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Gomez, Mary Elizabeth Eudy, Ph.D.

Rice University, 1988
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INVESTIGATIONS OF THE BIOSYNTHESIS OF SPARSOMYCIN

by

MARY ELIZABETH EUDY GOMEZ

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

DOCTOR OF PHILOSOPHY

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April, 1988
ABSTRACT

INVESTIGATIONS OF THE BIOSYNTHESIS OF SPARSOMYCIN

By Mary Elizabeth Eudy Gomez

Investigations of the biosynthesis of the antitumor antibiotic sparsomycin (1) by Streptomyces sparsogenes have been carried out. Incorporation studies employing (13C)-labeled precursors have shown that the monoxodithioacetal moiety (6) of the antibiotic arises from the step-wise introduction of a thiomethyl group into the S-methyl group of S-methyl-D-cysteine. The methyl group of (6) has its origin in the methyl group of L-methionine, but an experiment utilizing [methyl-3H, 35S]-L-methionine has demonstrated that intact incorporation of the thiomethyl group of this precursor does not occur.

The results of feeding experiments with (13C)- and (2H)-labeled forms of tryptophan have indicated that the uracil moiety (7) of sparsomycin is derived from the indole nucleus of tryptophan via aromatic ring cleavage followed by recyclization. Preliminary evidence for the intermediacy of N-formyl-anthranilic acid in the conversion of tryptophan to sparsomycin has been obtained.
ACKNOWLEDGEMENTS

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My fellow group members also deserve mention, particularly Dorita Arzu' and Volker Bornemann. Their companionship made the lab a more enjoyable place in which to work. I would also like to express my appreciation to Dr. Alan Kook for all the NMR work he carried out for me.

The National Cancer Institute, the Department of Health and Human Services, and the Robert A. Welch Foundation are gratefully acknowledged for their financial support of this research. I also thank the late Dr. Paul Wiley for providing us with an authentic sample of sparsomycin.
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INTRODUCTION

Sparsomycin (1) is a unique antitumor antibiotic which was first isolated from the fermentation broth of Streptomyces sparsogenes var. sparsogenes sp. n. in 1962 by Owen, Dietz, and Camiener at UpJohn.\textsuperscript{1} The compound was subsequently isolated in 1966 by Higashide and coworkers in Japan from Streptomyces cuspidosporus.\textsuperscript{2} It is noteworthy that the antibiotic tubercidin (2) has also been isolated from each of these organisms.\textsuperscript{2,3,4}

The structure of sparsomycin was elucidated by Wiley and MacKellar in 1970,\textsuperscript{5} and its absolute configuration was determined by Ottenheijm and coworkers in 1981.\textsuperscript{6} Three syntheses of sparsomycin have appeared in the literature.\textsuperscript{7,8}

Sparsomycin exhibits a wide range of biological activity in vitro. It is moderately active against a variety of gram-negative and gram-positive bacteria and fungi. It inhibits the growth of several types of tumors and is particularly active against KB human epidermoid carcinoma cells in tissue culture.\textsuperscript{1} Sparsomycin also inhibits the growth of certain viruses, e. g. pseudorabies virus, equine encephalitis virus, Newcastle disease virus, and fowl plague virus.\textsuperscript{9} Sparsomycin has also been reported to increase markedly the lethal effects of ionizing radiation on E. coli when present in high concentration. Low concentrations of sparsomycin were shown to protect E. coli slightly from those same effects.\textsuperscript{10}

Sparsomycin exerts its effects by inhibiting protein biosynthesis, specifically by interfering with the peptide bond-forming step.\textsuperscript{1,7} Studies by Flynn and Ash have suggested that sparsomycin accomplishes
Figure 1
this by occupying the A-site of the 50S ribosomal subunit.\textsuperscript{12} It then undergoes an irreversible Pummerer reaction with a nearby acylated histidine residue, thus terminating the growing polypeptide. In the model of Flynn and Ash, the R configuration at the sulfoxide sulfur of sparsomycin is essential for placement of the oxygen-sulfur bond in the correct position for this Pummerer reaction to occur.

Because sparsomycin proved toxic in clinical tests,\textsuperscript{13} there has been much interest in the preparation and testing of synthetic analogues of this compound.\textsuperscript{12,14,15} Few biologically-active examples have been found. Two analogues of sparsomycin which do inhibit protein biosynthesis are $\textit{g}$\textsuperscript{14} and $\textit{h}$.\textsuperscript{12}

Prior to the investigations described herein and the published results of these studies,\textsuperscript{16} nothing was known about the biosynthetic origins of sparsomycin. This compound is of particular interest because of its unusual structure which includes a highly-modified uracil moiety and an extremely rare monoxodithioacetal group. In fact, the only other known natural product containing this group is $\gamma$-glutamylmarasmin (\textit{g}),\textsuperscript{17} which has been isolated from the mushrooms \textit{Marasmius alliaceus}, \textit{Marasmius scorodonius}, and \textit{Marasmius prasiosmus}. $\gamma$-Glutamylmarasmin is the precursor of certain odorous components of these mushrooms. The monoxodithioacetal functionality has received attention from synthetic chemists because the carbamion of this group can serve as an acyl anion equivalent.\textsuperscript{8,18,19}

For the discussion of its biosynthesis, sparsomycin can be formally divided into two fragments: 1) the amino alcohol moiety (\textit{g}), and 2) the uracil moiety (\textit{z}) as shown in Scheme 1.

Initial speculation\textsuperscript{20} about the biosynthesis of sparsomycin dealt with the possible origins of the amino alcohol moiety (\textit{g}). One
Figure 2
Figure 3
Scheme 1
hypothesis is that this group is derived from L-cysteine. Scheme 2 illustrates possible pathways of conversion of L-cysteine (8) into amino alcohol 6. The thiohemiacetal 10 could be derived from L-cysteine by methylation with S-adenosyl methionine (SAM) to S-methyl-L-cysteine (9) followed by hydroxylation. Thiohemiacetal 10 could also arise from the reaction between L-cysteine and tetrahydrofolate.

SAM (14) and tetrahydrofolate are the two most common sources of one-carbon units in biological systems. Tetrahydrofolate can occur in various forms as shown in Figure 5. The carbon donated by SAM is, of course, the methyl group; this carbon has its origin in the methyl group of methionine. The carbon supplied by tetrahydrofolate is the one-carbon group borne by N-5 or N-10. This carbon atom ultimately derives from formate or C-3 of serine. Other possible sources of this one-carbon unit are C-2 of glycine, CO₂, or C-2 of histidine.

Dithiohemiacetal 12 could arise in one of three ways. The first possible pathway is by reaction of 10 with hydrosulfide anion. A second possibility is by reaction of 10 with cysteine to produce djenkolic acid (11). This amino acid has been isolated from *Pithecolobium labatum* (*Leguminosae*) and *Albizia lophanta* (*Leguminosae*). Djenkolic acid could then be cleaved as the pyridoxal phosphate adduct to produce 12. Pyridoxal phosphate (18) is a coenzyme which can catalyze the elimination of leaving groups from C-3 of amino acids. The structure of pyridoxal phosphate and the cleavage of its adduct with djenkolic acid are shown in Scheme 3. A third, and perhaps the most likely, hypothetical pathway is by the direct insertion of sulfur into the methyl group of S-methyl-L-cysteine (9). This type of insertion is well-documented and occurs by an unknown mechanism. The intermediacy of free radicals has been suggested.
Scheme 2
Figure 4
Figure 5
Scheme 3
Scheme 3 (cont'd)
Intermediate **12** could then be converted to dithioacetal **13** by methylation with SAM. Dithioacetal **13** could also be produced by reaction of **10** with methanethiol. Methanethiol could be produced from methionine.\(^{31,32}\) It could also arise from the hydrolytic degradation of S-methylcysteine (9) by an alkylcysteine lyase.\(^{31,33}\) There is evidence that the methylthio group of methionine has its origin in the methanethiol produced by this cleavage in yeasts and Neurospora.^{34}\)

Three additional transformations are required for the conversion of **13** into **6**: 1) inversion of configuration at C-2' (this step is unnecessary if the scheme begins with D-cysteine, since the configurations of sparsomycin and **6** correspond to that of D-cysteine), 2) reduction of the carboxyl group to an alcohol, and 3) oxidation of one sulfur atom to a sulfoxide. These transformations have arbitrarily been placed at the end of the sequence, but they could take place earlier.

In a second hypothesis,\(^{20}\) the amino alcohol moiety **6** derives from L-serine. The D-configuration at C-2' could simply be the L-center of serine "turned upside down." Scheme 4 outlines possible pathways leading from L-serine (19) to amino alcohol **6**. D-Cysteinol (20) could result from the reduction of the carboxyl group of serine followed by reaction with hydrosulfide ion. The reduction could also be followed by reaction with cysteine to produce amino acid **21** which could be cleaved to D-cysteinol via its pyridoxal phosphate adduct. D-Cysteinol could then be methylated with SAM to S-methyl-D-cysteinol (22). This intermediate could also be formed directly from serine by reduction and subsequent reaction with methanethiol. Oxidation of S-methyl-D-cysteinol would then lead to amino alcohol **23** which could also be produced by the reaction of D-cysteinol (20) with tetrahydrofolate. Amino alcohol **23** could then be converted to
Scheme 4
Scheme 4 (cont'd)
dithioacetal 26 in one of three ways: 1) reaction with cysteine to produce the djenkolic acid derivative 24 followed by pyridoxal phosphate cleavage and SAM methylation, 2) reaction with hydrosulfide ion to produce thiohemiacetal 25 which could be methylated with SAM (25 could also be formed from 22 by direct insertion of sulfur), or 3) reaction with methanethiol. Dithioacetal 26, once formed, merely requires oxidation of one sulfur atom to a sulfoxide to form amino alcohol 6.

Early speculation concerning the biosynthetic origin of the uracil moiety (Z) of sparsomycin centered on two major hypotheses. The first of these was formulated as shown in Scheme 5. 4-Methyluracil (31) could arise from the decarboxylation and methylation by SAM of orotic acid (30), the biosynthesis of which proceeds as shown from aspartic acid (27) and carbamoyl phosphate. 4-Methyluracil could then react with the pyridoxal phosphate adduct of serine to produce amino acid 32. This reaction is analogous to the formation of tryptophan from indole and L-serine which is catalyzed by tryptophan synthetase. (See Scheme 6.) Amino acid 32 could finally be transformed into uracil moiety Z by an ammonia lyase.

A second possible pathway for the biosynthesis of uracil moiety Z is outlined in Scheme 7. Formation of a double bond between carbons 3 and 4 of glutamic acid (33) followed by two successive methylations with SAM would produce 4-ethylidenglutamic acid (34). This amino acid has been isolated from Tulipa gesneriana (Liliaceae) and Tetrapleura tetraptera (Mimosaceae). The biosynthesis of this amino acid has not been investigated, but it probably proceeds as shown because the methyl group of methionine is the source of the carbon atoms in the ethylidene moiety present at C-24 of many plant sterols. (See Scheme 8.)
Scheme 5

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2. Materials and Methods
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4. Discussion
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6. References

Appendix

Figure Legends

Scheme 1

Scheme 2

Scheme 3

Scheme 4

Scheme 5

Scheme 6

Scheme 7
Scheme 5 (cont'd)
Scheme 6
Scheme 7
Scheme 7 (cont'd)
Scheme 8
Michael addition of water to 34 followed by oxidation would yield the ketone 36. This intermediate could also be formed by acetylation of glutamic acid with acetyl CoA. Transamination of 36 would lead to amino acid 37 which could condense with carbamoyl phosphate to form the dihydropyrimidine 38. This intermediate could then be oxidized to pyrimidine 39 which, after removal of ammonia by an ammonia lyase, would yield the modified uracil moiety (7).

The method used to test biosynthetic hypotheses such as those outlined above is to administer to the organism each potential precursor marked with a "label" which can be detected, if present, in the metabolite produced. The label which can be detected most easily is a radioactive isotope of carbon [14C], hydrogen [3H], or sulfur [35S]. These labels can be detected in the metabolite by the extremely sensitive technique of liquid scintillation counting.41 This method requires that the compounds of interest, or derivatives thereof, be able to be recrystallized to constant specific activity in order to determine accurately the extent of incorporation of a proposed precursor. Liquid scintillation counting also yields no information concerning the location of the label in the molecule. Specific incorporation of a precursor must be verified by chemically degrading the molecule in such a fashion that the site of the label can be isolated and the radioactivity measured in order to prove that all the incorporated activity resides at that position.

Both of these requirements, the ability to be recrystallized and efficient degradation methods, limit the usefulness of radiolabels in investigating the biosynthesis of sparsomycin. First, sparsomycin is a flocculent material which is very difficult to obtain in crystalline form. In addition, an examination of its structure reveals the difficulty of derivatizing it in order to obtain a crystalline compound. Second,
although much degradative chemistry of sparsomycin has been published,\textsuperscript{42} the isolation of its individual carbon atoms seemed a rather formidable task. Therefore, the results obtained from feedings of singly-labeled radioactive precursors would be used only for purposes of comparing the incorporation of one precursor to another. No conclusions about specific incorporation would be drawn.

More reliable information could probably be obtained from feeding experiments employing doubly-labeled radioactive precursors. In these experiments, a precursor labeled with two radioisotopes [\textsuperscript{3}H and \textsuperscript{14}C or \textsuperscript{35}S] in a known ratio is fed to the organism, and the isotope ratio of the metabolite is determined. Comparison of the isotope ratio of the sparsomycin to that of the precursor would be expected to yield information about the incorporation of the precursor. The results of an experiment in which the sparsomycin recovered possessed the same isotope ratio as the precursor (or in which the ratio equaled a calculable theoretical value corresponding to the loss of part of one label) could be taken as fairly conclusive evidence for the specific incorporation of the precursor because it is unlikely that such a result would be obtained if a labeled impurity were present.

We hoped to carry out most of the work in this investigation by means of stable isotope techniques. In this type of experiment, a postulated precursor labeled in an appropriate position with a stable isotope of carbon (\textsuperscript{13}C) is administered to the organism. The presence of the label in the metabolite is then determined by mass spectrometry or nuclear magnetic resonance spectrometry.\textsuperscript{43} Since it is generally very difficult to determine the location of a label from a mass spectrum, the detection method of choice is NMR spectrometry. This method is based upon the fact that the incorporation of a \textsuperscript{13}C-label derived from a
precursor produces an enhancement of the signal of the corresponding carbon atom in the $^{13}$C-NMR spectrum of the metabolite. Therefore, the label is located simultaneously with the confirmation of incorporation. Two requirements must be met in order to conduct biosynthetic studies of sparsomycin with $^{13}$C-labeled precursors. First, the $^{13}$C-NMR spectrum of sparsomycin must be assigned. We thought that this could be accomplished on the basis of chemical shifts and with the aid of various NMR techniques such as INEPT, DEPT, and 2-dimensional spectrometry, together with hints from precursor incorporation experiments. Second, incorporation of a precursor must be sufficiently high to produce a clear enrichment of the appropriate resonance in the $^{13}$C-NMR spectrum of sparsomycin. Herbert$^{44}$ sets the lower limit for such enrichment at 20% above natural abundance, or 0.2%. This requirement should not be a problem because compounds labeled with stable isotopes are highly enriched at the appropriate site, and a relatively large quantity of precursor labeled with a stable isotope can be fed much less expensively than the corresponding amount of radiolabeled precursor.

Deuterium ($^{2}$H) can also be used as the stable isotope in biosynthetic investigations.$^{45}$ Although its incorporation can be detected by mass spectrometry, $^{2}$H-NMR spectrometry is generally the preferred method for the location of deuterium labels.

Initial evaluation of the biosynthetic hypotheses pertaining to the "right-hand side" of sparsomycin, amino alcohol $^{6}$, could be accomplished by incorporation experiments utilizing labeled forms of L-cysteine and L-serine. It was quite possible that both of these precursors would be incorporated into sparsomycin because cysteine is formed in vivo from serine. There is also at least one known enzyme, serine sulphydrase,$^{46}$ capable of converting cysteine to serine. This enzyme is widely
distributed in nature and acts on cysteine to produce serine and hydrogen sulfide. However, Schemes 2 and 4 can be clearly distinguished by the results of a feeding experiment utilizing (3-$^{13}$C)-DL-cysteine. If one of the pathways outlined in Scheme 2 is in operation, the $^{13}$C-label should be located at C-3' of sparsomycin. If L-cysteine is incorporated via L-serine (Scheme 4), C-1' should be labeled.

Incorporation of (3-$^{13}$C)-DL-serine should give complementary results. If L-serine is incorporated into sparsomycin directly by one of the pathways shown in Scheme 4, C-1' of sparsomycin should be labeled. If L-serine is converted to L-cysteine before incorporation, the label should be located at C-3'.

The origins of C-4' and C-5' are indicated in Schemes 2 and 4 as SAM or tetrahydrofolate and SAM, respectively. Feeding experiments employing labeled forms of methionine and sodium formate can be carried out to determine the sources of these two carbon atoms.

