AN ABSTRACT OF THE THESIS OF

Shyh-Chen Ju for the degree of Doctor of Philosophy in Chemistry presented on September 7, 1988.

Title: Biosynthesis of Acivicin and 4-Hydroxyacivicin

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Abstract approved: _____
Steven J. Gould

In order to establish the biosynthetic pathway in *Streptomyces sviceus* leading to acivicin, **1**, and 4-hydroxyacivicin, **2**, ^{14}C-labeled glutamic acid, glutamine, glycine, and ornithine were fed to growing cultures and the metabolites were subsequently isolated by chromatographic procedures. Only the ornithine yielded radioactive **1** and **2**. A series of ornithines labeled with stable isotopes (^{13}C, ^{15}N, ^{2}H) were then synthesized and fed to cultures of *Streptomyces sviceus*. High field NMR (^{13}C or ^{2}H) analysis of the resulting acivicin and 4-hydroxyacivicin samples revealed that acivicin and 4-hydroxyacivicin are formed from ornithine and established key details of the biosynthetic pathway. In order to probe the fate of the three hydrogens at C-2 and C-3 of ornithine, a feeding of DL-[2,3,3-$^{2}H_3$]ornithine, **56b**, established that deuterium had been retained only at C-3; all label from C-2 had been lost. Further feedings using chirally deuterated ornithines **56c** and **56d** revealed that the 3-pro*S* hydrogen of ornithine was lost, while the 3-pro*R* hydrogen of ornithine was retained in the insertion of oxygen at C-3.

A fermentation in the presence of $^{18}O_2$ gas revealed that both oxygen atoms in 4-hydroxyacivicin were derived from $^{18}O_2$.

To identify the first intermediate in the pathway, deuterium-labeled E-β-hydroxy-L-[4,4-$^{3}H_2$]-ornithine, **74a**, and T-β-hydroxy-L-[4,4-$^{3}H_2$]ornithine, **75a**, were synthesized and fed. Neither of these were found to enrich acivicin or 4-hydroxyacivicin. These results were confirmed by isotope trapping experiments with T-β-hydroxyornithine, **75**, and E-β-hydroxyornithine, **74**. By feeding DL-[3,3,4,4-$^{2}H_4$]-ornithine, it was found that C-4 deuterium had been retained without exchange inside the cell in the biosynthesis of **1** and **2**.

δ-N-hydroxyornithine was next synthesized and prepared in labeled form for testing. A feeding of DL-[3,3,4,4-$^{4}H_2$]-δ-N-hydroxyornithine was carried out and the results clearly indicated that this compound was well incorporated. Thus, it appears that N-hydroxyornithine is the first intermediate in the pathway leading to **1** and **2**.

In order to determine the relationship between **1** and **2**, a feeding experiment with L-[5-^{14}C]acivicin was carried out. The result showed that *S. sviceus* had produced 4-hydroxyacivicin from acivicin.

Biosynthesis of Acivicin and 4-Hydroxyacivicin

by

Shyh-Chen Ju

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed September 7, 1988

Commencement June 1991

APPROVED:

Redacted for Privacy

Professor of Chemistry in charge of major

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Date thesis is presented: September 7, 1988

Typed by Jeanne Reisner for Shyh-Chen Ju

To my Parents

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Professor Steven J. Gould, my research director, for his patience, encouragement, constant support, and guidance without which I would not have completed this work. His profound and thorough knowledge always has been a key to solving many problems. The concepts he stressed to me to be a capable scientist and skills he demonstrated shall always be remembered.

My special thanks go to Dr. V. A. Palaniswamy for his technical assistance, helpful discussions and most of all for his friendship. Mr. Rodger Kohnert is warmly thanked for the NMR and mass spectral services.

Thanks to all members of the "Gould group" and the chemistry department for their help in making my stay at OSU a memorable one. Special acknowledgements go to my colleagues P. C. Prabhakaran, Peter Yorgey, and Drs. Karl De Jesus and James Kilgore, for their suggestions and assistance at various times.

I wish to thank my committee members for their help and suggestions. Ms. Jeanne Reisner is thanked for typing this thesis.

I also thank the National Institutes of Health for financial support.

Finally, I would like to thank my parents, family members and my fiancee for their unending patience, support and encouragement.

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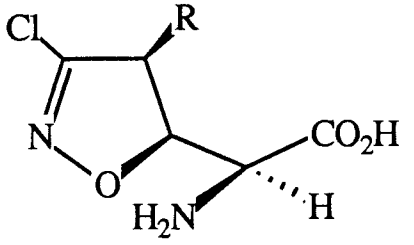
BIOSYNTHESIS OF ACIVICIN AND 4-HYDROXYACIVICIN

INTRODUCTION

Isolation and Structure Elucidation of Acivicin and 4-Hydroxyacivicin

Acivicin (previously known as AT-125), **1**, (Figure 1) was isolated by Martin and coworkers[1] in 1973 as a metabolite of *Streptomyces sviceus*.

The structure and absolute stereochemistry of **1** were first determined by X-ray crystallographic analysis[1] of a salt. The NMR results were consistent with the structure deduced from X-ray analysis. The proton NMR spectrum indicated the presence of four non-exchangeable protons consistent with a methylene group, an α amino acid residue, and an oxygen bearing carbon whose proton was coupled to the methylene and the methine protons. A significant observation from mass spectrometry was the apparent presence of chlorine in the ion fragments. Elemental analysis of **1** agreed well with the the empirical formula $C_5H_7ClN_2O_3$.



1: R = H

2: R = OH

Figure 1. Acivicin and 4-Hydroxyacivicin

4-Hydroxyacivicin[2], **2**, (Figure 1) is a related antitumor agent which was isolated from the same fermentation producing acivicin and was found to have a hydroxylated structure.[3] Mass spectrometry of the bis-t-butyldimethylsilyl derivative of this compound indicated a molecular weight of 410, compared to molecular weight 194 of the natural product, suggesting that an oxygenated analog had

been isolated. The 400 MHz NMR spectrum of a deuterium oxide solution of this amino acid clearly indicated the presence of two non-exchangeable protons on carbons bearing oxygen.

An X-ray crystallographic study of a salt of 4-hydroxyacivicin confirmed the structure and rigorously established the stereochemistry as α*S*,4S,5*R*-α-amino-3-chloro-4-hydroxy-4,5 dihydro-5-isoxazoleacetic acid.

Chemical and Physical Properties of Acivicin and 4-Hydroxyacivicin

Acivicin is a white crystalline solid that melts with decomposition at 201-202 °C. It is partially soluble in methanol, dimethylsulfoxide, dimethyl formamide and is freely soluble in water, but is insoluble in nearly all other organic solvents.

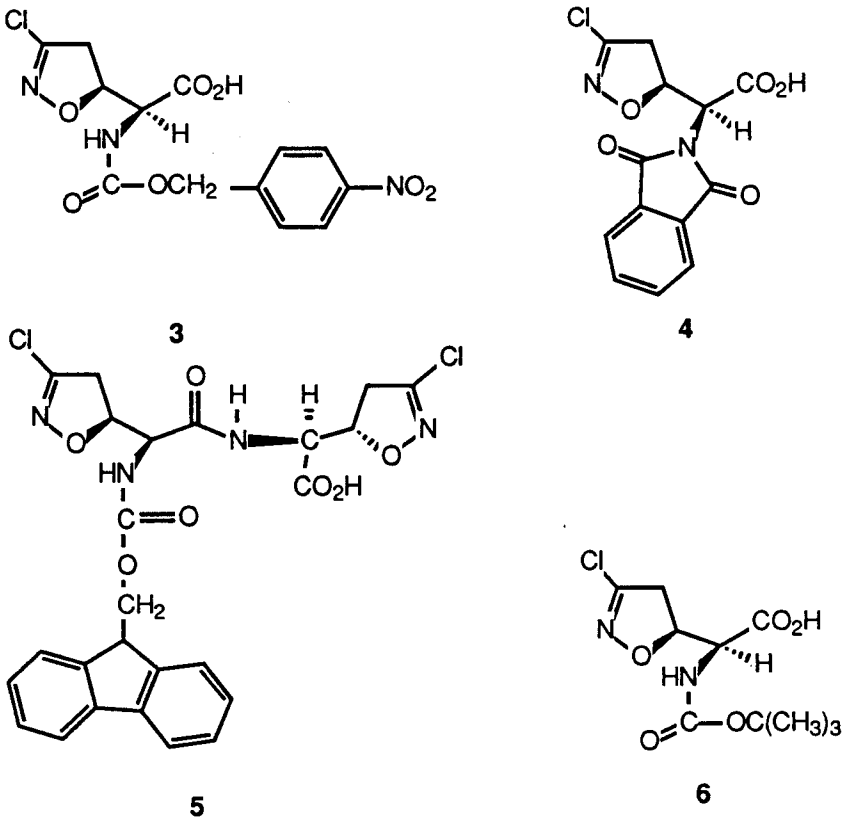


Figure 2. Derivatives of Acivicin

Four acivicin derivatives were prepared during the purification studies:[4] carbo-p-nitrobenzyloxy, **3**, phthalimido, **4**, fluorenyl-methoxycarbonyl, **5**, and t-butyloxy carbonyl, **6** (Figure 2). Acivicin is less stable under basic than acidic conditions. It can be hydrolyzed to trichlomic acid, **7**, (Figure 3) with 2N NaOH[5] at room temperature.

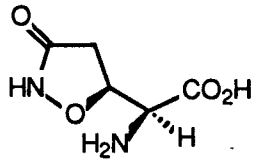


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Figure 3. Trichlomic Acid

The 400 MHz ^{1}H-NMR and 100.6 MHz ^{13}C-NMR data are listed in Table 1 and Table 2.

Table 1. 400 MHz ^{1}H-NMR Data of Acivicin

proton	chemical shift (ppm)	integration	multiplicity	J (Hz)
H-2	4.0	1H	d	3.2
H-3	5.2	1H	m	-
H-4	3.4	2H	m	-

Table 2. 100.6 MHz ^{13}C-NMR Data of Acivicin

carbon	chemical shift (δ)
1	170.2
2	56.4
3	80.7
4	40.2
5	152.6

Hydroxyacivicin is also a crystalline solid, melting with decomposition at about 152 °C. It can be recrystallized from water-methanol. This compound is soluble in water and insoluble in nearly all organic solvents. The hydroxylated amino acid,[3] unlike acivicin, shows a tendency to associate with up to one equivalent of water which can be removed by drying under reduced pressure, but may be reabsorbed from atmospheric moisture. The 400 MHz ^{1}H-NMR and 100.6 MHz ^{13}C-NMR data are listed in Table 3 and Table 4.

Table 3. 400 MHz ^{1}H-NMR Data of 4-Hydroxyacivicin

proton	chemical shift (ppm)	integration	multiplicity	J (Hz)
H-2	4.4	1H	d	4.1
H-3	5.2	1H	dd	3.7, 4.4
H-4	5.3	1H	d	8.5

Table 4. 100.6 MHz ^{13}C-NMR Data of 4-Hydroxyacivicin

carbon	chemical shift (δ)
1	170.4
2	53.1
3	81.7
4	77.2
5	154.7

Biological Activity of Acivicin and 4-Hydroxyacivicin

Acivicin exhibits a broad spectrum of biological activities.[2] It has antibacterial activity against *Bacillus subtilis* and *Escherichia coli* in addition to antifungal and antitumor activity.[6] The antibacterial activity of acivicin could be antagonized by a mixture of amino acids and partially antagonized with a combination

of purines, pyrimidines, or their nucleosides. In further studies, it was shown that this activity was rather specifically antagonized by L-histidine.[7]

The antitumor activity of acivicin in experimental mouse tumors has been reviewed.[8] Acivicin was active against interperitoneal (i.p.) -inoculated L-1210 leukemia cells when the agent was administered by the i.p., subcutaneous, or oral routes.

4-Hydroxyacivicin exhibited less than 0.1% the activity of acivicin when assayed against *B. subtilis* cultivated in a synthetic agar medium. However, its potency with respect to inhibition of growth of L-1210 cells in culture is roughly 20% that of acivicin. 4-Hydroxyacivicin was approximately 4-10 times less potent than acivicin in the treatment of L-1210 mouse leukemia, but the optimal therapeutic effects of the two agents were quite comparable.[9] Because of this reduced potency and the lack of evident therapeutic advantages, little development of 4-hydroxyacivicin has been carried out.

Antimetabolite Properties of Acivicin

Acivicin was detected in fermentation broths because of its antimetabolite activity towards bacteria. The antimicrobial activity of acivicin was antagonized rather specifically by L-histidine while L-glutamine had no effect.[7]

In an extension of those studies to mammalian cells, Cooney's group[10] found that L-histidine, at concentrations from 1-250 µg/ml, had no effect on the growth inhibitory activity of acivicin towards KB or L-1210 cells. The only amino acid which showed even a small effect was L-glutamine. For example, with L-1210 cells in the presence of 100 µg/ml L-glutamine, approximately 0.1 µg/ml of acivicin inhibited growth by 90%. The concentration of acivicin required for comparable growth inhibition when 1000 µg/ml L-glutamine was employed was approximately

three-fold higher.[11] Similar results were observed with KB cells. Further and more direct evidence of the activity of acivicin as a glutamine antagonist was provided by Jayaram and Cooney.[12] They showed that acivicin was a potent inhibitor of asparagine synthetase activity in mouse tumors. Their further studies indicated that acivicin was also a good inhibitor of several other enzymes catalyzing amide transfer from glutamine. A number of such enzymes are involved in the *de novo* biosynthesis of pyrimidines, purines, and their nucleotides (Figure 4). Because the agent may inhibit a variety of glutamine-dependent enzymes, it will inhibit cell growth by preventing the synthesis of RNA and DNA intermediates.

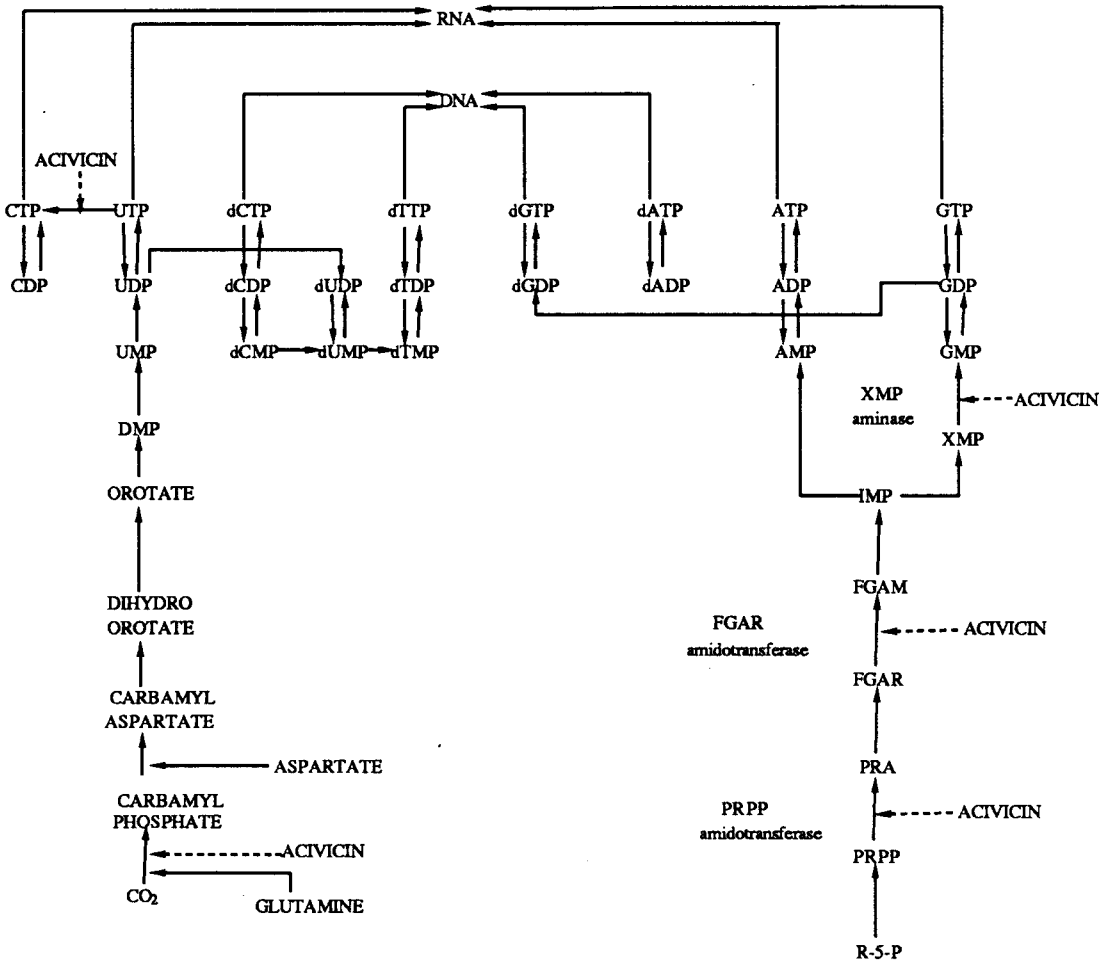


Figure 4. Biochemical Pattern of Enzyme Inhibition by Acivicin[10]

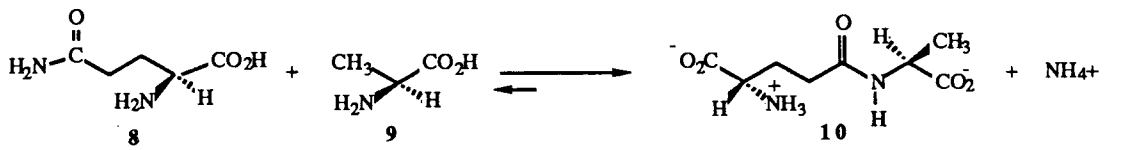
Clinical Studies with Acivicin

Preclinical studies revealed a significant increase in the lifespan induced by acivicin in the murine L-1210 ascitic leukemia model.[6] There was also activity in P388 murine ascites leukemia and human breast and colon xenografts in nude mice.[13] This activity was responsible for selection of acivicin as a promising agent for clinical trial. From clinical studies, beneficial antitumor responses were observed in some patients having gastric carcinogen,[14] melanoma epidermoid lung cancer,[15] pulmonary adenocarcinoma,[16] and small cell lung carcinoma.[17] These findings encouraged trials of the clinical efficacy of acivicin in the diseases where responses occurred. Unfortunately, there was a high incidence of serious side effects. Common occurrences at low doses[18] included lethargy, fatigue, asthenia, disorientation, depression, vivid dreams, nightmares, headaches, confusion, and mood changes. Further clinical work will be necessary in order to discover the full potential of acivicin for combination chemotherapy in human malignancies.

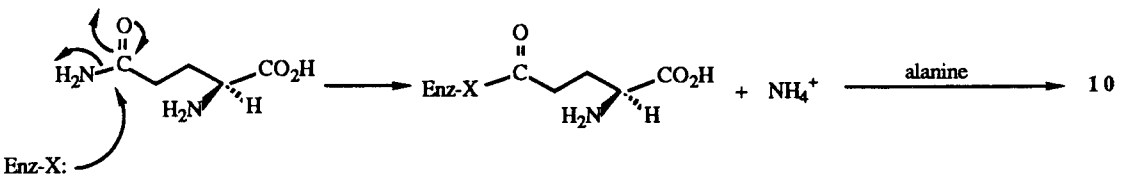
The Binding Mechanism of Glutathione and Acivicin to γ-Glutamyl Transferase

Rat kidney cells contain a membrane-bound enzyme that carries out γ-glutamyl transfers to a variety of physiological α-amino acids. It has been termed a γ-glutamyl transpeptidase. Because this activity is localized in the membranes facing the renal lumen, Meister[19] and his colleagues have suggested that the enzyme has a function in passage of amino acids across the kidney membrane by capturing them as γ-glutamyl derivatives.

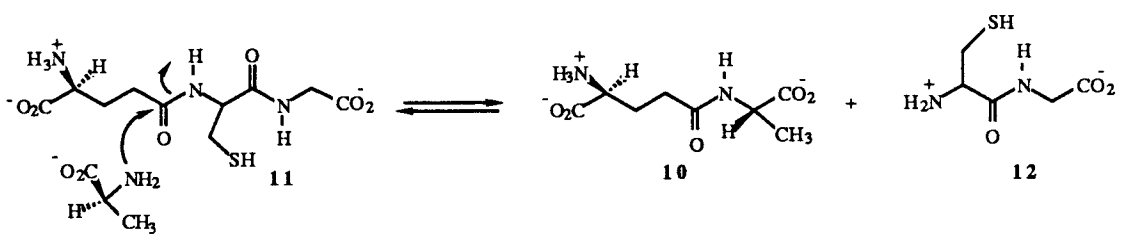
The purified enzyme accepts a large number of α-amino acids as acceptors.[20] As an illustration, γ-glutamyl transfer from glutamine, **8**, to alanine, **9**, forms



γ-glutamylalanine, **10**. The mechanism is unknown, although it may well be that the alanine amino group attacks a covalent γ-glutamyl-enzyme derivative.



In the absence of an amino acid as acceptor, the transpeptidase shows hydrolase activity, using water as acceptor. In fact, the preferred γ-glutamyl donor for this transpeptidase is not glutamine itself, but a ubiquitous γ-glutamyl-containing tripeptide, glutathione (γ-glutamylcysteinyl glycine, **11**). Thus, γ-glutamyl transfer to some α-amino acid nucleophile will generate, in addition to the new γ-glutamyl amino acid, the dipeptide fragment cysteinyl-glycine, **12** (Scheme 1).



Scheme 1

In 1983,[21] Reed's group reported that the glutamine analog acivicin inactivated γ-glutamyl transferase. The carboxyl group of acivicin is responsible for specific binding to γ-glutamyl transferase via the electrostatic interaction of their respective carboxyl group and an arginyl residue at the active site. However,

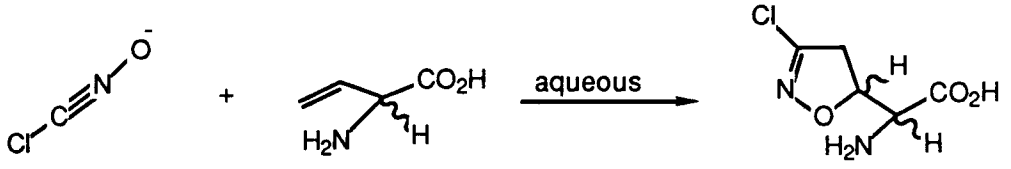
the inactivation of the γ-glutamyl transferase capability of acivicin was abolished by esterification of the carboxyl moiety but was eventually regained upon incubation of acivicin methyl ester with a carboxyl esterase.

Chemical Synthesis of Acivicin

Reported syntheses of acivicin may be divided into five major reaction classes. These involve the use of (1) dipolar cycloadditions, (2) intramolecular Michael reactions, (3) intramolecular alkylation of hydroxamic acids, (4) aldol condensations or (5) oxidative cleavage of cyclopentenes.

Synthesis from 1,3-Dipolar Cycloadditions

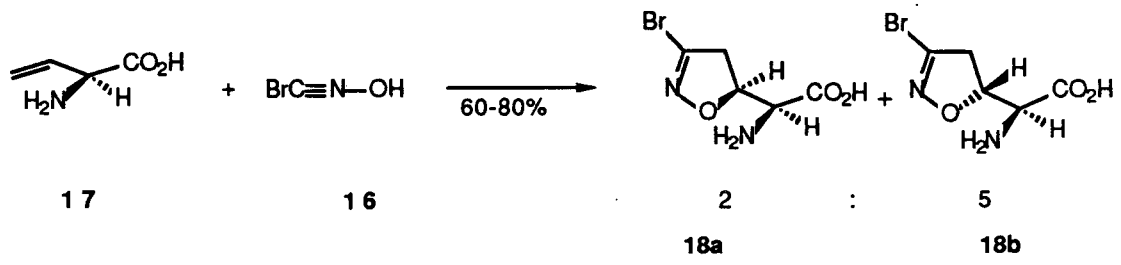
An attempted synthesis of chloroisoxazoline **13** by addition of chloronitrile oxide, **14**, to vinyl glycine, **15**, was unsuccessful[22] (Scheme 2). Apparently, the rate of 1,3-dipolar reaction was too slow to compete with furoxan formation.



14 **15** **13**

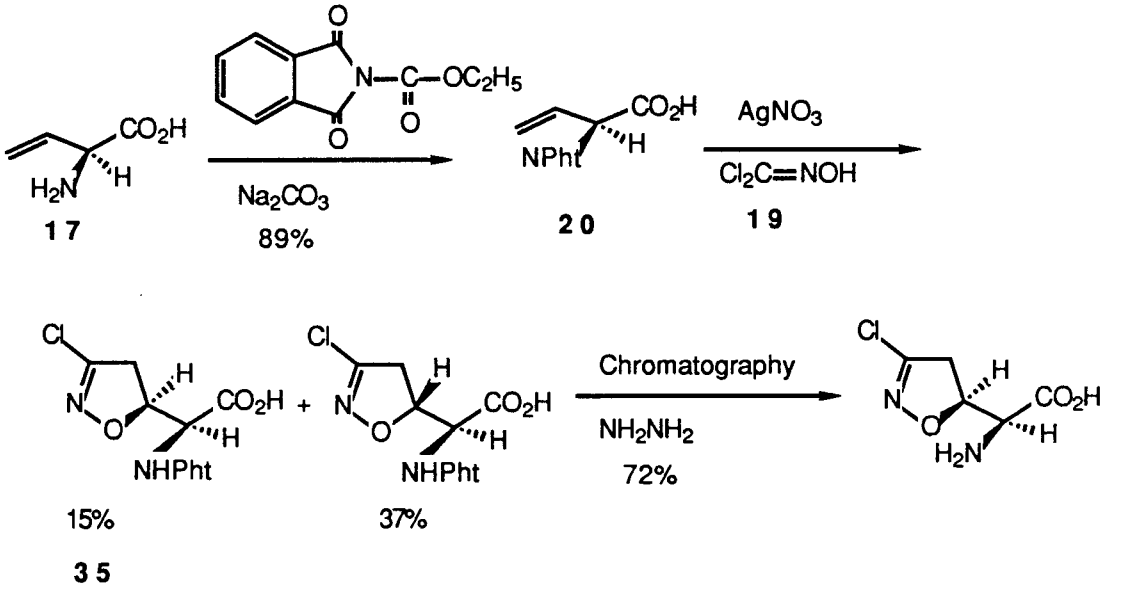
Scheme 2. An Unsuccessful Synthesis Toward Acivicin

Hagedorn and coworkers[23] have used the more reactive bromonitrile oxide, **16**, which, when treated with vinyl glycine, **17**, gave bromoacivicin, **18a,b** (Scheme 3). Unfortunately, the bromoacivicin could not be converted to acivicin at



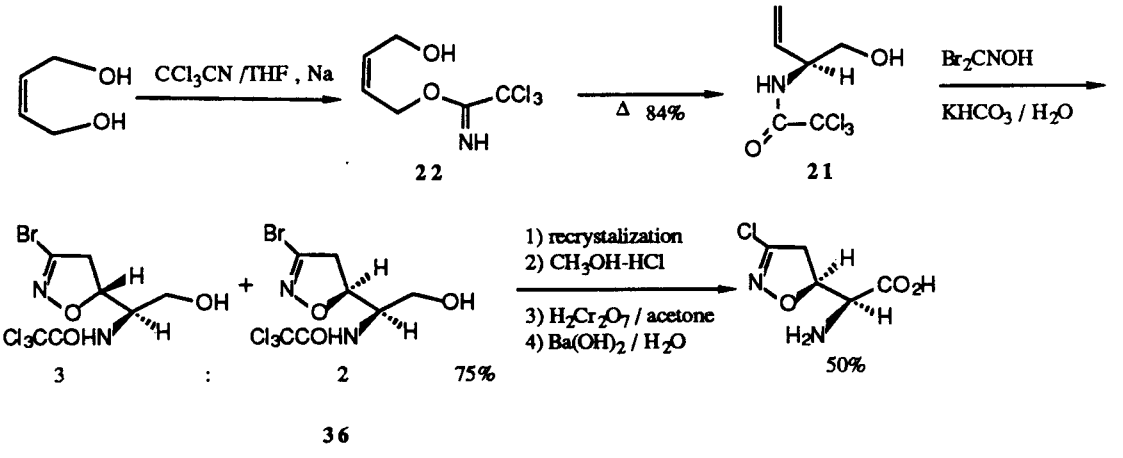
Scheme 3. Hagedorn's Synthesis of Bromoacivicin

this time. To activate 1,3 dipolar cycloadditions, Wade and coworkers[24] have carried out the synthesis of acivicin by addition of $AgNO_3$ to dichloroformadoxime, **19**, in the presence of phthalimido vinylglycine **20** as the key reaction. The reactions are summarized in Scheme 4.



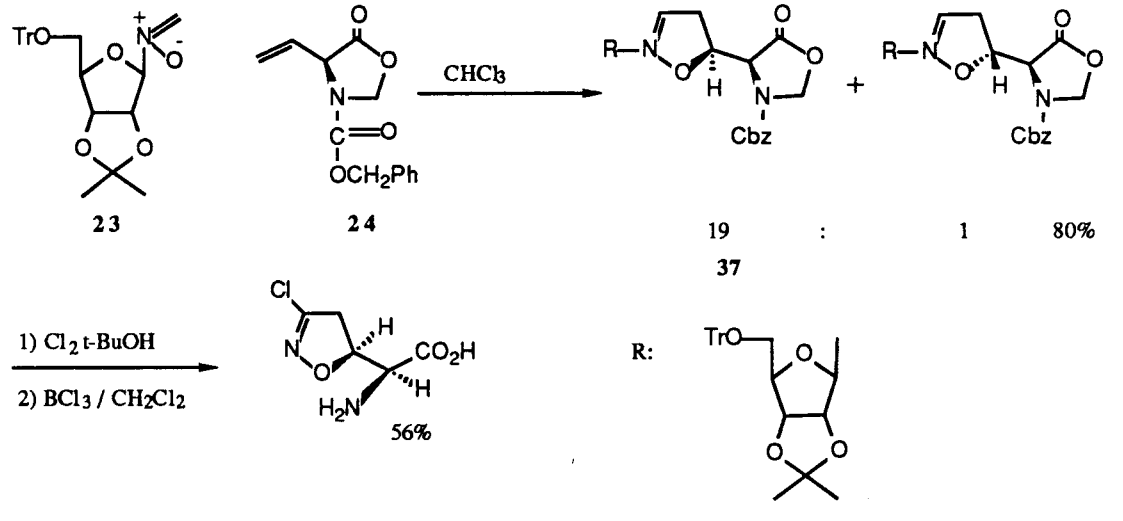
Scheme 4. Wade's Synthesis of Acivicin

Vyas and coworkers[25] have synthesized acivicin as outlined in Scheme 5. Their approach centers on the synthesis of **21** by a thermal [3,3]sigmatropic rearrangement of trichloroimidate **22** followed by a 1,3-dipolar cycloaddition to give racemic acivicin (Scheme 5).



Scheme 5. Vyas's Synthesis of Acivicin

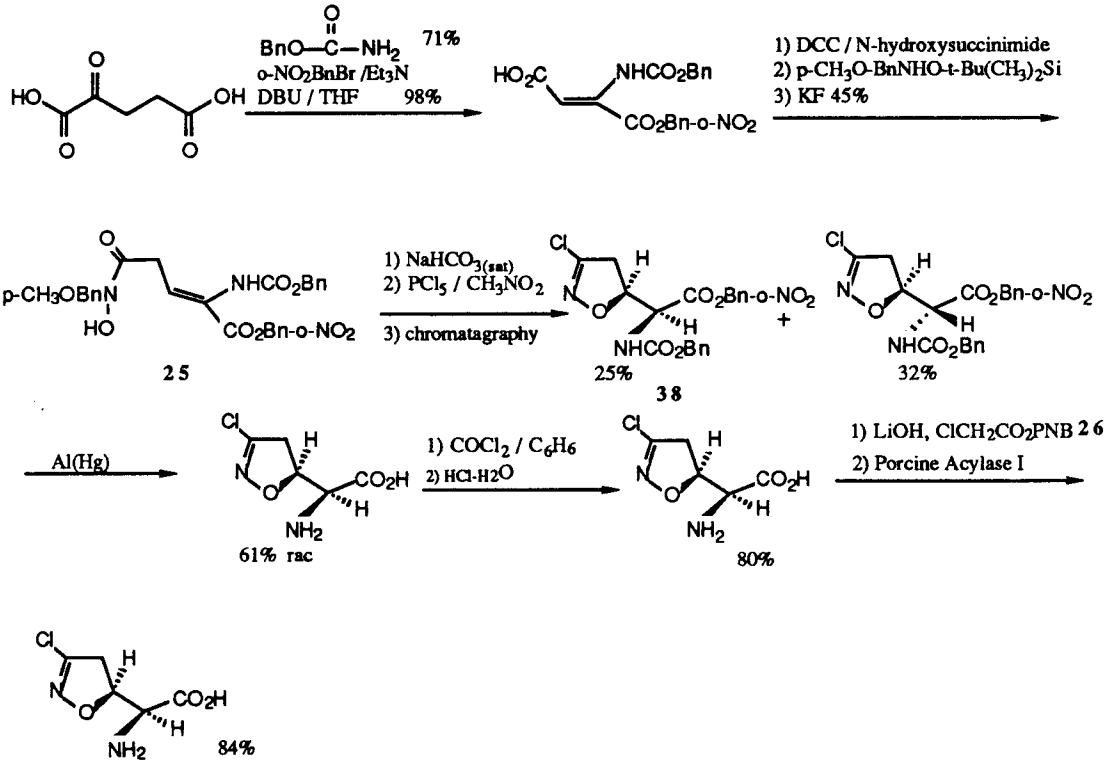
The main limitation of these synthetic approaches above has been the poor diastereofacial selectivity in the cycloaddition. The wrong C-3 stereoisomer has generally been obtained as the major product. However, Witney's[26] group has reported a short, highly stereoselective total synthesis of acivicin, based upon the principle of double asymmetric induction[27] in the reaction of chiral nitrone[28] **23** with L-vinyl glycine[29] derivative **24**. The reactions are summarized in Scheme 6.



Scheme 6. Witney's Synthesis of Acivicin

Synthesis from Intramolecular Michael Reactions

Baldwin and his coworkers[30] developed an alternative synthesis for acivicin via an intramolecular Michael cyclization of a protected α,β-dehydroglutamic acid γ-hydroxamate **25**. Separation of diastereoisomers and deprotection to racemic acivicin followed by enzymatic resolution of the N-chloro acetamide **26** provided the natural α*S*,5*S* isomer. Yield and reagents are given in Scheme 7.

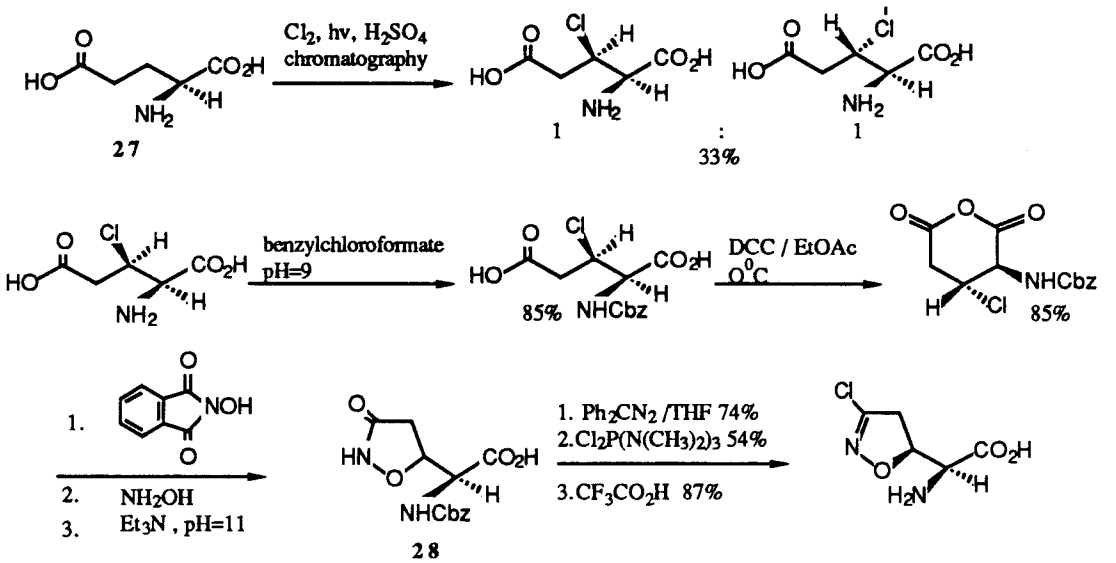


Scheme 7. Baldwin's Synthesis of Acivicin

Synthesis from Intramolecular Alkylation of Hydroxamic Acids

In 1981, Silverman and coworkers[31] reported a completely stereospecific synthesis of acivicin (Scheme 8). A key feature in the strategy to this end involved the photochlorination of L-glutamic acid, **27**, by a procedure of Kollonitsch et al.[32] After five steps, the isoxazole ring was formed stereospecifically by nucleophilic

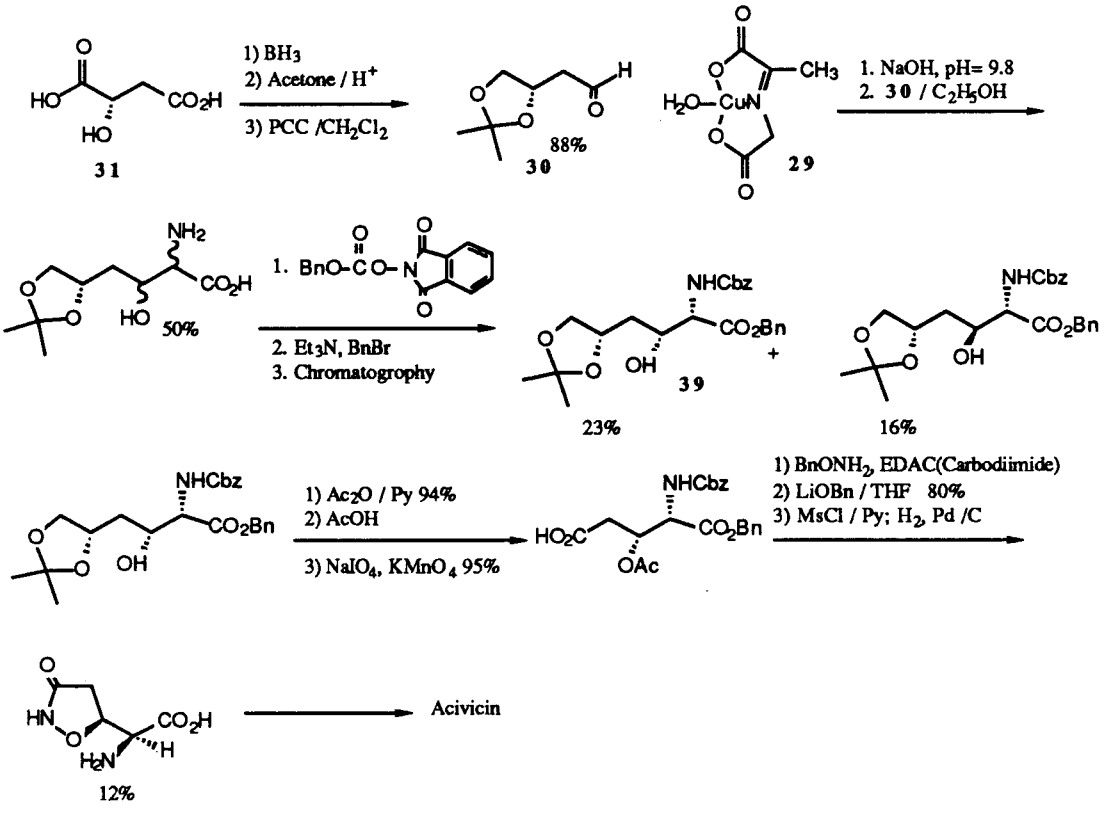
substitution of chloride to give the tricholomic acid derivative **28**. This derivative was then converted to acivicin by chlorination and deprotection (Scheme 8).



