INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or “target” for pages apparently lacking from the document photographed is “Missing Page(s)”. If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of “sectioning” the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.
Mueller, Jay Vincent

INVESTIGATIONS OF THE BIOSYNTHESIS OF ELAIOMYCIN

Rice University

University Microfilms
International 300 N. Zeeb Road, Ann Arbor, MI 48106
RICE UNIVERSITY

INVESTIGATIONS OF THE BIOSYNTHESIS OF ELAIOMYCIN

by

JAY VINCENT MUELLER

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

DOCTOR OF PHILOSOPHY

APPROVED, THESIS COMMITTEE:

R. J. Parry, Associate Professor of Chemistry
Chairman

W. E. Billups, Professor of Chemistry

Ronald L. Sass, Professor of Biology

HOUSTON, TEXAS
MAY, 1984
ABSTRACT

INVESTIGATIONS OF THE BIOSYNTHESIS OF ELAIOMYCIN

by Jay Vincent Mueller

The biosynthesis of the antibiotic elaiomycin (1), produced by Streptomyces gelaticus, has been investigated. Precursor incorporation studies using $^{13}$C- and $[^3H,^{14}C]$-labeled forms of serine have shown that carbons 2-4 of elaiomycin are derived from serine, with the hydroxymethyl carbon of serine being incorporated into C-4 of elaiomycin. An administration of (methyl-$^{13}$C)-methionine has proven the methyl carbon of methionine to be the source of the O-methyl carbon of elaiomycin. ($^{13}$C)- and $[^{14}C]$-labeled precursor incorporation experiments indicate the novel introduction of C-2 of acetate into C-1 of elaiomycin. The administration of precursors labeled with contiguous $^{13}$C and $^{15}$N atoms has shown the oxygen-bearing nitrogen atom in elaiomycin to be derived from p-octylamine and the other nitrogen atom to be derived from serine.
ACKNOWLEDGMENTS

Many people share responsibility for helping me complete my graduate studies. The person who bears the most credit, though, is Ron Parry. His sharp mind, general research expertise, and patience provided the foundation for my successes. His affable nature and diverse interests made it a pleasure to have him as my adviser these past five years.

My friends in my lab and throughout the department also deserve big mention for their importance to me. They lightened my tasks through their support and encouragement, and through sharing many good laughs with me.

Thanks must also be extended to my undergraduate friends, especially to the members of Brown College, where I taught for three years and was graciously made an associate. Their kindness towards me and their pride in me was always uplifting.

Finally, my parents are gratefully acknowledged for the moral and financial support they have given me throughout my education.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................. iii  
LIST OF FIGURES ....................................................... vi  
LIST OF SCHEMES ...................................................... viii  
INTRODUCTION .......................................................... 1  
RESULTS AND DISCUSSION ............................................. 13  
EXPERIMENTAL .......................................................... 50  

<table>
<thead>
<tr>
<th>Compound</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Benzylxypropanoic Acid (15)</td>
<td>51</td>
</tr>
<tr>
<td>2-Benzylxypropanoyl Chloride</td>
<td>52</td>
</tr>
<tr>
<td>1-Diazo-3-benzylxybutanone (16)</td>
<td>52</td>
</tr>
<tr>
<td>1-Methoxy-3-benzylxybutanone (17)</td>
<td>53</td>
</tr>
<tr>
<td>1-Methoxy-2-amino-3-benzylxybutane (18)</td>
<td>54</td>
</tr>
<tr>
<td>N,O-bis(p-bromobenzoyl)-3-amino-4-methoxy-2-butanol (Bis Derivative 19)</td>
<td>56</td>
</tr>
<tr>
<td>N-(n-octyl)-p-bromobenzamide (20)</td>
<td>58</td>
</tr>
<tr>
<td>Hydrogenolysis of Elaiomycin and Derivatization of Product Amines ..........</td>
<td>60</td>
</tr>
<tr>
<td>N-(p-bromobenzoyl)-3-amino-4-methoxy-2-butanol (Mono Derivative 23)</td>
<td>61</td>
</tr>
<tr>
<td>N-(p-bromobenzoyl)-3-amino-4-methoxybutanone (Ketone 24)</td>
<td>63</td>
</tr>
<tr>
<td>Kuhn-Roth Oxidation of Ketone 24</td>
<td>64</td>
</tr>
<tr>
<td>(3-13C)-DL-Serine</td>
<td>66</td>
</tr>
<tr>
<td>n-Heptyl Mesylate</td>
<td>68</td>
</tr>
<tr>
<td>(1-13C,15N)-n-Heptyl Cyanide</td>
<td>68</td>
</tr>
<tr>
<td>(1-13C,15N)-n-Octylamine Hydrochloride</td>
<td>69</td>
</tr>
<tr>
<td>(2-13C,15N)-Glycine Ethyl Ester Hydrochloride</td>
<td>70</td>
</tr>
</tbody>
</table>
Ethyl (2-\(^{13}\)C,\(^{15}\)N)-Hippurate ........................................ 70
Ethyl (2-\(^{13}\)C,\(^{15}\)N)-2-Formyl Hippurate (28) ................. 71
N-Benzoyl (2-\(^{13}\)C,\(^{15}\)N)-Serine Ethyl Ester ......................... 73
(2-\(^{13}\)C,\(^{15}\)N)-DL-Serine Hydrochloride ................................ 74
Preparation of Sporulation Agar Slants of \textit{S. gelaticus} ............... 75
Preparation of Fermentation Medium for \textit{S. gelaticus} ................... 76
Inoculation and Fermentation Procedure for \textit{S. gelaticus} ............. 77
Typical Administration of Labeled Compounds ................................ 77
Isolation of Elaiomycin ..................................................... 78
Large Scale Fermentation of \textit{S. gelaticus} ................................. 79

REFERENCES ............................................................................. 82
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Naturally Occurring Azoxy Compounds</td>
<td>2</td>
</tr>
<tr>
<td>2.</td>
<td>Naturally Occurring Hydrazine Derivatives</td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>$^{13}$C NMR Spectrum of Natural Elaiomycin</td>
<td>21</td>
</tr>
<tr>
<td>4.</td>
<td>$^{13}$C NMR Spectrum of Elaiomycin Derived from (1,2-$^{13}$C$_2$)-Acetate</td>
<td>23</td>
</tr>
<tr>
<td>5.</td>
<td>INEPT Spectra of Elaiomycin</td>
<td>27</td>
</tr>
<tr>
<td>6.</td>
<td>$^{13}$C NMR Spectrum of Elaiomycin Derived from (3-$^{13}$C)-DL-Serine</td>
<td>28</td>
</tr>
<tr>
<td>7.</td>
<td>$^{13}$C NMR Spectrum of Elaiomycin Derived from methyl-$^{13}$C-L-Methionine</td>
<td>31</td>
</tr>
<tr>
<td>8.</td>
<td>$^{13}$C NMR Spectrum of Elaiomycin Derived from (1,2-$^{13}$C$_2$)-Acetate</td>
<td>33</td>
</tr>
<tr>
<td>9.</td>
<td>$^{13}$C NMR Spectrum of Elaiomycin Derived from (2-$^{13}$C)-Acetate</td>
<td>35</td>
</tr>
<tr>
<td>10.</td>
<td>$^{13}$C NMR Spectrum of Elaiomycin Derived from (2-$^{13}$C)-Glycine</td>
<td>37</td>
</tr>
<tr>
<td>11.</td>
<td>$^{13}$C NMR Spectrum of Elaiomycin Derived from Pulsed Administration of (2-$^{13}$C)-Acetate</td>
<td>39</td>
</tr>
</tbody>
</table>
12. $^{13}\text{C}$ NMR Spectrum of Elaiomycin Derived from (1-$^{13}\text{C},^{15}\text{N}$)-N-Octylamine .......... 46

13. $^{13}\text{C}$ NMR Spectrum of Elaiomycin Derived from (2-$^{13}\text{C},^{15}\text{N}$)-DL-Serine ............... 49
# List of Schemes

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Hypothetical Pathways to Elaiomycin</td>
<td>5-6</td>
</tr>
<tr>
<td>2. Biosynthesis of Naturally Occurring N-Hydroxylamines</td>
<td>7</td>
</tr>
<tr>
<td>3. Hydrogenolysis of Elaiomycin</td>
<td>14</td>
</tr>
<tr>
<td>4. Synthesis of Crystalline Derivatives of Hydrogenolysis Products 14 and n-Octylamine</td>
<td>16</td>
</tr>
<tr>
<td>5. Suspected Incorporation of Threonine into Elaiomycin</td>
<td>18</td>
</tr>
<tr>
<td>6. Synthesis of (3-$^{13}$C)-DL-Serine</td>
<td>25</td>
</tr>
<tr>
<td>7. Results of Precursor Incorporation Experiment Utilizing [U-$^{14}$C,3-B,2-$^{3}$H]-DL-Serine</td>
<td>30</td>
</tr>
<tr>
<td>8. Results of Precursor Incorporation Experiment Utilizing [2-$^{14}$C]-Acetate</td>
<td>40</td>
</tr>
<tr>
<td>9. Proposed Mechanism of Incorporation of C-2 of Acetate into C-1 of Elaiomycin</td>
<td>42</td>
</tr>
<tr>
<td>10. Synthesis of (1-$^{13}$C,15N)-n-Octylamine Hydrochloride</td>
<td>45</td>
</tr>
<tr>
<td>11. Synthesis of (2-$^{13}$C,15N)-DL-Serine Hydrochloride</td>
<td>48</td>
</tr>
</tbody>
</table>
INTRODUCTION

Elaiomycin, \((2S,3S)-4\text{-methoxy}-3-(1'\text{-cis-octenyl-trans-azoxy})-2\text{-butanol}\) (1), is an oily antibiotic isolated from submerged culture filtrates of the bacterium *Streptomyces gelaticus*.\(^1\) First reported in 1954 by researchers at Parke-Davis\(^2\), elaiomycin exhibits unusual biological activity as it only exhibits strong in vitro inhibition of virulent and avirulent forms of the bovine and human strains of *Mycobacterium tuberculosis*.\(^3\) The antibiotic has been found to induce tumors in rats\(^4\) and to be therapeutically ineffective at subtoxic dosages against experimental tubercle infections of mice and rats.\(^3a\)

The gross structure of elaiomycin was elegantly elucidated by Stevens, et al.\(^5\) in 1958 and its D-threo configuration was reported in 1959 by the same group.\(^6\) The cis geometry of the carbon-carbon double bond was determined in 1969,\(^7\) and the trans geometry of the azoxy function was determined in 1972.\(^8\) An eighteen-step total synthesis from D-threonine in 0.55% yield was reported in 1977 by Moss.\(^9\)

Elaiomycin is one of only a small number of known naturally occurring azoxy compounds, all of which exhibit biological activity. The only other \(\alpha,\beta\)-unsaturated azoxy compound which has been found in nature is the antifungal agent LL-BH872\(\alpha\) (2), produced by *Streptomyces hinnulinus*.\(^7,8\)
FIGURE 1

1

2

2', 1'

3, $R = H$

4, $R = \beta$-D-primeverosyl

5, $R = \beta$-D-glucosyl

6
A set of azoxyglycosides is produced by the cycads. All are glycones of the potent toxin and carcinogen methylazoxymethanol (3),\textsuperscript{10} which itself occurs naturally in cycads. Macrozamin (4)\textsuperscript{11} and cycasin (5),\textsuperscript{11e,12} the most-studied compounds of the group, are toxic and carcinogenic.\textsuperscript{13,14} Cycasin is known to derive its harmful effects in rats from its hydrolysis to methylazoxymethanol by a $\beta$-glucosidase produced by intestinal bacteria. A final azoxy compound isolated from two species of Calvatia, p-carboxyphenylazoxycyanide (6),\textsuperscript{15} is an antifungal, antibacterial, and antitumor agent. Prior to the research reported herein, nothing was known regarding the biosynthesis of any of these compounds.

