BIOACTIVE METABOLITES FROM CULTURES OF TWO SPECIES OF HIGHER FUNGI

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Bioactive Metabolites from Cultures of Two Species of Higher Fungi

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Abstract

BIOACTIVE METABOLITES FROM CULTURES OF TWO SPECIES OF HIGHER FUNGI

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Higher fungi collected from Central and Southern Ethiopia were investigated. The ethyl acetate extracts of submerged cultures of fifty fungi were screened for antimicrobial activity and lethality to brine shrimp (*Artemia salina*).

Trametes pubescens and an *Agaricus* sp. were chosen for further chemical studies of the bioactive metabolites. Larger scale cultivations of these two species were made and the bioactive secondary metabolites were isolated, the structures of which were characterized by spectroscopic methods and chemical transformations.

T. pubescens yielded an antifungal compound which was characterized as *trans*-2,3-epoxy deca-4,6,8-triyn-1-ol. Literature search revealed this compound to be reported for the first time. The *Agaricus* sp. afforded a known compound responsible for the fungal culture´s lethality to brine shrimp and identified as monocerin, which appeared to be the first report from *Agaricus* species.

1.0. INTRODUCTION

1.1. General

Secondary metabolites produced by fungi have been of interest to man for a long time. The diverse types of fungal secondary metabolites have been documented in volumes I and II of Turner, 1971 [1] and Turner and Aldridge, 1983 [2]. Fungal secondary metabolites such as penicillin and cephalosporin, antibiotics from *Penicillium* species and *Cephalosporium acremonium*, respectively are two of the most outstanding medicinally valuable discoveries made. Amanita toxins and hallucinogens from mushroom fruitbodies [3, 4], toxins in foods and grains such as aflatoxins, ergot alkaloids which are pharmaceutically important, are some of the examples of fungal secondary products affecting man´s life. Some fungi are of economic interest because of their pathogenicity to crop plants as well as plants used as wood source [5]. On the other hand antibiotics of fungal source such as streptomycin, tetracycline, cycloheximide and griseofulvin have been used to control plant diseases [6]. It has been argued that use of fungal metabolites as pesticides has the advantage of their being biodegradable hence presenting less pollution hazards. Biotechnology based on lignocellulosic materials is an important area of research in the attempt to substitute fossil fuel resources with renewable sources of energy. Since wood degradation is carried out by and large by fungi, understanding the mechanism of action of the enzymes in the degradation of cellulose, hemicellulose and lignin is required in order to put them to proper utilization [7]. Fungi also find industrial uses notably in the food fermentation industry and in the research field of geneticists and biochemists as biological research tools [8].

1.2. Classification : Fungal systematics.

According to Whittaker [9] living organisms are now organized into five kingdoms; Animalia, Plantae, Fungi, Monera and the Protista. The classification is based on the mode of nutrition of the organisms. The kingdom Fungi has two divisions, the Pantanemomycota and the Eumycota. The division

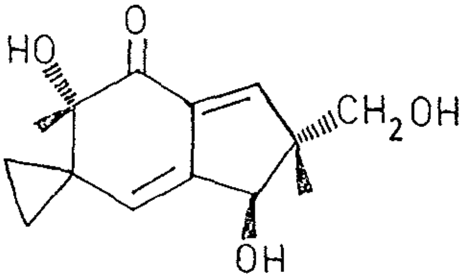
Zygomycotina, Ascomycotina, Basidiomycotina and Deuteromycotina. Of these subdivisions the Ascomycotina and Basidiomycotina are grouped together under the general heading of the Higher Fungi or the Macromycetes. The Macromycetes are comprised of about 46,000 species [10]. The grouping into the Ascomycotina and Basidiomycotina is based on the type of the sexual spores formed. The Ascomycotina which includes the ergot fungi, the cup fungi and the morels form ascospores within a membraneous cell called an ascus. The Basidiomycotina comprises of the bracket fungi, puffballs, jelly fungi, earth stars, bird's nest fungi stinkhorns and the familiar mushrooms and toadstools. The Basidiomycotina are characterized by their sexual spores, the basidiospores which are in most cases formed in groups of four on a club shaped cell called a basidium. The Basidiomycotina are considered to be the most highly advanced of the Fungi. The subdivision Basidiomycotina is further divided into three classes: the Teliomycetes, Hymenomycetes and Gasteromycetes [11]. The Hymenomycetes is the largest class. The polypores come under the Aphyllophorales within the family Polyporaceae of which *Trametes* is one genus and the *Agaricus* come under the order Agaricales within the family Agaricaceae [11, 12].

1.3. Systematic classification of antibiotics.

Systematic classification of antibiotics has been attempted by various groups according to effectiveness of the antibiotics in their clinical uses, according to mechanism of action, based on the biosynthetic pathway, and according to chemical structure [13]. Berdy suggests that a methodical systematization can be made according to the chemical structure since the relationship between structure and biological activity is obvious and chemically similar compounds do usually have the same biosynthetic pathways, and because that is the area that provides classification on a factual basis. A proposed key to the main antibiotic families is also given in the same publication. The systematic classification of antibiotics as well as biologically inactive secondary metabolites of fungi can also be used in chemotaxonomical studies. Fungal metabolic constituents sometimes serve to delineate unsettled fungal classifications [15-18].

1.4. Structure-biological activity relationships of bioactive compounds of higher fungi.

Attempts have been made to propose the structure activity relationships (SAR) of bioactive compounds. However mechanisms of action and the SAR are not explicitly known even for the clinically important antibiotics. Studies therefore continue for the elucidation of the mode of action of these important compounds, and some generalizations and hypotheses have been put forward. The presence of α,β-unsaturated carbonyl groups seems to be generally connected with antibiotic and cytotoxic activities [19]. A review of the biological activity of sesquiterpene lactones [20] states the presence of an exocyclic methylene conjugated to a γ-lactone, and of a functional group such as an epoxide, hydroxyl, chlorohydrin, α,β-unsaturated ketone or O-acyl adjacent to the α-CH_2 of γ-lactone which can enhance the reactivity of the conjugated lactone towards biological nucleophiles to be the principal requirements for biological activity. The presence of highly electrophilic functional groups that selectively alkylate nucleophiles such as thiols by Michael-type addition are responsible for the inhibitory action of sesquiterpene lactones [20, 21]. Reaction of endocyclic α,β,-unsaturated γ-lactones with thiols has also been suggested to play a key role in several biological growth regulatory phenomenon [22]. Illudin S (1), an antimicrobial agent isolated from *Clitocybe illudens* [23, 24] and *Lampteromyces japonicus* [25] inhibits in vivo synthesis of deoxyribonucleic acid (DNA) from thymidine [26]. Illudin S (1) possesses a cyclopropane ring and an α,β-unsaturated ketone function, both potentially good alkylating agents.



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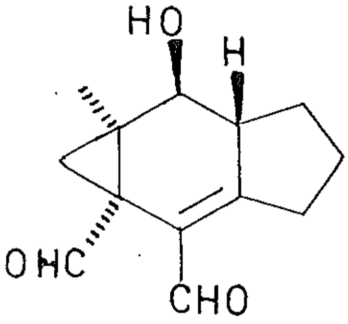
The SAR for unsaturated dialdehydes has been studied [27] using the secondary metabolite merulidial (2), an isolactarane sesquiterpene produced by the fungus *Merulius tremellosus.*



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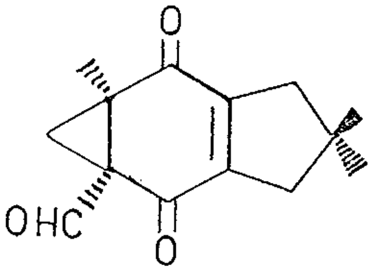

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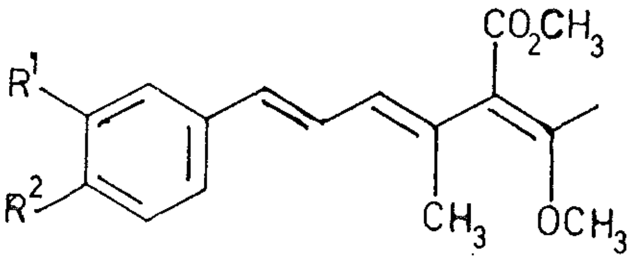
Merulidial has antibiotic, cytotoxic and mutagenic properties for which the presence of an unsaturated dialdehyde function is thought to be essential. Small changes in the structure influence the biological activity of merulidial and related compounds [27]. The reduced triol is completely inactive [28]. In another report [19] the aldehyde group of merulidial was oxidized to the homo-*p*-quinone (3) which however retained its activity. Another group of compounds, the strobilurins (**4**, **5**) which are antifungal agents, have been shown to depend on the presence of the terminal *E*-β-methoxyacrylate for their bioactivity. The synthetic compound with the (*Z*)-geometry at the terminal double bond is found to have no biological activity [19].



