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Investigations of the biosynthesis of sinefungin

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Rice University, 1989
RICE UNIVERSITY

INVESTIGATIONS OF THE BIOSYNTHESES OF SIN EFUNGIN

BY

ISIDORA YVONNE ARZU

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DOCTOR OF PHILOSOPHY

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HOUSTON, TEXAS
JULY, 1988
Abstract

Investigations of the Biosynthesis of Sinefungin

by

Isidora Yvonne Arzu

The biosynthesis of the antifungal, antiviral antibiotic, sinefungin (1), produced by Streptomyces griseolus has been investigated. Precursor incorporation studies using [U-14C], [5-3H], (5-13C) and (5-13C, 5-15N)-ornithine have shown that carbons 6'-10' were derived from the intact incorporation of ornithine with retention of the C-5 nitrogen and with the loss of one of the protons from C-5 of ornithine.

The results of the feeding experiments of [14C] and [3H] labelled adenosine have indicated that the adenine moiety of adenosine was incorporated intact. Administration of adenosine labelled on the ribose moiety indicated that adenosine was incorporated into sinefungin with 50% loss of protons from C-5' of adenosine.
ACKNOWLEDGEMENTS

Several people share responsibility for my completing my graduate studies. Dr. Ronald J. Parry deserves the lion share of the credit. His sharp mind, pleasant nature, expertise in chemistry and his patience provided the foundation for my success.

I would like to thank my husband, Henry, for his love, support and encouragement.

I cannot thank my parents, Charles and Frances Arzu, my brother and my sisters enough for their endless support, concern and interest in my progress.

Several members of the research group made my stay here very enjoyable and they deserve special mention. I extend special thanks to M. V. Naidu, Raghupathi Subramanian, Elizabeth Eudy Gomez, Robson Mafoti, and Rajesh Turakia
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Introduction

Sinefungin, 5'-deoxy - 5' - (1,4 - diamino - 4 - carboxybutyl) - adenosine (1) (Figure I), is an antifungal\(^1,\)\(^2\), antiamoebicidal\(^3,\)\(^4\) antibiotic isolated from the fermentation broth of the bacterium, *Streptomyces griseolus* NRRL 3739, a new strain of *Streptomyces* isolated from a soil sample collected in Cote d'Ivoire\(^5\). Sinefungin was first isolated by a group at Eli Lilly and Company along with its related metabolites: 4',5'-dehydrosinefungin, (A9145C) (2), sinefungin lactam, 5' - deoxy - 5' - (3'-aminopiperidin-2'-one - 6yl)adenosine (3), and 5' deoxy - 5' - (1-amino - 4 - ureido, 4 - carboxylamidobutyl)adenosine (4)\(^1,\)\(^6\) in 1973 during a search for compounds with antifungal activity (Figure II). The Eli Lilly group also elucidated the structures and published the complete spectral data of all the compounds they had isolated. In 1977, sinefungin and sinefungin lactam were also isolated from the fermentation broth of *Streptomyces incarnatus* by researchers at Rhone Poulenc\(^7\) in France.

Sinefungin is structurally related to S-adenosylhomocysteine (4) and S-adenosylmethionine (5)\(^8\) (Figure III). The antibiotic has some very interesting properties because of this similarity. Recently, sinefungin has been the subject of many studies focusing primarily on its ability to act as a potent inhibitor of S-adenosylmethionine dependent transferases\(^8-\)\(^19\). These are specific methyl transferases which use S-adenosylmethionine as a methyl donor. In these enzymatic systems, S-adenosylhomocysteine is the endogenous inhibitor. However, sinefungin is often a much more potent inhibitor.

Probably the most interesting property of sinefungin to humans is its very potent *in vivo* and *in vitro* antiparasitic activity against the malaria parasite *Plasmodium falciparum*\(^6,\)\(^19\), African *trypanosoma*\(^21\).
Figure I
Figure II
Figure III
and some species of *Leishmania*²³, 24-26.

Trypanosomaisis is endemic all over equatorial Africa infecting both man and wild and domesticated beast. Usually the disease starts with the infection of the blood and lymphatic system, but eventually the infection affects the central nervous system producing the characteristic sleeping sickness in its victims.

Leishmaniasis refers to a group of illnesses caused by a flagellated protozoan (which belong to species *Leishmania*) transmitted to humans by a fly's bite. The organism moves into the skin and bloodstream, localizes in the reticuloendothelial systems and then spreads from there²⁷. The *Leishmania* species used in the sinfungin studies is the causative agent of the cutaneous lesions in humans²⁵, ²⁸ who live in Central and, South America, the Mediterranean and Equatorial Africa.

Sinfungin also causes the inhibition of polyamine synthesis. S-Adenosylmethionine, a structural relative of sinfungin, not only serves as a methyl donor, but also as a precursor in the biosynthesis of polyamines which influence nucleic acid biosynthesis. The method of action seems to be sinfungin's interference with DNA replication and synthesis because S-adenosylmethionine is necessary for the methylation of DNA²⁹,³⁰ in the polyamines system. However, DNA polymerase of the *Trypanosomatidae* has been shown to be biochemically and immunologically distinct from its mammalian counterpart²⁸, ³¹-³³ Sinfungin appears to be antiparasitic at levels that are not toxic to mammalian cells. All these considerations, make sinfungin a rational target for chemotherapy²⁸ as an antiparasitic drug. Nevertheless, it had been reported in earlier literature that sinfungin was commercially unexploitable because of its nephrotoxicity to dogs³⁴.

There have been five partial syntheses³³-³⁷ and one complete
synthesis\textsuperscript{39} of sinefungin reported in the literature. There has been a concerted effort to find an analogue that might be more active with less toxicity because of its significant antifungal, antiparasitic, and antiviral\textsuperscript{10,18,41} activity.

On close inspection of this molecule, it becomes apparent that sinefungin might be biosynthesized by the condensation of the amino acid, ornithine (8), with the nucleoside, adenosine (7) (Scheme I). The researchers at Eli Lilly, in an effort to elucidate the origin of the carbon skeleton, administered several probable \textsuperscript{14}C labelled precursors to the fermentation broth of \textit{Streptomyces griseolus}. Very high levels of incorporation were reported for [U-\textsuperscript{14}C]-L-ornithine, [8-\textsuperscript{14}C]-adenosine, and [5-\textsuperscript{14}C]-DL-citrulline (Table I\textsuperscript{42}); however, very low incorporations were reported for [U-\textsuperscript{14}C]-L-arginine and no incorporation for [1-\textsuperscript{14}C]-D-ribose. The interpretation of these experiments is potentially flawed because the sinefungin isolated was never rigorously purified to ensure that activity measured arose from the incorporated label, nor was the sinefungin degraded to prove with certainty that the incorporation was indeed specific\textsuperscript{42}.

Our research was based on the published results by the Eli Lilly group. We started with the duplication of the experiments they had done, but their high levels of incorporation were not reproducible in our laboratory. However, adenosine and ornithine resulted in high enough levels of incorporation into sinefungin to pursue the study of its biosynthesis.

Sinefungin is the formal product of the carbon-carbon formation between C-5 of ornithine and C-5' of adenosine accompanied by the release of one mole of water (Scheme I). This bond formation is of the utmost interest because chemically this transformation would be
<table>
<thead>
<tr>
<th>$^{14}$C Substrate</th>
<th>% Total Incorporation$^a$</th>
<th>% Incorporation into Sinefungin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. [8-$^{14}$C]-Adenosine</td>
<td>52.6</td>
<td>33.1</td>
</tr>
<tr>
<td>2. [U-$^{14}$C]-ATP</td>
<td>38.1</td>
<td>28.3</td>
</tr>
<tr>
<td>3. [8-$^{14}$C]-Adenine</td>
<td>32.0</td>
<td>18.9</td>
</tr>
<tr>
<td>4. [U-$^{14}$C]-L-Ornithine</td>
<td>18.6</td>
<td>10.0</td>
</tr>
<tr>
<td>5. [5-$^{14}$C]-DL-Citrulline</td>
<td>16.8</td>
<td>10.9</td>
</tr>
<tr>
<td>6. [U-$^{14}$C]-L-Glycine</td>
<td>4.7</td>
<td>2.7</td>
</tr>
<tr>
<td>7. [U-$^{14}$C]-Glucose</td>
<td>3.6</td>
<td>1.9</td>
</tr>
<tr>
<td>8. [U-$^{14}$C]-L-Arginine</td>
<td>2.0</td>
<td>1.4</td>
</tr>
<tr>
<td>9. [U-$^{14}$C]-Acetate</td>
<td>1.7</td>
<td>0.97</td>
</tr>
<tr>
<td>10. [U-$^{14}$C]-L-Isoleucine</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>11. [Ureido-$^{14}$C]-L-Citrulline</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>12. [U-$^{14}$C]-L-Valine</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>13. [1,2-$^{14}$C]-Choline Cl</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>14. [U-$^{14}$C]-L-Glutamic Acid</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>15. [U-$^{14}$C]-L-Tyrosine</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>16. [U-$^{14}$C]-D-Ribose</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

$^a$ Reflects the incorporation into both A9145C and sinefungin

Table I
Scheme 1
extremely difficult unless both these molecules were sufficiently activated. For the purpose of this discussion on the probable mechanism by which sinefungin is biosynthesized, sinefungin can be divided into two moieties: ornithine and adenosine. Each moiety will be discussed separately.

First, there are two hypotheses for the conversion of ornithine into the appropriate nucleophilic species. We propose that under enzymatic conditions, ornithine (8) condenses with the coenzyme, pyridoxal phosphate (9) to form a Schiff's base (10) (Scheme II). The protons at C-5 of this reactive enamine would then exhibit enhanced acidity. The removal of one proton would allow alkylation by an electrophilic form of adenosine to afford a pyridoxal phosphate-sinefungin adduct (11). Subsequent to the alkylation, pyridoxal phosphate could then be removed by hydrolysis to give the desired sinefungin.

Another possible route for this conversion involves the transformation of ornithine (8) to glutamic-γ-semialdehyde (12) by ornithine transaminase (Scheme IIIa). This transformation would result in the loss of one of the protons at C-5 of ornithine and the loss of the nitrogen at C-5 of ornithine. The glutamic-γ-semialdehyde (12) could then react with thiamine pyrophosphate (13) to form an adduct (14). In the adduct, the remaining proton at C-5 of ornithine would exhibit enhanced acidity, again allowing alkylation by an electrophilic form of adenosine to form the thiamine-sinefungin complex (15) following the removal of the remaining proton. The elimination of thiamine pyrophosphate would afford 6'-oxosinefungin (16); the carbonyl group of 16 could then be transaminated to give the desired sinefungin (Scheme IIIa). However, if ornithine is converted to 17, the corresponding imine
(Scheme IIIb) of glutamic-γ-semialdehyde (15), then thiamine pyrophosphate could react with 17 to form an adduct (18). This adduct could then be alkylated with an electrophilic form of adenosine to afford the thiamine pyrophosphate - sinfungin complex (19) following the removal of the remaining proton from C-5 of ornithine. Sinfungin would be produced by the ensuing elimination of thiamine pyrophosphate followed by the reduction of the C-6' imine bond of 20.

It is conceivable that the nitrogen at C-5 of ornithine could be lost during the biosynthesis of sinfungin if sinfungin is formed via Scheme IIIa. The complete loss of the γ- nitrogen of ornithine would support the hypothesis of thiamine pyrophosphate as the possible pathway for the biogenesis of sinfungin. However, the nitrogen may be lost by a process that is unrelated to the biosynthesis of this antibiotic.

The retention of the γ-nitrogen of ornithine alone does not validate the proposal that the biosynthesis of sinfungin proceeds via thiamine pyrophosphate pathway because the nitrogen at C-5 of ornithine is retained during the biosynthesis of sinfungin via pyridoxal phosphate. The difference between the three pathways (Schemes II, IIIa and IIIb) for the activation of ornithine, is that both protons at C-5 of ornithine are lost if sinfungin is biosynthesized by either of the two thiamine pyrophosphate pathways, (Schemes IIIa and IIIb) and only one proton at C-5 from ornithine is lost and the nitrogen at C-5 of ornithine is retained during the biosynthesis of sinfungin via pyridoxal phosphate pathway (Scheme II).

