Isolation of antitrypanosomal compounds from Myanmar medicinal plants

(ミャンマー薬用植物からの抗トリパノソーマ剤の探索)

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Contents

List of abbreviation

AcOH	Acetic acid
ALAT	α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate
AMPA	Alanine amino transferase
BSA	Bovine serum albumin
^{13}C-NMR	^{13}C nuclear magnetic resonance
$CHCl_3$	Chloroform
δ	Chemical shift in parts per million
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EtOAc	Ethyl acetate
EtOH	Ethanol
EI-MS	Electron ionization mass spectroscopy
FAB-MS	Fast atom bombardment mass spectroscopy
^{1}H-NMR	Proton nuclear magnetic resource
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HMI-9	Hirumi-9 medium
HPLC	High performance liquid chromatography
HS	Horse serum
IC_{50}	50% inhibitory concentration
IMDM	Iscove's modified Dulbecco's medium
IR	Infrared absorption
$[M]^+$	Molecular ion

MDH	Malate dehydrogenase
MeOH	Methanol
MHz	Megahertz
µM	Micromolar
MS	Mass spectrometry
m/z	Mass-to-charge ratio
NaOH	Sodium hydroxide
$NaHCO_3$	Sodium bicarbonate
PS	Penicillin
PTLC	Preparative thin-layer chromatography
SiO_2	Silica gel
TLC	Thin-layer chromatography
UV	Ultraviolet absorption

List of Figures

List of Tables

List of Schemes

CHAPTER 1

INTRODUCTION

Trypanosomiasis

Trypanosomiasis, a serious parasitic disease affecting humans and animals in many countries, is caused by blood-dwelling *Trypanosoma* species. Trypanosomes are protozoan parasites grouped in the order “Kinetoplastida” and are a monophyletic group of obligate, unicellular parasitic flagellate protozoa. The genus *Trypanosoma* is divided into two main groups based on the mode of transmission by their insect vectors: stercoraria and salivaria (Fig. 1). Stercoraria parasite development takes place in the intestinal tract of the invertebrate vector, and the route of vertebrate infection is via feces. On the other hand, salivarian parasites colonize the stomach of their invertebrate vectors, but never pass to the intestinal tract. Instead, they migrate towards the salivary gland of the vector where the infectious form develops to invade their vertebrate hosts (Hoare et al., 1966, Wery et al., 1995).

A certain collection of salivarian trypanosomes can be further classified into the *Trypanozoon* group (Fig. 2). Within this subgenus, there are three major trypanosome species; namely *T. brucei*, *T. evansi* and *T. equiperdum* (Brun et al., 1998). *Trypanosoma brucei* has three subgenera; *T. b. rhodesiense* and *T. b. gambiense* are the causative agents of sleeping sickness in humans, and *T. b. brucei* is the causative agent of Nagana, a cattle disease in Africa. Among of the salivarian trypanosomes, *T. evansi* has the widest distribution of all species of trypanosomes and the greatest range of mammalian hosts (Hoare et al., 1972), making it one of the most economically important protozoan diseases present in the world today. For example, it causes a serious pathogenic protozoan infection

in animals, which is the pathogenic trypanosome first discovered. Dr. Griffith Evans discovered the organism in 1880 in the blood of horses and camels in India that were suffering from a disease called surra, which is of great economic threat in Africa, Asia, and Central and South America, where thousands of animals die from *T. evansi* infection in every year (Brun et al., 1998).

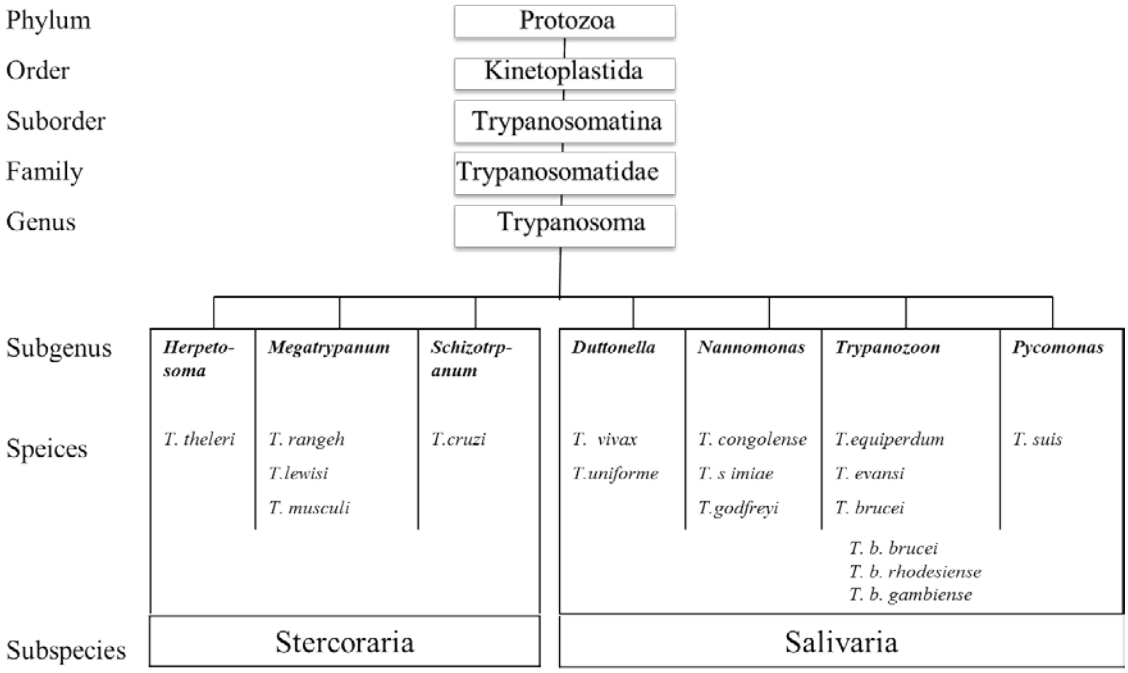


Fig. 1. Classification of trypanosomes.

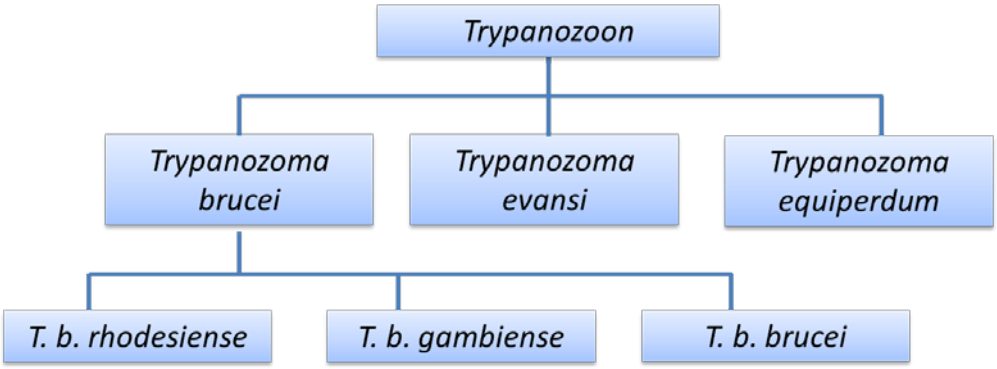


Fig. 2. Classification of the *Trypanozoon* group belonging to salivarian trypanosomes.

1.1 *Trypanosoma evansi*

1.1.1 Morphology and epidemiology

Trypanosoma evansi is an animal-pathogenic flagellated protozoan parasite and the most widespread pathogenic trypanosome globally (Cadioli et al., 2006). It infects a variety of animals and causes epidemics of a disease known as surra. The disease occurs not only in Africa, but also in Central and South America and Asia. *T. evansi* has arisen from *T. brucei brucei*, and it closely related to *T. brucei*. It is distinguished from *T. b. brucei* by its distinctive kinetoplast DNA (kDNA), which shows the lack of minicircle sequence heterogeneity and an absence of maxi circles (Hoare et al., 1956). A procyclic or insect stage does not exist in *T. evansi*, a phenotype that is attributed to the lack of maxicircles in the kinetoplast DNA (Borst et al., 1987). The size of *T. evansi* is between 15-36 µm in length, excluding the free flagellum (Stevens et al., 2004) (Fig. 3). Additionally, *T. evansi* is covered by a thick layer of a single glycoprotein, which is the primary immunogen eliciting antibody formation. Periodically, the organism alters the glycoprotein coating and evades defensive responses of the host (Brun et al., 1998).

*T. evans*i has a wide range of hosts and is pathogenic to most domestic, wild and laboratory mammals (Hoare et al., 1972). Camels are most often affected in the Middle East and Africa, horses in South America and horses, mules, buffaloes and deer in China. In Vietnam, the buffaloes and cattle commonly acquire surra with heavy clinical signs and die more frequently in the winter and the beginning of spring when they encounter many environmental stresses such as insufficient nutrition, hard work and cold weather. Southeast Asia, surra is mostly found in horses, cattle and buffaloes (Holland et al., 2004). The severity of the disease varies according to the strain of *T. evansi* and host factors such as general health and stress of the animal (Brun et al., 1998). Human infection by animal

species of *Trypanosoma* is usually impossible because of a trypanolytic factor in human serum. However, it has been found that *T. congolese* and *T. evansi* are capable of resisting human plasma in certain circumstances (Hawking et al., 1978). Recently, a human case of trypanosomiasis caused by *T. evansi* was identified in India (Joshi et al., 2005).

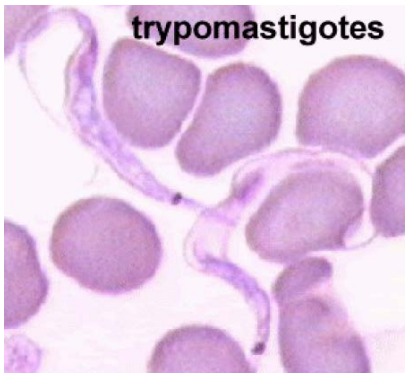


Fig. 3. Trypomastigotes form of *Trypanosoma evansi* in blood smear.

T. brucei differs fundamentally from *T. evansi* in complete cyclical development in the tsetse fly (Fig. 4). In the case of *T. brucei*, the tsetse ingests trypanosomes when it feeds on an animal infected with the parasite. The trypanosomes differentiate into several forms in the fly, culminating in the metacyclic form, which is able to infect mammalian hosts. When the infected fly next feeds on a healthy animal, these metacyclic trypanosomes are transmitted into the skin along with tsetse saliva. The parasites in this newly infected animal differentiate into a bloodstream form specially adapted to live in the mammalian blood (Foli et al., 1989). The bloodstream parasites multiply by binary fission and enter the animal's lymphatic and blood circulation. As flies feed on animals infected with the parasites, they take up blood containing trypanosomes, which then completes the life cycle.

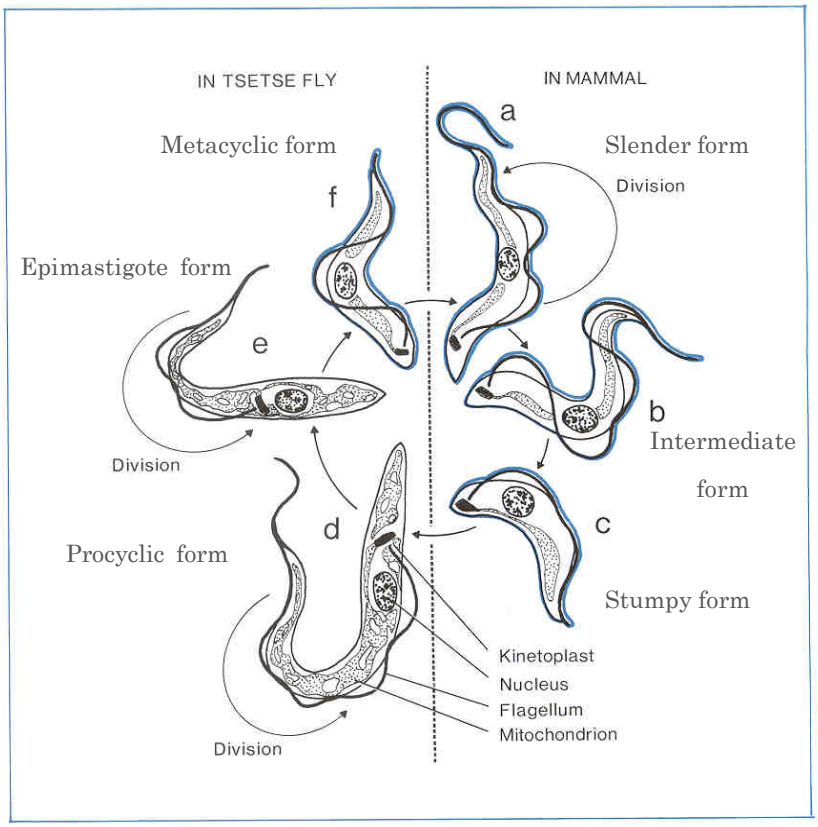


Fig. 4. Life cycle of *Trypanosoma brucei*.

(http://www.ilri.org/InfoServ/Webpub/fulldocs/Ilrad90/Trypano.htm)

Infection begins when trypanosomes are injected into the blood of a mammal by a tsetse fly as it feeds on the animal. In the animal host, slender forms of the parasites multiply by binary fission until large parasite numbers build up in the blood. The trypanosomes then transform first into intermediate forms and then into stumpy forms, the latter of which are able to infect tsetse flies. Stumpy forms of the parasites are ingested by a tsetse fly as it feeds on an infected animal. In the midgut of the tsetse fly, procyclic forms arise and undergo division, after which the parasites enter the proventriculus and later the salivary glands of the fly, where they assume epimastigote forms and undergo further division. Finally, metacyclic formsarise in the salivary glands. Metacyclics are able to infect mammals, and the life cycle is repeated.

1.1.2 Transmission

1.1.2.1 Mechanical transmission by biting

Although *T. evansi* is not transmitted by tsetse flies, they are transmitted in several other ways, via biting insects, sucking insects, and vampire bats (Fig. 5). Mechanical transmission by biting hematophagous insects, especially flies in the Tabanidae family such as horseflies (*Tabanus* spp.) and stable flies (*Stomoxys* spp.), *Haematopota*, and *Chrysops* spp. (Fig. 6), is the most important mode of transmission for *T. evansi*. Larvae of horseflies live in soil near water (Hoare et al., 1972). Mechanical transmission is a nonspecific process, which can take place when a biting insect initiates a blood meal on an infected host, starts to feed on infected blood, is interrupted by defensive movements of the host, or flies off from the infected host, and lands on another animal to begin its blood meal again. When the insect first attempts to feed on blood, its mouthparts can contain a small amount of blood via capillary strength, estimated at 1–12 nL in tabanids and 0.03 nL in *Stomoxys* (Foli et al., 1989). Trypanosomes do not generally survive for very long in biting insects. For example, their survival was estimated at 30 min with *T. vivax* in tabanids and even shorter in *Stomoxys* spp. (Ferenc et al., 1988). Experimental research shows that the transmission is efficient when there is a short time lapse between two interrupted blood meals, that is, less than 30 min (Sumba et al., 1998, Mihok et al., 1995). No intermediate hosts are present and there is no insect developmental cycle available for *T. evansi*. *T. evansi* reproduces by binary fission in the mammalian host. However, their vectors may vary depending on the conditions. For example, *Tabanus* species are the most efficient vector for transmission of this parasite in China and Indonesia (Luckins et al., 1988, Lun et al., 1993).

1.1.2.2 Other means of transmission

In addition to vector transmissions and the contamination of a wound, iatrogenic transmission caused by the use of nonsterile surgical instruments or needles may be of importance, especially during vaccination campaigns and mass treatments that could spread disease (Davila et al., 2000). Sexual transmission or transmission from dam to calf or foal can occur in particular cases, when mucosae are altered or in cases of very close contact (licking) with parasitized secretions (mucus, lacrimation, etc.). This may be responsible for occasional direct horizontal transmission, although the real impact has not been estimated. *T. evansi* may be transmitted by perioral contamination. This mechanism could obviously occur quite easily when the oral mucosae are damaged. This may be frequent when carnivores eat infected prey. Dogs and cats living in the vicinity of slaughterhouses could be infected by eating fresh meat, blood, offal, or bones. Hunting dogs and wild carnivores could be contaminated in this way. The observation of circus tigers infected by *T. evansi* also suggests that infection is most likely due to eating infected meat (Bhaskararao et al., 1995). Thus, the presence of *T. evansi* in French Guiana was revealed by the observation of a single case in a hunting dog (Desquesnes et al., 2013), which demonstrates the importance of the role of the dog as a sentinel for surra. In addition to mechanical transmission by insects and vampire bats, *T. evansi* can be transmitted through milk or during coitus (Brun et al., 1998, OIE., 2004).

1.1.2.3 The role of vampire bats

Transmission by the vampire bat (*Dosmodus rotundus*) is a newly appointed biological system that has been established in Latin America (Hoare et al., 1965). Vampire bats are infected by the oral route when they lick blood from an infected prey (most often

horses or cattle). As a host of *T. evansi*, bats may develop clinical symptoms and die during the initial phase of the disease. However, in the case of surviving bats, the parasite multiplies in the blood and is then found in the saliva of chronically infected bats or in bats that do not show any clinical symptoms. Later, infected bats can contaminate their congeners by biting, thus acting as reservoir hosts. They can also contaminate livestock, acting as permanent vectors, capable of contaminating their hosts for a long period of time. Lastly, in the case of bats, the trypanosome may be transmitted from biter to bitten or vice versa. Because the vampire bats can contaminate each other, a vampire colony can maintain *T. evansi* in the absence of the main host (e.g., the horse), which makes them a reservoir for the parasite. When feeding on horses or cattle, vampire bats are vectors, in as much as they initiate infection that biting insects can then spread to other susceptible animals (OIE., 2004, Desquesnes et al., 2013).

1.1.2.4 Detailed insight into transmission by biting

It was initially suspected that the transmission of trypanosomes was purely mechanical (as in the case for *T. evansi*, which is transferred by biting flies or when carnivores bite their prey and get oral wounds). In 1909, Kleine carried out transmission experiments from human patients to apes and monkeys. He showed that infected tsetse flies were vectors and that the parasite had to be present in the fly for a minimum period before it could cause infection. He subsequently showed that only the metacyclic forms in the salivary glands of the insects were infectious. When a tsetse fly bites an infected human patient, it can ingest the parasites. After their arrival in the insect stomach, many (99%) die because of interactions with specific lectins in the insect stomach; lectins are proteins that bind specifically to certain saccharides, such as those present on glycoproteins (Holland et

al., 2003). In man, the parasites multiply outside the cells, namely in the blood, lymph and cerebrospinal fluid. Others can survive in certain areas such as the plexus choroideus in the brain, from whence they cause flare-ups (WHO., 2012).

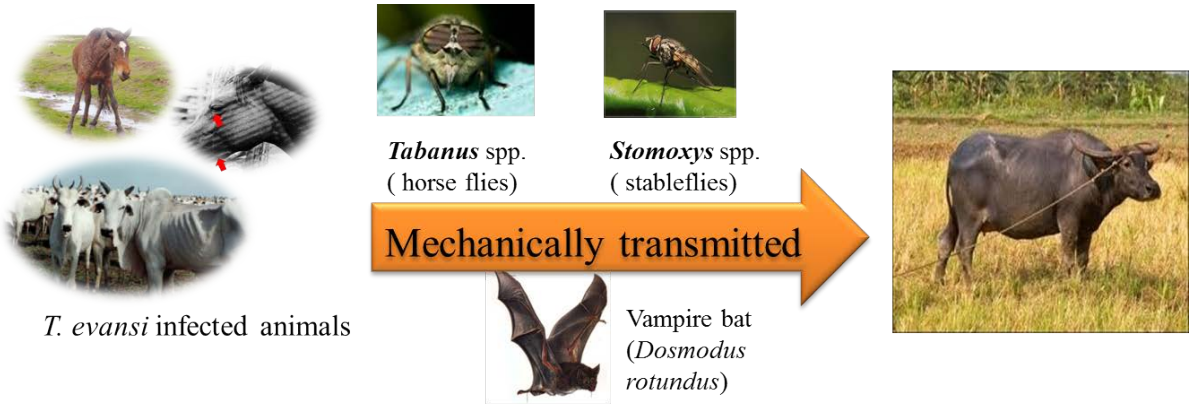


Fig. 5. Mechanical transmission of *T. evansi* by hematophagous insects (Bawm et al., 2010).

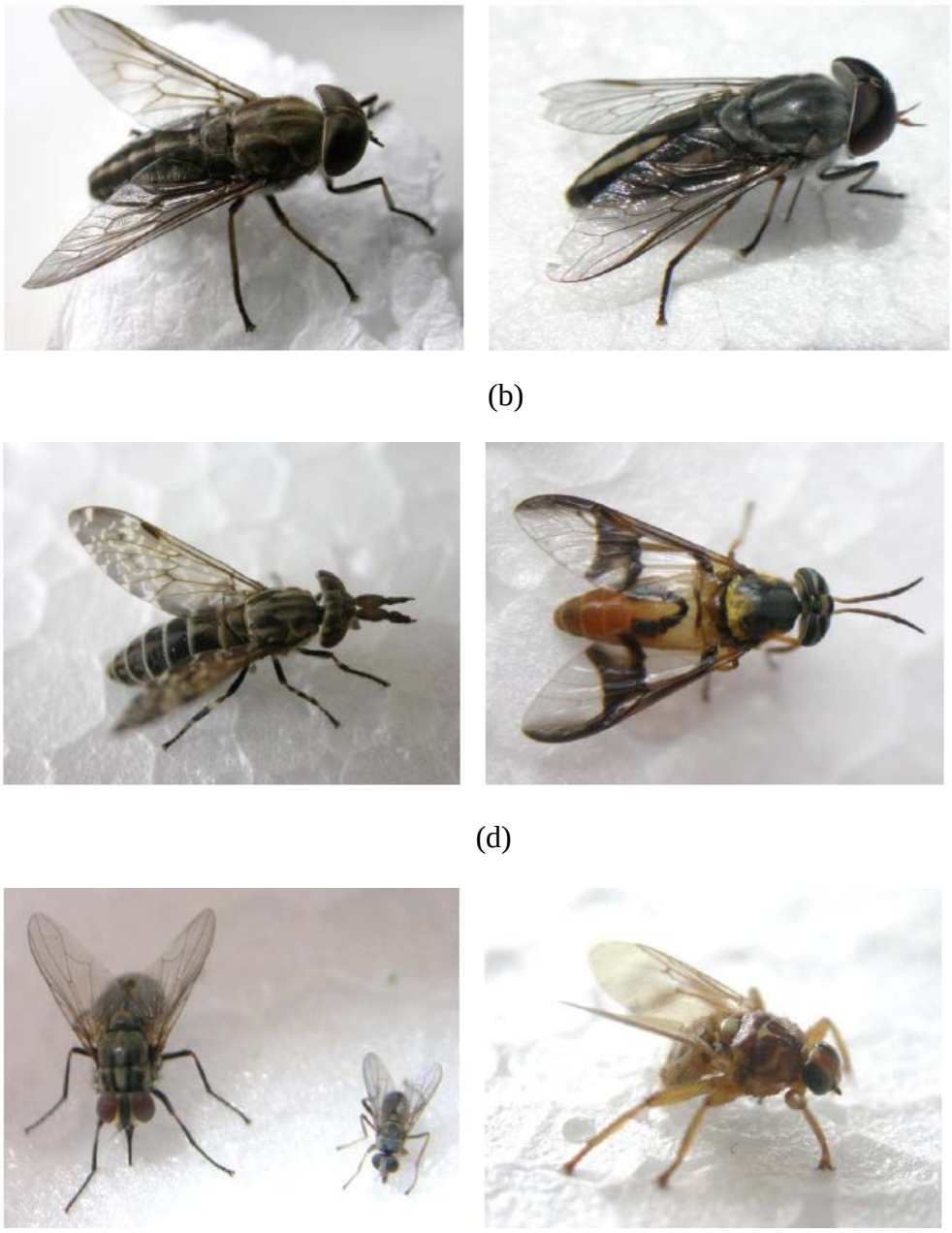


Fig. 6. Close-up of horseflies, some of the potential insect vectors of *T. evansi* in Thailand; (a) *Tabanus rubidus* (18-25 mm), (b) *Tabanus striatus* (13-18 mm), (c) *Haematopota* sp. (8-15 mm), (d) *Chrysops dispar* (9-16 mm), (e) *Stomoxys* sp. and *Haemotobia* sp. (5-9 mm and 2.5-4 mm) and (f) *Hippobosca* sp. (9-13 mm) (Desquesnes et al., 2013).

1.1.3 Clinical signs and distribution

Clinical signs of the disease can vary by individual or between species. The clinical signs of surra caused by *T. evansi* are characterized by fever and anemia, followed by emaciation, edema, cachexia and enlargement of the lymph nodes and spleen. Neurological symptoms occur late in the disease. The acute stage symptoms of this disease include abortion, central nervous system disorders, and even death. The chronic condition is characterized by decreased; working capacity and productivity of the animals (WHO, 2005). Acutely infected animals die within weeks or months. In contrast, some chronic infections of *T. evansi* may persist for several years (Brun et al., 1998, Boid et al., 1980). In horses, donkeys, and mules, the incubation period of the disease ranges from 5 to 60 days. The disease is often rapidly fatal in camels, buffaloes, horses, cattle and dogs, but mild and subclinical infections can also occur in these species. In cattle and water buffaloes, a wide range of other clinical signs recorded include fever, salivation, diarrhea, edema, jaundice, conjunctivitis, lacrimation, mucopurulent nasal discharge, dyspnea, alopecia, urticaria, swelling of superficial lymph nodes, abortion and infertility, decreased milk yield, incoordination and paralysis (Luckins et al., 2004). The disease is made more severe by factors such as poor nutrition, stress and over working. Some animals recover very slowly without treatment while others become sick, collapse and die after few months and show necrosis at the edges of the ears.

T. evansi, the causative agent of “surra” has spread far outside the tsetse regions of sub-Sahara Africa and is at present known to occur throughout the Sahel region of Africa, in North Africa, most near and Far East Asian countries and many countries in Latin American, from Argentina in the south to at least Venezuela and Colombia in the north. The situation in Central America is not well defined, but in the past the parasite has been reported in at least

one country, Panama. The endemic geographic distribution of this disease ranges from China, the Philippines and parts of Indonesia, across Southeast Asia and parts of the former U.S.S.R, into the Indian subcontinent (Fig. 7), the Middle East and Africa, until it reaches Central and South America (OIE, 2004).

Surra was reported from Myanmar in 1885. During the course of military action there, some 6,500 mules imported from the Yunnan Province in China infected with the disease in Myanmar. Some died in Myanmar, but nearly 5,000 survived and returned to China. In China, *T. evansi* was first introduced in 1885 from Myanmar into the Yunnan Province in the southwest and later to other parts of China. Then, trypanosomiasis of animals became widespread throughout most of the country. *T. evansi* was first detected in 1888 in mules from North Vietnam and further isolations from equids were reported in the ensuing years. The first cases of bovine trypanosomiasis were detected in South Annam in 1906. The prevalence of infection varied between 25% and 40% and epidemics of surra killed hundreds of camels (Fig. 8 and Table 1) (Luckins, 1988).

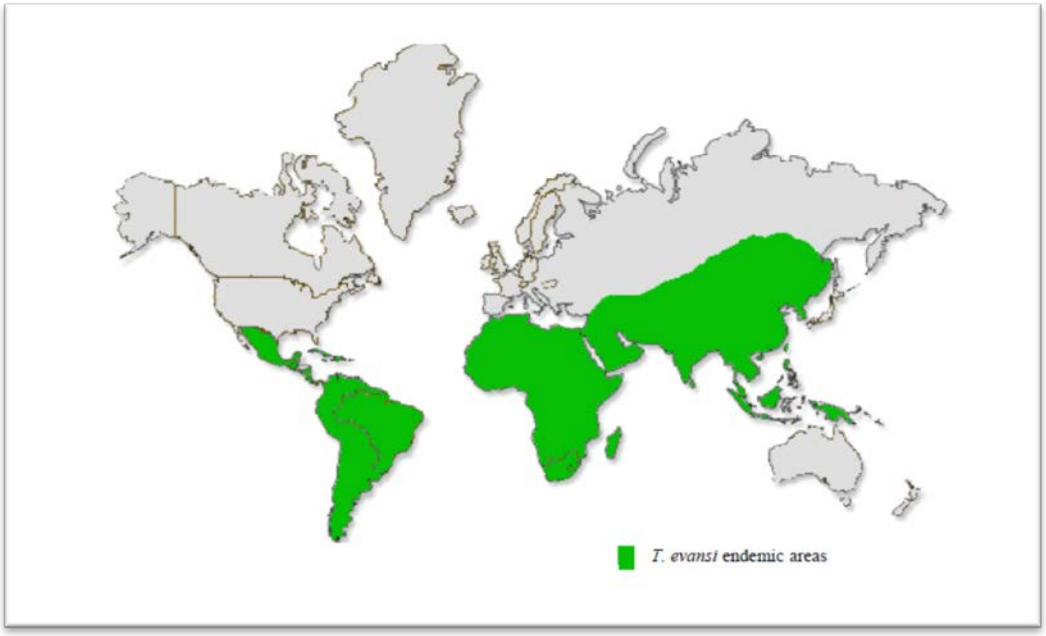


Fig. 7. Geographical distribution of endemic areas of *T. evansi* infection (OIE, 2004).

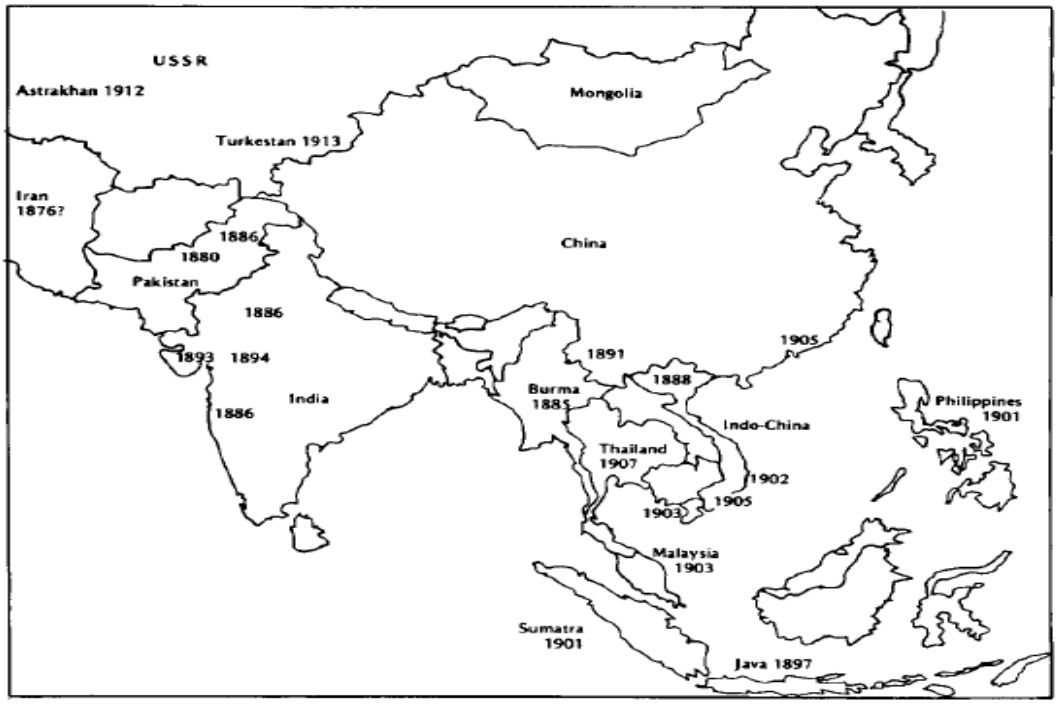


Fig. 8. Occurrence of *T. evansi* in Asia. The dates indicated correspond to the earliest reports of the disease associated with the demonstration of parasites in the animals (Luckins, 1988).

Table 1. Geographic distribution of *Trypanosoma* species.

Trypanosome species	**Domestic animals affected**	**Reservoir hosts**	**Geographic area**
T. congolense	Cattle, camels*, horses, dogs, sheep, goats, pigs	Several groups of wild mammals	Africa
T. simiae	Pigs	Wart hog, bush pig	Africa
T. vivax	Cattle, sheep, goats, domestic buffaloes, horses	Several groups of wild mammals	Africa and parts of South America
T. uniforme	Cattle, sheep, goats	Various wild ruminants	
T. brucei brucei	Horses, camels*, dogs, sheep, goats, cattle, pigs	Several groups of wild mammals	Sub-Saharan Africa
T. b. rhodesiense *T. b. gambiense*	Human sleeping sickness; affect domestic animals as *T. b. brucei***	Several groups of wild mammals (particularly *T. b. rhodesiense*)	
T. evansi	Camels, horses, dogs, domestic buffaloes, cattle	Several wild mammals in Latin America	Africa, Asia , Central and South America
T. equiperdum	Horses, donkeys, mules	-	North and South Africa

* Camels are highly susceptible to *T. congolense* and to *T. brucei*, but do not usually penetrate into tsetse country

** In particular, the behavior of *T. brucei rhodesiense* in domestic animals is quite similar to that of *T. b. brucei*, whereas *T. brucei gambiense* is typically more chronic (as it is in humans).

1.1.4. Chemotherapeutic treatment of trypanosomiasis

In general, the chemotherapeutic approach is used much more widely than vector control because it is easier to kill the trypanosomes than the flies (WHO., 1998). The chemotherapy of animal trypanosomiasis has made great progress and there are several highly active drugs that are easy to use. The use of trypanocides has consequently become widespread, and the number of trypanocidal treatments carried out every year in Africa is increasing. trypanocides can be divided into two categories. The “curative drugs” are used for treatment and have a short-term effect. They can kill the parasites, although they do not always eliminate 100% of them. The “curative/preventive drugs” are used for chemoprophylaxis. They not only kill parasites but also prevent any new infection or new circulation of parasites, due to the remnants of a sustainable curative dose in the serum of animals under chemoprophylaxis (Desquensnes et al., 2013). Curative drugs aim to eliminate parasites from a sick animal. A drug could be regarded as “curative” when the dose used is able to eliminate all parasites. The most widely used curative trypanocide against surra is diminazene aceturate (Berenil). Other drugs also can be used, such as suramin (Germanin), isometamidium chloride (Samorin) (both curative and preventive), homidium (Novidium), quinapyramine sulphate and cymerlarsan (MelCy) (so far, only recommended for curative treatment of camels), quinapyaramine (curative and/or preventive) (Fig. 9) (Table 2).

Diminazene aceturate (DA) (Fig. 9) is an aromatic diamidine used to control babesia and trypanosome infections in ruminants. A curative dose for DA is administered via intramuscular injection to obtain a high concentration of the chemical in the circulating blood. The withdrawal period for the consumption of produce from cattle treated with DA is 21 days for meat and 3 days for milk (Peregrine et al., 1993). However, the amount of

remnant drug actually suggests a longer withdrawal period of 30 and 21 days for meat and milk, respectively (Mdachi et al., 1995). The dose recommended for the treatment of infections caused by parasites belonging to the *Trypanozoon* subgenus is 7 mg/kg body weight (bw) of DA, via intramuscular injection. The reality in the field often reveals that a dose of 3.5 mg/kg bw is used to control surra. This could be for various reasons, including ignorance of the right dose or financial concerns. The use of DA for horses and dogs is limited due to poor efficacy and tolerance in trypanosome species. Diminazene aceturate has been used for a long time. Consequently, trypanosomes have developed chemoresistance in most parts of the world (OIE., 2004, Pregrine et al., 1993).

Suramin was introduced in the early 1920s produced by chemical modification of the coloring agent trypan red (Fig. 9). Suramin was originally synthesized as a dye and is principally used for the early treatment of East African *T. b. rhodesiense*, which causes sleeping sickness in humans, as well as treatment of *T. evansi* infection in animals. This naphthalene compound is soluble in water, but solutions deteriorate quickly in air. It is a relatively slow-acting trypanocide (>6 hours *in vitro*) with high clinical therapeutic activity against both *T. b. gambiense* and *T. b. rhodesiense*. Suramin reacts reversibly with a variety of biomolecules *in vitro*, inhibiting many trypanosomal and mammalian enzymes and receptors unrelated to its antiparasitic effects, including purinergic and AMPA (α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate) receptors (Suzuki et al., 2004). Suramin is also used as a treatment for trypanosomiasis caused by *T. evansi* in animals in India, and this treatment was considered to be the most appropriate for the consenting patient (Joshi et al., 2005). The most common problem encountered after several doses of suramin is renal toxicity, manifested by albuminuria, and delayed neurological complications, including headache, metallic taste, paresthesias and peripheral neuropathy

(Voogd et al., 1993).

Isometamidium (Samorin®, Trypamidium®) and homidium (chloride salt; Novidium®; bromide salt or ethidium bromide: Ethidium®) (Fig. 9) are phenanthridinium compounds, whose antitrypanosomal activity was first demonstrated more than 50 years ago (Browning et al., 1938). Isometamidium differs from homidium by an additional *m*-amidinophenyl-azo-amine moiety, which is in fact part of the diminazene molecule. Both isometamidium and homidium are active against *T. congolense* and *T. vivax*. Additionally, isometamidium is also effective against infections caused by *T. b. brucei* and *T. evansi* in donkeys, horses and camels (Kinabo et al., 1988). The mechanism of resistance against isometamidium is associated with reduced accumulation of the drug in the parasite (Sutherland et al., 1993). One of the major adverse properties of phenanthridinium drugs, particularly isometamidium, is tissue damage at the infection site (Kinabo et al., 1988). Homidium was extensively used in the 1960s and 1970s, but its usefulness has been greatly reduced due to widespread resistance (Scott et al., 1974).

Quinapyramine was discontinued due to its capacity to induce multi-drug resistance (Holmes et al., 2004) and can be used to treat the infection by subcutaneous injection at a dose of 5 mg/ kg bw (Fig. 9).

Recently, a new, water soluble trivalent arsenical agent, melarsamine hydrochloride (MelCy) (trade name: Cymelarsan) (Fig. 9), has been tested in camels artificially infected with *T. evansi* and shown to be effective against *T. b. brucei*, *T. evansi*, and *T. equiperdum* in camels, buffalo, goats and pigs *in vitro* (Otsulya et al., 1992, Zweygarth et al., 1992). The drug product is presented as a white powder highly soluble in water. Compared to suramin, isometamidium and diminazene, MelCy has shown to be more efficacious against *T. evansi* and *T. equiperdum* (Zhang et al., 1991). MelCy is a melaminophenylarsine made by

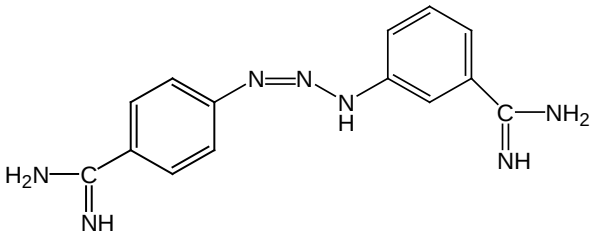
conjugating one equivalent of melarsen oxide with two equivalents of cysteamine. On incubation at room temperature, the MelCy content decreases steadily with an associated increase in the melarsen oxide and sodium melarsen contents (Berger et al., 1994). There are also indications that the drug may be effective in eliminating *T. b. gambience* and *T. b. rhodesiense* in their reservoir hosts such as cattle and swine (Kinabo, 1993). It is highly active against *T. evansi* infection, yet its downfalls revolve around its toxicity, expensive market price and global unavailability. While suramin, diminazene aceturate and quinapyramine have been utilized for more than 50 years, MelCy, belonging to the family of melaminophenyl arsenicals, was pharmaceutically developed within 20 years (Williamson et al., 1970; Zelleke et al., 1989).

1.1.4.1 The use of trypanocides in various host species

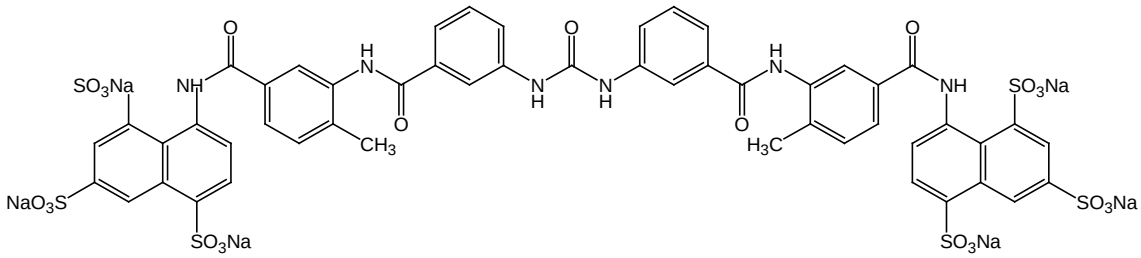
Buffaloes, cattle, and small ruminants infected with *T. evansi* can be treated with DA (preferred drug) at a dose of 7 mg/kg bw via intramuscular injection. The withdrawal period for meat consumption should be >30 days. In the case of strong clinical signs, especially when parasitemia is high, an initial injection of 3.5 mg/kg bw DA may be given to reduce the parasitemia and a second injection of 7 mg/kg bw can be given 5 days later to ensure that all the parasites are killed. Horses, dogs, and cats can be treated with DA or IMC despite being quite sensitive to the drugs. It is essential to provide an adequate water supply to avoid a toxic effect on the kidneys, which can be fatal because of this treatment. Similarly, in the case of very high parasitemia in cattle, half a dose of DA or IMC, followed by a normal dose 5 days later can be administered.

In camels, although a number of trypanocides have been used (DA, IMC, suramin, quinapyramine, etc.), melarsomine dihydrochloride is the ideal product (dose: 0.25 mg/kg

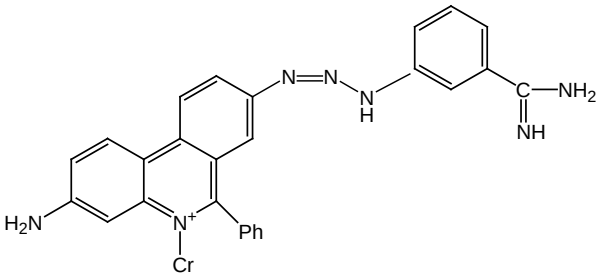
bw), which can be increased to 0.5 mg/kg bw if a fully curative (sterilizing) treatment is required for international trading. In enzootic areas, a dose of 3.5 mg/kg of DA can also be used. However, it can induce severe side effects and might not be sufficient to clear all parasites from the camel. In pigs, little information is available on the control practices used for African trypanosomes. Quinapyramine may be used, as well as DA, though it appears to be of limited efficacy (Tuntasuvan et al., 2003a). IMC and Cymerlarsan (MelCy) could also be used. However, experimental evaluations are necessary to validate the treatment protocols. In Asian elephants, several attempts have been made with DA. Lower doses, such as 5 mg/kg bw, resulted in relapses (Hin-On et al., 2004, Arjkumpa et al., 2012, Rodtian et al., 2012), while 8 mg/kg bw seems to be efficient (Table 2).



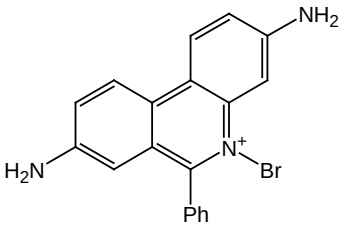
diminazene aceturate



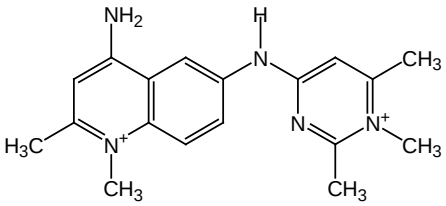
suramin



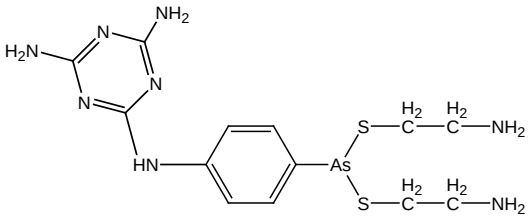
isometamedium chloride



homidium bromide



quinapyramine



cymelarsan

Fig. 9. Chemical structures of diminazene aceturate; an aromatic diamidine, suramin; a naphthalene compound, isometamedium, homidium, quinapyramine; a quinoline pyramidine, and cymelarsan.

Table 2. Trypanocide drugs currently available for use in domestic livestock (Holmes et al., 2004).

Trypanocidal drugs	Trade name	Activity		Toxic effects	
		Highly active	Less active	Good tolerance	Possible local and general reactions
Diminazene aceturate	Berenil	*T. congolence* *T. vivax*	*T. brucei* *T. evansi*	Donkeys, camels, cattle and small ruminants	Horses, camels
Suramin	Naganol	*T. evansi* *T. brucei*		Camels and Horses	
Isometamedium chloride	Samorin, Trypamidium, Verdium	*T. vivax* *T. congolense* *T. evansi*	*T. brucei*	Small ruminants, equidae and camels	Cattle
Homidium	Ethidium, Novidium	*T. vivax* *T. congolense*		Cattle, sheep, goat and pigs	Horses
Quinapyramin prosalt	Trypacide prosalt	*T. congolense* *T. vivax* *T. brucei* *T. evansi*		Equidae, pigs and dogs	Horses
Melarsenoxide cysteamine	Cymelarsan	*T. evansi*		Camels	

1.1.4.2 Current problem with trypanocides and a possible way to prevent the disease

Despite the fact that chemotherapy is the major means of disease control, development of new antitrypanosomal drugs has been more or less static over the last three decades, due to lack of interest by the pharmaceutical industry to invest into research and development of antitrypanosomal drugs (Gutteridge, 1985).

Several problems are associated with the current standard drugs that are available for the treatment of *T. evansi* infection due to side effects and ineffectiveness against drug resistant parasites in many regions (Kibona et al., 2006; Matovu et al., 2001). Drug resistance in trypanosomes arises through either the loss or alteration of certain transporter proteins, implying a decrease in the amount of drug entering the parasite. The most common problem of DA (Berenil), is severe side-effects, including anaphylactic-shock, severe cutaneous reactions, neurotoxic signs, and cases of renal failure (WHO 1998). Additionally, it causes severe toxicity in horses and mules after injection, with a minimal protective effect of the drug (Tuntasuvan et al., 2003b). Berenil was proved to be effective for the treatment of surra in cattle, buffaloes, sheep, pigs, and camels (Peregrine et al., 1993), but caused fatal reactions in camels, horses and dogs at doses considered to be normal and harmless in cattle (Sirivan et al., 1994). Therefore, there is an urgent need for new chemotherapeutic drugs that, are safe, effective, cheap and, easy to administer, for the treatment of *T. evansi* (surra), which mainly affects people in developing countries (Camacho et al., 2003). One possible alternative source of such affordable treatments lies in the use of natural products.

Natural products, including locally grown medicinal plants, are recognized as an important source of chemotherapeutic agents, particularly those are used to treat infectious diseases, and about half of the useful drugs today are derived from natural sources.

Extensive studies have shown that medicinal plants from several regions contain compounds active against protozoa diseases (Camacho et al., 2000). Today approximately 80% of the world's population relies on traditional medicinal plant for primary health care. The remaining 20% of the world's population also depends on the ingredients derived from plant sources for health care (Farnsworth et al., 1985). Plants and their extracts have been used for centuries to relieve pain, aid healing and kill bacteria and insects. They have also been employed in perfumery, cosmetics and religious rites. The number of natural products obtained from plants has now reached over 100,000 and every year new compounds are being discovered. Plants, microorganisms and marine organisms are potential sources for new drugs because they contain a countless quantity of ingredients with a great variety of structures and pharmacological activities (Newman et al., 2003). Numerous plant-derived natural products with antiprotozoal activities have been reported. There are publications reporting the activity of purified natural products against trypanosomes that are responsible for sleeping sickness in humans and nagana in domesticated animals (Hoet et al., 2004).

As described earlier, research toward the development of new compounds for the treatment of surra is very important. Myanmar is abundant in plant resources and Myanmar traditional medicinal practitioners have been using a variety of herbal preparations to treat different types of diseases including microbial infection and protozoan diseases. A previous study revealed that the crude extract of Myanmar medicinal plants showed *in vitro* inhibitory activity against *T. evansi* (Bawm et al., 2010). Several promising candidate plants such as *Vitis repens, Vitex arborea, Eucalyptus globules, Jatropha podagrica, Rhoeo discolor, Daucus carota, Combretum acuminatum, Phyllanthus simplex,* and *Andographis paniculata* were found to have the activity against *T. evansi*. Among of them, dry rhizome bark of *Vitis repens* showed the high activity against *T. evansi* with an IC_{50} = 8.6 µg/mL. Further research

on following medicinal plants and their inhibitory activities against *T. evansi* was conducted in this study.

1.1.5 Objectives of this study

The current study focused on *Trypansoma evansi*. As mentioned earlier in the section on treatment of *T. evansi*, some chemotherapeutic drugs reduce the severity and the mortality associated with the disease. However, these drugs carry with them several side effects such as anaphylactic-shock, neurotoxic signs and cases of renal failure. Therefore, the production of some of these drugs was recently stopped, and an alternative chemotherapeutic agent with fewer side effects is urgently needed for the treatment of *T. evansi* infection. One possible alternative source for affordable treatments lies in the use of plant extracts. Therefore, our research group has proposed that an antitrypanosomal component could be isolated from extracts of medicinal plants from Myanmar. The overall aim of this study was to identify the compounds with antitrypanosomal activity against *T. evansi* from the following traditional Myanmar medicinal plants; *Vitis repens* Wight & Arn., *Phyllanthus simplex* Retz., *Vitex arborea* Desf., and *Ampelopsis brevipedunculata* var. *heterophylla*.

