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.
ABSTRACT

THE BIOSYNTHESIS OF ASPARAGUSIC ACID
IN ASPARAGUS OFFICINALIS

ANITA E. MIZUSAWA

Asparagusic acid is a naturally occurring 1,2-dithiolane that has been isolated from both the roots and edible portions of Asparagus plants (Asparagus officinalis L.). The substance is a plant growth inhibitor exerting activity comparable to abscisic acid. Asparagusic acid also possesses potent nematicidal activity. The mode of biosynthesis of the 1,2-dithiolane ring of lipoic acid suggests that asparagusic acid may be biosynthesized from isobutyric acid. [1-\textsuperscript{14}C]Isobutyric acid was administered and found to be specifically incorporated into asparagusic acid. Doubly labelled forms of isobutyric acid were administered and the results show that Asparagus officinalis does not biosynthesize sulfur containing compounds via the same mechanism that is available to bacteria and fungi. Several sulfur containing compounds, [2-\textsuperscript{3}H, 1-\textsuperscript{14}C]-sodium 3-mercaptopoisobutyrate, [\textsuperscript{35}S, 3-\textsuperscript{3}H]-sodium 3-mercaptopoisobutyrate and [\textsuperscript{35}S, 3-\textsuperscript{3}H]S-(2-carboxy-n-propyl)-L-cysteine were synthesized and administered and the biosynthesis of asparagusic acid in Asparagus officinalis was determined.
to my brothers, for their love and support
Acknowledgements

I would like to thank my research advisor, Dr. Ronald J. Parry, for his guidance and support. His consistently pleasant nature made working for him very enjoyable. Dr. Parry's knowledge, insight, and dedication make him a valuable chemist and an asset to Rice University.

Many thanks go to Dr. M. V. Naidu for generously sharing his knowledge and expertise. His constant companionship in the lab made my four years of graduate school a very peaceful and happy existence.

I would also like to thank the organic faculty, Dr. Lewis, Dr. Engel, Dr. Fukuyama, and Dr. Billups, for encouraging me to continue my education in chemistry.

My greatest appreciation goes to my family, friends, and the Rice gym for keeping me sane and healthy for the past four years.

The National Science Foundation and the Robert A. Welch Foundation are gratefully acknowledged for providing the funds for this research.
# TABLE OF CONTENTS

Acknowledgements.................................................................................. iv  
List of Tables ......................................................................................... vii  
List of Schemes ....................................................................................... viii  
List of Figures ......................................................................................... ix  
Introduction ............................................................................................. 1  
Results and Discussion ........................................................................... 26  
Experimental .......................................................................................... 57  
  Administration of Radiolabelled Precursors ............................................ 59  
  Isolation of S-(2-Carboxy-n-propyl)cysteine from *Asparagus officinalis* ................................................................. 60  
  Synthesis of Ethyl Bis(hydroxymethyl)malonate ..................................... 61  
  Preparation of 95% Phosphoric Acid ...................................................... 62  
  Synthesis of α-(Iodomethyl)acrylic Acid ................................................ 62  
  Preparation of Aqueous Sodium Trithiocarbonate .................................. 64  
  Synthesis of Dihydroasparagusic Acid .................................................... 64  
  Synthesis of Asparagusic Acid ............................................................... 65  
  Synthesis of S,S′-Bis(p-phenylbenzyl)dihydroasparagusic Acid .......... 66  
  Synthesis of S,S′-Bis(p-phenylbenzyl)dihydroasparagusic Acid Anilide .... 68  
  Preparation of Raney Nickel Catalyst ..................................................... 69  
  Desulfurization of S,S′-Bis(p-phenylbenzyl)dihydroasparagusic Acid Anilide ................................................................. 70  
  Hydrolysis of Isobutyric Acid Anilide .................................................... 70  
  Schmidt Degradation of Isobutyric Acid ................................................ 71  
  Synthesis of Isopropylbenzamide ........................................................ 72  
  Schmidt Degradation of S-(2-Carboxypropyl)-L-cysteine ....................... 73  
  Synthesis of S-(2-Aminopropyl)-L-cysteine ......................................... 74  
  Synthesis of n-Butyl Propionate .......................................................... 75
**TABLE OF CONTENTS**

**Experimental**

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthesis of [3-3H]Trifluoroacetic Acid</td>
<td>76</td>
</tr>
<tr>
<td>Synthesis of Sodium [2-3H]Isobutyrate</td>
<td>76</td>
</tr>
<tr>
<td>Synthesis of Sodium [3-3H]Isobutyrate</td>
<td>78</td>
</tr>
<tr>
<td>Synthesis of [1-14C]Methacrylic Acid</td>
<td>80</td>
</tr>
<tr>
<td>Synthesis of Sodium [1-14C]3-Mercaptobisobutyrate</td>
<td>81</td>
</tr>
<tr>
<td>Synthesis of Sodium [2-3H]3-Mercaptobisobutyrate</td>
<td>82</td>
</tr>
<tr>
<td>Synthesis of Methyl 2,3-Dibromoisobutyrate</td>
<td>83</td>
</tr>
<tr>
<td>Synthesis of 3-Bromo-2-Methylpropenoic Acid</td>
<td>84</td>
</tr>
<tr>
<td>Synthesis of [3-3H]3-Bromo-2-Methylpropenoic Acid</td>
<td>85</td>
</tr>
<tr>
<td>Preparation of Sodium Amalgan</td>
<td>86</td>
</tr>
<tr>
<td>Synthesis of [3-3H]Methacrylic Acid</td>
<td>87</td>
</tr>
<tr>
<td>Synthesis of 3-Bromoisobutyric Acid</td>
<td>87</td>
</tr>
<tr>
<td>Synthesis of Sodium [3-3H]3-Mercaptobisobutyrate</td>
<td>68</td>
</tr>
<tr>
<td>Synthesis of Sodium [35S]3-Mercaptobisobutyrate</td>
<td>89</td>
</tr>
<tr>
<td>Synthesis of S-(2-Carboxypropyl)-L-Cysteine</td>
<td>90</td>
</tr>
<tr>
<td>Synthesis of [35S]S-(2-Carboxypropyl)-L-Cysteine</td>
<td>91</td>
</tr>
<tr>
<td>Synthesis of Isobutyric Acid Anilide</td>
<td>92</td>
</tr>
<tr>
<td>Synthesis of O,S-Bis(p-Bromophenacyl)3-Mercaptobisobutyrate</td>
<td>92</td>
</tr>
<tr>
<td>References</td>
<td>94</td>
</tr>
</tbody>
</table>
LIST OF TABLES

TABLE

I. Incorporation of Specifically Tritiate Dethiobiocytin into Biotin .................................................. 8

II. Incorporation of Labelled Octanoic Acid into Lipoic Acid .......................................................... 13

III. Precursor Incorporation Experiments in Asparagus officinalis ...................................................... 25

IV. Tabulated Results of the Precursor Incorporation Experiments ....................................................... 55
LIST OF FIGURES

FIGURE

1. Asparagusic Acid ................................................. 6
2. Lipoic Acid ....................................................... 6
3. 3-(Methylthio)isobutyric Acid ................................. 6
4. Biotin ............................................................ 6
5. Pimelic Acid ...................................................... 6
6. Dethiobiotin ...................................................... 6
7. Biotin Sulfone .................................................... 10
8. Biotin Intermediate Ruled Out .................................. 10
9. Octanoic Acid ...................................................... 10
10. p-Phenylbenzyldihydrolipoic Acid ............................... 10
11. Isobutyric Acid ................................................... 10
12. Methacrylic Acid .................................................. 20
13. 3,3'-Dimercaptoisobutyric Acid ................................ 20
14. 3-Mercaptoisobutyric Acid ....................................... 20
15. S-(2-Carboxy-n-propyl)-L-cysteine .............................. 20
16. S-(1,2-Dicarboxyethyl)-L-cysteine .............................. 20
17. Disulfide of S-(2-Carboxy-3-mercaptopropyl)-cysteine and 3-Mercaptoisobutyric Acid .......................... 21
18. Disulfide of S-(2-Carboxy-3-mercaptopropyl)cysteine .......... 21
19. Cysteine .......................................................... 21
LIST OF SCHEMES

SCHEME

I. Biosynthesis of Biotin ....................................... 5
II. Biosynthesis and Degradation
    of Lipoic Acid ........................................... 11
III. Biosynthesis of Isobutyric Acid ......................... 15
IV. Degradation of Asparagusic Acid .......................... 16
V. Synthesis of Asparagusic Acid .............................. 28
VI. Synthesis of Sodium [3-3H]Isobutyrate .................. 33
VII. Synthesis of Sodium [2-3H]Isobutyrate ................ 34
VIII. Synthesis of [1-14C]Methacrylic Acid
      and Feeding Results .................................. 38
IX. Hypothesized Biosynthetic Pathway to
     Asparagusic Acid ........................................ 40
X. Synthesis of Sodium [1-14C]3-Mercaptoisobutyrate ....... 41
XI. Synthesis of Sodium [2-3H]3-Mercaptoisobutyrate ....... 42
XII. Synthesis of Sodium [35S]3-Mercaptoisobutyrate ....... 44
XIII. Synthesis of Sodium [3-3H]3-Mercaptoisobutyrate ...... 46
XIV. Synthesis of [35S]S-(2-Carboxypropyl)-L-cysteine ...... 51
XV. Synthesis of S-(2-Carboxy-[3-3H]propyl)-L-cysteine ... 53
XVI. Elucidated Biosynthetic Pathway of Asparagusic Acid ... 54
INTRODUCTION

*Asparagus officinalis* is the most common of the 150 species that make up the genus *Asparagus*, belonging to the lily family (*Liliaceae*). The young shoots of *A. officinalis* are a popular culinary vegetable because of their flavor and their medicinal properties. The roots have been used as an aperient medicine and the fruits as a diuretic. The flavor of asparagus has been linked to the presence of sulfur-containing compounds and these compounds have also been found to be important in its defense system. Plants belonging to *Allium* also of the *Liliaceae* are known to contain a variety of sulfur containing compounds.

Thirty-two grams of a sulfur containing component was isolated from forty kilograms of asparagus aroma concentrate in 1948 by Jansen and the structure was found to be 3,3'-dimercaptopisobutyric acid. In 1972, Yanagawa et al. identified three sulfur containing acids from *Asparagus*, one being 1,2-dithiolane-4-carboxylic acid, "asparagusic acid" (1, p. 6).

Asparagusic acid (1, p. 6) is a naturally occurring 1,2-dithiolane that has been isolated from both the roots and edible portions of *Asparagus* plants (*Asparagus officinalis* L.). It has been shown to be a powerful growth inhibitor of lettuce, rice, rape, radish, and
barnyard grass. Its effects at different molar concentrations closely parallel that of abscissic acid and it is considerably more inhibitory to root elongation than lipoic acid (2, p. 6). Takasugi and coworkers found that asparagusic acid extracted from 10 year old *A. officinalis* roots inhibited second stage larva emergence from cysts of *Heterodera rostochiensis* and acted as a potent nematocide against emerged larva and adults of various worm species. This general toxic effect may be the result of lipoyl dehydrogenase inactivation by substrate competition and/or reduction of the catalytic sites of the enzyme by asparagusate. This effect is due to the presence of two sulfur atoms.

Little is known about the biosynthetic origin of dithiolanes. Asparagusic acid, because of its simplicity, appears to be a good model for determining the mechanism of sulfur introduction into the dithiolane ring structure of plants. The biological activity of asparagusic acid is heavily dependent on its disulfide linkage, which gives additional relevancy to the investigation of the mode of sulfur introduction.

In 1977, Tressl and coworkers administered [U-14C]L-valine to *Asparagus* tissue discs cut from the top of the *Asparagus* stem. The radiolabelled compound was incorporated into isobutyrate, methacrylate and into
3-(methylthio)isobutyrate (3, p. 6) and to a lesser extent into asparagus acid. [U-\textsuperscript{14}C]Cysteine and [U-\textsuperscript{14}C]acetate were not incorporated into asparagus acid. The results obtained from these experiments are inconclusive. The biosynthetic study of asparagus acid should include the whole plant, both the roots and the stems, since there is a possibility that there may be a change in the plant metabolism after cutting the plant. Furthermore, no degradation experiments were reported, so one could not tell whether these compounds were specifically incorporated or incorporated as a result of catabolism to other compounds.

The mechanism of sulfur introduction into the dithiolane and the thiophene ring structures of lipoic acid (2, p. 6) and biotin (4, p. 6) by \textit{Escherichia coli} and \textit{Aspergillus niger}, respectively, has been studied. It is appropriate to discuss biotin and lipoic acid not only because of their structural similarity to asparagus acid but also because the methodology used to study their biosynthesis can be applied to asparagus acid.

