On the Biosynthesis of Artemisia Ketone in Artemisia Annua.

Janell Broussard Simpson
Louisiana State University and Agricultural & Mechanical College

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_disstheses

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_disstheses/3464
INFORMATION TO USERS

This was produced from a copy of a document sent to us for microfilming. While the most advanced technological means to photograph and reproduce this document have been used, the quality is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help you understand 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 you of complete continuity.

2. When an image on the film is obliterated with a round black mark it is an indication that the film inspector noticed either blurred copy because of movement during exposure, or duplicate copy. Unless we meant to delete copyrighted materials that should not have been filmed, you will find a good image of the page in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed the photographer has followed a definite method in "sectioning" the material. 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 any illustrations that cannot be reproduced satisfactorily by xerography, photographic prints can be purchased at additional cost and tipped into your xerographic copy. Requests can be made to our Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases we have filmed the best available copy.
SIMPSON, JANELL BROUSSARD

ON THE BIOSYNTHESIS OF ARTEMISIA KETONE IN ARTEMISIA ANNUA

The Louisiana State University and Agricultural and Mechanical Col.

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106 18 Bedford Row, London WC1R 4EJ, England

PH.D. 1979
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark √.

1. Glossy photographs
2. Colored illustrations
3. Photographs with dark background
4. Illustrations are poor copy
5. Print shows through as there is text on both sides of page
6. Indistinct, broken or small print on several pages throughout
7. Tightly bound copy with print lost in spine
8. Computer printout pages with indistinct print
9. Page(s) lacking when material received, and not available from school or author
10. Page(s) seem to be missing in numbering only as text follows
11. Poor carbon copy
12. Not original copy, several pages with blurred type
13. Appendix pages are poor copy
14. Original copy with light type
15. Curling and wrinkled pages
16. Other
ON THE BIOSYNTHESIS OF ARTEMISIA KETONE
IN ARTEMISIA ANNUA

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biochemistry

by

Janell Broussard Simpson
B.S., Louisiana State University, 1974
December, 1979
EXAMINATION AND THESIS REPORT

Candidate: Janell Broussard Simpson

Major Field: Biochemistry

Title of Thesis: On the Biosynthesis of Artemisia Ketone In Artemisia Annua

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Date of Examination:

October 17, 1979
ACKNOWLEDGEMENTS

I wish to express my thanks to Dr. G. E. Risinger for his guidance during my graduate career.

I am grateful to the other members of my committee, especially Dr. George Newkome, for his early and continuing interest in my scientific development.

The financial assistance of the Biochemistry Department is appreciated.

Finally, I wish to thank my family and friends, especially my husband, Allan, for their continued support and encouragement.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vii</td>
</tr>
<tr>
<td>I. INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>II. EXPERIMENTAL</td>
<td>47</td>
</tr>
<tr>
<td>Synthesis and Reactions of Artemisia Ketone</td>
<td>49</td>
</tr>
<tr>
<td>Routes to Radioactive 3-Methylbutanal</td>
<td>54</td>
</tr>
<tr>
<td>Synthesis of Thiamine Compounds</td>
<td>58</td>
</tr>
<tr>
<td>Feeding Experiments</td>
<td>62</td>
</tr>
<tr>
<td>III. RESULTS AND DISCUSSION</td>
<td>79</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>117</td>
</tr>
<tr>
<td>VITA</td>
<td>125</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Incorporations of precursors into regular monoterpenes</td>
<td>10</td>
</tr>
<tr>
<td>Table 2</td>
<td>Irregular monoterpenes and their natural sources</td>
<td>19</td>
</tr>
<tr>
<td>Table 3</td>
<td>Incorporations of precursors into irregular monoterpenes</td>
<td>31</td>
</tr>
<tr>
<td>Table 4</td>
<td>PMR assignments of artemisyl compounds</td>
<td>75</td>
</tr>
<tr>
<td>Table 5</td>
<td>Essential oil analysis</td>
<td>76</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>Foliage of <em>A. annua</em> and structure of artemisia ketone</td>
<td>2</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Modes of dimerization of isoprene</td>
<td>4</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Biosynthesis of mevalonic acid from acetyl CoA-[2-14C]</td>
<td>6</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Conversion of MVA into monoterpenes</td>
<td>7</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>Monoterpenes</td>
<td>8</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Conversion of leucine and valine to DMAPP</td>
<td>15</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>Labelling patterns in geraniol</td>
<td>18</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>Mechanisms of artemisia ketone biosynthesis</td>
<td>24</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>Mechanisms of artemisia ketone biosynthesis</td>
<td>25</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>Mechanisms involving a cyclopropyl intermediate</td>
<td>27</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>Biosynthesis of artemisia alcohol</td>
<td>28</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>Unified approach to irregular monoterpenone biosynthesis</td>
<td>30</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>Incorporation of MVA into chrysanthemic acid</td>
<td>35</td>
</tr>
<tr>
<td>Figure 14.</td>
<td>Pools involved in asymmetric labelling</td>
<td>39</td>
</tr>
<tr>
<td>Figure 15.</td>
<td>Conversion of leucine to HMG CoA</td>
<td>41</td>
</tr>
<tr>
<td>Figure 16.</td>
<td>Conversion of HMG CoA to IPP</td>
<td>42</td>
</tr>
</tbody>
</table>
Figure 17. Labelling pattern in \( \delta \)-carotene from L-leucine-[\( 4^{-14} \)C] .......... 44
Figure 18. Labelling pattern in echinuline .......... 45
Figure 19. Substrates fed to Artemisia annua ................. 80
Figure 20. Irregular diterpene formed in yeast preparations containing geraniol, citral and thiamine .......... 82
Figure 21. Labelled irregular diterpene formed in yeast preparation .......... 83
Figure 22. Substrates for irregular monoterpene biosynthesis .......... 84
Figure 23. Mechanism of continuous-flow Reformatsky reaction .......... 86
Figure 24. Pathway to artemisia ketone .......... 88
Figure 25. Entry of the valine skeleton into leucine pathway .......... 91
Figure 26. \( \alpha \)-Hydroxy acids as substrates for A. annua .......... 93
Figure 27. Degradation of artemisia ketone by two methods .......... 96
Figure 28. Synthesis of 16 ................. 102
Figure 29. Synthetic route to 19 ................. 106
Figure 30. Biosynthesis of 19 by A. annua ................. 107
Figure 31. Biosynthesis of artemisia ketone .......... 111
Figure 32. Incorporation of 3-methylbutanal-[\( 1^{-14} \)C] into IPP .......... 115
ARTEMISIA KETONE IS AN IRREGULAR MONOTERPENE THAT
HAS BEEN SHOWN EXPERIMENTALLY TO HAVE AN ASYMMETRIC
LABELLING PATTERN WHEN MEVALONIC ACID IS FED TO WHOLE
PLANTS OF *ARTEMISIA ANNUA*. THE MAJORITY OF THE LABEL IS
ISOLATED IN THAT HALF OF THE MOLECULE DESIGNATED BY ANALYSIS TO REGULAR MONOTERPENES AS THE \( I \) UNIT. HOWEVER, CURRENT THEORIES OF IRREGULAR MONOTERPENE BIOSYNTHESIS
PREDICT THAT EACH HALF OF ARTEMISIA KETONE SHOULD CONTAIN
EQUAL AMOUNTS OF LABEL AND THAT MEVALONIC ACID SHOULD BE AN EFFICIENT PRECURSOR.

IN THIS RESEARCH PROJECT, SEVERAL FEEDING EXPERIMENTS WERE CARRIED OUT WITH WHOLE PLANTS OF *ARTEMISIA ANNUA* TO ELUCIDATE THE DETAILS OF THE ASYMMETRIC LABELLING
PHENOMENON. A PATHWAY IS PROPOSED INVOLVING THIAMINE PYRIPHOSPHATE AS A COFACTOR IN CONVERTING THE AMINO ACID LEUCINE TO A FIVE-CARBON PRECURSOR OF THE \( D \) UNIT OF ARTEMISIA KETONE. THE SUBSTRATES CHOSEN FOR FEEDING EXPERIMENTS WERE DESIGNED TO INTERACT WITH THE PROPOSED PATHWAY AND EFFICIENTLY LABEL THE \( D \) UNIT.

SUBSTRATES FED TO *A. ANNUA* INCLUDED THE AMINO ACIDS LEUCINE AND VALINE AND THEIR \( \alpha \)-HYDROXY ACID ANALOGS. WHILE THE TWO AMINO ACIDS WERE NOT EFFICIENTLY INCORPORATED INTO ARTEMISIA KETONE, THE \( \alpha \)-HYDROXY ACIDS WERE
incorporated in better yield than mevalonic acid! However, degradation of a sample of artemisia ketone from the feeding of a \( \alpha \)-hydroxyisocaproic acid showed that the majority of the label resided in the \( I \) portion of artemisia ketone. These substrates were metabolized via a mevalonoid pathway that was previously shown to be an important route of leucine incorporation into carotenoids via HMG CoA.

Other substrates examined as precursors to the proposed \( D \) pathway were 2-(1-hydroxy-3-methylbutyl-[\( \text{\textsuperscript{14}C} \)])-thiamine chloride hydrochloride and its thiazole analog, 2-(1-hydroxy-3-methylbutyl-[\( \text{\textsuperscript{14}C} \)])-4-methyl-5-(2-hydroxyethyl)thiazole. Both of these substrates yielded levels of incorporation into artemisia ketone comparable to those attained with mevalonic acid. Feeding experiments with two aldehydes, 3-methylbutanal-[\( \text{\textsuperscript{14}C} \)] and 3-methyl-2-butenal-[\( \text{\textsuperscript{14}C} \)], resulted in similar levels of radioactivity in artemisia ketone. Degradation of an artemisia ketone sample from a 3-methylbutanal-[\( \text{\textsuperscript{14}C} \)] feeding revealed an asymmetric labelling pattern with the majority of the label located in the \( I \) unit. Again, these substrates were metabolized via an existing mevalonoid pathway resulting in a labelling pattern similar to mevalonic acid.

The substrates selected as precursors to the \( D \) unit
of artemisia ketone did not follow the proposed route of metabolism involving thiamine pyrophosphate. They were utilized via an established pathway in which they were degraded and reincorporated into a mevalonoid precursor, HMG CoA. This compound gives rise to isopentenyl pyrophosphate which is then used to generate monoterpenes.
I. INTRODUCTION

"Of these worts that we name Artemisia, it is said that Diana did find them and delivered their powers and leechdom to Chiron the Centaur, who first from these Worts set forth a leechdom, and he named these worts from the name of Diana, Artemis, that is Artemisia." (1)

Artemisia annua (Figure 1) is an herb belonging to a group of plants in the Compositae called Wormwoods. The whole group, consisting of one hundred eighty species, is known for the extreme bitterness of all parts of the plant. Extracts and teas of the Wormwoods have been used throughout history in liqueurs such as absinthe and vermouth and in medicines for gout and digestion as well as an anthelmintic (1). Some species, such as A. dracunculus (taragon), are commonly found in herb gardens, whereas A. tridentata (sagebrush), covers large areas of the western plains of the United States and has forage value for wildlife only when a harsh winter has killed other vegetation (2). Artemisia annua is not native to this country (3), but grows abundantly in Turkey (4), Bulgaria (5), and parts of Russia (6).

The medicinal and aromatic properties of these herbs
Figure 1. Foliage of *Artemisia annua* and structure of artemisia ketone.
are attributed to essential oil components. The essential oil is a complex mixture of steam-volatile plant constituents normally deposited in either dead cells, oil cavities or subcuticular spaces of glandular hairs (7). Artemisia ketone is a pungent-smelling terpene which is the major constituent of the essential oil of A. annua. In 1917 (8) the compound was first reported as a ketonic molecule possessing two double bonds and having the molecular formula C₁₀H₁₆O. The structure (Figure 1) was supported by classical degradation methods (9). Ruzicka, et al (10) offered further confirmation of the originally proposed structure and commented on the irregularity of the terpene linkage.

As early as 1887, Wallach (11) had undertaken a study of essential oil components from which he postulated the "isoprene rule". This rule categorized the structure of many natural compounds as a head-to-tail or 4-1 dimerization of the basic five-carbon unit, isoprene (Figure 2). However, other possibilities such as the 4-2 dimerization noted above for artemisia ketone did not fit into the isoprene rule and were described as "irregular".

The isoprene rule was later modified by Ruzicka (12) to include a wide range of biosynthetic possibilities. This revised concept of terpene biosynthesis is called the biogenetic isoprene rule and states that naturally
Figure 2. Modes of dimerization of isoprene.
occurring terpenoids are derived either directly or by way of predictable stereospecific cyclizations, rearrangements and dimerizations from acyclic combinations of a basic five-carbon unit. The search for the biological equivalent of that five-carbon unit has been the major impetus of many research groups. A key discovery in the biosynthesis of terpenes and steroids was that mevalonic acid (MVA) was incorporated almost quantitatively into cholesterol in cell-free systems derived from either yeast or liver (13). The biosynthesis of mevalonic acid from acetate (14) is shown in Figure 3.

Conversion of MVA into isopentenyl pyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP) is shown in Figure 4. MVA-kinase catalyzes the phosphorylation of MVA and subsequent decarboxylation (15). The condensation of IPP and DMAPP then occurs via an $S_N2$-E2 reaction to give a "normal" monoterpane, geranyl pyrophosphate (GPP). In accordance with the biogenetic isoprene rule, GPP undergoes further rearrangements and condensations to produce a wide spectrum of known compounds (Figure 5). Thus, on biosynthetic grounds, each monoterpane can be described as containing two sections, a D section derived from DMAPP and an I section derived from IPP. It was envisioned that feeding of labelled MVA to plants would result in the isolation of labelled monoterpenes. How-
Figure 3. Biosynthesis of mevalonic acid from acetyl CoA-[2-14C].
Figure 4. Conversion of MVA into monoterpenes.
Figure 5. Monoterpenes.
ever, the label did not occur equally in the D and I units of the terpenes.

Incorporations of several key precursors into monoterpene are listed in Table 1. Among the regular terpenes, three types of labelling patterns are observed. The most common result is an asymmetric distribution of the label with the majority (80 - 90%) located in the I section of the monoterpenes. However, some examples have been reported where the majority resides in the D section. There have also been reports of symmetrical labelling in monoterpenes.

Banthorpe and Charlwood (16) introduced the concept of separate pools for the monoterpenic precursors, IPP and DMAPP, in order to explain adequately asymmetric labelling results. The terpenes (-)-thujone, (+)-isothujone and (+)-sabinene were biosynthesized from MVA-[2-14C] by Thuja, Tanacetum and Juniperous species, respectively. While actual 14C incorporation was low (0.004 - 0.008%), the bulk of the tracer (90 - 99%) was located in that part of the skeleton derived from IPP. The tracer pattern in (+)-sabinyl acetate was also asymmetric but the acetate group contained ten times more label than the terpenoid portion, indicating extensive degradation of MVA and reincorporation as two-carbon fragments.

