17 x 10^6 dpm ^{14}C/mmole, 154.9% specific incorporation of ^{14}C; Day 8/10mM (31 mgs) 4.6 +/- 0.36 x 10^6 dpm ^{14}C/mmole, 269.9% specific incorporation of ^{14}C; Day 9/5mM (14 mgs) 2.47 +/- 0.07 x 10^6 dpm ^{14}C/mmole, 144.9% specific incorporation of ^{14}C; Day 9/10mM (85 mgs, oil), 4.12 +/- 0.12 x 10^6 dpm ^{14}C/mmole, 241.8% specific incorporation of ^{14}C; Day 10/5mM (8.6 mgs) 2.26 +/- 0.09 x 10^6 dpm of ^{14}C/mmole, 132.6% specific incorporation of ^{14}C; Day 10/10mM (29 mgs), no crystals could be obtained.

A sample from each of the 5mM feedings was submitted for MS/^2H analysis. Then, the lasalocid A from all of the 5mM butyrate feedings was combined, precipitated as the Na-salt (14 mgs), submitted for MS analysis, and then sent to Carnegie-Mellon for high-field ^1H-NMR and ^2H-NMR spectroscopy. The lasalocid A from the 10mM feedings (18 mgs) was treated in the same way.

[1-^{13}C, 2-$^{2}H_2$, 1-^{14}C]-Butyrate Feeding

[1-^{13}C, 2-$^{2}H_2$]-Butyrate was admixed with [1-^{14}C]- butyrate (Amersham-Searle, 58 mCi/mmole). An aliquot was diluted, derivatized to the p-phenylphenacyl ester, and counted to constant specific activity: 7.74 x 10^5 dpm ^{14}C/mmole.

The experiment was carried out by feeding the butyrate solution at 12.5mM levels to 2 x 100 mL media/flask. The butyrate was fed on Days 5 and 6, and the flasks were harvested on Day 7. The flasks were worked-up as usual by EtOAc extraction and PLC. No lasalocid A was produced under these feeding conditions.

[1-^{13}C, 2-$^{2}H_2$, 1-^{14}C]-Butyrate Feeding (Repeat)

[1-^{13}C, 2-$^{2}H_2$]-Butyrate was admixed with [1-^{14}C]-butyrate (Amersham-Searle, 58 mCi/mmole). An aliquot was removed, diluted, derivatized to the p-phenylphenacyl ester and counted to constant specific activity: 8.8 x 10^5 dpm ^{14}C/mmole.

The experiment was carried out by feeding the following: sterile H_2O (2 x 100 mL media/flask); [1-^{13}C, 2-$^{2}H_2$, 1-^{14}C]-butyrate at 8.5mM (3 x 100 mL media/flask); cold butyrate at 8.5mM (2 x 100 mL media/flask). The flasks are fed on Days 5 and 6, and harvested on Day 7. The flasks were worked-up as usual by EtOAc extraction and PLC. Lasalocid A was produced, but in very low yields: 4 mg total in all three cases. None of the samples could be recrystallized. The lasalocid A from the labelled butyrate was analyzed by MS, ^{13}C-NMR (50 MHz, 100 MHz) and ^{2}H-NMR (30 MHz) spectroscopy.

[1-^{14}C]-Butyrate Feeding

Cold Na-butyrate was admixed with [1-^{14}C]-butyrate (Amersham-Searle, 58 mCi/mmole) to give a solution of 2.99 x 10^6 dpm ^{14}C/mmole. The experiment was carried out by feeding 3 x 50 mL media/flask of each of the following: sterile H_2O; 2mM butyrate solution; 3.5mM butyrate solution; 5mM butyrate solution. The flasks were fed over the normal four day period.

At the end of the fermentation, each flask was worked up separataely by EtOAc extraction, Sep-Pak™ separation, PLC and HPLC. The samples of lasalocid A were analyzed by measuring HPLC peak areas and counting an aliquot of the collected fractions containing the lasalocid A. The 2mM feeding yielded an average of 2.5 mg Lasalocid A/50 mL media with a specific incorporation of 24.6%. The 3.5mM feeding yielded an average of 6 mg lasalocid A/50 mL media with a specific incorporation of 79.6%. The 5mM feeding yielded an average of 4.8 mg lasalocid A/50 mL media with a specific incorporation of 105.8%. The H_2O controls yielded an average of 4.7 mg lasalocid A/50 mL media.

[1-^{13}C, 2-$^{2}H_2$, 1-^{14}C]-Butyrate Feeding (Repeat)

[1-^{13}C, 2-$^{2}H_2$]-Butyrate and [1-^{14}C]-butyrate (Amersham-Searle, 58 mCi/mmole) were admixed to give a solution of 2.03 x 10^6 dpm ^{14}C/mmole. The experiment was carried out by feeding the butyrate solution at 5mM levels to 18 x 50 mL media/flask. The flasks were fed over the normal four day period. At the end of the fermentation, the flasks were harvested and worked up as usual by

EtOAc extraction, Sep-Pak™ separation, PLC and HPLC. The HPLC purification was done in two stages: initially, the residue of 3 flasks was combined so that a total of 6 passages through the column were run, yielding a total of 118 mg of lasalocid A, 25 mg lasalocid homologs and 38 mg isolasalocid A. The lasalocid A fraction was then rechromatographed, yielding 79 mgs pure lasalocid A. The final sample was analyzed by MS and ^{13}C-NMR spectroscopy; the ^{14}C incorporation was not determined.

[1-^{13}C]-Isobutyrate Feeding

The [1-^{13}C]-isobutyrate (MSD Isotopes, 90% ^{13}C) was not admixed with any ^{14}C tracer. The experiment was carried out by feeding the isobutyrate solution at 5mM levels to 9 x 50 mL media/flask over the normal four day period.

At the end of the fermentation, the flasks were worked up as usual by EtOAc extraction, Sep-Pak™ separation, PLC and HPLC. A total of 37 mgs lasalocid A was recovered from 450 mL media. The lasalocid A was analyzed by MS and ^{13}C-NMR spectroscopy.

[2,3,3'-$^{14}C_3$]-Isobutyrate Feeding

Cold sodium isobutyrate and [2,3,3'-$^{14}C_3$]-isobutyrate were admixed to give a solution of 5.74×10^6 dpm ^{14}C/mmole. The experiment was carried out by feeding 3 x 50 mL media/flask each of the following: sterile H_2O; 1mM isobutyrate; 2.5mM isobutyrate; 5mM isobutyrate. The flasks were fed over the normal four day period.

Ater the fermentation the flasks were worked up as usual by EtOAc extraction, Sep-Pak™ separation, PLC and HPLC. The samples of lasalocid A were analyzed by HPLC and counting an aliquot of the collected fractions containing lasalocid A. The 1mM feeding yielded an average of 2.05 mg lasalocid A/50 mL media, with a specific incorporation of 31.2%. The 2.5mM feeding yielded an average of 2.85 mg lasalocid A/50 mL media with a specific incorporation of 88.8%. The 5mM feeding yielded an average of 1.8 mg lasalocid A/50 mL media with a specific incorporation of 140.9%. The H_2O controls yielded an average of 1.35 mg lasalocid A/50 mL media.

[2,3,3'-$^{13}C_3$]-Isobutyrate Feeding

[2,3,3'-$^{13}C_3$]-Isobutyrate (69 mg, 0.61 mmole) was admixed with [2,3,3'-$^{14}C_3$]-isobutyrate (21 mg, 0.19 mmole) to give a solution of 5.77 x 10^6 dpm ^{14}C/mmole. In this experiment the w/w ratio of $^{13}C/^{14}C$ labelled precursor was 3.29, (the ^{14}C precursor was used at greater than tracer level). The experiment was carried out by feeding the isobutyrate at 4mM levels to 2 x 50 mL media/flask on Days 6 and 7 only.

At the end of the normal fermentation, the flasks were harvested and worked up as usual by EtOAc extraction, Sep-Pak™ separation, PLC and HPLC. This experiment yielded 19 mg of pure lasalocid A which was analyzed by MS and ^{13}C-NMR spectroscopy.

[4-^{13}C] Butyrate Feeding

The [4-^{13}C]-butyrate (MSD Isotopes, 90.7 atom % ^{13}C) was not admixed with any ^{14}C tracer. The experiment was carried out by feeding the butyrate solution at 4.5mM levels to 2 x 50 mL media/flask over the normal four day period.

At the end of the fermentation, the flasks were worked up as usual by EtOAc extraction, Sep-Pak^{Tm} separation, PLC and HPLC. A total of 15 mgs lasalocid A was recovered from 100 mL media. The lasalocid A was analyzed by ^{13}C-NMR spectroscopy.

$^{2}H_{2}O$ Feeding

The seed cultures were grown in the standard SP/LO media. After 72 hours, a 2% v/v inoculum of the seed culture was transferred to fresh SP/LO media (8 x 50 mL media/flask) that had been prepared from 1:1 $^{2}H_{2}O$:$^{1}H_{2}O$. Starting 24 hours after inoculation, the cultures were then assayed every 24 hours during the normal fermentation checking wet cell mounts and TLC/bioautograms. After the fermentation, six of the flasks were harvested and worked-up as usual by EtOAc extraction and PLC. The yield from these flasks was 45 mg lasalocid A/300 mL media. The Lasalocid A from this experiment was used for various spectral analysis including: MS (high-res); ^{13}C-NMR (50 MHz, 118 MHz); ^{2}H-NMR (30 MHz, 92 MHz); {^{1}H, ^{2}H}^{13}C-NMR; {^{2}H}-INEPT; Tandem-SEFT.

The other two flasks were worked-up after the addition of 1 mCi $^{3}H_{2}O$/flask. After the EtOAc extraction of these two flasks, 95% of the counts remained in the broth, while 5% remained in the

EtOAc extract. After the first plating, the isolated lasalocid A contained only background radioactivity.

[U-^{14}C] Valine Feeding

L-Valine (cold) was admixed with [U-^{14}C]-valine (NEN, 272 mCi/mmole) in three different solutions: 1mM, 4.74 x 10^7 dpm ^{14}C/mmole; 5mM, 9.76 x 10^6 dpm^{14}C/mmole; 10mM 4.81 x 10^6 dpm ^{14}C/mmole.

The experiment was carried out by feeding the following: sterile H_2O (3 x 50 mL media/flask); 1mM (3 x 50 mL media/flask); 5mM (3 x 50 mL media/flask); 10mM (3 x 50 mL media/flask). The flasks were assayed by TLC only during the course of the fermentation. After Day 10, the flasks were harvested and worked-up as usual by EtOAc extraction and PLC. The weight of the recovered lasalocid A was recorded for each flask prior to recrystallizing and counting to constant specific activity. The specific incorporation of ^{14}C into lasalocid was: 16.9% (1mM); 77.9% (5mM); 116.5% (10mM).

[^{13}C]-$NaHCO_3$ Feeding

[^{13}C]-$NaHCO_3$ (KOR Isotopes, 90% ^{13}C) was admixed with [^{14}C]-$NaHCO_3$ to give a solution of 2.15 x 10^7 dpm ^{14}C/mmole.

The experiment was carried out by feeding the following: sterile H_2O (2 x 50 mL media/flask); 1mM (6 x 50 mL media/flask); 2.5mM (6 x 50 mL media/flask); 5mM (6 x 50 mL media/flask). The flasks were not assayed during the fermentation. After Day 10, the flasks were harvested and worked-up as usual with EtOAc

extraction, Sep-Pak™ separation, PLC and HPLC. The recovered weights of lasalocid A after HPLC were: 2.5 mg/300 mL media (2.5mM); 5.7 mg/300 mL media (5mM). (The sample from the 1mM feedings was lost when the prep-HPLC went down). No radioactivity counts were taken on the final lasalocid A. The two recovered samples were analyzed by ^{13}C-NMR spectroscopy.

RESULTS AND DISCUSSION

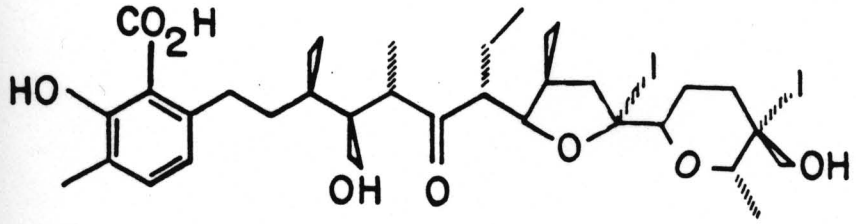
General background

In these investigations, the majority of the biosynthetic studies on lasalocid A used precursors labelled with stable isotopes that could, for the most part, be followed and quantitated using either MS or NMR spectral analysis. In the initial work with ^{14}C and ^{3}H labelled precursors, and in some cases with the stable isotopes, it was necessary to either derivatize or degrade the lasalocid A in order to locate the incorporated label. The biosynthetic studies by Westley et al. (1970) were done using ^{14}C labelled precursors and degrading lasalocid A in order to determine the sites of incorporation. In addition, the same group reported on several chemical transformations of lasalocid A (Westley et al., 1973a; 1973b) that were developed in order to test the pharmacological activities of lasalocid A derivatives. These reactions served as the basis for the derivatization and degradation reactions used in this study.

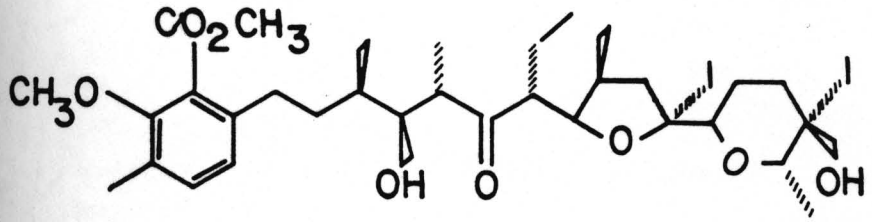
The reactions outlined in Figure 20 proved to be the most useful. The transformation of lasalocid A to its 1,3-bis-OMe derivative (**2**) and then to its 1,3-bis-OMe-11,22-bis-OTFA derivative (**3**) were especially useful in NMR analysis due to the added deshielding of the C-11 and C-22 resonances which are normally in crowded spectral regions. The other important reaction was the pyrolytic degradation of lasalocid A (Westley et

al., 1973b). By using the 1,3-bis-OMe-lasalocid A as the starting material for the pyrolysis, it is possible to isolate the two halves of the lasalocid A molecule. Aside from simple dehydration or base exchange reactions, further degradation of lasalocid A was not necessary for these studies.

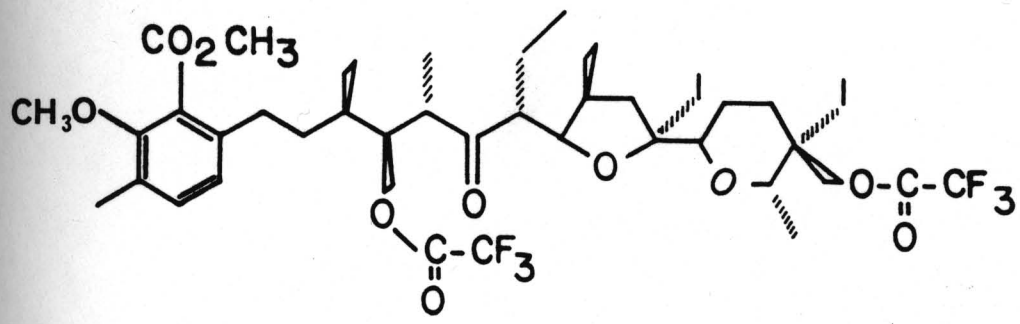
Figure 20. Derivatizations and Degradations of Lasalocid A


CO2H
HO
OH
O
O
O
OH

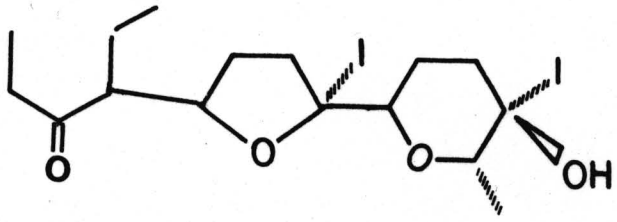
1


CO2CH3
CH3O
OH
O
O
O
OH

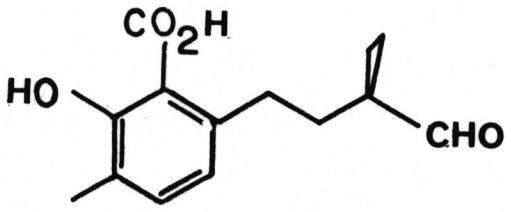
2


CO2CH3
CH3O
O
C-CF3
O
O
O
O
O-C-CF3
O

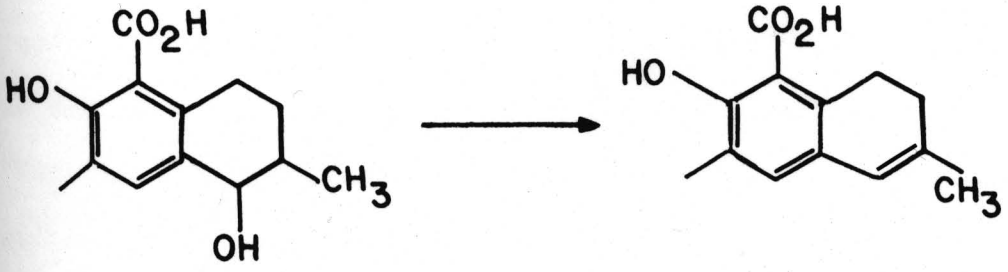
3



4 Ketone Fragment

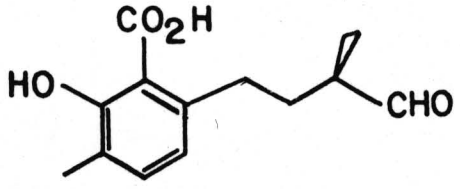


5 Aldehyde Fagment

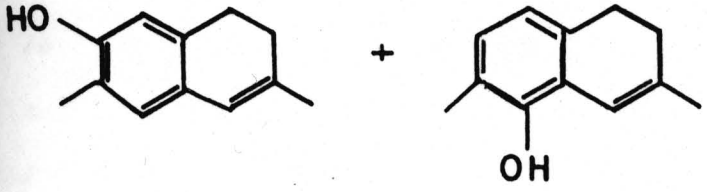


7 Acid Fragment

8



5



9a

9b

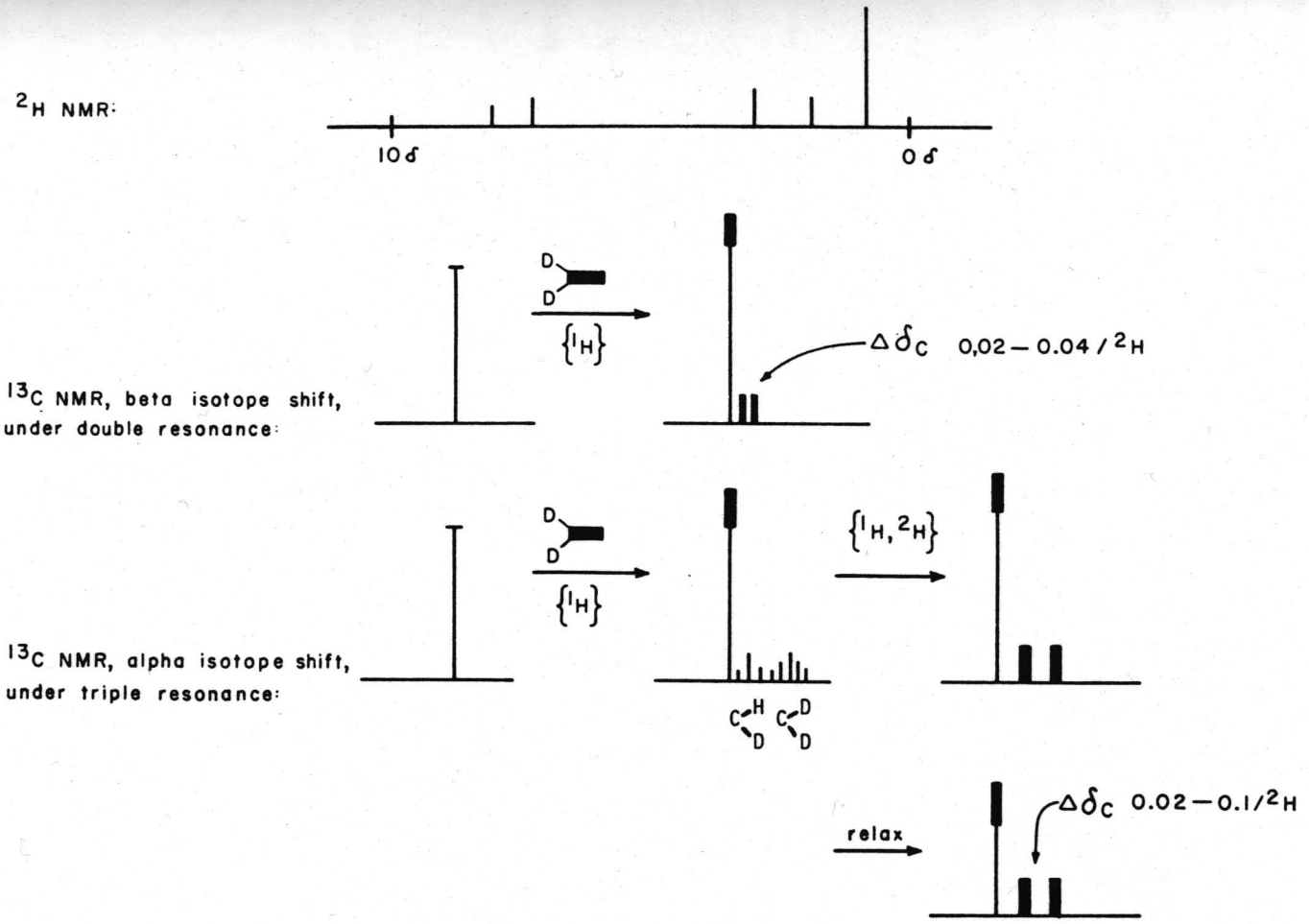
The biosynthetic studies that involved following only the incorporation of carbon atoms were analyzed by ^{13}C-NMR techniques, as described in Chapter One, and quantitated by determining the "Enrichment Factor", as described in the Materials and Methods section of this chapter. The majority of the labelled precursor studies however, involved using a multiple label of ^{13}C with ^{2}H or ^{18}O. It proved to be a considerable challenge to follow and quantitate the incorporation of stable isotopes, other than ^{13}C, into lasalocid A.

The incorporation of $^{13}C/^{18}O$ double labelled precursors was observed and quantitated by the ^{18}O induced isotope shift of the carbon resonance, which was accomplished by high-field ^{13}C-NMR spectroscopy. The observation of an ^{18}O induced isotopic shift is well documented in the nmr literature (see for example: Risley and Van Etten, 1979; 1980a; 1980b; Everett, 1982). Heavy-atom isotopic substitution generally causes an upfield shift of an nmr resonance, which is the case for all ^{18}O induced shifts reported so far. The magnitude of the ^{18}O induced shift is dependent primarily on the type of compound (the functional group and the hybridization) and the number of ^{18}O substituents present (Risley and Van Etten, 1980a; 1980b). Thus, an aldehyde or ketone functional group exhibit a greater shift than a carboxylic acid, which is greater than that for an alcohol or ether substituent. In the case of an ^{18}O substituted alcohol, the magnitude of the shift is in the range of ca. 0.016-0.035 ppm, as compared with

0.043-0.050 ppm for an aldehyde or ketone. While the primary factor affecting the magnitude of the isotope effect is the structure of the carbon-oxygen functional group, there are methods that will enhance the observed isotopic shift, especially for alcohols which normally exhibit small isotope shifts. If the alcohol is derivatized to an ester, in particular, the trifluoroacetic acid ester (TFA), two factors will serve to enhance the isotopic shift. First, the isotopic shift will be enhanced due to the added shielding effects of the TFA group, and second, the possibility for hydrogen bonding is decreased, which apparently can decrease the magnitude of the isotopic shift. This method was successfully employed in labelling experiments with lasalocid A.

The quantitation of incorporation of $^{2}H/^{13}C$ or ^{2}H labelled precursors into lasalocid A was more difficult. Three methods were used (Figure 21); all with severe limitations due to the low levels of ^{2}H retention. When ^{2}H retention was observed, it was usually on the order of 2-10%; the remainder of the label being lost in exchange processes, either specific, such as "Post-Malonate" exchange (McInnes et al., 1983), or non-specific, as would be expected from a continual cycling of intermediates through metabolic pathways. In order to establish a baseline for the observation of ^{2}H retention in lasalocid A, several NMR spectral experiments were done using two different model compounds.

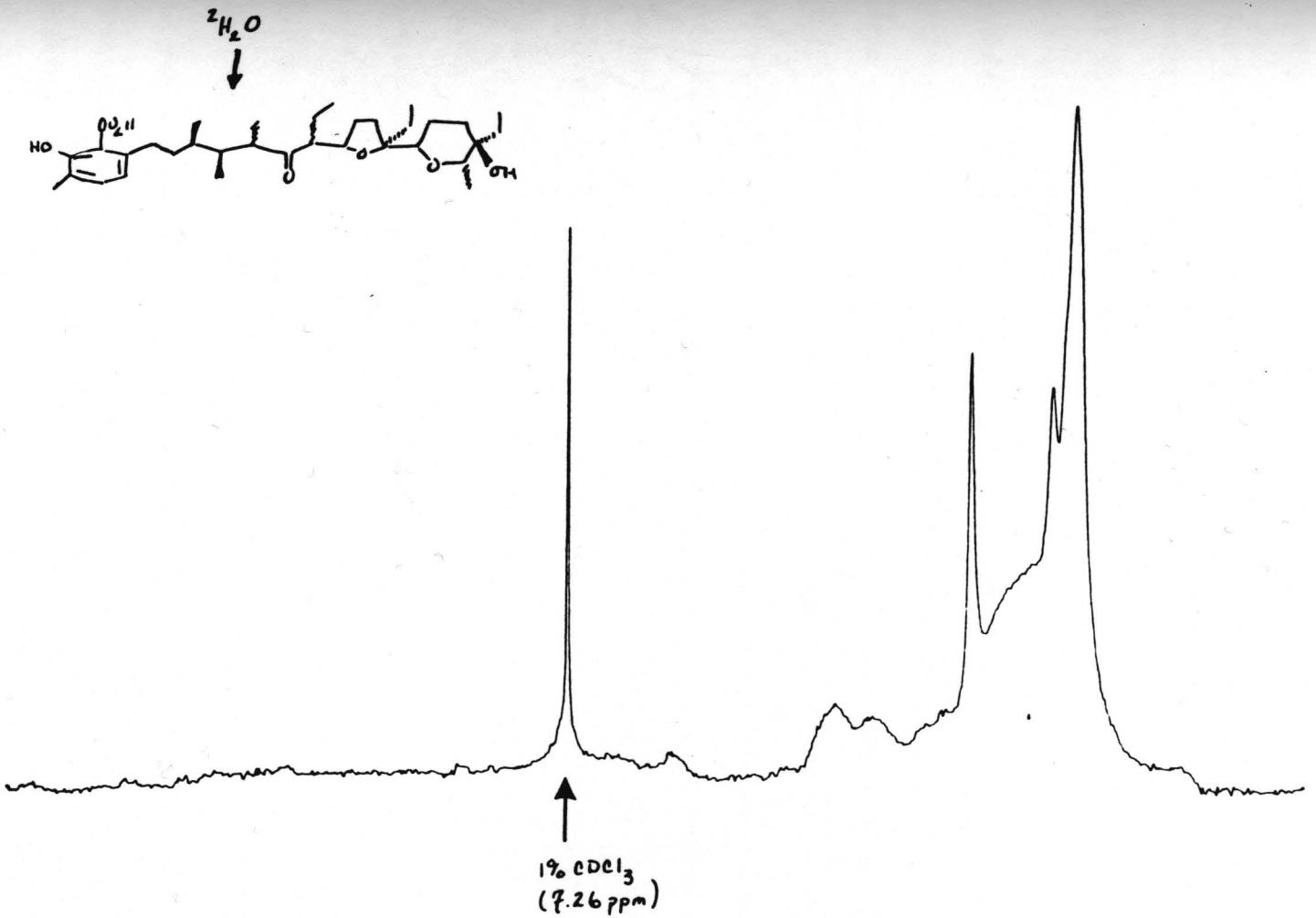
Figure 21. Three Methods For Quantitating ^{2}H in Biosynthetic Studies


^{2}H NMR:
10 δ
0 δ
^{13}C NMR, beta isotope shift, under double resonance:
D
D
{^{1}H}
Δδ$_C$ 0,02 – 0.04 / ^{2}H
^{13}C NMR, alpha isotope shift, under triple resonance:
D
D
{^{1}H}
{^{1}H, ^{2}H}
C–H
D
C–D
D
relax
Δδ$_C$ 0.02 – 0.1/^{2}H

Perdeuterated lasalocid A and the p-phenylphenacyl ester of [2-$^{2}H_{2}$]-butyric acid were the two model compounds used. In order to generate the perdeuterated lasalocid A, a biochemical labelling experiment was carried out in which S. lasaliensis was grown in SP/LO media containing 50:50 $^{1}H_{2}O$:$^{2}H_{2}O$ (Katz and Crespi, 1970), which yielded lasalocid A highly enriched with ^{2}H as verified by high-resolution MS; for example, the m/e 211 fragment contained 20% for d_{n}, $n > 8$; 15.2% d_{8}; 17.2% d_{7}; 17.6% d_{6}; 14.6% d_{5}; 15.4% for d_{n}, $n > 5$. The second model compound was prepared by derivatizing a sample of [2-$^{2}H_{2}$]-butyric acid (98% d_{2} by low-resolution MS analysis) to its p-phenylphenacyl ester and combining this derivatized sample with the p-phenylphenacyl ester of unlabelled butyrate in the following ratios of labelled to unlabelled compound: 1:1, 1:2 and 1:4 (w/w). This derivative was chosen because the oxygenation pattern is similar to the central portion of the lasalocid A molecule and because of the ease of preparation. These two compounds were then analyzed by the following NMR spectral techniques: ^{2}H-NMR, both low-field and high-field; β-isotope shift analysis; INEPT and {^{2}H}-INEPT (Rinaldi and Baldwin, 1982; 1983); and TANDEM-SEFT (Wesener et al., 1984); of these, ^{2}H-NMR and β-isotope shift analysis, along with the Triple Resonance Technique ($^{13}C\{^{1}H,^{2}H\}$, McInnes et al., 1980) were the methods used for isotope enrichment analysis in these investigations.

The simplest method for detection of ^2H enrichment is by direct observation using ^2H-NMR. However, there are two drawbacks to this method. The first is the inherent line broadening due to ^2H being a quadrupolar nucleus, which can make it impossible to accurately assign a resonance in a complex spectrum even though to a first approximation, $\delta^2H = \delta^1H$. The second drawback is a result of carrying out biosynthetic experiments in whole-cell systems which can result in considerable ^2H-exchange. Nevertheless, it was possible to use ^2H-NMR as a means of analyzing biosynthetically enriched samples of lasalocid A, although in the ^2H-NMR spectrum of the perdeuterated lasalocid A at both low-field (30 MHz) and high-field (90 MHz, Carnegie-Mellon) the ^2H enriched methyl groups dominated the spectrum and could not be individually assigned (Figure 22). In the case of the samples of lasalocid A enriched by the incorporation of specific precursors, there were two factors which made it possible to make accurate assignments. First, the ^2H incorporation was distributed over considerably fewer positions and as a result the spectra was less crowded. Second, by using the method of solvent induced chemical shifts (described in Chapter One, Results and Discussion) the resonances could be assigned on the basis of their chemical shift in the two different solvent systems.

Figure 22. 30 MHz ^{2}H-NMR of Perdeuterated Lasalocid A


^2H_2O
HO
OH
1% $CDCl_3$
(7.26 ppm)

A second technique for observing ^{2}H enrichment is the use of the isotopic shift. The deuterium isotope effect on chemical shifts ^{13}C-NMR spectra is well established (see for example: Colli et al., 1973; Tulloch and Mazurek, 1973); however, unlike ^{18}O induced shifts, ^{2}H induced shifts do not exhibit systematic behavior and therefore are more difficult to characterize. ^{2}H induced shifts have been reported for carbons 1-4 bonds removed from the isotopically substituted atom (Tulloch, 1977; Aydin et al., 1981); for having intrinsic "through-space" and long-range "through-bond" effects (Ernst et al, 1982; Christofides and Davies, 1982); and for being stereospecific, especially for carbons 3-4 bonds removed from the substituted atom (Aydin and Gunthar, 1981; Aydin et al., 1984). In most cases, deuteration results in a shielding effect; however, in a few cases, the isotope substitution has been reported to be deshielding (see for example, Ernst et al., 1982; Kurobane et al., 1978; Simpson and Stenzel, 1982). The magnitude of the isotope effect varies and the factors affecting it are not well documented. The presence of more than one shifted resonance can be due to either a difference in the number of isotopic substituents at a single atom, or due to isotopic substituents at more than one carbon, resulting in a combination of α-, β-, γ- or δ-shifts (Simpson and Stenzel, 1982; Abell and Staunton, 1981). Nevertheless, in biosynthetic studies, the β-isotope shift has been used more and more recently. The main advantage of analyzing a β-shift versus an α-shift is that a β

-shifted carbon is essentially a singlet, since ^{13}C-^{2}H coupling over more than one bond is negligible, whereas a carbon bearing a directly bonded isotope substituent suffers from decreased S/N ratio due to poor relaxation, loss of nuclear Overhauser effect and signal multiplicity (Abell and Staunton, 1981).

In the studies with the model compounds the β-isotope shift was observed using the series of p-phenylphenacyl butyrate derivatives. The $^{13}C\{^{1}H\}$ spectra were acquired at 50 MHz using a two-level decoupling program, where 16K data points were acquired and zero-filled to 32K in order to enhance the resolution. The line broadening was decreased from the theoretical minimum of 1.75 Hz to 1.0 Hz prior to the zero-fill. (Technically, this procedure could be argued based on the strict definition of maximum resolution (Rmax = Sweep Width/(0.5*Data Points); however, to a first approximation, this method is valid). The resulting spectra (Figure 23) clearly show a ^{2}H induced isotopic shift on the methylene carbon (ca. 18.5 ppm). In the 1:1 deuterated:non-deuterated mixture, there are two distinct β -shifted resonances in a ratio of 1:1, one is shifted 2.5 Hz (ca. 0.05 ppm), the other is shifted 5.0 Hz (ca. 0.1 ppm). As the deuterated species is further diluted with unlabelled compound, the resonance that is shifted by 0.05 ppm essentially disappears. The presence of two shifted resonances signifies that there is a mixture of deuterated species present. In this case, it would imply that the original deuterated butyrate was either a mixture

of mono- and di-deuterated butyrate, or that the deuterium exchange occured at more than one site. Since the latter case is highly unlikely, it must be assumed that some of the species was only mono-deuterated, and that the MS analysis was misleading. The NMR data are consistent with a mixture of mono- and di-deuterated species, where the majority is di-deuterated.

Figure 23. ^{2}H Beta-Isotopic Shifts in ^{13}C-NMR: Analysis of a Model Compound

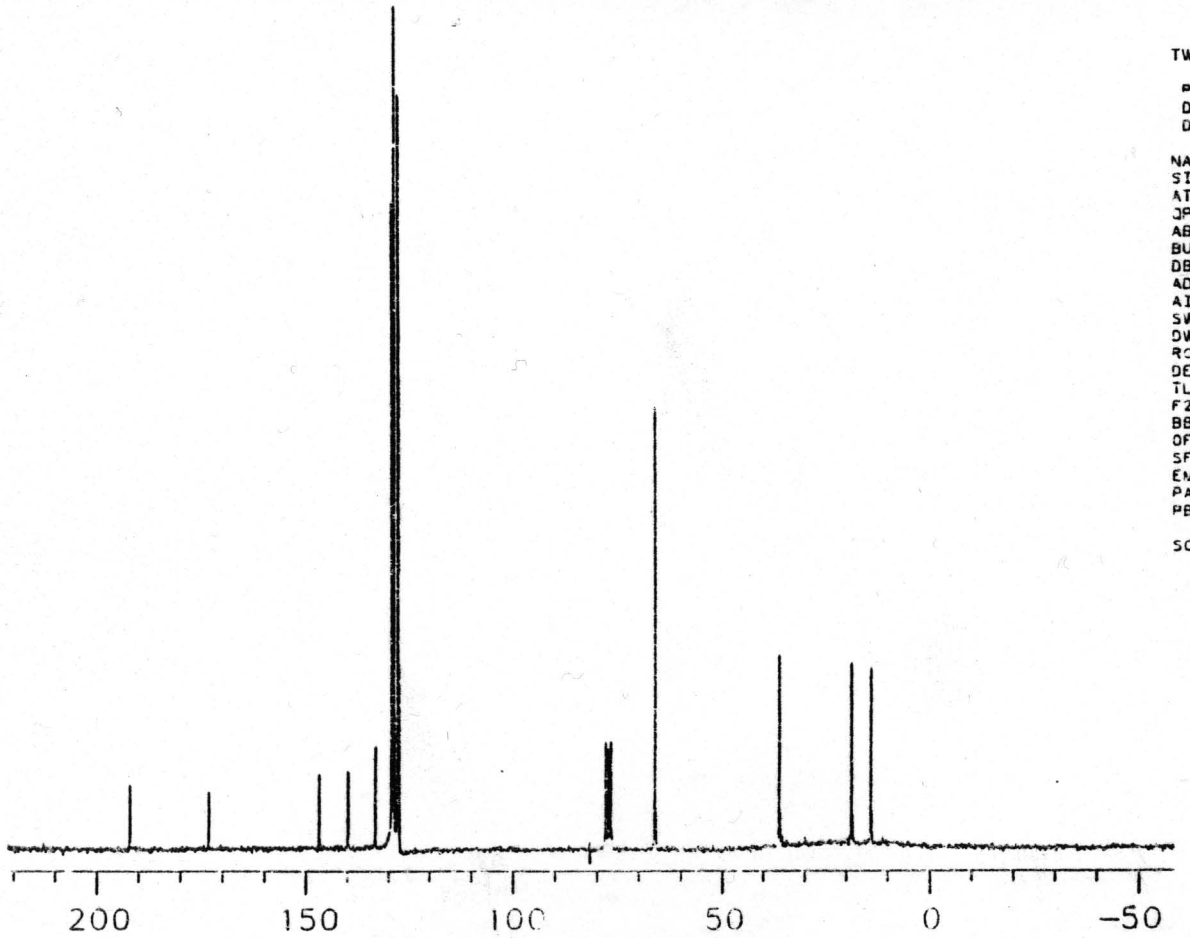
a) Full Spectra (50 MHz ^{13}C-NMR) of a 1:1 Mixture of Deuterated:Non-deuterated p-Phenylphenacyl Butyrate p. 184

b) Expansion (20 Hz/cm) of the ^{13}C-NMR Spectra of the 1:1 Mixture p. 185

c) Expansion (20 Hz/cm) of the ^{13}C-NMR Spectra of the 1:2 Mixture p. 186

d) Expansion (20 Hz/cm) of the ^{13}C-NMR Spectra of the 1:4 Mixture p. 187

MMS 500 MSHERMAN 21NOV84
MODEL CMPD (1 1)



TWO-LEVEL DECOUPLING

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D3= 1.00 MSEC
D5= 1.00 SEC

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AT = 581.63 MSEC
JPD ON = 1
ABC ON
BUTTERWORTH FILTER ON
DB ATT = 3
ADC = 12 BITS
AI = 6
SW = +/- 7042.25
DW = 71
RG = 10 USEC
DE= 71 USEC
TL HIGH POWER ON
F2= 200.067121
BB MODULATION ON
OF= 4034.48
SF= 50.310831
EM= 2.00
PA= 185.0
PB= 72.8

SCALE = 700.00 HZ/CM
= 13.9135 PPM/CM

MMS 500 MSHERMAN 21NOV84
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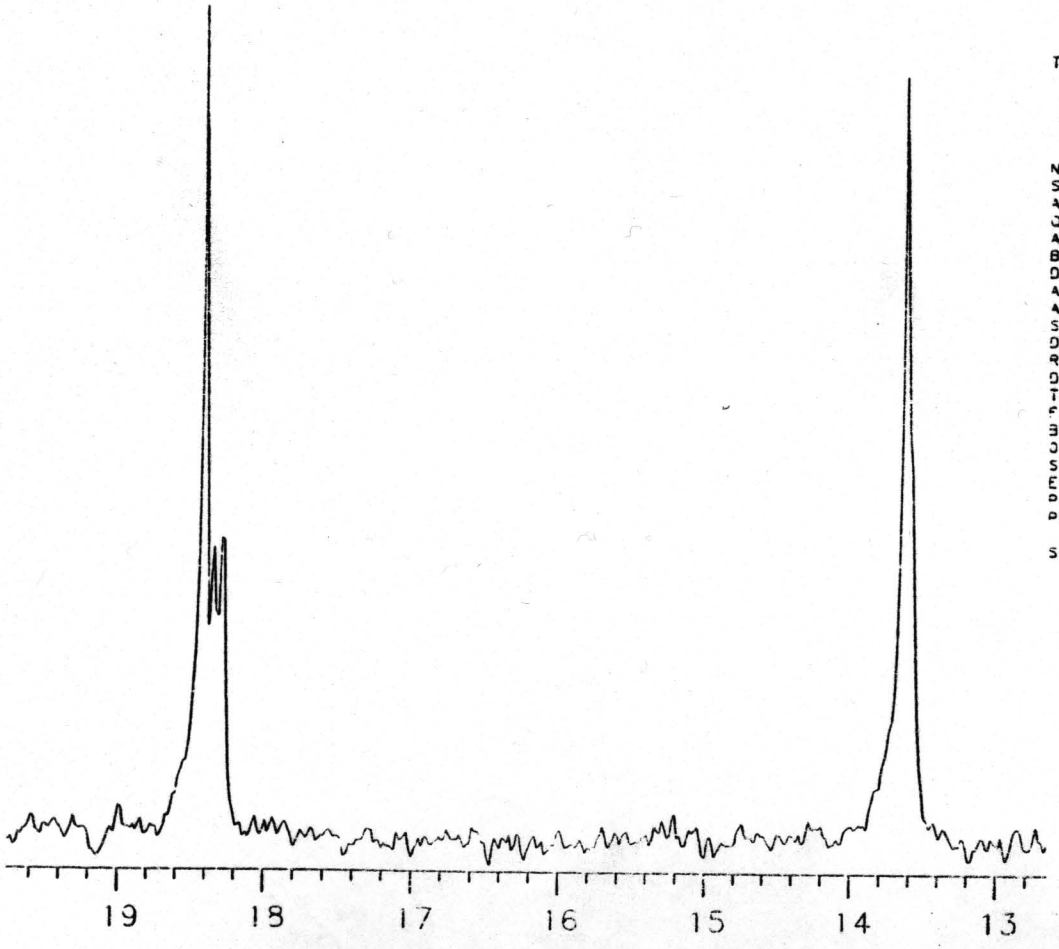

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SW = +/- 7042.25
DW = 71
RG = 10 USEC
DE= 71 USEC
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F2= 200.067121
BB MODULATION ON
OF= 4095.63
SF= 50.310831
EM= 1.00
PA= 184.3
PB= 73.3

SCALE 20.00 HZ/CM
= .3975 PPM/CM

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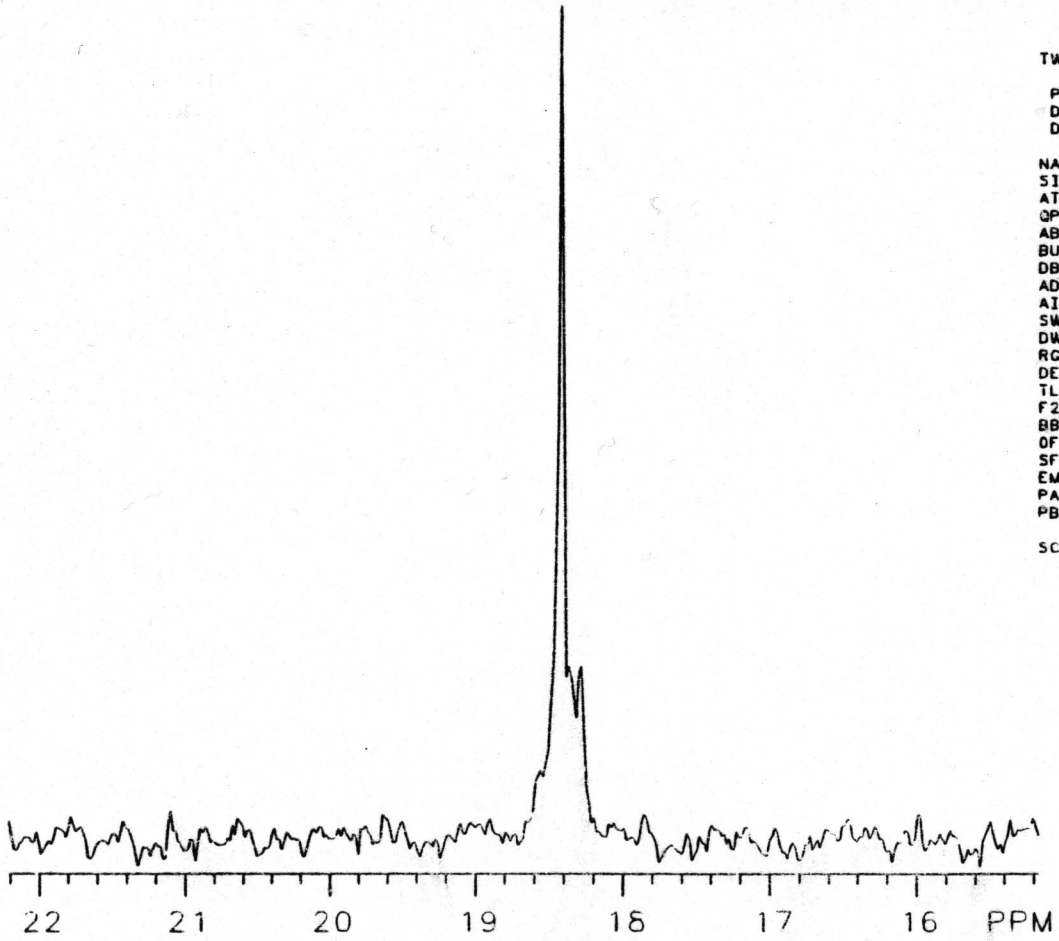

TWO-LEVEL DECOUPLING

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D5= 1.00 SEC

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ABC ON
BUTTERWORTH FILTER ON
DB ATT = 3
ADC = 8 BITS
AI = 9
SW = +/- 7042.25
DW = 71
RG = 10 USEC
DE= 71 USEC
TL HIGH POWER ON
F2= 200.067121
BB MODULATION ON
OF= 4095.72
SF= 50.310831
EM= 1.00
PA= 180.1
PB= 82.9

SCALE 20.00 HZ/CM
= .3975 PPM/CM

MMS 506 MSHERMAN 24NOV84
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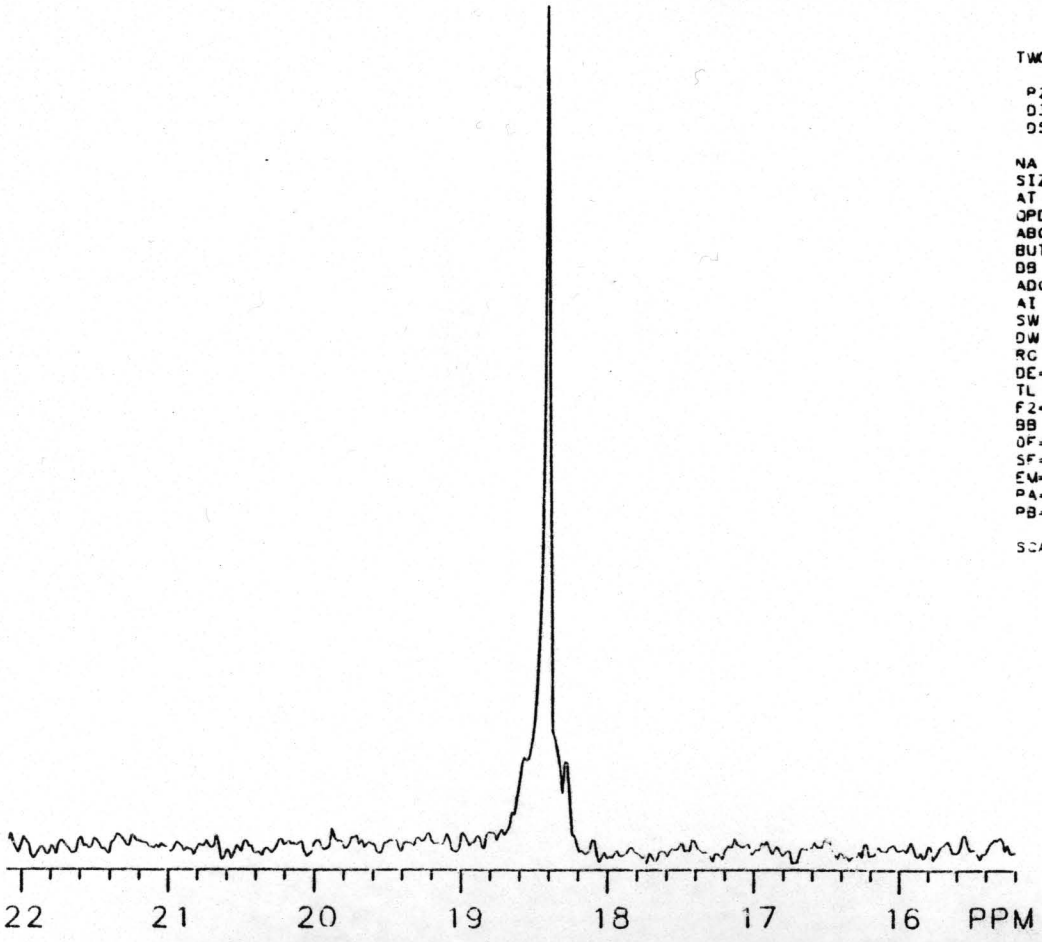

TWO-LEVEL DECOUPLING

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D5= 1.00 SEC

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ABC ON
BUTTERWORTH FILTER ON
DB ATT = 3
ADC = 8 BITS
AI = 10
SW = +/- 7042.25
DW = 71
RG = 10 USEC
DE= 71 USEC
TL HIGH POWER ON
F2= 200.067121
BB MODULATION ON
OF= 4095.55
SF= 50.310831
EM= 1.00
PA= 175.7
PB= 93.4

SCALE = 20.00 HZ/CM
= .3975 PPM/CM

In one report (Simpson and Stenzel, 1982), it was noted that the ^{2}H induced shifts were on the order of 0.08 ppm, and were additive; that is, a CD ($C-^{2}H$) group would shift the β-carbon by 0.08 ppm, while a CD_2 group would shift the β-carbon by 0.16 ppm. These data are consistent with what was observed with the model compounds in this study, where the shifts are on the order of 0.05 ppm/^{2}H atom. The fact that the resonance at 0.05 ppm upfield of the methylene carbon disappears upon dilution suggests that the mono-deuterated species is present in a lower concentration than the di-deuterated species, and that even though both species are being equally diluted, the mono-deuterated species is no longer within the limits of resolution in the 1:4 mixture.

The third technique that was routinely used during these studies was the Triple Resonance Experiment ($^{13}C\{^{2}H,^{1}H\}$) which was first used by McInnes et al. (1980) in biosynthetic studies. The experiment involves acquiring two different ^{13}C-NMR spectra, the first with ^{1}H decoupling only, the second with simultaneous ^{1}H and ^{2}H decoupling. Then, after correcting for the resonances due to ^{13}C bonded to ^{1}H, the remaining α-shifted resonances which are due to ^{13}C bonded to ^{2}H can be quantitated both for multiplicity of isotopic substituents as determined by the amount of α-isotope shift, and for the amount of incorporated label as determined by their intensity. The authors facilitated this experimental method by using a ^{19}F lock, and by adding $Cr(acac)_3$ to ensure relaxation and allow for accurate intensity measurements by integration. In

several of the earlier experiments during this study, the samples of labelled lasalocid A were sent to Halifax where they were analyzed by J.A. Walter and G.A. McInnes using their Triple Resonance Experiment. More recently, attempts have been made to run the experiment on the ^{2}H labelled samples of lasalocid A with the assistance of E.S. Mooberry in the Department of Biochemistry at Wisconsin. At this time the two main problems appear to be: the lack of good spectral resolution, necessary for analyzing low-level enrichment typical of biosynthetic experiments; and the lack an internal ^{19}F lock system, which resulted in a slight field drift and made spectral subtraction impossible.

Finally, attempts were made to analyze deuterium enrichment in lasalocid A by some of the newer pulse sequence techniques. Rinaldi and Baldwin (1982) first recognized that ^{13}C spectra of different deuterated molecular systems could be achieved by polarization transfer from deuterium in their paper on the $\{^{2}H\}$-INEPT pulse sequence. This method has recently been improved upon (Rinaldi and Baldwin, 1983; Bendall et al., 1984) and complementary spin-echo sequences developed (Wesener et al., 1984). The sample of perdeuterated lasalocid A was sent to Rinaldi for analysis by $\{^{2}H\}$-INEPT and to Gunther for analysis by the TANDEM-SEFT technique. In neither case were the spectral results useful for quantitating ^{2}H enrichment into lasalocid A, but whether this was a result of the experimental limitations, the sample or the NMR instrument is difficult to judge. It should be

noted however, that both the polarization transfer experiments and the spin-echo experiments have been refined since the perdeuterated lasalocid A sample was analyzed (Rinaldi and Baldwin, 1983; Wesener et al., 1984).

Oxygenation Pattern of Lasalocid A

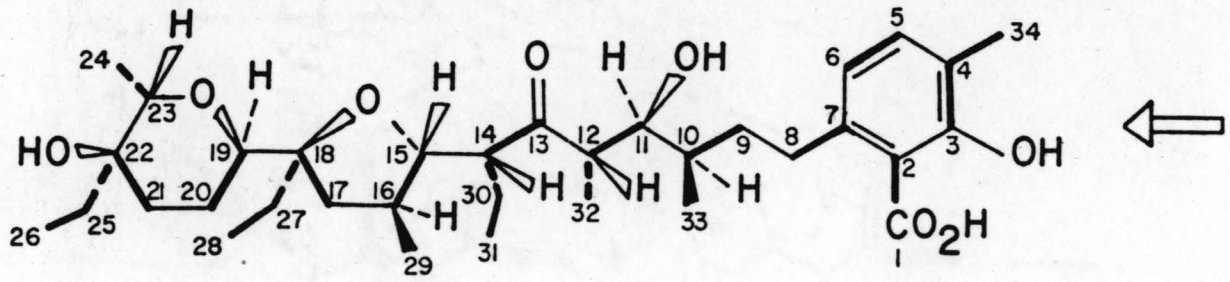
The polyether antibiotics are characteristically highly oxygenated compounds. Therefore, one question regarding the biosynthesis of these compounds is to consider whether or not the organism is capable of preferentially incorporating CO_2 into any one particular site in the molecule. The feeding experiment using [^{13}C]-$NaHCO_3$ as the precursor yielded lasalocid A that contained about 1-2% ^{13}C enrichment at all of the carbons originating from the carboxyl carbon of the acid precursors. These data are consistent with Westley's observations which suggests that the origin of the carbons in lasalocid A, and in particular the carboxyl carbon of the acid precursor, probably arise from the intact subunit.

The question of what role the oxygenation patterns of lasalocid A might play in determining the mechanism of assembly of the molecule, with particular attention to the stereocontrol of the assembly process, was further pursued. Stereochemical control in the biosynthesis of the polyether antibiotics is important if it is assumed that they are formed in a manner similar to fatty acids. In fatty acid biosynthesis the repetitive assembly of the C-2 subunits occurs by stereospecific reactions having a uniform

stereochemistry when viewed by isotopic labelling. In polyether antibiotics the chiral centers in the C-2 to C-4 subunits corresponding to the alpha and beta carbons of the C-2 subunits of fatty acids have different absolute stereochemistry. Thus, in the assembly of polyether antibiotics, stereochemical modifications must occur, either during the carbon chain assembly process or after the chain has been formed. This question can be approached by looking at possible mechanisms for the formation of the hydroxymethylene centers in the central portion of lasalocid A. According to Westley's hypothesis for the formation of lasalocid A, the three key C-O centers to consider would be at C-11, C-13 and C-15; the other oxygens in the molecule are either located at centers lacking a defined stereochemistry, such as the aromatic ring, or are believed to arise from molecular oxygen via oxidation of the putative diene intermediate (Figure 24).

Three possible mechanisms for controlling the stereochemistry of the hydroxymethylene centers in lasalocid A biosynthesis are (i) direct stereodivergent reduction of the keto group in the growing β-ketoacyl chain; (ii) reduction of the keto group followed by dehydration and then stereospecific rehydration of the resulting enone, occurring after carbon chain formation; or (iii) regiochemically controlled epimerization of the different centers, either during chain elongation or in a subsequent intermediate (Figure 25). The second mechanism can be distinguished from the other two by determining the biochemical origin of the oxygen

atoms at C-11, C-13 and C-15, since this mechanism involves a dehydration-rehydration and would therefore involve the possible loss of ^{18}O label by exchange with environmental H_2O. The first and third mechanisms are difficult to distinguish on a whole-cell level since both possibilities would involve retention of the oxygen label; therefore, further distinction would require studying the isolated enzymes. Based on this rationale, the biosynthetic feeding experiments with $^{13}C/^{18}O$ labelled precursors were carried out in order to gain more insight into the sterocontolled mechanism of assembly of lasalocid A.


H
24
O
23
H
HO
22
19
O
18
H
15
O
14
13
12
H
OH
11
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9
8
5
34
6
4
7
3
OH
2
21
20
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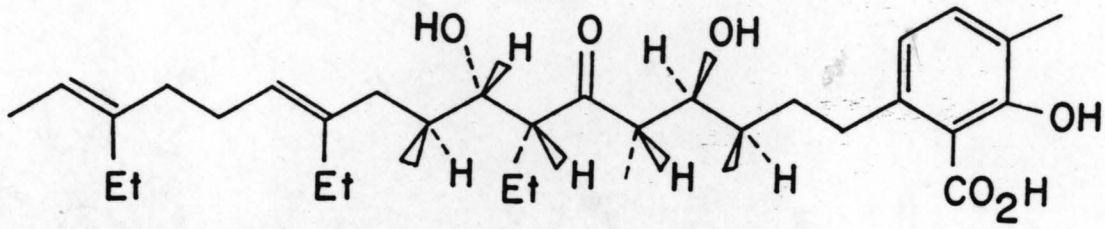
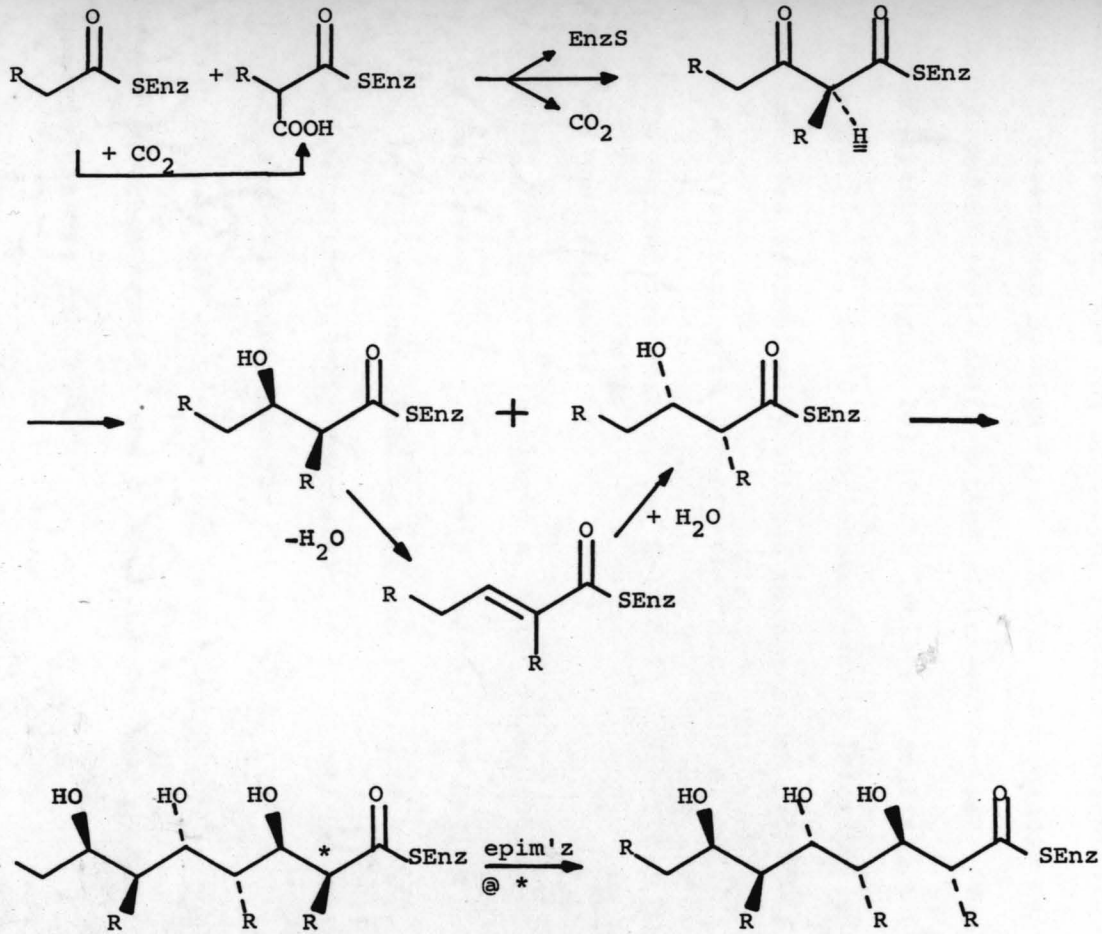
16
H
30
H
32
H
33
H
CO2H
1
26
25
27
28
29
31

2


HO
H
O
H
OH
OH
Et
Et
H
Et
H
H
H
CO2H


R
SEnz
+ R
SEnz
COOH
+ CO_2
EnzS
CO_2
R
SEnz
R
H
HO
R
SEnz
R
HO
R
SEnz
R
$-H_2O$
+ H_2O
R
SEnz
R
HO
HO
HO
R
*
SEnz
epim'z
@ *
HO
HO
HO
R
SEnz
R
R
R

Sodium [1-^{13}C, 1-^{18}O]-propionate and sodium [1-^{13}C, 1-^{18}O]-butyrate were prepared and fed to S. lasaliensis in separate experiments, and the resulting isotopic enrichments of lasalocid A determined by high-field ^{13}C-NMR spectroscopy using the ^{18}O induced isotopic shift method discussed earlier. The 100 MHz ^{13}C-NMR spectra (Figure 26) of the isolated lasalocid A (free acid) from the [1-^{13}C, 1-^{18}O]-propionate feeding initially showed only one resolved isotopically shifted resonance at C-15/O-15. On subsequent derivatization to 1,3-bis-OMe-lasalocid A, two isotopically shifted resonances, for C-15/O-15 and C-3/O-3, were resolved but upon derivatizing to 1,3-bis-OMe-11,22-bis-OTFA-lasalocid A, three isotopically shifted resonances were resolved for C-15/O-15, C-3/O-3 and C-11/O-11. The [1-^{13}C, 1-^{18}O]-butyrate feeding yielded lasalocid A which upon derivatization to the 1,3-bis-OMe-lasalocid A, showed two resolved isotopically shifted resonances for C-1 and C-13. The isotopic enrichment (Figure 27) was determined by measuring the intensities (I) of the enriched peaks in the ^{13}C-NMR spectra and calculating the isotopic ratios:

$$I(^{13}C\text{-}^{18}O)/(I(^{13}C\text{-}^{16}O) + I(^{13}C\text{-}^{18}O)).$$

The NMR spectral results were confirmed by MS analysis of the 1,3-bis-OMe-11,22-bis-OTFA lasalocid A derivatives for both experiments. For example, the MS analysis of the derivatized lasalocid A from the propionate experiment showed that the base

peak at m/e 571.292 exhibited isotopically labelled ions at m/e 581.319 with an intensity of 7.5 mole % of all the ionic species of the 571 peak. This is consistent with $^{12}C_5{}^{13}C_4H_{42}{}^{16}O_4{}^{18}O_3CF_3$.