Scheme 8. Silverman's Synthesis of Acivicin

Synthesis from Aldol Condensations

In 1987, Hanessian reported[33] the synthesis of acivicin using an aldol condensation between N-pyruviylidene glycinatoaquacopper(II), **29**, and an optically active aldehyde **30** derived from S-malic acid, **31**, as the key bond-forming reaction. Although a viable strategy was developed in this synthesis, no asymmetric induction was observed. The yields and reagents are given in Scheme 9.

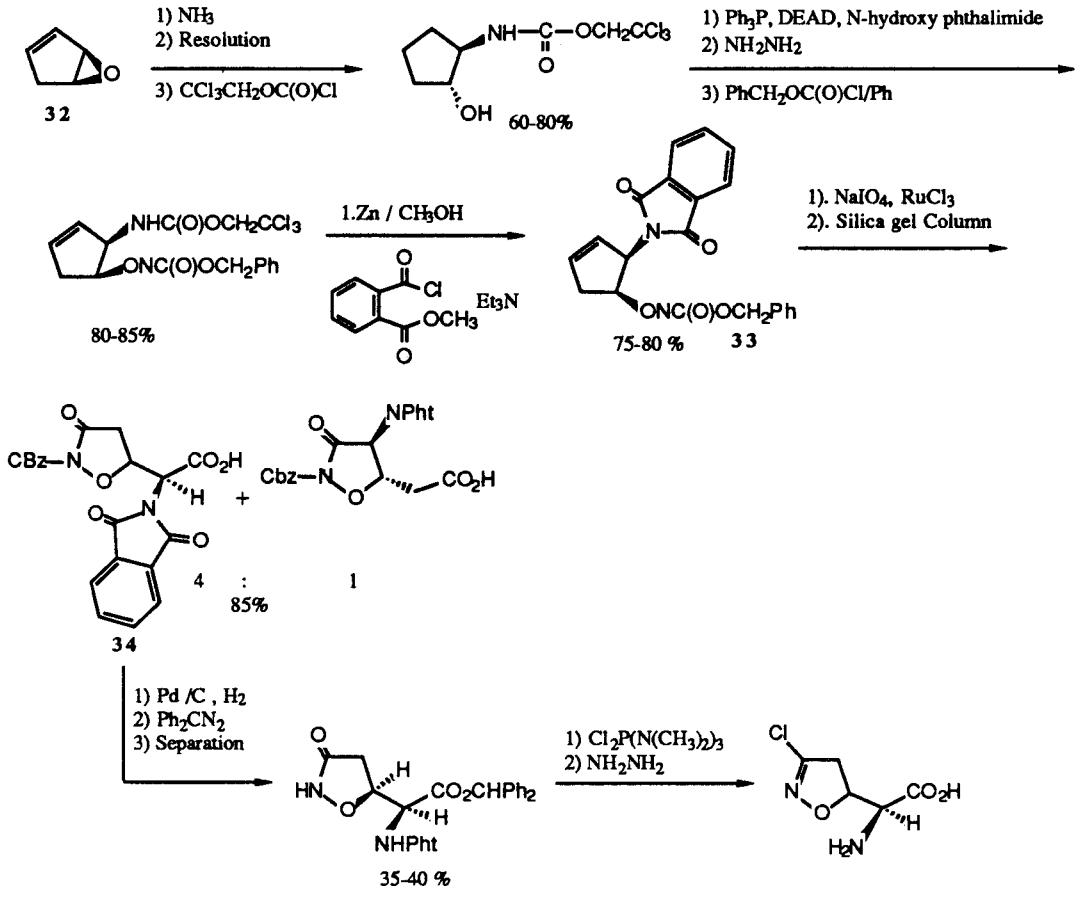


Scheme 9. Hanessian's Synthesis of Acivicin

Synthesis from Oxidative Cleavage of Cyclopentenes

Kelly and coworkers[34] have reported a stereoselective synthesis of optically pure acivicin which began with cyclopentadiene monoepoxide, **32**. In devising this plan, Kelly's group had anticipated that oxidative cleavage of the double bond in **33** would produce cyclized materials **34**. The complete synthesis is shown in Scheme 10.

A summary of syntheses with diastereoselectivity and overall yield from readily available starting materials are listed in Table 5.



Scheme 10. Kelly's Synthesis of Acivicin

Table 5. Summary of Synthetic Results of Acivicin

Starting material	Ratio E-isoxazolidine : T-isoxazolidine	Overall yield,* %	Ref.
Dibromoformaldoxime	1 : 2.5	**18a**, 8.5-17%	23
Dichloroformaldoxime	1 : 2.4	**35**, 13%	24
Cis-2-buten-1,4-diol	1 : 1.5	**36**, 25%	25
Chiral nitrone	19 : 1	**37**, 76%	26
α-Ketoglutamate	1 : 2.1	**38**, 7.8%	30
L-Glutamic acid	1 : 0	**28**, 0.32%	31
S-Malic acid	1 : 0	**39**, 0.02%	33
Cyclopentadiene mono epoxide	4 : 0	**34**, 0.28%	34

* From starting material to key intermediate.

Acivicin Analogs and Their Biosynthesis

In addition to those of *S. sviceus*, four metabolites with isoxazole rings at various levels of oxidation have been isolated from different microorganisms and mushrooms. They are tricholomic acid, **7**, ibotenic acid, **40**, muscimol, **41**, and D-cycloserine, **42** (Figure 5).

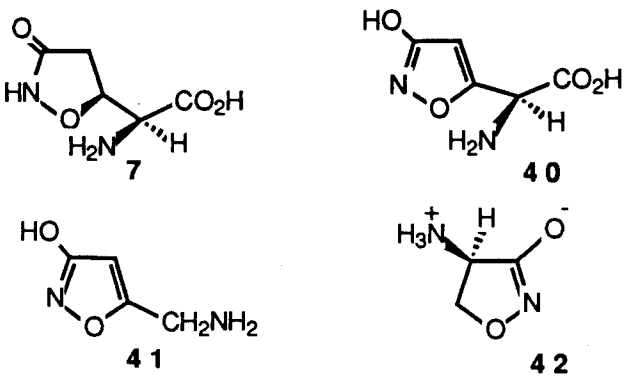
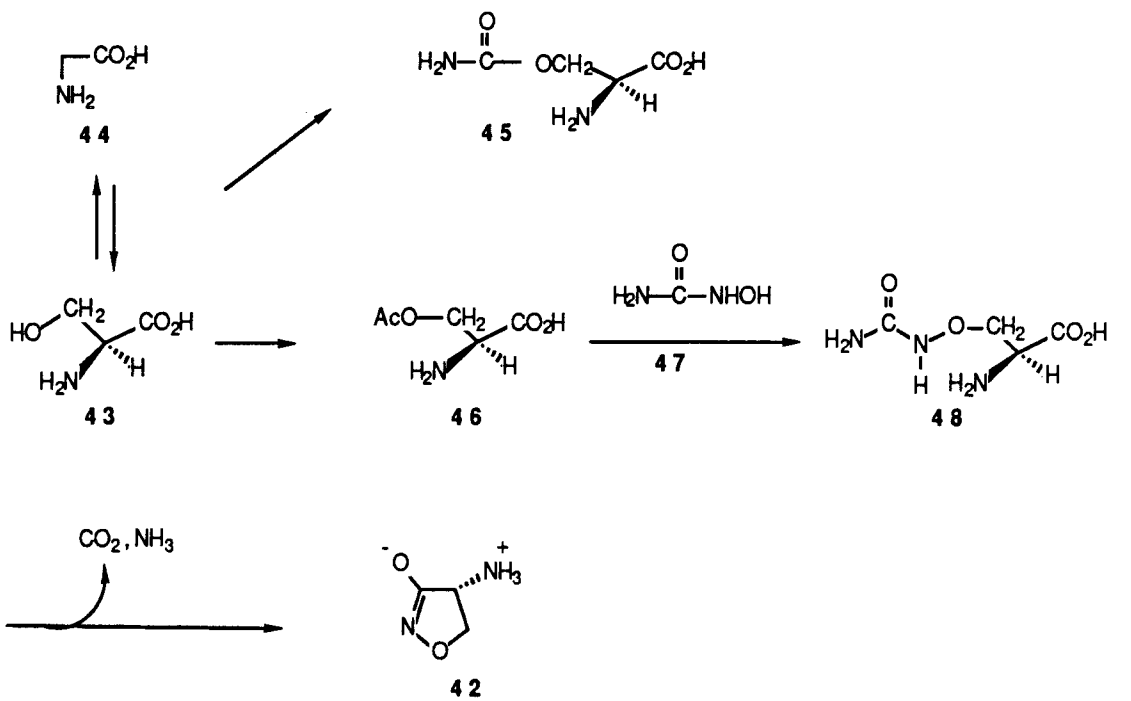


Figure 5. Acivicin Analogs

Tricholomic acid is an amino acid isolated by Takemoto et al[35] as a flycidal and tasty constituent of a mushroom, *Tricholoma muscarium* Kawamura. Its structure has been established through standard spectroscopic techniques as well as X-ray crystallography. This amino acid kills flies when the insect ingests the compound. Its chemical synthesis was described by Kamiya's group[36] in 1969. Isoxazole-derived muscimol and ibotenic acid were isolated in 1965[37] by Eugster and his colleagues and by other research groups independently.[38] Muscimol and ibotenic acid exhibit narcotic properties acting on the human central nervous system.[39] So far, there is no biosynthetic work reported for **7**, **40** and **41**. D-cycloserine, **42**, is another metabolite with an isoxazoline ring, and has been isolated from various *Streptomyces* spp. in different laboratories.[40] Its chemical synthesis has been described.[41] D-Cycloserine is therapeutically effective against gram-positive and gram-negative bacteria, rickettsiae and certain protozoa.[42]

The first biosynthetic study on D-cycloserine was carried out by Tanaka et al in 1963.[43] It was found that both DL-[3-^{14}C]serine, **43**, and [1-^{14}C]glycine, **44**, are biosynthetically incorporated into D-cycloserine in *Streptomyces*. On the other hand, D-O-carbamyl[3-^{14}C]serine, **45**, which was simultaneously produced by their organism, was not utilized as a precursor. Later, in 1981, Gatenbeck's group[44,45] found that O-acetyl-L-serine, **46**, and hydroxyurea, **47**, are intermediates in the biosynthesis of D-cycloserine. The enzymatic formation of O-ureido[U-^{14}C]serine, **48**, from O-acetyl-L-serine and hydroxyurea was demonstrated by incubating a cell-free preparation of *Streptomyces sp.* with [U-^{14}C]-labeled substrate. The results suggested the following pathway for D-cycloserine biosynthesis (Scheme 11).



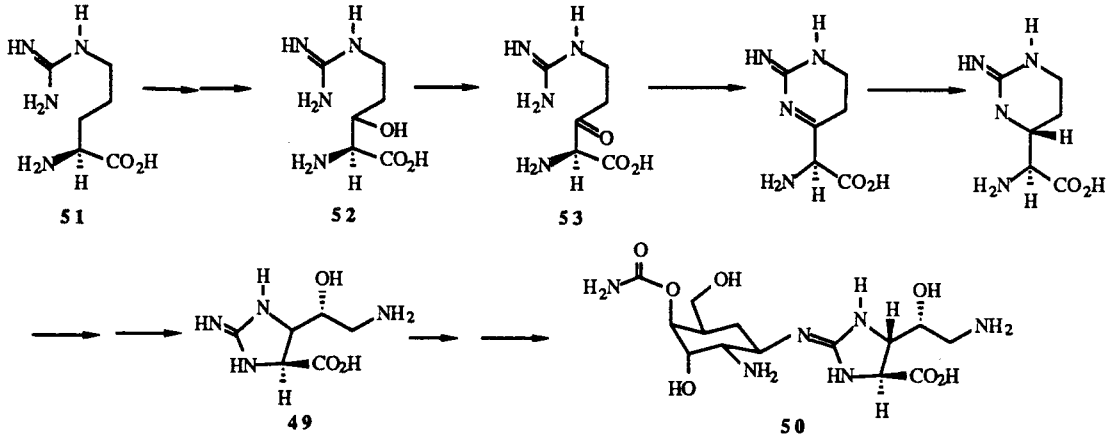
Scheme 11. Biosynthesis of D-cycloserine

Biosynthetic Methodology

There are available a number of excellent reviews and monographs on the methodology used in biosynthesis, which can be located through the *Specialist Periodical Reports on Biosynthesis*[46] and *Nuclear Magnetic Resonance*[47] and will not be discussed here. Clarifications of each technique used in this study are presented, as needed, in the "Results and Discussion" section.

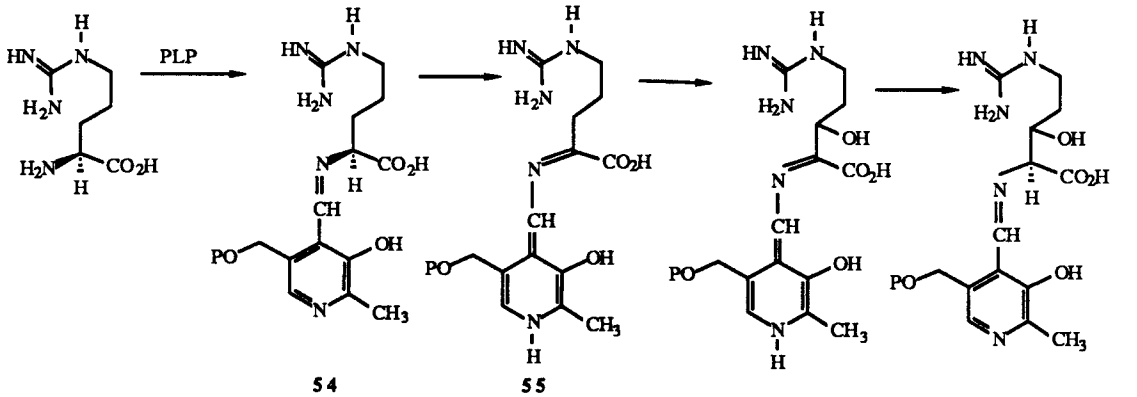
Purpose of the Present Study

The work of our group on the biosynthesis of the streptolidine[48] moiety **49** of streptothricin F, **50**, had indicated the intermediacy of arginine, **51**, and possibly that of β-hydroxy-arginine, **52**, and β-ketoarginine, **53**. Hydroxylation of isolated aliphatic carbon is well documented in biological systems, and such a reaction could be involved in the formation of **52** (Scheme 12). However, condensation of arginine



Scheme 12. Proposed Mechanism for Biosynthesis of Streptothricin

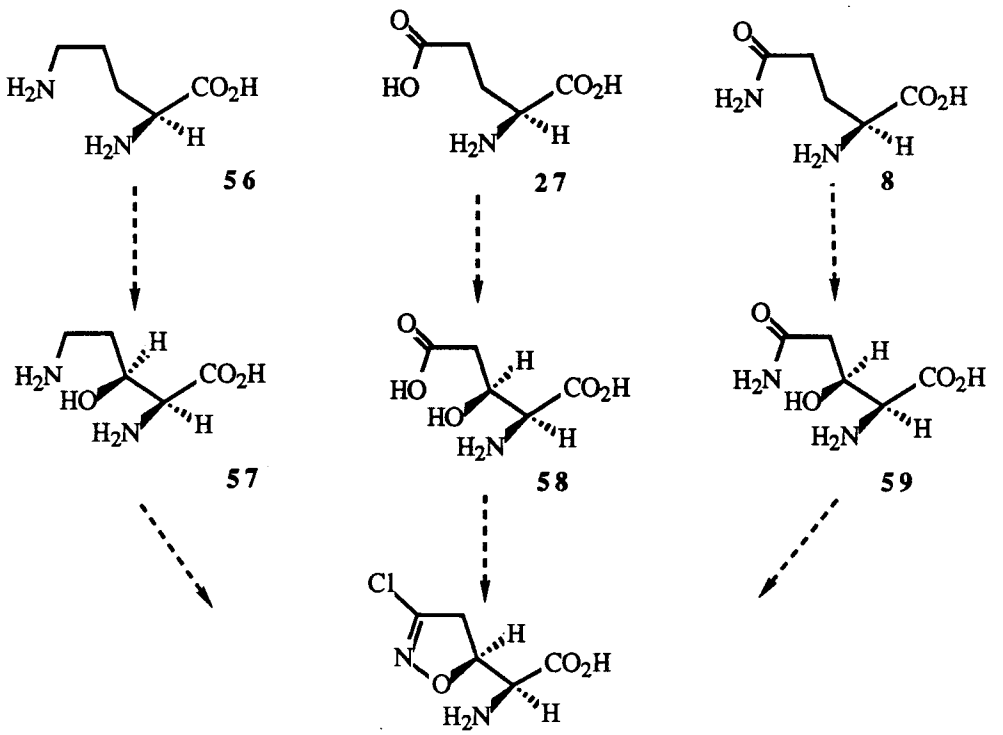
with a carbonyl compound, such as pyridoxal phosphate, to yield Schiff base, **54**, followed by tautomerization to imine **55** would yield a more reactive allylic β-carbon, as shown in Scheme 13.



Scheme 13. Proposed Mechanism for Biosynthesis of β-Hydroxyarginine

If correct, it would be possible to extend this generality to various β-hydroxyamino acids. Furthermore, since the antibiotic acivicin would be most easily understood as biosynthesized from a 5-carbon β-hydroxyamino acid, we started our investigation on the biosynthesis of acivicin. It was recognized that acivicin might be derived from L-glutamic acid, **27**, L-glutamine, **8**, or L-ornithine, **56**. These amino acids would then be oxidized to β-hydroxyamino acids **57**, **58**, **59** which would then cyclize to acivicin shown as Scheme 14.

Our research goals have been focused on determining (i) the primary metabolic precursor for the biosynthesis of acivicin and 4-hydroxyacivicin, (ii) the origin of the isoxazoline oxygen and the mechanism of its introduction, (iii) how the isoxazoline ring is formed biosynthetically.



Scheme 14. Proposed Retrobiosynthetic Analysis Based on 5-Carbon Amino Acids

Naturally Occurring β-Hydroxyamino Acids

β-Hydroxyamino acids are non-proteinogenic amino acids. A number of naturally occurring β-hydroxyamino acids have been isolated from hydrolyzates of peptides, or directly as free entities. Others are implied in the structures of natural products. Their number is steadily increasing as more and more new natural products are discovered. Some of them have been summarized by Wityak,[49] and Table 6 lists the β-hydroxy amino acids which have been found as constituents of natural products in the recent literature.

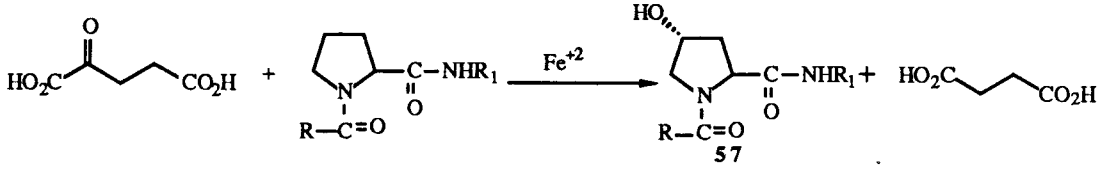
Table 6. List of β-Hydroxyamino Acids

Name	Comments	Reference
β-Hydroxyleucine	Constituent of leucinostatin D, C	50,51
β-Hydroxyphenylalanine	Constituent of biphenomycins	52
β-Hydroxyphenylalanine	Constituent of antibiotic M43 D	53
β-Hydroxyphenylalanine	Constituent of antibiotic M43 A,B	54
β-Hydroxyphenylalanine	Constituent of antibiotic A41030A	55
β-Hydroxyphenylalanine	Constituent of A51568B	56
β-Hydroxyphenylalanine	Constituent of acivicin A	57
β-Hydroxyphenylalanine	Constituent of antibiotic AAD-609	58
N-Methyl-β-hydroxy-phenylalanine	Constituent of phomopsine	59
N-Methyl-β-hydroxy-phenylalanine	Constituent of dityromycin	60
N,N-dimethyl-β-hydroxy-leucine	Constituent of antibiotic AAD-609	61

Biological Oxidation

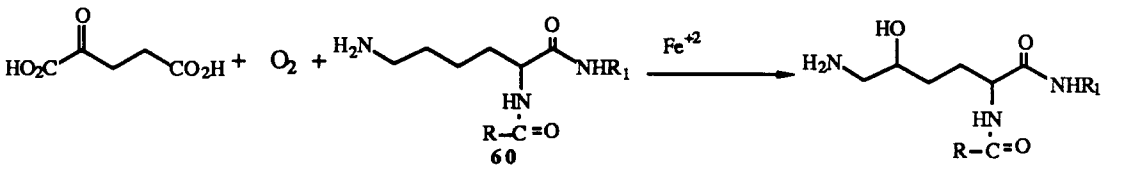
From the studies discussed, it would seem that formation of the isoxazoline ring of acivicin required an oxidative functionalization of the precursor α-amino acid. The subject of biological oxidation has been extensively reviewed.[62] Therefore, the following discussion will focus upon biological hydroxylation occurring at an unactivated carbon atoms.

The enzyme responsible for formation of the 4-hydroxy-prolyl residue in animal collagen and in plant cell wall proteins,[63] prolyl hydroxylase, has been shown to require O_2, Fe^{2+}, α-ketoglutarate and ascorbate (Scheme 15). The same cofactor



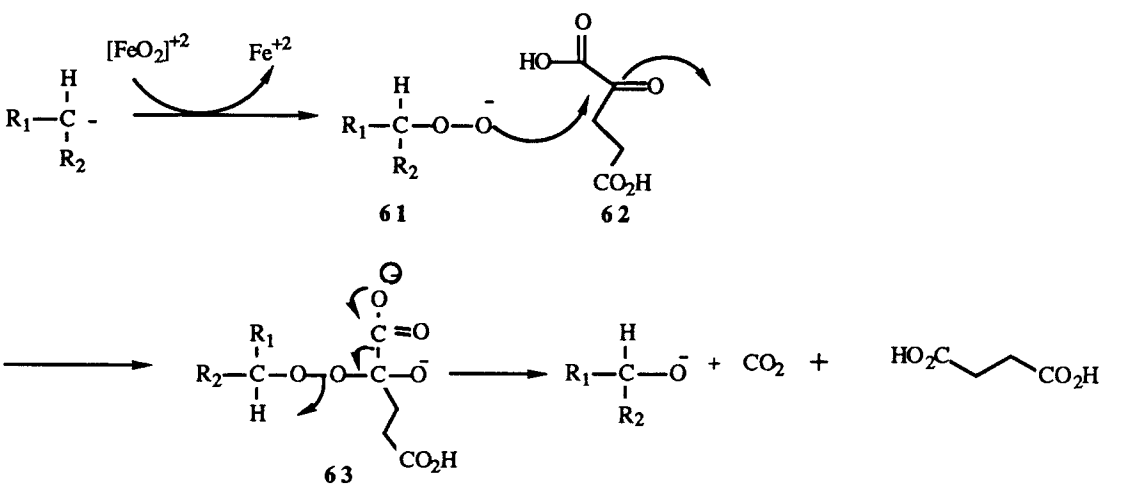
Scheme 15. Enzyme Hydroxylation of Proline

or cosubstrate are required for the hydroxylation of lysine, **60**, in proto-collagen (Scheme 16).[64]



Scheme 16. Enzyme Hydroxylation of Lysine

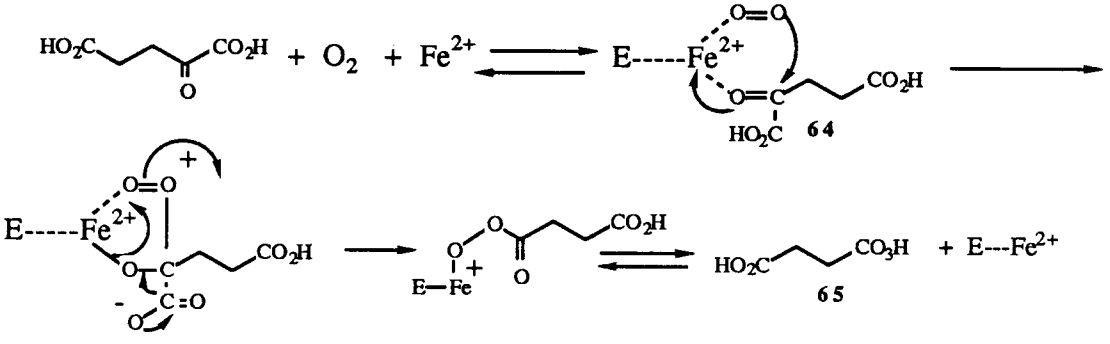
Linstedt[65] has proposed a mechanism to explain the above transformation which involved the initial formation of a substrate hydroperoxide at the methylene carbon to be hydroxylated. This was followed by attack of the peroxyanion **61** on the electrophilic carbonyl of α-ketoglutarate **62** to form a bridged peroxyspecies **63**.[66] Decarboxylation would then proceed with fission of the O-O peroxide linkage to yield the observed product (Scheme 17).



Scheme 17. Linstedt's Proposal for the Mechanism of α-Ketoglutarate

This proposal suffers from the drawback that the initial attack of the enzyme-bound form of O_2 at the unactivated methylene carbon of the prolyl residue is hard to visualize. A somewhat more attractive proposal has been put forth by Hamilton.[67]

This proposal requires initial formation of an α-ketoglutarate-oxygen Fe(II) complex **64** at the active site. Nucleophilic attack at the carbonyl produces a coordinated tetrahedral intermediate, which then decomposes to the peracid **65** and carbon dioxide (Scheme 18). The mechanistic details of carbon-hydrogen insertion of an oxenoid oxygen into the C-H bond will have to await further study.



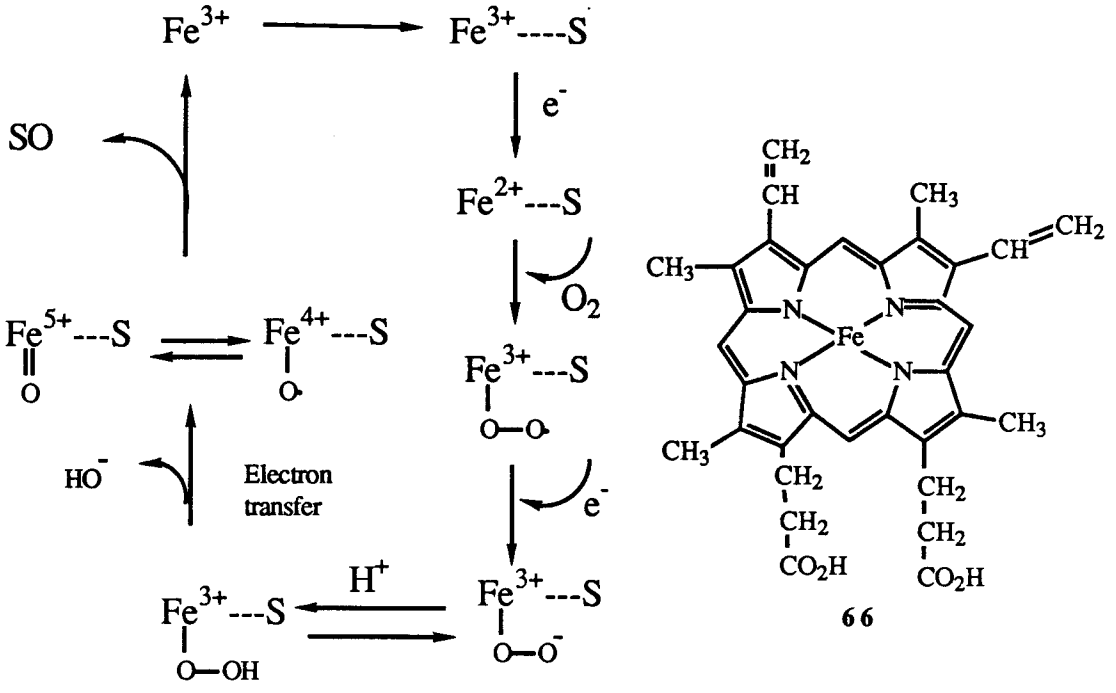
Scheme 18. Hamilton's Proposal for the Mechanism of α-Ketoglutarate Dependent Dioxygenase

Monooxygenases involved in the hydroxylation of steroids[68] and terpenes[69] have been reviewed. Irrespective of the source of the steroid hydroxylating enzyme, the available evidence suggests that it is an iron-containing cytochrome P-450 dependent species[70] which functions with the stoichiometry shown in Equation 1.

$$R\text{-}H + NADPH + O_2 \longrightarrow R\text{-}OH + NADP^+ + OH^- \quad \text{Equation 1.}$$

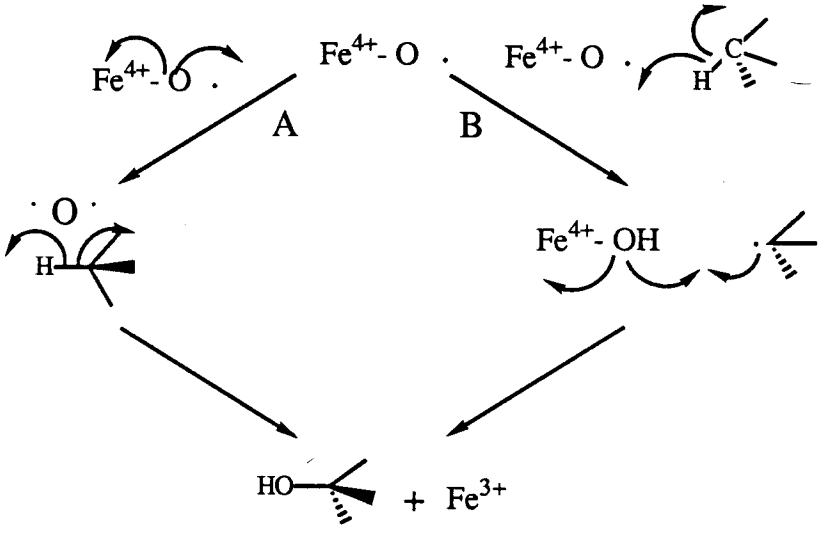
These enzymes incorporate one atom of molecular oxygen into the substrate and are classified as monooxygenases.

Much work on this class of enzymes has been performed using soluble enzymes from bacterial sources, such as the camphor hydroxylase from *Pseudomonas putida*.[71,72] The active site of cytochrome P-450 from *P. putida* contains an iron heme in the form of iron protoporphyrin IX, **66**, the resting state of cytochrome



Scheme 19. The Catalytic Cycle of Cytochrome P-450 Dependent Monooxygenase S: Substrate

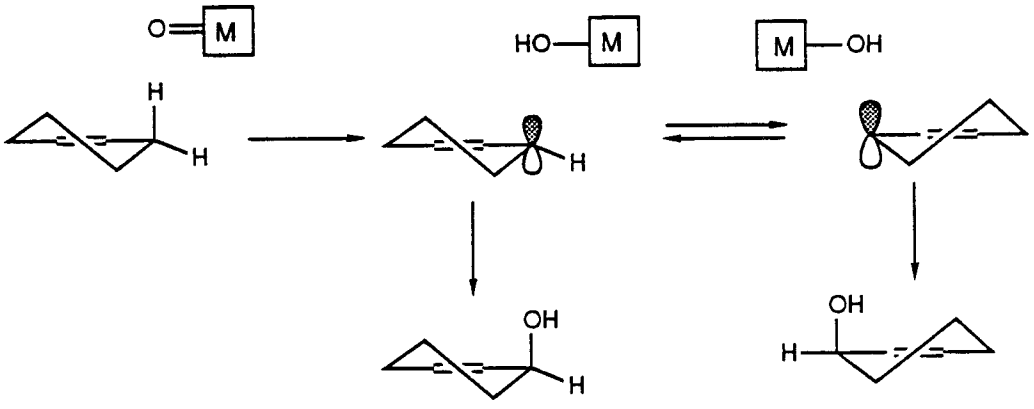
P-450 has iron in the ferric state. This catalytic cycle is shown in Scheme 19. The oxidizing species has been proposed to react with the substrate in two ways (paths A and B, Scheme 20). The first involves a direct insertion of the six-electron oxenoid



Scheme 20. Proposed Mechanism for Cytochrome P-450 Mediated Hydroxylation at a Saturated Carbon

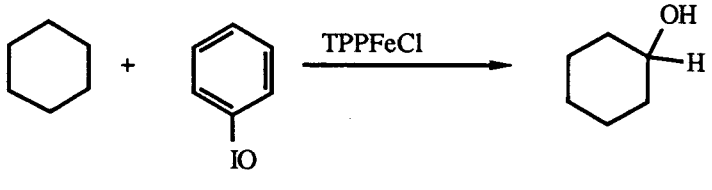
species into the C-H bond (pathway A).[73] The second is a variation of the first and involves homolysis of the substrate CH bond (path B).[74]

Later Grove's group[75] also found that a mechanism is suggested for allylic hydroxylation by cytochrome P-450 and by the metalloporphyrin model systems involving initial hydrogen atom abstraction from the allylic site followed by cage recombination of the incipient, allylic free radical (Scheme 21).



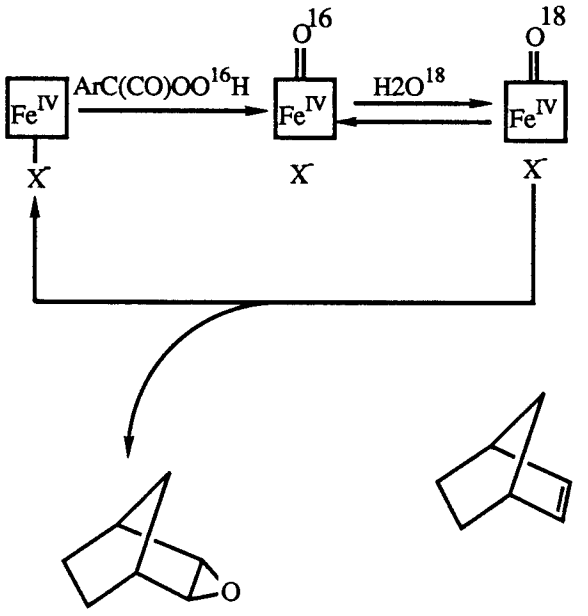
Scheme 21. Proposed Mechanism for Hydroxylation of Cyclohexane

In 1978, Grove's group discovered that synthetic iron(III) porphyrin complexes would catalyze the transfer of oxygens from iodosylbenzene to paraffins (Scheme 22).[76] Iodosylbenzene was chosen in part because it had been shown to be an acceptable oxygen source for cytochrome P-450. Subsequently, chromium and manganese porphyrin systems were also shown to be effective.



Scheme 22. Reaction of Cyclohexane

The isolation and characterization of reactive oxometalloporphyrins has begun to provide a rationale for this process and for the enzymic reaction as well.[77] The high chemical reactivity of oxometalloporphyrin toward hydrocarbons has indicated that it is kinetically competent to be the reactive species in the iron porphyrin catalytic systems. That the O-O bond is broken in the reactive complex is supported by the efficient incorporation of ^{18}O from water into the product epoxide in the presence of olefinic substrates. Peroxyacids don't exchange the peroxidic oxygen with water, whereas the oxoligand of metaloxo complexes do. Thus, a mechanism for oxygen transfer to olefins via oxometalloporphyrin is that shown in Scheme 23.[78,79] To date, the actual mechanism of the hydroxylation step has still not been determined.



Scheme 23. Proposed Mechanism for Oxygen Transfer to Olefin via Oxometalloprophrin

RESULTS AND DISCUSSION

Biochemical Work

As part of our biosynthetic studies, it was first necessary to become familiar with as well as check the reproducibility of the various techniques involved. Even though the fermentation and the bioassay conditions were optimized by Dr. David Martin of the Upjohn Company, the results were not reproducible in our laboratory and this caused serious problems during the work-up of fermentations. Hence, efforts were taken to optimize the bioassay, fermentation and isolation conditions.

Bioassay

The inherent variability of the biological system required a method to quantify antibiotic production. The approach chosen was a microbiological asasy for inhibition of the growth of an acivicin-sensitive bacterium.

Bacillus subtilis[7] UC-902 had been reported as the test organism for the assay of acivicin and this was carried out by the agar diffusion method.[80] The use of this organism was reinvestigated with the aid of Peter Yorgey. It was found that *B. subtilis* was a sensitive organism, giving clear zones of inhibition down to a concentration of 0.031 μg/ml.

