Bioactive Metabolites from the Cultures of *Phellinus* sp. and *Lentinus velutinus*

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ABSTRACT

Bioactive metabolites from the cultures of *Phellinus* sp. and *Lentinus velutinus*

by

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(Research Advisor, Dr Ermias Dagne)

The ethyl acetate extract of the fermentation culture broth of *Phellinus* sp. gave two compounds identified as 3-chloro-4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde. Both 3-chloro-4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde showed phytotoxic activity and lethality to brine shrimp (*Artemia salina* Leach) but 3,4-dimethoxybenzaldehyde showed weaker activity. Both 3-chloro-4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde were isolated for the first time from the genus *Phellinus*. The structures were elucidated by spectral analyses and synthesis. A fatty acid and a triglyceride were also isolated from the *Phellinus* sp. grown on rice.

The ethyl acetate extract of the fermentation culture broth of *Lentinus velutinus* gave rise to three compounds of which two were identified as the antibiotic compounds hypnophilin and velutinol and the third as the antitumour compound panepoxydon. Velutinol was reported earlier from the culture filtrate of the same fungus, however, hypnophilin and panepoxydon have not hitherto been reported from the genus *Lentinus*.

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1. INTRODUCTION

Fungi are heterotrophic[1] organisms, the vast majority of which are constructed of more or less microscopic, cylindrical filaments or hyphae with well defined cell walls. The hyphae branch profusely to form mycelium. Saprophytic or parasitic mode of life sets the fungi clearly apart from the autotrophic[2] green plants. Likewise, stress upon the hyphal form of construction separates the fungi from most bacteria, Actinomycetes and protozoa.

1.1 Classification of fungi

The development of mycelium, the kind of spores and sporangia, the nature of the life cycle and the presence or absence of sexual reproduction are the features employed in defining the major group of fungi. Accordingly fungi are grouped under five sub-divisions, namely, Mastigomycotina, Zygomycotina, Ascomycotina, Basidiomycotina, Deuteromycotina [1].

The Mastigomycotina is a composite grouping, covering all fungi that form motile, flagellated spores (zoospores). Zoospore structure is an important feature in the classification of Mastigomycotina. It is a primitive group of fungi and readily obtained from water, mud and wet soil.

The Zygomycotina reproduce asexually by non-motile sporangiospores and sexually by gametangial copulation which results in the formation of zygospores. This group is widely distributed in soil or dung.

The Ascomycotina (Ascomycetes) derive their name from the ascus, the sack-like vessel which contains the sexual spores. The mycelium is septate, and the most common asexual spores are conidia, borne on conidiophore. The ascus forming yeasts, some black molds and green molds, the powdery mildews, the cup fungi, morels and the truffles are among the best known examples of Ascomycetes.

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[1] Heterotrophic organisms do not prepare their own food, therefore, depend on others for food.

The Basidiomycotina (Basidiomycetes) bear their spores, called basidiospores, on a spore producing structure, the basidium. Many of the familiar larger fleshy fungi are members of this group,which include the toadstools, bracket fungi, fairy clubs, puff-balls, stinkhorns, earth-stars, bird's nest fungi and jelly fungi. Two important groups of plant pathogens, the rusts (Uresinales) and smuts (Ustilaginales) are also classified in the Basidiomycetes.

The Deuteromycotina (Fungi Imperfecti), are those fungi for which sexual (perfect) states are either unknown or may possibly be entirely lacking. They form asexual spores borne on conidiophores. An enormous number of common and industrially or environmentally important fungi such as *Penicillium, Aspergillus,* and *Fusarium* fall within this group [2].

It is estimated that there are more than 100,000 species of fungi, about one third of which are Basidiomycetes [3].

1.2 Uses of fungi

Fungi have two major uses in industry, as a source of chemicals and as a food or food processing agent. Fungi produce a wide range of useful compounds, of which antibiotics and enzymes are the most familiar example. Other activities of fungi which are exploited by industry include production of alcohol and/or carbon dioxide by the yeast, *Saccharomyces cerevisiae;* production of organic acids (mainly citric acid) by *Aspergillus niger*, production of glycerol by yeasts and lipids by *Aspergillus niger*. In addition, several anti-tumour agents have recently been extracted from fungi. Fungi are used as food (as mushroom crops) and also in the production of beer, wine and bread (e.g *Saccharomyces cerevisiae)* [4].

1.3 Secondary metabolites of fungi

The metabolic pathways of fungi can be divided into primary and secondary metabolism. The primary metabolism of an organism is the summation of an interrelated series of enzyme-catalysed chemical reactions which provides the organism with its energy, its synthetic intermediates and its key macrontolecules such as proteins and DNA. On the other hand, secondary metabolism involves mainly synthetic processes whose end products, the secondary metabolites, play often no obvious role in the economy of the organism [5].

In view of different characters of the synthesis in the successive growth phase, the term "balanced growth" or "trophophase" has been used to refer to the rapid growth stage in which the synthesis of primary metabolites takes place, and a "storage and maintenance phase" or "idiophase" is used to describe the stationary stage of growth in which the secondary metabolites are synthesized. Antibiotics produced by fungi are secondary metabolites, since they appear to play no essential role in the cell metabolism and are synthesized only after the organism has passed through part or all of the period of balanced growth (trophophase) [6].

Fungi, like other organisms and higher plants, are able to produce secondary metabolites. Of the various hypotheses as to why secondary metabolites are formed, the one which states that secondary metabolites are a measure of the fitness of the organism to survive is currently favoured by some workers [7,8].

Biosynthetically, fungal secondary metabolites could be divided into three major groups: the polyketides, the terpenes and the amino acid derivatives [9]. However, there are also fungal secondary metabolites derived directly from glucose without the intervention of acetate. Glucose is converted to triose, either by the Embden-Meyerof pathway or by the pentose phosphate cycle. The latter route makes available pentose, important in nucleotide biosynthesis, and a tetrose which can react with phosphoenol pyruvate to give shikimic acid which is the intermediate for aromatic acids and also for many aromatic secondary metabolites [5].

Linear condensation of acetyl CoA with several molecules of malonyl CoA leads to fatty acids. Long-chain fatty acids containing two or more double bonds are found both in abundance and variety in fungi [10]. The natural polyacetylenes can also be regarded as fatty acid derivatives. Polyketide metabolites are the most numerous secondary metabolites of fungi. They are formed by condensation of an acetyl unit, or other acyl unit, with malonyl units, with concomitant decarboxylation. The resulting polyketide (poly-fl-ketomethylene) possesses activated methylene groups which pan take part in internal aldoltype condensation to give aromatic compounds [5]. Terpenes and steroids are derived from the condensation of isoprene unit through the mevalonate pathway. Sesquiterpenes, diterpenes and tetracyclic triterpenes are common secondary metabolites of fungi. Monoterpenes have also been isolated as volatile components of fungal metabolites [11]. Tetracyclic triterpenoids of lanostane group are common components of polypores [12]. Nitrogen containing compounds are usually derived from the metabolism of amino acids, purines and pyrimidines [13].

The search for biologically active metabolites of fungi attracted greater attention with the discovery of the antibiotic penicillin from *Penicillium chrysogenum*. Thereafter, new antibiotics like cephalosporins from *Cephalosporin acremonium*, griseofulvin from *Penicillium griseofulvum*, streptomycin from *Streptomyces griseus* and macrolide antibiotics from many other fungi were discovered [14]. Up to present time, the two 6-lactam antibiotics, penicillins and cephalosporins, are the most important fungal antibiotics both in terms of bulk production and clinical effectiveness [15]. An enormous number of antibiotics such as tetracyclines, erythromycins and nystatin were discovered from Actinomycetes but the chances for isolating new antibiotics from this group have apparently decreased. Since the early screening for antibiotics from Basidiomycetes in 1945, it became apparent that Basidiomycetes are good sources of antibiotics. It is also considered that tropical Basidiomycetes and their metabolites are less thoroughly studied than temperate species [14].

1.4 Cultivation of Fungi

The most important aspect of the study of fungi is their identification, maintenance and cultivation. Reproducible experiments using a fungus necessitates a continuous source of the organism which requires keeping the organism alive, keeping it free from contamination and maintaining the biochemical property which is being studied. In order to keep an organism alive it must be transferred periodically to fresh medium. Unfortunately, fungi may show strain variation during transfer but to reduce the chance of strain variation, i.e. of subculturing from the "wrong" part of the parent culture, and of contamination by other organisms, the transfer must be as infrequent as possible. For most experiments fungi are grown on liquid media but for storage purposes a solid medium is more convenient i.e. agar medium which has been allowed to solidify in a slopping test tube. The media for growing fungi contain a carbon source, a nitrogen source, phosphate, sulphate, magnesium, potassium and in trace amounts the elements iron, zinc, molybdenum, and copper.

The great advantage of fungi as sources of secondary metabolites is their ability to produce the compounds on aqueous media. As a result, secondary metabolites of diverse type are conveniently available in the laboratory for chemical, biochemical and biological studies [5].

1.5 The biology and chemistry of *Phellinus sp*.

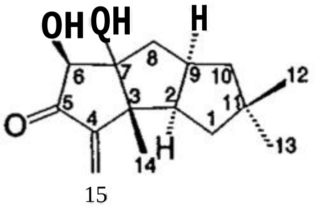
The genus *Phellinus* belongs to the class Basidiomycetes, order Hymenochaetales and family Hymenochetaceae. According to a recent survey 154 species and 67 forms and varieties of *Phellinus* are recognized. Species of *Phellinus* decay heart-wood, cause root rots and cankers of live standing trees and destroy slash and other woody residues. They are collectively responsible for more timber damage than any other genus of wood destroying fungi [16].

Types of compounds identified from earlier investigations on some *Phellinus* spp. are polysaccharides [6]; aromatic alcohols, esters of aromatic acids, and terpenes [11]; fatty acids and sterols [17]; 3,14’-bihipidinyl [18]; 7-phenylheptan-3-ones [19]; 2-carbomethoxyoxepin-1-carbomethoxybenzene-1,2-oxide [20] and cyclophellitol [21].

Different types of fatty acids such as palmitic, oleic, stearic, and linoleic acids have been identified in *P. pomaceus*. The major fatty acid in *P. pomaceus* is oleic acid [22]. Palmitic, stearic, oleic and linoleic acids were detected in all isolates of *P. weirii* [23]. Compounds isolated from the genus are listed in Appendix 1.

1.6 The biology and chemistry of *Lentinus velutinus*

The genus *Lentinus* belongs to the class Basidiomycetes, order Agaricales and family Polyporaceae. So far 63 species have been recognized in the genus *Lentinus*. The genus is essentially tropical in distribution. The number of species and their abundance becomes much reduced towards the polar regions and within temperate regions. Compounds reported from earlier investigations on some *Lentinus* spp. are listed in Appendix 2. *Lentinus velutinus* is possibly the only pantropical species and it probably represents the most common species of *Lentinus* in both West and East Africa. It grows either solitary or caespitosely on tree stumps, rooting trunks and branches or from buried wood, in both forest and open grassland conditions [24]. L. *velutinus* has been collected from Lepis forest, central Ethiopia, and its submerged culture was found to produce a bioactive secondary metabolite known as velutinol (1) [14].



