The previous study on the biosynthesis of acivicin (1) and 4-hydroxyacivicin (2) has concluded that ornithine is the primary precursor, and N⁵-hydroxyornithine is the first committed intermediate in the pathway. The rest of the pathway was to be elucidated. For this purpose, diethyl γ-([^18]O)N-oxide, N-p-methoxybenzylidene)-[4,4-²H₂]aminopropyl acetimidomalonate (55b), a precursor of N⁵-[¹⁸]O)hydroxy-[4,4-²H₂]ornithine (23b), [4,4-²H₂]tricholomic acid (5a), [4,4-²H₂]isoxazolidinylglycine (25a), and [4,4-²H₂]isoxazolinylglycine (26a) were synthesized.

Preparation of 55b started from a deuterium exchange reaction of 3-hydroxypropionitrile (49). O-Tosylation, and alkylation with acetimidomalonate gave 3-ethyl 2-acetamido-2-carboxethoxy-4-cyano[4,4-²H₂]-butanoate (51a). Hydrogenation followed by condensation with anisaldehyde gave imine 53a which was oxidized by
[18O]monoperoxyphthalic acid. Isomerization of the resulting oxaziridine yielded 55b.

[4,4-2H2]Tricholomic acid was prepared simply by hydrolysis of acivicin in NaOD/D2O. The key step in the syntheses of [4,4-2H2]isoxazolidinylglycine (25a), and [4,4-2H2]isoxazolinylglycine (26a) was a 1,3-dipolar cycloaddition reaction of [4,4-2H2]vinylglycine methyldene ester (64a) and a chiral nitrene (63) generated in situ from paraformaldehyde and 2,3-O-isopropylidene-5-O-trityl-D-ribose-oxime (74). Separation of the resulting diastereomers and removal of the protecting groups gave 25a. Oxidation of the first deprotection product of the cycloadduct followed by reaction with boron tris(trifluoroacetate) gave 26a.

In addition to synthetic work, a study of fermentation cultures of S. sviceus led to isolations of phenylalanine, tyrosine, tryptophan, leucine, methionine, and methionine sulfoxide.
Syntheses of Labeled Putative Intermediates in Acivicin Biosynthesis

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Typed by researcher for Nan Jiang
To my husband Xingjian and my parents
Acknowledgments

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Syntheses of Labeled Putative Intermediates in Acivicin Biosynthesis

Introduction

Isolation and Structure Determination of Acivicin and 4-Hydroxyacivicin

Acivicin ((αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; AT-125), 1, and 4-hydroxyacivicin ((αS,4S,5R)-α-amino-3-chloro-4-hydroxy-4,5-dihydro-5-isoxazoleacetic acid), 2, have been isolated from Streptomyces svicues.

\[ \text{Cl} \quad \text{R} \quad \text{CO}_2\text{H} \]
\[ \text{N} \quad \text{O} \quad \text{NH}_2 \]

1. \( R = \text{H} \)
2. \( R = \text{OH} \)

Acivicin was first discovered by Hanka and coworkers in 1973 by using an in vitro screening system for detection of antimetabolites with antitumor activity. It was isolated soon thereafter from the fermentation broth. The structure and absolute stereochemistry of acivicin was determined by conventional spectroscopic analysis and X-ray crystallographic study. In addition to the unique structural features, the X-ray study also indicated that there is strong hydrogen bonding between the carboxylate oxygens and the ammonium group. The ring oxygen and nitrogen, on the other hand, do not participate in the hydrogen bonding.

4-Hydroxyacivicin was isolated a year later from cultures of the same organism. It has less than 0.1% of the activity of acivicin against
Bacillus subtilis, the organism employed to monitor the isolation and purification.\textsuperscript{3} The structure and absolute stereochemistry of 2 was also determined by spectroscopic methods as well as X-ray crystallography. Conformational differences between 1 and 2 due to the extra hydroxyl group were clearly observed from the X-ray study. A possible hydrogen bonding between the ammonium nitrogen and the hydroxyl hydrogen was also suggested.

**Biological Activity and On-going Clinical Trials of Acivicin**

Acivicin is an antitumor antibiotic. It also has antifungal activity. Its antibacterial activity was tested against various organisms, but found only positive against *Bacillus subtilis* and *Escherichia coli*.\textsuperscript{1}

The antitumor activity of acivicin was first observed when it was tested against L1210 and P388 lymphoid (murine) leukemias in mice.\textsuperscript{4} Preclinical studies of acivicin soon revealed significant activity against human lung (LX-1) and mammary (MX-1) tumor xenograft systems in nude mice.\textsuperscript{5} It also caused complete regression in the subcutaneous M5076 ovarian carcinoma model. Based on this evidence, clinical trials were conducted, and beneficial treatments were observed in some patients having non-small lung cancer, colon cancer, and malignant glioma.\textsuperscript{6} The stabilization of diseases was observed in most of the cases. However, significant CNS (central nervous system) effects, such as lethargy, fatigue, asthenia, disorientation, depression, and confusion at lower doses, hallucinations, ataxia, amnesia and dizziness at higher doses were reported in all studies, ranging from 33 to 42% of patients tested.\textsuperscript{5}

The mechanism of the CNS effects induced by acivicin are still unknown. One possibility is that acivicin may stimulate or inhibit (the action of) one or more neurotransmitters.\textsuperscript{5} Ibotenic acid\textsuperscript{7} (3) and muscimol\textsuperscript{8} (4), which are acivicin structure analogs are known to have
CNS excitants activity. Other hypotheses have also been proposed, but there is relatively little published experimental data supporting any of them.\(^5\)

![Chemical structures](image)

The anticancer activity of acivicin is believed to be the result of its ability to inhibit some glutamine dependent amidotransferases on the pyrimidine and purine biosynthetic pathway. Before 1990, it was thought that the sites of these inhibition are numerous and critical, as shown in Fig. 1. For example, the irreversible inhibition of carbamoyl phosphate synthetase II (CPS II) which catalyzes the first step (rate limiting step) of pyrimidine biosynthesis would completely block the DNA synthesis of growing cells. A recent study has shown, however, that acivicin is not a potent inhibitor of CPS II but it strongly inhibits CTP and GMP synthetases and partially inhibits N-formylglycineamidine ribotide (FGAM) synthetase of the purine pathway.\(^9\)

In addition to biochemical pharmacology studies,\(^10,11\) substantial effort toward developing acivicin as a potential antitumor agent has lead to studies in areas such as pharmacokinetics,\(^12\) animal model studies,\(^13\) and optimal dosage.\(^14\) For example, combination chemotherapies of acivicin with PALA (N-(phosphonacetyl-L-aspartate)),\(^15\) which is an L-aspartate antagonist, and of acivicin with CDDP (cis-diammine-dichloroplatium II),\(^16\) a nucleic acid salvage inhibitor, have also been investigated.

Positive results have been obtained on the prevention of CNS toxicity
of acivicin by a concomitant use of an amino acid mixture. McGovren has postulated that the transportation of acivicin into the brain could be common to other neutral amino acids where a specific and saturable carrier mediated system is involved. Therefore, the increase of other amino acids may block or reduce the brain uptake of acivicin. The results of their animal experiments are in good agreement with this hypothesis. Among seven cats studied, only one showed symptoms of sedation, while none showed ataxia, a symptom of lost control (imbalance) in movement.
However in the control experiment, more than 75% of the cats exhibited the above behaviors.

In addition to its antitumor activity, acivicin was reported recently as an antivirus agent.\textsuperscript{18} It effectively killed both the vector and the host form of the parasitic protozoa, \textit{Leishmania domovain}, which is a causative species of visceral leishmaniasis, commonly known as Kala-azar.

\textbf{Naturally Occurring Acivicin Analogs}

Four naturally occurring acivicin analogs, ibotenic acid, 3, musimol, 4, tricholomic acid, 5, and D-cycloserine, 6, have been reviewed in terms of their structure, biological activity and biosynthesis.\textsuperscript{19} In addition to these compounds, lactivicin 7 and a group of isoxazolin-5-ones 8-16 have been reported.

\[
\begin{align*}
\text{5} & \quad \text{CO}_2\text{H} & \quad \text{NH}_2 \\
\text{6} & \quad \text{H}_3\text{N}^+ & \quad \text{O}^- \\
\end{align*}
\]

Lactivicin (7) was isolated from cultures of the soil bacteria \textit{Empedobacter lactamgenus}, YK-258 and \textit{Lysobacter albus}, YK-422.\textsuperscript{20} It's structure was determined by acid and alkaline hydrolysis and spectroscopic analysis, and was found to contain a D-cycloserine moiety and a $\gamma$-lactone ring. A full account of the spectroscopic data was published.\textsuperscript{21} In aqueous solutions, lactivicin exists as an approximate 1:1 mixture of two epimers. The absolute configuration of each was assigned by CD spectra and X-ray crystallography of its derivative, 4-amino-
lactivinic acid. Treatment of 7 with H$_2^{18}$O did not result in incorporation of O-18 into the molecule, thus excluding the possibility that isomerization occurs by hydrolytic cleavage of the lactone or by attack of the hydroxyl anion at the C-6 position. A formation of an imminium cation 17 which is stabilized by the carboxylate group was suggested as an intermediate.

![Fig. 2 Isomerization of lactivicin](image)

Fig. 2 Isomerization of lactivicin

Lactivicin is moderately active against a broad range of Gram-negative bacteria and highly active against Gram-positive bacteria. It's biological activity resembles that of the β-lactam antibiotics, in as much as it has an affinity for penicillin binding proteins and susceptibility to β-lactamases. This was the first non-β-lactam antibiotic found to have such activity. A series of lactivicin derivatives were synthesized recently and were found to have potent antibacterial activities. Attempts to modify the cycloserine moiety and the γ-lactone ring by using its aza analog 18 or γ-lactam analog 19, however, resulted in decreased activity.
Isoxazolin-5-one derivatives (8-16) were isolated from legume seedlings and were identified by chemical degradation and spectroscopic methods.\textsuperscript{27} Of nine derivatives reported, four were amino acids. The heterocyclic ring was found to be very sensitive to dilute alkali and to UV radiation. When 8 was irradiated at 254 nm, three degradation products were identified as shown in Scheme 1. The biological activities of isoxazolin-5-ones have not been fully explored, but 8 was tested on neurons of vertebrates and found to be a weak neuronal excitant of the glutamate type. The synthesis of 8 has been reported.\textsuperscript{28}

\textbf{Scheme 1}

\begin{align*}
8. & \quad R_1 = H, R_2 = CH_2CH(NH_2)CO_2H \\
9. & \quad R_1 = H, R_2 = CH_2CH_2NH_2 \\
10. & \quad R_1 = H, R_2 = CH_2CH_2CH(NH_2)CO_2H \\
11. & \quad R_1 = H, R_2 = CH_2CH-CN \\
12. & \quad R_1 = H, R_2 = CH_2CHNHCOCH_2\text{CH}_2CH(NH_2)CO_2H \\
13. & \quad R_1 = H, R_2 = CH_2CO_2H \\
14. & \quad R_1 = H, R_2 = CH_2CHCO_2H \\
15. & \quad R_1 = H, R_2 = \beta\text{-D-glucose} \\
16. & \quad R_1 = CH_2CH(NH_2)CO_2H, R_2 = \beta\text{-D-glucose}
\end{align*}

No attempt has been made to investigate the biosynthesis of
lactivicin, and only a little work has been done on the isoxazolin-5-ones. Murakoshi et al. studied the origin of the side chains of compounds 8, 15, and 16 by incubating isoxazolin-5-one, 20, and the appropriate side chain precursors with crude enzyme preparations. It was found that O-acetylserine was the donor of the alanine side chain\textsuperscript{29}(Scheme 2), whereas UDP-glucose was the donor of the glucoside substituent.\textsuperscript{30} After glucosidation of the ring-nitrogen, nucleophilic alaninylation then occurred at position C-4.\textsuperscript{31}

**Scheme 2**

\[
\begin{align*}
\text{UDP-glucose} & \quad \rightarrow \quad \text{CH}_3\text{OC(O)OCH}_2\text{CHCO}_2\text{H} \\
\beta-D\text{-glucose} & \quad \rightarrow \quad \text{CH}_2\text{CHCO}_2\text{H} \quad \text{NH}_2
\end{align*}
\]

**Biosynthesis of Acvicin and 4-Hydroxyacivicin**

Research on the biosynthesis of acivicin and 4-hydroxyacivicin has been carried out previously in our group,\textsuperscript{32} as an extension of studies of the \(\beta\)-amino acids.\textsuperscript{33} Among three radio-isotopically labeled five carbon amino acids tested as primary precursors, glutamine, glutamic acid and ornithine, only ornithine was incorporated into both antibiotics (Scheme 3, only 1 is shown). The \(N^6\)-amino group of ornithine was shown to be retained by feeding \textsuperscript{13}C and \textsuperscript{15}N doubly-labeled ornithine, 21a, and
analyzing the derived antibiotics by $^{13}$C NMR spectroscopy. When [2,3,3-$^{2}$H$_{3}$]ornithine, 21b, was tested with the organism, the complete loss of the C-2 deuterium and the partial retention of the C-3 deuterium were resulted. Further feeding experiments with [3R-$^{2}$H]ornithine 21c, and [3S-$^{2}$H]ornithine, 21d, revealed that it was the 3S deuterium that was partially retained, while the 3R deuterium was lost. The net result, therefore, is the retention of stereochemistry at C-3 in the introduction of the oxygen.

