CHAPTER FOUR

4. EXTRACTION AND ISOLATION OF TERPENOIDS

4.1 Introduction

The extraction and isolation of triterpenoids from *Ganoderma* spp. have been reviewed in chapter two. Most researchers adopted specific techniques of isolating the terpenoids by treating the crude extract with various chemical reactions. For example, Hirotani et al. (1985a, b, 1986, 1987) in their experiments acidified the crude extract with hydrochloric acid and then methylated with diazomethane before column chromatography.

Column chromatography has been widely used to separate natural products, including the terpenoids. This technique is based on both adsorptivity and solubility. It is a solid-liquid phase-partitioning technique, in which a mixture of compounds is separated by distributing between a stationary phase and a moving phase (Pavia *et al.*, 1982). The most commonly used stationary phase is the silica gel.

Like column chromatography, thin-layer chromatography (TLC) is also a solid-liquid partitioning technique. The TLC is very useful for the rapid separation and qualitative analysis of small amounts of material. The later is especially important for monitoring the compounds isolated from column chromatographic separation.

For structure elucidation, NMR spectroscopy is the most useful single technique. However, an integrated approach of techniques, such as mass spectroscopy is necessary for unambiguous interpretations of the result.

In this study, a general extraction procedure was adopted. The fruit body chips of *G. tsugae* were ground and soaked in distilled hexane to extract the non-polar compounds. The sample was then decanted and resoaked with distilled methanol for the extraction of the more polar compounds. No additional chemicals were added to the crude extracts before column chromatography.

G. tsugae was used in this study instead of *G. lucidum* because this is the main species being cultivated in the farm. Furthermore, the phytochemical content of this species is not well studied. Recently, Gan et al. (1998) reported a new lanostanoid ester glycoside and a known steroid isolated from this species showing strong cytotoxic effect via apoptosis against hepatoma cells.

Thus, the aims of this study are:

1. to extract and screen for phytochemical content of *G. tsugae*.
2. to separate and isolate the major compounds (in particular, the terpenoids) present in the crude extracts.
3. to characterize the isolated compounds by spectroscopic techniques.

4.2 Materials and Methods

4.2.1 Fruit Body

Approximately 2 kg of the mature fruit body of *G. tsugae* (after 2 months old) was collected in April 1999 from a mushroom farm in Semenyih, Selangor, Malaysia

4.2.2 Extraction of Terpenoids

The general extraction procedures of terpenoids were summarized in Figure 4.1. The dried fruit body chips of *G. tsugae* was initially ground into smaller pieces and soaked twice in distilled hexane at room temperature for two days and then decanted. The residue was further re-extracted twice again with distilled methanol. Both the hexane and methanol extracts were then dried *in vacuo* and their weight recorded.

4.2.3 Phytochemical Tests

The hexane and methanol crude extracts were individually screened for alkaloids, terpenoids, saponins and flavonoids content.

Alkaloid Test.

Dragendorff's reagent (Munier and Macheboef modification) was used to detect the alkaloids. Positive colour spots are red, orange, yellow or brown indicate the presence of alkaloids.

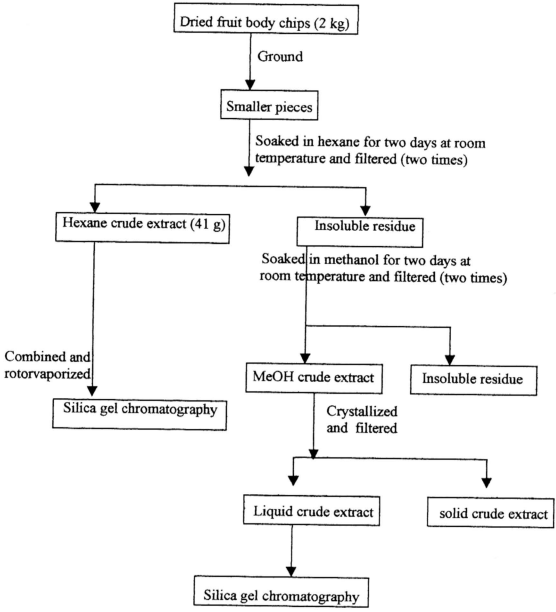


Figure 4.1: General scheme for the extraction of terpenoids from *G. tsugae*

Terpenoid Test.

Vanillin-sulphuric acid staining reagent was used to detect the terpenoids. TLC plates were heated to 110°C for 5 min. to develop colours- red, pink, purple, blue and grey for indication of various unsaturated terpenoids.

Saponin Test.

The crude extracts were boiled for 5 min in a test tube. The test tube was cooled and shaken very vigorously and set aside for 30 min. A 1-2 cm or more of stable foam is considered positive and no foam negative. A foam lasting 2, 3, and 4 h is given a relative scale of 2+, 3+ and 4+ respectively.

Flavonoid Test.

The crude extracts were dissolved with ether and shaken with equal volume of ammonia (1 vol. conc. NH_3 and 9 vol. water). A strong yellow colour in the ammonia layer indicates presenαof flavonoid and a purple colour indicates presenαof quinone or juglone

4.2.4 Chromatographic Separation of the Crude Extracts

Column chromatography and Thin Layer Chromatography (TLC) were used repeatedly in the process of isolation and separation of the crude extracts. The solvents used were distilled hexane, chloroform and methanol. The detailed isolation and separation of the crude extracts αre respectively shown in Figure 4.2 and 4.3.

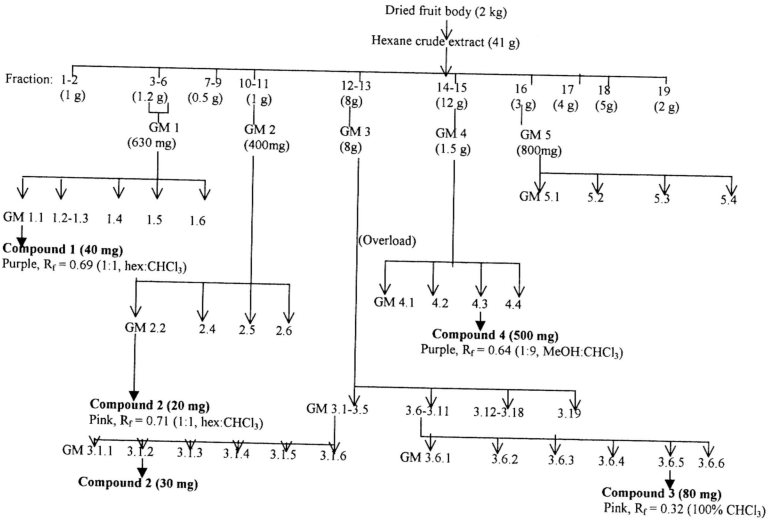


Figure 4.2: Detailed flow diagram of the isolation of terpenoids from hexane crude extract

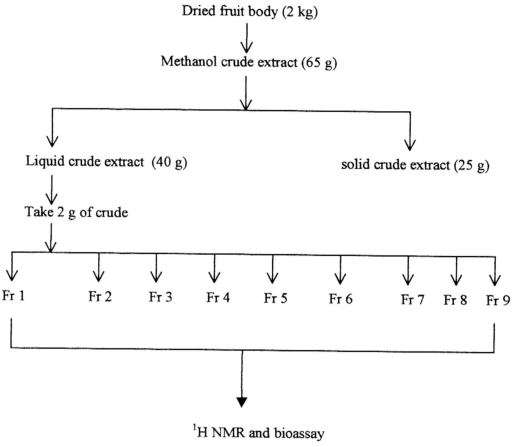


Figure 4.3: Detailed flow diagram of the isolation of terpenoids from methanol crude extract.

Column Chromatography of Hexane Crude Extract

A total of 41g of hexane crude extract was collected and chromatographed on silica gel (Merck, 230-400 mesh, 600g). Stepwise elution with hexane/chloroform/methanol in increasing polarity yielded 19 fractions (Fr.) (Table 4.1 and Fig 4.2). These fractions were monitored by TLC and ^{1}H NMR spectroscopy for terpenoids content and purity. Combination of fractions was based on TLC patterns (same R_f values) which yielded five main fractions: GM 1 (Fr 3-6), GM 2 (Fr10-11), GM 3 (Fr12-13), GM 4 (Fr14-15) and GM 5 (Fr 16) (Fig 4.2). Five of these fractions were rechromatographed individually to give subsequent purer fractions on a smaller column (Merck, 230-400 mesh, 10g).

Table 4.1: Solvent system and approximate volume used for elution of hexane crude

Fractions	Hex:$CHCl_3$:MeOH	Approximate Volume (L)
1-2	95:5:0	8.5
3-6	90:10:0	5.5
7-9	80:20:0	8.5
10-11	70:30:0	5.5
12	60:40:0	8.0
13-14	50:50:0	8.5
15	30:70:0	7.5
16	0:100:0	7.0
17	0:95:5	6.0
18	0:90:10	7.5
19	0:0:100	8.5

Column Chromatography of Methanol Crude Extract

Approximately 65 g of methanol crude extract was obtained and 2 g of crude sample was loaded to silica gel (Merck, 230-400 mesh, 30 g) packed column. Stepwise elution with hexane/chloroform/methanol of increasing polarity yielded nine fractions

(Fr1-Fr9) (Table 4.2 and Fig 4.3). The nine fractions were collected and monitored by TLC and ^{1}H NMR for terpenoids content and purity. Further purification of these nine fractions was not made due to time constraint. However, the nine fractions were used for biological assay.

Table 4.2: Solvent system and approximate volume used for elution of methanol crude.

Fractions	Hex:$CHCl_3$:MeOH	Approximate Volume (L)
1	70:30:0	1.5
2	60:40:0	0.5
3	50:50:0	0.8
4	20:80:0	1.5
5	0:95:5	0.5
6	0:90:10	1.0
7	0:80:20	0.5
8	0:60:40	0.5
9	0:0:100	0.6

Thin Layer Chromatography (TLC)

The TLC was used to monitor the presence and purity of terpenoids of the fractions collected from the column chromatography. The TLC plates were of precoated 5 x 10 cm glass plates with 0.25 mm thickness of silica gel 60F254 (Merck Art, 5719). A fine glass capillary tube was used to transfer a small volume of each fraction by spotting on the plate. The plate was then developed in paper-lined saturated chromatography tanks with appropriate solvent systems at room temperature. UV light (254 nm and 365 nm) and vanillin reagent were used to examine the developed plate.

4.2.5 Instrumentation

Mass Spectrometry (MS)

The pure compounds were thoroughly dried in a vacuum pump before recording the mass spectrum on a HP 60D electron impact mass spectrometer. The mass spectrometer operates at 70eV electron impact (EI) mode.

Nuclear Magnetic Resonance (NMR)

A 400 MHz JEOL-LAMDA 400 FT-NMR spectrometer was used to record proton spectrum for the examination of the purity of the terpenoids. All samples were dissolved in $CDCl_3$ and tetramethylsilane (TMS) was used as internal reference. ^{13}C NMR, DEPT, ^{1}H-^{1}H COSY, HETCOR spectrum were also recorded.

4.3 Results and Discussion

4.3.1 Phytochemical Tests

Various phytochemical tests were carried out to screen for other groups of compound besides terpenoids. The result showed that no saponins and flavonoids were detected in the hexane extract, while only a minor amount of saponins and flavonoids were present in the methanol extract (Table 4.3).

Alkaloids were detected in considerable amounts in the methanol extract, but at low level in the hexane extract (Table 4.3). Chang and But (1986) and Yu et al. (1990) have reported that alkaloids were present in the fruit body of *G. lucidum* and the mycelium of *G. capense* respectively. Although the alkaloids, saponins and flavonoids are known to possess interesting biological activities (Goh *et al.*, 1993), due to time constraint and the focus of this study, isolating these groups of compounds was not carried out.

