สารที่มีฤทธิ์ยับยั้งเอนไซม์ไลเปสจากพืชสมุนไพรไทยบางชนิด

นางสาวอริสรากรณ์ ศิรินามารัตนะ

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LIPASE INHIBITORS FROM SOME THAI MEDICINAL PLANTS

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อริสรากรณ์ ศิรินามารัตนะ : สารที่มีฤทธิ์ยับยั้งเอนไซม์ไลเปสจากพืชสมุนไพรไทยบางชนิด. (LIPASE INHIBITORS FROM SOME THAI MEDICINAL PLANTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ.ดร.สุญาณี พงษ์ธนานิกร, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร.รพีพล ภโววาท, รศ.ดร.ธิติมา เพ็งสุภาพ, 219 หน้า.

การคัดกรองฤทธิ์ยับยั้งเอนไซม์ไลเปสของสารสกัดจากพืชสมุนไพรไทย 28 ชนิด ที่ความเข้มข้น 5 ไมโครกรัมต่อมิลลิลิตร ผลการศึกษาพบว่าสารสกัดส่วนลำต้นของขุนไม้ (*Nageia wallichiana*) มีฤทธิ์แรงในการยับยั้งเอนไซม์ (ร้อยละ 86.60±0.66) จากการศึกษาเพื่อแยกสารออกฤทธิ์ สามารถแยกสารบริสุทธิ์ได้ 7 ชนิด ได้แก่ β-sitosterol, nagilactone A, inumakiol D, totarol, ferruginol, bis-2-ethylhexyl-phathalate และ nagilactone E-3-*O*-β-glucopyranoside การพิสูจน์โครงสร้างทางเคมีของสารเหล่านี้อาศัยข้อมูลต่างๆ ทางสเปกโตรสโคปี ได้แก่ UV, IR, MS และ NMR ร่วมกับการเปรียบเทียบกับข้อมูลของสารที่เคยมีการรายงานมาก่อน ผลการศึกษาพบว่ามีสาร 3 ชนิดที่มีฤทธิ์ยับยั้งเอนไซม์ไลเปส ได้แก่ totarol, ferruginol และ bis-2-ethylhexyl-phthalate ซึ่งมีค่าความเข้มข้นที่ออกฤทธิ์ยับยั้งได้ร้อยละ 50 (IC_{50} value) เท่ากับ 31.65±0.19, 30.98±2.24 และ 32.75±1.93 ไมโครโมลาร์ ตามลำดับ ซึ่งสารดังกล่าวมีฤทธิ์ยับยั้งเอนไซม์ไลเปสได้น้อยกว่า orlistat และ gallocatechin gallate ที่มีค่าความเข้มข้นที่ออกฤทธิ์ยับยั้งได้ร้อยละ 50 เท่ากับ 15.86±4.79 และ 198.27±1.47 นาโนโมลาร์ ตามลำดับ สาร totarol และ ferruginol มีโครงสร้างหลักคล้ายสารในกลุ่ม abietane-type diterpenoids ซึ่งเคยมีการรายงานฤทธิ์ยับยั้งเอนไซม์ไลเปส อย่างไรก็ตาม ยังไม่เคยมีการศึกษาฤทธิ์ยับยั้งเอนไซม์ไลเปสของสารที่มีฤทธิ์ทั้งสามชนิดโดยตรง ดังนั้นจึงเป็นการรายงานฤทธิ์นี้เป็นครั้งแรกกับสารดังกล่าว นอกจากนี้ยังเป็นการรายงานการค้นพบสารใหม่ ได้แก่ nagilactone E-3-*O*-β-glucopyranoside

สาขาวิชา เภสัชเคมีและผลิตภัณฑ์ธรรมชาติ ลายมือชื่อนิสิต.....
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The extracts from twenty-eight species of Thai medicinal plants were screened for anti-lipase activity at a final concentration of 5 µg/ml. The stems of *Neagia wallichiana* showed highly potent activity against pancreatic lipase (86.60±0.66%). After the isolation to determine the active compounds from this plant extract, seven pure compounds were isolated including of β-sitosterol, nagilactone A, inumakiol D, totarol, ferruginol, bis-2-ethylhexyl-phathalate, and nagilactone E-3-*O*-β-glucopyranoside. These chemical structures were elucidated based on extensive study of various spectroscopic data including UV, IR, MS and NMR, and comparison with previously reported data. The finding of this study found that the three active compounds were totarol, ferruginol, and bis-2-ethylhexyl-phthalate which were potent inhibitors with the IC_{50} values of 31.65±0.19, 30.98±2.24, and 32.75±1.93 µM respectively. However, these compounds were less potent than orlistat and gallocatechin gallate which their IC_{50} values were 15.86±4.79 and 198.27±1.47 nM respectively. Totarol and ferruginol had the same core skeleton of the structure as abietane-type diterpenoids which had previously been reported for anti-lipase activity. However, this was the first report for anti-lipase activities of these three active compounds. Furthermore, this was the first report on a novel compound as nagilactone E-*O*-β-glucopyranoside.

Field of Study : Pharmaceutical Chemistry and Natural Products	Student's Signature .....
	Advisor's Signature .....
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LIST OF ABBREVIATIONS

α	=	Alpha
ACN	=	Acetonitrile
β	=	Beta
br d	=	Broad doublet (for NMR spectra)
br dd	=	Broad doublets of doublets (for NMR spectra)
br ddd	=	Broad doublets of doublets of doublets (for NMR spectra)
br s	=	Broad singlet (for NMR spectra)
^{o}C	=	Degree Celsius
Calcd	=	Calculated
CC	=	Column chromatography
$CDCl_3$	=	Deuterated chloroform
C_5D_5N	=	Deuterated pyridine
CD_3OD	=	Deuterated methanol
CH_2Cl_2	=	Dichloromethane
$CHCl_3$	=	Chloroform
cm	=	Centimeter
cm^{-1}	=	reciprocal centimeter (unit of wave number)
^{13}C-NMR	=	Carbon-13 Nuclear Magnetic Resonance
d	=	doublet (for NMR spectra)
dd	=	doublet of doublets (for NMR spectra)
ddd	=	doublet of doublets of doublets (for NMR spectra)
dt	=	doublet of triplets (for NMR spectra)
dtd	=	Doublet of triplets of doublets (for NMR spectra)
δ	=	Chemical shift
ESI-MS	=	Electrospray Ionization Mass Spectrometry
EtOAc	=	Ethyl acetate
EtOH	=	Ethanol
g	=	Gram

h	=	Hour
^{1}H-NMR	=	Proton Nuclear Magnetic Resonance
^{1}H,^{1}H-COSY	=	Homonuclear (Proton-Proton) Correlation Spectroscopy
HMBC	=	Heteronuclear Multiple Bond Correlation
HSQC	=	Heteronuclear Single Quantum Coherence
Hz	=	Hertz
IC_{50}	=	Median Inhibitory Concentration
IR	=	Infrared Spectrum
J	=	Coupling constant
KBr	=	Potassium bromide
Kg	=	Kilogram
L	=	Liter
λ_{max}	=	Wavelength at maximal absorption
μg	=	Microgram
μg/ml	=	Microgram per milliliter
μl	=	Microliter
$[M]^{+}$	=	Molecular ion
m	=	Multiplet (for NMR spectra)
MeOH	=	Methanol
mg	=	Milligram
MHz	=	Megahertz
MIC	=	Minimum inhibitory concentration
min	=	Minute
ml	=	Milliliter
mm	=	Millimeter
mp	=	melting point
MS	=	Mass Spectrometry
MW	=	Molecular weight
m/z	=	Mass to charge ratio
Na	=	Sodium

ν_{max}	=	Wave number at maximal absorption
nm	=	Nanometer
NMR	=	Nuclear Magnetic Resonance
NOESY	=	Nuclear Overhauser Enhancement Spectroscopy
ppm	=	Part-per-million
s	=	Singlet (for NMR spectra)
sept	=	Septet (for NMR spectra)
t	=	Triplet (for NMR spectra)
td	=	Triplet of doublets (for NMR spectra)
TLC	=	Thin Layer Chromatography
UV	=	Ultraviolet

CHAPTER I

INTRODUCTION

1.1 Rationale

Obesity is a known health condition and its prevalence is rising globally (World Health Organization 2000). The condition is associated with increasing incidence of mortality (Stevens et al, 1998) and co-morbidities such as type 2 diabetes mellitus, gallblader disease, coronary artery disease, hypercholesterolemia, hypertension, osteoarthritis, and sleep apnea (Must et al, 1999). The important goals of the obesity treatment are weight loss and weight maintenance. Weight loss can be done by several ways and the drug therapy is an adjunct to diet and physical activity for the patients who have the body mass index (BMI) $\geq$ 27 kg/m^2 with co-morbidities.

Drug therapy is divided into two categories by the targets of action in brain and gastrointestinal targets (National Institute of Health National Heart Lung and Blood Institute, 2000). According to the Food Drug Administration (FDA), approval weight-loss drugs for short-term therapy are divided into two categories. The first category includes amphetamine and amphetamine derivatives such as phenmetrazine, phentermine, diethylpropion, phendimetrazine, benzphetamine and mazindol. Due to the addiction awareness and side effect concern, this category needs to be used in a short period for obesity treatment (Colman, 2005). The other category is serotonin agonists such as fenfluramine which is using for short-term therapy and dexfenfluramine for long-term therapy. However, the serotonin agonists were withdrawn from the market because they were associated with primary pulmonary hypertension and valvulopathy (Hutchinson and Ryder, 2007). Nowadays, sibutamine and orlistat are approved for long-term therapy in obesity treatment in conjunction with reduced-calorie diet. Sibutamine is the serotonin and noradrenaline reuptake inhibitor. However, sibutamine must be used cautiously in patients with high blood pressure and it is not recommended in patients

with heart failure, heart disease, arrhythmias, or stroke (Colman, 2005; St. Peter and Khan, 2005; Yeager et al, 2006).

Lipase inhibitors are one of the drugs of choices for long-term treatment for the obesity approved by the FDA (National Institute of Health National Heart Lung and Blood Institute, 2000). Orlistat is a prototype of lipase inhibitor which acts locally to block the absorption of fats by inhibiting pancreatic lipases in the gastrointestinal tract, resulting in weight reduction (Davidson et al, 1999). Although, orlistat has no effect on central nervous system (CNS) and minimally systemic absorption but orlistat can induce the gastrointestinal side effects which may cause the premature withdrawals (Sjöström et al, 1998). Recently, lipase inhibitors from plants such as saponins, polyphenolic compounds and terpenes have garnered increasing attention since they showed sufficient activity (Birari and Bhutani, 2007). There are several medicinal plants available in Thailand that has potential activity. In order to search for the new sources of lipase inhibitors, the present study investigated the lipase inhibitory activity of some Thai medicinal plants.

There are several methods to find out for pancreatic lipase inhibitors depending on the properties of substrates, the products and the methods to detect each product. Generally, most method developments aim to detect the products generated from the substrates more than to measure the decreasing amounts of the substrates. The development of the specific properties in the part of the substrates is easy to be detected by spectroscopic or radiometric method which gives more sensitivity than the conventional method which detects the generated free fatty acid by titration. Nowadays, the chromogenic, fluorogenic and radiometric substrates had been used and developed (Hermetter, 1999; Nakai et al, 2005; Sharma et al, 2005). Although, the sensitivity of these methods is high, the high cost of substrates and special techniques for protecting themselves from the radiation are needed. The tritimetric method is easier to develop but it requires more test sample amounts for testing. This method is similar to body system of animal and human; however, it has low sensitivity, consumes a lot of test sample amounts to determine the activity, and cannot be performed in the 96-well-plate.

Thus, this method is not suitable for screening a lot of test samples in a short time (Hermetter, 1999). The chromogenic and fluorometric substrates are more interesting because of the lower cost than radiometric substrates. In adition, it is easy to detect the products by spectroscopic method and can be developed the experiment in the 96-well plate (Hatano et al, 1997; Slanc et al, 2009). Recently, there were several studies which used *p*-nitorphenol palmitate and 4-methylumbelliferyl oleate as chromogenic and fluorogenic substrates for determining the lipase inhibitory activity of crude extracts or pure compounds from fungi (Slanc et al, 2004) and plants (Kurihara et al, 2003; Kwon et al, 2003; Slanc et al, 2009). However, the fluorometric method has higher sensitivity than chromogenic method. Therefore, in this study the fluorometric method was selected and 4-methylumbelliferyl oleate was used as substrate for bioassay-guided fractionation.

Bioassay-guided fractionations have been used for determining the active compounds for a long time. However, there are some limitations such as a few amount of purified fractions or pure compounds when re-isolation several times. The conventional bioassay guided fractionation may not guide the system for isolation of the active compounds in one step of the bioassay. The thin layer chromatography (TLC)-bioautography can solve these problems. This technique accompany with the isolation and the bioactivity determination of an extract or a pure compound. They can be performed with a small amount of sample in rapid time (Marston, 2011). TLC-bioautography has been developed for screening several bioactivities of the crude extracts or pure compounds such as antibacterial (Chomnawang et al, 2009), antifungal (Burkhead, Schisler and Slininger, 1995), antioxidant (Whittern, Miller and Pratt, 1984), free radical scavenging (Takao et al, 1994), and enzyme inhibition activities (Marston 2011). Recently, there were several enzyme systems that could be developed on TLC plate to screen for the inhibitory activity of the crude extracts such as acetylcholine esterase (Hostettmann et al, 2009), xanthine oxidase (Ramallo et al, 2006), and α- and β-glucosidase systems (Salazar and Furtan, 2007; Hostettmann et al, 2009). However, the system contained pancreatic lipase, a target enzyme for the treatment of obesity, has not been developed. So this study would like to develop the bioautography protocol for the screening the pancreatic lipase inhibitory effect of crude plant extracts.

1.2 Objectives

1. To determine the lipase inhibitory activity of Thai medicinal plants
2. To isolate the active compounds which have lipase inhibitory activity from Thai medicinal plants.
3. To elucidate the structure of the active compounds by spectroscopic method.
4. To develop novel method for screening lipase inhibitor by bioautographic assay.

CHAPTER II

LITERATURE REVIEW

2.1 Obesity

The World Health Organization (WHO) gives the definition of the overweight and obesity as abnormal or excessive fat accumulation that may impair health. In recently, obesity prevalence is rising worldwide at an alarming rate in both developed and developing countries. A cross-sectional random-digit telephone survey in 1991-1998, studied by The Center for Disease Control and Prevention and State Health Departments in the United State reported that the prevalence of obesity gradually increased from 12.0% in 1991 to 17.9% in 1998 (Mokdad et al, 1999). Subsequently, in 1999-2004 the National Health and Nutrition Examination Survey (NHANES) estimated the prevalences of overweight, obese, extremely obese in adults in 1960-1962 though 2007-2008. The trends were rather stable about 31.5-33.6% for overweight, increasing from 13.4% to 34.3% and 0.9% to 6.0% for obese and extremely obese respectively (Ogden et al, 2010b). In children and adolescents, the trends of obesity were increasing from 5.0% to 16.9% in 1963-1965 though 2007-2008 (Ogden et al. 2010a). In the South-East Asia, the prevalences of overweight and obesity in China in 1992 from the China National Nutrition Survey in 82,538 populations was conducted and it showed overweight prevelnce was 23.3% and obesity was less than 4% (Ge, 1997). Later, a cross-sectional nationwide survey of the Japan National Nutrition Survey was carried out in 1990-1994. The results of the prevalences of obesity grade 1 (BMI: 25.0-29.9 kg/m^2), grade 2 (30.0-39.9 kg/m^2), and grade 3 ($\geq$ 40.0 kg/m^2) was estimated to be 24.3%, 1.84% and 0.019% respectively in males, and 20.2%, 2.87% and 0.032% respectively in females (Yoshiike et al, 1998).

In Thailand, the prevalences of overweight and obesity are similar to those in USA. The first research was pioneered in 1990 by Tanphaichitr. The officers aged between 35-54 years were explored. The results showed that the BMI more than 29 kg/m^2 of the male officers and female officers were 2.2% and 3.0% respectively. The

next study in 1991, the First National Health Examination was conducted in 13,300 adults, aged $\geq$ 20 years. The results revealed that 12% of men and 19.5% of women had BMI 25-30 kg/m^2, whereas 1.7% of men and 5.6% of women had BMI >30 kg/m^2 (Thailand Health Research Institute, 1996). In 1995, the National Nutrition Survey of Thailand reported that overweight (BMI > 24.9 kg/m^2) in aged 15-19 years was 23.5% and obesity (BMI >25 kg/m^2) was 15.5%. Later in 2004, The Second National Health Examination Survey was conducted in 4,260 individuals aged 13-59 years. It was found that the overweight prevalences were 19.2% and 33.9% in men and women respectively, and the obesity prevalences were 3.5% and 8.8% in men and women respectively (Aekplakorn et al, 2004).

Obesity is an imbalance condition of energy intake and energy used. Two major factors that cause obesity that cause obesity include endogenous factors (genetic, physiological states, metabolic rate, ability of sensory receptors for receiving of smell and taste, and condition of gastrointestinal tract) and exogenous factors (cultural and religious beliefs and tradition about food that affect eating behaviors, socioeconomic status that reflects the ability to afford or access to food, medical conditions) which effect to imbalance of eating and energy expenditure. Some medical conditions may cause weight gain and obesity (Fankhauser and Kelly, 2009).

Obesity is the cause of several diseases, mortality and morbidity rates (Stevens et al, 1998; Must et al, 1999). These conditions increase the cost for treatment of own diseases and co-morbidities (Birmingham et al, 1999), so the weight reduction can help decrease in mortality, co-morbidities and save cost. It was recommended that the goal of weight loss therapy is to reduce body weight by approximately 8-10% of their initial weight within 6 months of therapy and to maintain this weight for a long time (National Institute of Health National Heart Lung and Blood Institute, 2000). The people who have BMI more than 25 kg/m^2 but less than 27 kg/m^2 and have no risk factor will loss the weight by diet therapy, physical activity, and playing sport for 6 months. If the weight losses less than the goal setting, they will take a medicine. The ones who have BMI $\leq$ 30 kg/m^2 and have no risk factor, or have BMI $\geq$ 27 kg/m^2 but not more than 30 kg/m^2 and

have only one risk factor will be treated by pharmacotherapy The obese who have BMI > 35 kg/m^2 but not more than 40 kg/m^2 and have only one risk factor, they will be treated with pharmacotherapy. The obese who have more than one risk factor or have BMI $\geq$ 40 kg/m^2, they will be treated by surgery.

2.2 Pharmacologic management of obesity

Drug treatment for obesity has been focused on modulating central and/or peripheral sites. So the drug may be divided into 2 main groups, as follow:

2.2.1 CNS acting agents

2.2.1.1 Noradrenergic agents

The mechanism acts through noradrenergic receptors in the lateral hypothalamus by stimulating releases or blocking reuptakes of norepinephine, epinephine and dopamine. Drugs in this class include phenteramine, amphetamine, phenylpropanolamine and ephedrine. Adverse effects of the drugs are arrhythmia, palpitation, insomnia, sweating, constipation, dry mouth, hypertension, and glaucoma. Moreover, derivatives of amphetamine especially metamphetamine causes addiction and abuse potential. Drugs in this class should be used in short-term therapy (St.Peter et al, 1999; Colman, 2005; Mancini and Halpern, 2006).

2.2.1.2 Serotonergic agents

The substances in this group such as fenfluramine and dexfenfluramine affect serotonin release and reuptake at the nerve ending. Although they were used to induce weight loss, they were withdrawn from the market because they caused the risk of primary pulmonary hypertension and left-side valvular degeneration (St.Peter et al. 1999; Colman, 2005).

2.2.1.3 Noradrenergic-serotonergic agents

Sibutramine is a centrally acting agent for antiobesity that inhibits the reuptakes of both serotonin and noradrenaline, and produces a felling of satiety or fullness, and therefore decrease in food intake. It is indicated for the management of

obesity, including weight loss and weight maintenance when used in conjunction with reduced-calorie diet (Smyth and Heron, 2006). Patients with poorly controlled blood pressure, heart failure, heart disease, arrhythmias, and stroke should not use sibutamine (St.Peter et al, 1999; Colman, 2005; Yeager et al, 2006).

2.2.1.4 Leptin analogues and leptin receptor agonists

Leptin, a hormone secreted by adipocytes, provides compelling evidence of a second adiposity signalThe effect of an increase of plasma leptin is decrease in the synthesis of neuropeptide Y and α-melanocyte stimulating hormone corticotrophin releasing hormone which can decrease an appetite. LY-355101 is the developing leptin analogue and leptin receptor agonist (Schwartz et al, 2000; Nawanopparatsakul, 2002).

2.2.1.5 Cannabinoid receptor antagonists

Rimonabant, the first selective CB_1-receptor blocker, is aimed at improving cardio-metabolic risk factors and assisting with weight loss and possibly smoking cessation (Yeager et al, 2006). Stimulation of the CB_1-receptors in fat cells promotes lipogenesis and inhibits the production of adiponectin, a cytokine derived from adipose tissue that has potentially important for anti-diabetic and anti-atherosclerotic properties (Després, Golay and Sjostrom, 2005).

2.2.2 Gastrointestinal acting agents

Orlistat acts locally to block the absorption of fat by irreversible inhibiting lipases in the gastrointestinal tract. It has no systemic effect, and its absorption by gastrointestinal tract is minimal when administered up to 800 mg daily (Colman, 2005). It inhibits absorption of lipids for 30%. Furthermore, it decreases blood pressure more than placebo and improves sugar level in diabetic patients. Side effects of orlistat are flatulence, diarrhea, oily spotting, liquid stools, fecal urgency, and abdominal cramping. The more fat intakes, the more side effects occur. Moreover, it may disturb the absorption of fat-soluble drugs and vitamins. Consequently, the dose of these drugs may be adjusted and fat-soluble vitamins may be supplemented (Guerciolini, 1997; James et al, 1997).

Orlistat is successful in inducing weight loss, because it acts out of the CNS. So it does not cause addiction or any systemic side effects. Several researchers supported for the efficacy of orlistat in order to loss weight. In 1999, Davidson et al (1999) found that taking orlistat 120 mg after meal three times per day for 2 years caused the reduction of low-density lipoprotein cholesterol (p<0.001) and insulin resistance (p=0.04) in 892 obeses (BMI 30-43 kg/m^2) when compared with placebo. Brakris et al. (2002) reported that orlistat (n=267) could decrease diastolic blood pressure more significantly than placebo (p=0.002). In addition, the combination with a mildly reduced-calorie diet, orlistat significantly reduced body weight, and improved glycemic control in 125 overweight and obese Chinese patients (BMI 25-40 kg/m^2) with newly diagnosed type 2 diabetes by decreasing fasting plasma glucose (p=0.0008) and improving HbA_{1c} (p=0.0003) (Shi et al, 2005).

2.3 Triacylglycerol lipases

Triacylglycerol lipases are classified among hydrolases and in subclass of carboxyesterases. Lipases can be found in many kinds of bacteria, fungi, animals and plants. Triacylglycerol lipases in human can be divided into two types according to their sites of action including tissues and gastrointestinal tract. Tissue lipases include lipoprotein lipase, hormone-sensitive lipase and hepatic lipase. For the lipase in human gastrointestinal tract, there are three kinds of lipases when classified by secretary organs. *Lingual lipase* is secreted by lingual serous glands. It hydrolyzes medium- and long chain triglycerides in the stomach (Hamosh, 1990). *Gastric lipase* is secreted by the chief cells which located in the fundic part of the stomach (Canaan et al, 1999). The enzyme is quite acid stable and the optimum pH for hydrolysis of triglycerides is 4.5-5.5. The other type is *pancreatic lipase* which consists of variety of lipases that are responsible for the majority of fat digestion. The most widely studied lipase is pancreatic triglyceride lipase. It is a carboxyl esterase that hydrolyzes acylglycerides but not phospholipids, cholesterol esters, or galactolipids. This enzyme cleaves a broad range

of acyl chain lengths of triglycerides (Whitcomb and Lowe, 2007). It is the major enzyme for triglyceride digestion in the gastrointestinal tract.

2.4 Lipase inhibitory activity from plants

Recently, many researchers have discovered that several medicinal plants have lipase inhibitory activity. Hatano et al (1997) found that dimeric flavans from *Cassia nomame* dried fruits and the semisynthesis of these derivatives could inhibit porcine pancreatic lipase. The IC_{50} value was in the range of 5.5 to more than 50 μM. In addition, Yamamoto et al (2000) investigated the inhibitory activity of the extract of *Cassia nomame* on rats fed with a high fat diet. They found that the lipase inhibitor (CT-II) extracted from the aerial part of *C. nomame* was effective in preventing and ameliorating obesity, fatty liver and hypertriglyceridemia in rats fed with a high fat diet and it could inhibit pancreatic lipase in the *in vitro* study.

Juhel et al (2000) reported that standardized green tea extract containing 25% catechins (Figure 2.1a) could significantly inhibit gastric and pancreatic lipases in the *in vitro* study. It was consistent with the study of Nakai et al (2005) who isolated the oolong tea polyphenols. It was found that polymerization of polyphenols from oolong tea had similar structure to dimeric flavans and flavan-3-ols. They had lipase inhibitory activity with IC_{50} values ranging from 0.092 to more than 20 μM. In addition, He and his colleagues (2007) found that polyphenol from green tea could inhibit α-amylase, pepsin, trypsin, and lipase. The inhibition ratios were 61%, 32%, 38%, and 54% respectively.

Tsutsumi et al (2000) studied the lipase inhibitory activity of the water extract of defatted rice bran. The results showed that the extract inhibited pancreatic lipase *in vitro*, decreased body weight and visceral fat weight of the male Sprague Dawley rat on the fourth week after fed with 10% extract. Yoshikawa et al (2002) reported that epigallocatechin (Figure 2.1c) and epicatechin-(4β->8)-(-)-4'-*O*-methylepigallocatechin (Figure 2.1e) inhibited pancreatic lipase activity with IC_{50} values of 88 and 68 mg/L respectively. Epicatechin, saponin and the tannin fractions inhibited lipoprotein lipase

activity with IC_{50} values of 81, 89, and 35 mg/L respectively. Mangiferin (Figure 2.1b) which belongs to xanthone family had some lipase inhibitory activity.

Kurihara et al (2003) examined the lipase inhibitory activity of *Cyclocarya paliurus* (Batal.) Iljinskaja extract on postprandial hyperlipidemia in mice. A single oral administration of 250 mg/kg of *C. paliurus* extract suppressed an increase in plasma triacylglycerol level when fed with 5 ml/kg of olive oil and lard. Furthermore, the extract showed pancreatic lipase inhibitory activity with an IC_{50} value of 9.1 μg/ml *in vitro*. Subsequently, Moreno et al (2003) assessed the effects of grape seed extract (GSE) on the fat-metabolizing enzymes (pancreatic lipase, lipoprotein lipase, and hormone-sensitive lipase) *in vitro*. They found that GSE showed inhibitory acitvity on pancreatic lipase (80%), lipoprotein lipase (30%) and hormone-sensitive lipase (28%) at the concentration of 1 mg/ml. In the same year, Kwon et al. reported that methanolic extract of *Dioscorea nipponica* Makino powder had potent inhibitory activity against porcine pancreatic lipase with an IC_{50} value of 5-10 μg/ml, where the enzyme activity was assayed using 4-methylumbelliferyl oleate (4-MUO) as substrate. After purification, the active compound was identified as dioscin (Figure 2.1d) and its aglycone (diosgenin) (Figure 2.1f) which belongs to the saponin family. Both dioscin and diosgenin suppressed the time-dependent increase of blood triglyceridel level in mice when orally administered with corn oil.

Mitra and Ray (2003) found that tannins from *Acacia auriculiformis* leaves extract inhibited lipase from *Labeo rohita* fish. So it could be affect the nutritional status and growth of the fish. Shin, Han and Kim (2003) isolated 3-methylethergalangin and 5-hydroxy-7-(4'-hydroxy-3'-methoxyphenyl)-1-phenyl-3-heptanone (Shin et al, 2004) which were flavone and diarylhapanoid from *Alpinia officinarum* could inhibit pancreatic lipase with an IC_{50} values of 1.3 mg/ml and 1.5 mg/ml respectively (Figure 2.2a and 2.2b). Ninomiya et al (2004) found the significant inhibitory effect on decrease in serum triglyceride in olive oil loaded mice when fed with methanolic extract from the leaves of *Salvia officinalis*. This extract showed inhibitory effect against pancreatic lipase in the *in*

vitro with the IC_{50} value of 94 μg/ml. The active component was carnosic acid (Figure 2.2c), a 4-abietan-type diterpene.

Lee and his group (2005) found that crocetin (Figure 2.2d) isolated from the water extract of *Gardenia jasminoides* fruits, showed significant inhibition of pancreatic lipase with an IC_{50} value of 2.1 mg/ml and significant inhibition of the increase in serum triglyceride level in Triton WR-1339-induced hyperlipidemic mice. This compound also showed hypolipidemic activity in hyperlipidemic mice induced by high cholesterol, high fat or high carbohydrate diets for 5 weeks. In the same year, Sharma et al. (2005) found three plants namely *Eriochloa villosa* (Thunb.) Kunth, *Orixa japonica* Thunb. and *Setaria italica* (L.) Palib. that exhibited strong *in vitro* anti-lipase activity by using a radioactive method. Moreover, Han et al (2005) reported that the purified chikusetsusaponins (Figure 2.2e) from *Panax japonicus* rhizomes showed inhibitory effect on pancreatic lipase activity determined by using triolein emulsified with lecithin. Kimura et al. (2006) reported that saponins from *Aesculus turbinate* BLUME could inhibit pancreatic lipase and the most active compound were escins (Figure 2.2f).

There are many plants and some active compounds such as phenolic compounds (flavonoids, xanthones, tannins and others), terpenoids, lignans (diarylhapanoids) and saponins, which have lipase inhibitory activity. Thailand locates in the tropical boundary and has abundantly tropical plants. Consequently, Thai medicinal plants can be the important sources of plants for developing novel lipase inhibitors.

2.5 Methods for determination of lipase inhibitors

The properties of the ideal bioassay for screening of enzyme inhibitors are inexpensive, sensitive to small amount of active materials, selective, simple to run and maintain, and moreover capable of high throughput (Rippin, 2003). There are many methods for screening lipase inhibitors.

2.5.1 Titrimetric method

This method uses emulsified triglycerides such as triolein (Juhel et al, 2000; Shin et al, 2003; Shin et al, 2004; Lee et al, 2005), tributyrin (Juhel et al, 2000; Kim and Kang, 2005), olive oil (He et al, 2007) and triacetin (Kimura et al, 2006) as substrates in aqueous phase. The ester of triacylglycerol is hydrolyzed at position 1 by the pancreatic lipase It also occurs at the water interface of emulsion droplets to release free fatty acid and diacylglycerol as products when the assay system is saturated with substrates. The free fatty acids are determined by titration with NaOH (Lowe, 1999). However, this method is time-consuming, uses high volume of the reaction, and is relatively insensitive because the size of emulsified droplets of substrate may give different results due to changing in the activity.

2.5.2 Radiometric method

This method uses radio label triglyceride such as [^{14}C] triolein (Sharma et al, 2005) or [^{13}H] triolein (Ramaswamy et al, 2002) as a substrate. After the reaction finishes, the radio label products (released free fatty acids) will be separated from diglycerides by thin layer chromatography or liquid-liquid extraction and determined the radio lable free fatty acid by liquid scintillation analyzer. This method has high sensitivity and uses small amount of sample, but it cannot be monitored continuously, needs time-consuming and the chromatographic or organic extraction. Moreover, the well-training is required because of some error from partition of the products (Lowe, 1999).

2.5.3 Turbidity method

This method uses triolein (Arai et al, 1999; Tsutsumi et al, 2000) as a substrate and the lipase activity is measured by determining the clearance of the turbidity of the triolein emulsion. The rate of clearance is measured by photometer and quantitated at the wavelength of 340 nm. This method has low sensitivity but it is convenient to use because of available commercial test kits (Lowe, 1999).

2.5.4 Colorimetric and fluorometric methods

These methods use naturally occurring chromophore or fluorophore tagged with fatty acids by ester bond or fluorescent fatty acids tagged with glycerol as substrates. These substrates express the less color or fluoresce in the assay condition before hydrolzed by pancreatic lipase. When the pancreatic lipase liberates substrates, it gives chromogenic or fluorogenic parts which are measured by spectroscopic method. The substrates for colorimetric method are *p*-nitrophenyl derivatives such as *p*-nitrophenyl palmitate, *p*-nitrophenyl stearate (Prim et al, 2003) and *p*-nitrophenyl butyrate (Kim et al, 2007). The substrates for fluorometric method are 4-methylumbelliferyl derivatives such as 4-methylumbelliferyl oleate (4-MUO) (Hatano et al, 1997; Bitou et al, 1999; Han et al, 2001; Kurihara et al, 2003; Kwon et al, 2003; Nakai et al, 2005; Kimura et al, 2006), 2,3-dimercaptopropanol tributyrate (Ninomiya et al, 2004), triacylglycerol esterified with parinaric acid (Beisson et al, 1999) and others. Both colorimetric and fluorometric methods have high sensitivity and most of the substrates can be determined with microplate and requires a short time. So they are suitable to be used for screening test. However, some short-chain fatty acids and medium-chain derivatives of chromogenic or fluorescent tag are lack of specificity for pancreatic lipase because they are also hydrolyzed by nonspecific carboxylesterases. Chromogenic and fluorescent tag of diacylglycerol or triacylglycerol with chromogenic or fluorescent tag of medium- or long-chain fatty acids is more specific. However, diacylglycerol and triacylglycerol tags need emulsion system to operate.

2.6 TLC-combining with bioassay (Bioautography)

Nowadays, thin-layer chromatography (TLC) in combination with bioassay is available for qualitative screening of the active compounds on various biological activities coupling with standard methods to confirm and avoid the time consuming (Rhee et al, 2001). In the past, TLC in combination with bioassay had been developed for guiding to isolate the active compounds, for example, antibacterial activity (Ahmad and Beg, 2001), antifungal activity (Burkhead et al, 1995; Rangasamy et al, 2007), and

antioxidant activity (Mimica-Dukic et al, 2004). Recently, the enzyme inhibitors played the important roles in the treatment of many diseases, so α-glucosidase inhibitory activity (SimÕes-Pires et al, 2009) and acetylcholine esterase inhibitory activity (Rhee et al, 2001) were developed. However, there is no research on the lipase inhibitory activity.

2.7 The genus *Nageia*

Nageia wallichiana (C.Presl) O. Kuntze is belonging to the Podocarpaceae family. There are 5 species of this genus. Their distributions were in the southern and eastern Asia and throughout Malaysia, the southern and northeastern India, the southern half of China, and the southern Japan to the eastern tip of New Guinea (Eckenwalder, 2009). There are two species in Thailand i.e. *N. motleyi* (Parlatore) De Laubenfels (Synonyms: *Podocarpus motleyi* (Parlatore) Dummer., *Dammara motleyi* Parlatore or *Decussocarpus motleyi* (Parlatore) De Laubenfels) and *N. wallichiana* (Synonyms: *Decussocarpus wallichianus* (Presl) De Laubenf., *Nageia blumei* (Enlicher) G. Gordon, *Podocarpus blumei* Endlicher or *Podocarpus wallichianus* Presl.) The vernacular name of *N. motleyi* was Sang chin (ซางจีน) (Smitinand and Larsen, 1970; Eckenwalder, 2009).

The distributions of *N. wallichiana* are in India, Burma, Vietnam, Laos, Cambodia, Malaysia, and Thailand. In Thailand, this plant can be found in Nan, Nakhon-Ratchasima, Chantaburi, Trat, Ranong, Nakhonsrithammarat, and Satun Provinces. The vernacular name is phayamai (พญาไม้) (South-eastern), and khunmai (ขุนไม้) (Peninsular) (Smitinand and Larsen, 1970; Eckenwalder, 2009).

The plant is an evergreen tree 10-50 m high, 70-150 cm in girth, branchless for half or more of its height and sometimes buttressed. The leaves are coriaceous and opposite or sub-opposite **(Figure 1)**. Their shapes are lanceolate-ovate, 10-18 cm long and 3-5 cm wide. The leave apex is caudate to cuspidate, while the base is acute, obtuse, rounded, or narrowly cuneate into a short petiole. There are 3-7 strobili in a fascicle on a short axillary peduncle. The female flowers are solitary on a terete receptacle. The seeds are globose, green or bluish-purple, about 1.5 cm in diameter on

a thick receptacle or nearly the same size of peduncle, all together 2-4 by 0.2-0.8 cm. (Smitinand and Larsen, 1970; Eckenwalder, 2009).

Figure 1 Leaves of *Nageia wallichiana*

The plant name, *N. wallichiana* and its synonyms were recorded about chemical constituents. Almost all compounds were terpenoids and benzenoids. All of the chemical constituents were shown in **Table 1**. Formerly, *N. wallichiana* was classified as the closely related to genus *Podocarpus* of the family Podocarpaceae. The chemical constituents of both genera were relatively similar. The chemical constituents of recorded *Podocarpus* spp. were summerized in the following part.

Table 1 Chemical constituents of *Nageia wallichina* and synonyms

Compound	Synonym	Plant part	References
A) Benzenoids			
Hydroquinone (1)	*Decussocarpus wallichianus*	Wood	Cambie et al, 1984
B) Terpenoids			
(I) Sesquiterpenes			
Blumenol A (2) (Vomifoliol)	*Podocarpus blumei*	Leaves	Galbraith and Horn, 1972
Blumenol B (3)	*Podocarpus blumei*	Leaves	Galbraith and Horn, 1972
Blumenol C (4)	*Podocarpus blumei*	Leaves	Galbraith and Horn, 1972

Table 1 (continued)

Compound	Synonym	Plant part	References
(II) Diterpenes			
(II.a) Podocarpane type diterpenoids			
Podocarpic acid (5)	*Decussocarpus wallichianus*	Wood	Cambie et al, 1984
(II.b) Totarane type diterpenoids			
4β-Carboxy-19-nortotarol (6)	*Decussocarpus wallichianus*	Wood	Cambie et al, 1984
19-Hydroxy totarol (7)	*Decussocarpus wallichianus*	Wood	Cambie et al, 1984
19-Oxototarol (8)	*Decussocarpus wallichianus*	Wood	Cambie et al, 1984
Totarol (9)	*Decussocarpus wallichianus*	Wood	Cambie et al, 1984
Totaryl acetate (10)	*Decussocarpus wallichianus*	Wood	Cambie et al, 1984
(II.c) Norditerpene dilactone			
2α-Hydroxy-nagilactone G (11)	*Podocarpus wallichiana*	Stem Bark	Ping et al, 1986
Nagilactone C (12)	*Podocarpus wallichiana*	Stem Bark	Ping et al, 1986
Nagilactone D (13)	*Decussocarpus wallichianus*	Wood	Cambie et al, 1984
	Podocarpus wallichiana	Stem Bark	Ping et al, 1986
Nagilactone E (14)	*Podocarpus wallichiana*	Stem Bark	Ping et al, 1986
Nagilactone G (15)	*Podocarpus wallichiana*	Stem Bark	Ping et al, 1986
(III) Triterpenoids			
β-Sitosterol (16)	*Decussocarpus wallichianus*	Wood	Cambie et al, 1984

2.8 Chemical constituents of the genus *Podocarpus*

Several phytochemical components have been found in *Podocarpus*, i.e. flavonoids **(Table 2A, Figure 2 and Figure 3)**, terpenoids **(Table 2B, Figure 4-Figure 7)**, and miscellaneous **(Table 2C, Figure 8)**. Terpenoids and biflavonoids are the main phytochemical compounds found in this genus. Terpenoids were divided into three main categories including sesquiterpenoids, diterpenoids, and triterpenoids.

Table 2 Chemical constituents of the genus *Podocarpus*

Compound	Source	Plant part	References
A) Flavonoids			
(I) all monoflavonoids			
Acacetin (17)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
Apigenin (18)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
Apigenin-7-*O*-glucoside (19)	*P. acutifolius* *P. hallii* *P. nivalis* *P. totara*	Foliages	Markham et al, 1985
Catechin (20)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
	P. macrophyllus var. *macrophyllus*	Leaves	Cheng et al, 2007
Cyanidin (21)	*P. nubigena*	Leaves and Twigs	Silva et al, 1973
Cyanidin-3-*O*-glucoside (22)	*P. elatus*	Fruits	Netzel et al, 2006
	P. lawrencii	Cones and Young Leaves	Crowden and Grubb, 1971
	P. polystachyus	Pink-tinted leaves	Lowry, 1968
	P. totara	Receptacles of female cone	Andersen, 1989
Cyanidin-3-*O*-rhamnoside (23)	*P. polystachyus*	Pink-tinted leaves	Lowry, 1968
Cyanidin-3-*O*-rutinoside (24)	*P. lawrencii*	Cones	Crowden and Grubb, 1971
	P. totara	Receptacles of female cone	Andersen, 1989

Table 2 (continued)

Compound	Source	Plant part	References
Delphinidin-3,5-*O*-diglucoside (25)	*P. polystachyus*	Foliages	Lowry, 1968
Delphinidin-3-*O*-glucoside (26)	*P. polystachyus*	Foliages	Lowry, 1968
Delphinidin-3-neohesperidoside (27)	*P. totara*	Receptacles of female cone	Andersen, 1989
Dihydrokaempferol (28) (Aromadendrin or (2*R*,3*R*)-3,5,7-trihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one)	*P. acutifolius* *P. hallii* *P. nivalis*	Foliages	Markham et al, 1985
Dihydrokaempferol-3-*O*-glucoside (29) (Aromadendrin-3-*O*-glucoside)	*P. nivalis*	Foliages	Markham et al, 1985
Dihydrokaempferol-7-*O*-glucoside (30) (Aromadendrin-7-*O*-glucoside)	*P. acutifolius* *P. hallii* *P. nivalis*	Foliages	Markham et al, 1985
Dihydroquercetin (31)	*P. nivalis*	Foliages	Markham et al, 1985
Dihydroquercetin-3-*O*--β-D-glucoside (32)	*P. nivalis*	Foliages	Markham et al, 1985
Dihydroquercetin-7-*O*-β-D-glucoside (33)	*P. nivalis*	Foliages	Markham et al, 1984
	P. acutifolius *P. hallii* *P. nivalis*	Foliages	Markham et al, 1985
7,4'-Dimethylaromadendrin (34)	*P. neriifolius*	Leaves	Mehdi and Rahman, 1974
7,4'-Dimethylaromadendrin 5-*O*-glucoside (35)	*P. neriifolius*	Leaves	Mehdi and Rahman, 1974
5-Hydroxy-7-methoxy-2-(4-methoxy-phenyl)-chromen-4-one (36)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
Isoorientin (37)	*P. nivalis*	Foliages	Markham et al, 1985
Kaempferol (38)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008

Table 2 (continued)

Compound	Source	Plant part	References
Kaempferol 3-*O*-glucoside (39)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
	P. acutifolius *P. hallii* *P. nivalis*	Foliages	Markham et al, 1985
Kaempferol-7-*O*-glucoside (40)	*P. acutifolius*	Foliages	Markham et al, 1985
	P. fasciculus	Stems and Leaves	Kuo et al, 2008
	P. hallii *P. nivalis*	Foliages	Markham et al, 1985
Luteolin (41)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
Luteolin-3'-*O*-glucoside (42)	*P. acutifolius* *P. nivalis*	Foliages	Markham et al, 1985
Luteolin-7-*O*-glucoside (43)	*P. acutifolius*	Foliages	Markham et al, 1985
	P. fasciculus	Stems and Leaves	Kuo et al, 2008
	P. hallii *P. nivalis*	Foliages	Markham et al, 1985
Luteolin-7-*O*-glucoside-3'-xyloside (44) (Luteolin 7-*O*-β-D-glucoside-3'-*O*-β-D-xyloside)	*P. nivalis*	Foliages	Markham et al, 1984 Markham et al, 1985
Luteolin-3'-*O*-xyloside (45) (Luteolin 3'-*O*-β-D-xyloside)	*P. nivalis*	Foliages	Markham et al, 1984 Markham et al, 1985
7-*O*-Methyl-aromadendrin 5-*O*-β-D-glucopyranoside (46)	*P. nivalis*	Foliages	Markham et al, 1984
7-*O*-Methyl-(2*R*:3*R*)-dihydroquercetin-5-*O*-β-D-glucopyranoside (47)	*P. nivalis*	Foliages	Markham et al, 1984
7-*O*-Methylkaempferol 5-*O*-β-D-glucopyranoside (48)	*P. nivalis*	Foliages	Markham et al, 1984
3-*O*-Methylmyricetin-7-*O*-rhamnoside-3'-*O*-xyloside (49)	*P. hallii*	Foliages	Markham et al, 1985
7-*O*-Methylquercetin-5-*O*-β-D-glucopyranoside (50)	*P. nivalis*	Foliages	Markham et al, 1984

Table 2 (continued)

Compound	Source	Plant part	References
Naringenin (51)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
	P. hallii	Foliages	Markham et al, 1985
Naringenin-7-*O*-glucoside (52)	*P. hallii* *P. nivalis*	Foliages	Markham et al, 1985
Naringin (53)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
Nubigenol (54)	*P. nubigena*	Leaves and Stems	Bhakuni et al, 1973
	P. saligna	Leaves and Stems	Matlin et al, 1984
Orientin (55) (Luteolin-8-*C*-glucoside)	*P. acutifolius*	Foliages	Markham et al, 1985
	P. fasciculus	Stems and Leaves	Kuo et al, 2008
	P. hallii *P. nivalis* *P. totara*	Foliages	Markham et al, 1985
Pelargonidin-3-glucoside (56)	*P. elatus*	Fruits	Netzel et al, 2006
Podocarpin A (57) (Cyanidin-3-neohesperidoside)	*P. lawrencii*	Female Cones	Crowden, 1974
	P. totara	Receptacles of female cone	Andersen, 1989
Quercetin (58)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
	P. macrophyllus var. *macrophyllus*	Leaves	Cheng et al, 2007
	P. nivalis	Foliages	Markham et al, 1985
Quercetin-3-*O*-glucoside (59)	*P. acutifolius*	Foliages	Markham et al, 1985
	P. fasciculus	Stems and Leaves	Kuo et al, 2008
	P. hallii *P. nivalis* *P. totara*	Foliages	Markham et al, 1985
Quercetin-7-*O*-glucoside (60)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
	P. nivalis	Foliages	Markham et al, 1985
2”-*O*-Rhamnosylorientin (61)	*P. acutifolius*	Foliages	Markham et al, 1985
2”-*O*-Rhamnosylvitexin (62)	*P. acutifolius*	Foliages	Markham et al, 1985
Rutin (63)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008

Table 2 (continued)

Compound	Source	Plant part	References
Tricetin (64)	*P. totara*	Foliages	Markham et al, 1985
Tricetin-3'-*O*-glucoside (65)	*P. totara*	Foliages	Markham et al, 1985
Vitexin (66) (Apigenin-8-*C*-glucoside)	*P. acutifolius,* *P. hallii* *P. nivalis* *P. totara*	Foliages	Markham et al, 1985
(II) Biflavonoids			
Amentoflavone (67)	*P. dacrydioides*	Leaves	Krauze-Baranowska et al, 2004
	P. fasciculus	Stems and Leaves	Kuo et al, 2008
	P. neriifolius	Leaves	Haider et al, 1974
	P. taxifolia	Leaves	Hameed et al, 1973
Bilobetin (68)	*P. dacrydioides*	Leaves	Krauze-Baranowska et al, 2004
	P. taxifolia	Leaves	Hameed et al, 1973
2,3-Dihydro-4',4'''-di-*O*-methylamentoflavone (69)	*P. macrophyllus* var. *macrophyllus*	Leaves	Cheng et al, 2007
2,3-Dihydrosciadopitysin (70)	*P. macrophyllus* var. *macrophyllus*	Leaves	Cheng et al, 2007
II-4''',I-7-Dimethoxy amentoflavone (71) (II-4',I-7-Di-*O*-methylamentoflavone)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
	P. taxifolia	Leaves	Roy et al, 1987
Ginkgetin (72)	*P. dacrydioides*	Leaves	Krauze-Baranowska et al, 2004
Heveaflavone (73)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
	P. taxifolia	Leaves	Roy et al, 1987
Hinokiflavone (74)	*P. dacrydioides*	Leaves	Krauze-Baranowska et al, 2004

Table 2 (continued)

Compound	Source	Plant part	References
Isoginkgetin (75)	*P. macrophyllus* var. *macrophyllus*	Leaves	Cheng et al, 2007
	P. dacrydioides	Leaves	Krauze-Baranowska et al, 2004
	P. neriifolius	Leaves	Haider et al, 1974
Kayaflavone (76)	*P. dacrydioides*	Leaves	Krauze-Baranowska et al, 2004
Podocarpus flavone A (77)	*P. dacrydioides*	Leaves	Krauze-Baranowska et al, 2004
	P. fasciculus	Stems/Leaves	Kuo et al, 2008
	P. neriifolius	Leaves	Haider et al, 1974
	P. taxifolia	Leaves	Hameed et al, 1973
	P. urbanii	Leaves/Twigs	Dasgupta et al, 1981
Podocarpus flavone B (78)	*P. dacrydioides*	Leaves	Krauze-Baranowska et al, 2004
	P. neriifolius	Leaves	Haider et al, 1974
Sciadopitysin (79)	*P. dacrydioides*	Leaves	Krauze-Baranowska et al, 2004
	P. macrophyllus var. *macrophyllus*	Leaves	Cheng et al, 2007
	P. urbanii	Leaves/Twigs	Dasgupta et al, 1981
Sequoiaflavone (80)	*P. dacrydioides*	Leaves	Krauze-Baranowska et al, 2004
	P. taxifolia	Leaves	Hameed et al, 1973
B) Terpenoids			
(I) Sesquiterpenoids			
Atractylon (81)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Bicyclogermacrene (82)	*P. spicatus*	Foliages(oil)	Lorimer and Rex, 1987
δ-Cadinene (83)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
α-Copaene (84)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Curzerenone (85)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987

Table 2 (continued)

Compound	Source	Plant part	References
δ-Elemene (86)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
(+)-Germacrene D (87)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
(+)-Longifolene (88)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Podoandin (89)	*P. andina*	Leaves	Kubo et al, 1992
Selina-4(14),7(11)-diene (90)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Vomifoliol (2) (Blumenol A)	*P. nagi*	Stem barks	Kubo and Ying, 1991a
(II) Diterpenoids			
(II.a) Abietane type diterpenoids			
8,9-Abieten-15-ol (91)	*P. lambertius*	Leaves	De Paiva et al, 1975
19-Acetoxy ferruginol (92)	*P. ferrugineus*	Heartwood	Cambie et al, 1984
Carnosol (93)	*P. koordersii*	Wood	Cambie et al, 1984
Ferruginol (94)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
15-Hydroxy dehydroabietic acid (95)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008
19-Hydroxy ferruginol (96)	*P. ferrugineus*	Heartwood	Cambie et al, 1984
Lambertic acids (97)	*P. lambertius*	Bark	De Paiva et al, 1975
Pododacric acid (98)	*P. neriifolius*	Wood	Cambie et al, 1983
Sugiol (99)	*P. andina*	Leaves	Kubo et al, 1992
	P. gracilior	Wood	Cambie et al, 1983
	P. hallii	Heartwood	Cambie and Russell, 1973
	P. nagi	Root bark	Ying and Kubo, 1991
(II.b) Bayerane type diterpenoids			
Beyerene (100)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
(II.c) Kaurane type diterpenoids			
Kaurene (101)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
(II.d) Labdane type diterpenoids			
16-Hydroxy communic acid (102)	*P. fasciculus*	Stems and Leaves	Kuo et al, 2008

Table 2 (continued)

Compound	Source	Plant part	References
(II.e) Pimarane type diterpenoids			
8β-Hydroxy isopimarene (103)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Isopimara-7,15-diene (104)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Isopimara 8,15-diene (105)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Isopimaric acid (106)	*P. lambertius*	Leaves	De Paiva Campello et al, 1975
Pimara-8(14),15-diene (107)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Rimuene (108)	*P. nubigena*	Leaves/Twigs	Silva et al, 1973
	P. saligna	Leaves/Stems	Matlin et al, 1984
	P. spicatus	Foliages (oil)	Lorimer and Rex, 1987
Rosadiene (109)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Sandaracopimaradiene (110)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
(II.f) Podocarpane type diterpenoids			
7-Hydroxy-1,4-α-dimethyl-1,2,3,4, 4a,9,10,10a-octahydro-phenan-threne-1-carboxylic acid (111)	*P. fasciculus*	Stems/Leaves	Kuo et al, 2008
Methylpodocarpate (112)	*P. acutifolius*	Heartwood	Bennett and Cambie, 1967
	P. nivalis	Heartwood	Bennett and Cambie, 1967
Podocarpa-8,11,13-trin-16-oic acid (113)	*P. fasciculus*	Stems/Leaves	Kuo et al, 2008
Podocarpic acid (5)	*P. acutifolius*	Heartwood	Bennett and Cambie, 1967
	P. fasciculus	Stems/Leaves	Kuo et al, 2008
	P. hallii	Heartwood	Cambie and Russell, 1973
	P. nivalis	Heartwood	Bennett and Cambie, 1967
(-)-Sclarene (114)	*P. spicatus*	Foliages (oil)	Lorimer and Rex, 1987
Sempervirol (115)	*P. neriifolius*	Wood	Cambie et al, 1983

Table 2 (continued)

Compound	Source	Plant part	References
(II.g) Phyllocladene type diterpenoids			
Isophyllocladene (116)	*P. lambertius*	Leaves	De Paiva et al, 1975
17-Isophyllocladenol (117)	*P. lambertius*	Leaves	De Paiva et al, 1975
Phyllocladene (118)	*P. lambertius*	Leaves	De Paiva et al, 1975
	P. spicatus	Foliages (oil)	Lorimer and Rex, 1987
(II.h) Totarane type diterpenoids			
4β-Carboxy-19-nortotarol (6)	*P. affinis*	Wood	Cambie et al, 1984
	P. falcatus	Wood	Cambie et al, 1984
	P. gracilior	Wood	Cambie et al, 1983
	P. koordersii	Wood	Cambie et al, 1984
	P. lambertius	Bark	De Paiva et al, 1975
	P. nagi	Root bark	Ying and Kubo, 1991
	P. neriifolius	Wood	Cambie et al, 1983
	P. polystachyus	Wood	Cambie et al, 1983
Cycloinumakiol (119)	*P. latifolius*	Root Barks	Devkota et al, 2011
3β-Hydroxy totarol (120)	*P. lambertius*	Bark	De Paiva et al, 1975
16-Hydroxy totarol (121)	*P. hallii*	Heartwood	Cambie and Russell, 1973
19-Hydroxy totarol (7)	*P. affinis*	Wood	Cambie et al, 1984
	P. gnidiodes	Wood	Cambie et al, 1983
	P. nagi	Root bark	Ying and Kubo, 1991
	P. sylvestris	Wood	Cambie et al, 1983
Inumakal (122)	*P. latifolius*	Root Barks	Devkota et al, 2011
Inumakoic acid (123)	*P. latifolius*	Root Barks	Devkota et al, 2011
16-Oxototarol (124)	*P. hallii*	Heartwood	Cambie and Russell, 1973
19-Oxototarol (8)	*P. gnidiodes*	Wood	Cambie et al, 1983
(Totaral)	*P. milanjianus*	Bark	Hembree et al, 1979
	P. polystachyus	Wood	Cambie et al, 1983
	P. sellowii	Bark	Hembree et al, 1979
Totaradiol (125)	*P. nagi*	Root bark	Ying and Kubo, 1991
Totaral (8) (19-Oxototarol)	*P. nagi*	Root bark	Ying and Kubo, 1991

Table 2 (continued)

Compound	Source	Plant part	References
Totarol (9)	*P. acutifolius*	Heartwood	Bennett and Cambie, 1967
	P. affinis	Wood	Cambie et al, 1984
	P. falcatus	Wood	Cambie et al, 1984
	P. gnidiodes	Wood	Cambie et al, 1983
	P. gracilior	Wood	Cambie et al, 1983
	P. hallii	Heartwood	Cambie and Russell, 1973
	P. koordersii	Wood	Cambie et al, 1984
	P. milanjianus	Bark	Hembree et al, 1979
	P. nagi	Root bark	Ying and Kubo, 1991
	P. nivalis	Heartwood	Bennett and Cambie, 1967
	P. neriifolius	Wood	Cambie et al, 1983
	P. polystachyus	Wood	Cambie et al, 1983
	P. sellowii	Bark	Hembree et al, 1979
	P. sylvestris	Wood	Cambie et al, 1983
Totaryl acetate (10)	*P. gnidiodes* *P. sylvestris*	Wood	Cambie et al, 1983
Podototarin (126) (macrophyllic acid)	*P. acutifolius*	Heartwood	Bennett and Cambie, 1967
	P. falcatus *P. koordersii*	Wood	Cambie et al, 1984
	P. neriifolius	Wood	Cambie et al, 1983
	P. nivalis	Heartwood	Bennett and Cambie, 1967
	P. polystachyus	Wood	Cambie et al, 1983
(II.i) Dimeric of diterpenes			
Podototarin monoacetate (127)	*P. acutifolius*	Heartwood	Bennett and Cambie, 1967
	P. nivalis	Heartwood	Bennett and Cambie, 1967
(II.j) Norditerpene dilactones			
2,3-Dehydro-16-hydroxynagilactone F (128)	*P. nagi*	Root bark	Ying et al, 1990
2,3-Dehydronagilactone A (129)	*P. nai*	Root barks	Ying and Kubo, 1993

Table 2 (continued)

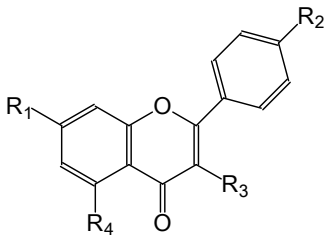
Compound	Source	Plant part	References
1-Deoxy-2,3-dehydronagilactone A (130)	*P. nagi*	Root barks	Ying and Kubo, 1993
1-Deoxy-2β,3β-epoxynagilactone A (131)	*P. nagi*	Seed Rinds	Zhang et al, 1992
1-Deoxy-2α-hydroxynagilactone A (132)	*P. nagi*	Endosperms	Zhang et al, 1992
1-Deoxynagilactone A (133)	*P. nagi*	Root barks	Ying and Kubo, 1993
2,3-Dihydropodolide (134)	*P. urbanii*	Leaves and Twigs	Dasgupta et al, 1981
3-Epinagilactone C (135)	*P. nagi*	Leaves	Kubo and Ying, 1991b
Hallactone B (136)	*P. macrophyllus* var. *maki*	Leaves	Park et al, 2004
16-Hydroxy nagilactone E (137)	*P. nagi*	Root bark	Ying et al, 1990
	P. nagi	Stem barks	Kubo and Ying, 1991a
2α-Hydroxy nagilactone F (138)	*P. nagi*	Root bark	Kubo et al, 1991
2α-Hydroxy nagilactone G (11)	*P. nagi*	Leaves	Kubo and Ying, 1991b
Inumakilactone (139)	*P. latifolius*	Root Barks	Devkota et al, 2011
Inumakilactone B (140)	*P. latifolius*	Root Barks	Devkota et al, 2011
Nagilactone C (12)	*P. fasciculus*	Stems/Leaves	Kuo et al, 2008
	P. nagi	Stem bark	Kubo and Ying, 1991a
	P. neriifolius	Twigs	Shrestha et al, 2001
	P. nubigena	Leaves/Twigs	Silva et al, 1973
	P. purdieanus	Bark	Wenkert and Chang, 1974
	P. urbanii	Leaves/Twigs	Dasgupta et al, 1981
	P. nagi	Seed Rinds	Zhang et al, 1992
Nagilactone D (13)	*P. nagi*	Stem barks	Kubo and Ying, 1991a
	P. nagi	Endosperms	Zhang et al, 1992
Nagilactone F (141)	*P. milanjianus* *P. sellowii*	Bark	Hembree et al, 1979
	P. latifolius	Root Barks	Devkota et al, 2011

Table 2 (continued)

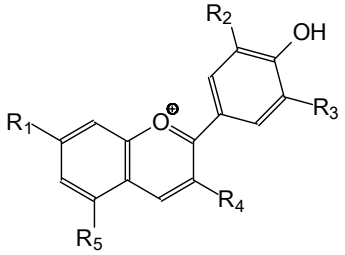
Compound	Source	Plant part	References
Nagilactone G (15)	*P. milanjianus* *P. sellowii*	Bark	Hembree et al, 1979
Nagilactone I (142)	*P. nagi*	Stem barks	Kubo et al. 1991b
		Root bark	Ying et al. 1990
Nagilactone J (143)	*P. nagi*	Stem bark	Kubo and Ying, 1991a
Nubilactone A (144)	*P. nubigena*	Leaves/ Twigs	Bhakuni et al, 1973
Nubilactone A monoacetate (145)	*P. nubigena*	Leaves/ Twigs	Bhakuni et al, 1973
Podolactone C (146)	*P. milanjianus*	Stem barks	Cassady et al, 1984
	P. macrophyllus var. *maki*	Leaves	Park et al, 2004
S_R-Podolactone D (147)	*P. macrophyllus* var. *maki*	Leaves	Park et al, 2003
	P. macrophyllus var. *maki*	Leaves	Park et al, 2004
S_S-Podolactone D (148)	*P. macrophyllus* var. *maki*	Leaves	Park et al, 2003
	P. macrophyllus var. *maki*	Leaves	Park et al, 2004
Podolide (149)	*P. urbanii*	Leaves and Twigs	Dasgupta et al, 1981
Rakanmakilactone A (150)	*P. macrophyllus* var. *maki*	Leaves	Park et al, 2004
Rakanmakilactone B (151)			
Rakanmakilactone C (152)			
Rakanmakilactone D (153)			
Rakanmakilactone E (154)			
Rakanmakilactone F (155)			
Salignone I (156)	*P. saligna*	Roots	Matlin et al, 1984
Salignone K (157)	*P. saligna*	Leaves/Stems	Matlin et al, 1984
Salignone L (158)	*P. saligna*	Leaves/Stems	Matlin et al, 1984
Salignone M (159)	*P. saligna*	Leaves/Stems	Matlin et al, 1984

Table 2 (continued)

Compound	Source	Plant part	References
Sellowin A (160)	*P. hallii*	Heartwood	Cambie and Russell, 1973
(III) Triterpenoids			
(III.a) Fernane type triterpenoids			
Fern-9(11)-ene (161)	*P. saligna*	Leaves	Silva et al, 1972
Isofernene (162)	*P. saligna*	Leaves	Silva et al, 1972
(III.b) Steroids			
Campesterol (163)	*P. nivalis*	Heartwood	Bennett and Cambie, 1967
Daucosterin (164) (β-sitosterol-3-*O*-glucoside)	*P. nubigena*	Leaves/Twigs	Silva et al, 1973
Ecdysterol (165)	*P. elata*	Seedlings	Sauer et al, 1968
	P. urbanii	Leaves/Twigs	Dasgupta et al, 1981
β-Sitosterol (16)	*P. acutifolius*	Heartwood	Bennett and Cambie, 1967
	P. lambertius	Leaves	De Paiva et al, 1975
	P. nivalis	Heartwood	Bennett and Cambie, 1967
	P. nubigena	Leaves/Twigs	Silva et al, 1973
	P. saligna	Leaves	Silva et al, 1972
	P. urbanii	Leaves/Twigs	Dasgupta et al, 1981
	P. ustus	Wood	Cambie et al, 1984
Stigmastan-3β,5α-diol-6-one (166)	*p. lambertius*	Leaves	De Paiva et al, 1975
C) Miscellaneous			
(I) Benzenoids			
Benzene-1,2,3,4-tetraol (167)	*P. fasciculus*	Stems/Leaves	Kuo et al, 2008
4-Ethylphenol (168)	*P. fasciculus*	Stems/Leaves	Kuo et al, 2008
5-(3-Hydroxy propyl)-2-methoxy phenol (169)	*P. fasciculus*	Stems/Leaves	Kuo et al, 2008
4-Methylphenol (170)	*P. fasciculus*	Stems/Leaves	Kuo et al, 2008
(II) Sugars			
Sequoyitol (171)	*P. sellowii*	Leaves	Mukherjee and De Medeiros, 1988
(III) Lignans			
Thujaplicatin methyl ether (172)	*P. saligna*	Roots	Matlin et al, 1984

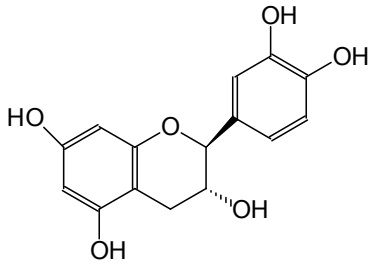


	R_1	R_2	R_3	R_4
Acacetin (17)	OH	OCH_3	H	OH
Apigenin (18)	OH	OH	H	OH
Apigenin-7-*O*-glucoside (19)	*O*-Glc	OH	H	OH
5-Hydroxy-7-methoxy-2-(4-methoxy-phenyl)-chromen-4-one (36)	OCH_3	OCH_3	H	OH
Kaempferol (38)	OH	OH	OH	OH
Kaempferol-3-*O*-glucoside (39)	OH	OH	*O*-Glc	OH
Kaempferol-7-*O*-glucoside (40)	*O*-Glc	OH	OH	OH
7-*O*-Methylkaempferol-5-*O*-β-D-glucopyranoside (48)	OCH_3	OH	OH	*O*-Glc

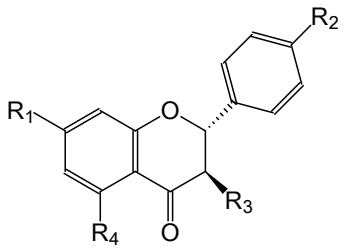


	R_1	R_2	R_3	R_4	R_5
Cyanidin (21)	OH	OH	H	OH	OH
Cyanidin-3-*O*-glucoside (22)	OH	OH	H	*O*-Glc	OH
Cyanidin-3-*O*-rhamnoside (23)	OH	OH	H	*O*-Rha	OH
Cyanidin-3-*O*-rutinoside (24)	OH	OH	H	*O*-Glc-(6←1)-Rha	OH
Delphinidin-3,5-*O*-diglucoside (25)	OH	OH	OH	*O*-Glc	*O*-Glc
Delphinidin-3-*O*-glucoside (26)	OH	OH	OH	*O*-Glc	OH
Delphinidin-3-neohesperidoside (27)	OH	OH	OH	*O*-Glc-(2←1)-Rha	OH
Pelargonidin-3-glucoside (56)	OH	H	H	*O*-Glc	OH
Podocarpin A (57) (Cyanidin-3-neohesperoside)	OH	OH	H	*O*-Glc-(2←1)-Rha	OH

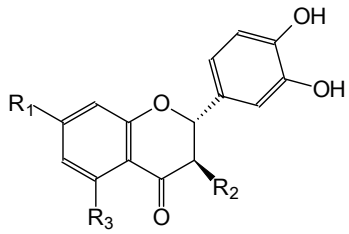
Figure 2 Chemical structures of monoflavonoids in the genus *Podocarpus*



Catechin (20)