CHAPTER 2

ANTITRYPANOSOMAL ACTIVITIES OF MYANMAR MEDICINAL PLANTS

2.1 Introduction

The Union of Myanmar is geographically situated in South-East Asia with supreme natural environment and abundant plant resources and well known for its green tropical vegetation and forests. It is bordered by India, Bangladesh and the Bay of Bengal on the west, China to the north and northeast, Laos and Thailand to the east and the Andaman Sea to the south. Traditional medicine in Myanmar is widely practiced by the majority of population, partly as a supplement and partly as an alternate to modern medicine. Myanmar traditional medicine has its origin from those of traditional medicine system of neighboring countries. It was enriched by the Myanmar traditions, adaptations and adoptions throughout centuries. Myanmar has inherited their own traditional medicine to maintain their health and treat various alignments such as malaria, diarrhea and fever for over millennia of history.

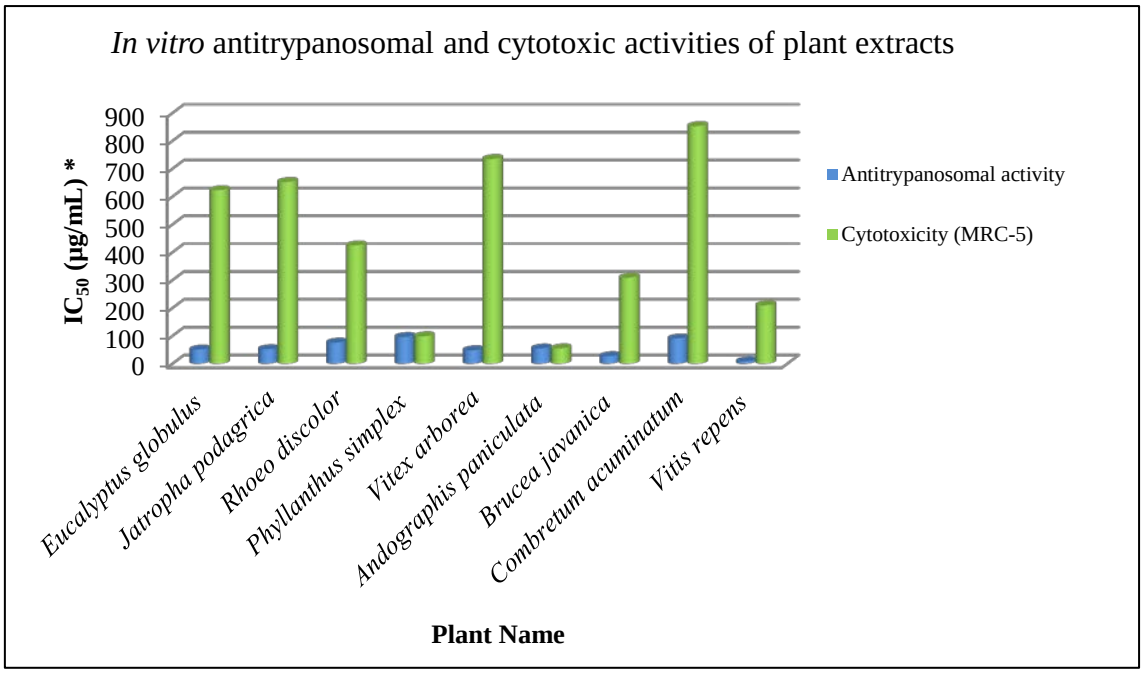
In Myanmar, people in rural area use folk medicine for the treatment of common infections. They use plant materials to cure various diseases because it can be easily available and cheap and they believe that it is more effective than modern medicine and it may have a reduce risk. Myanmar traditional medicinal practitioners have been using a variety of herbal preparations to treat different kinds of diseases including microbial infectious and protozoa diseases (Mon et al., 2008). Human trypanosomiasis is not endemic to Myanmar and medicinal plants had not been paid attention to this disease. However, agriculture and animal production (milk and meat) are the major industrial sectors in Myanmar. So that, control of livestock diseases is very important for the development of

national economy.

2.2 Antitrypansomal activities of Myanmar medicinal plants

This study dedicated to find antitrypanosomal ingredients from Myanmar medicinal plants. A previous study revealed that the crude extract of Myanmar medicinal plants showed *in vitro* inhibitory activities against *T. evansi* and cytotoxic activity against MRC-5 (Human lung diploid fibroblast) cells were determined (Bawm et al., 2010). Three of 55 fresh samples and 6 of 16 dry samples showed IC_{50} values with < 100 µg/ mL against *T. evansi* (Fig. 10A) with higher selectivity indexes (SI) (Fig. 10 B). Following showed plant names and their IC_{50} values. The crude extract of *Vitis repes* Wight & Arn, *Phyllanthus simplex* Retz., *Vitex arborea* Desf., and *Ampelopsis brevipedunculata* var. *heterophylla*, showed antitrypanosomal activity against *T. evansi* with the IC_{50} values of 8.6 µg/mL, 96.1 µg/mL, 48.6 µg/mL and 16.4 µg/mL, respectively (Fig. 10A). The isolation and structural elucidation of active ingredients in these plants were performed in this study.

(A)



* The IC_{50} value is the mean ± standard deviation of three independent experiments.

(B)

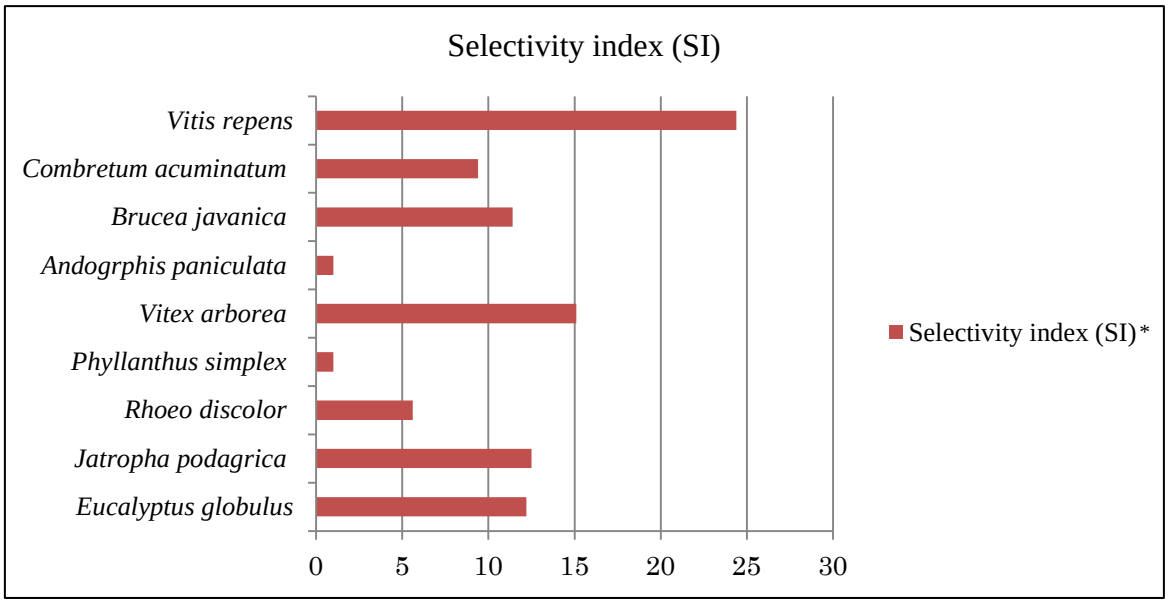


Fig. 10. Inhibition rates of Myanmar medicinal plant extracts against *T. evansi* (A), and their selectivity index (B).

* Selectivity index (SI) = $\left[\dfrac{\text{Antitrypanosomal activity}}{\text{Cytotoxicity}}\right]$

2.3 Collection and preparation of plant materials

The plant materials were collected at the Medicinal Plants Garden of Biotechnology Department Centre, Pathein University, Myanmar in December 2011 and identified by Associate Professor Daw Kin Kin Si at the Department of Botany, Pathein University, Ayeyarrwaddy Division. Plant materials were air dried under shade for several days. Dried plants were ground to small particle and then stored in a cold room at 4ºC. For each plant, ground sample was extracted with EtOH for 2 weeks, then passed through a filter paper, and concentrated to give a dried crude extract. The extracts were dissolved in DMSO and then tested for their antitrypanosomal activities against *T. evansi in vitro*. Extracts displaying adequate potency in the bioassay are then subjected to bioactivity-guided fractionation to isolate the active constituents.

2.4 Antitrypanosomal test

2.4.1 Parasite and culture medium

Bloodstream trypomastigotes form of *T. evansi* [H3 strain, isolated from deer in Thailand, provided by Professor Dr. Ken Katakura (Laboratory of Parasitolgy, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Japan)] were cultivated in HMI-9 medium (Hirumi-Hirumi et al., 1994) supplemented with 20% heat-inactivated horse serum (Sigma) (Table 4). Thaw horse serum (HS, 6 mL) is mixed with 40 mL of HMI-9 medium. HS and HMI-9 medium were mixed and stored it at 4°C before use. Take 3.6 mL of HMI-9 medium with HS and culture with 0.4 mL of *T. evansi*, then incubated at 37ºC in 5% CO_2 atmosphere for 72 h. Subculturing was performed every 3 days.. The compositions of culture medium are given in Table 3, and the method to make culture media was given in Fig. 11.

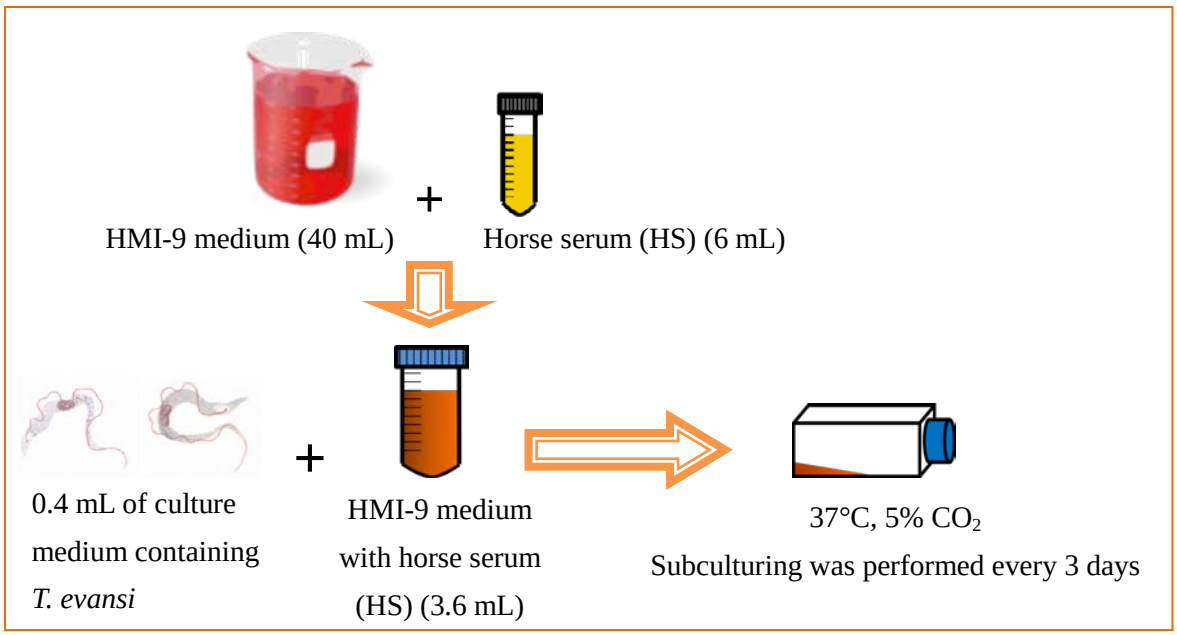


Fig. 11. Culturing method of *T. evansi*.

Table 3. The composition of HMI-9 culture medium.

Reagent	Item Number	Form	Preservation temperature	Amount of reagents[a]
$NaHCO_3$	WAKO 191-01301	solid	room temperature (RT)	2.17 g
IMDM (Iscove's Modified Dulbecco's medium)	SIGMA 17633	solid	4 °C	12.7 g
L-glutamine	WAKO 076-00521	solid	RT	0.26 g
bathcouproine disodium salt	SIGMA B1125	solid	4 °C	0.05 g
thymidine	WAKO 205-08091	solid	4 °C	0.04 g
BSA	SIGMA A2153	solid	4 °C	0.36 g
holo transferrin	SIGMA T1283	solid	4 °C	0.009 g
pyruvic acid	KANTO 32506-30	liquid	4 °C	78 µl
L-cystein	KANTO 07572-60	solid	RT	0.21 g
hypoxanthine	WAKO 080-03401	solid	-20 °C	1 mL[b]
HEPES	SIGMA H4034	solid	RT	12.9 g
penicillin (PS)	GIBCO 15140	liquid	-20 °C	900 µl
2-mercaptoethanol	WAKO 135-07522	liquid	4 °C	9 µl

[a] Reagents are dissolved with 200 mL H_2O and the solution is finally adjusted to 900 mL.

[b] Hypoxanthine (0.13 g) is dissolve into 0.8 ml of DDW with 0.2 ml of 10 M NaOH. If it is not dissolve yet, add small volume (or about 0.05 ml) of 10 M NaOH.

Table 4. The information of horse serum (HS).

Reagent	Item Number	Form	Preservation temperature
Horse serum*	GIBCO 16050-122	liquid	-20 °C

* The horse serum is inactivated by warming at 60°C from 40-45 min.

2.4.2 *In vitro* test for antitrypanosomal activity

In vitro antitrypanosomal tests were performed in a 96-well culture plate using the crude extracts. The extracts and isolated compounds were dissolved in dimethyl sulfoxide (100 µL) (DMSO) at a concentration of 10 mg/mL and make ten-fold serial dilutions (take 40 µL) with HMI-9 medium (360 µL) in the presence or absence of 0.5% DMSO. Equal volume of the culture liquid of bloodstream trypomastigote form of *T. evansi* (1 x 10^6 cell/mL) was added to the above mentioned solution. A 200 µL portion of culture medium of *T. evansi* in the presence of each compound was incubated in 96-well plate. The experiment was tested in triplicate. The plates were incubated at 37°C in 5% CO_2 atmosphere for 72 h (Fig. 12). The activity of the compounds was determined by counting the number of motile parasites in Neubauer haemocytometer [0.100 mm, Tiefe depth profindeur, and 0.0025 mm 2, [Hirschmann Laborgerate]. To determine the 50% inhibitory concentration (IC_{50}) on parasite growth for each compound, triplicate assays of the compounds at each concentration were prepared. The IC_{50} value was calculated by compouterized SigmaPlot Microsoft Excel (http://www.changbiosicence.com/stat/ec50.html).

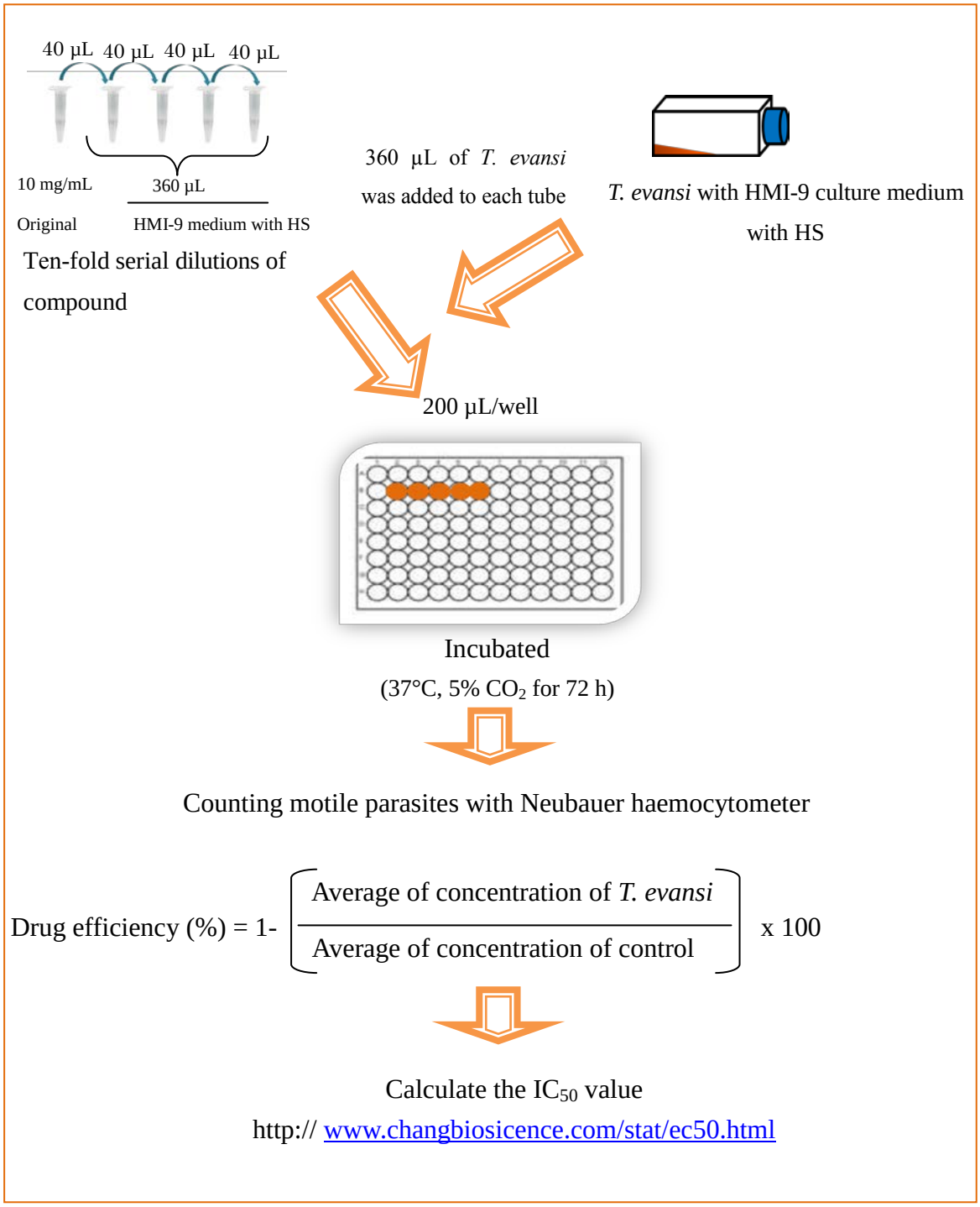


Fig. 12. Procudure of assay for *in vitro* antitrypanosomal activity.

CHAPTER 3

ISOLATION AND STRUCTURAL ELUCIDATION OF ANTITRYPANOSOMAL COMPOUNDS FROM *VITIS REPENS* WIGHT & ARN.

3.1 Biological description of *Vitis repens* Wight & Arn.

Fig. 13. Photograph of *Vitis repens* Wight & Arn.

Family : Vitaceae

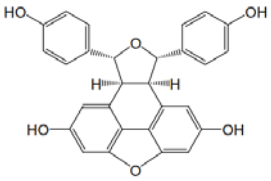
Scientific name : *Vitis repens* Wight & Arn.

Common name : Cissus, Voeur sanda

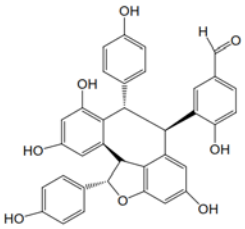
Myanmar name : Taung-Zee-Phyu

Vitis repens Wight & Arn., a plant species belong to the family Vitaceae. It is a scadent tendrilar climber with slender, glabrous, glaucous angular stems, and fusiform

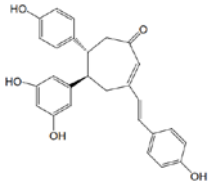
tuberculous rhizome. The tendrils are borne opposed to the leaf at the nodes and the tips are forked. Leaves are arranged in alternate, simple, beset with acuminate tips, serrulate margins and truncate to cordate bases. The upper surfaces of leaf blade in pale green colored with white patches. The flowers are small, yellowish green in colour and bracteates. The fruits are globose berries, dark red to shining black in fully ripe (Fig. 13). The fruits are very acid and leave a stinging sensation in the mouth which lasts for 24 hours. The plant is widely distributed in Shan state, eastern hills of Myanmar, where it is used in Myanmar folk medicines to treat sore, carbuncles, ulcers, hepatitis and jaundice, peptic ulcer, tumors and hypertention, and so on. In Myanmar, it is one of the effective antitumor herbal medicinal plants. Neither antiprotozoal nor antimicrobial activity of *V. repens* was found in the literature. The chemical studies on the genus *Vitis* commonly contains various oligomers of resveratrol, such as vitisinols A-D, (+)-ε-viniferin, (-)-viniferal, ampelopsin C, miyabenol A, (+)-vistin A, (+)-vistin C, glycosides, flavonoids, keto steroids, bergenin, and isolariciresinol (Fig. 14) (Li et al., 1996). Three different kinds of compounds such as bergenin, 1-[(3-methylbutyryl) phloroglocino]-β-D-glucopyranoside were isolated from the rhizome of *Vitis repens.*



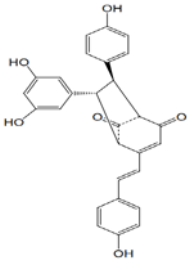
vitisinol A



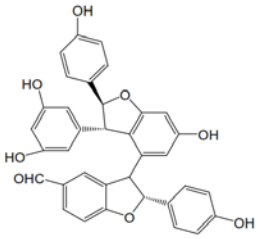
vitisinol B



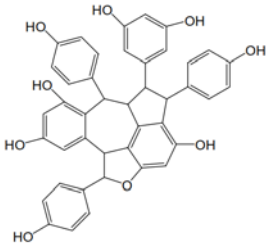
vitisinol C



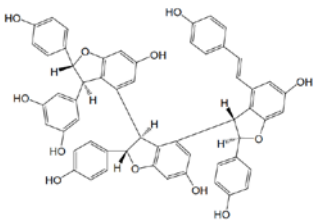
vitisinol D



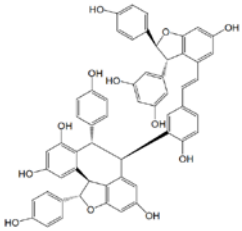
(-)-viniferal



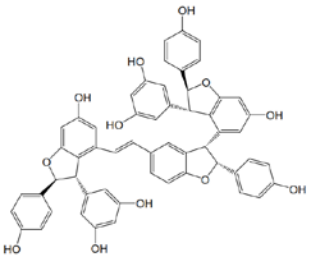
ampelopsin



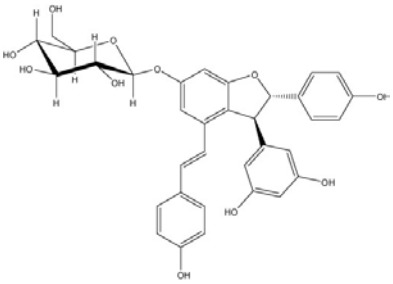
miyabenol A



(+)-vistin A



(+)-vistin C



(+)-ε-viniferin

Fig. 14. Chemical structures of various oligomers of resveratrol in *Vitis* species, vitisinols A-D, (-)-viniferal, ampelopsin C, miyabenol A, (+)-vistin A, (+)-vistin C, and (+)-ε-viniferin.

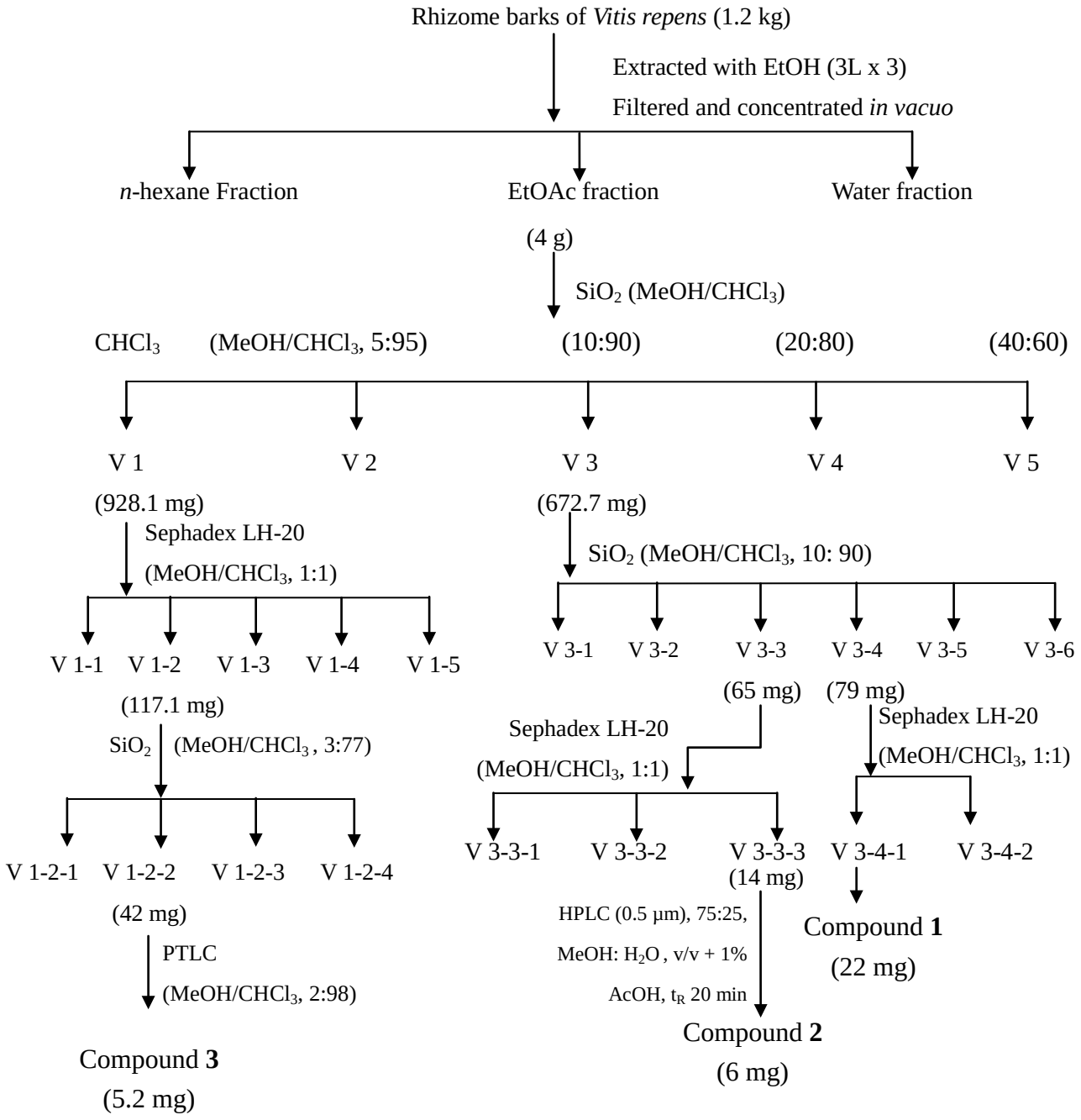
3.1.1 Extraction and isolation

The EtOAc extracts of *V. repens* rhizome barks were prepared as shown in Scheme 1. The dried rhizome barks of *V. repens* (1.2 kg) were pulverized and extracted three times with 3 L of EtOH at room temperature. The volatile components of the extract were evaporated to yield a brownish viscous residue. The EtOH extracts were further partitioned between organic solvents [*n*-hexane, EtOAc, and H_2O] (1 L each) successively. The ethyl acetate fraction of *V. repens* showed significant antitrypanosomal activity, but the biological activities of *n*-hexane and H_2O fractions were not as effective as that of EtOAc extract.

The EtOAc extract was dried over Na_2SO_4 and then concentrated under reduced pressure. The residue (4 g) was fractioned by silica gel column (400 g) chromatography, which was eluted with $CHCl_3$ (1 L), MeOH/$CHCl_3$ (5:95, 1 L), MeOH/$CHCl_3$ (10:90, 1 L), MeOH/$CHCl_3$ (20:80, 1 L) and MeOH/$CHCl_3$ (40:60, 1 L) to afford 5 fractions (V1 to V5). The eluents of MeOH/$CHCl_3$ (10:90, 1 L) (672.7 mg), V3 fraction, was rechromatographed using a silica gel column (100 g), which was eluted with the same solvent system to give 6 fractions (V3-1 to V3-6). The V3-4 (79 mg) fraction was further purified by Sephadex LH-20 (200 g), which was eluted with MeOH/$CHCl_3$ (1:1) to afford 2 fractions, V3-4-1 and V3-4-2. Fraction V3-4-1 was found to contain pure compound **1** (22 mg). The V3-3 (65 mg) fraction was separated by the Sephadex LH-20 (200 g), which was eluted with MeOH/$CHCl_3$ (1:1) to give 3 subfractions. The third sub-fraction V 3-3-3 (14 mg) was subjected to HPLC (Capcellpak C_{18}, 5 µm, 4.6 mm x 150 mm, Shiseido) using a solvent system composed of 75:25 = MeOH:H_2O (v/v) containing 1% AcOH at flow rate of 1 mL/min to give compound **2** (t_R 20 min, 6 mg).

The V1 fraction (928.1 mg) was purified by a Sephadex LH-20 (200 g) column to yield 5 sub-fractions (V1-1 to V1-5). The V1-2 (117.1 mg) sub-fraction was subjected to

silica gel (40 g) chromatography, which was eluted with MeOH/$CHCl_3$ (3:77) to yield 4 sub-fractions (V1-2-1 to V1-2-4). The V1-2-2 (42 mg) sub-fraction was subjected to preparative TLC (MeOH/ $CHCl_3$, 2:98) over silica gel to yield compound **3** (5.2 mg).



Scheme 1. Isolation procedure for active compounds from *V. repens*.

3.1.2 Structural elucidation of antitrypanosomal compounds (1-3)

Compound **1** was isolated as yellow amorphous, and its molecular formula was deduced from EI-MS and EI-HR-MS (Chart 1 and 2) as $C_{14}H_{12}O_3$ (*m/z* 228.0788 $[M]^+$, calc for $C_{14}H_{12}O_3$: 228.0786). The ^{1}H NMR signals (Chart 3) at δ_H 6.16 (1H, t, *J* = 4.3 Hz, H-4), 6.34, 6.35 (each 1H, d, *J* = 2.2 Hz, H2/6) revealed *meta* coupling, indicating a symmetrical 1,3,5-trisubstituted benzene ring. The resonances derived from two protons at δ_H 6.72 (1H, d, *J* =16.5 Hz, H-8) and 6.93 (1H, d, *J* = 16.5 Hz, H-7) showed *trans* configuration of the double bond. In addition, the resonances at δ_H 6.65, 6.78 (each 1H, d, *J* = 8.6 Hz, H-3′/5′) and δ_H 7.23, 7.37 (each 1H, d, *J* = 8.3 Hz, H-2′/6′) of the aromatic protons are evidence of a 1,4-disubstituted benzene ring. The ^{13}C NMR spectrum (Chart 4) of **1** showed resonances of aromatic quaternary carbon signals at δ_C 130.4 (C-1′), 141.3 (C-1), 158.4 (C-4′), 159.6 (C-3), and 159.7 (C-5); seven aromatic methyne carbon signals at δ_C 102.6 (C-4), 105.7 (C-2/6), 116.5 (C-3′/5′), and 128.8 (C-2′/6′); two olefinic carbon signals at δ_C 127.0 (C-8) and 129.4 (C-7). The direct connectivity between carbon and attached proton was revealed by HMQC (Chart 5) spectra. COSY correlations (Chart 6) were observed between the H-7 to H-8, H-2′/6′ to H-3′/5′, and H-2/6 to H-4. The oxygen atoms are attached to C-4′, C-3, and C-5. The connectivity of benzene ring with C-7 or C-8 was distinguished by a cross peaks between C-7 to H-2/6, which were revealed by HMBC spectra (Chart 7) (Fig. 15). Other HMBC correlations were observed between the H-8/C-7, H-7/8 to C-1, H-2′,6′/C-3′, 5′, 4, respectively. All of the findings above indicated that the partial structure of compound **1** was *trans*-3,4′,5-trihydroxystilbene (resveratrol). The structure was further confirmed by comparison of the literature data (Fulvio et al., 1995, Chen et al., 2002). Total assignments of ^{1}H and ^{13}C NMR spectra data were listed in Table 5.

Compound **2** was isolated as pale yellow amorphous, and its molecular formula was

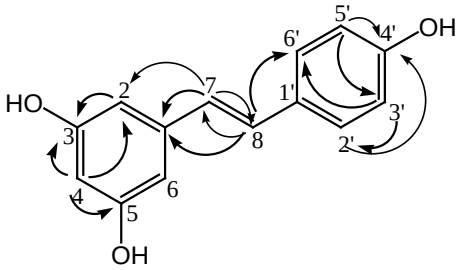
deduced from positive ion ESI-MS and EI-HR-MS data (Chart 8 and 9) as $C_{16}H_{18}O_{10}Na$ (*m/z* 393.07913 $[M+Na]^+$, calc for $C_{16}H_{18}O_{10}$: 370.0899). The value for specific rotation was -8.2° (*c* 0.3, MeOH). The ^1H NMR resonance (Chart 10) at δ_H 7.08 (1H, s, H-7) indicated the presence of one aromatic proton, and a group of signals at δ_H 4.98 (1H, d, *J* = 10.5 Hz, H-10b), 4.01 (1H, t, *J* = 9.5 Hz, H-4a), and δ_H 3.39-3.79 (each 1H, m, H-2/3/4) resembled a glycoside moiety. Two diastereotopic protons attached to oxygen atoms resonated at δ_H 4.16 (1H, m, H-11b) and 4.59 (1H, m, H-11a). In addition, the resonances of methyl signal assignable as one methoxy group and one acetyl group were found at δ_H 3.84 (3H, s, $-OCH_3$) and 2.13 (3H, s, $-CH_3$), respectively. The assignment of the ^{13}C NMR spectrum (Chart 11) of compound **2** indicated 16 carbon signals resonating at δ_C 172.6 (C=O), 165.6 (C-6), 152.5 (C-8), 149.4 (C-10), 142.3 (C-9), 119.5 (C-6a), 117.0 (C-10a), 111.2 (C-7), 81.3 (C-4a), 80.4 (C-2), 75.4 (C-4), 64.6 (C-11), 74.3 (C-10b), 60.8 ($-OCH_3$), 20.9 ($-CH_3$-C=O) and 71.9 (C-3). The methoxy carbon and acetyl methyl carbon resonated at δ_C 60.8 and 20.6, respectively.

The 1D NMR data of **2** was very similar to bergenin (Wang et al., 2005) except for the signals of an acetyl group at δ_C 172.6 and 20.9 and δ_H 2.13 (3H, s), suggesting that compound **2** is an acetyl derivative of bergenin. In addition, compared with the ^1H NMR data of bergenin, the downfield shift of H-11 is different from that of bergenin. These data confirmed that an acetyl group was connected to bergenin through C-11. The complete ^1H and ^{13}C NMR assignments and connectivity were established from a combination of COSY, HMBC, and HMQC (Chart 12, 13, and 14) data analyses. HMBC correlations were observed between H-10b/C-6a, C-10a, H-7/C-9, C-8, C-6, C-6a, and (C=O)/ ($-CH_3$). In the NOESY spectra (Chart 15) of **2**, the correlations between H-10b (δ_H 4.98), H-4 (δ_H 3.79) and, H-2 (δ_H 3.77), as well as between H-4a (δ_H 4.01) and H-3 (δ_H 3.39), were observed to reveal that the relative configuration of **2** was the same as that of bergenin. After assigning all of

the spectra data, the chemical structure of **2** was identified as 11-*O*-acetyl bergenin. In addition, 11-*O*-acetyl bergenin has been isolated from *V. repens* by Wang et al. (Wang et al., 2008). However, they did not examine the absolute configuration by measuring $[\alpha]_D$. Compound **2** was acetylated in a solution of pyridine (1 mL) and acetic anhydride (0.5 mL), which was subjected to a typical work up to give pentaacetylbergenin. The obtained compound showed $[\alpha]^{24}_D$ of -6.9^0 (*c* 0.2, MeOH). Authentic bergenin was also converted according to the same method to give pentaacetylbergenin, which showed a $[\alpha]^{24}_D$ of -6.4^0 (*c* 0.3, MeOH). Based on these results, we confirmed the absolute configuration of 11-*O*-acetyl bergenin (**2**), as shown in Fig 15. Total assignments of ^{1}H and ^{13}C NMR spectra data were listed in Table 6.

Compound **3** was isolated as white needles, and its molecular formula was deduced from EI-MS, EI-HR-MS, and FD-MS data (Chart 16, 17, and 18) as $C_{29}H_{48}O$ (*m/z* 412.3718 $[M]^+$, calc for $C_{29}H_{48}O$: 412.3707). The value for specific rotation was +73.3° (*c* 0.5, $CHCl_3$). The ^{1}H NMR spectrum (Chart 19) showed a series of methyl resonances at δ_H 1.16 (3H, s, H-19), 0.69 (3H, s, H-18), 0.90 (3H, d, *J* = 6.5 Hz, H-21), 0.83 (3H, d, *J* = 6.7 Hz, H-26), 0.80 (3H, d, *J* = 6.8 Hz, H-27), and 0.85 (3H, t, *J* = 6.7 Hz, H-29) that are clearly indicative of a steroid structure with a keto function at C-3. The ^{1}H NMR spectrum presented a singlet at δ 5.70 (1H, s, H-4), consistent with the presence of a vinylic proton (Fig. 15). The assignment of the ^{13}C NMR data of **3** (Chart 20) indicated 29 carbon signals, which were classified to six methyl groups [δ_C 11.9 (C-18), 12.0 (C-29), 17.4 (C-19), 18.7 (C-21), 19.0 (C-27), and 19.8 (C-26)], 11 methylene groups [δ_C 21.0 (C-11), 23.1 (C-28), 24.2 (C-16), 26.1 (C-23), 28.2 (C-15), 32.1 (C-7), 32.9 (C-6), 33.9 (C-22), 34.0 (C-2), 35.7 (C-1), and 39.6 (C-12)], four quaternary carbons [δ_C 38.6 (C-10), 42.4 (C-13), 171.5 (C-5), and 199.5 (C=O)] and eight methye groups [δ_C 29.2 (C-24), 35.6 (C-8), 36.1 (C-20), 45.9 (C-25), 53.8

(C-9), 55.9 (C-14), 56.0 (C-17), and 123.7 (C-4)] by DEPT spectrum data (Chart 21). The resonances of ^{1}H and ^{13}C NMR indicated the presence of a cholestane skeleton and an additional ethyl group at C-24 on the side chain. The HMBC correlations (Chart 22) were observed between H-19/C-1, C-10, C-5, C-9; H-18/C-12, C-13, C-14, C-17; H-21/C-20, C-22; H-26/C-24, C-25; H-27/C-24, C-25, and H-29/C-24. The detailed analyses of the COSY and HMQC spectra (Chart 23 and 24) of compound **3** (Fig. 15) provided the unambiguous assignment of all proton and carbon resonances, which were also confirmed by comparison to the literature data (Lee et al., 2010) of stigmastenone. Therefore, the structure of compound **3** was identified as stigmast-4-en-3-one (also known as stigmastenone). Total assignments of ^{1}H and ^{13}C NMR spectra data were listed in Table 7.



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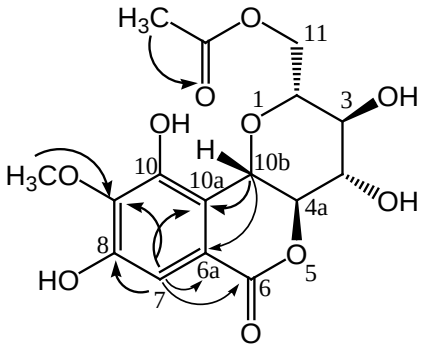

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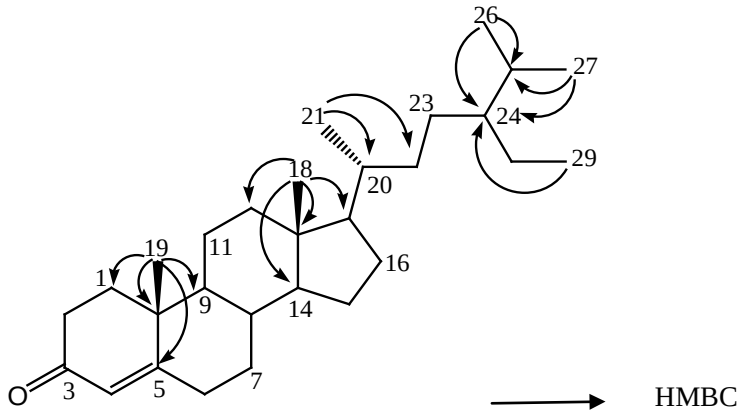


3

Fig. 15. Chemical structures of compound **1**(*E*-resveratrol), **2** (11-*O*-acetyl bergenin), and **3** (stigmast-4-en-3-one) with important correlations of HMBC.

Table 5. ^{1}H NMR and ^{13}CNMR spectra data of compound **1** (270 MHz, CD_3OD).

Position	Compound **1**	
	δ_H	δ_C
1	-	141.3
2	6.34 (1H, d, *J*=2.2 Hz)	105.7
3	-	159.6
4	6.16 (1H, t, *J*=4.3 Hz)	102.6
5	-	159.7
6	6.35 (1H, d, *J*=2.2 Hz)	105.7
7	6.93 (1H, d, *J*=16.5 Hz)	129.4
8	6.72 (1H, d, *J*=16.5 Hz)	127.0
1'	-	130.4
2'	7.23 (1H, d, *J*=8.3 Hz)	128.8
3'	6.65 (1H, d, *J*=8.6 Hz)	116.5
4'	-	158.4
5'	6.78 (1H, d, *J*=8.6 Hz)	116.5
6'	7.37 (1H, d, *J*=8.3 Hz)	128.8

Table 6. ^{1}H NMR (270 MHz, CD_3OD) and ^{13}CNMR spectra data of compound **2** (127.5 MHz, CD_3OD).

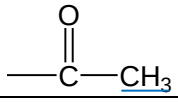
Position	Compound **2**	
	δ_H	δ_C
2	3.77 (1H, m)	80.4
3	3.39 (1H, m)	71.9
4	3.79 (1H, m)	75.4
4a	4.01 (1H, t, *J* = 9.5 Hz)	81.3
6	-	165.6
6a	-	119.5
7	7.08 (1H, s)	111.2
8	-	152.5
9	-	142.3
10	-	149.4
10a	-	117.0
10b	4.98 (1H, d, *J* = 10.5 Hz)	74.3
11	4.16, 4.59 (each 1H, m)	64.6
$-OCH_3$	3.84 (3H, s)	60.8
C=O	-	172.6
	2.13 (3H, s)	20.9

Table 7. ^{1}H NMR (270 MHz, $CDCl_3$) and ^{13}CNMR spectra data of compound **3** (500 MHz, $CDCl_3$).

Position	Compound **3**	
	δ_H	δ_C
1	2.06 (2H, m)	35.7
2	2.39 (2H, m)	34.0
3	-	199.5
4	5.70 (1H, s)	123.7
5	-	171.5
6	2.20 (2H, m)	32.9
7	1.06- 0.95 (2H, m)	32.1
8	1.42 (1H, m)	35.6
9	0.99 (1H, m)	53.8
10	-	38.6
11	1.65-1.42 (2H, m)	21.0
12	2.00 (2H, m)	39.6
13	-	42.4
14	0.95 (1H, m)	55.9
15	1.90 – 1.80 (2H, m)	28.2
16	1.65-1.42 (2H, m)	24.2
17	1.10 (1H, m)	56.0
18	0.69 (3H, s)	11.9
19	1.16 (3H, s)	17.4
20	1.40 (1H, m)	36.1
21	0.90 (3H, d, *J* = 6.5 Hz)	18.7
22	1.06- 0.95 (2H, m)	33.9
23	1.40-1.10 (2H, m)	26.1
24	1.65 (1H, m)	29.2
25	0.87 (1H, m)	45.9
26	0.83 (3H, d, *J* = 6.7 Hz)	19.8
27	0.80 (3H, d, *J* = 6.8 Hz)	19.0
28	1.40-1.10 (2H, m)	23.1
29	0.85 (3H, t, *J*= 6.7 Hz)	12.0

CHAPTER 4

ISOLATION AND STRUCTURAL ELUCIDATION OF ANTITRYPANOSOMAL COMPOUNDS FROM *PHYLLANTHUS SIMPLEX* RETZ.

4.1 Biological description of *Phyllanthus simplex* Retz.

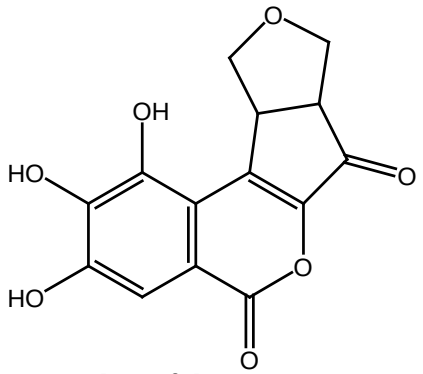
Fig. 16. Photograph of *Phyllanthus simplex* Retz.

Family	: Euphorbiaceae
Scientific name	: *Phyllanthus simplex* Retz.
Common name	: Seed-under-leaf
Myanmar name	: Taung-Zee-Phyu

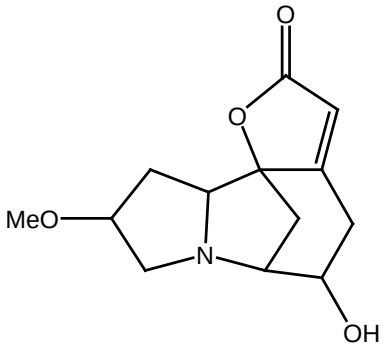
The genus *Phyllanthus* belongs to the family Euphorbiaceae, one of the largest families consisting of 300 genera and 6,000 species in which *P. accuminatus*, *P. amarus*, *P. pulcher*. *P. nirui*, *P. simplex*, and *P. urinaria* are included. In recent years, much research on the chemical constituents has been performed on plants of the genus *Phyllanthus*, which mainly contain terpenoids, alkaloids, flavonoids, lignans, polyphenols, tannins, coumarins,

and saponins. Previous reports indicate that triterpenoids possess anticancer property (Leonardus et al., 1990).

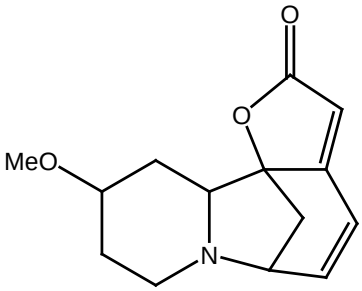
Among the *Phyllanthus* species, *Phyllanthus simplex* Retz., (Fig. 15) is indigenous to South East Asia, Southern India, and China. It is a glabrous twining perennial herb, which has a taproot and whose branches are compressed. In Myanmar, it is one of the medicinal plants used in the treatment of jaundice, liver disease, fever, and dysentery, and fresh leaves are also used to cure itching in children. The root is used as an external application for abscesses (Bagalkotkar et al., 2006). They possess well documented properties of being hepatoprotective, radical scavengers, and enzyme inhibitors. The plant is also reported to possess astringent, diuretic, and cathartic properties (Hemendra et al., 2011). The compound, 8,9-epoxy brevifolin (EBF), is a novel compound isolated from *P. simplex* (Fig. 17) and has been demonstrated to possess a hepatoprotective effect (Xiaofeng et al., 2008). In a previous study, simplexine (14-hydroxy-4-methoxy-13, 14-dihydronorsecurinine) and phyllanthine were isolated and purified from this herbal medicinal plant (Fig. 17) (Rajkishor et al., 1988), but relative and absolute configuration of the compounds were not elucidated in the literature. Research on *P. simplex* revealed that its anti-inflammatory and antioxidant activities (Hemendra et al., 2011).



8, 9-epoxy brevifolin (EBF)



simplexine



phyllanthine

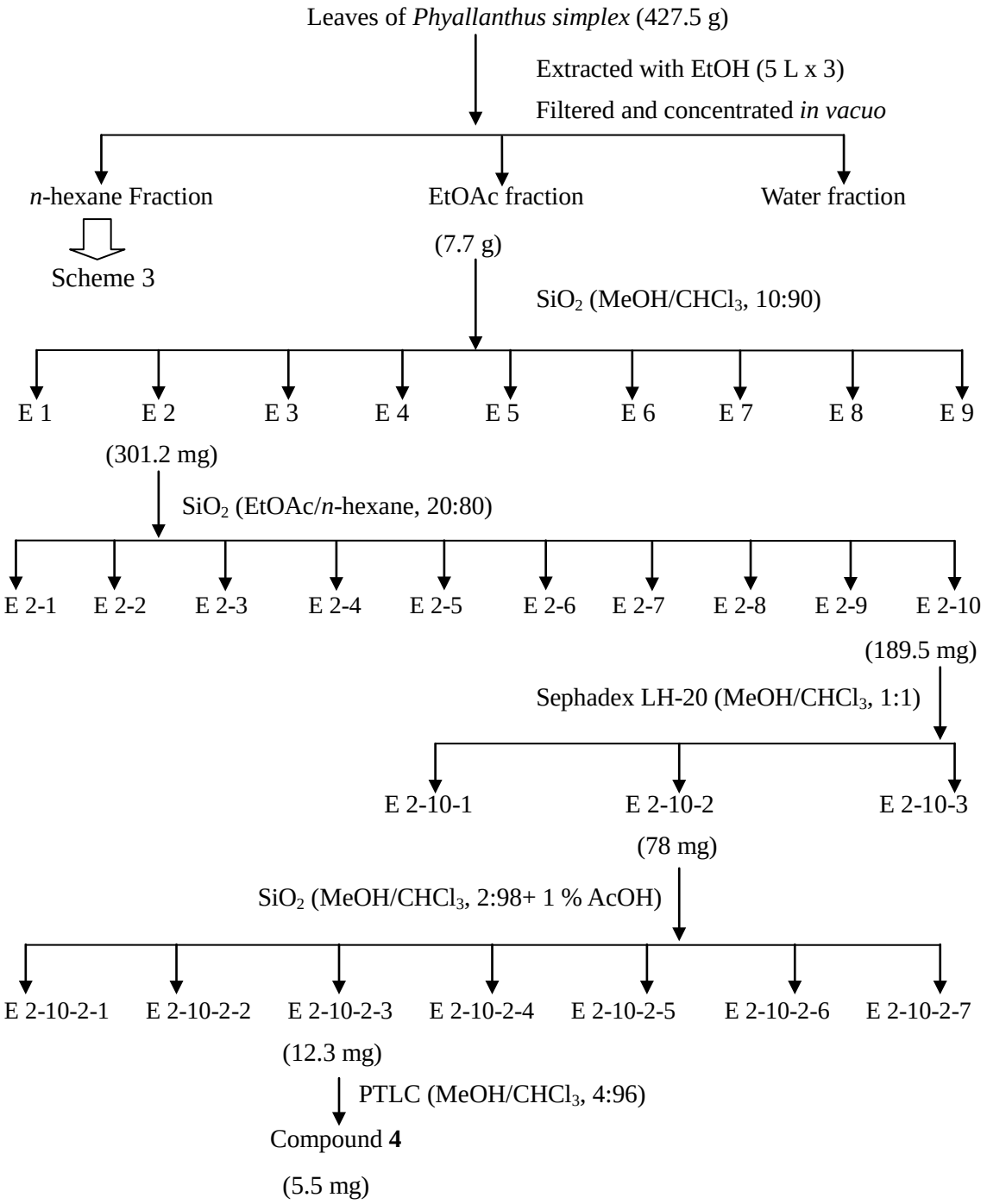
Fig. 17. Planar chemical structures of compounds*; 8,9-epoxy brevifolin (EBF), simplexine (14-hydroxy-4-methoxy-13, 14-dihydronorsecurinine), and phyllanthine, all isolated from *P. simplex* plant.