In a related experiment, Banthorpe and Baxendale (17)
<table>
<thead>
<tr>
<th>Plant</th>
<th>Terpene</th>
<th>Substrate</th>
<th>I</th>
<th>D%</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thuja</td>
<td>thujone</td>
<td>MVA-[2-(^{14})C]</td>
<td>90</td>
<td>10</td>
<td>(16)</td>
</tr>
<tr>
<td>Tanacetum</td>
<td>isothujone</td>
<td>&quot;</td>
<td>90</td>
<td>10</td>
<td>(16)</td>
</tr>
<tr>
<td>Juniperous</td>
<td>sabinene</td>
<td>&quot;</td>
<td>90</td>
<td>10</td>
<td>(16)</td>
</tr>
<tr>
<td>A. california</td>
<td>camphor</td>
<td>&quot;</td>
<td>89</td>
<td>13</td>
<td>(17)</td>
</tr>
<tr>
<td>Salvia</td>
<td>&quot;</td>
<td>&quot;</td>
<td>97</td>
<td>7</td>
<td>(17)</td>
</tr>
<tr>
<td>C. balsamita</td>
<td>&quot;</td>
<td>&quot;</td>
<td>85</td>
<td>4</td>
<td>(17)</td>
</tr>
<tr>
<td>Tanacetum</td>
<td>isothujone</td>
<td>IPP</td>
<td>88</td>
<td>10</td>
<td>(18)</td>
</tr>
<tr>
<td>Tanacetum</td>
<td>&quot;</td>
<td>DMAP</td>
<td>86</td>
<td>14</td>
<td>(18)</td>
</tr>
<tr>
<td>Tanacetum</td>
<td>&quot;</td>
<td>3,3-dimethylacrylic acid</td>
<td>80</td>
<td>18</td>
<td>(18)</td>
</tr>
<tr>
<td>P. graveolens</td>
<td>geraniol</td>
<td>&quot;</td>
<td>70</td>
<td>13</td>
<td>(18)</td>
</tr>
<tr>
<td>M. pulegium</td>
<td>pulegone</td>
<td>&quot;</td>
<td>91</td>
<td>11</td>
<td>(18)</td>
</tr>
<tr>
<td>P. roseum</td>
<td>geraniol</td>
<td>MVA-[2-(^{14})C]</td>
<td>81</td>
<td>10</td>
<td>(19)</td>
</tr>
<tr>
<td>P. roseum</td>
<td>citronellol</td>
<td>&quot;</td>
<td>68</td>
<td>10</td>
<td>(19)</td>
</tr>
</tbody>
</table>
Table 1 (Continued)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Terpene</th>
<th>Substrate</th>
<th>I</th>
<th>D%</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. vulgare</td>
<td>geraniol</td>
<td>IPP</td>
<td>65</td>
<td>34</td>
<td>(20)</td>
</tr>
<tr>
<td>T. vulgare</td>
<td>nerol</td>
<td>&quot;</td>
<td>68</td>
<td>33</td>
<td>(20)</td>
</tr>
<tr>
<td>T. vulgare</td>
<td>GPP</td>
<td>CO₂</td>
<td>-</td>
<td>98</td>
<td>(18)</td>
</tr>
<tr>
<td>C. camphora</td>
<td>linalool</td>
<td>leucine</td>
<td>18</td>
<td>81</td>
<td>(21)</td>
</tr>
<tr>
<td>C. camphora</td>
<td>&quot;</td>
<td>valine</td>
<td>30</td>
<td>70</td>
<td>(22)</td>
</tr>
<tr>
<td>P. roseum</td>
<td>geraniol</td>
<td>leucine</td>
<td>26</td>
<td>73</td>
<td>(23)</td>
</tr>
<tr>
<td>P. roseum</td>
<td>&quot;</td>
<td>valine</td>
<td>16</td>
<td>84</td>
<td>(23)</td>
</tr>
<tr>
<td>P. roseum</td>
<td>citronellol</td>
<td>leucine</td>
<td>17</td>
<td>83</td>
<td>(23)</td>
</tr>
<tr>
<td>P. roseum</td>
<td>&quot;</td>
<td>valine</td>
<td>12</td>
<td>88</td>
<td>(23)</td>
</tr>
<tr>
<td>tea rose</td>
<td>geraniol</td>
<td>MVA-[2-¹⁴C]</td>
<td>53</td>
<td>50</td>
<td>(24)</td>
</tr>
<tr>
<td>P. graveolens</td>
<td>geraniol</td>
<td>glucose</td>
<td>51</td>
<td>50</td>
<td>(18)</td>
</tr>
<tr>
<td>C. camphora</td>
<td>linalool</td>
<td>MVA-[2-¹⁴C]</td>
<td>54</td>
<td>46</td>
<td>(25)</td>
</tr>
<tr>
<td>C. moniliformis</td>
<td>geraniol</td>
<td>&quot;</td>
<td>49</td>
<td>53</td>
<td>(26)</td>
</tr>
</tbody>
</table>
Table 1 (continued)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Terpene</th>
<th>Substrate</th>
<th>I</th>
<th>D%</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. moniliformis</td>
<td>geraniol</td>
<td>leucine</td>
<td>50</td>
<td>54</td>
<td>(26)</td>
</tr>
<tr>
<td>C. moniliformis</td>
<td>geraniol</td>
<td>acetate</td>
<td>52</td>
<td>35</td>
<td>(26)</td>
</tr>
</tbody>
</table>
investigated the biosynthesis of camphor in *Artemisia californica*, *Salvia leucophylla* and *Chrysanthemum balsamita*. Again, the incorporation of MVA-[2-$^{14}$C] was uniformly low (0.002 - 0.2%) and the bulk (73 - 83%) of the tracer was located at C-6 in the I unit with the balance evenly distributed among the remaining nine carbon atoms.

In a later experiment, Allen *et al.* (18) extended the tracer work with (+)-isothujone in *Tanacetum vulgare* to include the substrates IPP-[4-$^{14}$C], DMAPP-[4-$^{14}$C] and 3,3-dimethylacrylic acid-[Me-$^{14}$C]. Isothujone biosynthesized from IPP and DMAPP contained 86% of the tracer at the carbonyl carbon. 3,3-Dimethylacrylic acid was also fed to *Pelargonium graveolens* and *Mentha pulegium* in attempts to label geraniol and (+)-pulegone. In each of the three monoterpenes, the bulk of the tracer was located in the IPP derived portion.

Similar results were obtained in labelling studies done by Suga and Shishibori (19). Geraniol and citronellol were biosynthesized from MVA-[2-$^{14}$C] by *Pelargonium roseum*. Degradation studies of these terpenes indicated that 70% of the tracer was located at C-4 in the I unit while C-8,10 contained less than 20% of the label.

Cell-free extracts of *Tanacetum vulgare* leaves contained enzymes for the biosynthesis of both geraniol and nerol (20). The combined yield of monoterpenes biosyn-
the synthesized from IPP-[4-\(^{14}\)C] was a remarkable 11.9%, while MVA-[2-\(^{14}\)C] was a less efficient substrate with combined yield of 2.4%. Surprisingly, this \textit{in vitro} system also produced asymmetrically labelled monoterpenes with 65 - 90% of the tracer located in the IPP portion of the skeleton.

An unusual labelling pattern was produced by exposing foliage of \textit{Tanacetum vulgare} to physiological levels of carbon dioxide-[\(^{14}\)C] for twenty days (18). A soluble enzyme system for the synthesis of geranyl pyrophosphate was prepared from the foliage. When isotopically normal IPP was added to the enzyme system, the product, GPP, was asymmetrically labelled; however, essentially all (98%) of the tracer was located in the DMAPP portion.

Suga (21-23) claimed to have labelled the DMAPP portion of geraniol, linalool and citronellol by feeding the amino acids leucine and valine to \textit{Cinnamomum camphora} and \textit{P. roseum}. Suga calculated the total radioactivity of five carbons of linalool to be equivalent to \(5/3\) of the label found in the three-carbon isopropylidene fragment. A similar rationale was employed in analysis of labelling data from geraniol and citronellol. The pathway he proposed for the incorporation of amino acids into monoterpenes is shown in Figure 6.

There have also been reports of symmetrically dis-
Figure 6. Conversion of leucine and valine to DMAPP.
tributed labelling patterns. These results were obtained from a study of geraniol biosynthesis in the hybrid tea rose by Francis et al. (24). When flower heads were incubated with dibenzylethlenediamine salt of MVA-[2-\(^{14}\)C], geraniol and nerol isolated one hour afterward contained 2.5 - 5% of the applied MVA. The purified compounds were then degraded in order to ascertain the location of the label. The tracer was distributed equally between C-4,5 and C-8,10.

Geraniol was also found to be symmetrically labelled by Allen et al. (18) in Pelargonium graveolens. In this case the substrate was glucose-[U-\(^{14}\)C] and geraniol incorporated 0.001% of the label. Degradation revealed the label to be equally distributed among the ten carbons.

Suga (25) conducted studies on the biosynthesis of linalool in C. camphora, in which the maximal incorporation of MVA-[2-\(^{14}\)C] into linalool was 0.022%. Degradation procedures established a distribution of radioactivity among carbons: C-7,8,10 (46%), C-1,2,3,4,5,9 (54%). Thus, he concluded that linalool was symmetrical.

Lanza and Palmer (26) investigated the production of monoterpenes via the fungal culture, Ceratocystis moniliformis. The monoterpenes excreted into the fungal growth medium were typical of those produced by higher plants and included citronellol, geraniol, geranial, nerol,
linalool, and α-terpineol. Of these, geraniol was studied to determine the extent of incorporation of three radioactive precursors, MVA-[2-14C], L-leucine-[4,5-3H] and acetate-[2-14C]. Levels of incorporation for each substrate into geraniol were: MVA, 0.36%; L-leucine, 0.13%; acetate, 0.74%. The higher incorporation of acetate was ascribed to permeability barriers which may have prevented mevalonic acid from entering the site of secondary metabolite synthesis. The distribution of radioactivity conformed in all cases to that theoretically expected for formation of geraniol via MVA (Figure 7). It was found that the label in geraniol was again equally distributed between the two five-carbon units.

Irregular terpenoids are a class of compounds that do not conform to either the classical isoprene rule or its biogenetic extension. Most are monoterpenes in which the tail of one isoprene unit is linked to one of the central atoms of the other unit, in contrast to the typical head-to-tail fashion (Figure 2). Irregular monoterpenes have an almost exclusive distribution in the Anthemideae tribe of the Compositae (27). Examples of the four major skeletal types and their natural sources are listed in Table 2.

Various speculations have been made as to the biosynthetic origins of artemisia ketone. The first two mecha-
Figure 7. Labelling patterns in geraniol.
| TABLE 2 |

IRREGULAR MONOTERPENES AND THEIR NATURAL SOURCES

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Natural Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemisyl</td>
<td>Artemisia japonica, ludoviciiana, annua (28)</td>
</tr>
<tr>
<td>Artemisia alcohol</td>
<td></td>
</tr>
<tr>
<td>Artemisia acetate</td>
<td>Artemisia annua (28)</td>
</tr>
<tr>
<td>Artemisia ketone</td>
<td>Achillea ageratum (32), millefolium (33); Artemisia annua, apicaceae, abruscula, frigida, japonica, ludoviciiana (28) princepts (28); Chrysanthemum tanacetum (28)</td>
</tr>
</tbody>
</table>
Table 2 (continued)

<table>
<thead>
<tr>
<th>Chemical Structure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="yomigi_alcohol" /></td>
<td>yomigi alcohol</td>
</tr>
<tr>
<td><img src="image" alt="arbolis_triene" /></td>
<td>artemisia triene</td>
</tr>
<tr>
<td><img src="image" alt="santolinyl" /></td>
<td>Santolinyl</td>
</tr>
<tr>
<td><img src="image" alt="Sanolina_triene" /></td>
<td>Sanolina triene</td>
</tr>
<tr>
<td><img src="image" alt="oxid_santolina_triene" /></td>
<td>Oxido Santolina triene</td>
</tr>
</tbody>
</table>

C. vulgare (28); Santolina chamaecyparissus (28); A. californica (34), vulgaris (35), pontica (36), feddi (28)

Artemisia arbuscula, tridentata (28); S. chamaecyparissus (28)

S. chamaecyparissus (28)

A. arbuscula, nova, tridentata (28); S. chamaecyparissus (28)

A. tridentata (38)
Table 2 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>artemisiaeole</td>
<td>A. tridentata (39)</td>
</tr>
<tr>
<td>Chrysanthemyl</td>
<td>13 species of Chrysanthemum, (28)</td>
</tr>
<tr>
<td>1R-3R-chrysanthemic acid</td>
<td></td>
</tr>
<tr>
<td>1R-3R-chrysanthemol</td>
<td>A. ludoviciana (40)</td>
</tr>
<tr>
<td>Lavandulyl</td>
<td>Lavandula officinalis, vera (28)</td>
</tr>
<tr>
<td>R-lavandulol</td>
<td></td>
</tr>
</tbody>
</table>
Table 2 (continued)

\[
\begin{align*}
\beta\text{-Cyclolavandulal} & \\
\text{Seseli indicum} (28) & \\
\end{align*}
\]
isms comply with the biogenetic isoprene rule in that the precursor to the irregular artemisyl skeleton is a normal monoterpane arrived at by rearrangements on the basic ten carbon compound, GPP. The mechanism proposed by Clemo (29) constructed the artemisyl skeleton by fission of 3-carene (Figure 8). Whereas, the mechanism proposed by Robinson (30) featured a ring-opening of a cyclopropyl intermediate which was derived from linalool.

Later proposals deviated from the normal 4-1 dimerization of "isoprene". Ruzicka (31) (Figure 9) proposed that the condensation of two molecules of 1,1-dimethylallylpyrophosphate in an abnormal 4-2 fashion would form the irregular structure. Richards and Hendrickson (14) proposed a variation of Ruzicka's mechanism in which condensation occurred between two molecules of 3,3-dimethylallylpyrophosphate, DMAPP.

Bates and Paknikar (41) (Figure 10) first proposed a biosynthetic relationship between artemisia ketone and another irregular terpene, chrysanthemic acid. Chrysanthemic acid occurs in ester form in pyrethrins I and II in flowers of Chrysanthemum cinerariaefolium. Since the establishment of presqualenyl pyrophosphate as a precursor of squalene (42, 43), the relationship between the chrysanthemyl structure and other irregular terpenes has been considered more viable. The cyclopropyl intermediates,
Figure 8. Mechanisms of artemisia ketone biosynthesis.
Figure 9. Mechanisms of artemisia ketone biosynthesis.
presqualenyl pyrophosphate and prephytoenyl pyrophosphate are considered structural links between higher terpenes and irregular monoterpenes. The cyclopropylcarbinyl cation generated by solvolytic removal of pyrophosphate in the mechanism of Bates and Paknikar (41) has been modelled by the alkoxyropyridinium iodide derivative of 1'R-3'R-chrysanthemol. Under normal solvolysis conditions, Poulter et al. (44) reported that 98% of the products had artemisyl skeletons and dihydrochrysanthemyloxpyridinium iodide yielded only products of a santolinyl skeleton upon hydrolysis (Figure 10). However, chrysanthemyl or santolinyl compounds were not detected when artemisia and yomigi dinitrobenzoates were subjected to hydrolytic conditions.