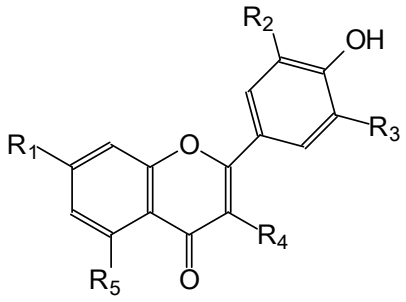


	R_1	R_2	R_3	R_4
Dihydrokaempferol (Aromadendrin) (28)	OH	OH	OH	OH
Dihydrokaempferol-3-*O*-glucoside (29) (Aromadendrin-3-*O*-glucoside)	OH	*O*-Glc	OH	OH
Dihydrokaempferol-7-*O*-glucoside (30) (Aromadendrin-7-*O*-glucoside)	*O*-Glc	OH	OH	OH
7,4'-Dimethoxylaromadendrin (34)	OCH_3	OCH_3	OH	OH
7,4'-Dimethoxylaromadendrin-5-*O*-glucoside (35)	OCH_3	OCH_3	OH	*O*-Glc
7-*O*-Methylaromadendrin-5-*O*-glucopyranoside (46)	OCH_3	OH	OH	*O*-Glc

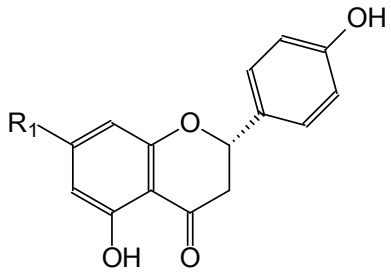


	R1	R2	R3
Dihydroquercetin (31)	OH	OH	OH
Dihydroquercetin-3-*O*-β-D-glucoside (32)	OH	*O*-Glc	OH
Dihydroquercetin-7-*O*-β-D-glucoside (33)	*O*-Glc	OH	OH
7-*O*-Methyl-(2*R*:3*R*)-dihydroquercetin-5-*O*-β-D-glucopyranoside (47)	OCH_3	OH	*O*-Glc

Figure 2 (continued)

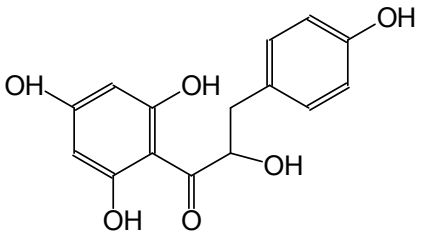


	R_1	R_2	R_3	R_4	R_5
Luteolin (41)	OH	OH	H	H	OH
Luteolin-3'-*O*-glucoside (42)	OH	*O*-Glc	H	H	OH
Luteolin-7-*O*-glucoside (43)	*O*-Glc	OH	H	H	OH
Luteolin-7-*O*-glucoside-3'-*O*-xyloside (44)	*O*-Glc	*O*-Xyl	H	H	OH
Luteolin-3'-*O*-xyloside (45)	OH	*O*-Xyl	H	H	OH
3-*O*-Methylmyricetin-7-*O*-rhamnoside-3'-*O*-xyloside (49)	*O*-Rha	*O*-Xyl	OH	OCH_3	OH
7-*O*-Methylquercetin-5-*O*-β-D-glucopyranoside (50)	OCH_3	OH	H	OH	*O*-Glc
Quercetin (58)	OH	OH	H	OH	OH
Quercetin-3-*O*-glucoside (59)	OH	OH	H	*O*-Glc	OH
Quercetin-7-*O*-glucoside (60)	*O*-Glc	OH	H	OH	OH
Rutin (63)	OH	OH	H	*O*-Rha-(1⟶6)-Glc	OH
Tricetin (64)	OH	OH	OH	H	OH
Tricetin-3'-*O*-glucoside (65)	OH	*O*-Glc	OH	H	OH

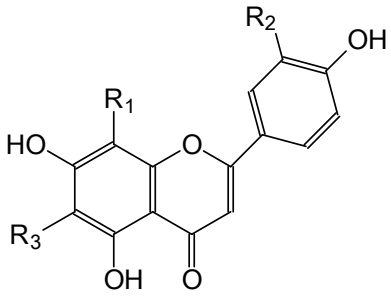


	R
Naringenin (51)	OH
Naringenin-7-*O*-glucoside (52)	O-Glc
Naringin (53)	*O*-Glc-(2⟵1)-Rha

Figure 2 (continued)

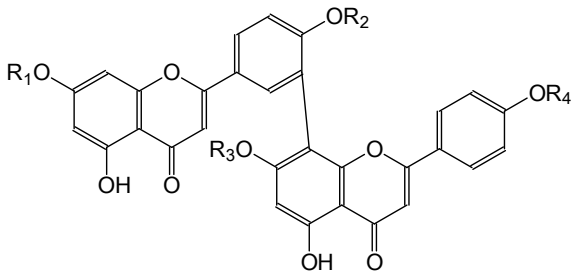


Nubigenol (54)

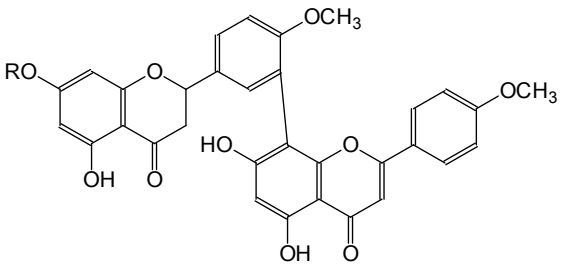


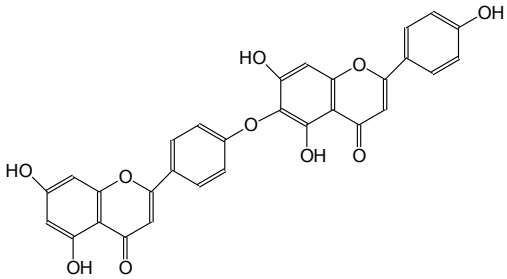
	R_1	R_2	R_3
Isoorientin (37)	H	OH	*C*-Glc
Orientin (55)	*C*-Glc	OH	H
2''-*O*-Rhamnosylorientin (61)	H	OH	*C*-Glc-(2←1)-Rha
2''-*O*-Rhamnosylvitrexin (62)	*C*-Glc-(2←1)-Rha	H	H
Vitrexin (Apigenin-8-*C*-glucoside) (66)	*C*-Glc	H	H

Figure 2 (continued)



	R_1	R_2	R_3	R_4
Amentoflavone (67)	H	H	H	H
Bilobetin (68)	H	CH_3	H	H
II-4‴,I-7-Dimethoxy amentoflavone (71) (II-4',I-7-Di-*O*-methylamentoflavone)	CH_3	H	H	CH_3
Ginkgetin (72)	CH_3	CH_3	H	H
Heveaflavone (73)	CH_3	H	CH_3	CH_3
Isoginkgetin (75)	H	CH_3	H	CH_3
Kayaflavone (76)	H	CH_3	CH_3	CH_3
Podocarpus flavone A (77)	H	H	H	CH_3
Podocarpus flavone B (78)	CH_3	H	H	CH_3
Sciadopitysin (79)	CH_3	CH_3	H	CH_3
Sequoiaflavone (80)	CH_3	H	H	H

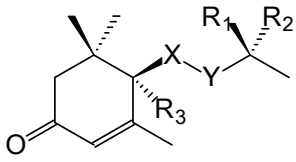




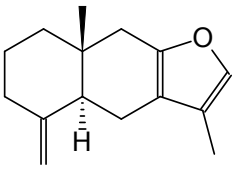
Hinokiflavone (74)

	R
2,3-Dihydro-4',4‴-di-*O*-methyl amentoflavone (69)	H
2,3-Dihydrosciadopitysin (70)	CH_3

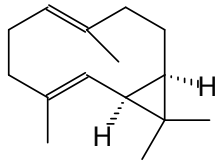
Figure 3 Chemical structures of Biflavonoids in the genus *Podocarpus*



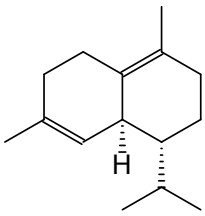
	X	Y	R1	R2	R3
Blumenol A (Vomifoliol) (2)	CH=CH		H	OH	OH
Blumenol B (3)	CH_2-CH_2		H	OH	OH
Blumenol C (4)	CH_2-CH_2		OH	H	H



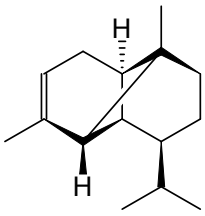
Atractylon (81)



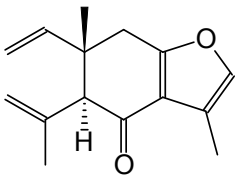
Bicyclogermacrene (82)



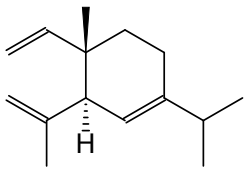
δ-Cadinene (83)



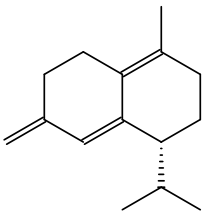
α-Copaene (84)



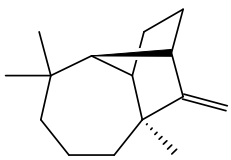
Curzerenone (85)



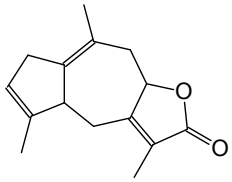
δ-Elemene (86)



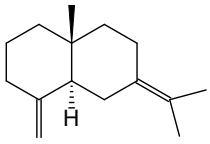
(+)-Germacrene D (87)



(+)-Longifolene (88)

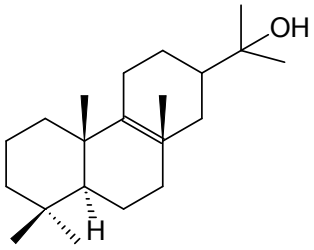


Podoandin (89)

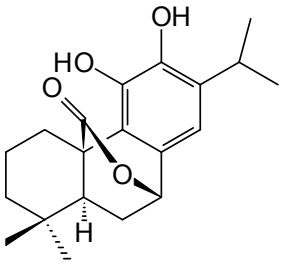


Selina-4(14),7(11)-diene (90)

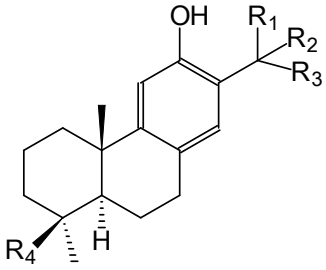
Figure 4 Chemical structures of Sesquiterpenes in the genus *Podocarpus*



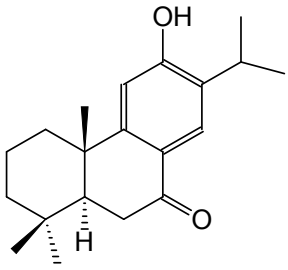
8,9-Abieten-15-ol (91)



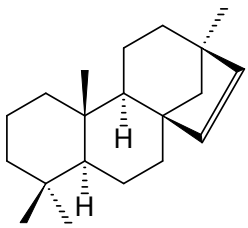
Carnosol (93)



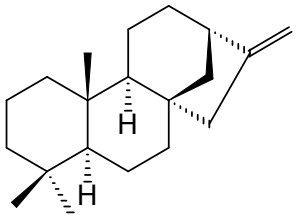
	R_1	R_2	R_3	R_4
19-Acetoxy ferruginol (92)	CH_3	H	CH_3	CH_2OAc
Ferruginol (94)	CH_3	H	CH_3	CH_3
15-Hydroxy dehydroabietic acid (95)	CH_3	OH	CH_3	COOH
19-Hydroxy ferruginol (96)	CH_3	H	CH_3	CH_2OH
Lambertic acid (97)	CH_3	H	CH_3	COOH
Pododacric acid (98)	CH_2OH	H	CH_2OH	COOH



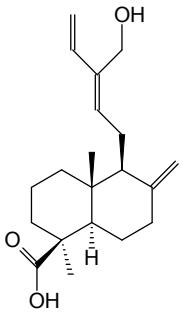
Sugiol (99)



Beyerene (100)

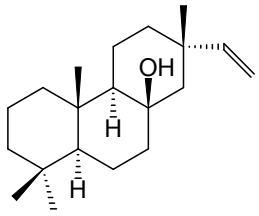


Kaurene (101)

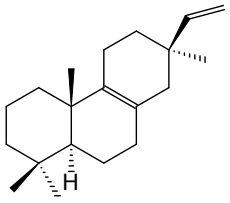


16-Hydroxy communic acid (102)

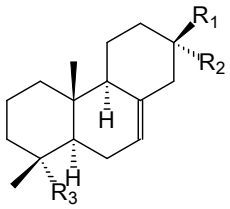
Figure 5 Chemical structures of diterpenoids in the genus *Podocarpus*



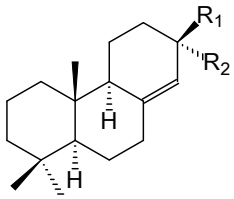
8β-Hydroxy isopimarene (103)

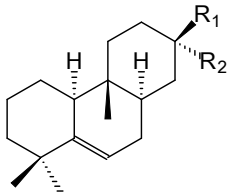


Isopimara-8,15-diene (105)

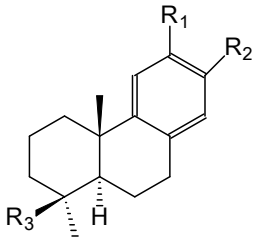


	R_1	R_2	R_3
Isopimara-7,15-diene (104)	$CH=CH_2$	CH_3	CH_3
Isopimaric acid (106)	CH_3	$CH=CH_2$	COOH



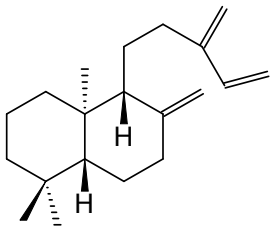


	R_1	R_2		R_1	R_2
Pimara-8(14),15-diene (107)	$CH=CH_2$	CH_3	Rimuene (108)	CH_3	$CH=CH_2$
Sandaracopimaradiene (110)	CH_3	$CH=CH_2$	Rosadiene (109)	$CH=CH_2$	CH_3

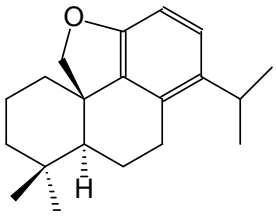


	R_1	R_2	R_3
7-Hydroxy-1,4-α-dimethyl-1,2,3,4,4a,9,10,10a-octahydro-phenanthrene-1-carboxylic acid (111)	H	OH	COOH
Methylpodocarpate (112)	OH	H	CO_2CH_3
Podocarpa-8,11,13-trin-16-oic acid (113)	H	H	COOH
Podocarpic acid (5)	OH	H	COOH
Sempervirol (115)	*iso*-propyl	OH	CH_3

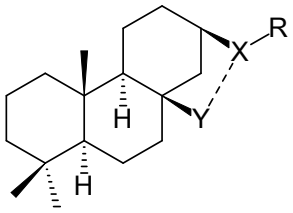
Figure 5 (continued)



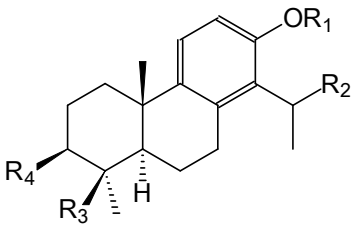
(-)-Sclarene (114)



Cycloinumakiol (119)

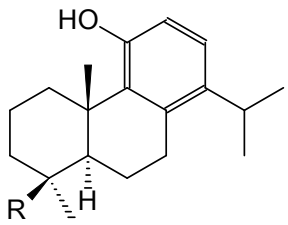


	X	Y	R
Isophyllocladene (116)	C=CH		CH_3
17-Isophyllocladenol (117)	C=CH		CH_2OH
Phyllocladene (118)	C-CH_2		=CH_2

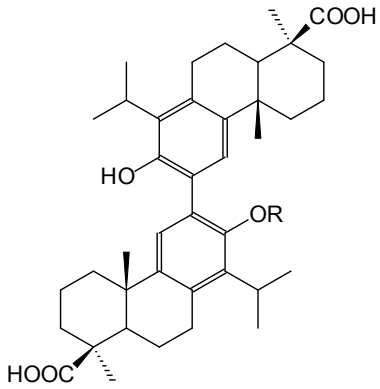


	R_1	R_2	R_3	R_4
4β-Carboxy-19-nortotarol (6)	H	CH_3	COOH	H
3β-Hydroxy totarol (120)	H	CH_3	CH_3	OH
16-Hydroxy totarol (121)	H	CH_2OH	CH_3	H
19-Hydroxy totarol (7)	H	CH_3	CH_2OH	H
16-Oxototarol (124)	H	COH	CH_3	H
19-Oxototarol (Totaral) (8)	H	CH_3	COH	H
Totaradiol (125)	H	CH_3	CH_3	OH
Totarol (9)	H	CH_3	CH_3	H
Totaryl acetate (10)	$COCH_3$	CH_3	CH_3	H

Figure 5 (continued)

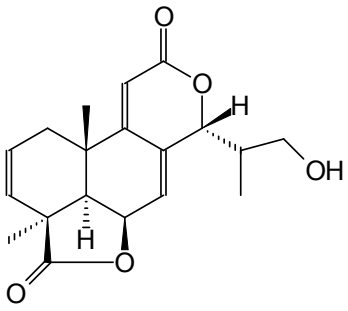


	R
Inumakal (122)	COH
Inumakoic acid (123)	COOH

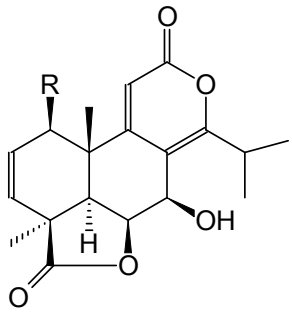


	R
Podototarin (126)	H
Podototarin monoacetate (127)	$COCH_3$

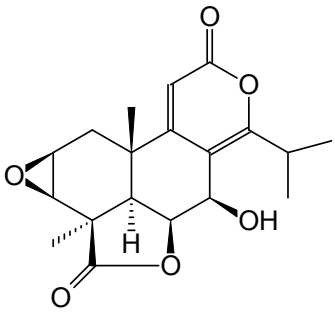
Figure 5 (continued)



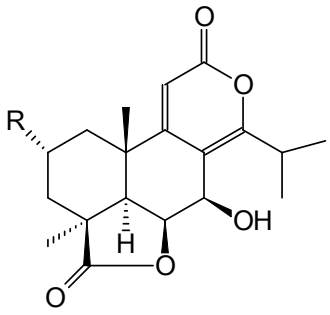
2,3-Dehydro-16-hydroxynagilactone F (128)



	R
2,3 Dehydronagilactone A (129)	OH
1-Deoxy-2,3-dehydronagilactone A (130)	H

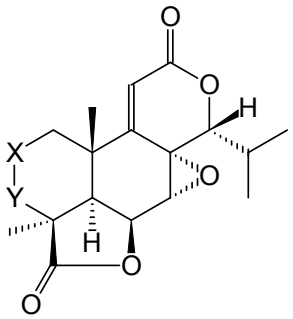


1-Deoxy-2β,3β-epoxynagilactone A (131)

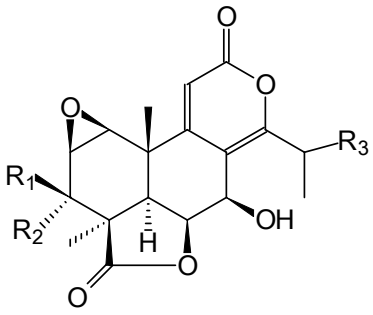


	R
1-Deoxy-2α-hydroxynagilactone A (132)	OH
1-Deoxynagilactone A (133)	H

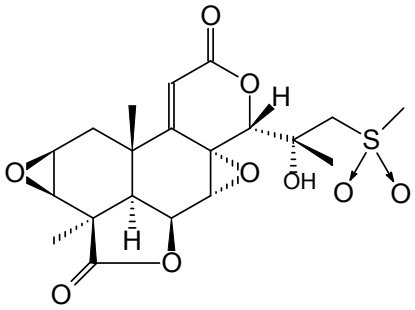
Figure 6 Chemical structures of norditerpene dilactones in the genus *Podocarpus*



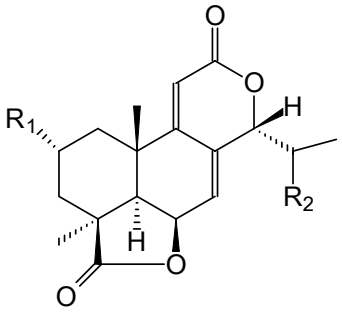
	X	Y
2,3-Dihydropodolide (134)	CH_2	CH_2
Podolide (149)	CH=CH	



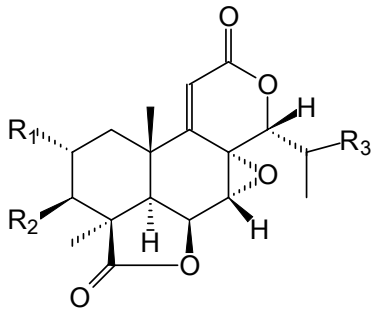
	R_1	R_2	R_3
3-Epinagilactone C (135)	H	OH	CH_3
Nagilactone C (12)	OH	H	CH_3
Nagilactone D (13)	OH	H	H



Hallactone B (136)

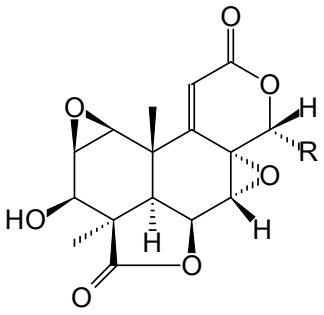


	R1	R2
2α-Hydroxynagilactone F (138)	OH	CH_3
Nagilactone F (141)	H	CH_3
Nagilactone I (142)	OH	CO_2CH_3

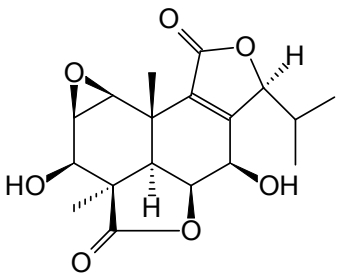


	R1	R2	R3
16-Hydroxy nagilactone E (137)	H	OH	CH_2OH
Nagilactone E (14)	H	OH	CH_3
2α-Hydroxynagilactone G (11)	OH	H	CH_3
Nagilactone G (15)	H	H	CH_3

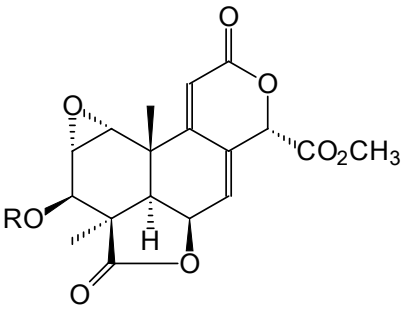
Figure 6 (continued)



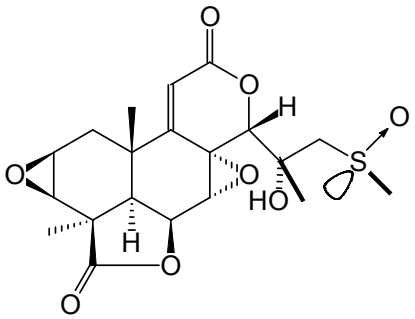
	R
Inumakilactone (139)	*iso*-Propyl
Inumakilactone B (140)	$CH=CH_2$



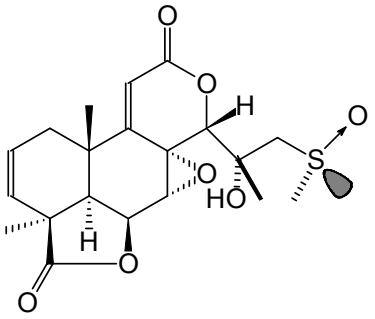
Nagilactone J (143)



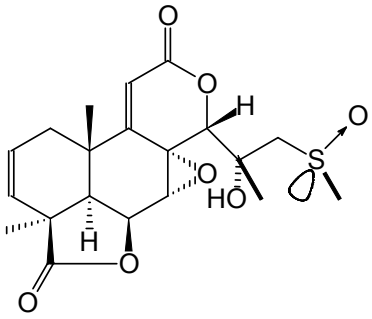
	R
Nubilactone A (144)	H
Nubilactone A monoacetate (145)	CH_3CO



Podolactone C (146)

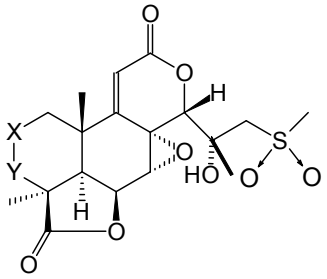


S_R-Podolactone D (147)

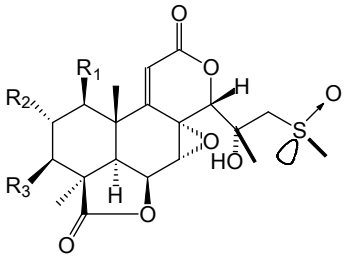


S_S-Podolactone D (148)

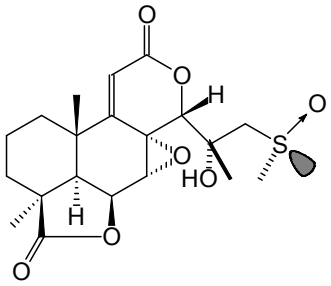
Figure 6 (continuted)



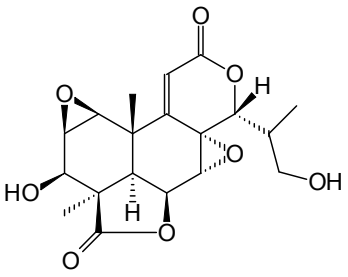
	X	Y
Rakanmakilactone A (150)	CH=CH	
Rakanmakilactone B (151)	CH_2	CH_2



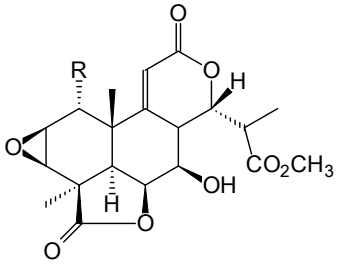
	R_1	R_2	R_3
Rakanmakilactone C (152)	H	H	H
Rakanmakilactone E (154)	H	Cl	OH
Rakanmakilactone F (155)	OH	H	H



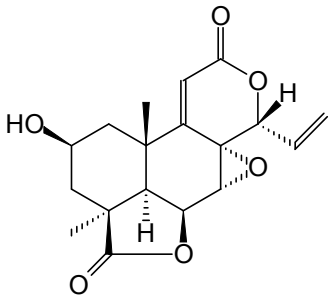
Rakanmakilactone D (153)



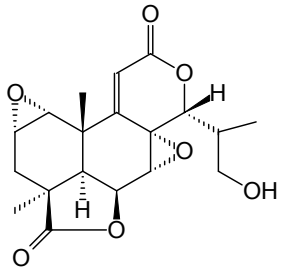
Salignone I (156)



	R
Salignone K (157)	H
Salignone L (158)	OH

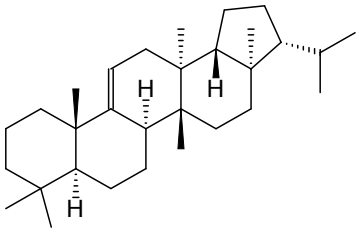


Salignone M (159)

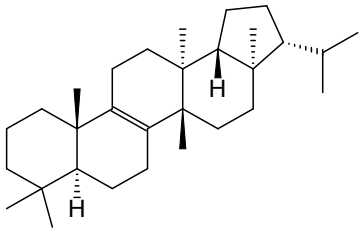


Sellowin A (160)

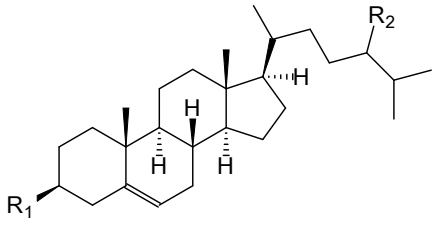
Figure 6 (continued)



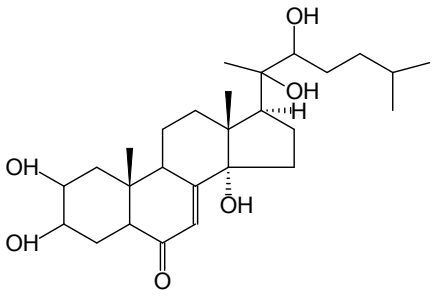
Fern-9(11)-ene (161)



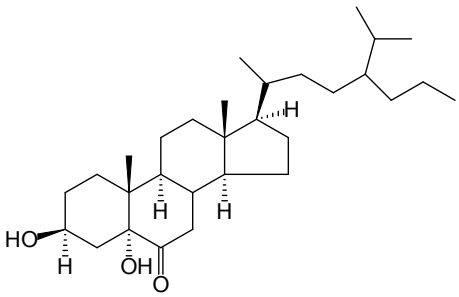
Isofernene (162)



	R_1	R_2
Campesterol (163)	OH	CH_3
Daucosterin (164)	*O*-Glc	CH_2CH_3
β-Sitosterol (16)	OH	CH_2CH_3

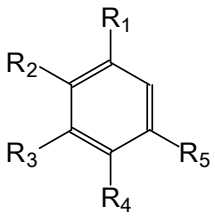


Ecdysterol (165)

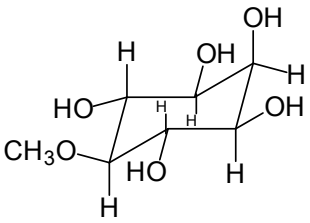


Stigmastan-3β,5α-diol-6-one (166)

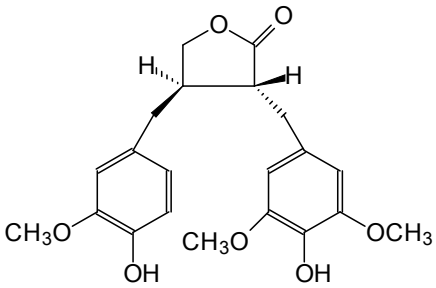
Figure 7 Chemical structures of steroids and triterpenoids in the genus *Podocarpus*



	R_1	R_2	R_3	R_4	R_5
Benzene-1,2,3,4-tetraol (167)	H	OH	OH	OH	OH
4-Ethylphenol (168)	CH_2CH_3	H	H	OH	H
5-(3-Hydroxy propyl)-2-methoxy phenol (169)	H	$CH_2CH_2CH_2OH$	H	OH	OCH_3
Hydroquinone (1)	OH	H	H	OH	H
4-Methylphenol (170)	CH_3	H	H	OH	H



Sequoyitol (171)



Thujaplicatin methyl ether (172)

Figure 8 Chemical structures of Benzenoids, Sugars and Lignans in the genus *Podocarpus*

2.9 Summary

Obesity is an important public health problem. Although the recommendation is weight reduction by diet control and increasing physical activity, but in some cases cannot achieve. So they need to use drugs. Pharmacologic management of obesity has been focused on modulating central and/or peripheral sites. The CNS acting drugs has many side effects so the use is limited. The gastrointestinal acting agent, lipase inhibitor, is an alternative for treatment of obesity. However, the price of this drug is too high because of the monopoly market. Therefore, research in development of this group of drug may be useful for obese patients. Many researchers have discovered that several medicinal plants have lipase inhibitory activity. In Thailand, there are several medicinal plants that have potential to give such activity. There are many methods for screening lipase inhibitors. A high sensitivity, rapid and convenient method is needed. In this study, TLC in combination with bioassay was also developed for screening lipase inhibitors from Thai medicinal plants.

CHAPTER III

MATERIALS AND METHODS

3.1 Sample Collection

Twenty-eight species of the plant materials were purchased or collected from the traditional medicine shops, the local markets, or from the forest from November 2006 to December 2007.

Seven species of plant materials were collected from the wild of Thailand including *Cryptolepis buchanani* Roem. & Schult., *Gnetum montanum* Markgr., *Nageia wallichiana* C. Presl Kuntze, *Neptunia triquetra* (Vahl) Benth., *Pinus kesiya* Royle ex Gordon, *Scaphium scaphigerum* (G. Don) Guib. & Planch., and *Scyphiphora hydrophyllacea* Gaertn.f. Eight cultivated plants were collected from garden in the provinces nearby Bangkok including *Emilia sonchaifolia* (L.) Dc. Ex Wight, *Coccinia grandis* (L.) Voigt, *Ixora grandifolia* Zoll.& Moritzi, *Luffa acutangula* (L.) Roxb., *Luffa cylindrica* (L.) M. Roem., *Morinda citrifolia* L., and *Passiflora foetida* L.. The other one garnered from upcountry was *Garcinia cowa* Roxb. ex DC.

Seven species of the cultivated plants were purchased from the commercial markets in fresh form including *Averrhoa bilimbi* L., *Citrus hystrix* DC., *Garcinia schomburgkiana* Pierre, *Gnetum gnemon* L. var. gnemon, *Neptunia oleracea* Lour., *Phyllanthus emblica* L., and *Polygonum odoratum* Lour. Six kinds of the cultivated plants were purchased in dried form including *Cymbopogon citratus* Stapf, *Gymnopetalum chinense* (Lour.) Merr., *Gymnopetalum integrifolium* (Roxb.) Kurz, *Oryza sativa* L. cv. Jao Hom Nin, *Randia horrida* Roem. & Schult. and *Sesamum indicum* L. The places where the material plants were purchased and collected were shown in **Table 3**.

All of the plants were identified by comparing with the herbarium specimens from the Royal Forest Department, Ministry of Natural Resources and Environment or identified by Assoc. Prof. Dr. Rapepol Bavovada. The plant materials were dried with oven at 45-60^{o}C, and voucher specimens were deposited in the herbarium of the

Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Table 3 The material plants in this study

Family	Scientific names (Thai name)	Part of plants	Sources (Province)
Asclepiadaceae	*Cryptolepis buchanani* Roem. & Schult. (เถาเอ็นอ่อน)	stems	Phuvour wildlife sanctuary (Nongkhai)
Asteraceae	*Emilia sonchaifolia* (L.) DC. Ex Wight (หูปลาช่อน)	arial parts	Pakkred District (Nonthaburi)
Clusiaceae	*Garcinia cowa* Roxb. Ex DC. (ชะมวง)	leaves	(Rayong)
	Garcinia schomburgkiana Pierre (มะดัน)	fruits	Pakkred market (Nonthaburi)
Cucurbitaceae	*Coccinia grandis* (L.) Voigt (ตำลึง)	arial parts and fruits	Koh Kred Island (Nonthaburi)
	Gymnopetalum chinense (Lour.) Merr. (กระดอม)	fruits	Chao Krom Poe Dispensary (Bangkok)
	Gymnopetalum integrifolium (Roxb.) Kurz (ขี้กาแดง)	fruits	Chao Krom Poe Dispensary (Bangkok)
	Luffa acutangula (L.) Roxb. (บวบเหลี่ยม)	arial parts	Koh Kred Island (Nonthaburi)
	Luffa cylindrica (L.) M. Roem. (บวบหอม)	arial parts, flowers, and fruits	Pakkred District (Nonthaburi)
Euphorbiaceae	*Phyllanthus emblica* L. (มะขามป้อม)	seeds	Tha Prachan pavement Market (Bangkok)
Gnetaceae	*Gnetum gnemon* L. (ผักเหมียง หรือ ผักเหลียง)	leaves	Market (Chumphon, Krabi)

Table 3 (Continued)

Family	Scientific names (Thai name)	Part of plants	Sources (Province)
	Gnetum montanum Markgr. (เมื่อย)	leaves	Phu Jong Nayoi National Park (Ubon Ratchathani)
Leguminosae	*Neptunia oleracea* Lour. (ผักกระเฉด)	whole plant	Pakkred market (Nonthaburi)
	Neptunia triquetra (Vahl) Benth. (กระเฉดบก)	arial parts	Thep Sathit District (Chaiyaphum)
Oxalidaceae	*Averrhoa bilimbi* L. (ตะลิงปลิง)	leaves and fruits	The market behind Mahathat temple (Bangkok)
Passifloraceae	*Passiflora foetida* L. (กระทกรก)	arial parts and fruits	Koh Kred Island (Nonthaburi)
Pedaliaceae	*Sesamum indicum* L. (งา)	seeds	Pakkred market (Nonthaburi)
Pinaceae	*Pinus kesiya* Royle ex Gordon (สนสามใบ)	leaves	Phu Jong Nayoi National Park (Ubon Ratchathani)
Poaceae	*Cymbopogon citratus* Stapf (ตะไคร้)	arial parts	Ampawan temple (Sing Buri)
	Oryza sativa L. cv. Jao Hom Nin (ข้าวหอมนิล)	seeds	Marketing Organization for Farmers market at Chatuchak
Podocarpaceae	*Nageia wallichiana* C. Presl Kuntze (ขุนไม้)	stems	Kao Soi Dao Wildlife Sanctuary (Chanthaburi)
Polygonaceae	*Polygonum odoratum* Lour. (ผักไผ่)	arial parts	Banphai market (Khon Kaen)
Rubiaceae	*Morinda citrifolia* L. (ยอบ้าน)	leaves and fruits	Koh Kred Island (Nonthaburi)
	Ixora grandifolia Zoll & Moritzi (เข็มเศรษฐี)	flowers and leaves	(Nonthaburi and Nakhon Pathom)
	Randia horrida Roem. & Schult. (คัดเค้า)	fruits	Chao Krom Poe Dispensary (Bangkok)

Table 3 (Continued)

Family	Scientific names (Thai name)	Part of plants	Sources (Province)
	Scyphiphora hydrophyllacea Gaertn.f. (สี้ง้ำ)	leaves and stems	Phu Jong Nayoi National Park (Ubon Ratchathani)
Rutaceae	*Citrus hystrix* DC. (มะกรูด)	leaves	Koh Kred Island (Nonthaburi)
Sterculiaceae	*Scaphium scaphigerum* (G.Don) Guib. & Planch. (พุงทะลาย)	leaves	Phu Jong Nayoi National Park (Ubon Ratchathani)

3.2 Screening for pancreatic lipase inhibitory activity of the plant samples

3.2.1 Preparation of crude extracts

Twenty grams of the dried and grinded plant materials were macerated with 200 ml of 80%MeOH (3 days for 3 times). The combined extracts were concentrated under reduced pressure to dryness. The crude extract were kept at -20°C before used.

3.2.2 Screening for pancreatic lipase inhibitory activity

The crude extracts were dissolved in DMSO to obtain 2 mg/ml of stock solution and kept at -20 °C. Before the assay, the stock solutions were diluted with distilled water to obtain 1%DMSO so the concentrations of the crude extracts were 20 µg/ml.

The assay was optimized from the study of Kurihara et al. (2003). The pancreatic lipase activity was measured by using 4-methylumbelliferyl oleate (4-MUO) as a substrate. Twenty-five microliters of 20 µg/ml of crude extract solution was dissolved in 1% DMSO in water (v/v) and 50 µl of 0.01 mM 4-MUO (Fluka®) was dissolved in a buffer consisting of 13 mM Tris–HCl, 150 mM NaCl, and 1.3 mM $CaCl_2$ (pH 8.0). The both solutions were mixed in the well of a microtiter plate, and then 25 µl of 50 U/ml of porcine pancreatic lipase Type II (PPL) (crude, Sigma®) in the above buffer was added to start the enzyme reaction. After incubation at 37°C for 20 min, 50 µl of 0.1

N HCl was added to stop the reaction and 100 µl of 0.1 mM sodium citrate (pH 4.2) was added for pH adjustment. The amount of 4-methylumbelliferone released by the PPL was measured with a microplate reader (Victor^3, Perkin Elmer, USA) at an excitation wavelength of 355 nm and an emission wavelength of 450 nm. The assay was run in triplicate for each plant extract. The blank was added with the same solutions as the test samples but the order of mixing was different that 0.1 N HCl was added before the enzyme. The 1%DMSO in water was used as solvent control. The enzyme inhibitory activity was reported as the mean of %inhibition ± standard deviation. The percentage of inhibition was expressed by following equation:

$$\%\text{inhibition} = \frac{[(FE-FEB)-(FS-FSB)]}{(FE-FB)} \times 100$$

where, FE and FS were fluorescence values of the normal enzymatic reaction at 20 min incubation of the solvent control and the sample plant extract, FEB and FSB were fluorescence values of the blank of solvent control and the sample plant extract. Orlistat (Sigma) and gallocatechin gallate (GCG) were used as positive controls. The stock solution of orlistat and GCG were prepared by dissolving in DMSO. The final concentration of DMSO in the reaction was 0.5% v/v which did not affect the assay system.

The plant extracts which had the percentage of inhibition more than 50% would be determined the IC_{50} values and the plant extract with high potency would be further determined for the active compounds.

3.2.3 IC_{50} value determination

The IC_{50} value was the concentration of the test sample which caused the enzyme inhibitory activity by 50%. The IC_{50} value was determined by varing the set of concentrations of each test sample and testing the activity. The IC_{50} value was obtained by plotted the graph between %inhibition (y-axis) versus concentrations of the test sample (x-axis).

3.3 General Techniques for Phytochemistry Study

3.3.1 Solvents

The commercial grade organic solvents were redistilled prior to use.

3.3.2 Chromatographic techniques

3.3.2.1 Analytical Thin-Layer Chromatography (TLC)

Technique : One dimension, ascending

Adsorbent : Silica gel 60 F_{254} (E. Merck) pre-coated plates

Layer thickness : 0.2 mm

Distance : 5.0 cm

Temperature : Laboratory temperature (25-30^{o}C)

Detection : 1) Visual detection under daylight

2) Visual detection under ultraviolet light (254 and 365 nm)

3) Spraying reagents

3.1) Spraying with anisaldehyde-sulphuric acid reagent (AS) and heating the plates at 110^{o}C for 5-15 min or

3.2) Spraying with 10%H_2SO_4 in EtOH and heating the plate at 100^{o}C for 3-5 min

3.3.2.2 Column chromatography

(1) Conventional column chromatography (CC)

Adsorbent : Silica gel 60 number 9385 (particle size 0.040-0.063 nm) and number 7734 (particle size 0.063-0.200 nm) (E. Merck)

Packing method : Wet packing: the adsorbent was slurried in the eluent and poured into a column and finally allowed to settle.

Solvent system : Various solvent systems depending on materials

Sample loading : The sample was mixed with silica gel after that allowed to dry or dissolved in a small amount of eluent and then gently applied on the top of column.

Detection : Fraction were combined by TLC technique in the same manner as described in section 3.3.2.1

(2) Gel filtration chromatography

Gel filter : Sephadex LH-20 (Pharmacia Biotech AB)

Packing method : Gel filter was suspended in the eluent and left standing to swell for 24 hours prior to use. It was then poured into the column and allowed to set tightly.

Solvent system : Various solvent systems depending on materials

Sample loading : The sample was dissolved in a small amount of eluent, and then gently applied on the top of column.

Detection : Fraction were combined by TLC technique in the same manner as described in section 3.2.1

3.3.3 Spectroscopy

3.3.3.1 Ultraviolet (UV spectra) spectra

UV spectra were obtained on a Milton Roy Spectronic 3000 Array spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmceutical Sciences, Chulalongkorn University).

3.3.3.2 Infrared (IR) spectra

IR spectra (KBr disc) were recorded on a Perkin Elmer FT-IR 1760X spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

3.3.3.3 Mass spectra (MS)

Mass spectra were obtained by an Electrospray Ionization Time of Flight mass spectra (ESITOFMS) made on a Micromass LCT mass spectrometer (The National Center for Genetic Engineering and Biotechnology, BIOTEC, Thailand).

3.3.3.4 Proton and carbon nuclear magnetic resonance (^{1}H-NMR and ^{13}C-NMR) spectra

^{1}H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a JEOL JMN-A 500 spectrometer, Varian Unity INOVA (Scientific and Technological Research Equipment Center, Chulalongkorn University).

The solvents for NMR spectra were deuterated solvents. The chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal.

3.4 Extraction and isolation of pure compounds

The plant materials were divided into two lots. The first lot was subjected to the general isolation process to obtain the main compounds from each fraction. The second lot was isolated by using bioassay-guided fractionation.

3.4.1 The first lot of plant materials

3.4.1.1 Source of plant materials

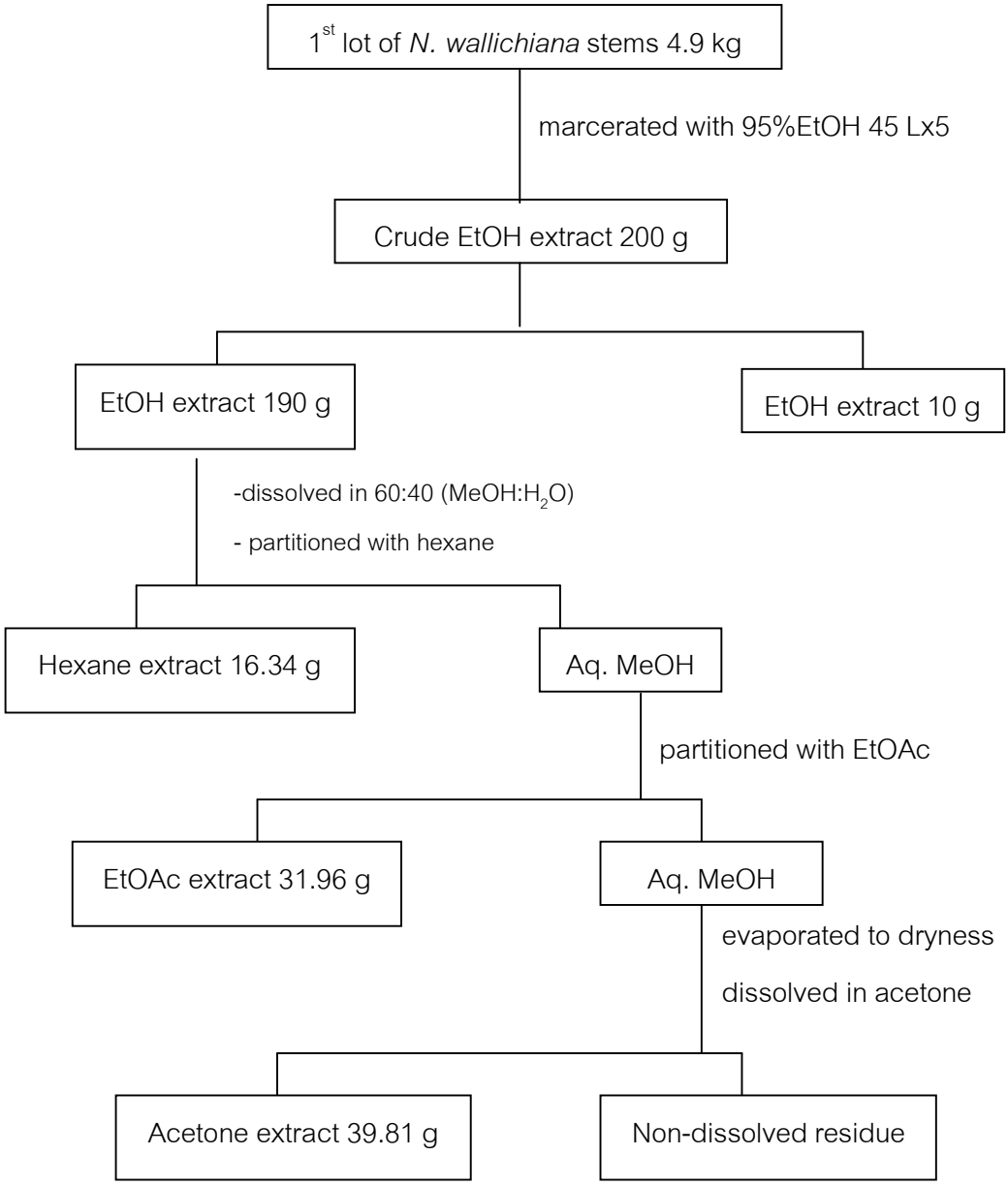
The plant material of the stem of *N. wallichiana* was collected at Khao Soi Dao Wildlife Sanctuary, Chantaburi Province in September 2008.

3.4.1.2 Extraction

The air-dried and grinded stem of *N. wallichiana* (4.9 kg) was extracted by maceration with 95% EtOH (45 L x 5 rounds). The pooled filtrate was evaporated under reduced pressure to afford the ethanol extract (200 g, 4.08 % of dried plant weight).

One hundred and ninety grams of ethanol extract was re-dissolved with 60% aqueous MeOH and partitioned with *n*-hexane to obtain hexane extract (16.34 g, 8.17%w/w of ethanol crude extract). The 60% aqueous MeOH residue was partitioned with ethyl acetate (EtOAc). The EtOAc layer was evaporated to obtain EtOAc extract (31.96 g, 15.98%w/w of ethanol crude extract).

The 60% aqueous MeOH layer was evaporated to dryness and re-dissolved in acetone to yield 39.81 g (19.91%w/w of ethanol crude extract) of the acetone extract, and 98.57 g of residue extract respectively. All partition extracts were purified before tested for the lipase inhibitory activity. The isolation process was shown in **Scheme 1**.



Scheme 1 Extraction and isolation process of *N. wallichiana* stems (1st lot)

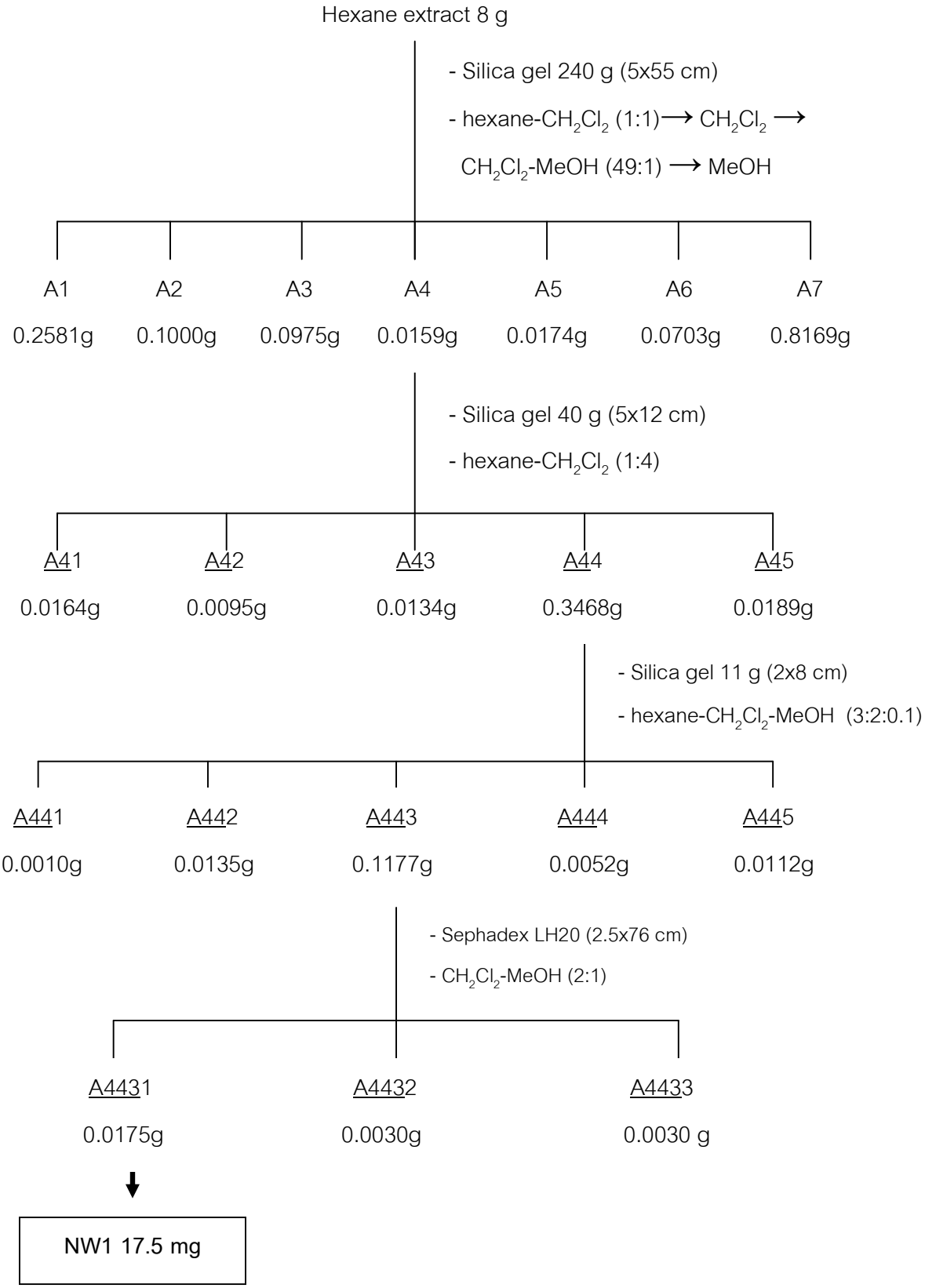
3.4.1.3 Isolation

(1) Hexane extract

The 8 g of hexane extract was subjected on a silica gel column chromatography (240 g, 5x55 cm), and gradient eluted with hexane-CH_2Cl_2 (1:1) ⟶ CH_2Cl_2 ⟶ CH_2Cl_2-MeOH (49:1) ⟶ MeOH. Fraction, each 50 ml, was collected and fractions with the same TLC patterns were pooled to obtain 7 fractions (A1-A7) **(Table 4)**. Fraction A4 was isolated by using silica gel (5x12 cm), eluted with hexane-CH_2Cl_2 (1:4) and collected 10 ml/fraction to obtain 5 fractions (A41-A45). Fraction A44 was re-columned on silica gel column chromatography (2x8 cm), eluted with hexane-CH_2Cl_2-MeOH (3:2:0.1) to obtain 5 fractions (A441-A445). Fraction A443 was re-columned by Sephadex LH20 (2.5x76 cm), eluted with CH_2Cl_2-MeOH (2:1) to obtain a pure compound NW1 (17.5 mg). The procedure of isolation was shown in **Scheme 2**.

Table 4 Combined fraction of the 1st column of the hexane extract of *N. wallichiana* stems (1st lot)

Fraction code	No. of eluate	Weight (g)
A1	1-15	0.2581
A2	16-43	0.1000
A3	44-77	0.0975
A4	78-121	0.0159
A5	122-143	0.0174
A6	144-176	0.0703
A7	177-182	0.8169



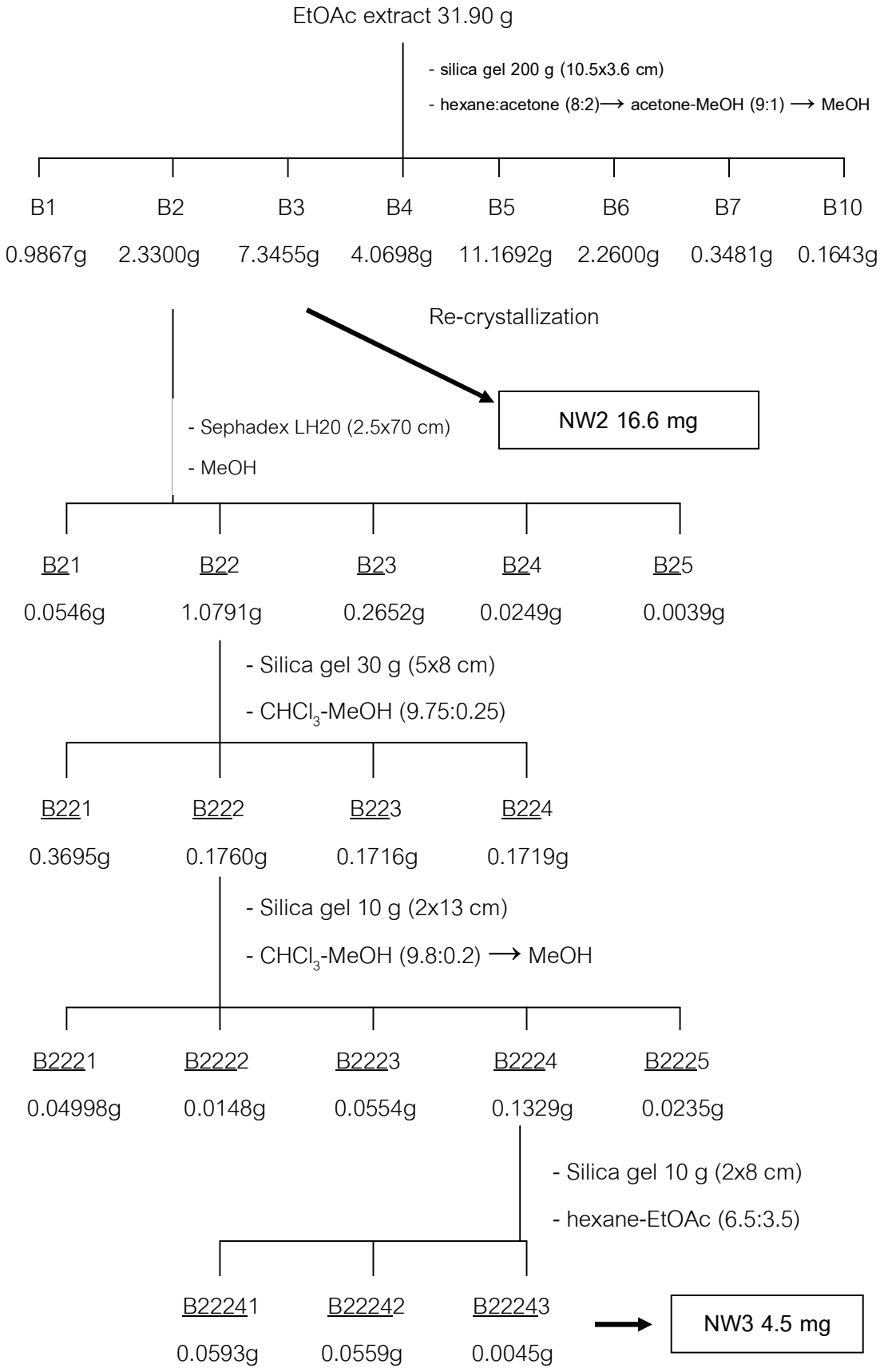
Scheme 2 Isolation of NW1 from hexane extract of *N. wallichiana* stems (1st lot)

(2) EtOAc extract

EtOAc extract (31.90 g) was purified by a flash column chromatography using 200 g of silica gel (10.5x3.6 cm), gradient eluted with hexane-acetone (8:2) ⟶ acetone-MeOH (9:1) ⟶ MeOH. Four hundred millilitres per fractions were collected and the fractions were combinded to obtain 10 fractions (B1-B10) **(Table 5)**. Fraction B3 was re-crystallized with MeOH to obtain a pure compound NW2 (13.0 mg). Fraction B2 was separated by Sephadex LH20 (2.5x70 cm) eluted with MeOH, to obtain 5 fractions (B21-B25). Fraction B22 was re-columned with three silica gel column chromatography, eluted with $CHCl_3$-MeOH (9.75:0.25), gradient eluted with $CHCl_3$-MeOH (9.8:0.2) ⟶ MeOH and hexane-EtOAc (6.5:3.5) to obtain a pure compound NW3 (4.5 mg). The procedure of isolation was shown in **Scheme 3**.

Table 5 Combined fraction of the first column of the EtOAc extract of *N. wallichiana* stems (1^{st} lot)

Fraction code	No. of eluate	Weight (g)
B1	1-8	0.9867
B2	9-23	2.3300
B3	24-40	7.3455
B4	41-51	4.0698
B5	52-62	11.1692
B6	63-79	2.2600
B7	80-84	0.3481
B8	85-96	1.0718
B9	97-104	1.8719
B10	105-111	0.1643



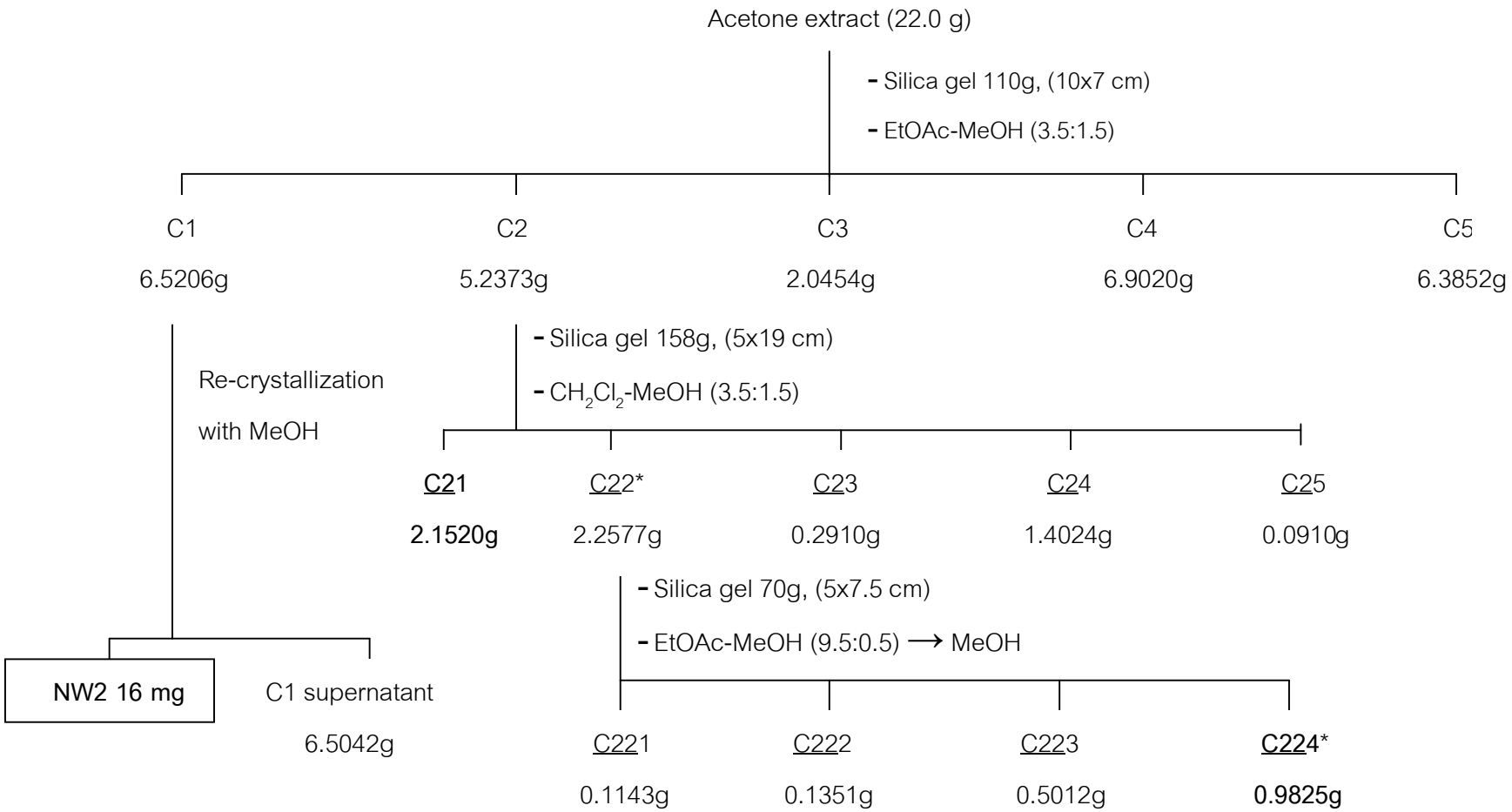
Scheme 3 Isolation of NW2 and NW3 from EtOAc extract of *N. wallichiana* stems (1st lot)

(3) Acetone extract

Acetone extract (22.0 g) was partially purified with a 110 g of silica gel column chromatography (10x7 cm). The column was eluted with EtOAc-MeOH (3.5:1.5), and 100 ml per fraction were collected. Fractions with the same TLC pattern were combined to obtain 5 fractions (C1-C5) **(Table 6)**. Fraction C1 was re-crystallized with MeOH to give 16.0 mg of a pure compound NW2. Fraction C2 was re-chromatographed over with silica gel (5x19 cm) and eluted with CH_2Cl_2-MeOH (3.5:1.5) to give 5 fractions (C21-C25). The isolation process was shown in **Scheme 4**. Fraction C21 was re-columned with Sephadex LH20 (2.5x42.5 cm) and followed by two silica gel columns to isolate some needle crystals. However, they were failed to purify because of too small amount in sub-fractions together with many impurities **(Scheme 5)**. Fraction C22 had a black spot on TLC when detected with 10%H_2SO_4 spraying reagent. Several columns were used to purify. However, the isolation and purification were not further performed for the pure black spot because it had low activity (0.09%inhibition) **(Scheme 4 and 6)**. For the fraction C2243 and C2244, the appropriate system for isolation could not be developed though these fractions were detectable greenish color with $FeCl_3$ TS solution which indicated that they may have phenolic compounds like catechol moiety in these fractions. Thus C2244 was alternatively purified by Sephadex LH20 column with acetone as eluent but the trial was failed because the constituents were persisted in the column and degraded after eluted with 1%v/v HCl in MeOH **(Scheme 6)**.