The results show a range of 87-98% retention of ^{18}O label at C-3, C-11, C-13 and C-15 of lasalocid A. To a first approximation, the average retention of ^{18}O label was 93% +/- 5%, which is within reasonable experimental error for complete retention. The data also show 52% retention of ^{18}O label for C-1, which is consistent with Westley's observation that butyrate is metabolized to acetate, thus providing $CH_3{}^{13}C{}^{18}O{}^{16}OH$ for isotopic labelling of C-1 of lasalocid A. The fact that approximately one half of the ^{13}C-^{18}O label was retained at this carbon is reconcilable with the hypothesis that the terminal carboxyl involved in the chain assembly process would have to undergo a hydrolysis of the thioester bond, which would introduce ^{16}O into C-1, in order to release the assembled carbon chain from the enzyme.

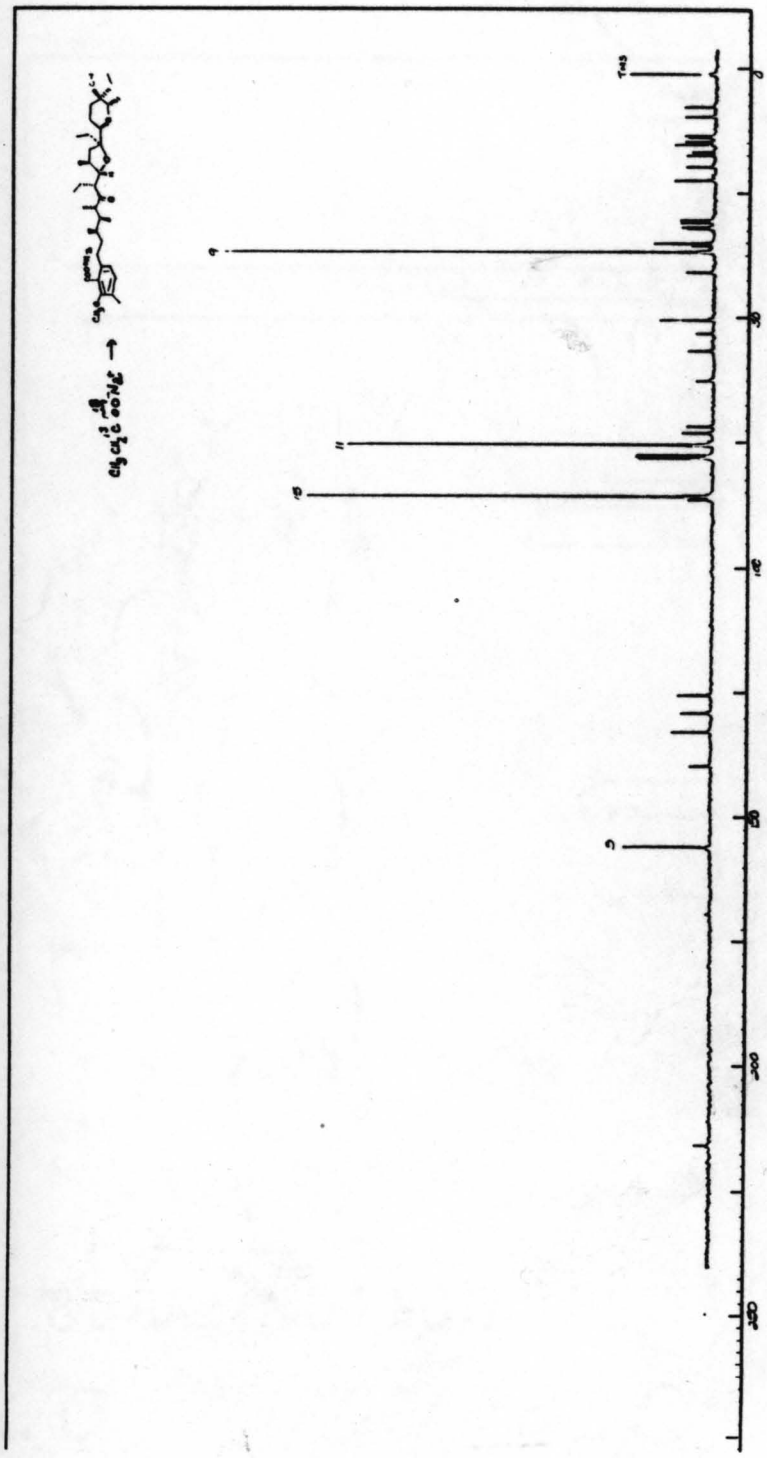
Figure 26. ^{13}C-NMR (100 MHz) Spectra of Lasalocid A Labelled by $^{13}C/^{18}O$ Precursors

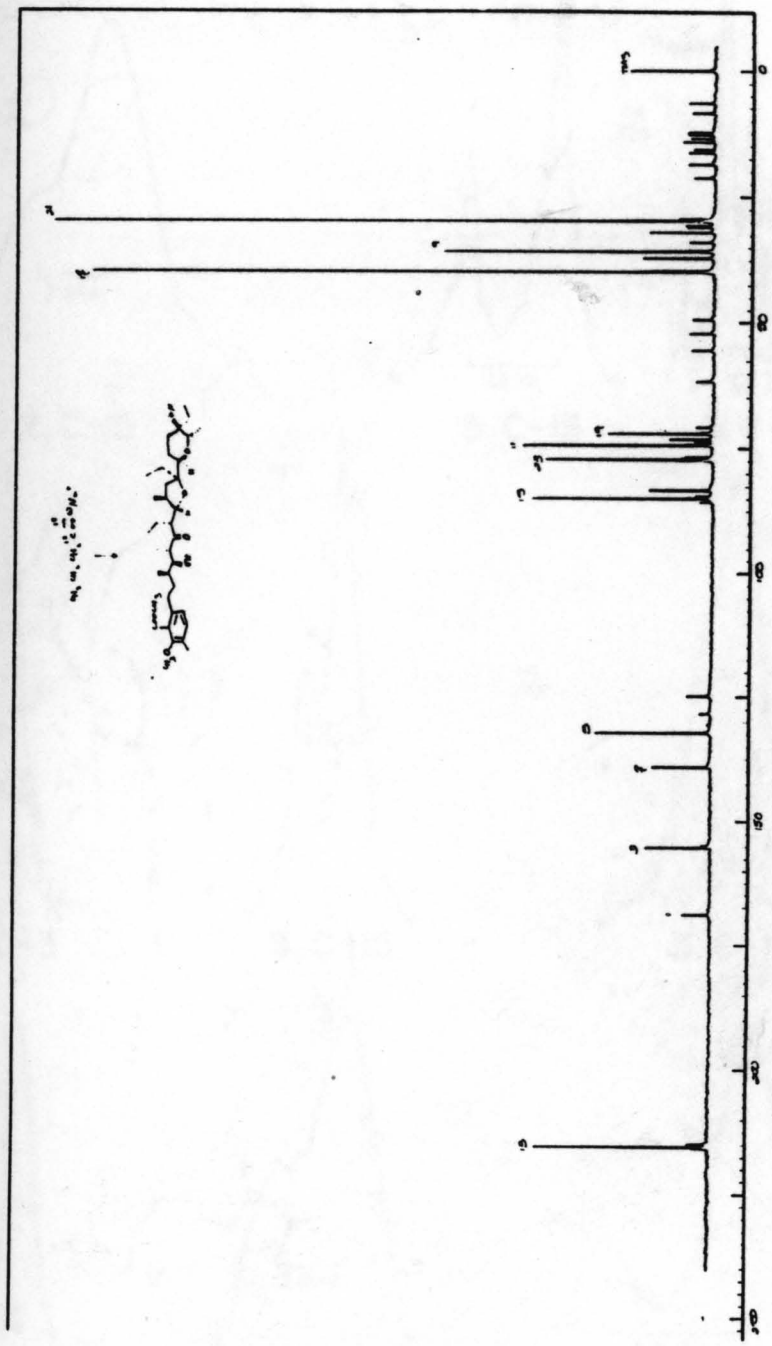
a) Lasalocid A from [1-^{13}C, 1-^{18}O]-Propionate Feeding p. 199

b) Lasalocid A from [1-^{13}C, 1-^{18}O]-Butyrate Feeding p. 200

c) Expansion of Carbons Labelled by $^{13}C/^{18}O$ Precursors p. 201

Lasalocid A (**2**)
bis-OMe-lasalocid A (5)
bis-OMe-bis-OTFA-lasalocid A (**6**)
(a), (c) and (d) are from Propionate Feeding; (b) is from Butyrate Feeding





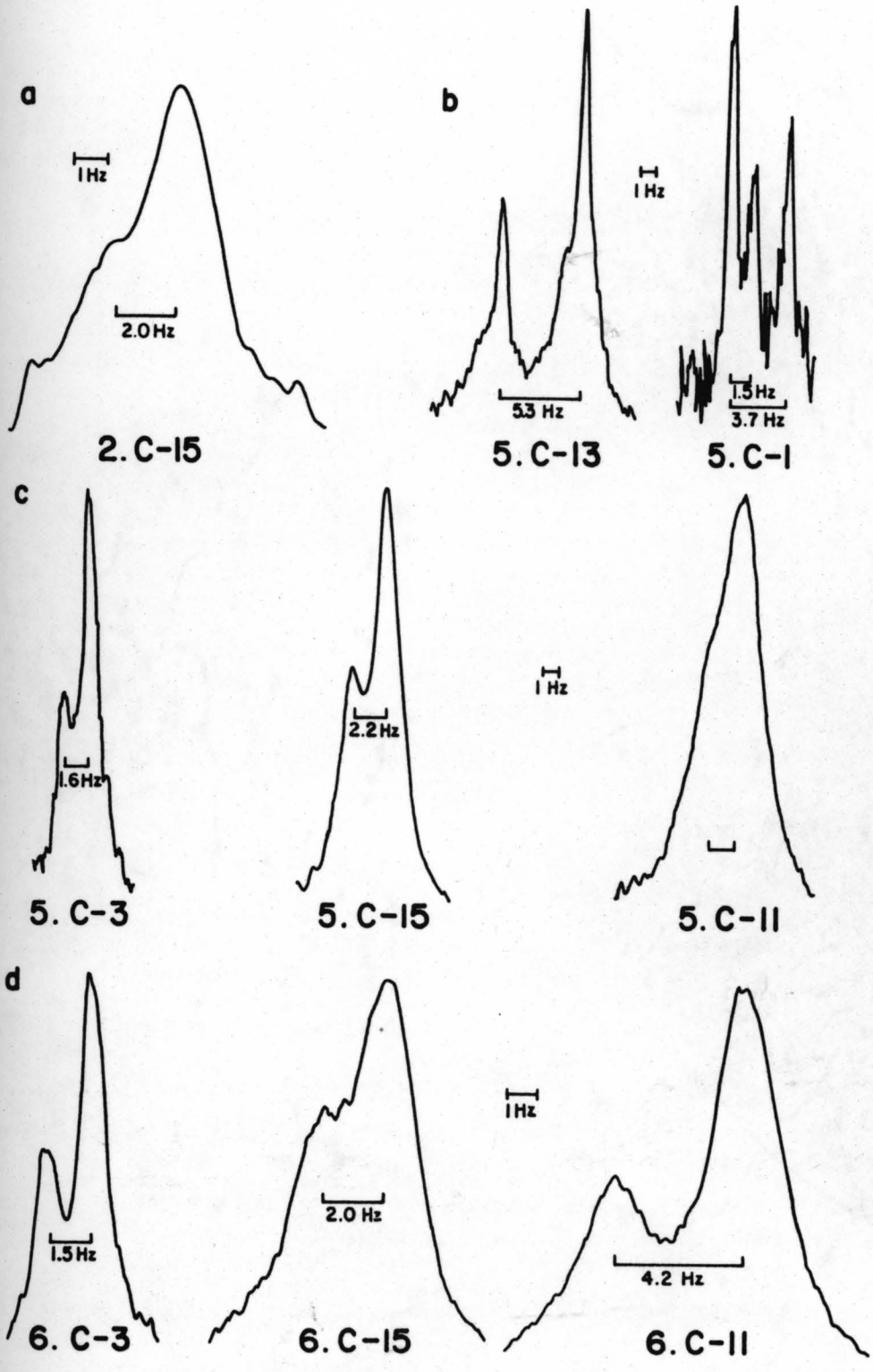

a
1 Hz
2.0 Hz
2. C-15
b
1 Hz
5.3 Hz
5. C-13
1.5 Hz
3.7 Hz
5. C-1
c
1.6 Hz
5. C-3
2.2 Hz
5. C-15
1 Hz
5. C-11
d
1.5 Hz
6. C-3
2.0 Hz
6. C-15
1 Hz
4.2 Hz
6. C-11

Figure 27. [^{13}C, ^{18}O] Content of Lasalocid A and Its Derivatives

Explanation of Footnotes:

a) ^{13}C-NMR Spectra (100.6 MHz)
b) Samples were ca. 0.1M solutions in $CDCl_3$. Chemical shifts are relative to internal Me_4Si.
c) Assignments are as reported by Seto et al., 1978.
d) Peak heights were corrected for the contribution due to natural abundance ^{13}C-^{16}O.
e) The intensity ratios were calculated as stated in text.
f) Calculated by dividing the isotopic ratio percentage of the [^{13}C, ^{18}O]-labelled site in lasalocid A or its derivative by the isotopic ratio percentage of the appropriate [1-^{13}C,1-^{18}O]-precursor.
g) Labelled by the incorporation of [1-^{13}C, 1-^{18}O]-propionate.
h) Labelled by the incorporation of [1-^{13}C, 1-^{18}O]-butyrate.
i) Chemical shifts were assigned based on the characteristic upfield shift on O-methylation relative to assigned values of lasalocid A.
j) The maximum ^{13}C,^{18}O-labelling probability of C-1 is 50% of the butyrate precursor, as explained in the text.
k) The chemical shift of C-11 in lasalocid A free acid is 73.4 ppm.

Table 1. [^{13}C,^{18}O]Content of Lasalocid A and Its Derivatives as Determined by ^{13}C NMR Spectroscopic Analysis.[a]

Compound	Carbon[b]	δ_C[b] (ppm)	$\Delta\delta_C$[c] (Hz)	Peak Heights ^{13}C-^{16}O[d] (cm)	Peak Heights ^{13}C-^{18}O (cm)	Isotopic Labeling Ratios[e] (%)	Retention of ^{18}O[f] (%)
2[g]	15	83.2	2.0	9.5	21.4	69	96
5[g]	3	155.4[i]	1.6	11.4	24.1	68	95
	15	85.1	2.2	12.8	25.5	67	92
5[h]	13	215.6	5.3	13.6	26.2	66	95
	1	169.1[i]	1.5 ($\underline{C}OCH_3$)	23.8	14.1	16[j]	52[j]
			3.7 ($\underline{C}$=O)		17.3	20[j]	
6[g]	3	155.6[i]	1.5	11.5	22.9	67	92
	15	86.4	2.0	9.7	23.1	70	98
	11	80.6[k]	4.2	14.2	24.0	63	87

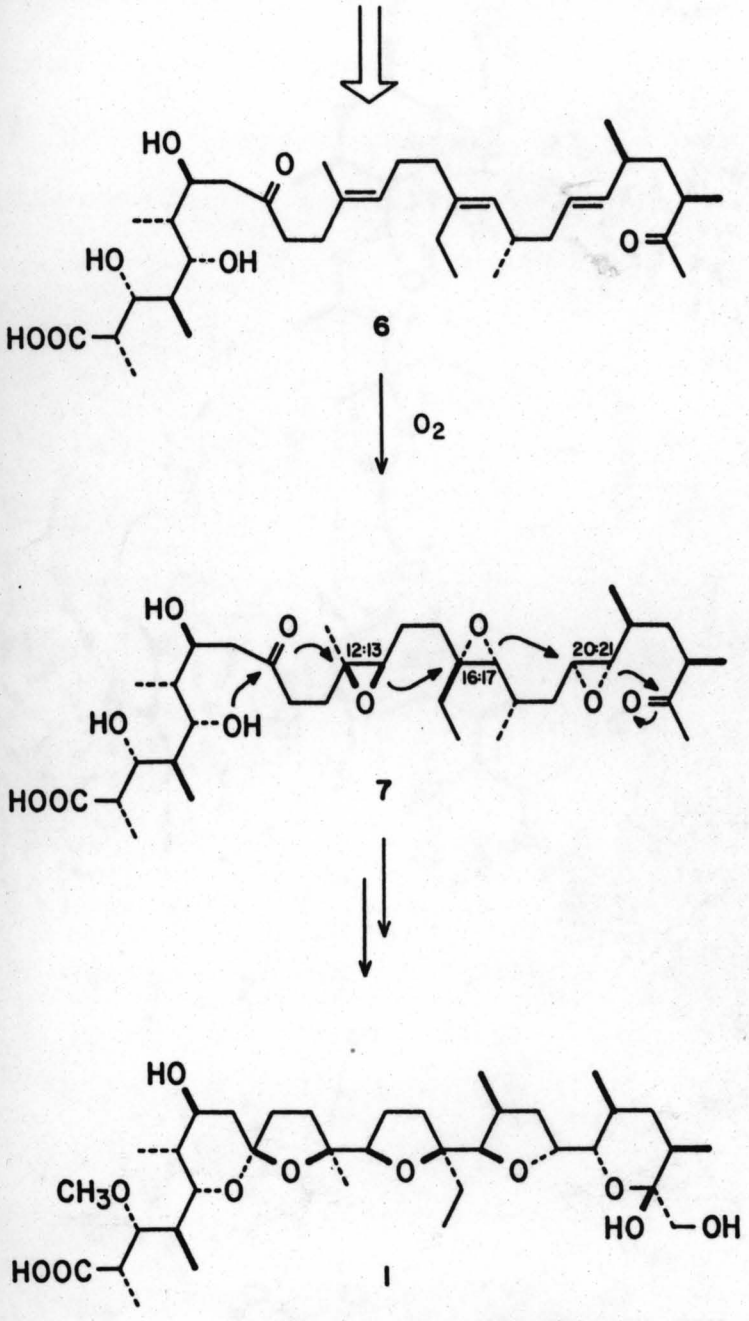
The results of these experiments show that intact carbon-oxygen fragments from [1-^{13}C, 1-^{18}O]-propionate incorporate into the hydroxymethylene groups of lasalocid A with different stereochemical configurations at C-11 and C-15. These data prove that the biosynthesis of lasalocid A cannot proceed via a dehydration-rehydration mechanism since the ^{13}C-^{18}O labels were incorporated intact and without dilution from environmental H_2O. Therefore, the stereocontrol exerted during the formation of lasalocid A at the hydroxymethylene centers must occur by stereodivergent reductases during carbon chain assembly, or by regiocontrolled epimerases, either during, or subsequent to, carbon chain assembly.

The data are also consistent with Westley's hypothesis that the terminal oxygens, located at C-19 and C-22, are introduced by air oxidation of an intermediate, since neither one of these oxygens was labelled with ^{18}O from propionate or butyrate. Ideally, the introduction of molecular oxygen into a metabolite is confirmed by growing the organism in an atmosphere of $^{18}O_2$ and looking for sites of enrichment. However S. lasaliensis is extremely sensitive to any changes in aeration during the fermentation process (Chapter One, Results and Discussion), so on a practical level, the experiment is impossible in this case. It is interesting to note, that Cane et al. (1982) were successful in tracing the origin of all the oxygens in the polyether antibiotic monensin A (Figure 28). The authors showed that three of the

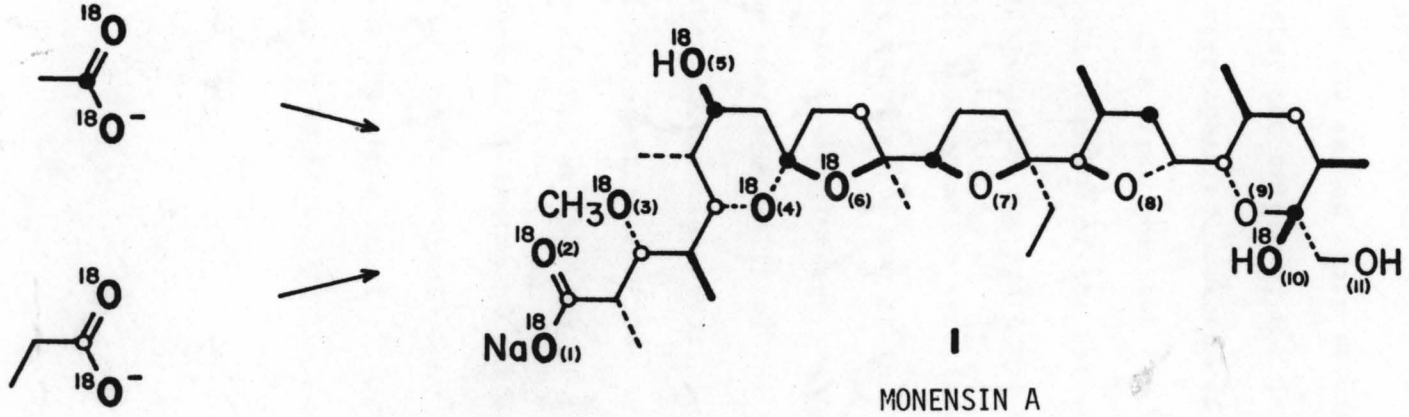
ether oxygens, O-7, O-8 and O-9, in the monensin molecule were derived from molecular oxygen, which supports their hypothesis that monensin is formed via a triene intermediate in a biosynthetic pathway similar to lasalocid A.

Figure 28. Biosynthesis of the Polyether Antibiotic Monensin

$CH_3COSCoA$, $CH_3CH_2COSCoA$, $CH_3CH_2CH_2COSCoA$



D.E.Cane, T-C. Liang and H. Hasler, JACS 1981, 103, 5962.



I

MONENSIN A

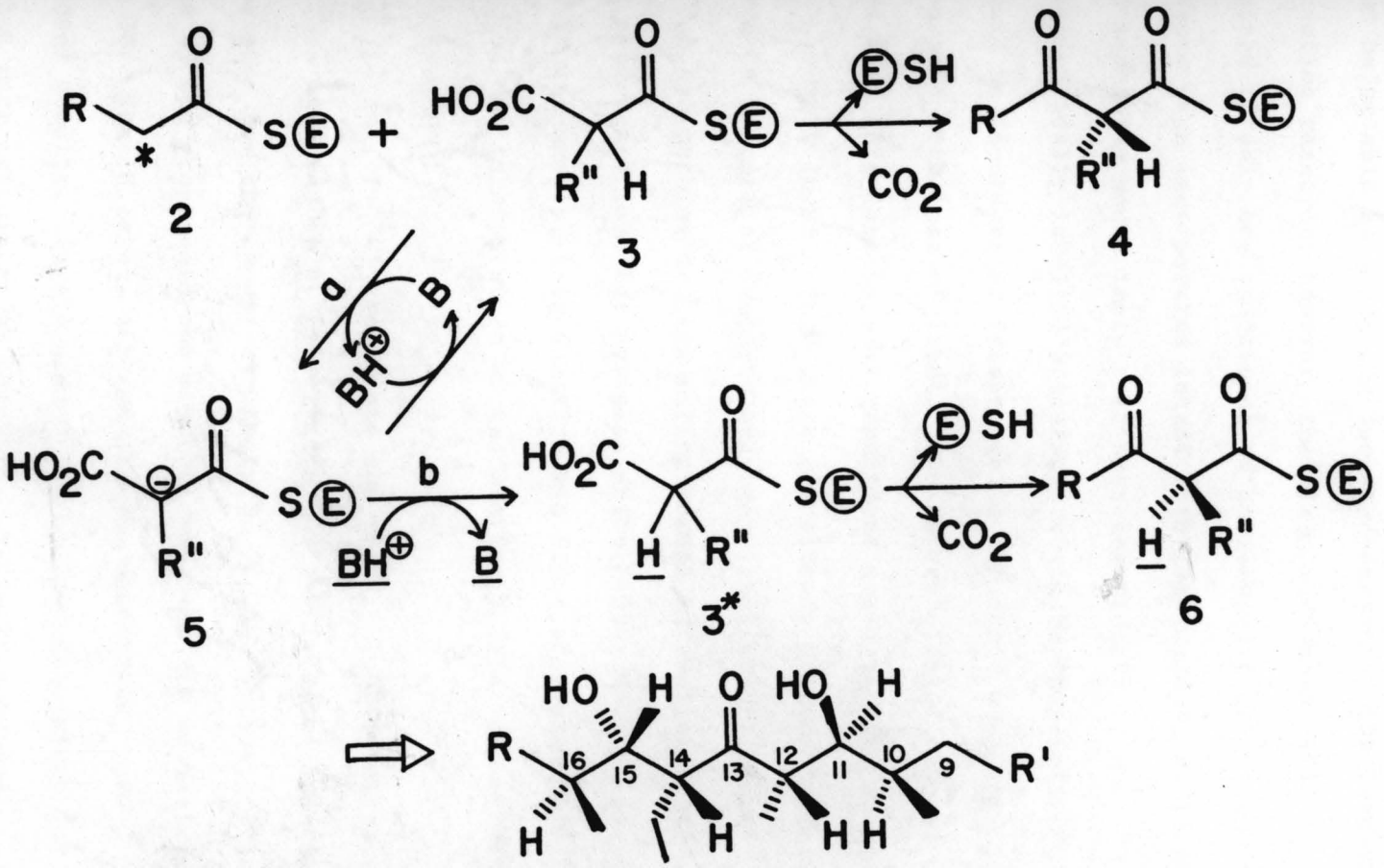
Carbon Chain Assembly of Lasalocid A

The remaining biosynthetic experiments in this study focused on two other aspects of the carbon chain assembly process of the lasalocid A biosynthesis; the origin of the C-2 to C-4 subunits of the molecule and the stereochemical control of methine centers found in lasalocid A. The first question was approached by determining the metabolic sources of the carbon atoms; the second, by following the α-methyl/methylene hydrogens of the different precursors during their incorporation into lasalocid A.

In considering the question of the stereocontrol of the carbon chain assembly, there are at least three mechanistic possibilities: (i) the stereochemistry of the subunit is determined prior to the condensation reaction, that is, the enzyme is stereospecific for one enantiomer of the subunit; (ii) the stereochemistry is determined at the time of the condensation reaction by the stereochemical course of the enzyme reaction; or (iii) the stereochemistry is determined after the carbon chain is assembled, either while the chain is still bound to the enzyme or in a free-intermediate, by specific epimerases (Figure 29).

Figure 29. Proposed Mechanism for Determination of Stereochemistry by Specific Epimerases

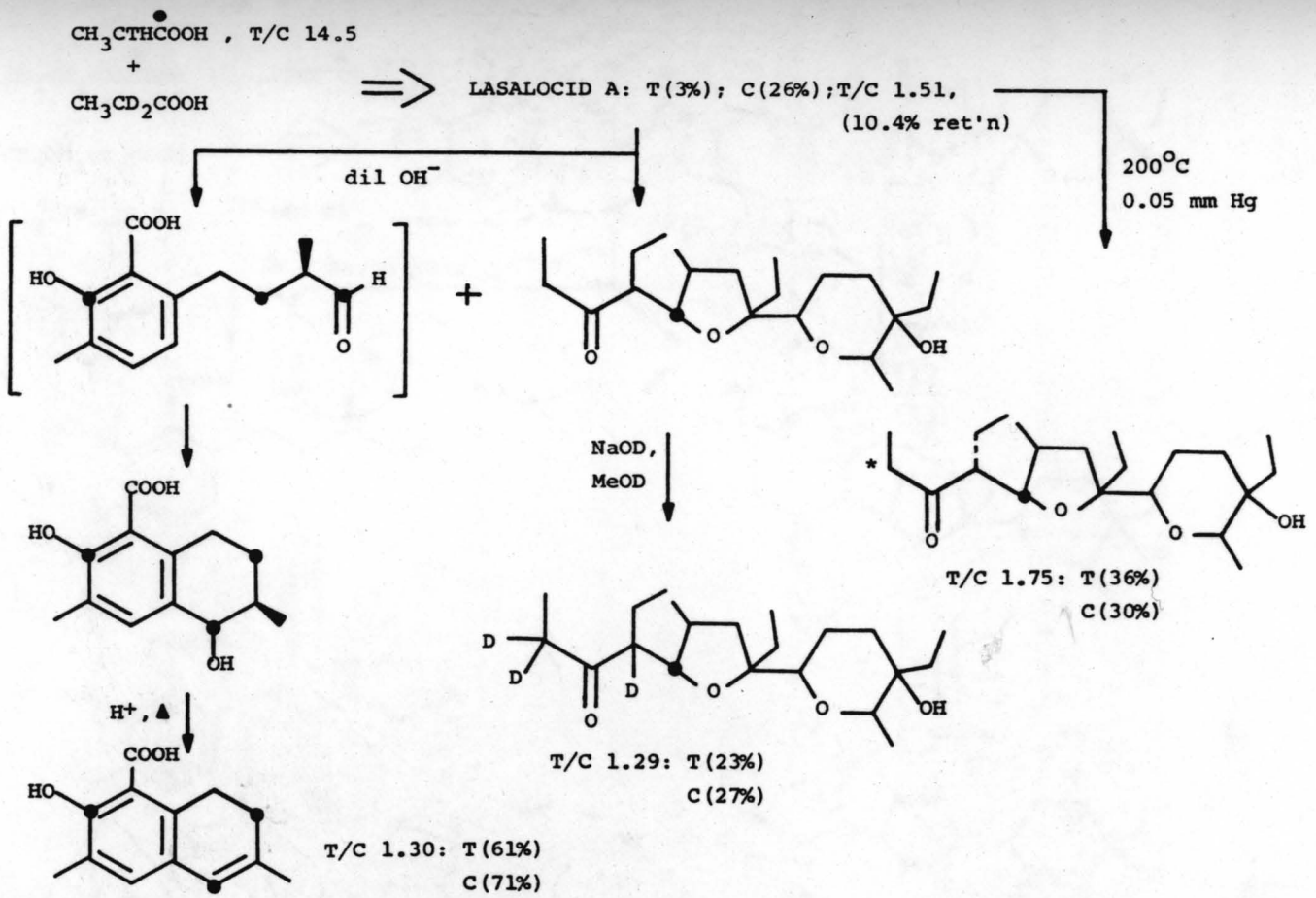
Scheme I

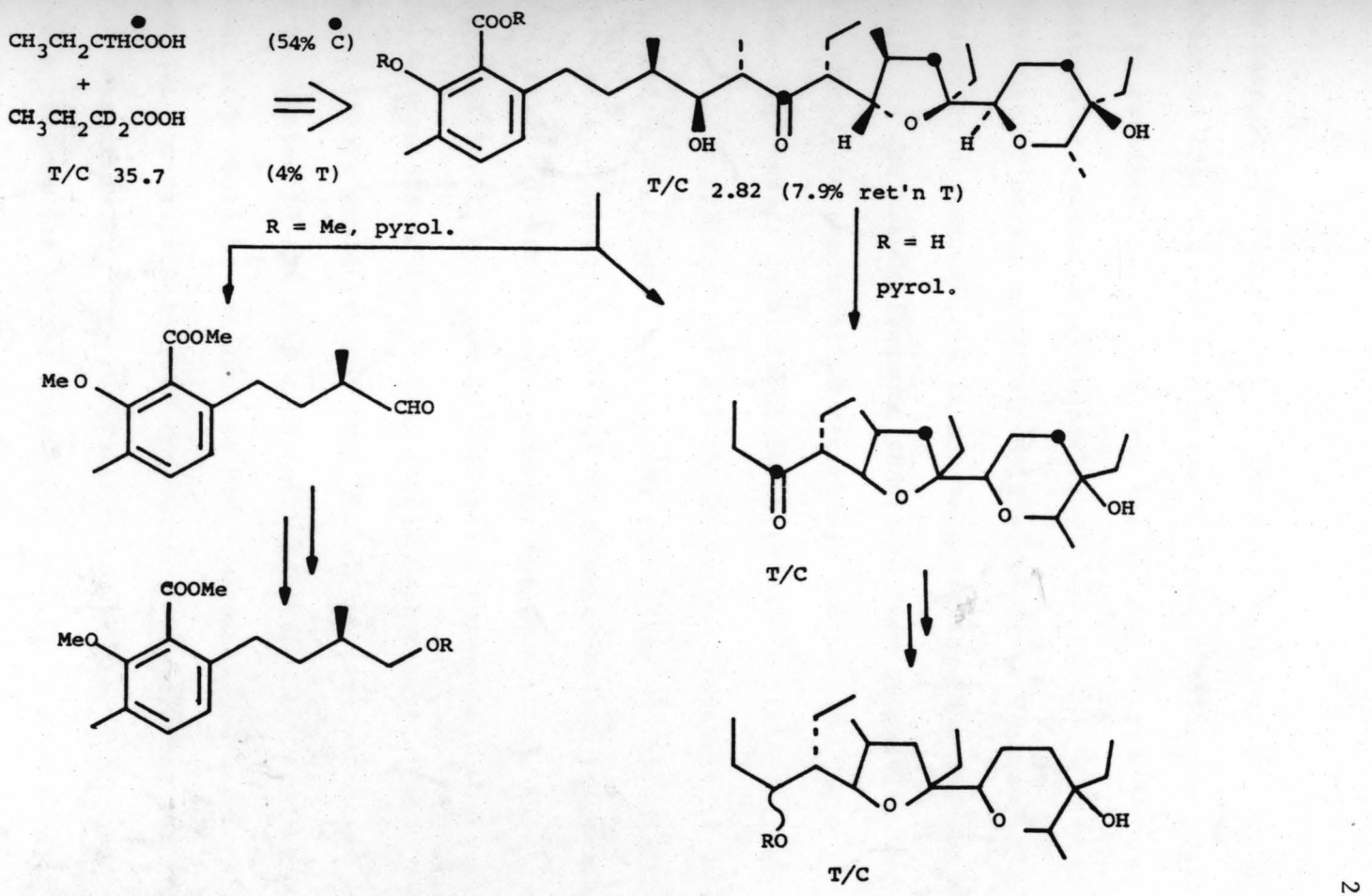

R
O
S E
*
2
+
HO2C
R" H
3
E SH
CO2
4
a
B
BH⊕
HO2C
R"
b
BH⊕
B
5
H
3*
6
HO
H
O
R
16
15
14
13
12
11
10
9
R'
H
H
H H

The distinction between these three mechanisms is difficult without being able to study the isolated enzyme(s) involved in the condensation reaction; however, the first mechanism could be determined if only one enantiomer of stereospecifically labelled precursors were incorporated intact into lasalocid A, while the second mechanism would imply that both enantiomers of the stereospecifically labelled precursors would be incorporated, but one would be incorporated intact, while the other would be incorporated with loss of label. The interpretation of the results from this last type of experiment would be very difficult on a whole-cell level, since there are already precedents for loss of variable amounts of isotopic hydrogen label at activated methylene and methine centers during normal metabolism of these simple precursors (see for example: White, 1980; McInnes et al., 1979). Consequently, this question was first approached by looking at the incorporation of radioactively labelled racemic precursors.

The [2-$^{3}H_2$, 1-^{14}C]-propionate and [2-$^{3}H_2$, 1-^{14}C]-butyrate precursors were fed to S. lasaliensis and the isolated lasalocid A was analyzed for the amount of retention of the ^{3}H label. The results of the propionate and butyrate feedings are summarized in Figure 30. The ^{3}H label, although easily detectable, gave unexpected results. In all cases, the theoretical values for ^{3}H retention were calculated on the basis of the hypothetical biosynthetic pathway for lasalocid A proposed by Westley et al.

Figure 30. Degradation of Lasalocid A Labelled by Radioactive Precursors


CH3CTHCOOH , T/C 14.5
+
CH3CD2COOH
LASALOCID A: T(3%); C(26%);T/C 1.51,
(10.4% ret'n)
200°C
0.05 mm Hg
dil OH⁻
COOH
HO
H
O
OH
NaOD,
MeOD
D
T/C 1.75: T(36%)
C(30%)
T/C 1.29: T(23%)
C(27%)
H+, ▲
T/C 1.30: T(61%)
C(71%)


CH3CH2CTHCOOH
+
CH3CH2CD2COOH
T/C 35.7
(54% C)
(4% T)
COOR
RO
OH
O
H
O
H
O
OH
T/C 2.82 (7.9% ret'n T)
R = Me, pyrol.
R = H
pyrol.
COOMe
MeO
CHO
T/C
COOMe
MeO
OR
RO
T/C

In the case of [2-^{3}H]-propionate, the theoretical value for ^{3}H retention is 25%; two out of a possible eight α-methylene protons should be retained, one at C-10 and one at C-12. After degradation of lasalocid A, theory would predict that 0% of the original ^{3}H label would be located in the dehydrated acid fragment, 0% in the base exchanged ketone fragment and 12.5% in the ketone fragment generated from pyrolysis (corresponding to the labelled α-carbonyl position). The experimental results gave 61% of the original ^{3}H label in the dehydrated acid fragment and 36% in the ketone fragment, of which 13% was at the α-carbonyl position by comparison of the specific radioactivity of the exchanged and unexchanged ketone fragments.

For the [2-^{3}H]-butyrate experiment, the theoretical ^{3}H retention is 16.7%, or 1 out of a possible 6 α-methylene protons should be retained at C-14. Then, after degradation of the molecule, theory would predict that all of the retained tritium would be located in the ketone fragment generated by pyrolysis, and that none would be retained in the base exchanged ketone fragment or the dehydrated acid fragment. Instead, what was found was 7.9% retention of ^{3}H in the lasalocid molecule, which upon pyrolysis yielded the ketone fragment with 2.5% ^{3}H retention; subsequent base exchange yielded the ketone fragment again with 2.5% ^{3}H retention, that is, no ^{3}H was apparently at C-14.

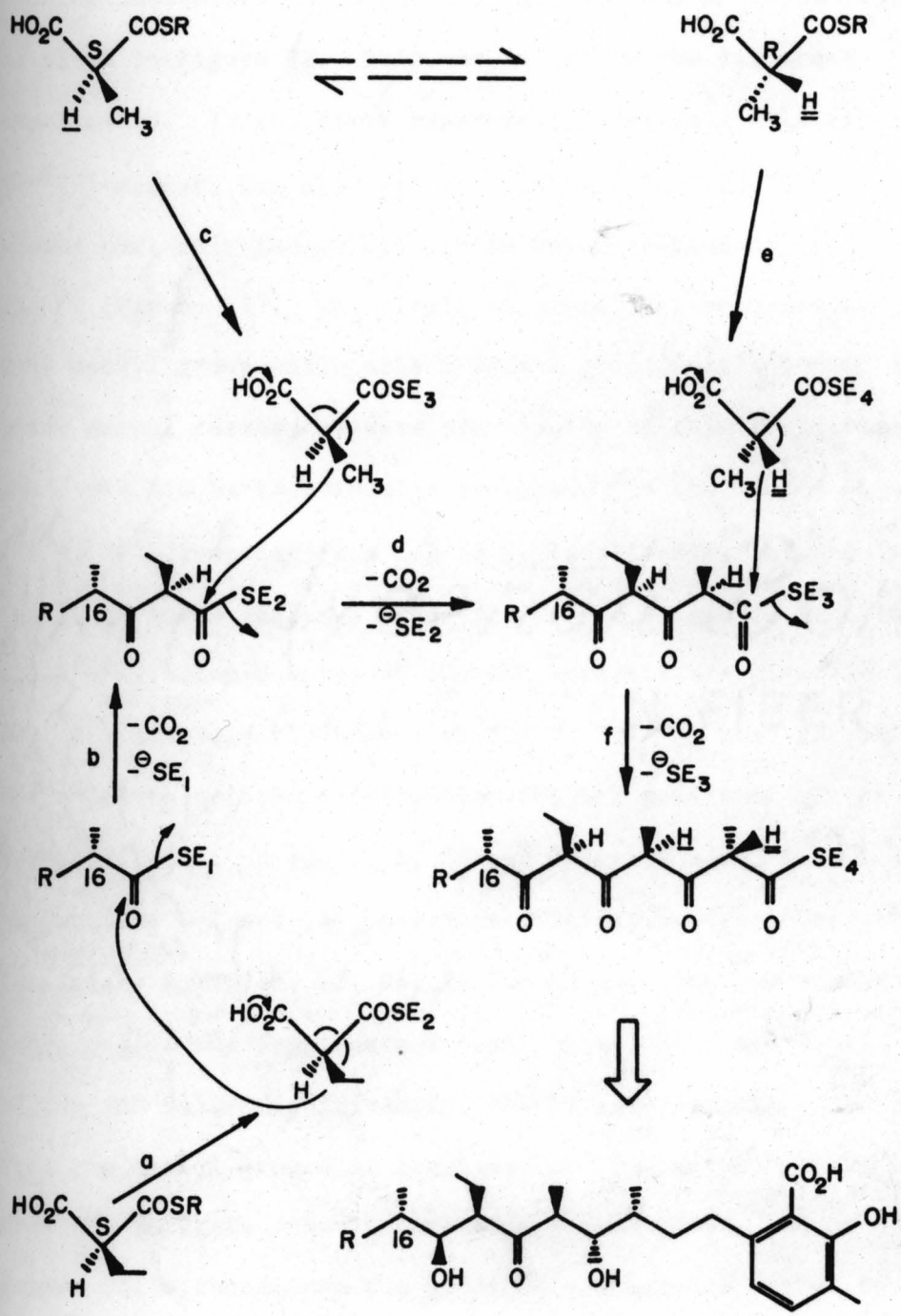
In both experiments, the ^{3}H retention was less than theory. In the propionate feeding the ^{3}H was distributed throughout the

molecule, while in the butyrate feeding the ^{3}H that was incorporated into the ketone fragment (the acid fragment was not analyzed) but apparently was not at the exchangeable α-methylene position. From these results it appears that considerable randomization of the ^{3}H label from these precursors occurs during the course of the fermentation, and as a consequence, it is difficult to make any conclusive statement about the mechanism of carbon chain assembly of lasalocid A.

At this point in the investigations, it was decided to focus on labelled precursor studies using the stable isotopes, ^{13}C and ^{2}H, and to follow their incorporation into lasalocid A by MS and NMR spectral techniques. This was done with the hopes of better understanding not only the stereochemical mechanism of carbon chain assembly, but also the metabolic pathways giving rise to the different subunits. The initial stable isotope experiments looked at the incorporation of acetate, especially in its role as the starter unit in the polyketide chain. The subsequent experiments focused on the question of the stereocontrol of the biosynthesis of the central portion of lasalocid A, composed of C-9 to C-16 (Figure 31), using propionate, succinate and butyrate as the labelled precursors. The central portion of lasalocid A represents the stereocomplexity of lasalocid A biosynthesis in that it involves the assembly of three propionate and one butyrate subunits which result in different stereochemical configurations at both the hydroxymethylene and methine centers. If lasalocid A

is assembled by the stereospecific incorporation of precursors into the growing carbon chain in a condensation reaction similar to fatty acid biosynthesis, as stated in one hypothesis, this would imply that the propionate subunits would arise from both (2R)- and (2S)-methylmalonyl-CoA, while the butyrate subunit would originate from (2S)-ethylmalonyl-CoA.

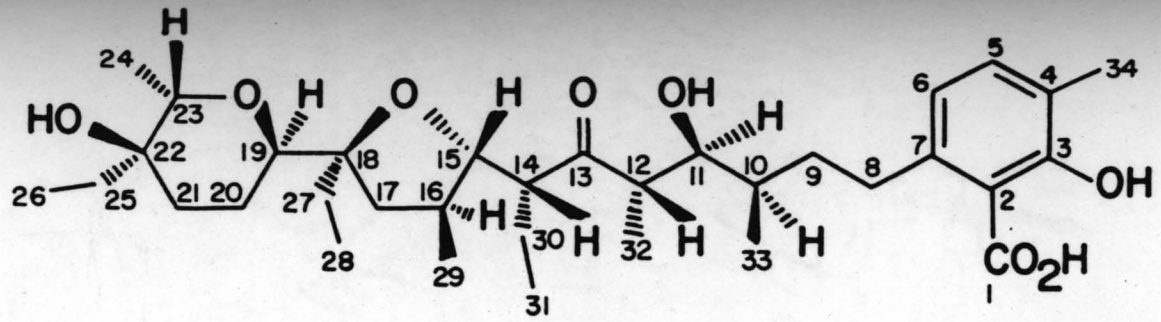
Figure 31. Proposed Incorporation of Chiral Precursors from Propionate, Succinate and Butyrate Subunits into the Central Portion of the Lasalocid A Molecule (C-9 to C-16)


HO2C
COSR
S
H
CH3
R
CH3
H
c
e
COSE3
COSE4
d
-CO2
-⊖SE2
SE2
SE3
16
O
b
-CO2
-⊖SE1
f
-CO2
-⊖SE3
SE1
SE4
COSE2
a
CO2H
OH

The incorporation of acetate was expected to label lasalocid A as shown in Figure 32. This was tested in two different experiments. In the first experiment, lasalocid A labelled by [2-$^{2}H_{3}$]-acetate was analyzed by low-field ^{2}H-NMR (30 MHz), which showed that only the methyl groups were labelled significantly by the ^{2}H (Figure 33). The single resolved resonance was for the C-34 methyl group which arises from a propionate subunit; all other methyl resonances were overlapping at this field strength and could not be individually assigned. In the second experiment, [2-^{3}H, 2-^{13}C]-acetate was fed to S. lasaliensis, and the isolated lasalocid A was analyzed using the Triple Resonance Experiment (25.1 MHz). The results of the NMR analysis are given in Figure 33. The data clearly show that [2-$^{2}H_{3}$, 2-^{13}C]-acetate labelled not only the carbons arising from the C-2 positions of the acetate subunit (Positions #2, 5, 8, 20 and 24), but also, to a lesser extent, the C-2 and C-4 positions originating from butyrate (Positions # 14, 18, 22, 26, 28 and 31) and the C-2 and C-3 positions of the propionate subunits (Carbons # 4, 10, 12, 16, 29, 32, 33 and 34). Significantly, the ^{2}H label was incorporated only into the methyl groups of lasalocid A. The methyl groups arising from the butyrate subunit contained either three, two or one ^{2}H atoms, while those from the propionate units had either two or one ^{2}H labels and the one methyl group from acetate, C-24, the starter unit, did not contain any ^{2}H label.

Figure 32. Biosynthetic Precursors of Lasalocid A:
Acetate as Precursor

The two carbon subunit arising from acetate should label the following carbons in lasalocid A: C-1/C-2; C-5/C-6; C-7/C-8; C-19/C-20; C-23/C-24.



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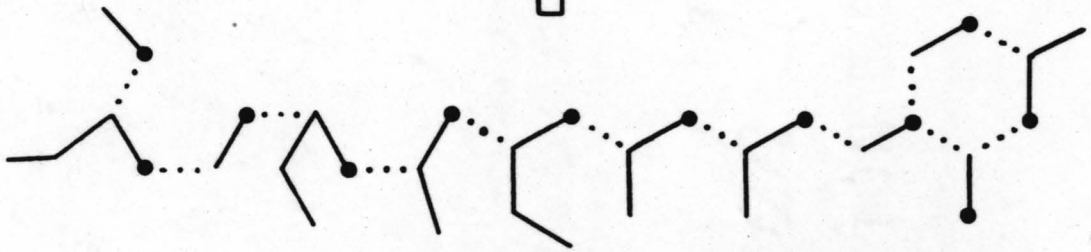


5 — CH_3CO_2H

4 — $CH_3CH_2CO_2H$

3 — $CH_3CH_2CH_2CO_2H$

Biosynthetic Precursors of Lasalocid A (I)

Figure 33. Spectral Results from the Acetate Feedings

a) Summary of $^{13}C/^{2}H$ enrichment from $[2\text{-}^{13}C, 2\text{-}^{2}H]$-acetate feeding. The % ^{13}C enrichment was calculated by the method of McInnes, et al., 1980; the method differs from the "Enrichment Factor" in that it corrects for the contribution of the $^{13}C\text{-}^{2}H$ species to the peak height of the ^{13}C enriched resonance p. 225

b) 30 MHz ^{2}H-NMR Spectra of lasalocid A enriched by $[2\text{-}^{2}H_2]$-acetate. p. 226

c) 100 MHz $^{13}C\{^{1}H\}$-NMR Spectra of lasalocid A enriched by $[2\text{-}^{2}H_2, 2\text{-}^{13}C]$-acetate. The spectra shows the ^{2}H induced -isotope shifts. p. 227

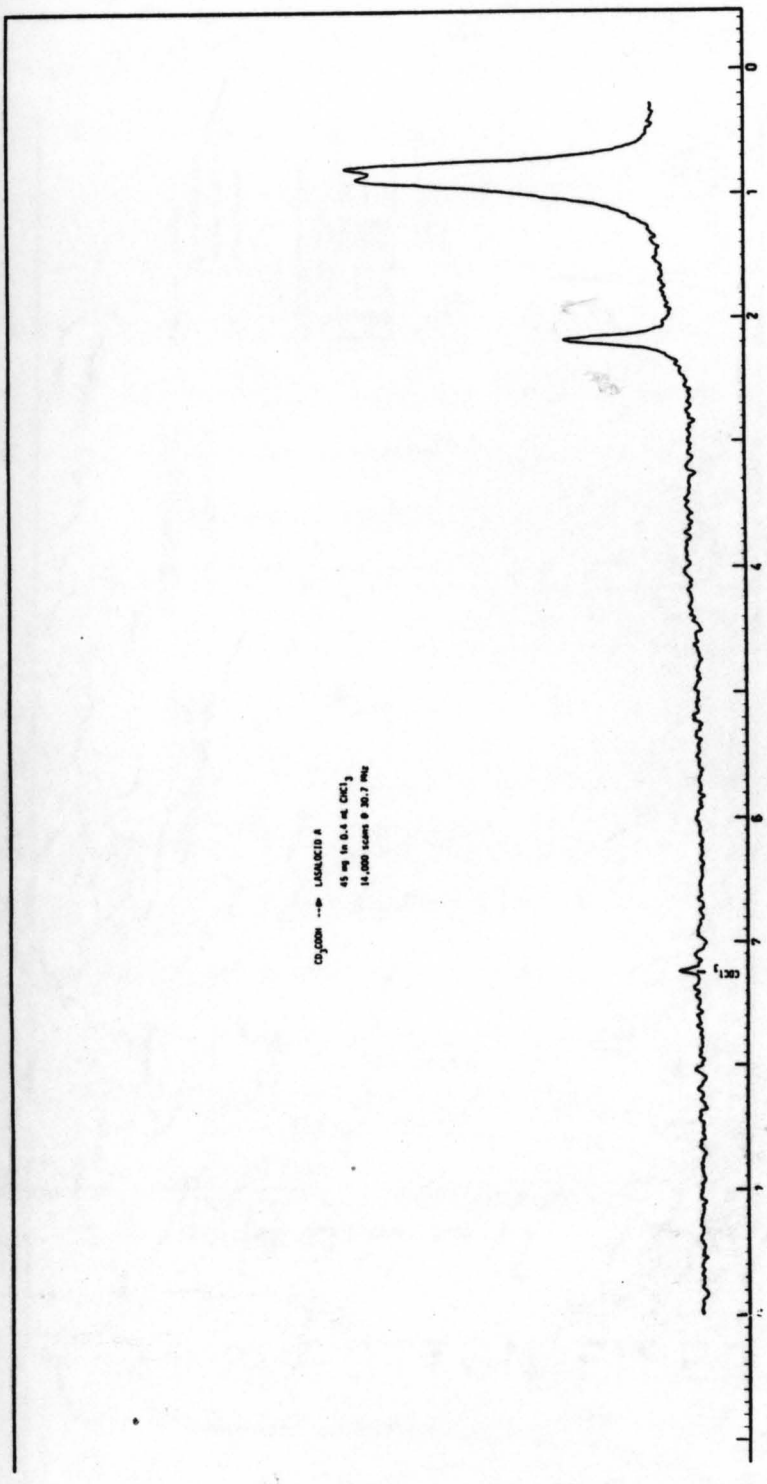
CARBON-13 ENRICHMENTS FOR LABELLING OF LASALOCID A

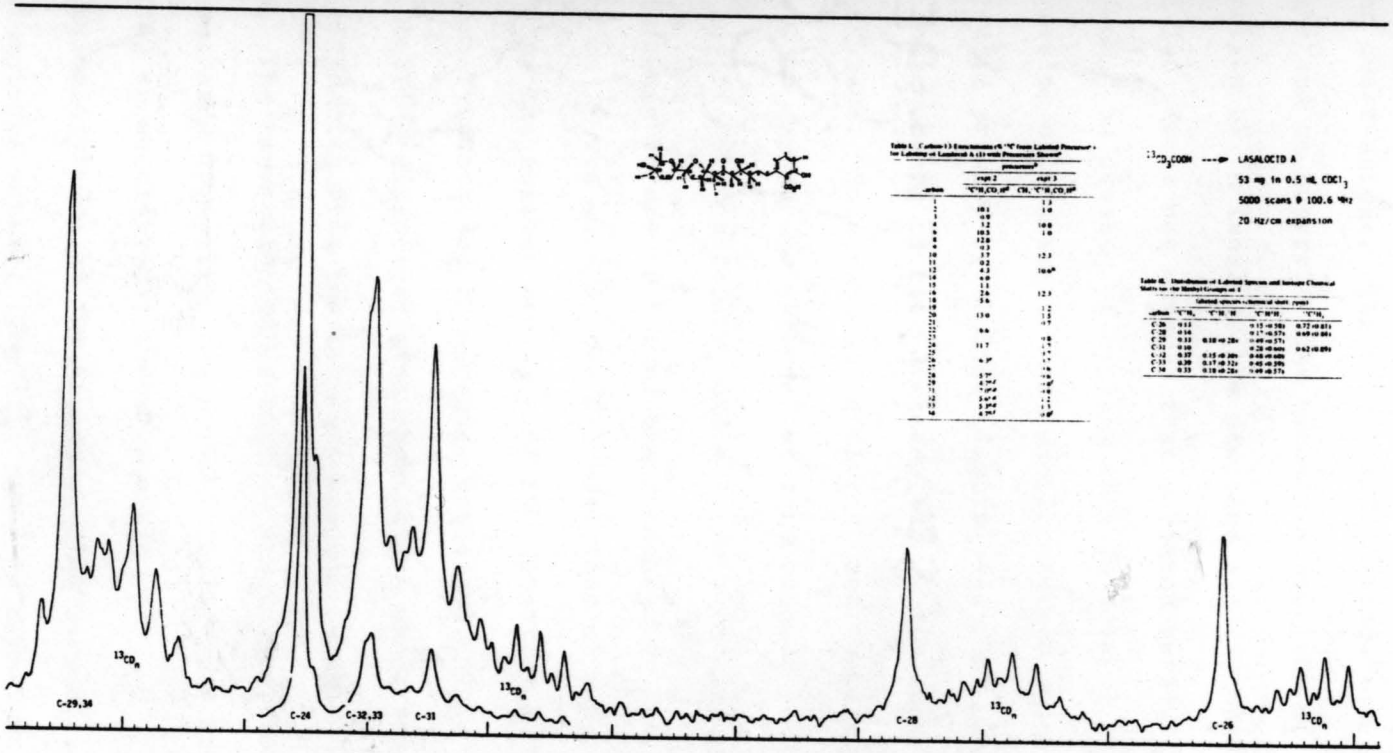
FROM [2-^{13}C, 2-$^{2}H_3$]-ACETATE

CARBON	% ^{13}C ENRICHMENT*
2	10.1
3	0.9
4	3.2
6	10.5
8	12.8
9	0.3
10	3.7
11	0.2
12	4.3
14	6.0
15	1.1
16	2.6
18	5.6
20	13.0
22	6.6
24	11.7
26	6.3
28	5.7
29	4.7
31	7.0
32	5.6
33	5.3
34	4.7

DISTRIBUTION OF LABELLED SPECIES FOR THE METHYL GROUPS

CARBON	($^{1}H_3$)	($^{1}H_2{}^{2}H_1$)	($^{1}H_1{}^{2}H_2$)	($^{2}H_3$)
26	0.13		0.15	0.72
28	0.14		0.17	0.69
29	0.33	0.18	0.49	
31	0.10		0.28	0.62
32	0.37	0.15	0.48	
33	0.39	0.17	0.45	
34	0.33	0.18	0.49	


CO2COOH --> LASALOCID A
45 mg in 0.4 mL CHCl3
14,000 scans @ 30.7 MHz
CHCl3
0
1
2
3
4
5
6
7



$^{13}CD_3COOH$ ⟶ LASALOCID A

53 mg in 0.5 mL $CDCl_3$

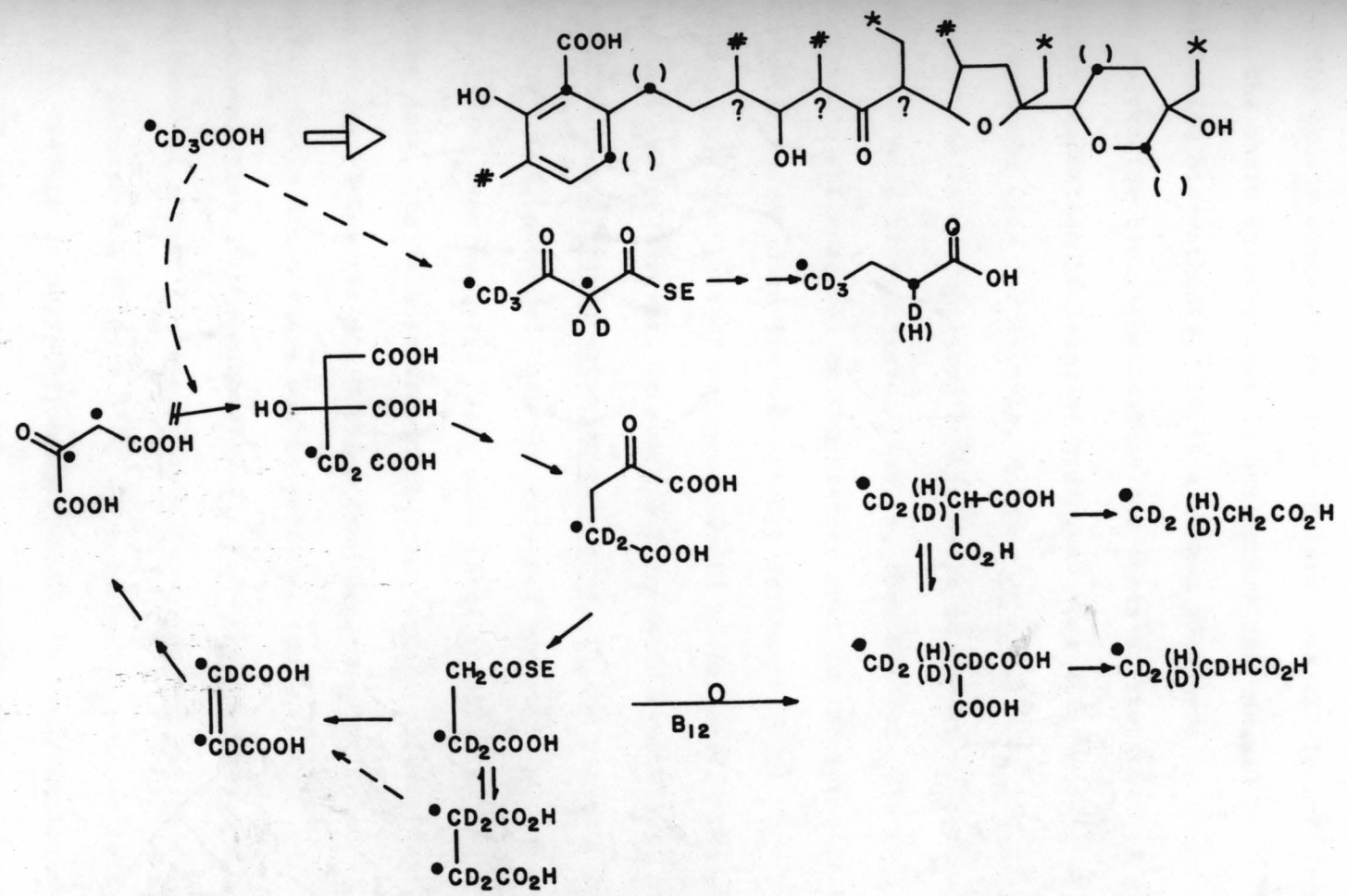
5000 scans @ 100.6 MHz

20 Hz/cm expansion

The results of the acetate incorporation experiments show two important observations. The first is the metabolism of acetate to propionate and to butyrate; the second is the lack of incorporation of ^{2}H label at the proposed starter unit methyl group, C-24. It is not unusual that a common metabolite like acetate would be capable of cycling through several pathways, and the conversion of acetate to both propionate and butyrate are well documented in primary metabolism (Figure 34). Two units of acetate can condense to form acetoacetate, which upon reduction yields butyrate. This pathway is direct, and therefore it is reasonable to expect a high level of retention of the ^{2}H label in the C-4 positions of butyrate, and a lower retention of ^{2}H at C-2 due to exchange before or during the condensation reaction. The fact that no ^{2}H was seen at the C-2 positions of butyrate can be explained by the further exchange of this position as result of the previously described ^{2}H exchange processes. This was observed in the labelling experiment with lasalocid A where the methyl groups originating from the butyrate subunit contained primarily 3 ^{2}H atoms. The conversion of acetate to propionate can be most easily explained by cycling the acetate through the Citric Acid Cycle (TCA) to succinyl-CoA then conversion to (2R)-methylmalonyl-CoA by the B_{12}-containing enzyme, methylmalonyl-CoA mutase. The (2R) and (2S) enantiomers of methylmalonyl-CoA can interconvert by the known epimerase. The loss of ^{2}H atoms from the labelled acetate into propionate can be

rationalized as follows: if the acetate cycles half way through the TCA cycle to succinate, and is then drained off for conversion to methylmalonyl-CoA prior to incorporation into the propionate subunits of lasalocid A, it would be expected that one or more ^{2}H atoms would be lost, depending on the extent of [1,2]-^{2}H shift in the mutase reaction. If on the other hand, the acetate was never converted to the free succinate (i.e. there is no equilibration of label between the C-2 and C-3 positions of succinyl-CoA) or the acetate passes through a complete cycle of the TCA, then it would be expected that all ^{2}H label would be lost from the propionate subunit. Finally, there is always the possiblity that another pathway exists in this microorganism which would serve to convert acetate to propionate in a more direct route. These mechanisms can explain why ^{2}H label was only seen in the methyl groups.