There are three hypotheses that can be formulated for the transformation of adenosine into an electrophilic moiety (Scheme IV). Any one of the proposed species may subsequently react with either the pyridoxal pyrophosphate or the thiamine pyrophosphate adduct of
ornithine in the aforementioned schemes. These nucleophilic forms of ornithine are represented as ornithine with a negative charge at C-5 (21). One electrophilic form of adenosine could be adenosine triphosphate (ATP) (12), which is a known substrate for some enzymatic systems. For example, it is well known that the coenzyme form of vitamin B₁₂ is synthesized in living systems by the reaction of adenosine triphosphate with the reduced form, vitamin B₁₂S. This transformation is catalyzed by enzyme cob(1)alamine adenosyltransferase⁴⁵. In biological systems, adenosine triphosphate is also the substrate for the enzyme, methionine adenosyltransferase which to catalyzes the formation of S-adenosylmethionine⁴⁶. If the electrophilic species is adenosine triphosphate, then there should be no perceivable loss of protons from C-5⁻ observed during the biosynthesis of sinefungin.

The second probable electrophilic species of adenosine could be 3⁻-oxo, 5⁻-deoxy-4⁻,5⁻-dehydroadenosine (23). This a reactive intermediate formed in the conversion of S-adenosylhomocysteine to adenosine and homocysteine catalyzed by the enzyme, S-adenosylhomocysteine hydrolase⁴⁷. If 23 were the intermediate involved in the sinefungin formation, then there would be a loss of the proton at C-4⁻; however, there would be no loss of protons observed from C-5⁻.

A third possibility would be the thioester of adenosine 5⁻-carboxylic acid (24). In this instance, there would be complete loss of protons from C-5⁻. The product from the reaction of the ornithine adduct with 21 would be 5⁻-oxosinefungin (25). The subsequent reduction of the ketone to a methylene group would produce sinefungin. The complete loss of protons from C-5⁻ of adenosine is insufficient in and of itself to
rule out the other two schemes as possible mechanisms by which sinefungin formed. The loss of the protons might be caused by process(es) unrelated to the biosynthesis of sinefungin.

Classically, the use of the radioisotopes, eg, $^3$H and $^{14}$C, is the most common method for investigating the biosynthesis of naturally occurring compounds. During the preliminary phase of the investigations $^{14}$C labelled precursors are administered to the producing organism followed by degradation of the isolated natural product to pin-point the exact location of the incorporated label$^{48}$. After these preliminary studies, the specificity of the label incorporation is tested by the administration doubly labelled precursors with a known $^3$H/$^{14}$C ratio. If the proposed precursor is incorporated intact, then the isolated labelled natural product and the precursor will have essentially identical ratios. However, a major change in the $^3$H/$^{14}$C ratio indicates a distintegration of the precursor.

This is a powerful tool in that only a very small amount of the generally or specifically labelled, hypothetical precursors is necessary for administration to the producing organism. The reason for this is that the detection of the incorporated label is accomplished by the extremely sensitive technique of liquid scintillation counting$^{48}$. Usually the degradation products of the natural product or a suitable derivative must be crystallized several times to a constant specific activity. This is important, in order to ensure that the activity measured originated from the natural product itself and not from a radiochemical impurity. Unfortunately, this technique was not useful for sinefungin because sinefungin proved very difficult to crystallize.

A more modern technique of investigation is the use of stable isotopes ($^2$H and $^{13}$C) as a probe for the specificity of incorporation
of the labelled precursors into the natural products of interest. Since it is very difficult to discern the exact location of the incorporated label by means of mass spectra without prior knowledge of the fragmentation patterns of the natural product, $^{13}$C-NMR and $^2$H-NMR are a more useful tools. Relatively large quantities of the $^{13}$C or $^2$H labelled precursors which are highly enriched at specific positions, are usually administered to the producing organism. Hopefully, "loading up" the fermentation permits the producing microorganism to efficiently take up the labelled precursor. Increased incorporation of the labelled precursor facilitates recognition of the enriched signal in the $^{13}$C-NMR spectrum of the natural product. The intensity of the enriched signal is contingent upon the ready convertibility of the pool of labelled precursors into the labelled natural product. There is usually a direct correlation between the intensity of the enriched signal and the proximity between the natural product product and the labelled precursor on the metabolic pathway leading to the natural product. The position of the incorporated label can be determined by the enhancement of the expected resonance, which would be greater than that due to natural abundance in the spectrum of the enriched natural product.

We propose to investigate the biosynthesis of sinuefungin through the feeding of both radiolabelled and $^{13}$C labelled precursors to the fermentation broth of *Streptomyces griseolus*. The issues which will be addressed in this thesis are: the mechanism by which adenosine is incorporated, the degree of proton loss from C-5 of ornithine and C-5' of adenosine, respectively, and finally, the question of whether or not the $\gamma$-amino group of ornithine is retained. To ascertain whether the nitrogen from C-5 is retained or lost during the formation of sinuefungin ($5^{13}$C, $5^{15}$N)-L-ornithine (36) could be administered to the fermentation broth
of *Streptomyces griseolus.* If the contiguous labels are incorporated intact, then \(^{13}\text{C}\) signal for C-6' of sinefungin would be split into a doublet by the adjacent \(^{15}\text{N}\), which has a spin of \(1/2\). The differentiation between these three schemes for the activation of ornithine by thiamine phosphate and the activation of ornithine by pyridoxal pyrophosphate would be accomplished by administration of (5RS)-[5-\(^{3}\text{H}\)]-L-ornithine (45) followed by the measurement of the degree of proton loss in the isolated sinefungin. In the hypothesis postulated in Scheme II, the loss of only one of the protons at C-5 would be observed in sinefungin. If the biosynthesis of sinefungin proceeds via Scheme IIIa or IIIb, then both protons at C-5 would be lost, in addition to the possible loss of the C-5 nitrogen.

The schemes postulated for the conversion of adenosine into suitable species for the biosynthesis of sinefungin could be differentiated from one another by the administration of (5'SRS) [5'-\(^{3}\text{H}\)]-adenosine (53) and/or [4'-\(^{3}\text{H}\)]-adenosine using [1'-\(^{14}\text{C}\)]-adenosine (60) as an internal reference. In Scheme IV if the biogenesis of sinefungin proceeded via either 22 or 23, then there would be no loss of protons at C-5' observed in sinefungin isolated. However, the proton at C-4' would be lost if sinefungin is formed via 23. There would be complete loss at C-5' if 24 is an intermediate during the biosynthesis of sinefungin.
RESULTS AND DISCUSSION

The early investigations of this project focused on finding a reliable and reproducible method for the isolation and purification of sinefungin. Eli Lilly and Company furnished a generous sample of sinefungin and a protocol by which sinefungin could be isolated and purified. We were unsuccessful in isolating and purifying sinefungin by the method that Eli Lilly had provided us and had to develop our own. The protocol that was devised is summarized in Scheme V.

Having found a reliable method by which sinefungin could be isolated, we then had to find a method by which we could monitor sinefungin production by high pressure liquid chromatography (HPLC). Investigators at Eli Lilly had developed a method for HPLC using a water jacketed 4.6 mm X 150 mm HPLC column which was filled with Bio-Rad Aminex-7 packing and was heated to 70°C. Sinefungin was eluted at a rate of 0.5 mL per minute with a solution of 5% isopropanol in 0.5 N ammonium acetate adjusted to pH 9.5. Under these conditions, sinefungin had a retention time of 7.2 minutes. This HPLC system was used to monitor antibiotic production during a time course study of the fermentation. Sinefungin production by Streptomyces griseolus was found to increase gradually over time reaching a maximum eight days after inoculation. In later experiments, this HPLC system was abandoned since it proved cumbersome and was sometimes unreliable. The HPLC system reported by Malina and Robert-Gero\textsuperscript{43} proved to be more convenient.

Although the researchers at Eli Lilly and Company reported that sinefungin was crystallized from a triphasic system of water, acetone and methanol\textsuperscript{50}, in our hands, sinefungin proved difficult to crystallize. Therefore sinefungin is not amenable to rigorous specific activity
*Streptomyces Griseolus*
8 days HPLC

mycelium → Centrifuge

Supernatant

\[ \text{Dowex 50 X 2} \]
\[ 0.05N \text{NH}_4\text{OH} \]

Crude Sinefungin

\[ \text{Cellulose} \]
\[ \text{BAW} \]

Sinefungin

**Scheme V**
acetone
dimethoxypropane
bis(4-nitrophenyl)phosphate

MeOH

Scheme VI
measurements because sinefungin and any derivatives prepared would have to be crystallized repeatedly to a constant specific activity, in order to ensure that the radioactivity measured was derived from sinefungin itself or its derivatives and not from a radiochemical impurity. Attempts to synthesize crystalline derivatives of sinefungin were unsuccessful. It was imperative that we develop methods by which we would be able to obtain pure sinefungin because the preliminary studies proposed were to be accomplished by the administration of small amounts of radiolabelled precursors.

In order to circumvent these difficulties, two derivatives were synthesized, each possessing distinctive chemical properties. 2',3' - O-Isopropylidenesinefungin (25) was prepared by a modification of a method of Hampton\(^6\) whereby sinefungin was stirred with acetone, 2,2-dimethoxypropane and bis(4-nitrophenyl)phosphate for 48 hours. Pure 2', 3' - O - isopropylidenesinefungin was isolated by paper chromatography and ion exchange chromatography. The second derivative, 2', 3' - O - isopropylidenesinefungin lactam (26), was prepared by refluxing 2',3'-O-isopropylidenesinefungin (25) in methanol for 72 hours (Scheme VI). 2', 3'-O-isopropylidenesinefungin lactam was purified by paper chromatography.

During the early stages of this work, *Streptomyces griseolus* produced approximately 25 mgs of sinefungin per liter of fermentation broth, but over time the cultures became much less productive. Sometimes the cultures produced as little as 1.5 mgs per liter or no sinefungin at all. Since we intended to analyze the sinefungin obtained from some of the studies by \(^{13}\)C- NMR, such low levels of antibiotic production were likely to create unnecessary difficulties. Since most *Streptomyces* cultures are not homogeneous, we decided to carry out one round of random strain selection on a *Streptomyces griseolus*
culture which produced no sinfungin. A spore suspension of the Streptomyces culture was made using a method by Hopwood et al\textsuperscript{52}. The spore suspension was successively diluted by factors of ten to a final dilution of $10^{-7}$. Each dilution was plated and allowed to grow in an incubator for 72 hours at 30°C. Individual colonies were scraped from the agar and allowed to grow in an inoculum medium for 48 hours. The inoculum was used to inoculate slants from each colony. One slant of each colony was chosen to inoculate small scale fermentations as described in Experimental. This led to the discovery of one colony which produced 18 mgs per liter.

Having found suitable derivatives, a culture which produced a reasonable amount of sinfungin, and a reliable HPLC system, we were now ready to initiate some precursor incorporation experiments in an effort to verify the results reported by the researchers at Eli Lilly and Company. Even though the research on both the origin of the amino acid moiety and the adenosine moiety was conducted simultaneously, each moiety will be discussed separately, for the purpose of clarity.

The Amino Acid Moiety

The Eli Lilly group reported impressive levels of incorporation into sinfungin when they administered $[8.14\text{C}]$-adenosine, $[U.14\text{C}]$-adenosine triphosphate, $[U.14\text{C}]$-L- ornithine and $[5.14\text{C}]$-DL- citrulline to Streptomyces griseolus. The Eli Lilly group measured the incorporation of these precursors into sinfungin by liquid scintillation counting of the eluant from an ion exchange resin. The eluant was concentrated to a small volume. The crude sinfungin concentrate was then developed by thin layer chromatography and the plates were then
subjected to scanning thin layer densitometry to calculate the $^{14}$C radioactivity. Our research commenced with the administration of $[\text{U}^{-14}\text{C}]$-L-ornithine to the fermentation of *Streptomyces griseolus*. This precursor resulted in no perceptable incorporation into the isolated sinefungin. This was puzzling to us because the Eli Lilly group reportedly found 10% incorporation of $[\text{U}^{-14}\text{C}]$-L-ornithine into sinefungin. The result, which was contradictory to our hypothesis, was interpreted as possibly due to a loss of the label by decarboxylation of the ornithine. Therefore, the incorporation of ornithine with a different labeling pattern was examined.

