THE

BIOGENESIS OF LYCOMARASMIN

AND ANTHERIDIOL

by

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ABSTRACT

Cultures of Fusarium oxysporum, f. lycopersici grown on a liquid medium with glucose as the principal carbon source produces, among other products, the phytotoxin, lycomarasmin. Acid hydrolysis of lycomarasmin results in the formation of aspartic acid, glycine, and pyruvic acid. Tracer studies showed that glycine-U-^{14}C, L-serine-U-^{14}C, DL-aspartic acid-4-^{14}C, DL-alanine-1-^{14}C, and glucose-U-^{14}C served as relatively efficient precursors of the lycomarasmin molecule. DL-Aspartic acid-4-^{14}C was incorporated into the 4-carbon fragment without label scrambling. Glycine was found to be the most efficient precursor of the 2-carbon fragment while phosphoenolpyruvate is the most probable direct route for the 3-carbon fragment.

Sexual reproduction in the aquatic fungus Achlya bisexualis is governed by antheridiol, a C_{29} sterol, secreted by the female plant and which initiates formation of antherdial hyphae on the male plant. The major sterols of this water mold were identified as fucosterol, 7-dehydro-fucosterol, 24-methylene cholesterol and cholesterol. Tracer studies showed that fucosterol supplied the necessary carbon skeleton for antheridiol. Labelling and trapping studies with a series of hypothetic sterol precursors have indicated the biological sequence involved in elaboration of the antheridiol side chain.

DEDICATED TO MY MOTHER
FOR ALL HER LOVE AND DEVOTION

ACKNOWLEDGEMENTS

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PART 1

LYCOMARASMIN

INTRODUCTION

Plant vascular wilt diseases are typically caused by two species of fungi, Fusarium and Verticillium, which enter the vascular system of the young root, either by penetration of the epidermis, or by direct ingress through wounds. Within the vessels the pathogen forms conidia or bud cells and by movement of these in the transpiration system, spreads upwards throughout the plant. By this means the pathogen becomes distributed throughout large plants at a rate which would be inconceivable by mycelial growth alone. The physiological activities of the pathogen within the vessels result in disordered host metabolism and development of the visible symptoms of disease. Only when the host reaches an advanced stage of disease, and the tissues surrounding the vessels become moribund, is there any substantial growth of the pathogen out from the vascular system.

There has been much controversy between those who consider that the symptoms of vascular diseases results from occlusion of the vessels by mycelium, or gums, or tyloses, or by the products of tissue breakdown resulting from the action of fungal enzymes[1], and those who believe that symptoms result from the action of toxins secreted by the fungus or produced by the host itself in response to infection[2].

In some diseases toxins are thought to cause a more rapid

and extensive invasion by the pathogen than would be the
case in the absence of a toxin; it has been suggested that
some parasites would be unsuccessful if the toxin did not
kill cells in advance of the fungus and permit it to establish
itself continually on dead or dying cells and produce more
toxin[3].

In other diseases toxins are thought to be translocated
from the centre of infection and produce conspicuous disease
symptoms which are thus of secondary origin, but of primary
consequence to the infected host. In either case a
mechanism for disease control is suggested; by rendering the
toxin inactive, one may cause the fungus to fail as a pathogen.

One of the most investigated organisms in this regard has
been Fusarium oxysporum lycopersici, causing wilting of
tomato. Three wilt inducing toxins have been isolated from
F. lycopersici in artifical cultures. These toxins bear no
chemical relationship with one another. Thus, lycomarasmin
(1), has an acyclic alkaloid type structure with a molecular
weight of 277[4]. Fusaric acid (2), the second toxin, is
5-n-butylpicolinic acid, a pyridine base alkaloid, with a
molecular weight of 179. It was first isolated by Yabuta
et al.[5] in 1934 from the culture filtrates of Fusarium
heterosporum.

$$HOOC-CH-NH-CH_2-CH-COOH$$

with vertical bonds to $HOOC-CH_2$ and $NH-CH_2CONH_2$

1

$$HOOC-\underset{N}{\bigcirc}-(CH_2)_3CH_3$$
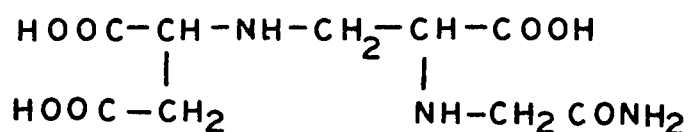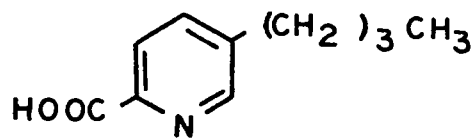
2

The third toxin, vasinfuscarin[6] has not yet been
completely purified and like fusaric acid is not specific to
F. lycopersici but is also produced by Gibberella fujikuroi.

In relation to every metabolic product of a pathogen
the question arises as to whether the pathogen produces it
solely in artificial culture, in which case it is an artifact,
or whether it is also synthesized in identical form inside
the host. Fusaric acid, or a substance very closely related
to it, has been detected in disease infected plants[7,8].
Lycomarasmin, however, has never been isolated from the
diseased plants. This is perhaps due to the fact that the
toxin, once secreted by the pathogen in the host, is
metabolized by the plant cells or converted into entirely
different substances. For this reason it might not be
possible to isolate lycomarasmin from the diseased plants
in its original form. Evidence of another kind is,
therefore, required to ascertain whether the metabolic
products of the pathogen in vivo bear any relationship with

the symptoms on the host.   This necessitated the need for a clear understanding of the mode of toxin production in vitro in order to correlate such studies with in vivo metabolism of the pathogen.   The objectives of the present work were, therefore, to determine the precursors of the lycomarasmin molecule, and to propose a mechanism for the biological formation of lycomarasmin from its precursors.

LITERATURE SURVEY

Several natural products with similar structural features to lycomarasmin have been characterised and their biosynthesis studied. Octopine (3), was isolated from the muscle of Octopus vulgaris by Morizawa in 1927[9], the structure of which was established by Moore and Wilson[10]. More recently, lysopine (4) was isolated in 1956 by Lioret[11] from the tissue of "crown gall", and the structure established in 1960 by Biemann[12]. Darling and Larsen[13] isolated and identified saccharopin (5) in 1961 from Neurospora crassa. This substance was synthesized by Kjaer and Larsen[14], and found to be an intermediate in the biosynthesis of lysine.

In 1956, the Société d' Etude and d' Applications Biologiques isolated three crystalline substances from the cultures of Aspergillus flavus oryzae, and proposed names for these substances, aspergillomarasmins A (6) and B (7) and anhydro-aspergillomarasmin B (8)[15,16].

The biosynthesis of octopine has been studied and found to involve a condensation of arginine and the keto-form of pyruvic acid followed by reduction in the presence of the enzyme octapine dehydrogenase (Fig. 1)[17]. The biosynthesis of saccharopin in yeast has been found to occur by a similar condensation between α-amino-adipic-δ-semialdehyde and glutamate, followed by reduction in the presence of saccharopine dehydrogenase (Fig. 2)[18].

$$H_2N-C-NH-CH_2-CH_2-CH_2-CH-COOH$$
$$\overset{\parallel}{NH} \qquad \overset{|}{NH}$$
$$CH_3-CH-COOH$$

3

$$H_2N-CH_2-CH_2-CH_2-CH_2-CH-COOH$$
$$\overset{|}{NH}$$
$$CH_3-CH-COOH$$

4

$$NH-CH_2-CH_2-CH_2-CH_2-CH-COOH$$
$$\overset{|}{CH}-CH_2-CH_2 \qquad \overset{|}{NH_2}$$
$$\overset{|}{COOH} \qquad \overset{|}{COOH}$$

5

$$CH_2 - CH - COOH$$

$$HOOC - CH - NH \quad NH$$

$$HOOC - CH_2 \quad R$$

6   R= -CH$_2$CH-COOH

                    NH$_2$

7   R= -CH$_2$-COOH

$$CH_2 - CH - COOH$$

$$HOOC - CH - N \qquad NH$$

$$HOOC - CH_2 \quad C - CH_2$$

$$\qquad\qquad\qquad O$$

8
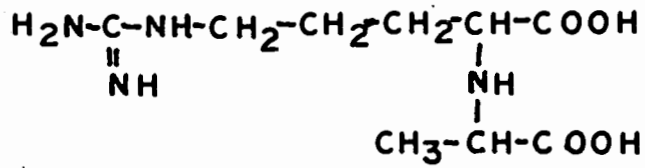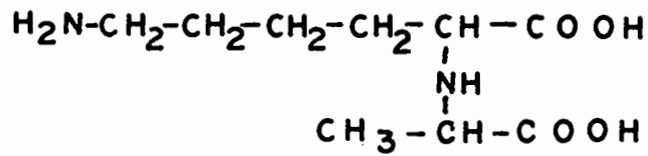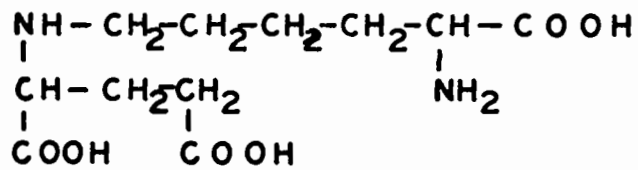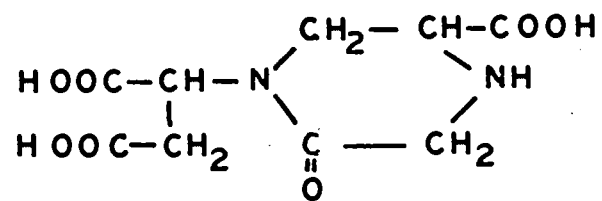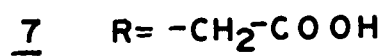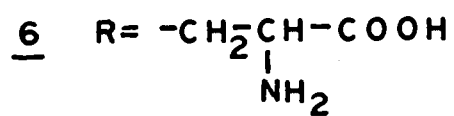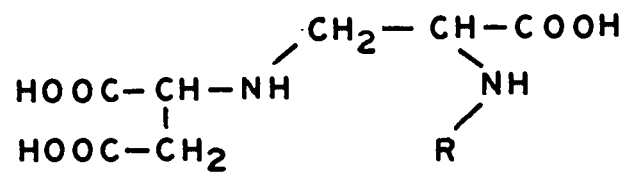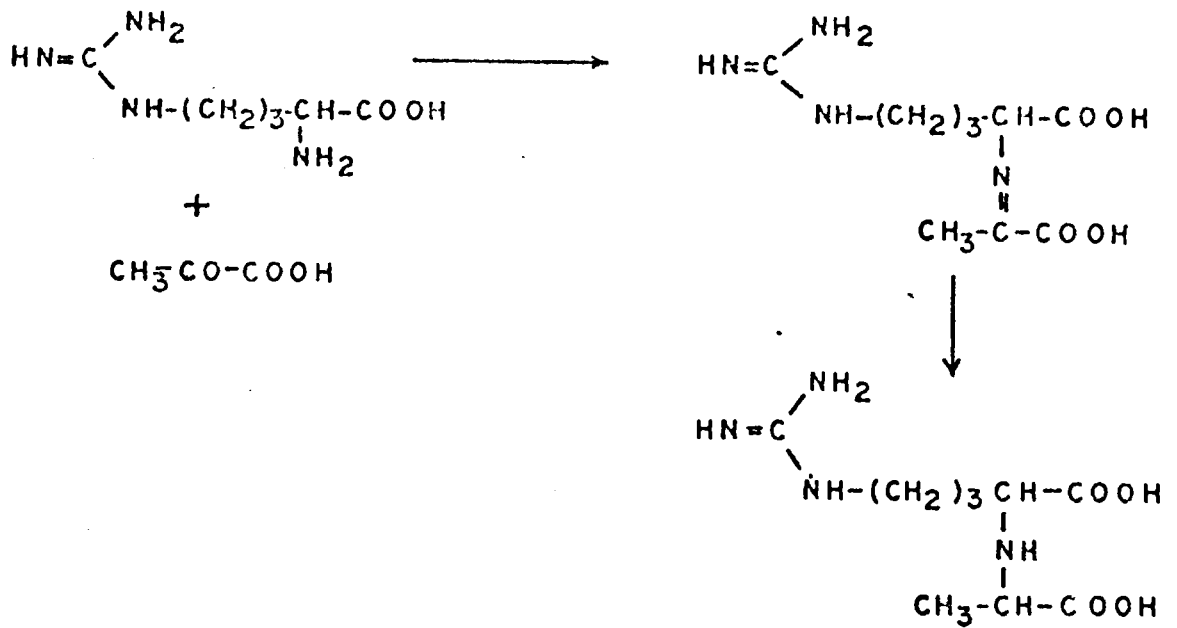
Figure 1. Biosynthesis of octapine
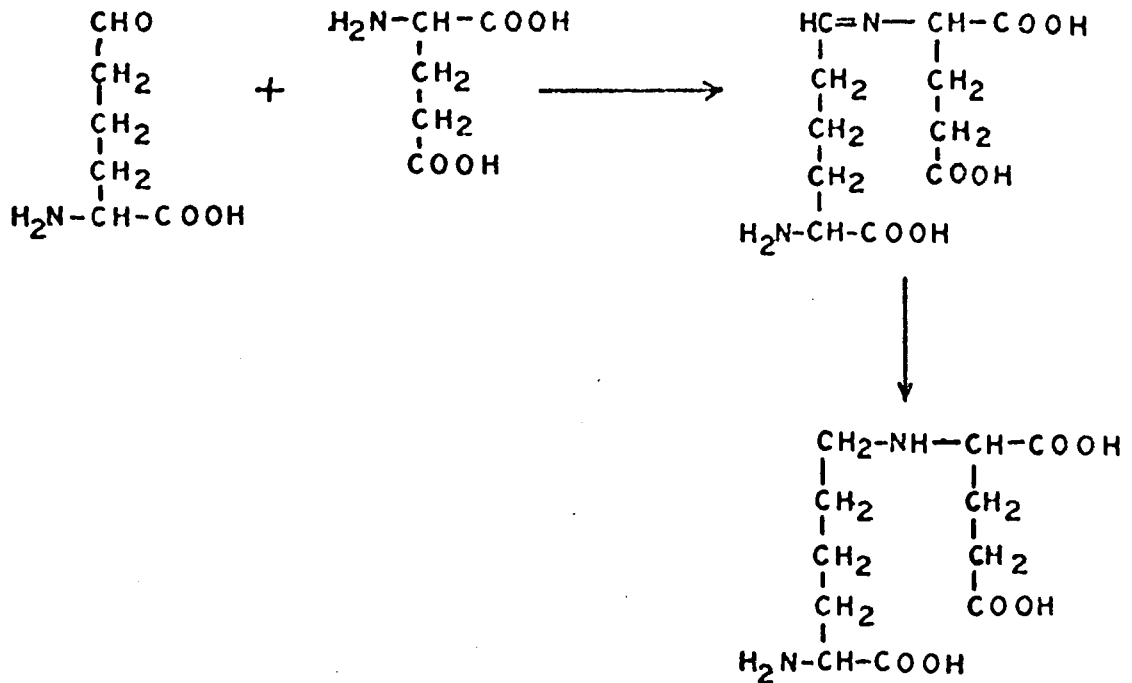
Figure 2. Biosynthesis of saccharopine

Lycomarasmin was first isolated by Plattner and Clauson-Kass[19] in 1944, who also determined a number of its properties, including its empirical formula and molecular weight. Woolley[20] later investigated the nature of lycomarasmin and proposed an incorrect structure. Final structural elucidation of lycomarasmin is attributed to the work of Hardegger et al.[4] in 1963 using partial synthesis and NMR studies. While considerable work has been done on the pathological and toxin actions, there is apparently no published literature on the biosynthetic origin apart from a communication by the author of this thesis[21].

The production of lycomarasmin in vitro is well established[4,19,22], and all workers agree that it can be isolated only from quiet cultures of F. lycopersici grown on Richard's medium which are at least 2 - 4 months old. Gaumann, however, reports that lycomarasmin can be demonstrated in the mycelium of F. lycopersici as early as the seventh day[23].

Since the lycomarasmin molecule contains only carbon, nitrogen, oxygen and hydrogen, the carbon and nitrogen metabolism of the pathogen concerned must play a decisive role in the biosynthesis of this compound.

Sanwall[24] has demonstrated that a quantitative change in the carbon and nitrogen ratio of the substrate medium causes a corresponding change in the quantity of toxins produced by F. lycopersici. Gottlieb[25] had earlier shown that various

amino acids could be used as the exclusive source of carbon in the medium for the growth of F. lycopersici. Experiments showed that with various amino acids as the carbon sources (eg. aspartic acid, glutamic acid) the fungus did not produce significant quantities of toxins even though growth took place. However, when 2.5% glycine was used as the sole carbon source, toxin production was quite comparable to that when glucose was the carbon source.

Analysis of the culture filtrates during the course of fermentation showed that maximum mycelial weight was attained within five weeks, and thereafter it decreased as lysis occurred. By the end of the eighth week, the lysis process was well under way. During the early growth period, the pH of the culture filtrate rose from an initial value of 4 to a value of 8 by the end of the fifth week. After glucose was depleted, polysaccharides increased slowly, rising as high as 1 mg/ml , a concentration on the threshold of toxicity[26]. The amino acids were produced in the medium from the beginning of the growth period, reached a peak at the end of 12 days and except for a decrease in production between the 12th and 21st day and again between the 33rd and 39th day, were always present at the peak level. Towards the end of the growth period, free amino acids rapidly increased in the medium. This is probably due to hydrolysis of cell proteins, once lysis has started.

Fluck and Richle[27] detected asparagine, aspartic acid, glutamic acid, serine, glycine and alanine in the culture filtrates of F. lycopersici regardless of incubation period. However, citrulline appeared in the 18th day of incubation, increased up to 24th day and then disappeared. Venkata Ram[28] also listed the occurrence of amino acids in the cultures of various species of Fusaria, although that of F. lycopersici is not available.

In a culture of F. lycopersici on Richard's nutrient solution containing nitrogen in the form of ammonium nitrate, analysis of the culture filtrate shows that the organism utilizes nitrate nitrogen in preference to ammonia nitrogen. In fact, ammonia increases steadily in the medium till the end of the growth period. In contrast, nitrates were rapidly taken up. Some of the excess ammonia may accumulate in the medium due to deamination of amino acids[24].

Lycomarasmin made its first appearance in the culture filtrate after lysis was clearly evident in the mycelial mat (Fig. 3). It is probable that lysis is a process which begins rather early during the growth of the mycelium, but which becomes evident in terms of mat weight only after mycelial growth ceases. If so, lysis had been under way for a considerable period before lycomarasmin first appeared in the culture filtrates. Other toxic products of fungus metabolism are known to be produced under similar conditions. Thus, streptomycin does not appear in the culture medium until

Streptomyces griseus approaches its maximum growth. The
peak of streptomycin production occurs considerably after the
peak of mycelial weight[29].
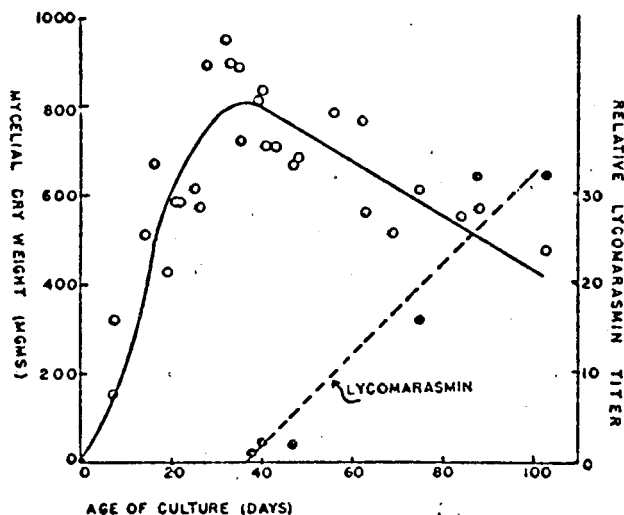
Figure 3.   Mycelial growth and lycomarasmin production of
            F. lycopersici. (Dimond & Waggoner[26])

Pertinent to the apparent rate at which lycomarasmin
appears in culture filtrates is the inherent instability of
the toxin.   On heating, lycomarasmin splits off NH_3 to form
biologically inactive anhydro-aspergillomarasmin B, or
anhydro-lycomarasmic acid [substance "J", (8)][4].

The heat of activation and the rate at which lycomarasmin
becomes inactive at 29°C have been studied[30].   Its half-life
has been estimated at 4.8 days in aqueous solution at pH 3.6.
However, at the hydrogen ion concentration of the fluid in
vessel elements in the tomato plant, pH 6.0, lycomarasmin,
while it decomposes at a measurable rate, is considerably more
stable.   As lycomarasmin decomposes to substance "J" it loses

both iron-chelating ability and wilt inducing ability. Lycomarasmin chelates other metals but these are not toxic to plants. These facts suggest that lycomarasmin is active on plant cells as the iron-lycomarasmin chelate complex.

HYPOTHESES OF LYCOMARASMIN BIOGENESIS

The structure of lycomarasmin indicates that at least
some parts of the molecule should be derived from simple
amino acids or their deaminated keto analogs. Sets of
different amino acids may be postulated as possible precursors
depending on how the molecule is subdivided into its apparent
structural units. Such a division of the toxin finds its
basis in the fact that acid hydrolysis of lycomarasmin gives
rise to L-aspartic acid, pyruvic acid, glycine and ammonia.
The mechanism of this hydrolysis commences with protonation
followed by β-elimination resulting in the formation of
aspartic acid and an enamine. The enamine isomerises to a
Schiff's base which on hydrolysis forms glycine and pyruvic
acid (Scheme 1)[31]. Aqueous hydrolysis on the other hand,
generates glycine, L-aspartic acid, DL-alanine and ammonia.
DL-alanine is derived by amination of pyruvate.