**Scheme 3**

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<tbody>
<tr>
<td>21a</td>
<td>$R = {^{13}}$CH$<em>{2}$${^{15}}$NH$</em>{2}$</td>
<td>1a</td>
</tr>
<tr>
<td>21b</td>
<td>$R = CH_{2}$NH$<em>{2}$, $H</em>{A} = H_{B} = H_{C} = {^{2}}H$</td>
<td>1b</td>
</tr>
<tr>
<td>21c</td>
<td>$R = CH_{2}$NH$<em>{2}$, $H</em>{A} = {^{2}}H$, H$<em>{B} = H</em>{C} = H$</td>
<td>1c</td>
</tr>
<tr>
<td>21d</td>
<td>$R = CH_{2}$NH$<em>{2}$, $H</em>{A} = H_{C} = H$, H$_{B} = {^{2}}H$</td>
<td>1d</td>
</tr>
</tbody>
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The source of the isoxazoline ring oxygen and the 4-hydroxyl oxygen of 2 was found to be derived from molecular oxygen by fermentation in the presence of $^{18}$O$_{2}$ (Scheme 4). The extent of oxygen-18 incorporation to the two positions was quite different. Whereas the 4-hydroxyl oxygen had a 51% enrichment, the ring oxygen was only enriched by 14%. Further, in

**Scheme 4**

$^{18}$O$_{2}$ fermentation

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<tr>
<td>1e</td>
<td>$R = H$, $\cdot O = ^{18}$O</td>
</tr>
<tr>
<td>2e</td>
<td>$R = OH$, $\cdot O = ^{18}$O</td>
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order to determine whether the hydroxylation of 2 was the last step of the biosynthesis, radiolabeled 1f was fed to the organism (Scheme 5). As expected, when 2f was isolated it had incorporated radioisotope indicating that it is indeed from 1f.

Scheme 5

\[
\text{Cl} \quad \text{CO}_2\text{H} \quad \text{NH}_2 \\
\text{Cl} \quad \text{CO}_2\text{H} \quad \text{NH}_2
\]

1f \( \cdot \) = \^{14}\text{C}, R = \text{H} \quad \text{2f} \ \Delta = \^{14}\text{C}, R = \text{OH} \\
2g \ \cdot \ = \^{14}\text{C}, R = \text{OH} \quad 1g \ \Delta = \^{12}\text{C}, R = \text{H}

From the results of the above feeding experiments it was suggested that hydroxylation at the β-carbon might be involved in the biosynthesis of 1 and the hydroxyl oxygen would eventually become ring oxygen. In addition, this hydroxylation step might be facilitated by the involvement of pyridoxal phosphate in yielding an allylic β-carbon (Scheme 6). The same mechanism has been proposed for arginine incorporation in streptothricin biosynthesis.\textsuperscript{33} In both cases, the loss of the C-2 deuterium was observed and could be well explained by this mechanism. The partial loss of the 3S deuterium of 21d and of the \(^{18}\text{O}\) label of the ring oxygen may also be accounted for by pyridoxal phosphate participation as shown in Scheme 6. Based on this hypothesis, deuterium-labeled erythro 3-hydroxyornithine, 22a, and threo 3-hydroxyornithine, 22b, were each synthesized and tested as an intermediate, again by whole cell feeding experiments (Scheme 7). Surprisingly, no incorporation was observed. One possibility to obtain such negative results was the loss of the deuteriums at C-4 position during
the biosynthesis. Therefore, [4,4-\textsuperscript{2}H\textsubscript{2}]ornithine, 21h, was fed to the organism. The incorporation of these deuteriums, however, was clearly observed on \textsuperscript{2}H NMR (Scheme 8).

Scheme 6

As an alternative hypothesis, the ring oxygen may be derived from other sources, for example, by hydroxylation at the \(\delta\) amino group of 21. Thus N-hydroxyornithine 23, might then be the first intermediate. When this hypothesis was tested by feeding 23a, a positive result was obtained
Scheme 7

\[
\begin{align*}
\text{R} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2 \quad \text{NH}_2 \\
\text{R'} & \quad \text{H} \\
\end{align*}
\]

22a. \( R' = \text{CH}_2\text{NH}_2, \quad R_1 = \text{OH}, \quad R_2 = \text{H}, \quad \text{H} = ^2\text{H} \)

22b. \( R' = \text{CH}_2\text{NH}_2, \quad R_1 = \text{H}, \quad R_2 = \text{OH}, \quad \text{H} = ^2\text{H} \)

This hypothesis was tested by feeding 23a, a positive result was obtained (Scheme 8). Therefore, it was established that 23 is the intermediate following 21 on the acivicin biosynthetic pathway.

Scheme 8

\[
\begin{align*}
\text{R} & \quad \text{H} \\
\text{R}_1 & \quad \text{R}_2 \\
\text{NH}_2 & \quad \text{CO}_2\text{H} \\
\text{R'} & \quad \text{H} \\
\end{align*}
\]

21h. \( R' = \text{CH}_2\text{NH}_2, \quad \text{H} = ^2\text{H}, \quad R = \text{H} \)

23a. \( R' = \text{CH}_2\text{NH}_2, \quad \text{H} = ^2\text{H}, \quad R = ^2\text{H} \)

Rationale for the Present Study

The present study was designed to establish the biosynthetic pathway of 1 and 2 beyond N\textsuperscript{6}-hydroxyornithine. Two hypotheses are shown in Schemes 9 and 10. Scheme 9 is based on the possibility that the oxygen of the hydroxylamino group (indicated by \( ^*\text{O} \)) of 23 is retained. A double inversion of stereochemistry at C-3 would have to take place to explain the retention of the 3S hydrogen of 21.

In Scheme 9, pathway a shows the hypothetical \( \beta \)-hydroxylation (through pyridoxal phosphate, not shown) of 23. Once hydroxylated,
nucleophilic attack of the N-hydroxyl group at C-3 of 24 would result in isoxazolidinyl ring formation to give 25. Further elaboration of 25 either through 5 or through 26, or through both, would result in 1.

Scheme 9

It should be noted that N-hydroxylamino acids, such as 23, have been reported to be labile and are usually stable only as solids. Like N-hydroxylamines, they are strong reducing agents; as a result, they themselves are easily oxidized to the corresponding oximes. In fact, free N-hydroxylamino acids have never been isolated from biological systems although they have been suggested as intermediates of various natural products. Therefore, the immediate oxidation of 23 may take place. The oxidation of the N-hydroxyl amino group of 23 (pathway c) would yield an oxime, 27, via a nitroso compound, whereas the oxidation of the adjacent
carbon would yield a hydroxymate 28 (pathway b). In the latter case, the stability of the NH-OH bond comes from the delocalization of the electron density through the N-hydroxy amide bond. Hydroxymates have been found frequently occurring in biological systems.\(^{34}\)

The possibility of 28 as an intermediate raises the question of why glutamine is not the primary precursor of 1, since N-hydroxylation of the amide group of glutamine would also yield 28. N-oxidations are generally catalyzed by NADPH-dependent monooxygenases, such as cytochrome P-450 monooxygenases and flavin containing monooxygenases.\(^{37}\) Cytochrome P-450 monooxygenases catalyze the oxidation of amide groups and amines that lack \(\alpha\)-carbon hydrogens, e.g., aromatic amines. Flavin containing monooxygenases, however, catalyze the oxidation of a vast majority of aliphatic amines or naturally occurring alkaloids. The substrate specificity of the latter enzymes is such that only nucleophilic amino groups can be oxidized through this mechanism. Aromatic amines, for instance, where the electrons on nitrogen are delocalized through the \(\pi\) system are not substrates of these enzymes. Therefore, the fact that only ornithine, 22, rather than glutamine is N-hydroxylated in the biosynthesis of 1 may be an indication of the presence of only flavin-containing monooxygenases in the cell. The study of the \(N^8\)-hydroxylation of lysine also suggested a P2(flavin containing monooxygenase)-mediated N-hydroxylation process.\(^{38}\)

Pathway c-a' in Scheme 9 represents the possibility that ring formation could occur through a dehydroamino acid 29. Numerous dehydroamino acids have been found as constituents of fungal metabolites.\(^{39}\) Its formation by dehydration of \(\beta\)-hydroxyamino acids or by a direct dehydrogenation of amino acids have all been suggested,\(^{39a}\) as proposed in the transformation of 27 to 29 and 30 to 29. The
tautomeration of 29 (Equation 1), or the addition, and elimination of \( \text{H}_2\text{O} \) may also account for the partial loss of the C-3 deuterium.

\[
\text{Eq. 1}
\]

The later steps of the biosynthesis may involve oxidation of 25 to isoxazoline 26 as had been mentioned before. A similar transformation was found as an enzymatic process in the study of biosynthesis of berberine. Molecular oxygen was shown to be the oxidant, which is subsequently reduced to hydrogen peroxide. The chlorination of 26 may also be an enzymatic process involving chloroperoxidase and \( \text{H}_2\text{O}_2 \) released from the last step. Alternatively, compound 25 or 26 may be converted to tricholomic acid, 5, and subsequently chlorinated to yield 1.

Scheme 10
If the ring oxygen of 1 does not derive from the N-hydroxyl group of 23, the biogenesis proposed in Scheme 10 would apply. This might be the circumstance, because the biological hydroxylations of sp\(^3\) hybridized carbon are often observed with retention of configuration.\(^{41}\) Subsequently, ring closure would result from the nucleophilic attack of the 3-hydroxyl group of 32, 33, or 34 on nitrogen at various oxidation states. For protonation of nitrogen of the N-hydroxylamino group of 33 low pH of the medium would be required,\(^{42}\) which would allow nucleophilic attack by the C-3 hydroxyl group to occur. Further elaboration of these compounds would then yield 1.

Oxygen exchange with the medium at a nitroso stage in the biosynthesis of 1 was also proposed to explain the fact that \(^{18}\)O\(_2\) was incorporated into the ring oxygen less than 1/3 compared to what was incorporated into the C-4 hydroxyl oxygen (Scheme 6).\(^{43}\) Aoyama et al. have studied oxygen exchange of aromatic and the tertiary C-nitroso compounds in the presence of H\(_2^{18}\)O.\(^{44}\) Under acidic conditions at room temperature, only negligible exchange (< 0.1%) was observed. The mechanism they proposed is shown in equation 2-5.

\[
\begin{align*}
R-N=O + H_3O^+ & \rightleftharpoons R-N^+OH + H_2O & \text{eq. 2} \\
R-N^+OH + H_2^{18}O & \rightleftharpoons R-N-OH^{18}OH_2 & \text{eq. 3} \\
R-N-OH^{18}OH_2 & \text{fast} \rightleftharpoons R-N^{18}OH & \text{eq. 4} \\
R-N^{18}OH & \text{fast} \rightleftharpoons R-N=^{18}O & \text{eq. 5}
\end{align*}
\]

One may argue that our higher fermentation temperature (32°C) may activate the oxygen exchange. However, it is known that nitroso
compounds possessing an α-hydrogen tautomerize to oximes.\textsuperscript{45} Therefore, the rate of tautomerization is probably much faster than exchange, making the oxygen exchange an unfavorable process.