Trost and LaRochelle (45) proposed the linking of two molecules of DMAPP to a sulfhydryl group on the enzyme and suggested that the resulting sulfonium ylide would form the irregular structure via a Stevens rearrangement (Figure 11). The feasibility of this proposal was examined by model studies (46). Solvolysis of S,S-dimethyl S-artemisyl sulfonium fluoborate in acetone generated almost exclusively yomigi alcohol. With methanol as solvent, the major product was methyl artemisyl ether. Minor constituents (less than 2%) were identified as the rearranged products having chrysanthemyl, santolinyl and head-to-head skeletons.
Figure 10. Mechanisms with a cyclopropyl intermediate.
Figure 11. Biosynthesis of artemisia alcohol.
Finally, Epstein and Poulter (28) have proposed a unified approach to irregular monoterpenic biosynthesis in which dimerization of two DMAPP units is initiated by attack of an electron-donating group, \(X\), at C-2. The intermediate structure I is depicted in Figure 12. Two modes of elimination of \(X\) from I are possible, one to give the lavandulol skeleton, and the second to form a cyclopropyl intermediate of the chrysanthemyl skeleton. This intermediate can directly form a cyclopropylcarbinyl cation by solvolysis of the pyrophosphate moiety. The cationic species is stabilized by breaking one of two bonds to form either the santolinyl or artemisyl skeletons.

Published experimental evidence on the biosynthesis of artemisia ketone up to the present time is based solely on the investigation of two research groups: Banthorpe et al. and Suga et al. Table 3 outlines the labelling patterns of artemisia ketone and chrysanthemic acid with various substrates.

The ornamental shrub Santolina chamaecyparissus has been reported to yield an oil that contained 65% (by weight) artemisia ketone (47). Labelling studies (48) with this shrub revealed extensive incorporation of MVA-[2-\(^{14}\)C] into \(\alpha\)-pinene, myrcene and three unidentified monoterpenes. However, artemisia ketone did not contain significant label; thus suggesting that the ketone was
Figure 12. Unified approach to irregular monoterpene biosynthesis.
<table>
<thead>
<tr>
<th>PLANT</th>
<th>TERPENE</th>
<th>SUBSTRATE</th>
<th>I</th>
<th>D%</th>
<th>REF.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. annua</td>
<td>artemisia ketone</td>
<td>MVA</td>
<td>92</td>
<td>10</td>
<td>(49)</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>80</td>
<td>13</td>
<td>(50)</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>IPP</td>
<td>96</td>
<td>5</td>
<td>(51)</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>DMAPP</td>
<td>89</td>
<td>10</td>
<td>(51)</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>geraniol-[2-14C]</td>
<td>60</td>
<td>-</td>
<td>(53)</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>nerol-[2-14C]</td>
<td>60</td>
<td>-</td>
<td>(53)</td>
</tr>
<tr>
<td>S. chamaecyparrisus</td>
<td>&quot;</td>
<td>IPP</td>
<td>93</td>
<td>-</td>
<td>(52)</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>DMAPP</td>
<td>93</td>
<td>-</td>
<td>(52)</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>DMVC</td>
<td>80</td>
<td>20</td>
<td>(52)</td>
</tr>
<tr>
<td>&quot;</td>
<td>trans-chrysanthemol</td>
<td>&quot;</td>
<td>62</td>
<td>38</td>
<td>(52)</td>
</tr>
<tr>
<td>C. cinerariaefolium</td>
<td>chrysanthemic acid</td>
<td>MVA</td>
<td>51</td>
<td>39</td>
<td>(54)</td>
</tr>
<tr>
<td>&quot;</td>
<td>&quot;</td>
<td>MVA</td>
<td>5</td>
<td>99</td>
<td>(55)</td>
</tr>
</tbody>
</table>
biosynthesized via a non-mevalonoid pathway.

In a later experiment with Artemisia annua, Banthorpe and Charlwood (49) determined that MVA-[2-¹⁴C] was indeed incorporated into artemisia ketone at low levels (0.001 - 0.01%). Degradation of the molecule revealed 92% of the tracer was located at C-3,9,10. Suga et al (50) confirmed the work (49) and found the level of incorporation of MVA-[2-¹⁴C] into artemisia ketone to be 0.001%. Again, the labelling pattern proved to be asymmetric with the internal methyls, C-9,10, containing 80% of the label while the terminal methyls, C-7,8, contained only 13% of tracer.

A more extensive study of artemisia ketone biosynthesis in A. annua and S. chamaecyparrisus produced similar results (51). MVA-[2-¹⁴C], IPP-[4-¹⁴C] and DMAPP-[4-¹⁴C] were studied as substrates. While A. annua incorporated all three substrates at optimum levels of 0.04 - 0.8%, S. chamaecyparrisus utilized only DMAPP and IPP as precursors of artemisia ketone, with 0.2 - 0.8% incorporation. Degradations established that the tracer in each case was asymmetrically located.

In vitro studies reported by Banthorpe et al (52) with A. annua and S. chamaecyparrisus summarized the incorporation of a variety of precursors into terpenes of irregular skeletons. The amount of tracer (0.2 - 5.4%)
incorporated into products by a cell-free system prepared from *A. annua* was inversely dependent on initial concentrations of substrate. The five-carbon precursors IPP, DMAPP and dimethylvinylcarbinol, DMVC, were incorporated asymmetrically into both artemisia ketone and trans-chrysanthemyl alcohol, in ratios of 80:20 and 62:38, respectively. Of major significance was the conversion of both cis and trans-chrysanthemyl alcohols and their pyrophosphates into artemisia ketone and its alcohol in good yield, 7.4%. The extract of *S. chamaecyparissus* effectively incorporated artemisyl alcohol into artemisia ketone (6.9%) and trans-chrysanthemyl alcohol (3.5%). Also of interest was the ability of the cell-free system to synthesize lavandulol and trans-chrysanthemyl alcohol, neither of which has been reported in the Compositae (23).

Finally, it was noted (53) that geraniol-[2-14C] and nerol-[2-14C] served as precursors to artemisia ketone in intact plants of *A. annua*. Extensive scrambling of the label occurred, with most of the label, 60%, located at C-2. Co-occurring regular monoterpenes, isothujone, pinocamphone and borneol were biosynthesized in a position-specific manner from the C-10 precursors.

Thus, the numerous labelling results (49-53) reported for artemisia ketone agreed, since the I unit contained the majority of the label when any one of a number of pre-
cursors was fed to either whole plants or cell-free systems. However, the labelling patterns of another irregular monoterpenic, chrysanthemic acid, are contradictory.

Crowley et al. (54) investigated the biosynthesis of chrysanthemic acid using both acetate-[2-14C] and MVA-[2-14C]. The terpenoid nature of chrysanthemic acid was established when they found MVA to be effectively (1.4%) incorporated. Acetate was incorporated to a lesser extent (0.4%). Degradation showed that the methyl groups of the cyclopropyl ring contained 51% of the tracer and those of the isopropylidene group contained 39%. Thus, they concluded that two units of mevalonic acid were incorporated symmetrically into chrysanthemic acid.

In contrast to these results, Pattenden and Storer (55) demonstrated asymmetric incorporation of MVA-4R-[4-3H] into chrysanthemic acid (Figure 13). Virtually all of the label was found in that portion of the molecule which corresponded to the unlabelled C-5 unit in artemisia ketone biosynthetic studies. He suggested (55) that the C-5 unit leading to the cyclopropane part of the molecule was non-mevalonoid in origin. The participation of 3,3-dimethylacrylic acid was indicated by preliminary evidence that cited levels of incorporation equivalent to MVA.

From the experimental work with labelling patterns
Figure 13. Incorporation of MVA into chrysanthemic acid.
in terpenes, two explanations have emerged. One idea proposed by the Banthorpe group (18) stated that pools of metabolites within the cell were restricted by compartmentation. Whereas, a second explanation (23) suggested to explain asymmetric labelling involved the participation of leucine in terpenoid biosynthesis.

The phenomenon of compartmentation within specialized structures was initially discussed by Rogers et al. (56). They described the factors regulating terpenoid synthesis of phytosterols and carotenoids in chloroplasts. Three separate compartments were hypothesized as influencing regulation of biosynthesis in germinating seedlings. A common compartment in which acetyl CoA was converted into mevalonic acid and higher terpenoids, was described as having access to two other compartments, extra-chloroplastidic and chloroplastidic. The former compartment was thought to be the synthetic site of phytosterols and pentacyclic triterpenes (C-30), while the latter compartment contained enzymes to make carotenoids (C-40) and phytols (C-20). The necessity for compartmentation was rationalized as follows: during germination, sterols are needed for formation of membranes and lamellae in extra-chloroplastidic structures. Mevalonic acid is formed from endogenous food resources and is prevented from entering the immature chloroplast to form unnecessary pigments.
Once above ground, the seedling rapidly develops chloroplasts which restrict mevalonic acid formed within from carbon dioxide fixation to the synthesis of chloroplast terpenoids.

The monoterpenes of plants of the Compositae family, with the exception of the Cichorieae, are synthesized in specialized structures (57). Storage of monoterpenes in the Pinus species occurs in leaf or wood resin ducts. In roses and C. cinerariaefolium, the site of synthesis and storage has been shown to be the flower head. It has been proposed (58) that in some species, terpene synthesis may occur in the plastids.

The nature of the secretory structure has received considerable attention from plant physiologists. The oil glands of Mentha piperata were examined by light and electron microscopy and were found to be multicellular modified epidermal hairs (59). The hairs originate from a single cell and envelope a globular head on a stalk. The oil gland can be described as a bag of related enzymes with associated storage space which has limited contact with the systemic circulation of the plant. The stalk cell wall appeared heavily cutinized while the membrane of the bulb was fragile and easily burst to release stored components.

Banthorpe et al. (18) applied the ideas of compartmentation to explain asymmetric monoterpenes labelling.
The maintenance of separate pools by selective membrane permeability as in carotenoids was ruled out by experimental results cited earlier in which geraniol was found to be asymmetrically labelled by a cell-free system (Page 14). Instead, they proposed that segregation of synthetic units is accomplished by protein-bound pools (Figure 14). Pool A is described as being free and sparsely filled while Pool B is essentially irreversibly protein-bound and larger than Pool A. More rapid synthesis and/or extended incubation periods allow both pools to incorporate precursor. An example of this condition would be the appearance of 98% of the label in the D unit of the isolated monoterpenes from a plant maintained on carbon dioxide-[\(^{14}C\)] (Page 14). It is concluded that similar pools are likely to exist for higher terpenes but that the indications of asymmetric labelling seen in monoterpenes biosynthesis would be obscured by relatively high rates of synthesis common for higher terpenes.

The second suggestion by Suga (23) was that the amino acids leucine and valine enter terpenoid biosynthesis via a direct conversion to an MVA metabolite, DMAPP (Figure 6). However, such a direct conversion is unlikely in view of other reports concerning the involvement of leucine in terpene biosynthesis.

Coon et al. (80, 81) investigated the metabolic fate of
Figure 14. Pools involved in asymmetric labelling.
branched-chain amino acids such as: leucine, isoleucine and valine. They considered these compounds important in completing the picture of natural product biosynthesis. Figure 15 summarizes the conversion of leucine to hydroxymethylglutaryl CoA (HMG CoA). It was further shown that an enzyme purified from pig heart catalyzed the cleavage of HMG CoA to acetoacetate and acetate (Figure 16). It should be noted that the carbon introduced by the biotin dependent carboxylation step in Figure 15 becomes a component of acetoacetate. Also, if HMG CoA was converted to MVA and on to IPP this new carbon would be lost as carbon dioxide. It has been suggested (82) that randomization of carbons could occur by known reactions after cleavage of HMG CoA by simple recombination of the two compounds. The second molecule of HMG CoA will lose acetate C-1 upon conversion to IPP that now contains the molecule of carbon dioxide introduced by biotin at C-1.

The importance of this leucine pathway in terpene biosynthesis is obvious in some of the earlier work with carotenoids. Leucine was found to stimulate carotenogenesis in the mold Phycomyces blakesleeanus (83) and its presence was necessary for carbon dioxide-[14C] to be fixed into β-carotene (84, 85) by the same organism. A cell-free system, prepared from P. blakesleeanus (87) incorporated acetate, HMG and mevalonic acid into
Figure 15. Conversion of leucine to HMG CoA.
Figure 16. Conversion of HMG CoA to IPP.
β-carotene.

The reports of labelling patterns in carotenes with leucine supplied as a radioactive substrate further substantiate the participation of this amino acid. Only C-4 of leucine was incorporated in sufficient quantity to be isolated from each isoprene unit of carotene in *P. blakesleeanus* (86), (Figure 17). It was concluded that leucine provided an "iso-C₃ fragment in which the 4-C is centrally located". This later observation agrees with the formation of acetoacetate from the second half of the leucine molecule and fixed carbon dioxide as in Figure 16. The incorporation of C-2 was to a lesser extent than C-4 but shared the same distribution pattern.

More extensive work by the same group (85) detailed the fate of each carbon from leucine. Acetoacetate cleaved from HMG CoA was recycled to MVA after attaining equilibrium with an active acetoacetate pool. Thus, in the carotenoids, considerable scrambling of leucine carbons occurs before incorporation into terpenoids as mevalonoid compounds.

A similar situation exists in leucine metabolism in the mold *Aspergillus amstelodami* (88). Feeding experiments with 90% enriched (4R)-leucine-[5-¹³C] resulted in preferential incorporation of the enriched carbon into the C-5 isoprenoid moieties of echinuline (Figure 18). The
Figure 17. Labelling pattern in $\beta$-carotene from L-leucine-$[4^{-14}C]$. 

$\beta$-CAROTENE
4-R-LEUCINE-[5-$^{13}$C]

\[ \text{Aspergillus amstelodami} \]

\[ \text{Echinuline} \quad \bullet \equiv ^{13}\text{C} \]

Figure 18. Labelling pattern in echinuline.
enrichment pattern is explained in terms of the same leucine pathway demonstrated in the carotenoids.
II. EXPERIMENTAL

Materials and Methods. All solvents were reagent or a better grade and were dried over 4 Å molecular sieves. 

$^1$H Nuclear magnetic resonance (PMR) spectra were performed on Varian A-60, Varian HA-100 or Bruker WP-200 spectrometers with 10% samples in deuterochloroform and tetramethysilane as internal standard (δ=0 ppm). In cases where deuterium oxide was the solvent, a tetramethysilane-carbon tetrachloride sample was used as external reference. Infrared recordings (IR) were obtained either neat or in carbon tetrachloride as solvent on a Perkin-Elmer Infracord. Mr. Ralph Seab of the LSU Chemistry Department performed elemental analyses on samples that were dried over phosphorus pentoxide under vacuum. Melting points were measured with a Hoover-Thomas Melt-temp capillary melting point apparatus and are uncorrected. Mass spectra (MS) were conducted on a Hewlett-Packard 5985 Quadrapole Mass Spectrometer by Mr. Don Patterson of the LSU Chemistry Department.

Silica gel 7GF for thin layer chromatography (TLC) was purchased from Supelco, Inc. Preparative chromatography (ThLC) was carried out on a 500 micron layer of silica gel spread on 20 x 20 cm glass plate developed once with the stipulated solvent. Analytical TLC separa-
tions were done on precoated plates from Quantum/Gram. Bands or spots were visualized under UV light or by staining with iodine vapor.

Radioactive supplies were purchased from ICN or New England Nuclear. A cocktail for counting radioactive samples was prepared by dissolving 2,5-diphenyloxazole (4 g) and 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (75 g) in scintillation-grade toluene (1 L). Quenching corrections for chemical derivatives were made by setting up a standard curve for each derivative.