Administration of $[\text{U}^{-14}\text{C}]-\text{L-ornithine}$ resulted in an incorporation of 0.12% after rigorous purification of the isolated sinefungin. This incorporation level was considerably lower than that which was reported by the Lilly group. At this point, we believed that a complete reinvestigation of sinefungin biosynthesis was warranted, and so we turned our attention to the testing of other probable precursors of the amino acid fragment.

The administration of both $[\text{U}^{-14}\text{C}]-\text{L-methionine}$ and $[\text{U}^{-14}\text{C}]-\text{L-aspartic acid}$ resulted in negligible incorporations (Table II). However, when $[\text{U}^{-14}\text{C}]-\text{L-serine}$ and $[\text{U}^{-14}\text{C}]-\text{pyruvate}$ were supplied to *Streptomyces griseolus* in separate experiments, they both resulted in moderate levels of incorporation which were, however, lower than that observed with ornithine. It is well known that serine is a major source of glycine and single carbon units needed for the synthesis of methyl and formyl groups. Serine is readily converted to glycine by the enzyme serine transhydroxymethylase$^{51}$. Glycine is a direct precursor in the biosynthesis of the adenine ring by the provision of the carbons which become C-4 and C-5 of adenine. Pyruvate is a primary metabolite which is utilized in many metabolic pathways.
<table>
<thead>
<tr>
<th>Expt.</th>
<th>Precursor (^{(3\text{H}/14\text{C})})</th>
<th>Incorporation into Sinefungin (^{(3\text{H}/14\text{C})})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>[1-(^{14}\text{C})]-L-Ornithine</td>
<td>0.0%</td>
</tr>
<tr>
<td>2.</td>
<td>[U-(^{14}\text{C})]-L-Ornithine</td>
<td>0.12%</td>
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<tr>
<td>3.</td>
<td>[U-(^{14}\text{C})]-L-Aspartic Acid</td>
<td>0.098%</td>
</tr>
<tr>
<td>4.</td>
<td>[U-(^{14}\text{C})]-L-Serine</td>
<td>0.013%</td>
</tr>
<tr>
<td>5.</td>
<td>[U-(^{14}\text{C})]-L-Methionine</td>
<td>0.002%</td>
</tr>
<tr>
<td>6.</td>
<td>[U-(^{14}\text{C})]-Pyruvate</td>
<td>0.013%</td>
</tr>
<tr>
<td>7.</td>
<td>[U-(^{14}\text{C})]-L-Arginine</td>
<td>0.31%</td>
</tr>
<tr>
<td>8.</td>
<td>(5RS)-[5-(^{3}\text{H})],[U-(^{14}\text{C})]-Ornithine (4.55)</td>
<td>0.12% (2.34) 51.4%(^{3}\text{H}_{\text{ret}})</td>
</tr>
<tr>
<td>9.</td>
<td>(5-(^{13}\text{C}))-L-Ornithine</td>
<td>4% (C-6')</td>
</tr>
<tr>
<td>10.</td>
<td>(5-(^{13}\text{C}),5-(^{15}\text{N}))-L-Ornithine</td>
<td>6.7% ((J_{\text{CN}} = 4.3\text{Hz}))</td>
</tr>
</tbody>
</table>

\(^{3}\text{H}_{\text{ret}}=\text{retention of the tritium label}\)
All the precursors tested so far resulted in such low levels of incorporation that we had no solid evidence for a specific precursor of the amino acid moiety of sinefungin. We were mystified by the lack of promising results obtained from the administration of the obvious precursors. Since ornithine produced the highest observed levels of incorporation, we decided to reexamine ornithine as the origin of the amino acid fragment by feeding specifically labelled ornithine. A synthesis of (5\(^{13}\)C)-L-ornithine was undertaken following a published procedure\(^6\)\(^4\). L-Methionine (27) was tosylated to afford N-tosylmethionine (28) followed by lactonization to N-tosylaminobutyrolactone (29). The lactone ring was opened with hydrobromic acid in acetic acid to give the bromide (30). The carboxylic acid moiety of the bromide 30 was protected as the methyl ester (31) followed by the protection of the amino group of 31 as the carboxbenzyloxy amide (32). The bromide moiety of 32 was displaced with \((^{13}\)C\()-cyanide to afford the protected nitrile (33). All the protecting groups of 33 were removed to give (5-\(^{13}\)C\)-L-ornithine (34) (Scheme VII).

The patent filed by both Eli Lilly and Rhone Poulenc provided \(^{13}\)C-NMR spectral data for sinefungin and its lactam. In the Rhone Poulenc patent, all the signals in the \(^{13}\)C-NMR of sinefungin are assigned. If (5-\(^{13}\)C) -L-ornithine were specifically incorporated, then the signal corresponding to C-6' at 48.2 ppm would be enriched. To our delight, administration of this (5-\(^{13}\)C)-L-ornithine resulted in a 4% enrichment at 48.2 (Figure IV). Unfortunately the fermentation to which the labelled ornithine was administered did not produce sinefungin well; nevertheless, the enrichment for the resonance the C-6' is clearly visible.

Encouraged by this result, we decided to ascertain whether the nitrogen at C-5 of ornithine is retained or lost during the biosynthesis of
Scheme VII
Figure IV. $^{13}$C-NMR of Sinfungin from (5-$^{13}$C)-Ornithine Feeding
Scheme VIII
sinefungin. (5-\textsuperscript{13}C, 5-\textsuperscript{15}N)-L-ornithine (36) was synthesized by a modification of the procedure just outlined. Using K\textsuperscript{13}C\textsuperscript{15}N, the bromide of 32 was displaced to give the doubly labelled nitrile (35). All the protecting groups were removed to afford (5-\textsuperscript{13}C, 5-\textsuperscript{15}N)-L-ornithine (36) (Scheme VIII). The \textsuperscript{13}C-NMR spectra of the (5-\textsuperscript{13}C, 5-\textsuperscript{15}N)-L-ornithine (36) revealed a doublet with a very small coupling constant of (J\textsubscript{CN} = 4.9 Hz) in the signal of C-5 of ornithine caused by the \textsuperscript{15}N label. If the C-5 nitrogen were retained on the administration of (5-\textsuperscript{13}C, 5-\textsuperscript{15}N)-L-ornithine to \textit{Streptomyces griseolus}, one would expect the coupling constants (J\textsubscript{CN}) of both the isolated sinefungin and the doubly labelled ornithine to be similar in the \textsuperscript{13}C-NMR spectra because the chemical environment of methylene group attached to the amine (-CH\textsubscript{2}NH\textsubscript{2}) of interest does not change drastically in the transformation of ornithine to sinefungin. The doubly labelled ornithine (36) was supplied to \textit{Streptomyces griseolus} which resulted in sinefungin that exhibited a doublet with 6.7% enrichment in the signal corresponding to C-6' in the \textsuperscript{13}C-NMR spectrum of the isolated sinefungin (Figure V). The doublet had a coupling constant of J\textsubscript{CN} = 4.3 Hz (Figure VI). This result demonstrates that the C-5 nitrogen of ornithine is retained during the biosynthesis of sinefungin.

The aforementioned results conclusively proved ornithine to be a specific precursor of sinefungin. On inspection of sinefungin, it is obvious that at least one of the protons at C-5 must be removed during the transformation of ornithine to sinefungin. In order to understand the stereochemical course of the transformation of ornithine to sinefungin, we proceeded to evaluate the extent of proton loss from C-5 of ornithine by the feeding of doubly labelled ornithine. If no protons were lost upon the administration of the labelled ornithine mixture, then the \textsuperscript{3}H/\textsuperscript{14}C
Figure 1. 13C-NMR of Sinelungin from (5-13C,5-15N)L-ornithine.
Figure VI. Doublet at 48.2 ppm (C-6') of Sinefungin
ratio of the isolated sinefungin and that of the labelled ornithine would be identical. However, if one or both protons were lost, then the $^3$H/$^{14}$C ratio of the isolated sinefungin would reveal a 50% or 100% tritium loss, respectively. An attempt was made to synthesize labelled 2(RS), 5(RS) - [5-$^3$H] - DL- ornithine (45) by a modification of a procedure by Townsend [66] (Scheme IX). $\gamma$, $\gamma$ - Dicarbethoxy - $\gamma$ - phthalimidobutyraldehyde (38) was prepared by a modification of a procedure by Moe and Warner, which was the condensation of freshly distilled acrolein with diethylphthalimidomalonate (37) [67]. The reduction of the aldehyde (38) by sodium borohydride resulted in the corresponding alcohol (39) which was then converted to its mesylate (40). The mesylate (40) was displaced with azide ion to give ethyl 2-carbethoxy-5-azidovalerate (41). Hydrolysis of 41 with concentrated hydrochloric acid and glacial acetic acid (1:1) resulted in the formation of several products (Scheme IX). Analysis of the reaction mixture revealed that one of the components had the same $R_f$ value as authentic ornithine. Repeated attempts at the purification of the crude ornithine by ion exchange chromatography and cellulose column chromatography failed to afford pure ornithine. Therefore, another method for the synthesis of 5(RS)-[5-$^3$H]-ornithine (45) had to be devised. 5(RS)-[5-$^3$H]-ornithine (45) was prepared by chemistry reported by Havrnek [64] and Satoh [65]. Methyl - L - $\alpha$ - benzyloxy carbonylamine - $\gamma$ - cyanobutyrate (43) was reduced with a mixture of sodium borotritide and cobaltous chloride in ethanol to afford the radiolabelled ornithine, methyl - N - carbobenzyoxy - 5(RS) [5-$^3$H]-L-ornithine (44). All the protecting groups of 44 were removed to give 5(RS)-[5-$^3$H]-L-ornithine (45) (Scheme X).

The experiment with doubly labelled ornithine was carried out using commercially available [U-$^{14}$C]-L-ornithine as an internal standard.
Scheme X
with the 5(RS)-[5-^3^H]-ornithine (45) we had prepared. A mixture of the
two labelled forms of ornithine with a ratio of 4.6 supplied to
*Streptomyces griseolus* resulted in tritium retention of 51.4% in the
isolated sinefungin. The labelled sinefungin was converted into 2',3' -
isopropylidenesinefungin and purified. It had a $^3$H/$^1^4$C ratio of 2.24
corresponding to 49.6% retention of the tritium at C-5 of ornithine.
These results combined with the data obtained from the $^{13}$C-NMR
spectra demonstrating that the C-5 nitrogen of ornithine is retained
during the biosynthesis of sinefungin, suggest that the amino acid
fragment of sinefungin is biosynthesized via the formation of a Schiff's
base between pyridoxal phosphate and ornithine. This adduct can then be
alkylated with an electrophilic form of adenosine.

The biogenesis of sinefungin has recently been studied in cell free
extracts$^{43}$ of *Streptomyces incarnatus* by the French group of Halina
Malina and Malka Robert-Gero. Sinefungin was produced when the
investigators incubated a cell-free extract with dithiothreitol,
adenosine triphosphate (ATP), arginine, pyridoxal phosphate and Mg$^{+2}$ or
Co$^{+2}$. When the cell-free extract was incubated with adenosine and
ornithine under identical conditions, no sinefungin was detected$^{43}$. Upon
the incubation of [$^1^4$C guanido] -L- arginine with the cell-free
system, the researchers detected no radioactivity in the isolated
sinefungin. Very little sinefungin was produced if one of the metal ions
(Mg$^{+2}$ or Co$^{+2}$) was not included in the cell-free system. The
researchers concluded that arginine, not ornithine, was the source of the
amino acid moiety of this antibiotic.

The reason that the cell-free extract cannot manufacture
sinefungin from ornithine and adenosine may be due to the absence of the
enzymes necessary for the transformation of ornithine into arginine and
of adenosine into adenosine triphosphate. It is well known that ornithine
is readily converted into arginine via the urea cycle. Ornithine (8) is transformed to citrulline (46) by the enzyme ornithine transcarbamoylase. Another enzyme, argininosuccinate synthase converts citrulline to argininosuccinate (47) which is converted to arginine (48) by the enzyme argininosuccinase (Scheme XI). The Eli Lilly group found almost identical levels of incorporation for both [5-\textsuperscript{14}C]-DL-citrulline and [U-\textsuperscript{14}C]-L-ornithine into sinefungin. However, it is significant to note that the Eli Lilly group had found no perceivable incorporation on administration of [U-\textsuperscript{14}C]-L-arginine to the fermentation broth of \textit{Streptomyces griseolus}. The results are surprising because of the close relationship between ornithine, citrulline and arginine.