Biotin (4, p. 6), \(\text{C}_{10}\text{H}_{16}\text{O}_{3}\text{N}_{2}\text{S}\), is a necessary growth factor. It is a vitamin which animals and some microorganisms cannot synthesize, but must acquire from their diet, although some lower life forms have the ability to synthesize their own. Biotin functions as the cofactor for a number of enzymatic carboxylation reactions. It is
now well established that a majority of fungi and bacteria synthesize the vitamin from pimelic acid (5, p. 6) via the steps indicated in Scheme I (p. 5).\textsuperscript{10}

The structure of biotin was elucidated in 1942 by DuVigneaud and coworkers,\textsuperscript{11,12} and was confirmed by chemical synthesis in 1943 by Harris and coworkers.\textsuperscript{13} Evidence for the conversion of dethiobiocin (6, p. 6) to biotin (4, p. 6) was very strong. When \textit{Saccharomyces cerevisiae} was incubated with dethiobiocin, DuVigneaud\textsuperscript{14} observed that the nutritional requirement for biotin was satisfied. Iwahara\textsuperscript{15} studied the amount of biotin production stimulated by additional dethiobiocin in several hundred microorganisms. Most strains revealed small changes in biotin production but a few yeasts and bacteria and many molds showed large increases in biotin yield.

When radioactive dethiobiocin was administered to cultures of \textit{A. niger}, the biotin was isolated as biotin sulfone (7, p. 10). An incorporation figure of 1-2\%\textsuperscript{16} was obtained. Therefore, the last stage in the sequence to biotin from pimelic acid appears to involve the biosynthetic conversion of (+)-dethiobiocin to (+)-biotin. This is of interest because of the unusual formation of the thiophane ring and its potential similarity to asparagusic acid.

Several tritiated forms of dethiobiocin were synthesized by Parry and coworkers and administered to \textit{A.
Scheme I.

\[
\text{CoA-S-CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \xrightarrow{L-\text{ALa}} \text{H}_2\text{N}-(\text{CH}_3)\text{CH}_{\text{CH}}\text{CH}_2-\text{CH}_2-\text{COOH}
\]

\[
\text{H}_2\text{N}-\text{CH}_2\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \xrightarrow{\text{HCO}_3^-/\text{ATP}} \text{H}_2\text{N}-\text{CH}_2\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH}
\]

\[
\text{H}_2\text{N}-\text{CH}_2\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH} \rightarrow \text{BIOTIN}
\]
niger. The results are shown in Table I (p. 8).\textsuperscript{17,18} A number of conclusions were drawn from the data.\textsuperscript{17} Parry stated that experiments 1 and 2 clearly demonstrate that the introduction of sulfur at C-1 and C-4 of (+)-dethiobiotin takes place without the loss of hydrogen from C-2 or C-3. It therefore seems unlikely that unsaturation is introduced at C-2 or C-3 during the biosynthesis of (+)-biotin from (+)-dethiobiotin; however the possibility of enzymatic removal of hydrogen from C-2 or C-3 followed by replacement of the hydrogen without exchange cannot be excluded. Experiment 3 shows that the incorporation of [1-$^3$H]-(+)-dethiobiotin into biotin proceeds with about 30% tritium loss. The nature of the reaction associated with the oxidation of the methyl group of (+)-dethiobiotin is unknown, but the tritium loss observed in experiment 3 is consistent with the removal of one hydrogen atom from the methyl group of dethiobiotin by a process which exhibits little or no isotope effect. Experiment 4 reveals that [4(RS)$^3$H]-(-)-dethiobiotin is incorporated into (+)-biotin with about 47% tritium loss. This figure is within experimental error of that expected (50%) for stereospecific removal of one hydrogen atom from C-4 of dethiobiotin during the formation of biotin. Experiment 5 rules out the unsaturated intermediate (8, p. 10).\textsuperscript{18} Thus, it appears that two hydrogen atoms are removed from (+)-dethiobiotin as
Table I. Incorporation of Specifically Tritiated Dethiobiocin into Biotin

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Precursor</th>
<th>Precursor $^3$H/$^{14}$C</th>
<th>Biotin (Sulfone Me Ester) $^3$H/$^{14}$C</th>
<th>Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>[2,3-$^3$H, 10-$^{14}$C]-$(+)$dtb $^a$</td>
<td>6.05</td>
<td>5.74</td>
<td>95</td>
</tr>
<tr>
<td>2.</td>
<td>[3-$^3$H, 10-$^{14}$C]-$(+)$dtb $^b$</td>
<td>2.89</td>
<td>3.04</td>
<td>105</td>
</tr>
<tr>
<td>3.</td>
<td>[1-$^3$H, 10-$^{14}$C]-$(+)$dtb</td>
<td>6.88</td>
<td>4.81</td>
<td>70</td>
</tr>
<tr>
<td>4.</td>
<td>[4(RS)-$^3$H, 10-$^{14}$C]-$(+)$dtb</td>
<td>5.88</td>
<td>3.10</td>
<td>53</td>
</tr>
<tr>
<td>5.</td>
<td>[5(RS)-$^3$H, 10-$^{14}$C]-$(+)$dtb</td>
<td>5.72</td>
<td>5.33</td>
<td>93</td>
</tr>
</tbody>
</table>

a. Precursor had 58% $^3$H at C-2, 42% $^3$H at C-3.
b. Precursor had 17% $^3$H at C-2, 83% $^3$H at C-3.
the result of its conversion to (+)-biotin.

In the late 1940's, α-lipoic acid was independently discovered in several laboratories as a growth factor for a number of microorganisms. It was isolated in 1951,19,20 and its structure established to be (+)-1,2-dithiolane-3-pentanoic acid (2, p. 6) in the following year.21 The absolute configuration of (+)-α-lipoic acid was determined in 1956.22 (+)-α-Lipoic acid is widely distributed among microorganisms, plants and animals. It was found to be an essential coenzyme for all systems of α-keto acid dehydrogenase complexes which catalyse the oxidative decarboxylation of α-ketoacids.23-25 Although considerable information is available concerning the mechanism of action of lipoic acid, the information concerning its biosynthesis is fairly recent.

Parry and coworkers26 verified Reed's unpublished observation27 that octanoic acid (9, p. 10) served as a specific precursor of (2, p. 6) in Escherichia coli by administration of sodium [1-14C]octanoate to shake cultures of E. coli (Crookes strain, ATCC 8739) and isolation of lipoic acid as the bis(p-phenylbenzyl) derivative (10, p. 10). A specific incorporation of 0.17% was obtained.26 The degradation pathway used is shown in Scheme II (p. 11).

Specifically tritiated forms of octanoic acid were synthesized by Parry and coworkers and the results are shown
Scheme II.

\[ \text{E. coli} \xrightarrow{\text{Na,NH}_3} \text{S-S} \xrightarrow{\text{OCH}_3} \text{COOH} \xrightarrow{\text{NaOH}} \text{COO-Me} \xrightarrow{\text{NHCONHPh}} \text{NH}_2 \]

\[ \text{Ph} \xrightarrow{\text{CH}_2S} \text{SCH}_2 \xrightarrow{\text{Ph}} \text{COO} \xrightarrow{\text{H}_2 \text{N}_3} \text{CO}_2 \]

\[ \text{8, 6, 5} \]

\[ \text{7} \]
in Table II (p. 13). A number of conclusions were drawn from the data. Parry stated that experiments 2 and 3 clearly show that the introduction of sulfur at C-6 and C-8 of octanoic acid takes place without loss of hydrogen from C-5 or C-7. It therefore seems unlikely that unsaturation is introduced at C-5 or C-7 during the biosynthesis of (+)-lipoic acid from octanoic acid; however, the possibility of enzymatic removal of hydrogen without exchange cannot be excluded. These results are similar to those obtained during the investigation of the mechanism of sulfur introduction during biotin biosynthesis. Experiment 4 shows that the incorporation of sodium[8-3H]octanoate into lipoate proceeds without tritium loss, within experimental error. This result is presumably the consequence of a substantial tritium isotope effect associated with the removal of a hydrogen atom from C-8 of octanoate. Experiment 5 reveals that sodium [6(RS)-3H]octanoate is incorporated into lipoic acid with about 50% tritium loss. This figure is precisely that expected for the stereospecific removal of one hydrogen atom from C-6 of octanoic acid as consequence of sulfur introduction, and it parallels the results obtained when [4(RS)-3H]dethiobiotin is transformed into biotin.

Investigation of disulfide introduction in a higher plant system seems necessary if one wished to formulate a
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Precursor</th>
<th>$^{3}H/^{14}C$ for Precursor</th>
<th>$^{3}H/^{14}C$ for Lipoic Acid</th>
<th>Labeling Pattern</th>
<th>% $^{3}H$ Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[1-$^{14}C$]-9</td>
<td>-</td>
<td>-</td>
<td>&gt;90% label at C-1</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>[5(RS)-$^{3}H,1^{14}C$]-9</td>
<td>4.05</td>
<td>4.11</td>
<td>-</td>
<td>102.0</td>
</tr>
<tr>
<td>3</td>
<td>[7(RS)-$^{3}H,1^{14}C$]-9</td>
<td>3.95</td>
<td>3.81</td>
<td>-</td>
<td>96.5</td>
</tr>
<tr>
<td>4</td>
<td>[8-$^{3}H,1^{14}C$]-9</td>
<td>5.02</td>
<td>4.81</td>
<td>-</td>
<td>95.8</td>
</tr>
<tr>
<td>5</td>
<td>[6(RS)-$^{3}H,1^{14}C$]-9</td>
<td>5.08</td>
<td>2.53</td>
<td>-</td>
<td>49.8</td>
</tr>
</tbody>
</table>
general claim that the mechanism of sulfur incorporation into organic rings occur via a biosynthetic pathway initiated at saturated carbon atoms. Previous research indicated that sulfur introduction in bacteria and fungi, two distinct forms of living matter are quite similar. Studying the biosynthesis of asparagusic acid in \textit{A. officinalis} would demonstrate whether higher plants biosynthesize sulfur compounds via the same unusual mechanism that is available to bacteria and fungi. Asparagusic acid is a structural analog of lipoate and one would think that precursors and the mode of sulfur introduction would be similar.

The potential precursor for asparagusic acid was hypothesized to be isobutyric acid (11, p. 10), by analogy with lipoic acid (2, p. 6) biosynthesis. Isobutyric acid is known to be derived from valine as shown in Scheme III (p. 15). Sodium $[1^{14}C]$isobutyrate was administered to \textit{Asparagus officinalis} and found to be specifically incorporated. The degradation pathway used is shown in Scheme IV (p. 16).

The approach chosen to investigate the nature of the intervening steps between isobutyric acid and asparagusic acid was to administer specifically tritiated forms of isobutyric acid labelled on carbon atoms 2 and 3, mixed with $[1^{14}C]$isobutyric acid, to \textit{Asparagus officinalis}. The
Scheme III.

\[
\text{NH}_2 \xrightarrow{\text{amino acid oxidase}} \text{NH}_3 \quad \text{H}_2 \text{O} \quad \text{COOH} \\
\text{hydrolysis} \quad \text{H}_2 \text{O} \quad \text{COOH} \\
\text{HN} \quad \text{COOH} \quad \text{CoASH} \\
\text{amino acid oxidase} \quad \text{H}_2 \text{O} \quad \text{COASH} \\
\text{HN} \quad \text{COOH} \quad \text{CoASH} \quad \text{A} \quad \text{CO}_2 \\
\text{amino acid oxidase} \quad \text{CoASH} \quad \text{A} \quad \text{CO}_2
\]
Scheme IV.

DEGRADATION OF ASPARAGUSIC ACID

\[
\begin{align*}
\text{PhNHH}_2 & \xrightarrow{\text{DCC}} \text{HN}_3 \\
\text{CH}_2\text{S} & \xrightarrow{\text{Na, NH}_3} \text{CH}_2\text{Cl} \\
\text{ArCH}_2\text{S} & \xrightarrow{\text{Raney Ni}} \text{CONHPh} \\
\text{SCH}_2\text{Ar} & \xrightarrow{1.\text{HCl}} \text{CONHPh} \\
\text{SCH}_2\text{Ar} & \xrightarrow{2.\text{NaOH}} \text{CONHPh} \\
\text{CO}_2 & + \text{CONHPh} \\
\end{align*}
\]
carbon-14 label serves as an internal reference; the decrease in the $^{3}H/^{14}C$ ratio indicates the amount of tritium loss relative to carbon-14. The amount of tritium lost in the conversion of specifically tritiated forms of isobutyric acid to asparagusic acid was expected to provide information concerning the nature of the reactions leading to the formation of the dithiolane ring system.

Administration of [2-$^{3}H,1-^{14}C$]isobutyrate to Asparagus plants should determine the oxidation level experienced by C-2 of isobutyric acid as the result of sulfur introduction at C-3 or C-3'. Oxidation of C-2 would be signaled by complete tritium loss, while absence of oxidation would be indicated by complete tritium retention.

