博士論文

論文題目　Search for anti-microbial compounds from cultured mycelia of *Basidiomycota* and *Ascomycota*

（担子菌門と子嚢菌門培養菌糸体の抗菌化合物の探索）

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Introduction

Animals, plants and fungi are eukaryote living life exist in the nature. Inter or intra kingdoms interact to each other. This results in the complicated ecosystem. The organisms produce primary and secondary metabolites for every purpose. We can summarize this relation briefly in Figure 1 (Tanaka O., 2002).

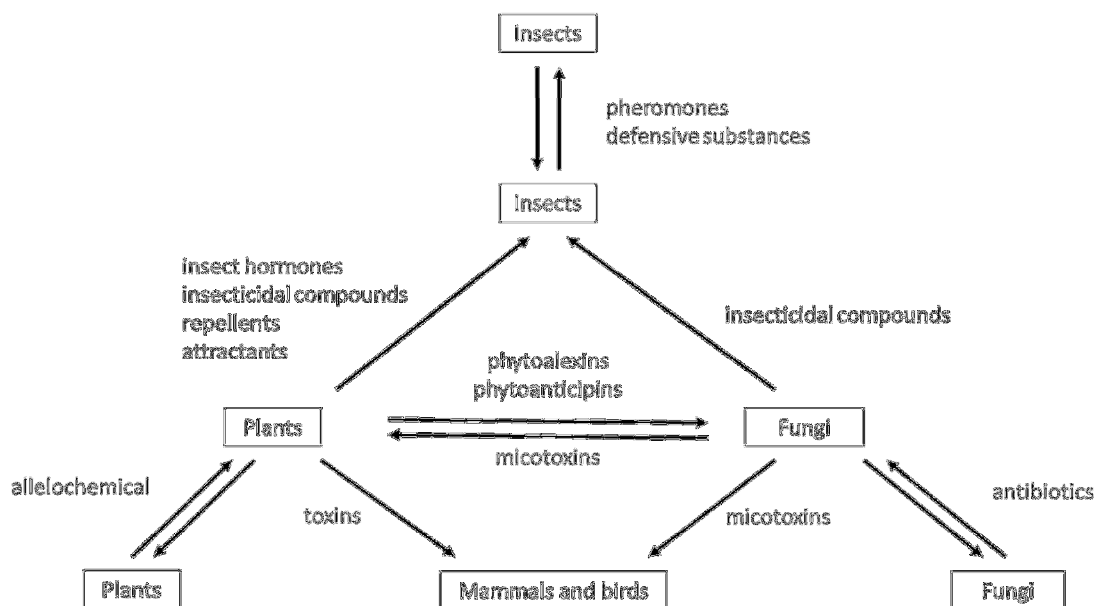


Figure1. Interaction among eukaryote with chemical compounds produced by them.

Mushrooms, especially *Basidiomycota*, produce many secondary metabolites which do not exist in other fungi (Yamazaki M., 1978). *Basidiomycota* mushrooms have well developed shikimate pathway biosynthesis. Also, *Basidiomycota* mushrooms own more secondary metabolites from mevalonate pathway than any other fungi, such as triterpenes and sesquiterpene. Moreover, polyacetylenes from malonate pathway are considered as unique product in *Basidiomycota* mushrooms among fungi（Mizuno T., 1992）.

Mushrooms are the creatures that do not move, therefore, it is a very important mechanism to produce protective compounds to protect themselves. However, many mechanisms are remained unknown, for example, the timing and the target of these compounds production and activation. In order to understand their mechanisms, we believe that it is our primary task to isolate and identify the secondary metabolites which contain in mushrooms.

To our knowledge, we understand that mushrooms produce many secondary metabolites with strong physiological activities, for example, metabolites from amino acid such as ibotenic acid and tricholomic acid are famous as the insecticides from the ancient ages. And the hetero-cyclic peptides produced by *Amanita virosa* mushrooms are well studied (Faulstich H., 1980).

However, the systematic researches are limited, many of the function and physiological meaning in mushrooms are remained unclear. Mushrooms are well used as herbal medicine and folk medicine, nevertheless, they are not considered as important medicinal resources until the discovery of anti-tumor drugs Lentinan from *Lentinula edodes* and Schizophyllan from *Schizophylum commune* in recent years. The reasons for succeed of Lentinan and Schizophyllan are, comparing to other mushrooms, these two mushrooms are easy for isolation, tissue culture and sample collection. Unfortunately, the fact is that many mushrooms are perennial and they take many years until their fruit bodies are formed. It is economically unrealistic to cultivate these mushrooms in artificial ways (Yamazaki M., 1978).

On the other hand, the methods of mycelia culture of high class mushrooms are well established in recent years. By means of controlling the culture condition, it is easy to obtain stable, qualitative and quantitative culture mycelia (Nakamura T., 2000). This method attracts many interests, for example, polysaccharides from the hot water extract of *Fomes yucatensis* cultured mycelia showed strong anti-cancer bioactivity. This finding was approved as an immune-enhancement drug by Korean healthy ministry in 1993 called [Mesima] (Kim, J.P., 1997).

Since mushrooms and its cultured mycelia won so many trophies, I focused on two classes of medicinal mushrooms, *Basidiomycota* and *Ascomycota*.

Basidiomycota

Basidiomycota mushrooms are promised material for drug discovery. The mentioned anti-tumor drugs Schizophyllan and Mesima were isolated from *Basidiomycota* mushrooms. Moreover, the anti-microbial activity has also been reported. When 317 strains, representing 204 species and 17 orders of *Basidiomycota* mushrooms were screened for antimicrobial activity on human pathogens, over 45% of the tested strains were positive for antimicrobial activity (Suay I, 2000). The medicinal properties of *Basidiomycota* mushrooms are frequently described in ancient literatures. In Asian, the holocarp of several genera are eaten, including garnish or boiled as teas and applied as therapies for a variety of human ailments ranging from the common cold to cure for certain forms of cancers (Loreto R., 2008). Recent researches showed they exhibit anti-cancer activity, antibacterial activity, antifungal activity and helminthicidal activity (Loreto R., 2008). Researchers (Alves M., 2013) especially focus on their antifungal activity because of the antifungal compounds with more or less strong activities could be isolated from many mushroom species and could be beneficial for humans. Many secondary metabolites with pharmaceutical activities were reported (Figure. 2), including aromatic compounds, terpenoids and amino acids (Chen Y., 2009).

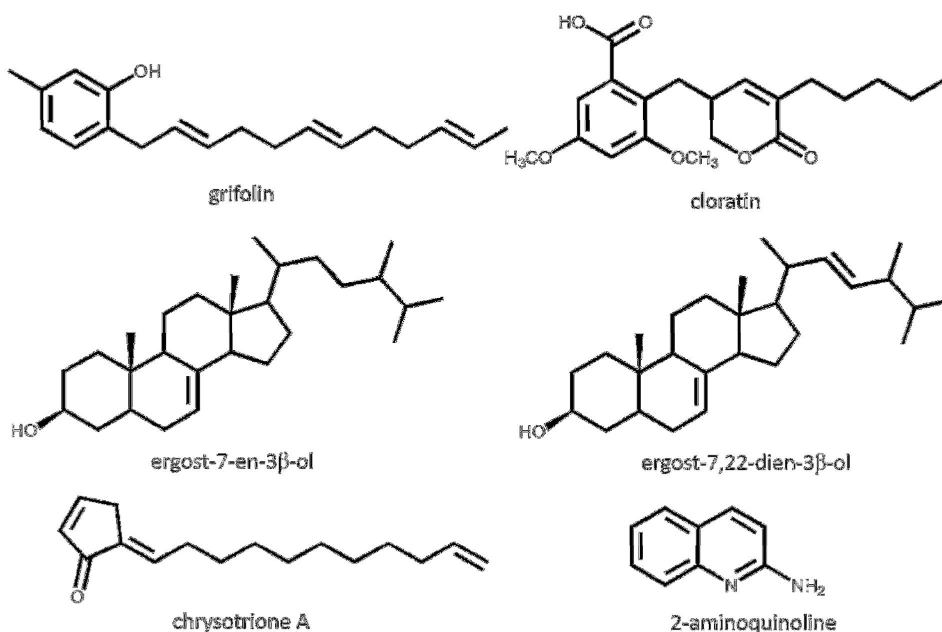


Figure 2. Chemical structure of the low-molecular-weight compounds with antifungal properties identified in mushrooms.

Ascomycota

On the other hand, *Ascomycota* medicinal mushrooms usually indicated as *Clavicipitaceae*, including *Cordyceps*, *Isaria* and *Pacilomyces.* They form parasitic complex of fungus and caterpillar, which has been used for medicinal purposes for centuries particularly in China, Japan and other Asian countries. The natural products of this family undoubtedly summoned the interest of first discoverers. The fungal spores infect and take over the host organism causing its eventual demise. The fungus continues to grow and emerges from the corpse of the host organism. Many subsequent studies were conducted using cultured *Clavicipitaceae* and have yielded positive results suggesting the cultivated fungi might possess the same health-promoting functions as the natural counterpart (Chen P.X., 2013). Recent researches specially mark on their anti-aging, reparative properties, anti-cancer, immuno-stimulation and anti-microbial activities (Chen P.X., 2013). Due to their various pharmaceutical activities and scarcity, *Cordyceps* mushrooms (*Clavicipitaceae*) are considered as high value items from the past. This phenomenon had a sharp increase following the severe acute respiratory syndrome (SARS) outbreak in China in 2003. The price of *C. sinensis* has increased 900% in a decade (1998-2008) (Winkler, D, 2008). These demands accelerate the research speed of the *Clavicipitaceae* mushrooms. Cordycepin, terpenoids, polysaccharides, nucleotides and amino acids were reported as

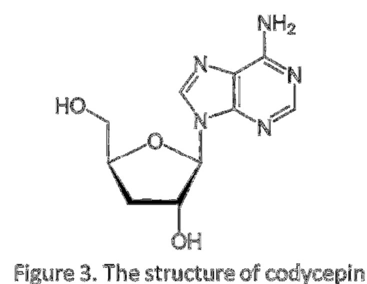


Figure 3. The structure of codycepin

their bioactive components (Figure 3) (Zhou X.W., 2009).

Dermatomycosis

Fungal infections of the skin and nails form the most numerous and widespread group of all mycoses. The prevalence of superficial mycotic infections has risen to such a level in the last decades that skin mycoses now affect more than 20-25% of world population (Male O., 1990). According to a survey by Ogasawara (Ogasawara Y., 2003), the prevalence of superficial mycotic infections was about 25% in Japan, it is equal to the world average even Japan is considered as one of the well-developed country. This might be because of the humid and hot weather in Japan. Nevertheless, antibiotic, salicylic acid and fatty acid type anti-fungal medicines were eliminated due to several reasons such as efficiency, side effects and the ability to making profits. Researchers believe that the tendency of elimination will accelerate in the future. Japanese government noticed and made several policies to solve this problem, however, the fact is the number of anti-fungal drugs was reduced from 10 to 7 in a decade (Isozumi K., 2013).

On the other hand, due to the development of transplanting therapy, population aging, reports of new fungi, the clinical cases of patients with deep mycosis are increasing. However, the number of clinically available antifungal drugs is very limited and each drug has some drawbacks compare to anti-biotic drugs. The main reason for the difficulty of anti-fungal drug development is that fungi are eukaryote, and is hard to ensure the selective toxicity. In Japan, even the official guideline for clinical evaluation was developed in recent years (Fukuoka T., 2004 and Havlickova B., 2008). As the results, it is important to develop and discover new anti-fungal compounds/drugs before these problems become severe.

There are approximately 40 different species of dermatophytes, charaterised by their capability to digest keratin and divided among three genera: *Trichophyton*, *Microsporum* and *Epidermophyton*. A majority of superficial fungal infections of the skin are caused by five or six species of dermatophyte (Kawano S., 2012). Among these dermatophytes, *Trichophyton rubrum* and *Microsporum canis* are two common species, which own more than 80% infection among the world (Gino A.V., 2012).

Due to the reasons, there were two main aims of this investigation. The first one was to investigate the anti-fungal compounds toward *M. canis* and *T. rubrum* (which are two of the common infective fungi) from the cultured mycelia of medicinal mushrooms. There are 46 kinds of different cultured mycelia in our laboratory. All of them were received from Dr. Hirotani (Kitasato University) or purchased from the Hakkou Research Center Ltd.. Many of them are famous for anti-bacteria, anti-fungal activity from the traditional usage. However, due to many reasons such as limited resource and unsufficient research, the bioactive compounds of each strain and their

mechanism are remained unknown, even though many of them are proofed to be effective to many infective pathogens. Moreover, I expect this study could give an example for anti-fungal drugs development.

The second aim of this investigation was to uncover the secondary metabolites which are produced by the cultured mycelia of medicinal mushrooms. As mentioned above, due to the limited natural resource, many of them are remained un-developed. We already know that mushrooms produce various kinds of secondary metabolites in order to protect themselves such as aromatic compounds, terpenoids, polyacetylenes, protein and enzymes. Some of them own specific bioactivities. For example, mescaline, ibotenic acid and so on. Therefore, it is reasonable to expect to isolate new compounds from the cultured mycelia.

Since the fungal infection is a worldwide issue. Many evidences indicate the necessity of anti-fungal drugs development is very important nowadays. Our final goal is to investigate the anti-fungal compounds which can be used in our daily life and protect us away from fungal infection, or to provide other options in treating fungal infection.

Chapter 1
Screening for anti-fungal activities of cultured mycelia of mushrooms

Section 1

Cultivation and extraction of anti-fungal components from *Agaricomycetes* medicinal mushrooms cultured mycelia

There are many kinds of medicinal fungi belonged to *Agaricomycetes*, including Kawariharatake, a well-known medicinal fungi as a food supplement in Japan. There are six stains of cultured mycelia of medicinal mushrooms which are belonged to *Agaricomycetes* in our laboratory. They are *Agaricus hortensis, A. bitorguis, A. rhodmanji, A. arvensis, A. subrufescens* and *Ligunosus rhinoceros*.

Mycelia of each mushrooms were cultured in liquid potato dextrose (PD) medium, under 25℃ in the dark for 6 weeks. After six weeks culture, the cultures were harvested and then separated into mycelia part and broth part. The mycelia part was extracted with methanol (MeOH) to obtain MeOH extract. The MeOH extract was partitioned between $CHCl_3$ and H_2O to obtain $CHCl_3$ fraction and H_2O fraction. On the other hand, broth part was extracted with ethyl acetate (EtOAc) to obtain EtOAc fraction and water fraction. The organic solvents were evaporated by evaporator, and the water fractions were freeze dried.

The prepared 24 samples were then applied to *Microsporum canis* for anti-fungal activity test by disk diffusion method. The results showed that the cultured broth of *Lignosus rhinoceros* had the strongest anti-fungal activity (Table 1)

Table 1. Antibacterial activity of *Agaricomycetes* extracts against *M. canis*

	Mycelium		Broth	
	$CHCl_3$	H_2O	EtOAc	H_2O
Agaricus hortensis	−	−	±	−
Agaricus bitorquis	±	−	−	−
Agaricus arvensis	−	−	−	−
Agaricus rhodmanji	±	−	−	−
Agarricus subrufesens	−	−	−	−
Lignosus rhonoceros	−	−	+	−

Agar diffusion assays were carried out using paper disks of 8 mm diameter spotted with sample dissolved in DMSO.
+ : inhibitory zone was formed.
± : incomplete inhibitory zone was formed.
− : no inhibitory zone was formed.

Section 2

Cultivation and extraction of anti-fungal components from *Clavicipitaceae* mushrooms cultured mycelia

There are six stains of cultured mycelia belonged to *Actonomycetes* in our laboratory. They are *Cordyceps ferruginosa, C. roseostromata, C. oxycephala, C. tricentri, C. prolifica* and *Isaria* sp..

Mycelia of each mushroom was cultured in liquid PD medium, under 25℃ in the dark for 6 weeks. After six weeks culture, the cultures were harvested and then separated into mycelia part and broth part. The mycelia part was extracted with methanol (MeOH) to obtain MeOH extract. The MeOH extract was partitioned between EtOAc and H_2O to obtain EtOAc fraction and H_2O fraction. The sample preparation in broth part was the same as section 1. The organic solvents were evaporated by evaporator, and the water fractions were dried by freeze dried.

The prepared 24 samples were then applied to *M. canis* for anti-fungal activity test by disk diffusion method. The results showed that the *C. oxycephala, C. prolifica* and *Isaria* sp. extracts had bioactivity (Table 2). Due to the slow growth of *C. oxycephala* and *C. prolifica,* I chose *Isaria* sp. as my study material.

Table 2. Antibacterial activity of *Clavicipitaceae* cultured extracts against *M. canis*

	Mycelium		Broth	
	EtOAc	H_2O	EtOAc	H_2O
Cordyceps ferruginosa	−	−	−	−
Cordyceps roseostromata	−	−	±	−
Cordyceps oxycephala	−	−	+	−
Cordyceps tricentri	−	−	±	−
Cordyceps prolifica	+	−	−	−
Isaria sp.	−	−	+	−

Agar diffusion assays were carried out using paper disks of 8 mm diameter spotted with sample dissolved in DMSO.
+ : inhibitory zone was formed.
± : incomplete inhibitory zone was formed.
− : no inhibitory zone was formed.

Section 3

Cultivation and extraction of anti-fungal components from *Ganodermataceae* medicinal mushrooms cultured mycelia

Ganoderma is a white rot wood-degrading basidiomycete with hard fruiting bodies. In this family, *G. lucidum* and *G. applanatum* are two of most well-known species. In Asian traditional medicine, the fruiting body of *G. lucidum*, called Ling-Zhi in Chinese and Reishi in Japanese language, has been used for many treatments. It is even more treasured than any other medicine in China. In ancient Chinese medicinal encyclopedias "Shen Nong's Ben Cao Jing" described that *G. lucidum* is non-toxin and can be used daily to improve health conditions. *Ganodermataceae* medicinal mushrooms are promised material for bioactive compounds discovery. Many compounds were reported in this family with various pharmaceutical activities such as antiallergenic constituents (Kino K., 1991), immunomodulatory action (Hem L.G., 1995), antitumor activity (Maruyama H., 1989), cardiovascular effects (Lee S.Y., 1990) and liver protection (Liu G.T., 1979).

There are five stains of cultured mycelia of medicinal mushrooms which are belonged to *Ganodermataceae* in our laboratory called *Ganoderma lucidum, G. mastoporum, G. applanatum, G. neo japonicum* and *G. boninense.* General culture conditions and sample preparation of *Ganodermataceae* medicinal mushrooms are the same as the one mentioned in section 1. Four samples of each species were prepared for anti-fungal activity test. As shown in Table 3, many of them showed antifungal activity. Due to the well-studied *G. lucidum* and *G. applanatum*, I choose *G. mastoporum* as my research material.

Table 3. Anti-fungal activity of *Ganodermataceae* extracts against *M. canis*

	Mycelium		Broth	
	EtOAc	H_2O	EtOAc	H_2O
Ganoderma lucidum	+	−	+	−
Ganoderma mastoporum	+	−	±	−
Ganoderma applanatum	+	−	−	−
Ganoderma neo japonicum	±	−	−	−
Ganoderma boninense	+	−	−	−

Agar diffusion assays were carried out using paper disks of 8 mm diameter spotted with sample dissolved in DMSO.
+ : inhibitory zone was formed.
± : incomplete inhibitory zone was formed.
− : no inhibitory zone was formed.

Section 4

Discussion

As shown in Table 1, 2 and 3, many cultured mycelia of mushrooms showed anti-fungal activity toward *M. canis* from their cultured broth extracts and mycelia extracts. Due to the reasons such as well-studied, growth rate and strength of anti-fungal activities, we chose *L. rhinoceros, Isaria* sp. and *G. mastoporum* as the research materials in this study.

In order to get access to understand what components inside these cultured mycelia of mushrooms exhibit anti-fungal activities, large scale culture, purification and structures determination of these three species will be carried out in the following chapters.

Chapter 2
Anti-fungal compounds from *Lignosus rhinoceros*

Section 1

About *Lignosus rhinoceros*

Lignosus rhinoceros, also called tiger milk mushroom, is belonged to *Polyporaceae*. It is distributed to Malaysia, Australia and south part of Japan (Okinawa). In traditional usage, it is used for diarrhea, rheumatic pains, cough, asthma, cardiac diseases and dysmenorrhea. This mushroom is one of the most popular medicinal mushrooms in Malaysia (Lee M.L., 2012 and Comer, E.J.H, 1989). However, due to the over harvesting, *L. rhinoceros* was indicated as an endangered mushroom by Malaysia (Eik, L.F. 2012) and Japanese government (www.biodic.go.jp). Therefore, there were only few studies on the pharmaceutical activities of *L. rhinoceros* in recent years because of lack of supply. In 2012, Shopana M. et al (Shopana M., 2012) investigated that the fruit body of *L. rhinoceros* has anti-fungal activity toward *C. albican*, however, no further studies of its bioactive compounds. In this study, I will work on the bioactive compounds of cultured *L. rhinoceros*.

Section 2

Isolation of bioactive compounds from *L. rhinoceros*

The results showed that the culture broth of *L. rhinoceros* showed anti-fungal activity to *M. canis*. As shown in Figure 4, large scale culture (20L) was performed using Roux flasks. The culture broth was collected after 6 weeks cultivation for further purification and isolation. The EtOAc extract (2g) of *L. rhinoceros* cultured broth was purified by silica gel column chromatography. By means of bioactivity-guided fractionation procedure, fr. 3 (370.8mg) from $CHCl_3$: MeOH(100:1) showed the strongest bioactivity. Analysis of fr. 3 (Figure 5) revealed that it had two major spots and a tailing part. This fraction was then purified by silica gel column chromatography again to obtain fr. 3-1 (62.3mg) and 3-2 (271.6mg). Fr. 3-1 was then purified by normal phase HPLC to obtain compounds **1** and **2**, where fr. 3-2 was purified by reversed phase HPLC to obtain compounds **3** to **8**. While applied these compounds for the anti-fungal activity test, compounds **1** (36.3mg) and **2** (18.2mg) showed significant inhibition zones (Table 4).

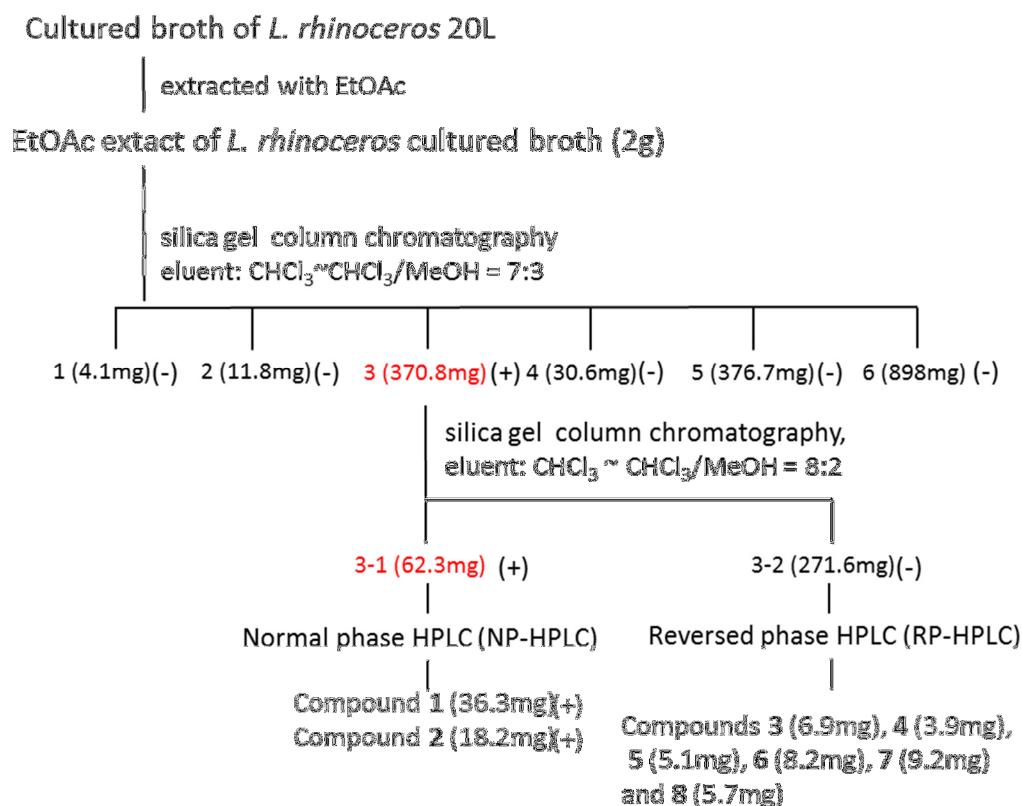


Figure 4. Isolation procedures of the anti-fungal compounds against *M. canis* from cultured broth of *L. rhinoceros*

TLC conditions

Plate; silica gel

Detection; spread 10% H_2SO_4 aq. and heated

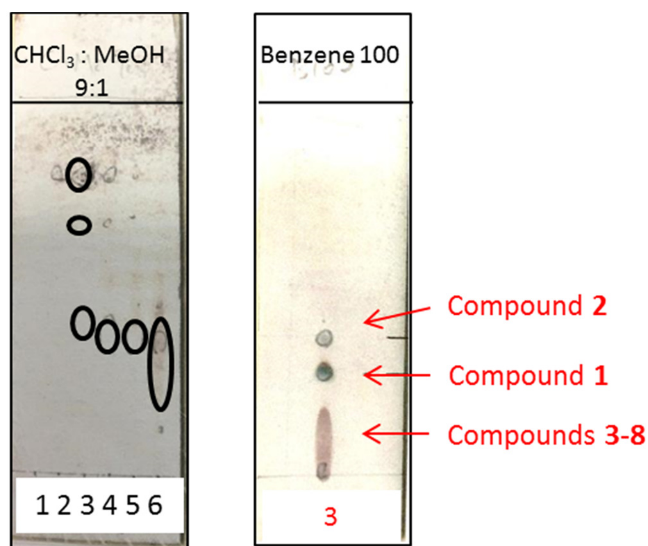


Figure 5. TLC analysis of fractions from *L. rhinoceros* cultured broth

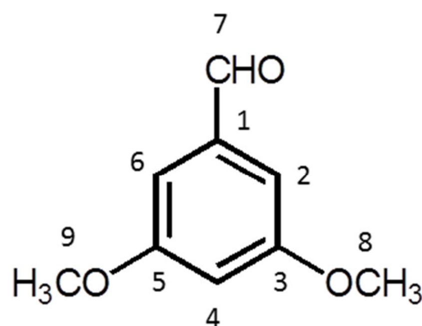
Table 4. Summary of the purification of the fractions/compounds of *L. rhinoceros* with anti-fungal activity against *M. canis*

Fraction	Weight (mg)	Inhibitory zone (diameter in mm)	Spotted amount (mg)
EtOAc fraction	2000	40	25
Fr. 3	370.8	22	5
Fr. 3-1	62.3	30.1	1
Compound **1**	42.3	18.8	0.4
Compound **2**	18.2	17.6	0.4

Section 3

Structure of compound **1**

According to the LR-EI-MS and LR-ESI-MS, compound **1** was found to have a molecular ion peak at *m/z* 166. And in the ^{13}C-NMR spectrum (Table 5), only six signals were observed. And DEPT spectrum showed it has a methyl signal, three methine signals and two quaternary carbon signals. Considering its molecular weight, it indicated that compound **1** is a compound with symmetric structure. The ^{1}H-NMR showed a doublet and a triplet signals at the aromatic region. The doublet one at δ 7.01 (2H, d, *J* = 2.4 Hz) was assigned to H-2 and H-6, and the triplet one at δ 6.70 (1H, t, *J* = 2.4 Hz) was assigned to H-4. And two singlet peaks at δ 9.91 (1H, s) and δ 3.85 (6H, s) indicated that compound **1** has an aldehyde and two methoxyl substitutes. Together with ^{13}C-NMR spectrum, it indicated that compound **1** is a 2,4,6-trisubstituted benzene structure. Compared the data with the reference (Lima L.S., 2003), compound **1** was identified as 3,5-dimethoxybenzaldehyde (Figure 6).



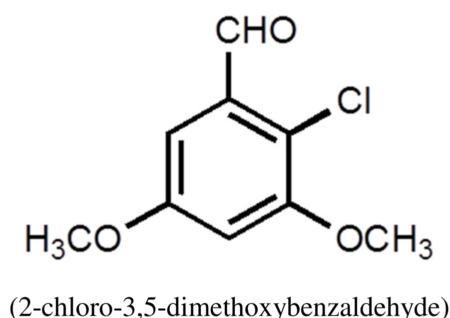
(3,5-dimethoxybenzaldehyde)

Figure 6. The structure of compound **1**

Section 4

Structure of compound **2**

According to the LR-EI-MS, compound **2** was found to have a molecular ion peak at *m/z* 200. The ^{1}H-NMR showed two doublet signals at the aromatic region. They are at δ 6.73 (1H, d, J = 2.3 Hz) and δ 7.01 (1H, d, J = 2.3 Hz). And three singlet peaks at δ 10.50 (1H, s), δ 3.91 (3H, s) and δ 3.87 (3H, s) indicated that compound **2** has an aldehyde and two methoxyl substitutes. All evidence indicated that compound **2** has similar structure to compound **1** but a different substitute. When take a close look at the LR-EI-MS spectrum of compound **2**, aside the major peak at *m/z* 200, there was a minor peak at *m/z* 202 appeared in a ratio of 3:1. This fact indicated that the existence of chlorine. Also, compound **2** was found to have a peak by HR-EI-MS at *m/z* 200.0252 with a molecular formula $C_9H_9O_3Cl$. The observation of cross-peaks in the HMBC (Figure 7) between the signal at 6.73 ppm (H-4) with the carbon signals corresponding to C-3 and C-5; the signal at 7.01 ppm (H-6) with the carbon signals corresponding to C-1, C-2 and C-5 indicated that chlorine is at C-2 position and the 2 methoxyl groups are at C-3 and C-5 positions. As the results, compound **2** was identified as 2-chloro-3,5-dimethoxybenzaldehyde (Figure 7).



(2-chloro-3,5-dimethoxybenzaldehyde)

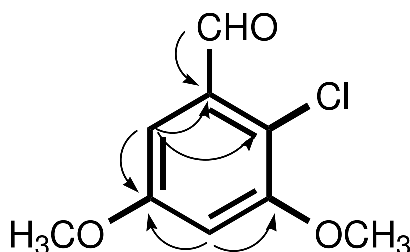


Figure 7. The structure of compound **2** and its HMBC correlation

Table 5. C-NMR data of compounds **1-6**

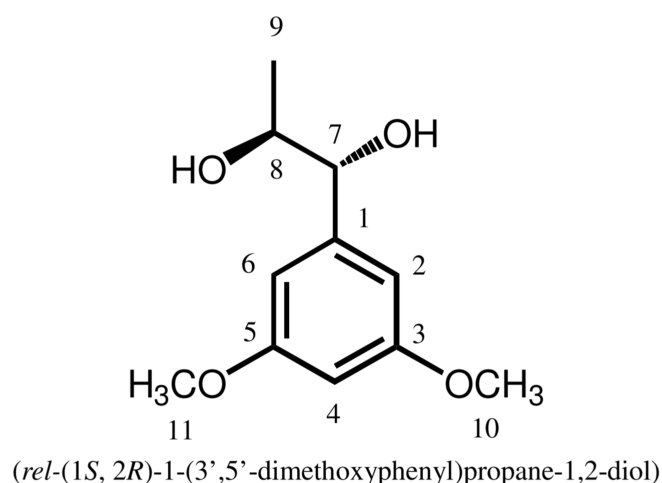
^{13}C-NMR data ($CDCl_3$)	δC	
	1	**2**
1	138.4	133.6
2	107.2	119.6
3	161.2	159.1
4	107.1	106.0
5	161.2	156.3
6	107.2	102.4
7	192.0	189.9
8	55.6	55.8
9	55.6	56.5

^{13}C-NMR data (CD_3OD)	δC			
	3	**4**	**7**	**8**
1	144.9	144.8	132.9	127.0
2	104.9	105.1	107.3	112.8
3	161.1	161.2	161.2	165.5
4	99.2	99.5	105.1	106.0
5	161.1	161.2	161.2	159.4
6	99.2	99.5	107.3	102.1
7	78.0	79.3	168.6	168.5
8	71.4	71.8	-	-
9	17.1	18.3	-	-
10	54.7	54.7	55.0	55.2
11	54.7	54.7	55.0	56.0

Section 5

Structure of compounds **3**

Compound **3** was observed as a light orange oil with $[\alpha]_D$ -6.3 (*c* 0.48, MeOH). According to LR-EI-MS, compound **3** was found to have a molecular ion peak at *m/z* 212. The ^{13}C-NMR spectrum (Table 5) showed only eight peaks, four of them were at aromatic region. The DEPT spectrum showed they are two methyl signals, four methine signals and two quaternary carbon signals. Similar to the reason of compound **1**, it pointed out that compound **3** may be a symmetric structure. The ^{1}H-NMR showed one doublet and one triplet signals at the aromatic region. They are at δ 6.54 (2H, d, *J* = 2.3 Hz) and δ 6.36 (1H, t, *J* = 2.3 Hz). And a singlet peak at δ 3.75 (6H, s) indicated that compound **3** has a benzene ring with three substitutes. And three peaks at δ 4.43 (1H, d, *J* = 4.4Hz), δ 3.79 (1H, dq, *J* = 4.4 and 6.2Hz) and δ 0.98 (3H, d, *J* = 6.2Hz) indicated that compound **3** has a propanediol side chain. Compared the data with the literature (Kamikawa K., 2000), which reported the NMR data of synthesized *rel*-(1*S*, 2*R*)-1-(3',5'-dimethoxyphenyl) propane-1,2-diol and matched to compound **3**. Therefore, compound **3** was identified as *rel*-(1*S*, 2*R*)-1-(3',5'-dimethoxyphenyl) propane-1,2-diol (Figure 8).