Phosphotransferases probably synthesize adenosine triphosphate in two steps: firstly, the conversion of adenosine to adenosine monophosphate and then secondly, adenosine triphosphate is synthesized by the transfer of a pyrophosphate group onto adenosine monophosphate. An explanation for the formation of only small quantities of sinefungin in the incubations devoid of Mg\textsuperscript{+2} may be due to the fact that Mg\textsuperscript{+2} is usually required for phosphate coordination during enzymatic reactions which utilize ATP.

The hypothesis for the biosynthesis of sinefungin proposed by Robert-Gero et al. was similar to that which we had postulated. Pyridoxal phosphate (9), activated by an enzyme, forms a Schiff's base with the terminal nitrogen of the guanido group of arginine (49) (Scheme XII\textsuperscript{43}). The protons at C-5 arginine would then exhibit enhanced acidity allowing the alkylation of the pyridoxal phosphate adduct by adenosine triphosphate with direct displacement of the triphosphate group. The pyridoxal phosphate sinefungin complex (50) would undergo hydrolysis to release one mole of urea, free pyridoxal phosphate, and sinefungin. If the biosynthesis of sinefungin were to proceed by this mechanism, one
would expect that the there would be a loss of one of the protons from C-5 of arginine and that the nitrogen at C-5 of arginine would be retained.

Prior to the publication of the Malka Robert-Gero's study on the biosynthesis of sinefungin we already had obtained results which proved ornithine to be a specific precursor of sinefungin. In light of the results of Robert-Gero, we decided to test the level of incorporation or arginine. Commercially available \([U-^{14}C] - L - arginine\) was administered to \(Streptomyces griseolus\) which resulted in an incorporation of 0.31% into sinefungin. This is not significantly different from the incorporation we had observed from the feeding of \([U-^{14}C] - L - ornithine\).

Our results, thus far, are consistent with those published by Malina and Robert-Gero.

**The Adenosine Moiety**

Having established the biogenetic origin of the five carbons of the amino acid moiety, we continued to reexamine the results of the Lilly group. Commercially available \([U-^{14}C] - adenosine\) was supplied to \(Streptomyces griseolus\) resulting in an incorporation of 0.4% into sinefungin (Table III). Again, we were unable to duplicate the high levels of incorporation observed by the Eli Lilly group. In their feeding of \([8-^{14}C] - adenosine\) they calculated a 33.1% incorporation into sinefungin. We decided to look at another possible biogenetic route for the synthesis of sinefungin.

The Eli Lilly study reported a good level of incorporation of \([U-^{14}C] - glucose\) into sinefungin. We decided to investigate it further.
[U-14C]-glucose was administered to *Streptomyces griseolus* resulting in 0.4% total incorporation into sinefugin. To evaluate whether the glucose was incorporated largely intact, a double labelled experiment was performed using a mixture of commercially available [3-3H]-glucose and [1-14C]-glucose. The rationale behind this experiment is that if glucose is incorporated into sinefugin with loss of one of the carbons and with no loss of protons or carbons, then the 3H/14C ratio of the isolated sinefugin would increase by 1/6 (3H/14C ratio of 4.28) relative to that of the administered precursor. A mixture of the labelled glucose with the 3H/14C ratio of 3.67 was supplied to the fermentation broth resulting in sinefugin with a ratio of 0.92 corresponding to 25% retention of the tritium label. This result had no obvious explanation. Therefore we decided to administer commercially available (1-13C) - glucose to *Streptomyces griseolus*, but there was no enrichment observed in the 13C-NMR spectra of the isolated sinefugin. Glucose was abandoned as a possible precursor because the results were not clear cut and definitive.

We turned our attention to the reexamination of adenosine because an incorporation of 0.40% was better than we had observed for the incorporation of ornithine into sinefugin. Undaunted, we examined the details of the incorporation of adenosine into sinefugin. To evaluate whether adenosine was specifically incorporated intact into sinefugin, a double labelled experiment was performed using a mixture of commercially available [8-14C]-adenosine and (5'RS) - [5'-3H] - adenosine prepared by a modification of procedure published by Schmidt56. 2',3'-O-Isopropylideneadenosine (51) was oxidized to the carboxylic acid 52. The carboxylic acid (52) was converted to a mixed anhydride which was then transformed into an acyl azide with sodium azide. The acyl azide was reduced with potassium borotritiide to yield
(5'-RS)-[5'-3H]-2',3'-O-isopropylideneadenosine (53). The isopropylidene group was removed by acid hydrolysis to afford (5'-RS)-[5'-3H]-adenosine (54) (Scheme XIII). We expected that adenosine would be incorporated intact into sinefungin, i.e., with no loss of protons, because the results published by Eli Lilly: [8-14C] - adenosine and [U-14C] - adenosine triphosphate had exhibited very high levels of incorporation. If the labelled adenosine is incorporated intact, i.e., with no loss of tritium, then the 3H/14C ratio of the isolated sinefungin would be identical to the ratio of the administered adenosine. The loss of protons would be revealed by a decrease in the tritium to carbon-14 ratio.

The radiolabelled adenosine mixture with a 3H/14C ratio of 4.7 was administered to Streptomyces griseolus. Much to our surprise, the isolated sinefungin had a 3H/14C ratio of 0.39. After conversion of sinefungin to its two derivatives, 2',3' isopropylidenesinefungin and the corresponding lactam, the ratio remain virtually unchanged (0.44 and 0.39 respectively), corresponding to 8.3% retention of the tritium. This result could signify one of two things: during the transformation of adenosine into sinefungin both protons at C-5' are lost or that during the biosynthesis of sinefungin, adenosine is being cleaved into adenine and ribose with the reincorporation of the adenine fragment. An explanation for this result could the operation of the purine salvage pathway, a route by which purine bases are conserved in living systems.

We investigated further the incorporation of adenosine into sinefungin by the administration of commercially available [2-3H]-adenosine with [U-14C] - adenosine as an internal standard. A mixture of the labelled adenosines with a 3H/14C ratio of 3.39 was supplied to the fermentation broth of Streptomyces griseolus, and this resulted in a 3H/14C ratio of 3.8 in the isolated sinefungin. The isolated sinefungin was then converted into 2',3'-O-isopropylidenesinefungin and the
Scheme XIII
corresponding lactam both of which exhibited a $^{3}\text{H}/^{14}\text{C}$ ratio of 4.53 and 4.59, respectively. These ratios corresponded to a 135% retention of the tritium label (Table III).

We were a little puzzled by the result because we had expected a $^{3}\text{H}/^{14}\text{C}$ ratio of either 3.39 (100% retention of the tritium label) or 6.78 ("200% retention"). If the ribose fragment of adenosine is not incorporated into sinfungin then five of adenosine's ten carbons or half of adenosine's carbons would be lost. A decrease of half of the carbon label would result in a doubling of the $^{3}\text{H}/^{14}\text{C}$ ratio; hence, the expected ratio should have been 6.78. If the adenosine had been incorporated intact, then the ratio would remain identical to that of the administered adenosine mixture. The ratio of 4.59 (135% retention) of the tritium label could represent a combination of intact incorporation plus partial incorporation of the adenine ring which in this experiment carried the ($^{3}\text{H}$) tritium label. If this hypothesis were correct, then one can calculate that 65% of the tritium label resulted from intact incorporation, while 35% of the tritium label resulted from the cleavage of the adenosine followed by the incorporation of the tritiated adenine.

The administration of a mixture [2-$^{3}\text{H}$]-adenosine and [8-$^{14}\text{C}$]-adenosine with a ratio of 4.35 to the fermentation of Streptomyces griseolus resulted in the incorporation into sinfungin and its derivatives possessing the ratio of 3.68, 3.83 and 3.92 or 90% retention of tritium. This confirms that adenine ring is incorporated intact.

In order to evaluate further the extent of proton loss at C-5'of adenosine, we decided to administer to Streptomyces griseolus a mixture of (5'RS)-[5-$^{3}\text{H}$]-adenosine, which had been synthesized earlier, and [U-$^{14}\text{C}$]-adenosine with a ratio of 4.09. Adenine and ribose (100 mgs each) were added to the fermentation broth in an attempt to block the
purine salvage pathway. The isolated sinefungin had a $^{3}\text{H}/^{14}\text{C}$ ratio of 0.98. The sinefungin was converted to $2',3',5'-\text{O}$-isopropylidenesinefungin and it had a $^{3}\text{H}/^{14}\text{C}$ ratio of 1.00 corresponding to 24.6% retention of the tritium label. It became clear that we needed to devise an experiment which would circumvent the purine salvage pathway.

One way to determine the extent of proton loss would be to place both labels in the ribose moiety of the adenosine molecule. We decided to supply *Streptomyces griseolus* with a mixture of (5′RS)-[5′-$^{3}\text{H}$]-adenosine and [1′-$^{14}\text{C}$]-adenosine. (5′RS)-[5′-$^{3}\text{H}$]-adenosine had been synthesized earlier. [1′-$^{14}\text{C}$]-Adenosine was not commercially available and a synthesis of it was therefore undertaken. Tetraacetyl-[1-$^{14}\text{C}$]-ribofuranose$^{58}$ (56) and tetraacetyl - [1-$^{14}\text{C}$] - ribopyranose (57) were prepared from commercially available [1-$^{14}\text{C}$]-ribose (55). Adenine was coupled with the 56 and 57 to give 2′,3′,5′-O-triacetyl-[1′-$^{14}\text{C}$]-adenosine$^{59}$ (58) and the six-membered ring analog 59. Both the triacetyl-[1-$^{14}\text{C}$]-adenosine (58) and 59 were deprotected by base hydrolysis and then separated by MPLC to afford [1′-$^{14}\text{C}$]-adenosine (60). The [1′-$^{14}\text{C}$]-adenosine prepared was not radiochemically pure when subjected to thin layer radioscanning. When it was mixed with (5′RS)-[5′-$^{3}\text{H}$]-adenosine, the mixture had a $^{3}\text{H}/^{14}\text{C}$ ratio of 3.43. A small portion of the doubly labelled mixture was diluted with unlabelled adenosine, an recrystallized repeatedly. In this manner, a corrected $^{3}\text{H}/^{14}\text{C}$ ratio of 4.62 was obtained.

The mixture of (5′RS)-[5′-$^{3}\text{H}$]-adenosine and [1′-$^{14}\text{C}$]-adenosine with a ratio of 3.43 was supplied to the *Streptomyces griseolus* and this resulted in a $^{3}\text{H}/^{14}\text{C}$ ratio of 2.14 in the isolated sinefungin which corresponds to a 62.4% retention of the tritium label. Using the corrected ratio of 4.62 for the administered precursor, sinefungin's $^{3}\text{H}/^{14}\text{C}$ ratio of 2.14 corresponds to a 46.3% retention of the tritium
<table>
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<th>Expt. Precursor ((^{3}\text{H}/^{14}\text{C}) ratio)</th>
<th>Incorporation into Sinefungin ((^{3}\text{H}/^{14}\text{C}))</th>
<th>(^{3}\text{H})ret*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. [8-(^{14}\text{C})]-Adenosine</td>
<td>0.40%</td>
<td></td>
</tr>
<tr>
<td>2. [U-(^{14}\text{C})]-Glucose</td>
<td>0.50%</td>
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<td>3. [1-(^{14}\text{C})]-D-Ribose</td>
<td>0.084%</td>
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<td>4. [3-(^{3}\text{H})],[1-(^{14}\text{C})]-Glucose (3.67)</td>
<td>0.040% (1.45)</td>
<td>25.1%</td>
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<td>5. (5'RS)-[5'-(^{3}\text{H})],[8-(^{14}\text{C})]-Adenosine (4.7)</td>
<td>0.60% (0.39)</td>
<td>8.3%</td>
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<td>6. [2,(^{3}\text{H})],[U-(^{14}\text{C})]-Adenosine (3.39)</td>
<td>0.66% (4.59)</td>
<td>135%</td>
</tr>
<tr>
<td>7. [2,(^{3}\text{H})],[8-(^{14}\text{C})]-Adenosine (4.35)</td>
<td>0.25% (3.92)</td>
<td>90.1%</td>
</tr>
<tr>
<td>8. (5'RS)-[5'-(^{3}\text{H})],[U-(^{14}\text{C})]-Adenosine (4.09)</td>
<td>(1.00)</td>
<td>24.7%</td>
</tr>
<tr>
<td>100 mgs each: adenine, ribose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9. (5'RS)-[5'-(^{3}\text{H})],[1-(^{14}\text{C})]-Adenosine (4.6)</td>
<td>(2.14)</td>
<td>46.2%</td>
</tr>
</tbody>
</table>

\(^{3}\text{H}\)ret = retention of the tritium label
label. The sinefungin was then converted into the 2',3'-O-isopropylidenesinefungin which had a $^{3}\text{H}/^{14}\text{C}$ ratio of 2.08. This corresponds to 60.1% tritium retention or 45.2% tritium label retention for the uncorrected and corrected $^{3}\text{H}/^{14}\text{C}$ ratios respectively.