The bioassay was done as follows. A stock spore suspension was prepared and stored at 4 °C. Prior to the bioassay, the synthetic agar to be used was inoculated with an appropriate amount of the spore suspension. Paper disks were used as a reservoir for the acivicin solution. A ten-fold dilution of the spore suspension into the agar and use of 8 mL of this inoculated agar per petri plate was found adequate. The use of 80 μl of the test solution per disk was found to be optimum. The standard bioassay curve was obtained by using different concentrations of authentic acivicin

and plotting the concentration (μg/ml) vs inhibition zone diameter. This gave a straight line as shown in Figure 6.

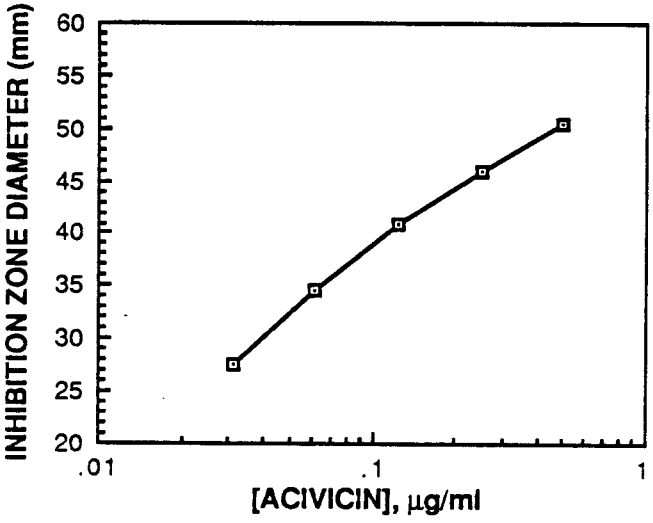


Figure 6. Acivicin Bioassay Curve

Fermentation of Acivicin and 4-Hydroxyacivicin

The acivicin and 4-hydroxyacivicin producer, *Streptomyces sviceus*, was stored on Hickey-Tresher agar[81] slants or plates at 4 °C in the cold room. However, such cultures have a lifetime of only about two months. Even though fermentations were tried by changing conditions like rpm, temperature, the nature of flask, different quantities of *S. sviceus* spores used to seed the cultures, etc., the maximum production of acivicin obtainable using these Hickey-Tresner agar slants was 2-5 μg/ml, which made the purification process difficult. This problem was overcome by using soil cultures of *Streptomyces sviceus*. The recipes of the seed medium and the production medium[10] are listed below.

Seed medium:

D-glucose	0.5 g
Peptone (Difco)	0.5 g
Yeast extract	0.13 g
dd, H_2O	50 mL (in 250 mL Erlenmeyer flask)

The pH of the solution was adjusted to 7.2 using 1N NaOH.

Production medium:

Cerelose	0.4 g
Washed dried yeast	0.5 g
Kaysoy 200c	4 g
Corn starch	2 g
NH_4Cl	1 g
Lard oil	1 mL
Tap water	200 mL (in 1 liter Erlenmeyer flask)[82]

The pH of the broth was adjusted to pH 7.2 prior to autoclaving.

The maximum production of acivicin with soil cultures for initiation of liquid fermentations was 15-22 μg/ml. The following conditions were found to be best:

Seed medium	:	the same as before
Flask	:	Erlenmeyer flask (250 mL)
Temperature, rpm, time	:	27 °C, 280 rpm, 69 h
Production medium	:	the same as before
Flask	:	Baffled flask (1L)[82]
% inoculum of seed	:	2.5% (v/v)
Temperature, rpm, time	:	32 °C, 250 rpm, 120 h

Generally, 200 mL of fermentation broth was used per 1 liter baffled flask, with the production of acivicin in the range of 12-17 μg/ml as determined by bioassay. A study was performed to monitor the production of acivicin as a function of

time and the results are shown in Figure 7. The appearance of acivicin started after 48 h of incubation and reached a maximal concentration by 120 hours.

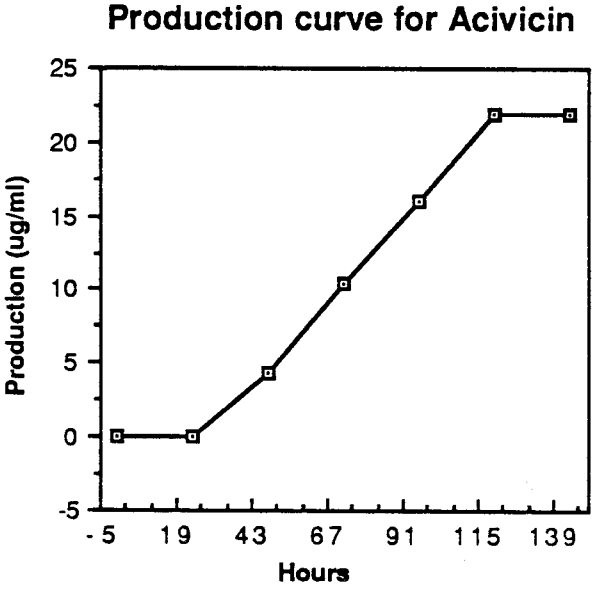


Figure 7. Production Curve of Acivicin

Isolation of Acivicin and 4-Hydroxyacivicin

After 120 hours of incubation the fermentation was harvested and centrifuged to remove the mycelia and other solid materials. Resulting pellets were washed with a minimum amount of deionized water, centrifuged, and the washings were combined with the original supernatant. The resulting clear supernatant, neutralized to pH 7.8 with 2N HCl was next passed through a cation exchange column (Dowex 50Wx8, H^+) and washed with deionized water. Acivicin and 4-hydroxyacivicin bound to this resin. The column was then eluted with 2.5N NH_4OH. Fractions containing acivicin and 4-hydroxyacivicin (determined by ninhydrin test) were combined, rotary evaporated at ambient temperature to remove ammonia, and then lyophilized. The concentrate was then adjusted to pH 7 and passed through an anion exchange column (AG3-X4A, OH^-). The column was washed with deionized water, 50% methanol-water, and 90%

methanol-water. The column was then finally eluted with methanol-water-glacial acetic acid (90:10:3) with a flow rate of 3 mL/min. Fractions containing acivicin and 4-hydroxyacivicin were combined, rotary evaporated at 45 °C to remove the solvent, and lyophilized. The residue from the acivicin and 4-hydroxyacivicin fractions was redissolved in water and evporated onto silica gel. The loaded silica gel was then poured on top of a silica gel column. The column was then eluted with methyl ethyl ketone-acetone-water (65:20:15) and the effluent was monitored by tlc on silica gel plates. Two fractions were obtained. Each fraction was recrystallized from methanol and water. They gave pure acivicin and 4-hydroxyacivicin as white crystals.

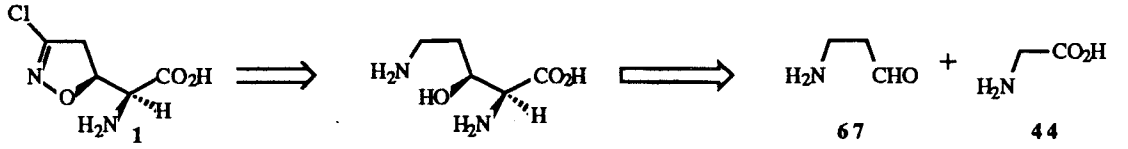
In the present study of the mechanistic and stereochemical aspects of the biosynthesis of acivicin and 4-hydroxyacivicin, deuterium labeled precursors have been used primarily, and the resulting acivicin and 4-hydroxy acivicin samples were analyzed by ^{2}H NMR spectroscopy.

In planning to synthesize isotopically labelled compounds, several important factors must be considered. The first is that the synthesis must be efficient, as low yields increase both cost and the scale necessary to produce the desired quantity of material. A second consideration is that it should be short, as again cost and scale are minimized. The third, and most important factor, is that the label be introduced as easily as possible into the synthetic scheme. Prior to the synthesis of labeled precursors, the reaction conditions were optimized by carrying out the reactions using unlabeled reactants and reagents.

Radioactive Feeding Experiment

It was initially recognized that acivicin and 4-hydroxyacivicin might be derived from L-glutamic acid, **27**, L-glutamine, **8**, or L-ornithine, **56**, as

mentioned earlier (Scheme 14). Another possibility was that acivicin and 4-hydroxyacivicin could be derived from glycine, **44**, by an aldol condensation with **67**, followed by an oxidative process centered on the nitrogen, as shown in Scheme 24.



Scheme 24. A Possible Precursor Derived from Glycine

Therefore, the incorporation of various ^{14}C labelled five carbon amino acids, as well as glycine, into acivicin and 4-hydroxyacivicin was investigated.

Feeding of DL-[1-^{14}C]Glutamic Acid and L-[U-^{14}C]Glutamine

From the time-course studies of acivicin production, it was observed that acivicin began to appear at 48 hours after inoculation and reached a maximum at approximately 120 hours after inoculation. Hence, DL-[1-^{14}C]glutamic acid and L-[U-^{14}C]glutamine were each fed to separate production broths, one flask each, at 48, 72 and 96 h after inoculation, and each fermentation was continued for a total of 120 h. All six experiments were worked up in standard fashion. Authentic acivicin and 4-hydroxyacivicin were added to each as carrier, and the material recovered in the work-up was recrystallized five times. In none of these experiments was either metabolite radioactive. Hence, the negative results indicated that neither glutamic acid nor glutamine was a precursor of acivicin and 4-hydroxyacivicin.

Feeding of [1-^{14}C]Glycine

[1-^{14}C]Glycine was fed to separate production broths, one flask each, at 48, 72 and 96 h after inoculation, and each fermentation was continued for a total of 120

h. All three experiments were worked up in standard fashion. Authentic acivicin and 4-hydroxyacivicin was again added to each as carrier. The recovered material was recrystallized five times. Again, in none of these experiments was either metabolite radioactive. The negative results indicated that glycine was not a precursor of acivicin and 4-hydroxyacivicin.

Feeding of DL-[2-^{14}C]Ornithine

When DL-[2-^{14}C]ornithine was fed at 48 h after inoculation, the isolated acivicin **1a** and 4-hydroxyacivicin **2a** were radioactive. The percentage incorporation for acivicin **1a** was 0.2%. For 4-hydroxyacivicin, it was 2.0%. This indicated that ornithine was taken up by *S. sviceus* and a portion utilized for acivicin and 4-hydroxyacivicin.[83] This seemed to represent a surprising apparent lack of metabolic economy since C-5 of ornithine - derived from the ε-carboxyl group of glutamic acid - must subsequently be reoxidized to the amide oxidation state.

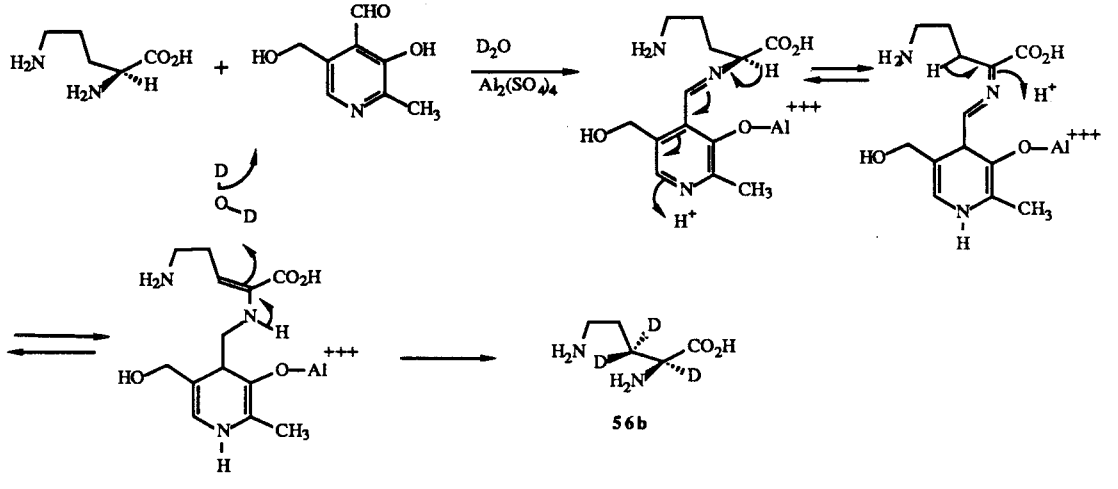
Feeding of DL-[5-^{15}C,5-^{15}N]Ornithine, **56a**[84]

In order to determine whether the ornithine incorporation was specific, and to simultaneously determine whether the δ-amino group was retained, **56a**[84] was fed. This feeding was done using 38.8 mg of **56a** mixed with 29.2 x 10^6 DPM of DL-[5-^{14}C]ornithine. The mixture was fed in equal portions to five 200 mL production broths at 48 h, and the resulting acivicin **1b** and 4-hydroxyacivicin **2b** was purified by the standard procedure. Radioactivity data for 4-hydroxyacivicin **2b** indicated a 2.64% incorporation of [5-^{14}C]ornithine based on both D and L isomers, and this corresponded to a 4.16 enrichment in ^{13}C. A 0.28% incorporation of [^{14}C]ornithine based on both D and L isomers corresponding to a 1.26% enrichment in ^{13}C was obtained for acivicin, **1b**. In fact, the 100.6 MHz ^{13}C NMR spectra of acivicin **1b**

and 4-hydroxyacivicin **2b** in D_2O each exhibited a spin-coupled doublet (J_{CN} = 2.7 Hz) for C-5 (152.6 and 154.7 ppm, respectively), as shown in Figures 8 and 9. The shoulder peak was interpreted as one of the two peaks of a spin coupled doublet, the other peak being buried under the natural abundance peak due to an downfield shift (~2.7 Hz) observed because of ^{13}C shielding due to the heavier ^{15}N nucleus. Normalization of the integrals against the resonance for C-1 gave an enrichment of 5.8% and 4.1% respectively. Thus, both isotopes were retained and ornithine was demonstrated to be the primary precursor to both metabolites.

Synthesis of DL-[2,3,3-$^2H_3$]Ornithine·HCl, 56b

In order to probe the fate of the three hydrogens at C-2 and C-3 of ornithine **56b**, into acivicin, **1**, and 4-hydroxyacivicin, **2**, feeding experiments with ornithine **56b** labeled with deuterium at C-2 and C-3 were necessary. Hence, this compound was our next synthetic target. This compound was prepared by the procedure of Lemaster and Richards.[85] The synthesis of **56b** involved a pyridoxal-catalyzed exchange with D_2O in the presence of Al^{+3}. The suggested mechanism is shown in Scheme 25.



Scheme 25. A Proposed Mechanism for DL-[2,3,3-$^2H_3$]Ornithine

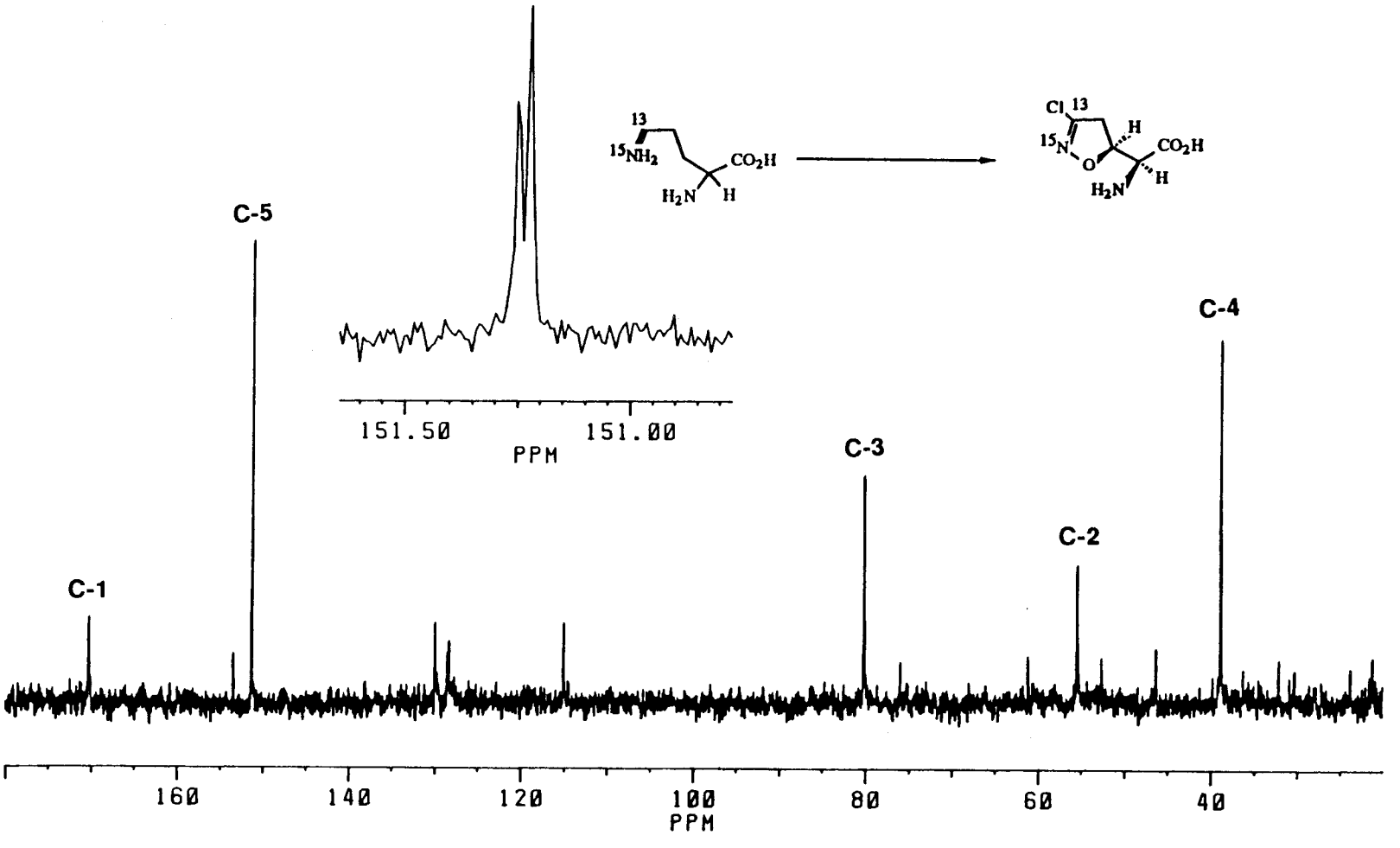


Figure 8. ^{13}C NMR of Acivicin **1b** from the Feeding of **5 6 a**

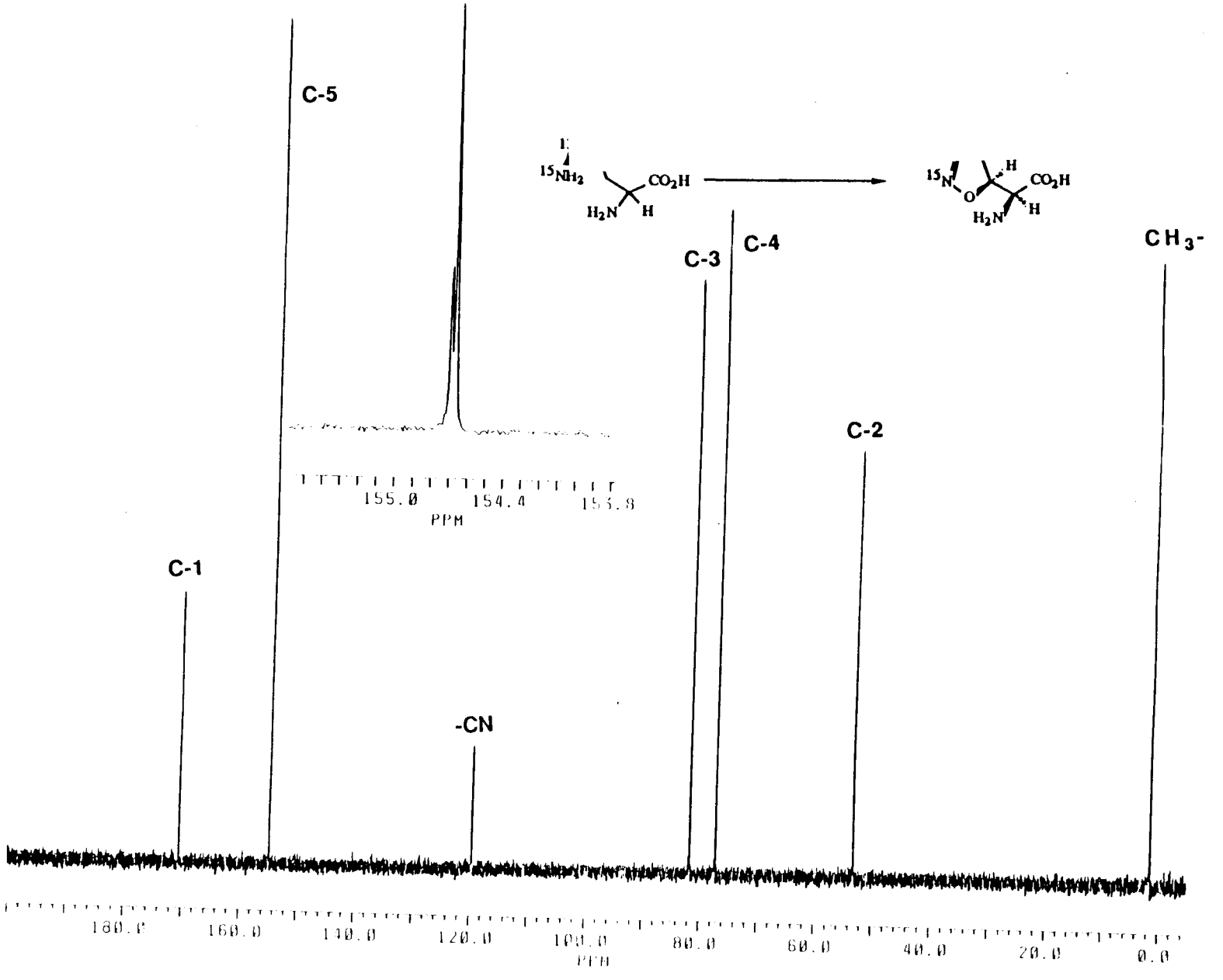


Figure 9. ^{13}C NMR of 4-Hydroxyacivicin **2b** from the Feeding of **5** **6a**

This exchange reaction was tried two times. The reaction was performed in a seal tube heated in an oil bath for two days. Deuterated ornithine was next purified by cation ion exchange chromatography. The column was washed with deionized water and eluted with O.1N NH4OH. Recrystallization of the product was then done from a water-ethanol mixture at pH 6-7. Proton NMR analysis of the ornithine indicated that the extent of α-deuteration was 100% with 57% β deuteration. It was necessary to replace the used D_2O with a fresh batch one more time to maximize the deuterium enrichment. After two consecutive exchanges at 125 °C, the deuterium enrichment was found to be 76% at β position and 100% at α position by ^1H NMR integration. Mass recovery was 51%.

Feeding of DL-[2,3,3-$^2H_3$]Ornithine, 56b

Labeled ornithine **56b** (88.92 mg) admixed with 26.7 x 10^6 DPM of DL-[5-^{14}C]ornithine was fed in equal portions to ten 200 mL production broths 48 h after inoculation with a vegetative seed culture. The resulting acivicin **1c** and 4-hydroxyacivicin **2c** were purified by the usual procedure, and showed a 1.30% incorporation of radioactivity for **2c** and 0.21% incorporation of radioactivity for **1c**. This feeding experiment afforded 3.6 mg of **1c** and 27 mg of **2c**. The 61.4 MHz ^2H NMR spectrum of **2c** showed resonances for residual HOD (δ 4.93), for t-butyl alcohol added as a chemical shift (δ 1.27) and deuterium quantitation reference, and for deuterium at C-3 (δ 5.19). Absolutely no deuterium was detectable at C-2, even though the signal to noise ratio would have allowed detection of 2% retention relative to C-3. By comparing the ^2H content (1.08 μmole) calculated from the ^{14}C incorporation to that obtained from integration at the NMR signal (0.41 μmole), it was clear that only 38% of the ^2H had been retained and the remaining 62% was lost at C-3 for **2c** (Figure 10).

The ^{2}H NMR spectrum of **1c** similarly showed retention of deuterium only at C-3 although, due to the small amount of sample, the signal to noise was much poorer (Figure 10).

It was noteworthy that a complete loss of deuterium had also been obtained during incorporation of a variety of arginines labeled with deuterium at C-2 in the biosynthesis of streptothricin F **50**[86] for which we had postulated the involvement of β-hydroxyarginine **52**. In both cases we believed that the loss of this hydrogen might have been mechanism-based and related to the hydroxylation.

Feeding of [2*RS*,3*R*]-[3-^{2}H]Ornithine, 56c, and [2*RS*,3*S*]-[3-^{2}H]Ornithine, 56d

The objective of these feedings was to understand which hydrogen of C-3 (Pro-*R* or Pro-*S*) of ornithine was lost in the biosynthesis of acivicin 1 and 4-hydroxyacivicin **2**. To study this, it was necessary to use ornithine chirally labeled with deuterium at C-3.[87] Separate feedings of each of the chirally deuterated ornithines were carried out.

One feeding was carried out using 90 mg of 3*S*-[^{2}H]-DL-ornithine, **56d** (85% chiral purity), along with 38.5 x 10^{6} DPM of DL-[5-^{14}C]ornithine. The resulting acivicin, **1d**, and 4-hydroxyacivicin, **2d**, showed a 1.32% incorporation of radioactivity for **2d** and 0.29% incorporation of radioacitivity for **1d**. This feeding experiment afforded 4.2 mg of **1d** and 17.6 mg of **2d**. The ^{2}H NMR spectrum of **2d** (Figure 11) showed a small peak at 5.3 ppm (H-3). By comparing the ^{2}H content (0.16 μmole) based on ^{14}C incorporation to that obtained from integration, 0.073 μmole at H-3, it was found that 45.6% of deuterium was retained at H-3 from the 15% optical impurity (3*R*) of the precursor. If the 0.073 μmole of deuterium was coming from 3*S*-hydrogen, this should be compared with the expected ^{2}H content of

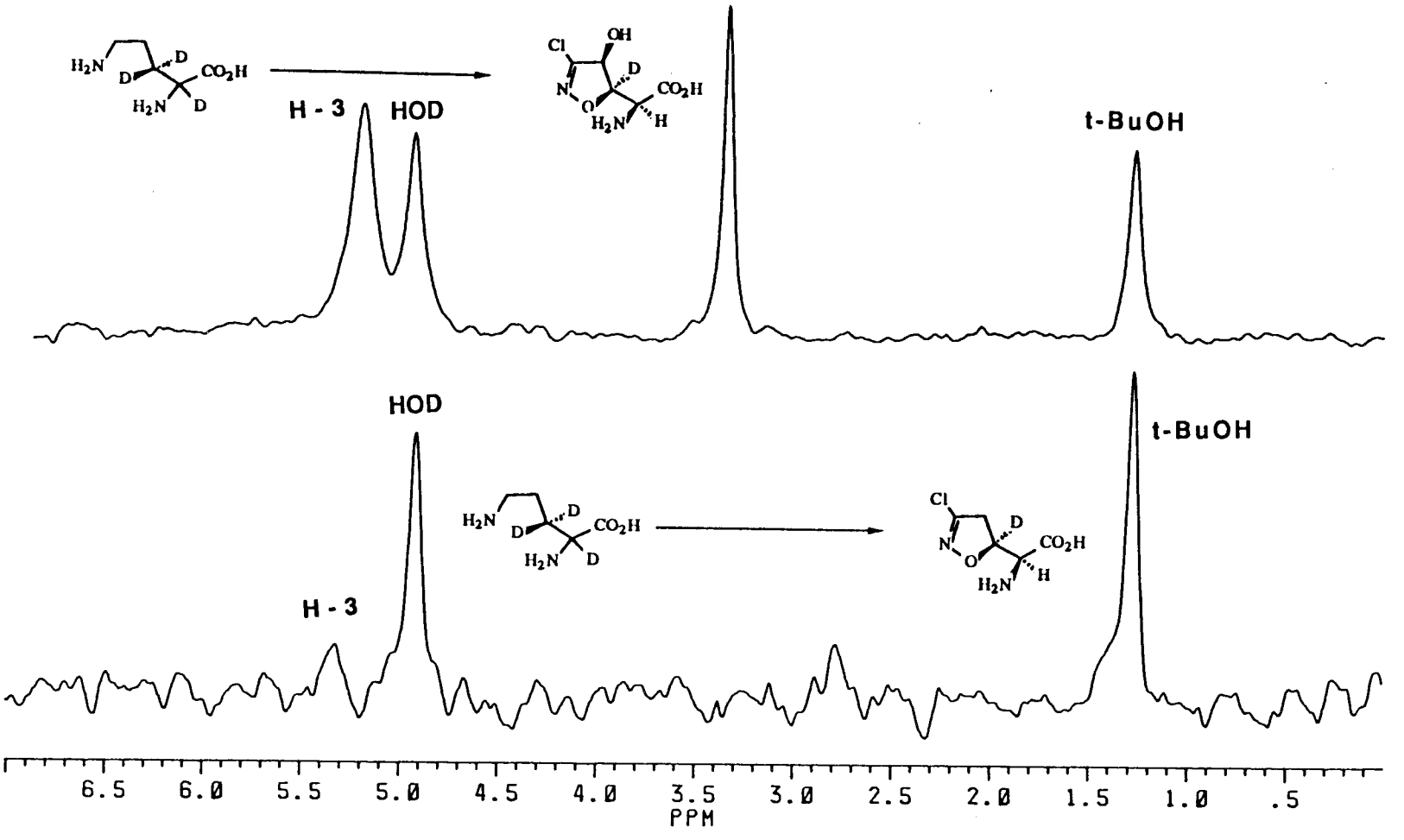


Figure 10. ^{2}H NMR of 4-Hydroxyacivicin **2 c** and Acivicin **1 c** from the Feeding of **5 6 b**

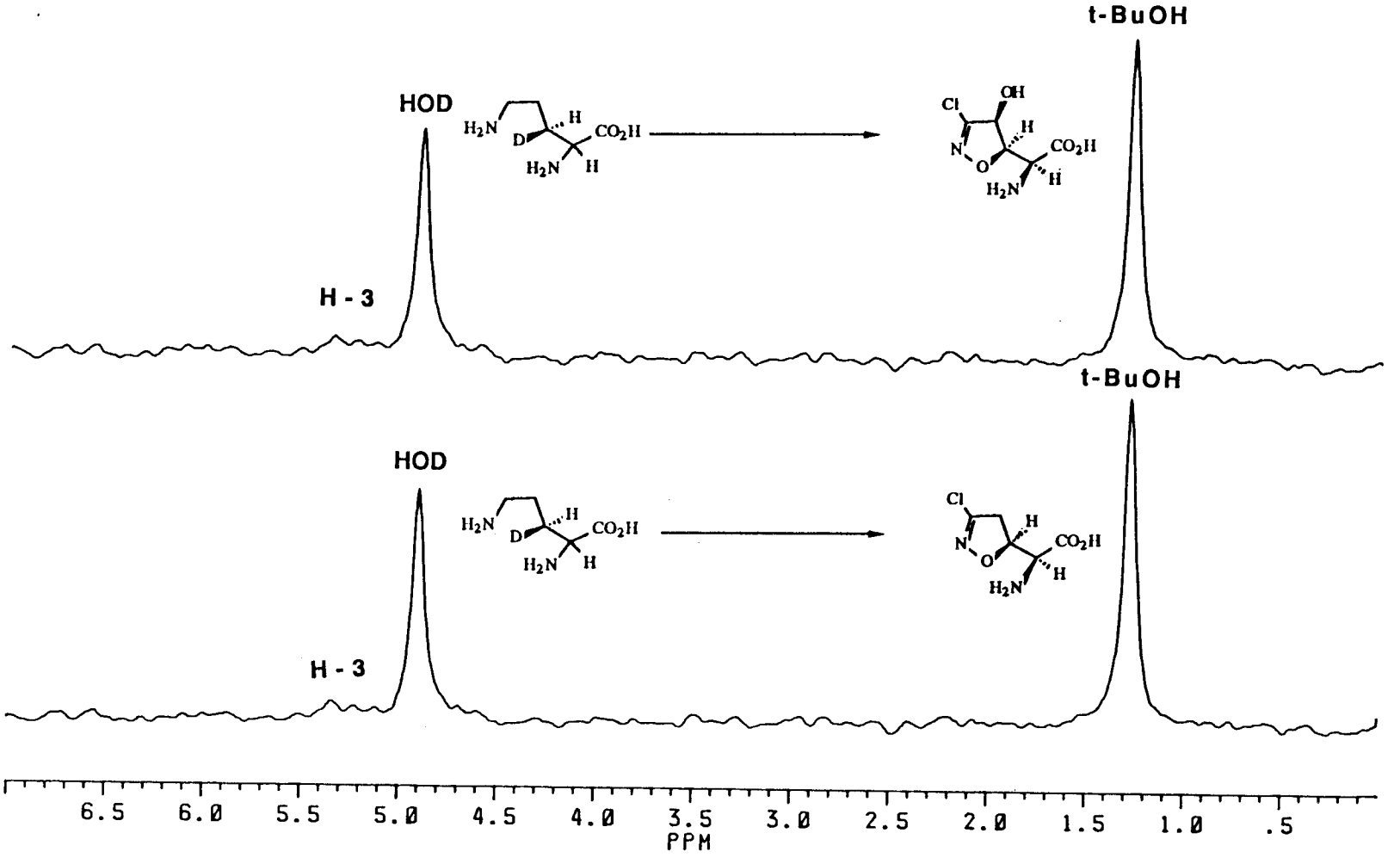


Figure 11. ^{2}H NMR of 4-Hydroxyacivicin **2d** and Acivicin **1d** from the Feeding of **56d**

H-3 (0.96 μmole) based on the ^{14}C incorporation of DL-5-^{14}C]ornithine. By comparing 0.073 μmole to 0.96 μmole, it was clear that only 7.6% of ^{2}H would have been retained. This result would be in great contrast to the DL-[2,3,3]ornithine feeding experiment which gave a 38% of ^{2}H in H-3 of **2c**. From the above discussions, it is clearly indicated that the 3*S* hydrogen was lost.

The ^{2}H NMR spectrum of **1d** (Figure 11) similarly showed retention of deuterium only at C-3 (δ 5.2 ppm) although due to the small amount of sample, the signal to noise was much poorer.

The other feeding was carried out using 91 mg of 3*R*-[^{2}H]-DL-ornithine (70% chiral purity), **56c**, along with 37.0 x 10^{6} DPM of DL-[5-^{14}C]ornithine. The resulting acivicin, **1e**, and 4-hydroxyacivicin, **2e**, were purified by the standard procedure and showed a 1.49% incorporation of radioactivity for **2e** and 0.29% incorporation of radioactivity for **1e**. This feeding experiment afforded 18 mg of **2e** and 11.1 mg of **1e**. Upon analysis of the ^{2}H NMR spectrum (Figure 12), **2e** showed a single peak at 5.3 ppm (H-3) in addition to those from t-BuOH (δ 1.27 ppm) and residual HOD (δ 4.9 ppm). By comparing the ^{2}H content (0.83 μmole) based on ^{14}C incorporation, to that obtained from integration, 0.39 μmole at H-3, it was found that only 47% of the ^{2}H at the 3*R* position of **56c** had been retained. The remaining 53% was lost at C-3 from **2e**. The ^{2}H NMR spectrum of **1e** (Figure 12) clearly indicated that 68.2% of the ^{2}H had been retained and the remaining 32% was lost at C-3.