The extent of oxidation of the methyl groups of isobutyrate as the result of sulfur introduction could be investigated by administration of [3,3'-$^{3}H,1-^{14}C$]isobutyric acid to Asparagus. If the intermediates between isobutyrate and asparagusic acid such that C-3 is raised to the oxidation level of an alcohol, then one would expect $\geq 67\%$ tritium retention depending on the magnitude of the tritium isotope effect associated with the oxidation step. If the intermediates involved in the introduction of sulfur at C-3 of isobutyrate are such that C-3 is raised to an aldehyde oxidation level, then a tritium retention between ca. 34-50% can be expected, assuming that the removal of
hydrogen accompanying the conversion on C-3 from the alcohol to the aldehyde oxidation level is stereospecific (0.67 x 0.5 = 0.34). In the unlikely event that C-3 of isobutyrate were oxidized to the oxidation level of a carboxylic acid during asparagusic acid formation, then complete tritium loss could be expected.

The labelling of isobutyric acid with tritium at positions C-2 and/or C-3 and subsequent administration would determine whether the introduction of unsaturation is involved in the biochemical transformation. Tritium label loss from C-3 only would suggest oxidation at C-3 whereas tritium loss from both C-2 and C-3 would suggest the intermediacy of an unsaturated compound (12, p. 20). Tritium loss at C-3 depends on the magnitude of the tritium isotope effect. Since the tritium carbon bond is stronger than the hydrogen carbon bond, there can be discrimination against the loss of tritium resulting in a tritium isotope effect. Large isotope effects are not uncommon (ie 20:1). \(^{29}\) It is also important to note that the three hydrogens of the methyl group cannot be distinguished by an enzyme.

If there were 100% retention of the tritium at C-2, then the conversion of isobutyric acid to asparagusic acid follows the pattern shown for biotin and lipoic with no unsaturation involved. If loss were detected at both C-2 and C-3, then \([1^{-14}C]\)methacrylic acid (12, p. 20) should be
administered to determine whether it is specifically incorporated into asparagusic acid.

Several sulfur containing compounds have been isolated from *Asparagus* plants and characterized. These compounds could be precursors along the biosynthetic pathway to asparagusic acid. Tressl and coworkers\textsuperscript{8} isolated several sulfur containing acids and esters from white asparagus (*Asparagus officinalis*). These acids included asparagusic acid (1, p. 6), its methyl and ethyl esters, 3,3'-dimercaptoisobutyric acid (13, p. 20), 3-mercaptoisobutyric acid (14, p. 20), and others. Kasai, Hirakuri and coworkers\textsuperscript{30} isolated and characterized two acidic cysteine derivatives and six acidic dipeptides from *Asparagus* shoots. Four of them gave positive reactions with both iodoplatinate and ninhydrin reagents. Two were identified as S-(2-carboxy-n-propyl)-L-cysteine (15, p. 20) and S-(1,2-dicarboxyethyl)-L-cysteine (16, p. 20). The other two were established as the mixed disulfide of S-(2-carboxy-3-mercaptopropyl)-L-cysteine and 3-mercaptoisobutyric acid (17, p. 21) and the disulfide of S-(2-carboxy-3-mercaptopropyl)cysteine (18, p. 21).

A second phase in the investigation of asparagusic acid biosynthesis can be initiated when the mechanism of sulfur introduction have been probed by the tritium labeling experiments discussed. This phase will involve attempts to
determine the nature of the intermediates between isobutyric acid and asparagusic acid. The approach will be similar to the approach taken with biotin in order to find intermediates between dethiobiotin and biotin.31 Potential intermediates will be synthesized and administered to A. officinalis in labeled form to determine if they are specifically incorporated into asparagusic acid. During this phase one can determine whether the sulfur is derived from a sulfur containing primary metabolite such as cysteine (19, p. 21) or from inorganic sulfur.

A potential intermediate, 3-mercaptopisobutyric acid, will be tested first because of its structural similarity to both asparagusic acid and isobutyric acid. It is also known to be a naturally occurring component in Asparagus. Administration of sodium[1-14C]3-mercaptopisobutyrate (14, p. 20), subsequent isolation of asparagusic acid (1, p. 6) and degradation would show whether 14 was along the biosynthetic pathway to 1. If 14 were found to be specifically incorporated, then [2-3H,1-14C]3-mercaptopisobutyric acid should also be administered to determine whether unsaturation occurred during the addition of the second sulfur to form asparagusic acid. In order to show that 3-mercaptopisobutyric acid does not eliminate to form methacrylic acid (12, p. 20) and then proceed on to asparagusic acid, [35S]3-mercaptopisobutyric acid should be
administered with [3-\textsuperscript{3}H]3-mercaptoisobutyric acid as an internal reference, since there should be no hydrogen loss at C-3 after sulfur is introduced. If 3-mercaptoisobutyric acid were incorporated intact, the $^{35}\text{S}$/\textsuperscript{3}H ratio should remain the same when asparagusic acid is isolated and counted (after allowing for the radioactive decay of $^{35}\text{S}$). If the ratio were not consistent, then this would indicate that elimination had occurred.

If the sulfur were derived from an amino acid, then a possible intermediate to be tested is S-(2-carboxypropyl)cysteine (15, p. 20). In order to show that it is biosynthesized from the same initial precursor, isobutyric acid, $[1$-$^{14}$C]isobutyric acid should be administered and \textsuperscript{15} isolated and degraded to show whether it is specifically incorporated. If \textsuperscript{15} were found to be a precursor of 1, then a double label experiment must be performed to show that S-(2-carboxypropyl)cysteine is incorporated into asparagusic acid without loss of the sulfur from cysteine proving that cysteine was not eliminated first. This could be accomplished by administration of $[^{35}\text{S}]$S-(2-carboxypropyl)cysteine with [3-\textsuperscript{3}H]S-(2-carboxypropyl)cysteine as an internal standard. If the ratio of $^{35}\text{S}$/\textsuperscript{3}H remains constant, then \textsuperscript{15} is incorporated intact and if the ratio is changed (i.e. complete loss of $^{35}\text{S}$ label), then elimination could have
occurred.

A summary of precursors to be tested is given in Table III (p. 25). Once these are tested we should understand the biosynthesis of asparagusic acid in *A. officinalis*. 
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Precursor</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$[1^{-14}C]$-Sodium isobutyrate</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>$[3,4^{-3}H,1^{-14}C]$-Sodium isobutyrate</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>$[2^{-3}H,1^{-14}C]$-Sodium isobutyrate</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>$[1^{-14}C]$-Sodium methacrylate</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>$[2^{-3}H,1^{-14}C]$-(±)-Sodium 3-mercaptoisobutyrate</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>$[^{35}S,3(RS)-^{3}H]$-(±)-Sodium 3-mercaptoisobutyrate</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>$[1^{-14}C]$-Sodium isobutyrate</td>
<td>16</td>
</tr>
<tr>
<td>8</td>
<td>$[^{35}S,3(RS)-^{3}H]$-2(RS)-S- (β-carboxy-n-propyl)-L-cysteine</td>
<td>1</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

The study of sulfur incorporation into the 1,2-dithiolane ring of asparagusic acid (1, p. 6) in *Asparagus officinalis* has been the main goal of this research project.

We first directed our attention to finding a specific precursor of asparagusic acid. Because of its structural similarity, isobutyric acid (11, p. 10) was the first to be tested. Sodium [1-\textsuperscript{14}C]isobutyric acid was tested and found to be incorporated intact into asparagusic acid.\textsuperscript{32} This was accomplished by first administering the radioactive compound, purchased from ICN, dissolved in water to *Asparagus* plants by the cotton wick method. This method involves uptake of the aqueous precursor through a silk thread, that had been placed through the stem of the plant, by capillary action. The ends of the threads were placed in feeding tubes attached to the stems directly below the threads and the aqueous solution placed in the tubes. After the solution containing the radioactive compound was taken up, more water was added to the tubes to assure complete uptake of the precursor. The plants were allowed to metabolize the precursor for 4 to 5 days. After this time the plants were harvested, blended with 95% ethanol, and the ground material was repeatedly extracted with 95% ethanol.
Asparagusic acid is present in Asparagus in minute amounts, so it was necessary to synthesize the acid for isotopic dilution purposes.\textsuperscript{32} Asparagusic acid was synthesized according to the procedure of Yanagawa and coworkers\textsuperscript{33} (Scheme V, p. 28). Diethyl malonate (20) was added to aqueous formaldehyde (21) and potassium carbonate. Ethyl bis(hydroxymethyl)malonate (22) was isolated after stirring and extracting. When 22 was treated with 57\% hydroiodic acid to yield diiodoisobutyric acid (23) a mixture of the diiodo and \( \alpha \)-iodomethylacrylic acids was obtained. This problem was solved by converting the \( \alpha \)-iodomethylacrylic acid (24) to the diiodoisobutyric acid (23) by treating the mixture with 95\% phosphoric acid and potassium iodide. The diiodoisobutyric acid was then converted to dihydroasparagusic acid (13) by treatment with aqueous 33\% sodium thithiocarbonate. Finally, 13 was dissolved in dimethylsulfoxide and heated to yield asparagusic acid (1). This procedure was followed several times and a few times the asparagusic acid was isolated as a viscous oil which proved to be identical to the solid, obtained from previous experiments, by NMR and tlc. Unlabelled asparagusic acid was added to the ethanol extract of Asparagus which was then evaporated. The crude extracted material was washed with aqueous sodium bicarbonate solution which was then acidified to pH 3 with concentrated hydrochloric acid. The acidified
SCHEME V

SYNTHESIS OF ASPARAGUS ACID

\[ \text{HCHO} + \text{K}_2\text{CO}_3 \rightarrow \text{OC}_2\text{H}_5 \text{OOC}_2\text{H}_5 + \text{COOH} \]

\[ \Delta \]

\[ \text{K}_2\text{CS}_2 \rightarrow \text{COOH} \]

\[ \Delta \]

\[ \text{Na}_2\text{CS}_2 \rightarrow \text{COOH} \]

\[ \Delta \]

\[ \text{KI, H}_3\text{PO}_4, \Delta \]

\[ \text{COOH} \]
aqueous solution was extracted with ethyl acetate. The organic phase was dried, evaporated, and the remaining material was derivatized and purified as the p-phenylbenzyl derivative (25).

The thioether (25) was then degraded to show the specific incorporation of the precursor (Scheme IV, p. 30). This was accomplished by first converting the thioether (25) to the anilide derivative (26) using DCC and aniline. This derivatization was performed to insure that the product of the Raney nickel desulfurization would be a solid. The anilide was transformed to isobutyric acid anilide (27) by treatment with Raney nickel. 27 was then hydrolysed and the resulting isobutyric acid then subjected to Schmidt degradation. After the loss of the labelled CO₂, the remaining portion of the molecule (isopropylamine, 28) was derivatized using benzoyl chloride to give isopropyl benzamide (29), a solid, that was recrystallized and counted to show that no radioactivity remained in that portion of the asparagusic acid. From this experiment, one can conclude that isobutyric acid is a specific precursor of asparagusic acid. The sodium [1-^{14}C]isobutyrate incorporation figure was 1.2%.28

Experiments with [2-^{3}H] and [3-^{3}H]isobutyric acid were then carried out to obtain clues to the mechanism of sulfur introduction. If oxidation at C-3 or the formation of a
Scheme IV.

DEGRADATION OF ASPARAGUS ACID
possible unsaturated intermediate, such as methacrylic acid (12, p. 20), occurred, then these experiments should reveal this fact. It will be recalled that an unsaturated intermediate was ruled out in biotin and lipoic acid biosyntheses. Loss of tritium at a carbon adjacent to the site of sulfur introduction was not found in either of these cases. Administration of $[2,3^{-3}\text{H}],[3^{-3}\text{H}]$, and $[5^{-3}\text{H}]-(-\text{dethiobiotin to } A. \text{ niger}$ and isolation of biotin sulfone showed no tritium loss. Administration of $[5^{-3}\text{H}]$ and $[7^{-3}\text{H}]$octanoic acid to $E. \text{ coli}$ and isolation of lipoic acid showed no tritium loss. Therefore the hypothesis of an unsaturated intermediate was proved incorrect.

The synthesis of sodium $[2^{-3}\text{H}]$isobutyrate was first attempted by reducing acetone to isopropanol with tritiated lithium aluminum hydride followed by bromination using phosphorus tribromide. The resulting 2-bromopropane was then converted to the acid by forming the Grignard reagent and subsequent treatment with carbon dioxide. This reaction sequence was unsatisfactory due to the volatility of the starting materials and intermediates which made their isolation difficult therefore causing the yields to be low.

Sodium $[3^{-3}\text{H}]$isobutyrate was first synthesized and administered and after a tritium retention of 96.7% was found, sodium $[2^{-3}\text{H}]$isobutyrate was tested. Both tritiated compounds were synthesized from n-butyl propionate (30).
The n-butyl ester was used to lower the volatility by increasing the molecular weight without causing steric problems during deprotonation at C-2 (i.e. t-butyl or cyclohexyl esters). n-Butyl propionate was synthesized by treatment of propionyl chloride with a pyridine, n-butanol mixture in methylene chloride. It was then alkylated following the procedure of Schlessinger et al. by treatment with LDA followed by alkylation of the resulting anion with $[^3\text{H}]$methyl iodide (Scheme VI, p. 33). The resulting n-butyl isobutyrate was hydrolysed to the acid, steam distilled, and titrated with dilute sodium hydroxide to yield sodium $[3-[^3\text{H}]]$isobutyrate (31).