The four carbon fragment of the lycomarasmin molecule
could conceivably be derived from aspartate, oxaloacetate or
fumarate. A quantitative estimate of the involvement as
precursors of one or more of these compounds can be obtained
by a feeding experiment with aspartic acid-4-^{14}C, followed
by degradation of lycomarasmin by acid hydrolysis into its
separate structural units and determination of their specific
activities. Experiments designed to distinguish between these
several possibilities are based on the following biochemical
inter-relationships:

$$\text{HOOC-CH-NH-CH}_2\text{-CH-COOH}$$
$$\text{HOOC-CH}_2 \qquad \text{NH-CH}_2\text{CONH}_2$$

$$\downarrow$$

$$\text{HOOC-CH-}\overset{+}{\text{N}}\text{-CH}_2\text{-CH-COOH}$$
$$\text{HOOC-CH}_2 \overset{H\ H}{}\qquad \text{NH-CH}_2\text{CONH}_2$$

$$\downarrow$$

$$\text{HOOC-CH-NH}_2 \ + \ \text{CH}_2\text{=C-COOH}$$
$$\text{HOOC-CH}_2 \qquad\qquad \text{NH-CH}_2\text{CONH}_2$$

$$\downarrow\ \text{+NH}_3$$

$$\text{CH}_3\text{-C-COOH}$$
$$\overset{\|}{\text{N-CH}_2\text{COOH}}$$

$$\downarrow$$

$$\text{CH}_3\text{-C-COOH}$$
$$\overset{\|}{\text{O}}$$
$$+$$
$$\text{NH}_2\text{CH}_2\text{COOH}$$
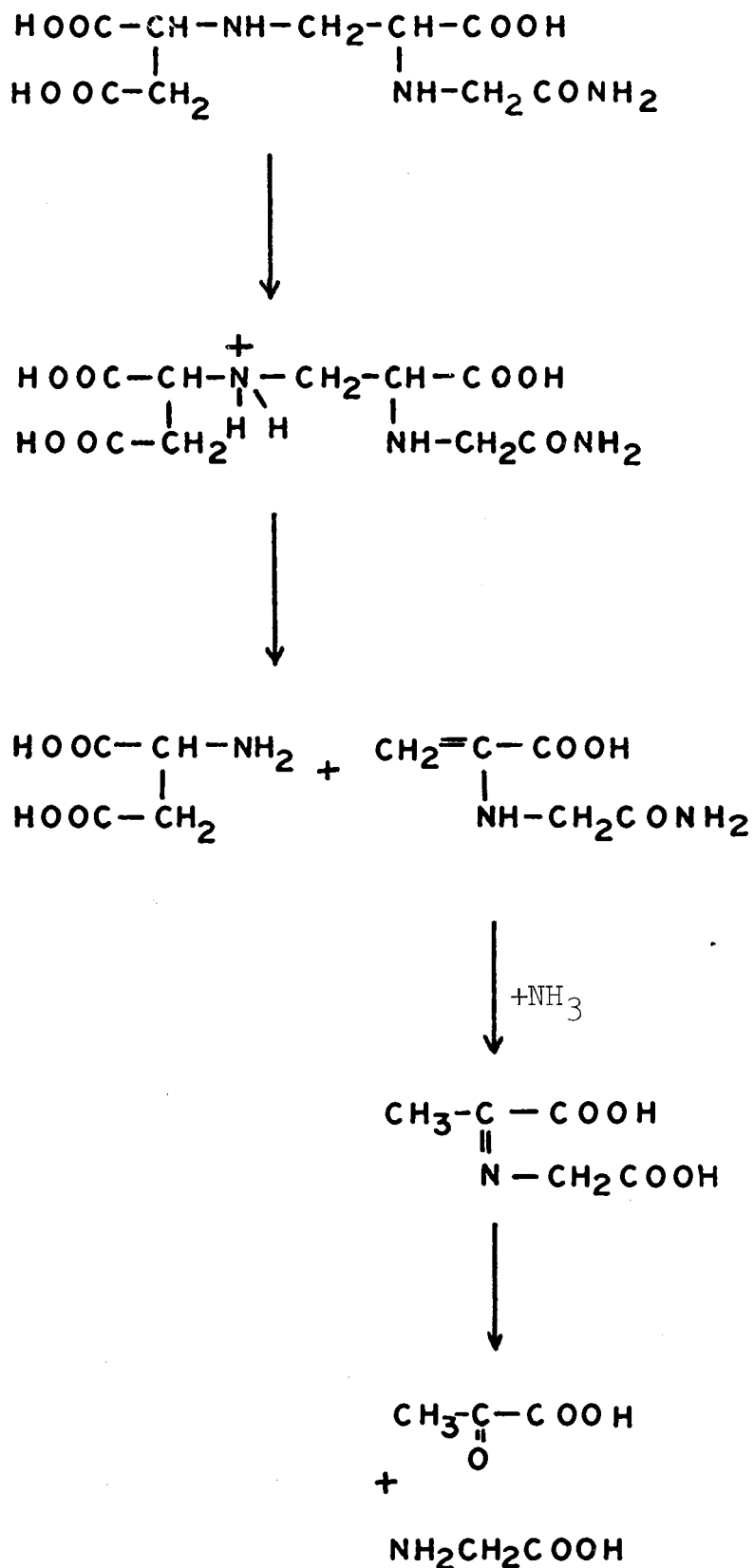
Scheme 1. Acid hydrolysis of lycomarasmin.

```
              COOH                      COOH
              |                         |
              CH-NH₂                    C=O
aspartate     |          ⇌              |          oxaloacetate
              CH₂                       CH₂
              |                         |
              COOH                      COOH



              ⇊                         ⇈



              COOH                      COOH
              |                         |
              CH                        CH-OH
fumarate      ‖          ⇌              |          malate
              CH                        CH₂
              |                         |
              COOH                      COOH
```
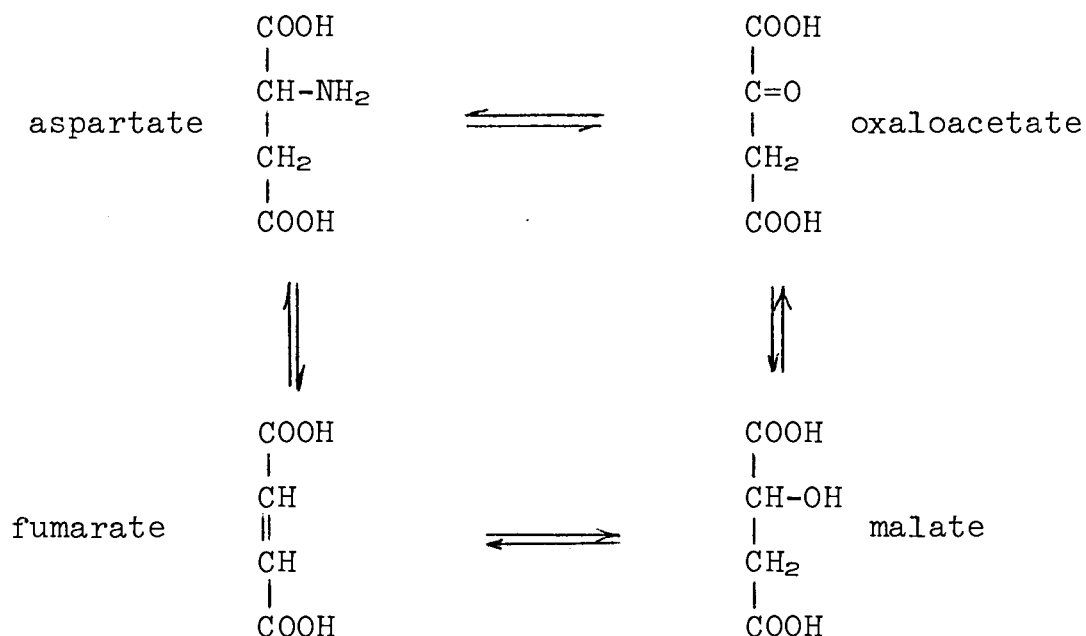
The feeding of aspartate-4-^{14}C to the organism and
determination of the position of label in the four carbon
fragment of lycomarasmin would distinguish between the
involvement of fumarate or oxaloacetate and aspartate.
If the four carbon unit of aspartate-4-^{14}C is incorporated as
fumarate then the label will be equally distributed between
C_1 and C_4 because of the symmetry of the fumarate molecule.
If, on the other hand, aspartate is incorporated as such
or as oxaloacetate, then the majority of label will be
retained at C_4.

To distinguish between the possibility of either
oxaloacetate or aspartate being incorporated, the use of
aspartate-2-^{3}H is necessary. If aspartate is incorporated
as such then it is reasonable to assume that tritium at C_2
would be retained whereas it would be lost if oxaloacetate

is involved.

There also exists several possibilities for the biogenetic origin of the three carbon fragment of lycomarasmin. Consideration of its origin indicates the possible involvement of a 3-carbon amino acid which contains a functional group at C_3, i.e. serine or cysteine, thus providing the necessary functionality for condensation with the four-carbon fragment. Another possibility is that the three carbon fragment is derived from a 3-carbon intermediate arising from the catabolism of glucose, examples being pyruvic acid, phosphoenolpyruvate, or 3-phosphohydroxypyruvate. Each of these suggested precursors are biochemically closely related to one another as described in Fig. 3, and a definite conclusion as to the precise contribution of each possible precursor to the three carbon fragment will be difficult to obtain.

Labelling experiments with ^{14}C-labelled glucose, alanine and serine will however indicate specificity of conversion of these precursors into lycomarasmin and may also furnish information as to the nature of the most immediate precursor.

Information concerning the mechanism of condensation of the four-carbon with the three carbon fragment may possibly be obtained from a dual labelling experiment. The C_3 of each of the possible precursors is derived from C_1 and C_6 of glucose. During glycolysis the glucose molecule is cleaved to glyceraldehyde-3-phosphate and dihydroxyacetone

GLUCOSE

⇅

⇅

COO~P
|
CHOH
|
CH₂O~P

1,3-diphosphoglycerate

⇅

COOH   COOH   COOH
| | |
CHOH ⇌ C=O ⇌ CH–NH₂
| | |
CH₂O~P   CH₂O~P   CH₂OH

3-phospho-   3-phosphohydroxy   serine
glycerate   pyruvate

⇅

COOH
|
CHO~P
|
CH₂OH

2-phosphoglycerate

↓

COOH
|
C-O~P
‖
CH₂

phosphoenolpyruvate

⟶ PYRUVATE

$(P = PO_3^{2-})$
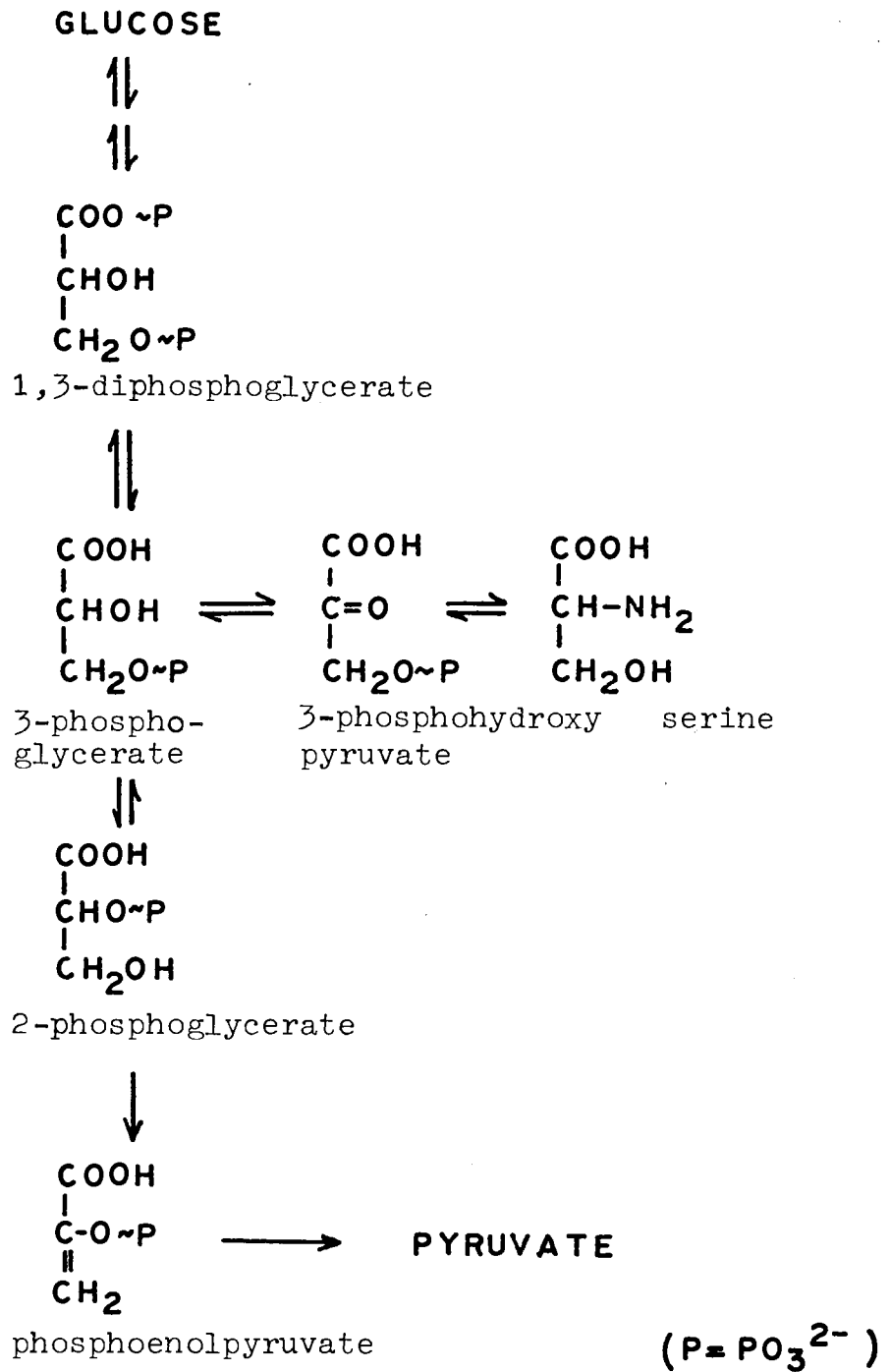
Figure 3. Catabolism of glucose.

phosphate:

$$
\begin{array}{ll}
1 & CH_2OPO_3{}^{2-} \\
2 & C=O \\
3 & HOCH \\
4 & CHOH \\
5 & CHOH \\
6 & CH_2OPO_3{}^{2-}
\end{array}
\rightleftharpoons
\begin{array}{ll}
1 & CH_2OPO_3{}^{2-} \\
2 & C=O \\
3 & CH_2OH \\
4 & CHO \\
5 & CHOH \\
6 & CH_2OPO_3{}^{2-}
\end{array}
$$
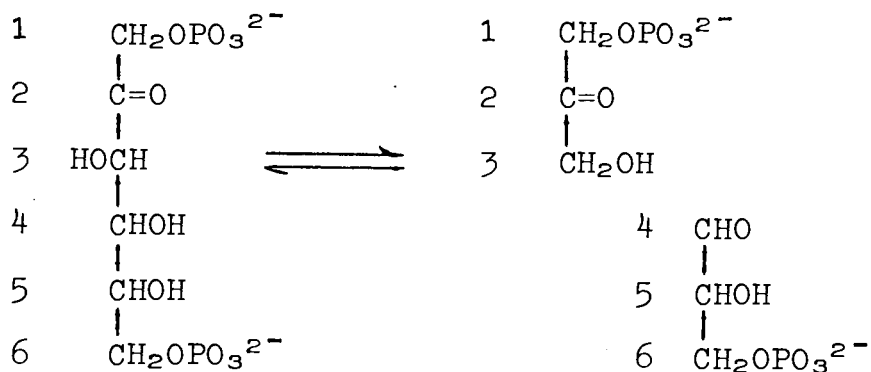
Only one of these compounds, namely glyceraldehyde-3-phosphate, can be directly degraded in the further reactions of glycolysis. The other, dihydroxyacetone phosphate, is reversibly converted to glyceraldehyde-3-phosphate:

$$
\begin{array}{lll}
1 & CH_2OPO_3{}^{2-} & 6 \\
2 & C=O & 5 \\
3 & CH_2OH & 4
\end{array}
\rightleftharpoons
\begin{array}{lll}
4 & CHO & 3 \\
5 & CHOH & 2 \\
6 & CH_2OPO_3{}^{2-} & 1
\end{array}
$$
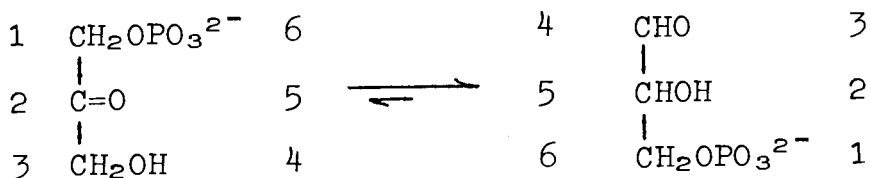
By this reaction carbon atoms 1, 2 and 3 of the starting glucose now have become indistinguishable from carbon atoms 6, 5 and 4 respectively. Thus, the glucose molecule is cleaved into two identical 3-carbon fragments.

Four possible mechanisms for the condensation of the four-carbon and three carbon fragments involve C_3 of the three carbon fragment:

(1)   R'NH$_2$ CH$_2$-CH-COOH $\xrightarrow{SN2}$ R'NHCH$_2$-CH-COOH

                 (P)O   R   R

(2)   (P)O-CH$_2$-$\overset{H}{\underset{R}{C}}$-COOH $\xrightarrow{-Pi}$ CH$_2$=C-COOH

                                 R'NH$_2$ R

(3)   HO-CH$_2$-C-COOH $\underset{+H\cdot}{\overset{+RNH_2}{\underset{-H_2O}{\xrightarrow{\hspace{1cm}}}}}$ HO-CH$_2$-CH-COOH $\xrightarrow{-H\cdot}$ OHC-CH-COOH

                   O   NHR   NHR

                                                          $\underset{+H\cdot}{\overset{+R'NH_2}{\underset{-H_2O}{\downarrow}}}$

                                 R'NHCH$_2$-CH-COOH

                                         NHR

                                         $\uparrow$ +H·

(4)   CH$_2$=C-COOH $\underset{+H_2O}{\overset{-Pi}{\underset{-2H\cdot}{\xrightarrow{\hspace{1cm}}}}}$ R'NHCH$_2$-C-COOH $\underset{-H_2O}{\overset{+RNH_2}{\xrightarrow{\hspace{1cm}}}}$ R'NHCH$_2$-C-COOH

   R'NH$_2$ O-(P)   O   NR
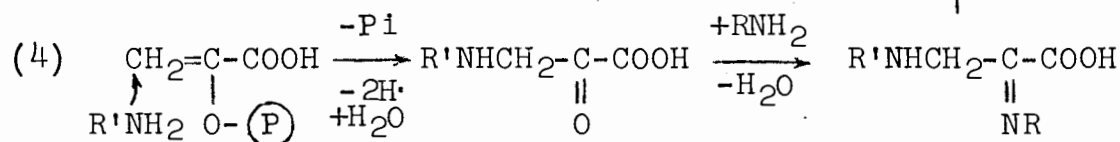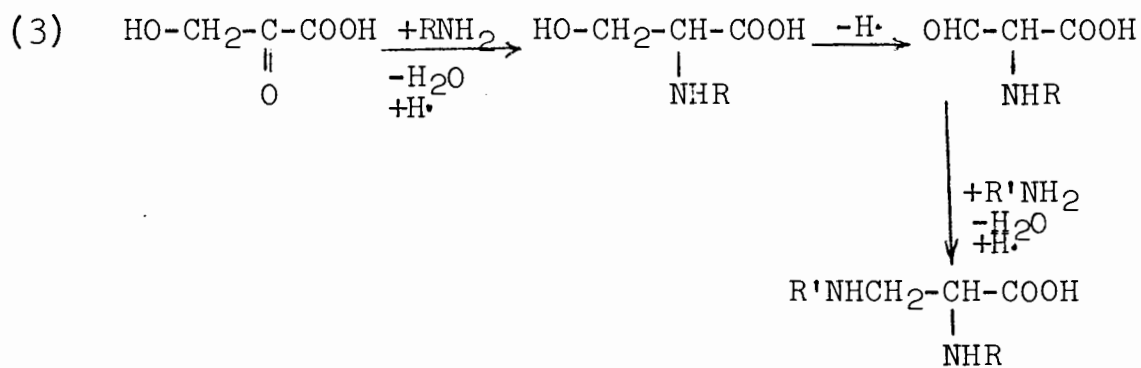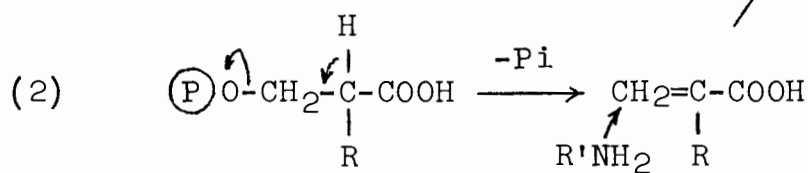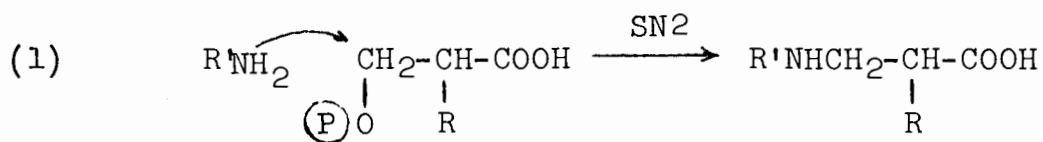
Scheme (1) involves a nucleophilic (SN2 type) attack of the amino group at C_3 in which phosphate would be a good leaving group. Scheme (2) would involve a similar attack on the α,β-unsaturated acrylyl system also possessing an α-amino (enamine form) substituent (glycine). Scheme (4) involves addition of amine followed by hydrolysis of phosphate with subsequent oxidation of the hydroxyl group to a ketone function. Scheme (3) requires oxidation to occur first at C_3 followed by condensation and reduction. A dual labelling experiment with tritium at C_3 (C_6 in the original glucose molecule) could, therefore, possibly distinguish between Schemes (1),(2), and (4), and Scheme (3). The success of such an experiment would of course largely depend on relatively rapid incorporation of precursor without prior biological or chemical exchange.

Based on the assumption that the precursors of lycomarasmin can be found among the amino acids, then the two-carbon fragment is most probably derived from glycine or glyoxylate. Feeding experiments with uniformly and specifically labelled glycine will, therefore, give an indication of the participation of glycine as a precursor of not only the 2-carbon fragment but also the 3-fragment in the event that the conversion of glycine → serine → 3-carbon precursor and/or glycine → serine → hydroxypyruvate → 3-carbon precursor are facile.

RESULTS AND DISCUSSION

Although the amino acid content of F. lycopersici has
been fairly well studied[27,28] there is apparently little
information as to the relative amounts of amino acids produced
by the fungus. Since this study was to involve administration
of ^{14}C-amino acids to cultures it was deemed necessary to
determine the variation in amino acid content of the culture
medium over the period of time during which labelling studies
were to be carried out.

This preliminary study therefore, involved the analysis
of filtrates of a series of cultures which had been growing
from 7 to 10 weeks. Each culture was worked-up in a manner
similar to that described for the isolation of lycomarasmin[4],
with the exception that the aqueous methanol solution was
retained and analysed using an amino acid analyser. The
results (Table 1) have been calculated as percentages of the
total amino acids present in each culture and show that the
percentage of glutamate steadily increased with a corresponding
decrease in alanine during the 7-9 weeks growth period. Table 1
indicates only the amino acids detected in the culture filtrate.

TABLE 1

Relative amounts of amino acids in culture filtrates
of F. oxysporum lycopersici.

Amino Acid	Age of culture (weeks)			
	7	8	9	10
Aspartic Acid	3.2	<2	<2	8.4
Threonine	6.4	5.4	7.7	8.6
Asparagine	2.0	1.7	2.9	<2
Glutamic Acid	17.7	39.5	50	38.7
Serine	3.8	2.7	4.7	16.0
Glycine	7.3	15.3	8.8	14.0
Alanine	59.5	28.6	16.7	14.4
Proline	<2	<2	<2	<2
Valine	"	"	9.6	"
Methionine	"	"	<2	"
Isoleucine	"	6.9	"	"
Leucine	"	<2	"	"
Tyrosine	"	"	"	"
Phenylalanine	"	"	"	"

Figures are expressed as percentages of total amino
acids present.

As a first approach toward determining the origin of the biogenetic lycomarasmin molecule, cultures of F. lycopersici were inoculated with ^{14}C-labelled amino acids and the specific activity of the toxin determined. This was followed by hydrolytic cleavage of the alkaloid and comparison of specific activities of the hydrolysis products.

As a regular procedure, the still cultures were maintained at 25° in the dark for 7 weeks after which 50μCi of ^{14}C-labelled amino acid were distributed between six cultures. Two series of experiments were performed in which the cultures were harvested after 5 and 24 hour contact with the labelled compounds. Crystalline lycomarasmin was isolated using a published isolation procedure[4]. The specific activities of the hydrolysis products of labelled toxin are shown in Tables 2, 3, and 4.

The results in Table 2 strongly indicate that the administered ^{14}C-labelled amino acids can serve as relatively efficient precursors. The label distribution observed in Tables 3 and 4 can be explained in terms of known enzymatic interconversions of the administered labelled amino acids, and will be considered in some detail in the succeeding portions concerned with the incorporation of specific precursors.