Terpenoids were found to be present in high concentrations in both the hexane and methanol crude extracts (Table 4.3). The total yield obtained was approximately 3.5-4.5% (w/w). This result correlates well with many of the reported literature, whereby the triterpenoids are one of the major compounds present in *Ganoderma* species (Willard, 1990; Jong and Birmingham, 1992; Mizuno *et al.*, 1995). Thus, it is in the interest of this study to further isolate and separate the terpenoids.

Table 4.3: Phytochemical screening of the hexane and methanol crude extracts of *G. tsugae*.

Phytochemical test	Hexane crude extract	Methanol crude extract
Saponins	0*	+1
Flavonoids	0	+1
Alkaloids	+1	+2
Terpenoids	+4	+4

*Relative scale: 0= no, +1= low, +2= moderate, +3= high, +4=very high.

4.3.2 Chemical-Guided Fractionation

Figures 4.2 and 4.3 show a flow-chart of the isolation of compounds from hexane and methanol crude extracts using various polarity of the solvent systems. The R_f values of the TLC (Tables 4.4, 4.5, 4.6, 4.7 and Figures 4.4, 4.5, 4.6 and 4.7) were used as the guide for fractionation. Fractions that showed the same pattern of R_f values and colours were recombined to obtain a higher yield, which could later be used in bioassay or for further purification work.

The target compounds for isolation were identified as major spots with clear intensity of colour on TLC plates. By focusing on these target compounds, four pure compounds from hexane extract were successfully isolated by repeated column chromatography. Each compound was observed as a single spot on the TLC plate, showing different colour and R_f value (Table 4.8). This approach of targeting major spots on the TLC plates will not only cut down the time of isolation, but also help to obtain enough yield (quantity) for further spectroscopic studies and biological assays. No compound was isolated from the methanol extract due to time constraint.

Table 4.4: R_f values and colours obtained from TLC plates (Figure 4.4)

Fractions	Colour (R_f value)/ comment
GM 1.1	Purple (0.69)
GM 1.2	Purple (0.69), sky blue (0.62)
GM 1.3	Purple (0.69), sky blue (0.62)
GM 1.4	Grey (0.60), with short tailing
GM 1.5	Green, long tailing
GM 1.6	Brown (0.10)
GM 2.2	Pink (0.71)
GM 2.4	Blue (0.36), green (0.28)
GM 2.5	Blue (0.36), green (0.28), pink with tailing
GM 2.6	Pink and brown tailing

Solvent system: 1:1, hex:$CHCl_3$

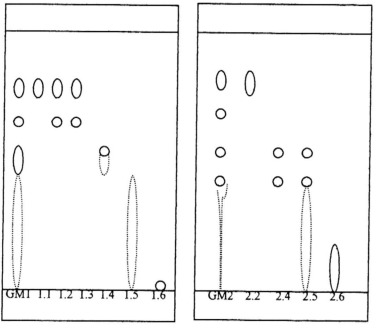


Figure 4.4: TLC patterns obtained from chromatographic separation of GM1 (Fr 3-6) and GM 2 (Fr10-11)

Table 4.5: R_f values and colours obtained from TLC plates (Figure 4.5)

Fractions	Colour (R_f value)/ comment
GM 3.1-3.5	Dark blue (0.62), sky blue (0.58), blue(0.25), sky blue (0.075)
GM 3.6-3.11	Dark blue (0.62), sky blue (0.58), yellow (0.30), pink (0.19), dark blue (0.10), brown, (0.075)
GM 3.12-3.18	sky blue (0.58), yellow (0.30), pink (0.19), dark blue (0.10), brown, (0.075)
GM 3.19	pink (0.19), dark blue (0.10), brown, (0.075)
GM 3.1.1	Dark blue (0.76), pink (0.74), grey (0.70)
GM 3.1.2	Pink (0.79)
GM 3.1.3	Blue/ grey (tailing)
GM 3.1.4	Dark blue (0.31) with tailing
GM 3.1.5	Dark blue (0.31)
GM 3.1.6	Dark blue (0.31) and brown tailing
GM 3.6.1	Dark blue (0.73), purple (0.66), grey (0.65)
GM 3.6.2	Dark blue (0.73), purple (0.66), grey (0.65)
GM 3.6.3	Sky blue (0.40), pink (0.32), with grey tailing
GM 3.6.4	Sky blue (0.40), brown (0.36), pink (0.30)
GM 3.6.5	Pink (0.30)
GM 3.6.6	Pink (0.30) with brown, blue overlapping

Solvent system: 100% $CHCl_3$

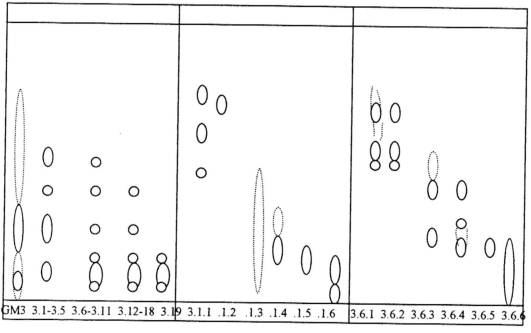


Figure 4.5: TLC patterns obtained from chromatographic separation of GM 3 (Fr 12-13)

Table 4.6: R_f values and colours obtained from TLC plates (Figure 4.6)

Fractions	Colour (R_f value)/ comment
GM 4.1	Dark blue (0.89), purple (0.68), grey (0.56)
GM 4.2	Dark blue (0.89), purple (0.68), grey tailing
GM 4.3	Purple (0.69)
GM 4.4	Grey (0.60), with short tailing
GM 5.1	Purple (0.81), blue (0.76), dark blue, green overlapping, dark blue (0.43), brown/ grey tailing, yellow (0.10)
GM 5.2	Purple (0.81), blue (0.76), dark blue, green overlapping, dark blue (0.43), pink (0.32) brown/ grey tailing, yellow (0.10)
GM 5.3	Green (0.54), dark blue (0.43) yellow (0.10)
GM 5.4	Dark blue (0.43)

Solvent system: 1:9, MeOH:$CHCl_3$

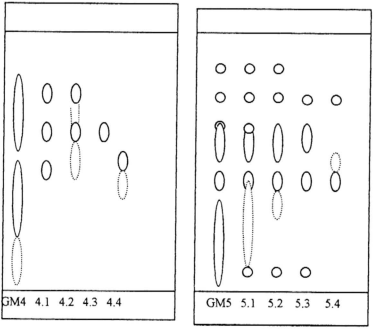


Figure 4.6: TLC patterns obtained from chromatographic separation of GM 4 (Fr 14-15) and GM 5 (Fr16)

Table 4.7: R_f values and colours obtained from TLC plates (Figure 4.7)

Fractions	Colour (Rf value)/ comment
Fr 1	Dark blue (0.75), purple (0.63) with grey tailing
Fr 2	Dark blue (0.75), blue (0.71), purple (0.63)
Fr 3	Dark blue (0.75), blue (0.71), purple (0.63), grey (0.56)
Fr 4	Purple (0.63), with fade green
Fr 5	Green (0.52), with blue tailing
Fr 6	Green (0.56), blue (0.53)
Fr 7	Green and pink overlapping (0.48), dark blue (0.42), yellow (0.21)
Fr 8	Fade pink with yellow (0.18)
Fr 9	Brown (0.06)

Solvent system: 1:9, MeOH:$CHCl_3$

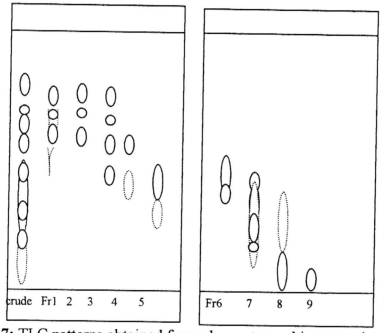


Figure 4.7: TLC patterns obtained from chromatographic separation of methanol crude extract

Fractions	Colour (R_f value)	Solvent system
GM 1.1	Purple (0.69)	1:1, hex:$CHCl_3$
GM 2.2	Pink (0.71)	1:1, hex:$CHCl_3$
GM 3.6.5	Pink (0.32)	100% $CHCl_3$
GM 4.3	Purple (0.69)	1:9, MeOH:$CHCl_3$

In addition to TLC, ^1H NMR was also recorded for each fraction collected to further confirm the content and purity of the terpenoids. All fractions showed terpenoids-like ^1H NMR spectrum, whereby the region between 0 ppm to approximately 2.5 ppm showed continuous peak. (Appendix III). This indicates that the methyl (CH_3), methylene (CH_2) and methine (CH) protons are all present in close relation to each other.

Isolation of compound 1 (GM1.1)

Fractions 3-6 from the first column chromatography were combined (named GM 1) and rechromatographed by slowly increasing the polarity of the chloroform. Six fractions were collected and analyzed by TLC. **Compound 1** (GM 1.1) was eluted with 5% chloroform in hexane and was observed as one bright purple spot at R_f value of 0.69 (in 1:1, hex:$CHCl_3$) on TLC plate (Figure 4.4). GM 1.2 and GM 1.3, which appeared as two spots of same colours (purple and sky blue) and R_f values (0.69 and 0.62 respectively) (Table 4.4 and Figure 4.4) were combined and rechromatographed to isolate more of GM1.1 for spectroscopic studies. The final yield obtained was approximately 40 mg (0.002% w/w)

Isolation of Compound 2 (GM 2.2)

Fractions 10-11 were combined (named GM 2) and rechromatographed by slowly increasing the polarity of chloroform. **Compound 2** (GM 2.2) was eluted at 10-20% of chloroform in hexane and observed as a pink spot with R_f value of 0.71 (in 1:1, hex:$CHCl_3$) on TLC plate. The yield obtained was approximately 50 mg (0.0025% w/w)

Isolation of Compound 3 (GM 3.6.5)

Fractions 12-13 were combined and rechromatographed using chloroform/ methanol solvent system with increasing methanol gradient. 19 fractions were obtained due to overloading of the column. These fractions were combined accordingly to their R_f values to give four subsequent fractions. From the first fraction (GM 3.1-3.5), **compound 2** was again obtained.

The second fraction (GM 3.6-3.11) was chromatographed on silica gel using hexane/ chloroform solvent system, with increasing the chloroform gradient slowly. From this column, **compound 3** (GM 3.6.5) was eluted at 60-70% of chloroform in hexane. It appeared as a pinkish spot with R_f value of 0.32 (in 100% $CHCl_3$) on the TLC plate. The yield obtained was approximately 80 mg (0.004% w/w)

Isolation of Compound 4 (GM 4.3)

Compound 4 (GM 4.3) was isolated from combined primary fraction of 14-15 (GM 4). It was eluted at 10-20% methanol in chloroform. The compound showed a purple colour spot, with R_f value of 0.38 (in 1:9, MeOH:$CHCl_3$) on TLC plate. The yield obtained was approximately 500 mg (0.025% w/w)

4.3.3 Characterization of Isolated Compounds

A new brominated triterpene, together with three known compounds were isolated from the hexane crude extract of the fruit body of *Ganoderma tsugae*. The structures of the isolated compounds were determined by spectroscopic means, including 2D-NMR. Three of the compounds (GM 1.1, GM 3.65, GM 4.3) were having the ergosta-type skeleton, while the other (GM 2.2) was from the phthalate family.