Once the origins of the carbon atoms of amino alcohol moiety $^6$ have been established, experiments to investigate the intermediacy of various compounds could be performed. The exact compounds evaluated as precursors would, of course, be determined by the results of the studies previously described, but any of the compounds shown in Schemes 2 and 4 could be fed to S. sparsogenes if obtainable in an appropriately-labeled form.

If L-cysteine proved to be the precursor of amino alcohol $^6$, two additional questions to be answered in this phase of the investigation were 1) the timing of the inversion of configuration at C-2' and 2) the timing of the reduction of the carboxyl group of cysteine to the hydroxyl group at C-1'.
Regardless of the precursor of carbons 1', 2', and 3', two questions to be addressed were: 1) the mechanism of sulfur introduction at C-4' and 2) the possibility of the intact incorporation of the thiomethyl group of methionine into the thiomethyl group of sparsomycin. This second possibility was especially intriguing because we had found no published examples of the incorporation of the thiomethyl group of methionine into a natural product. The incorporation of the thiomethyl group of methionine could be explored by a feeding experiment utilizing $[^{35}\text{S}^\text{H}]$-L-methionine.

The validity of the hypotheses outlined in Scheme 5 concerning the origin of the "left-hand side" of sparsomycin, uracil moiety Z, could be tested by incorporation experiments involving appropriately-labeled forms of aspartic acid. The results of the experiments with serine and methionine carried out to determine the origin of the "right-hand side" of sparsomycin might also provide insight into the origin of Z, since serine and methionine could possibly be incorporated into both portions of sparsomycin.

The hypothesis shown in Scheme 7 can be evaluated by administration of labeled forms of glutamic acid and sodium acetate to S. sparsogenes. Methionine is also a possible participant in this pathway.

Table 1 summarizes the preliminary incorporation experiments that we planned to carry out to begin the research described in this thesis.
### Table 1

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Fragment into which Incorporation is Postulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U-(^{14})C]-L-Cysteine</td>
<td>Amino-alcohol</td>
</tr>
<tr>
<td>[U-(^{14})C]-L-Serine</td>
<td>Both</td>
</tr>
<tr>
<td>[Methyl-(^{14})C]-L-Methionine</td>
<td>Both</td>
</tr>
<tr>
<td>(^{14})C]-Sodium Formate</td>
<td>Amino-alcohol</td>
</tr>
<tr>
<td>[U-(^{14})C]-L-Aspartic Acid</td>
<td>Uracil</td>
</tr>
<tr>
<td>[U-(^{14})C]-L-Glutamic Acid</td>
<td>Uracil</td>
</tr>
<tr>
<td>[U-(^{14})C]-Sodium Acetate</td>
<td>Uracil</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Work on this project began with efforts to grow *S. sparsogenes* and to isolate sparsomycin from the culture. An initial concern was the development of an HPLC system appropriate for the detection of sparsomycin in the fermentation broth. An HPLC system for the detection of sparsomycin was desired in order to ascertain whether or not our culture produced sparsomycin and to determine the timing of such production. The initial system used was a modification of another in use in our laboratory at that time and consisted of a reversed-phase column developed with the aqueous KH$_2$PO$_4$ buffer described under EXPERIMENTAL. We later discovered the HPLC system developed by Winograd *et al.*\(^4\) and began using it almost exclusively due to its greater convenience.

*Streptomyces sparsogenes* grew well on slants of Bennett's agar. Fermentations were then conducted according to the procedure of Owen, Dietz, and Camiener.\(^1\) Daily monitoring by HPLC of the sparsomycin content of a fermentation indicated that production began on or about day 3 and continued until day 10 or 11, when the sparsomycin content reached a maximum. (We were pleased to find that we could detect the presence of sparsomycin in filtered fermentation broth; partial work-up in order to concentrate the sparsomycin was unnecessary.) Later cultures of *S. sparsogenes* often did not begin producing sparsomycin until day 6 or 7. A few fermentations produced little or no sparsomycin at all. After the unfortunate feeding of a few precursors to fermentations which did not produce antibiotic, future administrations of precursor were not begun until sparsomycin production had commenced, as indicated by HPLC.
The isolation procedure of Argoudelis and Herr\textsuperscript{3} was initially followed closely, but modifications to improve the purity of the sparsomycin and to reduce the work-up time were gradually incorporated. The temperature at which fermentations were conducted was also raised to 32°C. from 28°C., because fermentations carried out at 32°C. reportedly produced more sparsomycin than those performed at a lower temperature.\textsuperscript{1} The isolation procedure ultimately used is shown in Scheme 9. Two-liter fermentations worked-up according to this scheme generally yielded four to twelve mg of sparsomycin. (Fermentations in which little or no antibiotic could be detected by HPLC were not worked-up.)

Although many of the experiments concerning the "right-hand" and "left-hand" portions of sparsomycin were actually carried out concurrently, the description of the research performed will be artificially divided along this line in order to clarify the discussion.

**Right-Hand Fragment**

Investigation of the biosynthesis of the right-hand fragment, amino alcohol moiety $\mathcal{G}$, began with feeding experiments utilizing the precursors postulated in Schemes 2 and 4, specifically $[U^{-14}C]^{-L}$-cysteine, $[methyl^{-3}H]^{-L}$-methionine, $[U^{-14}C]^{-L}$-serine, and $[^{14}C]^{-sodium}$ formate. Each precursor was administered to a separate fermentation of $S. sparsogenes$ as described under EXPERIMENTAL. The sparsomycin produced by each fermentation was isolated, and its activity was measured by liquid scintillation counting. Table 2 summarizes the results of these
Fermentation Broth
1. Adjust to pH 3.0
2. Filter

Filtrate
1. Stir with charcoal & Celite
2. Filter

Charcoal Mixture
1. Wash with H₂O
2. Wash with 20:80 acetone:H₂O (pH 8.0)

Solid
1. Stir with 50:50 acetone:H₂O pH 2.5
2. Filter

Filtrate
1. Adjust to pH 6.2
2. Concentrate
3. Lyophilize

Powder

Insoluble Material
Extract with MeOH at 40°C.

MeOH Solution

Discard

Cont'd

Scheme 9
MeOH Solution
1. Concentrate
2. Extract with butanone-McIlvaine's buffer solution

Butanone-H$_2$O Solution
1. Concentrate
2. MPLC (KH$_2$PO$_4$-MeOH buffer)

Fractions containing sparsomycin as shown by HPLC
1. Concentrate
2. Lyophilize

Powder

MPLC (85:15 MeOH:H$_2$O)

Fractions containing sparsomycin

Scheme 9 (cont'd)
Fractions containing sparsomycin

1. Concentrate
2. Lyophilize

Powder

1. Dissolve in MeOH
2. Precipitate with dry ether

Sparsomycin

Cellulose column
(6:1 n-butanol:H₂O)

Fractions containing sparsomycin as shown by TLC

1. Concentrate
2. Lyophilize

Very pure sparsomycin

Scheme 9 (cont'd)
preliminary experiments.

Table 2

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Specific Incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U-$^{14}$C]-L-Cysteine</td>
<td>1.0</td>
</tr>
<tr>
<td>[methyl-$^{3}$H]-L-Methionine</td>
<td>0.016</td>
</tr>
<tr>
<td>[U-$^{14}$C]-L-Serine</td>
<td>0.037</td>
</tr>
<tr>
<td>[$^{14}$C]-Sodium formate</td>
<td>0.00029</td>
</tr>
</tbody>
</table>

Specific incorporation is defined as the ratio of the specific activity (µCi/mol) of the sparsomycin isolated to that of the precursor administered and is reported as a percentage. From the tabulated results it is obvious that [U-$^{14}$C]-L-cysteine was incorporated into sparsomycin quite efficiently. Methionine and serine, although their specific incorporations were much lower than that of cysteine, could not be ruled out as precursors on this basis. It is especially difficult to envision a biosynthetic route to sparsomycin that does not include the incorporation of at least one methyl group from methionine, and the observed incorporation of serine was even higher than that of methionine! Although non-zero, the incorporation figure for sodium formate was so low that this compound was eliminated as a possible precursor.

Having obtained some evidence for the incorporation of cysteine, methionine, and serine into sparsomycin, we then began experiments utilizing $^{13}$C-labeled precursors. The first step was to obtain a $^{13}$C-NMR spectrum of sparsomycin. This spectrum is shown in Figure 6. A second spectrum was taken in the presence of the water-soluble relaxing agent
disodium iron (III) diethylenetriaminepentaacetic acid dihydrate. This agent caused the signals due to the three carbonyl carbons to appear in the spectrum which is shown in Figure 7 and also caused the height of the signals due to the two quaternary carbons to increase.

On the basis of these two spectra, the signals can be divided into five groups: 1. The signals at 16.20 ppm and 16.81 ppm represent the two methyl groups, C-1 and C-5'. 2. The signals at 46.72 ppm, 52.90 ppm, 54.75 ppm, and 63.09 ppm represent C-1', C-2', C-3', and C-4', not necessarily respectively. 3. The signals at 120.81 ppm and 132.47 ppm represent the two vinyl carbons, C-4 and C-5. 4. The signals at 105.91 ppm and 156.69 ppm represent the two quaternary carbons, C-2 and C-3. 5. The signals at 151.55 ppm, 164.74 ppm, and 169.27 ppm represent the three carbonyl carbons, C-6, C-7, and C-8.

The signal at 46.72 ppm was assigned to methine carbon C-2' by means of an NMR pulse program called INEPT (Insensitive Nuclei Enhanced by Polarization Transfer). This program produces spectra in which methyl and methine signals are phased in the normal manner, but methylene signals appear 180° out of phase, i. e. they point downward. As shown in Figure 8, the signal at 46.72 is the only resonance of the four assigned to C-1' through C-4' which points upward, so it must correspond to C-2'.

On the basis of chemical shifts and with the assistance of $^{13}$C correlation charts, the three methylene signals were tentatively assigned as indicated in Table 3.
Table 3

<table>
<thead>
<tr>
<th>Chemical Shift (ppm)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>52.90</td>
<td>C-3'</td>
</tr>
<tr>
<td>54.75</td>
<td>C-4'</td>
</tr>
<tr>
<td>63.09</td>
<td>C-1'</td>
</tr>
</tbody>
</table>

Table 4 summarizes the assignments of the $^{13}$C-NMR spectrum of sparsomycin thus far described:

Table 4

<table>
<thead>
<tr>
<th>Chemical Shift (ppm)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.21</td>
<td>C-1 or C-5'</td>
</tr>
<tr>
<td>16.81</td>
<td>C-1 or C-5'</td>
</tr>
<tr>
<td>46.72</td>
<td>C-2'</td>
</tr>
<tr>
<td>52.90</td>
<td>C-3'</td>
</tr>
<tr>
<td>54.75</td>
<td>C-4'</td>
</tr>
<tr>
<td>63.09</td>
<td>C-1'</td>
</tr>
<tr>
<td>105.91</td>
<td>C-2 or C-3</td>
</tr>
<tr>
<td>120.81</td>
<td>C-4 or C-5</td>
</tr>
<tr>
<td>132.47</td>
<td>C-4 or C-5</td>
</tr>
<tr>
<td>151.56</td>
<td>C-6, C-7, or C-8</td>
</tr>
<tr>
<td>156.69</td>
<td>C-2 or C-3</td>
</tr>
<tr>
<td>164.74</td>
<td>C-6, C-7, or C-8</td>
</tr>
<tr>
<td>169.27</td>
<td>C-6, C-7, or C-8</td>
</tr>
</tbody>
</table>

With a partial assignment of the $^{13}$C-NMR spectrum of sparsomycin in hand, we were then ready to begin incorporation experiments utilizing $^{13}$C-labeled precursors. The first $^{13}$C-labeled precursor fed to S. sparsogenes was (methyl-$^{13}$C)-L-methionine. This compound was chosen because it was commercially available and relatively inexpensive. Five
hundred milligrams of this precursor were administered in an attempt to "load up" the medium and ensure good incorporation in this initial experiment involving a $^{13}$C-labeled precursor. Figure 9 shows that good incorporation was indeed obtained. The spectrum of the sparsomycin biosynthesized in the presence of (methyl-$^{13}$C)-L-methionine showed an enrichment of 6.7% in the methyl group responsible for the signal at 16.21 ppm. (Percent enrichment was determined by comparison of the enriched signal with the unenriched, natural abundance signal. The natural abundance of $^{13}$C is approximately 1.1%.) Since the label was incorporated into only one methyl carbon atom, this signal was assigned to C-5'. We had postulated origins other than the methyl group of methionine for C-1, but not for C-5'.

Not surprisingly, the methyl group of methionine was also incorporated into C-4' to the extent of 2.7%. This result suggested the intermediacy of S-methylcysteine (9, Scheme 2). Experiments utilizing this postulated precursor will be described later. The enhancement of the carbon signal at 54.75 by the methyl group of methionine also supported our assignment of this signal to C-4' of sparsomycin, since it was extremely unlikely that this group was incorporated into C-1' or C-3'.

The next precursor administered in the investigation of the origin of the right-hand side of sparsomycin was (3-$^{13}$C)-DL-cysteine. This experiment was expected to give definitive support to either the hypothesis shown in Scheme 2 (cysteine as precursor of C-1', C-2', and C-3'), or that outlined in Scheme 4 (serine as precursor of C-1', C-2', and C-3'). At that time, no $^{13}$C-labeled forms of cysteine were commercially available, so (3-$^{13}$C)-DL-cysteine was synthesized by the method of Gasparini et. al.50 as shown in Scheme 10. Diethyl acetimidomalonate was converted to compound 41 by a Mannich-type reaction with piperidine
Scheme 10
and $^{13}$C-formaldehyde. Methiodide 42 resulted from the treatment of 41 with methyl iodide. Displacement of the N-methyl piperdyl moiety by benzyl mercaptide produced intermediate 43 which was hydrolyzed to the hydrochloride salt of (3-$^{13}$C)-S-benzyl-DL-cysteine (44) with concentrated hydrochloric acid. The desired precursor, (3-$^{13}$C)-DL-cysteine (45) was finally obtained by the cleavage of 44 with sodium in liquid ammonia.

The synthetic (3-$^{13}$C)-DL-cysteine was administered to S. sparsogenes, and the sparsomycin produced was isolated from the fermentation. The $^{13}$C-NMR spectrum of this material is shown in Figure 10. To our surprise, the spectrum showed enrichment at two positions. C-3' was enriched to the extent of 1.0%. This result was in accordance with our hypothesis outlined in Scheme 2. C-1' was clearly not enriched which cast doubt upon the postulated incorporation of cysteine into sparsomycin via serine. However, the signal corresponding to C-4' showed an enrichment of 0.6%. This result was very unexpected. It seemed to indicate the formation of a one-carbon fragment from C-3 of cysteine, which was completely unprecedented. We speculated that cysteine might be converted via a serine sulfhydrase-type enzyme (see INTRODUCTION) to serine. The formation of one-carbon units from C-3 of serine is well-documented,22 but these units are incorporated into metabolites via tetrahydrofolate. If C-3' of cysteine were incorporated into C-4' of sparsomycin via serine, [14C]-sodium formate should label this same position, and we had already shown that this precursor was only poorly incorporated. This speculation was not inconsistent with the previously-obtained incorporation of the methyl group of methionine into C-4' of sparsomycin because the methyl group of methionine derives from the tetrahydrofolate pathway.
Figure 10

22.5 MHz

MeOH
in an effort to clarify this unexpected incorporation of C-3' of cysteine into C-4' and to gain additional evidence for our hypothesis shown in Scheme 2, a feeding experiment utilizing (3-\(^{13}\)C)-DL-serine was performed. This precursor was commercially available but was prohibitively expensive, so it was synthesized by the method of King\(^{51}\) as outlined in Scheme 11.

Alcohol 46 resulted from the base-catalyzed condensation of diethyl acetamidomalonate with \((^{13}\text{C})\)-formaldehyde. Basic hydrolysis of the ester groups and decarboxylation of the resulting diacid produced amide 47. Hydrolysis of the amide gave rise to serine which was converted to serine ethyl ester (48) under the work-up conditions. This compound was hydrolyzed with acid back to (3-\(^{13}\)C)-DL-serine (49).

The (3-\(^{13}\)C)-DL-serine was administered to a fermentation of S. sparsogenes, but, to our surprise, the \(^{13}\text{C}\)-NMR spectrum of the isolated sparsomycin showed no enhanced signals. To verify this result, the administration of this precursor to the organism was repeated. Approximately 600 mg were fed to "load up" the medium as was done in the case of (methyl-\(^{13}\)C)-L-methionine. [3-\(^{3}\text{H}\)]-L-Serine was fed simultaneously to the same fermentation to serve as a more sensitive check on incorporation. The radioactively-labeled serine was incorporated to the extent of 1.9% after correction for dilution. This was the highest specific incorporation value ever obtained in this project. Once again, however, no enrichment of any of the signals was observed in the \(^{13}\text{C}\)-NMR spectrum of the sparsomycin produced.

The above results suggest that C-3 of (3-\(^{13}\)C)-DL-serine may be randomly incorporated into sparsomycin as one-carbon fragments produced by the degradation of the compound. The incorporation of C-3
Scheme 11
of (3-$^{13}$C)-DL-cysteine into C-4' of sparsomycin remains a baffling puzzle.