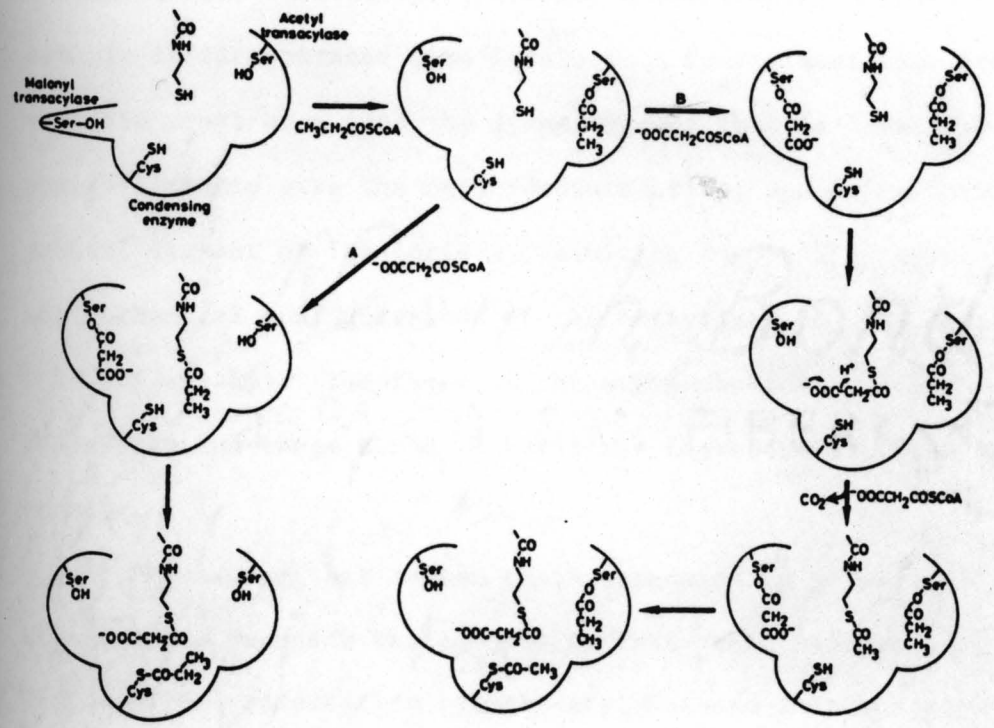
Figure 34. Metabolic Pathways of Acetate Leading to Propionate and Butyrate, and their Enrichment of Lasalocid A.


COOH
HO
OH
O
SE
D D
D
(H)
CD2 COOH
CH2COSE
CDCOOH
CO2H
B12

The second observation, that ^{2}H label was not incorporated into the chain starter unit is unexpected and unusual in polyketide biosynthesis. It is a common phenomena in fatty acid and polyketide biosynthesis that the chain starter unit retains a higher proportion of labelled hydrogens than at any other site in the molecule (see for example, McInnes et al., 1979; 1980). This observation is rationalized on the basis on the known mechanisms for fatty acid biosynthesis; that is, that the molecule of acetyl-CoA which serves as the starter unit is incorporated without conversion to its malonyl-CoA derivative, and consequently, no loss of hydrogens would be expected. There has been one report however, in which a fatty acid synthetase (FAS) isolated from Brevibacterium ammoniagenes, in the presence of propionyl-CoA, produced both the expected odd-numbered fatty acid and also an even-numbered fatty acid (Arai et al., 1982). In the latter case, the authors showed that the actual priming substrate was an acetyl-CoA residue derived from decarboxylation of malonyl-CoA. Since there was no evidence for direct decarboxylation of the malonyl-CoA, the authors proposed a hypothetical scheme for a propionyl-CoA-dependent decarboxylation of the malonyl-CoA (Figure 35). If a mechanism such as this one were to operate in polyether biosynthesis, it would explain the lack of ^{2}H label at the starter unit. The conversion of acetate to malonyl-CoA, via acetyl-CoA carboxylase, requires the loss of one ^{2}H label and the proposed enzyme bound decarboxylation of

malonyl-CoA to the priming substrate also requires the loss of a ^{2}H label. Any further loss of ^{2}H label could be rationalized by a second round of the interconversion of acetyl-CoA and malonyl-CoA. Whether or not this mechanism is a reasonable one for polyether antibiotic biosynthesis, the complete loss of ^{2}H in the starter unit in lasalocid A would suggest that some sort of priming reaction occurs. The observation that lasalocid A did not retain ^{2}H at the methyl of the starter unit was the first reported case in polyketide biosynthesis (Hutchinson et al, 1981) and while this is not a common phenomena for polyketides, it has recently been reported for another polyether antibiotic (Doddrell et al., 1984) as well as for the polyether antibiotic, salinomycin (H. Seto, personal communications).

Figure 35. Hypothetical Scheme for a Propionyl-CoA Dependent Decarboxylation of MalonylCoA in Fatty Acid Biosynthesis.



Path A: formation of heptadecanoic acid in vitro by B. ammoniagenes fatty acid synthetase.

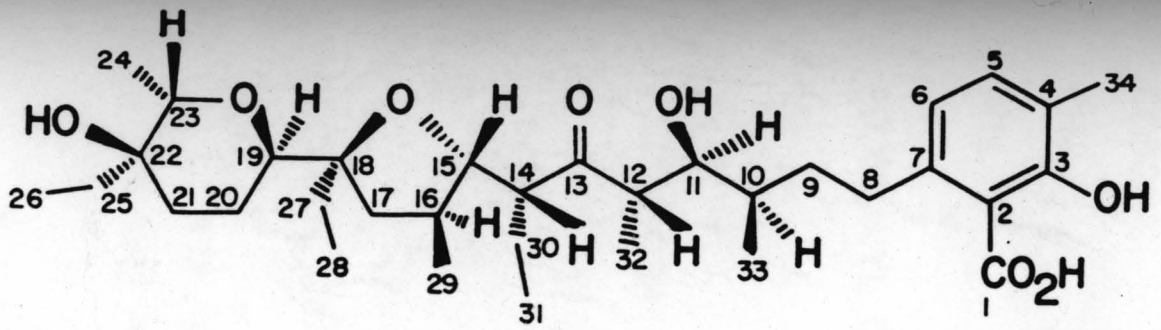
Path B: formation of stearic and oleic acids in same system.

(K. Arai, A. Kawaguchi, Y. Saito, N. Koike, Y. Seyama, T. Yamakawa and S. Okuda, J. Biochem. 1982, 91, 11.)

The investigation of the three carbon subunit precursor of lasalocid A was approached by analogous studies of both propionate and succinate. According to Westley's hypothesis, a three carbon subunit is incorporated into lasalocid A four times: once into the aromatic ring; once into the diene segment that is later oxidized and cyclized to give the tetahydrofuran ring; and twice into the central segment of lasalocid A, resulting in opposite absolute stereochemical configurations of the methylines at C-10 and at C-12 (Figure 36). The focus of the stereochemical studies concerning the three carbon subunit was therefore on these two centers.

It is assumed that carbon chain extension in polyketide biosynthesis proceeds analogously to fatty acid biosynthesis by a Claisen-type condensation between acyl-CoA and α-carboxyacyl-CoA esters. In the case of the three carbon subunit, the activated ester is methylmalonyl-CoA. Methylmalonyl-CoA has been studied exhaustively (see for example: Allen et al., 1964; Retey, 1982) and is known to be the common intermediate in the interconversion of propionate and succinate, where the reaction proceeds stereospecifically (Figure 37). Propionyl-CoA is carboxylated by propionyl-CoA carboxylase with retention of configuration at C-2, when viewed by isotopic labelling, to give (S)-methylmalonyl-CoA, while succinyl-CoA undergoes a B_{12} catalyzed rearrangement reaction to give (R)-methylmalonyl-CoA; the two enantiomers are interconverted by means of an epimerase. Therefore the

investigations of the three carbon subunit proceeded by studying the incorporation of an intact ^{13}C-^{2}H unit from propionate *versus* that from succinate in an attempt to determine the mechanism of stereocontrolled chain extension.



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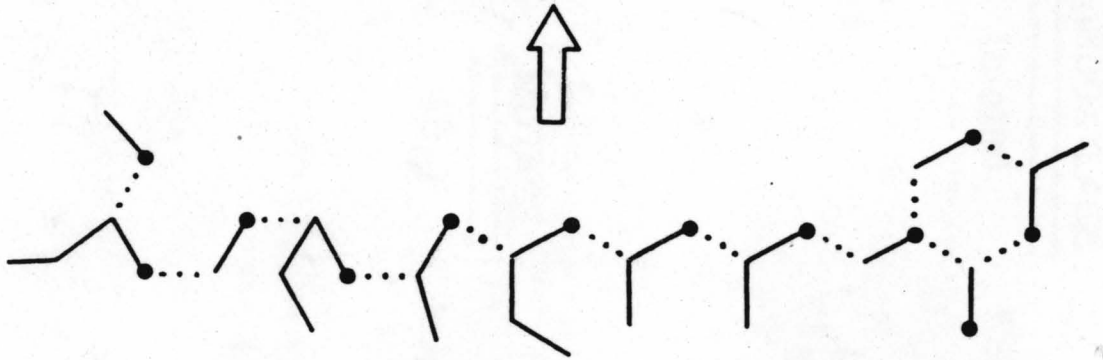
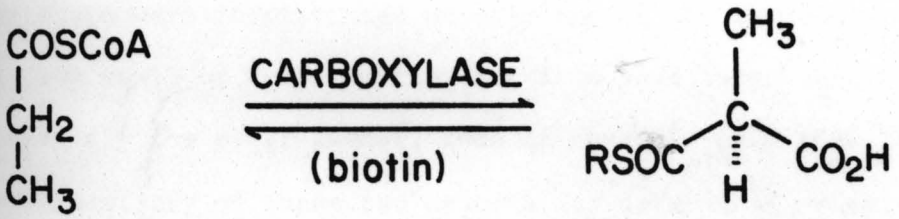


5 — CH_3CO_2H

4 — $CH_3CH_2CO_2H$

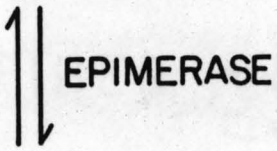
3 — $CH_3CH_2CH_2CO_2H$

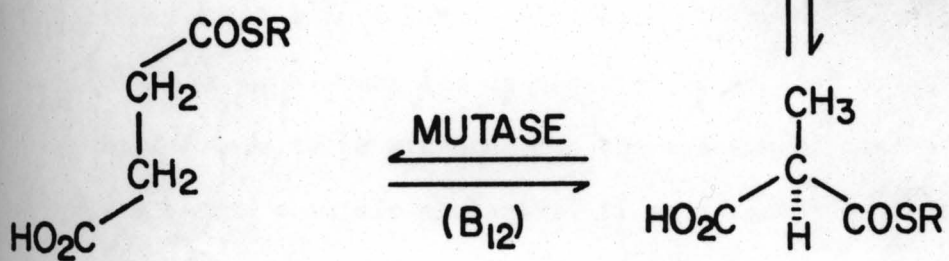
Biosynthetic Precursors of Lasalocid A (I)



PROPIONYL CoA

(2S) METHYL
MALONYL CoA





SUCCINYL CoA

(2R) METHYL
MALONYL CoA

The rationale for the experiments is as follows, where a racemization versus an epimerization is as defined by Walsh (1979). In the first mechanism, if an intact ^{13}C-^{2}H unit from propionate were incorporated at only one of the two centers in question (C-10 or C-12), while that from succinate labelled exclusively the other center, then it could be said that the stereochemistry of these two centers was determined prior to the condensation reaction. This could be possible if more than one condensing enzyme was involved, each capable of accepting only one enantiomer, or if the condensing enzyme itself could act on either enantiomer, but a loading reaction which is sterospecific determines which enantiomer is accepted. In both cases there would have to be a means for distinguishing in what order the enantiomers were to be accepted for the subsequent condensation.

In a second possible mechanism, if an intact ^{13}C-^{2}H from propionate, as well as from succinate, labelled both centers (or conversely, if the intact label was lost from both substrates), then it could be said that the stereospecificity of the reaction is determined at the time of condensation. It is possible that the condensing enzyme could accept either the R or the S enantiomer of methylmalonyl-CoA and then racemize the center just prior to condensation while the substrate is enzyme bound. The racemization would have to occur regardless of the enantiomer accepted for the condensation reaction, and would imply that the sequence of stereospecificity is determined by the order of

specific racemizations. If the racemization were to occur via proton abstraction in a single-base mechanism or if proton transfer was relatively fast and consequently did not allow for isotopic exchange with solvent, then the result would be an intact ^{13}C-^{2}H unit from both propionate and succinate. On the other hand, the racemization could occur via a two-base site mechanism, or proceed relatively slow and therefore allow for equilibration with solvent proton, both of which would result in loss of the intact ^{13}C-^{2}H unit. A parallel mechanism exists in which the stereospecificity is determined at the time of the condensation reaction. However, in this case the condensing enzyme accepts only one enantiomer of methylmalonyl-CoA, and the racemization occurs just prior to the condensation at a specified point in the carbon chain extension process. The results of this mechanism would be indistinguishable from the third mechanism (see below) in a whole-cell study.

The third mechanism implies that only one enantiomer of methylmalonyl-CoA is accepted for the condensation reaction, which proceeds like fatty acid biosynthesis to produce a stereochemically uniform molecule. Then later a regiospecific epimerase acts at certain centers to produce the opposite stereochemistry. The results of this mechanism would be that one substrate would lose all ^{2}H prior to condensation via the known epimerization of R and S methylmalonyl-CoA, while the other

substrate would retain the intact ^{13}C-^{2}H unit at only one of the two centers.

The studies with propionate as the subunit were approached with two different experimental methods. In the first experiment, [2-^{13}C, 2-$^{2}H_2$]-propionate was fed to S. lasaliensis, and the isolated lasalocid A was analyzed by $^{13}C\{^{1}H\}$-NMR (100 MHz) and $^{13}C\{^{1}H,^{2}H\}$-NMR (25.1 MHz). The results are given in Figure 38. The data from the Triple Resonance experiment were analyzed in the same manner as for the previously described acetate experiment. The results showed that C-4, C-10, C-12 and C-16 were enriched with ^{13}C approximately 10 fold over any other site. The only site in the molecule labeled with ^{2}H was C-12, which was enriched by 10.6% (+/- 2.8%). There are three other possible sites for ^{2}H enrichment from the propionate precursor: C-4, which would lose the ^{2}H upon aromatization of the ring; C-16, which would not be expected to retain ^{2}H if the molecule undergoes dehydration to the putative diene intermediate; and C-10, which is the center of opposite absolute stereochemistry. In order to verify these results, the experiment was repeated, and the results from the Triple Resonance experiment show 16% ^{2}H retention at C-12, no ^{2}H retention at C-10 and approximately 8% ^{13}C enrichment at the four major carbons (C-4, C-10, C-12 and C-16). The results were confirmed by high-resolution MS analysis of the m/e 57 peak which verified the presence of an ion containing ^{13}C and ^{2}H.

Figure 38. NMR Spectral Results of Lasalocid A Labelled by [2-^{13}C, 2-$^{2}H_2$]-Propionate

CARBON-13 ENRICHMENTS FOR LABELLING OF LASALOCID A

FROM [2-^{13}C, 2-$^{2}H_2$]-PROPIONATE

CARBON	% ^{13}C ENRICHMENT
1	1.3
2	1.0
4	10.8
6	1.0
10	12.3
12	10.6
16	12.3
19	1.2
20	1.5
21	0.7
23	0.8
24	1.7
25	0.7
26	0.7
27	0.6
28	0.6
29	0.8
31	0.8
32	1.2
33	1.3
34	0.8

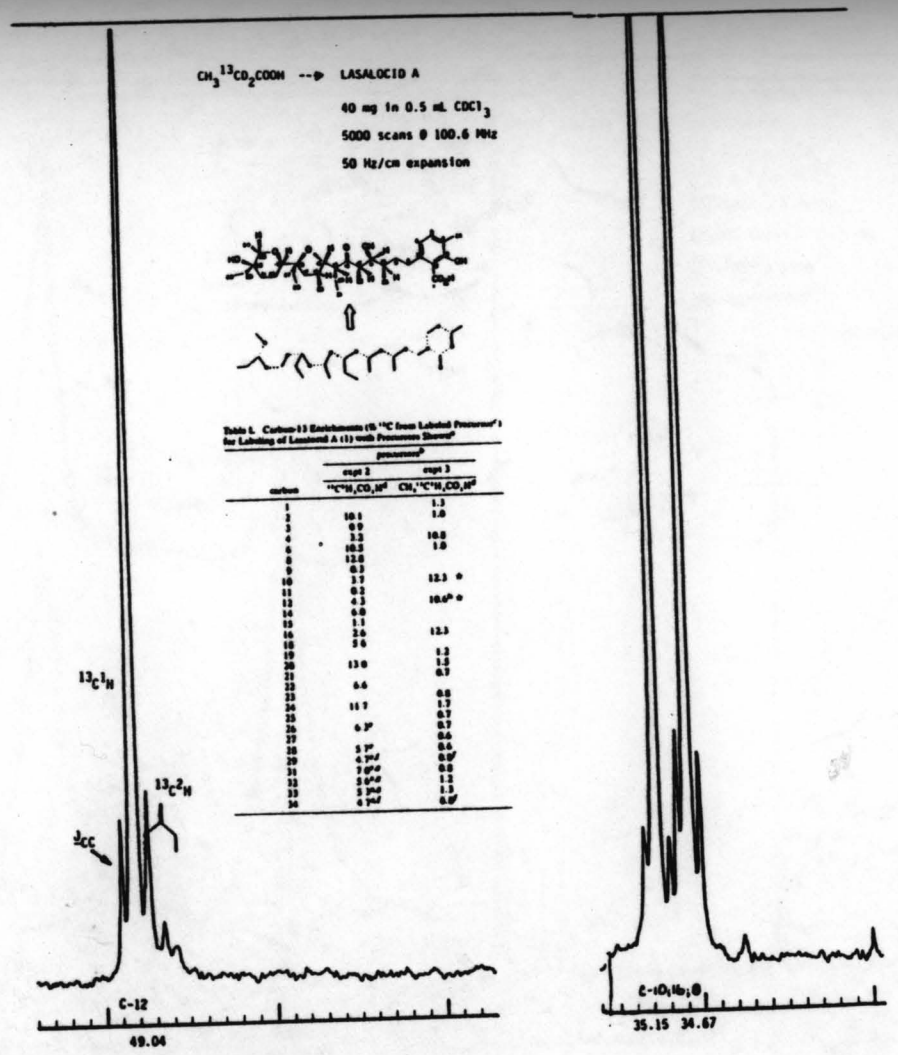
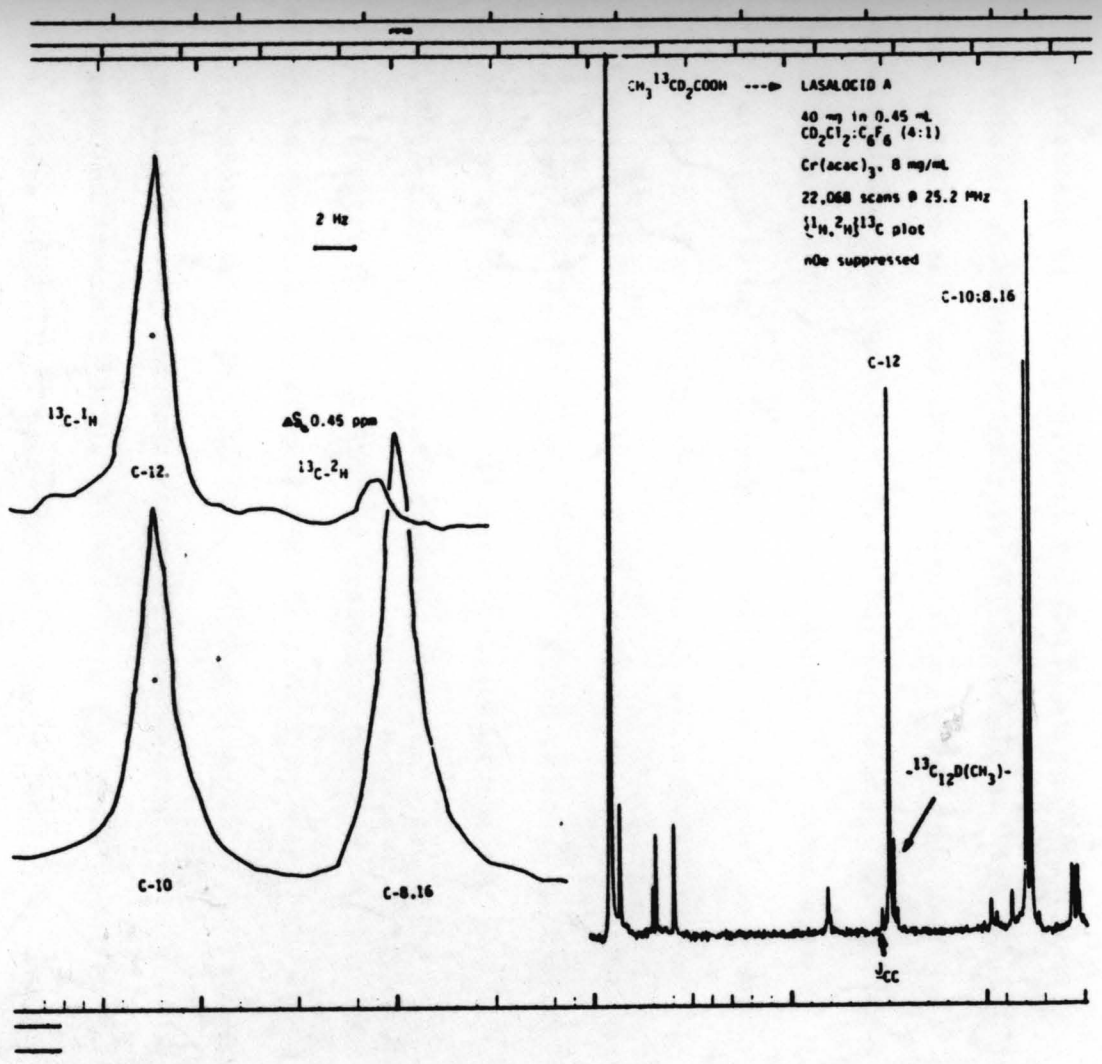


Table I. Carbon-13 Enrichments (% ^{13}C from Labeled Precursor) for Labeling of Lasalocid A (1) with Propionate Shown[a]

	precursor[b]	
	expt 2	expt 3
carbon	$^{13}CH_3CD_2CO_2H$	$CH_3{}^{13}CH_2CO_2H$
1		1.3
2	10.1	1.0
3	0.0	
4	1.2	10.8
5	10.2	1.0
6	12.0	
7	0.2	
8	1.7	12.3 *
9	0.2	
10	4.3	10.6[c] *
11	0.0	
12	1.1	
13	2.6	12.3
14	2.6	
15		1.2
16	13.0	1.2
17		0.7
18	6.6	
19		0.8
20	11.7	1.7
21		0.7
22	6.3[c]	0.7
23		0.6
24	3.3[c]	0.6
25	4.3[c,d]	0.8[d]
26	3.9[c,d]	0.8
27	3.6[c,d]	1.3
28	3.7[c,d]	1.3
29	4.2[d]	0.8[d]


ppm
$CH_3{}^{13}CD_2COOH$ ---▶ LASALOCID A
40 mg in 0.45 mL
$CD_2Cl_2:C_6F_6$ (4:1)
$Cr(acac)_3$, 8 mg/mL
22,068 scans @ 25.2 MHz
$\{^1H,^2H\}^{13}C$ plot
nOe suppressed
C-10:8,16
C-12
2 Hz
$^{13}C-^1H$
$\Delta\delta_0$ 0.45 ppm
$^{13}C-^2H$
C-12
C-10
C-8,16
$-^{13}C_{12}D(CH_3)-$
$^1J_{CC}$

The second approach was to look for a ^{2}H induced β-shift in the ^{13}C-NMR spectra. Initially, the experiment was done with [1-^{13}C, 2-$^{2}H_2$]-propionate. The ^{13}C-NMR spectral results showed ^{13}C enrichment at C-3, C-9, C-11 and C-15, about 3 fold greater than natural abundance. Analysis of the sample at high-field (125 MHz) failed to show any shifted resonances. The subsequent experiment was done with [3-^{13}C, 2-$^{2}H_2$]-propionate. In this case the ^{13}C-NMR (50 MHz) spectral results showed ^{13}C enrichment of C-34, C-33, C-32 and C-29 about 18 fold above natural abundance (Figure 39). The ^{13}C-NMR spectra also showed a β-shifted resonance for C-32 but not for C-33, corresponding to a deuterium being present at C-12 but not at C-10, which is consistent with the results of the Triple Resonance analysis of the two [2-^{13}C, 2-$^{2}H_2$]-propionate feeding experiments. The analysis of the ^{13}C-NMR spectra for the β-shifted resonance was done in the same manner as described previously for the model compound. The results showed an upfield shift of 0.098 ppm, which is similar to the model compound, and consistent with the literature values (Simpson and Stenzel, 1982). By measuring the areas under the peaks, there was 9.2% ^{2}H retention at C-12. It should be noted that the ^{2}H induced β-shift of the methyl carbon resonance in this labelling experiment was larger than what was seen for the model compound. In the case of the model compound, the ^{2}H is adjacent to a carbonyl which could result in a smaller isotope shift due to incomplete relaxation of the carbonyl carbon.

It also appears as if the [3-^{13}C]-propionate had been metabolized so as to introduce ^{13}C into other positions, in particular, into the C-4 and the C-2 positions of butyrate (corresponding to C-26, C-28, C-31, C-22, C-18 and C-14; the enrichments at C-22 and C-31 are not readily obvious, since these resonances are not well resolved in this spectral analysis) and into both positions of acetate (corresponding to: C-1/C-2, C-5/C-6, C-7/C-8, C-19/C-20, C-23/C-24). The metabolism of [3-^{13}C]-propionate into [4-^{13}C]- or [2-^{13}C]-butyrate or [2-^{13}C]-acetate is easy to rationalize. As it is already known that propionate and succinate readily interconvert, this would give succinate labelled at C-2 or C-3, which could be metabolized to [2-^{13}C]-acetate by partial reversal of the TCA induced by an accumulation of succinate, or by conversion of succinate to oxaloacetate, thence to pyruvate and acetate. The [4-^{13}C]- or [2-^{13}C]-butyrate could then be generated by condensation of the labelled acetate with unlabelled acetate to give acetoacetate and then butyrate. It should be noted however, that the enrichment at these other sites occurs at one-fifth to one-ninth of that at the positions corresponding to the C-3 position of propionate; or in other words, these pathways are operating at about one-seventh the efficiency of the main metabolic route.

Figure 39. NMR Spectral Results of Lasalocid A Labelled by [3-^{13}C, 2-$^{2}H_2$]-Propionate

a) Carbon-13 Enrichments of Lasalocid A p. 251

b) $^{13}C\{^{1}H\}$-NMR (50 MHz) Spectra of Lasalocid A - Full Spectra p. 252

c) Expansion at 20 Hz/cm of 12-18 ppm p. 253

d) Expansion at 5 Hz/cm of 13-14.8 ppm p. 254
C-24 is at 14.418 ppm
C-33 is at 13.982 ppm
C-32 is at 13.607 ppm *
C-31 is at 13.353 ppm

*β-shifted resonance at 0.098 ppm upfield

CARBON-13 ENRICHMENTS FOR LABELLING OF LASALOCID A

FROM [3-^{13}C, 2-$^{2}H_2$]-PROPIONATE

CARBON	ENRICHMENT FACTOR
1	3.6
2	4.8
3	1.9
4	1.6
5	2.1
6	1.3
7	2.8
8	3.3
9	1.7
10	1.8
11	1.6
12	1.8
13	1.0
14	2.5
15	1.8
16	3.3
17	1.3
18	2.1
19	2.9
20	3.2
21	1.2
23	2.2
24	3.4
25	0.9
26	2.6
27	0.9
28	3.5
29	20.5
30	1.7
32	16.9
33	17.8
34	16.7

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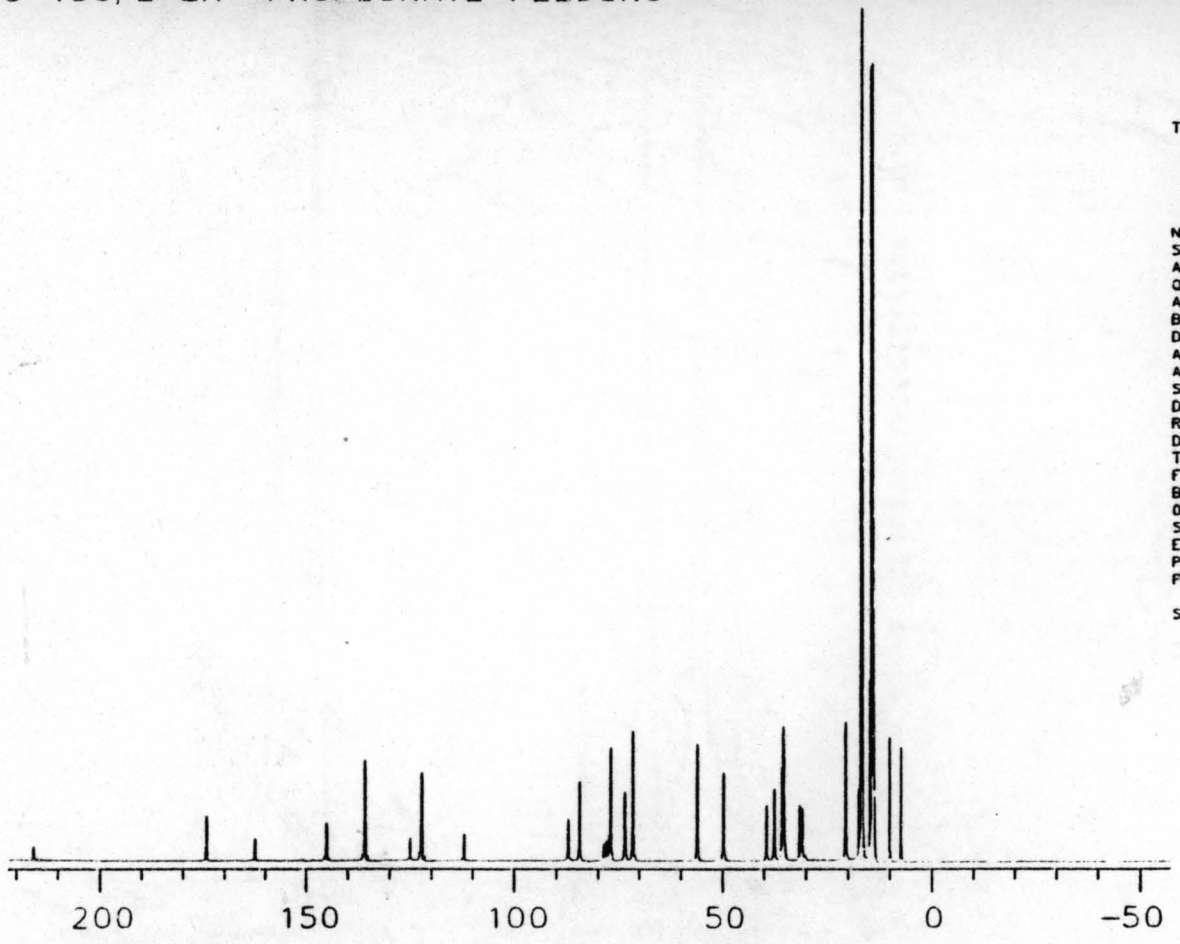
3-13C, 2-2H PROPIONATE FEEDING



TWO-LEVEL DECOUPLING

P2= 8.00 USEC
D3= 1.00 MSEC
D5= 1.00 SEC

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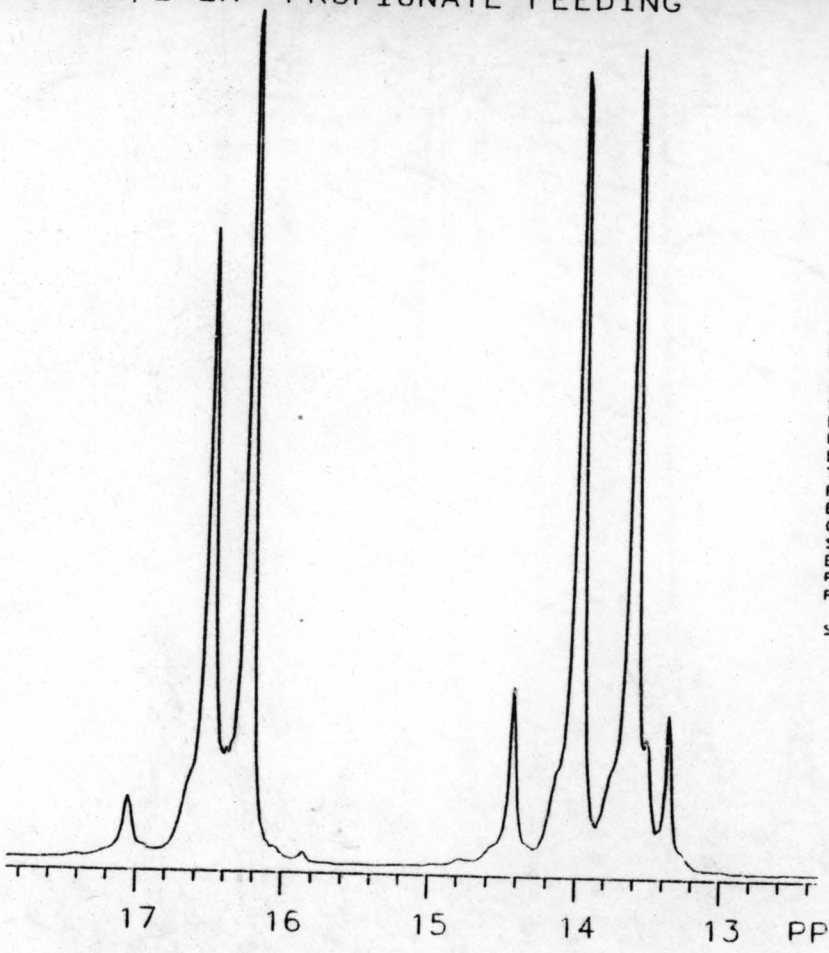

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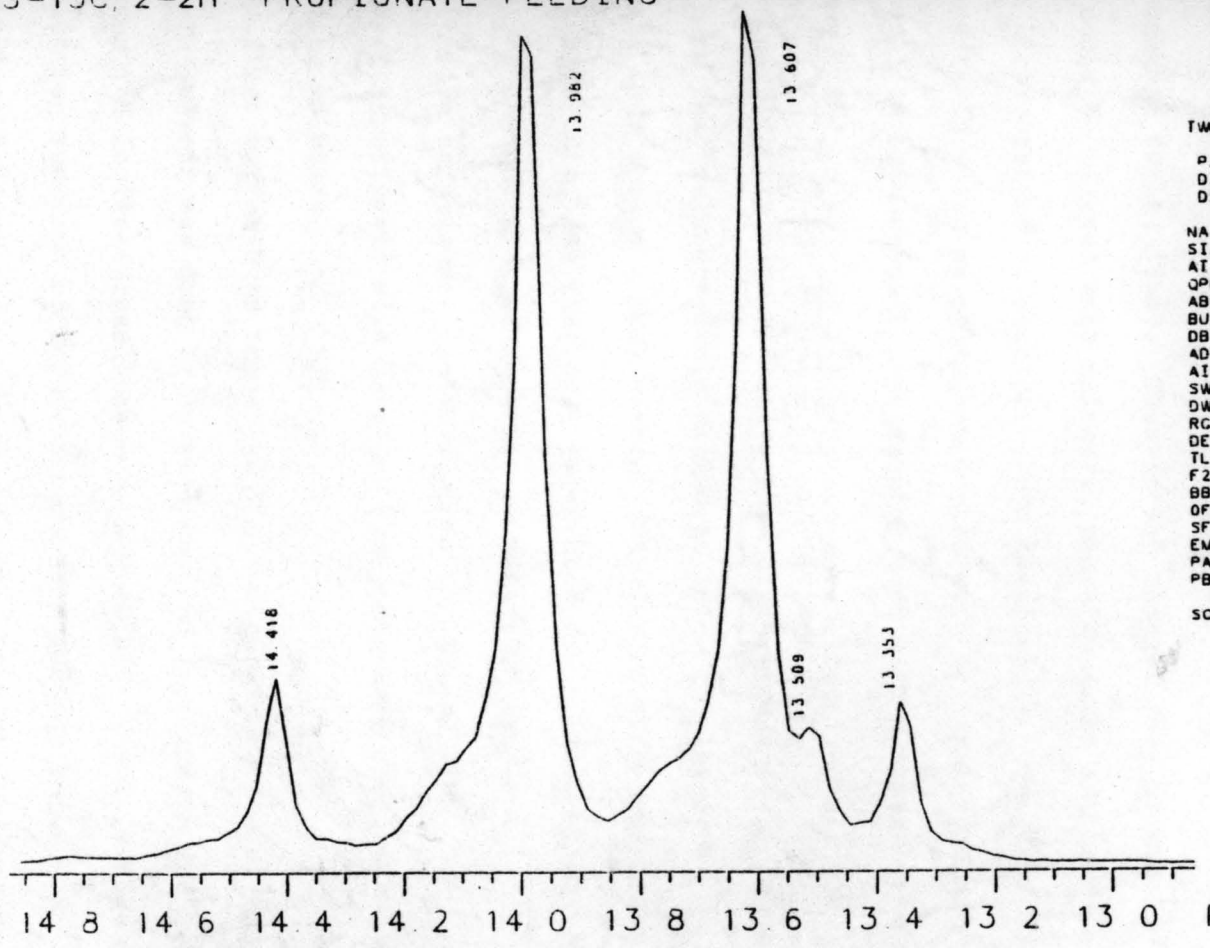

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The experiments with succinate as a precursor were approached by three different experimental techniques and involved considerable background work in order to establish the feeding conditions. The initial attempts with succinate feedings yielded low values for its specific incorporation into lasalocid A. It is known that malonate is a specific inhibitor of succinic dehydrogenase (Webb, 1966). Thus it was thought that if the TCA cycle could be blocked with malonate, there would be an increase in the incorporation of succinate into lasalocid A. Feeding experiments in which malonate was co-fed with succinate proved successful in this respect. Subsequent work with malonate showed that the disodium salt of malonate was more effective than the free acid which was more effective than diethyl malonate. It was also shown that malonate could be fed at twice the levels of succinate (5mM versus 2.5mM) without interfering with production of the antibiotic, and that this level produced a block of the TCA, since the addition of fumarate or malate at the same concentration as malonate could overcome the block. The subsequent experiments with labelled succinate were carried out by co-feeding malonate.

The first approach to investigating the incorporation of the succinate subunit was done by feeding [2,3-$^{2}H_{4}$]-succinate and analyzing the isolated lasalocid A by high-field ^{2}H-NMR (90 MHz). The resulting spectrum of sodium lasalocid A ($CDCl_{3}$) showed ^{2}H enrichment at 2.2 ppm and at about 0.9 ppm. Since the hydrogens

at C-10 and C-34 would be expected to overlap in ^{2}H-NMR under these conditions (the ^{1}H-NMR assignments are: 2.20 ppm for C-34 and 2.14 ppm for C-10; C-12 is distinct at 2.82 ppm), the spectrum was run again using C_6D_6 as the solvent, where the expected resonances would be at 2.47 ppm for C-34 and 2.09 for C-10, and C-12 at 2.80 ppm. The resulting spectrum showed two resonances: one at 2.53 ppm and the other at 2.14 ppm, however, upon further investigation a solvent impurity was found at 2.10 ppm. In a repeat of the feeding experiment, the lasalocid A was isolated as the free acid and again analyzed by high-field ^{2}H-NMR. The sample was run using C_6D_6 as the solvent, where in this case the expected resonances would be at 2.80 for C-12, 1.87 for C-10 and 2.29 for C-34. The resulting spectra showed ^{2}H at about 0.9 ppm and 2.2 ppm, which can be explained by enrichment at the methyl groups, as well as ^{2}H at about 1.3 ppm, which is unassignable in the lasalocid A spectrum. An independent check of the solvent showed no impurites in the ^{2}H-NMR spectra. The results from this approach suggest that there is no ^{2}H enrichment at the C-10 and C-12 methine centers.

The second approach was to feed [1,4-$^{13}C_2$, 2,3-$^{2}H_4$]-succinate to S. lasaliensis and analyze the isolated lasalocid A by high-field ^{13}C-NMR for any ^{2}H induced β-shifted ^{13}C resonances. In the first of these experiments, the isolated lasalocid A showed ^{13}C enrichment only at C-3, C-9, C-11 and C-15, the four carbons arising from the carboxyl of the three carbon precursor. All

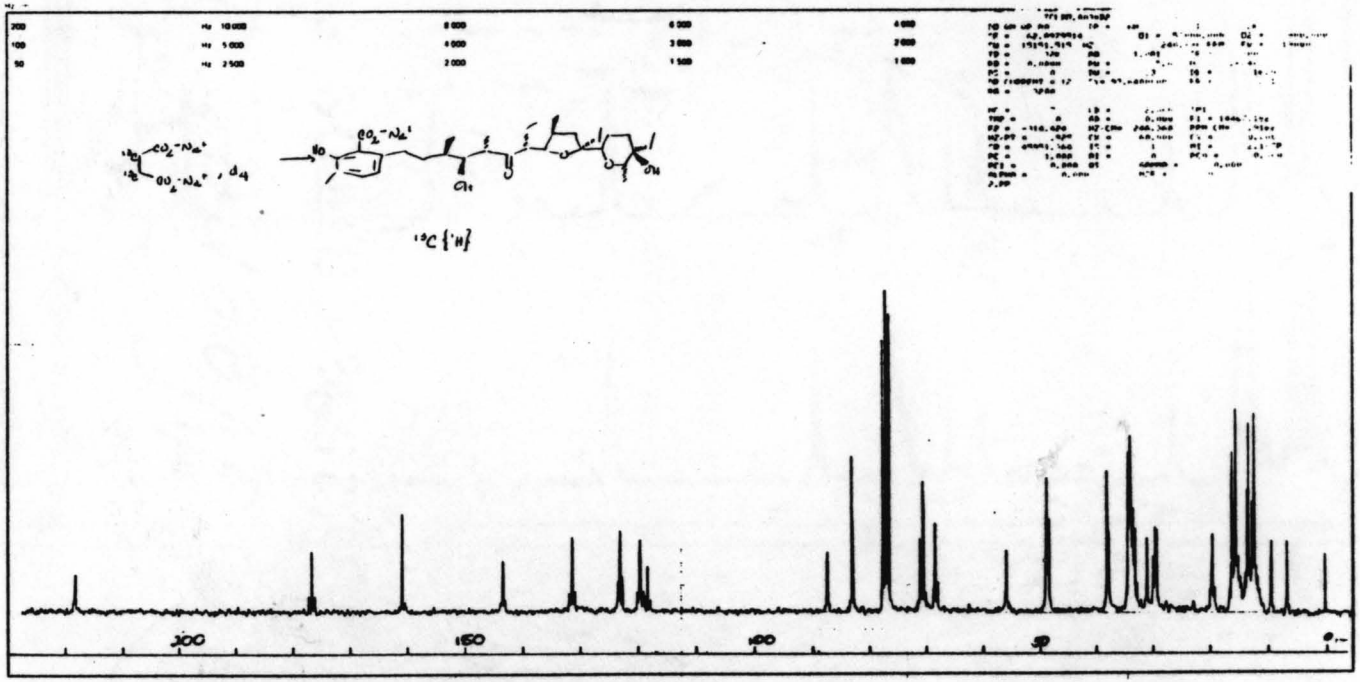
attempts to locate any β-shifted ^{13}C resonances failed. In the second experiment, the same precursor was fed, but at more frequent intervals. This was done in order to build up the available pools of labelled succinate, with the idea that it might increase the incorporation of succinate into lasalocid A. The resulting ^{13}C-NMR showed a low-level ^{13}C enrichment at all carbons arising from the carboxyl of their respective subunits; very similar to the results of the [^{13}C]-$NaHCO_3$ feeding experiment. Again, the results from this method suggest that no ^{2}H was retained at any of the methylene centers.

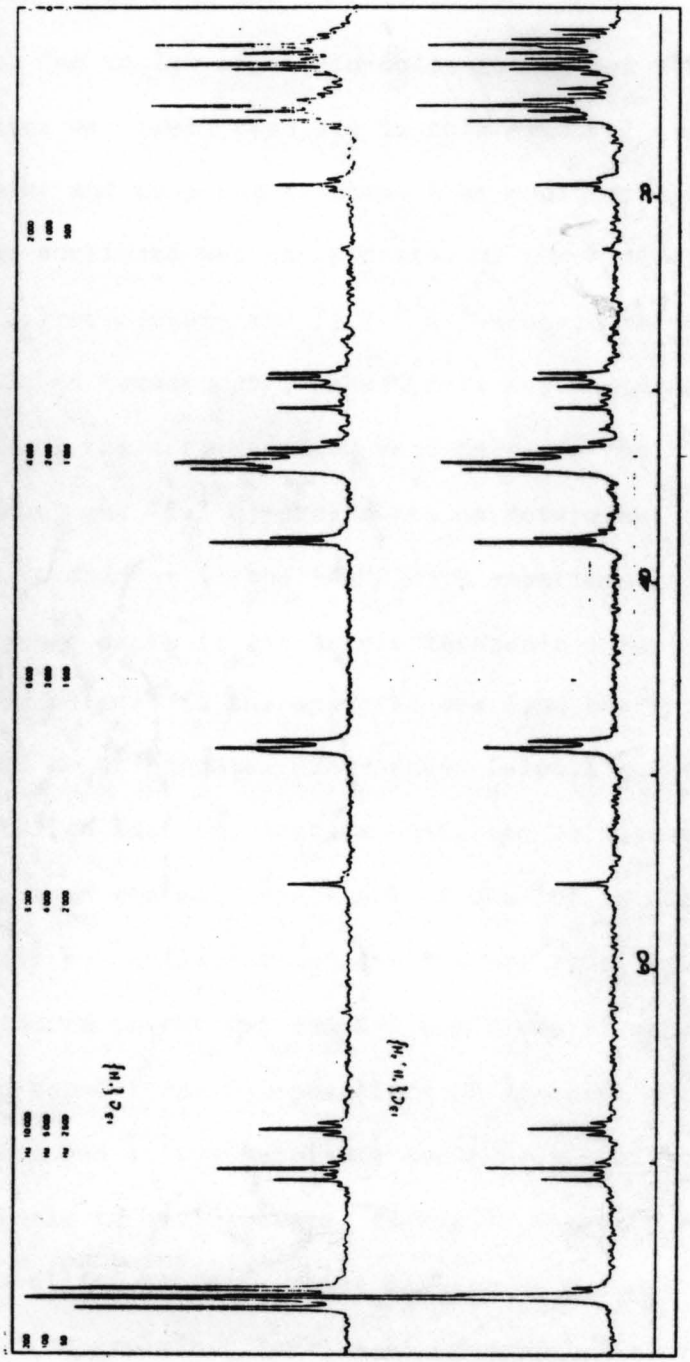
The third experimental approach used in studying the incorporation of succinate into lasalocid A involved two different experiments which were to be analyzed by the Triple Resonance experiment. In the first, [2,3-$^{13}C_2$, 2,3-$^{2}H_2$]-succinate was fed and the isolated lasalocid A was analyzed by ^{13}C-NMR spectroscopy using broadband decoupling, the Triple Resonance experiment and the INADEQUATE pulse sequence. The results of the Triple Resonance spectra showed ^{2}H incorporation for only the methyl groups; there was no ^{2}H at either C-10 or C-12 (Figure 40). The data from this NMR spectra also indicated that several of the carbons were enriched and ^{13}C coupled, more than what would be expected from direct incorporation of succinate into lasalocid A.

Figure 40. NMR Spectral Results of Lasalocid A Labelled by [2,3-$^{13}C_2$, 2,3-$^{2}H_4$]-Succinate

a) $^{13}C\{^{1}H\}$-NMR (67 MHz) Spectra p. 259

b) $^{13}C\{^{1}H\}$-NMR and $^{13}C\{^{1}H,^{2}H\}$-NMR Spectra - Expansion of 10-80 ppm p. 260





The coupling patterns observed above were for the most part confirmed by running the INADEQUATE experiment (^{13}C-NMR, 50 MHz); however, due to low signal-to-noise ratio, not all of the resonances were well resolved in this spectral experiment. The enrichment and coupling patterns that could be discerned suggested that the succinate was incorporated as three distinct units: [2,3-$^{13}C_2$]-propionate and [1,2-$^{13}C_2$]-acetate as determined by the ^{13}C enriched resonances in the Triple Resonance experiment and the presence of the corresponding resonances in the INADEQUATE experiment; and [1-^{13}C]-propionate as determined by the lack of coupled resonances in the INADEQUATE experiment corresponding to the enriched peaks in the Triple Resonance experiment. The first labelling pattern is the expected one from the interconversion of succinate to propionate. The second labelling pattern, incorporation into the acetate unit, can be rationalized by assuming that the malonate block of the TCA is only partial, and that after one cycle through the TCA the label can be redistributed to the C-1 and C-2 positions of succinate, and from there to the C-1 and C-2 positions of acetate, either by reversal of the TCA due to the succinate accumulation or by the conversion of succinate to oxaloacetate, thence to pyruvate and acetate. The third labelling pattern, which results in a single label, is more difficult to explain. Because of the complex enrichment and coupling patterns that resulted from this experiment, the second experiment was designed in order to simplify the results. [2-^{13}C,

2-$^{2}H_{2}$]-diethyl succinate was fed to S. lasaliensis and the isolated lasalocid A analyze by low-field ^{13}C-NMR (22.5 MHz). The results of the NMR spectra showed that little or no ^{13}C had been incorporated. Apparently the organism could not take up the diethyl ester of succinate, even though the incorporation of the diethyl succinate has been observed in other cases, including the polyether antibiotic monensin A (D.E. Cane, personal communication).

Overall, the results of the succinate experiments, while not always as clear cut as the propionate experiments, show that no ^{2}H is retained at the methine centers. These results and those from the propionate experiments suggest that the mechanism for stereocontrolled carbon chain assembly of lasalocid A proceeds as follows. Only the 2S enantiomer of methylmalonyl-CoA is used as the substrate by the condensing enzyme, and then one of two things occurs; either a racemization occurs at the site of the condensation in which the proton transfer involves exchange with solvent protons, or a regiospecific epimerization occurs after the carbon chain has been formed, where again, the proton transfer would have to involve exchange with solvent protons. These two mechanisms cannot be distinguished at the whole-cell level. It should be noted that similar results for the polyether antibiotic monensin A have recently been reported (Sood et al., 1984). The authors looked at the incorporation of [2-$^{2}H_{2}$]-, (S)-[2-$^{2}H_{1}$]-, and (R)-[2-$^{2}H_{1}$]-propionate into monensin A and found that in the first

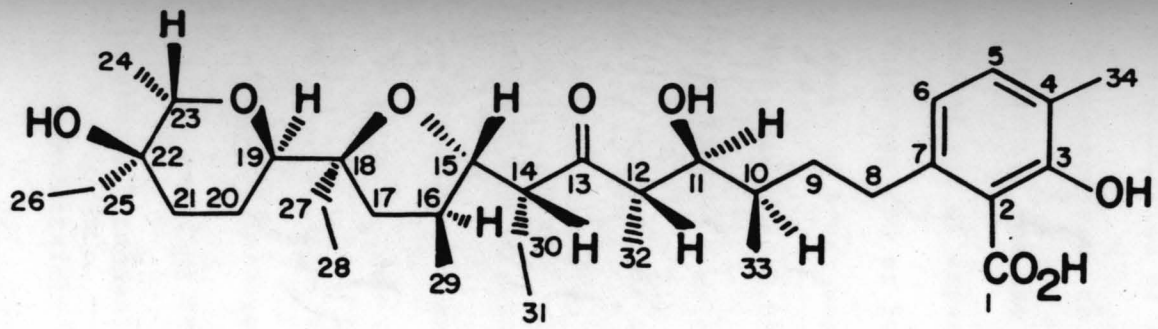
two cases, the ^{2}H label was retained at the methine centers in question, while no ^{2}H was retained from the 2(R) enantiomer as expected due to loss of the ^{2}H from the 2 pro R position during carboxylation of propionate.

The investigation of the four carbon subunit of lasalocid A was done by using butyrate as the precursor. Lasalocid A was one of the first secondary metabolites reported to incorporate an intact butyrate unit (Westley et al., 1972). It is presumed that the butyrate is incorporated into the carbon chain via the activated ester, 2-ethylmalonyl-CoA. Unlike the well studied methylmalonyl-CoA, not as much is known about the ethyl analogue; the absolute configuration of ethylmalonyl-CoA is unknown and there is no definitive evidence for the corresponding butyrylCoA-carboxylase or epimerase. However, it is known that propionylCoA-carboxylase will convert butyryl-CoA to ethylmalonyl-CoA at a reduced rate (see for example: Tietz and Ochoa, 1959; Kaziro et al, 1961; Halenz et al, 1962) and that methylmalonyl-CoA mutase will use ethylmalonyl-CoA as a substrate giving predominantly (2R)-methylsuccinyl-CoA as the product (Retey et al., 1978). Based on this evidence it is reasonable to assume that butyrate is metabolized similar to propionate, that is, butyryl-CoA is carboxylated to give (2S)-ethylmalonyl-CoA, which can be epimerized to the (2R) enantiomer; and that the carbon chain extension process of lasalocid A proceeds the same for the butyrate subunit as for the propionate subunit. With this

background, the rationale for the stereocontrolled incorporation of the butyrate subunit is the same as previously described for propionate. It would therefore be expected that the incorporation of labelled butyrate would proceed with retention of the ^{2}H label only at C-14 of lasalocid A. This is the only carbon arising from C-2 of butyrate that could retain a ^{2}H label, since the other two positions are tetrasubstituted, and this center has the same absolute stereochemistry as C-12 where ^{2}H was retained from the propionate feedings (Figure 41).

Figure 41. Biosynthetic Precursors of Lasalocid A:
Butyrate as Precursor

The four carbon subunit arising from butyrate should label the following carbons in lasalocid A:
C-13/C-14/C-30/C-31; C-17/C-18/C-27/C-28; C-21/C-22/C-25/C-26



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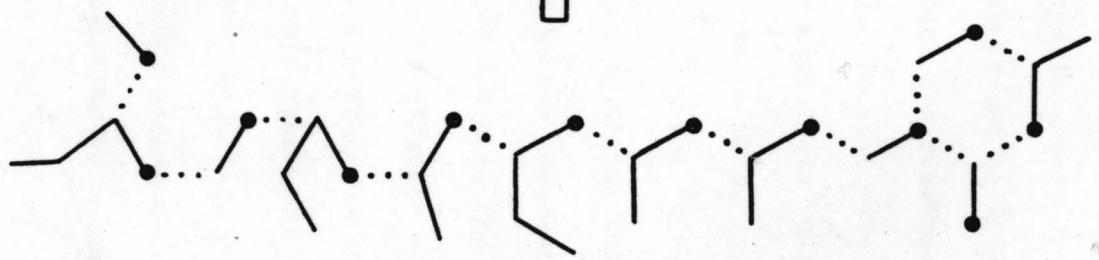


5 — CH_3CO_2H

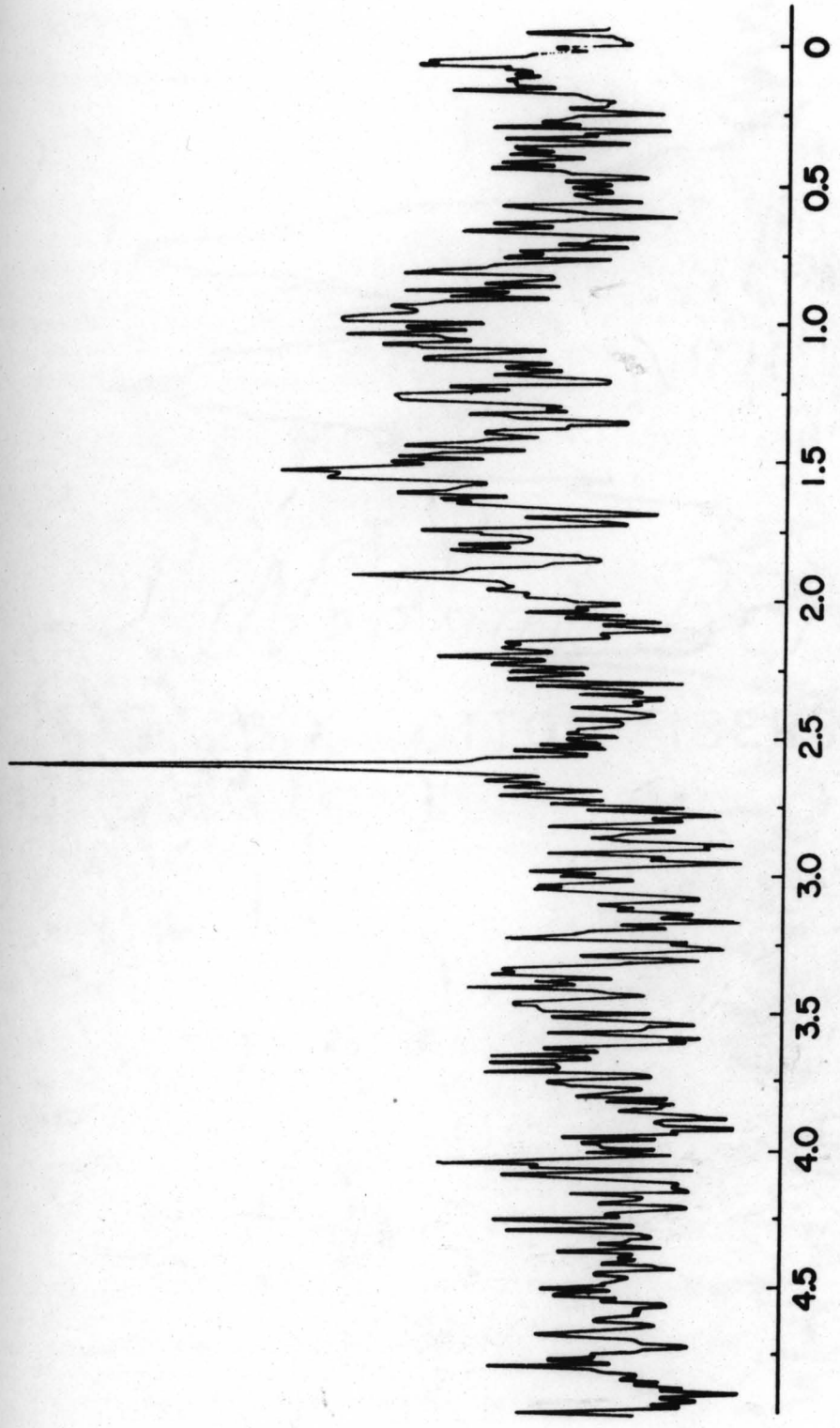
4 — $CH_3CH_2CO_2H$

3 — $CH_3CH_2CH_2CO_2H$

Biosynthetic Precursors of Lasalocid A (I)

The initial experiments with the butyrate precursor were complicated by the fact that the organism, S. lasaliensis, had essentially become a low-producer (see Results and Discussion, Chapter One), and any addition of the butyrate precursor inhibited all production of lasalocid A. The first experiment using [1-^{13}C, 2-$^{2}H_2$]-butyrate thus resulted in a very low yield of the antibiotic and the sample could not be accurately analyzed. The second set of experiments used the precursor [2-$^{2}H_2$]-butyrate which was fed at 5mM and 10mM levels. The isolated sodium lasalocid A was analyzed by high-field ^{2}H-NMR spectroscopy (90 MHz) using ca. 0.05M solutions in $CDCl_3$ (Figure 42). The data from the 5mM feedings showed only one ^{2}H resonance at 2.52 ppm, which corresponds to hydrogen at C-14. The results of the 10mM feedings showed in addition to the resonance at 2.52 ppm, two broadened peaks, one at about 0.9 ppm, the other at about 1.7 ppm. The data from these experiments strongly suggest that butyrate is incorporated into lasalocid A with retention of ^{2}H label at C-14, and that at high levels, either the butyrate is metabolized to acetate and incorporated into the methyl groups of lasalocid A or the ^{2}H resulting from the dehydrogenation of butyrate enters lasalocid A randomly via reductive exchange process. However, due to the uncertainty about the stability of the organism at the time that these experiments were carried out, these data must be interpreted with some degree of caution.