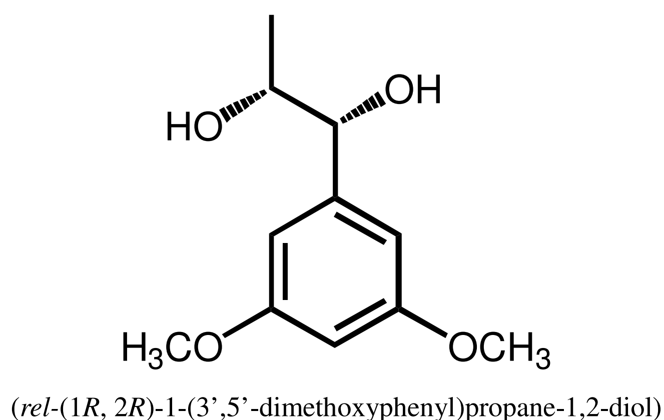
(*rel*-(1*S*, 2*R*)-1-(3',5'-dimethoxyphenyl)propane-1,2-diol)

Figure 8. The structure of compound **3**

Section 6

Structure of compounds **4**

Compound **4** was observed as a light orange oil with $[\alpha]_D$ 0 (*c* 1.1, MeOH). According to LR-EI-MS, compound **4** was found to have a molecular ion peak at *m/z* 212, which is the same with compound **3**. The ^{13}C-NMR spectrum (Table 5) and the DEPT spectrum showed compound **4** has similar pattern to compound **3** but only with slight different chemical shift. The ^{1}H-NMR showed one doublet and one triplet signals at the aromatic region. They are at δ 6.52 (2H, d, *J* = 2.3 Hz) and δ 6.38 (1H, t, *J* = 2.3 Hz). And a singlet peak at δ 3.76 (6H, s) indicated that compound **4** has a benzene ring with three substitutes. And three peaks were at δ 4.26 (1H, d, *J* = 7.1 Hz), δ 3.80 (1H, dq, *J* = 7.1, 6.3 Hz) and δ 0.97 (3H, d, *J* = 6.3 Hz). All the evidence indicated that compound **4** is a diastereomoer of compound **3**. Compared the data with the literature (Kamikawa K., 2000), which reported the NMR data of synthesized *rel*-(1*R*, 2*R*)-1-(3',5'-dimethoxyphenyl) propane-1,2-diol and matched to compound **4**. Therefore, compound **4** was identified as *rel*-(1*R*, 2*R*)-1-(3',5'-dimethoxyphenyl) propane-1,2-diol (Figure 9).



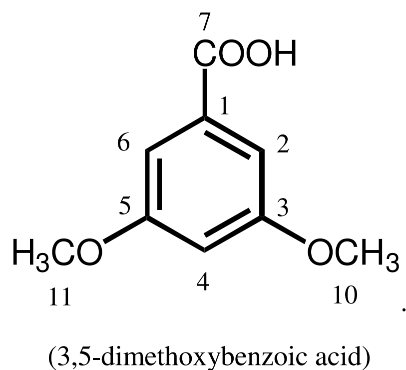
(*rel*-(1*R*, 2*R*)-1-(3',5'-dimethoxyphenyl)propane-1,2-diol)

Figure 9. The structure of compound **4**

Section 7

Structure of compound **5**

According to LR-EI-MS, compound **5** was found to have a molecular ion peak at *m/z* 182. In the ^{13}C-NMR spectrum (Table 5), only six signals were observed, a signal at δ 168.6 indicated the existence of a carboxylic acid. The DEPT spectrum showed it has a methyl signal, two methine signals and three quaternary carbon signals. Considering its molecular weight, it indicated that compound **5** is a compound with symmetric structure. The ^{1}H-NMR showed a doublet and a triplet signals at the aromatic region. The doublet one at δ 7.14 (2H, d, *J* = 2.3 Hz) was assigned to H-2 and H-6, and the triplet one at δ 6.68 (1H, t, *J* = 2.3 Hz) was assigned to H-4. And one singlet peak at δ 3.80 (6H, s) indicated that compound **5** has two methoxyl substitutes. Compared the data with reference (http://sdbs.db.aist.go.jp/), compound **5** was identified as 3,5-dimethoxybenzoic acid (Figure 10).



(3,5-dimethoxybenzoic acid)

Figure 10. The structure of compound **5**

Section 8

Structure of compound **6**

According to LR-EI-MS, compound **6** was found to have a molecular ion peak at *m/z* 216. The ^{1}H-NMR showed two doublet signals at the aromatic region. They are at δ 6.77 (1H, d, *J* = 2.8 Hz) and δ 6.70 (1H, d, *J* = 2.8 Hz). And two singlet peaks at δ 3.88 (3H, *s*) and δ 3.82 (3H, s) indicated that compound **5** has two methoxyl substitutes. Similar to the relation between compounds **1** and **2**, compound **6** has similar structure to compound **5**. When take a close look at the LR-EI-MS spectrum of compound **5**, a minor peak at *m/z* 218 aside major peak at *m/z* 216 appeared in a ratio of 3:1. Also, compound **6** was found to have a peak by HR-EI-MS at *m/z* 216.0177 with a molecular formula $C_9H_9O_4Cl$. As the results, compound **6** was identified as 2-chloro-3,5- dimethoxybenzoic acid (Figure 11).

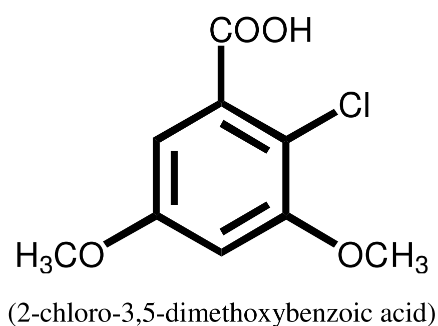


Figure 11. The structure of compound **6**

Section 9

Structure of compound **7**

Compound **7** was observed as a light orange oil with $[\alpha]_D$ 56.3 (*c* 0.6, MeOH). According to LR-EI-MS, compound **7** was found to have a molecular ion peak at *m/z* 184. The ^{1}H-NMR showed two doublet peaks. They are at δ 6.05 (1H, d, *J* = 2.3 Hz) and δ 5.53 (1H, t, *J* = 2.3 Hz). And a singlet peak at δ 3.84 (3H, s) indicated that it has a methoxyl group. And three peaks at δ 4.10 (1H, tq), δ 2.56 (2H, m) and δ 1.22 (3H, d, *J* = 6.8 Hz) indicated that compound **7** has a 2-propanol side chain. The ^{13}C-NMR spectrum (Table 5) showed nine peaks and the DEPT spectrum showed they are two methyl signals, one methylene signal, three methine signals and three quaternary carbon signals. The peak at δ 166.7 indicated that compound **7** has a lactone ring structure. The observation of cross-peaks in the HMBC (Figure 13) between the signal at δ 5.52 (H-5) with the carbon signals correspond to C-4, C-6 and C-7; the signal at δ 6.05 (H-7) with the carbon signals correspond to C-6 and C-8 indicated that 2-propanol side chain is at C-4, methoxyl group is at C-6. Compared with the literature (Zhou H., 2010) which reported the NMR data of synthesized 6-(2-hydroxypropyl)-4-methoxy-pyran-2-one, compound **7** was identified as 6-(2-hydroxypropyl)-4- methoxy-pyran-2-one (Figure 12).

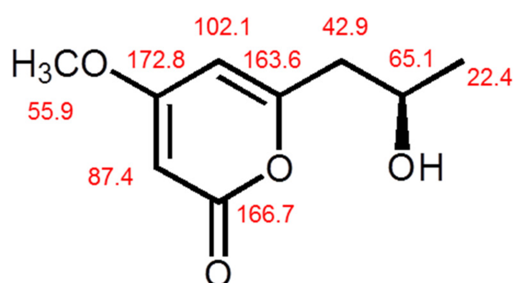


Figure 12. The structure of compound **7** and its ^{13}C-NMR chemical shifts in CD_3OD

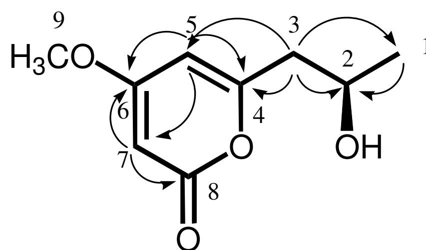
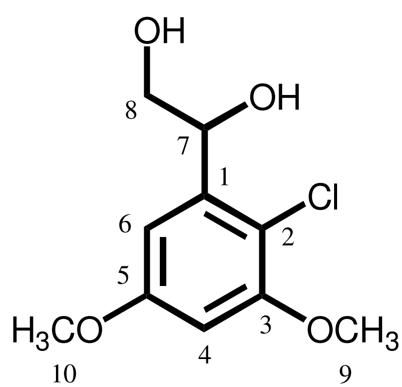


Figure 13. HMBC correlation of compound **7**

Section 10

Structure of compound **8**

Compound **8** was observed as light orange oil with $[\alpha]_D$ -23.9 (*c* 0.27, MeOH). According to the LR-EI-MS, compound **8** was found to have a molecular ion peak at *m/z* 232. In ^{13}C-NMR spectrum (Table 6), ten signals were observed. And DEPT spectrum showed it has two methyl signals, one methylene signal, three methine signals and four quaternary carbon signals. The ^{1}H-NMR showed two doublet signals at the aromatic region. They are at δ 6.80 (1H, d, *J* = 2.8 Hz) and δ 6.54 (1H, d, *J* = 2.8 Hz). And two singlet peaks at δ 3.85 (3H, *s*) and δ 3.80 (3H, s) indicated that compound **8** has two methoxyl substitutes. And three peaks at δ 5.13 (1H, dd, *J* = 7.5, 3.0 Hz), δ 3.69 (1H, dd, *J* = 11.5, 3.0 Hz) and δ 3.43 (1H, dd, *J* = 11.5, 7.5 Hz) indicated that compound **8** has an 1,2-ethane-diol side chain. The observation of cross-peaks in the HMBC (Figure 14) between the signal at δ 6.54 (H-4) with the carbon signals correspond to C-3, C-5 and C-6; the signal at δ 6.80 (H-6) with the carbon signals correspond to C-1 and C-5 indicated that the 2 methoxyl groups are at C-3 and C-5. The signal at δ 5.13 (H-7) with the carbon signals correspond to C-1 and C-2 indicated that chlorine is at C-2. Similar to compounds **2** and **6**, the LR-EI-MS spectrum (Figure 15) showed that there were two minor peaks at *m/z* 234 and 203 aside the major peaks at *m/z* 232 and 201. The signal intensity showed in a ratio of 1:3. This fact indicated that the existence of chlorine. Also, compound **8** was found to have a peak by HR-ESI-MS at *m/z* 255.0322 $[M+Na]^+$ with a molecular formula $C_{10}H_{13}O_5ClNa$. Together with HMBC spectrum, compound **8** was identified as a new compound named 1-(2-chloro-3,5-dimethoxy-phenyl)-ethane-1,2-diol (Figure 14).



(1-(2-chloro-3,5-dimethoxy-phenyl)-ethane-1,2-diol)

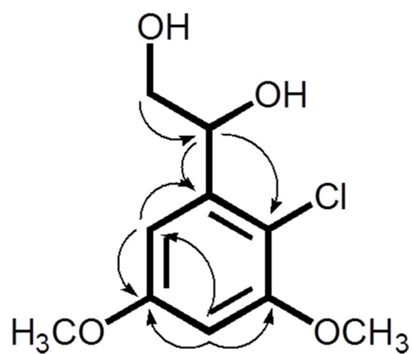


Figure 14. The structure of compound **8** and HMBC correlation

Table 6. ^{1}H-NMR and ^{13}C-NMR data of compounds **8** (Solvent CD_3OD)

Positions	δC		δH
1	111.9	C	
2	141.5	C	
3	152.8	C	
4	98.8	CH	6.54 (1H, d, *J* = 2.8 Hz)
5	159.7	C	
6	103.8	CH	6.80 (1H, d, *J* = 2.8 Hz)
7	71.7	CH_2	5.13 (1H, dd, *J* = 7.5, 3.0 Hz)
8	66.2	CH_2	3.69 (1H, dd, *J* = 11.5, 3.0 Hz) 3.43 (1H, dd, *J* = 11.5, 7.5 Hz)
9	55.7	CH_3	3.85 (3H, *s*)
10	55.0	CH_3	3.80 (3H, s)

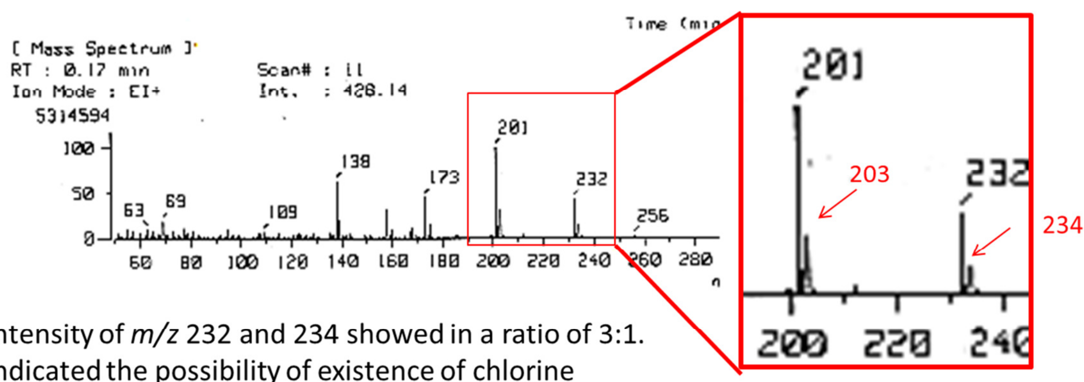


Figure 15. LR-EI-MS spectrum of compound **8**

Section 11

Anti-fungal and anti-microbial activities of compounds **1-8**

Compound **1-8** were applied to *Candida albicans*, *Microsporum canis* and *Trichophyton rubrum* for anti-fungal activity test. The results were shown in Table 7 and Table 8.

Table 7. Anti-fungal activities of compounds **1-8**

Compound		*C. albicans*		*M. canis*		*T. rubrum*	
		36 hours	9 days	6 days	14 days	6 days	14 days
1	0.2 mg	32.0 x 31.9	13.3 x 19.3 (not clear)	12.9 x 10.5	8 x 8	59.8 x 48.0	34.1 x 23.9 (not clear)
2	0.2 mg	8 x 8		17.6 x 17.6	14.0 x 14.6	31.7 x 27.6	15.7 x 14.9
3	0.4 mg	8 x 8		8 x 8		8 x 8	
4	0.4 mg	8 x 8		8 x 8		8 x 8	
5	0.4 mg	8 x 8		8 x 8		8 x 8	
6	0.4 mg	8 x 8		8 x 8		8 x 8	
7	0.4 mg	8 x 8		8 x 8		8 x 8	
8	0.4 mg	8 x 8		8 x 8		8 x 8	
Miconazole	0.002 mg		27.0 x 27.4	42.8 x 41.3	31.2 x 31.4	32.2 x 36.6	26.3 x 26.5

Microbial inoculation: 10^7cfu/ml. Paper disk size: 8 x 8 (mm)

Table 8. Benzaldehyle derivatives MICs (mg/L)

	Compound 1	Compound 2	Fluconazole	Miconazole
C. albicans	64		1	
M. canis	50	100		0.8
T. rubrum	25	50		0.8

Also, these compounds were applied to other bacteria such as methicillin-susceptible *Staphylococcus aureus* (MSSA), *Escherichia coli* and *Pseudomonas aeruginosa* for the anti-microbial activity tests (Table 9).

Table 9. Compounds **1-8** MICs (mg/L)

Compound	MIC		
	MSSA1	*E.coli*	*P. aeruginosa*
1	>128	>128	128
2	>128	>128	>128
3	>128	>128	>128
4	>128	>128	>128
5	>128	>128	>128
6	>128	>128	>128
7	>128	>128	>128
8	>128	>128	>128
Vancomycin	0.64		
Gentamycin		4	
Cephataxime			4

Section 12

Discussion

In this study, we demonstrated that cultured mycelia of *L. rhinoceros* produce anti-fungal benzaldehyle derivatives and release them into the broth. Allelopathy is a phenomenon usually reported among plants. However, similar phenomenon was also reported in many mushrooms (Aratani H., 2004). This study indicated that *L. rhinoceros* releases anti-fungal benzaldyhyde derivatives to protect their own life. The aim of this study is to investigate the anti-fungal compounds which could be used in our daily life to prevent us away from fungal infection. This study demonstrated that the isolates or the extract of cultured broth of *L. rhinoceros* may be a good choice for treating skin disease or daily supplements. Moreover, *L. rhinoceros* is widely used and well accepted in Malaysia and China from the early ages, in order to make this aim practically, tasks such as cytotoxicity and *in-vivo* toxicity are important for the future studies.

As mentioned in the introduction, there are only limited reports of *L. rhinoceros* because of the lack of supply. Recent report of its anti-fungal activity is demonstrated in 2012 (Shopana M., 2012), the crude extracts of fruit body of *L. rhinoceros* showed anti-fungal activity toward *C. albicans*. However, no more advanced studies were reported and phytochemistry of *L. rhinoceros* is remained pending. This study unveiled the compounds **1** and **2** (3,5-dimethoxybenzaldehyde and 2-chloro-3,5-dimethoxybenzaldehyde, respectively) produced by *L. rhinoceros* cultured mycelia are the resources for its anti-fungal activity.

Both bioactive compounds **1** and **2** are benzaldehyde derivatives. Prior studies showed that analogs of benzoic acids, cinnamic acids, benzaldehydes and common phenolics founds in living life inhibit biosynthesis of mycotoxins and growth of various fungi, both filamentous and yeasts (Tawata S., 1996 and Florianowicz T., 1998). And the benzaldehyde derivatives target cellular anti-oxidation components of fungi, such as superoxide dismutase, glutathione reductase, have potent antifungal activity against clinical strains of these yeasts. Such chemosensitization can reduce costs, lower resistance, and alleviate health risks associated with current antifungal therapy (Kim J.H., 2011).

While comparing the anti-fungal activities of compounds **1** and **2** (Table 7, 8), both of them showed significant anti-fungal activity toward *M. canis* and *T. rubrum*. Especially, *T. rubrum* seemed to be more sensitive to compounds **1** and **2** than *M. canis*, and compound **1** had stronger potency than compound **2** in the first week culture. Interestingly, after 2 weeks cultivation, compound **2** remained clear inhibition zones toward to both *M. canis* and *T. rubrum* while the inhibition zone of compound **1** was almost covered by fungi. The only difference between compound **1** and compound **2** is the chlorine substitute at C-2 position of compound **2**, this fact indicated that the chlorine substitute may be hard to be decomposed by fungi and results in the enhancement of the duration of anti-fungal activity. Previous studies indicated that chlorine atoms

intended to be biologically active (Naumann K., 2000) because of 1. High electrophilicity at the carbon connected to a chlorine atom, 2. Electronic effects caused by chlorine increase electrophilic reactivity at more remote carbon atoms, 3. Low bond energy in alkyl chlorides gives high reactivity and 4. Increase in lipophilicity of the molecule. On the other hand, there were 8 compounds isolated from the cultured broth of *L. rhinoceros* in this study. They are two benzaldehyde derivatives, two phenyl-1,2-propanediol derivatives, two benzoic acid derivatives, one phenyl-1,2-ethanediol derivative and one lactone derivative. Except the two benzaldehyde derivatives, none of any other compounds showed anti-fungal activities. Therefore, aldehyde group may be necessary for aromatic compounds to have anti-fungal activities.

In this study, a known lactone derivative and series of aromatic compounds including 6 known compounds and 1 new compound (named 1-(2-chloro-3,5-dimethoxyphenyl)-ethane-1,2-diol) were isolated from the cultured broth of *L. rhinoceros*. Six of the known compounds were the first report from the natural products. Three of them were containing a chlorine substitutes. Proposed biosynthetic pathway of these compounds was demonstrated in Figure 16. Acetyl CoA and malonyl CoA started by the polyketide biosynthesis, the intermediate 5-(2-propanol)-2,4-dihydroxy benzoic acid was formed. Followed by methylation, decarboxylation and hydroxylation, compounds **3** and **4** were formed. Then a demethylation occurred at the 1,2-propane-diol side chain, together with chlorination at C-2 to obtain compound **8**. From compound **8**, oxidative cleavage and oxidation occurred to obtain compounds **2** and **6**. On the other hand, oxidative cleavage and demethylation occurred on compounds **3** and **4** to obtain compound **1**, followed by an oxidation to obtain compound **5.**

Again, the studies on *L. rhinoceros* are still unsufficient due to the limited resource supply. This study demonstrated that cultured mycelia of *L. rhinoceros* has potential not only to be an anti-fungal material, but also to produce chlorine contained natural products and is a treasure for natural compounds discovering.

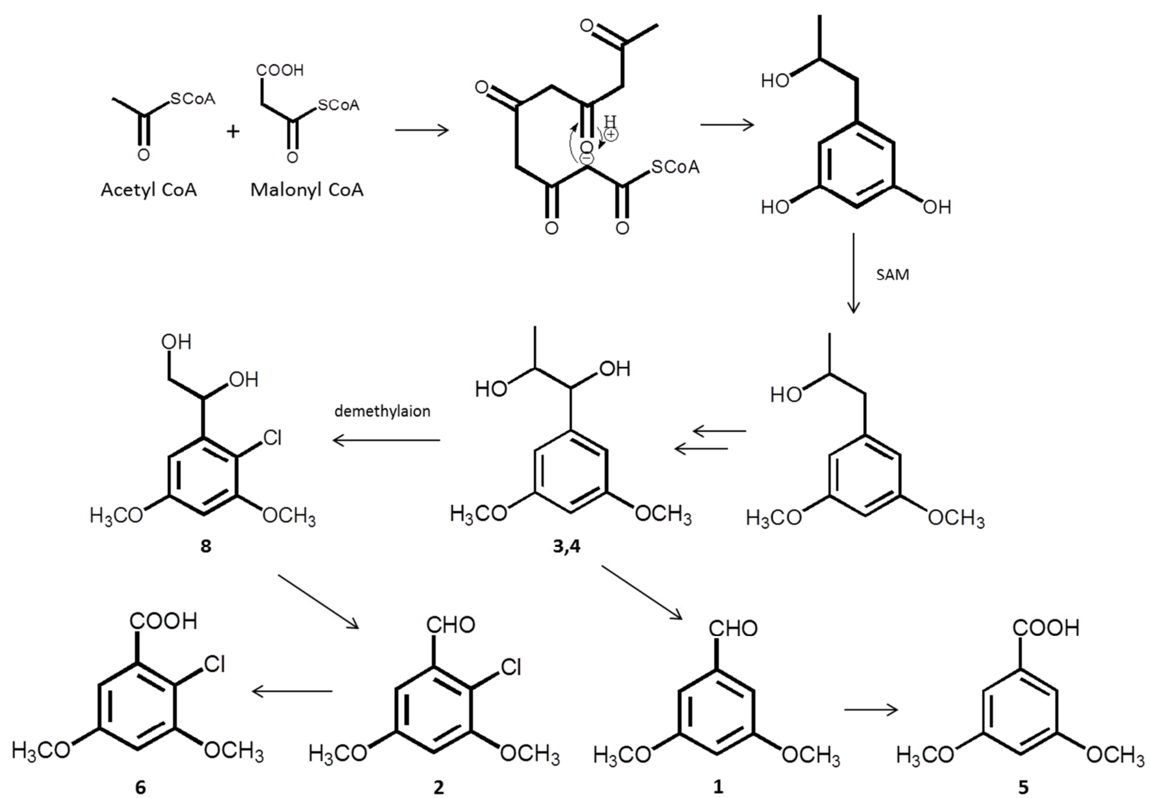


Figure 16. Proposed biosynthesis pathway of compounds **1-6** and **8**

Chapter 3

Anti-fungal study of *Isaria* sp.

Section 1

About *Isaria* sp. (Tosakasanagihanatake)

Isaria sp. is belonged to *Clavicipitaceae*. Species belonged to this family have unique parasitic life cycle. They are also called “Dong Chong Xia Cao“ (means “winter worm, summer grass”) in China and “Tochukaso” in Japan. The fungal spores or mycelia infect and take over the host organism, this eventually cause the host death. Then the fungal continues to grow and emerges from the corpse of the host organism (Figure 17). When people talk about “Tochukaso”, the first idea comes up with *Cordyceps*. Nevertheless, *Isaria* is particularly important since they show wide range of bioactive compounds (Haritakun R. 2007) such as immunosuppressant sphingoid ISP-I (Fujita T., 1994), antioxidative pseudo-dipeptide hanasanagin (Sakakura A., 2005) and three types of insecticidal cyclodepsipeptides (Vining L.C.. 1962; Baute R., 1981 and Briggs L.H., 1968).

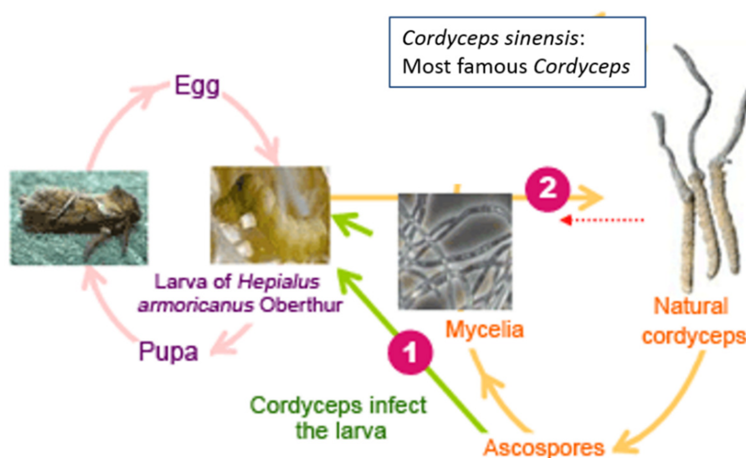


Figure 17. Life cycle of *Codyceps sinnensis*

Modified from http://www.cgb.com.tw

Section 2

Isolation of bioactive compounds from *Isaria* sp.

The results showed that the culture broth of *Isaria* sp., had anti-fungal activity to *M. canis*. Large scale culture (20L) was performed using Roux flask. The culture broth was collected after 6 weeks cultivation for further purification and isolation (Figure 18). The culture broth was then passed through HP-20 column chromatography. By means of bioactivity-guided fractionation procedure, fraction washed by 100% MeOH (fr. 3) showed the anti-fungal activity. Further purification was then carried out by silica gel column chromatography. And it showed that fr. 3-3 and 3-4 (Figure 19) from $CHCl_3$: MeOH (99.5:0.5) showed the bioactivity. fr. 3-3 and 3-4 were then put together (named Fr. 3-3') for further purification. As shown in the TLC (Figure 19), fr. 3-3'-4 appeared in a single spot. And HPLC analysis showed it had high purity (Figure 20).

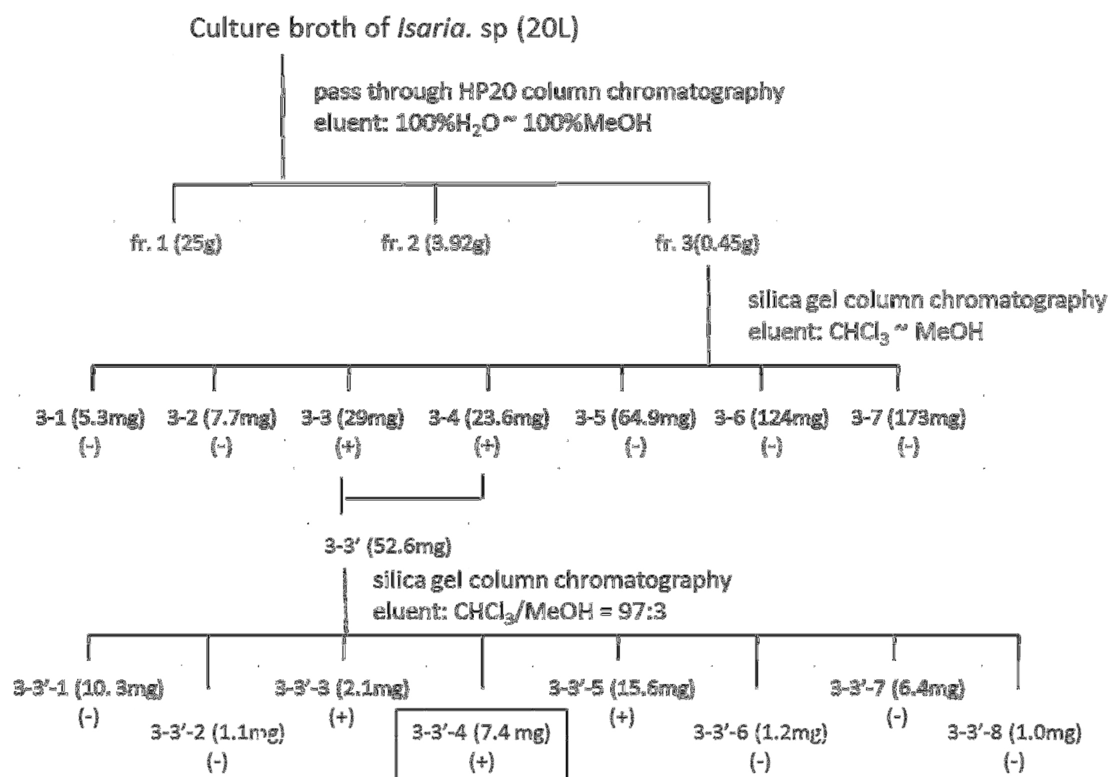


Figure 18. Separation procedures of bioactive fraction from *Isaria* sp. culture broth

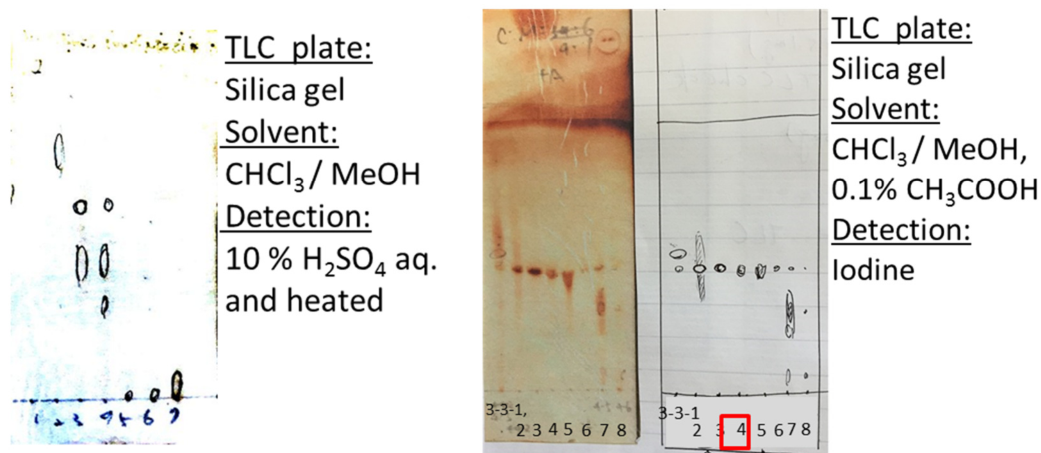


Figure 19. TLC analysis of fr. 3-1~3-7 (left) and fr. 3-3'-1~3-3'-7 (right)
of cultured broth of *Isaria* sp.