4 $R^1 = R^2 = H$
5 $R^1 = OH, R^2 = Cl$

1.5. Objectives and scope of this work

Tropical fungi have not been as extensively studied as the temperate fungi. Recently, a project on bioactive metabolites from fermentation cultures of Ethiopian Basidiomycetes [29], where 120 fungi were screened, has yielded eleven bioactive metabolites. The chemical structures of most of these metabolites have been elucidated. The present study is part of an ongoing collaborative project dealing with biological and chemical aspects of tropical fungi, particularly those collected from Ethiopia. In the course of this work 260 higher fungi (mostly Basidiomycetes) were collected from the highlands of Ethiopia in June-July, 1990, out of which 50 were screened for bioactivity. Here we describe the isolation of pure cultures, the screening and cultivation of fungal cultures with bioactivity, and the isolation and characterization of two bioactive metabolites from two fungi.

2.0. RESULTS AND DISCUSSION

2.1. Growth, cultivations of cultures and production of metabolites.

Cultures using yeast extract-maltose-glucose medium were made for 130 fungal species. Of these 61 species grew on laboratory media. The EtOAc extracts of 50 species were screened for bioactivity as described in the Experimental section and this resulted in the identification of 15 species with bioactivity. A few species were selected for further studies based on the activity and TLC profile of the crude EtOAc extract.

Cultivation of seven fungi, *Abortiporus bienniia, Agaricus* sp., *Cerena meyenni, Termitomyces microcarpus, Trametes pubescens* and two *Xylaria* spp., and isolation of the bioactive constituents was attempted. The cultivation was initially attempted on wort medium since the fungi grew well on it and because it was a cheap and locally available medium. However it was not possible to get consistent and reproducible results with this medium. Different standard media were then tried out and modified BAF medium was found to be the best. All further cultivations were carried out on modified BAF medium. The isolation and the structural elucidation of the bioactive compounds from submerged culture cultivations of two fungi, *Agaricus* sp. and *Trametes pubescens* is described.

2.2. *Trametes pubescens* (Fr.)

2.2.1. The producing fungus

Trametes pubescens (Fr.) Pil. is a polypore collected from Munesa Forest, S. Ethiopia in July 1990. It is a cosmopolitan fungus with a wide distribution in East Africa particularly in Ethiopia, Kenya and Tanzania [30]. Eighteen species in North America [31] and 20 species in Africa have been described [30] in the genus *Trametes. Trametes* spp. are known to cause white rot of hardwoods.

2.2.2. Culture cultivation and isolation of compound 6, an antifungal metabolite.

The fungus was cultivated in submerged culture in 1 l flasks. A 4 days old submerged culture (100 ml) in 250 ml flasks was used for inoculum. The culture filtrate was separated from the mycelia by suction filtration after 10 days of growth. This was subjected to extraction with EtOAc and the EtOAc extract was concentrated to afford 390 mg of crude extract. The TLC of the crude extract showed six spots out of which the major component happened to be the active principle. 25 mg of this active component was isolated by CC and its structure elucidated as described below.

2.2.3. Structure elucidation of *trans*-2,3-epoxy deca-4,6,8-triyn-1-ol (6).

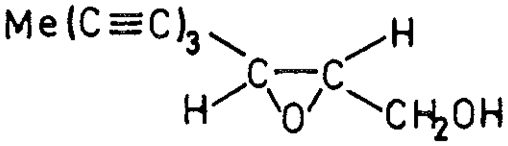
Compound **6** was found to be thermally unstable and showed a color change from pale yellow to blue and then violet when left in open air. The UV spectrum displayed finely structured finger-like absorption maxima which are known to be characteristic of polyacetylenes [32, 33]. However, when compared with the UV spectrum of a simple triyne (Table 1) the absorption maxima were slightly shifted to longer wavelength which suggested the presence of an oxygenated functional group [36, 37]. The IR spectrum showed absorption bands at 2250 cm^{-1} characteristic of carbon-carbon triple bonds, a band at 3400 for an OH group and at 1070 and 860 cm^{-1} which can be attributed to an epoxide moiety [38, 39].

Table 1: UV maxima of compound **6** and similar triynes.

$CH_3(C\equiv C)_3CH_3$	207	239	253	268	286	306 [34]
Compound **6**		242	262	276	293	312
$H(C\equiv C)_3CH{=}CHCH_2OH$	209	245	259	274	291	310 [35]

The high field ^{1}H NMR spectrum showed resonances at δ1.85 (3H, *s*), δ3.19 (1H, *m*), δ3.36 (*1H, d, J* = 2.5 Hz), δ3.52 (1H, *dd*, *J* = 13 and 4 Hz), δ3.69 (1H, *dd*,

J = 13 and 3 Hz). The singlet at δ1.85 was assigned to a terminal methyl group on an acetylenic chain. The sharp singlet at δ1.85 suggested that there were more than two triple bonds [40]. The doublet of doublets at δ3.52 and 3.69 can be assigned to geminal protons (Jgem = 13 Hz) on an oxygenated carbon atom and are both coupled with the one-proton multiplet at δ3.19. The multiplet at δ3.19 is further coupled with the one-proton doublet at δ3.36. The above NMR findings together with the UV and IR discussed above suggested the presence of an epoxide flanked between a triyne and CH_2OH group. Thus structure **6** was assigned to this fungal metabolite.

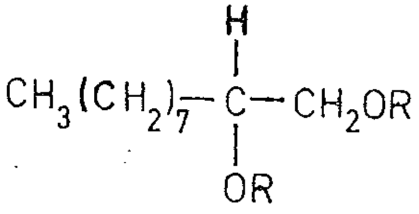


6

The above assignment was further supported by the ^{13}C NMR spectrum which portrayed at least eight carbon resonances. The DEPT spectrum confirmed the presence of two tertiary, one secondary and one primary carbon atoms. The primary carbon resonance which appeared at δ3.7 is in accord with a terminal methyl group attached to an sp hybridized carbon atom. Furthermore the chemical shifts (δ43.5 and 61.7) of the tertiary carbon atoms are in agreement with the presence of an epoxide and the secondary carbon resonating at δ61.4 is consistent with a CH_2OH group. Only four of the expected six acetylenic carbon resonances appeared at δ64.6, 74.5, 78.6 and 85.3 (signal overlapping). The HRMS showed a molecular ion peak at m/z 160 consistent with the molecular formula $C_{10}H_8O_2$ as suggested for compound **6**.

Further supporting evidence to the above structural assignment was obtained by subjecting compound **6** to catalytic hydrogenation using PtO_2 as a catalyst.

The ^{1}H NMR spectrum (Table 2, Fig. 1) of the hydrogenation product displayed a three-proton triplet at δ0.85 due to a terminal methyl group. The broad singlets at δ1.25, 1.4 and 1.6 integrate for a total of fourteen protons and suggest the presence of seven CH_2 groups. The remaining three-proton resonances appeared at δ3.43, 3.66, and 3.71. The signals at δ3.43 and 3.66 are geminally coupled (J = 11 Hz) and are both vicinally coupled (J = 7 and 3 Hz) with the one-proton multiplet at δ3.71. This NMR finding suggests the presence of a glycol and structure **7** was assigned to the hydrogenation product.



$$CH_3(CH_2)_7-\overset{\displaystyle H}{\underset{\displaystyle OR}{C}}-CH_2OR$$

7 R=H

8 R=Ac

The formation of **7** from **6** can be explained by hydrogenation of the triple bonds and hydrogenolysis of the epoxide at the C-3 oxygen bond. The ^{1}H NMR spectrum and the melting point of compound **7** are identical with those reported for 1,2-decanediol [41].

