BIOSYNTHETIC STUDIES ON THE p-BENZOQUINONES PRODUCED

BY <u>SHANORELLA</u> <u>SPIROTRICHA</u> BENJAMIN

by

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We accept this thesis as conforming to the

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THE UNIVERSITY OF BRITISH COLUMBIA

December, 1969

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ABSTRACT

From the culture medium of <u>Shanorella</u> <u>spirotricha</u> Benjamin, four p-benzoquinones have been isolated and identified. The major pigment is shanorellin (2,6-dimethyl-3-hydroxymethyl-5-hydroxy-1,4-benzoquinone). The other three pigments are the acetate, the ethyl ether and the dimer of shanorellin.

Shanorellin was found to be synthesized <u>via</u> the acetate-polymalonate pathway by tracer experiments with ^{14}C-labelled compounds and by chemical degradation of the labelled shanorellin produced.

A study was made of the optimal conditions required for the production of these benzoquinones. Significant factors were pH, temperature and sources of nitrogen and vitamins of the nutrient medium.

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INTRODUCTION

I. Natural Distribution of p-Benzoquinones

p-Benzoquinones are distributed widely in Nature. Two groups can be distinguished by their chemical structures: those with a polyprenyl side chain and those without a polyprenyl side chain. To the first group, which is also referred to as the isoprenoid quinones, belong the ubiquinones, plastoquinones, tocopherolquinones and rhodoquinones (1-3). The second group consists of a large number of variously substituted compounds, including a new benzoquinone, shanorellin (Fig. 1), produced by the fungus Shanorella spirotricha Benjamin, with which this thesis is concerned (4). The following discussion concentrates on this second group of benzoquinones only.
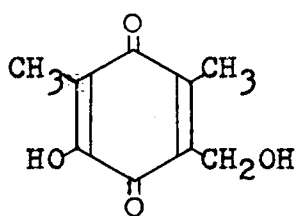
Fig. 1. Structure of Shanorellin.

Benzoquinones without a polyprenyl side chain have been isolated from fungi (5), lichens (6), higher plants (7,8) and arthropods (9,10). No compound of this type has been reported from animals other than the arthropods, gymnosperms, lower plants or bacteria. Some examples of benzoquinones occurring
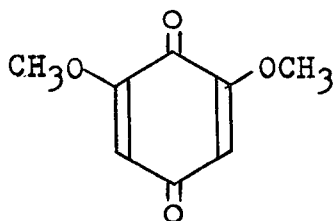
2,6-dimethoxy-
Benzoquinone

Ranunculaceae
Meliaceae
Simarubaceae
Apocynaceae
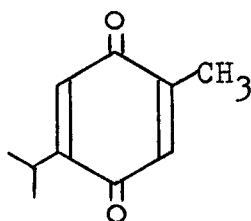
Thymoquinone

Umbelliferae
Cupressaceae
Labiatae
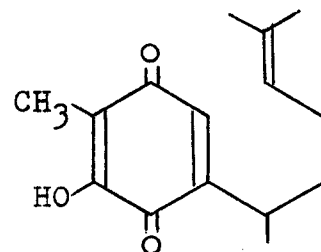
Perezone

Compositae

$R = C_{11}H_{23}$
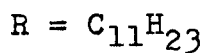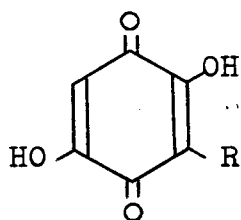
Embelin

Myrsinaceae

$R = C_{13}H_{27}$
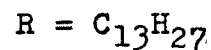
Rapanone

Myrsinaceae
Connaraceae
Oxalidaceae

$R' = C_{12}H_{43}$
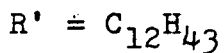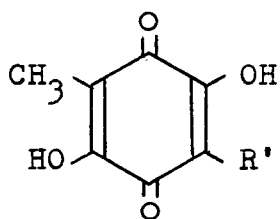
Polygonaquinone

Polygonaceae

$R' = (CH_2)_{13}CH=CHC_4H_9$
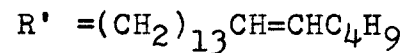
Maesaquinone

Myrsinaceae

Fig. 2. Examples of Naturally Occurring Benzoquinones
in Higher Plants.

in the angiosperms are illustrated in Fig. 2. The presence of the benzoquinones embelin, rapanone and maesaquinone in four genera of the Myrsinaceae distinguishes this family from the neighbouring family, Primulaceae (8). Ogawa and Natori, in their studies on the hydroxybenzoquinones of Myrsinaceous species from Japan, also concluded that the distribution of these benzoquinones is a chemotaxonomical characteristic of the Myrsinaceae (11).

Fungi produce the largest number of benzoquinones, and all of them are found in the Deuteromycetes, Ascomycetes and Basidiomycetes. These fungal metabolites can further be divided into three groups: monobenzoquinones, bibenzo-quinones and terphenylquinones (5,12). While the monobenzo-quinones and bibenzoquinones (Fig. 3) occur from all of the above three groups of fungi, the terphenyl derivatives (Fig. 3) are found principally in the Agaricaceae and Polyporaceae of the Basidiomycetes. The structures of fungal quinones isolated before 1963 are shown in the book 'List of Fungal Products' by Shibata, Natori and Udagawa (5). Since then, additional monobenzoquinones, apart from shanorellin, have been identified (13-17) and they are indicated in Fig. 4. From the lichens, polyporic acid has been isolated from Sticta and thelephoric acid from Lobaria.

Bibenzoquinones
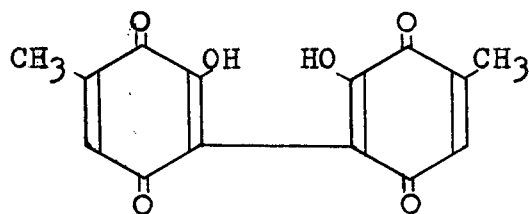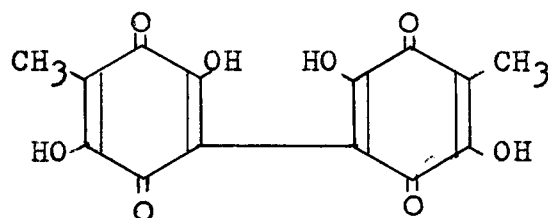
Phoenicin   Oosporein

Terphenylquinones
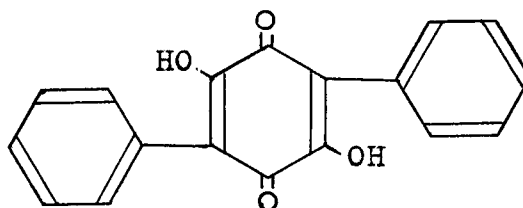
Polyporic Acid
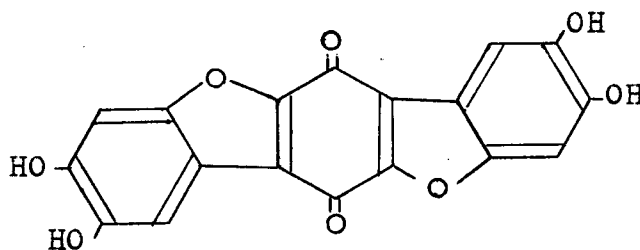
Theleporic Acid

Fig. 3.   Examples of Fungal Bibenzoquinones and
          Terphenylquinones.

Aspergillus fumigatus Fres.
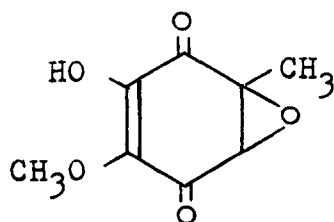
Fumigatin epoxide (13)

Lentinus degener Kalchbr.
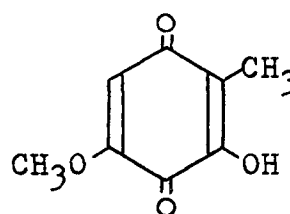
4-methoxy-6-hydoxyTQ

Helicobasidium mompa Tanaka
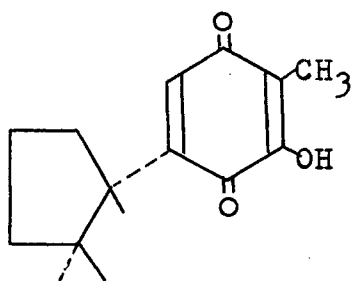
Deoxyhelicobasidin (15)

Penicillium spinulosum Thom

+ Fumigatin
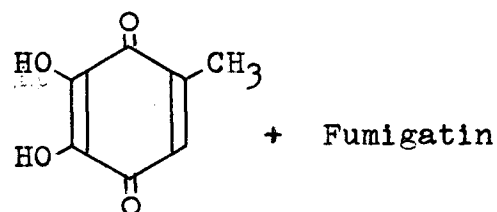
3,4-dihydroxyTQ (16)

Gliocladium roseum Bainer
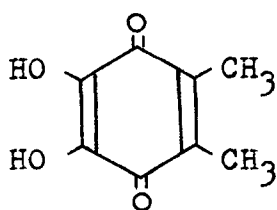
3,4-dihydroxy-6-methylTQ
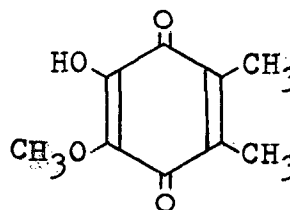
3-hydroxy-4-methoxy-6methylTQ (17)

Fig. 4. New Fungal Monobenzoquinones (TQ=Toluquinone).

II. Established Biosynthetic Pathways for p-Benzoquinones

There are three pathways established for the biosynthesis of benzoquinones. One of these, based on shikimic acid and its transformation products, leads to the biosynthesis of the quinone nucleus of the isoprenoid quinones (18). Two fungal benzoquinones, aurantiogliocladin (Fig. 9) and coprinin (Fig. 10) have also been reported to be formed via this route (7,19). Bolkart and Zenk (20), in their studies on the biosynthesis of methoxy-hydroquinone and 2,6-dimethoxy-hydroquinone glucosides in wheat seedlings, have shown that these two compounds (presumably the precursors for the corresponding quinones in wheat flour) are synthesized through oxidative decarboxylation of the similarly substituted benzoic acids as indicated in Fig. 5.

R	R$_1$	(I)	(II)	(III)
H	OCH$_3$	Ferulic acid	Vanillic acid	Methoxy-HQ
OCH$_3$	OCH$_3$	Sinapic acid	Syringic acid	2,6-dimethoxy HQ
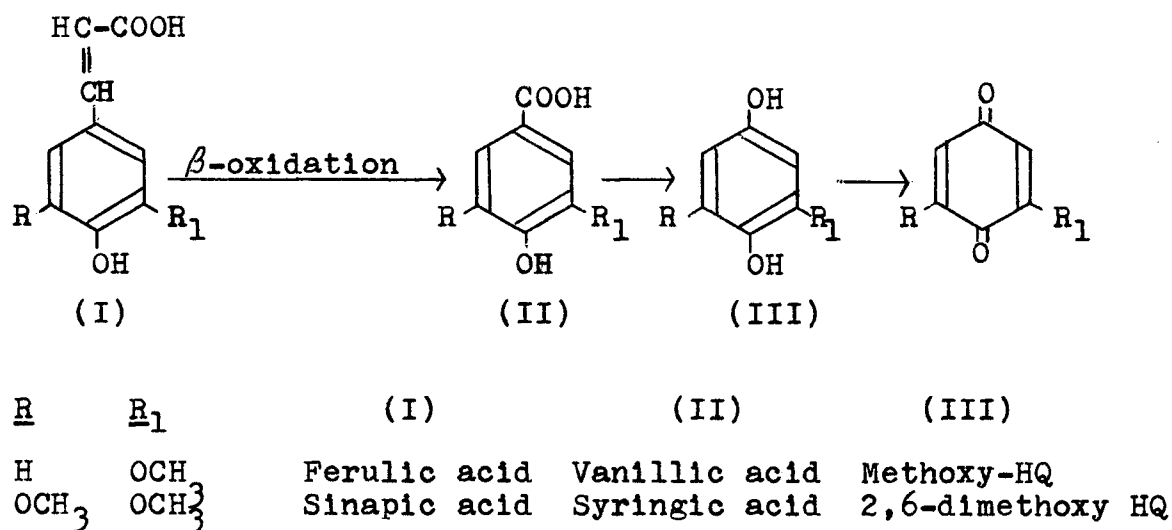
Fig. 5. Proposed Scheme for Benzoquinone Formation from Cinnamic Acid in Wheat Seedlings (HQ=Hydroquinone).

On the other hand, Kirk, Harkin and Cowling (21), using model compounds closely related to lignin, have demonstrated that cultures of wood rotting fungi, as well as purified p-diphenol oxidases from culture filtrates, are able to cleave the alkyl-aryl bond in substrates to form benzoquinone derivatives (Fig. 6). Caldwell and Steelink (22) have further obtained evidence suggesting that the depolymerization of lignin by these fungi is the result of a series of one-electron oxidation steps catalyzed by phenol oxidases. Thus, the wide distribution of 2,6-dimethoxy-benzoquinone reported from plants might indeed partly be due to the action of fungi rather than to the intrinsic biosynthetic capability of the plants.
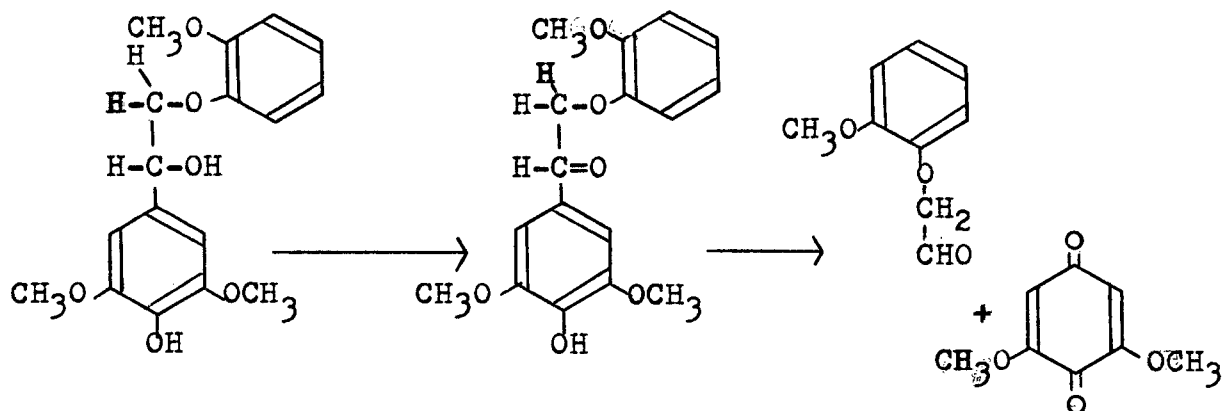
Fig. 6. Fungal Degradation of Lignin Model Compound Syringylglycol-β-Guaiacyl ether (22).

The second established pathway for benzoquinones bio-synthesis is based on the head-to-tail linkage of acetate units. It is by this route that most fungal monobenzoquinones are formed. The development of this acetate theory and the mechanisms of reactions proposed in respect to it will be discussed in detail later on.

The third established pathway is also based on acetate, but by way of mevalonate. The fungal benzoquinone, helico-basidin, has been shown to be synthesized as a sesquiterpene which undergoes subsequent ring formation and oxidation processes (15). Zenk has suggested a scheme for the formation of thymoquinone which occurs in the essential oil of Umbelliferae, Labiatae and Cupressaceae (23). All the intermediate compounds in this proposed scheme have been identified in the oil of _Monarda_ _fistulosa_ (Fig.7).
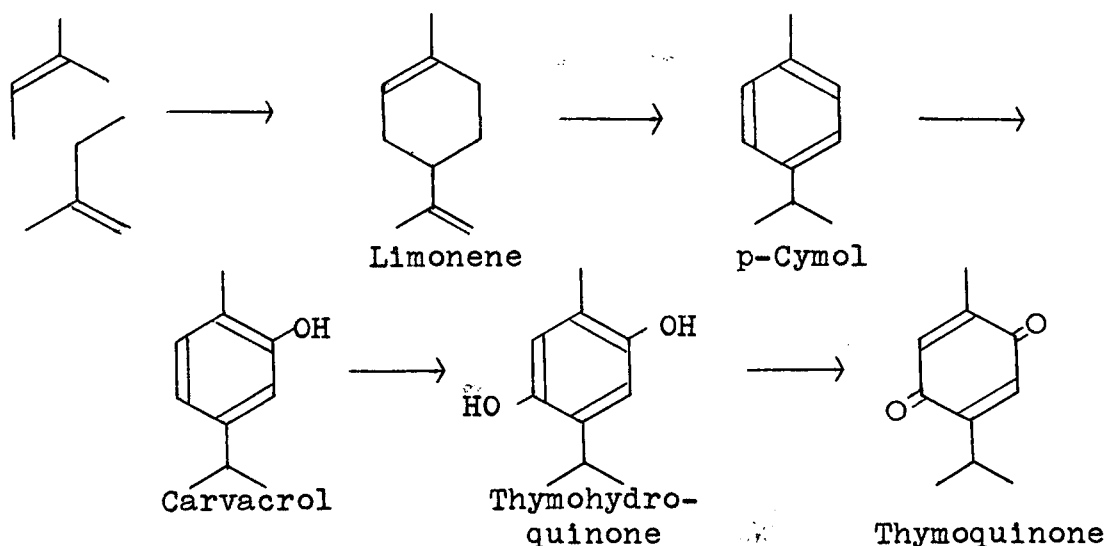
Fig. 7. Hypothetical Scheme for the Formation of Thymoquinone as proposed by Zenk.

III. Acetate-Polymalonate Pathway

(A) Historical Development

The possibility of the role of acetate in the biosynthesis of certain naturally occurring compounds was suggested by Collie in 1907 (24), but it was not until 1953 that Birch and Donovan proposed the hypothesis that these molecules were elaborated by head-to-tail linkage of acetate units (25). These compounds are marked by the frequent occurrence of oxygen atoms in β-positions to each other and β- to positions of ring closure. The first experimental proof for this hypothesis was the finding that sodium-1-^{14}C-acetate was incorporated into 6-methylsalicylic acid by Penicillium griseofulvum (26). Since then, the studies on the biosynthesis of many other fungal aromatic metabolites from 1- or 2-^{14}C-acetate have tended to confirm this overall pathway (27-32). With the increasing understanding in the biosynthesis of fatty acids and of sterols, Bassett and Tanenbaum (33) proposed that the activated form of acetate utilized would be acetyl-CoA and they obtained a cell-free extract from Penicillium patulum which converted acetyl-CoA into patulin, a metabolite known to be derived from 6-methylsalicylic acid (34,35). The idea that these aromatic compounds were formed by repeated condensation of acetyl-CoA was not entirely satisfactory due to the unfavourable equilibrium (37), and Lynen suggested that this difficulty could be overcome if malonyl-CoA were involved rather than acetyl-CoA (36). Lynen and Tada obtained from

Penicillium _patulum_ a soluble preparation which catalyzed the synthesis of 6-methylsalicylic acid from 1 mole of labelled acetyl-CoA and 3 moles of malonyl-CoA in the presence of NADPH (38). Lynen also proposed that the enzyme catalyzing the formation of the aromatic structure resembled the enzyme complex encountered in the studies of fatty acid synthesis in yeast and that the intermediates were bound to the complex by way of a thioester linkage, the product being liberated only after the process was completed (36). Later, in his investigations, Lynen found that at least two different types of sulphydryl groups are involved in the yeast fatty acid synthetase (78). If the initial steps in the polyketide biosynthesis do occur in steps parallel to that of fatty acid, the reaction sequence would occur as shown in Fig. 8.

Priming Reaction

$CH_3-COSCoA$ + $\begin{matrix} HS \\ HS \end{matrix}\rangle$ Enzyme $\longrightarrow$ $\begin{matrix} HS \\ CH_3-COS \end{matrix}\rangle$ Enzyme + HSCoA

Chain Lengthening Reactions

$\begin{matrix} HS \\ CH_3-COS \end{matrix}\rangle$ Enzyme $\xrightarrow{\text{malonyl-CoA}}$ $\begin{matrix} COOH \\ | \\ CH_2-COS \\ CH_3-COS \end{matrix}\rangle$ Enzyme + HSCoA

$\begin{matrix} COOH \\ | \\ CH_2COS \\ CH_3COS \end{matrix}\rangle$ Enzyme $\xrightarrow{\text{condensation}}$ $\begin{matrix} O \\ \| \\ CH_3-C-CH_2-COS \\ HS \end{matrix}\rangle$ Enzyme

+ CO_2

$$CH_3-\overset{O}{\overset{\|}{C}}-CH_2-CO\underset{HS}{\overset{S}{\diagup}}\hspace{-0.3em}\diagdown Enzyme \xrightarrow{\text{acyl transfer}} CH_3-\overset{O}{\overset{\|}{C}}-CH_2-CO\underset{\diagdown}{\overset{HS}{\diagdown}}\hspace{-0.2em}Enzyme$$

$$CH_3-\overset{O}{\overset{\|}{C}}-CH_2CO\underset{\diagdown}{\overset{HS}{\diagdown}}\hspace{-0.2em}Enzyme \xrightarrow{\text{+ n malonyl-CoA}} CH_3\overset{O}{\overset{\|}{C}}-(CH_2\overset{O}{\overset{\|}{C}})_{n-1}CH_2CO\underset{\diagdown}{\overset{HS}{\diagdown}}\hspace{-0.2em}Enz$$
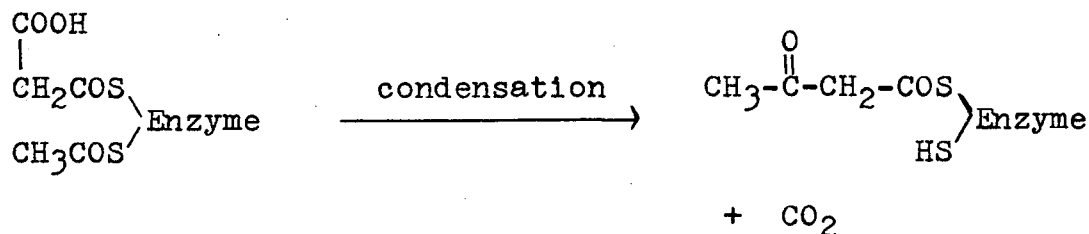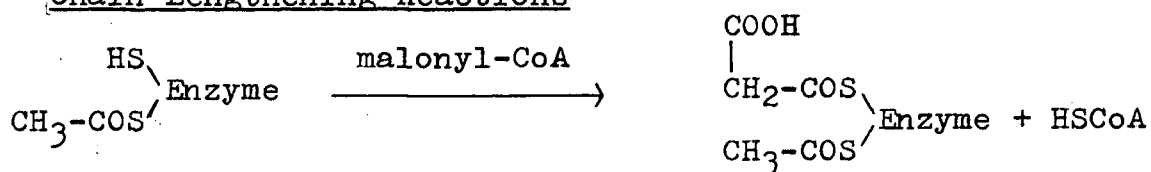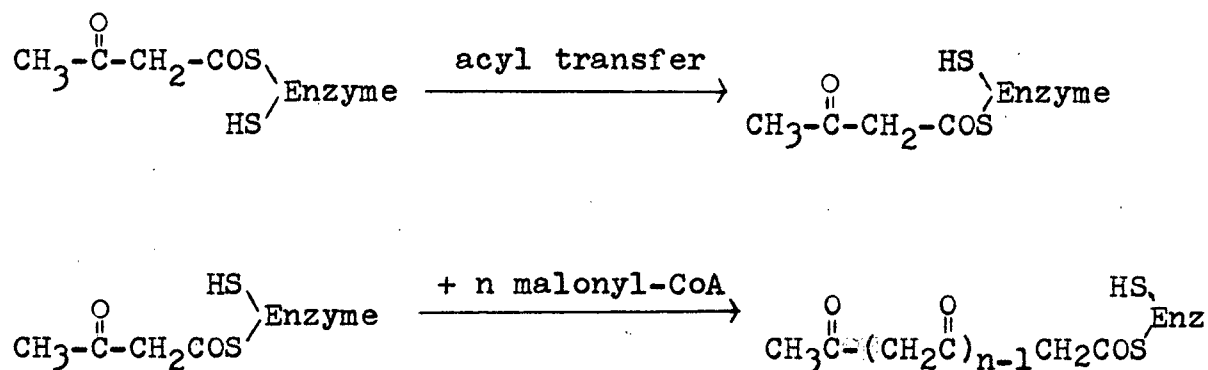
Fig. 8. Hypothetical Reaction Scheme for the Synthesis
of Polyketo-chain.

Experiments with ^{14}C-acetate and ^{14}C-malonate did show
that a wide range of fungal metabolites were derived by
repeated condensation of malonyl-CoA units with an acetyl-
CoA "starter", e.g. 6-methylsalicylic acid (35,40,42),
orsellinic acid (39), clavatol (44) and aurantiogliocladin
(43), (Fig. 9).

6-Methylsalicylic
Acid

Orsellinic Acid

Clavatol

Aurantiogliocladin
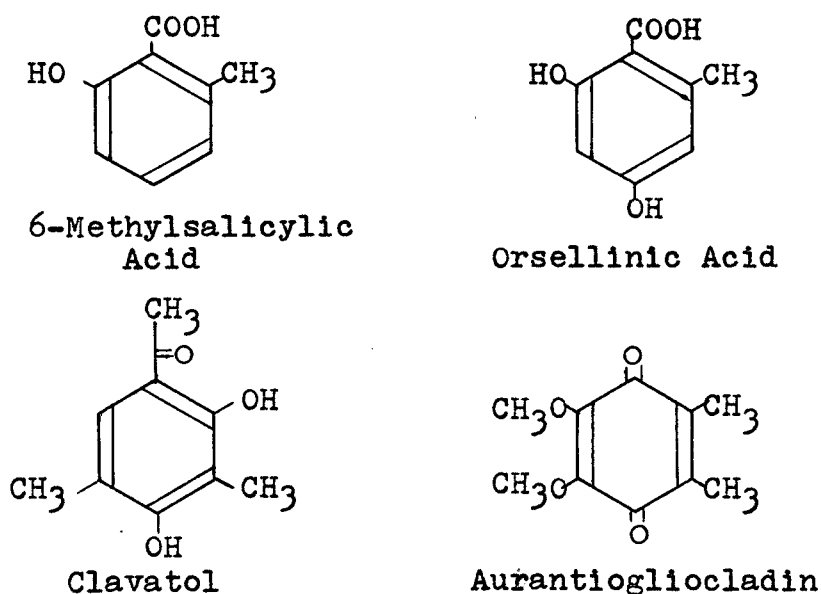
Fig. 9. Fungal Metabolites Biosynthesized from Acetate-
Polymalonate Pathway.