\textbf{Purpose of the Present Study}

The biological functions of acivicin and its unusual structural features have made it challenging to both biological and synthetic chemists. The total synthesis of 1 has been achieved a number of times.\textsuperscript{46} A thorough understanding of its synthesis \textit{in vivo} was our ultimate goal.

The various possibilities for the biosynthesis of 1 makes its study interesting. Although the biogenetic schemes proposed for 1 look complicated, there are only a few key intermediates, namely: tricholomic acid (5), isoxazolidinyl glycine (25), and isoxazolinyl glycine (26). The \textit{in vivo} tests of these three putative intermediates plus a test of whether the oxygen of the N-hydroxyl group of 23 is retained would simplify the whole picture. Therefore, the purpose of the present study has been to prepare the appropriately labeled 23, 5, 25, and 26 and, if possible, carry out these feeding experiments.
Results and Discussion

Synthetic Studies

Preparation of a Precursor of $N^6$-[18O]hydroxyornithine, 55b

To understand the biosynthetic origin of the oxygen atom in the isoxazoline ring of acivicin, preparation of O-18 labeled $N^6$-hydroxyornithine was the first target of this work. In addition to O-18, we also decided to introduce a deuterium label at an appropriate position. While 18O would be used to determine whether this oxygen is retained or lost in the biosynthesis of 1, the deuterium could serve as an internal standard for proof of the successful incorporation of 23.

The synthesis of 23 has been reported several times before. The key intermediates of these syntheses are shown in Scheme 11. Approach a used hydroxylamine derivative 35 as starting material which was alkylated in two steps to give 36. Removal of the protecting groups of 36 yielded 23. In approaches b and c, partial reduction of 38 or 39 followed by hydrolysis gave 23 in low overall yield. The oxygen atoms were introduced by the reaction of NaNO$_2$ with a bromo compound. Approach d involved an N-alkylation of anti-benzaldoxime, 40, with methyl 2-acetamido-5-iodovalerate, 41, to give nitron 42. The N-alkylation step was reported in low yield (41%). A similar reaction had been attempted in our group without success. Benzylaldoxime is commonly prepared from benzylaldehyde and hydroxylamine. Since neither the O-18 labeled hydroxylamine nor the O-18 labeled metal nitrites were readily available,
these approaches could not be our choice of synthesis.

The fifth approach (e) was used to successfully synthesize the tetradeuterated 23a. It was based on hydrolysis of nitrone 43. The oxygen of the nitrone group of 43 was introduced by oxidation of the imine functionality using monoperoxyphthalic acid (MPP), 45. This would be a suitable approach if an O-18 labelled peroxyacid could be prepared. A literature search indicated two possible methods. The first was the photooxidation of methyl p-formylbenzoate (46) in the presence of O2 to give
p-methoxycarbonyl perbenzoic acid (47). The second was the preparation of 45 from benzhydrol and H₂O₂. In the latter case, [¹⁸O₂]H₂O₂ was required. Initially, we decided to try the first method.

Scheme 12

Ohno et. al. used the a photooxidation method to prepare 47 from 46 in 80-95% yield (Scheme 12). Peracid 47 was tested in various epoxidation and Baeyer-Villiger reactions, and was found to have comparable reactivity with perbenzoic acid and monoperoxyphthalic acid. Compound 47 is a crystalline solid and is as stable as m-chloroperbenzoic acid. The attempts to prepare 47, however, all failed. Since we did not have a high pressure mercury lamp, a medium pressure mercury lamp was used. Both external and internal irradiation methods were tried, but only p-methoxybenzoic acid was obtained. Therefore we turned our attention to make [¹⁸O₂]H₂O₂.

[¹⁸O₂]H₂O₂ is actually commercially available. Considering the cost, however, we decided to make our own by the method of base-catalyzed autooxidation of benzhydrol (48) with ¹⁸O₂ (Scheme 13). This method utilizes molecular oxygen, the least expensive O-18 source, and does not require specialized equipment and long reaction times as some other methods do (Scheme 13).
Preparation of unlabeled H₂O₂ was first tried. In each trial, the oxygen uptake was fast and then stopped, yielding KOOH which precipitated out of the benzene solution. The reaction yield was calculated by dissolving a small portion of the dry KOOH in cold 1 N HCl containing 1 mM EDTA and titrating the resulting solution iodometrically. Moderate yields of 75 to 83% were obtained. The calculated yields of the reactions were probably lowered by decomposition of KOOH in the air, which was found to be very fast. A change from a yellow to a white solid with the simultaneous release of oxygen gas was noticed. When a large amount of KOOH was dissolved in 1 N HCl containing EDTA at 0 °C, the same decomposition process (O₂ evolution) took place dramatically, retaining only 20% of the original H₂O₂. It was found, however, when the same dry KOOH was dissolved in cold water (0 °C), less KOOH decomposition took place.

It was realized that acidification of KOOH to produce hydrogen
peroxide was not necessary, because the following step to make 45 from phthalic anhydride (49) only requires HOO⁻ (Scheme 14). Using KOOH generated from autooxidation, the reaction went smoothly to give 45 in 76% titrated yield. Experiments with O-18 were later carried out, and [¹⁸O₂]H₂O₂ and 45a were obtained in 59% and 75% yield, respectively. With the method of preparation of labelled MPP mastered, the unlabeled synthesis of 23 using approach e was then attempted and 23 was obtained in 16.4% overall yield.

Scheme 15

Feeling secure with each step of the synthesis, the preparation of doubly labelled 23b was then started (Scheme 15). Heating of 50 at reflux in methanol-OD and D₂O with a catalytic amount of NaCN resulted in 94%
deuterium exchange at the α-position as determined by $^1$H NMR spectroscopy. The nitrile 50a was then tosylated in 78% yield to give 51a, which was alkylated in liquid ammonia 53 with diethyl amidomalonate, 37, to give 52a. Almost half of the deuterium label was found to be lost during the alkylation, presumably due to the basicity of liquid ammonia being enough to deprotonate the proton α to the cyanide group. The use of NaOEt in DMSO was then tried. The mixture was heated at 60-70 °C for six days for complete reaction. $^1$H NMR spectrum of the purified product revealed, however, that 25% of the deuterium was still lost. The attempts to increase the deuterium content of the sample by treatment of 52a with potassium hydride followed by D$_2$O quench did not show noticeable improvement. Instead of trying other conditions, we decided to carry on with this material.

The nitrile 52a was reduced by catalytic hydrogenation under acidic conditions to the primary amine 53a, which was then allowed to condense with p-methoxybenzylaldehyde to give imine 54a. Without purification, 54a was oxidized by [18O$_2$]monoperoxyphthalic acid to produce oxaziridine. The oxaziridine could not be isolated but easily isomerized to nitrone 55b under acidic conditions. Some 55b was recrystallized from the reaction mixture after standing with the by-product phthalic acid for 1.5 days. Purification of the rest of the reaction mixture with silica gel chromatography completely isomerized the oxaziridine to afford 55b in 26% yield from 53a. The isotope content of 55b was determined by mass spectroscopy with comparison of unlabeled 55. It was revealed that 98% molecules contain O-18 label, 27.8% molecules contain two deuteriums and 62.4% molecules contain one deuterium at C-4.

Hydrolysis of 55b in 6 N HCl would then give N$^6$-
[\textsuperscript{18}O]hydroxyornithine. Since 23 is not a stable species, it is wise to make it just before use, hence the synthesis was stopped at 55b.

**Preparation of [4, 4-\textsuperscript{2}H\textsubscript{2}]Tricholomic acid, 5a**

Tricholomic acid, L-erythro-\(\alpha\)-amino-3-oxo-5-isoxazolidineacetic acid (5), was isolated from a mushroom, *Tricholoma muscarium* Kawamure, by Takemoto *et al.* in 1964.\textsuperscript{56} It was found to have flycidal properties. It has also been used as a seasoning because of its taste.\textsuperscript{57}

Preparation of suitably labelled 5 was one goal of our work. The amino acid 5 was first synthesized in low overall yield from diethyl threo-\(\beta\)-hydroxyglutamate, 56, via hydroxamic acid 57 as an DL mixture (Scheme 16).\textsuperscript{58} In a modified synthesis where the \(\alpha\)-amino group was protected to avoid side reactions, a 400\% improvement in yield was achieved.\textsuperscript{59} Tricholomic acid and its derivatives were also synthesized as the intermediates in the total syntheses of acivicin reported by Silverman,\textsuperscript{60} Hanessian,\textsuperscript{61} and Kelly.\textsuperscript{62}

**Scheme 16**

\[
\begin{align*}
\text{EtO}_2\text{C} & \text{OH} & \text{EtO}_2\text{C} & \text{Cl} \\
\text{NH}_2 & \text{HCl} & \text{NH}_2 & \text{HCl} \\
56 & & 57 & \rightarrow 5
\end{align*}
\]

Martin *et al.* have found that basic hydrolysis of acivicin gave tricholomic acid.\textsuperscript{63} This method seemed attractive since the basic
conditions used to produce 5 might lead to exchange of hydrogen at C-4. Indeed, hydrolysis of 1 in 5 N NaOD/D2O gave 5a in 36% yield (Scheme 17). Although the 1H NMR showed over 95% deuterium exchange, the presence of two diastereomers in a ratio of 2:1 favoring 5a was also observed.

Scheme 17

The presence of the two diastereomers may be due to epimerization at β position (Scheme 18) under either a basic conditions used for reaction or the acidic conditions used in workup and purification. Amberlite IRC-50 was used for purification and the pH of the column eluant containing 5a dropped to about 3 if the solution was adjusted to neutral pH prior to the column application. Epimerization at the α position is also a possibility but epimerization of amino acids under these conditions is usually a slow process.64

It was possible to reduce the extent of epimerization or to avoid it completely by performing the transformation at below room temperature and by applying a dilute alkaline solution of the product to the Amberlite IRC-50 column to give a neutral solution of 5a. Mass spectroscopic analysis of the resulting sample revealed that 75.2% molecules were dideuterated and 11.0% molecules were monodeuterated.
Preparation of labelled Isoxazolidinyl glycine and Isoxazolinyl glycine

As mentioned in the Introduction, compounds 25 and 26 are among the three proposed key intermediates in the biosynthesis of 1. Therefore, preparations of appropriately labelled 25 and 26 were the next goal. Whitney and coworkers have prepared 26 and compound 58, a potential precursor of 25, in the total synthesis of acivicin (Scheme 20).65 However, our initial strategy was to develop methods to synthesize these compounds using materials that were available from co-workers in our group.66 For example, it was hoped that 25 could be prepared by debenzylation of compound 59 or its methyl ester, 60, both of which were synthetic intermediates in our β-hydroxyornithine synthesis.67 One of the simple ways to prepare 26 may be dechlorination of acivicin.
Attempts in debenzylation of isoxazolidine derivative

For the preparation of 25, hydrogenolysis of 59 was studied. It is known that under prolonged (3 days) catalytic hydrogenation (Pd(OH)₂), 59 could be reduced completely to give β-hydroxyornithine. It was unclear whether debenzylation could be effected without N-O bond cleavage. Hydrogenation of 59 using Pd/C, EtOH at room temperature was followed using TLC. Three new products appeared on TLC regardless the reaction time, one of which had the same Rf value as β-hydroxyornithine. The other two were sensitive to UV detection indicating the benzyl or benzyloxy groups were present. Detection by ninhydrin spray showed all three compounds contained an amino group indicating possible cleavage of the N-O bond.

Ram and coworkers had reported a method of rapid debenzylations of N-benzylamino derivatives to amines using ammonium formate and Pd/C. The reported debenzylations were typically complete within ten minutes in refluxing methanol. However, under these conditions, 59 gave the same reaction mixture as in the catalytic hydrogenolysis of 59. Debenzylation of 60 and 61 with cerium ammonium nitrate gave only complex products. Treatment of 61 with TFA only hydrolyzed the methyl ester.

Use of α-chloroethylchloroformate (ACE-Cl), a reagent which selectively debenzylates tertiary amines to secondary amines seemed appropriate. Unfortunately, reaction of 60 only resulted in cleavage of the Cbz group even at elevated temperature.