* Relative and absolute configurations of the compounds were not discussed in the literature (Rajkishor et al., 1988).

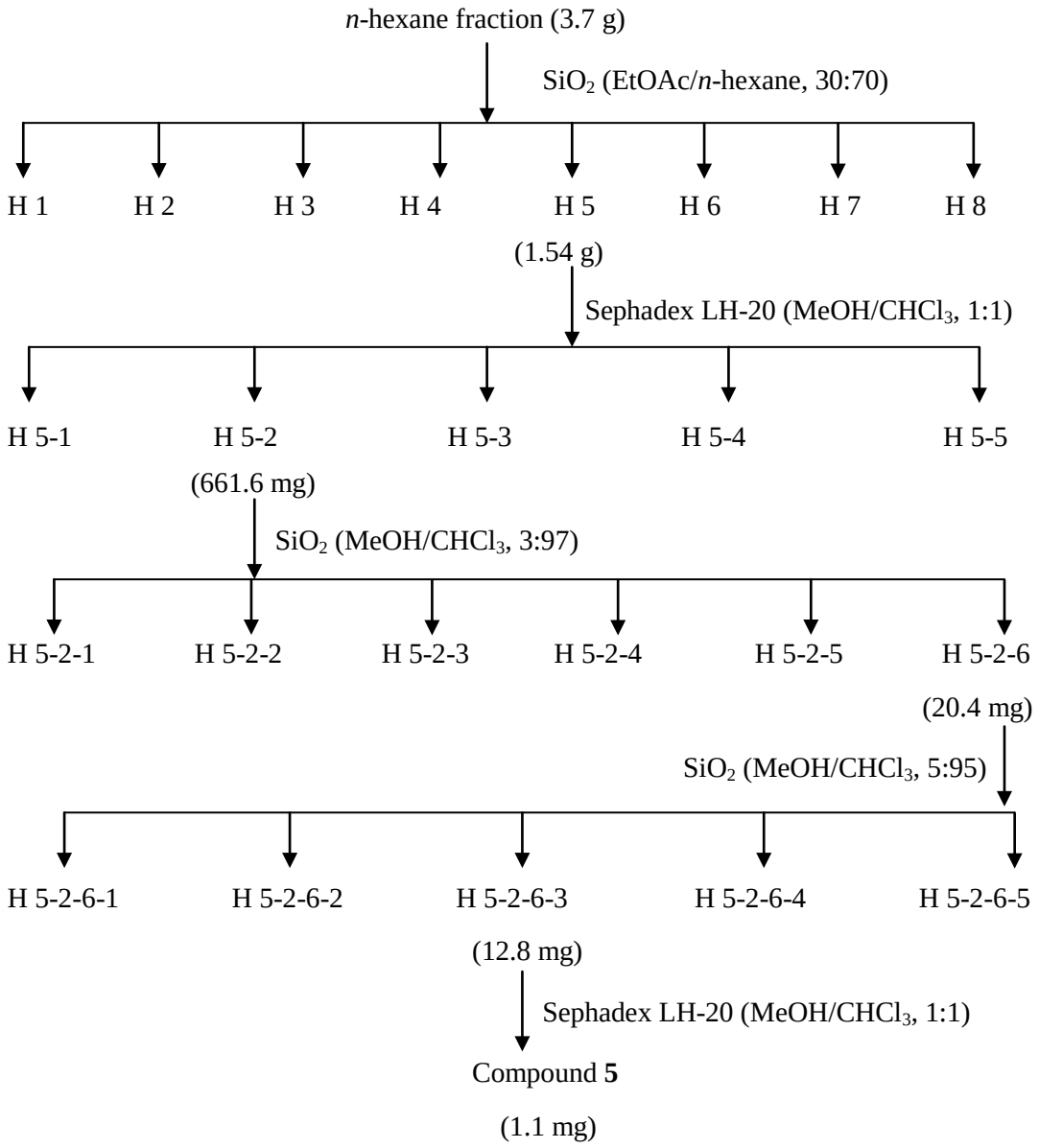
4.1.1 Extraction and isolation

The powdered leaves of *P. simplex* (427.5 g) were extracted with 5 L EtOH for three times at room temperature. After filtration, the EtOH extract was concentrated and partitioned between *n*-hexane, EtOAc, and water. According to the bioassay-guided fractionation, the active EtOAc and *n*-hexane layers were selected to continue for isolation of active compounds as shown in Schemes 2 and 3. The EtOAc residue (7.7 g) was subjected to a silica gel (950 g) column chromatography and successively eluted with MeOH/$CHCl_3$ (10:90). The antitrypanosomal active fraction E 2 (301.2 mg) was subjected to a silica gel (50 g) column chromatograph with EtOAc/*n*-hexane (20:80) to give 10 sub-fractions. The E 2-10 (189.5 mg) fraction was further purified by Sephadex LH-20 column (200 g) eluted with MeOH/$CHCl_3$ (1:1) to afford 3 fractions. The active fraction E 2-10-2 (78 mg) was further separated by a silica gel (10 g) column with MeOH/$CHCl_3$ (2:98 with 0.1 % AcOH) to give 7 fractions. The resulting active fraction E 2-10-2-3 (12.3 mg) was further subjected to preparative TLC with MeOH/$CHCl_3$ (4:96) and found to contain compound **4** (5.5 mg).

The *n*-hexane fraction (3.7 g) was subjected to a silica gel (450 g) column eluted with EtOAc/*n*-hexane (30:70) to afford 8 fractions. According to bio-assay guided fractionation, the active fraction H 5 (1.54 g) was purified by Sephadex LH-20 column (200 g) with MeOH/$CHCl_3$ (1:1) to give 5 sub-fractions. The fraction H 5-2 (661.6 mg) was separated by a silica gel (100 g) column with MeOH/$CHCl_3$ (3:97) to give 6 sub-fractions. The H 5-2-6 (20.4 mg) was further purified by using a silica gel (50 g) column with MeOH/$CHCl_3$ (5:95) to give 5 fractions. Compound **5** (1.1 mg) was isolated from the sub-fraction H 5-2-6-3 (12.8 mg) by using Sephadex LH-20 column (200 g) with MeOH/$CHCl_3$ (1:1).



Scheme 2. Isolation procedure for active compounds from *P. simplex*.



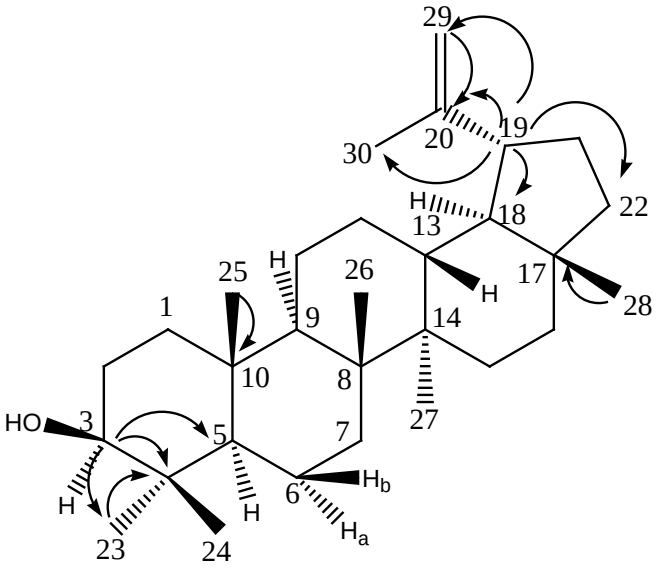
Scheme 3. Isolation procedure for active compounds from *P. simplex*.

4.1.2 Structural elucidation of antitrypanosomal compounds (4, 5)

Compound **4** was isolated as white pearl powder, and its molecular formula was deduced from EI-MS, EI-HR-MS, and FD-MS data (Chart 25, 26, and 27) as $C_{30}H_{50}O$ (*m/z* 426.3847 $[M]^+$, calc for $C_{30}H_{50}O$: 426.3864). The ^1H NMR spectrum (Chart 28) showed seven tertiary methyl singlets at δ 0.77, 0.78, 0.84, 0.96, 0.97, 1.03, and 1.71, and one secondary hydroxyl group as double doublet at δ 3.18. It also showed two olefinic proton signals at δ 4.56 and 4.69 representing the exocyclic double bond. An isoprenyl group is confirmed by the ^{13}C NMR signals (Chart 29) at δ 19.3 (C-30), 109.3 (C-29), and 151.0 (C-20). The ^{13}C NMR spectrum of compound **4** showed 30 signals for the terpenoid of lupane skeleton which included a carbon bonded to the hydroxyl group at C-3 position appearing at δ 79.0, while the olefinic carbons of the exocyclic double bond appeared at δ 151.0 and 109.3. The remaining ^1H and ^{13}C NMR signals were assigned from COSY spectra (Chart 30) in combination with and NOESY (Chart 31) spectra. The directly attached protons to carbon were determined by HMQC (Chart 32) spectra. The COSY spectrum exhibited correlations peaks such as between H-19/H-21, H-18, H-30. The NOESY spectrum showed the cross peaks of H-5 with H-9; H-9 with H-27; H-13 with H-19, H-26, H-28; H-18 with H-27; H-25 with H-26. The cross peaks between H-23 with H-3 and H-6a, which lead the resonance H-6a to be δ_H 1.54. In the HMBC spectra (Chart 33), the methine proton signal at δ_H 3.18 (H-3) showed cross peaks with a methyl carbon signal (δ_C 28.0, C-23), quaternary carbon signal (δ_C 38.7, C-4) and methine carbon signal (δ_C 55.3, C-5). Also the signal at δ_H 2.39 (H-19) showed cross peaks with two methylene carbon signals (C-22) and (C-29), a methine carbon signal (C-18), a methyl carbon signal (C-30) and a quaternary carbon signal (C-20). Other HMBC correlations observed between as H-25/C-10 and H-28/C-17. The above spectral data suggested that compound **4** is a lupane triterpene

having a secondary hydroxyl group (Fig 18). The forgoing spectral analysis and, comparison with reported data together with optical rotation [α_D + 27.4^0, *c* = 0.5, $CHCl_3$] (literature value: α_D + 26.4^0, *c* = 0.4, $CHCl_3$) (Jain et al., 2010) led us to deduce the structure of compound **4** as lupeol, a pentacyclic tri-terpenoid. Total assignments of ^{1}H and ^{13}C NMR spectra data were listed in Table 8.

Compound **5** was isolated as colourless needles, and its molecular formula was deduced from EI-MS and EI-HR-MS data (Chart 34 and 35) as $C_{30}H_{48}O$ (*m/z* 424.3683 $[M]^+$, calc for $C_{30}H_{48}O$: 424.3707). The ^{1}HNMR spectrum (Chart 36) of **5**, the resonances which were thought to be derived from six methyls [δ_H 0.77, 0.86, 0.90, 0.91, 0.99 (3H each, s), and 1.14 (3H, s)], one doublet methyl [δ_H 1.23 (3H, d, *J* = 6.5 Hz, H-29), and one olefinic proton [δ_H 4.62 (3H, d, *J*= 2.0 Hz)]. The ^{13}C NMR spectrum (Chart 37) of compound **5** revealed 30 carbons signals, which were assigned by DEPT experiments (Chart 38) as eight methyls, nine methylenes, six methines, and seven quaternary carbons. The ^{13}C NMR spectrum included a saturated carbonyl group in keto function at C-3 position, suggested the presence of lupane triterpene having a carbonyl group in its structure (Fig. 18). The placement of the double bond located at C-20 and C-21 was confirmed from the HMBC correlations (Chart 39) between H-19 to C-20 and C-21. Other HMBC correlations were observed as H-5/C-3, C-4, H-13/C-14, and H-2/C-1. Based on the COSY and HMQC (Chart 40 and 41) correlations, the structure of **5** was assigned as Ψ-taraxasterone and further supported by the coincidences with the reported physical and spectral data together with optical rotation [α_D + 80.0^0, *c* = 0.1, $CHCl_3$] (literature value: α_D + 80.8^0, *c* = 1.2, $CHCl_3$) (Anjaneyulu et al., 1999). Total assignments of ^{1}H and ^{13}C NMR spectra data were listed in Table 9.



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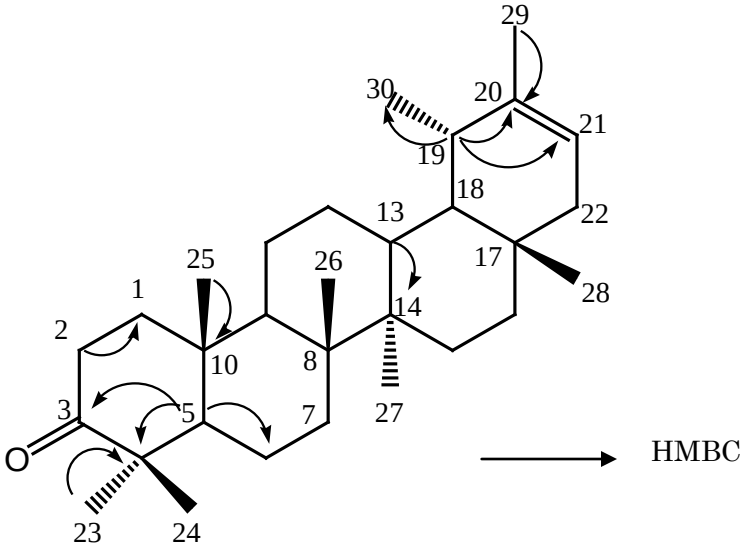

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Fig. 18. Chemical structures of compound **4** (lupeol) and **5** (Ψ-taraxasterone) with important correlations of HMBC.

Table 8. ^{1}H NMR and ^{13}CNMR spectra data of compound **4** (500 MHz, $CDCl_3$).

Position	Compound **4**	
	δ_H	δ_C
1	1.67 (1H, m), 0.91 (1H, m)	38.1
2	1.56 (2H, m)	25.2
3	3.18 (1H, dd, *J* = 5.4, 10.6 Hz)	79.0
4	-	38.7
5	0.69 (1H, m)	55.3
6a	1.54 (1H, m)	18.3
6b	1.38 (1H, m)	
7	1.37 (2H, m)	34.3
8	-	40.9
9	1.33 (1H, m)	50.5
10	-	37.2
11	1.42 (1H, m), 1.28 (1H, m)	20.9
12	1.07-1.67 (2H, m)	27.4
13	1.61 (1H, m)	40.1
14	-	42.9
15	1.68 (2H, m)	27.5
16	1.49 (2H, m)	35.6
17	-	43.0
18	1.39 (1H, m)	48.3
19	2.39 (1H, m)	48.0
20	-	151.0
21	1.25 (2H, m)	29.9
22	1.20 (2H, m)	40.9
23	0.97 (3H, s)	28.0
24	0.77 (3H, s)	15.4
25	0.84 (3H, s)	16.1
26	1.03 (3H, s)	16.0
27	0.96 (3H, s)	14.6
28	0.78 (3H, s)	18.0
29	4.69 (1H, s), 4.56 (1H, s)	109.3
30	1.71 (3H, s)	19.3

Table 9. ^{1}H NMR and ^{13}C NMR spectra data of compound **5** (500 MHz, $CDCl_3$).

Position	Compound **5**	
	δ_H	δ_C
1	0.96 (2H, m)	30.3
2	1.66 (2H, m)	37.4
3	-	207.6
4	-	41.8
5	0.70 (1H, m)	55.0
6	1.39 (1H, m), 1.53 (1H, m)	15.1
7	1.36 (2H, m)	33.9
8	-	41.1
9	1.34 (1H, m)	50.4
10	-	42.3
11	1.28 (2H, m)	22.1
12	1.13 (1H, m), 1.68 (1H, m)	27.2
13	1.58 (1H, m)	41.9
14	-	43.3
15	0.97 (2H, m)	27.7
16	1.27 (1H, m), 1.17 (1H, m)	38.8
17	-	36.7
18	0.92 (1H, m)	47.3
19	2.07 (1H, m)	36.5
20	-	139.5
21	2.43 (1H, m)	118.5
22	1.37 (2H, m)	38.9
23	0.99 (3H, s)	21.1
24	0.77 (3H, s)	17.9
25	0.86 (3H, s)	21.2
26	1.14 (3H, s)	14.5
27	0.91 (3H, s)	14.4
28	0.90 (3H, s)	16.0
29	1.23 (3H, d, J = 6.5 Hz)	26.4
30	4.62 (3H, d, J = 2.0 Hz))	27.0

CHAPTER 5

ISOLATION AND STRUCTURAL ELUCIDATION OF ANTITRYPANOSOMAL COMPOUNDS FROM *VITEX ARBOREA* DESF.

5.1 Biological description of *Vitex arborea* Desf.

Fig. 19. Photograph of *Vitex arborea* Desf.

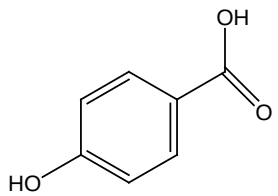
Family : Verbenaceae

Scientific name : *Vitex arborea* Desf.

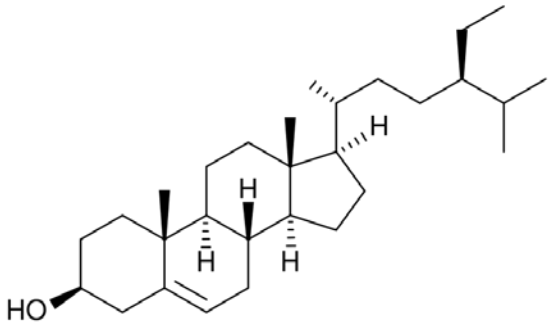
Common name : Pepper-vine, Chinese chaste tree

Myanmar name : Kyet-lal-san

Vitex arborea Desf. is a plant species belong to the family Verbenaceae and is a synonym of *Vitex negundo* var. *negundo*. It is a small to medium-sized evergreen tree, up to 6-18 m high. Leaves are palmately compound, arranged in opposite and decussate position. The flowers are numerous, small and bluish-violet color. *V. arborea* is native to Eastern and Southern Africa and Asia. In Myanmar, this plant is mostly distributed in Kayah and Kayin state and used as a folk medicine for the treatment of fever and diarrhea. There are no reports for isolation of the compounds from this plant. This is the first report on isolation of compounds from this plant. *p*-Hydroxybenzoic acid (*p*-HBA) and *β*-sitosterol were isolated from the hexane and methanol extract of the synonym plant of *V. negundo,* and molecular structures of compounds are as shown in Fig. 20 (Ram et al., 2009). The reports indicated that extracts of family Vebenaceae plant had volatile oil, resin, alkaloid, lichen acids, and glucoside and possessed antibacterial, antimicrobial (Khokra et al., 2008), antinoiceptive (Cheng-Jian et al., 2010), antitumor (Dewade et al., 2010), and anti-inflammatory property (Vishal et al., 2006).



p-hydroxybenzoic acid (*p*-HBA)

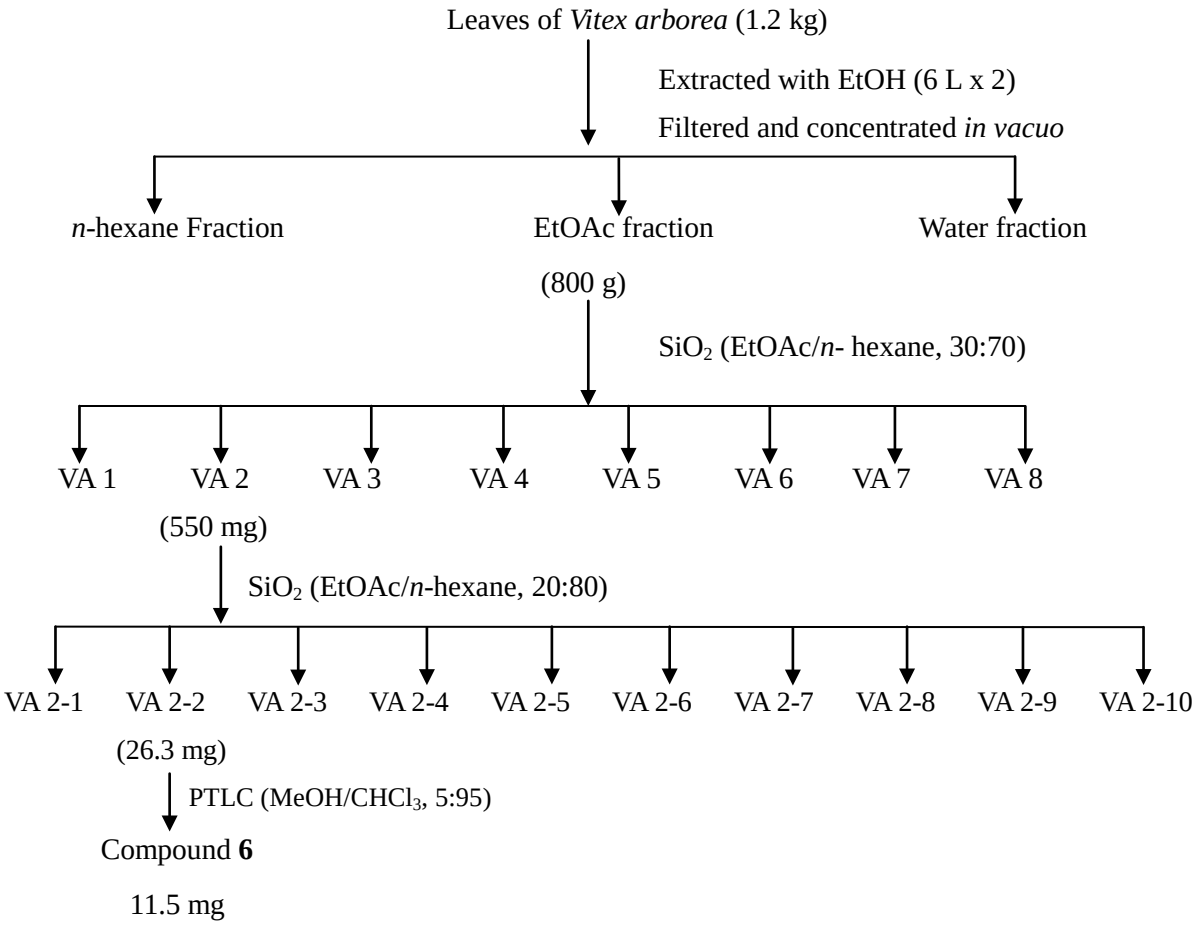


β-sitosterol

Fig. 20. Molecular structures of compounds; *p*-hydroxybenzoic acid (*p*-HBA), and *β*-sitosterol, isolated from the synonym plant (*V. negundo*) of *V. arborea*.

5.1.1 Extraction and isolation

Extraction and isolation of the active compounds were monitored by the antitrypanosomal assay. The powdered leaves of *V. arborea* (1.2 kg) were extracted with 6 L EtOH twice at room temperature. After filtration, the EtOH extract was concentrated *in vacuo*, and then separted between *n*-hexane, EtOAc, and water in succession. According to bioassay guided fractionation, the EtOAc fraction of *V. arborea* (800 g) was chromatographed on a silica gel (2,000 g) column eluted with EtOAc/*n*-hexane (30:70) to give 8 subfractions (VA 1 to VA 8). The VA 2 fraction (550 mg) was purified by a silica gel (220 g) column with EtOAc/*n*-hexane (20:80) to give 10 subfractions (VA 2-1 to VA 2-10). The VA 2-2 (26.3 mg) fraction was subjected to PTLC (MeOH/$CHCl_3$, 5:95) to afford compound **6** (11.5 mg).

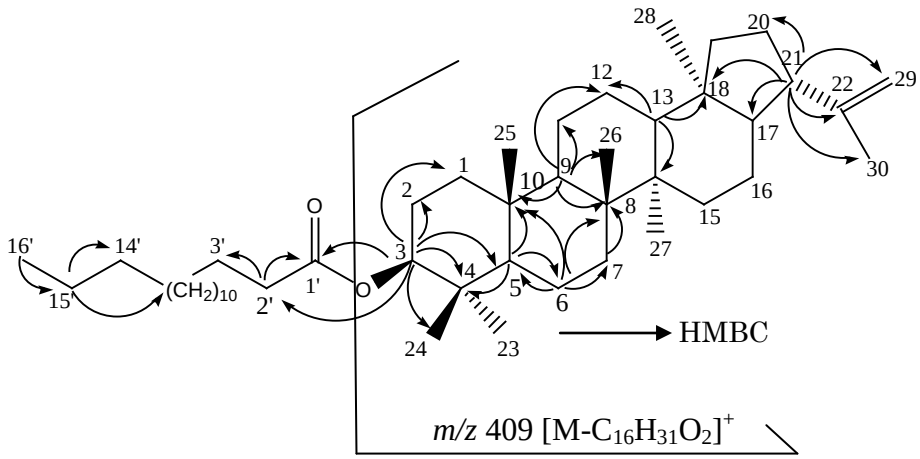


Scheme 4. Isolation procedure for active compounds from *V. arborea*.

5.1.2 Structure elucidation of antitrypanosomal compound (6)

Compound **6** was isolated as yellow oil, and its molecular formula was deduced from EI-MS, EI-HR-MS, and FD-MS data (Chart 42, 43, and 44) as $C_{46}H_{80}O_2$ (*m/z* 664.6159 $[M]^+$, calc for $C_{46}H_{80}O_2$: 664.6162). The ^1H NMR spectrum (Chart 45) showed the signals of six methyl singlets (δ 0.78, 0.81, 0.89, 0.91, 0.94, and 1.01), an oxymethine at δ 4.56, and an isopropenyl group inferred by the presence of a methyl singlet at δ 1.49 and a broad singlet at δ 4.67. The ^{13}C NMR signals (Chart 46) at δ 173.7 indicated the presence of an ester carbonyl group. The HMBC spectrum (Chart 47) of **6** showed the correlations of H-3/C-1, C-2, C-4, C-5, C-24; H-5/C-10, C-4; H-6/C-5, C-7, C-8, C-10; H-9/C-8, C-10, C-11, C-12, C-26; H-13/C-12, C-14, C-18; H-21/C-17, C-18, C-20, C-22, C-29, and C-30. The presence of the palmitoyloxy group was supported by the mass spectral fragment observed in the EI-MS at *m/z* 409 $[M-C_{16}H_{31}O_2]^+$ formed by the loss of palmitoyloxy side chain from the molecular ion together with HMBC and COSY correlations (Chart 48) as shown in Fig. 21. The connectivity of the palmitoyloxy group with the C-3 position was determined with HMBC correlations between H-3/C-1', C-2'. The proton directly attached to carbon was deduced by HMQC data (Chart 49). The NMR data of compound **6** was compared with the literature values, and it was found that spectral data of **6** was similar to moretenyl palmitate (Fig. 21) and hopenyl-3*β*-*O*-palmitate (Ya-Ching et al., 2003, Prakash et al., 2002). However, the absolute configuration of C-21 was different between moretenyl palmitate and hopenyl-3*β*-*O*-palmitate. The chemical structure of **6** was confirmed as hopenyl-3*β*-*O*-palmitate (Fig. 21) due to close resemblance of H-21 (δ 2.58) in **6** with that (δ 2.61) in hopenyl-3*β*-*O*-palmitate (Table 11) together with optical rotation [α_D + 38.0^0, *c* = 0.5, $CHCl_3$] (literature value: α_D + 37.8^0, *c* = 0.5, $CHCl_3$) (Prakash et al., 2002). This is the first report for the isolation of compound **6** from *V. arborea*. Total assignments of ^1H and ^{13}C

NMR spectra data were listed in Table 10.



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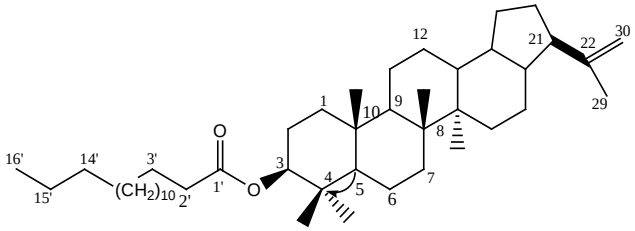


Moretenyl palmitate

Fig. 21. Chemical structures of compound **6** (hopenyl-3*β*-*O*-palmitate) with important correlations of HMBC and MS fragmentation, and moretenly palmitate.

Table 10. ^{1}H NMR (270 MHz, $CDCl_3$) and ^{13}CNMR spectra data of compound **6** (500 MHz, $CDCl_3$).

Compound **6**					
Position	δ_H	δ_C	Position	δ_H	δ_C
1	0.96 (2H, m)	38.0	20	1.35 (2H, m)	27.2
2	1.22 (1H,m), 1.25 (1H, m)	27.9	21	2.58 (1H, m)	47.9
3	4.40 (1H, m)	80.6	22	-	150.2
4	-	37.8	23	0.78 (3H, s)	28.0
5	0.79 (1H, m)	55.4	24	0.94 (3H, s)	18.3
6	1.28 (2H, m)	18.3	25	0.81 (3H, s)	16.1
7	1.43 (2H, m)	36.6	26	0.91 (3H, s)	16.0
8	-	42.8	27	1.01 (3H, s)	15.9
9	1.31 (1H, m)	50.3	28	0.89 (3H, s)	14.5
10	-	37.2	29	4.56 (1H, s), 4.67 (1H, s)	114.2
11	1.30 (2H, m)	21.0	30	1.49 (3H, s)	19.2
12	1.52 (2H, m)	25.1	1'	-	173.7
13	1.32 (1H, m)	48.2	2'	2.21 (2H, m)	34.8
14	-	42.9	3'	1.60 (2H, m)	23.7
15	1.41 (2H, m)	34.8	4'-13'	1.25 (2H, m)	29.2-29.7
16	1.34 (2H, m)	21.0	14'	1.25 (2H, m)	31.9
17	0.75 (1H, m)	58.3	15'	1.25 (2H, m)	22.6
18	-	42.9	16'	0.87 (3H, m)	14.1
19	1.33 (2H, m)	40.8			

Table 11. Spectra data of reference compounds of compound **6**.

Compounds	Position	δ_H
Compound **6**	21	2.58 (1H, m)
	22	-
Hopenyl-3*β*-*O*-palmitate	21	2.61 (1H, m)[a)]
	22	-
Moretenyl palmitate	21	2.30 (dt, 6.4, 7.8)[b)]
	22	-

[a)] Prakash et al., 2002.

[b)] Ya-Ching et al., 2003.

CHAPTER 6

ISOLATION AND STRUCTURAL ELUCIDATION OF ANTITRYPANOSOMAL COMPOUNDS FROM *AMPELOPSIS BREVIPEDUNCULATA* VAR. *HETEROPHYLLA*

6.1 Biological description of *Ampelopsis brevipedunculata* var. *heterophylla*

Fig. 22. Photograph of *Ampelopsis brevipedunculata* var. *heterophylla*.

Family : Vitaceae

Scientific name : *Ampelopsis brevipedunculata*

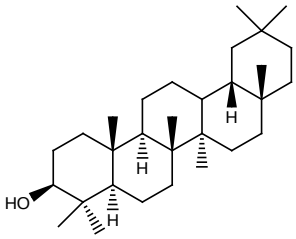
Common name : Porcelain vein

Myanmar name : Za-Pyit-Nwel

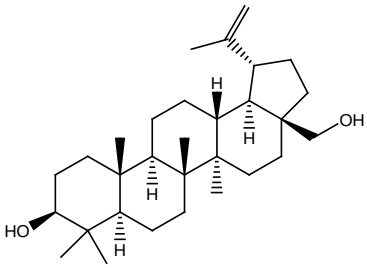
Vitis is a genus of 65 species of woody, deciduous tendril climbers, occasionally shrubs, occurring in woodland and woodland margins in northern temperate regions. The family of *Ampelopsis brevipedunculata* is Vitaceae and widely distributed in Shan state and Pyin Oo Lwin of Myanmar, where it is often used as a folk medicine. It is a deciduous,

woody, perennial vine with a large deep taproot and ridges on branching stems (Fig. 22). The tiny green flowers are produced in panicles from the leaf axils. Some species are edible and used to make wine. It does well train against a wall or over a trellis, or through a large tree. This species prefers full sun and tolerates heat, cold, and strong wind. This plant can grow in a variety of habitats including forest edges, riparian areas, thickets, and waste places. Stems and roots were traditionally used as an anti-imflammatories, diuretics, and anti-hepatotoxins agent in folk medicine. Phytochemical studies on this plant led to the isolation of *β*-amyrin, betulin, vanillic acid, ethyl gallate, kaempferol, aromadendrene, and resveratrol as shown in Fig. 23. Three novel oligostilbenes, ampelopsins A, B, and C, have been isolated from *Ampelopsis brevipedunculata* (Fig. 23) (Oshima et al., 1998).

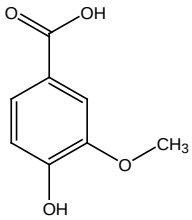
Cyclohexane and methanol extracts of leaves and inflorescences of *A. previpedunculata* are shown to exert significant cytotoxic action on both microbial and cancer (Xu et al., 1995, Kundakonic et al., 2008). The drug efficacy of this plant has been reported for liver disease, specifically ethanol extracts of fresh berries. The dried leaves and branches of *A. brevipedunculata* var. *heterophylla* have also been used as tea, whereas its fresh berries are usually immersed in alcohol in Japan (Nakamura et al., 2013). Biological studies showed that both alcoholic and water decoctions of the fruits, leaves, stems, and roots of *A. brevipedunculata* had an inhibitory action of collagen synthesis of liver cells and anti-fatty liver action (Oshima et al., 1998).



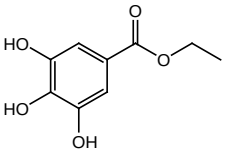
β-amyrin



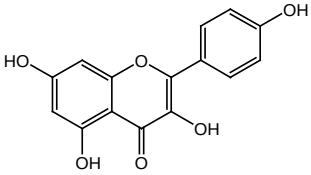
botulin



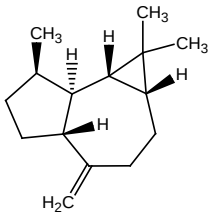
vanillic acid



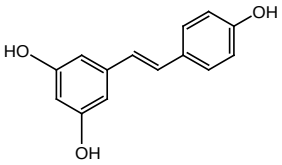
ethyl gallate



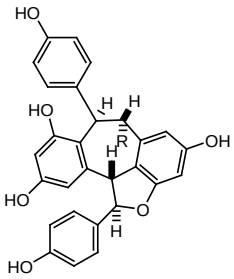
kaempferol



aromadendrene

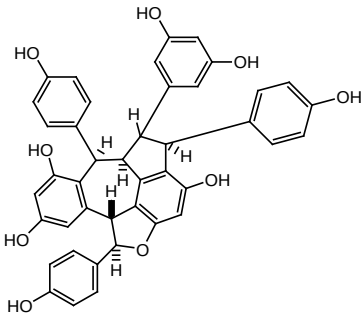


resveratrol



ampelopsin A R = OH

ampelopsin B R = H

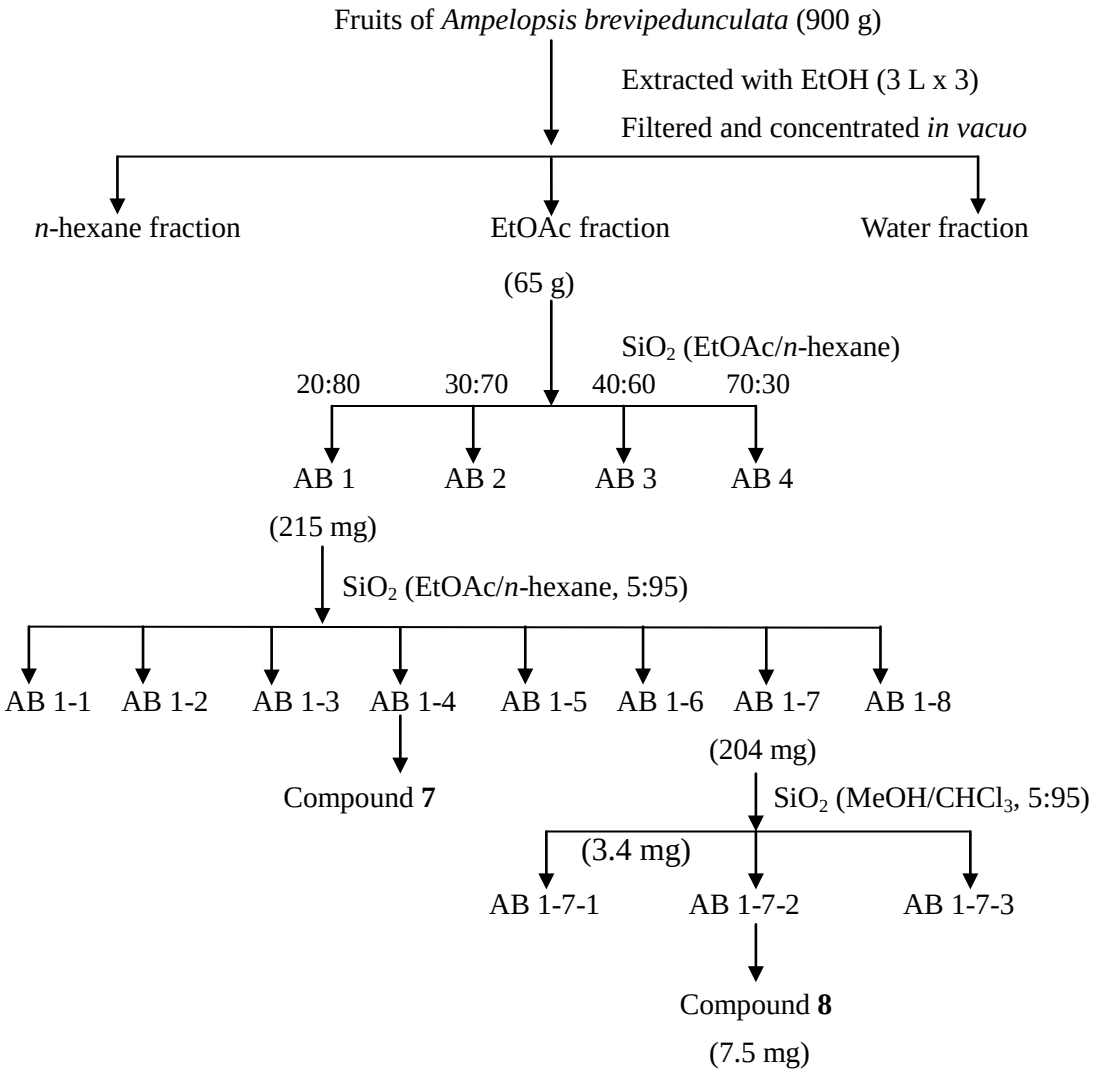


ampelopsin C

Fig. 23. Molecular structures of compounds; *β*-amyrin, betulin, vanillic acid, ethyl gallate, kaempferol, aromadendrene, resveratrol, and three novel oligostilbenes, ampelopsins A, B, and C, isolated from *A. brevipedunculata*.

6.1.1 Extraction and isolation

The extract of fruits of *A. brevipedunculata* was prepared following Scheme 5. The fruits of *A. brevipdeunculata* (900 g) were pulverized and extracted three times with 3 L of EtOH at room temperature. The components of the extract were evaporated and further partitioned between organic solvents; *n*-hexane, EtOAc, and water (1 L each) successively. Based on the activity, the EtOAc fraction of *A. bevipedunculata* (65 g) was separated by a silica gel (1,200 g) column and eluted with a gradient of EtOAc/*n*-hexane to yield 4 fractions (AB 1 to AB 4). AB 1 (215 mg) was fractionated by a silica gel (100 g) column, which was eluted with EtOAc/*n*-hexane (5:95) to give 8 subfractions (AB 1-1 to AB 1-8). The AB 1-4 fraction was revealed to contain compound **7** (3.4 mg). AB 1-7 (204 mg) fraction was further purified by a silica gel column (100 g, MeOH/$CHCl_3$, 5:95) to give 3 subfractions (AB 1-7-1 to AB 1-7-3). The AB 1-7-2 fraction was revealed to contain compound **8** (7.5 mg).



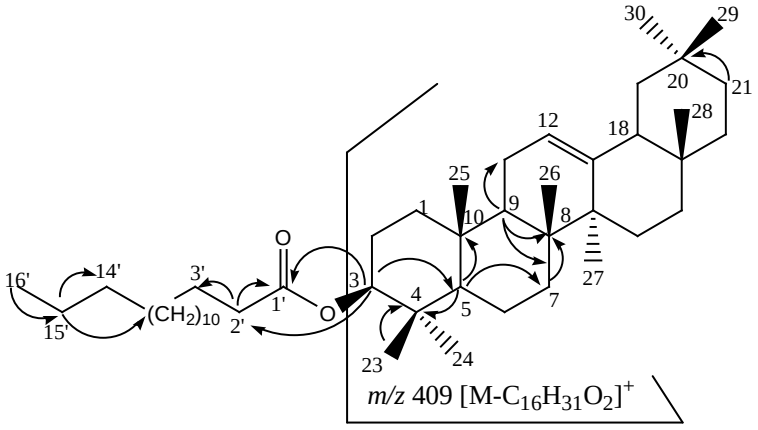
Scheme 5. Isolation procedure for active compounds from *A. brevipedunculata*.

6.1.2 Structural elucidation of antitrypanosomal compounds (7, 8)

Compound **7** was isolated as pale yellow oil, and its molecular formula was deduced from EI-MS and EI-HR-MS data (Chart 50 and 51) as $C_{46}H_{80}O_2$ (*m/z* 664.6143 $[M]^+$, calc for $C_{46}H_{80}O_2$: 664.6162). Mass fragment at *m/z* 409 indicated that it is an analogue of **6**. The ^1H and ^{13}C NMR spectra (Chart 52 and 53) suggested that compound **7** was triterpenoid with oleanane skeleton and established for 8 methyl groups (δ 0.70, 0.81, 0.86, 0.89, 0.94, 0.96, 1.02, and 1.71), methyl singal at δ_H 0.88 (δ_C 14.1), and unsaturated carbons at δ_C 123.6 and 135.1. The HMBC spectrum (Chart 54) of compound **7** showed a long range correlation between palmitoyloxy side chain (H-3/C-1', C-2'). Other HMBC correlations were observed between H-5/C-4, C-7, C-10; H-9/C-7, C-8, C-11; H-7/C-8; H-3/C-5; H-23/C-4; H-2'/C-1', C-3'; H-16'/C-15'; H-15'/C-14'. Based on 1D NMR, COSY (Chart 55), HMQC (Chart 56) spectra and mass fragment in EI-MS data, compound **7** was fatty acid ester derivative and eventually identified as *β*-amyrin palmitate (Fig. 24). Determination of the chemical structure of compound **7** was further supported by the reported data together with optical rotation [α_D + 26.7^0, *c* = 0.1, $CHCl_3$] (literature value: α_D + 26.3^0, *c* = 4.0, $CHCl_3$) (Marizeth et al., 2002). Total assignments of ^1H and ^{13}C NMR spectra data were listed in Table 12.

Compound **8** was isolated as yellow crystal that gave $[M]^+$ molecular ion at *m/z* 228 in the EI-MS spectrum (Chart 57). The ^1H NMR spectrum (Chart 58) of **8** consist 9 protons. The three aromatic protons at δ 6.16 (1H, t, *J* = 4.3 Hz), 6.34 and 6.35 (2H, d, *J* = 2.2 Hz) revealed *meta* coupling each other, indicating a symmetrical 1,3,5-trisubstituted benzene ring. The two olefinic protons at δ 6.83 and 6.72 with *J* = 16.5 Hz suggested *trans* configuration of double bond. The resonance signals at δ 6.65 and 6.68 (2H, d, *J* = 8.6 Hz) and 7.23 and 7.27 (2H, d, *J* = 8.3 Hz) of the aromatic protons are evidence of a 1,

4-disubstituted benzene. The ^{13}C NMR spectrum (Chart 59) of **8** showed the presence of 14 carbon atoms. Two aromatic quaternary carbon atoms are at δ 130.4 (C-1') and 141.3 (C-1). Seven aromatic CH carbon atoms observed at δ 102.6 (C-4), 105.7 (C-2/6), 116.5 (C-3'/5'), and 128.8 (C-2'/6'). Two olefinic carbon atoms with the *trans* configuration were found at δ 127.0 (C-8) and 129.4 (C-7). The resonance signals at δ 158.4 (C-4'), 159.6 (C-3), and 159.7 (C-5) indicated that the aromatic quaternary carbon atoms attached to the oxygen atoms. Chemical structure of compound **8** was estimated by ^{1}H and ^{13}C NMR, and also by comparison with those data of literature vales (Fulvio et al., 1995, Chen et al., 2002) to be *trans*- 3,4,5-trihydroxystilbene (**1**, *E*-resveratrol) (Fig. 24). Total assignments of ^{1}H and ^{13}C NMR spectra data were listed in Table 5.



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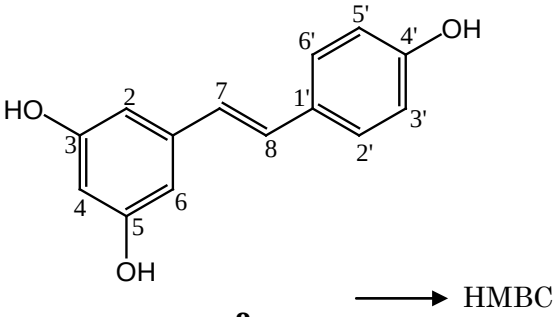

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Fig. 24. Chemical structures of compound **7** (*β*-amyrin palmitate) with important correlations of HMBC and MS fragmentation, and compound **8** (*E*-resveratrol).

Table 12. ^{1}H NMR and ^{13}CNMR spectra data of compound **7** (500 MHz, $CDCl_3$).

Compound **7**					
Position	δ_H	δ_C	Position	δ_H	δ_C
1	0.96 (2H, m)	38.2	20	-	29.8
2	1.22 (2H, m)	23.7	21	2.29 (2H, m)	35.1
3	4.12 (1H, m)	80.4	22	1.54 (2H, m)	39.3
4	-	35.1	23	0.70 (3H, s)	28.1
5	0.84 (1H, m)	49.2	24	0.94 (3H, s)	14.2
6	1.28 (2H, m)	18.0	25	0.81 (3H, s)	15.2
7	1.43 (2H, m)	33.1	26	0.89 (3H, s)	19.0
8	-	39.7	27	1.02 (3H, s)	25.8
9	1.31 (1H, m)	47.8	28	0.86 (3H, s)	28.0
10	-	36.6	29	0.96 (3H, s)	34.2
11	1.33 (2H, m)	20.1	30	1.71(3H, s)	25.0
12	5.14 (1H, m)	123.6	1'	-	173.2
13	-	135.1	2'	2.28 (2H, m)	34.9
14	-	40.1	3'	1.54 (2H, m)	23.7
15	1.46 (2H, m)	27.9	4'-13'	1.17 (2H, m)	29.2-29.7
16	1.33 (2H, m)	26.1	14'	1.17 (2H, m)	31.9
17	-	40.1	15'	1.17 (2H, m)	22.6
18	0.85 (1H, m)	47.1	16'	0.88 (3H, m)	14.1
19	1.18 (2H, m)	45.9			

CHAPTER 7

ANTITRYPANOSOMAL ACTIVITIES OF ISOLATED COMPOUNDS FROM MYANMAR MEDICINAL PLANTS

7.1 Antitrypanosomal activities of isolated compounds (1-7) from Myanmar medicinal plants

Bioactivity-guided fractionation of the ethanolic extract of Myanmar medicinal plants; *V. repens*, *P. simplex*, *V. arborea*, and *A. brevipedunculata*, led to the isolation of seven known compounds; resveratrol (**1**), 11-*O*-acetyl-bergenin (**2**), stigmast-4-en-3-one (**3**), lupeol (**4**), Ψ-taraxasterone (**5**), hopenyl-3*β*-*O*-palmitate (**6**), and *β*-amyrin palmitate (**7**).

Recent research reveals promising activity of resveratrol (**1**). It was uncovered that topical applications of resveratrol may provide protection from skin cancer (Moammir et al., 2005). Resveratrol has also shown promising results in studies for the treatment of colon cancer and esophageal tumors (Mohammad et al., 2007). It is produced by the plant as a defence against diseases. Resveratrol is present in many plants and fruits, including red grapes, eucalyptus, spruce, blueberries, mulberries, peanuts, and giant knotweed. Resveratrol is an antioxidant but its antioxidant properties are weaker than those of quercetin and epicatechin. Resveratrol also increases the activity of some antiretroviral drugs *in vitro*. Resveratrol protects our heart and blood vessels by scavenging oxidants which could cause oxidation of lipids, and by preventing apoptosis of endothelial cells (Satyanand et al., 2010). It may also help to prevent heart damage after a cardiac arrest. Mohammad et al., (2007) reported that reduced platelet aggregation has been attributes to resveratrol, thereby reducing the risk of atherosclerosis (Mohammad et al., 2007). One study showed that resveratrol was

able to increase the life span of mice on a high calorie diet (Baur et al., 2006). Resveratrol also reduced kidney damage of rats treated with the antibiotic gentamicin (Satyanand et al., 2010).