Crude enzyme preparations have recently been isolated
for the biosynthesis of 6-methylsalicylic acid from Peni-
cillium patulum (45) and orsellinic acid from Penicillium
madriti (46). For the 6-methylsalicylic acid synthetase
to function, a NADPH-generating system was required. With
the crude orsellinic acid synthetase system, if NADPH was
added, both orsellinic acid and fatty acids were detected;
the omission of NADPH from the reaction mixture resulted in
the loss of fatty acid synthesis, but that of orsellinic
acid was retained.

Additional proof for the occurrence of the acetate-
polymalonate pathway was presented by Gatenbeck and Mosbach
(47). By culturing Chaetomium cochliodes with ^{18}O-acetate,
the ^{18}O content of the carboxyl group was found to be half
that of each hydroxyl group, in accordance with the idea
that coenzyme A is involved in the biosynthesis and that
hydrolysis of the final acyl-CoA intermediate would introduce
the second oxygen from water.

(B) The Role of Acetate-Polymalonate Pathway in Fungal Benzo-
    quinone Biosynthesis

The fungal benzoquinones derived from the acetate-poly-
malonate pathway are characterized by the presence of at least
one methyl group (or its various oxidation states) on the
quinone nucleus, and invariably, this carbon has been proven
to be derived from the methyl carbon of acetate. Bentley (48)
has further noted that 6-methylsalicylic acid and orsellinic

acid, both formed by the condensation of 1 mole of acetate with
3 moles of malonate, could be intermediates in the biosynthesis
of other aromatic compounds. With regards to benzoquinone
biosynthesis, all the experimental evidence reported so far
indicates that these two acids are precursors of quinones in
which there is no extra methyl group added to the quinone
ring other than the one derived from the methyl group of acetate.
Examples of these benzoquinones are coprinin, its hydroxylated
derivative, terreic acid, fumigatin and spinulosin. Coprinin
(49), its hydroxylated derivative (50) and terreic acid (51)
have been shown to be synthesized from 6-methylsalicylic acid
(Fig. 10), and spinulosin (16) and fumigatin (62,52,69) from
orsellinic acid (Fig. 11). Examples of quinones with an
extra methyl group on the ring are those reported from
Gliocladium roseum: gliorosein, aurantiogliocladin and rubro-
gliocladin (Fig. 12). 5-Methylorcylaldehyde has been found
by Steward and Packter to be the first aromatic compound in-
volved in the biosynthesis of the quinones by the fungus.
Birch (7,49) and Pettersson (54) have studied the biosynthesis
of aurantiogliocladin in this fungus also, and their results
are indicated in Fig. 13.   The fact that 5-methylorcylald-
ehyde was incorporated into gliorosein to the extent of 36%
(53) as compared with the low incorporation of orsellinic
acid (0.07%) and 2,4-dihydroxy-5,6-dimethylbenzoic acid (0.9%)
into aurantiogliocladin (54), and that the introduction of
a methyl group is mechanistically favourable if it were
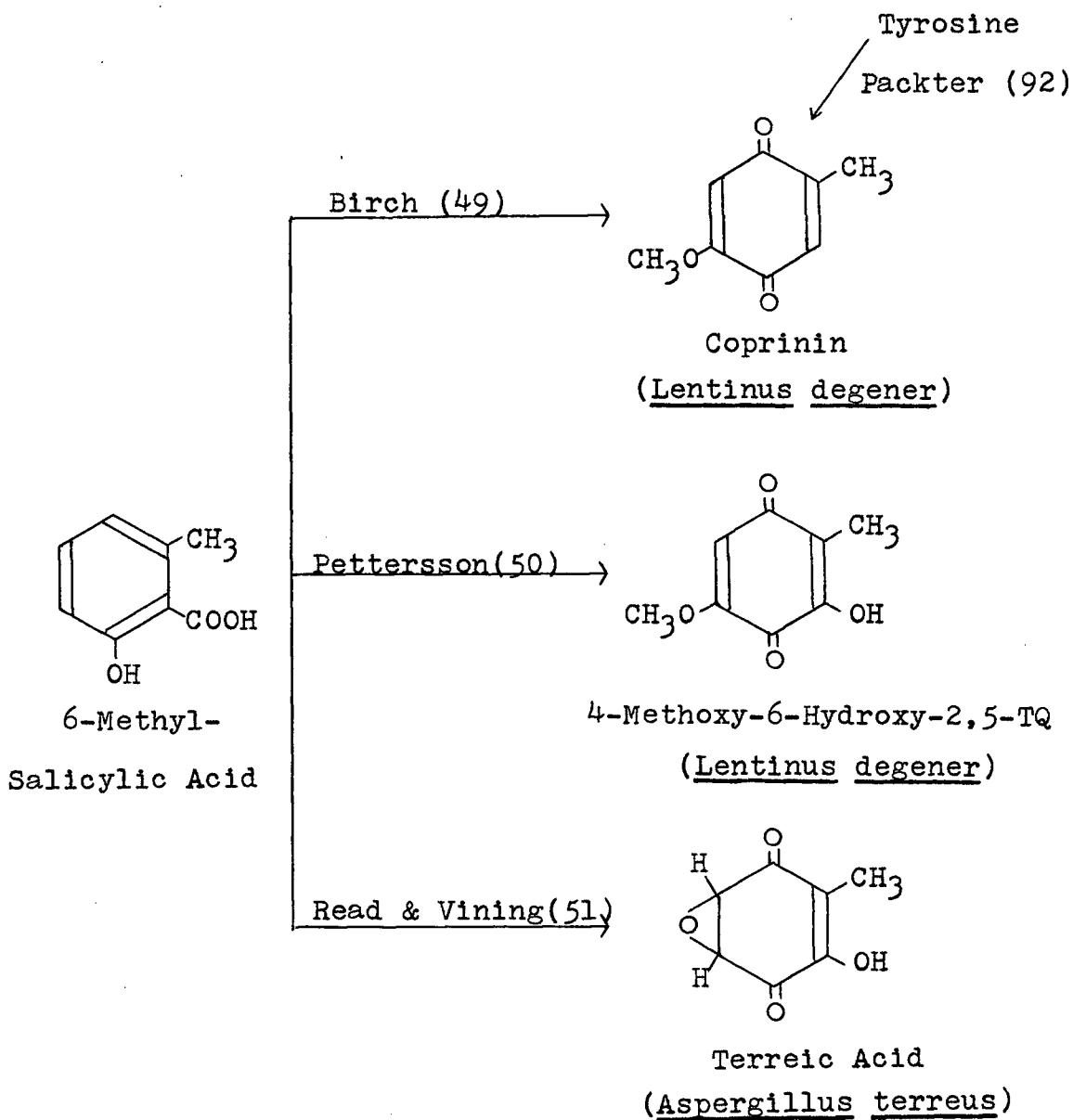
Fig. 10. Fungal Benzoquinones Derived from 6-Methyl-
Salicylic Acid. (TQ=Toluquinone)
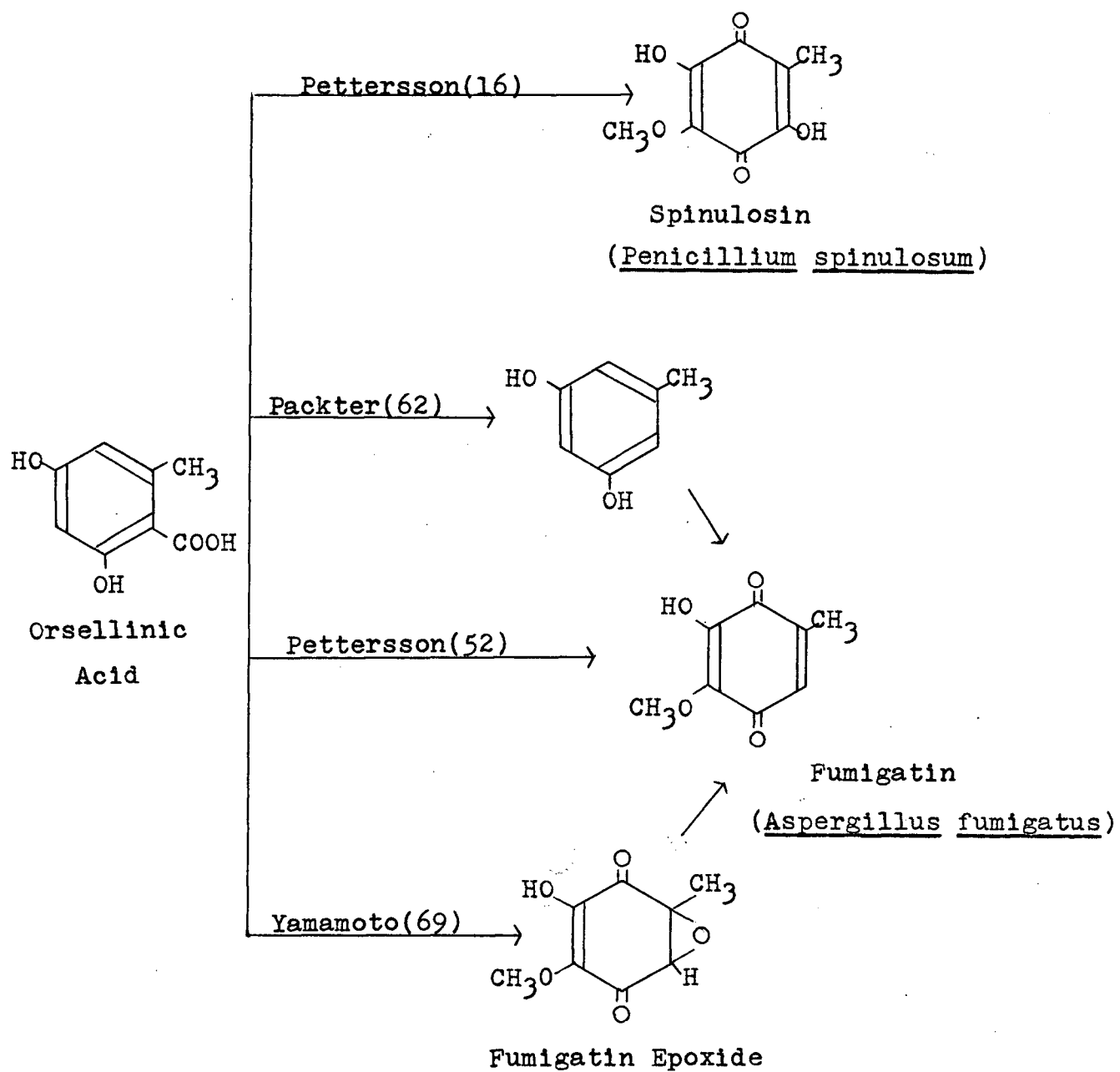
Fig. 11. Fungal Benzoquinones Derived from Orsellinic Acid.
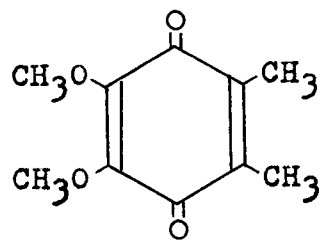
Fig. 12. Structures of Benzoquinones Produced by *Gliocladium* *roseum*.

Birch

    6-Methylsalicylic Acid (49)⌐

                        ⊢→ Aurantiogliocladin

    Phenylalanine (7)⌐
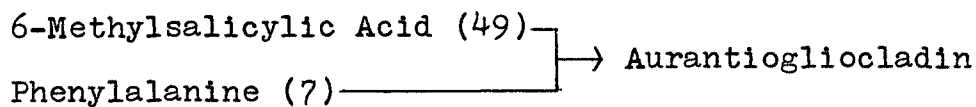
Pettersson (54)

Steward and Packter (63)

    Acetyl-CoA +

    3 Malonyl-CoA +

    S-Adenosylmethionine

Gliorosein
↓
Quinol
↓
Rubrogliocladin
↓
Aurantiogliocladin
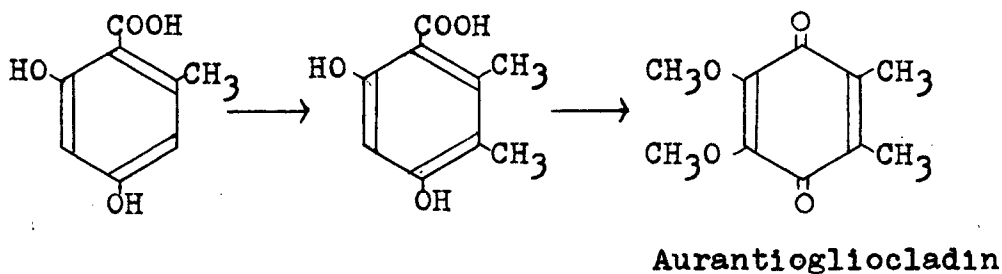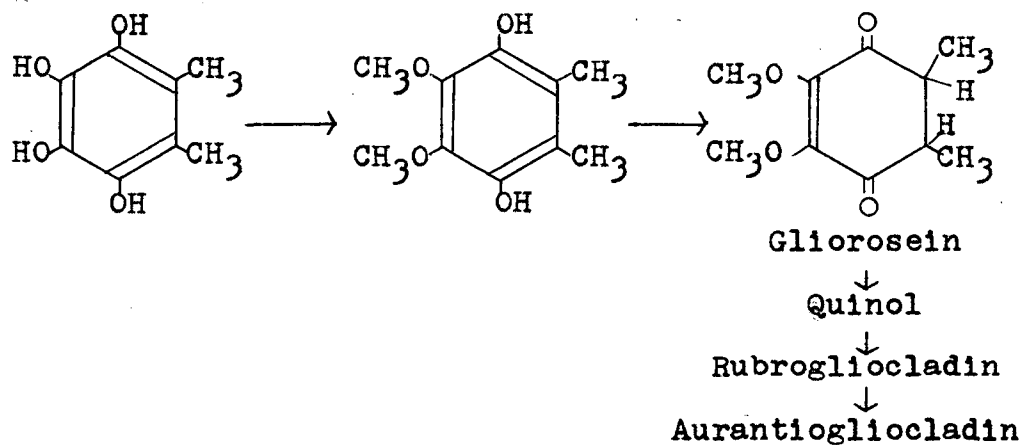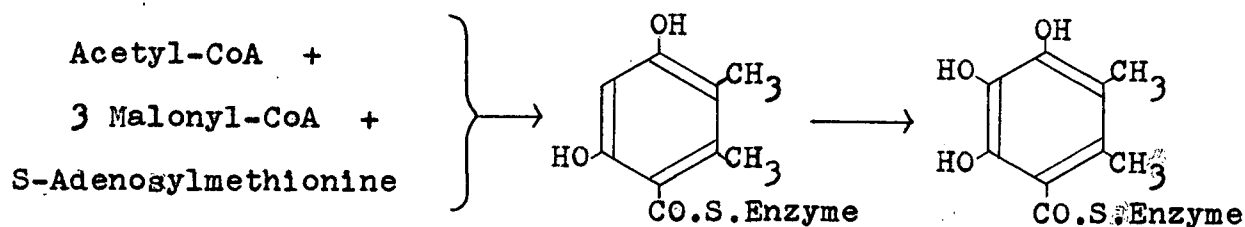
Fig. 13. Biosynthetic Pathways Proposed for the Quinones

from Gliocladium roseum.

to be added to a polyketo-chain rather than to an aromatic ring, render the pathway proposed by Steward and Packter more acceptable.

Subsequent to the formation of these aromatic acids, other reactions such as decarboxylation, hydroxylation and O-methylation take place. The oxidation of the hydroquinone to the quinone has generally been accepted as the final reaction in the biosynthesis process. These reactions together with the proposed mechanisms for polyketide bio-synthesis will now be considered.

Bu'Lock, in accordance with the current concept of fatty acid biosynthesis, has postulated a mechanism for the conden-sation of acetate (bound to an acyl carrier protein as in fatty acid biosynthesis) and malonate units (similarly bound to acyl carrier protein) in the formation of a β-polyketoacyl chain (Fig. 14). "The condensing enzyme," Bu'Lock suggested(55), "carries a single -SH group and metal ion. The thioacyl derivative of the enzyme is formed first, and this then accepts, by further coordination with the metal, the malonyl-CP (here written as the enol). Condensation-decarboxylation occurs within the complex, producing the acylacetyl product attached to the carrier protein by the thioester link of the original malonyl-CP, but still attached to the enzyme through the metal. For fatty acid synthesis, dissociation of this complex gives acylacetyl-CP from which the higher acyl-CP is generated, by reduction, etc. For polyketide synthesis, the

acylacetyl-CP-metal-thiolenzyme complex undergoes transacyla-
tion internally, giving the acylacetyl-derivative of the enzyme,
and this reacts again, as shown, with further molecules of
malonyl-CP. Each condensation is followed by the internal
transacylation, and each condensation effectively inserts a
(CH_2CO) group between the growing chain and the enzyme. When
a certain stage is reached, this growing loop of $(CH_2CO)_n$ units
itself becomes capable of occupying the coordination position
of the metal ion; in the simplest case this is attained with
four C_2 units, and the resultant complex is of the types
illustrated in (Fig. 14)." Bu'Lock stated that the construction
of models had shown that all the stereoelectronic requirements
for these reactions were elegantly met. He also noted that
polyketide assembly and polyketide cyclization were disting-
uishable processes, each subject to genetic control.

After the formation but before the aromatization of the
β-polyketoacyl chain, two reactions are found to be closely
integrated with polyketide biosynthesis (55,56). These two
reactions are alkylation and deoxygenation. Alkylation invo-
lves the participation of cations such as CH_3^+ (from S-adenosyl-
methionine) and $(CH_3)_2C=CHCH_2^+$ (from isopentenyl pyrophosphate),
and these cations will undergo electrophilic substitution with
compounds that would readily yield an anion. On the methylene
carbons of the β-polyketoacyl chain, the presence of active
hydrogens adjacent to a carbonyl function renders these carbons
very susceptible to electrophilic attack. So, it is expected

$$\underset{\text{Enz}}{\overset{\text{M}}{|}}\overset{\text{SH}}{|} \quad \xrightarrow{\quad CH_3CO.SR \quad} \quad RSH \quad + \quad \underset{CH_3}{\overset{M}{O=C}}\overset{}{\underset{\text{Enz}}{-S}}$$

$$\underset{CH_2COSR}{\overset{CO_2}{\diagdown}} \quad \xrightarrow{\hspace{3cm}}$$

$$\xrightarrow{\hspace{2cm}} \quad CO_2 \quad + \quad \underset{CH_3}{\overset{O}{\underset{\|}{C}}} \quad \underset{\text{Enz}}{\overset{M}{\underset{SH}{|}}} \quad CH=C-SR$$

CH$_3$COCH$_2$CO.SR + Enz-SH   Polyketide Biosynthesis

Reduction etc.   (continued on next page)

Fatty Acid Biosynthesis

M

O   O

CH₃—C—CH=C—S

Enz

+   RSH

$$\xrightarrow{\quad CO_2 \quad CH_2CO.SR \quad}$$

O⁻
|
O=C
|
CH
↑
M   C—SR
O

O

CH₃—C=CH   CO

Enz   S

$$\xrightarrow{\quad} CO_2 \; +$$

M

O   O

CH₃—C   CO   CH—SR

CH

Enz—SH

$$\xrightarrow{\quad CO_2 \quad CH_2CO.SR \quad}$$

O⁻
|
O=C
|
CH
↑
M   C—SR
O

O   O⁻

CH₃—C   C=CH   CO

CH   Enz   S

CO₂   +

M —— Enz—SH

O   O

CH₃—C   C—SR

CH   CH

CO   C—O⁻

CH

(A)

(A)

Orsellinic Acid   2-Acetyl-Phloroglucinol
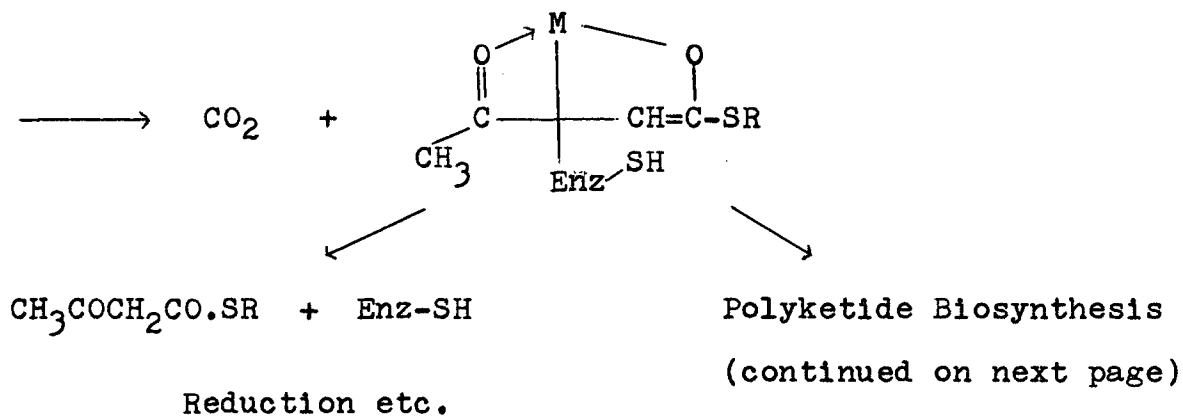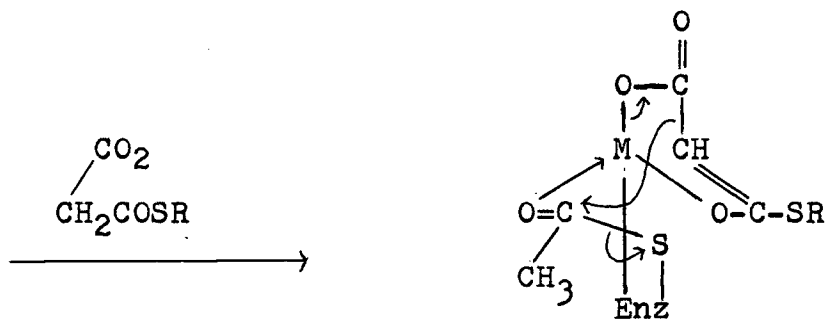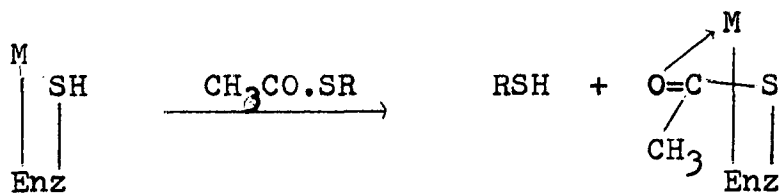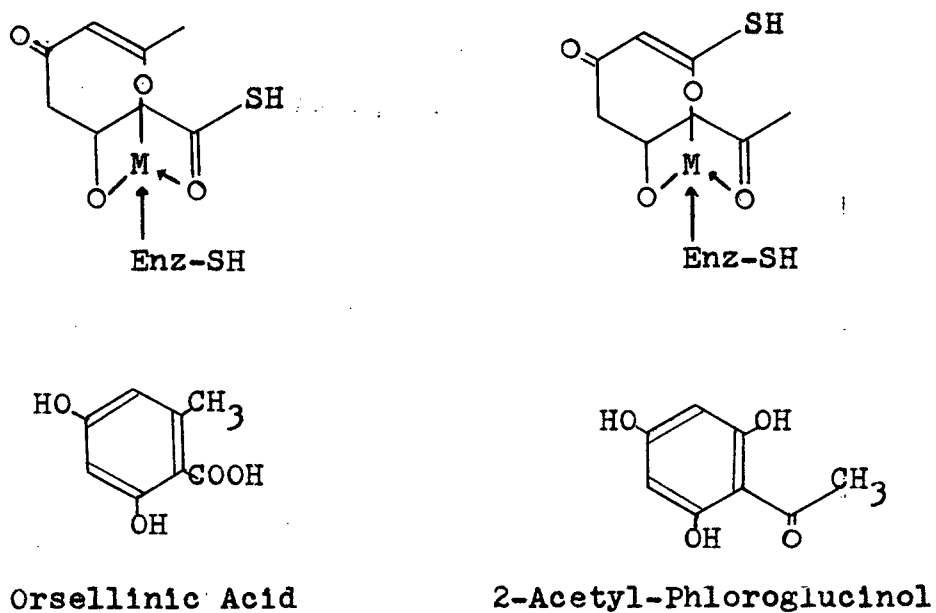
Fig. 14.   Mechanism for Formation of the Enzyme-Bound,
           Metal-Stabilized, Polyketide 'Loop' as Proposed
           by Bu'Lock.

that these methylene carbons are the site of alkylation reactions. This prediction is borned out by the observation that many polyketides are alkylated on the alternate, non-oxygenated atoms of the chain, e.g. clavatol in Fig. 9. Tracer studies with 6-methylsalicylic acid and orsellinic acid have shown that these two acids were not converted into gliorosein by Gliocladium roseum (19), but the methylated orsellinic derivative was (53). Labelled orsellinic acid has been shown to be the precursor of lecanoric acid but not of atranorin which possesses an additional C_1 unit on each ring of the lichen depside (57). Similarly, resaceto-phenone was not incorporated into clavatol by Aspergillus clavatus (44). Mutant studies in the tetracycline producing Streptomyces aurefaciens has demonstrated that the wild type could not transform the non-methylated cyclized intermediate into tetracycline (58). Recently, Gatenbeck, Eriksson and Hannson have isolated a crude protein fraction from Aspergillus flaviceps, a mould which produces a series of aromatic com-pounds such as 5-methylorsellinic acid, flavipin, and in low yield, orsellinic acid (59). Their results indicated that methylation occurred on a tetraacetic acid structure bound to the protein isolated, and not on the orsellinic acid. They suggested also that the protein to which the tetraacetic acid was attached might be involved in the formation of the polyketo-chain from acetyl-CoA and malonyl-CoA units. Thus, all the evidence would seem to establish that in polyketide synthesis

alkylation processes occur on the β-polyketoacyl structure

prior to cyclization and aromatization. Mention must also

be made, however, of an enzyme system for non-specific C-

methylation of an aromatic nucleus from <u>Streptomyces rimosus</u>

which was shown to convert resacetophenone and 3-methylres-

acetophenone to clavatol (44).