Figure 42: NMR Spectral Results of Lasalocid A Labelled by [2-$^2H_2$]-Butyrate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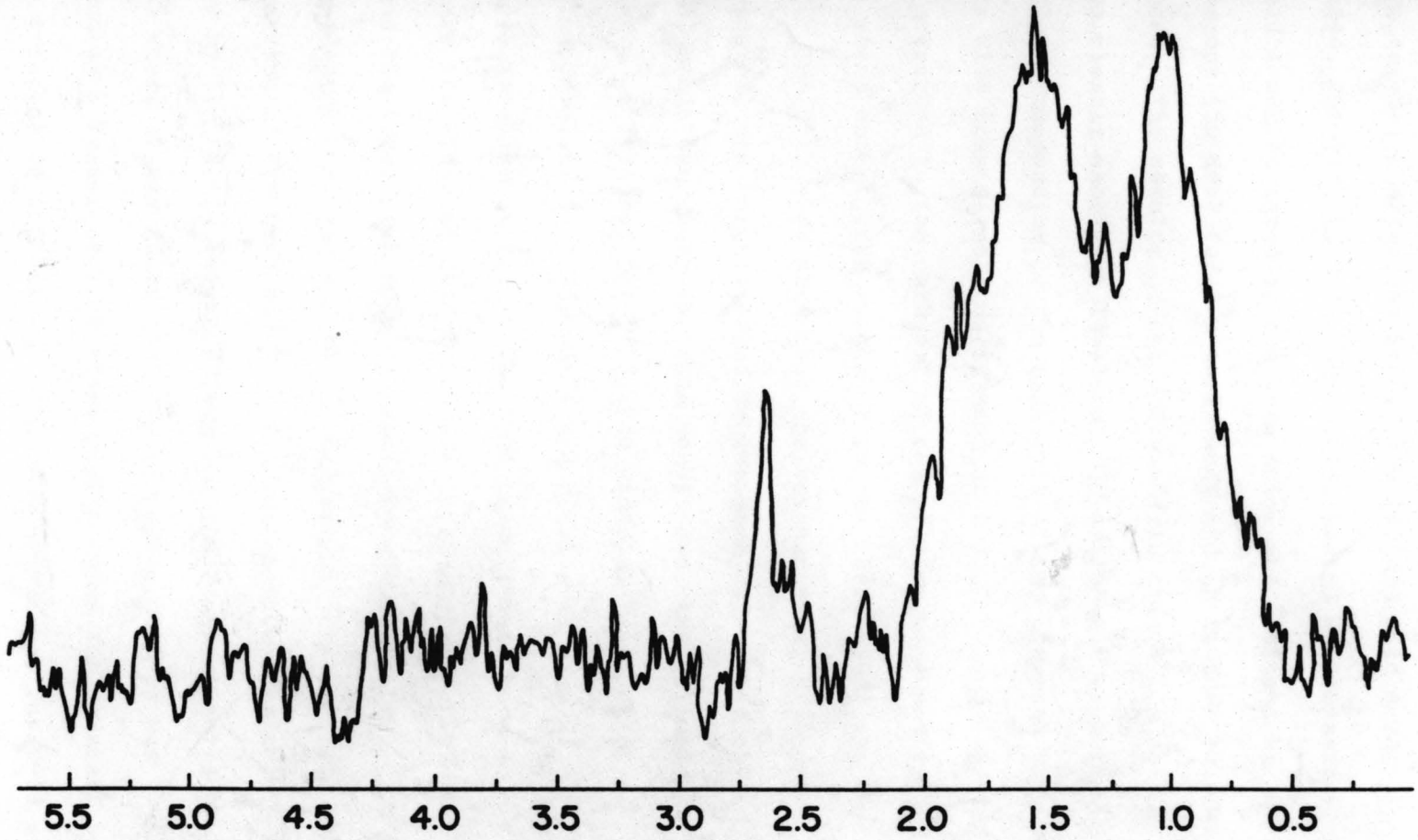
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4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5

More recently, after the production of lasalocid A was stabilized, the [1-^{13}C, 2-$^{2}H_2$]-butyrate feeding was repeated. The lasalocid A was isolated as the free acid, and analyzed by ^{13}C-NMR spectroscopy (50 MHz) for ^{13}C enrichment and ^{2}H induced shifted resonances. The results of the NMR analysis are in Figure 43. The data clearly show high levels of ^{13}C enrichment at C-13, C-17 and C-21, corresponding to the carboxyls of the butyrate subunit. There is also considerable enrichment at C-3, C-9, C-11 and C-15, which correspond to the carboxyl of the propionate subunit, and to a much lesser extent, the carbons from the carboxyl of the acetate subunit. The data also show that the resonances at C-13 and C-15 are broadened, and when compared to the same resonances of a standard sample run under the same conditions, the following ratios were determined: for C-13, the width at half-height of labelled:unlabelled is 2.25, while the total peak area of labelled:unlabelled is 2.18. The same calculations were made for C-15 where the width at half-height of labelled:unlabelled is 1.71, and the total peak area of labelled:unlabelled is 1.72. The line broadening that was due to ^{13}C enrichment only was calculated by comparison of the C-13 and C-15 resonances from lasalocid A labelled by [1-^{13}C]-isobutyrate (see ahead for experimental results) where it was found that the resonance at C-13 was broadened by a factor of 1.28 times greater than the standard and C-15 by a factor of 1.06 times. Thus the resonances for C-13 and C-15 of lasalocid A labelled by [1-^{13}C, 1-^{2}H]-butyrate are

broadened beyond what would be expected for ^{13}C enrichment by a factor of approximately 1.7 for C-13 and 1.6 for C-15 above natural abundance. Therefore, it is reasonable to assume from these results, that by some mechanism butyrate is efficiently metabolized to propionate, and that both the labelled butyrate and the labelled propionate can incorporate into the same molecule of lasalocid A. Thus, if ^{2}H were retained at C-14, the broadened resonances at both C-13 and C-15 could be explained as unresolved ^{2}H induced β-shifted ^{13}C resonances. These results, along with the ^{2}H-NMR analysis of the previous feeding experiment, can be interpreted as retention of ^{2}H at C-14 of lasalocid A. Thus, the stereochemical mechanism proposed for the carbon chain extension process of lasalocid A is consistent for both the three carbon subunit and the four carbon subunit.

Figure 43: NMR Spectral Results of Lasalocid A Labelled by [1-^{13}C, 2-$^{2}H_2$]-Butyrate

a) Carbon-13 Enrichments of Lasalocid A p. 274

* Enrichment at C-13 appears to be low, since the calculation for the "Enrichment Factor" does not account for the broadened resonance. See text for calculations of total peak area.

b) $^{13}C\{^{1}H\}$-NMR (50 MHz) Spectra p. 275

c) Expansion at 10 Hz/cm of 213-216 ppm of the labelled lasalocid A (C-13) p. 276

d) Expansion at 10 Hz/cm of 213-216 ppm of natural abundance lasalocid A, run under the same conditions (C-13) p. 277

e) Expansion at 10 Hz/cm of 82-85 ppm of the labelled lasalocid A (C-15) p. 278

f) Expansion at 10 Hz/cm of 82-85 ppm of natural abundance lasalocid A (C-15) p. 279

CARBON-13 LABELLED ENRICHMENTS FOR LABELLING OF LASALOCID A

FROM [1-^{13}C, 2-$^{2}H_2$]-BUTYRATE

CARBON	ENRICHMENT FACTOR
1	3.9
3	12.0
5	2.2
7	4.6
9	12.4
11	10.0
13	8.9 *
15	8.2
17	20.0
19	3.3
21	17.0
23	3.6

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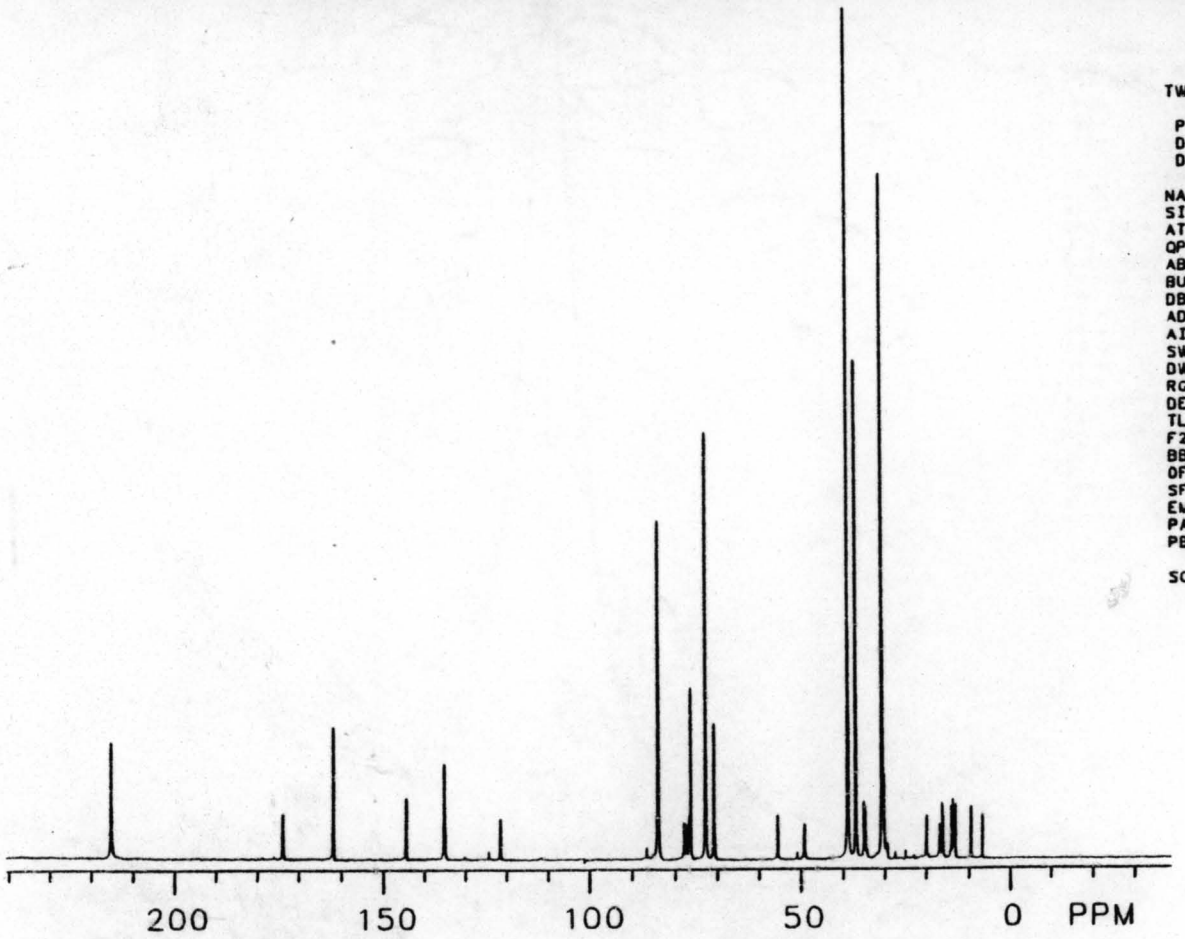

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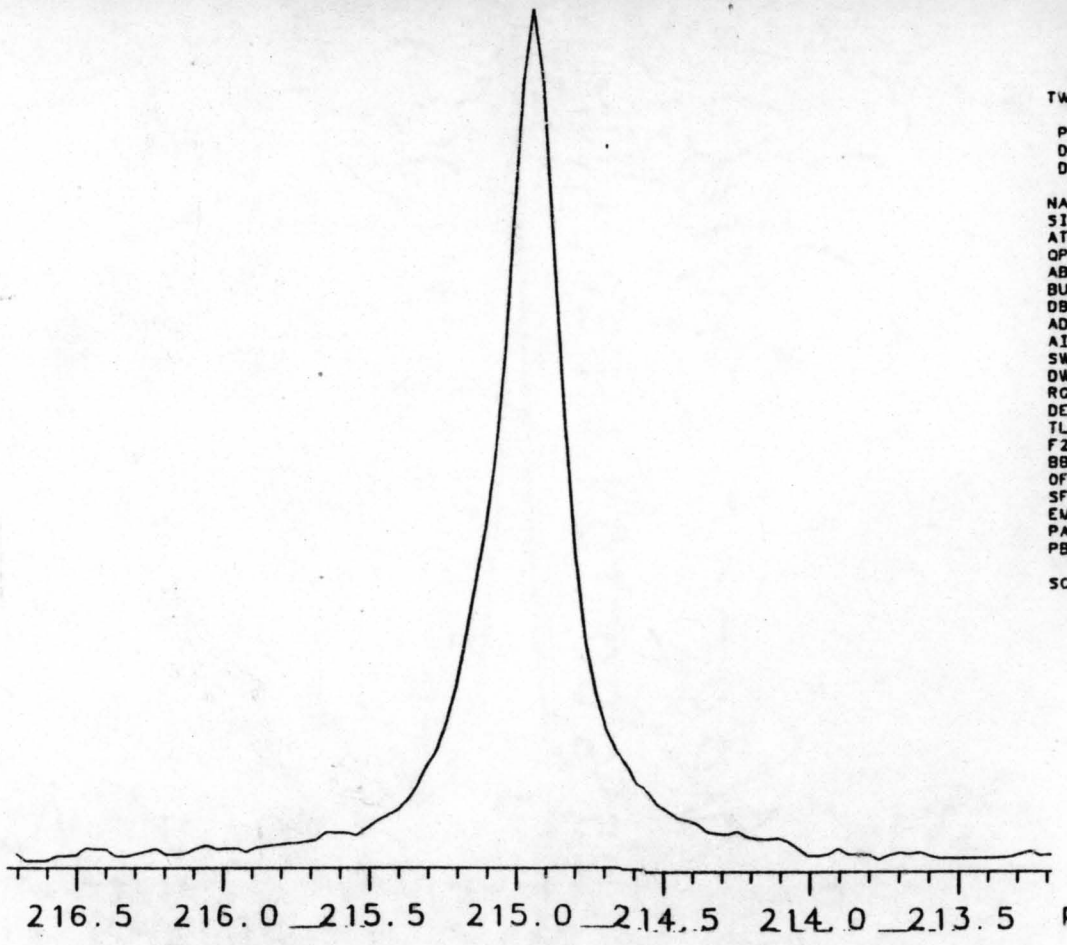

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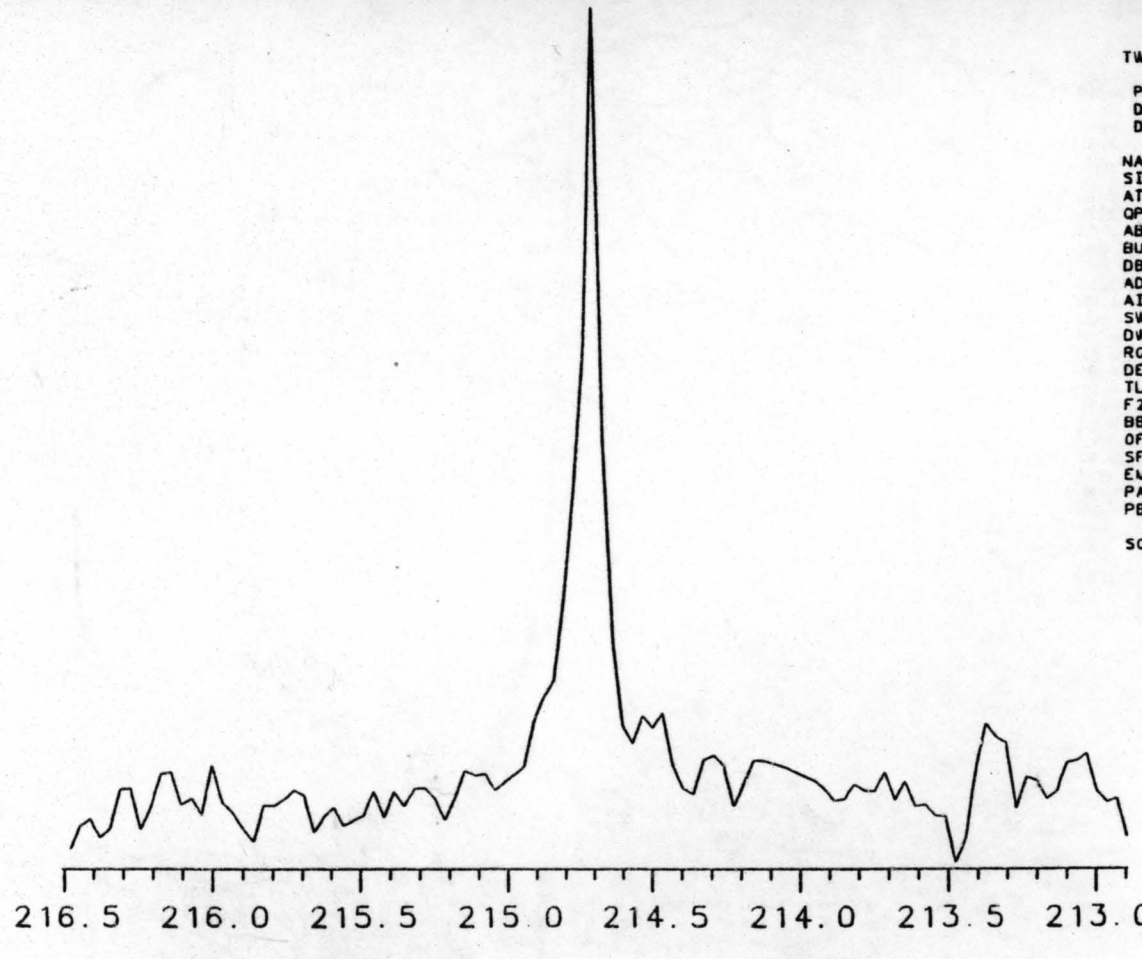

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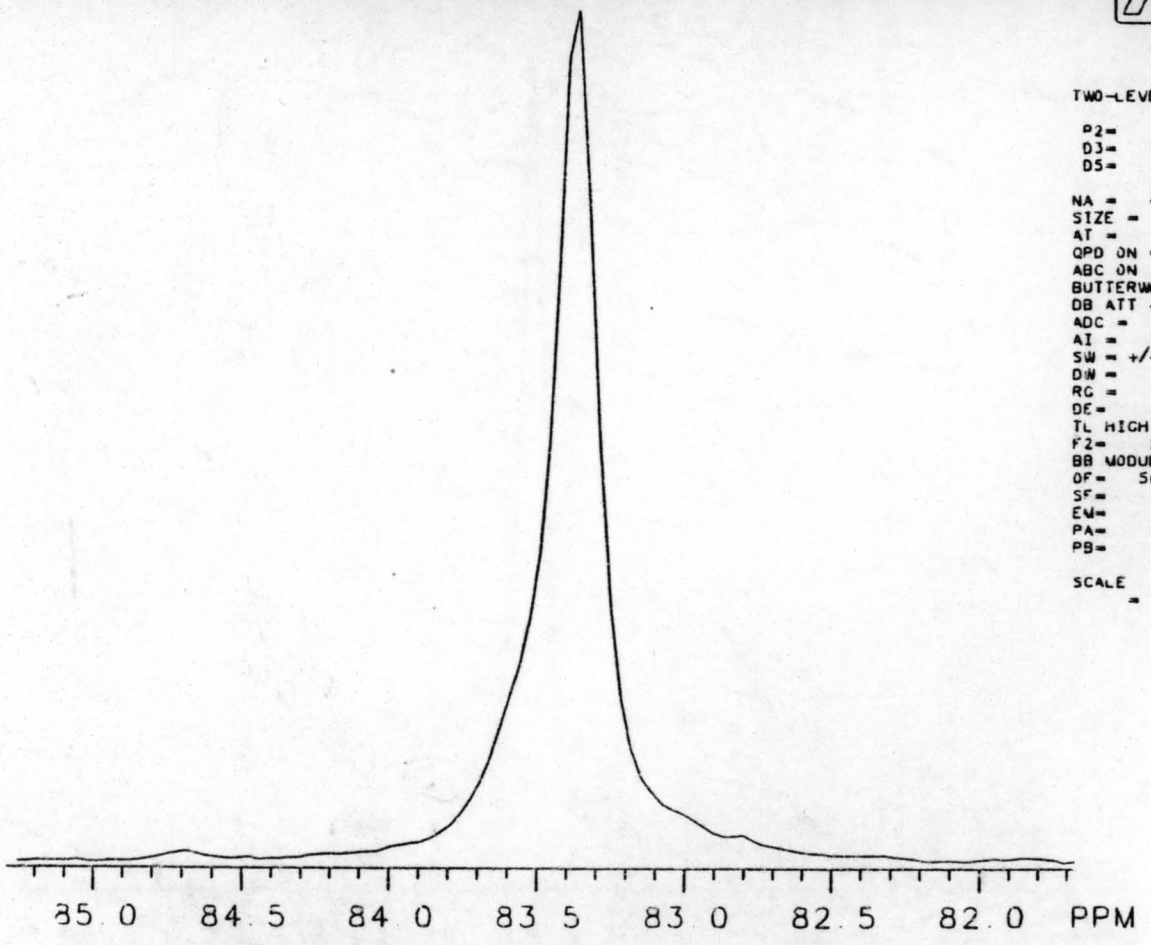

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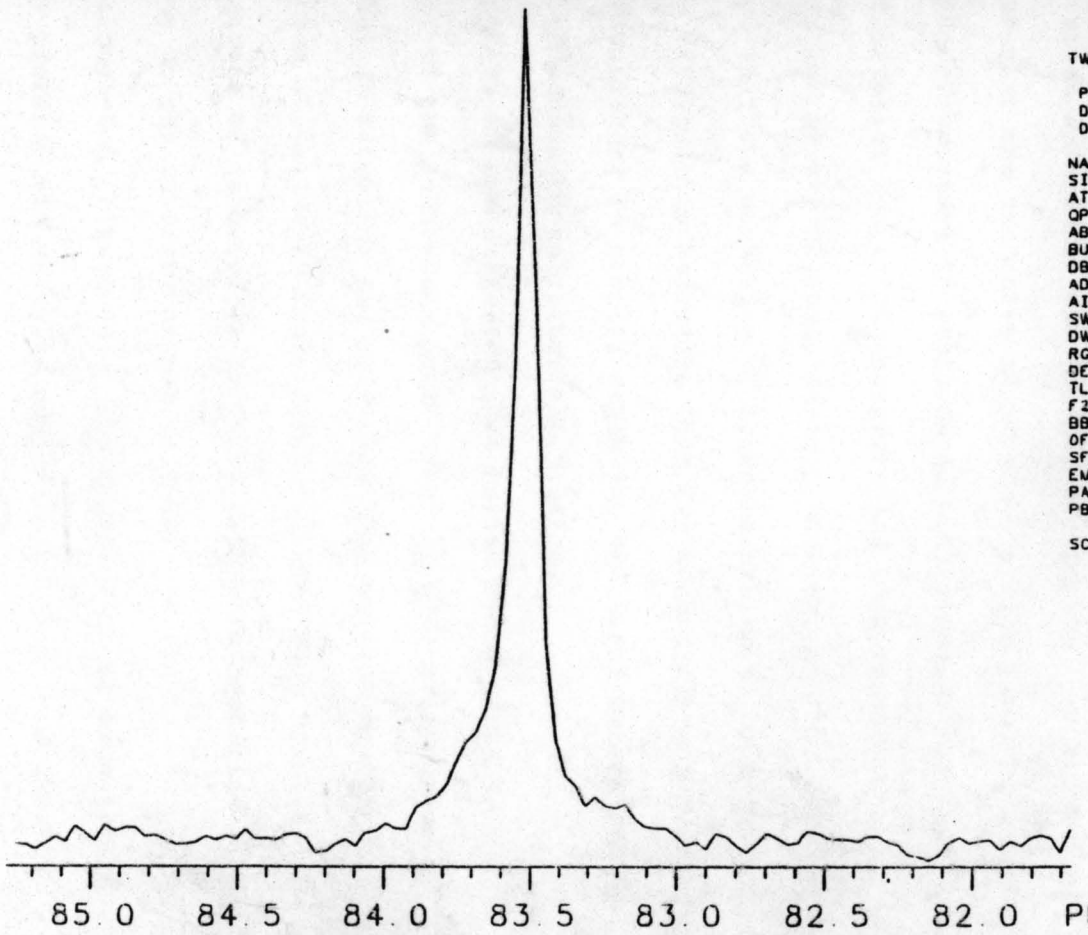

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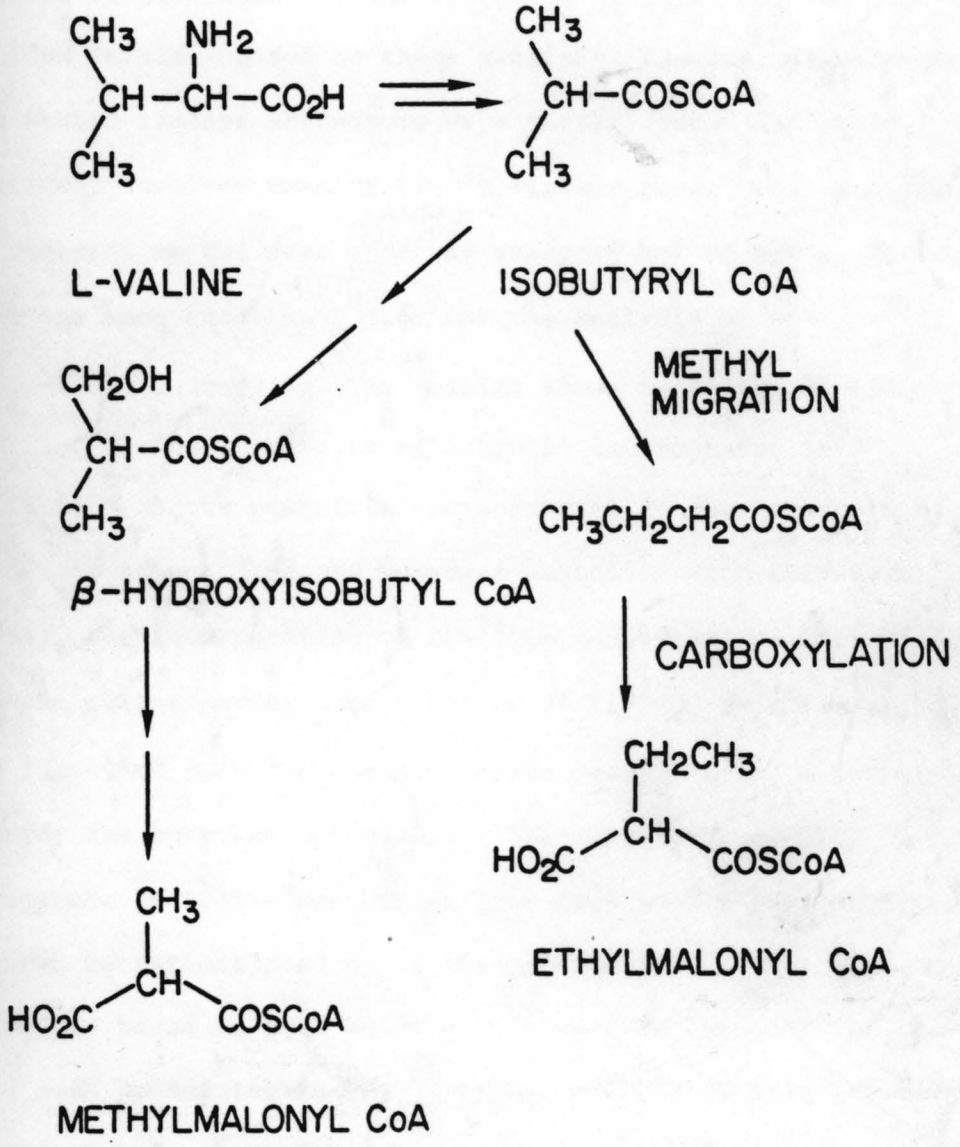
SCALE 10.00 HZ/CM
= .1987 PPM/CM

The other interesting information that came out of the [1-^{13}C]-butyrate incorporation is the ability of S. lasaliensis to efficiently metabolize butyrate to propionate with regiospecific retention of ^{13}C label. The conversion of propionate to butyrate has been seen in other polyether antibiotics, such as narasin and lysocellin (Liu, 1982). In narasin, it was noted that the primary enrichment by [4-^{13}C]-butyrate also led to a secondary incorporation apparently via [3-^{13}C]-propionate, and it was suggested that the mechanism of interconversion involved an α -oxidation of butyrate to propionate. Likewise, with lysocellin, the incorporation by [1-^{13}C]-butyrate led to a secondary incorporation by [1-^{13}C]-propionate. However, in this case it was suggested that the mechanism of interconversion is by ω-oxidation. It seems difficult to reconcile that two such different mechanisms can be functioning in similar organisms which are metabolizing similar compounds to polyether antibiotics.

Recently, it was suggested that valine could serve as the precursor of the n-butyrate unit (Vanek et al., 1982; Omura et al., 1983) in macrolide and polyether biosynthesis by the known degradation of valine to isobutyrate followed by a proposed rearrangement of isobutyrate to n-butyrate in a mechanism analogous to the interconversion of succinate and methylmalonyl-CoA (Figure 44). The degradation of isobutyrate normally proceeds via methylmalonyl-CoA and then into the TCA cycle therefore, if isobutyrate can also be metabolized to

n-butyrate, then this would be a reasonable mechanism to explain the interconversion of propionate and butyrate. In experiments with the macrolide antibiotic, tylosin, it was found that [2-^{13}C]-valine and [3,3'-^{13}C]-isobutyrate were incorporated into the molecule in the positions that would be expected for metabolism of these precursors to propionate and to butyrate (Omura et al., 1983). In similar experiments with monensin, Vanek et al (1983) showed that [1-^{13}C]-isobutyrate was incorporated into monensin A at the carbon corresponding to the carboxyl of the butyrate subunit. Based on this information it was decided to investigate the incoporation of isobutyrate into the polyether antibiotic laslaocid A.

Figure 44. Degradation of Valine to Isobutyrate and Methylmalonyl-CoA (Known Pathway) or to Ethylmalonyl-CoA (Proposed Pathway)


CH3 NH2
CH–CH–CO2H
CH3
CH3
CH–COSCoA
CH3
L-VALINE
ISOBUTYRYL CoA
METHYL
MIGRATION
CH2OH
CH–COSCoA
CH3
CH3CH2CH2COSCoA
β-HYDROXYISOBUTYL CoA
CARBOXYLATION
CH2CH3
CH
HO2C
COSCoA
CH3
CH
HO2C
COSCoA
ETHYLMALONYL CoA
METHYLMALONYL CoA

The initial experiments with the isobutyrate precursor involved feeding [U-^{14}C]-valine to S. lasaliensis at three different concentrations. The results of the feedings showed 17% specific incorporation of the valine at 1mM, 78% for 5mM and 116% for 10mM levels. Based on these promising results, experiments with stable isotope precursors were carried out. The first experiment involved feeding [1-^{13}C]-isobutyrate. The lasalocid A was isolated as the free acid and analyzed by ^{13}C-NMR spectroscopy under the same conditions used for the analysis of the [1-^{13}C, 2-$^{2}H_2$]-butyrate feeding. The results shown in Figure 45 indicate that [1-^{13}C]-isobutyrate is efficiently incorporated into lasalocid A at the positions corresponding to the carboxyls of the propionate subunit and the butyrate subunit. With the exception of C-13, the incorporation of [1-^{13}C]-isobutyrate is overall less than the corresponding incorporation of [1-^{13}C, 2-$^{2}H_2$]-n-butyrate by a factor of 1.75 for the propionate positions and a factor of 1.38 for the butyrate positions. The enrichment at C-13 from isobutyrate is 1.32 times higher than from n-butyrate; however, this can be rationalized since the calculation of the "Enrichment Factor" is based on peak heights, and does not account for the noted peak broadening due to ^{2}H retention. It is also interesting to note that for both [1-^{13}C]-butyrate and [1-^{13}C]-isobutyrate, the degradation of these precursors to [1-^{13}C]-acetate and its subsequent incorporation into lasalocid A occurs at a very low level, about one-fifth to one-fourth of the maximum incorporation

of butyrate or isobutyrate. Nevertheless, these results are consistent with the proposed pathway for the interconversion of propionate and butyrate, and for isobutyrate serving as an important intermediate in the metabolic pathways giving rise to the subunits of lasalocid A.

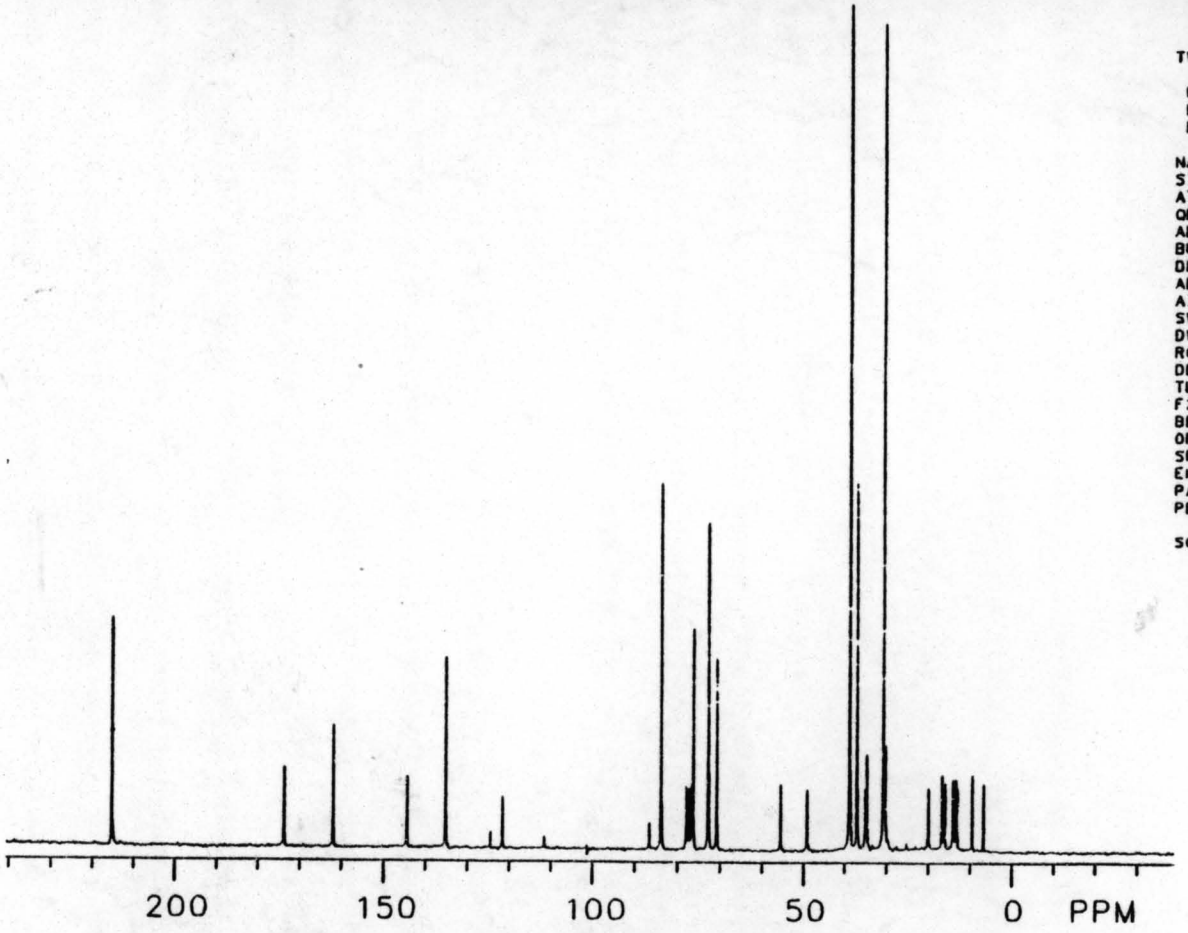
Figure 45. NMR Spectral Results of Lasalocid A Labelled by [1-^{13}C]-Isobutyrate

CARBON-13 ENRICHMENTS FOR LABELLING OF LASALOCID A

FROM [1-^{13}C]-ISOBUTYRATE

CARBON	ENRICHMENT FACTOR
1	4.5
3	7.2
5	2.9
7	3.5
9	6.1
11	5.1
13	11.7
15	5.9
17	13.2
19	3.1
21	13.7
23	3.1

MMS 601 MSHERMAM 07DEC84
1-13C - ISOBUTYRATE FEEDING



TWO-LEVEL DECOUPLING

P2= 8.00 USEC
D3= 1.00 MSEC
D5= 1.00 SEC

NA = 0
SIZE = 16384
AT = 581.63 MSEC
QPD ON = 1
ABC ON
BUTTERWORTH FILTER ON
DB ATT. = 3
ADC = 12 BITS
AI = 7
SW = +/- 7042.25
DW = 71
RG = 10 USEC
DE= 71 USEC
TL HIGH POWER ON
F2= 200.066700
BB MODULATION ON
OF= 5073.47
SF= 50.311808
EM= 2.00
PA= 185.6
PB= 69.0

SCALE 700.00 HZ/CM
= 13.9132 PPM/CM

The subsequent experiments with the isobutyrate precursor involved looking at the possible rearrangement mechanism. The idea was to look at the resulting carbon coupling patterns from the incorporation of [2,3,3'-$^{13}C_3$]-isobutyrate into lasalocid A in order to verify whether or not the rearrangement was intramolecular. The initial feeding experiment used [2,3,3'-$^{14}C_3$]-isobutyrate as the precursor, and the isolated lasalocid A was analyzed by incorporation of radioactivity. The precursor was fed at three concentrations and found to incorporate very efficiently: 1mM levels yielded 31% specific incorporation, 2.5mM levels gave 89% specific incorporation and 5mM levels resulted in 141% specific incorporation. The analogous ^{13}C precursor was synthesized in the same manner, but for an unknown reason the synthesis did not yield the desired product (see Materials and Methods); consequently, the feeding experiment gave useless results.

In one final experiment, the incorporation of [4-^{13}C]-butyrate was studied in order to better understand the interconversion of propionate and butyrate. If the isobutyrate pathway functions and propionate and butyrate are readily interconverted, then it would be expected that the precursor [4-^{13}C]-butyrate should label not only the resonances arising from the C-4 position of butyrate but also those arising from the C-3 position of propionate. If on the other hand the mechanism of interconversion were to involve ω -oxidation of butyrate to propionate, then the label would be

lost, and only the C-4 positions of butyrate would be enriched with ^{13}C. The third alternative, the proposed α-oxidation of butyrate to propionate has been ruled out in the previous experiments with [1-^{13}C]-butyrate and -isobutyrate, since in both experiments the ^{13}C label was efficiently incorporated into carboxyl positions arising from both the propionate subunit as well as the butyrate subunit.

The [4-^{13}C]-butyrate was fed and the isolated lasalocid A free acid analyzed by ^{13}C-NMR spectroscopy (50 MHz). The results shown in Figure 46 clearly show that the [4-^{13}C]-butyrate is efficiently incorporated into both the C-3 position of the propionate subunit as well as the C-4 position of butyrate. The data also show that [4-^{13}C]-butyrate is degraded to [2-^{13}C]-acetate, as would be expected from all the previous studies. It also appears as if the butyrate has been metabolized into the C-1 position of acetate, and possibly to C-2/C-3 double labelled propionate (note the intense ^{13}C-^{13}C couplings at C-12 in the expanded spectra from the [4-^{13}C]-butyrate feeding); however, it is not clearly understood how the organism can generate a double labelled propionate subunit from the single labelled butyrate, since this implies that a carbon bond has been broken and reformed intramolecularly with respect to the ^{13}C enriched carbon. As with the other precursors, both of these metabolic routes operate at a much lower frequency than the primary pathways. In any event, the interconversion of the propionate and butyrate subunits can be said to occur via the

intermediate isobutyrate most likely in a rearrangement mechanism that is similar to the interconversion of succinate and methylmalonyl-CoA.

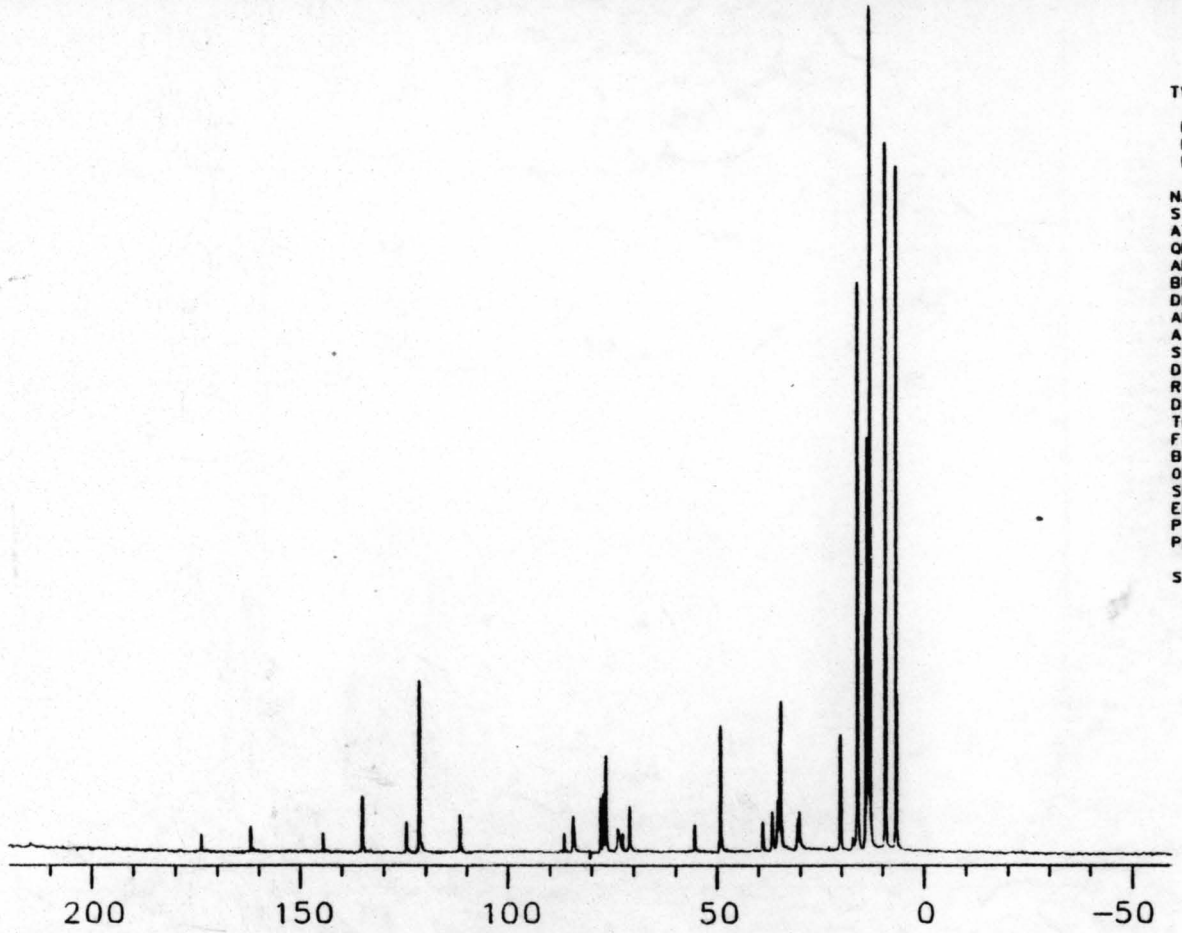
Figure 46. NMR Spectral Results of Lasalocid A Labelled by [4-^{13}C]-Butyrate

CARBON-13 ENRICHMENTS FOR LABELLING OF LASALOCID A

FROM [4-^{13}C]-BUTYRATE

CARBON	ENRICHMENT FACTOR
2	11.7
4	3.7
6	6.9
8	6.2
12	4.3
19	1.8
20	4.5
23	3.3
24	13.0
26	28.3
28	34.3
29	23.3
31	30.7
32	11.3
33	12.0
34	18.9

MMS 900 MSHERMAN 07FEB85
4-13C-BUTYRATE FEEDING
(EXPT #46)



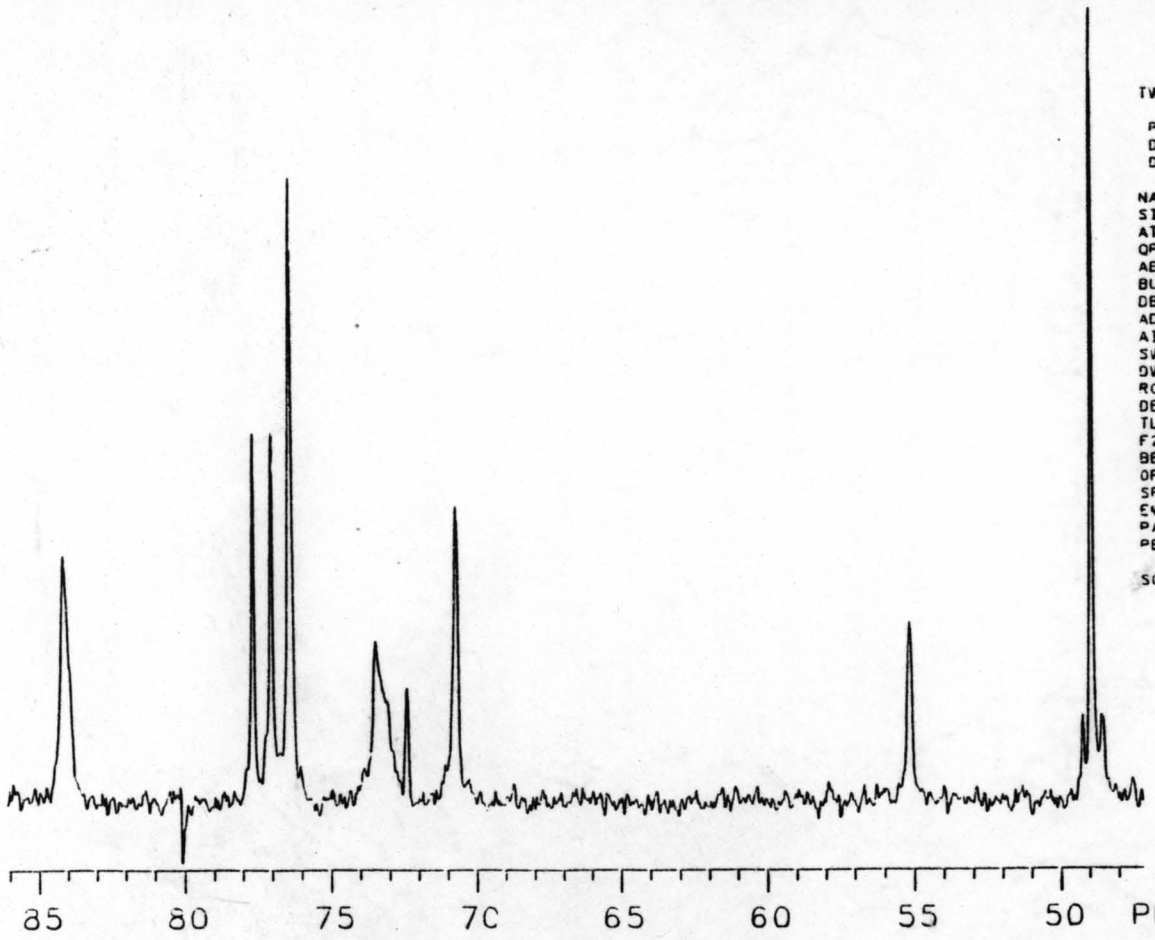
TWO-LEVEL DECOUPLING

P2= 8.00 USEC
D3= 1.00 MSEC
D5= 1.00 SEC

NA = 53500
SIZE = 16384
AT = 581.63 MSEC
QPD ON = 1
ABC ON
BUTTERWORTH FILTER ON
DB ATT = 3
ADC = 8 BITS
AI = 11
SW = +/- 7042.25
DW = 71
RC = 10 USEC
DE = 71 USEC
TL HIGH POWER ON
F2= 200.068000
BB MODULATION ON
OF= 4025.74
SF= 50.310758
EM= 2.00
PA= 188.5
PB= 96.4

SCALE = 700.00 HZ/CM
= 13.9135 PPM/CM

MMS 900 MSHERMAN 07FEB85
4-13C-BUTYRATE FEEDING
(EXPT #46)



TWO-LEVEL DECOUPLING

P2= 8.00 USEC
D3= 1.00 MSEC
D5= 1.00 SEC

NA = 53500
SIZE = 16384
AT = 581.63 MSEC
QPD ON = 1
ABC ON
BUTTERWORTH FILTER ON
DB ATT = 3
ADC = 8 BITS
AI = 11
SW = +/- 7042.25
DW = 71
RG = 10 USEC
DE= 71 USEC
TL HIGH POWER ON
F2= 200.068000
BB MODULATION ON
OF= 4026.15
SF= 50.310758
EM= 2.00
PA= 215.9
PB= 61.0

SCALE = 100.00 HZ/CM
= 1.9876 PPM/CM

The experiments presented in this chapter were carried out in order to better understand the biosynthesis of lasalocid A with regard to three broad question:

(i) What are the origins of the C-2 to C-4 subunits?

(ii) What determines the order of assembly of the subunits?

(iii) What process controls the absolute configuration of the chiral centers?

The results of the experiments have provided a great deal of insight into the first and third questions. At this time the second question can only be approached on a hypothetical basis, since there no experimental evidence is available.

The biosynthesis of lasalocid A depends on having access to metabolic pools of the three substrates: acetate, propionate and butyrate. All three substrates are available through normal primary metabolic pathways in particular from beta-oxidation of fatty acids, degradation of amino acids or the TCA cycle. However, what has become evident from these studies on the polyether antibiotic lasalocid A, and from studies on other antibiotics, is the fact that all the precursor pools are readily interconverted, and in some cases these interconversions cannot be explained by the known primary metabolic pathways.

The results of the precursor studies have shown that the interconversions between acetate and propionate, and acetate and

butyrate can be explained by primary metabolic pathways (see Figure 34, p. 226), and the interconversion of propionate and butyrate has been shown to occur probably via the common intermediate, isobutyrate. This then explains the primary labelling patterns seen in lasalocid A.

What cannnot be as easily explained are the minor pathways, such as the apparent conversion of [4-^{13}C]-butyrate to [2,3-$^{13}C_2$]-propionate. Until further work is done to rule out any possible artifacts in the spectral analysis, it is risky to propose new metabolic pathways. It should be noted however, that at lower precursor concentrations these pathways are not detected by NMR spectral techniques. This can be rationalized by two possible mechanisms: first, the enzymes responsible for these pathways are constitutive, but operate at a constant reduced rate relative to the primary pathways; thus at lower concentrations of precursor, these pathways are not observable within experimental limits. On the other hand, it is possible to imagine that these enzymes are inducible, and as a consequence, the minor pathways function only above a threshold level of a precursor. In view of the results of the second [1,4-$^{13}C_2$, 2,3-$^{2}H_2$]-succinate feeding experiment, where the precursor was fed at the same concentration, but at more frequent intervals, and the resulting lasalocid A showed a low level of ^{13}C enrichment at several sites, it is reasonable at this time to propose that the enzymes involved in these minor pathways are inducible.

The question pertaining to the stereocontrolled biosynthesis of lasalocid A has been as thoroughly studied as a whole-cell system will permit. The labelling studies with the acetate precursor have shown that the starter unit in lasalocid A biosynthesis is distinct from the normal polyketide mechanism, in that there was a complete loss of ^{2}H label at this site; thus it appears as if the starter unit has been activated prior to condensation. The feeding experiments with the $^{13}C/^{18}O$ labelled precursors clearly show that these units are incorporated intact into hydroxymethylene centers of opposite sterochemical configuration, which suggests that the stereocontrol exerted during the formation of the hydroxymethylene centers must occur by stereodivergent reductases during carbon chain assembly, or by regiocontroled epimerases, either during carbon chain assembly or on a subsequent free intermediate. The results of these experiments, and the analogous ones for monensin A, strongly support the biosynthetic hypothesis that these molecules are formed via a diene (or triene, in the case of monensin A) intermediate, which undergoes air oxidation and subsequent cyclization to give the cyclic ethers characteristic of the polyether antibiotics. Likewise, the studies on lasalocid A using labelled propionate, succinate and butyrate, and again the analogous work on monensin A, have shown that the carbon chain extension process occurs by condensation of the (2S) enantiomer of the activated CoA ester of the subunit to the growing carbon

chain, and that either a racemization occurs at the time of the condensation, or a regiospecific epimerization occurs after the carbon chain has been formed.

CHAPTER THREE

PRELIMINARY INVESTIGATIONS INTO THE PRODUCTION OF THE POLYETHER ANTIBIOTIC LASALOCID A

INTRODUCTION

The investigations into the biochemistry of the production of the polyether antibiotic lasalocid A were undertaken primarily as a result of several questions that came out of the precursor incorporation studies. During the course of the previously described investigations it was continually noted that the production of the antibiotic lasalocid A from liquid fermentation cultures was not reproducible, in particular, the quantity of antibiotic isolated varied with each precursor and with the concentration of the precursor in the grpwth medium. It was also unclear as to what conditions initiated the onset of antibiotic production, and at what time this began. From the NMR spectral analysis of labelled precursor incorporation studies it was apparent that S. lasaliensis is capable of efficiently metabolizing the exogenous precursors both by known metabolic routes and by routes that apparently involve inducible enzymes and are not well understood. All of these observations regarding what factors affect the production of the polyether antibiotic lasalocid A, along with the possibility of identifying any intermediates in the biosynthetic pathway between the simple C-2 to C-4 acid precursors and the final product, were a stimulus in initiating the studies described in this chapter.

Because there is very little literature precedence for this type of investigation, it was decided to attempt only a

preliminary study at this time in order to look at a variety of factors, rather than pursuing any one thing in depth. As a consequence the following five things were examined:

(i) the effect of precursor pressure as a result of adding various levels of exogenous substrates;

(ii) the effect of the fatty acid and polyketide synthetase inhibitor, cerulenin;

(iii) the effect of mono-oxygenase inhibitors, especially with regard to the putative diene biosynthetic intermediate;

(iv) the effect of protein synthesis inhibitors on the antibiotic synthetase enzymes;

(v) the effect of fluorinated substrate analogues.

All of these effects were studied using a whole-cell system, and despite the limitations of this system, these studies have provided useful and interesting information regarding the biochemistry of the production of lasalocid A.

MATERIALS AND METHODS

General Procedures

In all of the experiments Streptomyces lasaliensis NRRL 3382R wild type was used. The organism was maintained as 1 mL frozen spore suspensions at -80°C in 2G Solution which were prepared from an isolated single colony of NRRL 3382R. The details for preparing the frozen spore suspensions are given in Chapter One of this thesis.

For the fermentation experiments described in this chapter, one vial of the frozen spore suspension was used to inoculate 50 mL media/250 mL flask as a seed culture. This culture was grown (28-30°C, 250 rpm) to stationary phase (usually 72 hours) before using it to inoculate the fermentation media. MYM media or SP/LO media were both used for the seed cultures. The fermention media was either MYM media, SP/LO media or 14C media. Once the fermentation cultures had been started by placing them on the shaker, they remained in place during the entire fermentation wihtout stopping the shaker until ready to harvest.

Assays were done during the course of the fermentation by removing a 1.0 mL aliquot from the culture and placing it in a 13 x 100 mm tube. This aliquot was "worked-up" by a mini-extraction as follows: dd H_2O (ca. 0.5 mL) was added to each tube, the tubes were vortexed briefly, then spun down at 3000 rpm for 5 min. The aqueous layer was pipetted off into a second tube taking care not to disturb the cells. This process was repeated with ca. 1 mL of

fresh H_2O. $CHCl_3$ (1.0 mL) was added to the combined aqueous layers, vortexed well, then when the layers had settled, the aqueous layer was pipetted off and discarded, while the $CHCl_3$ was evaporated under N_2.

The cells were transfered to disposable aluminum weighing dishes with 2-3 H_2O rinses, dried at 90°C for 18 hours, then dried in vacuo at room temperature for an additional 12 hours before weighing. The results of this process is referred to as "dry cell weights" or "DCW". An alternative measurement of cell growth that was used is referred to as "packed cell volume" or "PCV". In this latter method, the cell mass was measured at the end of the fermentation by decanting the entire contents of a flask into a Corning disposable connical centrifuge tube and centrifuging at 3000 rpm for 15-20 min. The media was decanted for extraction and the cell mass measured against the tube calibrations. This method is not as accurate as the DCW method, and was used only for relative comparisons of total cell mass. (Any modification of the above procedure is known as "Sophia's Secret"; the details of which are unknown).

The evaporated sample was used for the HPLC analysis. The samples were dissolved in MeOH:$CHCl_3$ (3:1) and injected onto the analytical HPLC column. (Details of the HPLC assay are given in Chapter One of this thesis). The HPLC assay was used to quantitate the amount of lasalocid A present and to determine the incorporation of [^{14}C] precursors into lasalocid A. The base and

height of the HPLC peak were measured and a 1.0 mL aliquot of the fraction containing lasalocid A was counted. The remainder of the calculations were done using a spreadsheet, which was set up in the following order: (i) base, (ii) height, (iii) range (iv) amount injected, (v) cpm/mL, (vi) amount of lasalocid A (referred to as "AMT"), and (vii) counts/mg (C/MG). The calculation for the amount of lasalocid A was based on a standard curve and took into account corrections for differences in the range of the UV monitor and the amount of sample injected. The calculation for the radioactivity counts 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.

The last two values AMT and C/MG are summarized in separate tables of data for each experiment. The data for the third recorded value, the dry cell weights, also referred to as DCW, are summarized in another table under the heading of WT. Each experiment is referred to by a number in the form of "IS#"; this number is used in all the tables of data. The codes in the tables follow the general format: listing the concentration of inhibitor first, followed by the time of the assay ("2-6" refers to 2mM at 6 hours). The abbreviation, "CO", refers to control flasks. The experiments where this code varies are: the "Baseline Data Experiment" (A = acetate, P = propionate, S = succinate, B = butyrate, L = Lasalocid, H = Homologs, I = Isolasalocid); IS# 8 (C

= cerulenin, S = monofluoro succinic acid); IS#9 (A = 2-fluoropropionic acid, E = ethyl 2-fluoropropionate).

Inhibitors

Cerulenin (2,3-epoxy-4-oxo-7,10-dodecadienamide), cycloheximide (3-[2(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl] glutarimide) and p-fluoro-DL-phenylalanine were purchased from Sigma. SKF 525-A (Proadifen-HCl) was a gift from Smith Kline and French Laboratories. 2-Diethyl-GEB (3-beta-(2-diethylaminoethoxy)androst-5-en-17-one, hydrochloride; 2-diethyl-(U-1866A)) was a gift from The Upjohn Company. AY 994 (trans-1,4-bis(2-chloobenzylaminomethyl)cyclohexane dihydrochloride) was a gift from The DuPont Company. The fluorinated substrates: 2-fluorosuccinate, 2-fluoropropionate, and 2(S)-2-fluoropropionic acid and its ethyl ester, were synthesized as described below.

All inhibitors were prepared as aqueous solutions of known concentration which were filter sterilized using a Millipore disposable filter unit (Millex^{R}-GS, 0.22 μm), except in the case of the mono-oxygenase inhibitors and the protein inhibitors which were added to the seed cultures prior to sterilization following the procedure of Corcoran and Vygantas (1982). The sodium [1-^{14}C] acetate solutions used were prepared by dissolving the radioactive acetate (New England Nuclear) in H_2O, usually at a concentration of 5-10 uCi/mL, and filter sterilizing as before. All controls

were fed sterile H_2O in a volume comparable to the volume of the inhibitor solutions.

Inhibitor Synthesis

[U-^{14}C] 2(S)-2-Fluoropropionic acid and

-Ethyl 2-fluoropropionate

This was prepared according to the method of Olah (Olah, et al., 1974; 1979). L-[U-^{14}C] Alanine (NEN, 168.0 mCi/mmole; 100 uCi) was admixed with L-alanine (Sigma; 2.66 g, 29.9 mmoles) to give a theoretical mixture of 3.346 uCi/mmole. The alanine was dissolved in 75 mL of 70% polyhydrogen fluoride/pyridine (Aldrich) contained in a teflon reaction vessel. While stirring, $NaNO_2$ (dried at 140°C for 24 hours, 3.1 g, 45 mmole) was added slowly. The reaction was run at room temperature for 1 hour, then quenched with ice/H_2O. The reaction mixture was then extracted 3-4 times with equal volumes of Et_2O, the Et_2O layers washed with 10% $NaHCO_3$, then dried over anhydrous Na_2SO_4 and evaporated in vacuo. The residue was distilled by Kugelrohr at 66-68°C, yielding [U-^{14}C]-2(S)-2-fluoropropionate (1.3 gms, 12.2 mmoles, 31.6% yield). (In a sample of 2(S)-2-fluoropropionate, prepared by the same procedure, it was found that the material contained only 57% ee). The product was analyzed by ^{13}C-NMR in $CDCl_3$ (C-1, 130.5 ppm, d, J = 124 Hz; C-2, 85.2 ppm, d, J = 182.8 Hz, C-3, 18.12 ppm, d, J = 22.85 Hz); and a combined ^{1}H-NMR and ^{19}F-NMR analysis in $^{2}H_2O$ (assigned as an AMX_3 system: A = -184.56 ppm, doublet of

quartets, J_{AM} = 48 Hz, J_{AX} = 25.5 Hz; M = 4.95 ppm, doublet of quartets, J_{AM} = 48 Hz, J_{MX} = 7 Hz; X_3 = 1.37 ppm, J_{AX} = 25.5 Hz, J_{MX} = 7 Hz). The ^{19}F-NMR resonances were assigned relative to $CFCl_3$ at 0.0 ppm. An aliquot of the product was counted: 6.925 x 10^6 dpm/mmole.

[U-^{14}C]-2(S)-2-Ethyl fluoropropionate was prepared by esterifying [U-^{14}C]-2(S)-2-propionic acid (300 mg, 3.26 mmole) with $CH_3CH_2N_2$. $CH_3CH_2N_2$ was prepared from N-ethyl-N-nitrosourea (1 gm, 8.5 mmole) in 6 mL Et_2O and 4 mL 40% KOH; the EtN_2 and Et_2O were co-distilled into a cold solution of the 2-fluoropropionic acid in Et_2O. The distillate was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* at 0°C to yield [U-^{14}C]-2(S) ethyl-2-fluoropropionate (189 mg, 1.57 mmole, 48% yield). The esterification was verified by ^{1}H-NMR.

(+/-)-2-Fluoropropionic Acid

The racemic fluoropropionate was prepared by C.Q. Sun, using the same procedure as detailed above, except the starting material was cold DL-alanine. The structure was verified by ^{1}H-NMR and ^{19}F-NMR; the assignments matched those given in the above procedure.