The plant extracts containing bergenin and 11-*O*-acetyl bergenin (**2**) have long been used as a folk medicine in several parts of Asia. It exhibits antihepatotoxic, anti-HIV, antiarrhythmic, anti-inflammatory, and immunomodulatory properties (Singh et al., 2009). It also exhibits various biological activities such as antiulcer, antimalaria, antifungal, and burn wound healing effects (Nazir et al., 2011). Previous study also revealed that stigmast 4-en-3-one (**3**) exhibited the vasodepressor effect (Barla et al., 2006) and hypoglycemia effect (Alexanda et al., 2004).

Lupeol (**4**) has been shown to exhibit various pharmacological activities under *in vitro* and *in vivo* conditions, including inflammation, cancer, arthritis, diabetes, heart diseases, renal toxicity, and hepatic toxicity (Fernández et al., 2001). It is reported to exhibit pharmacological activities against various disease conditions (Saratha et al., 2011). Ψ-Taraxasterone (**5**) exhibits several pharmacological activities such as anti-HIV, anti-cancer, anti-inflammatory, hepatoprotective, spasmolytic, and spasmogenic amongst others. It is potentially an important ingredient of medicinal plant for mankind (Muley et al., 2009).

Previous reports indicated that hopenyl-3*β*-*O*-palmitate (**6**) has been shown to inhibit antibacterial and antifungal activities (Rojas et al., 2007), and *β*-amyrin palmitate (**7**) has anti-inflammatory activity (Kweifio et al., 1995). Several studies addressed on the molecular mechanism of the anti-inflammatory activity (Flavia et al., 2012). One study has shown its preventive or therapeutic anti-inflammatory potential in a murine model of trinitro-benzene-sulfonic acid (TNBS)-induced colitis, and it was found to be as efficacious

as dexamethasone in reversing the macroscopic and microscopic outcomes of TNBS-induced colitis through suppression of inflammatory cytokines and cyclooxygenase-2 levels, and by inhibition of NF-κB activation (Vitor et al., 2009). In other studies, *β*-amyrin ameliorated periodontal inflammation in rat model of ligature-induced periodontitis to reduce the neutrophil infiltration, oxidative stress and the production of pro inflammatory cytokine, TNF- α (Holanda et al., 2008).

Aforementioned compounds were isolated from Myanmar medicinal plants in this study and were evaluated inhibitory effect on *T. evansi in vitro* (Table 13). Evaluations of antitrypanosomal activity of compounds (**1-7**) had never been performed until this study was done.

Table 13. Antitrypanosomal activities of isolated compounds.

Compounds	IC_{50} (µg/ mL)
E-resveratrol (**1**)	31.4
11-*O*-acetyl bergenin (**2**)	61.2
stigmast-4-en-3-one (**3**)	62.8
lupeol (**4**)	98.4
Ψ-taraxasterone (**5**)	115.4
hopenyl-3*β*-*O*-palmitate (**6**)	68.2
β-amyrin palmitate (**7**)	60.8
diminazene aceturate *	3.51

* Control (The reference data from Bawm et al., 2010).

As shown in Table 13, the isolated compounds exhibited mild to moderate

antitrypanosomal activity against trypomastigotes of *T. evansi*. Among of the isolated compounds, resveratrol (**1**) showed the highest activity against *T. evansi*. In general, compared with the standard drug, diminazene aceturate, having IC_{50} value of 3.51 µg/mL, the isolated compounds (**1-7**) showed relatively weak antitrypanosomal activities. However, considering the potentially fatal side-effects of the standard drug, it is likely that the isolated compounds (**1-7**) might be good leads for treatment against to kill *T. evansi*. It appears that the antitrypanosomal activities of the crude plant extracts showed higher activity than those of isolated compounds. It seems that main antitrypanosomal activity in the crude extract may be lost during the isolation procedure. However, the isolation procedure in this study was according to the bioassay-directed fractionation. It was thought that the activity found in the crude plant extract was not lost during isolation procedure. This may be due to the synergistic effect of the coexisting compounds in the crude extract. We also need to pay attention that some active compounds are decomposed during isolation procedure such as hydroperoxides (Nagametsu et al., 2012, Li et al., 2013), which are difficult to isolate. Therefore, it is suggested that further research should be perform to investigate the isolation and identification of the antitrypanosomal compounds of Myanmar medicinal plant extracts.

CHAPTER 8

EXPERIMENTAL

8.1 General

Optical rotations were measured with a JASCO P-2200 polarimeter. Mass spectra were recorded a JEOL JMS-SX 102a and JMS-AX500 spectrometers. NMR spectra were recorded in a JEOL JNM-EX 270 FT-NMR spectrometer and on a Bruker AMX-500 FT-NMR spectrometer. Column chromatography was conducted with silica gel 60 (spherical, 70-140 mesh ASTM, Kanto Chemical). Analytical thin-layer chromatography was performed with silica gel 60 F_{254} (Merck).

8.2 Spectra data of the isolated compounds

Resveratrol (**1**); yellow amorphous: EI-MS *m/z* (rel. int.): *m/z* 228 $[M]^+$ (100), 181 (12) (Chart 1). EI-HR-MS *m/z*: 228.0788 $[M]^+$ (calc. for $C_{14}H_{12}O_3$: 228.0786) (Chart 2).

^{1}H NMR (270 MHz, CD_3OD) δ: 6.34 (1H, d, *J* = 2.2 Hz, H-2), 6.16 (1H, t, *J* = 4.3 Hz, H-4), 6.35 (1H, d, *J* = 2.2 Hz, H-6), 6.93 (1H, d, *J* = 16.5 Hz, H-7), 6.72 (1H, d, *J* = 16.5 Hz, H-8), 7.23 (1H, d, *J* = 8.3 Hz, H-2'), 6.65 (1H, d, *J* = 8.6 Hz, H-3'), 6.78 (1H, d, *J* = 8.6 Hz, H-5'), 7.37 (1H, d, *J* = 8.3 Hz, H-6') (Chart 3).

^{13}C NMR (270 MHz, CD_3OD) δ: 141.3 (C-1), 105.7 (CH-2), 159.6 (C-3), 102.6 (CH-4), 159.7 (C-5), 105.7 (CH-6), 129.4 (CH-7), 127.0 (CH-8), 130.4 (C-1'), 128.8 (CH-2'), 116.5 (CH-3'), 158.4 (C-4'), 116.5 (CH-5'), 128.8 (CH-6') (Chart 4).

HMQC (Chart 5). COSY (Chart 6). HMBC (Chart 7).

11-*O*-Acetyl bergenin (**2**); pale yellow amorphous: $[\alpha]^{28}_{D=}$ -8.2^0 (*c* 0.3; MeOH). EI-HR-MS *m/z* (rel. int.): 393.07913 $[M+Na]^+$ (calc. for $C_{16}H_{18}O_{10}$: 370.0899) (Chart 9).

^{1}H NMR (270 MHz, CD_3OD) δ: 3.77 (1H, m, H-2), 3.39 (1H, m, H-3), 3.79 (1H, m, H-4), 4.01 (1H, t, *J* = 9.54 Hz, H-4a), 7.08 (1H, s, H-7), 4.98 (1H, d, *J* = 10.5 Hz, H-10b), 4.16 and 4.59 (each 1H, m, H-11), 3.84 (3H, s, $-OCH_3$), 2.13 (3H, s, $-CH_3$) (Chart 10).

^{13}C NMR (127.5 MHz, CD_3OD) δ: 60.8 ($-OCH_3$), 20.9 ($-CH_3$), 172.6 (C=O), 80.4 (CH-2), 71.9 (CH-3), 75.4 (CH-4), 81.3 (CH-4a), 165.6 (C-6), 119.5 (C-6a), 111.2 (CH-7), 152.5 (C-8), 142.3 (C-9), 149.4 (C-10), 117.0 (C-10a), 74.3 (C-10b), 64.6 (CH_2-11) (Chart 11).

COSY (Chart 12). HMBC (Chart 13). HMQC (Chart 14). NOESY (Chart 15).

Pentaacetate bergenin from compound **2**

$[\alpha]_{D=}$ -6.9^0 (*c* 0.2; MeOH).

Pentaacetate bergenin from authentic bergenin

$[\alpha]_{D=}$ -6.4^0 (*c* 0.3; MeOH).

Stigmast-4-en-3-one (**3**); white needle**;** $[\alpha]^{26}_{D=}$ +73.3^0 (*c* 0.5; MeOH). EI-MS *m/z* (rel.int.): 412 $[M]^+$ (82.53), 398.0 (16.39), 370 (27.19), 289 (25.76), 271 (20.86), 229 (51.57), 147 (24.22), 135 (19.12), 124 (100), 95 (22.03), 55 (17.93) (Chart 16). EI-HR-MS *m/z*: 412.3718 $[M]^+$ (calc. for $C_{29}H_{48}O$: 412.3707) (Chart 17).

^{1}H NMR (270 MHz, $CDCl_3$) δ: 2.06 (2H, m, H-1), 2.39 (2H, m, H-2), 5.70 (1H, s, H-4), 2.20 (2H, m, H-6), 1.06-0.95 (2H, m, H-7), 1.42 (1H, m, H-8), 0.99 (1H, m, H-9), 1.65-1.42 (2H, m, H-11), 2.00 (2H, m, H-12), 0.95 (1H, m, H-14), 1.90-1.80 (2H, m, H-15), 1.65-1.42 (2H, m, H-16), 1.10 (1H, m, H-17),0.69 (3H, s, H-18), 1.16 (3H, s, H-19), 1.40 (1H, m, H-20), 0.90 (3H, d, H-21), 1.06-0.95 (2H, m, H-22), 1.40-1.10 (2H, m, H-23), 1.65 (1H, m,

H-24), 0.87 (1H, m, H-25), 0.83 (3H, d, *J* = 6.7 Hz), 0.80 (3H, d, *J* = 6.8 Hz), 1.40-1.10 (2H, m), 0.85 (3H, t, *J* = 6.7 Hz) (Chart 19).

^{13}C NMR (500 MHz, $CDCl_3$) δ: 35.7 (C-1), 34.0 (C-2), 123.7 (CH-4), 171.5 (C-5), 32.9 (C-6), 32.1 (CH_2-7), 35.6 (CH-8), 53.8 (CH-9), 38.6 (C-10), 21.0 (CH_2-11), 39.6 (CH_2-12), 42.4 (C-13), 55.9 (CH-14), 28.2 (CH_2-15), 24.2 (CH_2-16), 56.0 (CH-17),11.9 (CH_3-18), 17.4 (CH_3-19), 36.1 (CH-20), 18.7 (CH_3-21), 33.9 (CH_2-22), 26.1 (CH_2-23), 29.2 (CH-24), 45.9 (CH-25), 19.8 (CH_3-26), 19.0 (CH_3-27), 23.1 (CH_2-28), 12.0 (CH_3-29), 199.5 (C=O) (Chart 20).

DEPT (Chart 21). HMBC (Chart 22). COSY (Chart 23). HMQC (Chart 24).

Lupeol (**4**); white pearl powder; $[\alpha]_D$ = +27.4º (*c* = 0.5 , $CHCl_3$); EI-MS: *m/z* (rel. int.): 426 $[M]^+$ for formula $C_{30}H_{50}O$. EI-MS; *m/z* 203 (rel. int.): (37), 189 (50), 135 (64), 121 (71), 95 (78), 81 (36), 69 (68), 41 (64) (Chart 25). EI-HR-MS *m/z*: 426.3847 $[M]^+$ (calc. for $C_{30}H_{50}O$: 426.3864) (Chart 26).

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 1.67, 0.91 (2H, m, H-1), 1.56 (2H, m, H-2), 3.18 (1H, dd, *J* = 5.4, 10.6 Hz, H-3), 0.69 (1H, m, H-5), 1.54 (1H, m, H-6a), 1.38 (1H, m, H-6b), 1.37 (2H, m, H-7), 1.33 (1H, m, H-9), 1.42, 1.28 (2H, m, H-11), 1.07-1.67 (2H, m, H-12), 1.61 (1H, m, H-13), 1.68 (2H, m, H-15), 1.49 (2H, m, H-16), 1.39 (1H, m, H-18), 2.39 (1H, m, H-19), 1.25 (2H, m, H-21), 1.20 (2H, m, H-22), 0.97 (3H, s, H-23), 0.77 (3H, s, H-24), 0.84 (3H, s, H-25), 1.03 (3H, s, H-26), 0.96 (3H, s, H-27), 0.78 (3H, s, H-28), 4.69, 4.56 (2H, s, H-29), 1.71 (3H, s, H-30) (Chart 28).

^{13}C NMR (500 MHz, $CDCl_3$): δ_C 38.1 (C-1), 25.2 (C-2), 79.0 (C-3), 38.7 (C-4), 55.3 (C-5), 18.3 (C-6), 34.3 (C-7), 40.9 (C-8), 50.5 (C-9), 37.2 (C-10), 20.9 (C-11), 27.4 (C-12), 40.1 (C-13), 42.9 (C-14), 27.5 (C-15), 35.6 (C-16), 43.0 (C-17), 48.3 (C-18), 48.0 (C-19), 151.0

(C-20), 29.9 (C-21), 40.9 (C-22), 28.0 (C-23), 15.4 (C-24), 16.1 (C-25), 16.0 (C-26), 14.6 (C-27), 18.0 (C-28), 109.3 (C-29), 19.3 (C-30) (Chart 29).

COSY (Chart 30). NOESY (Chart 31). HMQC (Chart 32). HMBC (Chart 33).

Ψ-Taraxasterone (**5**); colorless needles; $[\alpha]_D$ = +80.0º (*c* = 0.1, $CHCl_3$) ; ESI-MS: *m/z* 424 $[M]^+$ for formula $C_{30}H_{48}O$. EI/MS: *m/z* (rel. int.): 424 $[M]^+$ (25), 205 (30), 189 (11) (Chart 34). EI-HR-MS *m/z*: 424.3683 $[M]^+$ (calc. for $C_{30}H_{48}O$: 424.3707) (Chart 35).

^{1}H NMR (500 MHz, $CDCl_3$): δ_H 0.96 (2H, m, H-1), 1.66 (2H, m, H-2), 0.70 (1H, m, H-5), 1.39, 1.53 (2H, m, H-6), 1.36 (2H, m, H-7), 1.34 (1H, m, H-9), 1.28 (2H, m, H-11), 1.13, 1.68 (2H, m, H-12), 1.58 (1H, m, H-13), 0.97 (2H, m, H-15), 1.27, 1.17 (2H, m, H-16), 0.92 (1H, m, H-18), 2.07 (1H, m, H-19), 2.43 (1H, m, H-21), 1.37 (2H, m, H-22), 0.99 (3H, s, H-23), 0.77 (3H, s, H-24), 0.86 (3H, s, H-25), 1.14 (3H, s, H-26), 0.91 (3H, s, H-27), 0.90 (3H, s, H-28), 1.23 (3H, d, *J* = 6.5 Hz, H-29), 4.62 (3H, m, H-30) (Chart 36).

^{13}C NMR (500 MHz, $CDCl_3$): δ_C 30.3 (C-1), 37.4 (C-2), 207.6 (C-3), 41.8 (C-4), 55.0 (C-5), 15.1 (C-6), 33.9 (C-7), 41.1 (C-8), 50.4 (C-9), 42.3 (C-10), 22.1 (C-11), 27.2 (C-12), 41.9 (C-13), 43.3 (C-14), 27.7 (C-15), 38.8 (C-16), 36.7 (C-17), 47.3 (C-18), 36.5 (C-19), 139.5 (C-20), 118.5 (C-21), 38.9 (C-22), 21.1 (C-23), 17.9 (C-24), 21.2 (C-25), 14.5 (C-26), 14.4 (C-27), 16.0 (C-28), 26.4 (C-29), 27.0 (C-30) (Chart 37).

DEPT (Chart 38). HMBC (Chart 39). COSY (Chart 40). HMQC (Chart 41).

Hopenyl-3*β*-*O*-palmitate (**6**); yellow oil. $[\alpha]_D$: +38.0º (*c* = 0.5, $CHCl_3$). EI-MS: *m/z* (rel. int.): 664 $[M]^+$ (25), 409 (10), 229 (18), 189 (100), 175 (84), 161 (34), 147 (33), 135 (37), 121 (45), 109 (42), 95 (56), 81 (35), 57 (23) (Chart 42). EI-HR-MS *m/z*: 664.616 $[M]^+$ (calc. for $C_{46}H_{80}O_2$: 664.6162) (Chart 43).

^{1}H NMR (270 MHz, $CDCl_3$): δ_H 0.96 (2H, m, H-1), 1.22, 1.25 (1H, m, H-2), 4.40 (1H, m, H-3), 0.79 (1H, m, H-5), 1.28 (2H, m, H-6), 1.43 (2H, m, H-7), 1.31 (1H, m, H-9), 1.30 (2H, m, H-11), 1.52 (2H, m, H-12), 1.32 (1H, m, H-13), 1.41 (2H, m, H-15), 1.34 (2H, m, H-16), 0.75 (1H, m, H-17), 1.33 (2H, m, H-19), 1.35 (2H, m, H-20), 2.58 (1H, m, H-21), 0.78 (3H, s, H-23), 0.94 (3H, s, H-24), 0.81 (3H, s, H-25), 0.91 (3H, s, H-26), 1.01 (3H, s, H-27), 0.89 (3H, s, H-28), 4.56, 4.67 (1H, s, H-29), 1.49 (3H, s, H-30), 2.21 (2H, m, H-2'), 1.60 (2H, m, H-3'), 1.25 (2H, m, H-4'-15'), 0.87 (3H, m, H-16') (Chart 45).

^{13}C NMR (500 MHz, $CDCl_3$): δ_C 38.0 (C-1), 27.9 (C-2), 80.6 (C-3), 37.8 (C-4), 55.4 (C-5), 18.3 (C-6), 36.6(C-7), 42.8 (C-8), 50.3(C-9), 37.2 (C-10), 21.0 (C-11), 25.1 (C-12), 48.2 (C-13), 42.9 (C-14), 34.8 (C-15), 21.0 (C-16), 58.3 (C-17), 42.9 (C-18), 40.8 (C-19), 27.2 (C-20), 47.9 (C-21). 150.2 (C-22), 28.0 (C-23), 18.3 (C-24), 16.1 (C-25), 16.0 (C-26), 15.9 (C-27), 14.5 (C-28), 114.2 (C-29), 19.2 (C-30), 173.7 (C-1'), 34.8 (C-2'), 23.7 (C-3'), 29.2-29.7 (C-4'~13'), 31.9 (C-14'), 22.6 (C-15'), 14.1 (C16') (Chart 46).

HMBC (Chart 47). COSY (Chart 48). HMQC (Chart 49).

β-Amyrin palmitate (**7**); pale-yellow color. $[\alpha]_D$: +26.7º (*c* = 0.1, $CHCl_3$), EI-MS: *m/z* (rel. int.): 664 $[M]^+$ (25), 409 (10), 229 (18), 189 (100), 175 (84), 161 (34), 147 (33), 135 (37), 121 (45), 109 (42), 95 (56), 81 (35), 57 (23) (Chart 50). EI-HR-MS *m/z*: 664.6143 $[M]^+$ (calc. for $C_{46}H_{80}O_2$: 664.6162) (Chart 51).

^{1}H NMR (500 MHZ, $CDCl_3$): δ_H 0.96 (2H, m, H-1), 1.22 (2H, m, H-2), 4.12 (1H, m, H-3), 0.84 (1H, m, H-5), 1.28 (2H, m, H-6), 1.43 (2H, m, H-7), 1.31 (1H, m, H-9), 1.33 (2H, m, H-11), 5.14 (1H, m, H-12), 1.46 (2H, m, H-15), 1.33 (2H, m, H-16), 0.85 (1H, m, H-18), 1.18 (2H, m, H-19), 2.29 (2H, m, H-21), 1.54 (2H, m, H-22), 0.70 (3H, s, H-23), 0.94 (3H, s, H-24), 0.81 (3H, s, H-25), 0.89 (3H, s, H-26), 1.02 (3H, s, H-27), 0.86 (3H, s, H-28), 0.96

(3H, s, H-29), 1.71 (3H, s, H-30), 2.28 (2H, m, H-2'), 1.54 (2H, m, H-3'), 1.17 (2H, m, H-4'-15'), 0.88 (3H, m, H-16') (Chart 45) (Chart 52).

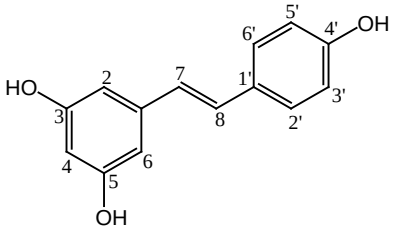
^{13}C NMR (500 MHz, $CDCl_3$): δ_C 38.2 (C-1), 23.7 (C-2), 80.4 (C-3), 35.1 (C-4), 49.2 (C-5), 18.0 (C-6), 33.1 (C-7), 39.7 (C-8), 47.8 (C-9), 36.6 (C-10), 20.1 (C-11), 123.6 (C-12), 135.1 (C-13), 40.1 (C-14), 27.9 (C-15), 26.1(C-16), 40.1 (C-17), 47.1 (C-18), 45.9 (C-19), 29.8 (C-20), 35.1 (C-21). 39.3 (C-22), 28.1 (C-23), 14.2 (C-24), 15.2 (C-25), 19.0 (C-26), 25.8 (C-27), 28.0 (C-28), 34.2(C-29), 25.0 (C-30), 173.2 (C-1’), 34.9 (C-2’), 23.7 (C-3’), 29.2-29.7 (C-4’~13’), 31.9 (C-14’), 22.6 (C-15’), 14.1 (C16’) (Chart 53).

HMBC (Chart 54). COSY (Chart 55). HMQC (Chart 56).

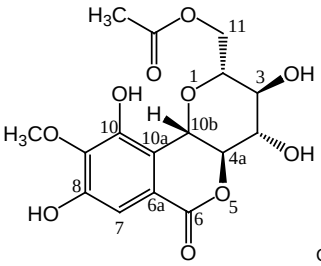
Summary

Trypanosomiasis, a serious parasitic disease affecting humans and animals in many countries, is caused by blood-dwelling *Trypanosoma* species. The genus *Trypanosoma* is divided into two main groups based on the mode of transmission by their insect vectors: stercoraria and salivaria. Among of the salivarian trypanosomes, *Trypanosoma evansi* has the widest distribution of all species of trypanosomes and the greatest range of mammalian hosts, making it one of the most economically important protozoan diseases present in the world today. *T. evansi* is an animal-pathogenic flagellated protozoan parasite and infects a variety of animals and causes epidemics of a disease known as surra, which is of great treat in Africa, Asia, and Central and South America. The parasites are mechanically transmitted by biting hematophagous insects, especially flies in the Tabanidae family such as horseflies (*Tabanus* spp.) and stable flies (*Stomoxys* spp.) from one infected host to other. Some antitrypanosomal drugs reduce the severity of clinical signs and the mortality associated with the disease. However, several problems are associated with these drugs due to their side effects and ineffectiveness against drug resistant parasites in many regions. Therefore, an alternative chemotherapeutic agent with fewer side effects is urgently needed to prevent and treat disease caused by *Trypanosoma* species. Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug discoveries because of the unmatched availability of chemical diversity. Myanmar is abundant in plant resources, and Myanmar traditional medicinal practitioners have been using a variety of herbal preparations to treat different types of diseases. A previous study revealed that the crude extract of Myanmar medicinal plants showed *in vitro* inhibitory activity against *T. evansi*. Bioactivity-guided fractionation of the ethanolic extract of Myanmar medicinal plants, *V. repens*, *P. simplex*, *V. arborea*, and *A. brevipedunculata*, led to

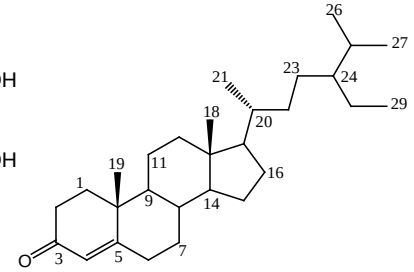
the isolation of seven known compounds, resveratrol (**1**), 11-*O*-acetyl-bergenin (**2**), stigmast-4-en-3-one (**3**), lupeol (**4**), Ψ-taraxasterone (**5**), hopenyl-3*β*-*O*-palmitate (**6**), and *β*-amyrin palmitate (**7**). The isolated compounds were tested *in vitro* for their antiprotozoal activities against *T. evansi*. The IC_{50} value of antitrypanocidal effect of resveratrol (**1**), 11-*O*-acetyl bergenin (**2**), stigmast-4-en-3-one (**3**), lupeol (**4**), Ψ-taraxasterone (**5**), hopenyl-3*β*-*O*-palmitate (**6**) and *β*-amyrin palmitate (**7**) exhibited 31.4, 61.2, 62.8, 98.4, 115.4, 68.2, and 60.8 µg/mL, respectively. The isolated compounds (**1-7**) exhibited the mild to moderate antitrypanosomal activity against *T. evansi*. Among of the isolated compounds, resveratrol (**1**) showed the highest activity against *T. evansi*. It is likely that the isolated compounds (**1-7**) might be good leads for treatment against to kill *T. evansi*. In conclusion, medicinal plants used in Myanmar may offer a potential use to prevent and treat disease caused by *T. evansi*.



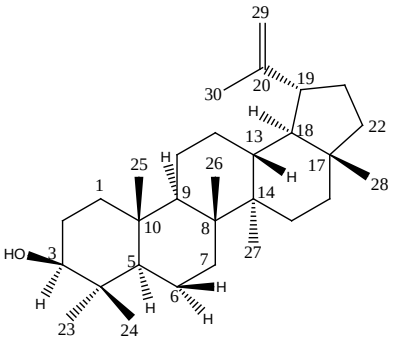
1, 8 (*E*-resveratrol)



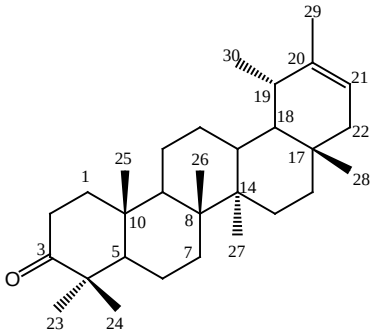
2 (11-*O*-acetyl bergenin)



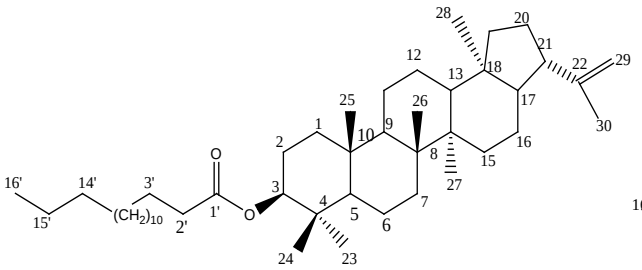
3 (stigmast-4-en-3-one)



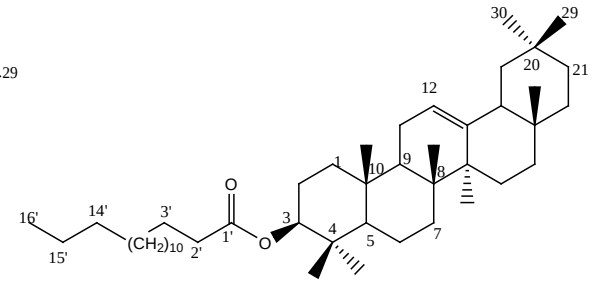
4 (lupeol)



5 (Ψ-taraxasterone)



6 (hopenyl-3*β*-*O*- palmitate)



7 (*β*-amyrin palmitate)

Fig. 25. Antitrypanosomal compounds (**1-7**) isolated from Myanmar medicinal plants.

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MelCy (Cymelarsan) in domestic animals infected with stocks of *Trypanosoma brucei brucei* and *Trypanosoma evansi*. *Tropical Medicine and Parasitology*, 43, 226-228.

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Last but not the least a sincere thanks to all my friends in Japan for being a family away from home.

LIST OF PUBLICATIONS

Publication in Refereed Journal

1. Nyunt K. S., Elkhateeb A., Tosa Y., Nabeta K., Katakura K., Matsuura H., 2012. Isolation of antitrypanosomal compounds from *Vitis repens*, a medicinal plant of Myanmar. *Natural Product Communications*, 7, 609-610.

Conferences

(Oral defense)

1. Nyunt K. S., Elkhateeb A., Tosa Y., Nabeta K., Katakura K., Matsuura H., March, 2012. Isolation of antitrypanosomal compounds from Myanmar medicinal plants. *Presentation in the Conference of Japan Society of Bioscience, Biotechnology and Agrochemistry*. Kyoto, Japan (ID No. 2A04a14).
2. Nyunt K. S., Elkhateeb A., Phay N., Takahashi K., Hara K., Miyazaki H., Katakura K., Matsuura H., March, 2014. Studies on antitrypanosomal activity of Myanmar medicinal plants. *Presentation in the Conference of Japan Society of Bioscience, Biotechnology and Agrochemistry*. Tokyo, Japan (ID No. 3A06p20).

(Poster section)

1. Nyunt K. S., Elkhateeb A., Phay N., Takahashi K., Hara K., Miyazaki H., Katakura K., Matsuura H., August, 2013. Studies on antitrypanosomal activity of Myanmar medicinal plants. *Presentation in the Conference of Japan Society of Bioscience, Biotechnology and Agrochemistry*. Asahikawa, Hokkaido (Poster Section).

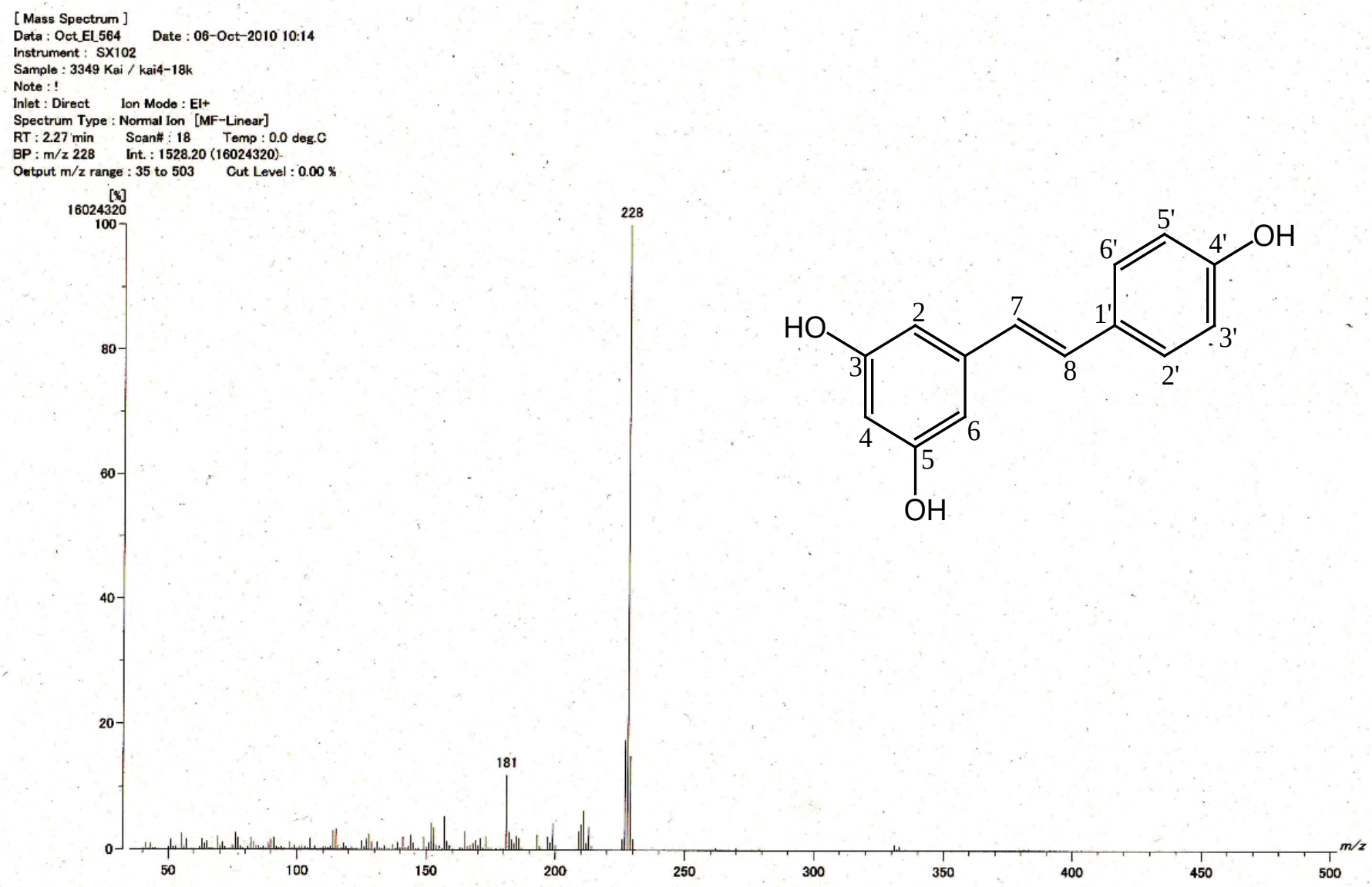
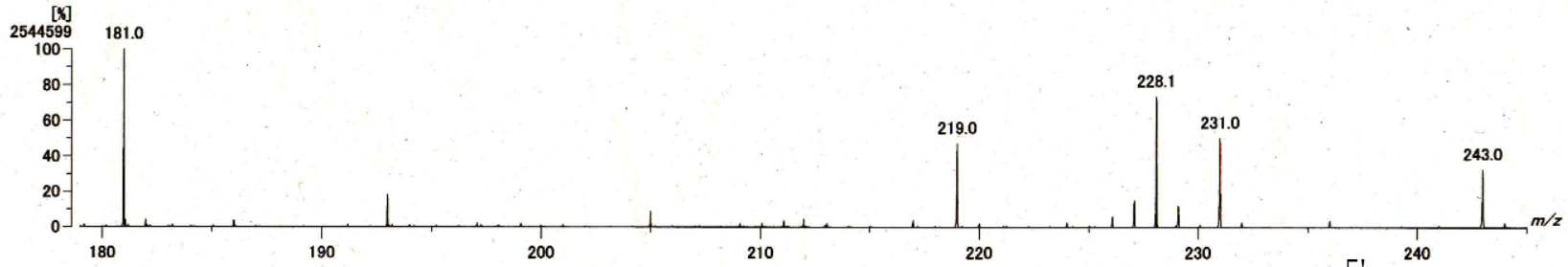


Chart 1. EI-MS spectrum of compound **1**.

[Mass Spectrum]
Data : Oct_EI_567 Date : 06-Oct-2010 10:39
Sample : 3349 Kai / Kai4-18k
Note : SX102A @ GC-MS Lab. Fac. Agric. Hokkaido Univ.
RT : 1.00 min Scan# : 5
Elements : C 50/0, H 80/0, N 4/0, O 10/0, Si 3/0
Mass Tolerance : 20ppm, 5mmu if m/z < 250, 10mmu if m/z > 500
Unsaturation (U.S.) : -0.5 – 20.0



	Observed m/z	Int%	Err[ppm / mmu]	U.S.	Composition
1	228.0788	73.53	-11.1 / -2.5	13.5	C17 H10 N
2			+6.6 / +1.5	9.5	C12 H10 N3 O2
3			+0.7 / +0.2	9.0	C14 H12 O3
4			+18.3 / +4.2	5.0	C9 H12 N2 O5
5			-19.2 / -4.4	0.5	C5 H14 N3 O7
6			-19.0 / -4.3	9.0	C11 H12 N4 Si
7			-7.2 / -1.6	4.5	C8 H14 N3 O3 Si
8			-13.1 / -3.0	4.0	C10 H16 O4 Si
9			+4.5 / +1.0	0.0	C5 H16 N2 O6 Si
10			-1.1 / -0.3	8.0	C13 H16 Si2
11			+16.5 / +3.8	4.0	C8 H16 N2 O2 Si2
12			-21.0 / -4.8	-0.5	C4 H18 N3 O4 Si2
13			-9.0 / -2.1	3.5	C7 H18 N3 Si3
14			-14.9 / -3.4	3.0	C9 H20 O Si3

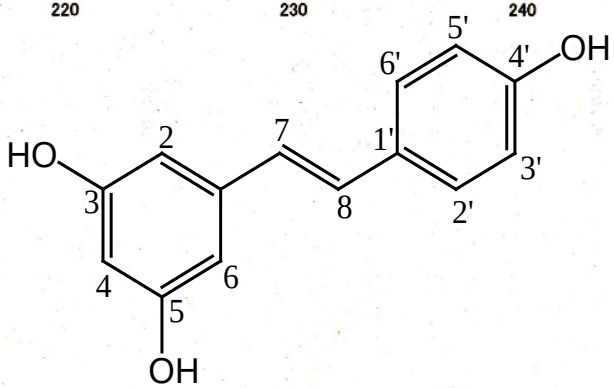


Chart 2. HR-EI-MS spectrum of compound **1**.

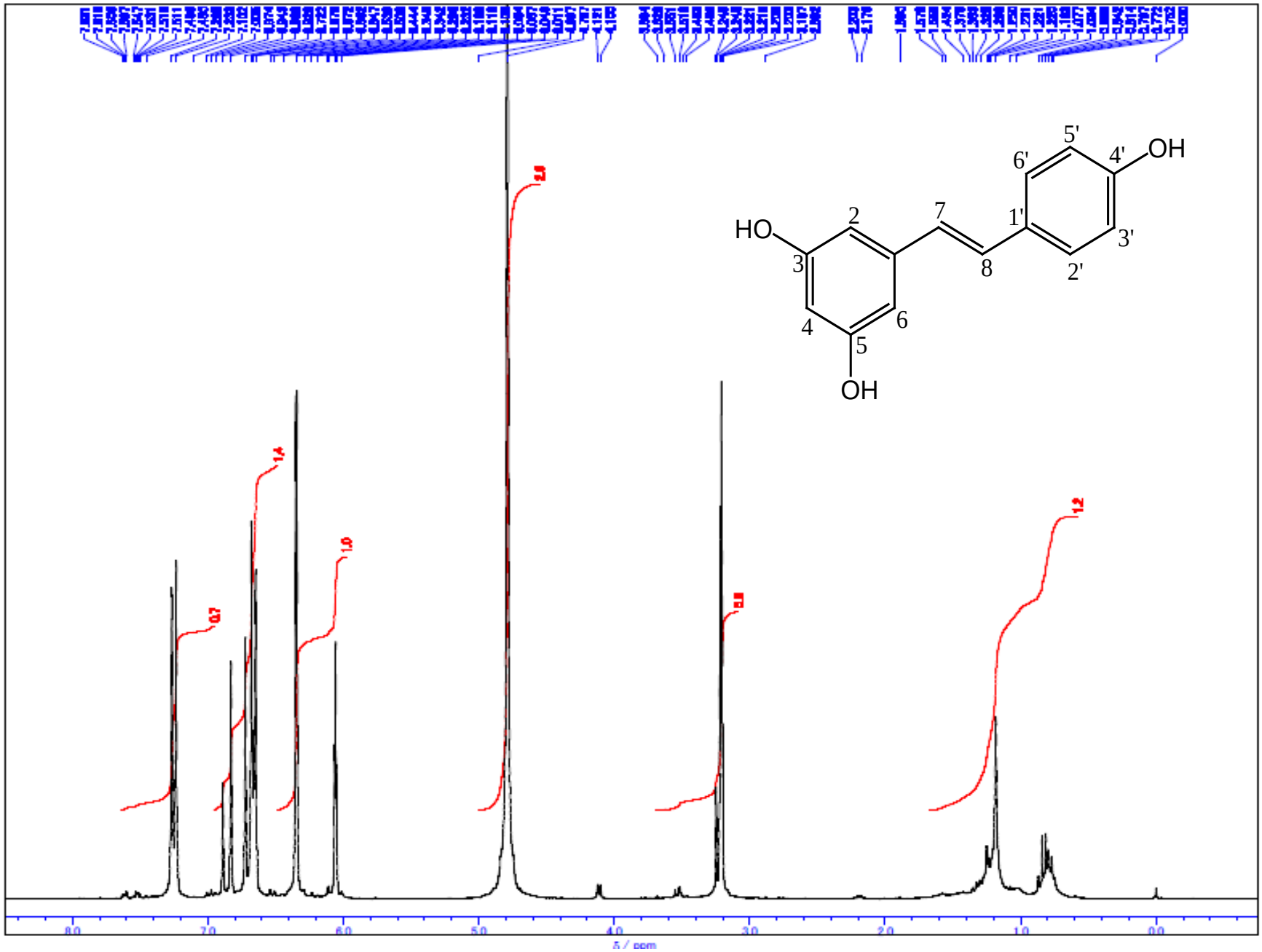


Chart 3. ^{1}H NMR spectrum of compound **1** (270 MHz, CD_3OD).

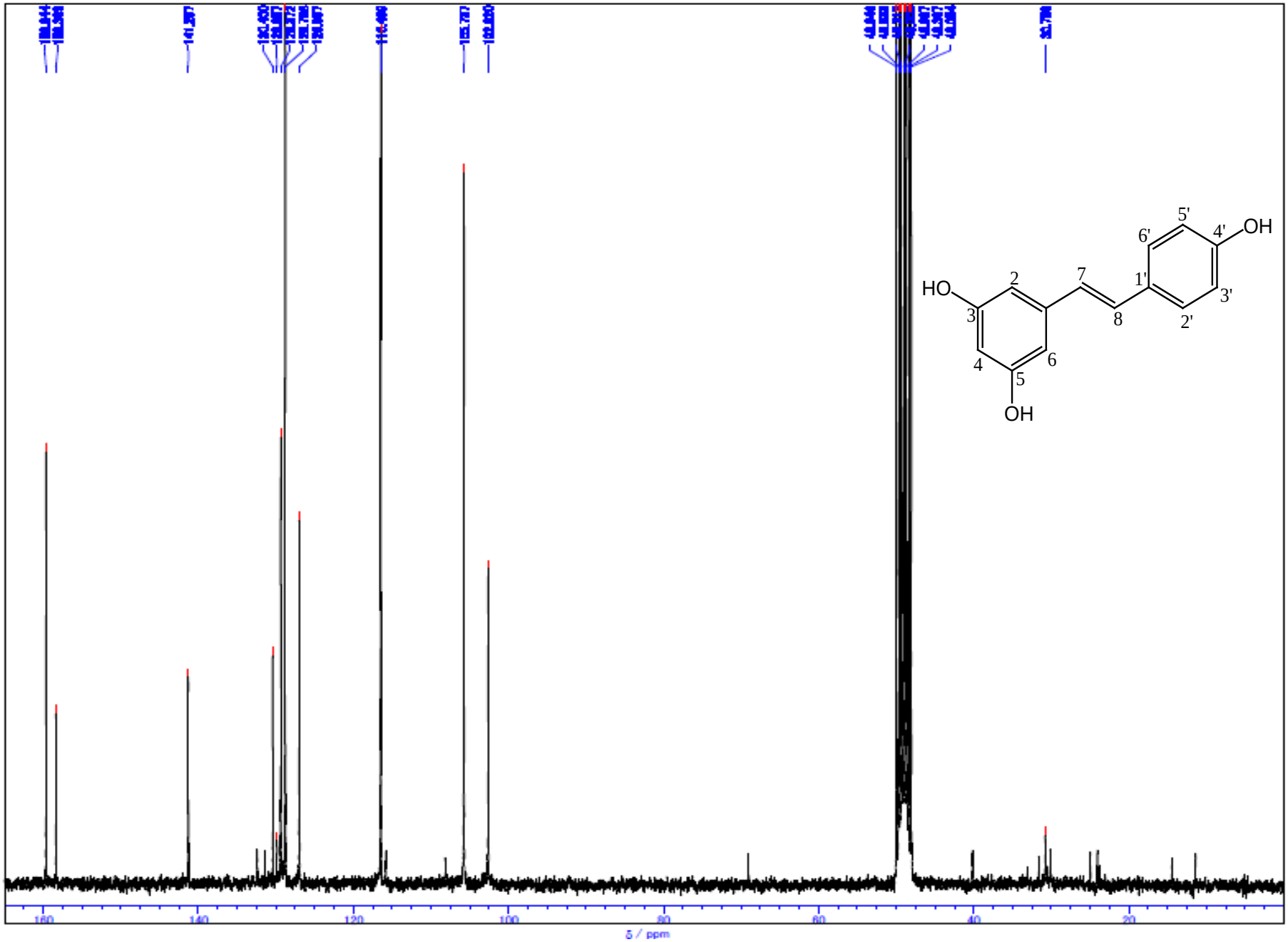


Chart 4. ^{13}C NMR spectrum of compound **1** (270 MHz, CD_3OD).

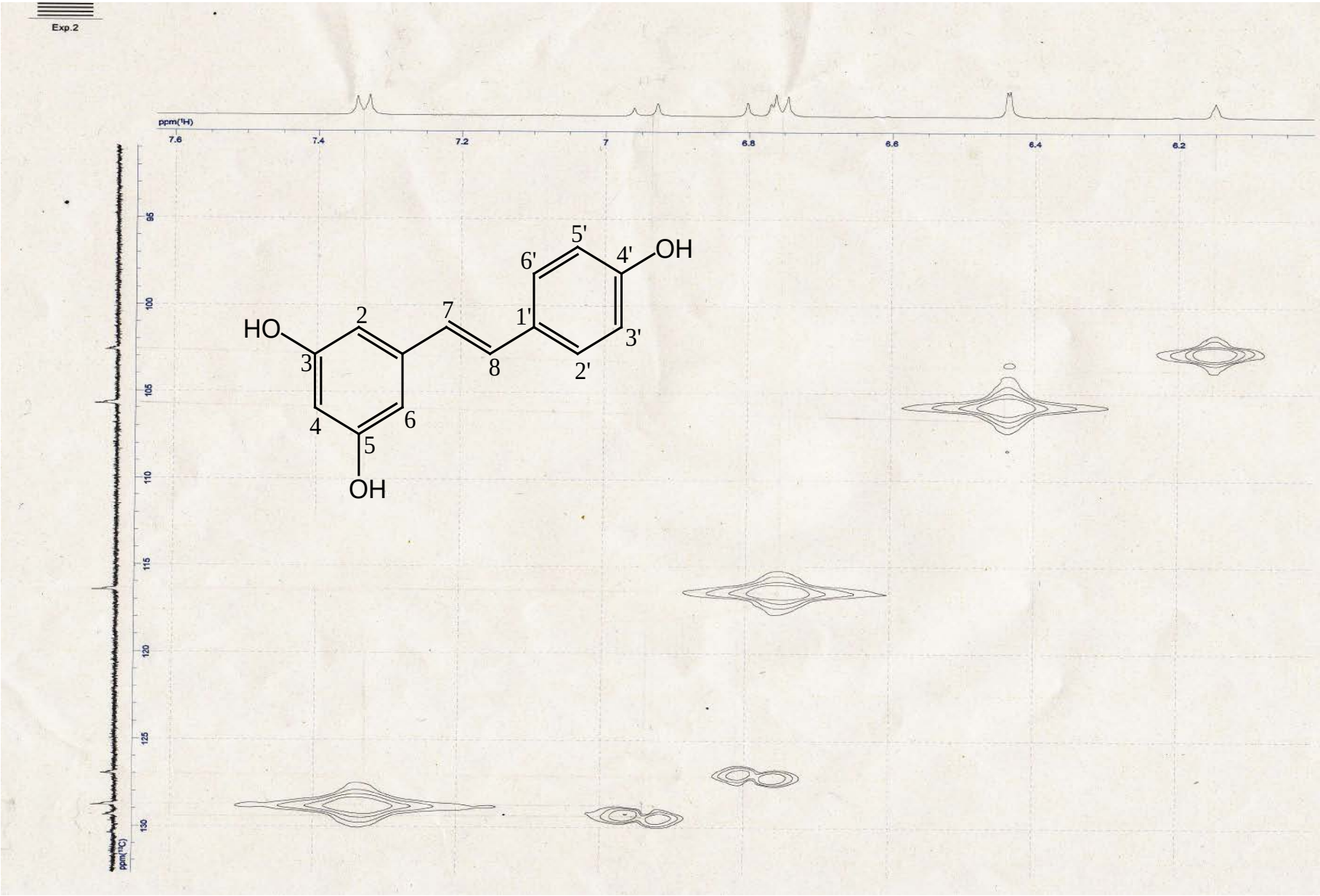


Chart 5. HMQC spectrum of compound **1** (270 MHz, CD_3OD).