Before considering the deoxygenation process, an exam-

ination of the mechanism proposed for the cyclization and

subsequent aromatization of β-ketoacyl chain would indicate

that deoxygenation occurs before aromatization. Two organic

reaction mechanisms have been suggested for the formation

of an aromatic ring (56,60): (1) Claisen-condensation

involving an ester group and an activated CH_2 leading to

β-polyketoesters or β-polyketones, and (2) Aldol-conden-

sation involving a reactive methylene and a carbonyl function

(Fig. 15). These two reactions would lead to the formation

of two basic forms of C_8 polyketides, the orsellinic and

the 2-acetyl-phloroglucinol types. There are two other groups

of C_8 polyketides which can be formed similarly but which have

one oxygen function missing. These are the 6-methylsalicylic

and 2-acetyl-resorcinol types of compounds (Fig. 16). As can

be seen in Fig. 14 and 15, the position at which deoxygenation

occurs is at the carbonyl that plays no part in the cyclizat-

ion of the β-polyketoacyl chain. Birch has proposed that

this absence of an oxygen function could result from reduction

of a carbonyl in an open-chain or cyclic, but non-aromatic,
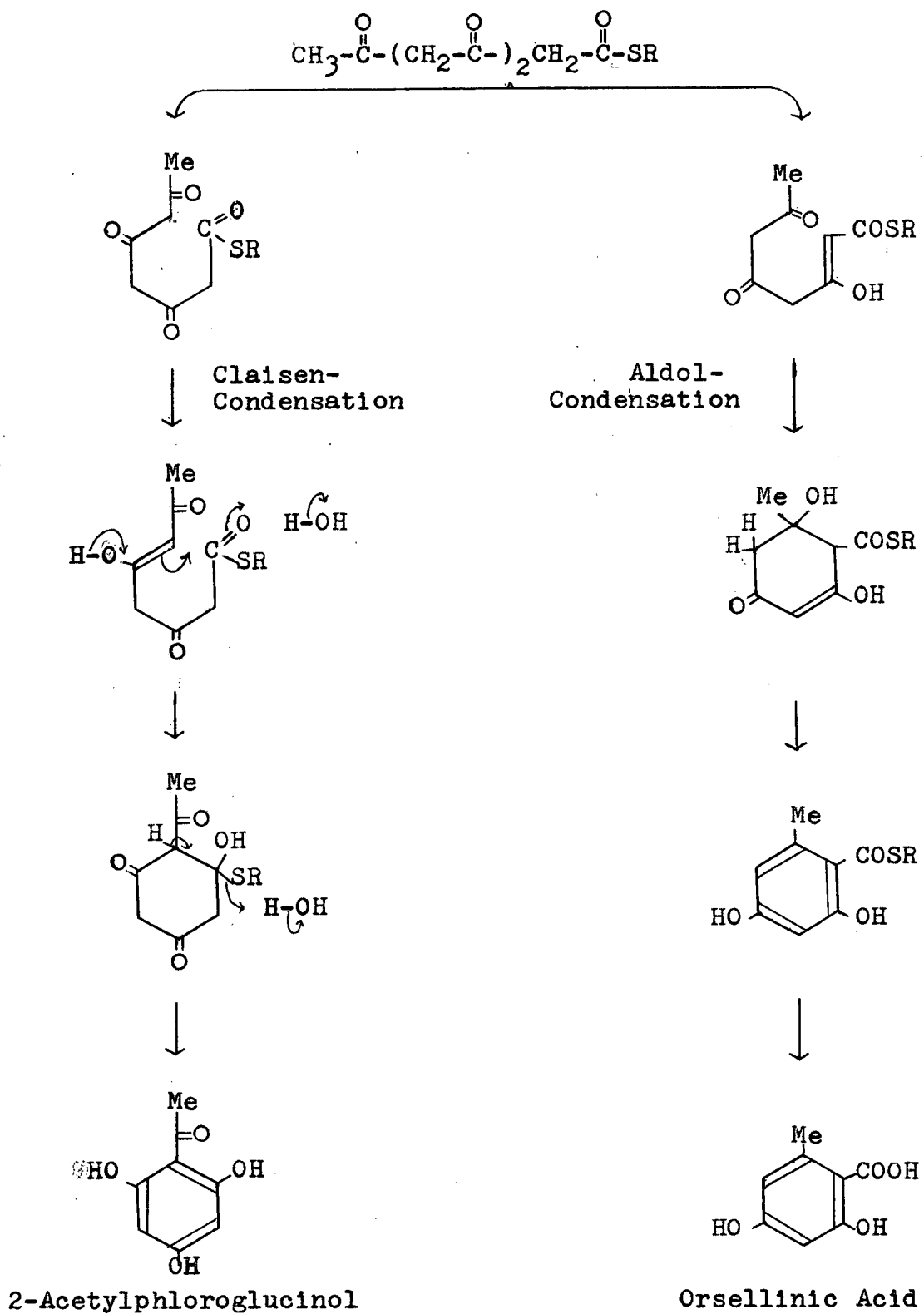
Fig. 15. Condensation Reactions Proposed for Cyclization of C_8 Chain in Polyketide Biosynthesis.

$$CH_3-\overset{O}{\overset{\|}{C}}-(CH_2-\overset{O}{\overset{\|}{C}}-)_2CH_2-\overset{O}{\overset{\|}{C}}-SR$$

Claisen-Condensation   Aldol-Condensation

2-Acetyl-Resorcinol   6-Methylsalicylic Acid
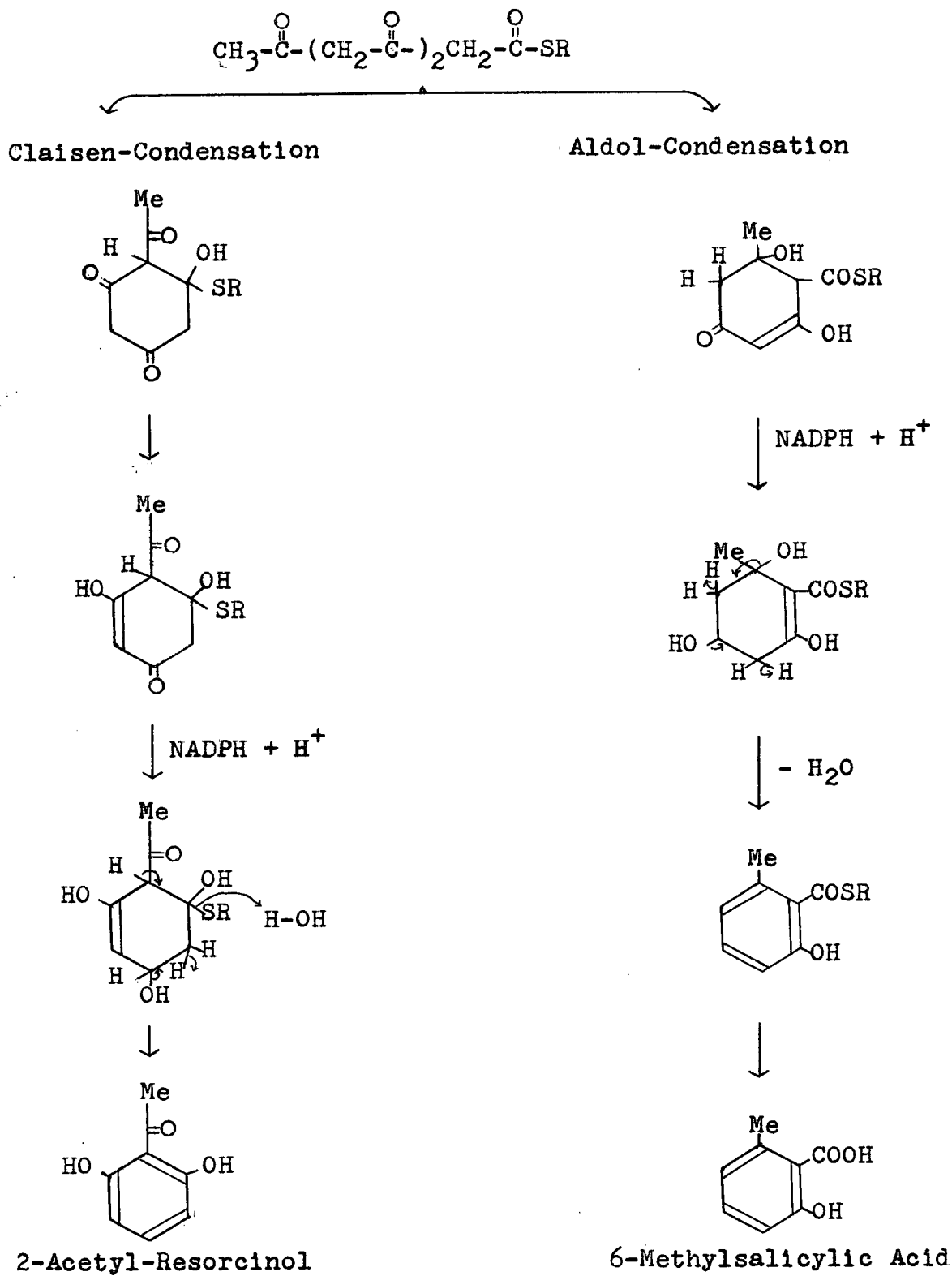
Fig. 16. Cyclization of C_8 Chain in Polyketide Biosynthesis with Reductive Steps.

precursor, aromatization being accomplished by dehydration rather than by enolization (60). No experimental proof has been put forth for this suggestion, but evidence for the synthesis of aromatic compounds from shikimate pathway does give support to this proposal. Dehydroshikimic dehydrase has been isolated for the formation of protocatechuic acid from Neurospora crassa (75) and prephenic acid aromatase for phenylpyruvic acid from Escherichia coli (76). Both studies have shown that these two acids were formed by a dehydration process involving the respective cyclic but non-aromatic precursors. Also, in microorganisms and higher plants, the keto-acids that give rise to phenylalanine and tyrosine from shikimic acid are known to branch from each other after prephenic acid (77). Similar types of reactions would be quite likely to occur in the biosynthesis of the above described oxygenated and deoxygenated types of compounds derived from the acetate-polymalonate pathway.

For the formation of the aromatic ring, Birch has suggested that proper activation of the appropriate carbonyl function together with an enzyme to bind the intermediate correctly would bring about ring closure at the appropriate carbon atoms (56). As illustrated earlier, Bu'Lock has proposed another mechanism involving a chelated metal complex for these reactions. Two other mechanisms have been postulated for the formation of an aromatic ring from acetate units (32,47), but due to lack of supporting evidence, they are generally disregarded.

Decarboxylation is a common process in the secondary metabolism of molds (74), and is involved in all fungal benzoquinone biosynthesis. Pettersson (52) showed that orsellinic acid, specifically labelled at C_2 was converted to fumigatin, presumably by a process of non-oxidative decarboxylation (Fig. 17). That this is indeed the process is supported by the finding that orincol was a good precursor of fumigatin (62).
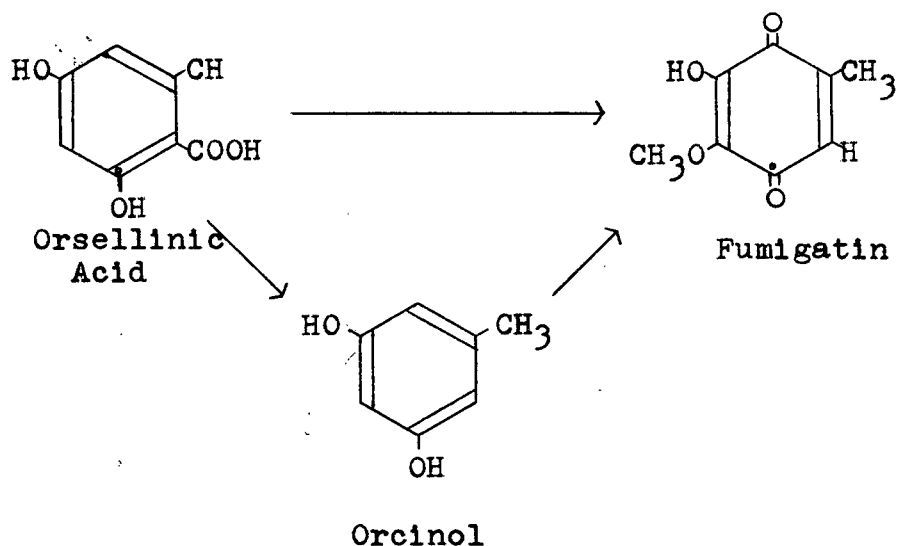
Fig. 17. Formation of Fumigatin from Orsellinic Acid and Orcinol.

With Gliocladium roseum, the formation of gliorosein
involves decarboxylation and oxidation at the same carbon
atom. 1,3-Dihydroxy-4,5-dimethylbenzene was found not to
be incorporated into gliorosein (54,63) but 5-methyl-orcylal-
dehyde, which presumably was converted to the acid derivative
in the organism, was incorporated (53,63). An oxidative
decarboxylation reaction was thus implied to have occurred,
though, a non-oxidative decarboxylase has been isolated from
the same organism (64). Pettersson has reported the failure
of incorporation of fumigatin into spinulosin by a certain
strain of Penicillium spinulosum which synthesizes both
fumigatin and spinulosin and both quinones have been shown to
be derived from orsellinic acid (65). This failure can be
explained if their metabolic pathways depart from each other
at the stage of decarboxylation. An oxidative decarboxylation
would give rise to spinulosin and a non-oxidative decarboxyl-
ation would give rise to fumigatin (Fig. 18A). A similar
explanation could account for the unsuccessful conversion of
coprinin hydroquinone to 4-methoxy-6-hydroxy-2,5-toluquinone
by Lentinus degener (65) (Fig. 18B).

Hydroxylation is another common metabolic process. It
occurs widely in microorganisms, especially in the degradation
of aromatic compounds (66). The conversion of 6-methylsali-
cylic acid to orsellinic acid has been reported by Packter
to occur in Aspergillus fumigatus, though 6-methylsalicylic
acid has never been detected in normal cultures (67). The

A.

Non-Oxidative
Decarboxylation

Fumigatin

HO—CH₃... (structures)

Orsellinic Acid

Oxidative
Decarboxylation

Spinulosin

B.

Non-Oxidative
Decarboxylation

Coprinin

6-Methylsalicylic
Acid

Oxidative
Decarboxylation

4-Methoxy-6-Hydroxy-
2,5-Toluquinone
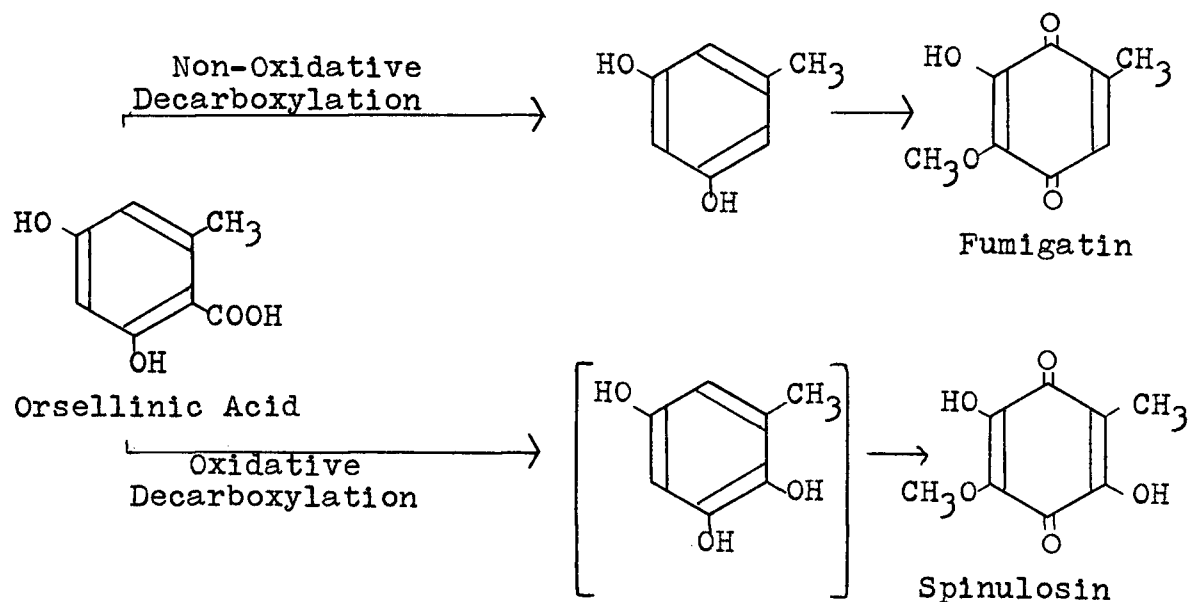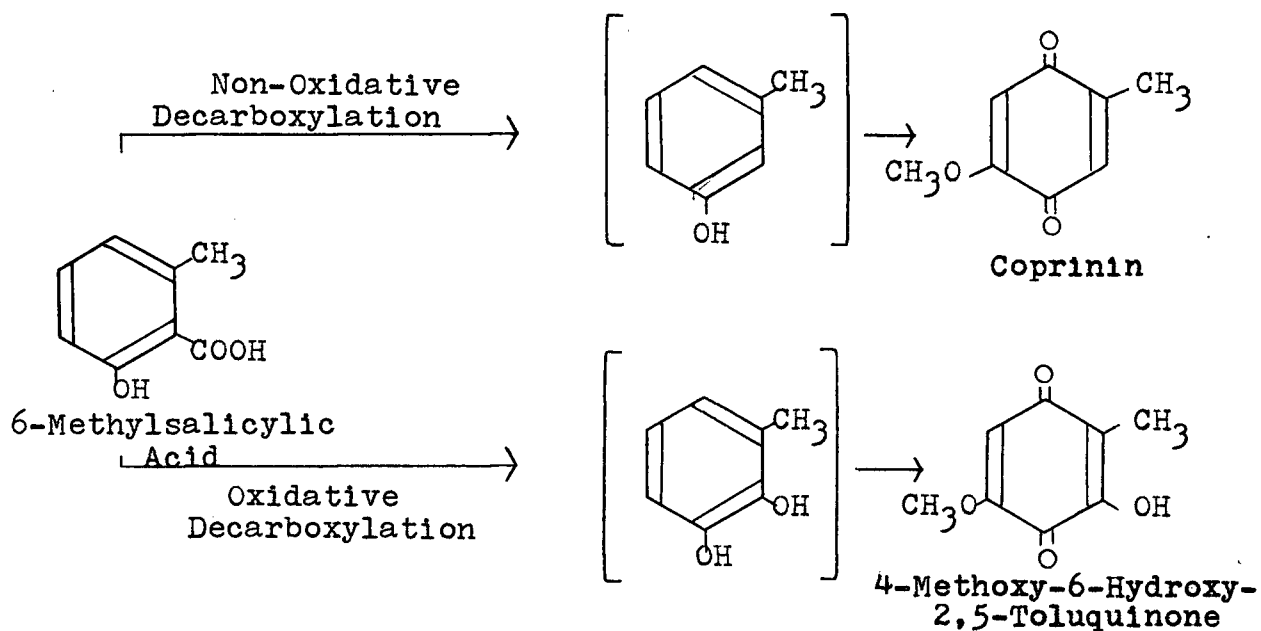
Fig. 18. Modes of Decarboxylation in the Biosynthesis

of Fungal Toluquinones.

conversion of orsellinic acid to 6-methylsalicylic acid has been mentioned by Glover, but his results have not been published (68). The origin of an epoxide oxygen on a benzoquinone ring is of particular interest. Read, Westlake and Vining (51) have established that atmospheric oxygen is the source of the epoxide oxygen in terreic acid, and the oxygen was introduced into an intermediate derived from 6-methylsalicylic acid. Yamamoto et al., have presented evidence that fumigatin epoxide is converted to fumigatol and fumigatin but not vice versa by a strain of Aspergillus fumigatus (Fig. 11) and that spinulosin arises as an artifact from this epoxide during isolation.

The methyl group of the methoxy function in fungal benzoquinones has been shown to originate from methionine and formate (28,63). The active methyl donor in this reaction is regarded as S-adenosylmethionine, the formate carbon being converted to the methyl carbon of methionine before incorporation. Steward and Packter have made a study of the O- and C-methylation processes in gliorosein biosynthesis with (Me-^{14}C) and (Me-^{3}H)-methionine (63). They reported that the methyl group was transferred intact in O-methylation but only two tritium atoms from the original methyl group was transferred in C-methylation. Similar results with respect to C-methylation have also been observed in ergosterol and tuberculostearic acid biosynthesis (70). In the same study, Steward and Packter also reported that the specific activities

of the labelled O-methyl and C-methyl groups showed an appreciable difference. The percentage difference between C-methyl and O-methyl was 14% in a methionine-$^{14}CH_3$ feeding, and 15% when formate-^{14}C was used. To explain this discrepancy, they suggested two possibilities. The first one was that two methyl group pools were involved. The second one was that, if the C-methylated precursor was already present in a much larger amount at the time when the labelled compounds were added, the O-methyl groups formed subsequently would have a higher specific activity.

It is generally agreed that the final step in fungal benzoquinone biosynthesis is the oxidation of the corresponding hydroquinone. Küster and Little (71) had proposed that fumigatin and spinulosin might be formed from the corresponding hydroquinones by the action of an endocellular phenolase system, but their experimental results have been disproved by Pettersson (72). In Pettersson's view, these quinones are derived from their respective hydroquinones by a non-enzymatic air oxidation process in the culture medium (65). He arrived at this conclusion by (1) determining the relative proportion of oxidized to reduced quinone in the medium during growth of the mold by potentiometry, (2) studying the kinetics of autooxidation of hydroquinones to quinones, (3) isolating and identifying the hydroquinones in the medium. On the basis of these criteria, his results from studies of cultures of Aspergillus fumigatus (72), Gliocladium roseum (54),

Penicillium spinulosum (16) and Lentinus degener (14) have
shown that all the quinones produced were present in the
reduced forms during the larger part of the production phase,
being converted into the quinone forms at a later stage in the
development of the molds. Packter and Steward, by correlating
the results obtained from the production curve of each
metabolite concerned with the specific activities of these
compounds from radioactive tracer experiments, came to a
somewhat different conclusion than that suggested by Pettersson
(73). After feeding Gliocladium roseum with acetate-1-^{14}C,
they isolated aurantiogliocladin only from the mycelium, and
gliorosein, the quinol and quinhydrone of aurantiogliocladin
from the medium. The amounts and specific activities were
found to increase in the order listed. Moreover, when labelled
aurantiogliocladin was added to the medium, 52% of it was
found to enter the mycelium, and labelled gliorosein and the
quinol were obtained on isolation. Also, gliorosein has been
found to isomerize to the quinone aurantiogliocladin (via the
quinol and quinhydrone) when allowed to incubate alone in
autoclaved medium. From these results, Packter and Steward
proposed a scheme (Fig. 19) to indicate the interrelationship
among these compounds and their probable sites of formation
(19). With similar type of studies with Aspergillus fumigatus,
Packter (62,67) concluded that although most of the fumigatin
present in the medium could arise from oxidation of fumigatol,
some fumigatin was secreted as such, but his interpretation
of his results was strongly refuted by Pettersson (50).

<u>Mycelium</u>

Gliorosein $\longleftrightarrow$ tautomerase rapid $\longrightarrow$ Quinol $\longleftarrow$

Gliorosein $\xrightarrow{\text{spontaneous}}$ Quinol $\longrightarrow$ Quinhydrone $\longrightarrow$ Quinone
slow
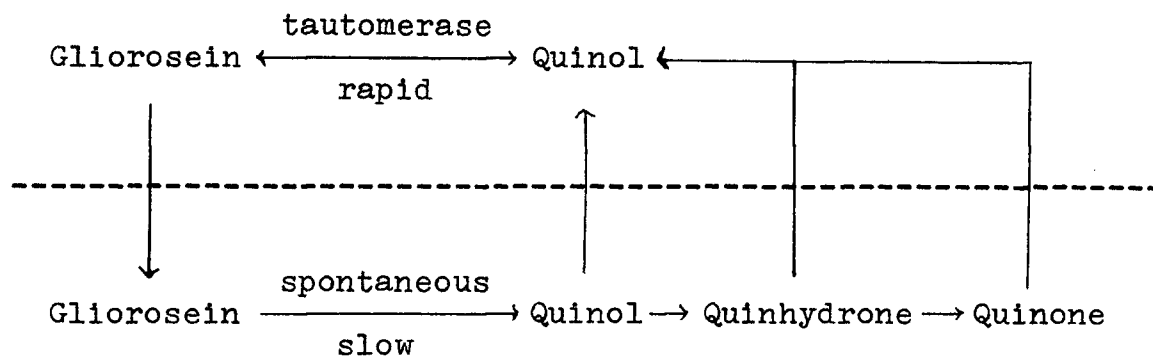
<u>Medium</u>

Fig. 19. Proposed Interrelationship of Quinones and

Hydroquinone from <u>Gliocladium</u> <u>roseum</u>

(Quinol = aurantiogliocladin hydroquinone

Quinhydrone = rubrogliocladin

Quinone = aurantiogliocladin

See Fig. 12 for structures)

Shanorella spirotricha: A p-Benzoquinone-Producing Ascomycete

Shanorella spirotricha was described by R.K. Benjamin who classified it in the Gymnoascaceae of the Ascomycetes (79). The culture used in this study (UBC 240) was kindly provided by Dr. R.J. Bandoni (Dept. of Botany, U.B.C.).

Shanorellin (2,6-dimethyl-3-hydroxymethyl-5-hydroxy-1,4-benzoquinone) was first detected as an extracellular purple pigment when S. spirotricha was cultured on malt extract agar. On examination of the medium, the most conspicuous pigment (shanorellin) was found to behave similarly to the hydroxybenzoquinones, fumigatin and spinulosin, isolated by Raistrick et al. from species of Aspergillus and Penicillium (80,81). The physical, chemical and spectroscopic properties of shanorellin were determined, and its derivatives were prepared. From these data, two isomeric structures were found to be possible. Chemical syntheses were carried out for the two isomeric forms, and simultaneously, the chloro-derivative was prepared and subjected to X-ray crystallographic analysis.

Experiments on environmental effects and nutritional requirement of the culture were performed. The biosynthetic pathway by which shanorellin was produced was studied with various ^{14}C-labelled compounds. From the mixture of pigments present in the medium, a second compound was isolated and identified as the acetyl-derivative of shanorellin, a third and a fourth were found to be ether derivatives of shanorellin.

Part A. Environmental Effects on Pigment Production
and Growth in Shanorella spirotricha

Shanorella spirotricha was first cultured on a liquid
medium of modified malt extract. In about five days, a
mycelial mat was formed which was white initially but
changed to golden yellow and orange red as it became
established on the surface. About the eighth day, the
medium started to turn purple and by the fifteenth day,
the originally transparent yellowish brown medium was a
dark purple. As the culture aged, the mycelial mat grad-
ually turned brown and abundant cleistothecia were formed.
At the same time, the purple pigment in the medium started
to disappear, and in about two months, the color of the
medium was brown.

When cultured on Czapek-Dox medium, the mycelium grew
very slowly. On addition of yeast extract (0.2%) to the
medium, growth and pigmentation resembling that described
with malt extract were observed. Czapek-Dox medium with
the addition of 0.2% yeast extract was used as the standard
medium in the investigations which followed.

Observations were made of the effects on mycelial
growth and pigment production of pH, temperature and
nitrogen and vitamin sources of the medium.

General Methods

Culture Media

(A) Modified Malt Extract - malt extract (Difco) 30g, soytone (Difco) 5g, yeast extract (Difco) 1g, distilled water 1 litre.