Attempts to dechlorinate acivicin

Attempts to dechlorinate 1 using AIBN and tributyltin hydride were also unsuccessful. Catalytic hydrogenolysis under neutral conditions
(Pd/C, 95% EtOH) cleanly gave erythro-β-hydroxy glutamine, 62. Apparently, N-O bond cleavage followed by hydrolysis of the resulting chloroimine gave 62 (Scheme 19). Interestingly, in contrast to this, catalytic hydrogenation of compound 1 with PtO₂ under acidic conditions gave erythro-β-hydroxy ornithine. Due to the failure of these experiments, attempts to prepare 25 and 26 from available materials were abandoned in favor of better approaches.

Scheme 19

![Scheme 19](image)

Preparation of [4,4-²H₂] Isoxazolidinyl Glycine (25a) and [4,4-²H₂] Isoxazolinyl Glycine (26a)

The next best approach was to use the method developed by Whitney, et al. Their synthesis depended upon a key 1,3-dipolar cycloaddition of nitrone 63 generated in situ and vinylglycine derivative 64 to construct the isoxazolidine ring (Scheme 20). A good diastereomeric selectivity (>19:1) was achieved in the cycloaddition reaction by using a double asymmetric induction method with a chiral auxilliary. Vinylglycine derivative 64 was prepared by pyrolysis of sulfoxide 65. We envisioned that the hydrogens adjacent to sulfur in the intermediate sulfoxide 65 might be prone to H-D
exchange under appropriate conditions. Thus both deuterium labelled compounds of 25 and 26 could be synthesized.

Scheme 20

Preparation of deuterium labelled sulfoxide derivative was first attempted. Using Whitney's method, L-methionine (67) was protected with benzyl chloroformate (Cbz-Cl) to give N-Cbz methionine (68) (Scheme 21), and then transformed to N-Cbz methionine methylidene ester (69). Compound 69 was oxidized by sodium periodate to the corresponding sulfoxide 65 (56% from 67). Unfortunately, attempts to exchange the hydrogens adjacent to the sulfoxide using potassium tert-butoxide in t-BuOD gave only decomposition products.

It was assumed that the cyclic methylidene ester group was sensitive to the basic conditions. Therefore, an earlier introduction of the sulfoxide functionality followed by exchange was attempted. Hence, 67 was oxidized directly with hydrogen peroxide to L-methionine-dl-sulfoxide (70). Treatment of sulfoxide 70 with NaOD in D₂O at 90 °C for three days led to
exchange at both the C-4 and C-6 positions as indicated by the $^1$H NMR spectrum. Therefore, the method was modified to obtain compound 65 from 67 in 40% overall yield (Scheme 22).

Pyrolysis of 65 in the presence of triethylphosphite went smoothly and subsequent purification gave 64 in 69% yield (Scheme 21). Compound 64 was found to be a rather stable species as opposed to 72, which easily isomerizes to the α,β-unsaturated ester. One may envision that since there is a considerable double bond character in the carbon nitrogen bond
of the carbobenzoxy group, isomerization of 64 to 73 to introduce a third sp$^2$ center into the five membered ring is likely to be an unfavorable process (Scheme 23).

Scheme 23

Having prepared 64, the preparation of the required oxime 74 was performed. The method used by Whitney et al. for the ketalization (acetone, sulphuric acid) of D-ribose, 75 to 76, gave a low reaction yield and used a complicated purification procedure.$^{76}$ A modified procedure using 2,2-dimethoxypropane (77) and a catalytic amount of toluenesulfonic acid in dimethylformamide followed by purification with flash chromatography gave 76 in 77% yield (Scheme 24).$^{77}$ The optimum molar ratio of 77 to 75 was found to be 2:1, further increases in 77 tended to give more of the diacetonide product 78.$^{78}$ Compound 75 was transformed further using Vasella's procedure$^{79}$ to give 74 in 46% overall yield.

The crucial cycloaddition step was performed by heating a mixture of paraformaldehyde, vinylglycine 64, and oxime 74, in chloroform at reflux (Scheme 20). Although Whitney had reported a diastereomeric ratio of over 19 to 1, a 10 to 1 ratio of diasteromers (determined by $^1$H NMR spectrum of 79) of 66 favoring the desired isomer was obtained from this reaction. Pure 66 could be isolated by flash chromatography. The optical
rotation of this compound was found to be +69.5 as opposed to +57.1 in the literature.

**Scheme 24**

Removal of the chiral auxiliary using 98% formic acid gave isoxazolidine 58, which was then oxidized by N-chlorosuccinimide in CH$_2$Cl$_2$ to afford isoxazoline 79 (Scheme 25). The $^1$H NMR spectrum of 79 was obtained at 50 °C which allowed good resolution of two diasteromers as mentioned by Whitney. Removal of Cbz and methyldene ester groups using boron tris(trifluoroacetate)$^{80}$ afforded 26 in 38% yield from 58.

Attempts to deprotect 58 using similar conditions gave a mixture of two products which were difficult to separate. The resulting mixture was
therefore derivatized using [2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON) followed by diazomethane. Isolation of compound 80 showed that 25 was one of the products in the mixture from the boron tris(trifluoacetate) reaction (Scheme 26). Different reaction conditions were therefore studied to avoid side reaction, and treatment of 58 with boron trichloride in CH$_2$Cl$_2$ afforded desired product 25 in 77% yield.

Compounds 25a, and 26a were prepared using the same method after introduction of deuterium labels by exchange reaction of methionine sulfoxide, 70. A relatively low diastereoselectivity (4:1, favoring the desired diastereomer) in the 1,3-dipolar cycloaddition step of 64a and 74 was
observed with the deuterated compounds. It is possible that the cycloaddition is faster due to a secondary isotope effect. Over the same length of reaction time as for the unlabeled reaction the kinetic product (desired diastereomer) is isomerized to the thermodynamic product (undesired diastereomer). The low diastereomeric ratio led to some difficulty in the purification step. A new solvent system for flash chromatography was finally developed (ethyl acetate/dichloromethane/toluene:1/8/9) to separate out most of the desired diastereomer. Subsequent deprotection and oxidation steps gave 25a and 26a from 66a in 51.5% and 38.9% yield, respectively. The deuterium content of 25a and 26a was determined from the mass spectrum of 58a. It was determined that 62.3% molecules contain two deuteriums while 26.0% molecules contain one deuterium.

**Fermentation Studies**

A prerequisite to feeding experiments in order to study the biosynthesis of acivicin, was the isolation and purification of 1 and 2 from the fermentation broth of *S. suiceus*. The isolation and purification procedure involved centrifugation to remove mycelia, filtration of the supernatant through celite, cation exchange chromatography followed by anion exchange chromatography, then flash chromatography to separate 1 and 2, and finally recrystallization. The concentration of 1 in the broth and after each purification step can be quantitated with bioassays using *B. subtilis* as the test organism. In our previous studies a yield of 12-17 mg/L of 1 was obtained from the production broth. Successful isolation and purification of 1 and 2 were carried out in the initial stages of the present work. However, it was found that production decreased to about 1
mg/L after the organism had been kept on soil for six years. The low yield was insufficient for feeding studies.

Substantial efforts were therefore made to increase the production. New soil cultures were made from the original soil cultures, from spores that had been preserved on agar plugs in liquid nitrogen, and from slants newly obtained from Upjohn Company. Fermentations were carried out to test these cultures, however, no improvement in production was observed as determined from bioassays. Fresh batches of several fermentation ingredients, such as peptone, kaysoy 200C, whole yeast, and lard oil were used to prepare the media. In order to exclude the possibility that the decease in production was a result of these changes, comparative studies with the original batches were carried out in each case, but none of these showed any noticeable effect. Large scale fermentations using the Microferm stirred fermentor were tried, with the thought that the production may increase due to efficient ventilation provided in the system. This was found not to be the case. The use of an alternative production medium (phytone medium) did not improve the yield, either.

In contrast to the bioassays, which indicated a low production of 1, TLC showed relatively large quantities of compounds with Rf values very similar to 1 and 2. Since it is known that 4-hydroxyacivicin (2) has a low activity against B. subtilis,3 it was thought possible that most of 1 had been converted into 2, which would account for the low yield demonstrated in the bioassays.

Isolation of 2 was thus attempted. Instead of 2, however, phenylalanine was first isolated by flash chromatography in large quantities. With additional use of paper chromatography,83 tyrosine and methionine sulfoxide were also obtained. Since there were several
metabolites having very similar Rf's to 1 and 2, a more effective solvent system for TLC was sought. On silica gel, solvent systems including chloroform/methanol/acetic acid (92/7/1), butanol/glacial acetic acid/water/pyridine (15/3/12/10), butanol/acetone/glacial acetic acid/5.6% ammonia/water (9/3/2/2/4), and phenol/water/28% ammonia (30/10/1) were studied. In addition, cellulose paper systems using methanol/pyridine/6

Table 1. The TLC Rf values of various amino acids in different solvent systems

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Solvent 1* (silica gel)</th>
<th>Solvent 2* (silica gel)</th>
<th>Solvent 3* (cellulose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acivicin</td>
<td>0.43</td>
<td>0.22</td>
<td>0.46</td>
</tr>
<tr>
<td>4-Hydroxy-acivicin</td>
<td>0.55</td>
<td>0.30</td>
<td>0.40</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.55</td>
<td>0.30</td>
<td>0.66</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.52</td>
<td>0.27</td>
<td>0.29</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.54</td>
<td>0.36</td>
<td>0.44</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.47</td>
<td>0.19</td>
<td>0.62</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.43</td>
<td>0.20</td>
<td>0.49</td>
</tr>
<tr>
<td>Methionine sulfoxide</td>
<td>0.25</td>
<td>0.15</td>
<td>0.24</td>
</tr>
</tbody>
</table>

* Solvent 1: 1-propanol/ethyl acetate/water, was the solvent used to monitor the purification. Solvent 2: methyl ethyl ketone/acetone/water: 65/20/15, was the solvent for flash chromatography. Solvent 3: chloroform/methanol/water: 3.2/2/0.65, was the new solvent developed to monitor the purification and for purification with cellulose media.
N HCl/water (80/10/4/26), chloroform/methanol/17% ammonia (2/2/1) were also investigated. It was found finally that chloroform/methanol/17% ammonia: 3.2/2/0.65 on cellulose paper gave the best separation. Column chromatography using this solvent system with microcrystalline cellulose, plus the use of preparative TLC then resulted in the isolation of methionine and tryptophan. Leucine was also obtained from the culture, but 2 was isolated only in very small quantities (< 1 mg/2L). It is not clear that isolation of these common amino acids would have any significance to the biosynthesis of 1. The $R_f$ values in different solvent systems, of the amino acids that are related to this study are listed in Table 1.

Conclusions and Areas for Further Study

Isotopically labelled 55b, 5a, 25a, and 26a were synthesized. Studies in fermentation cultures of S. suiceus allowed the isolation of phenylalanine, tyrosine, tryptophan, leucine, methionine, and methionine sulfoxide. Unfortunately, in contrast to our earlier studies, we could no longer produce sufficient quantities of 1 and 2.

The most urgent task for further study would be efforts to obtain a strain of the organism that produces enough of 1 and 2. Once the production of 1 and 2 is normalized, the feeding experiments with the above labelled compounds would need to be carried out. The result form the feeding experiment of 23b (prepared by hydrolysis of 55b) would clearly indicate which of the biogenetic proposals presented in Scheme 9 or 10 would be relevant to the biosynthesis. The result from feeding 25a would tells us whether or not 1 and 2 are biosynthesized from pathway a of Scheme 9 or pathway d of Scheme 10. And the results from feeding 5a and
26a would not only give us more detail about biosynthetic pathway of 1 and 2, but also provide an indication of the mechanism for chlorination of 1 and 2. Biohalogenation has been one of the active areas in biochemical studies, and a short review regarding its occurrence, mechanism, and application will be provided at the end of this section.

Although there are great possibilities that these feedings would let us completely understand the route of biosynthesis of 1, additional feeding experiments may be required. For instance, feeding 29 or 30 may be needed if the results indicated that both 23a and 26a, but not 25a, were incorporated into 1 and 2.