An examination of the structure of elaiomycin reveals that its carbon skeleton consists of two parts— an eight carbon unit bonded to the oxygen-bearing nitrogen atom, and a five carbon unit bonded to the other nitrogen atom. Parry\textsuperscript{16} has shown that C-1 of p-octylamine is specifically incorporated into elaiomycin as C-1', implying that all eight carbons of the "left side" of the molecule are derived from p-octylamine. It was subsequently shown that the generation of the carbon-carbon double bond in elaiomycin involves stereospecific removal of the pro-R hydrogens at C-1 and C-2 of p-octylamine.\textsuperscript{16} In the right side, the presence of two carbons bonded to oxygen adjacent to a
carbon bonded to nitrogen suggests an α-amino acid precursor. A number of hypotheses may accordingly be offered for the biogenesis of the azoxy linkage of the two units. One possibility would be the condensation between \( n \)-octylhydroxylamine (7) and an amino acid (8) or between \( n \)-octylamine and an \( N \)-hydroxyamino acid (9) to yield a hydrazine (10) (Scheme 1, Route A). \( N \)-hydroxy compounds are well-documented,\(^{17}\) and in one case, the mechanism of \( N \)-hydroxylation has been investigated. The biosynthesis of the antitumor agent hadacidin involves oxidation of glycine to \( N \)-hydroxyglycine (Scheme 2a). Experiments with \(^{18}O_2\) established that the hydroxyl function is derived from molecular oxygen.\(^{18}\) A similar mechanism has been suggested for the conversion of ornithine to \( \delta \)-\( N \)-hydroxyornithine in the biosynthesis of the iron-chelating cyclic peptide ferrichrome (Scheme 2b).\(^{19}\) Naturally occurring hydrazine derivatives seem to be less common than \( N \)-hydroxy compounds, but some such compounds have been isolated from microorganisms (Figure 2). While the mechanism of the formation of the \( N-N \) single bond in naturally occurring hydrazines has apparently never been investigated, there is evidence that the formation of azobenzenes from anilines by soil bacteria proceeds via reaction of a phenylhydroxylamine with an aniline to give a hydrazobenzene, which is subsequently oxidized to the corresponding azobenzene.\(^{21}\) An
SCHEME 1

Route A:

\[
R\text{-NHOH} + H_2N\text{-COOH} \rightarrow R\text{-NH}_{\text{2}} + HONH\text{-COOH} \rightarrow R\text{-N-NH-COOH}
\]

\[R = n-C_{6}H_{13}\]

\[R' = \text{amino acid side chain}\]

Route B:

\[
R\text{-NHOH} + HONH\text{-COOH} \rightarrow R\text{-N-N-COOH}
\]

\[
R\text{-N-N-COOH} \rightarrow R\text{-N-N-N-COOH} \rightarrow R\text{-N-N-N-COOH} \rightarrow R\text{-N-N-N-COOH}
\]

\[\sim\]

\[\sim\]
SCHEME 1 (cont'd)

\[ R = n-C_6H_{13} \]

\[ R' = \text{amino acid side chain} \]

Route C:

\[ R \text{CHO} + H_2NNH - \text{COOH} \rightarrow R \text{NNH} - \text{COOH} \]

\[ R \text{NNH}_2 + \text{COOH} \rightarrow R \text{NH} - \text{COOH} \]

\[ \text{tautomerization} \]

\[ R \text{N=N} - \text{COOH} \]

\[ R \text{N=N} - \text{OCH}_3 \]

\[ 1 \sim \]

\[ 12 \sim \]

\[ 13 \sim \]
FIGURE 2

5-Chloropiperazic Acid (ref. 20a)  N-Amino-D-Proline (ref. 20b)
(Streptomyces jamaicensis)  (Linum usitatissimum)

Spinamycin (ref. 20b)
(Streptomyces albospinus)

Agaratine (ref. 20c)
(Agaricus bisporus)

Gyromitrin (ref. 20d)
(Gyromitrella esculenta)

Negamycin (ref. 20e)
(Streptomyces purpeofuscus)
azo moiety, as in 11, resulting from an analogous oxidation by \textit{S. gelaticus} would presumably serve as the precursor of the azoxy function in elaiomycin. Introduction of the double bond in the C₈ moiety and elaboration of the right side might occur at any stage in the biosynthesis; these steps have arbitrarily been depicted as occurring late in the pathways shown.

Another possible route to elaiomycin involves direct reaction of two hydroxylamines (7 and 9) (Scheme 1, Route B). Such a coupling would produce azo compound 11 directly. Chemical analogy of this transformation exists\textsuperscript{22} but presently there seems to be no biochemical precedent.

A third hypothesis for the coupling step involves the condensation of a hydrazine compound with a carbonyl compound (Scheme 1, Route C). A C₈ aldehyde might condense with an \(\alpha\)-hydrazino acid or a C₈ hydrazine might condense with an \(\alpha\)-keto acid derived from the transamination of an amino acid. Biochemical tautomerization of the resultant hydrazone 12 or 13 to the azo compound 11, followed by oxidation, would generate the azoxy moiety of elaiomycin. Examples of naturally occurring hydrazine derivatives have already been shown in Figure 3. Of these, N-amino-D-proline and 5-chloropiperazic acid provide examples of \(\alpha\)-hydrazino acids found in nature. Precedence for the natural occurrence of hydrazones is also provided by one of the
compounds in Figure 2, gyromitrin.

There are two general methods of investigating the biosynthesis of a natural product, both involving the use of isotopic labels. The classical method for biosynthetic studies makes use of radioactive tracers such as $^{14}\text{C}$ and tritium. A hypothetical precursor in a generally or specifically labeled form is administered to the producing organism; a small amount of labeled material is suitable for this method, because detection of incorporated radiolabel is monitored by the very sensitive technique of liquid scintillation counting. If desired, the sites of the incorporated label(s) are located in the natural product through an appropriate degradation scheme. In such a scheme, the compounds to be counted must be able to be recrystallized to constant specific activity; this is essential in order to ensure the activity of each compound counted arises from the compound itself and not from a radiochemical impurity. A potential drawback to locating labels by degradation is the possibility that the degradation scheme may proceed in low yield, resulting in levels of total activity which are too low to be dealt with at late stages of the scheme.

A more modern method of biosynthetic investigation involves the use of stable isotopes such as $^{13}\text{C}$ and deuterium as labels and the use of NMR spectroscopy or mass
spectrometry to detect incorporation into the natural product of interest. In the simplest application of this technique using NMR detection, an incorporated label is expected to result in enhancement of the appropriate signals above and beyond the intensity derived from natural abundance. The compounds administered are highly enriched with the chosen isotope at the appropriate positions, enabling a much larger mass of precursor to be administered than for the analogous radiolabeled experiment. (In the case of a radiolabeled precursor, feeding a greater mass results in feeding inconveniently, and expensively, high levels of radioactivity, if the specific activity of the precursor is not to be diluted.) Therefore, stable isotopes offer investigators of microbial biosynthesis the advantage of being able to "load up" the organism's medium with precursor, in the hope that the organism may take up and metabolize more of it, hopefully in a manner efficient enough to result in discernible enrichment of the appropriate NMR signals. Along with the necessity of efficient conversion of precursor to natural product, successful exploitation of the stable isotope method requires that the extent of dilution of the labeled precursor by the pool of unlabeled precursor be insufficient to prevent incorporated label from providing the level of isotopic enrichment needed for detection.
The previously mentioned result of Parry immediately suggested a feeding of \((1^{13}\text{C},^{15}\text{N})-n\text{-octylamine}\) to \textit{S. gelaticus} to determine whether the nitrogen atom of octylamine is specifically incorporated into the oxygen-bearing nitrogen of elaiomycin. If the contiguous labels are incorporated intact, the C-1' signal of elaiomycin would be expected to be split into a doublet by the adjacent \(^{15}\text{N}\), which has a spin number of \(1/2\). Investigations focusing on the right side of elaiomycin could begin by testing amino acids uniformly labeled with \(^{14}\text{C}\) as precursors. Such experiments would hopefully implicate a particular amino acid as a precursor on the basis of relative incorporation levels. Further experiments involving this amino acid specifically labeled with \(^{14}\text{C}\) or \(^{13}\text{C}\) would then be conducted to prove specific incorporation. Once a specific precursor for the carbon skeleton of the right side of elaiomycin could be found, a \(^{13}\text{C},^{15}\text{N}\) experiment analogous to that conducted with octylamine could be performed. Results of these feedings might suggest further experiments using labeled hydroxylamines or hydrazines derived from the known precursors of either the left or right side of the antibiotic to determine the mechanism of coupling of the two sides of the molecule.
RESULTS AND DISCUSSION

The early investigations of this project were directed towards finding crystalline compounds which would provide the basis for any degradative work on elaiomycin to be undertaken in experiments involving the administration of radiolabeled precursors. As elaiomycin is an oil, it is not amenable to rigorous specific activity measurements; attempts to derivatize elaiomycin itself as a crystalline compound failed, presumably because the octyl moiety hinders facile crystallization. Stevens, et al., have shown that elaiomycin may be hydrogenolyzed to yield n-octylamine and amino alcohol 14 (Scheme 3). We hoped that the amino alcohol could be derivatized to yield a crystalline compound suitable for specific activity measurements prior to any possible degradative work on the right side of elaiomycin. It was desirable to initially approach such a derivative through a totally synthetic route for several related reasons. First, elaiomycin is a rather precious commodity; S. gelaticus produces as little as one milligram per liter of fermentation medium. As many as 12 milligrams per liter were produced in the early stages of the project, but over time our cultures were much less
SCHEME 3

\[ \text{H}_2, \text{PtO}_2, \text{HOAc} \]

\[ \text{C}_{12}\text{H}_{25} \begin{array}{c} \text{OCH}_3 \\ \text{HO} \end{array} \]

\[ \text{H}_2\text{N} \begin{array}{c} \text{OCH}_3 \\ \text{HO} \end{array} \]

\[ \text{C}_8\text{H}_{17} \begin{array}{c} \text{NH}_2 \end{array} \]
productive. Secondly, we thought it would probably be necessary to use a synthetic crystalline derivative as a diluent before proceeding with any degradative work on the right side fragment; this would allow us to run reactions on and to crystallize to constant specific activity amounts of material which were not inconveniently small. Such a derivative would of course also provide a reservoir of material from which diluent material for later stages of a degradative scheme could be synthesized.