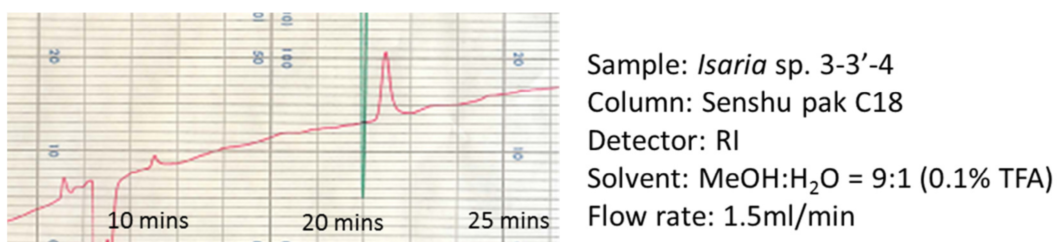


Figure 20. HPLC analysis of bioactive fraction 3-3'-4 from cultured broth of *Isaria* sp.

Since Fr. 3-3'-4 showed high purity. NMR spectrum was measured in order to understand the structure. However, as shown in Figure 21, fr. 3-3'-4 seems to exist as more than two conformers and was hard to understand the structure. Therefore, I tried to add 0.1% trifluoroacetic acid (TFA) in order to stabilize its structure. After treating TFA, the NMR spectrum showed its structure was changed (Figure 21). Unfortunately, this change was irreversible. While we applied the samples before/after TFA treating, the bioactivity was decreased to *C. albicans*, *M. canis* and *T. rubrum* (Table 10).

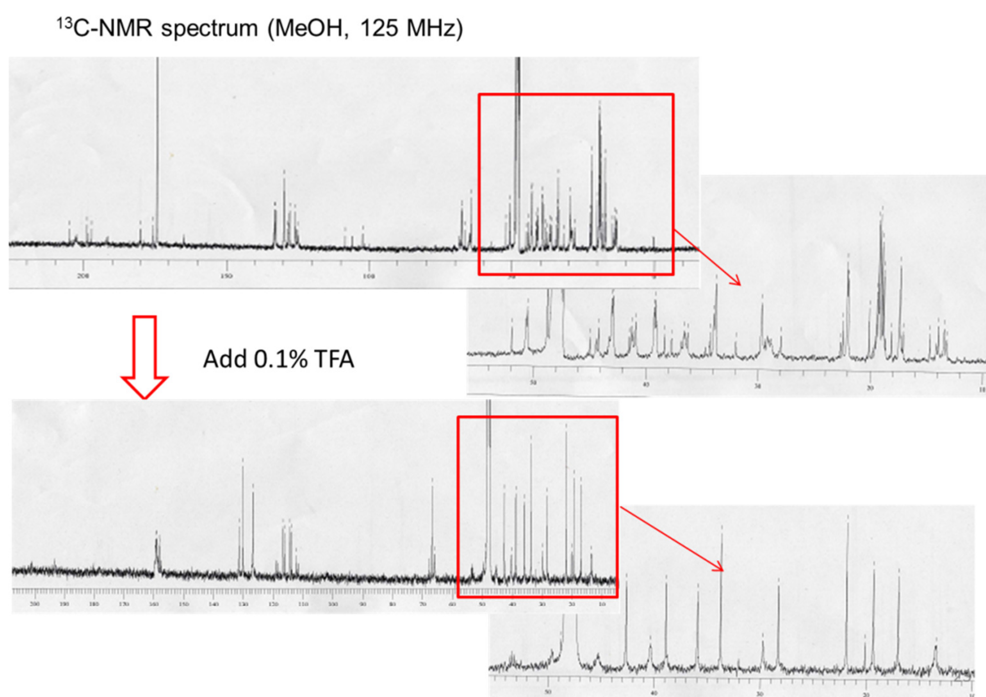


Figure 21. ^{13}C-NMR spectrum of *Isaria* sp. Fr. 3-3'-4 before/after TFA treating

Table 10. Bioactivities of *Isaria* sp. Fr. 3-3'-4 before/after TFA treating

Sample	Sample applied (mg)	Inhibition zone (mm)		
		C. albicans	*M. canis*	*T. rubrum*
Isria sp. Fr. 3-3'-4 before TFA treating	0.2	13.4 x 13.4	12.0 x 14.0	22.7 x 21.6
Isria sp. Fr. 3-3'-4 after TFA treating	0.2	10.3 x 10.3	8.2 x 8.0	9.8 x 9.6

Section 3

Isolation of compounds from *Isaria* sp. cultured mycelia

There were many kinds of secondary metabolites reported from the family Clavicipitaceae mushrooms, including nucleosides, polysaccharides, terpenoids and steroids (Chen P.X., 2013). In this section, the cultured mycelia of *Isaria* sp. were collected after 6 weeks cultivation. The cultured mycelia were freeze dried (136.7g) and then extracted with MeOH to obtain MeOH extract (15.4g). The MeOH extract was then partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ extract was purified by silica gel column chromatography and HPLC to obtain compounds **9** (14mg), **10** (7.7mg), **11** (4.2mg), **12** (3.3mg) and **13** (3.2mg).

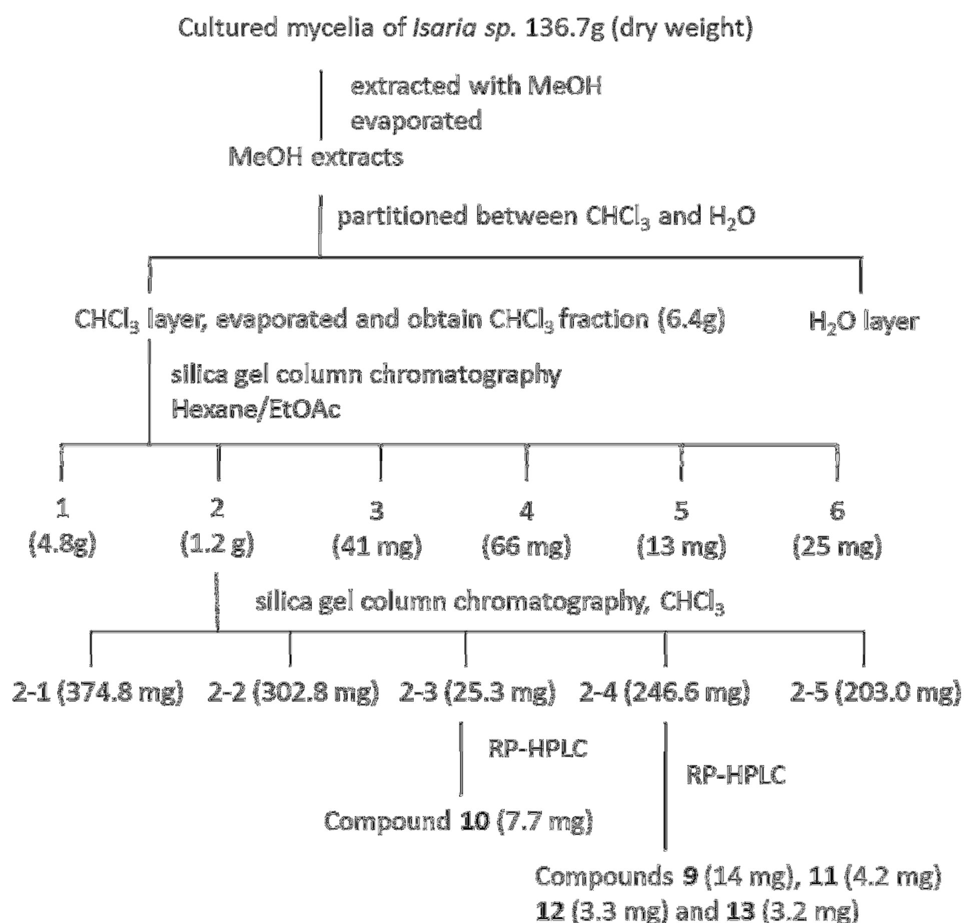
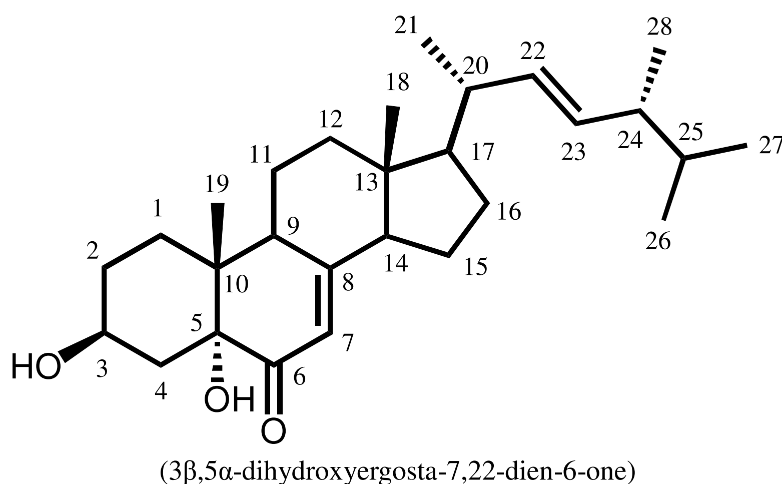


Figure 22. Separation procedures of compounds **9-13** from *Isaria* sp. cultured mycelia

Section 4

Structure of compound **9**

Compound **9** was observed as white powder with $[\alpha]_D$ 10.0 (*c* 1.0, $CHCl_3$). According to the LR-EI-MS, compound **9** was found to have a molecular ion peak at *m/z* 428. In ^{13}C-NMR spectrum (Table 11), 28 signals were observed and identified as a steroid derivative. And DEPT spectrum showed it has 6 methyl signals, 7 methylene signals, 10 methine signals and 5 quaternary carbon signals. Two additional oxygen atoms in the molecular weight of **9** in comparison to the molecular weight of ergosterol and a signal at 198.4 ppm showed one of the oxygen is a ketone group. And the ^{1}H-NMR of an olefin methine signal at 5.63 ppm appeared as a singlet peak indicates that there are two quaternary carbons connect to it. The observation of cross-peaks in the HMBC between the signal at δ 5.63 (H-7) and the carbon signals corresponding to C-5, C-9 and C-14 indicate that the ketone group is at C-6 position. And the downfield shift of the 3α proton at 4.02 ppm is consistent with the α stereochemistry for the 5α-hydroxy group (Anna A., 1990; Ismcs S., 1991 and Venkateswarlu Y., 1996). As the results, compound **9** was assumed to be 3β,5α-dihydroxyergosta- 7,22-dien-6-one. And the carbon chemical shift of compound **9** almost matched to the literature (Ishizuka T., 1992). Therefore, compound **9** was identified as 3β,5α-dihydroxyergosta-7,22- dien-6-one (Figure 23).



(3β,5α-dihydroxyergosta-7,22-dien-6-one)

Figure 23. The structure of compound **9**

Table 11. ^{13}C-NMR data of compounds **9-12**

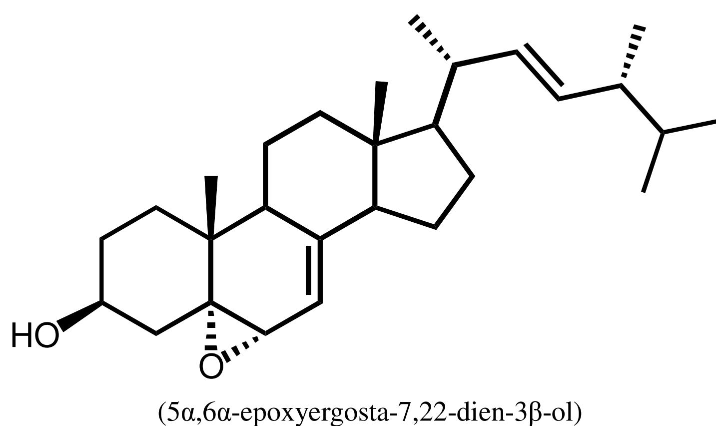
	δC			
	9[a]	**10**[a]	**11**[b]	**12**[c]
1	30.3	31.6	26.4	32.9
2	30.4	30.5	31.5	30.8
3	67.5	67.4	66.8	67.4
4	38.9	39.2	38.1	39.7
5	77.9	76.1	79.8	43.4
6	198.1	70.4	199.1	73.2
7	119.7	119.4	120.3	118.1
8	165.1	142.1	164.1	142.8
9	44.8	43.7	75.1	75.9
10	36.6	38.6	42.3	37.1
11	22.5	21.3	29.0	22.0
12	40.2	38.5	35.5	39.5
13	44.0	43.3	45.4	43.4
14	55.8	54.7	52.0	54.9
15	22.0	22.7	22.8	23.0
16	27.8	28.0	28.4	28.2
17	56.2	55.9	56.2	56.4
18	12.7	12.2	12.4	11.8
19	17.6	17.8	20.4	17.9
20	40.5	40.4	40.6	40.8
21	21.1	21.1	21.3	20.7
22	135.0	135.4	136.2	136.0
23	132.6	132.1	132.5	132.2
24	42.9	42.8	43.1	43.7
25	33.1	33.1	33.4	33.4
26	19.9	19.9	20.2	19.5
27	19.6	19.6	19.9	19.1
28	16.4	17.6	17.9	17.2

[a] Run in $CDCl_3$ [b] Run in d_5-pyridine [c] Run in CD_3OD

Section 5

Structure of compound **10**

Compound **10** was obtained as white powder with $[\alpha]_D$ 12.7 (*c* 0.3, MeOH). According to the LR-EI-MS, compound **10** was found to have a molecular ion peak at *m/z* 412. In ^{13}C-NMR spectrum (Table 11), 28 signals were observed and identified as a steroid derivative. And DEPT spectrum showed it has 6 methyl signals, 7 methylene signals, 11 methine signals and 4 quaternary carbon signals. One additional oxygen atom in the molecular weight of **10** in comparison to the molecular weight of ergosterol and the observation in the ^{1}H-NMR of a signal at 3.93 ppm and the olefin methine signal at 5.00 ppm is in agreement with this proposal. The observation of cross-peaks in the HMBC between the signal at 5.00 ppm (H-7) and the carbon signals corresponding to C-5, C-9 and C-14 and the cross peaks for the signal at 3.93 ppm (H-6) with the carbon signals corresponding to C-7, C-8 and C-10 indicate the epoxide is in the 5,6 position. Similar to the reason of compound **9**, the downfield shift of the 3α proton at 3.93 ppm is consistent with the α stereochemistry for the 5α-epoxy group. As the results, compound **10** was assumed to be 5α,6α-epoxyergosta-7,22-dien-3β-ol. And the carbon chemical shift of compound **10** almost matched to the literature (Bok J.W., 1999). Therefore, compound **10** was identified as 5α,6α-epoxyergosta-7,22-dien-3β-ol (Figure 24).



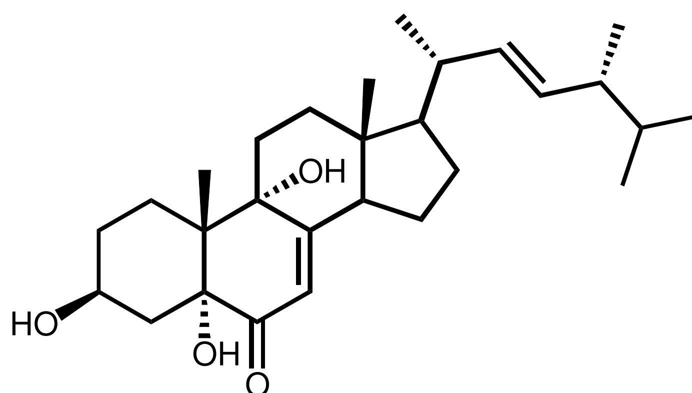
(5α,6α-epoxyergosta-7,22-dien-3β-ol)

Figure 24. The structure of compound 10

Section 6

Structure of compound **11**

Compound **11** was obtained as white powder with $[\alpha]_D$ -13.2 (*c* 0.28, MeOH). According to the LR-EI-MS, compound **11** was found to have a molecular ion peak at *m/z* 444. In ^{13}C-NMR spectrum (Table 11), 28 signals were observed and identified as a steroid derivative. And DEPT spectrum showed it has 6 methyl signals, 7 methylene signals, 9 methine signals and 6 quaternary carbon signals. One additional oxygen atom in the molecular weight of **11** in comparison to the molecular weight of compound **9** and a signal at 199.1 ppm showed one oxygen is a ketone group. And the ^{1}H-NMR of an olefin methine signal at 5.57 ppm appeared as a singlet peak indicates that the ketone is at C6 position like compound **9**. The observation of cross-peaks in the HMBC between the signal at 5.57 ppm (H-7) and the carbon signals corresponding to C-5 (79.8 ppm), C-9 (75.1 ppm) and C-14 indicate that the additional hydroxyl group is at C-9 position. And the downfield shift of the 3α proton at 3.92 ppm is consistent with the α stereochemistry for the 5α-hydroxy group. As the results, compound **11** was assumed to be a trihydroxyergosta- 7,22-dien-6-one compound. Together with the previous report (Cai H.H., 2013), the carbon chemical shift of compound **11** almost matched to the shift reported in the literature. Therefore, hydroxyl group at C-9 was considered as a α stereochemistry and compound **11** was identified as 3β,5α,9α- trihydroxyergosta-7,22-dien-6-one (Figure 25).



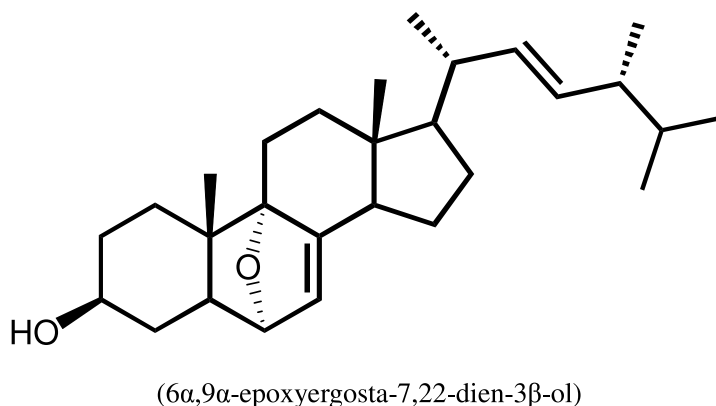
(3β,5α,9α- trihydroxyergosta- 7,22-dien-6-one)

Figure 25. The structure of compound **11**

Section 7

Structure of compound **12**

Compound **12** was obtained as white powder with $[\alpha]_D$ -29.6 (*c* 0.15, MeOH). According to the LR-EI-MS, compound **12** was found to have a molecular ion peak at *m/z* 412. In ^{13}C-NMR spectrum (Table 11), 28 signals were observed and identified as a steroid derivative. And DEPT spectrum showed it has 6 methyl signals, 7 methylene signals, 11 methine signals and 4 quaternary carbon signals. The molecular weight of **12** is the same as the molecular weight of **10.** The slight difference on ^{1}H-NMR and ^{13}C-NMR spectrum between compound **10** and **12** showed that compound **12** has similar structure to compound **10**. Compare to the data reported in the literature (Chen X.M., 2000). Compound **12** was identified as 6α,9α-epoxyergosta-7,22-dien-3β-ol (Figure 26).



(6α,9α-epoxyergosta-7,22-dien-3β-ol)

Figure 26. The structure of compound **12**

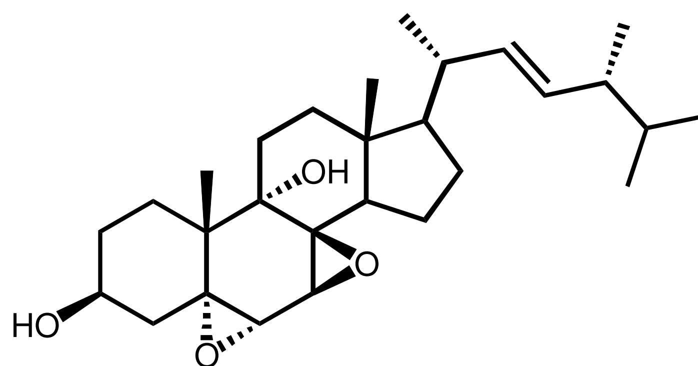
Section 8

Structure of compound **13**

Compound **13** (Figure 27) was obtained as white powder with $[\alpha]_D$ -43.8 (*c* 0.18, MeOH). Compound **13** was found to have a molecular ion peak by LR-EI-MS at *m/z* 444, and was also found to have a peak by HR-ESI-MS at *m/z* 467.3047 $[M+Na]^+$ with a molecular formula $C_{28}H_{44}O_4Na$. In ^{13}C-NMR spectrum (Table 12), 28 signals were observed and identified as a steroid derivative. And DEPT spectrum showed it has 6 methyl signals, 7 methylene signals, 10 methine signals and 5 quaternary carbon signals. Three additional oxygen atoms in the molecular weight of **13** in comparison to the molecular weight of ergosterol and the observation in the ^{13}C-NMR of 6 peaks at the region between 60 ppm and 70 ppm indicate that compound **13** has 2 hydroxy groups and 2 epoxides. In HMBC, 1.04ppm (d, *J*=6.8Hz) has cross-peaks with (CH, 40.7ppm), (CH,135.8ppm; 0.96ppm (d, *J*=6.8Hz) has cross-peaks with (CH, 132.4ppm), (CH,43.3ppm); 0.87ppm (d, *J*=6.8Hz) has cross-peaks with (CH, 43.3ppm), (CH, 33.4ppm); 0.76ppm (s) has cross-peaks with (CH2,32.9ppm), (C,40.9ppm), (CH,53.0) and (CH,53.4ppm). It showed similar chemical shift and HMBC correlation with other ergosterol derivatives. Therefore, compound **13** was identified with an ergostane structure compound. The observation in the ^1H NMR showed that signals on C-26 and C-27 were in the same coupling constant of 6.8 Hz indicate that the configuration of the methyl group at C-24 is to be *S* (Gunatilaka A., 1981). And the observation of cross-peaks in the HMBC (Figure 28) between the signal 1.57ppm (H-14) and the carbon signal corresponding to C-8 (65.7ppm); 1.38 ppm (H-19) and the carbon signals corresponding to C-1, C-5, C-9 and C-10; the cross peak for the signal at 4.15 ppm (H-6) with the carbon signal corresponding to C-7 and the cross peak for the signal at 3.08 ppm (H-7) with the carbon signal corresponding to C-8 indicate that C-5, C-6, C-7, C-8 and C-9 are with oxygen. Also, the signal 1.85ppm (H-11) and the carbon signals corresponding to 65.7ppm and 69.5ppm indicates that C-8 is next to C-9. The C-H coupling constant of C-7 is 175Hz and the observation of a correlation in the COSY (Figure 28) between H-6 (4.15ppm) and H-7 (3.08ppm) indicate that the 2 epoxides may at C5-C6 and C7-C8 position. And the observation of cross-peak in the NOESY (Figure 28) between the signal at H-19 (1.38 ppm) and H-6 (4.15 ppm) indicate that C5-C6 epoxide is α configuration, the downfield shift of the 3α proton at 3.82 ppm in agreement with this proposal. According to Karplus equation, the coupling constant of 2.2Hz at H-6 and H-7 protons indicate that C7-C8 epoxide is β configuration. As the results, compound **13** was identified as a new compound named 5α,6α:7β,8β-diepoxyergost-22-ene-3β,9α-diol (Figure 27).

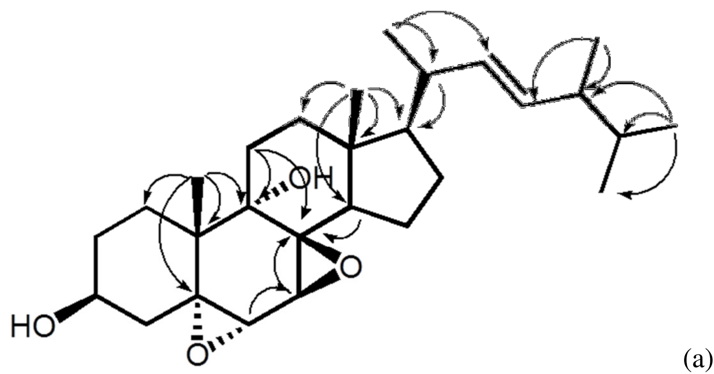
Table 12. NMR data of compounds **13**

Positions	δC		δH
1	28.2	CH_2	1.84
2	30.1	CH_2	2.05
3	67.9	CH	3.82
4	40.3	CH_2	1.21
5	66.5	C	
6	66.0	CH	4.15 (d, J = 2.2 Hz)
7	61.7	CH	3.08 (d, J = 2.2 Hz)
8	65.7	C	
9	69.5	C	
10	35.9	C	
11	22.2	CH_2	1.85
12	32.9	CH_2	1.52
13	40.9	C	
14	53.0	CH	1.57
15	23.2	CH_2	2.11
16	28.0	CH_2	1.65
17	53.4	CH	1.24
18	11.8	CH_3	0.76 (s)
19	20.9	CH_3	1.38 (s)
20	40.7	CH	2.03
21	20.7	CH_3	1.04 (d, J = 6.8 Hz)
22	135.8	CH	5.22 (dd, J = 15.0, 7.2 Hz)
23	132.4	CH	5.24 (dd, J = 15.0, 7.8 Hz)
24	43.3	CH	1.86
25	33.4	CH	1.49
26	19.5	CH_3	0.87 (d, J = 6.8 Hz)
27	19.1	CH_3	0.87 (d, J = 6.8Hz)
28	17.2	CH_3	0.96 (d, J = 6.8 Hz)

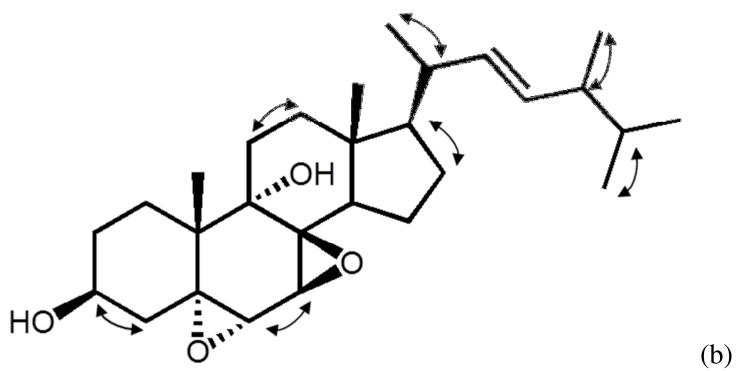


(5α,6α:7β,8β-diepoxy-ergost-22-ene-3β,9α-diol)

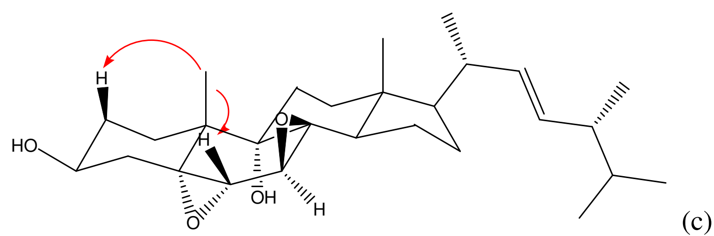
Figure 27. The structure of compound **13**



(a)



(b)



(c)

Figure 28. HMBC (a), COSY (b) and NOESY (c) correlation of compound **13.**

Section 9

Anti-microbial activities of compounds **9-13**

Compound **9-13** were applied to MSSA, *E. coli* and *P. aeruginosa* for the anti-microbial activities. The results were shown in Table 13.

Table13. Compounds **9-13** MICs (mg/L)

Compound	MIC		
	MSSA1	*E.coli*	*P. aeruginosa*
9	128	>128	>128
10	>128	>128	>128
11	>128	>128	128
12	>128	>128	128
13	>128	>128	>128
Vancomycin	0.64		
Gentamycin		4	
Cephataxime			4

Section 10

Discussion

In this study, we demonstrated that cultured mycelia of *Isaria* sp. produce anti-fungal components and release them into the culture broth. *Isaria* species are the imperfect stage of *Cordyceps*, which is belonged to *Clavicipitaceae* (White J., 2003). *Clavicipitaceae* mushrooms have special parasitic life style and attract so many interests. The most well-known specie in this family is *Cordyceps sinensis*, and reports of its secondary metabolites and pharmaceutical activities were described in many literatures (James F. White, 2003). Compare to the well-studied *Cordyceps* medicinal mushrooms, the studies about *Isaria* medicinal mushrooms are limited even though there have been reported to be effective as immunosuppressive agents (Fujita T., 1994 and Iijima M., 1992), antioxidative agent (Sakakura A., 2005) and insecticidal agent (Vining L.C., 1972).

In the early stage of our study, the extract of cultured broth from *Isaria* sp. revealed significant inhibition zones to *M. canis, T. rubrum* and *C. albicans*. To date, no studies were reported about the anti-fungal activities from cultured broth of *Isaria* mushrooms and this investigation was expected to be the first report of anti-fungal components from the cultured broth of *Isaria* mushrooms. However, followed the bioactive guided fractionation procedure, the bioactive components seem to be exist as more than 2 conformers and was hard to understand the structure. Methods including different isolation conditions, methylation (by trimethylsilyldiazomethane), acidic treating and different NMR solvents were carried out in order to understand its structure. Acidic treating with 0.1%TFA seemed to be able to stabilize its structure, however, unfortunately, this change seemed to be irreversible. The anti-fungal activities of bioactive components from *Isaria* sp. significantly decreased after treating with 0.1%TFA.

In order to get access to understand the structure of bioactive components from *Isaria* sp., customized culture conditions and preferred isolation methods are necessary to obtain sufficient material for further experiments. Nevertheless, extract of cultured broth of *Isaria* sp. exhibited the anti-fungal activity and has the potential to protect us away from fungal infections.

On the other hand, several of compounds were isolated from *Clavicipitaceae* medicinal mushrooms including cordycepin, terpenoids, polysaccharides, nucleotides and amino acids (Zhou X.W., 2009). It is worthy to study on their secondary metabolites for new compounds discovery. In this study, five steroid derivatives were isolated from the cultured mycelium of *Isaria* sp., one of them is a new compound named 5α,6α:7β,8β-diepoxy-ergost-22-ene-3β,9α-diol. The proposed biosynthetic pathway of these compounds was shown in Figure 29.

Start from mevalonate, ergosterol is synthesized by mevalonate pathway as an intermediate. An epoxidation is occurred on C5-C6 double bond to synthesize compounds **10** and **12**.

From compound **10**, further oxidation takes place at 5,6-epoxide to synthesize compound **9**, and then a hydroxylation takes place at C-9 to synthesize compound **11**. On the other hand, compound **13** is synthesized when a further epoxidation and hydroxylation take place on compound **10**.