Gas liquid chromatographic (GLC) analyses were performed on a Perkin-Elmer 990 equipped with flame-ionization detector; for preparative work, the instrument was modified by installing a 50:1 effluent splitter. The GLC was linked to a Perkin-Elmer Recorder, Model 56, and an Infotronics Automatic Digital Integrator, Model CRS-208. A single column (64 mm x 3 m stainless steel column, 30% Carbowax on Chromosorb P, AW, 60/80 mesh) was prepared and used for both analytical and preparative purposes. The majority of analyses were carried out at 130 or 145°C with nitrogen as carrier gas and optimum flow rate of 33.3 ml/minute. Samples were identified by comparison of retention times ($t_R$) with known standards or by collection and instrumental analysis of the sample. Semi-quantitative examinations of mixture components were made by comparing retention volumes ($V_R$) as a per-
The GLC collection apparatus was designed with the aid of Ms. Roxanne Dikeman, and consisted of a U tube, 5 cm on a side, of soft 3 mm glass connected to the effluent splitter port by means of a teflon sleeve. This collector could be cooled to (-78°C) in a Dewar containing dry ice and acetone. The efficiency (25 - 50%) of this method varied with sample volatility. Collection of peaks as close as 3 minutes in $t_R$ was possible without significant contamination.

Synthesis and Reactions of Artemisia Ketone

**SYNTHESIS OF 3-METHYL-2-BUTENOL.** Under a nitrogen atmosphere, a solution of sodium bis(2-methoxy-ethoxy)aluminum hydride (70%, Red-Al, 32 mL) was added via a constant-pressure addition funnel to 3,3-dimethyl-acrylic acid (7 g, 70 mmol) dissolved in benzene (150 mL). When addition was complete, the viscous solution was refluxed for two hours followed by stirring at room temperature overnight. Saturated ammonium chloride (30 mL) was added slowly while cooling. A granular white precipitate resulted, from which the benzene was easily decanted. The precipitate was washed with ether (100 mL). The combined organic solvent was extracted with water (100 mL) followed by saturated sodium chloride (until the washes were neutral), dried over sodium sulfate
and distilled to give 3-methyl-2-butanol: 6 g (56%); bp 62°C (30 mm); [lit bp 52°C (10 mm)]; PMR δ 2.0 (d, (CH₃)_2, J=4 Hz, 6H), 2.9 (s, OH, 1H), 4.1 (d, CH₂, J=7 Hz, 2H), 5.4 (br t, C=CH, J=7 Hz).

SYNTHESIS OF 1-BROMO-3-METHYL-2-BUTENE 2 (61). 3-Methyl-2-butanol (1.0 g, 13 mmol) was dissolved in carbon tetrabromide (8.6 g, 26 mmol) and anhydrous ether (25 mL) under nitrogen. Triphenylphosphine (6.3 g, 26 mmol) was added and the mixture refluxed for 1 hour. After cooling, the ether was decanted from the residue of triphenylphosphine oxide into petroleum ether to allow further precipitation of the oxide. Filtration and removal of the solvent yielded a pale yellow oil that rapidly darkened on exposure to air: 1.4 g (74%) bp 129 - 133°C [lit bp 130 - 133°C]; PMR δ 1.67 (d, (CH₃)_2, J=2 Hz, 6H), 3.90 (d, CH₂, J=8.5 Hz, 2H), 5.45 (br t, C=CH, J=8.5 Hz, 1H).

SYNTHESIS OF 3-METHYL-2-BUTENAL 3 (62). Chromium trioxide (7.2 g, 72 mmol) was added to a stirred dichloromethane (175 mL) solution of pyridine (11.4 g, 144 mmol). The flask was equipped with a calcium chloride drying tube and stirred for 15 minutes, then 3-methyl-2-butanol (1 g, 12 mmol) in dichloromethane (15 mL) was added. After stirring for another 15 minutes, the solvent
was decanted and the tarry precipitate was washed with ether (200 mL). The combined organic solvent was washed with 5% sodium hydroxide (3 x 100 mL), 5% hydrochloric acid (100 mL), 5% sodium bicarbonate (100 mL), and saturated aqueous sodium chloride. After drying over anhydrous sodium sulfate, the solvent was concentrated to give 3-methyl-2-butenal: 1 g (70%) bp 50 - 53°C (50 mm) [lit bp 133°C (730 mm)]; PMR δ 2.1 (d, Me₂, J=1.5 Hz, 6H), 5.9 (d, C=CH, J=8 Hz, 1H), 10.1 (d, CHO, J=8 Hz, 1H).

SYNTHESIS OF ARTEMISIA ALCOHOL (63). Zinc (100 g, 20 mesh) was activated by washing sequentially with the following reagents (50 mL each): 2% hydrochloric acid, water, 95% ethanol, acetone and anhydrous ether. After drying in vacuo at 100°C, zinc was poured into a column with tetrahydrofuran (25 mL) and brought to reflux temperature via an external heating tape. A solution of 3-methyl-2-butenal (84 mg, 1 mmol) and 1-bromo-3-methyl-2-butene (223 mg, 1.5 mmol) in tetrahydrofuran (25 mL) was slowly dripped through the column. After flushing the column with additional solvent (25 mL), the eluant was diluted with ether (59 mL). The organic phase was washed sequentially with ice-cold 5% sulfuric acid, 5% sodium bicarbonate and saturated aqueous sodium chloride (100 mL each). The solvent was concentrated
to give a fragrant yellow oil: 152 mg (99%); bp 194 - 196°C [lit bp 196°C]; PMR is listed in Table 4.

OXIDATION OF ARTEMISIA ALCOHOL TO ARTEMISIA KETONE 5,

METHOD A. CHROMIUM TRIOXIDE - PYRIDINE. The crude oil (5 g) of the Reformatsky reaction was oxidized with chromium trioxide in pyridine as described above. After workup and removal of solvent, GLC showed artemisia ketone 5 to be a minor component (29%) of the mixture. A second component (55%) with longer tR (GLC) was separated by a silica gel column eluted with petroleum ether:ether (9:1) and identified as 2,5,5-trimethyl-3,6-heptadien-2-ol 6. PMRs of 5 and 6 are listed in Table 4.

METHOD B. PYRIDINIUM CHLOROCHROMATE (64). Chromium trioxide (45 g, 450 mmol) was rapidly added to 6 M hydrochloric acid (82 mL) with stirring. Pyridine (36 mL, 450 mmol) was added over 10 minutes while maintaining the reaction temperature at 0°C. The reaction was stirred for an additional 15 minutes, then the crystals were filtered on a sintered glass funnel. The crystals were dried in vacuo at 50°C for 3 hours to give 31 g (52%) of pure pyridinium chlorochromate. Artemisia alcohol (1.7 g, 10 mmol) was treated with pyridinium chlorochromate (2 g, 15 mmol) and sodium acetate (237 mg, 3 mmol) in
dichloromethane (10 mL). After stirring at 25°C for 2 hours, the solvent was decanted from the black deposit and purified through a Florisil (60/80 mesh) column. After concentration of the mixture, GLC analysis of the crude reaction mixture showed \( \mathbf{4} \) had isomerized (90%) to \( \mathbf{6} \).

**METHOD C. MANGANESE DIOXIDE OXIDATION (65).** An aqueous solution of manganese sulfate was stirred and heated to 90°C. Concentrated aqueous potassium permanganate was slowly added until a slight pink coloration was evident when spotted on filter paper. Manganese dioxide was filtered and washed with additional hot water. The filter cake was dried at 120°C for 4 hours, with occasional grinding to facilitate drying.

The alcohol \( \mathbf{4} \) (700 mg, 4.5 mmol) was stirred with the freshly prepared manganese dioxide (7 g, 70 mmol) in dichloromethane (50 mL) for 4 hours. The mixture was then filtered with the aid of a Celite pad. GLC analysis showed artemisia ketone \( \mathbf{5} \) (18%, \( t_R \) 14.2 minutes), \( \mathbf{6} \) (23%, \( t_R \) 19.7 minutes) and the major peak, 3-methyl-2-butenal (47%, \( t_R \) 10 minutes).

**SYNTHESIS OF 5,6-DIHYDROARTEMISA KETONE \( \mathbf{7} \) (110).** Lithium metal (14 g, 2 mmol) was placed in a three-necked, round-bottomed flask equipped with an addition funnel, a Dewar condenser filled with dry ice and acetone, and a
glass-covered stirring bar. After flushing with argon, the apparatus was cooled to -75°C, then dry (KOH pellets) ammonia (100 mL) was introduced. Artemisia ketone (100 mg, 0.66 mmol) in ether (10 mL) was added to the bright blue solution and the mixture was stirred for 30 minutes. Excess lithium was quenched with saturated ammonium chloride. The reaction was allowed to warm to 25°C and excess ammonia evaporated. Water (10 mL) was added to the residual oil, then the solution was extracted with ether, washed with saturated sodium chloride until neutral and dried over anhydrous sodium sulfate. GLC analysis showed two major components: 5,6-dihydroartemisia ketone \( \mathcal{Z} \) \((t_R 8.15 \text{ minutes})\) and 5,6-dihydroartemisia alcohol \( \mathcal{S} \) \((t_R 14.4 \text{ minutes})\); PMRs of \( \mathcal{Z} \) and \( \mathcal{S} \) are listed in Table 4.

Routes to Radioactive 3-Methylbutanal

1,4-GRIGNARD WITH CUPROUS CHLORIDE (68). Methylmagnesium bromide (55 mL, 3 M/ether, 0.15 mol) was cooled to -5°C, then crotonaldehyde (4.1 mL, 50 mmol) in anhydrous ether (75 mL) was added. Cuprous chloride (175 mg, 1.8 mmol), prepared by the Gocmen procedure (69), was added in small aliquots over 1.5 hours. After the addition was complete, the deep green mixture was stirred for an additional 30 minutes. The mixture was hydrolyzed by addition of 5% hydrochloric acid and ice. The ether
layer was separated, washed sequentially with saturated sodium bicarbonate (50 mL), water (100 mL) and distilled to give three fractions. The major fraction gave 3-penten-2-ol \( \mathcal{Z} \): 3.1 g (72%); bp 115 - 119°C [lit bp 119 - 121°C]; PMR \( \delta 1.17 \) (d, \( \text{CH}_3\text{CH}=\text{CH}_2 \), J=6.5 Hz, 3H), 1.60 (d, \( \text{CH}_3\text{CHOH} \), J=5 Hz, 3H), 2.5 (s, OH, 1H), 4.17 (q, \( \text{CHOH} \), J=5 Hz, 1H), 5.4 - 5.7 (m, \( \text{CH}=\text{CH}_2 \), 2H).

1,4-GRIGNARD WITH N-\( \text{t-} \)-BUTYLCROTONIMINE \( \text{I} \) (70). To \( \text{t-} \)-butylamine (5.2 mL, 50 mmol) cooled to -5°C under nitrogen, crotonaldehyde (4.1 mL, 50 mmol) was added over 30 minutes, followed by stirring at 25°C for 12 hours. Potassium carbonate was added giving rise to a thick gold colloid: 1.5 g (75%); bp 129°C [lit (66) bp 129 - 130°C]; PMR \( \delta 1.1 \) (s, \( \text{Me}_3 \), 9H), 1.85 (d, \( \text{CH}_3 \), J=5 Hz, 3H), 6.1 (m, \( \text{CH}=\text{CH}_2 \), 2H), 7.7 (d, \( \text{CH}=\text{N} \), J=7 Hz, 1H).

To methylmagnesium bromide (20 mL, 60 mmol) under nitrogen, \( \text{I} \) in ether (20 mL) was slowly added over 45 minutes. After stirring for 12 hours, a buff-colored precipitate was visible. The reaction mixture was added to a vigorously stirred solution of ammonium chloride and ice. The ether phase was separated and washed with potassium carbonate and water. The bright orange organic phase was analyzed (GLC) to show 3-methylbutanal as a minor (8%) component. Preparative GC methods were unable to separate the desired product from closely
eluting components.

ATTEMPTED γ-HYDROGEN EXCHANGE ON ETHYL 3-METHYL-2-
BUTENOATE 11. 11 was prepared by stirring 3,3-dimethyl-
acrylic acid (5 g, 50 mmol) with concentrated sulfuric
acid (5 mL) in ethanol (20 mL) for 12 hours. An equal
volume of water was added, followed by extraction with
ether. The ether phase was washed with aqueous sodium
bicarbonate, dried over anhydrous sodium sulfate, and
distilled to give the ester 11: 4 mL (62%); bp 154 -
155°C [lit (67) bp 156°C]; PMR δ 1.25 (t, CH₃CH₂, J=7 Hz,
3H), 2.00 (d, Me₂, J=16 Hz, 6H), 4.14 (q, CH₂, J=7 Hz,
2H), 5.68 (m, CH, 1H).

The following catalysts were used unsuccessfully in
a variety of conditions to initiate exchange of the
γ-hydrogen with deuterium oxide: triethylamine, potassium
carbonate, 50% sulfuric acid, sodium metal and potassium
hydroxide.

γ-HYDROGEN EXCHANGE WITH 3. 3-Methyl-2-butenal 3
was prepared as above and subjected to similar conditions
in attempts to deuterate the γ-position. However, no
exchange was detected by integration of PMR spectra.
3-METHYL BUTANOIC ACID \( \text{II} \). 3-Methylbutanoic acid was prepared according to method described by Williams and Ott (71). 1-Bromo-2-methylpropane was distilled from calcium chloride onto molecular sieves before use. Magnesium turnings (840 mg, 35 mmol) were covered with anhydrous ether (5 mL), then 1-bromo-2-methylpropane (3.6 mL, 35 mmol) in ether (150 mL) was slowly added. After the addition was complete, the reaction was stirred at 25°C for one hour.

The Grignard reagent was cooled to -75°C and the system was placed under a slight vacuum. Carbon dioxide-[\(^{14}\text{C}\)] was first generated, via the apparatus designed by Gear and Spencer (72), by addition of 10% hydrochloric acid to barium carbonate-[\(^{14}\text{C}\)]. Generation of carbon dioxide from barium carbonate (1.15 g, 5.83 mmol) surrounding the vial followed and required 30 minutes. The stirred reaction mixture was maintained at -70°C to -50°C for a total of 2 hours. The Grignard was hydrolyzed with 10% hydrochloric acid (30 mL) with the development of a bright yellow color. The aqueous layer was removed and the remaining ether layer was extracted with 10% sodium hydroxide (2 x 20 mL). The basic wash was acidified with concentrated hydrochloric acid and then extracted with ether and dried over anhydrous sodium sulfate. Removal of the solvent yielded 3-methylbutanoic acid: 380 mg (64%); bp 93°C (60 mm) \([\text{lit (67) bp 176°C}]\); PMR \( \delta \) 0.98
(d, Me₂, J=6.5, 6H), 2.08 - 2.21 (m, CHCH₂, 3H), 11.0 (s, OH, 1H).

3-METHYL-2-BUTENOIC ACID 13. The Grignard reagent was generated in a manner similar to that described above. Methallyl chloride (14.2 mL, 138 mmol) was added, then refluxed for 5 hours. Carbonation proceeded over the same time interval as above. Extraction of the hydrolyzed product with 25% sodium hydroxide (2 x 100 mL) provided the salt of 3-methyl-2-butenoic acid. The product was isomerized to the desired compound by refluxing for 2.5 hours in 25% sodium hydroxide (200 mL). The basic solution was cooled and acidified with concentrated hydrochloric acid, followed by extraction with ether. The ether layer was dried with anhydrous sodium sulfate and concentrated to afford 3-methyl-2-butenoic acid: 320 mg (14%), mp 68 - 69°C [lit mp 68.5 - 69.5°C]; PMR δ 2.07 (d, Me₂, J=14.5 Hz, 6H), 5.69 (m, CH, 1H), 12.22 (s, OH, 1H).