The crystalline compound we sought was obtained through a scheme which paralleled work done by Stevens' group in their elucidation of the structure of elaiomycin (Scheme 4a). 2-Bromopropanoic acid was converted to 2-benzyloxypropanoic acid (15). After conversion of 15 to the acid chloride, treatment of the latter compound with diazomethane gave the diazo ketone 16. Exposure of 16 to boron trifluoride in methanol then effected conversion to the methoxy ketone 17. 17 was reductively aminated using a two-step procedure involving oximation followed by reduction with LiAlH₄, to afford benzyloxy amine 18, an O-protected version of hydrogenolysis product 14. Deprotection of 18, followed by diacylation of the amino alcohol using p-bromobenzoyl chloride, gave the crystalline bis derivative 19 as a mixture of diastereomers. Fortunately, the diastereomers could be separated by preparative TLC.
Additionally, n-octylamine, the hydrogenolysis product of the left side of elaiomycin, was found to yield the crystalline derivative 20 upon treatment with p-bromobenzoyl chloride (Scheme 4b). Elaiomycin was hydrogenolyzed and the products 14 and n-octylamine treated with p-bromobenzoyl chloride. TLC of the reaction mixture confirmed the presence of both the octyl derivative 20 and the diastereomer of 19 having a lower $R_f$. The diastereomer of 19 of lower $R_f$ was therefore assigned to the threo series, to which elaiomycin belongs. The threo diastereomer could be crystallized with difficulty from benzene-hexane. The threo diastereomer of 19 was subsequently used as diluent material when necessary.

Having found a crystalline derivative of the right side of elaiomycin, we were now ready to initiate some precursor incorporation experiments. As mentioned in the Introduction, an amino acid was presumed to serve as a precursor for the right side of elaiomycin. When this research commenced, it was thought highly likely that this amino acid was threonine (Scheme 5). Biochemical reduction of its carboxyl group, followed by *in vivo* O-methylation, would formally yield the structure of elaiomycin's right side. It was understood by us that both chiral centers of L-threonine, the amino acid's "natural" stereoisomer, would
SCHEME 5

H₂N-\begin{array}{c}O \text{H} \\ \text{HO} \end{array} \xrightarrow{S. \text{gelaticus}} n-C₆H₁₃\begin{array}{c}N \text{N} \\ -O \text{HO} \end{array}
have to be formally inverted for threonine to serve as a precursor for elaiomycin.

Administration of \([U-{^14}C}\)-L-threonine to \(S. \text{gelaticus}\) resulted in an incorporation level of just 0.0055%. The antibiotic was degraded to \(14\) and \(n\)-octylamine, which were then converted to their derivatives \(19\) and \(20\). Crystallization of these derivatives to constant specific activity showed the left side to have twice the specific activity of the right side. This result, contradictory to our hypothesis, was interpreted as being due to the fact that threonine is a primary metabolite, utilized in many pathways. It seemed plausible that a generally labeled form of it might have its label randomly distributed along many pathways in the two day fermentation period following the feeding. In this manner it was presumed that a significant amount of label was incorporated into the left side relative to the right side.

The case for threonine being a precursor for the right side because of its structural resemblance seemed strong enough to warrant additional effort in line with that hypothesis. A specifically labeled precursor seemed appropriate, as the apparent problem of randomization would be minimized if the label only occurred at one site in the administered molecule. Synthesis of racemic \([1-{^14}C]\)-threonine using a published procedure was
undertaken. Administration of this compound resulted in an incorporation figure of 0.035%. The higher incorporation figure was attributed to the presence of the D-isomer of threonine in the feeding mixture. As the degradative work necessary to isolate C-3 of elaiomycin, the expected site of specific incorporation, appeared formidable, a similar synthesis of racemic (1-$^{13}$C)-threonine was carried out. The $^{13}$C NMR spectrum of elaiomycin (Figure 3) could be partially analyzed on the basis of expected chemical shifts. The signals at 136.51 and 133.64 ppm arise from the two vinyl carbons, the central group of four signals arise from the four carbons adjacent to hetero atoms on the right side of the molecule, and the upfield group of six lines can be attributed to C-1 and the carbons in the n-hexyl chain. (It appeared that the tallest peak, at 29.20 ppm, was actually two coincident signals, as otherwise one signal would be "missing.") The ensuing feeding of the labeled threonine resulted in no enhancement of any of the signals in the $^{13}$C NMR spectrum of elaiomycin, however.

At this point, our efforts turned to testing other precursors. [U-$^{14}$C]-L-Serine was supplied to S. gelaticus, resulting in very low incorporation. [U-$^{14}$C]-Erythritol was administered, also resulting in negligible incorporation. [1-$^{14}$C]-DL-2-Aminobutanoic acid was also administered, with the same result. Sodium (1, 2-$^{13}$C$_2$)-acetate was
administered with the expectation that any acetate units incorporated in an intact manner into the right side of elaiomycin, most probably at C-1 and C-2, would be detected by the appearance of doublets in the $^{13}$C spectrum of the derived antibiotic resulting from $^{13}$C-$^{13}$C coupling. However, no coupling was exhibited among the signals derived from carbons of the right side (Figure 4). This experiment did serve as a check on the biosynthesis of the C$_8$ moiety, though. Extensive coupling was seen among the carbons in the saturated hydrocarbon section of the spectrum, as well as between the downfield vinyl carbons. This result confirmed the ultimate origin of the octyl chain in fatty acid biosynthesis, which involves the formal polymerization of acetyl units via acetyl- and malonyl-CoA.$^{26}$

Having no solid evidence for a specific precursor of the right side of elaiomycin, and rather puzzled by the lack of results from the feedings of what seemed to be reasonable precursors, we decided to retrace our steps, in a manner of sorts. [U-$^{14}$C]-L-Serine was referred to S. gelaticus, using a new experimental timetable. We decided to abandon our standard practice of administering the precursor 48 hours after inoculation of the fermentation medium and isolating the antibiotic four days after inoculation. Instead, the feeding was done 24 hours following inoculation, and the fermentation worked up three days after inoculation. We
made this change of procedure secure in the knowledge gained from a previous similar, but inadvertent, change of precursor administration and harvesting timing, in which the mistimed experiment still yielded elaiomycin. By administering serine using the revised timing, we obtained elaiomycin having an incorporation figure of 0.062%, our highest incorporation figure yet. Apparently, the timing of precursor administration is critical to the level of incorporation, at least in the case of serine. Henceforth, we used the new timing for our precursor incorporation experiments.

Encouraged by the incorporation level of serine, we decided to test a $^{13}$C labeled form of the amino acid as a precursor. (3-$^{13}$C)-DL-serine was synthesized according to the procedure of King (Scheme 6). The $^{13}$C-Formaldehyde was condensed with diethylacetamidomalonate to yield alcohol. The ester functions were saponified and the resulting diacid was decarboxylated. Hydrolysis of amide afforded serine, which was converted by the conditions of the workup to serine ethyl ester. Acidic hydrolysis of the ester gave back labeled serine, in 55% overall yield.

Concurrently with this synthesis, an analysis of the portion of elaiomycin's $^{13}$C NMR spectrum of relevance to the right side of molecule was undertaken. Using a pulse program called INEPT (Insensitive Nuclei Enhanced by
**Scheme 6**

$$\text{CH}_3\text{CONH} - \text{CO}_2\text{C}_2\text{H}_5 + \text{CH}_2\text{O} \quad \xrightarrow{\text{OH}^-} \quad \text{CH}_3\text{CONH} - \text{CO}_2\text{C}_2\text{H}_5 \quad \xrightarrow{\text{21}} \quad \text{CH}_3\text{CONH} - \text{CO}_2\text{C}_2\text{H}_5 \quad \xrightarrow{\text{22}} \quad \text{OH} - \text{COOH} \quad \xrightarrow{\text{HOAc, } \Delta} \quad \text{OH} - \text{NHCOCH}_3$$

1) OH $^-$
2) HOAc, $\Delta$

workup with EtOH under acidic conditions
Polarization Transfer), 28 in conjunction with Fourier transform NMR, we were able to make an assignment for the methylene (CH₂OCH₃) carbon, C-4 of elaiomycin. The version of INEPT used produces a spectrum in which methylene signals appear 180° out of phase, whereas methyl and methine signals are phased normally. As the only signal of the central group of four which pointed down was the one at 70.54 ppm (Figure 5a), we assigned it to the C-4 methylene. With the ¹³C spectrum of the right side of elaiomycin somewhat clarified, the (3-¹³C)-DL-serine was administered to S. gelaticus, resulting in the first conclusive evidence of a specific precursor for the right side; the C-4 signal showed obvious enrichment (Figure 6).

To evaluate whether all three carbons of serine are specifically incorporated intact into elaiomycin, a double label experiment was carried out using a mixture of [3RS-³H]-L-serine and [U-¹⁴C]-L-serine, both commercially available. The rationale behind this experiment involved the assumption that the tritium label would be incorporated intact. We had already shown that the carbon bearing the tritium was specifically incorporated, and had no reason to suspect any in vivo C-H bond cleavage at that carbon. If the three carbons of serine were incorporated specifically and in an intact manner, the ³H/¹⁴C ratio of the right side of the derived elaiomycin would be expected to be identical
a) INEPT spectrum of elaiomycin:

CH's and CH₃'s up, CH₂'s down

b) Partial INEPT spectrum

of elaiomycin: CH's only
to the value for the administered serine. If any of the other two carbons were not incorporated, the ratio would rise to a calculable new value. A mixture of the two labeled forms of serine having a $^{3}\text{H}/^{14}\text{C}$ ratio of 4.91 was fed to \textit{S. gelaticus} (Scheme 7), and the \textit{bis} derivative \textsuperscript{19} was isolated in the usual manner. Difficulties in crystallizing the \textit{bis} derivative led us to transesterify that compound to the mono derivative \textsuperscript{23} in 81\% yield, which was more easily crystallized, albeit with difficulty. \textsuperscript{23} was crystallized to a constant $^{3}\text{H}/^{14}\text{C}$ ratio of 4.86. This result indicated that serine provides carbons 2-4 of elaiomycin.