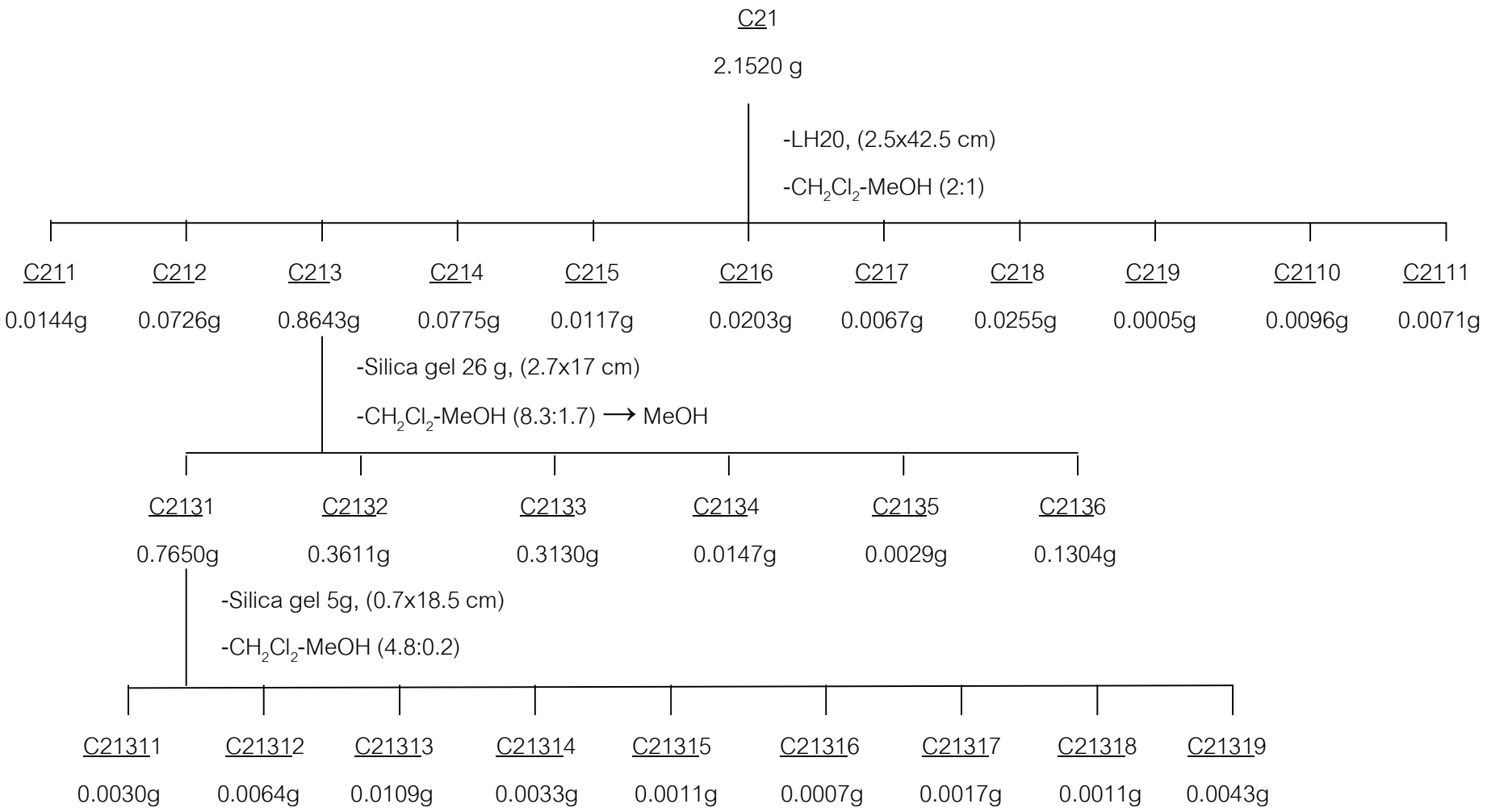
Table 6 Combined fraction of the first column of the acetone extract of *N. wallichiana* stems (1^{st} lot)

Fraction code	No. of eluate	Weight (g)
C1	1-5	6.5206
C2	6-17	5.2373
C3	18-25	2.0454
C4	26-41	6.9020
C5	42-104	6.3852

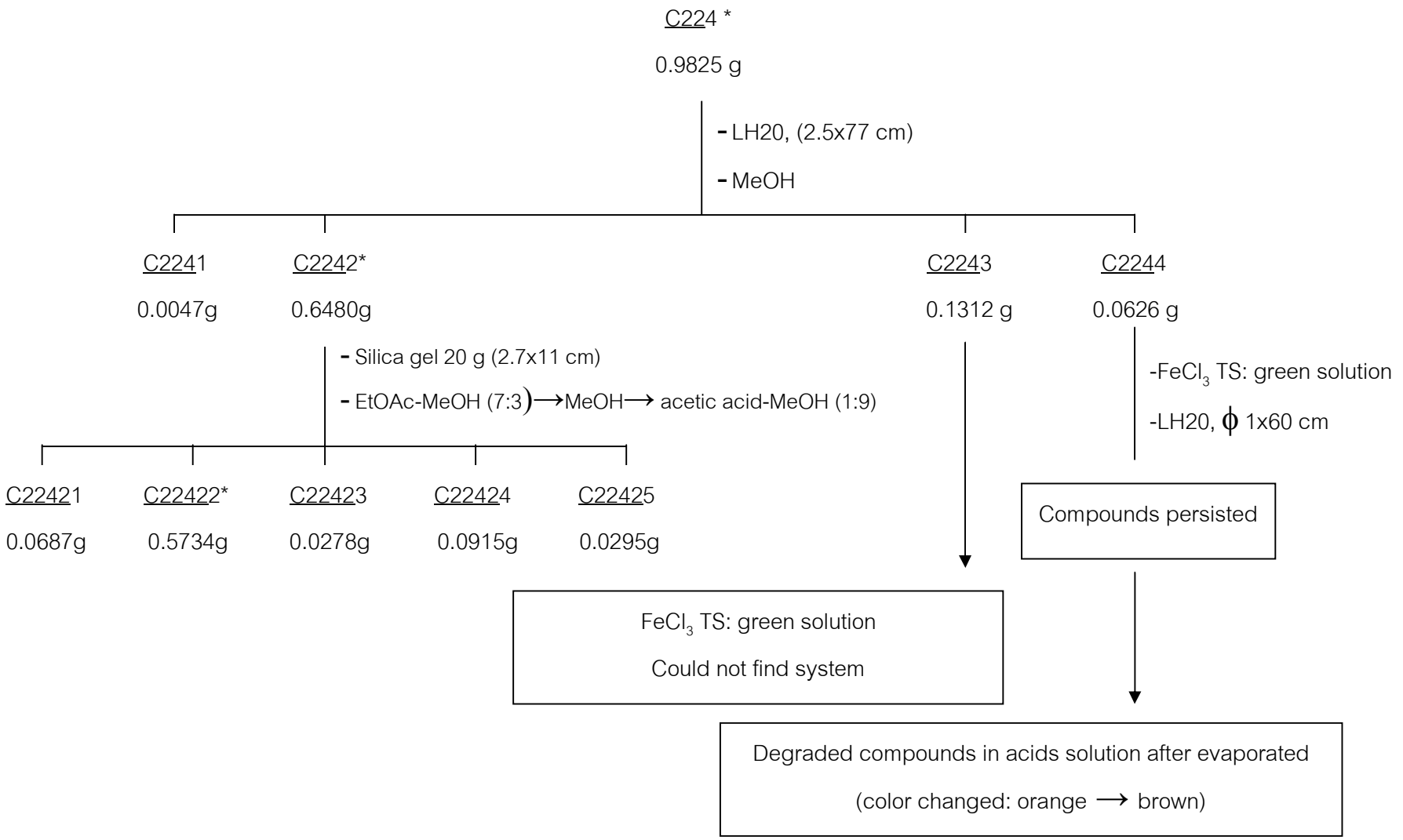


Scheme 4 Isolation of NW2 of acetone extract of *N. wallichiana* stems (1st lot),

* = containing a black spot on TLC detected with 10%H_2SO_4 in EtOH



Scheme 5 Isolation of C21 of acetone extract of *N. wallichiana* stems (1st lot)



Scheme 6 Isolation of C224 of acetone extract of *N. wallichiana* stems (1st lot)

* = containing a black spot on TLC detected with 10%H_2SO_4 in EtOH

(4) MeOH extract

Chemical constituents in the methanol extract were very polar. The suitable chromatographic system could not be developed.

3.4.2 The second lot of plant materials

3.4.2.1 Source of plant materials

The second lot of plant materials of the stems of *N. wallichiana* was collected from Khao Soi Dao Wildlife Sanctuary, Chantaburi Province in March 2010.

3.4.2.2 Extraction and semi-purified separation

Twelve kilograms of dried powder of *N. wallichiana* stems were macerated with 95%EtOH (50 L) for 3 times. After filtration and reduced pressure evaporation, 138 g of EtOH extract was obtained. Its lipase inhibitory activity was 94.98±0.20 %inhibition at the concentration of 12.5 µg/ml. EtOH extract was mixed with Kieselgur 32 g until to provide a dry powder, packed in a column and eluted with gradient increasing polarity of solvent from EtOAc, acetone and MeOH, respectively, to obtain 42.15 g of EtOAc fraction (60.59±1.06 %inh., 12.5 µg/ml), acetone fraction 33.00 g (96.07±0.14 %inh., 12.5 µg/ml), and methanol fraction 76.62 g (96.60±0.25 %inh., 12.5 µg/ml). Each eluting solvent was eluted until the color faded. The process was shown in **Scheme 7**.

3.4.2.3 Isolation

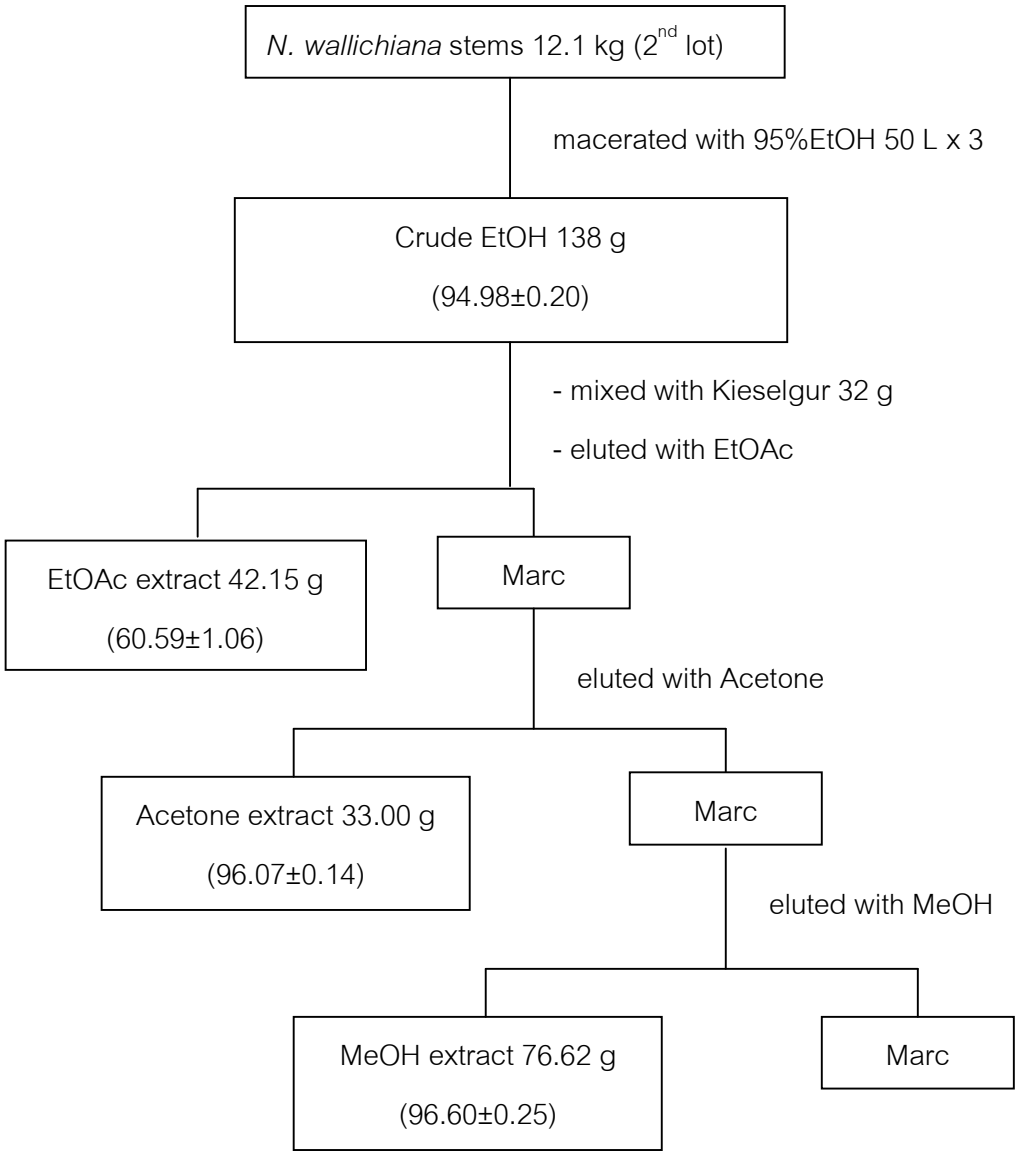
(1) EtOAc extract

The EtOAc fraction was separated by using a flash kieselgur column chromatography (800g, 15x6.5 cm), eluted with hexane ⟶ hexane-EtOAc (1:1) ⟶ EtOAc ⟶ EtOAc-MeOH (95:5⟶0:100), respectively. Each 500-ml fraction was pooled by the same TLC pattern to obtain 9 fractions (D1-D9, **Table 7**). All fractions were tested for the activity (**Scheme 8**). Fraction D6 and D7 expressed the strong activity but they were difficult to isolate any pure compounds. The lipase inhibitory activity of fraction D2 was more than 50%inhibition and this fraction had some main chemical constituents.

Consequently, it was further purified. This fraction was subjected on a silica gel column chromatography, eluted with gradient system of hexane-CH_2Cl_2 (3:7) to MeOH to give 17 fractions (D21-D217). The activities of all fractions were shown in **Scheme 8**. Fraction D22 was further purified because it had the strongest activity (85.65±0.36 %inh.). This fraction was separated by silica gel column, eluted with hexane-CH_2Cl_2 (2:3) to give 6 fractions (D221-D226). Sub-fractions D221 - D223 gave strong activity and had the same main spot so these fractions were re-isolated though several columns (**Scheme 10–12)** to give three pure compounds NW4 (43.9 mg), NW5 (3.5 mg) and NW6 (16.9 mg). Fraction D21 (57.90±1.37%inh.) was subjected to a silica gel column chromatography and eluted with hexane-CH_2Cl_2 (3:7) to give 11 fractions (D211-D2111). Following to the strongest activity fraction and several re-columned **(Scheme 9)**, a pure compound NW4 (24.3 mg) was purified.

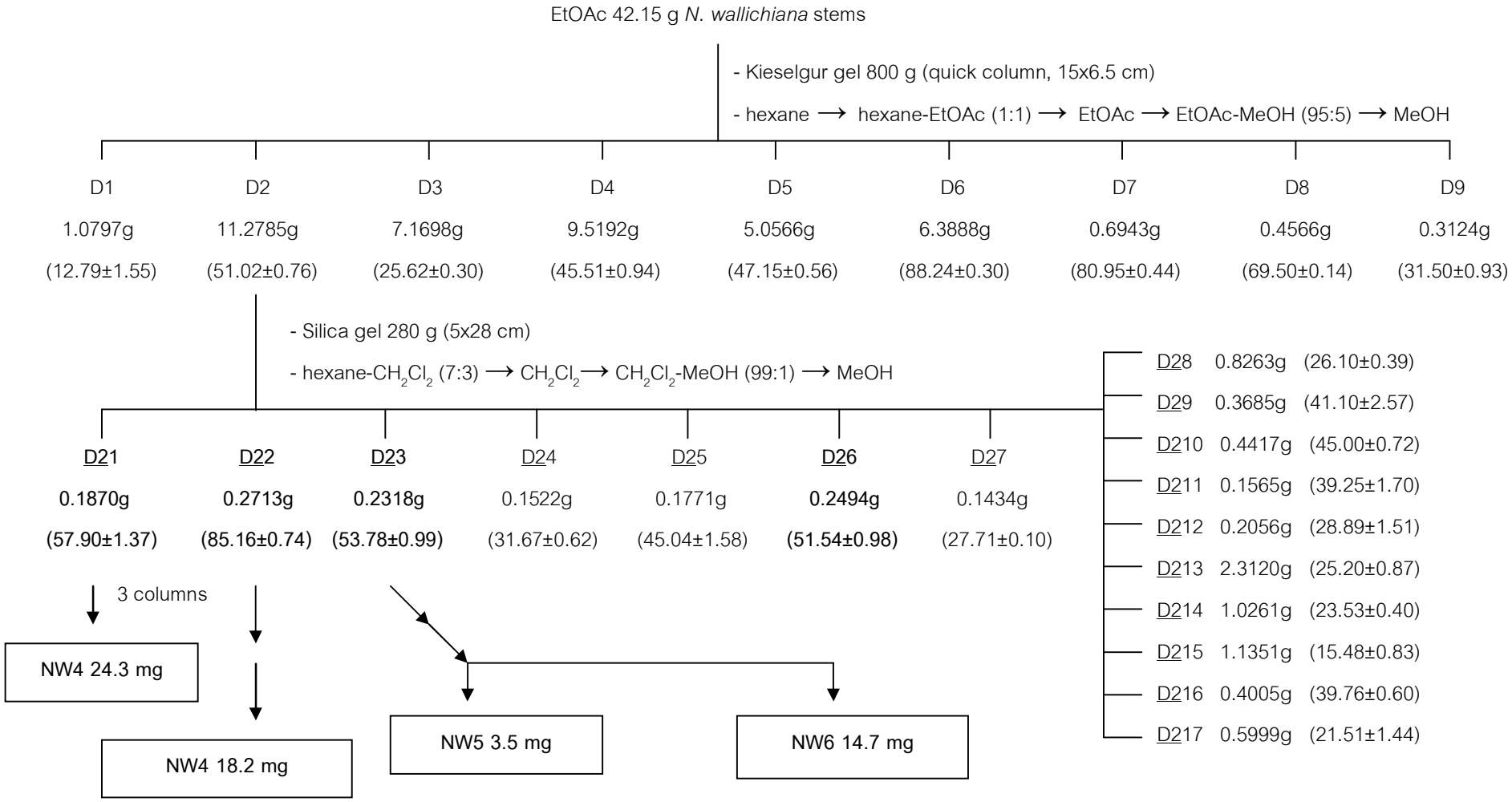
Table 7 Combined fraction of the first column of the EtOAc extract of *N. wallichiana* stems (2^{nd} lot)

Fraction code	No. of eluate	Weight (g)
D1	1-2	1.0797
D2	3-4	11.2785
D3	5-14	7.1698
D4	15-20	9.5192
D5	21-32	5.0566
D6	33-41	6.3888
D7	42-45	0.6943
D8	46-48	0.4566
D9	50	0.3124



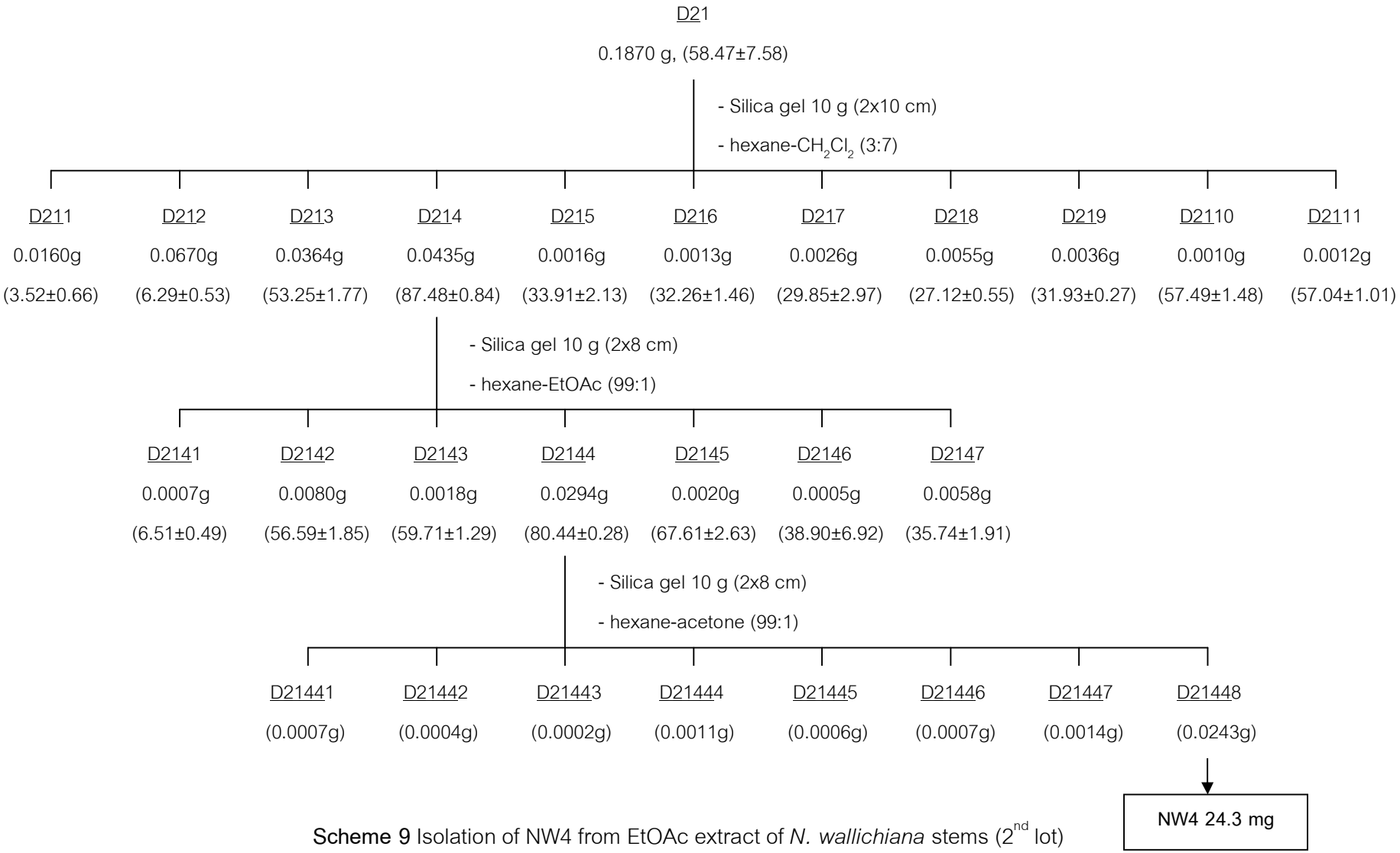
Scheme 7 Extraction and separation process of *N. wallichiana* stems (2nd lot),

( ) = Mean±SD of %inhibition at final concentration of 12.5 µg/ml



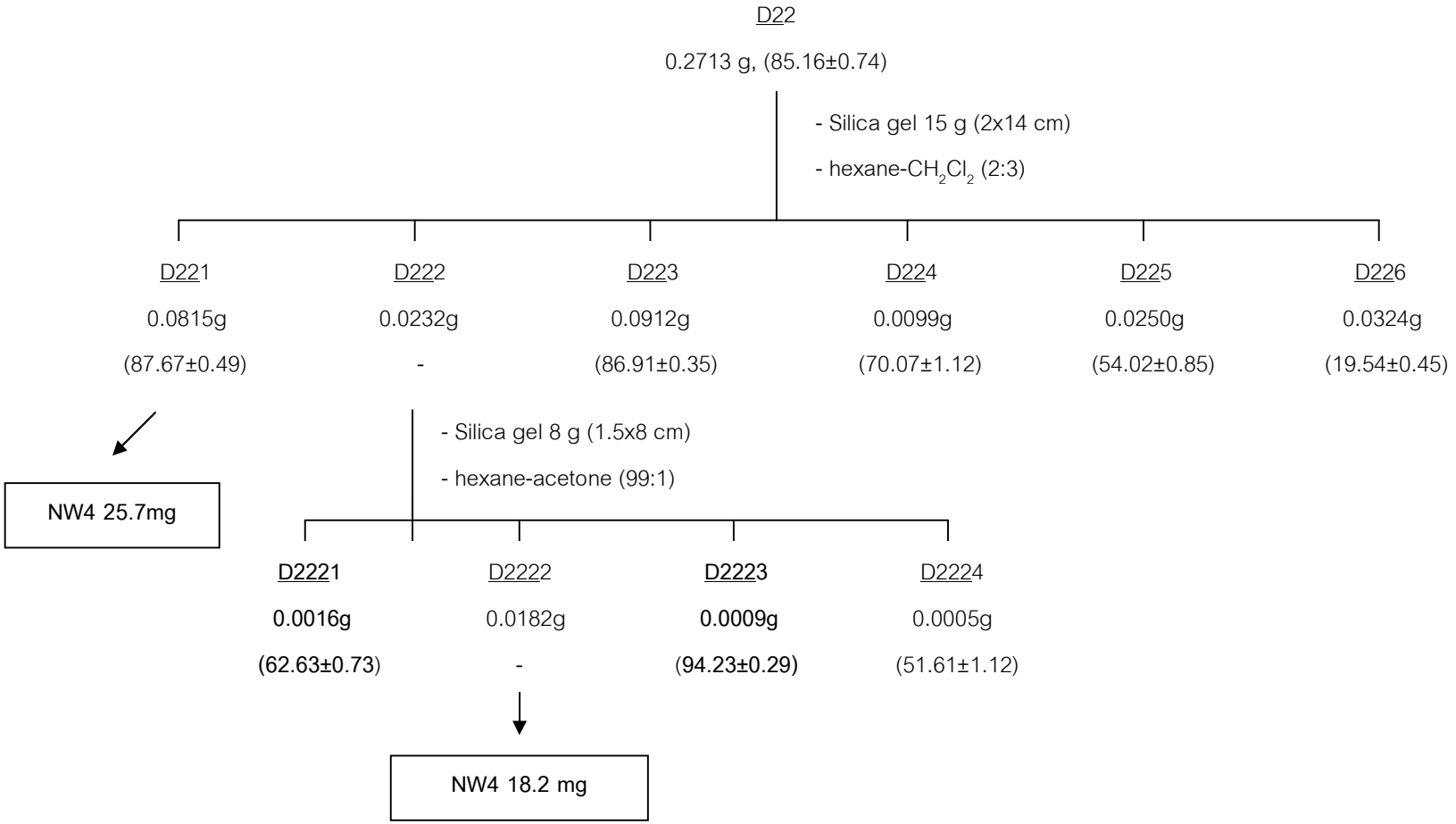
Scheme 8 Isolation of the active compounds from EtOAc extract from *N. wallichiana* stems (2nd lot)

( ) = Mean±SD of %inhibition at final concentration of 12.5 µg/ml



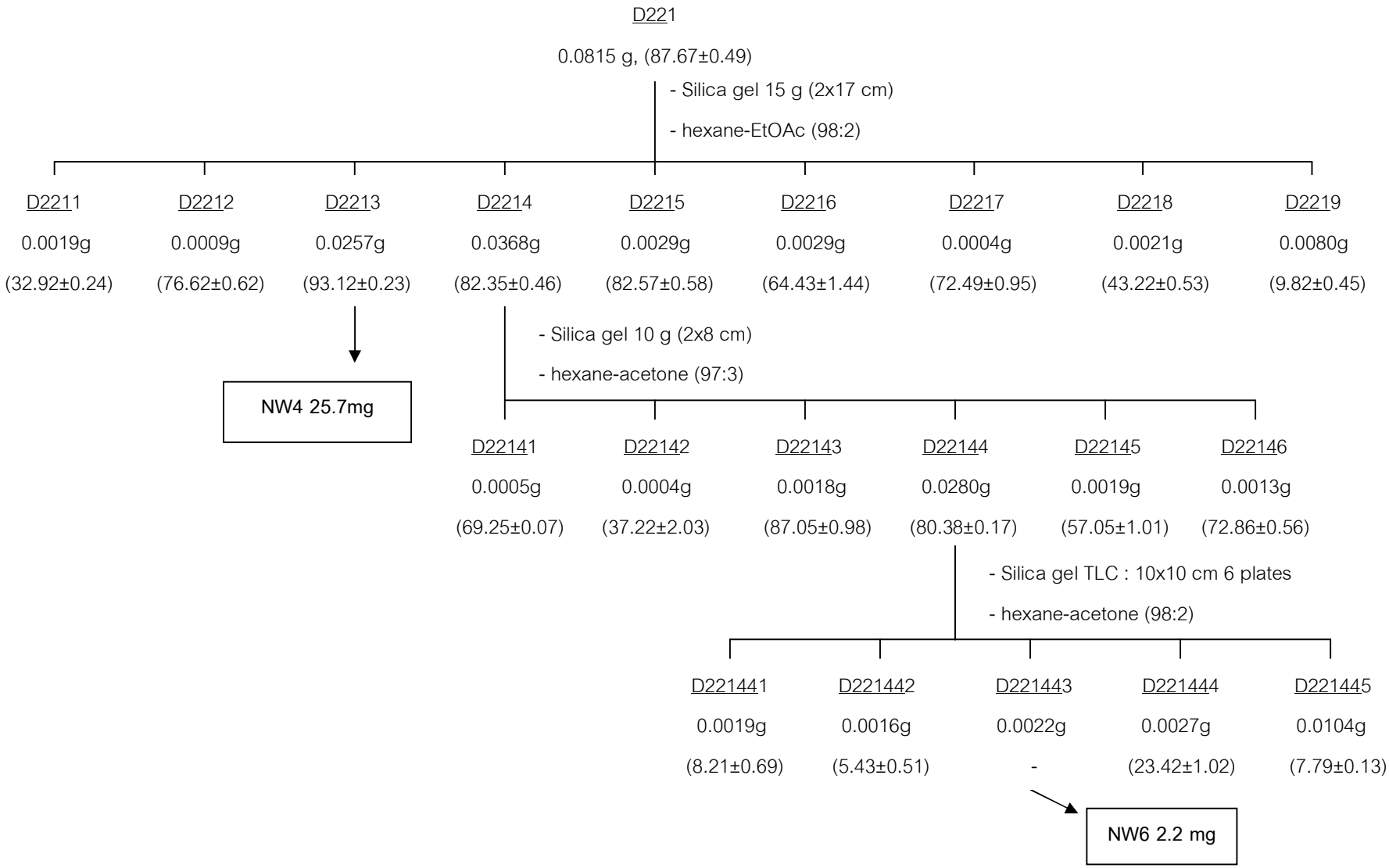
Scheme 9 Isolation of NW4 from EtOAc extract of *N. wallichiana* stems (2nd lot)

, ( ) = Mean±SD of %inhibition at final concentration of 12.5 µg/ml



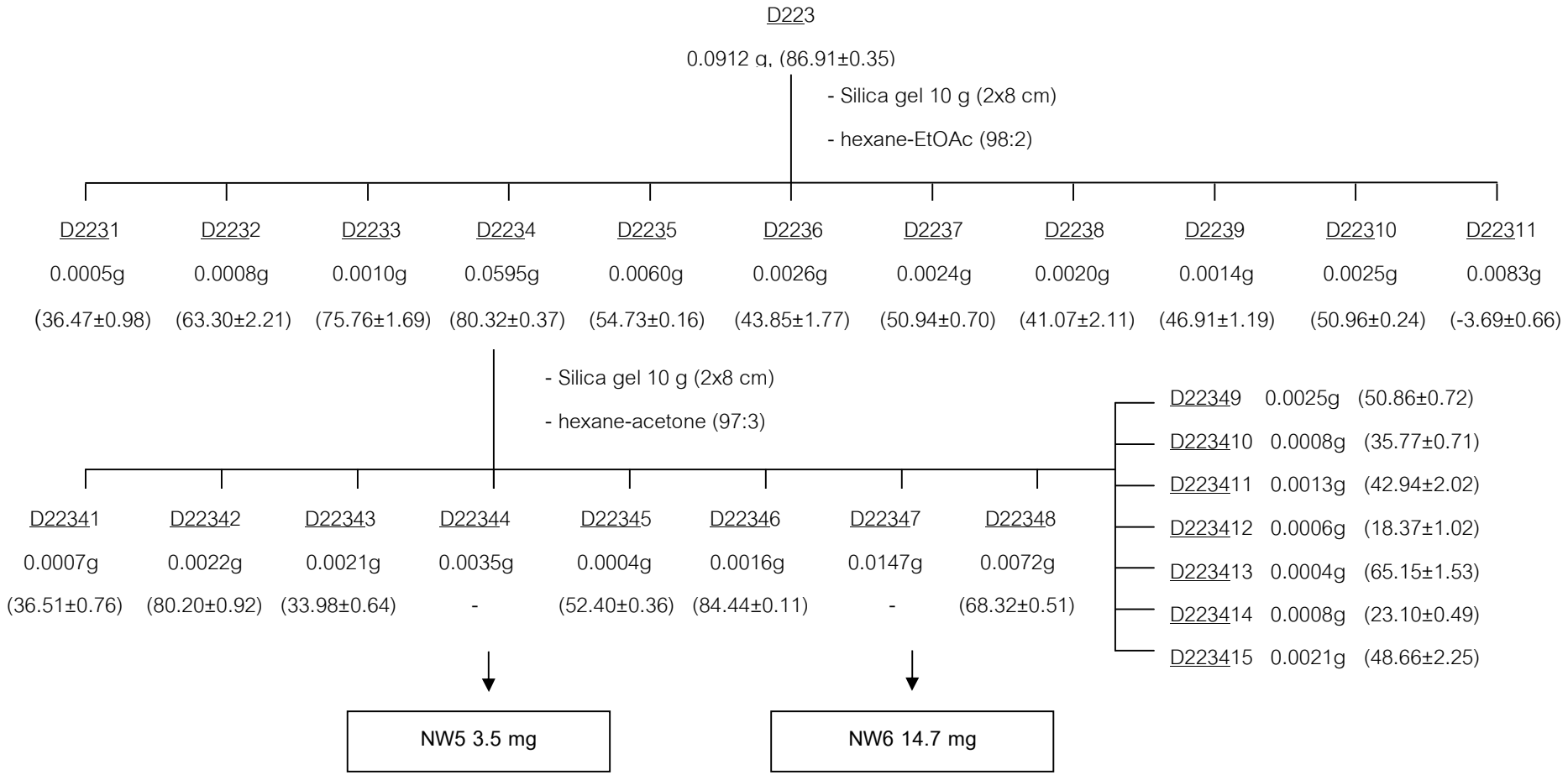
Scheme 10 Isolation of NW4 from sub-fraction D22 of EtOAc extract of *N. wallichiana* stems (2nd lot)

( ) = Mean±SD of %inhibition at final concentration of 12.5 µg/ml



Scheme 11 Isolation of NW4 and NW6 from sub-fraction D221 of EtOAc extract of *N. wallichiana* stems (2nd lot)

( ) = Mean±SD of %inhibition at final concentration of 12.5 µg/ml



Scheme 12 Isolation of NW5 and NW6 from sub-fraction D223 of EtOAc extract of *N. wallichiana* stems (2nd lot)

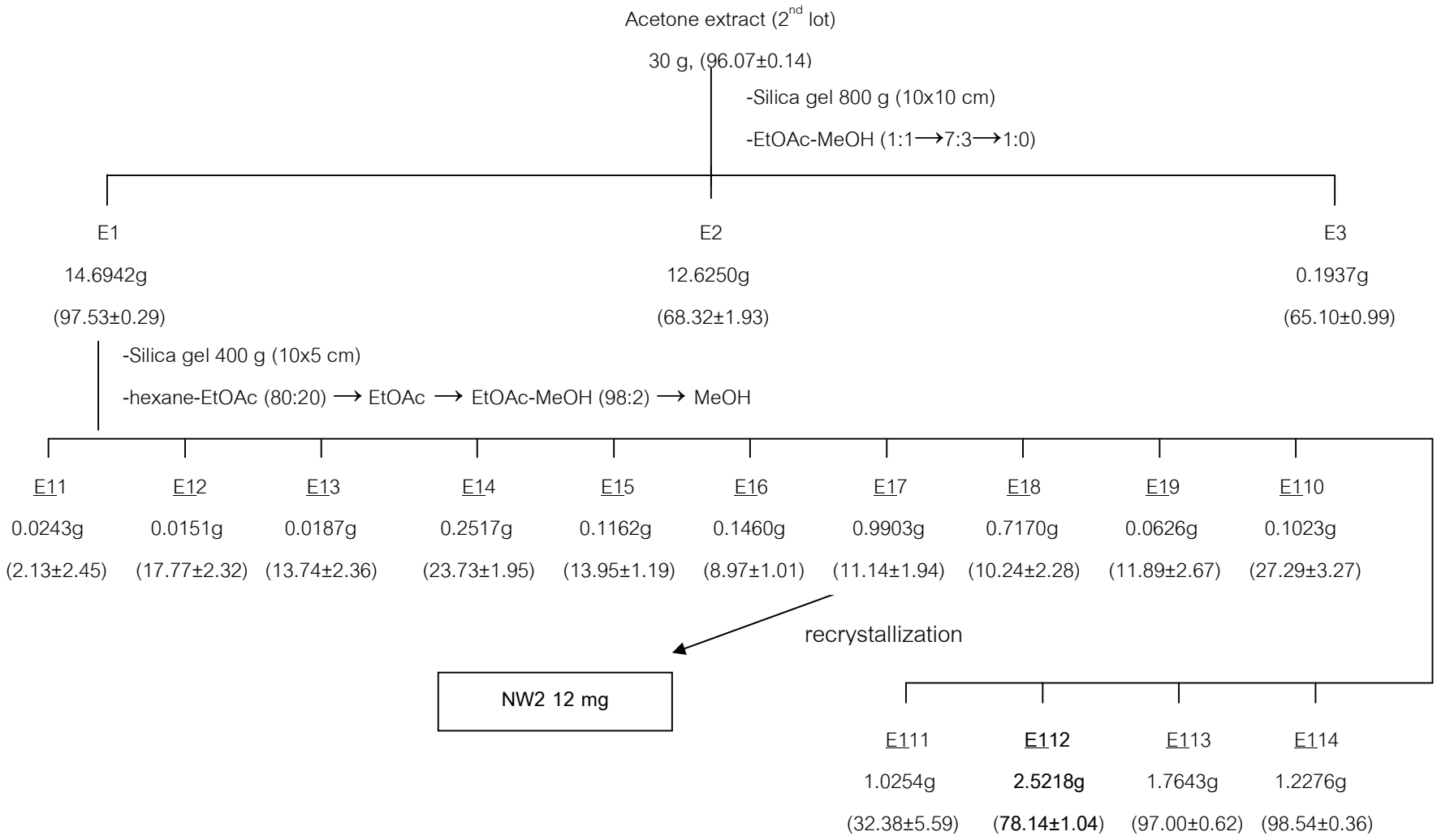
( ) = Mean±SD of %inhibition at final concentration of 12.5 µg/ml

(2) Acetone extract

The 30 g of acetone extract was loaded on a silica gel column chromatography (800g, 10x10 cm) and gradient eluted with EtOAc-MeOH (50:50) ⟶ MeOH. The fraction was received 100 ml per fraction. The fractions with the same TLC pattern were combined to give 3 fractions (E1-E3, **Table 8**). All fractions were tested for activity and the results were shown in **Scheme 13**. Fraction E1 expressed the strong activity (97.53±0.29%) at 12.5 µg/ml so it was further separated. This fraction was re-chromatography using with silica gel, gradient eluted with hexane-EtOAc (80:20) ⟶ MeOH to give 14 fractions (E11-E114). Fraction E17 was re-crystallization to obtain a pure compound NW2 (12 mg). Fractions E112, E113, and E114 which showed strong activity (78.14±1.04%, 97.00±0.62%, and 98.54±0.36 %inhibition respectively) so they were further isolated. Fraction E112 was subjected to a silica gel column chromatography, gradient eluted with $CHCl_3$-EtOH (6:4) ⟶ MeOH to give 8 fractions (E1121-E1128). Fraction E1122 was further purified with Sephadex LH20, eluted with MeOH and was re-columned with two silica gel column, eluted with EtOAc-MeOH (96:4) and $CHCl_3$-EtOH (9:1) to obtain a pure compound NW7 (2.5 mg) **(Scheme 14)**.

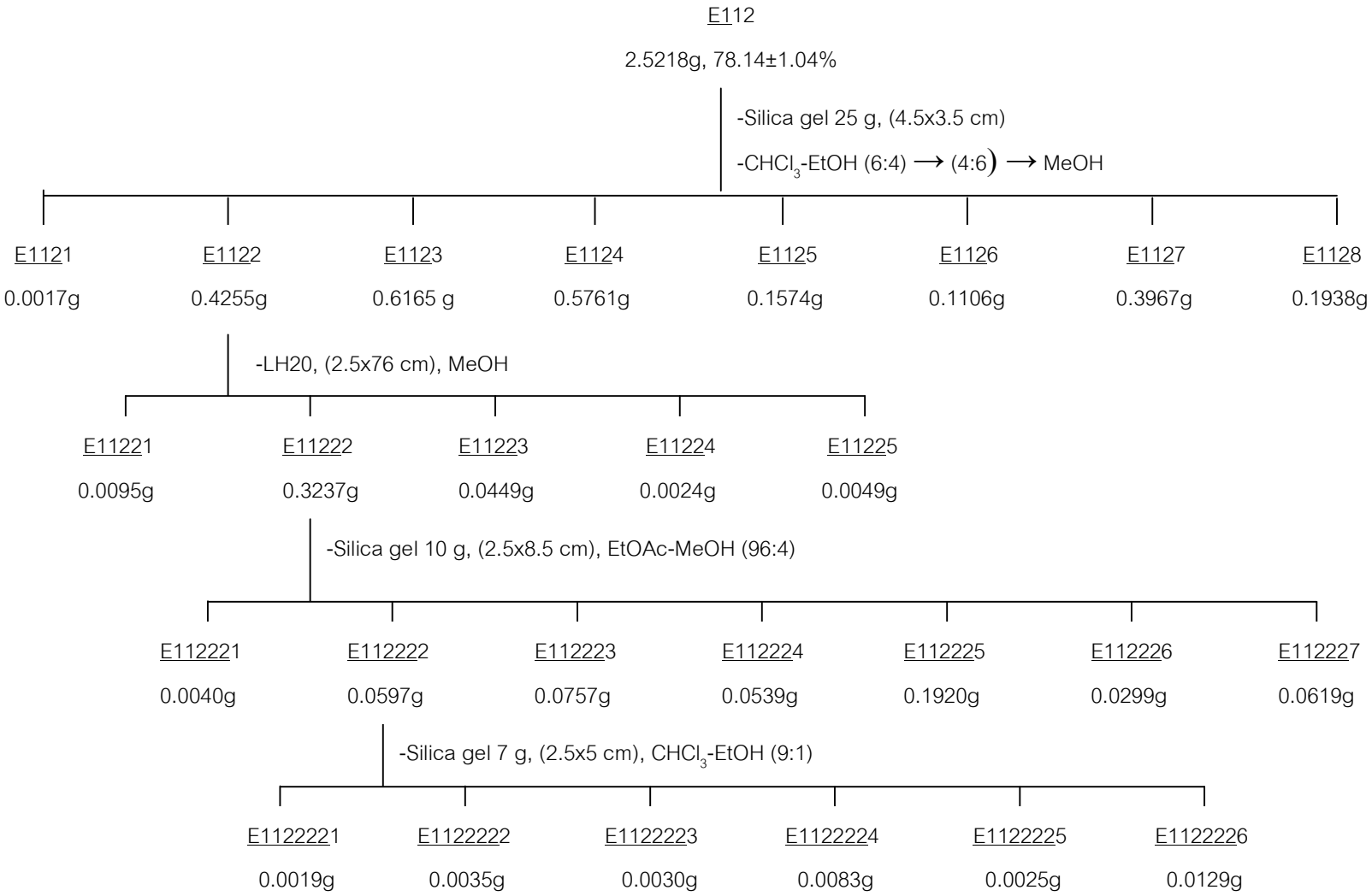
Table 8 Combined fraction of the first column of the acetone extract of *N. wallichiana* stems (2^{nd} lot)

Fraction code	No. of eluate	Weight (g)
E1	1-6	14.6942
E2	7-14	12.6250
E3	15	0.1937



Scheme 13 Isolation of NW2 from acetone extract of *N. wallichiana* stems (2nd lot),

( ) = Mean±SD of %inhibition at final concentration of 12.5 µg/ml



Scheme 14 Isolation of NW7 from sub-fraction E112 of acetone extract of *N. wallichiana* stems (2nd lot)

( ) = Mean±SD of %inhibition at final concentration of 12.5 µg/ml

(3) MeOH

Chemical constituents in the methanol extract were very polar. The suitable chromatographic system could not be developed.

3.5 Physical and Spectral Data of the Isolated Compounds

3.5.1 Compound NW1

Compound NW1 was obtained as clear rosette needle in CH_2Cl_2. This compound is soluble in CH_2Cl_2 and $CHCl_3$.

Mp: 136-138 °C

ESI-MS: *m/z*: 437 $[M+Na]^+$, 413 $[M-H]^+$; **Figure A1 (Appendix I).**

IR: ν_{max} cm^{-1} (KBr): 3426, 2960-2852, 1465, 1376, 1062-1054; **Figure A2 (Appendix I).**

^{1}H-NMR: δ ppm, 500 MHz, in $CDCl_3$; 0.66 (3H, *s*), 0.79 (3H, *d,* J = 7 Hz), 0.81 (3H, *d*, J = 7 Hz), 0.82 (3H, *t*, J = 7.5 Hz), 0.90 (3H, *d*, J = 6.5 Hz), 0.99 (3H, *s*), 3.50 (1H, *ddt*, J = 11, 11.25, 4.75 Hz) and 5.33 (1H, *dd*, J = 2.5 Hz); **Figure A3 (Appendix I).**

^{13}C-NMR: δ ppm, 125 MHz, in $CDCl_3$ 11.8, 12.0, 18.8, 19.0, 19.4, 19.8, 21.1, 23.1, 24.3, 26.1, 28.2, 29.1, 31.6, 31.9, 31.9, 33.9, 36.1, 36.5, 37.2, 39.7, 42.3, 42.3, 45.8, 50.1, 56.0, 56.8, 71.8, 121.7, and 140.7; **Figure A4 (Appendix I).**

3.5.2 Compound NW2

Compound NW2 was obtained as clear needle crystal in MeOH with little of pyridine. This compound is soluble in pyridine, DMSO and slightly soluble in MeOH.

Mp: 300-301 °C

UV: λ_{max} nm (MeOH): 233, 268, 331; **Figure A5 (Appendix I).**

ESI-MS: *m/z*: 371 $[M+Na]^+$, 349 $[M+H]^+$; **Figure A6 (Appendix I).**

IR: ν_{max} cm^{-1} (KBr): 3468, 3391, 1744, 1698, 1627, 1544, 1077; **Figure A7 (Appendix I).**

^{1}H-NMR: δ ppm, 500 MHz, in C_5D_5N; 1.24 (3H, *d*, J = 7 Hz), 1.30 (3H, *d*, J = 7 Hz), 1.31 (3H, *s*), 1.54 (1H, *dt*, J = 14.5, 7 Hz), 1.80 (1H, *d*, J = 5.5 Hz), 1.99 (3H, *s*), 2.00 (2H, *m*), 2.52 (1H, *dt*, J = 14.5, 7 Hz), 3.48 (1H, *sept*, J = 7 Hz), 4.10 (1H, *dt*, J = 12, 5.75 Hz), 5.16 (1H, *dd*, J = 8.5, 5.5 Hz), 5.65 (1H, *dd*, J = 8.5, 3.5 Hz), 6.75 (1H, *d*, J = 5.75 Hz), 7.36 (1H, *s*), 7.82 (1H, *d*, J = 3.5 Hz); **Figure A8 (Appendix I).**

^{13}C-NMR: δ ppm, 125 MHz, C_5D_5N; 16.1, 20.2, 20.7, 24.6, 28.0, 29.6, 29.8, 41.6, 43.2, 50.2, 60.5, 71.1, 74.9, 108.1, 112.0, 162.8, 166.1, 169.6, and 181.4; **Figure A9 (Appendix I).**

3.5.3 Compound NW3

Compound NW3 was obtained as clear needle crystal in MeOH. This compound is soluble in MeOH.

Mp: 134-135 °C

UV: λ_{max} nm (MeOH): 214, 232, 273, 295; **Figure A14 (Appendix I).**

ESI-MS: *m/z*: 355 $[M+Na]^+$; **Figure A15 (Appendix I).**

IR: ν_{max} cm^{-1} (KBr): 3391, 3156, 2984-2849, 1690; **Figure A16 (Appendix I).**

^{1}H-NMR: δ ppm, 500 MHz, in CD_3OD; 1.04 (3H, *s*), 1.14 (1H, *ddd*, J = 14.5, 13.5, 4 Hz), 1.28 (3H, *s*), 1.33 (3H, *d*, J = 7 Hz), 1.37 (1H, *br dd*, J = 13.5, 4 Hz), 1.40 (3H, *d*, J = 7 Hz), 1.58 (1H, *br ddd*, J = 14, 4, 3.5 Hz), 2.01 (1H, *dd*, J = 13.5, 2 Hz), 2.02 (1H, *ddd*, J = 14.5, 13.5, 3.5 Hz), 2.15 (1H, *ddd*, J = 16.5, 13.5, 3 Hz), 2.23 (2H, *br d*, J = 14 Hz), 2.27 (1H, *br d*, J = 14 Hz), 3.52 (1H, *sept*, J = 7 Hz), 4.92 (1H, *br s*), 6.64 (1H, *d*, J = 8.5 Hz), 6.96 (1H, *d*, J = 9 Hz); **Figure A17 (Appendix I).**

^{13}C-NMR: δ ppm, 125 MHz, in CD_3OD; 20.9, 21.0, 21.3, 22.9, 29.0, 29.1, 32.4, 38.6, 39.7, 41.0, 44.3, 46.2, 66.1, 117.7, 124.9, 134.2, 135.0, 141.1, 155.5, 181.9; **Figure A18 (Appendix I).**

3.5.4 Compound NW4

Compound NW4 was obtained as light yellow wax. This compound is soluble in CH_2Cl_2 and $CHCl_3$.

ESI-MS: *m/z*: 287 $[M+H]^+$, 309 $[M+Na]^+$; **Figure A23 (Appendix I).**

IR: ν_{max} cm^{-1} (KBr): 3461, 3049, 2939-2846; **Figure A24 (Appendix I).**

^{1}H-NMR: δ ppm, 500 MHz, in $CDCl_3$; 0.91 (3H, *s*), 0.94 (3H, *s*), 1.17 (3H, *s*), 1.21 (1H, *dd*, J = 13.5, 4 Hz), 1.26 (1H, *dd*, J = 13, 2 Hz), 1.31 (1H, *dd*, J = 14.5, 3.5 Hz), 1.33 (3H, *d*, J = 7 Hz), 1.34 (3H, *d*, J = 7 Hz), 1.46 (1H, *dtd*, J = 13, 3, 1.5 Hz), 1.57 (1H, *dquin*, J = 14, 3.5 Hz), 1.65 (1H, *m*), 1.70 (1H, *dddt*, J = 13.5, 13.5, 13.5, 3 Hz), 1.73 (*m*), 1.90 (1H, *br dd*, J = 13, 8 Hz), 2.22 (1H, *br d*, J = 12 Hz), 2.74 (1H, *ddd*, J = 18.25, 11.25, 8 Hz), 2.93 (1H, *dd*, J = 17.25, 6.25 Hz), 3.28 (1H, *sept*, J = 7 Hz), 4.43 (1H, *br s*), 6.50 (1H, *d*, J = 8.5 Hz), 6.99 (1H, *d*, J = 8.5 Hz); **Figure A25 (Appendix I).**

^{13}C-NMR: δ ppm, 125 MHz, in $CDCl_3$; 19.33, 19.46, 20.32, 20.34, 21.56, 25.15, 27.12, 28.74, 33.22, 33.25, 37.68, 39.54, 41.56, 49.55, 114.26, 122.97, 130.9, 133.9, 143.19, 151.92; **Figure A26 (Appendix I).**

3.5.5 Compound NW5

Compound NW5 was obtained as yellow wax. This compound is soluble in CH_2Cl_2 and $CHCl_3$.

UV: λ_{max} nm (MeOH): 242, 281; **Figure A31 (Appendix I).**

ESI-MS: *m/z*: 391 $[M+H]^+$, 413 $[M+Na]^+$; **Figure A32 (Appendix I).**

IR: ν_{max} cm^{-1} (KBr): 3071, 2959-2873, 1728, 1286, 1272; **Figure A33 (Appendix I).**

^{1}H-NMR: δ ppm, 500 MHz, in $CDCl_3$; 0.87 (3H, *t*, J = 7 Hz), 0.90 (3H, *t*, J = 7.5 Hz), 1.29 (2H, *m*), 1.33 (2H, *m*), 1.40 (2H, *qd*, J = 14, 7 Hz), 1.66 (1H, *sept*, J = 6 Hz), 4.18 (1H, *dd*, J = 11.5, 6 Hz), 4.22 (1H, *dd*, J = 11.5, 6 Hz), 7.51 (1H, *dd*, J = 5.75, 3.25 Hz), 7.69 (1H, *dd*, J = 5.75, 3.25 Hz); **Figure A34 (Appendix I)**.

^{13}C-NMR: δ ppm, 125 MHz, in $CDCl_3$; 11.0, 14.0, 23.0, 23.7, 29.0, 30.4, 38.7, 68.2, 129.0, 130.9, 132.5, 167.8; **Figure A35 (Appendix I)**.

3.5.6 Compound NW6

Compound NW6 was obtained as yellow wax. This compound is soluble in CH_2Cl_2 and $CHCl_3$.

UV: λ_{max} nm (MeOH): 234, 285; **Figure A39 (Appendix I)**.

ESI-MS: *m/z*: 285 $[M-H]^+$, 301 $[M+15]^+$; **Figure A40 (Appendix I)**.

IR: ν_{max} cm^{-1} (KBr): 3388, 2960-2846; **Figure A41 (Appendix I)**.

^{1}H-NMR: δ ppm, 500 MHz, in $CDCl_3$; 0.90 (3H, *s*), 0.93 (3H, *s*), 1.16 (3H, *s*), 1.19 (1H, *dd*, J = 14, 4 Hz), 1.21 (3H, *d*, J = 7 Hz), 1.23 (3H, *d*, J = 7 Hz), 1.30 (1H, *dd*, J = 12.5, 2.5 Hz), 1.36 (1H, *td*, 13, 4 Hz), 1.45 (1H, *dtd*, J = 13, 3, 1.5 Hz), 1.57 (1H, *dquin*, J = 14, 3.25 Hz), 1.65 (1H, *m*), 1.72 (1H, *dddt*, J = 13.25, 13.25, 13.66, 3.5 Hz), 1.84 (1H, *dddd*, J = 13, 7.25, 1.94, 2 Hz), 2.15 (1H, *br dd*, J = 12.5, 1 Hz), 2.76 (1H, *ddd*, J = 17, 11, 7.1 Hz), 2.85 (1H, *ddd*, J = 16.75, 6.6, 1.5 Hz), 3.10 (1H, *sept*, J = 7 Hz), 4.51 (1H, *br s*), 6.61 (1H, *s*), 6.82 (1H, *s*); **Figure 42 (Appendix I)**.

^{13}C-NMR: δ ppm, 125 MHz, in $CDCl_3$; 19.2, 19.3, 21.6, 22.5, 22.7, 24.8, 26.8, 29.7, 33.3, 33.42, 37.5, 38.8, 41.7, 50.3, 110.9, 126.6, 127.3, 131.4, 148.7, 150.6; **Figure 43 (Appendix I)**.

3.5.7 Compound NW7

Compound NW7 was obtained as colorless needles in MeOH. This compound is soluble in pyridine.

ESI-MS: *m/z*: 511 $[M+H]^+$, 533 $[M+Na]^+$; **Figure A44 (Appendix I)**.

IR: ν_{max} cm^{-1} (KBr): 3432, 2937-2904, 1775, 1758, 1712, 1077-997; **Figure A45 (Appendix I)**.

^{1}H-NMR: δ ppm, 500 MHz, in C_5D_5N; 0.99 (3H, *d*, J = 7 Hz), 1.15 (3H, *d*, J = 7 Hz), 1.22 (3H, *s*), 1.38 (1H, *td*, J = 12.5, 4.3 Hz), 1.59 (1H, *dt*, J = 13, 4.25 Hz), 1.69 (3H, *s*), 1.91 (1H, *d*, 4.5 Hz), 1.94 (1H, *dq*, J = 7, 3.5 Hz), 2.05 (1H, *dtd*, J = 14.5, 11.5, 3.8 Hz), 2.38 (1H, *dtd*, J = 14.25, 4.9, 5 Hz), 3.94 (1H, *dd*, J = 10.5, 5.5 Hz), 3.98 (1H, *ddd*, J = 8.25, 6, 2.3 Hz), 4.10 (1H, *t*, J = 8.25 Hz), 4.21 (1H, *t*, J = 8.5 Hz),4.22 (1H, *d*, J = 7.5 Hz), 4.25 (1H, *t*, J = 8.5 Hz), 4.39 (1H, *dd*, J = 11.75, 5.25 Hz), 4.55 (1H, *d*, J = 3.5 Hz), 4.57 (1H, *dd*, J = 11.25, 2.25 Hz), 4.93 (1H, *d*, J = 7.5 Hz), 5.11 (2H, *br d*, J = 4.5 Hz), 6.12 (1H, *s*); **Figure A46 (Appendix I)**.

^{13}C-NMR: δ ppm, 125 MHz, in C_5D_5N; 16.6, 21.3, 21.9, 24.2, 26.8, 27.3, 29.0, 36.3, 45.4, 46.3, 54.2, 58.9, 62.9, 71.5, 72.2, 75.5, 78.6, 78.6, 82.9, 83.2, 107.7, 117.1, 159.0, 163.8, 176.3 ; **Figure A47 (Appendix I)**.

3.6 Bioautography development for screening of lipase inhibitory activity

Although the fluorometrically method for screening of lipase inhibitors using 4-methylumbelliferyl oleate (4-MUO) caused a good sensitivity in 96-well plate assay, the high cost of this substrate was not reasonable to be used in the development of bioautographic assay. So *p*-nitrophenyl palmitate was another choice for using as substrate for bioautography assay. The advantage of *p*-nitrophyl palmitate is the yellow color product in alkaline solution which can be visualized. It can be dissolved in organic solvent which is easily to be removed from TLC plate. Moreover its cost is cheaper than 4-MUO.

3.6.1 Screening of crude plant extracts with colorimetric assay

3.6.1.1 Preparation of plant extracts

The 2 mg/ml stock solution of the plant extracts were dissolved in DMSO and kept in -20°C before used.

3.6.1.2 Screening of plant extract samples with colorimetric assay

The method of Slanc et al (2004) was optimized for enzymatic assay. *p*-nitrophenyl palmitate (PNP) (Sigma®) was dissolved in acetonitrile to give a stock soltution with a concentration of 10 mM. Ethanol was then added to a final composition of acetonitrile:ethanol (1:2), resulting in 3.33 mM PNP. The solution was stored at -20 °C. Porcine pancreatic lipase (type II, crude, Sigma®) was dissolved in 0.061 M of Tris-HCl buffer, pH 8.5 to a final concentration of 5 mg/mL. Two hundred microlitres of reaction mixture, containing 0.30 mg/mL of enzyme, 5 µg/mL of plant extract, 0.167 mM of PNP and 0.061 M of Tris-HCl buffer, pH 8.5, was incubated at 37 °C for 25 min in shaking water bath 100 rpm. The absorbance of released *p*-nitrophenol was measured at 405 nm using a microplate reader (Victor^3, Perkin Elmer, USA). The enzyme was replaced by Tris-HCl buffer and the extract was replaced by 5%DMSO in distilled water for the blank and the solvent controls. The percentage of inhibition was calculated by the following equation i.e.

$$\%\text{Inhibition} = \left[\frac{(AE-AEB)-(AS-ASB)}{(AE-AEB)}\right] \times 100$$

where AE and AS were the absorbance values of the normal assay condition of the solvent blank and sample plant extract after 25-min incubation respectively, AEB and ASB were the absorbance values of the blank of the solvent control and sample plant extract respectively. Pancreatic lipase inhibitory activity was performed in triplicate for each plant extract. The results were demonstrated by means ± standard deviations. Oristat (Sigma®) was used as positive control for the assay. Final concentration of DMSO in the assay was 0.25%v/v which did not affect the assay system.

3.6.2 Determination of the substrate and enzyme concentrations for bioautographic assay

The first trial condition was to prepare substrate and enzyme in the same concentration of the 96-well plate. The 3.8 mg of substrate was weighed and dissolved in 1 ml of acetonitrile then added 2 ml of EtOH to give 3.33 mM PNP. The 25 mg of enzyme was weighed and dissolved in 5.0 ml 0.061 M Tris-HCl buffer to obtain 5 mg/ml. The substrate and enzyme solutions were kept at -20°C and 4°C respectively before used. Before dipping 1.5x1.5 cm of TLC plate into the substrate solution, the substrate was diluted to 0.167 mM by the 0.061 M Tris-HCl buffer. After dipping the TLC plate into the substrate, TLC plate was evaporated to damply dry, and then dipped the TLC plate in the enzyme solution. After that, the TLC plate was put in the glass plate which had wet cotton around the TLC plate for keeping it moist and then incubated at 37°C in the incubator. The yellow color of the product (*p*-nitrophenol) was observed and recorded every 5-15 minutes. The control TLC plate was done by using 0.061 M Tris-HCl buffer instead of the enzyme solution. The experiment was run in duplicate. After 2-hour observation, if the yellow color did not occur, they were continued to incubate until 4 hours. If all TLC plate did not change to yellow color, the experiment was stopped. This result was implied that the reaction of the enzyme did not occur but in the 96-well was appeared dominant yellow solution on enzymatic reaction wells after incubation in 37°C shaking water bath. This may be done to the low concentration of the substrate and enzyme per area of the TLC plate. Therefore, the next experiment was tried to vary concentrations of substrate and enzyme to determine the suitable condition.

The substrate was varied in three concentrations including 0.83 mM, 1.67 mM, and 3.33 mM. Substrate was prepared by weighing 15.6 mg of PNP, dissolved in 4.0 ml of ACN followed by adding 8.0 ml of EtOH to give 3.33 mM PNP. And 2-fold dilution with ACN:EtOH (1:2) had been done for the other concentrations. All of substrates were kept at -20°C and thawed to room temperature (25°C) before used. The enzyme was varied in five concentrations including 0.032 (10 Units/ml), 2.5, 5, 10, 20 and 40 mg/ml respectively. The 40 mg/ml of enzyme stock solution was prepared by weighing 400 mg, dissolving in 0.061 M Tris-HCl buffer, and adjusting the volume to

10.0 ml. The other concentrations of enzyme solutions were 2-fold dilution from 40 mg/ml of the enzyme stock solution. All enzyme solutions were kept on ice bath before used. The control group had been done in the same way as above using 0.061 M Tris-HCl buffer instead of the enzyme solution. The experiment was done in duplicate. The process of this experiment was shown in following procedures.

Firstly, incubated the glass plate (reaction tank, ϕ 18 cm) was irrigated with some water at 37^{o}C in the incubator about 1 hour to adjust the temperature of the enzymatic reaction tank to 37^{o}C. After the preparation of enzyme and substrate solutions was finished, dipped each TLC plate (1.5 x 1.5 cm) into each concentration of the enzyme solution which was kept on the ice bath, put it in the incubated glass plate **(Figure 9)**, and continued to incubate the glass plate for an hour to reach the optimum temperature. Then the substrate solution at room temperature was sprayed on the TLC plate. Each concentration of substrate was sprayed three times per each TLC plate. Then close the cover of the glass plate when the TLC plate went damply dry. Finally, the glass plate tank was incubated at 37^{o}C and observed the yellow color.

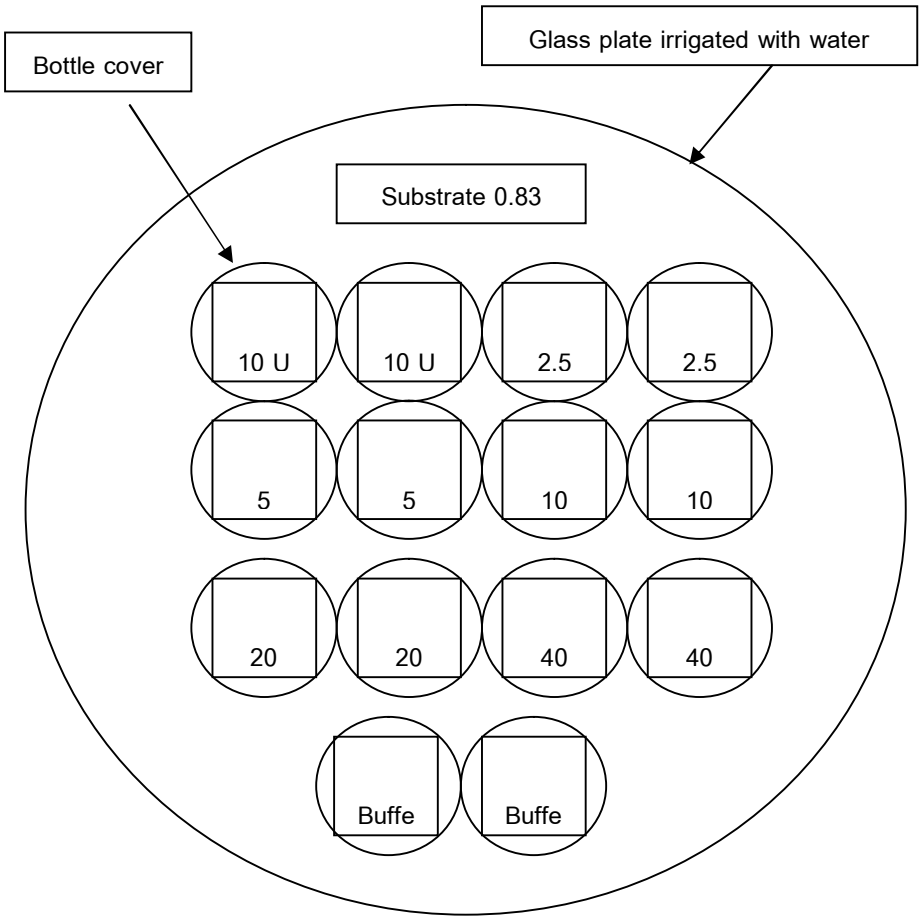


Figure 9 Sample of incubated preparation glass plate containing of the TLC plate (square shape) which put on the bottle cover (small circle shape). The bottle cover was stood in glass plate irrigated with water. The number in the square was the enzyme concentration or buffer which the TLC was dipped.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Screening for lipase inhibitors of Thai medicinal plants

There were 37 extracts from 28 species of the plants in this screening test. All extracts were tested at the concentration of 5 µg/ml. The lipase inhibitory activity was expressed as mean percentage of inhibition and standard deviation (**Table 9**). Activities of six extracts were greater than 50%, and those of 3 extracts from 2 species were more than 80%. These extracts were determined for the IC_{50} values. The highly active extracts were selected for further determining the active compounds. The lipase inhibitory activity of six selected extracts at 5 µg/ml were 51.44±3.30, 57.51±2.28, 68.71±0.24, 88.13±0.71, 86.60±0.66, and 88.71±0.57% for *P. kesiya* (leaves), *N. triquetra* (arial parts), *I. grandifolia* (leaves), *P. emblica* (seeds), and *N. wallichiana* (stems and leaves), respectively. Their IC_{50} values were 5.08±0.18, 4.88±0.29, 3.20±0.27, 1.64±0.10, 1.35±0.09, and 1.33±0.03 µg/ml respectively **(Table 10)**. When comparing the IC_{50} value, the pancreatic lipase inhibitory activity of the leaves part of *N. wallichiana* was equal to their stem. However, the leaves part had more chlorophyll which might disturb the isolation process. Thus the stem part of *N. wallichiana* was selected for the isolation of the active compounds.

Table 9 Inhibitory activity of porcine pancreatic lipase from plant extracts

Family	Species	Part	%inhibition (Mean±SD)
Asclepiadaceae	*Cryptolepis buchanani* Roem. & Schult.	stems	10.00±0.88
Asteraceae	*Emilia sonchaifolia* (L.) Dc. Ex Wight	arial parts	13.57±0.68
Clusiaceae	*Garcinia cowa* Roxb. ex DC.	leaves	23.00±1.22
	Garcinia schomburgkiana Pierre	fruits	12.79±0.77
Cucurbitaceae	*Coccinia grandis* (L.) Voigt	arial parts	15.21±1.18
		fruits	15.77±0.76

Table 9 (continued)

Family	Species	Part	%inhibition (Mean±SD)
	Gymnopetalum chinense (Lour.) Merr.	fruits	8.00±0.59
	Gymnopetalum integrifolium (Roxb.) Kurz	fruits	7.50±0.38
	Luffa acutangula (L.) Roxb.	arial parts	13.54±0.63
	Luffa cylindrica (L.) M.Roem.	arial parts	7.67±0.43
		flowers	3.13±0.03
		fruits	5.14±0.56
Euphorbiaceae	*Phyllanthus emblica* L.	seeds	88.13±0.71
Gnetaceae	*Gnetum gnemon* L.	leaves	22.46±1.39
	Gnetum montanum Markgr.	leaves	45.78±1.20
Leguminosae	*Neptunia oleracea* Lour.	whole plant	10.78±0.77
	Neptunia triquetra (Vahl) Benth.	arial parts	57.51±2.28
Oxalidaceae	*Averrhoa bilimbi* L.	leaves	34.07±2.52
		fruits	2.31±0.14
Passifloraceae	*Passiflora foetida* L.	arial parts	15.89±1.20
		fruits	13.42±0.49
Pedaliaceae	*Sesamum indicum* L.	seeds	8.60±0.44
Pinaceae	*Pinus kesiya* Royle ex Gordon	leaves	51.44±3.30
Poaceae	*Cymbopogon citratus* Stapf	arial parts	16.43±1.47
	Oryza sativa L. cv. Jao Hom Nin	seeds	4.67±0.50
Podocarpaceae	*Nageia wallichiana* C. Presl Kuntze	stems	86.60±0.66
		leaves	88.71±0.57
Polygonaceae	*Polygonum odoratum* Lour.	arial parts	21.30±1.57
Rubiaceae	*Morinda citrifolia* L.	leaves	6.74±0.64
		fruits	9.66±1.30
	Ixora grandifolia Zoll.& Moritzi	flowers	43.02±1.32
		leaves	68.71±0.24
	Randia horrida Roem. & Schult.	fruits	10.73±0.52

Table 9 (continued)

Family	Species	Part	%inhibition (Mean±SD)
	Scyphiphora hydrophyllacea Gaertn.f.	leaves	11.56±1.04
		stems	14.44±0.52
Rutaceae	*Citrus hystrix* DC.	leaves	13.47±0.30
Sterculiaceae	*Scaphium scaphigerum* (G.Don) Guib. & Planch.	leaves	8.03±0.73
	Orlistat [a]		53.00±0.41

The final concentration of each plant extract was tested at 5µg/ml.
Three measurements were carried out per sample (n=3).
[a] A final concentration of orlistat for this study was 8 ng/ml.