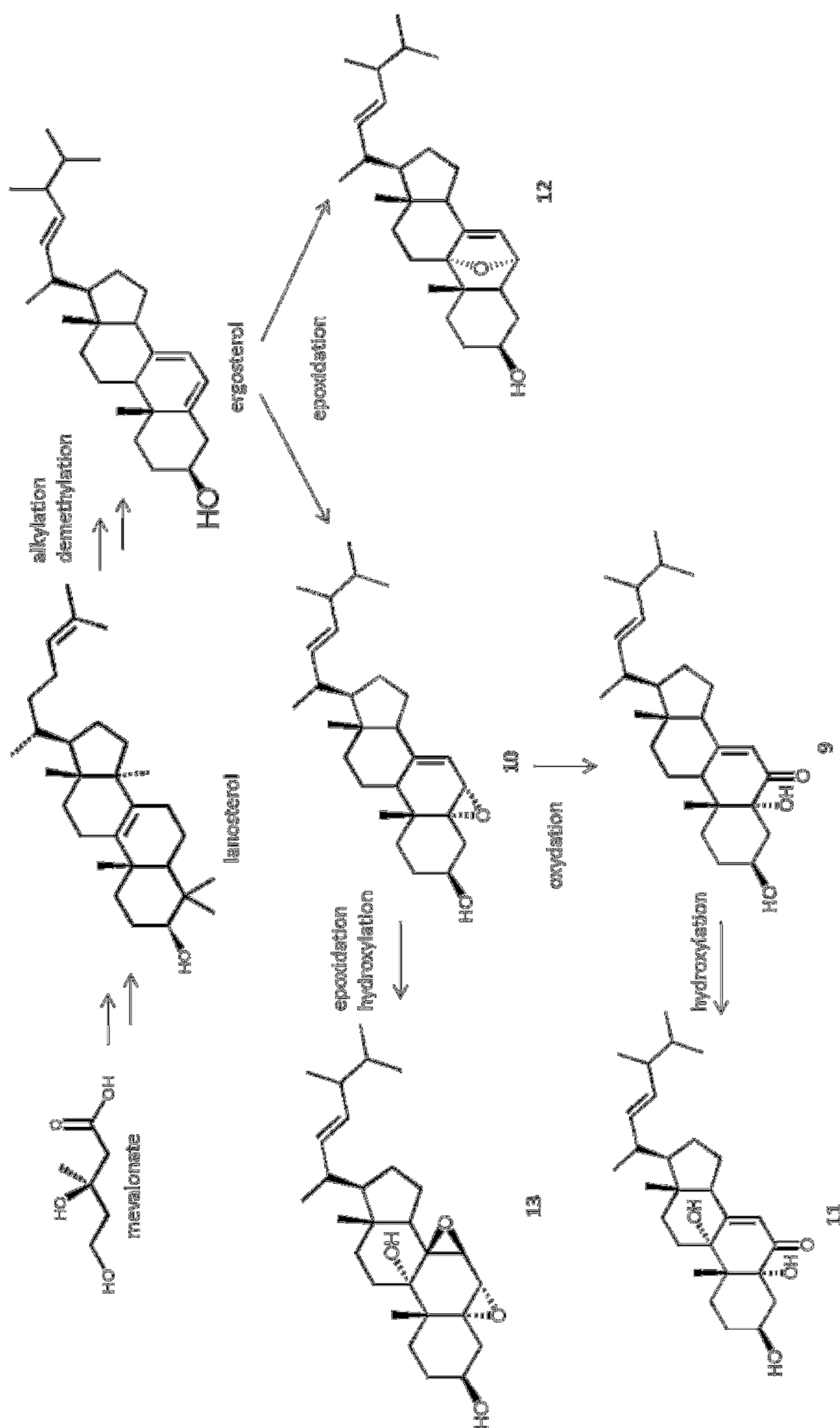


Figure 29. Proposed biosynthetic pathway of steroid derivatives from *Isaria* sp..

Chapter 4
Anti-fungal compounds from *Ganoderma mastoporum*

Section 1

About *Ganoderma mastoporum*

G. mastoporum (Figure 30) is a fungus widely distributed in Southern China, Northern Vietnam, Malaysia, Philippines (Thang T.D., 2013). Compare to *G. lucidum* and *G. applanatum*, it is not well-studied yet. Reports of *G. mastoporum* is limited and it produces some unique sesquiterpene such as ganomastenol (Figure 31) (Hirotani M., 1995, Ino C., 1993)

Figure 30. *Ganoderma mastoporum*

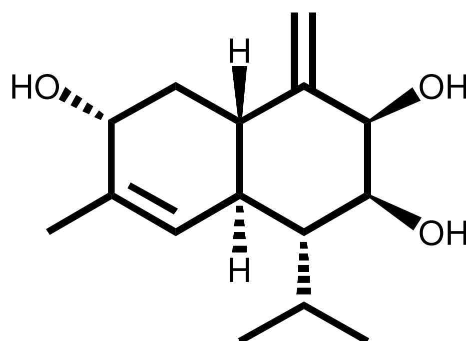


Figure 31. Ganomastenol

Section 2

Isolation of bioactive compounds from *G. mastoporum*

The results showed that the cultured mycelia of *G. mastoporum* have anti-fungal activity (Table 13). Large scale culture (20L) was performed using Roux flask. The cultured mycelia were collected after 6 weeks cultivation and freeze dried (95g) for further purification. Dried mycelia were extracted with MeOH to obtain MeOH extract (15.4g). The MeOH extract was then partitioned between $CHCl_3$ and H_2O to obtain $CHCl_3$ fraction (4g). The $CHCl_3$ was then purified by silica gel column chromatography. By means of bioactivity-guided fractionation procedure, fr. 3 (267.9mg) from $CHCl_3$: MeOH (99.5:0.5) showed the bioactivity. The further purification also performed by silica gel column chromatography to obtain bioactive fraction 3-4 and 3-5. These 2 fractions were purified by RP-HPLC to obtain compounds **14** (11mg), **15** (6mg), **16** (22.2mg), **17** (7.6mg) and **18** (9.2mg). While applied these compounds for the anti-fungal activity test, compounds **14** (11mg) showed anti-fungal activity toward *M. canis* and *T. rubrum*, and **17** (7.6mg) showed anti-fungal activity toward *T. rubrum*.

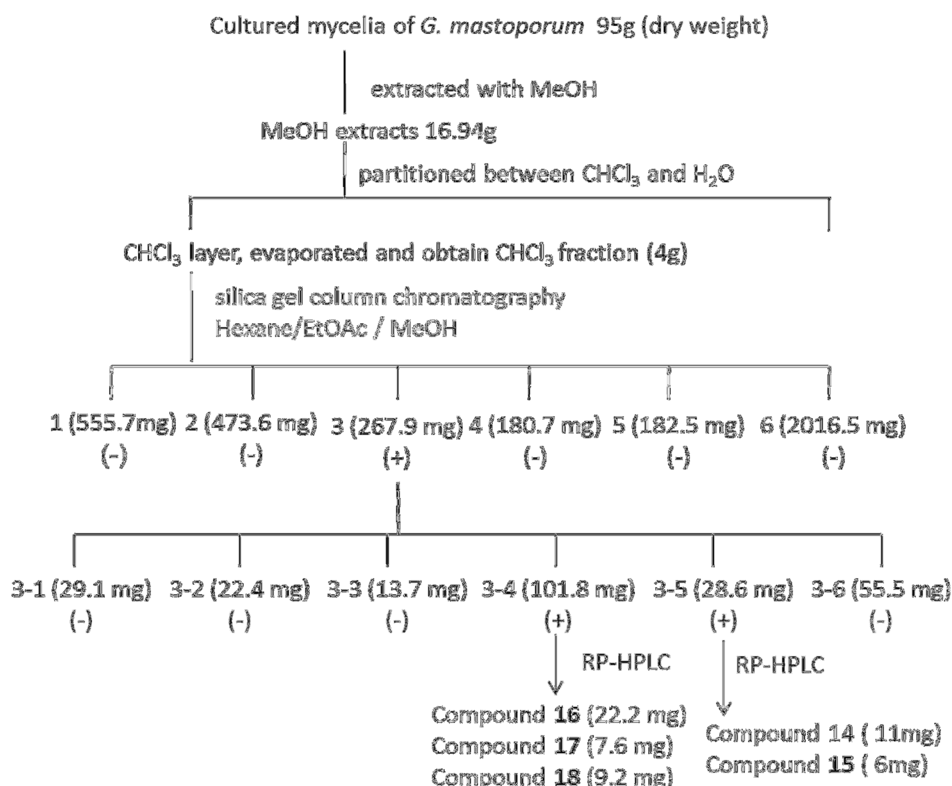


Figure 32. Separation procedures of bioactive compounds from *G. mastoporum*

Section 3

Structure of compound **14**

According to the LR-EI-MS, compound **14** was found to have a molecular ion peak at *m/z* 122. And in the ^{13}C-NMR spectrum (Table 14), only five signals were observed, and four of them are at aromatic region. Considering its molecular weight, it indicated that compound **14** is a compound with symmetric structure. A peak at 172.2 ppm in ^{13}C-NMR spectrum indicates that compound **14** has a carboxylic acid group. And three signals in ^{1}H NMR δ 8.11 (2H, d, J = 7.4 Hz), δ 7.60 (1H, t, J = 7.4 Hz) and δ 7.47 (2H, t, J = 7.4 Hz) indicated that compound **14** has only one substitute. As the results, compound **14** was identified as benzoic acid (Figure 33).

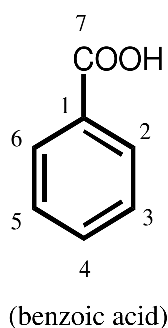


Figure 33. The structure of bioactive compound **14**

Table 14. ^{13}C-NMR data of compounds **14** and **15**

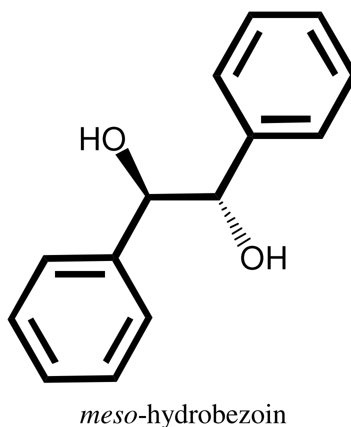
^{13}C-NMR data		
	δC	
	14[c]	**15**[a]
1	129.4	141.5
2	130.3	127.2
3	128.6	127.3
4	133.9	127.0
5	128.6	127.3
6	130.3	127.2
7	172.2	77.7

[a] Run in $CDCl_3$
[c] Run in CD_3OD

Section 4

Structure of compound **15**

Compound **15** was isolated from the same fraction to compound **14**. The LR-EI-MS found that compound **15** has a molecular ion peak at *m/z* 108. In the ^{1}H-NMR, the signal observed at 4.75ppm indicates compound **15** may have a benzene ring with an alkyl hydroxy substitute. However, the DEPT spectrum indicated that the only carbon on the substitute of compound **15** is a methine but not methylene. This fact conflicts to the hypothesis of alkyl hydroxy substitute. In order to clear this question, compound **15** was performed to LR-EI-MS again in a slow chamber heating rate of 8 and a peak at *m/z* 214 was found with this change. As the results, compound **15** was assumed to be hydrobenzoin. Compare to the ^{13}C-NMR reported in the literature (Griffith J., 2010) and standard, the chemical shift in compound **15** (Table 14) matched perfectly. Therefore, compound **15** was identified as *meso*-hydrobezoin (Figure 34).



meso-hydrobezoin

Figure 34. The structure of compound **15**

Section 5

Structure of compound **16**

Compound **16** was observed as white powder with $[\alpha]_D$ -49.1 (*c* 1.7, $CHCl_3$) According to the LR-EI-MS, compound **16** was found to have a molecular ion peak at *m/z* 396. In ^{13}C-NMR spectrum (Table 15), 28 signals were observed and identified as a steroid derivative. And DEPT spectrum showed it has 6 methyl signals, 7 methylene signals, 11 methine signals and 4 quaternary carbon signals. ^{13}C-NMR also indicates that compound **16** has 6 carbons in alkene region. Together with ^{1}H-NMR spectrum, two proton signals at 5.56ppm and 5.38ppm indicate that compound **16** has two double bonds at C5-C6 and C7-C8. These data indicate that compound 16 may be ergosterol. And the signals in ^{13}C-NMR of compound **16** almost match to ergosterol reported in the literature (William B. Smith, 1977). Therefore, compound **16** was identified as ergosterol (Figure 35).

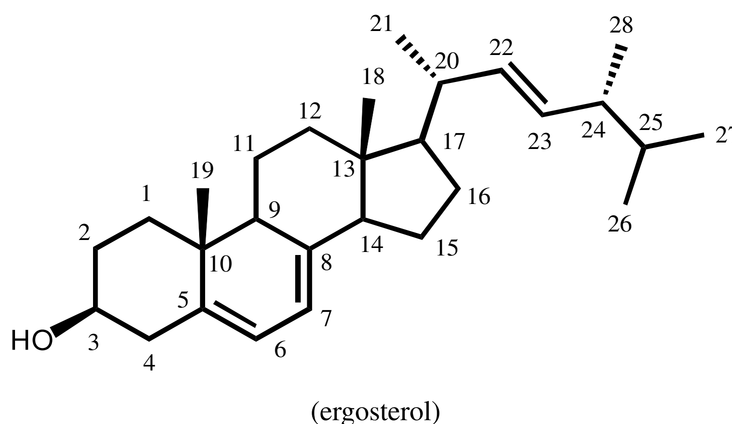


Figure 35. The structure of compound **16**

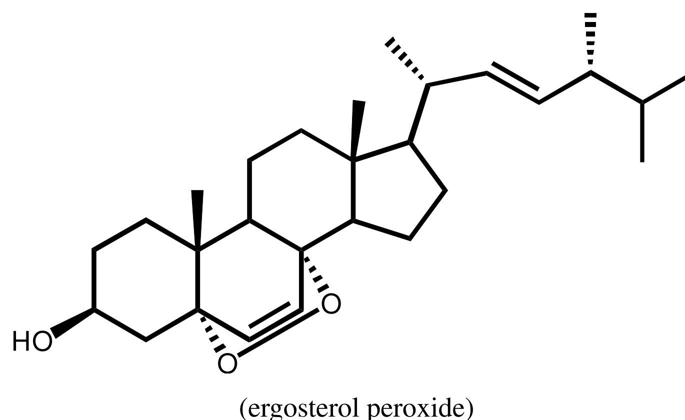
Table 15. ^{13}C-NMR data of compounds **16-18**

^{13}C-NMR data ($CDCl_3$)	δC		
	16	**17**	**18**
1	38.4	34.7	38.4
2	32.1	30.2	32.0
3	70.5	66.5	70.4
4	40.9	37.0	40.8
5	139.8	79.5	21.1
6	119.7	130.8	33.1
7	116.4	135.5	119.6
8	141.4	82.2	141.3
9	46.3	51.7	139.8
10	37.1	37.0	42.6
11	21.2	20.7	116.3
12	39.2	39.4	39.1
13	42.9	44.6	42.8
14	54.6	51.1	54.5
15	23.0	23.4	23.0
16	28.3	28.7	28.3
17	55.8	56.2	55.7
18	12.1	12.9	12.0
19	16.3	18.2	17.6
20	40.4	39.8	40.4
21	21.2	20.9	21.1
22	135.6	135.2	135.5
23	132.1	132.3	132.0
24	42.9	42.8	42.8
25	33.1	33.1	33.1
26	20.0	20.0	20.0
27	19.7	19.7	19.6
28	17.6	17.6	16.2

Section 6

Structure of compound **17**

Compound **17** was observed as white powder with $[\alpha]_D$ -17.8 (*c* 0.6, $CHCl_3$). According to the LR-EI-MS, compound **17** was found to have a molecular ion peak at *m/z* 428. In ^{13}C-NMR spectrum, 28 signals were observed and assumed as a steroid derivative. And DEPT spectrum showed it has 6 methyl signals, 7 methylene signals, 11 methine signals and 4 quaternary carbon signals. The difference in molecular weight between ergosterol **(16)** and compound **17** might be resulted from 2 additional oxygens, implying that compound **17** is a peroxidated derivative of ergosterol **(16)**. And the lack of one pair of double bond in ^{13}C-NMR supports this hypothesis. Compare to the ^{13}C-NMR spectrum reported in the literature (Shin Y., 2001), compound **17** was identified as ergosterol-peroxide (Figure 36).



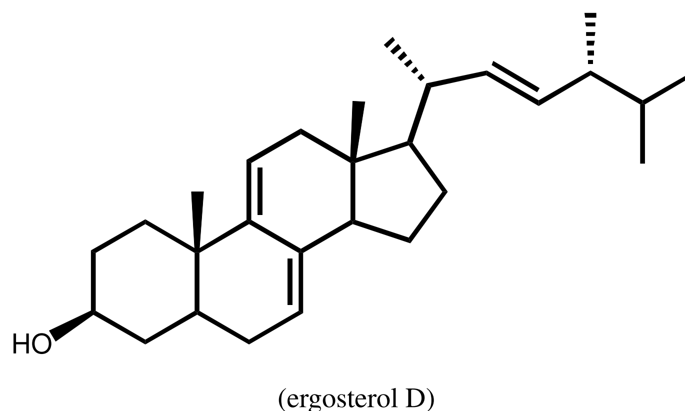
(ergosterol peroxide)

Figure 36. The structure of compound **17**

Section 7

Structure of compound **18**

Compound **18** was observed as white powder with $[\alpha]_D$ -101.8 (*c* 0.5, $CHCl_3$). According to the LR-EI-MS, compound **18** was found to have a molecular ion peak at *m/z* 396. The molecular weight is the same to ergosterol (**16**). However, the difference in ^{13}C-NMR and ^{1}H-NMR indicate that compound **18** has different structure to ergosterol (**16**). Interestingly, in ^{13}C-NMR spectrum (Table 15), only 26 signals were observed, the DEPT showed they are 6 methyl signals, 6 methylene signals, 11 methine signals and 3 quaternary carbon signals and the signals at 21.1ppm and 42.8ppm have stronger intensities than any other signals. This indicates that the 2 signals may overlap to other peaks. This phenomenon is in agreement with the previous study (Abraham R., 1974). Compare tp the ^{13}C-NMR reported in the literature (Huang G.J., 2012), compound **18** was identified as ergosterol D (Figure 37).



(ergosterol D)

Figure 37. The structure of compound **18**

Section 8

Anti-fungal and anti-microbial activities of compounds **14-18**

Compounds **14-18** were applied to *Candida albicans*, *Microsporum canis* and *Trichophyton rubrum* for anti-fungal activity test. The results were shown in Table 16. Also, compounds **14-18** were applied to MSSA, *E. coli* and *P. aeruginosa* for the anti-microbial activities. The results were shown in Table 17.

Table 16. Anti-fungal activities of compounds **14-18**

Compound		*C. Albicans*	*M. canis*		*T. rubrum*	
		6 days	6 days	14 days	6 days	14 days
14	0.6 mg	8 x 8	17.5 x 19.2	8 x 8	16.1 x 15.6	8 x 8
15	0.4 mg	8 x 8	8 x 8	8 x 8	8 x 8	8 x 8
16	0.4 mg	8 x 8	8 x 8	8 x 8	8 x 8	8 x 8
17	0.4 mg	8 x 8	8 x 8	8 x 8	16.3 x 14.5	8 x 8
18	0.4 mg	8 x 8	8 x 8	8 x 8	8 x 8	8 x 8
Miconazole	0.002 mg	27.0 x 27.4	42.8 x 41.3	31.2 x 31.4	32.2 x 36.6	26.3 x 26.5

Microbial inoculation: 10^7cfu/ml. Paper disk size: 8 x 8 (mm)

Table 17. Compounds **14-18** MICs (mg/L)

Compound	MIC		
	MSSA1	*E.coli*	*P. aeruginosa*
14	>128	>128	>128
15	>128	>128	>128
16	>128	>128	>128
17	64	>128	128
18	>128	>128	>128
Vancomycin	0.64		
Gentamycin		4	
Cephataxime			4

Section 9

Discussion

Ganodermataceae medicinal mushrooms are the promised resources for bioactive components discovery. Studies are focus on polysaccharides and triterpenes/triterpenoids compounds. They are proofed to have pharmacological activities such as anti-tumor and immune-modulating activities for hypoglycemosis, hepatoprotection, and the effect on blood vessel system (Zhou X.W., 2007). In this study, five compounds (Figure 38) were isolated from the cultured mycelia of *G. mastoporum*. While applied these compounds to *M. canis*, *T. rubrum* and *C. albicans*, the bioactivities of compounds **14** and **17** were confirmed by paper disk diffusion method.

Compound **14** was identified as benzoic acid, which showed anti-fungal activity toward *M. canis* and *T. rubrum*. As mentioned in the chapter 1, benzoic acid inhibits biosynthesis of mycotoxins and growth of various fungi, both filamentous and yeasts (Tawata S., 1996 and Florianowicz T, 1998). Moreover, benzoic acid shows its anti-fungal activity is caused by high concentration of benzoic acid lower the intracellular pH into the range where phosphofructokinase is sensitive (Krebs H., 1983). In Japan, benzoic acid is admitted by Ministry of Health, Labour and Welfare as a preservative, which could be used in margarine, cavier, soy source, drinks and so on (http://www.ffcr.or.jp/).

Compound **17** was identified as ergosterol-peroxide, which showed anti-fungal activity toward *T. rubrum* and anti-bacteria activity toward MSSA and *P. aeruginosa*. Literatures have reported that ergosterol-peroxide exhibits anti-fungal activities toward *Magnaporthe oryzae* (Zhao J.L., 2010), anti-bacteria activity toward *Mycobacterium tuberculosis* (Duarte N., 2007). Also, anti-tumor activity was also reported (Bok J.W., 1999).

Compound **18** was identified as ergosterol D. Although it didn’t show anti-microbial activities in this study, the previous report showed ergosterol D can block the reduction of serum NO level in CCl_4-treated mice as a liver protective agent (Huang G.J., 2012) and has the potential to be a liver protective food supplement.

In this study, the crude extract of cultured mycelia of *G. mastoporum* showed strong anti-fungal activity in the prior investigation. However, the bioactive fractions seemed to be spread out during the purification and isolation. Different structures of anti-fungal compounds in this study explained that the strong anti-fungal activity of the crude extract may come from more than two compounds from the cultured mycelia of *G. mastoporum*. Instead of pure compounds, the crude extract of *G. mastoporum* might be preferred as an anti-fungal material to prevent our daily life away from the fungal infection.

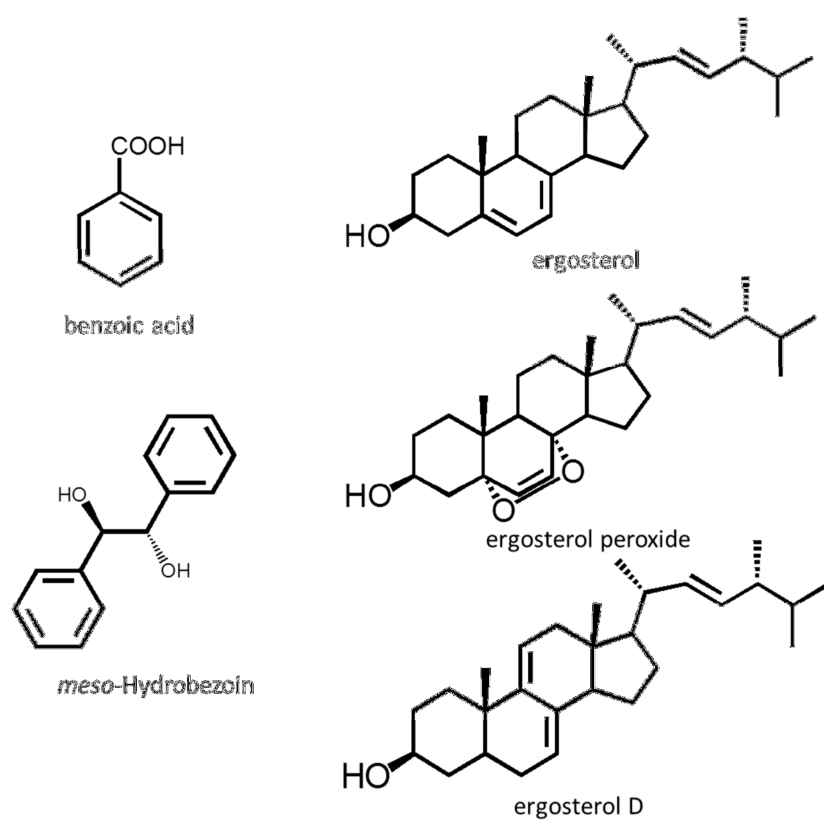


Figure 38. Compounds isolated from the cultured mycelia of *G. mastoporum*

Conclusion

Two anti-fungal compounds **1** (3,5-dimethoxybenzaldehyde) and **2** (2-chloro-3,5-dimethoxybenzaldehyde) were isolated from the cultured broth of *L. rhinoceros*. In addition, five known compounds (compounds **3-7**) and one new compound (compound **8**, named 1-(2-chloro-3,5-dimethoxyphenyl)-ethane-1,2-diol) were also isolated from the cultured broth. Six of the isolates are the first report from the natural products. The results of anti-fungal activities showed that compound **1** is able to provide strong anti-fungal activity but supposed to be decomposed quickly by fungi. On the other hand, compound **2** is less strong than compound **1**, but may harder to be decomposed by fungi. These results indicated that: 1, chlorine substitute may enhance the duration of anti-fungal activity. 2, aldehyde substitute may be necessary for aromatic compounds to exhibit anti-fungal activity. Moreover, three of the isolates (compounds **2**, **6** and **8**) were chlorine contained compounds, and the chlorine contained compounds are comparatively rare from the natural products. This study is the first anti-fungal study on *L. rhinoceros* secondary metabolites, and the results showed the isolates or the extract of cultured broth of *L. rhinoceros* may give us a new option to threat skin infection.

The cultured broth of *Isaria* sp. was proofed to have anti-fungal activity in this study. However, the anti-fungal components seem to have intriguing characteristic and is hard to understand their structure. Customized culture conditions and isolation properties are the primary tasks for further investigation. To this understanding, the extract of cultured broth of *Isaria* sp. might be preferred as an option to treat skin infection. Nevertheless, five steroid derivatives **9-13** were isolated from the cultured mycelia of *Isaria* sp., including four known compounds and one new compound named 5α,6α:7β,8β-diepoxy-ergost-22-ene-3β,9α-diol. Studies on *Isaria* species are remained unsufficient even though they have been proofed to exhibit various pharmaceutical activities. This study demonstrated some possibility of *Isaria* medicinal mushrooms on bioactivity and secondary metabolites.

Five known compounds were isolated from the cultured mycelia of *G. mastoporum*. They are benzoic acid (**14**), *meso*-hydrobezoin (**15**), ergosterol (**16**), ergosterol-peroxide (**17**) and ergosterol D (**18**). Compounds **14** showed anti-fungal activities, and compound **17** not only showed anti-fungal activity but also showed anti-bacterial activities toward MSSA and *P. aeruginosa*. In this study, we can understand that the anti-fungal activity from the cultured mycelia of *G. mastoporum* may result from different anti-fungal mechanisms. This result showed that the extract of cultured mycelia of *G. mastoporum* may be better for anti-fungal utilities.

Experimental

Apparatus and reagents used in the study in Chapter 1~4 are listed below

Apparatus

NMR: JNM-ECA500 (JEOL) and JNM-ECX500 (JEOL)

The chemical shifts are recorded as δ or ppm. The $CHCl_3$ signal at 7.24ppm, the lowest pyridine signal at 8.71ppm and MeOH signal at 3.30ppm are set as reference in ^{1}H-NMR. The $CDCl_3$ signal at 77.0ppm, the lowest d_5-pyridine signal at 149.9ppm and MeOH-d5 signal at 48.0ppm are set as reference in ^{13}C-NMR. Coupling constant (*J*) is represented as Hz. Signal patterns in the ^{1}H-NMR are represented as: singlet = s, doublet = d, triplet = t, quartet = q, multiplet = m, ^{13}C-NMR are represented as: quaternry carbon = s, methine = d, methylene = t, methyl =q.

EI-MS: JMS-SX102A (JOEL)

FAB-MS: JMS-700 (JOEL)

ESI-MS: JMS-T100LC-AccuTOF (JOEL)

HPLC

Pump: LC-10ADVP (Shimadzu)

Oven: CTO-10AVP/10ACVP (Shimadzu)

UV-VIS detector: SPD-M10AVP/10AVVP (Shimadzu)

Refractive index (RI) detector: RID-10A (Shimadzu)

Coloumn: Capcell PAK C18 (10.0 x 250 mm, Shiseido, Japan),
Pegasil-silica (10.0 x 250 mm, Senshu, Japan)

Polarimeter: JASCO P-1010

TLC: Silica gel 60 F254 plates, 1.05715.0009 (Merck)
Silica gel 60 F254, 0.5mm, 1.05744.0009 (Merck)

Silica gel column: Wakogel® C-200 (Wako)

HP20 column: Diaion HP20 (Mitsubishi Chemical Corporation, Japan)

Media for cultured mycelium of medicinal mushrooms

1. Potato dextrose (PD) medium

	Dissolved in 1L water
Potato starch	4.0g
Dextrose	20.0g
Add 15.0g agar to make agar medium (if necessary)	
Adding 5% KOH to adjust to final pH 5.6	

2. Yeast and malt (YM) medium

	Dissolved in 1L water
Yeast extract	3.0g
Malt extract	3.0g
Peptone	5.0g
Sucrose	10.0g
Add 20.0g agar to make agar medium (if necessary)	
Adding 5% KOH to adjust to final pH 6.2	

Microbial used for bioactivity tests

Candida albicans ATCC 44858
Microsporum canis TIMM 1502
Trychophyton rubrum IFO 058074
MSSA1 (methicillin-susceptible *Staphylococcus aureus*)
Escherichia coli W3110
Pseudomonas aeruginosa PAO1

MSSA1 stain is isolated from Kyushu university hospital

Media for microbial cultivation

Agar medium was prepared by adding 15g agar
All media were autoclaved under 121℃ for 15 minutes for sterilizing

LB10 medium

Bacton trypton	10g
Yeast extract	5g
NaCl	10g
Water	1L

Mueller-Hinton (MH)

Mueller-Hinton broth	21g
Water	1L

(Ca^{2+} solution 4.2ml and Mg^{2+} solution 2.0ml were added after sterilizing)

Sabouraud

Peptone	10g
Sucrose	40g
Water	1L

RPMI-1640

RPMI-1640	10.4g
MOPS buffer	34.53g
Water	1L

Paper-disk diffusion method

Paper-disk diffusion method was used for screening including sample extracts and purified samples during the isolation. The samples were dissolved in DMSO and then applied to the paper disk. 100μl broth of each cultured microbial was loaded to the agar medium equally, and then the paper-disks were set on the agar medium. The anti-microbial activities were confirmed if clear inhibition zones were observed. The culture conditions for each microbial was listed in the Table. 18.

Table 18. Culture conditions of microbial

	Medium	Temperature (℃)	Time (day)	Diameters of paper disks (mm)	Sample volume in DMSO (μl)
C. albicans	Sabouraud	37	2	8	40
M. canis	Sabouraud	37	7	8	40
T. rubrum	Sabouraud	37	7	8	40
S. Aureus	MH	37	1	8	40
E. Coli	MH	37	1	8	40
P. aeruginosa	MH	37	1	8	40

Broth microdilution method (MIC) (http://www.chemotherapy.or.jp/)

M. canis and *T. rubrum* were performed on test tubes, the others were performed on 96-well plates (Asone) for the broth microdilution method to obtain the MIC for each samples. A series of 2 time diluted samples were prepared and mixed with diluted microbial cultured broth. The concentration of each microbial was adjusted to 10^7 CFU/ml for test tubes and 1-5 x 10^5 CFU/ml for 96-well plates. MIC was recorded for the concentration while no precipitation was observed. The culture conditions for each microbial was listed in the Table. 19.

Table 19. Culture conditions of microbial

	Medium	Temperature (℃)	Time (day)	Positive controls
C. albicans	RPMI-1640	30	2	fluconazole
M. canis	Sabouraud	30	7	miconazole
T. rubrum	Sabouraud	30	7	miconazole
S. Aureus	MH	30	1	vancomycin
E. Coli	MH	30	1	gentamycin
P. aeruginosa	MH	30	1	cephataxime

Experiment for all chapters

Materials

In April, 2011, researcher Dr. Ino (from Kitasato University) provided 46 strains of cultured mycelia of medicinal mushrooms (Table 20) to our laboratory (Laboratory of Medicinal Plant Chemistry). All strains are cultured on potato dextrose (PD) agar medium or yeast-malt (YM) agar medium, 25℃ in the dark, and subculture for every 3 months.