Having established the biogenetic origin of three of the carbons of the right side of elaiomycin, we tried to discover the precursors of the other two carbons. It is well established that methionine (via \textit{S}-adenosyl methionine) serves as a primary donor of \textit{C\textsubscript{1}} units, including O-methyl groups, in biosynthesis.\textsuperscript{29} Accordingly, (\textit{methyl-}$^{13}\text{C}$)-\textit{L}-methionine was administered to \textit{S. gelaticus}. One of the central four signals in the $^{13}\text{C}$ NMR spectrum of the elaiomycin derived from this experiment showed enrichment; the signal at 58.74 ppm was about 40 times the height of the other natural abundance signals, indicating extensive enrichment (Figure 7). The positive assignment of this signal as the methoxyl carbon came from another INEPT
$$\text{SCHEME 7}$$

$$\text{T}$$

$$\text{H}_2\text{N}$$

$$\text{O}$$

$$\text{H}_2\text{O}$$

$$\text{N}$$

$$\text{O}$$

$$\text{C}_6\text{H}_{13}$$

$$\text{N}$$

$$\text{N}$$

$$\text{OCH}_3$$

$$\text{HO}$$

$$\text{1}\text{H/}^{14}\text{C} = 4.91$$

1) $$\text{H}_2\text{PtO}_2\text{, HOAc}$$

2) $$\text{p-BrPhCOCl}$$

$$\text{Br}$$

$$\text{CONH}$$

$$\text{OCH}_3$$

$$\text{19}$$

$$\text{Br}$$

$$\text{COO}$$

$$\text{NaOEt}$$

$$\text{Br}$$

$$\text{CONH}$$

$$\text{OCH}_3$$

$$\text{HO}$$

$$\text{23}$$

$$\text{3H/}^{14}\text{C} = 4.86$$
experiment. Using an INEPT pulse program which permits the observation of methine signals only, we noted the disappearance of the signal at 58.74 ppm and the signal at 70.54 ppm, previously assigned to C-4 (Figure 5b). We therefore concluded the signal at 58.74 ppm arises from the methoxyl carbon and that the labeled methionine feeding resulted in the labeling of the methoxyl carbon of elaiomycin.

The origin of one more carbon atom remained to be found, namely that of C-1 of elaiomycin. This proved to be a fairly elusive task. The search began with a hindsight discovery related to an experiment conducted when we were still trying to find any precursor at all for the right side. Upon reexamining the $^{13}$C NMR spectrum of elaiomycin obtained from the administration of sodium (1,2-$^{13}$C$_2$)-acetate (reproduced as Figure 8), we noted that the C-1 signal appeared enriched relative to the central group of four signals. The enrichment at C-1 was made all the more pronounced by a comparison of the intensity of its signal to that of the likewise enriched signal of C-8'. The intensity of the C-1 signal was 1.5 times that of the three lines (central natural abundance signal and two satellites representing $^{13}$C-$^{13}$C coupling) associated with the terminal methyl carbon of the C$_8$ unit. The assignment of these two signals was based on the INEPT spectrum (Figure 5a), along
with their upfield shifts. The distinction between them could be made on the basis of expected chemical shifts and the fact that the methyl group of the C₈ moiety is expected to exhibit coupling in such a double label experiment. The problem which now faced us was to determine which carbon of acetate produced the observed enrichment in the C-1 signal of elaiomycin.

Because C-2 of acetate is already in the "correct" oxidation state for incorporation into C-1 of elaiomycin, we expected it to be the source of the observed enrichment at that site. Sodium (2-¹³C)-acetate was therefore fed to S. gelasticus, and the ¹³C spectrum of the derived elaiomycin was examined (Figure 9). The intensity of the C-1 signal did appear enriched relative to the central group of four signals. As expected, C-2', C-8' and two other signals presumably arising from C-4' and C-6' were also enriched. The enrichment at C-1, however, was not of the order of the enrichment observed, for example, at C-4 in the labeled serine feeding. We were also somewhat disturbed that the relative enrichment of C-1 to C-8' observed in the sodium (1, 2-¹³C₂)-acetate experiment was not reproduced in this single label experiment. Even a second feeding of sodium (1, 2-¹³C₂)-acetate did not result in the same level of enrichment at C-1 relative to C-8' as in the first feeding.
Wondering whether we were pursuing a false lead, we tried administering two other potential sources of $C_1$ units. Sodium ($^{13}$C)-formate was fed with the expectation that the labeled carbon would enter the organism's pool of methylene-tetrahydrofolate, a common $C_1$ donor. No incorporation was detected, however. A recent report that C-2 of glycine could serve as a source of a $C_1$ unit suggested a feeding of (2-$^{13}$C)-glycine. In the ensuing experiment, no incorporation was observed at C-1 of elaiomycin, but the C-3 and C-4 signals were enriched, and even exhibited some coupling (Figure 10). The labeling at C-3 is understood from the known biosynthesis of serine from glycine and methylene-tetrahydrofolate. The labeling at C-4, and the observed coupling, may be rationalized by the known incorporation of C-2 of glycine into methylene-tetrahydrofolate, and the subsequent condensation of a glycine molecule with the labeled methylene-tetrahydrofolate to yield labeled or doubly labeled serine.

At this point, we turned our attention back to acetate. Believing that perhaps we were not timing our feedings properly for maximum incorporation of this precursor, a pulsed feeding of sodium (2-$^{13}$C)-acetate was conducted. The labeled compound was fed at the time of inoculation, and at 12, 24, and 36 hours after inoculation in an attempt to
introduce acetate at some time when *S. gelaticus* might utilize its C-2 more efficiently. As before, C-1 of elaiomycin appeared enriched relative to the other four signals arising from the right side of the molecule. In fact, the enrichment was greater than in the non-pulsed feeding (Figure 11).

As we were apparently discovering an unusual mode of introduction of a C-1 unit in a biosynthetic scheme, we felt it worthwhile to strengthen our case for its occurrence. To accomplish this, we turned to the more sensitive detection technique of scintillation counting and administered sodium [2-\(^{14}\)C]-acetate to *S. gelaticus*. Cleavage of the elaiomycin obtained and isolation of the carbons of the right side as the bis derivative 19 was followed by transesterification and PCC oxidation to give the easily crystallizable ketone 24 (Scheme 8). This compound was recrystallized to constant specific activity and a value of 1.30 x 10\(^5\) dpm/mmol was obtained. A Kuhn-Roth oxidation was then carried out on the ketone, whereby the methyl carbonyl moiety, formerly C-1 and C-2 of elaiomycin, was converted to acetic acid. The acetic acid was isolated as its crystalline p-bromophenacyl ester 25. This compound was recrystallized to a constant specific activity of 1.055 x 10\(^5\) dpm/mmol, indicating that 81% of the activity was present at C-1 and C-2 of elaiomycin. Because C-2 was known to be derived from serine, any activity in the
SCHEME 8

\[ \text{Specific activity} = 1.30 \times 10^5 \text{ dpm/mmol} \]

\[ \text{Specific activity} = 1.055 \times 10^5 \text{ dpm/mmol} \]
p-bromophenacyl acetate would be expected to reside in its methyl group, derived from C-1. As acetate is a precursor of very general utility in biosynthetic pathways, some incorporation of label into the other carbon atoms of the right side might be expected to occur, preventing us from observing 100% of the activity of the right side in the p-bromophenacyl acetate. The result obtained, however, together with the results of the $^{13}$C experiments, strongly indicates the specific incorporation of C-2 of acetate into C-1 of elaiomycin.

This result represents a novel introduction of a C$_1$ unit in a biosynthetic pathway. A possible mechanism of the introduction is given in Scheme 9. Conversion of acetyl-CoA to malonyl-CoA would allow for a nucleophilic attack by C-2 on an activated form of the carboxyl group of serine, possibly a mixed anhydride with phosphoric acid. Hydrolysis of the thioester linkage of the CoA prosthetic group followed by decarboxylation of the resulting $\beta$-keto acid 26 would generate ketone 27. 27 would then be further elaborated to yield the right side of elaiomycin. (For simplicity, O-methylation and the coupling of the two sides of the molecule have arbitrarily been shown as late steps.) Recently, Kingston's group has reported the introduction of C-2 of acetate as a C$_1$ unit in the biosynthesis of the peptolide antibiotic virginiamycin $\text{M}_{1}$. Their results
SCHEME 9

\[ \text{acetate} \rightarrow \text{SCoA} \xrightarrow{\text{CO}_2, \text{biotin}} \text{malonyl-CoA} \]

\[ \text{H}_2\text{N} - \text{HO} \xrightarrow{\text{ATP+ADP}} \text{H}_2\text{N} - \text{OP}_3 - \text{2} \]

\[ \xrightarrow{-\text{CO}_2, -\text{PO}_4 -3} \]

\[ \xrightarrow{\text{H}_2\text{O}} \]

\[ \text{H}_2\text{N} - \text{HO} \xrightarrow{\text{25}} \text{H}_2\text{N} - \text{SCoA} \]

\[ \xrightarrow{-\text{CO}_2} \]

\[ \xrightarrow{\text{27}} \]

\[ \xrightarrow{\text{30}} \text{product} \]
suggested an aldol-type nucleophilic attack of C-2 of acetate (as malonyl-CoA) on a keto group of a polyketide chain, followed by hydrolysis of the CoA linkage and decarboxylation.

Our results concerning the biosynthesis of the right side of elaiomycin are interesting in light of our original hypothesis that threonine served as the precursor of that portion of the antibiotic. In that hypothesis, we postulated formal inversions of configuration at the two chiral centers of L-threonine, and reductive O-methylation of its carboxyl group (Scheme 5). Our results indicate that *S. gelaticus* utilizes a more efficient scheme for synthesizing elaiomycin. The methoxyl function arises through unexceptional O-methylation of the existing hydroxyl group of serine. The \( \beta \) configuration at C-2 of elaiomycin arises via a simple stereospecific reduction of a keto group, rather than through a more complicated, presumably multistep, inversion of configuration at a hydroxyl-bearing carbon. It may also be noted that because serine is incorporated in the sense that its carboxyl carbon becomes C-2 of elaiomycin (i.e., it is incorporated in the opposite sense of what we hypothesized for threonine), no inversion of configuration is required at the \( \alpha \)-carbon of L-serine for incorporation to occur.
In addition to the elucidation of the origin of the right side carbon 'skeleton of elaiomycin, two important results concerning the biosynthesis of the azoxy linkage may be reported. As mentioned in the Introduction, when this research commenced, specific incorporation of C-1 of \( \text{p-octylamine} \) into C-1' of elaiomycin was already proven. Therefore, the feeding of \((^{13}\text{C},^{15}\text{N})-\text{p-octylamine}\) was undertaken to determine whether the nitrogen of octylamine was incorporated into the oxygen-bearing nitrogen atom of elaiomycin. \( \text{p-Heptanol} \) was converted to its mesylate, and then treated with commercially available \( K^{13}\text{C}^{15}\text{N} \) (Scheme 10). The resulting nitrile was reduced to the amine hydrochloride, which was then supplied to \( S. \text{gelaticus} \). The elaiomycin obtained from this fermentation gave a C-1' signal which appeared as a doublet \((J = 16 \text{ Hz})\), due to splitting from the adjacent \( ^{15}\text{N} \) (Figure 12). The smaller signal within the doublet represents natural abundance \( ^{13}\text{C} \) not contiguous to \( ^{15}\text{N} \). This experiment proved intact incorporation of the nitrogen of \( \text{p-octylamine} \), and ruled out the hypothesis (Scheme 1, Route C) that a \( C_8 \) aldehyde might condense with an \( \alpha \)-hydrazino acid in the biosynthetic step coupling the two sides of elaiomycin.