(+/-)-2-Fluorosuccinic Acid

The racemic fluorosuccinic acid was prepared by C.Q. Sun according to the method of Lowe (Lowe *et al.*, 1980). 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 cleaved with H_3O^+ to give the 2-fluorosuccinic acid, which was recrystallized from EtOAc (m.p. 145°C). The structure was verified by ^{1}H-NMR and ^{19}F-NMR of the succinic anhydride in D_2O (assigned as an AMX_2 system: A = -188.7 ppm, doublet of triplets, J_{AM} = 46.5 Hz, J_{AX} = 25.5 Hz; M = 5.4 ppm, doublet of triplets, J_{AM} = 46.5 Hz, J_{MX} = 5.0 Hz; X = 3.15 ppm, doublet of doublets, J_{AX} = 25.5 Hz, J_{MX} = 5.0 Hz). The ^{19}F-NMR assignments were relative to $CFCl_3$ at 0.0 ppm.

Inhibitor Studies

Cerulenin (IS #8)

The seed cultures for this experiment were started in the standard method by adding 1 vial frozen spore suspension (cell count: 3.0 x 10^6/0.1 mL) to 50 mL MYM and grown for 72 hours. Then, 18 flasks x 25 mL MYM media/125 mL flask were innoculated with 2% v/v seed culture and returned to the shaker.

All 18 flasks were assayed as follows: t = 72 (time of innoculation), t = 96, t = 102, t = 120, t = 144, and t = 167.5 hours (time of harvest). The inhibitor was added at t = 96 hours after removing the assay sample. The sodium [1-^{14}C]-acetate (0.066 µCi/flask) was added at t = 99 hours. All flasks were treated in triplicate as follows: 5, 10, 15, 20 and, 30 µg/mL cerulenin; sterile H_2O was used as the control.

The aliquots removed during the fermentation were extracted and assayed by HPLC and dry cell weights following the standard procedure. At t = 167.5 hours, all the flasks were removed from the shaker, sampled, then harvested as follows: measure pH, measure packed cell volume, extract media with 15 mL $CHCl_3$/25 mL media. The $CHCl_3$ was evaporated and the sample analyzed by HPLC (inject 1/20 total on analytical column). The final pH and packed cell volumes (PCV), recorded as the average of three flasks, are as follows: 5 µg/mL (pH 7.54, PCV 2.5 cc); 10 µg/mL (pH 7.46, PCV 1.83 cc); 15 µg/mL (pH 7.50, PCV 1.83 cc); 20 µg/mL (pH 7.36, PCV 2.5 cc); 30 µg/mL (pH 7.36, PCV 2.5 cc); sterile H_2O (pH 7.56, PCV 2.0 cc).

Protein Inhibitors and Mono-oxygenase Inhibitors (IS# 7)

The seed cultures for this experiment were started in the standard procedure by adding 1 vial frozen spore suspension (cell count: 3.0 x 10^6/0.1 mL) to each of 6 flasks containing 50 mL MYM media. The following inhibitors were added to five of the flasks prior to sterilizing the media: (i) 15 µg/mL GEB; (ii) 15 µg/mL AY 994; (iii) 15 µg/mL SKF 525A; (iv) 100 µg/mL p-Fluorophenylalanine; (v) 100 µg/mL cycloheximide. The sixth flask contained no additional ingredients. The seed cultures were grown for 72 hours, then 126 tubes (16 x 150 mm) with 5 mL MYM media/tube were inoculated with 2% v/v 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) which contained no additional ingredients. The tubes were then placed at ca. a 45° angle, in wire test tube racks, in the shaker at 28-30°C, 250 rpm.

All tubes were treated in triplicate, except the controls, of which there were six total. All of the inhibitors were added at 80 hours after transfer of seed culture as follows: 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 same concentrations) to 12 tubes of seed culture (ii) and 12 tubes of seed culture (vi); SKF 525A (at same concentrations) 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 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 innoculation, all 126 tubes were harvested and worked up using the standard procedure as for the 1 mL aliquot assay. The dry cell weights were measured and the entire $CHCl_3$ extract was assayed by HPLC.

Protein Inhibitors (IS# 11)

The seed cultures for this experiment were started in the standard procedure by adding 1 vial frozen spore suspension (cell

count $9.2 \times 10^7/0.1$ mL) to each of three flasks containing 50 mL MYM media. In addition, p-Fluorophenylalanine (100 µgm/mL) was added to one flask and Cycloheximide (100 µgm/mL) was added to a second flask prior to sterilization. The seed cultures were grown for 72 hours, then 36 flasks x 25 mL MYM media flask were inoculated (2% v/v) with the seed culture containing p-Fluorophenylalanine; 36 flasks were inoculated with the seed culture containing Cycloheximide; 12 flasks were inoculated with the seed culture having no additional ingredients. All the flasks were then returned to the shaker.

All fermentation flasks were treated in triplicate; the protein inhibitor solutions were administered at 100 µgm/mL, while the controls were fed sterile H_2O. All flasks had 0.0125 µCi/mmole sodium [1-^{14}C]-acetate added approximately 10 mins after addition of the inhibitor or H_2O. The inhibitor solutions were added as follows: at t = 0, p-Fluorophenylalanine to 9 flasks, Cycloheximide to 9 flasks and, H_2O to 3 flasks; at t = 24 hours, p-Fluorophenylalanine to 9 flasks, Cycloheximide to 9 flasks and H_2O to 3 flasks; at t = 48 hours, p-Fluoropehnylalanine to 9 flasks, Cycloheximide to 9 flasks and, H_2O to 3 flasks; at t = 72 hours, p-Fluorophenylalanine to 6 flasks, Cycloheximide to 6 flasks and H_2O to 2 flasks; at t = 96 hours, p-Fluorophenylalanine to 3 flasks, Cycloheximide to 3 flasks and H_2O to 1 flask.

At 72 hours, one set of flasks (3/inhibitor and 1/control) from each time period already started (in this case t = 0, 24, 48

hrs) was harvested and worked up. This was repeated at 96 hours and again at 120 hours. The work-up consisted of measuring packed cell volume for each set of triplicates and $CHCl_3$ extraction for each separate flask. The $CHCl_3$ extracts were analyzed by HPLC for peak area and counts.

(+/-)-2-Fluoropropionic Acid (IS# 3)

The seed cultures for this experiment were started in the standard procedure by addding 1 vial frozen spore suspension to 12 flasks with 50 mL SP/LO media. The cultures were grown for 68 hours, then they were spun down (7.5K, 10 min., Sorvall^R-RC-5B, SS34 rotor), washed twice with Na_2PO_4 buffer (50mM, pH 7.4), resuspended in 600 mL 14C Media, then dispensed into 24 flasks with 25 mL media/125 mL flask using sterile technique. The flasks were then returned to the shaker.

All flasks were treated in triplicate. Samples (1.0 mL aliquots) were removed from 21 of the 24 flasks at the following times: t = 0 (time of transfer), 6, 12, 18, 20, 22, 26, 30, 33.5, 48, 54, 58.5, and 70.5 hours (just prior to harvesting). In addition, three of the flasks were sampled only at t = 0 and 70.5 hours. There were three sets of controls: (i) three flasks were assayed only at the beginning and at the end and nothing was added to the culture (referred to as "CO"); (ii) three flasks were assayed at each time point and nothing was added to the culture (CO'); (iii) three flasks were assayed at each time point and the cultures were fed both sterile H_2O (pH 2.6) and sodium [1-^{14}C]-

acetate (CO"). The sodium [1-^{14}C]-acetate (5 uCi/flask) was added to 18 flasks at 18 hours, after removing the assay sample. Then at 20 hours, after the assay, the inhibitor was added at the following concentrations: 0 (sterile H_2O, pH2.6), 2, 5, 10, 20 and 50mM. Each concentration of the 2-fluoropropionic acid was prepared as a separate solution; the pH of the 10mM solution was 2.8.

The aliquots removed during the course of the fermentation were assayed by dry cell weight and HPLC (peak area and counts) following the standard procedure. After 70.5 hours, all 24 flasks were harvested and the triplicate flasks were combined. The final pH and packed cell volume for each set was recorded; then the media was extracted with 3 x 100 mL $CHCl_3$/flask and the $CHCl_3$ was dried over anhydrous Na_2SO_4 and evaporated. The residue from each $CHCl_3$ extract was weighed, assayed by HPLC (peak area and counts), and purified by HPLC for spectral analysis (^{19}F-NMR, 188 MHz).

(+/-)-2-Fluoropropionic Acid (IS# 5)

The seed cultures for this experiment were started by adding 1/3 vial frozen spore suspension to each of 21 flasks with 50 mL SP/LO media. The cultures were grown for 66 hours, then the cells were spun down, washed and resuspended in 1.0 L 14C Media in the same manner as described in the previous experiment. The media/cells mixture was dispensed into 21 flasks of 50 mL media. The flasks were then returned to the shaker.

All flasks were treated in triplicate. Samples (1.0 mL aliquots) were removed at the following times: 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. At 48 hours, after assaying, sodium [1-^{14}C]-acetate (5 uCi/flask) was added to each flask. At 54 hours, after assaying, the inhibitor solutions were added to each flask as follows: 0 (sterile H_2O, pH 7), 2, 3.5, 5, 6.5, 8 and 10mM. Each concentration of inhibitor was prepared as a separate solution.

pH 7.

The aliquots removed during the course of the fermentation were assayed by dry cell weight and HPLC (peak area and counts) following the usual procedure. After 123 hours, the flasks were sampled and then harvested. The final pH was recorded for each flask, then the triplicate flasks were combined for the remainder of the work-up. The packed cell volume was measured and the media was extracted with 3 x 100 mL EtOAc. The EtOAc was dried over anhydrous Na_2SO_4 and evaporated. The residue from each EtOAc extract was weighed, analyzed by HPLC (peak area and counts), then purified on the semi-prep HPLC for spectral analysis (^{19}F-NMR, 188 MHz).

(+/-)-2-Monofluoro Succinic Acid (IS# 8)

The seed cultures for this experiment were started in the standard procedure by adding 1 vial frozen spore suspension (cell count 3 x 10^6/0.1 mL) to 50 mL MYM Media. The seed cultures were grown for 72 hours, then used to inoculate (2% v/v) 18 flasks of

25 mL MYM Media/125 mL flask. The flasks were then returned to the shaker.

All flasks were treated in triplicate. The flasks were sampled (1.0 mL aliquots) at t = 72 (after inoculation), 96, 102, 120, 144 and 167.5 hours (just prior to harvesting). The inhibitor was added at 96 hours after assaying at the following concentrations: 0 (sterile H_2O, pH 7), 2, 3.5, 5, 6.5 and 10mM. The sodium [1-^{14}C]-acetate solution (0.066 uCi/flask) was added at 99 hours. The pH of the fluorosuccinate solution was 1.5.

The aliquots removed during the course of the fermentation were assayed by dry cell weight and HPLC (peak area and counts) by the standard procedure. After 167.5 hours, the flasks were assayed and then harvested. The pH and packed cell volume was measured for each flask. The contents of each flask was extracted with 15 mL $CHCl_3$/25 ml media and the $CHCl_3$ extracts were assayed by HPLC (peak area and counts).

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

[U-^{14}C] 2(S)-2-Ethyl Fluoropropionate (IS# 9)

The seed cultures for this experiment were started in the standard procedure by adding 1 vial frozen spore suspension (cell count: 3 x 10^6) 50 mL MYM Media and grown for 72 hours before being used to inoculate (2% v/v) 22 flasks of 50 mL MYM Media/250 mL flask. The flasks were then returned to the shaker.

Samples (1.0 mL aliquots) were removed from all 22 flasks at the following times: t = 72 (after inoculation), 82, 83.5, 96 and

108.5 hours. The inhibitor solutions were added at 82 hours, after assaying. The flasks were fed as follows: 2mM 2-fluoropropionic acid (4 flasks); 3.5mM 2-fluoropropionic acid (4 flasks); 5mM 2-fluoropropionic acid (4 flasks); 2mM ethyl 2-fluoropropionate (3 flasks); 3.5mM ethyl 2-fluoropropionate (3 flasks); sterile H_2O (pH 7, 4 flasks). The pH of the inhibitor solutions was adjusted to 7.4 and 2 x 0.1 mL were removed for counting purposes prior to sterilization. The [U-^{14}C]-2(S)-2-fluoropropionic acid counted at 4.17 x 10^5 dpm/mmole; the [U-^{14}C]-2(S)-ethyl 2-fluoropropionate was 1.13 x 10^5 dpm/mmole.

The aliquots removed during the fermentation were assayed by dry cell weight and HPLC (peak area and counts) by the standard procedure. The flasks were harvested at 124 hours, and worked-up by measuring the pH and packed cell volume of each flask. The media was extracted with 25 mL $CHCl_3$/50 mL media. The $CHCl_3$ extract was assayed by HPLC (peak area and counts).

Baseline Data Experiment

The seed culture for this experiment was started in the standard procedure by adding 1 vial frozen spore suspension (cell count: 2.8 x 10^8/0.1 mL) to 50 mL SP/LO media. The seed culture was grown for 72 hours, then used to inoculate (2% v/v) each of 39 flasks of 25 mL SP/LO media/125 mL flask. The flasks were returned to the shaker.

The feeding/inhibitor solutions used in this experiment were prepared by admixing the unlabelled precursor with the analogous

[^{14}C] labelled precursor. 2 x 0.1 mL aliquots were removed and diluted to 100 mL for counting purposes, then the remainder of the solution was filter sterilized using additional H_2O rinses to bring the total volume to the required amount. The sodium [1-^{14}C]-acetate solution was 1.858 x 10^6 dpm/mmole; the sodium [1-^{14}C]-propionate solution was 1.9114 x 10^6 dpm/mmole; the sodium [2,3-$^{14}C_2$]-succinate solution was 1.975 x 10^7 dpm/mmole; the sodium [1-^{14}C] butyrate solution was 9.25 x 10^6 dpm/mmole.

All flasks were treated in triplicate, adding the feeding/inhibitor solutions at 2, 5 and 10mM levels at 72, 96, 120 and 144 hours after innoculation (this is equivalent to a normal feeding experiment as explained in Chapter Two). Three flasks were used as controls and were fed sterile H_2O.

At 168 hours all flasks were harvested and worked up as follows: add to each flask 2.5 gm celite and 25 mL EtOAc, stir vigorously for 1-1.5 hours, then extract the aqueous phase with EtOAc (2 x 25 mL). The combined EtOAc extracts were washed with brine, dried over anhydrous Na_2SO_4, and evaporated <u>in vacuo</u>. The resulting residue from the EtOAc extract was filtered prior to loading the sample on the semi-prep HPLC column.

The HPLC peak area was used to quantitate the amount of Lasalocid A, Homologs and Isolasalocid A present, and 1 mL aliquots of the appropriate fractions were counted in order to determine the specific incorporation of each precursor.

RESULTS AND DISCUSSION

Precursor Pressure

During the course of the biosynthetic studies the most consistent observation of S. lasaliensis fermentation cultures was the continual irregularity in the production of the antibiotic lasalocid A. The methodology employed during these studies (Chapter One, Results and Discussion) used reasonable, but not rigorous, techniques to ensure a uniform culture; nevertheless, the levels of antibiotic production varied considerably, and to a certain degree, appeared to depend on the precursor and the concentration being used. The irregularity in production of secondary metabolites by other Streptomyces has been seen before, even under highly reproducible experimental conditions (W.A. Strohl, personal communications). The dependency of secondary metabolite production on exogenous precursors has also been noted previously (see for example: Day et al., 1973, regarding the polyether antibiotic monensin A; May and Formica, 1974). While this phenomena is generally referred to as "precursor pressure", there has been no attempt to define or quantitate it. Consequently, it was decided to look at the effect of the normal substrates used in the biosynthesis of lasalocid A upon its production.

The experiment carried out (referred to as the Baseline Data Experiment, or BDE) involved feeding acetate, propionate,

succinate and butyrate at a representative range of concentrations to cultures of S. lasaliensis under the standard conditions for a feeding experiment. At the end of the fermentation, each culture was analyzed for the amount of lasalocid A, lasalocid homologs and isolasalocid, as well as for the amount of incorporation of the precursor into each of the three products. The results shown in Figure 50 prove that there is a definite effect of the exogenous precursors on antibiotic production. The precursor acetate inhibited the antibiotic production by about 40% at 2mM levels and by approximately 50% at higher levels. At the same time the specific incorporation of acetate showed the expected increase in progressing from 2mm levels to 10mM levels. The precursor propionate, while overall inhibitory, showed a maximum level of production at the intermediate concentration of 5mM (about 30% inhibition at 5mM versus approximately 50% inhibition at 2mM and 10mM). Propionate like acetate showed the expected rise in specific incorporation into the products in going from lower concentrations to higher concentrations. The precursor succinate showed the greatest effect, where it was seen that the production of the antibiotic was increasingly inhibited in a range of 50-80% as the concentration of the precursor increased. Like the other precursors, the specific incorporation of succinate into the antibiotic increased with the increased concentration of the precursor. Butyrate was distinct from the other three precursors; at higher levels it was clearly stimulatory. The production of

lasalocid A in the presence of butyrate, relative to the control, progressed from 0.6 at 2mM to 1.3 at 5mM and 1.1 at 10mM. Again, the results for butyrate were distinct with regard to its effect on the specific incorporation into the products. This effect is particularly noticeable for the homologs and isolasalocid A. While the effect of the exogenous precursors is clearly shown in this experiment, it is difficult to rationalize all of the results. Only for the precursors succinate and butyrate can reasonable explanations be given.

In the case of succinate, the drastic inhibition of the antibiotic is attributed to a physiological effect on the energy-producing metabolism of the organism. That is, exogenous succinate appears to decrease the respiration rate of the cultures. This result was seen in an independent experiment, where the respiration of S. lasaliensis cultures was monitored with an O_2 electrode. The addition of succinate at concentrations of 0.8 - 2.5 mM resulted in a reproducible decrease in O_2 consumption by the cells. This effect has also been seen in other procaryotes, such as Arthrobacter (J. Ensign, personal communication), and probably is due to an inhibition of α -ketoglutarate dehydrogenase or citrate synthetase by succinyl-CoA produced from the succinate in vivo.

The results of the butyrate feeding corroborate Westley's hypothesis that a change in the ratio of available propionate/butyrate will alter the final ratio of lasalocid

A/lasalocid homologs composition. Westley et al. (1974c) had attributed an observed increase in the percentage of lasalocid homologs from low yielding fermentations to insufficient propionate in the fermentation media. However, there was no report of an attempt to further investigate or quantitate this effect. In this experiment, butyrate clearly stimulates the production of lasalocid A and at higher levels it also stimulates production of the lasalocid homologs. This effect seen on the lasalocid homologs is easy to rationalize based on the known structure of the homologs and the proposed biosynthetic pathway; that is, when the C-4 subunit is available in large excess over the C-3 subunit, it is used in place of the C-3 subunit occasionally during the carbon chain extension process.

Inhibition of Fatty Acid and Polyketide Synthesis

Cerulenin (Figure 47) was originally isolated as an antifungal antibiotic from Cephalosporium caerulens. Subsequent work on this antibiotic showed that it specifically inhibits the β-ketoacyl synthetase enzyme of fatty acid synthesis, and that it will also inhibit polyketide biosynthesis, presumably at the analogous condensation step (see for example: Omura, 1976; 1981). It has been noted that cerulenin will completely inhibit the production of polyketides in cultures until cerulenin itself is metabolized, at which point there is a release from inhibition and the production of the polyketide resumes (see for example: Omura, 1976; Martin and McDaniel, 1975). In this investigation (IS #8),

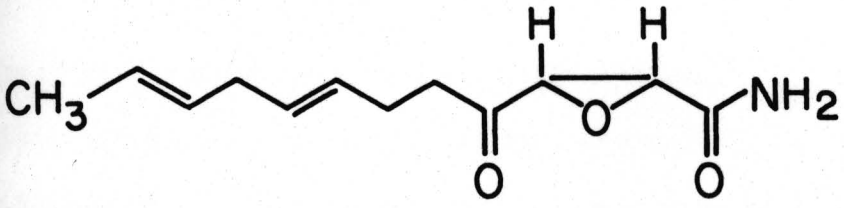
cerulenin was added to cultures of S. lasaliensis at concentrations of 5, 10, 15 and 20 µgm/mL. The results of the experiment (Figure 51) show exactly what would be predicted based on the previous studies with cerulenin. The production of the antibiotic lasalocid A falls off immediately following addition of the cerulenin; the release from inhibition occurs for the two lowest concentrations of cerulenin (5 and 10 µgm/mL), and then later for the other three concentrations. The conclusion that can be drawn from this experiment is that lasalocid A is produced in a manner similar to other polyketides, where the mechanism of carbon chain formation involves a condensation between acylthioesters and α-carboxyacylthioesters analogous to that for fatty acids.

Oxygenase Inhibitors

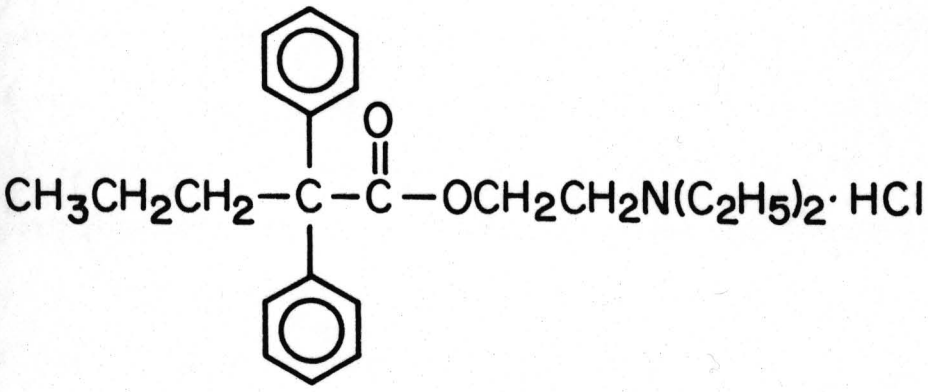
The hypothetical scheme for the biosynthesis of lasalocid A involves a putative diene intermediate that undergoes air oxidation to form a diepoxide that can rearrange to give the two terminal cyclic ethers. From the biosynthetic studies it was shown that $^{13}C/^{18}O$ labelled precursors did not incorporate ^{18}O into the oxygens attached to C-19 and C-22 of lasalocid A, thus lending support to this biosynthetic hypothesis. In these investigations it was decided to look at the effect of oxygenase inhibitiors on the production of lasalocid A. This was done in order to verify whether or not the biosynthesis of lasalocid A is dependent on air oxidation, and if so, would the putative diene intermediate accumulate in sufficient amount for its isolation and

identification. Initially three known oxygenase inhibitors were chosen. The inhibitor, SKF-525A (Figure 47), is a well known inhibitor of several reactions involved in drug metabolism, including side-chain oxidation and hydroxylation (see for example: Holmes and Bentz, 1960; Dennis and West, 1967; Reid, 1968). The inhibitor, GEB, has been shown to have an affect on the hydroxylase activity required for the biosynthesis of the macrolide antibiotic erythromycin A. In these experiments, a derivative of GEB was used (2-diethyl-GEB). The third inhibitor, AY-994, has been used in studying the oxidation reactions involved in sterol biosynthesis (see for example: Schroepfer, 1982).

Figure 47. Structure of the Fatty Acid and Polyketide Synthetase Inhibitor, Cerulenin; and the Mono-Oxygenase Inhibitor, SKF 525-A



CERULENIN



SKF 525-A

In one of the experiments with the oxygenase inhibitors (IS# 7, Figure 52), the seed cultures were started both with and without the inhibitors present, and then for all the cultures the inhibitors were added later during the fermentation at a range of concentrations from 10-30 ugm/mL. The cultures that had had the inhibitors added to the seed cultures showed a more pronounced inhibition of antibiotic production, where SKF-525A showed the greatest effect. The cultures that had been started with normal seed cultures showed a decrease in antibiotic production in progressing from inhibitor concentrations of 15-30 µgm/mL, whereas the cultures that were started with inhibitor present in the seed culture were not as dependent on concentration of the subsequent addition of inhibitor. In a subsequent experiment the inhibitor SKF-525A was used in concentrations of 50 and 100 µgm/mL, and was found to not only inhibit the antibiotic production but also caused a drastic decrease in total cell mass as measured by PCV. While none of these experiments yielded concrete evidence of possible enzyme free intermediates in the biosynthetic pathway, the fact that the oxygenase inhibitors decrease the antibiotic production adds one more piece of evidence to support the biosynthetic hypothesis that laslaocid A is formed via a diene intermediate.

Protein Synthesis Inhibitors

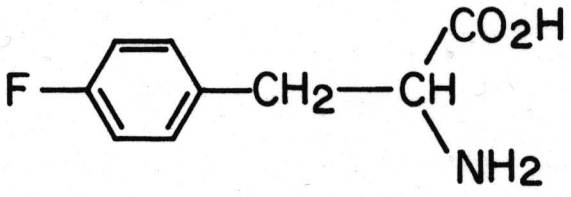
There have been several reports on the use of inhibitors of protein synthesis in secondary metabolite studies, most notably

with the polyketide 6-MSA and with fungal phenolic metabolites derived from acetate. In the studies on 6-MSA (Bu'Lock et al., 1968) it was found that the protein inhibitors, cycloheximide and p-fluorophenylalanine, could inhibit the production of the polyketide depending on what concentration was used and at what time in the fermentation it was added. It was concluded that 6-MSA synthetase is produced early in the fermentation, probably during replicatory growth in the trophophase, prior to actual synthesis of 6-MSA. It was also noted that once the synthetase was formed it was able to maintain synthesis of the polyketide even when additional protein synthesis had been blocked. From this it was suggested that the synthesis of 6-MSA is dependent on levels of enzyme and not on substrate. In a separate study (Light, 1970), it was shown that when inhibitors such as cycloheximide and p-fluorophenylalanine were added at levels that would only partially block protein synthesis, there was an observed stimulation in the production of 6-MSA. In one report which pertained to the effect of protein synthesis inhibitors on the production of phenolic metabolites in a fungus (Packter and Light, 1974) it was noted that the inhibitor p-fluorophenylalanine inhibited the secretion of acetate derived phenolic compounds, but not shikimate derived phenolic compounds. It was also observed that this inhibitor caused the secretion of 4-fluorophenylpyruvic acid, presumably by the transamination of the inhibitor. While it is difficult to thoroughly explain all of these noted effects,

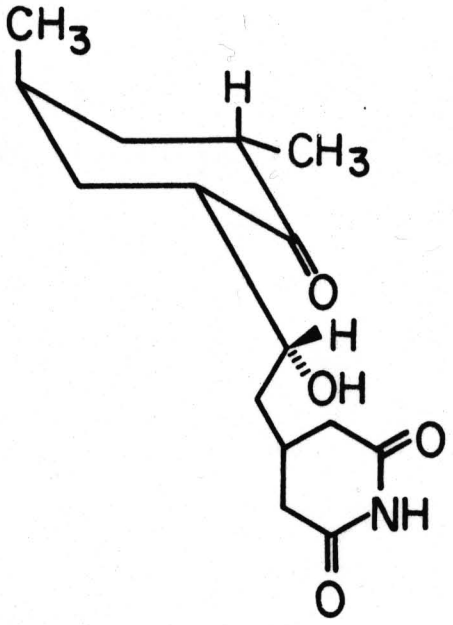
they have produced interesting information regarding regulation and production of secondary metabolites. As a consequence, it was decided to look at the effects of these two inhibitors (Figure 48) on the production of the polyether antibiotic lasalocid A.

In one experiment with the protein synthesis inhibitors (IS# 7, Figure 52), the experiment was set up analogous to the previously described oxygenase inhibitor study, except the inhibitors were used in a range of concentrations from 50-200 µgm/mL. The inhibition by p-fluorophenylalanine is clearly shown. The cultures with the inhibitor present in the seed culture were not inhibited to the same extent as those without inhibitor in the seed culture; probably due to metabolism of the p-fluorophenylalanine. There is also a notable release from inhibition at the highest concentration of p-fluorophenylalanine in both cases. One possible explanation is that at higher concentrations the p-fluorophenylalanine can induce enzymes that would metabolize or otherwise utilize the compound, and as a result less of it becomes available for acting as an inhibitor. On the other hand, the presence of cycloheximide in the seed culture causes a pronounced inhibition, while the cultures without cycloheximide in the seed culture exhibit an increase in production.

Figure 48. Structures of the Inhibitors of Protein Synthesis: p-Fluorophenylalanine and Cycloheximide



p – FLUOROPHENYLALANINE



L – CYCLOHEXIMIDE

In a second experiment, the protein synthesis inhibitors, at concentrations of 100 µgm/mL, were added to cultures of S. lasaliensis at different times throughout the fermentation. The results of this experiment (IS# 11, Figure 53) like the previous experiment, show that p-fluorophenylalanine is a more effective inhibitor of lasalocid A biosynthesis at the concentrations tested, and also indicate that the inhibition caused by p-fluorophenylalanine is alleviated with time, which could be explained by the metabolism of the inhibitor via normal amino acid degradation pathways. From the data it appears that the time between 48 and 72 hours after inoculation is the most critical for the onset of antibiotic production and the incorporation of labelled substrate into the antibiotic. These initial studies with the protein synthesis inhibitors, especially p-fluorophenylalanine, show promising results with regard to defining the regulation of lasalocid A synthesis.

Fluorinated Substrate Analogues

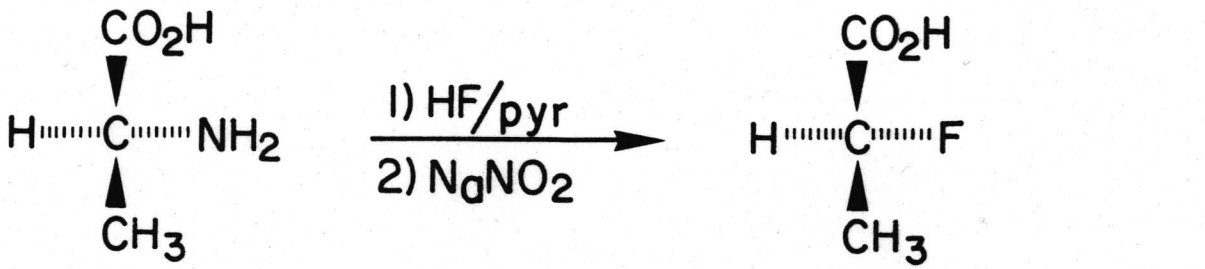
The use of fluorinated substrate analogues has become increasingly more important as a technique in the field of enzyme mechanistic studies (see for example: Walsh, 1983). However, the use of substrate analogues in whole-cell metabolic studies has been very limited. There was one report in which the authors investigated the effects of 2- and 3-fluoropropionic acid and their ethyl esters on the production of the macrolide antibiotic erythromycin (Pape and Grisebach, 1967). In this study it was

found that these substrate analogues caused an inhibiton of erythromycin synthesis at 1-15 mM concentrations of the inhibitors in the growth medium, with the largest effect seen for 3-fluoropropionic acid ethyl ester. One explanation for this inhibition is that the fluorinated analogue is acting as a suicide substrate inhibitor for propionyl-CoA carboxylase or at the point of condensation of a propionate subunit with the growing carbon chain (Stubbe and Abeles, 1977; Stubbe et al., 1979). Consequently, it was decided to look at the effect of fluorinated analogues on the synthesis of lasalocid A.

These studies involved looking at two different substrate analogues under different conditions. Initially, the effects of racemic 2-fluoropropionic acid was examined, both at a wide range of concentrations (IS# 3, Figure 54), and then in a narrow range of concentrations (IS# 5, Figure 55), followed by an analogous study with racemic 2-fluorosuccinic acid (IS# 8, Figure 51). A final study was done using [U-^{14}C]-(2S)-2-fluoropropionate and the corresponding ethyl ester (IS# 9, Figure 56). The (2S) enantiomer of the fluorinated analogue (Figure 49) would be expected to be carboxylated by propionyl-CoA carboxylase to give (2R)-2-fluoromethylmalonyl-CoA which would correspond to (2S)-methylmalonyl-CoA, if the enzyme does not differentiate between a fluorine atom and a hydrogen atom prior to catalysis. The results of this series of studies proved to be quite

unexpected; fluoropropionate stimulates the production of the polyether antibiotic lasalocid A under certain conditions.

Figure 49. Synthesis of (2S)-2-Fluoropropionic Acid



L-ALANINE

2-(S)-F-PROPIONIC ACID

The initial studies (IS# 3,5 and 8) used the free acid form of the substrates, which is very acidic. At higher concentrations of the fluoropropionic acid, and at lower and moderate concentrations of the fluorosuccinic acid, the acidity of the inhibitor solutions was such that the organism could not compensate for it. This was verified with controls that were run at the same pH as the inhibitor solutions (IS# 3) and at normal pH (IS# 5). Under the acid conditions, the antibiotic lasalocid A was found to be unstable and could no longer be detected by HPLC assay or by screening for radioactivity counts. At low to moderate concentrations of the fluoropropionic acid, the organism can compensate for the acidity and metabolize the substrate. Even under these conditions some stimulation in the production of the antibiotic can be seen (IS# 5 at levels of 2 and 3.5 mM). The most noticeable effect was seen in the final study where [U-^{14}C]-(2S)-2-fluoropropionate and the corresponding ethyl ester were used as the substrates. In this study, the fluoropropionate was titrated to pH 8 prior to addition to the culture. Both the fluoropropionate and its ethyl ester caused a marked, 2-8 fold, increase in the final concentration of the antibiotic lasalocid A at concentrations of 2-5 mM of the substrate analogue. At the same time, no substrate was incorporated into the antibiotic as assayed for by radioactivity and by ^{19}F-NMR spectroscopy. These results were totally unexpected and are undocumented in the literature. While it is beyond the scope of this study to

accurately analyze what caused the stimulation of antibiotic production, it is possible to suggest a reasonable rationale. It can be imagined that the fluorinated substrate analogue has acted on the enzymes responsible for the formation or availability of the normal substrates, for example, the enzymes involved in fatty acid or amino acid degradation or the carboxylation of propionate, and altered the regulation of these enzymes so as to cause an overproduction of the enzymes and the normal substrates. But, until the enzymes involved in the synthesis of the antibiotic are available to study, it cannot be said for certainty where or how the fluorinated substrate analogue acts.

The results presented in this chapter show that considerable information regarding the regulation and physiological effects of the production of the polyether antibiotic lasalocid A can be obtained in a whole-cell system. The studies presented here are not meant to be complete; however, they have laid the groundwork for further investigations.

Figure 50. Results of the Baseline Data Experiment

BASELINE DATA EXPERIMENT

BDE CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
2-A-L	1.24713	1.19135	1.36828	1.26892	.08847
2-A-H	.12151	.11069	.13283	.12168	.01107
2-A-I	.23345	.22276	.24771	.23464	.01247
5-A-L	1.12058	.70036	.93996	.92030	.21011
5-A-H	.09434	.06792	.08679	.08302	.01321
5-A-I	.17030	.12606	.14941	.14859	.02212
10-A-L	1.35894	.88761	1.01663	1.08773	.23566
10-A-H	.16377	.09660	.07044	.11027	.04667
10-A-I	.23001	.13000	.15138	.17046	.05001
2-P-L	1.21887	.66301	1.04759	.97649	.27793
2-P-H	.11119	.06415	.09509	.09015	.02352
2-P-I	.18578	.10321	.16587	.15162	.04128
5-P-L	1.41399	2.18168	1.57126	1.72231	.38385
5-P-H	.15622	NA	.12151	.13887	.01736
5-P-I	.22805	NA	.24771	.23788	.00983
10-P-L	1.12610	1.00926	1.41301	1.18279	.20188
10-P-H	.10038	.08101	NA	.09069	.00969
10-P-I	.17251	.14056	NA	.15654	.01597
2-S-L	1.00016	1.55848	.43349	.99738	.56250
2-S-H	.09560	.21031	.07044	.12545	.06994
2-S-I	.15482	.15973	.06488	.12647	.04743
5-S-L	.80505	1.20757	.91784	.97682	.20126
5-S-H	.06692	.09509	.09056	.08419	.01409
5-S-I	.08847	.14744	.13516	.12369	.02949
10-S-L	.62123	.23001	.26835	.37320	.19561
10-S-H	.12739	.07044	.03421	.07735	.04659
10-S-I	NA	.07471	.04423	.05947	.01524
2-B-L	.79472	2.21756	1.21396	1.40875	.71142
2-B-H	.09660	.10667	.09434	.09920	.00616
2-B-I	.13270	.36173	.18185	.22543	.11451
5-B-L	3.02949	NA	NA	3.02949	0.00000
5-B-H	.16302	NA	NA	.16302	0.00000
5-B-I	.34060	NA	NA	.34060	0.00000
10-B-L	3.20298	2.18807	2.19200	2.52769	.50745
10-B-H	NA	NA	.26515	.26515	0.00000
10-B-I	NA	.33175	.35141	.34158	.00983
S-25-L	1.87647	2.44168	2.92529	2.41448	.52441
S-25-H	.19080	NA	NA	.19080	0.00000
S-25-I	.29391	NA	NA	.29391	0.00000

BASELINE DATA EXPERIMENT

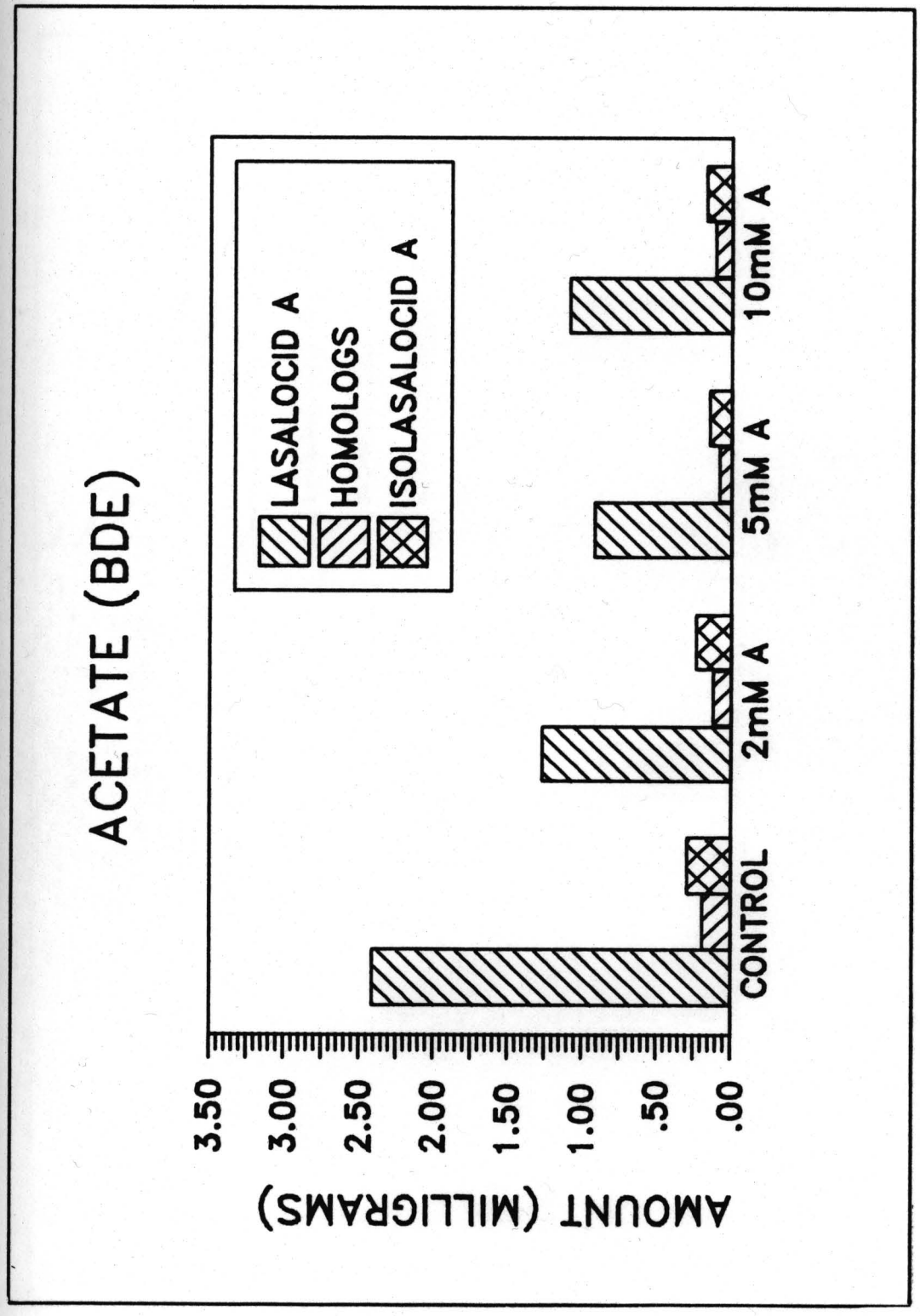
BDE CODE	C/MM(1)	C/MM(2)	C/MM(3)	AVG C/MM	(+/-)	SP INC
2-A-L	1084679	119323	1871053	1025018	875865	.5517
2-A-H	1060463	1071356	1820238	1317352	379887	.7090
2-A-I	981087	818401	2180825	1326771	681212	.7141
5-A-L	3727410	3142410	3264105	3377975	292500	1.8181
5-A-H	2517866	2293139	2749047	2520017	227954	1.3563
5-A-I	1617235	1662383	1786465	1688694	84615	.9089
10-A-L	6956556	5327863	6767114	6350511	814346	3.4179
10-A-H	5710751	4793115	8066002	6189956	1636444	3.3315
10-A-I	3570581	2268382	2815611	2884858	651100	1.5527
2-P-L	442038	446900	683270	524070	120616	.3917
2-P-H	206934	187620	543504	312686	177942	.2337
2-P-I	110518	NA	260365	185442	74924	.1386
5-P-L	911544	839696	1792233	1181158	476269	.8828
5-P-H	539295	NA	1468333	1003814	464519	.7502
5-P-I	279416	NA	641671	460544	181128	.3442
10-P-L	2052443	2909150	3382149	2781247	664853	2.0787
10-P-H	1886784	1866008	NA	1876396	10388	1.4024
10-P-I	685388	848713	NA	767050	81663	.5733
2-S-L	1983846	1663373	1996674	1881298	166650	.1361
2-S-H	3858562	2502915	4839602	3733693	1168344	.2701
2-S-I	1593746	1930330	4654488	2726188	1530371	.1972
5-S-L	5835614	4886830	5576278	5432907	474392	.3930
5-S-H	13166933	10034273	9799258	11000155	1683837	.7957
5-S-I	9607634	8796928	10233107	9545890	718090	.6905
10-S-L	2000694	1408225	6190913	3199944	2391344	.2315
10-S-H	7984065	3341988	29053573	13459875	12855792	.9736
10-S-I	NA	6041759	543440556	274741158	268699398	19.8728
2-B-L	4652595	3882000	4238818	4257804	385298	.6575
2-B-H	9985657	16839375	9148372	11991134	3845502	1.8516
2-B-I	10534652	6473645	7845130	8284476	2030504	1.2793
5-B-L	12756325	NA	NA	12756325	0	1.9698
5-B-H	51541326	NA	NA	51541326	0	7.9588
5-B-I	24225268	NA	NA	24225268	0	3.7408
10-B-L	21647410	29048078	38535385	29743624	8443988	4.5929
10-B-H	NA	NA	45494836	45494836	0	7.0251
10-B-I	NA	57791462	58268532	58029997	238535	8.9608

BASELINE DATA EXPERIMENT

EXPERIMENT	MG PRODUCED RELATIVE TO CONTROL		
	L	H	I
2mM ACETATE	.53	.64	.80
5mM ACETATE	.38	.44	.50
10mM ACETATE	.45	.58	.58
2mM PROPIONATE	.40	.48	.52
5mM PROPIONATE	.71	.73	.81
10mM PROPIONATE	.49	.48	.53
2mM SUCCINATE	.41	.66	.43
5mM SUCCINATE	.40	.45	.42
10mM SUCCINATE	.15	.41	.26
2mM BUTYRATE	.58	.52	.77
5mM BUTYRATE	1.26	.86	1.16
10mM BUTYRATE	1.05	1.40	1.10

BASELINE DATA EXPERIMENT

EXPERIMENT	MG PRODUCED RELATIVE TO EACH PRECURSOR			SP. INC. RELATIVE TO EACH PRECURSOR		
	L	H	I	L	H	I
2mM ACETATE	1.00	.10	.19	1.00	1.29	1.29
5mM ACETATE	1.00	.09	.16	1.00	.75	.50
10mM ACETATE	1.00	.10	.16	1.00	.97	.45
2mM PROPIONATE	1.00	.09	.16	1.00	.60	.35
5mM PROPIONATE	1.00	.08	.14	1.00	.85	.39
10mM PROPIONATE	1.00	.08	.13	1.00	.67	.28
2mM SUCCINATE	1.00	.13	.13	1.00	1.99	1.45
5mM SUCCINATE	1.00	.09	.13	1.00	2.03	1.76
10mM SUCCINATE	1.00	.21	.20	1.00	4.21	8.12
2mM BUTYRATE	1.00	.07	.16	1.00	2.82	1.95
5mM BUTYRATE	1.00	.05	.11	1.00	4.04	1.91
10mM BUTYRATE	1.00	.11	.14	1.00	2.53	1.95


ACETATE (BDE)
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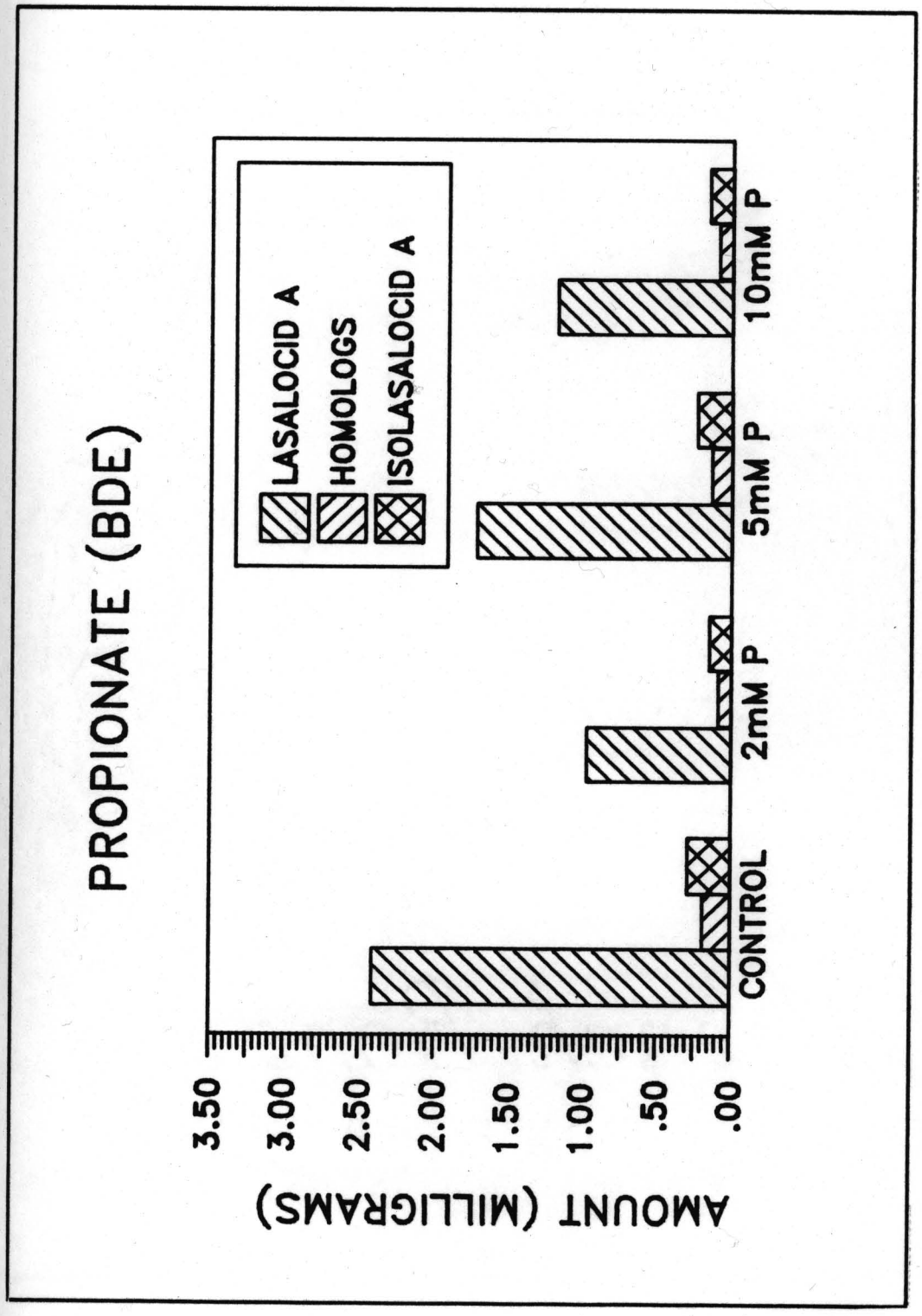
3.00
2.50
2.00
1.50
1.00
.50
.00
LASALOCID A
HOMOLOGS
ISOLASALOCID A
CONTROL
2mM A
5mM A
10mM A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HOMOLOGS
ISOLASALOCID A
AMOUNT (MILLIGRAMS)
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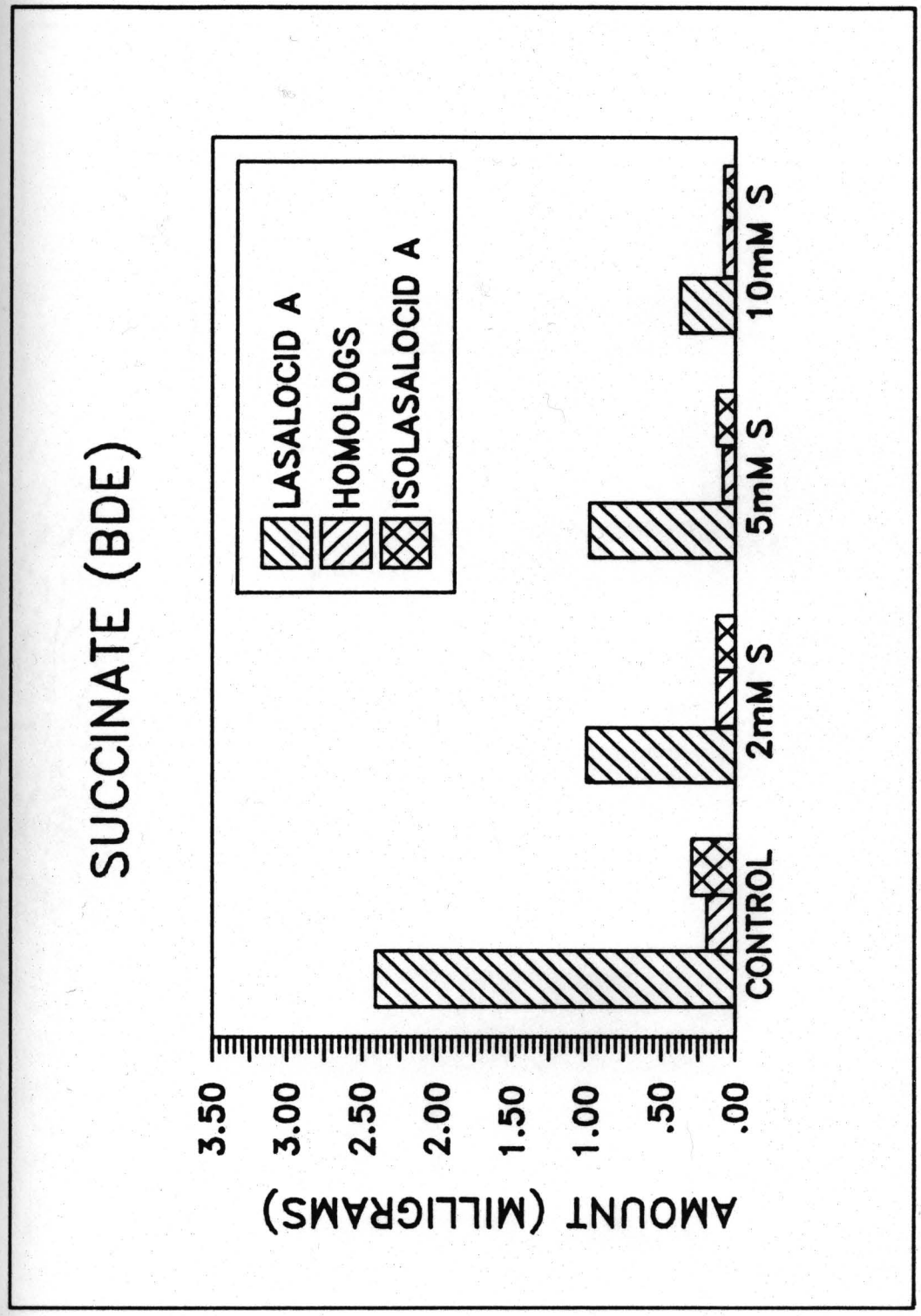
2.50
2.00
1.50
1.00
.50
.00
CONTROL
2mM P
5mM P
10mM P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ISOLASALOCID A
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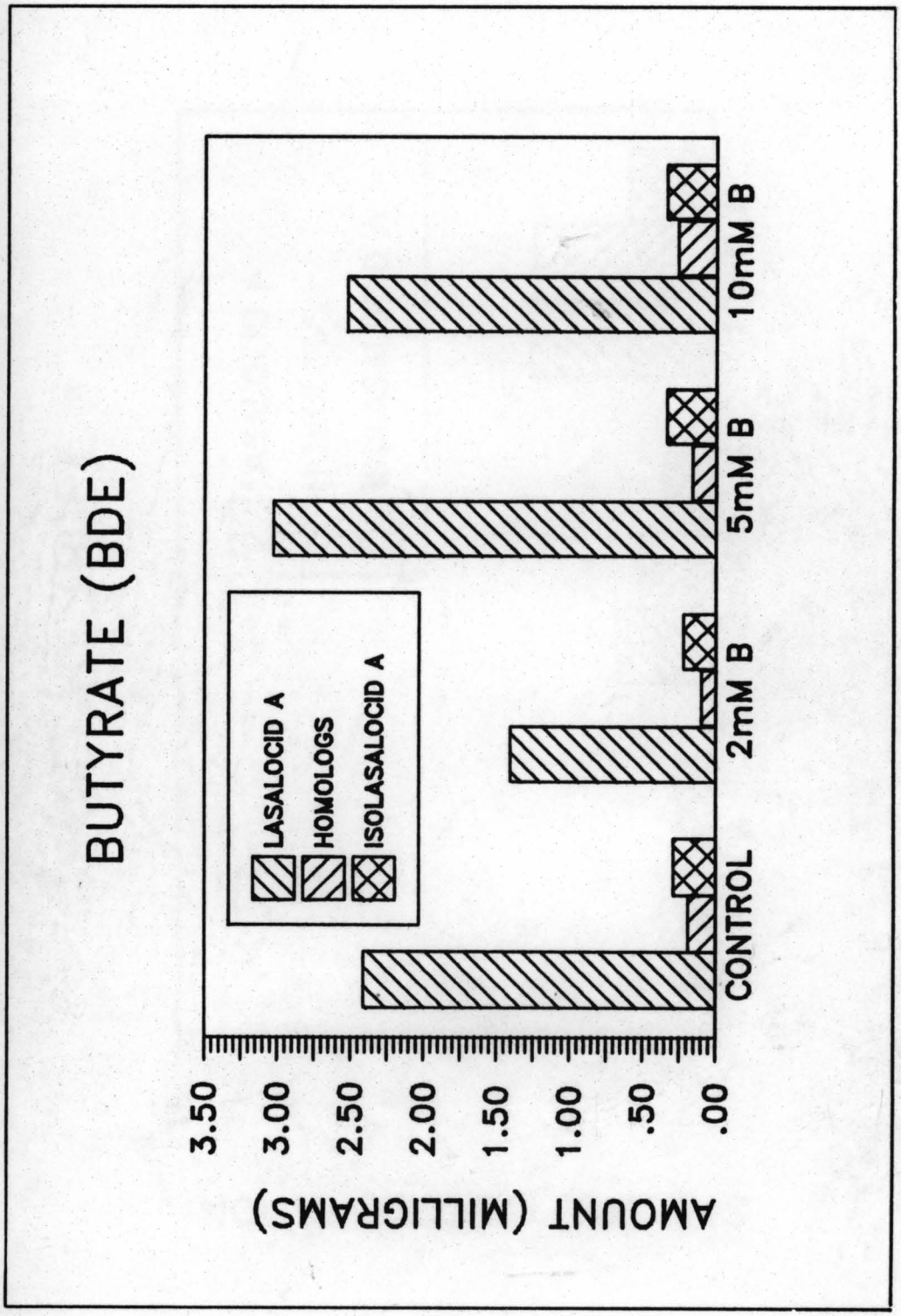
2.50
2.00
1.50
1.00
.50
.00
CONTROL
2mM S
5mM S
10mM S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HOMOLOGS
ISOLASALOCID A
AMOUNT (MILLIGRAMS)
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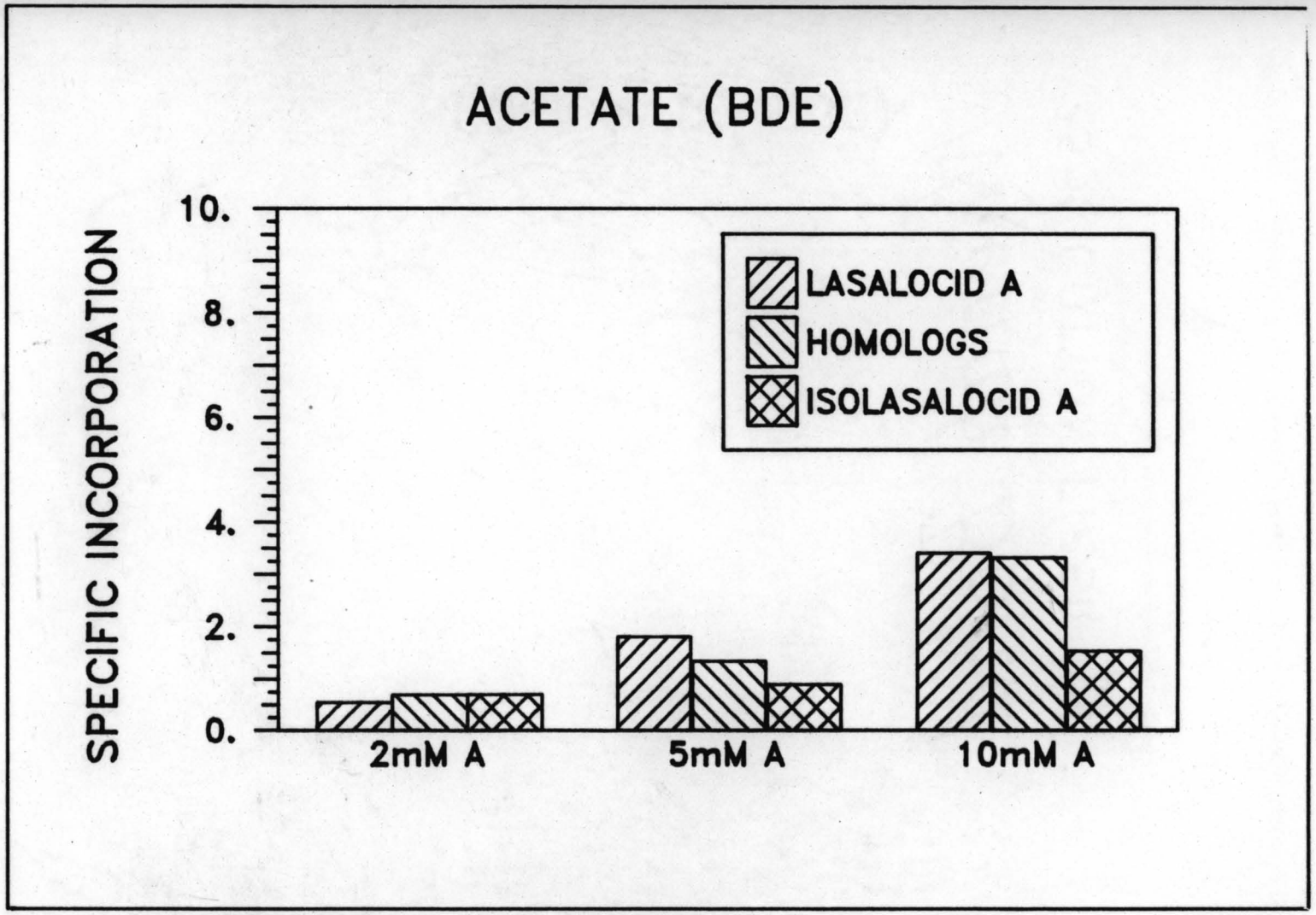
2.50
2.00
1.50
1.00
.50
.00
CONTROL
2mM B
5mM B
10mM B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SPECIFIC INCORPORATION
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8.
6.
4.
2.
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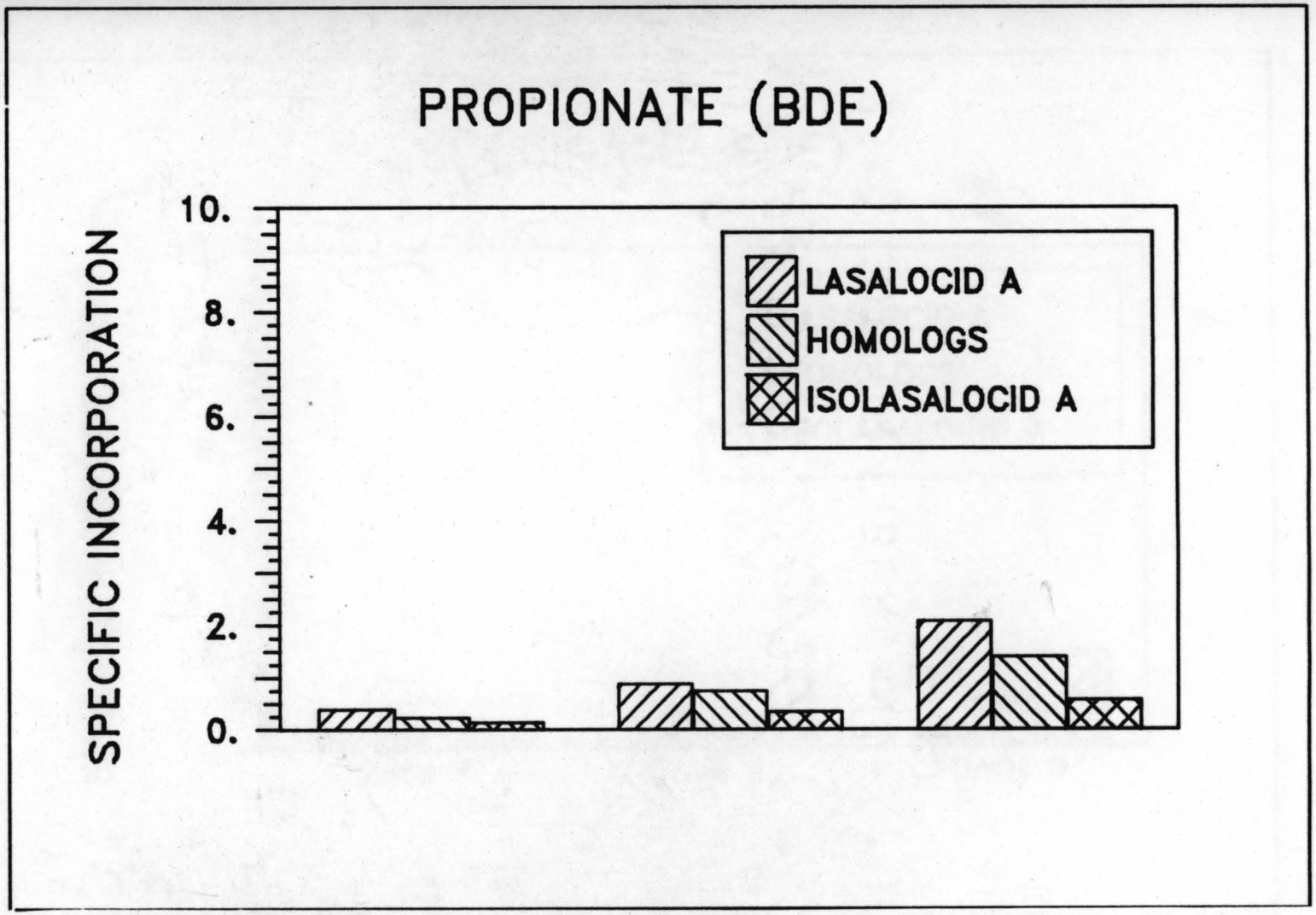
LASALOCID A
HOMOLOGS
ISOLASALOCID A
2mM A
5mM A
10mM A