The apparent 50% tritium loss is incompatible with the hypotheses postulated by Parry, the Eli Lilly researchers and Robert-Gero. On the basis of these hypotheses, it had been expected that there would be complete retention of all protons at C-5' of adenosine.

Sinefungin biosynthesized by Streptomyces griseolus also produces another metabolite, A9145C (2) which is 4',5'-dehydrosemiefungin. The researchers at Eli Lilly and Company reported that during the time course study of the fermentation broth of Streptomyces griseolus, A9145C appeared 168 hours (7 days) after inoculation whilst sinefungin production peaked 8 days after inoculation and then declined sharply on the 9th day. The microorganism produced very small amounts of A9145C compared to the 500 mgs per liter of fermentation broth reported for sinefungin. They observed that the loss of label from sinefungin was accompanied by an increased in the label incorporated in A9145C. The Eli Lilly group suggested that sinefungin is converted into A9145C. However, it is possible that the microorganism created A9145C first which was then reduced to sinefungin but for some unknown reason the organism ceased reduction and allowed A9145C to accumulate. The researchers would then observe an increase in concentration of A9145C in the fermentation broth.

Sinefungin could be formed by the formation of a Schiff's base between the C-5 nitrogen in arginine and pyridoxal phosphate. The pyridoxal phosphate - arginine adduct (49) could then be alkylated by 5'-adenosine aldehyde (60) after the removal of a proton from C-5 of
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arginine. Dehydration and hydrolysis of 61 would afford 4',5'-dehydrosinefungin, urea and pyridoxal phosphate (Scheme XV). The reduction of the 4',5' double bond would give the desired sinefungin. The sinefungin derived from the reduction of the double bond in A9145C (2) would result in the apparent loss of 50% of the tritium label. This hypothesis is inconsistent with the result reported by Robert-Gero because he found that no sinefungin was formed when he incubated adenosine with a cell-free extract. Another possible explanation for the loss of 50% of the tritium label could be the existence of an enzyme, sinefungin dehydrogenase, which could interconvert sinefungin and A9145C. It is also conceivable that during the conversion of adenosine triphosphate into sinefungin it loses 50% of the protons from C-5' by a process that is unrelated to the biosynthesis of sinefungin. Clearly, the biosynthesis of sinefungin appears to be more complex than previously imagined.

In the future, a screen for a strain of Streptomyces griseolus which produces large quantities of A9145C should be performed. Radiolabelled or $^{13}$C labelled A9145C could be made by fermentation, isolated, purified and administered to the culture currently in use to measure the level of incorporation into sinefungin. If sinefungin is a product of the reduction of the 4',5' double bond in A9145C, then the incorporation would be much higher than that observed for ornithine or adenosine. The hypothesis that the biosynthesis of sinefungin proceeds via the reduction of A9145C could also be tested by the administration of a mixture of [4',3H]-adenosine, which has been made enzymatically by Parry, and [1',14C]-adenosine. The sinefungin isolated in this experiment would exhibit complete loss of the tritium label.
Experimental

Proton NMR spectra were obtained using a Jeol FX-90Q spectrophotometer at 90 MHz. Carbon NMR ($^{13}$C-NMR) spectra were taken on an IBM AF 300 at 75.45 MHz and a Jeol FX-90Q at 22.5 MHz with dioxane or methanol as an internal standard. Chemical shift values are given in parts per million downfield from tetramethylsilane (TMS) in organic solvents or sodium 2,2,3,3,-d₄ 3-trimethylsilylpropionate (TSP) in D₂O. Mass spectra were run on Finnigan 3300 CEC 111021-110B mass spectrometer. Infrared spectra were recorded on a Perkin Elmer 1320 Infrared Spectrophotometer or 4230 spectrophotometer. All melting points were taken on a Fisher-Johns Melting Point Apparatus and are uncorrected.

Preparative thin layer chromatography (TLC) was accomplished using 0.75 mm layers of Merck silica gel, type PF-254. Analytical TLC was run on pre-coated Merck silica gel type 60, F-254, glass plates of 0.25 thickness. Flash column chromatography was carried out using silica gel, type 60A, 230-400 mesh as the stationary phase. Visualization was by means of short wavelength UV light and spraying with 5% phosphomolybdic acid in ethanol followed by heat treatment. Analytical cellulose chromatography was done on pre-coated Merck cellulose F-254 glass plates of 0.1 mm thickness. Visualization was accomplished by spraying the plates with 2% ninhydrin in ethanol followed by heating. Cellulose column chromatography was done on Merck Microcrystalline cellulose. Paper chromatography was performed on Whatman 3MM paper with visualization by short wavelength ultraviolet light. High performance liquid chromatography (HPLC) was conducted using either an Altex 110A pump or a Spectra Physics SP 8700
Solvent Delivery System. Monitoring was accomplished using either a Hitachi Model 100-40 Variable Wavelength Spectrophotometer equipped with a Varian Model 9176 Recorder, or an ISCO Model UA-5 Absorbance Monitor, set at 254 nm, using HPLC cells with 5mm pathlength. The liquid chromatography column, 4.6 mm X 150 mm, was packed with Altex Ultrasphere Octadecyl (C18) reverse phase packing and was equipped with a microguard ODS-5S precolumn. The column was eluted at a rate of 1.5 mL/min. with a gradient of 0.05N dihydrogen ammonium phosphate and methanol. Medium pressure liquid chromatography (MPLC) was carried out on an 11 mm X 480 mm column containing Baker Reverse Phase Octadecyl (C18) silane packing. The solvent was delivered by a Rainin B-100S Eldex Pump. The column was eluted at the rate of 1.5 mL per minute. Product elution was monitored using a ISCO Model UA-5 Absorbance Monitor set at 254 nm. Fractions were collected in an ISCO Cygnet Fraction Collector or an ISCO Model 273 Fraction Collector. Centrifugation was carried out using a Dupont Sorvall RC-5B Refrigerated Superspeed Centrifuge. A Corning Model 12 Research pH Meter was used to measure pH.

Radiolabelled compounds for feeding experiments and chemical synthesis were purchased from New England Nuclear Corporation or Amersham/Searle Corporation. Samples for liquid scintillation counting were weighed on a Perkin-Elmer Autobalanced AD-2 and counted on either a Beckman Model LS 301 or Beckman Model LS100C8 Liquid Scintillation Counter in Aquasol 2 or toluene scintillation fluids, purchased from New England Nuclear. Radiochemical purity was checked using a Berthold LB 22832 Automatic TLC Linear Analyzer, interfaced with an Apple IIE computer. All stable isotope labelled compounds were
purchased from either Cambridge Isotopes or Merck Isotopes.

The sterile environment necessary for microbial strain improvement was provided by a BBL Biohazard laminar flow cabinet. Incubation of Petri dishes were done statically in a Beckman incubator at 30°C. Centrifugation was done in a Beckman GFC centrifuge in sterile 50 mL tubes.

Preparation of Inoculum Medium

In 100 mL tap water, dextrose (1 g), grit extract (1 g), edible molasses (0.5 g), dextrin (3 g), and Amber BYF 300 (0.5 g, Amber Laboratories, Juneau, Wisconsin) were added. The pH of the cloudy dark brown solution was adjusted to 6.5 with 1N NaOH. A two mL aliquot of this solution was placed in a 150 mm x 20 mm screw top culture tube, autoclaved for 20 minutes and cooled. Using sterile technique, a lyophile was added to the medium and allowed to grow for 48 hours statically in a GCA/Precision Scientific Shaker Bath at 30°. Growth was visible by the increase of insoluble material, cloudiness of the broth and a ring of growth formed on top of the liquid.

Preparation of Grit Extract

In a 2000 mL Erlenmeyer flask, deionized water (400 mL) was added to Nutrisoy grits (70 g, soybean meal, Archer Midlands Daniel Company). The suspension was autoclaved at 121°C for 20 minutes. The pale yellow grainy mush was centrifuged at 9,000 rpm for 30 minutes to collect solids. The supernatant was decanted and lyophilised overnight yielding 14 g of solubles.
Preparation of Nadrisol Extracts

A suspension of Nadrisol (70 g, National Distillers Company) in glass distilled water (400 mL) was stirred for 30 minutes at room temperature in a 1 liter Erlenmeyer flask. The solids were collected by centrifugation at 9,000 rpm for 30 minutes. The supernatant was decanted and lyophilised overnight yielding 14.5 g of a dark brown solid.

Preparation of Slants

In a 1L Erlenmeyer flask, Nadrisol (1 g, National Distillers Company), edible blackstrap molasses (1 g), Nutrasoy flour (7.5 g, Archer Daniels Midland Company), calcium carbonate (0.5 g), and Dextrin 700 (5 g, A. E. Staley) were added to deionized water (500 mL). The pH of the pale brown suspension was adjusted to 6.5 by the addition of 1N NaOH. After the addition of Difco agar (12.5 g), the suspension was stirred and heated to boiling. Complete dissolution did not occur but the insolubles were well suspended. Eight mL aliquots of this solution were pipetted into 150 mm x 20 mm culture tubes which were then closed with the caps loosened one half turn. The culture tubes were autoclaved for 20 minutes. All insolubles settled at the bottom of each tube after autoclaving but they were evenly distributed, before solidification by vortexing. Slants were allowed to cool at an angle. The tilt angle was chosen so that the agar remained 5 cm below the mouth of the tube.

The agar slants were inoculated with two drops of the inoculum medium described previously. Inoculated slants were manually rotated so that the inoculum completely coated the agar slant surface. They were then statically incubated at 30°C in a GCA/Precision Scientific
Shaker Bath. White growth was clearly visible after 72 hours. The slants sporulated after 5-6 days.

Method of Random Strain Improvement

Preparation of Spore Suspension

To each well sporulated slant, 9 mL of sterile 0.1% Tween in water was added. The spores were suspended with a loop and the slant vortexed. The spore suspension was filtered through cotton wool (Figure VII) and centrifuged at 3000 rpm for 7.5 mins. The water/Tween was decanted as soon as the centrifuge stopped and the black pelleted spores were resuspended in sterile water, vortexed and centrifuged as mentioned above. The spores were vortexed in the one drop water left in the centrifuge tube after decantation. One mL of sterile 20% glycerol/water was added to the spore suspension and 0.1 mL of this solution was then diluted one hundred fold ($10^{-2}$). This first dilution was diluted successively by factors of ten to a final dilution of $10^{-7}$.

Agar (approximately 23 mL) prepared as described before was poured into sterile Petri dishes and allowed to cool. Once cooled, the dishes were opened and the water condensate allowed to evaporate in a laminar flow cabinet. Each dilution (100 μL) was spread on a Petri dish with a sterile glass rod bent to the shape of a hockey stick until all the liquid was absorbed into the agar layer. The Petri dishes were bound together by masking tape and placed upside down in the incubator.

