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CHEMICAL CONSTITUENTS OF *CLINACANTHUS SIAMENSIS* LEAVES

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จากการศึกษาองค์ประกอบทางเคมีของสารสกัดเมทานอลของใบลิ้นงูเห่า (*Clinacanthus siamensis* Brem.) โดยเทคนิคโครมาโตกราฟี ทำให้สามารถแยกสารพวก glycoglycerolipidsในรูปอนุพันธ์เปอร์อะซิเตท ซึ่งการพิสูจน์โครงสร้างทางเคมีของสารที่สกัดแยกได้กระทำโดยการวิเคราะห์ข้อมูลทาง spectroscopy โดยเฉพาะ 1D และ 2D-NMR เปรียบเทียบข้อมูลที่ได้กับสารที่มีการรายงานมาก่อน ร่วมกับการวิเคราะห์ข้อมูลจากก๊าซโครมาโตแกรมของ fatty acid methyl esters ที่ได้จากการไฮโดรไลซ์ด้วยกรดและเอ็นไซม์ พบว่าสารพวก glycoglycerolipids เป็นสารผสมของ monogalactopyranosyl diglycerides 1 ชนิด และ สารผสมของ digalactopyranosyl diglycerides อีก 2 ชนิด โดยมีหมู่ acyl เป็นกรดไขมันชนิด hexadecanoic acid (16:0), hexadecaenoic acid (16:1), octadecanoic acid (18:0) และ octadecatrienoic acid (18:3) เป็นองค์ประกอบ นอกจากนี้ยังพบสารผสมของ β-sitosterol-3-O-glucoside และ β-stigmasterol-3-O-glucoside อีกด้วย

ภาควิชา	เภสัชเวท	ลายมือนิสิต.....
สาขาวิชา	เภสัชเวท	ลายมือชื่ออาจารย์ที่ปรึกษา.....
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Investigation of the chemical constituents from the methanol extract of *Clinacanthus siamensis* Brem. leaves led to the isolation of glycoglycerolipids as the peracetyl derivatives. Structure elucidation and identification of the isolated compounds were mainly determined by spectroscopic data analysis especially 1D and 2D-NMR, comparison with previously reported data, together with analysis of gas chromatograms of fatty acid methyl esters from acid and enzymatic hydrolysis. The glycoglycerolipids are one mixture of monogalactopyranosyl diglycerides and two mixtures of digalactopyranosyl diglycerides. The acyl groups of the glycoglycerolipids are hexadecanoic acid (16:0), hexadecaenoic acid (16:1), octadecanoic acid (18:0) and octadecatrienoic acid (18:3). In addition, a mixture of β-sitosterol-3-O-glucoside and β-stigmasterol-3-O-glucoside is also isolated.

Department	Pharmacognosy	Student's signature……...
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CONTENTS

Page

CONTENTS (continued)

Page

CONTENTS (continued)

Page

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (continued)

Figure **Page**

LIST OF FIGURES (continued)

Figure **Page**

LIST OF SCHEMES

Scheme **Page**

LIST OF TABLES

Table **Page**

ABBREVIATIONS

δ	=	Chemical shift
ε_{max}	=	Molar absorptivity at maximum absorption
λ_{max}	=	Wave length at maximum absorption
μg	=	Microgram
μl	=	Microliter
μM	=	Micromolar
ν_{max}	=	Wave number at maximum absorption
^{13}C-NMR	=	Carbon-13 nuclear magnetic resonance
^{1}H-NMR	=	Proton nuclear magnetic resonance
$^{1}J_{HC}$	=	One bond proton-carbon coupling constant
1D	=	One dimensional (for NMR spectra)
2D	=	Two dimensional (for NMR spectra)
ax	=	Axial
br d	=	Broad doublet (for NMR spectra)
br s	=	Broad singlet (for NMR spectra)
br t	=	Broad triplet (for NMR spectra)
C_5D_5N	=	Deuterated pyridine
CCl_4	=	Carbon tetrachloride
$CDCl_3$	=	Deuterated chloroform
$CHCl_3$	=	Chloroform
cm	=	Centimeter
CMIR	=	Cell-mediated immune response
CO_2	=	Carbon dioxide
COSY	=	Correlation spectroscopy
CPE	=	Cytopathic effect
d	=	Doublet (for NMR spectra)
D_2O	=	Deuterium oxide
dd	=	Doublets of doublet (for NMR spectra)
DEPT	=	Distortionless enhancement by polarization transfer
DH	=	DNA hybridization
DMSO-d_6	=	Deuterated dimethyl sulfoxide
dt	=	Doublets of triplet (for NMR spectra)

ABBREVIATIONS (continued)

ED_{50}	=	50% Effective dose
EIMS	=	Electron impact mass spectrometry
eq	=	Equatorial
ESIMS	=	Electrospray ionization mass spectroscopy
EtOAc	=	Ethyl acetate
EtOH	=	Ethanol
eV	=	Electron volt
FABMS	=	Fast atom bombardment mass spectroscopy
FT	=	Fourier transformed
FTIR	=	Fourier transformed infrared
g	=	Gram
GC	=	Gas chromatography
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation
HMQC	=	^{1}H-detected heteronuclear multiple quantum coherence
hr.	=	hour
HSV-1	=	Herpes simplex virus type 1
HSV-2	=	Herpes simplex virus type 2
Hz	=	Hertz
ID_{50}	=	50% Inhibition dose
IR	=	Infrared
J	=	Coupling constant
KBr	=	Potassium bromide
kg	=	Kilogram
l	=	liter
LD_{100}	=	100% Lethal dose
LD_{50}	=	50% Lethal dose
LD_{84}	=	84% Lethal dose
m	=	Multiplet (for NMR spectra)
m/z	=	Mass to charge ratio
M^{+}	=	Molecular ion
$[M+H]^{+}$	=	Protonated molecular ion
MEM	=	Modified Eagle Medium

ABBREVIATIONS (continued)

MeOH	=	Methanol
mg	=	Milligram
MHz	=	Megahertz
MIC	=	Minimum inhibitory concentration
ml	=	Milliliter
ml/min	=	Milliliter per minute
MM	=	Maintenance medium
mm	=	Millimeter
MS	=	Mass spectrometry
$^{n}J_{HC}$	=	Proton-carbon long range coupling constant
nm	=	Nanometer
NMR	=	Nuclear magnetic resonance
No.	=	Number
NOE	=	Nuclear overhauser effect correlation spectroscopy
$^{\circ}C$	=	Degree Celsius
PBS	=	Phosphate buffer saline
PFU	=	Plaque forming unit
ppm	=	Part per million
PRA	=	Plaque reduction assay
Pyridine-d_5	=	Deuterated pyridine
q	=	Quartet
s	=	Singlet (for NMR spectra)
sp.	=	Species (singular)
spp.	=	Species (plural)
t	=	Triplet (for NMR spectra)
THF	=	Tetrahydrofuran
TI	=	Therapeutic index
TLC	=	Thin layer chromatography
UV	=	Ultraviolet
VZV	=	Varicella zoster virus

CHAPTER I

INTRODUCTION

Thailand is rich with valuable herbs and medicinal plants with a high potential for the development into healthcare products for domestic use and for export. To be successful in the product development, a great number of researches on medicinal plants need to be conducted in the aspects of phytochemistry, pharmacology, toxicology and clinical uses.

The genus *Clinacanthus* belongs to the family Acanthaceae. There are 3 species of this genus: *C. nutans*, *C. siamensis* and *C. spirei*. The Forest Herbarium in the Thai Plant Names Tem Smitinand had reported only 2 species as follows (The Forest Herbarium, Royal Forest Department, 2001):

1. *C. nutans* : The plant is known in Thailand by the following names: Phaya yo, Phaya plong dam, Phaya plong thong (central), Phak man kai or Phak lin khait (Chiang Mai), Phaya plong kham (Lampang), Pho-so-chaang (Karen-Mae Hong Son) and Salaed pang phon tua mia (Phitsanulok) (The Forest Herbarium, Royal Forest Department, 2001).

Clinacanthus nutans (Burm. f.) Lindau is an important Thai medicinal plant, the fresh leaves of which have been used as antipyretic, anti-inflammatory, anti-snake venom, anti-dysentery and as treatment for aphthous-ulcer, insect bites and rashes (นันทวรรณ บุณยะประภัศร และ อรนุช โชคชัยเจริญพรม, 2542). *C. nutans* is a small shrub native to tropical Asia. The use of *C. nutans* has been mentioned in Thai folk medicine. Its fresh leaves have been used domestically for the treatment of herpes simplex skin infection and shingles, and for the relief of pain from insect bites (กระทรวงสาธารณสุข, สำนักงานปลัดกระทรวง, 2537). Extract from the leaves was reported to possess anti-inflammatory activity (Kittisiripornkul, 1984; Tanasomwong, 1986), antiviral activity against varicella zoster virus (Thawaranantha *et al.*, 1992) and herpes simplex virus type 2 (Jayavasu *et al.*, 1992a)

Clinical trials for the treatment of genital herpes and varicella zoster were reported for *C. nutans* preparations (Jayavasu *et al*., 1992b; สมชาย แสงกิจพรและคณะ, 2536). The glycerine and tincture preparations of *C. nutans* extract were used for the treatment of aphthous ulcer or herpes stomalitis (สุภาภรณ์ ปิติพร และ อุไรวรรณ โชติเกียรติ, 2532).

Since a number of experiments have reported that its leaf extract displayed antiviral and anti-inflammatory activities, Phaya yo is a herbal drug selected as one of the herbs of choice in the National List of Essential Drugs A.D. 1999 (List of herbal medicine products) (คณะกรรมการแห่งชาติด้านยา, 2542). The plant is suggested to be used against herpes simplex virus, varicella-virus, rashes and allergy.

2. *Clinacanthus siamensis* Brem. : This plant is known in Thailand by the name “Lin ngu hao” meaning “cobra’s tongue”.

It is found cultivated or grown wild throughout Thailand, for examples in Chantaburi (Makham) and Rayong Province (Chamao). In traditional medicine, leaves or roots of *C. siamensis* crushed with water are topically applied to relieve painful swelling or abscesses, including inflammation from centipede or scorpion bites (Chuakul *et al*., 1997).

Several phytochemical studies on *C. nutans* have been reported but none on *C. siamensis*. However, screening of pharmacological activities of the ethyl acetate extract of *C. siamensis* has shown significant anti-herpes simplex activity (Jamtaweekul, 2001). Isolation and identification of the active compounds of *C. siamensis* to be compared with *C. nutans* are therefore of interest.

The main objectives in this investigation are as follows:

1. To isolate and purify chemical compounds from the leaves of *Clinacanthus siamensis* Brem.
2. To determine the chemical structure of each isolated compounds.
3. To evaluate the isolated compounds for anti-herpes simplex activity.

CHARPTER II

LITERATURE REVIEW

1. Characteristics of *Clinacanthus siamensis* Brem.

The plants in the family Acanthaceae are herbaceous or climbing, rarely small trees; leaves opposite, often with distinct cystoliths; stipules absent; flowers zygomorphic, often with conspicuous bracts; calyx-segments or lobes 4 or 5, imbricate or valvate, rarely the calyx reduced to a ring ; corolla sympetalous, 2-lipped or sometimes 1-lipped, lobes imbricate or contorted; stamens 4, didynamous, or 2, inserted on the corolla-tube and alternate with its lobes; filaments free , or partially connate in pair; anthers 2-locular or 1-locular by reduction, loculi confluent or separated, sometimes one much smaller than the other, opening lengthwise; disk present; ovary superior, sessile on the disk, 2-locular; style simple; ovules axile, 2 or more in each loculus; fruit a capsule, often club-shaped, mostly elastically dehiscent from the apex downwards, the valves recurved and leaving the central axis; seed mostly with indurated funicle; endosperm rarely present; embryo large (Hutchinson, 1959)

The genus *Clinacanthus* is a small genus belongs to the family Acanthaceae. Two species (*C. nutans* and *C. siamensis*) are cultivated throughout Southern China and Malaysia. The characteristic of plants in the genus *Clinacanthus* are flowers not enclosed within an involucre; calyx and corolla glandular-pubescent; corolla red with yellow; segments much shorter than the tube. Shrub, erect-drooping or straggling upwards (Backer and Bakhuizen van den Brink, 1965).

According to Index Kewensis and its supplements, there are only three species of the genus *Clinacanthus* as follows (Hill, 1931-1935);

1. *Clinacanthus nutans* (Burm. f.) Lindau

This indigenous plant is well known in Thai as Phaya yo(พญายอ), Phaya plong dam (พญาปล้องดำ, Central), Phaya plong thong (พญาปล้องทอง, Central) and Salaed pang phon tua mia (เสลดพังพอนตัวเมีย, Phisanulok) (Smitinand, 2001). The plant is a herbaceous climbing plant. Its stems and branches are vanish and 1-3 m in height. Leaves are simple, opposite, narrowly elliptic oblong or lanceolate, 2.5-13 cm long, 0.5-1.5 cm wide. Leaves have apex acute or acuminate, margin exsculptate-dentate or subentire, base cuneate, obtuse, rounded or truncate often oblique, pubescent on the nerves, petiole 3-15 mm long. Flowers are in dense cymes at the top of the branches and their branchlets, cymes 5-∝ flowered, often terminating on drooping horizontal branches but themselves erect, subsecund, and combined into a large lax, leafy panicle. Each flower has calyx densely patently glandular-pubescent, about 1 cm long, corolla glandular-pubescent, about 3.5 cm, dull red with green base, lower lip (turned upwards) with yellow streaks, apically yellow or greenish yellow. Stamen 2, inserted in the throat, more or less appressed against the upper lip. Ovary is compressed, 2-celled, 2-ovules in each cell, having style filiform, shortly bidentate. Capsule is oblong, basally contracted into a short, solid stalk, 4-seeded (Backer and Bakhuizen van den Brink.1965, Benoist, 1935).

2. *Clinacanthus spirei* R. Ben.

Tiges cylindriques, striees longitudinalement, glabres. Feuilles brievement petioles lanceolees, arrondies, a la base, acuminees au sommet, a bord entire, recourbe en dessous, glabres, mais un peu rudes sur les 2 faces, longues de 6-10 cm. sur 1.5-3 cm Inflorescence: fleurs en epis denses, terminaux, penches; bractees largement lanceolees, a sommet arrondi, obtus, pubescentes-glanduleuses, longues de 20-22 mm. Sepales 5, lineaires, pubescents-glanduleux, libres des la base, longs de 10 mm. environ. Corolle longe de 35-40 mm, le tube legererement courbe et selargissant graduellement jusqu a la gorge; levre superrieure lanceolee, echancree au sommet; l inferieure brievement trilobee. Etamines 2, a filets poilus. Ovaire et base du style poilus (Hill, 1931-1935; Benoist, 1935).

3. *Clinacanthus siamensis* Brem.

Caulis ramique an initio glabri, subteretes, leviter striate; internodia 1-4 cm longa et 2.5-3.0 mm diam. Folia petiole glabro plerumque 2-3 cm longo instructa; lamina lanceolata, plerumque 11-16 cm. longa et 3-5 cm lata, apice acuminate, basi acuta vel subacuta, rarius paulum asymmetrica, casu quo uno latere subrotundata, utrimque latere costae plerumque 5 vel 6. Inflorescentia corymbosa, nutans, rachidibus pedicellisque puberulis. Flores resupinati, bracteis linearibus 5-7 mm longis suffulti. Pedicelli circ. 2 mm longi. Calycis lobi lineares, 12 mm longi, ut bracteae pilis capitatis puberulo-hirtelli. Corolla 5 cm longa, extus pilis capitatis parce et vix notabile puberula, intus prope basin breviter pubescens, labiis 15 mm longis, supero deltoideo, ad basin 6 mm lato, apice emarginato, infero ad medium 9 mm lato, lobis 5 mm longis, basi 2.5 mm latis, apice obtusis, mediano conduplicato. Granula pollinis globosa, eis generic *Pseuderanthemi similiora*, plurima parva et sterilia, aliquae tamen normalia, 46 µ diam. Discus annularis glaber. Ovarium glabrum, 2.5 mm altum; stylus glaber 4.5 cm longa; stigma breviter bilobatum. Capsula nondum visa (Dansk botanisk forening, 1961).

Scandent shrub, 1.5-4 m high, branchlets erect-drooping climbing over other plants. Leaves simple, opposite, lanceolate or lanceolate-oblong, 2.5-4 cm wide, 7-12 cm long. Inflorescence in terminal dense cyme; flowers dull red with green base, tubular, bilabiate. Fruit capsule (Chuakul *et al.*, 1997).

Figure 1. ***Clinacanthus siamensis*** **Brem.**

2. The chemical constituents and biological activity of plants in genus *Clinacanthus*

Previous phytochemical studies and biological activity of plants in this genus have been reported mainly on *C. nutans*. The results of these investigations are summarized as follows:

2.1 Chemical constituents

A number of phytochemical studies on *C. nutans* have been reported. The compounds isolated include steroids and terpenoids (Dampawan, 1977; Lin, Li and Yu, 1983), glycosylflavonoids (Teshima *et al*., 1997), sulfur-containing glucosides (Teshima *et al*., 1998) and compounds related to chlorophyll a and chlorophyll b (Dechatiwonge Na Ayudhya *et al*., 2001).