Treatment of compound **7** with Ac_2O/Py gave the diacetate **8**. The ^{1}H NMR spectrum of **8** (Table 2, Fig. 2) showed a singlet at δ2.05 integrating for six protons corresponding to two acetyl groups. In addition, two 1H doublet of doublets and a 1H multiplet appeared at δ4.02, 4.22 and 5.08, respectively. The signals at δ4.02 and 4.22 are geminally coupled with each other (J = 11.85 Hz) and are both vicinally coupled (J = 6.7 and 3.3 Hz) with the multiplet at δ5.08. Compared with the corresponding proton resonances of compound **7**, acetylation shifts of 0.59, 0.56 and 1.37 ppm are recognized for the same protons in compound **8**.

Table 2: ^1H NMR data of **7** and **8** (300 MHz, $CDCl_3$)

H	**7**	**8**
H_A	3.66 *dd*	4.22 *dd*
H_B	3.43 *dd*	4.02 *dd*
H_C	3.71 *m*	5.08 *m*
$(CH_2)_7$	1.40 *brs* (2H)	1.58 *brs* (4H)
	1.60 *brs* (2H)	1.28 *brs* (10H)
	1.25 *brs* (10H)	
CH_3	0.85 *t*	0.89 *t*
OAc		2.05 *s*

The above presented evidence establishes structure **6** for the fungal metabolite. The stereochemistry of the epoxide is tentatively assigned as *trans* from the rather small coupling constant (J = 2.5 Hz) for H_C and H_D [42] and based on the biogenetic consideration that the precursor to compound **6** would be a *trans* olefin.

A search in the chemical literature revealed that this is the first finding of compound **6** as a natural product.