(B) Modified Czapek-Dox Medium - glucose 50g, $NaNO_3$ 2g, K_2HPO_4 1g, KCl 0.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01g, yeast extract (Difco) 2g, distilled water 1 litre.

For agar plates, 1.5% agar was added to the above media.

Preparation of Inoculum

An ascospore from an old culture grown on modified malt extract agar was isolated with a micro-manipulator and transferred onto a modified Czapek-Dox agar plate. The culture which developed from this single spore was used for subsequent subculturing. A stock culture was kept on a modified malt extract slant.

Culture Conditions

All cultures were maintained in the dark at 25° in a Sargent Incubator unless otherwise stated.

I. Effects of pH

Method

Each sample consisted of one 125ml flask containing 50ml modified Czapek-Dox medium. The pH of the medium was adjusted by taking two autoclaved samples, titrating one with 0.5N HCl (autoclaved) and the other with 0.5N NaOH (autoclaved), to find the required volume of the acid or base to bring the medium to the pH values ranging from 4.2 to 9.0. The mycelial growth and pigment production were observed after 7, 12 and 17 days of incubation.

Results and Discussion

After 7 days of growth, surface mycelia began to appear in the solutions with initial pH 4.6 to 9.0. On the 12th day, purple pigmentation was apparent in flasks with initial pH ranging from 6.7 to 7.6. In the cultures starting with pH 6.5, 8.1 and 9.0, the purple pigment was already present in relatively large amount by this time (Fig. 20). By the 17th day, the media in flasks with the initial pH ranging from 6.0 to 9.0 were opaque with purple coloration, and pigment was beginning to appear in the culture with the starting pH of 5.3. Only slight pigmentation was observed in the two flasks with the lowest initial pH (Fig. 21). The final hydrogen ion concentrations of these media after 17 days of growth were measured and are indicated in Table I.

Fig. 20.   Appearances of S. spirotricha Cultures
with Initial pH 4.2 to 9.0 after 12 Days
of Incubation.
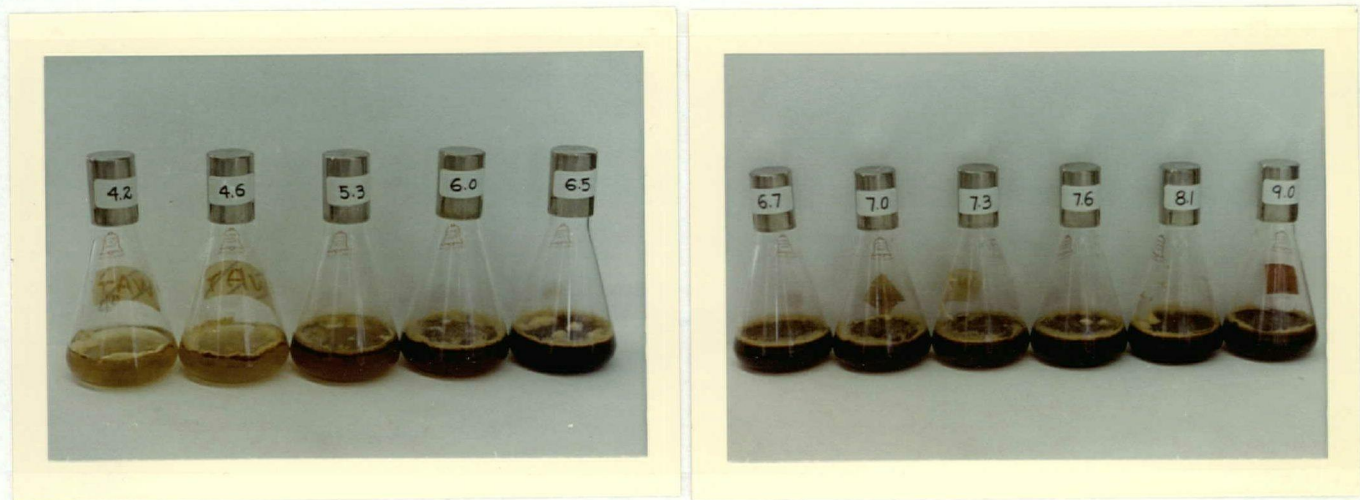
Fig. 21.   Appearances of S. spirotricha Cultures
with Initial pH 4.2 to 9.0 after 17 Days
of Incubation.

Initial pH	Final pH
4.2	4.6
4.6	5.7
5.3	6.2
6.0	6.4
*6.5	6.6
6.7	6.8
7.0	6.9
7.3	7.0
7.6	7.1
8.1	7.2
9.0	7.4

Table I. Changes in pH Values of Media Inoculated
with S. spirotricha after 17 Days of
Incubation. (* - normal pH of modified
Czapek-Dox Medium)

As can be observed from the color photographs, all
the flasks showed similar mycelial development after 17
days of incubation, except the one with the normal pH
of Czapek-Dox medium, i.e. pH 6.5. This flask showed a
more copious mycelial growth as well as an early onset of
pigment production. An early onset of pigment production
was also shown by the two flasks with initial pH of 8.1
and 9.0. Since the oxidation of hydroquinone to the quinone
form by air in a buffer solution at pH 8 is a standard
procedure for the chemical synthesis of benzoquinone (82),
one might expect an early appearance of quinoid pigment
in the medium at an alkaline pH. However, this explanation
does not account for the results observed in the culture
with an initial pH of 6.5.

It is evident that more experiments must be performed
before the effects of different pH on mycelial growth and
pigment production in S. Spirotricha could be explained.
It was concluded from the above observations that alter-
ations of the pH of the modified Czapek-Dox medium do not
give better results in terms of mycelial growth or pigment
production.

II. Effects of Temperature

Method

S. spirotricha was cultured in 125ml flasks with 50ml
of modified Czapek-Dox medium per flask. Each set consisted
of three flasks and one set was placed in one of the
following temperature chambers: 5°, 15°, 24°, 28°, 32°
and 35°. Mycelial growth and pigment production were
observed after 2 weeks of incubation.

Results and Discussion

The appearances of the cultures after two weeks of
incubation at different temperatures are shown in Fig. 22.

Fig. 22. Appearances of S. spirotricha Cultures
Incubated at Different Temperatures.
(From left to right: 5°, 15°, 24°, 28°,
32° and 35°)

There was no mycelial growth in flasks kept at 5°. A slight amount of surface mycelium was developed in flasks incubated at 15°, but no pigment was observed in the media. The cultures grown at 24° and 28° clearly showed an abundant production of the quinoid pigment in the media together with a normal development of mycelial mats. In flasks incubated at 32° and 35°, the mycelia showed a scanty growth and were greyish brown in color rather than reddish orange, and no extracellular pigment was produced. Thus, the temperature range of 24° to 28° appeared to be most appropriate for the growth of and the pigment production by this fungus. The incubation temperature of 25° was maintained in subsequent work.

III. Effects of Nitrogen Source

Method

The basic medium used was modified Czapek-Dox medium without sodium nitrate. Nitrogen containing compounds used as substitutes for nitrate nitrogen are listed in Table II. Three agar plates were prepared for each compound. Mycelial growth and pigment production were observed on the 7th, 12th and 17th day of growth.

Nitrogen Sources	Concentration (g/l)
1. Ammonium acetate	1.81
2. Ammonium tartrate	2.16
3. Sodium nitrate	2.00
4. Sodium nitrite	1.62
5. Ammonium sulfate	1.55
6. Sodium azide	0.51
7. Urea	0.71
8. Asparagine,hydrated	1.76
9. Casein hydrolysate	2.00
10. Peptone	2.00

Table II. List of Nitrogenous Compounds and their Concentrations used in the Culturing of S. spirotricha. (concentrations were calculated on the basis of percentage of nitrogen in each compound, taking 2g of sodium nitrate as standard)

Results and Discussion

One of the components of the basic medium used in this investigation was yeast extract, and since yeast extract contains protein, it can also serve as a source of nitrogen. It must be pointed out, therefore, that the results of this experiment only indicate the effects on mycelial growth and pigment production of the substitution of nitrate by other nitrogenous compounds in the presence of yeast extract protein.

That yeast extract (#11) can serve as a source of nitrogen is indicated in Fig. 23 and 24. The addition of nitrate (#3), nitrite (#4) and peptone (#10) to the medium gave similar results in respect to fungal growth and pigment production (Fig. 23 and 24).

As sodium azide (#6) is a respiratory inhibitor, it is not surprising that no growth occurred on the medium prepared with this compound. The cultures grown on urea (#7) and asparagine (#8) showed a thicker mycelial mat and the appearance of intracellular and extracellular pigments were delayed as compared to that observed with yeast extract (Fig. 23 and 24). The ammonium compounds gave variable results. With ammonium sulfate, mycelial growth and pigment production observed on the 12th day of culture were similar to those observed with yeast extract, but the extracellular pigment seemed to decrease from 12th day to 17th day of growth, in contrast to the culture with yeast extract in which it increased. The mycelial development of cultures with ammonium acetate and ammonium tartrate was similar to that

Fig. 23. Appearances of 12 Day Old Cultures of
S. spirotricha Grown on Different Nitrogen
Sources. (numbers refer to nitrogen compounds
listed in Table II, and # 11 is the control)

Fig. 24. Appearances of 17 Day
Old Cultures of S. spirotricha
Grown on Different Nitrogen
Sources. (numbering of plates:

```
 1   2   3
 4   5   6
 7   8   9
10  11
```

Numbers refer to nitrogen compounds
listed in Table II, and #11 is the
control)

observed with urea and asparagine. On pigment production, intracellular and extracellular pigments were apparent on the 17th day of growth with ammonium acetate, and these pigmenations were not observed with ammonium tartrate.

The general conclusions that may be drawn from these observations were that there seemed to be a correlation between intracellular and extracellular pigment formation, and that, for the culture of S. spirotricha, nitrate nitrogen is among those nitrogenous compounds that, together with yeast extract protein, give the best results in terms of growth and pigment production.

IV. Effects of Vitamins

Methods

Agar plates were prepared with Czapek-Dox medium with the addition of the various vitamins listed in Table IV. The concentrations of the vitamins are indicated in Table III. Spores from an old culture plate with abundant cleistothecia were transferred to the prepared plates with sterilized cotton swabs. Observations were made on the 2nd and 17th day of growth.

Vitamins	Concentration (mg/100ml)
Nicotinic Acid	0.060
Thiamine HCl	0.024
D-Biotin	0.500×10^{-3}
Pyridoxine HCl	0.100
Riboflavin	0.008

Table III. Concentrations of the Vitamins used
as Supplement to Czapek-Dox Medium.

Results and Discussion

A comparison of the extent of mycelial growth and
pigment production when Czapek-Dox medium was supplemented
with the vitamins listed in Table IV to that of yeast
extract on the 2nd and 17th day of growth of S. spirotricha
is shown in Table V. The plates in Fig. 25 are representa-
tive examples of the appearance of the cultures, and Fig. 26
shows the extent of mycelial formation after 17 days of
incubation when yeast extract was substituted by other
vitamins.

These results clearly demonstrated that the substitution
of yeast extract by the vitamins studied brought about a
great retardation of mycelial growth. The scanty growth
of mycelium in these plates might be taken as an indication
that there are some other growth factors in the yeast extract
that are required by the fungus for proper development.

Plate No.	Nicotinic Acid	Thiamine HCl	Biotin	Pyridoxine HCl
1	+			
2		+		
3			+	
4				+
5	+	+		
6	+		+	
7	+			+
8		+	+	
9		+		+
10			+	+
11	+	+	+	
12		+	+	+
13	+		+	+
14	+	+	+	+
15	+	+	+	+
		and Riboflavin		
16	-	-	-	-
Y	Yeast Extract			

Table IV. List of Vitamins Used in the Supplementation
of Czapek-Dox Medium.

	2nd Day		17th Day	
Plate No.	Mycel. Growth	Ex. Pigm	Mycel. Growth	Ex. Pigm
1	±	–	+	±
2	±	–	+	±
3	±	–	+	±
4	±	–	+	+
5	±	–	+	±
6	±	–	+	±
7	±	–	+	+
8	±	–	+	±
9	±	–	+	+
10	±	–	+	+
11	±	–	+	±
12	±	–	+	+
13	±	–	+	+
14	±	–	+	+
15	±	–	+	+
16	±	–	+	±
Y	++	–	+++++++	++++

Table V. Results of Mycelial Growth and Extracellular Pigment
Formation on Various Vitamin Sources in Cultures
of S. spirotricha. (– no pigment observed; ± slight
growth and pigment observed; + growth and pigment
observed)

All the media in which pyridoxine was present possessed
a greater amount of purple coloration as compared with those
in which other vitamins were added (Fig. 25). Thus it
seemed that pyridoxine has some effect on the extracellular
pigment formation process.

Fig. 25. Appearances of
17 Days Old Cultures of
S. spirotricha Grown on
Various Vitamin Sources.
(number refer to vitamins
added as listed in Table
IV)

Fig. 26. Extent of Mycelial
Growth of S. spirotricha on
Plate #3.

Part B. <u>Production of Extracellular Colored and Phenolic</u>
<u>Compounds in Relation to pH and Glucose Concen-</u>
<u>tration of the Medium and Changes in Mycelial</u>
<u>Dry Weight</u>

<u>Methods</u>

<u>Culture Condition</u>

The organism was cultured in Roux bottles with 100ml
modified Czapek-Dox medium per bottle.

<u>General Procedure and Isolation of Shanorellin</u>

Each sample consisted of three Roux bottles. The medium
was separated from the mycelial mat by filtration through
three layers of cheese cloth (preweighed). The pH of the
filtrate was measured and the solution diluted to 500ml with
distilled water. An aliquot of 10ml was taken for glucose
determination. The remaining solution was acidified with
dilute HCl to pH 2 and extracted with ethyl acetate. After
the removal of ethyl acetate with a flash evaporator, the
residue was dissolved in 1 ml ethanol. 0.05ml of the
solution was subjected to thin-layer chromatography for
detection of colored and phenolic compounds. The remainder
of the ethanolic solution was evaporated to dryness and
the residue dissolved in $CHCl_3$. This solution was passed
through a silicic acid column with $CHCl_3$ as the eluting
solvent. The shanorellin band, which appeared as the third
colored one on the column, was collected. The residue
obtained on removal of the solvent from this fraction was

sublimed at 100° under vacuum. The sublimate was further purified by thin-layer chromatography with solvent system A and the product resublimed. Its concentration was determined by u.v. spectrophotometry.

Determination of Absorption Curve of Shanorellin

The absorption of shanorellin in chloroform at 272nm at different concentrations was measured with a Unicam SP. 800 Ultraviolet Spectrophotometer. Solutions of shanorellin were found to obey the Beer-Lambert Law at concentrations of 0 to 0.9 X 10^{-2} mg per ml. (Fig. 27).

Determination of Mycelial Dry Weight, pH and Glucose

The mycelial mat on the Büchner funnel after separation from the medium in the above procedure was dried overnight on a petri dish in an oven at 90° and weighed. The pH of the medium was measured with a Leeds & Northrup pH meter. Glucose concentration was determined by the method of Nelson (83).

Detection of Colored and Phenolic Compounds

The thin-layer plates were developed two-dimensionally with solvent systems B and C. After observation under u.v. light, the plates were sprayed with a reagent to detect the presence of phenolic compounds.

Chromatography

(I) Column Chromatography   Silicic acid columns were
        prepared by mixing three parts of silicic acid
        (Baker Analyzed reagent) to one part of Hyflo
        Super Cel (Fisher Laboratory Chemical). Chloroform

(analytical grade) was used as the eluent solvent.

(II) <u>Thin-Layer Chromatography</u> Thin-layer plates were prepared by mixing 40g of silica gel G (Merck) with 100ml of distilled water. This mixture was applied with a spreader (Desaga) onto the plates to give a thickness of 0.5mm. The plates were activated at 110° for 1/2 hr and kept at 80° until used. The solvent systems used were as follows:

(A) Benzene : Acetic Acid 9 : 1 (v/v)

(B) Chloroform : Acetic Acid 500 : 37.5 (v/v)

(C) Cyclohexane : Ethyl acetate : Acetic Acid
    20:10:1 (v/v/v)

Phenolic compounds were detected by spraying with a freshly prepared mixture of the following solution first:

0.3% p-nitroaniline in 8% HCl   5ml

5% sodium nitrite   1ml

20% sodium acetate   15ml

The plates were then oversprayed with 2N NaOH.
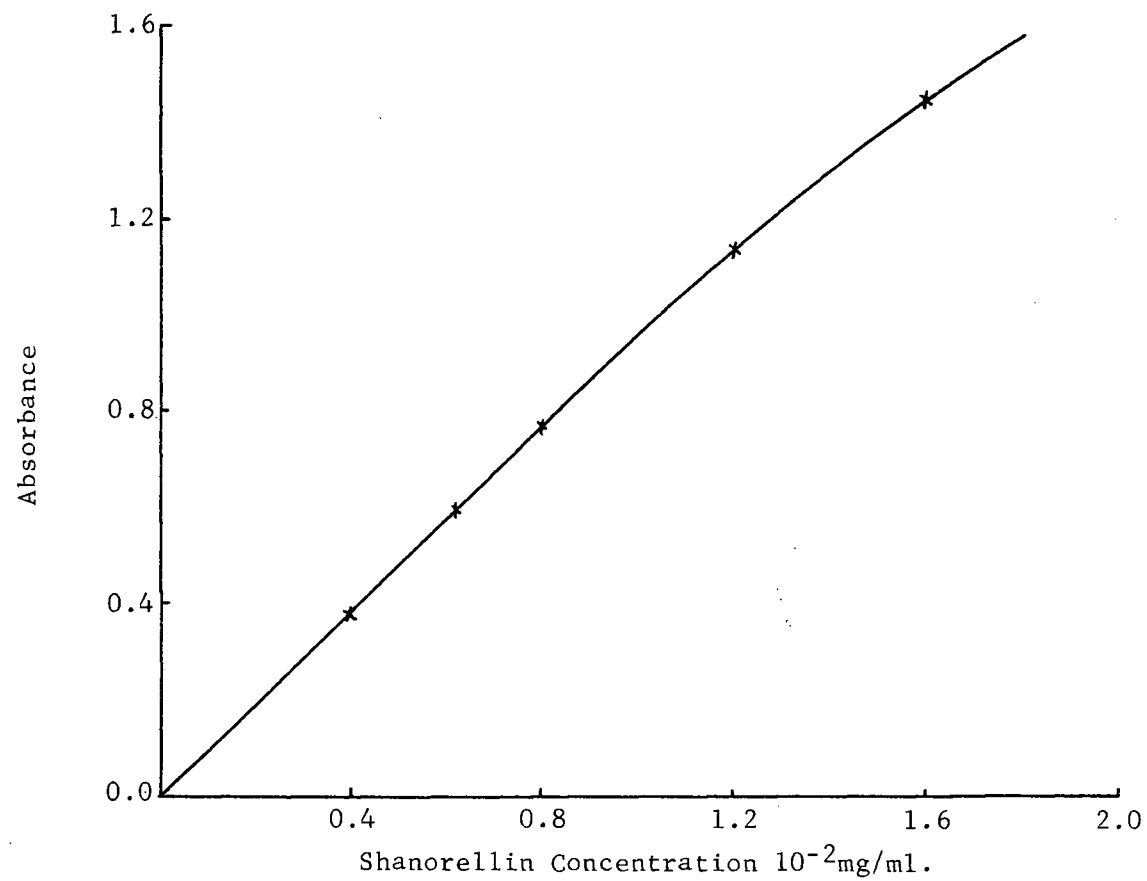
Fig. 27. Absorption Curve of Shanorellin in $CHCl_3$ at 272 nm.

<u>Results and Discussion</u>

In the first eight days of growth, there was a rapid increase of mycelial dry weight, a rise in pH and rapid utilization of glucose from the medium (Fig. 28). During this period, only traces of phenolic compounds were detected by thin-layer chromatography. On the 8th day, shanorellin began to appear, the pH of the medium decreased, and the mycelial growth rate started to decrease. By the 18th day, the shanorellin concentration in the medium was at its maxium. After this, it gradually disappeared indicating that it was being metabolized further to other compounds. The slight rise of pH between the 27th and 45th day suggests that there was a production of new metabolites by the organism.

There were four other spots in addition to shanorellin present in conspicuous amounts on thin-layer plates (Fig. 29-35). A number or letter, as shown in Fig. 36, is given for each spot to facilitate their identification on the plates and for the purpose of discussion.

Spots A and C and shanorellin are yellow and easily observed on the plates (Fig. 35). They appear dark purple under u.v. light. Spot A was found to consist of two compounds (Compound A and Compound B). These two compounds, together with Compound C (spot C), were isolated. Their chemical properties will be discussed in detail in Part C together with the structural determination of shanorellin.
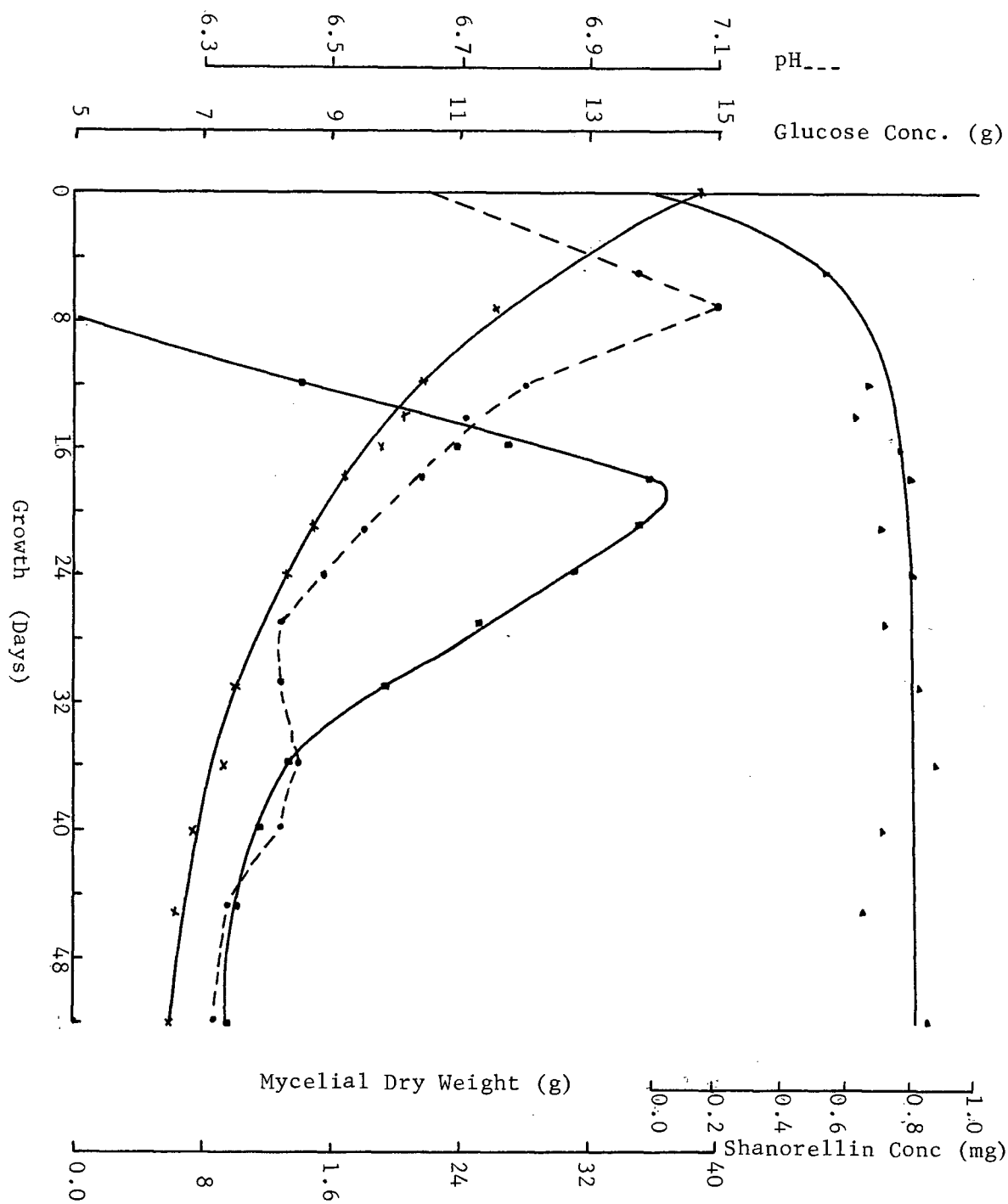
Fig. 28. Changes in pH, Glucose and Shanorellin Concentrations in the Medium and Mycelial Dry Weight with Growth in _Shanorella spirotricha_. (Total of 3 Roux Bottles)

pH ---

Glucose Conc. (g)

Growth (Days)

Mycelial Dry Weight (g)

Shanorellin Conc (mg)

Compounds I and II were the major compounds detected on the plates with the phenolic reagent spray. Compound I was present in the medium before shanorellin on the 7th day of culture and persisted throughout the period studied. Compound II appeared later and its concentration seemed to increase and then decrease as the culture became older. Efforts to isolate compound I and II for structural determinations were unsuccessful because they seemed to be absorbed by silica gel G and only trace amounts could be eluted from the plates.