TABLE 2

Specific Activity of Lycomarasmin

Precursor		cpm./mM$\times 10^{-4}$
	5 hr	24 hr
Glycine-U-^{14}C	0.22	3.34
L-Serine-U-^{14}C	0.64	3.25
DL-Aspartic Acid-4-^{14}C	0.17	1.67
DL-Alanine-1-^{14}C	0.10	2.01

TABLE 3

Specific Activity of Hydrolysis Products

of 5 hr. ^{14}C-Labelled Lycomarasmin

Precursor	Hydrolysis products cpm/mM×10^{-3}		
	Glycine	Aspartic Acid	Pyruvate (as 2,4-DNPH)
Glycine-U-^{14}C	0.54	0.31	1.52
L-Serine-U-^{14}C	2.20	0.74	3.78
DL-Aspartic Acid-4-^{14}C	0.1	0.61	1.05
DL-Alanine-1-^{14}C	0.1	0.10	1.12

TABLE 4

Specific Activity of Hydrolysis Products

of 24 hr. ^{14}C-Labelled Lycomarasmin

Precursor	Hydrolysis products cpm/mM$\times 10^{-4}$		
	Glycine	Aspartic Acid	Pyruvate (as 2,4-DNPH)
Glycine-U-^{14}C	1.04	0.37	1.54
L-Serine-U-^{14}C	0.97	0.44	1.44
DL-Aspartic Acid-4-^{14}C	0.07	1.12	0.41
DL-Alanine-1-^{14}C	0.05	0.99	0.97

Glycine-U-C^{14}, Glycine-2-^{14}C, and Serine-U-^{14}C

Labelling Experiments.

These two amino acids are considered together because
of their close metabolic relationship.

Tables 2 and 4 show that glycine and serine-U-^{14}C
were incorporated into the three fragments of the lycomarasmin
molecule to approximately the same extent after a 24 hour
labelling period. This label distribution might be expected
on the basis of the glycine-serine interconversion via the
biological aldol-retro-aldol reaction. It seems apparent
that glycine may be incorporated as such or may be deaminated
and the generated glyoxylate in turn condense with the
3-carbon fragment. A simplified sequence might be envisioned
as follows:

(a)

$$R-\overset{\overset{\textstyle O}{\|}}{C}-R \xrightarrow{\text{glycine}} $$

$$R-CH-R$$
$$\quad\;\; |$$
$$\quad NHCH_2COOH$$

(b)

$$R-CH-R \xrightarrow{\text{glyoxylate}}$$
$$\quad\;\; |$$
$$\quad NH_2$$

C_3-C_2 fragment
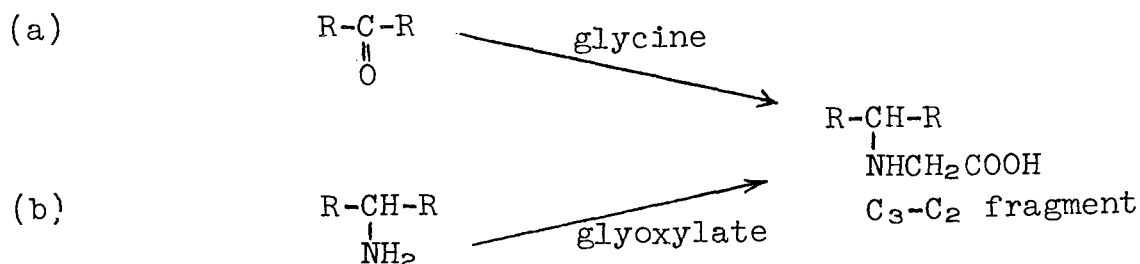
Incorporation of glycine and serine-U-^{14}C into the 4-
carbon fragment (aspartic acid) can occur by two alternate
pathways (Fig. 4). The first, involves conversion of
glycine to serine followed by either transamination to
3-hydroxypyruvate or deamination and dehydration to pyruvate.

CH_2COOH
|
NH_2
glycine

$CH_2 CH COOH$
| |
OH NH_2
serine

$CHO COOH$
glyoxylate

acetylCoA

CH_2 $C-COOH$
| ||
OH O
hydroxypyruvate

$CH_3 C-COOH$
      ||
      O
pyruvate

$COOH$
|
$CHOH$
|
CH_2
|
$COOH$
malate

$COOH$
|
$C=O$
|
CH_2
|
$COOH$
oxaloacetate
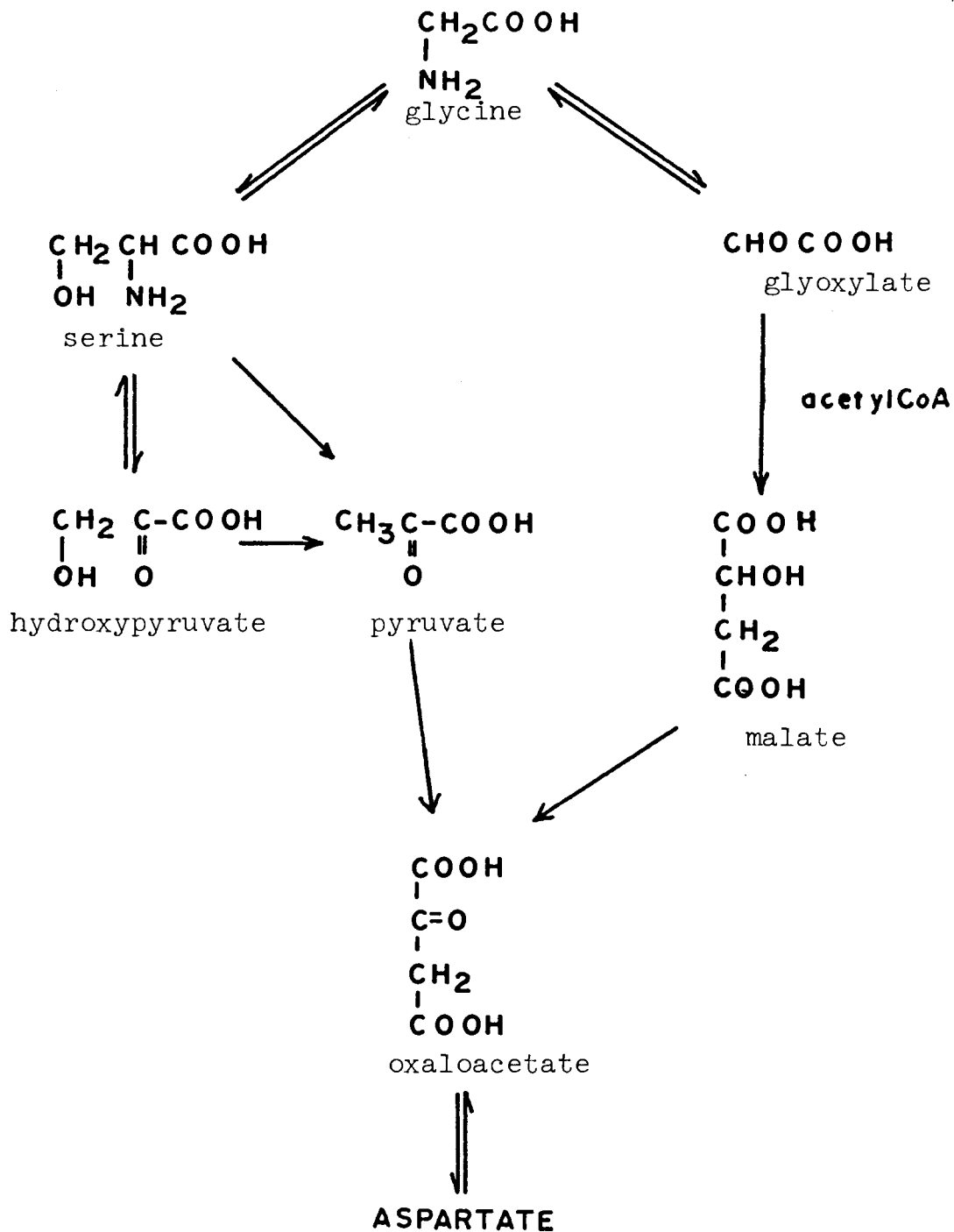
$ASPARTATE$

Figure 4. Incorporation of glycine into aspartate

The pyruvate so formed can readily undergo carboxylation by pyruvate carboxylase to form oxaloacetate from which aspartate is derived by transamination. The second pathway involves transamination of glycine to glyoxylate which then condenses with acetyl-CoA to form malate. Oxidation of malate via the TCA cycle produces oxaloacetate which is then transaminated as before.

A labelling experiment with specifically labelled glycine-2-^{14}C was designed to establish the origin of the 2C-fragment. Six cultures, 10 weeks old, were labelled for 48 hours with 100μC glycine-2-^{14}C from which the isolated lycomarasmin had a specific activity of 1.67 x 10^5 cpm/mM and the specific activities of the hydrolysis products are shown in Table 5.

The glycine was degraded by oxidation with ninhydrin and carbon 1, counted as CO_2, was found to contain 28% of the label, and carbon 2, counted as the dimedone derivative of formaldehyde, contained 72% of the label. This distribution of label can be rationalized on the basis of randomization of label in the TCA cycle and regeneration of glycine by transamination of glyoxylate from the glyoxylate cycle.

Despite this randomization of label, glycine-2-^{14}C was incorporated with relatively high efficiency into the 2C-fragment. Comparison of the ratios of the specific activities of the 2C and 3C-fragments in the 24 hr glycine-U-^{14}C

TABLE 5

Specific Activities of Hydrolysis Products of

Lycomarasmin from Cultures Inoculated with Glycine-2-[14]C

	cpm/mM$\times 10^{-4}$
Glycine	7.75
Aspartic Acid	1.83
Pyruvic Acid	4.04

experiment $(2:3)$ (Table 4) to that in the glycine-2-^{14}C experiment $(2:1)$ (Table 5), show that glycine-2-^{14}C is less efficiently incorporated into the 3C-fragment in the latter experiment. However, this may be primarily attributed to the difference in age of the cultures when the experiments were performed. Cultures 10 weeks old, used for the glycine-2-^{14}C experiment, contain a different pool size of amino acids than cultures 7 weeks old which were used for the first experiment (Table 1).

In view of the results of the labelling experiments with uniformly labelled glycine and serine and also with specifically labelled glycine, it is reasonable to propose that glycine is the precursor to the 2C-fragment. These experiments do not however, yield unequivocal information as to whether glycine participates as such or is first deaminated to glyoxylate before condensation with the 3C-fragment. The fact that the administered glycine-2-^{14}C is utilized in which 72% of the incorporated label is in C-2 indicates that the amino acid is primarily incorporated without extensive label randomization (into C-1). Kinetic considerations indicate that glycine as such condenses with a suitable substrate (e.g. keto compound).

Aspartic Acid-4-^{14}C Labelling Experiment.

The experimental results (Table 2 and 4) indicate
that aspartate can serve as the four-carbon source and may
also contribute to the 3C- fragment. The reason for the
relatively low specific activity of the lycomarasmin from
the aspartic acid-4-^{14}C labelling experiment may be
attributed to its generally accepted high biological lability.
It is significant to note that almost 70% of the label which
is incorporated into lycomarasmin enters the 4C-fragment and
25% into the 3C-fragment while very little activity ($\sim$5%)
enters the 2C-fragment. The observed incorporation of label
into the 3C-fragment could readily occur via fumarate
($C_4 \equiv C_1$) → malate → oxaloacetate → phosphoenolpyruvate (PEP)
→ 3-hydroxypyruvate → serine.

The low distribution (5%) of label into the 2C-fragment
(glycine) compared with 25% label distribution into the
3C-fragment (pyruvic acid) when labelled aspartic acid-4-^{14}C
is administered is of considerable interest. This
observation suggests that conversion of aspartate into a
suitable 3C-source does not proceed past 3-hydroxypyruvate
since conversion of this compound into serine should then lead
to incorporation of label into C-1 of glycine. The
transformation of glycine ⇌ serine was observed as would be
expected, when labelled glycine and serine were administered.

Although 3-phosphohydroxypyruvate is an attractive biochemical intermediate for the subsequent condensation with a suitable 4-carbon and 2-carbon precursor, PEP is equally attractive, if not more so as a direct 3C-source. Facile biogenetic condensations may be written involving PEP, or 3-phosphohydroxypyruvate:

(a)

$$\underset{\substack{R-NH-X \\ (4C \; precursor)}}{\text{(P)}O-CH_2-\underset{O}{\overset{\parallel}{C}}-COOH} \quad \underset{\substack{R'-NH-X \\ (2C \; precursor)}}{} \xrightarrow[\substack{-H_2O \\ +2H\cdot \\ -Pi}]{} \quad \underset{\substack{NH-R' \\ lycomarasmin}}{R-NH-CH_2-CHCOOH}$$

(b)

$$\underset{NH-R}{\text{(P)}O-CH_2-CH-COOH} \xrightarrow[\substack{+H_2O \\ -Pi \\ -2H\cdot}]{} \underset{\substack{H \quad NH-R \\ R-NH-X}}{\overset{O}{\underset{}{\parallel}}C-CH-COOH} \xrightarrow{+2H\cdot} lycomarasmin$$

(c)

$$\underset{\substack{R-NH-X}}{CH_2=C-COOH} \xrightarrow[\substack{+H_2O \\ -Pi \\ -2H}]{} \underset{\substack{NH \quad O \\ R \qquad R'-NH-X}}{CH_2-\overset{\parallel}{C}-COOH} \xrightarrow{} lycomarasmin$$
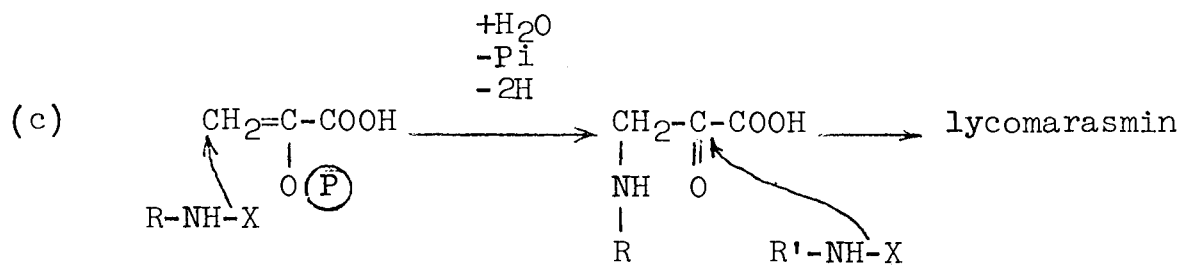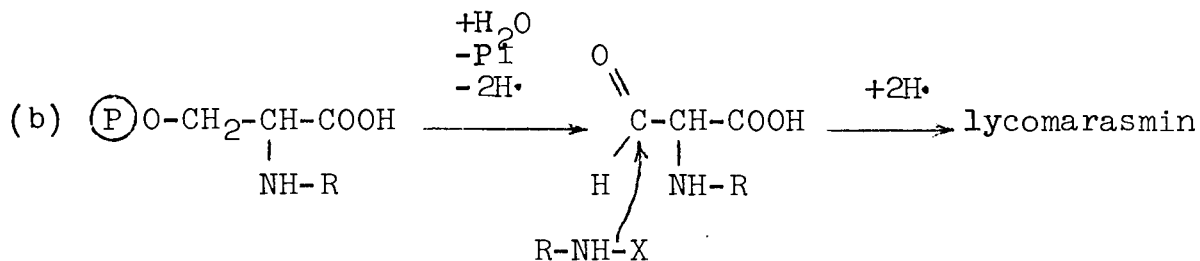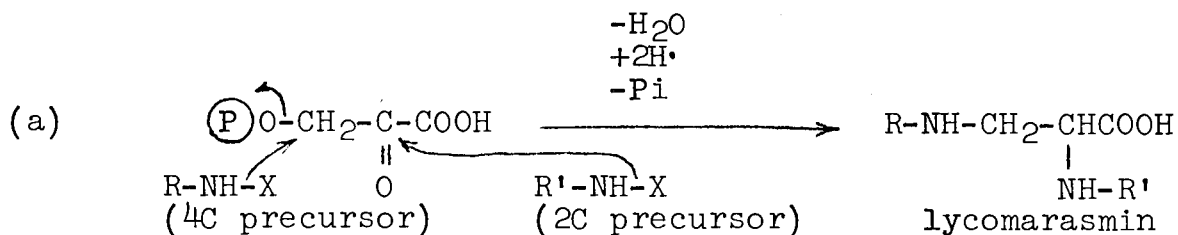
X=cofactor

Alanine-1-^{14}C Labelling Experiment.

Both the D- and L- isomers of alanine can be used as the sole carbon source for several species of Fusaria, and pyruvic acid has been shown to function as the central compound in the metabolism of this mold[31]. Incorporation of label from alanine-1-^{14}C into aspartate (the four-carbon fragment) could occur via pyruvate → oxaloacetate → fumarate. Oxaloacetate thus derived by carboxylation may give rise to phosphoenolpyruvate which can eventually give rise to 3-hydroxypyruvate via a similar pathway as indicated for aspartic acid in the previous section, and so enter the 3C-fragment (Fig. 5).

The low distribution (25%) of alanine-1-^{14}C into the 2-carbon fragment is of the same magnitude as that observed for aspartic acid-4-^{14}C. Based on the assumption that the label from both these compounds enters the 3-carbon fragment by a similar pathway, then the results from both labelling experiments with alanine and aspartate agree with the previous suggestion that conversion of these compounds into a 3-carbon source does not proceed past 3-hydroxypyruvate. If 3-hydroxypyruvate does serve as such as the 3-C precursor, this would imply, based on observed results with ^{14}C-serine, that the amination step leading from 3-hydroxypyruvate to serine is very slow in this organism while deamination to give the hydroxyketo acid proceeds with reasonable facility.
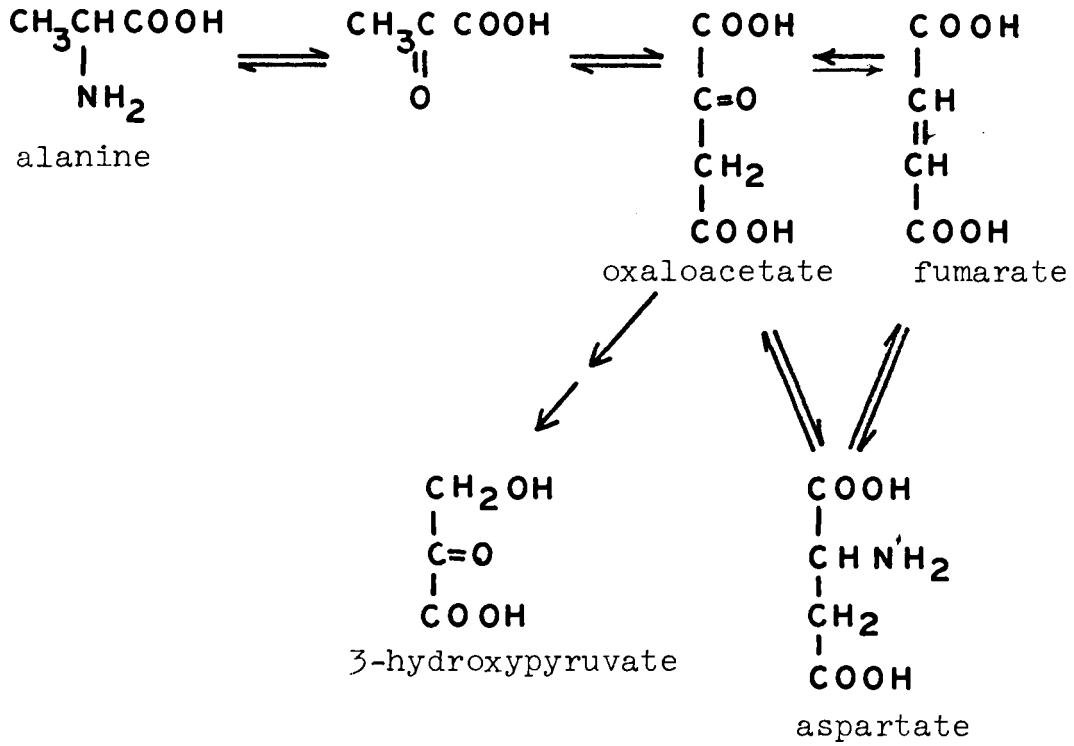
Figure 5. Incorporation of alanine into aspartate

If one involves PEP, then of course this pathway involving
3-hydroxypyruvate need not be operative to any significant
extent.

Examination of the catabolism of alanine-1-^{14}C via the
glyoxylate cycle by the pathway:

alanine → pyruvate $\xrightarrow{CO_2}$ acetyl-CoA $\xrightarrow{+\text{oxaloacetate}}$ isocitrate →
glyoxylate + succinate
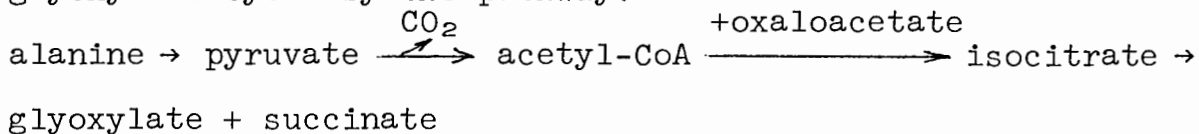
shows that loss of label results by the decarboxylation of
pyruvate. However, in the case of aspartic acid, the
label from aspartic acid-4-^{14}C would be expected to be
incorporated into the 2-carbon fragment via the glyoxylate
pathway.


```
   COOH              COOH              CH2COOH           CH2COOH
    |                 |                 |                 |
  CHNH2             C=O              HO-C-COOH          CH-COOH
    |                 |                 |                 |
   CH2               CH2               CH2              CHOH
  * |               * |               * |              * |
   COOH              COOH              COOH             COOH

 aspartate         oxaloacetate       citrate           |
                                                         ↓
                                                       CH2COOH
                                                         |
                                                       CH2COOH
                                                         +
                        *                              CHO
                      CH2COOH          ←              * |
                        |                              COOH
                       NH2
                     glycine                         glyoxylate
```
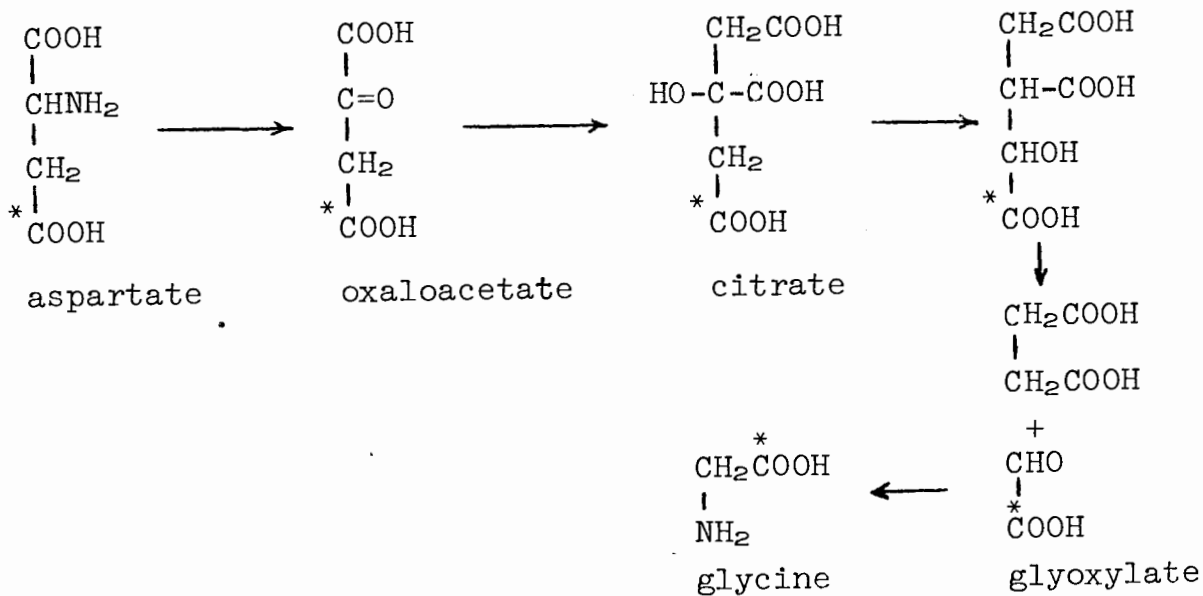
The result from the aspartic acid-^{14}C labelling
experiment, in which very low incorporation of label was
obtained in the two carbon fragment, therefore, implies
that the glyoxylate cycle is not operative to any appreciable
extent over a 24 hour period.