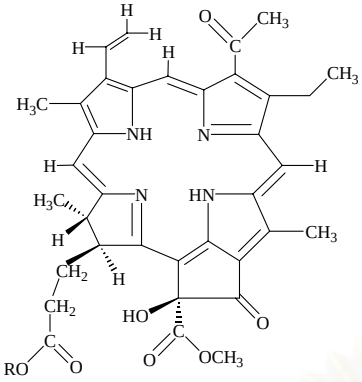
Table 1. Chemical constituents of *Clinacanthus* plants

Chemical constituents	Category	Plants part	References
13^2-hydroxy-(13^2-S)-phaeophytin b **[1]**	Chlorophylls	Leaves	Dechatiwonge Na Ayudhya *et al*., 2001
Phaeophorbide a **[2]**	Chlorophylls	Leaves	Dechatiwonge Na Ayudhya *et al*., 2001
Purpurin 18 phytyl ester **[3]**	Chlorophylls	Leaves	Dechatiwonge Na Ayudhya *et al*., 2001
(*E*)-3-methylsulfonyl-2-propenyl *β*-D-glucoside (clinacoside A) **[4]**	Glucosides	Stem and leaves	Teshima, *et al*., 1998
(*E*)-3-methylsulfinyl-2-propenyl *β*-D-glucoside (clinacoside B) **[5]**	Glucosides	Stem and leaves	Teshima, *et al*., 1998
Clinacoside C **[6]**	Glucosides	Stem and leaves	Teshima, *et al*., 1998

Table 1. (continued)

Chemical constituents	Category	Plants part	References
Cycloclinacoside A1 **[7]**	Glucosides	Stem and leaves	Teshima, *et al*., 1998
Cycloclinacoside A2 **[8]**	Glucosides	Stem and leaves	Teshima, *et al*., 1998
Isomollupentin 7-*O*-*β* -glucopyranoside **[9]**	Glycosylflavones	Stem and leaves	Teshima, *et al*., 1997
Isoorientin **[10]**	Glycosylflavones	Stem and leaves	Teshima, *et al*., 1997
Isovitexin **[11]**	Glycosylflavones	Stem and leaves	Teshima, *et al*., 1997
Orientin **[12]**	Glycosylflavones	Stem and leaves	Teshima, *et al*., 1997
Shaftoside **[13]**	Glycosylflavones	Stem and leaves	Teshima, *et al*., 1997
Vitexin **[14]**	Glycosylflavones	Stem and leaves	Teshima, *et al*., 1997
Betulin **[15]**	Terpenoids	Root	Lin, *et al*., 1983
Lupeol **[16]**	Terpenoids	Root stem	Lin, *et al*., 1983 Dampawan, *et al*., 1977
Stigmasterol **[17]**	Steroids	Root	Lin, *et al.*, 1983
β-sitosterol **[18]**	Steroids	Root stem	Lin, *et al*., 1983 Dampawan, *et al*., 1977

Compounds related to chlorophyll



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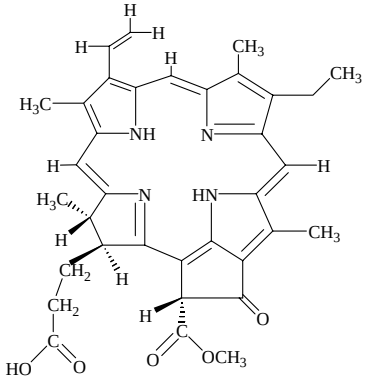

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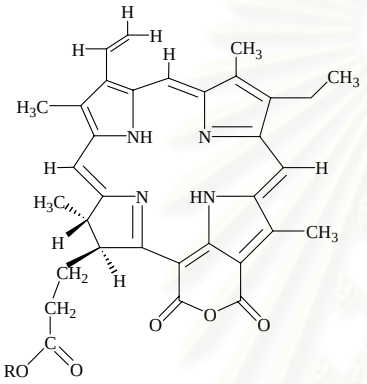
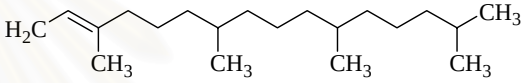
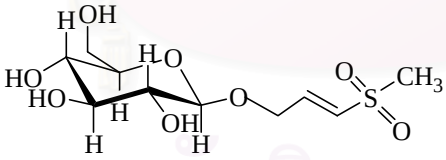


R = 

[3]

Glucosides



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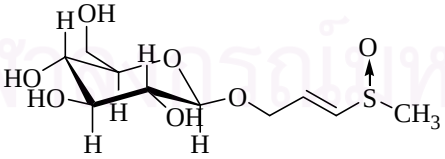

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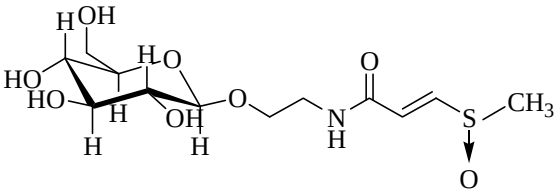
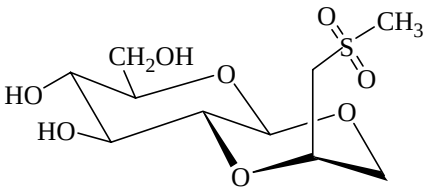


[6]

Glucosides (continued)



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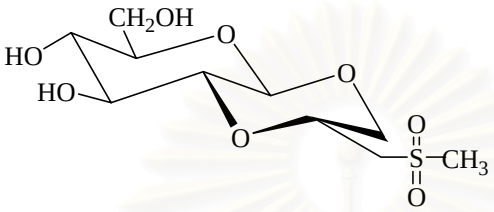
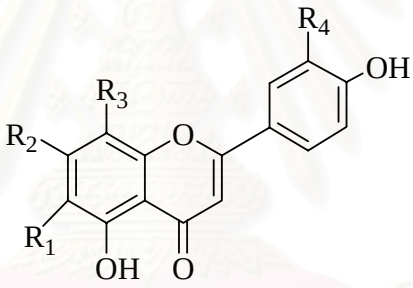


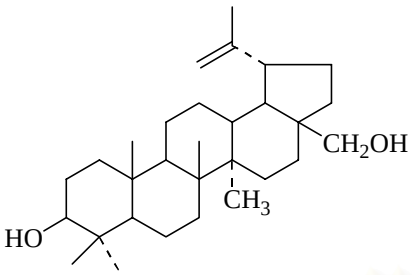
[8]

Glycosylflavones



	R_1	R_2	R_3	R_4
[9] Isomollupentin 7-O-β –glucopyranoside	Ara	O-Glc	H	H
[10] Isoorientin	Glc	OH	H	OH
[11] Isovitexin	Glc	OH	H	H
[12] Orientin	H	OH	Glc	OH
[13] Shaftoside	OH	Glc	H	H
[14] Vitexin	H	OH	Glc	H

Terpenoids



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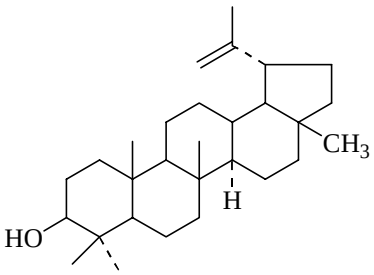
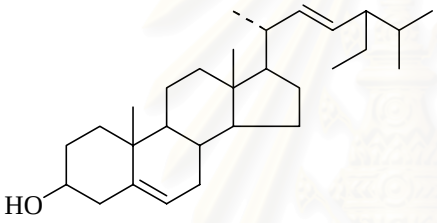


[16]

Steroids



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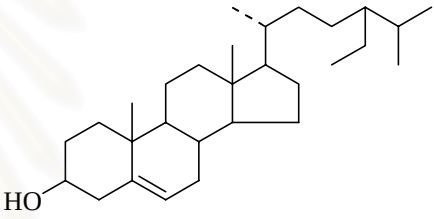


[18]

2.2 Biological activities

2.2.1 Anti-cobra venom activity

The effect of *C. nutans* extract against cobra venom was examined by intravenous injection of cobra venom to the tested mice. The intravenous LD_{16}, LD_{50} and LD_{84} of cobra venom were 0.13, 0.22 and 0.38 mg/kg body weight, respectively. Alcoholic extract of *C. nutans* (2g/ kg) administered intravenously following cobra venom significantly reduced the lethal effect of LD_{50} from 50% to 10%. But this amount of the extract did not change the number of dead mice from LD_{84} of the venom, although various routes of administration (intravenous, intraperitoneal and oral routes) were tried. Significant increase in the mortality of the experiment mice from the control (48%) to 70%, 60% and 70% were observed when cobra venom (0.38 mg/kg) was administered subcutaneously followed by alcoholic extract of *C. nutans* (2g/kg) via the intravenous, intraperitoneal and oral routes, respectively (Thongtharb and Tejasen, 1977).

2.2.2 Human cell-mediated immune response

The effects of *C. nutans* extract on lymphocyte proliferation, function of natural killer (NK) cells, and production of interleukin-2 (IL-2) and interleukin-4 (IL-4) were investigated. Lymphocyte proliferation was significantly increased at the extract concentrations of 0.5, 2.5 and 5 µg/ml, while the response was significantly reduced at 2.5 and 5 mg/ml. of *C. nutans* extract. The activity of NK cells was significantly decreased at the concentrations of 1 and 5 mg/ml. Furthermore, the level of IL-2 released from *C. nutans*-treated mononuclear cells was undetectable, whereas that of IL-4 was shown to be induced by the extract at the concentrations of 2.5 and 5 mg/ml. These data suggested that the effect of *C.nutans* on human CMIR *in vitro* may be partially due to the release of IL-4 from peripheral blood mononuclear cells (Sriwanthana, Chavalittumrong and Chompuk, 1996).

2.2.3 Anti-herpes simplex and herpes zoster virus activity

The extract of *C. nutans* was investigated for virucidal effect against herpes simplex virus (HSV). The activity was measured *in vitro* using a plaque reduction assay of HSV-2 in baby hamster kidney cell line. *C. nutans* extract completely inhibited plaque formation by HSV-2 at the concentration more than 1:2,400 and harvesting time for 45 minutes. However, it could not inhibited penetration of HSV-2 to the cultured cells (pre-treatment) and no activity was observed when the cultured cells were already infected (post-treatment) (Jayavasu *et al*., 1992a).

The antiviral effect of crude extract of *C. nutans* was also determined against varicella-zoster virus (VZV) by plaque reduction assay and DNA hybridization, compared with acyclovir as the positive control. *C. nutans* was found to be more active at reacting on virus directly (inactivation assay) than inhibition of viral penetration (pre-treatment) or inhibition of the infected cell (post-treatment). Via DNA hybridization, the ID_{50} (50% inhibitory dose of *C. nutans*) levels of pre-treatment, post-treatment, and inactivation assay were at dilution of 1:2,000, 1:6,000, and >1:18,000, respectively, whereas by plaque reduction assay, they were 1:2,000, 1:4,800 and 1:9,600, respectively. Since significant inhibition could be detected in the inactivation assay, the anti-VZV activity of the extract might be based on a direct interaction between active compounds in *C. nutans* and the virus, which is different mode of action difference from that of acyclovir (Thawaranantha *et al*., 1992).

In 1999, the extract of *C.nutans* was investigated for virucidal effects against herpes simplex virus type 2 strain G (standard strain) and five clinical HSV-2 isolates by inactivation kinetics assay, plaque inhibition assay (pre-treatment) and yield reduction assay (post-treatment). The results indicated that *C. nutans* extract could inactivate HSV-2 (G) at the concentration range from 253 to 2500 µg/ml. In plaque inhibition assays, acyclovir was used as positive control. *C. nutans* extracts was found to be inactive in this assay (Yoosook, 1999).

Clinical trial to evaluate the activity of *C. nutans* in 77 genital herpes-suspected patients was performed cream base developed from the extract of *C. nutans* compared with acyclovir and placebo. The patients who used *C. nutan*s, as well as

those who used acyclovir, developed crust lesion within 3 days and healing within 7 days while the crusting time of the placebo groups was in 4-7 days and healing time was 7-14 days or more. The trial showed *C. nutans* was efficient in shortening the duration of infection and reducing the severity. *C. nutans* cream also causes no sticky, burning, stinging pain during use, unlike acyclovir. No change in the level of eosinophils was detected during topical use of this cream (Jayavasu *et al*., 1992b).

In another trial, 163 patients with recurrent genital herpes simplex-type 2 virus infection were divided into 3 groups: experiment group using *C. nutans* cream, positive control group using acyclovir cream and negative control group using placebo. Patients entered within 48 hours of the onset of lesions, and the studied medication was applied 4 times daily for 6 days. The results were similar to the previous clinical trial (Jayavasu *et al*., 1992b). There was no side effect of *C. nutans* extract during treatment (สมชาย แสงกิจพร และคณะ, 2536).

2.2.4 Anti-inflammatory activity

Anti-inflammatory activity of *C. nutans* was examined using the carrageenin-induced hind paw edema and granuloma pouch technique to compare with other anti-inflammatory agents in animals models. It was found that the n-butanol extract *C. nutans* at the dose of 100 mg/kg was equally active to phenylbutazone and aspirin at 90 mg/kg and 270 mg/kg, respectively, while the extract at 540 mg/kg had equal anti-inflammatory activity to 4 mg/kg of indomethacin (Kittisiripornkul, 1984; Tanasomwong, 1986).

3. Toxicity tests

3.1 Acute toxicity

Acute toxicity study of ethanolic extract of *C. nutans* leaves in mice showed that the extract at the highest dose of 1.3 g/kg of body weight (g/kg BW), which is equivalent to 5.44 g/kg BW of dried powder leaves, given orally, subcutaneous or

intraperitoneally did not produce any signs of toxicity in the animals (Chavalittumrong *et al.*, 1995).

3.2 Subchronic toxicity

Subchronic toxicity study was performed in rats by giving the extract daily for 90 days at the oral doses of 0.01, 0.1 and 1 g/kg BW, equivalent to dried leaves 0.042, 0.42 and 4.18g/kg BW, respectively. The body weights of male rats receiving1.0 g/kg BW of the extract were found to be significantly lower than those of the control group ($P<0.05$), whereas food consumption of the control group and the extract-treated groups was not different (Chavalittumrong *et al.*, 1995).

3.3 Hematological and blood chemistry study

Platelet counts of the mice of both sexes receiving 1.0 g/kg BW of the extract were significantly higher than the control group ($P<0.05$) and all extract-treated groups had lower creatinine level lower than the control group ($P<0.05$). However, histopathological examination of internal organs did not show abnormalities that could be due to the effect of the extract (Chavalittumrong *et al.*, 1995).

3.4 Gastric ulcerogenicity

Butanolic extract of *C. nutans* orally administered to mice at the highest dose of 2.34 g/kg showed ulcerogenicity but to a lesser extent than aspirin given at 100 mg/kg. After 6 weeks of oral administration of the extract at 270 and 540 mg/kg, gastric ulcers could be found but less than those from 100 mg/kg of aspirin (Kittisiripornkul, 1984).

3.5 Skin irritation

The 95% ethanolic extract of *C. nutans* applied on the skin of rabbits and rats did not irritate the skin of the rabbits at the doses of 100 and 200 g%, although the dose of 100g% was found to irritate rat's skin. In sensitization test, the doses of 200 g% of extract caused skin rashes in rats (Kittisiripornkul, 1984).

Presently there is no report on phytochemical and biological activity of *C. siamensis*. Judging from many interesting biological activities of *C. nutans*, *C. siamensis* has thus been selected for investigation in this study.

CHAPTER III

EXPERIMENTAL

1. Source of plant material

The leaves of *Clinacanthus siamensis* Brem. were collected from a garden in the outlying area of Bangkok, Thailand in November 2002. Authentication was performed by comparison with herbarium specimens (BKF No. 130056) at the National Park Wildlife and Plant Conservation Department, Ministry of Natural Resources and Environment. The dried leaves (6 kg) were obtained after drying at the temperature of about 45°C and ground for extraction.

2. General techniques

2.1. Thin-layer chromatography (TLC)

Technique	:	One dimension, ascending
Adsorbent	:	Silica gel GF_{254} (E. Merck) and RP18 silica gel GF_{254} (E. Merck)
Layer thickness	:	250 μm
Distance	:	6 cm.
Temperature	:	Room temperature (30-35°C)
Detection	:	1. Ultraviolet light at wavelength of 254 and 365 nm. 2. Anisaldehyde reagent and heat until colors developed. 3. Visual detection in iodine vapor.

2.2. Column chromatography

2.2.1. Gel filtration chromatography

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

Packing method : Sephadex LH-20 gel was suspended in the eluent and left standing to swell overnight prior to use. It was then poured into column and allowed to set tightly.

Sample loading : The sample was dissolved in a small volume of the eluent and loaded on top of the column.