Once serine was found to be a precursor of the right side, an analogous \( ^{13}\text{C},^{15}\text{N} \) experiment was conducted with it. \((2-^{13}\text{C},^{15}\text{N})-\text{DL-Serine} \) was synthesized starting with
SCHEME 10

\[
\begin{align*}
\text{OH} & \xrightarrow{\text{MsCl, TEA}} \text{OMs} \\
& \xrightarrow{\text{K}^{13}_{15}\text{C}^{15}\text{N}, \text{Bu}_3\text{N}, \text{H}_2\text{O}} \text{CN} \\
& \xrightarrow{\text{H}_2, \text{PtO}_2, \text{CHCl}_3} \text{NH}_3\text{Cl}
\end{align*}
\]
commercially available \((2^{-13}C,^{15}N)\)-glycine \((\text{Scheme 11})\). After conversion of this material to glycine ethyl ester hydrochloride, it was N-benzoylated to afford ethyl hippurate. \(\alpha\)-Formylation using ethyl formate gave enol \(28\). Reduction of the enol with aluminum amalgam in moist ether produced N-benzoyl serine ethyl ester, which was subsequently hydrolyzed to serine hydrochloride. Feeding of the doubly labeled compound to \(S.\ gelaticus\) yielded elaiomycin which exhibited an enriched doublet at 64.82 ppm \((J = 3.3 \text{ Hz})\), proving intact incorporation of the nitrogen of serine's amino group into the right side nitrogen atom of elaiomycin \((\text{Figure 13})\). This result ruled out the coupling hypothesis involving the condensation of a \(C_8\) hydrazine with an \(\alpha\)-keto acid \((\text{Scheme 1, Route C})\). Further experiments might show whether an amine and a hydroxylamine, or two hydroxylamines are involved in the coupling step.
EXPERIMENTAL

$^1$H NMR spectra were obtained using either a Varian EM-390 or a Jeol FX-90Q machine at 90 MHz. $^{13}$C NMR spectra were taken on a Jeol FX-90Q at 22.5 MHz. Chemical shifts for proton spectra are given in parts per million downfield from tetramethylsilane (TMS) in CDCl$_3$, unless otherwise noted. $^{13}$C spectra taken in $d_6$-benzene use the middle solvent signal at 128.00 ppm as the internal reference. All elaiomycin spectra were taken in $d_6$-benzene. $^{13}$C spectra taken in D$_2$O use the methyl signal of CH$_3$CN at 1.3 ppm as the internal reference. High resolution mass spectra were run on a CEC 111021-110B mass spectrometer. Gas chromatography was run on a Hewlett Packard 5710A machine using a 10% SE-30 glass column (6 ft, 2 mm ID). All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected.

Radiolabeled compounds for feeding were purchased from New England Nuclear and Amersham. Compounds labeled with stable isotopes were obtained from KOR, Merck, or Cambridge Isotope Laboratories. Samples for liquid scintillation counting were weighed on a Perkin Elmer AD-2 Autobalance and counted using a Beckman LS 100-C or a Packard 3003 liquid scintillation counter in Aquasol or toluene scintillation fluids. These solvents were either purchased from New
England Nuclear or prepared from materials supplied by New England Nuclear. Radiochemical purity was checked using a Berthold LB-2723 radiochromatogram scanner.

Preparative thin layer chromatography (TLC) was accomplished using 0.75 mm layers of Merck silica gel, type 60 with PF-254 indicator.

2-Benzyloxypropanoic Acid (15)\textsuperscript{35}

6.4 g (0.278 mol) sodium was added to 185 mL distilled benzyl alcohol and stirred with exclusion of moisture until all the sodium had reacted. 11.4 mL (19.38 g, 0.127 mol) 2-bromopropanoic acid was then added to the warm solution. The reaction mixture was left to stand overnight at room temperature. The following day, the precipitated NaBr was filtered out with difficulty through a sintered glass funnel. The benzyl alcohol was evaporated on a rotary evaporator using a vacuum pump and a hot water bath. The residue was dissolved in 70 mL water, and then extracted with ether (3 x 35 mL). The combined ethereal phases were backwashed with brine, and then dried with MgSO\textsubscript{4}. Evaporation of the solvent in vacuo and distillation of the crude acid at 123° using a oil pump operating at full vacuum afforded 16.3 g (79%) of the colorless liquid product.
$^1$H NMR (90 MHz, CDCl$_3$):

1.45 (3H, d, $J = 7$ Hz, CH$_3$)
4.05 (1H, q, $J = 7$ Hz, CHOBrzl)
4.40 - 4.75 (2H, ABq, $J_{AB} = 11$ Hz, OCH$_2$Ph)
7.27 (5H, s, ArH)

2-Benzyloxypropanoyl Chloride

18.17 mL (29.64 g, 0.25 mol) thionyl chloride was added in one portion to 15.27 g (0.085 mol) 2-benzyloxypropanoic acid. The mixture was refluxed with stirring for 0.5 h. The excess thionyl chloride was removed in vacuo, and the product was distilled at 67º using an oil pump operating at full vacuum, affording 14.35 g (79.6%) of the desired product, a colorless oil.

$^1$H NMR (90 MHz, CDCl$_3$):

1.52 (3H, d, $J = 6$ Hz, CH$_3$)
4.12 - 4.80 (3H, m, CHOCH$_2$Ph)
7.32 (5H, s, ArH)

1-Diazo-3-benzyloxybutanone (16)$^5$

A mixture of 250 mL ether and 75 mL 40% aqueous KOH was chilled to 5º. 26 g (0.25 mol) N-nitroso-N-methyl urea$^{36}$ was added as fast as foaming allowed while the solution was swirled in an ice-salt bath inside a hood. The yellow ethereal phase was decanted and dried for 2 h over KOH in
the refrigerator. 11.78 g (0.059 mol) 2-benzylxypropanoyl chloride was added dropwise to the ethereal solution of diazomethane with stirring and cooling in an ice bath. Once addition was complete, the excess diazomethane was purged by bubbling nitrogen through the solution. The ether was removed in vacuo and the product was Kugelrohr distilled at 93°, using an oil pump operating at full vacuum. The yield was 10.69 g (88.3%) of a yellow liquid, which showed major IR absorption peaks both for a carbonyl group (1680 cm⁻¹) and for a diazo structure (2115 cm⁻¹).

1-Methoxy-3-benzylxybutanone (17)⁵

8.85 g (0.043 mol) diazoketone 16 was dissolved in 150 mL methanol previously dried over 4A molecular sieve. The solution was chilled in an ice bath and 0.2 mL distilled boron trifluoride etherate was added. Mild evolution of nitrogen gas resulted. The reaction was allowed to proceed 15 h, coming to room temperature as the ice melted. The methanol was removed in vacuo and the residue taken up in 40 mL ether and extracted twice with 10 mL saturated aqueous NaHCO₃. The ethereal phase was then washed with 10 mL brine and dried using MgSO₄. The ether was evaporated in vacuo, leaving a residue which was Kugelrohr distilled using an oil pump operating at full vacuum (95-104° Kugelrohr temperature) to give 8.09 g (89%) of the desired ketone, a
colorless oil. Its IR spectrum showed disappearance of the diazo band and the appearance of a new carbonyl band at 1730 cm\(^{-1}\).

\(^1\)H NMR (90 MHz, CDCl\(_3\)):

1.32 (3H, d, J = 7 Hz, CCH\(_3\))
3.33 (3H, s, OCH\(_3\))
4.02 (1H, q, J = 7 Hz, CHO\(_2\)Bz)
4.23 (2H, v. tight d, OCH\(_2\)Ph)
4.47 (2H, s, O=CCH\(_2\)O)
7.20 (5H, s, ArH)

\(^1\)-Methoxy-2-amino-3-benzylxybutane (18)\(^5\)

4.003 g (0.014 mol) ketone 17 was dissolved in 20 mL 95% ethanol. 2.13 (0.03 mol) hydroxylamine hydrochloride was added, followed by a solution of 1.42 g (0.035 mol) NaOH in 5 mL water. A yellow color was produced and NaCl precipitated. Another 5 mL water was added, causing the yellow color to disappear. The solution was refluxed 2.3 h. The ethanol was removed in vacuo until salts began precipitating out, at which point 20 mL water was added to the residue. Extraction three times with 20 mL portions of ether, drying of the ethereal extracts with MgSO\(_4\), and in vacuo removal of the ether afforded 4.14 g of crude oxime.
The crude oxime was dissolved in 20 mL ether and added dropwise to a suspension of 3.62 g (0.095 mol) LiAlH₄ in 30 mL ether. The solution was refluxed for 21 h. The excess LiAlH₄ was destroyed using water, added dropwise while cooling the reaction flask in an ice bath. 10 mL of a saturated aqueous solution of Rochelle salt was added to the mixture, and the solution was filtered through paper and a Buchner funnel. The phases were separated and the aqueous phase extracted four times with 10 mL ether. The volume of the combined ethereal phases was reduced to about 20 mL, and was then extracted three times with 10 mL 3 N HCl. The combined aqueous phases were then made basic with aqueous NaOH, and extracted with three 50 mL portions of ether. The ethereal phases were dried using sodium sulfate, and the ether removed in vacuo. The residue was Kugelrohr distilled using an oil pump operating at full vacuum (ca. 100⁰ Kugelrohr temperature), yielding 2.912 g (73%) of the desired product, a slightly yellowish oil.

¹H NMR (90 MHz, CDCl₃):

1.18 (3H, pair of overlapping d, J = 6.1 Hz, CCH₃)
1.73 (2H, s, NH₂)
2.76 - 3.67 (7H, m with sharp s at 3.32, CH₂OCH₃, CHOBzl, CHNH₂)
4.18 - 4.72 (2H, m, OCH$_2$Ph)  
7.30 (5H, s, ArH)  

**N$_2$O-bis(p-bromobenzoyl)-3-amino-4-methoxy-2-butanol**  
(Bis Derivative 19)  

158 mg (0.756 mmol) benzyl alcohol amine 18 was dissolved in 1.3 mL absolute ethanol. 61 mg 10% palladium on carbon and 0.26 mL concentrated HCl were added. The mixture was hydrogenated on a Parr apparatus for 3.5 h at 50 psi. The catalyst was filtered, the solvent removed *in vacuo*, and the residue pumped on using a high vacuum pump. The product amino alcohol hydrochloride was an exceedingly hygroscopic oil; attempts to isolate the free base through titration with aqueous barium sulfate and through ion exchange methods were unsuccessful. $^1$H NMR of the hydrochloride form indicated that debenzylation had occurred, however.  