A feeding experiment involving (2,3-$^{13}$C)-DL-serine might give a clearer result because of the enhanced sensitivity due to the $^{13}$C-$^{13}$C coupling in the $^{13}$C-NMR spectrum of sparsomycin; this effect would be observed, however, only if the bond between C-2 and C-3 of serine is not broken before incorporation. This precursor could be prepared from commercial (2-$^{13}$C)-diethyl acetamidomalonate by the same route shown in Scheme 11.

Eager for more conclusive experimental results, we began to explore the role of S-methylcysteine (9) as a precursor of sparsomycin. The possible participation of S-methylcysteine in the biosynthesis of sparsomycin was suggested by the fact that the methyl group of methionine labeled C-4' of sparsomycin, and the role of S-methylcysteine as a precursor had already been hypothesized as shown in Scheme 2. (Methyl-$^{13}$C)-S-methyl-L-cysteine (50) was prepared as shown in Scheme 12 from L-cystine by cleavage of the disulfide bond by sodium in liquid ammonia followed by quenching with ($^{13}$C)-methyl iodide.$^{52}$

The $^{13}$C-NMR spectrum (Figure 11) of the sparsomycin biosynthesized in the presence of this precursor showed a clear enrichment of 0.3% at C-4', as expected. Since C-5' was not labeled, intact incorporation of (methyl-$^{13}$C)-S-methyl-L-cysteine was assumed. (Cleavage of the sulfur-$^{13}$C bond and subsequent incorporation of the labeled methyl group into C-4' should also have labeled C-5'.) This assumption could be verified by a feeding experiment utilizing [3-$^3$H, methyl-$^{14}$C]-S-methyl-L-cysteine. Isolation of sparsomycin having the same $^3$H/$^{14}$C ratio as the precursor would conclusively prove intact incorporation of S-methyl-L-cysteine.
Scheme 12
Having discovered the basic building blocks of the right-hand portion of sparsomycin, we began to consider some of the mechanistic questions mentioned in the INTRODUCTION. The first of these concerned the timing of the inversion of configuration at C-2 of S-methyl-L-cysteine (the configuration at C-2' of sparsomycin corresponds to that of the D-isomer.) To investigate this problem, (methyl-\(^{13}\)C)-S-methyl-D-cysteine was synthesized from D-cystine by the same method as was used for the preparation of the L-isomer. Administration of this precursor to \textit{S. sparsogenes} produced sparsomycin which yielded the \(^{13}\)C-NMR spectrum shown in Figure 12. This spectrum displays an even more pronounced enrichment (0.6\%) at C-4', and, once again, no enhancement of the signal due to C-5'.

Specific incorporation of both the L and D isomers of S-methylcysteine indicated that the inversion of the L-center at C-2 of this precursor to the D-configuration found at C-2' of sparsomycin \textit{can} occur before further elaboration of this precursor takes place. The D-enantiomer appeared to be a slightly better precursor than the L-compound.

The second mechanistic question explored in this phase of the project was the timing of the reduction of the carboxyl group of cysteine (via S-methylcysteine) to the hydroxyl group at C-1' of sparsomycin. A feeding experiment utilizing (methyl-\(^{13}\)C)-S-methyl-D-cysteinol was conducted in order to answer this question. As shown in Scheme 13, this precursor was prepared from (methyl-\(^{13}\)C)-S-methyl-D-cysteine which was synthesized as previously described. (Methyl-\(^{13}\)C)-S-methyl-D-cysteine (50) was converted to its methyl ester hydrochloride (51). Reduction of 51 with NaBH\(_4\)\(^{53}\) led to the desired precursor (52).
Scheme 13
The compound was administered to *S. sparsogenes*, but the $^{13}$C-NMR spectrum of the sparsomycin isolated from the fermentation showed no signal enhancement. This result tended to support the hypothesis that 52 is not an intermediate in the biosynthesis of sparsomycin, i. e. that reduction of the carboxyl group of S-methyl-D-cysteine to an alcohol takes place after the incorporation of this precursor. It was possible that the (methyl-$^{13}$C)-S-methyl-D-cysteinol was incorporated at a level too low to produce enhancement in the $^{13}$C-NMR spectrum or that the precursor was not taken up and utilized by the organism.

A third aspect of the biosynthesis of the right-hand side of sparsomycin we wished to investigate was the mechanism of sulfur introduction at C-4'. We had already established the intermediacy of S-methyl-D-cysteine in the biosynthesis of sparsomycin. Amino alcohol moiety 6 was, therefore, apparently biosynthesized by the oxidative introduction of a thiomethyl group into the methyl group of S-methyl-D-cysteine. This transformation appeared to be somewhat similar to the biosynthesis of biotin (54) from dethiobiobitin (53) and to that of lipoic acid (56) from octanoic acid (55). (Scheme 14.) In each case, one hydrogen atom of the methyl group is replaced by a sulfur atom, and no hydrogen atoms are lost from the adjacent carbon atom, i. e. no unsaturated intermediates are involved.28 Biosynthetic studies of these conversions have revealed that the methyl functionalizations are non-stereospecific, and it has been suggested that they occur via radical intermediates.30 The methylene groups are oxidized with retention of configuration in the case of 53 and with inversion of configuration in the case of 55.28 A radical mechanism has also been proposed for the formation of the penicillins, e. g. penicillin N (Scheme 15), from their precursors.29
Scheme 15

Penicillin

\[ \text{HOOC} \quad \text{NH}_2 \quad \text{CH}_3 \quad \text{COOH} \]

\[ \text{HOOC} \quad \text{NH} \quad \text{N} \quad \text{S} \quad \text{CH}_3 \]

\[ \text{HOOC} \quad \text{NH} \quad \text{N} \quad \text{CH}_3 \quad \text{COOH} \]
To gain insight into the mechanism of sulfur introduction at C-4', we attempted to determine the number of hydrogen atoms removed from the methyl group of S-methyl-D-cysteine during its conversion to sparsomycin. In an experiment designed to provide this information, we synthesized \((\text{methyl}^{13}\text{C}, \text{methyl}^{2}\text{H}_3)\)-S-methyl-D-cysteine from D-cystine and \((\text{methyl}^{13}\text{C}, \text{methyl}^{2}\text{H}_2)\)-methyl iodide by the same procedure previously described for the preparation of \((\text{methyl}^{13}\text{C})\)-S-methyl-D-cysteine. This precursor was administered to \textit{S. sparsogenes} and was expected to produce sparsomycin containing a \(^{13}\text{C}\)-label at the 4' position and having zero, one, or two deuterium atoms attached to that carbon atom. The number of deuterium atoms present could be determined by examination of the \(^{13}\text{C}\)-NMR spectrum of the sparsomycin.

However, the \(^{13}\text{C}\)-NMR spectrum of the sparsomycin isolated from this feeding showed no apparent \(^{13}\text{C}\)-enrichment, and \(^2\text{H}\)-NMR spectrometry indicated the presence of no deuterium atoms. A new sample of the same precursor was synthesized and fed, but the same result was obtained. The lack of incorporation of this precursor was attributed to an isotope effect caused by the three deuterium atoms. Future plans for this project include an incorporation experiment involving \([\text{methyl}^{3}\text{H}, ^{35}\text{S}]\)-S-methyl-L-cysteine. (Only \(^{35}\text{S}\)-L-Cystine is commercially available, so the D-isomer could not be synthesized.) Comparison of the \(^3\text{H}/^{35}\text{S}\) ratio of the sparsomycin produced to that of the precursor should provide information about the number of hydrogen atoms lost or about the validity of the isotope effect hypothesis.

A fourth mechanistic question pertaining to the right-hand side of sparsomycin concerned the possible intact incorporation of the thiomethyl group of methionine into the thiomethyl group of sparsomycin.
As mentioned previously, we were able to find no published examples of the intact incorporation of the thiomethyl group of methionine into a natural product. There is evidence, however, for the intact incorporation of the thiomethyl group of S-methyl cysteine into metabolites.21,32,33

Experimental investigation of this problem proved to be quite difficult. The obvious approach, of course, was to feed \([methyl^{3}{H},^{35}{S}]\)-L-methionine. Maintenance of the \(^{3}{H}/^{35}{S}\) ratio from the precursor through to sparsomycin could not be expected, however, for two reasons. The first was the fact that the methyl group of methionine labels C-4' as well as C-5' of sparsomycin. The second reason was that some of the \(^{35}{S}\) would probably be incorporated into the sulfoxide sulfur. As outlined in Scheme 16, the sulfur atom of methionine can be incorporated into cysteine via the conversion of methionine to homocysteine (57) which can react with serine to form cystathionine (58). Cystathionine can then be cleaved to produce cysteine.54 We had already demonstrated the incorporation of cysteine into sparsomycin. These two complications rendered the expected \(^{3}{H}/^{35}{S}\) ratio of the sparsomycin produced from \([methyl^{3}{H},^{35}{S}]\)-L-methionine incalculable.

To circumvent this problem, we expended considerable effort in attempts to find a method of isolating in a countable form the thiomethyl group of sparsomycin. Because of our scant supply of sparsomycin, all of this work was first carried out with model compounds. Our first approach was to try to reduce the sulfoxide sulfur to obtain a dithiaoacetal which should hydrolyze more cleanly than the monoxodithiaoacetal of sparsomycin. The thiomethyl group would be liberated as methanethiol which could be trapped in basic solution and derivatized with
2,4-dinitrochlorobenzene.

The model compound chosen for use in establishing the reduction conditions was N-(benzyloxy carbonyl)-S-oxo-S-[(methylthio)methyl]-L-cysteine (63). This model compound possessed all the relevant structural features of sparsomycin and was synthesized as shown in Scheme 17. L-Cystine was converted to its methyl ester hydrochloride (59) by treatment with hydrogen chloride in methanol.55 The amino group was then protected with benzyl chloroformate.56 Methyl ester 60 was then reduced to the alcohol with sodium borohydride and lithium iodide.57 During this reduction, the disulfide bond was also reduced, so the disulfide bond was regenerated by treatment with iodine which resulted in alcohol 61. This intermediate was then cyclized to oxathiolane 62 by reaction with acetic acid and N-chlorosuccinimide.57 The oxathiolane was then cleaved with the anion of dimethyl sulfide to produce the desired substituted cysteineol 63.57

After a literature search, the reagent chosen for the reduction of the model compound was tripotassium enneachloroditungstate, K₃W₂Cl₉.58 This reagent was prepared from tungstic acid as shown in Scheme 18 and was tested by reduction of simple sulfoxides. Treatment of the model compound with this reagent under the test conditions, however, apparently decomposed the model compound. After various unsuccessful attempts to remedy this situation, this reagent was abandoned.

We then tried a second reducing agent on a simpler model compound, methyl methylthiomethyl sulfoxide (64). We attempted to reduce this compound with tris(dimethyl amino phosphine) and iodine. This method had been reported to reduce sulfoxides cleanly and selectively,59 but it apparently decomposed this model compound.
Scheme 17
H₂WO₄ $\xrightarrow{1. \text{ K}_2\text{CO}_3, \text{ HCl}}$ \[\xrightarrow{2. \text{ Sn}}\] K₃W₂Cl₉

**Scheme 18**
Figure 13
We then tried several methods to directly decompose the monoxodithioacetal functionality. In the first attempt, N-(benzyloxycarbonyl)-S-oxo-S-[(methylthio)methyl]-L-cysteinol was treated with benzyl bromide. We thought that a sulfonium salt might be produced which would either "fall apart" spontaneously to produce benzyl methyl sulfide or could be easily hydrolyzed. The benzyl methyl sulfide would contain the thiomethyl group of the model compound and could be derivatized as the sulfone. However, the model compound would not react with benzyl bromide.

We then attempted to hydrolyze methyl methylthiomethyl sulfoxide directly with sulfuric acid\(^\text{18}\) and trap any methanethiol generated as its sodium salt, but the compound proved stable to acid. The model compound was then treated with acetic anhydride in an attempt to form a Pummerer product, hydrolysis of which would yield methanethiol, but again, no methanethiol was produced. Scheme 19 outlines the general Pummerer rearrangement mechanism.\(^\text{60}\)

At this point in the investigation, a possible way to prevent methionine from labeling C-4′ of sparsomycin occurred to us. Since this carbon was also labeled by the methyl group of S-methylcysteine, adding a large amount of this precursor, unlabeled, to the medium, might suppress the incorporation of the methyl group of methionine into C-4′ and allow its incorporation only into C-5′. This proposition was tested by a experiment in which \((\text{methyl}\text{-}^{13}\text{C})\)-L-methionine and five hundred milligrams of unlabeled S-methyl-L-cysteine were simultaneously fed to \(S.\ sparsogenes\). The \(^{13}\text{C}\)-NMR spectrum (Figure 14) of the sparsomycin produced shows that the desired result was indeed obtained. The signal due to C-5′ was still greatly enriched (23%), but the signal due to C-4′
Scheme 19
was not enriched at all. The label was effectively "blocked-out" from C-4'.

Since we then had a method to suppress labeling of C-4' of sparsomycin by the methyl group of methionine, we conducted a feeding of \([\text{methyl-}^3\text{H},^{35}\text{S}]\)-L-methionine. Five hundred milligrams of S-methyl-L-cysteine were simultaneously fed to the organism. The \(^3\text{H}/^{35}\text{S}\) ratio of the methionine was 5.40; the ratio in the recovered sparsomycin was 4.37.\(^{61}\) The fact that the ratio had decreased indicated either a loss of tritium or an excess incorporation of sulfur. The latter was considered more likely since, as has already been shown, the sulfur atom of methionine can become that of cysteine.

We then searched for a way to determine the percentage of the total \(^{35}\text{S}\) activity located in the sulfoxide sulfur atom. Knowledge of this value would allow us to calculate, from the overall \(^3\text{H}/^{35}\text{S}\) ratio, the \(^3\text{H}/^{35}\text{S}\) ratio of the thiomethyl group of sparsomycin. Comparison of this \(^3\text{H}/^{35}\text{S}\) ratio to that of the doubly-labeled methionine fed to the organism would provide evidence for or against the intact incorporation of the thiomethyl group of methionine into the thiomethyl group of sparsomycin.

We found that Wiley and MacKellar had reported the isolation of a compound they claimed to be di-O,N-acetylcysteinol (65) by acidic hydrolysis of sparsomycin followed by acetylation of the water-soluble residue.\(^{42}\) We were able to isolate a compound exhibiting the same properties from sparsomycin by following their procedure. We then had a method of determining the \(^{35}\text{S}\) activity in the sulfoxide sulfur of sparsomycin: Sparsomycin biosynthesized from \([\text{methyl-}^3\text{H},^{35}\text{S}]\)-L-methionine and unlabeled S-methyl-L-cysteine could be hydrolyzed under acidic conditions, and the left-hand portion could be filtered off as
Figure 15
the acid. The residue could then be treated with acetic anhydride and pyridine to produce di-O,N-acetylcysteinol, which could be counted to determine the percentage of the total $^{35}$S activity located in the sulfoxide sulfur atom.

Due to the low yield of di-O,N-acetylcysteinol from sparsomycin, accurate determination of the $^{35}$S content of this degradation product necessitated the addition of unlabeled material as carrier to obtain an amount of compound large enough for recrystallization to constant specific activity. We therefore tried to synthesize this compound by another route.

Our first attempt (Scheme 20) began with cysteine ethyl ester hydrochloride (66). This compound was converted with aqueous sodium carbonate to the free amine (67) which was then reduced with sodium borohydride. The crude cysteinol (68) obtained was acetylated with acetic anhydride and pyridine. The product of this sequence was not the same compound as that obtained from the hydrolysis and acetylation of sparsomycin, and it was determined to be tri-O,N,S-acetylcysteinol (69). An attempt to hydrolyze off the S-acetyl group apparently removed both the S- and O-acetyl groups.

Discouraged by these results, we reexamined the procedure of Wiley and MacKellar. We discovered that they had taken no precautions to preclude the formation of a disulfide bond. We therefore suspected that the compound they, and we, actually obtained from the hydrolysis and acetylation of sparsomycin was the disulfide, di-O,N-acetylcystinol. We prepared this compound by the route shown in Scheme 21.

Cysteine ethyl ester hydrochloride (66) was reduced to cysteinol (68) with sodium borohydride. Iodine treatment of the crude cysteinol
Scheme 20
Scheme 21
led to the disulfide, cystinol (71), which was acetylated to produce di-O,N-acetylcystinol (72).

The di-O,N-acetylcystinol was shown to be identical to the compound isolated from sparsomycin by both $^1$H- and $^{13}$C-NMR spectrometry. The mass spectrum of 72 also gave the correct molecular ion for the disulfide.

The degradative route by which di-O,N-acetylcystinol was obtained from sparsomycin can now be presented.\(^{42}\) (Scheme 22) The amide linkage of sparsomycin (1) was hydrolyzed with hot aqueous hydrochloric acid. The left-hand portion was filtered off as the insoluble acid, $\beta$-[(E)-1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidine]-acrylic acid (73). The water-soluble residue contained cysteinol (68) and/or cystinol (71) and was subjected to acetylation with acetic anhydride and pyridine. Di-O,N-acetylcysteineol (72) was isolated from the reaction mixture by chloroform extraction and chromatography on silica gel.