**Biohalogenation: Occurrence, Mechanism, and the Application**

The largest source of halogenated organic compounds is marine organisms. Over 700 marine halometabolites have been isolated and characterized. From terrestrial microbial sources, various compounds such as chloramphenicol and pyrrolnitrin, and of course acivicin were identified.

The enzymes capable of catalyzing biohalogenations are chloroperoxidases, myeloperoxidases, bromoperoxidases, and lactoperoxidase. While the first two enzymes can catalyze biohalogenation with all halide ions except F in the presence of H2O2, the latter two can utilize only Br and I as donors for this type of reaction.

Chloroperoxidases are by now the best studied enzymes for halogenation in terms of substrate specificity, reaction mechanism, and enzyme stability. These enzymes can catalyze the halogenation of a large number of substrates including β-keto acids, α, β-unsaturated carboxylic acids, cyclic β-diketones, steroids, substituted phenols, thiols, alkynes, cycloalkanes, nucleic bases (uracil and pyrazole) and
pyridine derivatives, and anilines as well as N-oxidation of p-chloroaniline. These halogenation reactions were found to be non-stereospecific.

It was established that the halogenation agents in enzymatic halogenation reactions are electrophilic. The exact nature of this electrophilic halogenation agent is not known yet. Based on studies using different substrates, either enzyme-bound species or free molecules, such as molecular halogens, halonium cation, and hypohalites have been proposed as halogenating intermediates. A comparison of the enzymatic and chemical reactions were the basis for each study.

Chloroperoxidases have been used in the preparation of a number of organic compounds. Both pyrazole and pyridine derivatives were chlorinated on a synthetically useful scale. Higher yields relative to chemical reactions were obtained in all reactions.
Experimental

General

Melting points were obtained on a Buchi melting point apparatus and are uncorrected. Infrared spectra (IR) were recorded on either a Nicolet 5DXB-FT-IR or a Nicolet 5PC FT-IR spectrometer. $^1$H NMR and $^{13}$C NMR were obtained on either a Bruker AM-400 or a Bruker AC-300 spectrometer using tetramethylsilane (δ 0.0) or t-BuOH (δ 1.27), and occasionally H$_2$O (δ 4.9 in D$_2$O) as internal standard. Mass spectra were taken on a Varian MAT CH7. High resolution mass spectra were obtained on a Kratos MS 50 TC spectrometer. Optical rotations were determined on a Perkin-Elmyer 243 polarimeter. Elemental analysis was performed by Desert Analytics, Tucson, Arizona.

Analytical thin layer chromatography (TLC) was conducted on the precoated silica gel 60 F-254 or the precoated cellulose TLC plates manufactured by E. Merck, Scientific. Column chromatography was carried out with E. Merck silica gel 60 (230-400 mesh ASTM). Ion exchange resins were obtained from Sigma Chemical Company and were converted to the necessary ionic form prior to use.

Preparative thin layer chromatography was done on the glass plate precoated with silica gel 60 F-254. Paper chromatography was performed on Whatman Grade No. 17 Chrom. (thickness 0.88 mm) paper.

Dichloromethane, xylenes, and in some cases, benzene were distilled from calcium hydride. Other solvents were used as purchased. Starting materials were obtained commercially and used without further purification unless noted. Oxygen-18 gas ($^{18}$O: 98%) and D$_2$O ($^2$H: 99.9%)
used for reactions were purchased from Cambridge Isotope Laboratories, and 40% NaOD/D$_2$O was obtained from aldrich Chemical Company, Inc. For isolation of reaction products, the solvent was removed by rotary evaporation at water-aspirator pressure unless otherwise mentioned, and the residual solvent was removed by high vacuum (less than 1 torr). Flasks and syringe were dried in an oven (at 110 °C) overnight and cooled in a dessicator over anhydrous calcium sulfate prior to use. Drying agent refers to sodium sulfate. Reactions were routinely carried out under an inert atmosphere of nitrogen or argon.

**Synthesis of Labelled Precursors**

3-Hydroxy[2,2-2H$_2$]propionitrile (50a).

To a solution of NaCN (0.32 g, 6.53 mmol) in D$_2$O (25 mL) and methanol-OD (15 mL) was added 3-hydroxypropionitrile (50, 5 g, 4.8 mL, 70.34 mmol), and the resulting solution was heated at reflux for 23 hours. This solution was then concentrated to 10 mL, saturated with NaCl, and extracted with EtOAc. The EtOAc solution was dried over Na$_2$SO$_4$, filtered and concentrated to yield 2.03 g (66.7%) of 50a. TLC (CHCl$_3$-CH$_3$OH-AcOH 92:7:1 on silica gel, I$_2$ detection) R$_f$ 0.21; IR (neat) 3414 (br.), 2253, 1122 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 3.85 (2H, s) 2.60 (m, 0.12H, 90% deuterium).

3-Tosyl-[2,2-2H$_2$]propionitrile (51a).

This was prepared using sokellarios method$^{104}$ except as follows. To a solution of 50a (2.95 g, 40.4 mmol) in dry pyridine (40 mL) at 0 °C was added p-toluenesulfonyl chloride (10.7 g, 56.1 mmol, purified by recrystallization). The reaction mixture was stirred at 0 °C for five hours
and then was kept in a refrigerator overnight, poured to 500 mL ice-water. The resulting solid was collected and recrystallized from ether/hexane to gave 7.2 g (78.2%) of 51a. TLC (silica gel, Et2O/CHCl3; 5/1) Rf 0.41; IR (KBr) 2257, 1597, 1350, 1192; mp 63-64 °C (lit.105 63 °C); 1H NMR (300 MHz, CDCl3) δ 7.82 (2H, d, J=8.3 Hz), 7.38 (2H, d, J=8.0 Hz), 4.21 (s, 2H), 2.74 (m, 91% deuterium), 2.47 (s, 3H).

3-Ethyl 2-Acetamido-2-carboethoxy-4-cyano[4,4-2H2]-butanoate (52a).

This was prepared by the method of Wityak54a except as follows. To a solution of diethyl acetamidomalonate (5.13 g, 23.6 mmol) in liquid ammonia (50 mL) at -78 °C was added 51a (4.5 g, 19.7 mmol). The reaction mixture was stirred for 2.5 hours at -78 °C, and then allowed to warm to room temperature. The resulting residue was dissolved in water (30 mL) and extracted with CHCl3 (3 x 30 mL). The CHCl3 solution was dried, concentrated, and recrystallized from CH2Cl2/hexane to give 3.85 g of 52a. Chromatographic purification (silica gel, CHCl3/CH3OH: 9/1) gave another 0.8 g of 52a (total 86.8%). TLC (CHCl3/CH3OH 87/13, silica gel) Rf 0.74; mp 91.5-93 °C (lit.106 92-94 °C); IR 3354, 2985, 1737, 1664 cm⁻¹; 1H NMR (300 MHz, CDCl3) δ 6.84 (1H, br s) 4.29 (2H, q, J=7.15 Hz), 4.28 (2H, q, J=7.15 Hz), 2.70 (2H, d, J=7.16) 2.33 (m, 49.5% deuterium), 2.08 (3H, s), 1.28 (6H, t, J=7.13 Hz).

Diethyl γ-amino[4,4-2H2]propyl Acetamidomalonate (53a).

This was prepared by the method of Wityak54a except as follows. A solution of 52a (3 g, 11.02 mmol) in 95% ethanol (100 mL) containing conc. hydrochloric acid (6 mL) was exposed to a hydrogen atmosphere over PtO2.H2O (0.4 g, 13% w.w) for 8 hours, the solution was filtered through celite and solvent was evaporated to obtain 3.89 g (100%) of 53a as a
colorless hygroscopic solid. TLC (silica gel, CHCl₃/MeOH: 87/13) Rf 0.13; IR(neat) 3383, 2982, 1740, 1655, 1516, 1239 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) 4.35 (4H, q, J=7.2 Hz), 3.07 (2H, d, J=7.2 Hz), 2.34 (2H, d, J=7.5 Hz), 2.15 (3H,s), 1.65 (m, 64% deuterium), 1.32 (6H, t, J=7.2 Hz)

[^18O₂]Potassium Hydrogenperoxide

This was prepared by the method of Foote⁵², except as follows. The flask containing potassium tert-butoxide (3.36 g, 30 mmol) was evacuated and exposed to a ^¹⁸O₂ atmosphere. A solution of benzyldrol (48, 5.52 g, 30.0 mmol) in dry benzene (80 mL) was added at 0 °C and the resulting mixture was stirred under ^¹⁸O₂ at room temperature. After 2 hours, the oxygen-18 uptake was stopped (702 mL ^¹⁸O₂ was consumed). The resulting suspension was centrifuged slightly, and the yellow solid washed once with benzene. After removing the liquid by a pasteur pipet, the yellow solid was dried under reduced pressure. To this solid at -8 °C was added 100 mL ice-water, and 3 mL of the resulting solution was titrated iodometrically using sodium thiosulfate.¹⁰⁷ The content of the peracid was calculated according to the following equation. It was determined that 1.35 g KOOH (59%) was obtained.

\[
W_{\text{peracid}} (g) = \frac{1}{2} \times MW_{\text{peracid}} \times M_{\text{Na}_2\text{S}_2\text{O}_3} \times V_{\text{Na}_2\text{S}_2\text{O}_3}
\]


To the crude solution of[^18O]H₂O₂ (1.35 g, 17.11 mmol) in H₂O (97 mL) at -8 °C was added sodium bicarbonate monohydrate (0.5 g, 4.0 mmol) and phthalic anhydride (30.4 g, 20.52 mmol, purified by sublimation). The reaction mixture was stirred vigorously at -8 °C--5 °C for 30 minutes, added 20 mL cold ether, and acidified with cold 17% sulfuric acid until
Congo red. The aqueous layer was separated and extracted with ether until no 45a was left (tested by potassium iodide starch paper). The organic solution was titrated by the above method (in the preparation of [\(^{18}\text{O}\)H\(_2\)O\(_2\)) and concentrated to about 20 mL for the next reaction. Titration showed that 2.40 g (75.3%) of 45a was obtained.

**Diethyl γ-(N-[\(^{18}\text{O}\)oxide, N-p-methoxybenzylidene)amino-[4,4-\(^2\text{H}_2\)propyl Acetamidomalonate (55b).**

This was prepared by the method of Ju\(^{108}\) except as follows. To 53a (3.89 g, 11.02 mmol) was added benzene (100 mL), triethylamine (25 mL), and anisaldehyde (2.26 g, 2.01 mL, 16.60 mmol). The reaction mixture was stirred at reflux for 10 hours using a Dean-Stark apparatus, and then filtered, and concentrated to give 5.83 g of an orange oil. The crude schiff base was dissolved in 50 mL dry ether and was added a solution of \([\text{\(^{18}\text{O}\)MPP (2.39 g, 12.84 mmol) in ether (20 mL) obtained above precooled to 0 °C. The reaction mixture was stirred for 30 minutes, and was kept in a refrigerator overnight. The ether solution was separated from a precipitated yellow oil, concentrated, and purified by flash chromatography (silica gel, EtOAc, then CHCl\(_3\)/CH\(_3\)OH: 9/1). Further recrystallization from benzene afforded 0.76 g of 55b as a white solid.

To the precipitated yellow oil was added CH\(_2\)Cl\(_2\) (50 mL), and this was washed with 50% NaHCO\(_3\) solution (2 x 40 mL), dried and concentrated. Recrystallization gave 35 mg of a white solid, identified as 55b. Chromatographic purification of the combined mother liquor afforded anther 0.36 g (total 25.3% from 53a) of 55b. TLC (silica gel, CHCl\(_3\)/CH\(_3\)OH: 9:1) R\(_f\) 0.48; mp 147-148 °C (lit.\(^{108}\) 149–149.5 °C); IR(neat) 3400, 2984, 1740, 1672, 1604, 1507, 860 cm\(^{-1}\); \(^1\)H NMR (300 MHz, CDCl\(_3\)) δ 8.21 (2H, d, J = 8.9 Hz), 7.30 (1H, s), 6.93 (2H, d, J=8.9 Hz), 6.86 (1H, s), 4.24 (4H, q, J=7.1 Hz), 3.86
(2H, d, J=9 Hz), 3.85 (3H, s), 2.39 (2H, d, J=8.6 Hz), 2.04 (3H, s), 1.86 (m, 56% deuterium), 1.23 (6H, t, J=7.1 Hz); ElMS (70ev), m/z (relative intensity) 412.0 (9.34), 411.0 (15.92), 410.0 (1.54), 338.0 (32.83), 148.0 (100). The isotope content calculated was: 98%-18O, 27.8%-d$_2$, 62.4%-d$_1$.