Colonies of bacterial growth were visible after 48 hours. The Petri dishes inoculated with the most dilute spore suspension ($10^{-7}, 10^{-6}$) had almost no growth. Single colonies were visible on the others. To
Figure VII
each Petri dish, containing 50 μg, 100 μg, 200 μg, 300 μg, 400 μg and 500 μg per mL concentration of sinefungin dissolved in agar prepared as aforementioned, 100 μL of the spore suspension (10^-5 dilution) was added and absorbed. They were then placed in the incubator and allowed to grow. However, growth was virtually identical on all the Petri dishes. Using a microbiological loop, twenty individual colonies, which were chosen at random, were scraped from the Petri dishes, and each colony was allowed to grow in 2 mL of the inoculum medium previously described in a 20 mm X 150 mm sterile test tube equipped with a fitted plastic cap. The inoculated test tubes were placed in a New Brunswick G-25 or G-25R incubator where they were shaken at a slant in a test tube rack at 250 rpm for 48 hours at 30°C. Two drops of each inoculum were used to inoculate two slants from each colony. One of the two slants produced from each colony was used to inoculate twenty small fermentations (100 mL broth), each in a 500 mL wide mouth Erlenmeyer flask. The content of each flask was sampled and analyzed by HPLC on the fourth and the eighth day after inoculation to assess antibiotic production. The colonies which demonstrated the ability to produce larger quantities of sinefungin were preserved as lyophiles or on soil as described in the following section.

Preservation of the Microorganism, *Streptomyces griseolus*

Preparation of Lyophiles

*Streptomyces griseolus* was preserved as lyophiles, and the broth for lyophilization was prepared in the following manner: glucose (10 g)
and dehydrated nutrient broth (Difco, 0.43 g) were dissolved in deionized water (33 mL). To desiccated horse serum (Difco), water (10 mL) was added. The horse serum and the nutrient broth mixture (3.3 mL) were sterilized by filtration through a sterile Millipore filter (0.2 µm pore size) into a sterile 50 mL Erlenmeyer flask to make the mist desicans solution.

To each well sporulated slant (approximately 15 days old), 2.0 mL aliquot of mist desicans was added. The spores were scraped from the agar surface with a microbiological loop. The spore suspension (0.3 mL) was added to six sterile ampoules (2.0 mL capacity). These were attached to the lyophilizer via a sterile adapter. The ampoules were frozen and lyophilized for 24 hours. They were then sealed under vacuum with an oxygen-natural gas torch. The lyophiles were stored at 4°C in the refrigerator.

**Soil Preservation**

Sieved garden potting soil (1 kg) was spread in a 9 inch X 13 inch pyrex baking dish which was covered with aluminium foil then autoclaved for one hour. The pyrex dish containing the soil was placed in an oven at 37°C and allowed to stand overnight. The dish was returned to the autoclave and once again sterilized for 60 minutes. In each of one dozen screw capped, 13 mm X 100 mm culture tubes was placed the sterile soil (1 g) which was then autoclaved for one hour. Brain heart infusion (1 mL) (Difco, 37 g in 1 L of H₂O) was placed in two of the twelve tubes to test the sterility of the soil. The tubes containing the brain heart infusion broth were heated in the incubator at 30°C for 48
hours. If the broth became cloudy the soil was unsterile.

To each well sporulated slant, YM broth (3 mL) (Difco, 21 g in 1000 mL of water) was added. The spores were scraped from the slant with a loop. The spore suspension was transferred to a sterile 150 mm X 20 mm culture tube which was then shaken at 250 rpm in an incubator for 48 hours at 30° C. The suspension (0.2 mL) was added to each of the remaining sterile soil tubes (10) which were then closed with the caps loosened one half turn. The inoculated tubes were allowed to dry at room temperature for one week. The tubes were then stored at 4°C in the refrigerator.

Preparation of Fermentation Medium

To 2000 mL of glass distilled water was added Proflo Oil (80 g, crude cottonseed oil, Procter and Gamble), Nutrisoy grits (10 g, 20-80, Archer Daniels Midland Company), tyrosine (10.8 g), calcium carbonate (4 g), dibasic sodium phosphate (Na$_2$HPO$_4$, 0.4 g), and cobalt chloride (0.02 g). Using 1N NaOH, the pH of the suspension was adjusted to 7.2. Ten 1000 mL Erlenmeyer flasks each containing 200 mL of the broth were stoppered with foam plugs and autoclaved for 20 minutes.

Inoculation of the Fermentation Medium

To each of two well sporulated slants (8 days old), sterile glass distilled water (5 mL) was added. The spores of the slants were suspended using a loop. The spores, which are resistant to wetting, were evenly distributed among the ten flasks prepared as described above.
Sterile technique was maintained throughout the inoculation procedure. The flasks were placed in a New Brunswick G-25 or G-25R incubator where they were shaken at 250 rpm for 8 days at 30°C.

Monitoring Antibiotic Production

Each day, starting 48 hours after inoculation, 1 mL of the fermentation broth was taken from each flask. The samples were combined and filtered to remove all the insolubles. Ten µL of the filtered broth was injected onto a 4.6 mm X 150 mm HPLC ODS reverse phase column. The gradient used to separate the compounds was composed of solvent A (0.05M ammonium hydrogen phosphate [(NH₄)₂HPO₄] buffer adjusted to pH 6) and solvent B (MeOH). The following elution program was used: 100% of solvent A for 5 minutes, 100% - to 80% of solvent A and 0% to 20% of solvent B over 10 minutes, 80% of solvent A with 20% of solvent B for 5 minutes, 80% to 100% of solvent A and 20% to 0% of solvent B over 10 minutes, then 100% of solvent A for 5 minutes. Under these conditions, Sinfungin had a retention time of 4.2 minutes. Sinfungin production was detectable by HPLC 48 hours after inoculation. The concentration of the antibiotic steadily increased until the production of sinfungin peaked on the eighth day after inoculation. Degradation of the antibiotic began on the ninth day.
Isolation of Sinfungin

The isolation of sinfungin was done by a modification of the procedure provided by The Eli Lilly Company.

Eight days after inoculation the cloudy, dark brown fermentation was worked up. The contents of all ten flasks were combined in polyethylene bottles fitted with sealed caps and centrifuged using a Sorval-Dupont GS-3 rotor at 9,000 rpm for 40 minutes. The supernatant was decanted and filtered through a piece of glass wool placed in a large glass funnel. The clear, brown filtrate (1400 mL) was placed in a glass column containing 550 mL ion exchange resin (Dowex 50 X 2, 100-200 mesh, NH$_4^+$ form). The column was washed with one liter of water or until the eluant was clear. Then the sinfungin was eluted with 0.05N NH$_4$OH. The collection of eluant started after the eluant had turned basic and a total of 1.5 liters were collected. After the removal of ammonia on a rotary evaporator, the 1.5 liters were frozen in round bottom flasks and lyophilized overnight.

The crude sinfungin, a dark brown resin, was further purified by chromatography on a 165 mm X 15 mm cellulose column which was first washed with one bed volume water to remove most of the coloured impurities. The sinfungin was then eluted with butanol: water: glacial acetic acid (BAW)$_3$ (25: 4: 10). Any slight impurity left was then removed by loading the sinfungin on a column containing Dowex 50W X 2, 100-200 mesh, resin (NH$_4^+$, 4 mL). The sinfungin was eluted with 0.05N NH$_4$OH. Because sinfungin was difficult to crystallize, two derivatives of sinfungin were prepared: 2',3', - O - isopropylidenedesinefungin, and 2',3', -O- isopropylidenedesinefungin lactam.
These derivatives were employed to purity the sinefungin obtained from all the feedings of $^{14}$C labelled precursors.

**Typical Administration of Precursors**

Before the precursors were administered to the fermentation medium, the fermentation broth was first monitored for the production of the antibiotic, sinefungin, by HPLC as described earlier. Monitoring by HPLC was carried out 72 hours and 96 hours after an inoculation. The labelled precursors was administered only if the antibiotic production was satisfactory.

The labelled precursors were dissolved in 20 mL distilled water. The feedings were pulsed: half (10 mL) of the precursor solution was administered 72 hours after inoculation and the remaining half after 96 hours. Each time, 10 mL of the solution was taken up into a syringe and the contents sterilized by passing through a sterile Millipore filter (0.45 pore size) and the solution distributed equally among the fermentation flasks. After the addition, the syringe and filter were flushed with distilled water (4 mL) which was put into the last flask.

**Synthesis of 5' (RS)-[5'-$^3$H]-Adenosine**

*Synthesis of 2', 3'-O-Isopropylidene adenosine-5'-carboxylic Acid (51)*

This synthesis was a modification of procedure Schmidt et al.5
In a large Erlenmeyer flask (5 L), 2',3'-O-isopropylidene-adenosine (25 g, 81.4 mmol) was dissolved in boiling water (4.5 L). The mixture was stirred and allowed to cool overnight. Potassium permanganate (52 g, 329.0 mmol) was added slowly over two hours with vigorous stirring. The reaction was then stirred for five days. After this time, the reaction was filtered through Celite, and the filtrate concentrated to less than one liter volume on a cyclic evaporator. The pH of the concentrate was acidified to 4.5 with concentrated hydrochloric acid. The resultant precipitate was collected by suction filtration, and then dried in vacuo over phosphorus pentoxide affording a white solid which weighed 15.7 g (51.1 mmol, 63%).

Synthesis of (5'SR)-[5'-3H]-2',3'-O-isopropylidene-adenosine (52)\textsuperscript{56}

To the cooled (0°C), stirred solution of 2',3'-O-isopropylidene-adenosine carboxylic acid (100 mg, 0.309 mmol) and triethylamine (0.15 mL, 1.07 mmol) in acetone (7.5 mL) was added ethyl chloroformate (0.075 mL, 0.78 mmol) and the suspension stirred for 3 hours at 0°C. Sodium azide (50 mg, 0.77 mmol), dissolved in 2 drops of water was then added to the suspension. The reaction was stirred at 0° for an additional 2 hours. Volatiles were removed on a rotary evaporator with the water bath heated and kept below 20°C. Anhydrous ether (5 mL) was added and removed in vacuo. The resultant yellow precipitate was triturated with anhydrous ether (3 X 5 mL) then filtered. The solid was suspended in water/ethanol (3:2). Potassium borotritide (100 mCi, 0.80 mg) was added and the reaction was stirred for one hour. Unlabelled sodium borohydride (40 mg, 1.06 mmol) was then added over one hour. The reaction was stirred overnight at room temperature. Volatiles were
removed under reduced pressure. The residue was extracted with hot chloroform (3 X 5 mL). The extracts were combined, washed with brine, dried over magnesium sulfate and evaporated to dryness under reduced pressure. The slightly yellow solid was triturated with ether (3 X 5 mL), filtered, recrystallized from hot water, then dried in vacuo over phosphorous pentoxide yielding 28.5 mg (12.7 mCi).

**Synthesis of 5' (RS)- [5'-^3H]- Adenosine (53)**

In a 5 mL round bottom flask, trifluoroacetic acid (90%, 1.28 mL), water (0.14 mL) and 5'- (RS) [5'-^3H]- 2',3'-0-isopropylidene- adenosine (28.45 mg, 0.093 mmol) were added and allowed to stir at room temperature for forty minutes. The acids were removed under reduced pressure and the residue neutralize with methanolic ammonia (3 mL). The methanol was removed and the residue, which was dissolved in water, loaded onto column of Dowex 50 W X 8, 20-50 mesh resin. The column was first washed with water and the adenosine was eluted with 1N NH₄OH. After the addition of cold adenosine (30 mgs) to the hot adenosine produced in this reaction, the adenosine was recrystallized from hot water affording 30.15 mg (5.68 mCi, 6% radiochemical yield).

**Synthesis of [1'-^14C]- Adenosine**

**Synthesis of 1,2,3,5 O- Tetraacetyl -D-ribofuranose (56)**

This synthesis is a modification of a procedure by Brown et al. A vigorously refluxing solution of anhydrous sodium acetate (40
mgs, 0.50 mmol) and acetic anhydride (0.40 mL, 4.2 mmol) was added to [1-\textsuperscript{14}C]-D-ribose (250 \mu Ci, 0.66 mg) with unlabelled ribose (10 mg) which were lyophilized overnight in a 3 mL reaction vial. In three portions, ribose (90 mg, 0.67 mmol) was added to refluxing mixture, and the reaction was allowed to reflux for one minute after each addition. The reaction was refluxed for an additional two minutes after the last addition of ribose. The hot mixture was poured into ice where the product oiled out of solution and then crystallized upon stirring. The crystals were dissolved in methanol, the solution was decolorized with charcoal, and then filtered through Celite. The filtrate was concentrated to a small volume, seeded with an authentic ribose tetraacetate crystal (Sigma) and allowed to crystallize for several hours in the freezer. The crystals were collected and dried over \textsubscript{5}P_{2}O_{5} in vacuo affording 86.4 mgs (0.27 mmol, 40%). The crystals were a mixture of the ribofuranose and the ribopyranose tetraacetate. However the majority of the crystals were ribofuranose tetraacetate because during the crystallization the solution was seeded.