We then knew the identity of the compound isolated from the hydrolysis and acetylation of sparsomycin, and had enough of it available to use as carrier. Since by this time much of the $^{35}$S activity of the sparsomycin obtained from the feeding of [methyl-$^3$H,$^{35}$S]-L-methionine had been lost due to the relatively short half-life of $^{35}$S, we repeated the feeding of this precursor in conjunction with unlabeled S-methyl-L-cysteine.

A sample of [methyl-$^3$H,$^{35}$S]-L-methionine having a $^3$H/$^{35}$S ratio of 10.22 was administered to a fermentation of S. sparsogenes along with 500 mg of unlabeled S-methyl-L-cysteine. The $^3$H/$^{35}$S ratio of the sparsomycin produced was 4.25. The sparsomycin was diluted with unlabeled sparsomycin, and the sample was subjected to acidic
Scheme 22
hydrolysis. Di-O,N-acetylcystinol was then obtained by acetylation of the water-soluble residue. This fragment was diluted with unlabeled synthetic material and its approximate specific activity was determined. (The material, for unknown reasons, would not recrystallize to true constant activity or isotope ratio.) After back-correction for dilution, the fragment was calculated to possess 35% of the specific $^3$H activity and 55% of the specific $^{35}$S activity of the sparsomycin. The fact that the fragment contained tritium cast doubt upon the validity of this experiment because we had not expected this fragment to contain any $^3$H activity. Nevertheless, the calculations were carried out; correction of the sparsomycin $^3$H/$^{35}$S ratio by the activities of the di-O,N-acetylcystinol yielded a $^3$H/$^{35}$S ratio of 6.14 for the thiomethyl group. Comparison of this ratio, 6.14, to that of the precursor methionine, 10.22, led us to conclude that intact incorporation of the thiomethyl group of methionine into sparsomycin does not occur.

**Left-Hand Fragment**

Our investigations of the biosynthesis of the left-hand side of sparsomycin, uracil moiety Z, began with evaluation of the precursors suggested by Schemes 5 and 7. Scheme 5 proposes aspartic acid, serine, and the methyl group of methionine as precursors of this fragment. A feeding experiment employing [U-$^{14}$C]-L-aspartic acid gave a specific incorporation figure of 0.001%. This low value cast doubt upon the validity of this hypothesis. The incorporations of serine and the methyl
group of methionine had already been examined in the investigation of the origin of the right-hand portion of sparsomycin, and neither had been found to be a precursor of the left-hand fragment. On the basis of these results, this hypothesis was discarded.

Glutamic acid and sodium acetate or the methyl group of methionine were postulated as precursors of uracil moiety 7 in Scheme 7. Evaluation of [U-\(^{14}\)C]-L-glutamic acid gave an extremely low specific incorporation of 0.00045%. A value of 0.0022% was obtained for the specific incorporation of [U-\(^{14}\)C]-sodium acetate. As mentioned above, we had already demonstrated that the methyl group of methionine was not incorporated into the left-hand side of sparsomycin. Thus, the hypothesis outlined in Scheme 7 was rejected.

Having gained no experimental support for either of our hypotheses concerning the biosynthetic origins of uracil moiety 7, we proposed others. One of these was formulated as shown in Scheme 23.\(^{20}\) Uracil moiety 7 could be derived from tyrosine in the following manner: Tyrosine (74) could be oxidized to 3,4-dihydroxyphenylalanine (75). Oxidative ring cleavage would lead to amino acid 76 which, after the addition of water, would yield amino acid 77. This intermediate could be decarboxylated to alcohol 78 which could be oxidized to triacid 79. Removal of ammonia by an ammonia lyase followed by addition of water and oxidation would produce intermediate 81 which could be decarboxylated to ketone 82. Formal addition of urea followed by ring closure in conjunction with the loss of two moles of water would result in uracil moiety 7.

Two feeding experiments utilizing [U-\(^{14}\)C]-L-tyrosine were conducted in an attempt to evaluate this hypothesis, but unfortunately
Scheme 23
Scheme 23 (cont'd)
Scheme 23 (cont'd)
neither fermentation produced enough sparsomycin to isolate. We then considered the possibility that uracil itself, 83, might be elaborated to form the uracil moiety of sparsomycin. This hypothesis was tested by the administration of commercial [2-14C]-uracil to S. sparsogenes. An incorporation value of only 0.00078% into sparsomycin was obtained, so uracil was ruled out as a precursor.

As a "shot in the dark," we then administered [1-14C]-D-ribose to S. sparsogenes. This compound is a precursor of tubercidin (2)62 (See Scheme 24), which, as previously mentioned, is biosynthesized along with sparsomycin by both S. sparsogenes and S. cuspidosporus. This interesting production of the two compounds by two different organisms prompted us to speculate that their biosynthetic origins might be somehow related. We obtained a specific incorporation of 0.0068% of [1-14C]-D-ribose into sparsomycin. This value was deemed too low for further investigation of ribose as a precursor of sparsomycin. A possible explanation for the non-zero incorporation of ribose into sparsomycin will be provided later.

At this point in the investigation a new precursor for the left-hand side of sparsomycin occurred to us. We postulated that this fragment could be derived from tryptophan as shown in Scheme 25. This pathway began with the well-known conversion of tryptophan (83) to N-formylkynurenine (84).63 Cleavage of the side chain to a carboxyl group would produce N-formyl-anthranilic acid (85). Oxidative ring cleavage would lead to intermediate 86. This compound would require several modifications to produce uracil moiety Z, including the introduction of the second ring nitrogen, change of configuration about the side-chain double bond, oxidation of one aldehyde to an acid, and
Scheme 24
Scheme 25
reduction of the other aldehyde to a methyl group.

An alternate pathway proceeds through anthranilic acid as shown in Scheme 26. This route also invokes the intermediacy of N-formylkynurenine (84). The hydrolytic removal of the N-formyl group and the cleavage of the resulting kynurenine (87) by kynureninase to anthranilic acid (88) are well-established. Oxidative ring-cleavage of 88 would lead to intermediate 89. To produce uracil moiety Z, 89 must undergo, in addition to the transformations listed for the conversion of 86 to Z, the insertion of a one-carbon unit to complete the pyrimidine ring.

This hypothesis was tested by a feeding experiment employing [5-3H]-L-tryptophan. We were very pleased with the resulting specific incorporation of 0.17%. Table 5 summarizes the results of the preliminary incorporation experiments performed to investigate the biosynthesis of uracil moiety Z.

Table 5

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Specific Incorporation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U-14C]-L-Aspartic Acid</td>
<td>0.001</td>
</tr>
<tr>
<td>[U-14C]-L-Glutamic Acid</td>
<td>0.00045</td>
</tr>
<tr>
<td>[U-14C]-Sodium Acetate</td>
<td>0.0022</td>
</tr>
<tr>
<td>[2-14C]-Uracil</td>
<td>0.00078</td>
</tr>
<tr>
<td>[1,14C]-D-Ribose</td>
<td>0.0068</td>
</tr>
<tr>
<td>[5-3H]-L-Tryptophan</td>
<td>0.17</td>
</tr>
</tbody>
</table>

An experiment utilizing [5-3H,U-14C]-L-tryptophan was then
Scheme 26
Scheme 26 (cont'd)
conducted to confirm the role of tryptophan as a specific precursor of the left-hand fragment of sparsomycin. If the biosynthetic pathway proceeds as shown in Scheme 25, complete retention of tritium and retention of eight of the carbon atoms of tryptophan would be expected. If the N-formyl group of N-formyl-anthranilic acid were lost, as postulated in Scheme 26, only seven carbon atoms would be retained. The $^{3}\text{H}/^{14}\text{C}$ ratio of the doubly-labeled tryptophan fed was 5.45. If all the tritium and eight carbon atoms were retained, a $^{3}\text{H}/^{14}\text{C}$ ratio of 7.49 would be predicted for the sparsomycin isolated. If only seven carbon atoms were retained, the sparsomycin should possess a $^{3}\text{H}/^{14}\text{C}$ ratio of 8.56. The actual $^{3}\text{H}/^{14}\text{C}$ ratio of the sparsomycin produced in this experiment was 7.96. Since this value fell between the two theoretical values, we could not decide whether seven or eight carbon atoms of tryptophan had been incorporated into sparsomycin, i.e. we could not distinguish between Schemes 25 and 26. It was clear, however, that tryptophan served as a specific precursor of sparsomycin. Evidence to further substantiate the specific incorporation of tryptophan and to provide support for one of the two proposed pathways was then sought.

The first experiment designed to yield such evidence again utilized [5-$^{3}\text{H}$]-L-tryptophan as a precursor. The $^{3}\text{H}$-label was expected to be incorporated into C-5 of sparsomycin. A sample of tryptophan containing one millicurie of tritium was fed to $S$. sparsogenes. We thought that perhaps enough activity would be incorporated into the sparsomycin produced to allow us to locate the label by $^{3}\text{H}$-NMR spectrometry. Although a specific incorporation of 0.22% was obtained, the total activity recovered was only nine microcuries. We subsequently learned that approximately one hundred microcuries are needed for $^{3}\text{H}$-NMR
spectrometry.

We then carried out the synthesis of (2-$^{13}$C)-DL-tryptophan. The results of an incorporation experiment utilizing this precursor would provide evidence for (Scheme 25) or against (Scheme 26) the incorporation of tryptophan with retention of C-2 (the N-formyl group of N-formyl-anthranilic acid (85). It was prepared as shown in Scheme 27.

(Formyl-$^{13}$C)-acetic formic anhydride (90) resulted from the condensation of acetyl chloride and ($^{13}$C)-sodium formate.$^{65}$ Reaction of this anhydride with o-toluidine then yielded (formyl-$^{13}$C)-N-formyl-o-toluidine (91).$^{66}$ This compound was then cyclized to (2-$^{13}$C)-indole (92) by treatment with potassium t-butoxide and heat.$^{67}$ (2-$^{13}$C)-Gramine (93) then resulted from the Mannich-type reaction of the labeled indole with formaldehyde and dimethylamine.$^{68}$ Diester (94) then arose from the reaction of the (2-$^{13}$C)-gramine with diethyl acetamidomalonate.$^{69}$ This intermediate was then deesterified and decarboxylated, and the amino-protecting group was removed to form (2-$^{13}$C)-DL-tryptophan (95).$^{68}$

The $^{13}$C-NMR spectrum (Figure 17) of the sparsomycin biosynthesized from this precursor shows that the label was located in the carbon atom responsible for the signal at 151.55 ppm. This signal was enriched to the extent of 22%. We had assigned this signal to C-6, C-7, or C-8. Due to the enrichment of this signal by C-2 of tryptophan, we assigned this signal to C-8 of sparsomycin. We now had evidence in favor of Scheme 25, and Scheme 26 appeared less likely.

To prove conclusively the intact incorporation of the indole moiety of tryptophan, it was only necessary to determine the site of one additional carbon atom of tryptophan in sparsomycin. Scheme 25 predicts
Scheme 27
that C-3 of tryptophan becomes C-3 of sparsomycin. We therefore undertook the synthesis of \((3^{13}C)\)-DL-tryptophan. This preparation is shown in Scheme 28. \((2^{13}C)\)-Sodium acetate would be converted to \((2^{13}C)\)-acetyl bromide \((96)\) by heating with benzoyl bromide and benzoic acid.\(^{70,71}\) The bromide could then be displaced with cyanide ion to yield \((2^{13}C)\)-pyruvonitrile \((97)\) which could be hydrolyzed with aqueous acid to \((3^{13}C)\)-pyruvamide \((98)\).\(^{71}\) Reaction of this intermediate with phenylhydrazine hydrochloride and subsequent hydrolysis would lead to \((3^{13}C)\)-pyruvic acid phenylhydrazone \((99)\) which, after esterification with diazomethane and acid-catalyzed cyclization, would yield methyl \((3^{13}C)\)-indole-2-carboxylate \((100)\).\(^{72}\) Hydrolysis of the ester group followed by decarboxylation with cupric oxide and quinoline would result in \((3^{13}C)\)-indole \((102)\).\(^{72}\) The remainder of the sequence leading to \((3^{13}C)\)-DL-tryptophan \((103)\) was identical to that described for the synthesis of \((2^{13}C)\)-DL-tryptophan from \((2^{13}C)\)-indole.

This sequence was carried out on unlabeled material. Because of its length and the low yields of some of its steps, this synthetic route was deemed too expensive to be performed with labeled material. While we were considering ways to improve the yield and lower the cost of this route, a precursor which could be administered to \(S.\) \(s\)parsogenes instead of \((3^{13}C)\)-DL-tryptophan occurred to us. This compound was \((5^{2}H_{1})\)-DL-tryptophan. A feeding experiment utilizing this compound should yield the same information we expected to obtain from our previous feeding of one millicurie of \((5^{3}H_{1})\)-L-tryptophan, i. e. the label should be located at C-5 of sparsomycin. The presence of deuterium at that position could be determined by \(^{2}H\)-NMR spectrometry.
Scheme 28
Scheme 28 (cont'd)
Scheme 28 (cont'd)
(5-²H₁)-DL-Tryptophan was prepared by the method shown in Scheme 29. 5-Bromo-DL-tryptophan was converted to (5-²H₁)-DL-tryptophan (106) by reaction with deuterium gas.³³ Palladium on calcium carbonate was used as the catalyst. We found it necessary to prepare this catalyst ourselves, as commercial material proved to be virtually inactive.⁷⁴

The synthetic (5-²H₁)-DL-tryptophan was administered to S. sparsogenes, and the sparsomycin isolated was examined by ²H-NMR spectrometry. The spectrum (Figure 18) displayed a deuterium signal at 7.09 ppm. The ¹H-NMR spectrum (Figure 19) of the material contained an extra singlet overlapping the signal corresponding to the proton attached to C-4. This singlet represented the population of sparsomycin molecules in which deuterium was attached to C-5 and in which the signal due to the proton borne by C-4 was no longer split. We now had definitive proof of the biosynthesis of the left-hand portion of sparsomycin from the indole nuclues of tryptophan. The ²H-enrichment of the proton attached to C-5 was determined to be 6% by the procedure described the APPENDIX.

An explanation for the non-zero incorporation (0.0068%) of [1-¹⁴C]-D-ribose could then be proposed in light of the described results of feeding experiments employing labeled forms of tryptophan. C-1 and C-2 of ribose are incorporated into C-2 and C-3, respectively, of tryptophan during its biosynthesis.⁷⁵ (See Scheme 30.) In our feeding experiment involving [1-¹⁴C]-D-ribose, the label was incorporated into sparsomycin via tryptophan.

Two-dimensional NMR spectrometry of the sparsomycin (Figure 20) indicated that C-5, the carbon labeled by (5-²H₁)-DL-tryptohan,
Scheme 29

\[ \text{Pd/CaCO}_3 \]

\[ \text{D}_2 \]
Figure 18
Scheme 30
corresponded to the signal at 120.81 ppm and C-4 corresponded to the signal at 132.47 ppm in the $^{13}$C-NMR spectrum. A more complete assignment of the $^{13}$C-NMR spectrum of sparsomycin could now be made as shown in Table 6.

**Table 6**

<table>
<thead>
<tr>
<th>Chemical Shift (ppm)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.21</td>
<td>C-5'</td>
</tr>
<tr>
<td>16.81</td>
<td>C-1</td>
</tr>
<tr>
<td>46.72</td>
<td>C-2'</td>
</tr>
<tr>
<td>52.90</td>
<td>C-3'</td>
</tr>
<tr>
<td>54.75</td>
<td>C-4'</td>
</tr>
<tr>
<td>63.09</td>
<td>C-1'</td>
</tr>
<tr>
<td>105.91</td>
<td>C-2 or C-3</td>
</tr>
<tr>
<td>120.81</td>
<td>C-5</td>
</tr>
<tr>
<td>132.47</td>
<td>C-4</td>
</tr>
<tr>
<td>151.56</td>
<td>C-8</td>
</tr>
<tr>
<td>156.69</td>
<td>C-2 or C-3</td>
</tr>
<tr>
<td>164.74</td>
<td>C-6 or C-7</td>
</tr>
<tr>
<td>169.27</td>
<td>C-6 or C-7</td>
</tr>
</tbody>
</table>

In an initial experiment in the investigation to elucidate the nature of the intermediates between tryptophan and sparsomycin, [ring-U-$^{14}$C]-N-formyl-anthranilic acid was synthesized and evaluated as a precursor. This material was prepared by formylation of [ring-U-$^{14}$C]-anthranilic acid with formic acid.\(^7\)\(^6\) (See Scheme 31.) This precursor was incorporated into sparsomycin to the extent of 0.96%. Work to prepare this compound labeled with a stable isotope in order to confirm its specific incorporation into sparsomycin is currently underway.
Scheme 31
SUMMARY AND CONCLUSIONS

At the conclusion of the research described in this thesis, the fundamental "building blocks" of sparsomycin had been discovered, and some of the details of the biosynthesis of the antibiotic from these precursors had been elucidated. Table 7 summarizes the major experimental results obtained.