During the alkylation procedure, dialkylation leading to pivalic acid was encountered. Its formation was controlled by monitoring the time allowed for the enolate formation. The reaction sequence was performed, adjusting reaction times, and the pivalic acid formation was monitored by gas chromatography and kept to a minimum. The amount of radioactivity due to pivalic acid was determined by comparing the $[^3\text{H}]$/14C ratio of the salts to the ratio of the isobutyric anilide derivatives synthesized from a diluted sample of the radioactive precursor. The amount due to pivalic acid was found to be negligible.

Sodium $[2-[^3\text{H}]]$isobutyrate was prepared in a similar fashion from n-butyl propionate (Scheme VII, p. 34). The
SCHEME VI

\[
\begin{align*}
\text{n-BuO} & \xrightarrow{1. \text{LDA}} \text{n-BuO} \\
& \xrightarrow{2. \text{H}_2\text{SO}_4} \text{n-BuO} \\
& \xrightarrow{2. \text{NaOH}} \text{COONa}
\end{align*}
\]
SCHEME VII

\[
\begin{align*}
&\text{n-BuO} \\
&\text{1. LDA} \\
&\text{2. CF}_3\text{COOT} \\
&\text{3. COONa} \\
\end{align*}
\]

\[
\begin{align*}
&\text{n-BuO} \\
&\text{1. H}_2\text{SO}_4 \\
&\text{2. NaOH} \\
\end{align*}
\]
ester (30) was first treated with LDA and quenched with tritiated trifluoroacetic acid, which was prepared from tritiated water and trifluoroacetic anhydride. The incorporation of the label was first tested by using deuterated water and analysing the product by NMR spectroscopy. The ratio of the alpha hydrogen to the methyl hydrogens should be 1:6 but was found to be approximately 1:15. n-Butyl [2-3H]propionate was again deprotonated with LDA taking advantage of the tritium isotope effect assuming that the base would selectively remove the remaining hydrogen over the tritium atom. This step was also tested by treating n-butyl [2-2H]propionate with LDA, then alkylating it with methyl iodide and verifying that the deuterium was retained by NMR spectroscopy. The resulting anion of n-butyl [2-3H]propionate was quenched with unlabelled methyl iodide to produce n-butyl [2-3H]isobutyrate which was then hydrolysed, steam distilled, and titrated as previously described. Sodium [2-3H]isobutyrate (32) was obtained.

Each of the specifically tritiated sodium isobutyrate 31 and 32 was mixed with sodium [1-14C]isobutyrate purchased from ICN. Each of the tritium to carbon-14 ratios was determined by liquid scintillation counting of the salts and checked by derivatization of a portion of each mixture and measurement of the ratio of the derivative. This was done
by converting the salt to the anilide by treatment with DCC and aniline. A minute portion of the radiolabelled material was mixed with unlabelled sodium isobutyrate and then derivatized. The anilides were then recrystallized and counted until a constant ratio was obtained. In each case, the tritium to carbon-14 ratios obtained were identical within experimental error with the ratios obtained by recrystallization of the anilides. The two samples of doubly labelled sodium isobutyrate were then administered to *Asparagus* and the asparagusic acid isolated as the bis(p-phenylbenzyl)asparagusic acid (25), as previously described. The derivative was then recrystallized until a constant ratio was obtained which was compared to the original ratio. For the sodium [3-\(^3\)H]isobutyrate (31) the original ratio was 5.72 and the final ratio for the derivative (25) was 5.33 corresponding to a 96.7\% retention of \(^3\)H. This implied that incorporation of 31 into asparagusic acid proceeded without tritium loss within experimental error. A substantial tritium isotope effect associated with the removal of a hydrogen atom from each of the methyl groups of isobutyrate could account for this high degree of tritium retention. A similar effect was also observed in the conversion of [8-\(^3\)H]octanoic acid into lipoic acid\(^{17,18}\) (Table II, p. 13).

The sodium [2-\(^3\)H]isobutyrate ratio was 6.36 prior to
the feeding and after the feeding, the ratio of the isolated thioether (25) was 0.16. This implies a total loss of all the tritium label which is in complete contrast to the behaviour observed during lipoic acid biosynthesis. For the lipoic acid case, no tritium is lost from carbon atoms adjacent to the sites of sulfur introduction (Table II, p. 13). Therefore it seems that the 1,2-dithiolane ring formation in plants does not mimic the mechanism found in fungi and bacteria. A plausible explanation for the loss of tritium from sodium [2-\textsuperscript{3}H]isobutyrate would involve an unsaturated intermediate. This intermediate could come from dehydrogenation of isobutyric acid to methacrylic acid, a conversion that has been reported in both plants and in microorganisms.\textsuperscript{35,36} Consequently [1-\textsuperscript{14}C]methacrylic acid (33) was the next precursor tested.

Sodium [1-\textsuperscript{14}C]methacrylate was synthesized from 2-bromopropene via the Grignard reagent and labelled barium carbonate (Scheme VIII, p. 38). It was administered to Asparagus for five days and asparagusic acid was isolated as the thioether (25), which was degraded as previously described (Scheme IV, p. 30). [1-\textsuperscript{14}C]Methacrylic acid was found to be specifically incorporated into asparagusic acid (0.38%) which suggests a biosynthetic pathway involving an unsaturated intermediate. Dehydrogenation of isobutyric acid or isobutyryl CoA to methacrylic acid could be followed
SCHEME VII

Asparagus

\[
\text{MgBr}
\]

\[
\text{CO}_2
\]

\[
\text{NHCOPh}
\]

\[
0.38\%
\]

33°
by a Michael-type addition of a sulfur nucleophile. A second dehydrogenation step would then lead to another unsaturated acid that would undergo addition of a second mole of a sulfur nucleophile to lead to asparagusic acid (Scheme IX, p. 40). Several possible precursors could be tested to evaluate this Scheme, but the most plausible is 3-mercaptoisobutyric acid (14), because of its similarity to both isobutyric acid and asparagusic acid.

Sodium $[1-^{14}C]$3-mercaptoisobutyrate (34) was synthesized from $[1-^{14}C]$methacrylic acid which was prepared as previously described. The methacrylic acid was treated with thiolacetic acid and the Michael addition product was then hydrolysed by alkaline hydrolysis to the $[1-^{14}C]$3-mercaptoisobutyric acid (Scheme X, p. 41).37 $[2-^3H]$3-Mercaptoisobutyric acid (35a) was synthesized in the same way by Michael addition of thiolacetic acid to methacrylic acid in tritiated water (Scheme XI, p. 42). This experiment was first performed in deuterated water and checked by NMR spectroscopy to show that the deuterium was incorporated in the alpha position only. The S-acetyl $[2-^3H]$3-mercaptoisobutyric acid (35b) was then hydrolysed. The resulting $[2-^3H]$3-mercaptoisobutyric acid was mixed with the $[1-^{14}C]$3-mercaptoisobutyric acid, the tritium to carbon-14 ratio determined, and a portion was checked by derivatization with p-bromophenacylbromide and found to be
Scheme IX

\[
\begin{align*}
\text{COOH} & \rightarrow \text{COOH} & \rightarrow \text{COOH} \\
\text{SR} & \rightarrow \text{COOH} & \rightarrow \text{COOH}
\end{align*}
\]
SCHEME X

$\text{MgBr}$ → $\text{CO}_2$ → $\text{KOH}$ → $\text{COOK}$

$\text{KOH}$ → $\text{SH}$

$\text{COOH}$ → $\text{NaOH}$ → $\text{COONa}$

$\Delta$

34
identical within experimental error to the underivatized precursor. The original ratio was found to be 6.76. The doubly labelled precursor was then administered to *Asparagus*. Counting of the isolated thioether (25) showed a final ratio of 0.15, which implied that the tritium label at C-2 was lost during the incorporation process. The thioether was degraded (Scheme IV, p. 30) to show specific incorporation 0.35% of [1-\(^{14}\text{C}\)]3-mercaptoisobutyric acid into asparagusic acid.

Two conclusions can be drawn from this experiment. First, that 3-mercaptoisobutyric acid is a specific precursor of asparagusic acid and second, that the incorporation proceeds with the introduction of unsaturation between C-2 and C-4. The latter fact is demonstrated by tritium label loss at C-2 similar to that which occurred when isobutyric acid was transformed into asparagusic acid. In order to rule out the possibility that sodium 3-mercaptoisobutyrate is incorporated into asparagusic acid by reversion to methacrylic acid, another doubly labeled precursor was administered, namely, [3-\(^{3}\text{H},^{35}\text{S}\)]3-mercaptoisobutyric acid.

Sodium [\(^{35}\text{S}\)]3-mercaptoisobutyrate (36) was synthesized from treatment of 3-bromoisoobutyric acid (37) with [\(^{35}\text{S}\)]hydrogen sulfide purchased from Amersham (Scheme XII, p. 44).\(^{38}\) [\(^{35}\text{S}\)]Hydrogen sulfide was vacuum transferred into
a frozen solution of sodium hydroxide. The solution was then mixed with 3-bromoisobutyric acid prepared from methacrylic acid and hydrogen bromide. The reaction was driven to completion by addition of radioactive hydrogen sulfide dissolved in a sodium hydroxide solution. The resulting $^{35}$S-3-mercaptoisobutyric acid was distilled, purified, and titrated to give the sodium salt.

The sodium [3-$^3$H]3-mercaptoisobutyrate (38) was prepared from methyl 2,3-dibromoisoibutyrate (39) (Scheme XIII, p. 46). 39 was obtained by the addition of bromine to methyl methacrylate.39,40 The dibromo compound (39) was dehydrobrominated using DBU and the resulting ester (40) was then hydrolysed to yield 3-bromo-2-methylpropenoic acid (41).41 Incorporation of tritium label was first tested by using deuterium. The NMR spectra of 3-bromo-2-methylpropenoic acid shows a proton signal at delta 7.71 due to the vinyl hydrogen. When 41 was treated with t-butyllithium in THF followed by deuterated water, there was a decreased in size of this signal relative to the other signals. This implied a replacement of the vinyl hydrogen with deuterium. The experiment was then performed using tritiated water36 and [3-$^3$H]3-bromo-2-methylpropenoic acid (42) was obtained after work-up. [3-$^3$H]methacrylic acid (43) was then prepared by treating 42 with sodium amalgam. A few problems were encountered at this point due
Scheme XI

\[ \text{DBU} \rightarrow \text{COO}^+ \text{Me} \]

\[ \Delta \rightarrow \text{COO}^- \text{Me} \]

\[ \text{Br} \]

\[ \text{COOH} \]

\[ \text{Na}^- \text{(Bu)} \]

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

\[ \text{S}^- \]

\[ \text{COO}^- \text{Na} \]

\[ \text{R} = \text{CH}_3\text{CO}, \text{C}_6\text{H}_4\text{Br} \]

\[ \text{Phenylphenacyl bromide} \]

\[ \text{Br} \]

\[ \text{COOH} \]

\[ \text{1.1-BuLi} \]

\[ \text{2.1H}_2\text{O} \]

\[ \Delta \rightarrow \text{SH}^- \]

\[ \Delta \rightarrow \text{SH}^- \]
to overreduction of the $[3^{-3}H]3$-bromo-2-methylpropenoic acid (42) to isobutyric acid (11, p. 10). When the reaction was carried out for shorter periods, 42 was not fully converted to 43. At lower temperatures, below $-10^0C$, the solution turned to ice and it was difficult to mix in the sodium amalgam. The problem was resolved by adding the sodium amalgam in small portions to a frozen solution and then letting the solution warm to $0^0C$ after each addition. The ratio of isobutyric acid to methacrylic acid was determined by the ratio of the vinyl methyl proton at 2.01 ppm to the methyl protons of isobutyric acid. The ratio of these protons should be 2:1 and using this information the ratio was found to be 3:1. $[3^{-3}H]3$-mercaptoisobutyric acid was then prepared by treating this mixture of $[3^{-3}H]$isobutyric acid and $[3^{-3}H]$methacrylic acid with thiolacetic acid, hydrolysing the resulting thioester (44) and isolating the free acid using silica gel chromatography in an inert atmosphere. It was titrated to give sodium $[3^{-3}H]3$-mercaptoisobutyrate (38, Scheme XIII, p. 46). 38 was then used as an internal standard for the sodium $[^{35}S]3$-mercaptoisobutyrate (36) since the two C-3 hydrogens should be retained throughout the conversion of mercaptoisobutyric acid to asparagusic acid. $[3^{-3}H, ^{35}S]3$-Mercaptoisobutyric acid was prepared and counted and a small portion derivatized with p-bromophenacyl
bromide. The structure of the derivative was shown to be \textit{45} (p. 46) by mass spectroscopy and NMR. The derivative was counted and recrystallized until a constant ratio, 0.341, was obtained. This was identical to that of the salt, within experimental error. Since the half-life of \textit{\textsuperscript{35}S} is 87.4 days, the doubly labelled derivative was saved to allow determination of the new ratio resulting from radiodecay of the \textit{\textsuperscript{35}S} during the time expended for the precursor incorporation experiment. The ratio of the asparagusic acid biosynthesized from doubly labeled [\textit{\textsuperscript{3-H}}, \textit{\textsuperscript{35}S}]3-mercaptoisobutyric acid was determined to be 0.322. After correcting the ratio for radioactive decay, it remained essentially unchanged from that before administration. This implied that the 3-mercaptoisobutyric acid was incorporated intact into asparagusic acid and did not eliminate first to form methacrylic acid.