Compound 2 (GM 2.2)

GM 2.2 was the first compound to be characterized. It was obtained as pale yellow crystalline needles. The electron impact mass spectroscopy (EIMS) (Figure 4.8) showed the presence of molecular ion peak at m/z 390, corresponding to the formula $C_{24}H_{38}O_4$. The major fragments showed were 279, 261, 149, 113, 71 and 57. These fragments, especially the 279 and 149 peaks, were characteristic fragmentation pattern of di-(2-ethylhexyl) phthalate (DEHP) (Cohen *et al.*, 1991).

^{1}H NMR (Figure 4.9) and ^{13}C NMR (Figure 4.10) for GM 2.2 were in accordance with the standard DEHP and reported data (Cohen *et al.*, 1991). The ^{1}H NMR showed that the molecule has a plane symmetry, with two methine protons (δ 7.70 and δ 7.52), exhibiting the AA'BB' system typical of a phthalate. The chemical shift at 4.22 ppm is the AB part of the ABX pattern formed by the three protons in the O-CH_2-CH group, giving rise to a multiplet in ^{1}H NMR (Figure 4.9).

The ^{13}C NMR (Figure 4.10) indicated only 12 carbons, with very high intensity peaks. This is because the molecule has a plane symmetry, giving rise to overlapping carbon signals. The carbonyl carbon was furthest down field, which was observed at δ 167.64. A quaternary carbon and two methine carbons also appeared in low field region with δ 132.42, 130.78 and 128.72 respectively. The methylene carbon attached to oxygen showed a peak at δ 68.08. All of these carbon resonances correlated well to the standard and reported data DEHP (Cohen *et al.*, 1991). Table 4.9 and Table 4.10 summarizes the data for ^{1}H NMR and ^{13}C NMR and DEPT (Figure 4.11) spectrum respectively.

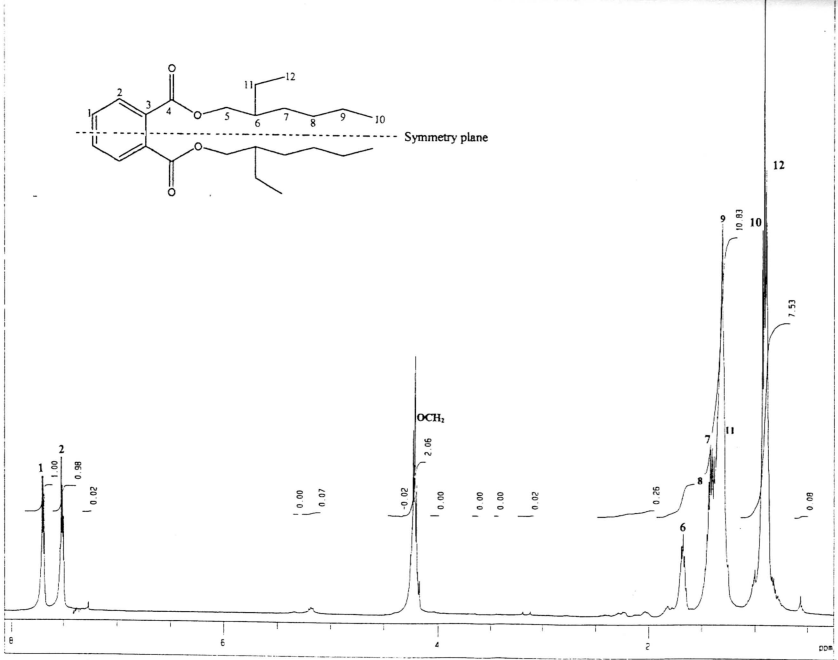
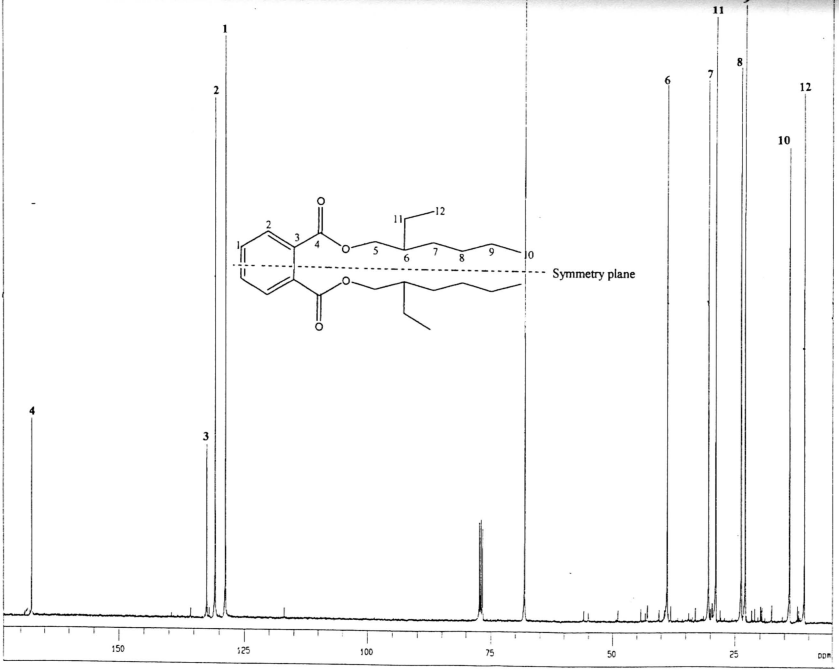


Figure 4.9: ^{1}H NMR spectrum of DEHP (GM 2.2)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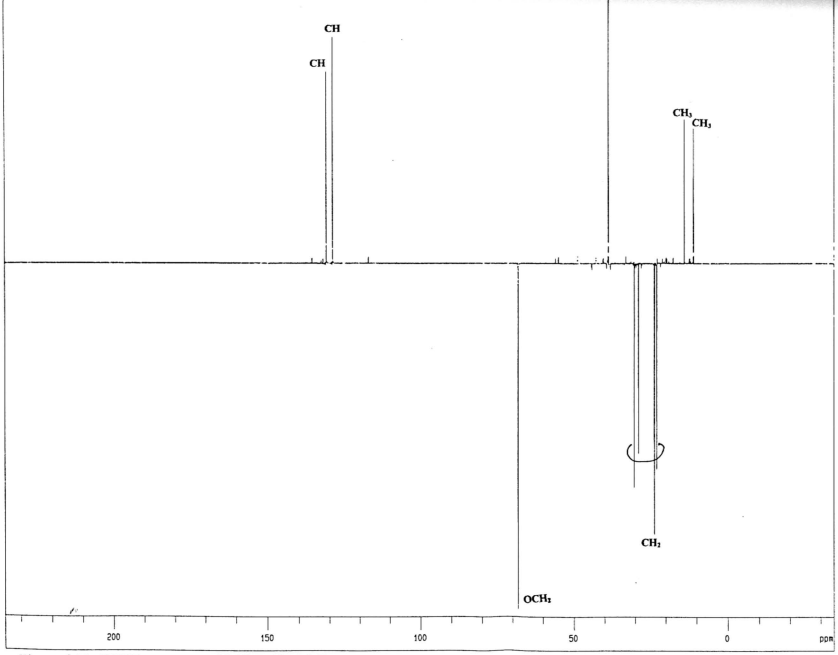


Figure 4.11: DEPT spectrum of DEHP (GM 2.2)

Table 4.9: ^{1}H NMR (400 MHz) spectral data for DEHP (GM 2.2) in $CDCl_3$

Position	δH (ppm)/ Multiplicity	Reported data (Cohen *et al.*, 1991)
1	7.71 /dd*	7.70
2	7.51 /dd	7.53
3	-	-
4	-	-
5	4.22 /m	4.22
6	1.61 /m	1.41
7	1.45 /dt	1.50
8	1.48 /m	1.51
9	1.26 /m	1.27
10	0.93 /t	0.92
11	1.24 /m	1.25
12	0.86 /t	0.85

* dd= double doublet, d= doublet, t= triplet, m= multiplet.

Table 4.10: ^{13}C NMR (400 MHz) and DEPT spectral data for DEHP (GM 2.2) in $CDCl_3$

Position	δC (ppm)	Reported data (Cohen *et al.*, 1991)	DEPT
1	128.70	128.89	CH
2	130.78	130.89	CH
3	132.43	132.66	C
4	167.65	167.75	COO
5	68.06	68.28	OCH_2
6	38.70	38.94	CH
7	30.32	30.54	CH_2
8	28.87	23.94	CH_2
9	22.90	23.06	CH_2
10	13.95	14.02	CH_3
11	28.87	29.06	CH_2
12	10.87	11.04	CH_3

In addition, COSY (Figure 4.12) and HETCHOR (Figure 4.13) were also included to further confirm the structure. The ^{1}H-^{1}H COSY enables the proton linkage of H1 to H2 to be observed. The linkage was terminated by a quatanary carbon at C3. The proton-proton connectivities resumed from H5 to H6, H6 to H11, H12 and H7, H8, H9 and H10 (Figure 4.12).

The HETCOR spectrum (Figure 4.13) showed the linkage of carbon to proton signals. As can be seen from the spectrum, linkage of carbon (C1) to proton (H1), C2 to H2, C5 to OCH_2 (5), C6 to H6 and so on. This step by step elucidation of the two-dimensional NMR signals further confirmed the establishment of the DEHP structure.

This is the first time that DEHP is being isolated from the *Ganoderma* spp. Di-(2-ethylhexyl) phthalate (DEHP) is the most widely used plasticizer (comprising 50% of all phthalate ester plasticizers) that softens resins. It is used for making the polyvinyl chloride (PVC) utilized in building, construction and packaging, and for medical device components. Smaller amounts are used in industrial paints and as dielectric fluid in condensers (IPCS, 1992).

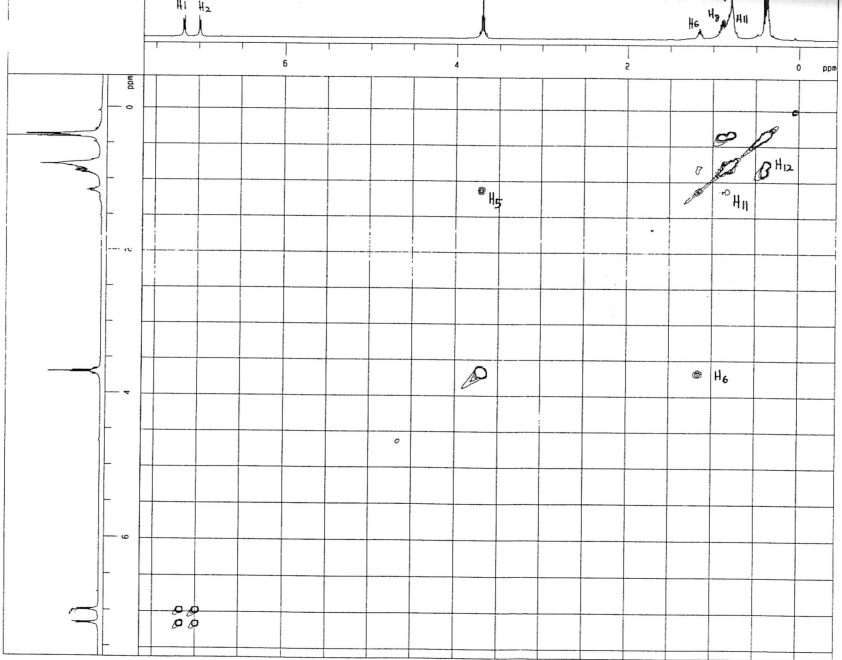


Figure 4.12: COSY spectrum of DEHP (GM 2.2)