Degradation of Aspartic Acid.

In order to differentiate between the two possibilities
of the 4-carbon fragment being derived directly from either
fumarate or aspartate, aspartic acid isolated from
lycomarasmin was first degraded by deamination to malic acid
followed by decarboxylation to acetaldehyde.

The total carboxyl activity of malic acid and also the
activity at C_1 were determined in separate experiments, and
the activity at C_4 obtained by difference.

Table 6 shows the distribution of ^{14}C in the carbon
atoms of aspartic acid (specific activity = 1.12 x 10^4 cpm/mM)
obtained from lycomarasmin isolated from cultures which had
been labelled 24 hours with DL-aspartic acid-4-^{14}C. The
distribution is expressed as a percentage of the total
activity detected.

The overall pattern, therefore, strongly suggests that
the entry of C_1, C_2, C_3 and C_4 of aspartic acid occurs

TABLE 6
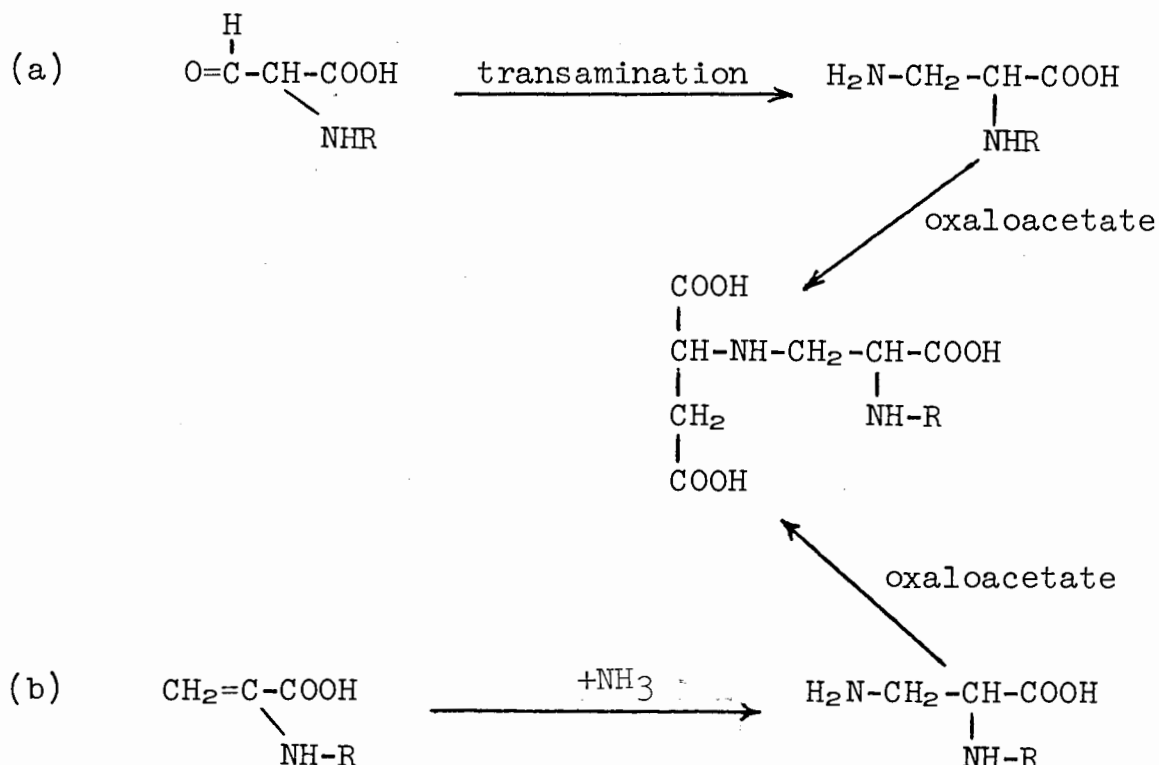
Distribution of Label in Aspartic Acid

Carbon atom of aspartate		Distribution
1	COOH	6
2	CHNH$_2$	<5
3	CH$_2$	<5
4	COOH	94

Figures are expressed as percentages of total activity detected.

mainly as an intact or at least isotopically identically reconstituted carbon chain into the 4-carbon component of lycomarasmin.

The relatively specific incorporation of aspartic acid-4-^{14}C, therefore, eliminates the possibility of direct participation of fumarate and suggests that aspartate may be incorporated directly either (1) by condensation with the 3C-fragment by the schemes suggested previously, or (2) as oxaloacetate in which an oxidized 3C-fragment is first transaminated as follows:

(a)
$$O=\overset{\overset{\displaystyle H}{|}}{C}-CH-COOH \xrightarrow{\text{transamination}} H_2N-CH_2-CH-COOH$$
$$\qquad\qquad\backslash NHR \qquad\qquad\qquad\qquad\qquad | \; NHR$$

oxaloacetate

$$COOH$$
$$|$$
$$CH-NH-CH_2-CH-COOH$$
$$|\qquad\qquad\qquad |$$
$$CH_2 \qquad\qquad NH-R$$
$$|$$
$$COOH$$

oxaloacetate

(b)
$$CH_2=C-COOH \xrightarrow{\quad +NH_3 \quad} H_2N-CH_2-CH-COOH$$
$$\qquad\backslash NH-R \qquad\qquad\qquad\qquad\qquad | \; NH-R$$

Labelling experiments with ^{14}C, therefore, do not differentiate between participation of aspartate and oxaloacetate.

L-Aspartic Acid-2,3-^{3}H Feeding Experiment.

The object of this experiment was to determine whether aspartic acid-2,3-^{3}H was incorporated as such into lycomarasmin or if it was first deaminated to oxaloacetate and then incorporated.

Aspartic acid isolated from lycomarasmin was degraded in a similar way as in the previous experiment, with the exception that acetaldehyde was further degraded by the iodoform reaction to give C_2 as formate.

L-Aspartic acid-2,3-^{3}H (1.00 mC) was equally distributed between six cultures. It was found necessary to extend the feeding period to 14 days in order to increase the incorporation of ^{3}H to a measurable quantity for degradation experiments. Carrier lycomarasmin (200 mg) was added during the work-up procedure to give a final yield of 250 mgs which on acid hydrolysis gave 114 mgs of aspartic acid and 60 mgs glycine. The results of this feeding experiment are summerized in Table 7. It was not found possible to determine the specific activity of the 2,4-DNPH derivative of pyruvic acid due to severe colour quenching in the tritium channel.

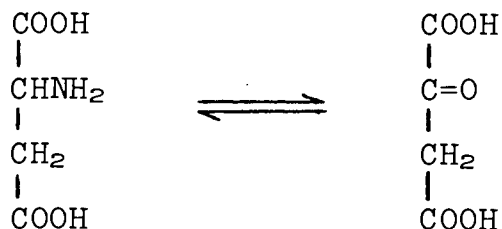Degradation of the isolated aspartic acid resulted in only 1.5% of the label of malate being detected in the formic acid derived from C_2 of aspartate. This low level of activity
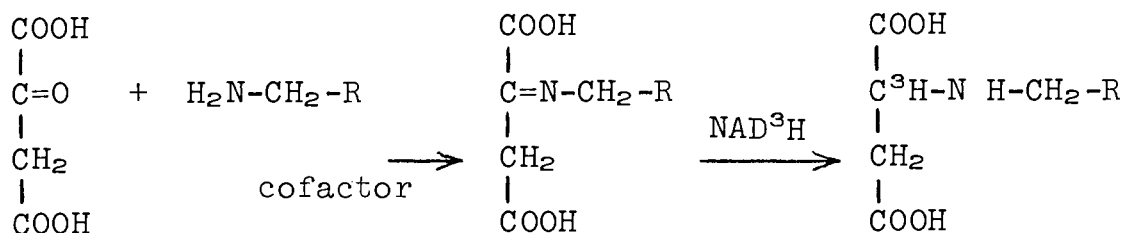
TABLE 7

Specific Activities from L-Aspartic

Acid-2,3-^{3}H Experiment

	Specific Activity cpm/mM
Lycomarasmin	3.14×10^5
Glycine	6.0×10^3
Aspartic Acid	7.92×10^3

at C_2 is most probably the result of this amino acid
undergoing deamination at a faster rate than its direct
incorporation into lycomarasmin:

```
COOH                        COOH
 |                           |
CHNH₂      ⇌                C=O
 |                           |
CH₂                         CH₂
 |                           |
COOH                        COOH
```

Nevertheless, if the four-carbon fragment was derived
from oxaloacetate then all the tritium at C_2 in aspartic
acid-2,3-^{3}H would necessarily be lost before incorporation
into lycomarasmin, in which case, despite the low level of
activity detected, this result would suggest that aspartate
is in fact incorporated as such. There exists the
possibility, however, due to the catabolism of aspartate,
of the formation in the cultures of tritium labelled NADH.
The condensation of oxaloacetate with an amino group on the
3C-fragment via a Schiff's base followed by reduction could,
therefore, result in the re-incorporation of tritium at C_2
in the 4C-fragment:

```
COOH                      COOH                      COOH
 |                         |                         |
C=O    +  H₂N-CH₂-R       C=N-CH₂-R                 C³H-N H-CH₂-R
 |                         |            NAD³H         |
CH₂                       CH₂         ──────→        CH₂
 |        cofactor         |                          |
COOH      ──────→         COOH                       COOH
```

This experiment, therefore, although suggesting the incorporation of aspartate as such, does not conclusively differentiate between the participation of aspartate from that of oxaloacetate. The biogenetic reaction sequence involved followed by the requisite chemical degradations involved may be summarized as in Fig. 6.

The tritium from aspartic acid-2,3-^{3}H can be incorporated into the 2C-fragment (glycine) via the glyoxylate cycle operative over the extended feeding period (see scheme on page 37).
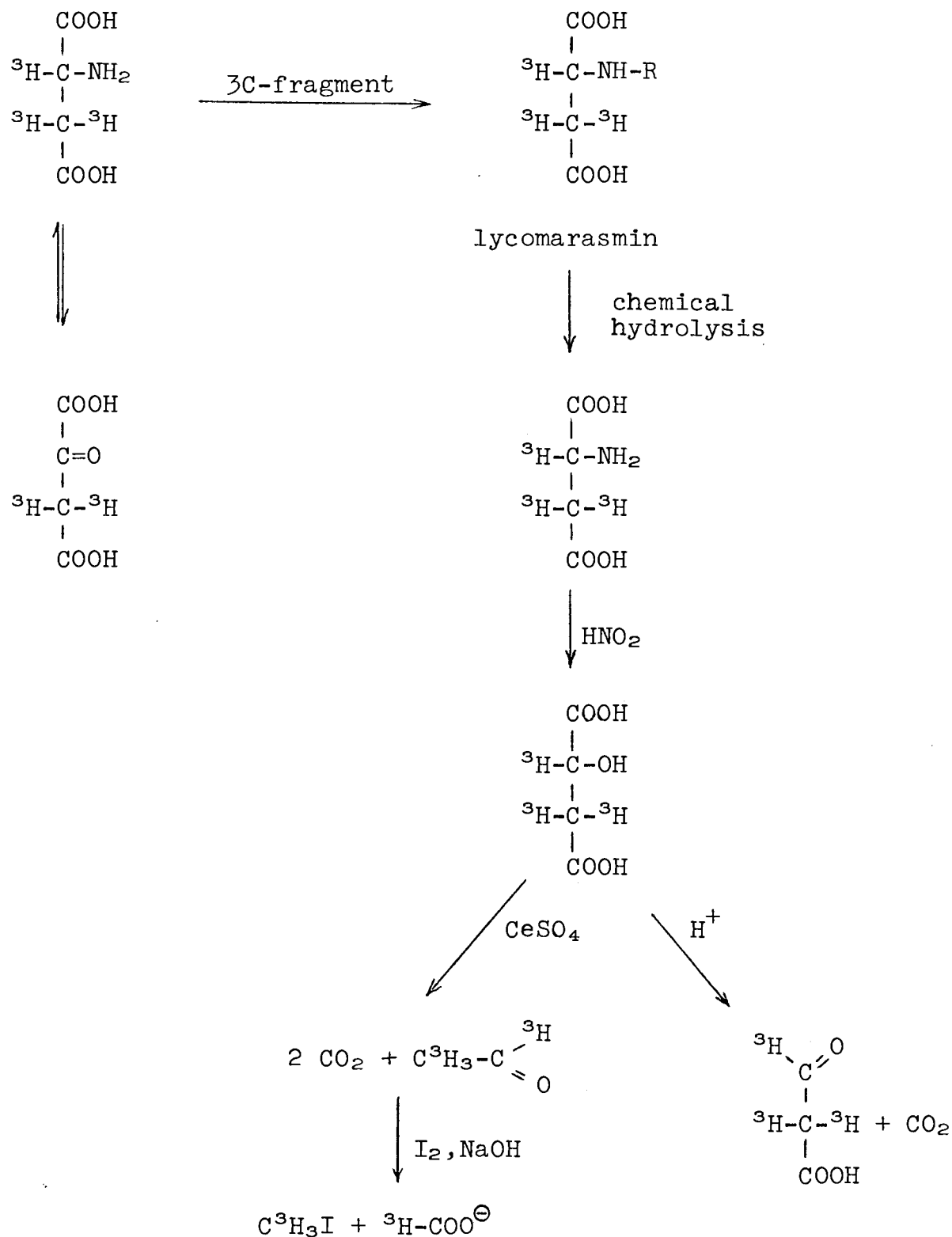
```
        COOH                                    COOH
         |                                       |
   ³H-C-NH₂         3C-fragment            ³H-C-NH-R
         |         ──────────────►               |
   ³H-C-³H                                  ³H-C-³H
         |                                       |
        COOH                                    COOH

                                            lycomarasmin

       ↓↑                                        │ chemical
                                                 ↓ hydrolysis


        COOH                                    COOH
         |                                       |
        C=O                                ³H-C-NH₂
         |                                       |
   ³H-C-³H                                  ³H-C-³H
         |                                       |
        COOH                                    COOH

                                                 │ HNO₂
                                                 ↓

                                                COOH
                                                 |
                                           ³H-C-OH
                                                 |
                                           ³H-C-³H
                                                 |
                                                COOH
```

CeSO₄ ↙   ↘ H⁺

$$2\ CO_2 + C^3H_3\text{-}C{\overset{\displaystyle ^3H}{\underset{\displaystyle =O}{\diagup}}}$$

$$\overset{\displaystyle ^3H}{\underset{\displaystyle C}{\diagdown}}{\overset{\displaystyle =O}{}}$$
$$^3H\text{-}C\text{-}^3H + CO_2$$
$$|$$
$$COOH$$

│ I₂,NaOH
↓

C³H₃I + ³H-COO⊖

Figure 6. Biogenetic reaction sequence and chemical
          degradation of aspartic acid.

D-Glucose-U-^{14}C Labelling Experiment

Based on our previous findings that aspartic acid and glycine furnish the carbon atoms for the four-carbon and two carbon fragments respectively, possibilities for the origin of the three carbon fragment are a 3-carbon amino acid such as serine, or a 3-carbon compound derived from glucose such as 3-hydroxypyruvate, phosphoenolpyruvate or pyruvate. With a view to determining the participation of the latter three compounds a labelling experiment with glucose-U-^{14}C was carried out. D-Glucose-U-^{14}C (500 μ C) was distributed between six cultures 10 weeks old and the lycomarasmin isolated after a 24 hour labelling period. The specific activities of lycomarasmin and the hydrolysed products have been divided by 10 for comparison purposes with those from the amino acid labelling experiments before entry into Table 8.

The results in Table 8 suggest that either glucose is a more immediate precursor than the amino acids or that it penetrates to the site of synthesis more easily. However, previous analytical studies of the culture medium have shown that the organism is depleted in glucose after 30 days growth[24], thus largely eliminating the possibility that glucose serves as an immediate precursor at such a time. Further, Dimond and Waggoner[26] showed that lycomarasmin

TABLE 8

Specific Activities from D-Glucose-U-^{14}C

Labelling Experiment

	cpm/mMx10^{-4}
Lycomarasmin	12.6
Glycine	3.16
Aspartic Acid	2.47
Pyruvic Acid	5.0

appeared, and steadily increased, in the culture medium at about the same time that glucose totally disappeared. External application of ^{14}C-labelled amino acids results in their dilution by the relatively large pool of unlabelled amino acids in the medium[24] prior to their transport to the site of synthesis resulting in relatively low specific activity of lycomarasmin. Glucose, on the other hand, will undergo rapid catabolism inside the cells resulting in production of numerous 2- and 3-carbon compounds and provided they undergo transamination near the site of synthesis, the specific activity of lycomarasmin will be relatively high. Unless this transamination process occurs near the site of synthesis, the release of the freshly derived amino acids into the general amino acid pools would consequently give specific activities not too different from direct administration of labelled amino acids.

Cockrane et al.[32] supplied F. oxysporum with glucose-U-^{14}C and subsequently demonstrated that less than 5% of the glucose label remains in the medium. The present finding of a relatively higher incorporation of glucose into lycomarasmin as compared with the amino acids may therefore simply indicate that permeability barriers exist.

Nevertheless, the finding that glucose readily supplies the carbon atoms to the 2C- and 3C-fragments suggests that this hexose is rapidly catabolized to glycine. Two possible
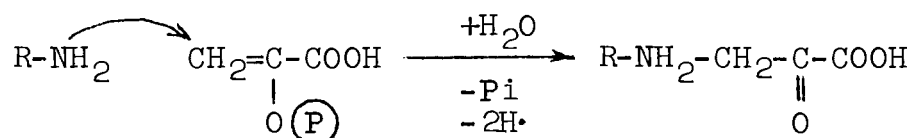
pathways exist for the synthesis of glycine from glucose.

Enzymes have been isolated from E. Coli[33] and Salmonella

typhimurium[34] that synthesize glycine by the pathway:

glucose → 3P-glycerate → 3-hydroxypyruvate → serine → glycine

in which 3P-glycerate is the branch-point from the main

glycolosis pathway. The second pathway in micro-organisms

for glycine biosynthesis from glucose involves the glyoxylate

cycle in which glycine is formed by transamination of

glyoxylate. Data from the aspartate labelling experiment,

however, has indicated that the glyoxylate pathway is

operative to only a limited extent over a 24 hour period,

suggesting, therefore, that the former is the major pathway

by which glycine is formed from glucose in this organism.

Since the alanine and aspartate labelling experiments

have shown that label enters the 3C-fragment, then based

on the data from the glucose feeding experiment, it would

be expected that if the label enters 3P-glycerate then it

would also enter the 2C-fragment. The low incorporation of

label from alanine and aspartate into the 2C-fragment,

therefore suggests that conversion of the 3C-source does not

proceed past PEP and implies the involvement of either

pyruvate or PEP in the formation of the 3C-fragment.

Of these two possibilities, the logical precursor to

the 3C-fragment would be PEP. Experiments with isotopic

oxygen on the mechanism and pathway of enzymatic transferring

reactions between ADP and various phosphate donors have

proved that it is the phosphoryl group $\left(-P\begin{smallmatrix}O^-\\\parallel\\O\\\diagdown O^-\end{smallmatrix}\right)$ that is

transferred[35]. Condensation of aspartic acid and PEP

followed by hydrolysis of phosphate with subsequent oxidation

of the hydroxyl group would therefore result in carbonyl

formation at C_2 providing the necessary functionality for

condensation with the 2C-fragment:

$$R-NH_2 \qquad CH_2=\underset{\underset{O\;\textcircled{P}}{|}}{C}-COOH \quad \xrightarrow[\substack{-Pi \\ -2H\cdot}]{+H_2O} \quad R-NH_2-CH_2-\underset{\underset{O}{\parallel}}{C}-COOH$$

Dual Labelling Experiment with D-Glucose-U-^{14}C-6^{3}H.

It was reasoned that the information gained from a dual

labelling experiment would elucidate the mechanism by which

the 4-carbon fragment condenses with the 3-carbon fragment

(Schemes 1, 2, 3 and 4, p.20).

For the detection of tritium in the three carbon

fragment, it was found necessary to count this fragment as

alanine rather than pyruvic acid because of the intense

colour quenching caused by the 2,4-DNPH derivative of pyruvic

acid. For this reason, the lycomarasmin was degraded by

aqueous hydrolysis resulting in glycine, alanine and aspartic

acid.

It was also necessary to extend the feeding period
from 24 hours to 41 hours in order to increase the
incorporation of ^{14}C and ^{3}H to a measurable quantity for the
calculation of the ^{3}H/^{14}C ratio.

The results of this dual labelling experiment in which
500 μC of D-glucose-U-^{14}C and 1.0 mC of D-glucose-6-^{3}H
were distributed between six cultures are summerized in
Table 9. During the work-up procedure   50 mgs of carrier
lycomarasmin were added to give a final yield of 330 mgs.

Each ^{14}C/^{3}H   ratio was divided by the ^{14}C/^{3}H ratio
of the original glucose to give the ratio that would have been
obtained if the ^{14}C/^{3}H ratio in the glucose had been 1:1
before entry into Table 9.

The three carbon fragment can be derived from D-glucose
via 3-phosphoglycerate by the pathway described in Fig. 3.
Consideration of the pathway by which the 3C-fragment is
derived from glucose leads to the prediction therefore, that
retention of ^{3}H at C$_3$ will result in a similar ^{14}C/^{3}H ratio
as was started with, 1:1 corrected. Similarly, loss of one
tritium atom at C$_3$ will result in a ratio of about 2:1
corrected. The experimental value of ^{14}C/^{3}H in alanine
derived from pyruvate is 10.2:1 corrected, showing a
substantial loss of tritium.