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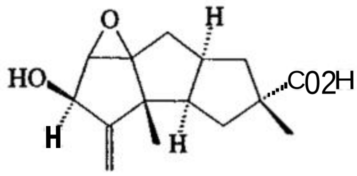
Velutinol is a sesquiterpenoid antibiotic, with a hirsutane skeleton [25]. It is effective against gram-positive bacteria and relatively ineffective against gram-negative bacteria and yeast. When tested by agar diffusion assay it inhibited the growth of many fungi. Velutinol is very effective against the common mold fungi particularly against *Mucor miehei, Paecilomyces varioti, Aspergillus ochraceus, Neurospora ccrassa, Penicillium islandicum and P. notation*. Germination of spores of mold fungi was completely inhibited between 5-20 ptg/ml. It inhibited the growth of Ehrlich Ascitic Carcinoma (ECA) cells at a concentration between 10-20 /xg/ml [14].

Several theories have been advanced for the enzymatic construction of the hirsutane skeleton from famesyl pyrophosphate. Experimental evidence to support the theories has been obtained from the ^{13}C labelling in hirsutic acid (2) and complicatic acid (3).

In one study the compounds were isolated from cultures of *Stereum complication* supplemented with singly labelled [1-^{13}C] acetate [26]. The results were consistent with the mechanism involving prior humulene (4) formation and proceeds through carbonium ion



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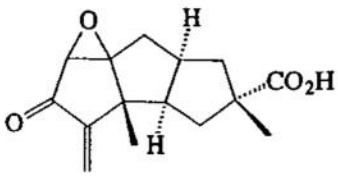

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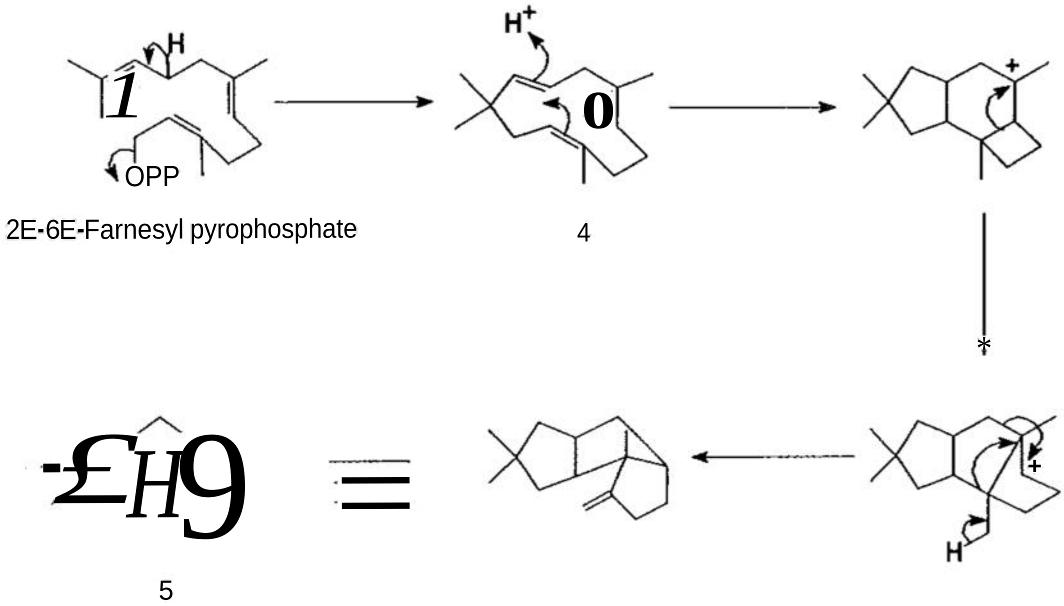
intermediates. The recent isolation of the hydrocarbon hirsutene (5) from *Coriolus consors* supports this biogenetic hypothesis [26]. Scheme 1 shows the proposed biogenetic route to the hirsutane skeleton.



Scheme 1. Biogenesis of the hirsutane skeleton

1.7 Objective of the present study

Among the producers of secondary metabolites the Basidiomycetes offer a great potential for new biologically active metabolites. Chances to find new types of compounds acting on a new target are promising [27]. Tropical Basidiomycetes and their metabolites are less thoroughly studied than temperate species [14]. This study, on the cultures of *Phellinus* sp. and *Lentinus velutinus*, is part of an ongoing program to isolate and characterize biologically active secondary metabolites from Basidiomycetes collected in Ethiopia.

2. RESULTS AND DISCUSSION

2.1 Phytotoxic and cytotoxic compounds from the fermentation culture of *Phellinus* sp.

Phellinus sp., collected from Menagesha State Forest on decaying wood, was maintained on agar slants in Dr D. Abate's laboratory in the Biology Department. The fungus was cultured for 11 days on medium B (see Experimental). The EtOAc extract of the culture broth showed phytotoxicity and lethality to brine shrimp. This prompted us to isolate and characterize the bioactive constituents. The results of our work are described below.

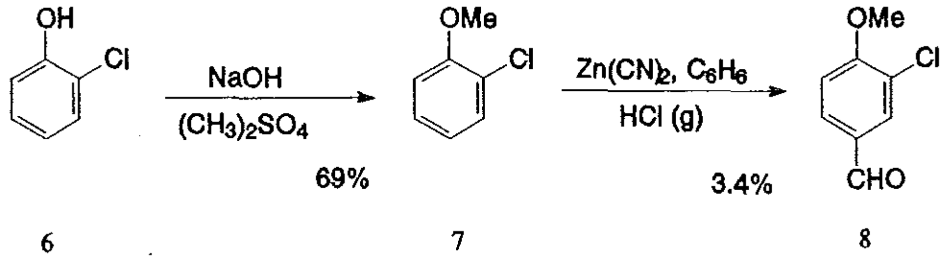
2.1.1 Isolation of compounds

The culture filtrate of *Phellinus* sp. grown for 11 days on medium B was extracted with equal volume of EtOAc and the solvent removed *in vacuo* to give 218 mg of crude extract. Fractionation and purification by column chromatography and chromatotron of the crude extract resulted in the isolation of compounds **8** and **10** which turned out to be phytotoxic and lethal to brine shrimp larvae.

2.1.2 3-Chloro-4-methoxybenzaldehyde (8)

Compound **8** has been identified as 3-chloro-4-methoxybenzaldehyde (**8**) based on spectroscopic data. In order to confirm this, **8** was synthesized starting from *o*-chlorophenol (**6**) in two steps, i.e., methylation with dimethyl sulphate followed by Gattermann aldehyde synthesis. The synthetic procedures for the methylation of phenol and Gattermann aldehyde synthesis of anisole were adopted from *Vogel's Text Book of Practical Organic Chemistry* [28] with some modification.

The UV spectrum (in EtOH) of the synthetic compound exhibited λ_{max} at 271 and 227 nm which is in good agreement with the spectrum of the natural product which showed λ_{max} at 272 and 228 nm. The mass spectra of both the natural product and the synthetic compound showed $[M]^+$ at *m/z* 170 (60%) and $[M + 2]^+$ 172 (19%) since the chlorine atom consists of ^{35}Cl and ^{37}Cl in the ratio approximately 3:1. The presence of one chlorine



Scheme 2. Synthesis of 3-chloro-4-methoxybenzaldehyde

atom was also clear from the relative intensities of $[M]^+$ and $[M + 2]^+$ Peaks. The natural product and the synthetic compound had an identical melting point of 55-56° which is in a good agreement with the literature [29] melting point. ^{1}H NMR spectrum of the synthetic compound showed a three-proton singlet at δ 4.0 and a one-proton singlet at δ 9.85 assignable to a methoxyl and an aldehyde functional groups, respectively. The three aromatic proton signals at δ 7.05 (*d*, $J = 9$ Hz), 7.75 (*dd*, $J = 9$, 2 Hz) and 7.8 (*d*, $J = 2$ Hz) revealed the presence of a 1,2,4-trisubstituted aromatic ring. The above data was found to be the same for the natural product and in good agreement with that reported in the literature [29]. The ^{13}C NMR spectrum of the synthetic compound disclosed the presence of 7 carbon resonances corresponding to one primary, four tertiary and two quaternary carbons. Furthermore, the DEPT spectrum suggested that the signal due to C-1 might have overlapped with the signals of either C-2 or C-6. Table 2.1 shows that the ^{13}C NMR of the natural and the synthetic products are comparable with the calculated values.

Table 2.1 ^{13}C NMR spectral data for the natural and the synthetic 3-chloro-4-methoxybenzaldehyde (**8**) (22.5 MHz, $CDCl_3$).

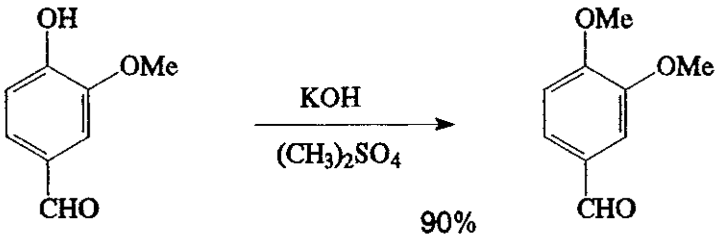
C	*Nat. prod.*	*Synth.*	calc.
1	130.5	---	130.7
2	131.2	130.8	131.2
3	123.8	123.6	120.9
4	159.9	159.7	165.8
5	111.8	111.7	116.0
6	130.3	130.3	128.9
OMe	56.5	56.3	
CHO	189.4	189.3	

The above spectral data clearly indicated that the two compounds, the isolated and the synthetic, are identical. The natural product from *Phellinus* sp. is therefore 3-chloro-4-methoxybenzaldehyde. This compound has not been encountered before in the genus *Phellinus*.

The aldehyde, 3-chloro-4-methoxybenzaldehyde, was isolated from the culture of the fungus *Lepista diemii* Singer, and its structure was established by comparison of its spectral data with that of the synthetic product [29]. It was isolated only in trace amounts from the culture filtrate and the bioactivity was not reported.

2.1.3 3,4-Dimethoxybenzaldehyde(10)

Compound **10** isolated from the culture filtrate of *Phellinus* sp. was identified as 3,4-dimethoxybenzaldehyde (**10**) based on its ^{1}H NMR spectrum. To confirm this, **10** was synthesized by methylating vanillin (**9**) with dimethyl sulphate [28].



9 10

Scheme 3. Synthesis of 3,4-dimethoxybenzaldehyde

The synthetic compound was found to be identical to the natural product by co-TLC. In addition the UV spectrum (in $CHCl_3$) of the synthetic compound showed k_{max} at 310 and 281 nm which is in agreement with that of the natural product which showed k_{max} at 310 and 280 nm. The mass spectra of the natural product and the synthetic compound exhibited molecular ion peaks at *m/z* 166 consistent with the molecular formula $C_9H_{10}O_3$. The ^{1}H NMR spectrum of the synthetic compound showed two singlets of three protons each at δ 4.0 and 3.96 assignable to two methoxyl groups. The singlet at δ 9.85 was assigned to an aldehyde functional group. The aromatic protons signals appeared at δ 6.9 (*d*, J = 9 Hz), 7.4 (*s*), 7.45 (*dd*, J = 9, 2 Hz). This ^{1}H NMR spectrum was found to be identical with that of the natural product. The above discussed spectral data indicated that the two

Fig 1 ^{1}H NMR spectrum of 3-chloro-4-methoxybenzaldehyde.