The 3-mercaptoisobutyric acid incorporation experiments suggest that the sulfur donor in asparagusic acid biosynthesis is sulfide. However, there is another possibility, namely, that cysteine (19, p. 21), is the immediate sulfur donor. This possibility arises from the fact that S-(2-carboxy-n-propyl)-L-cysteine (15, p. 20) has been reported to occur in both onion and garlic plants.\textit{42,43,44} Both of these plants are \textit{Allium} species that are members of the \textit{Liliaceae}. Martha Washington \textit{Asparagus}
plants are of the *Asparagus* genus which is also a member of the *Liliaceae*. Therefore there is a high probability that S-(2-carboxy-n-propyl)-L-cysteine might also occur in *Asparagus officinalis*. In order to test this hypothesis, [1-14C]isobutyric acid was administered to *Asparagus* plants and after 5 days radioactive S-(2-carboxy-n-propyl)cysteine was isolated by isotopic dilution techniques. The unlabelled S-(2-carboxy-n-propyl)cysteine, used as carrier, was prepared from methacrylic acid and cysteine hydrochloride.\(^\text{45}\) Degradation of \(15\) via a Schmidt reaction to the diamino acid (46, p. 50) proved that the incorporation was specific. Carboxylic acids alpha to amino groups do not undergo Schmidt degradation,\(^\text{46}\) therefore only the C-1 carbon was lost. The incorporation was found to be 0.04% and the diamino compound (46) was found to be void of radioactivity. Shortly after this experiment had been performed, the isolation of S-(2-carboxy-n-propyl)cysteine from *A. officinalis* was reported by Kasai and coworkers\(^\text{30}\). \(^{35}\)S and \(^{3}\)H forms of 15 were next synthesized to evaluate the compound as a precursor of asparagusic acid. \(^{35}\)S-(2-carboxy-n-propyl)cysteine was synthesized by treatment of methacrylic acid with \(^{35}\)S-cysteine hydrochloride (Scheme XIV, p. 51). The corresponding \(^{3}\)H compound was prepared by treatment of \(^{3}\)Hmethacrylic acid with cysteine hydrochloride (Scheme
The two labeled forms were mixed and the ratio (0.254) was found to be identical within experimental error to that of a small amount of radioactive mixture which had been diluted with unlabelled S-(2-carboxy-n-propyl)cysteine and repeatedly recrystallized. The mixture of two labelled amino acids were then administered to Asparagus plants. The asparagusic acid ratio was 0.256, which showed that the sulfur was retained. This experiment revealed that the amino acid was specifically incorporated into asparagusic acid with retention of the sulfur atom derived from cysteine.

The combined data from experiments 5-8 can be interpreted in two ways. The first is that both 3-mercaptopisobutyric acid (14) and S-(2-carboxy-n-propyl)cysteine (15) precede asparagusic acid in its biosynthesis with 14 lying closer on the pathway than 15. The second explanation is that the two thio compounds are interconverted in vivo (Scheme XVI, p. 54). If this is the case, then only one of the two compounds need lie on the pathway in order to explain the results. The interconversion between the two thio compounds could presumably be mediated by pyridoxal phosphate.

From the results of the experiments in Table IV (p. 55), it is possible to say that the biosynthesis of the 1,2-dithiolane ring of asparagusic acid is now largely understood and it has been found to be completely different
<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Precursor</th>
<th>Precursor Isotope Ratio</th>
<th>% Incorp. and/or Isotope Ratio in Product</th>
<th>Labeling Pattern or % Isotope Retention in Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$[1^{-14}C]$-Sodium isobutyrate</td>
<td>-</td>
<td>1.2% into $\text{I}$</td>
<td>No label at C-2 to C-4 of 29</td>
</tr>
<tr>
<td>2</td>
<td>$[3,4^{-3}H,1^{-14}C]$-Sodium isobutyrate</td>
<td>$\text{H}^{14}C = 5.72$</td>
<td>$\text{H}^{14}C = 5.33$ for 25</td>
<td>96.7% $^3H$ retention in 25</td>
</tr>
<tr>
<td>3</td>
<td>$[2^{-3}H,1^{-14}C]$-Sodium isobutyrate</td>
<td>$\text{H}^{14}C = 6.36$</td>
<td>$\text{H}^{14}C = 0.16$ for 25</td>
<td>2.5% $^3H$ retention in 25</td>
</tr>
<tr>
<td>4</td>
<td>$[1^{-14}C]$-Sodium methacrylate</td>
<td>-</td>
<td>0.38% into $\text{I}$</td>
<td>No label at C-2 to C-4 of 29</td>
</tr>
<tr>
<td>5</td>
<td>$[2^{-3}H,1^{-14}C]$-($\pm$)-Sodium 3-mercaptopoisobutyrate</td>
<td>$\text{H}^{14}C = 6.76$</td>
<td>0.35% into $\text{I}$</td>
<td>2.3% $^3H$ retention in 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\text{H}^{14}C = 0.154$ for 25</td>
<td>No label at C-2 to C-4 of 29</td>
</tr>
<tr>
<td>6</td>
<td>$[^{35}S,3(RS)^{-3}H]$-($\pm$)-Sodium 3-mercaptopoisobutyrate</td>
<td>$^{35}S/^{3}H = 0.341$</td>
<td>1.1% into $\text{I}$</td>
<td>94.4% $^{35}S$ retention in 25</td>
</tr>
<tr>
<td>7</td>
<td>$[1^{-14}C]$-Sodium isobutyrate</td>
<td>-</td>
<td>0.04% into 15</td>
<td>No label at C-2 to C-7 of 46</td>
</tr>
<tr>
<td>8</td>
<td>$[^{35}S,3(RS)^{-3}H]$-2(RS)-S-(β-carboxy-n-propyl)-L-cysteine</td>
<td>$^{35}S/^{3}H = 0.254$</td>
<td>1.2% into $\text{I}$</td>
<td>101% $^{35}S$ retention in 25</td>
</tr>
</tbody>
</table>
from the pathway found in bacteria. The proposed biosynthetic pathway is shown in Scheme XVI (p. 54) and the results tabulated in Table IV (p. 55).
EXPERIMENTAL

Nuclear magnetic resonance spectra were obtained using Varian EM-390 and JEOL FX-90Q 90MHz spectrometers. Chemical shifts are in parts per million (d) downfield from tetramethylsilane, which was used as a zero reference. Finnigan 3300 and CEC 1110 21-110B mass spectrometers were used to obtain mass spectral data. Perkin Elmer 3920 (6ft UCW-96 on Chromosorb) and Hewlett Packard .5710A (6ft x 2mm I.D., 10% W-98 glass column) gas chromatographs were used for gas chromatographic identification and separation.

All radioactive compounds were weighed on a Perkin Elmer Autobalance AD-2 or a Mettler Micro-Gram-Atic balance and counted with a Beckman LS 100-C liquid scintillation counter using Aquasol or toluene scintillation fluids. These solvents were purchased or made from materials supplied by New England Nuclear. A Varian 6000-1 radiochromatogram scanner was used to check for radiochemical impurities. The internal standards, \(^3\)H-toluene and \(^14\)C-toluene, were purchased from New England Nuclear (NEN) and \(^35\)S-dioctylsulfide was purchased from Amersham. These radiolabelled compounds were used to check the efficiency of the Beckman LS 100-C. The radioactive compounds that were purchased were obtained from either NEN,
Amersham, or ICN.

Asparagus roots were obtained from Burpee Co., and the plants maintained in a greenhouse until two weeks prior to a feeding at which time they were kept in a LAB-LINE Biotronette Mark III Environmental Chamber. The plants were blended in a Waring commercial blender.

Preparative thin layer chromatography (tlc) was performed on Merck silica gel, type 60, PF-254 (0.75mm) and analytical tlc was done on Merck Polygram SIL G/UV254 (0.25mm) plates. Column chromatography was performed using either Grace silica gel, Grade H, Mallinckrodt SilicAR CC-4, or microcrystalline Cellulose EM-2331. Visualization was accomplished by shortwave UV, phosphomolybdic acid stain, bromocresol green spray, iodine stain, or ninhydrin spray.

Melting points were taken on a Fischer-Johns Melting Point Apparatus and are uncorrected.

A melting point and a literature reference showing the reported value for the melting point is given for all previously synthesized solid compounds. All previously synthesized liquid compounds are identified by NMR and a reference is provided showing its synthesis. The structures of all new compounds were verified by NMR and either high resolution MS or elemental analysis.
ADMINISTRATION OF RADIOLABELLED PRECURSORS

Asparagus roots, purchased from Burpee Co., were planted and maintained in a greenhouse. Two weeks prior to a feeding, the plants were transferred to an environmental chamber that was set for a long day (12 hours)/short night (12 hours) cycle. The plants were allowed two weeks to acclimate. Three days before the feeding, watering was discontinued to insure complete uptake of the solution of the compound during administration. Feeding tubes were attached to three healthy stems of the plant and the radioactive compound was administered by the cotton wick method. Metabolism of the precursor was allowed to proceed for four to five days. The plants were harvested, and they were then homogenized in a Waring Commercial Blender with 95% ethanol. The ground plant material was placed in a column with a sintered glass frit and 12 liters of 95% ethanol was passed through the plant material over three days.

Unlabeled asparagusic acid carrier was added to the eluant which was then evaporated. The residue was dissolved in ether and extracted with aqueous sodium bicarbonate (3x100ml). The aqueous layer was acidified with concentrated hydrochloric acid to pH 3 and reextracted with ethyl acetate (5x). The ethyl acetate was evaporated and
the recovered material derivatized using sodium, liquid ammonia and chloromethylbiphenyl. The bis(p-phenylbenzyl)dihydroasparagusic acid was purified by silica chromatography and recrystallized to constant radioactivity.

ISOLATION OF S-(2-CARBOXY-n-PROPYL)CYSTEINE (15, p. 20)
FROM ASPARAGUS officinalis

Sodium [1-\(^{14}\)C]isobutyric acid was administered to A. officinalis in the same manner as previously described, but the isolation of S-(2-carboxy-n-propyl)cysteine was accomplished by a different procedure. The Asparagus plants were blended and placed in a column equipped with a sintered glass frit. Twelve liters of 95% ethanol was passed through the column and S-(2-carboxy-n-propyl)cysteine dissolved in water was added to the eluant. The ethanol was then evaporated and the residue was dissolved in water and washed with ether and chloroform. The aqueous layer was lyophilized and the amino acid isolated by paper chromatography (butanol/acetic acid/water, 12:5:5). The amino acid was extracted from the paper using water which was then evaporated under reduced pressure. S-(2-carboxy-n-propyl)cysteine being an amino acid, proved
very difficult to drive out of aqueous solution. Crystallization was finally accomplished by using a minimum amount of water with acetone. The purified S-(2-carboxy-n-propyl)cysteine was then degraded using the Schmidt reaction.

SYNTHESIS OF
ETHYL BIS(HYDROXYMETHYL)MALONATE (22, Scheme V, p. 28)\textsuperscript{47}

6N Aqueous potassium carbonate (6ml, 0.04 moles) was added to 37% aqueous formaldehyde (173g, 2.1 moles). The solution was cooled in an ice bath and stirred as diethyl malonate (160g, 1 mole) was added dropwise over fifty minutes. The solution turned cloudy after forty minutes and additional potassium carbonate (200mg, 1.45 mmoles) was added to bring the pH to 8. After complete addition of diethyl malonate, potassium carbonate (200mg, 1.45 mmoles) was added again and the solution cleared after stirring for one hour at room temperature. Saturated aqueous ammonium sulfate (400ml) was added and an oil liberated, which was extracted with anhydrous diethyl ether (300ml). The aqueous layer was separated and reextracted with ether. The ether fractions were combined, dried over anhydrous magnesium sulfate and the solvent was removed under reduced pressure.
The crude ethyl bis(hydroxymethyl)malonate was heated to 35°C while connected to a vacuum pump to remove excess solvent and unreacted starting materials. The viscous oil was chilled to 0°C which produced a gummy white solid that was filtered and dried by suction. The solid was crystallized from warm benzene and petroleum ether to give ethyl bis(hydroxymethyl)malonate (67.9g, 0.31 moles, 31% yield, mp 48°C, lit. mp 50°C\(^\text{47}\)).

**PREPARATION OF .95% PHOSPHORIC ACID\(^\text{48}\)**

Phosphorous pentoxide (6.5g, 46 mmoles) was placed in an oven-dried flask and 85% phosphoric acid (13.5ml, 214 mmoles) slowly added. The reaction being very exothermic was controlled by cooling on ice. The mixture was stirred three hours and the resulting 95% phosphoric acid stored tightly stoppered to prevent water absorption.