Previous labelling experiments with glucose-U-^{14}C
have indicated that PEP is the probable precursor for the

TABLE 9

Hydrolysis Products of Lycomarasmin from

Dual Labelling Experiment

	cpm/mg of ^{14}C	cpm/mg of ^{3}H	^{14}C/^{3}H
Glycine	5650	1105	10.2:1
Aspartic Acid	2501	1175	4.3:1
Alanine	6350	1340	9.5:1

3C-fragment and pyruvate has been shown to function as the central compound in the metabolism of this mold[36]. Relatively rapid conversion of PEP to pyruvate compared to a slow rate of incorporation of PEP into lycomarasmin, followed by fast turnover in the TCA cycle and regeneration of PEP by decarboxylation of oxaloacetate would, therefore, result in this substantial loss of tritium at C_3 in the 3C-fragment (C_6 in the original glucose) (Figure 8).
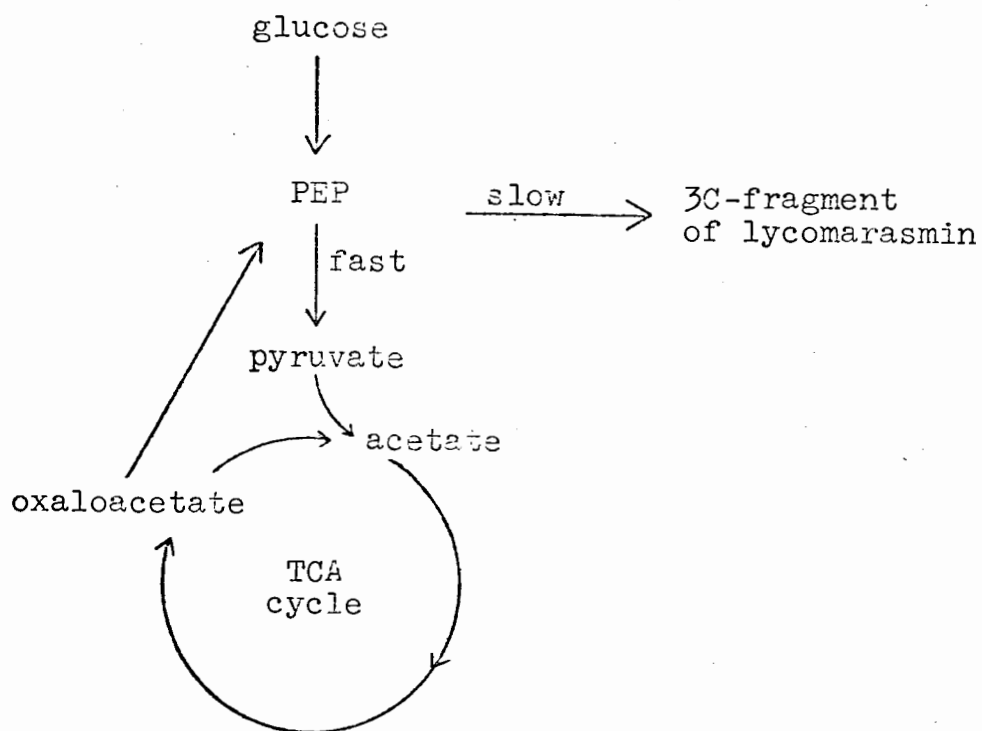
Figure 8. Incorporation of 3C-fragment into lycomarasmin

Labelling Experiments with ^{14}C-intermediates.

Although lycomarasmin itself has not been chemically synthesized, there exists several published procedures for the synthesis of derivatives of lycomarasmin[36]. Some of these derivates have been found active in the test for wilting of tomato plants[36]. Since the biosynthesis of lycomarasmin may involve a series of intermediates in which either the 2C- and 3C-fragments or the 4C- and 3C-fragments are involved, it was decided to synthesize two of the most likely intermediates labelled with ^{14}C and feed them to the fungus. Specific incorporation of either of the intermediates would give information as to the sequence in which the fragments are incorporated into lycomarasmin.

The synthesis of α-carboxymethylamino-β-alanine (9), was carried out according to the published procedure[37], and was labelled with ^{14}C by using glycine-2-^{14}C as starting material.

The published procedure[38] was used also to prepare ^{14}C-labelled N-(β-carboxy-β-aminoethyl)-aspartic acid (10) from aspartic acid-4-[14]C.

$$NH_2-CH_2-CH-COOH$$
$$NH-^{14}CH_2-COOH$$

$$HOOC-CH-NH-CH_2-CH-COOH$$
$$HOO^{14}C-CH_2 \qquad NH_2$$
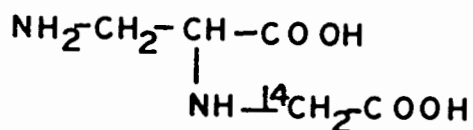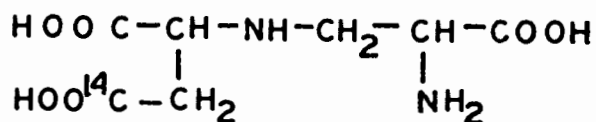
9 10

Inoculation of cultures with these two intermediates using a similar method as described for amino acids, resulted in no activity being detected in the lycomarasmin.

SUMMARY AND CONCLUSIONS

It is difficult, in view of the tracer experiments, to construct a definite biogenetic sequence whereby the toxin molecule is generated. However, the most significant results obtained have involved feeding glycine-2-^{14}C and aspartic acid-4-^{14}C. The administrated glycine-2-^{14}C was primarily incorporated without extensive label randomization and kinetic considerations indicate that glycine as such condenses with a suitable 3C-keto compound. Feeding of aspartic acid-4-^{14}C showed that the entry of C_1, C_2, C_3 and C_4 of aspartate occurs mainly as an intact carbon chain into the 4C-fragment of lycomarasmin, although experiments with aspartic acid-2,3-^{3}H were inconclusive in differentiating between the participation of oxaloacetate from that of aspartate.

The labels from alanine-1-^{14}C and aspartic acid-4-^{14}C were incorporated to only a small extent into the 2C-fragment of lycomarasmin indicating the conversion of these compounds does not proceed past 3-hydroxypyruvate. Taken in conjunction with results from a glucose-U-^{14}C feeding experiment, the above experiments indicate that the probable precursor of the 3C-fragment is a compound readily derived from glucose.

The condensation of aspartate with PEP would give an attractive C_4 - C_3 precursor which could consequently condense with glycine to give the required carbon skeleton as well as

the amino functions. It is also possible that the $C_4 - C_3$ condensation with the 2C-precursor could involve glycinamide directly thus generating lycomarasmin directly and not necessitating subsequent amide formation. PEP is favoured as the 3C-precursor because it is firstly an intermediate in the glycolysis pathway and secondly, it can be formed by the well established decarboxylation of oxaloacetate.

The prime difficulty in establishing a more highly defined mechanism is the very biochemically labile nature of the most probable immediate precursors. Our original objectives of establishing the precise identity of all intermediates as well as the nature of the condensations was not fully realized.

EXPERIMENTAL

Growth Conditions of Fungus

Fusarium oxysporum Schl. emend. Sny. et Hans.
f. lycopersici (Sacc.) Sny. et Hans, Strain no. 5414,
kindly supplied by Dr. S. Naef-Roth, Institut fur Spezielle
Botanikder Eidgenossischen Technischen Hochschule, Zurich,
was used in all experiments. Stock cultures were maintained
on potato dextrose agar slants.

Culture media was inoculated by transferring the
mycelium to 2ℓ. Erlenmeyer flasks containing 500 ml.
modified Richards' medium[19] and maintained (still culture)
at 25°C in the dark.

Analysis of amino acids.

A batch of 4 cultures were inoculated and grown for 7
weeks under the conditions described. Each successive week
thereafter, one culture was filtered and $Ba(OH)_2$ (1g)
dissolved in H_2O(~10mls) was added to the filtrate. After
centrifugation the filtrate was evaporated to small volume
(~20ml) and 10 x vol. of MeOH added. The residue was
centrifuged and the solution evaporated to dryness. The
residue was dissolved in 6N HCl (10ml), filtered to remove

any residue and a portion analysed for amino acids using a
Beckman 119 amino acid analyser.

Radioisotopic Techniques

    a) Radioactive materials and administration

DL-Aspartic acid-1-^{14}C, DL-alanine-1-^{14}C, L-serine-U-^{14}C,
glycine-U-^{14}C, glycine-2-^{14}C, D-glucose-U-^{14}C, D-glucose-6-^{3}H,
L-aspartic acid-2,3-^{3}H, were obtained from the International
Chemical and Nuclear Corporation, California, and New England
Nuclear , Massachusetts.

    An aqueous solution of each radioactive tracer was
prepared in a standard volumetric flask and sterilized for
30 minutes at 130°C. Aliquots of the appropriate solution
were administered to cultures which had been growing for 2-4
months using a sterilized hypodermic syringe fitted with a 12
inch needle. The solution containing the radioactive compound
was injected into the culture medium below the mycelial pad.

    b) Assay of Radioactivity

      i) Water soluble compounds (amino acids).

    The weighed sample (10-20 mg) of the crystallized
compound was dissolved in water in a 5 ml volumetric flask.
An aliquot (0.2 ml) of this solution was transferred to a
glass counting vial containing 15 ml of scintillator solution
consisting of naphthalene (100g) and 2,5-diphenyloxazole

(PPO) (5g) dissolved in dioxane (1ℓ).

The samples were counted in a Beckman LS200B-automatic liquid-scintillation counter under the specified conditions. Background corrections were made and the observed count rate was converted to an absolute value. The counting efficiency obtained was 91% for ^{14}C and 30% for [3]H. Dual labelled amino acids were counted as previously described and the absolute count rate determined by the use of internal standards[39]. Sample activities were determined in duplicate with a counting error of 3% or less in each determination.

  ii) 2,4-Dinitrophenylhydrazone derivative of

    pyruvic acid.

The weighed sample (20.00 mg) of crystalline derivative in a 10 ml. volumetric flask was dissolved in acetone (2 ml) and the volume made up to 10 ml with toluene. An aliquot (0.10 ml) was mixed, in a counting vial, with 15 ml. of scintillator solution consisting of PPO (4g) and (POPOP) (50 mg) per litre of toluene, and counted as before. Quenching corrections were made by subtraction of a background count of an inactive sample of similar concentration. The counting efficiency of ^{14}C using this method was 86%.

  iii) Lycomarasmin

A weighed sample of the toxin (10 mg) was dissolved in 0.2N sodium hydroxide (1 ml) and the volume adjusted to 10 ml with water. An aliquote (0.2 ml) of this solution

was counted as previously described for the determination of activity in the amino acids.

    iv) Radioactivity of $^{14}CO_2$ was assayed using the procedure described by Jeffay and Alvarez[40].

The scintillation medium used was a toluene: ethylene glycol monomethylether solution (2:1 v/v) containing 5.5 grams per litre of 2,5-diphenyloxazole, stored in the the dark in an amber bottle.

    A solution of ethanolamine in ethylene glycol monomethyl ether (3 mls) (1:2 v/v) was used to trap the carbon dioxide. After completion of the experiment this ethanolamine solution was transferred to a counting vial containing 15 ml of the scintillation solution.

Isolation of Lycomarasmin

    Lycomarasmin was isolated, after requisite growth intervals, from the culture filtrates according to the procedure described by Hardegger et al.[4]. The barium salt of lycomarasmin was precipitated from the filtrate with methanol and purified by recrystallization from acid solution at pH 2.6, m.p. 225-228° (decomp.), literature m.p. 227-229°[4].

Yields varied from 0-50mg/litre of culture filtrate.

Mass spec. of triethyl ester of Substance "J" found 344.

Calcd. for $C_{15}H_{24}N_2O_7$ requires mol. wt. 344.

Acid Hydrolysis of Lycomarasmin

Lycomarasmin (100 mgs), dissolved in 6 $\underline{N}$ hydrochloric
acid (5 ml) was heated in a sealed tube for 7 hours at
140-150°C. After cooling the solution was transferred to a
50 ml flask and connected to the vacuum pump via a dry
ice-acetone cold-trap (-78°C). The flask was warmed carefully
to completely remove the solvent (the remaining residue
contained amino acids) and the condensate in the trap added
to a solution of 2,4-dinitrophenylhydrazine (50 mg) in hot
6 $\underline{N}$ hydrochloric acid (5 ml). The resulting solution was
heated on a water bath for 5 minutes and then stored at 4°C
for 15 hours during which time the 2,4-DNPH derivative of
pyruvic acid crystallized. The derivative was collected
(centrifugation) and dissolved in a solution (5 ml) of 2$\underline{N}$
sodium carbonate. The insoluble reagent was removed by
filtration and the filtrate was carefully acidified with 50%
hydrochloric acid and the resulting precipitate collected.
The 2,4-DNPH derivative was washed with 1-2 ml. of water and
dried under vacuum (27 mg, 28%) m.p. 215-217°C, m.m.p.214-216°C.

The residue of amino acids was, after addition of water
(10 ml), again concentrated to dryness in vacuo to effect
removal of residual HCl. The mixture of amino acids was
dissolved in water (2-3 ml) and separated using a Dowex
1(x8) acetate column (1 X 10 cm). Glycine was eluted with

water (35 ml) and aspartic acid with 0.5 N acetic acid
(60 ml).

Each fraction was evaporated to dryness. The residues
were dissolved in water (2-3 ml) and after addition of charcoal
filtered through a 2 mm. layer of Celite 535. The filtrate
was concentrated to a small volume (1-3 ml) upon which the
amino acid crystallized by the addition of ethanol (1-5 ml).
For aspartic acid, glacial acetic acid (0.5 ml) was also
added.

Aqueous hydrolysis of lycomarasmin

Lycomarasmin (200 mg), suspended in water (7 ml),
was heated in a sealed tube for $2\frac{1}{2}$ hours at 200°C. After
cooling, the solution was removed and transferred to a 50 ml
flask and evaporated to dryness under vacuum. An aqueous
solution of the residue was decolourised with charcoal.
After filtering through a pad of Celite, the mixture of amino
acids was partially separated on a Dowex 1(x8) acetate column
(1 x 10 cm.). Glycine and alanine were eluted with water
(35 ml) and aspartic acid with 0.5 N acetic acid (60 ml).
Aspartic acid was crystallized as described previously.

The mixture of alanine and glycine was resolved on a
Dowex 50(x8)(H$^+$) column[41]. The amino acids were eluted with
1 N hydrochloric acid at a rate of 8 ml./hr. and collected in

10 ml fractions in which glycine was first eluted followed
by alanine. The fractions containing the individual amino
acids, as detected by paper chromatography, were combined
and evaporated to dryness in vacuo. The HCl was removed
from the amino acid by elution with water through a Dowex
1(x8) (acetate) column and the amino acid crystallized from
aqueous ethanol.

Paper Chromatography

The purity of each amino acid, isolated by hydrolysis
of lycomarasmin, was checked by paper chromatography (Whatman
no. 1) using three different solvent systems[4]. Dried chromatograms
were sprayed with 0.25% ninhydrin solution in acetone followed
by heating in an oven at 105° C for 15 minutes.

Skeletal Degradation of Amino Acids and Malic acid

The chemical degradation of amino acids and malic acid
for localization of radioactivity was carried out according
to published procedures.

i) Aspartic acid was converted to malic acid via
deamination with nitrous acid and the malic acid cocrystallized
with inert carrier from a solution of dry benzene and acetone[42]

ii) Glycine was oxidised with ninhydrin to CO_2 and

formaldehyde. The CO_2 was assayed as described[40] and formaldehyde trapped as the dimedone derivative[43].

    iii) Malic acid

        Oxidation of malic acid with ceric ammonium sulphate resulted in carbon atoms 1 and 4 being liberated as CO_2, assayed as described[40], and carbons 2 and 3 as acetaldehyde, trapped as the bisulphite adduct[43]. Treatment of malic acid with conc. H_2SO_4 at 50-60°C for 1 hr. resulted in carbon 1 being evolved as CO which was oxidised to CO_2 by passage through a tube containing CuO at 400°[43].

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PART 2

ANTHERIDIOL

INTRODUCTION

The genus <u>Achlya</u> belongs to a family of aquatic fungi known commonly as water molds. These molds form a major part of the fungous flora of ponds and lakes, where they grow on twigs, fruits, dead insects, and other organic debris fortuitously provided. Members of the genus <u>Achlya</u> are frequently found in association with diseased and moribund fish and fish eggs. Whether they are the primary cause of disease or are only opportunists has not been established. Among the species of <u>Achlya</u> isolated from infected fish, <u>A. bisexualis</u> and <u>A. flagellata</u> are the only ones which have been shown able to grow and develop on experimentally wounded fish, a demonstration which seems to implicate these species as agents of disease[1].

Water molds are most abundant in shallow, sheltered water where oxygen and food are plentiful. Here vegetative growth is best. Water molds have colourless, filamentous cells that are large enough to be seen with the unaided eye. The cells - or hyphae, as they are called - are multinucleate and lack crosswalls, except for those delimiting the reproductive organs. A hypha elongates only at its tip. Occasionally, a hyphal tip may branch, but normally branches arise laterally.

The asexual and sexual reproductive organs are formed at

the tips of hyphae and hyphal branches. The antheridium
(male sex organ) is cylindrical or lobed, whereas the
oogonium (female) is more or less spherical. Shortly after
the oogonial initial is formed, the antheridial branch begins
to grow toward it. Upon reaching the female initial, the
branch curves around it, and the antheridium is delimited
by a cross wall which forms near the tip of the branch. A
cross wall at the base of the oogonial initial delimits the
oogonium, and within this organ from one to twenty uninucleate
oospheres (eggs) are produced. Fertilization is accomplished
by the passage of nuclei through fertilization tubes that
extend from the antheridium to each egg.

In most species of Achlya the oogonia and antheridia are
borne on the same thallus. There are two species however,
in which the cooperation of two individuals is needed for
the production of sex organs. If kept apart, each is usually
sterile, but when mycelia of opposite sex are allowed to grow
near one another, antheridial branches will develop on one and
grow to the oogonial initials produced by the other.

A. bisexualis was used by John R. Raper[2] in his classic
experiments showing that sexual reproduction in certain species
of Achlya is initiated and controlled by diffusible substances
reciprocally secreted by the sexually reacting pair. Raper
found that lateral branches were produced within 4 hours on
the hyphae of the male when Seitz-filtered water in which a

female had grown was poured over the male. When the water in which a male had grown was poured over the female, there was no response. However, when the water in which the male hyphae had reacted by branching was filtered and poured over a female, oogonial initials were produced. Clearly, a secretion of the vegetative hyphae of the female initiated the sexual reaction by inducing the formation of antheridial branches, and the sexually active male then secreted a substance that induced the formation of oogonial initials on the female. The female secretion was called "hormone A" and the male secretion was called "hormone B". The growth of the antheridial branches to the oogonial initials and the formation of antheridia, Raper concluded, are controlled by a specific substance secreted only by the oogonial initials, called "hormone C".

The cleavage that gives rise to oospheres was attributed to the action of a fourth substance, called "hormone D", secreted by the antheridium. Thus it appeared that each stage in the formation and conjugation of the sex organs was controlled by a different hormone. In later experiments, working with purified natural "hormone A", Barksdale[3] showed that the specific actions which had previously been attributed to hormones A, C, and D were, in fact, caused by only one hormone. Arsenault et al.[4] were the first to characterize "hormone A" as a sterol and named it antheridiol (1), the structure of which has since been proved by synthesis[5]. In addition to

antheridiol, the biologically inactive 23-deoxyantheridiol
(2), has also been isolated from culture liquids of A.
bisexualis[6].
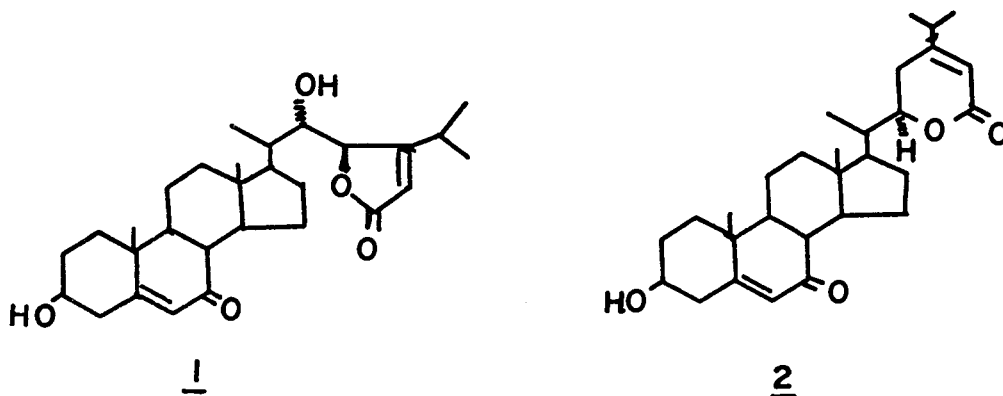
Although it has been well established that algae and
fungi produce a wide range of sterols essential for their
growth and reproduction[7], this finding of a specific action
of a sterol as a plant sex hormone was the first identification
of the biochemical function of a sterol in plants.

Two compounds with similar side chain structural
characteristics to antheridiol have been isolated from a
plant. The C_{29} ecdysterols 3 and 4 were both isolated from
Cyanthula capitata and their biosynthesis postulated to occur
by enzymatic hydroxylation followed by further modifications
to form the lactones[8].

$\underline{3}$ $\qquad$ $\underline{4}$
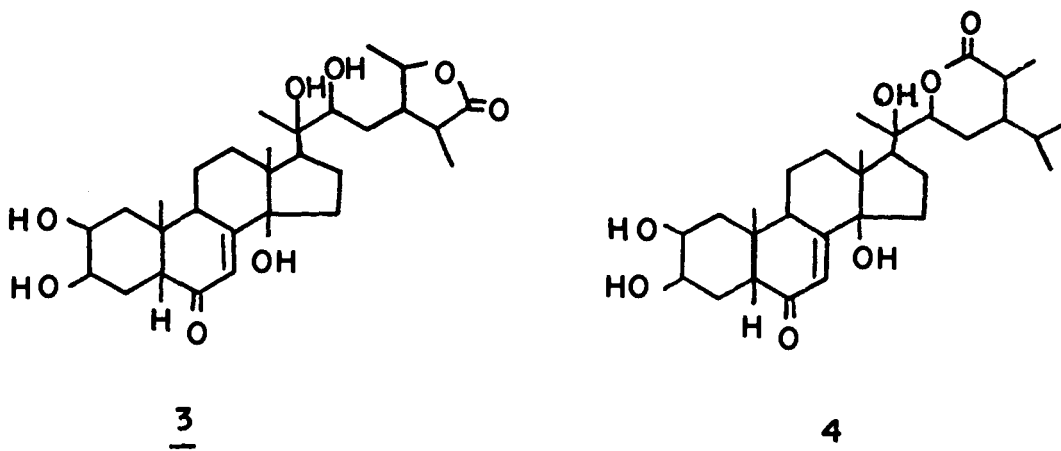
Despite research into the precursors of the ecdysterols[9], the formation of the lactone ring remains to be investigated. In recent years interest in phytosterol biosynthesis has focused principally upon the mechanism of side chain alkylation and the intermediates involved in the elaboration of the Δ^5-phytosterols[10]. The finding of the antheridiols ($\underline{1}$ and $\underline{2}$) and the ecdysterols ($\underline{3}$ and $\underline{4}$) possessing unique oxygenated side chains therefore raises the question as to their origin in the plant species. It was decided therefore to undertake a biosynthetic study of antheridiol with the intention of elucidating the steps leading to lactone formation in the side chain.

Upon consideration of the carbon skeleton of antheridiol and on the basis of previous biosynthetic studies of steroidal metabolites[9], it is reasonable to propose that the hormone's biogenetic origin involves a C_{29} sterol precursor. The object of the present work was to:

1. determine the major sterols of <u>A. bisexualis</u>,

2. synthesize some possible intermediates involved
   in the biosynthetic pathway leading to antheridiol,

and 3. perform labelling and trapping experiments to
   determine the sequence of events involved in
   elaboration of antheridiol.

LITERATURE SURVEY

There is much biological evidence for hormonal control
of sexual reproduction in a wide range of fungi, but only
three hormones besides antheridiol have been chemically
characterized.

The sexual reproduction of the water mold Allomyces
involves the formation of motile male and female gametes by
the same fungus, and the subsequent fusion of actively
swimming males with the sluggish females. Machlis[11] has shown
that the male gametes are chemotactically attracted to the
female gametes and has named the attractant sirenin (5), the
synthesis of which has been acheived by Nutting et al.[12]. By
growing the female hydrid under conditions designed to control
the formation and release of the gametes a yield of 1_μM-sirenin
can be obtained in the culture filtrate.