Similar studies on changes in pH, glucose concentration and mycelial growth in relation to benzoquinone formation in fungi have been carried out by Pettersson with *Aspergillus fumigatus* (72, 84) and by Read, Westlake and Vining with *Aspergillus terreus* (61). Since the medium used in each culture was different, a comparison of the factors mentioned and benzoquinone formation in these fungi could not be made.
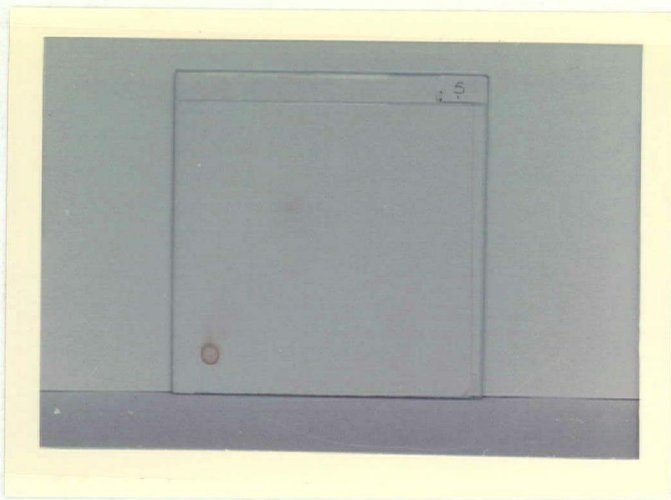
Fig. 29.
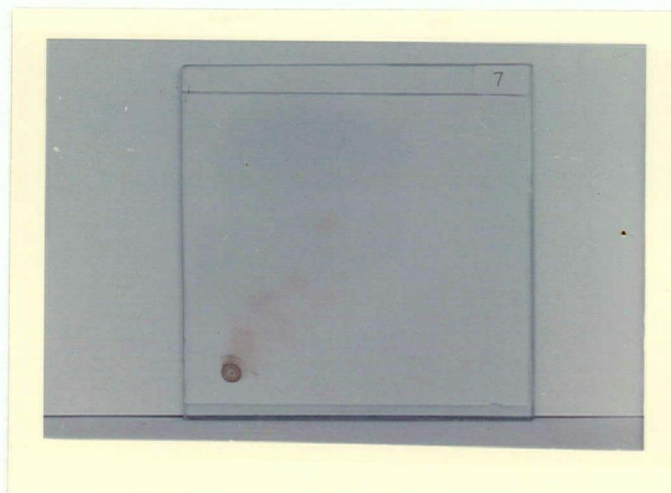
Fig. 30.
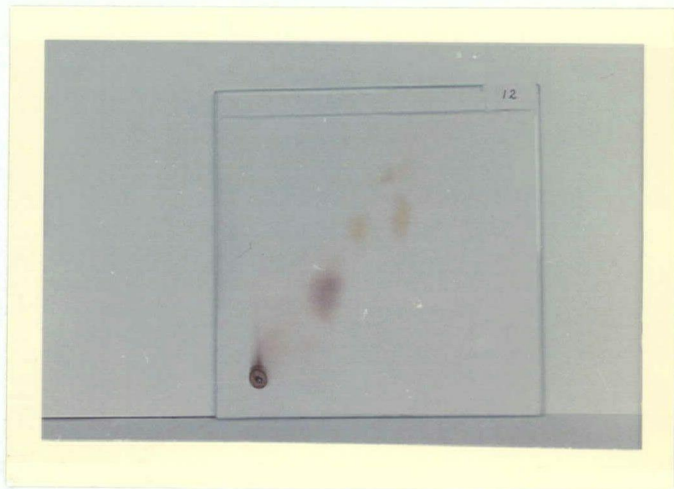
Fig. 31.

Fig. 32
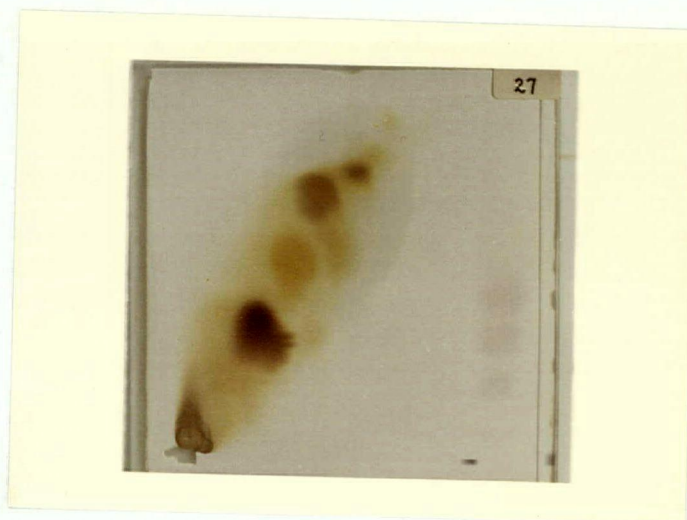
Fig. 33

Fig. 34

Fig. 29-34. Detection of Colored and Phenolic Compounds in the Culture Medium during Growth of S. spirotricha by Thin-layer chromatography. (number on plate indicates the days of incubation; all plates were sprayed with p-nitroaniline reagent)
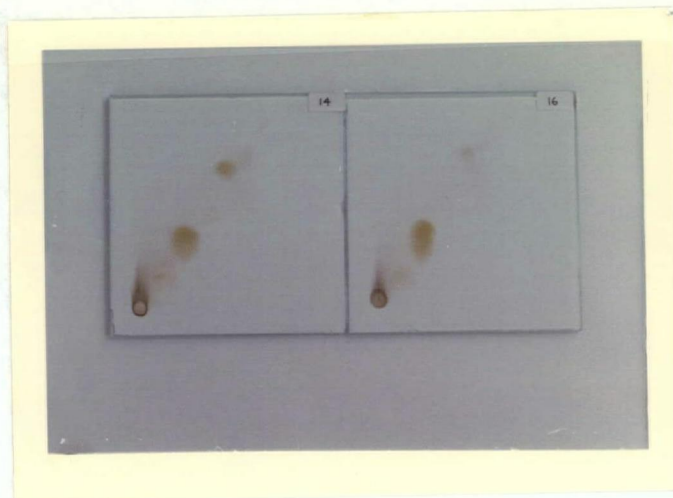
Fig. 35. Similar to Fig. 29-34 but without spraying
with p-nitroaniline reagent.
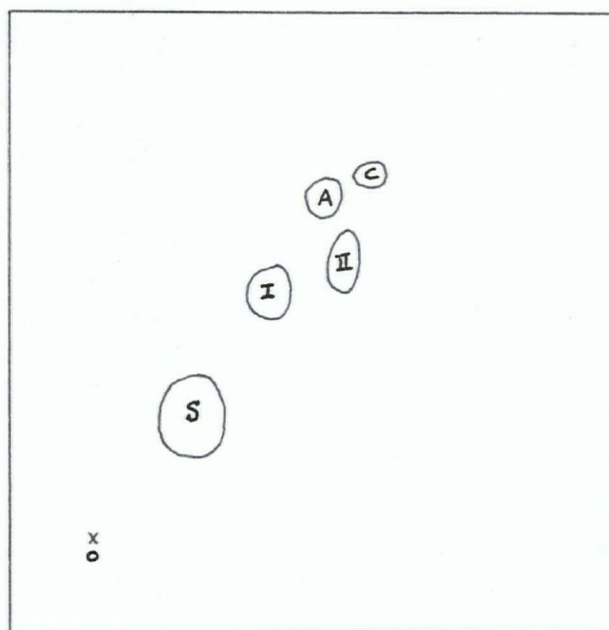
Fig. 36. Typical Thin-Layer Plate Illustrating the
Relative Positions of the Compounds Present
in Conspicuous Amount.

(S = Shanorellin, A and C = Benzoquinones,
I and II = Phenolic Compounds, o = origin)

Part C. <u>Elucidation of the Chemical Structures of</u>
<u>Shanorellin, its Monoacetate (Compound A)</u>
<u>and its Ether Derivatives (Compound B and</u>
<u>Compound C)</u>

The partial structural determination of shanorellin
was accomplished by chemical and spectroscopic studies (4).
The final structure was determined by X-ray diffraction
crystallography carried out by Dr. E. Subramanian and
Dr. J. Trotter (Chemistry Dept., U.B.C.). Preliminary
examination of Compound A, B and C indicated that they
were closely related to shanorellin and sufficient amounts
of these three compounds were isolated for u.v., i.r., n.m.r.
and mass spectroscopic analyses. From these results,
chemical structures can be proposed for them.

<u>Methods</u>

<u>Culture Conditions</u>

For isolation of metabolites produced in the medium,
1 litre flasks with 400ml of modified Czapek-Dox medium
per flask were used. All the other conditions were the
same as those described in the general procedure of Part A.

<u>Isolation of Metabolites</u>

The isolation and purification procedures as described
in Part B for shanorellin were followed. Compound A, B and
C appeared together as the second yellow band on a silicic
acid column and were subsequently separated from one another

by tlc with solvent system A (Part B) and solvent system D
(Chloroform:acetic acid 9:1 v/v). All compounds were
crystallized from benzene-light petroleum (b.r. 65-120°).

<u>Instrumentation</u>

Melting Point : Thomas Hoover Capillary Melting Point
            Apparatus. (All readings were uncorrected).

U.V. Spectrophotometry: Unicam SP 800 Ultraviolet
            Spectrophometer.

I.R. Spectrophotometry : Unicam SP. 200G Infrared Spect-
            trophotometer.

N.M.R. Spectroscopy : Varian Model HA-100 and T-60,
            Joel C60H. In all samples, $CDCl_3$ was
            used as solvent with tetramethylsilane
            as internal standard (Chemistry Dept.,
            U.B.C.).

Mass Spectroscopy : Associated Electrical Industries
            Model M89 Instrument (Chemistry Dept.,
            U.B.C.).

<u>Elemental Analysis</u>

Elemental analyses were carried out by Dr. C. Daessle
of Montreal.

<u>Preparation of Derivatives</u>

(1) <u>Shanorellin diacetate</u>. To shanorellin (100mg) in acetic
    anhydride (2ml) was added conc. H_2SO_4 (2 drops) and the
    solution warmed for a few seconds. The diacetate separ-
    ated out as a brownish yellow oil on addition of ice
    water and was extracted with ethyl ether. On evaporation

of the ether, a viscous brownish yellow oil (93.8mg) was obtained which could not be crystallized from either methanol or light petroleum. Tlc on silica gel G with solvent system A showed the presence of one compound at Rf 0.55. (n.m.r. spectrum: 2 CH_3 at τ 7.87 and 8.03; CH_2 at τ 5.4 and 2 CH_3CO at τ 7.68 and 7.98).

(2) <u>Shanorellin tetra-acetate</u>. Shanorellin (100mg) in ethanol (5ml) was treated with $NaBH_4$ until the yellow color disappeared. Water (20ml) was added, and the solution extracted four times with ethyl ether (20ml). The ether extract was dried with anhydrous sodium sulfate and the solvent removed. Acetylation with acetic anhydride (2ml) and pyridine (2ml) overnight at room temperature followed by addition of ethanol (10ml) and evaporation <u>in</u> <u>vacuo</u> below 40° until the pyridine was removed gave a slightly yellowish gummy residue. Crystallization from methanol afforded the colorless tetra-acetate. (m.p. 112°, yield 47mg. n.m.r. spectrum: 2CH_3 at τ 7.87 and 8.05; CH_2 at τ 4.95; 4 CH_3CO at τ 7.7, 7.74, 7.78 and 8.03).

(3) <u>Shanorellin chloride</u>. Thionyl chloride (65.45mg) was added dropwise to shanorellin (91mg) in chloroform (20ml) and pyridine (39.5mg) in an ice bath. After the addition the solution was warmed to room temperature and then refluxed for 2.5 hr. The solvent was removed <u>in</u> <u>vacuo</u> and the residue chromatographed on silica gel G plates with solvent system A as the developing system. The chloride band had

an Rf of 0.6. It was crystallized from hexane and chloroform giving orange-yellow needles. (m.p. 69°, yield 46mg. MW 200.5. Found: C, 53.67; H, 4.4; Cl, 17.66. $C_9H_9O_3Cl$ required. C, 53.86; H, 4.48; Cl, 17.71 per cent. n.m.r. spectrum: 2 CH_3 at τ 7.8 and 8.03; CH_2 at τ 5.53; OH at τ 2.985).

Results and Discussion

All hydroxybenzoquinones produced by fungi have been detected as purple pigments in the medium (5, 80,81). The solubility of these compounds in water is due to the disso-ciation of the acidic hydrogen at the hydroxyl group situated at the carbon α to the carbonyl function. Other character-istics of this type of compound include the ease of sublima-tion and oxidation of the reduced form in a buffer solution at pH 8 by aeration. Shanorellin, Compounds A, B and C were shown to possess these properties. The chemical and spectro-scopic studies on each compound will be discussed individually.

I. Shanorellin

Shanorellin crystallizes as bright orange-yellow needles, m.p. 121°, and sublimes at 100° under vacuum. Its solutions are decolorized by sodium borohydride or sodium dithionite, and on adjusting the pH to 8 and aeration, a purple color appears indicating the quinone is formed. It is unstable in highly alkaline solution, e.g. 2N NH_4OH or 0.5N NaOH, turning brown after standing for 15 minutes.

From the elemental analysis (Found: C, 58.81; H, 5.56

per cent) and the molecular weight of 182 from the mass spec-
trum (Fig. 37), the molecular formula of shanorellin is cal-
culated to be $C_9H_{10}O_4$ (Required: C, 59.3; H, 5.5 per cent).
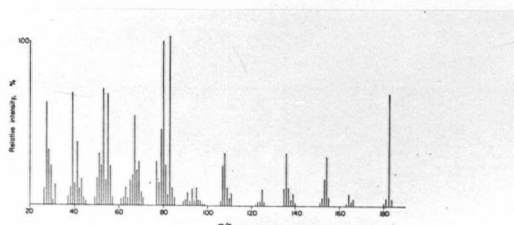
Fig. 37. Mass Spectrum of Shanorellin.

The u.v. absorption spectra of hydroxybenzoquinones
variously substituted with methyl groups show two regions
of absorption: (1) a strong band in the range of 263-280nm
($\log\epsilon$ ca 4) and (2) a weak band in the range of 379-409nm
($\log\epsilon$ 2-3) (85). Shanorellin with absorption bands at
272nm ($\log\epsilon$ 4.05) and 406nm ($\log\epsilon$ 2.07) in chloroform
(Fig. 38) shows that it has similar absorption properties
to the hydroxy-alkylbenzoquinones. In ethanol, the band with
λ_{Max} 372 in chloroform is shifted to 269.5nm and the one
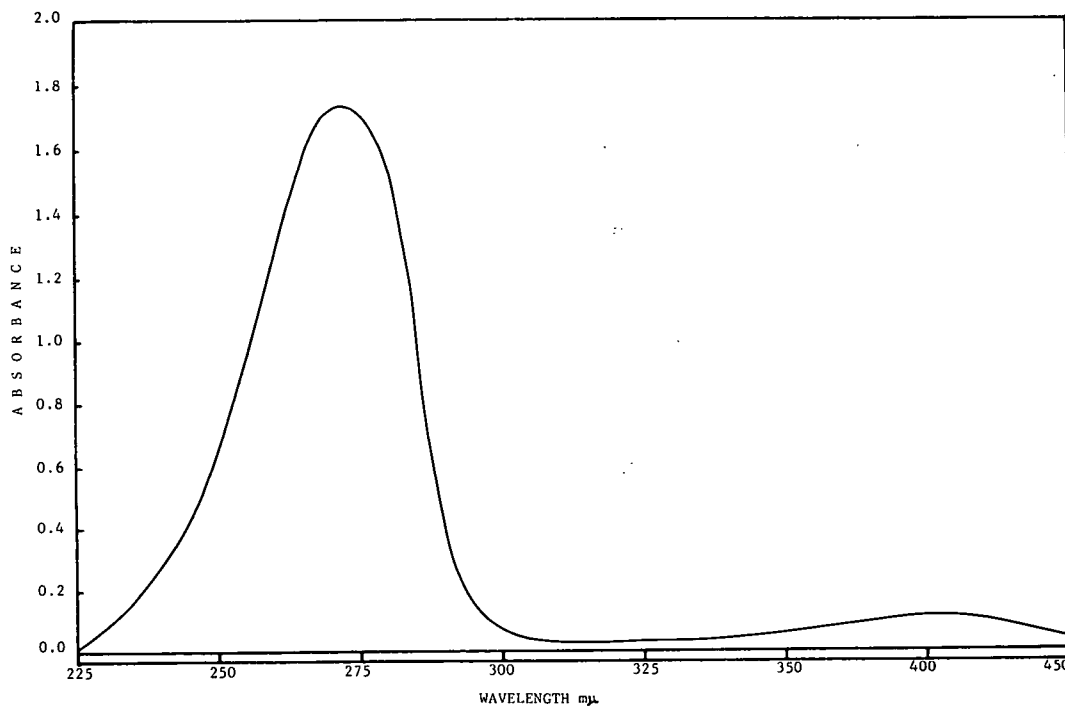
Fig. 38. Ultraviolet Spectrum of Shanorellin in $CHCl_3$.

at 406nm remains the same. On addition of $NaBH_4$, the band at 269.5nm disappears, and, if the spectrum is taken immediately, a band at 355nm appears, and on standing, this band is gradually replaced by another at 300nm.

The i.r. spectrum of p-benzoquinones have been studied by Yates, Ardao and Fieser (86). Two bands are observed in the carbonyl stretching region in these compounds, and in shanorellin, they appear at 1660 and 1640 cm^{-1}. Other bands in the spectrum of shanorellin are the O-H stretching vibration at 3180 and 3450 cm^{-1}, C=C stretching at 1620 cm^{-1}, and alkane C-H stretching in the region of 2900-3000 cm^{-1} (Fig. 39).
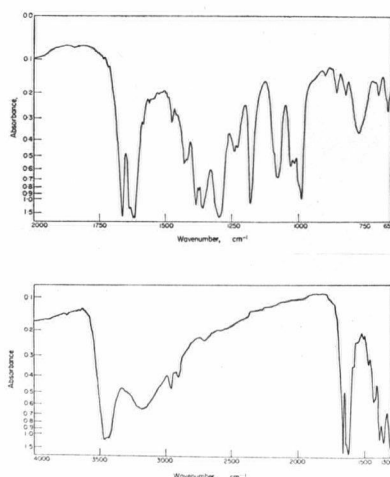
Fig. 39. Infrared Spectrum of Shanorellin in KBr Disc.

The n.m.r. spectrum of shanorellin reveals the presence
of five types of protons in the proportion of 1:2:1:3:3
(τ, 3.06, 5.475, 7.75, 7.885 and 8.075, Fig. 40). On
addition of D_2O the peak at τ 3.06 and the broad band
around τ 7.75 disappear indicating the presence of two
exchangeable protons. Of the remaining peaks, that at
τ 5.475 could be assigned to methylene protons and the other
two, at τ 7.885 and 8.075, to protons of two methyl groups.
On expansion of the sweep width of the spectrum (10X), the
methylene protons appear as a quartet, and the methyl protons
at τ 7.885 as a triplet. These two groups of protons are
thus coupled with each other (coupling constant J = 0.5Hz).
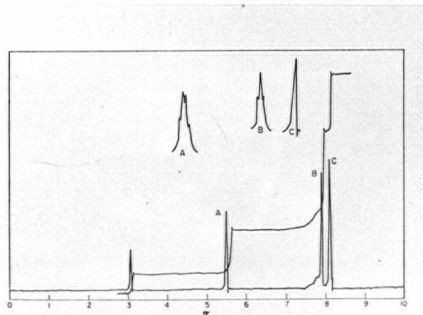
Fig. 40. N.M.R. Spectrum of Shanorellin in CDCl$_3$.

The coupling constant is too small for a CH$_3$CH$_2$ function but could account for a methyl and methylene group on adjacent carbons, on the same side of the quinone nucleus. The n.m.r. spectrum of tauranin (I), a benzoquinone produced by Oospora aurantia (87), is of particular relevance to shanorellin because this compound possesses an hydroxy as well as a hydroxymethyl function. The τ values of the hydroxyl protons and the methylene protons are very close to those observed in shanorellin:
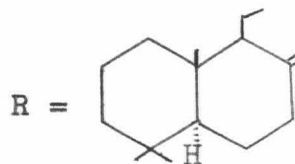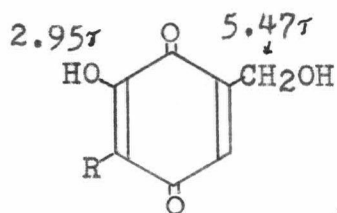
(I)

Thus, the band with τ at 3.06 could be assigned to the nuclear hydroxyl proton while the band at 7.75 to the hydroxyl proton of the hydroxymethyl group.

A diacetate and a tetra-acetate of shanorellin were prepared and the n.m.r. spectra obtained. The preparation of these derivatives confirmed the presence in the molecule of two hydroxyl functions in addition to the two carbonyl function of the benzoquinone nucleus.

With th above information, it is possible to assign either of the two isomeric structures shown below to shanorellin:
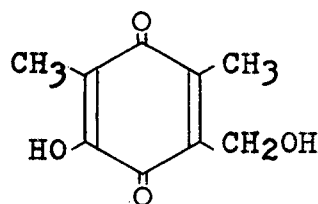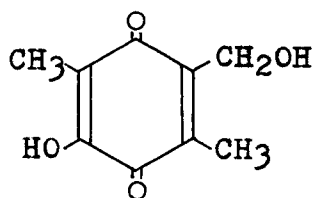
(II)   (III)

The mass spectrum of shanorellin (Fig. 37) could not be used to distinguish between these two isomeric forms either. From the known fragmentation pattern of benzoquinones (88), these two isomers would give similar ions:
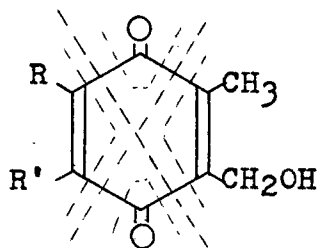
Compound	(II)	(III)
R	CH_3	OH
R'	OH	CH_3

A common feature of the mass spectra of benzoquinones is the occurrence of the ions corresponding to the loss of one and of two molecules of carbon monoxide (89). In shanorellin, they are represented by the peaks at masses 154 and 126. The base peak at mass 83 could be accounted for by the 1,2- and 4,5-bonds cleavages with the loss of a hydride ion (Fig. 41A). The intense peak of mass 80 could be derived from the ion with mass 154 by dehydration and further cleavage of the 5 membered ring (Fig. 41B). The dehydration of benzyl alcohol with an ortho methyl group has been studied by Aczel and Lumpkin (90). In this type of compound, the peak due to $(p - H_2O)$ ion is often one of the most prominent ones in the spectra, and it is possible due to the formation of a hydrogen bond prior to fragmentation. To account for the appearance of some of the major peaks in the mass spectrum of shanorellin, the fragmentation patterns shown in Fig. 41A and 41B are proposed.

The final structure of shanorellin (II) was obtained by X-ray crystallographic analysis of the chloro-derivative of shanorellin (2,6-dimethyl-5-hydroxy-3-chloromethyl-1,4-benzoquinone). This was determined by Dr. E. Subramanian and Dr. J. Trotter of the Chemistry Dept., U.B.C.
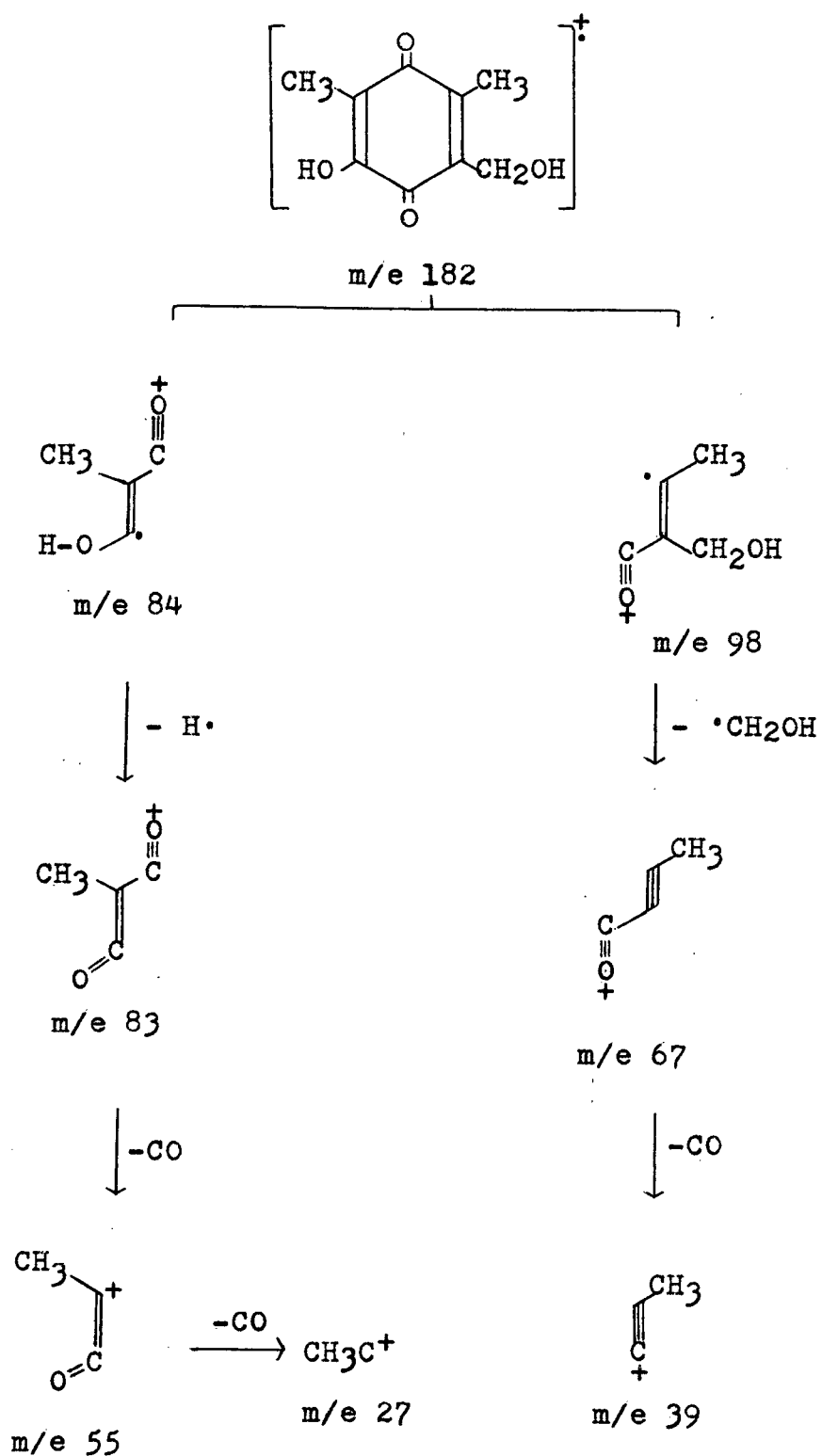
Fig. 41A. Some of the Possible Bond Cleavages in Shanorellin upon Electron Impact.
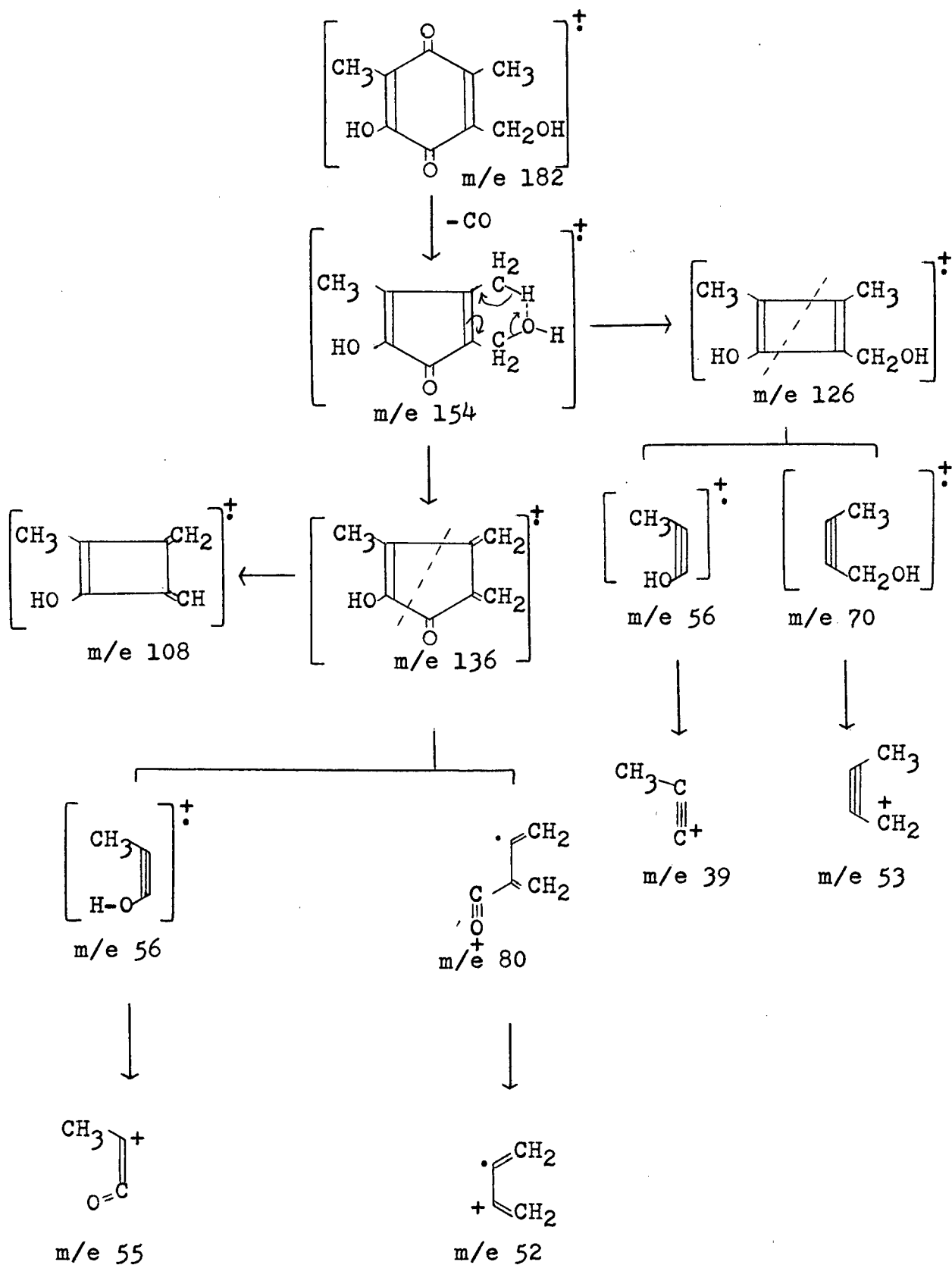
Fig. 41B. Some of the Possible Bond Cleavages in Shanorellin upon Electron Impact.