**N$^6$-hydroxyornithine (23).**

This was prepared by the method of Maehr$^{47d}$ except as follows. A mixture of 55 (698 mg, 1.71 mmol) in 6 N hydrochloric acid (30 mL) was stirred at 100 °C for 6 hours, and concentrated until nearly dry. To the residue was added fresh 6 N HCl (30 mL), and the mixture was stirred for 4 hours. The resulting solution was concentrated to remove HCl, diluted with water (30 mL), and filtered through celite. The filtrate was adjusted with 1N NaOH to pH 4.5 and loaded onto a cation exchange column (Dowex 50W x 4, 200-400 mesh, H$^+$ form, 15 cm x 200 cm), which was washed with water, and eluded with 1.5 N HCl. The fractions containing 23 were then pooled and concentrated. Further lyophilization resulted in 298.7 mg (77%) of 23 dihydrochloride as a green hygroscopic solid. Part of this material was recrystallized from CHCl$_3$/MeOH/17% NH$_4$OH to give a white solid. TLC (silica gel, CHCl$_3$/MeOH/ 28% ammonia: 1/4/0.5) R$_f$ 0.36; $^1$H NMR (300 MHz, D$_2$O) \( \delta \) 4.14 (1H, t, J= 6.5 Hz), 3.34 (1H, t, J= 6.5 Hz), 1.99 (4H, m).

**2S, 5'S)-α-Amino-3-oxo-5-isoxazolidineacetic Acid (Tricholomic Acid, 5).**

A solution of 1 (50 mg, 2.8 mmol) in 5 N NaOH (1 mL) was stirred at room temperature for 10 hours, and diluted to 10 mL with water. The diluted solution was passed through a cation exchange column (IRC-50,
16-50 mesh, H\(^+\) form, 10 mm x 55 mm), and the column was washed with water (50 mL). The combined eluent and washings were freeze-dried to yield a white solid (74.5 mg), which was recrystallized from methanol to afforded 11.7 mg (26%) of 5. TLC (silica gel, n-PrOH/EtOAc/H\(_2\)O: 4/4/3) \(R_f\) 0.13 (the solution pH: 12); \(R_f\) 0.37 (pH: 7); \(^1\)H NMR (300 MHz, D\(_2\)O) 5.22 (1H, d, t, \(J=8.7, 3.3\) Hz), 3.14 (1H, d, d, \(J=17.1, 8.5\) Hz), 3.07 (1H, d, d, 17.1, 9.1 Hz); \(^1\)C NMR (75 MHz, D\(_2\)O) 174.6, 171.3, 79.8, 56.9, 35.1; FAB MS (neg. FAB, glycerol/H\(_2\)O) 159.0 (M-1, 7.9).

(2S, 5'S)-\(\alpha\)-Amino-3-oxo-5-[4,4-\(^2\)H\(_2\)]isoxazolidineacetic Acid (5a).

This was prepared as above except as follows. A solution of 1 (110 mg, 0.616 mmol) in 5 N NaOD/D\(_2\)O (1.5 mL, diluted from commercial 40% NOD/D\(_2\)O solution) was stirred at room temperature for 24 hours, and then was diluted to 8 mL with water. Ion exchange chromatography and subsequent recrystallization afforded 53 mg (53%) of 5a which was found to contain 10% of another diastereomer. \(^1\)H NMR (300 MHz, D\(_2\)O) \(\delta\) 5.23 (1H, d, \(J=3.1\) Hz), 4.13 (d, 1H, J=3.3), 3.13 (m, 96.6% deuterium); FAB MS (neg. FAB, glycerol/H\(_2\)O) 161.1 (M-1, 7.4, d\(_2\)), 160.0 (M-1, 1.1, d\(_2\)). Calculated deuterium content: 75.2%-d\(_2\), 11.1%-d\(_1\).

\(L\)-erythro-\(\beta\)-hydroxyglutamine (62).

A suspension of 1 (5 mg, 0.028 mmol) and Pd-C (1 mg, 20% w/w) in 95% EtOH (3 mL) was exposed to a hydrogen atmosphere over for 8 hours. The solution was filtered through celite and concentrated to give a white solid. Recrystallization from EtOH afforded 3.5 mg (76%) of 62. TLC (silica gel, n-PrOH/EtOAc/H\(_2\)O: 4/4/3) \(R_f\) 0.40; IR(KBr) 3387, 1617, 1334, 1108 cm\(^{-1}\); \(^1\)H NMR (300 MHz, D\(_2\)O, t-BuOH as internal standard) \(\delta\) 4.50 (1H, d, d, d,
J=8.4, 5.1, 2.8 Hz), 4.18 (1H, d, J=2.95 Hz), 3.10 (1H, d, d, J=17.0, 8.8 Hz), 3.0 (1H, d, d, J=17.0, 5.0 Hz).

**L-Methionine-dl-Sulfoxide (70).**

To a suspension of L-methionine (5 g, 33.5 mmol) in H₂O (50 mL) at 0 °C was slowly added 30% hydrogen peroxide (4.17 mL, 36.7 mmol). The solution was stirred for 3 hours at room temperature and freeze-dried. Recrystallization from 1N NaOH gave 5.9 g (94%) of 70 sodium salt as a white solid. TLC (silica gel, i-PrOH/EtOAc/H₂O: 4/4/3) Rf 0.43; IR: 2952 (br), 2119(weak), 1624, 1582 cm⁻¹; ¹H NMR: δ 3.77(1H, m), 2.9-3.16 (2H, m), 2.77 (3H, s), 2.2-2.3 (2H, m); mp 101-102.5 °C (lit.¹⁰⁹a 239 °C for (S)c(R)s-form) [α]D²⁵ = +12.3 ° (c, 1 in H₂O), lit.¹⁰⁹b +25 ° (d-sulfoxide: 50.6%, c, 1, in H₂O).

**L-[4,4,5,5,5,-2H₅]Methionine-di sulfoxide (70a).**

A solution of the sodium salt of 70 (24.12 g, 128.8 mmol) in 2.5 N NaOD/D₂O (100 mL) was heated at 90-95 °C (oil bath) for 10 days. The extend of exchange was frequently checked by ¹H NMR spectroscopy, and the presence of 96.3% deuterium at C-6 and 65.7% deuterium at C-4 was observed at the end. The reaction mixture was diluted with H₂O and freeze-dried.

To the resulting solid (5.7 g, 36.9 mmol) was added D₂O (25 mL) and the solution was adjusted to pH 12.5 with 40% NaOD/D₂O. The reaction mixture was heated at reflux for another seven days and used directly for the next reaction. ¹H NMR: 3.43 (1H, t, J=6.5 Hz), 2.95 (87.2% deuterium, m), 2.75 (99% deuterium, m), 2.15-1.94 (2H, m).
(αS)-N-(Benzyloxy carbonyl)methionine Sulfoxide (71).

To a solution of NaHCO₃ (12 g, 142.9 mmol) in H₂O (200 mL) was added 70 (9.5 g, 50.6 mmol), and benzyl chloroformate (9.5 g, 8.0 mL, 55.65 mmol) at room temperature. The mixture was stirred for 5 h, washed with ether (2 x 50 mL), acidified with conc. HCl to Congo Red, and extracted with dichloromethane (4 x 100 mL). The organic solution was dried and concentrated to obtain 12.8 g (84.2%) of 71 as a colorless oil. A portion of the resulting oil was recrystallized from CHCl₃/Benzene. TLC (silica gel, i-PrOH/EtOAc/H₂O: 4/4/3) Rf 0.64; IR(neat) 3307, 2923, 1714, 1223; ¹H NMR: (300 MHz, CD₃OD) δ 7.3 (m, 5H), 5.09 (2H, m), 4.32 (1H, m), 2.88(2H, m), 2.61(3H, s), 2.3(1H, m), 2.1(1H, m). ¹H NMR (CH₂Cl₂) δ 7.35 (5H, m), 6.75 (1H, br s), 6.0 (0.53H, d, J=7.3), 5.88 (0.47H, d, J=7.3), 5.09 (2H, s), 4.4 (1H, m), 2.8 (2H, m), 2.67, 2.61 (6H, 2s), 2.38 - 2.2 (2H, m). ¹³C NMR: δ 174.5, 158.6, 138.1, 129.5, 129.1, 128.9, 67.7, 54.5, 54.1, 51.2, 51.1, 38.2, 38.0, 26.0, 25.9.

(αS)-N-Benzyloxy carbonyl)-[4,4,5,5,5-2H₅] methionine Sulfoxide (71a).

This was prepared as above except as follows. The reaction mixture of 70a (5.7 g, ~36.9 mmol) in NaOD/D₂O (25 mL) was diluted to 120 mL with water. To this solution was added NaHCO₃ (4 g, 47.6 mmol). Benzyl chloroformate (11.9 g, 10 mL, 69.7 mmol) was also added in three portions at 0 °C. The reaction mixture was stirred at room temperature overnight, and subsequent work-up to give 10.3 g (91.3%) of 71a (as a light yellow oil). IR (neat) 3306, 1709, 1535, 1226; ¹H NMR (300 MHz, CD₃OD), δ 7.3 (5H, m), 5.09 (2H, ABq), 4.32 (1H, m), 2.85 (m, 88.6% deuterium), 2.58 (s, 97.5% deuterium), 2.3 (1H, m), 2.1 (1H, m).
(aS)-N-(Benzyloxycarbonyl)methionine Sulfoxide Methyldene Ester (65).

A mixture of 71a (3.0 g, 9.99 mmol), paraformaldehyde (1.6 g, 53.3 mmol), and p-toluenesulfonic acid (0.140 g, 0.74 mmol) in benzene (100 mL) was stirred at reflux overnight using a Dean-Stark apparatus. The resulting solution was concentrated to 30 mL and diluted with dichloromethane to 100 mL, washed with 5% NaHCO₃ (3 x 100 mL), dried, and concentrated to give 2.01 g (64.5%) of 65 as an oil. TLC (silica gel, EtOAc/MeOH: 95/5) Rf 0.31; IR(neat) 1798, 1709 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 7.36 (5H, m), 5.51 (1H, s), 5.24-5.12 (3H, m), 4.42 (1H, t, J=6.0 Hz), 2.79 (2H, br s), 2.50 (3H, s), 2.35 (2H, m).

(aS)-N-(Benzyloxycarbonyl)[4,4,5,5,5-²H₅]methionine Sulfoxide Methyldene Ester (65a).

This was prepared as above except as follows. A mixture of 71a (10.3 g, 33.7 mmol), paraformaldehyde (6 g, 200 mmol), and p-toluenesulfonic acid (320 mg, 1.68 mmol) in benzene (200 mL) was stirred at reflux for 5 hours using a Dean Stark apparatus, and worked-up to afford 6.05 g (56.5%) of 65a as an oil. ¹H NMR (300 MHz, CDCl₃) δ 7.36 (5H, m), 5.55 (1H, s), 5.30-5.13 (3H, m), 4.42 (1H, t, J=6.0 Hz), 2.79 (2H, br s), 2.50 (3H, s), 2.35 (2H, m).

(aS)-N-(Benzyloxycarbonyl)vinylglycine Methyldene Ester (64).

This was prepared by the method of Whitney⁶⁵ except as the follows. To a solution of 64 (0.80 g, 2.56 mmol) in dry xylenes (16 mL) was added triethylphosphite (0.82 g, 0.85 mL, 4.96 mmol) under a nitrogen atmosphere. The reaction mixture was stirred at reflux for 2 days and worked-up to give a crude oil. Chromatographic purification (silica gel,
EtOAc/Hexane: 2/8, Rf 0.32) afforded 341.6 mg (53.5%) of 64 as an colorless oil. IR 1800, 1715, 760; [α]D 25 = + 74.5° (c,1, CHCl₃; lit.65 + 78°); ¹H NMR (300 MHz, CDCl₃) δ 7.36 (5H, s), 5.86 (1H, d, d, d, J=17, 10, 5 Hz), 5.59 (1H, br s), 5.38-5.46 (2H, m), 5.25 (1H, d, J=4.6 Hz), 5.19 (2H, ABq, J=12 Hz), 4.82 (1H, br d, J=4.5 Hz).