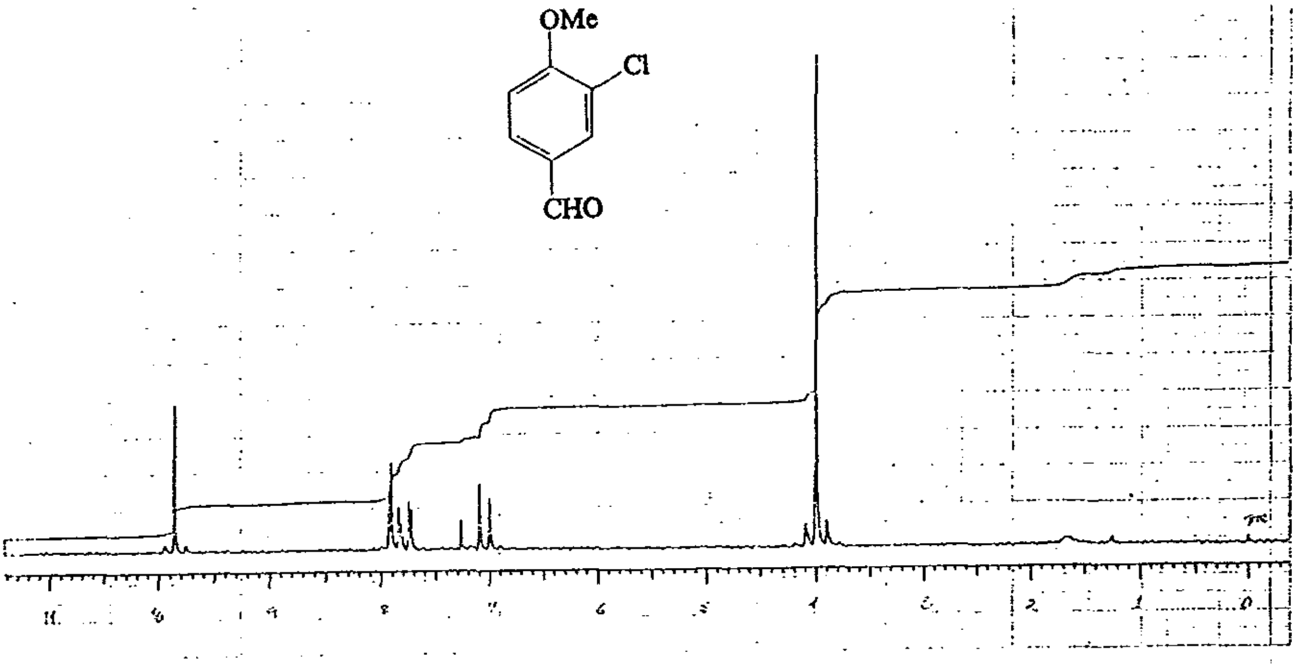


Fig 2 ^{13}C NMR spectrum of 3-chloro-4-methoxybenzaldehyde (natural product).

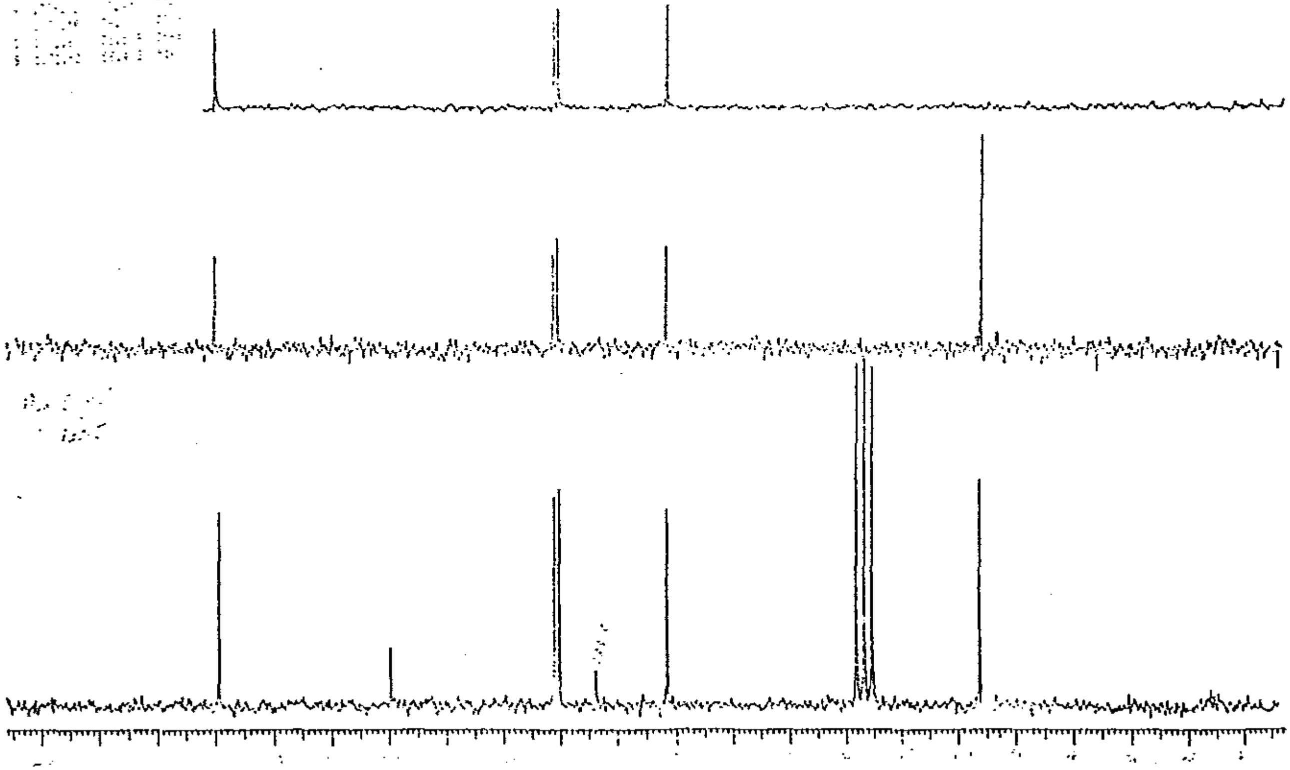


Fig 3 ^{13}C NMR spectrum of 3-chloro-4-methoxybenzaldehyde (synthetic compound).

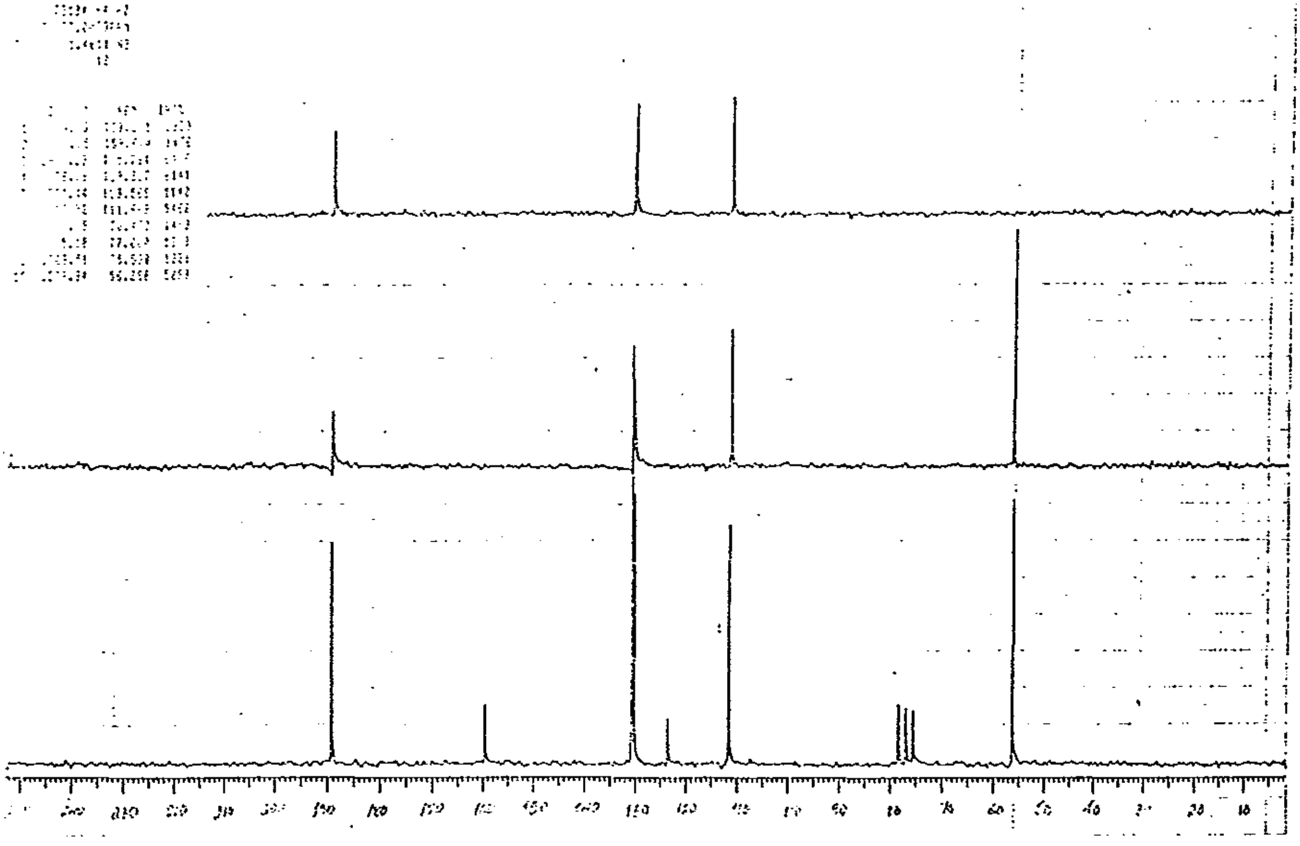
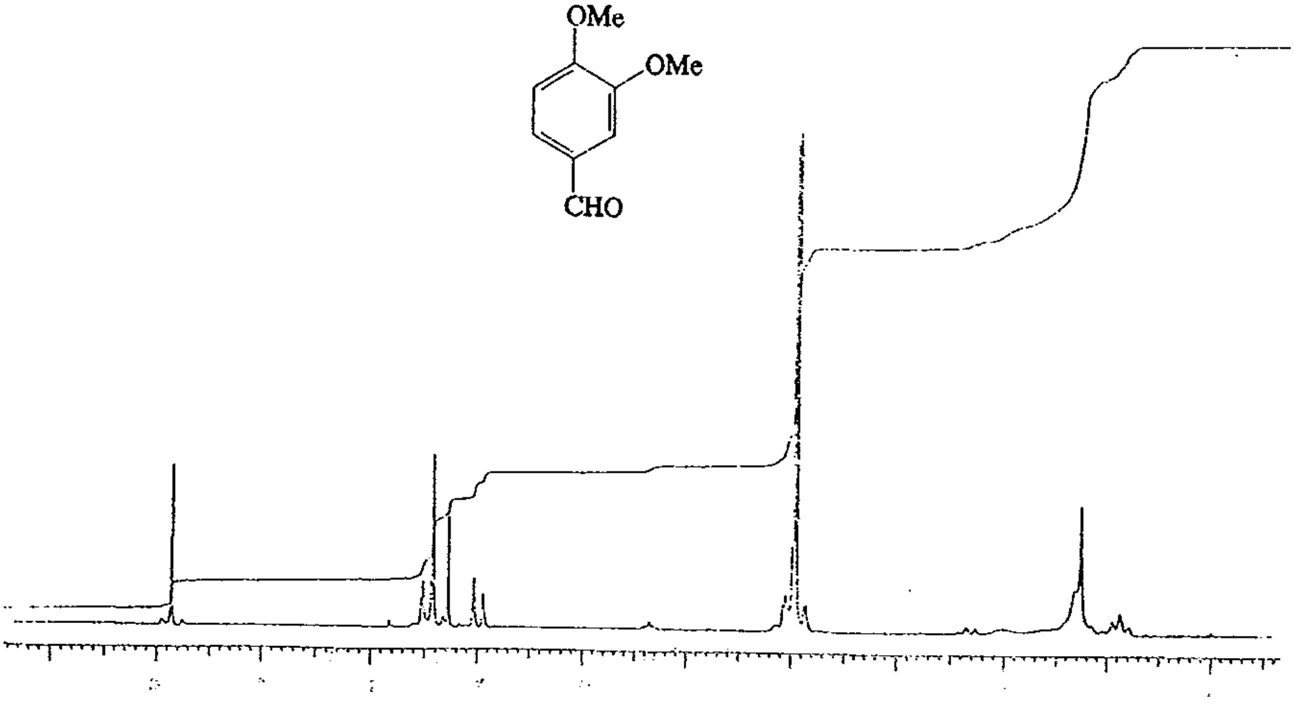