5
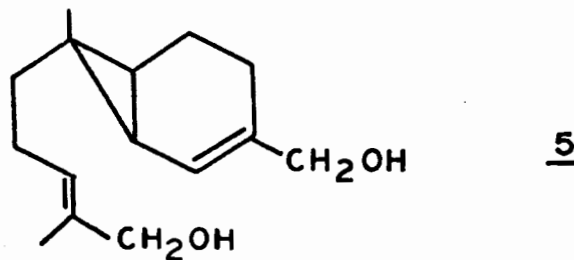
The involvement of hormones controlling sexual
reproduction in the Mucorales was first demonstrated by
Burgeff[13] in 1924. In this group of fungi sexual reproduction
occurs between morphologically indistinguishable mating types,
(+) and (-), and Burgeff showed that diffusible substances are
responsible for the formation of sexual hyphae when two mating

types meet. Although Burgeff and Plempel[14] obtained an extract from the culture filtrate of mated M. mucedo that had hormone activity, the hormone was not fully characterized. It was not until 1967, working with a closely related fungus Blakeslea trispora, that van der Ende[15] isolated two compounds each of which was active in the sex hormone bioassay with M. mucedo. It was shown that these compounds were the trisporic acids B and C ($\bar{6}$).
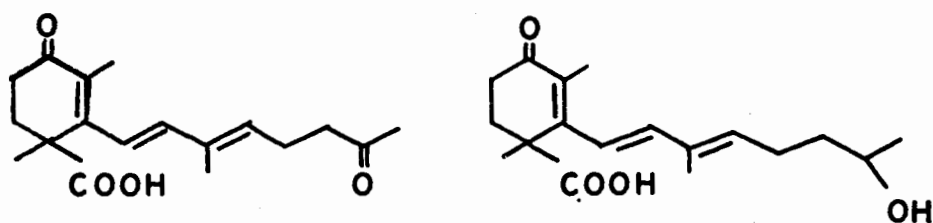
6

At about the same time, Gooday[16] obtained from mycelia harvested from mixed cultures of "mating types" of M. mucedo a substance which had sex hormone activity in both the plus and minus strain. Subsequently, it has been established that the active material consists mostly of triscorpic acid C[17]. Evidence has been obtained from biosynthetic experiments that the trisporic acids are biosynthesized by cleavage and oxidation of β-carotene via retinal and trisporal C, the primary alcohol corresponding to trisporic acid C[18].

Ectocarpin (7) is the most recent sex hormone to be fully characterized chemically[19].
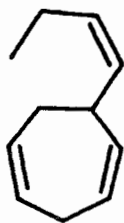
7

In contrast to the other sex hormones described, ectocarpin is volatile and can be removed from the culture medium of <u>Ectocarpus siliculosus</u> by an air stream and condensed in a cold trap. The action of this sex hormone is similar to that of sirenin in that it is excreted by the female gametes resulting in attraction and fertilization by the male gametes.

Antheridiol has been isolated from several strains of A. bisexualis[6]. The small quantity of this hormone produced by <u>Achlya</u>, however, presents problems in the isolation procedure. Antheridiol (2 mg) was first isolated from 320 litres of culture filtrate from one strain of female A. bisexualis[20] and another strain was found to produce only 2 mg. of antheridiol from 500 litres[6]. It is therefore impractical to isolate antheridiol in usable quantities, and as a result, a great deal of attention has been directed recently to the synthesis of pure antheridiol.[21,22,23] The most practical synthesis[23] yields a mixture of the four possible stereoisomers in respect to the asymmetric carbons

22 and 23. Antheridiol has been found to possess the 22S,23R stereochemistry[22] and the synthesis yields the isomers (22S,23R), (22R,23S), (22R,23R) and (22S,23S) in a ratio of 2:10:2:1 respectively and in an overall yield of approximately 20%.

23-Deoxyantheridiol (2) exists in culture filtrates in about the same concentration as does antheridiol ($\sim$3-7μ g-litre). Although the synthesis of 23-deoxyantheridiol in its natural 22R configuration has not been achieved, the unnatural 22S isomer has been synthesized[6].

The importance of sterols to permeability, reproduction and growth functions of many fungi has been well documented[7]. Of significant importance are the studies on species of the fungal genera Pythium and Phytophthora which show purely vegetative growth in media which lack sterols. However, in media to which certain sterols are added, vegetative growth is increased and sexual organs (oogonia and antherida) develop and oospores are formed[24]. Experiments performed to determine the structural features of the sterol molecule necessary to induce this physiological activity in the case of Phytophthora cactorum have shown that the sterol nucleus is required with a hydroxyl group at carbon 3, a double bond in the B ring, and a side chain of more than five carbon atoms.

The most striking result obtained, however, relates to the position of the 29th carbon atom. The double bond linking carbons 24 and 28 holds C_{29} in the E-position in fucosterol(8).

and in the alternate $\underline{Z}$-position in isofucosterol ($\underline{9}$)[25]. Of

the sterols tested, which included cholesterol, β-sitosterol,

$\underline{8}$ $\underline{9}$
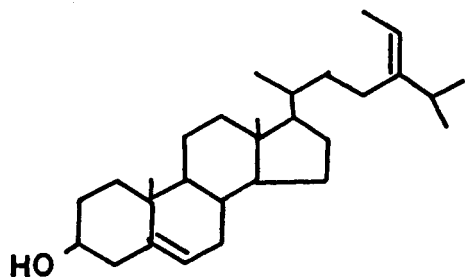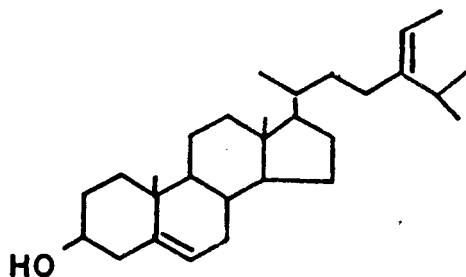
ergosterol, Δ^5-cholenol, Δ^5-cholesten-3-one, and Δ^7cholestenol,

fucosterol was found to be the most active in the production

of oospores with isofucsterol being slightly less active.

With the indentification of antheridiol as a sex hormone

in Achlya it is reasonable to propose that steroids which

have previously been assigned hormonal activity in various

fungi, are in fact, precursors of steroidal hormones analogous

to antheridiol.

Although the sterols produced by a number of Phycomycetes

have been investigated by McCorkindale et al.[26] and found to

contain varying proportions of cholesterol, desmosterol,

24-methylene cholesterol and fucosterol, the sterol content

of only one species of Achlya was studied. A. caroliniana

was shown to contain a mixture of cholesterol, (7.2%),

demosterol (27%), and 24-methylene cholesterol (1%).

HYPOTHESIS OF ANTHERIDIOL BIOGENESIS

Our preliminary investigation of the sterols of
A. bisexualis showed that fucosterol ($\underline{8}$) was the major
sterol and 7-dehydrofucosterol ($\underline{10}$), a logical precursor to
$\underline{8}$, was also detected. The presence of fucosterol, in water
mold can therefore provide the necessary carbon skeleton for
subsequent modification of the side chain and nucleus.

The formation of the antheridiol side chain from
fucosterol can be envisaged to occur by oxidation to a carboxyl
group at C_{29} followed by opening of a $C_{22,23}$ epoxide by
protonation and a concerted SN_2 type reaction of the carboxyl
anion at C_{23} (Fig. 1). This type of mechanism would be
expected to result in a mixture of δ- and γ- lactones by
cyclisation to either C_{22} or C_{23} respectively.
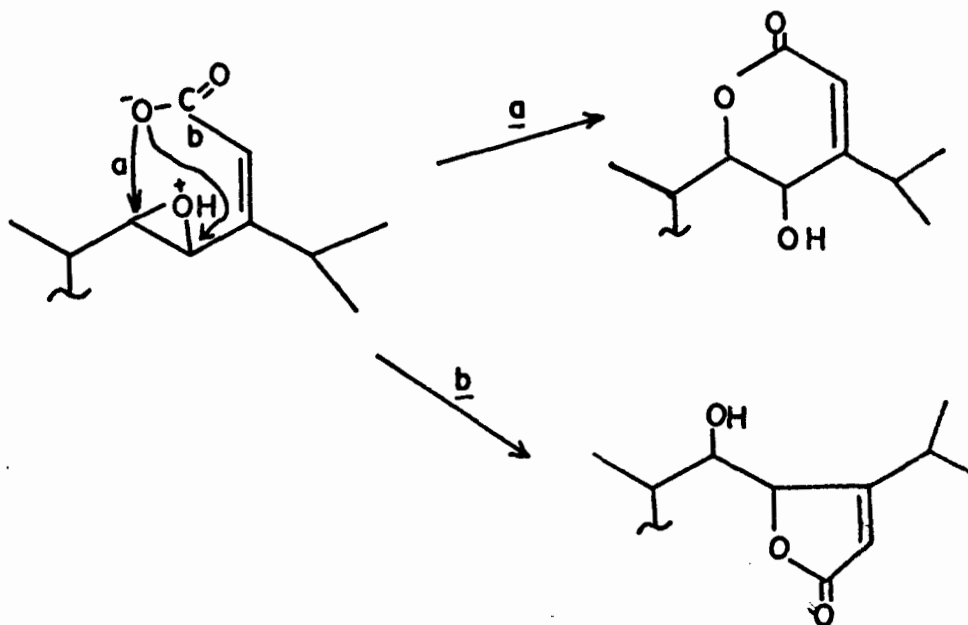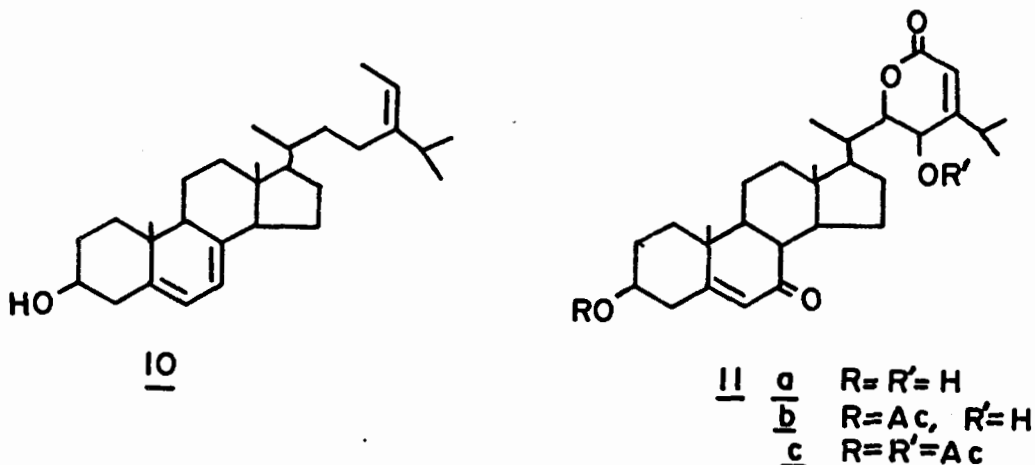

Figure 1. Formation of δ- and γ- lactones

A similar result would be obtained if a $C_{22,23}$ diol was involved. It follows therefore that the 23-hydroxy-δ-lactone isomer, isoantheridiol (11) would be a likely substrate in the elaboration of antheridiol. In agreement with this type of mechanism is the occurrence of both γ- and δ- lactone derivatives in A. bisexualis. It seems highly probable that the biosynthesis of 23-deoxyantheridiol (2) involves either a 22,23-diol or a 22,23-epoxide precursor as most certainly would be the case of antheridiol (1). In the case of 2, the absence of the 23-hydroxyl presents no difficulties in terms of metabolic reactions.

The enzymatic conversion of fucosterol to antheridiol requires 4 general transformations:

  1. Oxidation in the B-ring at C_6,

  2. Hydroxylation or oxidation at C_{22}, C_{23},

3. Oxidation at C_{29},

4. Lactone formation.

Some of these transformations can be multistep enzymatic processes. If one considers the individual enzymatic reactions to be mutually independent they could conceivably occur in any order. The total number of potential metabolic intermediates involved in the fucosterol to antheridiol conversion would then depend on the total number of possible combinations of enzymatic steps involved.

In order to simplify the overall picture and to reduce the total number of potential intermediates to a reasonable number, several reaction sequences were combined in one-step transformations. Also, previous biosynthetic studies of phytosterols have shown that enzymes responsible for steroidal modification have considerable latitude with respect to substrate structure[27]. It seems likely therefore that the organism will modify the side chain regardless of the state of transformation of the nucleus.

With these conditions in mind, we decided to synthesize some hypothetical radioactive precursors and carry out a series of feeding experiments to determine the incorporation of the intermediates into antheridiol (Fig. 2). Although these experiments will show which of the precursors are metabolized to antheridiol, they will not establish the involvement of individual intermediates in the biogensis of
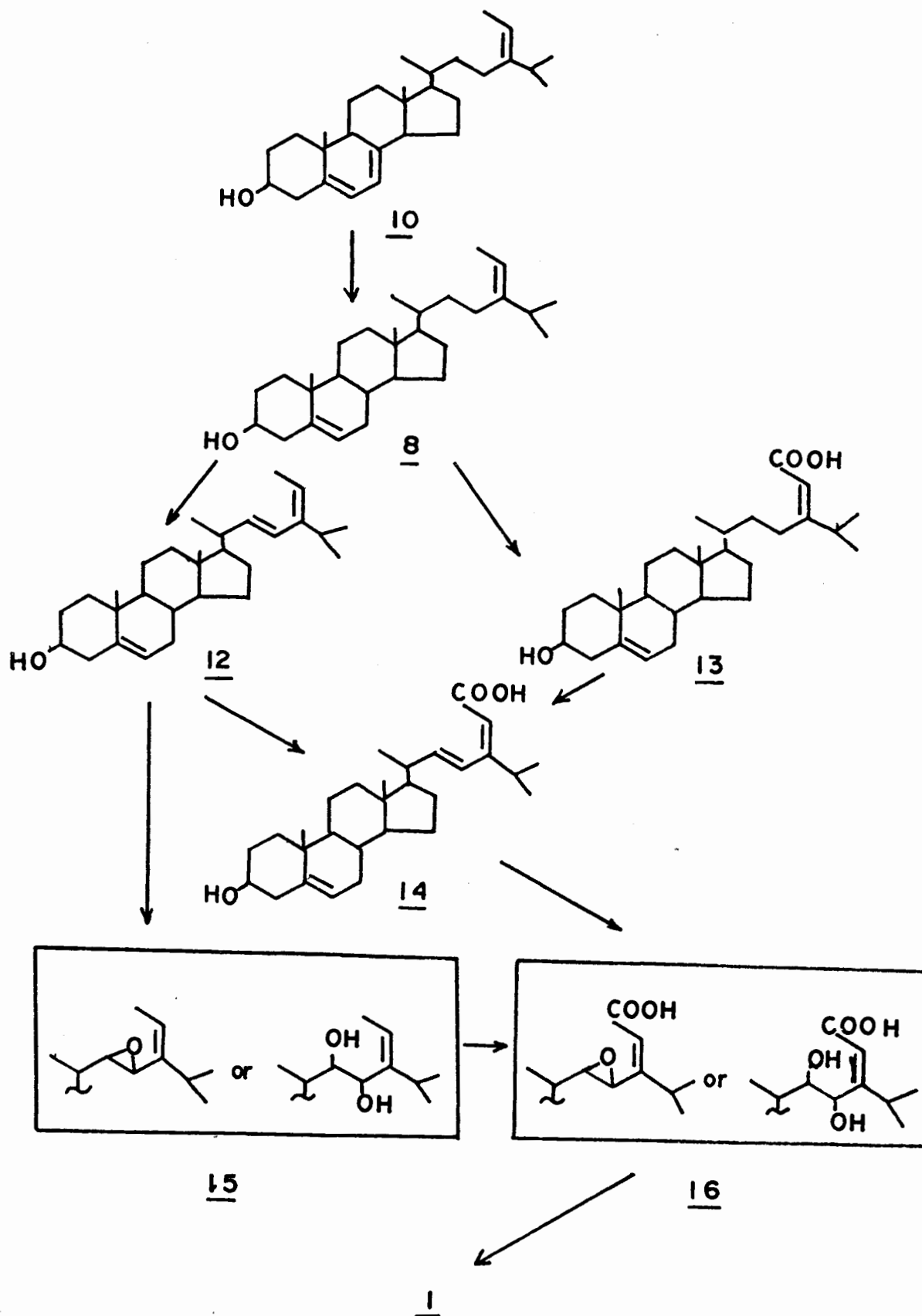
Figure 2. Intermediates involved in elaboration of the antheridiol side chain

antheridiol. In order to determine the involvement of specific intermediates we designed trapping experiments in which a precursor of antheridiol was fed to the organism and unlabelled intermediate (carrier) added during the work-up procedure. The intermediate would be re-isolated and any radio-activity found would furnish strong evidence that the particular compound was an intermediate in the biosynthetic pathway.

In conjunction with these incorporation studies, we decided to attempt the synthesis of antheridiol along a fashion in which the biosynthesis might be expected to occur. Our intention was to synthesize the 22,23-epoxy-29-carbethoxy compound (17) or the 22,23-diol (18), and it was reasoned that acid hydrolysis of the ester function in either 17 or 18 would result in a mixture of the corresponding γ- and δ-lactones.

COOEt   COOEt

17   18
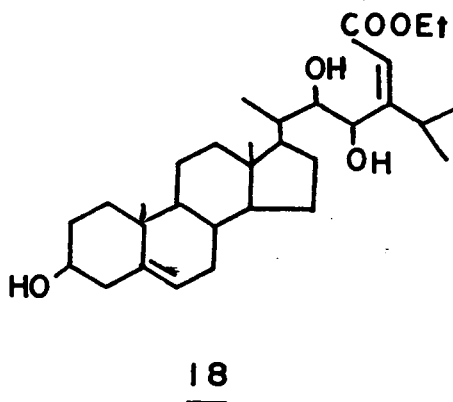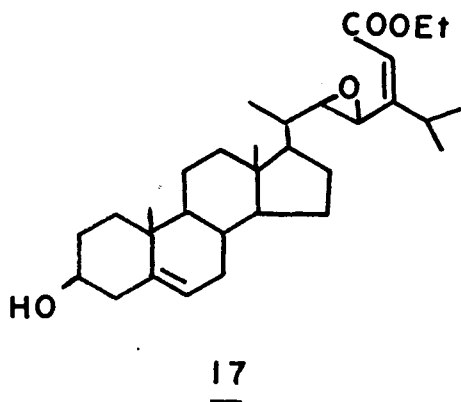
The synthesis of antheridiol by this mechanism could possibly give further information regarding the biosynthetic sequence involved in the elaboration of the hormone. Similarly, the formation of the 23-hydroxy-δ-lactone isomer (11) from the precursors 17 or 18 would indicate that it also may be elaborated by the microorganism, the possibility of which, could be studied by a trapping experiment.

RESULTS AND DISCUSSION

1. Identification of Major Sterols of A. Bisexualis

The mold cultures were grown in 500 ml of media as previously described[20] and the mycelia collected after 7 days. The air dried mycelia was saponified with ethanolic KOH and the lipids extracted with hexane. The non-saponifiable material was chromatographed on alumina (activity III) and squalane and other hydrocarbons (up to 80% of the non-saponifiable lipid) eluted with light petroleum. Further elution with diethyl ether gave a fraction containing sterols, which were subsequently acetylated and separated by preparative TLC on silica gel impregnated with silver nitrate.

Gas-liquid chromatrography of the sterol acetate mixture showed four major components, Rt (retention time relative to cholesteryl acetate), 1.00, 1.28, 1.51 and 1.67, in a ratio of 3:17:70:10 respectively, based on peak areas, and three minor components < 1% each, with Rt, 1.11, 1.20 and 1.40. The Rt values of the four major components correspond to previously published values for cholesteryl acetate, 24-methylene cholesteryl acetate, fucosteryl acetate and an unidentified steroid, respectively[28].

Two distinct bands were abserved on the TLC plates in daylight with Rf values 0.47 - 0.51 and 0.60 - 0.70. Under U.V. light another two bands were visible with Rf values,

0.19 - 0.33 and 0.70 - 0.74. Each of the four bands were

eluted with ether and examined separately.

Band Rf 0.60 - 0.70 was recrystallized from methanol as

plates, m.p. 118-119°, mixed m.p. with fucosteryl acetate

118-119°. Over injection with fucosteryl acetate on QF-1

column showed one peak with Rt = 1.51. The mass spectrum and

NMR of the isolated steroid were identical in every respect

with authentic fucosteryl acetate. The NMR spectrum clearly

distinguished between fucosterol and its isomer,

28-isofucosterol in that the proton resonance signal of the

C-25 hydrogen appears at δ 2.2 and 2.8,respectively.

Band Rf 0.47 - 0.51 was recrystallized from methanol as

plates, m.p. 131-2°, mixed m.p. with 24-methylene cholesteryl

acetate, 130-2°. Over injection with 24-methylene cholesteryl

acetate on QF-1 column showed one peak with Rt = 1.28. The

mass spectrum, NMR and IR of the isolated steroid were

identical in every respect to authentic 24-methylene cholesteryl

acetate.

Band 0.19 - 0.33, on GLC examination showed a major

component with Rt = 1.67 with a number of small impurities.

It was therefore subjected again to preparative TLC using 10%

ethyl acetate-benzene as solvent and recrystallization from

methanol gave 1 mg of colourless crystals, m.p. 127-130°.

Literature m.p. for 7-dehydrofucosteryl acetate 129-131°[29].

The mass spectrum, NMR, UV spectra and retention time on GLC

examination of the isolated steroid were identical in every respect to authentic 7-dehydrofucosteryl acetate.

Band Rf 0.70 - 0.74 was difficult to distinguish even under UV light because of overlapping with fucosteryl acetate. This band was therefore subjected again to preparative TLC using 20% hexane-benzene as solvent. The band which co-chromatographed with cholesteryl acetate was examined by GLC which showed the major component corresponding to cholesteryl acetate and two smaller peaks, about 3% each, with Rt 1.29 and 1.51. Further attempts at separation were not made because of lack of material. Confirmation of cholesteryl acetate was obtained by GLC on 1% QF-1 and mass spectrographic analysis of the major component showed a peak of M^+ less CH_3COOH at $^m/e$ 368. Over injection of this sample with cholesteryl acetate showed no change in the mass spectrum.

A. bisexualis therefore contains a mixture of cholesterol, 24-methylene cholesterol, fucosterol, and 7-dehydrofucosterol as the major sterols.

These results have been published elsewhere[30].