Table 10 IC_{50} values of crude extract which had more than 50% inhibition

Crude extracts	Plant Part	% Inhibition	IC_{50} values (µg/ml)
Pinus kesiya	leaves	51.44±3.30	5.08±0.18
Neptunia triquetra	arial parts	57.51±2.28	4.88±0.29
Ixora grandifolia	leaves	68.71±0.24	3.20±0.27
Phyllanthus emblica	seeds	88.13±0.71	1.64±0.10
Nageia wallichiana	stems	83.65±1.41	1.35±0.09
	leaves	88.71±0.57	1.33±0.03

4.2 Extraction and isolation of the active compounds from the selected plant

4.2.1 The first lot of *N. wallichiana* stems

The stems of *N. wallichiana* were completely extracted with 95%EtOH. The ethanolic extract was further partitioned successively with hexane, EtOAc, and acetone. All extracts were processed through separation and isolation of the pure compounds. All of the extracts and pure compounds were determined for the lipase inhibitory activity by modifying method from Kurihara et al. (2003). The results were shown in **Tables 11-Table 14**.

The lipase inhibitory activities of crude EtOH, hexane, EtOAc, acetone extracts, and remaining residue of *N. wallichiana* stems were 89.74±1.30%, 19.33 ± 0.73%, 83.58±0.97%, 85.75±0.67%, and 92.17±2.70% respectively **(Table 11)**. The results showed that the medium to high polarity of extracts expressed more potent

activities than non-polar extracts. However, the activity of hexane extract increased from about 19% to 20-50%inhibition after purification **(Table 12)**. Consequently, the next lot of *N. wallichiana* stems was isolated by starting with hexane.

Table 11 Lipase inhibitory activity of all partition extracts of *N. wallichiana* (1st lot) at 12.5 µg/ml (n=3)

Samples*	%inhibition (Mean ± SD)
orlistat 8 ng/ml	50.23 ± 0.81
EtOH extract before partition	89.74 ± 1.30
Hexane extract	19.32 ± 0.72
EtoAc extract	83.58 ± 0.97
Acetone extract	85.75 ± 0.67
EtOH residual extract	92.17 ± 2.69

*Each sample was tested in triplicate.

From the isolation of hexane extract, the main pure compound was isolated and coded as NW1 (17.5 mg). NW1 was elucidated as β-sitosterol. The lipase inhibitory activity of this compound at the concentration of 3.02 µM was 10.49±0.24%. Therefore, the IC_{50} value was more than 3.02 µM. The real IC_{50} value of this compound could not be determined because of the limitation of dissolution in DMSO.

From the EtOAc extract, the semi-purification of crude EtOAc extract through the first silica gel column, the potency of lipase inhibitory activities of the semi-purification fractions were directly varied with the polarity of the fractions (**Table 13**). Two pure compounds isolated from this extract included NW2 (16.6 mg) and NW3 (4.5 mg) which were identified as nagilactone A and inumakiol D respectively. Nagilactone A was isolated from the fraction B3 which had 55.19%inhibition at concentration of 12.5 µg/ml. But its activity was too low to be implied by the high IC_{50} value more than 1.44 mM (>500 µg/ml). The finding indicated that the main compound (nagilactone A) in fraction B3 was not the active compound in this fraction. Inumakiol D was isolated from fraction B2 which showed some lipase inhibitory activity of 32.59%inhibition at a concentration of 12.5

µg/ml. However, sub-fraction B22243 which gave inumakiol D expressed low activity (-8.13%inhibition) at a concentration of 12.5 µg/ml. Therefore, this compound was not the active compound in B2 fraction.

Table 12 Lipase inhibitory activity of hexane sub-fractions of *N. wallichiana* (1st lot) at 12.5 µg/ml (n=2)

Fractions	Mean of %inhibition	Fractions	Mean of %inhibition
Orlistat 7 ng/ml	52.00	A44	24.19
A1	74.43	A45	11.29
A2	56.10	A441	38.21
A3	53.24	A442	8.55
A4	25.43	A443	-
A5	31.42	A444	8.91
A6	45.18	A445	10.52
A7	24.06	A4431	11.29
A41	50.04	A4432	49.08
A42	42.65	A4433	10.56
A43	41.42		

- = not test for the lipase inhibitory activity

From acetone extract, all sub-fractions (C1-C5) from the first column had strong lipase inhibitory activity (>85%inhibition) at a concentration of 12.5 µg/ml (**Table 14**). However, an isolated pure compound (NW2) was not the active compound in this fraction. And the fractions C22243 and C22244 which had potent lipase inhibitory activity (67.46% and 97.65%inhibition) were failed to isolate.

Three isolated pure compounds from this lot of plant material expressed low lipase inhibitory activity. The finding was indicated that the isolated pure compounds by non-bioassay guided fractionation did not meet the aim to find the active components. Consequently, the second lot was isolated by bioassay guided fractionation.

Table 13 Lipase inhibitory activity of EtOAc sub-fractions of *N. wallichiana* (1st lot) at 12.5 µg/ml (n=2)

Fractions	Mean of %inhibition	Fractions	Mean of %inhibition
Orlistat 7 ng/ml	56.81	B221	23.30
B1	24.50	B222	47.39
B2	32.59	B223	41.50
B3	55.19	B224	33.32
B4	66.42	B2221	12.20
B5	86.39	B2222	42.61
B6	94.96	B2223	37.70
B7	96.69	B2224	68.97
B8	95.52	B2225	41.65
Orlistat 7 ng/ml	54.12	Orlistat 7 ng/ml	52.00
B21	44.07	B22241	44.00
B22	10.83	B22242	53.94
B23	34.55	B22243	-8.13
B24	88.79		
B25	83.61		

Table 14 Lipase inhibitory activity of acetone sub-fractions of *N. wallichiana* (1st lot) at 12.5 µg/ml (n=2)

Fractions	Mean of %inhibition	Fractions	Mean of %inhibition
Orlistat 7 ng/ml	52.00	Orlistat 7 ng/ml	63.79
C1	87.46	C2131	9.37
C2	92.52	C2132	7.53
C3	94.60	C2133	-0.54
C4	94.43	C2134	20.03
C5	96.47	C2135	10.68
Orlistat 7 ng/ml	70.19	C2136	4.54
C21	12.91	Orlistat 7 ng/ml	-
C22	90.55	C21311-C21319	-
C23	NF	Orlistat 7 ng/ml	70.19
C24	NF	C221	93.90
C25	NF	C222	13.39
Orlistat 7 ng/ml	63.79	C223	71.47
C211	-3.59	C224	89.81
C212	42.37	Orlistat 7 ng/ml	51.70
C213	-1.14	C2241	66.13
C214	3.63	C2242	6.70
C215	68.66	C2243	67.46
C216	66.23	C2244	97.65
C217	83.90	Orlistat 7 ng/ml	70.19
C218	80.54	C22421	1.95
C219	96.27	C22422	0.09
C2110	87.33	C22423	-3.06
C2111	92.94	C22424	-1.70
		C22425	-2.25

NF = not find the fraction, - = not test for the lipase inhibitory activity

4.2.2 The second lot of *N. wallichiana* stems

There were three active compounds which were isolated from EtOAc extract including NW4, NW5, and NW6. These compounds were elucidated as totarol, bis-2-ethylhexyl-phthalate, and ferruginol. The two pure compounds from acetone extract with weak lipase inhibitory activities included NW2 and NW7 which were elucidated as nagilactone A and nagilactone E-3-*O*-β-glucopyranoside.

Seven pure compounds were isolated from both lots of the material plants. There were 6 known compounds i.e. β-sitosterol, nagilactone A, inumakiol D, totarol, ferruginol and bis-2-ethylhexyl phthalate, and 1 novel compound was nagilactone E-*O*-β-glucopyranoside. The lipase inhibitory activities of the pure compounds were discussed in the topic of elucidation part.

4.3 Identification of the pure compounds

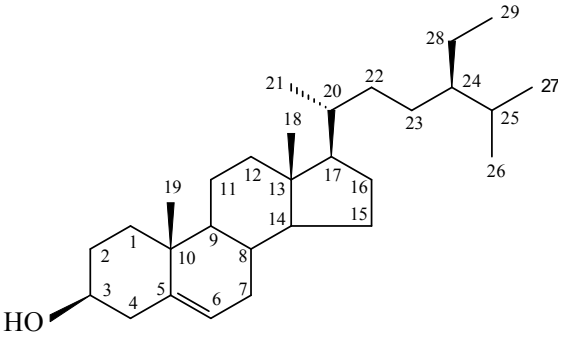
4.3.1 Identification of compound NW1 (β-Sitosterol)

Compound NW1 was identified as β-sitosterol. It was clear needles. Its green positive color with the Liebermann-Burchard test indicated the steroidal skeleton. It gave two layers of colors in one spot upon spraying with anisaldehyde-H_2SO_4, an outer layer was purple and an inner layer was dark pink, which was the characteristic of β-sitosterol. The ESIMS spectrum **(Figure A1 in Appendix)** showed $[M+Na]^+$ ion peak at *m/z* 437, corresponding to molecular formula of $C_{29}H_{50}O$. The IR spectrum displayed the OH stretching at 3425 cm^{-1} **(Figure A2)**.

The ^{1}H-NMR spectrum of NW1 was shown in **Figure A3**. This compound exhibited six methyl signals which are characteristic of steroid nucleus. They were two methyl singlets at δ 0.66 (H-18) and 0.99 (H-19), three methyl doublets at δ 0.79 (J = 7 Hz, H-26), 0.81 (J = 7 Hz, H-27), and 0.90 (J = 6.5 Hz, H-21), and a methyl triplet at δ 0.82 ppm (J = 7.5 Hz, H-29). The proton spectrum also showed a tri-substituted olefinic signal at H-6 and a methylene proton (H-7) at δ 5.33 ppm (*dd*, J = 2.5 Hz, H-6). The

other downfield methine proton at δ 3.50 ppm (*ddt*, J = 11, 11.25, 4.75 Hz) represented a geminal proton to a hydroxyl group at position 3 of a 3β-hydroxy sterol.

The ^{13}C-NMR spectrum was shown in **Figure A4** and was compared with previous report of β-sitosterol (De-Eknamkul and Potduang, 2003) in **Table 15.** This spectrum showed 29 carbon signals, including six methyl carbons at δ 11.8 (C-18), 12.0 (C-29), 18.8 (C-21), 19.0 (C-27), 19.4 (C-19) and 19.8 (C-26), eleven methylene carbons at δ 21.1 (C-11), 23.1 (C-28), 24.3 (C-15), 26.1 (C-23), 28.2 (C-16), 31.7 (C-2), 31.9 (C-7), 34.0 (C-22), 37.3 (C-1), 29.8 (C-12) and 42.3 (C-4), nine methine carbons at δ 29.1 (C-25), 31.9 (C-8), 36.1 (C-20), 45.8 (C-24), 50.1 (C-9), 56.0 (C-17), 56.8 (C-14), 71.8 (C-3) and 121.7 (C-6), and three quaternary carbons at δ 36.5 (C-10), 42.3 (C-13) and 140.7 ppm (C-5), respectively. The olefinic carbon of tri-substituted at C-5 and C-6 were expressed at δ 121.7 (C-6) and 140.7 (C-5). And the hydroxyl substituted at C-3 was shown at 71.8 ppm.



β- Sitosterol

The pancreatic lipase inhibitory activity of β-sitotsterol has been reported (Watanabe et al, 2008). Its activity was 20.87±11.30%inhibition at 242 μM (100 μg/ml). This finding indicated that β-sitosterol may be poor in decreasing absorption of triglycerides. However, the other study indicated that this compound tended to decrease triglyceride in the patients with borderline of triglyceride level in meta-analysis

study (Wu et al, 2009). Consequently, β-sitosterol may decrease triglyceride level by other pathways.

Table 15 ^{13}C-NMR spectral data of NW1 and β-Sitosterol

Position	δ (ppm)	
	NW1*	β-Sitosterol**
	^{13}C	^{13}C
1	37.2	37.2
2	31.6	31.6
3	71.8	71.8
4	42.3	42.2
5	140.7	140.7
6	121.7	121.7
7	31.9	31.9
8	31.9	31.9
9	50.1	50.1
10	36.5	36.5
11	21.1	21.1
12	39.7	39.7
13	42.3	42.3
14	56.8	56.7
15	24.3	24.3

Position	δ (ppm)	
	NW1*	β-Sitosterol**
	^{13}C	^{13}C
16	28.2	28.2
17	56.0	56.0
18	11.8	11.8
19	19.4	19.4
20	36.1	36.1
21	18.8	18.8
22	33.9	33.9
23	26.1	26.0
24	45.8	45.8
25	29.1	29.1
26	19.8	19.8
27	19.0	19.0
28	23.1	23.0
29	12.0	12.0

*Solvent: $CDCl_3$; 125 MHz

**De-Eknamkul and Potduang, 2003; solvent: $CDCl_3$; 125 MHz

4.3.2 Identification of compound NW2 (Nagilactone A)

Compound NW2 was a clear needle crystal, soluble in pyridine and slightly soluble in MeOH. The structure of NW2 was elucidated by interpretation of spectroscopic data comparing with nagilactone A (Hayashi et al, 1977; Ying and Kubo, 1993) as shown in the **Table 16**. The ESI mass spectrum showed the intensity of pseudomolecular ion $[M+Na]^+$ peaks at *m/z* 371 which established the molecular formula as $C_{19}H_{24}O_6$ **(Figure A6)**. The UV spectrum showed the absorption of two lactone

groups at λ_{max} 268 nm (γ-lactone) and 331 nm (α-pyrone) **(Figure A5)**. The IR spectrum **(Figure A7)** indicated the presence of two lactone groups and an α-pyrone group at ν_{max} 1698, 1627 and 1545 cm^{-1}, a γ-lactone group at 1744 cm^{-1}, and the two hydroxyl groups at ν_{max} 3468 and 3391 cm^{-1}.

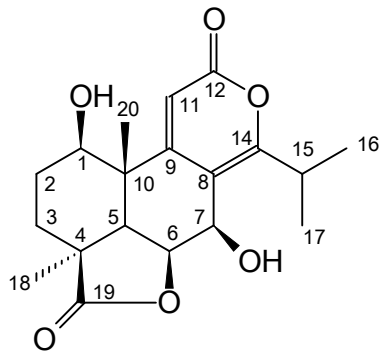
From ^{13}C-NMR spectrum and DEPT spectra **(Figure A9)**, nineteen carbon signals can be differentiated into four methyl carbons, two methylene carbons, six methine carbons, and seven quaternary carbons. There are two carbonyl lactone at δ 181.4 ppm (γ-lactone, C-19) and 162.8 ppm (α-pyrone lactone, C-12). Five conjugated carbons of the α-pyrone ring compose of three quaternary carbons at δ 112.0 (C-8), 166.1 (C-9) and 169.6 ppm (C-14), and one methine at δ 108.1 ppm (C-12).

Analysis of the ^{1}H-NMR spectrum **(Figure A8)** and ^{1}H,^{1}H-COSY spectrum **(Figure A11 and Table 17)** of NW2 showed the presence of an isopropyl protons at δ 3.48 ppm (*septet*, J = 7 Hz, H-15), 1.30 ppm (*d*, J = 7 Hz, H-16), and 1.24 ppm (*d*, J = 7 Hz, H-17) based on their coupling patterns. Two fragments of partial structure were identified. The first fragment, the coupling signals between H-1 (4.14 ppm, *dd*, J = 12 and 6.5 Hz) and H-2 (δ 2.00 ppm, *m*), and H-2 and H-3 (1.54 ppm, *dt*, J = 14 and 6.5 Hz) indicated that H-1 connected to H-2 and H-2 connected to H-3. The second fragment was indicated from the consequent coupling signal of H-5 (1.81, *d*, J = 5.5 Hz), H-6 (5.16 ppm, *dd*, 8.5, 5.5 Hz), and H-7 (5.6 ppm, *dd*, J = 8.5, 3.5 Hz).

Connection of the two fragments was determined base on the HMBC spectrum **(Figure A12)**. The correlations from H-2, H-3 and H-5 to the quaternary carbon at position 4 (C-4) indicated that the first fragment at position 3 connected to the second fragment at position 5 via the quaternary carbon at position 4. The correlation signals between H-1, H-2, 1-OH, H-5 and H-6 with the quaternary carbon at position 10 (C-10) indicated that the first fragment at position 1 connected to the second fragment at position 5 via quaternary carbon at position 10 (C-10). The correlations between H-1 with C-10 and C-9 not C-8, H-6 with C-7 and C-8 not C-9, and H-7 with C-8 and C-9 indicated that C-10 connected with C-9, C-7 connected with C-8, and C-8 and C-9 bonded together. The isopropyl group located on the quaternary carbon at C-14

because H-15, H-16, and H-17 had long-ranged correlations with quaternary carbon at C-14. C-14 bonded with C-8 because of the correlations between H-15 to C-14 and C-8. The correlations from H-11 to C-8, C-9, C-10, and C-12 confirmed the core structure of this compound. CH_3 at position 18 and carbonyl lactone at position 19 connected to the quaternary C-4 based on the correlations from H-18 with C-3, C-4, C-5, and C-19; and from the H-3, H-5, and H-18 to C-19. The other methyl substituted on the quaternary carbon C-10 based on the correlation between the H_3-20 and C-10 and the correlations from H-1 and H-5 to C-20.

The stereochemistry of this compound was determined by NOESY spectrum **(Figure A13)** and $^3J_{H\text{-}H}$ values **(Table 17)**. Ring A and ring B fused with *trans* configuration because H_3-20 had not NOE correlation to H-5. Consequently, if the H-20 were β-oriented, the H-5 were α-oriented. This assumption was implied by the biosynthesis of the norditerpene dilatones in plants which the precursor was started from 12-hydroxytotarol. And the stereochemistry of 12-hydroxytotarol had been identified (Barrero et al, 2003). The H-5 had NOE correlation to H-1, H-6, and H-18. The H-6 also had NOE correlation to H-7 and H-18. So H-1, H-6, H-7 and H-18 were α-orientation, and the 1-OH was β-orientation. The 1-OH had NOE correlation to 7-OH. In addition, the coupling constant between H-6 and H-7 was 8.5 Hz. This implied that their dihedral angle was zero degree. So the 7-OH was lied in β-orientation. These results were related with previous report (Hirotsu et al, 1975) that showed the absolute configuration of nagilactone A diacetate by x-ray crystallography which ring A and ring B junction was *trans*; H-5/H-6, H-6/H-7 was *cis*. The 7-OAc was *cis* to CH_3-20 and the γ-lactone ring. The 1-OAc was in an equatorial position. Ring A was a distorted chair conformation.



Nagilactone A

Nagilactone A and its derivatives had been found mainly in the genus of *Podocarpus* (Zhang et al, 1992; Ying and Kubo, 1993; Xuan et al, 1995). Some studies about activities of nagilactone A had been reported including anti-feedants of guinea pigs (Hayashi et al, 1992), plant growth inhibitory activity of dwarf peas (Galbraith et al, 1972), insecticidal activity (Barrero et al, 2003), cytotoxic activity against Yoshida Sarcoma cell line at IC_{50} value 3.2 μM (Hayashi et al, 1979). Moreover, some derivatives of nagilactone A such as 1-deoxy-2β,3β-epoxynagilactone A and 1-deoxy-2α-hydroxynagilactone A could prolong development periods to the third instar (Zhang et al, 1992). However, it has never been tested for lipase inhibitory activity.

Table 16 ^{1}H- and ^{13}C-NMR spectral data of NW2 and Nagilactone A

Position	NW2*		Nagilactone A	
	δ (ppm)		δ (ppm)	
	^{1}H (multiplicity, *J* in Hz)	^{13}C	^{1}H**[a] (multiplicity, *J* in Hz)	^{13}C**[b]
1	4.10 (*dt*, 12, 5.75)	71.1	4.10 (*br*)	71.1
2	2.00 (m)	29.8	-	28.9
3α	1.54 (*dt*, 14.5, 7)	28.0	-	29.9
3β	2.52 (*dt*, 14.5, 7)		-	
4	-	43.2	-	43.3
5	1.80 (*d*, 5.5)	50.2	1.83 (*d*, 5.7)	50.3
6	5.16 (*dd*, 8.5, 5.5)	74.9	5.17 (*dd*, 8.6, 5.7)	74.9
7	5.65 (*dd*, 8.5, 3.5)	60.5	5.67 (*d*, 8.6)	60.6
8	-	112.0	-	112.0
9	-	166.1	-	169.7
10	-	41.6	-	41.7
11	7.36 (*s*)	108.1	7.38 (*s*)	108.2
12	-	162.8	-	162.7
13	-	-	-	-
14	-	169.6	-	166.1
15	3.48 (*sept*, 7)	29.6	3.51 (*m*, 6.6)	29.6
16	1.30 (*d*, 7)	20.7	1.26 (*d*, 6.6)****	20.2
17	1.24 (*d*, 7)	20.2	1.26 (*d*, 6.6)****	20.8
18	1.31 (*s*)	24.6	1.33 (*s*)	24.6
19	-	181.4	-	181.4
20	1.99 (*s*)	16.1	2.01 (*s*)	16.1
1-OH	6.75 (*d*, 5.75)		-	
7-OH	7.82 (*d*, 3.5)		-	

*Solvent: C_5D_5N; 500 MHz (^{1}H-NMR); 125 MHz (^{13}C-NMR)

**[a] Hayashi et al, 1977; solvent C_5D_5N, ****Overlapped signals

**[b] Ying and Kubo, 1993

Table 17 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR, ^{1}H,^{1}H-COSY, HMBC and NOESY spectral data of NW2 in C_5D_5N

Position	NW2				
	δ (ppm)		COSY	HMBC	NOESY
	^{1}H (multiplicity, *J* in Hz)	^{13}C			
1	4.10 (*dt*, 12, 5.75)	71.1	1-OH, H-2	C-2, C-3, C-5, C-9, C-10, C-20	H-5, H-11
2	2.00 (*m*)	29.8	H-1, H-3	C-1, C-4, C-3, C-10	H-1
3α	1.54 (*dt*, 14.5, 7)	28.0	H-2	C-1, C-2, C-4, C-5, C-18, C-19	H-18
3β	2.52 (*dt*, 14.5, 7)				H-20
4	-	43.2	-	-	-
5	1.80 (*d*, 5.5)	50.2	H-6	C-1, C-4, C-9, C-10, C-18, C-19, C-20	H-1, H-6, H-18
6	5.16 (*dd*, 8.5, 5.5)	74.9	H-5, H-7	C-5, C-7, C-8, C-10	H-5, H-18
7	5.65 (*dd*, 8.5, 3.5)	60.5	7-OH, H-6	C-5, C-6, C-8, C-9, C-14	H-6, H-15
8	-	112.0	-	-	-
9	-	166.1	-	-	-
10	-	41.6	-	-	-
11	7.36 (*s*)	108.1	-	C-8, C-9,C-10, C-12, C-14	H-1
12	-	162.8	-	-	-
13	-	-	-	-	-
14	-	169.6	-	-	-
15	3.48 (*sept*, 7)	29.6	H-16, H-17	C-8, C-14, C-16, C-17	H-7, H-16, H-17
16	1.30 (*d*, 7)	20.7	H-15	C-14, C-15, C-17	H-15
17	1.24 (*d*, 7)	20.2	H-15	C-14, C-15, C-16	H-15, H-16
18	1.31 (*s*)	24.6	-	C-3, C-4, C-5,C-19	H-3α, H-5, H-6
19	-	181.4	-	-	-
20	1.99 (*s*)	16.1	-	C-1, C-5, C-9, C-10	H-3β, 1-OH
1-OH	6.75 (*d*, 5)		H-1	C-1, C-2, C-10	H-20, 7-OH
7-OH	7.82 (*d*, 5.75)		H-7	C-7, C-8	1-OH

4.3.3 Identification of compound NW3 (Inumakiol D)

Compound NW3 was isolated as amorphous solid (4.5 mg). On the TLC, this compound appeared as a dark spot under UV 254 nm, a quenching spot under UV 365 nm and a light pink color spot after detected with anisaldehyde spraying reagent. The molecular formula was determined to be $C_{20}H_{28}O_4$ from the $[M+Na]^+$ peak at *m/z* 355 in ESI mass spectrum **(Figure A15)**. UV spectra showed absorption about 273-295 nm (broad band) of phenol ring **(Figure A14)**. The IR adsorption spectrum **(Figure A16)** showed the strong and broad adsorption band of hydroxyl group overlapped with hydroxyl group of carboxylic acid at 3391 cm^{-1}, strong adsorption of carbonyl group at 1690 cm^{-1}, aromatic C-H stretching at 3156 cm^{-1} and aliphatic C-H stretching at 2984-2849 cm^{-1}.

The ^{1}H-NMR spectrum **(Figure A17)** showed two aromatic protons at δ 6.64 ppm (*d*, J = 8.5 Hz, H-12) and 6.96 ppm (*d*, J = 9 Hz, H-11), oxymethylene proton at δ 4.92 ppm (*br s*, H-7), isopropyl group at δ 3.52 ppm (*sept*, 7, H-15), 1.40 ppm (*d*, J = 7, H-16), 1.33 ppm (*d*, J = 7, H-17), and two singlet methyl groups at δ 1.04 ppm (H-20) and 1.28 ppm (H-18). There were twenty-five protons from the integration of ^{1}H-NMR spectrum. So these confirmed that the twenty-eight protons and four oxygen atoms were in the calculated molecular formula.

Based on carbons from the ^{13}C-NMR spectrum, DEPT spectra, and HSQC spectra **(Figure A18 and Figure A19)**, twenty carbons were divided into one carboxylic carbon at δ 181.9 ppm, six aromatic carbons (4 C and 2 CH) at δ 117.7 (C-12), 124.9 (C-11), 134.2 (C-14),135.0 (C-8), 141.1 (C-9), and 155.5 ppm (C-13), one oxymethine at δ 66.1 ppm (C-7), the other two quaternary at δ 44.3 ppm (C-4) and 39.7 ppm (C-10), two methine carbons at δ 46.2 ppm (C-5) and 29.0 ppm (C-15), four methylene carbons at δ 21.3 (C-2), 32.4 (C-6), 38.6 (C-3) and 41.0 ppm (C-1), and four methyl carbons at δ 20.9 (C-16), 21.0 (C-17), 29.1 (C-18) and 22.9 ppm (C-20).

From the ^{1}H,^{1}H-COSY spectra **(Figure A20a-b)**, four fragments of partial structure were elucidated. The first fragment contained three methylene groups at position 1, 2, and 3. The second fragment was a methine group at position 5, a

methylene group at position 6 and a methine group at position 7. The third fragment contained two aromatic methine groups at position 11 and 12. The last fragment was an isopropyl group.

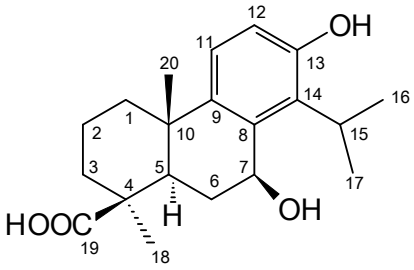
Based on HMBC experiment, quaternary carbons at δ 44.3, 135.0, 141.1, 39.7, 155.5, 134.2, 181.9 could be assigned at positions 4, 8, 9, 10, 13, 14, and 19, respectively which the HMBC spectra were showed in **Figure A21a-c**. Two methyl groups at δ 29.1 (C-18) and 22.9 (C-20) were attached to C-4 and C-10 respectively based on the HMBC correlations between H-3 and H-5 to C-18, and from H-1 and H-5 to C-20. In addition, the methyl proton at δ 1.28 ppm (H_3-18) had correlations to C-3, C-4, C-5 and C-19, and proton at δ 1.04 ppm (C-20) had correlations to C-1, C-5, C-9 and C-10. The HMBC correlations observed between the protons H-7, H-12, H-15, H-16, H-17 and the carbon C-14 and between the proton H-11, H-12, H-15 and the carbon C-13 indicated that the isopropyl group was at C-14 and the phenolic hydroxyl group was at C-13.

The stereochemistry was proved by the NOESY experiment **(Table 18 and Figure A22a-b)**. The NOE between the methyl protons H_3-18 and the methine proton H-5 implied that the methyl group at C-4 and the carboxyl group at C-4 were α- and β- orientation, respectively. The NOE between H-1β and the H-20 and between H-1α and H-5 indicated that the ring junction of A/B was *trans*. H-7 did not have NOE correlation to H-20. This implied that hydroxyl group at C-7 was in β-orientation.

All chemical shifts of the ^{1}H- and ^{13}C-NMR were compared with the reference report of inumakiol D (Sato et al., 2008) in **Table 19**. The chemical shifts of NW3 were a little shift from the reference because of the different solvents. From all of the interpretation and NMR comparing data, it was concluded that NW3 was inumakiol D.

Inumakiol D has been reported for a very weak anti-bacterial against *Streptococcus mutans* MT8148R, *Streptococcus sobrinus* 6715, *Actinomyces viscosus* ATCC15987, *Porphyromonas gingivalis* ATCC33277, *Fusobacterium nucleatum* JCM

8532, *Actinobacillus actinomycetemcomitans* ATCC29522, *Streptococcus aureus* IID671, and *Streptococcus pyogenes* by the MIC ≥ 50 ppm (Sato et al, 2008).



Inumakiol D

Table 18 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR, ^{1}H,^{1}H-COSY, HMBC, and NOESY spectral data of NW3 in CD_3OD

Position	NW3				
	δ (ppm)		COSY	HMBC	NOESY
	^{1}H (multiplicity, J in Hz)	^{13}C			
1α	2.23 (*br d*, 14)	41.0	H-2	C-2, C-5, C-20	H-5
1β	1.37 (*br dd*, 13.5, 4)				-
2α	1.58 (*br ddd*, 14, 4, 3.5)	21.33	H-1, H-3	C-4, C-10	H-3α
2β	2.02 (*ddd*, 14.5, 13.5, 3.5)				H-20
3α	1.14 (*ddd*, 14.5, 13.5, 4)	38.6	H-2	C-1, C-2, C-4, C-5, C-18, C-19	H-1α, H-2α
3β	2.23 (*br d*, 14)				H-1β, H-20
4	-	44.3	-	-	-
5	2.01 (*dd*, 13.5, 2)	46.2	H-6	C-1, C-3, C-6, C-7, C-10, C-18, C-19, C-20	H-1α, H-3α, H-18
6α	2.27 (*br d*, 14)	32.4	H-5, H-7	C-5, C-7, C-8, C-10	H-7α
6β	2.15 (*ddd*, 16.5, 13.5, 3)				H-7α

Table 18 (continued)

Position	NW3				
	δ (ppm)		COSY	HMBC	NOESY
	^{1}H (multiplicity, J in Hz)	^{13}C			
7	4.92 (*br s*)	66.11	H-6	C-5, C-6, C-8, C-9, C-14	H-6α, H-6β, H-15
8	-	135.0	-	-	-
9	-	141.1	-	-	-
10	-	39.7	-	-	-
11	6.96 (*d*, 9)	124.9	H-12	C-8, C-10, C-13	H-1β, H-20
12	6.64 (*d*, 8.5)	117.7	H-11	C-9, C-13, C-14, C-15	-
13	-	155.5	-	-	-
14	-	134.2	-	-	-
15	3.52 (*sept*, 7)	29.0	H-16, H-17	C-8, C-13, C-14, C-16, C-17	H-16, H-17
16	1.40 (*d*, 7)	20.9	H-15	C-14, C-15, C-17	-
17	1.33 (*d*, 7)	21.0	H-15	C-14, C-15, C-16	-
18	1.28 (*s*)	29.1	-	C-3, C-4, C-5, C-19	H-5
19	-	181.9	-	-	-
20	1.04 (s)	22.9	-	C-1, C-5, C-9, C-10	H-2β, H-3β, H-6β, H-11

Table 19 ^{1}H- and ^{13}C-NMR spectral data of NW3 and Inumakiol D

Position	NW3*		Inumakiol D**	
	δ (ppm)		δ (ppm)	
	^{1}H (multiplicity, J in Hz)	^{13}C	^{1}H (multiplicity, *J* in Hz)	^{13}C
1α	2.23 (*br d*, 14)	41.0	2.26 (d, 12.4)	40.3
1β	1.37 (*br dd*, 13.5, 4)		1.22 (*m*)	
2α	1.58 (*br ddd*, 14, 4, 3.5)	21.33	1.57 (*m*)	20.9
2β	2.02 (*ddd*, 14.5, 13.5, 3.5)		2.35 (*m*)	
3α	1.14 (*ddd*, 14.5, 13.5, 4)	38.6	1.09 (*ddd*, 13.1, 13.1, 3.9)	38.4
3β	2.23 (*br d*, 14)		2.55 (*d*, 13.1)	
4	-	44.3	-	43.7
5	2.01 (*dd*, 13.5, 2)	46.2	2.47 (*d*, 12.8)	45.6
6α	2.27 (*br d*, 14)	32.4	2.74 (*m*)	32.7
6β	2.15 (*ddd*, 16.5, 13.5, 3)		2.91 (*d*, 14.3)	
7	4.92 (*br s*)	66.11	5.49 (*s*)	65.5
8	-	135.0	-	136.6
9	-	141.1	-	140.3
10	-	39.7	-	39.2
11	6.96 (*d*, 9)	124.9	7.16 (*d*, 8.5)	124.4
12	6.64 (*d*, 8.5)	117.7	7.19 (*d*, 8.5)	117.3
13	-	155.5	-	155.7
14	-	134.2	-	134.0
15	3.52 (*sept*, 7)	29.0	4.25 (*m*)	28.5
16	1.40 (*d*, 7)	20.9	1.86 (*d*, 7.0)	21.3
17	1.33 (*d*, 7)	21.0	1.72 (*d*, 7.0)	21.2
18	1.28 (*s*)	29.1	1.55 (*s*)	29.1
19	-	181.9	-	180.4
20	1.04 (s)	22.9	1.40 (*s*)	23.1
1-OH	-		-	-
7-OH	-		6.47 (*br s*)	-
13-OH	-		10.8 (*s*)	-

*Solvent: CD_3OD; 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR)

**Sato et al, 2008; solvent: C_5D_5N; 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR)

4.3.4 Identification of compound NW4 (Totarol)

Compound NW4 was a yellow wax (68.2 mg). This compound was pink color spot on TLC when detected with anisaldehyde spraying reagent. The molecular formula was $C_{20}H_{30}O$ from the $[M+H]^+$ and $[M+Na]^+$ peaks at *m/z* 287 and 309 in ESI mass spectrum **(Figure A23)**. The IR spectrum **(Figure A24)** showed the strong adsorption bands of the hydroxyl group, aromatic C-H stretching and aliphatic C-H stretching at 3461, 3049, and 2965-2846 cm^{-1}. ^{1}H- and ^{13}C-NMR spectra of compound NW4 were compared with the reference (Ying and Kubo, 1991; Miyake et al., 2007) and it was identified as totarol **(Table 20)**.

The ^{1}H-NMR spectrum **(Figure A25)** showed two aromatic protons at δ 6.50 ppm (*d*, J = 8.5 Hz, H-12) and 6.99 ppm (*d*, J = 8.5 Hz, H-11), isopropyl group at δ 3.28 ppm (*sept*, 7, H-15), 1.33 ppm (*d*, J = 7, H-16), 1.34 ppm (*d*, J = 7, H-17), and three singlet methyl groups at δ 0.91 (H-19), 0.94 (C-18) and 1.17 ppm (H-20).

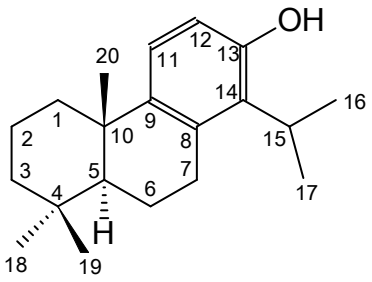
There were twenty carbons from the ^{13}C-NMR spectrum. From the ^{13}C-NMR, DEPT spectra and HSQC spectra **(Figure A26 and Figure A27a-b)**, they were divided into six aromatic carbons (4 C and 2 CH) at δ 114.3 (C-12), 123.0 (C-11), 130.9 (C-14),133.9 (C-8), 143.2 (C-9), and 151.9 ppm (C-13), two quaternary at δ 33.3 ppm (C-4) and 37.7 ppm (C-10), two methine carbons at δ 49.6 ppm (C-5) and 27.1 ppm (C-15), five methylene carbons at δ 19.3 (C-6), 19.5 (C-2), 28.7 (C-7), 39.6 (C-1) and 41.6 ppm (C-3), and five methyl carbons at δ 20.3 (C-16 and C-17), 21.6 (C-19), 25.2 (C-20) and 33.2 ppm (C-18).

Four fragments of partial structure including three methylene groups at position 1, 2, and 3, methine group at position 5 with methylene group at position 6 and 7, two methine aromatic groups at position 11 and 12, and isopropyl group, were interpreted by the ^{1}H,^{1}H-COSY spectrum **(Figure A28a-b)**.

The structure of NW4 was similar to NW3 (Inumakiol D). The differences between totarol and inumakiol D were the change of 19-COOH to 19-CH_3 and the lack of the 7-OH in totarol. Therefore, the ^{1}H-NMR spectrum of NW4 would increase a methyl

proton signal (δ 0.91 (s)) from four signals in inumakiol D to five signals in totarol and the H-7 was splitted into two nonequivalence methylene proton signals instead of one methine proton signal in inumakiol D. In addition, the chemical shifts of H-6 and H-7 were more up-field and the splitting patterns were changed to 1.65 ppm (*m*, H-6β), 1.90 ppm (*br dd*, J = 13 and 8 Hz, H-6α), 2.93 ppm (*dd*, J = 17.25 and 6.25 Hz, H-7β) and 2.74 ppm (*ddd*, J = 18.25, 11.25, 8 Hz, H-7α). The ^{13}C-NMR spectrum of NW4 was also changed. The chemical shifts of the C-4, C-6 and C-7 were more up-field at δ 33.2, 19.3 and 28.7 ppm respectively. The chemical shifts of C-3 and C-5 of totarol were more downfield (δ 41.2 and 49.6 ppm) because of the lacking of anisotropy of 19-COOH.

All assignments of carbons, protons and stereochemistry of this structure were confirmed by HMBC and NOESY experiments **(Figure A29a-b and Figure A30a-c)**. In HMBC spectrum H-19 had correlations to C-3, C-4, C-5 and C-18. And there was NOE between H-20 and H-19, but not between H-19 and H-18. These concluded that H-19 was at the same β-orientation as 19-COOH of inumakiol D.



Totarol

Table 20 ^{1}H- and ^{13}C-NMR spectral data of NW4 and Totarol

Position	NW4*		Totarol	
	δ (ppm)		δ (ppm)	
	^{1}H (multiplicity, *J* in Hz)	^{13}C	^{1}H**[a] (multiplicity, *J* in Hz)	^{13}C**[b]
1α	1.31 (*d*, 3.5)	39.6	1.32 (*ddd*, 12.8, 12.4, 3.0)	39.6
1β	2.22 (*br d*, 12)		2.25 (*br d*, 12.4)	
2α	1.59 (*m*)	19.5	1.49 (*ddd*, 13.7, 3.8, 3.0)	19.5
2β	1.73 (*m*)		1.65 (****)	
3α	1.21 (*dd*, 13.5, 4)	41.6	1.14 (*ddd*, 13.7, 13.3, 3.8)	41.6
3β	1.46 (*dtd*, 13, 3, 1.5)	41.6	1.40 (*br d*, 13.3)	41.6
4	-	33.3	-	33.2
5	1.26 (*dd*, 13, 2)	49.6	1.26 (*d*, 12.0)	49.6
6α	1.90 (*dd*, 13, 8)	19.3	1.89 (*dd*, 12.0, 8.1)	19.4
6β	1.65 (*m*)		1.67 (****)	
7α	2.74 (*ddd*, 17.5, 11.5, 7.4)	28.7	2.81 (*ddd*, 16.7, 8.1, 6.4)	28.7
7β	2.93 (*dd*, 17.25, 6.25)		3.04 (*dd*, 16.7, 6.4)	
8	-	133.9	-	134.0
9	-	143.2	-	143.2
10	-	37.7	-	37.7
11	6.99 (*d*, 8.5)	123.0	7.10 (*d*, 8.5)	123.0
12	6.50 (*d*, 8.5)	114.3	7.01 (*d*, 8.5)	114.3
13	-	151.9	-	151.9
14	-	130.9	-	131.0
15	3.28 (*quint*, 7)	27.1	3.46 (*br*)	27.1
16	1.33 (*d*, 7)	20.3	1.64 (6.8)	20.3
17	1.34 (*d*, 7)	20.3	1.67 (*d*, 6.8)	20.4
18	0.94 (*s*)	33.2	0.93 (*s*)	33.3
19	0.91 (*s*)	21.6	0.89 (*s*)	21.6
20	1.17 (*s*)	25.2	1.21 (*s*)	25.2
13-OH	4.43 (*br s*)	-	10.65 (*s*)	-

*Solvent: $CDCl_3$; 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR),
**[a]Ying and Kubo, 1991; solvent: C_5D_5N; 500 MHz, ****Overlapped with other proton signals
**[b] Miyake et al, 2007; solvent: $CDCl_3$; 100 MHz

Totarol is well-known to possess anti-bacterial activity against methicilin-resistant *Staphylococcus aureus* (Muroi and Kubo, 1996), penicillin-resistant *Streptococcus pneumoniae* (Evans and Furneaux, 2000), erythomycin-resistant *Streptococcus pyogenes*, high-level-gentamicin-resistant *Enterococcus faecalis* (Evans et al, 2000), and *Propionibacterium acnes* (Kubo et al, 1994). Anti-acne was reported in a 14-year-old male using totarol in the form of an alcohol-based topical medication and following with a totarol-containing moisturizer for the treatment of acne vulgaris. The patient applied this product twice daily for 4 weeks and then once daily for 2 weeks. The inflammation and the size of the lesion were decreased (Nixon and Hobbs, 2006). Totarol also has anti-oxidative effect. It inhibited auto-oxidation of linoleic acid and mitochondrial and microsomal lipid peroxidation induced by Fe (III)-ADP/NADH or Fe (III)-ADP/NADPH (Haraguchi et al, 1996). Recently, Totarol™ has been patent in supercritical fluid extraction process for commercial pure compound for topical anti-inflammatory agent (Abdillahi et al, 2010). However, the purposes of this commercial brand are anti-bacterial, anti-acne, and anti-oxidant in cosmetics (Mende Biotech Ltd., 2006). Furthermore, totarol showed the best anti-mycobacterial activity with *Mycobacterium tuberculosis* H37Rv with the MIC of 73.7 μM (Gordien et al, 2009), anti-parasitic (*Leishmania donovani*), nematicidal (*Caenorhabditis elegans*), and anti-fouling (*Artemia salina*) with IC_{50} values of 3.5 μg/ml, 80 μg/ml and 1 μg/ml respectively (Samoylenko et al, 2008). Anti-fibrotic activity of totarol has been determined by Lee et al, 2008. Totarol dramatically reduced cell proliferation of rat hepatic stellate cell line (HSC-T6) in dose-dependent manner. Hepatic stellate cell lines are activated and act as myofibroblast-like phenotype during liver fibrogenesis. Consequently, they play a key role to increase in proliferation and extracellular matrix synthesis. So totarol may decrease pathogenesis of liver fibrosis. In addition, totarol has antiplasmodial activity against a chloroquine-resistance strain of *Plasmodium falciparum* at an IC_{50} value of 4.29 μM (Clarkson et al, 2003). Totarol showed larvicidal activity against mosquito larvae (second- and fourth- instar of *Culex pipiens*) at the LC_{50} value of 0.25 and 0.37 μg/ml respectively (Lee et al, 2000), and gastroprotective effect when induced by HCl/EtOH

(Areche et al, 2007). However, lipase inhibitory activity of this compound has never been performed.

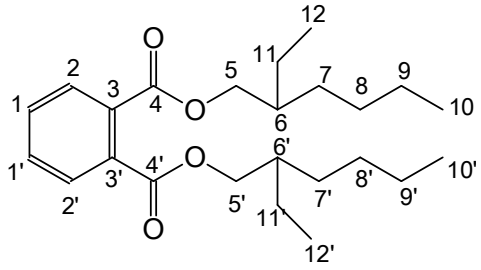
4.3.5 Identification of compound NW5 (Bis-2-ethylhexyl-phthalate)

Compound NW5 was a yellow wax (5.7 mg), soluble in CH_2Cl_2 and $CHCl_3$. The UV spectrum **(Figure A31)** showed the adsorption of aromatic conjugated with carbonyl ester at λ_{max} 280 nm. The IR spectrum **(Figure A33)** showed the strong adsorption bands of the carbonyl ester (the C=O and C—O stretching vibration) at 1728 and 1286-1272 cm^{-1}. The ESI mass spectrum **(Figure A32)** displayed an $[M+H]^+$ and $[M+Na]^+$ peaks at *m/z* 391 and 413 corresponding to the molecular formula of $C_{24}H_{38}O_4$. The ^{13}C-NMR spectrum and DEPT 90^o and 135^o spectra **(Figure A35)** displayed twelve carbon resonances composed with a carbonyl ester carbon at δ 167.8 ppm (C-4), three aromatic carbons including quaternary carbons at δ 129.0 (C-2), 130.8 (C-1) and 132.5 ppm (C-3), one another methine at δ 38.7 ppm (C-6), five methylene carbons at δ 23.0 (C-9), 23.7 (C-11), 29.0 (C-8), 30.4 (C-7) and 68.2 ppm (C-5), and two methyl carbons at δ 11.0 (C-12) and 14.0 ppm (C-10). From ^{13}C-NMR spectrum, all DEPT spectra and molecular formula, it was suggested that structure of this compound had plan of symmetry as mirror image.

The ^{1}H-NMR spectrum **(Figure A34)** confirmed the presence of oxy-methylene proton at δ 4.18 (*dd*, J = 11.5 and 6 Hz, H-5a and H-5'a) and 4.22 ppm (*dd*, J = 11.5 and 6 Hz, H-5b and H-5'b), four methyl protons at δ 0.87 (*t*, = 7 Hz, H-10 and H-10') and 0.90 ppm (*t*, = 7.5 Hz, H-12 and H-12'), and four aromatic protons at δ 7.51 (*dd*, J = 5.75 and 3.25 Hz, H-1 and H-1') and 7.69 ppm (*dd*, J = 5.75 and 3.25 Hz, H-2 and H-2'). The rest proton resonances indicated the pattern of a 1,6-disubstitued aromatic compound at δ 7.51 (*dd*, J = 3.3, 5.8 Hz, H-1 and H-1') and 7.68 (*dd*, J = 3.3, 5.8 Hz, H-2 and H-2').

A carbonyl ester was assigned at C-4 based on the HMBC correlation between H-2 and C-4, and between H-5 and C-4. The partial structure of C-5 to C-12 was assigned based on ^{1}H,^{1}H-COSY and HMBC spectra **(Figure A37a-b and Figure**

A38a-b). From $^{1}H,^{1}H$-COSY data, were assigned the partial structure of H-11 of ethyl group (H-11 and H-12) adjoined with H-6. This assignment on long chain hydrocarbon was confirmed by HMBC data that H-6 had correlation with C-5, C-7, C-8, C-9, C-11 and C-12, H-7 had correlation with C-8, C-9 and C-11 (weak signal), H-10 had correlation with only with C-8 and C-9, and H-12 had correlation with only C-6 and C-11. The methylene at position 8 and 9 cannot be identified by COSY experiment, so it may need the NOE experiment by irradiating H-7 and observing of H-8 and irradiating H-10 observing of H-9. And all NMR spectroscopic data were shown in **Table 21**.



Bis-2-ethylhexyl-phthalate

Table 21 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR, ^{1}H,^{1}H-COSY, HMBC, and NOESY spectral data of NW5 in $CDCl_3$

Position	NW5			
	^{1}H δ (ppm) (multiplicity, *J* in Hz)	^{13}C δ (ppm)	COSY	HMBC
1,1'	7.51 (*dd*, 5.75, 3.25)	130.9	H-2	C-2, C-3, C-4
2,2'	7.69 (*dd*, 5.75, 3.25)	129.0	H-1	C-1, C-3, C-4
3,3'	-	132.5	-	-
4,4'	-	167.8	-	-
5,5'	Ha 4.18 (*dd*, 11.5, 6) Hb 4.22 (*dd*, 11.5, 6)	68.2	H-6	C-4, C-6, C-7, C-11
6,6'	1.66 (*sept*, 6)	38.7	H-5, H-7, H-11	C-5, C-7, C-8, C-9, C-11, C-12
7,7'	1.33 (*m*)	30.4	H-6	C-8, C-9, C-11
8,8'	1.29 (*m*)	23.0	H-10	C-7, C-10
9,9'	1.29 (*m*)	29.0	H-10	C-7, C-10
10,10'	0.87 (*t*, 7)	14.0	H-8, H-9	C-8, C-9
11,11'	1.40 (*qd*,14, 7)	23.7	H-6, H-12	C-5, C-6, C-7, C-12
12,12'	0.90 (*t*, 7.5)	11.0	H-11	C-6, C-11

Table 22 ^{1}H- and ^{13}C-NMR spectral data of NW5 and Bis-2-ethylhexyl-phthalate

Position	NW5*		Bis-2-ethylhexyl-phthalate**	
	δ (ppm)		δ (ppm)	
	^{1}H (multiplicity, *J* in Hz)	^{13}C	^{1}H (multiplicity, *J* in Hz)	^{13}C
1,1'	7.51 (*dd*, 5.75, 3.25)	130.8	7.91 (*dd*, 5.8, 2.0)	131.1
2,2'	7.69 (*dd*, 5.75, 3.25)	129.0	7.79 (*dd*, 5.8, 2.0)	129.0
3,3'	-	132.5	-	132.6
4,4'	-	167.8	-	168.0
5,5'	Ha 4.18 (*dd*, 11.5, 6) Hb 4.22 (*dd*, 11.5, 6)	68.2	4.19 (*m*)	66.4
6,6'	1.66 (*sept*, 6)	38.7	1.66 (*m*)	38.9
7,7'	1.33 (*m*)	30.4	1.40 (*m*)	30.6
8,8'	1.29 (*m*)	23.0	1.22-1.23 (*m*)	29.1
9,9'	1.29 (*m*)	29.0	1.29 (*m*)	23.2
10,10'	0.87 (*t*, 7)	14.0	0.89 (*m*)	14.3
11,11'	1.40 (*m*)	23.7	1.40 (*m*)	23.9
12,12'	0.90 (*t*, 7.5)	11.0	0.91 (*m*)	11.2

*solvent: $CDCl_3$; 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR)

**Al-Bari et al, 2006; solvent: $CDCl_3$; 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR)

Spectroscopic data of NW5 were compared with literature reviews (Al-Bari et al, 2006), and it was identified as bis-2-ethylhexyl-phthalate **(Table 22)**. This compound was used as a plasticizer for increasing workability, flexibility, or extensibility of the polyvinyl chloride (PVC) plastics, polyvinyl acetate, rubbers, cellulose plastics, and polyurethane resins (United Stated Consumer Product Safety Commission, 2010). It could be isolated from some plants such as *Calotropis gigantea* flower (Rowshanul and Rezaul, 2009) and *Arbutus unedo* root (Dib et al, 2010). Besides plants it was found in actinobacteria such as *Nocardia levis* (Kavitha et al, 2009).

Bis-2-ethylhexyl-phthalate has been reported in toxicity to endocrine activity on estrogens metabolism in female Fisher 344 rats and androgens production in Leydig cell hyperplasia, and structural and functional changing in thyroid. However, bis-

2-ethylhexyl-phthalate was largely negative for mutagenic or other genotoxic effects when tested in bacterial, eukaryotic, and mammalian *in vitro* systems with or without metabolic activation (United Stated Consumer Product Safety Commission, 2010). This compound had anti-bacterial activity, anti-fungal activity (Kavitha et al, 2009), and anti-fouling effect (Qi et al, 2009). However, there was no study on anti-lipase inhibitory activity.

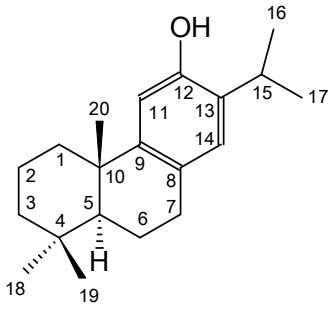
4.3.6 Identification of compound NW6 (Ferruginol)

Compound NW6 was a yellow wax (16.9 mg) and it was appeared in old rose color when detected with anisaldehyde spraying reagent. The UV spectrum **(Figure A39)** showed the adsorption of phenol moiety at λ_{max} about 285 nm. The pseudomolecular ion peak $[M-H]^+$ and $[M+15]^+$ at *m/z* 285 and 301 indicated the molecular formular as $C_{20}H_{30}O$ in ESI mass spectrum **(Figure A40)**. The IR adsorption spectrum **(Figure A41)** showed the strong adsorption bands of the hydroxyl group and aliphatic C-H stretching at 2960-2846 cm^{-1}.

The ^{1}H-NMR spectrum **(Figure A42)** showed two para aromatic protons at δ 6.61 ppm (*s*, H-11) and 6.82 ppm (*s*, H-14), isopropyl group at δ 3.10 ppm (*sept*, 7, H-15), 1.21 ppm (*d*, J = 7, H-16), 1.23 ppm (*d*, J = 7, H-17), and three singlet methyl groups at δ 0.90 (H-19), 0.93 (C-18) and 1.16 ppm (H-20).

There were twenty carbons from the ^{13}C-NMR spectrum. From the ^{13}C-NMR, DEPT spectra **(Figure A43)**, the carbons were divided into six aromatic carbons (4 C and 2 CH) at δ 111.1 (C-11), 127.3 (C-8), 131.4 (C-13), 133.9 (C-8), 148.7 (C-9), and 150.6 ppm (C-12), two quaternary carbons at δ 33.4 ppm (C-4) and 37.5 ppm (C-10), two methine carbons at δ 50.3 ppm (C-5) and 26.8 ppm (C-15), five methylene carbons at δ 19.2 (C-2), 19.3 (C-6), 29.7 (C-7), 38.8 (C-1) and 41.7 ppm (C-3), and five methyl carbons at δ 22.5 (C-16), 22.7 (C-17), 33.3 (C-18), 21.6 (C-19) and 24.7 ppm (C-20).

When comparing the ^{1}H- and ^{13}C-NMR spectra of NW6 with the references **(Table 23)** and the other spectroscopic data, it was indicated that this compound was ferruginol.



Ferruginol

Table 23 ^{1}H- and ^{13}C-NMR spectral data of NW6 and Ferruginol

Position	NW6*		Ferruginol**	
	δ (ppm)		δ (ppm)	
	^{1}H, (multiplicity, *J* in Hz)	^{13}C	^{1}H, (multiplicity, *J* in Hz)	^{13}C
1α	1.36 (*td*, 13, 4)	38.8	1.37 (*td*, 13.5, 3.5)	38.9
1β	2.15 (*br dd*, 12.5, 1)		2.15 (*dtd*, 13.5, 3.5, 1.5)	
2α	1.57 (*dquint*, 14, 3.25)	19.2	1.58 (*d*quint, 13.5, 3.5)	19.4
2β	1.72 (*dddt*, 13.25, 13.25, 13.66, 3.5)		1.72 (*qt*, 13.5, 3.5)	
3α	1.19 (*dd*, 14, 4)	41.7	1.23 (*td*, 13.5, 3.5)	41.8
3β	1.45 (*dtd*, 13, 3, 1.5)		1.46 (*dtd*, 13.5, 3.5, 1.5)	
4	-	33.4	-	33.5
5	1.30 (*dd*, 12.5, 2.5)	50.3	1.31 (*dd*, 12.5, 2)	50.4
6α	1.65 (*m*)	19.3	1.66 (*dddd*, 13.5, 12.5, 11, 7)	19.3
6β	1.84 (*dddd*, 13, 7.25, 1.94, 2)		1.85 (*ddt*, 13.5, 7.5, 2)	
7α	2.76 (*ddd*, 17, 11, 7.1)	29.7	2.76 (*ddd*, 16.5, 11, 7.5)	29.8
7β	2.85 (*ddd*, 16.75, 6.6, 1.5)		2.85 (*ddd*, 16.5, 7, 2)	
8	-	127.3	-	127.3
9	-	148.7	-	148.7
10	-	37.5	-	37.5
11	6.61 (*s*)	110.9	6.61 (*s*)	111.0
12	-	150.6	-	150.7
13	-	131.4	-	131.4
14	6.82 (*s*)	126.6	6.81 (*s*)	126.6

Table 23 (continued)

Position	NW6*		Ferruginol**	
	δ (ppm)		δ (ppm)	
	^{1}H, (multiplicity, *J* in Hz)	^{13}C	^{1}H, (multiplicity, *J* in Hz)	^{13}C
15	3.10 (*sept*, 7)	26.8	3.11 (*sept*, 7)	26.8
16	1.21 (*d*, 7)	22.5	1.22 (*d*, 7)	22.6
17	1.23 (*d*, 7)	22.7	1.24 (*d*, 7)	22.8
18	0.93 (*s*)	33.3	0.93 (*s*)	33.3
19	0.90 (*s*)	21.6	0.91 (*s*)	21.7
20	1.16 (*s*)	24.8	1.16 (*s*)	24.8
12-OH	4.51 (*br s*)	-	4.60 (*br s*)	-

*solvent: $CDCl_3$; 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR)

** Tezuka et al, 1998; solvent: $CDCl_3$; 400 MHz (^{1}H-NMR) and 100 MHz (^{13}C-NMR)

Ferruginol almost had the same activities as totarol such as anti-bacterial activity against gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Enterococcus faecalis* and methicillin-resistant *Staphylococcus aureus* (Matsushita et al, 2006; Li et al, 2008), anti-parasitic (*Leishmania donovani*) with the IC_{50} value of 3.5 µg/ml (Samoylenko et al, 2008), mosquito lavicidal activity against *Aedes aegypti* with the LC_{50} value of 64.1 µg/ml (Gu et al, 2009), and gastro-protective effect (Rodríguez et al, 2006; Areche et al, 2007). For gastro-protective effect, ferruginol inhibited the gastric lesions by 60% with similar effect to lansoprazole at 20 mg/kg in the model of gastric lesions induced by HCl/EtOH in mice. Ferruginol may prevent the gastric ulcer and heal the lesion by increasing prostaglandin-E_2 level in the culture human gastric epithelial cells (AGS) and stimulation of proliferation of MRC-5 fibroblast cell and AGS cell cultures (Rodríguez et al, 2006). Ferruginol and totarol could reduce HCl/EtOH- induced gastric lesion about 55% and 59% respectively. (Areche et al, 2007). In addition, ferruginol has other interesting activities such as inhibitory activity of androgen receptor transcriptional in prostate cancer cells (Tu et al, 2007) and cytotoxic activity against human pancreatic (MIAPaCa-

2) cell lines with IC_{50} value of 25.9 μM (Chen et al, 2011; Fronza et al, 2011). There was no study on lipase inhibitory activity of ferruginol.

4.3.7 Identification of compound NW7 (Nagilactone E-3-*O*-β-glucopyranoside)

Compound NW7 was a clear needle crystal, soluble in pyridine and slightly soluble in MeOH. The IR spectrum **(Figure A45)** indicated the presence of two lactone groups and hydroxyl groups, an α-pyrone group at ν_{max} 1758, 1712, and 1639 cm^{-1}, a γ-lactone group at 1776 cm^{-1}, C-O stretching at ν_{max} 1077-997 cm^{-1}, C-H stretching of aliphatic at ν_{max} 2937-2904 cm^{-1} and the hydroxyl groups at ν_{max} 3432 cm^{-1}. The ESI mass spectrum **(Figure A44)** showed the pseudomolecular ion $[M+H]^+$ and $[M+Na]^+$ peak at 511 and 533, respectively, which indicated the molecular formular as $C_{25}H_{34}O_{11}$.

Analysis of the ^{1}H-NMR spectrum **(Figure 46)** of NW7 showed the presence of an isopropyl group at δ 1.94 ppm (*dq*, J = 7, 3.5 Hz, H-15), 0.99 ppm (*d*, J = 7 Hz, H-17), and 1.15 ppm (*d*, J = 7 Hz, H-16). One downfield olefenic proton was observed at δ 6.12 ppm (*s*, H-11). Two singlet methyl signals were appeared at δ 1.22 ppm (H-20) and 1.69 ppm (H-18). ^{1}H-NMR showed one anomeric proton of monosaccharide at δ 4.93 ppm (*d*, J = 7.5 Hz, H-1') and the other hydroxyl methine protons at δ between 3.98 to 4.93 ppm.

The ^{13}C-NMR spectrum **(Figure A47)**, twenty-five carbon signals can be identified as four methyl carbons at δ 16.5 (C-16), 21.3 (C-17), 21.9 (C-18) and 24.2 ppm (C-20), three methylene carbons at δ 29.0 (C-1), 27.3 (C-2) and 62.9 ppm (C-6'), twelve methine carbons at δ 26.3 (C-15), 45.4 (C-5), 54.2 (C-7), 71.5 (C-4'), 72.2 (C-6), 75.5 (C-2'), 78.5 (C-3'), 78.6 (C-5'), 82.9 (C-14), 83.2 (C-3), 107.0 (C-1') and 117.0 ppm (C-11). The rests were quaternary carbons which were two carbonyl lactone at δ 176.0 ppm (γ-lactone, C-19) and 163.8 ppm (α-pyrone lactone, C-12), and the other four quaternary carbons at δ 36.3 (C-10), 46.3 (C-4), 58.9 (C-8) and 159.0 ppm (C-9).

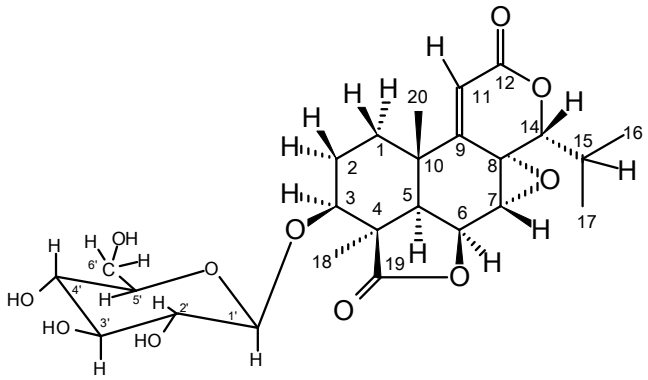
From ^{1}H,^{1}H-COSY spectra **(Figure A49a-b)**, four fragments of partial structure were identified. The first fragment was the connection between H-1 to H-2 and

H-2 to H-3. The second fragment was the connection of H-6 to H-5 and H-7 respectively. The third fragment was isopropyl group (H-14 to H-15, H-15 to H-16 and H-17). The last fragment was the sugar part.

From the HMBC spectra **(Figure A50a-d)**, the four fragments could be connected. The correlation between the H-2, H-3 and H-5 to C-4 indicated that position 3 of the first fragment connected to the position 5 of the second fragment via the quaternary carbon at position 4. And the correlation signals from H-1, H-2, H-5, H-6 and H-11 to C-10 indicated that the position 1 of the first fragment connected to the position 5 of the second fragment via quaternary carbon at position 10 (C-10). The correlations between H-1 and C-10, H-6 and C-7, C-8 and C-10 indicated that C-10 and C-7 were connected to C-9 and C-8, respectively, and C-8 and C-9 bonded together. The isopropyl group attached on the methine carbon at C-14 based on the connections from H-15, H-16, and H-17 to the quaternary carbon at C-14. The bond between C-14 and C-8 was suggested because of the correlation between H-14 and C-8. The correlations from H-11 to C-8, C-10, and C-12 confirmed the core structure of this compound. The CH_3-18 and carbonyl lactone at position 19 attatched to quaternary C-4 based on the correlations from H_3-18 to C-3, C-4, C-5, and C-19; and from the H-3, H-5, and H_3-18 to C-19. The CH_3-20 attached on the quaternary carbon at C-10 because the correlations between the H_3-20 and the quaternary carbon at C-1, C-5, C-9 and C-10, and the correlations from H-1 and H-5 to C-20 were observed. In sugar part, anomeric proton (H-1') had correlation with C-3, C-3'/C-4' and C-5'. This indicated that sugar part was connected to core structure of diterpenoids at C-3. Sugar part was *O*-glycosidic linkage with core structure of diterpenoids because the chemical shift of anomeric carbon was down field in the range of 90-112 ppm. The structure of glucose was confirmed with HMBC after assigned the position with $^1H,^1H$-COSY spectra.

Comparing the ^1H- and ^{13}C-NMR spectra of NW7 with the references **(Table 24)**, it was indicated that the two parts of core structure of this compound were nagilactone E and β-D-glucose.

The stereochemistry of this compound was determined by NOESY spectra **(Figure A51a-c)**. Ring A and ring B fused with *trans* configuration because the H_3-20 did had not NOE with H-5. The H_3-20 was β-orientation and H-5 was α-orientation as same as nagilactone A. H-3, H-6 and H_3-18 were α-orientation because of the NOEs of these protons and H-5. The H-7 and H-14 had the NOEs with H_3-20 so the epoxide at position 7 and 8, and isopropyl group at position 14 were α-orientation. The NOESY spectra of glucose were confirmed configuration by H1' with H5', H-3' with H-5', and H-5' with H-1' and H-3'. This confirmed β-orientation of anomeric carbon. And all NMR spectroscopic data were shown in **Table 25**.