Table 20. Cultured mycelia maintained in our laboratory

Agaricus arvensis	シロオオハラタケ	*Fuscoporia obliqu*	カバノアナタケ	*Ophiocordyceps tricentri*	アワフキムシタケ
Agaricus bitoriguis		*Ganoderma applanatum*	コフキサルノコシカケ	*Paecilomyces tenuipes*	
Agaricus campestris	ハラタケ	*Ganoderma boninense*	シママンネンタケ	*Perenniporia fraxinea*	ベッコウタケ
Agaricus hortensis		*Ganoderma lucidum*	マンネンタケ	*Perenniporia ochroleuca*	
Agaricus rhodmani		*Ganoderma mastoporum*	クロガネマンネンタケ	*Phellinus linteuss*	メシマコブ
Agaricus subrufescens	ハワリハラタケ	*Ganoderma neo japonicum*	マゴジャクシ	*Phellinus yucutensis*	メシマコブ
Agarocybe cylindrica	ヤナギマツタケ	*Isaria farinosa*	コナサナギタケ	*Piptoporus coloniensis*	カイメンタケ
Cordyceps ferruginosa	サビイロクビオレタケ	*Isaria sp.*	トサカハナサナギタケ	*Polyporus umbellatus*	チョレイマイタケ
Cordyceps gracilioides	ウスイロタンポタケ	*Lentinus sp.*	ナンヨウブクリュウ	*Prenniporia ochroleuca*	ウズラタケ
Cordyceps pseudolongissima	イリオモテセミタケ	*Lentinus tuberregium*	シイタケ	*Trametes orientalis*	クジラタケ
Cordyceps heteropoda	オオゼミタケ	*Lignosus rhinoceros*	ヒジリタケ	*Tyromyces sambuceus*	シロカイメンタケ
Cordyceps oxycephala	トガリスズメハチタケ	*Onygena equina*		*Wolfiporia cocos*	マツホド
Fomes fomentariius	ツリガネタケ	*Ophiocordyceps crinalis*		*Wolfiporia extensa*	

Anti-fungal activity screening

The cultured mycelia of medicinal mushrooms were transferred to 500ml Erlenmeyer (with flask bottom diameter 10mm) flasks containing 100ml PD medium or YM medium for submerged micro-propagation. The cultured mycelia were then grown at 25℃ in the dark for 6 weeks. All cultures were separated to mycelium part and broth part after 6 weeks cultivation. The mycelium part was freeze dried and then extracted with MeOH to obtain mycelia extracts, while the broth part was extracted with EtOAc to obtain broth extracts. And the weights of all extracts were recorded in order to evaluate the efficiency. All extracts were then screened for anti-fungal activity by disk-paper diffusion method. The anti-fungal activity was approved if inhibition zone was observed.

Experiment for Chapter 2

Materials

In Chapter 2, six kinds of cultured mycelia of Agaricomycetes medicinal mushrooms (*Agaricus hortensis, A. bitorguis, A. rhodmanji, A. arvensis, A. subrufescens* and *Lignosus rhinoceros*) were cultured for anti-fungal activity screening. And large scale culture of *L. rhinoceros* was performed in order to isolate the bioactive compounds.

Culture methods

Cultured mycelium of *L. rhinoceros* was cultured on PD agar medium in Petri dishes at 25℃ in the dark for 3 weeks as pre-culture. The 10-mm plugs were cut from the culture and seeded with 5 plugs to each Roux flask containing 200ml PD medium for submerged culture. The culture mycelia were grown at 25℃ in the dark for 6 weeks. After 6 weeks cultivation, the mycelia were removed and the culture broth was concentrated under reduced pressure to 2L and then extracted with EtOAc (2L x 3 times). The EtOAc extract was evaporated to dryness to afford 2g crude extract.

Isolation and purification

The crude extract was then passed through silica gel column chromatography and eluted with solvents in the order of $CHCl_3$, $CHCl_3$-MeOH (100:1), $CHCl_3$-MeOH (9:1), $CHCl_3$-MeOH (8:2) and $CHCl_3$-MeOH (7:3) to obtain fr.1 (from $CHCl_3$, 4.1mg), fr.2 (from $CHCl_3$, 11.8mg), fr.3 (from $CHCl_3$-MeOH (100:1), 370.8mg), fr.4 (from $CHCl_3$-MeOH (9:1), 30.6mg), fr.5 (from $CHCl_3$-MeOH (8:2), 376.7mg) and fr.6 (from $CHCl_3$-MeOH (7:3), 898mg). Each fraction was applied to *M. canis* to trace the bioactive fraction, only fr.3 showed significant inhibition zone (22mm) with 5mg sample applied. When detected the fr.3 with TLC plate by eluting with 100% benzene, two major spots and one tailing part were observed.

Further purification was performed by silica gel column chromatography again in order to separate the 2 spots from the tailing part. Eluting was performed in the order of $CHCl_3$ then $CHCl_3$-MeOH (8:2) to obtain fr.3-1 (from $CHCl_3$, 62.3mg) and fr.3-2 (from $CHCl_3$-MeOH (8:2), 271.6mg). The result of anti-fungal test showed that fr.3-1 showed 30.1mm inhibition zone with 1mg sample applied.

Fr.3-1 was purified by NP-HPLC (column: Pegasil silica 5µm 10 x 250mm, solvent: Hexane : Isopropanol = 100:1, detector: refractive index) to obtain compound **1** (36.3mg) and compound **2** (18.2mg). When applied compounds **1** and **2** to test for the anti-fungal activity, both compounds **1** and **2** showed significant inhibition zone of 18.8mm and 17.6mm, respectively. And fr.3-2 was purified by RP-HPLC (column: Capcell PAK C18 5µm 10 x 250mm, solvent: MeOH: H_2O (0.1%TFA) = 4:6, detector: refractive index) to obtain compounds **3** (6.9mg), **4** (3.9mg), **5** (5.1mg), **6** (8.2mg), **7** (9.2mg) and **8** (5.7mg).

3,5-dimethoxybenzaldehyde (1)

Observed as light yellow oil

EI-MS *m/z* 166$[M]^+$

^{1}H-NMR ($CDCl_3$) δ

3.85 (6H, s), 6.78 (1H, t, *J* = 2.4 Hz), 7.01 (2H, d, *J* = 2.4Hz), 9.9 (1H, s)

^{13}C-NMR ($CDCl_3$) δ

55.7 (q), 107.2 (d), 107.3 (d), 138.5 (s), 161.3 (s), 192.0 (d)

2-chloro-3,5-dimethoxybenzaldehyde (2)

Observed as white powder

EI-MS *m/z* 200 $[M]^+$

HR-EI-MS *m/z* 200.0252 $[M]^+$, with a molecular formula $C_9H_9O_3Cl$

1H-NMR ($CDCl_3$) δ

3.84 (3H, s), 3.91 (3H, s), 6.73 (1H, d, *J* = 2.3Hz), 7.01 (2H, d, *J* = 2.3Hz), 10.5 (1H, s)

13C-NMR ($CDCl_3$) δ

55.8 (q), 56.5 (q), 102.4 (d), 106.0 (d), 119.6 (s), 133.5 (s), 156.3 (s), 159.1 (s), 189.9 (d)

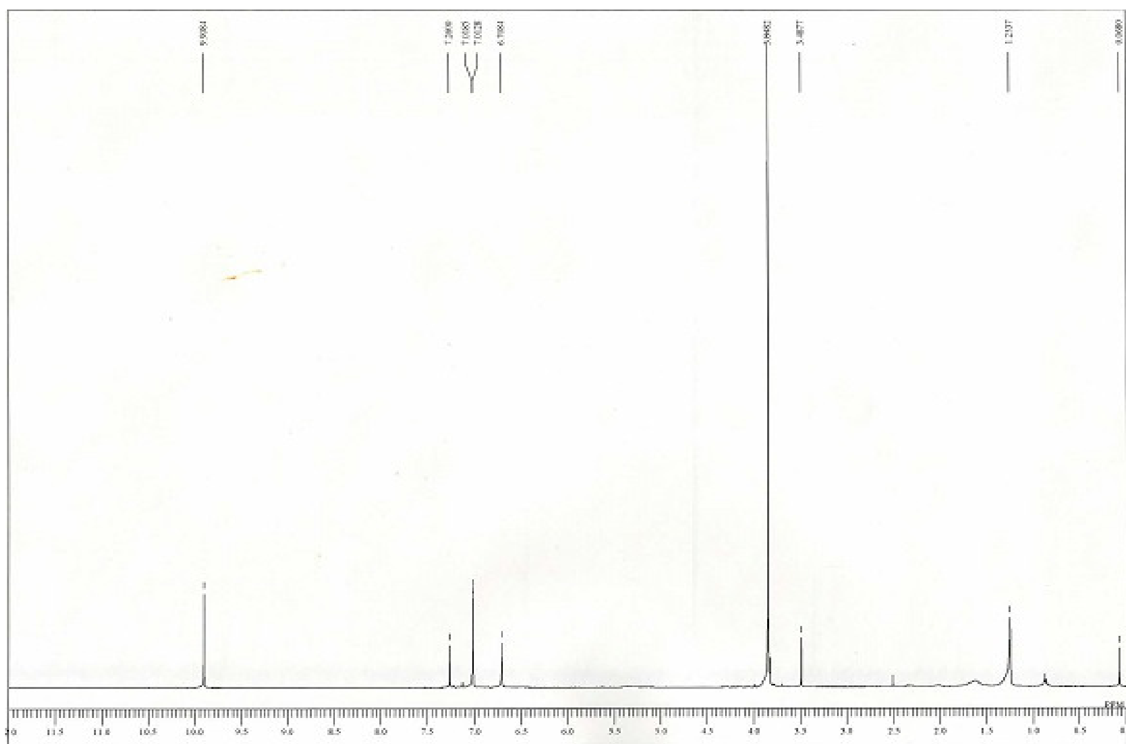


Figure 39. ^{1}H-NMR spectrum of compound **1** in $CDCl_3$

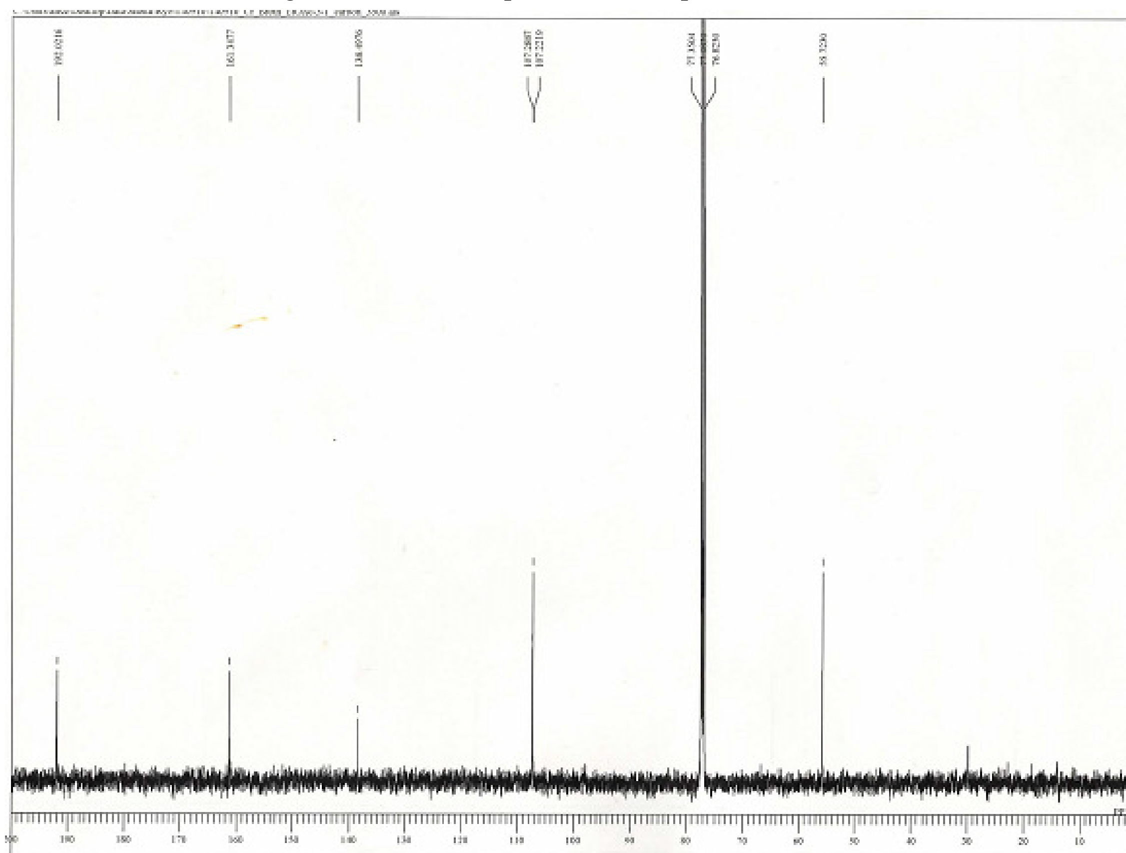


Figure 40. ^{13}C-NMR spectrum of compound **1** in $CDCl_3$

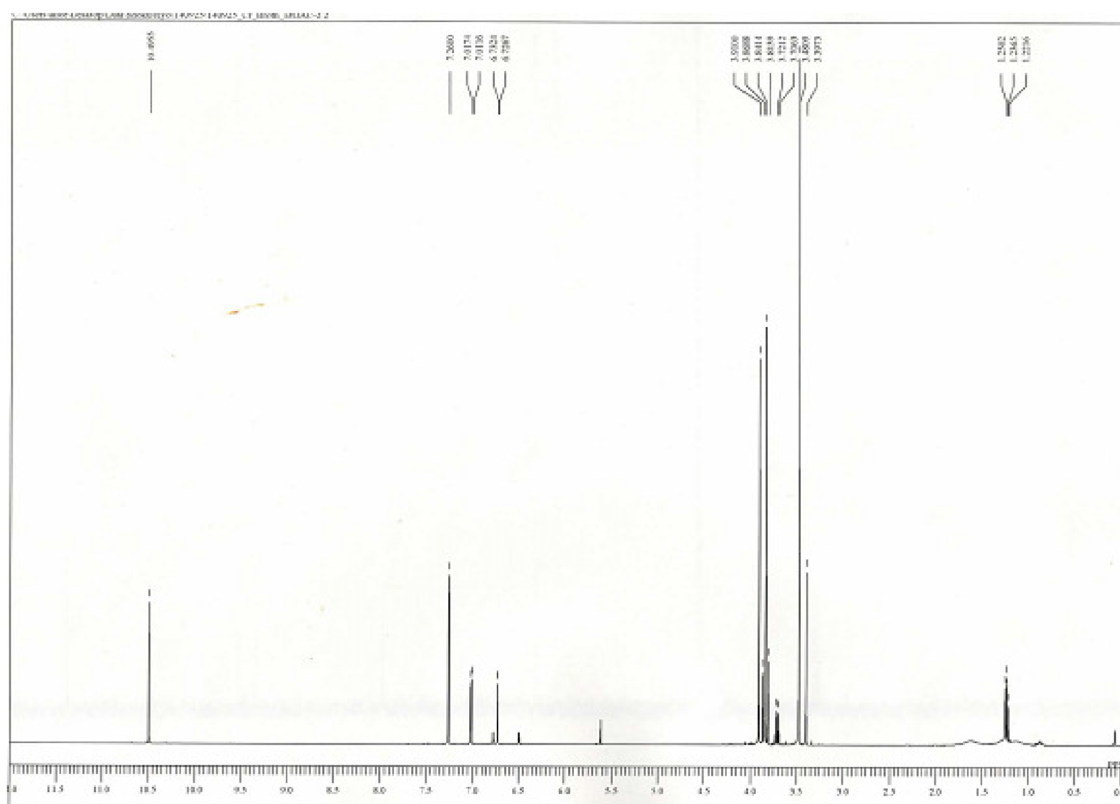


Figure 41. ^{1}H-NMR spectrum of compound **2** in $CDCl_3$

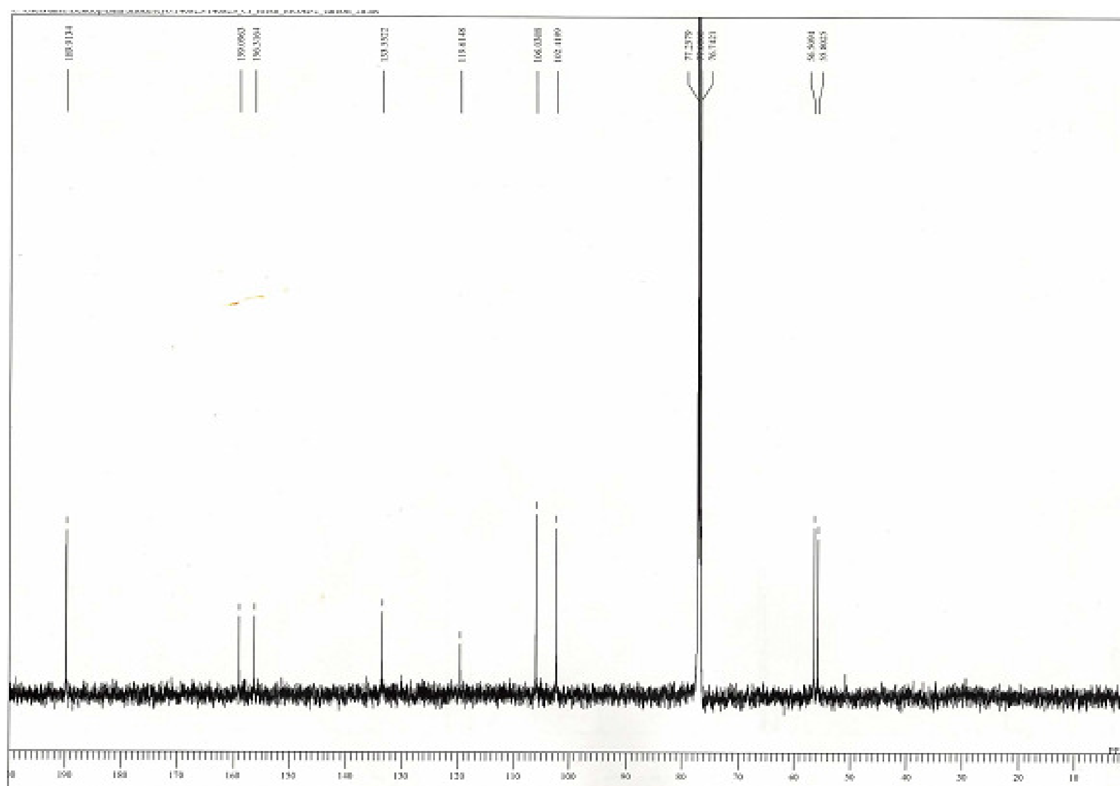


Figure 42. ^{13}C-NMR spectrum of compound **2** in $CDCl_3$

***rel*-(1*S*, 2*R*)-1-(3',5'-dimethoxyphenyl)propane-1,2-diol (3)**

Observed as light orange oil, $[\alpha]_D$ -6.3 (*c* 0.48, MeOH)
EI-MS *m/z* 212 $[M]^+$

^{1}H-NMR (CD_3OD) δ

1.11 (3H, d, *J* = 6.4Hz), 3.75 (6H, s), 3.83 (1H, dq, *J* = 4.4 and 6.4Hz), 4.43 (1H, d, *J* = 4.4Hz), 6.36 (1H, t, *J* = 2.4Hz), 6.54 (2H, d, *J* = 2.4Hz)

^{13}C-NMR (CD_3OD) δ

17.1 (q), 54.7 (q), 71.3 (d), 78.0 (d), 99.2 (d), 104.9 (d), 144.9 (s), 161.1 (s)

***rel*-(1*R*, 2*R*)-1-(3',5'-dimethoxyphenyl)propane-1,2-diol (4)**

Observed as light orange oil, $[\alpha]_D$ 0 (*c* 1.1, MeOH)
EI-MS *m/z* 212 $[M]^+$

^{1}H-NMR (CD_3OD) δ

0.98 (3H, d, *J* = 6.2Hz), 3.76 (6H, s), 3.79 (1H, dq, *J* = 4.4 and 6.2Hz), 4.26 (1H, d, *J* = 4.4Hz), 6.37 (1H, t, *J* = 2.3Hz), 6.52 (2H, d, *J* = 2.3Hz)

^{13}C-NMR (CD_3OD) δ

18.3 (q), 54.7 (q), 71.8 (d), 79.3 (d), 99.5 (d), 105.1 (d), 144.8 (s), 161.2 (s)

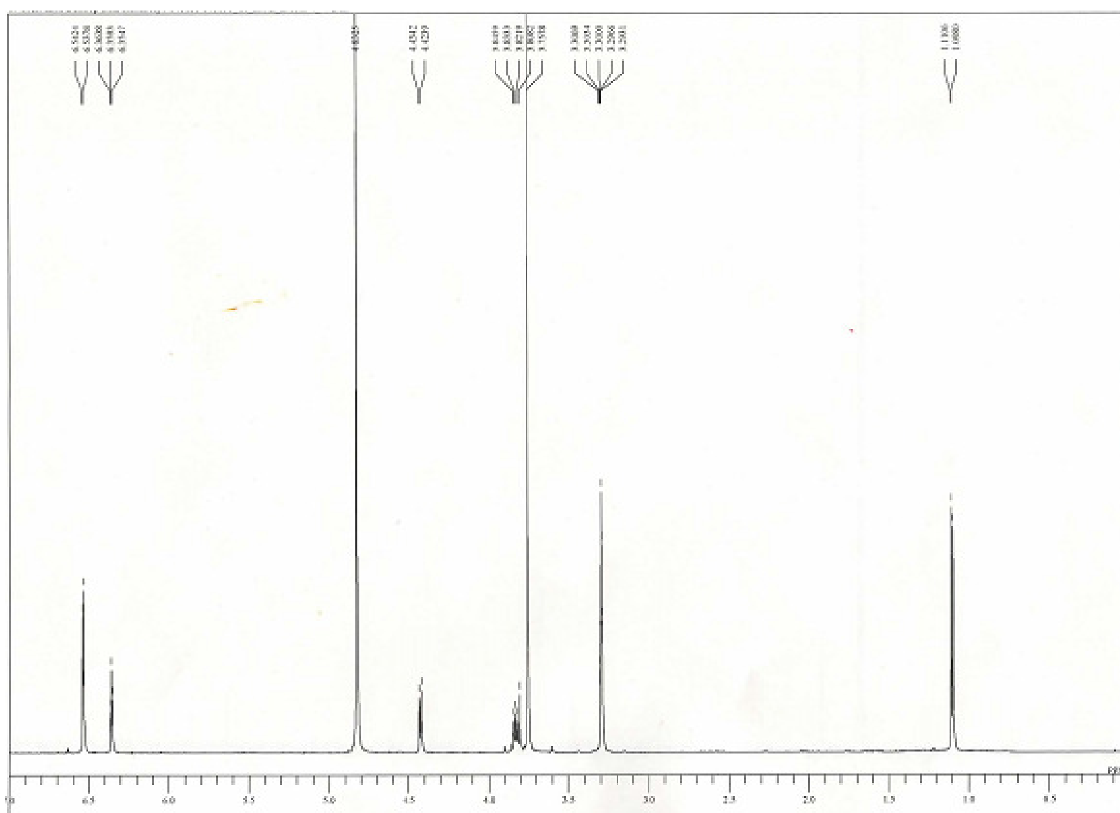


Figure 43. ^{1}H-NMR spectrum of compound **3** in CD_3OD

Figure 44. ^{13}C-NMR spectrum of compound **3** in CD_3OD

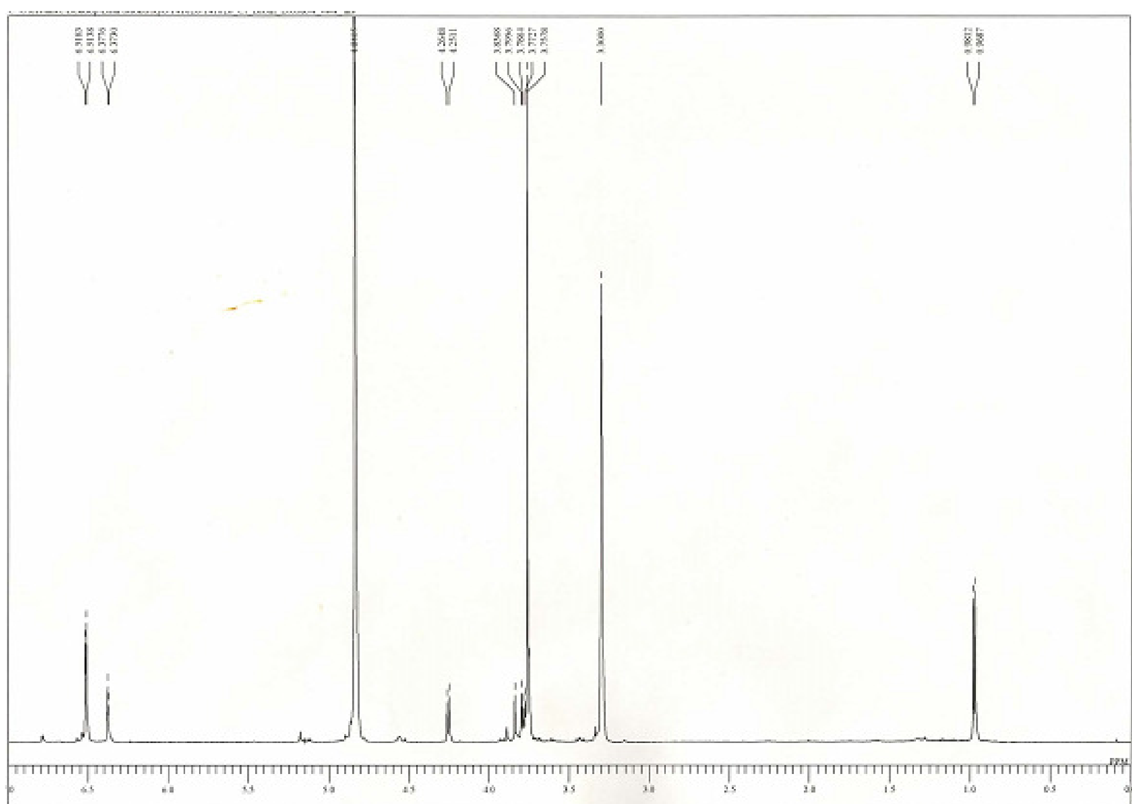


Figure 45. ^{1}H-NMR spectrum of compound **4** in CD_3OD

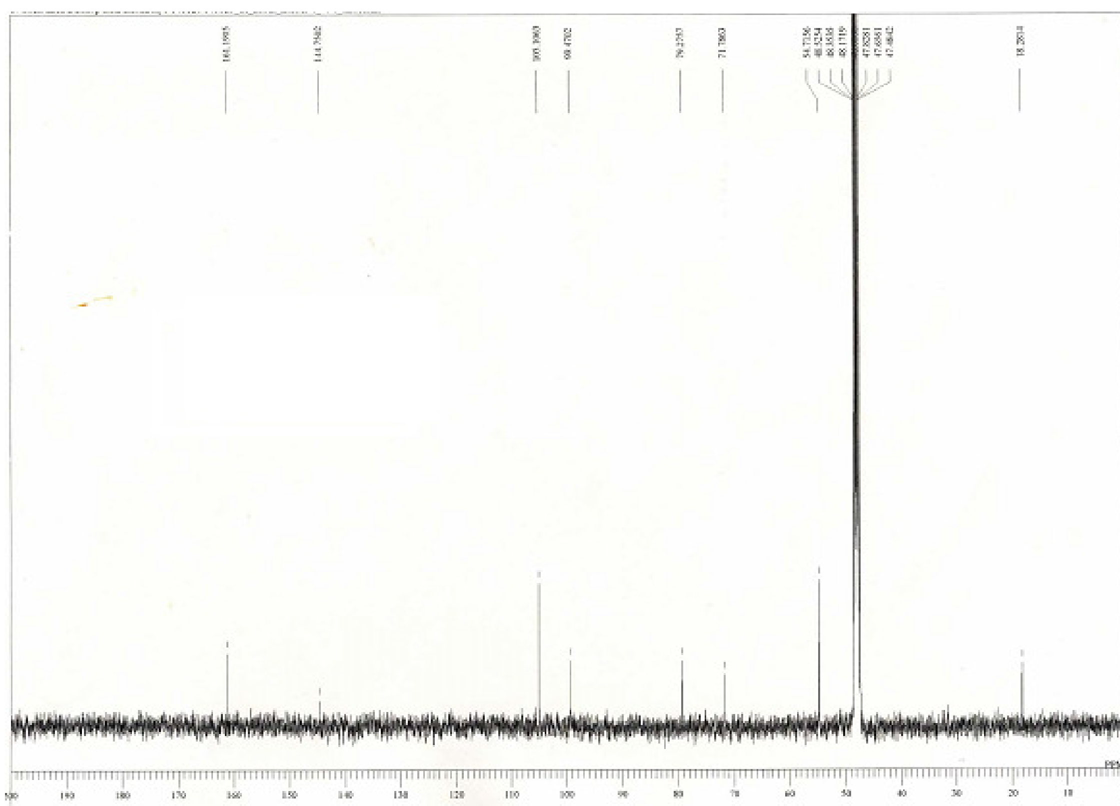


Figure 46. ^{13}C-NMR spectrum of compound **4** in CD_3OD

3,5-dimethoxybenzoic acid (5)

Observed as light yellow powder

EI-MS *m/z* 182 $[M]^+$

^{1}H-NMR (CD_3OD) δ

3.80 (6H, s), 6.68 (1H, t, *J* = 2.3 Hz), 7.14 (2H, d, *J* = 2.3 Hz)

^{13}C-NMR (CD_3OD) δ

55.0 (q), 105.1 (d), 107.3 (d), 132.9 (s), 161.2 (s), 168.6 (s)

2-chloro-3,5- dimethoxybenzoic acid (6)

Observed as light yellow powder

EI-MS *m/z* 216 $[M]^+$

HR-EI-MS *m/z* 216.0177 $[M]^+$, with a molecular formula $C_9H_9O_4Cl$

^{1}H-NMR (CD_3OD) δ

3.80 (3H, s), 3.86 (3H, s), 6.70 (1H, d, *J* = 2.8Hz), 6.77 (1H, d, *J* = 2.8Hz)

^{13}C-NMR (CD_3OD) δ

55.2 (q), 56.0 (q), 102.1 (d), 106.0 (d), 112.8 (s), 127.0 (s), 159.4 (s), 165.5 (s), 168.5 (s)