**SYNTHESIS OF**  
\(^\text{α-}(\text{IODOMETHYL})\text{ACRYLIC ACID (24, Scheme V, p. 28)}\)^{33,49}

Ethyl bis(hydroxymethyl)malonate (60g, 0.3 moles) was dissolved in 57% hydroiodic acid (243g, 1.1 moles). The
clear yellow reaction mixture was gradually heated to 120°C and kept at this temperature until the solution turned dark red (approximately forty-five minutes). The solution was cooled to room temperature and allowed to stand for twelve hours. Yellow crystals precipitated and were collected on a sintered glass funnel. The solid was washed with a minimum amount of chilled water and the resulting pale yellow solid was dissolved in hot carbon tetrachloride and the cooled solution was dried over anhydrous magnesium sulfate. The solution was concentrated until crystals appeared, then chilled to precipitate more solid (25.73g). The solid was found to be a mixture of both α-(iodomethyl)acrylic acid and β,β′-diiodoisobutyric acid. This mixture of iodo acids (25.73g) was ground with potassium iodide (32.1g, 0.193 moles) and chilled 95% phosphoric acid (54.6g, 0.53 moles) to a smooth yellow paste. The mixture was heated six hours at 100°C, cooled to room temperature, then poured into an ice water mixture. The product was collected on a sintered glass funnel, washed with sodium thiosulfate (0.5g, 3.2 mmoles) in water (25ml) to remove traces of iodine, and dried over phosphorous pentoxide. The crude yellowish orange product was dissolved in ethyl acetate, filtered and concentrated. Chilled petroleum ether was added to initiate crystallization and 3,4-diiodoisobutyric acid obtained (31g, 0.09 moles, 31% yield, mp 128-130°C, lit mp 127-129.5°C).
Diiodoisobutyric acid (23, p. 28)

NMR: 2.88-3.22 (1H, quintet, methine proton),
      3.52-3.70 (4H, doublet, CH₂I)

α-(Iodomethyl)acrylic acid (24, p. 28)

NMR: 4.18 (2H, singlet, CH₂I), 6.15
      (1H, doublet, vinyl proton of acrylic acid,
      cis to CH₂I), 6.47 (1H, doublet, vinyl
      proton of acrylic acid, cis to carboxylic acid)

PREPARATION OF AQUEOUS SODIUM TRITHIOCARBONATE

Sodium sulfide (103g, 0.43 moles) was dissolved in
water (50ml) at 35-40°C. Carbon disulfide (35.6g, 0.47
moles) was added and the heating continued for six hours.
After this time the yellow solution had turned deep red.
The excess carbon disulfide was removed by evaporation under
reduced pressure and water was added to obtain total volume
of 200ml (33% solution).

SYNTHESIS OF DIHYDROASPARAGUSIC ACID (13, p. 28)
Aqueous 33% sodium thiosulfate (42g, 89 mmoles) was slowly added under nitrogen to diiodoisobutyric acid (10g, 29 mmoles). The reaction mixture was heated at 50°C for five hours and acidified to pH 3 with 6N sulfuric acid. The aqueous layer was extracted with ethyl acetate (3x30ml). The organic layer was dried with anhydrous magnesium sulfate and concentrated under a stream of nitrogen to give a viscous yellow oil (59% yield). The structure was verified by NMR spectroscopy.

NMR: 1.49 (2H, triplet, absent on D2O shake), 2.80 and 2.90 (5H, masked doublet and a masked quintet)

SYNTHESIS OF ASPARAGUSIC ACID (1, p. 28)33

The noncrystalline dihydroasparagusic acid (2.55g, 17 mmoles) was dissolved in dimethylsulfoxide (25.5ml, 0.36 moles) and the solution stirred under nitrogen at 70-75°C for five hours until analytical tlc showed no starting material (silica, benzene/ethyl acetate, 10:3). The reaction mixture was poured into ice water and extracted with benzene (3x80ml). The yellow organic layer was
extracted with water to remove traces of dimethylsulfoxide and it was then filtered through anhydrous magnesium sulfate. The benzene was removed under reduced pressure to yield a yellow solid (566mg), which was dissolved in hot benzene and crystallized by adding chilled cyclohexane. After scratching and chilling to 5°C, asparagusic acid was obtained (546mg, 3.6 mmoles, 21% Yield, mp 72-76°C, lit. mp 76.5-77.5°C\textsuperscript{33}).

NMR: 2.52-2.82 (5H, multiplet)

SYNTHESIS OF
S,S'-BIS(p-PHENYLBENZYL)DIHYDROASPARAGUSIC ACID (25, p. 30)\textsuperscript{26}

Liquid ammonia (25ml) was refluxed over sodium metal for fifteen minutes to remove impurities and traces of water. It was then redistilled into a three-necked flask (approximately 15ml). Crude plant extract (approximately 1g, containing 250mg of carrier asparagusic acid, 1.67 mmoles) dissolved in dry THF (2ml) was added alternately with small pieces of sodium metal. After addition of the plant extract, a small amount of synthetic asparagusic acid
was added to the solution to quench the free electrons. Purified (silica column chromatography, benzene/petroleum ether, 1:20) p-chloromethylbiphenyl (.677g, 2 eq.) was added and the solution was refluxed for one hour at room temperature until it turned muddy yellow. The condenser was removed and the liquid ammonia was evaporated. The solvent and traces of ammonia were removed using the vacuum pump. Water was added to the residue and the solution was acidified to pH 2. It was extracted with chloroform (3x25ml) and the chloroform was dried with anhydrous magnesium sulfate. Evaporation of the solvent gave S,S'-bis(p-phenylbenzyl)asparagusic acid and other products which were separated by preparative thin layer chromatography (silica, ethyl acetate/benzene, 1:10). The purified derivative was crystalized from benzene/cyclohexane to give white crystals (120mg, 0.25 mmoles, 15% yield, mp 135-136°C).

High Resolution Mass Spectrometry Report for C_{30}H_{28}O_{2}S_{2}

Observed Ion 1 = M^+ - 167 = C_{17}H_{17}O_{2}S_{2}

Expected Mass (amu) = 317.066985
Observed Mass (amu) = 317.0671

Observed Ion 2 = M^+ - 285 = C_{13}H_{11}S

Expected Mass (amu) = 199.058145
Observed Mass (amu) = 199.0583

Observed Ion 3 = M^+ - 317 = C_{13}H_{11}

Expected Mass (amu) = 167.086075
Observed Mass (amu) = 167.0858
NMR: 2.78 (5H, multiplet), 3.73 (4H, singlet), 7.0-8.1 (18H, broad band multiplet)

SYNTHESIS OF
S,S'-BIS(p-PHENYL BENZYL)DIHYDRO ASPARAGUSIC ACID ANILIDE (26, p. 30)\textsuperscript{52}

Freshly distilled oxalyl chloride (0.24ml, 2.75 mmoles) was added to S,S'-bis(p-phenylbenzyl)asparagusic acid (130mg, 0.269 mmoles) in dry benzene (4.7ml), at room temperature under nitrogen. The solution was refluxed at 80\textdegree C for fifteen minutes and allowed to stand for two hours at room temperature. The benzene and excess oxalyl chloride were removed under vacuum and the yellow residue repeatedly dissolved in benzene and evaporated, to remove all traces of the oxalyl chloride. The residue, dissolved in benzene, was cooled in an ice water bath and aniline (0.26ml, 2.85 mmoles) was added to it. The resulting solution was stirred overnight. The reaction mixture was extracted with 6\% hydrochloric acid (3x15ml), water (3x15ml). The organic layer was dried over anhydrous magnesium sulfate and the solvent removed. The remaining solid residue was recrystallized from benzene/cyclohexane to yield S,S'-bis(p-phenylbenzyl)asparagusic acid anilide (103mg, 0.18 mmoles, 67\% yield, mp 167-168\textdegree C).
NMR: 2.13 (1H, broad singlet, absent on D2O shake), 2.66 (5H, multiplet),
3.7 (4H, singlet), 7.4 (23H, multiplet)

IR (NaCl soln cell; CHCl3) -- 1710 cm⁻¹, 3450 cm⁻¹

High Resolution Mass Spectrometry Report for \( \text{C}_36\text{H}_{33}\text{S}_2\text{N} \)

Observed Ion 1 = \( \text{M}^+ - 167 = \text{C}_{23}\text{H}_{22}\text{OS}_2\text{N} \)

Expected Mass (amu) = 392.114270
Observed Mass (amu) = 392.1139

Observed Ion 2 = \( \text{M}^+ - 392 = \text{C}_{13}\text{H}_{11} \)

Expected Mass (amu) = 167.086075
Observed Mass (amu) = 167.0858

PREPARATION OF RANEY NICKEL CATALYST

Aluminum-nickel alloy (114g, 1.33 moles) was added in small quantities to aqueous sodium hydroxide (3.8M, 600ml) over a two hour period keeping the temperature below 25°C to control foaming. The catalyst was left for fifteen hours at room temperature until the evolution of hydrogen slackened. The metal-base mixture was heated on a steam bath for 1.5 hours until the hydrogen evolution ceased and the base was decanted from the metal. The original volume was made up with distilled water and the catalyst transferred to a
smaller flask. The water was decanted and the metal shaken with 2.5N sodium hydroxide (200ml) which was also decanted. The catalyst was washed with distilled water until the pH of the wash water was neutral. The activated catalyst was rinsed with water (10x), 95% ethyl alcohol (3x) and 100% ethyl alcohol (3x). The activated catalyst was stored in absolute ethanol at 5°C.

Raney Nickel Desulfurization Of
S,S'-Bis(p-Phenylbenzyl)Dihydroasparagusic Acid Anilide (26, p. 30)²⁶

Raney nickel catalyst (3g) was added to S,S'-bis(p-phenylbenzyl)asparagusic acid anilide (113.8mg, 0.20 mmoles) in 100% ethanol (10ml). The mixture was heated for seven hours at 90°C and the catalyst filtered off. The solution was evaporated and the crude product chromatographed on preparative silica tlc plates (ethyl acetate/toluene, 1:4). Isobutyric acid anilide was obtained (27mg, 0.17 mmoles, 85% yield, mp 106-107°C, lit. mp 105°C₅⁴).

Hydrolysis of Isobutyric Acid Anilide (27, p. 30)⁵⁵
Isobutyric acid anilide (65mg, 0.4 mmoles) was dissolved in 20% hydrochloric acid (4ml) and heated at 95°C for five hours. The water/HCl mixture was evaporated under reduced pressure to dryness, more water was added and the solution was evaporated again. The residue was dissolved in water and the pH was adjusted to 11 with 0.5N sodium hydroxide. The aqueous solution was first extracted with diethyl ether to remove any aniline and it was then evaporated. The residue was stirred with 100% ethanol (3ml) and the ethanol was filtered and evaporated to yield sodium isobutyrate (50mg, 0.45 mmoles, 113% yield, indicating the presence of sodium hydroxide). The mixture was then subjected to Schmidt degradation.

SCHMIDT DEGRADATION OF ISOBUTYRIC ACID (11, p. 10)²

Sodium isobutyrate (50mg, 0.45 mmoles) was chilled to 0°C in a 5ml round bottom flask and 100% sulfuric acid (0.27ml) was added with cooling on ice. Sodium azide (50mg, 0.77 mmoles) was then added and the reaction mixture was connected to a series of traps and a nitrogen inlet. Carbon dioxide was removed from nitrogen by passing the nitrogen through an Ascarite trap before using it to flush the system. This was continued for ten minutes. The reaction
mixture was gradually heated to 70°C and held at that temperature for one hour. The carbon dioxide gas that evolved was passed through a gas dispersion tube into a 5% potassium permanganate/.5N sulfuric acid solution to remove organic contaminants prior to passage through two .5N sodium hydroxide traps. After one hour carbon dioxide free nitrogen was again flushed through the system for fifteen minutes at 70°C. The reaction pot was then cooled, stoppered and stored at -15°C. A saturated barium chloride solution was added to the sodium hydroxide traps to precipitate carbon dioxide as barium carbonate (11mg, 0.056 mmole, 12.4% yield) which was collected quickly on a sintered glass funnel to prevent contamination by atmospheric carbon dioxide. It was washed with water and dried in an Abderhalden at 100°C overnight.

SYNTHESIS OF ISOPROPYLBENZAMIDE (29, p. 30)

The reaction residue from the Schmidt degradation was cooled in an ice water bath and benzoyl chloride (0.12ml, 1 mmole) was added. The solution was stirred for thirty minutes after the pH was adjusted to 12 with 5N aqueous sodium hydroxide. More benzoyl chloride (0.12ml, 1 mmole) was added and the solution stirred for fifteen minutes.
Chloroform was added to the reaction mixture. The organic layer was separated and washed with sodium bicarbonate and water, and then dried over anhydrous magnesium sulfate. Removal of the chloroform gave crude material that was purified on preparative silica plates (ethyl acetate/benzene, 1:10). The identity of the solid as isopropylbenzamide (20mg, 0.12 mmoles, 27% yield, mp 104-105°C), was verified by melting point and analytical tlc against authentic material (lit mp 100°C).