II.   Compound A

Compound A crystallizes as bright orange-yellow needles, m.p. 128° and sublimes at 100° under vacuum. As with shanorellin, its solutions are decolorized by sodium borohydride and sodium dithionite, and on adjusting the pH to alkalinity, a purple color appears on aeration.

The u.v. spectrum of Compound A in chloroform shows a strong absorption band at 269nm with a shoulder at 274nm and a weak one at 395nm (Fig. 42). These bands are within the regions of absorption shown by hydroxybenzoquinones and with similar extinction coefficients (85). In ethanol, these bands are shifted to 267.5nm and 297nm. The shoulder that appears in chloroform is not observed. On addition of NaBH$_4$, as in shanorellin, the band at 267.5nm is first replaced by one at 360nm then by one at 295nm.

Fig. 42.   Ultraviolet Spectrum of Compound A in CHCl$_3$.
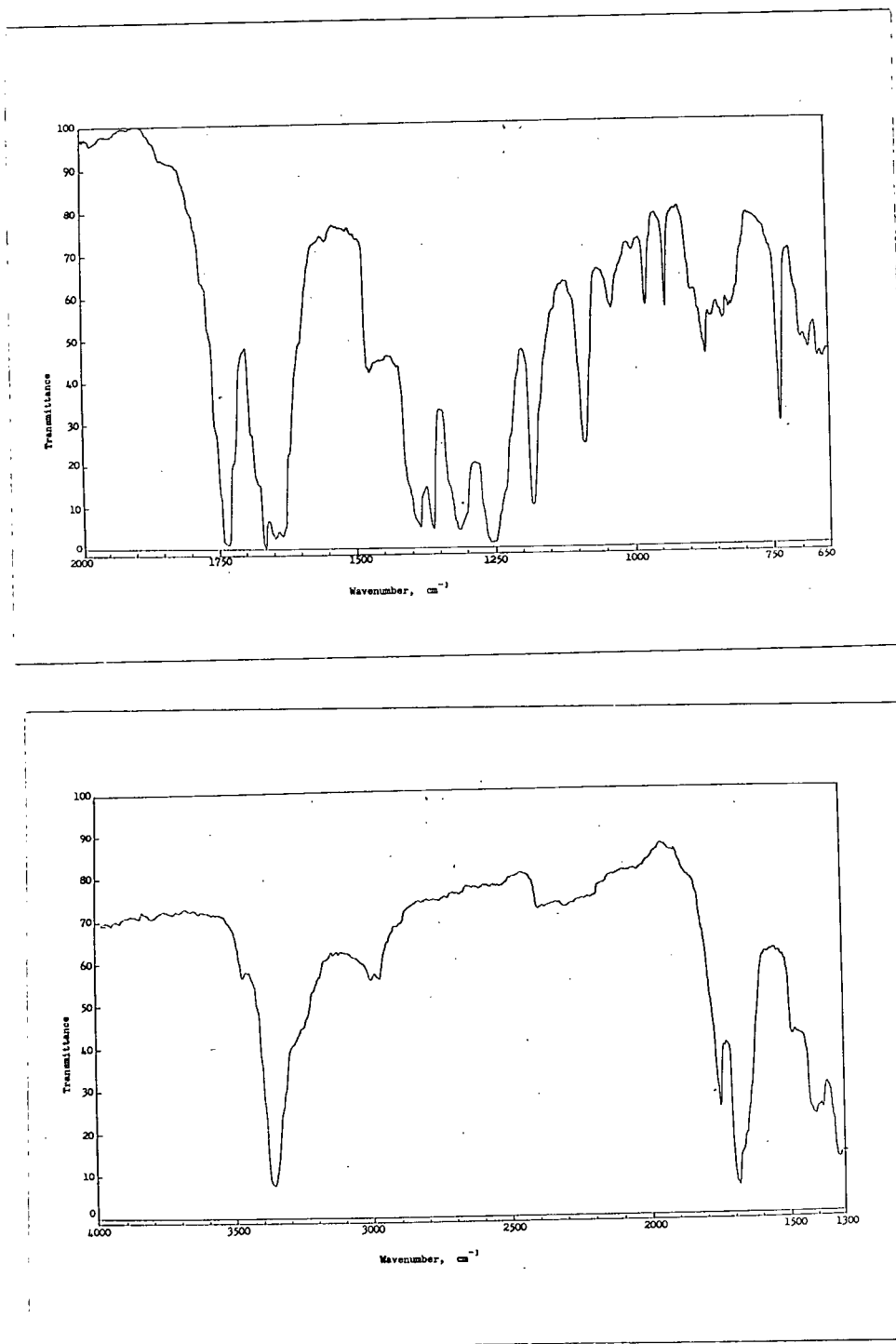
Fig. 43. Infrared Spectrum of Compound A in KBr Disc.

The i.r. spectrum of Compound A (Fig. 43) indicates the following functional groups: quinone C=O stretching at 1665 and 1645 cm^{-1}, C=C stretching at 1635 cm^{-1}, alkane C-H stretching in the region of 2900-3000 cm^{-1}, ester C=O stretching at 1740 cm^{-1} and ester C-O stretching at 1255 cm^{-1}. A comparison of this spectrum with that of shanorellin shows that apart from the ester function bands, Compound A contains bands that are characteristic of shanorellin. Thus an ester of shanorellin is indicated.

That this compound is an ester derivative of shanorellin is confirmed by the n.m.r. spectrum and mass spectrum. The n.m.r. spectrum (Fig. 44) reveals that there are 5 types of protons in the proportion of 1:2:3:3:3 at τ 3.04, 5.00, 7.86, 7.97 and 8.07.
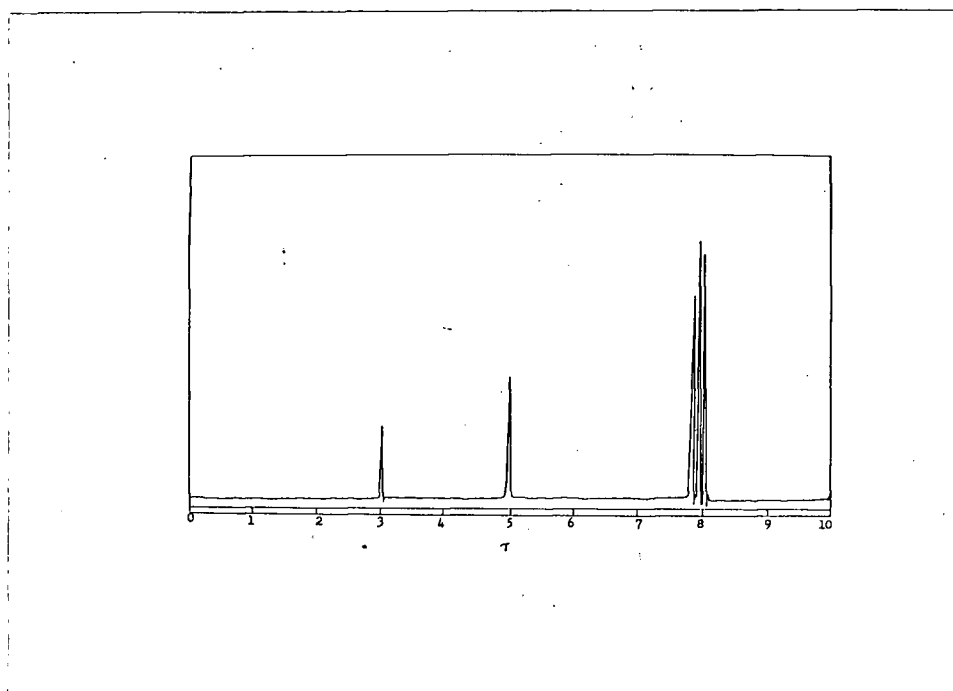
Fig. 44. N.M.R. Spectrum of Compound A in CDCl$_3$.

The peak at τ 3.04 disappears on addition of D_2O. The value of the three protons at τ 7.86 indicates that it could be due to an acetyl function as in shanorellin diacetate, whose acetyl protons give similar τ value.

The mass spectrum of Compound A (Fig. 45) gives the molecular peak at mass 224, the molecular weight of the monoacetate derivative of shanorellin, with the acetate ion (mass 43) as the base peak. The peaks prominent in shanorellin are also observed in this spectrum: masses at 53, 55, 67, 80, 83, 108, 136, 154 and 182.
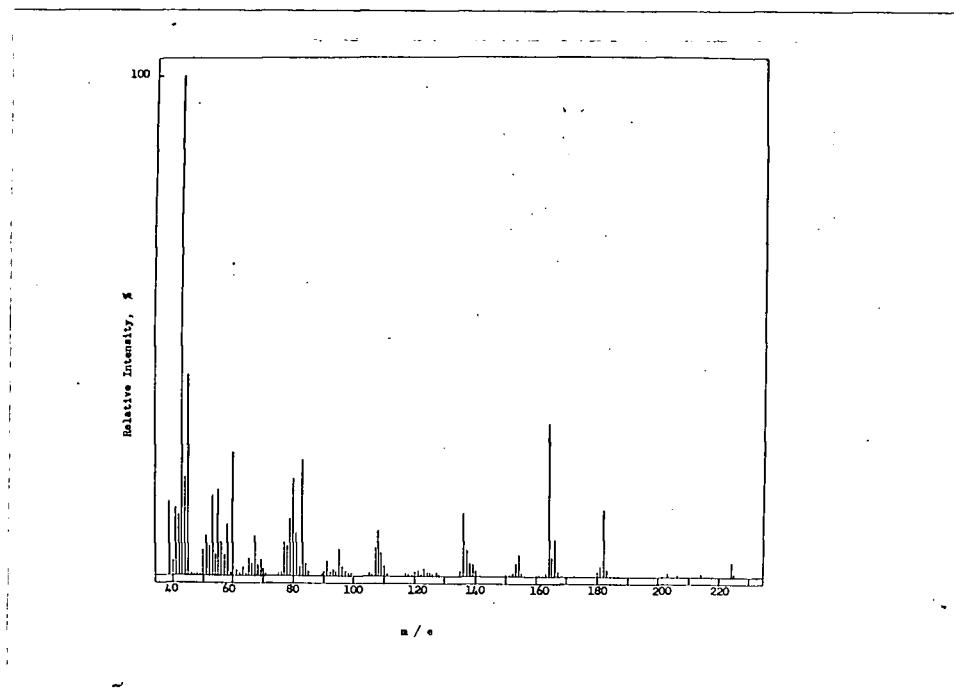
Fig. 45. Mass Spectrum of Compound A.

The peaks of masses 164 and 60 could be accounted for by the following fragmentation:

m/e 164   m/e 60
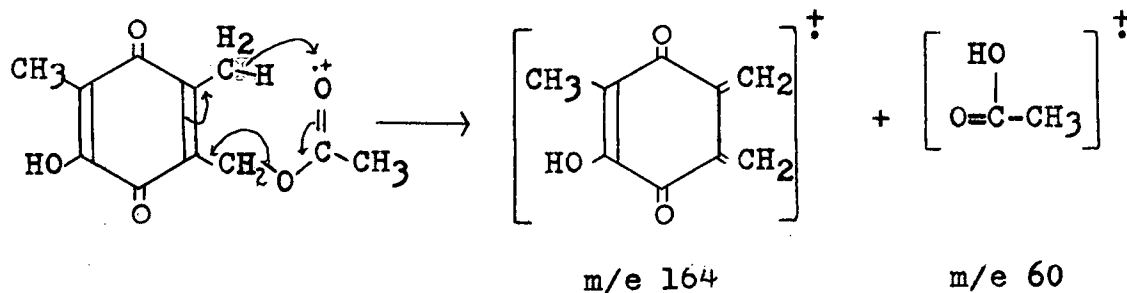
To confirm the presence of the ester function, a hydrolysis reaction was carried out on Compound A with dilute NaOH for one minute. The resulting compound gave the same u.v. spectrum and Rf values on thin-layer plates as shanorellin (Rf values of Compound A in solvent system A: 0.55, system B: 0.79, system C: 0.45, system D 0.79).

Thus, the following structure is proposed for Compound A:
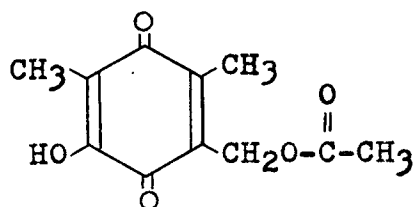
Since ethyl acetate was used in the isolation procedure, it was considered possible that Compound A was an artifact of the extraction procedure used in isolating shanorellin. The extraction procedure was repeated with a sample of shan-

orellin. The residue obtained after removal of the solvent
was chromatographed on thin-layer plate and developed in
solvent system A. In addition to shanorellin, a samll
amount of another yellow spot with the same Rf as Compound A
was present on the plate. This yellow compound turned to a
purple color on exposure to ammonia vapour and had the same
u.v. spectrum as Compound A. Similar results was also
observed when shanorellin (10mg) in ethyl acetate (20ml) in
the presence of conc. HCl (2 drops) and anhydrous sodium
sulfate was allowed to stand for one week at room temperature.
Thus Compound A could be an artifact. One way to avoid this
complication arising from solvent is to substitute ether for
ethyl acetate. It should be pointed out that Compound A,
as well as Compounds B and C were not produced by every batch
of culture.

III. Compound B

Compound B crystallizes as fine dull orange yellow needles, m.p. 170°, and sublimes at 145° under vacuum. It is soluble in alkaline medium giving a purple coloration and on acidification and extraction with chloroform, the original compound is recovered as shown by its u.v. spectrum and Rf values on thin-layer plates (Rf values of Compound B in solvent system A: 0.49, system B: 0.77, system C: 0.44, system D: 0.76).

Compound B possesses two bands in the u.v. spectrum (Fig. 46), with λ_{max} in chloroform at 268nm (log ϵ 4.48) and at 405nm (log ϵ 3.10). In ethanol, these bands are shifted to 267nm and 406nm. On addition of $NaBH_4$, the band at 267nm is displaced first to 362nm, and after one hour, this band is further replaced by one at 305nm.
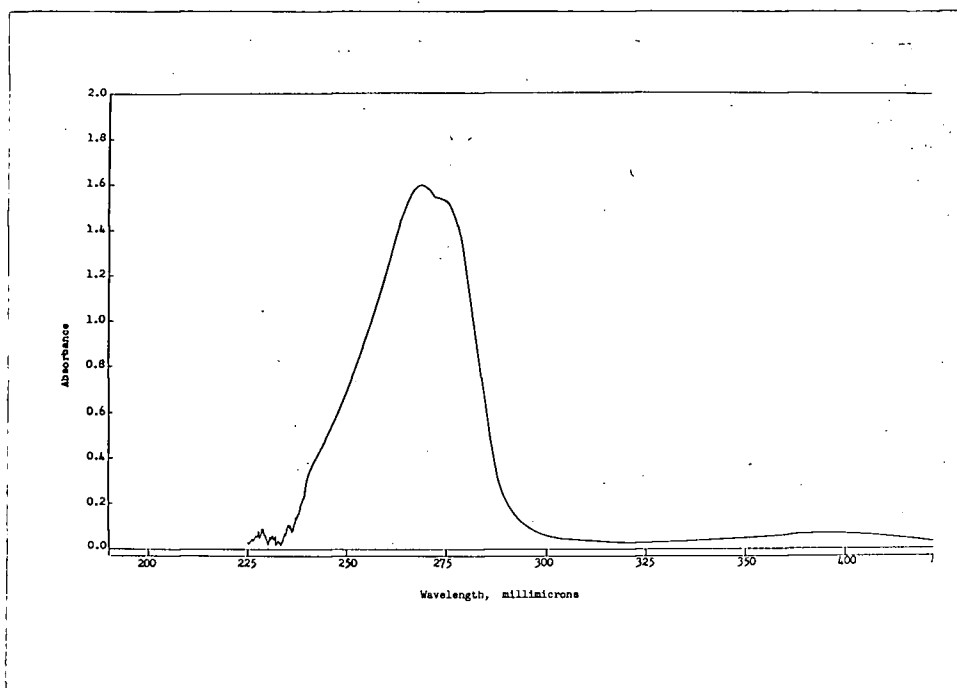

Fig. 46. Ultraviolet Spectrum of Compound B in $CHCl_3$.
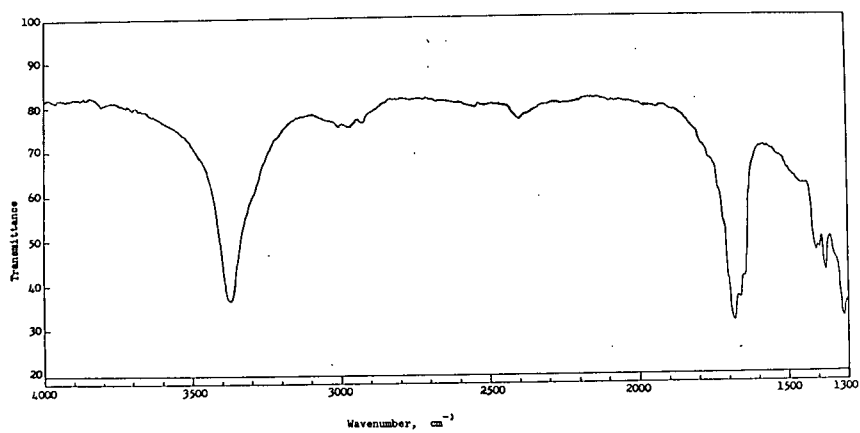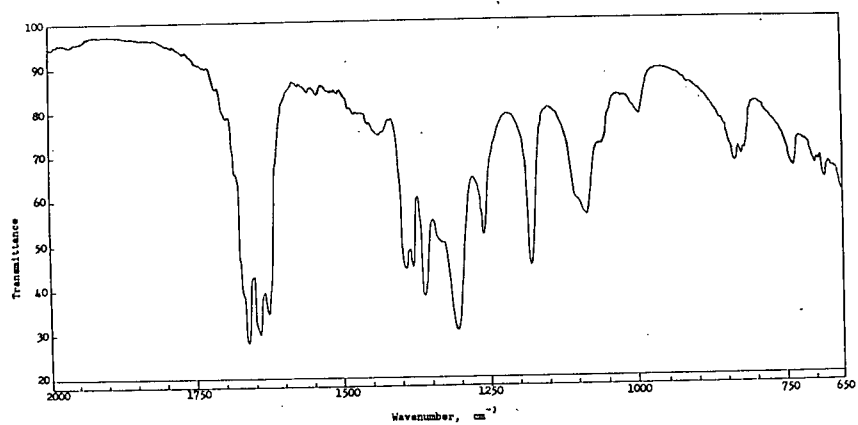
Fig. 47. Infrared Spectrum of Compound B in KBr Disc.

The i.r. spectrum of Compound B (Fig. 47) shows the following bands: O-H stretching at 3330 cm^{-1}, C=C stretching at 1620 cm^{-1}, quinone C=O stretching at 1660 and 1640 cm^{-1}, and ether C-O stretching at 1085 cm^{-1}. A comparison of the finger-print region of this spectrum with that of shanorellin indicates that Compound B possesses a number of bands that are present also in shanorellin, e.g. O-H bending and C-O stretching of the tertiary alcohol at 1180 and 1355 cm^{-1}, the C-H alkane bending bands in the region between 1400 and 1500 cm^{-1}.

The n.m.r. spectrum of Compound B (Fig. 48) shows four types of protons at τ 2.95, 5.5, 7.825, 8.02 in the proportion of 1:2:3:3. The band at τ 2.95 disappears on addition of D_2O. These τ values closely resemble those given by shanorellin.
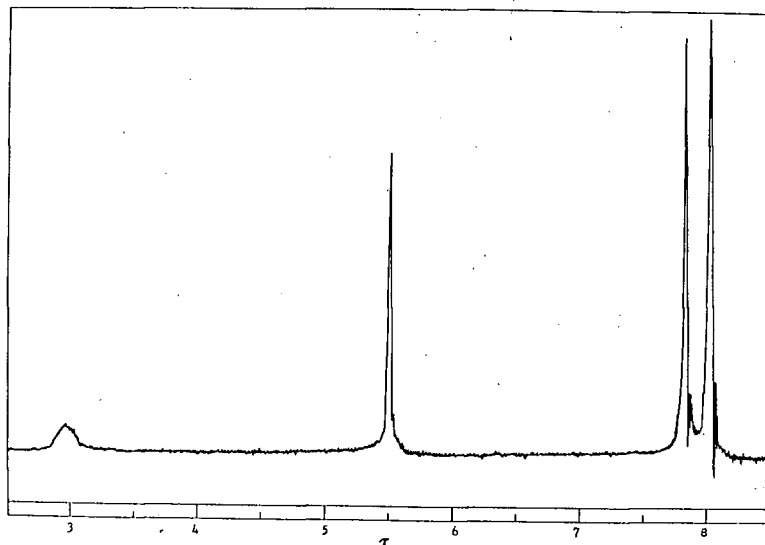
Fig. 48. N.M.R. Spectrum of Compound B in CDCl$_3$.

Using this information, the following structure is pro-
posed for Compound B:
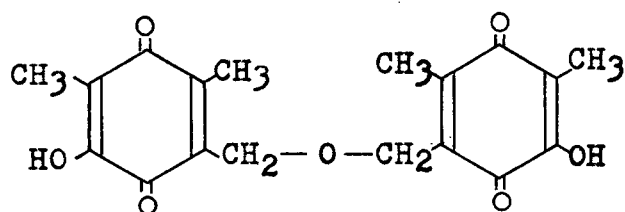
The proposed structure of Compound B is confirmed by
its mass spectrum which shows the molecular peak at mass
346 (Fig. 49).
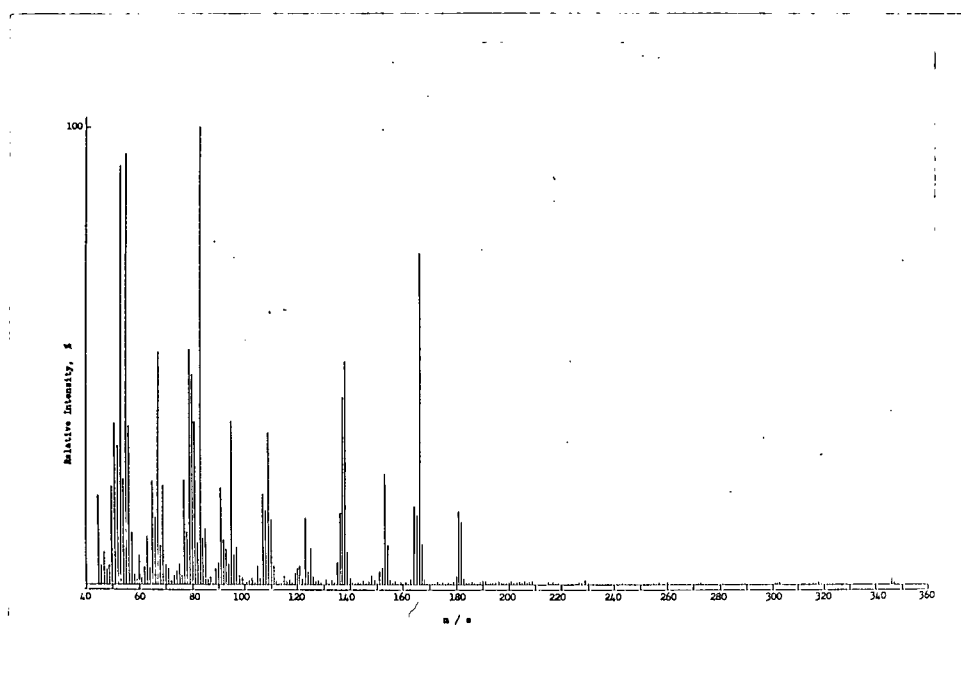
Fig. 49. Mass Spectrum of Compound B.