Detection : Fractions were detected by TLC techniques as described in Section 2.1

2.2.2. Vacuum liquid column chromatography

Adsorbent : Silica gel (No. 1.07734), particle size 0.063-0.200 mm. (70-230 mesh ASTM) (E. Merck).

Packing method : Dry packing in the sintered glass filter column

Sample loading : The sample was dissolved in a small volume of organic solvent, mixed with a small quantity of adsorbent, triturated, dried and then applied gently on top of the column.

Detection : Fractions were detected by TLC techniques as described in Section 2.1

2.2.3. Flash column chromatography

Adsorbent : Silica gel (No. 1.07734), particle size 0.063-0.200 mm (70-230 mesh ASTM) (E. Merck).

RP18 silica gel (No. 1.10167), particle size 43-60 μm (E. Merck).

Packing method : The adsorbent was suspended in the eluent. The slurry of the adsorbent was poured into the column and then allowed to settle overnight.

Sample loading : The sample was dissolved in a small volume of the eluent and loaded on top of the column

Detection : Fractions were detected by TLC technique as described in the section 2.1.

2.3. Gas chromatography (GC)

Column oven	:	205/205 °C		
Inj Port A	:	250/250 °C		
FID-1	:	260/260 °C		
Flow	:	1. Control mode	:	split
		2. Column Press (Kpa)	:	282/282
		3. Column flow (ml/min)	:	1.5/1.5
		4. Linear velocity (cm/s)	:	30/30
		5. Total flow (ml/min)	:	52/52
		6. Split ratio	:	25/25
		7. Column length (m)	:	60
		8. Column diameter (mm):		0.25

Reference standard : Fatty acid methyl ester mixture #13 (35034), Restek corporation (Figure 61).

The gas chromatograms were obtained on a GC-17A Gas chromatograph Shimadzu (Division of Biotechnology, School of Bioresources and Technology, King's Mongkut University of Technology, Thonburi).

3. Spectroscopy

3.1. Proton and carbon nuclear resonance (^{1}H and ^{13}C NMR) spectroscopy

^{1}H and ^{13}C NMR, DEPT 90 and 135, ^{1}H-^{1}H COSY, HMQC and HMBC spectra were obtained on a Bruker Advance DPX-300 FT-NMR spectrometer, operating at 300 MHz for protons, 75 MHz for carbons. Solvents for NMR spectra were deuterated pyridine (pyridine-d_5) and deuterated chloroform ($CDCl_3$). Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal. Proton-detected heteronuclear correlations were measured using HMQC (optimized for $^{n}J_{HC}$ = 145 Hz) and HMBC (optimized for $^{n}J_{HC}$ = 4 and 8 Hz) pulse sequences.

3.2. Ultraviolet (UV) absorption spectroscopy

UV (in methanol) spectra were obtained on a Milton Roy Spectronic 3000 Array spectrophotometer.

3.3. Infrared (IR) absorption spectroscopy

IR spectra (film) were obtained on a Perkin Elmer 2000 FT-IR 1760X spectrometer.

The measurement of UV, IR and NMR properties were performed at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

4. Chemical reactions

4.1. Acetylation

The sample dissolved in pyridine (0.5ml) was treated with acetic anhydride (0.5 ml). The solution was stirred at room temperature for 12 hours under nitrogen. The reaction mixture was stopped by adding 10 ml of water into the flask. The aqueous solution was partitioned with 15 ml of $CHCl_3$ three times. The $CHCl_3$ extract was dried under reduced pressure to give the acetylated compound (Rho *et al*., 1997).

4.2. Formation of fatty acid methyl ester

4.2.1. Diazomethane treatment (Esterification)

The fatty acid was treated with diazomethane (CH_2N_2) to produce the fatty acid methyl ester for GC analysis. To prepare CH_2N_2, 0.4 ml of cold 50% aqueous KOH was added to 10 ml of diethyl ether (Et_2O_2), then cooled the solution in ice bath to 5°C. The solution was stirred very slowly by magnetic stirrer while 500 mg of 1-methyl-3-nitro-1-nitrosoguanidine ($C_2H_5N_4O_3$) was added in small portions over an hour. CH_2N_2 generated by the reaction of $C_2H_5N_4O_3$ with 50% KOH was poured into

a volumetric flask filled with 2 g of KOH pellet. The solution was stored in the freezer for 2 hours before using (Kitagawa, Hayashi and Kobayashi, 1989; Jung, Lee and Kang, 1996).

4.2.2. Acid hydrolysis (Transmethylation)

One mg of glycolipids was dissolved in 4 ml of 0.5N anhydrous methanolic HCl, capped under argon, and incubated at 98°C for 2 hour. After cooling to room temperature, the reaction mixture was extracted with hexane and injected directly into the GC column (Esch *et al*., 1999)

4.2.3. Enzymatic hydrolysis

The sequence of fatty acid residues of glycolipids was determined by regio-selective enzymatic hydrolysis, which occurred at the *sn*-1 position of the glycerol moiety without acyl migration. The sample (1 mg) was dissolved in 100 μl dioxane-water (1:1), then 15 units (0.5 mg) of lipase type XIII from *Pseudomonas* sp. (Lot 36H068, Sigma) were added and the mixture incubated at 37°C for 3 hours. The reaction was quenched by adding 5% acetic acid (125 μl), and EtOH was added to the reaction mixture. Solvent was removed under reduced pressure and the resulting residue dissolved in THF. The THF solution was added with a small volume of the CH_2N_2 solution and the reaction mixture was extracted with hexane. The hexane layer was condensed under reduced pressure and analyzed by GC (Kobayashi *et al*., 1992; Jung, Lee and Kang, 1996).

5. Solvents

All commercial grade solvents were redistilled prior to use and analytical grade solvents were used for chemical reaction, respectively.

6. Anti-herpes simplex activity

The assay for anti-herpes simplex activity was performed at the Department of Microbiology, Faculty of Pharmaceuticals Sciences, Chulalongkorn University. Selected fractions and isolated compounds were examined for anti-herpes simplex virus types 1 and 2 activity by plaque reduction method as follows.

Antiviral activity against HSV-1 (KOS) and HSV-2 (186) was evaluated using the plaque reduction assay (inactivation). Briefly, virus (30 PFU/25 μL) was mixed with 25 μL of complete medium containing various concentrations of test compound and then incubated at 37°C for 1 hr. After incubation, the mixtures were added into Vero cells ($6x10^5$ cells/well) in 96-well microtiter plates and incubated at 37°C for 2 hr. The overlay medium containing various concentrations of test compound was added to the Vero cells and incubated at 37°C in humidified CO_2 incubator for 2 days. Then, virus growth inhibition was evaluated by counting the virus plaque forming on Vero cells compared with the controls. The cells also were stained with 1% crystal violet in 10% formalin for 1 hr. The percent plaque inhibition was determined. Acyclovir was used as positive control.

Antiviral activity was expressed as a score of % inhibition (+4 = 100%, +3 > 50%, +2 = 50% and +1 < 50%) and 50% effective concentration (ED_{50}), the concentration of extract that inhibited plaque formation by 50%, compared with control as calculated by the median effect method (Abou-Karam and Shier, 1990; Lipipun, *et al*., 2000).

7. Extraction and isolation

7.1. Extraction of the leaves of *Clinacanthus siamensis* Brem.

The dried leaves of *C. siamensis* (6 kg) were macerated with 20 L methanol for 3 times. The obtained extract was evaporated under reduced pressure at temperature not exceeding 50°C to give 3 L of the concentrated methanol extract (F001). The extract was dissolved with methanol, and then water was added to make

20% methanol in water solution. The aqueous methanol solution was partitioned with 10 L of ethyl acetate. The ethyl acetate solution was evaporated to give 360 g of the ethyl acetate extract (F002), which was dissolved in 10 L of methanol and partitioned with 10 L of hexane to yield 300 g of the hexane extract (F004) and 60 g of the methanol extract (F005) (Scheme 1). Each extract was subjected to with anti-herpes simplex activity assay (as described in Section 6). The methanol extract showing anti HSV-1 and HSV-2 at concentration of 50 μg/ml was chosen for further separation.

7.2. Isolation of Compound CSF 1

The methanol extract (F005) was chromatographed on a vacuum liquid column using a sintered glass filter column of silica gel (No. 7734, 270 g). F005 (60 g) was dissolved in $CHCl_3$, mixed with 60 g of kieselguhr, dried and then ground. The interesting fraction was detected by anisaldehyde spraying reagent which showed positive as violet color after heated. Elution was performed in a polarity gradient manner with mixtures of chloroform-methanol ($CHCl_3$, 2%, 5%, 10%, 20% methanol in $CHCl_3$ and 100% methanol). The eluates were collected as 150 ml fractions and examined by TLC (silica gel, $CHCl_3$-MeOH = 4:1), spraying with anisaldehyde and heating. Fractions (a total of 62 fractions) with similar chromatographic pattern were combined to yield 4 fractions: F006 (22.0 g), F007 (6.0 g), F008 (8.2 g) and F009 (2.6 g).

F007 (6.0 g) equally divided into 4 portions, was further purified by using a column of Sephadex LH20 (3.5 x 45 cm) with $CHCl_3$-MeOH (1:1) as the eluent. The eluates were collected as 20 ml fractions and examined by TLC (silica gel, $CHCl_3$-MeOH = 4:1) sprayed with anisaldehyde. Combination of fractions showing similar chromatographic pattern gave 3 fractions: F010 (260 mg), F011 (4.3 g) and F012 (700 mg). F011, appearing as waxy mass, was purified by dissolving in a small volume of $CHCl_3$, then adding methanol into the solution, which was left standing at –20°C until the precipitated settled. The mother-liquor was repeatedly precipitated until no further crop was observed. The precipitate was washed with a small volume of methanol to yield CSF 1 (32 mg) (Scheme 1) and F011′ was further separated.

7.3. Isolation of Compound peracetyl CSF 3

F008 (8.2 g) showed violet spots on TLC (silica gel GF_{254}) after spraying with anisaldehyde reagent. F008 was fractionated by gel filtration chromatography using a column of Sephadex LH20 (3.5 x 50 cm) with EtOAc-MeOH (1:1) as the eluent. The eluates were collected as 20 ml fraction and examined by TLC (silica gel, $CHCl_3$-MeOH = 4:1), spraying with anisaldehyde. Fractions were combined to yield 5 fractions: F016 (530 mg), F017 (490 mg), F018 (3.5 g), F019 (740 mg) and F020 (70 mg).

From TLC pattern, F018 (3.5 g) was chosen for separation by flash column chromatography (silica gel 60 No. 1.07734, 85 g). Elution was performed in a polarity gradient manner with mixtures of $CHCl_3$-MeOH ($CHCl_3$, 2%, 5%, 10%, 20% methanol in $CHCl_3$ and 100% methanol). The eluates were collected as 20 ml per fractions and combined to yield 3 fractions: F043 (575 mg), F044 (1.7 g) and F045 (89.5 g).

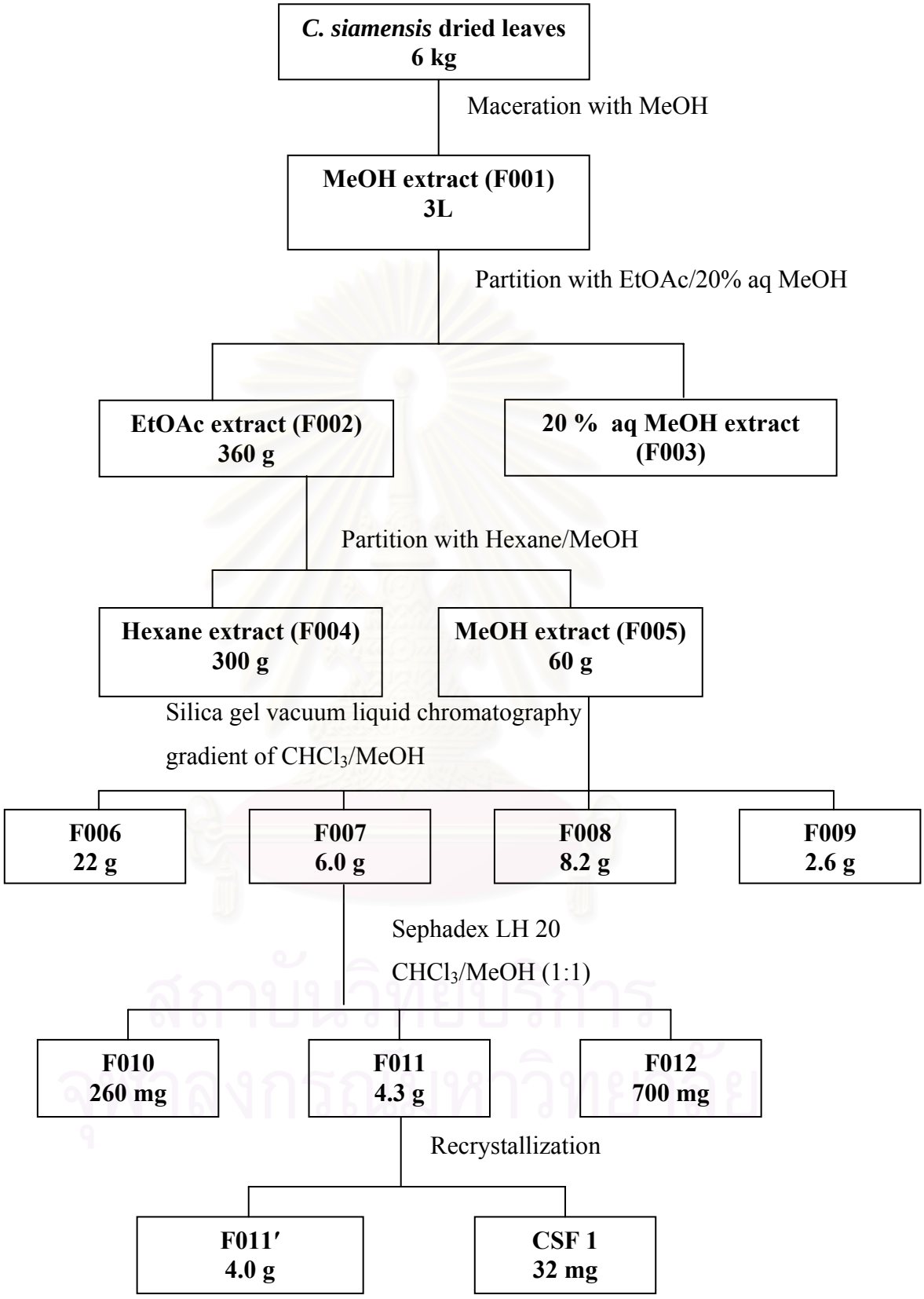
F045 was chromatographed by using RP18 silica gel G60 flash column chromatography with MeOH-THF-H_2O (5:3:2) as the eluent. The sample was dissolved in a small amount of the eluent and loaded into the column. The eluates were examined by TLC (silica gel, $CHCl_3$-MeOH = 4:1). Three combined fractions: F046 (33 mg), F047 (365 mg) and F048 (110 mg) were obtained. F047 (365 mg) was separated on a Sephadex LH20 column (EtOAc-MeOH = 4:1) to yield 3 fractions: F049 (73 mg), F050 (139 mg) and F051 (40 mg). F050 showed one violet spot on TLC (silica gel, $CHCl_3$-MeOH = 4:1) after spraying with anisaldehyde. Then, a portion (20 mg) of this fraction was subjected to acetylation (as described in 4.1) and the acetylated products were used for determination of their chemical structure. The acetylated compounds were isolated by silica gel column chromatography using EtOAc:hexane (2:3) as the eluent to obtain peracetyl derivative of CSF 3 (12 mg) (Scheme 2).