1 mL dry benzene was added to the rather insoluble amino alcohol hydrochloride. 413 mg p-bromobenzoyl chloride (1.88 mmol, 2.44 equiv based on quantitative formation of amino alcohol 14 in the hydrogenation step) was then introduced. The mixture was refluxed for 24 h, after which only a small amount of insoluble material remained. After the benzene was removed *in vacuo*, the residue was taken up in 10 mL chloroform and extracted with 5 mL saturated aqueous NaHCO$_3$. The organic phase was dried using MgSO$_4$,
and evaporated in vacuo. The residue was taken up in CH₂Cl₂ and subjected to preparative TLC (80:45 hexanes:EtOAc, silica, UV). Two bands very close to each other were eluted giving a total yield of 283 mg (66%) of the product as a mixture of diastereomers. The diastereomers could be separated by preparative TLC (100:45 hexane:EtOAc, silica, developed successively until diastereomers are separated as shown by UV). The lower diastereomer had the same R_f as the threo compound obtained from derivatization of the hydrogenolysis products of elaiomycin. It could be crystallized with difficulty from benzene-hexane, mp = 109-111°. The erythro diastereomer could be crystallized more easily from benzene, mp = 175-177°.

¹H NMR (90 MHz, CDCl₃) of threo diastereomer:
1.42 (3H, d, J = 6.6 Hz, CCH₃)
3.34 (3H, s, OCH₃)
3.52 - 3.72 (2H, m, CH₂OMe)
4.33 - 4.63 (1H, m, CHNHCOPh)
5.24 - 5.67 (1H, m CHOCOPh)
6.67 (1H, br d, NHCOPh)
7.44 - 7.96 (8H, m, ArH)

¹H NMR (90 MHz, CDCl₃) of erythro diastereomer:
1.44 (3H, d, J = 6 Hz, CCH₃)
3.32 (3H, s, OCH₃)
3.40 - 3.76 (2H, m, OCH₂)
4.38 - 4.72 (1H, m, CHNHCOPh)
5.16 - 5.50 (1H, m, CHOCOPh)
6.82 (1H, br d, NH)
7.44 - 8.00 (8H, m, ArH)

High Resolution Mass Spectrum Report

Observed ion 1 = M⁺ C_{19}H_{19}NO_{4}^{79}Br_{2}
Expected mass (amu) = 482.967985
Observed mass (amu) = 482.9689

Observed ion 2 = M⁺ C_{19}H_{19}NO_{4}^{79}Br_{81}Br
Expected mass (amu) = 484.965985
Observed mass (amu) = 484.9667

Observed ion 3 = M⁺ C_{19}H_{19}NO_{4}^{81}Br_{2}
Expected mass (amu) = 486.963985
Observed mass (amu) = 486.9645

N-(n-octyl)-p-bromobenzamide (20)

This reaction was carried out on the acetate salt of n-octylamine in order to simulate the anticipated derivatization of the acetate salt of n-octylamine afforded by hydrogenolysis of elaiomycin in acetic acid.

To 69 mg (0.518 mmol) 97% n-octylamine was added 0.05 mL (0.85 mmol) glacial acetic acid. After removing the excess acetic acid in vacuo, 0.8 mL dry benzene and 147 mg (0.67 mmol, 1.29 equiv) p-bromobenzoyl chloride was added. The solution was heated at 90° with stirring for 7.5 h.
Some precipitated crystalline material was observed. An additional 0.5 mL dry benzene was then added and the mixture heated an additional 1.75 h. The reaction mixture was left at 25\(^\circ\) overnight, and the benzene was then removed \textit{in vacuo}. The residue was taken up in 6 mL EtOAc, and the solution extracted twice with 3 mL 1 N HCl and twice with 3 mL saturated aqueous NaHCO\(_3\). After drying the organic phase with MgSO\(_4\), the EtOAc was removed \textit{in vacuo}, leaving a white solid. The product could be crystallized from hexane, affording 138 mg (85\%) of a white solid, mp = 69-70\(^\circ\).

\(^1\)H NMR (90 MHz, CDCl\(_3\)):

0.87 (3H, br t, CH\(_3\))

1.02 - 1.80 (12H, br s with downfield shoulder, C(CH\(_2\))\(_6\)C)

3.20 (2H, m, CH\(_2\)NH)

6.06 (1H, br s, NH)

7.44 - 7.72 (4H, m, ArH)

High Resolution Mass Spectrum Report

Observed ion 1 = M\(^+\) C\(_{15}\)H\(_{22}\)NO\(_{79}\)Br

Expected mass (amu) = 311.088430

Observed mass (amu) = 311.0888

Observed ion 2 = M\(^+\) C\(_{15}\)H\(_{22}\)NO\(_{81}\)Br

Expected mass (amu) = 313.086430

Observed mass (amu) = 313.0870
Hydrogenolysis of Elaiomycin and Derivatization of Product Amines

To 7.5 mg (0.029 mmol) elaiomycin in a 5 mL flask was added 0.5 mL glacial acetic acid and 1.3 mg PtO₂. The flask was capped with a septum stopper, and a balloon filled with hydrogen was connected to the flask by means of a needle inserted through the septum. The air in the flask was purged for several minutes, using a second needle as an exit port. The exit needle was then removed. The balloon was refilled with hydrogen and reconnected to the reaction flask. The reaction was stirred under hydrogen for 2.2 h, after which TLC (1:1 hexane:EtOAc, silica, UV) showed no elaiomycin left. Products identical in Rf to authentic n-octylamine and the synthetic amino alcohol were shown to be present by TLC (BAW 4:1:5, upper phase, silica, ninhydrin). The catalyst was removed by filtration, and the filtrate evaporated in vacuo to yield a residue containing the acetate salts of the two amines.

The mixture of the acetate salts of the two product amines was dissolved in 0.5 ml dry benzene, and 42 mg (0.19 mmol) recrystallized p-bromobenzoyl chloride was added. The solution was refluxed with stirring 22 h. After this period neither of the starting amines was observed by TLC. After the benzene was removed in vacuo, the residue was taken up
in methylene chloride and subjected to preparative TLC (50:80 EtOAc:hexane, silica, UV). The use of synthetic 19 and 20 as standards enabled identification of the desired products on the chromatogram. The yield of bis derivative 19 was 3.8 mg (27.0% from elaiomycin) and that of octyl derivative 20 was 3.2 mg (35.3% from elaiomycin).

\[ \text{N-}(p\text{-bromobenzoyl})\text{-3-amino-4-methoxy-2-butanol} \]

(Mono derivative 23)

To 34 mg (.054 mmol) of the three diastereomer of the bis derivative 19 was added 1 mL (1.34 equiv) of an ethoxide solution prepared from 12 mL absolute ethanol and 18 mg sodium. After stirring with exclusion of moisture at room temperature for 15 min, the yellowish solution was taken to pH 1 using aqueous 1 N HCl, the solution becoming colorless in the process. The solution was taken to dryness in vacuo, 1 mL water was added, and the mixture was extracted three times with 2 mL ether. The combined ethereal extracts were dried with MgSO\(_4\), evaporated in vacuo, and subjected to preparative TLC (1:9 hexanes:EtOAc, silica, UV), affording 17 mg (80.8%) of the three diastereomer of alcohol 23. It could be crystallized with difficulty from ether-hexane, affording short white needles, mp = 129 - 131°.
By running this reaction with the unseparated diastereomers of bis derivative 19, a 70% yield of the alcohol 23 as a mixture of diastereomers could be obtained. The diastereomeric mixture of 23 gave a similar $^1$H NMR as the threeo diastereomer, with the C-methyl group becoming a pair of overlapping doublets, and the NH signal appearing as a broad "triplet." The diastereomeric mixture of 23 was suitable for conversion to the ketone and subsequent crystallization to constant specific activity (in the case of the [2-¹⁴C]-acetate experiment), because oxidation to the ketone eliminates one of the asymmetric centers. Therefore, 19 derived from elaiomycin obtained from the [2-¹⁴C]-acetate administration was diluted with the unseparated diastereomers of synthetic 19.

$^1$H NMR (90 MHz, CDCl₃) of threeo diastereomer:

1.32 (3H, d, J = 5 Hz, CCH₃)
3.38 (3H, s, OCH₃)
3.52 - 4.02 (5H, m, CHO, OCH₂, CHNHCOAr)
6.92 (1H, br d, NH)
7.48 - 7.90 (4H, m, ArH)

High Resolution Mass Spectrometry Report

Observed ion 1 = $^+$ C₁₂H₁₆NO₃$^{79}$Br
Expected mass (amu) = 301.031300
Observed mass (amu) = 301.0307
Observed ion 2 = $^+$ C₁₂H₁₆NO₃$^{81}$Br
Expected mass (amu) = 303.029300
Observed mass (amu) = 303.0289

\textbf{N-(p-bromobenzoyl)-3-amino-4-methoxybutanone (Ketone 24)}

34.5 mg (0.115 mmol) diastereomeric 23 was dissolved in 0.7 mL methylene chloride. 150 mg (0.696 mmol) pyridinium chlorochromate, prepared\textsuperscript{37} in the past 24 h period, was added. The reaction flask was capped and stirred for 15.25 h, with a black precipitate forming within the first hour. 3 mL ether were added, and the supernatant solution was passed through a short plug of 2.4 g Florisil, which retained the colored chromium salts. The black gum left from the reaction was triturated with 2 mL ether five times, and the extracts were also run through the Florisil. The clear eluate was evaporated \textit{in vacuo} and purified using preparative TLC (EtOAc, silica, UV), affording 25 mg (72.4\%) of ketone 24. The product could be crystallized from benzene-cyclohexane, giving small white prisms, mp 131-132\°.