Nagilactone E-*O*-β-glucopyranoside

Table 24 ^{1}H- and ^{13}C-NMR spectral data of NW7, Nagilactone E, and β-D-glucose

Position	NW7* (diterpenoid part)		Nagilactone E	
	δ (ppm)		δ (ppm)	
	^{1}H, (multiplicity, *J* in Hz)	^{13}C	^{1}H**[a], (multiplicity, *J* in Hz)	^{13}C**[b]
1α	1.38 (*td*, 12.5, 4.3)	29.0	-	29.3
1β	1.59 (*dt*, 13, 4.25)		-	
2α	2.38 (*dtd*, 14.25, 4.9, 5)	27.3	-	28.6
2β	2.05 (*dtd*, 14.5, 11.5, 3.8)		-	
3	3.94 (*dd*, 10.5, 5.5)	83.2	3.65 (*br m*)	72.5
4	-	46.3	-	45.7

Table 24 (continued)

Position	NW7* (diterpenoid part)		Nagilactone E	
	δ (ppm)		δ (ppm)	
	^{1}H, (multiplicity, *J* in Hz)	^{13}C	^{1}H**[a], (multiplicity, *J* in Hz)	^{13}C**[b]
5	1.91 (*d*, 4.5)	45.4	1.88 (*d*, 4.5)	44.3
6	5.11 (*br d*, 4.5)	72.2	5.01 (*dd*, 1.5, 4.5)	72.6
7	4.22 (*d*, 7.5)	54.2	3.99 (*d*, 1.5)	54.3
8	-	58.9	-	59.0
9	-	159.0	-	159.1
10	-	36.3	-	36.6
11	6.12 (*s*)	117.1	5.98 (*s*)	117.1
12	-	163.8	-	163.8
13	-	-	-	-
14	4.55 (*d*, 3.5)	82.9	4.45 (*d*, 4.0)	82.9
15	1.94 (*dq*, 7, 3.5)	26.8	-	26.9
16	1.15 (*d*, 7)	16.6	-	16.6
17	0.99 (*d*, 7)	21.3	-	21.3
18	1.69 (*s*)	21.9	-	24.3
19	-	176.3	-	178.0
20	1.22 (*s*)	24.2	-	22.0
	NW7 (sugar part)		β-D-glucose***	
1’	4.93 (*d*, 7.5)	107.7	5.16 (*d*, 7.8)	105.5
2’	4.10 (*t*, 8.25)	75.5	4.04	75.4
3’	4.25 (*t*, 8.5)	78.5	4.20	78.5
4’	4.21 (*t*, 8.5)	71.5	4.20	71.4
5’	3.98 (*ddd*, 8.25, 6, 2.3)	78.6	3.90	78.2
6’a	4.39 (*dd*, 11.75, 5.25)	62.9	4.32	62.5
6’b	4.57 (*dd*, 11.25, 2.25)		4.46	

*solvent: $CDCl_3$; 500 MHz (^{1}H-NMR) and 125 MHz (^{13}C-NMR)

**[a] Hayashi et al, 1972; solvent: $CDCl_3$

**[b] Kubo and Ying, 1991b; solvent: C_5D_5N

Table 25 ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR, ^{1}H,^{1}H-COSY, HMBC and NOESY spectral data of NW7 in C_5D_5N

Position	NW7				
	δ (ppm)		COSY	HMBC	NOESY
	^{1}H (multiplicity, J in Hz)	^{13}C			
1α	1.38 (*td*, 12.5, 4.3)	29.0	H-2	C-2, C-3, C-10, C-20	H-5
1β	1.59 (*dt*, 13, 4.25)			C-2, C-3, C-5, C-10, C-18	H-20
2α	2.38 (*dtd*, 14.25, 4.9, 5)	27.3	H-1, H-3	C-3	H-3
2β	2.05 (*dtd*, 14.5, 11.5, 3.8)			C-3, C-4, C-10	H-20
3	3.94 (*dd*, 10.5, 5.5)	83.2	H-2	C-2, C-4, C-18, C-19, C-1'	H-2α, H-18
4	-	46.3	-	-	-
5	1.91 (*d*, 4.5)	45.4	H-6	C-1, C-4, C-9, C-10, C-18, C-19, C-20	H-1α, H-6, H-18
6	5.11 (*br d*, 4.5)	72.2	H-5, H-7	C-7, C-8, C-10	H-18
7	4.22 (*d*, 7.5)	54.2	H-6	C-5, C-6	H-15, H-20
8	-	58.9	-	-	-
9	-	159.0	-	-	-
10	-	36.3	-	-	-
11	6.12 (*s*)	117.1	-	C-8, C-10, C-12	H-1α, H-1β
12	-	163.8	-	-	-
13	-	-	-	-	-
14	4.55 (*d*, 3.5)	82.9	H-15	C-8, C-15, C-16, C-17	H-20
15	1.94 (*dq*, 7, 3.5)	26.8	H-16, H-17	C-16, C-17	H-7
16	1.15 (*d*, 7)	16.6	H-15	C-14, C-15, C-17	-
17	0.99 (*d*, 7)	21.3	H-15	C-14, C-15, C-16	-

Table 25 (continued)

Position	NW7				
	δ (ppm)		COSY	HMBC	NOESY
	^{1}H (multiplicity, J in Hz)	^{13}C			
18	1.69 (*s*)	21.9	-	C-3, C-4, C-5, C-15, C-19	H3, H-5, H-6
19	-	176.3	-	-	-
20	1.22 (*s*)	24.2	-	C-1, C-5, C-9, C-10	H-1α, H-1β, H-2β
1’	4.93 (*d*, 7.5)	107.7	H-2’	C-3, C-2’, C-5’	C-3’, C-5’
2’	4.10 (*t*, 8.25)	75.5	H-3’	C-1’, C-3’, C-4’	-
3’	4.25 (*t*, 8.5)	78.5	H-3’, H-4’	C-1’, C-2’, C-4’, C-6’	C-5’
4’	4.21 (*t*, 8.5)	71.5	H-3’, H-4’, H-5’	C-2’, C-6’	-
5’	3.98 (*ddd*, 8.25, 6, 2.3)	78.6	H-3’, H-4’, H-6’	C-1’, C-2’	C-1’, C-3’
6’a	4.39 (*dd*, 11.75, 5.25)	62.9	H-5’	C-4’, C-5’	-
6’b	4.57 (*dd*, 11.25, 2.25)			C-4’	-

***Hernández-Carlos et al, 2011; solvent : C_5D_5N; 300 MHz (^{1}H-NMR) and 75 MHz (^{13}C-NMR)

4.4 Lipase inhibitory activity of the pure compounds

All the pure isolated compounds were tested for lipase inhibitory activity. The results were shown in **Table 26**. The determination of the IC_{50} values of positive control and all pure compounds were shown in **Table 28**. And the dose-response curves of the active compounds were shown in **Figure 10**.

Table 26 Lipase inhibitory activity of the pure isolated compounds

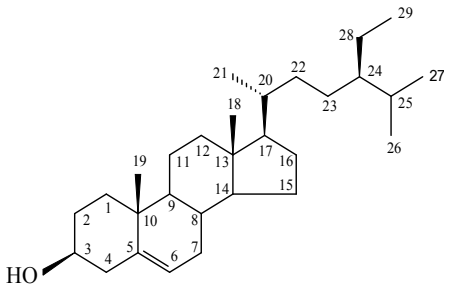
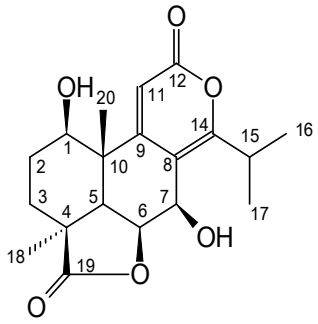
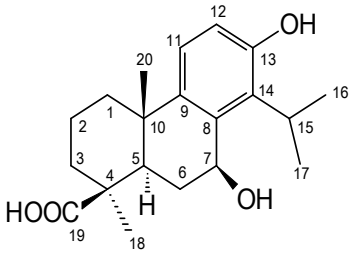
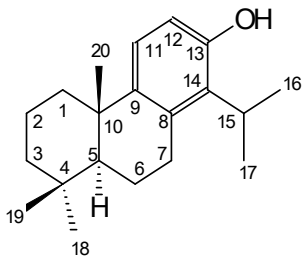
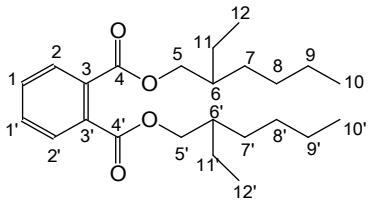
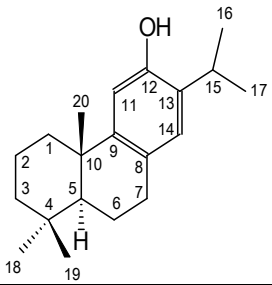
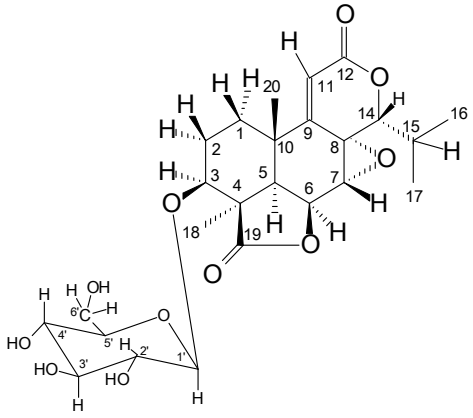
Compounds	IC_{50} (μM)
β-sitosterol 	>3.02
Nagilactone A 	>1436.78
Inumakiol D 	>1129.52
Totarol 	31.65±0.19

Table 26 (continued)

Compounds	IC_{50} (μM)
Bis-2-ethylhexyl-phthalate 	32.75±1.93
Ferruginol 	30.98±2.24
Nagilactone E-3-*O*-β-glucopyranoside 	>196.08
Orlistat*	0.016±0.005
Gallocatechin gallate (GCG)*	0.198±0.001

* = positive control

Totarol, bis-2-ethylhexyl-phthalate, and ferruginol were found to possess lipase inhibitory activity. Ferruginol and totarol displayed nearby the same activity with the IC_{50} values of 31.65±0.19 μM and 32.99±4.51 μM respectively. Consideration on their chemical structures, moving of hydroxyl and isopropyl groups from position 13 and 14 of totarol to position 12 and 13 of ferruginol respectively, did not affect the activity. Comparing the structure of inumakiol D with totarol, an addition of a hydroxyl group at

position 7 and oxidation of C-19 to a carboxylic functional group were found to reduce activity. Nagilactone A and nagilactone E-3-*O*-β-glucopyranoside were the other inactive compound. Under pH 8.0 of the assay condition, its lactone ring D might be hydrolyzed to a carboxyl group (Fassberg and Stella, 1992; Hanson et al, 2003). Then the product possessed a similar structure to inumakiol D. This confirmed the suggestion that carboxyl group at C-4 dramatically decreased the lipase activity.

Totarol and inumakiol D were classified as the same type diterpene but they were different type from ferruginol. However, their overall core structure was rather the same abietane-type diterpenes. Lipase inhibitory activity of some compounds in abietane-type diterpene group, such as carnosic acid and carnosol, has been previously reported (Ninomiya et al, 2004). Their lipase inhibitory activities were shown in **Table 27**. The result of this study supported the importance of abietane skeleton on this activity and gained more information on its structure-activity relationships. Moreover, norditerpene dilactones has never been reported on lipase inhibitory activity so this is the first report of this type of diterpenoids including nagilactone A and nagilactone E-3-*O*-β-glucopyranoside for this activity. The three active compounds were lower potency on lipase inhibitory activity than both positive controls which the IC_{50} values were in the ranges of ng/ml.

In vivo experiment on the isolated compounds or the extract of this plant should be further studied for development as an alternative choice for anti-obesity supplements.

Table 27 Structure and the IC_{50} values of carnosol and carnosic acid (Ninomiya et al, 2004)

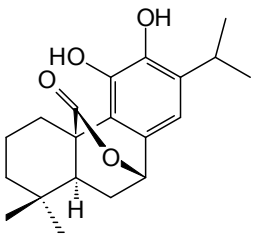
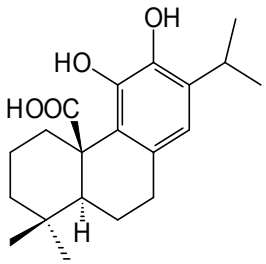
Compounds	IC_{50} (μM)
Orlistat	0.003
Carnosol (7) 	13
Carnosic acid (8) 	36

Table 28 IC_{50} values of positive control (Orlistat and Gallocatechin gallate (GCG)) and pure compounds

Sample	Final conc. [µg/ml]	% Inhibition			Mean±SD
		Exp 1	Exp 2	Exp 3	
Orlistat	0.00156	9.67	7.10	2.30	6.36 ± 3.74
Molecular weight = 495.7	0.00625	44.74	42.24	21.49	36.16 ± 12.76
	0.0125	55.99	59.35	59.24	58.19 ± 1.91
	0.05	88.97	85.62	91.29	88.63 ± 2.85
	1	98.52	97.65	98.70	98.29 ± 0.56
IC_{50} value (ng/ml)		7.20	5.90	10.5	7.87 ± 2.37
IC_{50} value (nM)		14.50	11.90	21.19	15.86 ± 4.79
GCG	0.0005	0.58	0.56	0.56	0.57 ± 0.01
Molecular weight = 458.4	0.005	1.63	1.67	1.63	1.64 ± 0.02
	0.05	3.64	3.64	3.62	3.63 ± 0.01
	0.2	39.92	40.21	40.05	40.06 ± 0.14
	0.4	62.44	61.14	61.84	61.81 ± 0.65
	0.6	83.57	82.90	82.23	82.90 ± 0.67
	0.8	87.75	88.87	87.00	87.87 ± 0.94
	1	90.20	90.90	91.54	90.88 ± 0.67
	10	94.82	94.99	94.12	94.64 ± 0.46
IC_{50} value (ng/ml)		90.21	90.91	91.55	90.89 ± 0.67
IC_{50} value (nM)		196.79	198.31	199.72	198.27 ± 1.47
β-sitosterol (NW1)					
Molecular weight = 414					
3.02 µM	1.25	10.360	10.77	10.35	10.49 ± 0.24
Nagilactone A (NW2)	1.76	6.57	6.54	5.35	6.15 ± 0.69
Molecular weight = 348	35.16	6.06	6.43	3.26	5.25 ± 1.73
	70.31	5.13	4.75	6.16	5.35 ± 0.73
	140.63	6.34	5.66	4.74	5.58 ± 0.80
	281.25	7.38	7.56	6.09	7.01 ± 0.80
	375.00	10.19	9.22	7.79	9.06 ± 1.21
1,436.78 µM	500.00	11.08	10.09	9.493	10.22 ± 0.80

Table 28 (continued)

Sample	Final conc. [µg/ml]	% Inhibition			Mean±SD
		Exp 1	Exp 2	Exp 3	
Inumakiol D (NW3)	9.89	3.37	3.83	3.20	3.47 ± 0.33
Molecular weight = 332	39.55	7.70	6.23	8.06	7.33 ± 0.97
	79.10	10.19	10.89	8.50	9.86 ± 1.23
	158.20	20.25	18.51	16.58	18.45 ± 1.84
	210.94	22.78	24.23	20.51	22.51 ± 1.87
	281.25	36.50	33.48	31.45	33.81 ± 2.54
1,129.52 µM	375.00	43.88	45.16	40.65	43.23 ± 2.32
Totarol (NW4)	0.08	1.93	1.57	0.99	1.50 ± 0.47
Molecular weight = 286	0.88	2.40	2.21	1.33	1.98 ± 0.57
	1.76	2.64	2.63	1.69	2.32 ± 0.54
	3.52	3.44	3.07	1.70	2.74 ± 0.92
	7.03	21.07	20.34	19.52	20.31 ± 0.78
	9.38	59.37	58.04	57.88	58.43 ± 0.82
	12.50	79.23	78.47	77.59	78.43 ± 0.82
	75.00	99.11	99.14	99.17	99.14 ± 0.03
	100.00	99.38	99.37	99.37	99.38 ± 0.01
IC_{50} value (µg/ml)		8.99	9.08	9.09	9.05 ± 0.05
IC_{50} value (µM)		31.44	31.75	31.77	31.65 ± 0.19
Bis-2-ethylhexyl-phthalate (NW5)	0.00156	-0.82	-0.79	-0.96	-0.86 ± 0.09
Molecular weight = 390	0.156	-0.72	-0.84	-0.99	-0.85 ± 0.14
	1.56	6.96	7.44	7.43	7.28 ± 0.28
	3.13	17.74	18.60	18.74	18.36 ± 0.54
	6.25	33.56	33.83	35.03	34.14 ± 0.78
	12.50	44.85	46.58	45.20	45.54 ± 0.91
	25.00	64.44	69.27	66.26	66.65 ± 2.44
	50.00	79.18	81.62	79.71	80.17 ± 1.28
	100.00	86.43	89.11	89.52	88.35 ± 1.68
IC_{50} value (µg/ml)		12.81	12.00	13.50	12.77 ± 0.75
IC_{50} value (µM)		32.85	30.77	34.62	32.75 ± 1.93

Table 28 (continued)

Sample	Final conc. [µg/ml]	% Inhibition			Mean±SD
		Exp 1	Exp 2	Exp 3	
Ferruginol (NW6)	0.08	2.42	0.96	-0.49	0.97 ± 1.45
Molecular weight = 286	0.88	1.82	2.94	1.32	2.03 ± 0.83
	1.76	3.44	2.28	1.59	2.44 ± 0.93
	3.52	3.21	3.04	4.35	3.53 ± 0.71
	7.03	33.66	40.01	24.47	32.72 ± 7.81
	9.38	51.91	58.58	53.74	54.74 ± 3.44
	12.50	72.48	76.95	70.12	73.18 ± 3.47
	75.00	97.62	98.07	97.66	97.78 ± 0.25
	100.00	98.83	95.52	99.17	97.84 ± 2.02
IC_{50} value (µg/ml)		9.12	8.13	9.33	8.86 ± 0.64
IC_{50} value (µM)		31.88	28.43	32.62	30.98 ± 2.24
Nagilactone E-3-*O*-β-	0.00156	3.96	1.68		2.82
glucopyranoside	0.15625	5.01	-0.38		2.32
(NW7)	1.5625	0.52	-0.19		0.17
Molecular weight = 510	3.125	3.21	3.05		3.13
	6.25	6.18	5.42		5.80
	12.5	5.68	4.57		5.13
	25	4.69	4.52		4.60
	50	10.11	6.83		8.47
196.08 µM	100	7.65	12.56		10.11

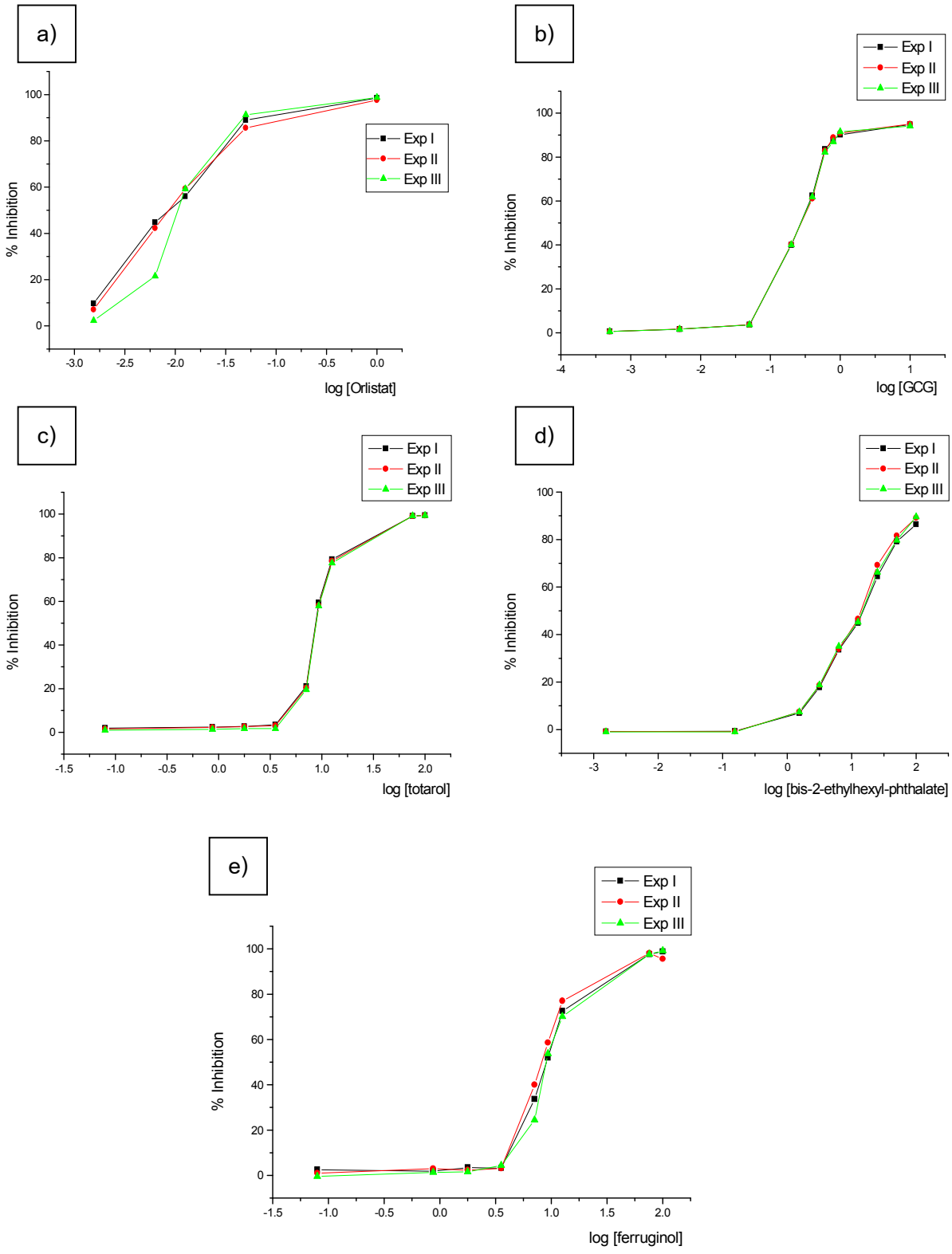


Figure 10 Dose-response curves of positive control and some active compounds. Pictures a), b), c), d), and e) were dose-dependent curves of orlistat, gallocatechin gallate, totarol, bis-2-ethylhexyl-phthalate, and ferruginol respectively (n=3).

4.5 Bioautography development

p-Nitrophenyl palmitate (PNP) was the substrate selected for development of bioautographic assay of lipase inhibitory activity because its cost was reasonable for preparing large amounts for spraying reagent and could be developed the yellow color products which was easily to detect by visual. To change substrate to PNP, this assay system would be tested for comparing with the prior method which used 4-MUO as substrate by screening all plant extracts with this assay.

4.5.1 Screening of crude extract using *p*-nitrophenol palmitate as substrate

All plant extracts were screening with alternative assay using PNP as substrate (Slanc et al, 2004) with the same concentration of plant extracts (5 µg/ml) to compare the correlation of %inhibition with previous assay. The results **(Table 29)** found that almost of the extracts gave the same trend of %inhibition in the both assay using different substrates. These were implied that both substrates had well correlation on lipase inhibitory activity so the PNP could be used instead of 4-MUO to screen plant extracts on the development of bioautographic assay. However, some plant extracts such as *P. emblica* (seeds) and *N. wallichiana* (leaves and stems) expressed dramatically reversed on lipase inhibitory activities which were implied by the high differences in %inhibition of both substrates about 87.72%, 83.72%, and 94.27% for *P. emblica* (seeds) and *N. wallichiana* (leaves and stems) respectively. This effect might be occurred from the long term keeping of the crude extracts and/or the chemical constituents in crude extracts might quench the fluorescence detection of 4-methylumbelliferone, the enzymatic reaction product of 4-MUO. This effect might be proved in the further study.

4.5.2 Determination of the substrate and enzyme concentration for bioautographic assay

The secondary experiment which substrate and enzyme concentrations were varied was different from the first experiment in the process of incubating the enzyme solution on TLC plate to reach the optimum temperature at 37^{o}C before reacting with the room temperature substrate solution. This process was better than previous

experiment that it gave rapid development of yellow color within short time (1-5 min) after spraying substrate. This effect occurred in all concentrations of substrate. The more increase in substrate concentration, the more rapid yellow color developed. The 3.33 mM PNP gave yellow color within 1-2 minutes after spraying but it was 5 minutes for 0.83 mM PNP.

The intensity of yellow color from various concentrations of substrate and enzyme was determined. The results showed that 0.8 mM PNP gave more pale yellow color than 1.67 and 3.33 mM PNP in all concentrations of enzyme **(Figure 11)**. And each concentration of substrate, the strong yellow color was more intense when the concentration of enzyme was increased, especially 20 and 40 mg/ml. The intensity of color was stable in all concentrations of enzyme and substrate after the TLC plate was incubated at 37°C for 30 min except for the enzyme concentration of 2.5 mg/ml that was stable for 1-hour incubation. At 10 U/ml of the enzyme concentration, the yellow color did not appear thoroughly 14-hour incubation. No yellow color was observed in the control group (buffer). The result indicated that the yellow intensity had directly relation to the increase of substrate and enzyme concentrations.

The finding of this study indicated that the optimum concentrations of substrate and enzyme which caused well visual yellow color development in appropriate incubation times (not more than 1-2 hours of incubation period) and using the less concentration of the enzyme were 3.33 mM PNP and 2.5 mg/ml enzyme solution after incubating the TLC plate at 37°C for 1 hour. However, the optimum concentration of the enzyme in this preliminary study was too high about 787.5 U/ml (2.5 mg protein/ml x 315 U/mg protein when using olive oil as substrate), compared with other reports **(Table 30)**. The high consuming of the enzyme concentration in the enzymatic reaction caused the low sensitivity of the assay. Because the more amounts of the enzyme used in the reaction, the more amounts of the positive compounds or other lipase inhibitors used. Therefore, the further study may determine the minimum concentration of the enzyme which can cause visual yellow color to increase the sensitivity of the assay. The following problems of the decrease in concentration of the enzyme might be the pale yellow color development and the prolonged incubation time to increase the intensity of

yellow color. For solving these problems, the alternative substrates which the by-product can react with other compounds to give dark tone of the color than yellow color such as purple color will be used.

The previous report of bioautographic assay of α- and β-glucosidase inhibitors (SimÕes-Pires et al, 2009), the optimal conditions for α- and β-glucosidase inhibitors were compared between using *p*-nitrophenyl derivative substrates such as *p*-nitrophenyl-α-D-glucopyranoside (α-glucosidase) or *p*-nitrophenyl-β-glucopyranoside (β-glucosidase) and 2-napthyl derivative substrates such as 2-napthyl-α-D-glucopyranoside (α-glucosidase) or 2-napthyl-β-glucopyranoside (β-glucosidase) with the fast blue B salt in the mixed ratio of 1:1 (for α-D-glucosidase) or 1:4 (for β-D-glucosidase). The mixtures of 2-napthyl derivative substrates and the fast blue B salt were sprayed onto the plate to give a purple background after incubation of the TLC plate with enzyme at room temperature for 60 min for α-glucosidase and 37°C for 20 min for β-glucosidase. It was found that the *p*-nitrophynyl derivative substrates gave less clear inhibition zones than the 2-napthyl derivative substrates. In addition, the 2-naphthyl derivative substrate increased limit of detection of positive compounds (conduritol B epoxide, castanospermine, and miglitol) for inhibiting α-glucosidase and β-glucosidase down to 0.1, 0.05, and 0.005 µg respectively. To improve the clear color detection from white spot on the yellow background to a white spot on purple background during decreased the enzyme concentration, the 2-naphthyl derivative substrates with the fast blue B salt were suggested for further study.

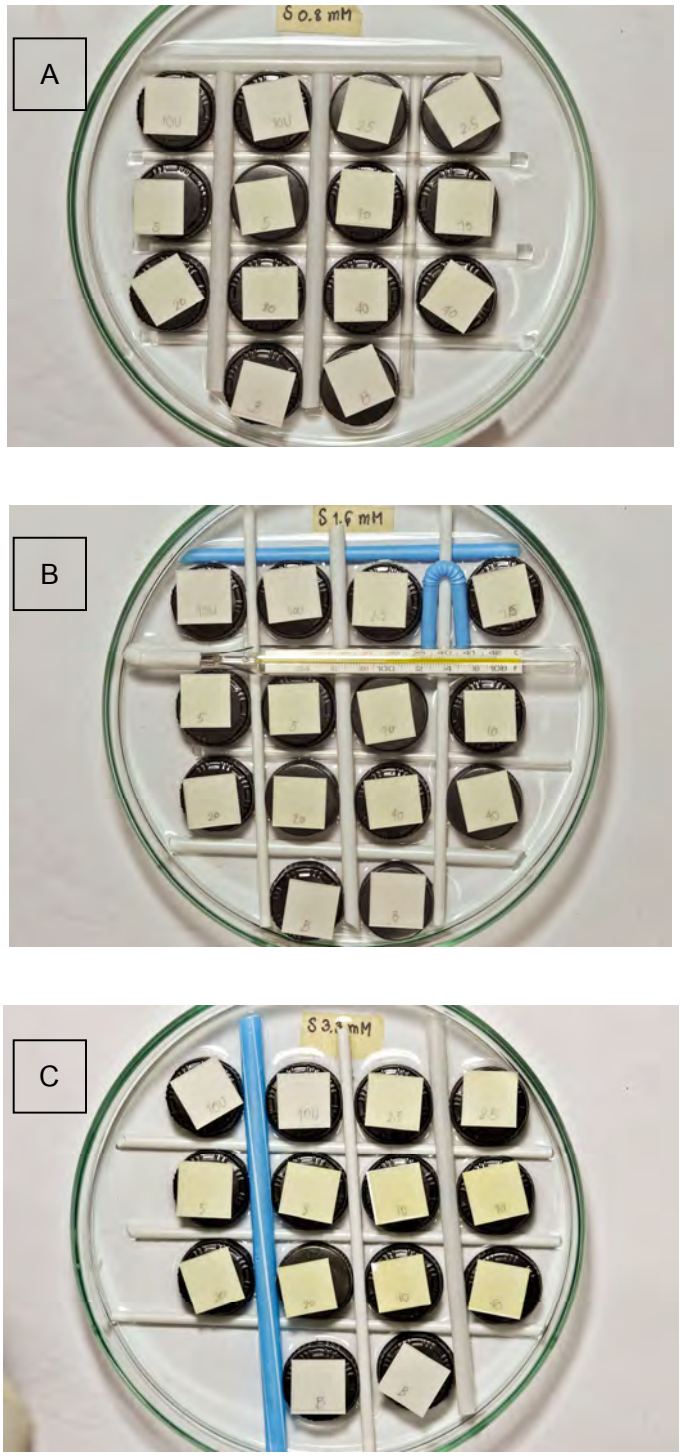


Figure 11 The result of bioautography by various concentrations of *p*-nitrophenyl palmitate (PNP) and porcine pancreatic lipase incubated at 37°C for 1 hour. Picture A, B, and C were the results of various concentrations of substrate from 0.83, 1.67, and 3.33 mM respectively. The number on TLC plate in each picture was shown the concentrations of enzyme including 10 U, 2.5, 5, 10, 20, 40 mg/ml, and B meaning of 10 Units/ml, 2.5, 5, 10, 20, 40 mg/ml, and 0.061 M Tris-HCl buffer pH 8.5 (control group) respectively.

Table 29 Pancreatic lipase inhibitory activity of Thai medicinal plants using 4-methyl-umbelliferyl oleate and *p*-nitropheylpalmitate as substrates

Family	Species	Part	4-MUO %inhibition (mean±SD)	PNP %inhibition (mean±SD)
Asclepiadaceae	*Cryptolepis buchanani* Roem. & Schult.	stems	10.00±0.88	39.05±0.38
Asteraceae	*Aster cordifolius* L.	arial parts	13.57±0.68	16.74±0.93
Clusiaceae	*Garcinia cowa* Roxb. ex DC.	leaves	23.00±1.22	12.73±0.83
	Garcinia schomburgkiana Pierre	fruits	12.79±0.77	10.94±0.84
Cucurbitaceae	*Coccinia grandis* (L.) Voigt	arial parts	15.21±1.18	48.64±0.54
		fruits	15.77±0.76	45.25±0.70
	Gymnopetalum chinense (Lour.) Merr.	fruits	8.00±0.59	49.92±0.72
	Gymnopetalum integrifolium (Roxb.) Kurz	fruits	7.00±0.38	41.27±1.65
	Luffa acutangula (L.) Roxb.	arial parts	14.00±0.63	48.50±0.19
	Luffa cylindrica (L.) M.Roem.	arial parts	7.67±0.43	49.28±1.91
		flowers	3.13±0.03	44.27±0.47
		fruits	5.00±0.56	26.61±0.75
Euphorbiaceae	*Phyllanthus emblica* L.	seeds	88.13±0.71	0.41±0.15
Gnetaceae	*Gnetum gnemon* L.	leaves	22.46±1.39	0.16±1.25
	Gnetum montanum Markgr.	leaves	45.78±1.20	37.34±0.39
Leguminosae	*Neptunia oleracea* Lour.	whole plant	11.43±0.77	11.02±0.28
	Neptunia triquetra (Vahl) Benth.	arial parts	57.51±2.28	5.48±0.22
Oxalidaceae	*Averrhoa bilimbi* L.	leaves	34.07±2.52	21.23±0.91
		fruits	2.31±0.14	29.84±0.26
Passifloraceae	*Passiflora foetida* L.	arial parts	15.89±1.20	33.24±3.24
		fruits	13.42±0.49	24.26±1.70
Pedaliaceae	*Sesamum indicum* L.	seeds	8.60±0.44	44.81±3.01
Pinaceae	*Pinus kesiya* Royle ex Gordon	leaves	51.44±3.30	44.52±4.76
Poaceae	*Cymbopogon citratus* Stapf	arial parts	16.43±1.47	45.62±0.98
	Oryza sativa L. cv. Jao Hom Nin	seeds	4.67±0.5	7.60±0.75
Podocarpaceae	*Nageia wallichiana* C. Presl Kuntze	leaves	88.71±0.57	4.99±0.39
		stems	86.60±0.66	-7.67±3.51

Table 29 (continued)

Family	Species	Part	4-MUO %inhibition (mean±SD)	PNP %inhibition (mean±SD)
Polygonaceae	*Polygonum odoratum* Lour.	arial parts	21.30±1.57	9.45±0.67
Rubiaceae	*Morinda citrifolia* L.	leaves	7.00±0.64	-5.11±3.43
		fruits	10.00±1.30	7.89±0.58
	Ixora grandifolia Zoll.& Moritzi	flowers	43.02±1.32	17.42±0.41
		leaves	68.71±0.24	5.15±0.18
	Randia horrida Roem. & Schult.	fruits	10.73±0.52	44.32±1.17
	Scyphiphora hydrophyllacea Gaertn.f.	leaves	11.56±1.04	44.20±0.85
		stems	14.00±0.52	42.27±0.39
Rutaceae	*Citrus hystrix* DC.	leaves	13.47±0.30	54.14±1.75
Sterculiaceae	*Scaphium scaphigerum* (G.Don) Guib. & Planch.	leaves	8.03±0.73	58.64±3.37
	Orlistat [IC_{50} value (ng/ml)]		9.37±0.09	14.15±1.60

The final concentration of each plant extract was tested in the concentration of 5 µg/ml.

Three measurements were carried out per sample (n=3).

Table 30 Enzymatic reaction for bioautographic assay

Substrate	Enzyme	Incubation condition (positive detection)	Limit of detection	Reference
esculin 0.2%w/v	β-glucosidase 8.3 mU/ml	Incubated TLC plate with enzyme in agar overlay at 37°C for 120 min after immersed in substrate at 37°C for 120 min (lacking β-glucosidase activiy: clear spot was observed against a dark brown background) Principle : esculetin (product from enzyme reaction) form complex with $FeCl_3$ to give brown complex	conduritol B epoxide 100 ng	Salazar and Furlan, 2007
4-methoxyphenyl acetate 43 mM	acetylcholinesterase (from electric eel) 1 U/ml	37°C for 20 min (lacking acetylcholinesterase activity: light yellow spot against aquamarine blue background was observed) Principle : by product from the enzyme reaction (4-methoxy phenol) reacts with potassium ferricyanide and iron chloride hexahydrate to make aquamarine blue color	Huperzine A 0.1 ng Physostigmine 1 ng	Yang et al, n/a-n/a

Table 30 (continued)

Substrate	Enzyme	Incubation condition (positive detection)	Limit of detection	Reference
1-naphthyl acetate 1 mg/ml	a) acetylcholinesterase (from electric eel) 6.7 U/ml b) butyrylcholinesterase 3.3 U/ml	Enzyme and TLC plate were incubated at 37°C for 20 min before detected with substrate and fast blue B salt (lacking acetylcholinesterase activity: clear spot against purple background) Principle : 1-naphthal (product from the enzyme reaction) reacts with Fast Blue B Salt to give azo dye (purple)	a) physostigmine 1 ng galantamine 10 ng b) physostigmine 1 ng galantamine 100 ng	Marston et al, 2002
a) 2-napthyl-α-D-glucopyranoside b) 2-napthyl-β-glucopyranoside 6.53 mM	a) α- glucosidase b) β-glucosidase 10 U/ml	a) room temperature for 60 min b) 37°C for 20 min (lacking α- or β-glucosidase activity: clear spot against a purple background) Principle : 2-naphthol from enzyme reaction reacts with Fast Blue B Salt to form azo dye (purple)	a) conduritol B epoxide 100 ng castanospermine 50 ng miglitol 5 ng b) conduritol B epoxide 100 ng	SimÕes-Pires et al, 2009

Table 30 (continued)

Substrate	Enzyme	Incubation condition (positive detection)	Limit of detection	Reference
xanthine 3 mM	xanthine oxidase 68 mU/ml	38°C for 20 min (lacking xanthine oxidase activity: clear zone against a dark purple background) Principle : superoxide from enzyme reaction which reduce the pale yellow tetrazolium salt to a formazan (purple)	allopurinol 5 ng	Ramallo et al, 2006

CHAPTER V

CONCLUSION

Thai medicinal plants had potential for anti-lipase activity. *Nageia wallichiana* was one of the studied plants which had potent activity and the active compounds were totarol, ferruginol, and bis-2-ethylhexyl-phathalate isolated from EtOAc extract. The other pure compounds from acetone extract including nagilactone A and nagilactone E-3-*O*-β-glycopyranoside were not active. Many active fractions were found in the polar part, but this purification was not successful because of many mixtures and complicated system for isolation with silica gel or reverse phase column chromatography.

There are several studies of totarol about anti-bacterial, anti-acne and anti-inflammatory and antiseptic, whereas gastro-protective effect was reported for ferruginol. Bis-2-ethylhexyl-phthalate is used as a plasticizer and is concerned with toxicity to consumers. Totarol and ferruginol have main structure like abietane-type diterpenoids which have been reported on the lipase inhibitory activity. This is a first time of report on this activity for bis-2-ethylhexyl-phthalate.

Neagia wallichiana is a reservation plants and has not been used in ethnobotany. So it has not been developed to commercial products for curing diseases because it is poor in safety evidences. However, it is a good source for isolation of many diterpenoids compounds.

Preliminary study for bioautographic assay has been developed for pancreatic lipase activity test. The suitable substrate and condition were 3.33 mM *p*-nitrophenol palmitate at pH 7.5 and 37^{o}C incubation for 30 min in humidity tank. The concentration of enzyme was determined by using as less as amounts of enzyme concentration which could generate the visual color. To increase the sensitivity of the assay, it was suggested that the substrate might be changed to 2-naphthyl derivatives and using the fast blue B salt to give the reaction products that be easily observed by the intense purple color.

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APPENDICES

APPENDIX A

(SPECTROSCOPIC SPECTRA)

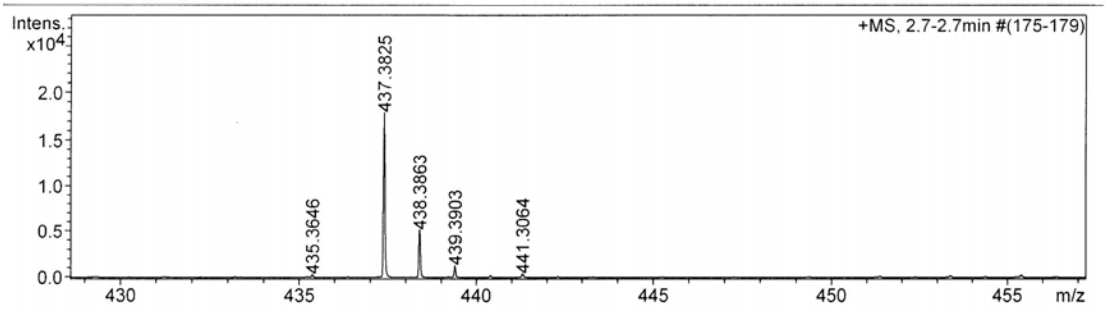


Figure A1 ESI Mass Spectrum of NW1 (β-Sitosterol)

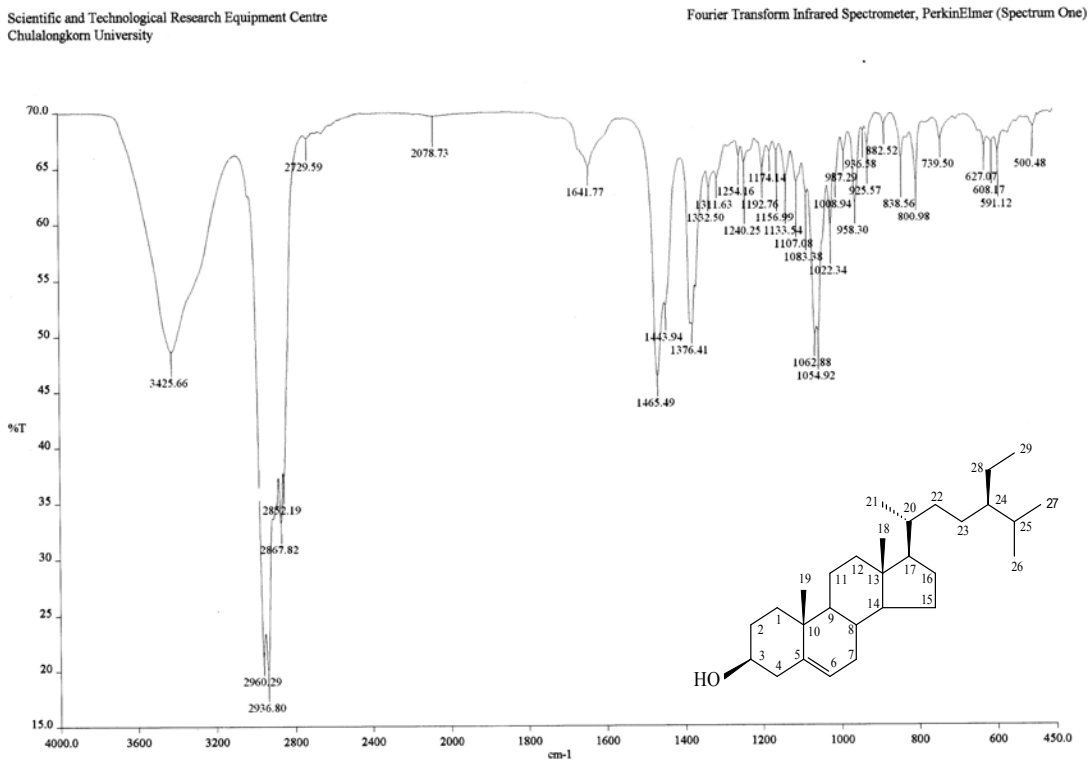


Figure A2 IR Spectrum of NW1 (β-Sitosterol) (KBr)

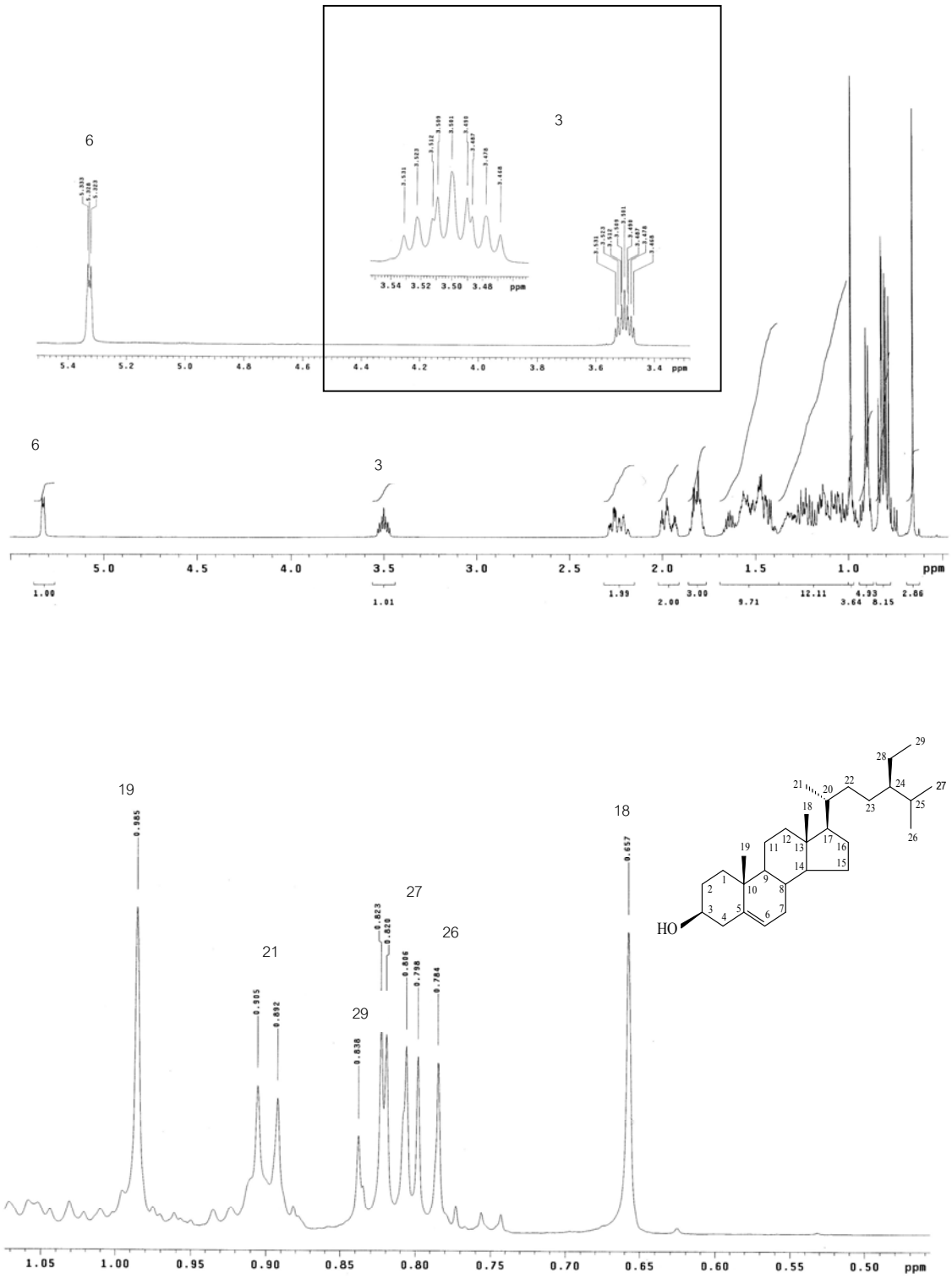


Figure A3 ^{1}H-NMR (500 MHz) Spectrum of NW1 (β-Sitosterol) in $CDCl_3$

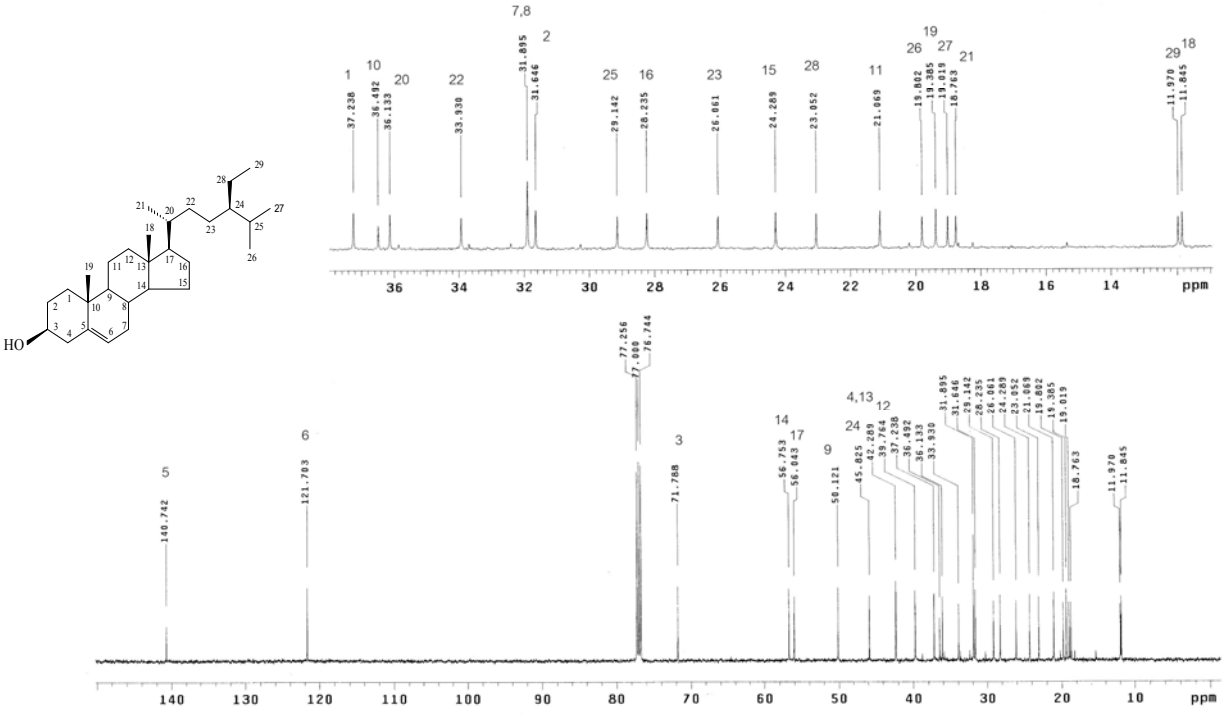


Figure A4 ^{13}C-NMR (125 MHz) Spectrum of NW1 (β-Sitosterol) in $CDCl_3$

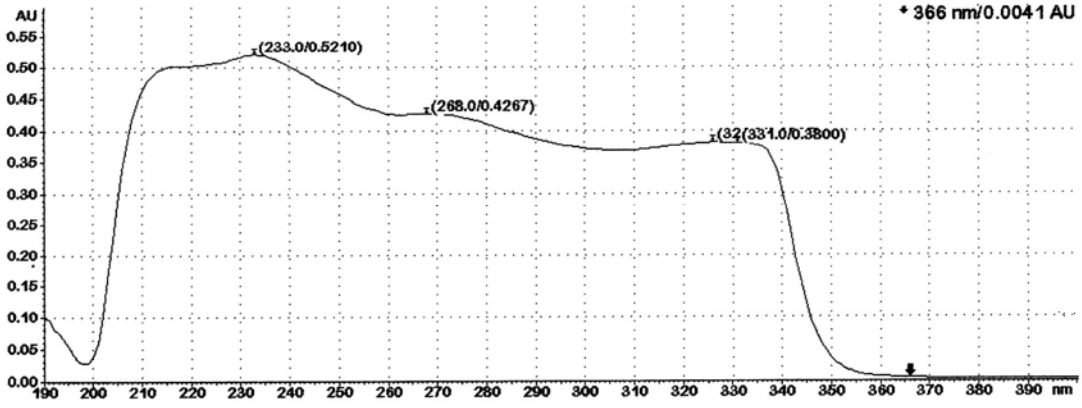


Figure A5 UV Spectrum of compound NW2 (Nagilactone A) in MeOH

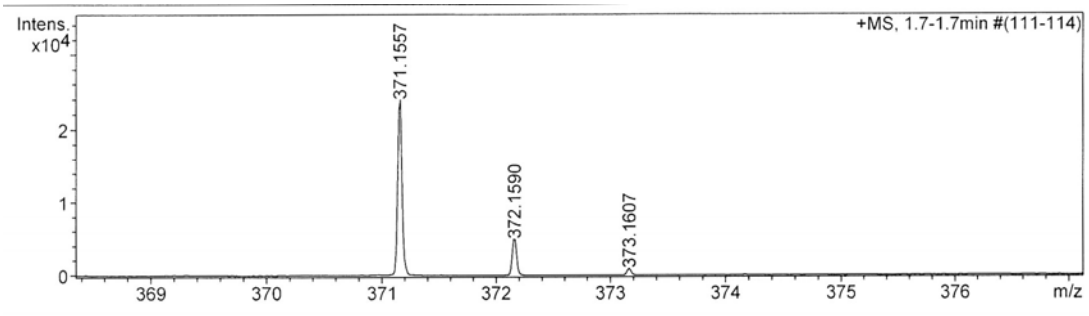


Figure A6 ESI Mass spectrum of compound NW2 (Nagilactone A)

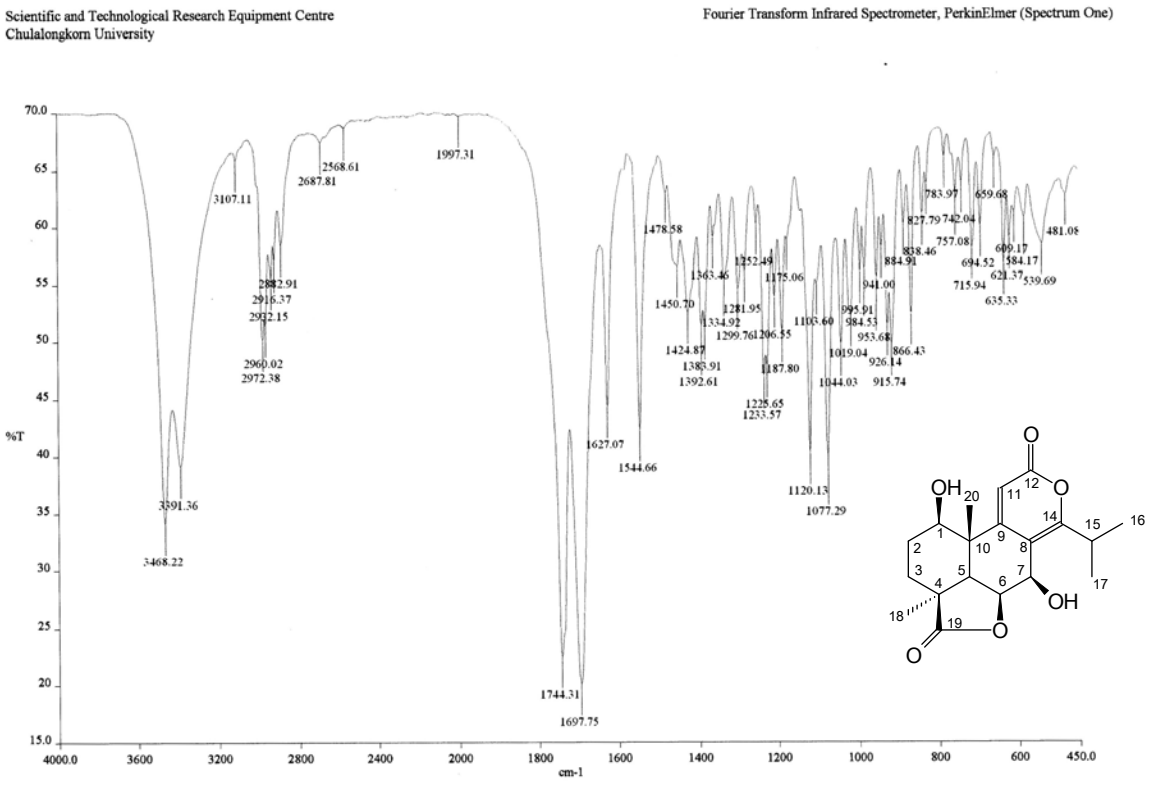


Figure A7 IR spectrum of compound NW2 (Nagilactone A) (KBr)

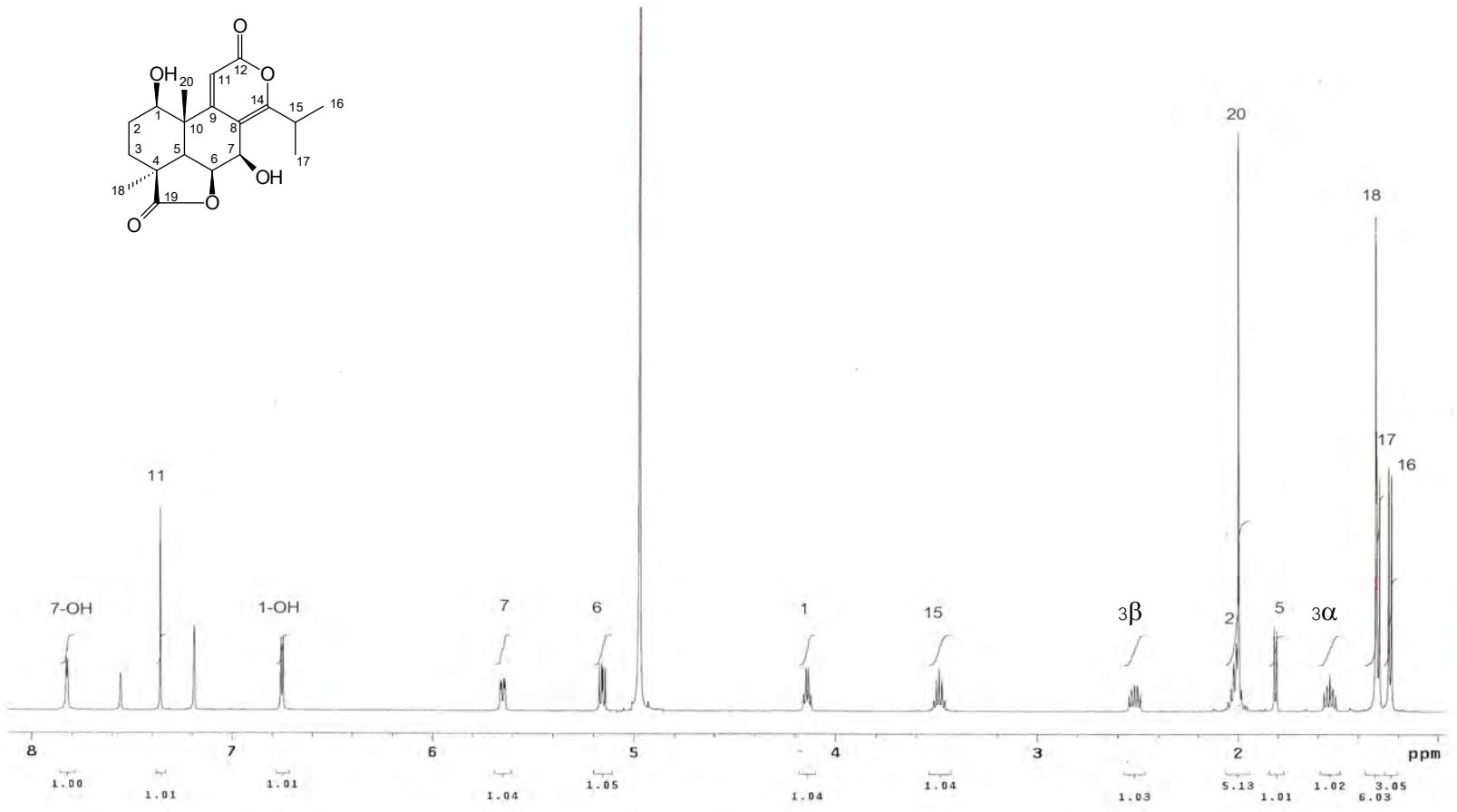


Figure A8 ^{1}H-NMR (500 MHz) Spectrum of NW2 (Nagilactone A) in C_5D_5N

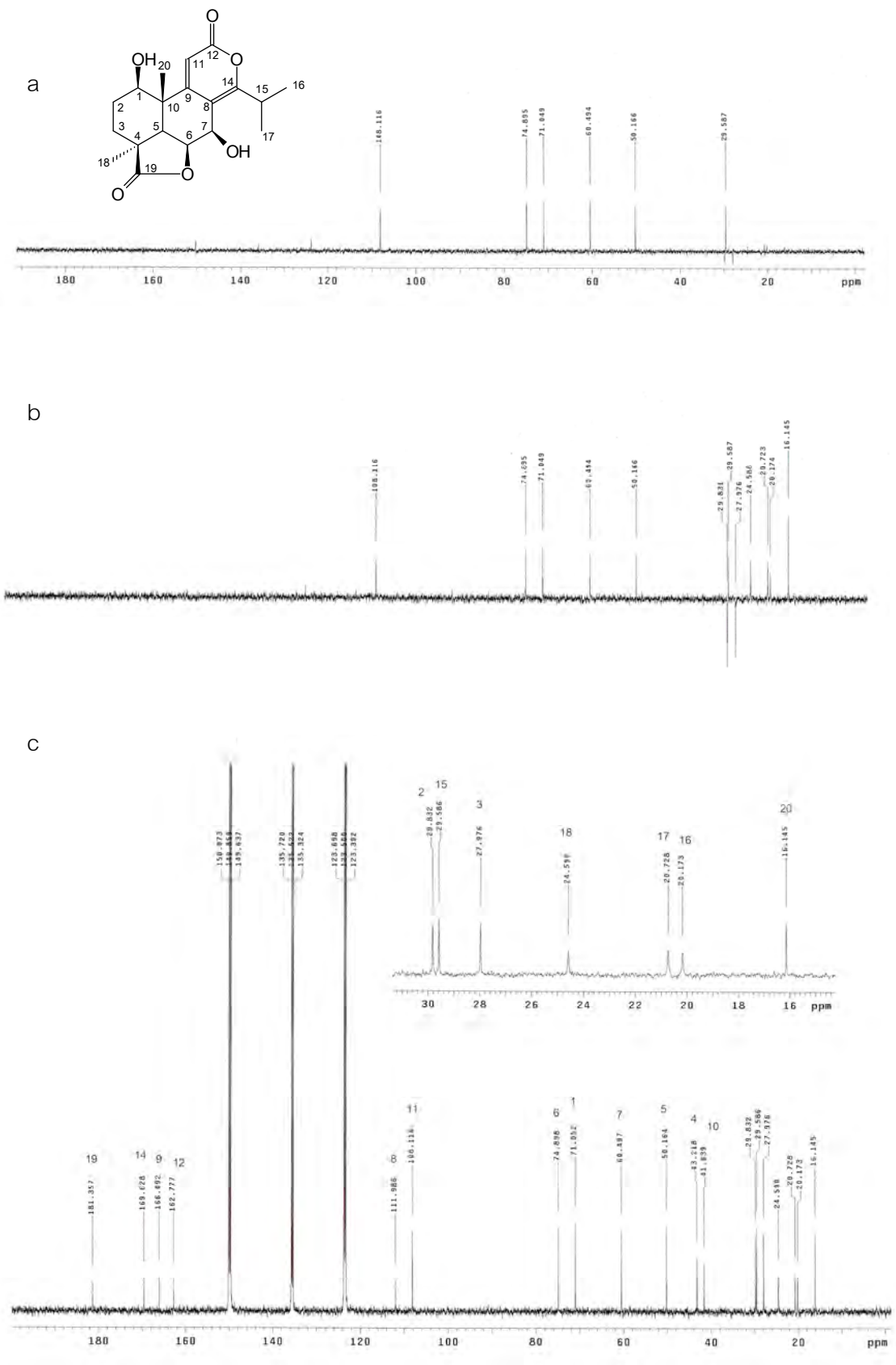


Figure A9 DEPT90° (a), DEPT135° (b), and ^{13}C-NMR (c) (125 MHz) Spectra of NW2 (Nagilactone A) in C_5D_5N