Synthesis of Thiamine Compounds

SYNTHESIS OF 2-(1-HYDROXY-3-METHYLBUTYL)THIAMINE CHLORIDE HYDROCHLORIDE 16 (73). Thiamine chloride hydrochloride (4 g, 11.9 mmol) was added to absolute ethanol (120 mL), followed by addition of 3-methylbutanal (1.6 g, 14 mmol). After stirring at -4°C for 30 minutes, sodium ethoxide prepared from sodium (543 mg, 23.6 mmol) in
ethanol (30 mL) was slowly added. The opaque yellow solution was stirred for 5 hours, then acidified to pH 3 by hydrogen chloride gas generated from sodium chloride and sulfuric acid. Filtration through a medium frit removed precipitated salts and thiamine. Excess ethanol was removed under reduced pressure. The remaining yellow oil yielded crystals when stirred with cold ether (250 mL). The crystals were filtered and dried in vacuo over phosphorus pentoxide to give 16: 1.76 g (35%); mp 155°C (dec); PMR δ 1.00 (d, Me₂, J=5.5 Hz, 6H), 1.70 - 2.01 (m, CH₂CHMe₂, 3H), 2.47 (s, py, 3H), 2.68 (s, thiazole CH₃, 3H), 3.23 (t, CH₂CH₂OH, J=6 Hz, 2H), 3.57 (t, thiazole CHOH, J=7 Hz, 1H), 3.94 (t, CH₂OH, J=6 Hz, 2H), 5.60 (s, py-CH₂-thiazole, 2H), 7.47 (s, pyH, 1H). Anal. calcd for C₇H₁₈Cl₂N₄O₂S·2H₂O: C, 44.44; H, 6.80; N, 12.20. Found: C, 44.81; H, 6.65; N, 12.45.

SYNTHESIS OF 2-(1-HYDROXY-3-METHYLBUTYL)-4-METHYL-5-(2-HYDROXYETHYL)THIAZOLE 17 (74). Sodium sulfite (1.7 g, 13.4 mmol) was dissolved in water (10 mL) and adjusted to pH 5 with concentrated hydrochloric acid, then 16 (850 mg, 2 mmol) was added with stirring under nitrogen for 20 hours.

The resulting opaque solution was adjusted to pH 10 by addition of 5% sodium hydroxide (15 mL); and, as the pH increased, the precipitate dissolved. The solution
was extracted with dichloromethane (2 x 20 mL), dried over anhydrous sodium sulfate and concentrated to give 17:
260 mg (60%); Rf (ether) 0.37; PMR δ 0.95 (d, Me2, J=5.5 Hz, 6H), 1.70 - 2.01 (m, CH2CHMe2, 3H), 2.38 (s, thiazole CH3, 3H), 2.91 (t, CH2CH2OH, J=6 Hz, 2H), 3.45 (t, thiazole CHO, J=7 Hz, 1H), 3.78 (t, CH2CH2OH, J=6 Hz, 2H); mol wt. (MS) m/e 223 (M+).

SYNTHESIS OF 2-(1-OXO-3-METHYLBUTYL)-4-METHYL-5-(2-HYDROXYETHYL)THIAZOLE 18. A mixture of 17 (500 mg, 2.2 mmol) and manganese dioxide (5 g, 57 mmol) in dichloromethane (25 mL) was refluxed for 5 hours. The manganese dioxide was filtered from the reaction to give 18 as a yellow oil: 480 mg (96%); Rf (ether) 0.67; PMR δ 1.01 (d, Me2, J=6.5 Hz, 6H), 1.70 (m, CHMe2, 1H), 2.40 (s, thiazole CH3, 3H), 2.85 (d, COCH2, J=5 Hz, 2H), 3.05 (t, CH2CH2OH, J=6 Hz, 2H), 3.90 (t, CH2CH2OH, J=6 Hz, 2H); mol wt. (MS) m/e 226 (M+).

SYNTHESIS OF 2-(3,3,6-TRIMETHYL-4-HYDROXY-1-HEPTENYL-4-METHYL-5-(2-HYDROXYETHYL)THIAZOLE 19 (63). A solution of 18 (500 mg, 2.2 mmol) and 2 (492 mg, 3.3 mmol) in tetrahydrofuran (25 mL) was passed through a heated column of granular zinc (20 mesh). Workup in a manner similar to that described for artemisia alcohol afforded 19: 420 mg (63%); Rf 0.75 [ether:hexane (10:2)]; PMR δ 0.95 (d,
\[
\text{Me}_2\text{CH, 6H), 1.01 (s, C-3-CH}_3, 3\text{H), 1.04 (s, C-3-CH}_3, 3\text{H), 1.5 - 1.95 (m, CH}_2\text{CHMe}_2, 3\text{H), 2.35 (s, thiazole CH}_3, 3\text{H), 2.97 (t, CH}_2\text{CH}_2\text{OH, J=6 Hz, 2H), 3.62 (s, OH, 1H), 3.86 (t, CH}_2\text{CH}_2\text{OH, J = 6 Hz, 2H), 4.90 - 5.20 (octet, CH=CH}_2, J_{AB}=1.5 \text{ Hz, 2H), 6.10 (q, CH=CH}_2, J_{AX}=10 \text{ Hz, J}_{BX}=18 \text{ Hz, 1H).}
\]

\[\alpha\text{-HYDROXYISOCAPROIC ACID 20 (75). L-Leucine (26 \mu \text{M, 0.2 micromole) in 0.01 N hydrochloric acid was cooled, and concentrated hydrochloric acid (0.4 mL) was added, followed by 1.5 M sulfuric acid (1 \mu \text{L). An aqueous solution of sodium nitrite (0.5 M, 3 x 1 \mu \text{L}) was added in 15-minute intervals. The solution was maintained at 0°C for a total of 1.5 hours, then was allowed to stand at 25°C for 2.5 hours. A spot test with ninhydrin was negative. Extraction with ether (3 x 1 mL) gave the hydroxy acid 20: 4.03 microgram (15.3%).}
\]

Paper chromatography (76) confirmed the formation of 20 (R_f 0.95). No leucine (R_f 0.10) was detected. The chromatogram was run on Whatman #1 paper and developed in the upper phase of ethyl acetate:water:formic acid (60:35:5). The spots were visualized by a bromocresol green spray.

\[\alpha\text{-HYDROXYISOVALERIC ACID 21. L-Valine was treated in same manner as L-leucine to give 21 (R_f 0.95). No}
\]
valine ($R_f$ 0.05) was detected in a ninhydrin spot test or on the chromatogram.

Feeding Experiments

YEAST PREPARATION (77, 78). Fleischman's active dry yeast (70 g) was powdered at low speed in a Waring blender. The yeast was suspended in 0.066 M ammonium phosphate buffer (200 mL, pH 7.2) for 4 hours followed by centrifugation at 15,000 rpm (0°C) for 1 hour. The supernatant (120 mL) was decanted into ammonium sulfate (56.4 g) and stirred at 0°C for 5 minutes. The enzymes were precipitated by centrifuging at 4500 rpm for 30 minutes. After decantation of the liquid, the precipitated protein was dissolved in a sodium phosphate buffer (30 mL, 0.06 M, pH 7.2). The following compounds were added sequentially to the buffered enzyme: magnesium sulfate (590 mg, 2.4 mmol), sodium adenosine triphosphate (1.15 g, 2.4 mmol), Triton X-100 (0.6 mL), $\mathbf{1}$ (51 mg, 0.06 mmol) and $\mathbf{16}$ (272 mg, 0.6 mmol). The enzymatic reaction was incubated at 37°C for 3 hours, followed by heating at 70°C with 20% potassium hydroxide (10 mL) in ethanol to denature the enzymes. Extraction of the protein mixture with ether:acetone (1:1) separated the terpenoid products from proteins and salts. Preliminary GLC analysis indicated the major component to be one of the unchanged starting materials, $\mathbf{1}$. Remaining GLC peaks did not
correspond to either artemisia ketone \( \delta \) or 5,6-dihydro-
artemisia ketone \( \gamma \).

CULTIVATION OF ARTEMISIA ANNUA. Seeds of Artemisia
annua were initially obtained as a gift of the Royal
Botannic Gardens, Kew. Several plants from these seeds
were allowed to mature to flowering stage and dry in the
greenhouse. These plants were stored in a plastic bag
to provide seeds for the majority of experiments.

From June, 1977, until December, 1977, plants were
grown in a greenhouse under the supervision of
Dr. James Fontenot of the LSU Horticulture Department.
The average summer temperature in the greenhouse was
36\(^{\circ}\)C; during winter months, heat was supplied to main-
tain temperature above 25\(^{\circ}\)C.

Beginning January, 1978, the plants were grown
indoors under Sylvania Life Line artificial light with
14-hour days. The lights were suspended from a frame
1.2 m high and were adjusted as the plants matured to
maintain 10 cm distance from the tips of the plants.
Seven 40-watt bulbs were used to illuminate 2.3 m\(^2\) of
growing space. Average temperature was maintained at
ambient temperature (25\(^{\circ}\)C).

Germination of seeds usually required 48 hours. A
25 x 50 cm plastic tray with drainage holes was filled
with commercial potting soil. A liberal quantity of
dried plant material containing seeds was sprinkled on the damp soil and covered with a layer of peat moss. The entire tray was then covered with a sheet of clear plastic to retain moisture. Ten plants could be satisfactorily grown in one tray containing a 5 cm layer of soil. A soluble fertilizer was applied with each watering.

In January, 1979, supplemental plants were cultivated in a greenhouse under Dr. J. P. Snow of LSU Plant Pathology. These plants were to be used to obtain a sufficient quantity of essential oil for instrumental analysis. It was observed that the plants exhibited a different growth pattern when allowed to mature in crowded conditions. Although they were sprayed regularly for fungi and caterpillars, several groups of plants developed withered leaves and died. The plants also had more spindly stalks and were smaller than those grown indoors. However, the profile of essential oil content was similar to that of previous groups.

Plants were selected for feeding experiments at nine weeks and had an average height of 30 cm. The stems were cut close to the ground and dirt was brushed from the lower leaves. Each stem was then carefully cut again with a sharp knife so as not to crush the stem. They were then dipped in dilute sodium perchlorate, rinsed in distilled water and dried. The plants were bundled so that the cut ends were level and placed in a small beaker with radio-
active substrate dissolved in nutrient solution (15). This solution (10 mL) was absorbed in one to two hours and followed by additional quantities of nutrient solution. The nutrient solution was a 1 mM potassium phosphate buffer (pH 7.6) supplemented with 0.3 mM glucose and sodium adenosine triphosphate (1 mg/mL). Radioactivity was found in the tips of the plants within an hour for several different substrates. The plants were then maintained for specified time periods on the nutrient solution under continuous fluorescent illumination. No bacterial growth was evident even after 48-hour feedings.

The essential oil was prepared according to the method of Falk et al. (33). Fresh plant material (200 g) was chopped and coarsely ground in a Waring blender. The oil was obtained by steam distillation of the macerate until 1 liter of distillate was collected. The oily distillate was saturated with sodium chloride followed by extraction with ether (3 x 50 mL). The ether layer was successively washed with 5% sodium hydroxide (50 mL), 5% sodium bicarbonate (50 mL) and water (20 mL). Upon concentration, essential oil (190 mg) was isolated and analyzed (GLC) to possess the following:
<table>
<thead>
<tr>
<th>Peak #</th>
<th></th>
<th>t&lt;sub&gt;Pr&lt;/sub&gt; (min)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td>camphene</td>
<td>4.4</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>myrcene</td>
<td>5.2</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>1,8-cineole</td>
<td>7.8</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td>artemisia ketone</td>
<td>12.0</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>artemisia alcohol</td>
<td>19.8</td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td>unidentified</td>
<td>35.0</td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td>unidentified</td>
<td>55.2</td>
</tr>
</tbody>
</table>

2-(1-HYDROXY-3-METHYLBUTYL-[1-<sup>14</sup>C])THIAMINE CHLORIDE HYDROCHLORIDE 16. The hydrochloride 16 was prepared from 3-methylbutyric acid-[1-<sup>14</sup>C] (purchased from ICN) via reduction to 3-methylbutanol with Red-Al, followed by oxidation to 3-methylbutanal with chromium trioxide and pyridine. 16 was isolated (9.2%) and identical in all respects to previously prepared unlabelled material.

Hydrochloride 16 (350 mg, 0.83 mmol) was dissolved in buffer (pH 7.2) and fed to 12-week-old plants raised in the greenhouse. After 48 hours, the plants had absorbed a total of 250 ml of buffer and were worked up as described above to yield the labelled essential oil (200 mg).

Artemisia ketone was collected by preparative GLC and counted in a liquid scintillation counter. Isovaleraldehyde was identified by removal from the oil with aqueous saturated sodium bisulfite. The volatile alde-
hyde was not isolable by GC due to the small quantity present in relation to other components of the oil. A control distillation carried out with unlabelled \(_{16}\) released no isovaleraldehyde into the distillate.

The majority of radioactivity remained in the plant material, which was divided into two parts. The first batch was acidified to pH 2.5 by the addition of concentrated hydrochloric acid and then steam distilled. The ether extract of the distillate contained a small quantity of artemisia ketone possessing no significant radioactivity. The major component furfural was not radioactive and identical to a known sample: \(\text{bp} \ 90^\circ\text{C} \ (65 \text{ mm}) \ [\text{lit (67) bp} \ 161.5^\circ\text{C}] ; \text{PMR} \ \delta \ 6.52 (q, H-4, J=1.5 \text{ Hz}, 1\text{H}), 7.11 (d, H-3, J=3.5 \text{ Hz}, 1\text{H}), 7.61 (s, H-5, 1\text{H}), 9.63 (s, \text{CHO}, 1\text{H}).

The second batch of plant material was stirred in acetone for 1 hour. The oil isolated following steam distillation contained only traces of artemisia ketone.

The experiment was repeated with substrate prepared from barium carbonate-[\(^{14}\text{C}\)] by carbonation of the Grignard reagent as described above to give 3-methylbutyric acid-[\(^{1-14}\text{C}\)]. \(_{16}\) was then prepared in 6% yield based on barium carbonate.

Hydrochloride \(_{16}\) (309 mg, 0.73 mmol) was fed to nine-week-old plants raised indoors. The workup via steam distillation yielded an essential oil of higher
total radioactivity than the previous feeding. 3-Methylbutanoic acid was characterized in the base washes of the oil workup as well as a minor (1%) component of the oil itself.

2-(1-HYDROXY-3-METHYLBUTYL-[1-14C])-4-METHYL-5-(2-HYDROXYETHYL)THIAZOLE 17. Thiazole 17 (57 mg, 0.25 mmol), prepared from 16 by sodium sulfite cleavage, was fed to plants (130 g wet wt.) for 48 hours. The procedure for isolation of monoterpenes was developed with the help of Dr. N. H. Fischer, of the LSU Chemistry Department. The stems were washed to remove buffer and blended in bulk ethanol (2 L). The alcoholic macerate was boiled for 30 minutes and then filtered through Celite to remove stems and leaf tissue. The filtrate volume was reduced in vacuo to 500 mL, then treated with 5% lead acetate (500 mL). The gummy precipitate was removed by filtration through a Celite pad. The resultant yellow filtrate was extracted with ether (4 x 50 mL). Separation (ThLC) of the ethereal oil eluting with ether:hexane (10:2) revealed several fractions. The fraction corresponding to 17 (Rf 0.15) accounted for 64% of radioactivity in the oil. Two other fractions contained significant radioactivity; Rf 0.74 corresponding to 18 and Rf 0.90 corresponding to the mixed terpenes of the essential oil.
3-METHYL BUTANAL-[1-14C]. 3-Methylbutanal-[1-14C] was prepared by the barium carbonate and Grignard reaction sequence. The aldehyde (95 mg, 1.1 mmol) was dissolved in 50% aqueous ethanol (5 mL) and fed to the plants for 36 hours. After the oil was obtained in the usual manner, it was treated with saturated aqueous sodium bisulfite (5 mL) to remove unchanged starting material.