If the deuterium content of **1e** and **2e** were coming from the 3*S* hydrogen, the expected ^{2}H content should have been 0.36 μmole and 0.22 μmole for **2e** and **1e** at H-3, respectively. By comparing the deuterium content based on ^{14}C incorporation to that obtained from integration, it indicated that over 100% of ^{2}H would have been

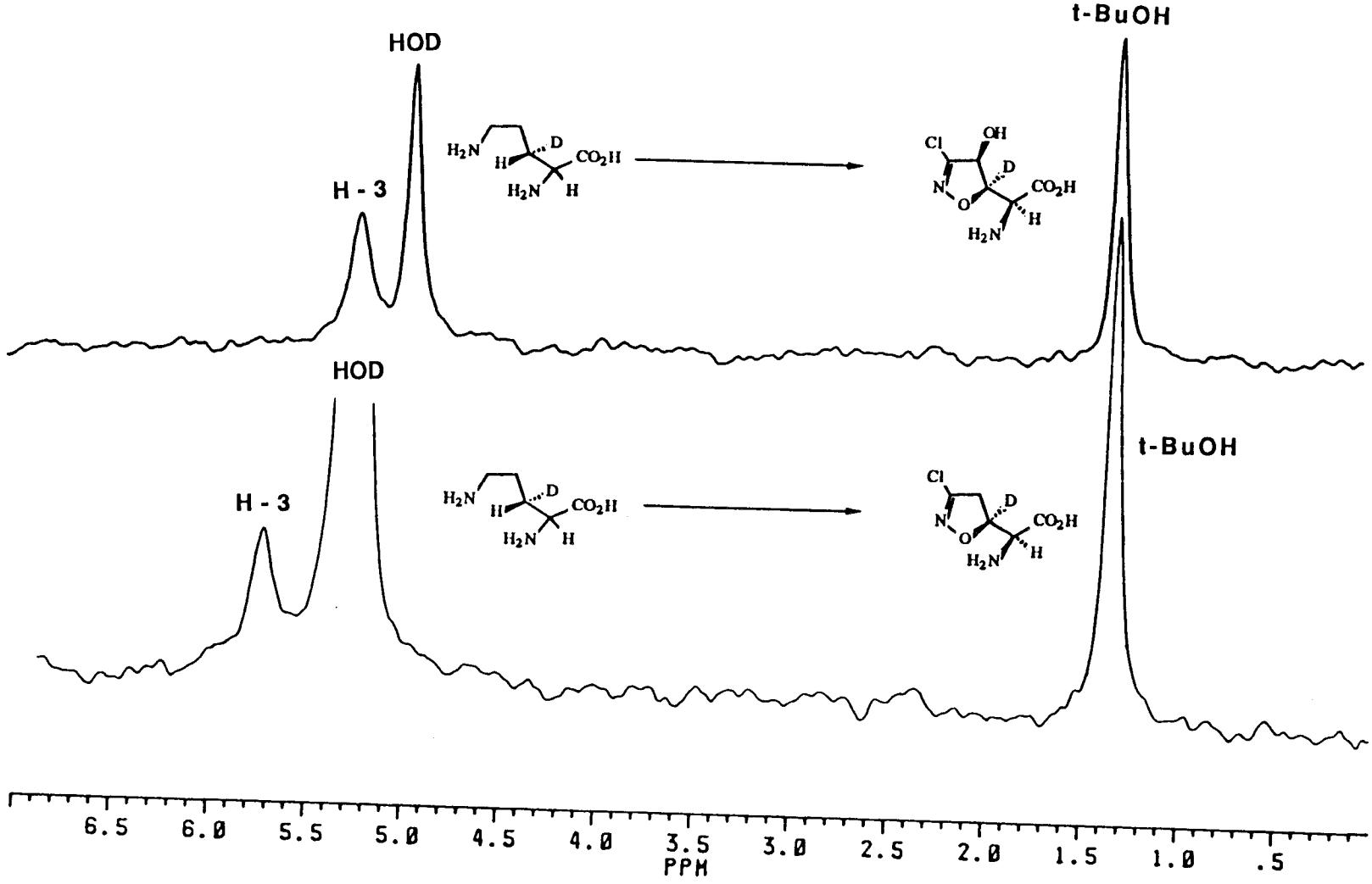
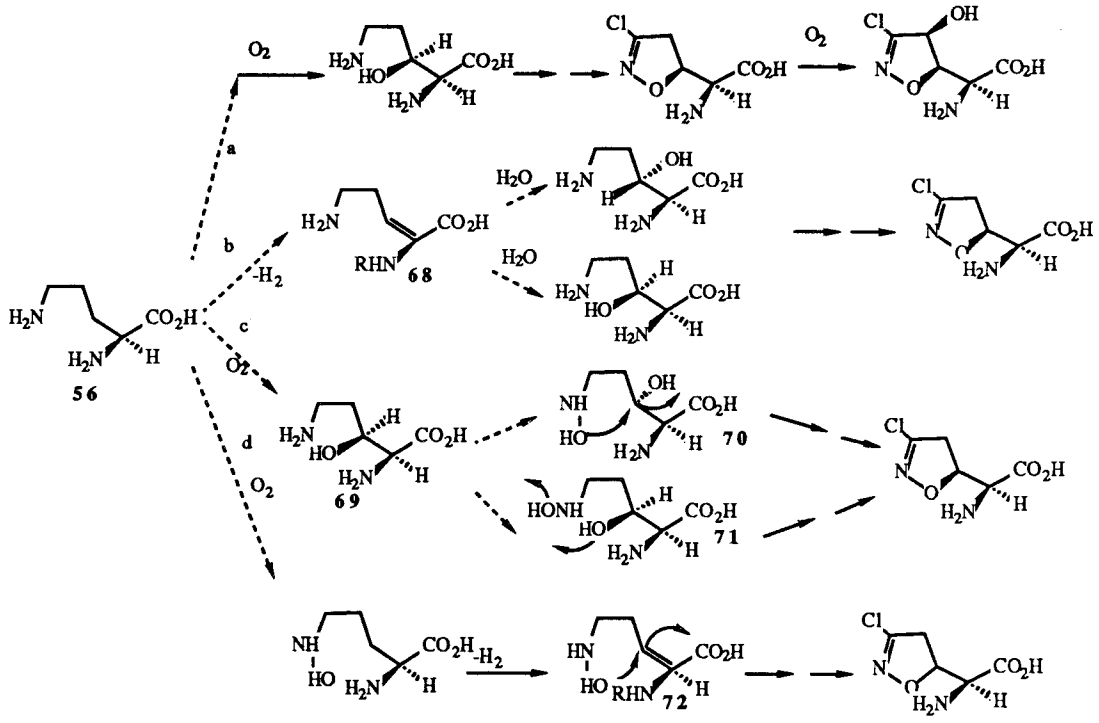


Figure 12. ^{2}H NMR of 4-hydroxyacivicin **2e** and Acivicin **1e** from the Feeding of **56c**

retained. These results clearly indicated it was the 3*S* hydrogen that was lost in the biosynthesis of **1e** and **2e**.

The experiments mentioned so far clearly indicated that ornithine was the primary precursor to both metabolites. Here, four possible retro-biosynthetic analyses, based on ornithine, **56**, are possible and these are shown in Scheme 26.



Scheme 26. Possible Retrobiosynthetic Analysis Based on Ornithine

Thus, in pathway a the isoxazoline oxygen might be derived from molecular O_2 by an enzyme-catalyzed direct insertion to the C-H bond at C-3. The ring could then be formed by an oxidative process centered on the nitrogen.

Alternately, the oxygen could be derived from water by addition to a dehydroornithine **68** (pathway b). Another possibility would involve an N-hydroxyamino acid intermediate **69**, which then could be oxidized to N-hydroxy-β-hydroxyornithine, **70** or **71**. This could then cyclize to acivicin biosynthetically

(pathway c). The last possibility would involve an N-hydroxy-dehydroamino acid **72**. This would then cyclize to acivicin **1** (pathway d).

Based on this proposal (Scheme 26), the next objective of this study was two fold: (1) to determine the source of oxygen for the isoxazoline ring and (2) to elucidate the biosynthetic sequence of the key intermediates. In order to elucidate the source of the isoxazoline oxygen in the biosynthesis of acivicin **1** and 4-hydroxyacivicin **2**, an oxygen-18 feeding experiment was carried out.

Oxygen-16 Fermentations

In an attempt to determine the origin of the oxygen atoms in acivicin, **1**, and 4-hydroxyacivicin, **2**, a fermentation utilizing $^{18}O_2$-enriched gas was planned. Incorporation can usually be determined by analysis of the ^{13}C NMR spectra of the derived metabolites. If an oxygen atom was derived from molecular oxygen, then two resonances should be observed for the corresponding carbon atom in the ^{13}C NMR spectrum when a strong magnetic field is used. The natural abundance ^{13}C-^{16}O resonance should be observable along with an isotopically upfield shifted ^{13}C-^{18}O resonance. This phenomenon was first observed independently by two research groups,[88,89] and has since been used to determine the origin of oxygen atoms in numerous biosynthetic studies.[90]

In our studies, in order to decrease the dead volume of nearly two liters (for cost efficiency) and optimize oxygen uptake, a fermentation bubbler apparatus (Figure 13) was built. This design was based on personal communications with Dr. J. C. Vederas of the University of Alberta. Trial operations with standard medium gave extensive foaming within 48 hours. Should this foaming have continued unabated, it would surely have resulted in damage to the aquarium pump. Addition of antifoaming agents proved fruitless.

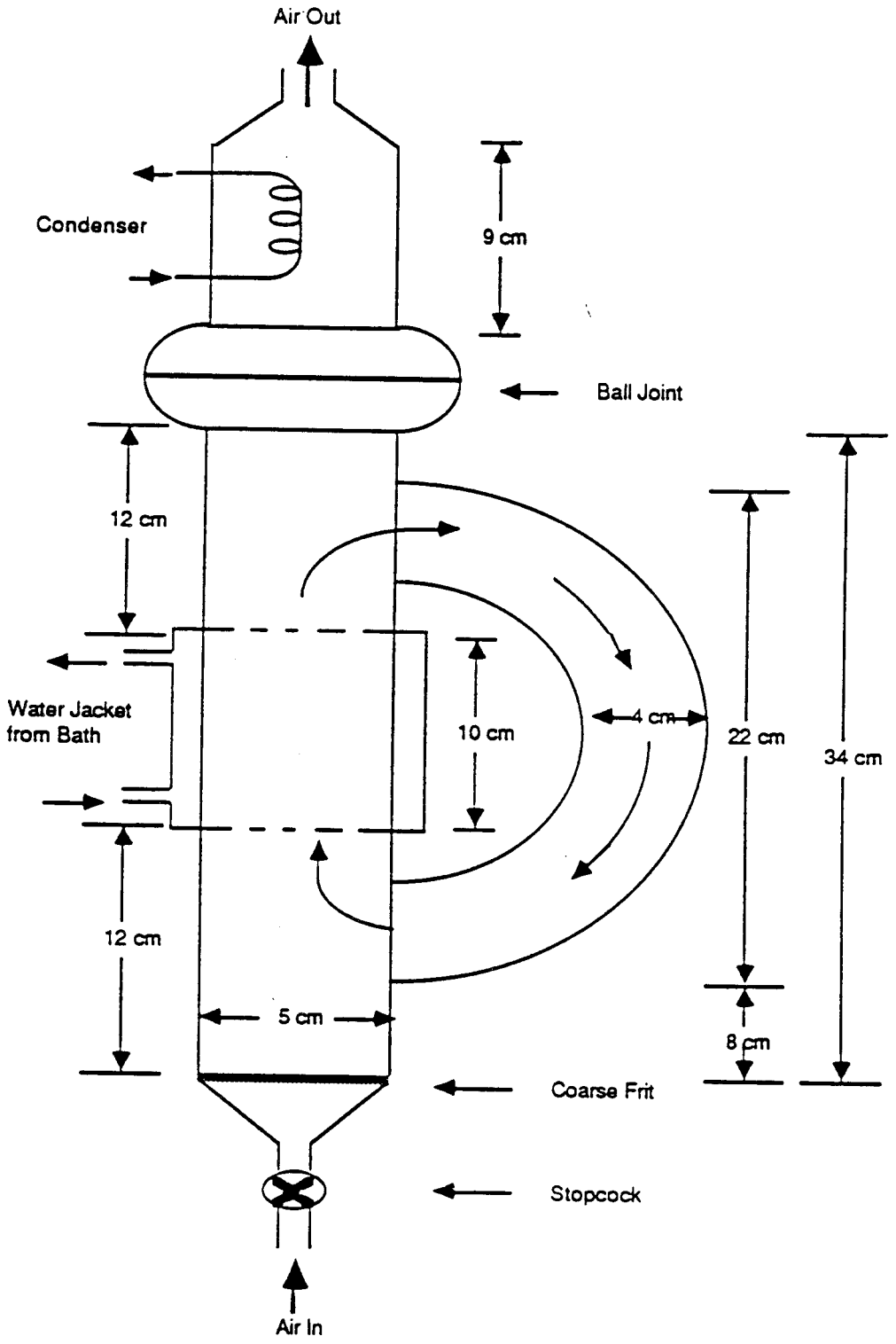


Figure 13. Vederas Apparatus

At this time we also considered another system for the fermentation. A closed system using two ball joint flasks joined in series as shown in Figure 14 was

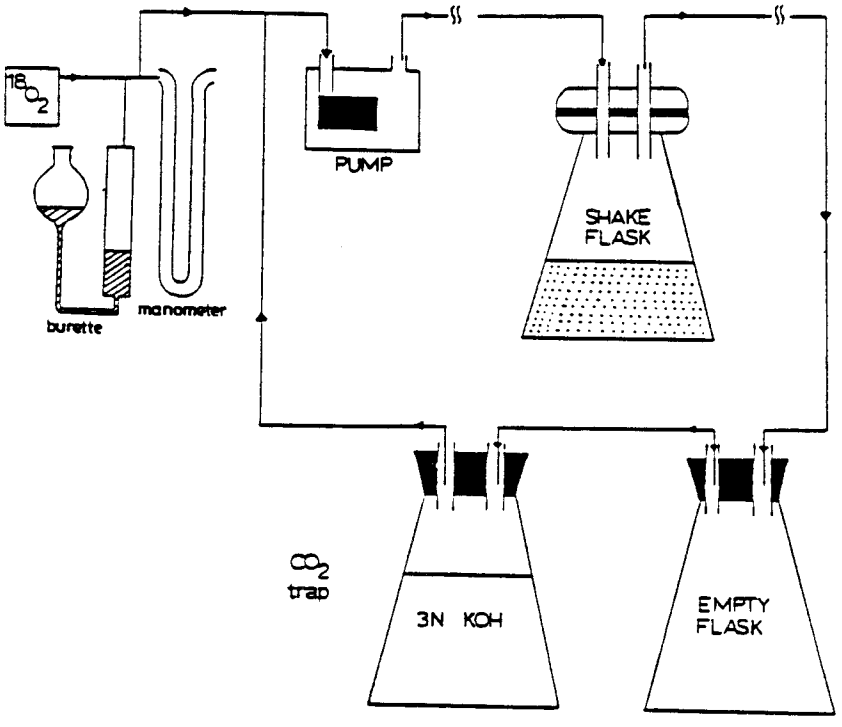


Figure 14. Ball Joint Fermentation Layout

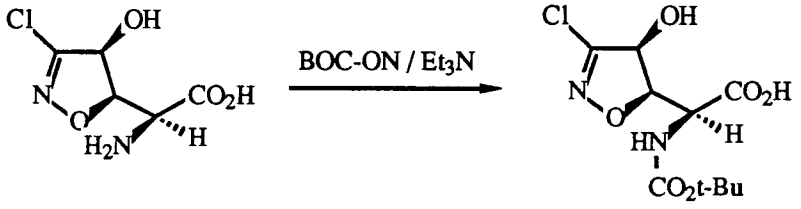
initially investigated. However, the production was always less than 1 μg/ml. Other unscucessful tactics included fluctuation of the gas flow rate (from 2 l/min to 0.5 l/min) and the addition of ornithine to the medium to check whether the production could be increased. However, none of these attempts gave satisfactory production; the production was always less than 1 μg/ml. Finally, 0.1 g of $NaHCO_3$ per 200 mL production medium was added as a CO_2 source.[91] After 120 h, the fermentation was terminated and the production was found to be 6 μg/ml based on bioassay results. In this case 3.5 l of oxygen was consumed from 48 h to 120 h after inoculation and the

fermentation broth was then purified using standard procedure. Work-up gave 6.9 mg of hydroxyacivicin, which was analyzed by ^{13}C NMR spectroscopy.

In view of this successful result, we then followed the above procedure for O-18 feeding experiments.

Oxygen-18 Fermentation

A fermentation using the original ball joint baffled flask[92] method was performed. For the initial 46 hours and final 45 hours, $^{16}O_2$ gas was used to minimize waste of $^{18}O_2$ gas. The standard work-up after 120 hours afforded 12.5 mg of 4-hydroxyacivicin **2f**. Analysis of the 100.6 MHz ^{13}C NMR spectrum of 10 mg of **2f** revealed that the C-4 oxygen was derived from $^{18}O_2$ (Figure 15). A 1.2 Hz upfield isotope shift for the ^{13}C-^{18}O was observed. However, the source of oxygen at C-3 could not be identified since the C-3 peak was too broad. This may have been due to the existence of various hydrogen-bonded forms in D_2O.[89a] Therefore the BOC-derivative of 4-hydroxyacivicin **73** (Scheme 27) was prepared for analysis



73

Scheme 27. Derivative of 4-Hydroxyacivicin

by mass spectroscopy.[93] The mass spectral (positive, FAB) data showed incorporation of more than one ^{18}O into **2e**. The ratio of the relative abundances (Table 7) of the ions at m/e 293, 295, 297, and 299 led to the conclusion that both of the oxygen atoms in **2e** were derived from $^{18}O_2$. The C-4 hydroxy group showed a 51% enrich-

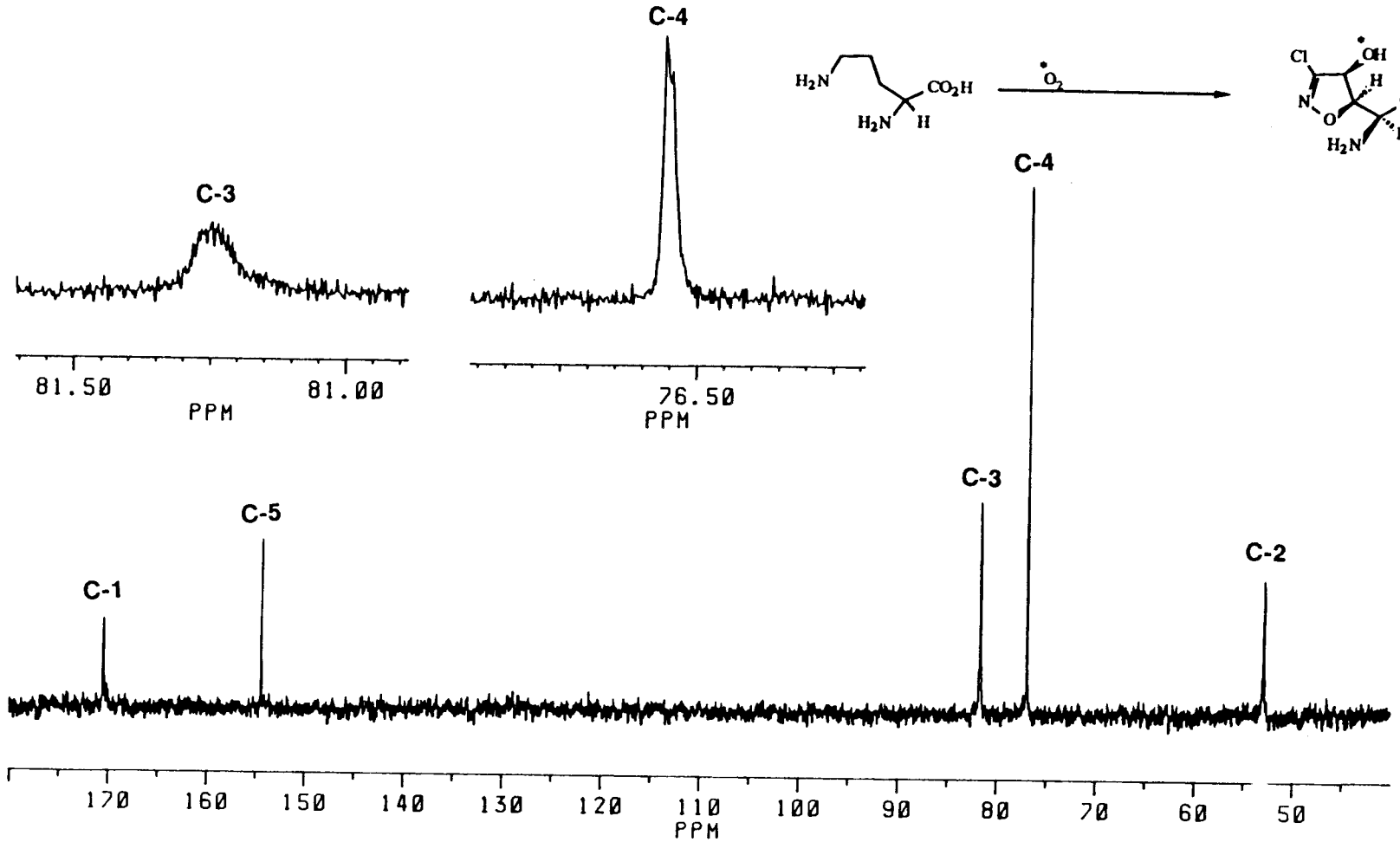
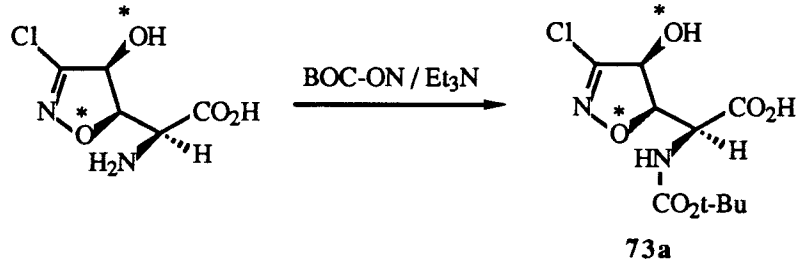


Figure 15. 100.06 MHz ^{13}C NMR Spectrum of 4-Hydroxyacivicin **2f** Produced under an $^{18}O_2$ Enriched Atmosphere

ment, while the other oxygen (C-3) in 4-hydroxyacivicin showed a 14% enrichment (Scheme 28).



73a

Scheme 28. Derivative of Oxygen-18 4-Hydroxyacivicin

Table 7. Relative abundance ratios for t-butyloxycarbonyl 4-hydroxyacivicin

Sample	MS	ions	ratios
unlabeled **2**	FAB (positive)	293:295:297:299	100:32.7:0.3:0.1
labeled **2f**	FAB (positive)		100:84.6:24.8:2.9

Based on the above findings and Scheme 26, five key intermediates could be proposed as Figure 16 shows. They are E-β-hydroxyornithine, **74**, T-β-hydroxy-ornithine, **75**, N-hydroxyornithine, **69**, N-hydroxy E-β-hydroxyornithine, **70**, and N-hydroxy T-β-hydroxyornithine, **75**. The identity of naturally occurring β-hydroxy amino acids was discussed in the Introduction.

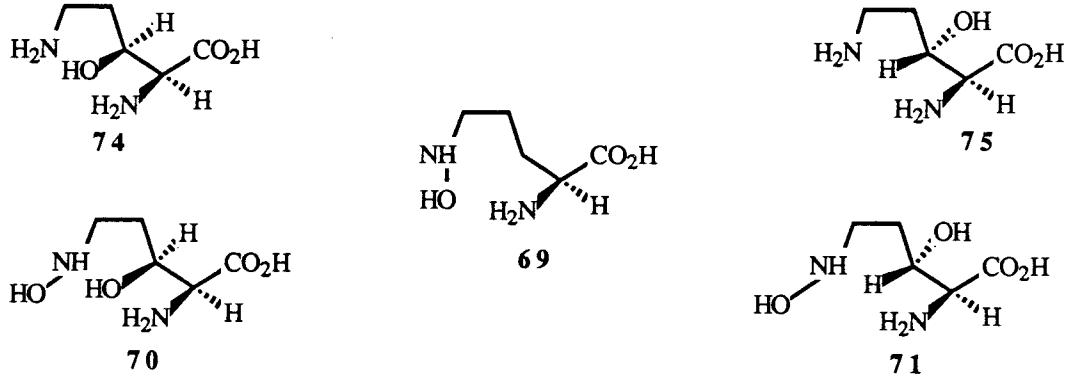


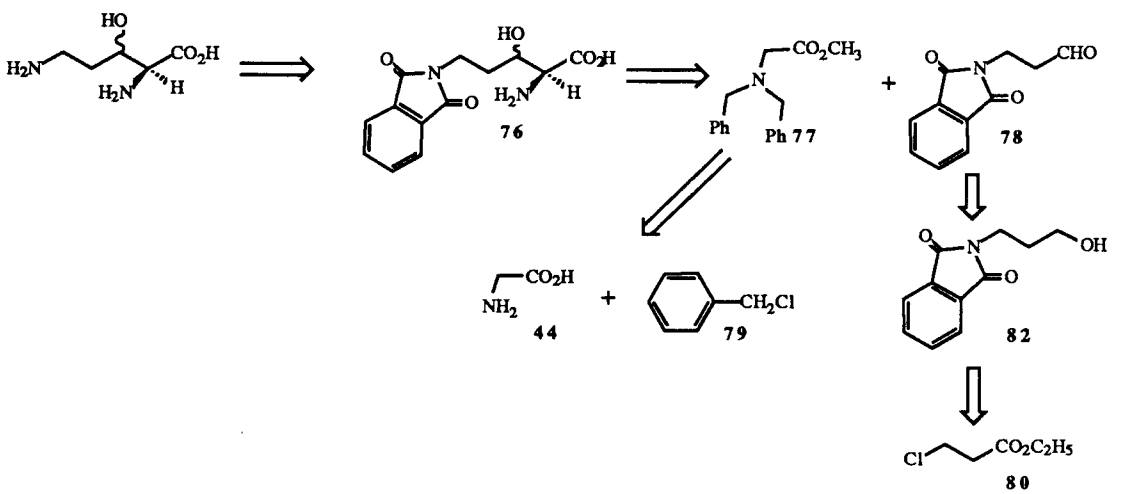
Figure 16. Structures of Possible Intermediates for Acivicin and 4-Hydroxyacivicin

Synthesis of the Erythro- and Threo-β-Hydroxy-L-[4,4-$^2H_2$]Ornithine

The objective of this synthesis was to identify the key intermediates in the biosynthesis of acivicin, **1**, and 4-hydroxyacivicin, **2**. The first question addressed was whether **1** and **2** were coming from E-β-hydroxyornithine, **74**, or from T-β-hydroxyornithine, **75**, in the advanced stages. Hence, these compounds were our synthetic targets.

Approach 1: The erythro- and threo-β-hydroxy-DL-ornithines, **74** and **75**, have been synthesized twice previously via the glycine enolate methodology.[94] However, these syntheses were either lengthy or not amenable to the introduction of isotope labels. Hence, a synthetic strategy was designed that would allow the preparation of properly labeled **74** and **75**, as shown in Scheme 29.

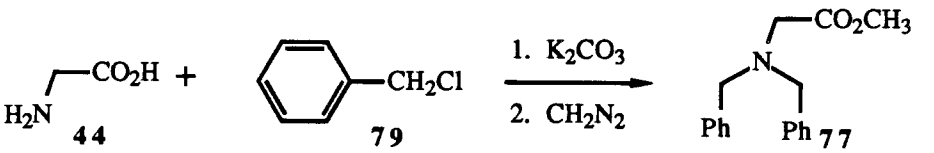
Here it was hoped that phthalimido adduct **76** could be formed by an aldol condensation[95] of **77** and **78**.



Scheme 29. β-Hydroxy-Ornithine Retrosynthetic Analysis

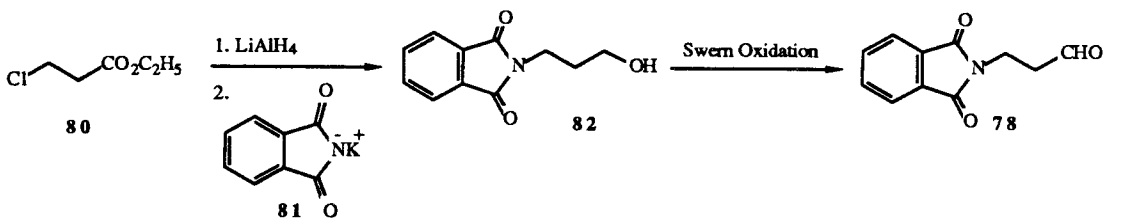
Methyl N,N-dibenzyl glycinate,[96] **77**, was synthesized in approximately 63.4% yield by reaction of commercially available glycine, **44**, with three

equivalents of benzyl chloride, **79**, followed by methylation with diazomethane as shown in Scheme 30.



Scheme 30. Synthesis of N,N-Dibenzyl Methyl Glycinate

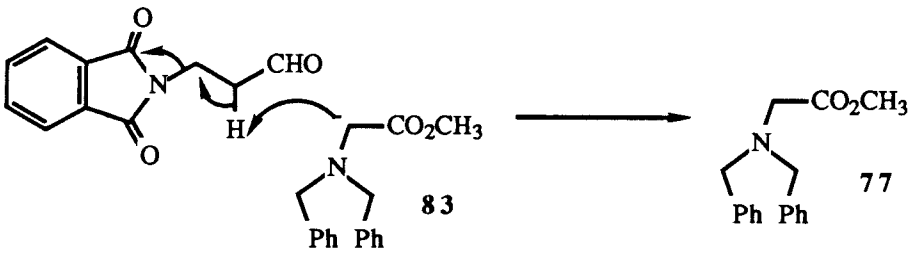
Aldehyde **78** was readily prepared from ethyl 3-chloropropionate, **80**, in three steps as shown in Scheme 31. Reduction of the ester **80** using $LiAlH_4$ followed



Scheme 31. Synthesis of N-(3-Propanol)phthalimide

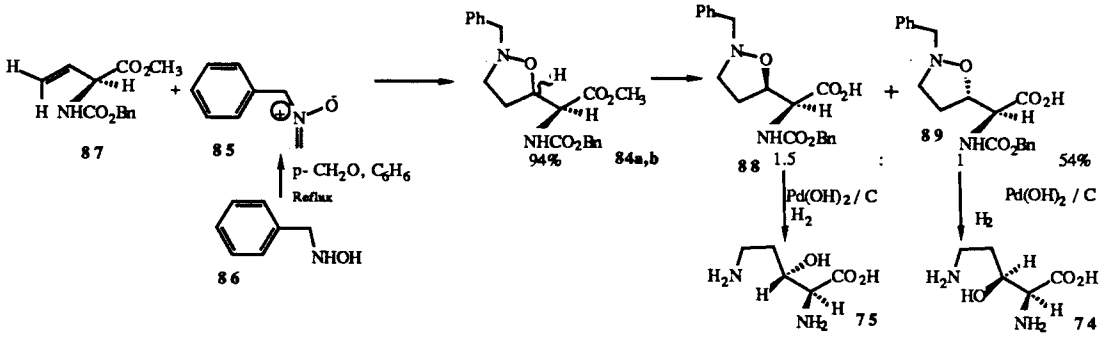
by reaction with potassium phthalimide, **81**, gave phthalimidopropanol, **82**, in 40% yield. Employing the procedure of Swern et al,[97] the alcohol **82** was quantitatively oxidized to aldehyde **78**.

Having the aldehyde **78** and methyl glycinate **77** at hand, the aldol condensation coupling reaction was attempted. Unfortunately, none of the combinations tried afforded the desired products. These unsuccessful conditions included use of bases such as $NaOCH_3$ and LDA, but these gave only recovered starting glycinate. This was probably arising from elimination of aldehyde **78** by anion **83**, as shown in Scheme 32.



Scheme 32. Possible Mechanism of Elimination of Aldehyde

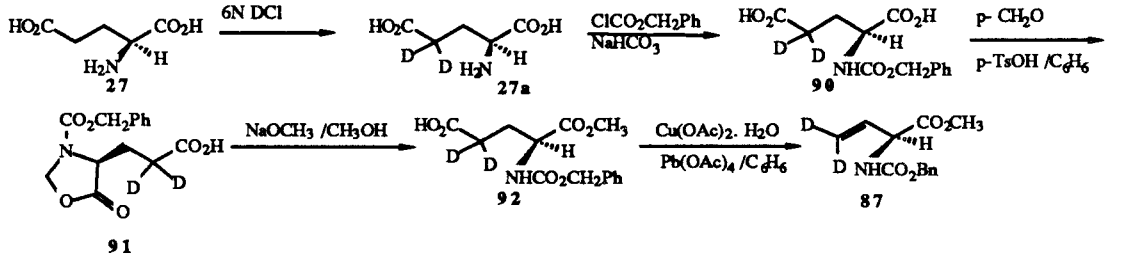
Approach 2: It was then decided to carry out the synthesis by adapting methodology previously developed in our laboratory.[98] This is shown in Scheme 33.



Scheme 33. Synthesis of T-β-Hydroxyornithine and E-β-Hydroxyornithine

As envisioned, the desired erythro- and threo-β-hydroxy ornithines, **74** and **75**, were obtained via **84a,b**, as a result of a 1,3-dipolar cycloaddition of in situ generated nitrone, **85** (from **86** with p-CH_2O), with 2(*S*)-vinylglycine[99] **87** in 93%. Saponification of the esters **84a,b** under the conditions specified by Corey[100] afforded the carboxylic acid, **88** and **89** in 54% yield after purification by flash chromatography. Reduction of **88** using Pearlman's catalyst gave threo-β-hydroxyornithine **75** in quantitative yield. Reduction of **89** similarly afforded the erythro-β-hydroxyornithine **74** in 78% yield.

Vinylglycine, **87a**, was prepared by the method of Hanessian and, as shown in Scheme 34, deuterium was introduced by exchange of glutamic acid.



Scheme 34. Synthesis of Vinylglycine Derivative

Protection of L-glutamic acid, **27**, as its Cbz derivative **90** was done by treatment with 1.1 eq benzyl chloroformate under basic conditions and this gave a 76% yield. Subsequently, the reaction of glutamic acid derivative **90** with formaldehyde in acidic conditions resulted in a 92% yield of oxazolidinone **91**. Saponification of **91** with sodium methoxide afforded an 85% yield of glutamic acid derivative **92**. Difficulties were encountered with this route in the next steps, as the reported yield could not be obtained in the crucial decarboxylation step. The reported yield was 60%. However, yields of 35-40% were more typically observed for the production of **87**.

Following the above procedure, L-[4,4-$^2H_2$]glutamic acid, **27a**, was converted into erythro-β-hydroxy-[4,4-$^2H_2$]ornithine, **74a**, and threo-β-hydroxy-[4,4-$^2H_2$]ornithine, **75a**.

L-[4,4-$^2H_2$]glutamic acid, **27a**, was prepared from L-glutamic acid, **27**, by the acid catalyzed exchange of γ-hydrogen using 6N DCl at elevated temperature in a seal tube.[101] It was necessary to replace the used 6N DCl with a fresh batch twice in order to maximize the deuterium enrichment. The deuterium enrichment was found to be 95% by ^1H NMR integration.

Feeding of E-β-Hydroxy-L-[4,4-$^2H_2$]Ornithine 74a and T-β-Hydroxy-L-[4,4-$^2H_2$]Ornithine 75a

Separate feedings of E-β-hydroxy-L-[4,4-$^2H_2$]ornithine, **74a**, and T-β-hydroxy-L-[4,4-$^2H_2$]ornithine, **75a**, were carried out. A feeding was carried out using 51.6 mg of **75a** along with 24.0 x 10^6 DPM of DL-[5-^{14}C]ornithine as a control. The resulting acivicin, **1g**, and 4-hydroxyacivicin, **2g**, showed percent incorporations of radioactivity of 0.23 and 3.6 respectively, based on the DL-[5-^{14}C]ornithine fed. This feeding experiment afforded 27.8 mg of hydroxyacivicin and 6.7 mg of acivicin. The ^2H NMR spectra of **1g** and **2g** indicated no enrichment of deuterium (Figure 17).

Another feeding was carried out using 50 mg of **74a** along with 11.66 x 10^6 DPM of DL-[5-^{14}C]ornithine as a control. The resulting acivicin, **1h**, and 4-hydroxyacivicin, **2h**, were purified by the standard procedure and showed a 5.6% incorporation of radioactivity for **2h** and a 0.50% incorporation of radioactivity for **1h**. This feeding experiment afforded 19.4 mg of 4-hydroxyacivicin and 4.3 mg of acivicin. However, on analysis of the ^2H NMR spectra (Figure 18), again no enrichment of deuterium was seen.

Feeding of DL-[3,3,4,4-$^2H_4$]ornithine, 56e[102]

The experiments mentioned so far indicated clearly that L-[4,4-$^2H_2$]-β-hydroxy ornithine was not incorporated into acivicin or 4-hydroxyacivicin during the biosynthetic process. However, some questions remained to be answered before reaching such a final conclusion. One question was whether the deuterium labeling in E-β-hydroxyornithine **74a** and/or T-β-hydroxyornithine **75a** was lost inside the cell by way of exchange,[103] such as that shown in Scheme 35.