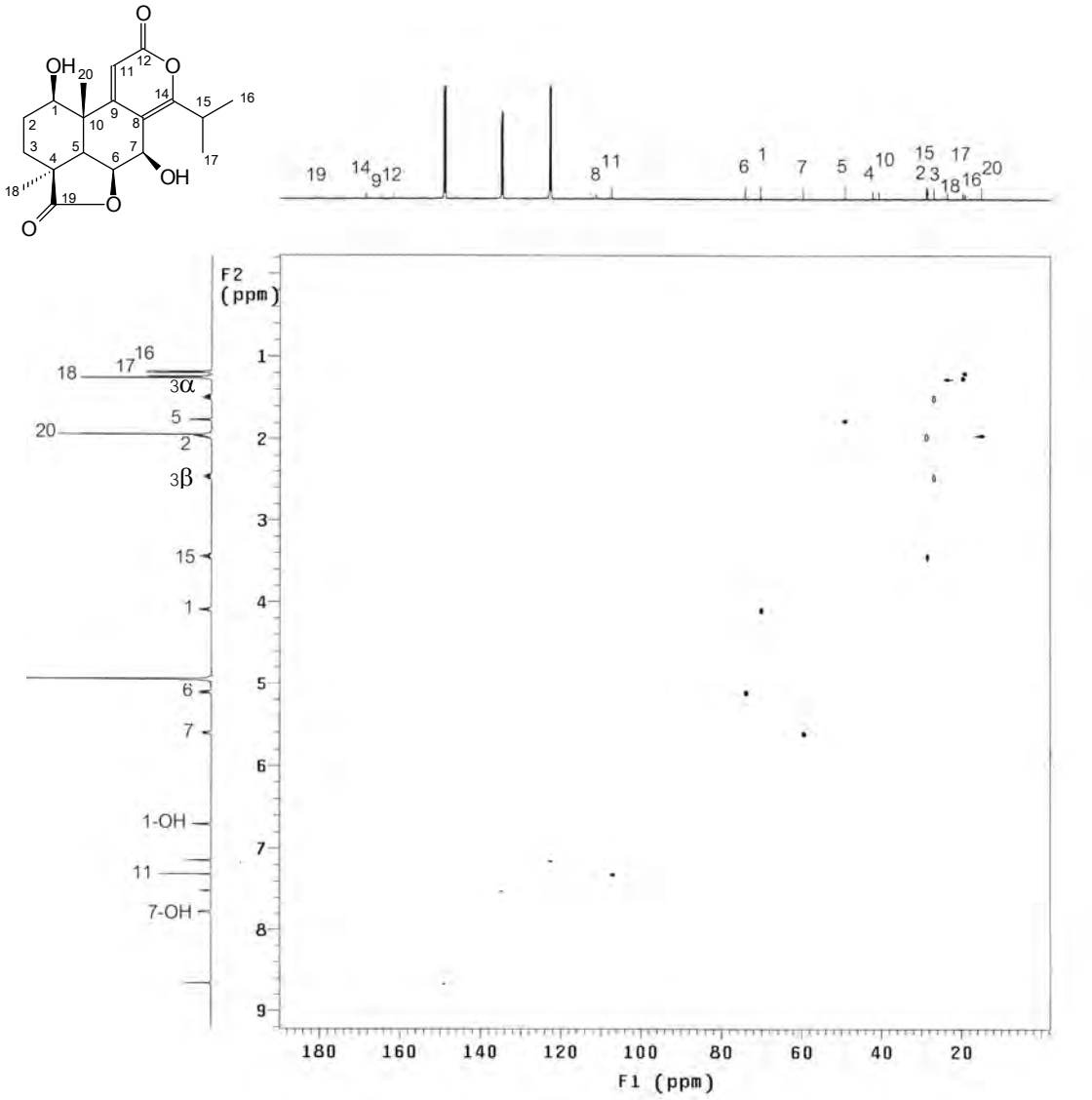


Figure A10 HSQC Spectrum of NW1 (Nagilactone A) in C_5D_5N

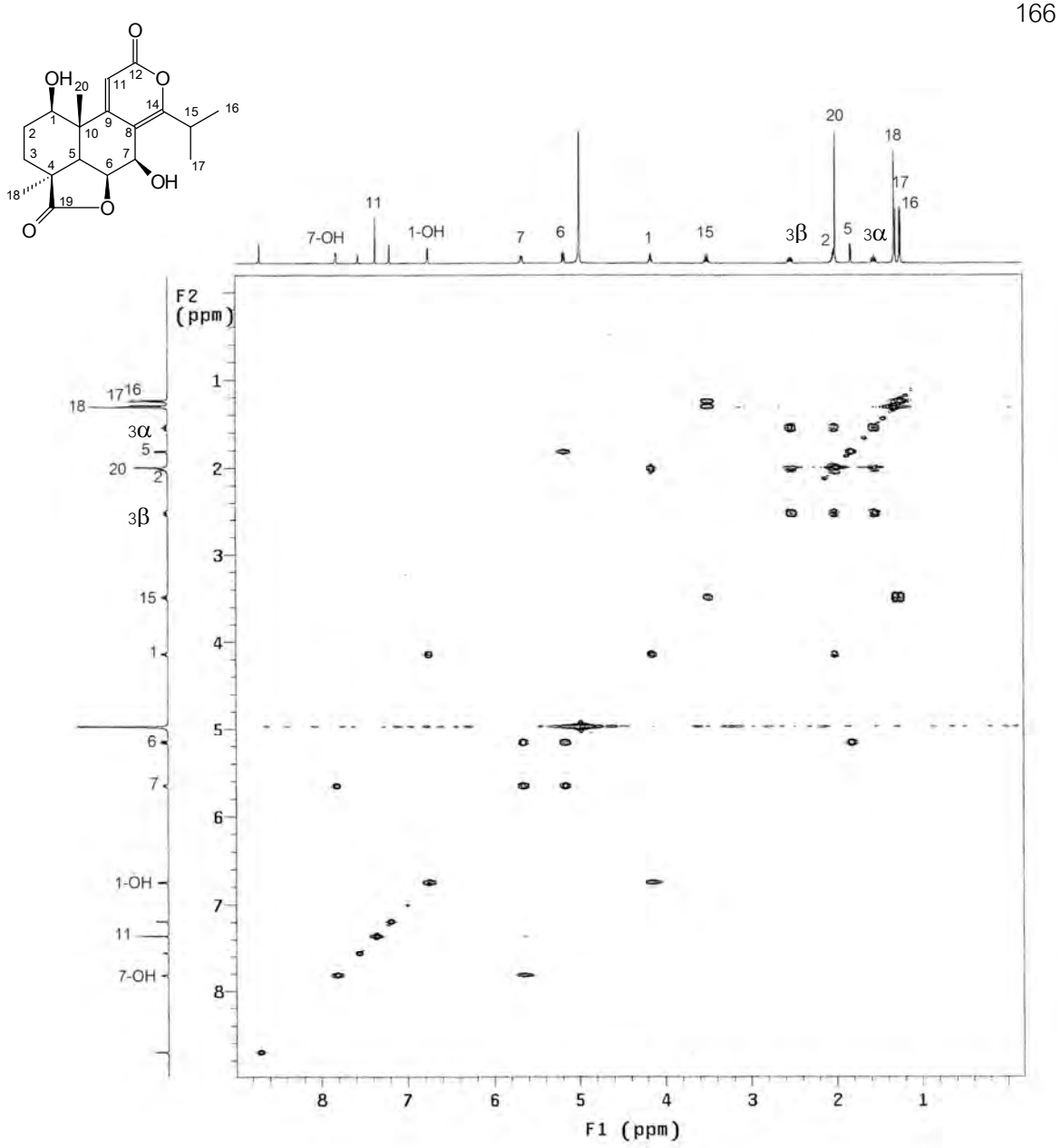


Figure A11 ^{1}H-^{1}H-COSY Spectrum of NW1 (Nagilactone A) in C_5D_5N

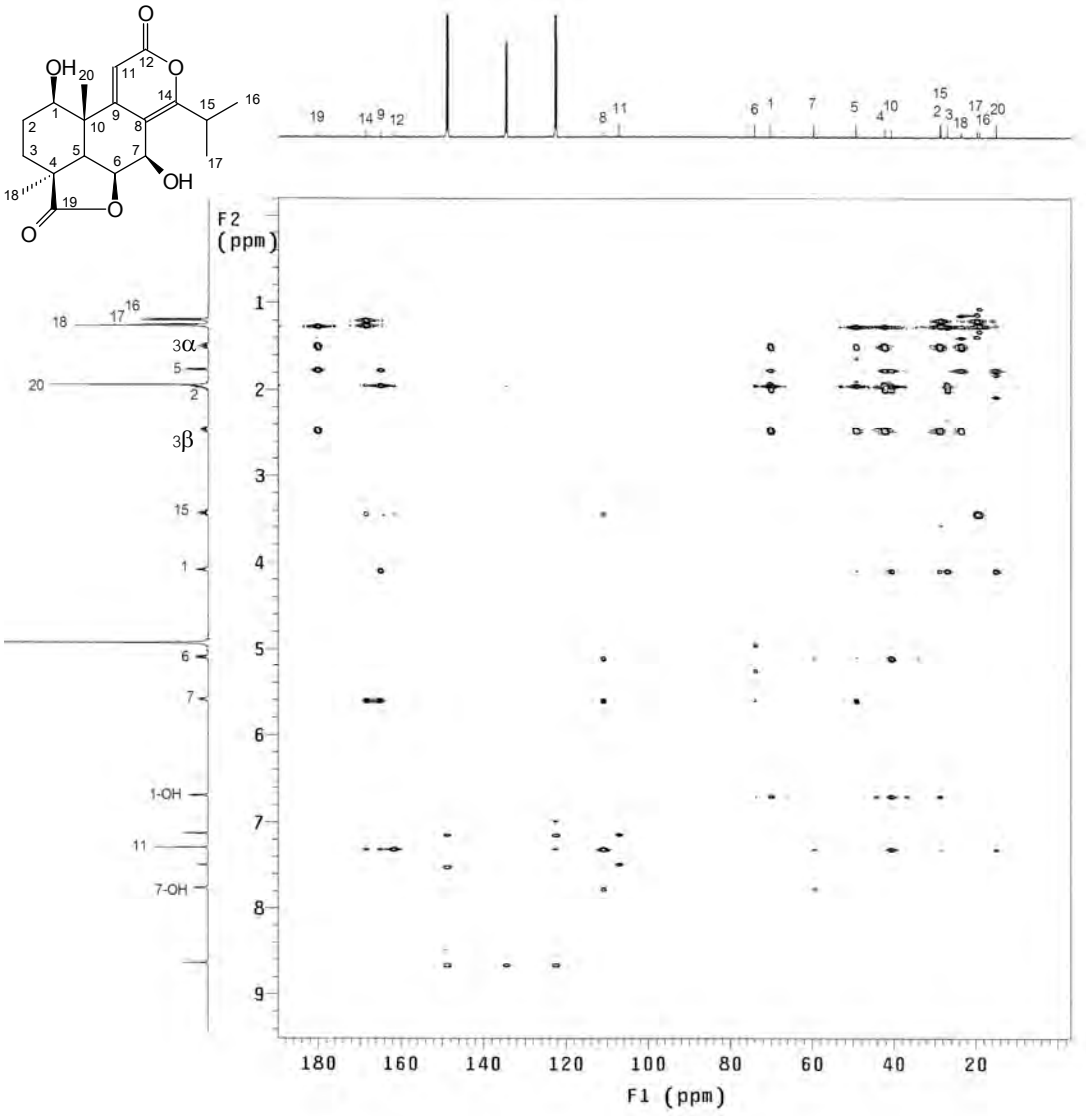


Figure A12 HMBC Spectrum of NW1 (Nagilactone A) in C_5D_5N

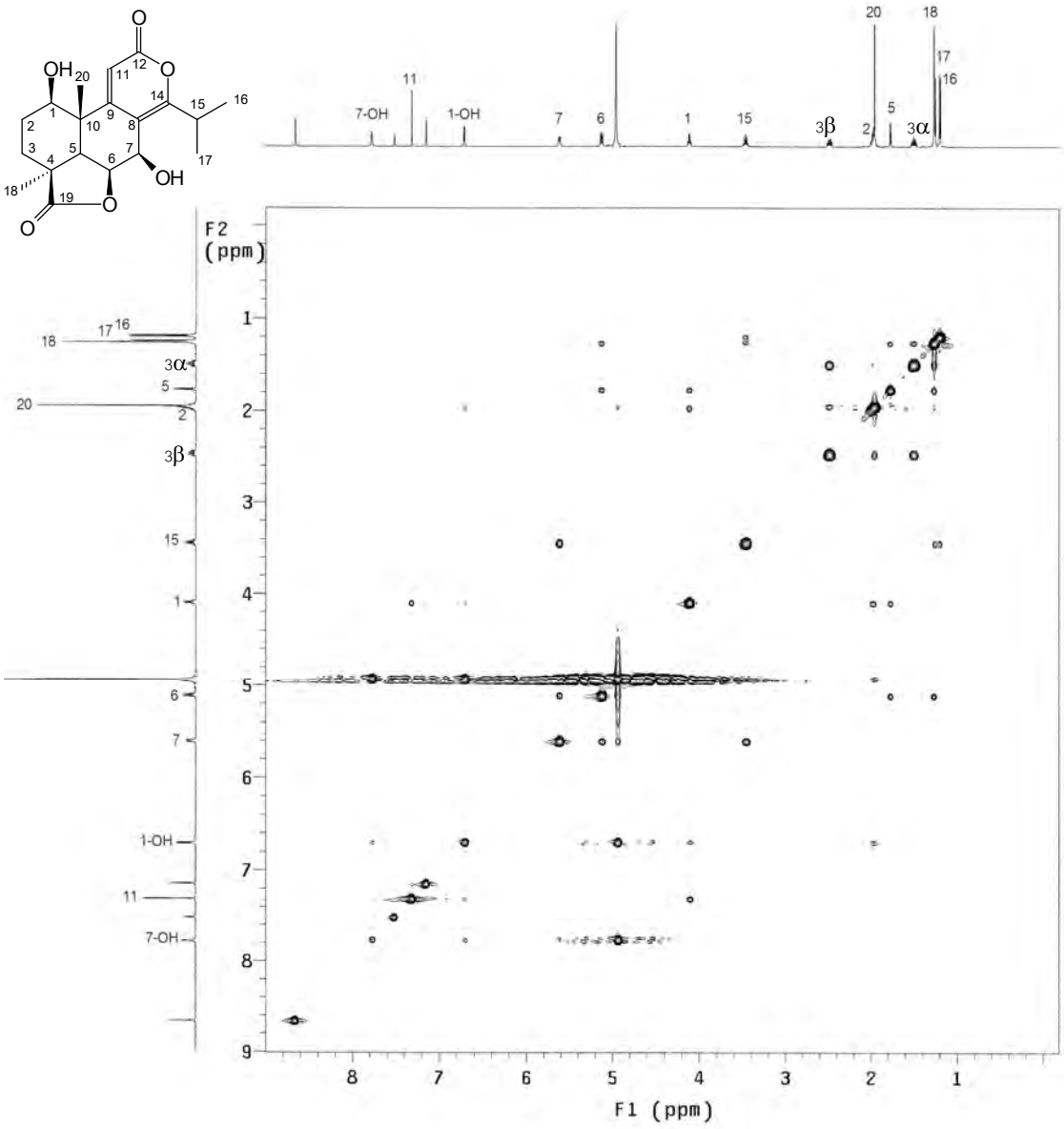


Figure A13 NOESY Spectrum of NW1 (Nagilactone A) in C_5D_5N

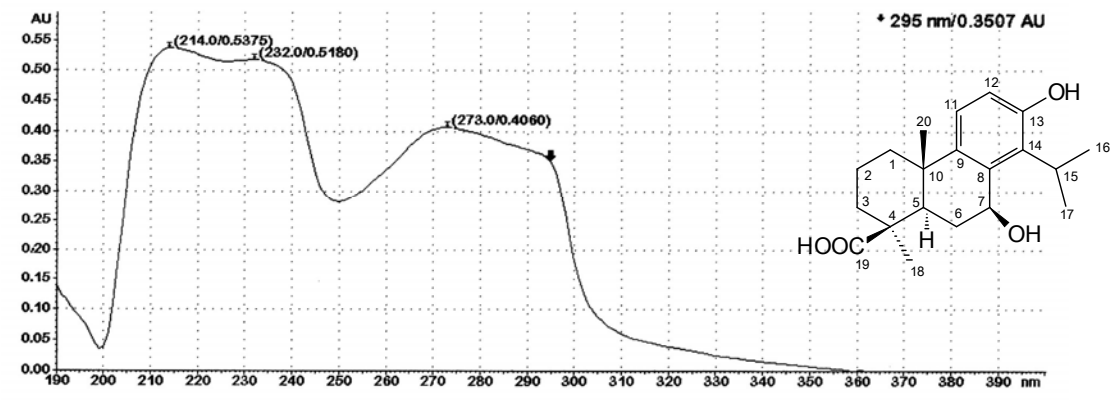


Figure A14 UV Spectrum of NW3 (Inumakiol D) in MeOH

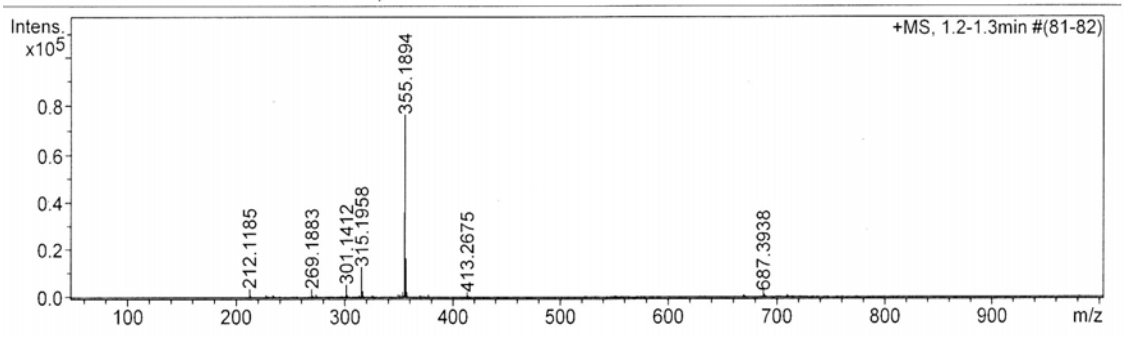


Figure A15 ESI Mass Spectrum of NW3 (Inumakiol D)

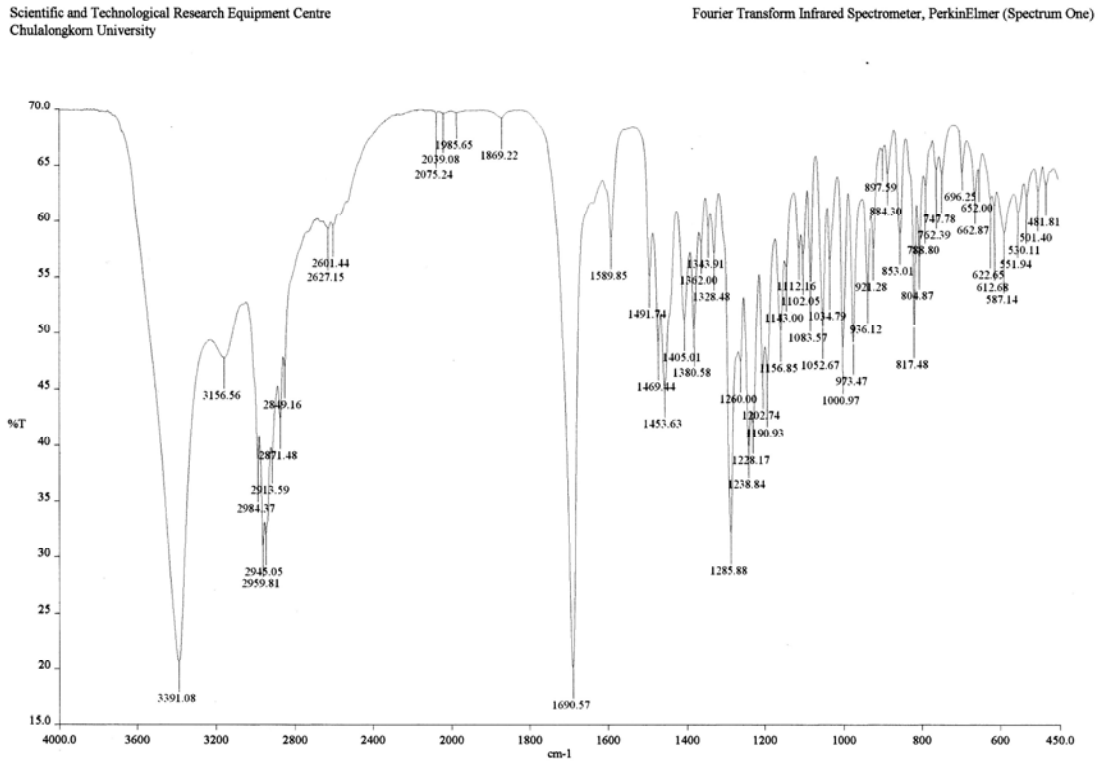


Figure A16 IR Spectrum of compound NW3 (Inumakiol D) (KBr)

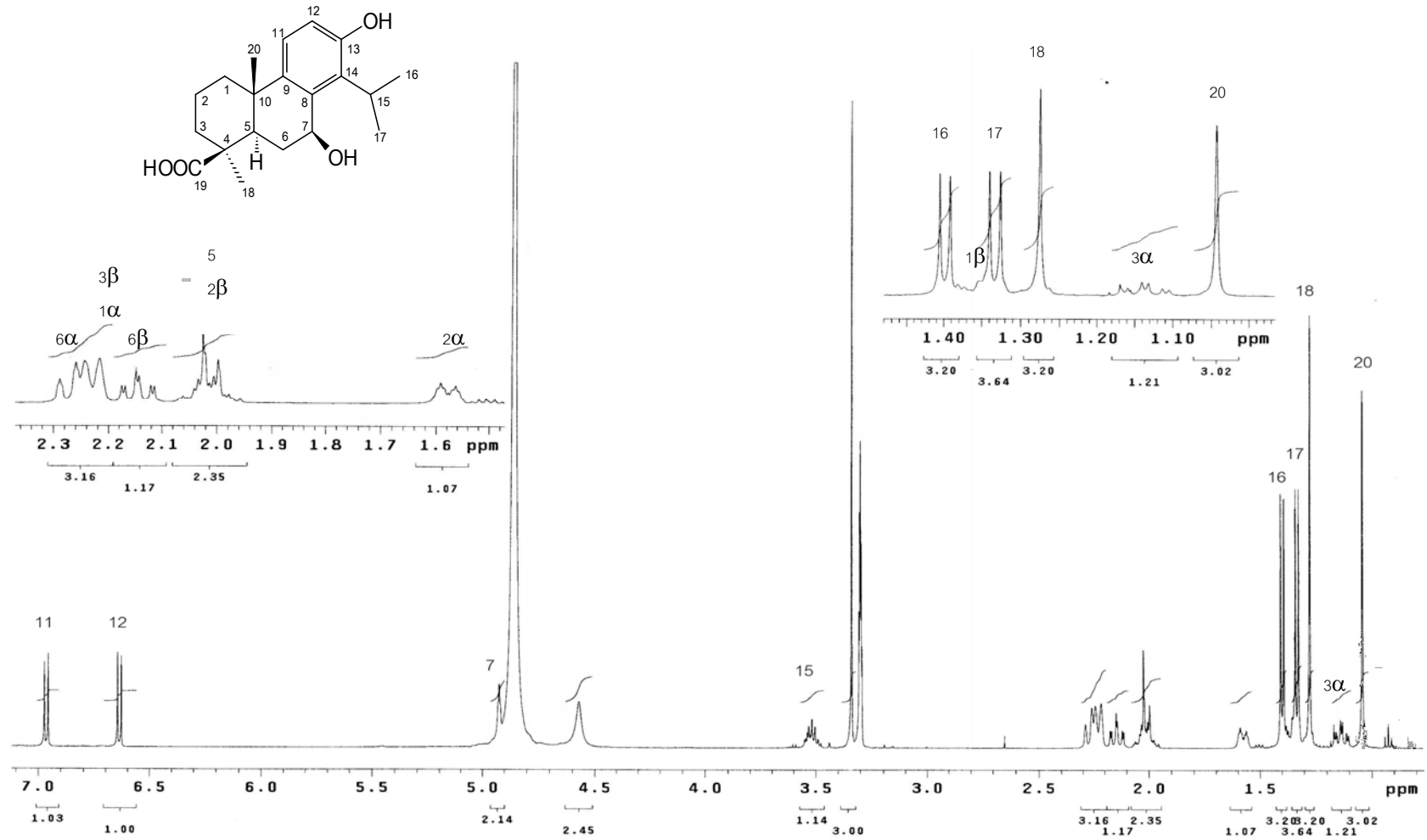


Figure A17 ^{1}H-NMR (500 MHz) Spectrum of compound NW3 (Inumakiol D) in CD_3OD

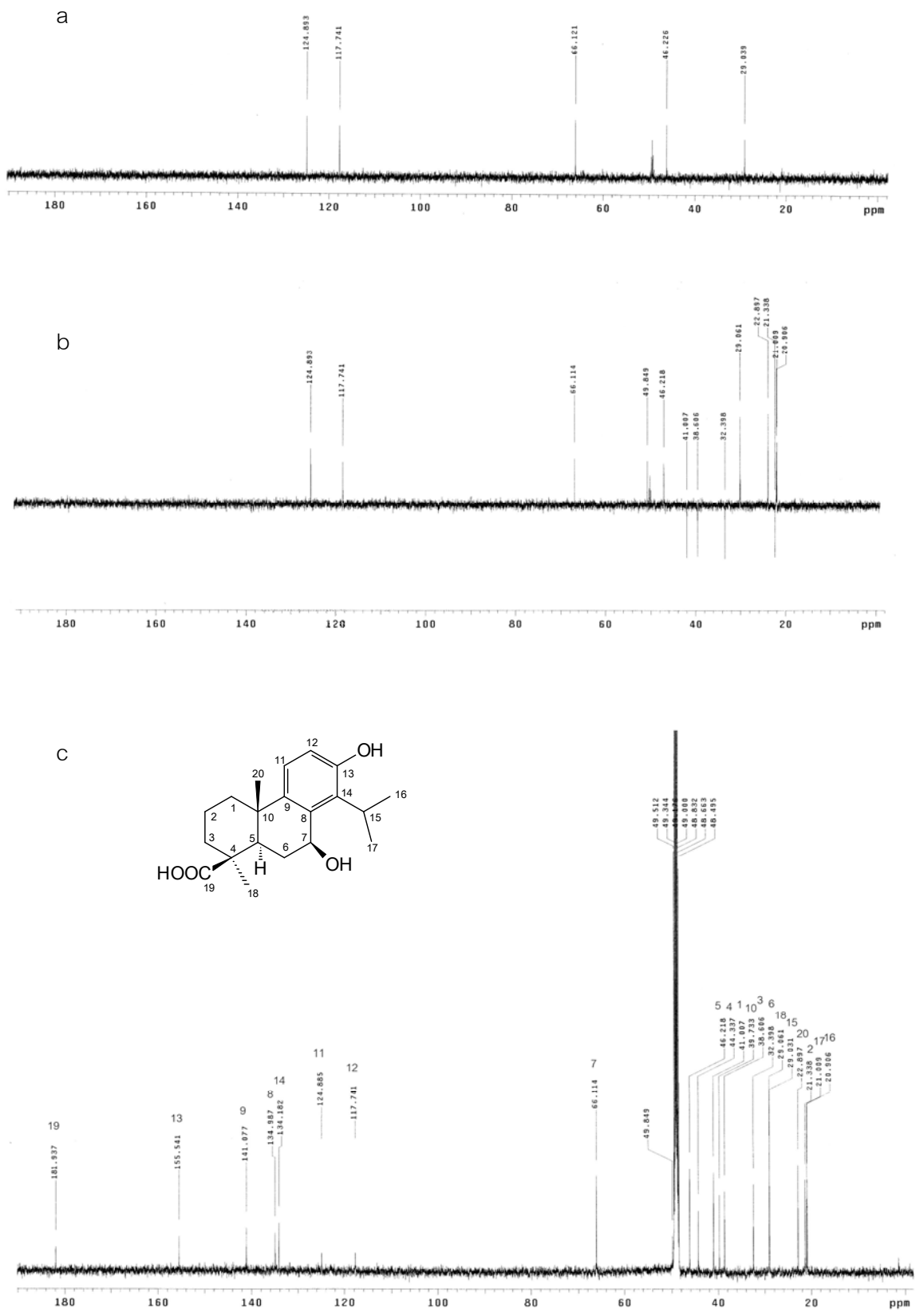


Figure A18 DEPT90° (a), DEPT135° (b), and ^{13}C-NMR (c) (125 MHz) Spectra of NW3 (Inumakiol D) in CD_3OD

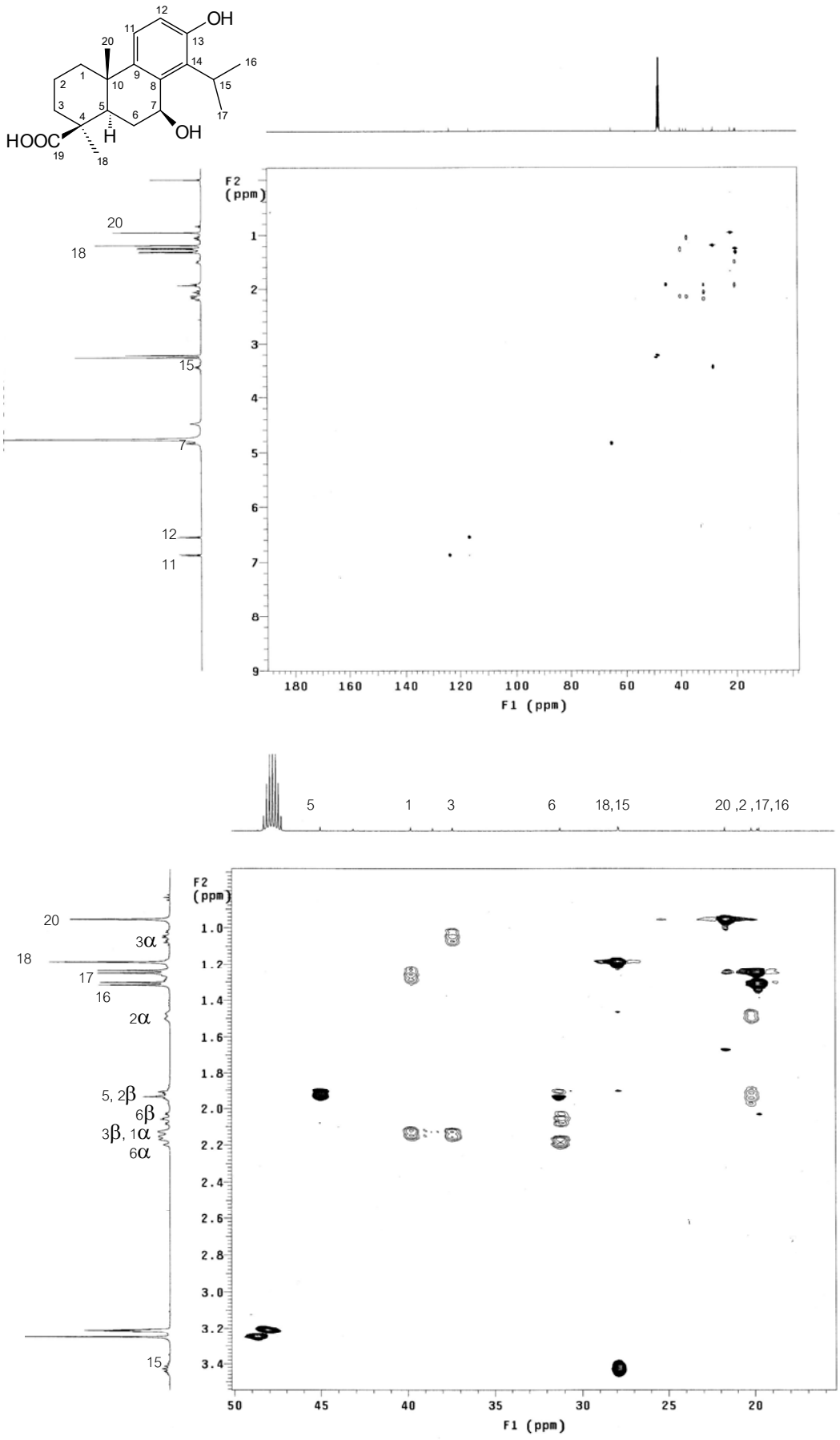


Figure A19 HSQC Spectra of NW3 (Inumakiol D) in CD_3OD

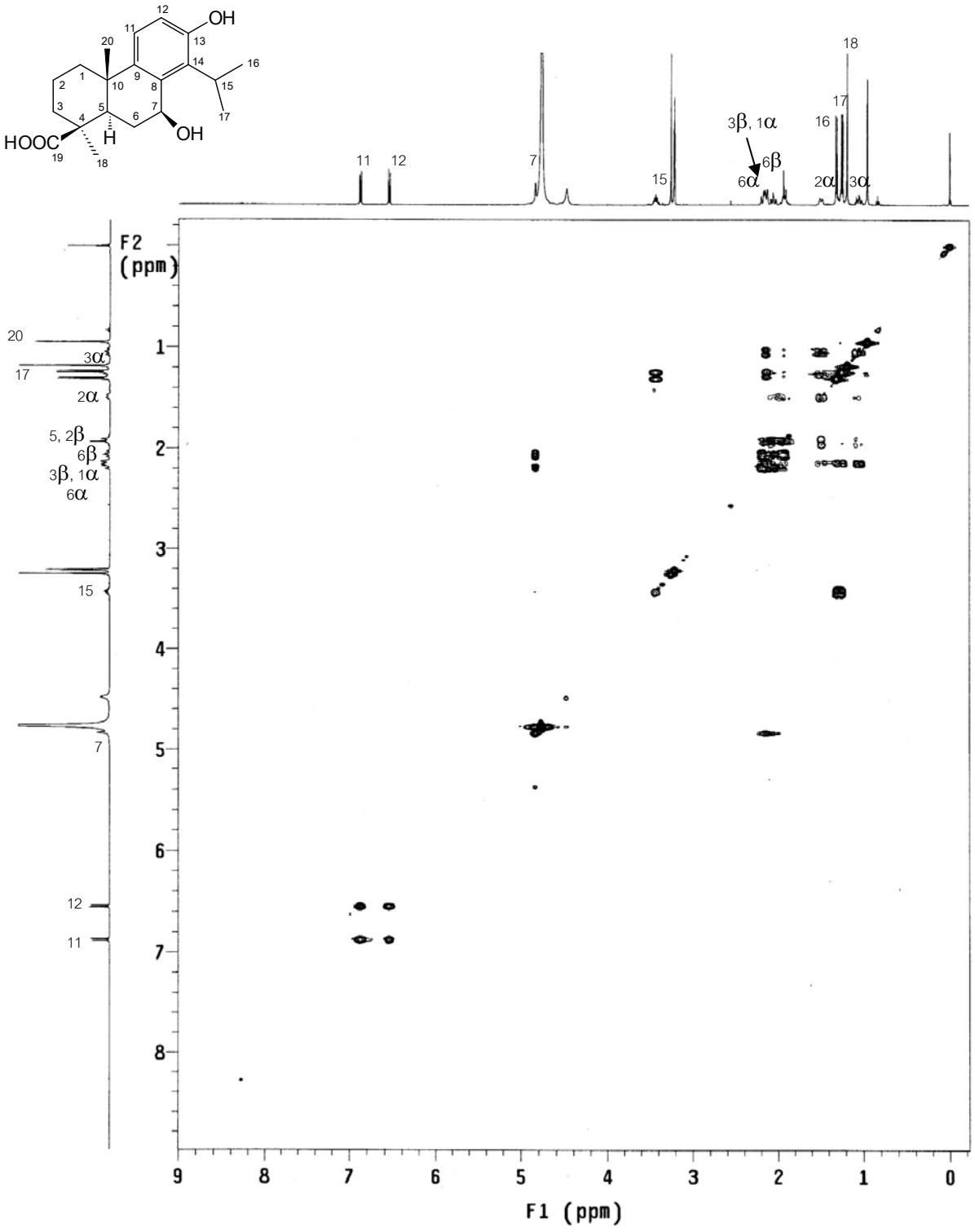


Figure A20a ^{1}H-^{1}H-COSY Spectra of NW3 (Inumakiol D) in CD_3OD

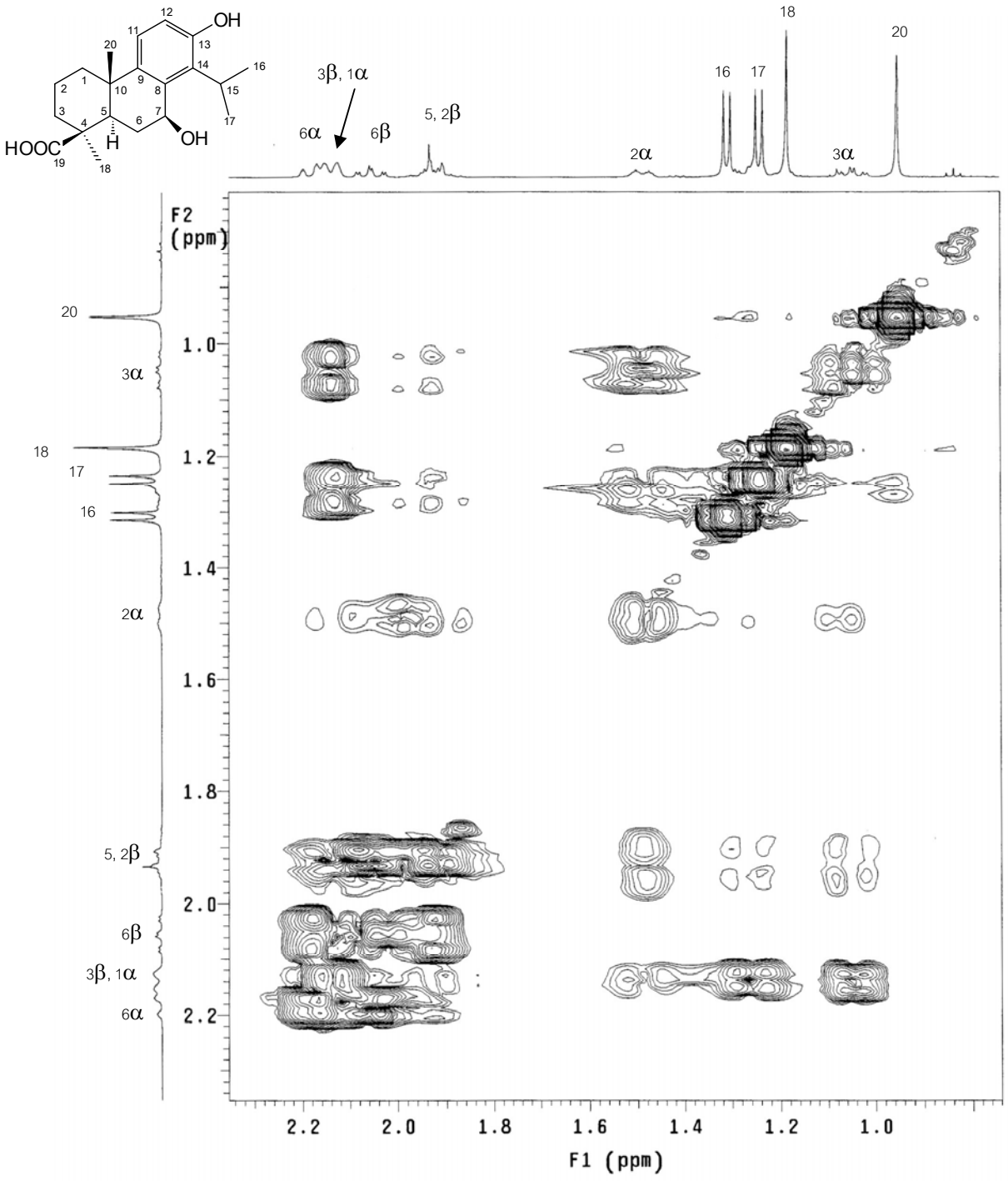


Figure A20b ^{1}H-^{1}H COSY Spectra of NW3 (Inumakiol D) in CD_3OD (continued)

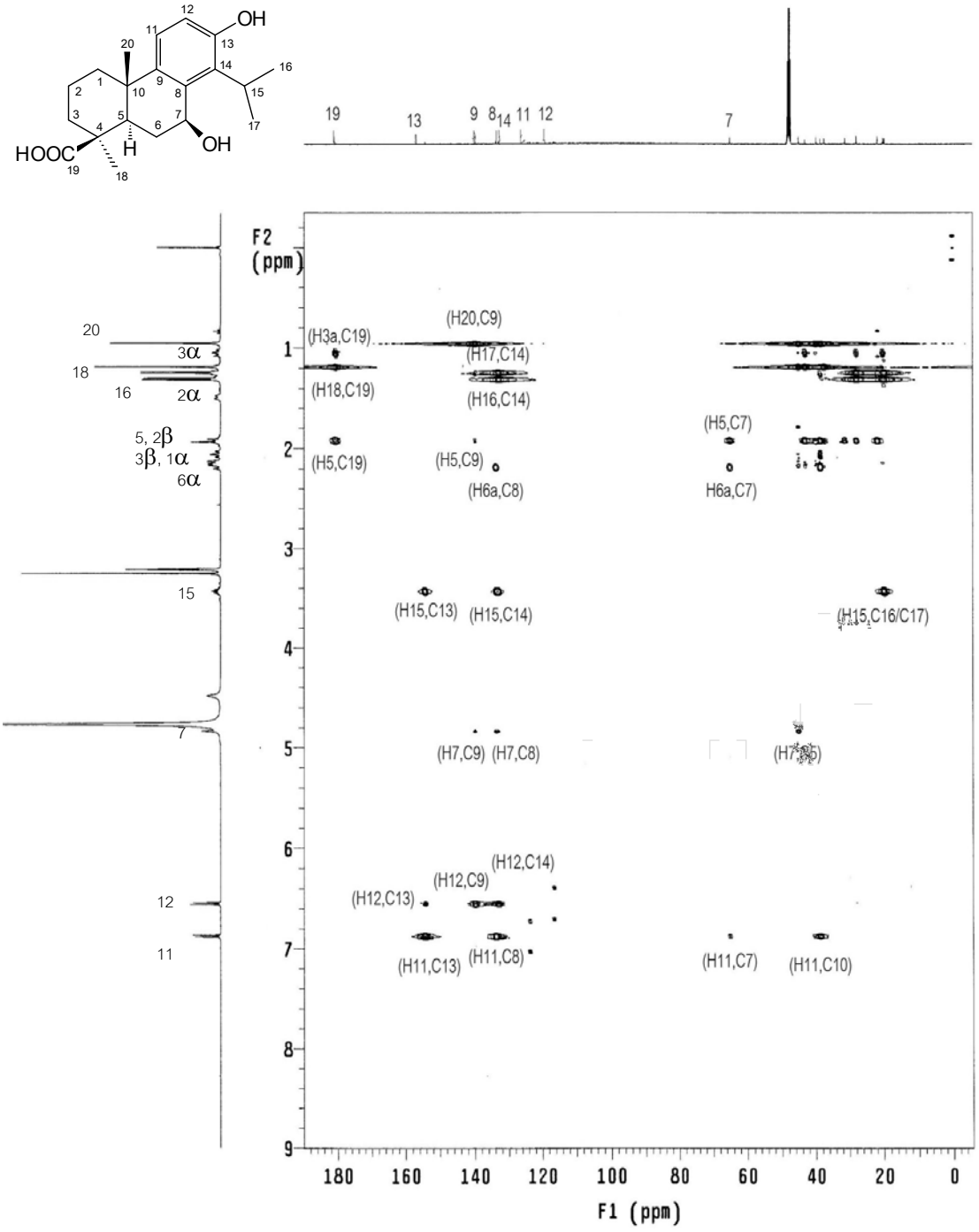


Figure A21a HMBC Spectra of NW3 (Inumakiol D) in CD_3OD

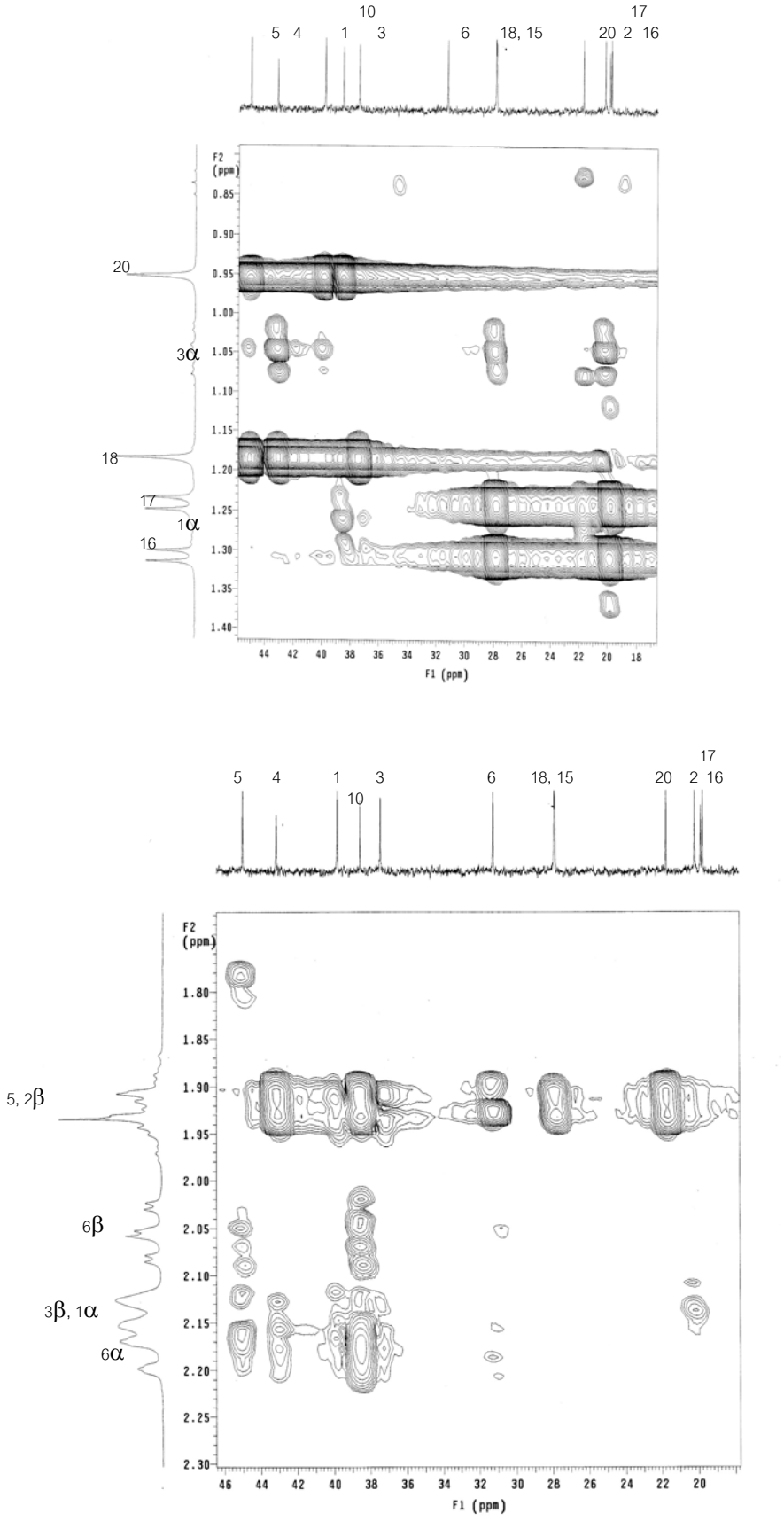


Figure A21b HMBC Spectra of NW3 (Inumakiol D) in CD_3OD (continued)

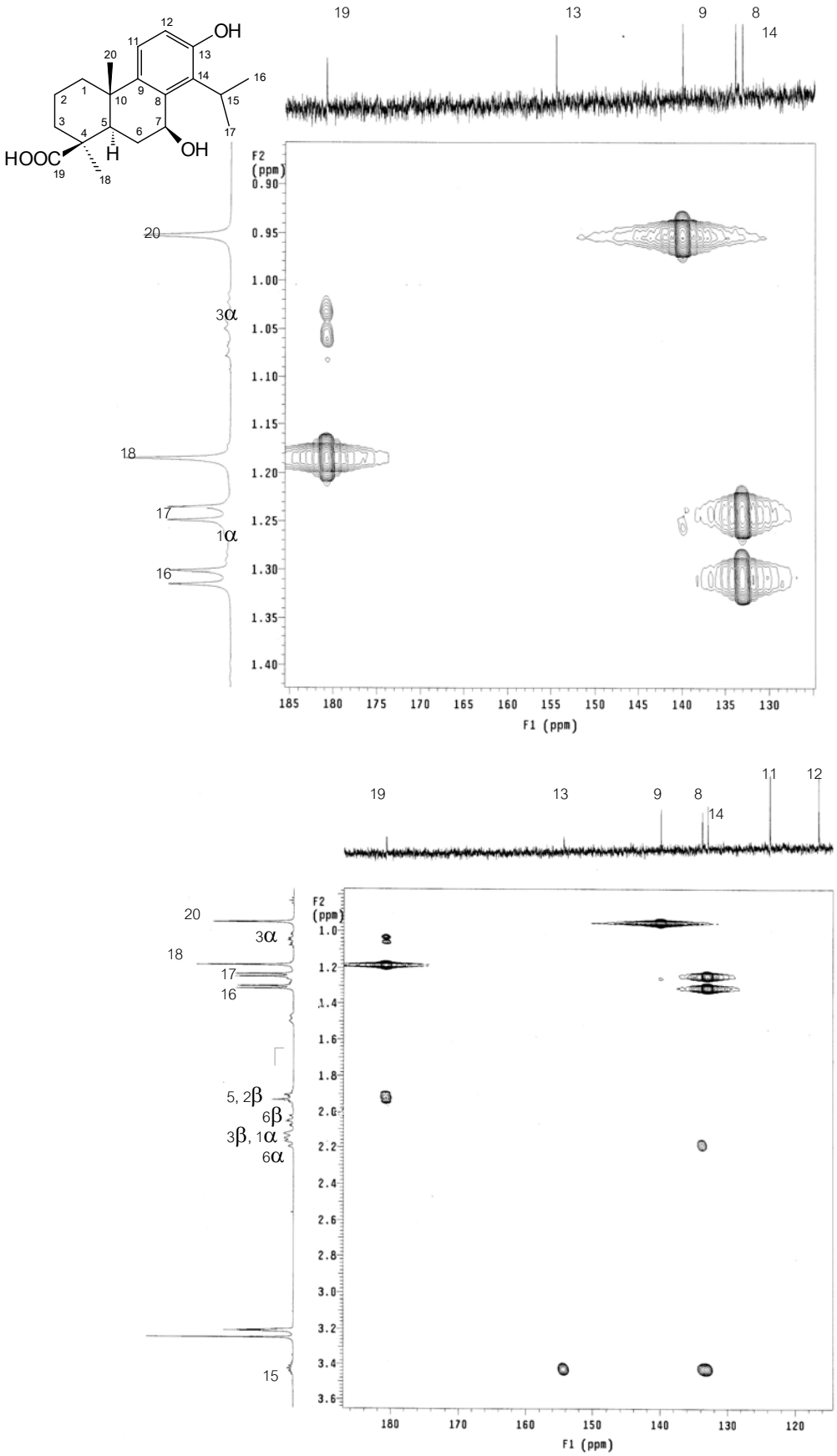


Figure A21c HMBC Spectra of NW3 (Inumakiol D) in CD_3OD (continued)

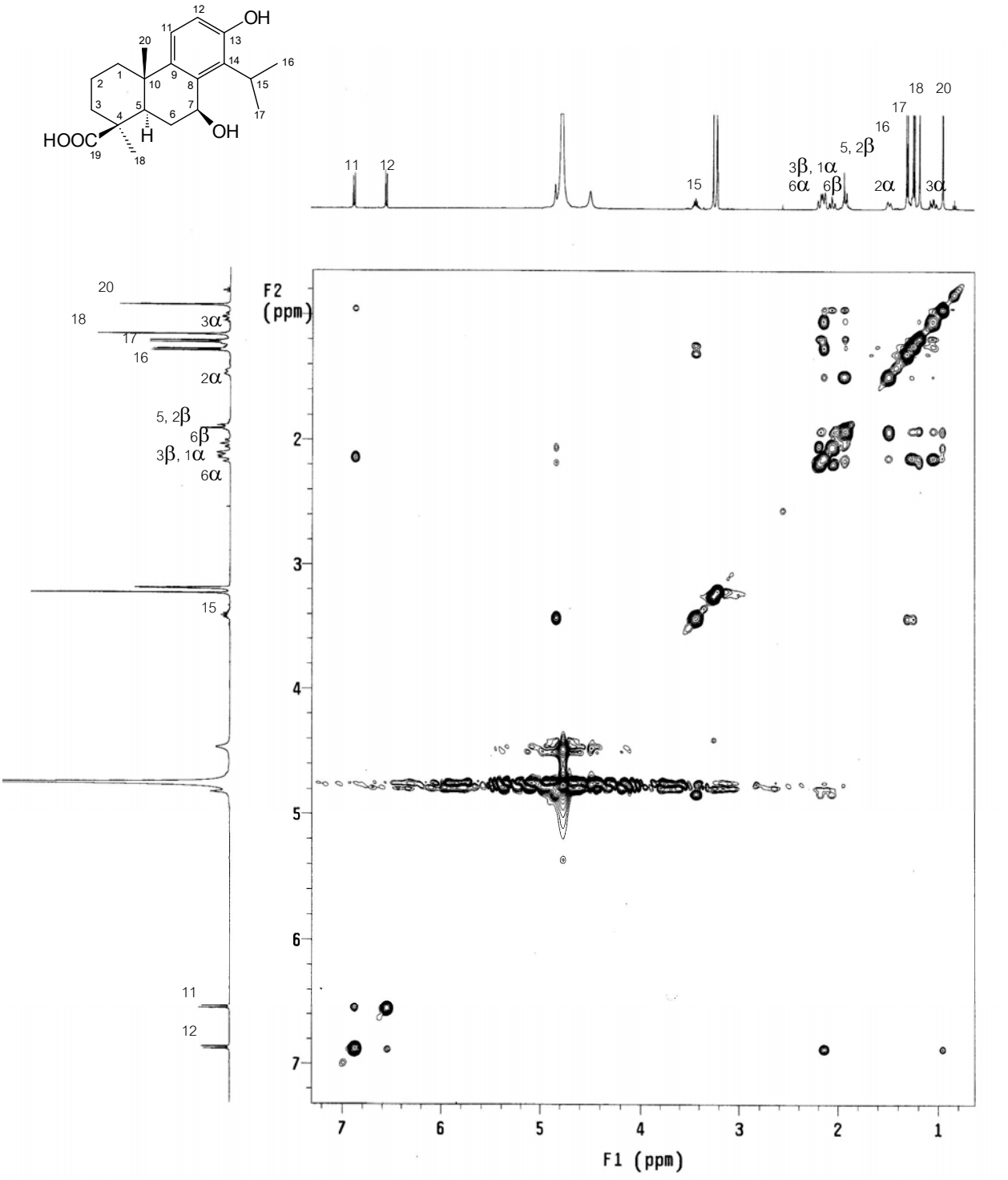


Figure A22a NOESY Spectra of NW3 (Inumakiol D) in CD_3OD

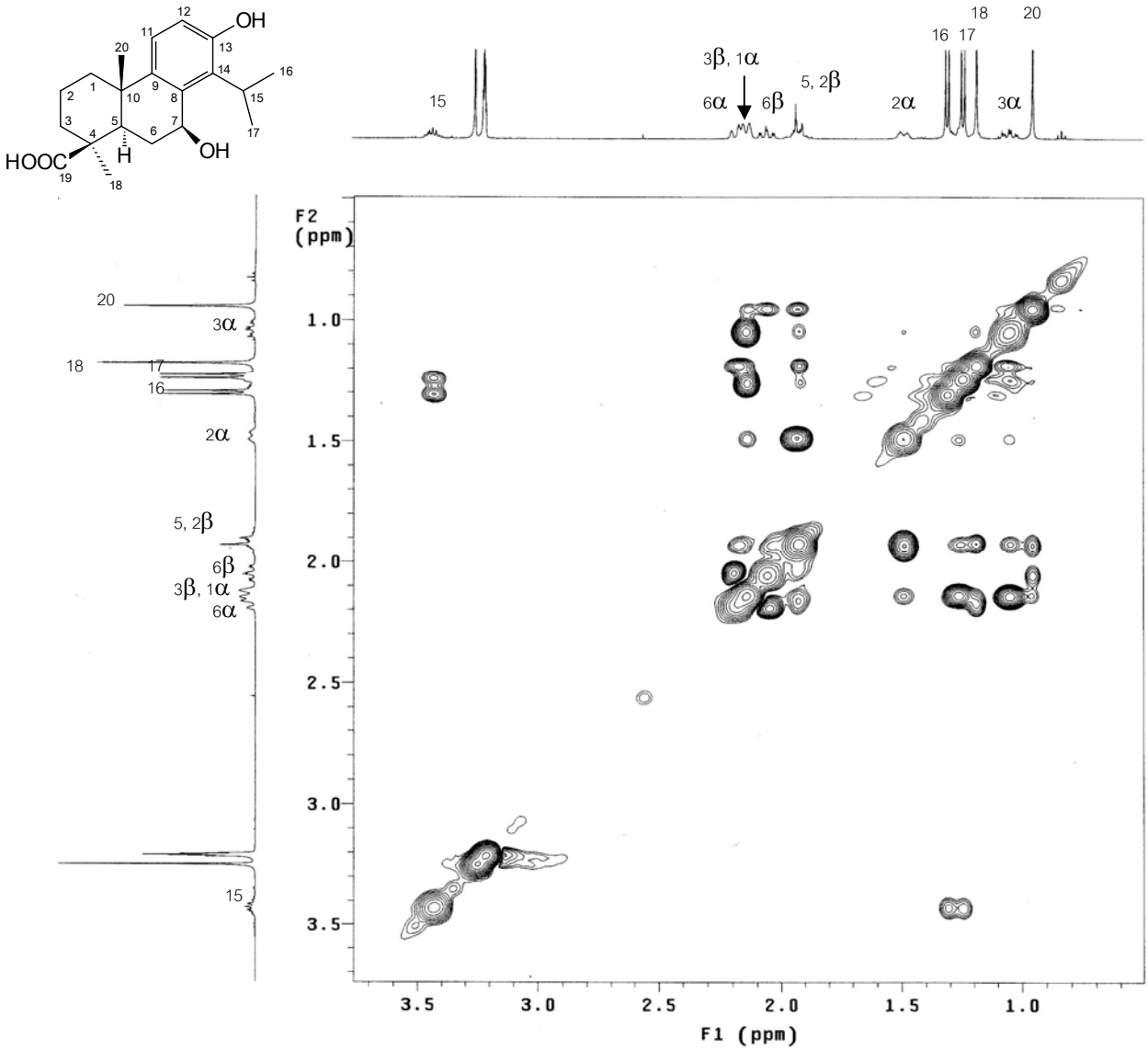


Figure A22b NOESY Spectra of NW3 (Inumakiol D) in CD_3OD (continued)

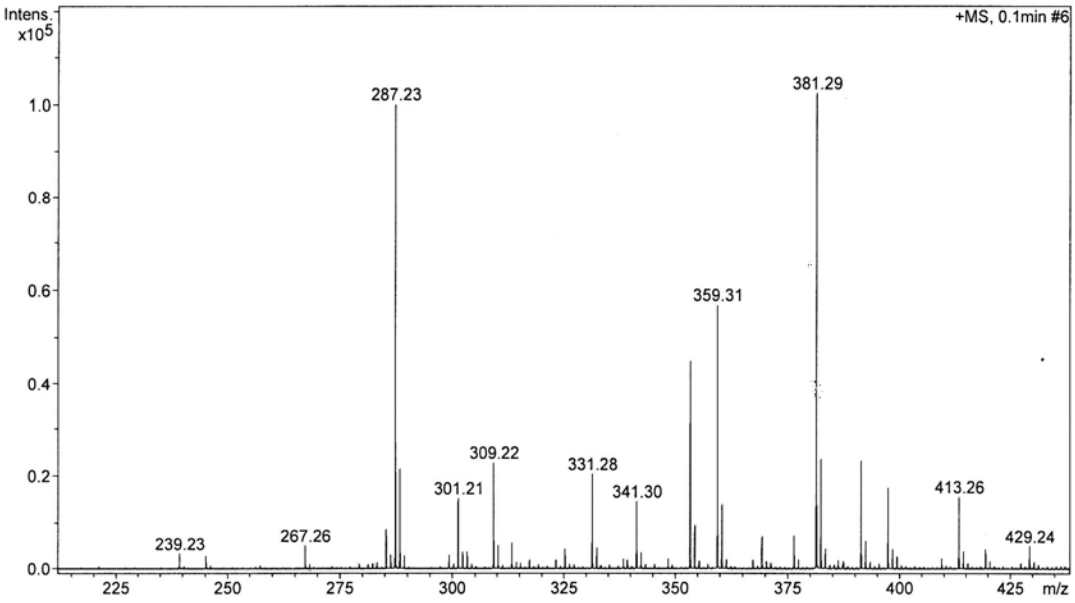


Figure A23 ESI Mass Spectrum of NW4 (Totarol)

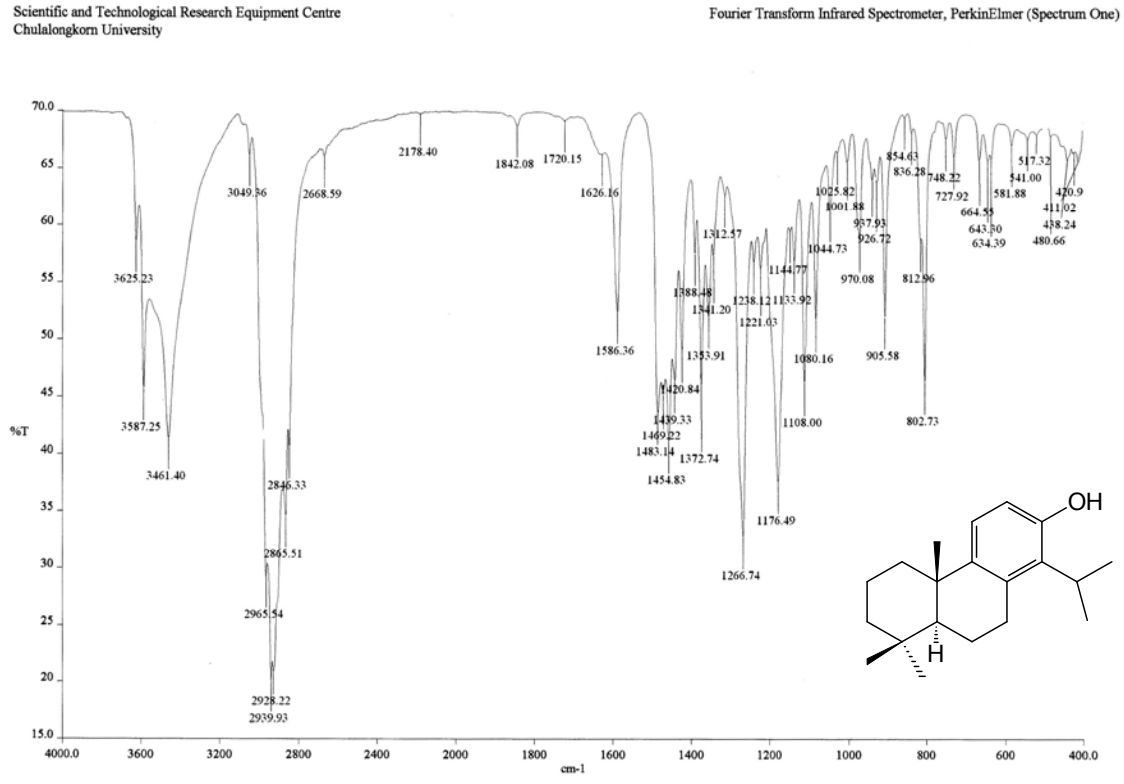


Figure A24 IR Spectrum of NW4 (Totarol) (KBr)

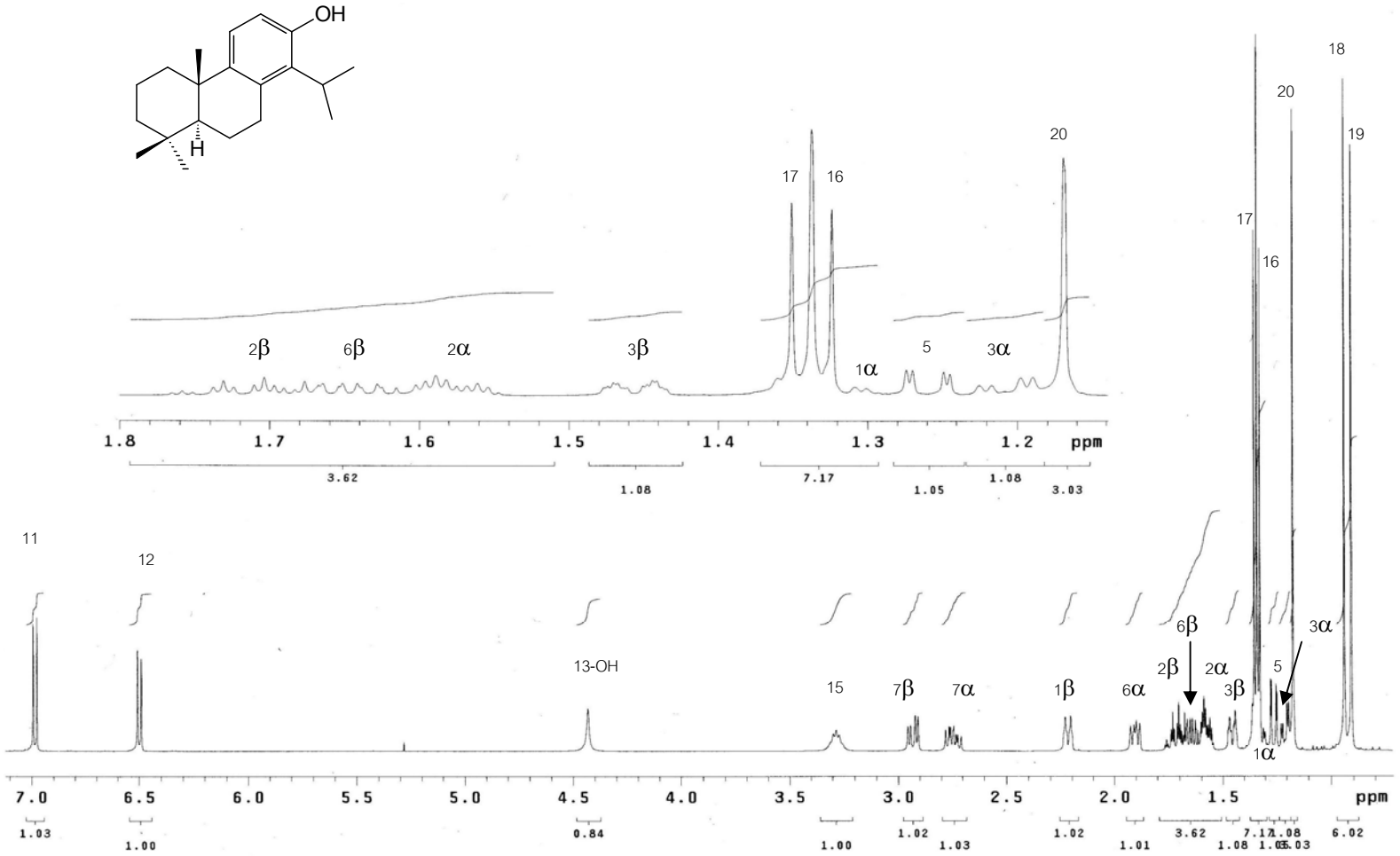


Figure A25 ^{1}H-NMR (500 MHz) Spectrum of NW4 (Totarol) in $CDCl_3$

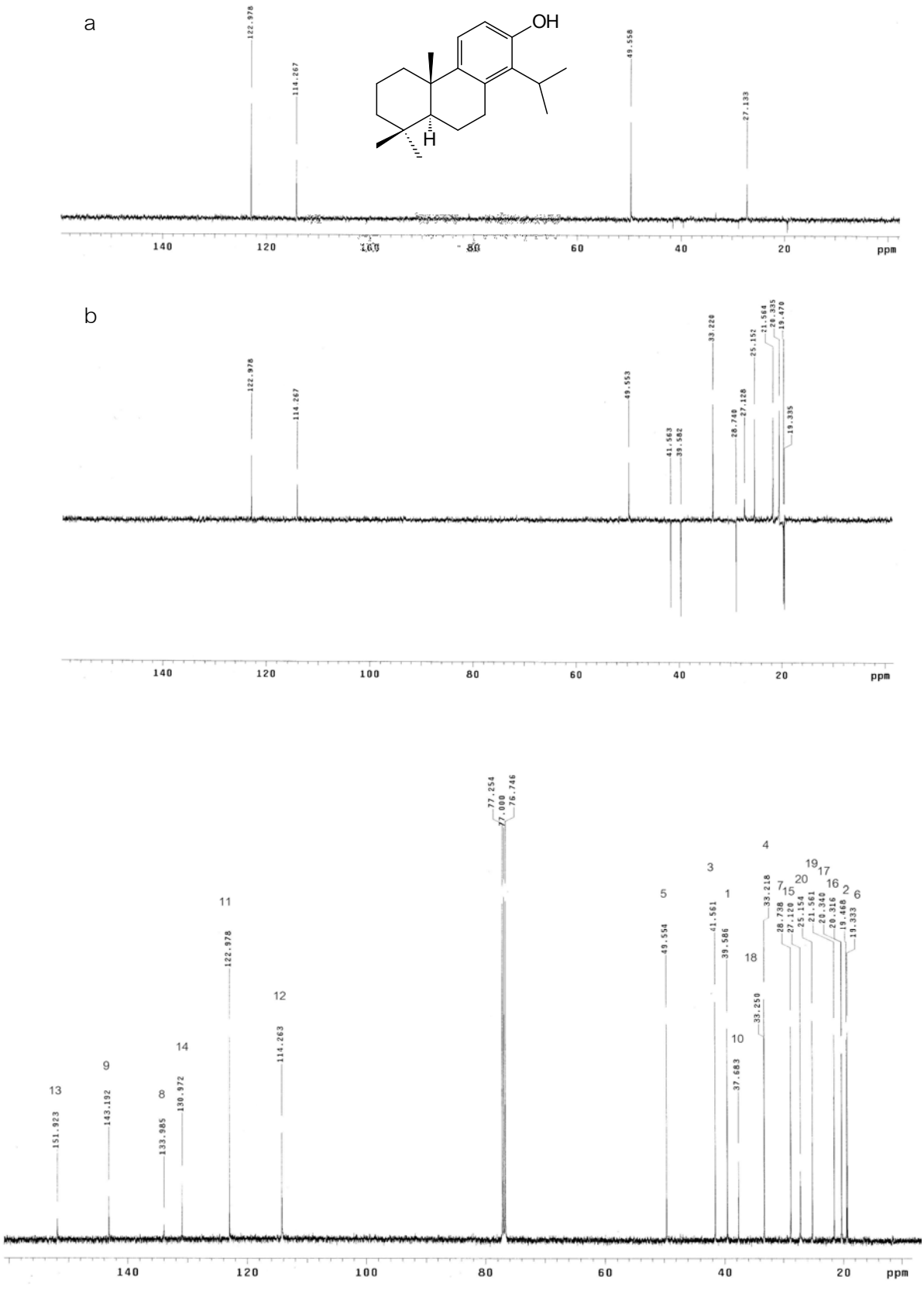


Figure A26 DEPT90° (a), DEPT135° (b), and ^{13}C-NMR (c) (125 MHz) Spectra of NW4 (Totarol) in $CDCl_3$