(αS)-N-(Benzyloxy carbonyl)[4,4-²H₂] vinylglycine Methylidene Ester Vinyl (64a).

This was prepared as above except as follows. A solution of 65a (6.0 g, 19.0 mmol) and triethylphosphite (6.31 g, 6.5 mL, 38.0 mmol) in xylenes (100 mL, distilled) was stirred at reflux for 2.5 days. Subsequent work-up and chromatographic purification yielded 3.3 g (69.1%) of 64a as a colorless oil. [α]D 25 = + 77.3°. ¹H NMR (CDCl₃) δ 7.36 (5H, m), δ 5.86 (1H, s), 5.59 (1H, s), 5.38-5.46 (m, deuterium 86.4%), 5.25 (1H, d, J=4.6 Hz), 5.19 (2H, ABq, J=12 Hz), 4.82 (1H, br d, J=4.5 Hz).

2,3-O-Isopropylidene-D-ribose (76).

To a solution of D-ribose (20 g, 133.2 mmol) in N,N-dimethylformamide (80 mL) containing a catalytic amount of p-toluenesulfonic acid (0.3 g, 1.53 mmol) was added 2,2-dimethoxypropane (27.75 g, 32.76 mL). The mixture was stirred overnight, and was added 2.3 mL anion exchange resin (IRA-410, OH⁻ form). After stirring 10 minutes, the solution was filtered, concentrated by high vacuum to yield the crude acetonide which, upon chromatographic purification (silica gel, EtOAc/Hexane: 6/5) gave 19.40 g (76.7%) of 76 as a colorless oil. TLC (silica gel, EtOAc/Hexane: 6/5) Rf 0.24, (EtOAc) Rf 0.57; IR(neat) 3394, 2943, 1211, 1060 cm⁻¹; ¹H NMR (300 MHz, DMSO-d₆) δ 6.52 (1H, d, J=6 Hz), 5.15 (1H, d,
51 Hz), 4.94 (1H, t, J=5.4 Hz), 4.68 (1H, d, J=6.0 Hz), 4.43 (1H, d, J=6.0 Hz), 3.98 (1H, d, d, J=7.0, 5.27 Hz), 3.4 (2H, m), 1.37 (3H, s), 1.24 (3H, s); 13C NMR (75 MHz, DMSO-d6) δ 110.94, 101.74, 86.26, 85.82, 81.77, 62.61, 26.25, 24.56.

2,3-O-isopropylidene-5-O-trityl-D-ribose-oxime (74).

This was prepared by the method of Vasella82 except as follows. A solution of 76 (19.7 g, 103.6 mmol) and trityl chloride (triphenylchloromethane, 32.35 g, 116.0 mmol) in pyridine (65 mL, stored over molecular sieves, 4A) was stored for 4 days in dark at room temperature. Methanol (8.5 mL) was added and the solution was kept in dark for three more days. To the resulting solution was added hydroxylamine hydrochloride (12.56 g, 178.2 mmol). The reaction mixture was stirred for 6 hours at room temperature, concentrated by high vacuum, diluted with water and extracted with dichloromethane. The organic solution was dried and evaporated three times with benzene to obtain a reddish residue. The crude 74 was recrystallized repeatedly from EtOAc/Hexane to afford 31.45 g (67.7%) of 74. TLC (silica gel, EtOAc/Hexane: 3/7) Rf 0.23; mp 93.5-95 °C (lit.82 89 °C); IR 3600-3200, 3086, 3059, 1593, 1493, 1446, 1377, 1219, 1165, 1065; 1H NMR (300 MHz, CDCl3) E-oxime: 8.45 (br s), 7.49-7.40, (m), 7.32-7.19 (m), 4.77(d, d, J=7.1, 6.2 Hz), 4.22 (d, d, J=9.2, 6.2 Hz), 3.75 (m), 3.32 (m), 2.82 (br s), 1.38 (s), 1.34 (s), Z(-oxime), 7.49-7.40 (m), 7.32-7.19(m), 6.80 (d, J=5.7 Hz), 5.34 (t, J=6 Hz), 4.41 (t, J=6 Hz), 3.75 (m), 3.32 (m), 2.82 (br s), 1.38 (s), 1.34 (s), E/Z: 4/1 (lit.82 58/42); 13C NMR E isomer, 148.4, 143.6, 128.6, 127.1, 109.8 86.8, 75.2, 68.8, 64.8, 27.6, 25.4; Z isomer, 150.3, 143.8, 128.6, 127.7, 126.9, 109., 78.1, 70.9, 70.1, 60.4, 27.2, 25.0.
(αS,5S)-2-(2',3'-O-Isopropylidene-5'-O-trityl-α-D-ribofuranosyl)-α-
[(benzyloxy carbonyl) amino] 5-isoxazolidineacetic Acid Methylidene Ester
(66).

This was prepared by the method of Whitney except as follows. A
mixture of 64 (167.7 mg, 0.673 mmol), 74 (436.9 mg, 0.774 mmol), and
paraformaldehyde (30.3 mg, 1.01 mmol) in chloroform (10 mL) was stirred
at reflux for 35 hours and concentrated. Chromatographic purification
(silica gel, EtOAc/hexane: 3/7, Rf 0.27) afforded two fractions (70% total
yield), No. 1: 151.2 mg of 66 as a single diastereomer. mp 81-82 °C (lit. 75-78
°C); [α]D25 = + 69.5 ° (c, 0.93, CHCl3; lit. + 57.1 °, c, 0.98, CHCl3); IR 2944,
1801, 1717 cm⁻¹; 1H NMR (CDCl3) δ: 7.38-7.20 (20 H, m), 5.53 (1 H, d, J = 4
Hz), 5.28 (1 H, d, J = 4 Hz), 5.20 (2 H, ABq, J = 12 Hz), 4.83 (1 H, d, J = 6 Hz),
4.78 (1H, d, J = 6 Hz), 4.72 (1H, m, H-5), 4.36 (2 H, s, H-4' + H-α), 4.32 (1 H,
d, J = 2 Hz, H-'1), 3.66 (1 H, dd, J = 8, 2 Hz, H-5'), 3.54 (1 H, dd, J = 8, 2 Hz,
H-5'), 3.24 (1 H, m, H-3), 3.13 (1 H, m, H-3), 2.68 (1 H, m, H-4), 1.47 (3 H, s),
1.31 (3 H, s) No. 2: 179.0 mg as a mixture of diastereomers (ee of 66,
75.6%).

(αS,5S)-2-(2',3'-O-Isopropylidene-5'-O-trityl-α-D-ribofuranosyl)-α-
[(benzyloxy carbonyl) amino] 5-[4',4'-2H] isoxazolidineacetic Acid
Methylidene Ester (66a).

This was prepared as above except as follows. A solution of 64a (2.42
g, 9.63 mmol), 74 (4.70 g, 10.48 mmol), and paraformaldehyde (440 mg,
14.67 mmol) in CHCl₃ (50 mL) was stirred at reflux for 36 hours,
concentrated, and purified by chromatography (silica gel,
EtOAc/CH₂Cl₂/toluene: 1/8/9, Rf 0.3). From a total of 5.89 g (86.3% yield) of
cycloadduct, 2.83 g of pure 66a was obtained. [α]D25 = + 83.5 ° (c, 1, CHCl₃);
$^1$H NMR (CDCl$_3$) δ: 7.47-7.20 (20 H, m), 5.48 (1 H, br. m), 5.15 (3 H, m), 4.66 (1 H, br, m), 4.50 (1 H, dd, J = 6, 2 Hz), 4.53 (1 H dd, J = 6, 2 Hz), 4.46 (1 H, d, J=2 Hz), 4.32 (1 H, m), 4.23 (1 H, ddd, J = 6, 6, 2Hz), 3.22 (2 H, m), 3.10 (1 H, s), 2.48 (0.086 H, m, 91.4% 2H), 2.31 (0.124 H, m, 90% 2H), 1.49 (3 H, s), 1.30 (3 H, s). $^{13}$C NMR (CDCl$_3$) 169.4, 152.3, 143.8, 135.3, 128.7, 128.4, 127.8, 127.1, 113.0, 98.3, 86.8, 85.6, 83.6, 81.8, 78.0, 68.1, 64.3, 57.2, 48.4, 28.9 (deuterium coupling) 26.9, 25.3.

(aS,5S)-α-(benzyloxy carbonyl amino)-5-isoxazolidineacetic Acid Methylidene Ester (58).

This was prepared by the method of Whitney$^{65}$ except as follows. To 66 (1.945 g, 2.75 mmol) at 0 °C was added cold 96% formic acid (17 mL). The mixture was stirred for 15 hours at room temperature and concentrated. To the residue was added cold ether (20 mL) and ice cold 2 N hydrochloride acid (10 mL). The aqueous portion was washed with cold ether (2 x 20 mL), neutralized with sodium bicarbonate, saturated with sodium chloride, and extracted with dichloromethane (5 x 20 mL). The organic solution was dried and concentrated to give 642.5 mg (77.3%) of 58 as an oil. A fraction of it was purified by flash chromatography (silica gel, EtOAc/hexane: 6/5); TLC (EtOAc/hexane: 6/5) R$_f$ 0.21; IR 1796, 1714; $^1$H NMR (CDCl$_3$) δ 7.37 (5H, m), 5.54 (1H, br s), 5.33 (1H, d, J=4 Hz), 5.19 (2H, ABq, J=12 Hz), 4.54 (1H, br m), 4.47 (1H, s), 4.05 (1H, br m), 3.26 (1H, m), 3.17 (1H, m), 2.62 (1H, m), 2.42 (1H, br s), EIMS: 293.1 (M$^+$, 100).

(aS,5S)-α-[(benzyloxy carbonyl) amino]-5-[4',4'-$^2$H$_2$]isoxazolidineacetic Acid Methylidene Ester (58a).

This was prepared as above except as follows. To 66a (2.8 g, 39.5
mmol) at 0 °C was added cold 96% formic acid (22 mL). The mixture was stirred for 16 hours at room temperature and was worked-up to give 0.802 g (67%) of 58a as an oil. [α]D\textsuperscript{25} = 177.76 ° (c, 0.49, CHCl\textsubscript{3}; lit.\textsuperscript{65} 176 °, c, 1.0, CHCl\textsubscript{3})); IR 1796, 1714; \textsuperscript{1}H NMR (CDC\textsubscript{3}) 7.37 (5H, m), 5.54 (1H, br s), 5.33 (1H, d, J=4 Hz), 5.19 (2H, ABq, J=12 Hz), 4.83 (1H, br s), 4.54 (1H, br m), 4.47 (1H, s), 3.26 (1H, d, J=15 Hz), 3.2 (2H, ABq, J=15 Hz), 3.12 (1H, d, J=15 Hz), 2.62 (m, 93.4% D), 2.42 (br s, 93.2% D); \textsuperscript{13}C NMR: 169.6, 152.3, 135.3, 128.7, 128.6, 128.3, 79.4, 78.1, 68.0, 57.3, 49.1, 31.6, EIMS 295.1 (M\textsuperscript{+}, 24.1, d\textsubscript{2}), 294.1 (M\textsuperscript{+}, 11.5, d\textsubscript{1}). Calculated deuterium content: 62.26%-d\textsubscript{2}, 26.01%-d\textsubscript{1}.

(αS, 5S)-α-Amino-5-isoxazolidineacetic Acid (25).