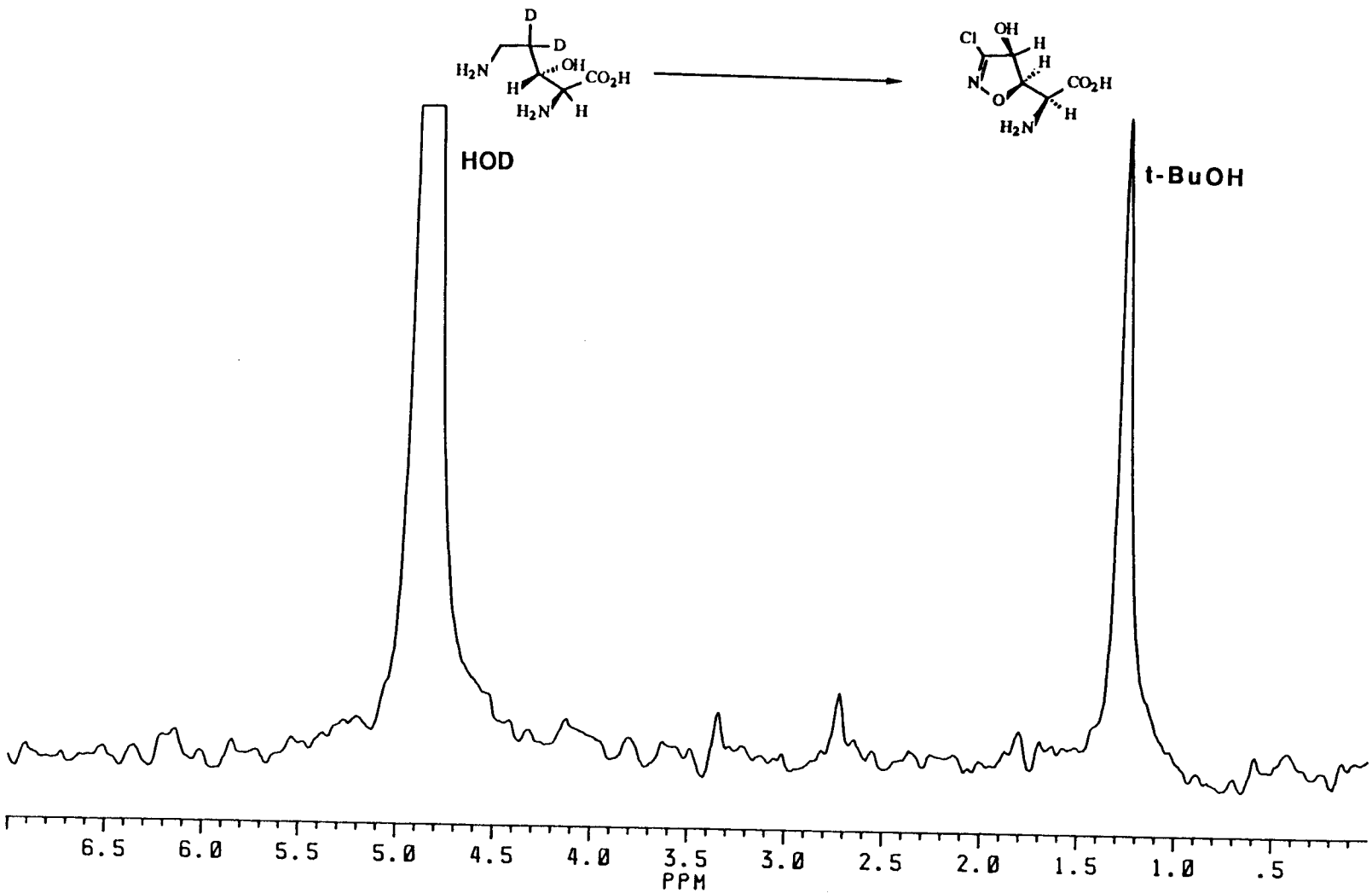


Figure 17. ^{2}H NMR of 4-Hydroxyacivicin **2g** from the Feeding of **75a**

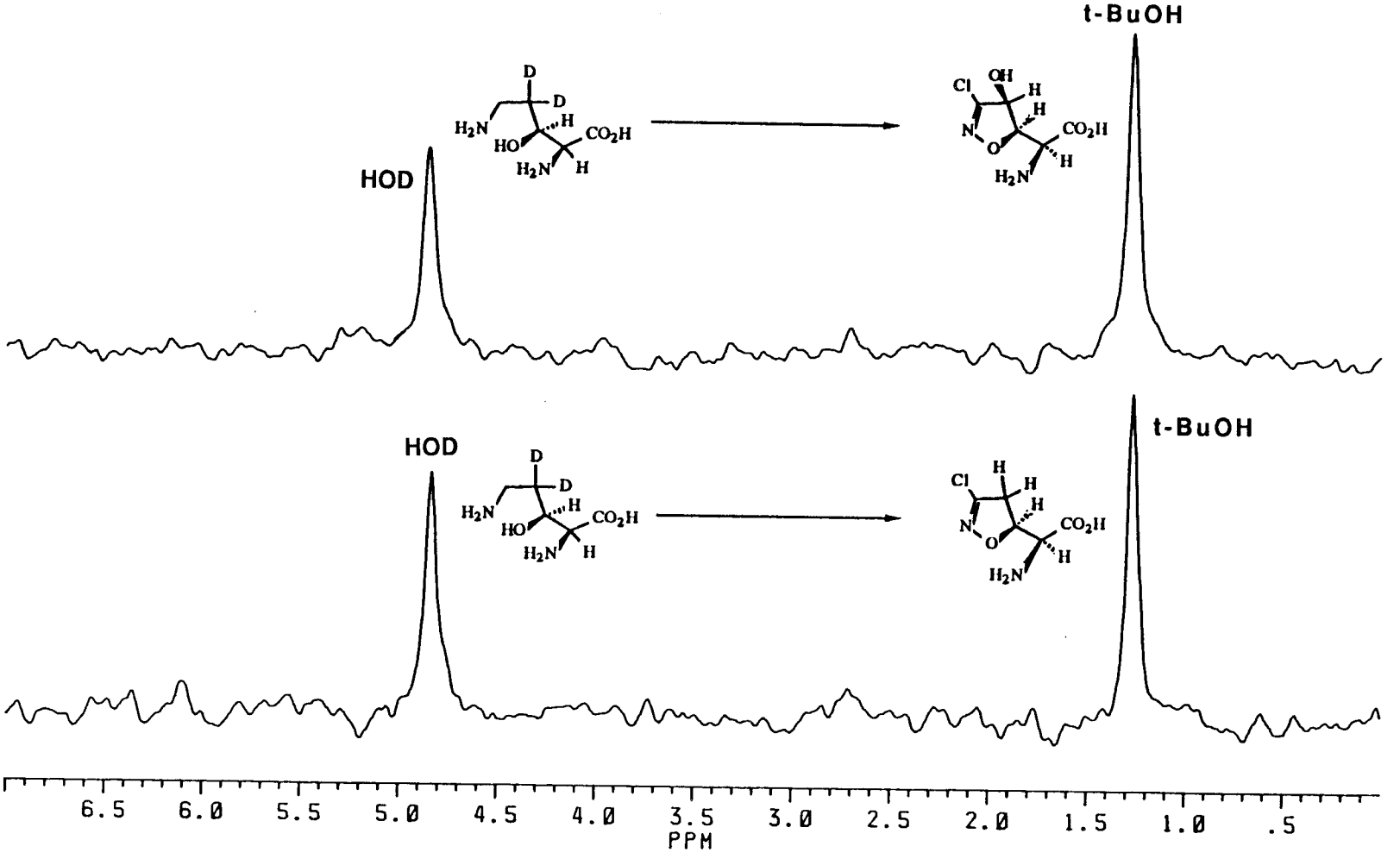
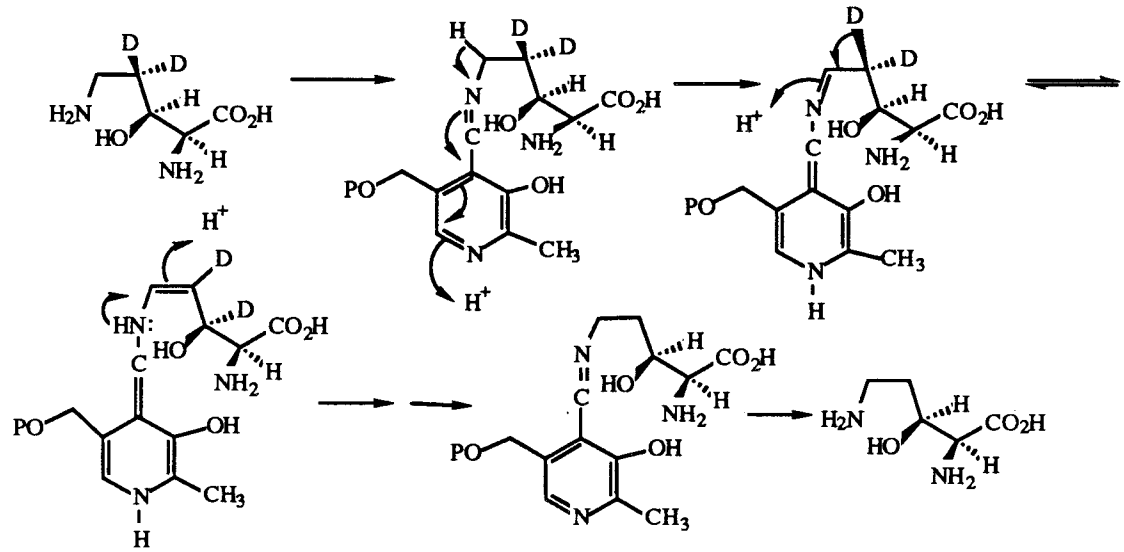


Figure 18. ^2H NMR of 4-Hydroxyacivicin **2 h** and Acivicin **1 h** from the Feeding of **7 4 a**



Scheme 35. Proposal for the Mechanism of Exchange Reaction

To test this, a feeding was carried out using 75 mg of DL-[3,3,4,4-$^2H_4$]ornithine[102] **56e** along with 69.56 x 10^6 DPM of DL-[5-^{14}C]ornithine. The resulting acivicin, **1i**, and 4-hydroxyacivicin, **2i**, showed a 0.62% incorporation of radioactivity for **2i** and a 0.47% incorporation of radioactivity for **1i**. This feeding experiment afforded 23.5 mg of **2i** and 4.3 mg of **1i**. Upon analysis of ^{2}H NMR spectrum (Figure 19), **1i** showed a peak at 5.2 ppm (H-3) and another at 3.4 ppm (H-4). By comparing the predicted ^{2}H content (0.32 µmole at H-3 and 0.14 µmole at H-4), based on ^{14}C incorporation, to that obtained from integration of the NMR signals, 0.118 µmole at H-3 and 0.115 µmole at H-4, it was found that approximately all of the C-4 deuterium had remained intact without loss, and 37% of deuterium at H-3 was retained.

The ^{2}H NMR spectrum of **2i** (Figure 19a) gave a similar result. This was obtained by comparing the ^{2}H content (0.49 µmole at H-3 and 0.23 µmole at H-4), based on ^{14}C incorporation to that obtained from integration, 0.23 µmole at H-3 and 0.23 µmole at H-4. It was found that approximately one of the C-4 deuterium labels had remained intact and 46% of deuterium at H-3 was retained.

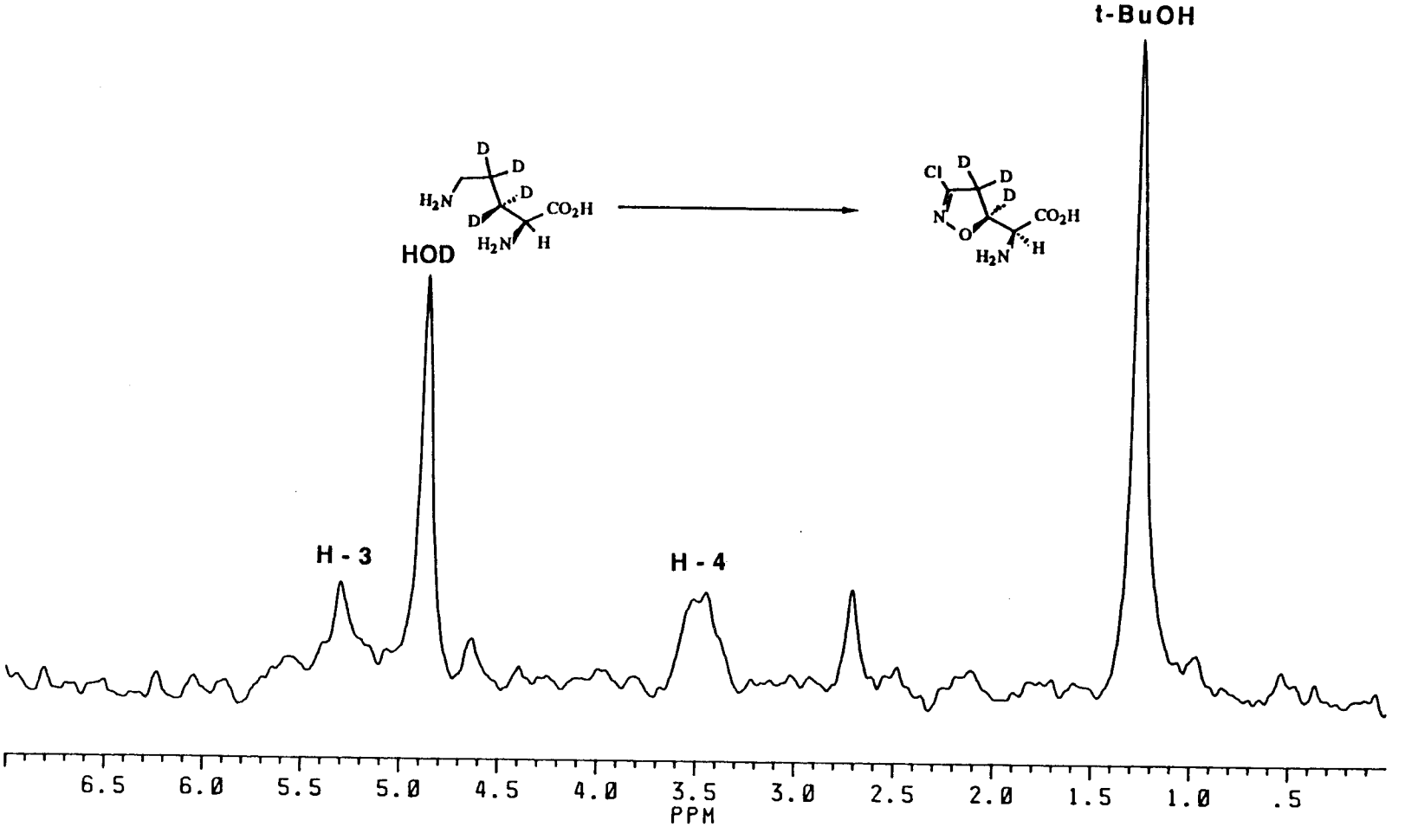


Figure 19. ^{2}H NMR of Acivicin **1 l** from the Feeding of **56e**

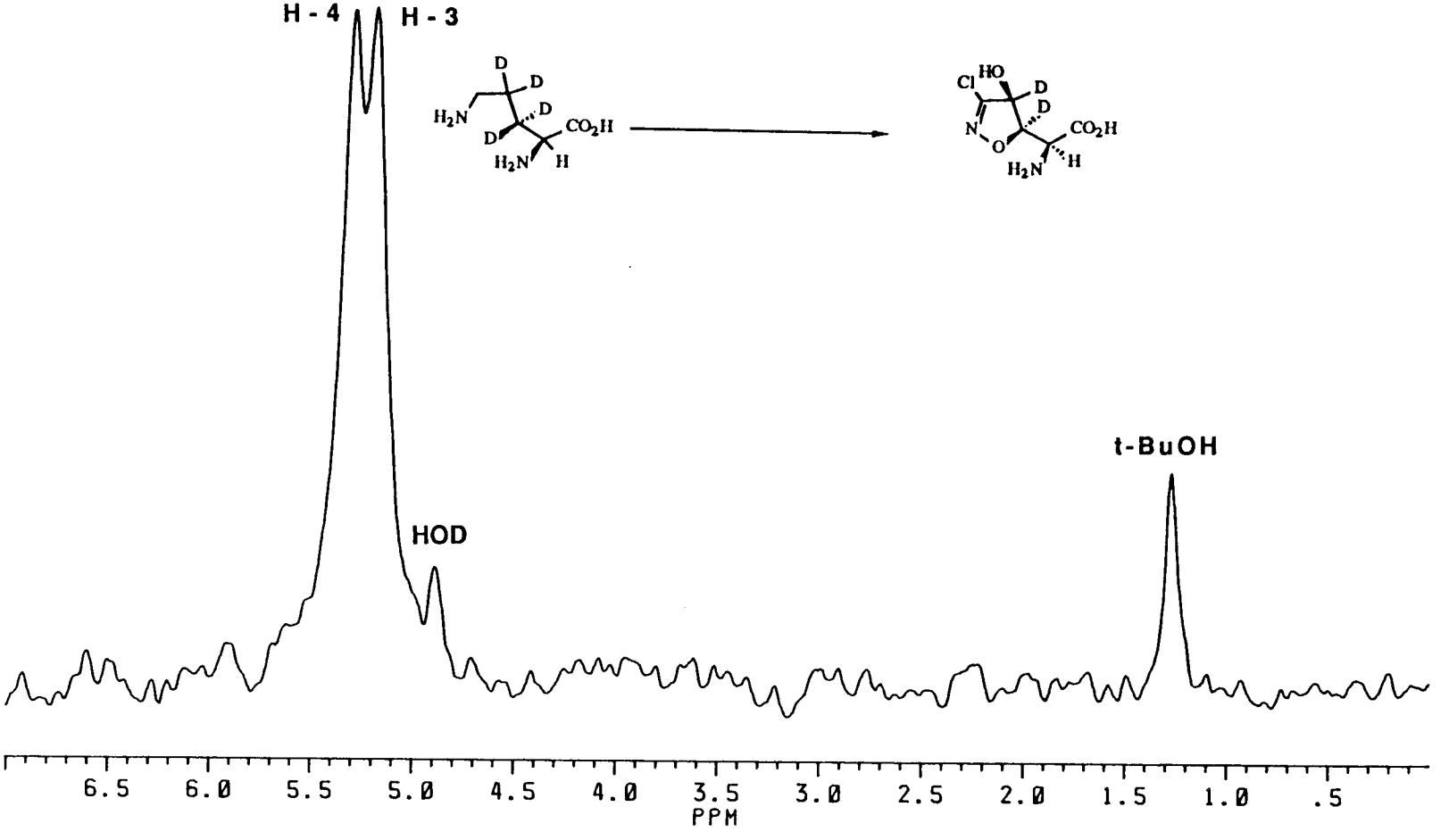


Figure 19a. ^{2}H NMR of 4-Hydroxyacivicin **2l** from the Feeding of **56e**

Erythro and Threo-β-hydroxy-L-ornithine Isotope Trapping Experiments

Preliminary considerations. One question remained to be answered before reaching a final conclusion. This was whether the deuterium labeled E-β-hydroxyornithine **74a** and T-β-hydroxyornithine **75a** might not be able to enter the cell, even though one of these (in non-labeled form) might be formed by the organism. To understand this, an isotopic trapping experiment was used.

In preparing for the trapping attempts, it was clear that a procedure for the recovery of β-hydroxyornithine from the fermentation mixture needed to be developed. A small (100 mL) production fermentation was harvested after 72 hours. Following sonication and filtration, β-hydroxyornithine (11 mg) was added and the filtrate passed through a column of Dowex 50Wx4. The bound materials were washed with deionized water and then displaced using a 2N HCl gradient. This afforded a mixture of various components. Using analytical tlc (n-BuOH/H_2O/AcOH 3:1:1 on cellulose), and developing the chromatogram three times in succession, it was found that β-hydroxyornithine could be separated from the mixture.

The production of acivicin started approximately 48 hours after inoculation, and reached a maximum concentration at 120 hours. Assuming a biological pool of relatively small size, it was reasoned that if β-hydroxyornithine was produced, its synthesis must begin early and continue well into the fermentation. It was decided on this basis to administer DL-[5-^{14}C]ornithine at an early stage of the fermentation and to work up the fermentation at different times.

Erythro and Threo-β-hydroxy-L-ornithine Isotope Trapping Experiments

In the first experiment to trap the erythro diastereomer, 25.74 x 10^6 DPM, DL-[5-^{14}C]ornithine was administered after 48 hours and the isolation began 15 hours later with the addition of 11.4 mg of authentic **74**. Using the isolation protocol just described, crude E-β-hydroxyornithine **74** was recovered. This was then diluted with an additional 31.4 mg of pure **74** and recrystallized repeatedly. No radioactivity was found in this material.

A similar experiment was conducted to investigate the possible intermediacy of T-β-hydroxyornithine **75**. Much like the previous experiment, 24.64 x 10^6 of DL-[5-^{14}C]ornithine was fed to a 100 mL fermentation broth. Using the standard isolation procedure, T-β-hydroxyornithine **75** was then obtained in which less than 0.04% of the original radioactivity was retained after five recrystallizations.

In the second experiment, DL-[5-^{14}C] ornithine (62.12 x 10^6 DPM) was added to a 200 mL fermentation of *S. sviceus* at 55 h. Three hours later, the broth was divided in two. One half received a small quantity (11.4 mg) of T-β-hydroxy-ornithine, **75**, and the other received a small quantity (11.4 mg) of E-β-hydroxy-ornithine, **74**. After cell disruption by sonication to release any endogenous labeled isomer and centrifugation to remove the solid, each supernatant was purified as usual. The crude T-β-hydroxyornithine was diluted with an additional 46 mg of **75** and recrystallized repeatedly. The resulting T-β-hydroxyornithine had retained less than 0.03% of the radioactivity (after seven recrystallizations). The crude E-β-hydroxyornithine was diluted with an additional 31.4 mg of **74** and recrystallized for five times. However, further recrystallization could not be done due to the very small quantity of E-β-hydroxyornithine remaining. The percentage activity remaining decreased in every recrystallization and hence no positive results could be

derived from this experiment but 0.1% still remained after the fifth recrystallization. Therefore, this experiment was repeated using a larger quantity of carrier (49.3 mg) of **74**. Thus, DL-[5-^{14}C]ornithine (19.6 x 10^6 DPM) was fed to a 200 mL fermentation of *S. sviceus* at 55 h. Three hours later, the fermentation was stopped. A similar experiment shown as above was then carried out. After seven recrystallizations the recovered E-β-hydroxyornithine had retained less than 0.03% of the radioactivity that had been fed. Because the levels of radioactivity obtained were close to background, it was unlikely that either diastereomer had been formed by the organism.

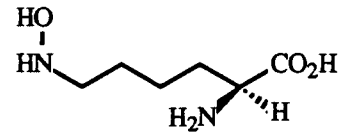
Nonetheless, at this time it was considered whether the timing for harvesting was important or not. To test this, a feeding experiment by using 39.23 x 10^6 DPM DL-[5-^{14}C]ornithine was carried out. This was fed at 55 hours. Six hours later, the broth was divided in two. One half received a small quantity (12.5 mg) of **74** and the other a small quantity (12.6 mg) of **75**. The hydroxyornithines were isolated in the usual way. The experimental data showed that the radioactivity gradually decreased and again approached zero (<0.03% of the specific activity of that fed). This led us to conclude that neither E-β-hydroxyornithine nor T-β-hydroxyornithine was a key intermediate to acivicin and 4-hydroxyacivicin.

We next considered the possibility that δ-N-hydroxyornithine was the first intermediate.

Naturally Occurring N-Hydroxy Amino Acids and Their Biosynthesis

Although free N-hydroxy amino acids have not been found in living cells, these compounds have been isolated from parent hydroxamics, N-acyl derivatives of N-hydroxyamino acids, after hydrolysis. Two of the most frequently isolated

N-hydroxy amino acids are δ-N-hydroxyornithine, **69**, and ε-N-hydroxylysine, **93** (Figure 20). Table 8 lists the natural products which include N-hydroxyornithine.



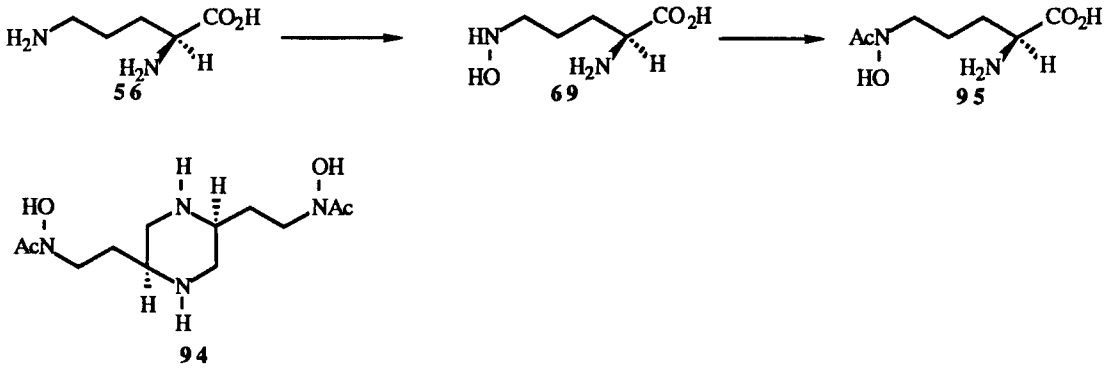
93

Figure 20. Structure of ε-N-Hydrolysine

Table 8. δ-N-Hydroxyornithine containing natural products

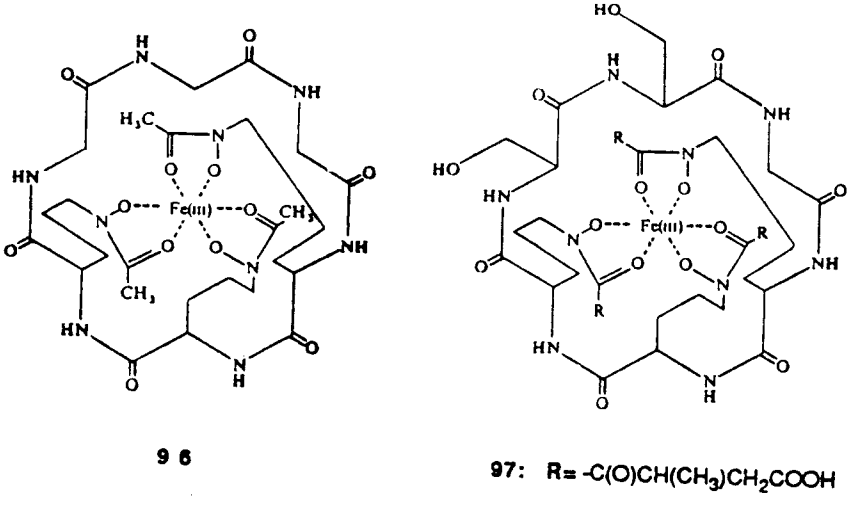
Name	Organism	Reference
Fusarinine A, B	*Fusarium roseum*	104
Fusarinine	*Fusarium* sp.	105
Rodotorulic acid	*Rhodotorula pilimanae*	104, 106
Dimeric acid	*Fusarium dimerum*	107
Ferrichrome A	*Ustilago sphaerogena*	108
Fusigen	*Penicillium variable*	109
Albomycin	*Actinomyces subtropicus*	110, 111
Ferrichrocin	*Aspergillus mellena*	112
Coprogen	*Neurospora Crassa*	113
DDF	*Aspergillus ochraceous*	114
Neocoprogen	*Curvularia lunata*	115

In 1972, Neiland's group[116] indicated that biosynthesis of rodotorulic acid, **94**, was coming from **56** and δ-N-acyl-L-δ-N-hydroxyornithine, **95**, as shown in Scheme 36. Later, Emery[108] found that the biosynthesis of ferrichrome, **96**, and



Scheme 36. Biosynthesis of Rhodotorulic Acid

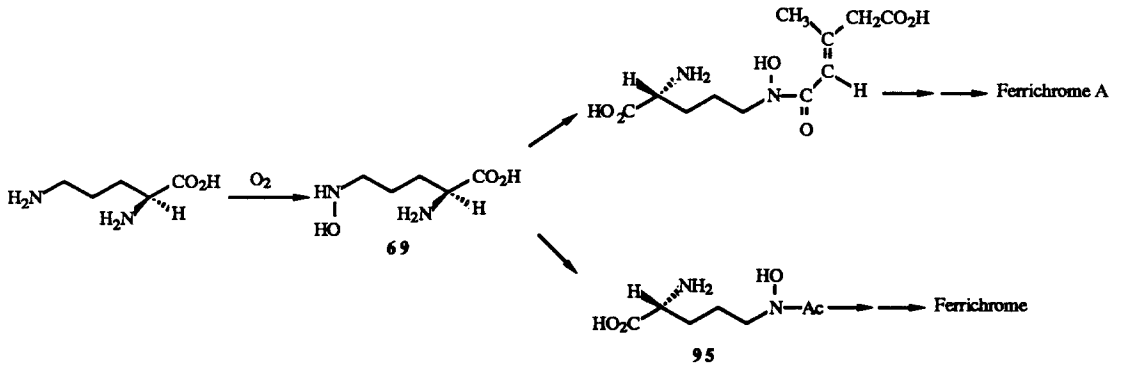
ferrichrome A, **97** (Figure 21) was coming from **69** formed in *Ustilago sphaerogena* (Scheme 37).



96

97: R= -C(O)CH(CH$_3$)CH$_2$COOH

Figure 21. Ferrichrome and Ferrichrome A



Scheme 37. Biosynthesis of Ferrichrome and Ferrichrome A

ε-N-Hydroxylycine, **93**, has been found only in aerobactin,[117,118] mycobactin,[119] and cobactin.[120]

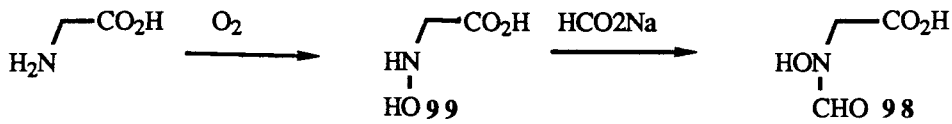
α-N-Hydroxy amino acids appear very rarely and, until now, the presence of only a few α-N-hydroxy amino acids in nature has been confirmed. Table 9 lists the α-N-hydroxy amino acids known today.

Table 9. List of α-N-hydroxyamino Acids

Name	Comments	Reference
α-N-hydroxyglycine	Constituent of hadacidin	121
α-N-hydroxytyrosine	Constituent of mycelianamide	122
α-N-hydroxyalanine	Constituent of mycelianamide	122
α-N-hydroxyisoleucine	Constituent of aspergillic acid	123
α-N-hydroxyleucine	Constituent of pulcherrimin	124
α-N-hydroxyaspartic acid	Constituent of peptide	125
α-N-hydroxyserine	Constituent of azinothricin	126

The primary precursors of hadacidin, **98**, were well-established in 1966. Studies utilizing [^{14}C]-glycine and [^{14}C]-sodium formate revealed these compounds to be excellent precursors. Also, they found that the α-N-hydroxyglycine moiety, **99**, of **98** was biosynthesized from α-N-hydroxyglycine **99**. Experiments with

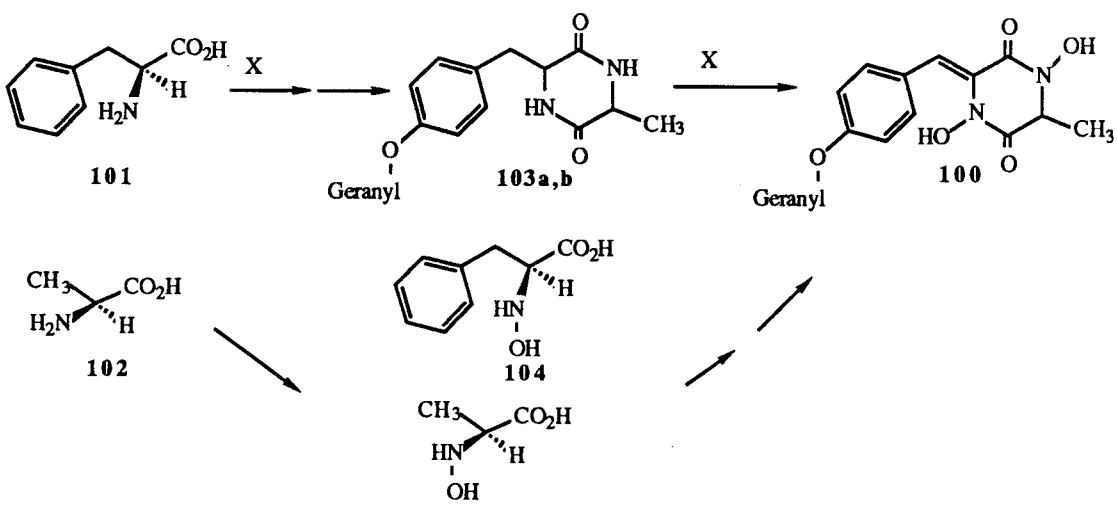
molecular oxygen showed that the hydroxyamino oxygen atom of hadacidin was derived from molecular oxygen[127] as shown in Scheme 38. This was done by analyzing the



Scheme 38. Biosynthesis of Hadacidin

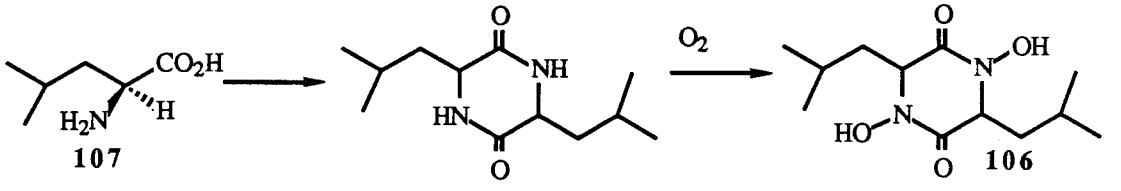
distillate ($H_2{}^{18}O$) which came from reducing oxygen-18 labeled hadacidin, and formylglycine with mass spectroscopy.

Mycelianamide, **100**, is a diketopiperazine formed from tyrosine, **101**, and alanine, **102**. However, the appropriate radioactivity labeled diketopiperazines, cyclo-(L-alanyl-L-tyrosyl) and cyclo-(L-alanyl-d-tyrosyl), **103a,b** failed to act as precursors. This suggested that either the diketopiperazine was formed from α-N-hydroxyamino acids, **104** and **105**, or that the diketopiperazine formed remained enzyme-bound until after further reaction, with no equilibrium occurring with unbound (radioactive) diketopiperazine[128] (Scheme 39).



Scheme 39. Biosynthesis of Mycelianamide

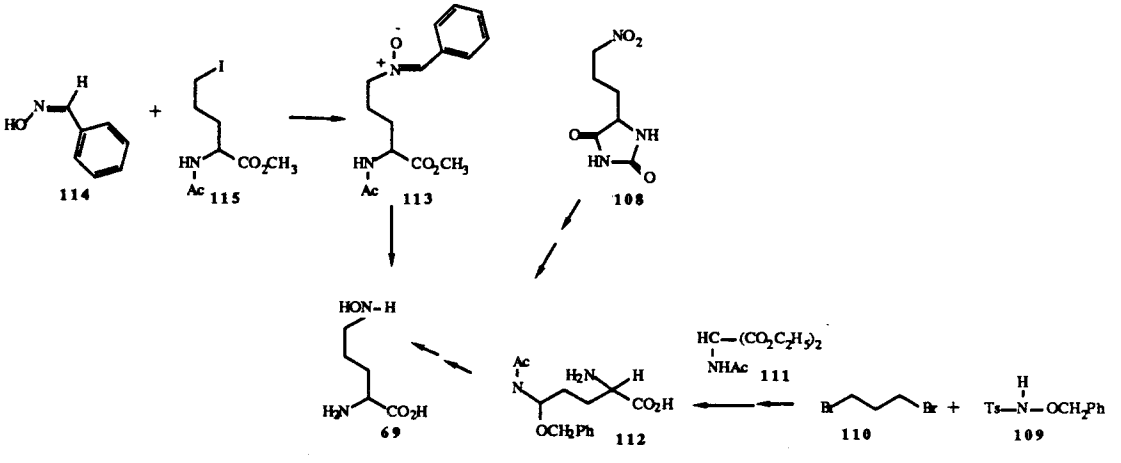
Pulcheriminic acid,[129a] **106**, is, like mycelianamide, **100**, an N,N-dihydroxy diketopiperazine. It has been concluded that it is derived by the hydroxylation of diketopiperazine,[129b] formed from two molecules of leucine, **107** (Scheme 40).



Scheme 40. Biosynthesis of Pulcheriminic Acid

Synthesis of DL-[3,3,4,4-$^2H_4$]-δ-N-hydroxyornithine, 69

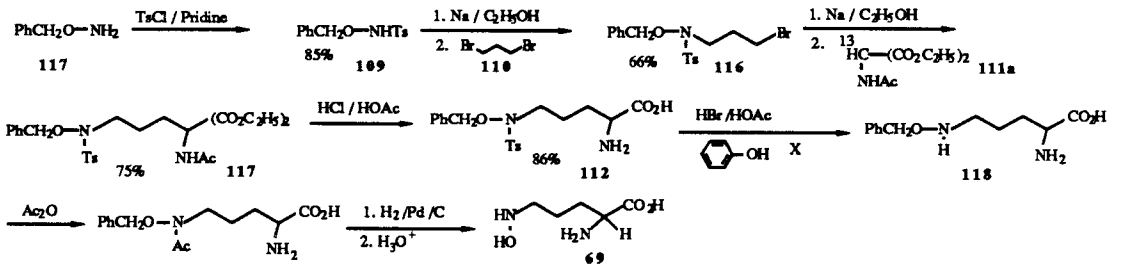
δ-N-hydroxyornithine **69** has been synthesized three times previously.[130] The first reported approach,[130c], based on partial reduction of **108** followed by hydrolysis, gave racemic N-hydroxyornithine **69** in very low overall yield. Alternatively, alkylation of O-benzyl-N-tosyl hydroxylamine[130a] **109** with 1,3-dibromopropane, **110** followed by condensation with diethyl acetamidomalonate, **111**, hydrolysis, decarboxylation, detosylation, and acetylation furnished racemic



Scheme 41. Previous Syntheses of N-Hydroxyornithine

112. This served as a substrate for hydrogenation and eventually afforded N-hydroxyornithine **69**. The third synthesis of N-hydroxyornithine **69**[130b] involved the hydrolysis of nitrone **113**, prepared by N-alkylation of anti-benzaldoxime **114** with methyl 2-acetamido-5-iodovalerate, **115**, as shown in Scheme 41.

We decided to carry out the synthesis based on one of the literature procedures[130a] to prepare N-hydroxyornithine **69** with some hope to get better than original yields of the steps involving the labeled compound (Scheme 42).



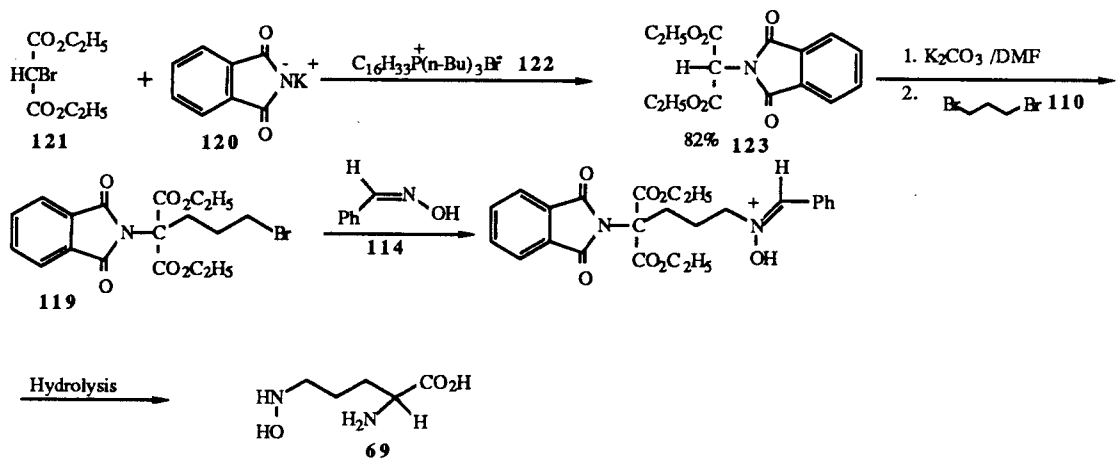
Scheme 42. Synthesis of N-Hydroxyornithine

Our plan was to introduce the label at the stage of alkylation of intermediate **116** using [2-^{13}C]-acetamidomalonate **111a**. O-Benzylhydroxyl amine **117** was first converted into the tosylate derivative **109** in 85% yield. Tosylate **109** was then alkylated with 1,3-dibromopropane, **110**, to γ-(N-tosyl-N-benzyloxy)-amino-propyl bromide, **116**. This was allowed to react with diethyl sodio-acetamido malonate, and the product **117** was heated with concentrated hydrochloric acid to yield racemic N$^{\delta}$-tosyl-N$^{\delta}$-benzyloxyornithine, **112**. The yields are shown in Scheme 42 The next objective to be addressed was the reductive-oxidative cleavage[131] of the tosyl group from the nitrogen. Treatment of **112** with 36% HBr in acetic acid in the presence of phenol at room temperature for 50 h gave none of the

desired product. Other detosylation conditions were tried but they did not afford the desired product, either. These unsuccessful conditions are listed below.

	Reaction Condition	Result
(a)	1 eq, 2.7 eq phenol in 36% HBr-HOAc, 35 °C, 50 h	starting material
(b)	1 eq, 2.7 eq phenol in 48% HBr, Δx, 6 h	ornithine
(c)	1 eq, 2.7 eq phenol in 48% HBr, RT, 52 h	starting material
(d)	1 eq, 2.9 eq phenol in 40% HBr-HOAc, rt, 52 h	starting material

Finally, this problem was solved by carrying out the reaction at 80 °C in 36% HBr-HOAc for 14 h. A 62% yield of **118** was obtained. However, these conditions could not be used in large scale reactions. When a scale of 0.5 g was used, the only product present by tlc was ornithine, **56**. Due to the many difficulties encountered, this route was abandoned. Hence, at this time we designed an alternate route (Scheme 43).