Table 7

<table>
<thead>
<tr>
<th>Precursor</th>
<th>% Incorporation, or % Enrichment</th>
<th>Labeling Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Methyl-(^{13})C)-L-Methionine</td>
<td>2.7 (C-4')</td>
<td>C-4', C-5'</td>
</tr>
<tr>
<td></td>
<td>6.7 (C-5')</td>
<td></td>
</tr>
<tr>
<td>(3-(^{13})C)-DL-Cysteine</td>
<td>1.0 (C-3')</td>
<td>C-3', C-4'</td>
</tr>
<tr>
<td></td>
<td>0.6 (C-4')</td>
<td></td>
</tr>
<tr>
<td>(Methyl-(^{13})C)-S-Methyl-L-Cysteine</td>
<td>0.3</td>
<td>C-4'</td>
</tr>
<tr>
<td>(Methyl-(^{13})C)-S-Methyl-D-Cysteine</td>
<td>0.6</td>
<td>C-4'</td>
</tr>
<tr>
<td>[Methyl-(^{3})H, (^{35})S]-L-Methionine (10.22)</td>
<td>6.14</td>
<td>- - - -</td>
</tr>
<tr>
<td>[5-(^{3})H, U-(^{14})C]-L-Tryptophan (5.45)</td>
<td>7.96</td>
<td>- - - -</td>
</tr>
<tr>
<td>(2-(^{13})C)-DL-Tryptophan</td>
<td>22</td>
<td>C-8</td>
</tr>
<tr>
<td>(5-(^{2})H(_1))-DL-Tryptophan</td>
<td>6</td>
<td>C-5</td>
</tr>
<tr>
<td>[Ring-U-(^{14})C]-N-Formyl-Anthranilic Acid</td>
<td>0.96</td>
<td>- - - -</td>
</tr>
</tbody>
</table>

The methyl group of (methyl-\(^{13}\)C)-L-methionine was incorporated into C-4' and C-5' of sparsomycin. (3-\(^{13}\)C)-DL-Cysteine was shown to label C-3' of sparsomycin. These two results suggested the intermediacy of S-methylcysteine. Both the L- and D-isomers of (methyl-\(^{13}\)C)-S-methyl-cysteine were incorporated. They both labeled
sparsomycin at C-4', and the D-isomer appeared to be a slightly more efficient precursor. Experiments utilizing labeled forms of serine failed to demonstrate specific incorporation of this precursor into sparsomycin. This failure and the incorporation of C-3 of cysteine into C-4' of sparsomycin suggested that unusual C-1 chemistry occurs in *S. sparsogenes*.

Other results acquired in the investigation of the right-hand portion of sparsomycin indicated that the methylthio group of methionine was not incorporated intact into the methylthio group of the antibiotic. In addition, reduction of the carboxyl group of S-methylcysteine to the alcohol group present at C-1' of sparsomycin apparently does not occur until after incorporation and elaboration of this precursor. Experiments designed to provide information concerning the loss of hydrogen atoms from the methyl group of S-methylcysteine during its conversion to sparsomycin yielded no meaningful results.

The left-hand portion of sparsomycin was shown to derive from the indole moiety of tryptophan. Intact incorporation of the indole nucleus was demonstrated by the following two results: 1) (2-13C)-DL-tryptophan labeled sparsomycin at C-8 and 2) (5-2H1)-DL-tryptophan labeled sparsomycin at C-5. An experiment employing (ring-U-14C)-N-formyl-anthranilic acid suggested that the pathway leading from tryptophan to sparsomycin proceeds through N-formyl-anthranilic acid. Future work on this project will focus on confirmation of this result and further elucidation of the nature of the biosynthetic intermediates between tryptophan and sparsomycin.
EXPERIMENTAL

Proton nuclear magnetic resonance spectra were taken either on a Jeol FX-90 spectrometer at 90 MHz or on an IBM AF300 at 300.133 MHz. Chemical shifts are given in parts per million downfield from tetramethylsilane (0.0 ppm) for spectra taken in CDCl₃ and from sodium 3-trimethylsilyl propionate-2,2,3,3-d₄ (0.0 ppm) for spectra taken in deuterium oxide. ¹³C-NMR spectra were taken on the same two instruments at 22.5 MHz and 75.469 MHz, respectively. Methanol (49.0 ppm) was used as an internal reference. Deuterium spectra were taken on the IBM AF300 at 46.072 MHz. Deuterium-depleted water was used both as solvent and as reference. High-resolution mass spectra were obtained on a Finnigan 3300 mass spectrometer.

Analytical thin layer chromatography was done on glass plates precoated with Cellulose F or Merck silica gel type 60, F-254. Cellulose plates were visualized with short wavelength ultraviolet light or by spraying with a 2% solution of ninhydrin in ethanol and heating. Silica gel plates were visualized with short wavelength ultraviolet light or by spraying with a 5% solution of phosphomolybdic acid in ethanol and heating. Gas chromatography was carried out on a Hewlett Packard 5710A machine using a 10% SE-30 glass column (6 ft., 2 mm ID). Column chromatography was performed on Baker silica gel (60-200 mesh or 200-400 mesh), Merck silica gel (grade 60, 60Å), or Avicel microcrystalline cellulose. Solvents were delivered to both silica gel and cellulose columns via a reservoir attached to the top of the column.

Medium pressure liquid chromatography (MPLC) was performed on a column (11 mm ID X 48 cm) of Baker reversed-phase octadecylsilane bonded to silica gel (average particle diameter of 40 μm). Solvents were
delivered with a Rainin Model B-100-S Eldex pump. The column output was monitored with an ISCO Model UA-5 absorbance-fluorescence detector with an Isco Type 6 optical unit, 2 mm flow cells, and a built-in recorder. Fractions were automatically collected with either an ISCO Model 273 or an ISCO Cygnet fraction collector.

High performance liquid chromatography (HPLC) was carried out on an Altex Ultrasphere octadecylsilane reversed-phase column (4.6 mm ID X 15 cm) preceded by a Micro-Guard ODS-5S pre-column with a Spectrophysics SP Model 8700 solvent delivery system or an Altex Model 110A pump. The column output was monitored with either an Isco Model V4 variable wavelength absorbance monitor with a built-in recorder, the Isco Model UA-5 absorbance-fluorescence monitor described above, or an Hitachi Model 100-40 variable wavelength spectrophotometer with a Varian Model 9176 recorder.

Melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Measurement of pH was done with a Corning Model 12 research pH meter with a combination electrode. Small-scale centrifugation was done in a Dynac centrifuge. Spore suspensions were prepared on a Vortex-Genie mixer. Slants were grown in a Scientific Products Model B7001-3 water bath. Shake cultures were grown in a New Brunswick Model G-25 or Model G-25R incubator-shaker.

Radiochemicals were purchased from Amersham Corporation, Dupont NEN Research Products, or Sigma Chemical Co. Samples for liquid scintillation counting were weighed on a Perkin-Elmer AD-2 autobalance and were counted with a Beckman Model LS 3801 or a Beckman Model LS 100C liquid scintillation counter in Aquasol 2 or toluene scintillation fluid. The scintillation fluids were purchased from Dupont NEN. TLC plates were scanned with a Berthold LB 22832 Automatic TLC Linear
Analyzer which was interfaced with an Apple Ile microcomputer.

Compounds labeled with stable isotopes were purchased from MSD Isotopes, Cambridge Isotope Laboratories, Aldrich Chemical Co., or Stohler/KOR Stable Isotopes.

Ethyl ether was freed from peroxides and water by passage through a column of neutral activated alumina followed by distillation from sodium. Benzene was purified by distillation from sodium. Methanoli was distilled neat or, in cases where absolute dryness was necessary, from magnesium turnings. Ethyl acetate and hexane were distilled neat. Nitrogen was dried by passage through a tower containing concentrated H₂SO₄. Ammonia was purified by collection over sodium and subsequent evaporation into the reaction vessel, where it was recondensed. All other solvents and reagents were either used as purchased or were purified as described below.

**Growth of Streptomyces sparsogenes on Slants**

*Streptomyces sparsogenes* (ATCC #25498) was grown on slants of Bennett's agar: 1.0 g Difco yeast extract, 1.0 g Difco beef extract, 2.0 g N-Z-amine Type A (an enzymatic digest of casein from Sheffield Products), and 10.0 g glucose were dissolved in one liter of glass-distilled water. The pH of the solution was adjusted to 7.0 with 1 N NaOH. Eight mL of this solution (hereafter referred to as "broth") were pipetted into each of four 150 mm X 20 mm screw-cap culture tubes. Fifteen grams of Difco agar were added to the remainder of the broth which was then heated and stirred magnetically until the agar dissolved. The hot agar solution was pipetted into culture tubes in 8-10 mL aliquots. The tubes were autoclaved at 121°C for 20 minutes. The tubes containing
agar solution were then allowed to cool at an angle so that the agar remained 4-5 cm below the neck of each tube. The tubes containing broth and the agar slants were stored at room temperature and used within a few months of preparation.

The tubes of broth were inoculated from lyophiles of *Streptomyces sparsogenes* by dissolving the lyophilized material in a small volume of broth and pipetting the solution back into the tube from which the broth was taken. All procedures involving slants and inoculation were performed using sterile technique. The inoculated broth was grown for three days in a water bath at 30°C. with the screw cap loosened one-half turn to allow for exchange of gases. The cloudy growth was then suspended by agitation on a Vortex mixer, and 0.5 mL of it was spread evenly over the surface of each of several Bennett's agar slants. The slants were then grown in a 30°C. water bath with the screw caps loosened one-half turn. White growth appeared after one day. After two to three days the growth became yellow and then gradually turned purple by the tenth to twelfth day. Black growth was observed on slants older than twelve days. Slants were used when between ten and twelve days old. Cultures grown from older slants produced little or no antibiotic, even when the slants had been kept refrigerated.

**Long-Term Maintenance of *Streptomyces sparsogenes***

*Streptomyces sparsogenes* was best maintained as lyophiles. The lyophilizing solution was prepared in the following manner: 10.0 g glucose and 0.43 g Difco dehydrated nutrient broth were dissolved in 33 mL water. Ten mL of Difco desiccated horse serum were dissolved in 10 mL water. The horse serum solution and 3.3 mL of the nutrient broth
solution were added through sterile Millipore filters (0.20 μm pore size) to the same pre-sterilized flask. The resulting solution was known as "mist. desiccans."

Three mL "mist. desiccans" were added to a slant of *Streptomyces sparsogenes*. The spores were scraped off the surface of the agar with a sterile wire loop and suspended in the liquid by agitation on a Vortex mixer. Three-tenths mL of spore suspension was added to each of six sterile lyophilization ampoules. These were attached to a lyophilizer via a special sterilized adapter, frozen, and lyophilized for 24 hours. The ampoules were then sealed with an oxygen-natural gas torch while still under vacuum and could be stored indefinitely in the refrigerator.

**Preparation and Inoculation of Seed Medium**

Fermentations of *Streptomyces sparsogenes* were conducted according to the procedure of Owen, Dietz, and Camiener.\(^1\)

The medium in which an inoculum of the organism was grown contained 4.55 g glucose (equivalent to 5.0 g glucose monohydrate) and 5.0 g coarsely-ground cottonseed meal (Southern Cotton Oil Co.) in 200 mL tap water. The pH of the mixture was adjusted to 7.2 with 1 N NaOH. The medium was distributed equally between two 500 mL Erlenmeyer flasks which were stoppered with foam plugs and autoclaved at 121°C. for 30 minutes. The flasks were allowed to cool before inoculation.

Ten mL sterile deionized water were added to a ten- to twelve-day-old slant of *Streptomyces sparsogenes*. A spore suspension was prepared by scraping the spores off the surface of the slant with a sterile wire loop and agitating the slant on a Vortex mixer. Five mL of spore
suspension were added to each of the two flasks containing seed medium. The flasks were then incubated at 32°C. and 250 rpm for three days.

Preparation and Inoculation of Fermentation Medium

The fermentation medium contained 18.18 g glucose (equivalent to 20 g glucose monohydrate), 15.0 g dextrin (A. E. Staley), 15.0 g cottonseed meal, 15.0 g distiller's solubles (Brown-Forman, crude protein 27%, crude fat 8%, crude fiber 4%), and 8.0 g lard oil (#2, George Piau's Sons, Inc.) in 2 liters of tap water. The pH of the mixture was adjusted to 7.2 with 1 N NaOH. The mixture was divided equally (200 mL portions) between ten 1000 mL Erlenmeyer flasks which were stoppered with foam plugs and autoclaved at 121°C. for 30 minutes. The flasks were allowed to cool before the introduction of inoculum.

The flasks containing three-day-old inoculum were swirled vigorously by hand to shake down the growth on their sides. Ten mL of inoculum were added to each fermentation flask via a sterile wide-mouthed pipet. The flasks were incubated at 32°C. and 250 rpm for the duration of the fermentation.

HPLC Monitoring and Administration of Precursors

High performance liquid chromatography was a convenient way to monitor the production of sparsomycin by the organism. Samples of fermentation broth were withdrawn using sterile technique from two or more flasks, combined, and filtered. Samples (20 µL) of the filtrate were injected onto the HPLC column described previously.
Two different HPLC solvent systems were used to examine the fermentation broth. The first of these was developed in this laboratory and was prepared by mixing four parts 0.01 M aqueous KH$_2$PO$_4$ (adjusted to pH 5.5 with 1 N NaOH) with one part 4:1 methanol:water. A flow rate of 1.0 mL per minute was used. The retention time of sparsomycin in this system was approximately nine minutes.

The second HPLC system$^{47}$ was used more often due to its greater ease of solvent preparation. The solvent was an 85:15 mixture of water and methanol, and a flow rate of 1.0 mL per minute was used. Sparsomycin had a retention time of approximately ten minutes in this system.

These same two systems were also used to follow the isolation of sparsomycin, especially for checking column fractions.

The HPLC column was monitored at 254, 302 (the UV$_{max}$ of sparsomycin$^3$), or 310 nm, depending on the purpose of the injection and the monitor used.

Due to the extremely variable timing of production of sparsomycin by *Streptomyces sparsogenes* and its frequent failure to produce antibiotic at all, the feeding of precursors was always correlated with sparsomycin production as determined qualitatively by HPLC. Each precursor was "pulse-fed" over a period of two to four days, with the first feeding occurring just after the beginning of antibiotic production. Sparsomycin first appeared three to six days after the inoculation of the fermentation medium. The remainder of the precursor was administered in one or two more "pulses" over the next one to three days, depending on the rate of sparsomycin production. The fermentation was then continued until the sparsomycin production leveled off, generally nine to eleven
days after inoculation.

For administration to the fermentation, each precursor was dissolved in 10 mL water (or more if necessary to dissolve the compound). One-half or one-third of the solution (depending on the number of "pulses") was taken up in a syringe and sterilized by passage through a sterile Millipore filter (0.20 μm pore size) during addition of equal aliquots to each fermentation flask. The filter was rinsed with water which was also added to one of the flasks.

Isolation of Sparsomycin

Sparsomycin was isolated by a modification of the procedure of Argoudelis and Herr. The contents of all the fermentation flasks were combined. Each flask was rinsed with approximately 200 mL water, and these rinses were added to the fermentation broth. The pH of the mixture was adjusted to 3.0 with concentrated H₂SO₄. Some additional material precipitated out of solution during the acidification. The resultant mixture was then filtered with the aid of acid-washed Celite. The filter cake was washed with 150 mL water, and the pH of the combined filtrate and wash was adjusted to 8.0 with 1 N NaOH. The solution darkened considerably during this adjustment. The solution was stirred mechanically for 1 hour with 30.0 g acid-washed Celite and 20.0 g Darco G-60 charcoal. The mixture was filtered, and the carbon cake was washed with 135 mL water and then with 100 mL 20:80 acetone:water (adjusted to pH 8.0 with dilute aqueous NaOH). The carbon cake was then stirred frequently by hand for 1 hour (mechanical stirring caused evaporation of the acetone) with 800 mL 50:50 acetone:water (adjusted to pH 2.5 with 1 N HCl). The mixture was filtered, and the bright yellow filtrate was
adjusted to pH 6.2 with 1 N NaOH. The acetone was evaporated under reduced pressure, and the remaining aqueous solution was then lyophilized.

The brown solid obtained was slurried with 15.0 mL methanol at 40°C. for 1 hour. The mixture was filtered, and the insoluble material was washed with a little warm methanol. The combined filtrate and wash were evaporated under reduced pressure to an orange solid.

McIlvaine's buffer\textsuperscript{78} (pH 6.0) was then prepared by mixing 0.2 M aqueous Na\textsubscript{2}HPO\textsubscript{4} solution and 0.1 M aqueous citric acid solution in a 12.6/7.4 ratio of volumes. This solution was shaken with an equal volume of methyl ethyl ketone, and the mixture was separated into two layers-"upper phase" and "lower phase."

The orange solid was dissolved in 10 mL of "lower phase." This solution was extracted with eight 100 mL portions of "upper phase." The combined organic layers were evaporated under reduced pressure to a small amount of yellow oil.

The oil was then chromatographed on the MPLC column described previously using the aqueous KH\textsubscript{2}PO\textsubscript{4}-methanol solvent described in the section on HPLC. The column output was monitored at 310 or 254 nm, and 3-mL fractions were collected at a flow rate of 1.5 mL per minute. Sparsomycin was typically found in fractions 25-34. These fractions were combined and concentrated \textit{in vacuo} to a small volume of yellow oil.