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

2.31 (3H, s, CH\textsubscript{3}C=O)
3.37 (3H, s, OCH\textsubscript{3})
3.67 - 4.06 (2H, q of d, ABX system,
J = 3.5 Hz, 10.1 Hz, OCH\textsubscript{2})
4.72 - 4.92 (1H, m, CHNH)
7.18 (1H, br d, NH)
7.52 - 7.78 (4H, m, ArH)

High Resolution Mass Spectrometry Report

Observed ion 1 = \( M^+ \text{C}_{12}\text{H}_{14}\text{NO}_3 \text{Br}^{79}\)
Expected mass (amu) = 299.015650
Observed mass (amu) = 299.0150

Observed ion 2 = \( M^+ \text{C}_{12}\text{H}_{14}\text{NO}_3 \text{Br}^{81}\)
Expected mass (amu) = 301.013650
Observed mass (amu) = 301.0132

Kuhn-Roth Oxidation of Ketone 24

21 mg ketone 24 (0.070 mmol) was placed in a 25 mL three-neck flask fitted tightly with a reflux condenser and an addition funnel having a Teflon stopcock and no side arm. A ground glass stopper was tightly inserted in the remaining neck of the reaction flask. No grease was used for any of the joints. A solution 1.8 g CrO₃, 7.2 mL water, and 1.8 mL concentrated sulfuric acid was admitted through the addition funnel, and the mixture was refluxed 40 min. After allowing the mixture to cool, the reflux condenser was replaced with two Kjeldahl traps connected in series and wrapped in glass wool. The traps, in turn, were connected to a water-jacketed distillation condenser and a receiving flask cooled in an ice bath. As before, no joints were greased.
The reaction mixture was distilled at an oil bath temperature of 190-200°C, adding water from the addition funnel as necessary to maintain an 8-10 mL volume of liquid in the three-neck flask. About 50 mL of distillate was collected. The distillate was very slightly yellow, indicating a small amount of chromic acid may have distilled over. After adding one drop 1% ethanolic phenolphthalein to the cold distillate, dilute aqueous K₂CO₃ (prepared from 200 mg K₂CO₃ and 25 mL water) was added until a permanent pink color was produced. The titrated distillate was then frozen by swirling in a dry ice-acetone bath, and lyophilized. The remaining fluffy purple solid (24 mg) was dissolved in water, transferred to a 5 mL flask, and relyophilized.

To the dry solid, 20 mg (0.072 mmol) p-bromophenacyl bromide, 3 mg dibenzo-18-crown-6, and 1.2 mL acetonitrile (dried overnight over 3Å molecular sieve) were added. The mixture was refluxed for 1 h. A white solid was filtered out, and the filtrate evaporated in vacuo to a residue which was subjected to preparative TLC (1:9 EtOAc:hexane once, then 10:45 EtOAc:hexane twice, silica, UV) to yield 14 mg (83%) p-bromophenacyl acetate (25). The product could be crystallized from hexane, giving fluffy white crystals, mp = 86°C, lit.38 = 85°C.
(3-\textsuperscript{13}C)-DL-Serine\textsuperscript{27}

To 1.475 g (6.79 mmol) diethyl acetamidomalonate was added a solution of 0.21 g (6.77 mmol) (\textsuperscript{13}C)\textsubscript{-}formaldehyde (90 atom% \textsuperscript{13}C) in ca. 1.5 mL water. 95 microliters 1 N NaOH were added and the solution was stirred for 5.25 h. 10.2 mL 1.4 N NaOH was then introduced and the mixture was stirred for 46.5 h. After this period, 2.8 mL glacial HOAc was added, and the solution was refluxed 1 h. The solvent was then evaporated \textit{in vacuo} and the oily residue pumped on using an oil pump operating at full vacuum. To remove residual water, the residue was dissolved in absolute EtOH, the EtOH was evaporated \textit{in vacuo}, and the residue pumped on once again. White solid material, presumably a mixture of NaCl and serine hydrochloride, began precipitating.

The residue was leached with 20 mL absolute EtOH by swirling the mixture as the EtOH refluxed. The solution was filtered through a sintered funnel. This leaching procedure was repeated twice. The first filtrate was orange, and the next two were colorless. An additional 10 mL leaching gave a barely positive ninhydrin test; therefore, it was concluded that the separation of serine ethyl ester from the NaCl was complete. The combined leachings were evaporated to an oil \textit{in vacuo} and pumped on. 3.4 mL concentrated aqueous HCl was added to the residue, and the mixture was refluxed with stirring for 1 h. The orange-brown solution
was then refluxed with 20 mg acid-washed charcoal; the charcoal was filtered out and washed with 3 mL hot water three times. The charcoal treatment was repeated, giving a yellowish solution which was concentrated in vacuo. The concentrated solution of serine hydrochloride was run down a 16 cm column of 23 g Amberlite IRA-45 weakly basic ion exchange resin (16 - 50 mesh, free base form), using distilled water as the eluant. The first two column volumes removed most of the product, which was detected by ninhydrin. The eluate containing the serine was lyophilized, leaving a pale yellow solid. This material was recrystallized from water-ethanol, using the solvents in a 2:5 ratio of volumes. The crystals were washed with absolute EtOH, followed by ether. The total yield of two crops was 390 mg (54.8% from formaldehyde). The first crop of crystals was pale yellow and shiny, and the second was rather amorphous. The labeled product gave a single spot identical to authentic serine by TLC (BAW 25:4:10, cellulose, ninhydrin detection). When it was added to an NMR tube containing unlabeled serine in D₂O, the ¹³C signal at 60.65 ppm became enhanced. No new ¹³C signals were observed.
**n-Heptyl Mesylate**

1.52 mL (1.25 g, 10.75 mmol) n-heptanol and 2.3 mL (1.67 g, 16.5 mmol) distilled triethylamine were dissolved in 30 mL methylene chloride. The mixture was cooled to near 0º using an ice bath and 0.93 mL (1.38 g, 12.02 mmol) mesyl chloride was added dropwise with stirring. White triethylamine hydrochloride precipitated. Stirring at near 0º was continued for 40 min. The reaction mixture was then extracted successively with 25 mL 0.1 N HCl, 10 mL saturated aqueous NaHCO₃, and 10 mL water. The organic phase was dried with MgSO₄ and the ether was evaporated in vacuo to yield 1.98 g (94%) of the desired product, a colorless oil.

1H NMR (90 MHz, CDCl₃):

- 0.87 (3H, br t, J = 6 Hz, terminal CH₃)
- 1.08 - 1.92 (10H, m, C(CH₂)₅C)
- 2.93 (3H, s, OSO₂CH₃)
- 4.20 (2H, t, J = 6 Hz, CH₂OMs)

**(1-¹³C, ¹⁵N)-n-Heptyl Cyanide**

The following procedure is an adaptation of the method of Reeves and White.³⁹ 240 mg K¹³C¹⁵N (3.58 mmol, 90.6 atom% ¹³C, 99.4 atom% ¹⁵N) was dissolved in 4.59 mL water. 676 mg (3.48 mmol) n-heptyl mesylate and 154 microliters distilled tributylamine was added. The mixture was refluxed for 3.25
h, and then allowed to cool. The mixture was extracted twice with EtOAc. The combined organic phases were washed once with 1 N HCl, and then dried with MgSO₄. Evaporation of the EtOAc in vacuo afforded 488 mg crude material, which was Kugelrohr distilled at 100°C (ca. 24 mm) to yield 391 mg (88%) of the labeled nitrile. The product had an identical GC retention time as authentic nitrile and showed a strong doublet in its ¹³C spectrum at 119.57 ppm (J = 16.6 Hz, d₆-benzene). Its ¹³C NMR spectrum was otherwise identical to that of authentic n-heptyl cyanide.

(1⁻¹³C,⁻¹⁵N)-n-Octylamine Hydrochloride

The following procedure is an adaptation of the method of Secrist and Logue.⁴⁰

391 mg (3.08 mmol) doubly labeled n-heptyl cyanide was dissolved in 77 mL absolute EtOH. 1.55 mL (2.31 g, 19.3 mmol) chloroform and 88 mg PtO₂ was added. The mixture was hydrogenated for 9.75 h on a Parr hydrogenation apparatus at 45 psi. The mixture was filtered through a fritted funnel using Celite, and the filter cake washed with absolute EtOH. The filtrate was evaporated in vacuo at a temperature below 35°C to yield a residue which was crystallized from ethanol-ether. The total yield of two crops was 373 mg (72%) of the colorless amine hydrochloride, identical in TLC behavior to authentic material (BAW 4:1:5, upper phase,
silica, ninhydrin). When a small amount of the product was added to an NMR tube containing an authentic sample of n-octylamine hydrochloride in D₂O, a strong doublet arose about the ¹³C signal at 41.87 ppm (J = 4.9 Hz); no other new ¹³C signals were observed.

(2-¹³C,¹⁵N)-Glycine Ethyl Ester Hydrochloride

To 1.006 g (13.1 mmol) (2-¹³C,¹⁵N)-glycine, previously dried overnight in vacuo at 70°C over phosphorus pentoxide, was added 10 mL absolute EtOH saturated with dry HCl. The mixture was refluxed with stirring for 2.5 h. Upon cooling, a white crystalline mass formed, which was collected on a sintered glass funnel and rinsed with 2 mL cold absolute EtOH. The crystals were dried over phosphorus pentoxide at 70°C and weighed about 1.6 g. Evaporation of the mother liquor in vacuo below 35°C, pumping on the residue to remove as much HCl as possible, and crystallization from absolute EtOH-ether afforded two more crops of crystals for a total yield of 1.798 g (97.3%) of the labeled product, mp = 143°C, lit. ¹⁴ = 142-143°C.

Ethyl (2-¹³C,¹⁵N)-Hippurate

To 1.798 g (12.71 mmol) labeled glycine ethyl ester hydrochloride was added 9.3 mL benzene previously distilled from sodium. 1.64 mL (1.99 g, 14.1 mmol) distilled benzoyl
chloride was added dropwise to the stirred suspension and the mixture was refluxed for 18 h. The clear yellowish solution was then evaporated in vacuo and Kugelrohr distilled at ca. 175° using an oil pump. The very viscous distillate, which smelled of benzoyl chloride, was crystallized from ether. The crystals were washed with petroleum ether (bp 35 - 60°) to remove traces of benzoyl chloride. The combined yield of two crops and product obtained by preparative TLC of the mother liquor (1:1 hexane:EtOAc, silica, UV) afforded 2.547 g (96%) of the product, mp = 58-59°, lit.41 = 59-61°.

¹H NMR (90 MHz, CDCl₃):

1.33 (3H, t, J = 6 Hz, CH₃)
4.17 - 4.41 (4H, m, OCH₂ and CH₂C=O)
6.68 (1H, br s, NH)
7.38 - 7.96 (5H, m, ArH)

**Ethyl (2-¹³C,¹⁵N)-2-Formylhippurate (28)**

11.3 g (49.1 mmol) sodium was dissolved in 24.8 mL ethanol previously distilled from magnesium ethoxide. The sodium ethoxide solution was cooled in an ice bath and 4.0 mL (3.67 g, 4.96 mmol) purified²³ ethyl formate was added dropwise with stirring over 15 min. Stirring was stopped, and the solution was allowed to stand for 3.7 h at room temperature. A small amount of gas was evolved and a small
amount of a white solid precipitated over this period. 2.547 g (12.2 mmol) ethyl (2-$^{13}$C, $^{15}$N)-hippurate was then added in several portions with stirring. The solution was allowed to stand at room temperature without stirring, protected from moisture. After 24 h, the reaction mixture, which had become a thick orange slurry, was chilled in an ice bath for 45 min. The chilled mixture was filtered through a sintered glass funnel and sucked quite dry. The filter cake was washed successively with 10, 7, and 5 mL anhydrous ether, and then with 10 and 7 mL petroleum ether. The cream colored sodio derivative of the desired product was then dried in vacuo at 25°C.