Scheme 43. An Approach to Synthesis of N-Hydroxyornithine

This route depended on the N-alkylation of antibenzaldoxime,[132,133] **114**, with the diethyl ester of (γ-bromopropyl)-N-phthalimidomalonic acid, **119**.

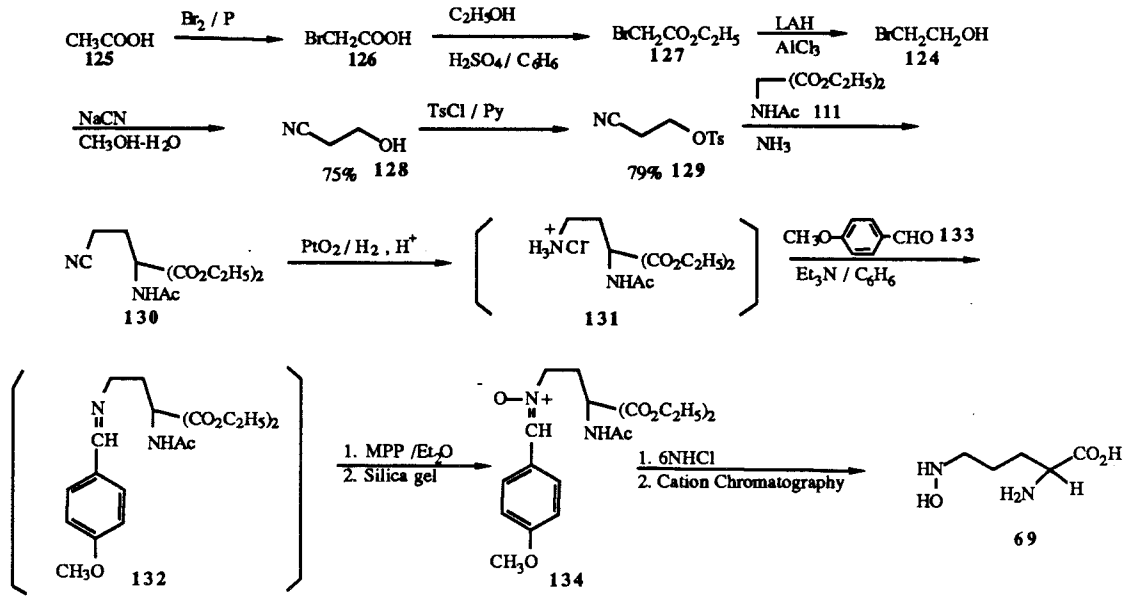
Potassium phthalimide, **120**, reacted with diethylbromomalonate, **121** in the presence of the phase transfer reagent hexadecyl tributyl phosphonium bromide, **122**,[134] to give **123** in 82% yield. Compound **119** was prepared by heating (85-95 °C), with continuous stirring, a mixture of **123**, anhydrous postassium carbonate and 1,3-dibromopropane, **110**, in DMF.[135] The desired product **119** was then obtained in 76% yield.

Having the compound **119** at hand, the nucleophilic coupling reaction was attempted. Unfortunately, difficulties were encountered with this route also. None of the combinations tried afforded useful products. These unsuccessful conditions are listed below.

Reaction Condition	Result
1 eq **119** in DMF, 1 eq **114**, in 1 eq $NaOC_2H_5/C_2H_5OH$[136]	no reaction
1 eq **119** in DMF, 1 eq **114**, in 1 eq $NaOC_2H_5/C_2H_5OH$, NaI (cat)	no reaction
1 eq **119** in DMF, 1 eq **114**, in 1 eq $NaOC_2H_5/C_2H_5OH$, 70 °C, 10 h	no reaction

At this time we designed an alternate route (Scheme 44) for the synthesis of **69**. This route involved the literature preparation of 2-bromo ethanol **124** from acetic acid[137], **125**. Bromoacetic acid, **126**, was prepared in the presence of red phosphorous in 88% yield, and was next subjected to esterification to give **127**, followed by reduction with LAH and aluminum chloride to afford **124** (50% overall yield from acetic acid). Subsequently, displacement of bromide with sodium cyanide[138] gave **128** in 74% yield. Tosylation of **128** gave **129** in 80% yield. Alkylation was then effected in ammonia with to obtain the cyano adduct **130** in 77% yield. The standard protocol[84a] of reduction of **130** involved the use of ethanol as solvent containing two equivalents of hydrochloric acid (to prevent catalyst poisoning

and promote formation of the primary amine). This gave a hygroscopic residue which was assumed to be the aminodiester hydrochloride **131**.



Scheme 44. Synthesis of N-Hydroxyornithine

Attempts to convert **131** to **132** with p-methoxybenzaldehyde **133** were tried and they included use of different solvents such as dichloromethane, chloroform, and ethanol. None of these methods, however, proved to give a good yield in this case, based on the tlc analysis.

Finally, this problem was solved by carrying out the reaction under the following conditions. Compound **131** was converted to Schiff base[139] **132** by the action of p-methoxybenzaldehyde **133** in benzene under basic conditions. The water was removed by an azeotropic method. However, the intermediate could not be isolated, either by using a chromatography or crystallization method. Therefore, Schiff base **132** was oxidized directly by the method of Polonski and Chimiak,[140] followed by isomerization with a silica gel column to give a mixture of compounds **134** and **133**. These were separated with preparative TLC to afford the desired N-

benzylidene-N-hydroxy ethyl ester **134** (32% overall yield from **130**). Hydrolysis of **134** in 6N HCl at reflux gave N-hydroxyornithine, **69**, in 61.4% yield. The product was purified by cation exchange chromatography using Dowex 50Wx4 (H^+) resin and compound **69** could not be cyclized in the acidic condition. This was mentioned by the Emery group.[108]

It was envisioned that use of acetic acid-d_4, **125a**, during the bromination would yield compound **126a**. This would then be converted to **124a** by esterification and reduction with lithium aluminum deuteride to afford **124a**.

Subsequent displacement of bromide with sodium cyanide in methanol and water would give **128a**. However, after following this protocol it was revealed by ^1H NMR spectroscopy that most of the deuterium label at C-2 of **128a** had been lost. The deuterium enrichment of **128a** was 100% and 10% at the β and α positions, respectively. Following the above procedure **130a** was converted into 2*RS*-δ-N-hydroxy[3,3,4,4-$^2H_4$]ornithine, **69a**.

Feeding of DL-δ-N-hydroxy[3,3,4,4-$^2H_4$]ornithine, 69a

To test whether **69** is the first intermediate in acivicin biosynthesis, a total of 114.92 mg (0.57 mmole) of **69a** was administered to a production broth in pulses at 48 h and 60 h after inoculation. After a total of 120 h, the fermentation was worked up using the standard procedure. This afforded 43.1 mg of 4-hydroxyacivicin **2j** and 8.0 mg of acivicin **1j**. The ^2H NMR spectrum of **1j** (6.32 mg) (Figure 22) showed resonances at δ 3.4 (H-4) and δ 5.2 (H-3) relative to t-BuOH, integrating for 0.41 μmole and 1.54 μmole, respectively. Incorporation was determined to be 2.01% for H-4 and 1.46% for H-3 by application of the following formulae:

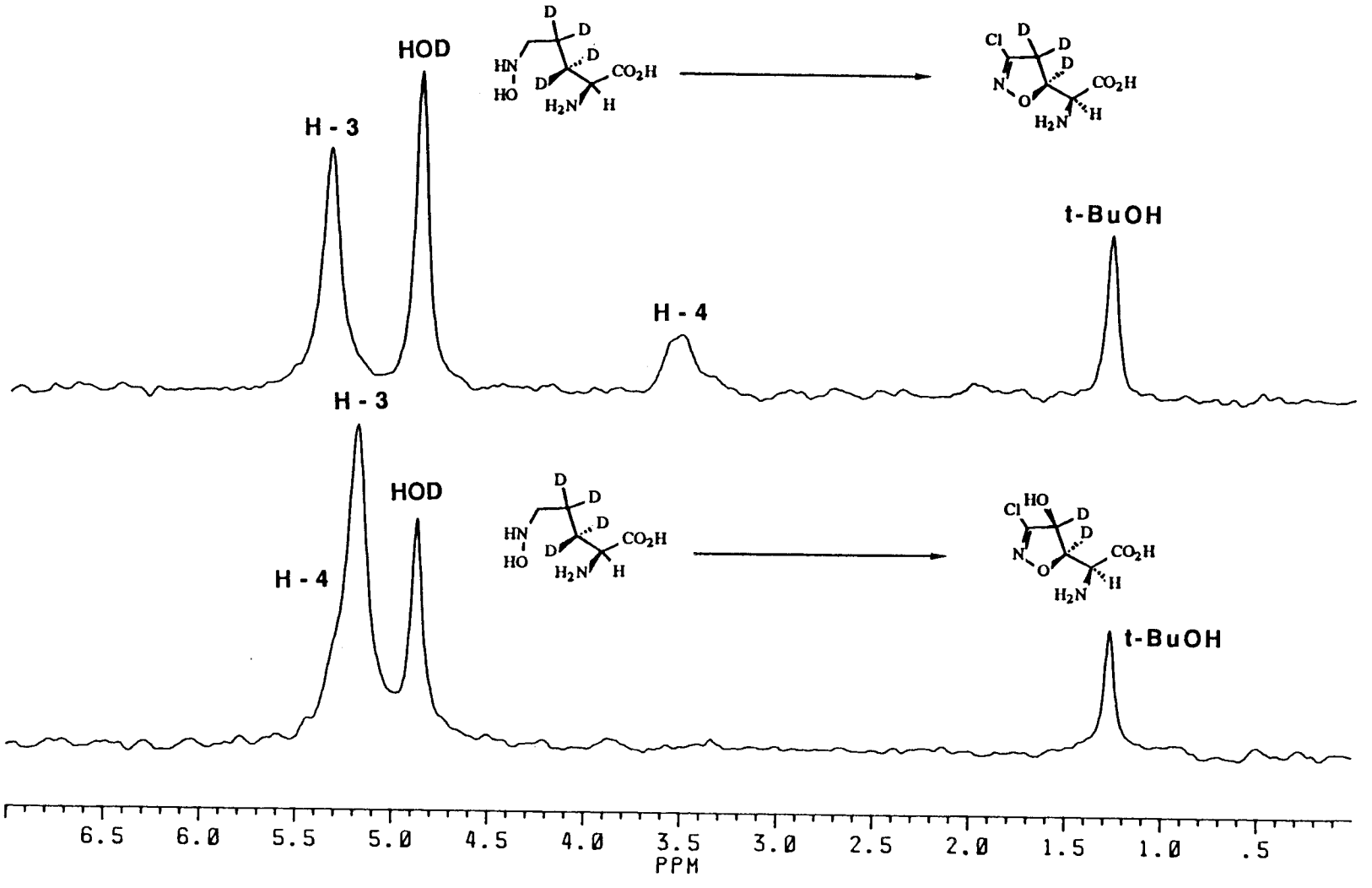


Figure 22. ^{2}H NMR of 4-Hydroxyacivicin **2j** and Acivicin **1 j** from the Feeding of **69a**

25 μL t-BuOH = 0.38 μmole of ^2H

H-4: Integration of the peak area at δ 3.4 gave a value of 108% that of the t-BuOH peak

0.38 μmole of ^2H in solvent x 1.08% = 0.41 μmole

$$\% \text{ enrichment (H-4)} = \frac{\text{specific activity isolated}}{\text{specific activity fed}} = \frac{\text{μmole of deuterium in acivicin/μmole of acivicin}}{\text{μmole of deuterium in N-hydroxyornithine/ μmole of N/hydroxy ornithine fed}}$$

$$= \frac{0.4/\mu\text{mole} \;/\; (6.32/178) \times 1000}{(114.92/220) \times 2 \times 0.1 \;/\; 114.92/200} = 5.8$$

$$\text{Dilution} = \frac{100}{\text{enrichment}} = 17$$

$$\% \text{ incorporation (H-4)} = \frac{\text{μmole of acivicin produced}}{\text{μmole substrate fed x dilution}} \times 100\% =$$

$$\frac{31.8/178.5}{114.92/220 \times 17} \times 100\% = 2.01\%$$

H-3: Integration of the peak area at δ 5.2 gave a value of 4.05% that of the t-BuOH peak.

0.38 μmole of ^2H in solvent x 4.05% = 1.54 μmole

$$\% \text{ enrichment} = \frac{1.54 \;/\; (6.32/178.5) \times 1000}{(114.92/220) \times 1 \;/\; 114.92/220} = 4.30$$

$$\text{Dilution} = \frac{100}{\text{enrichment}} = \frac{100}{4.30} = 23.3$$

$$\%\ \text{incorporation} = \frac{31.8/178.5}{114.92/220 \times 23.3} \times 100\% = 1.46\%$$

By comparing the incorporation of H-3 (1.46%) to incorporation of H-4 (2.0%), it was found that only 70% of ^2H had been retained.

The ^2H NMR spectral data of **2j** similarly showed retention of deuterium at H-3 and H-4 (Figure 22). Incorporation was shown to be 3.86% for H-4 and 2.80% for H-3 based on the ^2H NMR spectral data.

These led us to conclude that N-hydroxyornithine was a key intermediate for acivicin and 4-hydroxyacivicin.

Feedings of L-[5-^{14}C]Acivicin 1k and L-4-Hydroxy[5-^{14}C]acivicin 2 k

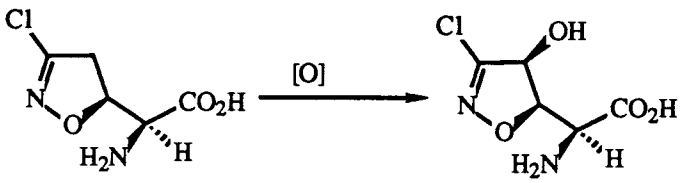
The objective of these feedings was to understand whether acivicin **1** was a direct precursor to 4-hydroxyacivicin or - rather unlikely - the biosynthetic pathway was proceeding in the opposite direction (Scheme 45).

1 **2**

Scheme 45. Hypothetical Relationships of Acivicin and 4-Hydroxyacivicin

A total of 4.5 x 10^4 DPM of L-[5-^{14}C]acivicin[141a] was fed to a 200 mL fermentation of *S. sviceus* at 76 h, 88 h, and 100 h. The broth was harvested and the

metabolite isolated in the usual manner. From the data obtained, it was shown that *S. sviceus* had produced 4-hydroxyacivicin from acivicin with incorporation of 15% of the L-[5-^{14}C]acivicin fed (Scheme 46).



Scheme 46. Biosynthesis of 4-Hydroxyacivicin from Acivicin

L-4-Hydroxy[5-^{14}C]acivicin[141b] (3.6 x 10^5 DPM) was fed in the same fashion as the acivicin feeding. This was worked up as usual. The results showed there was no radioactivity in the acivicin after purification. 4-Hydroxyacivicin was not a direct precursor to acivicin.

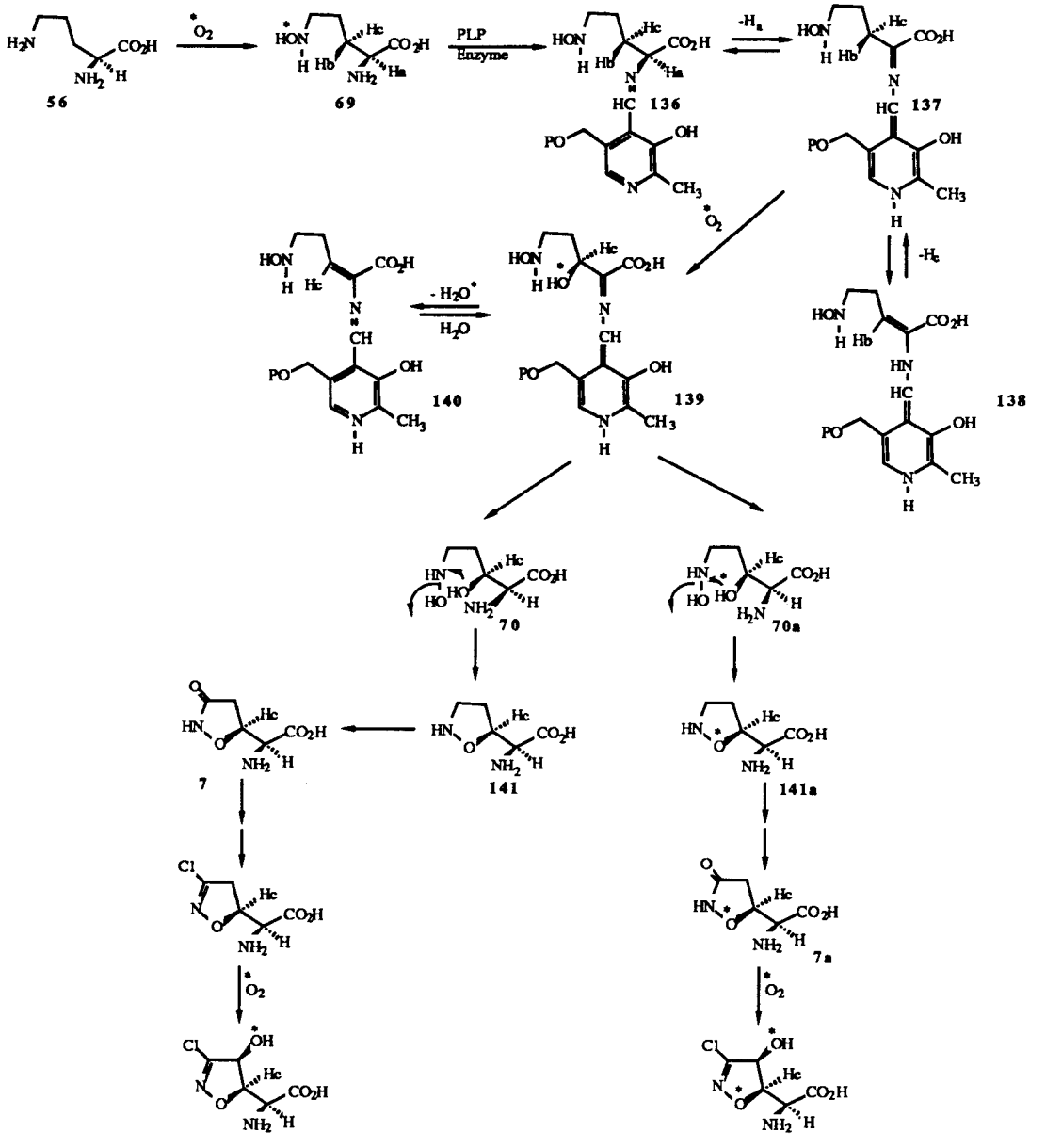
Conclusions and Areas for Further Study

The results of our feeding experiments are summarized in Table 10.

Table 10. Summary of Biosynthetic Experiments

Compound fed	4-Hydroxyacivicin	Acivicin
DL-[2-^{14}C]ornithine	% incorporation: 2%	% incorporation: 0.2%
DL-[5-^{13}C,5-^{15}N]ornithine	Both isotopes were retained	Similar result
DL-[2,3,3-$^{2}H_3$]ornithine	No deuterium at C-2 38% of ^{2}H retained at C-3	Similar result
DL-3*R*-[$^{2}H_1$]-ornithine	47% of deuterium retained at C-3	Similar result
DL-3*S*-[$^{2}H_1$]-ornithine	Deuterium was lost	Similar result
Oxygen-18	51% enrichment at C-4 14% enrichment at C-3	———
E-β-Hydroxy-L-[4,4-$^{2}H_2$]-ornithine	No enrichment	No enrichment
T-β-Hydroxy-L-[4,4-$^{2}H_2$]-ornithine	No enrichment	No enrichment
DL-[3,3,4,4-$^{2}H_4$]-ornithine	One H-4 deuterium retained	Both H-4 deuteriums retained
DL-[3,3,4,4-$^{2}H_4$]-δ-N-hydroxyornithine	% incorporation: 3.86% (C-4), 2.80% (C-3) 72% of ^{2}H retained at C-3	% incorporation: 2.01% (C-4), 1.46% (C-3) 70% of ^{2}H retained at C-3
L-[5-^{14}C]acivicin	15% incorporation	———
L-[5-^{14}C]hydroxyacivicin	———	No incorporation
E-β-hydroxyornithine trapping experiment	Result:	
(a) 48 h → 63 h	No incorporation	
(b) 55 h → 58 h	No incorporation	
(c) 55 h → 61 h	No incorporation	
T-β-hydroxyornithine trapping experiment		
(a) 48 h → 63 h	No incorporation	
(b) 55 h → 58 h	No incorporation	
(c) 55 h → 61 h	No incorporation	

The pathway shown in Scheme 47 is consistent with experimental results.

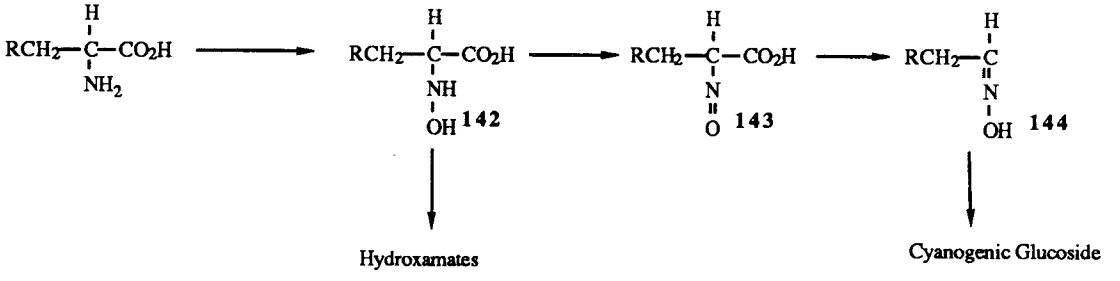


Scheme 47. Proposed Mechanism in Biosynthesis of Acivicin and 4-Hydroxyacivicin

Initially, ornithine, **56**, could be enzymatically oxidized to δ-N-hydroxyornithine, **69**, with molecular oxygen.[142] This would be followed by reaction of δ-N-hydroxyornithine, **69**, with pyridoxal phosphate to afford Schiff base **136**.[103] A tautomeric shift to **137** would result in loss of Ha. Further, the proton (Hc) could be exchanged stereospecifically in **137** to **138**. Hydroxylation of isolated

aliphatic carbon is well documented in biological systems and such a reaction could be involved in the formation of **139** from **137**. Compound **139** facilitates α,β-elimination and could equilibrate with **140**. At this stage, labeled oxygen-18 would be partially exchanged with $H_2{}^{16}O$. Subsequently N-hydroxyamino acid **139** could be converted to **141**, **141a** by tautomerization, hydrolysis and cyclization of **70** and **70a**. Compound **141**, **141a** could be oxidized to tricholomic acid **7**, **7a**. This would then be converted to acivicin. Finally, acivicin could be oxidized to 4-hydroxyacivicin.

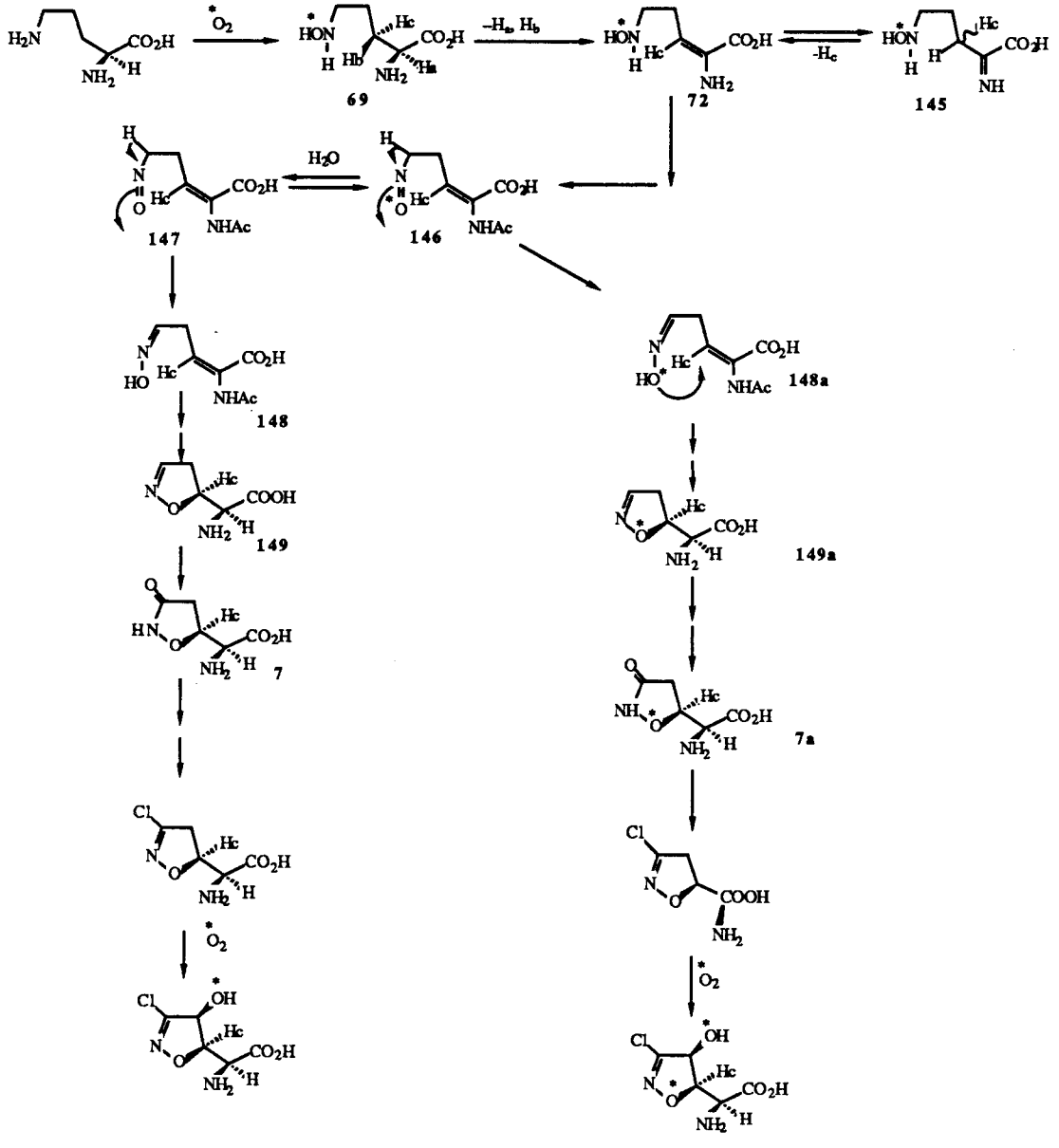
An alternative possibility would involve a nitroso amino acid and an aldoxime in the biosynthesis of acivicin and 4-hydroxyacivicin. Some assumptions could be made based on the biosynthesis of cyanogenic glucosides (Scheme 48), which has been proposed[143] to involve N-hydroxyamino acid **142**, α-nitroso acid **143**, and aldoxime **144**, as intermediates.



Scheme 48. Possible Intermediate in the Biosynthesis of Cyanogenic Glucoside

From this perspective the biosynthetic routes to acivicin shown in Scheme 49 could be proposed. N-hydroxyornithine, **69**, would undergo dehydrogenation to **72**. Although little is known about the formation of dehydroamino acid units in peptides and cyclopeptides, a number of possible pathways have been discussed by Schmidt et al.[144] Dehydrogenation could be followed by a tautomeric shift to **145** resulting

in partial loss of Hc. This N-hydroxy dehydro amino acid **72** could then be oxidized to δ-nitroso amino acid **146**. The δ-nitroso amino acid **146** could undergo partial

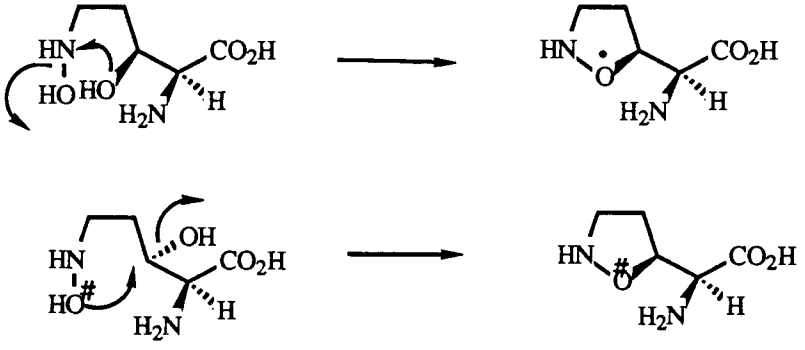


Scheme 49. Proposed Mechanism in Biosynthesis of Acivicin and 4-Hydroxyacivicin

exchange with $H_2{}^{16}O$ to **147**. This phenomenon could explain the data previously given which showed that the enrichment at C-3 was 14%, while the enrichment at C-4 was 51%. **147** could then be converted to aldoxime **148**, **148a** followed by a

Michael cyclization to give **149, 149a**. Subsequently hydration/oxidation of **149, 149a** would afford tricholomic acid **7, 7a** and this could be further transformed to acivicin **1** by chlorination.

Additional experiments such as feedings of labeled oxygen-18 δ-N-hydroxyornithine **69b** can be proposed. This would clearly indicate whether δ-N-hydroxyornithine cyclizes to the isoxazole ring by the displacement of an original β-oxygen or in the opposite fashion shown as Scheme 50.



Scheme 50. Proposed Mechanism for Isoxazolidine Ring Formation

Other advanced intermediates, such as the dehydroamino acid, should be tested. The experiment designs would depend on the oxygen-18-δ-N-hydroxyornithine feeding experiment.

EXPERIMENTAL

General

^{1}H and ^{13}C NMR spectra were recorded either on a Bruker Am 400 (400.13 MHz and 100.6 MHz, respectively) or on a Varian FT-80A (80 MHz) spectrometer. ^{2}H NMR spectra were obtained on the Bruker AM 400 at 61.4 MHz. All chemical shifts for ^{1}H and ^{13}C spectra are reported in parts per million (ppm) relative to external tetramethylsilane ($(CH_3)_4Si$, δ 0.00). Chemical shifts for ^{2}H spectra are relative to the natural abundance deuterium resonance of t-BuOH (δ 1.23). Infrared spectra (IR) were recorded in wavenumbers on a Nicolet 5DXB FT-IR spectrometer. Low resolution mass spectra were taken on a Varian MAT CH-7 spectrometer with a System Industries 150 data system. High resolution mass spectra were taken on a Kratos MS 50TC spectrometer. Melting points were taken on a Buchi melting point apparatus and are uncorrected Elemental analyses were performed by R. Johnson at MicAnal (Tucson, AZ).

Flash chromatography was carried out on silica (EM Reagents, Keiselgel 60, 230-400 mesh). Analytical thin layer chromatography (TLC) was carried out on precoated Keiselgel 60 F_{254} (0.2 mm on aluminum sheets) and visualized by long and/or short wave UV. Preparative thin layer chromatography (PLC) was carried out on precoated keiselgel 60 F_{254} glass plates (20 cm x 20 cm x 2 mm). Radioactivity measurements were carried out using a Beckman model LS 7800 Liquid Scintillation Counter with automatic quench correction external standardization to yield disintegrations per minute.

Materials

Solvents for the routine acquisition of NMR spectra were purchased from either Aldrich Chemical Company, Inc. (Milwaukee, WI), Stohler/KOR Stable Isotopes (Cambridge, MA), or Cambridge Isotope Laboratories (Woburn, MA). $^{18}O_2$ gas (50% enriched) was purchased from Cambridge Isotope Laboratories; ethanol-d (99.5 atom % D), lithium deuteride (98 atom % D), acetic acid-d_4 (99.5 atom % D) were purchased from Aldrich Chemical Company, as was deuterium oxide (99.8 atom % D) and deuterium-depleted water (natural abundance x 0.0046%, lot 4602 CIML).

Radioactive isotopes used for feeding were purchased from Research Products International Corporation (Mount Prospect, IL) or New England Nuclear (Boston, MA).

All solvents were reagent grade and used directly as purchased except the following. Dry tetrahydrofuran (THF) was distilled over sodium using benzophenone ketyl as the indicator, and dimethylsulfoxide (DMSO), dimethyl-formamide (DMF) and dichloromethane were distilled over CaH_2.

All other reagents for synthetic studies were used without further purification unless noted, and were obtained from either Aldrich, Sigma, VWR Scientific, or Fluka Chemical Corp.

Synthetic Studies

DL-[2,3,3-$^2H_3$]Ornithine, 56b.[85] In a 125 mL round bottom flask with a neck approximately 8 cm long and with a 0.5 cm diameter were placed L-ornithine (1.68 g, 10 mmole), pyridoxal:HCl (0.2036 g, 1 mmole), ^2H_2O (5 mL) and aluminum sulfate (0.157 g, 0.25 mmole). The mixture was wrapped with aluminum foil, frozen, and lyophilized. After lyophilization, pyridine (0.42 mL, 5

mmole) and ^2H_2O (10 mL) were added. The solution was frozen again, and the flask was sealed under vacuum and heated at 125 °C for two days. After cooling to room temperature the flask was opened and the contents poured into a 250 mL Erlenmeyer flask containing H_2O (200 mL), sodium oxalate (0.27 g, 2.0 mmole), collidine (0.66 mL, 5 mmole) and conc HCl (0.42 mL). The solution was loaded onto a cation column (Dowex 50Wx8, H^+, 100-200 mesh, 3 x 25.5 cm). The ion exchange resin was washed with distilled water and then eluted with 0.2 N NH_4OH. Fractions giving a positive reaction with ninhydrin were pooled, rotovaped, and lyophilized to yield an off-white solid. Recrystallization from water-ethyl alcohol (pH = 6-7) afforded 1.1 g (66%) of pure white crystals: mp 232° dec (lit.[145] 230-232 °C dec); R_f = 0.58 (1-butanol-acetone-diethylamine-water 70:70:35:70 on celluose); IR (1% KBr) 3040-2820, 2105, 1626, 1568 cm^{-1}; ^1H NMR (^2H_2O) δ 3.16 (2H, t, J=7.7 Hz), 1.75-1.95 (2H, m), 1.96-2.05 (0.48H, t).

Methyl N,N-Dibenzyl Glycinate,[96] 77. Glycine (7.5 g, 0.1 mole) was dissolved in 1N K_2CO_3 (100 mL). The aqueous solution was heated at reflux in an oil bath, then 34.5 mL (0.3 mole) of benzyl chloride, along with 300 mL of 1N K_2CO_3, was added dropwise to the glycine solution. After the final addition, the mixture was heated at reflux at 168 °C overnight. After work-up, the aqueous solution was adjusted to pH 2 with 6N HCl and the product was filtered. Then the crystals were washed with ethylacetate to remove the benzyl chloride. N,N-dibenzylglycine was obtained in 63.4% yield (13.0 g). TLC ($CHCl_3$-CH_3OH 5:5 on silica gel) R_f 0.5; mp 180-182 °C; IR (1% KBr) 2500-3300, 1715, 1440, 1280 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 7.5 (10H, s), 4.75 (4H, s), 3.8 (2H, s).

N,N-dibenzylglycinate (3.23 g, 12.7 mmole) was dissolved in 1:1 (ether:CH_3OH) and treated with excess diazomethane. Then the excess diazomethane

was removed by nitrogen gas. The solvent was evaporated under vacuum. Purification of the residue by flash chromatography (n-hexane-EtOAc 7:3, 3.5 x 18 cm column) gave 3 g (88%) of **77**. TLC (n-hexane/EtOAc 7:3 on silica gel) R_f 0.67; mp 41-43 °C; IR (neat) 3028, 2950, 1738, 1453 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.25 (10H, m), 3.8 (4H, s), 3.6 (3H, s), 3.25 (2H, s).

N-(3-Hydroxypropyl)phthalimide, 82. To a stirred suspension of $LiAlH_4$ (2.7 g, 0.065 mole) in anhydrous ether (200 mL) at 0 °C under N_2, ethyl 3-chloropropionate, **90**, (15 g, 0.11 mole) was added dropwise over 30 min while keeping the temperature at 0 °C. The mixture was warmed to room temperature and stirred for 3 h, and then cooled to 5 °C in an ice bath. Excess $LiAlH_4$ was quenched by successive addition of H_2O (2.5 mL), NaOH (15% solution, 2.4 mL) and H_2O (7.2 mL).[146] The mixture was stirred for 30 min, allowed to settle for 2 h and the resulting precipitate was removed by vacuum filtration. The residue was thoroughly washed with ether and the combined filtrates were evaporated under reduced pressure.

The resulting crude 3-chloropropanol was reacted with potassium phthalimide (35.3 g, 0.19 mole) in dry DMF (100 mL) at 120 °C for 4 h.[147] After cooling to room temperature, $CHCl_3$ (150 mL) was added and the mixture was poured into water (500 mL). The organic layer was repeatedly washed with water and dried (Na_2SO_4), and upon removal of solvent under reduced pressure gave a white, crystalline solid which was rcrystallized from 95% EtOH. This yielded 8 g (36% from chloropropionate): TLC (100% EtOAc on silica gel, UV detection) R_f 0.44; mp 75-76 °C (lit[147] 75-79 °C); IR ($CHCl_3$) 3350, 1725, 1450 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 7.77 (4H, m), 3.86 (2H, t, J=6.4 Hz), 3.59 (2H, t, J=6 Hz), 2.55 (1H, br s), 1.87 (2H, q, J=6 Hz).

N-(3-Oxopropyl)phthalimide, 78. Oxalyl chloride (1.87 mL, 20.57 mmole) was taken up in dry CH_2Cl_2 (40 mL) and stirred under N_2 at -60 °C. To this was added dry DMSO (2.9 mL, 34.03 mmole) in dry CH_2Cl_2 (10 mL) and the resulting mixture was stirred for 5 min.[149] A solution of **92** (4 g, 19.32 mmole in 10 mL dry CH_2Cl_2) was then added to the reaction flask with continuous stirring, while the temperature was maintained below -50 °C. After 25 min, Et_3N (11.69 mL, 81.32 mmole) was added dropwise and the resulting paste was allowed to warm to room temperature. After stirring for 5 min, water (50 mL) was added, the phases were separated and the organic layer was washed successively with 1% HCl, 50% Na_2CO_3 and H_2O. This was then dried (Na_2SO_4) and concentrated in vacuo to give a white solid. Recrystallization from CH_2Cl_2-hexane gave 3.28 g (82.5%) of aldehyde **78**: TLC (100% EtOAc, silica gel, UV detection) R_f 0.62; mp 126-127 °C (lit.[150] 125-126 °C); IR (KBr 3025, 2980, 1772, 1715 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 9.82 (1H, t, J=1.3 Hz), 9.70-7.90 (4H, m), 4.04 (2H, t, J=7 Hz), 2.87 (2H, dt, J=7, 1.3 Hz).