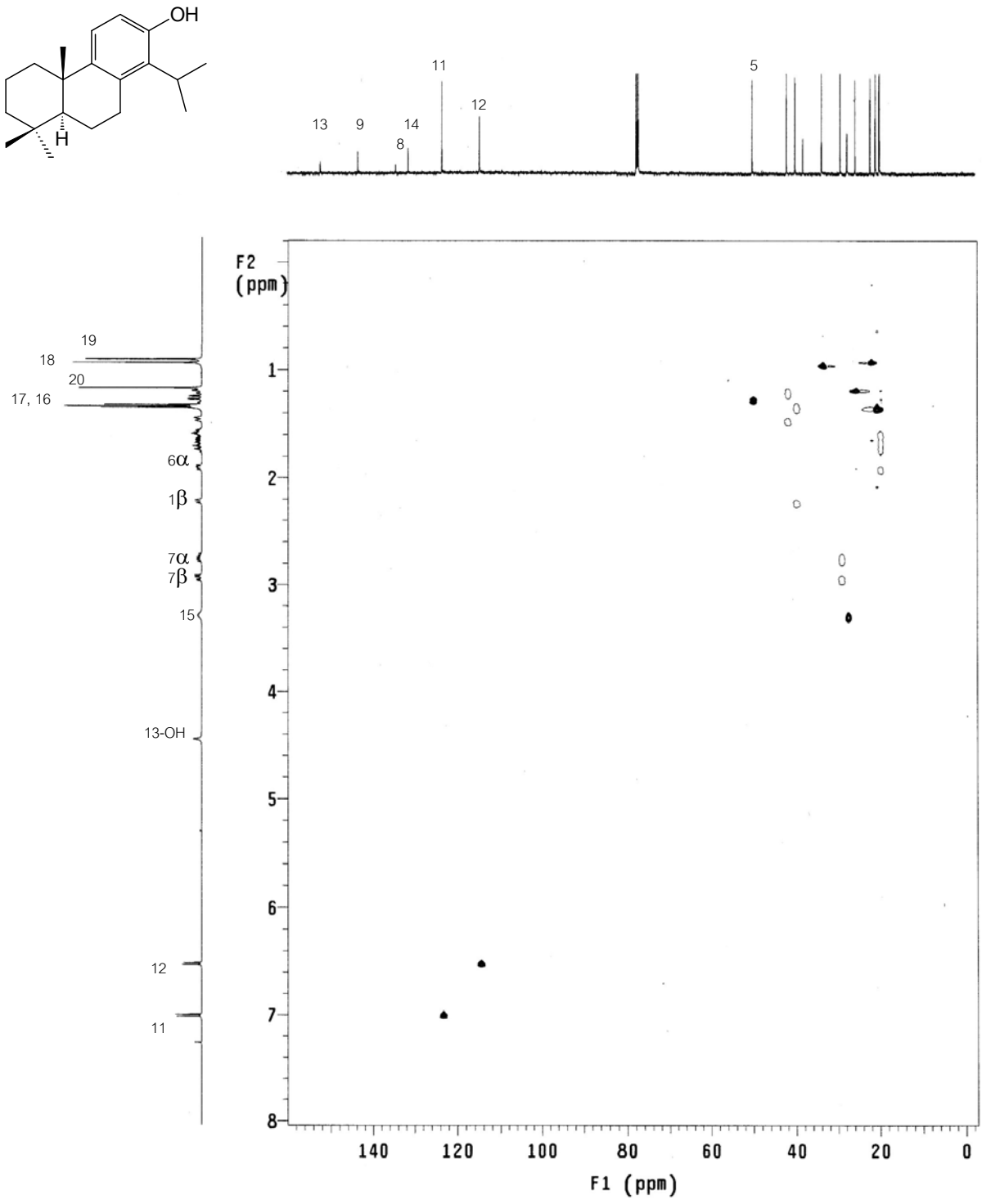


Figure A27a HSQC Spectra of NW4 (Totarol) in $CDCl_3$

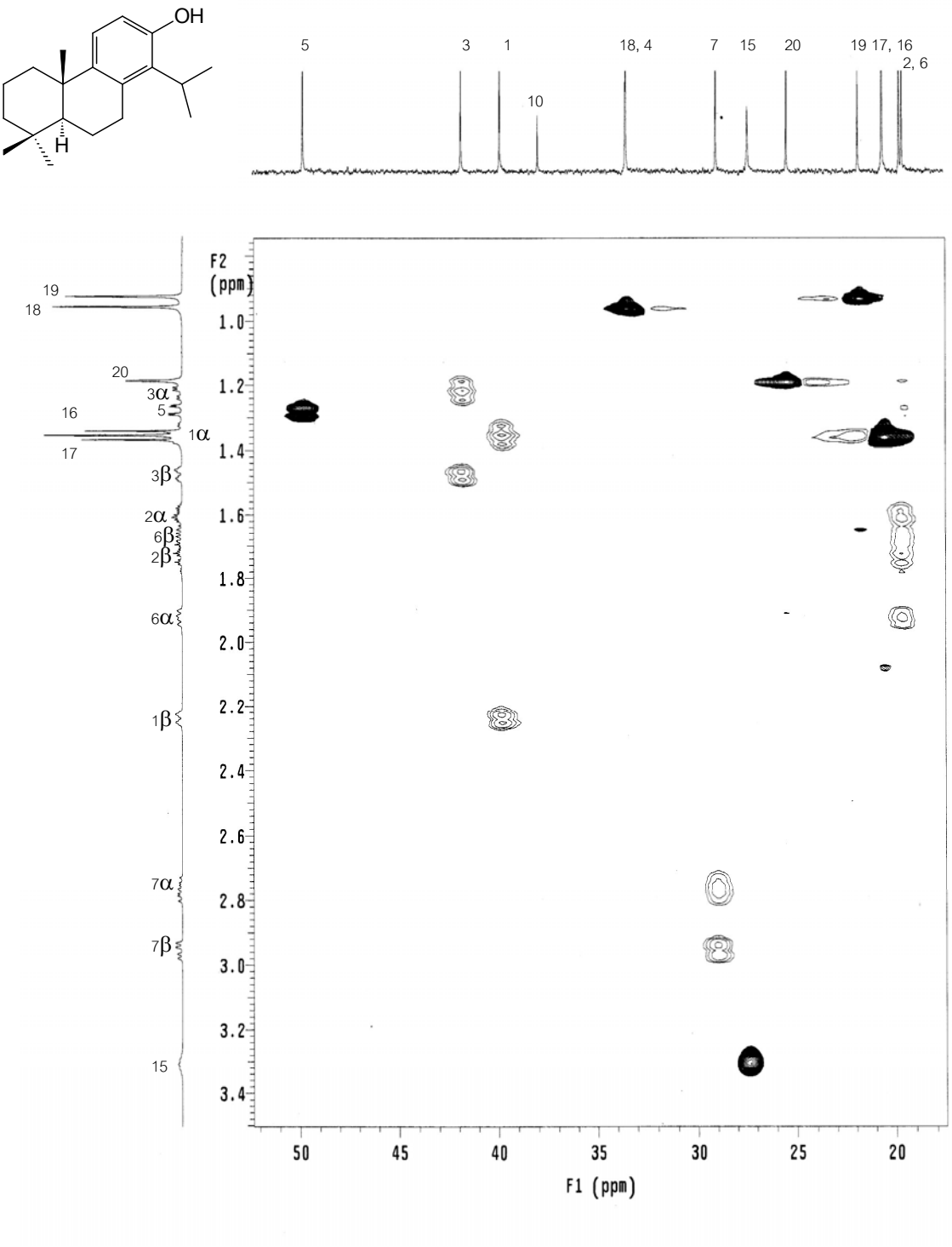


Figure A27b HSQC Spectra of NW4 (Totarol) in $CDCl_3$ (continued)

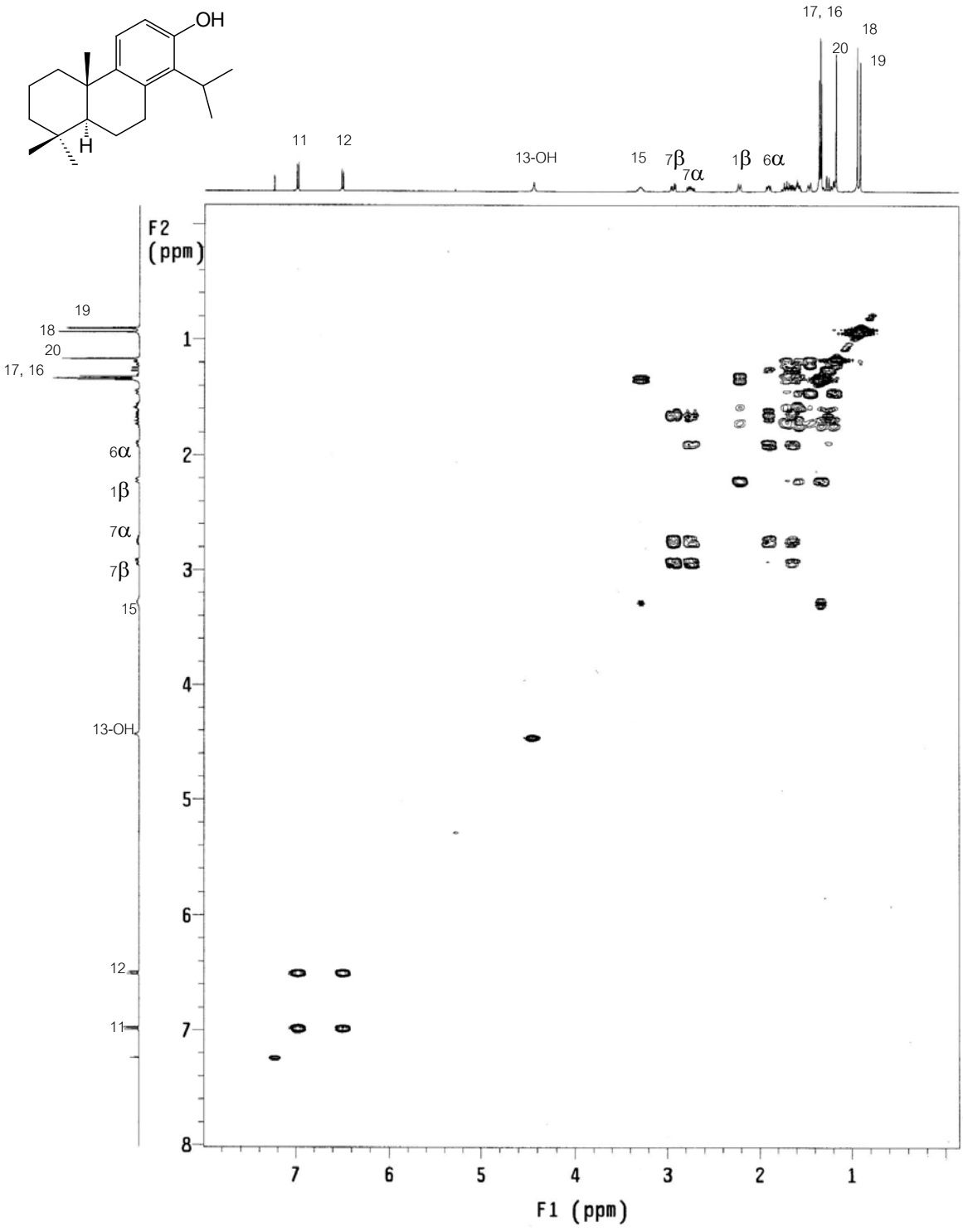


Figure A28a ^{1}H-^{1}H-COSY Spectra of NW4 (Totarol) in $CDCl_3$

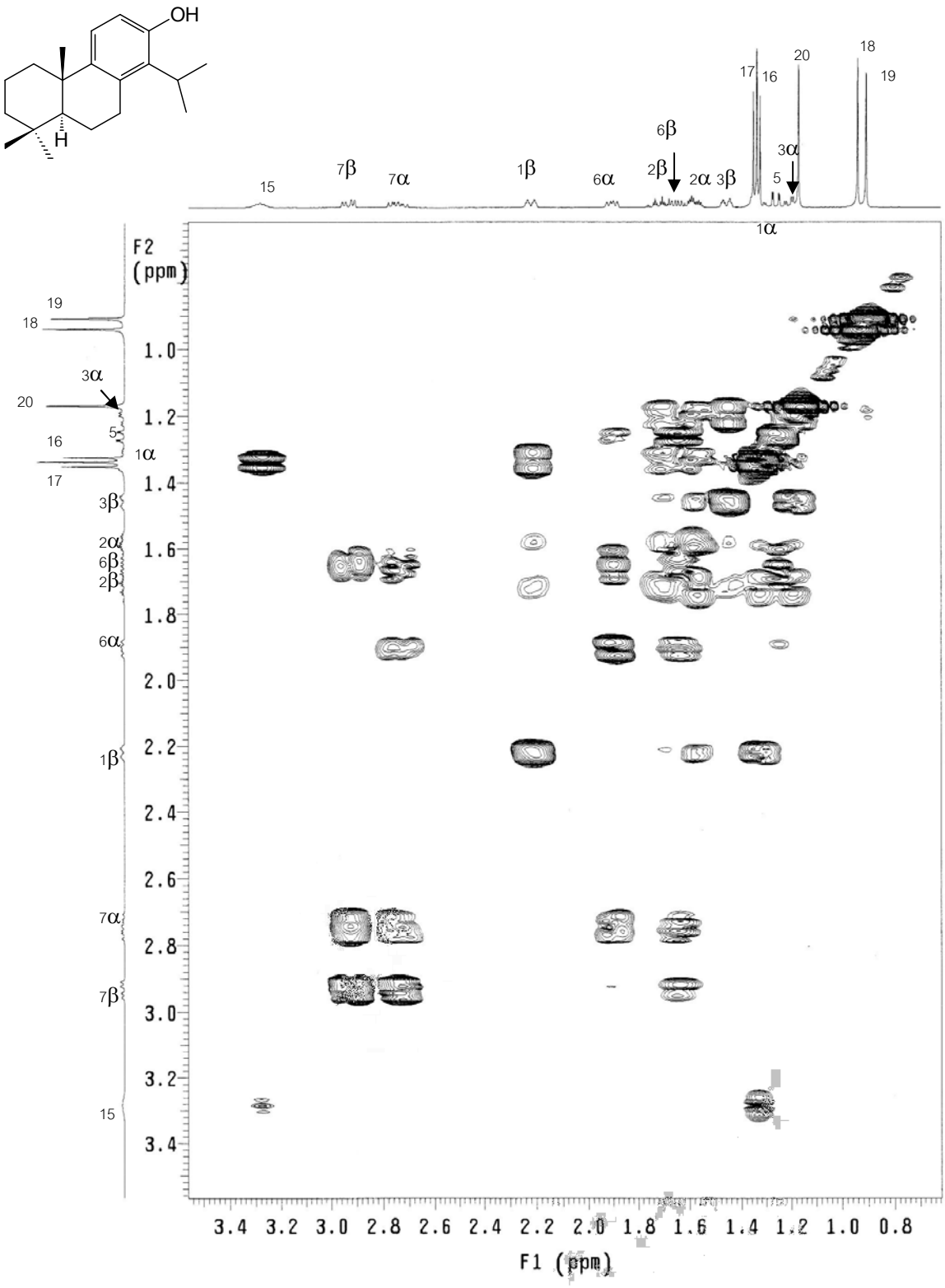


Figure A28b ^{1}H-^{1}H-COSY Spectra of NW4 (Totarol) in $CDCl_3$ (continued)

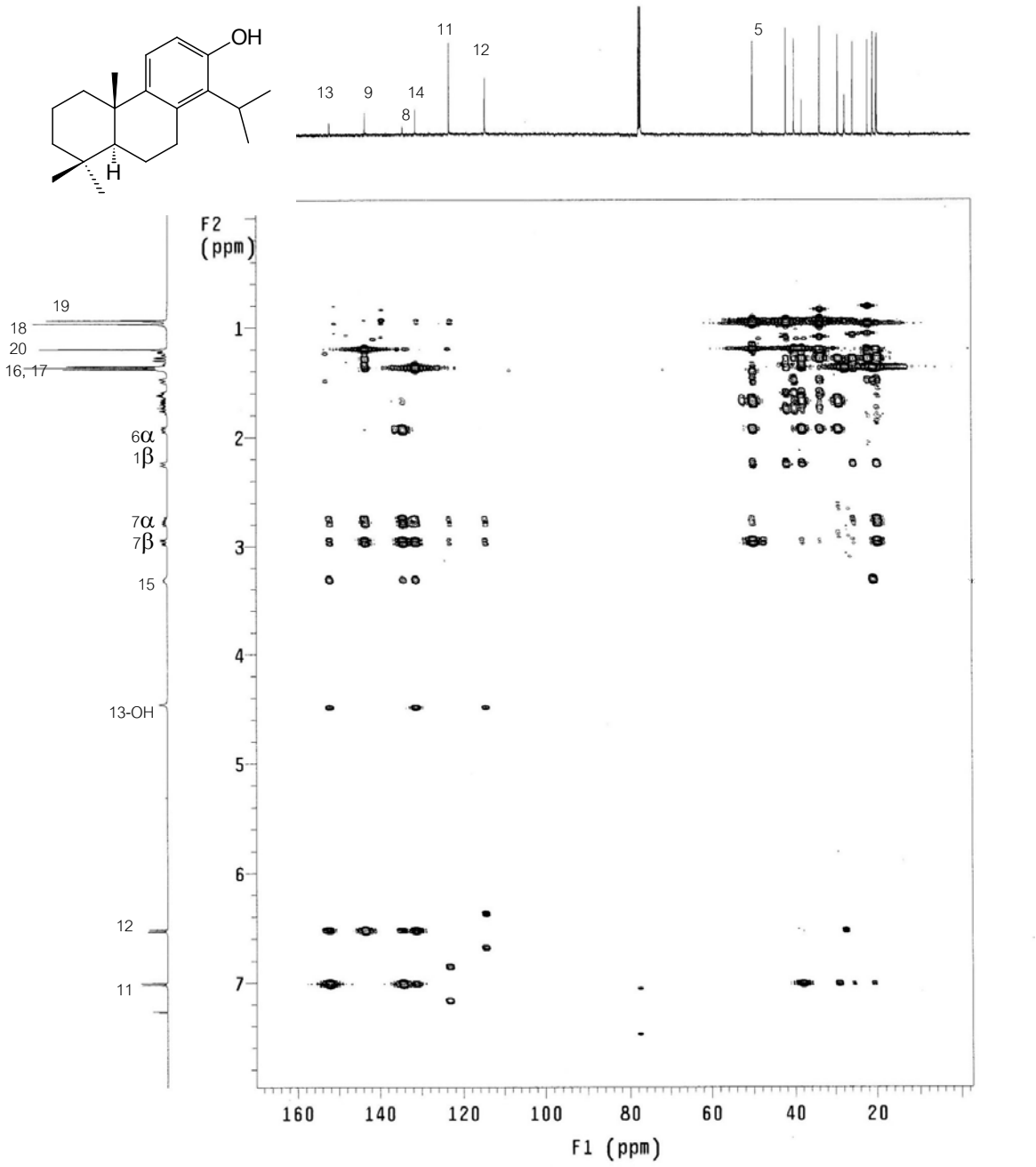


Figure A29a HMBC Spectra of NW4 (Totarol) in $CDCl_3$

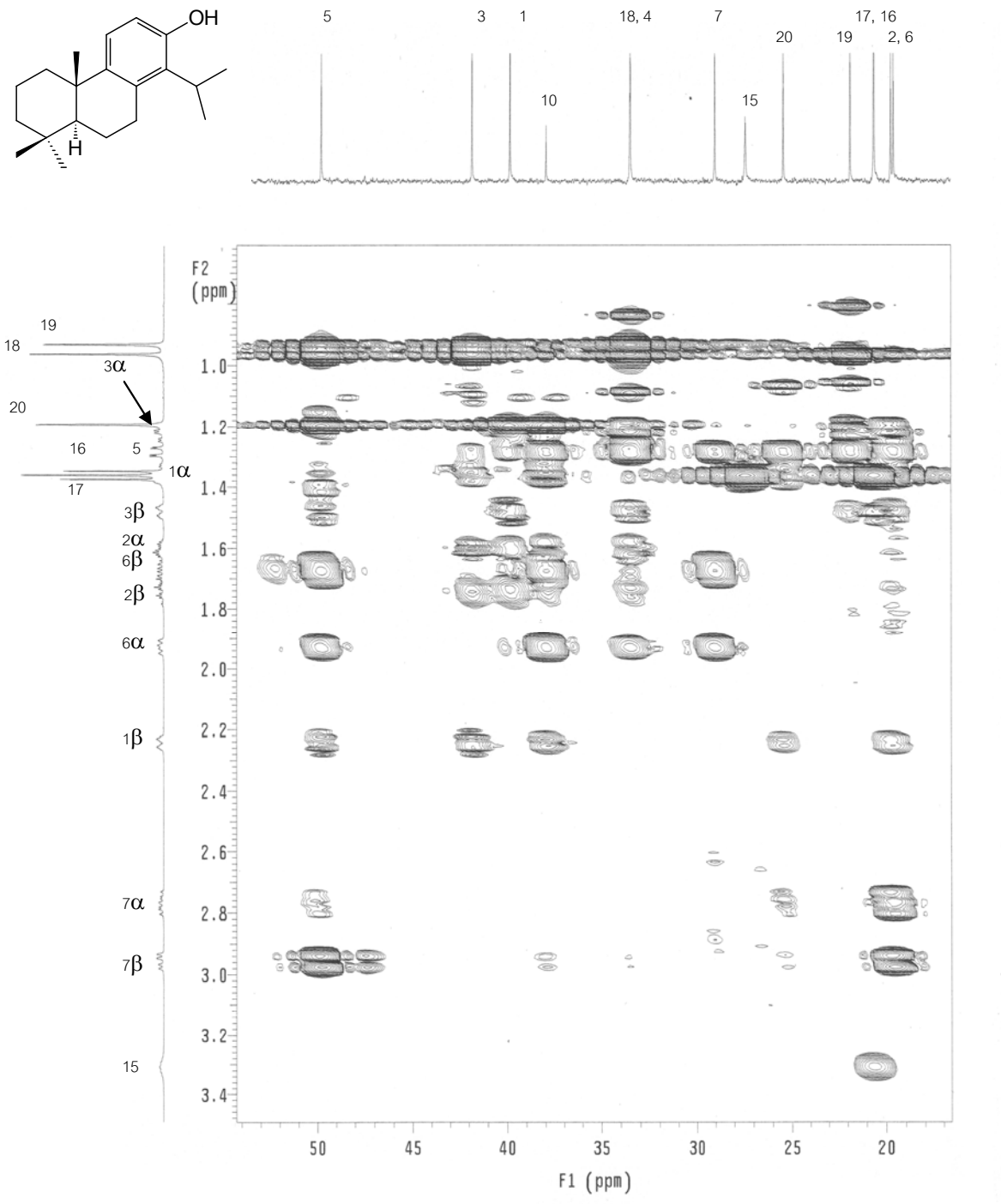


Figure A29b HMBC Spectra of NW4 (Totarol) in $CDCl_3$ (continued)

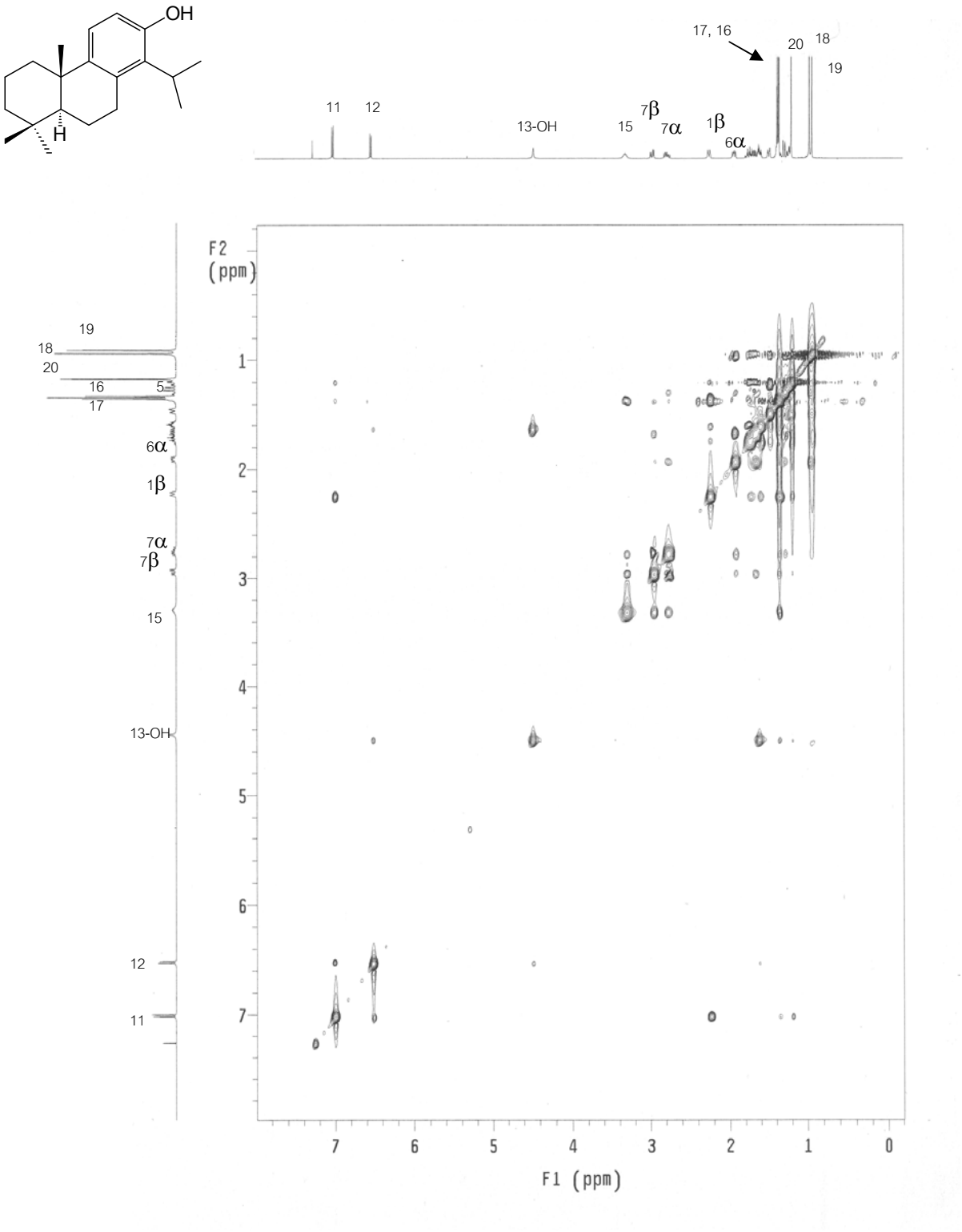


Figure A30a NOESY Spectrum of NW4 (Totarol) in $CDCl_3$

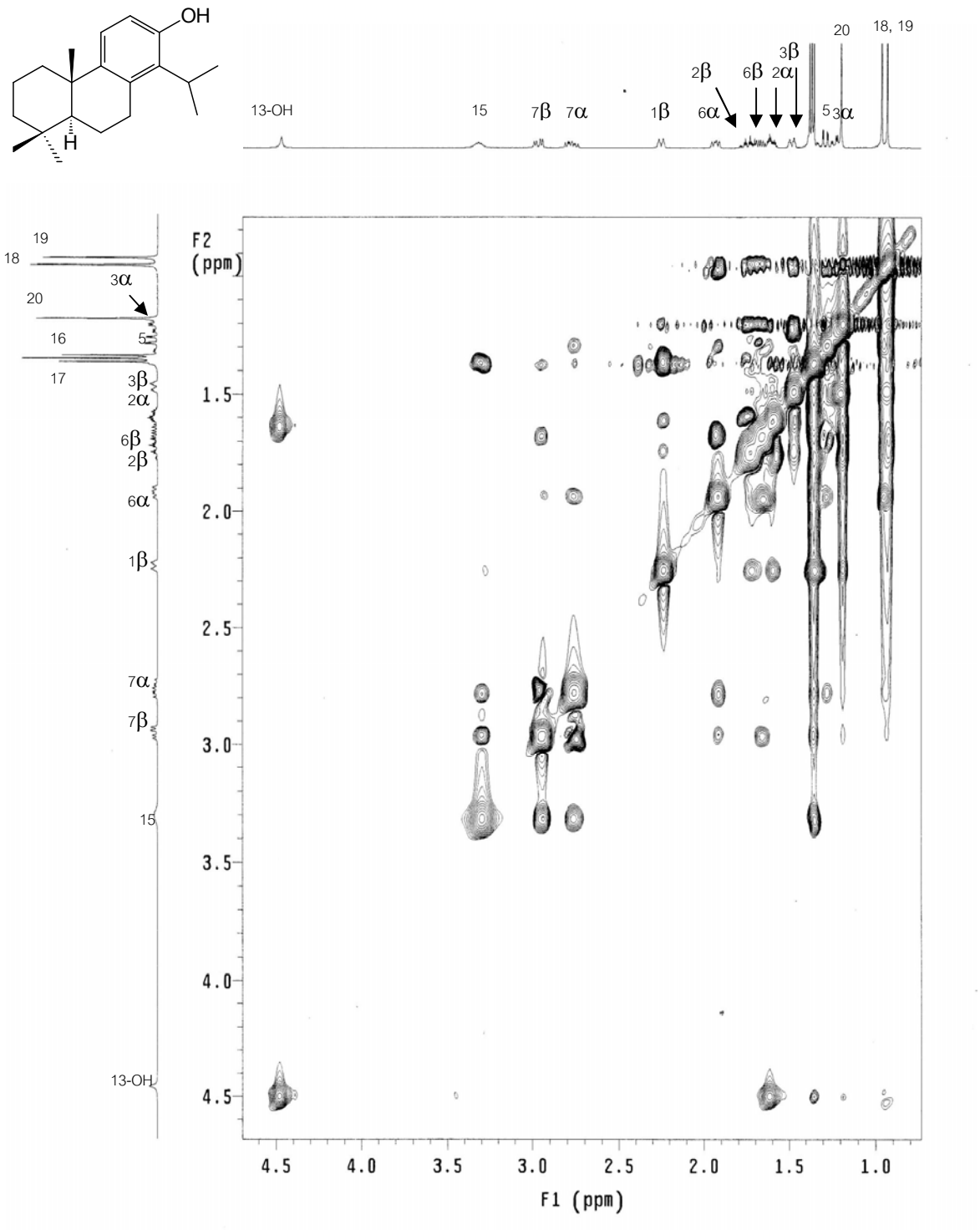


Figure A30b NOESY Spectrum of NW4 (Totarol) in $CDCl_3$ (continued)

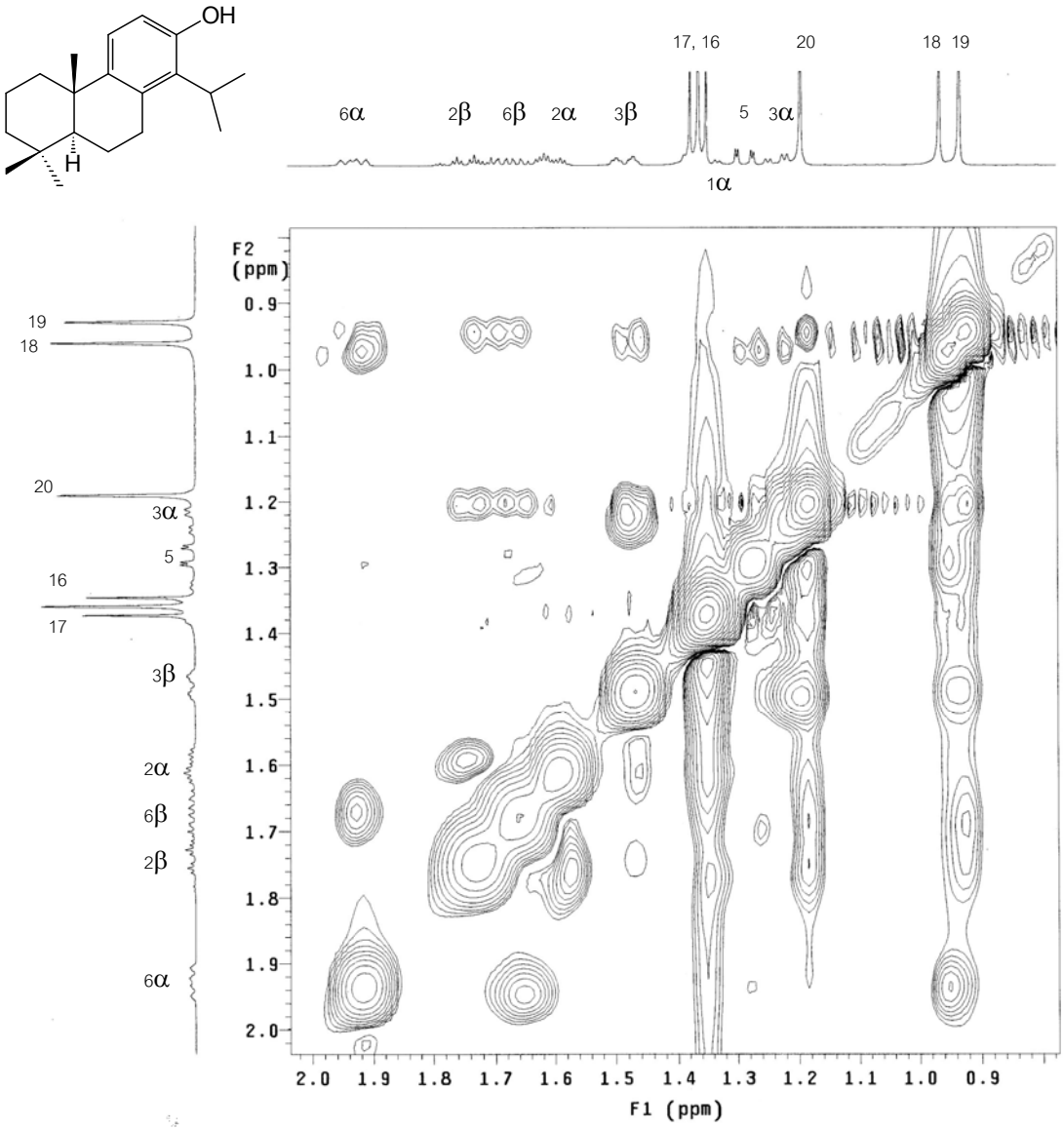


Figure A30c NOESY Spectrum of NW4 (Totarol) in $CDCl_3$ (continued)

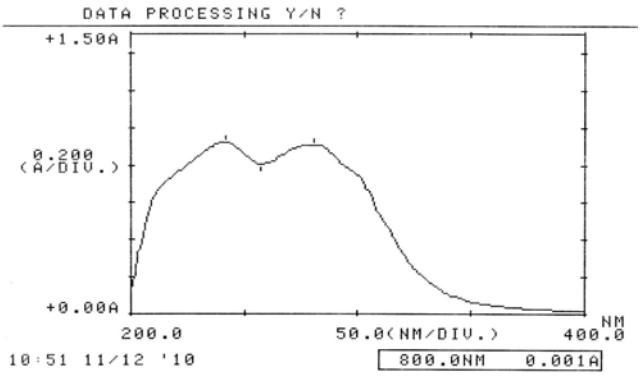


Figure A31 UV Spectrum of NW5 (Bis-2-ethylhexyl-phthalate) in MeOH

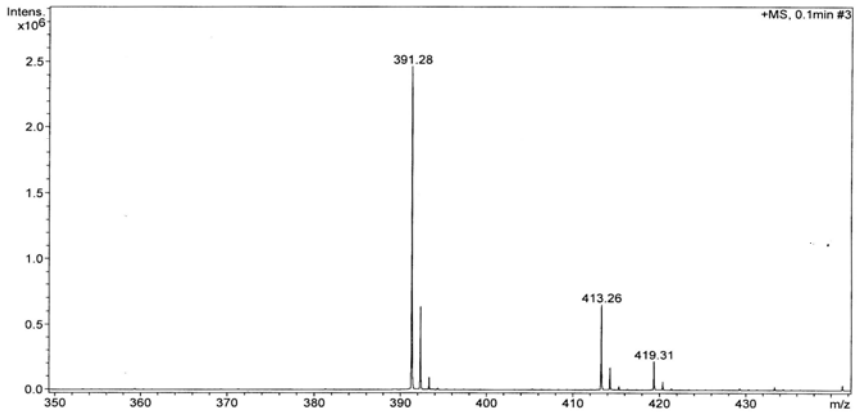


Figure A32 ESI Mass Spectrum of NW5 (Bis-2-ethylhexyl-phthalate)

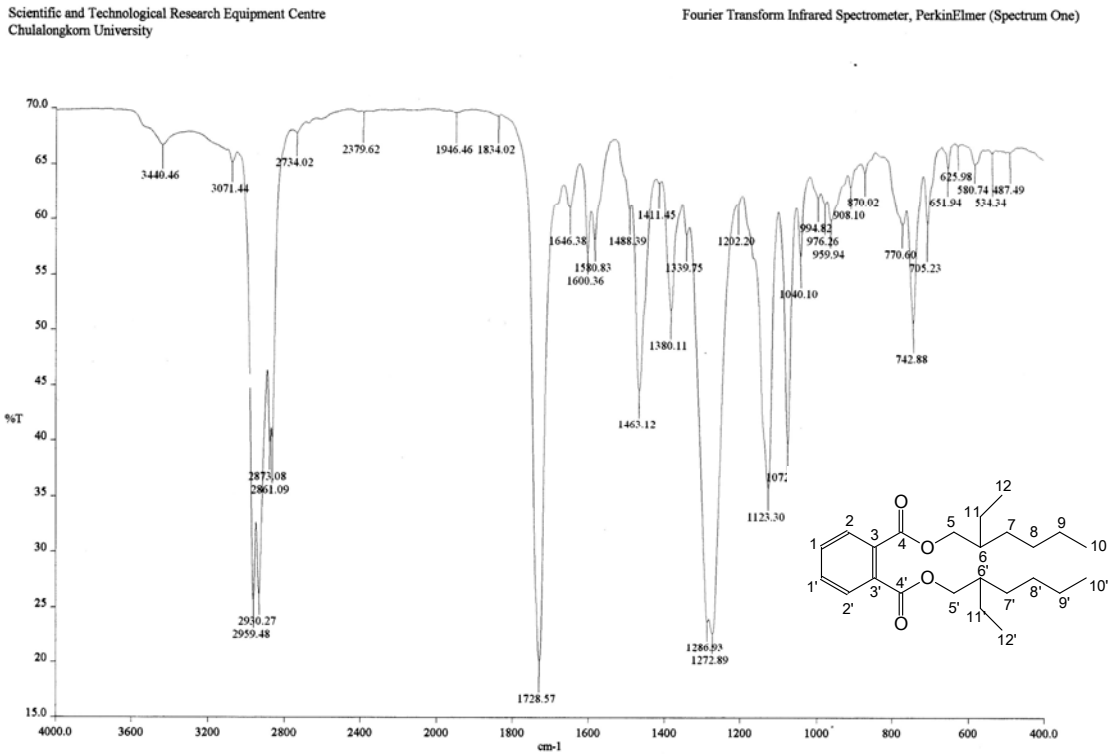


Figure A33 IR Spectrum of NW5 (Bis-2-ethylhexyl-phthalate) (KBr)

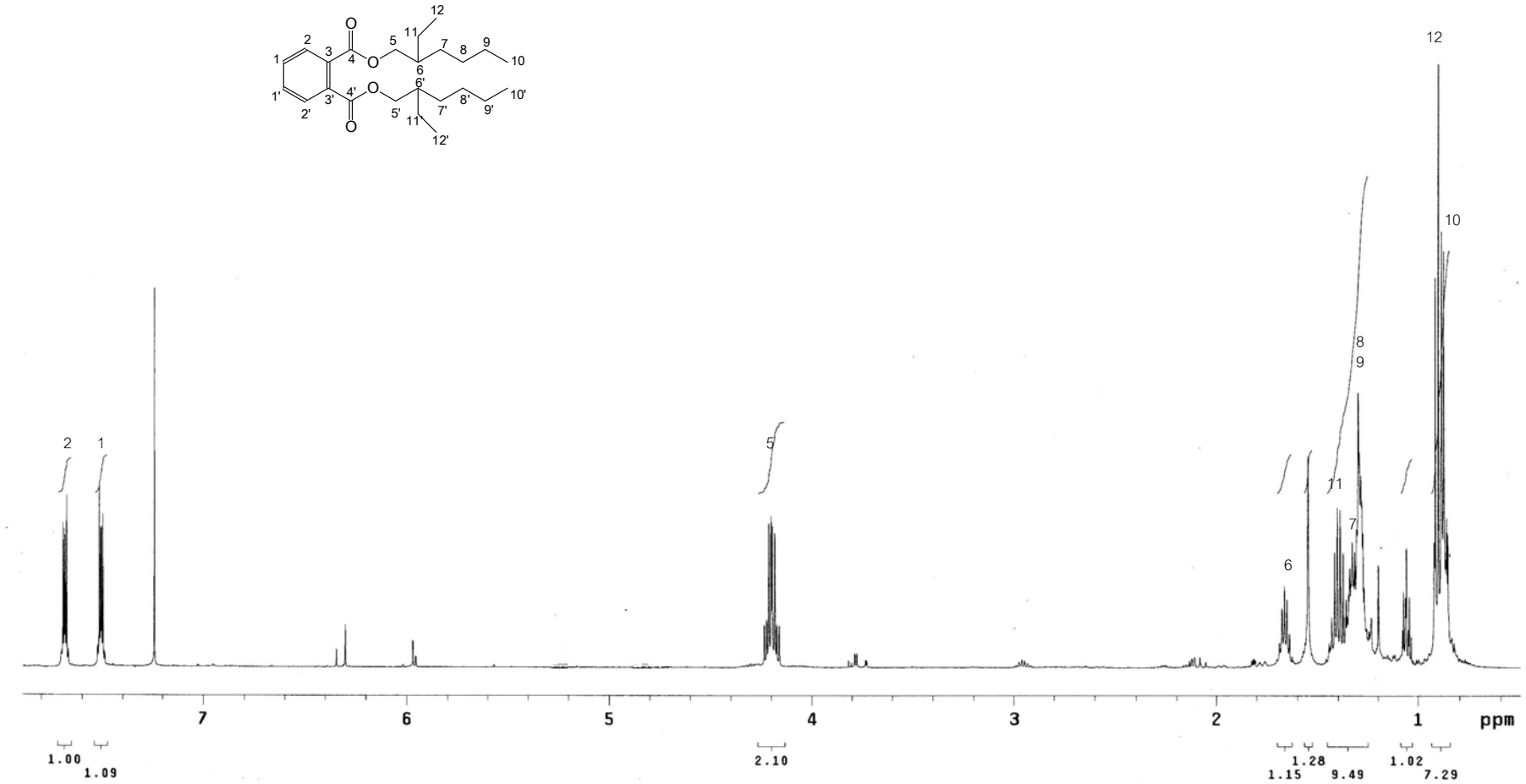


Figure A34 ^{1}H-NMR (500 MHz) Spectrum of NW5 (Bis-2-ethylhexyl-phthalate) in $CDCl_3$

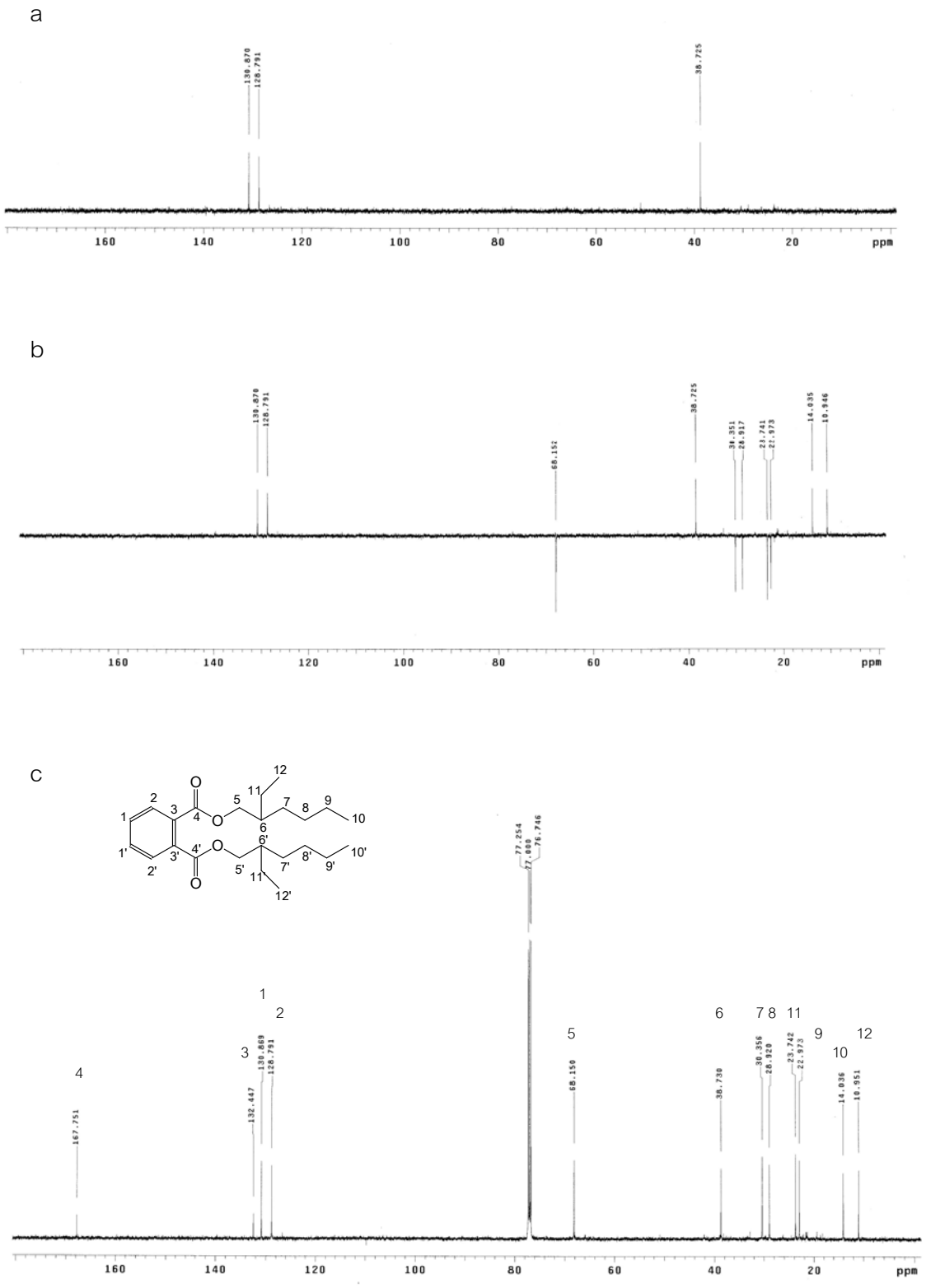


Figure A35 DEPT90° (a), DEPT135° (b), and ^{13}C-NMR (c) (125 MHz) Spectra of NW5 (Bis-2-ethylhexyl-phthalate) in $CDCl_3$

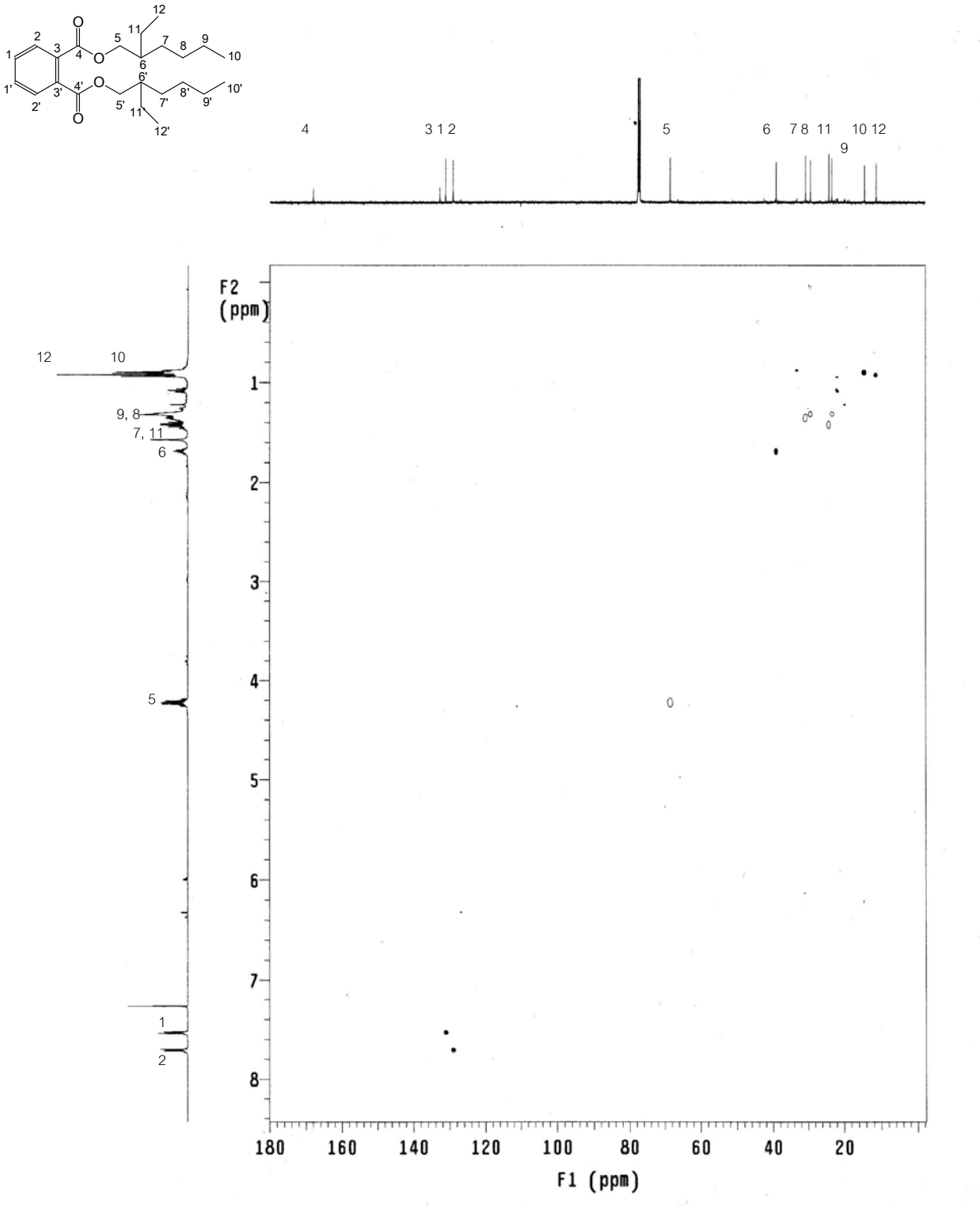


Figure A36a HSQC Spectra of NW5 (Bis-2-ethylhexyl-phthalate) in $CDCl_3$

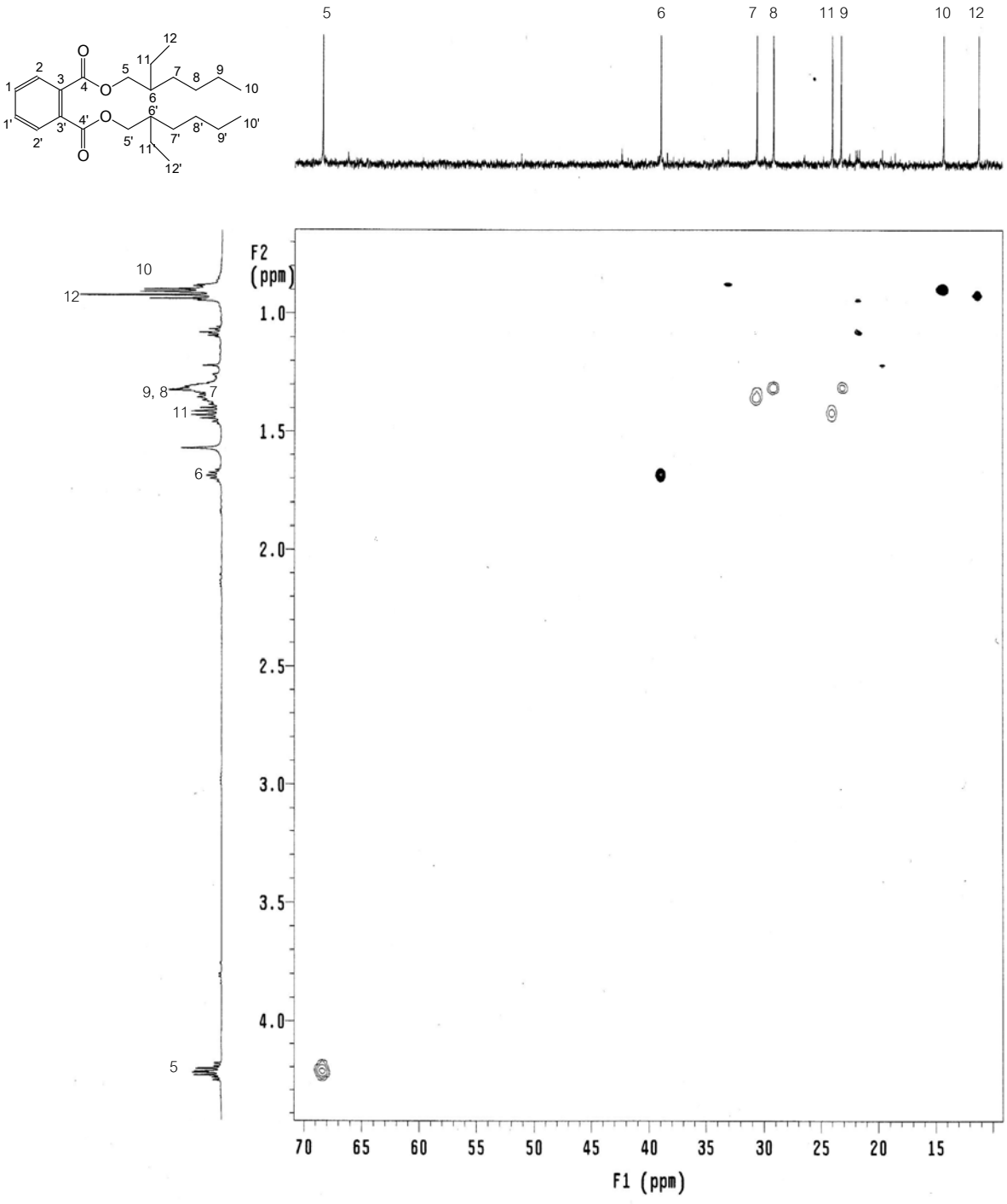


Figure A36b HSQC Spectra of NW5 (Bis-2-ethylhexyl-phthalate) in $CDCl_3$ (continued)

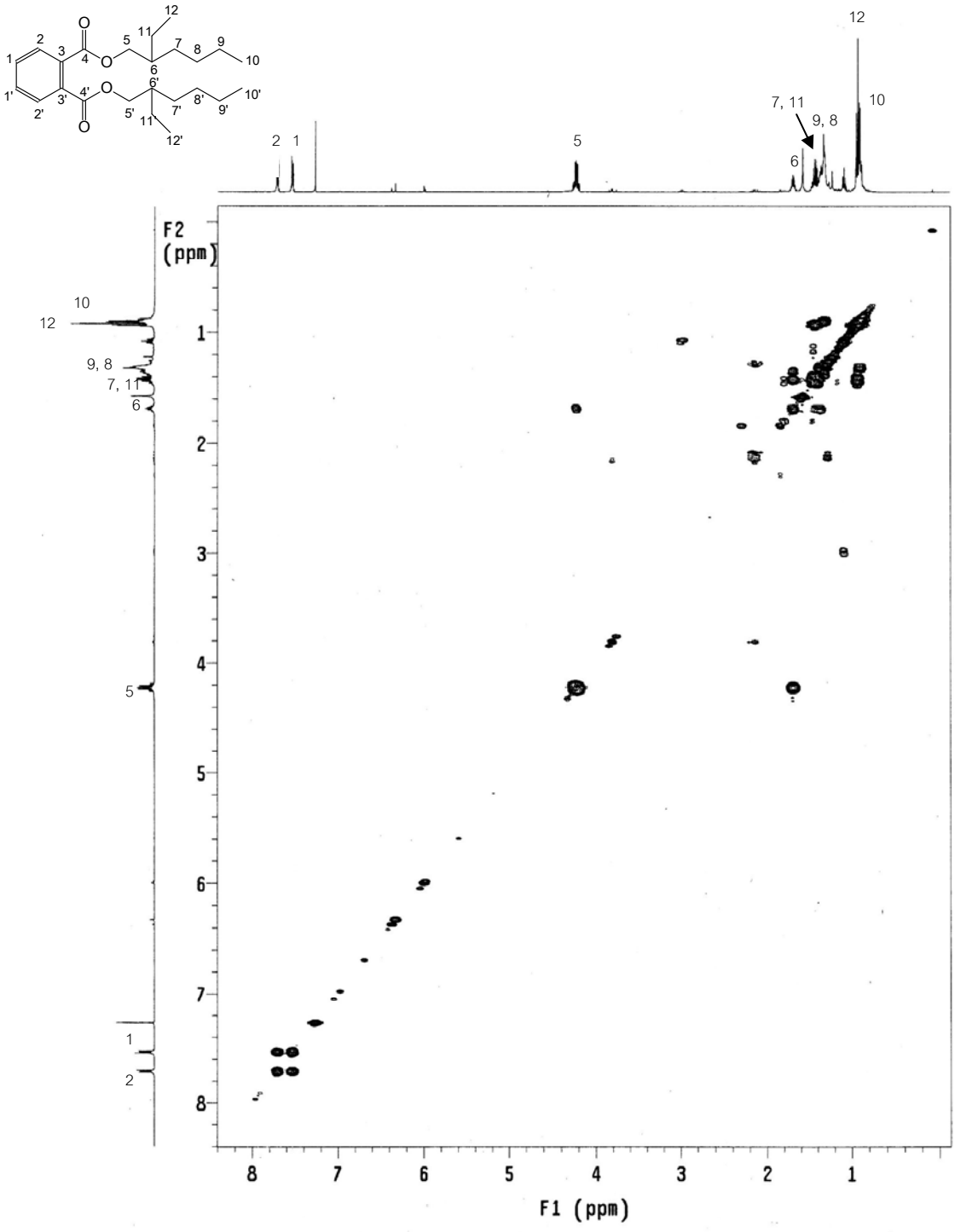


Figure A37a ^{1}H-^{1}H-COSY Spectra of NW5 (Bis-2-ethylhexyl-phthalate) in $CDCl_3$

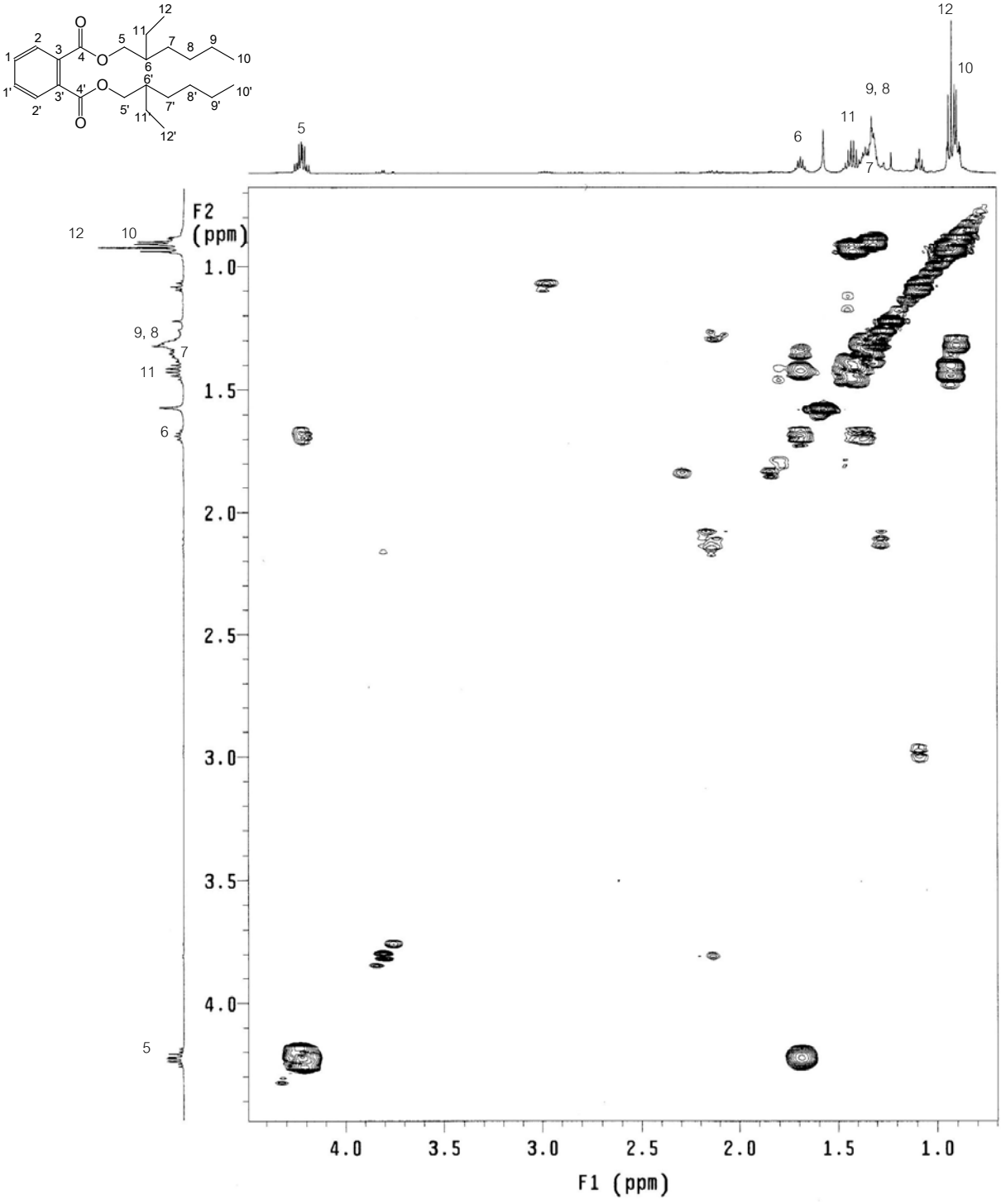


Figure A37b ^{1}H-^{1}H-COSY Spectra of NW5 (Bis-2-ethylhexyl-phthalate) in $CDCl_3$ (continued)

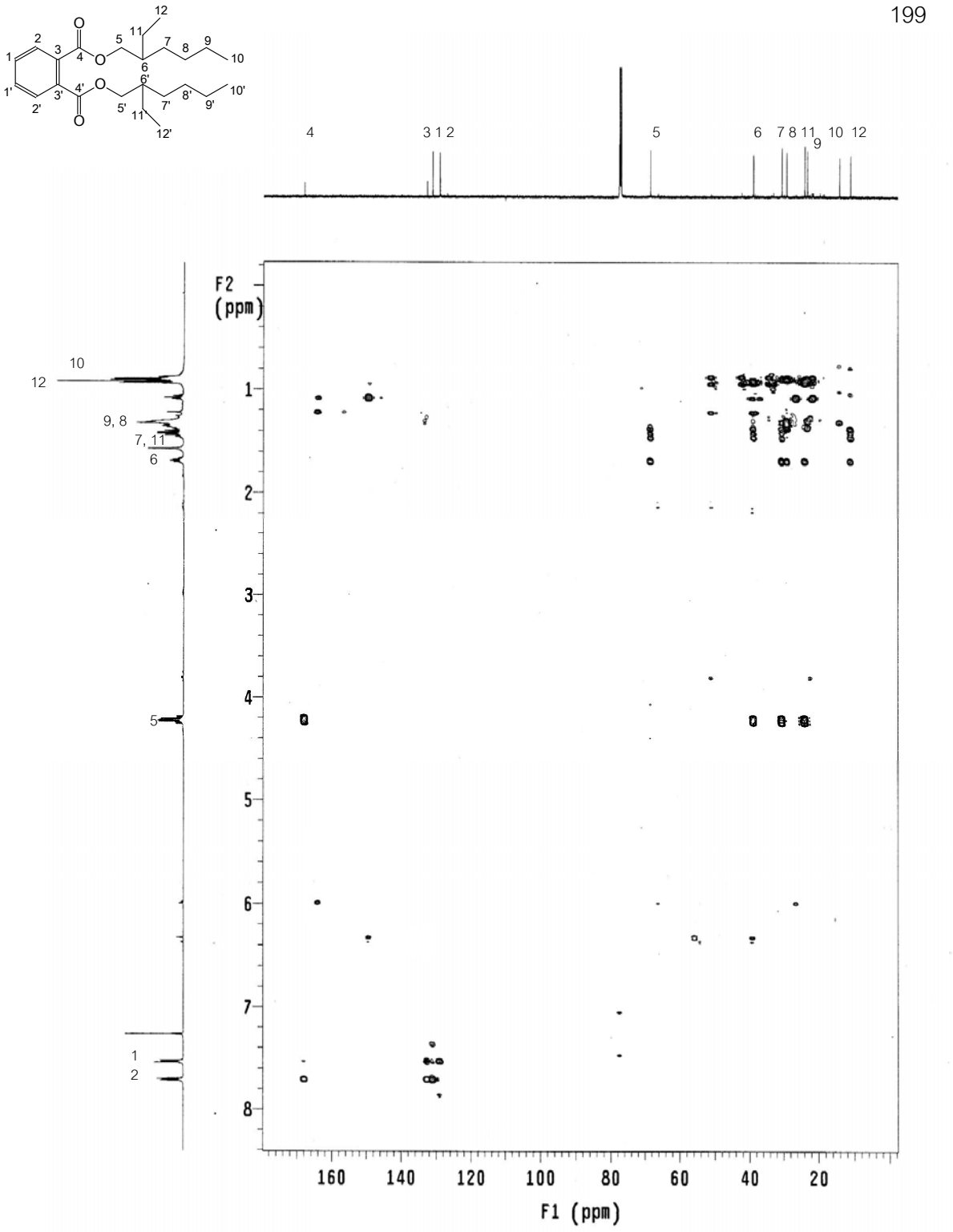


Figure A38a HMBC Spectra of NW5 (Bis-2-ethylhexyl-phthalate) in $CDCl_3$

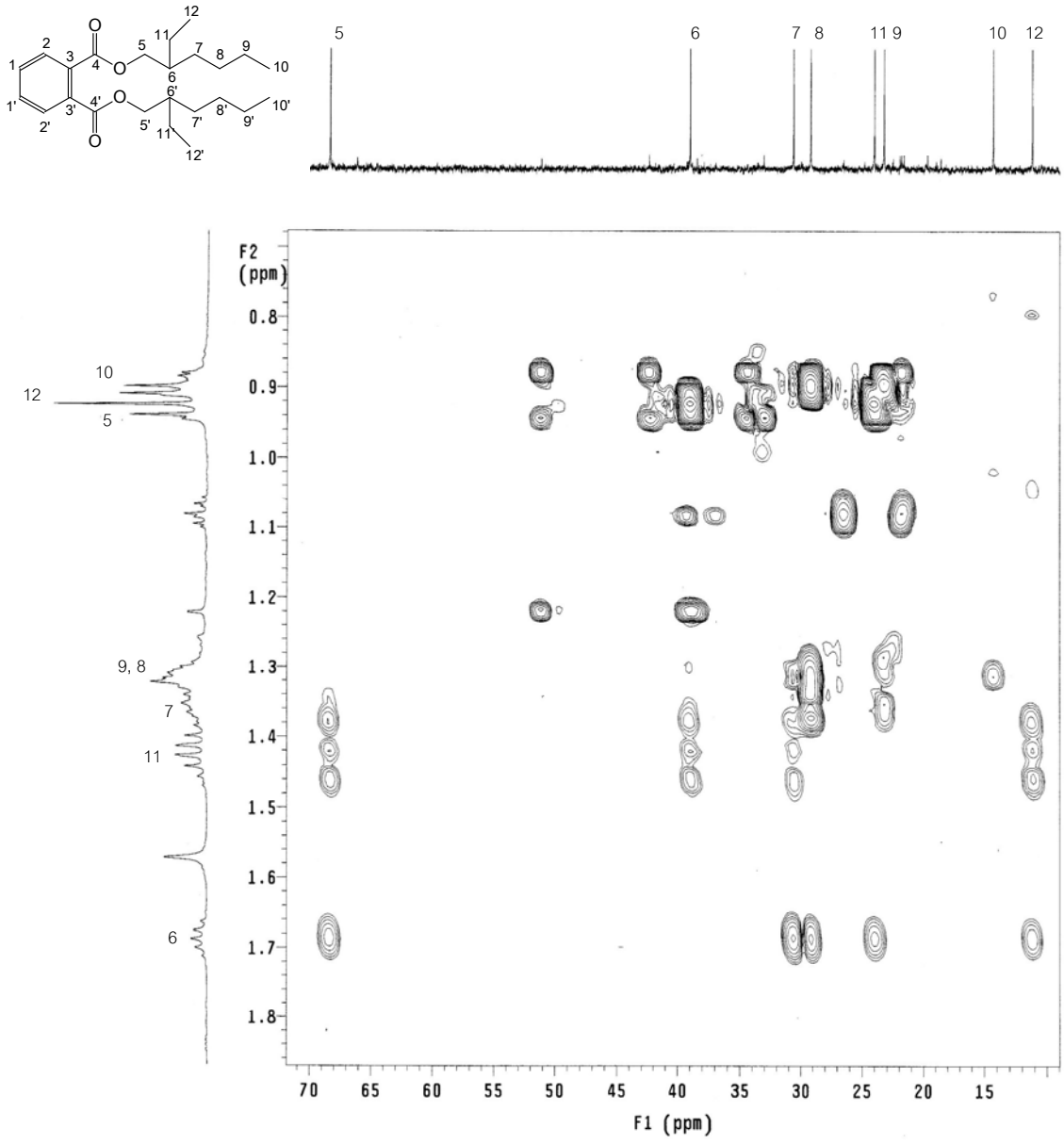
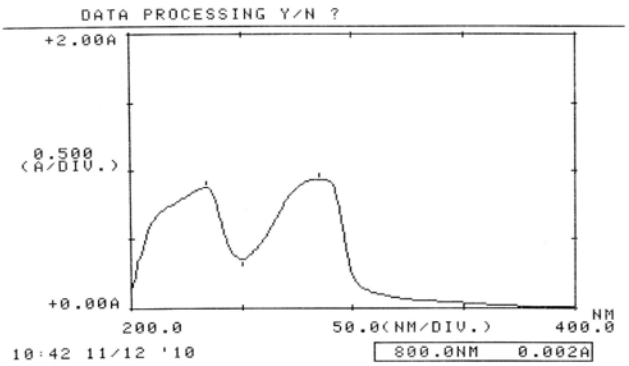