2. Synthesis of Intermediates (Fig.3)

Synthesis of the intermediates was carried out after careful consideration as to the most favourable precursor to the required compounds 11, 12 and 13. Synthesis of 14 had
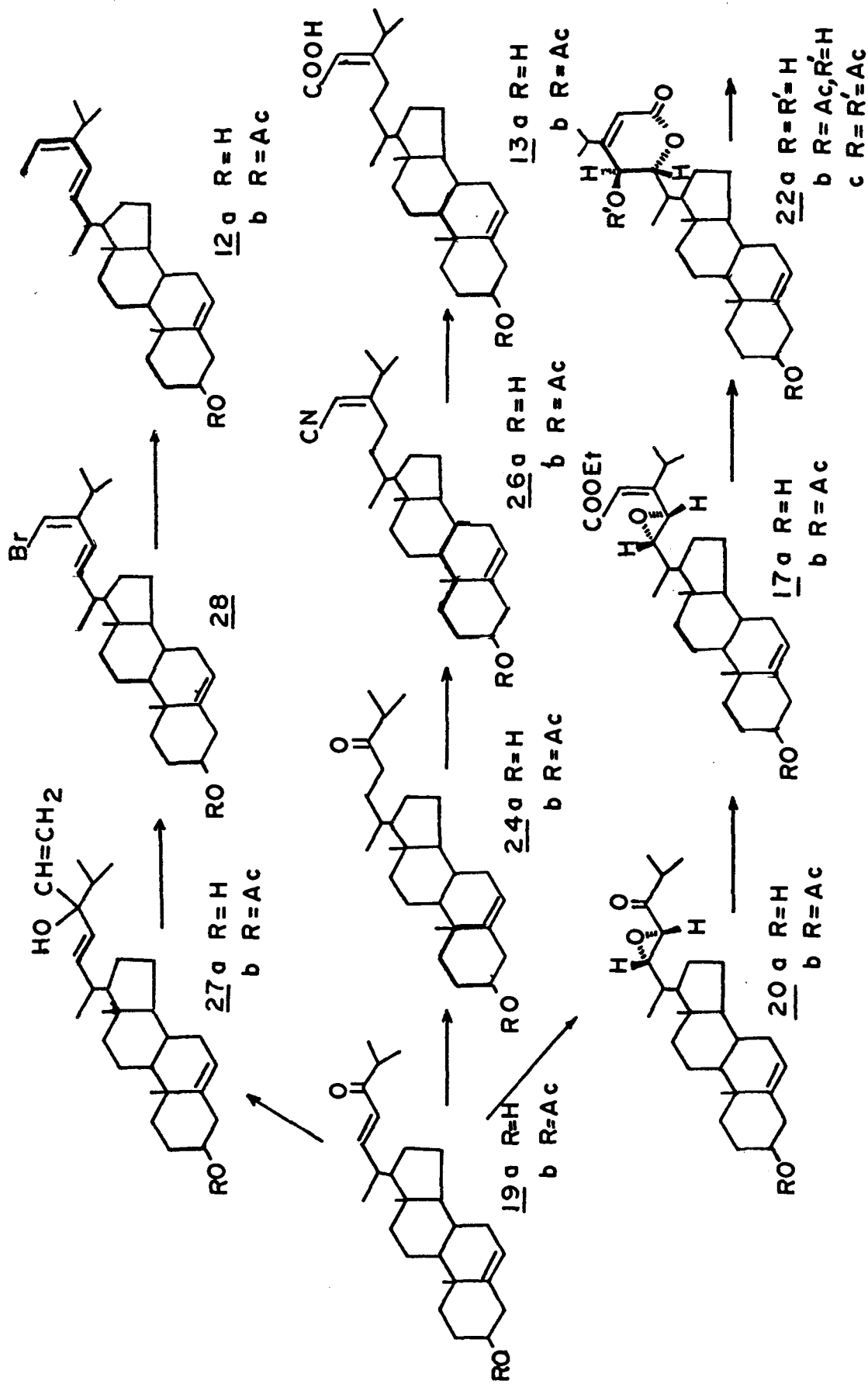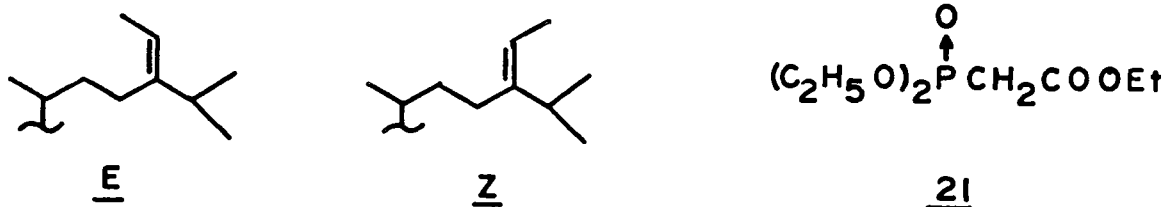
Figure 3. Syntheses of intermediates

been previously reported[5]. The starting material chosen for the syntheses was the α,β-unsaturated ketone, 19, derived from readily available stigmasterol[31]. This C_{27} compound (19) was favoured because it possesses a Δ^{22} double bond required in 12, and which provides the necessary functionality for 22,23-epoxide formation in 20. Also, the ketone at C_{24} is suitably positioned for a Wittig reaction required to introduce the extra two carbon atoms in 11, 12, and 13.

Synthesis of isoantheridiol (11)

Alkaline epoxidation of 19 with hydrogen peroxide in methanol gave a mixture of epoxy acetate 20b and alcohol 20a. This reaction is known to be highly stereoselective giving the 22S,23R isomer[32]. Deacetylation of the mixture gave the epoxy alcohol 20a in 70% yield. Attempts to open the epoxide (20) under acidic or basic conditions to form the diol were unsuccessful.

A Wittig reaction[33] of the carbanion of diethyl carbethoxymethyl phosphonate (21) with epoxy ketone 20a, was used to generate 17a in 30% yield. Compound 17 can have two possible side chain isomeric forms[34]. The E-configuration was assigned to 17 based on the following evidence. The Z-configuration would require isomerization to E as a prerequisite to lactone formation. Such a step would involve protonation at C-28 to give the C-24 carbonium ion and allow free rotation about the 24-28 bond. In the presence of D_2O,

the C-28 olefinic proton, in the case of the Z isomer, would be expected to exchange. In the conversion of 17 to 22 in the presence of D_2O, no reduction in the intensity of the C-28 proton signal was observed. This would be expected for the E-configuration of 17 in which isomerization of the $C_{24,28}$-double bond is not required for lactone formation.

the structures labeled **E** and **Z**, and structure **21**: $(C_2H_5O)_2\overset{O}{\overset{\uparrow}{P}}CH_2COOEt$

Treatment of 17a with aqueous perchloric acid in methanol at r.t. resulted in rapid formation of the highly insoluble δ-lactone 22a in quantitative yield. This substance (22a) gave one spot on TLC as did the monoacetate 22b and diacetate 22c. Since the 22S,23R and 22R,23S isomers of the corresponding γ-lactone have been separated by TLC[22], this indicated the product 22a was a single epimer. No formation of the γ-lactone was detected in the reaction. The monoacetate 22b was noticeably more soluble in organic solvents than the corresponding 3β-alcohol, 22a. For this reason the NMR spectrum for the monoacetate is recorded in preference to that of 22a. It is significant to note that the coupling constant of the $C_{23}\underline{H}$ (J = 10 Hz) is strongly indicative of trans C_{22}, C_{23} hydrogens thus further substantiating the assigned E configuration of 17 and is also supportive of the suggested

mechanism of δ-lactone formation. All conventional methods which were used in an attempt to open the epoxide ($\underline{17}$) and place a halogen atom or acetate group at C_{22} and hydroxyl at C_{23} (e.g. anhydrous HBr, acetic acid-HCl[35]) failed, and resulted in formation of the δ-lactone. The intention had been to cause γ-lactone formation by blocking the C_{22} position.

Compound $\underline{22a}$ was readily converted into the (22R,23S) 23-hydroxy δ-lactone isomer of antheridiol, $\underline{11a}$ by hematoporphyrin-sensitized photo-oxygenation and oxidative rearrangement[5] in 50% yield.

The stereochemical assignments at C_{22} follow from a comparison of the circular dichroism spectra of the δ-lactones $\underline{11}$ and $\underline{22}$ and parasorbic acid $\underline{23}$ in which the methyl group is known from NMR measurements to be equatorial[36]. The CD curve of $\underline{11}$ (in methanol) revealed a strong negative peak at 260 nm ($[\theta] = -8440°$). Since parasorbic acid exhibits a positive CD curve at 260 nm[37] due to the chirality of the nonplanar enone system, it follows that both the δ-lactones ($\underline{11}$ and $\underline{22}$) have the opposite stereochemistry to parasorbic acid. Since the C_{22} stereochemistry of both jaborosalactone A($\underline{24a}$) and withaferin A($\underline{24b}$) have been determined by this method (the assignments were subsequently found to agree with those determined by x-ray measurements[38,39], we feel justified in assigning the 22R configuration to the δ-lactones $\underline{11}$ and $\underline{22}$.
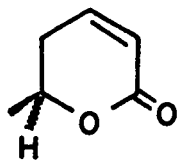
23
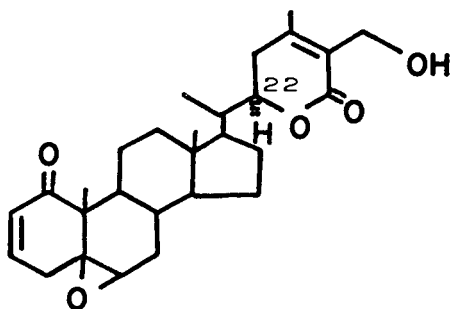
24a
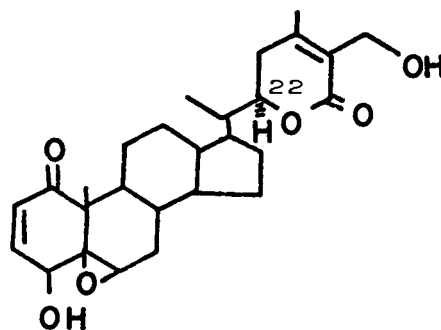
24b

The assignment of the R configuration at C_{22}, and the fact that only the δ-lactone is formed, is based on the n.m.r. evidence for trans C_{22}, C_{23} hydrogens (J = 10 Hz). We further interpreted these observations to indicate a relatively concerted epoxide ring opening resulting in the formation of the δ-lactone exclusively. Epoxide opening to yield a relatively free carbocation (C_{22}) would be expected to give an R,S configuration at C_{22} of the eventual δ-lactone. A plausible route could involve hydrolysis of the α,β-unsaturated ester function with concom ittant protonation of the epoxide followed by a concerted epoxide ring opening (nucleophilic attack at C_{22}) to give the δ-lactone.

The mass spectrum of the 23-hydroxy δ-lactone, 11a is similar to that reported for antheridiol[4]. The molecular ion (a) undergoes a retro-Diels-Alder fragmentation[15] to furnish a very intense peak ($^m/e$ 344, b) and fragment c.
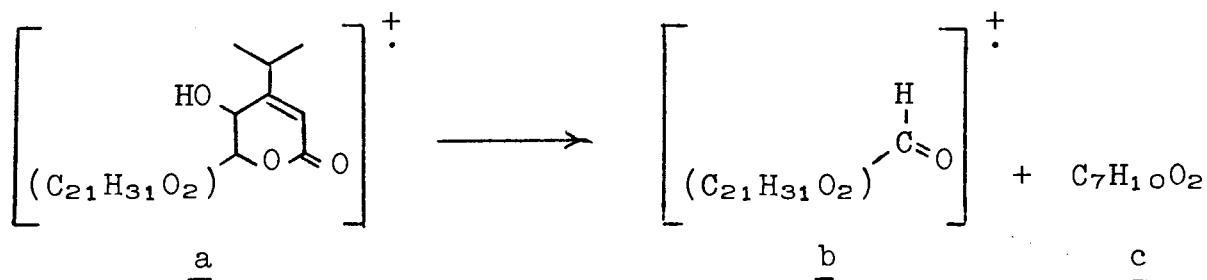

The 23-hydroxy δ-lactone, 11a is characterised by its melting point, 240-243°, a broad absorption band in the uv at 230 nm (ε = 16,500), and a strong absorption band in the ir at 1685 cm^{-1}. When the ethanolic solution which had been used for the uv spectrum was refluxed with hydrochloric acid the uv spectrum changed to λ_{max} 216, 279 nm resulting from extended conjugation of the $\Delta^{3,5}$-7-ketone.

Preparation of 3β-hydroxy stigmasta-5, E 24(28)-dien-29-oic acid (13).

Hydrogenation of the α,β-unsaturated ketone (19b) with a palladium catalyst poisoned with barium sulphate resulted in a >90% yield of 24b[40]. The intention was to synthesize 13 using the modified Wittig reaction between 24 and the carbanion of diethyl carbethoxymethyl phosphonate (21) as previously described for the synthesis of 17. However, no reaction was observed by this method in spite of increasing the reaction

time and temperature. A literature search revealed that
previous workers had compared the reactivity of the
phosphonates 21 and 25 with ketones in various positions in
the steroid nucleus and showed that 21 reacted only with
3-keto steroids, whereas 25 led to unsaturated nitriles from
steroids with the keto group at C_3, C_{17}, and C_{20}[41].

$$\overset{\overset{\text{O}}{\|}}{(C_2H_5O)_2 \, P \, CH_2 \, CN}$$
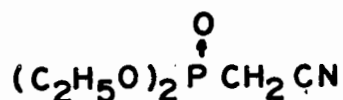
25

Attempts to synthesize 26b from a Wittig reaction
between 24b and 25 were successful and resulted in a yield of
68% of 26b. The 24(28) E-configuration was assigned to 26
based on the absence of C_{25}-proton resonance at δ 2.8 in the
NMR spectrum[25]. Conversion of the nitrile 26 to the acid 13
was achieved by alkaline hydrolysis.

Preparation of 3β-acetoxy stigmasta-5,22, E 24(28)-triene(12)

The C_{28}-isomer of 12 has previously been synthesized by
a Wittig reaction between 19b and ethyl triphenyl phosphonium
iodide[31]. The authors reported the C_{25} proton resonance at δ
2.8 indicative of the 24(28) Z-configuration[25].

In order to synthesize the required E-isomer it was
necessary to use the method described by Sucrow[42]. The vinyl
carbinols 27a and b were prepared from 19a and vinyl magnesium
bromide in THF. Transformation of 27b under allylic
rearrangement to the allyl bromide (28) by phosphorus

tribromide followed by reduction with lithium aluminium hydride gave the required compound 12.

3. Labelling Studies

The finding of 7-dehydrofucosterol (10) and fucosterol (8) in A. bisexualis suggested the possibility that a precursor-product relationship might exist between the two since the normal sequence of double bond changes in the biosynthesis of phytosterols is $\Delta^7 \to \Delta^{5,7} \to \Delta^5$ [43]. At the outset of this biosynthetic study it was decided to confirm this relationship by a tracer experiment with ^{3}H-7-dehydrofucosterol. $(2,4-^3H_3)$ 7-Dehydrofucosterol (11 mg) specific activity (5.3×10^4 cpm/mg) was distributed between 30, 3 day old cultures of A. bisexualis and the sterols extracted from the mycelia as previously described after a further 3 day growth period. Fucosterol (100 mg) was added as carrier to the extract and after acetylation, separated by preparative thin-layer chromatography on silver nitrate impregnated silica gel, followed by recrystallization to constant activity. The complete results of this experiment are recorded in Table 1, and confirm that the transformation (10) → (8) occurs in this organism.

Both antheridiol (1) and 23-deoxyantheridiol (2) have been isolated from culture liquids of A. bisexualis after a 3 day growth period[20]. Barksdale has reported time course studies with this organism using a bioassay method and demonstrated that

TABLE 1

Incorporation of 7-Dehydrofucosterol into Fucosterol

Total cpm of Precursor			cpm/mg of Fucosterol
5.83x10^5	1st recrystallization		488
	2nd	''	466
	3rd	''	483
	4th	''	483

Specific Activity of fucosterol based on added carrier (100mg)
=4.83x10^4 cpm/mg, incorporation, 8%

hormonal activity steadily increased between 24 and 72 hours from time of inoculation after which it remained constant[44]. Based on this information it was decided therefore to study the biosynthesis of antheridiol by administration of labelled precursors and work-up of the cultures within a 3 day period.

The radioactive sterol precursor (10-60 mg), emulsified in acetone-water with Tween 80, was equally distributed between 60, 24 hour old cultures of A. bisexualis under aseptic conditions and at the end of a 72 hour growth period, the cultures worked-up as described previously[6]. Since it was not possible to isolate antheridiol from 30 l. of culture liquid, due to the small amount produced by the organism, it was necessary to perform a trapping experiment. Usually, synthetic antheridiol (50 mg) dissolved in methanol containing 1% Tween 80 was added to the culture filtrate before extraction with methylene chloride. The extract was chromatographed on silica gel with 1:1 ethyl acetate/petroleum ether, and the antheridiol purified by preparative TLC on silica gel with 6% MeOH/CHCl$_3$ before determination of the activity.

The antheridiol used as carrier was a mixture of two isomers and it was not possible therefore to recrystallize to constant activity. The two isomers did however chromatograph together in the solvent system used and the criteria of purity was therefore taken when rechromatography resulted in constant activity. Antheridiol was then acetylated and again purified

by preparative TLC using 1% MeOH/CHCl$_3$ and if the specific
activity of the diacetate was found to be similar to that of
the deacetylated material, this was taken as evidence that
the precursor had been metabolized to antheridiol.

Each of the compounds 8, 12, 13, and 14 were tested as
precursors by the method just described and the results are
summarized in Table 2, in which the specific activity of
antheridiol is based on the amount of added carrier. The
results show that compounds 8, 12, and 14, were metabolized by
the organism to antheridiol.

With a view to determining the involvement of
isoantheridiol (11a)in the metabolism of the micro-organism,
a similar trapping experiment was performed with ^{3}H-fucosterol
(30 mg) as precursor. Difficulty was experienced, however, in
separating isoantheridiol (11a)from antheridiol (1) because of
their similar Rf values on silica gel in several solvent
systems. Separation was finally achieved by continuous thin-
layer chromatography over a 24 hour period with 2% MeOH-CHCl$_3$
in which isoantheridiol was slightly more polar. After
chromatography in this way (3x), isoantheridiol was found to
be devoid of activity, thus indicating that it is not a
substrate produced by water mold.

Having shown that compounds 8, 12, and 14 were metabolized
to antheridiol it was necessary to determine, by trapping
experiments, the involvement of each of these compounds as

TABLE 2

Incorporation of Intermediates into Antheridiol

Precursor	Total cpm added	Specific Activity* of Antheridiol cpm/mg	%
(2,4-$^3H_3$) Fucosterol ($\underline{8}$)	7.95x10^7	8.0x10^2	0.001
(23,25-$^3H_3$) Stigmasta-5,22, $\underline{E}$ 24(28)-trien-3β-ol ($\underline{12}$)	1.91x10^6	2.0x10^2	0.01
(29-^{14}C) 3β-Hydroxy-stigmasta -5,22,$\underline{E}$ 24(28)-trien-29-oic acid ($\underline{14}$)	2.40x10^5	2.5x10^2	0.1
(23,25-$^3H_3$) 3β-Hydroxy-stigmasta-5,$\underline{E}$ 24(28)-dien-29-oic acid ($\underline{13}$)	3.70x10^6	<100	

*Based on added carrier

specific intermediates in the biosynthetic pathway. The results of these studies are summarized in Table 3. In each experiment the mycelia was subjected to base hydrolysis and the lipid fraction extracted with hexane. Carrier material was added and the intermediates separated by chromatography, followed by recrystallization to constant activity.

Compounds 8 and 12 were not separated as their acetates under normal conditions of preparative TLC on silica gel/ AgNO₃ with benzene. Separation was achieved, however, by continuous TLC over a 5 day period with 20% benzene-hexane in which 12 was slightly more polar. The extraction of 14 was carried out after the hydrolysate had been acidified with dilute HCl. Compound 14 was obtained from the extract by chromatography on a silica gel column and elution with increasing concentrations of methanol in chloroform and finally purified by preparative TLC on silica gel with 8% MeOH/CHCl₃.

In order to determine whether the added intermediates were degraded by the micro-organism to simpler molecules (acetate) and then became re-incorporated into steroids, fucosterol was isolated from the feeding experiment with compound 13. After separation and purification, fucosterol was found to be devoid of activity thus indicating that compound 13 had not been catabolized by the organism. Based on this experiment, it is reasonable to assume that the other intermediates tested behaved likewise and that the transformations studied had indeed occurred.

Precursor	Total cpm added	Intermediate	Specific * Activity cpm/mg	Incorporation %
^{3}H-Fucosterol (8)	3.99×10^7	Stigmasta-5,22,24(28) trien-3β-ol (12)	3.0×10^2	0.07
^{3}H-Stigmasta-5,22, 24(28)-trien-3β-ol (12)	1.91×10^6	3β-Hydroxy-stigmasta- 5,22,24(28)-trien-29- oic acid (14)	1.24×10^2	0.06

*Based on added carrier

TABLE 3. Results of Trapping Experiments

CONCLUSION

This investigation has successfully shown that $\underline{A}$. **bisexualis** contains a mixture of fucosterol, 7-dehydro-fucosterol, cholesterol, and 24-methylene cholesterol as the major sterols, and labelling experiments have shown that fucosterol supplied the necessary carbon skeleton for antheridiol. The synthesis of intermediates $\underline{12}$, $\underline{13}$ and $\underline{14}$ combined with tracer experiments indicated that the sequence of events leading to elaboration of the γ-lactone in antheridiol involves dehydrogenation at $C_{22,23}$ followed by oxidation at C_{29}. Oxidation at C_{29} can be assumed to follow the sequence: $CH_3 \rightarrow CH_2OH \rightarrow CHO \rightarrow COOH$, similar to that which occurs in the removal of 4-dimethyl groups from lanosterol and cycloartanol[45]. The non-incorporation of compound $\underline{13}$ into antheridiol indicates that oxidation at C_{29} does not precede dehydrogenation.

The trapping of compounds $\underline{12}$ and $\underline{14}$ in the mycelium indicate that oxidation at C_{29} and dehydrogenation at $C_{22,23}$ take place prior to oxidation in the ring. However, if our original assumption is correct, that modification of the side chain occurs regardless of the nucleus, then the above conclusion is not valid.

The synthesis of a C_{29} lactone via a pathway similar to that which was postulate to occur naturally by oxidation at C_{29} and oxidation at $C_{22,23}$, resulted in exclusive formation of isoantheridiol (δ-lactone), and trapping experiments showed this compound was not a substrate of the organism. The significance of these results, combined with the fact that a side chain with a structure similar to that which is indicated in compound 16 is unstable and cyclises spontaneously, may indicate that a $C_{22,23}$ epoxide or diol are not true intermediates in the formation of the γ-lactone. If this is the case, then it can be postulated that the final steps involved in elaboration of the antheridiol side chain occur concommitantly eg. oxidation at $C_{22,23}$ and cyclisation to γ-lactone.

EXPERIMENTAL

Materials, Methods and Instrumentation

Fucosterol was isolated from a species of Fucus[46] collected from the beach at Vancouver, B.C. 24-Methylene cholesterol was synthesized by the reported procedure[47]. Cholesterol was obtained commercially. Achlya bisexualis, 14524, was obtained from American Type Culture Collection.

Spectra were obtained on the following instruments: Perkin-Elmer 457 (IR), Unicam SP800 (UV), Varian A56/60 and XL100 (NMR), Hitachi-Perkin-Elmer RMU-7 double focussing mass spectrometer connected to a Varian 140 GLC, and a Varian model 2100 gas chromatograph fitted with 1% QF-1 column and operated as reported[28].

Extraction and Separation of Steroids

The mycelia from Achlya bisexualis was filtered off and air dried at r.t. The dried mycelia (50g) was macerated in a blender with 500 ml 50% EtOH-H_2O. A further 250 ml EtOH and soln. of 75g KOH in 100 ml H_2O were added and the mixture refluxed under N_2 for 3 hrs. The soln. was centrifuged and the residue washed with EtOH. The soln. was diluted to 2 x its vol. with H_2O and extracted 4 x with hexane. The hexane soln.

was washed until neutral with H_2O and dried over $MgSO_4$.
Evaporation of the hexane extract in vacuo gave 450 mg of a
yellow oil.

In a typical separation 450 mg of non-saponifiable lipid
was chromatographed on 10g alumina (activity III) and the
hydrocarbons (360mg) eluted with 100 ml light petrol; the
column was then eluted with 100 ml Et_2O to give a fraction
(90mg) containing sterols. The sterol fraction was
consequently acetylated with 5 ml pyridine-Ac_2O (2:1) at r.t.
for 12 hrs. and worked up in the normal way.

TLC was carried out on plates 0.75 mm thick made from a
slurry containing 40 g silica gel G, 18g $AgNO_3$, 80 mg
rhodamine 6G, 25 ml EtOH and 60 ml H_2O. Plates were prepared
fresh each day before use and developed with benzene unless
stated otherwise.