To a solution of 58 (570 mg, 1.89 mmol) in 15 mL dry dichloromethane at 0 °C under a nitrogen atmosphere was added 1N boron trichloride in dichloromethane (12 mL, 12 mmol). The mixture was stirred for 20 hours at room temperature, and quenched with water (10 mL) at 0 °C. The organic layer was separated from the aqueous layer and was extracted with water (3 x 25 mL). The combined aqueous solution was concentrated by rotary evaporator at 30 °C to dryness. The resulting solid was redissolved in 1 N HCl (35 mL), loaded onto a cation exchange column (Dowex 50 x 4, 200~400 mesh, 1 cm x 20 cm). The column was washed with 150 mL water, and then eluded with 0.5 N ammonium hydroxide. The ninhydrin positive fractions were combined, and freeze dried to yield 202.5 mg (76.8%) of 25 as a powder. A portion of 25 was recrystallized from ethanol to give white crystals. TLC (silica gel, i-PrOH/EtOAc/H\textsubscript{2}O: 4/4/3) R\textsubscript{f} 0.32; [α]D\textsuperscript{25} = + 1.43 ° (c, 0.91, H\textsubscript{2}O); mp 144 °C (dec.); IR 3175, 3170~2500, 1653, 1523; \textsuperscript{1}H NMR (D\textsubscript{2}O, t-BuOH) δ 4.5 (1H, d, d, d, J=10.8, 8.5, 3.8 Hz), 4.1 (1H, d, J=3.8 Hz), 3.3 (1H, d, d, d, J=11.6, 8.2, 5.0 Hz), 3.1 (1H, m), 2.4 (1H, d, d, d, J=15.7, 13.0, 8.4 Hz), 2.2 (1H, m); \textsuperscript{13}C NMR (D\textsubscript{2}O, t-
BuOH) 172.6, 79.6, 56.7, 49.3, 31.4; Anal. Calcd for C$_{5}$H$_{10}$N$_{2}$O$_{3}$: C, 41.09; H, 6.90; N, 19.17. Found: C, 40.96; H, 7.11; N, 19.25.

(αS, 5S)-α-Amino-5-[4,4-2H$_{2}$]isoxazolidineacetic Acid (25a).

This was prepared as above except as follows. To a solution of 58a (230 mg, 0.76 mmol) in dry dichloromethane (15 mL) under nitrogen atmosphere was added 6 mL (6.0 mmol) of 1N boron trichloride in CH$_{2}$Cl$_{2}$ at 0 °C. The mixture was stirred for 18 hours and quenched with water. Subsequent work-up and recrystallization afforded 69.2 mg (61.8%) of 25a as a white solid. $^{1}$H NMR (300 MHz, D$_{2}$O, t-BuOH) δ 4.51 (1H, m), 4.09 (1H, d, J=3.8 Hz), 3.28 (1H, m), 3.07 (1H, m), 2.36 (m, 89%, deuterium), 2.21 (m, 89.6%, deuterium).

(αS,5S)-α-[benzyloxycarbonyl]amino)-4,5-dihydro-5-isoxazoleacetic Acid Methylidene Ester (79).

This was prepared by the method of Whitney except as follows. A mixture of 58 (256.4 mg, 0.85 mmol) and N-chlorosuccinimide (136 mg, 1.02 mmol) in dichloromethane (15 mL) was stirred at room temperature for 1 hour, concentrated, and resuspended in carbon tetrachloride (5 mL). The mixture was filtered and purified by flash chromatography (silica gel, EtOAc/Hexane: 4/6) to give 194 mg (76.2%) of 79 as an oil. TLC (silica gel, EtOAc/hexane: 1/1) R$_{f}$ 0.42; IR(neat) 1800, 1716 cm$^{-1}$; $^{1}$H NMR (CHCl$_{3}$, 297 K) δ 7.38 (5H, m), 7.17 (1H, s), 5.55 (1H, br s), 5.39 (1H, d, d, J=4 Hz), 5.21 (2H, ABq, J=12 Hz), 5.10 (1H, br, m), 4.33 (1H, br s), 3.51 (1H, d, d, J=18 Hz), 2.36 (m, 89%, deuterium), 2.21 (m, 89.6%, deuterium).
(αS,5S)-α-((benzyloxycarbonyl)amino)-4,5-dihydro-5-[4,4-H_2] isoxazoleacetic Acid Methylidene Ester (79a).

This was prepared as above except as follows. A mixture of 58a (540 mg, 1.77 mmol) and N-chlorosuccinimide (240 mg, 1.80 mmol) in dichloromethane (15 mL) was stirred for 1 hour at room temperature, concentrated and purified directly by flash chromatography (silica gel, EtOAc/Hexane: 4/6) to afford 444.9 mg (82.9%) of 79a. \([\alpha]_D^{25} = +246.5^\circ (c, 1, CHCl_3); \text{lit.} +251.5^\circ (c, 0.944, CHCl_3); \text{IR} (\text{neat}) 1800, 1716 \text{cm}^{-1}. \)<br />

1H NMR (CHCl_3, 297 K) \(\delta 7.38 (5H, m), 7.17 (1H, s), 5.55 (1H, br s), 5.39 (1H, d, d, J=4 \text{ Hz}), 5.21 (2H, ABq, J=12 \text{ Hz}), 5.10 (1H, br, s), 4.33 (1H, br s), 3.51 (1H, d, J=4, 7 \text{ Hz}), 3.21 (1H, br, m); ^1\text{H NMR} (323 K), 7.35 (5H, m), 7.10 (1H, s), 5.53 (1H, d, J=4 \text{ Hz}), 5.36 (1H, d, J=4 \text{ Hz}), 5.21 (2H, ABq, J=12 \text{ Hz}), 5.03 (1H, m), 4.29 (1H, s), 3.48 (m, 86.4% deuterium), 3.14 (m, 84% deuterium).

(αS,5S)-α-Amino-4,5-dihydro-5-isoxazolidineacetic Acid (26).

This was prepared by the method of Whitney except as follows. To a solution of 79 (65.2 mg, 0.217 mmol) in trifluoroacetic acid (2 mL) under a nitrogen atmosphere was slowly added 0.8 M boron tris(trifluoroacetate) \(^{310}\) in trifluoroacetic acid (1.2 mL) at 0 °C. The reaction mixture was stirred for one hour at 0 °C, concentrated, and treated three times with methanol and evaporated to dryness. The resulting mixture was added 2 N hydrochloride acid (3 mL), filtered, and loaded onto a cation exchange column (Dowex 50 x 4, 200-400 mesh, 5 mm x 120 mm). The column was washed with water, eluted with 0.5 N ammonia hydroxide. The ninhydrin positive fractions were pooled and freeze-dried to give 18.2 mg (58.3%) of 26. TLC (i-prOH/EtOAc/H_2O: 4/4/3/ on silica gel); \(R_f\) 0.40; ^1H
NMR (D$_2$O) 7.44 (1H, s), 5.10 (1H, d, d, d, J=11.5, 7.7, 3.5 Hz), 4.10 (1H, d, J=3.6 Hz), 3.35 (1H, d, d, d, J=18.8, 11, 3.5 Hz), 3.23 (1H, d, d, d, J=18.8, 7.8, 3.5 Hz).

(αS, 5S)-α-Amino-4,5-dihydro-5-[4,4-$^2$H$_2$]isoxazolidineacetic Acid (26a).

This was prepared as above except follows. To a solution of 79a (112.3 mg, 0.372 mmol) in trifluoroacetic acid (5 mL) under a nitrogen atmosphere was slowly added 0.8 M boron tris(trifluoacetate)$^{310}$ in trifluoroacetic acid (2.8 mL) at 0 ºC. The reaction mixture was stirred for one hour at 0 ºC, and subsequently worked-up to give 38.4 mg (70%) of 26a. $^1$H NMR 7.44 (1H, s), 5.09 (1H, m), 4.10 (1H, d, J=3.7 Hz), 3.3 (m, 81% deuterium), (98.6% ee); $^{13}$C NMR 172.7, 151.7, 78.2, 58.4, 39.0 (shows deuterium coupling).


To the solution of crude 3 (10 mg) in 50% aqueous dioxane (200 mL) was added triethylamine (30 mL), followed by Boc-ON reagent (60 mg). The mixture was stirred overnight at r.t.. The dioxane was removed by rototary evaporator, and the remaining aqueous was washed with EtOAc, acidified, and again extracted with chloroform (3 x 2 mL). The crude oil of 10 mg was obtained. Without purification, this material was dissolved in 2 mL MeOH, and was added dropwise to an ether solution of diazomathane until a light yellow solution was obtained. It was then concentrated and purified by flash chromatography (EtOAc : Hexane = 4 : 7) to give 6.5 mg material. IR 3349, 2977, 1714, 1162 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$=297
K: δ 5.44 (1H, d, J=8.5); 4.48(1H, m); 4.3(1H, m); 3.8-3.7( 1H, m); 3.77 (3H, s), 3.53-3.45 (1H, m), 2.47-2.28 (2H, m), 1.49, 1.44 (18 H, 2 x s). T=323 K: 4.48 (1 H, d,d, J=8.5, 4.6 Hz); $^{13}$C NMR: δ 169.6, 157.0, 81.9, 80.1, 52.2, 47.1, 30.0, 28.1, 28.0.

Biochemical Studies

**Fermentation of S. suiceus**

Seed medium and production medium for the acivicin and 4-hydroxyacivicin are listed in Table 2.

Seed medium (2 x 50 mL) was prepared in 250 mL erlenmeyer flasks. After autoclaved at 132 °C for 20 minutes, it was inoculated with S. suiceus spores preserved as a soil culture. One loop of such a soil culture was usually used. The seed culture was incubated in a rotatory shaker at 26-27 °C, 285 rpm for 69 hours. Sterile production media (10 x 200 mL) contained in the baffled flasks were then inoculated by the seed culture (5 mL, 2.5% v/v) and were incubated at 30 °C, 250 rpm for 120 hours.

<p>| Table 2. Ingredients of Fermentation Media |</p>
<table>
<thead>
<tr>
<th>Seed Medium</th>
<th>Production Medium</th>
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<tbody>
<tr>
<td>Glucose</td>
<td>0.5 g</td>
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<tr>
<td>Peptone (Difco)</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>0.13 g</td>
</tr>
<tr>
<td>Milli-Q Water</td>
<td>50 mL</td>
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Purification of Phenylalanine, Methionine and Tyrosine from *S. sviceus*.

The fermentation broth was centrifuged (9 K x 20 min.), and the supernatant was collected along with the H₂O washings of the mycelium. The combined broth was filtered through celite and adjusted pH to 7.8 before loading on a cation exchange column (Dowex 50 x 8, 100-200 mesh, H⁺ form, 55 mm x 20 mm). The column was washed with water and eluded with 2.5 N ammonium hydroxide. The eluded fractions were tested by ninhydrin spray on a silica gel plate. Ninhydrin positive fractions were combined. After removal of ammonia by a rotary evaporator, the solution was dried by a freeze drier. The resulting solid (7-8 g) was then added 120 mL water, and centrifuged to remove fine solid. After adjusting the pH to 7.0, this solution was loaded to an anion exchange column containing AG 3 x 4 resin (100-200 mesh, OH⁻ form, 20 mm x 200 mm). The column was washed with water, 50% ethanol, 90% ethanol successively, and eluded by methanol/water/acetic acid (90/10/3). The ninhydrin positive fractions were pooled and concentrated. Further drying using freeze dryer gave about 2 g solid material which was separated by flash chromatography (methyl ethyl ketone/acetone/water: 65/20/15) to obtain phenylalanine and a mixture of other amino acids. The mixture was separated by the use of paper chromatography (n-butanol/acetic acid/water: 12/3/5) using a descending method. After development, a strip of the chromatography paper was tested by ninhydrin spray. The ninhydrin positive bands were cut out and eluded with water. The resulting solutions were freeze dried, and pure tyrosine and methionine sulfoxide were obtained.
**Purification of Leucine**

The water washings of anion exchange chromatography was collected and was freeze dried. Flash chromatography was then carried out as same as before to afford leucine.

**Purification of Tryptophan and Methionine**

On a different run of fermentation, the elute from an anion exchange chromatography was loaded to a cellulose column preconditioned with solvent chloroform/methanol/17% ammonia: 3.2/2/0.65. Eluting the column with the same solvent gave phenylalanine and a mixture of other compounds. The mixture was developed by PTLC on silica gel using the solvent n-PrOH/EtOAc/H₂O: 4/4/1.5. The separated bands were washed with methyl ethyl ketone/acetone/water (65:20:15) to give methionine and tryptophan. The TLC and the ¹H NMR of these amino acids were as same as those of the authentic samples.
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66. Materials obtained from Ju, S-C, Wityak, J; Acivicin was obtained
from the Department of Biochemistry, Oregon State University.