The second feeding of 3-methylbutanal-[1-14C] (37 mg, 0.43 mmol) continued for 12 hours.

3-METHYL-2-BUTENAL-[1-14C]. 3-Methyl-2-butenal-[1-14C] (16.8 mg, 0.2 mmol) was prepared from the barium carbonate and Grignard reaction sequence with methallyl chloride and was fed to plants for 24 hours.

L-LEUCINE-[U-14C]. L-Leucine-[U-14C] (71 micrograms, 0.5 micromoles) was fed to plants in buffer (pH 7.2, 5 mL) for 48 hours. Workup of the plants in the usual manner yielded the essential oil (102 mg).

The experiment was repeated with L-leucine-[U-14C] (21 micrograms, 0.16 micromoles). One hour after the stems were placed in the radioactive solution, leaves were removed at 10 cm and 30 cm from the base of the stem. These samples were bleached in 30% hydrogen peroxide and counted. Each leaf contained significant radioactivity.
L-VALINE-[U-\(^{14}\)C]. L-Valine-[U-\(^{14}\)C] (30 micrograms, 0.25 micromole) was fed to 100 g of plants in 5 mM phosphate buffer (5 mL, pH 7.2) for 24 hours. After this time period, the plants appeared dehydrated and were no longer absorbing liquid.

The experiment was repeated with the same amount of L-valine as before; but the substrate was dissolved in 1 mM phosphate buffer (pH 7.2), and the plants were maintained on buffer solution for 48 hours.

L-\(\alpha\)-HYDROXYISOCAPROIC ACID-[U-\(^{14}\)C] 20. L-\(\alpha\)-Hydroxyisocaproic acid-[U-\(^{14}\)C] (4.0 micrograms, 0.03 micromole), prepared by nitrous acid reaction (75), was fed to 100 g of plants for 12 hours.

The experiment was repeated with L-\(\alpha\)-hydroxyisocaproic acid-[U-\(^{14}\)C] (0.62 microgram, 5 pmol) and mevalonic acid (2.5 mg) being fed to the plants for the same time period.

L-\(\alpha\)-HYDROXYISOVALERIC ACID-[U-\(^{14}\)C] 21. L-\(\alpha\)-Hydroxyisovaleric acid-[U-\(^{14}\)C] (0.23 microgram, 2.0 pmol) and mevalonic acid (2.5 mg) were fed to 85 g of plants for 12 hours.

DEGRADATION OF ARTEMISIA KETONE FROM L-\(\alpha\)-HYDROXYISOCAPROIC ACID (105, 106). Artemisia ketone (41 mg) was separated from the oil by chromatography (ThLC)
eluting with benzene to give six bands, $R_f$: 0.95, 0.81 (artemisia ketone), 0.62, 0.50, 0.32 (artemisia alcohol), 0.14. The purity of artemisia ketone was verified by GLC.

To a stirred solution of aqueous sodium hydroxide (150 mL, pH 12.2) and potassium permanganate (632 mg, 4 mmol) at 0°C, artemisia ketone (83 mg, 0.93 mmol) was added in $p$-dioxane (2 mL). After 30 minutes, no permanganate ion was evident and 30% formic acid (1 mL) was added to complete the reaction to manganese dioxide. The black precipitate was filtered on a celite pad, and the manganese dioxide cake was washed with additional water. Sodium periodate (3.4 g, 15.8 mmol) was added to the filtrate and stirred at 25°C in the dark for 1 hour. Excess periodate was treated with sodium arsenite (8.7 g) and 2% hydrochloric acid (15 mL). 5,5-Dimethyl-1,3-cyclohexanedione (500 mg) in 95% ethanol (20 mL) was added, and crystals formed overnight. The dimedone derivative was filtered and recrystallized from 95% ethanol to give methylene-dimedone: 140 mg (58%); mp 188 – 190°C [lit (79) mp 191 - 191.5°C].

The filtrate was divided into two equal volumes, one of which was distilled to obtain an aqueous acetone solution which was treated with 4-phenylsemicarbazone (150 mg, 1 mmol) and allowed to crystallize overnight. The product, acetone-4-phenylsemicarbazide was
isolated by filtration: 20 mg (32.5%); mp 153 - 155°C [lit (89) mp 160°C]. The second portion of the original filtrate was adjusted to pH 4 and refluxed for 1.5 hours. Carbon dioxide was collected in ethanolamine under a stream of nitrogen.

Isobutyric acid was identified (TLC) in the ether extract of the aqueous solutions: \( R_f \) 0.68 (ether).

<table>
<thead>
<tr>
<th>Fragment</th>
<th>DPM</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>formaldehyde</td>
<td>55</td>
<td>0.7</td>
</tr>
<tr>
<td>acetone</td>
<td>296</td>
<td>3.7</td>
</tr>
<tr>
<td>carbon dioxide</td>
<td>453</td>
<td>5.8</td>
</tr>
<tr>
<td>isobutyric acid</td>
<td>7000</td>
<td>89</td>
</tr>
</tbody>
</table>

DEGRADATION OF ARTEMISIA KETONE FROM 3-METHYLIBUTANAL-[\(1-^{14}\)C]. Artemisia ketone was isolated and degraded by potassium permanganate and sodium periodate as above.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>DPM</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>formaldehyde</td>
<td>100</td>
<td>1.4</td>
</tr>
<tr>
<td>acetone</td>
<td>392</td>
<td>5.6</td>
</tr>
<tr>
<td>carbon dioxide</td>
<td>46</td>
<td>0.7</td>
</tr>
<tr>
<td>C-2,3,9,10</td>
<td>6500</td>
<td>92</td>
</tr>
</tbody>
</table>

DEGRADATION OF ARTEMISIA KETONE FROM L-\(\alpha\)-HYDROXY-ISOCAPROIC ACID-[\(U-^{14}\)C] AND L-\(\alpha\)-HYDROXYISOVALERIC ACID-[\(U-^{14}\)C]. Artemisia ketone was isolated from each of
these feedings and combined for degradation by the method developed by Charlwood (90). Artemisia ketone (69 mg, 0.45 mmol) was stirred with aqueous potassium permanganate (142 mg, 0.9 mmol) in water (9.5 mL) for 1 hour. Another aliquot of potassium permanganate (82 mg, 0.52 mmol) in water (5 mL) was added and stirred for 4 hours. Manganese dioxide was removed by filtration through a celite pad. The filtrate was adjusted to pH 10 and distilled to obtain an aqueous acetone solution (1 mL). The distillate was treated with 4-phenylsemicarbazone (150 mg) dissolved in 50% ethanol. Acetone-4-phenylsemicarbazide was isolated after recrystallization from a minimal amount of 95% ethanol: 7 mg (8%); mp 147 - 149°C.

The remaining aqueous solution was evaporated in a stream of hot air. The solid products were acidified with 5 N sulfuric acid and extracted with ether (3 x 2 mL). The combined extract was dried over sodium sulfate and chromatographed (ThLC) eluting with a mixture of ethanol:water:ammonium hydroxide (85:11:4). Spots were visualized with bromocresol green spray. Standards and reaction component R_f: isobutyric acid, 0.64; formic acid, 0.51; dimethylmalonic acid, 0.09.
<table>
<thead>
<tr>
<th>Fragment</th>
<th>DPM</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>formic acid</td>
<td>16</td>
<td>1.4</td>
</tr>
<tr>
<td>acetone</td>
<td>56</td>
<td>4.8</td>
</tr>
<tr>
<td>isobutyric acid</td>
<td>946</td>
<td>82</td>
</tr>
<tr>
<td>dimethylmalonate</td>
<td>111</td>
<td>9.6</td>
</tr>
</tbody>
</table>
### TABLE 4

**PMR ASSIGNMENTS OF ARTEMISYL COMPOUNDS**

<table>
<thead>
<tr>
<th>PROTON</th>
<th>4.</th>
<th>5.</th>
<th>6.</th>
<th>7.</th>
<th>8.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1</td>
<td>4.85-5.20 Octet, 2H $J_{AB}=1.5$ Hz</td>
<td>4.91-5.20 Octet, 2H $J_{AB}=1.5$ Hz</td>
<td>5.01-5.10 Quintet, 2H $J_{AB}=1.5$ Hz</td>
<td>4.91-5.20 Octet, 2H $J_{AB}=1.5$ Hz</td>
<td>4.85-5.20 Octet, 2H $J_{AB}=1.5$ Hz</td>
</tr>
<tr>
<td>H-2</td>
<td>5.85 q, 1H $J_{AX}=10$ Hz $J_{BX}=18$ Hz</td>
<td>5.95 q, 1H $J_{AX}=10$ Hz $J_{BX}=18$ Hz</td>
<td>5.78 q, 1H $J_{AX}=10$ Hz $J_{BX}=18$ Hz</td>
<td>5.95 q, 1H $J_{AX}=10$ Hz $J_{BX}=18$ Hz</td>
<td>5.80 q, 1H $J_{AX}=10$ Hz $J_{BX}=18$ Hz</td>
</tr>
<tr>
<td>H-4</td>
<td>3.93 d, 1H $J=9$ Hz</td>
<td>-</td>
<td>2.74 d, 1H $J=2.5$ Hz</td>
<td>-</td>
<td>3.20 br t, 1H $J=5.5$ Hz</td>
</tr>
<tr>
<td>H-5</td>
<td>5.95 d, 1H $J=2.5$ Hz</td>
<td>6.25 m, 1H</td>
<td>2.87 d, 1H $J=2.5$ Hz</td>
<td>1.50-1.80 m, 3H</td>
<td>1.48-1.75 m, 3H</td>
</tr>
<tr>
<td>H-6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>H-7</td>
<td>1.70 s, 3H</td>
<td>2.12 s, 3H</td>
<td>1.29 s, 3H</td>
<td>0.95 d, 6H</td>
<td>0.95 d, 6H</td>
</tr>
<tr>
<td>H-8</td>
<td>1.67 s, 3H</td>
<td>1.89 s, 3H</td>
<td>1.20 s, 3H</td>
<td>1.05 d, 6H $J=6.5$ Hz</td>
<td>1.05 d, 6H $J=6.5$ Hz</td>
</tr>
<tr>
<td>H-9,10</td>
<td>1.00 s, 6H</td>
<td>1.25 s, 6H</td>
<td>1.04 s, 3H</td>
<td>1.05 s, 3H s, 3H</td>
<td>1.20 s, 6H</td>
</tr>
</tbody>
</table>

*Note: All values are in ppm.*
### TABLE 5
**ESSENTIAL OIL ANALYSIS**

<table>
<thead>
<tr>
<th></th>
<th>DPM</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L-LEUCINE-[U-(^{14})C]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25 microcuries; 5.55 \times 10^7dpm; 48 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oil</td>
<td>14,000</td>
<td>0.025</td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>2,530</td>
<td>0.0046</td>
</tr>
<tr>
<td>25 microcuries; 5.55 \times 10^7dpm; 36 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oil</td>
<td>13,930</td>
<td>0.025</td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>1,700</td>
<td>0.003</td>
</tr>
<tr>
<td>artemisia alcohol</td>
<td>2,290</td>
<td>0.004</td>
</tr>
</tbody>
</table>

| **L-VALINE-[U-\(^{14}\)C]** |        |        |
| 17 microcuries; 3.74 \times 10^7dpm; 24 hours |        |        |
| oil                 | 898    | 0.0024 |
| 17 microcuries; 3.74 \times 10^7dpm; 48 hours |        |        |
| oil                 | 4,360  | 0.012  |
| artemisia ketone    | 1,980  | 0.005  |

<p>| <strong>L-(\alpha)-HYDROXYISOCAPROIC ACID-[U-(^{14})C]</strong> | 20 |        |
| 7.66 microcuries; 1.7 \times 10^7dpm; 12 hours |        |        |
| oil                 | 106,000| 0.624  |
| artemisia ketone    | 41,630 | 0.245  |
| artemisia alcohol   | 9,600  | 0.056  |</p>
<table>
<thead>
<tr>
<th>Compound</th>
<th>Radioactivity</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mg MVA; 1.17 microcuries; 2.6 x 10^6dpm; 12 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oil</td>
<td>3,230</td>
<td>0.124</td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>300</td>
<td>0.012</td>
</tr>
<tr>
<td>L-α-HYDROXYISOVALERIC ACID-[U-14C] 21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 mg MVA; 0.52 microcuries; 1.14 x 10^6dpm; 12 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oil</td>
<td>7,230</td>
<td>0.63</td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>580</td>
<td>0.051</td>
</tr>
<tr>
<td>2-(1-HYDROXY-3-METHYLIBUTYL-[1-14C]THIAMINE CHLORIDE HYDRO-CHLORIDE 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.2 microcuries; 2.04 x 10^7dpm; 48 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oil</td>
<td>45,670</td>
<td>0.22</td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>3,840</td>
<td>0.019</td>
</tr>
<tr>
<td>3-methylbutanal</td>
<td>9,700</td>
<td>0.047</td>
</tr>
<tr>
<td>17.6 microcuries; 3.9 x 10^7dpm; 48 hours</td>
<td></td>
<td></td>
</tr>
<tr>
<td>oil</td>
<td>415,000</td>
<td>1.06</td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>3,860</td>
<td>0.0099</td>
</tr>
<tr>
<td>artemisia alcohol</td>
<td>11,400</td>
<td>0.029</td>
</tr>
<tr>
<td>3-methylbutanal</td>
<td>272,000</td>
<td>0.705</td>
</tr>
<tr>
<td>3-methylbutanoic acid</td>
<td>199,000</td>
<td>0.51</td>
</tr>
</tbody>
</table>
2-(1-HYDROXY-3-METHYLBUTYL-[\textsuperscript{14}C])4-METHYL-5-(2-HYDROXY-ETHYL)THIAZOLE \textsubscript{17}

10.8 microcuries; 2.4 \times 10^7 \text{dpm}; 48 hours

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity (microcuries)</th>
<th>DPM (10^7)</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oil</td>
<td>360,000</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>\textsubscript{18}</td>
<td>3,280</td>
<td>0.014</td>
<td></td>
</tr>
<tr>
<td>terpenes</td>
<td>700</td>
<td>0.003</td>
<td></td>
</tr>
</tbody>
</table>

3-METHYLBUTANAL-[\textsuperscript{14}C]

46.8 microcuries; 1.04 \times 10^8 \text{dpm}; 36 hours

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity (microcuries)</th>
<th>DPM (10^8)</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oil</td>
<td>106,000</td>
<td>0.102</td>
<td></td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>10,100</td>
<td>0.0097</td>
<td></td>
</tr>
<tr>
<td>artemisia alcohol</td>
<td>10,800</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>3-methylbutanal</td>
<td>120,000</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>3-methylbutanoic acid</td>
<td>2,978</td>
<td>0.003</td>
<td></td>
</tr>
</tbody>
</table>

37.1 microcuries; 8.24 \times 10^7 \text{dpm}; 12 hours

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity (microcuries)</th>
<th>DPM (10^7)</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oil</td>
<td>175,000</td>
<td>0.213</td>
<td></td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>53,000</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td>artemisia alcohol</td>
<td>40,600</td>
<td>0.049</td>
<td></td>
</tr>
</tbody>
</table>

3-METHYL-2-BUTENAL-[\textsuperscript{14}C]

0.064 microcuries; 1.42 \times 10^5 \text{dpm}; 24 hours

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity (microcuries)</th>
<th>DPM (10^5)</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oil</td>
<td>2,900</td>
<td>2.05</td>
<td></td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>500</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>artemisia alcohol</td>
<td>300</td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>3-methyl-2-butenal</td>
<td>7,480</td>
<td>5.3</td>
<td></td>
</tr>
</tbody>
</table>
III. RESULTS AND DISCUSSION

Current concepts (28, 41, 45) of irregular terpene biosynthesis feature condensations between two molecules of mevalonoid origin. According to these theories, MVA should be incorporated both efficiently and symmetrically into artemisia ketone. Table 3 (Page 31) presented the experimental data from two research groups on artemisia ketone biosynthesis. In each case (49 - 53), MVA was not an effective precursor and the label was asymmetrically incorporated with the majority located in the I portion.