PROPIONATE (BDE)
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8.
6.
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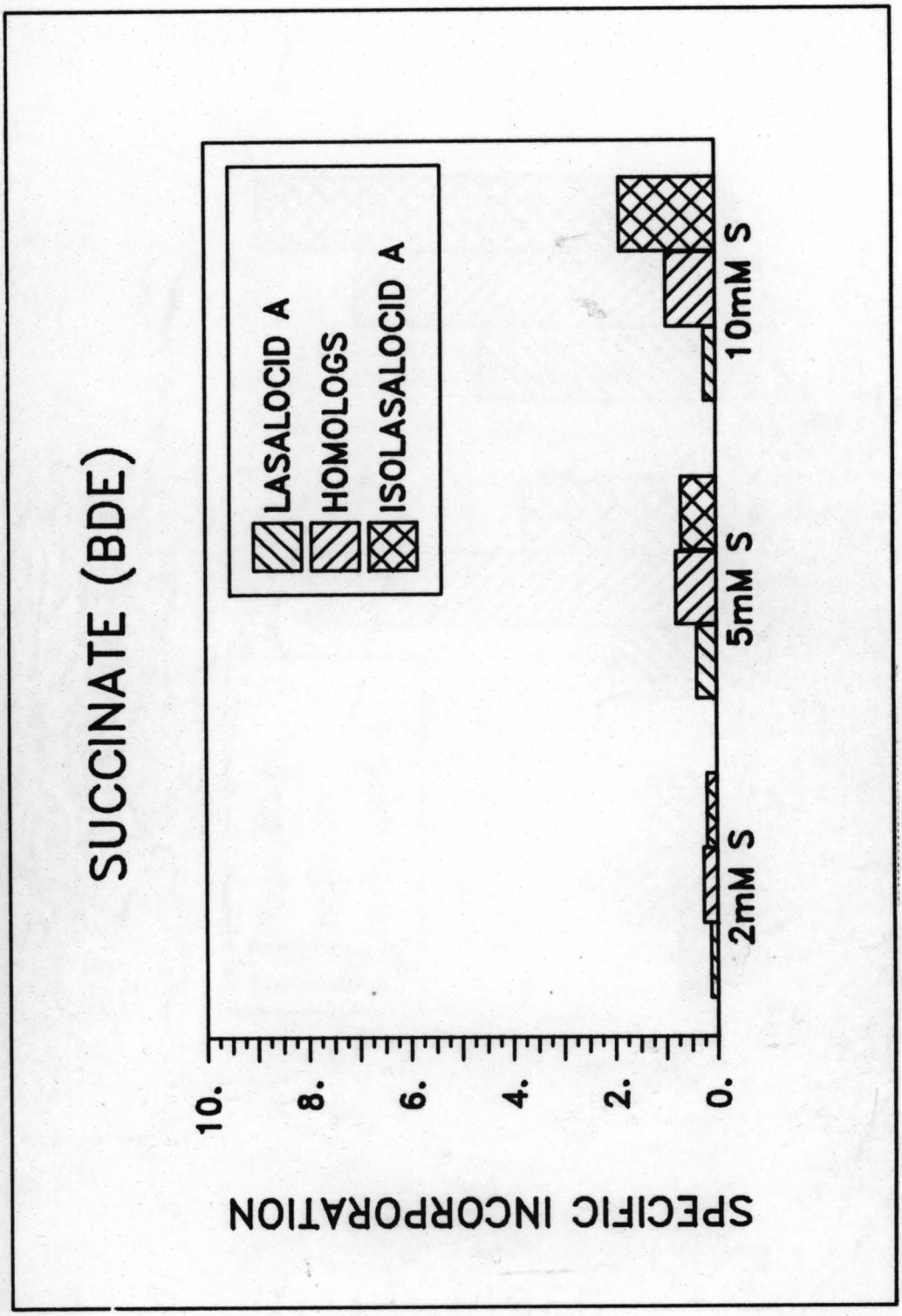
2.
0.
LASALOCID A
HOMOLOGS
ISOLASALOCID A


SUCCINATE (BDE)
SPECIFIC INCORPORATION
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8.
6.
4.
2.
0.
LASALOCID A
HOMOLOGS
ISOLASALOCID A
2mM S
5mM S
10mM S

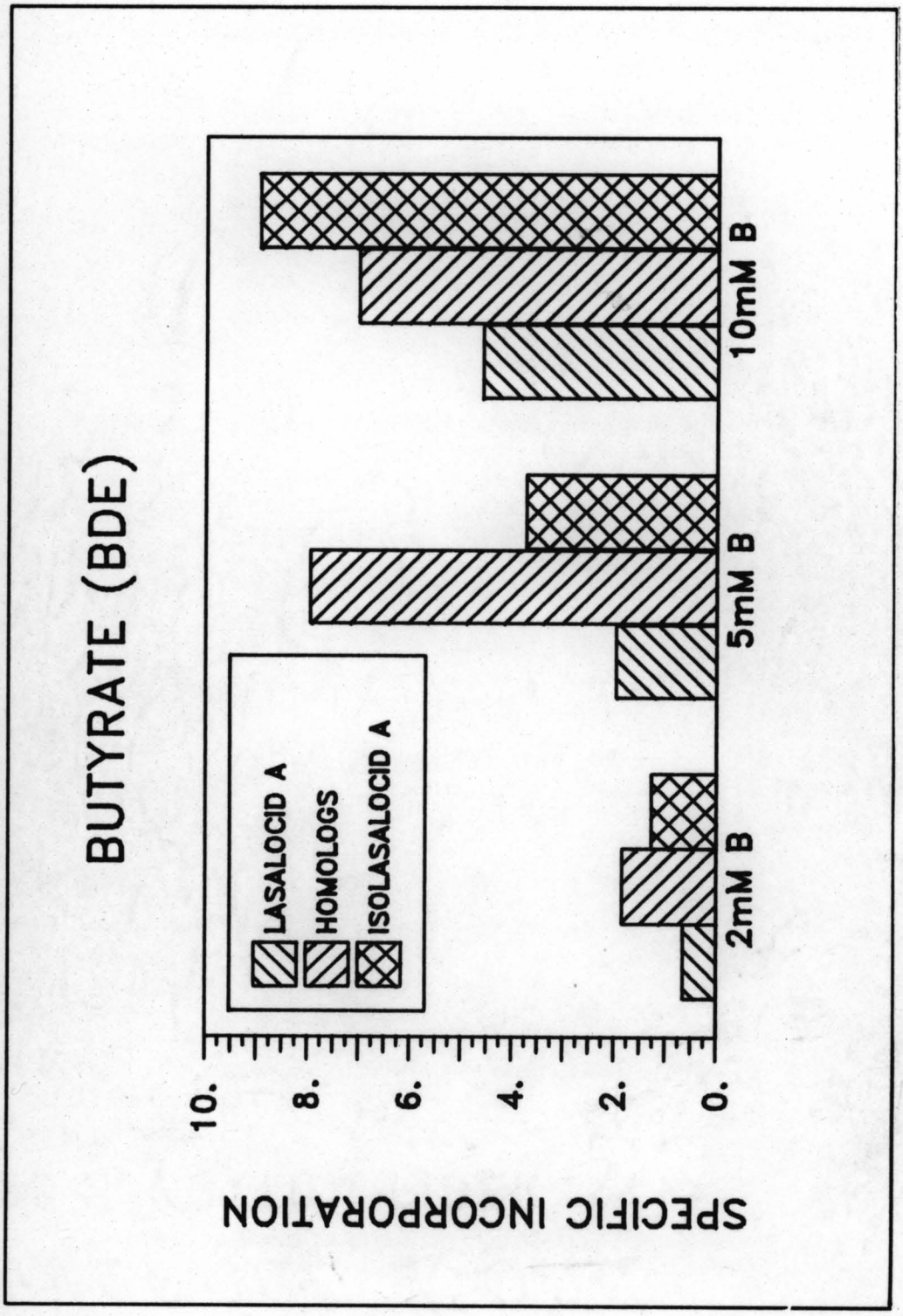
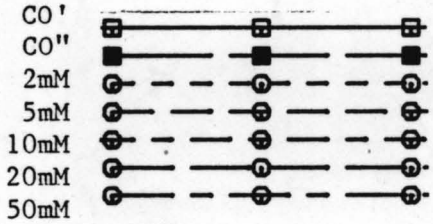

BUTYRATE (BDE)
SPECIFIC INCORPORATION
10.
8.
6.
4.
2.
0.
LASALOCID A
HOMOLOGS
ISOLASALOCID A
2mM B
5mM B
10mM B

Figure 54. Results of Inhibitor Study # 3:
(+/-)-2-Fluoropropionic Acid

Codes:



FLUOROPROPIONATE (IS# 3)

IS 3 CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
CO-0	.01109	.01234	.00841	.01061	.00197
CO-70.5	.00978	.01184	.00875	.01013	.00155
CO'-0	.01010	.00959	.00847	.00939	.00082
CO'-6	.00583	.00953	.00859	.00799	.00185
CO'-12	.00709	.00659	.00666	.00678	.00025
CO'-18	.00978	.01091	.00838	.00969	.00127
CO'-20	.06547	.01184	.01059	.02930	.02744
CO'-22	.00753	.00859	.00772	.00795	.00053
CO'-26	.00797	.00772	.00859	.00809	.00044
CO'-30	.01316	.01016	.01203	.01178	.00150
CO'-33.5	.01016	.00959	.00859	.00945	.00078
CO'-48	.01156	.01322	.01484	.01321	.00164
CO'-54	.01109	.01297	.01559	.01322	.00225
CO'-58.5	.01109	.01497	.01578	.01395	.00234
CO'-70.5	.01259	.01784	.01422	.01489	.00262
CO"-0	.01016	.00625	.00703	.00781	.00195
CO"-6	.00472	.00784	NA	.00628	.00156
CO"-12	.00578	.00703	.00922	.00734	.00172
CO"-18	.00809	.01081	.00998	.00963	.00136
CO"-20	.00922	.01016	.01184	.01041	.00131
CO"-22	NA	NA	NA	NA	NA
CO"-26	.00516	.00737	NA	.00627	.00111
CO"-30	NA	NA	NA	NA	NA
CO"-33.5	NA	NA	NA	NA	NA
CO"-48	NA	NA	NA	NA	NA
CO"-54	NA	NA	NA	NA	NA
CO"-58.5	NA	NA	NA	NA	NA
CO"-70.5	NA	NA	NA	NA	NA
2-0	.00734	.00922	.00797	.00818	.00094
2-6	.00584	NA	.00847	.00716	.00131
2-12	.00734	.00766	.01234	.00911	.00250
2-18	.00922	.00978	.01109	.01003	.00094
2-20	.00959	.01172	.01184	.01105	.00113
2-22	.00797	.00809	.00547	.00718	.00131
2-26	.00703	.00953	.00809	.00822	.00125
2-30	.00847	.01203	.01016	.01022	.00178
2-33.5	.00797	.01016	.01009	.00941	.00109
2-48	.01009	.01016	.00959	.00995	.00028
2-54	.00875	.00734	.01294	.00968	.00280
2-58.5	.00959	.01034	.00866	.00953	.00084
2-70.5	.00875	.01072	.01334	.01094	.00230
5-0	NA	.00828	.00584	.00706	.00122
5-6	.00997	.00847	.00797	.00880	.00100
5-12	.00766	.00594	.00666	.00675	.00086
5-18	.01047	.00984	.00959	.00997	.00044
5-20	.01359	.01072	.01034	.01155	.00162
5-22	.00622	.00659	.00847	.00709	.00113

IS 3 CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
5-26	.00634	.00666	.00666	.00655	.00016
5-30	.00891	.00928	.00806	.00875	.00061
5-33.5	.00709	.00759	.05469	.02313	.02380
5-48	.00659	.00841	.00841	.00780	.00091
5-54	.00809	.00772	.00734	.00772	.00037
5-58.5	.00959	.00909	.00634	.00834	.00162
5-70.5	.00984	.00578	.00841	.00801	.00203
10-0	NA	.00534	.00928	.00731	.00197
10-6	.00922	.00766	.00984	.00891	.00109
10-12	.00859	.00766	.00866	.00830	.00050
10-18	.01797	.01034	.01578	.01470	.00381
10-20	.01016	.00809	.00972	.00932	.00103
10-22	NA	NA	.00484	.00484	0.00000
10-26	.00491	.00622	NA	.00556	.00066
10-30	NA	NA	.00659	.00659	0.00000
10-33.5	NA	NA	.00641	.00641	0.00000
10-48	NA	NA	.00709	.00709	0.00000
10-54	NA	NA	.00766	.00766	0.00000
10-58.5	NA	NA	.00453	.00453	0.00000
10-70.5	NA	NA	.00622	.00622	0.00000
20-0	.00659	.00656	.00884	.00733	.00114
20-6	.00953	.00859	.00734	.00849	.00109
20-12	.00697	.00666	.00709	.00691	.00022
20-18	.00859	.00909	.00909	.00893	.00025
20-20	.00909	.00984	.00978	.00957	.00037
20-22	.00697	NA	NA	.00697	0.00000
20-26	NA	NA	NA	NA	NA
20-30	NA	NA	NA	NA	NA
20-33.5	NA	NA	NA	NA	NA
20-48	NA	NA	NA	NA	NA
20-54	NA	NA	NA	NA	NA
20-58.5	NA	NA	NA	NA	NA
20-70.5	NA	NA	NA	NA	NA
50-0	.00634	.00703	.00659	.00666	.00034
50-6	.00859	.00809	.00634	.00768	.00113
50-12	.00646	.00922	.00759	.00776	.00138
50-18	.00909	.00909	.00909	.00909	0.00000
50-20	.00928	.00884	.00984	.00932	.00050
50-22	NA	NA	NA	NA	NA
50-26	NA	NA	NA	NA	NA
50-30	NA	NA	NA	NA	NA
50-33.5	NA	NA	NA	NA	NA
50-48	NA	NA	NA	NA	NA
50-54	NA	NA	NA	NA	NA
50-58.5	NA	NA	NA	NA	NA
50-70.5	NA	NA	NA	NA	NA

FLUOROPROPIONATE (IS# 3)

IS 3 CODE	C/MG(1)	C/MG(2)	C/MG(3)	AVG C/MG	(+/-)
CO-0	NA	NA	NA	NA	NA
CO-70.5	NA	NA	NA	NA	NA
CO'-0	NA	NA	NA	NA	NA
CO'-6	NA	NA	NA	NA	NA
CO'-12	NA	NA	NA	NA	NA
CO'-18	NA	NA	NA	NA	NA
CO'-20	NA	NA	NA	NA	NA
CO'-22	NA	NA	NA	NA	NA
CO'-26	NA	NA	NA	NA	NA
CO'-30	NA	NA	NA	NA	NA
CO'-33.5	NA	NA	NA	NA	NA
CO'-48	NA	NA	NA	NA	NA
CO'-54	NA	NA	NA	NA	NA
CO'-58.5	NA	NA	NA	NA	NA
CO'-70.5	NA	NA	NA	NA	NA
CO"-0	NA	NA	NA	NA	NA
CO"-6	NA	NA	NA	NA	NA
CO"-12	NA	NA	NA	NA	NA
CO"-18	NA	NA	NA	NA	NA
CO"-20	96431	112586	81157	96725	15714
CO"-22	NA	NA	NA	NA	NA
CO"-26	NA	NA	NA	NA	NA
CO"-30	NA	NA	NA	NA	NA
CO"-33.5	NA	NA	NA	NA	NA
CO"-48	NA	NA	NA	NA	NA
CO"-54	NA	NA	NA	NA	NA
CO"-58.5	NA	NA	NA	NA	NA
CO"-70.5	NA	NA	NA	NA	NA
2-0	NA	NA	NA	NA	NA
2-6	NA	NA	NA	NA	NA
2-12	NA	NA	NA	NA	NA
2-18	NA	NA	NA	NA	NA
2-20	108985	141293	180437	143572	35726
2-22	108762	112920	37029	86237	37946
2-26	107904	51698	138857	99486	43580
2-30	92059	93301	113450	99603	10696
2-33.5	33544	65398	53967	50970	15927
2-48	70952	71513	64519	68995	3497
2-54	31937	95683	88591	72070	31873
2-58.5	70007	88553	72910	77157	9273
2-70.5	66189	107748	71680	81872	20780
5-0	NA	NA	NA	NA	NA
5-6	NA	NA	NA	NA	NA
5-12	NA	NA	NA	NA	NA
5-18	NA	NA	NA	NA	NA
5-20	121804	121791	162359	135318	20284
5-22	28764	36341	64800	43302	18018

IS 3 CODE	C/MG(1)	C/MG(2)	C/MG(3)	AVG C/MG	(+/-)
5-26	72993	45228	70783	63001	13882
5-30	121415	87709	79116	96080	21150
5-33.5	37110	28978	19625	28571	8742
5-48	55587	41674	88407	61889	23366
5-54	68386	62526	97246	76053	17360
5-58.5	73665	65394	74908	71322	4757
5-70.5	87223	333691	81181	167365	126255
10-0	NA	NA	NA	NA	NA
10-6	NA	NA	NA	NA	NA
10-12	NA	NA	NA	NA	NA
10-18	NA	NA	NA	NA	NA
10-20	158378	170465	173356	167400	7489
10-22	NA	NA	35954	35954	0
10-26	47740	11940	NA	29840	17900
10-30	NA	NA	63060	63060	0
10-33.5	NA	NA	NA	NA	NA
10-48	NA	NA	81547	81547	0
10-54	NA	NA	69473	69473	0
10-58.5	NA	NA	68077	68077	0
10-70.5	NA	NA	56768	56768	0
20-0	NA	NA	NA	NA	NA
20-6	NA	NA	NA	NA	NA
20-12	NA	NA	NA	NA	NA
20-18	NA	NA	NA	NA	NA
20-20	181039	153051	129393	154494	25823
20-22	NA	NA	NA	NA	NA
20-26	NA	NA	NA	NA	NA
20-30	NA	NA	NA	NA	NA
20-33.5	NA	NA	NA	NA	NA
20-48	NA	NA	NA	NA	NA
20-54	NA	NA	NA	NA	NA
20-58.5	NA	NA	NA	NA	NA
20-70.5	NA	NA	NA	NA	NA
50-0	NA	NA	NA	NA	NA
50-6	NA	NA	NA	NA	NA
50-12	NA	NA	NA	NA	NA
50-18	NA	NA	NA	NA	NA
50-20	111273	227067	185486	174609	57897
50-22	NA	NA	NA	NA	NA
50-26	NA	NA	NA	NA	NA
50-30	NA	NA	NA	NA	NA
50-33.5	NA	NA	NA	NA	NA
50-48	NA	NA	NA	NA	NA
50-54	NA	NA	NA	NA	NA
50-58.5	NA	NA	NA	NA	NA
50-70.5	NA	NA	NA	NA	NA

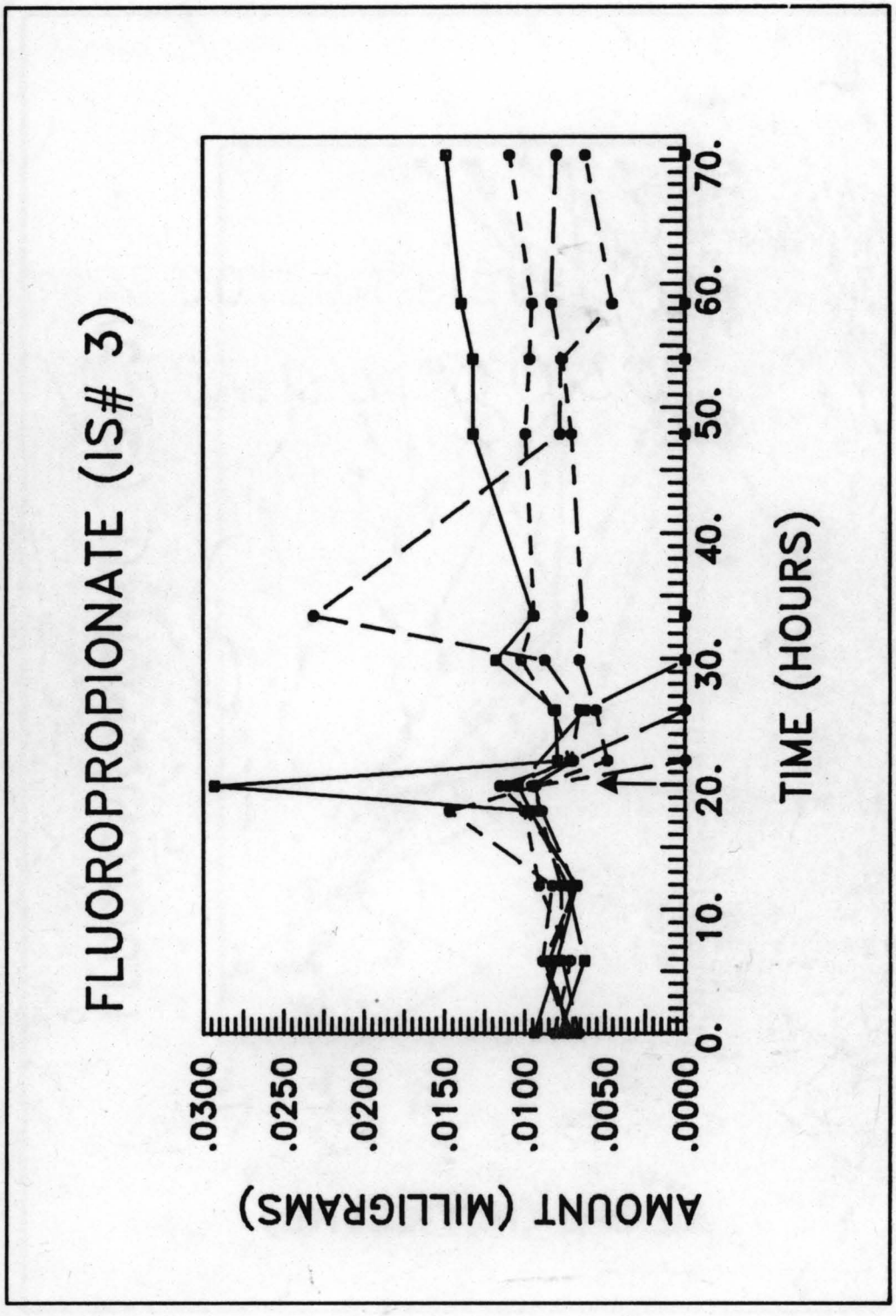
FLUOROPROPIONATE (IS# 3)

IS 3 CODE	WT(1)	WT(2)	WT(3)	AVG WT	(+/-)
CO-0	12.3	12.64	13.02	12.65	.36
CO-70.5	10.13	10.7	10.41	10.41	.28
CO'-0	13.52	13.77	12.61	13.30	.58
CO'-6	7.58	9.82	15.13	10.84	3.78
CO'-12	13.62	12.68	14.63	13.64	.98
CO'-18	12.5	10.9	13.17	12.19	1.13
CO'-20	12.48	12.78	13.08	12.78	.30
CO'-22	15.15	12.26	10.96	12.79	2.09
CO'-26	12.2	12.03	12.37	12.20	.17
CO'-30	11.33	11.93	11.51	11.59	.30
CO'-33.5	11.62	12.56	11.27	11.82	.65
CO'-48	9.89	9.69	10.33	9.97	.32
CO'-54	6.59	9.19	10.11	8.63	1.76
CO'-58.5	8.72	9.38	9.87	9.32	.57
CO'-70.5	11.2	NA	11.16	11.18	.02
CO"-0	13.53	12.6	13.98	13.37	.69
CO"-6	16.52	15.12	15.18	15.61	.70
CO"-12	13.94	14.68	12.47	13.70	1.10
CO"-18	12.49	12.4	13.57	12.82	.58
CO"-20	13.38	11.63	12.46	12.49	.88
CO"-22	10.95	9.58	10.98	10.50	.70
CO"-26	10.14	11.33	12.04	11.17	.95
CO"-30	11.27	10.08	10.15	10.50	.59
CO"-33.5	11.11	7.27	9.75	9.38	1.92
CO"-48	10.2	8.99	8.53	9.24	.83
CO"-54	9.13	10.26	9.4	9.60	.56
CO"-58.5	8.74	9.47	9.13	9.11	.37
CO"-70.5	12.54	11.69	11.91	12.05	.42
2-0	13.49	14.23	14.01	13.91	.37
2-6	11.33	14.99	16.05	14.12	2.36
2-12	11.94	13.09	15.02	13.35	1.54
2-18	12.08	12.02	12.75	12.28	.37
2-20	11.58	12	12.91	12.16	.67
2-22	8.69	9.12	12.5	10.10	1.91
2-26	9.27	9.47	11.83	10.19	1.28
2-30	8.32	9.78	11.06	9.72	1.37
2-33.5	9.75	9.82	10.44	10.00	.34
2-48	6.93	7.7	NA	7.31	.39
2-54	7.72	6.73	8.74	7.73	1.00
2-58.5	6.9	7.44	7.01	7.12	.27
2-70.5	9.38	9.83	8.69	9.30	.57
5-0	13.09	12.54	13.34	12.99	.40
5-6	14.32	13.52	15.45	14.43	.96
5-12	13.5	13.27	12.93	13.23	.29
5-18	12.21	13.49	12.23	12.64	.64
5-20	13.46	11.4	12.16	12.34	1.03
5-22	9.38	10.08	10.79	10.08	.70

IS 3 CODE	WT(1)	WT(2)	WT(3)	AVG WT	(+/-)
5-26	8.27	10.35	9.2	9.27	1.04
5-30	9.14	9.83	9.39	9.45	.34
5-33.5	8.19	9.12	10.15	9.15	.98
5-48	7.46	7.85	7.95	7.75	.25
5-54	7.16	6.18	8.08	7.14	.95
5-58.5	6.62	7.16	6.8	6.86	.27
5-70.5	8.18	7.16	8.93	8.09	.88
10-0	13.81	13.22	13.94	13.66	.36
10-6	14.98	14.88	14.81	14.89	.08
10-12	13.52	15.28	13.77	14.19	.88
10-18	13.38	13	13.76	13.38	.38
10-20	12.76	11.66	12.07	12.16	.55
10-22	9.54	10.48	11.77	10.60	1.12
10-26	7.46	9.22	8.84	8.51	.88
10-30	5.71	9.44	10.12	8.42	2.20
10-33.5	5.46	8.81	9.82	8.03	2.18
10-48	4.71	7.55	8.91	7.06	2.10
10-54	8.38	7.72	7.42	7.84	.48
10-58.5	5.9	7.35	7.26	6.84	.72
10-70.5	11.15	9.93	9.07	10.05	1.04
20-0	12.92	16.79	12.82	14.18	1.98
20-6	13.76	15.82	14.17	14.58	1.03
20-12	14.96	14.91	13.94	14.60	.51
20-18	13.18	12.01	12.66	12.62	.58
20-20	10.89	12.17	12.68	11.91	.89
20-22	11.21	12.23	11.55	11.66	.51
20-26	9.22	11.88	11.47	10.86	1.33
20-30	9.8	11.17	9.82	10.26	.68
20-33.5	9.54	10.04	10.53	10.04	.50
20-48	9.09	9.84	9.42	9.45	.38
20-54	9.59	7.24	10.07	8.97	1.42
20-58.5	8.6	8.81	10.11	9.17	.75
20-70.5	10.06	12.53	10.4	11.00	1.23
50-0	12.37	11.92	12.24	12.18	.22
50-6	14.54	14.15	14.16	14.28	.19
50-12	13.96	13.87	13.49	13.77	.24
50-18	12.73	12.74	6.2	10.56	3.27
50-20	11.71	11.17	11.05	11.31	.33
50-22	10.92	13.84	10.4	11.72	1.72
50-26	10.53	11.49	10.18	10.73	.66
50-30	10.56	8.83	10.64	10.01	.91
50-33.5	9.03	8.27	10.25	9.18	.99
50-48	12.37	8.38	11.6	10.78	1.99
50-54	11.01	9.92	11.69	10.87	.88
50-58.5	11.38	6.79	8.19	8.79	2.30
50-70.5	9.37	8.94	9.07	9.13	.21

FLUOROPROPIONATE (IS# 3)

IS 3 CODE	pH	TOTAL PCV
CO	6.99	23 cc
CO'	6.98	12.5 cc
CO"	2.42	4 cc
2mM	7.16	12.5 cc
5mM	7.04	12.5 cc
10mM	4.93	7.5 cc
20mM	3.54	5 cc
50mM	2.77	4 cc


FLUOROPROPIONATE (IS# 3)
AMOUNT (MILLIGRAMS)
.0300
.0250
.0200
.0150
.0100
.0050
.0000
0.
10.
20.
30.
40.
50.
60.
70.
TIME (HOURS)

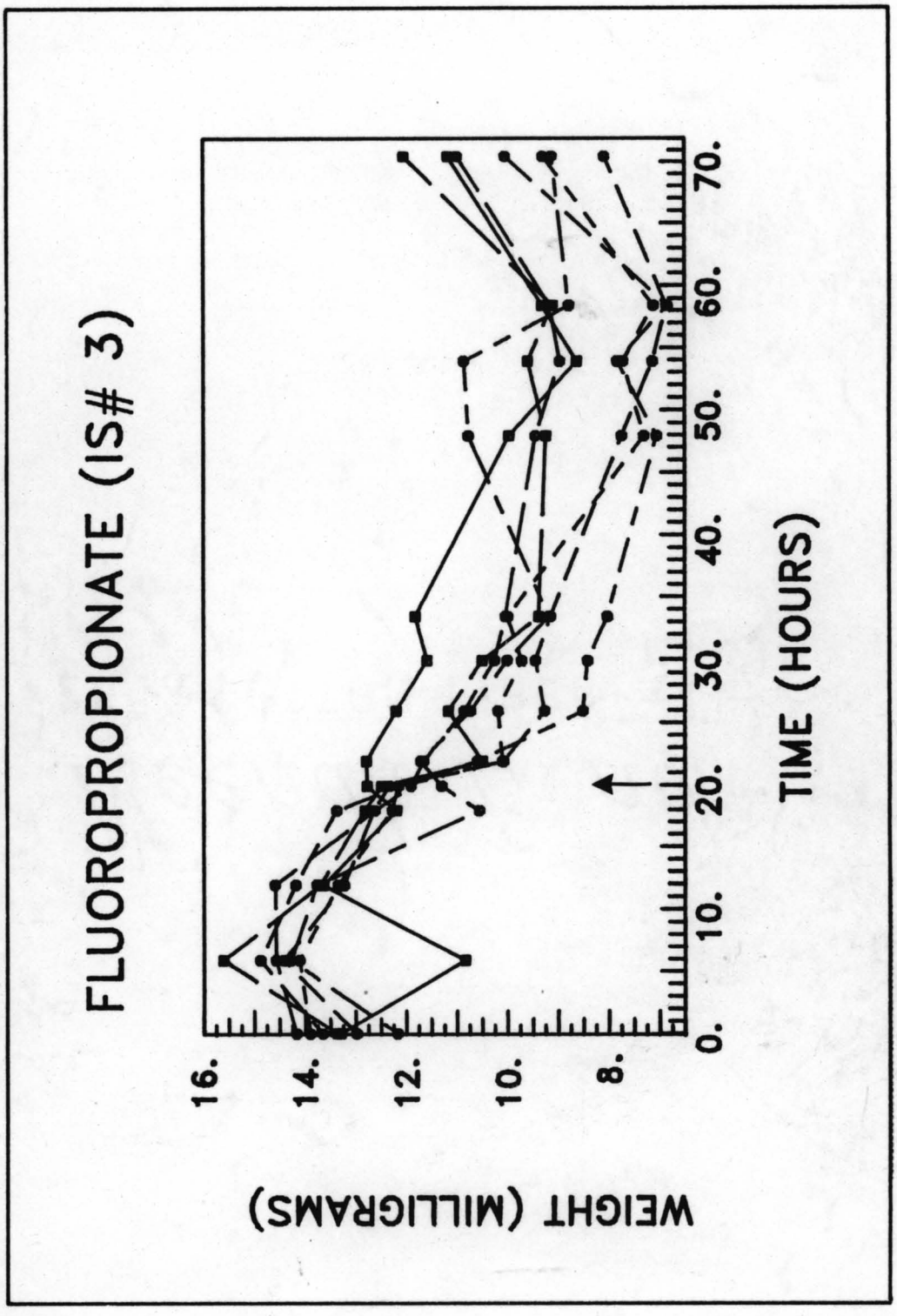
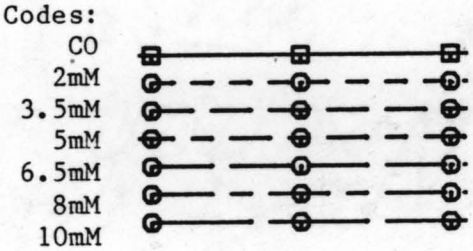

FLUOROPROPIONATE (IS# 3)
WEIGHT (MILLIGRAMS)
16.
14.
12.
10.
8.
0.
10.
20.
30.
40.
50.
60.
70.
TIME (HOURS)

Figure 55. Results of Inhibitor Study # 5:
(+/-) 2-Fluoropropionic Acid

Codes:



FLUOROPROPIONATE (IS# 5)

IS 5 CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
2-35	NA	NA	NA	NA	NA
2-48	.00434	.00444	NA	.00444	.00005
2-54	.00684	.00859	.00797	.00780	.00088
2-56	.00509	.00634	.00847	.00664	.00169
2-70.5	.00841	.00978	.01497	.01105	.00328
2-81.5	.01322	.01047	.02009	.01459	.00481
2-94.5	.01547	.01316	.01709	.01524	.00197
2-105	.01009	.01391	.01372	.01257	.00191
2-123	.01672	.00959	.02113	.01581	.00577
3.5-35	NA	NA	NA	NA	NA
3.5-48	.00641	.00666	NA	.00666	.00013
3.5-54	.00781	.00922	.00634	.00779	.00144
3.5-56	.00734	.00703	.00766	.00734	.00031
3.5-70.5	.00847	.01109	.00734	.00897	.00187
3.5-81.5	.00887	NA	.00781	.00834	.00053
3.5-94.5	.01297	.02147	.01547	.01664	.00425
3.5-105	.00772	.01584	.00847	.01068	.00406
3.5-123	.01609	.02197	.01597	.01801	.00300
5-35	NA	NA	NA	NA	NA
5-48	NA	NA	.00622	.00622	0.00000
5-54	.00641	.00609	.00734	.00661	.00063
5-56	.00484	.00847	.00734	.00689	.00181
5-70.5	.00584	.00797	.00887	.00756	.00152
5-81.5	.00703	.00609	.01234	.00849	.00313
5-94.5	.00669	.00566	.00797	.00677	.00116
5-105	.00547	NA	.00884	.00716	.00169
5-123	.00875	.00909	.00922	.00902	.00023
6.5-35	NA	NA	NA	NA	NA
6.5-48	.00547	NA	.00600	.00573	.00027
6.5-54	.00959	.01172	.00622	.00918	.00275
6.5-56	.00828	.00809	.00688	.00775	.00070
6.5-70.5	.00684	.00809	NA	.00747	.00063
6.5-81.5	.00659	.00809	.00725	.00731	.00075
6.5-94.5	.00866	.00641	.00531	.00679	.00167
6.5-105	.00703	.00634	.00622	.00653	.00041
6.5-123	.00891	.00703	.00609	.00734	.00141
8-35	NA	NA	NA	NA	NA
8-48	.00584	.00781	.00809	.00725	.00113
8-54	.01103	.01034	.00972	.01036	.00066
8-56	.00734	.00547	.00659	.00647	.00094
8-70.5	.00578	.00584	.00634	.00599	.00028
8-81.5	.00669	.00703	.00641	.00671	.00031
8-94.5	.00766	.00559	.00659	.00661	.00103
8-105	.00666	.00734	.00734	.00711	.00034
8-123	.00459	.00737	.00609	.00602	.00139

IS 5 CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
10-35	NA	NA	NA	NA	NA
10-48	.00922	.00622	.00622	.00722	.00150
10-54	.00997	.00797	.00734	.00843	.00131
10-56	.00469	NA	NA	.00469	0.00000
10-70.5	.00578	.00516	NA	.00547	.00031
10-81.5	.00641	.00644	NA	.00642	.00002
10-94.5	.00684	NA	NA	.00684	0.00000
10-105	.00622	.00578	NA	.00600	.00022
10-123	.00922	.00734	.00622	.00759	.00150
CO-35	NA	NA	NA	NA	NA
CO-48	.00528	.00666	.00566	.00586	.00069
CO-54	.00859	.00734	.00734	.00776	.00063
CO-56	.00562	.00753	.00659	.00658	.00095
CO-70.5	.00978	.00884	.00984	.00949	.00050
CO-81.5	.00859	.00866	.00684	.00803	.00091
CO-94.5	.01297	.00809	.00950	.01019	.00244
CO-105	.01766	.01103	.01359	.01409	.00331
CO-123	.01428	.01672	.01059	.01386	.00306

FLUOROPROPIONATE (IS# 5)

IS 5 CODE	C/MG(1)	C/MG(2)	C/MG(3)	AVG C/MG	(+/-)
2-35	NA	NA	NA	NA	NA
2-48	NA	NA	NA	NA	NA
2-54	20811	25763	49638	32071	14413
2-56	4240	6278	28694	13071	12227
2-70.5	9796	14906	16685	13796	3444
2-81.5	10877	8318	12026	10407	1854
2-94.5	10298	15341	14492	13377	2521
2-105	9897	10096	16385	12126	3244
2-123	4724	2814	11950	6496	4568
3.5-35	NA	NA	NA	NA	NA
3.5-48	NA	NA	NA	NA	NA
3.5-54	28426	41662	39476	36521	6618
3.5-56	11581	32928	15958	20156	10673
3.5-70.5	9246	15515	NA	12381	3135
3.5-81.5	15592	NA	12442	14017	1575
3.5-94.5	10878	11507	13091	11825	1106
3.5-105	8133	6689	15702	10174	4507
3.5-123	8724	8542	7228	8165	748
5-35	NA	NA	NA	NA	NA
5-48	NA	NA	NA	NA	NA
5-54	3688	10080	59837	24535	28074
5-56	3763	33954	40994	26237	18616
5-70.5	1040	3473	31107	11873	15034
5-81.5	2688	8197	26740	12542	12026
5-94.5	3432	2148	26598	10726	12225
5-105	NA	NA	15723	15723	0
5-123	NA	1132	12740	6936	5804
6.5-35	NA	NA	NA	NA	NA
6.5-48	NA	NA	NA	NA	NA
6.5-54	50377	36461	28438	38425	10969
6.5-56	26817	17930	6578	17108	10119
6.5-70.5	51189	25686	NA	38438	12752
6.5-81.5	29073	19432	5400	17968	11836
6.5-94.5	28774	23391	NA	26083	2691
6.5-105	12672	9789	NA	11231	1441
6.5-123	4699	11424	NA	8061	3363
8-35	NA	NA	NA	NA	NA
8-48	NA	NA	NA	NA	NA
8-54	86216	67214	72509	75313	9501
8-56	43200	42089	31530	38940	5835
8-70.5	48804	29917	50542	43088	10313
8-81.5	23518	36768	56898	39061	16690
8-94.5	28653	20755	20372	23260	4141
8-105	32146	21968	34009	29374	6020
8-123	14841	13271	41206	23106	13967

IS 5 CODE	C/MG(1)	C/MG(2)	C/MG(3)	AVG C/MG	(+/-)
10-35	NA	NA	NA	NA	NA
10-48	NA	NA	NA	NA	NA
10-54	84978	59379	66454	70270	12800
10-56	19296	NA	NA	19296	0
10-70.5	53124	19767	NA	36446	16679
10-81.5	35087	33449	NA	34268	0
10-94.5	12625	NA	NA	12625	0
10-105	23228	8640	NA	15934	7294
10-123	5858	7353	12591	8601	3367
CO-35	NA	NA	NA	NA	NA
CO-48	NA	NA	NA	NA	NA
CO-54	55217	61951	NA	58584	3367
CO-56	24120	13534	2969	13541	10576
CO-70.5	23187	16792	NA	19989	3198
CO-81.5	31968	27136	2071	20392	14948
CO-94.5	24879	21016	2487	16127	11196
CO-105	20950	NA	199	10574	10376
CO-123	15739	10497	NA	13118	2621

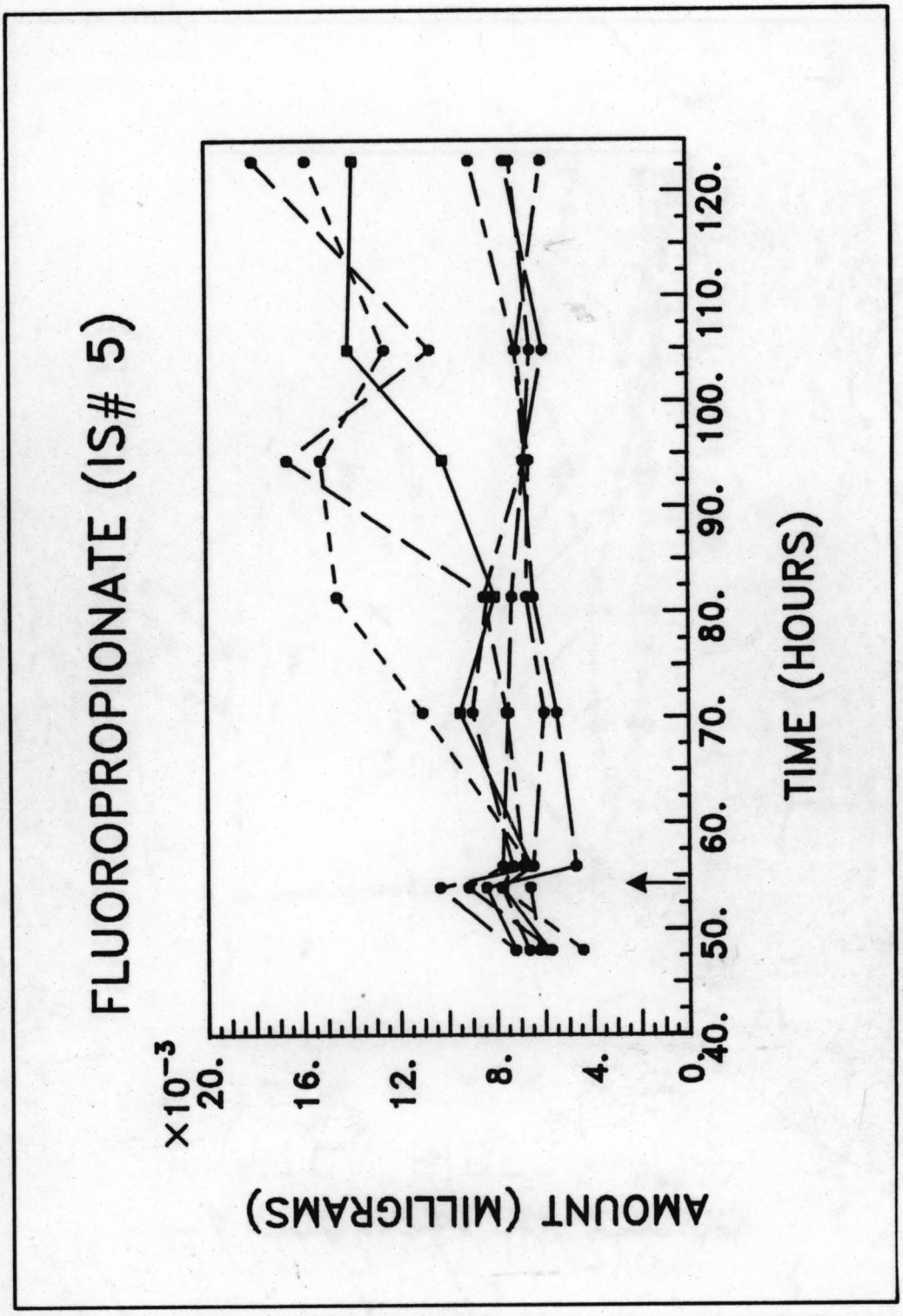
FLUOROPROPIONATE (IS# 5)

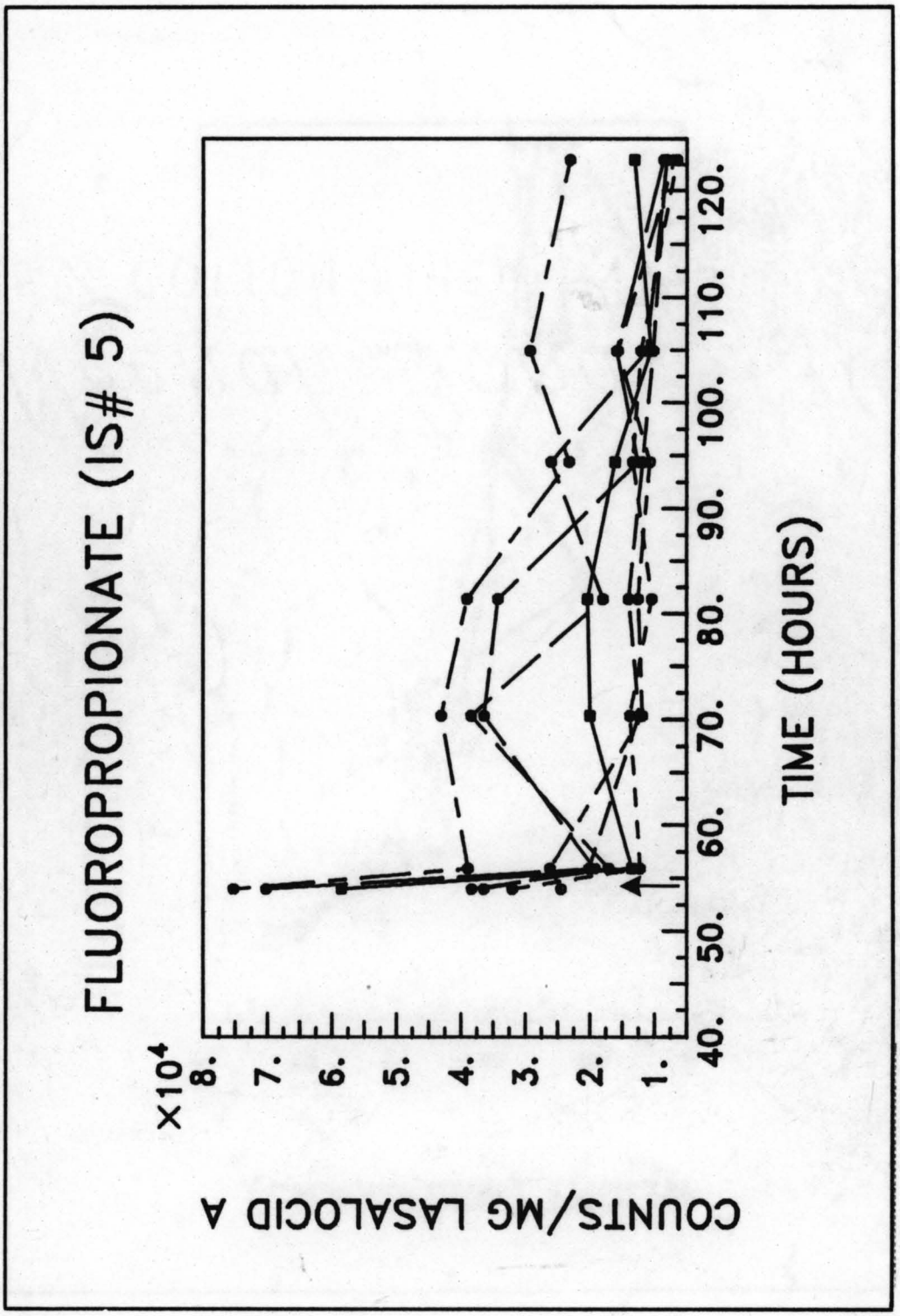
IS 5 CODE	WT(1)	WT(2)	WT(3)	AVG WT	(+/-)
2-35	13.77	13.08	12.3	13.05	.73
2-48	12.92	11.89	11.46	12.09	.73
2-54	11.36	10.39	9.58	10.44	.89
2-56	10.05	9.04	9.23	9.44	.51
2-70.5	8.89	9.36	8.49	8.91	.43
2-81.5	8.47	7.57	8.57	8.20	.50
2-94.5	.72	7.18	7.91	5.27	3.60
2-105	6.89	6.71	7.09	6.90	.19
2-123	6.83	7.14	7.98	7.32	.58
3.5-35	11.62	12.19	13.43	12.41	.91
3.5-48	12.53	12.55	11.04	12.04	.76
3.5-54	9.67	12.04	9.71	10.47	1.18
3.5-56	9.69	10.25	9.08	9.67	.58
3.5-70.5	7.13	8.9	8.46	8.16	.89
3.5-81.5	.74	8.69	8.36	5.93	3.97
3.5-94.5	7.06	8.81	8.31	8.06	.88
3.5-105	7.27	7.09	6.97	7.11	.15
3.5-123	7.19	7.19	8.19	7.52	.50
5-35	13.56	11.87	13.13	12.85	.85
5-48	11.5	12.55	12.49	12.18	.53
5-54	9.27	12.55	10.45	10.76	1.64
5-56	7.41	9.67	10.25	9.11	1.42
5-70.5	7.81	7.79	8.53	8.04	.37
5-81.5	7.41	7.37	8.88	7.89	.76
5-94.5	8.47	7.82	8.03	8.11	.33
5-105	7.32	6.82	7.48	7.21	.33
5-123	8.08	6.71	7.58	7.46	.69
6.5-35	13.21	13.83	9.52	12.19	2.16
6.5-48	11.91	12.03	13.37	12.44	.73
6.5-54	10.75	10.44	10.68	10.62	.16
6.5-56	9.49	9.56	9.91	9.65	.21
6.5-70.5	7.93	8.93	8.78	8.55	.50
6.5-81.5	8.58	8.26	8.94	8.59	.34
6.5-94.5	7.12	7.95	8.25	7.77	.56
6.5-105	6.11	6.51	6.71	6.44	.30
6.5-123	6.57	7.45	5.89	6.64	.78
8-35	12.87	13.31	12.97	13.05	.22
8-48	11.93	13.28	12.23	12.48	.67
8-54	10.77	9.66	9.63	10.02	.57
8-56	10.09	10.51	9.09	9.90	.71
8-70.5	9.84	10.01	8.96	9.60	.52
8-81.5	8.74	8.68	.75	6.06	4.00
8-94.5	8.43	7.62	7.11	7.72	.66
8-105	6.52	6.35	6.69	6.52	.17
8-123	7.62	6.17	5.81	6.53	.91

IS 5 CODE	WT(1)	WT(2)	WT(3)	AVG WT	(+/-)
10-35	13.35	13.78	13.47	13.53	.21
10-48	12.77	12.52	12.03	12.44	.37
10-54	9.92	9.48	10.57	9.99	.54
10-56	8.75	9.68	7.78	8.74	.95
10-70.5	8.25	10.24	10.65	9.71	1.20
10-81.5	8.47	8.76	.77	6.00	4.00
10-94.5	5.05	6.91	6.48	6.15	.93
10-105	5.59	6.04	.64	4.09	2.70
10-123	6.37	6.57	5.68	6.21	.45
CO-35	13.08	14.6	13.97	13.88	.76
CO-48	12.33	11.58	11.8	11.90	.38
CO-54	10.02	11.09	11.61	10.91	.79
CO-56	8.51	10.93	11.32	10.25	1.41
CO-70.5	8.58	7.97	9.34	8.63	.69
CO-81.5	8.04	7.51	8.78	8.11	.63
CO-94.5	7.21	7.46	8.96	7.88	.88
CO-105	7.23	6.89	8.63	7.58	.87
CO-123	5.86	7.34	7.73	6.98	.94

FLUOROPROPIONATE (IS# 5)

IS 5 CODE	pH(1)	pH (2)	pH (3)	AVG pH	TOTAL PCV
CONTROL	7.36	7.28	7.33	7.32	30 cc
2mM	7.04	7.25	7.15	7.15	30 cc
3.5mM	7.15	7.05	7.14	7.11	27.5 cc
5mM	6.90	7.03	7.22	7.05	27.5 cc
6.5mM	6.97	6.81	6.60	6.79	27.5 cc
8mM	6.73	6.43	6.67	6.61	27.5 cc
10mM	6.54	6.54	7.76	6.95	30 cc


FLUOROPROPIONATE (IS# 5)
AMOUNT (MILLIGRAMS)
X10⁻³
20.
16.
12.
8.
4.
0.
40.
50.
60.
70.
80.
90.
100.
110.
120.
TIME (HOURS)


FLUOROPROPIONATE (IS# 5)
COUNTS/MG LASALOCID A
X10⁴
8.
7.
6.
5.
4.
3.
2.
1.
40.
50.
60.
70.
80.
90.
100.
110.
120.
TIME (HOURS)

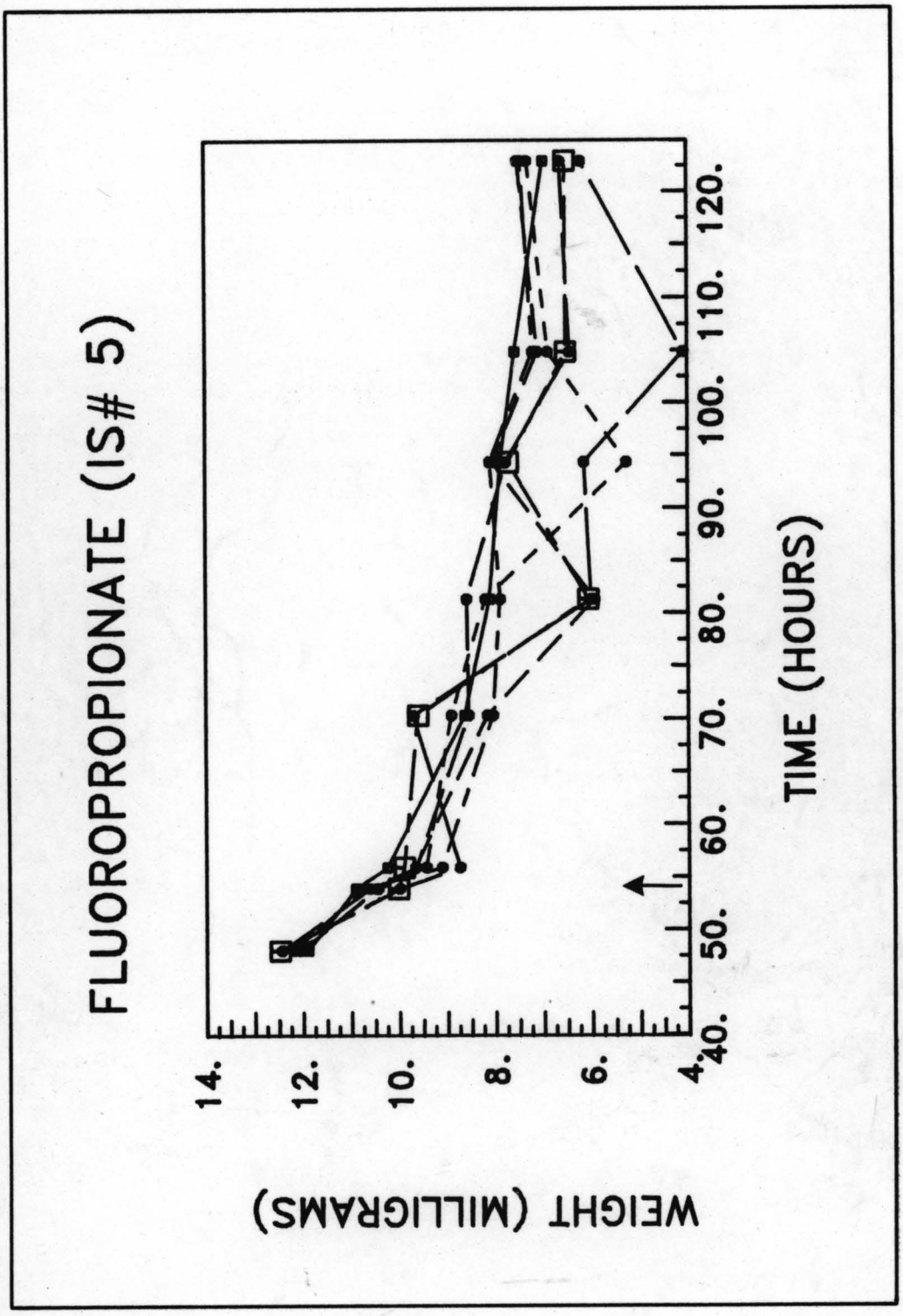
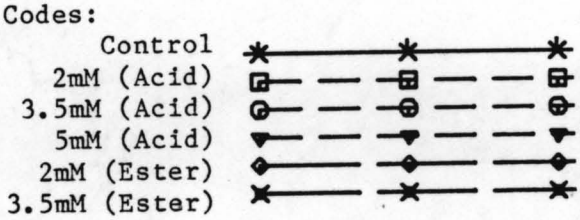

FLUOROPROPIONATE (IS# 5)
WEIGHT (MILLIGRAMS)
14.
12.
10.
8.
6.
4.
40.
50.
60.
70.
80.
90.
100.
110.
120.
TIME (HOURS)

Figure 56. Results of Inhibitor Study #9:
(2S)-2-Fluoropropionate
and its Ethyl Ester

Codes:



FLUOROPROPIONATE (IS# 9)

IS 9 CODE	AMT(1)	AMT(2)	AMT(3)	AMT(4)	AVG AMT	(+/-)
A2-72	NA	.01003	.00836	.01130	.00990	.00147
A2-82	.00747	.01356	.01486	.01010	.01150	.00370
A2-83.5	NA	.01770	.01730	.01504	.01668	.00133
A2-96	.02119	.03559	.02848	.02000	.02632	.00779
A2-108.5	.02261	.03259	.04364	.02703	.03147	.01052
A2-F	NA	1.20437	.96134	.90531	1.02367	.14953
A3.5-72	.00705	.00817	.03649	.00688	.01465	.01481
A3.5-82	.01303	.00882	.01248	.01135	.01142	.00211
A3.5-83	.01392	.01853	.01787	.01722	.01688	.00231
A3.5-96	.02271	.02040	.02271	.01401	.01995	.00435
A3.5-108.5	.03617	.02241	.03273	.01216	.02587	.01200
A3.5-F	.92005	.89744	NA	.46199	.75983	.22903
A5-72	.00451	.00855	.00861	.00822	.00747	.00205
A5-82	.01274	.00850	.01240	.01163	.01132	.00212
A5-83.5	.01221	.00908	.01661	.01596	.01347	.00376
A5-96	.02511	.02123	.04640	.02514	.02947	.01258
A5-108.5	.02462	.02182	.03730	.02907	.02820	.00774
A5-F	.44233	.69741	.95151	.88073	.74300	.25459
CO-72	.00950	.00531	.00610	.00284	.00594	.00333
CO-82	.02159	.01101	.00784	.00725	.01192	.00717
CO-83.5	.01757	.01084	.00747	.00786	.01094	.00505
CO-96	.02865	.02241	.01239	.00983	.01832	.00941
CO-108.5	.02241	.02167	.01474	.01305	.01797	.00468
CO-F	.11796	.15334	.11550	.11206	.12471	.02064
E2-72	.01661	.02821	.00383		.01622	.01219
E2-82	.01347	.02128	.00491		.01322	.00818
E2-83.5	.01376	.01789	.00521		.01229	.00634
E2-96	.01809	.01629	.01121		.01519	.00344
E2-108.5	.02831	.03106	.01180		.02372	.00963
E2-F	.47575	.66055	NA		.56815	.09240
E3.5-72	.01455	.02025	.01494		.01658	.00285
E3.5-82	.01380	.01345	.01784		.01503	.00219
E3.5-83.5	.02076	.03145	.02082		.02435	.00535
E3.5-96	.01681	.02595	.03981		.02752	.01150
E3.5-108.5	.02946	.03067	.02841		.02951	.00113
E3.5-F	.93480	.22608	.26491		.47526	.35436

FLUOROPROPIONATE (IS# 9)