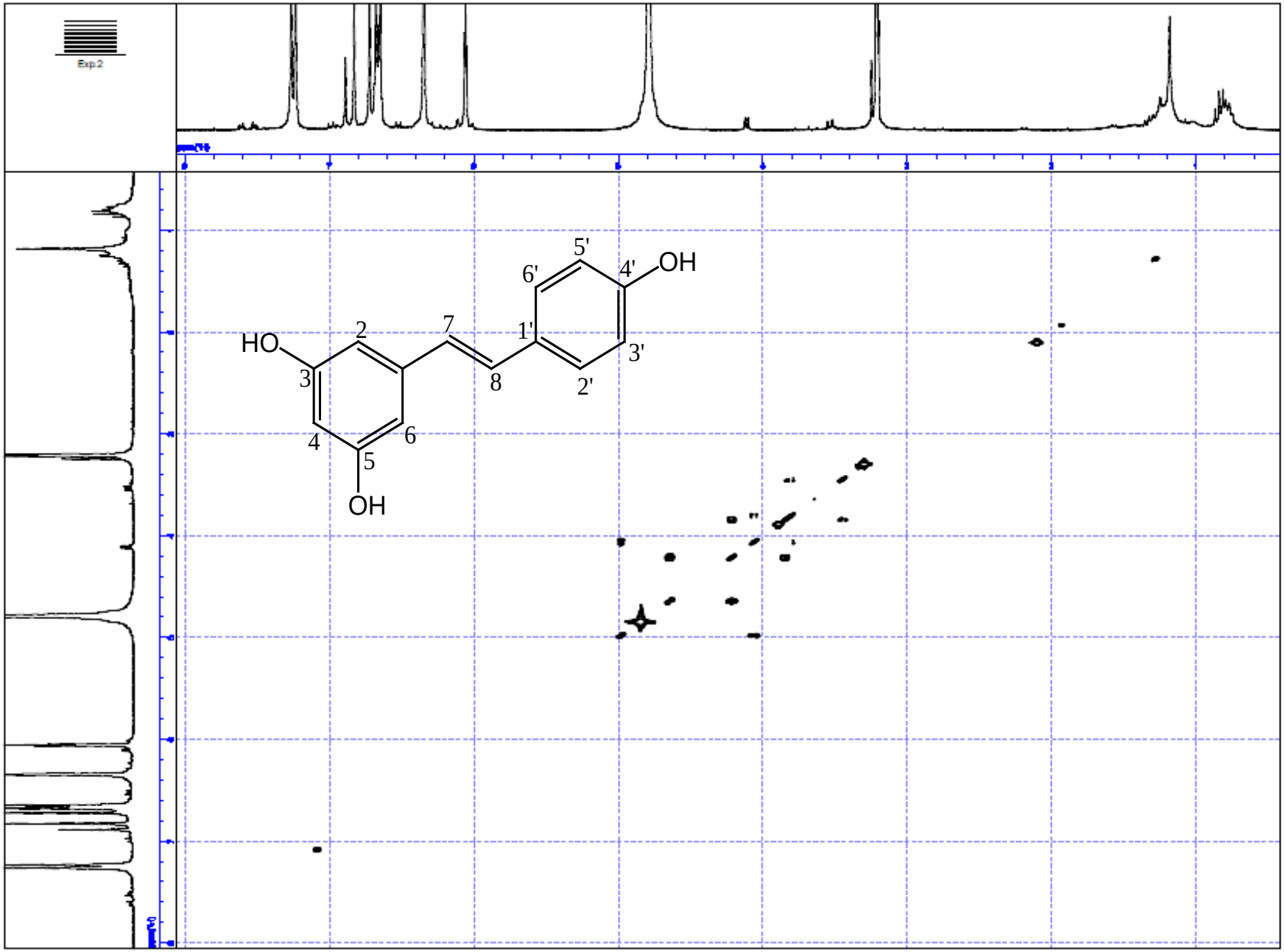


Chart 6. COSY spectrum of compound **1** (270 MHz, CD_3OD).

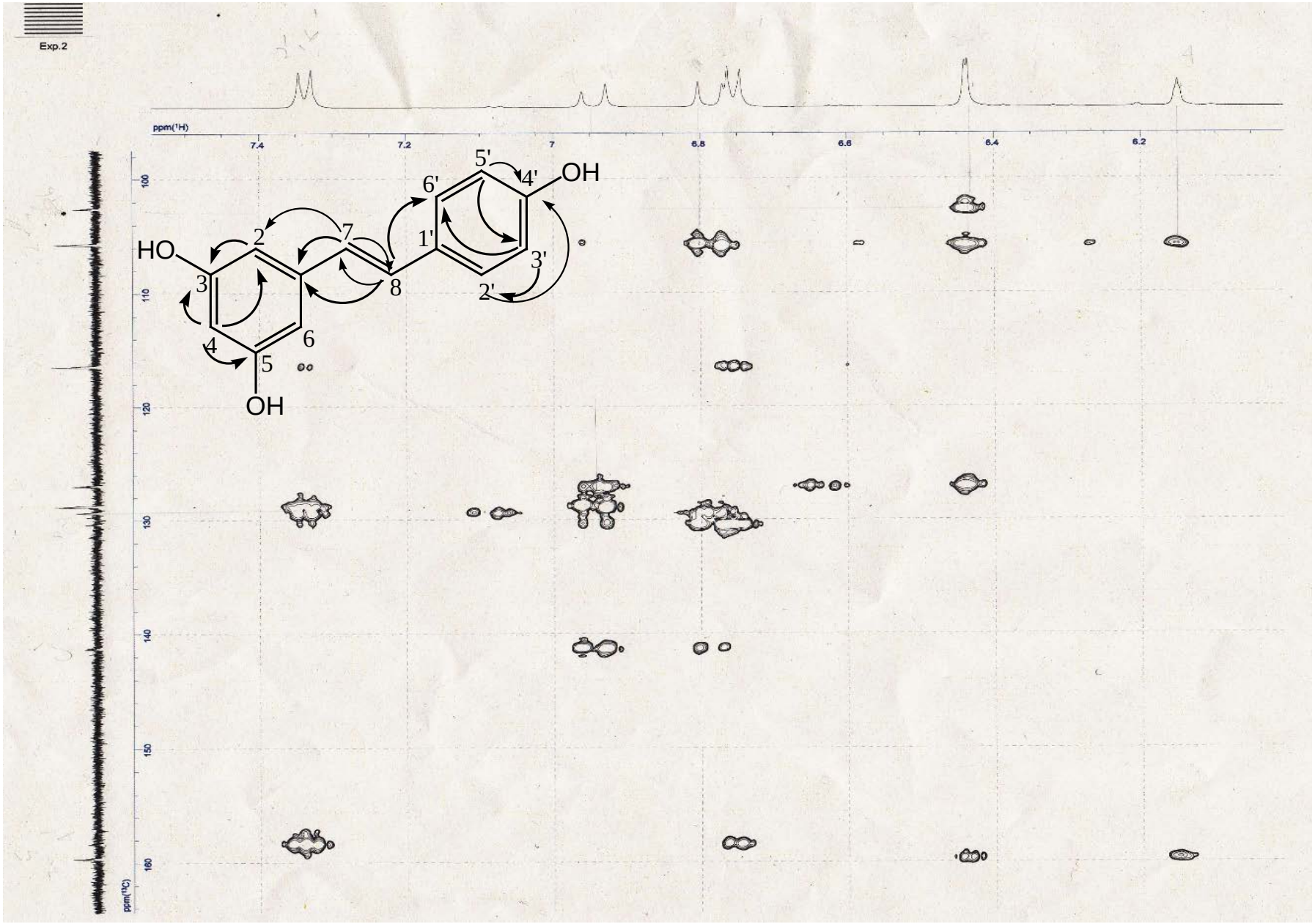


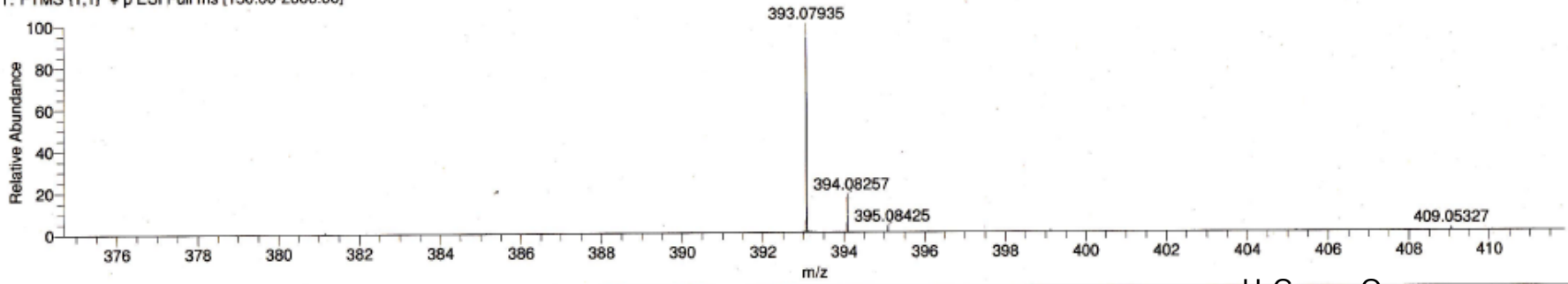
Chart 7. HMBC spectrum of compound **1** (270 MHz, CD_3OD).

Sample No. : C:\Xcalibur\...\0425\230177_C_3_3_H_pn
Operator name : tokumitsu ai
Date : 4/25/2011 9:55:25 AM
Instrumental method : C:\Xcalibur\methods\HESI_100ul\pn_H80_S30.meth
Instrumental Analysis Division, Equipment Management Center, Creative Research Institution Sousei, Hokkaido University

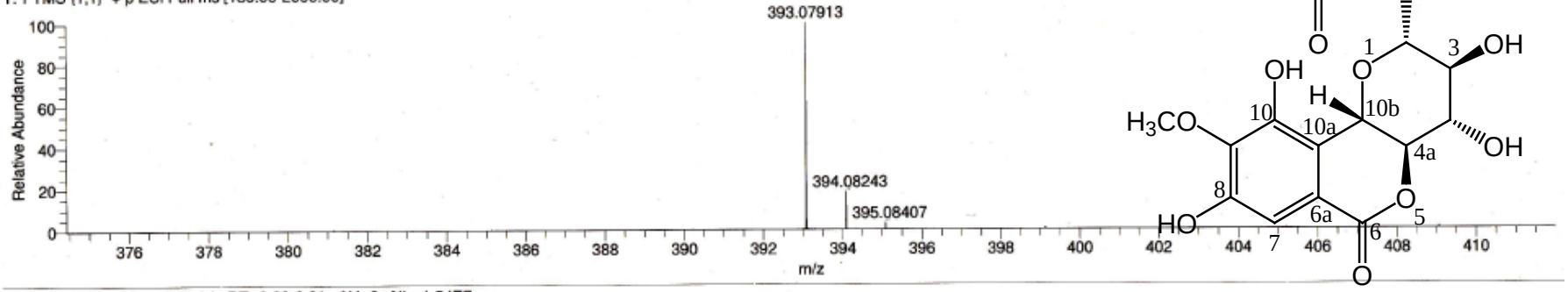
Instrument : Exactive

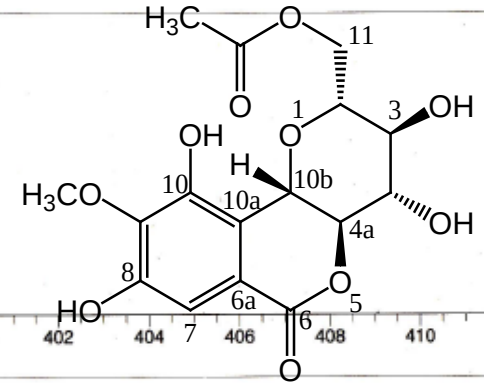
LC solvent : MeOH
Sample solvent : 提出液のまま

230177_C_3_3_H_pn #13-16 RT: 0.19-0.22 AV: 2 NL: 4.86E6
T: FTMS {1,1} + p ESI Full ms [150.00-2000.00]



230177_C_3_3_H_pn #17-21 RT: 0.24-0.28 AV: 3 NL: 1.53E7
T: FTMS {1,1} + p ESI Full ms [150.00-2000.00]





230177_C_3_3_H_pn #21-24 RT: 0.28-0.31 AV: 2 NL: 1.54E7
T: FTMS {1,1} + p ESI Full ms [150.00-2000.00]

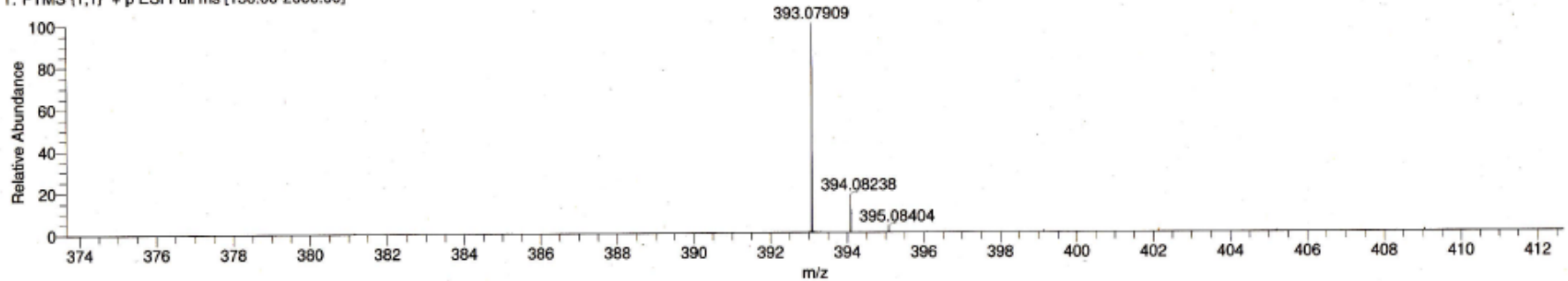


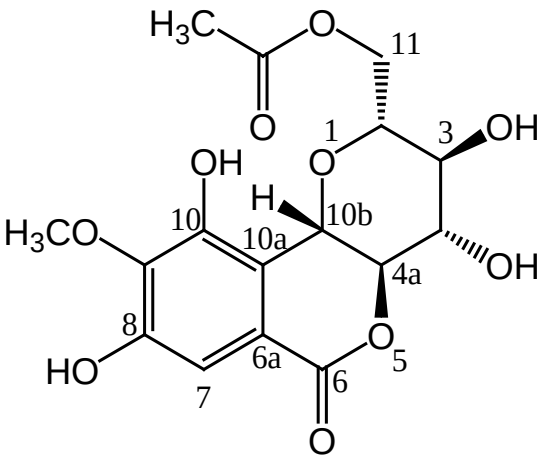
Chart 8. ESI-MS spectrum of compound **2**.

Elemental composition search on mass 393.07913

m/z = 388.07913-398.07913

Isotope	Min	Max
O-16	0	15
C-12	0	70
H-1	0	110
Na-23	0	1
N-14	0	5
P-31	0	2
S-32	0	2

Charge 1
Mass tolerance 5.00 ppm
Nitrogen rule not used
RDB equiv -1.00 - 100.00
max results 50



m/z	Theo. Mass	Delta (ppm)	RDB equiv.	composition
393.07913	393.07912	0.02	14.5	$C_{22}H_{17}O_5S$
	393.07905	0.19	2.5	$C_{11}H_{23}O_4N_4NaPS_2$
	393.07905	0.20	15.0	$C_{20}H_{17}O_2N_3P_2$
	393.07922	-0.22	7.5	$C_{16}H_{18}O_{10}Na$
	393.07925	-0.31	1.5	$C_{11}H_{22}O_{13}P$

Chart 9. HR-EI-MS spectrum of compound **2**.

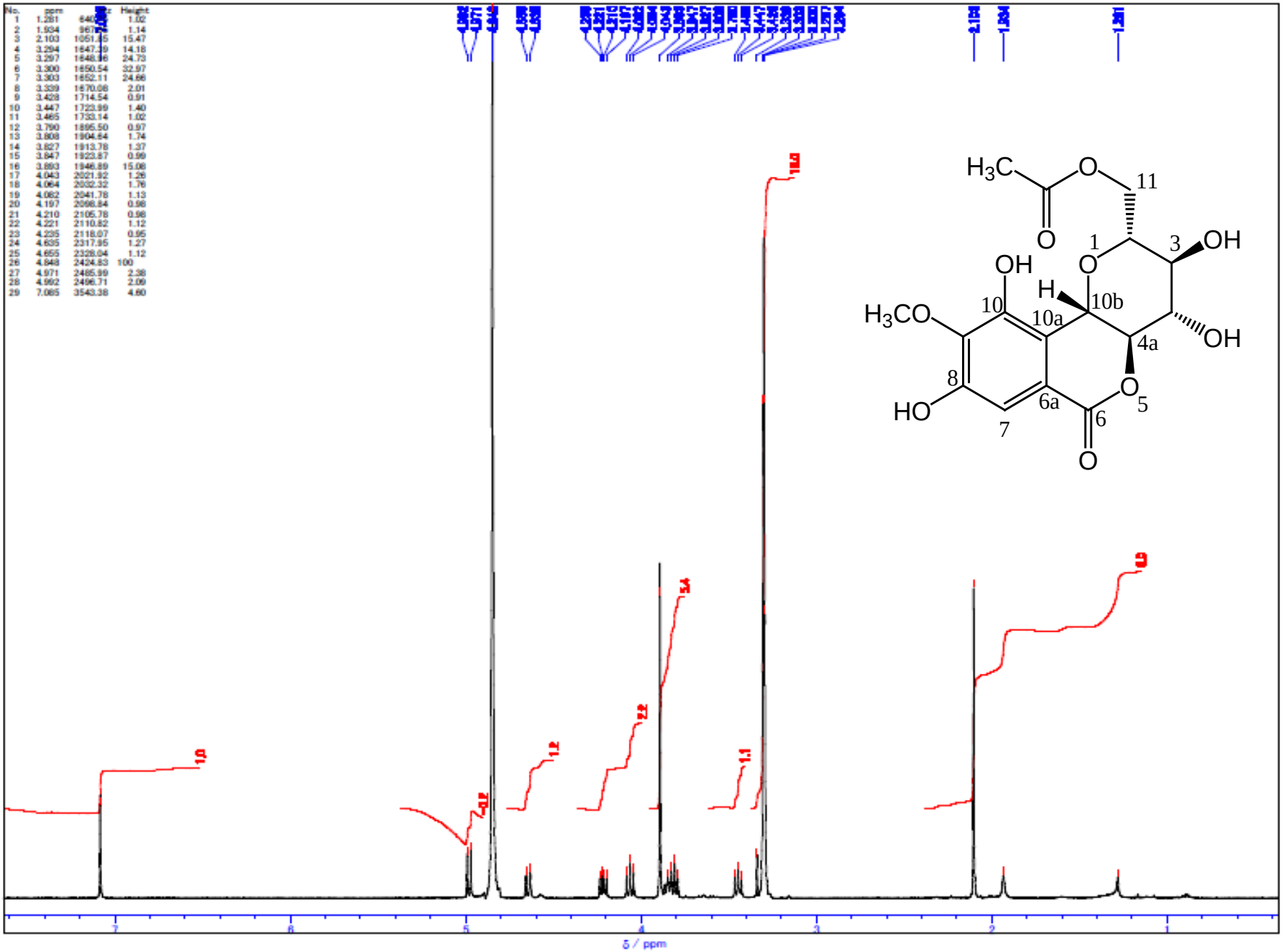


Chart 10. ^{1}H NMR spectrum of compound **2** (270 MHz, CD_3OD).

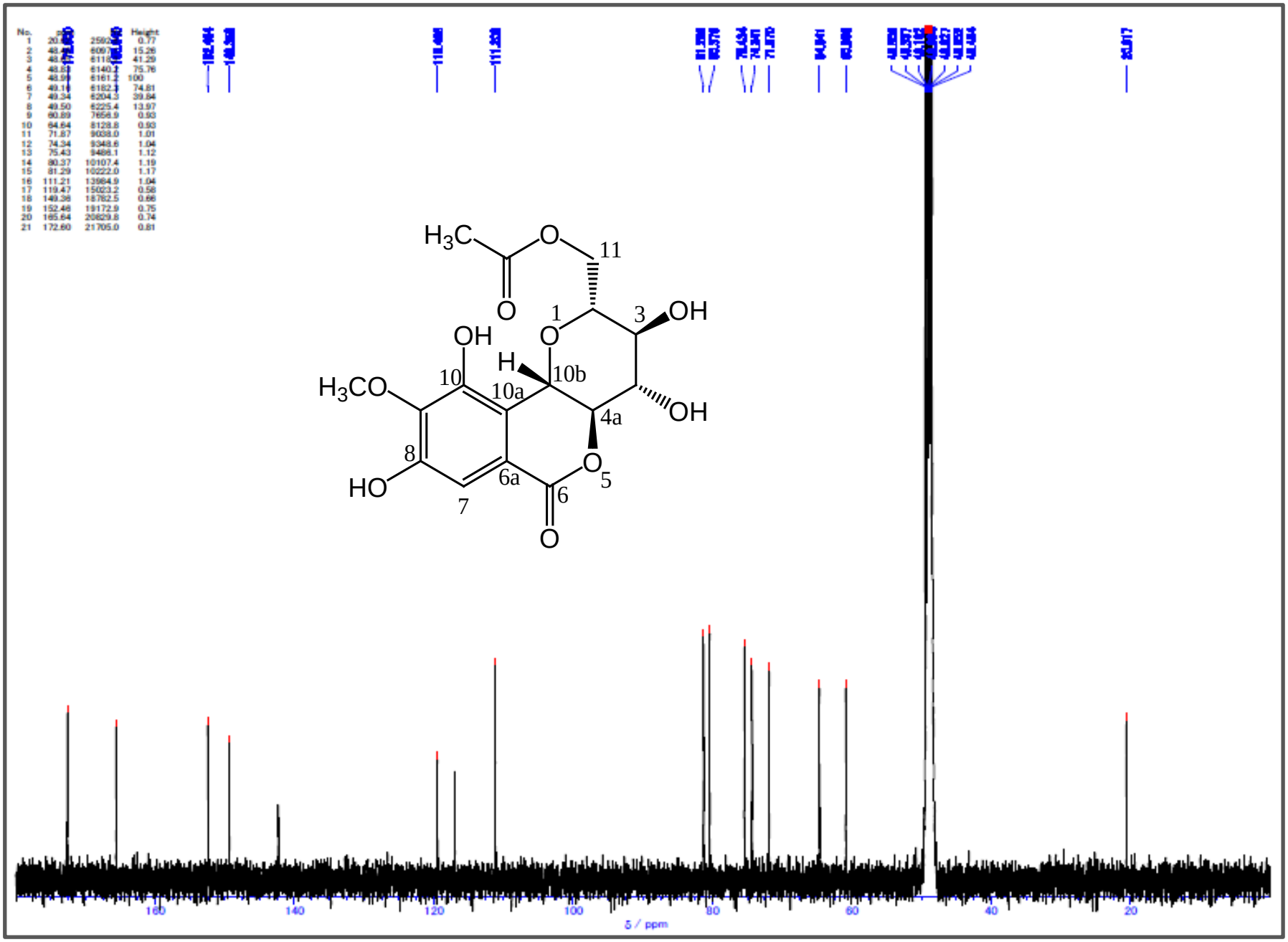


Chart 11. ^{13}C NMR spectrum of compound **2** (125 MHz, CD_3OD).

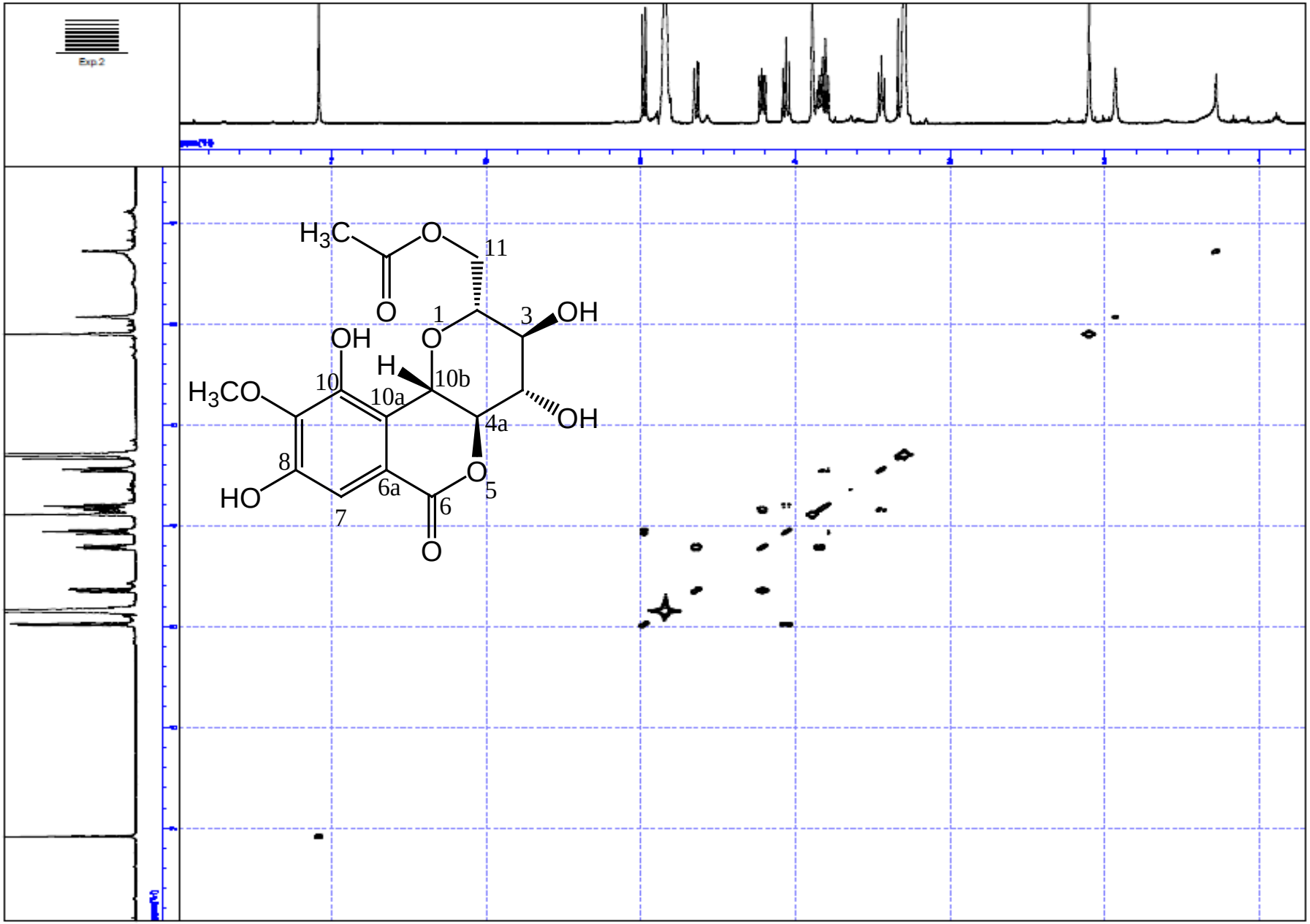


Chart 12. COSY spectrum of compound **2** (270 MHz, CD_3OD).

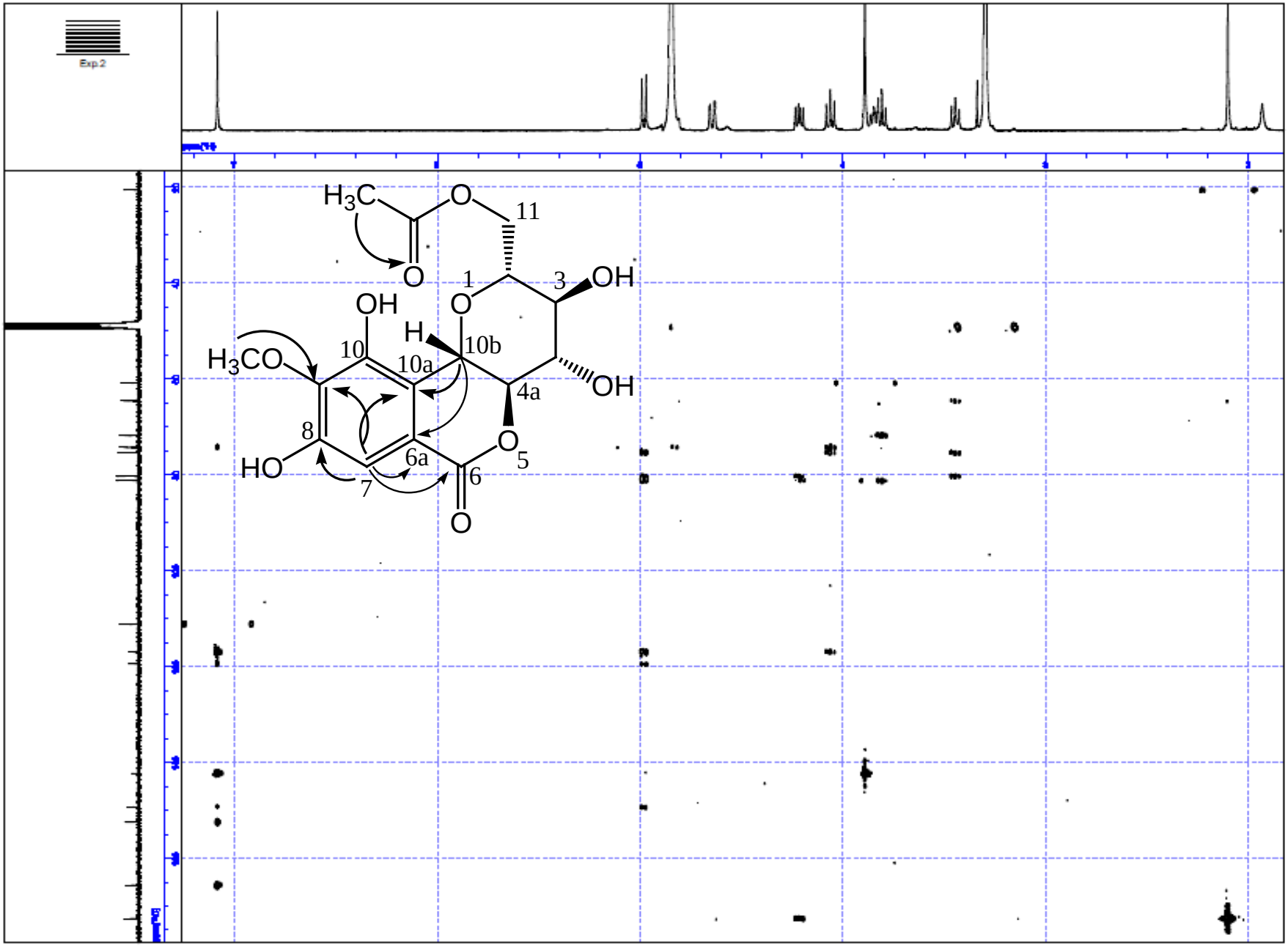


Chart 13. HMBC spectrum of compound **2** (270 MHz, CD_3OD).

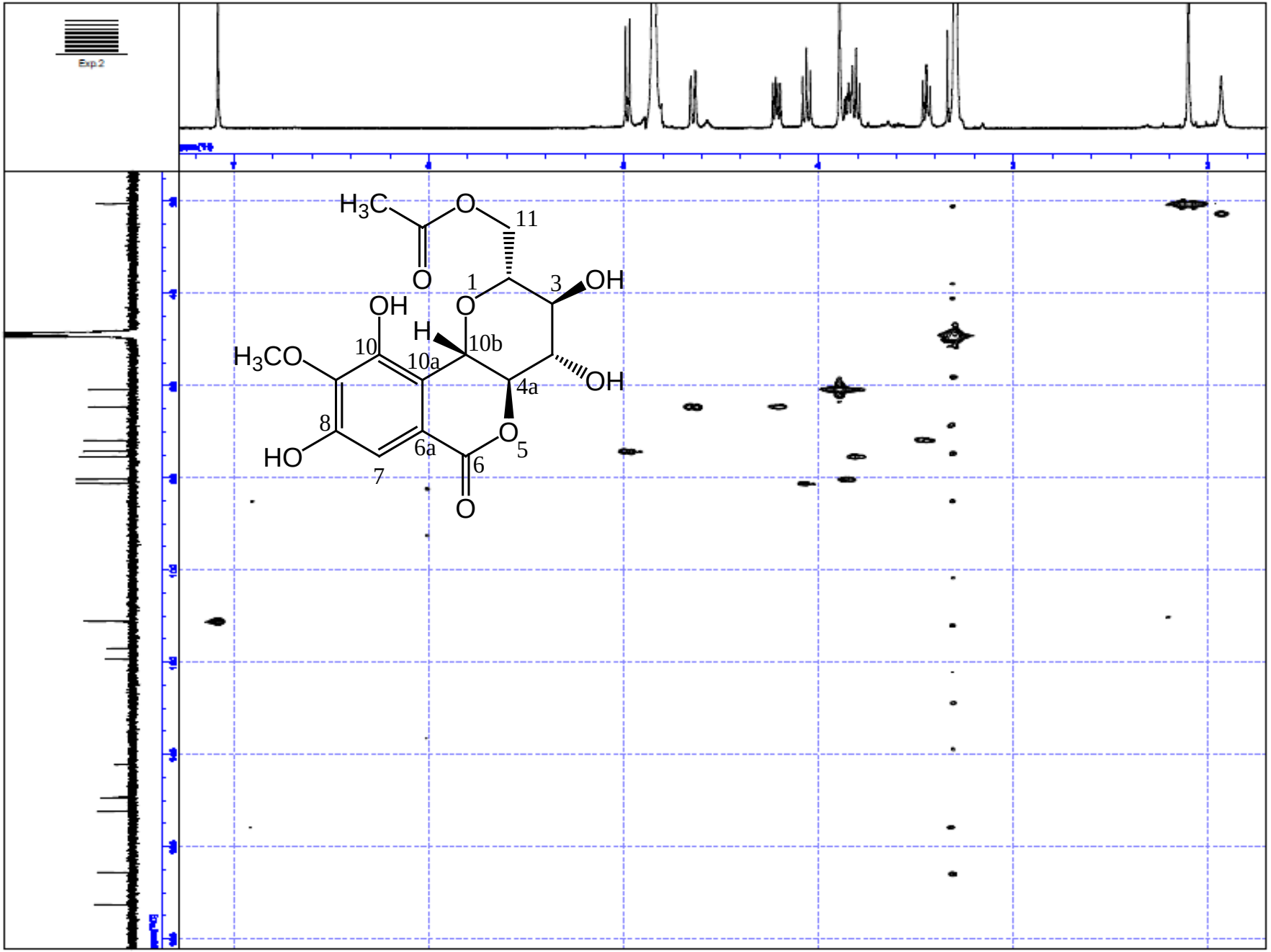


Chart 14. HMQC spectrum of compound **2** (270 MHz, CD_3OD).

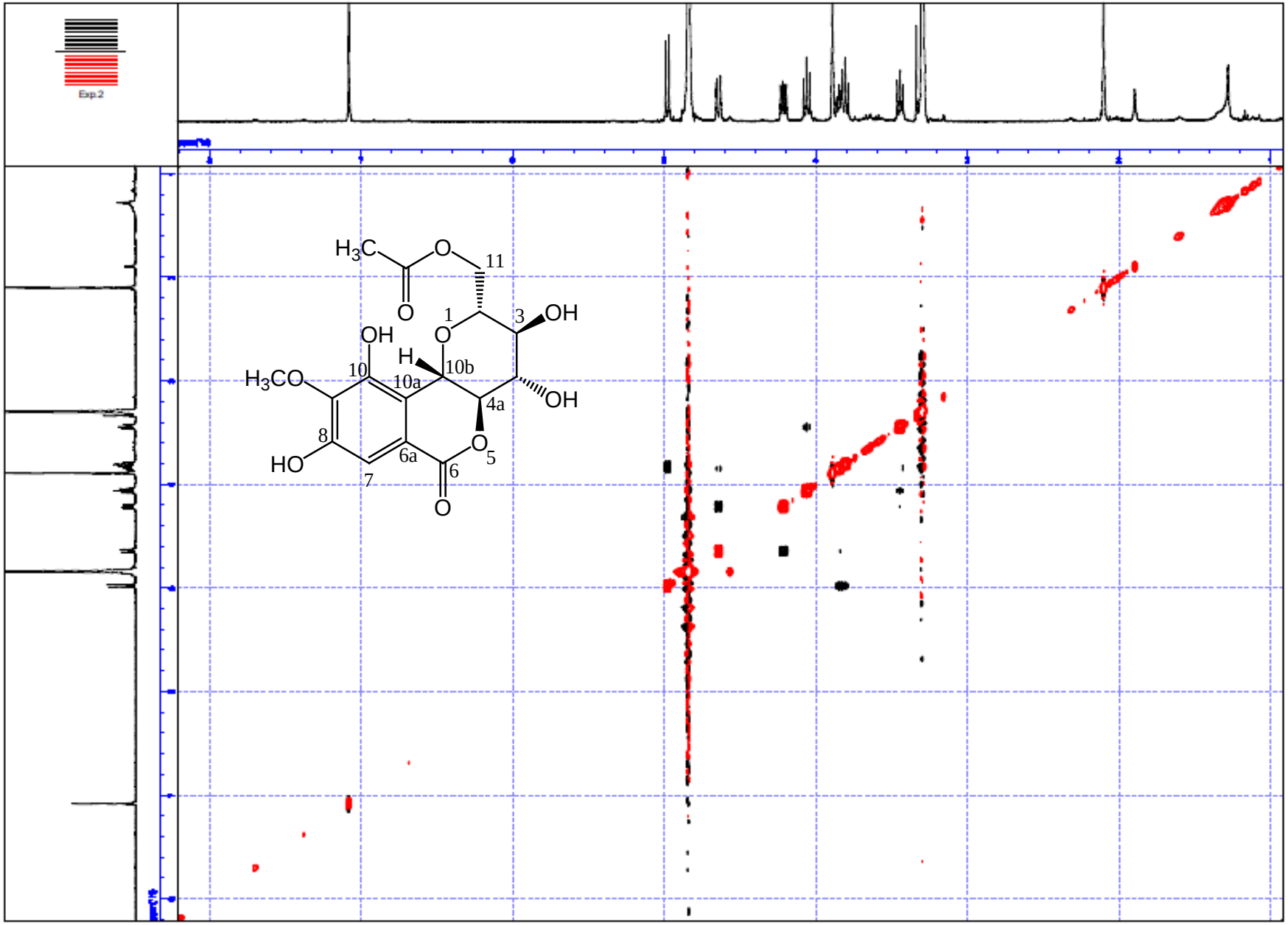


Chart 15. NOESY spectrum of compound **2**(270 MHz, CD_3OD).

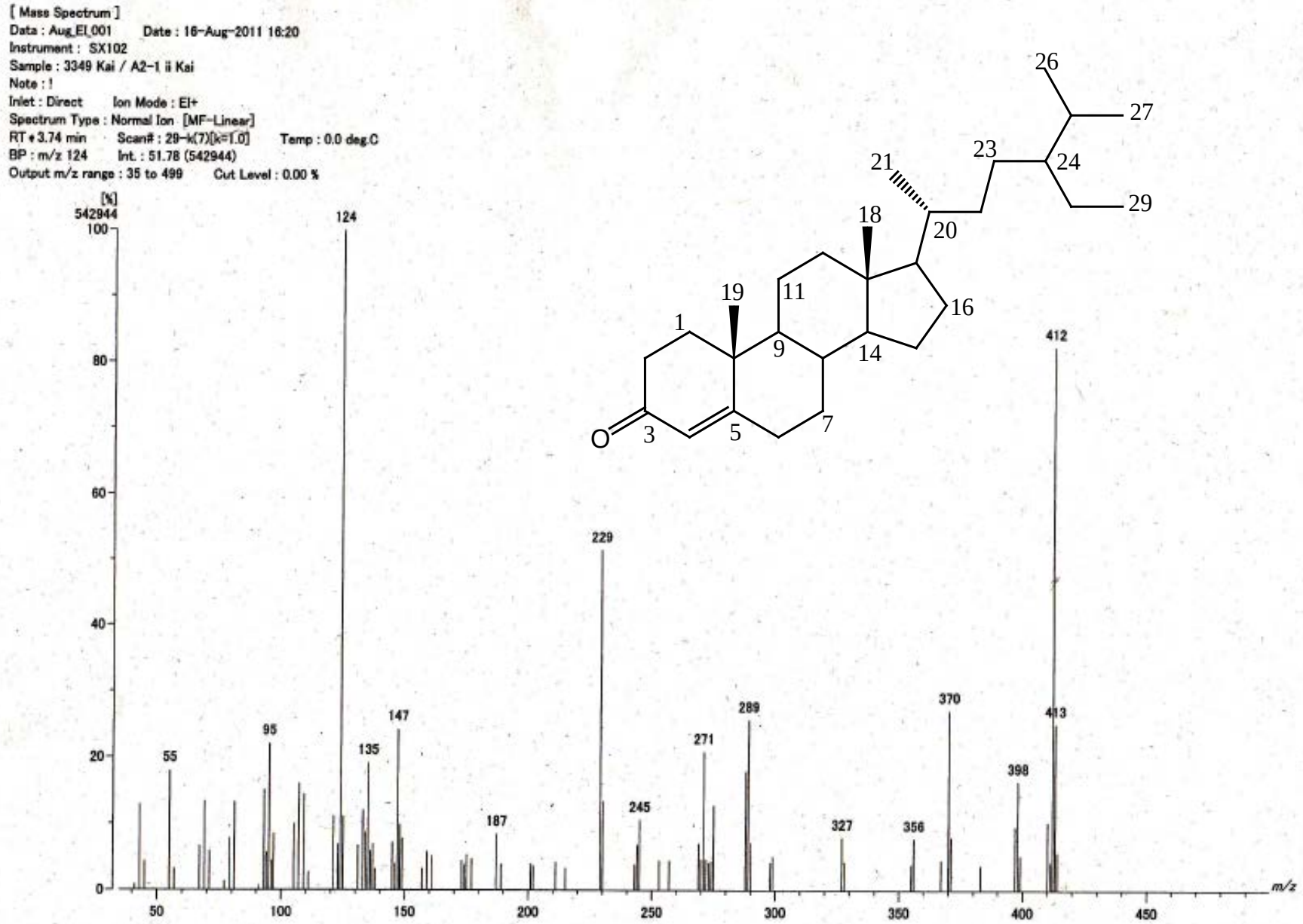


Chart 16. EI-MS spectrum of compound **3**.

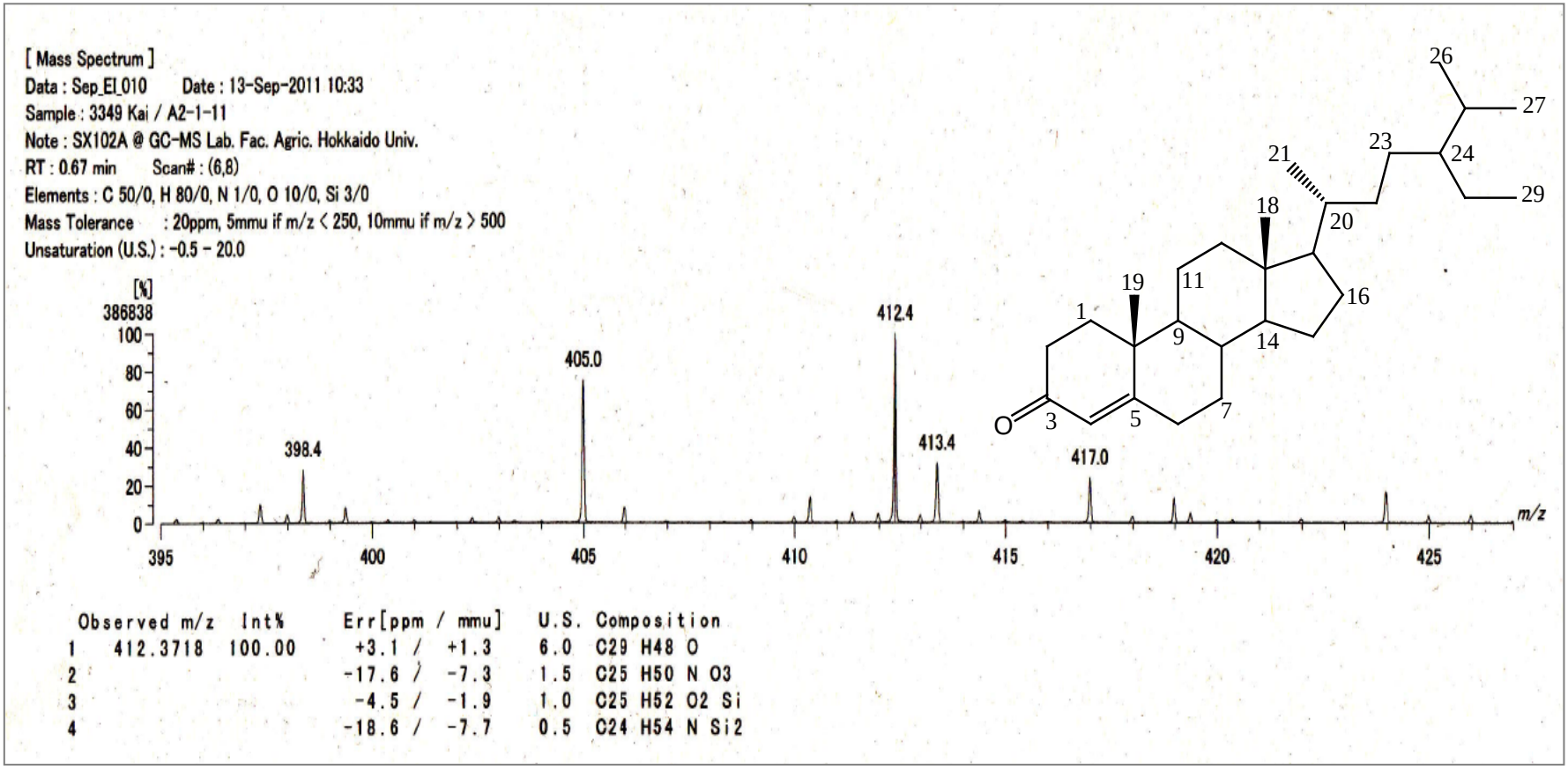


Chart 17. HR-EI-MS spectrum of compound **3**.

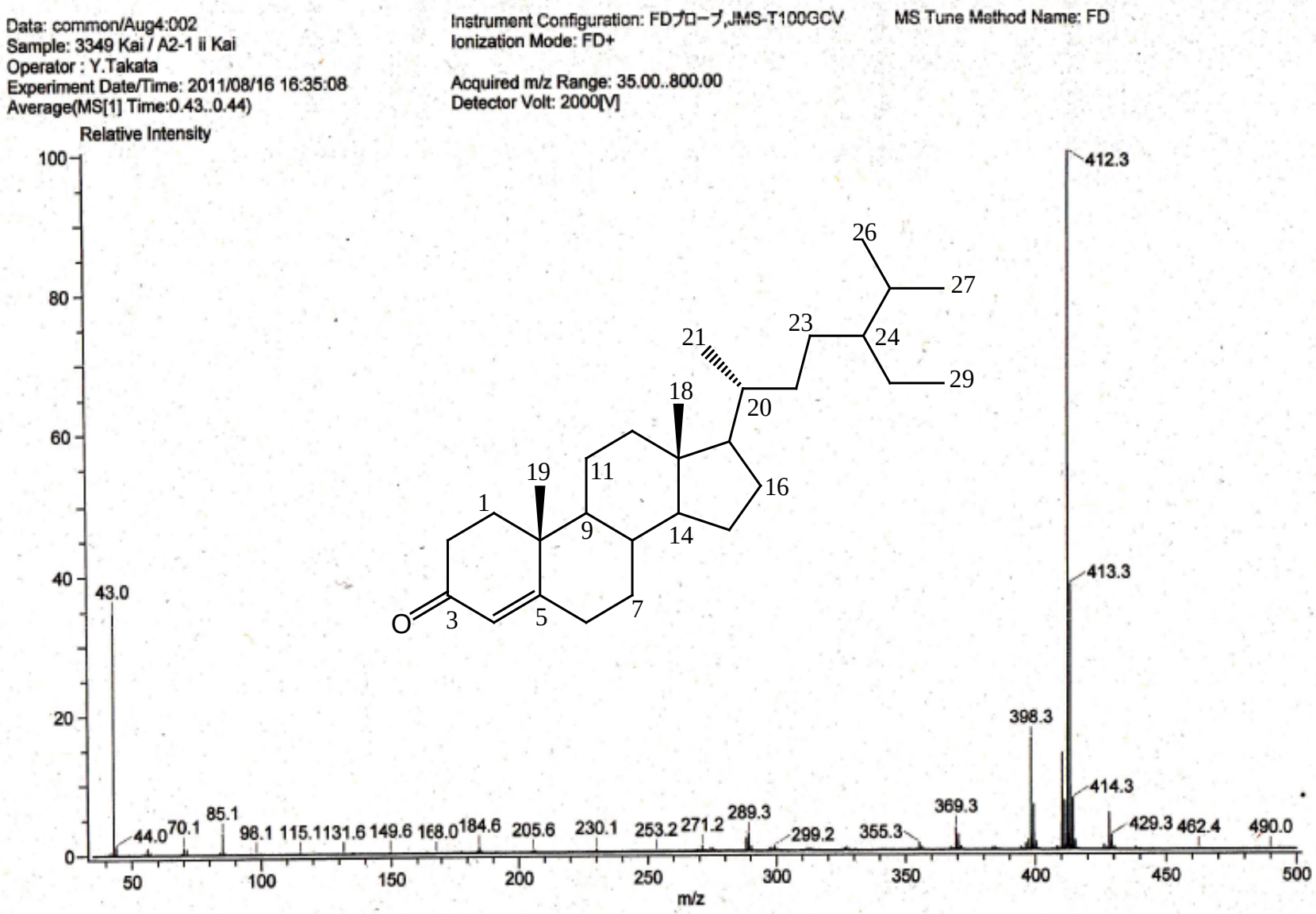


Chart 18. FD-MS spectrum of compound **3**.