The dry sodio derivative thus obtained was dissolved in 25 mL water, and acidified with concentrated HCl to pH 3, liberating a gummy oil. The gum was extracted with difficulty into 20 mL ether, and the aqueous phase was extracted three more times with 20 mL ether. After evaporation in vacuo to a volume of about 30 mL, the combined ethereal phases were dried with MgSO$_4$ and suction filtered through a sintered glass funnel; the limited solubility of the product in ether and the volatility of ether dictated a reasonably rapid filtration. In vacuo removal of the ether afforded 2.243 g (77.7%) of the crude product, a thick amber oil. The product gave a strong
positive FeCl₃ test,⁴⁴ indicating the presence of an enol moiety.

¹H NMR (90 MHz, CDCl₃):

1.29 (3H, t, CH₃)
4.20 (2H, q, CH₂)
4.60 - 6.00 (1H, br absorption with br s at 5.15, C=CHOH)
7.15 - 8.00 (6H, m, ArH, NH)

N-Benzoyl (2-¹³C,¹⁵N)-Serine Ethyl Ester

2.243 g (9.46 mmol) crude doubly labeled ethyl 2-formylhippurate was dissolved in 32 mL ether saturated with water. 1.7 g freshly prepared aluminum amalgam⁴⁵ was added, and the mixture stirred. On each of the next four days 2-3 drops of water were added to the mixture, and ether was added as necessary to maintain an approximately constant volume. On the fourth day, 1.7 g aluminum amalgam of the same lot used to start the reaction were rinsed in water, found to be active (evolution of hydrogen), and added wet to the reaction mixture. On the fifth day, two more drops of water were added. On the sixth day, a FeCl₃ test⁴⁴ of the solution gave a very weak violet color, indicating near-complete reduction of the enolic starting material. The opaque, grey reaction mixture was suction filtered through a medium porosity sintered glass funnel, with the
product crystallizing directly from the filtrate. The grey sludge remaining in the funnel was washed with ether several times. The sludge was then refluxed 30 min with 25 mL ether, filtered, and the process repeated. This leaching procedure typically afforded more product than the initial filtrate of the reaction mixture. The sludge could subsequently be leached with a mixture of 25 mL ether and 5 mL EtOAc and with a mixture of 25 mL ether and 10 mL EtOAc, but these leachings recovered much less product than the previous leachings, and contained some material giving a positive FeCl₃ test. The product was crystallized from ether, with the mother liquor being subjected to preparative TLC (3:1 EtOAc:hexane, silica, UV). The total yield was 1.472 g (65.1%), mp = 81°, lit.⁴¹ = 80°.

(2-¹³C,¹⁵N)-DL-Serine Hydrochloride

1.472 g (6.16 mmol) labeled N-benzoyl serine ethyl ester was refluxed with 29.3 mL 7% aqueous HCl with stirring for 12 h. Benzoic acid precipitated upon cooling. The mixture was extracted once with 12 mL ether and twice with 3 mL ether. The very acidic aqueous phase was concentrated in vacuo to a volume of ca. 10 mL, and then run down a short column of 6.5 g Amberlite IRA-45 weakly basic anion exchange resin (16 – 50 mesh, free base form); this treatment raised the pH of the solution to between 3 and 4. Lyophilization
of the solution yielded a pale yellow powder. The aqueous phase showed only one spot upon TLC analysis, identical in Rf to authentic serine (BAW 25:4:10, cellulose, ninhydrin). The product gave a positive test for chloride ion when treated with aqueous silver nitrate. When a sample of the product was added to an NMR sample of authentic serine in D2O, a strong doublet (J = 6.1 Hz) arose around the 13C signal at 56.74 ppm. No new 13C signals were observed. The yield of labeled serine hydrochloride was 680 mg (76.9% for the hydrolysis step, 36.3% from glycine).

Preparation of Sporulation Agar Slants of S. gelaticus

To 500 mL glass-distilled water were added 0.5 g Difco beef extract, 0.5 g BBL yeast extract, 1.0 Difco tryptose, 5.0 g D-glucose, 7.5 g Difco agar, and a trace of FeSO4. The mixture was stirred in a one-liter flask while heated in a hot water bath. Complete dissolution of the agar did not occur, but the material became suspended well enough for the mixture to be conveniently delivered via a pipet to 150x20 mm screw cap culture tubes. 8-9 ml of the suspension was added to each culture tube. The hole at the delivery end of the pipet was enlarged by breaking off the narrow section at that end of the pipet. The culture tubes were then closed, and the caps loosened one-half turn. The tubes were autoclaved 121° for 20 min. Autoclaving clarified the agar
mixture in the tubes. Soon after autoclaving, the culture tubes were tilted in a test tube rack and allowed to cool. The tilt angle was chosen so that the agar remained 5 cm below the mouth of the tube.

The agar slants were inoculated from *S. gelaticus* slants obtained from Parke-Davis. 2 mL sterile glass-distilled water were added to a Parke-Davis slant, and a spore suspension was produced using a loop. 0.1 mL of the spore suspension was then added to each of several sporulation agar slants using sterile technique. Once the first suspension is exhausted, 2 more mL of sterile water was added to the Parke-Davis slant. The slant was vortexed and the resulting suspension of the remaining spores used to similarly inoculate more slants. The inoculated slants were manually rotated to coat as much of the agar surface as possible with the spore suspension. The slants were then statically incubated at 28°C for 1 week in a GCA/Precision Scientific shaker bath. Grey growth and darkening of the agar was noted. The slants were then stored at 4°C in a refrigerator until used.

**Preparation of Fermentation Medium for *S. gelaticus***

To 2000 mL glass-distilled water were added 20 g maltose, 10 g Vita-Food debittered Brewers yeast, 10 g reagent grade NaCl, and 10 g Difco Casamino acids. All
components but the yeast were brought into solution by stirring, and the pH was taken to 7.5 using concentrated aqueous NaOH. 200 mL of the medium was added to each of ten one-liter flasks containing 200 mg CaCO$_3$. The flasks were then plugged with foam stoppers and autoclaved at 121° for 20 min and set aside to cool until inoculated.

Inoculation and Fermentation Procedure for S. gelaticus

To each of 2 sporulation agar slants of S. gelaticus were added 10 mL sterile glass-distilled water. The spores of each slant were suspended using a loop, and then vortexed. The spores, which are quite resistant to wetting, were then distributed about evenly among the 10 flasks prepared as described above. Sterile technique was maintained throughout the inoculation procedure.

The flasks were then shaken at 200 rpm in a New Brunswick G-25 or G-25R incubator shaker for the length of the fermentation period. The temperature was maintained as closely to 26° as possible.

Typical Administration of Labeled Compounds

The labeled compound was taken up in about 10 mL distilled water and the solution drawn into a 10 mL sterile disposable syringe. The solution of labeled material was then distributed about equally among 10 flasks by passage
through a sterile Luer-Lok-fitting Millipore filter disc having a 45 micron pore size. The filter disc was then removed, and several milliliters of water were drawn into the syringe. The filter disc was replaced, and the extra water used to rinse any compound residing in the "dead volume" of the filter disc into one of the flasks. Sterile technique was maintained throughout the entire feeding procedure.

Isolation of Elaiomycin

72 or 96 h after inoculating the flasks, the now-murky fermentation was worked up. The contents of the flasks were centrifuged in polyethylene bottles fitted with sealing tops using a Sorvall-Dupont GS-3 rotor at 7000 rpm for 20 min. The supernatant was decanted through a piece of Miracloth set in a large glass funnel. The Miracloth was supported by a wad of glass wool inserted at the top of the funnel's stem. The brown decantate was brought to pH 7.0 using 1 N HCl. The liquid was then extracted three times with 300 mL EtOAc, in the case of the usual 10-flask fermentation described above. Typically, little organic phase separated in the first extraction. The combined extracts contained a significant amount of an emulsion, which was separated by repeated vacuum filtration through Celite using a coarse porosity sintered funnel. The slower the filtration was
carried out, the better the phases separated. The combined organic phases were evaporated in vacuo to a volume of ca. 50 mL at or near room temperature. They were then dried with MgSO₄, gravity filtered, and evaporated in vacuo. The residue was then subjected to TLC (1:1 hexane:EtOAc, silica, UV). Elaiomycin has an Rf of ca. 0.5. If a ¹³C spectrum was to be taken of the elaiomycin, it was found to be a good precaution to use distilled EtOAc to elute the antibiotic from the silica gel. This procedure minimized the problem of spurious signals in our ¹³C spectra.

**Large Scale Fermentation of S. gelaticus**

In order to obtain elaiomycin with which to dilute material obtained from precursor incorporation experiments, we found it convenient to conduct large scale fermentations of *S. gelaticus*.

660 mL of fermentation medium was prepared as above, reducing all components proportionally. Approximately 220 mL of the medium was placed in each of three one-liter flasks containing 220 mg CaCO₃. The flasks were closed with foam plugs, autoclaved at 121°C for 20 min, and then allowed to cool.

10 mL of sterile water was added to sporulation agar slants of *S. gelaticus*, and the spores were suspended as above. 3 mL of this suspension was then added to each of
the three flasks of sterile fermentation medium, using sterile technique. The cultures were then set in an incubator shaker set at 160 rpm and 26-27°C.

Two days after the start of this fermentation, 10 L of the usual fermentation medium was prepared as above, increasing all components proportionally. This was poured into the fermentation vessel of a New Brunswick Model MF-128S Microferm fermentor through a large funnel inserted through the inoculation port. The pH of the medium was adjusted to 7.5 by addition of concentrated NaOH through the inoculation port. The pH could be conveniently monitored by testing the pH of samples withdrawn from the bulk of the medium through the micro sampling port. The inoculation port was closed and the medium was sterilized at 121°C for ca. 30 min, and then allowed to cool overnight to 25°C.

68 hr after the three one-liter flasks were inoculated, these cultures were used to inoculate the medium in the Microferm fermentor. The inoculum was poured through a large funnel set in the inoculation port. The mouths of the three flasks were flamed prior to pouring, and the funnel had been previously sterilized in an autoclave. This larger fermentation was allowed to progress four days, sparged at a rate of 11L/min at 25°C. The impeller was set at 200 rpm, and antifoam agent was added a drop at a time in a non-sterile manner through the inoculation port as needed.
over the course of the fermentation. The fermentation was then worked up in the usual manner.
REFERENCES

1. This species of Streptomyces was initially called S. hepaticus, and this name has persisted in the most recent literature. See L. E. Anderson, J. Ehrlich, S. H. Sun, and P. R. Burkholder, Antibiot. Chemother., 6, 100 (1956).


14. Several other glycosides of methylazoxymethanol have been isolated from cycads in small quantities. These compounds are called neocycasins and are presumed to have similar biological effects as cycasin and macrozamin if in vivo hydrolysis occurs. See ref. 13(c).