**Synthesis of 2',3',5'-O-Triacetyl [1-\textsuperscript{14}C]- adenosine (58)**

This procedure was based on a synthesis published by Saneyoshi et al. 59

In a dry 25 mL round bottom flask, 1, 2, 3, 5 -O-tetraacetyl-[1-\textsuperscript{14}C]-D-ribofuranose (86.4 mg, 0.27 mmol) and adenine (38 mg, 0.28 mmol) were suspended in dry acetonitrile (12 mL). A very dry, fixed needle gas tight syringe, was used to add tinctetrachloride (63 \mu L, 0.054 mmol) to the stirring suspension. All solids disappeared after 30
minutes. The reaction was allowed to stir overnight at room temperature. The mixture was concentrated to a volume of 0.5 mL, then a solution of sodium bicarbonate (175 mgs) in one mL of water was added. After the vigorous evolution of carbon dioxide was completed, the solvent is removed at reduced pressure. The residue was extracted with hot chloroform (3 X 20 mL). The combined extracts were filtered, dried over sodium sulfate and evaporated to dryness. The residue was loaded on a silica gel column which was first washed with CHCl₃/EtOAc (9:1, v/v), and the product mixture was eluted with CHCl₃/MeOH (9:1, v/v) affording a white foam which weighed 95 mg (0.24 mmol, 89 %).

Synthesis of [1'-¹⁴C]-Adenosine

In a screw capped Cole Palmer reaction tube, 2', 3', 5'-[1'-¹⁴C] adenosine triacetate (95 mg, 0.24 mmol) was dissolved in a stirred, cooled (0°C) solution of ammonia saturated methanol (5 mL). The reaction was heated at 50°C for 10 hours. The volatiles were removed at reduced pressure. The solid residue was purified by dissolving in water (2 mL) and loading on a reverse phase MPLC column. The adenosine was eluted with a solution of 8% methanol in water then recrystallized from hot water to afford 12 mg (0.05 mmol, 20%, 2.5% radiochemical yield)

Synthesis of (5-¹³C)-L-Ornithine

Synthesis of N-Tosyl-L-Methionine
A solution of p-toluenesulfonyl chloride (12 g, 62.9 mmol) in ether (60 mL) was added to a solution of L-methionine (9 g, 60.3 mmol) dissolved in 1N NaOH (120 mL). The reaction was stirred for 5 hours during which time a two phase system developed. The ether layer was separated and discarded. The aqueous phase was washed with ether (2 x 100 mL) then acidified with concentrated hydrochloric acid to pH 1. The product separated out of solution as an oil. The oil was dissolved in ethyl acetate, washed with brine and dried over sodium sulfate. The solvent was removed at reduced pressure yielding a viscous liquid which weighed 14.1 g (46.5 mmol, 78%).

**Synthesis of N-Tosyl-L-α-aminobutyrolactone (29)**

To a stirred solution of N-tosyl-L-methionine (9 g, 29.7 mmol), acetic acid (9 mL) and formic acid (80%, 18 mL) in a 100 mL round bottom flask, iodomethane (4.5 mL, 72.3 mmol) was added. The resultant mixture was stirred overnight, in complete darkness. After concentration at reduced pressure, the residue, a viscous reddish-brown liquid, was heated with 1N NaOH (30 mL) at 90° for 3.5 hours. During the course of the reaction, the pH of the heated solution was maintained between 6-7 by the addition of 2N NaOH. After cooling, the lactone crystals which had formed, were separated by suction filtration. The collected crystals were dissolved in EtOAc, washed with brine and dried over anhydrous sodium sulfate. The solvent was evaporated to dryness, and the lactone was crystallized from EtOAc/petroleum ether affording 4.5 g (17.7 mmol, 60%).
NMR (90 MHz) in d$_6$-acetone: δ 1.7-1.8 (2H, m, J=3.1 Hz, CH$_2$), 2.0 (3H, s, PhCH$_3$), 2.4 (1H, t, J=3.14 Hz, NCH), 4.2 (2H, t, J=4.5 Hz, CH$_2$O), 7.2-7.8 (4H, m, J=13.5 Hz, C$_6$H$_4$)

**Synthesis of L-α-Amino- γ-bromobutyric Acid Hydrobromide (30)**

In a pressure bottle, N-tosyl L- γ-aminobutyrolactone (4.8 g, 18.8 mmol), phenol (1.58 mL, 18 mmol), and hydrobromic acid /acetic acid (30-32%, Baker, 45 mL) were heated at 75° for 2.5 hours. After cooling, the reddish-brown precipitate formed was washed from the bottle with glacial acetic acid (40 mL). The resultant suspension was evaporated to dryness at reduced pressure. The residue was triturated repeatedly with ether (4 X 15 mL) to remove the brown impurities leaving a white powder which weighed 3.4 g (13.3 mmol, 69%).

NMR (90 MHz) in D$_2$O: δ 2.65 (2H, m, J=3.1 Hz, CH$_2$), 3.8 (2H, t, J=3.14 Hz, CH$_2$Br), 4.6 (1H, t, J=3.85 Hz, NCH)

**Synthesis of Methyl L-α-Amino-γ-bromobutyrate Hydrochloride (31)**

A stream of dry HCl gas was bubbled through a solution of L-α-amino- γ- bromobutyric acid hydrobromide (3.5 g, 13.3 mmol) in methanol (55 mL) at such a rate as to maintain the reaction temperature between 35°-40°C without cooling, for 1.5 hours. Removal of the
solvent at reduced pressure gave a yellow solid. The residue was then triturated with ether (3 x 20 mL) affording a white powder which weighed 3.0 g (12.9 mmol, 97%).

NMR (90 MHz in D₂O): δ 2.65 (2H, m, J=3.1 Hz, CH₂), 3.8 (2H, t, J=3.14 Hz, CH₂Br), 4.1(3H, s, OCH₃), 4.58 (1H, t, J=3.85, NCH)

Synthesis of Methyl benzylxycarbonyl-L-α-amino-γ-bromobutyrate (32)

To a stirred, cooled (0° C) solution of sodium bicarbonate (2.37 g, 28.2 mmol), methyl L-α-amino-γ-bromobutyrate hydrochloride (3.0 g, 12.9 mmol) and H₂O (14.5 mL) was added benzyl chloroformate (2.2 mL, 15.4 mmol), dropwise over 10 minutes. The reaction was stirred for 20 minutes at room temperature. During this time, a two layer system developed. After the addition of ether (40 mL), the organic was separated then washed successively with dilute sodium bicarbonate, 3N hydrochloric acid, water, brine and then dried over magnesium sulfate. Removal of the solvent at reduced pressure afforded a semi-solid crude product which was purified by column chromatography (silica gel, 2% ethanol/benzene). The product was crystallized from ethyl acetate/petroleum ether gave a white solid which weighed 3.2 g (9.7 mmol, 75%).

NMR (90MHz in CDCl₃): δ 2.3 (2H, m, J=3.85 Hz, CH₂), 3.3 (2H, t,
J=3.49 Hz, CH₂Br), 3.7 (3H, s, OCH₃), 4.5 (1H, t, J=3.5 Hz, NCH), 5.06 (2H, s, Ph-CH₂O), 5.45 (1H, b, NH), 7.3 (5H, s, C₆H₅)

**Synthesis of Methyl-L-α-benzylloxycarbonylamino-(4⁻¹³C)-cyanobutyrate (34)**

A suspension of methyl-α-benzylloxycarbonyl-L-α-amino-γ-bromobutyrate (1 g, 3.0 mmol) and K₁³CN (99.4 atom % ¹³C, 0.218 g, 3.3 mmol) were stirred in dimethylsulfoxide (4.5 mL) for 72 hours at room temperature. The reaction was monitored by TLC (2% ethanol/benzene) and was essentially complete after 72 hours. The reaction was quenched with water (5 mL). The product was extracted with ether (4 X 15 mL). The ether extracts were combined, washed with brine, dried over sodium sulfate and the solvent removed at reduced pressure. The residue was recrystallized from ethyl acetate/petroleum ether to afford 703 mg (2.6 mmol, 87%).

NMR (90MHz in CDCl₃): δ 2.4 (4H, m, J=9.64 Hz, C₂H₄CN), 3.8 (3H, s, OMe), 4.5 (1H, t, J=3.1 Hz, NCH), 5.2 (2H, s, O-CH₂), 5.4 (1H, b, NH), 7.4 (5H, s, C₆H₅)

¹³CMR (300 MHz): 53, 56, 67, 119 (enriched), 128, 171

**Synthesis of (5⁻¹³C)-L-Ornithine monohydrochloride (34)**

In a Parr apparatus bottle, (5⁻¹³C)-5-cyanobutyrate (0.80 g, 2.9
mmol) dissolved in glacial acetic acid (5 mL) was hydrogenated with Adam's catalyst (PtO₂, 267.5 mg) for 24 hours at 20 psi of hydrogen. The catalyst was filtered off and the filtrate acidified with concentrated hydrochloric acid (10 mL). The resultant mixture was heated to reflux for 3.5 hours under nitrogen. After concentration in vacuo, the crude ornithine was loaded on a column of Dowex 50 X 8, 20-50 mesh, resin (80 mL volume). The column was first washed with water and free ornithine eluted with 2N NH₄OH. The fractions containing ornithine were reduced in vacuo. The free ornithine was converted to the monohydrochloride salt by adjusting the pH of the ornithine solution to 3. Ornithine monohydrochloride was crystallized from water/ethanol. The yield was 300 mgs [1.8 mmol, 62%]. The ¹³C-NMR corresponded to the published spectra for ornithine monohydrochloride salt.

Synthesis of (5-¹³C, 5-¹⁵N)-L-Ornithine

The synthesis is based on a procedure published by Havranek et. al⁶⁴

Synthesis of Methyl-L-α-benzylxocarbonylamino-γ-(4-¹³C, 5-¹⁵N)-cyanobutrate (36)

A suspension of methyl-L-α-benzylxocarbonylamino-γ-bromobutyrate (1.5 g, 4.5 mmol) and Na¹³C¹⁵N (99 atom% ¹³C, 99 atom% ¹⁵N, 0.327 g, 4.95 mmol) in dimethylsulfoxide (6 mL) was stirred in a 10 mL round bottom flask for 72 hours at room temperature. During this time, the reaction was monitored by TLC (2% ethanol/benzene) and it was essentially complete after 72 hours. The reaction was quenched with
water (5 mL). The nitrile was extracted with ether (4 X 15 mL). The extracts were combined, dried over magnesium sulfate, and evaporated to dryness. The product was recrystallized from ethyl acetate/petroleum ether to give 1.1 g (3.9 mmol, 88%).

$^{13}$CMR (300 MHz): 53, 56, 67, 119 (d, $^1J_{CN} = 4.9$ Hz), 128, 171

**Synthesis of (S-$^{13}$C, S$^{15}$N)-L-Ornithine monohydrochloride (37)**

In a Parr apparatus bottle, ($^{13}$C$^{15}$N)-cyano butyrate (35) (1.1 g, 3.9 mmol) dissolved in glacial acetic acid (7.5 mL) was hydrogenated over Adam's catalyst (PtO$_2$, 401 mg) for 24 hours at 20 psi. The catalyst was removed by suction filtration, the filtrate was acidified with concentrated hydrochloric acid (15 mL), and then the mixture was refluxed for 3.5 hours under nitrogen. After concentration to a small volume, the concentrate was loaded onto a column of Dowex 50 X 8, 20-50 mesh, resin (120 mL). The column was first washed with water, and the free ornithine was then eluted with 2N NH$_4$OH. The fractions containing ornithine were concentrated *in vacuo*. Ornithine monohydrochloride was prepared by adjusting the pH of the free ornithine solution to 3. The monohydrochloride salt was crystallized from EtOH/H$_2$O to give 400 mg (2.4 mmol, 62%).