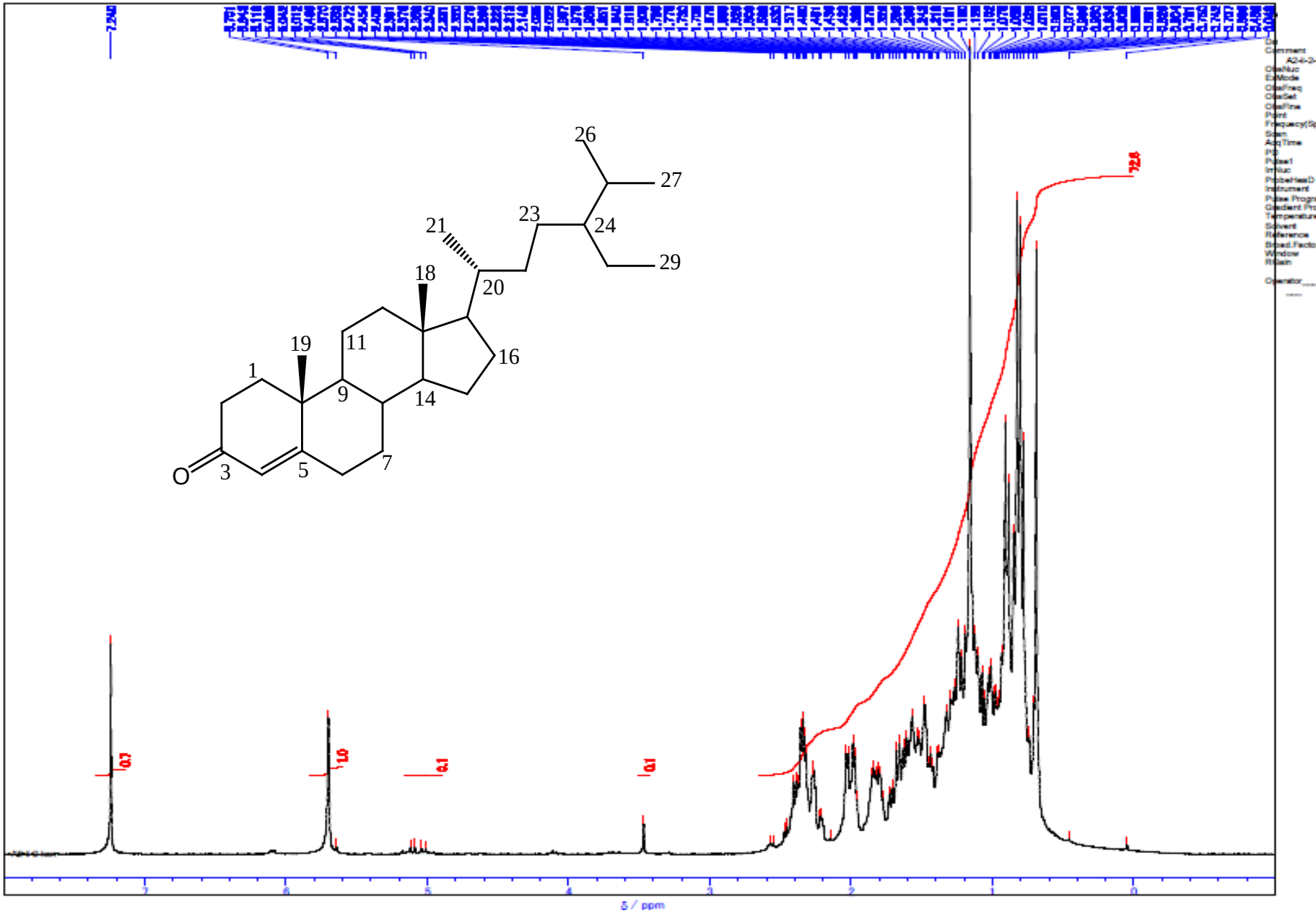


Chart 19. ^{1}H NMR spectrum of compound **3** (270 MHz, $CDCl_3$).

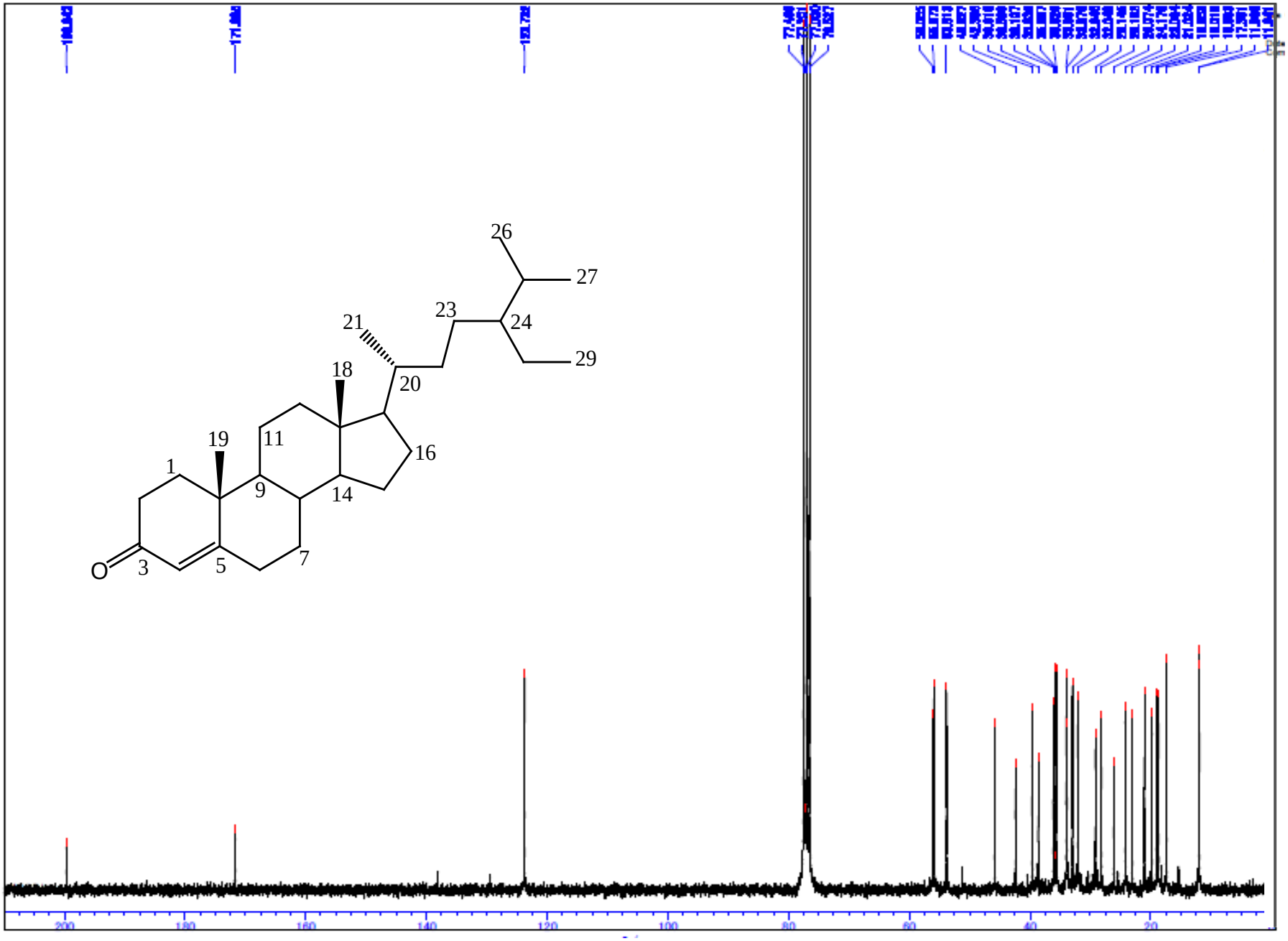


Chart 20. ^{13}C NMR spectrum of compound **3** (270 MHz, $CDCl_3$).

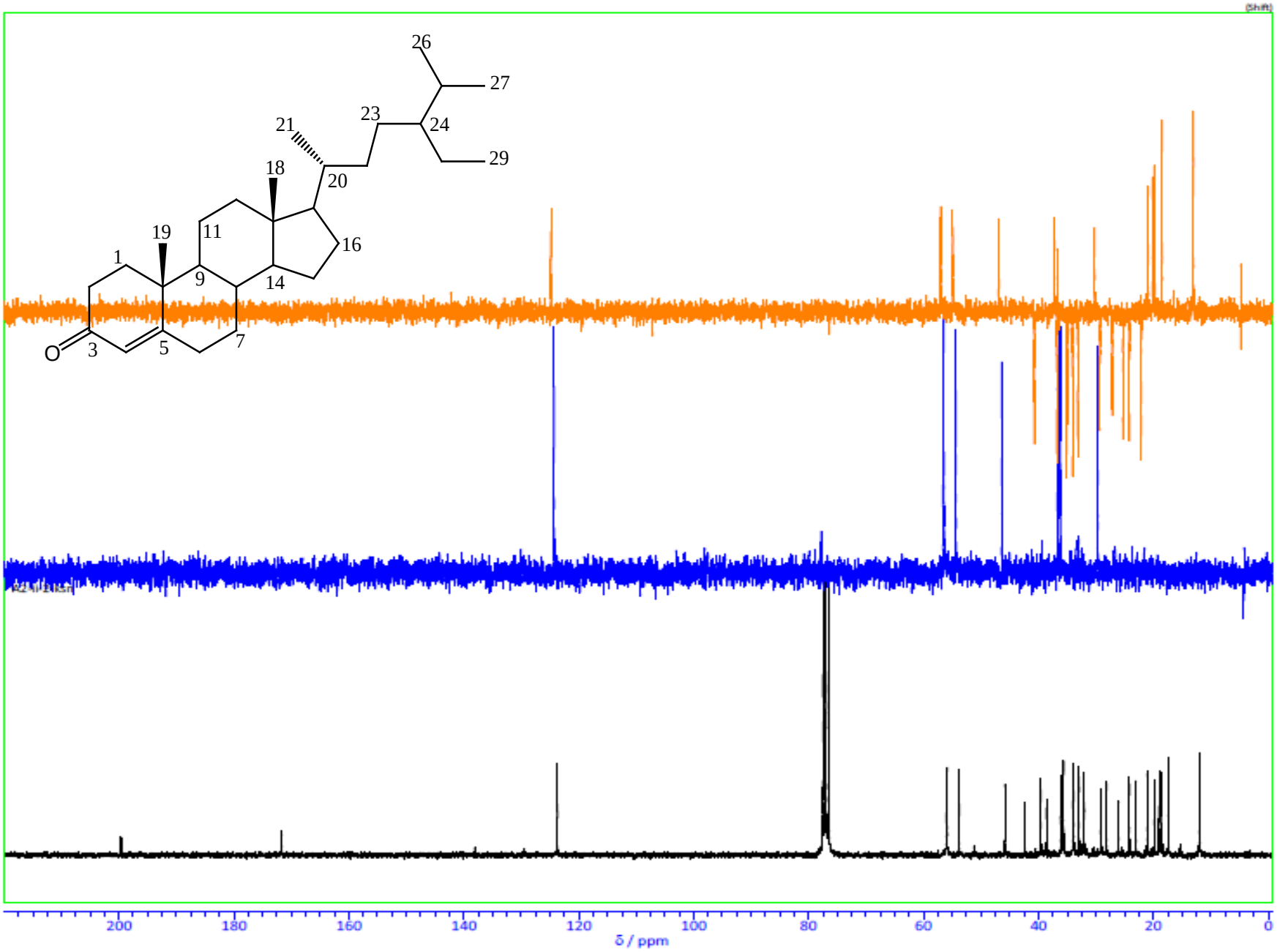


Chart 21. DEPT spectrum of compound **3** (270 MHz, $CDCl_3$).

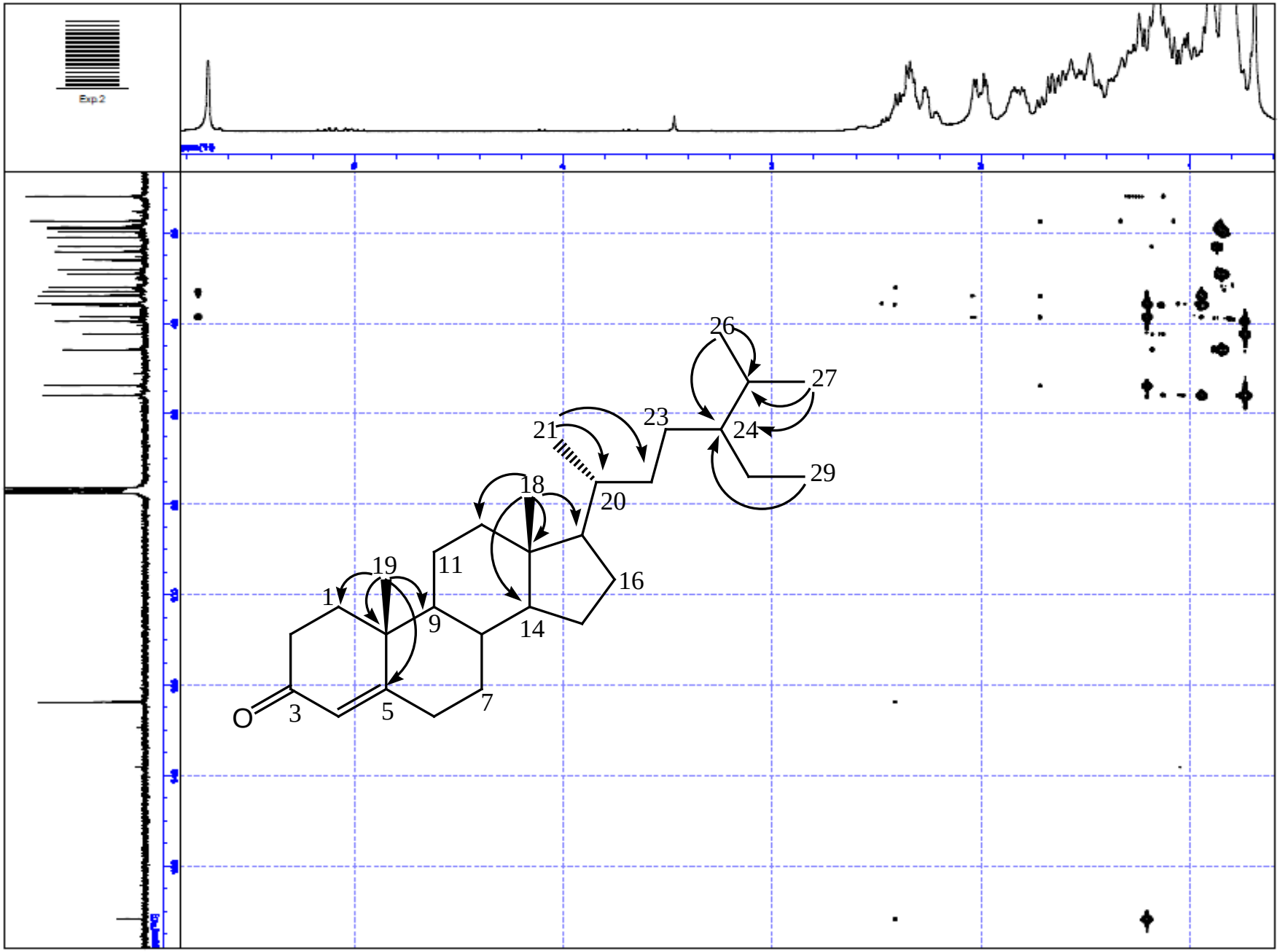


Chart 22. HMBC spectrum of compound **3** (500 MHz, $CDCl_3$).

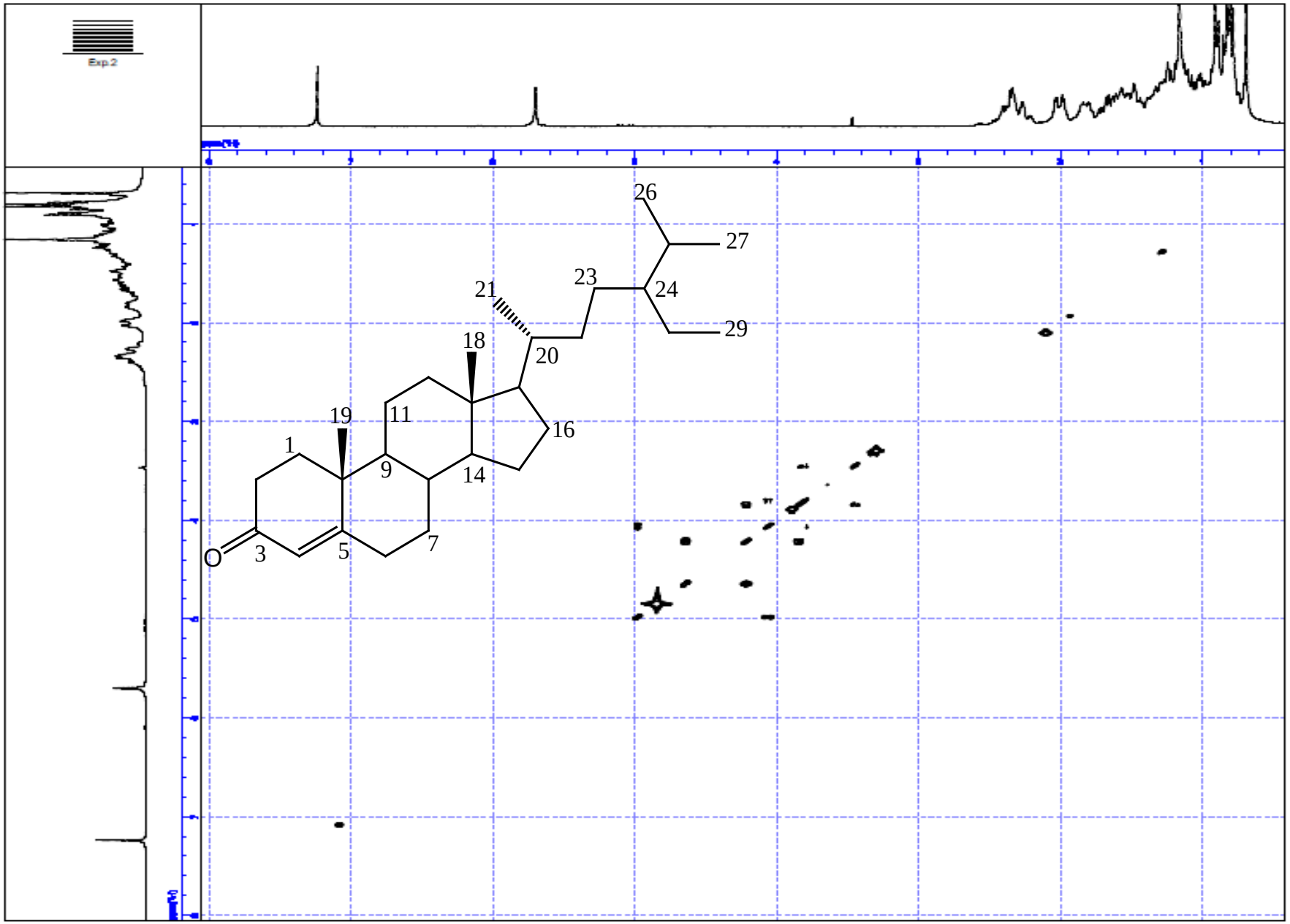


Chart 23. COSY spectrum of compound **3** (500 MHz, $CDCl_3$).

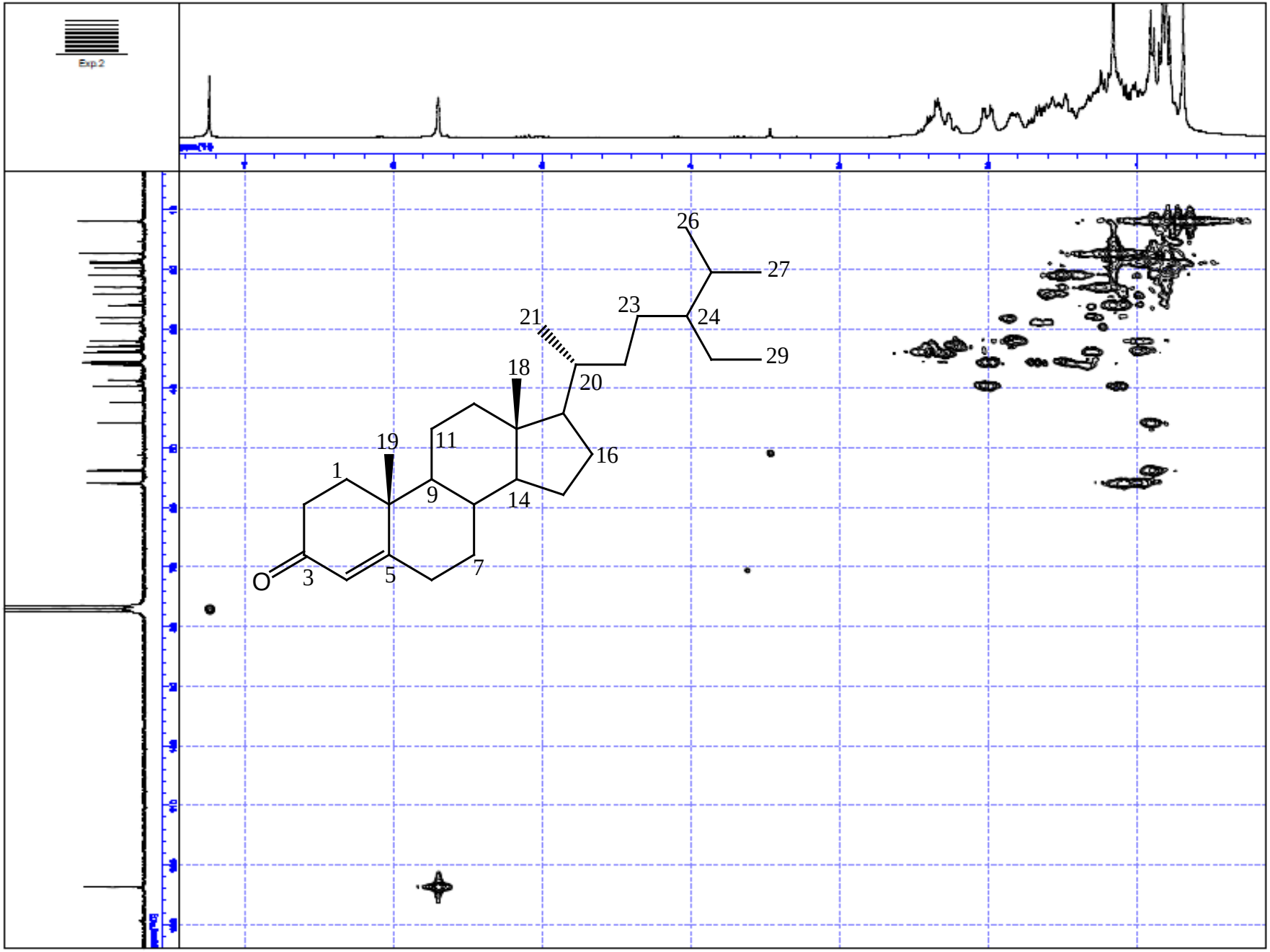


Chart 24. HMQC spectrum of compound **3** (500 MHz, $CDCl_3$).

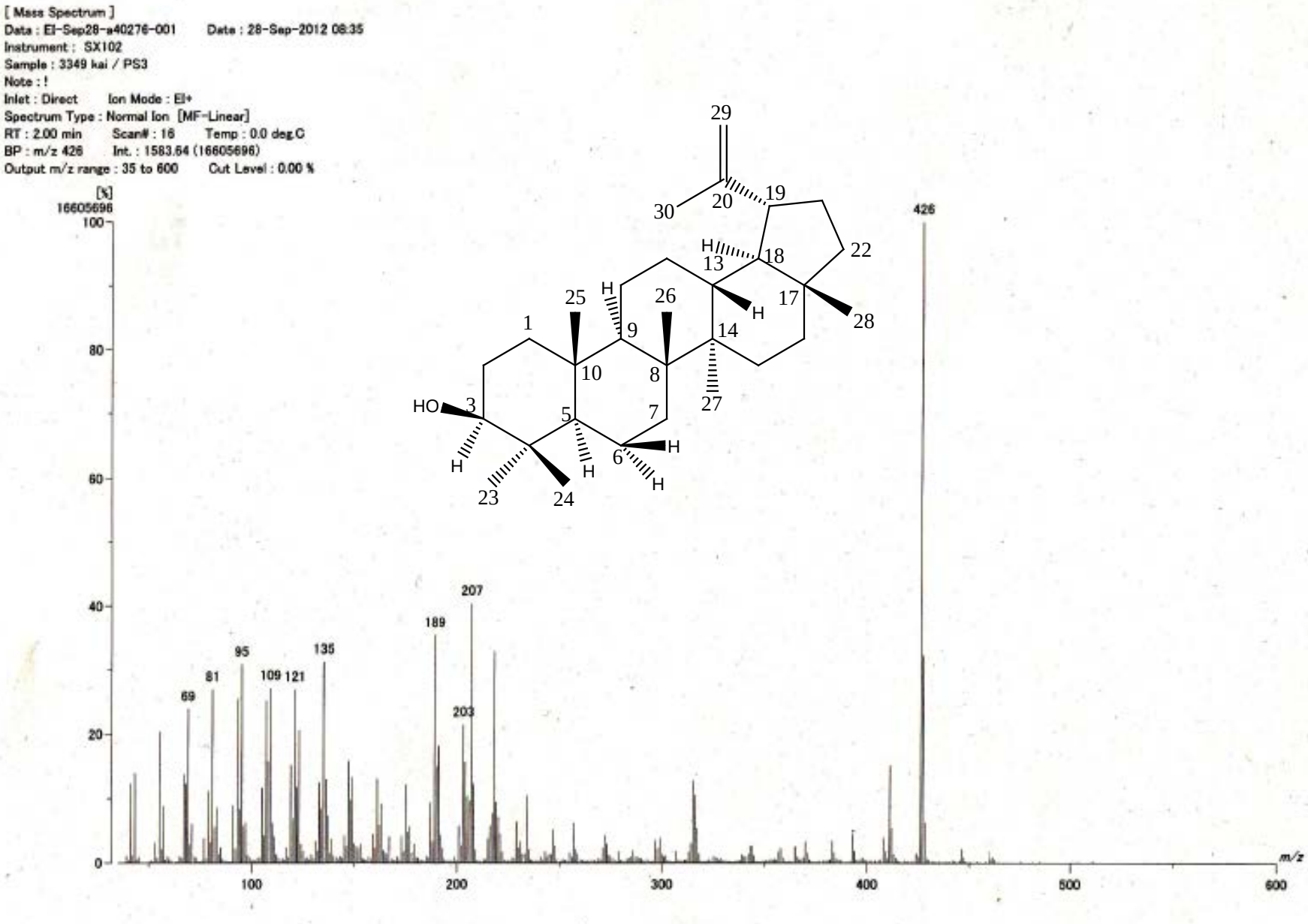
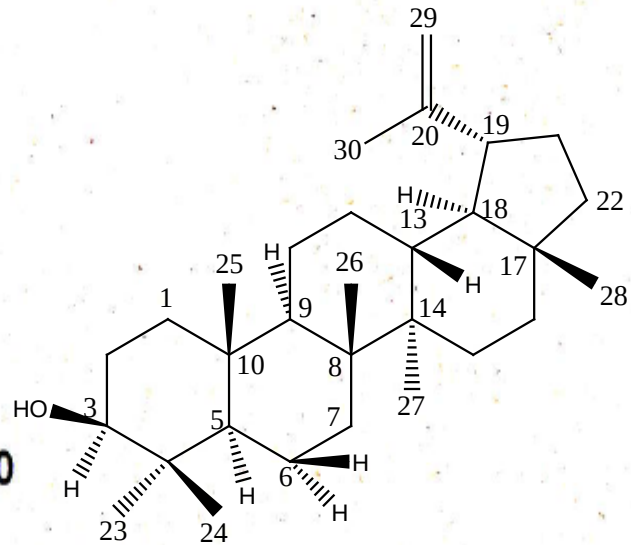


Chart 25. EI-MS spectrum of compound **4**.

Data : EI-Jan-031 Date : 03-Feb-2014 09:48
Instrument : SX102
Sample : a50845 2495 Khine / PS-3
Note : SX102A @ GC-MS Lab. Fac. Agric. Hokkaido Univ.
Inlet : Direct Ion Mode : EI+
RT : 0.29 min Scan# : 2
Elements : C 50/0, H 80/0, O 10/0
Mass Tolerance : 20ppm, 5mmu if m/z < 250, 10mmu if m/z > 500
Unsaturation (U.S.) : -0.5 - 10.0



	Observed m/z	Int%	Err[ppm / mmu]	U.S.	Composition
1	426.3847	100.00	-3.4 / -1.5	6.0	C30 H50 O

Chart 26. HR-EI-MS spectrum of compound **4**.

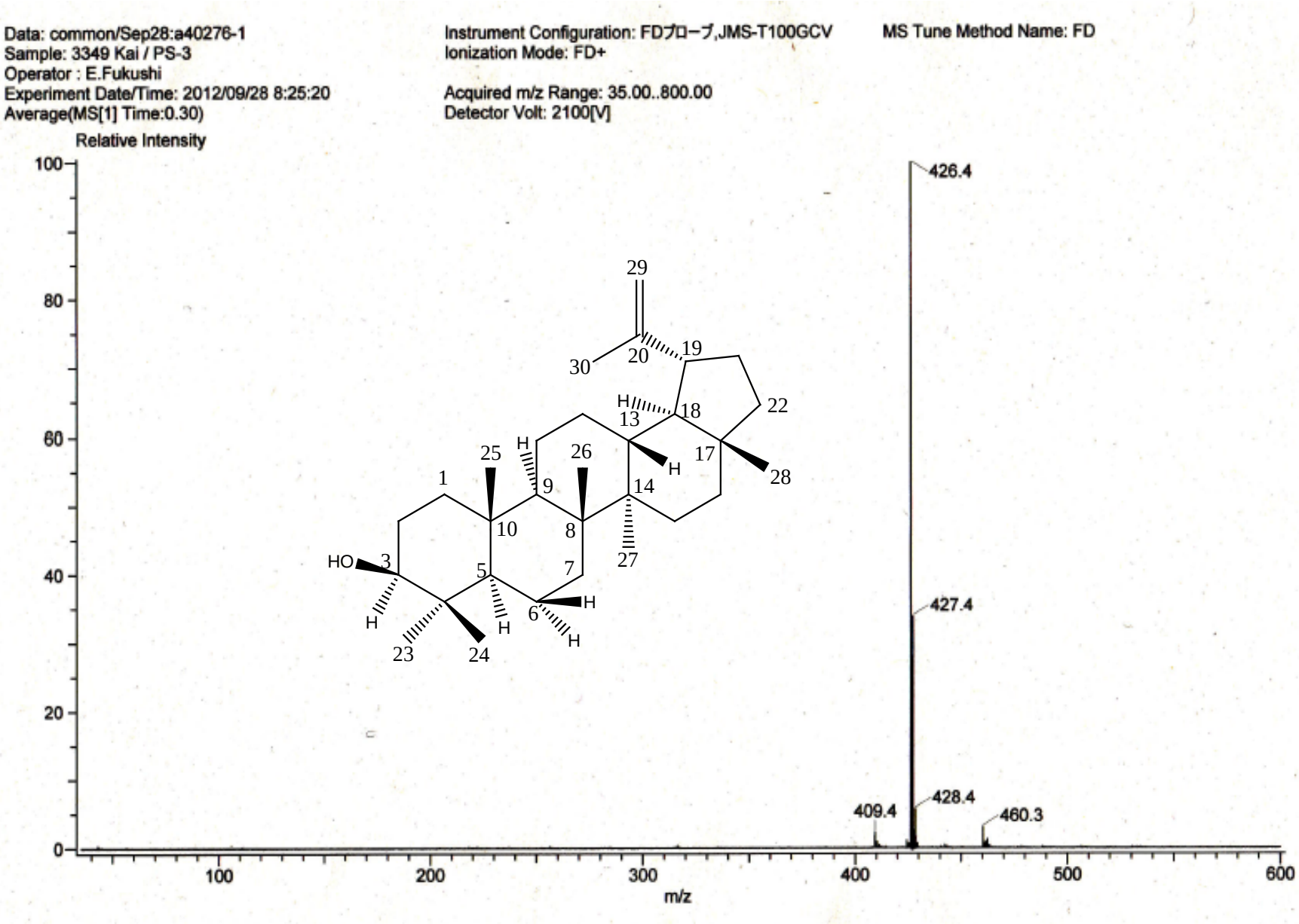


Chart 27. FD-MS spectrum of compound **4**.

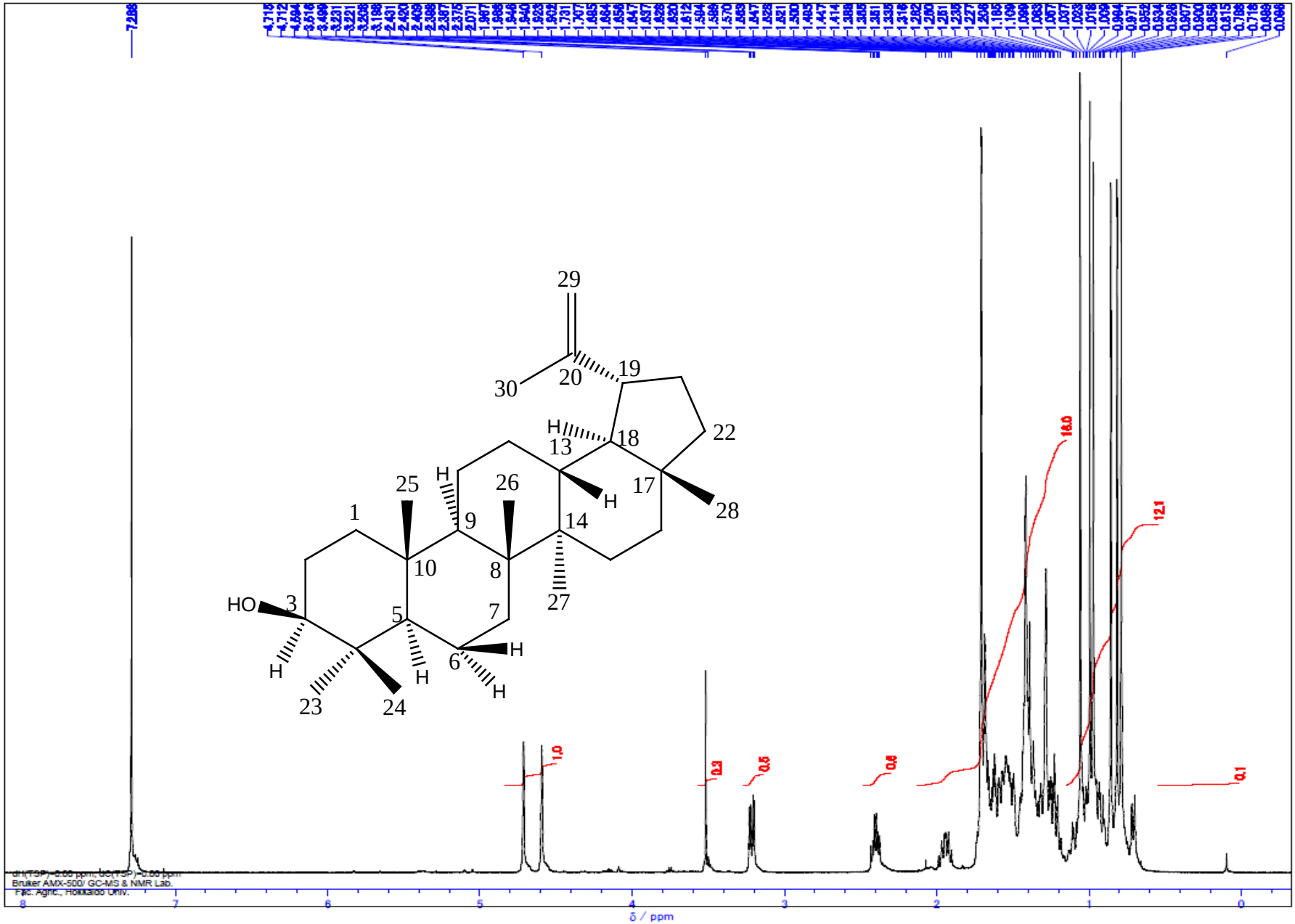


Chart 28. ^{1}H NMR spectrum of compound **4** (500 MHz, $CDCl_3$).

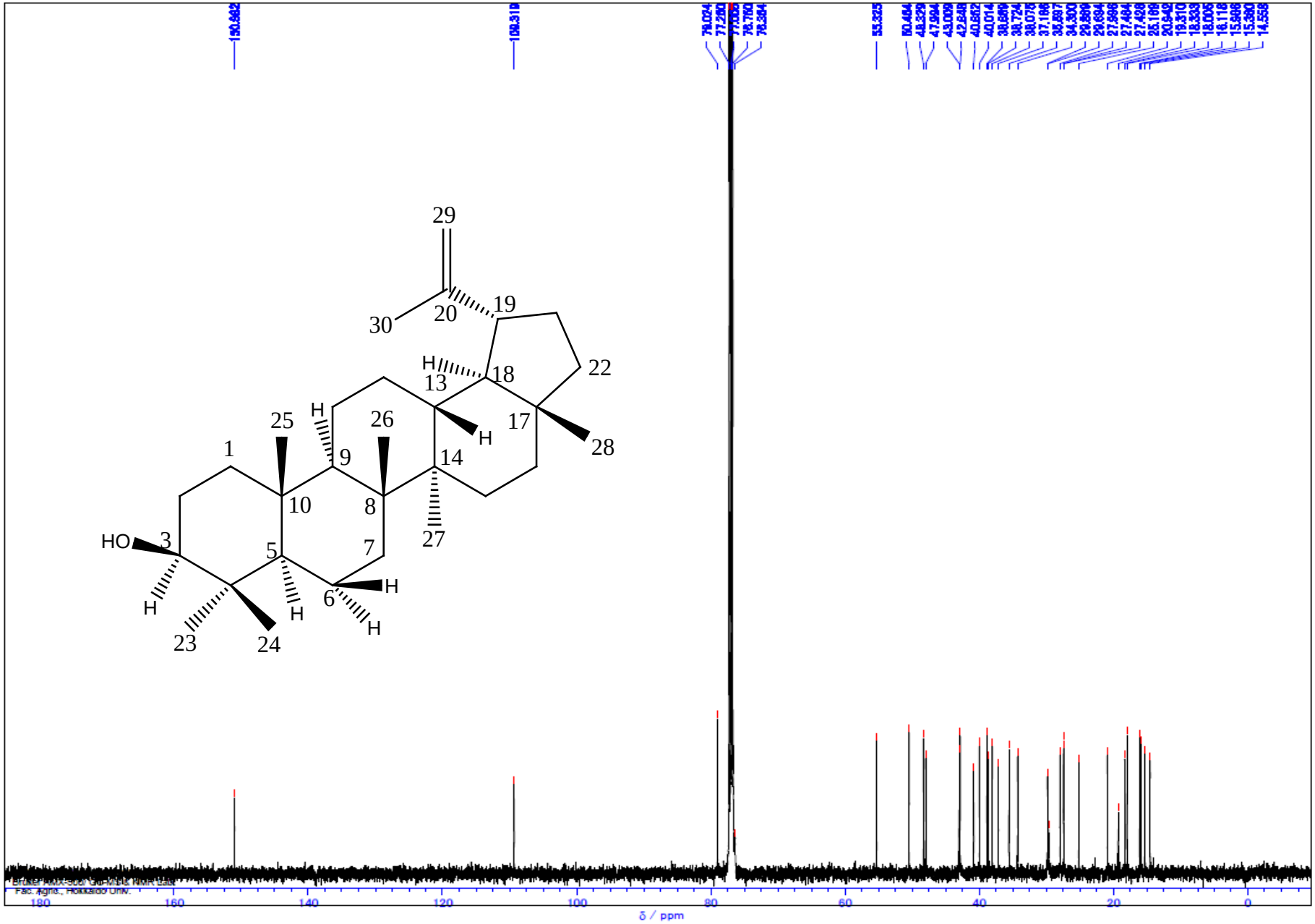


Chart 29. ^{13}C NMR spectrum of compound **4** (500 MHz, $CDCl_3$).

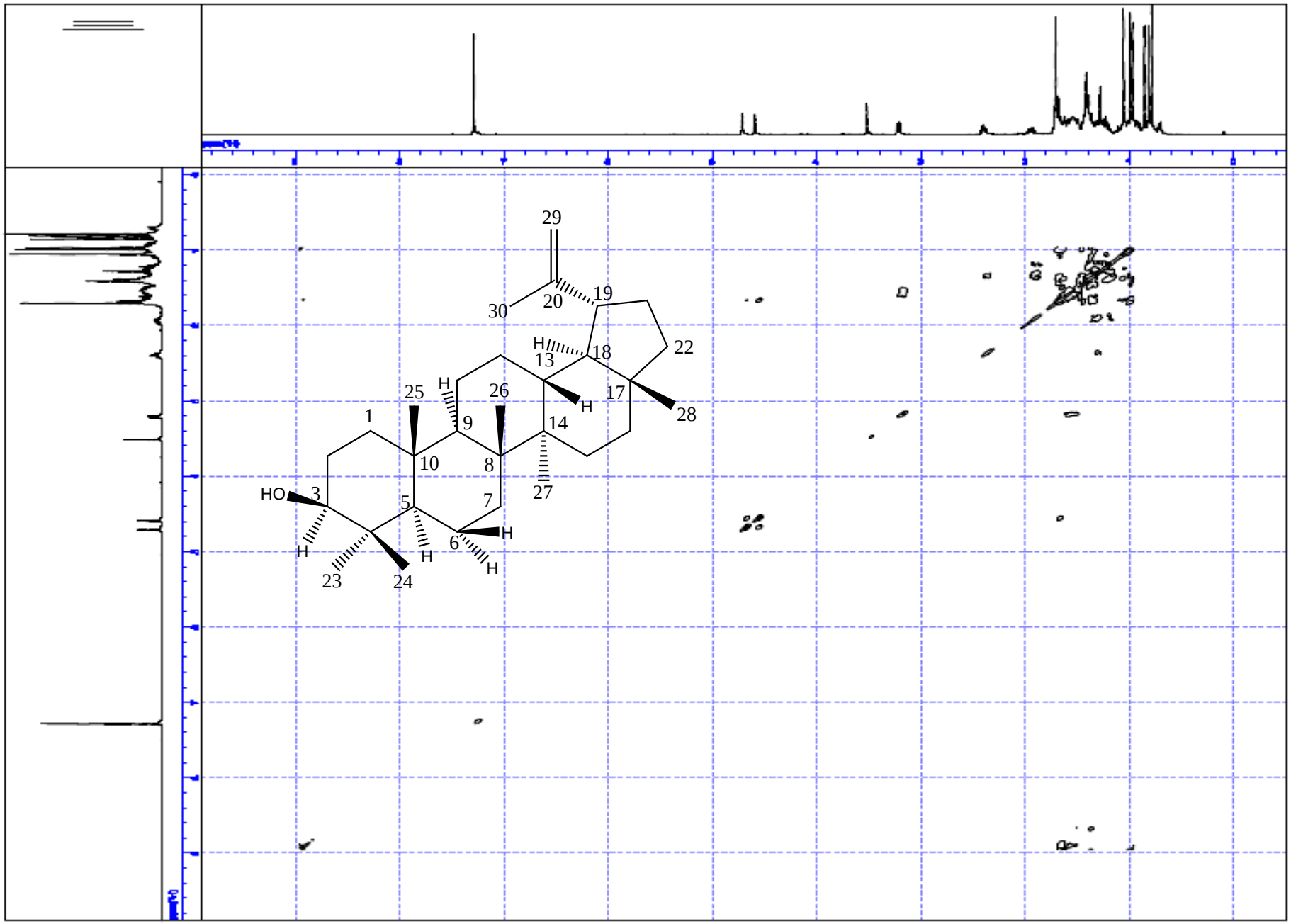


Chart 30. COSY spectrum of compound **4** (500 MHz, $CDCl_3$).

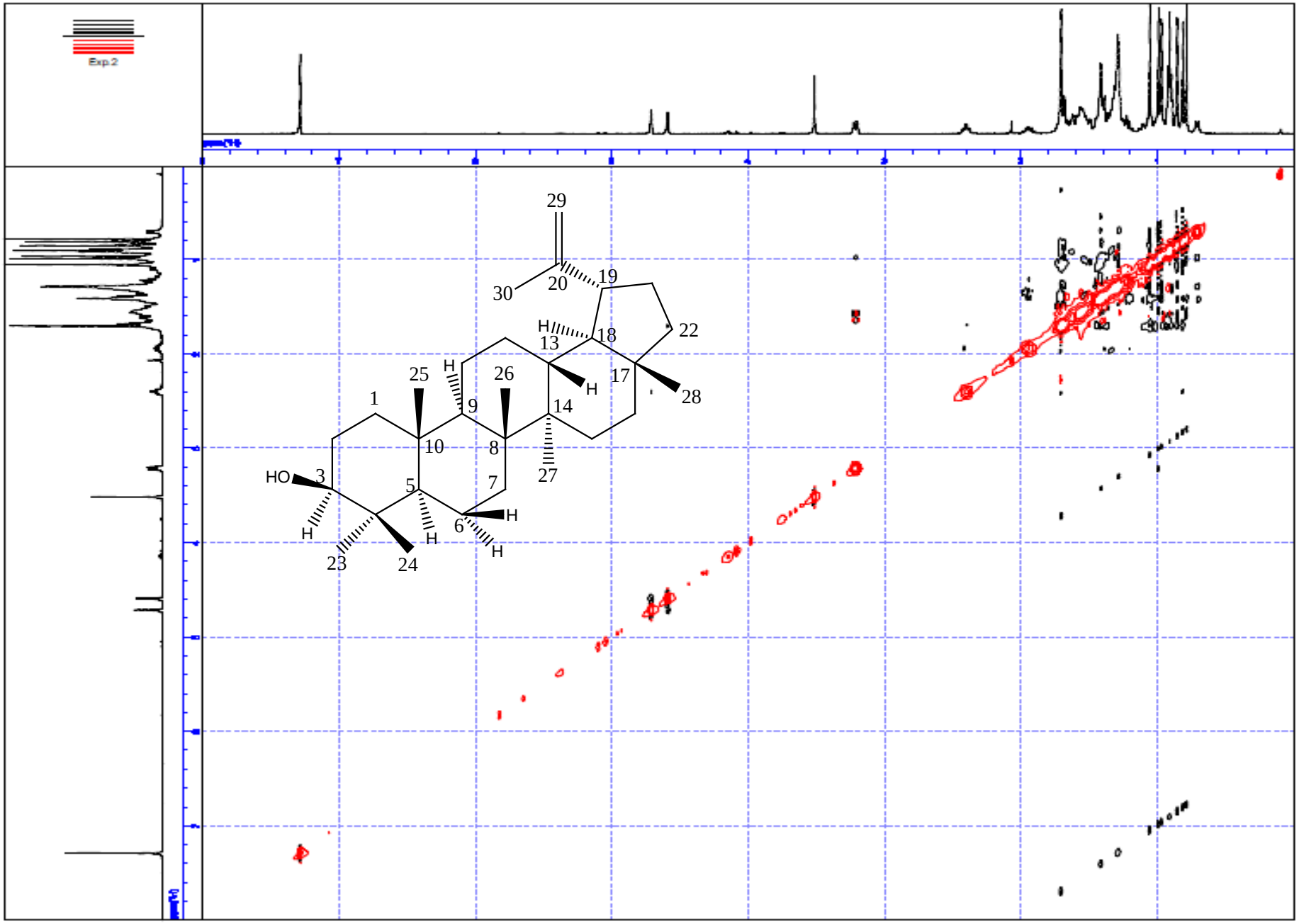


Chart 31. NOESY spectrum of compound **4** (500 MHz, $CDCl_3$).

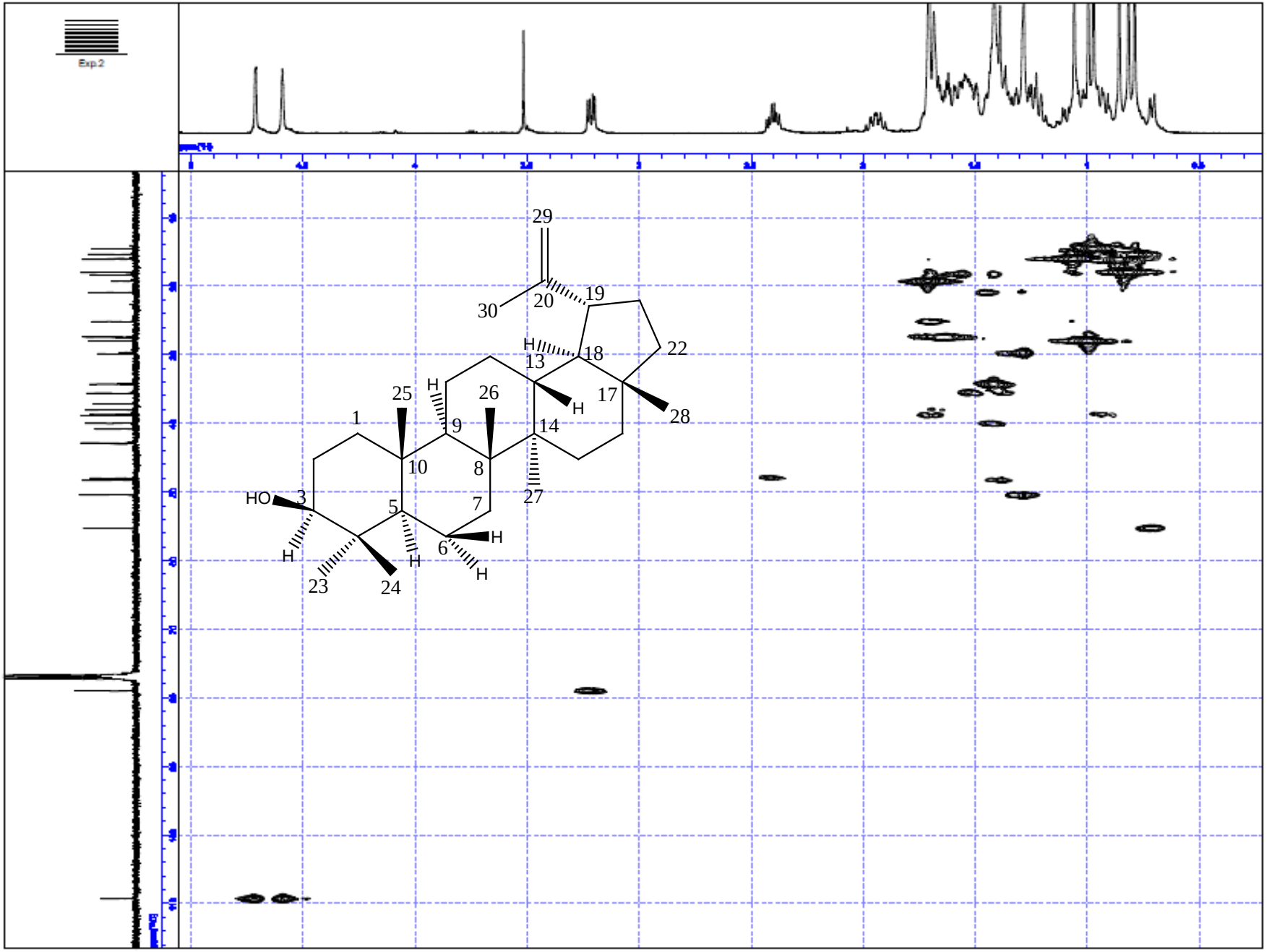


Chart 32. HMQC spectrum of compound **4** (500 MHz, $CDCl_3$).