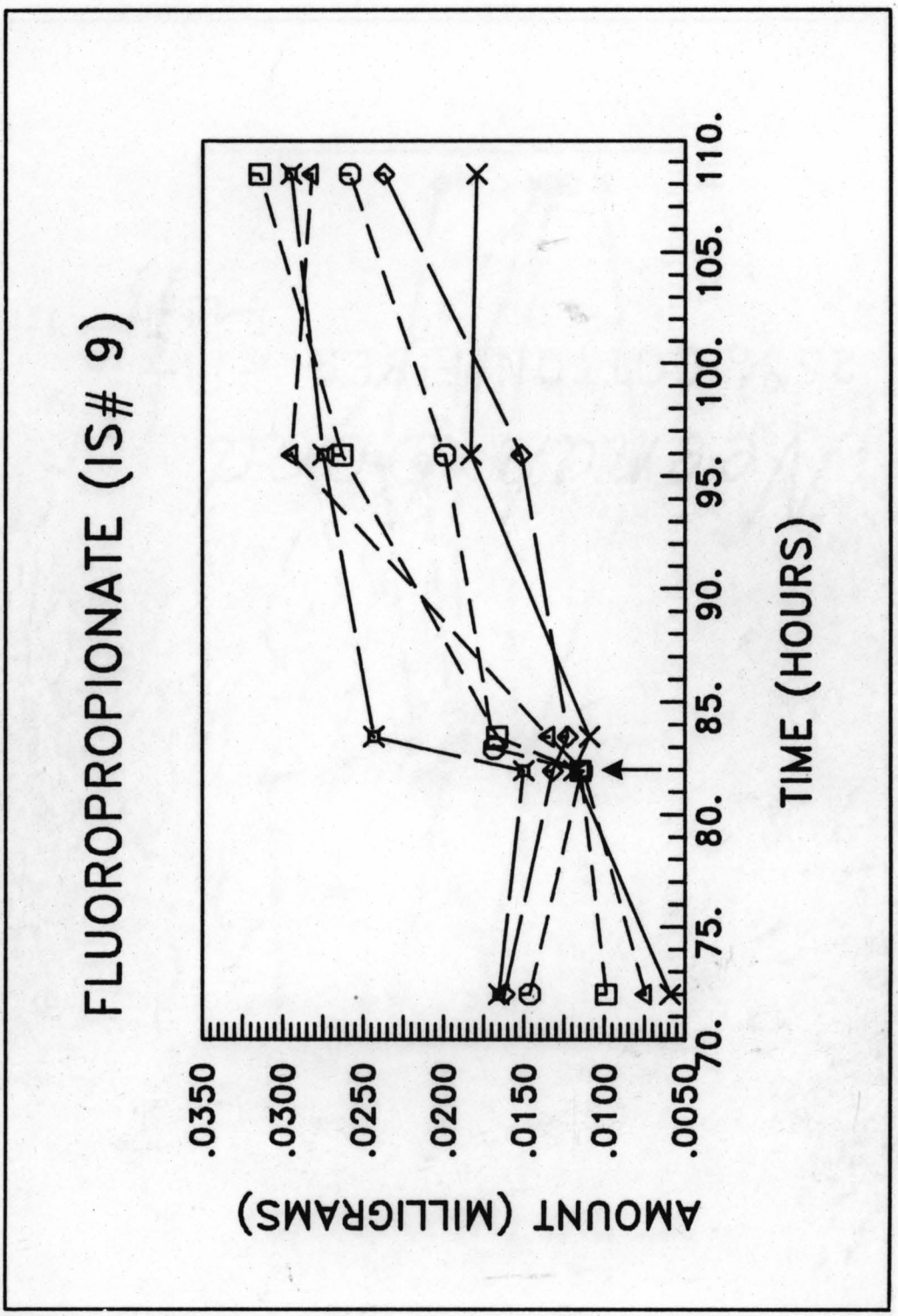
IS 9 CODE	WT(1)	WT(2)	WT(3)	WT(4)	AVG WT	(+/-)
A2-72	3.22	3.46	2.33	2.92	2.98	.56
A2-82	2.67	3.4	2.4	3.54	3.00	.57
A2-83.5	3.47	2.75	2.7	3.27	3.05	.39
A2-96	2.55	3.29	3.35	3.24	3.11	.40
A2-108.5	3.07	3.15	2.93	2.15	2.83	.50
A3.5-72	3.3	2.75	2.8	3.73	3.15	.49
A3.5-82	3.42	3.79	3.8	3.51	3.63	.19
A3.5-83.5	3.84	3.13	3.04	3.73	3.44	.40
A3.5-96	3.72	3.34	3.89	3.51	3.62	.28
A3.5-108.5	2.93	3.05	2.06	2.89	2.73	.49
A5-72	4.03	4.05	4.05	3.79	3.98	.13
A5-82	4.4	4.59	3.76	4.17	4.23	.42
A5-83.5	4.29	3.89	4.05	3.37	3.90	.46
A5-96	3.37	3.59	7.06	3.3	4.33	1.88
A5-108.5	3.08	2.65	3.15	2.45	2.83	.35
CO-72	4.47	4.06	2.28	2.31	3.28	1.09
CO-82	3.8	3.43	1.91	2.66	2.95	.94
CO-83.5	4.49	3.46	2.25	1.57	2.94	1.46
CO-96	2.36	2.67	2.11	1.96	2.27	.35
CO-108.5	3.43	3.33	1.18	2.1	2.51	1.12
E2-72	3.82	4.3	5.38		4.50	.78
E2-82	4.7	4.49	4.56		4.58	.10
E2-83.5	3.61	3.31	4.11		3.68	.40
E2-96	3.87	3.69	4.07		3.88	.19
E2-108.5	3.55	3.65	3.37		3.52	.14
E3.5-72	3.44	4.04	3.85		3.78	.30
E3.5-82	3.36	3.5	3.58		3.48	.11
E3.5-83	3.07	3.38	3.72		3.39	.33
E3.5-96	3.6	3.12	3.58		3.43	.24
E3.5-108.5	2.63	3.1	3.02		2.92	.24

FLUOROPROPIONATE (IS# 9)

IS 9 CODE	pH (1)	pH (2)	pH (3)	pH (4)	AVG pH
A2	6.60	6.59	6.59	6.59	6.59
A3.5	6.57	6.57	6.57	6.58	6.57
A5	6.59	6.58	6.58	6.58	6.58
CONTROL	6.59	6.59	6.64	6.62	6.61
E2	6.57	6.56	6.55		6.56
E3.5	6.53	6.54	6.54		6.54

PACKED CELL VOLUME (cc)

IS 9 CODE	(1)	(2)	(3)	(4)	AVG
A2	7.5	10.0	10.0	7.5	8.8
A3.5	7.5	10.0	7.5	10.0	8.8
A5	10.0	10.0	7.5	7.5	8.8
CONTROL	7.5	7.5	7.5	7.5	7.5
E2	7.5	7.5	7.5		7.5
E3.5	10.0	7.5	7.5		8.3


FLUOROPROPIONATE (IS# 9)
AMOUNT (MILLIGRAMS)
.0350
.0300
.0250
.0200
.0150
.0100
.0050
70.
75.
80.
85.
90.
95.
100.
105.
110.
TIME (HOURS)

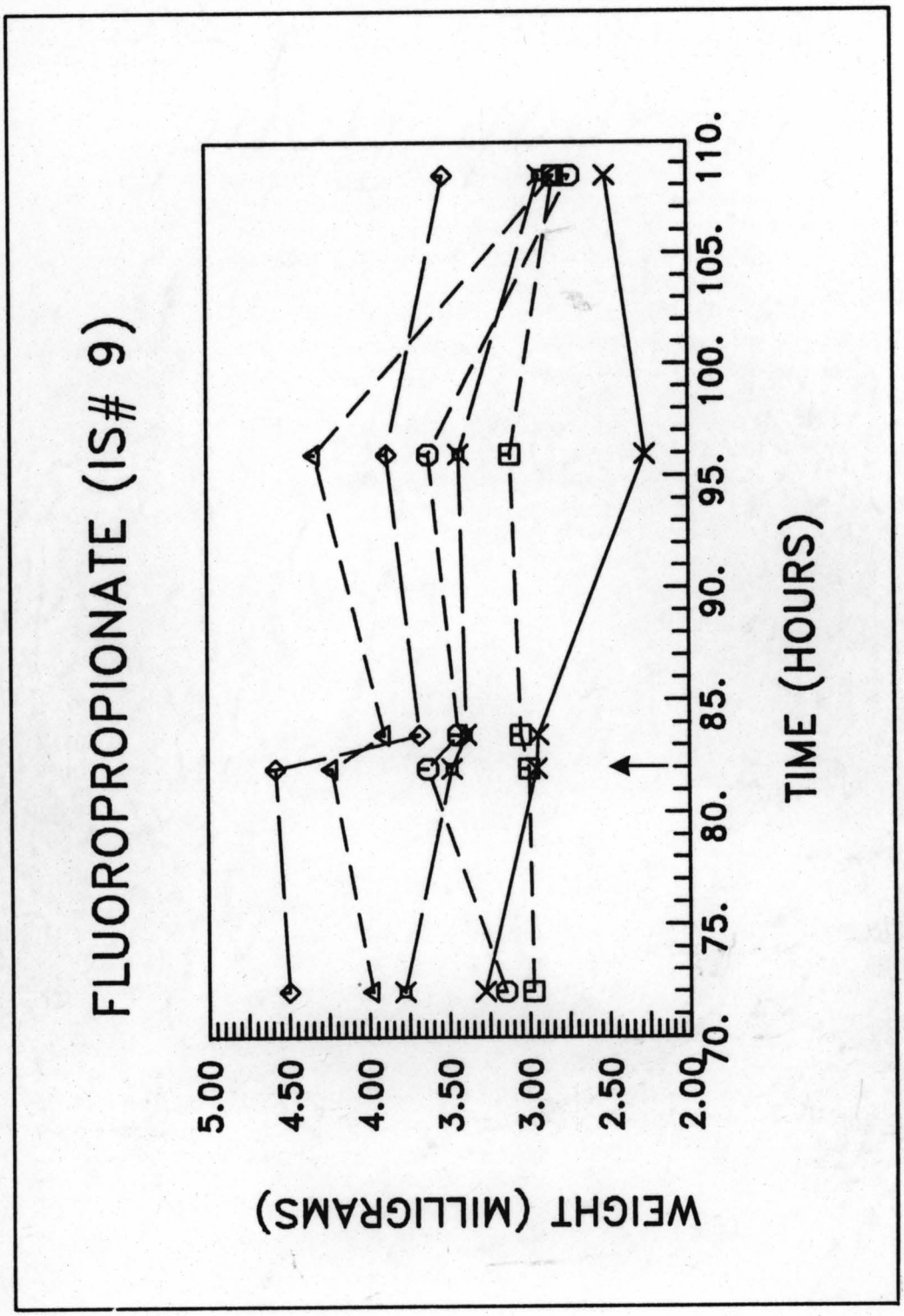
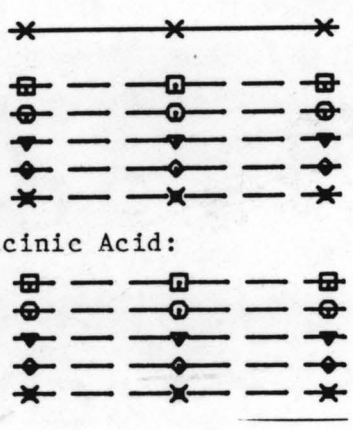

FLUOROPROPIONATE (IS# 9)
WEIGHT (MILLIGRAMS)
5.00
4.50
4.00
3.50
3.00
2.50
2.00
70.
75.
80.
85.
90.
95.
100.
105.
110.
TIME (HOURS)

Figure 51. Results of Inhibitor Study # 8:
Cerulenin and 2-Fluorosuccinic Acid

Codes:



CERULENIN AND FLUOROSUCCINATE (IS# 8)

IS8 CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
C5-72	.02378	.02109	.02284	.02257	.00134
C5-96	.03709	.04109	.04559	.04126	.00425
C5-102	.01130	.01629	.01298	.01352	.00249
C5-120	.01858	.01522	.01734	.01704	.00168
C5-144	.01659	.01754	.01529	.01647	.00112
C5-167.5	.02326	.02212	.02019	.02185	.00153
C5-F	.41297	.33434	.32259	.35664	.04519
C10-72	.01559	.01559	.02359	.01826	.00400
C10-96	.04634	.02581	.04203	.03806	.01027
C10-102	.01592	.01290	.01401	.01428	.00151
C10-120	.02186	.01170	.01784	.01713	.00508
C10-144	.01968	.01487	.02123	.01859	.00318
C10-167.5	.07189	.01420	.02711	.03774	.02884
C10-F	.28859	.30197	.33959	.31005	.02550
C15-72	.03266	.01984	.01709	.02320	.00778
C15-96	.03559	.02109	.03734	.03134	.00813
C15-102	.01298	.01415	.00929	.01214	.00243
C15-120	.01300	.01327	.01526	.01384	.00113
C15-144	.01761	.01714	.02016	.01831	.00151
C15-167.5	.01543	.01135	.01431	.01370	.00204
C15-F	.27922	.25859	.19659	.24480	.04131
C20-72	.02141	.04159	.04459	.03586	.01159
C20-96	.02722	.03797	.07109	.04543	.02194
C20-102	.01651	.01789	.03268	.02236	.00808
C20-120	.01376	.01145	.02801	.01774	.00828
C20-144	.01596	.01761	.01858	.01739	.00131
C20-167.5	.01486	.01335	.01654	.01492	.00160
C20-F	.22672	.21266	.36984	.26974	.07859
C30-72	.03684	.02941	.02666	.03097	.00509
C30-96	.09209	.02091	.02893	.04731	.03559
C30-102	.01100	.02408	.02418	.01975	.00659
C30-120	.00958	.02212	.02406	.01859	.00724
C30-144	.01858	.00550	.00870	.01093	.00654
C30-167.5	.01060	.01327	.01610	.01332	.00275
C30-F	.19953	.30059	.41634	.30549	.10841
S2-72	.02697	.01953	.02422	.02357	.00372
S2-96	.01457	.01625	.03527	.02203	.01035
S2-102	.00418	.00369	.00197	.00328	.00111
S2-120	.00330	.00197	.00094	.00207	.00118
S2-144	.00074	.00434	.00252	.00253	.00180
S2-167.5	.00413	.00275	.00626	.00438	.00175
S2-F	.13422	.19062	.16859	.16448	.02820

IS 8 CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
S3.5-72	.02547	.02503	.02459	.02503	.00044
S3.5-96	.00690	.01822	.01642	.01385	.00566
S3.5-102	.00108	.00224	.00168	.00167	.00058
S3.5-120	.00079	.00098	.00079	.00085	.00010
S3.5-144	.00205	.00151	.00118	.00158	.00044
S3.5-167.5	.00527	.00118	.00195	.00280	.00204
S3.5-F	.07109	.09109	.10984	.09068	.01938
S5-72	.02325	NA	.05247	.03786	.01461
S5-96	.01100	.03848	.00971	.01973	.01439
S5-102	.00071	.00260	.00184	.00171	.00094
S5-120	.00063	.00088	.00102	.00085	.00020
S5-144	.00084	.00049	.00128	.00087	.00039
S5-167.5	.00079	.00138	.00110	.00109	.00029
S5-F	.07797	.09734	.03609	.07047	.03062
S6.5-72	.02809	.03297	.05703	.03936	.01447
S6.5-96	.01026	.01309	.01172	.01169	.00142
S6.5-102	.00069	.00227	.00317	.00204	.00124
S6.5-120	.00021	.00354	.00582	.00319	.00281
S6.5-144	.00047	.00097	.00065	.00070	.00025
S6.5-167.5	.00083	.00035	.00053	.00057	.00024
S6.5-F	.02372	.02759	.06859	.03997	.02244
S10-72	.03509	.03359	.05647	.04172	.01144
S10-96	.00770	.00971	.00823	.00855	.00101
S10-102	.00079	.00195	.00153	.00142	.00058
S10-120	.00184	.00275	.00244	.00234	.00045
S10-144	.00086	.00079	.00039	.00068	.00024
S10-167.5	.00039	.00031	.00051	.00041	.00010
S10-F	.02859	.05359	.06891	.05036	.02016
CO-72	.03953	.03687	.00847	.02829	.01553
CO-96	.00943	.01120	.02854	.01639	.00956
CO-102	.03539	.03145	.04620	.03768	.00737
CO-120	.03121	.03195	.03502	.03272	.00190
CO-144	.03317	.02634	.03760	.03237	.00563
CO-167.5	.03539	.03631	.03784	.03651	.00123
CO-F	NA	1.29781	.97547	1.13664	.16117

CERULENIN AND FLUOROSUCCINATE (IS# 8)

IS8 CODE	C/MG(1)	C/MG(2)	C/MG(3)	AVG C/MG	(+/-)
C5-72	NA	NA	NA	NA	NA
C5-96	NA	NA	NA	NA	NA
C5-102	8068	3522	9202	6931	2840
C5-120	4586	3352	9931	5956	3290
C5-144	5930	3764	5572	5089	1083
C5-167.5	5237	3445	13492	7391	5023
C5-F	3266	2875	8104	4748	2614
C10-72	NA	NA	NA	NA	NA
C10-96	NA	NA	NA	NA	NA
C10-102	3052	NA	NA	3052	0
C10-120	5901	3129	4977	4669	1386
C10-144	3842	484	2091	2139	1679
C10-167.5	NA	NA	5799	5799	0
C10-F	4815	1369	3564	3249	1723
C15-72	NA	NA	NA	NA	NA
C15-96	NA	NA	NA	NA	NA
C15-102	NA	NA	NA	NA	NA
C15-120	NA	NA	NA	NA	NA
C15-144	NA	NA	833	833	0
C15-167.5	NA	NA	NA	NA	NA
C15-F	937	1111	1782	1277	423
C20-72	NA	NA	NA	NA	NA
C20-96	NA	NA	NA	NA	NA
C20-102	NA	NA	NA	NA	NA
C20-120	5494	NA	578	3036	2458
C20-144	7517	NA	NA	7517	0
C20-167.5	10093	NA	NA	10093	0
C20-F	11901	93	706	4233	5904
C30-72	NA	NA	NA	NA	NA
C30-96	NA	NA	NA	NA	NA
C30-102	NA	NA	NA	NA	NA
C30-120	NA	NA	NA	NA	NA
C30-144	NA	NA	NA	NA	NA
C30-167.5	NA	NA	NA	NA	NA
C30-F	201	319	238	253	59
S2-72	NA	NA	NA	NA	NA
S2-96	NA	NA	NA	NA	NA
S2-102	NA	NA	109872	109872	0
S2-120	16895	5799	212368	78354	103285
S2-144	NA	20715	51878	36297	15582
S2-167.5	37351	71068	98220	68880	30435
S2-F	23228	16156	36781	25388	10312

IS 8 CODE	C/MG (1)	C/MG (2)	C/MG(3)	AVG C/MG	(+/-)
S3.5-72	NA	NA	NA	NA	NA
S3.5-96	NA	NA	NA	NA	NA
S3.5-102	NA	NA	NA	NA	NA
S3.5-120	NA	NA	NA	NA	NA
S3.5-144	NA	NA	NA	NA	NA
S3.5-167.5	11957	16786	NA	14372	2414
S3.5-F	25825	4749	4026	11533	10900
S5-72	NA	NA	NA	NA	NA
S5-96	NA	NA	NA	NA	NA
S5-102	NA	NA	NA	NA	NA
S5-120	NA	NA	NA	NA	NA
S5-144	38778	NA	NA	38778	0
S5-167.5	NA	NA	NA	NA	NA
S5-F	6510	12734	10107	9784	3112
S6.5-72	NA	NA	NA	NA	NA
S6.5-96	NA	NA	NA	NA	NA
S6.5-102	NA	NA	NA	NA	NA
S6.5-120	NA	NA	NA	NA	NA
S6.5-144	NA	NA	NA	NA	NA
S6.5-167.5	NA	NA	NA	NA	NA
S6.5-F	NA	NA	5808	5808	0
S10-72	NA	NA	NA	NA	NA
S10-96	NA	NA	NA	NA	NA
S10-102	NA	NA	NA	NA	NA
S10-120	NA	NA	NA	NA	NA
S10-144	NA	NA	NA	NA	NA
S10-167.5	NA	NA	NA	NA	NA
S10-F	2203	313	575	1030	945
CO-72	NA	NA	NA	NA	NA
CO-96	NA	NA	NA	NA	NA
CO-102	475417	479107	576387	510304	50485
CO-120	458002	410978	358102	409027	49950
CO-144	559470	521892	362251	481204	98610
CO-167.5	349709	434762	430562	405011	42527
CO-F	NA	77056	134641	105849	28792

CERULENIN AND FLUOROSUCCINATE (IS# 8)

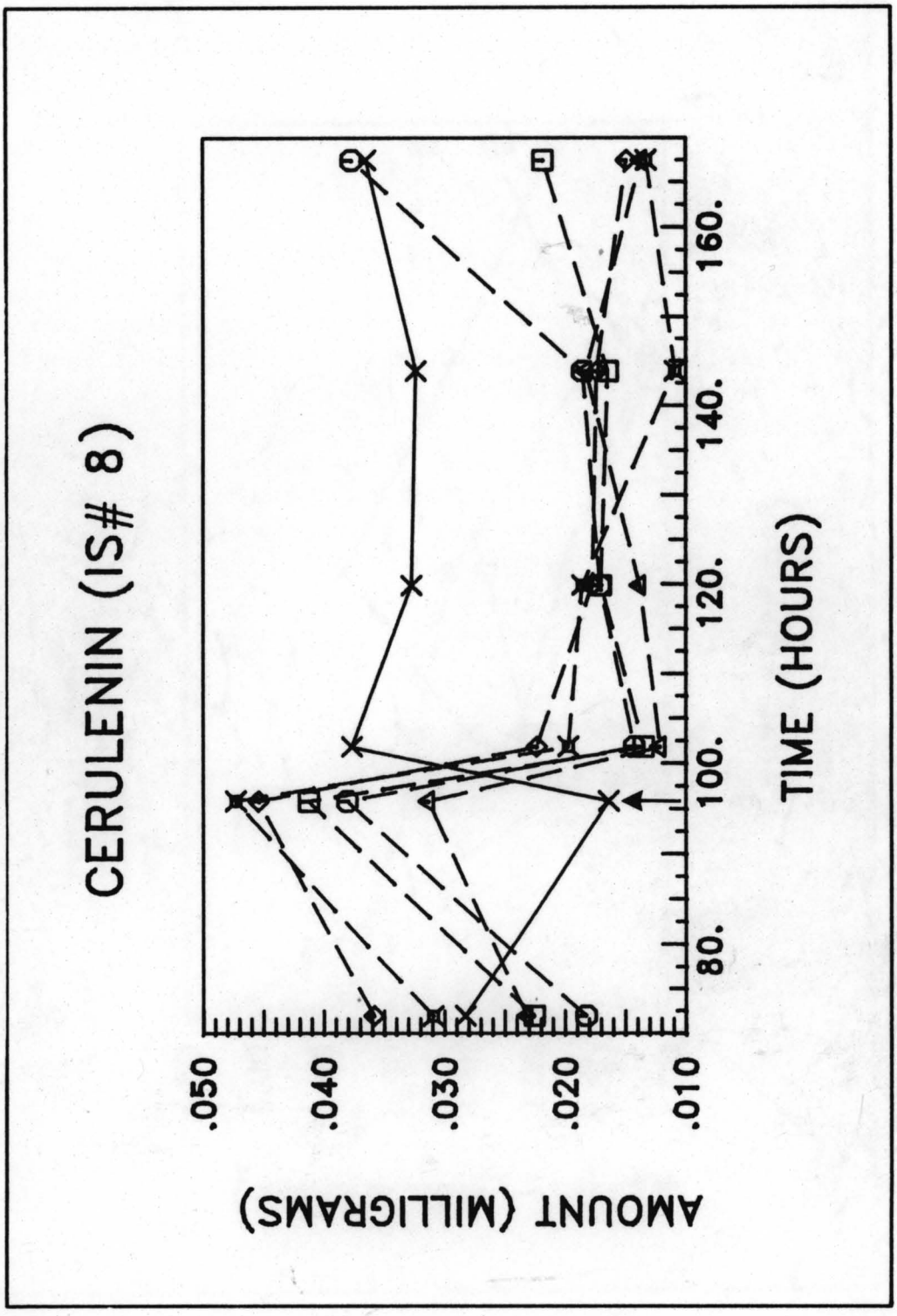
IS 8 CODE	WT(1)	WT(2)	WT(3)	AVG WT	(+/-)
C5-72	3.32	3.39	3.36	3.36	.04
C5-96	3.35	2.54	2.69	2.86	.41
C5-102	2.94	.92	2.21	2.02	1.01
C5-120	2.71	2.53	1.72	2.32	.49
C5-144	1.78	1.47	5.43	2.89	1.98
C5-167.5	2.9	2.54	2.88	2.77	.18
C10-72	2.93	2.44	2.98	2.78	.27
C10-96	2.42	2.04	2.67	2.38	.31
C10-102	2.06	1.14	2.41	1.87	.64
C10-120	1.7	1.68	1.99	1.79	.16
C10-144	1.09	.63	1.71	1.14	.54
C10-167.5	2.61	3.05	2.5	2.72	.27
C15-72	2.35	3.51	.86	2.24	1.32
C15-96	2.34	3.33	2.24	2.64	.54
C15-102	1.3	2.7	2.31	2.10	.70
C15-120	1.81	1.77	1.69	1.76	.06
C15-144	.92	1.16	1.08	1.05	.12
C15-167.5	2.03	2.15	2.26	2.15	.11
C20-72	1.91	.95	4.58	2.48	1.81
C20-96	2.75	1.9	3.87	2.84	.99
C20-102	3.06	1.84	3.59	2.83	.87
C20-120	1.87	2.43	3.41	2.57	.77
C20-144	1.84	1.18	1.51	1.51	.33
C20-167.5	2.45	1.66	2.7	2.27	.52
C30-72	2.28	3.85	2.47	2.87	.79
C30-96	3.54	3.1	1.5	2.71	1.02
C30-102	2.51	2.28	1.89	2.23	.31
C30-120	2.81	1.96	1.9	2.22	.46
C30-144	2.2	1.57	2.09	1.95	.32
C30-167.5	3.27	2.11	2.69	2.69	.58
S2-72	2.09	1.41	2.89	2.13	.74
S2-96	1.06	1	2.38	1.48	.69
S2-102	2.02	2.28	2.6	2.30	.29
S2-120	1.59	1.94	2.16	1.90	.29
S2-144	2.09	1.89	3.18	2.39	.65
S2-167.5	3.29	4.18	3.18	3.55	.50

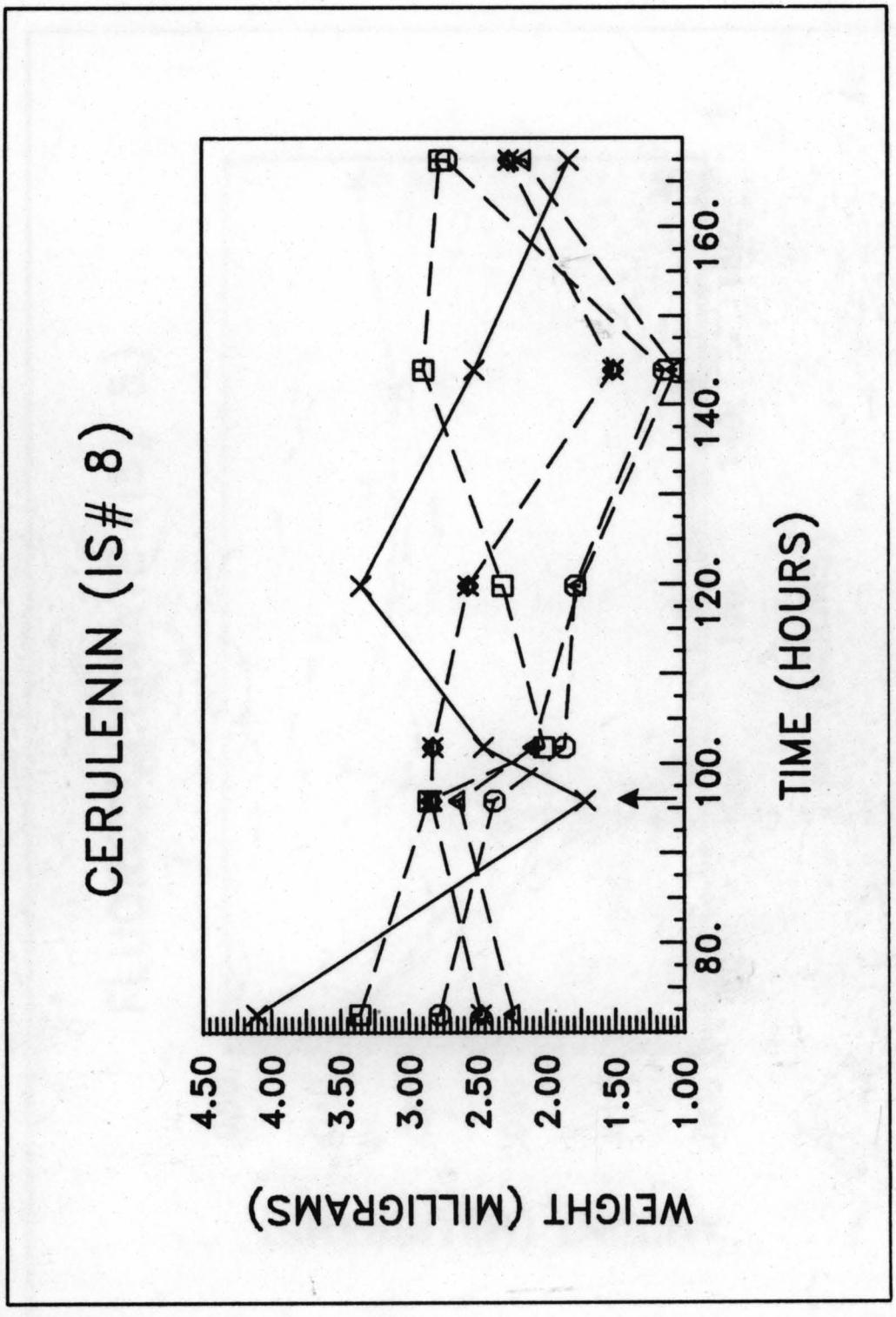
IS 8 CODE	WT(1)	WT(2)	WT(3)	AVG WT	(+/-)
S3.5-72	NA	2.78	3.03	2.90	.12
S3.5-96	2.44	3	3.41	2.95	.49
S3.5-102	2.13	3.13	2.77	2.68	.50
S3.5-120	1.63	2.38	2.42	2.14	.40
S3.5-144	2.32	2.84	3.22	2.79	.45
S3.5-167.5	2.91	3.24	3.24	3.13	.17
S5-72	2.44	4.2	5.71	4.12	1.64
S5-96	3.29	1.44	3.3	2.68	.93
S5-102	2.95	2.79	2.63	2.79	.16
S5-120	2.42	1.93	1.58	1.98	.42
S5-144	3.04	2.9	2.59	2.84	.23
S5-167.5	2.22	2.24	3.44	2.63	.61
S6.5-72	4.37	4.04	2.75	3.72	.81
S6.5-96	3.41	2.67	1.21	2.43	1.10
S6.5-102	3.56	2.82	2.49	2.96	.53
S6.5-120	3.79	2.81	3.2	3.27	.49
S6.5-144	2.94	2.6	4.48	3.34	.94
S6.5-167.5	2.77	3.43	3.18	3.13	.33
S10-72	4.42	3.41	5.16	4.33	.88
S10-96	3.68	1.18	2.56	2.47	1.25
S10-102	3.68	2.36	2.98	3.01	.66
S10-120	3.62	2.47	2.55	2.88	.57
S10-144	3.46	2.35	4.6	3.47	1.12
S10-167.5	4.13	2.74	2.77	3.21	.69
CO-72	5.68	3.4	3.24	4.11	1.22
CO-96	1.41	1.31	2.43	1.72	.56
CO-102	2.66	2.3	2.43	2.46	.18
CO-120	4	2.29	3.75	3.35	.85
CO-144	1.77	2.18	3.6	2.52	.92
CO-167.5	1.65	1.8	2.04	1.83	.20

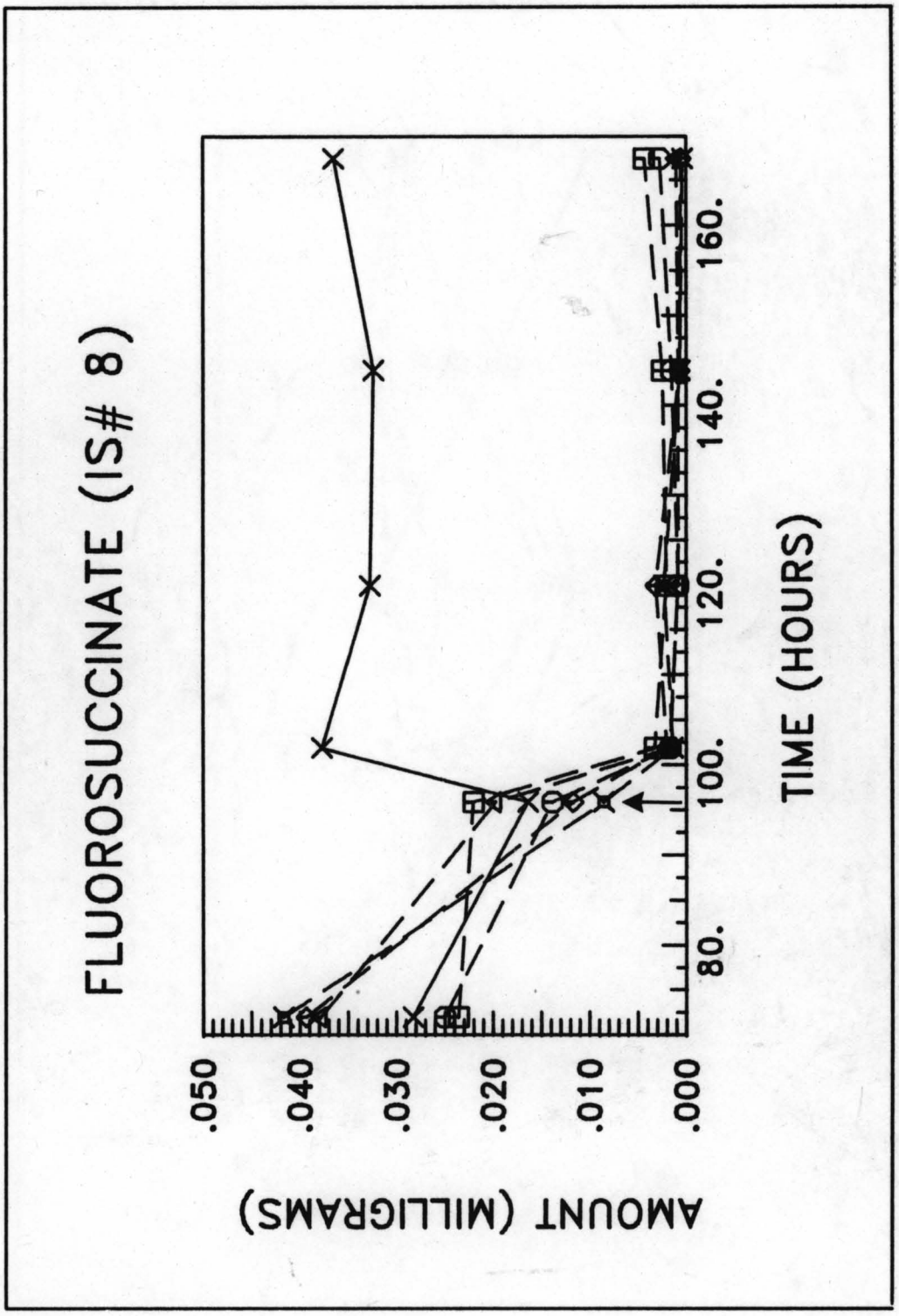
CERULENIN AND FLUOROSUCCINATE (IS# 8)

IS 8 CODE	pH (1)	pH (2)	pH (3)	AVG pH
C5	7.63	7.48	7.50	7.54
C10	7.42	7.49	7.48	7.46
C15	7.48	7.55	7.48	7.50
C20	7.34	7.35	7.38	7.36
C30	7.45	7.32	7.32	7.36
S2	4.88	4.62	4.74	4.75
S3.5	4.16	4.18	4.09	4.14
S5	3.79	3.90	3.89	3.86
S6.5	3.60	3.60	3.62	3.61
S10	3.24	3.22	3.16	3.21
CONTROL	7.43	7.62	7.64	7.56

	PACKED CELL VOLUME (cc)			
IS 8 CODE	(1)	(2)	(3)	AVG
C5	3.5	1.5	2.5	2.5
C10	2.0	1.5	2.0	1.8
C15	1.5	2.0	2.0	1.8
C20	2.0	1.5	4.0	2.5
C30	4.0	2.0	1.5	2.5
S2	1.5	1.0	1.0	1.2
S3.5	1.0	1.0	1.0	1.0
S5	1.0	1.0	1.0	1.0
S6.5	1.0	1.0	1.0	1.0
S10	1.0	1.0	1.0	1.0
CONTROL	2.0	2.0	2.0	2.0


CERULENIN (IS# 8)
AMOUNT (MILLIGRAMS)
.050
.040
.030
.020
.010
80.
100.
120.
140.
160.
TIME (HOURS)


CERULENIN (IS# 8)
WEIGHT (MILLIGRAMS)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
80.
100.
120.
140.
160.
TIME (HOURS)


FLUOROSUCCINATE (IS# 8)
AMOUNT (MILLIGRAMS)
.050
.040
.030
.020
.010
.000
80.
100.
120.
140.
160.
TIME (HOURS)

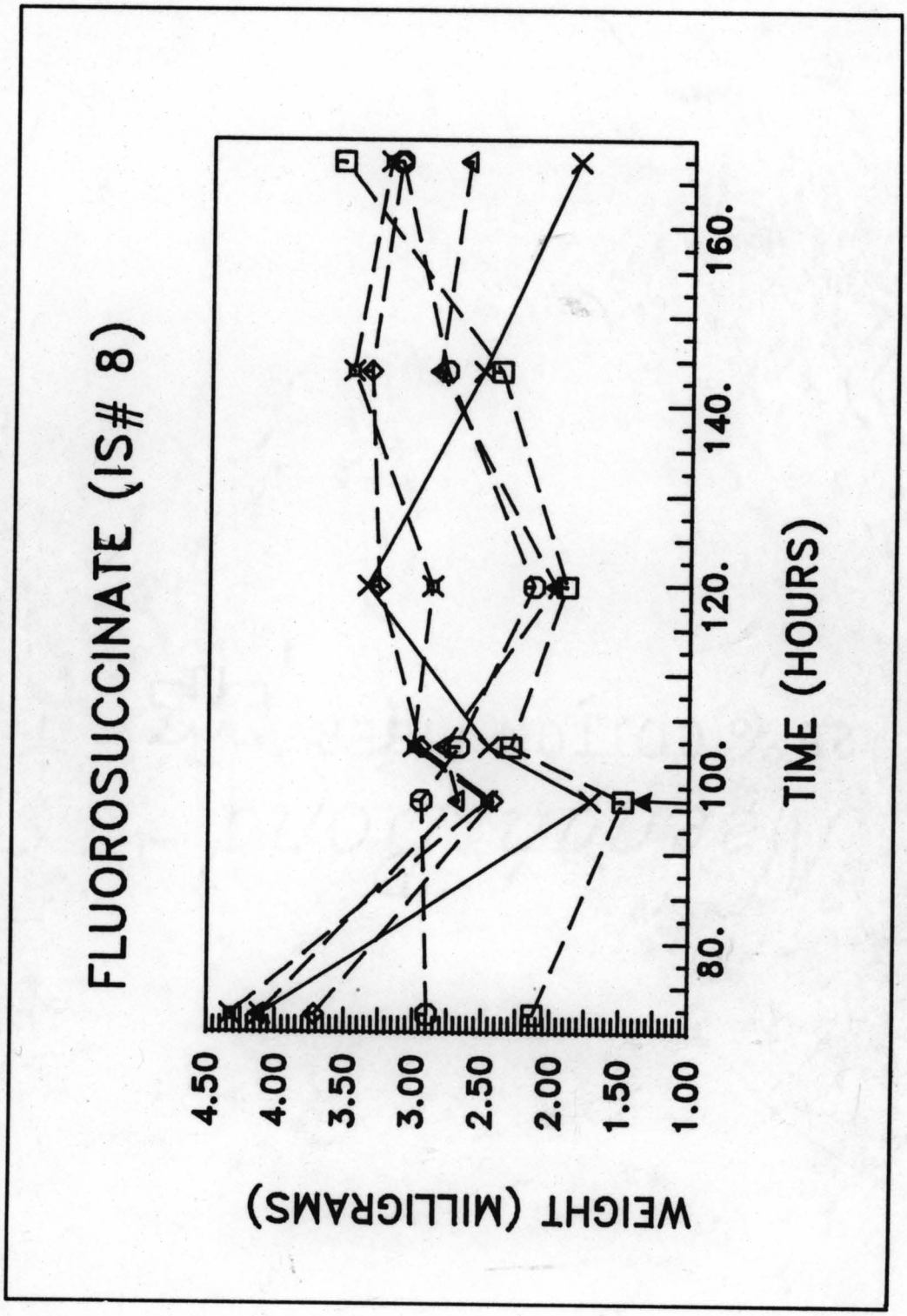

FLUOROSUCCINATE (IS# 8)
WEIGHT (MILLIGRAMS)
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
80.
100.
120.
140.
160.
TIME (HOURS)

Figure 52. Results of Inhibitor Study # 7:
Mono-Oxygenase and Protein Synthesis Inhibitors

Codes:

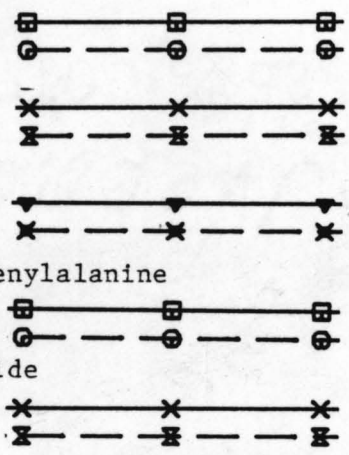
2-Diethyl-GEB
GE
GE (S)*

AY 994
AY
AY (S)

SKF 525-A
SK
SK (S)

p-Fluorophenylalanine
PF
PF (S)

Cycloheximide
CH
CH (S)



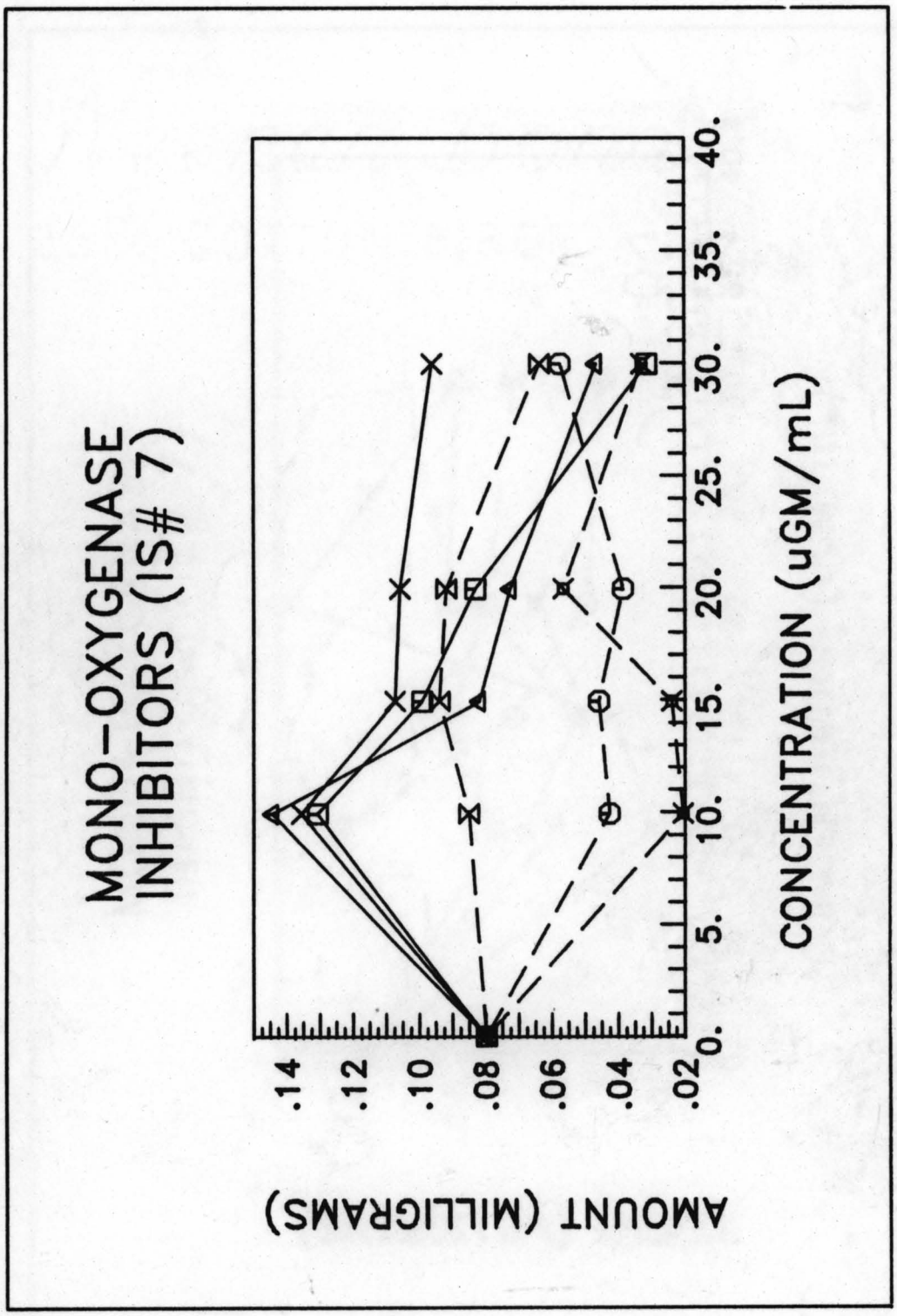
* (S) = Inhibitor also present in Seed Culture

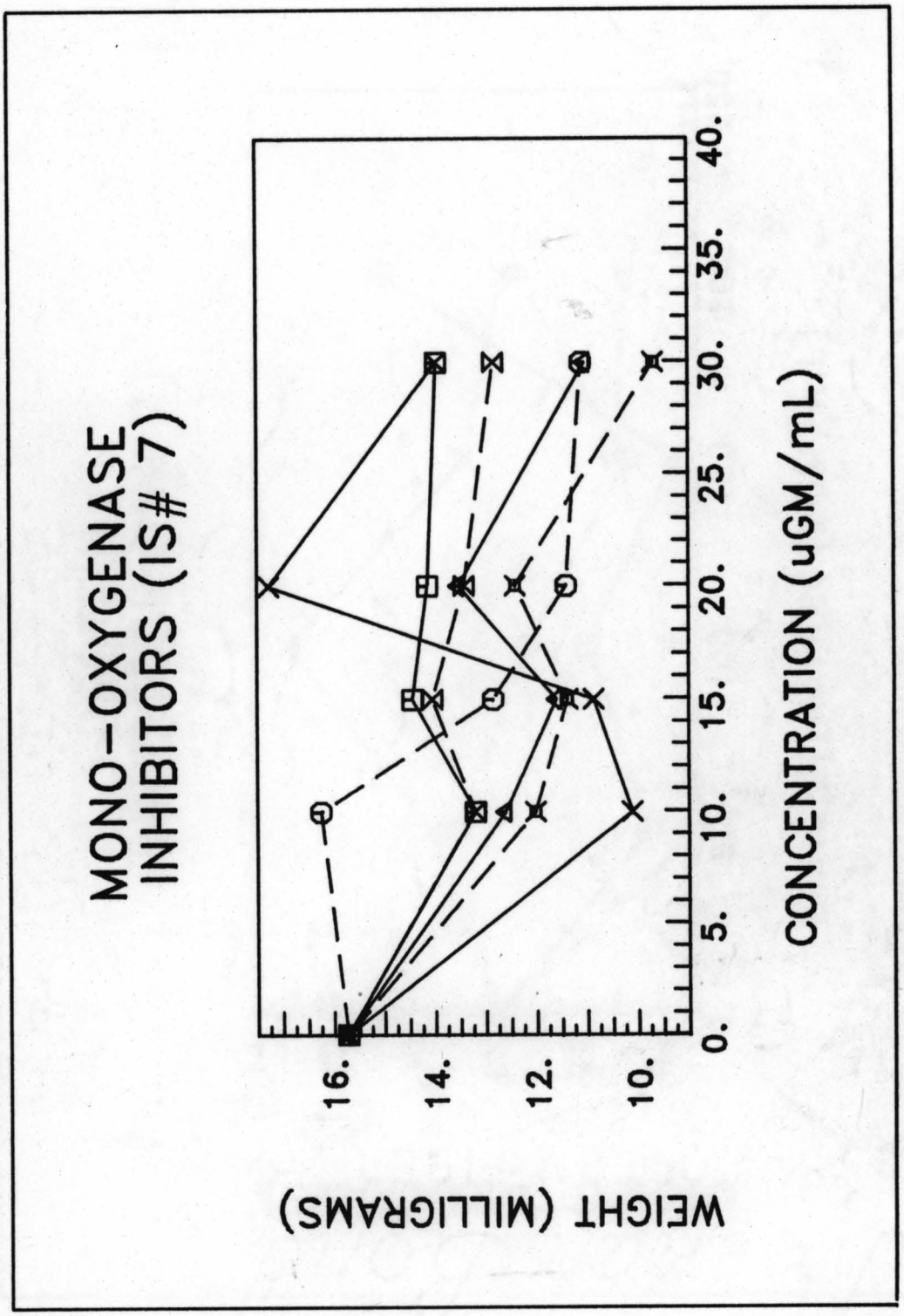
MONO-OXYGENASE AND PROTEIN INHIBITORS (IS# 7)

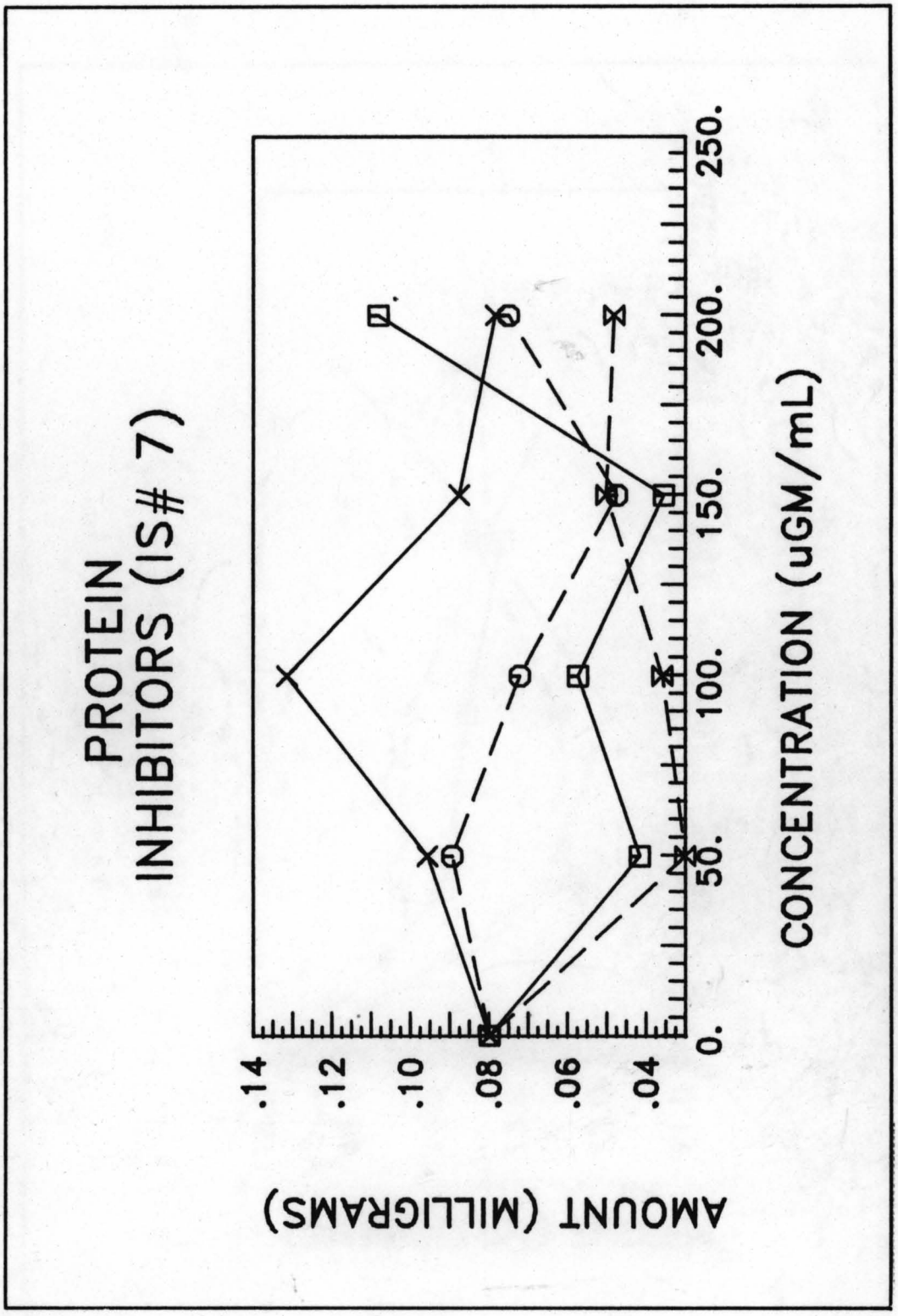
IS 7 CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
CONTROL	.09578	.10359	.04559	.08166	.02900
CONTROL	NA	.07359	.08275	.07817	.00458
GE-10	.14984	.09816	.14431	.13077	.02584
GE-15	.12844	.12159	.04766	.09923	.04039
GE-20	.04766	.04203	.15978	.08316	.05887
GE-30	.02472	.01709	.05234	.03139	.01763
GE-10(S)	.05803	.04391	.02634	.04276	.01584
GE-15(S)	.05759	.05234	.02797	.04597	.01481
GE-20(S)	.03597	.06237	.01759	.03865	.02239
GE-30(S)	.07109	.06219	.03934	.05754	.01588
AY-10	.13953	.10613	.16109	.13558	.02748
AY-15	.10612	.07959	.13641	.10738	.02841
AY-20	.09297	.09859	.12609	.10589	.01656
AY-30	.04234	.19905	.04702	.09614	.07835
AY-10(S)	.01372	.08063	.16109	.08515	.07369
AY-15(S)	.12859	.08609	.06734	.09401	.03063
AY-20(S)	.11359	.07359	.08859	.09193	.02000
AY-30(S)	.04959	.04344	.09816	.06373	.02736
SK-10	.15256	.14884	.13147	.14429	.01055
SK-15	.10359	.07159	.07053	.08191	.01653
SK-20	.10063	.09859	.01859	.07260	.04102
SK-30	.05703	.03172	.05109	.04661	.01266
SK-10(S)	.02703	NA	.01422	.02063	.00641
SK-15(S)	.03406	.01484	.02328	.02406	.00961
SK-20(S)	.04797	.08703	.03500	.05667	.02602
SK-30(S)	.02484	.01625	.05797	.03302	.02086
PF-50	.07141	.03359	.01984	.04161	.02578
PF-100	.02009	.02609	.12578	.05732	.05284
PF-150	.03459	.02803	.04384	.03549	.00790
PF-200	.13222	.16172	.03059	.10818	.06556
PF-50(S)	.13484	.04972	.08359	.08939	.04256
PF-100(S)	.08859	.06959	.05816	.07211	.01522
PF-150(S)	.05406	.06684	.02147	.04746	.02269
PF-200(S)	.03609	.09922	.08984	.07505	.03156
CH-50	.15359	.10812	.02547	.09573	.06406
CH-100	.14934	.05422	.19059	.13139	.06819
CH-150	.12578	.02459	.11234	.08757	.05059
CH-200	.03172	.06609	.13719	.07833	.05273
CH-50(S)	.01409	.01559	.06059	.03009	.02325
CH-100(S)	.06734	.02609	.01034	.03459	.02850
CH-150(S)	.07266	.01294	.06484	.05015	.02986
CH-200(S)	.01666	.08488	.04297	.04817	.03411

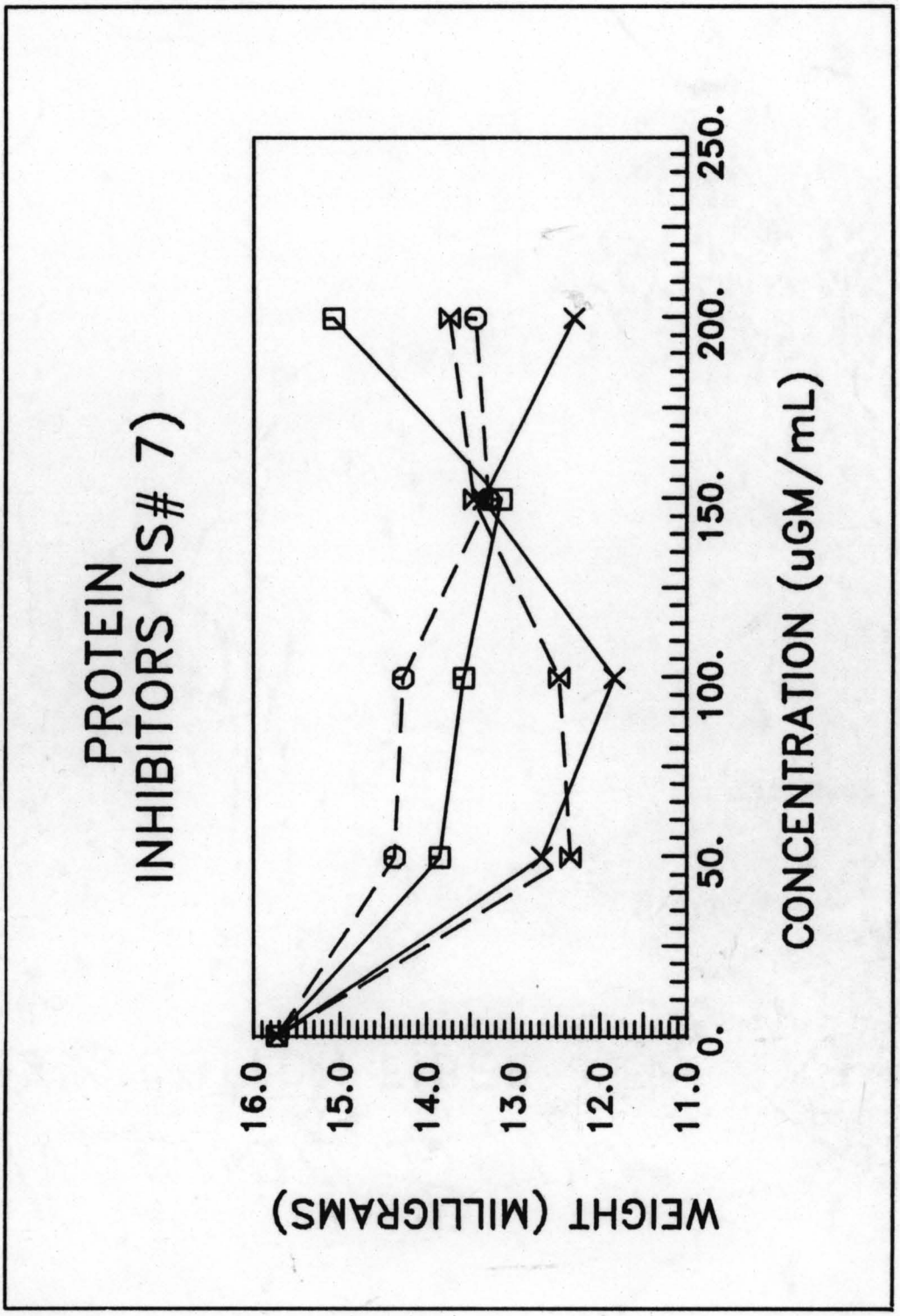
MONO-OXYGENASE AND PROTEIN INHIBITORS (IS# 7)

IS 7 CODE	WT(1)	WT(2)	WT(3)	AVG WT	(+/-)
CONTROL	14.14	15.2	16.04	15.13	.95
CONTROL	15.28	18.79	14.92	16.33	1.93
GE-10	13.38	12.85	13.37	13.20	.27
GE-15	14.51	13.82	14.98	14.44	.58
GE-20	14.02	14.73	13.68	14.14	.53
GE-30	16	12.16	13.73	13.96	1.92
GE-10(S)	13.81	12.58	22.37	16.25	4.90
GE-15(S)	14.47	13.39	10.69	12.85	1.89
GE-20(S)	14.11	9.85	10.26	11.41	2.13
GE-30(S)	10.22	11.45	11.66	11.11	.72
AY-10	8.83	10.23	11.31	10.12	1.24
AY-15	11.58	11.36	9.68	10.87	.95
AY-20	13.65	15.27	22.88	17.27	4.61
AY-30	14.03	13.86	14.02	13.97	.08
AY-10(S)	13.73	13.76	12.14	13.21	.81
AY-15(S)	16.68	9.88	15.59	14.05	3.40
AY-20(S)	12.6	13.72	13.94	13.42	.67
AY-30(S)	13.31	13.19	12.02	12.84	.65
SK-10	12.36	14.42	11.06	12.61	1.68
SK-15	11.42	11.86	11.51	11.60	.22
SK-20	14.7	12.11	13.78	13.53	1.29
SK-30	10	11.53	11.6	11.04	.80
SK-10(S)	11.34	13.13	11.6	12.02	.90
SK-15(S)	10.66	9.95	13.54	11.38	1.79
SK-20(S)	11.38	12.99	12.9	12.42	.80
SK-30(S)	10.65	8.75	9.68	9.69	.95
PF-50	14.3	12.61	14.63	13.85	1.01
PF-100	12.51	14.18	14.03	13.57	.83
PF-150	13.63	13.11	12.68	13.14	.48
PF-200	15.22	13.03	17	15.08	1.99
PF-50(S)	14.72	15.3	13.1	14.37	1.10
PF-100(S)	15.56	14.45	12.79	14.27	1.39
PF-150(S)	12.69	14.26	12.91	13.29	.79
PF-200(S)	13.55	13.27	13.49	13.44	.14
CH-50	11.68	13.71	12.61	12.67	1.02
CH-100	11.65	13.17	10.67	11.83	1.25
CH-150	10.68	18.34	11.02	13.35	3.83
CH-200	12.4	12.64	11.85	12.30	.40
CH-50(S)	12.68	13.61	10.74	12.34	1.43
CH-100(S)	13.5	13.52	10.4	12.47	1.56
CH-150(S)	14.01	12.6	13.76	13.46	.71
CH-200(S)	13.19	12.74	15.3	13.74	1.28


MONO-OXYGENASE
INHIBITORS (IS# 7)
AMOUNT (MILLIGRAMS)
.14
.12
.10
.08
.06
.04
.02
0.
5.
10.
15.
20.
25.
30.
35.
40.
CONCENTRATION (uGM/mL)


MONO-OXYGENASE
INHIBITORS (IS# 7)
WEIGHT (MILLIGRAMS)
16.
14.
12.
10.
0.
5.
10.
15.
20.
25.
30.
35.
40.
CONCENTRATION (uGM/mL)