The peak at mass 181 could be assigned to the ion produced from the cleavage of the ether linkage (90):

m/e 181
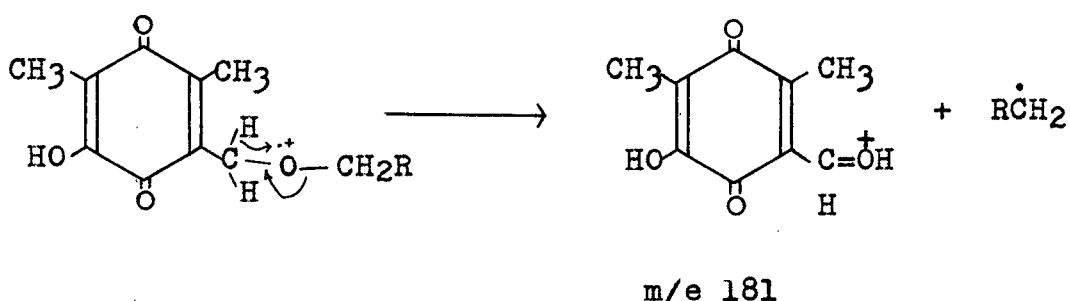
The base peak at mass 83 and those at masses 53, 55, 67, 108 and 136 also occur in shanorellin's spectrum. They could be accounted for by the subsequent fragmentation of the ion derived from the cleavage of the ether bond by processes similar to that described for shanorellin. The peak at mass 166 could be derived by similar bond cleavage and hydride transfer as proposed for bibenzyl ether (90):

m/e 166
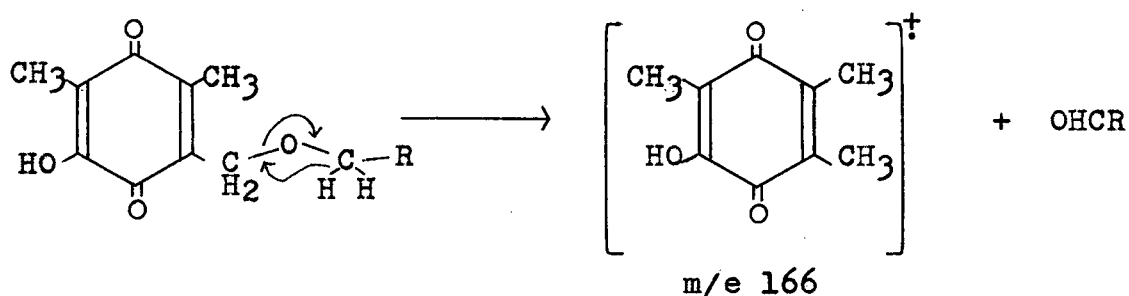
and the further loss of a carbon monoxide from this ion of mass 166 would give the peak at mass 138. Additional loss of a hydride ion and a carbon monoxide would give the peak at mass 109 and the loss of a •CH_2 would give the mass 95.

The acetyl derivative of Compound B had been prepared by the method described for shanorellin diacetate. The product was purified on silica gel G plate with solvent system D (Rf 0.85, m.p. 105°. n.m.r. spectrum: CH_2 at τ 5.16; 2 CH_3 at τ 7.83 and 8.00; CH_3CO at τ 7.60).

IV.   Compound C

Compound C is a viscous, orange-yellow liquid which evaporates readily at 78° under vacuum. It is soluble in alkaline medium to give a purple solution,,and, on acidification and extraction with chloroform, is recovered unchanged as indicated by its u.v. spectrum and Rf values on thin-layer plates (Rf values of Compound C in solvent system A: 0.6,.system B: 0.83, system C: 0.51, system D: 0.85).

The u.v. spectrum of Compound C (Fig. 50) in chloroform shows the two bands typical of benzoquinones : λ_{max} 269.5nm (logϵ 4.49) and 404nm (logϵ 3.10). In ethanol, these bands are shifted to 267.5nm and 407nm. On addition of $NaBH_4$, as in previously described compounds, the band at 267.5nm is replaced first by one at 359nm and later by another at 279nm.

The i.r. spectrum (Fig. 51) indicates the presence of O-H stretching vibration at 3400 cm^{-1}, alkane C-H stretching at 2895, 2940 and 2985 cm^{-1}, quinone C=O stretching at 1655 and 1645 cm^{-1}, C=C stretching at 1630 cm^{-1} and the ether C-O stretching at 1105 cm^{-1}.

The n.m.r. spectrum (Fig. 52) indicates the presence of an ethyl function in addition to those closely resembling that of Compound B. The methylene protons at τ 6.43 (quartet, J = 7Hz) and the methyl protons at τ 8.80 (triplet, J = 7Hz)

are evidently coupled with each other. The band at τ 2.86 could be assigned to a nuclear hydroxyl proton, and this assignment is further supported by its disappearance from the spectrum after the addition of D_2O. The band at τ 5.58 could be due to methylene protons and the ones at τ 7.83 and 8.07 to methyl protons. Thus the following structure is proposed for Compound C:
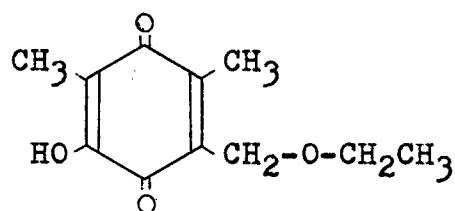
The mass spectrum (Fig. 53) gives the molecular peak at mass 210, the molecular weight of the structure shown above. The general grouping and some of the major mass peaks of this spectrum show great similarities to that of shanorellin, e.g. peaks at masses 39, 53, 55, 67, 80, 83, 108 and 136. The base peak of mass 31 could be derived as in ethyl butyl ether by a α, β cleavage with a single hydrogen transfer: (91)
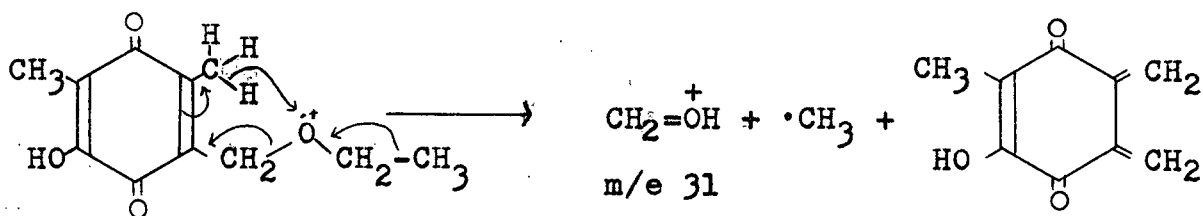
The peaks at masses 181, 165, 153, 45 and 29 could be due to ions formed by the following cleavages:
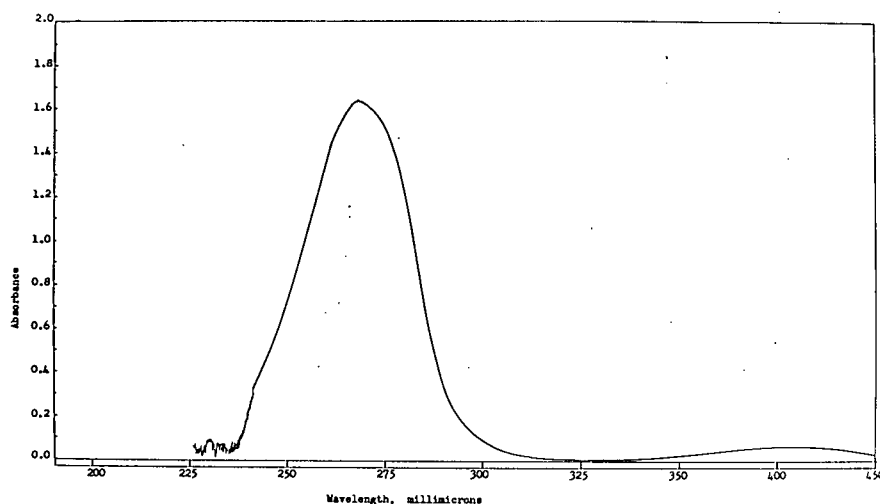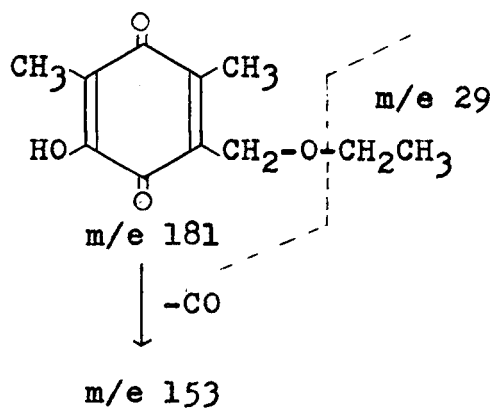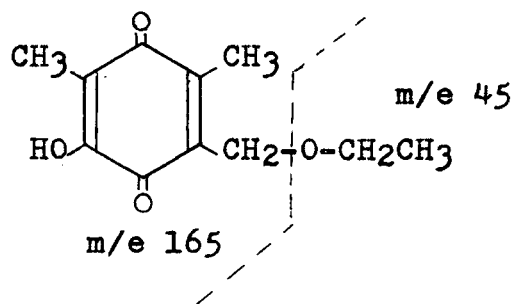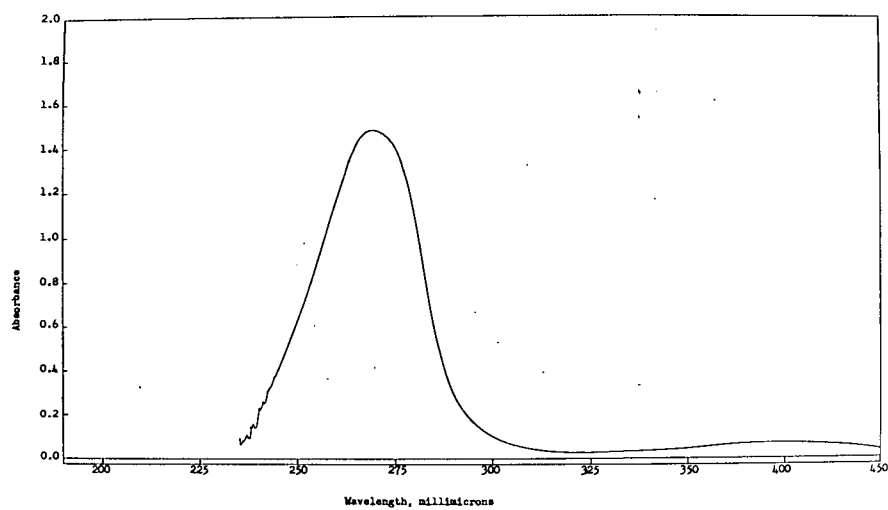
Fig. 50. Ultraviolet Spectrum of Compound C in $CHCl_3$.
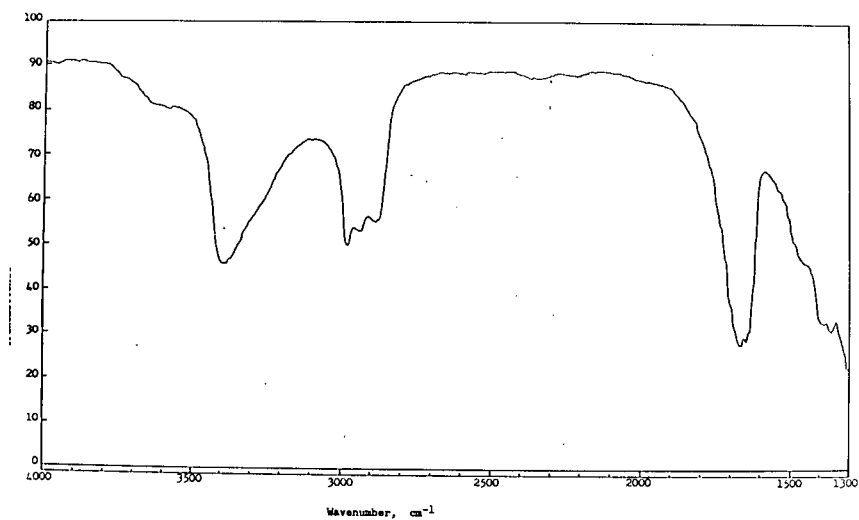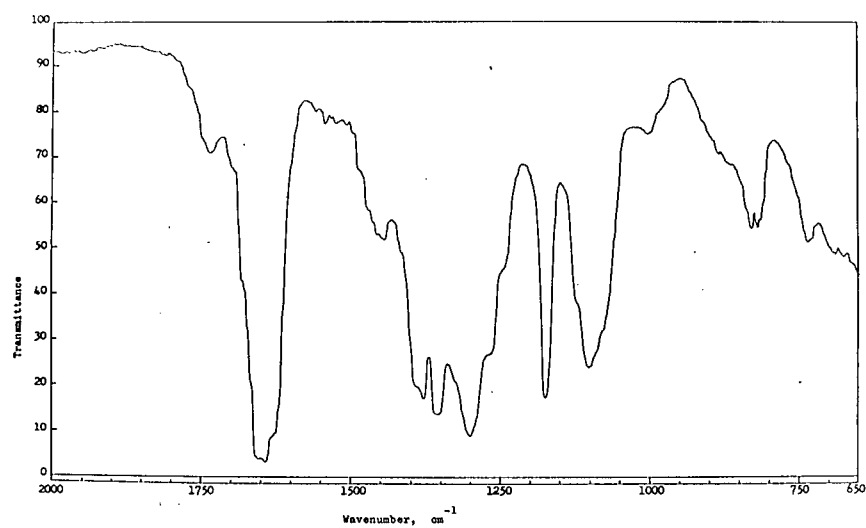
Fig. 51.   Infrared Spectrum of Compound C in KBr Disc.
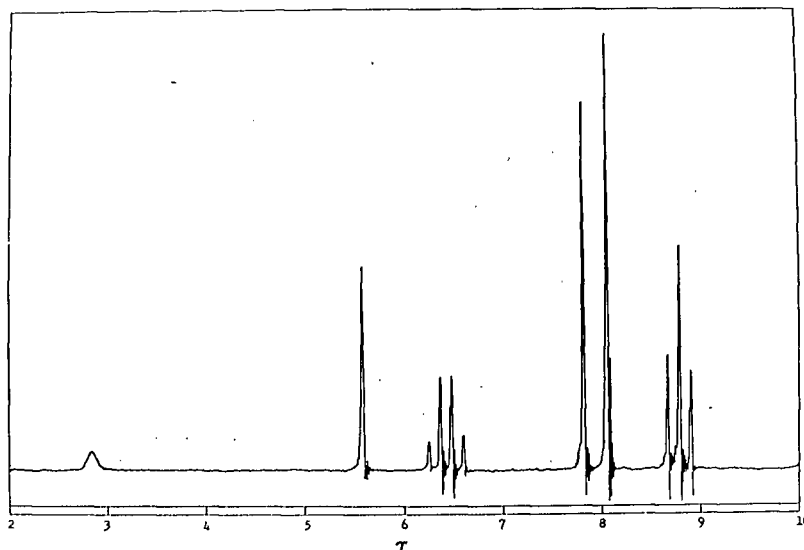
Fig. 52.   N.M.R. Spectrum of Compound C in CDCl$_3$.
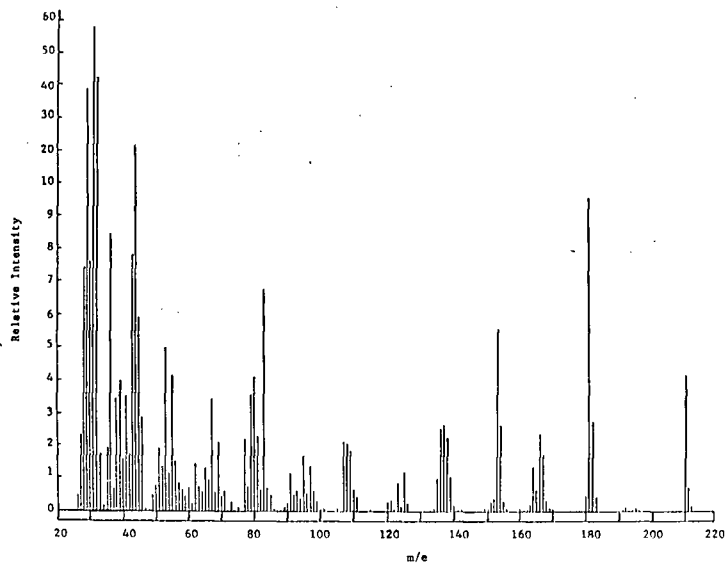
Fig. 53.   Mass spectrum of Compound C.

Part D. <u>The Biosynthesis of Shanorellin</u>

As stated in the introduction, three metabolic pathways have been shown to lead to the biosynthesis of p-benzoquinones. From the structure of shanorellin, the absence of any isoprene unit precludes its formation from mevalonate. The biosynthesis of the benzoquinone aurantiogliocladin (7) and coprinin hydroquinone (92) have been reported to be derived from compounds <u>via</u> the shikimic acid pathway. Therefore, ^{14}C-labelled shikimic acid, phenylalanine and tyrosine were fed to <u>S</u>. <u>spirotricha</u> and their incorporation into shanorellin determined. Propionate has been shown to be utilized by <u>Streptomyces</u> in the biosynthesis of macrolide antibiotics (93) and by <u>Penicillium</u> <u>baarnense</u> to give homoorsellinic acid (94). Since the number of carbon atoms in shanorellin is a multiple of three, the possibility of the participation of propionate in shanorellin production, though remote, was tested. The roles of acetate, malonate and the C_1 donors, methionine and formate, were also investigated. The origins of the carbon atoms of shanorellin were determined by the chemical degradation of shanorellin-^{14}C obtained in feeding experiments. Based on the results obtained, a possible route to its biosynthesis is suggested.

Methods

Culture Conditions

Cultures of S. spirotricha were grown in 125ml flasks or in Roux bottles as stated in each experiment. The preparation and conditions of cultures were the same as described in the general methods in Part A.

Preparation of Radioactive Compounds

All ^{14}C-labelled compounds were dissolved in water to give the appropriate concentrations and activities. For short-term feeding, i.e. less than 6 hr, the solutions were added without being autoclaved. For feedings of two days duration, the solutions were autoclaved before being used, except for malonic acid and oxalic acid which were sterilized by filtration through Millipore filters (0.22u pore size).

Isolation of Shanorellin

For comparative studies on the incorporation of various labelled precursors, the isolation procedure described in Part B was followed up to column chromatography. The shanorellin band from the column (37cm X 2½cm) was sublimed and the activity of the sublimate was determined. Shanorellin that was used in degradation reactions was further purified on preparative thin-layer plates, developed first with solvent system B and then with system C (Part B), and sublimed again. The sublimate was crystallized repeatedly from benzene-petroleum ether until constant specific activity was obtained.

Determination of the Activities of ^{14}C-labelled Compounds

All ^{14}C-labelled compounds were counted with a Nuclear Chicago Liquid Scintillation System 724 and 725 in 15ml of scintillator (PPO 6g/l, POPOP 0.1g/l, toluene 250ml, ethanol 150ml). All readings were corrected with an efficiency curve calibrated from standard quenching solutions by the channel ratio method. All shanorellin samples were dissolved in 10ml of $CHCl_3$ and an aliquot of 0.01ml was used in the determination of its activity. The quenching effect of shanorellin (0.01ml) had been tested with the following concentrations: 40mg, 20mg, and 10mg per 10ml of $CHCl_3$. The activity of 0.01ml n-hexadecane-1-^{14}C was used as the standard in the above scintillator. The efficiencies calculated from the readings by the channel ratio method coincided with those obtained from the standard quenching solutions. The quenching due to 2-phenylethylamin and acetic acid in aqueous solutions in the amounts used in the experiments had also been similarly tested. The efficiencies of the countings were found to be very close to the results obtained from the standard quenching solutions.

Degradation of ^{14}C-labelled Shanorellin

The purified shanorellin was first degraded by the Kuhn-Roth method as described by Eisenbraun, McElvain and Aycock (95). The acetate obtained was subsequently degraded by the Schmidt reaction as described by Phares (96). The carbon dioxide evolved in the latter reaction was trapped in an

aqueous solution of 80% 2-phenylethylamine instead of 0.5N NaOH as described.

Autoradiography

The developed thin-layer plates were allowed to be in contact with Kodak Blue Brand Medical X-ray films for two weeks.

Results and Discussion

To find the most appropriate age of the culture and duration of feeding of labelled compounds, two preliminary experiments were performed. The first experiment was to find the % incorporation of acetate-1-^{14}C into shanorellin when it was added to the medium of different stages of growth of the organism for 2 days. The use of acetate-1-^{14}C was chosen because it was considered to be the most likely precursor of shanorellin, and the duration of feeding was arbitrarily taken as 2 days. After finding the approximate stage of growth of the organism when acetate was best utilized to form shanorellin, the length of feeding time was again tested with acetate-1-^{14}C. The results are shown in Table VI and Table VII.

Table VI indicates that the specific activity of shanorellin decreased as the culture aged, but the % activity incorporated into shanorellin was highest around the eighteenth day of growth (approximately 13%). Thus, the most appropriate time to add the labelled compounds in order to obtain a high incorporation is about one week after the

Growth (Days)	pH of Medium	Mycelial Dry Weight (mg)	Amount (mg)	Shanorellin Activity (uc)	Specific Activity (uc/mg)
10	6.75	31.50	3.19	0.70	0.175
11	6.70	30.40	6.29	1.00	0.160
12	6.60	32.46	11.30	1.85	0.160
13	6.71	34.95	13.70	1.68	0.123
14	6.69	32.86	11.43	1.60	0.139
16	6.61	43.52	20.81	2.27	0.109
18	6.58	33.11	22.00	1.90	0.086
20	6.55	40.18	11.80	1.08	0.091
23	6.40	52.87	16.30	1.59	0.095
26	6.30	36.95	11.50	0.94	0.081

Table VI. Incorporation of Acetate-1-^{14}C into Shanorellin after Different Periods of Growth of S. spirotricha. Each sample consisted of three Roux bottles. To each bottle, 5uc of acetate-1-^{14}C was added on the day of growth as indicated. Incubation period was 2 days. The results are expressed as total of three Roux bottles.

Duration of Incubation (Days)	Amount (mg)	Shanorellin Activity (uc)	Specific Activity (uc/mg)
1	4.15	0.30	0.073
2	4.50	0.42	0.093
3	5.85	0.45	0.077
5	11.30	0.58	0.052
8	17.50	0.47	0.027

Table VII. Incorporation of Acetate-1-^{14}C into Shanorellin by S. spirotricha with Different Incubation Times. Each sample consisted of three 125ml flasks with 5 uc of acetate-1-^{14}C per flask added on the 15th day of growth. Results are expressed as the total of three flasks.

appearance of shanorellin. The specific activity of shanorellin
isolated during this time, though lower than that isolated
from earlier stages, is sufficiently high for degradation
reactions. From the results shown in Table VII, it is
apparent that the shanorellin isolated on the second day
after the addition of acetate-1-^{14}C to the medium gave the
highest specific activity. So, for testing the incorporation
of other possible ^{14}C-labelled precursors, the experiment
was carried out one week after the appearance of shanorellin
in the medium, and the incubation period was two days. The
results of this experiment are shown in Table VIII.

The results listed in Table VIII clearly show that
shanorellin is derived from the acetate-polymalonate pathway,
with methionine as a one-carbon donor. The slight activity
of shanorellin labelled from phenylalanine and tyrosine
could come from the acetate produced during the degradation
of these two amino acids (97). Sodium propionate and 2-
methylmalonic acid were definitely not precursors of shanorellin.
The activity of shanorellin labelled from malonic acid was
lower than that from acetate-2-^{14}C, but significant enough
to be taken that it was utilized in shanorellin production.
Sodium formate-^{14}C had been fed to another batch of S.
spirotricha, and as with methionine, it was efficiently
incorporated into shanorellin (6.9% incorporation).

As stated in the introduction of this thesis, there are
four prototypes of aromatic compounds formed from the acetate-
polymalonate pathway. The absence of any acetyl function on

^{14}C-Compound	Amount(mg)	Shanorellin Specific Activity(uc/mg)
acetate-1-^{14}C	8.26	0.036
acetate-2-^{14}C	11.93	0.050
malonic-2-^{14}C	9.10	0.019
shikimic-U-^{14}C	9.00	0.000
phenylalanine-U-^{14}C	9.00	0.002
tyrosine-U-^{14}C	7.40	0.005
propionate-1-^{14}C	8.70	0.000
2-methyl-^{14}C-malonic	9.20	0.000
methionine-^{14}CH$_3$	8.70	0.140

Table VIII. Incorporation of Various ^{14}C-labelled
Compounds into Shanorellin by S.
spirotricha. Each sample consisted
of three Roux bottles. The specific
activity of all the compounds were
adjusted to 2uc/uM/ml, and 4uc were
added to each bottle of a 14 day old
culture. Incubation time was 2 days.
All results are expressed as the total
of three bottles.

the nucleus of shanorellin eliminates its formation by way
of the acetyl-phloroglucinol and acetyl-resorcinol types of
ring closure. Processes similar to those in the biosynthesis
of orsellinic acid and 6-methylsalicylic acid are more
probable in shanorellin biosynthesis. The labelling patterns
of orsellinic acid and 6-methylsalicylic acid from acetate
have been well established (41, 47, 26, 98), and the carbon
atoms in shanorellin could similarly be labelled. The
pathways shown in Fig. 54 and Fig. 55 are designed to
indicate the possible sources of the carbon atoms in shanor-
ellin without considering the _in vivo_ biosynthetic sequence.