igure 1: ¹H-NMR spectrum of 7

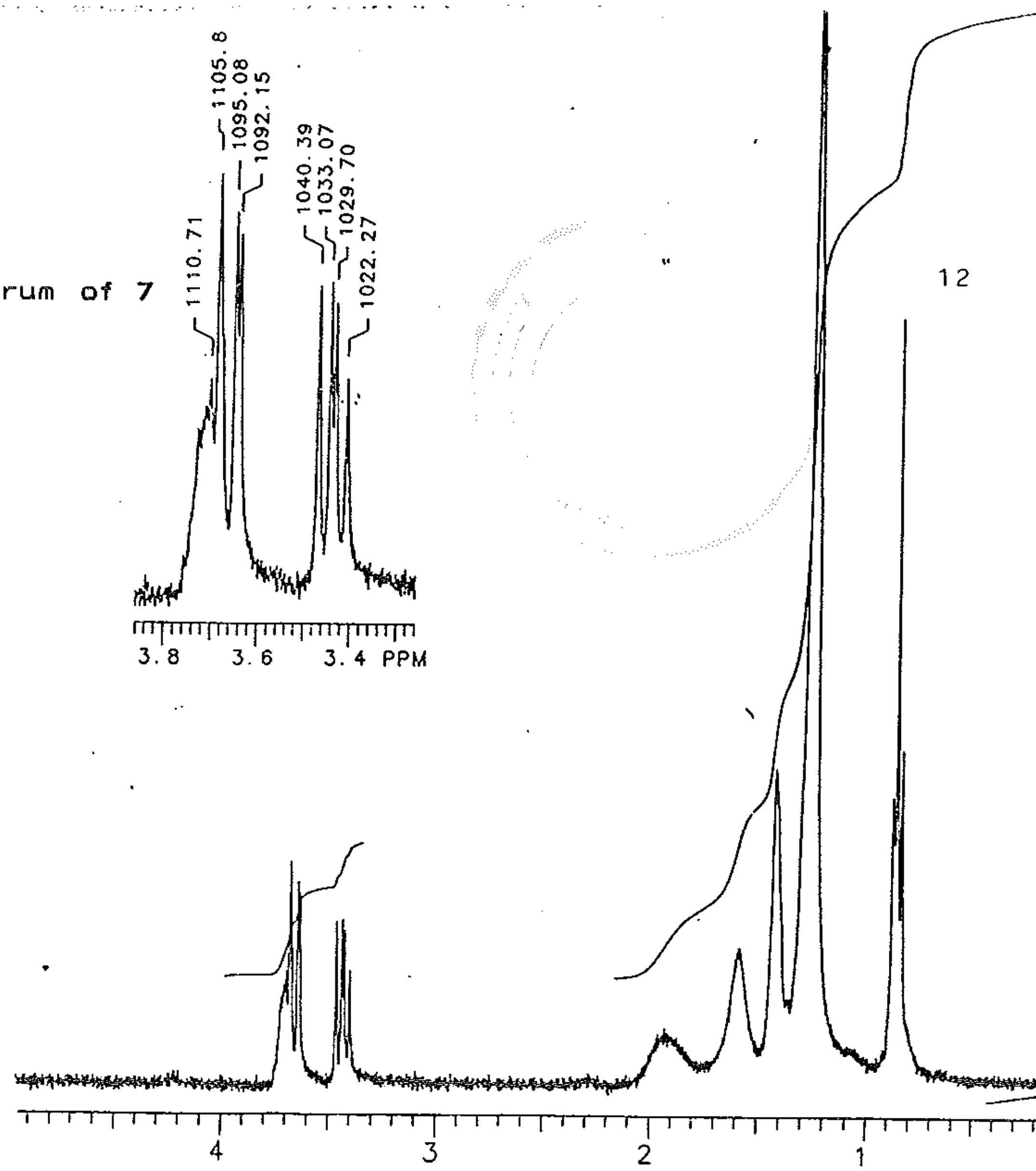


Figure 2: ¹H-NMR spectrum of 8

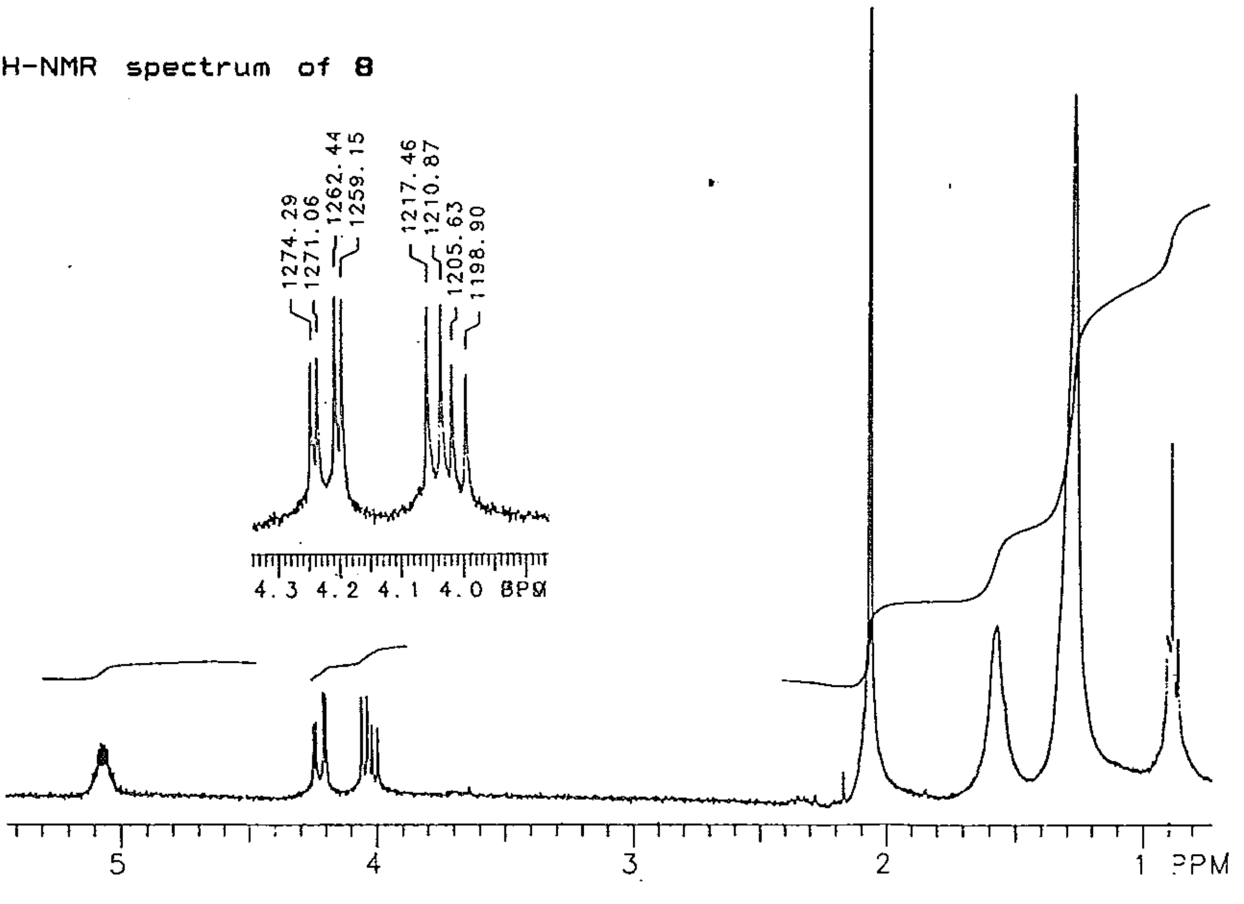


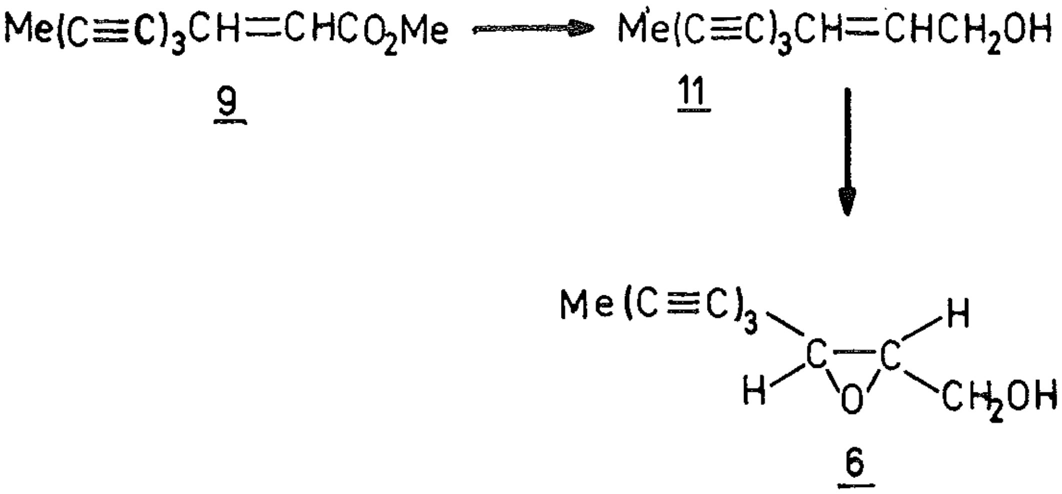
Table 3: C_9 and C_{10} compounds from fungi

Compound	Source	Reference
$CH_3(C\equiv C)_3CH=CHCO_2H$	*Polyporus anthracophilus*	44
	Pleurotus ulmarius	45
$CH_3(C\equiv C)_3CH=CHCH_2OH$	*Polyporus anthracophilus*	44
	Pleurotus ulmarius	45
	Poria sinuosa	40
	Tricholoma grammopodium	40
	Clitocybe obbatta	40
	C. candida	40
$CH_3(C\equiv C)_3CH=CHCHO$	*Pleurotus ulmarius*	45
$HOCH_2(C\equiv C)_3CH=CHCO_2H$	*Pleurotus ulmarius*	45
$HOCH_2(C\equiv C)_3CH=CHCH_2OH$	*Poria sinuosa*	40
	Coprinus quadrifidus	39
$HOC(C\equiv C)_3CH=CHCH_2OH$	*Coprinus quadrifidus*	39
	" "	"
$H(C\equiv C)_3CH=CHCH_2OH$	*Coprinus quadrifidus*	39
	Poria sinuosa	40
	Polyporus biformis	40
$HO_2C(C\equiv C)_3CH=CHCO_2H$	*Poria sinuosa*	40
$H(C\equiv C)_3CH=CHCO_2H$	*Poria sinuosa*	40
$H(C\equiv C)_3CH=CHCH_2OH$	*Coprinus quadrifidus*	39
$H(C\equiv C)_3CH(OH)CH(OH)CH_2OH$	*Coprinus quadrifidus*	39
$CH_3CH=CH(C\equiv C)_2CH=CHCO_2R$	*Polyporus anthracophilus*	44
$CH_3CH=CH(C\equiv C)_2CH=CHCH_2OH$	*Polyporus anthracophilus*	44
$HOCH_2CH=CH(C\equiv C)_2CH=CHCO_2R$	*Polyporus anthracophilus*	44
$RO_2CCH=CH(C\equiv C)_2CH=CHCO_2R$	*Polyporus anthracophilus*	44
$HOCH_2CH_2CH_2(C\equiv C)_2CH=CHCO_2R$	*Polyporus anthracophilus*	44
$HO_2CCH_2CH_2(C\equiv C)_2CH=CHCO_2R$	*Polyporus anthracophilus*	44
	Merulius lacrymans	45
$HO_2C(C\equiv C)_3CH_2CH_2CO_2H$	*Merulius lacrymans*	45

R = H, Me

In fungi odd-numbered carbon chains usually have a terminal ethynyl group whereas related even numbered compounds have a methyl or the oxygenated alcohol, aldehyde, or carboxylic acid terminus. Loss of the terminal C-atom occurs quite often, presumably *via* the sequence $CH_2OH \rightarrow CHO \rightarrow CO_2H$ followed by enzymic decarboxylation of acetylenic acid to yield a terminal acetylene. Supportive evidence has been obtained using cell free extracts from *C. quadrifidus* a fungus which produces both terminally oxidized C_{10} compounds and terminal ethynyl C_9 compounds [35, 39, 49, 52].

From the established biogenetic pathway discussed above for fungal polyacetylenes it can be concluded that compound **6** might also originate from the commonly encountered dehydromatricarianol (**11**), by epoxidation of the *trans* double bond (Scheme 2). The dehydromatricarianol might in turn have come from the ester 9 following the route given in Scheme 1.



Scheme 2: Possible biogenetic pathway for the formation of 6

2.2.5. Biological activity of compound 6 and its derivatives.

Polyacetylenic compounds generally show powerful biological activity but are also very toxic to be of any therapeutic use [32, 51].

Serial dilution assay was carried out to determine the antibiotic spectrum of compound 6 (Table 4). Compound 6 did not show antimicrobial activity against the gram positive bacteria *Staphylococcus aureus* and *Bacillus cereus* or the gram negative bacterium *Agrobacterium tumefaciens* upto concentrations of 100 µg/ml. However it showed antifungal properties as shown by the MIC ´s (Table 4) for *Candida albicans* and *C. tropicalis*, *Rhodotorula* sp. and inhibition of spore germination of *Aspergillus* species tested.

Table 4: Antimicrobial spectrum of compound 6
(serial dilution assay)

Test organism	MIC (µg/ml)	Incubation temp. (°C)
Bacteria		
Bacillus cereus	>100	27
Staphylococcus aureus	>100	27
Agrobacterium tumefaciens	>100	37
Fungi		
Candida albicans	50-100	37
C. tropicalis	50-100	37
Rhodotorula sp.	10-20	27
Aspergillus niger (spores)	1-5	27
A. ochraceus (spores)	1-5	27

The biological activity of **6** was followed by agar diffusion assay method using *Aspergillus niger* during screening of the fungal culture and isolation of the active metabolite.

Compound **6** was also shown to be active against brine shrimp. It caused almost 100% death of the brine shrimp at a concentration of 100 μg/ml. The hydrogenation product **7** and the diacetate **8** showed no activity against the fungi *A. niger* and *C. albicans* upto conc. of 100 μg/ml.

2.3. *Agaricus* sp.