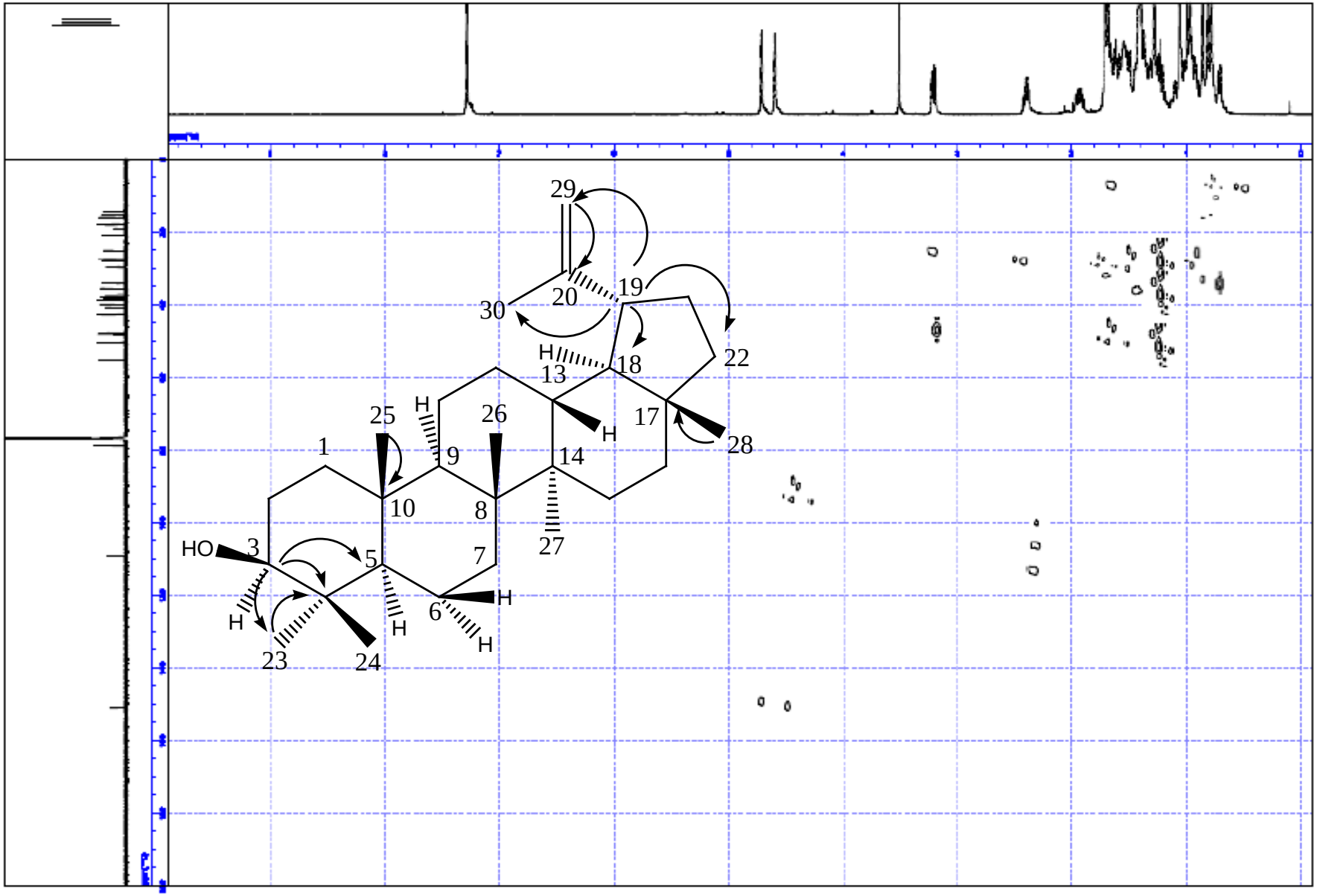


Chart 33. HMBC spectrum of compound **4** (500 MHz, $CDCl_3$).

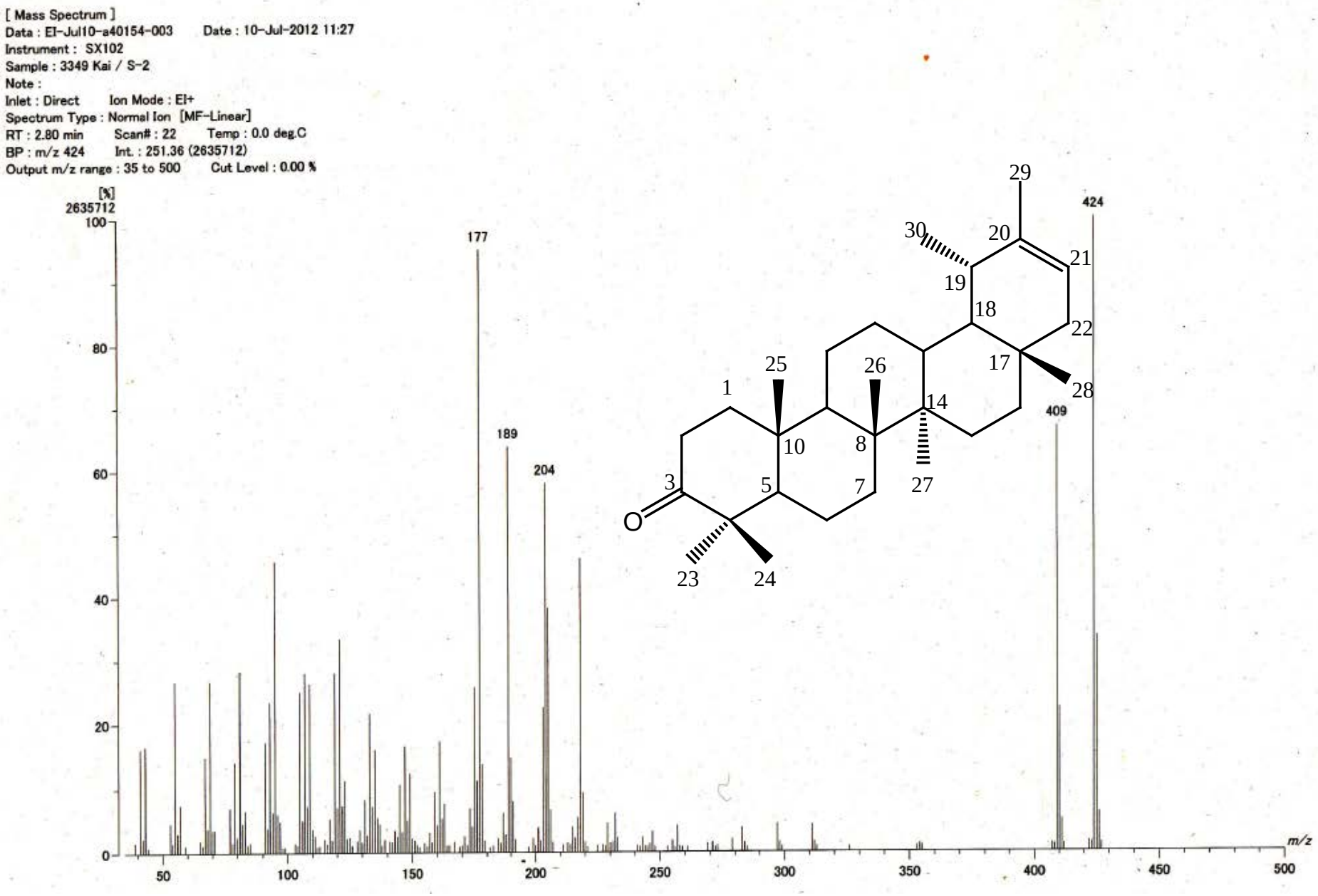
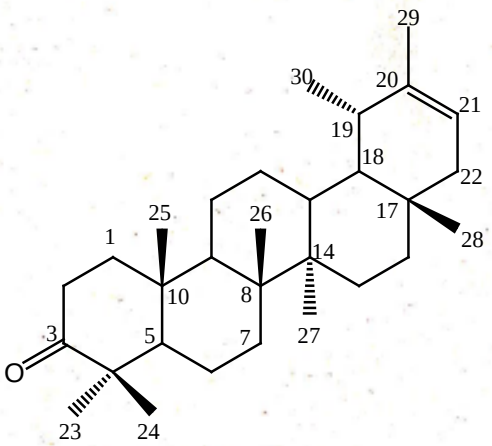


Chart 34. EI-MS spectrum of compound **5**.

Data : EI-Jan-035 Date : 03-Feb-2014 10:08
Instrument : SX102
Sample : a50846 2495 Khine / PS-2
Note : SX102A @ GC-MS Lab. Fac. Agric. Hokkaido Univ.
Inlet : Direct Ion Mode : EI+
RT : 1.99 min Scan# : 8
Elements : C 50/0, H 80/0, O 10/0
Mass Tolerance : 20ppm, 5mmu if m/z < 250, 10mmu if m/z > 500
Unsaturation (U.S.) : -0.5 - 10.0



	Observed m/z	Int%	Err[ppm / mmu]	U.S.	Composition
1	424.3683	29.54	-5.2 / -2.2	7.0	C30 H48 O

Chart 35. HR-EI-MS spectrum of compound **5**.

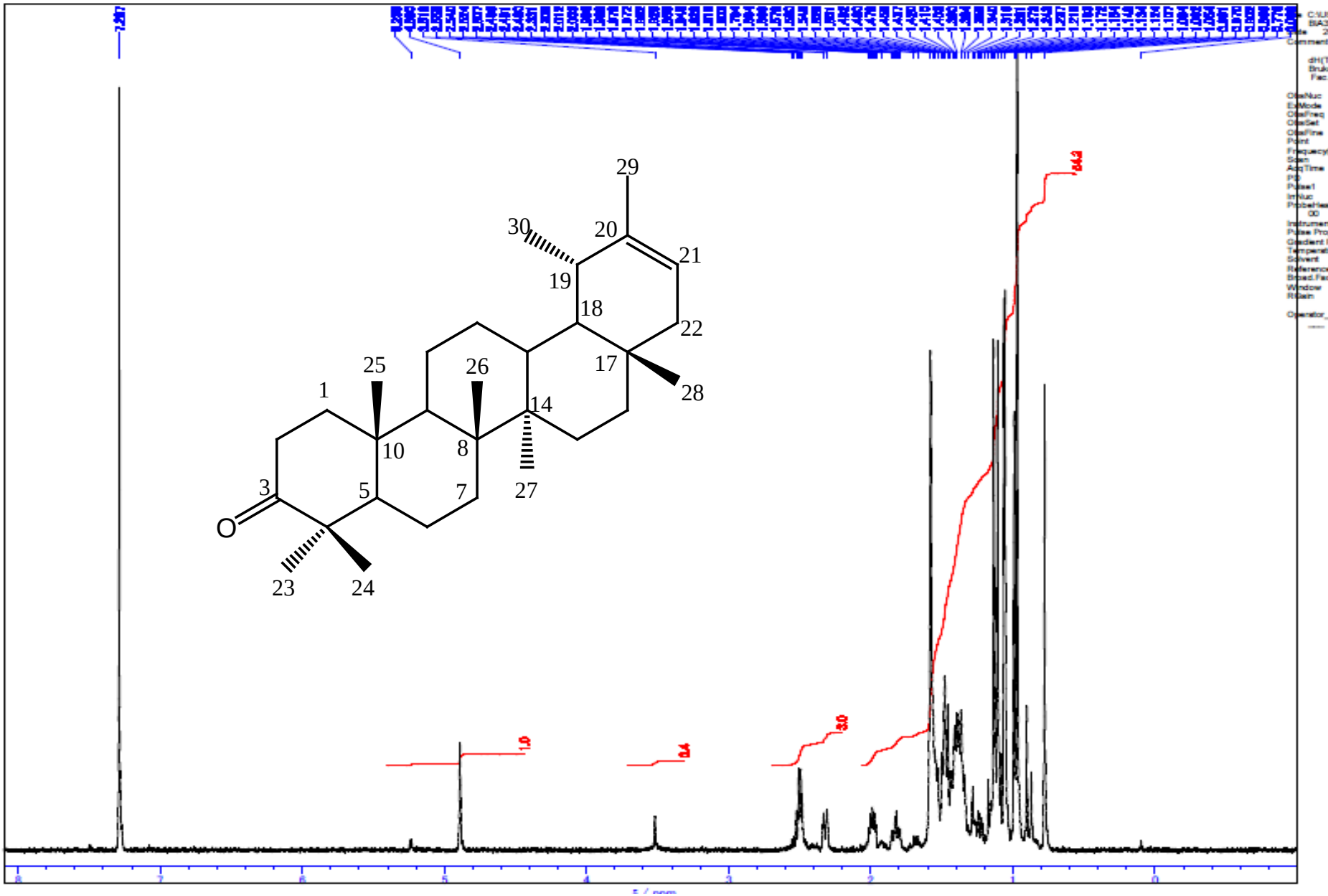


Chart 36. ^{1}H NMR spectrum of compound **5** (500 MHz, $CDCl_3$).

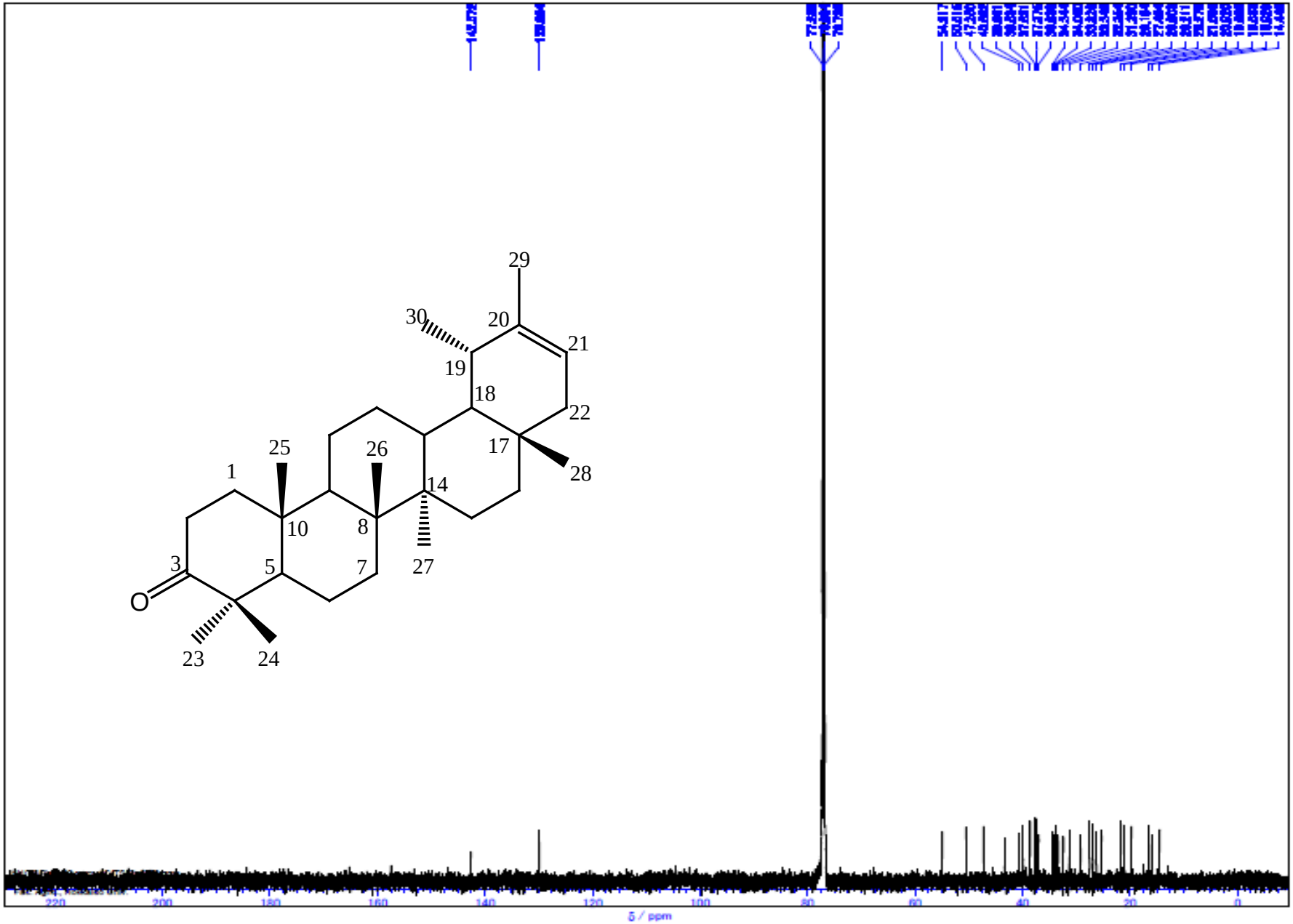


Chart 37. ^{13}C NMR spectrum of compound **5** (500 MHz, $CDCl_3$).

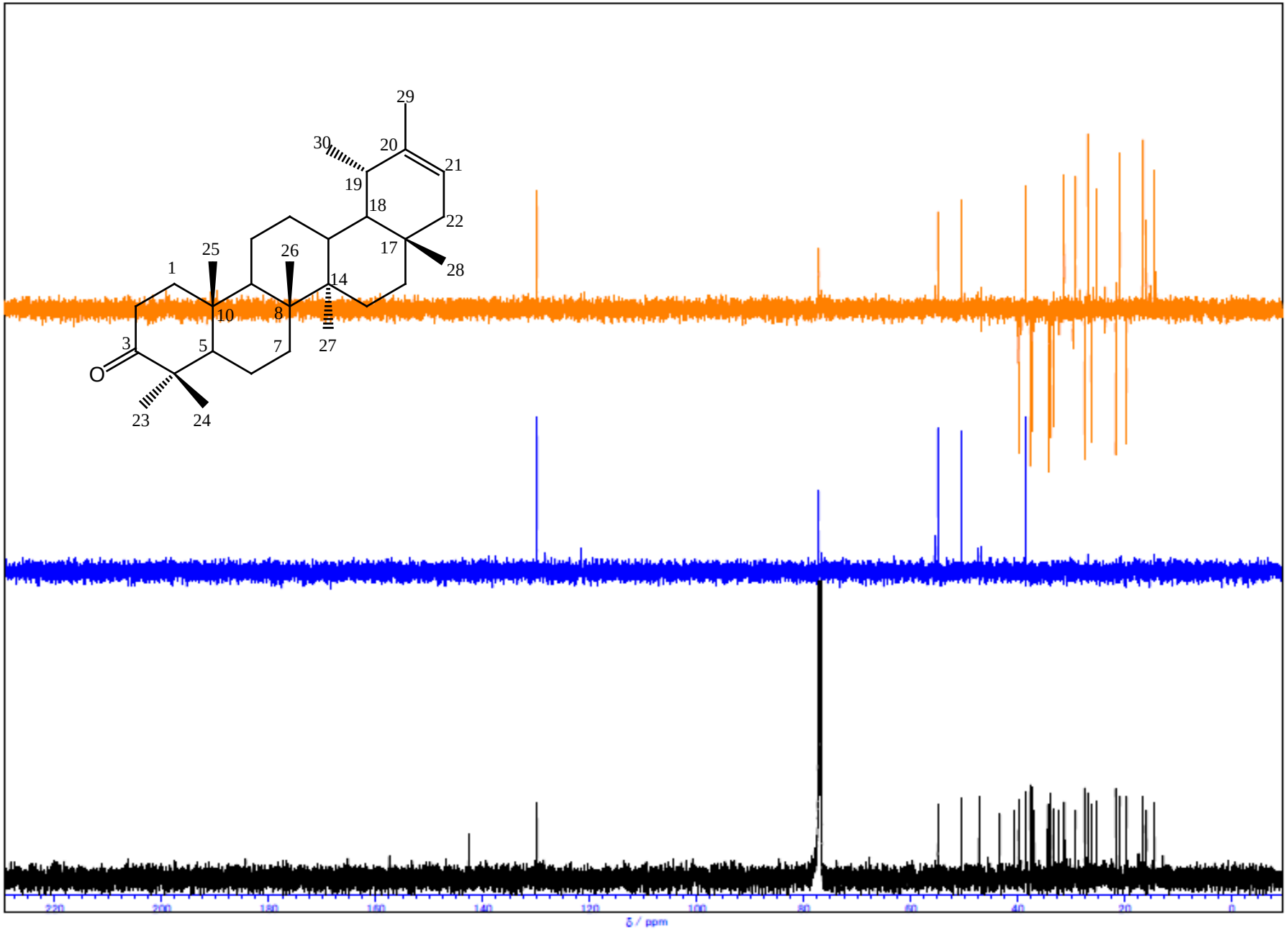


Chart 38. DEPT spectrum of compound **5** (500 MHz, $CDCl_3$).

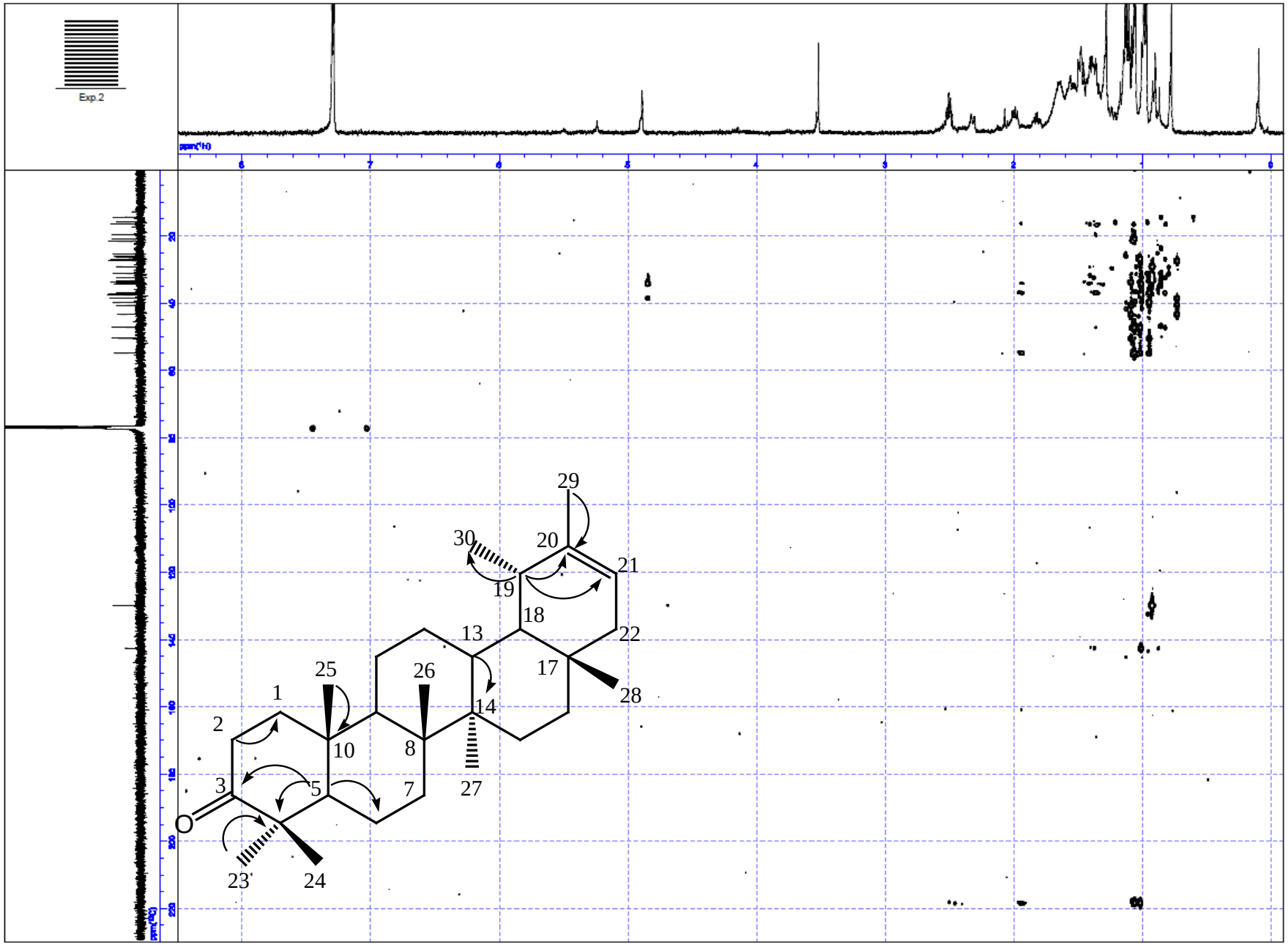


Chart 39. HMBC spectrum of compound **5** (500 MHz, $CDCl_3$).

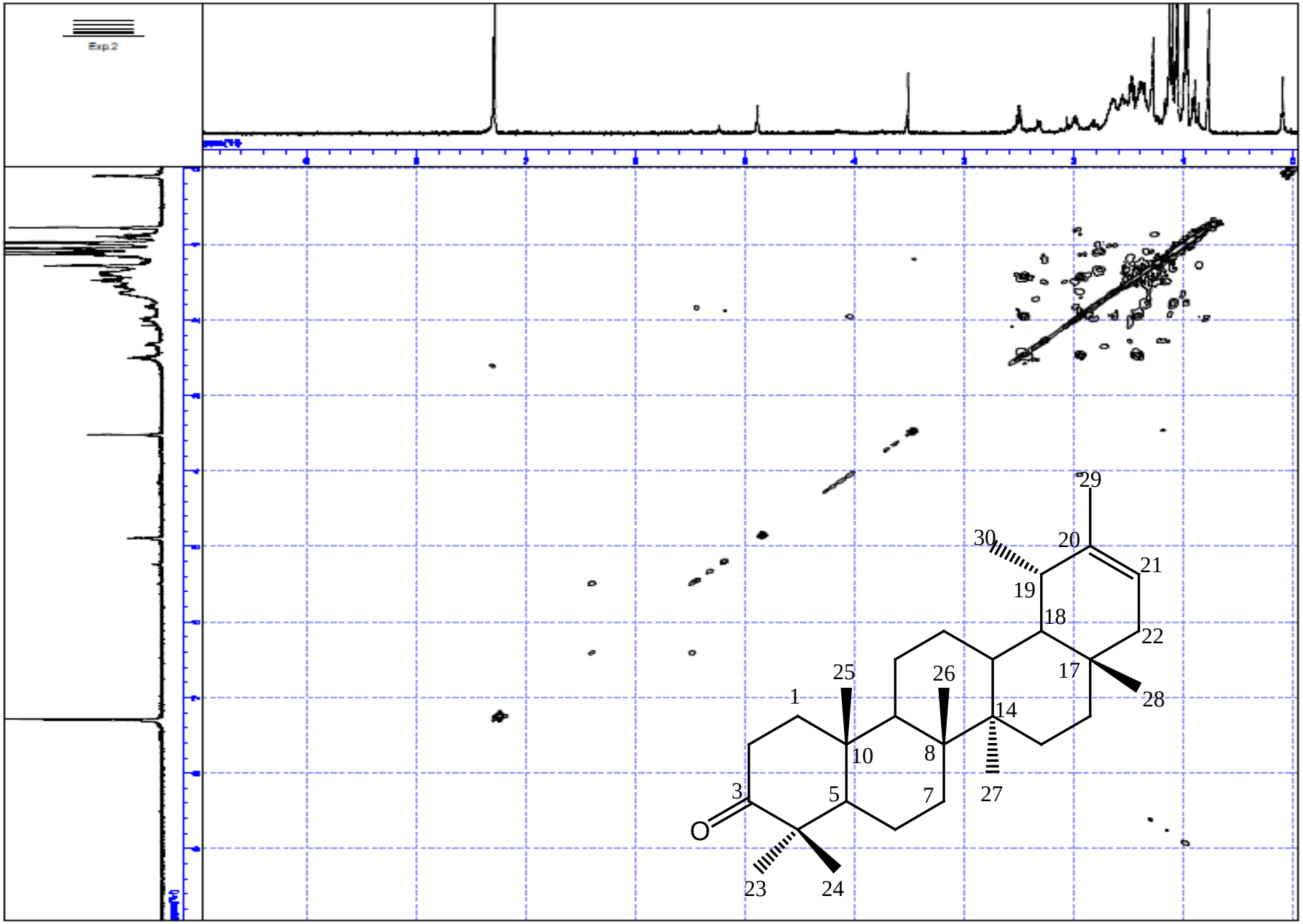


Chart 40. COSY spectrum of compound **5** (500 MHz, $CDCl_3$).

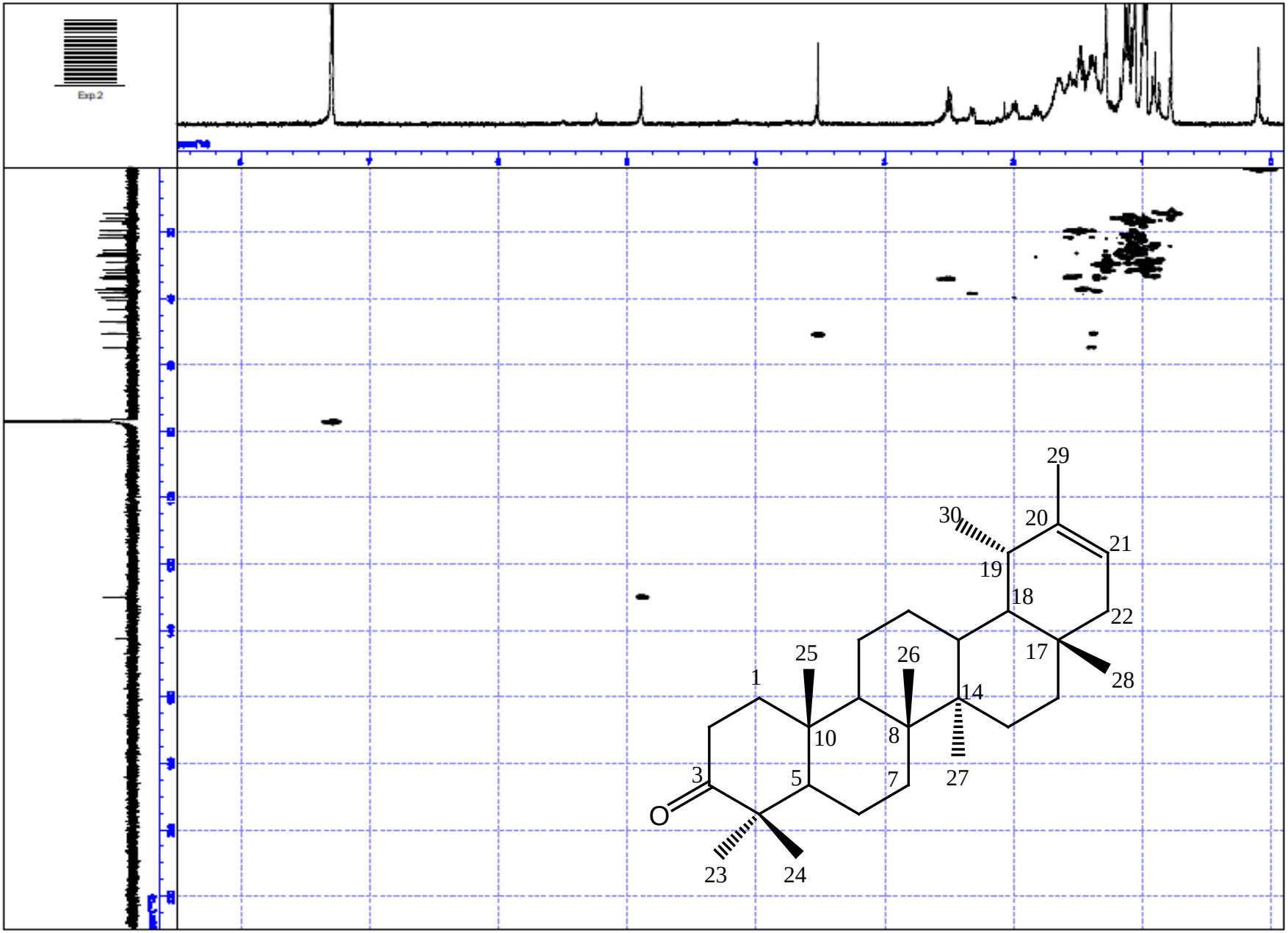


Chart 41. HMQC spectrum of compound **5** (500 MHz, $CDCl_3$).

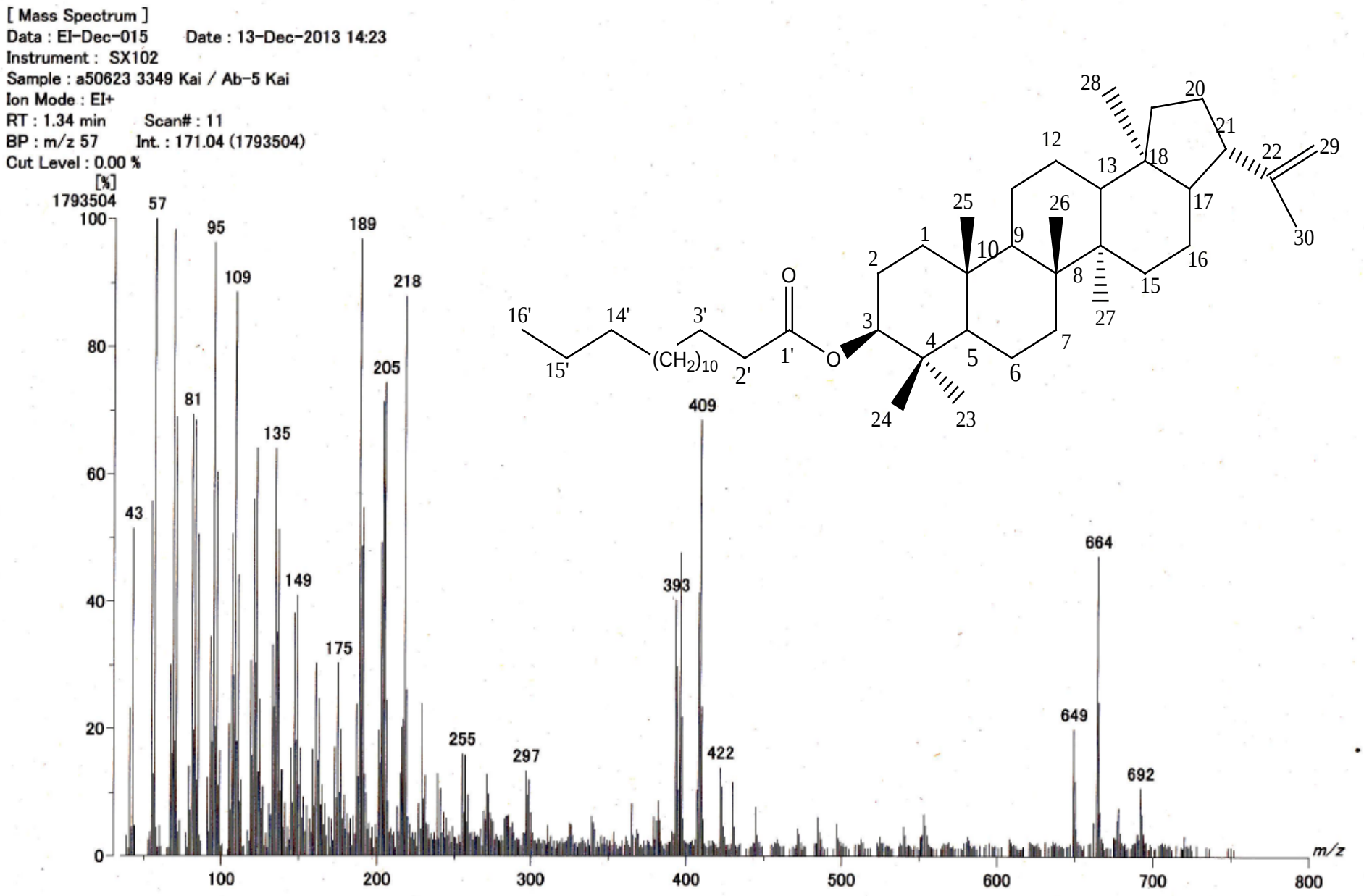
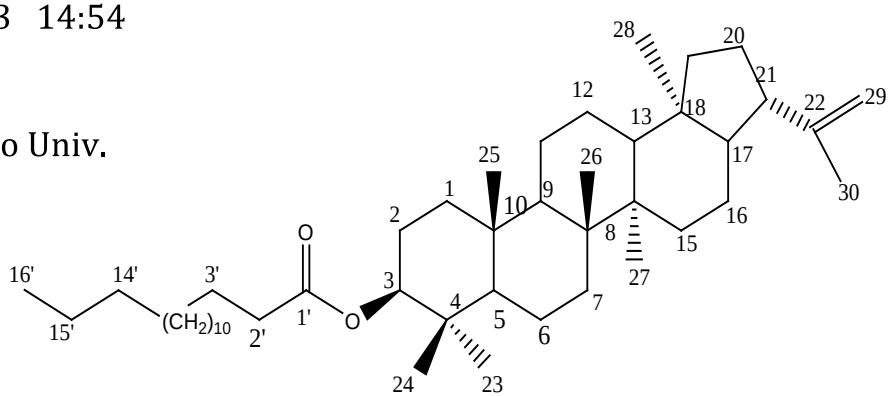


Chart 42. EI-MS spectrum of compound **6**.

Data : EI-Sep-014 Date : 24-Sep-2013 14:54
Instrument : SX 102
Sample : a50354 3349 Kai/ VAE-2-pi
Note : SX102A @ GC-MS Lab. Fac. Agric. Hokkaido Univ.
Inlet : Direct Ion Mode : EI+
RT : 0.85 min Scan# : 4
Elements : C 100/0, H 200/0, N 4/0, O 10/0
Mass Tolerance : 10mmu
Unsaturation (U.S.) : -0.5-50.0



	Observed m/z	Int %	Err [ppm / mmu]	U. S.	Composition
1.	664.6159	100.00	+ 2.1 / + 1.4	7.5	C44 H78 N3 O
2.			+ 0.1 / + 0.1	7.0	C46 H80 O2
3.			_ 10.7 / _ 7.1	3.0	C40 H80 N4 O3
4.			_ 12.8 / _ 8.5	2.5	C42 H82 N O4
5.			+ 6.2 / + 4.1	3.0	C41 H80 N2 O4

Chart 43. HR-EI-MS spectrum of compound **6**.

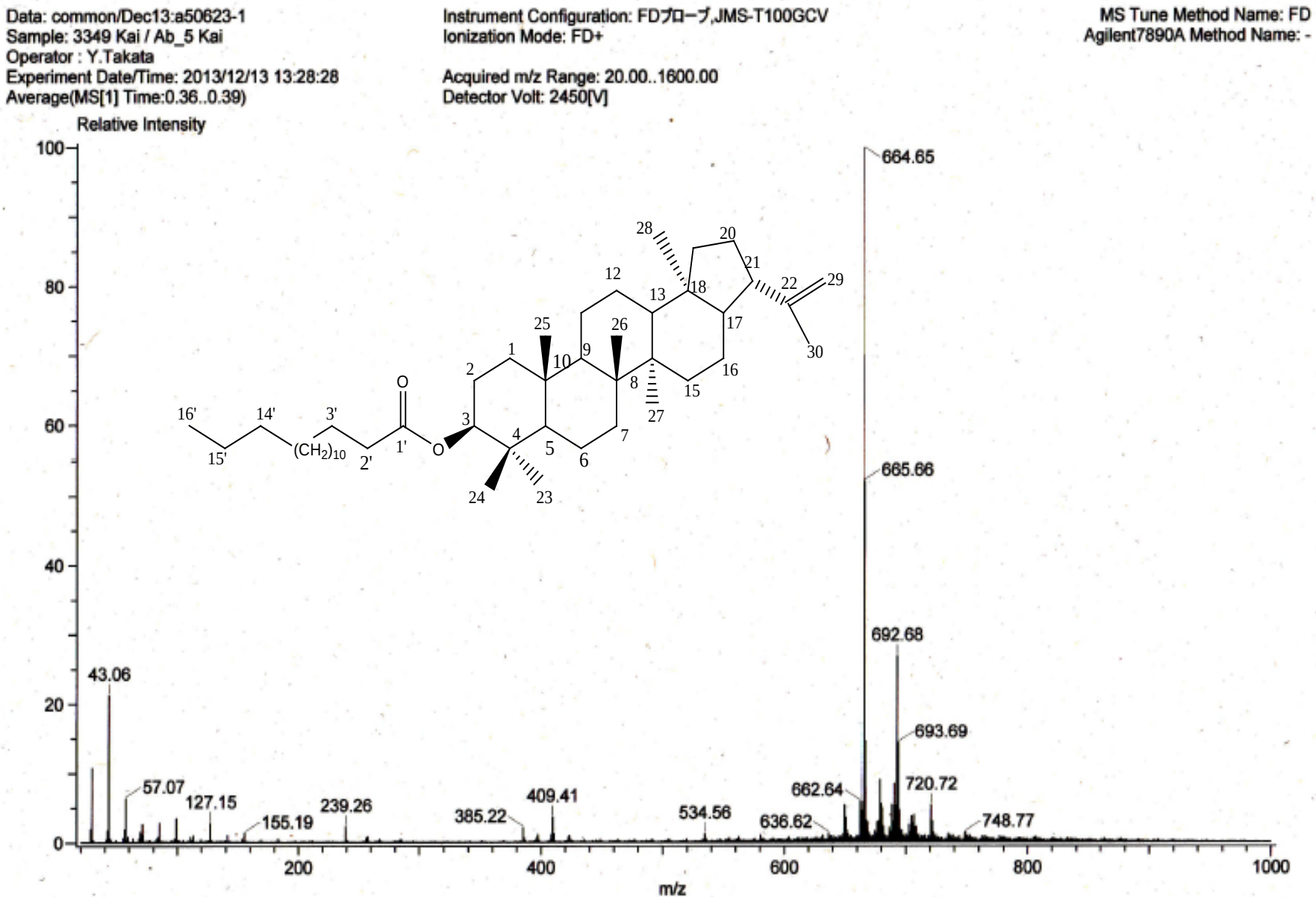


Chart 44. FD-MS spectrum of compound **6**.

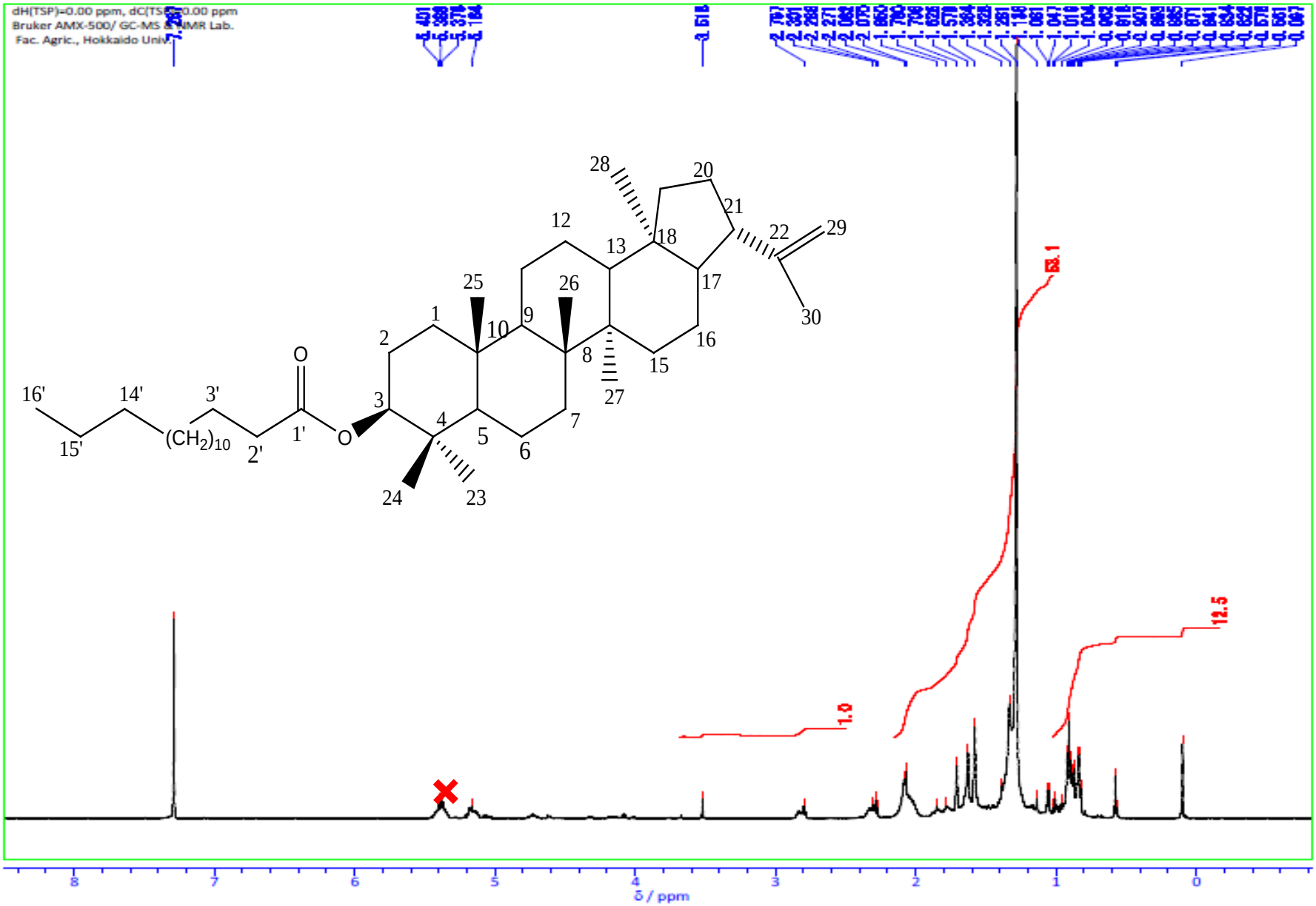


Chart 45. ^{1}H NMR spectrum of compound **6** (270 MHz, $CDCl_3$).

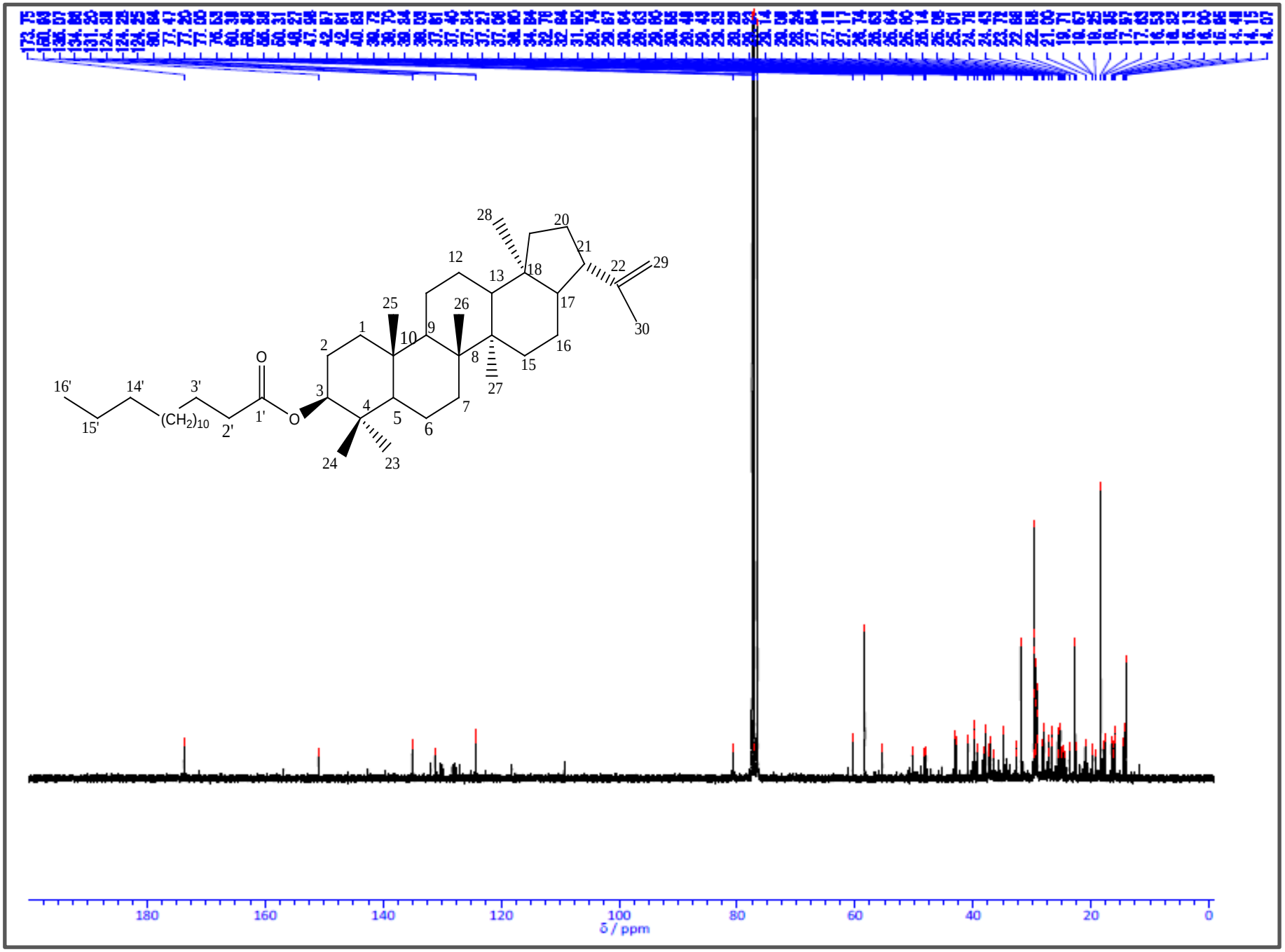


Chart 46. ^{13}C NMR spectrum of compound **6** (500 MHz, $CDCl_3$).