7.4. Isolation of Compound peracetyl CSF 2 And CSF 4

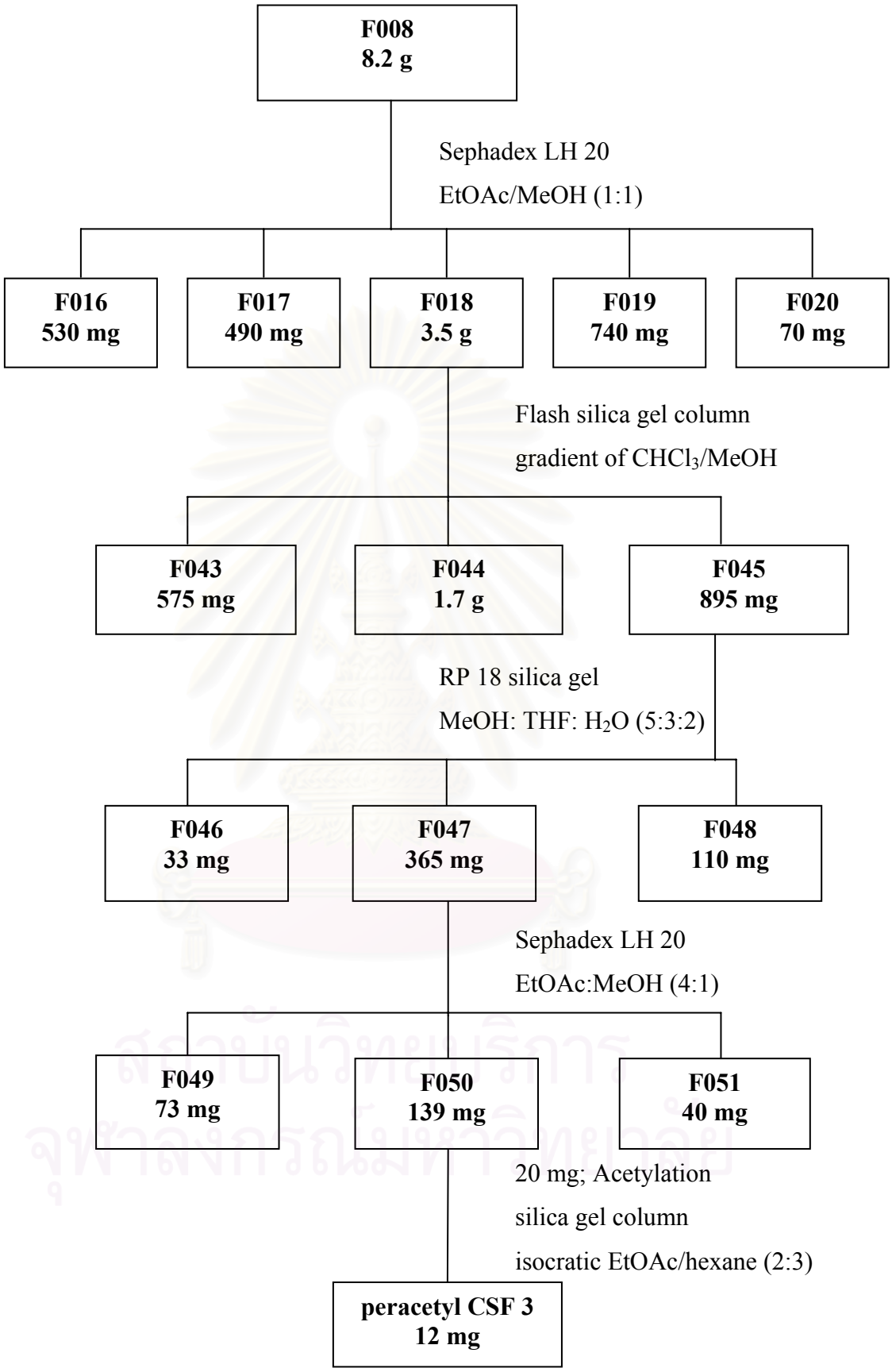
F011′ (4.0 g) showing violet spots after spraying with anisaldehyde reagent, was selected for further separation by gel filtration chromatography, using a column of Sephadex LH20 with $CHCl_3$-MeOH (1:1) as the eluent. The eluates were collected into 20 ml fractions and examined by TLC (silica gel, $CHCl_3$-MeOH = 4:1) sprayed with anisaldehyde. Three combined fractions: F013 (890 mg), F014 (2.2 g) and F015 (980 mg) were obtained. F014 (2.23 g) was further separated by another Sephadex LH20 column, eluted by ethyl acetate, into 4 fractions: F021 (140 mg), F022 (310 mg), F023 (1.3 g) and F024 (230 mg). F023 showed several violet spots on TLC (RP 18 silica gel GF_{254}: MeOH-THF-H_2O = 7:2:1) after spraying with anisaldehyde.

From TLC pattern, F023 (1.3 g) was chosen for separation using RP18 silica gel G60 flash column chromatography with MeOH-THF-H_2O (7:2:1) as the eluent. The sample was dissolved in a small amount of MeOH-THF-H_2O (7:2:1) and loaded into the column. The eluates were examined by TLC (silica gel, $CHCl_3$-MeOH = 4:1). Five combined fractions: F052 (400 mg), F053 (140 mg), F054 (250 mg), F055 (87 mg) and F056 (195 mg) were obtained.

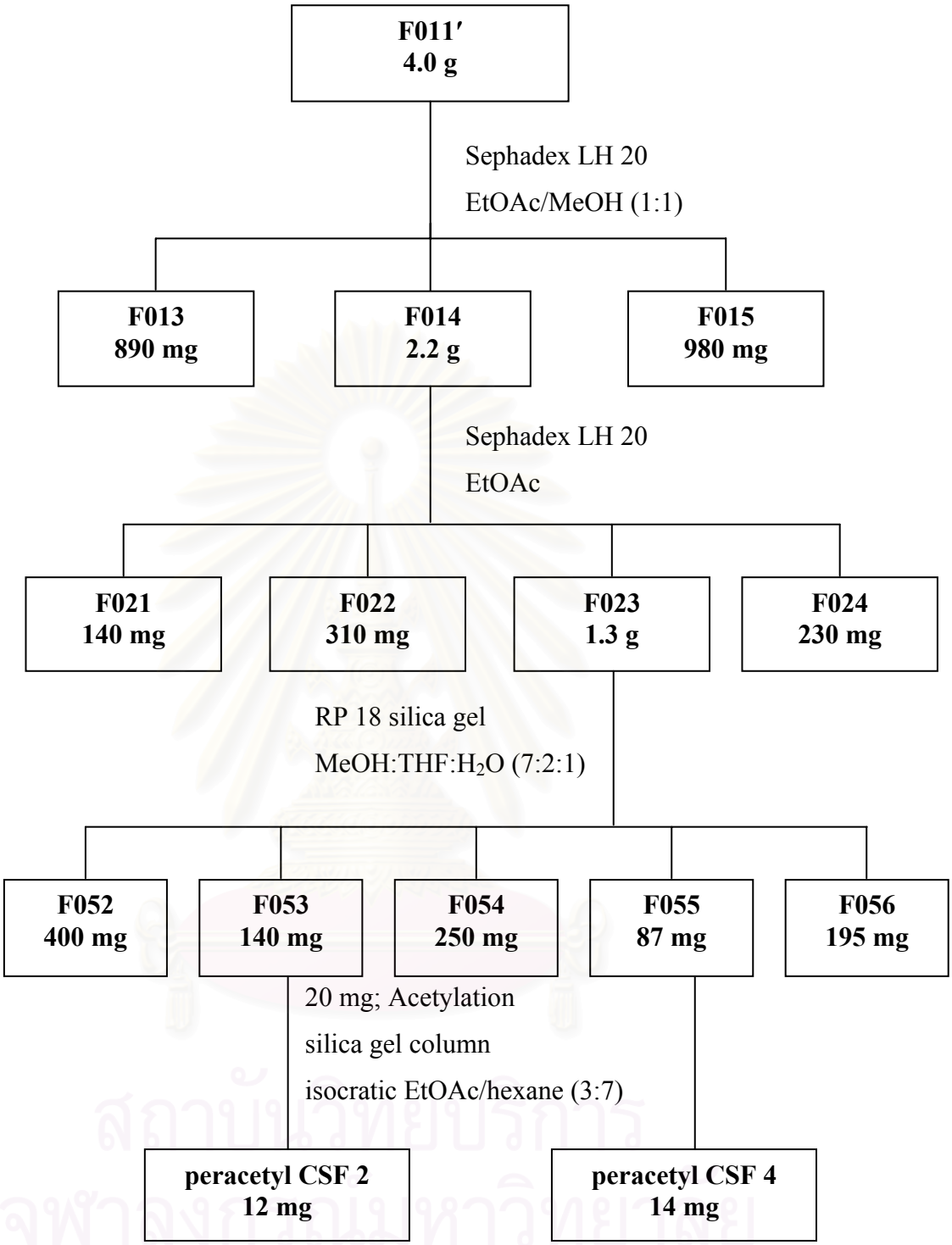
F053 and F055 showed several violet spots on TLC (RP18 silica gel GF_{254}) after spraying with anisaldehyde. Therefore, a portion (20 mg) of these fractions was subjected to acetylation and the acetylated products were used for determination of their chemical structures. The acetylated compounds were isolated by silica gel column chromatography using EtOAc:hexane (3:7) as the eluent to yield the peracetyl derivatives of CSF 2 (12 mg) and CSF 4 (14 mg) (Scheme 3).



Scheme 1. Fractionation of *C. siamensis* (F001-CSF 1)



Scheme 2. Fractionation of *C. siamensis* (F008-peracetyl CSF 3)



Scheme 3. Fractionation of *C. siamensis* (F011-peracetyl CSF 2 and CSF 4)

CHAPTER IV

RESULTS AND DISCUSSION

The methanol extract of *C. siamensis* leaves (F001) was partitioned with ethyl acetate and 20% aqueous methanol. The ethyl acetate extract was then partitioned between hexane and methanol. The methanol extract exhibited significant anti-herpes simplex virus activity and was further fractionation using several chromatographic techniques to afford four compounds including CSF1, CSF2, CSF3, and CSF4. The compounds were obtained in the yields of $5.3x10^{-6}$, $2.3x10^{-5}$, $2.3x10^{-5}$, and $1.4x10^{-5}$ % w/w of the dried leaves, respectively.

The structures of all isolates were determined by interpretation of their UV, IR, NMR, and GC data, and further confirmed by comparison with literature values.

1. Structure determination of the isolated compounds

1.1. Structure determination of compound CSF 1

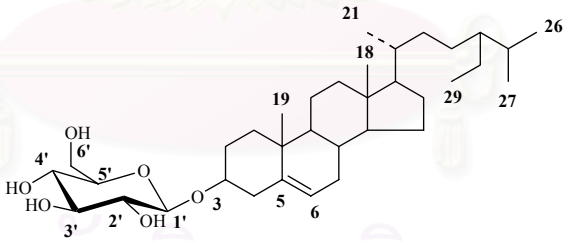
Compound CSF 1 appeared as colorless amorphous powder giving pink color with anisaldehyde reagent on silica gel TLC plate. The IR spectrum displayed absorption bands at 3,431 (O-H stretching), 2,929 (C-H stretching), 1,048 cm^{-1} (symmetric C-O-C stretching) (Figure 9). The ESIMS of this compound exhibited two pseudomolecular ion peaks $[M+Na]^+$ at *m/z* 597.3616 and 599.4213, implying the presence of two components with the molecular formulas $C_{35}H_{58}O_6$ (MW= 574) and $C_{35}H_{60}O_6$ (MW= 576)(Figure 10).

The ^1H NMR spectrum displayed signals for five methyl groups at δ 0.71, 0.91, 0.92, 0.94 and 0.98. Signals for several methine and methylene protons appeared at δ 0.90-2.00 ppm. In addition, a proton signal at δ 5.35 (1 H, br s) was assigned to H-6 of β-sitosterol-3-*O*-glucoside along with β-stigmasterol-3-O-glucoside in minor quantities. The olefinic proton signals appearing at δ 5.07 (0.3H, dd, *J*= 15.3, 8.4 Hz) and 5.20 (0.3 H, dd, *J*=15.3, 8.4 Hz) were assigned to H-22* and

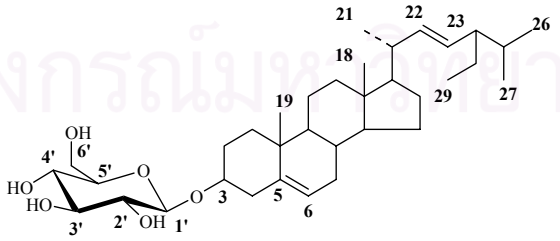
H-23* of β-stigmasterol-3-O-glucoside. The observed vicinal coupling constant of anomeric proton of sugar moiety at δ 5.07 (d, *J*=7.5 Hz) between the *trans* diaxial oxymethine proton $H_{1'}$ and $H_{2'}$ indicated the β-configuration of the glycosidic linkage. Integration of H-6, H-22* and H-23* was approximately in the ratio of 2.75:1:1. Therefore, it could be deduced that CSF 1 was a mixture of β-sitosterol-3-O-glucoside and β-stigmasterol-3-O-glucoside in the ratio of 7:4 (Figure 11).

The ^{13}C NMR spectrum of CSF1 displayed anomeric carbon at δ 102.26 ppm whereas signals between 80-60 ppm accounted for the presence of a glucose moiety. The downfield shift of anomeric carbon indicated that the sugar was attached to sitosterol and stigmasterol with *O*-linkage (δ 70-80 ppm for *C*-glycoside and 90-112 ppm for *O*-glycoside) (Agrawal, 1992). The small signals at δ138.77 and 129.47 ppm were assignable to C-22* and C-23* of β-stigmasterol-3-O-glucoside (Figure11 and 12).

These ^{1}H and ^{13}C data of compound CSF1 were in good agreement with those reported for the mixture of β-sitosterol-3-O-glucoside and β-stigmasterol-3-O-glucoside (Navickiene and Lopes, 2001; Pires *et al*,. 2002) (Table 2).



β-sitosterol-3-O-glucoside



β-stigmasterol-3-O-glucoside

Figure 2. Chemical structures of β-sitosterol-3-O-glucoside and β-stigmasterol-3-O-glucoside

Table 2. The ^{13}C NMR spectral data of β-sitosterol-3-O-glucoside, β-stigmasterol-3-O-glucoside, and compound CSF1

Position	β-sitosterol-3-O-glucoside[a] (ppm)	β-stigmasterol-3-O-glucoside[b] (ppm)	CSF1[c] (ppm)
1	37.2	37.6	37.7
2	31.8	30.4	30.5
3	77.9	78.2	78.2
4	39.7	39.5	40.2
5	140.7	141.1	140.8
6	121.7	122.1	121.9
7	31.9	32.3	32.3
8	29.9	32.2	32.4
9	50.1	50.5	50.5
10	36.6	37.1	37.1
11	21.0	21.5	21.5
12	39.0	40.0	39.5
13	42.7	42.5	42.7
14	56.5	57.0	57.0
15	24.2	24.7	24.7
16	28.2	28.7	28.7
17	56.0	56.2	56.4
18	12.2	12.3	12.2
19	18.9	19.2	19.6
20	36.1	41.0	36.6,41.0
21	18.7	19.6	19.27
22	33.9	139.0	34.4,140.9
23	26.1	129.6	26.6,124.1
24	45.8	51.6	39.5

Table 2. (continued)

Position	β-sitosterol-3-O-glucoside[a] (ppm)	β-stigmasterol-3-O-glucoside[b] (ppm)	CSF1[c] (ppm)
25	29.2	32.4	29.7
26	19.1	21.7	19.5
27	19.7	19.4	20.2
28	23.1	25.9	23.6
29	12.4	12.7	12.4
1′	102.3	102.7	102.6
2′	75.0	75.5	75.4
3′	78.3	78.8	78.6
4′	71.4	71.8	71.8
5′	78.1	78.7	78.7
6′	62.5	63.0	63.0

[a] measured in 500 MHz (C_5D_5N), (Navickiene and Lopes, 2001)

[b] measured in 500 MHz (C_5D_5N), (Pires, *et al*., 2002)

[c] measured in 300 MHz (C_5D_5N)

2. Structure determination of compound CSF 2

Fraction F053 from the methanol extract, showing violet spot upon spraying with anisaldehyde reagent, was subjected to be investigated for the anti-herpes simplex compounds. The ^{1}H NMR spectrum of fraction F053 suggested that it was a mixture of glycolipids. The broad signal at chemical shifts between 3.3 and 5.6 ppm revealed the presence of the sugar moieties. The two terminal methyl signals (δ 0.85, 3H, br t and δ 0.97, 3H, t, *J*= 7.5 Hz), and a broad methylene signal at δ 1.35 ppm indicated long chain fatty acids moieties (Figure 14).

Several attempts to isolate these complex glycolipids from this fraction by conventional methods had been unsuccessful. Therefore, to purify these glycolipid compounds, fraction F053 was acetylated prior to separation with chromatographic techniques. Compound peracetyl CSF2 was isolated from the acetylated fraction, and subjected to chemical structure determination by spectroscopic techniques.

The UV spectrum of peracetyl CSF 2 in MeOH exhibited λ_{max} at 268 nm (Figure 15). The IR spectrum displayed absorption bands at 3,000-2,840 (C-H stretching) and 1,740 cm^{-1} (C=O stretching) (Figure 16). In the 300 MHz ^{1}H-NMR and ^{13}C-NMR spectra of the peracetyl CSF2, the presence of resonances of a sugar, glycerol and aliphatic hydrocarbon with double bond strongly suggested glycolipid nature of the compound.

Analysis of the ^{1}H-NMR, ^{13}C-NMR, DEPT, ^{1}H-^{1}H COSY and HMQC spectra allowed the assignments of all the ^{1}H-NMR and ^{13}C-NMR signals for the sugar and the glycerol moieties as shown Table 3.

The ^{13}C-NMR spectrum of peracetyl CSF 2 exhibited many signals in the region between 20-40 ppm (alkyl chain), 8 signals between 60-75 ppm (sugar and glycerol methines and methylenes), one signal at 101.5 ppm (anomeric carbon) and six signals near 170 ppm (carbonyl) (Figures 19 and 20).