2.3.1. The producing fungus

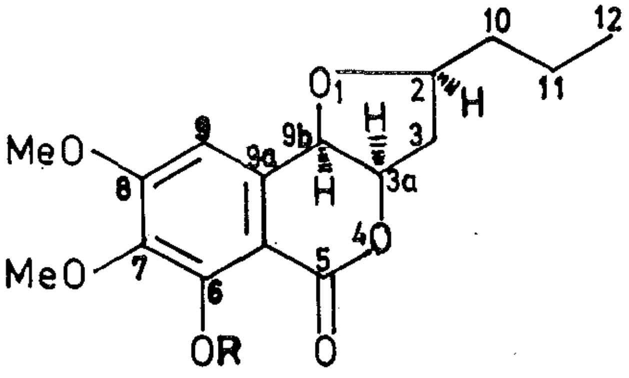
Agaricus sp. was collected from the Science Faculty campus of the Addis Ababa University in June 1990.

2.3.2. Culture cultivation and isolation of compound 12.

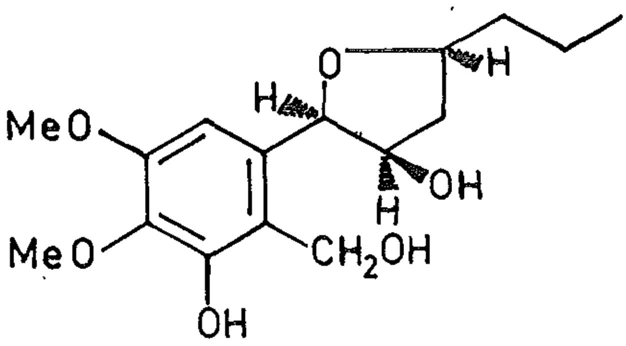
The submerged culture was cultivated in modified BAF medium in 1 l erlenmeyer flasks where a 100 ml of culture grown in the same media in 250 ml flasks for 4 days was used as inoculum. The culture filtrate from which the mycelia had been removed by filtration after 10 days of growth was extracted with EtOAc and the solvent removed under reduced pressure. The TLC of the crude extract showed at least 14 spots. The mixture was separated by prep TLC and the fractionated constituents were tested for lethality against brine shrimp. The major component (R_f = 0.8, solvent system $CHCl_3$:MeOH 95:5) was found to be the active constituent. The major compound was isolated in larger quantities by column chromatography of the crude extract and structure 12 was assigned to it as discussed below.

2.3.3. Structure elucidation of monocerin (12).

The HRMS spectrum of compound 12 showed a molecular ion at *m/z* 308 consistent with the molecular formula $C_{16}H_{20}O_6$. The IR spectrum showed a band at 1670 cm^{-1} assignable to a chelated ester carbonyl group. This was further supported by the presence of a downfield singlet in the ^1H NMR (J = 11.29) attributable to a chelated OH group. The ^1H NMR spectrum further suggested the presence of two aromatic methoxy groups, two methine protons on oxygenated carbon atoms, an aromatic proton, six methylene protons and a terminal methyl group. The presence of three primary, three secondary, four tertiary and six quaternary carbon atoms was derived from the ^{13}C NMR spectrum. Out of the four tertiary carbon atoms only one turned out to be aromatic and the remaining three are aliphatic and are also oxygenated. The presence of an ester carbonyl was deduced from the carbon resonance at δ171.4. Based on the above spectroscopic data structure 12 was assigned to this natural product. Comparison of the above presented spectroscopic data with those reported for monocerin (12) [53, 54, 55] showed a very close agreement except for the H-2 signal which is presumably buried under the OCH_3 signal.



12, R = H
14, R = Me



13

Pople and Grove [55] and Simpson et al [56] have established the absolute stereochemistry of monocerin to be 2S, 3aR, 9bR, on the basis of chiroptical and NMR studies. The specific rotation obtained for 12 ($[\alpha]_D$ = + 57°, c = 0.86, MeOH) was found to be very close to the reported values ($[\alpha]_D$ = + 58.8°, c = 0.37, MeOH) [53]; $[\alpha]_D$ = + 53°, c= 0.85, MeOH [54]).

The LAH reduction of compound 12 afforded compound 13. The UV spectrum of 13 showed absorption maxima that are shifted to shorter wavelength compared to the spectrum of 12 suggesting the reduction of the lactone carbonyl group. Comparison of the IR spectra of the compounds 12 and 13 showed the absence of the absorption band at 1670 cm^{-1} in the spectrum of 13. Furthermore, the chelated hydroxy signal at δ11.29 in the ^{1}H NMR spectrum of 12 disappeared in the spectrum of 13 (Table 5). Thus the ester carbonyl group in 12 must have been reduced and the lactone ring must have opened up. Further confirmation to the opening up of the lactone ring was provided by the switch in chemical shifts of the H-9b doublet and H-3a multiplet in the ^{1}H NMR spectra of compounds 12 and 13. In the ^{1}H NMR spectrum of 12, the H-9b and H-3a resonances appeared at δ4.53 and 5.04, respectively. H-9b, although benzylic, resonated upfield relative to H-3a and this has been attributed to the fact that the lactone and tetrahydrofuran rings are cis fused [54]. However, in the spectrum of **13**, the corresponding proton resonances appeared at δ4.88 and 4.45, respectively, with H-9b resonating downfield from H-3a as expected. This is in agreement with the opening up of the lactone ring to the diol as depicted in structure **13**.

In addition to the above, the ^1H NMR spectrum of **13** displayed two geminally coupled (J = 12.86 Hz) doublets at δ4.36 and 4.98 which are assignable to the methylene protons of the CH_2OH group at C-6a. The remaining proton resonances agree very well with the proposed structure. The H-2 signal was not however discernible in the $CDCl_3$ spectrum. In an attempt to observe the H-2 resonance, the ^1H NMR spectrum was done in C_6D_6. The OCH_3 signals shifted upfield relative to their chemical shifts in the $CDCl_3$ spectrum but the H-2 signal was not observed. Thus in both spectra, it is assumed that the H-2 signal is buried under an OCH_3 signal.

Table 5: ^1H NMR data of **13** (90 MHz)

H	$CDCl_3$	C_6D_6
H-11, H-10, H-3β	1.15-2.00, *m*	1.15-2.00, *m*
12-CH_3	0.90, *t*	0.92, *t*
6a-CH_2	4.39, *d* 4.89, *d*	4.36, *d* 4.98, *d*
H-3α	2.41, *m*	2.15, *m*
H-3a	4.48, *m*	4.45, *m*
H-9	6.78, *s*	7.09, *s*
H-9b	4.92, *d*	4.88, *d*
OMe	3.87, *s*	3.48, *s*
OMe	3.87, *s*	3.61, *s*

Compound **12** was methylated using MeI/K_2CO_3 in dry acetone, to afford compound 14. The IR spectrum of **14** showed a strong absorption band at 1720 cm^{-1} consistent with an unchelated lactone carbonyl group. The ^1H NMR spectrum was similar to that of monocerin (**12**) except for the appearance of an additional methoxyl resonance, a disappearance of the chelated OH signal and a downfield shift of the aromatic proton resonance. The H-2 signal was clearly observed and appeared as a one-proton multiplet at δ4.12. The IR and ^1H NMR spectra agree very well with those reported in the literature [53, 55] for compound **14.**

Monocerin (**12**) was first isolated from *Exerohilum monoceras* Drechsler (= *Drechslera monoceras* = *Helminthosporium monoceras*) [54]. It was later isolated from *Fusarium larvarum* Fukel [55]. The synthesis of monocerin has been achieved by Mori and Takaishi [53].