Fig 4 ^{1}H NMR spectrum of 3,4-dimethoxybezaldehyde.



compounds, the isolated and the synthetic, are identical. This helped to confirm that the natural product is 3,4-dimethoxybenzaldehyde. This is the first report of 3,4-dimethoxybenzaldehyde from the genus *Phellinus*.

Russel *et al.* [30] isolated 3,4-dimethoxybenzaldehyde and 3,4-dimethoxybenzyl alcohol from cultures of *Polystictus versicolor*, but considered these compounds to be lignin-degradation products of saw dust present in the medium. In another study, 3,4-dimethoxybenzyl alcohol was isolated from the cultures of a ligninolytic fungus *Phanerochaete chrysosporium*. Further investigation with cultures containing ^{14}C-glucose as a sole carbon source revealed that the alcohol is synthesized *de novo* from glucose [31]. Aromatic compounds including 3,4-dimethoxybenzaldehyde and 3,4-dimethoxybenzyl alcohol were also isolated from the cultures of a Basidiomycete *Bjerkandera adusta* [32]. 3,4-Dimethoxybenzaldehyde was found to be effective against *Trichomonas vaginalis* when tested against some bacteria [33].

2.1.4 Phytotoxicity and cytotoxicity of 3-chloro-4-dimethoxybenzaldehyde and 3,4-dimethoxybenzaldehyde.

The seed germination inhibitory effect of 3-chloro-4-methoxybenzaldehyde, 3,4-dimethoxybenzaldehyde and monocerin (11) (as standard) were tested on *Eragrostis teff*, *Brassica napus* and *Guizotia abyssinica*.

Monocerin when tested for phytotoxic effects on leaves (Dea cv) and root growth (pregerminated radish seedling) gave ID_{50} value around 308 μg/ml while on root cap cells (maize) and mesophyll protoplast (maize) ID_{50} values were 77 - 152 μg/ml and 24.6 μg/ml, respectively [34].

Monocerin reduced the growth of the test seeds by about 50% at the concentration of 20 μg/ml. The seed germination inhibitory effects of 3-chloro-4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde were 20-50 and 100-200 μg/ml, respectively, and these are comparable to the effect exhibited by monocerin. Seed inhibitory effect of the compounds at different concentrations are given in Table 2.2.

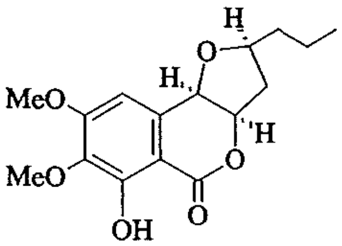


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Table 2.2 Effect of monocerin, 3-chloro-4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde on the germination of *Eragrostis teff*, *Brassica napus* and *Guizotia abyssinica*.

	Concentrations in μg/ml			
Test seeds/ Compounds	200	100	50	20
1. *Eragrostis teff*				
Monocerin	-	-	-	++
3-Chloro-4-methoxybenzaldehyde	-	-	-	+++
3,4-Dimethoxybenzaldehyde	-	+++	++++	++++
2. *Brassica napus*				
Monocerin	-	-	++	++
3-Chloro-4-methoxybenzaldehyde	-	-	++	++++
3,4-Dimethoxybenzaldehyde	-	+++	++++	++++
3. *Guizotia abyssinica*				
Monocerin	-	-	+	++
3-Chloro-4-methoxybenzaldehyde	-	-	++	+++
3,4-Dimethoxybenzaldehyde	-	++	++++	++++

Key:		
	-	no germination
	+	about 75% reduction in growth
	++	about 50% reduction in growth
	+++	about 25% reduction in growth
	++++	the same as control

Cytotoxic effect of 3-chloro-4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde was tested on brine shrimp larvae (*Artemia salina* Leach). The LC_{50} of 3-chloro-4-methoxybenzaldehyde is found to be 40 μg/ml and that of 3,4-dimethoxybenzaldehyde is greater than 100 μg/ml. Percent deaths at different concentrations are given in Table 2.3.

Table 2.3 Effect of 3-chloro-4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde on brine shrimp larvae (*Artemia salina* Leach) in percent deaths.

	concentration in μg/ml				
Compounds	100	75	50	40	30
3-chloro-4-methoxybenzaldehyde	100	90	60	50	0
3,4-dimethoxybenzaldehyde	20	0	0	0	0

2.2 A fatty acid and a triglyceride from *Phellinus* sp. grown on rice.

Phellinus sp. was grown on 250 g rice for 17 days. It was ground, dried and then the dried moldy rice was extracted with $CHCl_3$ to give 6 g brown oily crude extract. The crude extract was chromatographed by column chromatography and fractions were purified by PTLC to give two major compounds. Interestingly, the two compounds turned out to be different from 3-chloro-4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde, which were the compounds obtained from the broth culture filtrate of *Phellinus* sp. Spectroscopic analyses suggested these compounds to be a fatty acid and a triglyceride.

The ^1H NMR spectrum of the fatty acid showed a distorted triplet at δ 0.89 attributable to a terminal methyl group. The triplet at δ 2.35 together with the broad singlet at δ 10.7 indicated the presence of a carboxyl group at the other terminal. A large broad signal at δ 1.25 integrating for *ca* 12 protons suggested the presence of 6 methylene groups. The signal at δ 5.45 is assignable for olefinic protons. The signal at δ 2.0 is assigned for $-CH=CH-C\underline{H}_2$ protons and that at δ 2.75 for $-CH=CH-C\underline{H}_2-CH=CH-$ protons. The ^{13}C spectrum revealed one primary carbon (δ 14) assignable to the terminal methyl. The quaternary carbon signal at δ 180 is attributed to the carboxylic carbon. The six tertiary carbon signals which appeared between δ 128-131 are assigned to six olefinic carbons. Ten secondary carbon signals appeared between δ 22.6-34.1 and are assigned for the remaining methylene carbons. The IR spectrum of this compound showed a strong band at 1746 due to the carbonyl stretching of the carboxyl group and the UV λ_{max} at 232 nm indicated the occurrence of two conjugated double bonds. The above spectroscopic finding suggests that this compound is a C-18 fatty acid with three double bonds of which two are conjugated. Assignment of a complete structure to this compound is not possible at this stage because of lack of clear MS data.

Comparison of the ^{1}H NMR and ^{13}C NMR spectra of the two compounds indicated that the other compound is a triglyceride of the fatty acid described above. The ^{1}H NMR spectrum showed additional signal at δ 4.2 (*m*) assignable to protons on oxygenated carbons derived from a glycerol. In addition the signal at δ 10.7 disappeared suggesting ester formation. The ^{13}C spectrum showed two carbonyl signals at δ 173 and 172.6 and signals for carbons next to the carbonyl appeared at δ 34.1 and 33.9 which clearly displayed the steric effect in triglycerides [35]. Furthermore, additional signals appeared for oxygenated carbons at δ 69 (tertiary) and 62 (secondary). Although it is clear that this compound is a triglyceride, it is not possible to suggest a complete structure at present because the carboxylic acid from which it is derived has not been fully characterized.

2.3 Antibiotic and antitumour metabolites from the cultures of *Lentinus velutinus*.

Lentinus velutinus was collected from Lepis forest in Arsi and a strain was maintained on agar slants in Dr D. Abate's laboratory in the Biology Department. The fungus was cultured on medium B for 15 days and the culture filtrate was extracted with EtOAc. Screening of the crude EtOAc extract for antibiotic activity demonstrated that there was a compound, different from the known compound velutinol, which exhibited some antimicrobial activity. Velutinol and two other compounds were isolated and characterized as described below.

2.3.1 Isolation of compounds

The culture filtrate of *L. velutinus* grown for 15 days on medium B was extracted with equal volume EtOAc and concentrated to give 460 mg of crude extract. The crude extract was chromatographed on a column of silica gel with petrol/$CHCl_3$ mixture of increasing polarity and finally with methanol. Compounds **1**, **12** and **13** were obtained after purification of petrol/$CHCl_3$ (7:3), petrol/$CHCl_3$ (1:1) and methanol fractions, respectively.

2.3.2 Velutinol (1)

Compound 1 exhibited a UV absorption maximum (MeOH) at 233 nm which is comparable to that of velutinol (235 nm). The IR spectrum showed characteristic bands at 3448 (OH), 1732 (C=O, cyclopentanone) and 1636 cm^{-1} (-C=C-). The UV and IR spectra indicate the presence of an α,β-unsaturated carbonyl group. The mass spectrum of 1 revealed a molecular ion peak at *m/z* 250 which corresponds to the molecular formula of velutinol ($C_{15}H_{22}O_3$). Other peaks appeared at *m/z* 175 (100%) and 232 (M^+ - H_2O). These have also been reported for velutinol [25].

The ^1H NMR spectrum of 1 displayed signals at δ 6.05 and 5.23 which are assignable to exomethylene protons, the signal at δ 3.43 is ascribed to C<u>H</u>OH proton and signals at δ 0.92, 1.12 and 1.16 (three protons each) demonstrate the presence of three methyl groups. Table 2.4 compares the ^1H NMR spectrum of 1 with that reported for velutinol [25].

Table 2.4 ^{1}H NMR spectral data of **1** (300 MHz, $CDCl_3$) and velutinol [25].