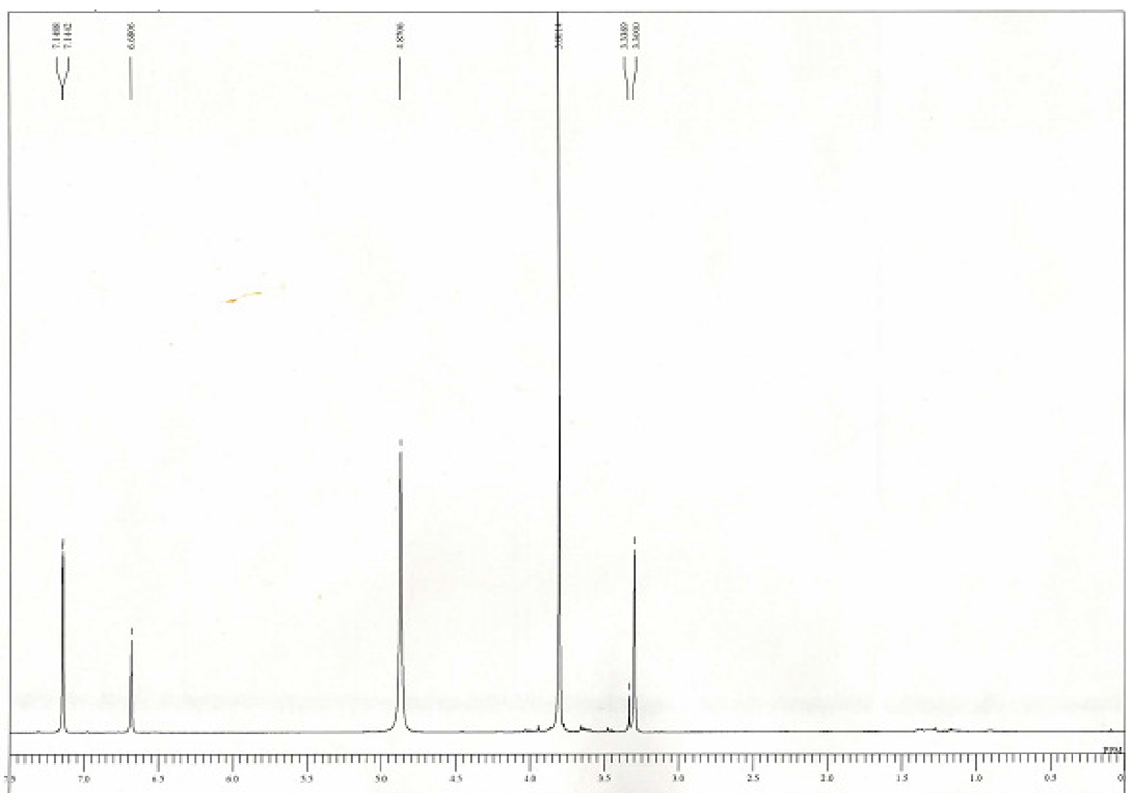


Figure 47. ^{1}H-NMR spectrum of compound **5** in CD_3OD

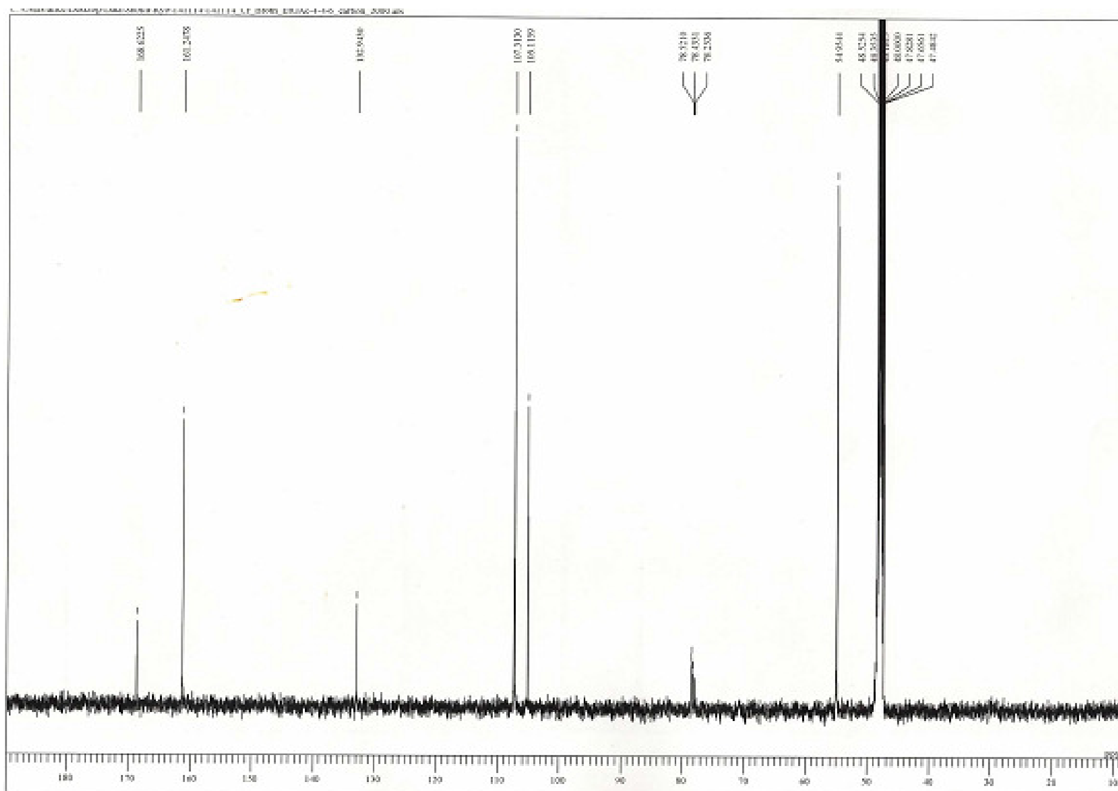


Figure 48. ^{13}C-NMR spectrum of compound **5** in CD_3OD

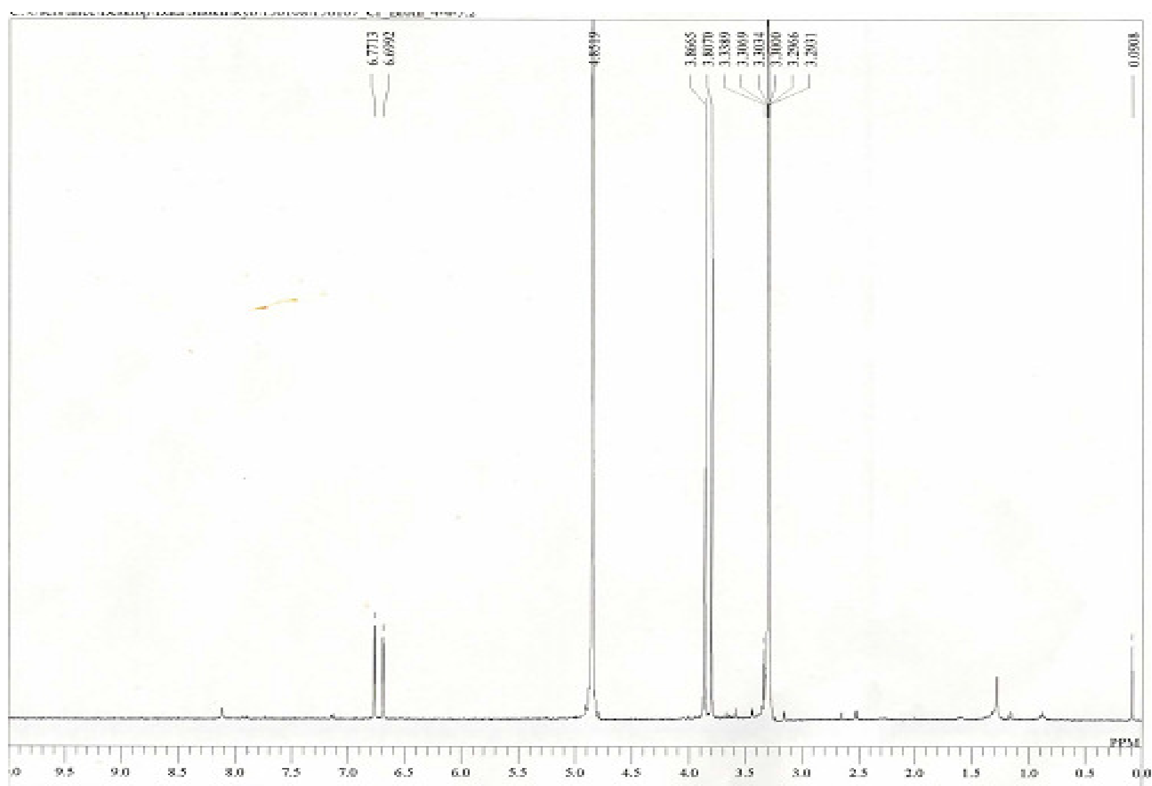


Figure 49. ^{1}H-NMR spectrum of compound **6** in CD_3OD

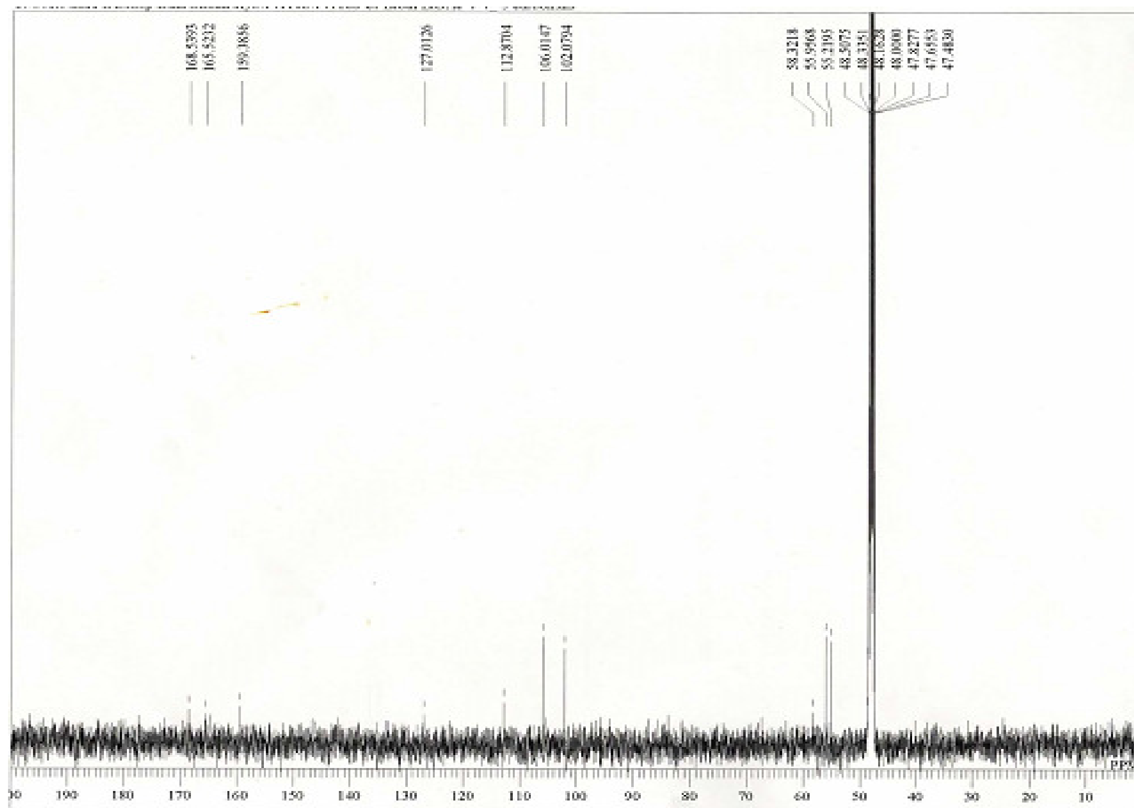


Figure 50. ^{13}C-NMR spectrum of compound **6** in CD_3OD

6-(2-hydroxypropyl)-4-methoxy-pyran-2-one (7)

Observed as light orange oil, $[\alpha]_D$ 56.3 (*c* 0.6, MeOH)
EI-MS *m/z* 184 $[M]^+$

^{1}H-NMR (CD_3OD) δ

1.22 (3H, d, *J* = 6.8 Hz), 2.56 (2H, m), 3.82 (3H, s), 4.10 (1H, tq), 5.53 (1H, d, *J* = 2.3 Hz), 6.05 (1H, d, *J* = 2.3 Hz)

^{13}C-NMR (CD_3OD) δ

22.4 (q), 55.9 (q), 42.9 (t), 65.1 (d), 87.4 (d), 102.1 (d), 163.6 (s), 166.7 (s), 172.8 (s)

1-(2-chloro-3,5-dimethoxy-phenyl)-ethane-1,2-diol (8)

Observed as light orange oil, $[\alpha]_D$ -23.9 (*c* 0.27, MeOH)
EI-MS *m/z* 232 $[M]^+$

HR-ESI-MS *m/z* 255.0322 $[M+Na]^+$, with a molecular formula $C_{10}H_{13}O_5ClNa$

^{1}H-NMR (CD_3OD) δ

3.43 (1H, dd, *J* = 11.5, 7.5 Hz), 3.69 (1H, dd, *J* = 11.5, 3.0 Hz), 3.80 (3H, s), 3.85 (3H, *s*), 5.13 (1H, dd, *J* = 7.5, 3.0 Hz), 6.54 (1H, d, *J* = 2.8 Hz), 6.80 (1H, d, *J* = 2.8 Hz)

^{13}C-NMR (CD_3OD) δ

55.0 (q), 55.7 (q), 66.2 (t), 71.7 (t), 98.8 (d), 103.8 (d), 111.9 (s), 141.5 (s), 152.8 (s), 159.7 (s)

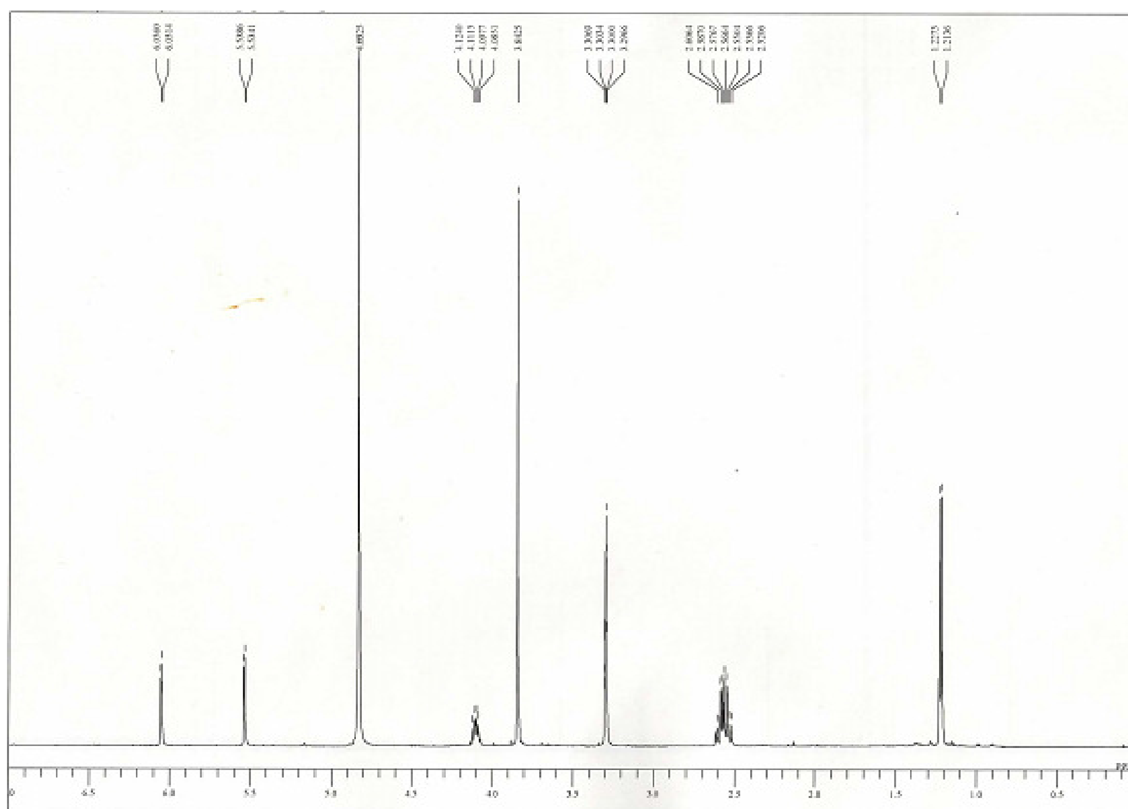


Figure 51. ^{1}H-NMR spectrum of compound **7** in CD_3OD

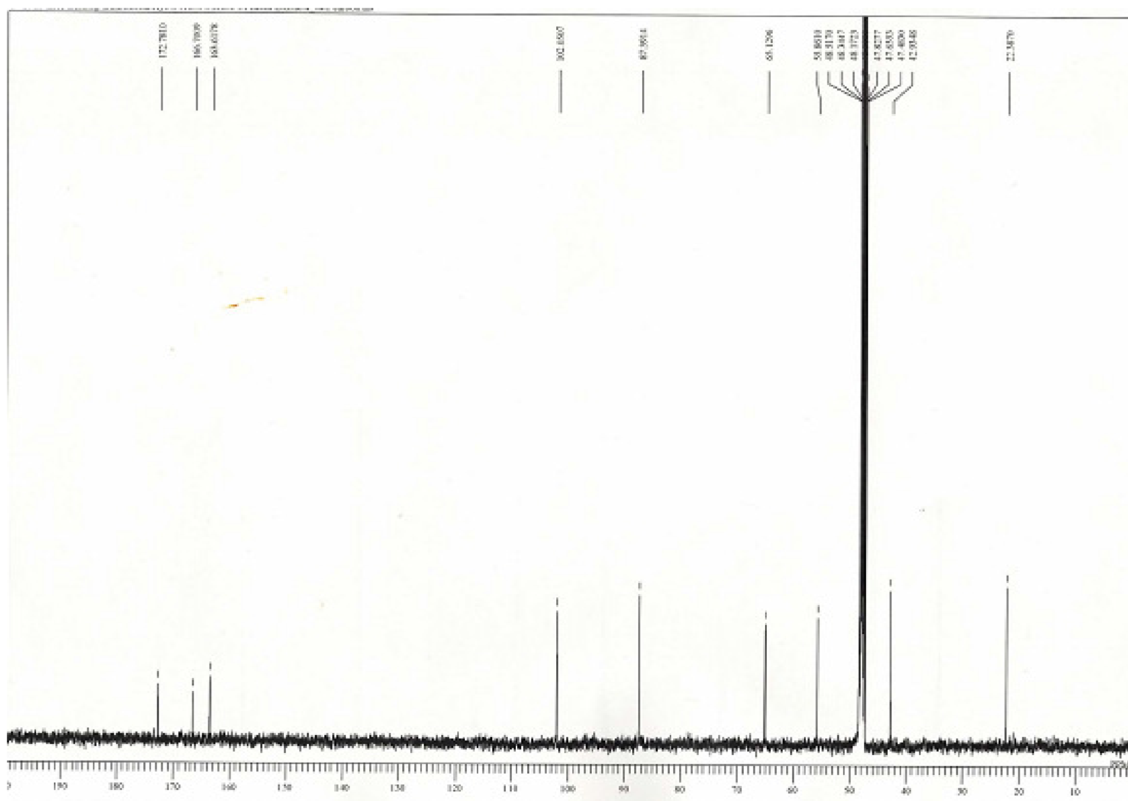


Figure 52. ^{13}C-NMR spectrum of compound **7** in CD_3OD

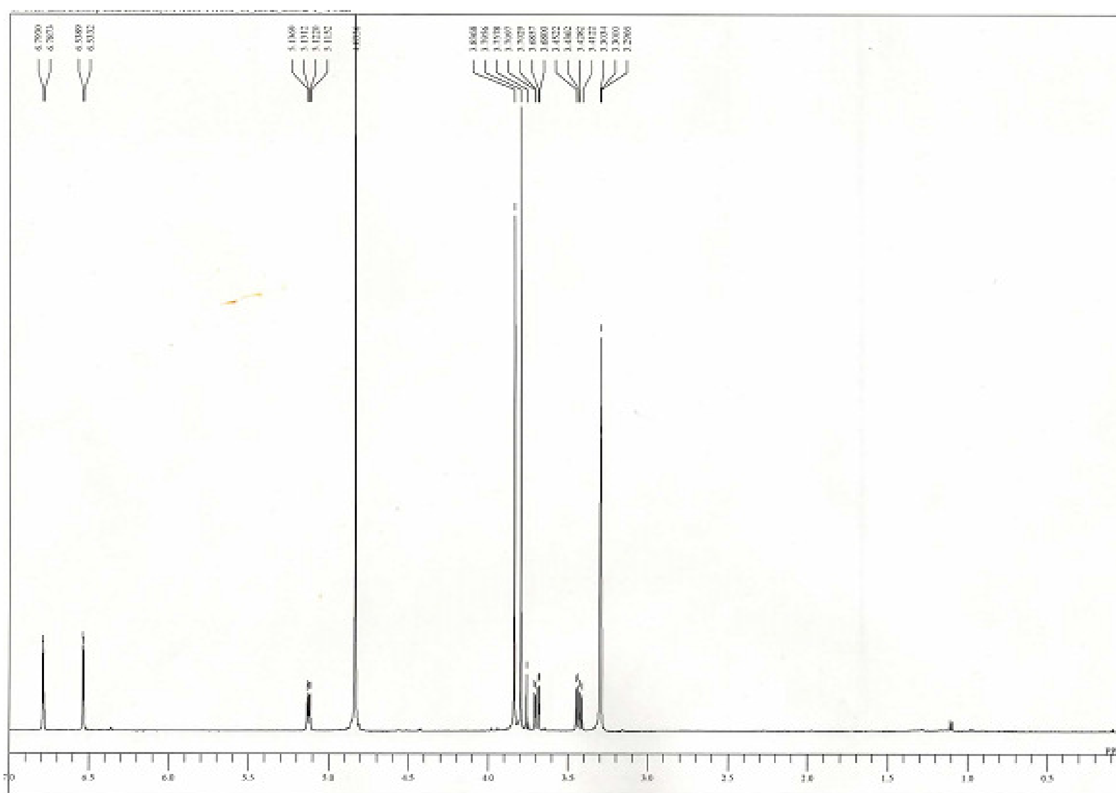


Figure 53. ^{1}H-NMR spectrum of compound **8** in CD_3OD

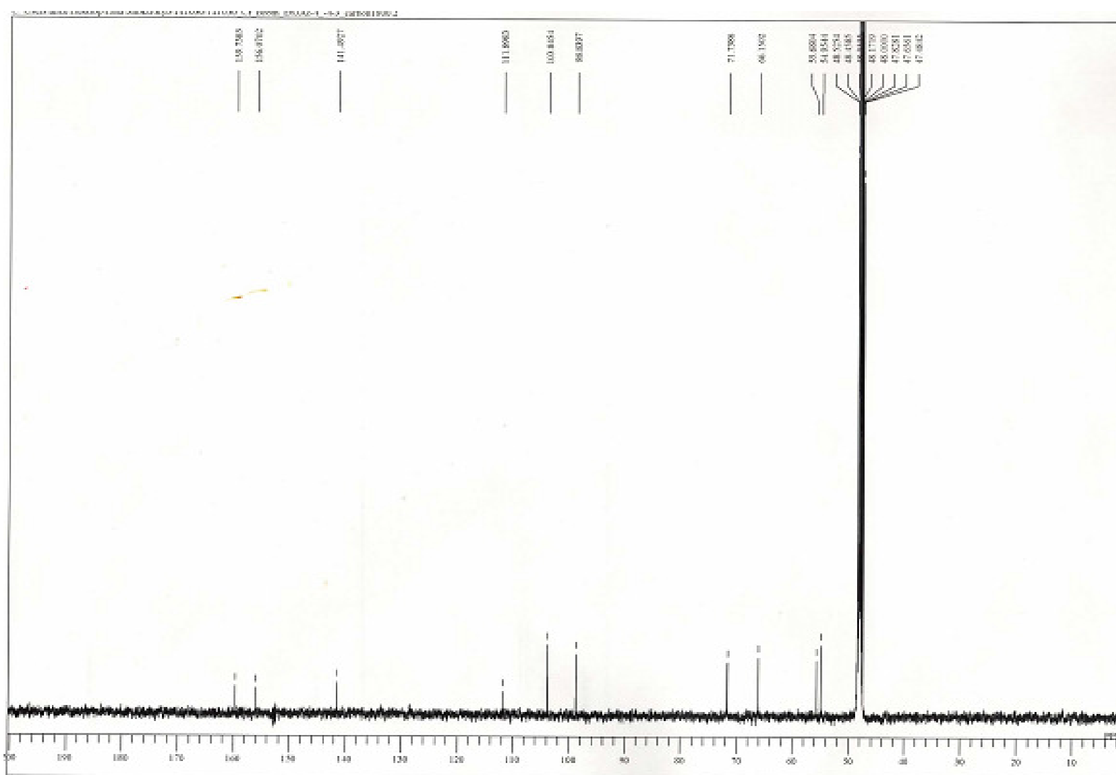


Figure 54. ^{13}C-NMR spectrum of compound **8** in CD_3OD

Experiment for Chapter 3

Materials

In Chapter 3, six kinds of cultured mycelia of *Clavicipitaceae* mushrooms (*Cordyceps ferruginosa, C. roseostromata, C. oxycephala, C. tricentri, C. prolifica* and *Isaria* sp.) were cultured for anti-fungal activity screening. And large scale culture of *Isaria* sp. was performed in order to isolate the bioactive compound and other secondary metabolites.

Culture methods

Cultured mycelia of *Isaria* sp. were cultured on PD agar medium in Petri dishes at 25℃ in the dark for 3 weeks as pre-culture. The 10-mm plugs were cut from the culture and seeded with 5 plugs to each Roux flask containing 200ml PD medium for submerged culture. The culture mycelium was grown at 25℃ in the dark for 6 weeks. After 6 weeks cultivation, the cultured mycelia were freeze dried and then extracted with MeOH to obtain MeOH extract 17.53g for secondary metabolites study; the cultured broth was passed through the HP20 column chromatography for the bioactivity study.

Isolation and purification

The cultured broth was passed through HP20 column chromatography and eluted with solvent in the order of Water, Water-MeOH (1:1) and MeOH to obtain fr.1 (from Water, 25g), fr.2 (from Water-MeOH, 3.92g) and fr.3 (from MeOH, 0.45g). Each fraction was applied to *M. canis* to trace the bioactive fraction, only fr.3 showed significant inhibition zone (15mm) with 1mg sample applied.

Further purification was performed by silica gel column chromatography, and eluted with solvents in the order of $CHCl_3$, $CHCl_3$-MeOH (95:5), $CHCl_3$-MeOH (9:1), $CHCl_3$-MeOH (8:2), $CHCl_3$-MeOH (7:3), $CHCl_3$-MeOH (6:4) and MeOH to obtain fr.3-1 (from $CHCl_3$, 5.3mg), fr.3-2 (from $CHCl_3$-MeOH (95:5), 7.7mg), fr.3-3 (from $CHCl_3$-MeOH (9:1), 29mg), fr. 3-4 (from $CHCl_3$-MeOH (8:2), 23.6mg), fr.3-5 (from $CHCl_3$-MeOH (7:3), 64.9mg), fr.3-6 (from $CHCl_3$-MeOH (6:4), 124mg) and fr.3-7 (from MeOH, 173.2mg). The result of anti-fungal test showed that fr.3-3 and fr.3-4 have bioactivity. The fr.3-3 and fr.3-4 were conbined and passed through the silica gel column chromatography again and eluted with $CHCl_3$-MeOH (97:3) to obtain fr. 3-3'-4.

As to mycelium part, the MeOH extract was partitioned between $CHCl_3$ and water to obtain $CHCl_3$ extract (6.4g). The $CHCl_3$ extract was then passed through the silica gel column chromatography, and eluted in the order of hexane, hexane-EtOAc (3:1), hexane-EtOAc (1:1), hexane-EtOAc (2:3), EtOAc, and then washed by MeOH to obtain fr.1 (4.8g), fr.2 (from hexane-EtOAc (3:1), 1.2g), fr.3 (41mg), fr.4 (66mg), fr.5 (13mg) and fr.6 (25mg). Due to the good identity of the spots of fr.2, further isolation was performed by silica gel column chromatography again and eluted with $CHCl_3$ to obtain fr.2-1 (374.8mg), fr.2-2 (302.8mg), fr.2-3 (25.3mg), fr.2-4 (246.6mg) and fr.2-5 (203.0mg). Further purification was carried out by RP-HPLC (column: Capcell PAK C18 5μm 10 x 250mm, solvent: MeOH: H_2O = 95:5~90:10, detector: refractive index) to obtain compound **10** (7.7mg) from fr.2-3 and compounds **9** (14mg), **11** (4.2mg), **12** (3.3mg) and **13** (3.2mg) from fr.2-4.