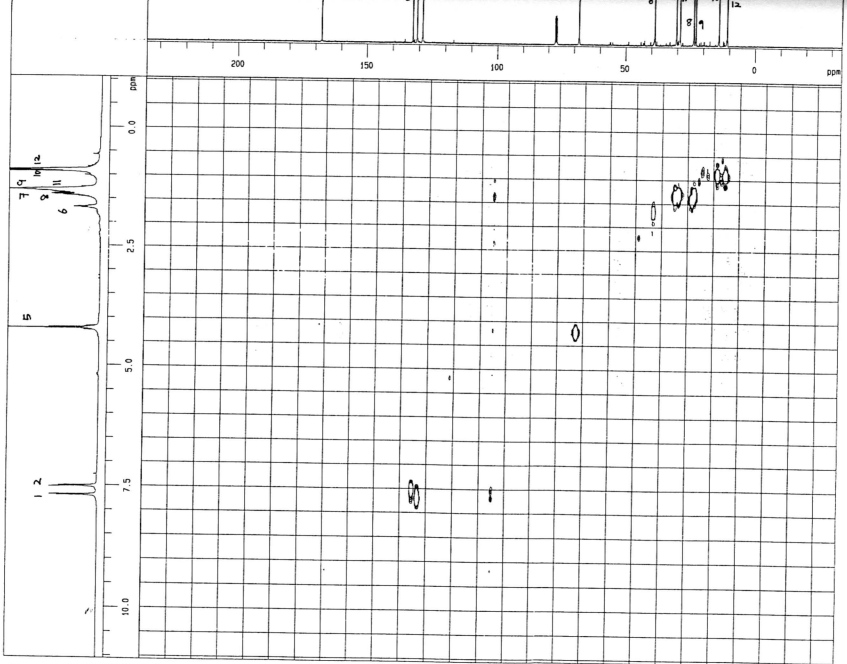


Figure 4.13: HETCHOR spectrum of DEHP (GM 2.2)

Di-(2-ethylhexyl) phthalate (DEHP) exists widely in the environment and have been reported in wide variety of substances (oil, soil, plants, and animals) (IPCS, 1992). Most occurrences have anthropogenic origins but some could be of natural origin (Mathur, 1974a, b). The nature of origin is further complicated by the fact that sampling techniques often lead to contamination of samples via contamination from plastic bags or bottles.

Mathur (1974a) critically reviewed the origin of phthalates and concluded that the possibility of the phthalic acid esters (PAEs) found in biological and geochemical samples being of biosynthetic origin cannot be ruled out. A study was conducted based on the assumption that if PAEs occur naturally, the microorganisms would utilize it during their normal role in the cyclization of carbon in nature. The results showed that PAEs were being used by soil and water microorganisms after 14 weeks (Mathur, 1974b).

On the other hand, the anthropogenic origin of DEHP found in this mushroom may be postulated coming from the plastic bag. During pasteurization of the substrate, the temperature is brought up to 80°C for 6 to 8 hours. Such long period of heating may resulted in the dissociation of DEHP from the plastic bags. Since DEHP has a high octanol-water partition coefficient, the equilibrium between water and an organic rich substrate is in favour of the substrate (IPCS, 1992). Furthermore, DEHP has been known to be present in high concentration, with approximately 40% (w/w) or more in plastic (IPCS, 1992). Thus, it is possible that the DEHP dissociated from the plastic polymer during pasteurization and adsorbed onto the organic substrate.

The *Ganoderma* spp. belonging to the Basidiomycetes has long known to bioaccumulate substances from their natural environment (Hudson, 1985). If DEHP is adsorbed onto the substrate, uptake of this compound is possible during the four to six weeks of cultivation. Many reported literatures found that a variety of living organisms such as insect, fish, animals are also able to bioaccumulate the DEHP (IPCS, 1992). This is partly because the degradation of DEHP is found to be low due to its long chain of hydrocarbon. Only 40-90% of DEHP is degraded after 10-35 days incubation with various bacteria and fungi under aerobic condition (ECETOC, 1985).

The concentration of DEHP in biota has been reported varies from less than 1 to 7000 μg/kg (IPCS, 1992). In this study, DEHP was estimated to be approximately 0.0025% w/w (or 25 mg/kg). DEHP has been found in various type of food such as fish, shell fish, eggs and cheese. In a study by Antonyuk (1975), DEHP migration from PVC materials into foodstuffs was noted following 7 days of contact. Levels of 4-16 mg DEHP /kg were detected in cheese, sausage, meat, flour, and rice while after 30 days levels of 30-150 mg/kg were found in sunflower oil.

The permissible level of DEHP migration to foodstuffs was considered to be 2.0 mg/kg (IPCS, 1992). Hence, the current finding showed that in this batch of *Ganoderma*, the level of DEHP has exceeded the permissible level. Limited information is available on the effects of DEHP on humans. Only mild gastric disturbances has so far been reported, but no other deleterious effects were reported

for two subjects given in 5 g to 10 g of DEHP (IPCS, 1992). However, DEHP has been well established to cause reproductive and hepatocarcinogenic effects in rats and mice (Kluwe *et al.*, 1982; Rao *et al.*, 1990). Currently, there is no sufficient evidence to suggest that DEHP is a potential human carcinogen (IPCS, 1992).

The above postulation of the source of DEHP is not yet conclusive. Further investigation should be carried out into the detection of DEHP in the substrate. Since only one batch of sample was used in this study, the possibility of one-batch off contamination cannot also be ruled out. Hence, without further confirmation of the presence of the DEHP from the substrate and few other batches or other bags of *Ganoderma* from other places of cultivation, this result should not be used against the mushroom industry as a whole.

Compound 4 (GM4.3)

GM 4.3 was obtained as white crystalline needles and identified as ergosta-5,7,22E,trien-3β-ol or 24R-methylcholesta-5,7,22-trien-3β-ol (ergosterol, provitamin D_2) by comparing the spectral data with that reported data (Kac *et al.*, 1984, Huang *et al.*, 1985). The EIMS (Figure 4.14) showed the presence of molecular ion peak at m/z 396 corresponding to the formula $C_{28}H_{44}O$. The major fragment peaks were 376, 363, 337, 300, 271 and 213. The fragment peak at m/z 337 is characteristic of $\Delta^{5,7}$-sterols (Yokokawa and Mitsuhashi, 1981).

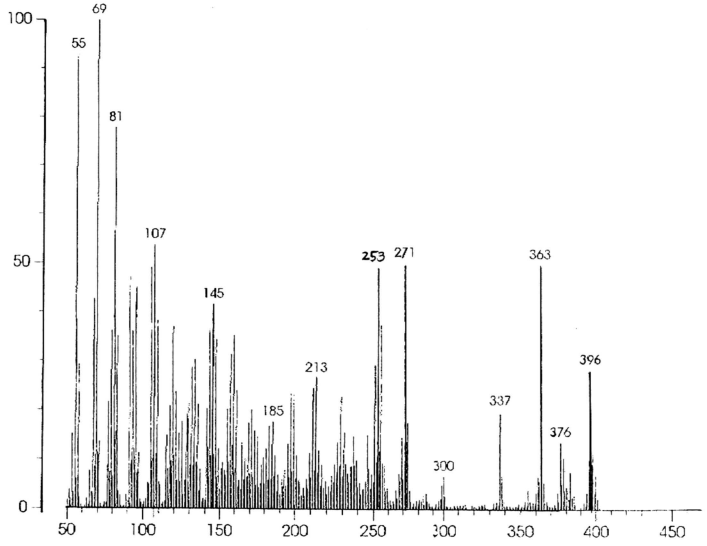


Figure 4.14: EIMS spectrum of ergosterol (GM 4.3)

The ^{1}H NMR (Figure 4.15a, b and Table 4.11) spectrum showed a multiple methyl peaks, with signals δ 0.63 (3H, C-18), 0.94 (3H, C-19), 1.05 (3H, C-21), 0.88 (3H, C-26), 0.86 (3H, C-27), and 0.91 (3H, C-28). Two double doublets at δ 5.57 and 5.38 attributable to two olefinic protons at C-6 and C-7 respectively. The other two olefinic protons at C-22 and C-23 on the chain showed a multiplet at δ 5.16. The C<u>H</u>-OH methine proton also displayed a multiplet at δ 3.62.

From the ^{13}C NMR (Figure 4.16 and Table 4.12) and DEPT (Figure 4.17) experiments, six methyl carbons, 7 methlyene carbons and 11 methine carbons were observed. Two quaternary carbons at C-5 and C-8 were displayed at δ 139.81 and 140.81 respectively. In addition, four methine carbons were also observed at low field, with δ 135.69 (C-22), 131.91 (C-23), 119.61 (C-6), and 116.31 (C-7). These results suggested that the molecule have three double bonds, which further confirmed that GM 4.3 was ergosterol (provitamin-D_2).

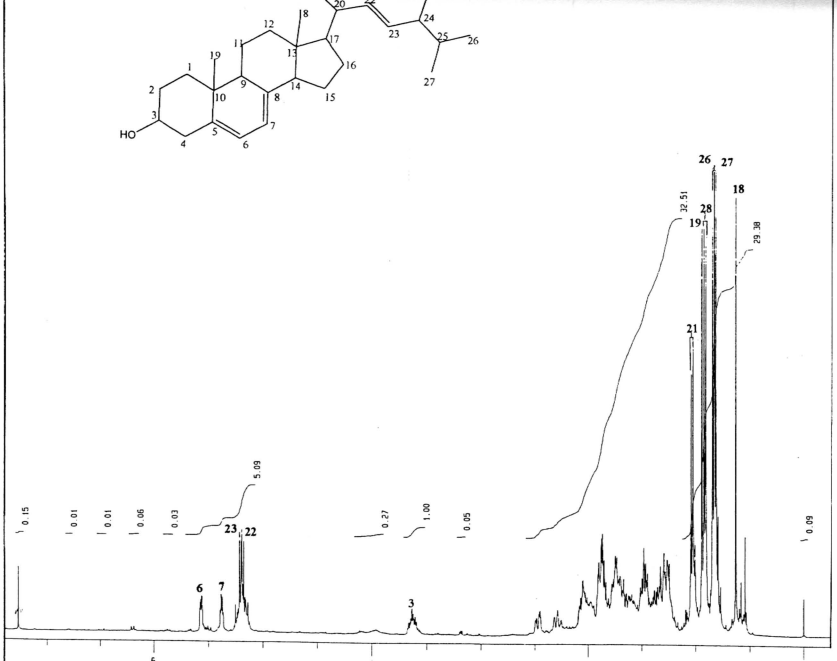


Figure 4.15a: ^{1}H NMR spectrum of ergosterol (GM 4.3)

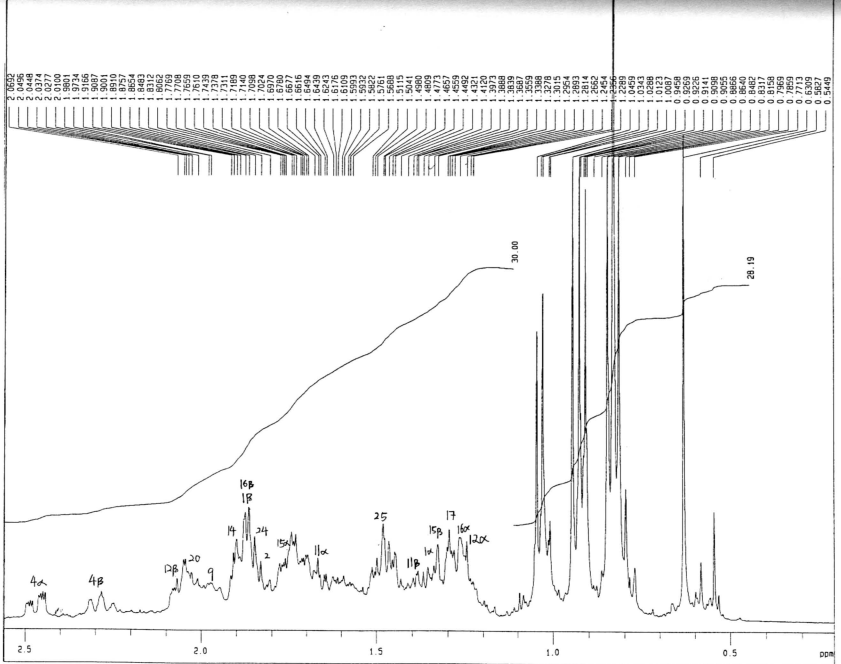


Figure 4.15b: Expanded section of ^{1}H NMR spectrum of ergosterol (GM 4.3)