The oil was rechromatographed on the same MPLC column with 85:15 water:methanol as solvent. This procedure "desalted" the sparsomycin as well as effecting further purification. The column was monitored and fractions were collected as described above. Sparsomycin was typically found in fractions 29-43. These fractions were combined
and concentrated *in vacuo* to an aqueous solution which was then lyophilized to an off-white solid.

The solid was dissolved in a small amount of methanol, and the solution was transferred to a tared Craig tube. The methanol solution was concentrated to approximately 0.3 mL by evaporation in a stream of nitrogen. Dry ether was then added to precipitate the sparsomycin. The solid was centrifuged down, the liquid was decanted, and the solid was dried *in vacuo* over P$_2$O$_5$ at room temperature for 1 hour. Four to twelve mg of sparsomycin were obtained. At this point, the material was usually sufficiently pure for $^{13}$C- or $^2$H-NMR spectrometry. When a radioactive precursor had been fed, the ether precipitation and sometimes both MPLC passes were repeated until the sparsomycin reached constant specific activity and/or isotope ratio.

In some cases, the sparsomycin still contained an unknown impurity which could be detected only by NMR spectrometry. This impurity was removed by chromatographing the sparsomycin on a column of 5 g microcrystalline cellulose (11 mm X 13 cm) with 6:1 n-butanol:water as solvent. Three-mL fractions were collected and checked by TLC on cellulose plates developed with the same solvent and visualized with short wavelength ultraviolet light. Sparsomycin had a $R_f$ of 0.32 in this system and was typically found in fractions 5 and 6. The fractions containing sparsomycin were combined, concentrated *in vacuo*, and finally lyophilized to obtain pure sparsomycin.
Synthesis of (3-$^{13}$C)-DL-Cysteine

A. (3-$^{13}$C)-S-Benzyl-DL-Cysteine Hydrochloride (44)$^{50}$

Diethyl acetamidomalonate (5.73 g, 26.4 mmol) and 2.85 mL (28.8 mmol) freshly-distilled piperidine were mixed together at 0°C. The mixture was removed from the cold bath and allowed to stir until it reached room temperature. After ten additional minutes of stirring, 200 mg (6.45 mmol) (13C)-formaldehyde (20% in aqueous solution) were added, and stirring was continued for 30 minutes. The reaction mixture was extracted four times with 15 mL ether. The combined organic layers were evaporated under reduced pressure to 3.82 g white solid.

This material was stirred for 72 hours in the dark with 80 mL (1.28 mol) freshly-distilled methyl iodide. After removal of the solvent in vacuo, 5.28 g crude yellow methiodide remained.

Four hundred mg sodium were dissolved in 100 mL absolute ethanol, and 4.2 mL freshly-distilled benzyl mercaptan were added to the solution. Forty mL of the resulting solution were added to the crude methiodide, and the mixture was refluxed under nitrogen for four hours, at which time another 20 mL of the sodium benzyl mercaptide solution were added (a total of 21.5 mmol sodium benzyl mercaptide was employed). Refluxing was continued for another hour.

The reaction mixture was allowed to cool, and it was then concentrated in vacuo to a brown oil. Fifteen mL water were added, and the solution was extracted five times with 15 mL benzene. The combined organic layers were evaporated under reduced pressure to give 1.12 g of an off-white semi-solid which was refluxed for 7 hours with 30 mL concentrated HCl. The solution was allowed to cool and was then washed
with ether. The aqueous solution was evaporated in vacuo to 1.91 g of an off-white solid.

This material was purified by chromatography on a column of 19 g microcrystalline cellulose (15 mm × 45 cm) with 25:4:10 n-butanol:acetic acid:water (BAW) as solvent. Four-mL fractions were collected and checked for the presence of product by TLC on cellulose plates developed with the same solvent as the column. The product was found in fractions 19-28. The combined fractions were evaporated in vacuo, and the white solid obtained was recrystallized several times from 25:4:10 BAW. A total of 595 mg (2.39 mmol, 36.9% yield based on $^{13}$C-formaldehyde) of ($^{3-13}$C)-S-benzyl-DL-cysteine hydrochloride was obtained. The identity of this compound and the location of the label were confirmed by comparison to the authentic unlabeled material by NMR and TLC (red spot of $R_f$ 0.55 on cellulose developed with 25:4:10 BAW).

B. ($^{3-13}$C)-DL-Cysteine (45)$_b^{50}$

($^{3-13}$C)-S-benzyl-DL-cysteine (595 mg, 2.39 mmol) was placed in a 3-necked flask fitted with a dry ice condenser which was protected from moisture by a KOH tube. Gaseous ammonia was introduced until 50 mL liquid ammonia had collected in the flask. Small pieces of sodium were added until the solution remained blue for twenty minutes, after which time the color faded. The ammonia was allowed to evaporate. The off-white residue remaining was dissolved in water, and the solution was adjusted to pH 7.0 with dilute HCl. The solvent was removed in vacuo, and the white solid remaining was recrystallized several times from water to yield 231 mg (1.89 mmol, 79.2% yield) ($^{3-13}$C)-DL-cysteine. The
structure and purity of the compound were analyzed by comparison of its NMR spectra and TLC behavior (yellow spot of $R_f$ 0.08 on cellulose developed with 25:4:10 BAW) with authentic unlabeled material. The $^{13}$C-NMR spectrum confirmed the location of the label at C-3.

**Synthesis of (3-$^{13}$C)-DL-Serine (49)**

Diethyl acetamidomalonate (1.48 g, 6.80 mmol) was suspended in a solution of 200 mg (6.45 mmol) ($^{13}$C)-formaldehyde in 1.0 mL water. The clear solution which formed after the addition of 95 $\mu$L (0.095 mmol) 1 N NaOH was stirred for 5.25 hours. A 1.4 N aqueous solution of NaOH (10.2 mL, 14.3 mmol) was then added, and stirring was continued for 46 hours. Glacial acetic acid (2.8 mL, 49 mmol) was added, and the solution was refluxed for one hour. The solvent was removed *in vacuo*, and 15 mL concentrated HCl were added. The mixture was refluxed for one hour, and it was then concentrated to a yellow oil under reduced pressure.

The oil was refluxed for 15 minutes with 15 mL absolute ethanol. The white solid which precipitated was collected by filtration and refluxed for 15 minutes with 15 mL absolute ethanol. The mixture was filtered, and the solid collected was subjected to ethanol extraction once more. The combined ethanol extracts were concentrated *in vacuo* to a yellow oil. Concentrated HCl (3.4 mL) was added, and the mixture was refluxed for one hour. The solution was then refluxed for 30 minutes with 25 mg acid-washed charcoal. The charcoal was removed by filtration and washed three times with 3-mL portions of hot water. The combined filtrate and washings were refluxed for 30 minutes with a fresh 25 mg of acid-washed charcoal. The charcoal was washed with three 3-mL portions of hot water, and the combined filtrate and washings were
concentrated in vacuo.

The concentrated solution was then loaded on a column of 23 g Amberlite IRA-45 weakly basic ion exchange resin (free base form, 16-50 mesh, 15 mm X 18 cm). The column was eluted with water, and the eluate was checked for the presence of serine with ninhydrin. All the serine was contained in the first 200 mL of water. The aqueous solution was lyophilized, and the white solid remaining was recrystallized from 2:5 water:ethanol. White crystals (427 mg, 4.03 mmol, 62.5% yield) of (3-\textsuperscript{13}C)-DL-serine were obtained. The \textsuperscript{1}H-NMR spectrum of the product was identical to that of authentic serine, and it also exhibited identical TLC behavior (orange spot of R\(_f\) 0.18 on cellulose developed with 25:4:10 BAW). The \textsuperscript{13}C-NMR spectrum confirmed the location of the label.

\textbf{Synthesis of (Methyl-\textsuperscript{13}C)-S-Methyl-L-Cysteine (50)\textsuperscript{52}}

L-Cystine (731 mg, 3.04 mmol) was placed in a 3-necked flask fitted with a dry ice condenser which was protected from moisture by a KOH tube. Gaseous ammonia was passed through the system until 10 mL liquid ammonia had collected in the flask. Small pieces of sodium were added until the solution remained blue for several minutes. (\textsuperscript{13}C)-Methyl iodide (1.0 g, 7.00 mmol, 1.15 equivalents) was slowly added, and stirring was continued for fifteen minutes. The ammonia was allowed to evaporate. The white residue was dissolved in water, the resulting solution was filtered, and the filtrate was made slightly acidic with 50% hydroiodic acid. The solution was warmed and diluted to three times its volume with warm ethanol. The crystals which gradually formed on cooling were collected by filtration and recrystallized from 2:1 ethanol:water. Two crops of crystals were recovered. A small amount of
starting material was removed from the first crop by chromatography on a microcrystalline cellulose column with 25:4:10 BAW as solvent. A total of 197 mg (1.44 mmol, 23.7% yield) \((\text{methyl}^{13}\text{C})-\text{S}-\text{methyl-L-cysteine}\) was obtained.

The product was identical to authentic S-methyl-L-cysteine by TLC (dark red spot of \(R_f\) 0.33 on cellulose developed with 25:4:10 BAW), and the \(^1\text{H}-\text{NMR}\) spectrum was the same as that of the authentic material. The \(^{13}\text{C}-\text{NMR}\) spectrum confirmed the location of the label in the methyl group.

**Synthesis of \((\text{Methyl}^{13}\text{C})-\text{S-Methyl-D-Cysteine}\)\(^{52}\)**

This compound was synthesized from D-cystine and \(^{13}\text{C}\)-methyl iodide in 57.4% yield by the same procedure used to prepare \((\text{methyl}^{13}\text{C})-\text{S}-\text{methyl-L-cysteine}\) and was characterized in the same way. Optical activity measurements showed that the D-cystine used contained a 92.9% enantiomeric excess of the D-isomer.

**Synthesis of \((\text{Methyl}^{13}\text{C}, \text{Methyl}^2\text{H}_3)-\text{S-Methyl-D-Cysteine}\)\(^{52}\)**

This compound was synthesized from D-cystine (92.9% enantiomeric excess) and \(^{13}\text{C}, ^2\text{H}_3\)-methyl iodide in 42.1% yield by the same procedure used to prepare \((\text{methyl}^{13}\text{C})-\text{S}-\text{methyl-L-cysteine}\). The structure and purity of the compound were established in the same manner.
Synthesis of (Methyl-\(^{13}\)C)-S-Methyl-D-Cysteinel

A. (Methyl-\(^{13}\)C)-S-Methyl-D-Cysteine Methyl Ester Hydrochloride (51)

(Methyl-\(^{13}\)C)-S-methyl-D-cysteine (606 mg, 4.45 mmol) was suspended in 8.0 mL dry methanol. Gaseous HCl was passed through the mixture for twenty minutes, and the mixture was then stirred overnight at room temperature. TLC showed that no starting material remained. The solvent was removed \textit{in vacuo}, and the product was washed three times with dry ether. The product (696 mg, 3.73 mmol, 83.8% yield) had a melting point of 136\(^{\circ}\)C.-139\(^{\circ}\)C. and was characterized by NMR spectrometry.

\(^1\)H NMR (\(\text{D}_2\text{O}, 90\) MHz): 1.38 (3H, s), 3.17 (2H, d, J=4.5 Hz), 3.88 (3H, s), 4.35 (1H, t, J=6.3 Hz), 5.50 (1H br. s)

\(^{13}\)C NMR (\(\text{D}_2\text{O}, 22.5\) MHz): 14.80 (enriched), 30.40, 52.04, 53.93

B. (Methyl-\(^{13}\)C)-S-Methyl-D-Cysteinol (52)\(^{53}\)

(Methyl-\(^{13}\)C)-S-methyl-D-cysteine methyl ester hydrochloride (696 mg, 3.73 mmol) was dissolved in 9.8 mL 50:50 ethanol:water. This solution was added dropwise to a solution of 1.00 g (26.5 mmol) NaBH\(_4\) in 9.8 mL 50:50 ethanol:water at 0\(^{\circ}\)C. The reaction mixture was stirred at 0\(^{\circ}\)C. for four hours and then at room temperature overnight. A gray solid was removed by filtration. The filtrate was concentrated \textit{in vacuo} to an aqueous solution which was extracted four times with an equal volume of chloroform. The combined organic layers were dried over anhydrous Na\(_2\)SO\(_4\). The drying agent was removed by filtration, and the filtrate was evaporated \textit{in vacuo} to 253 mg (2.07 mmol, 55.5% yield) of a colorless oil.
The product was characterized by NMR spectrometry.

$^1$H NMR (D$_2$O, 90 MHz): 1.38 (3H, s), 3.17 (2H, d, J=4.5 Hz), 3.88 (3H, s), 4.35 (1H, t, J=6.3 Hz), 5.50 (1H, br. s)

$^{13}$C NMR (D$_2$O, 22.5 MHz): 14.80 (enriched), 30.40, 52.04, 53.93

Synthesis of
N-(Benzylloxycarbonyl)-S-Oxo-S-[(Methylthio)methyl]-L-Cysteinol

A. Cystine Dimethylester Dihydrochloride (59)$^{55}$

A suspension of 4.99 g (20.78 mmol) L-cystine in 125 mL dry methanol was saturated with dry gaseous HCl and stirred at room temperature overnight. The solution was chilled to 0°C., and 250 mL dry ether were added. The white solid which separated was collected by filtration. The product was dissolved in 55 mL warm dry methanol, and the solution was decolorized with charcoal which was subsequently removed by filtration. The filtrate was concentrated in vacuo to approximately 25 mL, and the product was precipitated by the addition of three volumes of dry ether. Shiny white crystals (6.30 g, 18.45 mmol, 88.8% yield) which had a melting point of 161°C. (lit. 170°C.) were obtained.

$^1$H NMR (D$_2$O, 90 MHz): 3.47 (4H, d, J=5.4 Hz), 3.91 (6H, s), 4.62 (2H, t, J=5.4 Hz)
B. N,N'-bis(Benzyloxy carbonyl)-L-Cystine Methyl Ester (60)$^{56}$

Benzyl chloroformate (2.35 g, 13.77 mmol) was added dropwise over the course of 50 minutes to a stirred mixture of 2.17 g (6.35 mmol) L-cystine dimethylester dihydrochloride, 2.13 g NaHCO$_3$, 24 mL water, and 11 mL ether. The resulting mixture was stirred at room temperature for 2 hours. The layers were separated, and the organic layer was taken up in 16 mL ethyl acetate. The aqueous layer was washed twice with 13 mL ethyl acetate. The combined organic layers were washed twice with 50 mL saturated aqueous NaCl solution and dried over anhydrous MgSO$_4$. The drying agent was removed by filtration, and the filtrate was evaporated in vacuo to a very viscous, translucent liquid. Ten mL ether were added, and the solution was refrigerated overnight. The crystals which formed were recrystallized from 10:1 ether:methanol. The product crystallized as clumps of white needles which were washed with dry ether and pentane. A yield of 1.26 g (2.35 mmol, 37.0% yield) product (m. p. 59°C.-60°C., lit.$^{79}$ 57°C.-61°C.) was obtained.

$^1$H NMR (CDCl$_3$, 90 MHz): 3.17 (4H, d, J=5.4 Hz), 3.74 (6H, s), 4.68 (2H, t, J=5.4 Hz), 5.11 (4H, s), 5.65 (2H br. d, J=8.1 Hz), 7.34 (10H, s)

C. N,N'-bis(Benzyloxy carbonyl)-L-Cystinol (61)$^{57}$

A solution of 0.51 g (13.52 mmol) NaBH$_4$ and 1.79 g (13.40 mmol) LiI in 45 mL dry 1,2-dimethoxyethane (freshly-distilled from LiAlH$_4$) was chilled to -78°C. N,N'-Bis(benzyloxy carbonyl)-L-cystine methyl ester (1.13 g, 2.11 mmol) was added in one portion, and the reaction mixture
was stirred at room temperature until TLC (6:94 methanol:methylene chloride on silica gel, starting material's R_f=0.74) showed reaction was complete. Three hours were required. The pH was adjusted to 7.0 with concentrated HCl, and stirring was continued for another hour.

The mixture was concentrated under reduced pressure to one-half its volume. A solution of methanol 0.1 M in iodine and 0.2 M in pyridine was added to the milky white suspension until the color of iodine persisted. A few crystals of Na_2S_2O_5 were added to destroy the excess iodine. The solvent was removed in vacuo, and 20 mL water and 20 mL methylene chloride were added to the residue. The layers were separated, and the aqueous layer was extracted three times with 15 mL methylene chloride and twice with 15 mL ethyl acetate. The combined organic layers were dried over anhydrous Na_2SO_4. The drying agent was removed by filtration, the filtrate was evaporated in vacuo, and the residue was recrystallized from ethyl acetate. A yield of 0.89 g (1.85 mmol, 87.7% yield) product (m.p. 77°C.-79°C.) was obtained. This material exhibited the reported TLC behavior (R_f 0.17 on silica gel developed with 6:94 methanol:methylene chloride), and its ¹H-NMR spectrum matched the published data.