3β,5α-dihydroxyergosta-7,22-dien-6-one (9)

Observed as white powder, $[\alpha]_D$ 10.0 (*c* 1.0, $CHCl_3$)
EI-MS *m/z* 428 $[M]^+$

^{1}H-NMR ($CDCl_3$) δ

0.60 (3H, s), 0.82 (3H, *J* = 6.5 Hz), 0.84 (3H, *J* = 6.5 Hz), 0.91 (3H, d, *J* = 6.5 Hz), 0.94 (3H, s), 1.03 (3H, d, *J* = 6.5 Hz), 1.33 (2H), 1.34 (H), 1.42 (2H), 1.48 (2H), 1.61 (2H), 1.62 (2H), 1.7 (2H), 1.86 (1H), 2.03 (1H), 2.11 (2H), 2.12 (H), 4.02 (1H, m), 5.21 (1H, dd, *J* = 15.0, 8.5Hz), 5.22 (1H, dd, *J* = 15.0, 8.0Hz), 5.63 (1H, s)

^{13}C-NMR ($CDCl_3$) δ

12.7 (q), 16.4 (q), 17.6 (q), 19.6 (q), 19.9 (q), 21.1 (q), 21.9 (t), 22.5 (t), 27.8 (t), 30.2 (t), 30.4 (t), 33.0 (d), 36.3 (t), 38.8 (t), 40.3 (d), 40.5 (s), 42.8 (d), 43.8 (s), 44.7 (d), 55.8 (d), 56.0 (d), 67.4 (d), 77.6 (s), 119.7 (d), 132.5 (d), 135.0 (d), 165.5 (s), 198.7 (s)

5α,6α-epoxyergosta-7,22-dien-3β-ol (10)

Observed as white powder, $[\alpha]_D$ 12.7 (*c* 0.3, MeOH)
EI-MS *m/z* 412 $[M]^+$

^{1}H-NMR (CD_3OD) δ

0.63 (3H, s), 0.87 (3H, *J* = 6.9 Hz), 0.89 (3H, *J* = 6.9 Hz), 0.97 (3H, d, *J* = 6.5 Hz), 1.00 (3H, s), 1.07 (3H, d, *J* = 6.5 Hz), 1.33 (2H), 1.37 (H), 1.52 (2H), 1.54 (1H), 1.55 (2H), 1.68 (2H), 1.79 (2H), 1.88 (1H), 2.06 (1H), 2.08 (2H), 2.20 (H), 3.93 (1H, m), 3.93 (1H, d, *J* = 2.0 Hz), 5.00 (1H, d, *J* = 2.0 Hz), 5.23 (1H, dd, *J* = 14.0, 7.5Hz), 5.26 (1H, dd, *J* = 14.0, 7.0Hz)

^{13}C-NMR (CD_3OD) δ

12.7 (q), 16.4 (q), 17.6 (q), 19.6 (q), 19.9 (q), 21.1 (q), 21.9 (t), 22.5 (t), 27.8 (t), 30.2 (t), 30.4 (t), 33.0 (d), 36.3 (t), 38.8 (t), 40.3 (d), 40.5 (s), 42.8 (d), 43.8 (s), 44.7 (d), 55.8 (d), 56.0 (d), 67.4 (d), 77.6 (s), 119.7 (d), 132.5 (d), 135.0 (d), 165.5 (s), 198.7 (s)

Figureu 55. ^{1}H-NMR spectrum of compound **9** in CD_3OD

Figure 56. ^{13}C-NMR spectrum of compound **9** in CD_3OD

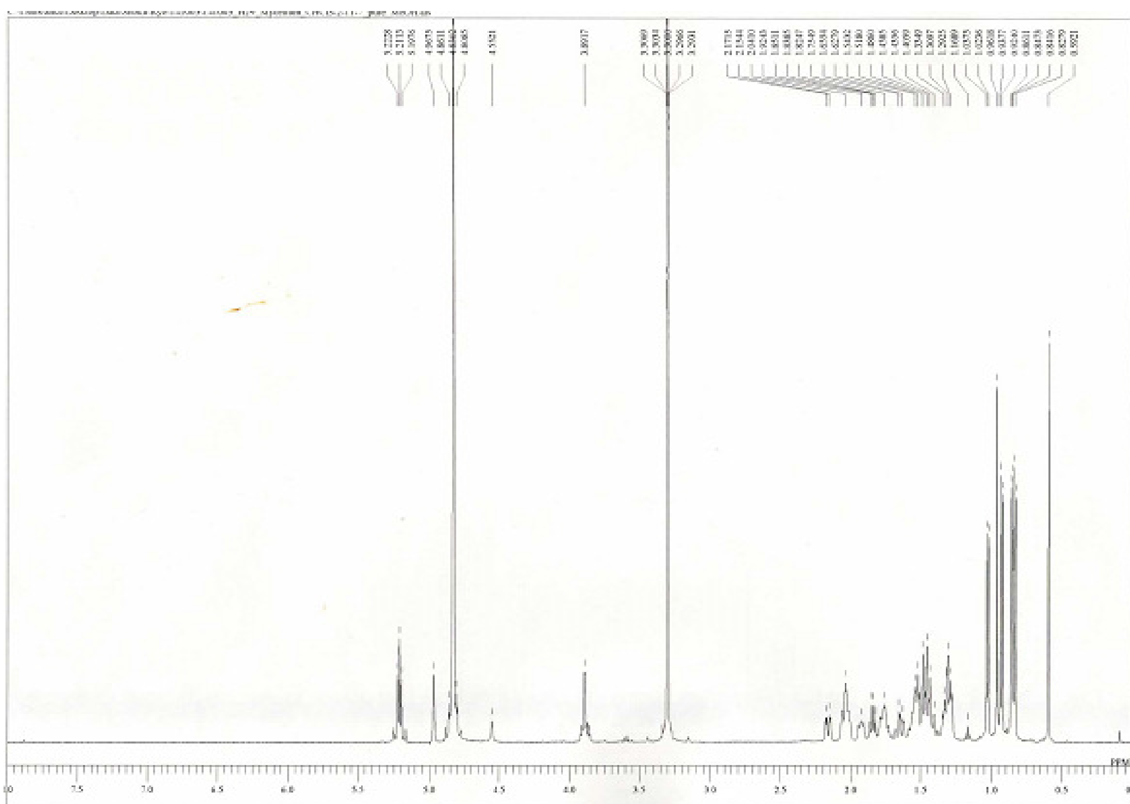


Figure 57. ^{1}H-NMR spectrum of compound **10** in CD_3OD

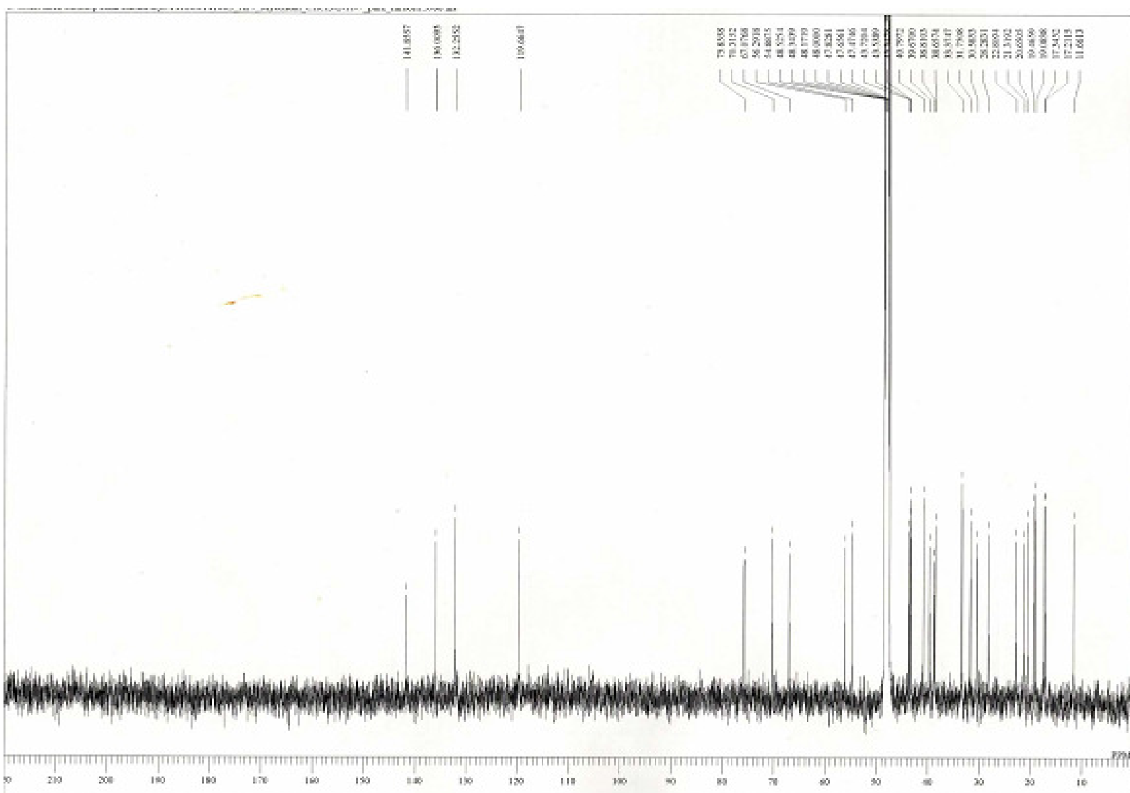


Figure 58. ^{13}C-NMR spectrum of compound **10** in CD_3OD

3β,5α,9α- trihydroxyergosta- 7,22-dien-6-one (11)

Observed as white powder, $[\alpha]_D$ -13.2 (*c* 0.28, MeOH)
EI-MS *m/z* 444 $[M]^+$

^{1}H-NMR (d_5-pyridine) δ

0.63 (3H, s), 0.85 (3H, *J* = 6.8 Hz), 0.86 (3H, *J* = 6.8 Hz), 0.95 (3H, d, *J* = 6.8 Hz), 1.04 (3H, d, *J* = 6.8 Hz), 1.15 (3H, s), 1.30 (2H), 1.34 (H), 1.47 (2H), 1.48 (2H), 1.61 (2H), 1.64 (2H), 1.86 (2H), 1.86 (1H), 1.98 (1H), 2.05 (2H), 4.02 (1H, m), 5.21 (1H, dd, *J* = 15.0, 8.5Hz), 5.22 (1H, dd, *J* = 15.0, 8.0Hz), 5.63 (1H, s)

^{13}C-NMR (d_5-pyridine) δ

12.4 (q), 17.9 (q), 19.8 (q), 20.1 (q), 20.4 (q), 21.3 (q), 22.7 (t), 26.4 (t), 28.3 (t), 29.0 (t), 30.0 (t), 33.4 (d), 35.2 (t), 36.2 (t), 40.7 (d), 41.8 (s), 43.3 (d), 45.2 (s), 51.8 (d), 56.1 (d), 68.8 (d), 75.0 (s), 79.8 (s), 120.3 (d), 132.4 (d), 136.0 (d), 164.1 (s), 199.1 (s)

6α,9α-epoxyergosta-7,22-dien-3β-ol (12)

Observed as white powder, $[\alpha]_D$ -29.6 (*c* 0.15, MeOH)
EI-MS *m/z* 412 $[M]^+$

^{1}H-NMR (CD_3OD) δ

0.63 (3H, s), 0.83 (3H, *J* = 6.8 Hz), 0.85 (3H, *J* = 6.8 Hz), 0.93 (3H, d, *J* = 6.8 Hz), 1.03 (3H, d, *J* = 6.3 Hz), 1.06 (3H, s), 1.28 (2H), 1.32 (H), 1.47 (2H), 1.50 (1H), 1.57 (2H), 1.70 (2H), 1.75 (2H), 1.85 (1H), 2.04 (1H), 2.08 (2H), 2.11 (H), 3.54 (1H, d, *J* = 4.8 Hz), 3.93 (1H, m), 5.19 (1H, dd, *J* = 15.0, 8.0 Hz), 5.23 (1H, dd, *J* = 15.0, 8.0 Hz) 5.26 (1H, d, *J* = 4.8 Hz)

^{13}C-NMR (CD_3OD) δ

11.8 (q), 17.2 (q), 17.9 (q), 19.1 (q), 19.5 (q), 20.7 (q), 22.0 (t), 23.0 (t), 28.2 (t), 30.8 (t), 32.9 (t), 33.4 (d), 37.1 (t), 39.5 (t), 39.7 (d), 40.8 (s), 43.4 (d), 43.4 (s), 43.7 (d), 54.9 (d), 56.4 (d), 67.4 (d), 73.2 (s), 75.9 (s), 118.1 (d), 132.2 (d), 136.0 (d), 142.8 (s)

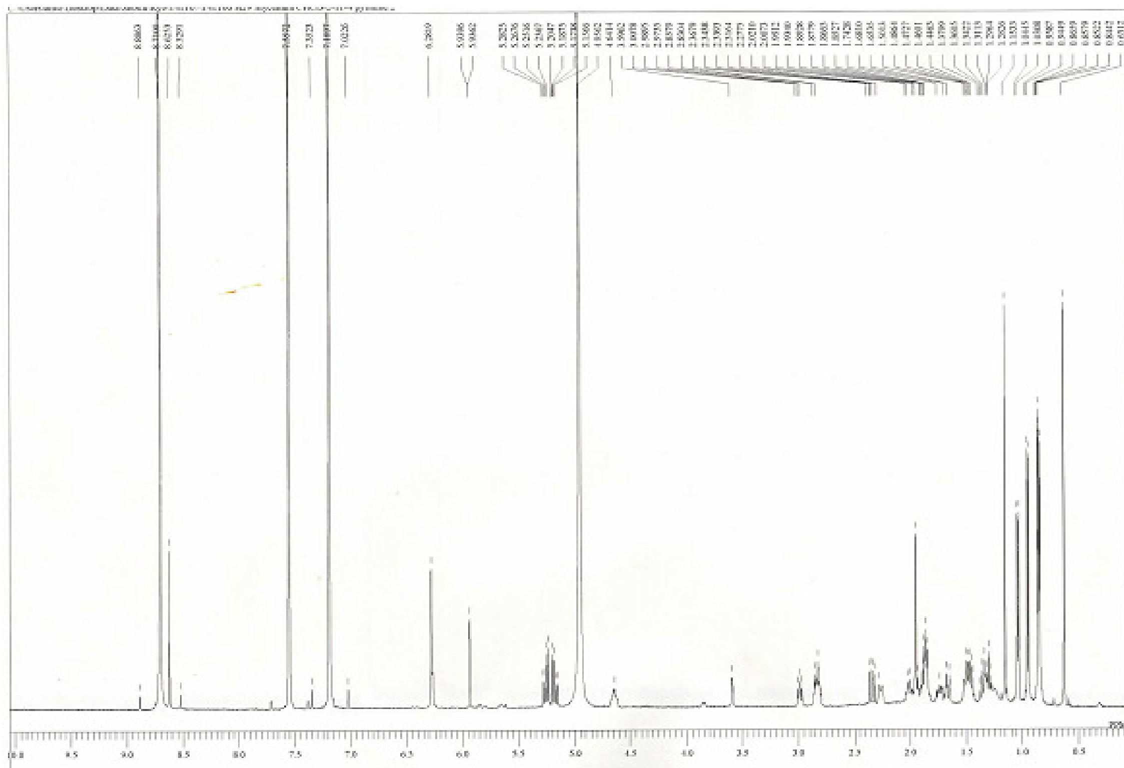


Figure 59. ^{13}C-NMR spectrum of compound **11** in d_5-pyridine

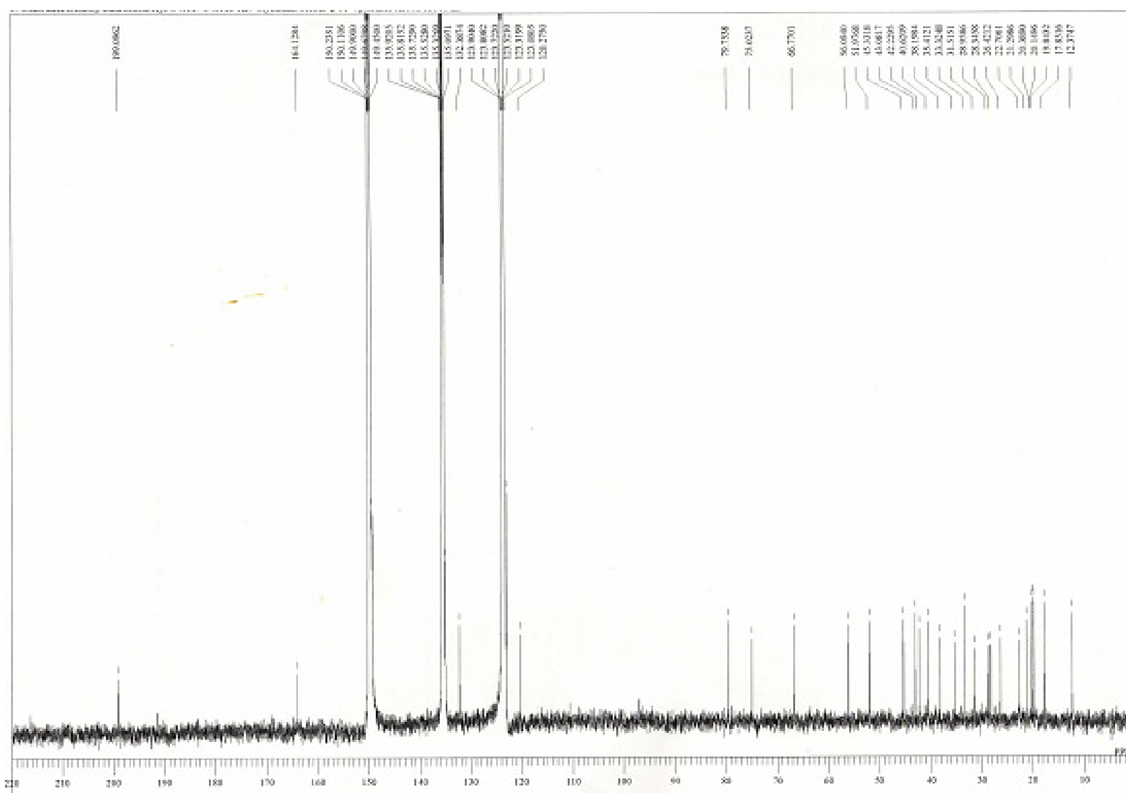


Figure 60. ^{13}C-NMR spectrum of compound **11** in d_5-pyridine

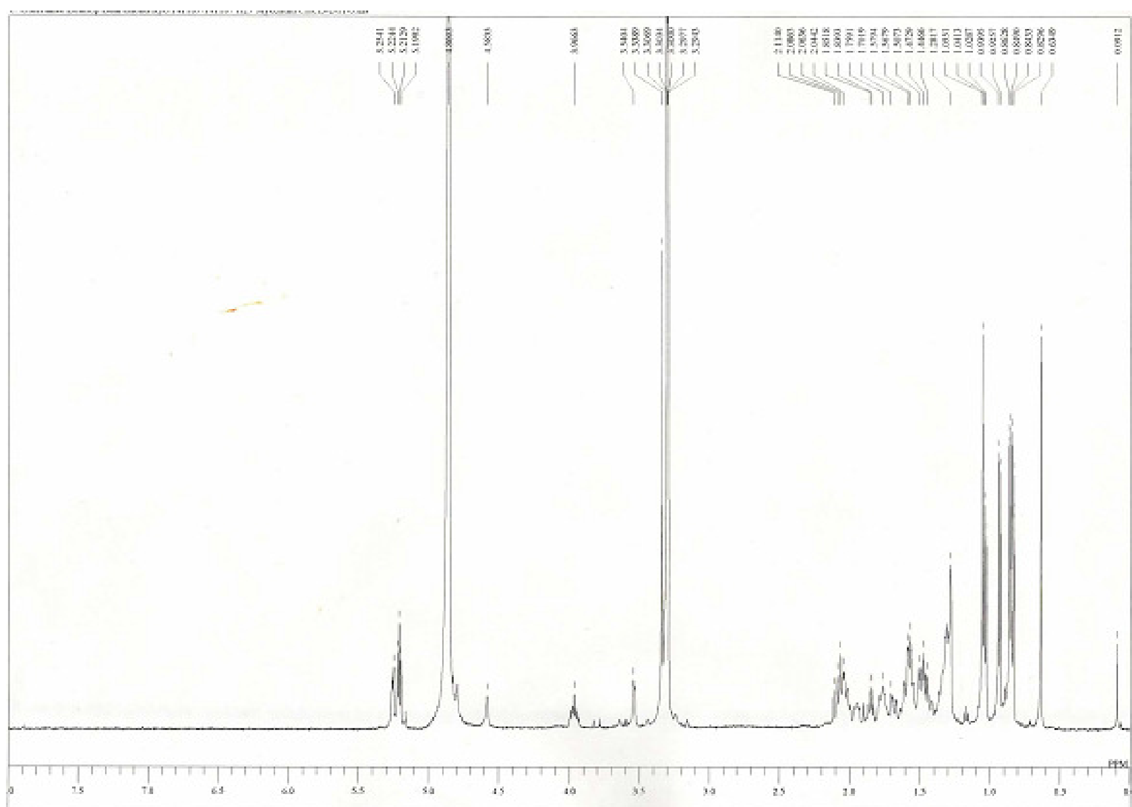


Figure 61. ^{1}H-NMR spectrum of compound **12** in CD_3OD

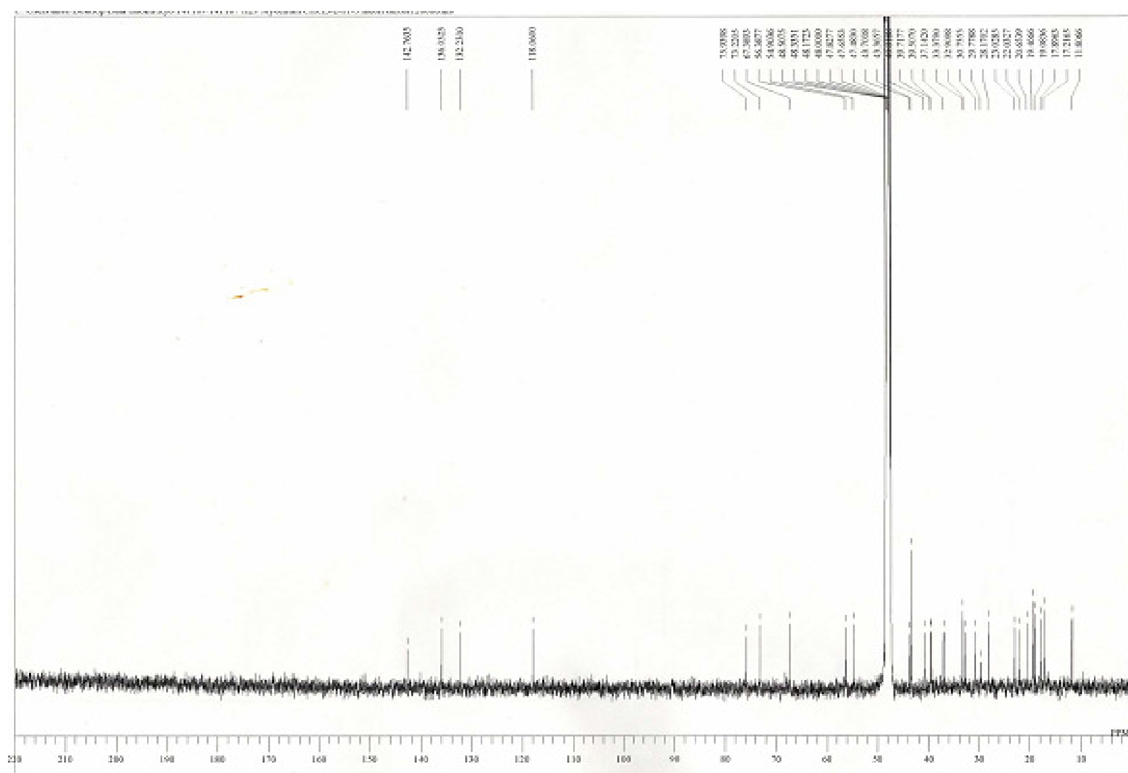


Figure 62. ^{13}C-NMR spectrum of compound **12** in CD_3OD

5α,6α:7β,8β-diepoxy-ergost-22-ene-3β,9α-diol (13)

Observed as white powder, $[\alpha]_D$ -43.8 (*c* 0.18, MeOH)

EI-MS *m/z* 428 $[M]^+$

FAB-MS *m/z* 451 $[M+Na]^+$

HR-ESI-MS *m/z* 467.3047, with a molecular formula $C_{28}H_{44}O_4Na$

^{1}H-NMR (CD_3OD) δ

0.76 (3H, s), 0.87 (3H, *J* = 6.8 Hz), 0.87 (3H, *J* = 6.8 Hz), 0.96 (3H, d, *J* = 6.8 Hz), 1.04 (3H, d, *J* = 6.8 Hz), 1.12 (2H), 1.21 (2H), 1.24 (H), 1.38 (3H, s), 1.49 (1H), 1.57 (1H), 1.65 (2H), 1.84 (2H), 1.84 (2H), 1.86 (H), 2.01 (2H), 2.03 (H), 2.18 (2H), 3.08 (d, *J* = 2.2 Hz), 3.82 (1H, m), 4.15 (d, *J* = 2.2 Hz), 5.22 (1H, dd, *J* = 15.0, 7.2 Hz), 5.24 (1H, dd, *J* = 15.0, 7.8Hz)

^{13}C-NMR (CD_3OD) δ

11.8 (q), 17.2 (q), 19.1 (q), 19.5 (q), 20.7 (q), 20.8 (q), 22.2 (t), 23.2 (t), 28.0 (t), 28.2 (t), 30.5 (t), 32.9 (t), 33.4 (d), 35.9 (s), 40.3 (t), 40.7 (d), 40.9 (s), 43.3 (d), 53.0 (d), 53.4 (d), 61.7 (d), 65.7 (s), 66.0 (d), 66.5 (s), 67.9 (d), 69.5 (s), 132.4 (d), 135.7 (d)

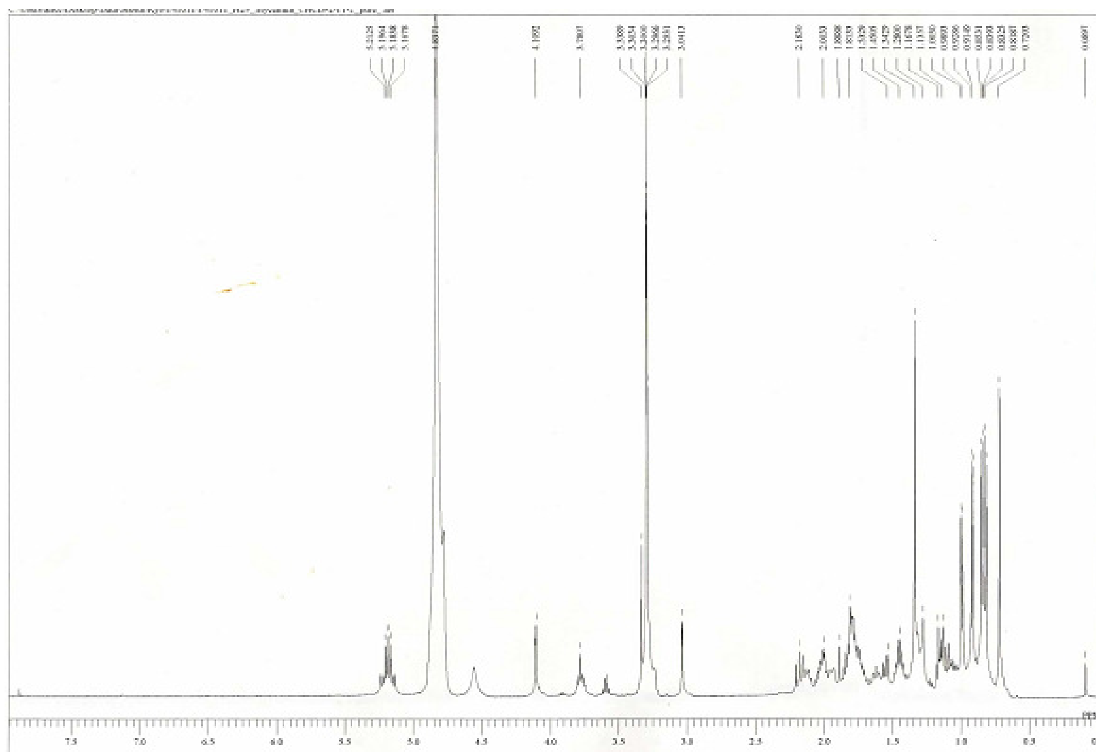


Figure 63. ^{1}H-NMR spectrum of compound **13** in CD_3OD

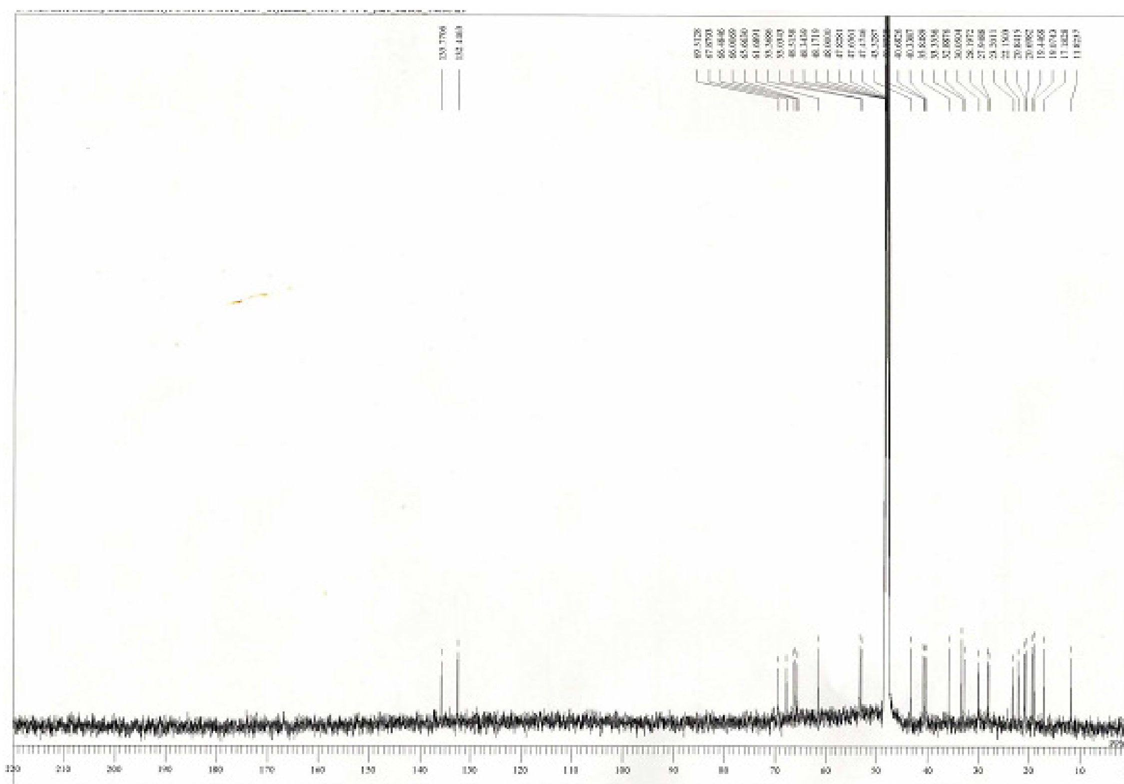


Figure 64. ^{13}C-NMR spectrum of compound **13** in CD_3OD

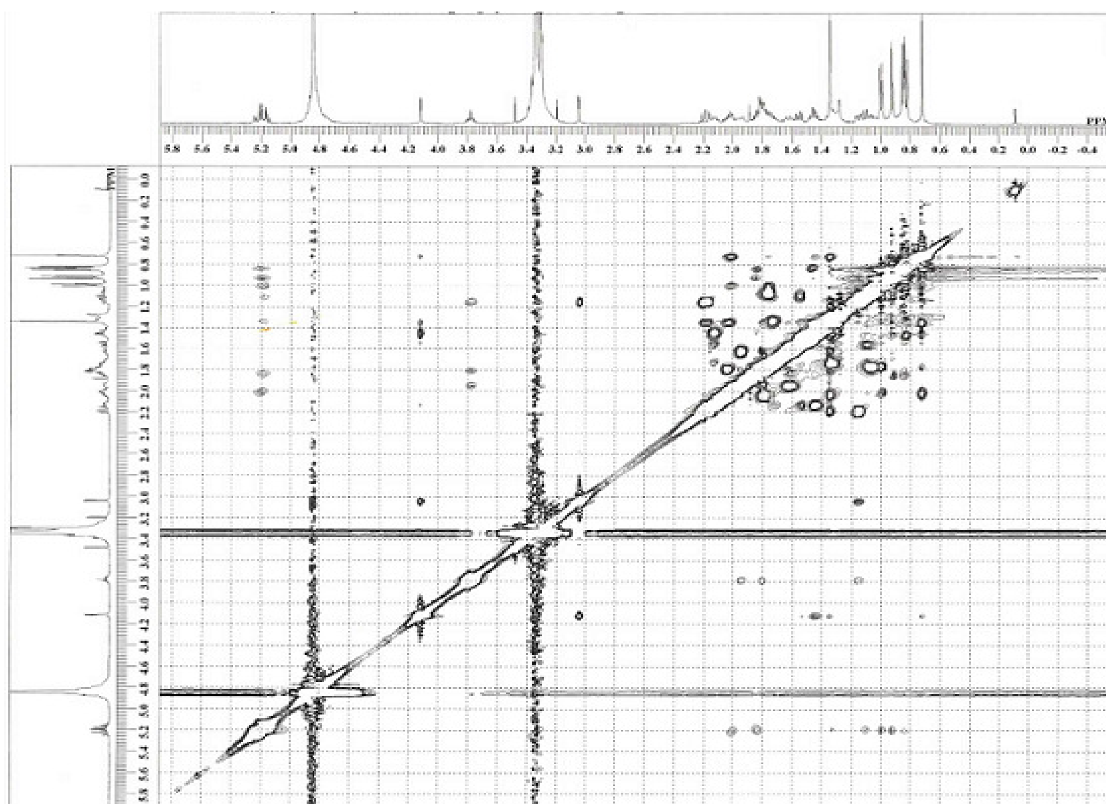


Figure 65. NOESY of compound **13** in CD_3OD

Experiment for Chapter 4

Materials

In Chapter 4, five kinds of cultured mycelia of Ganodermataceae medicinal mushrooms (*Ganoderma lucidum, G. mastoporum, G. applanatum, G. neo japonicum* and *G. boninense.*) were cultured for anti-fungal activity screening. And large scale culture of *G. mastoporum* was performed in order to isolate the bioactive compounds.

Culture methods

Cultured mycelia of *G. mastoporum* were cultured on PD agar medium in Petri dished at 25℃ in the dark for 3 weeks as pre-culture. The 10-mm plugs were cut from the culture and seeded with 5 plugs to each Roux flask containing 200ml PD medium for submerged culture. The culture mycelia were grown at 25℃ in the dark for 6 weeks. After 6 weeks cultivation, the cultured mycelia were collected and freeze dried (95g). The freeze dried sample was extracted with MeOH (3L x 3 times). The MeOH extract was evaporated to dryness to afford 16.94g crude extract.

Isolation and purification

The MeOH crude extract was then partitioned between $CHCl_3/H_2O$, followed by $EtOAc/H_2O$ and then $BuOH/H_2O$ to obtain $CHCl_3$ extract (5g), EtOAc extract (0.6g) and BuOH extract (1.2g). These extracts were applied to *M. canis* and *T. rubrum* for anti-fungal activity test. The results showed that $CHCl_3$ extract had significant bioactivity (8mg, inhibition zone: 25mm). Isolation was carried out by silica gel column chromatography and eluted with solvents in the order of hexane-EtOAc (4:1), hexane-EtOAc (1:1), hexane-EtOAc (2:3), EtOAc and MeOH to obtain fr.1 (from Hexane-EtOAc, 555.7mg), fr.2 (from hexane-EtOAc (4:1) and hexane-EtOAc (1:1), 473.6mg), fr.3 (from hexane-EtOAc (1:1), 267.9mg), fr.4 (from hexane-EtOAc (1:1), 180.7mg), fr.5 (from hexane-EtOAc (1:1) and hexane-EtOAc (2:3), 182.5mg) and fr.6 (from EtOAc and MeOH, 2016.5mg). Each fraction was applied to *M. canis* and *T. rubrum* to trace the bioactivity, fr.3 showed significant inhibition zone (13mm) with 1.5mg sample applied. Further purification was carried out by silica gel column chromatography again and eluted with solvents in the order of $CHCl_3$, $CHCl_3$-MeOH (100:5), $CHCl_3$-MeOH (9:1), $CHCl_3$-MeOH (8;2) and MeOH to obtain fr.3-1 (from $CHCl_3$, 29.1mg), fr.3-2 (from $CHCl_3$-MeOH (100:5), 22.4mg), fr.3-3 (from $CHCl_3$-MeOH (9:1), 13.7mg), fr.3-4 (from $CHCl_3$-MeOH (8:2), 101.8mg), fr.3-5 (from $CHCl_3$-MeOH (8:2), 28.6mg) and fr.3-6 (from MeOH, 55.5mg). The result of anti-fungal test showed that fr.3-4 and fr.3-5 showed anti-fungal activity.