H	1	Velutinol
1	1.51 *m*	1.70 *m*
2	2.39 *dt* (11.4, 9.0)	2.54 *dt* (11.5, 9.0)
6	3.43 *s*	3.62 *s*
8	1.99 *d* (8.5)	2.19 *d*
9	2.71 *m*	2.92 *m*
10α	1.23 *m*	1.37 *m*
10ß	1.8 *ddd* (12.7, 7.6)	2.00 *ddd* (12.5,7.5,1)
12 CH_3	0.92 *s*	1.12 *s*
13 CH_3	1.16 *s*	1.37 *s*
14 CH_3	1.12 *s*	1.31 *s*
15a	5.23 *s*	5.46 *s*
15b	6.05 *s*	6.24 *s*

The ^{13}C NMR spectrum of **1** showed the presence of a carbonyl carbon at δ 198.1 and olefinic carbons at δ 120 and 153.2. This combined with the ^{1}H NMR spectrum further supported the presence of α,ß-unsaturated carbonyl function. The carbon signals at δ 61 and 76.6 are assignable to hydroxyl substituted carbons (C-6 and C-7). Table 2.5 compares the ^{13}C NMR spectrum of **1** with that reported [25] for velutinol. There is a very good agreement between the two except for the signal due to C-2.

The ^{13}C NMR spectrum of **12** showed a signal at δ 197.6 due to a carbonyl group. In addition the occurrence of an exomethylene double bond (δ 121.6 and 153.7), epoxide substituted carbons (δ 61.1 and 76.6) and a hydroxyl substituted carbon (δ 81.1) were evident from the spectrum. Comparison of the ^{13}C NMR data of **12** with that of hypnophilin [36] (Table 2.7) demonstrated a good agreement.

Table 2.7 ^{13}C NMR data (100 MHz) of **12** compared with that of hypnophilin [36].

C	**12**	Hypnophilin
1	81.2 *d*	81.3 *d*
2	56.1 *d*	56.2 *d*
3	45.5 *s*	45.6 *s*
4	153.7 *s*	153.8 *s*
5	197.6 *s*	197.7 *s*
6	61.1 *d*	61.2 *d*
7	76.0 *s*	76.1 *s*
8	30.7 *t*	30.8 *t*
9	34.5 *d*	34.6 *d*
10	46.1 *t*	46.2 *t*
11	44.1 *s*	44.2 *s*
12, 13 & 14	17.7, 19.6 & 26.4 (each *q*)	17.7, 19.7 & 26.4 (each *q*)
15	121.6 *t*	121.7 *t*

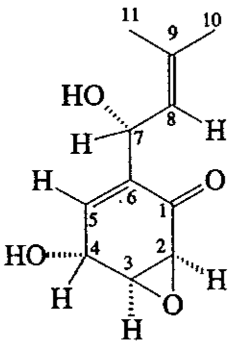
On the basis of the above discussed spectral data, compound **12** was identified as hypnophilin (**12**). This is the first report of the isolation of hypnophilin from the genus *Lentinus*.

Hypnophilin, a sesquiterpenoid antibiotic metabolite, was first isolated from the cultures of the fungus *Pleurotellus hypnophilus*. It is a colourless, unstable oil [36]. When tested by serial dilution assay, hypnophilin showed antimicrobial activity against the *Aerobacter* *aerogenes*, *Corynebacterium insidiosum*, *Mycobacterium phlei*, *Nematospora coryli* with MIC between 1-10 μg/ml. Hypnophilin also acts as a plant growth inhibitor and shows *in vitro* antitumour activity against ECA cells [37].

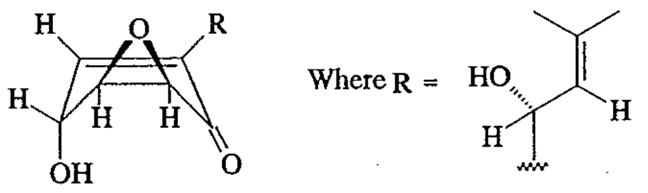
2.3.4 Panepoxydon (13)

Compound **13** exhibited a UV absorption maximum in methanol at 242 nm and an IR absorption band at 1680 cm^{-1} which suggested the presence of α,ß-unsaturated ketone. The IR spectrum also showed the presence of hydroxyl (3387 cm^{-1}), gem-dimethyl (1444 and 1380 cm^{-1}) [38] and an epoxide (1241 and 932 cm^{-1}) groups. In the ^{1}H NMR spectrum of **13** the multiplet at δ 6.7 was assigned for H-5. The two doublets at δ 5.3 (J = 9 Hz) and 4.96 (J = 4.5 Hz) were assigned for H-7 and H-4, respectively. Epoxymethine protons (H-3 and H-2) appeared at δ 3.8 (*m*) and 3.48 (*dd*, J = 4, 1 Hz), respectively. ^{1}H NMR also showed the presence of two methyl groups at δ 1.72 (*brs*) and an olefinic proton at δ 5.0 (*dm*, J = 9 Hz) suggesting the presence of a $-CH=C(CH_3)_2$ group.

The ^{1}H-^{1}H COSY spectrum of **13** revealed a more intense cross peak for H-3 and H-5 coupling than for H-3 and H-4. This together with the coupling constant and the coupling pattern in the ^{1}H NMR discussed above suggest the conformation **14**.



13



14

The ^{13}C NMR spectrum of **13** indicated the presence of 11 carbon resonances corresponding to two primary, six tertiary and three quaternary carbons. Assignment of all hydrogen containing carbon resonances was achieved with the help of ^{1}H-^{13}C hetronuclear correlation (HETCOR) spectrum. The presence of an α,ß-unsaturated

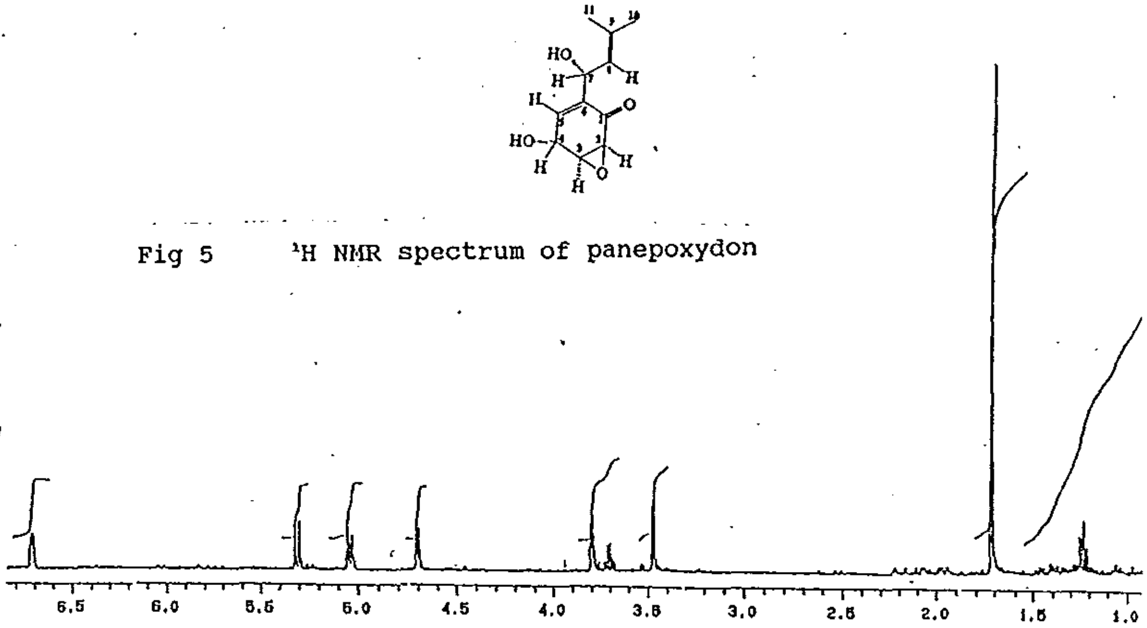
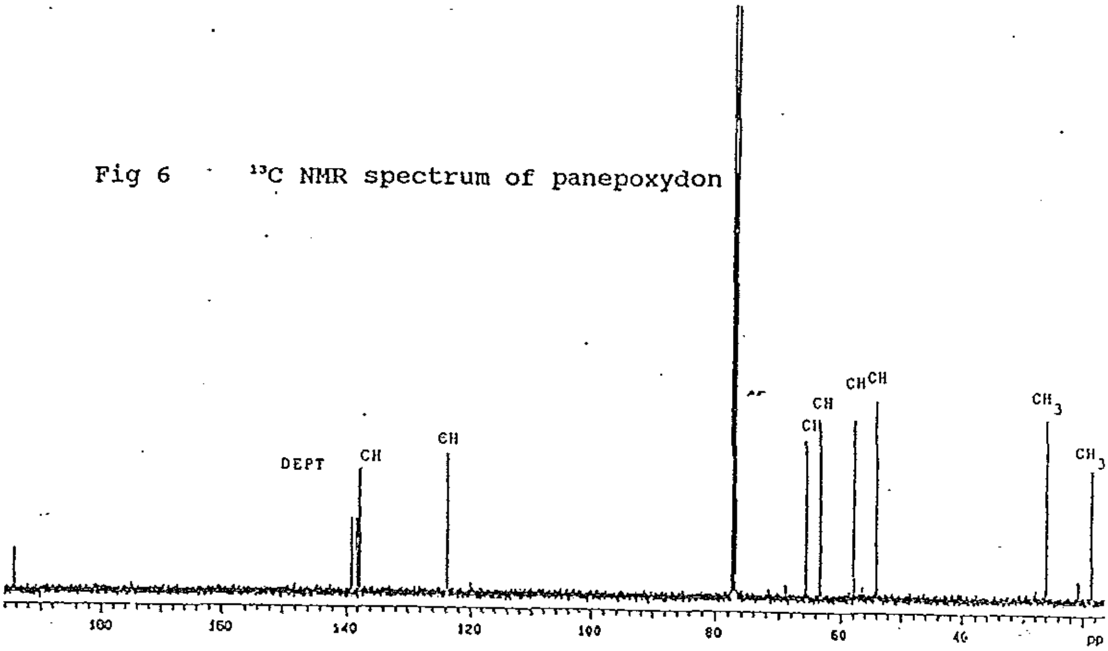


Fig 5 ¹H NMR spectrum of panepoxydon

Fig 6 ¹³C NMR spectrum of panepoxydon



The signals at δ 54.3 and 58.1 were assigned to C-2 and C-3, respectively. Hydroxyl substituted carbons C-4 and C-7 appeared at δ 63.6 and 65.8, respectively. The signal at δ 138.1 was assignable to C-5 which is ß to the carbonyl group. The signal at δ 124.1 was assigned to C-8 and the signals at δ 16.8 and 26.2 were assignable to C-10/C-11.

Based on the above discussed spectral data and by comparison this with that reported by Kis *et al.* [39] compound **13** was identified as panepoxydon. This is the first report of the isolation of panepoxydon from the genus *Lentinus*. The ^{13}C NMR spectrum of panepoxydon is also reported here for the first time.

Panepoxydon has previously been isolated and characterized from Basidiomycetes *Panus rudis* and *Panus conchatus* [39]. Panepoxydon was found to inhibit the growth of mouse-mast cell tumour P-815 at the concentration of 1 μg/ml.