Table 4.11: ^{1}H NMR (400 MHz) spectral data for ergosterol (GM 4.3) in $CDCl_3$

Position	δH (ppm)/ Multiplicity	Reported data (Perrier and McGlinchey, 1985)
1α	1.36 /t*	1.35
1β	1.89 /t	1.87
2α,β	1.90 /dt	1.90
3	3.62 /m	4.50
4α	2.48 /dd	2.50
4β	2.29 /t	2.35
5	-	-
6	5.57 /d	5.54
7	5.38 /d	5.35
8	-	-
9	1.97 /t	2.02
10	-	-
11α	1.66 /dt	1.68
11β	1.41 /dt	1.50
12α	1.28 /t	1.23
12β	2.08 /t	2.08
13	-	-
14	1.92 /t	1.88
15α	1.69 /dt	1.60
15β	1.35 /dt	1.37
16α	1.25 /dt	1.24
16β	1.87 /dt*	1.74
17	1.30 /dt	1.31
18	0.63 /s	0.68
19	0.94 /m	0.92
20	2.04 /m	2.05
21	1.05 /d	1.11
22	5.16 /m	5.18
23	5.18 /m	5.21
24	1.62 /m	1.80
25	1.48 /m	1.48
26	0.88 /d	0.82
27	0.86 /d	0.81
28	0.91 /d	0.92

* t= triplet, dd= double doublet, d= doublet, m= multiplet

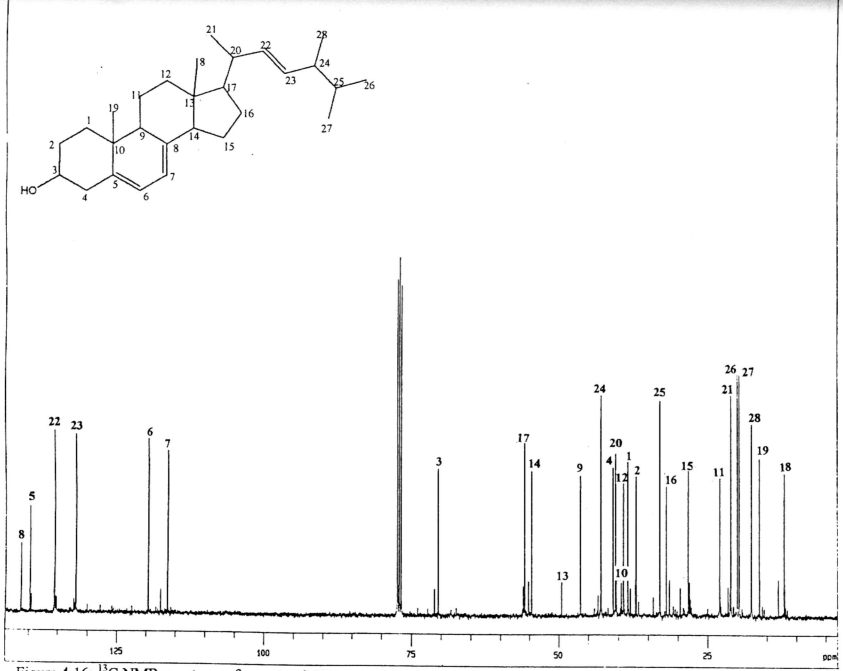


Figure 4.16: ^{13}C NMR spectrum of ergosterol (GM 4.3)

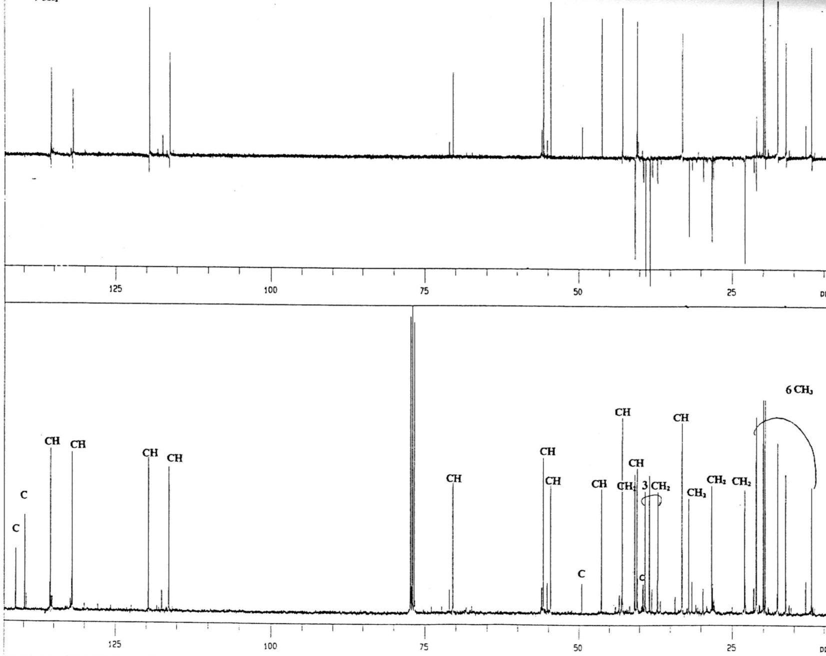


Figure 4.17: DEPT spectrum of ergosterol (GM 4.3)

Table 4.12: ^{13}C NMR (400 MHz) and DEPT spectral data for ergosterol (GM 4.3) in $CDCl_3$

Position	δC (ppm)	Reported data (Seo *et al.*, 1988)	DEPT
1	38.40	38.33	CH_2
2	37.05	31.75	CH_2
3	70.47	69.98	CHOH
4	139.8	40.54	CH_2
5	140.81	139.52	C
6	119.60	119.09	CH
7	116.31	115.87	CH
8	141.36	140.67	C
9	46.28	46.21	CH
10	39.48	37.00	C
11	23.01	21.14	CH_2
12	39.12	39.06	CH_2
13	49.49	42.74	C
14	54.57	54.42	CH
15	28.29	22.99	CH_2
16	31.49	28.19	CH_2
17	55.77	55.67	CH
18	12.10	12.06	CH_3
19	16.30	16.27	CH_3
20	40.29	40.24	CH
21	21.12	21.07	CH_3
22	135.69	135.03	CH
23	131.91	131.81	CH
24	43.32	42.68	CH
25	33.11	33.04	CH
26	19.96	19.89	CH_3
27	19.66	19.61	CH_3
28	17.61	17.57	CH_3

The 2D NMR such as HETCHOR (Figure 4.18 a, b) and COSY (Figure 4.19) allowed most of the assignment of proton signals. Figure 4.18 a, b illustrated the result of ^{1}H-^{13}C shift presented as contours. Methine carbons, such as C-14, exhibit a single peak while methylene carbons, such as C-4 show correlations to both of the protons (α and β) attached to the same carbon atom (Figure 4.18a).

The ^{1}H assignments were further determined using the COSY experiment, which allowed the ^{1}H-^{1}H connectivities to be established through scalar coupling between protons (Breitmaier, 1993). However, the COSY experiment does not directly distinguish between α and β protons in a given methylene group (Perrier and McGlinchey, 1988).

Since NOE difference spectroscopy measurement was not made, the α and β protons presented in this compound (Table 4.11) were distinguished by comparing with the reported literature (Perrier and McGlinchey, 1988).

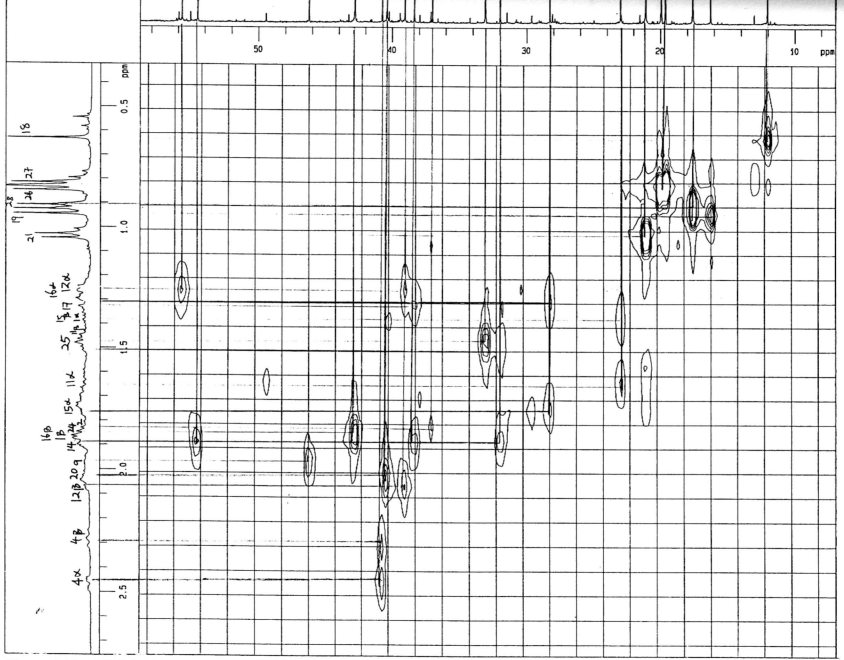


Figure 4.18a: HETCHOR spectrum of ergosterol (GM 4.3)

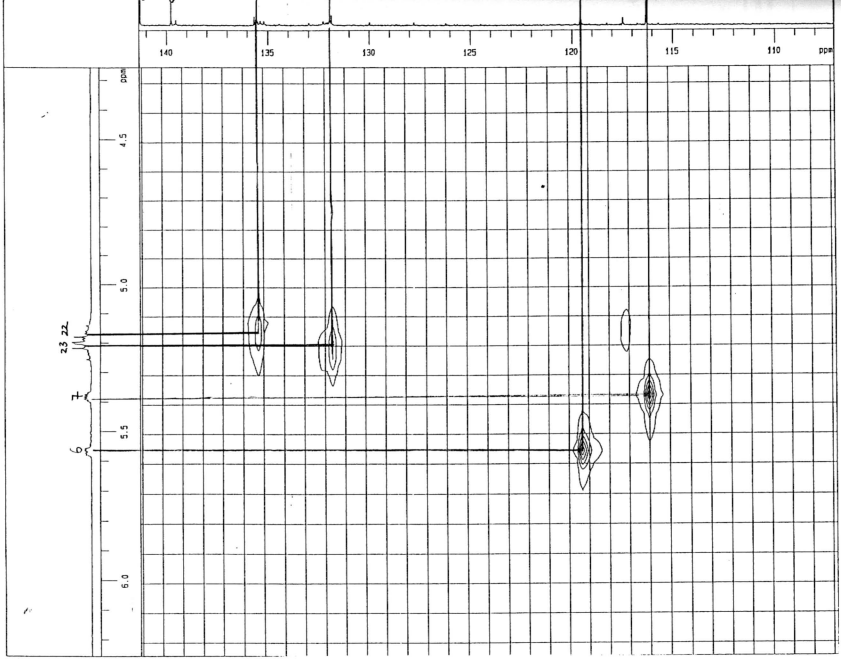


Figure 4.18b: HETCHOR spectrum of ergosterol (GM 4.3)