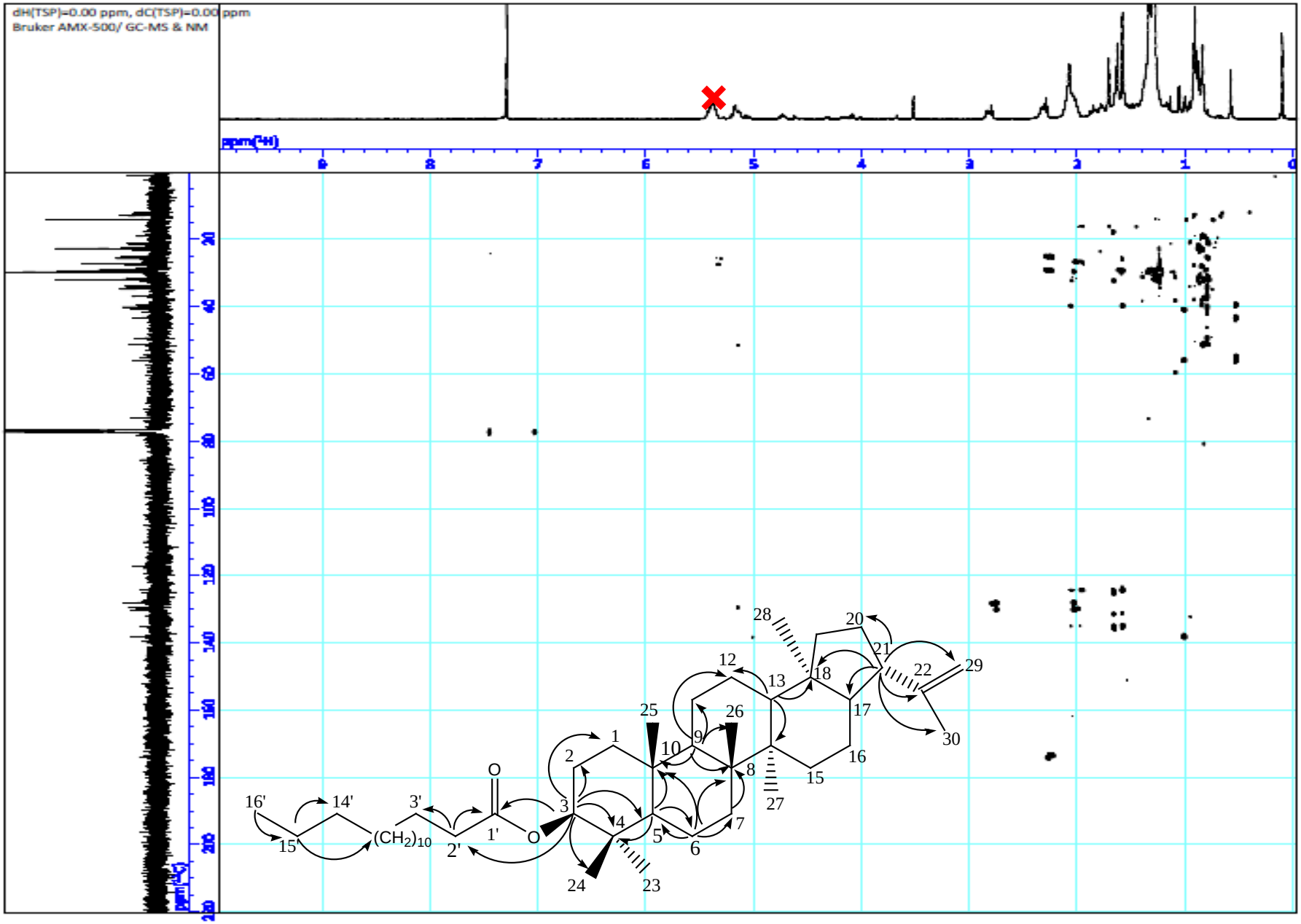


Chart 47. HMBC spectrum of compound **6** (500 MHz, $CDCl_3$).

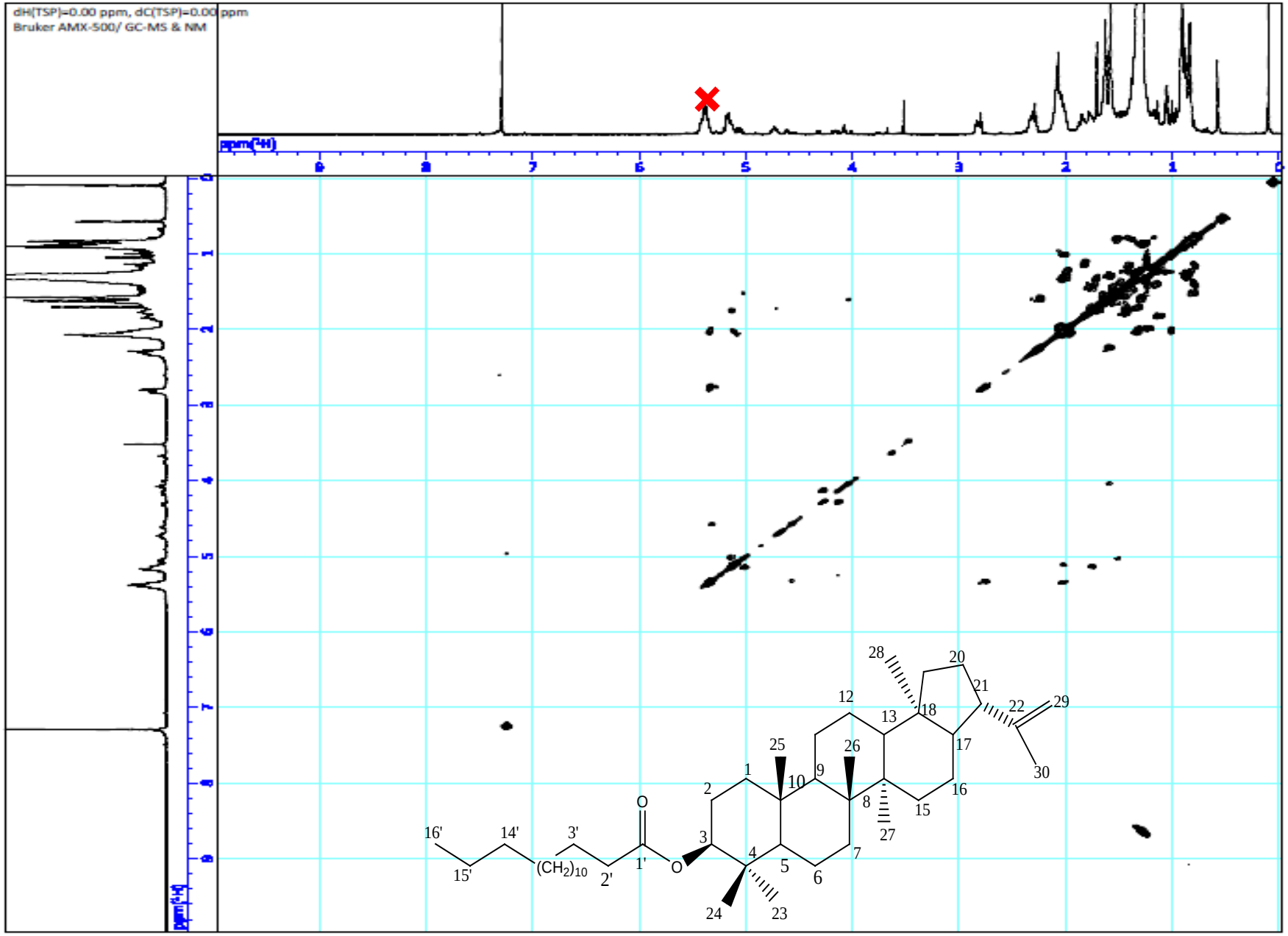


Chart 48. COSY spectrum of compound **6** (500 MHz, $CDCl_3$).

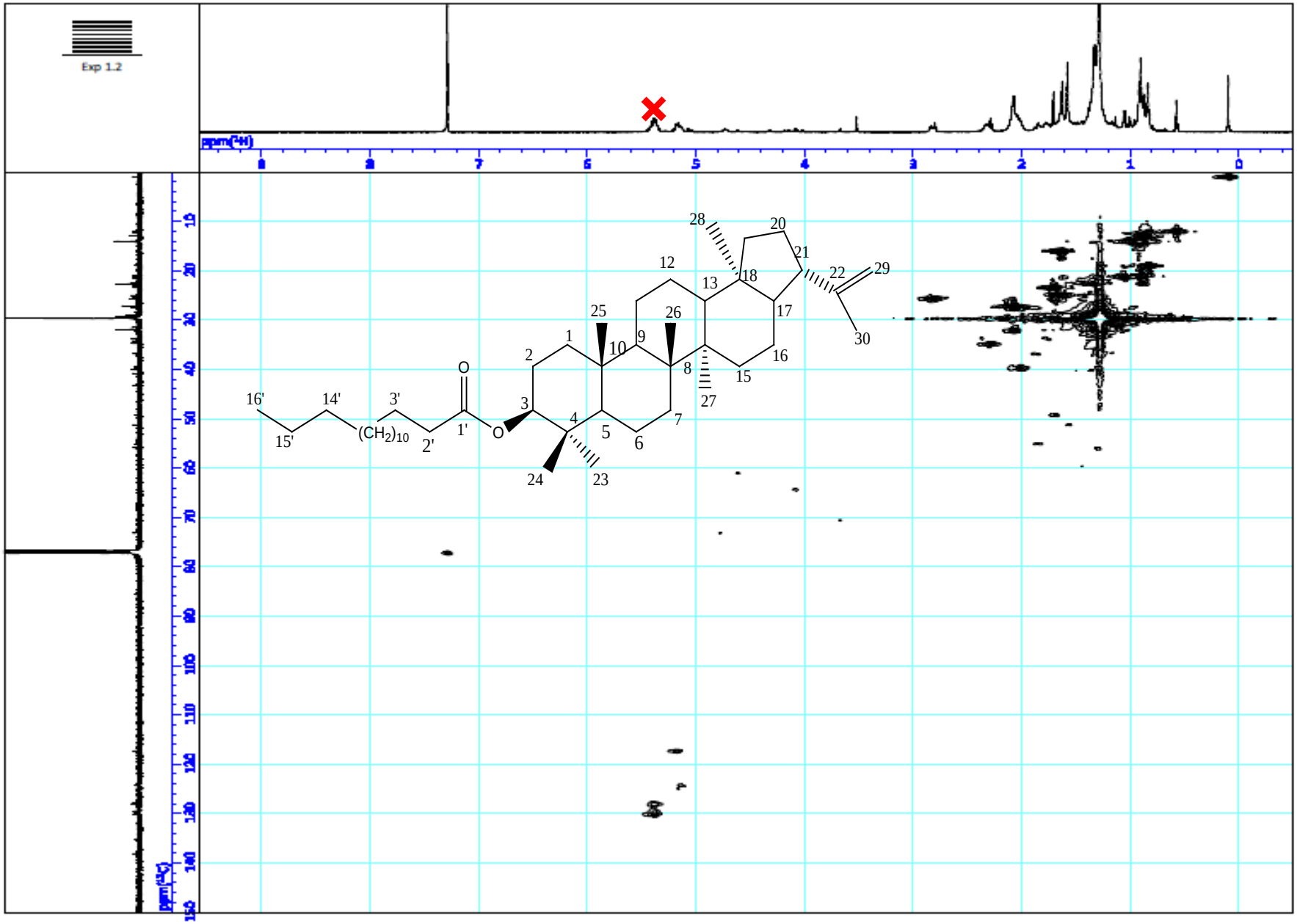


Chart 49. HMQC spectrum of compound **6** (500 MHz, $CDCl_3$).

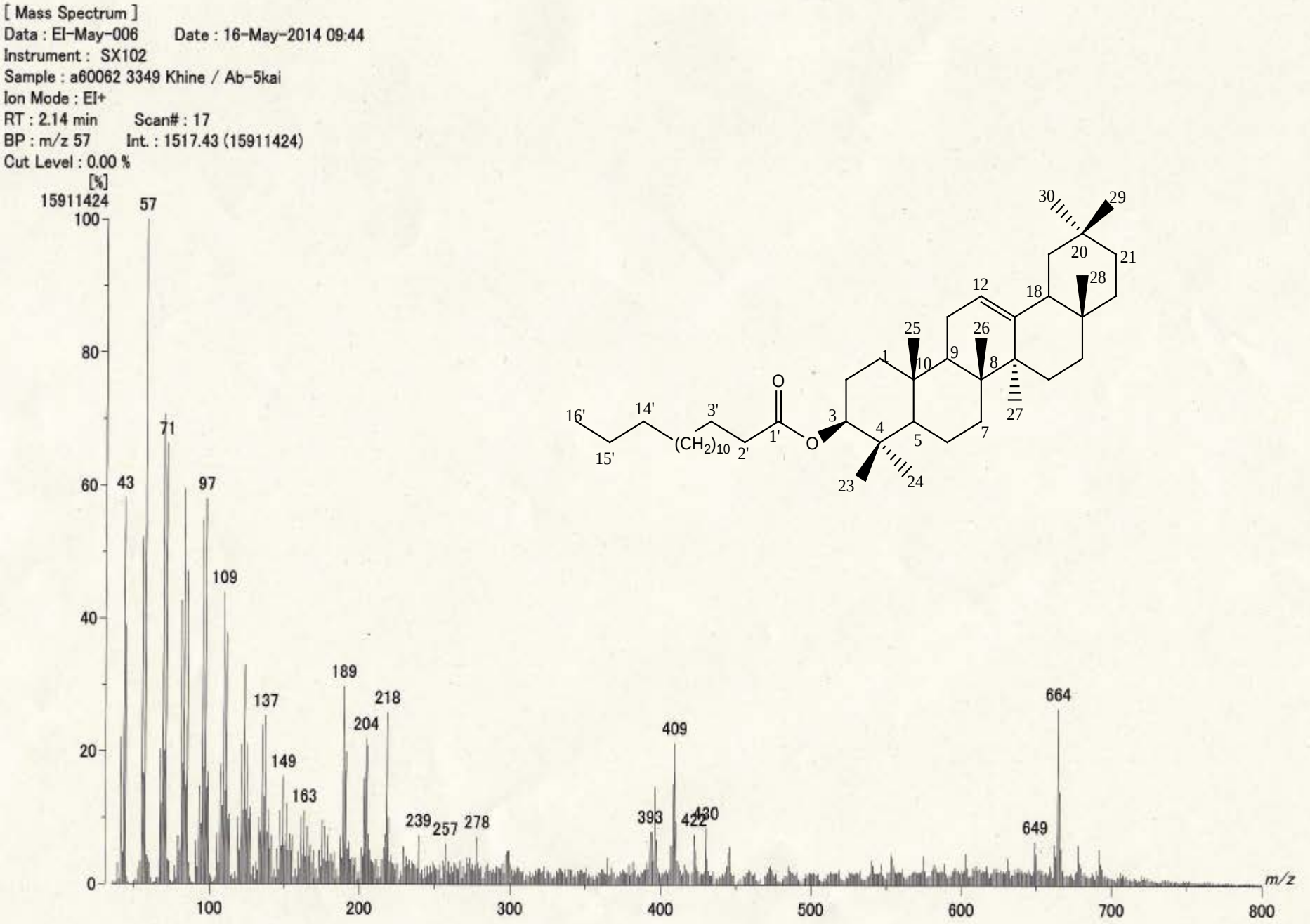
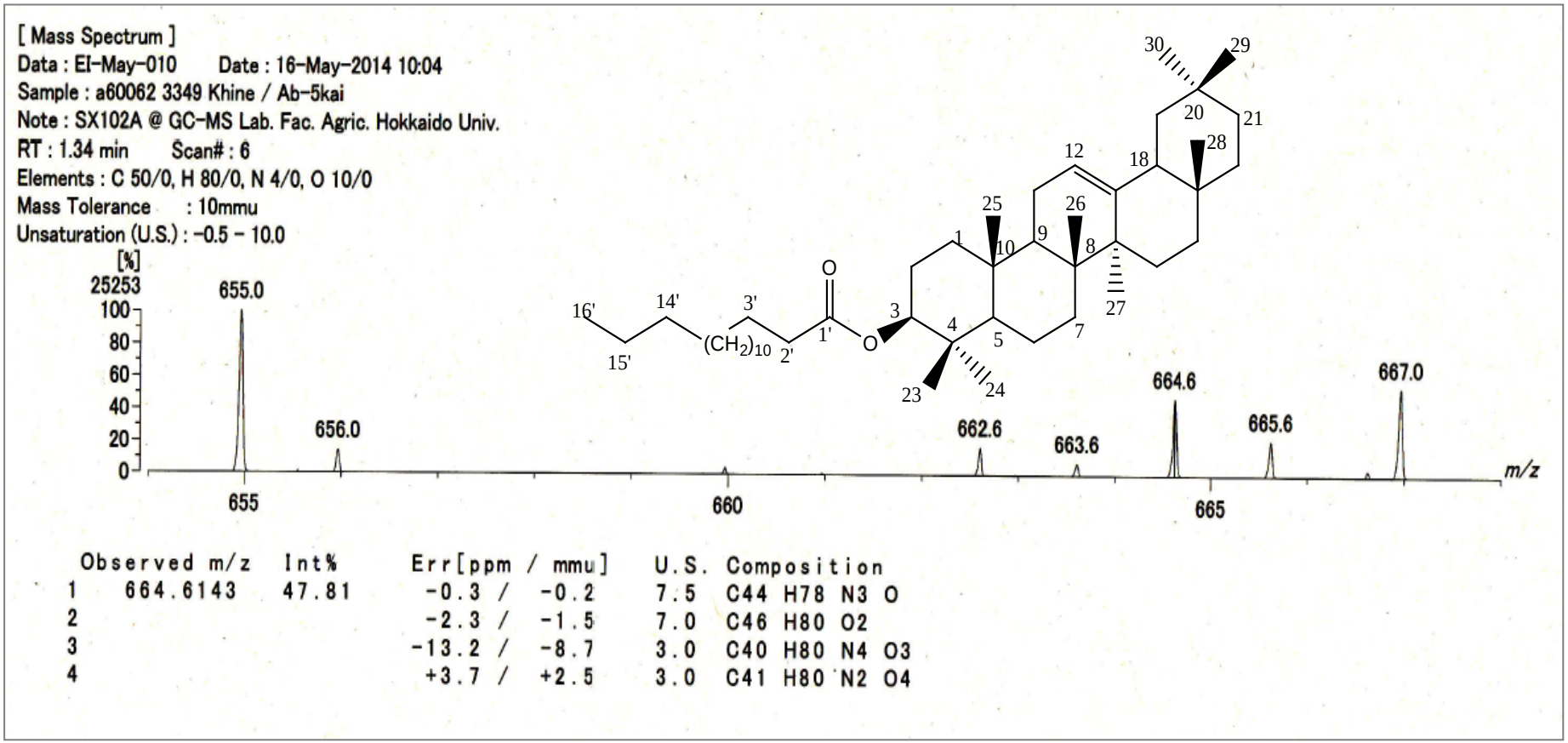


Chart 50. EI-MS spectrum of compound **7**.



	Observed m/z	Int%	Err[ppm / mmu]	U.S.	Composition
1	664.6143	47.81	-0.3 / -0.2	7.5	C44 H78 N3 O
2			-2.3 / -1.5	7.0	C46 H80 O2
3			-13.2 / -8.7	3.0	C40 H80 N4 O3
4			+3.7 / +2.5	3.0	C41 H80 N2 O4

Chart 51. HR-EI-MS spectrum of compound **7**.

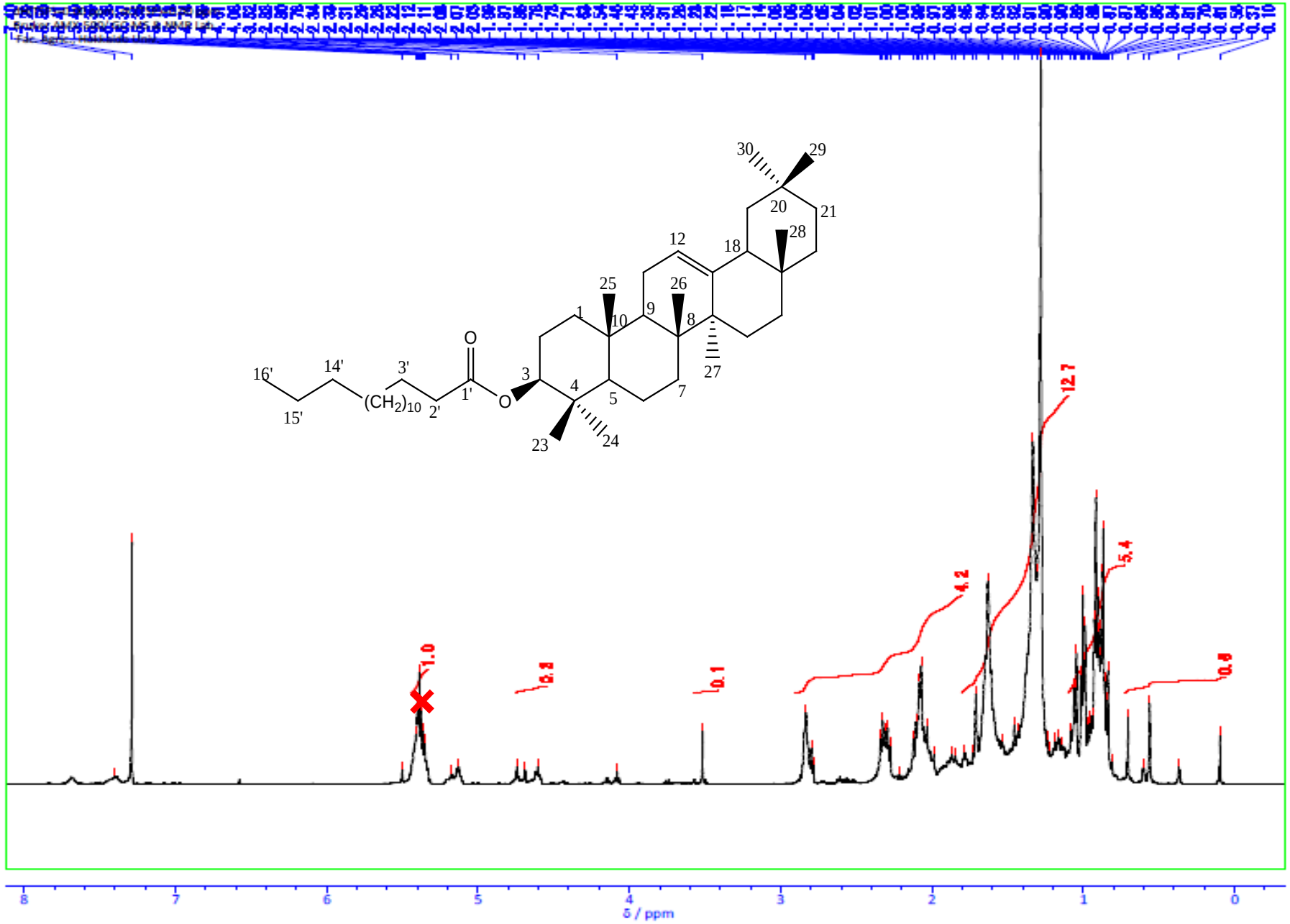


Chart 52. ^{1}H NMR spectrum of compound **7** (500 MHz, $CDCl_3$).

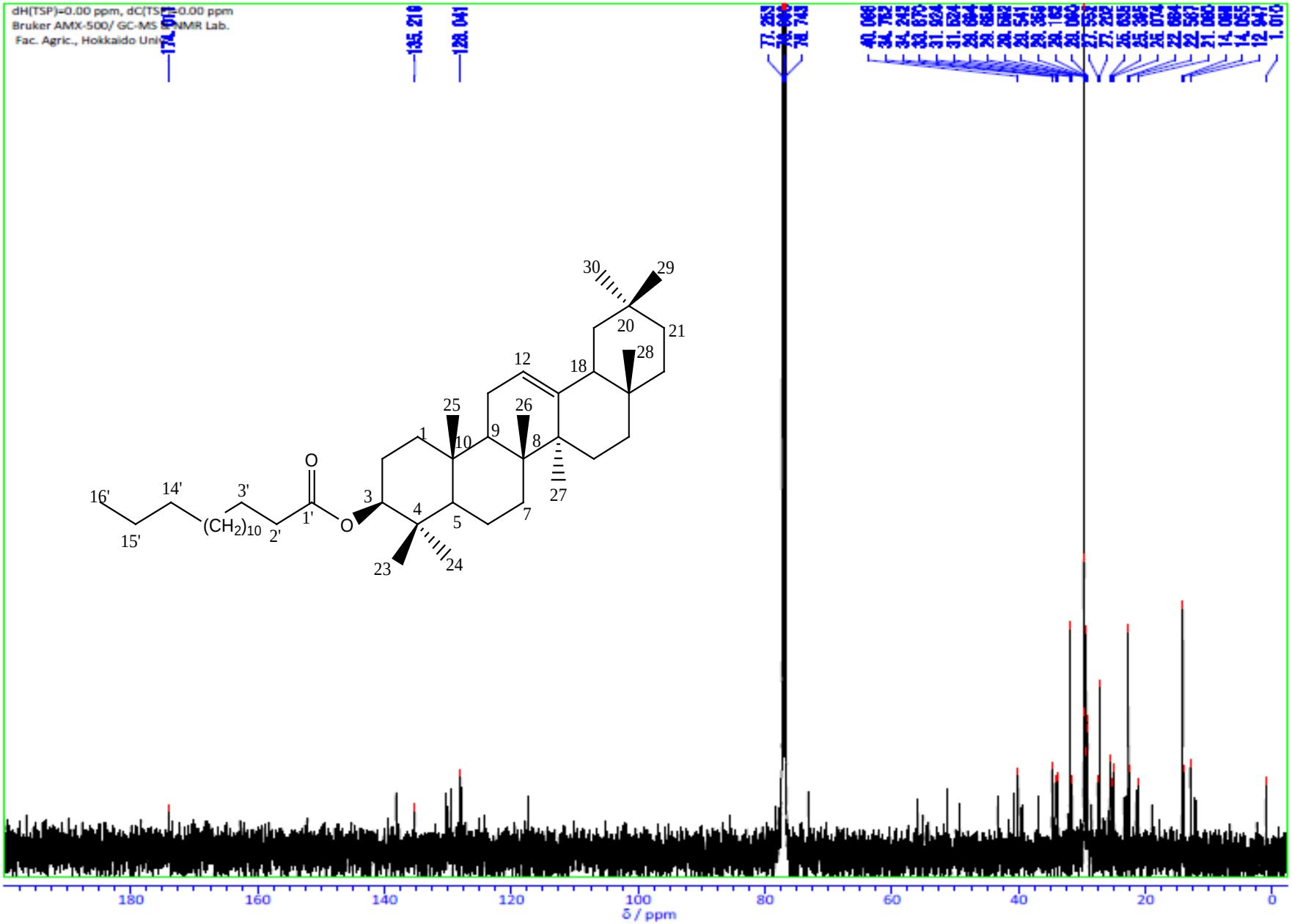


Chart 53. ^{13}C NMR spectrum of compound **7** (500 MHz, $CDCl_3$).

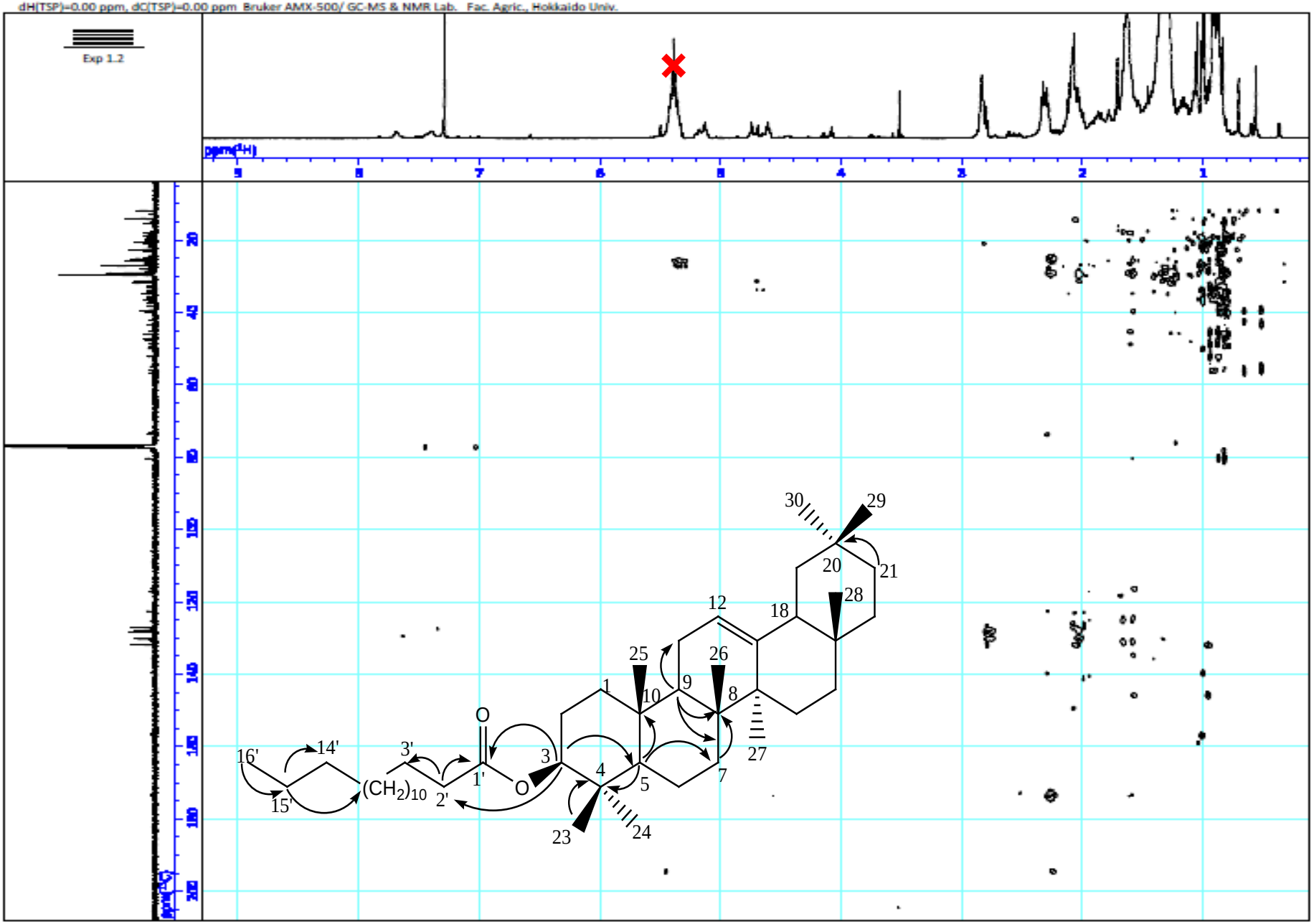


Chart 54. HMBC spectrum of compound **7** (500 MHz, $CDCl_3$).

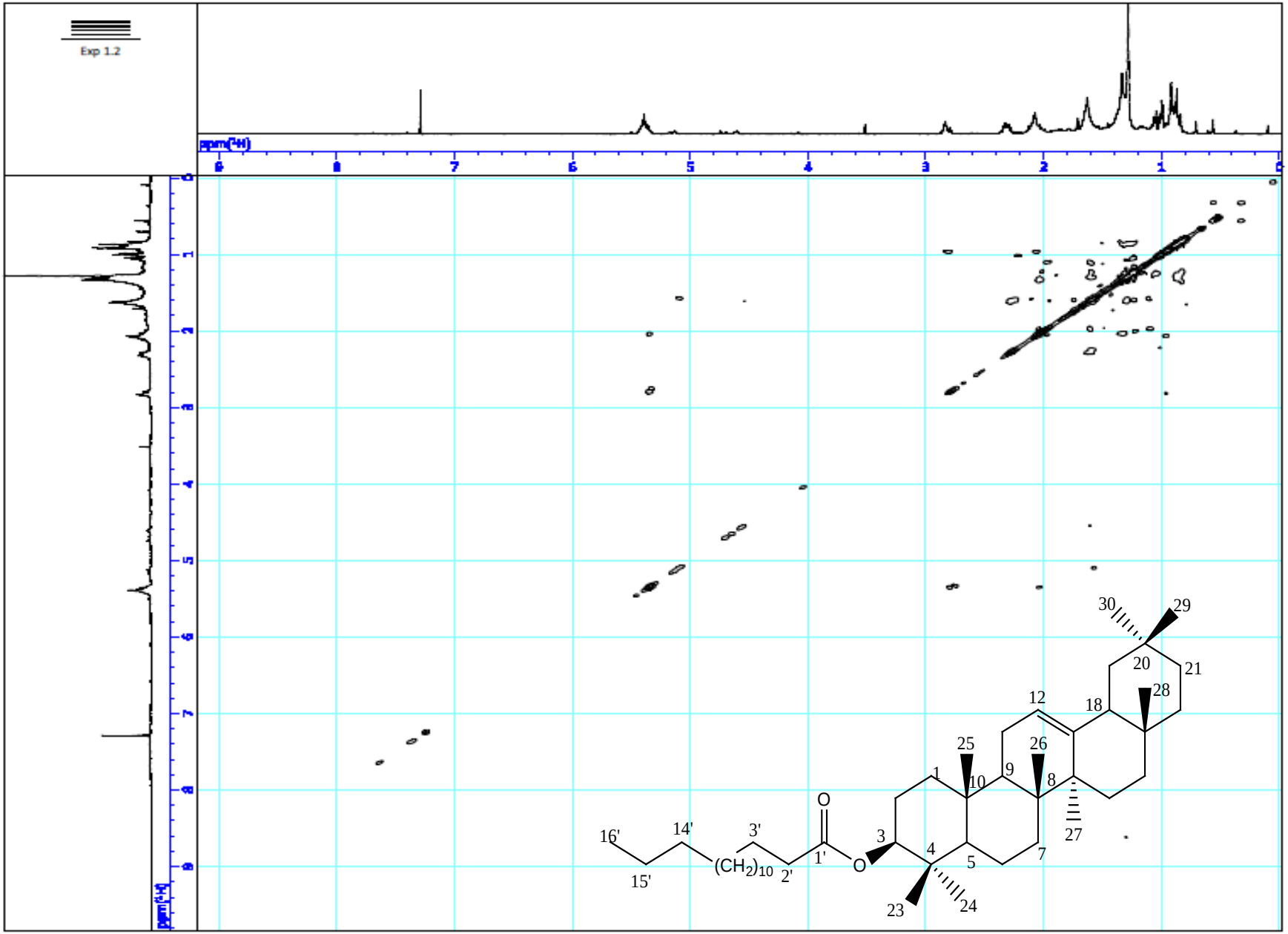


Chart 55. COSYspectrum of compound **7** (500 MHz, $CDCl_3$).

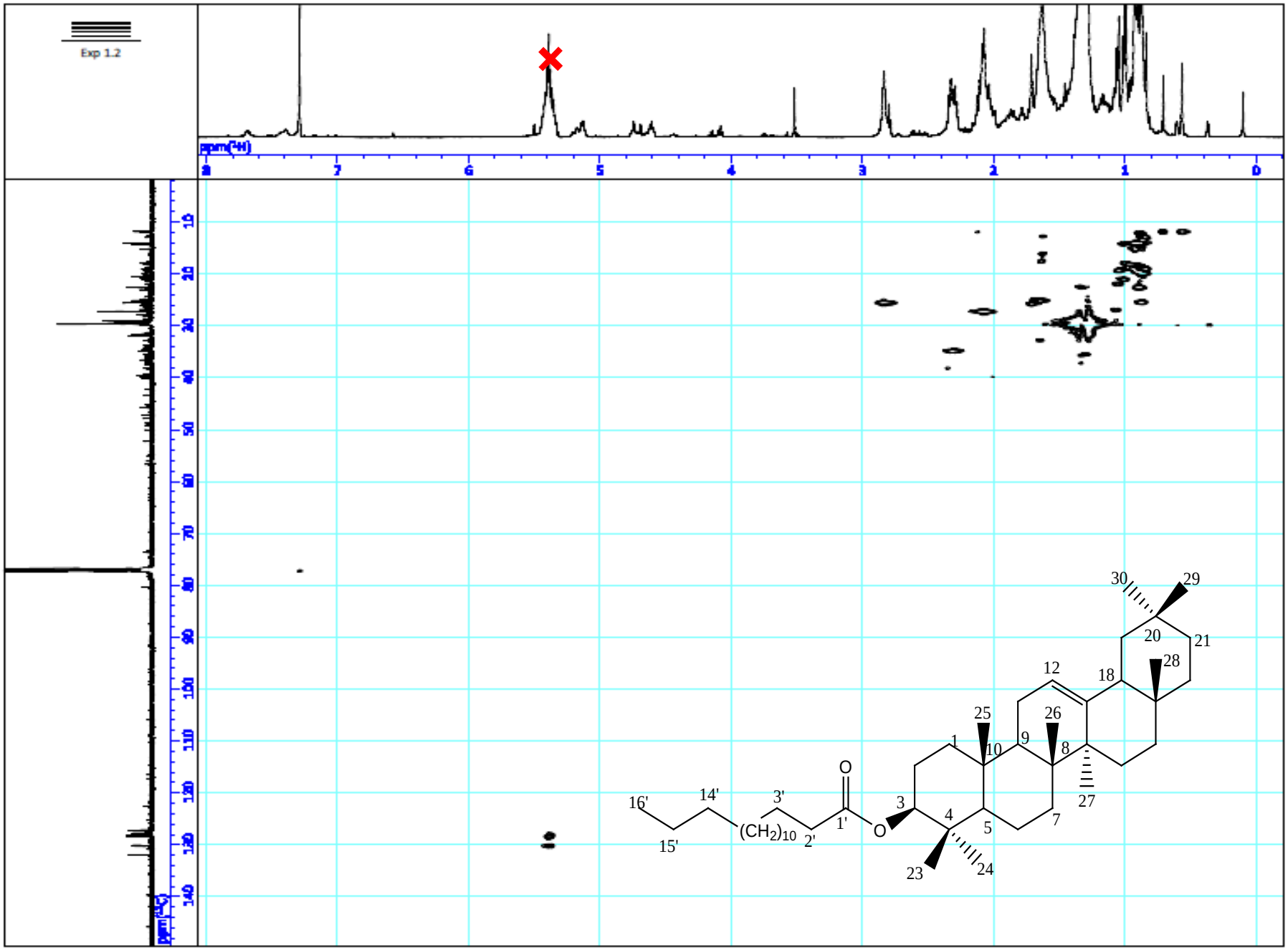


Chart 56. HMQC spectrum of compound **7** (500 MHz, $CDCl_3$).

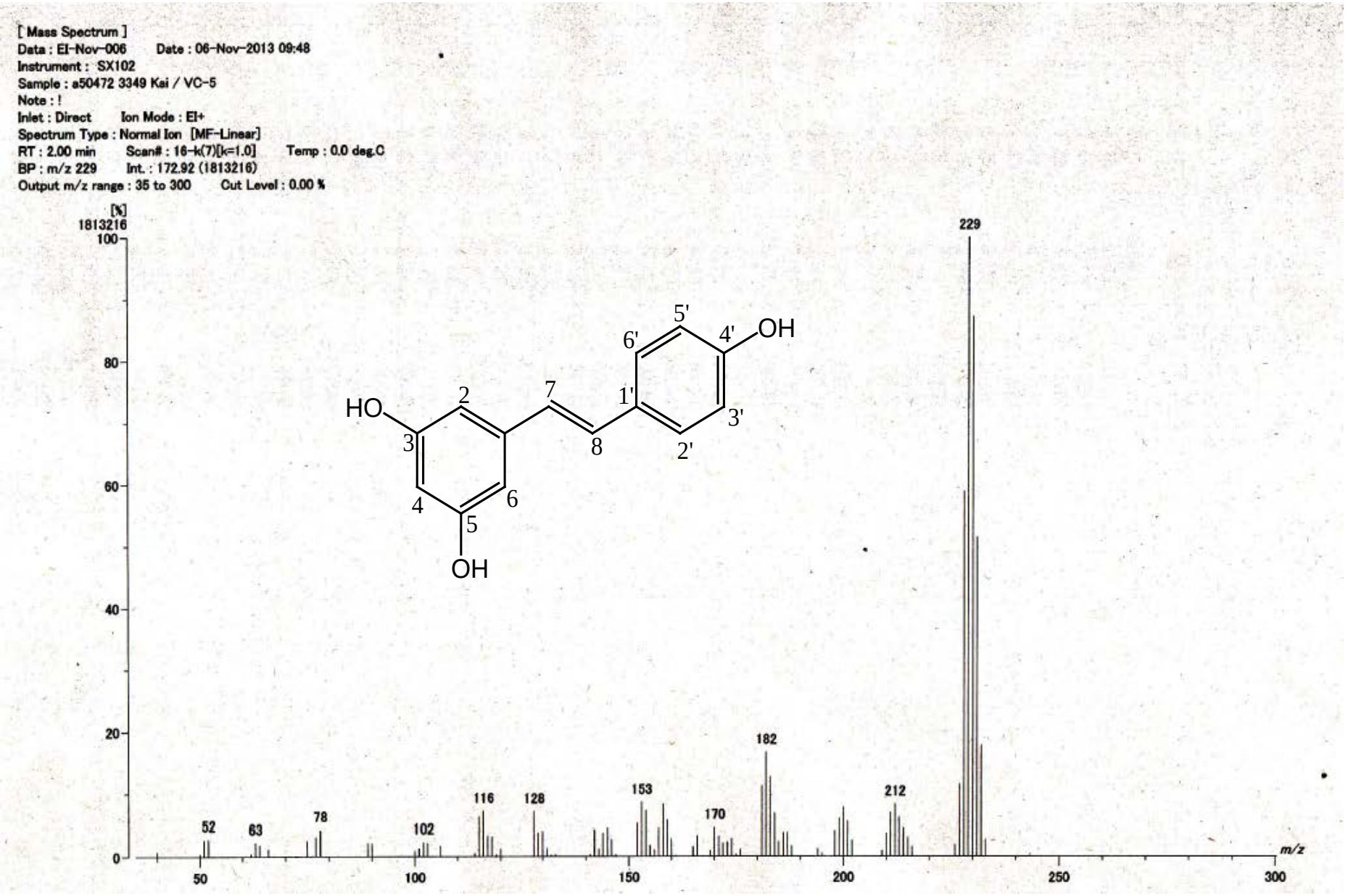


Chart 57. EI-MS data of compound **8** (compound 1).

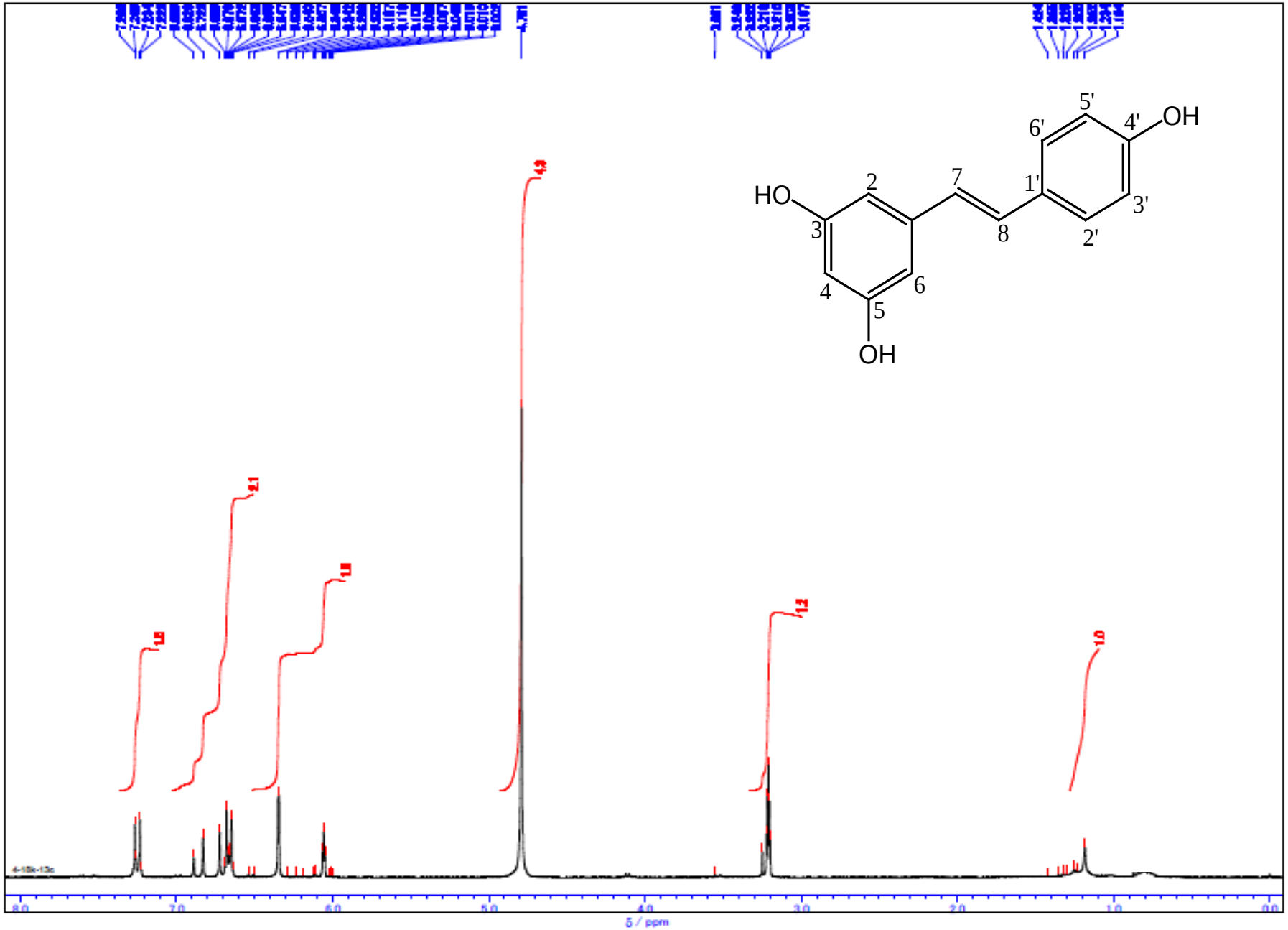


Chart 58. ^{1}H NMR spectrum of compound **8** (500 MHz, CD_3OD).

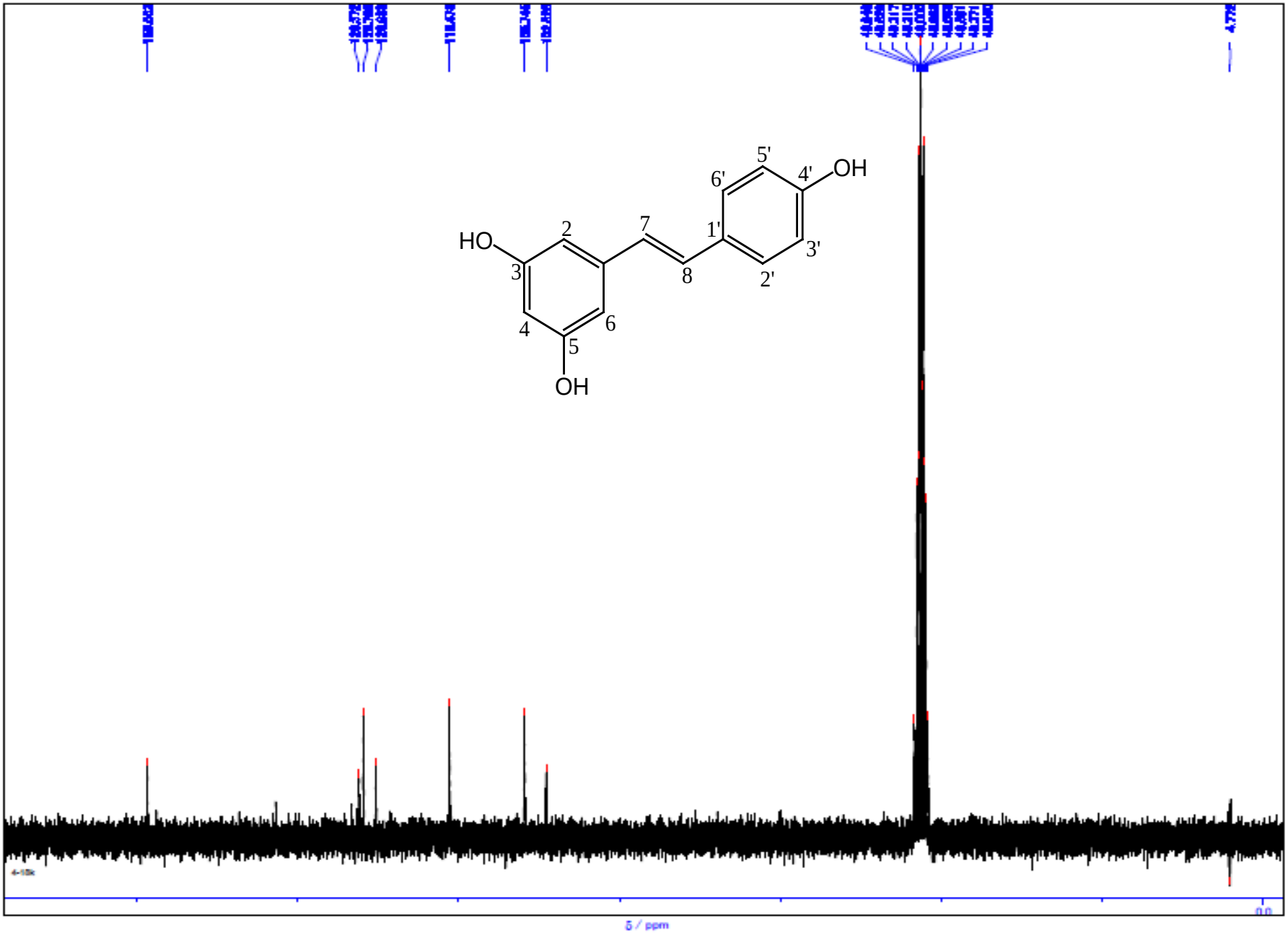


Chart 59. ^{13}C NMR spectrum of compound **8** (500 MHz, CD_3OD).