The Kuhn-Roth oxidation reaction is used routinely in
the tracer studies of methylated benzoquinones (51, 54, 63,
67, 92), the ring methyl group becoming the methyl group
of acetic acid. Shanorellin, when subjected to such a
reaction would give two moles of acetic acid for each mole
of shanorellin used, the other carbon atoms would most
probably be lost as carbon dioxide:
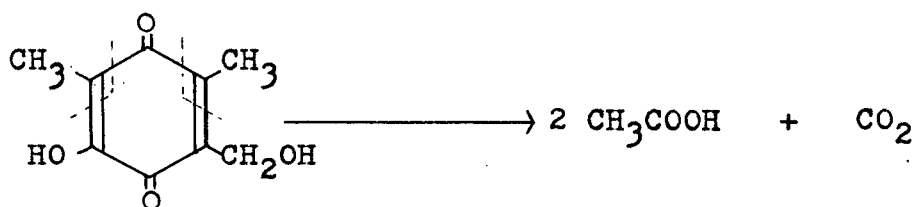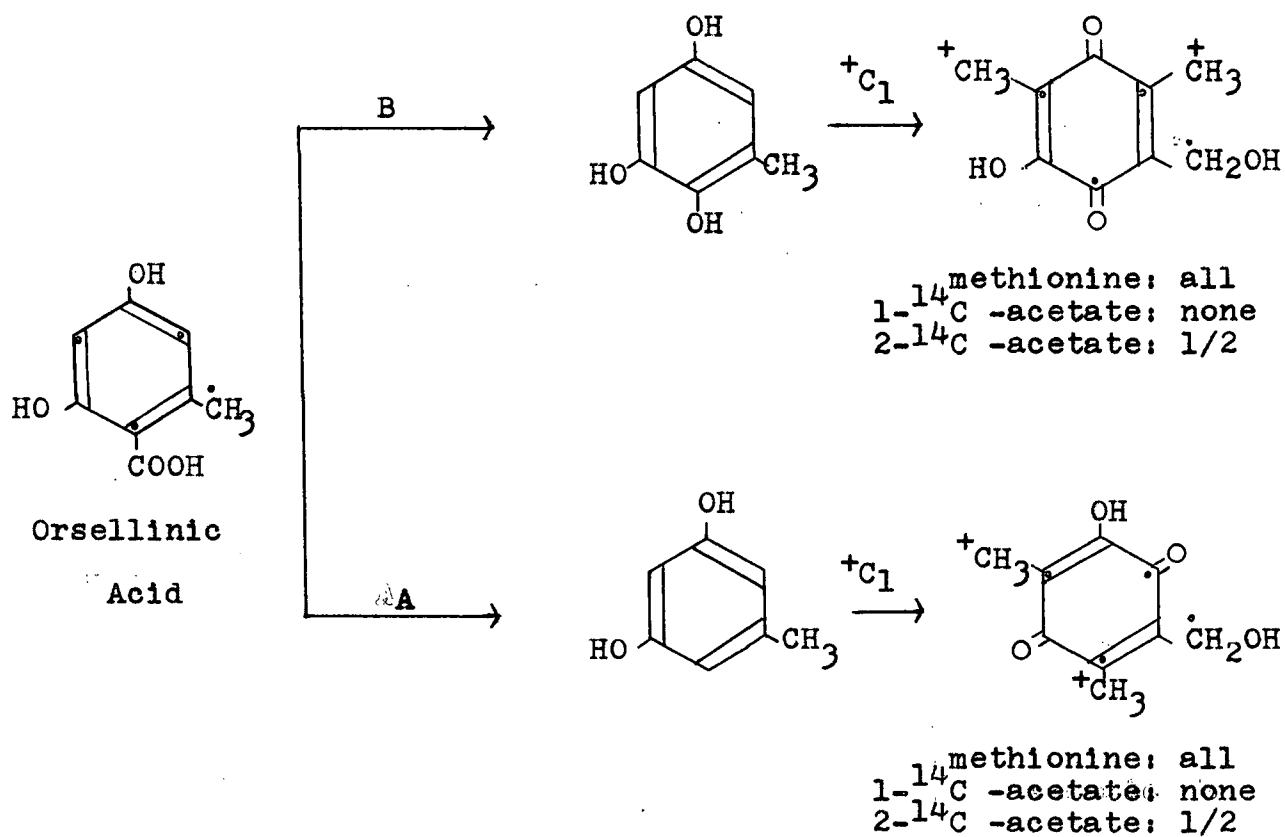
Fig. 54. Possible Origins of Carbon Atoms in Shanorellin

Based on Orsellinic Acid.

(A = non-oxidative decarboxylation

B = oxidative decarboxylation

. = carbon from methyl carbon of acetate

+ = carbon from C_1 donors)
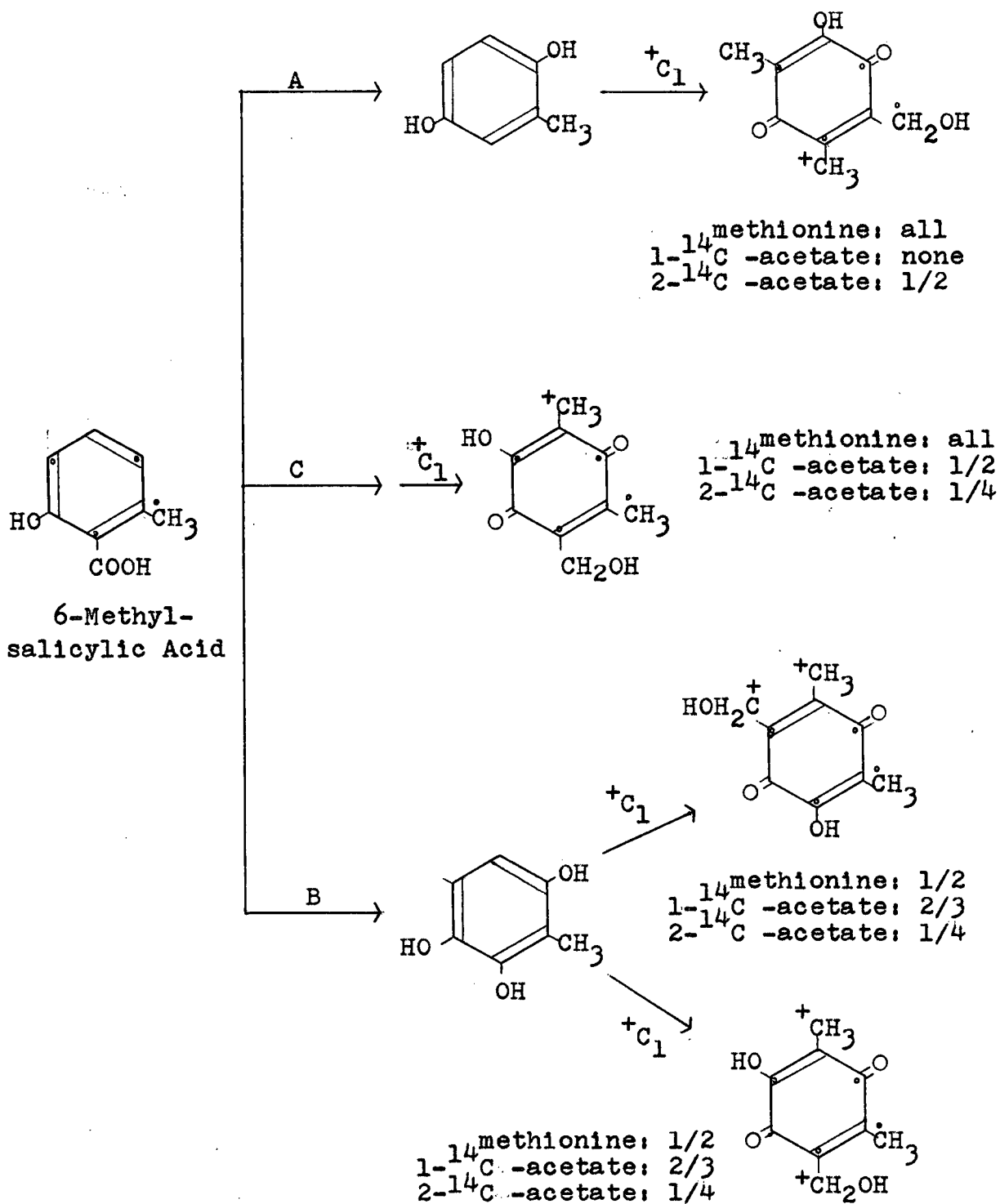
Fig. 55. Possible Origins of Carbon Atoms in Shanorellin

Based on 6-Methylsalicylic Acid.

(C = no decarboxylation.

For other symbols, see Fig. 54)

Trial experiments with unlabelled shanorellin did give such a yield of acetic acid (98-99%) on titration with 0.05N NaOH. If shanorellin, separately labelled from acetate-1-^{14}C, acetate-2-^{14}C and methionine-^{14}CH$_3$ are degraded in this way, the proportion of activity recovered in the acetic acid would give an indication of the origin of the carbon atoms. The theoretical values of the proportion of activity recovered in the acetic acid from shanorellin labelled with these three precursors and derived from the different pathways proposed, are shown also in Fig. 54 and 55.

The degradation results of shanorellin labelled from acetate-1-^{14}C, acetate-2-^{14}C and methionine -^{14}CH$_3$ by Kuhn-Roth oxidation are shown in Table IX. The percentage of recovery of activity in the acetic acid from shanorellin labelled from acetate-2-^{14}C could be taken as 50%. In one experiment a recovery of 49% had been obtained. The excess 5% could be due to the presence of unlabelled acetyl-CoA and short chain polyketo intermediate already formed in the mycelium. On addition of the labelled acetate, it would be incorporated to the latter part of the molecule, thus, giving higher activity in the degradation results. Similarly, the percentage of recovery of activity in the acetic acid from shanorellin labelled from methionine- ^{14}CH$_3$ could be taken as 100%. The 10% discrepancy could be accounted for if the mono-methylated polyketo intermediate was present in the mycelium when this labelled compound was added. In

Activity of Shanorellin	Activity Recovered[+] in Acetic Acid	% Recovery[*]
A. From Acetate-1-^{14}C		
0.97uc	0.00uc	0.00
1.13	0.00	0.00
B. From Acetate-2-^{14}C		
0.23	0.11	48.7
0.33	0.19	57.1
1.31	0.72	55.7
C. From Methionine-^{14}CH$_3$		
0.18	0.16	90.2
0.19	0.17	86.4
0.46	0.41	91.3

Table IX. Results of the Kuhn-Roth Degradation of
Shanorellin Labelled from Acetate-1-^{14}C,
Acetate-2-^{14}C and Methionine-^{14}CH$_3$.
(+, activity of acetic acid isolated from
75ml of distillate and diluted to 100ml
solution; 0.5ml of the solution was used
in the radioactive assay and 75ml were
pipetted for titration with 0.05N NaOH.
*, % recovery was calculated from the
specific activity of shanorellin before
degradation and the specific activity of
shanorellin calculated from the activity
and number of mmoles of acetic acid
recovered).

addition to these factors, the experimental errors due to technique and measurement should also be added. Shanorellin labelled from acetate-1-^{14}C gave unlabelled acetic acid. Thus, these degradation results and the predicted values shown in Fig. 54 and 55 indicate that the sources of carbon atoms of shanorellin are as follows:

$\cdot$ = methyl carbon of acetate

+ = methyl carbon of methionine
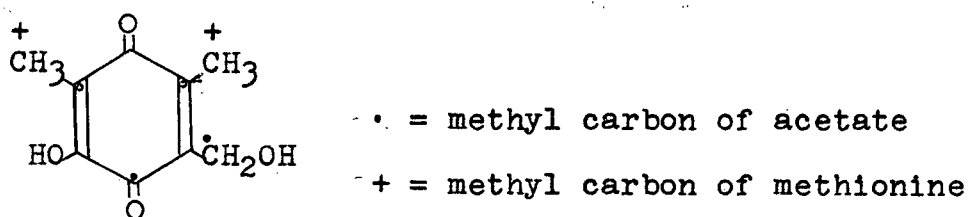
Fig. 56. Origins of Carbon Atoms in Shanorellin.

To confirm this labelling pattern, the acetate isolated from Kuhn-Roth oxidation of shanorellin was further degraded by the Schmidt reaction:

$$CH_3COOH \longrightarrow CH_3NH_2 + CO_2$$

The results of the Schmidt degradation of the acetates from shanorellin labelled from acetate-2-^{14}C and methionine-$^{14}CH_3$ are shown in Table X.

In trial experiments with commercial acetate-1-^{14}C and acetate-2-^{14}C, it was noted that when these solutions were subjected to lyophilization to remove the water present, 15-30% of the activity was lost during the process. The addition of 3 drops of 0.5N NaOH prevented such loss of activity. The Schmidt degration of acetate-2-^{14}C gave over 95% of the

	Activity Isolated (% Recovery)	
Activity of Acetate	CH_3NH_2 [*]	CO_2
A. From Shanorellin Labelled with Acetate-2-^{14}C		
0.13	0.051(38%)	0.062(46%)
0.13	0.049(37%)	0.058(43%)
0.03	0.014(46%)	0.009(30%)
B. From Shanorellin Labelled with Methionine-^{14}CH$_3$		
0.16	0.158(98%)	0.000(0%)
0.16	0.157(98%)	0.000(0%)
0.12	0.116(97%)	0.000(0%)

Table X. Schmidt Degradation of Acetate Isolated from Kuhn-Roth Oxidation of Shanorellin Labelled from Acetate-2-^{14}C and Methionine-^{14}CH$_3$. To each sample, 0.2mmoles of cold sodium acetate, 3 drops of 0.5N NaOH were added. ([*], activity of the residue remaining in the reaction flask)

activity in the methyl carbon (activity remaining in the flask) and none in the carboxyl carbon (activity in the trapping solution); but with acetate-1-^{14}C, a range of 50-80% of the total activity was isolated in the trapping solution and 2-5% remained in the flask. There was still 20-40% of the activity which could not be accounted for. This may be due to the inefficiency of the trapping solution for carbon dioxide, although phenethylamine has been reported to be one of the most efficient trapping reagents for carbon dioxide (99). Another reason may be due to the presence of sodium hydroxide in the reaction mixture which was not present in the reaction described in the original literature, and its presence may cause undesirable side reaction(s). When the data shown in Table X was obtained, another source of error in the degradation procedure was realized. After the acetic acid was isolated by steam distillation in the Kuhn-Roth oxidation, it was titrated with 0.05N NaOH with phenolphthalein as indicator. Since phenolphthalein contains two phenolic hydroxyl functions, in the presence of 100% H_2SO_4, it could react with the acetic acid present to give esters, thus retaining some of the activity as acetate in the flask. The result of Schmidt reaction with commercial acetate-1-^{14}C with 3 drops of 0.5N NaOH, 2 drops of phenolphthalein confirmed this source of error: 36% of the activity was recovered in the flask and 40% in the trapping solution. Thus, another Kuhn-Roth degradation of shanorellin labelled from acetate-2-^{14}C was carried out. The acetic acid isolated

was assayed for its activity (55% of the total activity in shanorellin was present in the acetic acid solution) and the solution was then made alkaline with 0.05N NaOH to pH 12 as tested with litmus paper. The Schmidt degradation of this sample gave 4.7% activity remaining in the flask and 56.9% activity in the trapping solution. This result is within the range of activities recovered with commercial acetate-1-^{14}C. Thus, it could be taken that most of the activity in the acetic acid isolated from shanorellin labelled from acetate-2-^{14}C resided in the carboxyl carbon. Together with the result obtained from the degradation of the acetic acid derived from shanorellin labelled from methionine-^{14}CH$_3$, it is conclusive to state that the labelling pattern shown in Fig. 56 is correct.

The metabolism of acetate-1-^{14}C and acetate-2-^{14}C, and the incorporation of the radioactivities into compounds of the C_1 pool have been observed by Birch et al. (28) and by Bentley and Lavate (43) in the biosynthesis of aurantiogliocladin. Birch suggested that the incorporation of activity from acetate-1-^{14}C into the methoxyl group of aurantiogliocladin occurred via $^{14}CO_2$. The fixation of CO_2 via β-carboxylation of pyruvate to oxaloacetate by the enzyme systems of phosphoenolpyruvic carboxylase and phosphoenolpyruvic carboxykinase have been demonstrated to occur in fungi (100). The entry of oxaloacetate into the tricarboxylic acid cycle and glyoxylate cycle would eventually label the glyoxylate. The conversion of glyoxylate to formate via oxalate has been

observed in bacteria and fungi (101). The involvement of
acetate-2-^{14}C in the tricarboxylic acid cycle and glyoxylate
cycle would also lead to the labelling of the C_1 pool. For
the two differently labelled acetate species to enter the
C_1 pool, oxalate would be the common precursor. Thus, to
test the possibility of the occurrence of these pathways
in $\underline{S}$. $\underline{spirotricha}$, oxalic-1,2-^{14}C-acid was fed to three
cultures grown in Roux bottles for 2 days (5uc/bottle). No
activity was detected in the shanorellin isolated. This
result may be taken as an indication that the randomization
of label of acetate by eventual incorporation into the C_1
pool $\underline{via}$ the above routes does not occur in $\underline{S}$. $\underline{spirotricha}$,
or, if it does occur, it is only to an insignificant extent.

As indicated in Fig. 54 and 55, three routes (pathway A
in Fig. 55, pathways A and B in Fig. 54) to the biosynthesis
of shanorellin are possible. Since the methylation process
occurs most likely at the polyketo-chain level for reasons
already discussed in the introduction, the pathway from
6-methylsalicylic acid, i.e. pathway A in Fig. 55, and the
one $\underline{via}$ non-oxidative decarboxylation of orsellinic acid,
i.e. pathway A in Fig. 54, may be eliminated. The presence
of 6-methylsalicylic acid and orsellinic acid could not be
detected in the culture medium at any stage of growth by
thin-layer chromatography. 3.5-Dimethylorsellinic acid has
been isolated from $\underline{Mortierella}$ $\underline{ramannian}$ (102). A sample was
kindly sent to us by Dr. W. W. Andres. It was not detected
in the fungal medium however. The relative positions of

some of the standard aromatic compounds to the two major
phenolic compounds detected on thin-layer plates are
shown in Fig. 57.
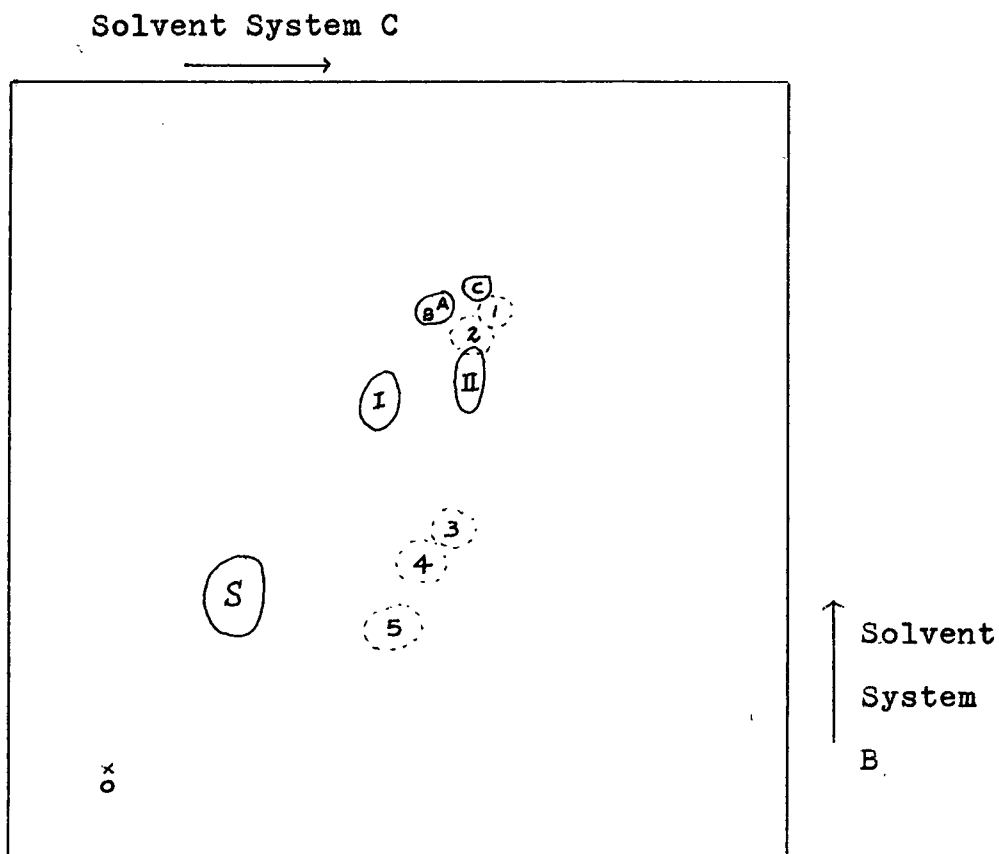
Fig. 57. The Relative Positions of the Benzoquinones and
Phenolic Compounds Detected in the Medium and
the Aromatic Acid Standards.

S - shanorellin

A - Compound A

B - Compound B

C - Compound C

I and II - Phenolic Compounds

0 - origin

1 - 6-methylsalicylic Acid

2 - 3,5-dimethyl-orsellinic acid

3 - orsellinic acid

4 - 3-methylorsellinic Acid

5 - 3,4-dimethyl-5-hydroxy-phenol

These observations thus add support to the postulation
that the methylation steps in polyketide biosynthesis occur
before the aromatization of the ring. The absence of 3,5-
dimethyl-orsellinic acid in the medium might be taken as an
indication that the oxidation of the methyl group to hydroxy-
methyl function occurs early in the biosynthesis of shanorell-
in. A compound with structure IV might be the first aromatic
compound formed:

$$\begin{array}{c}
\text{COOH} \\
\text{HO} \quad \quad \text{CH}_2\text{OH} \\
\text{CH}_3 \quad \quad \text{CH}_3 \\
\text{OH}
\end{array}$$
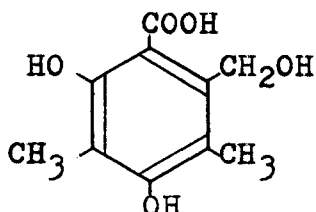
The phenolic compound II (Fig. 57) had been detected
by autoradiographs to be labelled with ^{14}C-acetate and
methionine. Its Rf values on thin-layer plates are very close
to that of 3,5-dimethyl-orsellinic acid (Fig. 57), and on
spraying with p-nitroaniline reagent, it appears as orange
brown, a color reaction also given by 3,5-dimethyl-orsellinic
acid. That this phenolic compound II has the structure IV
shown above is not unlikely. Subsequent oxidative decarbox-
ylation of IV and oxidation of the hydroquinone formed would
give shanorellin.

Part E. General Discussion

The occurrence of p-benzoquinones with structures
similar to shanorellin is not uncommon in fungi (5, 13-17).
Tauranin produced by Oospora aurantiaca possesses a hydroxy-
methyl and a hydroxyl function on the quinone ring (87) and
the oxidation of a methyl group to a primary alcohol has been
shown to occur in the biosynthesis of oosponol (102). The
acetylation of a primary alcohol function has been reported,
e.g. gliotoxin acetate and cephalosporin C (103). The
structures of the ether derivatives of shanorellin (Compound
B and C) are unique. Compounds with an ether linkage at
two aliphatic carbons have not been reported from the fungi.
Compound B could be regarded as a dimer of shanorellin.
Fungal metabolites such as the bibenzoquinones and terphenyl-
quinones are linked through the ring carbons (104); the
benzophenone derivatives, the spirans, the diphenyl ethers
and the depsidones all have a carbonyl and/or oxygen function
situated between the two rings (105). The nearest equivalent
to a structure like Compound B is the lignan pinoresinol which
occurs in Pinus and Picea (106). With regards to Compound C,
the ethyl ether of shanorellin, no such equivalent has been
found in fungi or in plants. Glycerol ethers have been
isolated from lipids of animals and a bacterium (107-109).
The chain length of these ethers ranges from 14 to 22 carbons.

The biosynthesis of shanorellin has been discussed. The
biosyntheses of Compounds A, B and C have not been studied

systematically, but some indication as to their mode of
formation may be suggested from those studies carried out
on shanorellin.

During the tracer studies of shanorellin, it was part of
the routine to take an aliquot of the ethyl acetate extract
of the medium and subject it to thin-layer chromatography
and autoradiography. In experiments where the labelled
compounds were fed for 2 days, precursors that labelled
shanorellin were found to label Compound A, B and C also.
So, Compounds A, B and C are synthesized from the acetate-
polymalonate pathway. With short-term feedings with acetate-
$2-^{14}C$ and methionine-$^{14}CH_3$ for 20 min, the autoradiographs
of the thin-layer plates developed two dimensionally with
solvent systems B and C showed three radioactive areas:
one for shanorellin, one for phenolic compound II (Fig. 57)
and one in the area of Compound A and B. Most probably the
last radioactive compound is the acetate of shanorellin
(Compound A), and is an artifact of the isolation procedure.
The ethyl ether of shanorellin (Compound C) became labelled
in experiments in which the period of administration of
radioactive substrates was 40 min or longer. That the appear-
ance of the ethyl ether of shanorellin as a radioactive comp-
ound falls behind that of shanorellin suggests that it is
formed from shanorellin. Labelled shanorellin was fed to a
culture of S. spirotricha, but unfortunately, that particular
batch did not produce these derivatives of shanorellin.

The acetyl carbons of sclerotiorin have been shown to be derived from acetate (110), and similar biosynthetic process in S. spirotricha would give shanorellin acetate, if this compound is produced by the fungus and not present as an artifact of the isolation procedure. The reduction of the carbonyl function could then give rise to the ethyl ether of shanorellin (Compound C). Another possibility for the biosynthesis of Compound C is that the hydroxymethyl function of shanorellin is methylated twice with methionine. Two successive methylations to give an ethyl function have been shown in the biosynthesis of the plant sterols, e.g. β -sitosterol and fucosterol (111). Unfortunately, this could not be tested as sufficient amounts of this compound were not recovered from tracer experiments with methionine.

For Compound B, it is most likely formed by the condensation of two moles of shanorellin or its hydroquinone or while shanorellin is in its quinhydrone form. If Compound B is synthesized in a similar pattern to lignins (112), it is formed via a free radical mechanism. In the presence of peroxidase and hydrogen peroxide, shanorellin, or its hydroquinone or quinhydrone, would react to give Compound C. Or, Compound C is synthesized by a process similar to those of glycerol ethers isolated from animals. An enzyme has been isolated from preputial tumors in mice which is capable of converting intact $1-^{14}C$ fatty alcohol, but not the acid or aldehyde, into alkyl glyceryl ethers (113).

In conclusion, a biogenetic scheme is proposed for the synthesis of shanorellin and its derivatives isolated from S. spirotricha (Fig. 58).

1 mole Acetyl-CoA

3 moles Malonyl-CoA

2 moles S-Adenosylmethionine

$$CH_3-\overset{\overset{O}{\|}}{C}-CH_2-\overset{\overset{O}{\|}}{C}-CH_2-\overset{\overset{O}{\|}}{C}-CH_2-COSR$$

Oxid^n C_1 C_1

$$\begin{array}{c} COOH \\ HO \quad CH_2OH \\ CH_3 \quad CH_3 \\ OH \end{array}$$

Oxidative
Decarboxylation

Compound A

Reduction

$$\begin{array}{c} O \\ HO \quad CH_2O-\overset{\overset{O}{\|}}{C}-CH_3 \\ CH_3 \quad CH_3 \\ O \end{array}$$

Acetyl-
CoA

2 C_1
Units

Compound C

$$\begin{array}{c} O \\ HO \quad CH_2O-CH_2CH_3 \\ CH_3 \quad CH_3 \\ O \end{array}$$

$$\begin{array}{c} OH \\ HO \quad CH_2OH \\ CH_3 \quad CH_3 \\ OH \end{array}$$

Oxidation

Shanorellin

$$\begin{array}{c} O \\ HO \quad CH_2OH \\ CH_3 \quad CH_3 \\ O \end{array}$$

Compound B

$$\begin{array}{c} O \qquad\qquad O \\ HO \quad CH_2-O-CH_2 \quad OH \\ CH_3 \quad CH_3 \quad CH_3 \quad CH_3 \\ O \qquad\qquad O \end{array}$$

Fig. 58. Suggested Biogenetic Scheme for the Synthesis of Shanorellin and its Derivatives

in S. spirotricha.

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