D. 4-[[Benzylxocarbonyl]amino]-1,2-Oxathiolane-2-Oxide (62)\(^{57}\)

N,N'-Bis(benzylxocarbonyl)-L-cystinol (0.15 g, 0.31 mmol) was dissolved in 3.3 mL glacial acetic acid. To this solution was added dropwise a solution of 0.13 g (0.94 mmol) N-chlorosuccinimide in 4.7 mL glacial acetic acid. The resulting solution was stirred overnight at room temperature. TLC (6:94 methanol:methylene chloride on silica gel,
starting material's \( R_f = 0.17 \) showed reaction was complete. Much of the acetic acid was removed \textit{in vacuo}, and 12.5 mL methylene chloride and 0.47 mL water were added to the residue. The layers were separated, and the organic layer was dried over anhydrous MgSO\(_4\). The drying agent was filtered out, and the filtrate was evaporated \textit{in vacuo}.

The residue was chromatographed on a column of 5 g silica gel (11 mm X 25 cm) with 0.5:99.5 methanol:methylene chloride as solvent. Five-mL fractions were collected and checked by TLC (6:94 methanol:methylene chloride on silica gel). Fractions 7-10 gave spots of \( R_f 0.63 \); fractions 15-35 gave spots of \( R_f 0.30 \). These groups of fractions were combined separately and evaporated \textit{in vacuo}. The residues were recrystallized from 2:1 hexane:ethyl acetate. Fractions 7-10 yielded 37.8 mg white crystals (m. p. 81°C.-82°C., lit. 87°C.) of the \( R_S \) diastereomer (R configuration about the sulfur atom). Fractions 15-35 yielded 39.7 mg white crystals (m. p. 60.5°C.-62.5°C.) of the \( S_S \) diastereomer (S configuration about the sulfur atom). The total yield was 97.0%. The two diastereomers were distinguished by their NMR spectra and TLC behavior.

E. \textit{N-(Benzyloxy carbonyl)-S-Oxo-S-[Methylthio)methyl]-L-Cysteinol} (63)

A flask fitted with a septum and containing 2.50 mL (6.0 mmol) 2.40 M \( n \)-butyllithium in hexane solution was cooled to 0°C. \( N,N,N',N' \)-Tetramethylethylenediamine (0.91 mL, 6.0 mmol), 0.44 mL (6.0 mmol) freshly-distilled dimethyl sulfide, and 2.0 mL dry THF (freshly-distilled from sodium and benzophenone) were added to the cold solution. The resulting solution was stirred at room temperature for 4
hours. It was then cooled to -30°C. and added dropwise to a -78°C.
solution of 0.49 g (1.94 mmol) 4-[(benzyloxy carbonyl)amino]-1,2-
oxathiolane-2-oxide in 6.0 mL dry THF. The mixture was stirred at -78°C.
for 30 minutes and then at room temperature for 30 minutes. It was then
cooled to 0°C., and 5 mL saturated aqueous KHSO₄ solution were added.
The pH of the mixture was adjusted to 8.0 by the addition of solid Na₂CO₃.

After the addition of 10 mL ethyl acetate, the layers were
separated, and the aqueous layer was extracted four times with 5 mL
ethyl acetate. The combined organic layers were dried over anhydrous
Na₂SO₄. The drying agent was removed by filtration, and the filtrate was
evaporated under reduced pressure.

The residue was chromatographed on a column of 23 g silica gel
(15 mm X 60 cm) with 5:95 methanol:methylene chloride as solvent.
Two-mL fractions were collected and checked by TLC on silica gel
developed with 1:9 methanol:methylene chloride. Fractions 55-67 gave
spots of Rf 0.34. These fractions were combined, and the solvent was
removed in vacuo. The residue was recrystallized from methylene
chloride-hexane. White crystals (0.25 g, 0.77 mmol, 40.0% yield) of
product were obtained. The¹H-NMR spectrum of the product showed it to
be a mixture of two diastereomers (R and S configuration about the
sulfoxide sulfur atom). They were not separated because of their
virtually identical TLC behaviors.
Preparation of Tripotassium Ennachloroditungstate and
Reduction of Model Sulfoxides

A. Preparation of Tripotassium Ennachloroditungstate

Potassium carbonate (6.0 g, 43.4 mmol) was dissolved in 6.0 mL of water, and 5.6 g (22.4 mmol) H₂WO₄ were added. Stirring was continued until the color of the suspension changed from yellow to white (approximately one hour). The K₂CO₃·H₂WO₄ mixture was added dropwise to 120 mL concentrated aqueous HCl over the course of 1.25 hours. Gaseous HCl was continuously passed through the aqueous HCl during this time. Four 5 mL portions of concentrated HCl were also added during the last 30 minutes.

The mixture was filtered to remove a small amount of white solid. The blue-green filtrate was placed in a 40°C water bath, and 19 g (160 mmol) metallic tin (20 mesh) were added over the course of 1.5 hours. The color of the mixture gradually changed from blue-green to blue to purple to red-brown to green. The mixture was cooled to -78°C., and 300 mL absolute ethanol were added. After 30 minutes, the mixture was filtered, and the product was washed with absolute ethanol and dry ether. A green product (7.31 g, 40.6% yield) was obtained. This material was further purified immediately before use by dissolution in 40 mL of 0.5 M HCl. After filtration of the solution, the filtrate was chilled to -10°C., and gaseous HCl was passed through the solution for one hour. The product was collected by filtration and dried in vacuo. The reducing activity of the product was determined as described below.
B. Reduction of Dimethyl Sulfoxide with \( \text{K}_3\text{W}_2\text{Cl}_9 \)^{80}

The activity of \( \text{K}_3\text{W}_2\text{Cl}_9 \) was tested by using it to reduce dimethyl sulfoxide to dimethyl sulfide. The reaction was monitored by gas chromatography using the previously-described column run isothermically at 100°C. Dimethyl sulfoxide had a retention time of 1.6 minutes; the product, dimethyl sulfide, had a retention time of 0.5 minutes.

Dimethyl sulfoxide (0.09 mL, 1.27 mmol) was added to a stirred mixture of 0.71 g (0.88 mmol) \( \text{K}_3\text{W}_2\text{Cl}_9 \), 5.0 mL water, and 0.5 mL methanol at 60°C. in a nitrogen atmosphere, and the resulting mixture was stirred under nitrogen at 60°C. for three hours. The color of the reaction mixture changed from green to very dark blue. Water (7.5 mL) was added, and the mixture was extracted three times with 5-mL portions of chloroform. The organic layers were combined, and an aliquot was injected onto the GC column. The desired product (and no other) was present, and no starting material was detected.

C. Reduction of Methyl Methylthiomethyl Sulfoxide by \( \text{K}_3\text{W}_2\text{Cl}_9 \)^{80}

Tripotassium enachloroditungstate was further tested by its reduction of methyl methylthiomethyl sulfoxide. The reaction was monitored using the same GC column as described above run isothermically at 150°C. The starting material had a retention time of 2.2 minutes; the product had a retention time of 0.7 minutes.

Methyl methylthiomethyl sulfoxide (0.13 mL, 1.25 mmol) was added to a stirred mixture of 1.40 g (1.74 mmol) \( \text{K}_3\text{W}_2\text{Cl}_9 \), 10.0 mL water, and 1.0 mL methanol at 60°C. under nitrogen. The reaction mixture was
stirred at 60°C under nitrogen for three hours, during which time the color of the reaction mixture changed from green to brown. Fifteen mL water were added, and the mixture was extracted three times with 10 mL chloroform. The organic layers were combined, and an aliquot was injected onto the GC column. A peak due to the desired product, \textit{bis}(methylthio)methane, was observed, and no starting material remained.

\textbf{D. Treatment of N-(Benzyloxy carbonyl)-S-Oxo-S-[(Methylthio)methyl]-L-Cysteinol with K}_3\textit{W}_2\textit{Cl}_9

Reduction of this model compound using K\textsubscript{3}W\textsubscript{2}Cl\textsubscript{9} was attempted. Tripotassium enchloroditungstate (37.1 mg, 0.046 mmol) was suspended in a solution of 0.26 mL water and 0.03 mL methanol under nitrogen. The mixture was heated to 60°C and 10.2 mg (0.032 mmol) N-(benzyloxy-carbonyl-S-oxo-S-[(methylthio)methyl]-L-cysteinol were added. The mixture was stirred at 60°C under nitrogen for three hours and gradually turned from green to brown-black.

Water was added to the reaction mixture, and the mixture was extracted six times with an equal volume of chloroform. TLC (silica gel developed with 1:9 methanol:methylene chloride) of the combined organic layers showed several spots. The aqueous layer was concentrated \textit{in vacuo} and extracted four more times with chloroform. All the organic layers were combined and evaporated \textit{in vacuo}. The residue gave several spots in various TLC systems.

Preparative TLC of the residue was done on silica gel with 5:95 ethyl acetate:hexane as solvent. Five bands appeared under short wavelength ultraviolet light. Each band was scraped off the plate separately, and the silica gel was extracted with chloroform. The
chloroform was evaporated under reduced pressure, and the residues were
dried in vacuo. Only the residues from the two bands with the highest
$R_f$'s had significant weights. Neither residue was the desired product as
shown by $^1$H-NMR spectrometry.

This reaction was repeated with varying reaction times without
success.

Other Attempts at Reduction or Degradation of Model Compounds

A. Treatment of Methyl Methylthiomethyl Sulfoxide with
   Tris(dimethylaminophosphine)/Iodine Reagent

Dry tris(dimethylaminophosphine) (0.95 mL, 5.0 mmol, 
freshly-distilled from CaH$_2$) was added over the course of forty minutes
to a stirred solution of 1.27 g (5.0 mmol) iodine in 5.0 mL acetonitrile.
The mixture was chilled in an ice bath, and a solution of 0.52 mL (5.0
mmol) methyl methylthiomethyl sulfoxide in 2.5 mL acetonitrile was
added dropwise over the course of ten minutes. The reaction mixture was
stirred for two hours at room temperature.

The mixture was taken up in 50 mL ether, and the solution was
washed twice with 30 mL 10% aqueous Na$_2$S$_2$O$_3$ solution, 30 mL water,
and 30 mL saturated aqueous NaCl solution. The ether layer gave a spot on
TLC that appeared to be the desired product, bis(methylthio)methane. The
ether layer was evaporated in vacuo to 0.50 g light yellow solid which
was extracted four times with ethyl acetate and once with ether. The
combined organic layers were evaporated in vacuo to a faintly yellow oil.
The $^1$H-NMR spectrum of this material did not resemble that of the
desired product, and its structure was not determined.

B. Treatment of N-(Benzylxocarbonyl)-S-Cxo-S-[(Methylthio)methyl]-L-Cysteinol with Benzyl Bromide

The model compound (4.4 mg, 0.014 mmol) was treated dropwise with an excess of benzyl bromide (5 drops). The reaction mixture was checked by TLC for the disappearance of starting material (silica gel developed with 1:9 methanol:methylene chloride; starting material's $R_f=0.34$) and for the formation of benzyl methyl sulfide (silica gel developed with hexane; benzyl methyl sulfide's $R_f=0.37$). Neither event occurred, even after warming and stirring overnight. The solvent was evaporated in vacuo, and the identity of the residue as starting material was confirmed by its $^1$H-NMR spectrum.

C. Treatment of Methyl Methylthiomethyl Sulfoxide with Sulfuric Acid$^{18}$

Methyl methylthiomethyl sulfoxide was treated with sulfuric acid in an attempt to hydrolyze it and generate methyl mercaptan which could be trapped.

Methyl methylthiomethyl sulfoxide (133.4 mg, 1.07 mmol) was dissolved in 2 mL absolute ethanol. Three drops 9 N $\text{H}_2\text{SO}_4$ were added, and the reaction was monitored by TLC (silica gel developed with ethyl acetate; starting material's $R_f=0.18$) for the disappearance of starting material. Even after 72 hours stirring and warming, the starting material was stable.
D. Subjection of Methyl Methylthiomethyl Sulfoxide to Pummerer Conditions$^{81}$

Methyl methylthiomethyl sulfoxide (0.52 g, 4.2 mmol) and 5.0 mL freshly-distilled acetic anhydride were placed in a flask fitted with a reflux condenser. The top of the condenser was connected to a trap containing 0.34 g (8.5 mmol) NaOH in 5.0 mL water. The mixture was refluxed for thirty minutes, at which time TLC (silica gel developed with ethyl acetate) showed that all the starting material ($R_f$ 0.18) had disappeared. Water (0.07 mL, 3.9 mmol) was added, and heating was continued for a few minutes.

2,4-Dinitrochlorobenzene (0.86 g, 4.2 mmol) was added to the solution in the trap along with a few drops of methanol to help the 2,4-dinitrochlorobenzene dissolve. The mixture was warmed briefly, allowed to cool, and filtered. The solid recovered was identified by $^1$H NMR as unreacted 2,4-dinitrochlorobenzene. The filtrate was evaporated in vacuo to an oil which was chromatographed on a column of 6 g silica gel (15 mm X 17 cm) with 1:4 ethyl acetate:hexane as eluant. Three-mL fractions were collected and checked by TLC (same system as above). Fractions 12-32 gave spots of $R_f$ 0.06. The combined fractions were evaporated in vacuo to 7.1 mg of an oil. The $^1$H-NMR spectrum of this material showed that it was not the desired 2,4-dinitrophenylmethyl sulfide. Additional unreacted 2,4-dinitrochlorobenzene was also recovered from the column.
Isolation of Di-O,N-Acetylcysteine (72) from Sparsomycin

Sparsomycin (19.3 mg, 0.050 mmol) was suspended in 1.0 mL 2 N HCl. A reflux condenser was attached to the flask, and the mixture was heated on a steam bath for two hours. The hot solution was diluted to twice its volume with water and transferred to a Craig tube. It was allowed to cool to room temperature and was then chilled. The crystals which formed were centrifuged down, and the supernatant was decanted. The crystals were dried in vacuo over P₂O₅. A yield of 1.5 mg (0.008 mmol, 15.3% yield) of β-[(E)-1,2,3,4-tetrahydro-2,4-dioxo-5-pyrimidine]-acrylic acid was obtained. The identity of the product was confirmed by its ¹H-NMR spectrum.

The supernatant was evaporated repeatedly in vacuo with frequent additions of methanol. Seventeen mg of an oily, off-white residue were obtained. One mL of dry pyridine (freshly-distilled from CaH₂) and 0.2 mL of freshly-distilled acetic anhydride were added to the residue, and the mixture was stirred at room temperature overnight while protected from moisture by a CaCl₂ tube. The mixture darkened to reddish-brown. The reaction mixture was evaporated repeatedly to dryness in vacuo with frequent additions of methanol. An oil (34.9 mg) which contained fine needle-like crystals was obtained.

The oil was mixed with 2 mL water, and the resulting mixture was extracted four times with 2-mL portions of chloroform. The organic layers were combined and concentrated in vacuo to a small volume of yellow oil. This material was chromatographed on a column of 3 g silica gel (15 mm x 8 cm). The column was eluted with chloroform until five 1-mL fractions had been collected. The solvent was then changed to 98:2
chloroform:methanol. After a total of sixty 1-mL fractions had been collected, the solvent was changed to 85:15 chloroform:methanol. The fractions were checked by TLC on silica gel developed with 5:3:2 cyclohexane:ethyl acetate:95% ethanol and visualized with short wavelength ultraviolet light and phosphomolybdate spray. Fractions 16-18 gave multiple spots under UV, and no fractions gave phosphomolybdate-active spots.

The fractions were combined in groups of approximately ten fractions. Each group was evaporated to dryness under reduced pressure, and the residue was examined by $^1$H-NMR spectrometry. The residues from fractions 40-49 and 50-60 were found to be the desired compound. These two residues were combined and recrystallized from hot dry benzene. White crystals (2.2 mg, 0.01 mmol, 22.6% yield) which had a melting point of 92°C.-94°C. (lit.99°C.- 100°C.) were obtained.

$^1$H NMR (CDCl$_3$, 90 MHz): 2.04 (6H, s), 2.14 (6H, s), 2.84-3.08 (4H, m), 4.16-4.26 (6H, m), 6.42 (2H, br. d)

$^{13}$C NMR (CDCl$_3$, 22.5 MHz): 20.69, 23.18, 40.09, 48.38, 64.05

**Synthesis of Di-O,N-Acetylcyctinol (72)**

Cystine diethyl ester dihydrochloride (2.00 g, 10.77 mmol) was dissolved in 28 mL 50:50 ethanol:water. This solution was added dropwise to a solution of 2.90 g (76.72 mmol) NaBH$_4$ in 28 mL 50:50 ethanol:water at 0°C. The resulting mixture was stirred at 0°C. for four hours and was then stirred at room temperature overnight. After cooling in an ice bath and adjustment to pH 7.0 with concentrated aqueous HCl,
the mixture was stirred for another hour at room temperature and was then concentrated to an aqueous solution in vacuo. A methanolic solution which was 0.1 M in iodine and 0.2 M in pyridine was added dropwise until the color of iodine persisted. Approximately 25 mL were required. A few crystals of Na$_2$S$_2$O$_5$ were added to destroy the excess iodine. The solvent was removed in vacuo.