N-Benzylhydroxylamine, 86. This was prepared by the method of Cope and Haven[151] except for the following. To a solution of benzyl chloride (45.45 g, 0.395 mole) in 70% ethanol (200 mL) was added hydroxylamine hydrochloride (14.0 g, 0.202 mole) and Na_2CO_3 (60.0 g, 0.556 mole). Crystallization from ethanol gave 24.1 g (62.3%) of N,N-dibenzylhydroxylamine: TLC (CH_2Cl_2 on silica gel) R_f 0.28; mp 123-124 °C (lit.[152] 123 °C); IR ($CHCl_3$) 3580, 3225, 2850, 1440, 1335 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 7.25 (10H, s), 6.19 (1H, s), 3.73 (4H, s).

A portion of this hydroxylamine (18.2 g, 85.3 mmole) was placed in ether (200 mL) and yellow HgO (37.4 g, 0.172 mole) was added in four portions. After 1

h, this resulted in 16.8 g (93%) of N-benzylbenzaldoxime: TLC (ether-n-hexane 4:1 on silica gel) R_f 0.24; mp 82-83 °C (lit.[151] 81.5-83.5 °C); IR (1% KBr) 1580, 1450, 1140, 930 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 8.2 (2H, m), 7.4 (9H, m), 5.04 (2H, s).

The nitrone (16.8 g, 79 mmole) was then hydrolyzed in conc HCl (40 mL) and purified by steam distillation, affording 8.6 g (88%) of hydroxylamine after crystallization from hexane. TLC (hexane-Et_2O 1:3 on silica gel) R_f 0.23; mp 58-59 °C (lit.[151] 56-58.5 °C); IR ($CHCl_3$) 3550, 3250, 1500, 1460 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 7.31 (5H, s), 5.2 (1H, br), 4.0 (2H, s).

L-[4,4-$^2H_2$]-Glutamic Acid, 27a. A solution of L-glutamic acid, **27**, (15.6 g, 84.81 mmole) in 6N DCl (50 mL) was heated at 95-105 °C in a heavy-walled pressure tube for seven days after which the liquid was removed by rotary evaporation. A small aliquot of the sample was checked by ^1H NMR and showed only 50% ^2H enrichment at C-4. Hence a fresh batch of 6N DCl was added and the exchange was continued for another week. After repeating this one more time, the resulting solution was concentrated under vacuum to a white crystalline residue which was filtered and washed with $CHCl_3$, yielding 14.4 g (91.70%) of **27a**: TLC (n-Butanol-AcOH-H_2O 3:1:1 on cellulose, 0.25% ethanolic ninhydrin spray) R_f 0.32; ^1H NMR (D_2O, 400 MHz) δ 3.0 (1H, t), 1.25-1.5 (0.1H, s), 1.0 (2H, d), Integration of the peak at 1.25-1.5 showed 95% removal of the C-4 hydrogens.

N-Benzyloxycarbonyl-L-[4,4-$^2H_2$]glutamic Acid, 90a.[98] To an iced cold solution of **27a** (13.4 g, 72.2 mmole) and $NaHCO_3$ (15.16 g, 180 mmole) in D_2O (72 mL) was added over a period of 2 h a solution of benzyl chloroformate (10.32 mL, 71.91 mmole) and 2.5N NaOD (28.8 mL). The mixture was stirred

vigorously with a mechanical stirrer. After 5 h, the mixture was treated with additional benzylchloroformate (10.32 mL, 71.91 mmole) and 2.5N NaOD (28.8 mL). After 5 h more, the mixture was extracted with EtOAc and ether (200 mL); the aqueous was then adjusted to pH 2 and extracted with EtOAc (300 mL). The organic solvent was removed under vacuum, and the product recrystallized from hexane to give 17.5 g (84%): TLC (AcOH-$CHCl_3$ 5:95 on silica gel) R_f 0.3; ^1H NMR (D_2O, NaOD, 400 MHz) δ 7.4 (5H, s), 5.2 (2H, s), 3.8 (1H, t), 2.1 (0.11H, bs), 1.9-2.0 (1H, m), 1.7-1.85 (1H).

3(S)-[3-(Benzyloxy)carbonyl-5-oxo-4-oxazolidinyl]-[2,2-$^2H_2$] Propionic Acid, 91a. This was prepared according to the literature procedure[153] except for the following. A mixture of p-toluenesulfonic acid (0.2 g, 1. mmole), paraformaldehyde (0.915 g, 30.5 mmole) and Cbz-glutamic acid **90a** (5 g, 18 mmole) in benzene (130 mL) afforded 4.4 g (85%) of the desired oxazolidine **91a**: TLC (CH_3OH-$CHCl_3$ 4% on silica gel) R_f 0.27; ^1H NMR ($CDCl_3$; 400 MHz) δ 7.36 (5H, s), 5.54 (1H, d, J=4.7 Hz), 5.26 (1H, d, J=4.7 Hz), 5.19 (2H, s), 4.40 (1H, m), 2.5 (0.11 H, br), 2.1-2.4 (2H, m).

N-(Benzyloxy)carbonyl-L-[4,4-$^2H_2$]glutamic Acid α-Methyl Ester, 92a. This was prepared according to the literature method[154] except as follows. A solution of $NaOCH_3$ was prepared by placing Na (0.655 g, 28 mmole) in methanol (85 mL) at 5 °C. To this was added a solution of oxazolidine **91a** 4.10 g, 13.98 mmole) in methanol (43 mL). After 3 h at 5 °C, the pH was adjusted to 6.0 with 6N HCl, and the mixture concentrated in vacuo. The resultant residue was partitioned between 5% HCl and ethyl acetate. The layers were separated and the aqueous layer extracted with dichloromethane. Concentration of the combined, dried

($MgSO_4$) organic extracts gave a syrup, which slowly crystallized upon standing at room temperature under n-hexane. The crystalline mass was crushed in a mortar and pestle and dried, affording 4.10 g (87.9%): mp 64-66 °C; TLC (C_6H_6-EtOH-AcOH 69:30:1 on silica gel) R_f 0.25; ^1H NMR (methanol-d_4, 400 MHz) δ 7.2 g (5H, s), 5.1 (2H, s), 4.38 (1H, m), 3.69 (3H, s), 2.3-2.4 (0.3H, m), 1.8-2.2 (2H, m).

N-(Benzyloxy-carboxyl-L-[4,4-$^2H_2$] Vinyl Glycine Methyl Ester, 87a. This was prepared according to the literature method[154] except as follows. To acid **92a** (5 g, 16.93 mmole) and $Cu(OAc)_2 \cdot H_2O$ (0.85 g, 4.25 mmole) in benzene (200 mL) was added $Pb(OAc)_4$ (15 g, 33.80 mmole). Purification by flash chromatography (n-hexane-EtOAc 9:1, 3 x 22 cm) gave 1.9 g (45%) of olefine **87a**: TLC (n-hexane-EtOAc 1:1 on silica gel) R_f 0.72; ^1H NMR ($CDCl_3$ 400 MHz) δ 7.35 (5H, s) 5.90 (1H, m), 5.5 (1H, br), 5.2-5.4 (0.35H, m), 4.95 (1H, m), 3.76 (3H, s).

2(S)-[2-Benzyl-5(S)-[4,4-$^2H_2$]isoxazolidinyl]-[N-((benzyloxy)carbonyl)amino] acetic acid, 89 and 2(S)-[2-Benzyl-5(R)-[4,4-$^2H_2$]isoxazolidinyl]-[N-((benzyloxy)carbonyl)amino] acetic acid, 88. Olefin **87a** (3.82 g, 15.19 mmole) paraformaldehyde (2.29 g, 76.48 mmole), **95** (2.0 g, 16.45 mmole) and 4Å molecular sieves (4.7 g) were placed in benzene (250 mL) and heated at a gentle reflux for 12 h. The sieves were removed by filtration and the filtrate concentrated in vacuo to a syrup, which was purified by flash chromatography (C_6H_6-EtOAc-CH_3OH 95:4:1, 3 x 22 cm) affording 5.73 g (97%) of the partially separated isoxazolidines **84a,b** as a light yellow oil: the mixture of esters **84a,b** (5.7 g, 14.82 mmole) was dissolved in

tetrahydrofuran (184 mL) and cooled to 5 °C in an ice-water bath. To this was added 0.56 N LiOH (61.5 mL), and the reaction stirred vigorously for 2 h. The mixture was concentrated in vacuo to a volume of 50 mL, followed by adjustment to pH 5.6 with 10% HCl and saturation with $(NH_4)_2SO_4$. Extraction of the resulting mixture with ethyl acetate, and concentration of the combined, dried ($MgSO_4$) extracts gave a yellow syrup which was purified by flash chromatography ($CHCl_3$-CH_3OH-AcOH 92:7:1, 4 x 34 cm). Combination of the appropriate fractions followed by crystallization of each diastereomer from methanol/water afforded 3.39 g of the threo isomer **88** and 2.40 g of erythro isomer **89** (54% combined). Data for erythro isomer **89**: mp 122 °C; TLC (92:7:1 $CHCl_3/CH_3OH$/AcOH on silica gel) R_f 0.24; ^1H NMR (methanol-d_4, 400 MHz; 270K, major conformer) δ 7.3 (10H, m), 5.07 (2H, s), 4.65 (1H, m), 4.41 (1H, d, J=5.3 Hz), 3.96 (1H, d, J=12.8), 3.81 (1H, d, J=12.8), 3.13 (1H, m), 2.94 (1H, m), 2.27-2.50 (0.36 H, m).

Data for threo isomer **88**: mp 129 °C; TLC R_f 0.30; ^1H NMR (methanol-d_4;; 400 MHz, 270K, major conformer) δ 7.3 (10H, m), 5.09 (2H, d, J=2.4 Hz), 4.9 (1H, m), 4.42 (1H, d, J=2.8 Hz), 4.01 (1H, d, J=12.6 Hz), 3.76 (1H, d, J=12.6 Hz), 3.07 (1H, m), 2.95 (1H, m), 2.56 (0.18H, m), 2.10 (0.18H, m).

Threo-β-hydroxy-L-[4,4-$^2H_2$]ornithine, 70a. To the isoxazolidine acetic acid **88a** (1 g, 2.68 mmol) was added 20% $Pd(OH)_2/C$ (1 g), absolute ethanol (50 mL) and 6N HCl (850 μL). The mixture was then hydrogenated at atmospheric pressure and room temperature. After four days the reaction was stopped, filtered through celite, and the solids washed with water. The pH was then adjusted to 6.7 with NH_4OH followed by concentration in vacuo to a volume of 1 mL. To this was added ethanol, which caused the amino acid to crystallize, affording 0.5 g (100%) of **75a**: mp 122.5-123 °C; TLC (n-BuOH-H_2O-AcOH 4:2:1 on cellulose, 0.3% ethanolic

ninhydrin spray) R_f 0.33; ^1H NMR (D_2O, 400 MHz) δ 4.22 (1H, d, J=5.2 Hz), 3.72 (1H, d, J=5.2 Hz), 3.23 (2H, dd, J=10 Hz), 2.08 (0.18 H, m), 1.97 (1H, m).

Erythro-β-hydroxy-L-[4,4-$^2H_2$]ornithine, 74a. This was prepared as per **75a** except as follows. The isoxazolidine acetic acid **89** (1 g, 2.68 mmole) afforded 0.38 g (78%) of **74a**: mp 230-232 °C; TLC (n-BuOH-H_2O-AcOH 4:2:1 on cellulose, 0.3% ethanolic ninhydrin spray) R_f 0.34; ^1H NMR (D_2O 400 MHz) δ 4.25 (1H, d, J=3.6 Hz), 3.87 (1H, d, J=3.6 Hz), 3.18 (2H, dd, J=12 Hz).

O-Benzyl-N-Tosylhydroxylamine, 109.[130a] To a chilled solution of o-benzylhydroxyamine **117** (3.31 g, 20.73 mmol) in pyridine (10 mL) a solution of p-toluenesulfonyl chloride (3.95 g, 20.73 mmole) in pyridine (5 mL) was added slowly over 2 h, and the mixture was allowd to stand overnight. A precipitate that had formed was filtered off and the filtrate was evaporated in vacuo. The residual oil was extracted with ethyl acetate (200 mL) and the organic layer was washed with 2N HCl and with water, dried over sodium sulfate, and then evaporated in vacuo. The residue was recrystallized from ethyl acetate/petroleum ether to yield 4.85 g (85%): mp 94-96 °C; TLC (CH_2Cl_2 on silica gel) R_f 0.42; IR ($CHCl_3$) 3320, 3080, 1300, 1176 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 7.75 (2H, d, J=8 Hz), 7.6-7.3 (7H, m), 5.0 (2H, s), 2.5 (3H, s).

γ-(N-Tosyl-N-Benzyloxy)-aminopropyl Bromide, 116. To a solution of sodium (0.3321 g, 14.44 mmole) in ethanol (17.2 mL) was added **109** (4 g, 14.44 mmole) with vigorous stirring at 70 °C. After a clear solution was obtained, 1,3-dibromopropane (2.93 mL, 28.88 mmole) was added. The mixture

which was extracted with ethyl acetate. The organic layer was thoroughly washed with water and dried over sodium sulfate. Evaporation of the solvent afforded an oily product which was crystallized from ethyl acetate/n-hexane to yield 3.77 g (66%): mp 73-75 °C; TLC ($CHCl_3$-n-hexane 6:4 on silica gel) R_f 0.5; IR ($CHCl_3$) 3040, 1300, 1176 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 7.75 (2H, d, J=8 Hz), 7.27 (7H, m), 5.0 (2H, s), 3.5 (2H, t, J=8 Hz), 3.0 (2H, t, J=4 Hz), 2.4 (3H, s), 1.95 (2H, q, J=4 Hz).

Diethyl γ-(N-Tosyl-N-Benzyloxy)-aminopropyl Acetamido-malonate, 117. To a solution of sodium (0.28 g, 9.9 mmole) in ethanol, (18.5 mL) was added diethyl acetamidomalonate (2.15 g, 9.9 mmol) while stirring. After 5 min, **116** (3.94 g, 9.9 mmole) was added and the mixture was heated at reflux for 8 h. The solvent was then removed in vacuo and the residue was extracted with chloroform. The chloroform solution was successively washed with water, dilute hydrochloric acid and water, dried over magnesium sulfate, and evaporated in vacuo. The oily residue was crystallized from ethyl acetate/petroleum ether and then from ethanol to yield 3.97 g (75.19%): mp 135-136 °C; TLC (CH_2Cl_2-EtOAc 9:1 on silica gel, UV detector) R_f 0.36; IR ($CHCl_3$) 3376, 2982, 1740, 1676, 1364, 1167 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 7.75 (2H, d, J=8 Hz), 7.27 (5H, s), 7.26 (2H, d, J=6 Hz), 6.75 (NH, br), 5.0 (2H, s), 4.5 (4H, q, J=4 Hz), 2.6-2.9 (2H, t, J=4 Hz), 2.25 (3H, s), 2.24-2.05 (2H, m), 1.95 (3H, s), 1.2 ppm (8H, m).

δ-N-Tosyl-δ-N-Benzyloxy-DL-ornithine, 112. A solution of **117** (3.97 g, 7.44 mmole) in a mixture of acetic acid (29.13 mL) and concentrated hydrochloric acid (11.5 mL) was heated under gentle reflux for 8 h. The solution was evaporated in vacuo and 2 mL of 14% aqueous ammonia was added to the residue.

The resulting solid was collected by filtration, washed with water, and recrystallized from acetic acid/water to yield 2.38 g (82%): mp 207-209 (dec); TLC (4:4:3 n-PrOH-EtOAc-H_2O 4:4:3 on silica gel, UV detector) R_f 0.74; IR ($CHCl_3$) 3138-3048, 1598, 1407, 1164 cm^{-1}; ^1H NMR (TFA, 80 MHz) δ 7.8 (2H, d, J=5 Hz), 7.25-7.5 (7H, d,s), 5.1 (2H, s), 4.25 (1H, t), 3.0 (2H, br), 2.5 (3H, s), 2.25 (2H, br), 1.75 (2H, br).

Diethyl Phathalimido Malonate, 123[155]. Diethyl bromomalonate **121** (9 g, 37.6 mmole) was added to potassium phthalimide (6.1 g, 33 mmole) and hexadecylbutylphosphonium bromide, **122** (1.75 g, 3.45 mmole) in anhyd toluene (100 mL). The mixture was stirred 20 h, then filtered and filtrate conc in vacuo. The residue was taken up in $CHCl_3$ (20 mL) and filtered through a column of silica gel (3 x 20 cm) eluted with $CHCl_3$ to remove the catalyst. Product-containing fractions were pooled and concentrated to dryness, and the product recrystallized from ethyl ether/petroleum ether to yield 8.32 g (82%) of **123**: TLC (9.5:0.5 $CHCl_3$-EtOAc 9.5:0.5 on silica gel, UV detector) R_f 0.44; mp 75 °C (lit.[156] 75-76 °C); IR ($CHCl_3$) 3008, 2987, 1760, 1756, 1696 cm^{-1}; ^1H ($CDCl_3$, 80 MHz) δ 7.75 (4H, m), 5.45 (1H, s), 4.3 (4H, q, J=7 Hz), 1.38 (6H, t, J=7 Hz).

Diethyl(γ-bromopropyl)-N-phthalimidomalonate, 119. This was prepared according to the literature method[157] except as follows. The diethyl ester of (γ-bromopropyl)-N-phthalimidomalonic acid **119** was prepared by heating (85-95 °C), with continuous stirring, a mixture of **123** (0.5 g, 1.63 mmole), anhydrous potassium carbonate (0.5 g, 3.61 mmole), 1.3-dibromopropane (1.25 mL, 12.28 mmole) and dimethylformamide (10 mL) until the reaction mixture turned from an orange-red to a white color. The solvent was removed under vacuum

and the residue was then purified by flash chromatography (CH_2Cl_2-EtOAc 9.9:0.1, 3 x 20 cm) to give 0.692 g (57%) of oily **119**: TLC (9.9:0.1 CH_2Cl_2-EtOAc) R_f 0.48; IR ($CDCl_3$) 1780, 1760-1720, 1660; ^1H NMR ($CDCl_3$, 80 MHz) δ 7.7 (4H, m), 4.2 (4H, q, J=5 Hz), 3.2 (2H, t, J=5 Hz), 2.6 (2H, m), 2.0 (2H, m), 1.8 (2H, m), 1.2 (6H, t, J=5 Hz).

Bromoacetic Acid, 126. This was prepared by the method of Ward[158] except as follows: Red phosphorous (0.1 g, 3 mmole) was placed in acetic acid (4.79 g, 79.9 mmole). To this mixture was added Br_2 (4.7 mL, 91 mmole) over 1 h. After heating at ~95 °C for 1 h, the resulting solid was kugelrohr distilled (50 °C, 1.25 mm Hg), giving 10.09 g (88%) of the bromide: IR ($CHCl_3$) 3200-3060, 1727, 860 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 3.80 (3H, s), 8.5 (1H, s).

Bromoacetic Acid-d_3, 126a. This was prepared as above except as follows. Red phosphorous (0.2 g, 6 mmole) was placed in acetic acid-d_4 (10.24 g, 0.16 mole). To this mixture was added Br_2 (9.37 mL, 0.182 mole), affording 20.67 g (91%) of **126a** after kugelrohr distillation.

Ethyl Bromoacetate, 127. This was prepared as directed[159] except as follows. Bromoacetic acid **126** (10.09 g, 72.5 mmole), absolute ethanol (6.84 mL, 0.12 mole) and conc H_2SO_4 (17 μL) were placed in benzene (30 mL). The mixture was heated at reflux for 5 h. The mixture was washed successively with water (20 mL), sat $NaHCO_3$ (20 mL), and water (30 mL), and dried over anhydrous Na_2SO_4. Concentration in vacuo gave 8.85 g (73.14%) of the ester **127**: TLC ($CHCl_3$ on silica gel, UV detector) R_f 0.74; IR ($CHCl_3$) 2984, 1741, 1282 cm^{-1}; ^1H NMR ($CDCl^3$, 80 MHz) δ 4.23 (2H, q, J=7 Hz), 3.81 (2H, s), 1.25 (3H, t, J=7 Hz).

Ethyl Bromo[2,2-$^2H_2$]acetate, 127a. This was prepared as above except as follows. Bromoacetic acid-d_3 **126a** (20.60 g, 0.145 mole), ethanol-d (13.7 mL, 0.23 mole), and conc H_2SO_4 (34.9 μL) were placed in benzene (50 mL). A Dean-Stark trap was fitted, and the mixture heated at reflux for 4 h, after which 3.6 mL of water had been collected. A supplemental addition of ethanol-d (2.7 mL, 46.6 mmole) was made, and the reaction cooled to room temperature after an additional 0.5 h. The mixture was washed successively with water (50 mL), 1% $NaHCO_3$ (50 mL), and water (30 mL), and dried over anhydrous Na_2SO_4. Concentration in vacuo gave 21.82 g (89%) of the ester **127a**: TLC ($CHCl_3$ on silica gel, UV detector) R_f 0.74; ^1H NMR ($CDCl_3$, 80 MHz) δ 4.23 (2H, 1, J=7 Hz), 1.25 (3H, t, J=7 Hz).

2-Bromoethanol, 124. This was prepared according to the literature procedure.[160] To a mixture of $LiAlH_4$ (1.05 g, 27.7 mmole) and $AlCl_3$ (3.63 g, 27.2 mmole) in dry ether (45 mL) at -78 °C was added ethyl bromoacetate **127** (2.98 mL, 26.9 mmole). After 2 h at -78 °C, the excess hydride was destroyed by the sequential addition of ethyl acetate (5 mL) and water (4.54 mL). After warming to room temperature, the resulting mixture was centrifuged and the supernatant collected. Extraction of the salts with ether (45 mL) followed by drying of the combined extracts over anhydrous $MgSO_4$ and concentration, afforded 2.2 g (65%): TLC (20% hexanes-$CHCl_3$ on silica gel) R_f 0.17; IR ($CHCl_3$) 3420-3200, 2962, 1423 cm^{-1}; ^1H NMR ($CDCl_3$) 3.85 (2H, m), 3.50 (2H, m), 2.27 (1H, bs).

2-Bromo[1,1,2,2-$^2H_4$]ethanol, 124a. This was prepared as above except for the following. To a mixture of $LiAlD_4$ (5.6 g, 0.13 mole) and $AlCl_3$ (17.04 g, 0.1305 mole) in dry ether (150 mL) at -78 °C was added **127a** (21.82

g, 0.13 mmole). After 2 h at -78 °C, the excess hydride was destroyed by the sequential addition of ethyl acetate (22 mL), and water (23 mL). The resulting mixture was centrifuged and the supernatant collected. Extraction of the salts with ether (50 mL) followed by drying of the combined extracts over anhydrous $MgSO_4$ and concentration, afforded 7.72 g (49%): TLC (20% hexane-$CHCl_3$ on silica gel) R_f 0.17; ^1H NMR ($CDCl_3$, 80 MHz) δ 2.5 (s).

3-Hydroxypropionitrile, 128. To a solution of NaCN (0.921 g, 18.80 mmole) in water (10 mL) was added a solution of 2-bromoethanol **124** (2.13 g, 17.04 mmol) in methanol (10 mL), and the resulting mixture was heated at reflux overnight. After concentration to 5 mL, the remaining water was azeotropically distilled with ethanol, and the residue extracted with ethyl acetate (100 mL). These extracts were dried over anhydrous $MgSO_4$, and concentrated in vacuo. The resulting oil was kugelrohr distilled (4 mm, 80 °C), giving 1.0 g (74%) of the nitrile: TLC ($CHCl_3$-CH_3OH-AcOH 92:7:1 on silica gel, I_2 detection) R_f 0.21; IR (neat) 3434, 2960, 2254, 1060 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz) δ 3.50 (3H, t, J=6.1 Hz), 2.5 (2H, t, J=6.1 Hz).

3-Hydroxy[2,2,3,3-$^2H_4$]propionitrile, 128a. This was prepared as above except as follows. **124a** (7.72 g, 0.0598 mole) was placed in methanol (20 mL) and added to a solution of NaCN (3.23 g, 0.066 mole) in H_2O (20 mL). This resulted in 3.357 g (75%) of the desired nitrile: TLC (92:7:1 $CHCl_3/CH_3OH$/AcOH on silica gel), R_f 0.21; ^1H NMR ($CDCl_3$, 80 MHz), δ 2.5 (s). The enrichment was measured at the next stage.

3-Tosylpropionitrile, 129. This was prepared by the method of Sokellarios[161] except as follows. Nitrile **128** (5 g, 70.42 mmole) was placed in dry pyridine (50 mL, 1.2 mole) along with p-toluenesulfonyl chloride (18.64 g, 97 mmole). The mixture was kept at 5 °C overnight, and work-up afforded 13.11 g (88%) of tosylate **129**: TLC (5:1 $Et_2O/CHCl_3$ on silica gel) R_f 0.41; mp 62-63 °C (lit[19] 63 °C); IR ($CHCl_3$) 3062, 2260, 1407, 1180; ^1H NMR ($CDCl_3$, 80 Mhz) δ 7.75 (2H, m), 7.25 (2H, m), 4.25 (2H, t, J=6.5 Hz), 2.75 (2H, t, J=6.5 Hz), 2.46 (3H, s).

3-Tosyl-[2,2,3,3-$^2H_4$]propionitrile, 129a. This was prepared as above except as follows. To nitrile **128a** (3.36 g, 0.0047 mole) in dry pyridine (35 mL) was added p-toluenesulfonyl chloride (11.83 g, 0.06 mole), resulting in 766 g (79.7% of tosylate **129a**: TLC (5:1 $Et_2O/CHCl_3$ on silica gel) R_f 0.41. Examination of the ^1H NMR spectrum ($CDCl_3$, 80 MHz) revealed complete deuteration at C-3 (δ 4.25) but only 10% deuteration at C-2 (δ 2.75).

Ethyl 2-Acetamide-2-carboethoxy-4-cyanobutanoate 130. This was prepared by the method of Shimo[162] except as follows. To a solution of diethyl-acetamidomalonate (5 g, 23.05 mmole) in liquid NH_3 (250 mL) at -78 °C was added tosylate **129**. After 2 h, the ammonia was evaporated and the resulting residue crystallized from CH_2Cl_2/n-hexane, giving 4.17 g (76%) of the nitrile **130**: TLC (87:13 $CHCl_3/CH_3OH$) R_f 0.36; mp 92-93.5 (lit.[163] 94 °C); IR ($CHCl_3$ 3400, 2250, 1740, 1664 cm^{-1}; ^1H NMR ($CDCl_3$, 80 MHz), δ 6.73 (1H, bs), 4.28 (4H, q, J=7.1 Hz), 2.7 (2H, m), 2.25 (2H, m), 2.07 (3H, s), 1.27 (6H, t, J=7.1 Hz).

Ethyl 2-Acetamido-2-carboethoxy-4-cyano[3,3,4,4-$^2H_4$]-butanoate, 130a. This was prepared as above except as follows. To a solution of diethyl acetamidomalonate (7.53 g, 34.7 mmole) in liquid NH_3 (100 mL) at -78 °C was added **129a** (7.66 g, 33.4 mmole). After 2 h, the ammonia was evaporated and the resulting residue crystallized from CH_2Cl_2/n-hexane, giving 7 g (76.4%) of the nitrile **130a**: TLC (87:13 $CHCl_3/CH_3OH$ on silica gel) R_f 0.36. Examination of the ^1H NMR spectrum ($CDCl_3$, 80 MHz) revealed complete deuteration at C-3 (δ 2.7) but only 10% deuteration at C-2 (δ 2.25).

Diethyl γ-(N-oxide,N-p-methoxybenzylidene)aminopropyl acetamidomalonate, 134. Nitrile **130** (3.21 g, 11.88 mmole) and $PtO_2 \cdot H_2O$ (0.485 g, 1.98 mmole) were placed in absolute ethanol (180 mL) containing conc HCl (12 mL) and hydrogenated at atmospheric pressure and room temperature for 5 h. After filtration and washing of the catalyst with ethanol, the combined filtrate was concentrated in vacuo, giving a hygroscopic residue (3.71 g). To a suspension of this residue in 96 mL benzene was added anisaldehyde (2.18 mL, 17.91 mmole) and triethylamine (33 mL, 238.8 mmole). These were allowed to stir for 4 h at 110 °C. Using a Dean-Stark trap, the water was then removed by azeotropic distillation. After evaporation of the benzene, the residue was placed on a high vacuum pump to remove trace amounts of triethylamine. This crude Schiff base was added to a solution of monoperoxyphthalic acid magnesium salt (8.17 g, 16.53 mmole) in 40 mL dry ether and 20 mL absolute ethanol at 0 °C and stirred for 3 h. The mixture was then kept in the refrigerator overnight. The precipitated phthalic acid was filtered and washed with $CHCl_3$, solvent was removed at 30 °C under vacuum. To the residue was added 40 mL $CHCl_3$ and the remaining phthalic acid removed after an additional 30 min cooling. The filtrate was then evaporated on silica gel (30 g) and placed on a

column (4 x 28 cm), which was eluted with $CHCl_3/CH_3OH$ (9:1). The product, contaminated with p-methylbenzaldehyde, was obtained by combination and concentration of the appropriate fractions. This mixture was then purified by PLC (silica gel, developed with ethylacetate) eluting the desired product with $CHCl_3$-CH_3OH (9:1), to give 1.5 g (32%) of **134**: TLC (9:1 $CHCl_3/CH_3OH$) R_f 0.48; mp 149-149.5 °C; IR ($CHCl_3$) 3400, 3040, 1738, 1671, 1602, 850 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 8.25 (2H, d, J=7 Hz), 7.35 (1H, s), 6.95 (2H, d, J=7.0 Hz), 6.85 (1H, s), 4.25 (4H, 1, J=7.1 Hz), 3.9 (2H, t, J=7.2 Hz), 3.8 (3H, s), 2.4 (2H, m), 2.05 (3H, s), 1.85 (2H, m), 1.25 (6H, t, J=7.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) 169.31, 167.74, 161.17, 134.44, 130.66, 123.28, 113.84, 66.06, 65.98, 62.73, 55.33, 29.37, 23.06, 22.17, 13.94; EIMS (70 ev), m/z (relative intensity) 408 (M^+, 48.9), 335 (100); Anal. calcd for $C_{20}H_{28}O_7N_2$: C, 58.82; H, 6.86; N, 6.86. Found: C, 58.84; H, 6.92; N, 6.80.

Diethyl γ-(N-oxide,N-p-methoxybenzylidene)[3,3,4,4-$^2H_4$]-aminopropyl Acetamidomalonate, 134a. Nitrile **130a** (3.21 g, 11.88 mmole) and PtO_2 (0.446 g, 1.98 mmole) were placed in absolute ethanol (90 mL) containing concd HCl (6 mL) and hydrogenated at atmospheric pressure and room temperature for 3.5 h. After filtration and washing of the catalyst with ethanol, the combined filtrate was concentrated in vacuo, giving a hygroscopic residue (3.8 g). To a suspension of this residue in 100 mL benzene was added anisaldehyde (2.26 mL, 8.56 mmole) and triethylamine (33 mL, 238.8 mmole). These were allowed to stir for 12 h at 110 °C while water was removed with a Dean-Stark trap. The crude Schiff base was added to a solution of monoperoxyphthalic acid Mg salt (8.17 g, 16.53 mmole) in dry ether (40 mL) and absolute ethanol (25 mL) at 0 °C and stirred for 3 h. Isolation as above gave 1.53 g (32%) of **134a**: TLC ($CHCl_3$-CH_3OH 9:1 on silica

gel) R_f 0.48. Examination of the ^1H NMR spectrum ($CDCl_3$, 80 MHz) revealed complete deuteration at C-3 (δ 2.4) but only 10% deuteration at C-4 (δ 1.85).

δ-N-Hydroxy-DL-Ornithine, 69. A solution of nitrone **134** (1 g, 2.54 mmole) in 6N hydrochloric acid[130b] (50 mL) was heated in an oil bath at 130 °C for 7 h, and the solution was then concentrated to dryness under reduced pressure. The residue was neutralized to pH 4.5 and loaded to a column of Dowex 50Wx4 (H^+) (200-400 mesh, 3 x 20 cm). The column was washed with water until neutral, then eluted with 1.5N HCl. Fractions containing N-hydroxyornithine were combined, concentrated at ambient temperature by rotary evpoaration to remove HCl, and lyophilized. This gave 0.21 g (40%) of **69** as a white glossy solid which could not be recrystallized because of its very high hygroscopic nature: TLC (chlorofrom-methanol-concentrated ammonium hydroxide-water 1:4:2:1 on silica gel) R_f 0.72 (lit.[23] R_f 0.72); ^1H NMR (D_2O, 80 MHz) δ 4.25 (1H, t), 3.50 (2H, t), 2.0 (4H, m); Ninhydrin, triphenyltetrazolium tests were all positive.

δ-N-Hydroxy-DL-[3,3,4,4-$^2H_4$]ornithine, 69a. Via the procedure described above, the tetradeuterio **134a** (1.0 g, 2.6 mmole) was heated at reflux with 6N HCl (200 mL), yielding 0.35 g (61.4%) of tetradeuterio-N-hydroxy ornithine **69a** after ion exchange chromatography: TLC same as that of **69**; ^1H NMR (D_2O, 80 MHz) δ 4.25 (1H, s), 3.50 (2H, t), 2.0 (1.86 H, bt).

Biosynthetic Studies

Bioassay. Preparation of Endospore Suspension of *Bacillus subtilis.* The endospores of *B. subtilis* were prepared with the help of Peter Yorgey. The procedure provided by Shirley Gerpheride (Upjohn Company, Kalamazoo, MI) for the

preparation of *B. subtilis* spores was adopted. All these operations were carried out under sterile conditions.

B. subtilis UC-902 (obtained from the Upjohn Company) maintained on Nutrient Agar at 4 °C was used for the preparation of endospores. It was streaked on a BHI agar plate [containing 20 mL of sterile 5.2% brain heart infusion agar dehydrated media (Diffco) in dd H_2O], using a sterile inoculating loop and incubated upside down at 37 °C for 24 h. Two isolated colonies were scraped off the agar using the inoculating loop and placed in 5 mL BHI broth (consisting of 3.7% brain heart infusion broth dehydrated media in dd H_2O), and vortexed to get a homogeneous solution. A portion (1.2 mL) of this was pipetted into two Roux bottles, each containing 100 mL sporulating agar [prepared from 3% trypticase soy broth (BBL), 1% $MnCl_2 \cdot 4H_2O$ and 1.5% Bacto Agar (Diffco) in dd H_2O], spread evenly on the surface and incubated at 32 °C. After two days, when the growth was found to be good, the incubation was shifted to 37 °C (to induce sporulation) and incubated for 6 days, at which time the sporulation was found to be optimum (microscopic examination of the spores by staining with crystal violet; endospores are resistant to staining). The spores were harvested as follows. Sterile 0.9% saline (5-10 mL) was placed into each Roux bottle and the spores were carefully scraped off the surface using a sterile inoculating loop and poured into sterile 50 mL centrifuge tubes. The agar surface was rinsed with saline solution (2 x 10 mL) and the combined spore suspensions centrifuged at 10000xg. The resulting pellets were resuspended in saline solution (a total of 30 mL), vortexed and centrifuged again. The resulting pellets were diluted to 50 mL with sterile saline, vortexed and 3 mL of this suspension was placed in each of 15 steile screw cap slant tubes. They were pasteurized at 70 °C in a water bath for 90 min with gentle agitation and stored in 4 °C for further use.

Preparation of Bioassay Plates. Sterile peptone agar (consisting of 100 mL solution A at pH 6.2, 1 mL of solution B, 0.1 mL solution C and 0.2 mL solution D) equilibrated at 50 °C in a water bath was inoculated with 1% v/v of diluted stock spore suspension of *B. subtilis*: to minimize the error during the transfer, 0.5 mL of the stock spore was initially diluted to 5 mL using sterile saline and 1 mL of this suspension was used per 100 mL of peptone agar. Eight milliliters of the resulting agar was dispensed on each petri plate, allowed to solidify and used for the bioassay. These plates could be stored for a maximum of one week at 4 °C, after which the bioassay values were found to deviate from the standard.

The constituents of solutions A, B, C and D are listed below.

Solution A

$Na_2HPO_4 \cdot 7H_2O$	1.7 g
KH_2PO_4 (anhydrate)	2.0 g
$(NH_4)_2SO_4$ (anydrate)	1.0 g
$MgSO_4$	0.1 g
Peptone agar	15 g
dd H_2O	1 L

The pH of the solution was adjusted to 6.2 using 1N NaOH.

Solution B

Glucose	50 g
dd H_2O	250 mL

Solution C

$FeCl_2 \cdot 4H_2O$	200 mg
dd H_2O	100 mL

Solution D	
$NaMoO_4 \cdot 2H_2O$	25 mg
$CoCl_2 \cdot 6H_2O$	25 mg
$CuSO_4$ (anydrate)	50 mg
$MnSO_4$	0.25 g
$CaCl_2$	2.5 g
$ZnCl_2$	0.25 g
dd H_2O	500 mL

The Bioassay. Paper bioassay disks (740-E)[164] were placed evenly on the agar plate prepared as above and 80 μL of the solution to be assayed was placed on each disk and incubated at 37 °C for 21 h. The diameter of the inhibition zone was measured and used to calcaulte the antibiotic equivalent from the standard curve. Generally the assay was done in duplicate. In the case of fermentations using the baffled flask, the broth was diluted 10 to 100 fold to get the inhibition zone in the proper range.

Fermentation. Maintenance of S. sviceus. Initially, *S. sviceus* was maintained on Hickey-Tresner agar[81] slants at 4 °C. However, the production of acivicin was found to decrease as the slant aged (~two months). Spores were generated on new slants prepared from the original slant (from Upjohn Company) and were transferred to 5 mL sterile 0.9% NaCl containing 0.01% Tween 80. This spore suspension was then vortexed until a reasonably even suspension was achieved. 0.5 mL of this spore-saline suspension was transferred to sterile soil in a culture tube, incubated at 26 °C for 2 weeks, and then stored at 4 °C.