In order to elucidate more details of the asymmetric labelling phenomenon, a series of feeding experiments were designed. Several non-mevalonoid substrates (Figure 19) were chosen as precursors to artemisia ketone in Artemisia annua and were designed to efficiently label the D unit via the cofactor thiamine pyrophosphate (TPP). If this route were successful, an immediate explanation could be offered for the asymmetric labelling and low MVA incorporation seen in artemisia ketone: the D unit is non-mevalonoid.

Participation of thiamine pyrophosphate as a cofactor in a pathway to irregular terpenoid compounds was implicated by earlier work in this laboratory (93). These investigators found that an equimolar mixture of thiamine
Figure 19. Substrates fed to *Artemisia annua*.
pyrophosphate, geraniol and citral was converted by a standard yeast preparation into a C-20 hydrocarbon, \( \text{22} \), as shown in Figure 20. An irregular terpenoid structure was assigned to this compound based on mass spectral data.

Synthesis of \( \text{22} \) proved difficult (115). Further, it was possible to employ the standard yeast preparation to synthesize \( \text{23} \), a saturated analog of \( \text{22} \) as shown in Figure 21. Karimian (115) supplied the yeast enzymes with 2-(1-hydroxy-3,7-dimethyl-1-oct-6-enyl)thiamine chloride hydrochloride \( \text{24} \) and geraniol-[\(^3\)H], and the radio-labelled product subsequently isolated in 7.6% yield was identical in chromatographic properties to \( \text{23} \). This experiment established the involvement of thiamine in the biosynthesis of a novel irregular diterpene.

As a preliminary part of this project, the same yeast preparation was supplied with 3-methyl-2-butenol \( \text{1} \) and 2-(1-hydroxy-3-methylbutyl)thiamine chloride hydrochloride \( \text{16} \) in order to determine if the cell-free system could prepare a ten-carbon analog of \( \text{22} \) or \( \text{23} \) (Figure 22). The details of the synthesis of 2-alkylthiamine derivatives, such as \( \text{16} \), were discussed previously by Karimian (115). The hydrochloride \( \text{16} \) was not a substrate for the yeast enzymes as \( \text{24} \) had been, and neither artemisia ketone nor 5,6-dihydroartemisia ketone \( \text{7} \) was detected in the reaction products.
Figure 20. Irregular diterpene formed in yeast preparations containing geraniol, citral and thiamine.
Figure 21. Labelled irregular diterpene formed in yeast preparation.
Figure 22. Substrates for irregular monoterpene biosynthesis.
The two expected products of the yeast preparation were synthesized by a continuous-flow Reformatsky column developed by Ruppert and White (63). The mechanism of the condensation between 3-methyl-2-butenal and 1-bromo-3-methyl-2-butene is shown in Figure 23. The transition state consists of a six-membered structure involving the allylic double bond of the halide. Collapse of the intermediate results in a tail-to-middle linkage of the two five-carbon units. Artemisia alcohol is obtained by subsequent hydrolysis of the salt.

Conventional methods of oxidation, such as chromium trioxide and pyridine or manganese dioxide, produced artemisia ketone in low yields. Further investigation revealed that the major compound isolated from the oxidation reaction mixture was a tertiary alcohol, 2,5,5-trimethyl-3,6-heptadiene-2-ol 6, formed by isomerization of artemisia alcohol. Alcohol 6 was identified on the basis of tR and PMR spectrum. This tertiary alcohol had a slightly longer retention time than artemisia alcohol. The PMR spectrum of artemisia alcohol shows a characteristic pattern at δ 5.90 for the α-vinyllic proton, whereas 6 showed new peaks at δ 2.73 (d, J=2.5 Hz, 1H) and δ 2.83 (d, J=2.5 Hz, 1H) for the vinyl protons. The most satisfactory method for oxidation of artemisia alcohol to artemisia ketone was chromium trioxide and pyridine.
Figure 23. Mechanism of continuous-flow Reformatsky reaction.
While some isomerization of artemisia alcohol to 6 did occur, the reaction time was short enough to minimize the formation of this unwanted product.

A second expected product of the yeast preparation was 5,6-dihydroartemisia ketone 7. This compound was synthesized by a 1,4-reduction of artemisia ketone with lithium in liquid ammonia. The method was chosen to selectively reduce the \( \alpha,\beta \)-unsaturated olefin as opposed to the terminal methylene which is reduced by conventional hydrogenations (109).

The inability of the yeast system to prepare a ten-carbon analog of larger irregular terpenes can be attributed to the specificity of the yeast enzymes for larger substrates. A feasible alternative was a study of 16 and similar compounds as substrates for \( A. \ annua \) to determine if the \( \Delta \) unit of artemisia ketone could be labelled.

Before MVA was accepted as a terpenoid precursor, many compounds were discussed (116) as candidates for the isoprenoid skeleton, and among them were isovaleraldehyde, methylbutenal, isoamyl alcohol, leucine and valine.

A pathway to artemisia ketone (Figure 24) involving the amino acid leucine as the non-mevalonoid component of artemisia ketone was proposed (94) to explain the lack of
Figure 24. Pathway to artemisia ketone.
MVA incorporation into the D unit. The proposed entry of leucine into the pathway follows accepted biochemical conversions. Pyridoxal phosphate catalyzes the conversion of leucine to α-ketoisocaproic acid. This transamination is the first step in the oxidation of many amino acids to intermediates of the citric acid cycle (95). The α-keto acid is then decarboxylated by TPP in a reaction analogous to the decarboxylation of pyruvic acid to generate acetaldehyde (96). The enamine 16 formed by decarboxylation can react by an $S_{N2}$ mechanism with DMAPP. An enamine alkylation of this type occurs in the thiamine catalyzed conversion of xylulose-5-phosphate to sedoheptulose-7-phosphate in the transketolase reaction (97). TPP is finally regenerated by release of the ten-carbon irregular terpene.

The release of a carbonyl compound from the C-2 position of thiamine is seen in the alcoholic fermentation pathway when acetaldehyde is released from 2-hydroxyethylthiamine (97). The conversion of 5,6-dihydroartemisia ketone to the naturally occurring α,β-unsaturated derivative is similar to flavin oxidations of other biological compounds. The oxidation of succinate to fumarate (98) is one example of such an oxidation.

To begin examining the feasibility of this pathway, L-leucine-[U-$^{14}$C] was fed to plants of *A. annua* for two different time intervals, forty-eight and thirty-six
hours. The oil isolated from each feeding contained a constant percentage of radioactivity. Similarly, there was no significant difference in the percentage of radioactivity found in artemisia ketone isolated from the two oils. The results of these feedings (Table 5, Page 76) are comparable to the data reported for earlier work with MVA in *A. annua*.

Neither 3-methylbutanal nor 3-methylbutanoic acid was detected in the oil. Earlier work on leucine metabolism in *Proteus vulgaris* reported the isolation of 3-methylbutanal from the organism via the 2,4-dinitrophenylhydrazine derivative (113).

Prospects for L-valine as a substrate for artemisia ketone biosynthesis were two-fold. The valine skeleton is a precursor of leucine as shown in the biosynthetic scheme of Figure 25 (102). The α-ketoacid derivative of valine is combined with acetate to give a seven-carbon diacid. The leucine skeleton is formed by decarboxylation of the carboxyl group from valine. The resulting α-ketoisocaproic acid can enter the pathway to artemisia ketone as proposed in Figure 24.

Secondly, valine can be oxidatively deaminated to 3,3-dimethylacrylic acid as proposed by Suga (23) (Figure 6). Subsequent reduction of the acid to the aldehyde would allow the valine skeleton a more direct
Figure 25. Entry of the valine skeleton into leucine pathway.
entry into the artemisia ketone pathway.

Results (Table 5) of the two feeding experiments with valine did not point to a direct involvement in the pathway. Although the label in artemisia ketone represented a larger proportion of the total radioactivity of the oil, the percentage of label incorporated from this amino acid was of the same level as leucine. In fact, oils from feedings with either amino acid contained such low amounts of radioactivity that it can be concluded that either these compounds are diverted for use in other pathways, or that the charged amino acids are unable to be transported into the plant cell.

To examine the latter possibility, L-α-hydroxyisocaproic acid-[U-\(^{14}\)C] \(^{20}\) was prepared by a classical procedure (75) from L-leucine-[U-\(^{14}\)C]. Deamination was shown (91) to proceed by an \(S_{N}1\) mechanism, and the \(α\)-carboxylate group was attributed to the maintenance of configuration. Once transported inside the cell, the \(α\)-hydroxy acid should be easily converted by plant enzymes to an \(α\)-ketoacid (Figure 26). Two enzymes, L-amino acid oxidase, a flavin-containing enzyme (101), and L-\(α\)-hydroxy acid oxidase (106, 107) are known to catalyze the oxidation reaction.

Initial results (Table 5) from the feeding experiment with L-hydroxyisocaproic acid-[U-\(^{14}\)C] showed a rela-
Figure 26. $\alpha$-Hydroxy acids as substrates for A. annua.
tively high amount of label in the oil (0.624%) and a large amount of radioactivity in artemisia ketone (0.245%). These results are much higher than those observed by Banthorpe and Charlwood (49) and Suga, et al. (50) for feeding experiments with MVA. Typical values for incorporations of mevalonoid substrates into artemisia ketone by A. annua are given below:

<table>
<thead>
<tr>
<th>Reference</th>
<th>Substrate</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>MVA</td>
<td>0.001 - 0.01</td>
</tr>
<tr>
<td>50</td>
<td>&quot;</td>
<td>0.001</td>
</tr>
<tr>
<td>51</td>
<td>&quot;</td>
<td>0.04</td>
</tr>
<tr>
<td>51</td>
<td>IPP</td>
<td>0.81</td>
</tr>
<tr>
<td>51</td>
<td>DMAPP</td>
<td>0.12</td>
</tr>
<tr>
<td>51</td>
<td>geraniol</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Was the efficient incorporation of L-α-hydroxyisocapric acid due to its participation in the non-mevalonoid pathway and labelling of the D unit? This question could only be answered by degradation of artemisia ketone.

Potassium permanganate is the classical reagent for degrading compounds containing double bonds (103). Under standard conditions, artemisia ketone released only one fragment of C-6,7,8 as acetone while the remaining fragment eluded attempts at characterization.

A procedure for oxidizing olefinic double bonds in
an aqueous solution of periodate containing catalytic amounts of permanganate was reported by Lemieux and von Rudloff (105). Under these conditions also, artemisia ketone released one mole of acetone, while the remaining fragment could not be identified. Apparently, the kinetics of the acetone formation allowed that fragment to be released before the terminal methylene reacted. The nature of the remaining seven-carbon fragment prevented further oxidation.

To react both olefinic bonds of artemisia ketone, it was necessary to treat the molecule initially with cold, dilute permanganate, followed by cleavage of the resulting diols with periodate (Figure 27, Reaction 1). Artemisia ketone from the L-\(\alpha\)-hydroxy acid feeding was degraded in this manner. Formaldehyde was easily identified and counted as the dimedone derivative. Acetone yielded a white, crystalline compound with 4-phenylsemicarbazone. The remaining fragment was oxidized to the diacid and decarboxylated to yield carbon dioxide and isobutyric acid. Results of this degradation showed the majority of the label (89%) to be found in the isobutyric acid fragment composed of C-2,3,9 and 10.

The degradation indicates two conclusions: a) the \(\alpha\)-hydroxy acid was incorporated into the \(\alpha\) unit of artemisia ketone in a manner similar to mevalonoid substrates
Figure 27. Degradation of artemisia ketone by two methods.
and b) the substrate was degraded and reincorporated as fragments because C-1 of artemisia ketone from this experiment contained less than 1% of the label. If the six-carbon substrate were directly converted to a five-carbon mevalonoid unit, each carbon should contain one-fifth of the total label in the I unit.

As outlined in Figures 15 and 16 (Pages 41 and 42), the conversion of leucine to HMG CoA and IPP is well-known in carotenoid biosynthesis (80 - 87). Thus, it might be expected that a portion of the substrate would be diverted to the mevalonoid pathway. The next two feeding experiments were designed to block entry of the label into the I or mevalonoid pathway and restrict the substrate to the proposed D pathway. This would be accomplished by supplying the plant simultaneously with the labelled substrates and cold MVA.

Thus, all mevalonoid pools would be filled with substrates, blocking entry of the fragments of the labelled hydroxy acids into the mevalonoid pathway. The applied substrates would not be subjected to degradation and reincorporation of fragments via MVA, but directed towards the proposed non-mevalonoid pathway. The appearance of an even higher level of incorporation in artemisia ketone, under these conditions, would affirm the non-mevalonoid pathway from a metabolic point of view.
Unfortunately, high incorporation seen initially with α-hydroxyisocaproic acid was not repeated when MVA was added to the nutrient solution of two different substrates, α-hydroxyisocaproic acid and α-hydroxyisovaleric acid, a valine analog. It is possible that the lower incorporation is due to regulation of artemisia ketone biosynthesis by excess MVA (117).

In these two experiments, artemisia ketone derived from α-hydroxyisocaproic acid incorporated 0.012% of the label and that derived from α-hydroxyisovaleric acid incorporated 0.051% of the label. It should be noted that incorporation of the valine derivative is four times that of the leucine derivative. This amount is still one order of magnitude lower than the incorporation of α-hydroxyisocaproic acid fed under normal conditions. A comparison of substrates under the disturbed conditions of these two experiments shows that the valine analog, α-hydroxyisovaleric acid, is a much better precursor.

A degradation was carried out on a sample of artemisia ketone isolated from the essential oils from feedings of α-hydroxyisocaproic acid and α-hydroxyisovaleric acid combined with unlabelled MVA. It was necessary to combine the oils to obtain artemisia ketone of high specific activity. The method (106) for this degradation was described by Charlwood (90) and obtained from a personal communica-
tion with Professor Banthorpe. The molecule was degraded by concentrated aqueous permanganate at room temperature followed by a second aliquot of permanganate. After stirring for five hours, the fragments indicated in the second reaction of Figure 27 were isolated. The majority (82%) of the radioactivity was located in the isobutyric acid fragment. A small amount of dimethyl malonate remained which did not decarboxylate, and this compound contained 9.6% of the label. At least 91.6% of the label from these experiments was found in the same carbons as in the previous degradations.