The glycerol part was established by a five-spin system at δ 4.30 (dd, *J*=12.0, 3.3 Hz, H-1b), 4.15 (m, H-1a), 5.15 (m, H-2), 3.69 (dd, *J*=10.9, 5.4 Hz, H-3a) and

3.92 ppm (dd, *J*=10.9, 5.0 Hz, H-3b) (Figure 18). The connectivities of these protons to the oxygenated carbons at δ 62.25 (C-1), 70.00 (C-2) and 67.59 (C-3) were confirmed by the correlations in the HMQC spectrum (Figure 25). Carbonyl carbon signals arising from the acyl groups were observed at 173.07 ppm. In addition, characteristic proton signals was observed at δ 2.29 (t, *J*= 7.4 Hz) and 2.76 ppm (t, *J* = 5.7 Hz), the chemical shifts of which matched well with those of methylene hydrogens lying between double bonds and next to carbonyl functionalities, respectively (Figures 18 and 26). The carbon signals at δ 131.83, 130.10, 128.18, and 127.65 ppm in the ^{13}C-NMR spectrum can be attributed to isolated double bonds, probably in fatty acid residues, correlating with the ^{1}H-multiplets centered at δ 5.34 ppm (Figure 24). The geometry of the double bond within the fatty acid moieties was presumed to be *cis*-configuration based on the chemical shift of methylene carbons next to a double bond (δ 27-28 ppm, *trans*-configuration appear at 32-33 ppm) (Stothers, 1972).

In the ^{1}H NMR spectrum, a huge multiplet between 1.20 and 1.80 ppm corresponds to aliphatic methylene proton. The signals in the 3.5 - 5.5 ppm region accounted for the presence of a monosaccharide moiety (Figure 18). The vicinal coupling constants in the acetylated sugar were as follows:

$J_{1',2'}$ = 7.8, diaxial

$J_{2',3'}$ = 10.4, diaxial

$J_{3',4'}$ = 3.4, axial-equatorial

These vicinal coupling constants indicated that the sugar was assigned as galactopyranose (Baruah *et al*., 1983). The 7.8 Hz coupling constant of anomeric proton signal at δ 4.45 indicated the *β*-configuration of the glycosidic linkage ($H_{1'}$ axial). The connectivity of the sugar moiety to the glycerol moiety was confirmed by a long-range correlation between $H_{1'}$ to C-3 of the glycerol moiety in HMBC ($^{n}J_{C\text{-}H}$= 8 Hz) spectrum (Figures 3, 26 and 27).

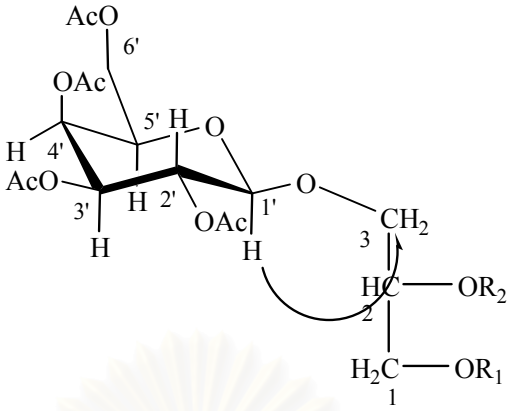


Figure 3. **The ^{1}H-^{13}C long-range correlation in the HMBC spectra of peracetyl CSF 2**

Analysis of the chemical shifts and vicinal coupling constants in the ^{1}H NMR spectrum (Figure 18) , together with 2D ^{1}H-^{1}H COSY (Figure 23) clearly pointed out that CSF 2 is monogalactosyl diacylglycerol.

Table 3. **The ^{1}H and ^{13}C NMR spectral data of the glycerol, sugar, and acetate parts for peracetyl CSF 2 and 1,2-acetyl-3-*O*-(β-D-2′,3′,4′,6′-tetraacetyl-galactopyranosyl)-*sn*-glycerol (MGalDAcG)**

Position	peracetyl CSF 2[a]		MGalDAcG[b]
	δ ^{1}H (multiplicity, *J* in Hz)	δ ^{13}C (ppm)	δ ^{1}H (multiplicity., *J* in Hz)
H-1a	4.15 (3H, m)	61.25	in 4..15 (3H, m)
H-1b	4.30 (1H, dd, 12.0, 3.3)		4.31 (1H, dd, 12.0, 3.5)
H-2	5.15 (1H, m)	70.00	5.19 (1H, m)
H-3a	3.69 (1H, dd, 10.9, 5.4)	67.59	3.68 (1H, dd, 11.0, 5.0)
H-3b	3.92 (1H, dd, 10.9, 5.0)		3.95 (1H, dd, 11.0, 5.0)
H-1′	4.45 (1H, d, 7.8)	101.50	4.48 (1H, d, 8.0)
H-2′	5.15 (1H, dd, 7.8, 10.3)	68.66	5.19 (1H, dd, 8.0, 10.5)
H-3′	4.97 (1H, dd, 10.4, 3.4)	70.78	5.01 (1H, dd, 3.5, 10.5)
H-4′	5.33 (1H, br d, 3.5)	66.99	5.39 (1H, br d, 3.5)
H-5′	4.13 (1H, br t, 6.5)	70.78	3.90 (1H, br t, 6.0)
H-6a′	4.13 (1H, m)	61.27	in 4.15 (3H, m)
H-6b′	4.13 (1H, m)		in 4.15 (3H, m)
-CO-$\underline{CH_3}$	2.14, 2.04, 2.03, 1.96	21.02-20.68	2.15, 2.06, 2.04, 1.98
-$\underline{C}$O-CH_3		170.15, 169.97, 169.87, 169.14	
$\underline{C}$=O		173.07 (2)	

[a] measured in 300 MHz, $CDCl_3$

[b] measured in 270 MHz, $CDCL_3$ (Baruah, *et al*., 1983)

As for the structure determination of the hydrophobic portion of glycolipids, NMR spectroscopy offers only limited use in the study of long alkyl chains since, both in ^{1}H and ^{13}C NMR spectra, signals of methylene groups are superimposed upon each other (Constantino, 2001).

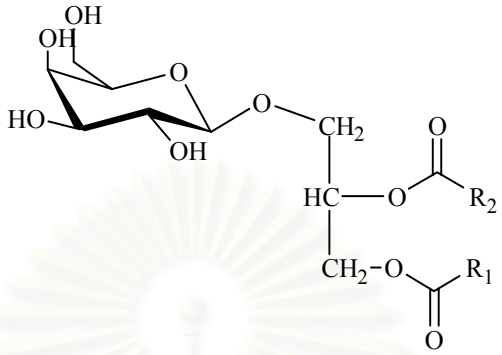
The structure of the long chain fatty acids was clarified by acid treatment and enzymatic hydrolysis. The fatty acid composition in CSF2 was determined by GC analysis and compared with authentic fatty acid methyl ester references, i.e. hexadecanoate (palmitate, 16:0), hexadecaenoate (palmitoleate, 16:1 (9z)), octadecanoate (stearate, 18:0), octadecadienoate (linoleate, 18:2 (9z, 12z)) and octadecatrienoate (linolenate, 18:3 (9z, 12z, 15z)). GC analyses of the above mentioned fatty acid methyl esters defined the composition as a mixture of hexadecanoyl (16:0), hexadecaenoyl (16:1), octadecanoyl (18:0) and octadecatrienoyl (18:3) (Figures 28 and Table 4). The percentage ratio of those three fatty acids is shown in Table 4.

Table 4. The acyl chain compositions of the peracetyl CSF 2

Acyl chains	Acid hydrolysis (%)	Enzymatic hydrolysis (%)
16:0	48.2	100
16:1	25.0	0
18:0	12.5	0
18:3	14.3	0
Total	100	100

Enzymatic hydrolysis using Lipase type XIII (from *Pseudomonas sp*.) liberated regioselective partial hydrolysis of CSF2, which occurred at the *sn*-1 position of the glycerol moiety without acyl migration. After treating with diazomethane to yield fatty acid methyl ester, the fatty acids were identified by gas chromatography (Figures 29 and Table 4). The gas chromatogram revealed that hexadecanoate was still the main composition. It was concluded that hexadecanoate attached at sn-1 position, whereas hexadecaenoate, octadecanoate or octadecatrienoate attached at sn-2 position. Based on the above evidence, the glycoglycerolipid CSF2

was a mixture of glycoglycerolipids having the basic skeleton 1,2-*O*-diacyl-3-*O*-(β-galactopyranosyl)-*sn*-glycerol as shown in Figure 4 (Rho *et al*., 1997; Son, 1990; Jung, Lee and Kang, 1996).



	R_1	R_2	
Major	Hexadecanoyl (16:0)	Hexadecaenoyl (16:1)	(≈ 50%)
Minor	Hexadecanoyl (16:0)	Octadecanoyl (18:0)	(≈ 25%)
	Hexadecanoyl (16:0)	Octadecatrienoyl (18:3)	(≈ 25%)

Figure 4. Mixture of acyl chains of compound CSF 2

3. Structure determination of compound CSF 3

Fraction F050 showed characteristic signals of the glycolipids in the ^{1}H NMR spectrum similar to fraction F053, it was therefore subjected to further isolation of glycolipids that might have anti-herpes simplex virus activity.

The broad signals in the ^{1}H NMR spectrum between the chemicals shifts of 3.5 and 5.5 ppm indicated the sugar moieties and the signals at δ 0.95 (3H, t, *J*= 7.3 Hz) and δ 0.85 (3H, br t) revealed the presence of terminal methyl of long chain fatty acid moieties (Figure 30). Similar to fraction F053, several attempts to isolate complex glycolipids from this fraction by conventional methods had been unsuccessful. Therefore, to purify these glycolipid compounds, fraction F050 was acetylated and then separated with chromatographic techniques. Compound peracetyl CSF3 was subjected to chemical structure determination by spectroscopic data.

The UV spectrum of peracetyl CSF3 in MeOH exhibited λ_{max} at 267 nm (Figure 31). The IR spectrum displayed absorption bands at 3,000-2840 (C-H stretching) and 1,731 cm^{-1} (C=O stretching) (Figure 32). In the ^{1}H-NMR and ^{13}C-NMR spectra of the peractyl CSF 3 indicated the presence of sugar, glycerol and aliphatic hydrocarbon with double bond, moieties of glycolipids could be observed.

Analysis of the ^{1}H-NMR, ^{13}C-NMR, DEPT, ^{1}H-^{1}H COSY, HMQC, and HMBC spactra allowed the assignments of all the ^{1}H-NMR and ^{13}C-NMR signals of the sugar and the glycerol moieties as shown in the Tables 5 and 6.

The NMR spectra revealed a number of oxymethines and methylenes (δ_H 4.09-5.43, δ_C 61.62-71.64), together with two anomeric methines (δ_H 4.45, 1H, d, *J*= 7.8 Hz and δ_H 4.92, 1H, d, *J*=3.2 Hz) suggesting the presence of the disaccharide units and correlating with the presence of the two anomeric carbons in the ^{13}C NMR spectrum at δ101.60 and 96.66 ppm (Figure 36).

In the ^{1}H NMR spectrum, the glycerol part was established by a five-spin system at δ 4.28 (dd, *J*=12.1, 3.0 Hz, H-1b), 4.15 (m, H-1a), 5.15 (m, H_2), 3.64 (dd, *J*=11.0, 3.0 Hz, H-3a) and 3.95 ppm (dd, *J*=11.0, 4.8 Hz, H-3b) (Figure 34). The

connectivities of these protons to the oxygenated carbons at δ 62.28 (C-1), 69.94 (C-2) and 67.48 (C-3) were established by the correlations in the HMQC spectrum (Figure 41).

The vicinal coupling constants in the peracetyl sugar units were observed as follows:

Unit A;	$J_{1',2'}$	=	7.8,	diaxial
	$J_{2',3'}$	=	9.9,	diaxial
	$J_{3',4'}$	=	3.1,	axial-equatorial
Unit B;	$J_{1',2'}$	=	3.4,	equatorial-axial
	$J_{2',3'}$	=	10.8,	diaxial
	$J_{3',4'}$	=	3.1,	axial-equatorial

On the basis of vicinal coupling constants in the ^{1}H NMR spectrum together with 2D ^{1}H-^{1}H COSY spectrum, the sugars were assigned as digalactopyranose (Figures 34 and 39) (Baruah, *et al*., 1983). The anomeric proton $H_{1'}$ was in the β-configuration of the glycosidic linkage ($H_{1'}$-axial), as shown by the coupling constant of $J_{1',2'}$ 7.8 Hz, whereas the 3.4 Hz coupling constant of $H_{1''}$ indicated the α-glycosidic linkage ($H_{1''}$- equatorial). The carbon signal of C-6′ was shifted downfield (δ 65.60) compared to that of CSF2 (δ 61.27), indicating a glycosidic linkage between C-1′′ and C-6′. The proton signals of $H_{5'}$ (3.82 ppm), $H_{6'a}$ (3.41 ppm) and $H_{6'b}$ (4.09 ppm) were also shifted up field in the ^{1}H NMR spectrum (Jung, Lee and Kang, 1996).

The connectivity of unit B as the terminal α-galactose unit to unit A as the β-galactose unit via a (1′′→6′)-*O*-glycosidic linkage was confirmed by a long range correlation between $H_{1''}$ to C-6′ and $H_{1'}$ to C-3 in the HMBC ($^nJ_{C\text{-}H}$ =8 Hz) spectrum (Figures 5, 42 and 43).

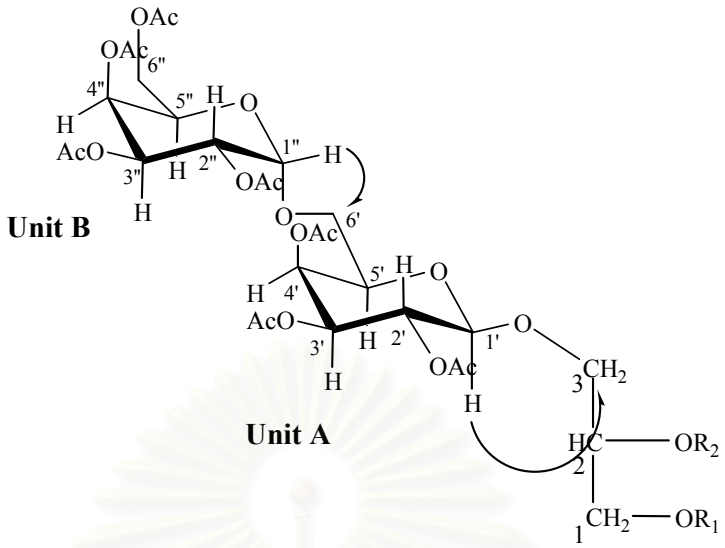


Figure 5. The ^{1}H-^{13}C long-range correlations in the HMBC ($^{n}J_{C-H}$ =8 Hz) spectra of peracetyl CSF 3

Based on the above evidence, the basic skeleton of CSF 3 was determined as digalactosyl diacylglycerol.