Forty mL of pyridine (freshly-distilled from BaO) and 8.0 mL of freshly-distilled acetic anhydride were added to the white residue, and the resulting mixture was stirred overnight at room temperature while protected from moisture by a drying tube containing CaCl$_2$.

Some of the liquid was removed in vacuo with frequent additions of methanol. The concentrated solution was taken up in 40 mL of water, and the resulting solution was extracted four times with 40-mL portions of chloroform. The combined organic layers were dried over anhydrous MgSO$_4$. The drying agent was removed by filtration, and the filtrate was concentrated in vacuo to a yellow oil.

The oil was chromatographed on a column of 10 g silica gel (15 mm X 26 cm). The column was developed with chloroform until sixty 4-mL fractions had been collected. The solvent was then changed to 1:9 methanol:chloroform. The fractions were checked by TLC on silica gel plates developed with 5:3:2 cyclohexane:ethyl acetate:95% ethanol and visualized by spraying with phosphomolybdate solution and heating. Fractions 62-64 gave spots of R$_f$ 0.20. These fractions were combined and concentrated in vacuo to a small volume of oil which was recrystallized with difficulty from hot dry benzene. The product (52.9 mg, 0.14 mmol, 1.3% yield) was identical in all respects to the previously-isolated di-O,N-acetylcystinol. The product also gave the
correct molecular ion (380) when subjected to mass spectrometry. The mass spectrum of the compound is shown in Figure 21.

We made no attempt to improve the yield of this synthesis because we needed only a few milligrams of the product for use as carrier. The yield was probably so low because many other oxidative processes could occur during the iodine treatment. In fact, the major product (as determined by TLC) appeared to remain in the aqueous layer during the chloroform extraction.

SYNTHESIS OF (2-\textsuperscript{13}C)-TRYPTOPHAN

A. (Formyl-\textsuperscript{13}C)-Acetic Formic Anhydride (90)\textsuperscript{65}

Sodium (\textsuperscript{13}C)-formate (3.06 g, 44.3 mmol) and 2.6 mL dry ether were placed in an ice-cold 3-necked flask fitted with an addition funnel, a thermometer, and a condenser protected by a CaCl\textsubscript{2} tube. Freshly-distilled acetyl chloride (2.75 mL, 38.7 mmol) was placed in the addition funnel and added dropwise to the stirred reaction mixture over the course of ten minutes. The reaction mixture was stirred for 5.5 hours; an ice bath was used as necessary to keep the mixture at room temperature. A white solid was removed by filtration and washed with a little dry ether. The combined filtrate and wash were distilled at 39 mm Hg, and the liquid distilling above 40°C. was collected and used immediately. A yield of 1.97 g (22.1 mmol, 57.1% yield)
Figure 21
(formyl-\textsuperscript{13}C)-acetic formic anhydride was obtained.

\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 90 MHz): 2.17 (3H, s), 8.97 (1H, s)

**B. (Formyl-\textsuperscript{13}C)-N-Formyl-o-Toluidine (91)**

This compound was synthesized by a procedure similar to that reported by Hurd and Roe\textsuperscript{66} for preparing formanilide.

Freshly-prepared (formyl-\textsuperscript{13}C)-acetic formic anhydride (1.97 g, 22.1 mmol) was placed in an ice-cooled flask, and 2.3 mL (21.4 mmol) o-toluidine (freshly-distilled from KOH) were added dropwise. The reaction mixture was checked immediately by TLC (silica gel developed with 2:1 hexane:ethylacetate; o-toluidine's R\textsubscript{f}=0.24) for the presence of o-toluidine. None remained. The reaction mixture was concentrated \textit{in vacuo}. The residue was dissolved in ethyl acetate, and the solution was extracted twice with an equal volume of 10% aqueous NaHCO\textsubscript{3} solution. The organic layer was evaporated \textit{in vacuo}, and the white solid remaining was purified by bulb-to-bulb distillation at 12 mm Hg (b. p. 174\textdegree C.). A yield of 2.36 g (formyl-\textsuperscript{13}C)-N-formyl-o-toluidine (17.3 mmol, 80.8% yield) which had a melting point of 50\textdegree C.-54\textdegree C. (lit. 62\textdegree C.) was obtained.

\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 90 HMrz): 2.30 (3H, s), 7.08-7.30 (4H, m), 7.90 (1H, br. d, J=9.0 Hz), 8.40-8.62 (1H, br. d, J=10.8 Hz)

\textsuperscript{13}C NMR (CDCl\textsubscript{3}, 22.5 MHz): 17.65, 120.68, 123.02, 125.51, 126.05, 127.08, 127.13, 130.55, 131.25, 159.30, (enriched), 163.29 (enriched)

It appeared that there was hindered rotation about the nitrogen
atom so that some of the carbon atoms, including the formyl carbon, gave
two lines in the $^{13}\text{C}$-NMR spectrum.

C. $(2^{-13}\text{C})$-Indole (92)$^{67}$

A 3-necked flask was fitted with a reflux condenser, and the
system was flushed with dry nitrogen. Dry t-butanol (20.8 mL, 221 mmol,
freshly-distilled from sodium) and 0.96 g (24.6 mmol) potassium were
placed in the flask which was gently warmed under nitrogen in an oil bath
until the potassium had dissolved. Freshly-prepared
$(\text{formyl-}^{13}\text{C})$-N-formyl-o-toluidine (2.25 g, 16.6 mmol) was added, and
the condenser was exchanged for a distillation head. The oil bath was
exchanged for a Wood's metal bath, and the temperature of the reaction
mixture was slowly increased until all the excess t-butanol had distilled
over. The residue was then heated slowly, under a nitrogen atmosphere,
to 350°C., and was kept between 350°C. and 360°C. for twenty minutes.
The reaction mixture, which had become very dark brown in color, was
allowed to cool to room temperature under nitrogen. Ten mL of water
were added, and the mixture was extracted with several portions of ether
(a total of 190 mL). The combined ether extracts were concentrated in
vacuo to a dark brown oil.

The oil was chromatographed on a column of 20 g silica gel (15
mm X 53 cm) with 9:1 hexane:ethyl acetate as solvent. Two-mL fractions
were collected and checked by TLC on silica gel developed with the same
solvent as the column. Indole had an $R_f$ of 0.15 in this system and was
found in fractions 45-86. The combined fractions were evaporated in
vacuo to a light green oil which was converted by bulb-to-bulb
distillation at reduced pressure to a pale yellow solid. Bulb-to-bulb
distillation at reduced pressure of this solid removed all the color and
yielded 0.59 g (5.0 mmol, 30.0% yield) of (2-^{13}C)-indole (m.p.
50\degree C.-51\degree C., lit. 52.5\degree C.). The \textsuperscript{1}H-NMR spectra of the product and
authentic indole were identical, and the \textsuperscript{13}C NMR spectrum of the product
showed enrichment at C-2.

D. (2-^{13}C)-Gramine (93)\textsuperscript{68}

Dimethylamine (0.62 g of a 40\% aqueous solution, 5.5 mmol) was
chilled to 0\degree C., and 0.71 mL (12.4 mmol) glacial acetic acid and 0.53 g (6.6
mmol) 37\% aqueous formaldehyde solution were added. This mixture was
added rapidly at 0\degree C. to 0.57 g (4.9 mmol) (2-^{13}C)-indole. The mixture
was stirred at room temperature for 18 hours. The yellow-brown
reaction mixture was added dropwise to a cold solution of 0.74 g (18.5
mmol) NaOH in 10.5 mL water. The yellow precipitate which formed was
collected by filtration, washed with water, and dried \textit{in vacuo}. A yield of
0.81 g (4.6 mmol, 94.6\% yield) off-white (2-^{13}C)-gramine (m.p.
124\degree C.-128\degree C., lit 138\degree C.-139\degree C.) was obtained. The product was
identical to authentic gramine by TLC (R\textsubscript{f} 0.10 on silica gel developed
with methanol). The \textsuperscript{1}H-NMR spectra of the product and authentic
material were identical, and the \textsuperscript{13}C-NMR spectrum of the product
confirmed the location of the label.

E. Ethyl \alpha-Carbethoxy-\alpha-Acetamido-\beta-[3-(2-^{13}C)-
Indoly]-Propionate (94)\textsuperscript{69}

Sodium (0.13 g, 5.7 mmol) was dissolved in 11.1 mL absolute
ethanol in a 3-necked flask equipped with a septum under nitrogen flush.
(2-$^{13}$C)-Gramine (0.80 g, 4.5 mmol) and 0.98 g (4.5 mmol) diethyl acetamidomalonate were added. The mixture was cooled in an ice bath, and 0.86 mL (9.1 mmol) dimethyl sulfate was added through the septum via a syringe. The mixture was stirred for five minutes at 0°C. and then for four hours at room temperature. The reaction mixture was poured into 35 mL ice water, and the resulting mixture was allowed to stand 30 minutes. The product was collected by filtration and recrystallized from ethanol-water. A pale yellow product (0.66 g, 1.9 mmol, 42.5% yield) which had a melting point of 145.5°C.-148.5°C. (lit. 153°C.) was obtained. (Yields as high as 99% were obtained in unlabeled runs.) The product had an $R_f$ of 0.19 on silica gel developed with 1:1 hexane:ethyl acetate.

$^1$H NMR (CDCl$_3$, 90 MHz): 1.34 (6H, t, J=7.2 Hz), 1.80 (2H, s), 2.02 (3H, s), 4.26 (4H, q, J=7.2 Hz), 6.68 (1H, s), 6.95-7.56 (5H, m), 8.36 (1H, br. s)

$^{13}$C NMR (CDCl$_3$, 22.5 MHz): 13.92, 23.07, 27.95, 62.53, 111.20, 118.45, 118.63, 119.44, 121.99, 123.18 (enriched)

F. (2-$^{13}$C)-DL-Tryptophan (95)$^{68}$

Ethyl α-carbethoxy-α-acetamido-β-[3-(2-$^{13}$C)-indolyl]-propionate (0.65 g, 1.9 mmol) was refluxed under nitrogen in a solution of 0.37 g (9.3 mmol) NaOH in 3.1 mL water for four hours. The reaction mixture was allowed to cool to room temperature and was then cooled in an ice bath. A small amount of water was added, and the pH of the mixture was adjusted to 4.0 with concentrated HCl.

After the addition of 10 mL 2 N H$_2$SO$_4$, the mixture was refluxed
under nitrogen for four hours. The reaction mixture was allowed to cool slightly, and its pH was adjusted to 7.0 with 0.1 M Ba(OH)$_2$. The hot mixture was filtered to remove BaSO$_4$. The filtrate was evaporated under reduced pressure to a pale yellow solid which was recrystallized from hot water. Three crops of white crystals totalling 0.21 g (1.0 mmol, 53.7% yield) of (2-$^{13}$C)-DL-tryptophan were obtained. The product was identical to authentic tryptophan by TLC (maroon spot of R$_f$ 0.50 on cellulose developed with 12:3:5 BAW) and by $^1$H-NMR spectrometry. The $^{13}$C-NMR spectrum confirmed the location of the label at C-2.

Preparation of 10% Palladium on Calcium Carbonate

Calcium chloride (2.55 g, 23.0 mmol) was dissolved in 30 mL water, and 2.40 g (22.6 mmol) Na$_2$CO$_3$ were added to the stirred solution. The white precipitate which formed was removed by filtration, washed thoroughly with water, and resuspended in 15 mL water. A solution of 417 mg (2.35 mmol) PdCl$_2$ and 1 mL 6 N HCl in 15 mL water was added with vigorous stirring. The product was collected by filtration and washed with water until all the chloride ion had been removed. The presence of chloride ion was detected by adding a few drops of wash to a few drops of dilute aqueous AgNO$_3$ and checking for the precipitation of white AgCl. The product was then washed with absolute ethanol and dry ether and dried in vacuo. A light brown product (1.21 g) was obtained.
Synthesis of \((5^2H_1)-DL\)-Tryptophan (106)$$^7$$

Deuterium gas was slowly passed through a 3-necked flask, of which one neck was connected via a stopcock to a deuterium-filled balloon. Nine mL 3% methanolic KOH and 800 mg freshly-prepared 10% palladium on CaCO$_3$ were placed in the flask. The mixture rapidly changed color from brown to gray. 5-Bromo-DL-tryptophan (0.80 g, 2.82 mmol) was added to the flask. The flow of deuterium was stopped, and the stopcock to the balloon was simultaneously opened. The reaction mixture was stirred for two hours.

A gray solid was removed by filtration and washed well with water. The combined filtrate and wash were adjusted to pH 7.0 with 0.1 N HCl. The solvent was evaporated \textit{in vacuo}, and the residue was recrystallized from hot water. Two crops totalling 0.28 g (1.39 mmol, 49.1% yield) were obtained. This material was identical to authentic tryptophan by TLC. The location of the label was confirmed by $^1$H- and $^{13}$C-NMR spectrometry.

$^1$H NMR (DCl-$D_2$O, 300 MHz): 3.40 (2H, m, J=9.0 Hz), 4.32 (1H, t, J=6.0 Hz), 7.20 (1H, d, J=12.0 Hz), 7.26 (1H, s), 7.46 (1H, d, J=12.0 Hz), 7.62 (1H, s)

The line at 119.24 ppm in the $^{13}$C-NMR spectrum (DCl-$D_2$O, 75.469 MHz) was a tiny triplet.
Synthesis of N-Formyl-[Ring-U-\textsuperscript{14}C\textsubscript{14}]-Anthranilic Acid (107)\textsuperscript{76}

Fifty \(\mu\text{Ci}\) (0.0044 mmol, sp. act.= 11.4 mCi/mmol) [ring-U-\textsubscript{14}C\textsubscript{14}]-anthranilic acid were dissolved in ethyl acetate, and the solution was added to a flask containing 7.32 mg (0.053 mmol) unlabeled anthranilic acid (a total of 0.57 mmol anthranilic acid). The solvent was removed \textit{in vacuo}, and 0.5 mL 98\% formic acid was added. The mixture was refluxed for 3 hours while protected from moisture by a drying tube containing CaCl\textsubscript{2}. The solvent was removed \textit{in vacuo}, and the residue was recrystallized from dilute aqueous ethanol. A yield of 6.48 mg (0.039 mmol, 68.4\% chemical yield) white product was obtained. The product (m.p. 164\textdegree C.-165.5\textdegree C., lit. 167\textdegree C.) gave only one spot on TLC (\(R_f\) 0.11 on silica gel developed with 2:1 acetone:hexane), and only one peak appeared on the radioscan of the TLC plate. The product was recrystallized to a constant specific activity of 7.35 X 10\textsuperscript{2} \(\mu\text{Ci}/\text{mmol}\) (57.3\% radiochemical yield).

\textsuperscript{1H} NMR (\(D_2\)O, 300 MHz):  7.30 (1H, dd),  7.51 (1H, d, \(J=9.0\) Hz), 7.63 (1H, dd), 8.03 (1H, d, \(J=8.1\) Hz), 8.16 (1H, d, \(J=8.3\) Hz), 8.37 (1H, s)
APPENDIX

This appendix describes the calculation of the deuterium content of the sparsomycin biosynthesized from (5-\textsuperscript{2}H\textsubscript{1})-DL-tryptophan. Because of the weak natural abundance signals in the \textsuperscript{2}H-NMR spectrum of the sample (the natural abundance of deuterium is only 0.015\%), the percent enrichment could not be determined by direct comparison with an unenriched signal, which is the method used to measure \textsuperscript{13}C-enrichments.

It was therefore necessary to compare the peak in the \textsuperscript{2}H-NMR spectrum of the sparsomycin to a standard. t-Butanol was used as the standard compound because it contains nine protons and would give a relatively intense natural abundance peak. Because we were unsure of the direct proportionality of peak size to t-butanol concentration, spectra of samples covering a range of t-butanol concentrations were taken. The graph shown in Figure 22 was then prepared from the raw data of concentrations and peak heights. As can be seen, the relationship between t-butanol concentration and natural abundance deuterium peak intensity was linear with a correlation coefficient of 0.98.

The intensity of the peak in the \textsuperscript{2}H-NMR spectrum of the labeled sparsomycin was then measured under the same conditions as those used for collection of the t-butanol spectra, and the t-butanol concentration to which the sparsomycin peak height was equivalent was determined from the graph. Calculations to convert this value (0.91 M) into the number of millimoles of deuterium (4.91 \times 10^{-4} mmol) contained in the sparsomycin sample were then performed. Division of this figure by the total number
Molarity vs. Peak Height

\[ y = 0.840 + 0.249x \quad R = 0.98 \]

Figure 22
of millimoles of sparsomycin present in the sample (8.97 \times 10^{-3} \text{ mmol}) and conversion of the result to a percentage yielded an enrichment of 6\%.
REFERENCES


20. R. J. Parry, unpublished observations.


22. *ibid.*, p. 496.


37. *ibid.;* p. 558.


44. *ibid.*, p. 21.


61. The $^{3}\text{H}/^{35}\text{S}$ ratios of the precursor methionine and the sparsomycin were determined simultaneously to avoid calculations to correct for the decay of $^{35}\text{S}$.


64. *ibid.*, pp. 453, 868-869.