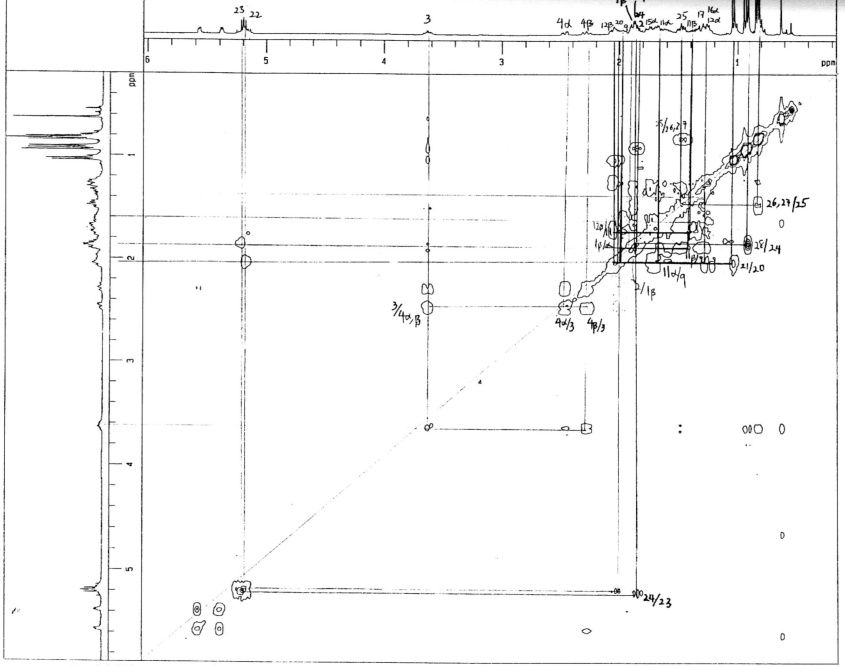


Figure 4.19: COSY spectrum of ergosterol (GM 4.3)

Ergosterol has been known as a major sterol in Basidiomyctes (Takaishi *et al.*, 1989). In this study, the yield of ergosterol obtained from *G. tsugae* was approximately 0.025% (w/w). This concentration is slightly lowered that the concentrations reported in *G. lucidum*, which was 0.3-0.4% (Kac *et al.*, 1984). The chemistry of ergosterol has received much attention because it is a precursor of vitamin D_2 (Harborne, 1973).

Ergosterol differs from cholesterol (which is the major sterol of the plasma membranes of most animals) by being methylated at C-24 of the side chain. A consequence of having ergosterol as opposed to cholesterol in membranes is increased fluidity (Griffin, 1994). The high concentration of ergosterol found in *G. tsugae* may play a role in its adaptability of higher temperature range (33-40°C) as compared to *G. lucidum* in the previous study (Figure 3.1). However, further investigation need to be made to confirm this observation.

Compound 3 (GM 3.6.5)

GM 3.6.5 was obtained as white crystalline. The EIMS (Figure 4.20a, b) gave an exact mass of 398 corresponding to the formula $C_{28}H_{46}O$. Other fragment ions at m/z 397, 382, 354, 270, 245 and 147 were also observed. This pattern of fragmentation was in good agreement with that of ergosta-7,22-dien-3-ol or 24-

methylcholesta-7,22-dien-3-ol (stellasterol) (Yokokawa and Mitsuhashi, 1981, Kac *et al.*, 1984), except the slight different of one mass unit. The compound can easily lost a proton ion at the initial bombardment by a bean of electrons. Further fragmentation of 397 to 382, 354, 270, 245 and 147 were consistent with the structure. The marked intensity of ion peak at m/z 270 (271) corroborates the Δ^7 double bond (Yokokawa and Mitsuhashi, 1981). Furthermore, the absence of ions at m/z 129 excludes the possibility of Δ^5-unsaturation (Takaishi *et al.*, 1989).

^{1}H NMR (Figure 4.21) and ^{13}C NMR (Figure 4.22) showed similar pattern as ergosterol. The ^{1}H NMR showed signals of 18-Me (δ0.54, 3H, s), 19-Me (0.85, 3H, s), terminal dimethyl (0.83 and 0.82, 6H, d), 21-Me (1.00, 3H, d) and three olefinic protons at C-7 (δ 5.20, 1H, dt) and C-22 and C-23 (δ 5.18 and 5.16 2H, m). The C<u>H</u>-OH methine proton was observed at δ 3.60 (m), which was slightly upfield as compared to C-3 of ergosterol (δ 3.62) due to the absence of one double bond at C-5.

The ^{13}C NMR (Figure 4.22) and DEPT experiment (Figure 4.23) were in good agreement with that of 24-methylcholesta-7,22-dien-3β-ol, with 6 methyl carbons, 8 methylene carbons, 10 methine carbons and 3 quaternary carbons. The ^{1}H NMR and ^{13}C spectra data and assignments are shown in Table 4.13 and 4.14 respectively.

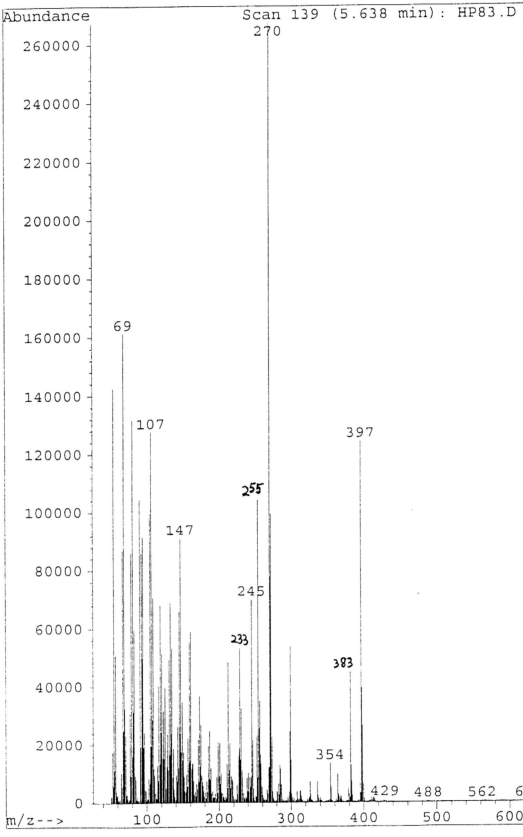


Figure 4.20a: EIMS spectrum of stellasterol (GM 3.6.5)

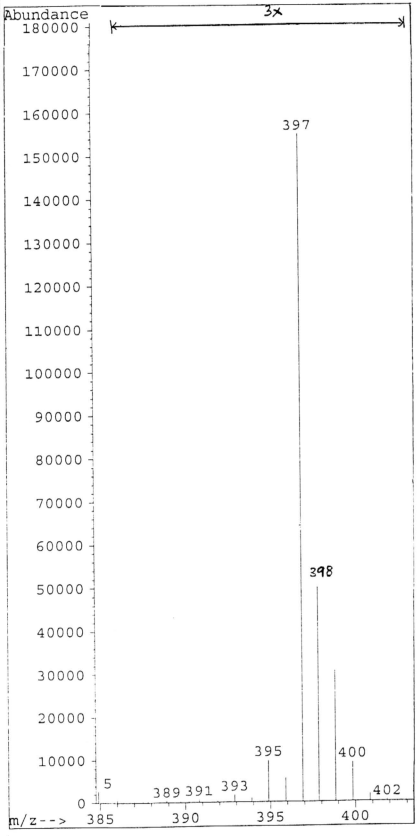


Figure 4.20b: EIMS spectrum of stellasterol (GM 3.6.5) (expanded)

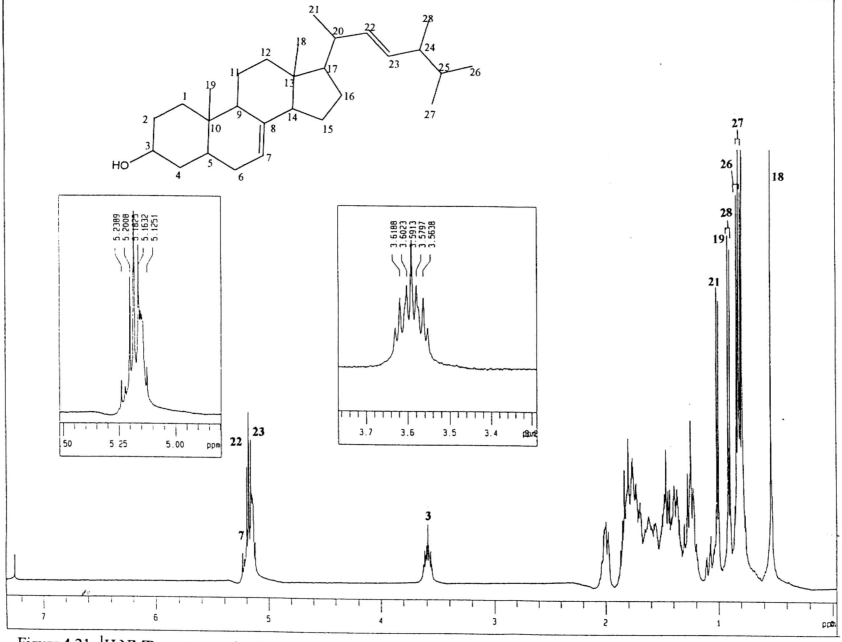


Figure 4.21: ^{1}H NMR spectrum of stellasterol (GM 3.6.5)

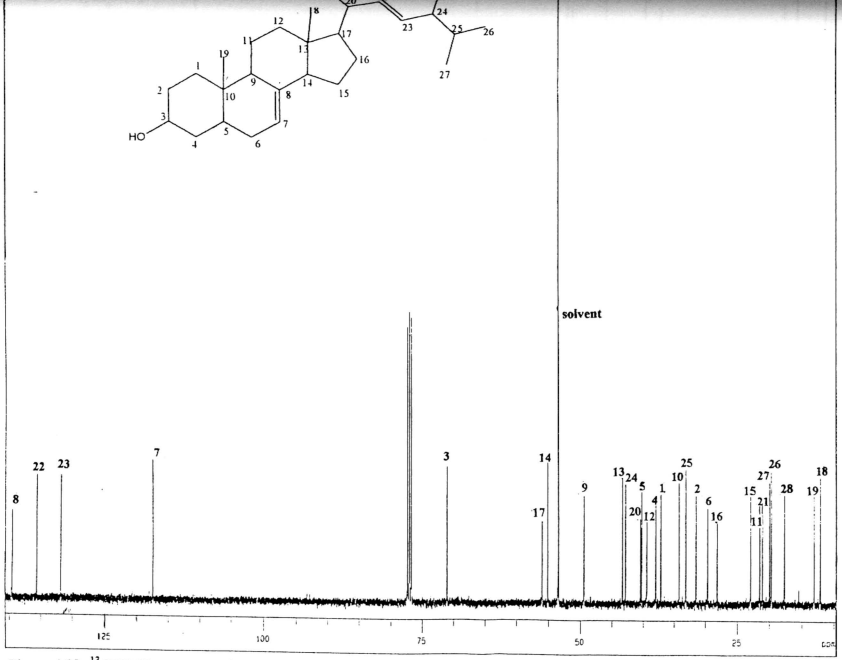


Figure 4.22: ^{13}C NMR spectrum of stellasterol (GM 3.6.5)