Table 5. The ^{1}H and ^{13}C NMR spectral data of the glycerol, sugar, and acetate parts for peracetyl CSF 3 and 1,2-acetyl-3-*O*-(*α*-D-2″,3″,4″,6″-tetraacetyl-galactopyranosyl-(1″→6′)-*O*-(*β*-D-2′,3′,4′-triacetyl-galactopyranosyl)-*sn*-glycerol (DGalDAcG)

Position	peracetyl CSF 3[a]		DGalDAcG[b]	
	δ ^{1}H (multiplicity, *J* in Hz)	δ ^{13}C (ppm)	δ ^{1}H (multiplicity, *J* in Hz)	δ ^{13}C (ppm)
H-1a	4.15 (1H, m)	62.28	4.15 (m)	62.50
H-1b	4.28 (1H, dd, 12.1, 3.0)		4.31 (dd, 12.0, 3.5)	
H-2	5.15 (1H, m)	69.94	5.19 (m)	
H-3a	3.64 (1H, dd, 11.0, 3.0)	67.48	3.68 (dd, 10.5, 3.5)	67.26
H-3b	3.95 (1H, dd, 11.0, 4.8)		3.98 (dd, 10.5, 5.0)	
H-1′	4.45 (1H, d, 7.8)	101.60	4.50 (d, 8.0)	101.59
H-2′	5.15 (1H, dd, 7.8, 9.9)	68.66	5.19 (m)	
H-3′	4.97 (1H, dd, 9.9, 3.1)	70.91	5.02 (dd, 10.5, 3.5)	
H-4′	5.39 (1H, br d, 3.1)	67.21	5.43 (br d, 3.5)	
H-5′	3.82 (1H, br t, 5.9)	71.63	3.87 (br t, 6.0)	
H-6a′	3.41 (1H, dd, 9.7, 7.2)	65.60	4.10 (m)	65.63
H-6b′	4.09 (1H, dd, 9.7, 5.2)		4.10 (m)	
H-1″	4.92 (1H, d, 3.4)	96.66	4.97 (d, 4.0)	96.69
H-2″	5.09 (1H, dd, 10.8, 3.4)	67.94	5.12 (dd, 11.0, 4.0)	
H-3″	5.26 (1H, dd, 10.8,3.1)	67.41	5.30 (dd, 11.0, 3.5)	
H-4″	5.43 (1H, br d, 3.1)	67.54	5.46 (br d, 3.5)	
H-5″	4.15 (1H, m)	66.63	4.21 (br s)	
H-6a″	4.09 (1H, dd, 7.0, 2.7)	61.62	3.44 (dd, 10.0, 6.5)	61.60
H-6b″	4.09 (1H, dd, 7.0, 2.7)		3.79 (dd, 10.0, 6.5)	
-CO-$\underline{CH_3}$	2.10 (2), 2.06, 2.04 (2), 2.03, 1.94	21.00-20.53	2.13, 2.13, 2.08, 2.07, 2.07, 2.06, 1.97, 1.97	
-$\underline{C}$O-CH_3		169.17, 169.70, 169.80, 169.80, 169.91, 170.15, 170.38		169.38, 169.92, 170.00, 170.00, 170.13, 170.13, 170.35
$\underline{C}$=O		173.07, 173.11		170.52, 170.57

[a] measured in 300 MHz, $CDCl_3$
[b] measured in 270 MHz, $CDCl_3$ (Baruah *et al*., 1983)

Table 6. **The ^{1}H and ^{13}C NMR assignments, ^{1}H-^{1}H COSY, and HMBC correlations of the glycerol, sugar and acetate parts of peracetyl CSF 3**

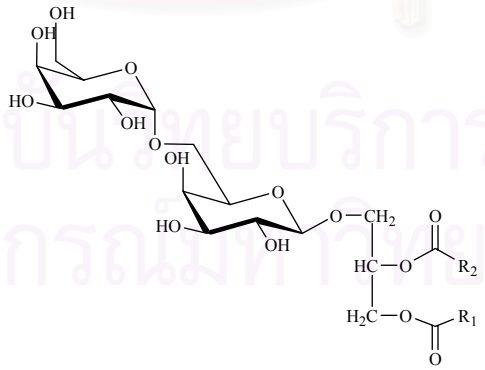
Position	δ ^{13}C (ppm)	δ ^{1}H (multiplicity, *J* in Hz)	^{1}H-^{1}H COSY	HMBC
1a	62.28	4.15 (1H, m)	H_{1b}, H_2	C_3
1b		4.28 (1H, dd, 12.1, 3.0)	H_{1a}, H_2	
2	68.66	5.15 (1H, m)	H_{1a}, H_{1b},	
3a	67.48	3.64 (1H, dd, 11.0, 3.0)	H_2, H_{3b}	
3b		3.95 (1H, dd, 11.0, 4.8)	H_2, H_{3a}	
1′	101.60	4.45 (1H, d, 7.8)	$H_{2'}$	C_3
2′	69.95	5.15 (1H, dd, 7.8, 9.9)	$H_{1'}$, $H_{3'}$	$C_{4'}$
3′	70.91	4.97 (1H, dd, 9.9, 3.1)	$H_{2'}$, $H_{4'}$	
4′	67.21	5.39 (1H, br d, 3.1)	$H_{3'}$	
5′	71.64	3.82 (1H, br t, 5.9)	$H_{6a'}$, $H_{6b'}$	
6a′	65.60	3.41 (1H, dd, 9.7, 7.2)	$H_{5'}$, $H_{6b'}$	
6b′		4.09 (1H, dd, 9.7, 5.2)	$H_{5'}$, $H_{6a'}$	
1″	96.66	4.92 (1H, d, 3.4)	$H_{2''}$	$C_{6'}$
2″	67.94	5.09 (1H, dd, 10.8, 3.4)	$H_{1''}$, $H_{3''}$	$C_{4''}$
3″	67.41	5.26 (1H, dd, 10.8,3.1)	$H_{2''}$, $H_{4''}$	$C_{5''}$
4″	67.54	5.43 (1H, br d, 3.1)	$H_{3''}$	
5″	66.63	4.15 (1H, m)	$H_{6a''}$, $H_{6b''}$	$C_{3''}$
6a″	61.62	4.09 (1H, dd, 7.0, 2.7)	$H_{5''}$, $H_{6b''}$	
6b″		4.09 (1H, dd, 7.0, 2.7)	$H_{5''}$, $H_{6a''}$	
-CO-$\underline{CH_3}$	21.00-20.53	2.10 (2), 2.06, 2.04 (2), 2.03, 1.94		
-$\underline{C}$O-CH_3	169.17, 169.70, 169.80, 169.80, 169.91, 170.15, 170.38			
$\underline{C}$=O	173.07, 173.11			

The long chain fatty acids of peracetyl CSF3 were determined by the same process as those of peracetyl CSF2. The peracetyl CSF3 was subjected to non-selective acid hydrolysis to obtain fatty acid methyl esters which were then analyzed by GC. The acyl composition of peracetyl CSF3 was identified as hexadecanoyl, hexadecaenoyl and octadecatrienoyl (Figure 44). The percentage ratio of those three fatty acids is shown in Table 7.

Table 7. The acyl chain compositions of the peracetyl CSF 3

Acyl chains	Acid hydrolysis (%)	Enzymatic hydrolysis (%)
16:0	50.5	100
16:1	26.8	0
18:3	22.7	0
Total	100	100

The gas chromatogram showed that the amount of hexadecanoate was still the main composition. It was concluded that the hexadecanoate attached at sn-1 position whereas hexadecaenoate or octadecatrienoate attached at sn-2 position. Based on the above evidence, the glycoglycerolipids CSF 3 was identified as a mixture of glycoglycerolipids having the basic skeleton 1,2-*O*-diacyl-3-*O*-(α-galactopyranosyl)-(1″→6′)-*O*-(β-galactopyranosyl)-*sn*-glycerol as shown in Figure 6 (Son, 1990; Falch *et al*., 1995; Jung, Lee and Kang, 1996).



R_1	R_2	
Hexadecanoyl (16:0)	Hexadecaenoyl (16:1)	(≈ 50%)
Hexadecanoyl (16:0)	Octadecatrienoyl (18:3)	(≈ 50%)

Figure 6. Mixture of acyl chains of compound CSF3

4. Structure determination of compound CSF 4

Fraction F055 showed characteristic signals of the glycolipids in the ^{1}H NMR spectrum similar to fraction F053 and F050, it was therefore subjected to further isolation glycolipids that might have anti-herpes simplex virus activity.

The broad signal of the ^{1}H NMR spectrum between the chemicals shifts of 3.5 and 5.5 ppm suggested sugar moieties and the signals at δ 0.95 (3.5H, t, *J*= 7.5 Hz) and δ 0.85 (2.5H, br t, *J* =6.4 Hz) revealed the presence of terminal methyl of long chain fatty acid moieties (Figure 46). Similar to fraction F050, several attempts to isolate these complex glycolipids from this fraction by conventional method, had been unsuccessful. Therefore, to isolate these glycolipid compounds, fraction F055 was acetylated and then separated with chromatographic techniques. Compound peracetyl CSF4 was subjected to chemical structure determination by spectroscopic data.

The UV spectrum of peracetyl CSF4 in MeOH exhibited λ_{max} at 267 nm (Figure 47). The IR spectrum displayed absorption bands at 3,000-2840 (C-H stretching) and 1,731 cm^{-1} (C=O stretching) indicated the presence of methylene groups and ester functionalities in its molecule (Silverstein, 1998) (Figure 48). The ^{1}H NMR spectral data of peracetyl CSF4 were essentially the same as those for CSF3, except for the integration of the signals of fatty acid residues (Figure 49).

The NMR spectra revealed a number of oxymethines and methylenes (δ_H 4.09-5.43, δ_C 61.62-71.64), together with two anomeric methines (δ_H 4.45, 1H, d, *J*=7.8 Hz and δ_H 4.92, 1H, d, *J*=3.2 Hz) suggesting the presence of the disaccharide units and correlating with the presence of the two anomeric carbons in the ^{13}C NMR spectrum at δ101.68 and 96.79 ppm (Figures 49 and 51).

The vicinal coupling constants in the peracetyl sugar units were observed as follows:

Unit A;	$J_{1',2'}$	=	7.8,	diaxial
	$J_{2',3'}$	=	10.3,	diaxial
	$J_{3',4'}$	=	3.2,	axial-equatorial
Unit B;	$J_{1',2'}$	=	3.4,	equatorial-axial

$J_{2',3'}$ = 10.8, diaxial

$J_{3',4'}$ = 3.1, axial-equatorial

On the basis of vicinal coupling constants in the ^{1}H NMR spectrum, together with 2D ^{1}H-^{1}H COSY spectrum, the sugars were assigned as digalactopyranose similar to CSF3 (Figures 50 and 54). The anomeric proton $H_{1'}$ was in the β-configuration of the glycosidic linkage ($H_{1'}$-axial), as shown by the coupling constant of $J_{1',2'}$ 7.8 Hz, whereas the 3.4 Hz coupling constant of $H_{1''}$ indicated the α-glycosidic linkage ($H_{1''}$- equatorial). The carbon signal of C-6′ was shifted downfield (δ 65.54) compared to that C-6′ of CSF2 (δ 61.55), indicating a glycosidic linkage between C-1′′ and C-6′. The proton signals of $H_{5'}$ (3.82 ppm), $H_{6'a}$ (3.41 ppm) and $H_{6'b}$ (3.95 ppm) were also shifted upfield in the ^{1}H NMR spectrum.

The connectivity of unit B as the terminal α-galactose unit to unit A as the β-galactose unit via a (1′′→6′)-*O*-glycosidic linkage was confirmed by a long range correlation between $H_{1''}$ to C-6′ and $H_{1'}$ to C-3 in the HMBC ($^nJ_{C\text{-}H}$ =8 Hz) spectrum (Figures 7 and 58).

Based on the above evidence, the basic skeleton of CSF 4 was determined as diacyl digalactosylglycerol.

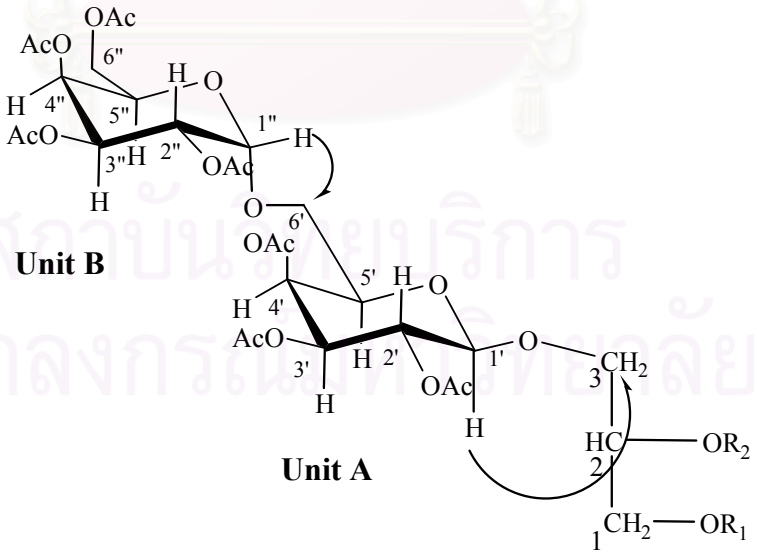


Figure 7. The ^{1}H-^{13}C long-range correlations in the HMBC spectra of peracetyl CSF 4

Table 8. **The ^{1}H and ^{13}C NMR assignments, ^{1}H-^{1}H COSY, and HMBC correlations of the glycerol, sugar and acetate parts of peracetyl CSF 4**

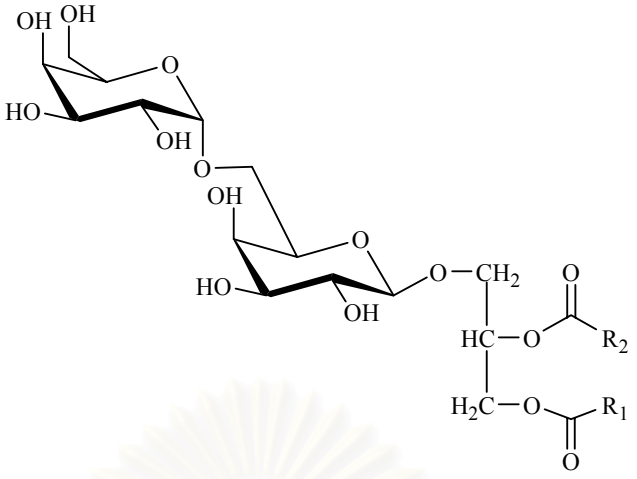
Position	δ ^{13}C (ppm)	δ ^{1}H (multiplicity, *J* in Hz)	^{1}H-^{1}H COSY	HMBC
1a	62.29	4.15 (1H, m)	H_{1b}, H_2	
1b		4.29 (1H, dd, 12.0, 3.1)	H_{1a}, H_2	C_3
2	68.37	5.16 (1H, m)	H_{1a}, H_{1b},	
3a	67.38	3.62 (1H, dd, 10.6, 5.2)	H_2, H_{3b}	
3b		3.96 (1H, dd, 10.6, 4.8)	H_2, H_{3a}	
1′	101.60	4.45 (1H, d, 7.8)	$H_{2'}$	C_{3a}, C_{3b}
2′	69.95	5.16 (1H, dd, 7.8, 10.3)	$H_{1'}$, $H_{3'}$	$C_{4'}$
3′	70.91	4.97 (1H, dd, 10.3, 3.2)	$H_{2'}$, $H_{4'}$	
4′	67.12	5.40 (1H, br d, 3.2)	$H_{3'}$	$C_{2'}$
5′	71.54	3.82 (1H, br t, 6.3)	$H_{6a'}$, $H_{6b'}$	$C_{3'}$
6a′	65.54	3.41 (1H, dd, 9.8, 7.4)	$H_{5'}$, $H_{6b'}$	
6b′		3.95 (1H, dd, 9.8, 5.2)	$H_{5'}$, $H_{6a'}$	
1″	96.79	4.92 (1H, d, 3.4)	$H_{2''}$	$C_{6'}$
2″	67.89	5.09 (1H, dd, 10.8, 3.4)	$H_{1''}$, $H_{3''}$	
3″	67.89	5.26 (1H, dd, 10.8,3.1)	$H_{2''}$, $H_{4''}$	$C_{5''}$
4″	67.49	5.43 (1H, br d, 3.1)	$H_{3''}$	$C_{2''}$, $C_{6''}$
5″	66.59	4.18 (1H, m)	$H_{6a''}$, $H_{6b''}$	$C_{3''}$
6a″	61.55	4.09 (1H, m)	$H_{5''}$, $H_{6b''}$	
6b″		4.09 (1H, m)	$H_{5''}$, $H_{6a''}$	
-CO-$\underline{CH_3}$	20.65-20.59	2.11(2), 2.07, 2.05(2), 2.04 , 1.95		
-$\underline{C}$O-CH_3	169.38,169.93(2), 170.04, 170.13, 170.39, 170.64			
$\underline{C}$=O	172.81, 173.32			

The long chain fatty acids of peracetyl CSF4 were determined by the same process as those of peracetyl CSF2 and peracetyl CSF3. The peracetyl CSF4 was subjected to non-selective acid hydrolysis to obtain fatty acid methyl esters which were then analyzed by GC. The acyl compositions of peracetyl CSF4 were identified as a mixture of hexadecanoyl, octadecanoyl and octadecatrienoyl (Figure 59). The percentage ratio of those three fatty acids is shown in Table 9.

Table 9. The acyl chain compositions of the peracetyl CSF 4

Acyl chains	Acid hydrolysis (%)	Enzymatic hydrolysis (%)
16:0	41.5	70.6
18:0	36.6	29.4
18:3	21.9	0
Total	100	100

The gas chromatogram showed that the amount of octadecatrienoate was disappeared while those of hexadecanoate and octadecanoate were still the main composition. It was concluded that the octadecatrienoate attached the glycerol at sn-2 position while hexadecanoate or octadecanoate bound both positions. Based on the above evidence, the chemical structure of the glycoglycerolipids CSF 4 was a mixture of glycoglycerolipids having the basic skeleton 1,2-*O*-diacyl-3-*O*-(α-galactopyranosyl)-(1″→6′)-*O*-(β-galactopyranosyl)-*sn*-glycerol as shown in Figure 8 (Son, 1990; Falch *et al*., 1995; Jung, Lee and Kang, 1996).



	R_1	R_2
Major	Hexadecanoyl (16:0)	Hexadecanoyl (18:0)
Minor	Hexadecanoyl (18:0)	Hexadecanoyl (16:0)
	Hexadecanoyl (16:0)	Octadecatrienoyl (18:3)
	Hexadecanoyl (18:0)	Octadecatrienoyl (18:3)
	Hexadecanoyl (16:0)	Hexadecanoyl (16:0)
	Hexadecanoyl (18:0)	Hexadecanoyl (18:0)

Figure 8. Mixture of acyl chains of compound CSF 4

Glycolipids are present in all living organisms and occur either as glycosphingolipids or acylated glycoglycerols. They are present in the cellular membrane, and their biological function depends on their concentration. Glycosyldiacylglycerides are the major representatives present in the chloroplasts and comprise about 50-80% of total lipids that constitute the membrane (Sassaki, 1999; Roughan and Batt, 1969). Up to now, anti-herpes simplex activity of glycoglycerolipids has been reported only from *Clinacanthus nutans* (Burm. f.)Lindau leaves (Satakhun, 2001). In this research, the structures of three galactosyldiacylglycerides obtained from *C. siamensis* leaves were described.