2.3.5 Antibiotic effects of 1 and 12

Antimicrobial activity was tested for *Bacillus cereus*, *Staphylococcus aureus* (bacteria) and *Saccharomyces cerevisiae* (yeast). Compounds **1** and **12** showed good activity against *Bacillus cereus*. Compound **12** was relatively more effective against the yeast, *Saccharomyces cerevisiae*. The anti- microbial effect of the compounds is shown in Table 2.8

Table 2.8 Antimicrobial effect of **1** and **12**.

	MIC	
Test organism	**1**	**12**
Bacillus cereus	20-30	30-50
Staphylococcus aureus	50	50-100
Saccharomyces cerevisiae	>100	50-100

Kupchan *et al.* [40] were among the first to stress the biological importance of the exocyclic methylene group adjacent to a carbonyl function. It was demonstrated that the methylene group easily undergoes a Michael-type addition reaction with nucleophiles, like the thiol group of cysteine, and thus interferes with the active sites of certain enzymes which are important for the growth of the organism.

3. EXPERIMENTAL

3.1 General

The ^1H NMR spectra (90, 300 and 400 MHz, $CDCl_3$) were recorded using TMS as internal standard. ^{13}C NMR spectra were recorded at 22.5 MHz and 100 MHz in $CDCl_3$. ^1H-^1H and ^1H-^{13}C COSY spectra were recorded at 300 MHz in $CDCl_3$. Melting points were determined on Thomas Hoover capillary melting point apparatus and are uncorrected. The UV spectra were recorded in ethanol, $CHCl_3$ and methanol using Milton Roy Spectronic 1001 Plus. LRMS were conducted on Finnigan Mat. 1125 and VG-MASSLAB 12-250. FT-IR were recorded using Perkin Erlmer (model 1600) instrument. Analytical thin layer chromatography (TLC) was run on a 0.2 mm silica gel 60 F_{254} layer and the spots were detected by their UV fluorescence. Column chromatography, centrifugal preparative TLC and preparative TLC were performed on silica gel 60 $PF_{254+366}$ (Merck) and silica gel 60 (230-400 mesh ASTM) (Merck).

3.2 Fungal Material

3.2.1 Unidentified *Phellinus* sp.

Phellinus sp. was collected from Menagesha State Forest by Dr Dawit Abate and voucher specimen of the fruit body was deposited under the cipher ADA-419 at the National Herbarium, Addis Ababa University. Culture was made from the fresh specimen and is maintained on 3% malt extract (Oxoid) as ADA-419 in the fungal culture collection of the Biology Department.

3.2.2 *Lentinus velutinus*

L. velutinus was identified and collected by Dr Dawit Abate of the Biology Department from Lepis forest in Shoa province and voucher specimen of the fruit body is deposited under the cipher ED-4 at the National Herbarium, Addis Ababa University. The strain is maintained on 3% malt extract (Oxoid) as ED-4 in the fungal culture collection of the Biology Department.

3.3 Cultivation of *Phellinus* sp. in submerged culture and isolation of compounds.

The fungus was aseptically transferred from slants to petri dish containing 20 ml of medium A (g/l: 10 g malt extract, 4 g yeast extract, 4 g glucose and 20 g agar in distilled water). Then the grown fungus (white colony) was cut into pieces under sterile condition and transferred into fifteen 250 ml Erlenmeyer flasks each containing 100 ml of a medium B (g/l: 10 g maltose, 10 g glucose, 2 g peptone, 1 g yeast extract, 500 mg KH_2PO_4, 5 mg $CaCl_2$, 10 mg $FeCl_3$ and 2 mg $ZnSO_4$ in distilled water). The flasks were shaken for 4 days on the shaker at 120 rpm at room temperature and used as inoculum for fifteen 1 l Erlenmeyer flasks each containing medium B (300 ml). The larger flasks were also placed on a shaker and shaken at 120 rpm at room temperature for 11 days. The culture broth was filtered and the filtrate was found to have a pH of 4.86. The filtrate was extracted with equal volume of EtOAc and the solvent removed *in vacuo* to give crude extract (218 mg).

The crude extract was chromatographed on a column of silica gel and eluted with petrol in increasing proportions of EtOAc. Fractions were collected and further purified by chromatotron to yielded compounds **8** (24 mg) and **10** (3 mg). These compounds showed phytotoxicity and lethality to brine shrimp.

3.4 Synthesis of 3-chloro-4-methoxybenzaldehyde (8).

A mixture of *o*-chlorophenol (6) (0.04 mole) and NaOH solution (1.8 N, 16 ml) was stirred in a 3-necked flask fitted with a reflux condenser and a separatory funnel. The warm mixture was cooled and $(CH_3)_2SO_4$ (5 ml) added dropwise during 1 hr with stirring. After being heated under reflux for 2 hr with stirring, the mixture was cooled and water (30 ml) was added. The organic layer separated and the residual aqueous solution extracted 3 times with ether. The combined organic layer was washed with water and dilute H_2SO_4, then dried with $CaCl_2$, concentrated *in vacuo* and vacuum distilled. The distillate was found to have traces of the starting material with *o*-chloroanisole (7). The distillate was purified by combining with dilute NaOH solution and extracting it with $CHCl_3$. The extract was dried and concentrated to give 7 (0.0276 mol, 69%).

A mixture of **7** (0.0276 mol), benzene (16 ml) and powdered $Zn(CN)_2$ (4.5 g) was placed in a 3-necked flask. The contents were cooled and a fairly rapid stream of HCl gas was passed for 1 hr and 45 min with stirring. Dry $AlCl_3$ (5 g) was added slowly with stirring and was followed by a slow stream of HCl gas while heating the mixture at 35-45° (oil bath) for 4 hr. The mixture was cooled to room temperature and poured with stirring into dilute HCl (250 ml). Then refluxed for 1 hr and steam distilled to give a distillate (350 ml). The organic layer was separated and the aqueous layer extracted with benzene. The combined organic layer was dried, the solvent was removed *in vacuo* and the residue vacuum distilled to give a distillate (2.5 ml). The distillate was purified by column chromatography to yield 3-chloro-4-methoxybenzaldehyde (**8**) (0.93 mmol, 3.4%).

3.5 Synthesis of 3,4-dimethoxybenzaldehyde(10).

Vanillin (9) (9.8 mmol) was placed in a 100 ml 3-necked flask equipped with a reflux condenser and magnetic stirrer and was then melted by warming on a water bath and stirred vigorously. Aqueous KOH solution (11 N, 5 ml) and dimethyl sulphate (3 ml) were added dropwise at about the same rate, keeping the KOH concentration in slight excess. The external heating was stopped when the mixture started to reflux gently. About 40 min after the start of the addition the mixture became turbid. The remaining reagents were added within about 25 min and the resulting yellow mixture was poured into a small porcelain basin. It was allowed to cool without disturbance overnight. The hard crystalline mass was filtered and ground in ice cold water (15 ml). It was filtered by suction and placed in a vacuum desiccator to dry. The yield of 3,4-dimethoxybenzaldehyde (10) was 0.82 mmol (90%).

3.6 Cultivation of *Phellinus* sp. on solid substrate and isolation of compounds.

The fungus was grown for 10 days in petri dishes on medium A. The fungus was cut into pieces under sterile condition and transferred into a 250 ml Erlenmeyer flask containing medium B (100 ml). The flask was placed on a shaker and shaken at 120 rpm at room temperature for 6 days.

Water (120 ml) was added to rice (250 g) in a 2 l Erlenmeyer flask. The rice was allowed to soak for 1 hr and then sterilized at 121° for 30 min. The culture of *Phellinus* sp. inoculum (100 ml), prepared as indicated above, was added to the sterile moist rice. The flask was shaken by hand to prevent clumping. When all seeds were invaded and the mycelial mat had turned white, 17 days after inoculation, the moldy rice was ground with a mortar and pestle. Then dried in an oven at 35° overnight and for 5 hr in an open air. The dried moldy rice was extracted with $CHCl_3$ to give a brown oily crude extract (6 g). The crude extract was applied on a column of silica gel and eluted with petrol containing increasing proportions of EtOAc. Purification of fraction 4 and 6 gave two major compounds (150 and 160 mg, respectively).

3.7 Cultivation of *L. velutinus* in submerged culture and isolation of compounds.

L. velutinus was aseptically transferred from slants to petri dishes containing medium A and grown for 6 days. It was then cut into pieces under sterile conditions and transferred into fifteen 250 ml Erlenmeyer flasks each containing medium B (100 ml). The flasks were placed on a shaker and shaken at 120 rpm at room temperature for 8 days. The fungus grown in these flasks was used as inoculum to fifteen 1 l Erlenmeyer flasks each containing medium B (300 ml). The larger flasks were shaken at 120 rpm at room temperature for 15 days. The culture broth was filtered and the filtrate (pH 4.8) was extracted with an equal volume of EtOAc. The solvent was removed *in vacuo* to give a brown oily residue (460 mg).

The crude extract was chromatographed on a column of silica gel. The column was eluted with mixtures of petrol-$CHCl_3$ of gradually increasing polarities, and finally with methanol. The petrol-$CHCl_3$ (7:3) fraction was purified by PTLC to give compound 1 (18 mg). The petrol-$CHCl_3$ (1:1) fractions were purified by PTLC to give compound 12 (50 mg). The methanol fractions gave compound 13 (35 mg) after purification.

3.8 Biological assays

3.8.1 Bioautography

The crude extracts showing biological activity in the agar diffusion assay were analyzed by TLC. The active spots were determined by first separating the crude extract into its constituents on TLC and then cutting the plates into small pieces and placing them facing down on the agar media seeded with test organisms. After incubation at an appropriate temperature for 48 hr, inhibition effects were recorded.

3.8.2 Phytotoxicity tests

Pure compounds (20, 50, 100 and 200 μg) were applied on filter paper discs of 13 mm diameter by serial dilution. After the paper discs had dried they were placed in small vials and moistened with distilled water (150 μl). Each of the different test seeds (5-10 in number depending on the size of the seed) were placed on each moistened disc. The vials were kept in a moistened container and incubated for four days at room temperature. The effect of the compound on germination of seeds was then recorded and compared with the control. The controls were in duplicates.

3.8.3 Brine shrimp bioassay

Brine shrimp eggs (*Artemia salina* Leach) were hatched in a rectangular dish (10.5 cm x 7 cm) filled with artificial sea water which was prepared by dissolving a commercial sea salt mixture (Instant Ocean from Aquarium Systems) with distilled water (38 mg/ml). A plastic divider was clamped in the dish to make two unequal compartments. The eggs were sprinkled into the larger compartment which was darkened, while the smaller compartment was illuminated. After 48 hr the brine shrimp larvae were collected from the lighted side.

Samples were prepared by dissolving 2 mg of the compound in 2 ml of methanol. Appropriate amounts of solution for 100, 75, 50, 45, 40 and 30 μg were transferred with micropipette to microtiter plate, and then dried in air.

Ten shrimps were transferred to each compartments of the microtiter plate using pipette, and an artificial sea water was added to make 1 ml. Survivors were counted after 24 hr, the percent deaths at each dose and control were determined.

3.8.4 Antibiotic test

The antifungal and antibacterial activity were tested by serial dilution assay. The test bacteria were grown in nutrient broth and the fungus in Sabouraud's broth. The test organisms (loop full microorganisms in 1 ml broth) were incubated with different amounts of the test substance for 24 hr. The minimal inhibition concentration (MIC) was defined as the lowest concentration of a substance which inhibited growth (visible turbidity). After 24 hr MIC was recorded by comparing with the control.