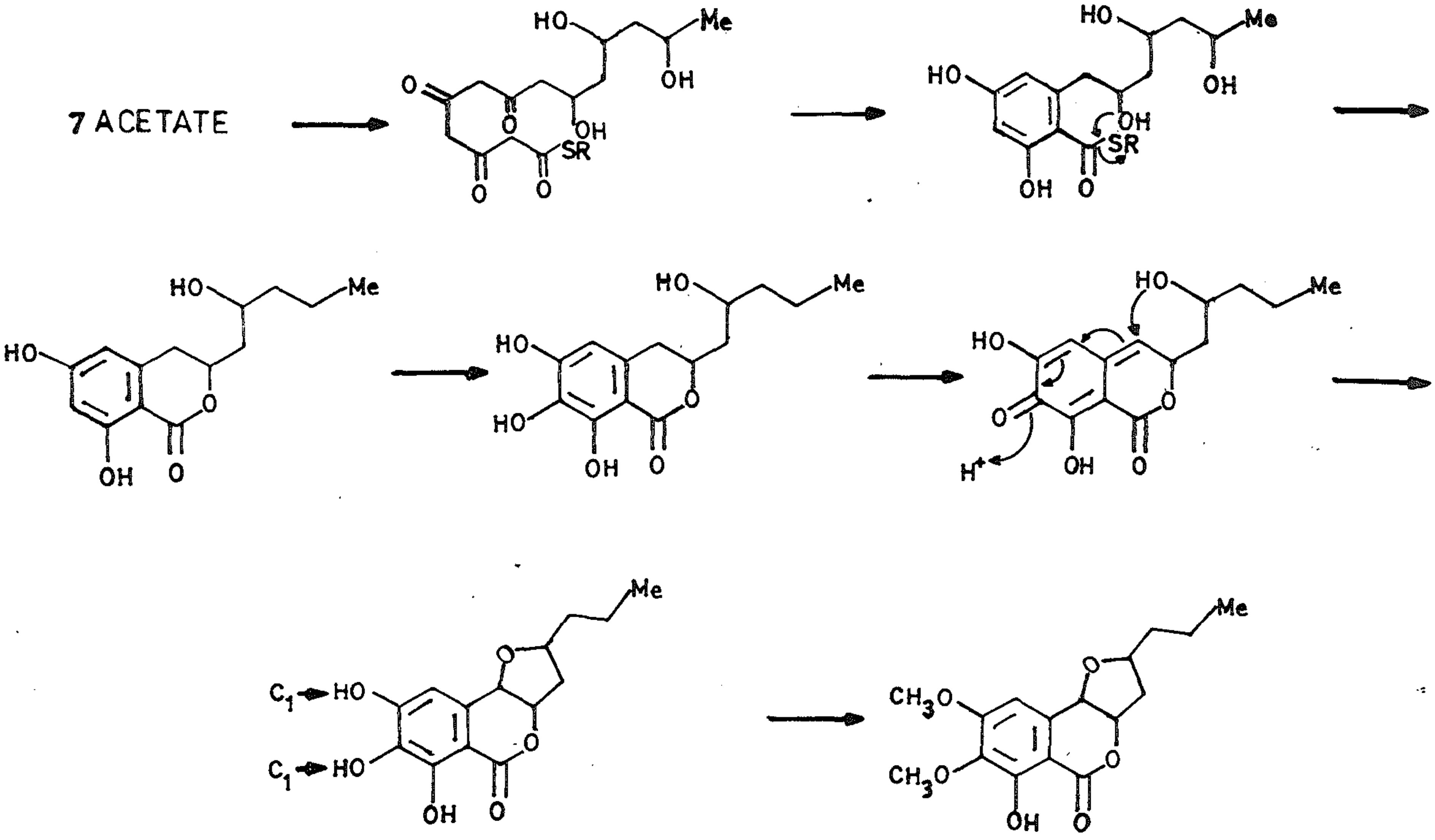
To our knowledge, this is the first report of isolation of monocerin from an *Agaricus* sp. or any other higher fungi.

2.3.4. Biogenesis of monocerin 12.

Biosynthetic studies of monocerin showed it to be of heptaketide origin. Simpson et al. [56] demonstrated that the biosynthesis of monocerin (**12**), in fungal cultures proceeds *via* the acetate malonate pathway by a feeding experiment. Analysis of the incorporation of ^2H, ^{14}C, and ^{18}O labelled acetates was used to suggest the pathway depicted in Scheme 3.

2.3.5. Biological activity

We have found that monocerin (**12**) is lethal to brine shrimp, with an LC_{50} of 50 μg/ml. However it showed no activity against the gram positive bacteria *Staphylococcus aureus, Bacillus cereus* and the mold *Aspergillus niger* and the yeast *Candida albicans* upto concentrations of 100 μg/ml.



Scheme 3: Biogenesis of Monocerin 12 [56]

The LAH reduction product of monocerin (**13**) and the methyl ether (**14**) showed no activity against brine shrimp upto concentrations of 200 μg/ml.

Monocerin is known to have antifungal activity against the powdery mildew *Erisyphe graminis* [54]. It was also found to be an insecticidal component of the fungus *Fusarium larvarum* [55]. Phytotoxic properties have also been reported for it [53].

Conclusion

This study describes the isolation and characterization of two bioactive components from submerged cultures of higher fungi of tropical origin. Compound **6**, *trans*-2,3-epoxy deca-4,6,8-triyn-1-ol, was isolated from *T. pubescens* and an *Agaricus* sp. yielded monocerin (**4**). Compound **6** turned out turned out to be a novel natural product. Monocerin **4** is also reported from an *Agaricus* for the first time. Attempts have been made to evaluate the biological activities of both of these compounds. These results indicate the need for more thorough studies of the chemistry and bioactivity of tropical higher fungi.

3.0. EXPERIMENTAL

3.1. Instruments , Chromatography and media.

Instruments

UV:	1001A-Plus, Milton Roy; On-line photodiode array detector, LKB.
IR:	Infrared spectrophotometer 727B , Perkin Elmer.
^{1}H NMR:	90 MHz, Jeol FX90Q; 300 MHz, Varian; 400 MHz, Bruker WM. δ values are given in ppm, multiplicities: *s* = singlet, *d* = doublet, *t* = triplet, *m* = multiplet.
^{13}C NMR:	22.5 MHz, Jeol FX90Q.
HRMS:	A.E.I. MS 50, 70 eV.
Optical rotation:	Polarimeter 241, Perkin Elmer.
Melting point:	Micro heating table, Boetius; hot stage melting point apparatus, Kofler; mp. uncorrected.
Hydrogenation:	Hydrogenation Parr Apparatus, Parr Instruments.

Chromatography

Thin Layer Chromatography:	Analytical and preparative chromatography silica gel plates F$_{254}$ 60, Merck, Darmstadt.
Spray reagents:	Vanillin:Sulfuric acid.
Column Chromatography:	Silica gel 60, Merck, Darmstadt.
Gas Chromatography:	SE-54 capillary column, Varian.
HPLC:	
Column:	Partisil 5, 250 mm x 4 mm i.d., particle size 5 μm, Whatman.
Pumps:	2150 HPLC pumps, LKB.
Controller:	2152 LC Controller, LKB.
Injector:	Model 7152 injector, Rheodyne.

Detector: 2151 variable wavelength monitor, 2140 Rapid spectral detector, LKB, interfaced with PC-6 NCR computer.
Recorder: 2220 Recording integrator, LKB.
Mobile Phase: Chloroform:methanol 95:5, 1.2 ml/min.

Media

Medium 1 (YMG -medium)
Yeast extract 4 g; Malt extract 10 g; Glucose 4 g; Tap water 1 l.
Medium 2 (3% malt extract medium)
Malt extract 30 g; Tap water 1 l.
Medium 3 (Modified BAF medium)
Maltose 20 g; Glucose 10 g; Peptone 2 g; Yeast extract 1 g; $MgSO_4.H_2O$ 1 g; KH_2PO_4 500 mg; $CaCl_2$ 50 mg; $FeCl_3$ 10 mg; $ZnSO_4$ 12 mg; Distilled water 1 l.
Medium 4 (Nutrient broth, Oxoid)
Nutrient broth 25 g; Distilled water 1 l.
Medium 5 (Minimal medium)
Glucose 20 g; NH_2SO_4 12 g; KH_2PO_4 500 mg; $MgSO_4$ 100 mg; $CaCl_2$ 10 mg; Distilled water 1 l.
Medium 6 (Wort broth)
Wort (Addis Ababa Brewery) 250 ml; Tap water 750 ml.

3.2. Collection, identification and cultivation of Basidiomycetes.

Collection and identification.

Fruitbodies of higher fungi were collected from the highlands of Ethiopia in June-July 1990. Herbarium specimens (available at the Biology Department, AAU) were air dried in most cases or in an oven at 50 °C.