Administration of Radioactive Precursors

The radioactive sterol of known activity (10-60mg) was
emulsified in Tween 80 ($\sim$ 500 mg) and dissolved in acetone.
The mixture was diluted with dist. water to $\sim$ 30 ml and
distributed aseptically between the cultures.

Counting of Radioactive Compounds

Radioactive measurements were made on a Beckman LS200B liquid scintillation counter. The sterol acetates were weighed into counting vials and dissolved in 15 mls of a toluene base scintillation solution containing 2.5-diphenyloxazole (5g) and PPO (0.3 g) per 1 l. Antheridiol was dissolved in hot methanol (0.5 ml) before addition of 15 mls of scintillation solution.

Preparation of 3β-acetoxy-24-oxo-cholest-5-en-22,23-epoxide(20b)

To a solution of 19a (3.0 g, 6.8 mole) in 100 ml acetone and 300 ml methanol, was added 20 ml of 38% hydrogen peroxide. The solution was stirred at room temperature and 4N NaOH (3 ml) added at such a rate as to keep the solution alkaline over a period of 3 hrs , and the solution was then left to stand at 4°C for 5 hrs. Most of the solvent was removed in vacuo and water added to the residue. The solution was extracted twice with ether and removal of the solvent in vacuo gave a non-crystalline residue, which was shown by TLC (SiO$_2$, 2% MeOH/CHCl$_3$) to be a mixture of 20a and 20b. The mixture was refluxed for ½ hr. with 100 ml 2% K$_2$CO$_3$ in 10% H$_2$O/MeOH. The solvent was removed (in vacuo) and water added to the residue, and the product extracted with ether. The ether layer was dried over MgSO$_4$ and removal of the solvent gave a crystalline solid. Recrystallization from methanol

gave 20a m.p. 180-181°C, (70%).

Acetylation of 20a with acetic anhydride/pyridine gave (20b).

Recrystallization from methanol gave m.p. 125-126°C; ν_{max}(CHCl$_3$): 2940, 1715, 1250, and 1030 cm^{-1}; NMR (CDCl$_3$): δ 0.65 (CH$_3$-C$_{18}$,s), 1.01 (CH$_3$-C$_{19}$,s), 1.01, 1.06, (2xd, J=7Hz, 2CH$_3$-C$_{25}$), 2.01 (CH$_3$OCO-,s), 2.5-2.9 (2H; sept. centred at 2.69, H-C$_{25}$; m centred at 2,80, H-C$_{22}$), 3.25 (H-C$_{23}$, d, J=2Hz), 4.4-4.8 (H-C$_3$, broad m), 5.51 (H-C$_6$, narrow m).

Anal. Calcd. for C$_{29}$H$_{44}$O$_4$ (mol.wt. 456): C, 76.27; H, 9.71. Found (^{m}/e, 456): C, 76.23; H, 9.64.

Preparation of (22S, 23R) 3β-acetoxy-24-carbethoxy-methylene-cholest-5-en-22,23-epoxide(17b).

A solution of 21 (2 g, 0.01 mole), prepared from ethyl bromoacetate and triethyl phosphite[48], in THF (10 ml) (distilled over LiAlH$_4$) was added dropwise with stirring to a suspension of sodium amide (0.4 g, 0.01 mole) in dry THF (10 ml) at 20°C. The solution was then stirred at room temperature under a slow stream of N$_2$ to sweep out the evolved ammonia. To the resulting yellowish-green solution was added, 20a (0.8 g, 2 m.mole) in THF (10 ml) over a 1 hr. period at 20-25°C after which the solution was left for a further period of one hour. The solvent was removed in vacuo, and water added to the residue. The aqueous solution was

extracted with ether, the extract dried over $MgSO_4$ and evaporated to give a colourless oil which was purified by chromatography (SiO_2, benzene) to give 17a which crystallised as needles from pet. ether (30:60), m.p. 117-118° (40%). Mass Spec. Found 484. $C_{31}H_{48}O_4$ requires: mol.wt. 484. When this Wittig reaction was carried out with acetate 20b, the product was found by TLC (SiO_2, $CHCl_3$) to be a mixture consisting mainly of 17a and 17b. Acetylation of 17a with acetic anhydride/pyridine gave 17b as an oil which crystallised as needles from ethanol, m.p. 117-118°; ν_{max} (KBr) 2970, 1730, 1710, 1630, 1245, 1210, 1172 and 1030 cm^{-1}; λ_{max} (EtOH) 231 nm (ϵ = 14,800); NMR ($CDCl_3$): δ 0.68 (CH_3-C_{18},s), 1.03 (CH_3-C_{19},s), 1.06 ($H-C_{21}$,d, J= 7Hz). 1.28 (C_2H_5OOC-, t, J=7Hz), 4.18 (C_2H_5OOC-, q, J=7Hz), 4.32 ($H-C_{23}$, broad s), 4.3-4.8 ($H-C_3$, broad m), 5.35 ($H-C_6$, narrow m), 5.78 (-OOC-CH=,s).

Anal. Calcd. for $C_{33}H_{50}O_5$ (mol.wt. 526): c, 75.29; h, 9.50. Found (^{m}/e, 526): C, 75.41; H, 9.64.

Preparation of (22R,23S) 3β,23-dihydroxy-24-carboxymethylene-cholest-5-en-19,22-lactone (22a).

To 17a (500 mgs) dissolved in methanol (50 ml), 5 ml 30% perchloric acid were added. After 5 min. the solution was concentrated in vacuo to small volume and left to stand at room temperature for crystallisation to give 22a m.p.

267-270° (>90%). ν_{max}(KBr): 3360, 2930, 1685, and 1380,

cm^{-1}; λ_{max}EtOH 210 nm (ϵ = 11,800). CD(c, 0.04, MeOH)

$[\theta]_{300}$ $\pm$0°, $[\theta]_{285}$ - 677°, $[\theta]_{280}$ - 1360°,$[\theta]_{275}$ - 2940°,

$[\theta]_{270}$ - 4960°, $[\theta]_{265}$ - 7210°, $[\theta]_{260}$ - 8440°, $[\theta]_{255}$ -

7660°, $[\theta]_{250}$ - 3160°, $[\theta]_{245}$ + 3400°, $[\theta]_{240}$ + 12,000°,

$[\theta]_{233}$ - 18,000°, $[\theta]_{225}$ + 10,200°, $[\theta]_{220}$ - 4520°. Anal.

Calcd. for $C_{29}H_{44}O_4$ (mol.wt. 456): C, 76.27: h, 9.71.

Found (^{m}/e, 456): C, 76.34; H, 9.73.

The monoacetate 22b, was prepared in a similar way from

17b. Recrystallisation from benzene gave m.p. 249-252° (d).

NMR (CDCl$_3$): δ 0.72 (CH$_3$-C$_{18}$, s), 1.03 (CH$_3$-C$_{19}$, s), 1.04

(H-C$_{21}$, d, J=6.5Hz). 1.17 (2CH$_3$-C$_{25}$, d, J=7Hz), 2.02

(CH$_3$OCO-,s), 2.87 (H-C$_{25}$, sept., J=7Hz), 4.16 (H-C$_{22}$, d,

J=10Hz), 4.2-4.8 (2H, m; addition of D$_2$O gives d at 4.37

J=10Hz, H-C$_{23}$ and H-C$_3$), 5.36 (H-C$_6$, m), 5.72 (lactone

olefinic-H,s). Mass. Spec. Found: 498. $C_{31}H_{46}O_5$ requires:

mol.wt. 498. Compound 22b was also prepared using 60%

perchloric acid (2.5 ml) - D$_2$O (2.5 ml). The NMR (CDCl$_3$) of

the product was identical to that reported above, i.e. no

exchange occurred at C$_{28}$.

Acetylation of 22b or 22a with acetic anhydride/pyridine

gave diacetate 22c. Recrystallisation from methanol gave

m.p. 207-209°. Mass Spec. Found: 540. $C_{33}H_{48}O_6$ requires:

mol.wt. 540. TLC of compounds 22a, 22b, and 22c on SiO$_2$

using 10% EtOAc/C$_6$H$_6$ as solvent showed each compound as a

single spot.

Preparation of (22R,23S) 3β,23-dihydroxy-24-carboxymethylene-5-en-7-one-29,22-lactone (11a).

A stirred solution of 22a (100 mgs) in 20 ml pyridine containing 10 mg hematoporphyrin was irradiated in a water-cooled jacket with seven 15 Watt fluorescent lamps (General Electric F15T12), during which time O_2 was bubbled through the solution. The reaction was monitored by TLC (SiO_2/ 4% MeOH in $CHCl_3$). After 24 hrs. the mixture was diluted with ether, treated with 20 mg charcoal, filtered through celite and the ether evaporated. Cupric acetate (70 mg) was added to the pyridine solution and the mixture was stirred for 5 hrs. and diluted with ethyl acetate. The solution was washed with dilute phosphoric acid, dil. $NaHCO_3$ and H_2O, dried over $MgSO_4$ and evaporated to dryness to give 11a. Recrystallization twice from ethyl acetate gave m.p. 240-243° (50%). ν_{max}(KBr) 3390, 2940, 1685, 1380, 1270 and 1050 cm^{-1}; λ_{max} EtOH 230 nm (ϵ = 16,500). The solution of 11a which had been used for the uv spectrum (2 mg in 50 ml EtOH) was treated with a few drops of 6N NCl and refluxed for 4 hrs. The uv spectrum now gave maxima at 279 and 216 nm. Mass spec. (inlet temp. 270°): ^{m}/e (relative intensity) 470 (45, M$^+$), 452 (18), 345 (42), 344 (41), 287 (29), 269 (26), 245 (41), 126 (62), 43 (100); CD (c, 0.0345, MeOH)$[\theta]_{310}$ ±0°, $[\theta]_{300}$ - 538°, $[\theta]_{295}$ - 1080°, $[\theta]_{290}$ - 2160°, $[\theta]_{285}$ - 2960°, $[\theta]_{280}$ - 4300°, $[\theta]_{275}$ - 7000°, $[\theta]_{270}$ - 10,000°, $[\theta]_{265}$ - 14,800°, $[\theta]_{260}$ - 18,850°, $[\theta]_{255}$ - 20,460°, $[\theta]_{250}$ - 18,850°;

$[\theta]_{245}$ - 12,120°, $[\theta]_{240}$ - 4050°, $[\theta]_{225}$ - 18,850°, $[\theta]_{220}$-70,000°. Anal. Calcd. for $C_{29}H_{42}O_5$: C, 74.01; H, 8.99. Found: C, 74.21; H, 9.00.

The monoacetate 11b was prepared in a similar manner from 22b. Recrystallisation from benzene gave m.p. 290-295°C. NMR (pyridine) δ 0.68 (CH_3-C_{18},s), 1.09 (CH_3-C_{19},2CH_3-C_{25}, d, J=7Hz), 1.13 (H-C_{21}, d, J=6.5 Hz), 2.03 (CH_3OCO-.s), 3.20 (H-C_{25}, sept., J=7Hz), 4.44 (H-C_{22}, d, J=10Hz), 4.5-4.8 (2H,m; 23-H and H-C_3), 5.74 (H-C_6, broad s). 5.90 (lactone olefinic-H, broad s). Mass. Spec. Found: 512. Calcd. for $C_{31}H_{44}O_6$ requires mol.wt. 512.

Acetylation of 11a or 11b with acetic anhydride/pyridine gave diacetate 11c. Recrystallisation from methanol gave m.p. 215-216°C. Mass Spec. Found: 554. Calcd. for $C_{33}H_{46}O_7$ requires mol.wt. 554.

Preparation of 3β-acetoxy stigmasta-5,22,28-trien-24-ol (27b)

To 2ml of Grignard reagent prepared from magnesium turnings (1.20 g) and vinyl bromide (5.80 g) in dry THF (30 ml), was added drop wise a solution of 19a (500 mg) in THF (2 ml). The solution was left for 1 hour with occasional warming to 50°. Excess reagent was decomposed with a sat. soln. of ammonium chloride, the product extracted with ether and dried over $MgSO_4$. Evaporation of the solvent gave 27a (32%), recrystallized from ether/pet. ether, m.p. 150-155°C.

Acetylation with acetic anhydride/pyridine gave 27b recrystallized from ether/pet.ether, m.p. 138-140°; ν_{max} (KBr): 3470, 1730, 1368, 1260, 970 and 915 cm^{-1}. NMR (CDCl$_3$) δ 0.70 (CH$_3$-C$_{18}$, s), 1.02 (CH$_3$-C$_{19}$, s), 2.01 (CH$_3$OCO-, s), 4.3-4.8 (H-C$_3$, broad m), 4.9-5.6 (5H, H-C$_{23}$, -CH=CH$_2$, H-C$_6$, m), 5.96 (H-C$_{23}$, d of d, J=7Hz and J=17Hz). Mass. Spec. Found: 468. Calcd. for C$_{31}$H$_{48}$O$_3$ requires mol.wt. 468.

Preparation of 3β-acetoxy stigmasta-5,22,E 24(28)-triene-29-bromide (28).

To a solution of phosphorus tribromide (0.2 ml) in pet. ether (2 ml) was added pyridine (0.2 ml) and the mixture cooled to 0°. To this solution was added 27b (60 mg) and the resulting mixture stirred for 10 min. at 0°, diluted with ether, and finally washed several times with H$_2$O. The extract was dried over MgSO$_4$ and the solvent removed to give 28 as a colourless oil.

NMR (CDCl$_3$) δ 0.75 (CH$_3$-C$_{18}$,s), 1.02 (CH$_3$-C$_{19}$, s), 1.01 (2 CH$_3$-C$_{25}$, J=7Hz) 2.01 (CH$_3$OCO-, s), 4.12, 4.17 (CH$_3$-C$_{29}$, 2 xd, J=8.5Hz), 4.5-4.8 (H-C$_3$, broad m), 5.42 (H-C$_6$, narrow m), 5.5-6.1 (3H, m; C$_{22}$-H, C$_{23}$-H, C$_{28}$-H).

Preparation of 3β-acetoxy stigmasta-5,22,E 24(28)-triene (12b)

To a solution of 28 (100 mg) in THF (3 ml) was added lithium aluminium hydride (80 mg) and the mixture refluxed for 2 hrs. The solution was diluted with ether, washed with H_2O, and dried over $MgSO_4$. The residue, after removal of the solvent, was acetylated, and purified by TLC on silica gel -$AgNO_3$ (benzene). The product, 12b, was recrystallized from MeOH, m.p. 106-107°.

ν_{max}(KBr) 2940, 1740, 1365, 1250, 1110, 1035, and 955 cm^{-1}; λ_{max}(EtOH) 236 nm (ϵ=19000); NMR ($CDCl_3$): δ 0.72 (CH_3-C_{18}, s), 1.02 (CH_3-C_{19}, s), 1.01 (2CH_3-C_{25}, d, J=7Hz), 1.71 (CH_3-C_{29}, d, J=7Hz), 2.01 (CH_3OCO- ,s), 4.3-4.8 (H-C_3, broad m), 5.2-6.5 (4H, H-C_{22},C_{23},C_{28}, and C_6).

Mass. Spec. Found: 452. Calcd. for $C_{31}H_{48}O_2$ requires mol.wt. 452.

Preparation of 3β-acetoxy stigmasta-5,E 24(28)-dien-28-nitrile (26b).

A solution of diethyl cyanomethyl phosphonate (1.8 g) in dry THF (4ml) was added drop wise, with stirring, to a suspension of sodium amide (0.4g) in dry THF (6ml), at 0-5°C. The resulting mixture was stirred at r.t. under a slow stream of N_2 for 3 hrs. To the resulting yellowish-green solution was added 24a (2.5 g in THF, 4 ml), and the solution stirred for a further 20 hrs. The solvent was removed in vacuo, water added to the residue and the aqueous solution extracted

with ether followed by drying over $MgSO_4$ and evaporated to dryness. The residue was acetylated with acetic anhydride/pyridine to give 26b (68%) which was recrystallized from methanol, m.p. 108-109°C; ν_{max}(KBr) 2940, 2210, 1715, 1480, 1370, and 1255 cm^{-1}; λ_{max}(EtOH) 222 nm (ϵ=9000) and 260 nm (ϵ=2800); NMR ($CDCl_3$): δ 0.69 (CH_3-C_{18}, s), 1.02 (CH_3-C_{19}, s), 1.09 ($2CH_3$-C_{25}, d, J=7Hz), 1.12 (H-C_{21}, d, J=7Hz), 2.02 (CH_3OCO-, s), 4.3-4.8 (H-C_3, broad m), 5.10 (CN-CH=,d, J=1Hz), 5.38 (H-C_6, narrow m).

Anal. Calcd. for $C_{31}H_{47}O_2N$ (mol.wt.465): Found ($^m/e$,465).

Preparation of 3β-acetoxy stigmasta-5,E 24(28)-diene-29-oic acid (13b).

Hydrolysis of 26b (150 mg) was carried out by refluxing in 40% NaOH in 40% H_2O/MeOH for 4 hrs. After cooling, the solution was acidified with dilute HCl, extracted with ethyl acetate and the combined extract dried over $MgSO_4$ and evaporated to dryness to give 13a, which upon recrystallization from acetone had m.p. 180-182°.

Acetylation of 13a in the same manner gave 13b m.p. 74-75°; ν_{max}(KBr) 3400, 2940, 1760, 1375, 1280, 1115, and 1032 cm^{-1}; λ_{max}(EtOH) 208 nm (ϵ=5100); NMR ($CDCl_3$): δ 0.69 (CH_3-C_{18},s), 1.02 (CH_3-C_{19}, s), 2.02 (CH_3OCO-, s), 4.3-4.8 (H-C_3, broad m), 5.3-6.0 (2H, =CH-COO and H-C_6, narrow m). Mass. Spec. Found: 484. Calcd. for $C_{31}H_{48}O_4$ requires mol.wt. 484.

Preparation of antheridiol.

Antheridiol was synthesized by the method of McMorris et al[23]. The erythro isomers (RS) and (SR) were separated from the threo isomers, but since attempts at separation of the erythro isomers resulted in only small quantities of the required isomer (22S,23R), it was decided to use the isomeric mixture (erythro) in trapping experiments. Both isomers had similar Rf values on silica gel in several solvent systems and co-chromatographed with authentic antheridiol*.

Preparative TLC of antheridiol was performed using silica gel '254' (20 x 20 cm, 1mm thick) with 6% MeOH/CHCl$_3$.

Preparation of tritium labelled compounds.

(2, 4-^{3}H$_3$) Fucosterol was prepared by the method of Thompson et al.[49]. The product was purified by TLC (silica gel/AgNO$_3$-benzene) and recrystallized from MeOH, had a specific activity of 1.33 X10^6 cpm/mg.

Tritium-labelled compounds 12 and 13 were prepared by the methods described above from the corresponding (23,25-^{3}H$_2$) ketone 19, obtained by tritium exchange on basic alumina deactivated with tritiated water as described by Hofmann

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* Kindly supplied by Dr. T.C. McMorris, The New York Botanical Garden, Bronx, N.Y.

et al.[50]. Compounds 12 and 13 had specific activities of 6.37 x 10⁴ and 1.85 x 10⁵ cpm/mg respectively.

Preparation of (29-^{14}C) 3β-hydroxy stigmasta - 5,22, E-24(28)-trien-29-oic acid (14) was carried out by the method of Edward et al.[5]. The side chain (27) was prepared from acetic acid-1-^{14}C (1.0 mC) by the method described[51]. Aldol condensation of 3β-acetoxy 22,23-bisnorchol-5-en-24-al(26) [m.p.137-139°] at -78° in THF with the anion of ethyl trans-3,4-dimethyl-2-pentenoate (27)[b.p. 46-48° (0.7 mm)] generated by treatment of 27 with lithium triphenylmethide in THF at -20° followed by warming the reaction mixture to 0° (45-90 min.) and work-up by acidification afforded the α,β-unsaturated lactone 28 (23%) [m.p. 208-212°]. Sequential treatment of lactone (28) with boiling 2% methanolic NaOH (5 hr.) and dilute HCl in aqueous methanol (15 min. at 20°) resulted in the formation of the (29-^{14}C) 3β-hydroxy-22,23-trans-dienoic acid (14) with a specific activity of 1.6x10⁴ cpm/mg.
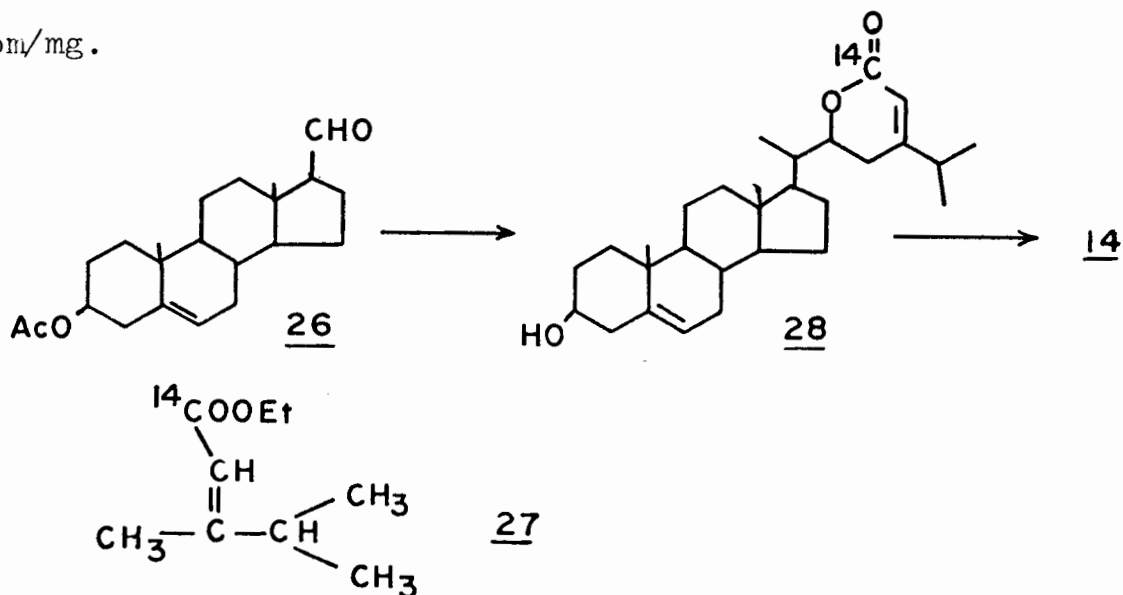
Synthesis of $(2,4-^3H_3)$ 7-dehydro fucosterol.

$(2,4-^3H_3)$ Fucosterol $(1.79 \times 10^5$ cpm/mg) (300 mg) was diluted with carrier fucosterol (700 mg) and benzoylated. A solution of fucosteryl benzoate (1g) in CCl_4 (25 ml) was heated to reflux and NBS (400 mg) added. The mixture was refluxed for 8 minutes, then cooled in ice and filtered. The filtrate was added drop wise to a boiling solution of trimethylphosphite (1.3 g) in xylene (8 ml). After refluxing for 90 minutes, the xylene was removed in vacuo and the residue hydrolysed by refluxing for 1 hr. in alcoholic KOH (20 ml). The residue was acetylated and the product purified on a silica gel column impregnated with $AgNO_3$ and eluted with benzene-hexane 1:1. 7-Dehydrofucosteryl acetate was further purified by preparative TLC on silica gel/$AgNO_3$ and hydrolysed by refluxing in 2% K_2CO_3 in 10% H_2O-MeOH for $\frac{1}{2}$ hour. Work-up in the usual way and recrystallization from MeOH gave 12 mg of 7-dehydrofucosterol m.p. 120-121°, reported 119-121°[29], with specific activity 5.3×10^4 cpm/mg.

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