3.9 Physicochemical data

Natural product.

3-Chloro-4-methoxybenzaldehyde (**8**) White solid; Mp 55-56°; Rf = 0.8 (petrol:$CHCl_3$, 1:1); UV λ_{max}(EtOH)nm: 272, 228; ^{1}H NMR (90 MHz, $CDCl_3$) δ: 4.0 (3H, *s*, OCH_3), 7.05 (1H, *d*, J = 9 Hz, H-5), 7.75 (1H, *dd*, J = 9, 2 Hz, H-6), 7.8 (1H, *d*, J = 2 Hz, H-2), 9.85 (1H, *s*, CHO); ^{13}C NMR (22.5 MHz, $CDCl_3$) see Table 1.; MS *m/z* (rel. int.): 172 $[M+2]^+$ (19), 170 $[M]^+$ (60), 171 (33), 169 (100), 141 (17), 99, 77, 63, 51.

3,4-Dimethoxybenzaldehyde (**10**) Rf = 0.8 ($CHCl_3$) UV λ_{max}($CHCl_3$)nm: 310, 280; ^{1}H NMR (90 MHz, $CDCl_3$) δ: 4.0 (3H, s, OCH_3), 3.96 (3H, s, OCH_3), 6.9 (1H, d, J = 9 Hz, H-5), 7.4 (1H, br s, H-2), 7.45 (1H, dd, J = 9, 2 Hz, H-6), 9.85 (1H, s, CHO); MS *m/z* (rel. int): 166 $[M]^+$ (20), 165 (100), 151 (23), 137 (10), 119 (10), 95, 77, 65, 51, 41.

Synthetic

3-Chloro-4-methoxybenzaldehyde (**8**) White solid; Mp 55-56° (lit [28] 56-57°); Rf = 0.8 (petrol:$CHCl_3$ 1:1).; UV λ_{max}(EtOH)nm: 271, 227 ; ^1H NMR (90 MHz, $CDCl_3$) is the same as the natural product; ^{13}C NMR (22.5 MHz, $CDCl_3$) See Table 1.; MS *m/z* (rel. int.) the same as the natural product.

3,4-Dimethoxybenzaldehyde (**10**) Rf = 0.8 ($CHCl_3$); UV λ_{max}($CHCl_3$)nm: 310, 281; ^1H NMR (90 MHz, $CDCl_3$) identical with the natural product; MS *m/z* (rel int.) the same as the natural product.

Velutinol (**1**) Colourless oil; Rf = 0.6 (petrol:$CHCl_3$, 1:1).; IR $\upsilon_{max}cm^{-1}$: 3448, 2926, 2865, 1732 and 1636; UV λ_{max}(MeOH)nm: 233; ^1H NMR (300 MHz, $CDCl_3$) given in Table 2.4; ^{13}C NMR (100 MHz, $CDCl_3$) given in Table 2.5; MS *m/z* (rel. int.): 250 $[M]^+$ (5), 232 (11), 214 (21), 175 (100).

Hypnophilin (**12**) Colourless oil; Rf = 0.7 ($CHCl_3$:EtOAc, 7:3); IR $\upsilon_{max}cm^{-1}$: 3471, 2936, 2867, 1728, 1633, 1253, 888; UV λ_{max}(MeOH)nm: 233.6; ^1H NMR (90 MHz, $CDCl_3$) given in Table 2.6; ^{13}C NMR (100 MHz, $CDCl_3$) given in Table 2.7; Ms *m/z* (rel int.): 248 $[M]^+$ (11), 233 (7), 231 (8), 230 (10), 219 (13), 215 (11), 201 (12), 177 (60), 158 (45).

Panepoxydon (**13**) Brown oil; Rf = 0.4 ($CHCl_3$:MeOH, 9:1); IR $\upsilon_{max}cm^{-1}$: 3387, 2961, 2925, 1680, 1444, 1380, 1241, 1041, 931, 850; UV λ_{max}(MeOH)nm: 242; ^1H NMR (400 MHz, $CDCl_3$) δ: 6.7 (1H, *m*, H-5), 5.3 (1H, *d*, $J = 9$ Hz, H-7), 5.03 (1H, *dm*, $J = 9$ Hz, H-8), 4.69 (1H, *d*, $J = 4.5$ Hz, H-4), 3.8 (1H, *m*, H-3), 3.48 (1H, *dd*, $J = 4, 1$ Hz, H-2) and 1.72 (6H, *t*, $J = 1$ Hz, 2 x CH_3); ^{13}C NMR (100 MHz, $CDCl^3$) δ: 16.8, 26.2, (each q, C-10/C-11), 54.3, 58.1, 63.6, 65.8 (each t, C-2, C-3, C-4 & C-7), 124.1, 138.1 (each t, C-8 & C-5), 138, 138.6 (each *s*, >C=C<) and 194 (*s*, C-1)

APPENDICES

Appendix 1. Compounds reported from the genus *Phellinus*.

Str.No.	Name of compound	Source organism	Ref.
15	Trametenolic acid	*Phellinus gilvus*	41
16	Ergosterol	"	"
		P. ignarus	42
		P. pomaceus	17
		P. robustus	42
17	5,6-Dihydroergosterol	*P. gilvus*	41
		P. pomaceus	17
18	Phenylethyl alcohol	*P. ignarius*	43
		P. laevigatus	"
		P. tremulae	"
19	Benzyl alcohol	*P. ignarius*	"
		P. laevigatus	"
		P. tremulae	"
20	Methyl benzoate	*P. ignarius*	"
		P. laevigatus	"
		P. tremulae	"
21	Ethyl benzoate	*P. ignarius*	"
		P. tremulae	"
22	Methyl salicylate	*P. ignarius*	"
		P. laevigatus	"
		P. tremulae	"
23	Phenol	*P. ignarius*	"
		P. tremulae	"
24	Linalool	*P. ignarius*	"
		P. tremulae	"
25	Hispidin	*P. pomaceus*	18
26	3,14'-Bihispidinyl	"	"

Contd . . .

27	Ergosta-7-en-3ß-ol	"	17
28	Ergosta-5,7-dien-3ß-ol	"	"
29	Oleic acid	"	"
		P. weirii	23
30	Linolenic acid	*P. pomaceus*	17
		P. weirii	23
31	Javerolic acid	*P. pomaceus*	44
32	Phellinic acid	"	"
33	7-Phenylheptan-3-one	*P. tremulae*	45
34	5-Hydroxy-7-phenylheptan-3-one	"	"
35	7-Phenyl-3-hydroxyheptane	"	"
36	7-Phenyl-4t-heptene-3-one	"	"
37	1-Phenylheptan-1,5-dione	"	46
38	Palmitic acid	*P. weirii*	23
39	Stearic acid	"	"

Appendix 2. Compounds reported from the genus *Lentinus*

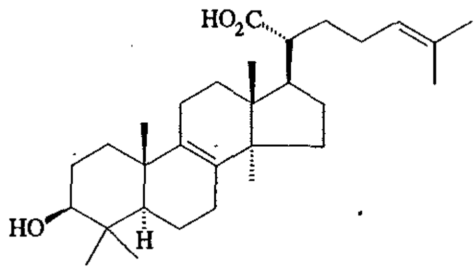
Str.No.	Name of compounds	Source organism	Ref.
15	Trametenolic acid	*Lentinus lepideus*	47
16	Ergosterol	"	"
		L. edodes	48
28	Ergosta-5,7-dien-3ß-ol	"	"
40	Eburicoic acid	*L. dactyloids*	49
		L. lepideus	47
41	Dehydroeburicoic acid	*L. dactyloids*	49
42	4-Methoxytoluquinone	*L. degener*	50
43	6-Hydroxy-4-methoxytoluquinone	"	51
44	Deoxyeritadenine	*L. edodes*	52
45	4-(amino-9H-purin-9-yl) propanoic acid	"	"

Contd . . .

47	Nicotianine	"	54
48	Saccharophine	"	55
49	SE-3	"	56
50	Lentinic acid	"	57
51	1,3-Dithietane	"	58
52	1,3,5-Trithiane	"	"
53	1,2,3,5-Tetrathiane	"	"
54	1,2,4,6-Tetrathiane	*L. edodes*	58
55	1,2,4,5,7-Pentathiocane	"	"
56	1,2,3,5,6,8-Hexathionane	"	"
57	2,3,5,6-Tetrathiaheptane	"	"
58	1,2,4,5-Tetrathiane	"	"
59	Lenthionine	"	59
60	1,2,4,5-Tetrathiane	"	"
61	1,2,4-Trithiolane	"	"
62	1-Octen-3-ol	"	"
63	2-Octen-1-ol	"	"
64	Methyl cinnamate	*L. lepidedus*	60
		L. ponderosus	61
65	Methyl p-coumarate	*L. Lepidedus*	62
66	Methyl p-methoxycinnamate	*L. lepidedus*	"
67	Methyl isoferulate	"	63
68	Methyl anisate	"	60
		L. ponderosus	61
69	δ-Cadinol	*L. lepidedus*	64
70	(-)-T-Muurolol	"	"
71	(+)-T-cadinol	"	"
72	(-)-α-Cadinol	"	"
73	Cubenol	"	"
74	Epicubenol	"	"
75	Trans, trans-farnesol	"	"
76	Bicyclofarnesol	"	"
[illegible]	[illegible]	"	[illegible]

Contd . . .

78	γ-muurolene	"	"
79	Lentideusether	"	66
80	Isolentideusether	"	"
81	Muurolene ether	"	"
82	Terrestrol	"	"



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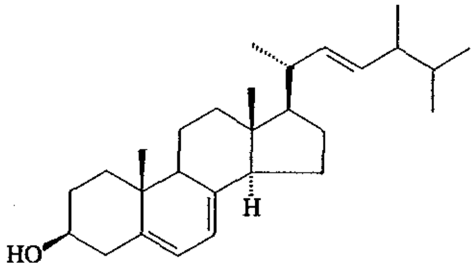

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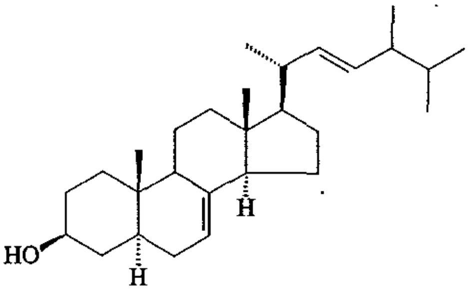

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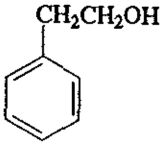

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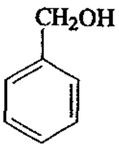

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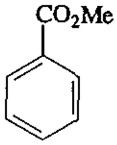

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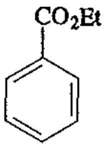

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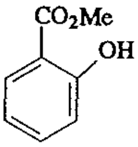

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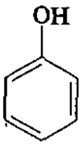

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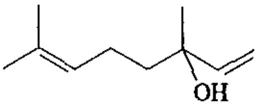