Identifications were made by Dr Dawit, Biology Department of Addis Ababa University, and Dr Ryvarden, University of Oslo, Norway.

Fungal cultures and maintenance media.

Culture were made from the pileus and/or the stipe of the fresh fruitbodies. Fungi were maintained on slants of malt extract, yeast extract, glucose, agar medium.

3.3. Submerged culture cultivation and screening

Growth media. Media 1-3, and 6 were used for cultivation of fungi.

Screening of fungal cultures.

Three 250 ml Erlenmeyer flasks containing media 1,2 and 6 (100 ml each) were inoculated with pieces of fungal culture previously grown on solid malt extract yeast extract glucose plates. The cultures were grown on a Gallenkamp rotatory shaker (110 rpm) at room temperature. 8-12 days later the mycelia were removed by suction filtration and the clear broth extracted with an equal volume of chloroform and dried with anhydrous Na_2SO_4. After evaporation of the solvent the residue was dissolved in 1 ml of MeOH.

Antifungal and antimicrobial bioassay.

40 µg each of the crude extract was absorbed on filter paper discs (6 mm) allowed to dry and placed on agar media plates seeded with different test organisms followed by incubation at 37 °C for 24 h. Biological activity was assessed by the appearance of a zone of inhibition around the disc (Agar Diffusion Assay). The test organisms used were *Aspergillus niger, Bacillus cereus, Bacillus subtilis, Candida albicans, Staphylococcus aureus.*

Brine shrimp lethality bioassay.

Fungal extracts were also tested for bioactivity using the shrimp lethality test on Artemia salina at concentrations of 100 µg, 200 µg and 500 µg of crude extract using Meyer et. al's method [57] with slight modifications. Brine shrimp eggs were purchased from San Francisco Bay Brand Inc. and hatched in a shallow rectangular (10.5 cm x 7 cm) container filled with artificial sea water. The sea water was prepared with a commercial salt mixture (Instant Ocean from Aquarium systems) and doubly distilled water. The dish was divided into two unequal compartments with a small slit at the bottom of the divider. The eggs were placed in the larger compartment which was kept in the dark and lidded, while the smaller compartment was lit. The nauplii were collected by a pipette from the lighted side. The unhatched eggs remained in the larger dark compartment while the phototrophic nauplii drifted into the lighted section.

The samples to be tested were dissolved in a DMSO:Water (1:1) solution. Ten shrimp each were added to sample vials and artificial sea water was added to make the volume up to 1 ml. The vials were kept under illumination at 27 °C for 24 h. The survivors were then counted and the per cent death at each dose and control were determined.

3.4. Bioautography and thin layer chromatography.

The crude extracts showing biological activity in the agar diffusion assay were analysed by TLC. Solvent systems of composition, toluene:acetone:acetic acid 70:30:1 and chloroform with 5-15% methanol were used to develop the chromatograms. The active spots or constituents were determined by first separating the crude extract into its constituents on a TLC plate and then cutting the TLC plate into small pieces and placing them face down on agar media seeded with test organisms against which they were found to be most effective (Bioautography), in the case where the extracts showed antimicrobial or antifungal activities. Where the activity was against brine shrimp, the crude extract was subjected to PTLC and the individual bands scraped were tested individually as described above.

3.5. Culture cultivation

Bioactive fungi were cultivated in 1 l Erlenmeyer flasks containing 400 ml of broth in Medium-6 (wort medium) which was a locally available and cheap medium. However this medium had to be abandoned eventually because it was not possible to get consistent results from the point of view of constituents and biological activity of extracts obtained using this medium. Media 1-3 were chosen for further cultivations. Submerged liquid culture cultivation was used in almost all cases. After 8-12 days of growth the culture fluid was separated from the mycelia by filtration. The culture filtrate was then extracted with an equal volume of ethyl acetate and the solvent removed under reduced pressure. The crude extracts were then subjected to various chromatographic techniques where isolation was desired.

3.6. Isolation of bioactive metabolites

3.6.1. *trans*-2,3-epoxy deca-4,6,8-triyn-1-ol (6) from *Trametes pubescens*.

Trametes pubescens (voucher no. ADA-202) is a polypore collected from Munesa forest, Southern Ethiopia in July 1990. The fungus was identified by Dr Ryvarden, University of Oslo.

Extraction and isolation of 6.

The screening tests showed the metabolites of this fungus to have antifungal properties. The extracts were active against *Aspergillus niger* and *Candida albicans*. The fungus was cultivated on a larger scale using fifteen 400 ml shake cultures in 1 l flasks. The culture was filtered after 10 days of growth to yield 5.8 l of filtrate with a pH of 4.23. The biomass had a dry weight of 8.2 g. The culture filtrate was extracted with an equal volume of ethyl acetate twice and solvent removed in vacuo to give crude extract (390 mg) which was chromatographed on silica gel. The column was eluted with increasing concentrations of methanol in dichloromethane. Twenty fractions were collected.

Fractions 16-20 were combined and concentrated to give a residue which was subjected to PTLC (silica gel 0.25 mm, toluene:acetone;acetic acid 70:30:1). 25 mg of 6 was obtained.

Compound ***6***

Yellow crystals (unstable)

Melting point: 98-102 °C (decomp.)

R_f = 0.64 (toluene:acetone:acetic acid 70:30:1); brown color upon reaction with vanillin:sulfuric acid spray reagent;

t_R (HPLC) = 3.47 min
UV ν_{max} nm: 242, 262, 270, 293, 312

IR λ_{max} cm^{-1}: 3400, 2250, 1385, 1070, 860

^1H NMR (90 MHz, $CDCl_3$): δ1.85 (3H, *s*, CH_3), 3.19 (1H, *m*, H_C), 3.36 (1H, *d*, *J* = 2.5 Hz, H_D), 3.52 (1H, *dd*, *J* = 13, 4 Hz, H_B), 3.69 (1H, *dd*, *J* = 13, 3 Hz, H_A)

HRMS *m/z* (rel. int): 160.0517 $[M]^+$ (11.8) (calc. for $C_{10}H_8O_2$: 160.0522), 130 $[M^+- CH_2O]$ (10.5), 100 $[M^+- C_2H_4O_2]$ (100)

^{13}C NMR (22.5 MHz, MeOD): δ3.7 (C-10), 43.5 (C-3), 61.7 (C-2), 61.4 (C-1), 64.6, 74.5, 78.6, 85.3 $(-C\equiv C_3-)$

Compound ***7***

Hydrogenation of ***6.***

Compound **6** (15 mg) was dissolved in MeOH:EtO_2 (1:1) and PtO_2 was added. The reaction flask was mounted on a Parr hydrogenation apparatus and the reaction was allowed to proceed under hydrogen atmosphere. The progress of the reaction was monitored by TLC. After 3h, no more of the starting material remained, the reaction was discontinued and the mixture was filtered. Removal of the solvent afforded 10 mg of a light yellow oil, which was recrystallized from methanol (**7**).

Melting point: 47-49 °C

R_f = 0.93 ($CHCl_3$:MeOH 99:1); orange color with vanillin:sulphuric acid spray reagent

IR λ_{max} cm^{-1}: 3300, 2955, 2845, 1460, 1090, 1060, 865

^1H NMR (90 MHz, $CHCl_3$): δ0.85 (3H, *t*, CH_3), 1.25 (10 H, *brs*, $(CH_2)_{5-9}$), 1.4 (2H, *brs*, H-3), 1.6 (2H, *brs*, H-4), 3.43 (1H, *dd*, *J* = 11, 7 Hz, H_B), 3.66 (1H, *dd*, *J* = 11, 3 Hz, H_A), 3.71 (1H, *m*, H_C).

*Compound **8***

Acetylation of 7.

A mixture of compound **7** (7 mg), acetic anhydride (0.5 ml) and pyridine (0.5 ml) was kept at room temperature with stirring for 7 h, then diluted with water, and extracted with $CHCl_3$. The $CHCl_3$ solution was dried over anhydrous Na_2SO_4 and evaporated to dryness. The reaction product (**8**, 5 mg), was analysed by TLC and GC and was found to be homogenous.