Again, the results indicated that the label is incorporated via a mevalonoid pathway with the label predominating in the I unit. The experiments using amino acids and their analogues were unable to label the D unit of artemisia ketone by the proposed pathway.

The next feeding experiment was done with 16, a substrate closer to the product of the proposed pathway. The 2-alkyl thiamine derivative 16 should be a specific precursor for the D unit of artemisia ketone and result in efficient incorporation. To obtain labelled 16, it was necessary to develop a synthetic scheme to labelled 3-methylbutanal. A number of reactions were attempted as potential routes to radio-labelled 3-methylbutanal. The first attempted reaction was a 1,4-Grignard with crotonal-
dehyde as starting material. Cuprous chloride was used to
catalyze the 1,4-addition of methylmagnesium bromide-$^{[14C]}$. This catalyst was first used by Kharasch and Tawney (68) to direct addition of methylmagnesium bromide to the β-position of isophorone in 80% yield. However, the aldehyde function of crotonaldehyde proved too reactive and the 1,2-addition product, 3-pentene-2-ol, predominated as had been previously reported in related systems (69). Successful 1,4-addition reactions with citral and alkyl-
copper lithium have been recently reported to proceed in 60 - 90% yield (92).

Further attempts to synthesize radioactive 3-methyl-
butanal involved the N-t-butylimine derivative of crotonal-
dehyde. It was rationalized that the increased bulk at
the imine position would direct the nucleophilic attack
of methylmagnesium bromide to the β-position. Such addi-
tions of larger Grignard reagents had been successful
with N-t-butylcrotonimine (70). However, the low yield of
3-methylbutanal by this method rendered the reaction
undesirable for radioactive work. The inherent reactivity
of the radioactive starting material, methylmagnesium bro-
mide, made the task of separating the small amount of
desired product from other radioactive products next to
impossible.

An ester, ethyl 3-methyl-2-butenoate, and an alde-
hyde, 3-methyl-2-butenal, were then studied as substrates for \( \gamma \)-hydrogen exchange with tritiated water. After tritium was introduced into the molecule, it could be reduced to 3-methylbutanol followed by oxidation to 3-methylbutanal-\([4,5-^3\text{H}]\). However, model studies with deuterium oxide showed this method to be an impractical route based on the inability to prepare materials of high specific activity.

The aldehyde, 3-methylbutanal, was finally prepared in good yield and high specific activity in a multi-step synthetic scheme. A Grignard reagent prepared from 1-bromo-2-methylpropane was reacted with barium carbonate-\([^{14}\text{C}]\) to give 3-methylbutanoic acid. Reduction of the acid with Red-Al resulted in formation of 3-methylbutanol, which could be easily isolated from residual acid. The aldehyde was obtained by oxidation of the alcohol with chromium trioxide and pyridine.

Once the radioactive aldehyde was prepared, it was possible to synthesize labelled \(^{16}\)O to use as a substrate for \textit{A. annua}. The overall reaction sequence from barium carbonate-\([^{14}\text{C}]\) is shown in Figure 28. Due to the volatility of 3-methylbutanal, it was impossible to remove the solvents (dichloromethane and ether) used in the oxidation reaction without simultaneous loss of product. Reaction times of twenty-four hours compensated for the
Figure 28. Synthesis of 16.
presence of additional solvents (ethanol, dichloromethane, ether).

The level of incorporation of 16 for two separate feeding experiments with *A. annua* was 0.019 and 0.0099%. Assuming that the enzyme selects one of the two stereoisomers composing the racemic mixture fed to the plant, the actual amount of incorporation of 16 is between 0.038 and 0.020%. Other terpenes of the oil contained comparable amounts of label. For example, 1,8-cineole contained 0.025% and artemisia alcohol contained 0.029% of the label.

Also isolated from the essential oil were 3-methylbutanoic acid and 3-methylbutanal. Since 16 was found to be stable under conditions of steam distillation and workup, it is proposed that these compounds arose from enzymatic action on 16 to release 3-methylbutanal, which was then oxidized to 3-methylbutanoic acid. It is probable that 16 was transported to a metabolism site within the plant and was recognized by an enzyme capable of catalyzing the release of the side chain.

Neither 3-methylbutanal nor 3-methylbutanoic acid has been reported as normal components in the essential oil of *A. annua*. Reports of these compounds in other aromatic plants are numerous. The aldehyde has been observed in American and French peppermint oils, in oil of Eucalyptus globulus, Java citronella and East Indian
sandalwood. The acid occurs in oil of valerian, citronella, cypress, spearmint and laurel leaves (99). It was also reported as one of the major constituents of several species of Siberian Artemisia (100).

It was hoped that incorporation levels relatively higher than those seen with MVA could be achieved by supplying the plant with a substrate closer to the end point of the artemisia ketone pathway than MVA. One possibility was that low level incorporation was due to degradation and reincorporation of fragments of the released isovaleryl side chain. A single conclusion was apparent from the initial experiments with 16. The substrate applied exogenously did not have direct or preferential access to the biosynthetic pathway of artemisia ketone.

An analog of 16, 2-(1-hydroxy-3-methylbutyl-[1-\textsuperscript{14}C]-4-methyl-5-(2-hydroxyethyl)thiazole 17, was then tried as a substrate for A. annua in another attempt to direct the label to the artemisia ketone pathway. Because the positive charge at nitrogen in the thiazole ring was no longer present, it was believed that 17 would be stable to enzymatic catalysis of the release of the isovaleryl side chain (118). Thus, any significant incorporation of label would proceed through entry of the thiazole intermediate into the D pathway. At the same
time, the lack of positive charge at nitrogen also would
prevent the release of the monoterpenic side chain from the
thiazole moiety resulting in a new product 19.

Thiazole 17 was prepared easily by sodium sulfite
cleavage of the pyrimidine ring from 16. Synthesis of
the new plant product 19 is shown in Figure 29. The pro-
posed mechanism for the biosynthesis of 19 is shown in
Figure 30.

It appeared unlikely that 19 would be a component
of the steam-distilled essential oil of the plant mace-
rate. An alternative procedure for isolation of the ter-
penoid plant products that would include 19 was developed.
The profile of terpenes in the ethereal oil isolated by
this alternate method was identical to the components of
the steam-distilled oil, and 19 was stable under condi-
tions of isolation.

The resulting ethereal oil contained 64% of the
applied substrate 17. The mixed terpenoid fraction incor-
porated 0.003% of the label, while the band corresponding
to 19 contained 0.014% of the label. The method of TLC
separation employed for the oil consistently yielded high
background due to the presence of the high specific activ-
ity substrate 17. Mass spectral analysis of the oil
component corresponding to 19 revealed no predominant
structural pattern. If the plant was able to synthesize
Figure 29. Synthetic route to 19.
Figure 30. Biosynthesis of 19.
19 under conditions of the experiment, it was either degraded during isolation and storage or present in such small amounts as to preclude analysis.

Isolation of the large amount of starting material suggests two possibilities. First, 17 is not adaptable to existing plant transport mechanisms and enzymes. While 17 lacks the charge and bulk of the pyrimidine ring found in 16, the molecular size of both compounds may prevent membrane transport. If 16 occurs as an intermediate in the pathway to artemisia ketone, it is probably located within the cell and is enzyme-bound. Thus, a specific transport mechanism from the extracellular fluid into the cell would be unnecessary.

Second, the proposed non-mevalonoid pathway does not operate via the thiamine intermediate. The nonspecific nature of the incorporations of 16 and 17 does not allow a firm conclusion to be drawn about the role of thiamine derivatives in the pathway.

The remaining experiments were directed towards finding a five-carbon non-mevalonoid compound that would yield high incorporation rates with A. annua. Smaller compounds should adapt readily to plant transport systems and may have direct access to the biosynthetic site which the larger compounds could not reach.

The first five-carbon substrate, 3-methylbutanal-
[\textsuperscript{1-\textsuperscript{14}C}], was dissolved in 50% ethanol before supplying the cut plants with the substrate. Allen et al. (51) treated water-insoluble compounds in a similar manner and found no tissue damage or difference in incorporation between a control feeding and one with ethanol. The substrate was fed to the plants for two different time intervals, twelve and thirty-six hours. Shorter feeding times resulted in a slightly higher percentage of radioactivity in artemisit ketone. The percentage incorporation for the thirty-six hour feeding is near that reported for artemisia ketone with \textsuperscript{16}. This indicates that the incorporation data reported with \textsuperscript{16} may, in fact, be due to label supplied by the released isovaleryl side chain.

The shorter feeding time considerably increased the amount of 3-methylbutanal recovered in the oil. The difference in 3-methylbutanal content between the two oils indicates the existence of a degradation pathway for 3-methylbutanal. The percentage incorporations of the two separate feedings are summarized below:

<table>
<thead>
<tr>
<th></th>
<th>\textsuperscript{12} hours</th>
<th>\textsuperscript{36} hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>oil</td>
<td>0.213</td>
<td>0.102</td>
</tr>
<tr>
<td>artemisia ketone</td>
<td>0.064</td>
<td>0.0097</td>
</tr>
<tr>
<td>3-methylbutanal</td>
<td>5.0</td>
<td>0.02</td>
</tr>
</tbody>
</table>
An artemisia ketone sample from the 3-methylbutanal-[\(^{14}\text{C}\)] feeding was degraded (Page 95) to determine if the label was located in the D unit. The results indicated a labelling pattern similar to that seen with \(\alpha\)-hydroxy acids. The majority (92\%) of the label was located in the isobutyric acid fragment derived from the I portion of artemisia ketone. Again, the substrate was not an efficient precursor of the proposed pathway.

One final compound, 3-methyl-2-butenal, was tried after an examination of the originally proposed pathway (Figure 24). One of the weak points of that pathway is the sequence of oxidation of 5,6-dihydroartemisia ketone to the \(\alpha,\beta\)-unsaturated derivative. The 5,6-dihydro compound has not been reported as a naturally occurring compound in \(A.\ annua\) or other species that produce artemisia ketone. An alternative to the original pathway is shown in Figure 31. In this proposal the alkyl side chain is cleaved from \(16\) and oxidized followed by formation of a 2-alkenylthiamine derivative. This compound now reacts with DMAPP to directly form artemisia ketone.

The first reaction in which the alkyl side chain is cleaved from thiamine was shown to exist in the feeding experiment with \(16\) where significant amounts of 3-methylbutanal were isolated in the oil. To examine the possibility of the oxidation step occurring at the point shown
Figure 31. Biosynthesis of artemisia ketone.
in Figure 31, the aldehyde, 3-methyl-2-butenal-[1-14C] was fed to the plants. However, the amount of radioactivity (500 dpm, 0.35%) was not great enough to allow degradation of the sample to determine label location.

A summary of the results of incorporation studies of various substrates as precursors to artemisia ketone reveals no solid evidence for the participation of thiamine derivatives in the D unit of artemisia ketone. Neither 16 nor 17 is an efficient precursor of artemisia ketone. In fact, results of later experiments with 3-methylbutanal indicate that the incorporation of label from 16 is due to the aldehyde alone. The amino acids, leucine and valine, are also poor substrates for artemisia ketone because they are not transported into the cell. The best substrate was α-hydroxyisocaproic acid. The experiments in which the plants were dosed with unlabelled MVA did not result in high levels of incorporation, probably due to shutting down of the pathway by high levels of MVA. In this case, α-hydroxyisovaleric acid was a better substrate than α-hydroxyisocaproic acid.

Degradation of artemisia ketone to locate the label contributes additional information about the use of these substrates in terpenoid biosynthesis. First, all samples of artemisia ketone are asymmetrically labelled. The ratios of label found in the I and D units for various
substrates are summarized below:

<table>
<thead>
<tr>
<th>Substrate</th>
<th>I</th>
<th>D%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 3-methylbutanal</td>
<td>93</td>
<td>6</td>
</tr>
<tr>
<td>2. α-hydroxyisocaproic acid</td>
<td>89</td>
<td>7</td>
</tr>
<tr>
<td>3. α-hydroxyisocaproic acid and α-hydroxyisovaleric acid with MVA</td>
<td>93</td>
<td>5</td>
</tr>
</tbody>
</table>

The distributions of the label in these samples of artemisia ketone are similar to the results cited in Table 3 for mevalonoid substrates.

A second conclusion from the degradation concerns the metabolism of the substrates before incorporation of the label. Considerable scrambling of the label occurs for each substrate.

A pathway which explains the metabolism and reincorporation of α-hydroxyisocaproic acid was discussed in the introduction (Page 40). Figures 15 and 16 outline the conversion of leucine to HMG CoA and subsequent cleavage of HMG CoA to acetoacetate and acetate. These two compounds are then recombined in a specific manner to form a second molecule of HMG CoA. The substrate, α-hydroxyisocaproic acid, enters the pathway (Figure 15) after oxidation to α-ketoisocaproic acid, and conversion to HMG CoA in a normal fashion follows. HMG CoA is then cleaved and the units of acetoacetate and acetate rear-
ranged. In the case of \( \alpha \)-hydroxyisocaproic acid, the dotted carbon in the figures is unlabelled, while the remaining carbons are labelled. Thus, the second molecule of HMG CoA forms IPP with unlabelled C-1. The validity of this pathway is confirmed by the experimental results of the degradation which showed C-1 of artemisia ketone to contain only 0.7% of the label.

The asymmetric incorporation of C-1 of 3-methylbutanal-[\( ^{14} \text{C} \)] is not as simple to account for. If the same rationale is used for this substrate as for \( \alpha \)-hydroxyisocaproic acid, 3-methylbutanal would enter the pathway (Figure 15) after oxidation to 3-methylbutanoic acid and esterification to CoASH. If HMG CoA is directly converted to IPP, then all of the label resides in C-1 of IPP and finally in C-1 of artemisia ketone. Experimentally, this is not the case as only 1.4% of the label is located at that position, while 92% is located at C-2,3,9 and 10.

Another alternative for HMG CoA is cleavage to acetoacetate and acetate. When these fragments are recombined to form HMG CoA (Figure 16), the labelled carbon from 3-methylbutanal is decarboxylated when IPP is formed. However, if acetate-[\( ^{14} \text{C} \)] dimerizes to acetoacetate, then label is incorporated into HMG CoA at C-1 and C-3 (Figure 32). A few turns around the cycle dilutes the label at C-1 with the acetate pool, but comparatively
Figure 32: Incorporation of 3-methylbutanal-[\text{1-14C}] into IPP.
concentrates the label at C-3. Finally, IPP is formed with C-3 labelled. Results of this type were found with leucine-[2-\textsuperscript{14}C] in \textit{P. blakesleeanus} (86).

Several conclusions are apparent from the results of this project. The whole plant is a dynamic biosynthetic system in which each compound participates in several pathways. Each pathway is under rigorous metabolic control which is disturbed by the introduction of exogenous substrates. The substrates in this study were intended to label the "non-mevalonoid" half of artemisia ketone. What has been demonstrated here is that these compounds are metabolized to "mevalonoid" compounds by established pathways. The route of incorporation for these substrates results in the same asymmetric labelling pattern as for mevalonoid substrates.
REFERENCES


3. Personal communication from National Seed Laboratory, Colorado State University.


Janell Broussard Simpson was born in New Iberia, Louisiana, where she lived with her parents, Richard and Joyce Broussard, until graduation from New Iberia Senior High School in 1971. At that time, she entered Louisiana State University and was awarded a Bachelor of Science in Biochemistry in 1974. Also in that year, she was married to Allan Simpson. Ms. Simpson is presently a candidate for the Doctorate of Philosophy in the Biochemistry Department.