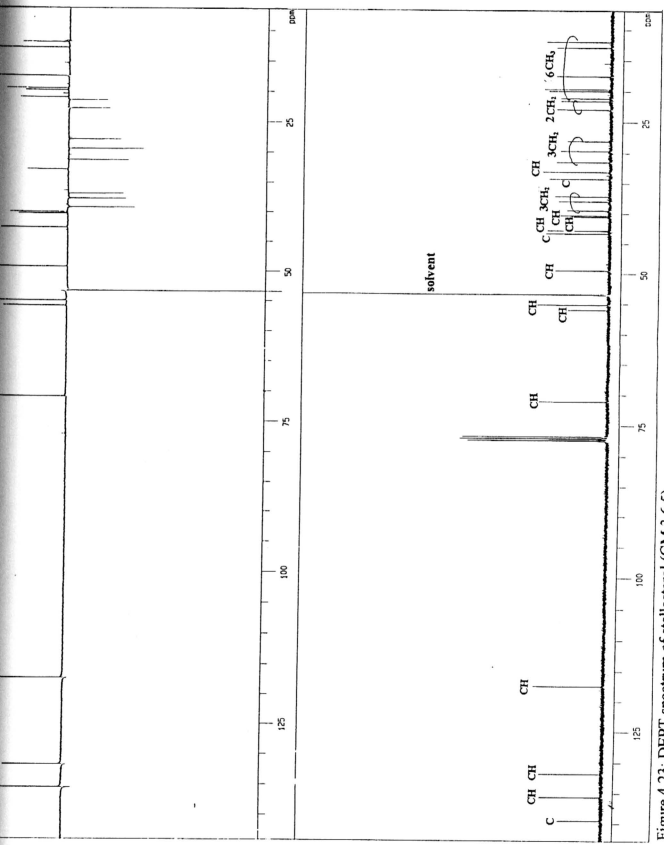


Figure 4.23: DEPT spectrum of stellasterol (GM 3.6.5)

Table 4.13: Partial ^{1}H chemical shifts of stellasterol (GM 3.6.5) in $CDCl_3$

Position	δH (ppm) /Multiplicity	Reported data (Lu *et al.*, 1985)
3	3.60 /m*	3.60
7	5.20 /dt	5.22
18	0.54 /s	0.55
19	0.85 /s	0.81
21	1.00 /d	1.02
22	5.18 /m	NA**
23	5.16 /m	NA
26	0.83 /d	0.84
27	0.82 /d	0.83
28	0.91 /d	0.91

*m= multiplet, dd= double doublet, d= doublet, t= triplet. **Not available

Table 4.14: ^{13}C NMR (400 MHz) and DEPT spectral data for stellasterol (GM 3.6.5) in $CDCl_3$

Position	δC (ppm)	Reported data (Takaishi *et al.*, 1989)	DEPT
1	37.09	37.7	CH_2
2	31.43	32.3	CH_2
3	71.00	70.3	CHOH
4	37.94	38.9	CH_2
5	40.22	40.7	C
6	29.59	30.1	CH_2
7	117.41	118.0	CH
8	139.81	139.6	C
9	49.41	49.8	CH
10	34.17	34.6	C
11	21.50	21.8	CH_2
12	39.41	39.7	CH_2
13	43.25	43.5	C
14	55.06	55.4	CH
15	22.88	23.3	CH_2
16	28.05	28.5	CH_2
17	55.92	56.2	CH
18	12.02	12.3	CH_3
19	12.97	13.2	CH_3
20	40.43	40.8	CH
21	21.05	21.4	CH_3
22	135.63	136.2	CH
23	131.83	132.1	CH
24	42.77	43.1	CH
25	33.05	33.3	CH
26	19.58	19.9	CH_3
27	19.88	20.1	CH_3
28	17.53	17.8	CH_3

Stellasterol has recently been reported as the major steroid in *G. lucidum*, while ergosterol and 24-methylcholesta-7-en-3β-ol are subcomponents (Kac *et al.*, 1984; Mizuno *et al.*, 1995). In this study, stellasterol was found to be approximately 0.004% (w/w). This concentration is far less than those ergosterol, 0.025%(w/w) reported earlier. The concentration of steroids will most probably be different depending on the stage and species of *Ganoderma* and the environment in which it has grown (Jong and Birmingham, 1992).

Compound 1 (GM 1.1)

GM 1.1 was obtained as white needles. The EIMS (Figure 4.25a, b) gave a parent ion of 639, corresponding to the proposed molecular formula $C_{38}H_{55}O_3Br$. Bromine was detected from the mass spectroscopy due to its characteristic isotopic abundance effect. Further confirmation of the isotopic effect was observed in Figure 4.25c when **compound 1** was again subjected to mass spectroscopy.

Characteristic fragment peak at 509, 495, 380, 365, 337, 282 and 253 corresponds well with the reported ergosta-type structure. The fragment ion at m/z 337 and 253 observed in ergosterol was also found in GM 1.1. These fragments are characteristic of the $\Delta^{5,7}$-sterols (Yokokawa and Mitsuhashi, 1981).

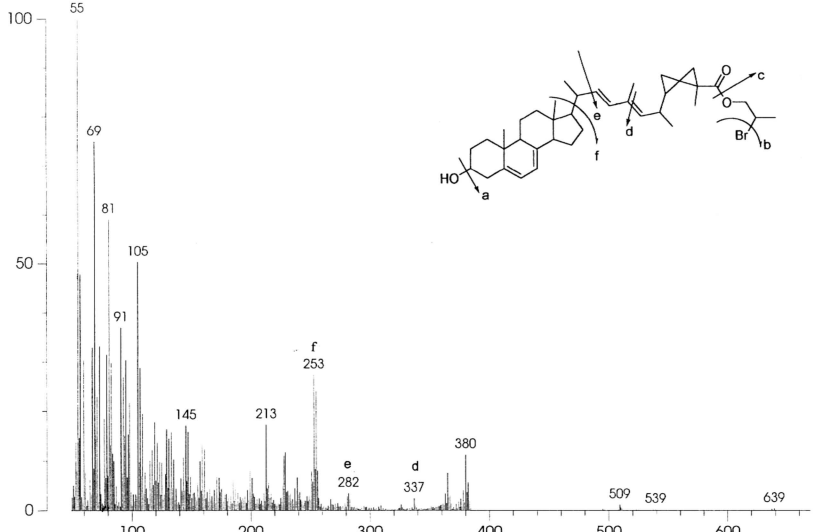


Figure 4.25a: EIMS spectrum of **compound 1** (GM 1.1)

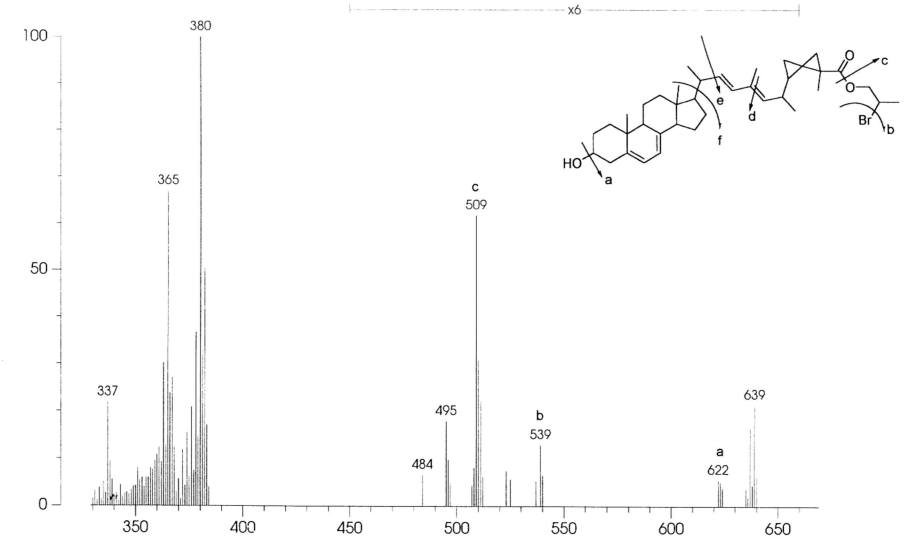


Figure 4.25b: EIMS spectrum of **compound 1** (GM 1.1)(expanded)

CHC0004 Scan 202 RT=3:32 100%=138976 mv 12-Apr-1999 09:36
HRP +EI GL 1.1 EI(70) LRP 0.7 S/D

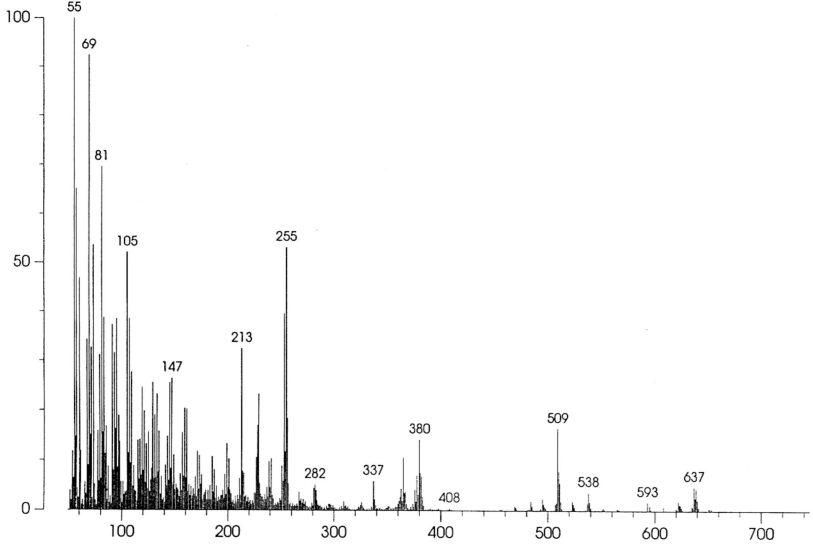


Figure 4.25c: Confirmation of EIMS spectrum of **compound 1** (GM 1.1) (repeat run)

Comparison of the ^{1}H-NMR (Figure 4.26) spectrum with that of ergosterol indicated the presence of hydroxyl group at C-3. However, due to the presence of extra two double bonds in the long chain, the methine protons attaching to the OH-group was further shifted down field, giving δ 4.73 as compared to ergosterol (δ 3.62)

The non-polar nature of this compound corresponded well with the number of double bonds present in the molecule. The ^{13}C-NMR (Figure 4.27) and DEPT experiment (Figure 4.28a, b, c) afforded 5 methine protons and 3 quaternary carbons in the low field region, which may be corresponded to the two double in the ring (at C-5 and C-7) and two double bonds in the long chain. An ester group was also observed at the lower field giving δ 173.52.

Overall, 7 methyl carbons (CH_3), about 10 methylene carbons (CH_2), 13 methine carbons (CH), and 8 quantenary carbons (C) were observed from the ^{13}C-NMR (Figure 4.27) and DEPT spectrum (Figure 4.28a, b, c). Based on all these spectroscopic data, the structure was tentatively proposed as shown in Figure 4.29. The mass fragmentation of this proposed structure agrees well with the EIMS spectrum (Figure 4.25 a, b).