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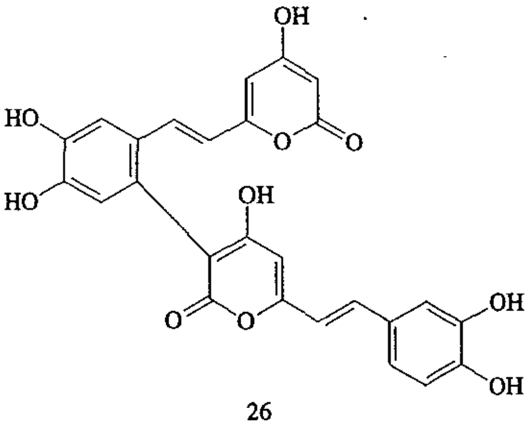

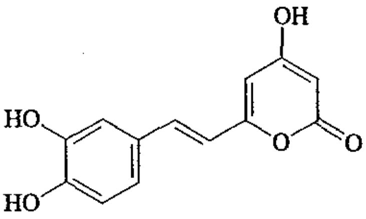

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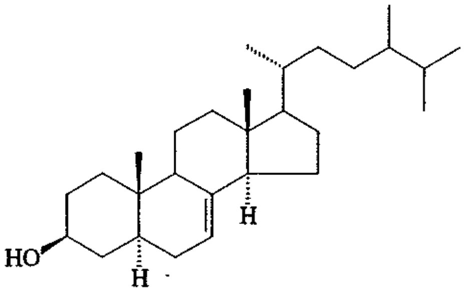

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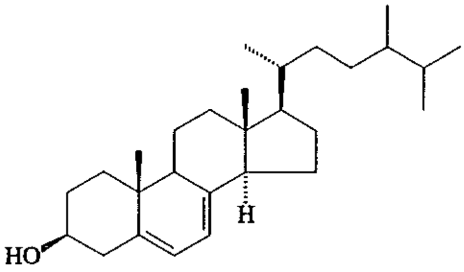


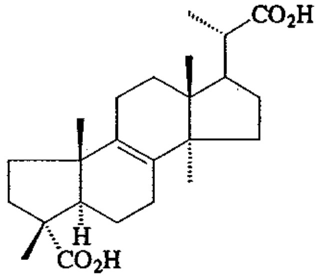
28

$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$

29

$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$

30



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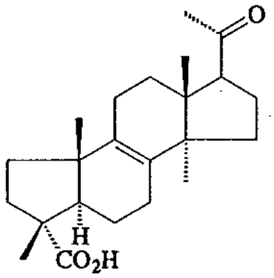

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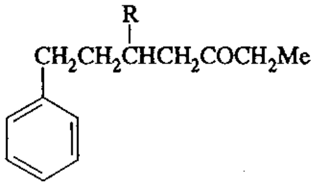
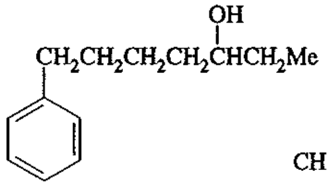


$CH_2CH_2\overset{R}{C}HCH_2COCH_2Me$

33 R = H

34 R = OH

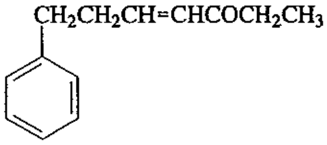


$CH_2CH_2CH_2CH_2\overset{OH}{C}HCH_2Me$

35

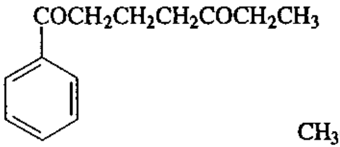
$CH_3(CH_2)_{14}COOH$

38



$CH_2CH_2CH{=}CHCOCH_2CH_3$

36

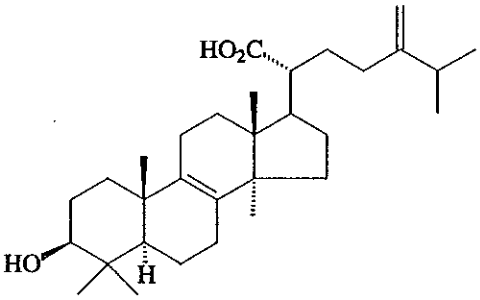


$COCH_2CH_2CH_2COCH_2CH_3$

37

$CH_3(CH_2)_{16}COOH$

39



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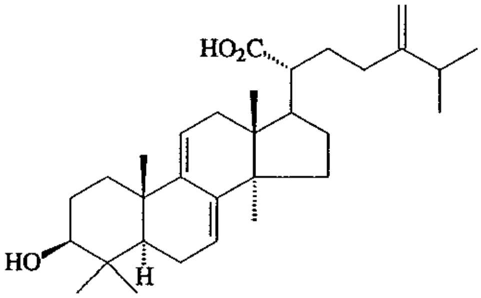

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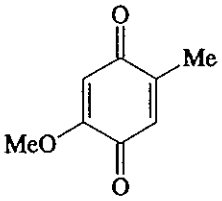

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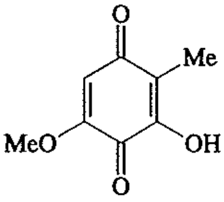

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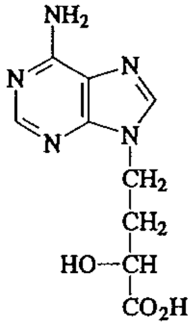

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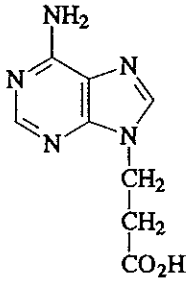

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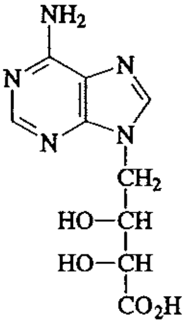

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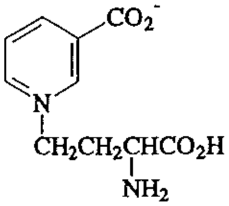


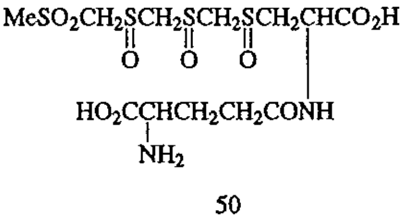
47

$HO_2C\underset{NH_2}{C}H(CH_2)_4NH\underset{CO_2H}{C}H(CH_2)_2CO_2H$

48

$MeSO_2CH_2OCH_2SCH_2SOMe$

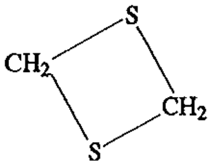
49



$MeSO_2CH_2\underset{\underset{O}{\|}}{S}CH_2\underset{\underset{O}{\|}}{S}CH_2\underset{\underset{O}{\|}}{S}CH_2\underset{|}{C}HCO_2H$

$HO_2C\underset{NH_2}{C}HCH_2CH_2CONH$

50



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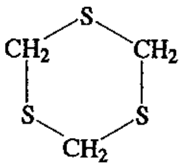

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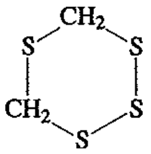

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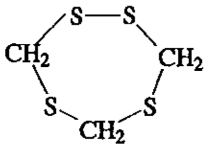

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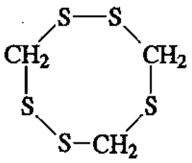

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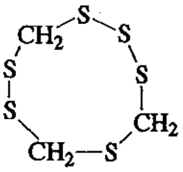


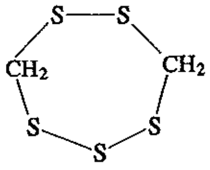
56

$CH_3SSCH_2SSCH_3$

57

$CH_3SSCH_2SCH_2CH_2SCH_2SSCH_3$

58



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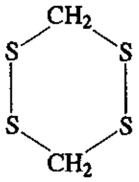

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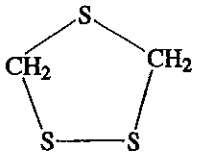

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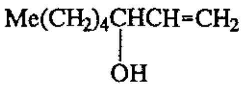
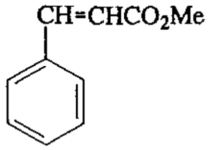


$Me(CH_2)_4CH(OH)CH{=}CH_2$

62

$Me(CH_2)_4CH{=}CHCH_2OH$

63



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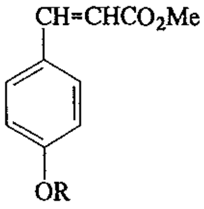
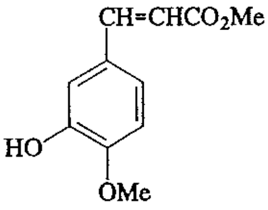


65 R = H

66 R = Me



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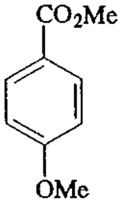

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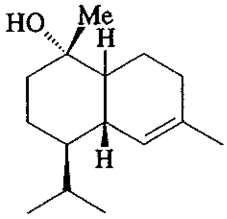

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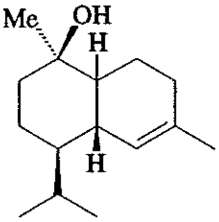

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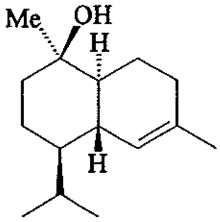

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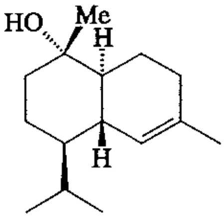

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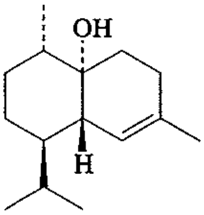

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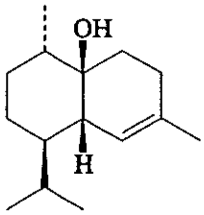

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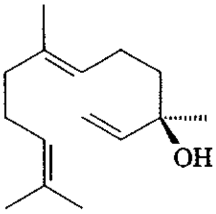

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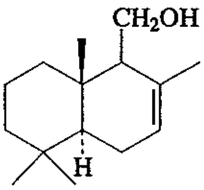

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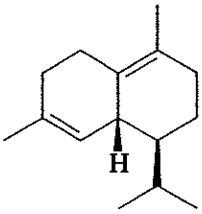

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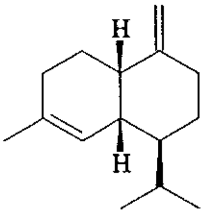

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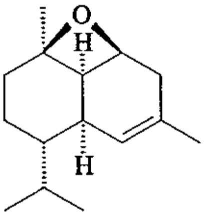

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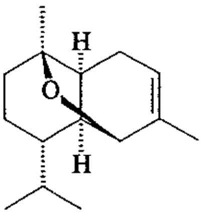

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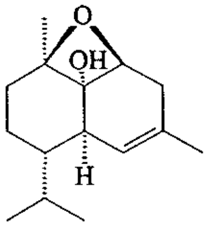

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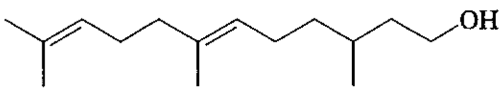

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