SCHMIDT DEGRADATION OF
S-(2-CARBOXYPROPYL)-L-CYSTEINE (15, p. 20)\textsuperscript{46,56}

S-(2-carboxypropyl)-L-cysteine (95mg, 0.46 mmoles) was chilled to 0°C in a 5ml round bottom flask and concentrated sulfuric acid (0.17 ml) was added and the mixture was stirred until the diacid dissolved. Sodium azide (35mg, 0.54 mmoles) was added and the reaction mixture connected to a series of traps and a nitrogen inlet. Carbon dioxide free nitrogen was obtained by passing nitrogen through an Ascarite trap. The system was then flushed with this purified nitrogen for ten minutes. The reaction mixture was stirred for thirty minutes then heated on a steam bath for three hours. The carbon dioxide gas that evolved was passed
through a gas dispersion tube into a 5% potassium permanganate/.5N sulfuric acid solution to remove organic contaminants prior to passage through two .5N sodium hydroxide traps. The reaction mixture turned dark yellow. Carbon dioxide free nitrogen was again flushed through the system for fifteen minutes. The reaction pot was cooled, stoppered and stored at -15°C. A saturated barium chloride solution was added to the sodium hydroxide traps to precipitate carbon dioxide as barium carbonate (82mg, 0.42 mmoles, 91% yield, 77% of the total radioactivity), which was collected quickly in a sintered glass funnel to prevent contamination by atmospheric carbon dioxide. It was dried in an Abderhalden at 100°C overnight.

SYNTHESIS OF
S-(2-AMINOPROPYL)-L-CYSTEINE (46, p. 50)46,56

The reaction residue from the Schmidt degradation was cooled in an ice water bath. Benzoyl chloride (0.1ml, 0.86 mmoles) and benzene (6ml) were then added. The solution was stirred for four hours at 95°C. The benzene was evaporated and the reaction mixture was dissolved in ether. The ether solution was extracted with water, dried over anhydrous magnesium sulfate and evaporated. The crude material was
purified on preparative silica plates (ethyl acetate/hexanes 1:2, 2x). The benzoyl derivative was then heated with 1N sodium hydroxide (3ml) for 7 hours at 95°C. The solution was acidified to pH 1 with hydrochloric acid and extracted with ethyl acetate(3x). The water phase was evaporated the crude product was then purified by chromatography on a cellulose column (butanol/acetic acid/water, BAW, 12:5:5). The resulting S-(2-aminopropyl)-L-cysteine (16mg, 0.09 mmole, 20% yield, decomposes at 220°C) contained less than 1 dpm/mg radioactivity.

High Resolution Mass Spectrometry Report for C₆H₁₄N₂O₂S

Observed Ion 1 = M⁺ = C₆H₁₄N₂O₂S

Expected Mass (amu) = 178.077580
Observed Mass (amu) = 178.0779

SYNTHESIS OF N-BUTYL PROPIONATE (30, p. 34)⁵⁸

Propionyl chloride (9.25g, 0.1 moles) dissolved in methylene chloride (10.7ml) was slowly added over one hour to a chilled solution of pyridine (8.06ml, 0.1 moles), n-butanol (9.15ml, 0.1 moles) and methylene chloride (10.7ml). The solution was stirred in an ice bath for four hours and then overnight at room temperature. The reaction mixture was extracted with water and the organic phase dried
using anhydrous magnesium sulfate and the solvent was removed. The resulting liquid was distilled to yield n-butyl propionate (12.89g, 99% yield, bp 140–150°C at 360mm).

NMR: 0.77–1.8 (10H, multiplet of overlapping signals) 2.27 (2H, quartet), 4.1 (2H, triplet)

SYNTHESIS OF [3H]TRIFLUOROACETIC ACID

[3H]Trifluoroacetic acid was generated by reacting trifluoroacetic anhydride (0.47ml, 3.34 mmoles) with tritiated water (30 mCi, 6ul) at -78°C. The mixture was then stirred overnight at room temperature and was then used to synthesize [2-3H]isobutyric acid.

SYNTHESIS OF SODIUM [2-3H]ISOBUTYRATE (32, p. 34)

N-Butyl propionate was treated with lithium diisopropylamide (LDA) in tetrahydrofuran (THF)/hexamethylphosphoramide (HMPA) following the procedure of Schlessinger et al. Diisopropylamine (0.56ml, 4 mmoles)
in THF (10ml)/HMPA (0.1ml) was stirred at -78°C under nitrogen for fifteen minutes. LDA was generated in situ by addition of butyllithium (2.34M in hexanes, 4 mmoles) at -78°C. The solution was warmed to 0°C over thirty minutes to allow complete formation of LDA and was then recooled to -78°C. n-Butyl propionate (0.6ml, 4 mmoles) was added and the solution was stirred for one hour at -78°C. The resulting anion was quenched at this temperature with [3H]trifluoroacetic acid (6 mmoles, 30 mCi). The solution was stirred for thirty minutes at -78°C, then at room temperature for one hour, and the THF removed by bulb to bulb distillation. The yellow residue dissolved in ether was washed with 5% hydrochloric acid, water, sodium bicarbonate, brine, and dried over anhydrous magnesium sulfate. The ether was removed and the resulting liquid (340mg) was purified by silica gel column chromatography (benzene/hexanes 1:1). The fractions were monitored for n-butyl [2-3H]propionate by gas chromatography. After purification the tritiated ester (64mg, 0.49 mmoles, 12% yield) was diluted with nonradioactive n-butyl propionate (62.75mg, 0.48 mmoles).

Deprotonation of n-butyl [2-3H]propionate was accomplished with LDA. The resulting enolate was alkylated with unlabelled methyl iodide to yield n-butyl [2-3H]isobutyrate which was isolated and purified by silica
gel chromatography as previously described for n-butyl propionate. The ester (120mg, 0.83 mmoles) was hydrolysed by stirring at room temperature with 1N sodium hydroxide (3ml) until the solution became one phase (20 hours). The solution was then acidified to pH 1 with dilute sulfuric acid and subjected to steam distillation. 300ml of water were collected and brought to pH 6 with addition of a dilute sodium hydroxide solution. The water was removed by evaporation under reduced pressure and the resulting sodium [2-\textsuperscript{3}H]isobutyrate was checked by analytical cellulose tlc (isopropanol/2N ammonium hydroxide 3:1) and visualized by bromocresol green. The yield of sodium isobutyrate was 88.7mg. (0.81 mmoles, 83% yield, Sp. act.= 0.015 mCi/mg, 4.4% radiochemical yield).

NMR: 1.22 (6H, doublet), 2.60 (1H, quintet),
11.55 (1H, medium broad singlet)

SYNTHESIS OF [3-\textsuperscript{3}H]SODIUM ISOBUTYRATE (31, p. 33)\textsuperscript{34,60}

[\textsuperscript{3}H]Methyl iodide (100mCi, Amersham) was vacuum transferred into a flask containing nonradioactive methyl iodide (0.15ml, 2.4 mmoles) and HMPA (0.1ml, 0.57 mmoles).
After releasing the vacuum, the flask was stoppered and stored at -20°C.

n-Butyl propionate was alkylated following the procedure of Schlessinger et al. LDA was prepared, as described for the sodium [2-3H]isobutyrate, by addition of butyllithium (1.23ml, 2 mmoles) to diisopropylamine (0.28ml, 2 mmoles) in THF (1.7ml) at -78°C under nitrogen. The solution was warmed to 0°C over thirty minutes to allow complete formation of the LDA and it was then recooled to -78°C. n-Butyl propionate (0.3ml, 2 mmoles) was added and the solution was stirred for one hour at -78°C. The previously prepared tritiated methyl iodide/HMPA solution was then syringed into the reaction flask at -78°C to quench the anion. The mixture was stirred for thirty minutes while allowing the solution to warm to room temperature. The THF was removed and the residue was dissolved in ether. The organic layer was washed with 10% hydrochloric acid, sodium bicarbonate, saturated sodium chloride, and dried over anhydrous magnesium sulfate. The ether was removed to yield n-butyl [3-3H]isobutyrate (194.2mg, 1.35 mmoles, 67.4% yield) which was checked for purity by gas chromatography. The ester was hydrolysed by refluxing it for 7.5 hours with dilute sodium hydroxide. The solution was acidified to pH 1 with sulfuric acid and subjected to steam distillation. The distillate was brought to pH 8 with a dilute solution of
sodium hydroxide. The water was then removed to yield sodium $[3^{3}H]i$so$bu$tyrate (70.75mg, 0.64 mmoles, 48% yield, Sp. act. = 0.239 mCi/mg, 17% radiochemical yield).

NMR: 1.22 (6H, doublet), 2.60 (1H, quintet), 11.55 (1H, medium broad singlet)

SYNTHESIS OF $[1-{^{14}}C]$METHACRYLIC ACID (33, p. 38)$^{62,63}$

2-Bromopropene (8.89ml, 0.1 moles) in dry ether (36ml) was added dropwise over one hour to a flask containing magnesium metal (26.7g, 1.1 moles) in dry ether (36ml). The solution was refluxed at 45°C for one hour and it was then decanted from the excess magnesium turnings. The molarity of the solution containing the Grignard reagent in ether was determined by titration with 1N sodium hydroxide. It was found to be 0.67M. $[^{14}C]$Carbon dioxide, which was generated by addition of concentrated sulfuric acid to $[^{14}C]$barium carbonate (52.7mg, 0.27 mmole, 38 mCi/mmole), was vacuum transferred into a solution of the Grignard reagent (4.4 mmoles) in ether. Unlabelled carbon dioxide was generated in the same way from barium carbonate (148.3mg, 0.75 mmoles) and vacuum transferred to force the reaction to completion.
Ether was evaporated from the reaction mixture and the solution acidified to pH 3 with sulfuric acid. Hydroquinone (1mg, 0.01 mmole) was added and the reaction mixture was subjected to steam distillation. The distillate was titrated with potassium hydroxide to pH 8. Water was removed and potassium $^{14}$C methacrylate (19.5mg, 0.16 mmole, 16% yield, Sp. act. = 0.028 mCi/mg, 5.37% radiochemical yield) was obtained.

NMR: 1.97 (3H, doublet), 5.72 (1H, complex series of lines), 6.30 (1H, doublet), 11.57 (1H, broad singlet)

SYNTHESIS OF
SODIUM $[1-^{14}$C]3-MERCAPTOISOBUTYRATE (34, p. 41)$^{37}$

Potassium $[1-^{14}$C] methacrylate (25mg, 0.2 mmole, 0.5 mCi) was dissolved in water (50ul) and placed in a one milliliter Reactivial. Thiolacetic acid (75ul, 1.05 mmole) and 1.45N sulfuric acid (50ul) were added and the vial was sealed and heated for three hours at 80-85°C. An additional amount of thiolacetic acid (40ul, 0.56 mmole) was added and the solution was heated again for three hours. To hydrolyse
the thioester, 5N sodium hydroxide (0.5ml, 0.25 mmoles) was added to the vial and the mixture was heated at 90-95°C for three hours. It was then cooled, acidified to pH 4 and the [1-14C]3-mercaptoisobutyric acid was extracted into ether. The crude product was purified by preparative thin layer chromatography (silica, benzene/ethyl acetate 1:1). The acid (21.6mg, 0.18 mmoles) was converted to the sodium salt by titration with a dilute sodium hydroxide solution to give sodium [1-14C]3-mercaptoisobutyrate (24.3mg, 0.17 mmoles, 85.0% yield, Sp. act. = 0.017 mCi/mg, 82.6% radiochemical yield).

NMR: 1.32 (3H, doublet), 1.70 (1H, multiplet),
2.80 (3H, multiplet)

SYNTHESIS OF
SODIUM [2-3H]3-MERCAPTOISOBUTYRATE (35a, p. 42)37

[2-3H]3-Mercaptoisobutyrate was prepared by heating a mixture of methacrylic acid (0.12ml, 1.4 mmoles) and thiolacetic acid (0.1ml, 1.4 mmoles) under nitrogen in the presence of tritiated water (20ul, 0.1 Ci, 1 mmole) for four hours at 80-90°C. The resulting thioester was hydrolysed by
addition of aqueous sodium hydroxide (2 eq.) to the reaction mixture, followed by heating under argon for six hours at 90-95°C. The solution was acidified to pH 4 with sulfuric acid and extracted with ether. The ether was dried, evaporated and the residue was purified by silica gel chromatography (benzene/ethyl acetate, 1:1). The acid was titrated with sodium hydroxide to give the sodium salt (62mg, 0.44 mmoles, 31.4% yield, 3.1% radiochemical yield, bp 112-114°C at 10mm, Sp. act. = 0.05 mCi/mg).