Fr.3-4 and fr.3-5 was purified by RP-HPLC (column: Capcell PAK C18 5μm 10 x 250mm, solvent: MeOH: H_2O (0.1%TFA) = 6:4, detector: refractive index) to obtain compounds **16** (22.2mg), **17** (7.6mg), **18** (9.2mg) from fr.3-4 and compounds **14** (11mg), **15** (6mg) from fr.3-5.

benzoic acid (14)

Observed as white powder
EI-MS *m/z* 122 $[M]^+$

^{1}H-NMR ($CDCl_3$) δ

8.11 (2H, dd, *J* = 7.4, 1.1 Hz), 7.60 (1H, td, *J* = 7.4, 1.1 Hz), 7.47 (2H, t, *J* = 7.4 Hz)

^{13}C-NMR ($CDCl_3$) δ

128.5 (d), 129.3 (s), 130.2 (d), 133.8 (d), 172.1 (s)

***meso*-hydrobezoin (15)**

Observed as white powder
EI-MS *m/z* 214 $[M]^+$

13C-NMR ($CDCl_3$) δ

78.1 (d), 127.1 (d), 128.1 (s), 128.2 (d), 139.8 (d)

ergosterol (16)

Observed as white powder, $[\alpha]_D$ -49.1 (*c* 1.7, $CHCl_3$)
EI-MS *m/z* 396 $[M]^+$

^{1}H-NMR ($CHCl_3$) δ

0.63 (3H, s), 0.80 (3H, d, *J* = 7.2 Hz), 0.84 (3H, d, *J* = 7.2 Hz), 0.92 (3H, d, *J* = 6.8 Hz), 0.95 (3H, s), 1.00 (3H, d, *J* = 6.8 Hz), 3.64 (1H, m), 4.15 (d, *J* = 2.2 Hz), 5.18 (1H, dd, *J* = 15.0, 7.5 Hz), 5.22 (1H, dd, *J* = 15.0, 8.0Hz), 5.38 (1H, d, *J* = 7.6 Hz), 5.57 (1H, d, *J* = 7.6 Hz)

^{13}C-NMR ($CHCl_3$) δ

12.1 (q), 16.3 (q), 17.6 (q), 19.7 (q), 20.0 (q), 21.2 (q), 21.2 (t), 23.0 (t), 28.3 (t), 32.1 (t), 33.1 (d), 37.1 (s), 38.4 (t), 39.2 (t), 40.4 (d), 40.9 (t), 42.9 (d), 42.9 (d), 46.3 (d), 54.5 (d), 55.7 (d), 70.5 (d), 116.4 (d), 119.7 (d), 132.1 (d), 135.6 (d), 139.8 (s), 141.4 (s)

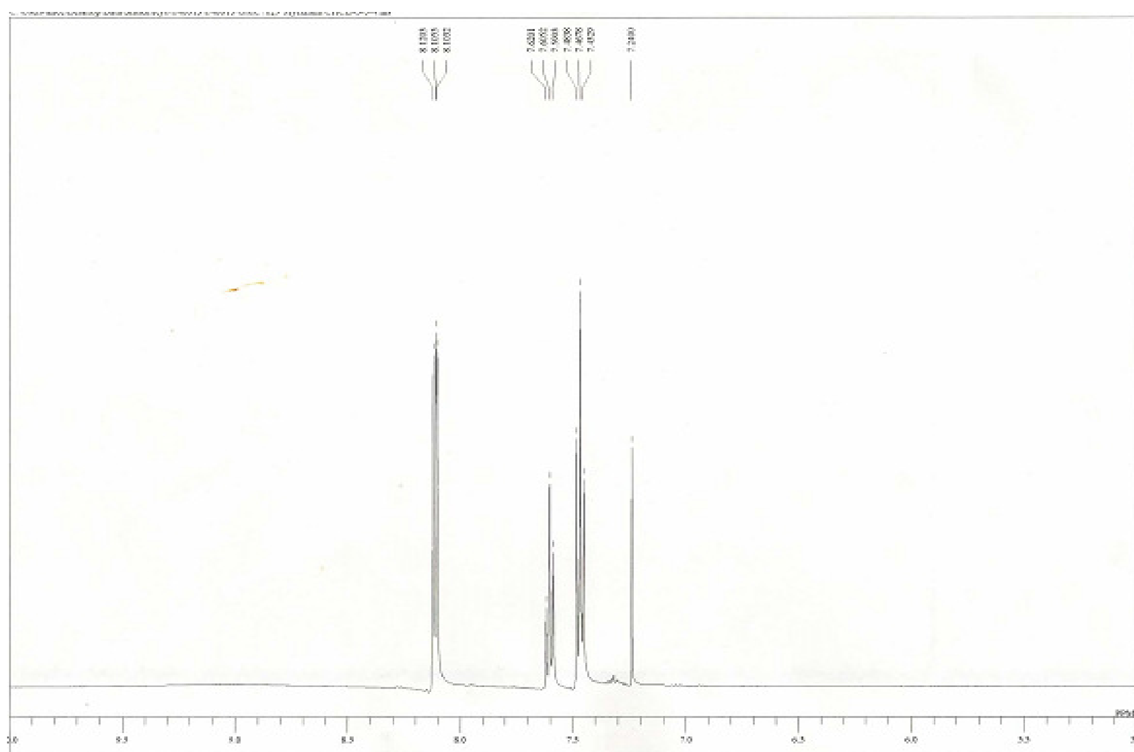


Figure 66. ^{1}H-NMR spectrum of compound **14** in $CDCl_3$

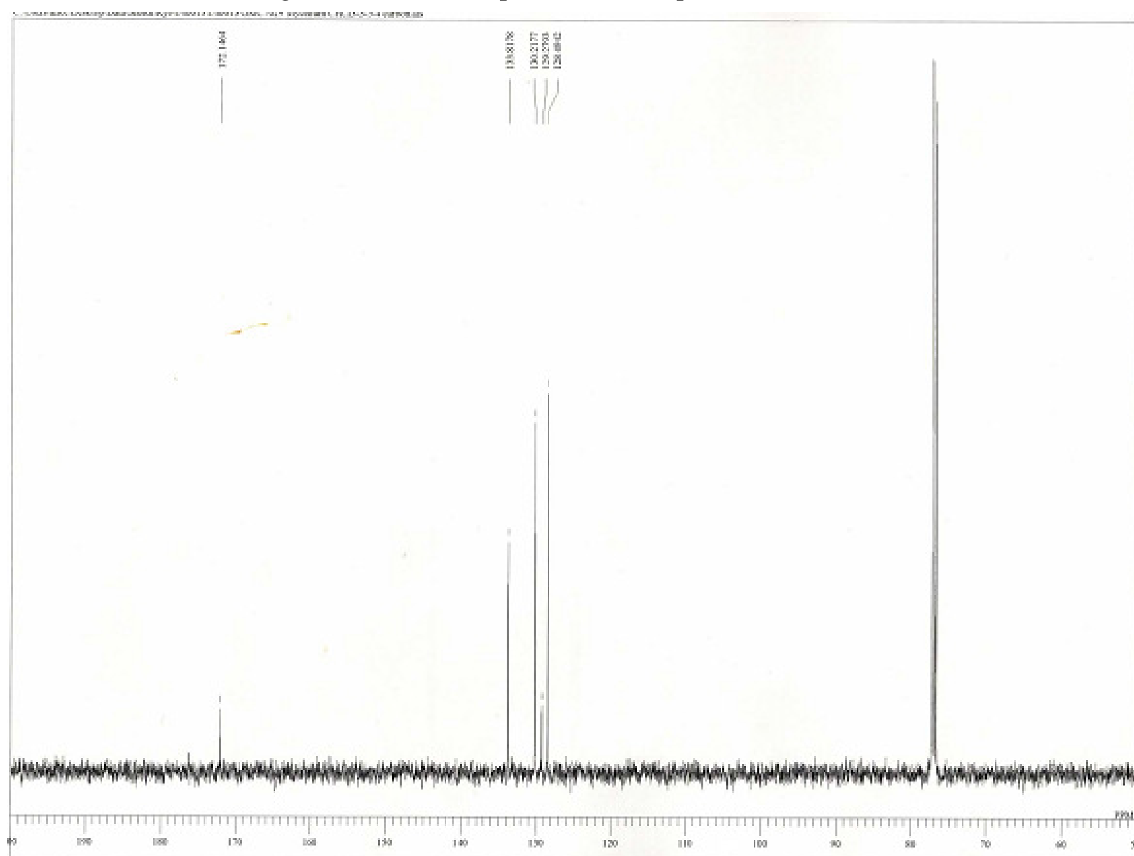
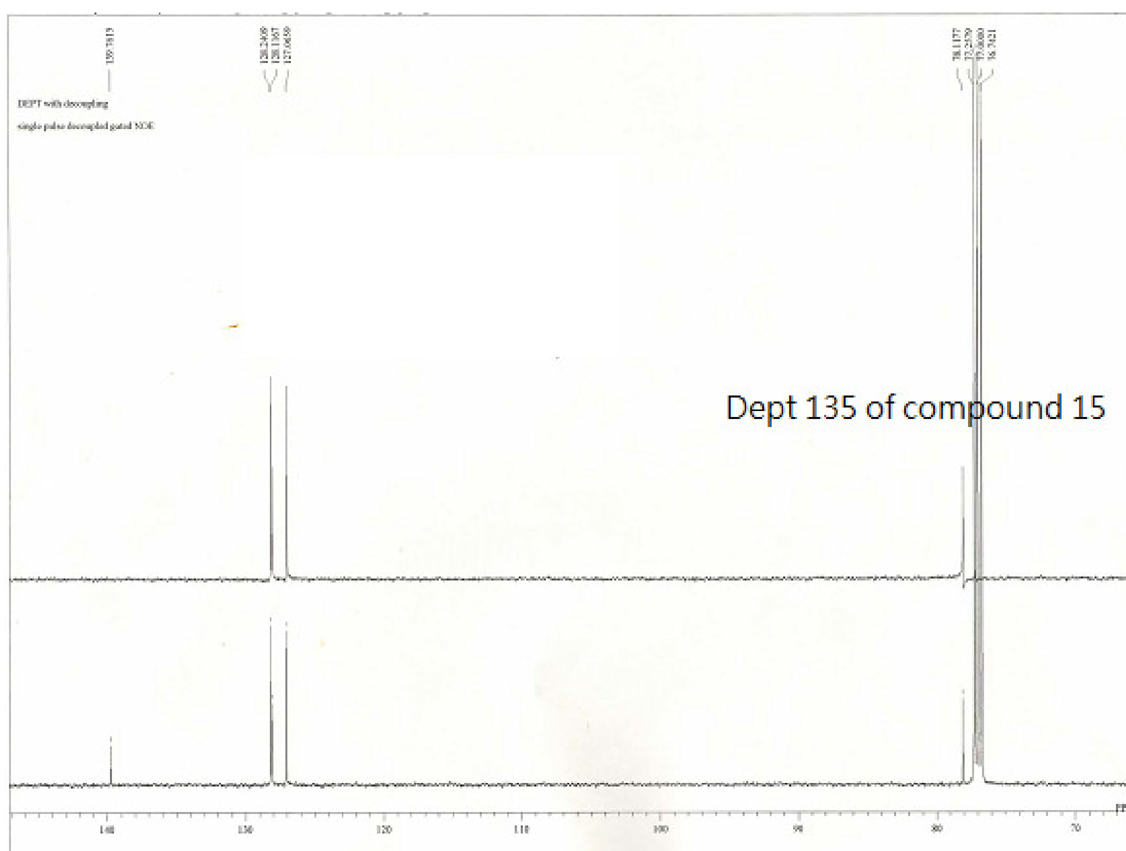


Figure 67. ^{13}C-NMR spectrum of compound **14** in $CDCl_3$



Figrure 68. ^{13}C-NMR and DEPT spectrums of compound **15** in $CDCl_3$

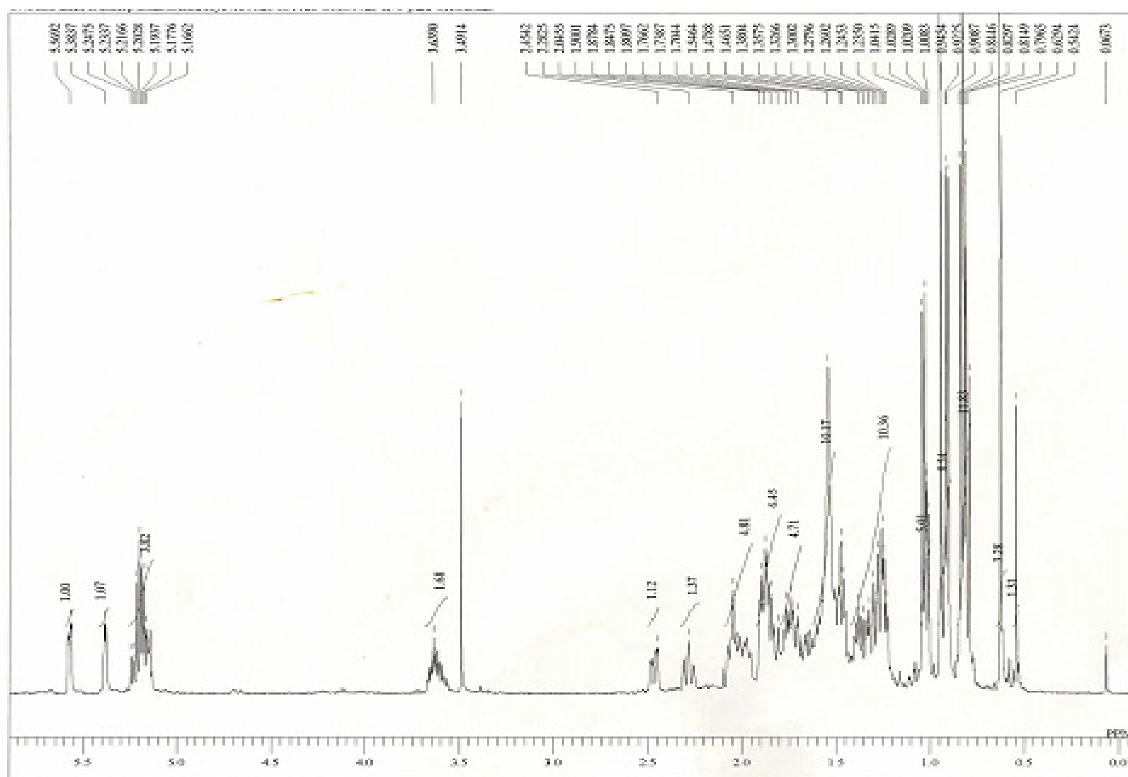


Figure 69. ^{1}H-NMR spectrum of compound **16** in $CDCl_3$

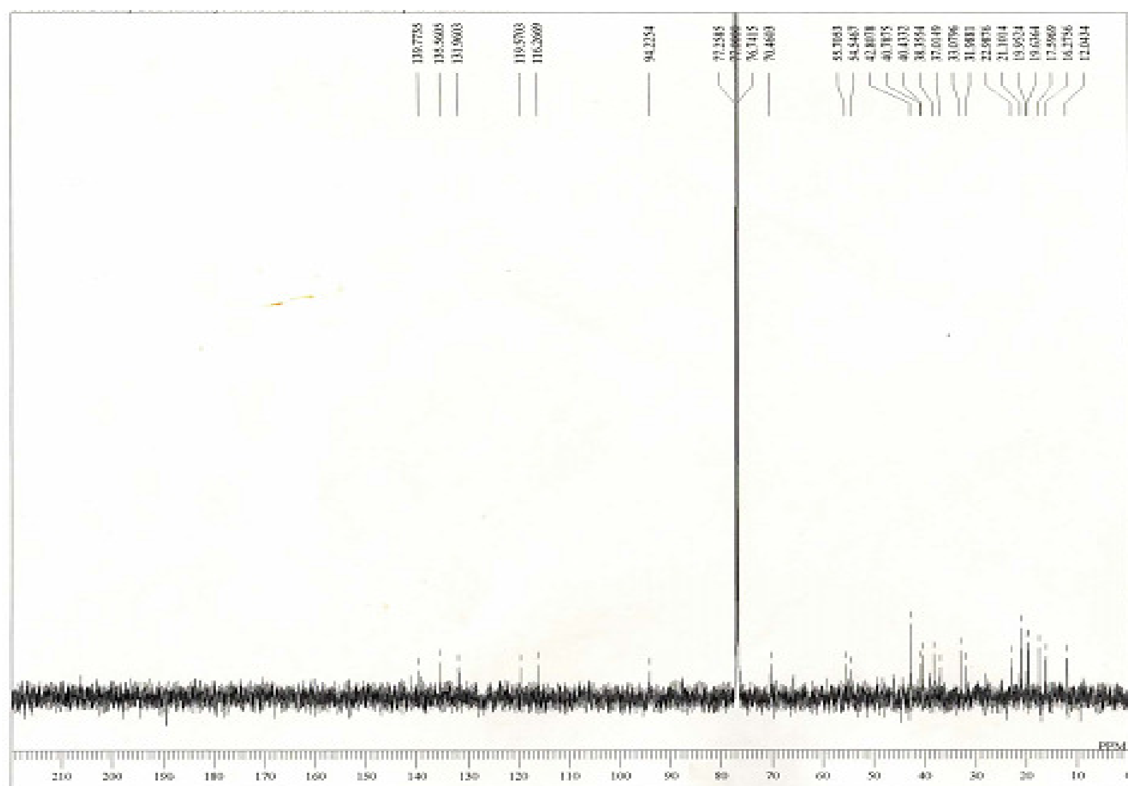


Figure 70. ^{13}C-NMR spectrum of compound **16** in $CDCl_3$

ergosterol peroxide (17)

Observed as white powder, $[\alpha]_D$ -17.8 (*c* 0.6, $CHCl_3$)
EI-MS *m/z* 428 $[M]^+$

^{1}H-NMR ($CHCl_3$) δ

0.81 (3H, d, *J* = 6.7 Hz), 0.82 (3H, s), 0.84 (3H, d, *J* = 6.7 Hz), 0.88 (3H, s), 0.91 (3H, *J* = 6.8 Hz), 1.00 (3H, d, *J* = 6.8 Hz), 3.97 (1H, m), 5.13 (1H, dd, J = 15.3, 7.8 Hz), 5.21 (1H, dd, J = 15.3, 7.4 Hz), 6.23 (1H, d, J = 8.5 Hz), 6.49 (1H, d, J = 8.5 Hz)

^{13}C-NMR ($CHCl_3$) δ

12.9 (q), 17.6 (q), 18.2 (q), 19.7 (q), 20.0 (q), 20.7 (q), 20.9 (t), 23.4 (t), 28.7 (t), 30.2 (t), 33.1 (d), 34.7 (t), 37.0 (s), 37.0 (t), 39.4 (t), 39.8 (d), 42.8 (d), 44.6 (s), 51.1 (d), 51.7 (d), 56.2 (d), 66.5 (d), 79.5 (s), 82.2 (s), 130.8 (d), 132.3 (d), 135.2 (d), 135.5 (d)

ergosterol D (18)

Observed as white powder, $[\alpha]_D$ -101.8 (*c* 0.5, $CHCl_3$)
EI-MS *m/z* 396 $[M]^+$

^{13}C-NMR ($CHCl_3$) δ

12.0 (q), 16.2 (q), 17.6 (q), 19.6 (q), 20.0 (q), 21.1 (q), 21.1 (d), 23.0 (t), 28.3 (t), 32.0 (t), 33.1 (t), 37.0 (d), 38.4 (t), 39.1 (t), 40.4 (d), 40.8 (t), 42.8 (d), 42.8 (s), 46.2 (s), 54.5 (d), 55.7 (d), 70.4 (d), 116.3 (d), 119.6 (d), 132.0 (d), 135.5 (d), 139.8 (d), 141.3 (s)

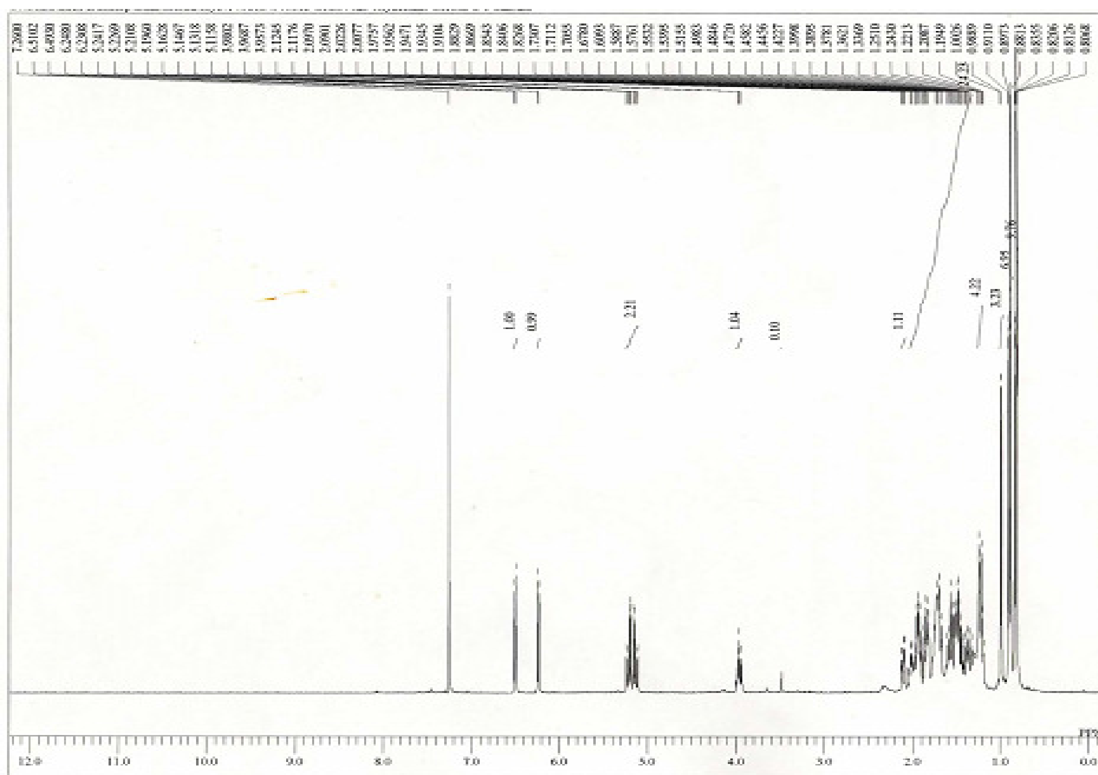


Figure 71. ^{1}H-NMR spectrum of compound **17** in $CDCl_3$

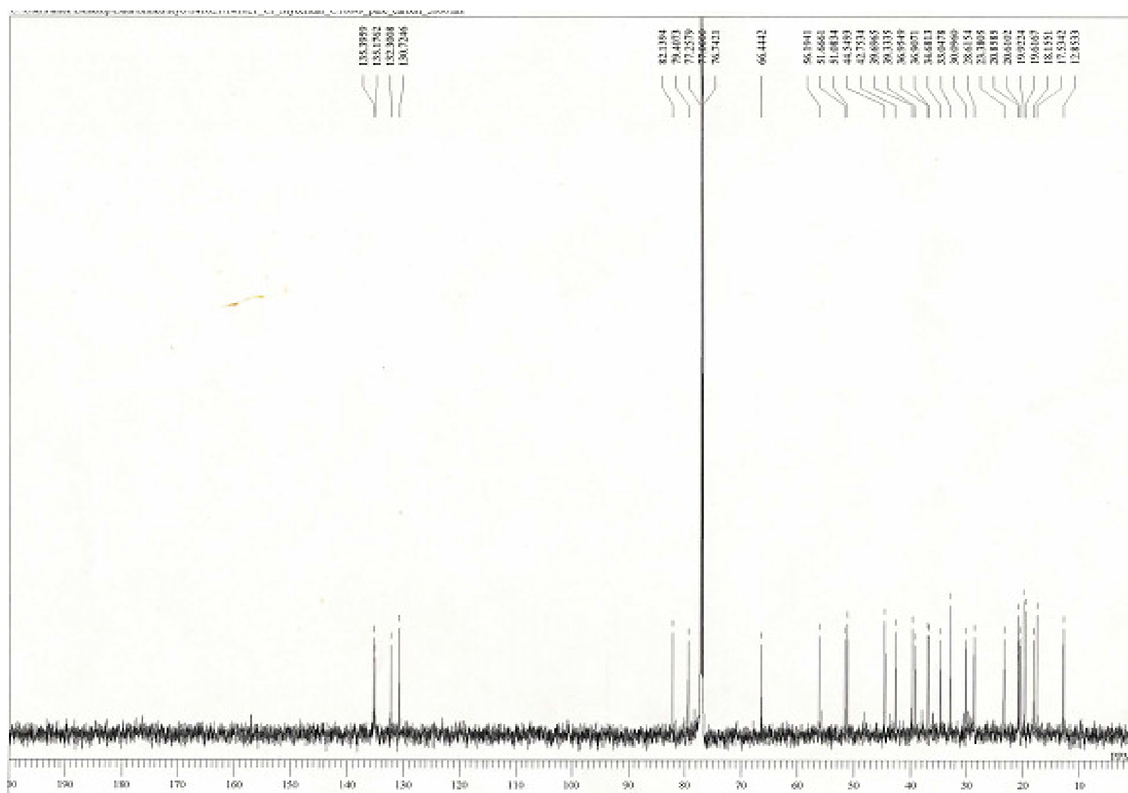


Figure 72. ^{13}C-NMR spectrum of compound **17** in $CDCl_3$

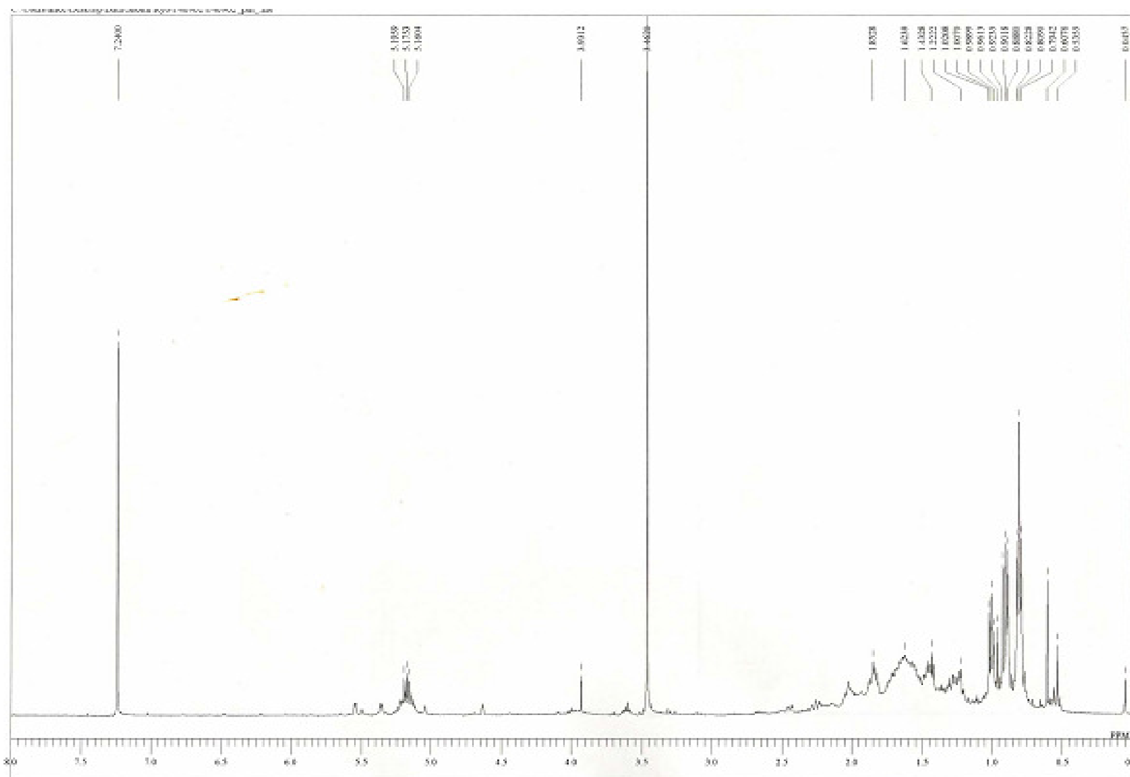


Figure 73. ^{1}H-NMR spectrum of compound **18** in $CDCl_3$

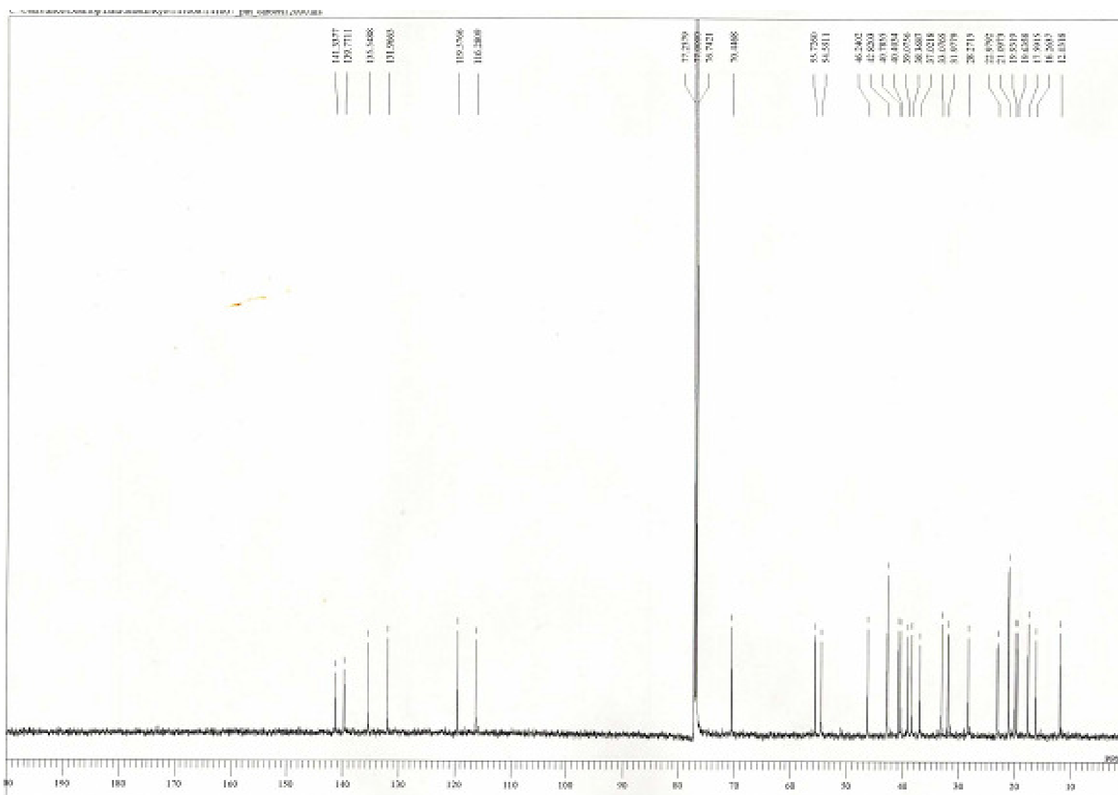


Figure 74. ^{13}C-NMR spectrum of compound **18** in $CDCl_3$

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