2. Anti-herpes simplex activity

Herpes simplex viruses (HSV) are among the most common infection agents of man. There are at least two distinct serotypes (HSV-1 and HSV-2), which have different modes of transmission. HSV-1 is transmitted chiefly via a nongential route, whereas HSV-2 is most often transmitted venereally or from a mother's genital infection to the new born. The mode of spread of each of the two virus types is reflected by its relative prevalence at different ages and by its pattern of clinical distribution within the host. Thus HSV-1 infections occur most frequently during childhood and usually affect body sites above the waist. HSV-2 infections, on the other hand, occur most often during adolescence and young adulthood and involve body sites below the waist, primarily the genitals. Most infections in newborn are also caused by HSV-2 (Nahmias and Josey, 1983).

In this study, evaluations of anti-herpes simplex activity of pure compounds and crude extracts were performed using the plaque reduction assay (inactivation) (Lipipun *et al.*, 2000; Abou-karam and Shier, 1990). The methanol extract from the leaves of *C. siamensis* at 50 μg/ml showed 90 % and 90 % inhibition for HSV-1 and HSV-2, respectively. Pure compounds were tested for anti-HSV activity at 50 µg/ml. Acyclovir was used as positive control and ED_{50} of acyclovir against HSV-1 and HSV-2 were 0.06 μg/ml and 0.50 μg/ml, respectively.

Four compounds from *C. siamensis* were evaluated for anti-herpes simplex activity. CSF1 showes no activity and CSF2 showes weak activity. CSF3 and CSF4 show moderated activity against both types of herpes simplex virus whereas peracetyl CSF2, peracetyl CSF3 and peracetyl CSF4 show no activity against both types of herpes simplex viruses as compared with acyclovir (Table 10).

Table 10. The percentage of virus inhibition by glycoglycerolipids isolated from *C. siamensis*

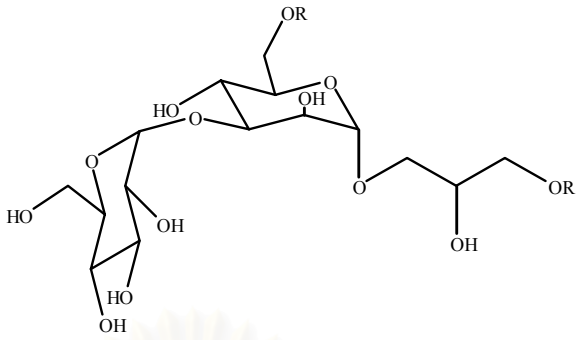
Compounds	Concentration (*μ*g/ml)	% inhibition	
		HSV-1	HSV-2
F005	50	90	90
CSF 1	50	neg	neg
CSF 2	50	26.9 (+1)	15.1 (+1)
CSF 2	100	32.1 (+1)	37.6 (+1)
peracetyl CSF 2	50	neg	neg
CSF 3	50	46.2 (+1)	56.1 (+2)
CSF 3	100	90.2 (+3)	95 (+3)
peracetyl CSF 3	50	neg	neg
CSF 4	50	46.2 (+1)	40.2 (+1)
CSF 4	100	85 (+3)	80 (+3)
peracetyl CSF 4	50	neg	neg

+4 = 100% inhibition, +3 > 50% inhibition, +2 = 50% inhibition, +1 < 50% inhibition, neg = negative

Glycolipids are ubiquitous membrane constituents of animals and plants which have attracted broad interest in the last few decades. The structure investigation of natural glycolipids, as well as the study of their biological properties providing exciting challenges for scientists (Constantino, Fattorusso and Mangoni, 2001). Glycolipids isolated from natural sources have recently shown interesting biological activity such as; anti tumor activities of 1-*O*-acyl-3-[α-*O*-glucopyranosyl-(1-3)-(6-*O*-acyl-α-mannopyranosyl)]glycerol with 14-methyl-hexadecanoic acid and 12-methyl-tetradecanoic acid positioned at C-6 of mannose unit and at the glycerol moiety from a marine sponge- associated *Mycobacterium* species **[19]** (Wicke et al., 2000): neuritogenic activity for treating Alzheimer's disease of termitomycesphins A-D **[20]-[23]** from the edible Chinese mushroom *Termitomyces albuminosus* (Berk.) Heim. (Qi, Ojika and Sakagami, 2000): plant growth-promoting activity of calonyctin **[24]**

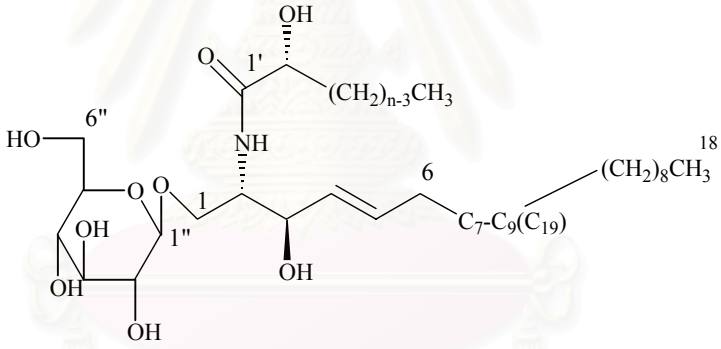
from the leaves of *Calonyction aculeatum* L. House (Furukawa *et al*., 2000); Antiviral activity of cycloviracin B_1 **[25]** from the actinomyces strain *Kibdelosporangium albatu*m so. nov.(R761-7) (Fürstner, Mlynarski and Albert, 2002) : cytolytic activity toward the heart cells of oysters of (2S)-1-*O*-3,6,9,12,15-octadecapaentaenoyl-2-*O*-6,9,12,15-octadecatetraenoyl-3-*O*-[α-D-galactopyranosyl-(1″-6‴)-*O*-β-D-galactopyranosyl]- *sn*-glycerol **[26]** from a culture marine dinoflagellate *Heterocapsa circularisquama* (Hiraga *et al*., 2002).

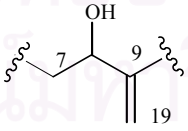
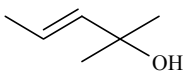
Synthetic glycolipids have displayed several bioactivities such as DNA polymerase inhibitor from 2-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)-glyceride with stearic acid **[27]**(Murakami *et al.,* 2003) and cancer chemoprevention activity of 1,6′-diacylderivatives of 2-*O*-β-D-glucosyl-*sn*-glycerol (a-d) **[28]-[31]** and 2-*O*-β-D-galactosyl-*sn*-glycerol (a-d) **[32]-[35]** (Colombo *et al*., 2001; Colombo *et al*., 2002).

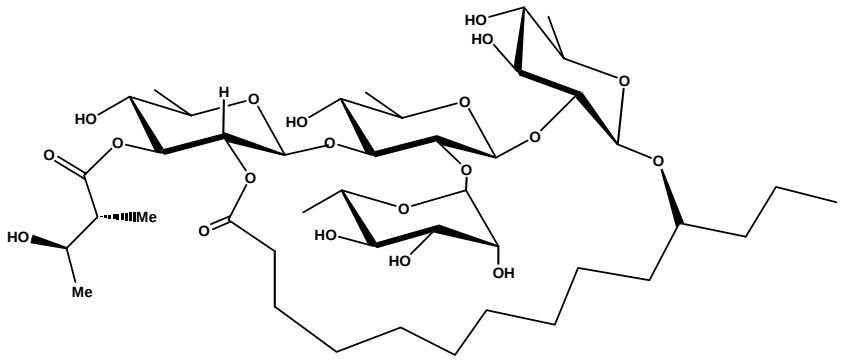


R = 14-methyl-hexadecanoic acid and 12-methyl-tetradexanoic acid

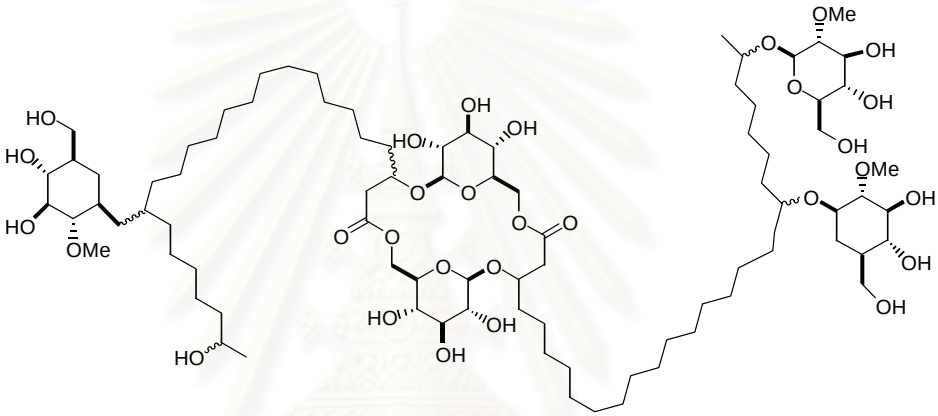
[19] 1-*O*-acyl-3-[α-*O*-glucopyranosyl-(1-3)-(6-*O*-acyl-α-mannopyranosyl)] glycerol



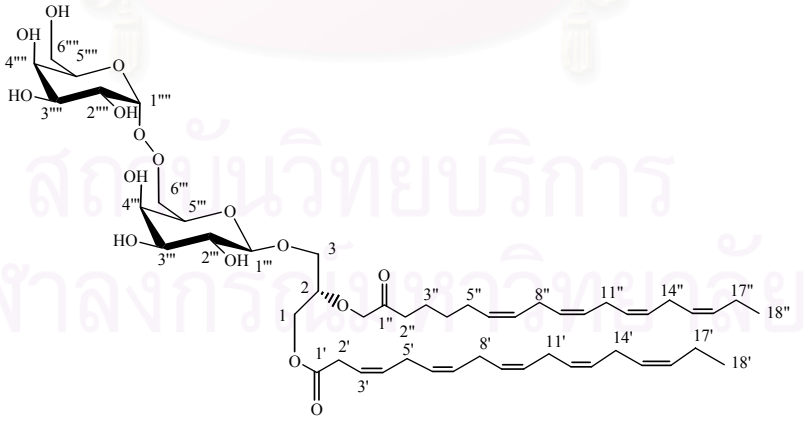
Termitomycesphin		**C_7-C_9 (C_{19})**	**n**
[20]	A		16
[21]	B		18
[22]	C		16
[23]	D		18



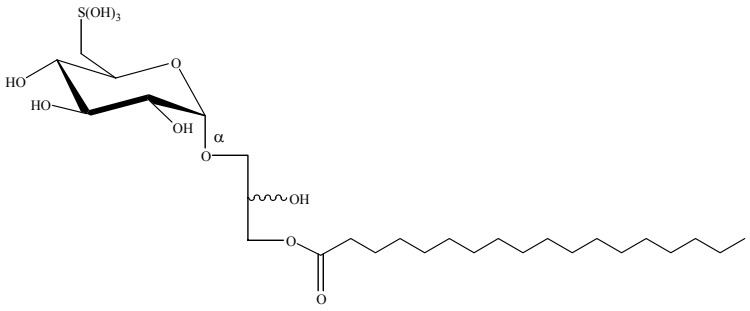
[24] Calonyctin A2



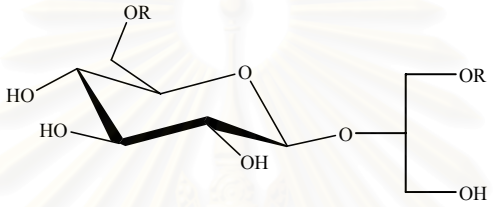
[25] Cyclovirabin B_1



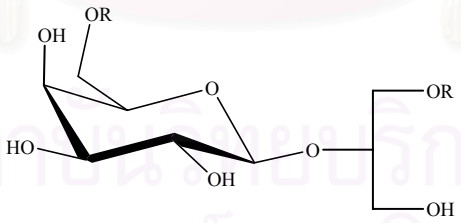
[26] (2S)-1-*O*-3,6,9,12,15-octadecapentaenoyl-2-*O*-6,9,12,15-octadecatetraenoyl-3-*O*-[α-D-galactopyranosyl-(1‴→6‴)-*O*-β-D-galactopyranosyl]-*sn*-glycerol



[27] 2-mono-*O*-acyl-3-*O*-(α-D-sulfoquinovosyl)-glyceride with stearic acid



		R
[28]	a	Butanoyl
[29]	b	Hexanoyl
[30]	c	Octanoyl
[31]	d	Decanoyl



		R
[32]	a	Butanoyl
[33]	b	Hexanoyl
[34]	c	Octanoyl
[35]	d	Decanoyl

CHAPTER V

CONCLUSION

In this investigation, the methanol extract from the leaves of *Clinacanthus siamensis* Brem. was mainly isolated by chromatographic techniques including silica gel quick column, Sephadex LH-20 column and RP-18 flash column. These techniques led to the isolation of one sterol glucoside and three mixtures of glycoglycerolipids.

The chemical structures of glycoglycerolipids were elucidated and identified through their acetylated derivatives. The sugar and glycerol moieties were determined by extensive spectroscopic studies mainly 1D and 2D NMR properties and comparison with previous reported data. The acyl chains were identified by GC analysis of the fatty acid methyl esters acquired from total acid hydrolysis and regioselective enzymatic hydrolysis. The basic skeleton of glycerolipids were then classified as 1,2-*O*-diacyl-3-*O*-(*β*-galactopyranosyl)-*sn*-glycerol and 1,2-diacyl-3-*O*-(*α*-galactopyranosyl)-(1″→6′)-*O*-(*β*-galactopyranosyl)-*sn*-glycerol. The fatty acid chains were identified as hexadecanoic acid (16:0), hexadecaenoic acid (16:1), octadecanoic acid (18:0) and octadecatrienoic acid (18:3).

In addition, compound CSF2 showed weak activity against HSV-1 and HSV-2 while compound CSF3 and CSF4 showed moderate activity. All of the peracetyl compounds inactive against both types of virus.

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APPENDIX

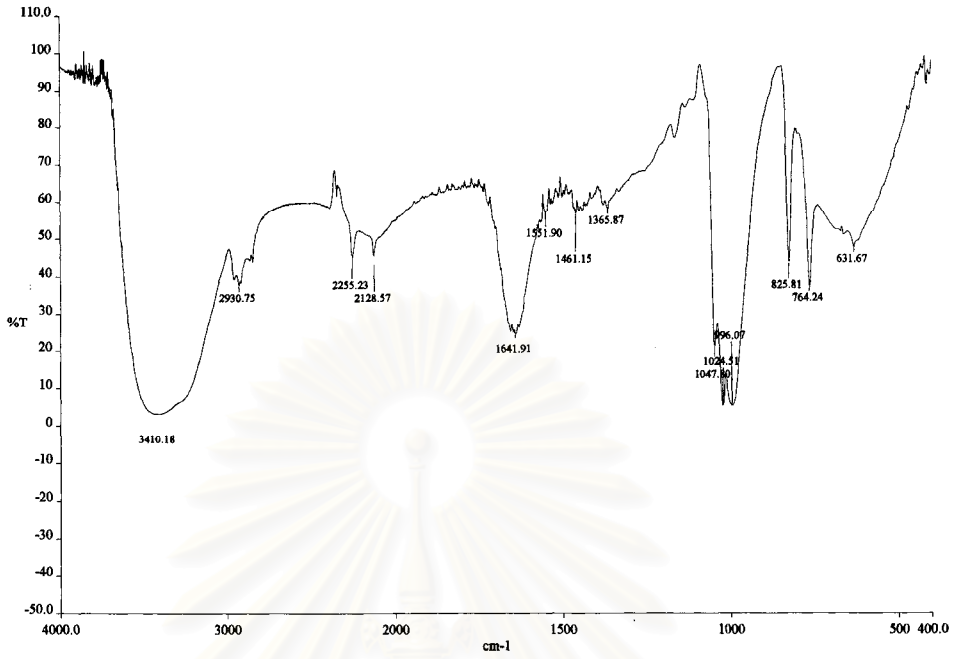

Figure 9. IR Spectrum of compound CSF 1 (film)