$^{13}$CNMR (300 MHz): $\delta$ 24.5, 29.2, 41.1 (d, $J=4.9$ Hz), 56.6, 179.4
Synthesis of 5(RS)-[5-3H]-L- Ornithine

*Synthesis of 5(RS)-[5-3H]- Methylbenzyloxy carbonyl-L-α, γ- dianinopentanoate hydrochloride* (43)

To a solution of unlabelled cyano butyrate (42) (150 mg, 0.54 mmol) and cobaltous chloride (259 mg, 1.08 mmol)\(^6\) in ethanol (4.8 mL) was added sodium borotritide (25 mCi, 2.64 mg), and the reaction mixture was allowed to stir for 10 minutes. Unlabelled sodium borohydrate (215 mg, 5.4 mmol) was added in portions and the mixture stirred for 20 minutes. The evolution of hydrogen gas was observed and a black precipitate appeared during the addition of sodium borohydrate. Six mL of 3N hydrochloric acid was added and the reaction stirred until the black precipitate had dissolved (45 minutes - 1 hour). After the removal of the solvent at reduced pressure, the hydrochloride salt was extracted with ether (3 X 15 mL). The combined ether extracts were washed with brine, dried over sodium sulfate and evaporated to dryness yielding a white solid which weighed 110 mg (0.35 mmol, 65%).

NMR (90 MHz in CDCl\(_3\)): 1.8 (6H, m, C\(_3\)H\(_6\)CN), 3.7 (3H, s, OCH\(_3\))

4.0 (1H, t, NCH), 5.1 (2H, s, PhCH\(_2\)O), 7.4 (5H, s, C\(_6\)H\(_5\))

*Synthesis of 5 (RS)-[5-3H]- Ornithine monohydrochloride*\(^6\)

In a Parr apparatus bottle the 5-(RS)-[5-3H]- methylbenzyloxy carbonyl - L - α, γ-diaminopentanoate hydrochloride (110 mg, 0.35 mmol) in glacial acetic acid (1.5 mL) was hydrogenated on
Adam's catalyst (PtO₂, 36 mg) at 45 psi of hydrogen for one hour. The catalyst was removed by suction filtration and the filtrate was acidified with concentrated hydrochloric acid (3 mL). The reaction mixture was gently refluxed for 3.5 hours under nitrogen. After concentration to a small volume, the concentrate was loaded on a column of Dowex 50 X 8, 20-50 mesh, resin, and free ornithine was eluted with 2N NH₄OH. The free ornithine was transformed into the hydrochloride salt by adjusting the pH of the dissolved ornithine solution to 3. The product was crystallized from ethanol/water. The hot ornithine was diluted with 10 mgs of cold ornithine to give 26 mg (15 μCi/mg, 2% radiochemical yield).

Alternate Attempted Synthesis of 2(RS), 5(RS) - [5-³H] - Ornithine

The synthesis is a modification of a procedure by Craig Townsend⁶⁶.

Synthesis of γ,γ'-Dicarboxy-γ- phthalimidobutyraldehyde (38) ⁶⁷

To a cooled solution (0° C) of sodium (50mg, 1 mmol) in ethanol (60 mL) was added diethyl phthalimidomalonate (6.1 g, 20 mmol) after the complete dissolution of the sodium. Using a dry syringe, a solution of freshly distilled acrolein (1.5 ml, 22 mmol) in ethanol (5 mL) was introduced dropwise. When most of the phthalimidomalonate had been consumed, the reaction mixture was allowed to warm to room temperature, and the reaction was then stirred for one hour. The reaction mixture was neutralized with glacial acetic acid (3 drops).
After the removal of the solvent *in vacuo* the product was purified by column chromatography (silica gel, ethyl acetate/hexane, 6:4) to give 2.5 g (6.9 mmol, 35%).

NMR (90MHz in CDCl$_3$): δ 1.29 (6H, t, J=6.3 Hz, 2CH$_3$), 2.79 (4H, m, J=3.6 Hz C$_2$H$_4$), 4.31(4H, q, J=7.2 Hz 2-OCH$_2$), 7.81 (4H m, J=5.3 Hz ArH), 9.70 (1H, s, CHO)

*Synthesis of Ethyl 2-carbethoxy -2- phthalimido- 5- L-hydroxyvalerate (39)*

To a vigorously stirred solution of γ,γ dicarbethoxy-γ-phthalimidobutyraldehyde (4.55 g, 26 mmol) in a mixture of ether/ water (60:10), sodium borohydride (478.5 mg, 12.6 mmol) was added in portions over two hours. Water (30 mL) was used to destroy the unreacted hydride and the aqueous layer was extracted with ether (5 X 50 mL). The ethereal phases were combined and dried over sodium sulfate. The solvent was removed at reduced pressure to give a clear oil. The residue was purified by column chromatography (ethyl acetate/hexane 6:4) to give a colourless oil which weighed 1.82 g (1.8 mmol, 70%)

NMR (90MHz in CDCl$_3$): δ 1.28 (6H, t, J=6.3 Hz, 2CH$_3$), 1.53 -1.78 (2H, m, J=3.1 Hz, C-4), 2.48 -2.68 (2H, m, J=3.6 Hz C-3), 3.66 (2H, t, J=6.3 Hz, ), 4.31(4H, q, J=7.2 Hz 2-OCH$_2$), 7.79 (4H m, J=5.4 Hz ArH)
Synthesis of Ethyl 2-carbethoxy-2-phthalimido-5-methane sulfonyloxy valerate (40)

To a cooled (0°C) solution of ethyl 2-carbethoxy-2-phthalimido-5-hydroxy valerate (630 mg, 1.74 mmol) and triethylamine (0.31 mL, 2.2 mmol) in methylene chloride (12.2 mL), a solution of methanesulfonyl chloride (0.175 mL, 2.2 mmol) in methylene chloride (1 mL) was added dropwise. The reaction mixture was stirred at room temperature for 30 minutes. After the removal of the volatiles at reduced pressure, the residue was dissolved in ethyl acetate/hexane (6:4). On standing, triethylamine hydrochloride salt precipitated out of the solution and was removed by suction filtration. The filtrate was evaporated to dryness under reduced pressure. Purification of the residue was accomplished by column chromatography (ethyl acetate/hexane, 6:4) to give 588 mg (1.34 mmol, 77%)

NMR (90MHz in CDCl₃): δ 1.28 (6H, t, J=6.3 Hz), 1.7 - 2.2 (2H, m, J=3.1 Hz, C-4), 2.48-2.68 (2H, m, J=3.6 Hz, C-3), 3.2 (3H, s, SCH₃), 4.3 (4H, q, J=7.1 Hz, 2-OCH₂), 7.79 (4H, m, J=5.4 Hz, ArH)

Synthesis of Ethyl 2-carbethoxy-2-phthalimido-5-azidovalerate (41)

Sodium azide (351.4 mg, 5.4 mmol) and the mesylate (479 mg, 1.09 mmol) were suspended in dimethylsulfoxide (5.4 mL) and then heated at 100°C for 18 hours. After cooling, the reaction was quenched with water (2 mL). The crude product was extracted with methylene chloride (3 X 15 mL). The extracts were combined, washed with brine, dried over sodium sulfate and the solvent evaporated to dryness in vacuo. The oily
residue was purified by column chromatography (ethyl acetate/hexane, 6:4) to afford 273 mg (0.70 mmol, 65%)

NMR (90MHz in CDCl3): δ 1.28 (6H, t, J=6.3 Hz, 2CH3), 1.53-1.9 (2H, m, J=3.1 Hz, C-4), 2.36-2.68 (2H, m, J=3.6 Hz, C-3), 3.32 (2H, t, J=3.6 Hz, CH2N3), 4.3(4H, q, J=7.1 Hz, 2-OCH2), 7.79 (4H m, J=5.4 Hz, ArH)

Synthesis of 2(RS), 5(RS)-DL- Ornithine Hydrochloride (42)

To a solution of ethyl 2-carbethoxy-2-phthalimido-5-azidovalerate (273 mg, 0.70 mmol) dissolved in glacial acetic acid (2 mL) was added concentrated hydrochloric acid (2 mL). The mixture was heated to vigorous reflux under nitrogen for 8 hours. The mixture was concentrated at reduced pressure and the concentrate loaded on a column of Dowex 50 X 8, 20 - 50 mesh, resin (36 mL). The column was washed with water until the eluant was colourless. Then the column was washed with 0.5N NH4OH (200 mL), and 1N NH4OH (50 mL). The ornithine was eluted with 2N NH4OH to give 48 mg (0.30 mmol, 43%). Although, the product mixture which was monitored by analytical cellulose TLC (BAW, 25: 4: 10) indicated the ornithine had been formed, we were unsuccessful in our attempts to isolate pure ornithine. Ornithine monohydrochloride was made by the method described earlier; however, the crystalline hydrochloride salt was never obtained. For the purposes of synthesizing radioactive ornithine with the tritium label, this synthetic scheme would be useless because of the inability to obtain pure ornithine.
Synthesis of Derivatives of Sinefungin

Synthesis of 2',3'-O-Isopropylidenesinefungin (25)

This synthesis is a modification of a procedure by Hampton.68

In a tightly stoppered 5 mL round bottom flask, a solution of sinefungin (20 mg, 0.052 mmol), bis(4-nitrophenyl)phosphate (57.1 mg, 0.17 mmol) and 2,2-dimethoxypropane (0.2 mL, 1.6 mmol) in acetone (2 mL) was stirred at room temperature for 48 hours. The reaction was quenched with tributylamine (100μL) and volatiles removed in vacuo. The yellow-orange residue was stirred in benzene (2 mL) for 2 hours. The product was filtered and washed with additional benzene. The solid was dissolved in water and then loaded on a plug (3-4 mL volume) of Dowex 50 X 2, 100-200 mesh, resin (NH₄⁺ form). The column was washed with water and isopropylidenesinefungin eluted with 2N NH₄OH.

After removal of the solvent at reduced pressure, the residue was dissolved in a minimum amount of water then purified by paper chromatography (Whatman 3MM paper, BAW, 25: 4: 10) to afford 14.5 mg (0.034 mmol, 66%), Rf=.6.

$^{13}$CMR (300 MHz in H₂O): δ 152.3 (C-2), 148.0 (C-4), 118.3 (C-5)
154.8 (C-6), 139.7 (C-8), 88.3 (C-1'), 73.4 (C-2', 3'), 80.3(C-4'),
36.7 (C-5'), 48.4 (C-6'), 28.2 (C-7'), 30.1 (C-8'), 55.0 (C-9'),
177.1 (C10'), 25.0 (C-11'), 114.9 (C-12'), 26.6 (C-13')

High Resolution Mass Spectrometry

Expected mass (amu) = 421.20734
Observed mass (amu) = 403.19741
Synthesis of 2',3'-O-Isopropylidenesinefungin Lactam (26)\textsuperscript{5}

A solution of 2',3'-O-isopropylidenesinefungin (14 mg, 0.034 mmol) in dry methanol (5 mL) was heated to reflux for 72 hours. After cooling, the solvent was removed at reduced pressure. The residue was purified by paper chromatography (Whatman 3MM, BAW, 24 : 4 : 10) R\textsubscript{T}=0.5. The band containing the lactam was cut into tiny strips, stirred in water for 30 minutes and then filtered. The filtrate was evaporated to dryness in vacuo affording 10 mg (0.025 mmol, 74%)

\(^{13}\text{CMR}\) (300 MHz in H\textsubscript{2}O):
\(\delta\)
153.3 (C-2), 149.0 (C-4), 119.3 (C-5)
156.1 (C-6), 140.0 (C-8), 88.1 (C-1'), 73.9 (C-2', 3'), 81.1 (C-4'),
40.1 (C-5'), 48.9 (C-6'), 26.3 (C-7'), 25.8 (C-8'), 50.7 (C-9'), 173.8
(C10'), 25.0 (C-11'), 114.9 (C-12'), 26.6 (C-13')

High Resolution Mass Spectrometry

Expected Mass (amu): 403.196755
Observed Mass (amu): 403.195574
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