PROTEIN
INHIBITORS (IS# 7)
AMOUNT (MILLIGRAMS)
.14
.12
.10
.08
.06
.04
0.
50.
100.
150.
200.
250.
CONCENTRATION (uGM/mL)


PROTEIN
INHIBITORS (IS# 7)
WEIGHT (MILLIGRAMS)
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15.0
14.0
13.0
12.0
11.0
0.
50.
100.
150.
200.
250.
CONCENTRATION (uGM/mL)

Figure 53. Results of Inhibitor Study # 11:
Protein Synthesis Inhibitors

PROTEIN INHIBITORS (IS# 11)

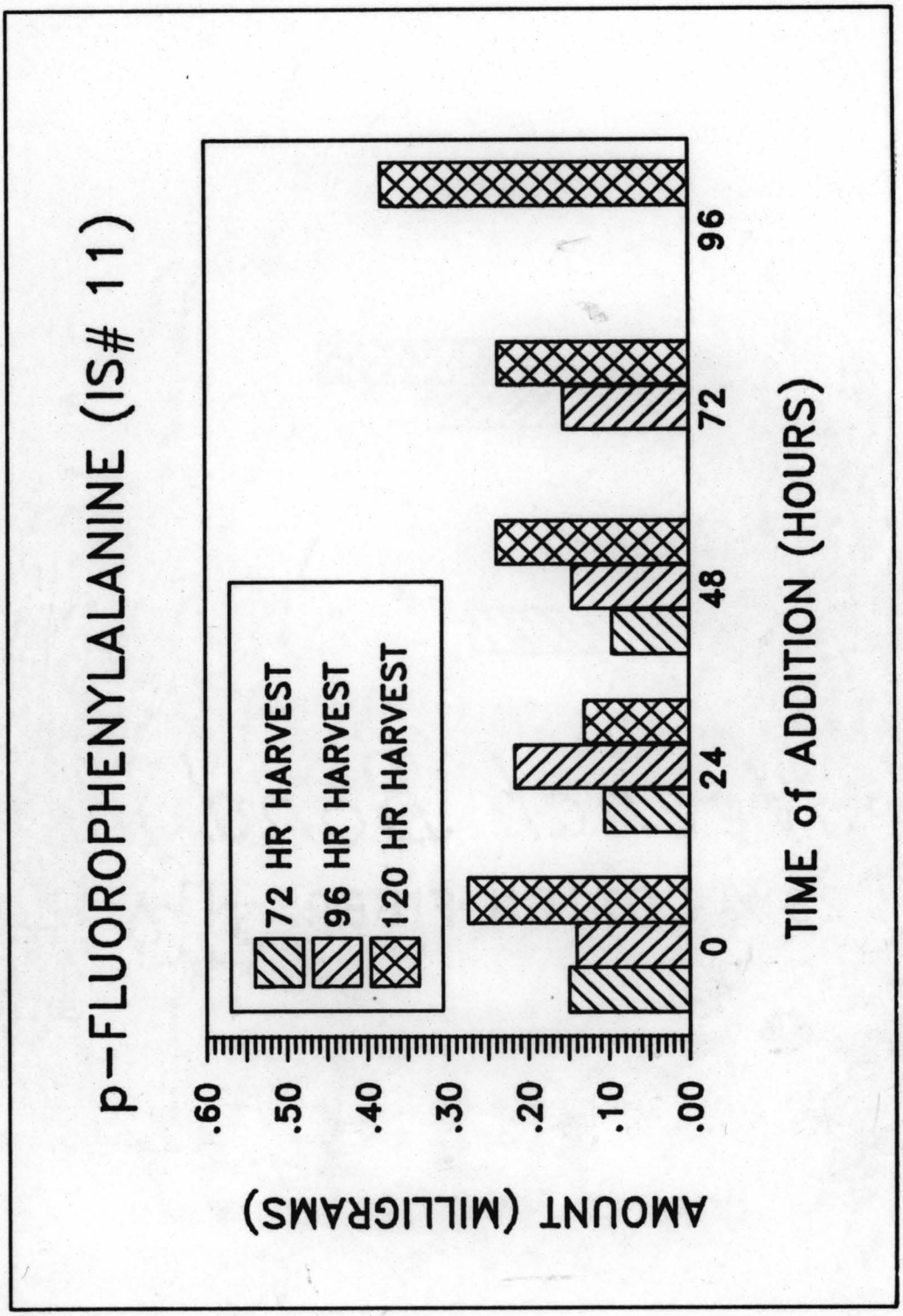
IS11 CODE	AMT(1)	AMT(2)	AMT(3)	AVG AMT	(+/-)
P-0,72	.13929	.15718	.15334	.14993	.00894
P-0,96	.11560	.10419	.20220	.14066	.04900
P-0,120	.29391	.33027	.20170	.27529	.06429
P-24,72	.12779	.09083	.09830	.10564	.01848
P-24,96	.11009	.26304	.27720	.21678	.08355
P-24,120	.13516	.10616	.14951	.13028	.02167
P-48,72	.09436	.05308	.14155	.09633	.04423
P-48,96	.14744	.17084	.11796	.14541	.02644
P-48,120	.28781	.27602	.15413	.23932	.06684
P-72,96	.19817	.13840	.13211	.15623	.03303
P-72,120	.22706	.25950	.22608	.23755	.01671
P-96,120	.41678	.34679	NA	.38178	.03499
C-0,72	.19463	.41481	.44115	.35020	.12326
C-0,96	.45806	.27842	.27572	.33740	.09117
C-0,120	.29980	.25410	.16219	.23870	.06881
C-24,72	.16612	.26835	.20888	.21445	.05111
C-24,96	.26540	.37510	.27326	.30459	.05485
C-24,120	.43879	.32320	.23788	.33329	.10046
C-48,72	.27130	.14705	.24771	.22202	.06212
C-48,96	.41678	.32241	.36802	.36907	.04718
C-48,120	.31750	.40301	.26835	.32962	.06733
C-72,96	.28486	NA	NA	.28486	0.00000
C-72,120	.29194	.15098	.34915	.26402	.09908
C-96,120	.20052	NA	.41992	.31022	.10970
CO-0,72	.08552				
CO-0,96	.17988				
CO-0,120	.31612				
CO-24,72	.05308				
CO-24,96	.22598				
CO-24,120	.27228				
CO-48,72	.21301				
CO-48,96	.37844				
CO-48,120	.17300				
CO-72,96	.06389				
CO-72,120	.06094				
CO-96,120	.15740				

PROTEIN INHIBITORS (IS# 11)

IS11 CODE	C/MG(1)	C/MG(2)	C/MG(3)	AVG C/MG	(+/-)
P-0,72	168.00	267.22	129.12	188.11	69.05
P-0,96	134.95	109.41	118.70	121.02	12.77
P-0,120	8.17	123.53	50.57	60.76	57.68
P-24,72	98.60	85.88	140.39	108.29	27.26
P-24,96	NA	NA	71.43	71.43	0.00
P-24,120	NA	124.34	108.36	116.35	7.99
P-48,72	1646.81	2238.14	1521.76	1802.24	358.19
P-48,96	1379.51	519.79	544.27	814.52	429.86
P-48,120	289.77	2543.36	700.71	1177.95	1126.79
P-72,96	2398.00	2627.15	1494.21	2173.12	566.47
P-72,120	3091.64	2508.65	3569.51	3056.60	530.43
P-96,120	1910.38	1460.26	NA	1685.32	225.06
C-0,72	NA	44.84	35.36	40.10	4.74
C-0,96	157.18	23.70	139.27	106.72	66.74
C-0,120	116.08	120.43	51.79	96.10	34.32
C-24,72	NA	40.25	68.94	54.59	14.35
C-24,96	45.21	102.37	120.76	89.45	37.77
C-24,120	134.00	129.96	138.73	134.23	4.39
C-48,72	460.01	518.19	552.27	510.16	46.13
C-48,96	417.49	321.95	314.66	351.37	51.42
C-48,120	171.97	257.56	234.77	221.43	42.79
C-72,96	1937.78	NA	NA	1937.78	0.00
C-72,120	1042.00	850.43	1022.49	971.64	95.78
C-96,120	798.91	NA	1658.88	1228.89	429.99
CO-0,72	84.19				
CO-0,96	123.41				
CO-0,120	146.15				
CO-24,72	203.47				
CO-24,96	31.86				
CO-24,120	101.37				
CO-48,72	1940.78				
CO-48,96	1060.67				
CO-48,120	860.11				
CO-72,96	3690.57				
CO-72,120	2028.11				
CO-96,120	2011.81				

PROTEIN INHIBITORS (IS# 11)

IS11 CODE	TOTAL PCV
P-0,72	6.5 cc
P-0-96	6.5 cc
P-0,120	6.5 cc
P-24,72	6.5 cc
P-24,96	5 cc
P-24,120	6.5 cc
P-48,72	6.5 cc
P-48,96	6.5 cc
P-48,120	6.5 cc
P-72,96	6 cc
P-72,120	7.5 cc
P-96,120	6 cc
C-0,72	8.5 cc
C-0,96	7 cc
C-0,120	7.5 cc
C-24,72	7.5 cc
C-24,96	7.5 cc
C-24,120	7.5 cc
C-48,72	6.5 cc
C-48,96	7.5 cc
C-48,120	7 cc
C-72,96	5 cc
C-72,120	6.5 cc
C-96,120	5 cc
CO-0,72	2.5 cc
CO-0,96	2.5 cc
CO-0,120	2.5 cc
CO-24,72	2.5 cc
CO-24,96	2.5 cc
CO-24,120	2.5 cc
CO-48,72	2.5 cc
CO-48,96	2.5 cc
CO-48,120	2.5 cc
CO-72,96	2.5 cc
CO-72,120	2.5 cc
CO-96,120	2.5 cc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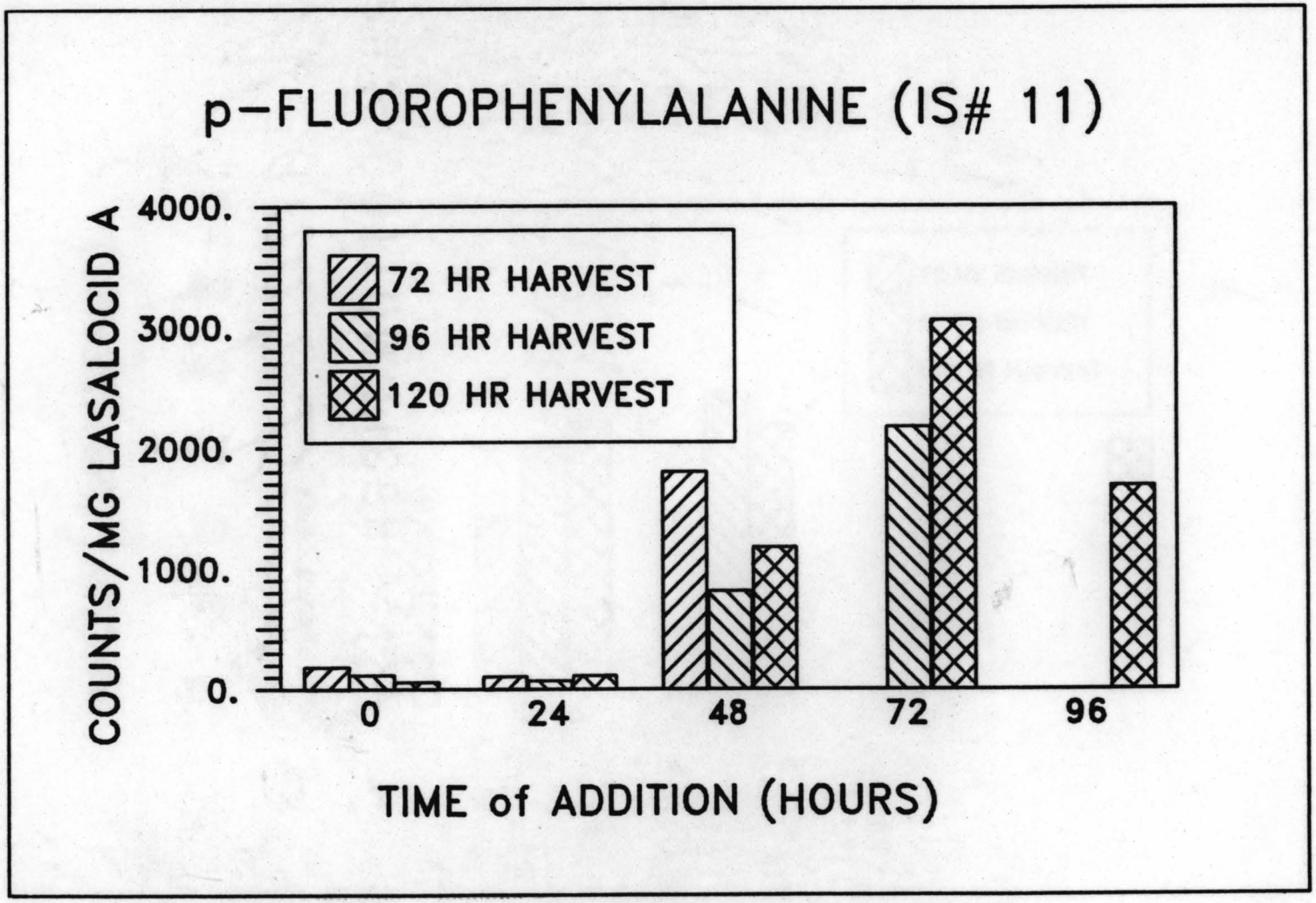
96 HR HARVEST
120 HR HARVEST
.60
.50
.40
.30
.20
.10
.00
AMOUNT (MILLIGRAMS)
0
24
48
72
96
TIME of ADDITION (HOURS)


p-FLUOROPHENYLALANINE (IS# 11)
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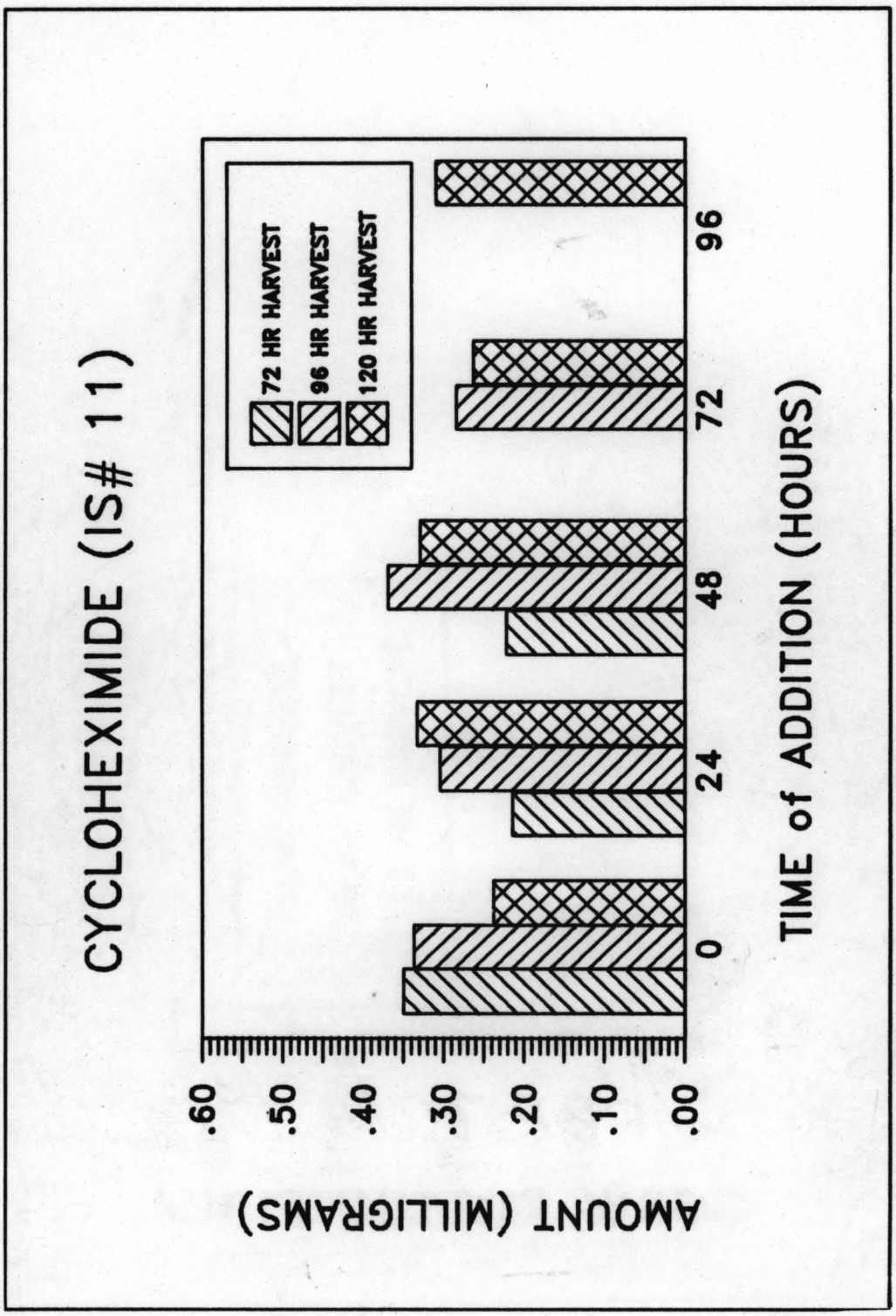
3000.
2000.
1000.
0.
72 HR HARVEST
96 HR HARVEST
120 HR HARVEST
0
24
48
72
96
TIME of ADDITION (HOURS)


CYCLOHEXIMIDE (IS# 11)
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96 HR HARVEST
120 HR HARVEST
AMOUNT (MILLIGRAMS)
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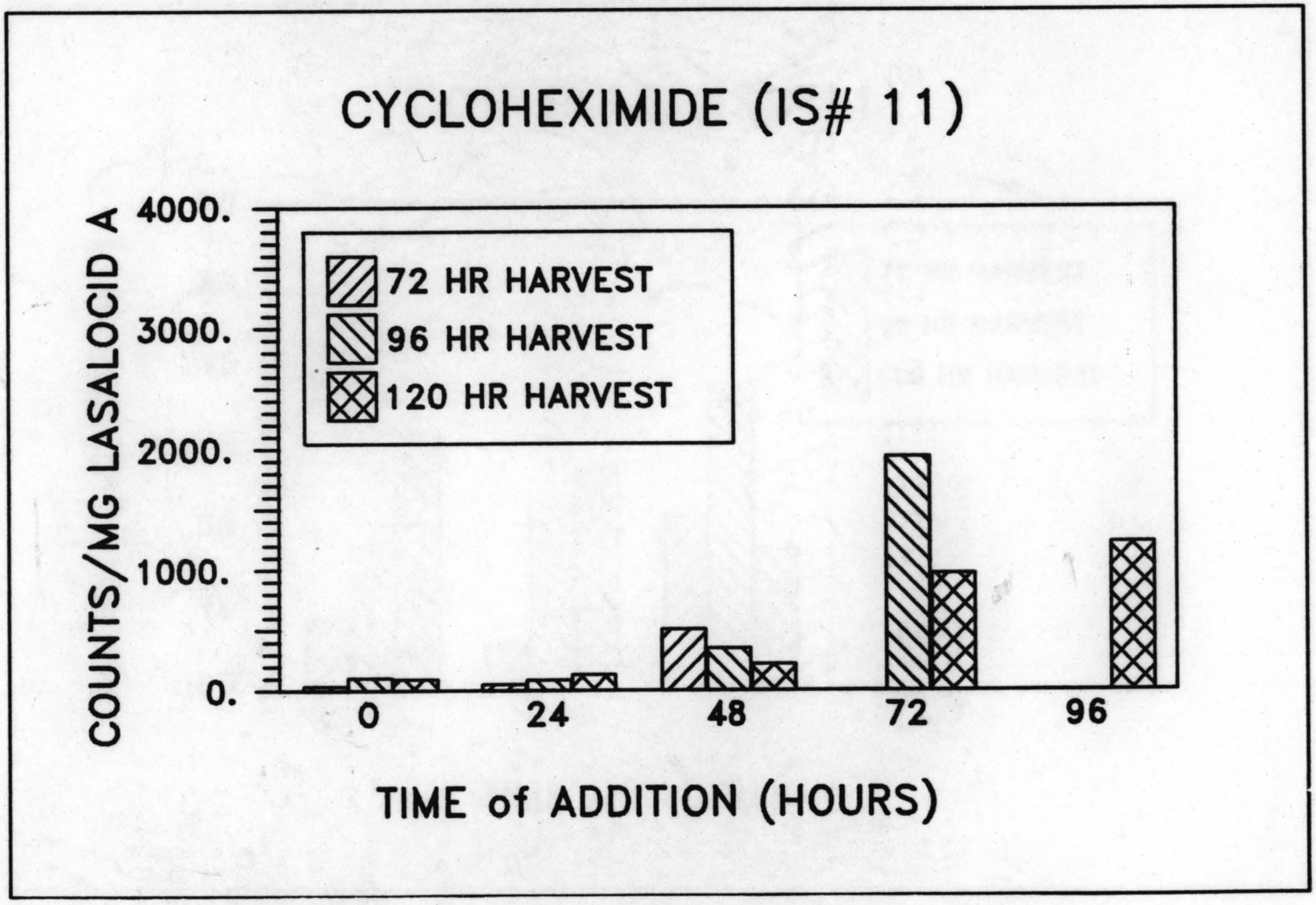
.50
.40
.30
.20
.10
.00
0
24
48
72
96
TIME of ADDITION (HOURS)


CYCLOHEXIMIDE (IS# 11)
COUNTS/MG LASALOCID A
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3000.
2000.
1000.
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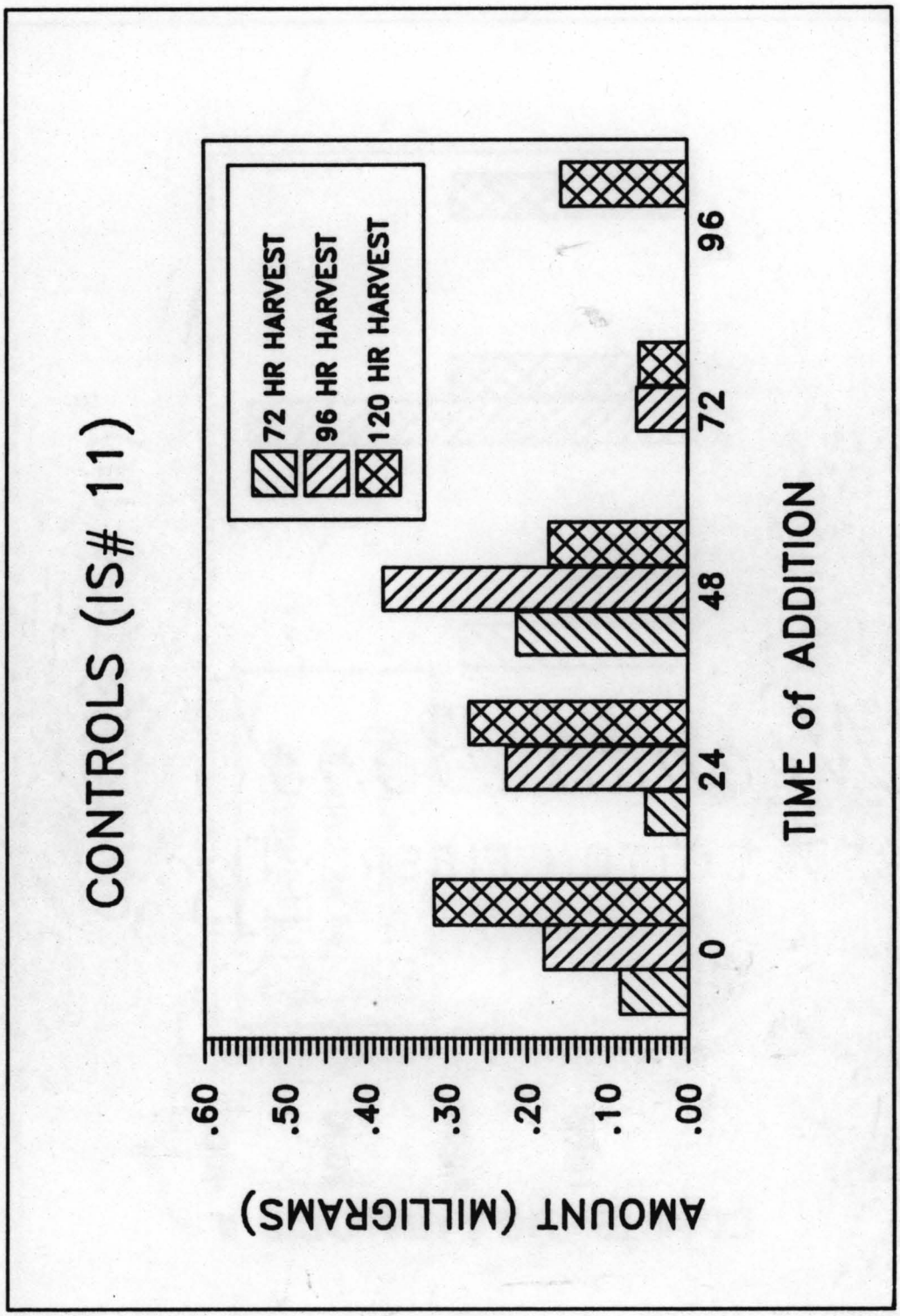
72 HR HARVEST
96 HR HARVEST
120 HR HARVEST
0
24
48
72
96
TIME of ADDITION (HOURS)


CONTROLS (IS# 11)
72 HR HARVEST
96 HR HARVEST
120 HR HARVEST
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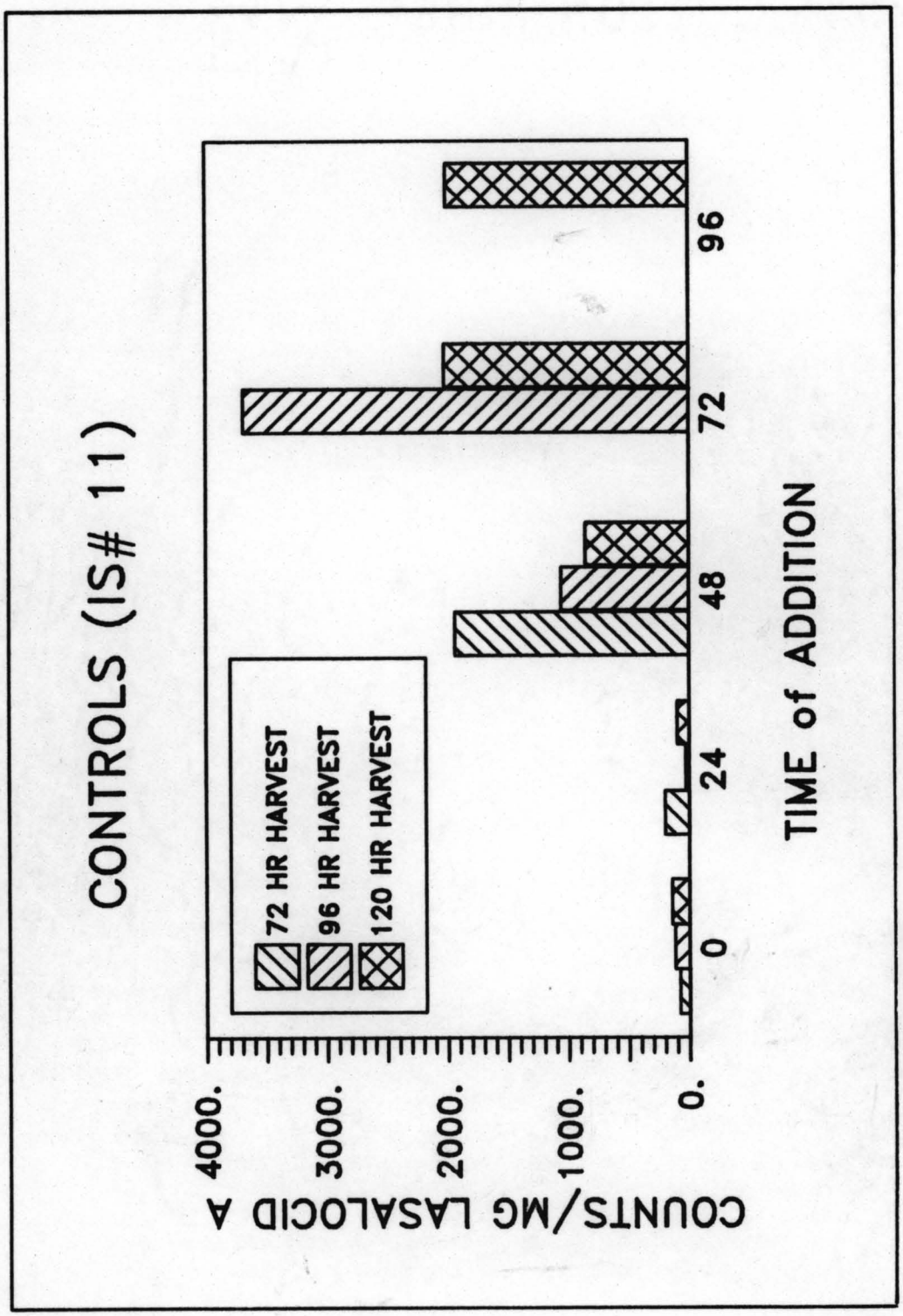
.50
.40
.30
.20
.10
.00
AMOUNT (MILLIGRAMS)
0
24
48
72
96
TIME of ADDITION


CONTROLS (IS# 11)
72 HR HARVEST
96 HR HARVEST
120 HR HARVEST
COUNTS/MG LASALOCID A
4000.
3000.
2000.
1000.
0.
0
24
48
72
96
TIME of ADDITION

BIBLIOGRAPHY

1) Abell, C. and J. Staunton. The use of ^{2}H N.m.r. Spectroscopy and Beta-Isotopic Shifts in the ^{13}C N.m.r. Spectrum to Measure Deuterium Retention in the Biosynthesis of the Polyketide 6-Methylsalicylic Acid. J.C.S. Chem. Comm. 1981, 856-858.

2) Agtarap. A., Chamberlin, J.W., Pinkerton, M., and L. Stienrauf. 1967. The Structure of Monensic Acid. J. Am. Chem. Soc. 89, 5737-5739.

3) Alberts, A.W. and M.D. Greenspan. 1984. Animal and Bacterial Fatty Acid Synthetase: Structure, Function and Regulation. In S. Numa (ed.) Fatty Acid Metabolism and Its Regulation. Vol. 7 of New Comprehensive Biochemistry. pp. 29-58.

4) Allen, S.H.G., Kellermeyer, R.W., Stjernholm, R.L. and H.G. Wood. 1964. Purification and Properties of Enzymes in the Propionic Acid Fermentation. J. Bacteriol. 87, 171-185.

5) Alpha, S.R. and A.H. Brady. 1973. Optical Activity of the Cation Carrier X537A. J. Am. Chem. Soc. 95, 7043-7049.

6) Anteunis, M.J.O. 1976. Solution Conformation of Lasalocid and Lasalocid-Na^{+} (X-537A). Bioorganic Chem. 5, 327-337.

7) Arai, K., Kawaguchi, A., Saito, Y., Koike, N., Seyama, Y., Yamakawa, T., and S. Okuda. Propionyl-CoA Induced Synthesis of Even-Chain-Length Fatty Acids by Fatty Acid Synthetase from Brevibacterium ammoniagenes. J. Biochem. 91, 11-18.

8) Arndt, F. 1943. Nitrosomethyl Urea I. (From Methylamine Hydrochloride). Org. Syn., Coll. Vol. 2, 461-462.

9) Arnstadt, K-I., Schindlbeck, G. and F. Lynen. 1975. Zum Mechanismus der Kondensationsreaktion der Fettsaurebiosynthese. Eur. J. Biochem. 55, 561-571.

10) Aydin, R. and H. Gunther. 1981. Chemical Applications of NMR Isotope Effects: Ring Inversion and Conformational Equilibrium in [$^{2}H_{1}$]-Cyclohexane. Angew. Chem. Int. Ed. Engl. 20, 985-986.

11) Aydin, R., Wesener, J.R., Gunther, H., Santillan, R.L., Garibay, M.-E. and P. Joseph-Nathan. 1984. On the Stereospecificity of Intrinsic $^{2}H/^{1}H$ NMR Isotope Effects on ^{13}C Chemical Shifts in Cyclohexanes. J. Org. Chem., 49, 3845-3847.

12) Bendall, R.M., Pegg, D.T., Wesener, J. and H. Gunther. 1984.

Complete Editing of ^{13}C Spectra of CD_nH_m Groups Using the UPT Sequence plus Gated Decoupling. J. Mag. Res. 59, 223-236.

13) Berger, J., Rachlin, A.I., Scott, W.E., Sternbach, L.H. and M.W. Goldberg. 1951. The Isolation of Three New Crystalline Antibiotics from Streptomyces. J. Am. Chem. Soc. 73, 5295-5298.

14) Birch, A.J. and F. W. Donovan. 1953. Studies in Relation to Biosynthesis. I. Some Possible Routes to Derivatives of Orcinol and Phloroglucinol. Austr. J. Chem. 6. 360-368.

15) Bissell, E.C. and I.C. Paul. 1972. Crystal and Molecular Structure of a Derivative of the Free Acid of the Antibiotic X-537A. J.C.S. Chem. Comm. 1972, 967-968.

16) Bloch, K. and D. Vance. 1977. Control Mechanisms in The Synthesis of Saturated Fatty Acids. Ann. Rev. Biochem. 46, 263-298.

17) Brindley, D.N.,Matsumaura, S. and K. Bloch. 1969. Mycobacterium phlei Fatty Acid Synthetase - A Bacterial Multienzyme Complex. Nature 224, 666-669.

18) Bu'Lock, J.D., Shepard, D. and D.J. Winstanley. 1968. Regulation of 6-methylalicylate and patulin synthesis in Penicillium urticae. Can. J. Microbiol., 15, 279-285.

19) Cane, D.E., Liang, T.-C. and H. Hasler. Polyether Biosynthesis. 2. Origin of the Oxygen Atoms of Monensin A. J. Am. Chem. Soc. 104, 7274-7281.

20) Colli, H.N., Gold, V. and J.E. Pearson. 1973. Deuterium Isotope Effects on Carbon-13 Nuclear Magnetic Resonance Spectra. J.C.S. Chem. Comm. 1973, 408-409.

21) Collie, J.N. 1907. Derivatives of the Multiple Keten Group. J. Chem. Soc. 91 1806-1813.

22) Christofides, J.C. and D.B. Davies. 1982. ^{1}H and ^{13}C N.M.R. Observation of ^{2}H Isotope Effects Transmitted Through Hydrogen Bonds. J.C.S. Chem. Comm., 1982, 560-562.

23) Corcoran, J.W. and A.M. Vygantas. 1982. Accumulation of 6-Deoxyerythronolide B in a Normal Strain of Streptomyces erythreus and Hydroxylation at Carbon 6 of the Erythranolide Ring System by a Soluble Noninduced Cell-Free Enzyme System. Biochemistry, 21, 263-269.

24) Dain, J.G. and R. Bentley. 1971. The Role of Malonate in the Bacterial Biosynthesis of 6-Methylslicylic Acid. Bioorganic Chem. 1, 374-379.

25) Day, L.E., Chamberlin, J.W., Gordee, E.Z., Chen, S., Gorman, M., Hamill, R.L., Ness, T., Weeks, R.E. and R. Stroshone. 1973. Biosynthesis of Monensin. Antimicrob. Agents and Chemother. 4, 410-414.

26) Dennis,D.T. and C.A. West. 1967. Biosynthesis of Gibberellins. 1967. J. Biol. Chem. 242, 3293-3300.

27) Dimroth, P., Walter, H. and F. Lynen. 1970. Biosynthese von 6-Methylsalicylsaure. Eur. J. Biochem. 13, 98-110.

28) Dimroth, P., Ringelmann, E. and F. Lynen. 1976. 6-Methylsalicylic Acid Synthetase from Penicillium patulum. Eur. J. Biochem. 68, 591-596.

29) Doddrell, D.M., Laue, E.D., Leeper, F.J., Staunton, J., Davies, A., Davies, A.B. and G.A.F. Ritchie. 1984. Biosynthesis of the Polyether Antibiotic ICI139603 in Streptomyces longisporoflavus: Investigation of Deuterium Retention after Incorporation of $CD_3{}^{13}CO_2H$, $^{13}CD_3CO_2H$, and $CH_3CD_2{}^{13}CO_2H$ using ^2H N.M.R. and Edited ^{13}C N.M.R. Spectra. J.C.S. Chem. Comm., 1984, 1302-1304.

30) Dotzlaf, J.E., Metzger, L.S. and M.A. Fogelsong. 1984. Incorporation of Amino Acid-Derived Carbon into Tylactone by Streptomyces fradiae GS14. Antimicrob. Agents and Chemother. 25, 216-220.

31) Ernst, L., Eltamany, S. and H. Hopf. 1982. Deuterium Isotope Effects on Carbon-13 Chemical Shifts in Cyclophanes. Deshielding Intrinsic Through-Space and Through-Bond Effects. J. Am. Chem. Soc. 104, 299-300.

32) Everett, G.W., Parker, S.B and R.J.P. Williams. 1983. Binding of Calcium(II) and Lanthanum(III) by a Microbial Inophore. Evidence for a Dynamic, Intramolecular Donor Exchange in Complexes of Lasalocid A. Biochemistry 22, 6149-6155.

33) Everett, J.R. 1982. Oxygen-18 Isotope Effects on ^{13}C NMR Signals. Org. Magn. Res. 19, 86-88.

34) Flatman, S. and N.M. Packter. 1983. Partial Purification of Fatty Acid Synthetase from Streptomyces coelicolor. Biochem. Soc. Trans. 11, 597.

35) Galitzer, S.J. and F.W. Oehme. 1984. A Literature Review on the Toxicity of Lasalocid, A Polyether Antibiotic. Vet. Hum. Toxicol. 26, 322-326.

36) Grandjean, J. and P. Laszlo. 1982. Complexation of Pr^{+3} by Two Different Ionophores Enhances Markedly its Transport Rate. Biochem. Biophys. Res. Comm. 104, 1293-1297.

37) Hagel, R.B. 1978. Liquid Chromatographic Determination of Lasalocid in Premixes. J. Assoc. Off. Anal. Chem. 61, 1070-1073.

38) Halenz, R., Feng, J.-Y., Hegre, C.S. and M.D. Lane. 1962. Some Enyzmic Properties of Mitochondrial Propionyl Carboxylase. J. Biol. Chem. 237, 2140-2147.

39) Hanna, D.A., Yeh, C., Shaw, J., and G.W. Everett. 1983. Gadolinium (III) and Manganese (II) Binding by a Polyether Ionophore. Influence of Cation Charge and Solvent Polarity on the Binding Sites of Lasalocid A (X-537A). Biochemistry. 22, 5619-5626.

40) Harned, R., Hidy, P.H., Corum, C.J. and K.L. Jones. 1951. Nigericin, a New Crystalline Antibiotic from an Unidentified Streptomyces. Antibiot. Chemother. I, 594-596.

41) Holmes, W.L. and J.D. Bentz. 1960. Inhibition of Cholesterol Biosynthesis in Vitro by beta-Diethylaminoethyl Diphenylpropylacetate Hydrochloride (SKF 525-A). J. Biol. Chem. 235, 3118-3122.

42) Hopwood, D.A. 1983. Genetic Manipulation of Streptomycetes, An EMBO Practical Course, John Innes Institute, Norwich, England.

43) Hutchinson, C.R., Sherman, M.M., McInnes, A.G., Walter, J.A. and J.C. Vederas. 1981. Biosynthesis of Macrolides. 6. Mechanism of Stereocontrol during the Formation of Lasalocid A. J. Am. Chem. Soc. 103, 5956-5959.

44) Ireland, R.E., Thaisrivongs, S. and C.S. Wilcox. 1980. Total Synthesis of Lasalocid A (X537A). J. Am. Chem. Soc. 102, 1155-1157.

45) Ireland, R.E., Anderson, R. Badoud, R., Fitzsimmons, B.J., McGarvey, G.J., Thaisrivongs, S. and C.S. Wilcox. 1983. The Total Synthesis of Ionophore Antibiotics. A Convergent Synthesis of Lasalocid A (X-537A). J. Am. Chem. Soc. 105, 1988-2006.

46) Johnson, S.M., Herrin, J., Liu, S.J. and I.C. Paul. 1970. The Crystal and Molecular Structure of the Barium Salt of an Antibiotic Containing a High Proportion of Oxygen. J. Am. Chem. Soc. 92, 4428-4435.

47) Katz, J.J. and H.L. Crespi. 1970. Isotope Effects in Biological Systems. In C.J. Collins and N.S. Bowman (eds.) Isotope Effects in Chemical Reactions, an ACS Monograph. Van Nostrand Reinhold Co., 286-363.

48) Kaziro, Y., Ochoa, S., Warner, R.C. and J.-Y. Chen. Metabolism of Propionic Acid in Animal Tissues VIII. Crystalline Propionyl Carboxylase. J. Biol. Chem. 236, 1917-1923.

49) Kubota, T, Matsutani, S., Shiro, M. and H. Koyama. 1968. The Structure of Polyetherin A. J.C.S. Chem. Comm. 1968, 1541-1543.

50) Kurobane, I., Vining, L.C. and A.G. McInnes. 1978. A New Secalonic Acid. Linkage Between Tetrahydroxanthone Units Determined from Deuterium Isotope ^{13}C Chemical Shifts. Tetrahedron Lett. 47, 4633-4636.

51) Lancini, G. and F. Parenti. 1982. Antibiotics, An Integrated View. Springer-Verlag.

52) Light, R.J. 1967. The Biosynthesis of 6-Methylsalicylic Acid. J. Biol. Chem. 242, 1880-1886.

53) Light, R.J. 1970. Enzymatic Studies on the Polyketide Hypothesis. J. Agr. Food Chem. 18, 260-267.

54) Lowe, G. and B.V.L. Potter. 1980. Synthesis, Absolute Configuration, and Circular Dichroism of the Enantiomers of Fluorosuccinic Acid. J.C.S. Perkin I. 2029-2032.

55) Liu, C.-m. 1982. Microbial Aspects of fPolyether Antibiotics: Activity, Production, and Biosynthesis. In J.W. Westley (ed.) Polyether Antibiotics, Vol. 1:Biology. Marcel Dekker, pp.43-102.

56) Lynen, F. 1980. On the Structure of Fatty Acid Synthetase of Yeast. Eur. J. Biochem. 112, 431-442.

57) Lynen, F. and M. Tada. 1961. Die biochemischen Grundlagen der "Polyacetat-Regal". Angew. Chem. 73, 513-519.

58) Maier, C. and I.C. Paul. 1971. X-Ray Crystal Structure of a Silver Complex of Antibiotic X-537A; a Structure Enclosing Two Metal Ions. J.C.S. Chem. Comm. 1971, 181-182.

59) Martin, J.F. and L.E. McDaniel. 1975. Specific Inhibition of Candicidin Biosynthesis by the Lipogenic Inhibitor Cerulenin. Biochim. Biophys. Acta, 411, 186-194.

60) McInnes, A.G., Walter, J.A. and J.L.C. Wright. 1979. Differential Hydrogen Exchange During the Biosynthesis of Fatty Acids in Anacystis nidulans: the incorporation of [2,2,2-$^{2}H_3$, 2-$^{13}C_{0,1}$]-Acetate. Tetrahedron Lett. 35, 3245-3248.

61) McInnes, A.G., Walter, J.A. and J.L.C. Wright. 1980. Biosynthesis of Hydrocarbons by Algae: Decarboxylation of Stearic Acid to N-Heptadecane in Anacystis nidulans Determined by ^{13}C- and ^{2}H-Labeling and ^{13}C Nuclear Magnetic Resonance. Lipids 15, 609-615.

62) McInnes, A.G., Walter, J.A. and J.L.C.Wright. 1983 Regiochemical Distribution of Deuterium During Fatty Acid Biosynthesis Following Incorporation of [2-^{13}C, 2-$^{2}H_3$] Acetate. Tetrahedron 39, 3515-3522.

63) Nakata, T., Schmid, G., Vranesic, B., Okigawa, M., Smith-Palmer, T. and Y. Kishi. 1978. A Total Synthesis of Lasalocid A. J. Am. Chem. Soc. 100, 2933-2935.

64) O'Hagen, D., Robinson, J.A. and D.L. Turner. 1983. Biosynthesis of the Macrolide Antibiotic Tylosin. Origins of the Oxygen Atoms in Tylactone. J.C.S. Chem. Comm. 1983, 1337-1340.

65) Olah, G.A., Welch, J.T., Vankar, Y.D., Nojima, M., Kerekes, I. and J.A. Olah. 1979. Synthetic Methods and Reactions. 63. Pyridinium Poly(hydrogen fluoride) (30% Pyridine-70% Hydrogen Fluoride): A Convenient Reagent for Organic Fluorination Reactions. J. Org. Chem. 44, 3872-3881.

66) Olah, G.A. adn J. Welch. 1974. Synthetic Methods and Reactions XII. Preparation of alpha-Fluorocarboxylic Acids from alpha-Amino Acids via Diazotization in Polyhydrogen Fluoride/Pyridine Solution. Synthesis 1974, 652.

67) Omura, S. 1976. The Antibiotic Cerulenin, a Novel Tool for Biochemistry as an Inhibitor of Fatty Acid Synthesis. Bacteriol. Rev. 40, 681-697.

68) Omura, S. 1980. Cerulenin. Meth. Enzymol. 72, 520-532.

69) Omura, S., Nakagawa, A., Takeshima, H., Miyazawa and C. Kitao. 1975. A ^{13}C Nuclear Magnetic Resonance Study of the Biosynthesis of the 16-Membered Macrolide Antibiotic Tylosin.

Tet. Lett. 50, 4503-4506.

70) Omura, S., Takeshima, H., Nakagawa, A., Kanemoto, N. and G. Lukacs. 1976. Studies on Carboxylic Acid Metabolism in a Macrolide-Producing Microorganism Using Carbon-13 Magnetic Resonance. Bioorganic Chem. 5, 451-454.

71) Omura, S., Takeshima, H., Nakagawa, A., Miyazawa, Piriou, F. and G. Lukacs. 1977. Studies on the Biosynthesis of 16-Membered Macrolide Antibiotics Using Carbon-13 Nuclear Magnetic Resonance Spectroscopy. Biochemistry 16 2860-2866.

72) Omura, S., Tanaka, H. and M. Tsukui. 1982. Biosynthesis of Tylosin: Oxidations of 5-O-Mycaminosylprotylonolide at C-20 and C-23 with a Cell-Free Extract from Streptomyces fradiae. Biochem. Biophys. Res. Comm. 107, 554-560.

73) Omura, S., Tanaka, Y., Mamada, H. and R. Masuma. 1983. Ammonium Ion Suppresses The Biosynthesis of Tylosin Aglycone by Interference with Valine Catabolism in Streptomyces fradiae. J. Antibiotics 1983, 1792-1794.

74) Omura, S., Tsuzuki, K., Tanaka, Y., Sakakibara, H., Aizawa, M. and G. Lukacs. 1983. Valine as a Precursor of n-Butyrate Unit in the Biosynthesis of Macrolide Aglycone. J. Antibiot. 36, 614-616.

75) Omura, S., Taki, A., Matsuda, K. and Y. Tanaka. 1984. Ammonium Ion Suppresses the Amino Acid Metabolism Involved in the Biosynthesis of Protylonolide in a Mutant of Streptomyces fradiae. J. Antibiotics 1984, 1362-1369.

76) Osborne, M.W. and M.R. Cohen. 1979. Method of Treating Hypertension. US Patent No. 4,161,520.

77) Packter, N.M. and J.S. Collins. 1974. Effect of Inhibitors of Protein Synthesis on the Formation of Phenols Derived from Acetate and Shikimic Acid in Aspergillus fumigatus. Eur. J. Biochem. 42, 291-302.

78) Pape,H. and H. Grisebach. 1967. Hemmung der Erythromycinsynthese bei S. erythreus durch alpha- und beta-Fluorpropionsaure. Z. Naturfor. 22B, 831-834.

79) Pospisil, S., Sedmera, P., Havranek, M., Krumphanzl, V. and Z. Vanek. 1983, Biosynthesis of Monensins A and B. J. Antibiot. 36, 617-619.

80) Pressman, B.C., Harris, E.J., Jagger, W.S. and J.H. Johnson. 1967. Antibiotic-Mediated Transport of Alkali Ions Across

Lipid Barriers. Biochemistry 58, 1949-1956.

81) Prosser, B.L.T. and N.J. Palleroni. 1982. Taxonomy of the Polyether Antibiotic-Producing Organixms. In J.W. Westley (ed.) Polyether Antibiotics, Vol 1:Biology. Marcel Dekker, pp. 21-41.

82) Rainwater, D.L. and P.E. Kolattukudy. 1985. Fatty Acid Biosynthesis in Mycobacterium tuberculosis var. bovis Bacillus Calmette-Guerin. J. Biol. Chem. 260, pp. 616-623.

83) Reid, W.W. 1968. Accumulation of Squalene-2,3-Oxide During Inhibition of Phytosterol Biosynthesis in Nicotiana tabacum. Phtochemistry 7, 451-452.

84) Retey, J., Smith, E.H. and B. Zagalak. 1978. Investigation of the Mechanism of the Methylmalonyl-CoA Mutase Reaction with the Sustrate Analogue: Ethylmalonyl-CoA. Eur. J. Biochem. 83, 437-451.

85) Retey, J. 1982. Methylmalonyl-CoA Mutase. In D. Dolphin (ed.) B_{12}, Vol 2. Wiley, pp. 357-379.

86) Richardson, F.S. and A. Das Gupta. 1981. Spectroscopic Studies on the Interaction of the Antibiotic Lasalocid A (X537A) with Lanthanide (III) Ions in Methanol. J. Am. Chem. Soc. 103, 5716-5725.

87) Rinaldi, P.L. and N.J. Baldwin. 1982. $^{13}C\{^{2}H\}$ Insensitive Nuclei Enhanced by Polarization Transfer (INEPT): A New NMR Strategy for Isotopic Labeling Studies. J. Am. Chem. Soc. 104, 5791-5793.

88) Rinaldi, P.L. and N.J. Baldwin. 1983. $^{13}C\{^{2}H\}$ Polarization Transfer Studies: New NMR Methods for Isotopic Labeling Studies. J. Am. Chem. Soc. 105, 7523-7527.

89) Risley, J.M. and R.L. Van Etten. 1979. An ^{18}O Isotope Shift upon ^{13}C-NMR Spectra and Its Application to the Study of Oxygen Exchange Kinetics. J. Am. Chem. Soc. 101, 252-253.

90) Risley, J.M. and R.L. Van Etten. 1980. ^{18}O-Isotope Effect in ^{13}C Nuclear Magnetic Resonance Spectroscopy. 2. The Effect of Structure. J. Am. Chem. Soc. 102, 4609-4614.

91) Risley, J.M. and R.L. Van Etten. 1980. ^{18}O-Isotope Effect in ^{13}C Nuclear Magnetic Resonance Spectroscopy. 3. Additivity Effects and Steric Effects. J. Am. Chem. Soc. 102, 6699-6702.

92) Ruff, M.D. 1982. Veterinary Applications. In J.W. Westley (ed.),Polyether Antibiotics, Vol. I., Marcel Dekker, pp. 303-332.

93) Schroepfer, G.L. 1982. Sterol Biosynthesis. Ann. Rev. Biochem., 51, 555-85.

94) Schmidt, P.G., Wang, A.H.-J. and I.C. Paul. 1974. A Structural Study on the Sodium Salt of the Ionophore, X-537A (Lasalocid), by X-Ray and Nuclear Magnetic Resonance Analysis. J. Am. Chem. Soc. 96, 6189-6191.

95) Schweizer, E. 1980. Fatty Acid Synthetase Complexes. In Bisswanger and Schmincke-Ott (eds.) Multifunctional Proteins, John Wiley and Sons, pp. 197-216.

96) Schweizer, E. Werkmeister, K., and M.K. Jain. 1978. Fatty Acid Biosynthesis in Yeast. Molec. and Cell. Biochem. 21, 95-107.

97) Scott, A.I., Phillips, G.T. and U. Kircheis. 1971. Biosynthesis of Polyketides. The Synthesis of 6-Methylsalicylic Acid and Triacetic Acid Lactone in Penicillium patulum. Bioorganic Chem. 1, 380-399.

98) Scott, A.I., Beadling, L.C., Georgopapadakou, N.H. and C.R. Subbarayan. 1973. Biosynthesis of Polyketides. Purification and Inhibition Studies of 6-Methylsalicylic Acid Synthase. Bioorganic Chem. 3, 238-248.

99) Sedgwick, B. and J.W. Cornforth. 1977. The Biosynthesis of Long-Chain Fatty Acids. Stereochemical Differentiation in the Enzymic Incorporation of Chiral Acetates. Eur. J. Biochem. 75, 465-479.

100) Sedgwick, B. and C. Morris. 1980. Stereochemical Course of Hydrogen Transfer Catalyzed by the Enoyl Reductase Enzyme of the Yeast Fatty Acid Synthetase. J.C.S. Chem. Comm. 1980, 96-97.

101) Sedgwick, B. Morris, C. and S.J. French. 1978. Stereochemical Course of Dehydration Catalyzed by the Yeast Fatty Acid Synthetase. J.C.S. Chem. Comm. 1978, 193-194.

102) Sedgwick, B., Cornforth, J.W., French, S.J., Gray, R.T., Kelstrup, E. and P. Willadsen. 1977. The Biosynthesis of Long-Chain Fatty Acids. Incorporation of Radioactivity from Stereospecifically Tritiated Malonyl Thiol Esters, and the Stereochemistry of the Acetyl-CoA Carboxylase Reaction. Eur. J. Biochem. 75, 481-495.

103) Seto, H., Westley, J.W. and R.G. Pitcher. 1978. The Complete Assignment of the ^{13}C NMR Spectra of Lasalocid and the Sodium Salt-Complex of the Antibiotic. J. Antibiotics 31, 289-293.

104) Shastri, B.P. and K.R.K. Easwaran. 1984. Conformations of Lasalocid A-Lithium Complexes in Acetonitrile. Int. J. Biol. Macromol. 6, 219-223.

105) Silverstein, R.M., Bassler, G.C.and T.C. Morrill. 1974. Spectrometric Identification of Organic Compounds, Third Edition, John Wiley and Sons, pp. 12-14.

106) Simpson, T.J. and D.J. Stenzel. 1982. Application of ^{2}H Beta-Isotopic Shifts in ^{13}C N.M.R. Spectra to Biosynthetic Studies. Incorporation of [1-^{13}C,$^{2}H_3$]-Acetate into O-Methylasparvenone in Aspergillus parvulus. J.C.S. Chem. Comm. 1982, 1074-1076.

107) Sood, G.R., Robinson, J.A. and A.A. Ajaz. 1984. Biosynthesis of the Polyether Antibiotic Monensin-A. Incorporation of [2-$^{2}H_2$]-, (R)-[2-$^{2}H_1$]- and (S)-[2-$^{2}H_1$]-Propionate. J.C.S. Chem. Comm. 1984, 1421-1423.

108) Steinrauf, L.K., Pinkerton, M.and J.W. Chamberlin. 1968. The Structure of Nigericin. Biochem. Biophys. Res. Comm. 33, 29-31.

109) Stempel, A. and J.W. Westley. 1973. Derivatives of Antibiotic X-537A. US Patent No. 3,715,372.

110) Stubbe, J. and R.H. Abeles. 1977. Biotin Carboxylations - Concerted or Not Concerted? That is the Question! J. Biol. Chem. 252, 8338-8340.

111) Stubbe, J., Fish, S. and R.H. Abeles. 1979. Are Carboxylations Involving Biotin Concerted or Nonconcerted? J. Biol. Chem. 255, 236-242.

112) Tietz, A. and S. Ochoa. 1959. Metabolism of Propionic Acid in Animal Tissues V. Purification and Properties of Propionyl Carboxylase. J. Biol. Chem. 234, 1394-1400.

113) Tulloch, A.P. 1977. Deuterium Isotope Effects and Assignment of ^{13}C Chemical Shifts in Spectra of Methyl Octadecanoate and the Sixteen Isomeric Oxooctadecanoates. Can. J. Chem., 55, 1135-1142.

114) Tulloch, A.P. and M. Mazurek. Deuterium Isotope Effects on

^{13}C Chemical Shifts in Long-chain Aliphatic Compounds. J.C.S. Chem. Comm. 1973, 692-693.

115) Vanek, Z., Pospisil, S., Tax, J., Klanova, K., Cimburkova, E., Tichy, P., Vorisek, J., Cudlin, J. and V. Krumphanzl. 1982. Biogenesis of Oligoketides. In H. Umezawa, A.L. Demain, T. Hata and C.R. Hutchinson (eds.) Trends in Antibiotic Research. Japan Antibiotics Research Association, pp. 213-219.

116) Vining, L.C. (ed.). 1983. Biochemistry and Genetic Regulation of Commercially Important Antibiotics. Addison Wesley.

117) Vogel, G. and F. Lynen. 1975. 6-Methylsalicylic Acid Synthetase. Methods Enzymol. 43, 520-530.

118) Volpe, J. J. and R. P. Vagelos, 1973. Saturated Fatty Acid Biosynthesis and Its Regulation. Ann. Rev. Biochem. 42, 21-60.

119) Vu-Trong, K., Bhuwapathanapun, S. and P.P. Gray. 1980. Metabolic Regulation in Tylosin-Producing Streptomyces fradiae: Regulatory Role of Adenylate Nucleotide Pool and Enzymes Involved in Biosynthesis of Tylonide Precursors. Antimicrob. Agents and Chemother. 17, 519-525.

120) Wakil, S.J. and J. K. Stoops. 1983. Structure and Mechanism of Fatty Acid Synthetase. In Paul D. Boyer (ed.) The Enzymes, Vol XVI. Academic Press, pp. 3-61.

121) Wakil, S.J., Stoops, J.K. and V.C. Joshi. 1983. Fatty Acid Synthesis and Its Regulation. Ann. Rev. Biochem. 52 537-79.

122) Walsh, C. 1979. Enzymatic Reaction Mechanisms. W.H. Freeman and Co., pp. 570-571.

123) Walsh, C. 1983. Fluorinated Substrate Analogs: Routes of Metabolism and Selective Toxicity. Adv. Enzymol. 55, 197-289.

124) Wang, C.C. 1978. Biochemical and Nutritional Aspects of Coccidia. In Avian Coccidiosis, P.L. Long, K.N. Boorman, and B.M. Freeman (eds.), British Poultry Sciences Ltd., pp.135-184.

125) Webb, J.L. 1966. Enzyme and Metabolic Inhibitors, Vol. II. Academic Press, pp. 1-244.

126) Weber, J.M. 1977. MS Thesis, UW-School of Pharmacy.

127) Wesener, J.R., Schmitt, P. and H. Gunther. Spin-Echo ^{13}C NMR Spectroscopy for the Analysis of Deuterated Carbon Compounds. J. Am. Chem. Soc. 106, 10-13.

128) Westley, J.W., Evans, R.H., Williams, T. and A. Stempel. 1970. Structure of Antibiotic X-537A. J.C.S. Chem. Comm. 1970, 71-73.

129) Westley, J.W., Evans, R.H., Preuss, D.L. and A. Stempel. 1970. Biosynthesis of Antibiotic X-537A. J.C.S. Chem. Comm. 1970, 1467-1468.

130) Westley, J.W., Oliveto, E.P., Berger, J., Evans, R.H., Glass, R., Stempel, A., Toome, V. and T. Williams. 1973a. Chemical Transformations of Antibiotic X-537A and Their Effect on Antibacterial Activity. J. Med. Chem., 16, 397-403.

131) Westley, J.W., Evans, R.H., Williams, T. and A. Stempel. 1973b. Pyrolytic Cleavage of Antibiotic X-537A and Related Reactions. J. Org. Chem. 38, 3431-3433.

132) Westley, J.W., Evans, R.H., Harvey, G., Pitcher, R.G., Preuss, D.L., Stempel, A. and J. Berger. 1974a. Biosynthesis of Lasalocid. I. Incorporation of ^{13}C and ^{14}C Labelled Precursors into Lasalocid A. J. Antibiotics 27, 288-297.

133) Westley, J.W., Blount. J.F., Evans, R.H., Stempel, A. and J. Berger. 1974b. Biosynthesis of Lasalocid. II. X-Ray Analysis of a Naturally Occurring Isomer of Lasalocid A. J. Antibiotics 27, 597-604.

134) Westley, J.W., Benz, W., Donahue, J., Evans, R.H., Scott, C.G., Stempel, A. and J. Berger. 1974c. Biosynthesis of Lasalocid. III. Isolation and Structure Determination of Four Homologs of Lasalocid A. J. Antibiotics 27, 744-753.

135) Westley, J.W. 1977. Polyether Antibiotics: Versatile Carboxylic Ionophores Produced by Streptomyces. Advances in Appl. Microbiol. 22, 177-223.

136) Westley, J.W., Evans, R.H. and J.F. Blount. 1977. Optical Resolution of Asymmetric Amines by Preferential Crystallization as Lasalocid Salts. J. Am. Chem. Soc. 99, 6057-6061.

137) Westley, J.W. 1978. Therapeutic Agent for Improving Cardiovascular Function. US Patent No. 4,083,968.

138) Westley, J.W., Seto, H. and N. Otake. 1979. Further Proposals for Polyether Antibiotic Notation. J. Antibiot. 32,

959-960.

139) Westley, J.W. (ed.). 1982. Polyether Antibiotics, Vol. 1:Biology. Marcel Dekker.

140) Westley, J.W. (ed.). 1983. Polyether Antibiotics, Vol. 2:Chemistry. Marcel Dekker.

141) White, R.H. 1980. Stoichiometry and Stereochemistry of Deuterium Incorporated into Fatty Acids by Cells of Escherichia coli Grown on [methyl-$^{2}H_{3}$] Acetate. Biochem. 19, 9-15.

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PUBLICATIONS 1) M. M. Sherman, R. P. Borris, M. Ogura, G. A. Cordell, N. R. Farnsworth, Phytochemistry, 1980, 19, 1499. 2) C. R. Hutchinson, M. M. Sherman, J. C. Vederas, T. T. Nakashima, J. Am. Chem. Soc., 1981, 103, 5963. 3) C. R. Hutchinson, M. M. Sherman, J. C. Vederas, A. G. McInnes, J. C. Walter, J. Am. Chem. Soc., 1981, 103, 5956. 4) M. M. Sherman, C. R. Hutchinson, in Bulletin of Magnetic Resonance, Proceedings of the Eighth Triennial Meeting of ISMAR, August, 1983.

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