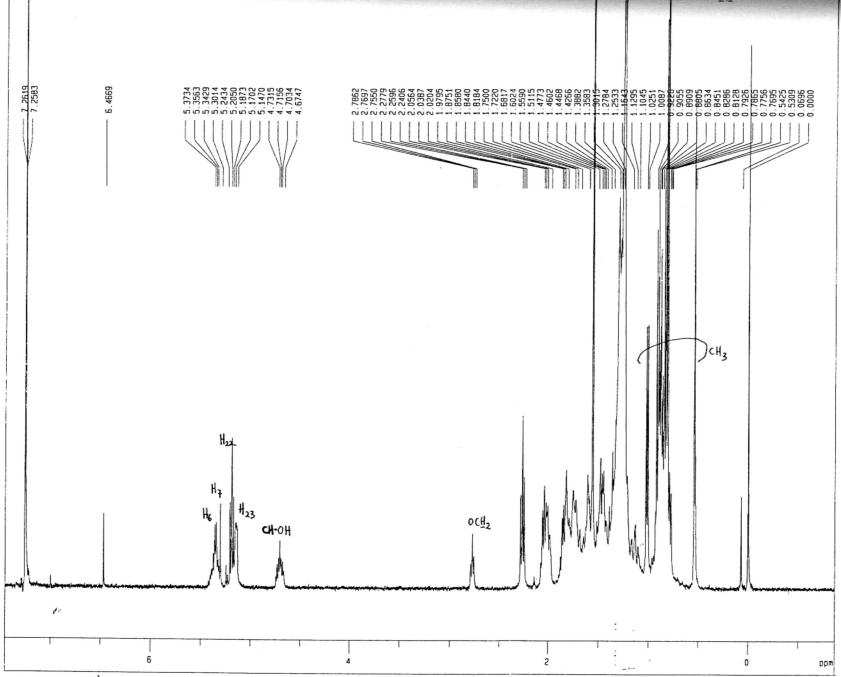


Figure 4.26: ^{1}H NMR spectrum of compound **1** (GM-1-1)

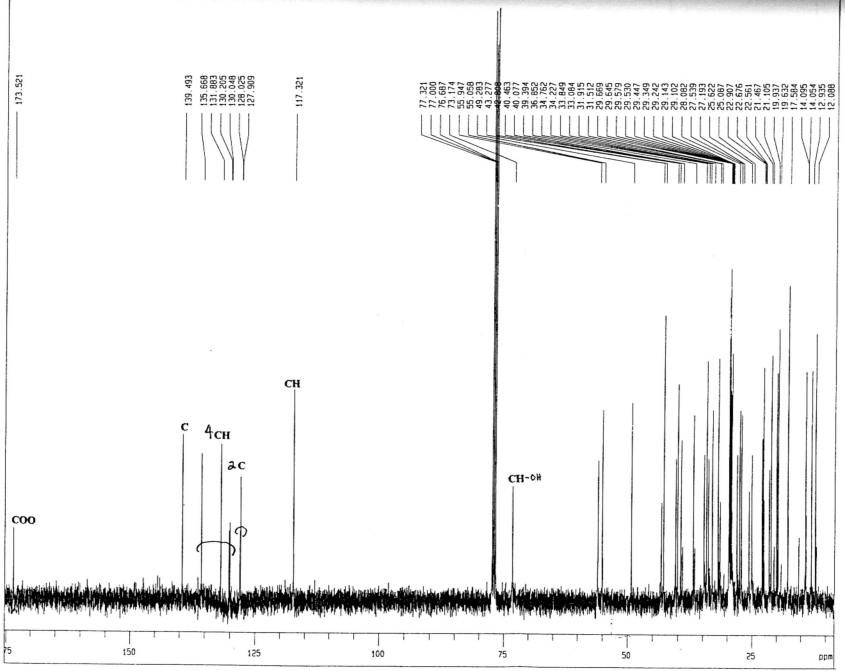


Figure 4.27: ^{13}C NMR spectrum of compound 11 (GM 1.1)

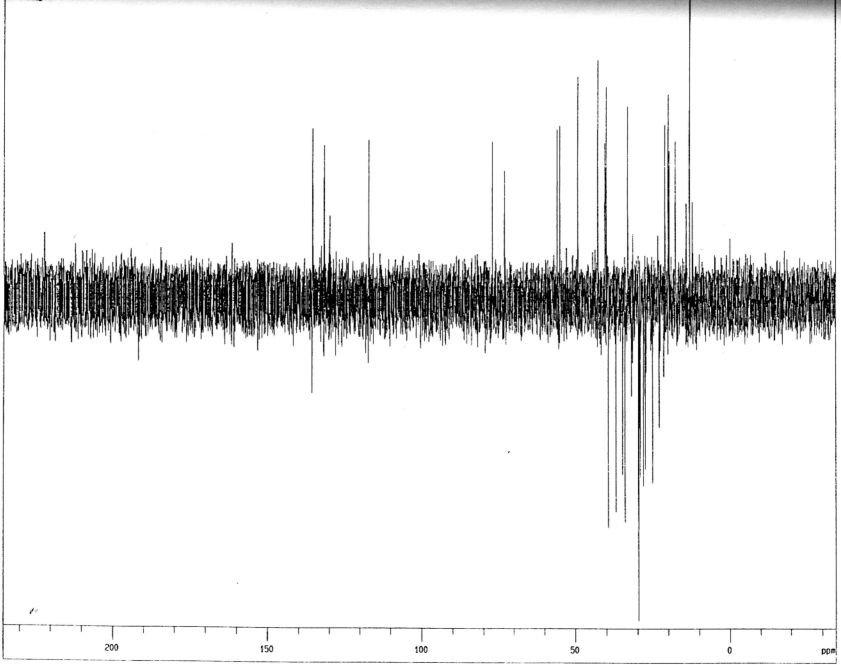


Figure 4.28a: DEPT spectrum of **compound 1** (GM 1.1)

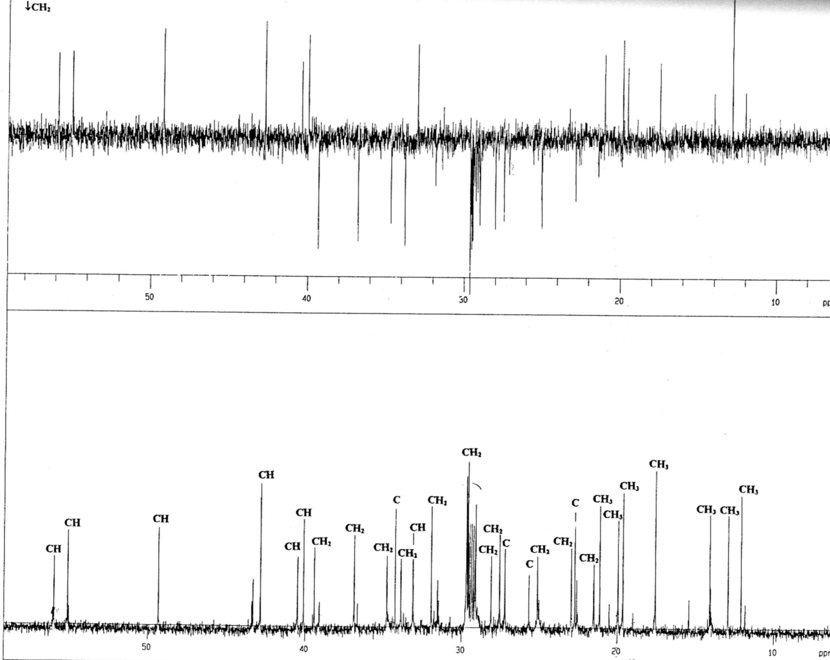
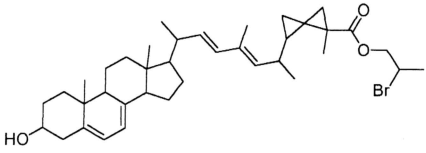


Figure 4.28c: DEPT spectrum of **compound 1** (GM 1.1) (expanded)

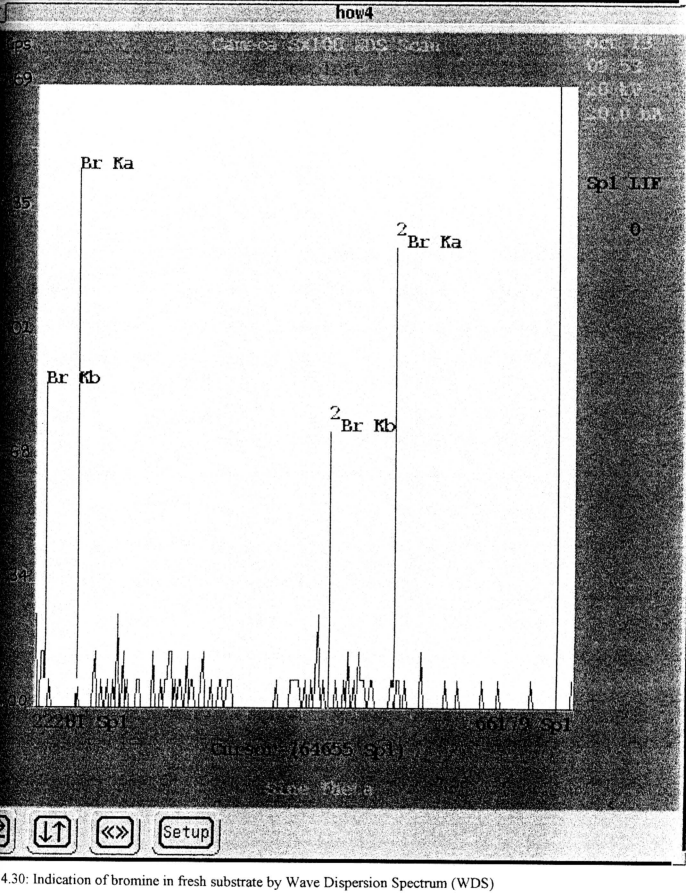


$C_{38}H_{55}BrO_3$

Figure 4.29: Proposed structure of **compound 1**

To our best knowledge, this is the first report on the isolation of a brominated sterol from a mushroom. The yield of **compound 1** was found to be low, with approximately 0.002% (w/w). Brominated compounds are usually found in the marine organisms such as marine sponges (Chuah, 1999). Sponges of the genus *Aplysina* (syn. *Verongia*) have been shown to contain bromotyrosine-derived metabolites, some of which may be artifacts of the isolation process (Faulkner, 1977).

Initial investigation of the fresh substrate by wave dispersion spectrum (Figure 4.30) indicated the presence of low level of bromine (less than 40 cps). However, no bromine was detected in substrate that had kept for two weeks (not shown). The possible origin of bromine present in the substrate may be due to fumigation of logs by methyl bromine. Fumigation of methyl bromine as an insecticide in the sawmill industry has been a common practice in some of the countries (Tanaka *et al.*, 1991).



4.30: Indication of bromine in fresh substrate by Wave Dispersion Spectrum (WDS)

In Malaysia, it is not sure whether this fumigation of logs was carried out in the local sawmill. Since the rubber wood sawdust were from several sawmills around the state (Kuan, 1999), tracking the source of the contaminated sawdust was not possible.

If bromine residue is present in the substrate, biosorption by *G. tsugae* during the coarse of cultivation is possible. The bromine residues may be taken up and somehow incorporate into **compound 1**. Manganese and copper accumulations have been reported in *Ganoderma* species (Blanchette, 1984; Muraleedharan and Venkobachar, 1990). However, to our best knowledge, no bromine uptake has so far been reported from the Basidiomycetes.

The absence of bromine residue in older substrate as compared to the presence of bromine residue in fresh substrate suggested the instability of bromine present in the substrate. The bromine residues may be quite volatile and easily escape from the substrate. Thus, one suggestion can be made from this observation is that the farmer should not use the fresh substrate, but the substrate should be kept for at least a month before using them. Furthermore, since the substrate was not composted prior to cultivation of mushroom, another suggestion is to compost the substrate before using them.