Oil

R_f =0.32 (toluene:acetone:HOAc 70:30:1)
Gas Chromatography: SE-54 capillary column, injection temperature 210 °C, detection temperature 260 °C, 70 -250 °C, 10 °C/min, retention time 12.5 min

IR λ_{max} cm^{-1}: 2950, 2850, 1730, 1440, 1360, 1210

^1H NMR (90 MHz, $CDCl_3$): δ0.89 (3H, *t*, CH_3), 1.28 (10 H, *brs*, $(CH_2)_{5-9}$), 1.58 (4H, *brs* $(CH_2)_{3-4}$), 2.05 (6H, *s*, OAc), 4.02 (1H, *dd*, J = 11.85, 6.7 Hz, H_B), 4.22 (1H, *dd*, J = 1.85, 3.3 Hz, H_A), 5.08 (1H, *m*, H_C).

*Biological activity of compound **6** and its derivatives.*

The biological activity of compound **6** against eight test organisms (Table 4) was measured using the serial dilution assay method. The inoculum used were the hyphae in all cases except the *Aspergillus* spp. where spore suspensions were used. Readings were taken after 24 h of incubation at the respective temperatures given in Table 4.

The activity of compound **6** against brine shrimp was also determined.

The derivatives, compounds **7** and **8** were tested against *Aspergillus niger* and *Candida albicans* using the agar diffusion assay, but showed no activity.

3.6.2. Monocerin (12) from *Agaricus* sp.

Agaricus sp. (voucher no. ADA-2) was collected from the Science Faculty campus in Addis Ababa in June 1990. It was identified as an *Agaricus* by Dr Dawit, Addis Ababa University.

*Extraction and isolation of **12***

The crude extract obtained from the culture filtrate of this fungus showed activity against brine shrimp in the screening tests. The fungus was then cultivated on a larger scale using ten 400 ml shake cultures in 1 l flasks. The culture was filtered by suction after 10 days of growth to yield 3.6 l of filtrate with a pH of 2.97. The biomass had a dry weight of 12.1 g. The culture filtrate was extracted with an equal volume of ethyl acetate twice and the solvent removed in vacuo to give crude extract (1.1 g) which was chromatographed on silica gel. The column was eluted with increasing concentrations of methanol in chloroform. A total of thirty-eight fractions were collected.

Fractions 11-12 were combined and evaporated to give a residue (148 mg) which was rechromatographed on silica gel eluting with increasing amounts of methanol in chloroform. Fifteen fractions were collected. Fractions 9-14 were pooled and yielded a residue which was applied onto PTLC (silica gel, 0.25 mm, $CHCl_3$:MeOH 95:5) to give compound **12** which was further purified on a chromatotron by elution with dichloromethane (yield, 48 mg).

A second batch of submerged culture with the same conditions described was cultivated and after 14 days of growth the fungal culture filtrate was passed through an Amberlite XAD-1180 (Serva) column. The organic matter was eluted with methanol. The crude extract obtained was 2 g. Monocerin (**12**) (162 mg) was isolated using the same chromatographic methods described above.

Compound **12** was found to be lethal to the brine shrimp, *Artemia salina*.

Compound 12

White crystals

Melting point: 52-54 °C (54-55 °C [53]; 53-55 °C [54]) $[\alpha]_D = +57°$ (c = 0.86, MeOH)

R_f = 0.8 (chloroform:methanol 95:5)

t_R (HPLC) = 2.80 min

UV ν_{max} nm: 220, 270, 300

IR λ_{max} cm^{-1}: 3000, 2975, 2910, 1670, 1640, 1600, 1450, 1400, 1270, 1130

^{1}H NMR (90 MHz, $CDCl_3$): δ0.92 (3H, *s*, 12-CH_3), 1.15 (4H, *m*, H-11, H-10), 2.13 (1H, *ddd*, *J* = 14.2, 6.3, 1.6, H-3β), 2.60 (1H, *ddd*, *J* = 14.2, 8.2, 6.3, H-3α), 3.90 (3H, *s*, OMe), 3.98 (3H, *s*, OMe), 4.53 (1H, *d*, *J* = 3.2 Hz, H-9b), 5.05 (1H, *m*, H-3a), 6.60 (1H, *s*, H-9), 11.29 (1H, *s*, OH)

HRMS *m/z* (rel. int.): 308.1263 [M]$^+$ (100) (calc. for $C_{16}H_{20}O_6$: 308.1255).

^{13}C NMR: δ15.1 (CH_3), 20.3 (C-11), 39.3 (C-10), 40.5 (C-3), 57.6 (OMe), 61.8 (OMe), 75.7 (C-2), 79.9 (C-3a), 103.5 (C-6a), 106.1 (C-9), 127.0 C-9a), 132.7 (C-7), 157.6 (C-6), 161.0 (C-8), 171.4 (C-5)

*Compound **13***

*Reduction of compound **12**.*

In a three-necked round bottom flask attached with a dropping funnel, a reflux condenser and a $CaCl_2$ guard tube, dry THF was placed and $LiAlH_4$ was added. The mixture was allowed to reflux and compound **12** dissolved in dry THF was added dropwise. After the addition was complete refluxing continued for 6h. The mixture was then allowed to cool and EtOAc was added followed by the addition of a small amount of water. Filtration over celite followed by removal of the solvent afforded **13**.

Light yellow oil

R_f = 0.72 (toluene: acetone: HOAc 70:30:1)

UV ν_{max} (MeOH) nm: 205 (sh), 226, 275

IR λ_{max} cm^{-1}: 3400, 3000, 2975, 2900, 1630, 1600, 1510, 1470, 1440, 1350, 1250, 1130

^{1}H NMR (90 MHz, $CDCl_3$, C_6D_6) see Table 5.

*Compound **14***

*Methylation of compound **12**.*

Compound **12** (72 mg) was dissolved in anhydrous acetone and anhydrous K_2CO_3 and MeI were added. The mixture was stirred at room temperature following the progress of the reaction by TLC. After 72 h the mixture was filtered and the solvent removed to afford compound **14** (57 mg).

Faint yellow oil

R_f = 0.62 ($CHCl_3$:MeOH 95:5)

IR λ_{max} cm^{-1}: 3000, 2950, 1720, 1620, 1600, 1500, 1460, 1360, 1250, 1100

^1H NMR (90 MHz, $CDCl_3$): δ0.86 (3H, *t*, 12-Me), 1.1-1.8 (4H, *m*, H-11, H-10), 2.15 (1H, *m*, H-3β), 2.48 (1H, *m*, H-3α), 3.85 (3H, *s*, OMe), 3.90 (3H, *s*, OMe), 3.92 (3H, *s*, OMe), 3.98-4.25 (1H, *m*, H-2), 4.48 (1H, *d*, *J* = 3.2, H-9b), 4.90 (1H, *m*, H-3a), 6.74 (1H, *s*, H-9).

*Compound **15***

A second compound **15** (5 mg) was isolated from fractions 7-10 of CC of the crude EtOAc extract. However it was not possible to elucidate its structure. The following data were generated for compound **15**.

R_f = 0.77 ($CHCl_3$:MeOH 95:5)
t_R (HPLC) = 2.85 min
UV ν_{max} nm: 231, 242, 273, 307

^{1}H NMR (90 MHz, $CDCl_3$): δ0.94 (2H, *dd*), 1.15 (3H, *s*), 1.2 (3H, *s*), 1.4 (1H, *d*), 2.4 (1H, m), 2.65 (1H, *d*), 2.8 (1H, *dd*), 3.71 (6H, *s*), 6.3 (2H, *t*)

HRMS *m/z* (rel. int.): 308.1275 [M^+] $C_{16}H_{20}O_6$ (18.5).

Biological activity of monocerin (12) and its derivatives.

Monocerin (**12**) was tested for bioactivity against, *Staphylococcus aureus, Bacillus cereus, Aspergillus niger* and *Candida albicans* using the agar diffusion assay method. No antimicrobial and /or antifungal activity was observed. Its lethality to brine shrimp was measured for triplicates of different concentrations of the pure compound following the above described steps for brine shrimp lethality.

Neither the LAH reduction product (**13**) nor the methyl ether (**14**) of monocerin (**12**) showed activity against brine shrimp.

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