Production of Acivicin and 4-Hydroxyacivicin. Sterile seed broth (50 mL) in a 250 mL Erlenmeyer flask was inoculated with *S. sviceus* spores from the soil

culture using a sterile inoculating loop. The seed culture was incubated at 28 °C and 270 rpm in a gyrotatory shaker for 69 h, at which time it showed dense growth. Using a sterile pipette, 5 mL of this seed culture (2.5% v/v) was transferred to 200 mL of sterile production broth prepared in a 1 L baffled flask and this was then incubated at 32 °C and 250 rpm in the incubator shaker. The production was found to be maximum at 120 h.

Feeding Protocol. Labeled ornithines and β-hydroxyornithines were fed in a single pulse at 48 h after inoculation. Labeled N-hydroxyornithines were fed in two pulses, at 48 h and 60 h, respectively, after inoculation of the production broth. Labeled acivicin and hydroxyacivicin were fed in three pulses at 76 h, 88 h, 100 h, respectively. All the fermentations were carried out using baffled flasks. Generally, the precursor to be fed was weighed out, mixed with the appropriate radioactive labeled compound in a 10 mL volumetric flask and diluted to the exact mark with dd H_2O. Duplicate samples of 50 µL each were diluted to 5.0 mL in a volumetric flask. Duplicate samples of 100 µL each were taken for scintillation counting and the remainder was used for the feeding. The precursor solution in a syringe was slowly injected into the production broth at the appropriate time, in a sterile manner by filtering through a Gelman membrane filter (product No. 4192, size 0.2 µm).

Purification of Acivicin and 4-Hydroxyacivicin. The production broth (2 L), harvested after 120 h of inoculation, was centrifuged at 9000xg for 20 min to remove the solid materials. The pellets were washed with a minimum amount of water and the washings were combined. The total volume of the broth was noted and a sample of ~2 mL was taken for bioassay and - when appropriate - radioactivity

counting. Similarly, samples were taken after each stage of purification for the same purpose.

The supernatant was adjusted to pH ~7.8 using 2N HCl and passed through a cation exchange column (Dowex 50x8, H^+, 100-200 mesh, 5 x 47 cm). After removing impurities by washing with water (1500 mL), acivicin and 4-hydroxyacivicin were eluted off the column using 2.5 N NH_4OH. Fractions containing acivicin (determined by ninhydrin test and TLC analysis) were combined and rotoevaporated at ambient temperature to 50 mL. The concentrate was then adjusted to pH 7 with 2N HCl and passed through an anion exchange column (AG3-X4A, 100-200 mesh, 3 x 12 cm). The column was washed with deionized water (1500 mL), 50% CH_3OH (2 L), and 90% MeOH (2.5 L). The column was finally eluted with methanol-H_2O-glacial acetic acid (90:10:3 v/v). Fractions containing acivicin and 4-hydroxyacivicin (determined by ninhydrin test and TLC analysis) were combined, rotoevaporated at 45 °C to remove the solvent and then lyophilized. The residues from these fractions were redissolved in water (10 mL) and evaporated onto a minimum quantity silica gel (0.4 g). The loaded silica gel was then poured on the top of a silica gel column (flash grade, 3 x 22 cm). The column was then eluted with methyl ethyl ketone-acetone-water (65:20:15) and the effluent was monitored by thin-layer chromatography on silica gel plates. Two fractions were obtained. Each fraction was recrystallized from methanol-water, yielding pure acivicin and 4-hydroxacivicin as white crystals. The acivicin and 4-hydroxyacivicin was detected by ninhydrin spray and confirmed by TLC (PrOH-EtOAc-H_2O 4:4:3 on silica gel, R_f 0.6 for 4-hydroxyacivicin, R_f 0.51 for acivicin). Radioactive samples were repeatedly recrystallized until constant specific activity was obtained (± 3%). Samples for radioactivity counting as well as for ^2H NMR spectroscopic analysis were

dried for 24 h in an Abderhalden dryer under vacuum and heated with acetone at reflux.

Calculations in Feeding Experiments. Various calculations were performed to determine the % incorporation, enrichment, dilution and specific activity based on the amount of precursor fed, bioassay and radioactivity data. The formulas used for these calculations are given below.

$$\text{Specific activity fed} = \frac{\text{dpm of the precursor fed}}{\text{mmole of precursor fed}}$$

$$\text{Specific activity isolated} = \frac{\text{dpm of isolated metabolite}}{\text{mmole of isolated metabolite}}$$

$$\text{Dilution} = \frac{\text{Specific activity fed}}{\text{Specific activity isolated}}$$

$$\%\ \text{incorporation} = \frac{100 \times \text{mmole of metabolite isolated}}{\text{mmole of precursor fed} \times \text{dilution}} \times 100$$

$$\text{Enrichment} = \frac{}{\text{Dilution}}$$

$$^2\text{H expected (}\mu\text{mole)} = \frac{\text{enrichment}^*}{100} \times \mu\text{mole of metabolite used for } ^2\text{H NMR}$$

*: based on radioactivity

Feedings. DL-[1-^{14}C]Glutamic Acid. DL-[1-^{14}C]Glutamic acid was fed to separate production broths at 48 h (total fed 32 x 10^6 dpm), 72 h (total fed 30.7 x 10^6 dpm), and 96 h (31.60 x 10^6 dpm). The 96 hour feeding was terminated after a total of 120 h; the broth was then centrifuged, 5.0 mg acivicin added to the supernatant as carrier and worked up in standard fashion. The results are given below:

Source	Acivicin (mg)	Total Activity (dpm)	% of Total Fed
Centrifugate	3.10	12.9 x 10^6	41
After H^+ column	9.0	12.5 x 10^6	39.4
After OH^- column	7.5	12.4 x 10^4	0.4
After silica gel column			
(a) hydroxyacivicin	—	72.4	2.2 x 10^{-4}
(b) acivicin	3	247.8	7.8 x 10^{-4}

Work-up of feedings done at 48 h and fed at 72 h were not finished, since the percent radioactivity remaining was lower than 1% after the cation chromatography.

L-[U-^{14}C]Glutamine. L-[U-^{14}C]Glutamine (34.01 x 10^6 dpm) was fed to a 200 mL production broth at 48 h. After an additional 72 h, the broth was centrifuged and 5.3 mg acivicin was added to the supernatant as carrier. Results are given below.

Source	Acivicin (mg)	Total Activity (dpm)	% of Total Fed
Centrifugate	1.88	25 x 10^5	7.3
After H^+ column	8.16	84.6 x 10^4	2.4
After OH^- column	7	81.1 x 10^3	0.2
After silica gel column			
(a) hydroxyacivicin	—	43.4 x 10^2	1.8 x 10^{-2}
(b) acivicin	3.75	42.8 x 10^2	1.8 x 10^{-2}

Additional experiments were carried out with feedings at 72 h (24.32×10^6 dpm) and at 96 h (25.42×10^6 dpm) These experiments were not finished since after the OH^- column the percentage of radioactivity remaining was very low which is less than 0.2% of the total fed.

[1-^{14}C]Glycine. [1-^{14}C]Glycine (42.21×10^6 dpm) was fed to a 200 mL production broth 48 h after inoculation. Work-up was as described above; again 5 mg acivicin was added as carrier. Results are given below.

Source	Acivicin (mg)	Total Activity (dpm)	% of Total Fed
Centrifugate	0.79	48×10^5	11.4
After H^+ column	4.12	37.4×10^5	9.1
After OH^- column	4.70	22.1×10^4	0.5
After silica gel column			
(a) acivicin	—	22.2×10^3	5.0×10^{-2}
(b) hydroxyacivicin	3.1	15.3×10^3	3.6×10^{-2}

Work-up of a second feeding experiment (38.96×10^6 dpm fed at 72 h) was not finished since after the OH^- column the percentage of radioactivity remaining was low which is less that 0.34%.

DL-[2-^{14}C]Ornithine. Radioactive acivicin **1a** and 4-hydroxyacivicin **2a** were obtained from feeding DL-[2-^{14}C]-ornithine. The fermentation/feeding data are given below.

Fermentation size	200 mL
Total activity of DL-[2-^{14}C]ornithine	20.75 x 10^6 dpm
Feeding mode/time	single pulse at 48 h
Total **1a** produced	1.08 mg
Total **2a** produced	4.7 mg
Acivicin added as carrier	5.0 mg

The radioactivity data obtained during the purification are given in Table 11.

Table 11. Radioactivity Data of **1a** and **2a**

Source	Bioassay	Total Activity (dpm)	% of Total Fed
Centrifugate	1.08 mg	11 x 10^6	53.23
After H^+ column	6.2 mg	14.0 x 10^5	6.75
After OH^- column	4.75 mg	9.0 x 10^5	4.33
After silica gel column			
(a) **1a**	3 mg	9.5 x 10^4	0.46
(b) **1b**	—	4.7 x 10^4	2.28

At this point 25.3 mg of **1** was added as additional carrier and the mixture was recrystallized from CH_3OH-H_2O to a constant specific activity, and the values are given below.

1st recrystallization	1.0 x 10^4 dpm/mmole
2nd recrystallization	7.7 x 10^4
3rd recrystallization	—
4th recrystallization	7.6 x 10^4
5th recrystallization	7.7 x 10^4

Average dpm/mmole of acivicin:	
(mean of 2nd, 4th, 5th recrystallization)	7.67×10^4
Total activity isolated	4.15×10^4 dpm
% incorporation	0.2% (based on both D- and L-isomers)

25.1 mg of **2** was added as carrier and recrystallized from CH_3OH-H_2O to a constant specific activity, and values are given below.

1st recrystallization	26.8×10^5 dpm/mmole
2nd recrystallization	28.5×10^5
3rd recrystallization	27.2×10^5
4th recrystallization	27.0×10^5
5th recrystallization	26.5×10^5
Average dpm/mmole of **2a**:	26.9×10^5 dpm/mmole
Total activity isolated	4.12×10^5 dpm/mmole
% incorporation	1.98% (based on both D- and L-isomers)

Work-up of additional feedings done at 72 h and at 96 h were not finished. After the OH^- column the percentage of radioactivity remaining was less than the 4.33% obtained when ornithine was fed at 48 h.

DL-[5-^{13}C,5-^{15}N]Ornithine, **56a**. 4-Hydroxyacivicin **2b** and acivicin **1b** were obtained from feeding DL-[5-^{13}C,5-^{15}N]ornithine **56a** and details of the fermentation are given below.

Fermentation size	5 x 200 mL
Amount fed	38.8 mg, 0.23 mmole

Total activity of DL-[5-^{14}C]-ornithine	29.20 x 10^6 dpm
Feeding mode/time	single pulse at 48 h
Specific activity fed	127 x 10^6 dpm/mmole
Total **1b** produced	9.17 mg
Total **2b** produced	29.18 mg
Specific activity isolated:	
(a) **1 b**	1.6 x 10^6 dpm/mmole
(b) **2 b**	5.1 x 10^6 dpm/mmole
Pure material obtained after recrystallization:	
(a) **1 b**	6 mg, 0.034 mmole
(b) **2 b**	11.9 mg, 0.06 mmole

The radioactivity data obtained during the purification are given in Table 12.

Table 12. Radioactivity Data of **56a** Feeding

Source	Bioassay	Total Activity (dpm)	% of Total Fed
Centrifugate	9.17 mg	1.46 x 10^7	50
After H^+ column	9.0 mg	10.4 x 10^6	35.7
After OH^- column	7.5 mg	1.2 x 10^5	4.2
After silica gel column			
(a) acivicin	3.8 mg	1.76 x 10^5	0.51
(b) 4-hydroxyacivicin	—	8.0 x 10^5	2.68

Dilution value:

(a) **1 b**	79
(b) **2 b**	24.86

% incorporation:

(a)	**1b**	0.28%
(b)	**2b**	2.64%

The ^{13}C NMR spectra of acivicin **1b** and 4-hydroxyacivicin **2b** were obtained using 11.7 mg of **2b** and 6 mg of **1b** in 450 μL D_2O, respectively. The spectrum of **2b** showed a 4.16% enrichment for C-5 (154.7 ppm, J_{CN}=2.7 Hz overlapping the natural abundance peak). The spectrum of **1b** showed a 1.26% enrichment for C-5 (152.6 ppm, J_{CN}=2.7 Hz overlapping the natural abundance peak). The spin coupled doublets were clearly revealed by substraction of the relevant natural abundance ^{13}C NMR spectrum.

DL-[2,3,3-$^2H_3$]Ornithine, **56b**. 4-Hydroxyacivicin **2c** and acivicin **1c** were obtained from feeding **56b** and the feeding/fermentation data are summarized below.

Fermentation size	10 x 200 mL
Amount of **56b** fed	88.92 mg, 0.52 mmole
Total activity of DL-[5-^{14}C]ornithine	26.7 x 10^6 dpm
Feeding mode/time	single pulse at 48 h after inoculation
Specific activity fed:	51.35 x 10^6 dpm/mmole
Total produced	
(a) **1c**	11.80 mg, 0.066 mmole
(b) **2c**	27 mg, 0.139 mmole
Specific activity isolated:	
(a) **1c**	8.4 x 10^5 dpm/mmole
(b) **2c**	13.75 x 10^5 dpm/mmole

Pure material obtained after recrystallization:

(a) **1c** 3.6 mg, 0.020 mmole

(b) **2c** 27 mg, 0.139 mmole

The radioactivity data obtained during the purification are given in Table 13.

Table 13. Radioactivity Data of **56b** Feeding

Source	Bioassay	Total Activity (dpm)	% of Total Fed
Centrifugate	11.80 mg	11.52×10^6	43.15
After H^+ column	10 mg	42.4×10^5	15.87
After OH^- column	11 mg	6.24×10^5	2.34
After silica gel column			
(a) **1c**	6.5 mg	8.0×10^4	0.30
(b) **2c**	—	3.7×10^5	1.40

Dilution value:	(a)	**1c**	61
	(b)	**2c**	37.3
% incorporation:	(a)	**2c**	1.30%
	(b)	**1c**	0.21%
Amount of **2c** used for ^2H NMR			19.1 mg, 0.098 mmole
^2H expected			1.08 µmole

The ^2H NMR spectral data of **2c** are given in Table 14.

Table 14. ^2H NMR Data of **2c**

δ (ppm)	Assignment	Integration (µmole of ^2H)
1.27	t-BuOH	0.38
4.4	H-2	0
5.3	H-3	1.08 µmole

The ^{2}H NMR spectra of **1c** similarly showed retention of deuterium only at C-3 although, due to the small amount of sample, the signal to noise was much poorer.

DL-[3*S*-$^{2}H_{1}$]Ornithine, **56d**. Acivicin **1d** and 4-hydroxyacivicin **2d** were obtained from feeding **56d** and the fermentation/feeding data are summarized below.

Fermentation size	5 x 200 mL
Amount of **56d** fed	90 mg, 0.53 mmole (85% 3*S*-^{2}H, 15% 3*R*-^{2}H)
Total activity of DL-[5-^{14}C]Ornithine	39 x 10^{6} dpm
Feeding mode/time	single pulse at 48 h
Specific activity fed:	73 x 10^{6} dpm/mmole
Total produced	
(a) **1d**	21.48 mg, 0.121 mmole
(b) **2d**	61.6 mg, 0.317 mmole
Specific activity isolated:	
(a) **1d**	1.0 x 10^{6} dpm/mmole
(b) **2d**	1.8 x 10^{6} dpm/mmole
Pure material obtained after recrystallization:	
(a) **1d**	4.2 mg, 0.024 mmole
(b) **2d**	17.6 mg, 0.090 mmole

Details of the radioactivity data obtained during the purification are given in Table 15.

Table 15. Radioactivity Data of **56d** Feeding

Source	Bioassay	Total Activity (dpm)	% of Total Fed
Centrifugate	21.48 mg	1.5×10^7	38.46
After H^+ column	21.00 mg	1.2×10^7	31.40
After OH^- column	21.5 mg	9.3×10^5	2.38
After silica gel column			
(a) **1d**	12.5 mg	6.9×10^4	0.18
(b) **2d**	—	4.3×10^5	1.12

Dilution value:	(a)	**1d**	77.3
	(b)	**2d**	46
% incorporation:	(a)	**1d**	0.29%
	(b)	**2d**	1.32%
Amount of material used for ^2H NMR			
	(a)	**1d**	4.2 mg, 0.024 mmole
	(b)	**2d**	10.2 mg, 0.053 mmole
^2H expected	(a)	**1d**	0.26 μmole
	(b)	**2d**	0.96 μmole

The ^2H NMR spectral data of **2d** are given in Table 16.

Table 16. ^2H NMR Data of **2d**

δ (ppm)	Assignment	Integration (μmole of ^2H)
1.27	t-BuOH	0.38
5.3	H-3	0.07 μmole (due to the 15% of 3*R* isomer present in the **56d** fed)

The ^2H NMR spectra of acivicin showed retention of deuterium only at C-3 although, due to the small amount of sample, the signal to noise was much poorer.

DL-[3*R*-$^{2}H_{1}$]-ornithine, **56c**. Acivicin **1e** and 4-hydroxyacivicin **2e** were obtained from feeding **56c** and the fermentation/feeding data are summarized below.

Fermentation size	10 x 200 mL
Amount of **56c** fed	91 mg, 0.54 mmole (70% 3*R*-^{2}H, 30% 3*S*-^{2}H)
Total activity of DL-[5-^{14}C]Ornithine fed	37 x 10^{6} dpm
Specific activity fed:	6.87 x 10^{6} dpm/mmole
Total material produced	
(a) **1e**	22.24 mg
(b) **2e**	73.3 mg
Pure material obtained after recrystallization:	
(a) **1e**	11.1 mg, 0.062 mmole
(b) **2e**	18 mg, 0.093 mmole

Details of the radioactivity data obtained during the purification are given in Table 17.

Table 17. Radioactivity Data of **56c** Feeding

Source	Bioassay	Total Activity (dpm)	% of Total Fed
Centrifugate	22.24 mg	14.7 x 10^{6}	39.8
After H^{+} column	19 mg	11 x 10^{6}	29.9
After OH^{-} column	17.5 mg	7.2 x 10^{5}	1.95
After silica gel column			
(a) **1e**	—	8.8 x 10^{4}	0.23
(b) **2e**	—	3.62 x 10^{5}	0.98

Dilution value:	(a)	**1e**	80.8
	(b)	**2e**	47
% incorporation:	(a)	**1e**	0.29%
	(b)	**2e**	1.49%
Amount of material used for ^2H NMR			
	(a)	**1e**	6.8 mg, 0.038 mmole
	(b)	**2e**	11.0 mg, 0.057 mmole
^2H expected:	(a)	**1e**	0.52 μmole
	(b)	**2e**	0.83 μmole

The ^2H NMR spectral data of **1e** and **2e** are given in Table 18.

Table 18. ^2H NMR Data of **1e** and **2e**

Compound	δ	Assignment	Integration (μmole of ^2H)
Acivicin	5.2	H-3	0.35
4-Hydroxyacivicin	5.3	H-3	0.39

Retention of 3*R*-^2H: 47% (based on comparison of NMR integral with ^{14}C incorporation).

Oxygen-18 Fermentation. A seed culture of *S. sviceus* was prepared in the usual fashion and used to inoculate two production broths (200 mL each in 1 L baffled flasks equipped with ball joints). $NaHCO_3$ (0.1 g) in 10 mL water was sterilized by micropore filtration and added to each production broth as a CO_2 source. The broths were connected in series and then attached to a closed system by two sterile cotton filters. The system contained a burette which was initially filled with $^{16}O_2$, a small aquarium pump, and a CO_2 trap (aqueous 3M KOH). Air was circulated at the rate of 2 L/min while the fermentation flasks were shaken at 32 °C (250 rpm.

The burette was filled with $^{18}O_2$ at 44 h, refilled periodically until the 76th hour, and thereafter refilled with $^{16}O_2$.

A total of 3.39 L $^{18}O_2$ had been consumed. The fermentation was worked up as usual after 120 h. The broth was then purified by using the standard procedure. 4-Hydroxyacivicin **2f** (12.7 mg) was obtained: ^{13}C NMR (100.6 MHz, D_2O) δ 170.2 (C-1), 154.7 (c-5), 81.7 (C-3), 77.2 (C-4), 53.1 (C-2). In addition to the ^{13}C-^{16}O resonance for C-4 (77.2 ppm), an upfield ^{13}C-^{18}O resonance at 77.2 ppm (isotope shift 1.2 Hz) was observed (46% ^{18}O enrichment).

N-t-Butyloxycarbonyl 4-Hydroxyacivicin, 73. 4-Hydroxy-acivicin, **2**, (10 mg, 0.05 mmole) was dissolved in 50% aq. dioxane (200 μL) at room temperature. Triethylamine (17.77 μL, 0.128 mmole) was added followed by BOC-ON (25.32 mg, 0.103 mmole) was added and the mixture stirred at room temperature overnight. H_2O (10 mL) was then added and the mixture extracted with EtOAc (50 mL). The aqueous phase was adjusted to pH 3.82 and extracted with EtOAc. The extracts were dried over Na_2SO_4 and concentration of the combined organic extracts gave 15 mg (55%) of desired product as an oil: TLC (8:2 EtOAc/CH_3OH) R_f 0.42; ^1H NMR ($CDCl_3$, 80 MHz) δ 7.8 (2H, br), 5.6 (1H, d), 5.1 (1H, br), 4.5 (1H, m), 1.4 (10H, s); ^{13}C ($CDCl_3$, 100 MHz) δ 172.61, 156,37, 152.15, 84.22, 81.63, 79.51, 52.46, 28.20. FABMS (glycerol, positive ion), m/e (relative intensity) 293 (100), 295 (32.7); High resolution mass spectrum calcd for $C_{10}H_{15}N_2O_3Cl$: 293.05402; found: 293.05166.

Oxygen-18 Labeled N-t-Butyloxycarbonyl-4-Hydroxyacivicin, 73a. The above mentioned procedure was followed to convert oxygen-18 labeled 4-hydroxyacivicin, **2f**, (10.1 mg, 0.056 mmole) into 16 mg (51%) of oxygen-18 N-

t-butyloxycarbonyl 4-hydroxyacivicin: FABMS (positive ion): m/e (relative intensity) 293 (M-1, 100), 205 (84.6), 297 (24.8), 299 (2.9).

E-β-Hydroxy-L-[4,4-$^{2}H_{2}$]ornithine, 74a. Acivicin **1h** and 4-hydroxyacivicin **2h** were obtained from feeding **74a** and fermentation/feeding data are summarized below.

Fermentation size	10 x 200 mL
Amount of **74a** fed	50 mg, 0.33 mmole
Total activity of DL-[5-^{14}C]Ornithine	11.2 x 10^{6} dpm
Feeding mode/time	single pulse at 48 h
Specific activity fed:	41.33 x 10^{6} dpm/mmole
Total material produced	
(a) **1h**	21.23 mg, 0.119 mmole
(b) **2h**	89.7 mg, 0.46 mmole
Specific activity isolated	
(a) **1h**	4.68 x 10^{5}
(b) **2h**	1.37 x 10^{6}
Pure material obtained after recrystallization:	
(a) **1h**	4.3 mg, 0.024 mmole
(b) **2h**	19.4 mg, 0.1 mmole

Details of the radioactivity data obtained during the purification are given in Table 19.

Table 19. Radioactivity Data of **74a** Feeding

Source	Total Activity (dpm)	% of Total Fed
Centrifugate	5.60×10^6	50
After H^+ column	3.65×10^6	32.58
After OH^- column	5.9×10^5	5.3
After silica gel column		
(a) **1h**	6.1×10^4	0.5
(b) **2h**	5.3×10^5	4.7

Dilution value:	(a)	**1h**	80.0
	(b)	**2h**	30.16
% incorporation:	(a)	**1h**	0.5%
	(b)	**2h**	5.6%
Amount of material used for ^2H NMR:			
	(a)	**1h**	4.3 mg, 0.024 mmole
	(b)	**2h**	12 mg, 0.061 mmole
^2H expected:	(a)	**1h**	0.24 μmole
	(b)	**2h**	1.61 μmole

On analysis of the ^2H NMR spectra of acivicin **1h** and 4-hydroxyacivicin **2h**, no enrichment of deuterium was seen.

T-β-Hydroxy-L-[4,4-$^2H_2$]-ornithine, 75a. Acivicin **1h** and 4-hydroxyacivicin **2h** were obtained from feeding **75a** and the fermentation/feeding data are summarized below.

Fermentation size	10 x 200 mL
Amount of **75a** fed	51.6 mg, 0.279 mmole

Total activity of DL-[5-^{14}C]-ornithine	24.2 x 10^6 dpm
Feeding mode/time	single pulse at 48 h
Specific activity fed:	86.7 x 10^6 dpm/mmole
Total material produced	
(a) **1 g**	19.7 mg, 0.11 mmole
(b) **2 g**	68 mg, 0.38 mmole
Specific activity isolated	
(a) **1 g**	5.3 x 10^5 dpm/mmole
(b) **2 g**	2.5 x 10^6 dpm/mmole
Pure material obtained after recrystallization:	
(a) **1 g**	6.7 mg, 0.03 mmole
(b) **2 g**	27.8 mg, 0.14 mmole

Details of the radioactivity data obtained during the purification are given in Table 20.

Table 20. Radioactivity Data of **75a** Feeding

Source	Total Activity (dpm)	% of Total Fed
Centrifugate	1.25 x 10^6	51.6
After H^+ column	8.8 x 10^6	36.3
After OH^- column	9.35 x 10^5	4.2
After silica gel column		
(a) **1 h**	7.2 x 10^4	0.3
(b) **2 h**	8.9 x 10^5	3.7

Dilution value:	(a)	**1 g**	163
	(b)	**2 g**	34.7

% incorporation:	(a)	**1g**	0.23%
	(b)	**2g**	3.6%
Amount of material used for ^2H NMR:			
	(a)	**1g**	6.7 mg, 0.038 mmole
	(b)	**2g**	11.0 mg, 0.056 mmole
^2H expected:	(a)	**1g**	0.18 μmole
	(b)	**2g**	1.29 μmole

On analysis of the ^2H NMR spectra of acivicin **1h** and 4-hydroxyacivicin **2h**, no enrichment of deuterium was seen.

Protocol for Erythro- and Threo-β-Hydroxy-L-Ornithine Trapping Experiments. The following description affords a general protocol for the trapping experiments. A small production fermentation (100 mL) was harvested after the desired duration. The broth was centrifuged and the supernatant was decanted. The pellet was resuspended in water (100 mL) and sonicated (output control 6, 90% duty cycle) for five minutes with cooling to break the cells. The supernatant was centrifuged at 19800xg; the combined supernatants were then lyophilized, the residue dissolved in 50 mL DH_2O, and 5 mg T- or E-β-hydroxy ornithine was added. After adjustment to pH 4.3, the solution was loaded onto a cation ion exchange column (Dowex 50Wx4, H^+, 100-200 mesh, 2 x 15 cm). After loading, the column was washed with deionized water, and then eluted with a O-2N HCl gradient solution (500 mL total volume) at a flow rate of 1.5-20 mL/min. Cellulose PLC developed with BuOH:H_2O:AcOH (3:1:1 v/v) was used to further purify this material. The nearly pure T- or E-β-hydroxyornithine was diluted with an additional 30-40 mg of pure compound and the mixture recrystallized repeatedly.

Trial 1.

E-β-hydroxyornithine 74 trapping experiment (fed at 48 h, worked up at 63 h)

In the first test, DL-[5-^{14}C]ornithine was administered 48 hours after inoculation, and the fermentation worked up at 63 hours. The results of this experiment are summarized in Table 21.

Table 21. Results of the 15 Hour Trapping of erythro-β-hydroxy-L-ornithine

Fermentation size:	100 mL
Total activity fed DL-[5-^{14}C]ornithine	25.19 x 10^6 dpm
Carrier added after centrifugate	11.4 mg
Amount of carrier added	31.4 mg

Source	Total Activity (dpm)	% of Total Fed
After centrifugate	10.5 x 10^6	42
After H^+ column	5.7 x 10^6	22.7
After PLC	10.5 x 10^6	4.2

Recrystallization	Specific Activity (dpm/mmole)	% Activity Remaining
1	3.7 x 10^5	10.7
2	4.9 x 10^4	5.6
3	6.6 x 10^3	0.2
4	0	0
5	0	0

T-β-hydroxyornithine trapping experiment (fed at 48 h, worked up at 63 h)

DL-[5-^{14}C]Ornithine was administered 48 h after inoculation, and the fermentation worked up at 63 hours. The results of this experiment are summarized in Table 22.

Table 22. Results of the 15 Hour Trapping of T-β-hydroxy-L-ornithine

Fermentation size:	100 mL
Total activity fed DL-[5-^{14}C]ornithine	24.8 x 10^6 dpm
Carrier added after centrifugate	10.1 mg
Amount of carried added after PLC	39.3 mg

Source	Total Activity (dpm)	% of Total Fed
After centrifugate	9.5 x 10^5	38.5
After H^+ column	4.5 x 10^5	18.1
After PLC	10.4 x 10^5	4.2

Recrystallization	Specific Activity (dpm/mmole)	% Activity Remaining
1	2.1 x 10^6	2.3
2	1.1 x 10^6	1.2
3	4.5 x 10^5	0.5
4	1.2 x 10^5	0.1
5	0.4 x 10^5	0.04

Trial 2.

E-β-hydroxyornithine trapping experiment (fed at 55 h, worked up at 58 h)

DL-[5-^{14}C]Ornithine was administered 55 h after inoculation, and worked up at 58 hours. The results of this experiment are summarized in Table 23.

Table 23. Results of 3 hours trapping of E-β-hydroxy-L-ornithine

Fermentation size:	100 mL
Total activity fed DL-[5-^{14}C]ornithine	17.5 x 10^6 dpm
Carrier added after centrifugate	15.1 mg
Amount of carrier added after PLC	49.3 mg

Source	Total Activity (dpm)	% of Total Fed
After centrifugate	8.2×10^6	48
After H^+ column	4.6×10^6	20.6
After PLC	2.1×10^6	12

Recrystallization	Specific Activity (dpm/mmole)	% Activity Remaining
1	1.20×10^6	2.9
2	6.45×10^5	1.6
3	1.80×10^5	0.44
4	6.10×10^4	0.15
5	5.65×10^4	0.14
6	1.65×10^4	0.04
7	1.45×10^4	0.036

T-β-hydroxyornithine trapping experiment (fed at 55 h, worked up at 58 h)

DL-[5-^{14}C]Ornithine was administered 55 h after inoculation, and worked up at 58 h. The results of this experiment are summarized in Table 24.

Table 24. Results of 3 hours trapping of T-β-hydroxy-L-ornithine

Fermentation size:	100 mL
Total activity fed DL-[5-^{14}C]ornithine	31.4×10^6 dpm
Carrier added after centrifugate	11.4 mg
Amount of carrier added after PLC	46 mg

Source	Total Activity (dpm)	% of Total Fed
After centrifugate	2.41×10^7	76.9
After H^+ column	15.3×10^6	48.9
After PLC	8.32×10^6	

Recrystallization	Specific Activity (dpm/mmole)	% Activity Remaining
1	3.9×10^6	4.8
2	2.6×10^6	3.2
3	2.0×10^6	2.4

4	8.4×10^5	1.0
5	1.9×10^5	0.2
6	2.7×10^4	0.03

Trial 3.

E-β-hydroxyornithine trapping experiment (fed at 55 h, worked up at 61 h)

DL-[5-^{14}C]Ornithine was adminstered 55 h after inoculation, and worked up at 61 h. The results of this experiment are summarized in Table 25.

Table 25. Results of 6 hours trapping of E-β-hydroxy-L-ornithine

Fermentation size:	100 mL
Total activity fed DL-[5-^{14}C]ornithine	19.6×10^6 dpm
Amount of carrier added after PLC	42.4 mg
Carrier added after centrifugate	12.5 mg

Source	Total Activity (dpm)	% of Total Fed
After centrifugate	14×10^6	72
After H^+ column	5.8×10^6	30
After PLC	2.4×10^6	12.2

Recrystallization	Specific Activity (dpm/mmole)	% Activity Remaining
1	9.7×10^3	2.6
2	4.5×10^3	1.2
3	1.4×10^3	0.37
4	7.4×10^2	0.20
5	2.3×10^2	0.06
6	1.1×10^2	0.03

T-β-hydroxy-L-ornithine feeding experiment (fed at 55 h, worked up at 61 h)

DL-[5-^{14}C]Ornithine was administered 55 h after inoculation, and worked up at 61 h. The results of this experiment are summarized in Table 26.

Table 26. Results of 6 hours trapping of T-β-hydroxy-L-ornithine

Fermentation size:	100 mL
Total activity fed DL-[5-^{14}C]ornithine	19.6 x 10^6 DPM
Carrier added after centrifugate	12.6 mg
Amount of carrier added after PLC	41 mg

Source	Total activity (dpm)	% of total fed
After centrifugate	14 x 10^6	72.3
After H^+ column	80.7 x 10^5	41.2
After PLC	2.9.8 x 10^5	15.1

Recrystallization	Specific Activity (dpm/mmole)	% Activity Remaining
1	3.3 x 10^6	6.0
2	9.8 x 10^5	1.8
3	4.8 x 10^5	0.88
4	1.6 x 10^5	0.29
5	6.8 x 10^4	0.12
6	1.8 x 10^4	0.03

DL-[3,3,4,4-$^2H_4$]Ornithine 56e. Acivicin **1i** and 4-hydroxyacivicin **2i** were obtained from **56e** and the fermentation/feeding data are summarized below.

Fermentation size	10 x 200 mL
Amount of **56e** fed	75 mg, 0.45 mmole
Enrichment of material fed	100% at H-3, 44% at H-4
Total activity of DL-[5-^{14}C]ornithine	70.1 x 10^6 dpm
Feeding mode/time	single pulse at 48 h
Specific activity fed:	16 x 10^7 dpm/mmole

Total material produced

(a) **1i** 15.0 mg

(b) **2i** 64.3 mg

Specific activity isolated

(a) **1i** 3.9×10^6 dpm/mmole

(b) **2i** 1.3×10^6 dpm/mmole

Pure material obtained after recrystallization:

(a) **1i** 2.4 mg, 0.013 mmole

(b) **2i** 23.5 mg, 0.121 mmole

Details of the radioactivity data obtained during the purification are given in Table 27.

Table 27. Radioactivity Data of **56e** Feeding

Source	Bioassay	Total Activity (dpm)	% of Total Fed
Centrifugate	14.96 mg	8.1×10^6	11.56
After H^+ column	11 mg	2.8×10^6	4
After OH^- column	10.5 mg	4.7×10^5	0.67
After silica gel column			
(a) **1i**	—	6.1×10^4	0.10
(b) **2i**	—	3.5×10^5	0.50

Dilution value:

(a) **1i** 41.2

(b) **2i** 126

Amount of material used for ^2H NMR:

(a)	4-hydroxyacivicin	12 mg, 0.062 mmole
(b)	acivicin	2.4 mg, 0.0135 mmole

^2H expected:

(a)	acivicin	0.32 µmole (H-3), 0.14 µmole (H-4)
(b)	4-hydroxyacivicin	0.49 µmole (H-3) 0.22 µmole (H-4)

The ^2H NMR spectrum data of acivicin **1i** and 4-hydroxyacivicin **2i** are given in Table 28.

Table 28. ^2H NMR Data of Acivicin **1i** and 4-Hydroxyacivicin **2i**

Compound	δ	Assignment	Integration
Acivicin	5.2	H-3	0.12 µmole
	3.4	H-4	0.12 µmole
4-Hydroxyacivicin	5.2	H-3	0.23 µmole
	5.3	H-4	0.23 µmole

L-[5-^{14}C]Acivicin, 1k. 4-Hydroxyacivicin was obtained from the feeding of L-[5-^{14}C]acivicin, **1k**. The fermentation/feeding data are given below.

Fermentation size	200 mL
Total activity of **1k** fed	4.4 x 10^4 dpm
Feeding mode/time	three pulses / 76 h, 88 h, 100 h
Total 4-hydroxyacivicin produced	15.2 mg, 0.078 mmole

The 4-hydroxyacivicin obtained (15.2 mg) was diluted with authentic 4-hydroxyacivicin (5.2 mg) as a carrier. The mixture was then recrystallized from CH_3OH-H_2O to a constant specific activity and the values are given below.

1st recrystallization	4.54 x 10^4 dpm/mmole
2nd recrystallization	5.06 x 10^4 dpm/mmole
3rd recrystallization	5.10 x 10^4 dpm/mmole
4th recrystallization	5.14 x 10^4 dpm/mmole
Specific activity isolated	5.10 x 10^4 dpm/mmole
% incorporation	15.33%

L-4-Hydroxy[5-^{14}C]acivicin, 2k. L-4-Hydroxy[5-^{14}C]acivicin, **2k**, (3.6 x 10^5 dpm) was fed to a 200 mL fermentation broth at 76 h, 88 h and 100 h. This was worked up as usual. There was no radioactivity in the acivicin obtained after the silica gel column chromatography.

DL-δ-N-Hydroxy[3,3,4,4-$^2H_4$]ornithine, 69a. Acivicin **1j** and 4-hydroxyacivicin **2j** were obtained from **69a** and the fermentation/feeding data are summarized below.

Fermentation size	10 x 200 mL
Amount of **69a** fed	114.92 mg, 0.52 mmole
Enrichment of material fed	100% at H-3, 10% at H-4
Feeding mode/time	two pulses, 48 h and 60 h
Total material produced	
(a) **1j**	31.8 mg, 0.178 mmole
(b) **2j**	88.2 mg, 0.45 mmole

Pure material obtained after recrystallization:

(a) **1j** 8 mg, 0.0449 mmole

(b) **2j** 43.1 mg, 0.222 mmole

The ^2H NMR spectral data of acivicin **1j** and 4-hydroxyacivicin **2j** are given below (Table 29).

Table 29. ^2H NMR Data of Acivicin **1k** and 4-Hydroxyacivicin **2k**

Compound	δ	Assignment	Integration
Acivicin	5.2	H-3	1.54 μmole
	3.4	H-4	0.41 μmole
4-Hydroxyacivicin	5.2	H-3	2.23 μmole
	5.3	H-4	2.23 μmole

Dilution value: (a) **1j** 17 (for H-4)

23.2 (for H-3)

(b) **2j** 22.5 (for H-4)

30.3 (for H-3)

% incorporation: (a) **1j** 2.01% (C-4),

1.46% (C-3)

(b) **2j** 3.86% (C-4),

2.80% (C-3)

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