NMR: 1.32 (3H, doublet), 1.70 (1H, multiplet),
     2.80 (3H, multiplet)

SYNTHESIS OF
METHYL 2,3-DIBROMOISOBUTYRATE (39, p. 46)\(^{39,40}\)

Bromine (10.30ml, 0.2 moles) was added dropwise over three hours to a chilled solution of methyl methacrylate (21.37ml, 0.2 moles). The solution was bright orange after being stirred for twelve hours indicating the presence of bromine. An additional amount of methyl methacrylate (2ml, 0.02 moles) was added but the solution remained orange. The mixture was stirred overnight with no color change detected.
The solution was washed with aqueous sodium thiosulfate to remove excess bromine and the resulting cloudy white solution was distilled. Methyl 2,3-dibromoisobutyrate was obtained (30g, 0.12 moles, 60% yield, bp 86°C at 15 mm\textsuperscript{39,40}).

NMR: 2.03 (3H, singlet), 3.83 (3H, singlet), 4.16 (1H, singlet), 4.27 (1H, singlet)

SYNTHESIS OF 3-BROMO-2-METHYLPROPENOIC ACID (40, p. 46)\textsuperscript{41}

Methyl 2,3-dibromoisobutyrate (30g, 0.12 moles) was dissolved in THF (100ml) and treated with 1,5-diazabicyclo[5,4,0]undec-5-ene (DBU, 17g, 1.1 mole eq.). The solution was refluxed at 80-85°C for one hour. The viscous milky white solution was cooled, poured into ice-water and extracted with ether. The organic phase was washed with 1N hydrochloric acid, saturated brine and dried over anhydrous magnesium sulfate. The ether was removed to yield methyl 3-bromo-2-methylpropenoate. Hydrolysis of the ester\textsuperscript{64} was accomplished by treatment with 2N aqueous sodium hydroxide (100ml) and methanol (20ml) for three hours at 50°C. The solution was cooled, acidified with concentrated hydrochloric acid and extracted with ether. The organic
phase was dried over anhydrous magnesium sulfate and evaporated. The 3-bromo-2-methylpropenoic acid (10.4g, 0.063 moles, 54.6% yield) was recrystallized from hot petroleum ether (20-40°C) to yield white needles that melted sharply at 63°C. The reported melting point is 60-62°C.

NMR: 2.01 (3H, doublet), 7.71 (1H, quartet)
11.45 (1H, broad singlet)

SYNTHESIS OF
[3-3H]3-BROMO-2-METHYLPROPENOIC ACID (42, p. 46)

3-Bromo-2-methylpropenoic acid (427.5mg, 2.6 mmoles) was dissolved in THF (15ml) under nitrogen and cooled to -78°C. t-Butyllithium (5.19ml, 7 mmoles) was syringed into the reaction flask and the solution stirred for one hour. Tritiated water (0.1ml, 500 mCi) was then added and the dark yellow solution became lighter as it was warmed to 0°C. Water (0.5ml) was added at 0°C and the solution was warmed to room temperature. The THF was removed, the water was acidified, and extracted with ether. The ether was washed with brine and dried over anhydrous sodium sulfate. Ether was removed to give [3-3H]3-bromo-2-methylpropenoic acid
(383mg, 2.3 mmoles, 88.5% yield, Sp. act. = 0.06 mCi/mg, 
4.6% radiochemical yield, mp 60-62°C, lit. mp 60-62°C\textsuperscript{36})

[3\textsuperscript{-2}H]3-Bromo-2-methylpropenoic Acid

NMR: 2.01 (3H, singlet), 11.45 (1H, broad singlet)

PREPARATION OF SODIUM AMALGAM\textsuperscript{65}

Sodium amalgam was prepared following the procedure described in Fieser and Fieser\textsuperscript{66} and the ratio of sodium to mercury supplied by Buckles and Mock\textsuperscript{65}. Sodium (10g, 0.43 moles) was placed in a three-necked flask equipped with an addition funnel charged with mercury (11.8ml, 0.80 moles) and a nitrogen inlet and outlet. The system was carefully flushed with nitrogen and the mercury was added to the sodium. The reaction was very exothermic. When all of the mercury was added, the hot solution solidified as it cooled. The solid was heated by a flame until it liquified and was then poured into a mortar. The resolidified sodium amalgam was crushed and stored in a tightly sealed bottle in dry ether under nitrogen.
SYNTHESIS OF [3-\(^3\)H]METHACRYLIC ACID (43, p. 46)^67

[3-\(^3\)H]3-Bromo-2-methylpropenoic acid (383mg, 2.3 mmoles, 23 mCi) was dissolved in aqueous .4N sodium hydroxide solution (10ml) and chilled to -20°C. Sodium amalgam (8.2g, 3.7 % sodium) was added in small portions at -20°C over two hours and the solution was allowed to warm from -10°C to 0°C after each addition. After all the sodium amalgam was added, the mixture was stirred an additional two hours at 0°C to -20°C. The liquid phase, which was decanted from the remaining solid sodium amalgam and liquid mercury, was washed with water, acidified to pH 1, and extracted with ether. The ether was evaporated and the product contained a 3:1 mixture of methacrylic acid and isobutyric acid (195mg total).

NMR: 1.97 (3H, doublet), 5.72 (1H, complex series of lines), 6.30 (1H, doublet), 11.57 (1H, broad singlet)

SYNTHESIS OF 3-BROMOISOBUTYRIC ACID (37, p. 44)^38

Freshly distilled methacrylic acid (43g, 0.5 moles) was
dissolved in chloroform (90ml), previously dried by passage through a column of Grade I activated alumina. The solution was cooled in an ice bath, saturated with dry hydrogen bromide gas and allowed to stand for forty-eight hours. At the end of this time, the solution was again saturated with HBr gas and left for another forty-eight hours. The chloroform and excess HBr were then distilled off. The remaining liquid was distilled under vacuum to give 3-bromoisobutyric acid (60g, 0.36 moles, 72% yield, bp 116-118°C at 13mm\textsuperscript{38}).

NMR: 1.5 (3H, doublet), 2.93 (1H, sextet), 3.5 (2H, AB, qd, octet)

SYNTHESIS OF
SODIUM [3-\textsuperscript{3}H]3-MERCAPTOISOBUTYRATE (38, p. 46)\textsuperscript{37}

[3-\textsuperscript{3}H]3-Mercaptoisobutyric acid was prepared by treating the methacrylic acid/isobutyric acid mixture (145mg/109mg methacrylic acid, 1.77 mmoles) with thiolacetic acid (200ul, 2.8 mmoles) under nitrogen at 80-90°C for four hours. The resulting thioester was hydrolysed by addition of two equivalents of aqueous sodium hydroxide followed by
heating under argon at 90-95°C for six hours. The solution was then acidified to pH 4 with sulfuric acid and extracted with ether. The ether was evaporated and the residue purified by silica gel chromatography to give free acid which was titrated with sodium hydroxide to give sodium [3-3H]3-mercaptopoisobutyrate (24.7mg, 0.17 mmoles, 7.39% yield, Sp. act.= 0.05 mCi/mg, 5.4% radiochemical yield from [3-3H]3-bromo-2-methylpropenoic acid).

**NMR:** 1.32 (3H, doublet), 1.70 (1H, multiplet),
2.80 (3H, multiplet)

SYNTHESIS OF
SODIUM [35S]3-MERCAPTOISOBUTYRATE (36, p. 44)38

[35S]Hydrogen sulfide (8.9 mCi, Amersham), that had been vacuum transferred into a 3.75N solution of sodium hydroxide (0.2ml), was added to B-bromoisobutyric acid (0.208g, 1.25 mmoles) dissolved in 1.67M sodium hydroxide solution (0.38ml, 0.63 mmoles). The mixture was heated to 95°C for ninety minutes. An additional amount of 3.75N sodium hydroxide solution (0.25ml) saturated with unlabelled hydrogen sulfide was added and heating was continued for
another ninety minutes. The reaction flask was cooled and the solution was acidified to pH 1 with dilute sulfuric acid. It was then extracted with ether and the ether was dried over anhydrous sodium sulfate. The solvent was evaporated and the crude product (159mg) was purified by bulb to bulb distillation. The distillate was further purified by silica gel chromatography (ethyl acetate/benzene 1:1) to yield 3-mercaptoisobutyric acid (20.6mg, 0.17 mmoles, 13.6% yield) which was titrated with sodium hydroxide to give sodium $[^{35}S]$3-mercaptoisobutyrate (24.1mg, 0.17 mmoles, Sp. act. = 0.015 mCi/mg, 4.1% radiochemical yield).

NMR: 1.32 (3H, doublet), 1.70 (1H, multiplet),
2.80 (3H, multiplet)

SYNTHESIS OF
S-(2-CARBOXY-[3-$^3$H]PROPYL)-L-CYSTEINE (Scheme XV, p. 53) 45

The mixture of [3-$^3$]methacrylic acid and isobutyric acid (100mg, 1.16 mmoles) was dissolved in a 2N sodium hydroxide solution (1ml) and L-cysteine hydrochloride (140mg, 0.89 mmoles) was added. The solution was stirred
under argon and heated to 95°C for 4 to 4.5 hours. Glacial acetic acid was added to the cooled reaction mixture to bring the pH to 4. Water was then removed and the residue purified by cellulose chromatography (BAW 12/5/5) followed by paper chromatography, using the same solvent system, to yield S-(2-carboxy-[3-H]propyl)cysteine (23.7mg, 0.1 mmoles, 11.23% yield, Sp. act. = 0.002mCi/mg, mp 193°C, lit. mp 194°C).45

SYNTHESIS OF

\[ ^{35}S \]S-(2-CARBOXYPROPYL)-L-CYSTEINE (Scheme XIV, p. 51)45

A mixture of \[^{35}S\]L-cysteine-hydrochloride (26mg, 0.16 mmole, 500 uCi, Amersham) and methacrylic acid (40mg, 0.47 mmoles) was stirred under a nitrogen atmosphere while 2N sodium hydroxide was added to bring the pH to 8. The resulting solution was heated at 95°C for eight hours, cooled, and brought to pH 2 with glacial acetic acid and the residue was purified by cellulose chromatography (BAW 12:5:5) to yield \[^{35}S\]S-(2-carboxypropyl)-L-cysteine (26.5mg, 0.13 mmoles, 80.0% yield, mp 195°C with decomposition, lit. mp 194°C with decomp.45, Sp. act. = 0.01 mCi/mg, 53.0% radiochemical yield).
SYNTHESIS OF ISOBUTYRIC ACID ANILIDE (27, p. 30)\textsuperscript{68,69}

Aniline (0.25ml, 2.75 mmoles) was added to a chilled solution of dicyclohexylcarbodiimide (0.562g, 2.75 mmoles) in acetonitrile (2ml). Isobutyric acid (0.25ml, 2.75 mmoles and a small amount of the radiolabelled isobutyric acid) was added dropwise to the chilled reaction flask. A white precipitate formed almost immediately and the solution was stirred for an additional five hours. Five drops of glacial acetic acid was then added to decompose any excess DCC and the solution was filtered through a sintered glass funnel to remove the insoluble urea. The filtrate was evaporated and the yellow residue, dissolved in ethyl acetate, was washed with 6% hydrochloric acid and then 5% aqueous sodium bicarbonate solution. The ethyl acetate was evaporated and the solid recrystallized benzene/petroleum ether (40-60\textdegree C) to yield white needles (270mg, 1.66 mmoles, 60.4% yield, mp 103-105\textdegree C, lit. mp 105\textdegree C\textsuperscript{54}) of isobutyric acid anilide.

SYNTHESIS OF

O,S-Bis(p-BROMOPHENACYL)3-MERCAPTOISOBUTYRATE

(45, p. 46)\textsuperscript{70}

3-Mercaptoisobutyrate (0.73g, 6.1 mmoles) in
acetonitrile (20ml) was stirred with potassium carbonate (0.84g, 6.1 mmoles) for thirty minutes. Dibenzo-18-crown-6 (0.21g, 0.59 mmoles) and p-bromophenacyl bromide (2.42g, 12.2 mmoles) were added to the mixture which was then heated at 60°C overnight. The solution was filtered, the acetonitrile was evaporated and the resulting residue was purified by silica gel chromatography (toluene/hexanes, 2:1). The derivative was recrystallized from chloroform/hexanes to a constant activity (695.22mg, 1.36 mmoles, 22.26% yield, mp 96.5-97.5°C).

NMR: 1.28 (3H, doublet), 2.78 (3H, broad multiplet),
3.76 (2H, singlet), 5.20 (2H, singlet),
8.60 (8H, doublet of doublets)

High Resolution Mass Spectrometry Report for $C_{20}H_{18}O_4Br_2S$
Observed Ion 1 = $M^+ = C_{20}H_{18}O_4Br_2S$
Expected Mass (amu) = 511.929160
Observed Mass (amu) = 511.9293

Observed Ion 2 = $M^+ + 2 = C_{20}H_{18}O_4^{81}Br^{79}BrS$
Expected Mass (amu) = 513.927160
Observed Mass (amu) = 513.9258
REFERENCES


15. S. Iwahara, S. Takasawa, T. Tochikura, and K. Ogata, Agric. Biol. Chem. (Tokyo), 30,
385 (1966).