Figure A38b HMBC Spectra of NW5 (Bis-2-ethylhexyl-phthalate) in $CDCl_3$ (continued)



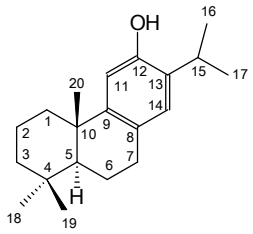


Figure A39 UV Spectra of NW6 (Ferruginol) in MeOH

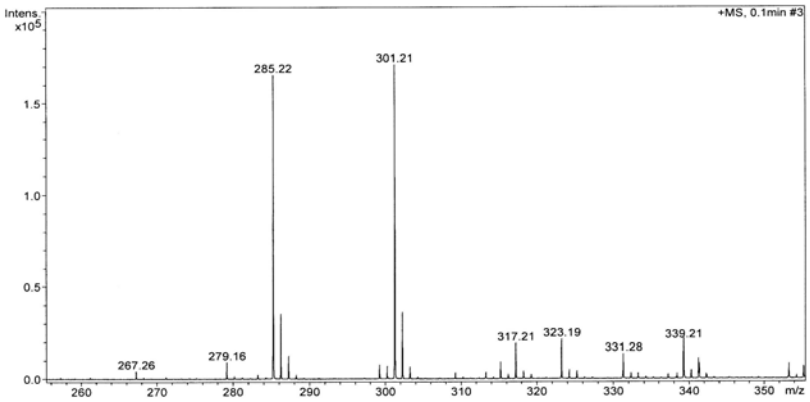


Figure A40 ESI Mass Spectra of NW6 (Ferruginol)

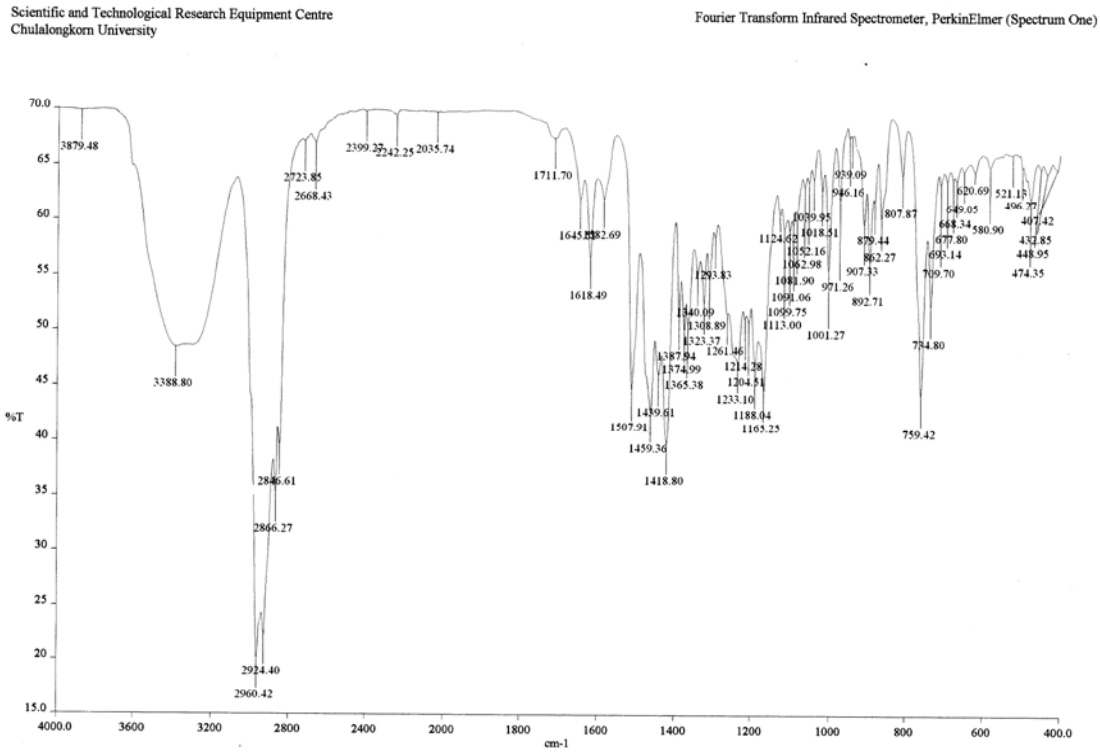


Figure A41 IR Spectra of NW6 (Ferruginol) (KBr)

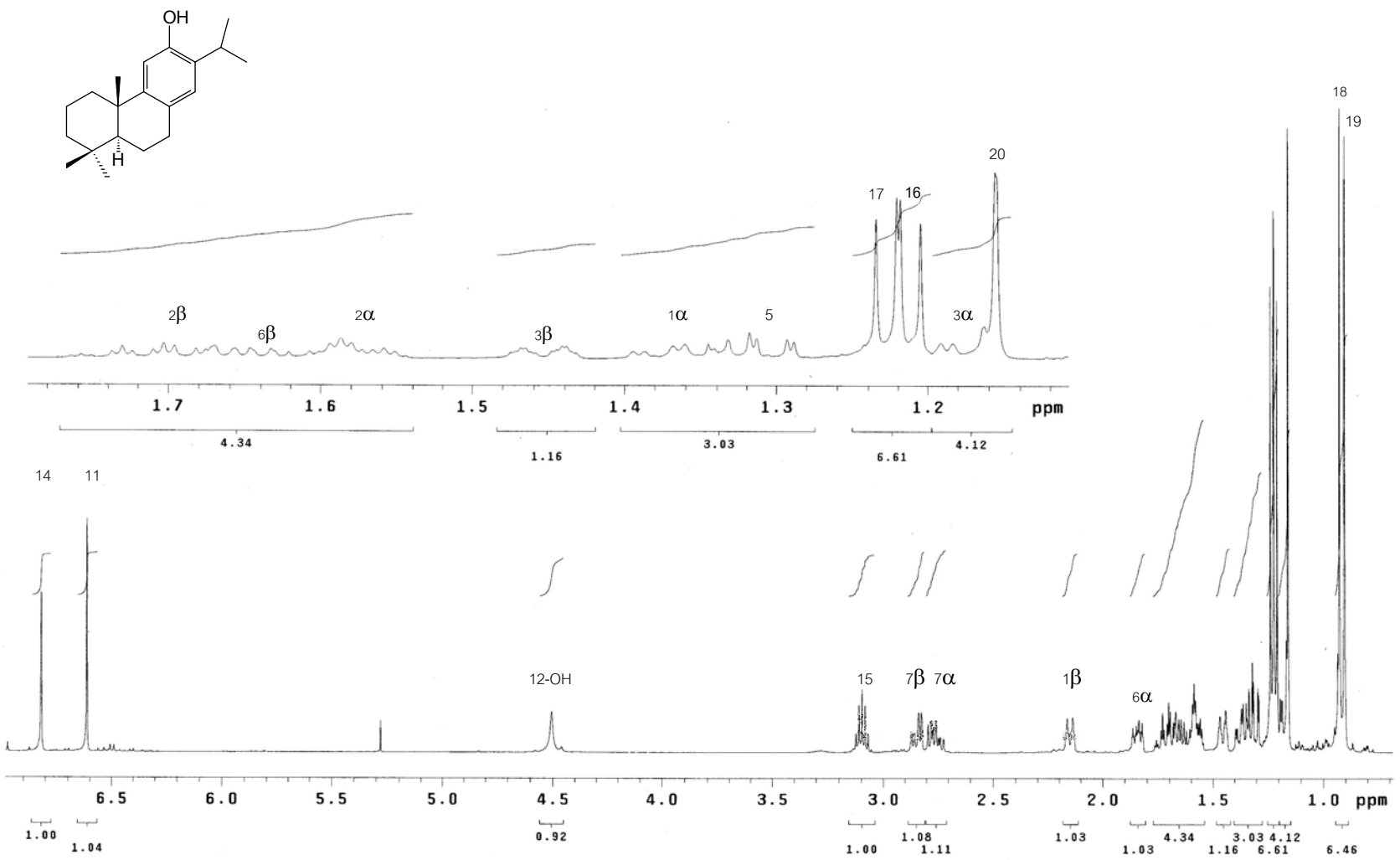


Figure A42 ^{1}H-NMR (500 MHz) Spectra of NW6 (Ferruginol) in $CDCl_3$

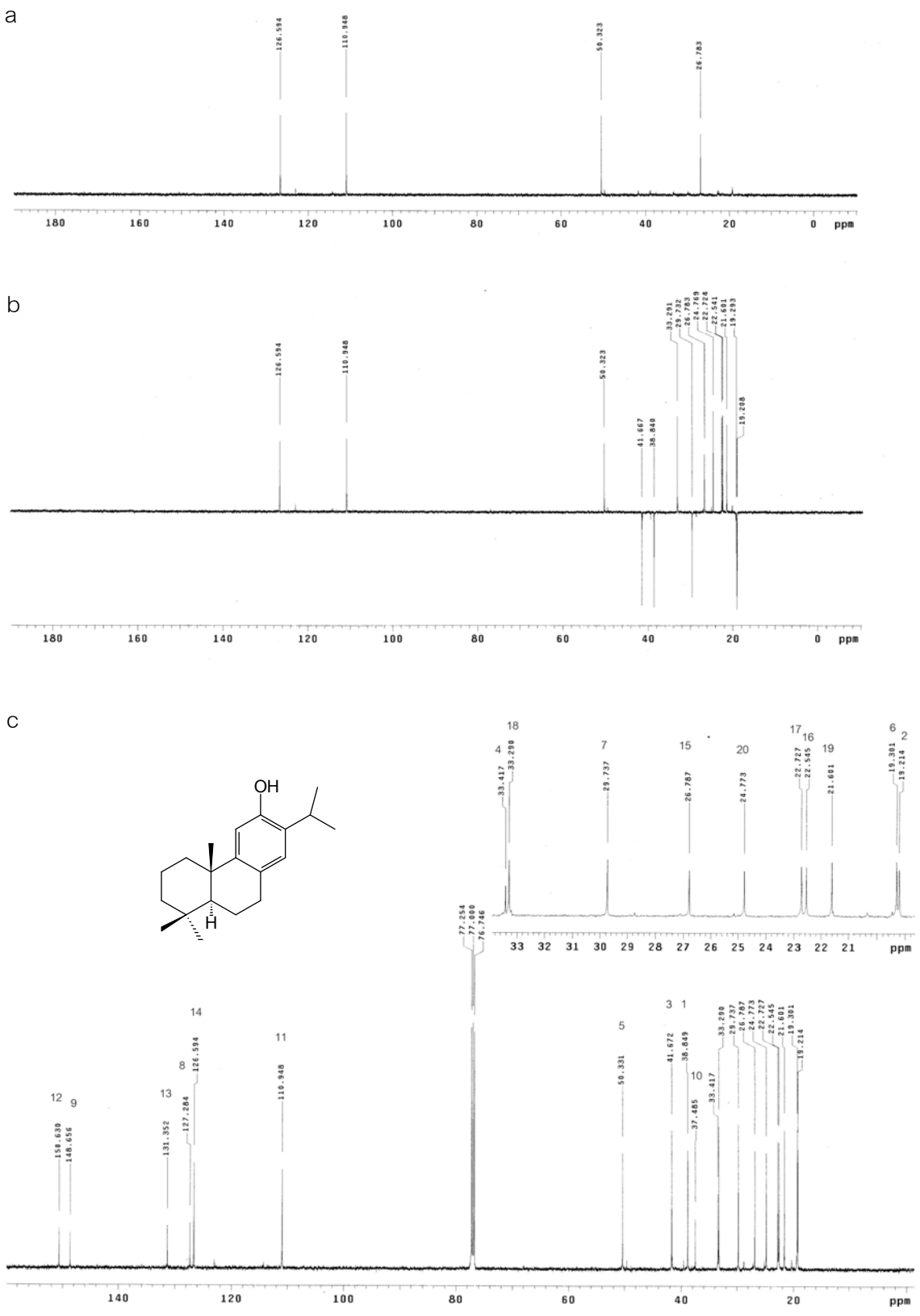


Figure A43 DEPT90° (a), DEPT135° (b), and ^{13}C-NMR (c) (125 MHz) Spectra of NW6 (Ferruginol) in $CDCl_3$

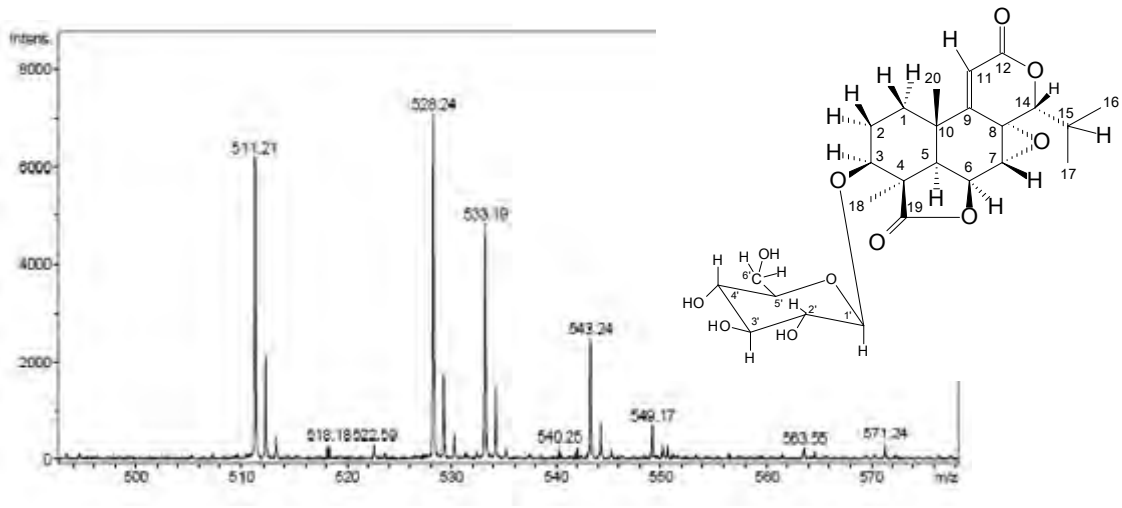


Figure A44 ESI Mass Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside)

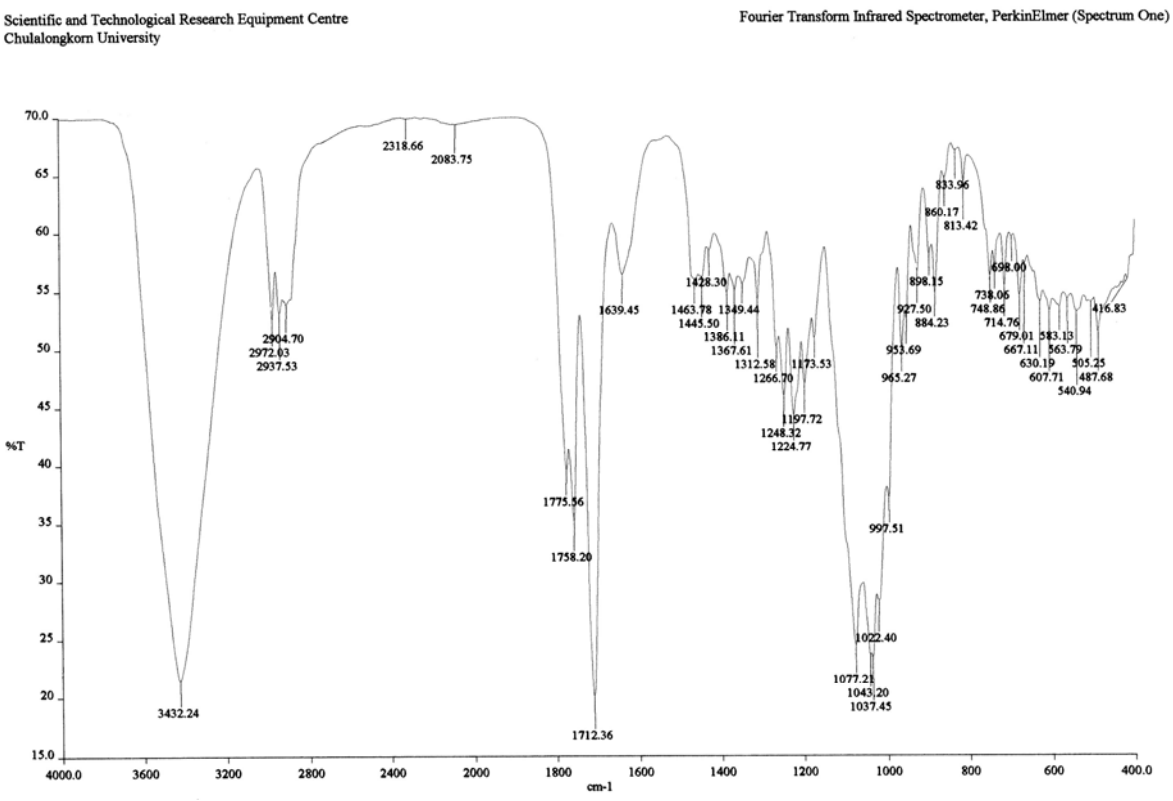


Figure A45 IR Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) (KBr)

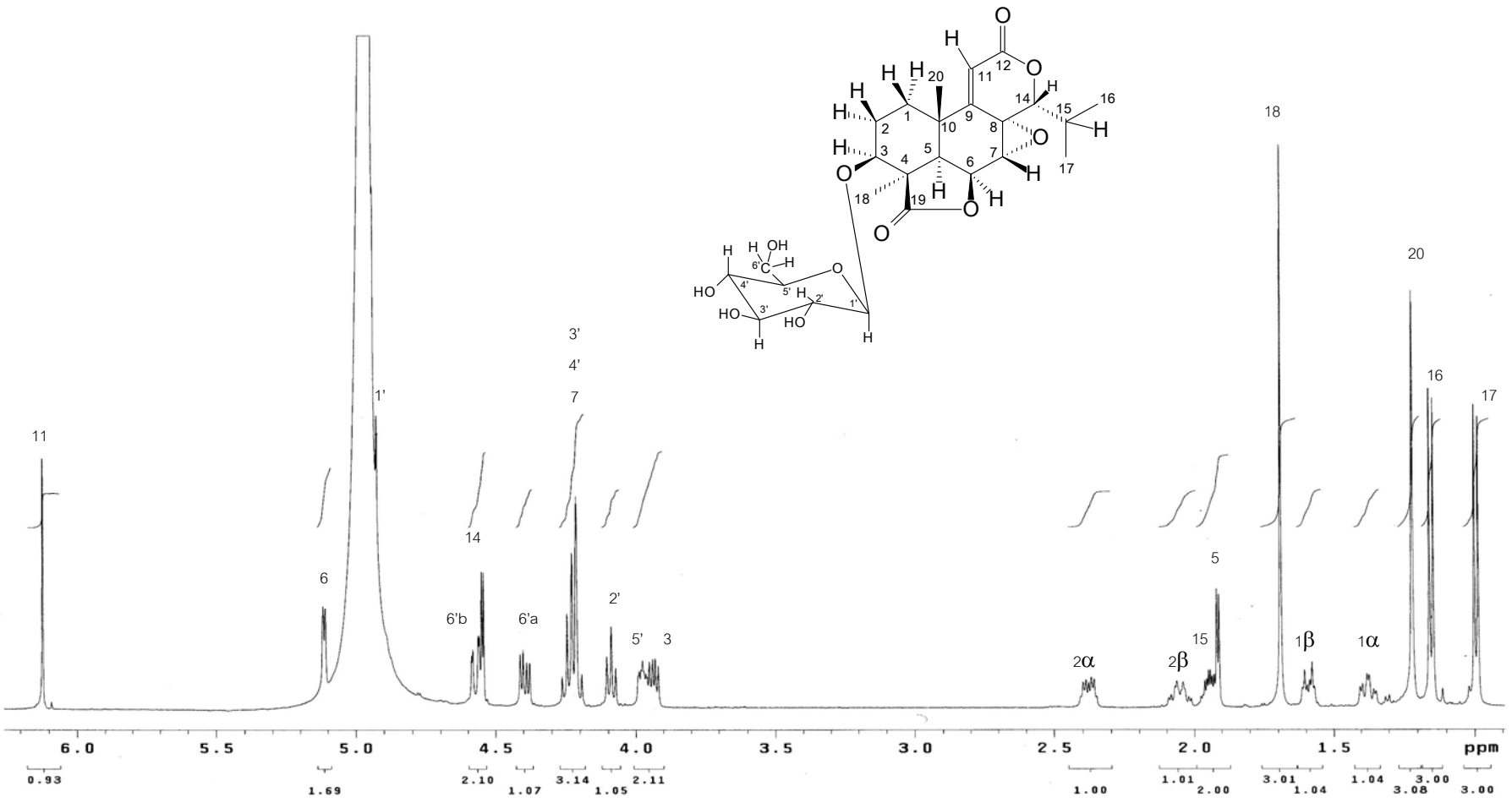


Figure A46 ^{1}H-NMR (500 MHz) Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N

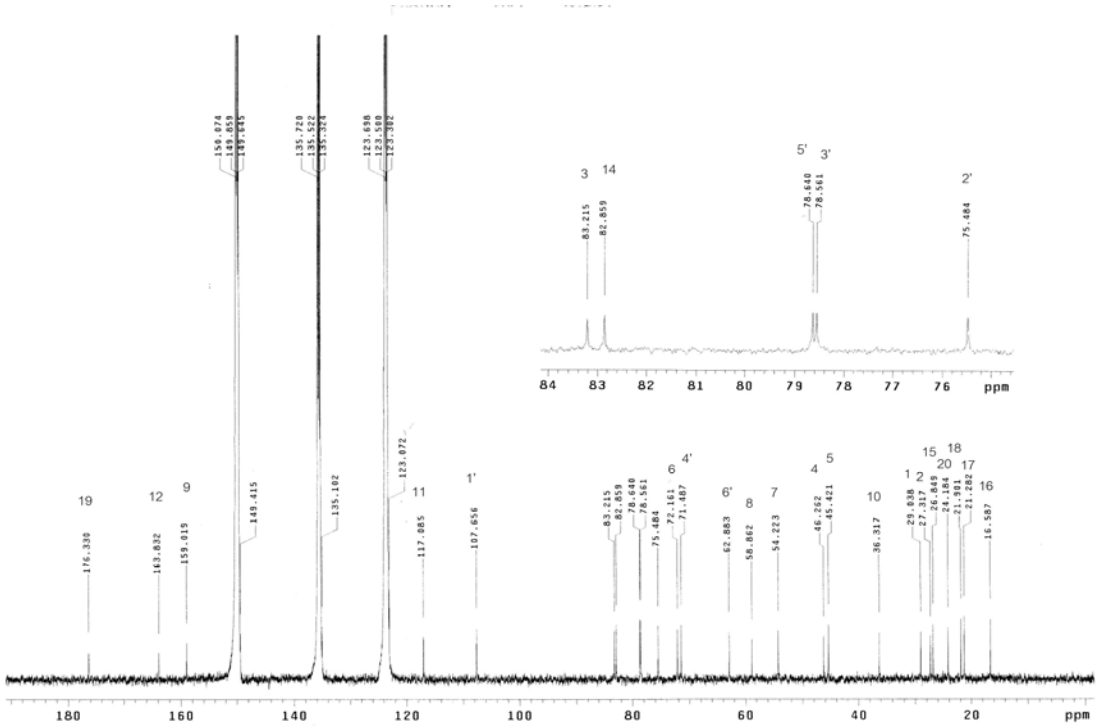


Figure A47 ^{13}C-NMR (125 MHz) Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N

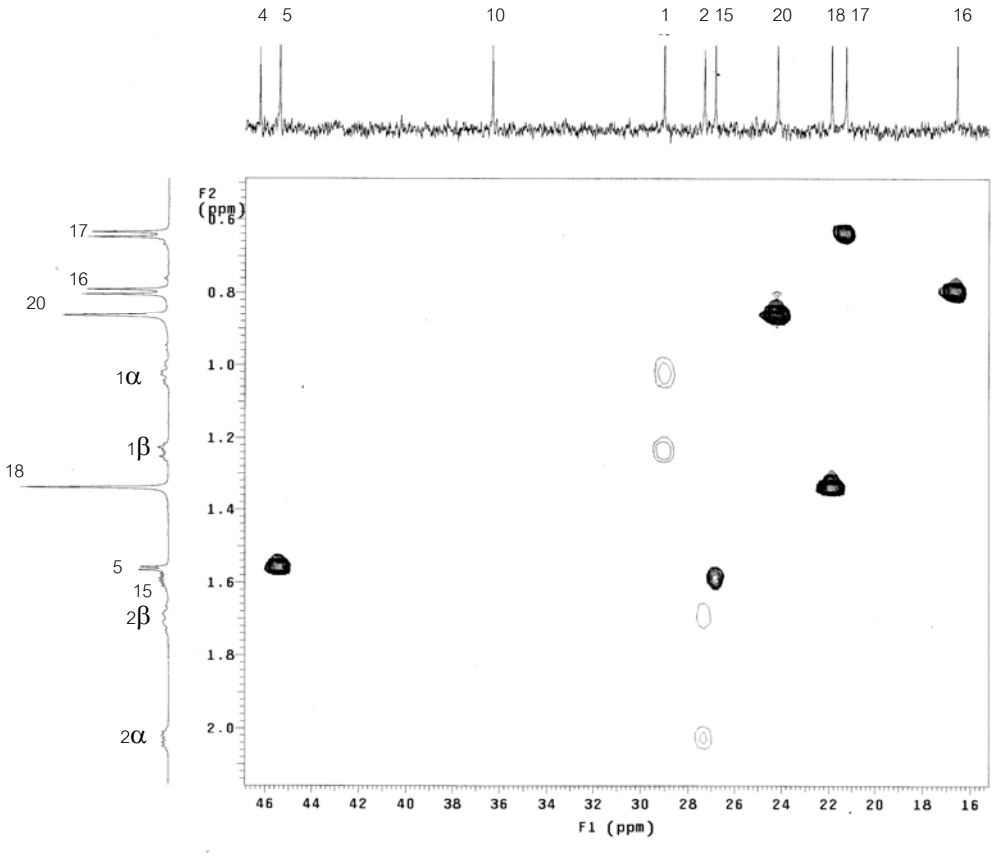


Figure A48a HSQC Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N

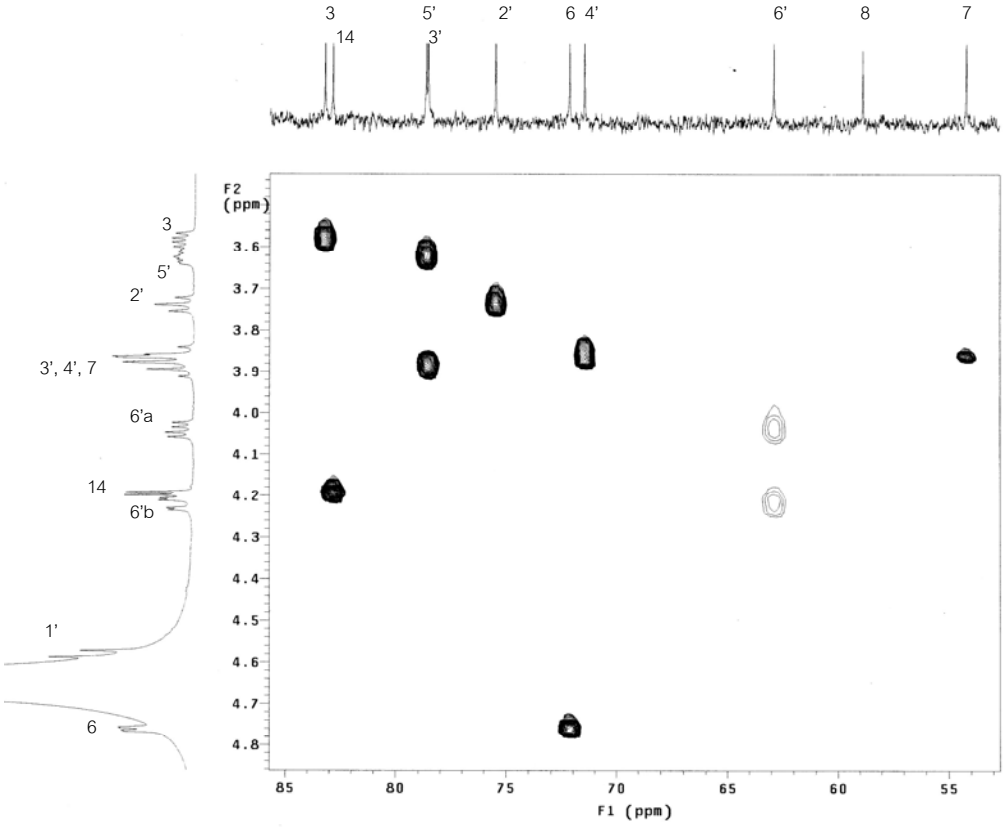


Figure A48b HSQC Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N (continued)

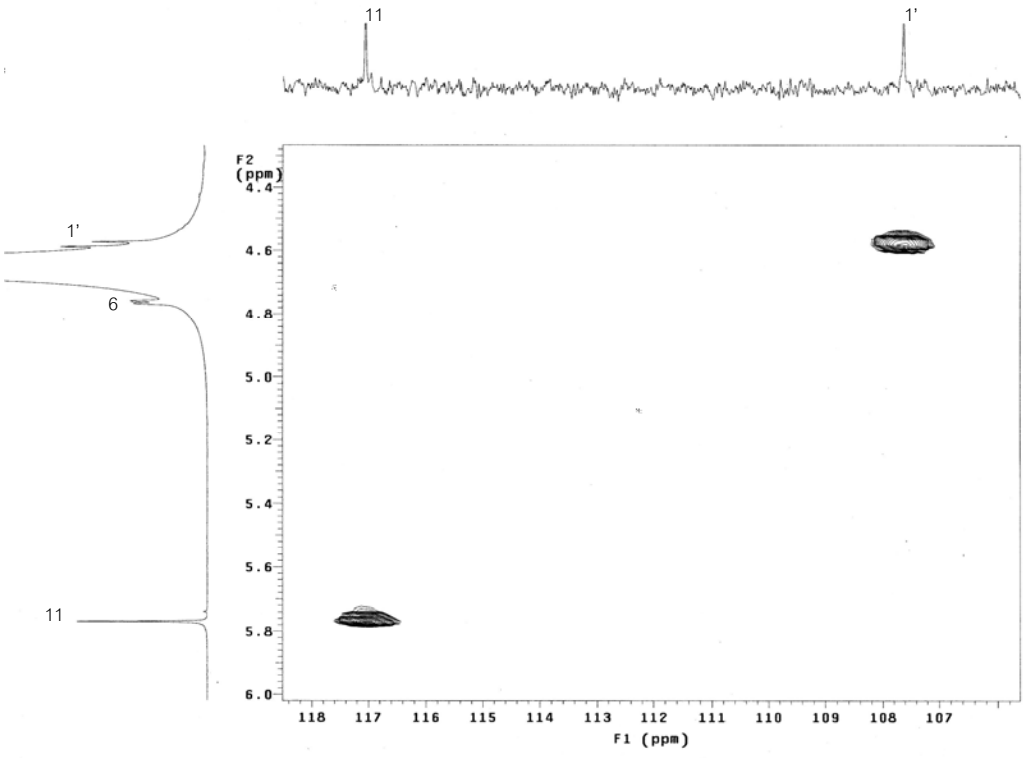


Figure A48c HSQC Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N (continued)

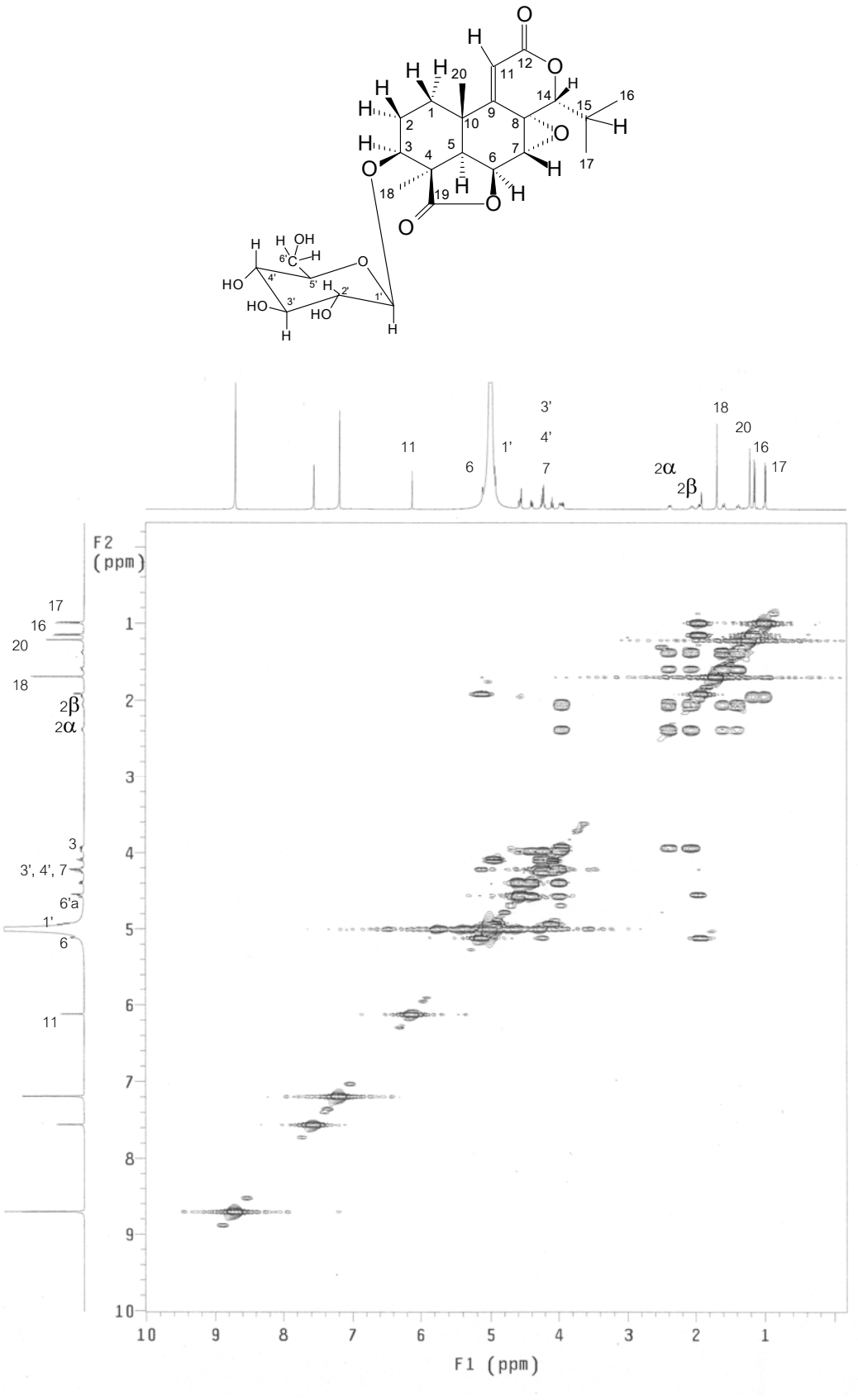


Figure A49a ^{1}H-^{1}H-COSY Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N

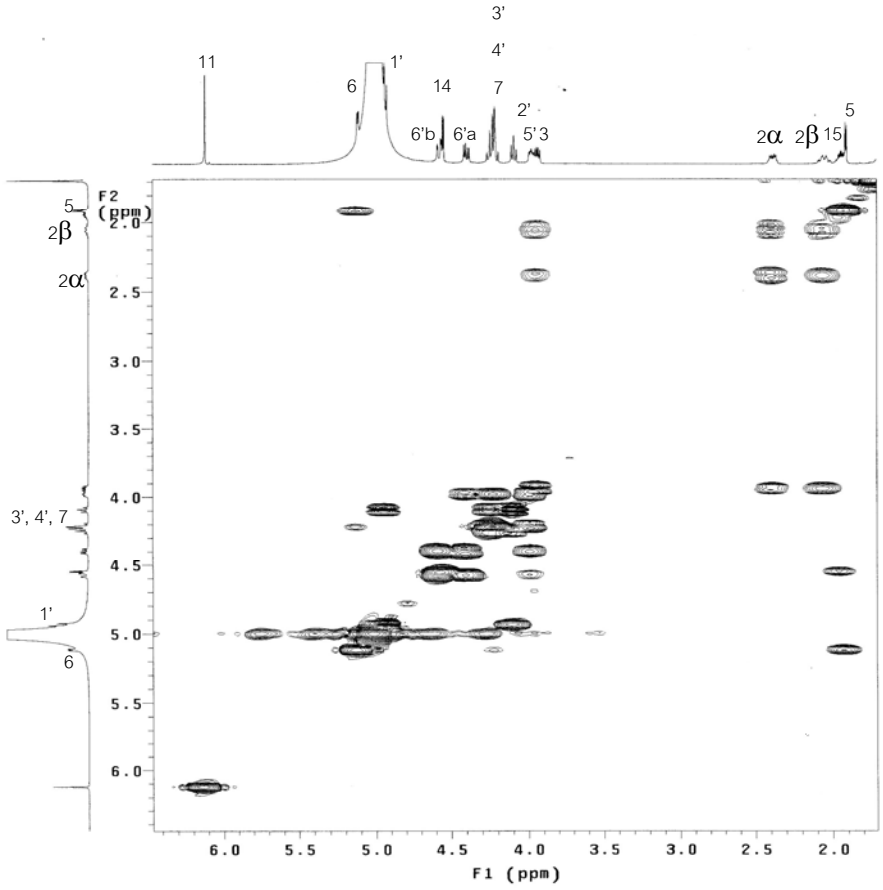


Figure A49b ^{1}H-^{1}H-COSY Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N (continued)

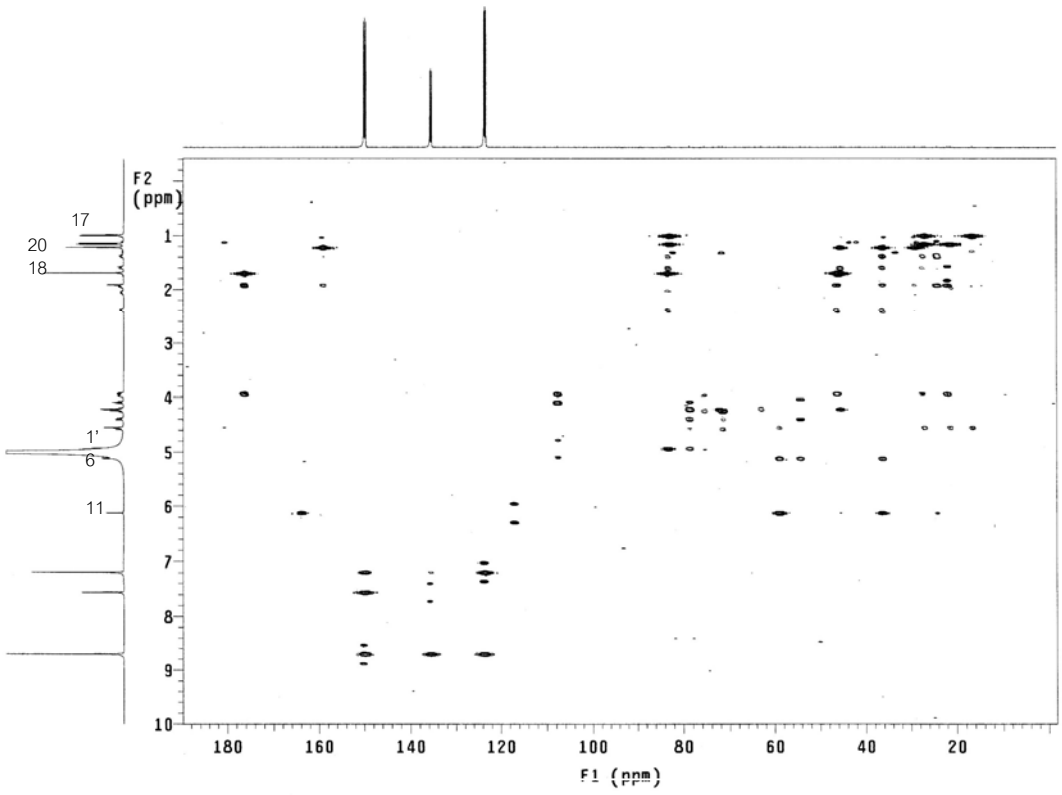


Figure A50a HMBC Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N

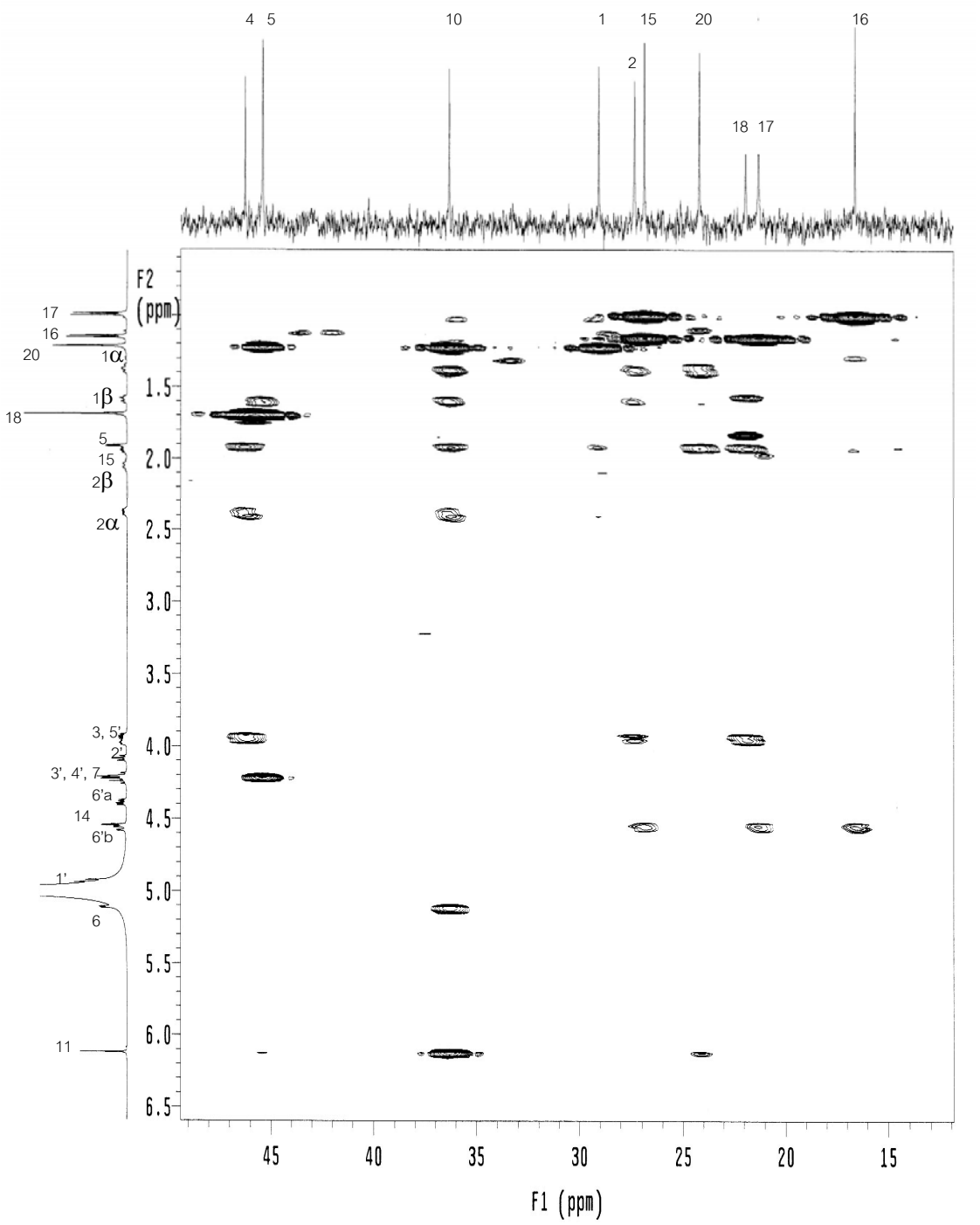


Figure A50b HMBC Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N (continued)

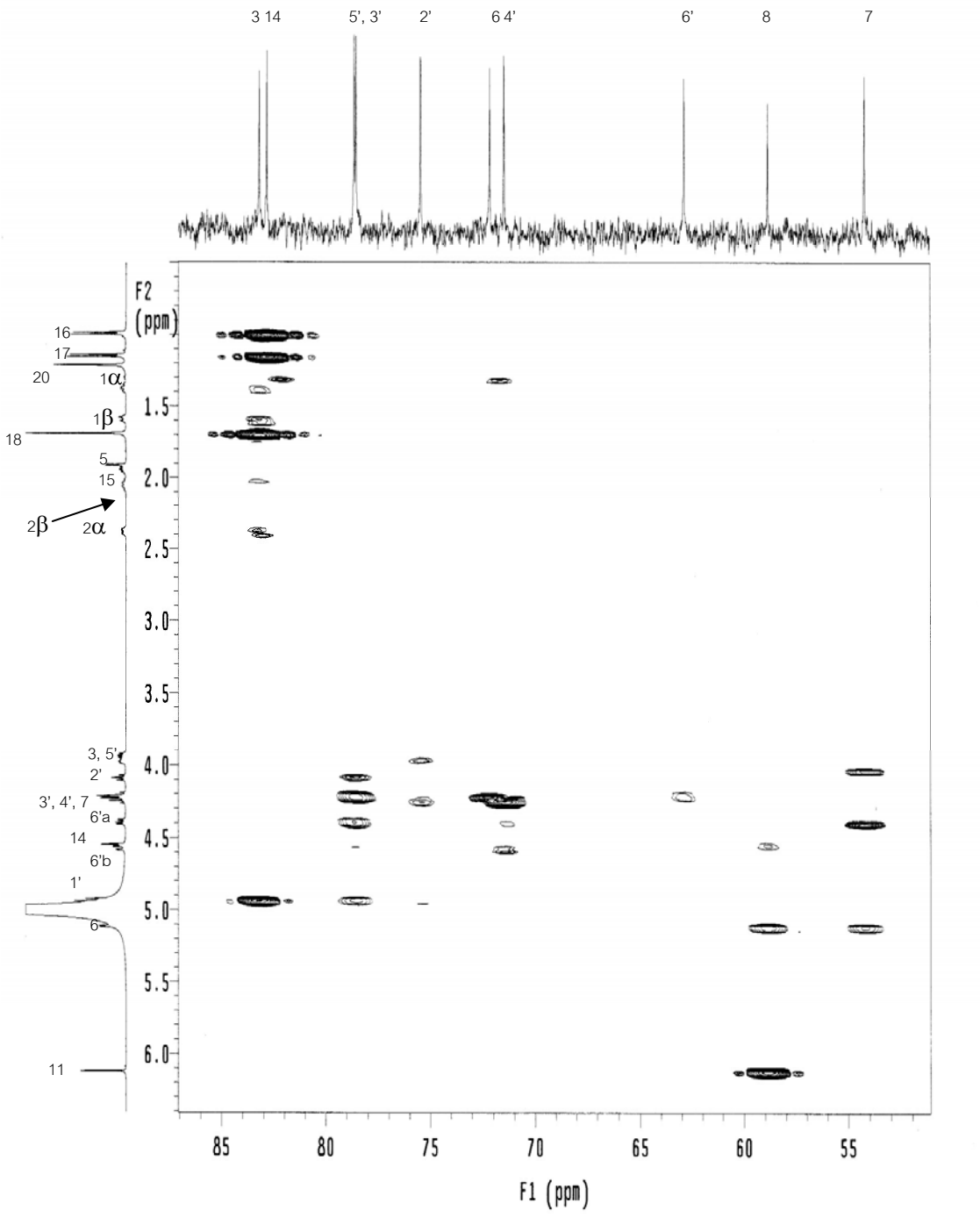


Figure A50c HMBC Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N (continued)

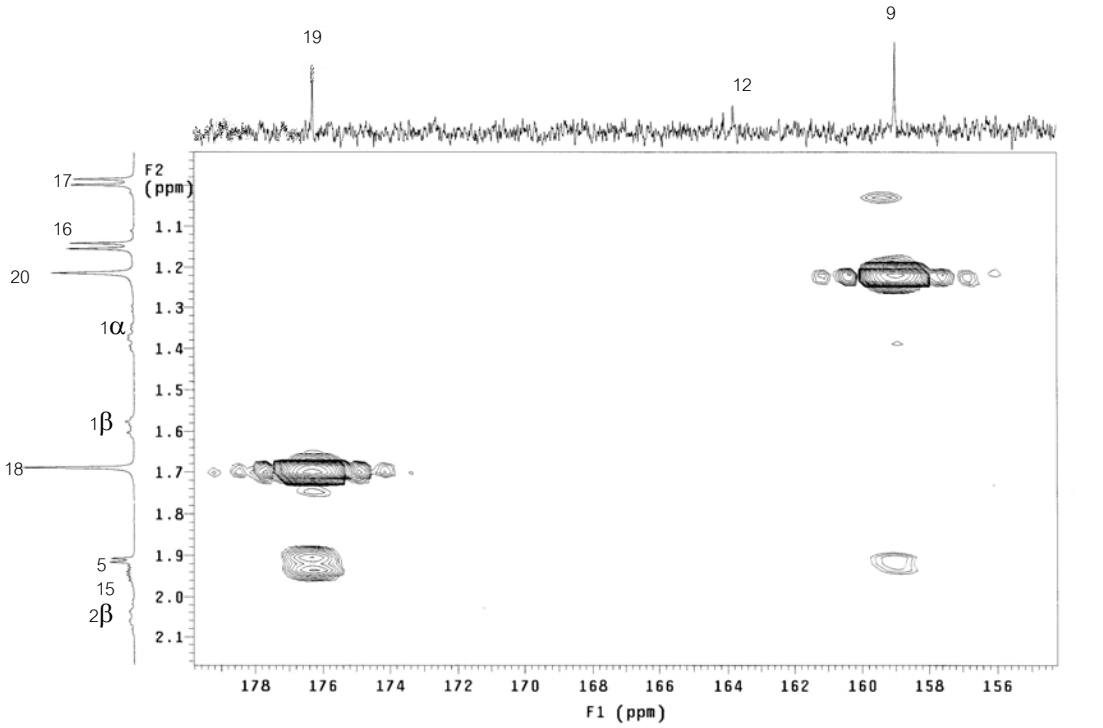


Figure A50d HMBC Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N (continued)

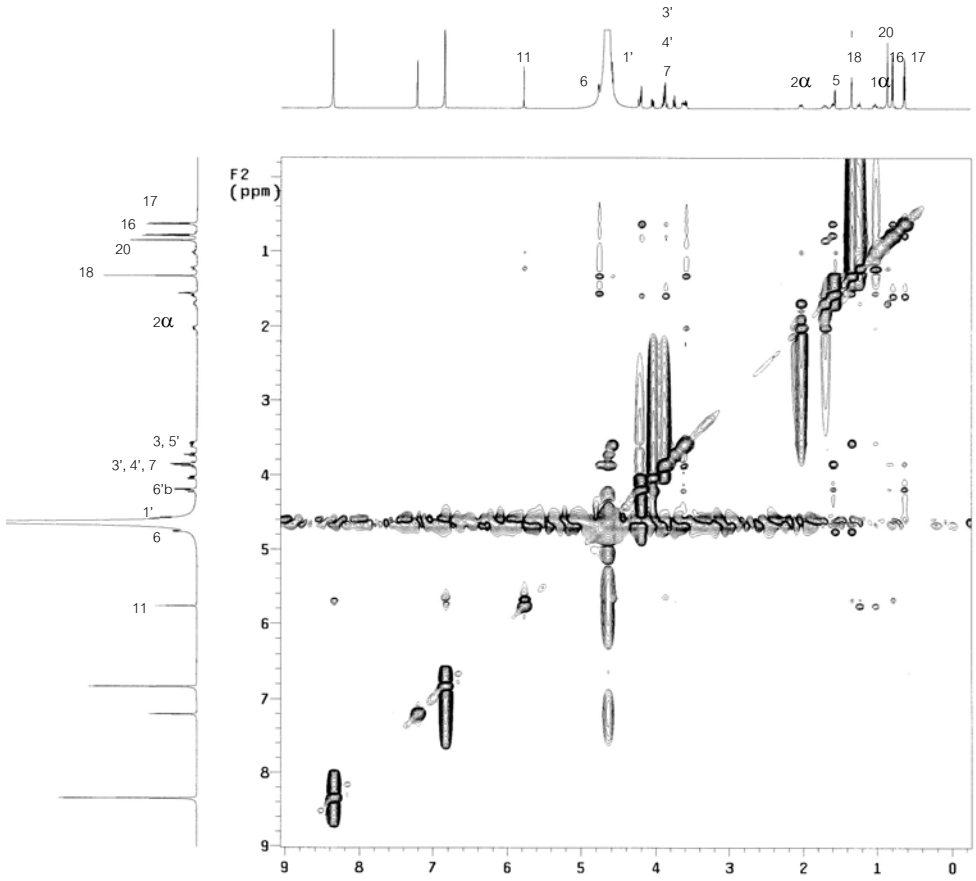


Figure A51a NOESY Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N

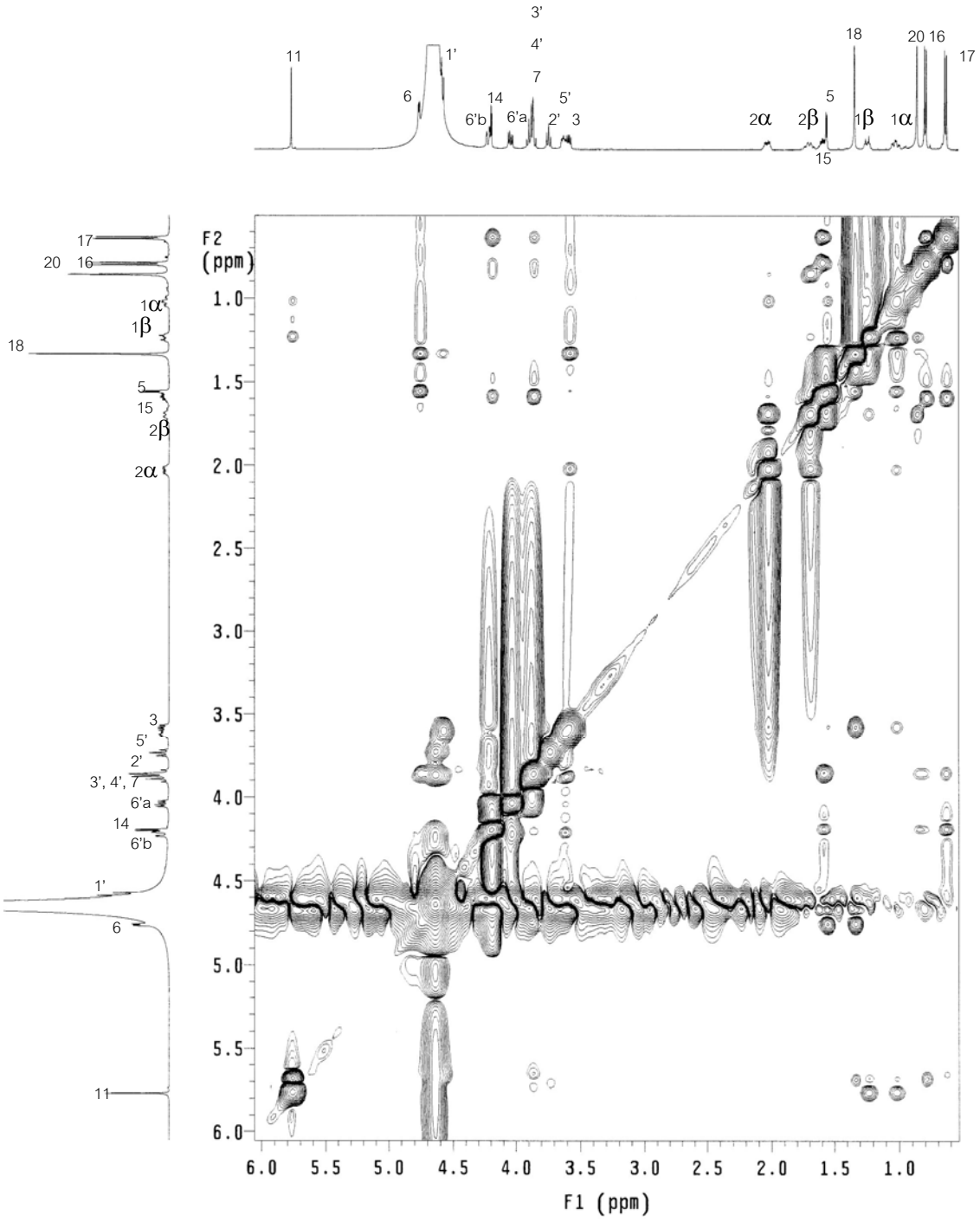


Figure A51b NOESY Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N (continued)

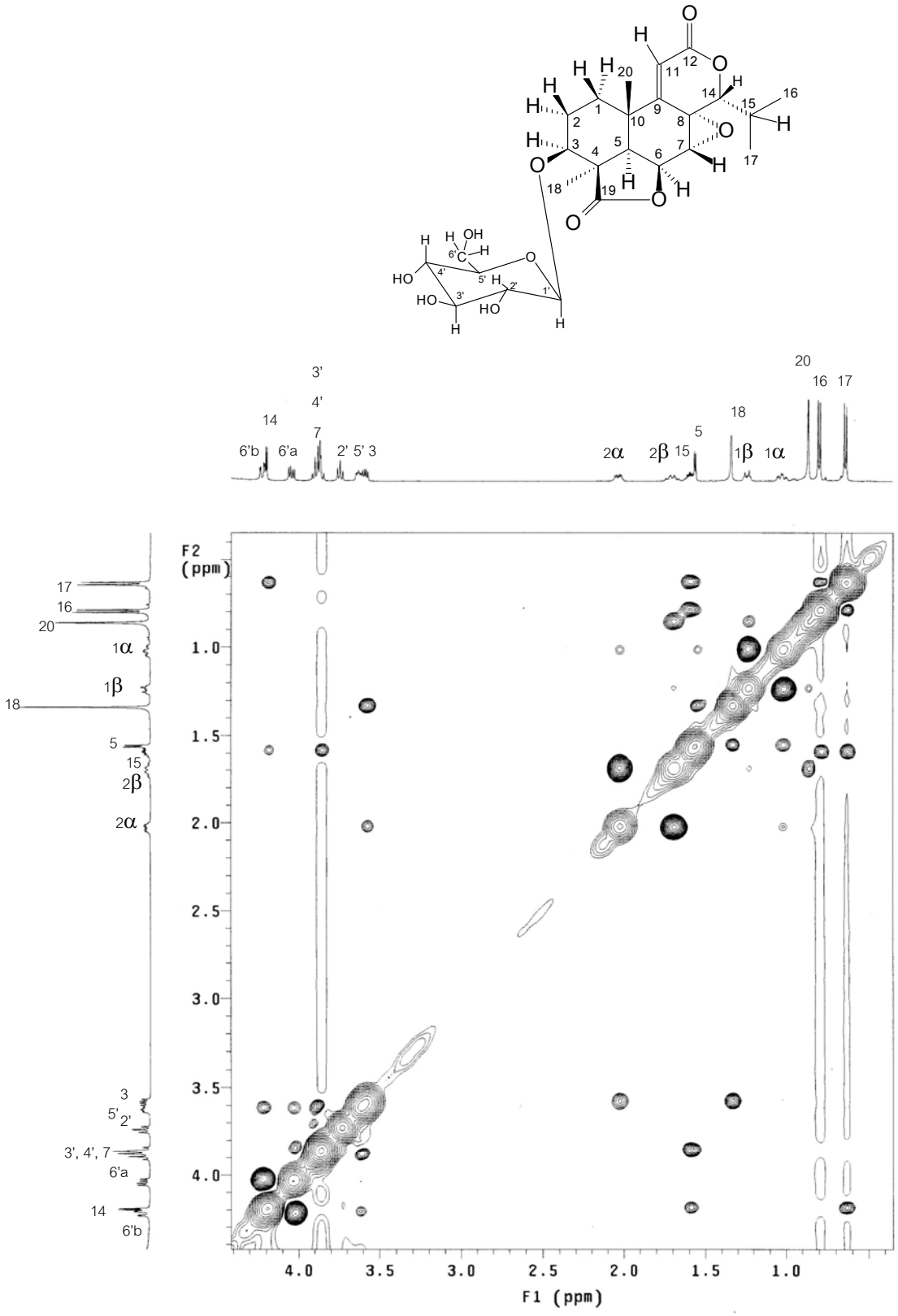


Figure A51c NOESY Spectra of NW7 (Nagilactone E-3-*O*-β-glucopyranoside) in C_5D_5N (continued)

APPENDIX B

(PREPARATION OF SOLUTIONS AND REAGENTS)

1) Buffers

1.1) 13 mM Tris-HCl, 150 mM NaCl, 1.3 mM $CaCl_2$ buffer pH 8.0 (500 ml)

Weighed Tris-HCl 1.0244 g

NaCl 4.3875 g

$CaCl_2.2H_2O$ 0.0956 g

Dissolved in distillated water 750 ml and

adjusted pH to 8.0 with NaOH

Adjusted distillated water q.s. to 500 ml

1.2) 0.1 M Sodium citrate buffer pH 4.2 (250 ml)

Weighed Sodium citrate dihydrate 7.3525 g

Dissolved in distillated water 188 ml and

adjusted pH to 4.2 with 0.1 N HCl

Adjusted distillated water q.s. to 250 ml

1.3) 0.061 M Tris-HCl buffer pH 8.5 (500 ml)

Weighed Tris-HCl 4.8068 g

Dissolved in distilled water 375 ml and

adjusted pH to 8.5 with 0.1 N NaOH

Adjusted distillated water q.s. to 500 ml

1.4) 0.1 N HCl 100 ml

Prepared 5%w/v HCl in distillated water by measuring off 3.4 ml of concentrated hydrochloric acid in distillated water and adjusted distillated water to 25 ml

Prepared 0.1 M HCl from 5%w/v HCl by measuring off 7.3 ml of 5%w/v HCl and adjusted distillated water q.s. to 100 ml

1.5) 0.1 N NaOH 100 ml

Weighed NaOH 0.4 g dissolved in distilled water q.s. to 100.0 ml

2) Spraying Reagents (Wagner and Bladt, 1996)

2.1) Anisaldehyde-sulphuric acid reagent (AS)

Preparation

0.5 ml anisaldehyde is mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml concentrated sulphuric acid, in that order.

Detection

The TLC plate is sprayed with the reagent, heated at 110°C for 5-15 min, evaluation in visual or UV-365 nm.

Stability

The reagent has only limited stability and is no longer useable when the color has turned to red-violet.

Detection

Terpenoids, propylpropanoids, pungent and bitter principles, saponins.

2.2) 10%H_2SO_4 in EtOH

Preparation

Slowly pour 10 ml of concentrated sulphuric acid into 75 ml of EtOH and adjusted the volume to 100 ml.

Detection

The TLC plate is sprayed with the reagent, heated at 100°C for 3-5 min, evaluation in visual.

Stability

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Detection

e.g. Cardiac glycosides, lignans.

VITA

Miss Arissarakorn Sirinamarattana was born in Nonthaburi on March 1, 1979. She received her Bachelor's degree of Science in Pharmacy in 2000 from Silpakorn University, Nakhon-pathom, Thailand. Since her graduation, she has been working as a lecturer at Faculty of Pharmaceutical Science, Silpakorn University.