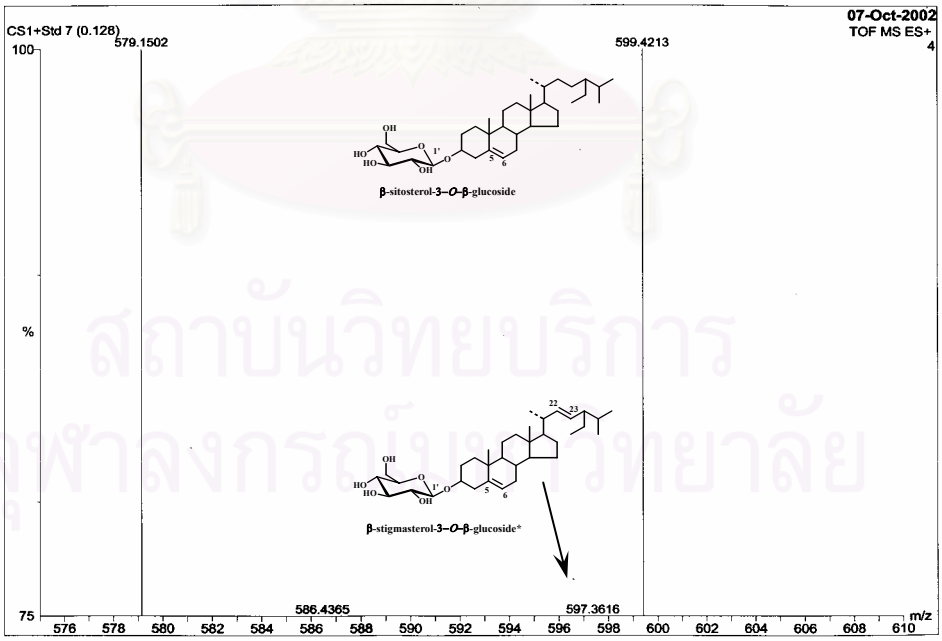

Figure 10. Mass spectrum of compound CSF 1

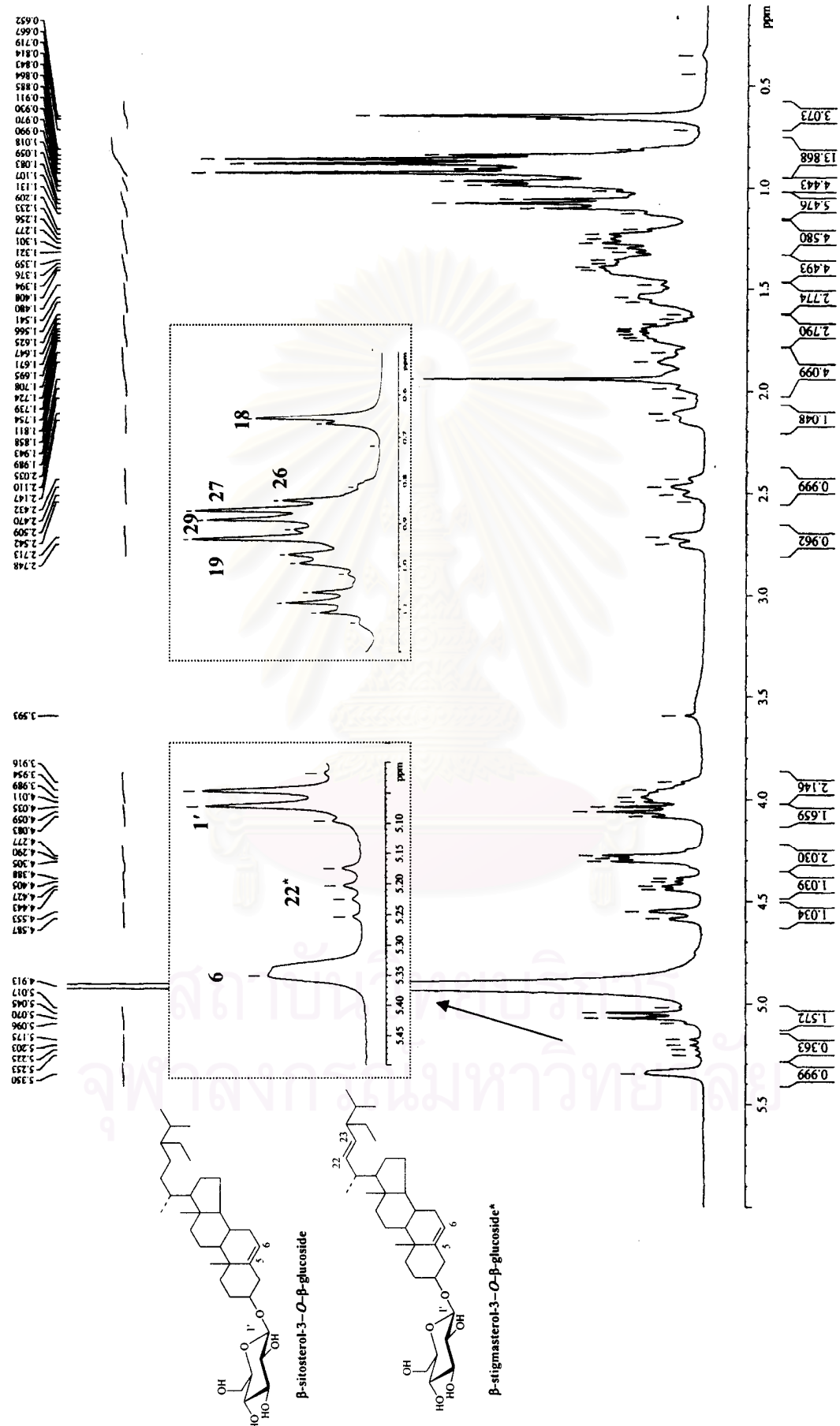

Figure 11. ^{1}H NMR (300 MHz) Spectrum of compound CSF1 (Pyridine-d_5)

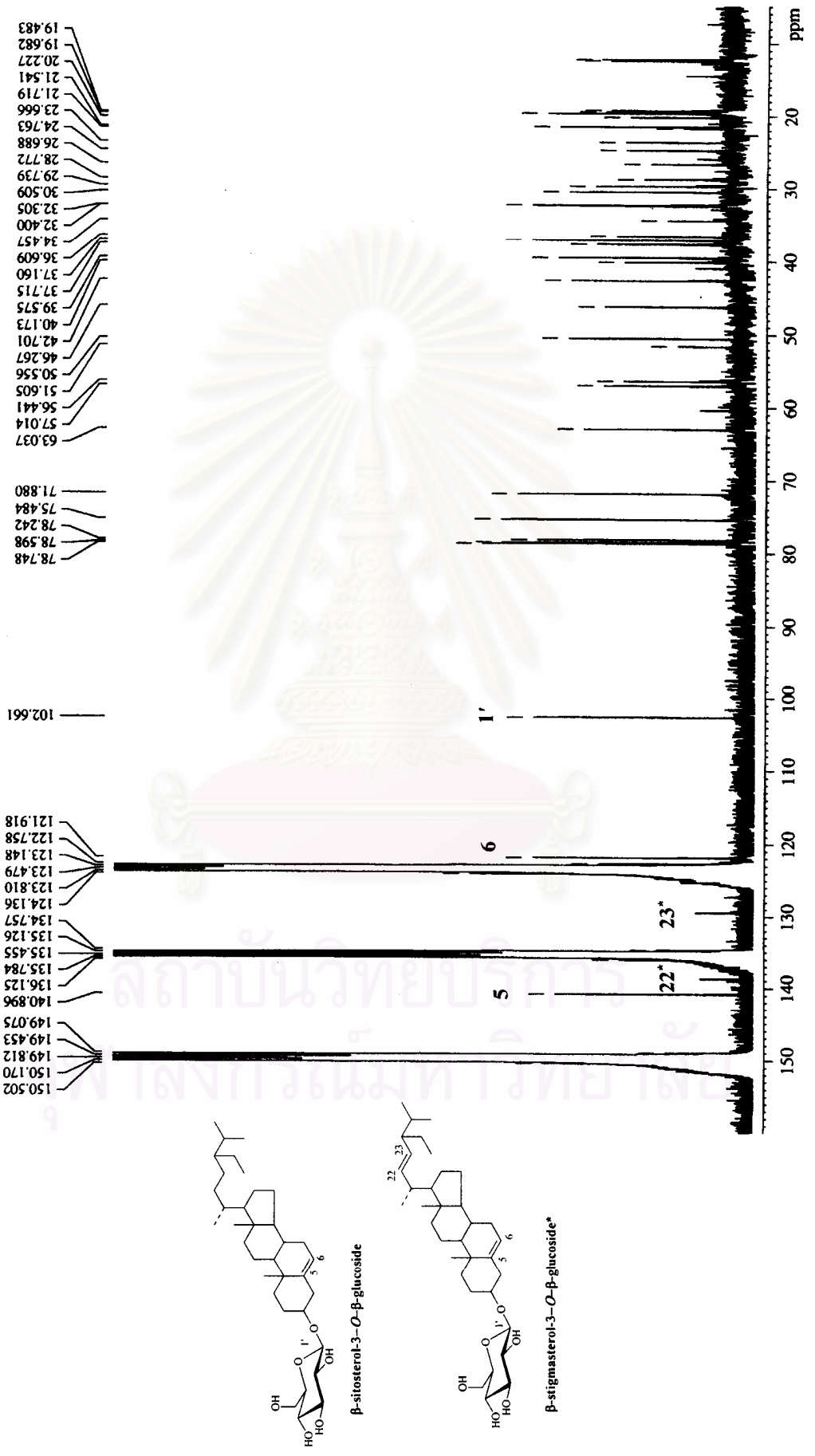

Figure 12. ^{13}C NMR (75 MHz) Spectrum of compound CSF 1 (Pyridine-d_5)

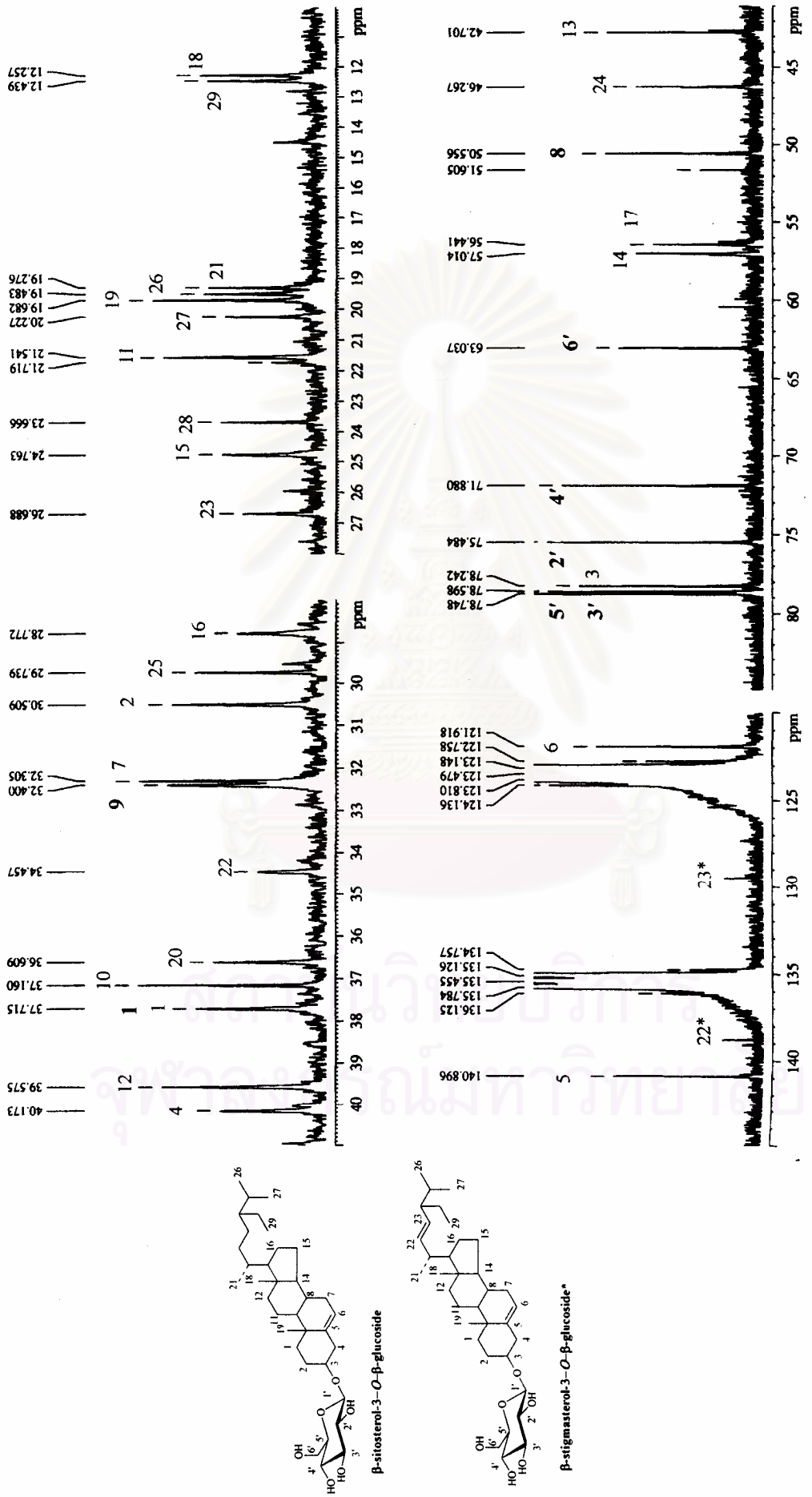

Figure 13. Expanded ^{13}C NMR (75 MHz) Spectrum of compound CSF 1 (Pyridine-d_5)

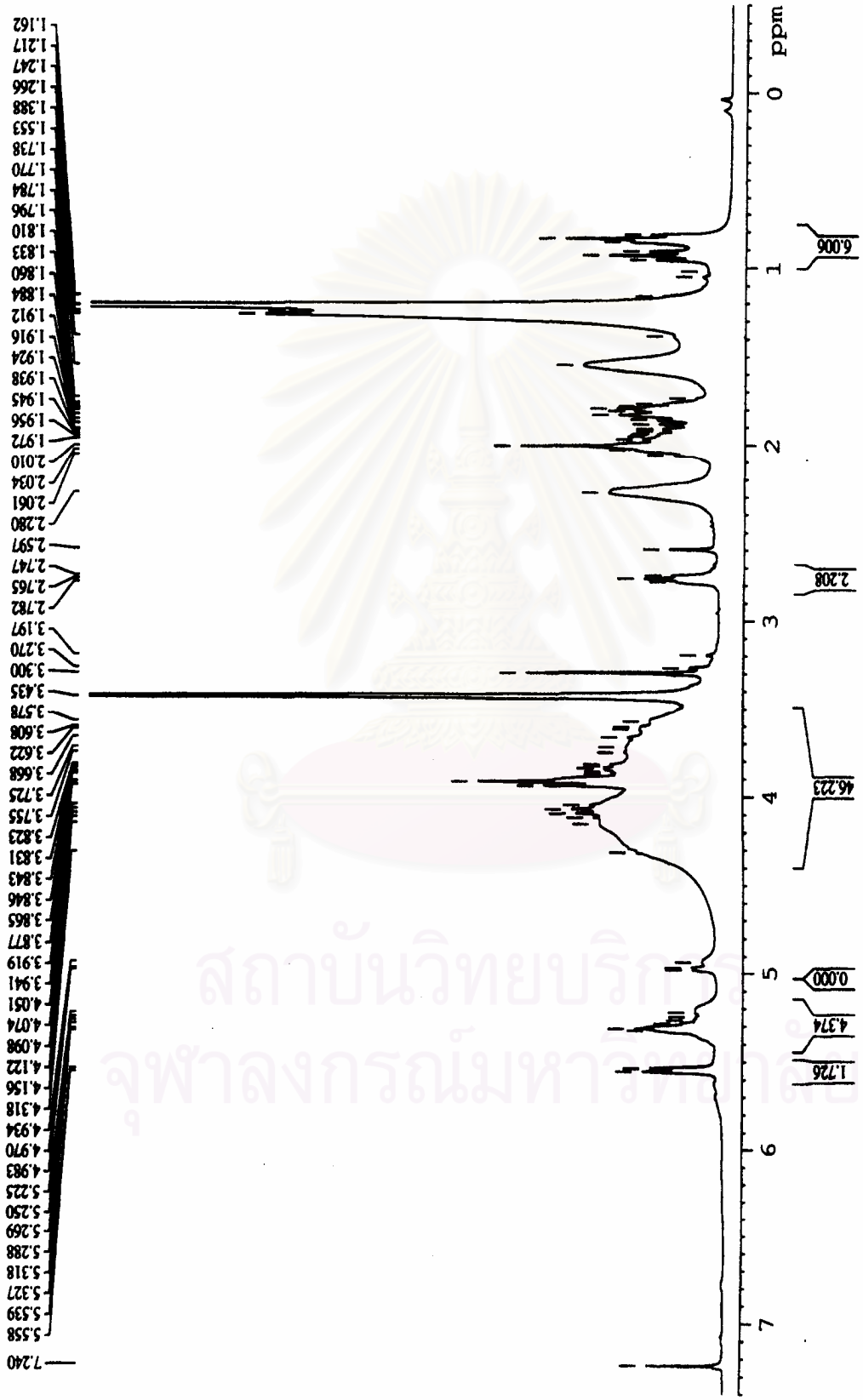

Figure 14. ^{1}H NMR (300 MHz) Spectrum of compound CSF 2 ($CDCL_3$)

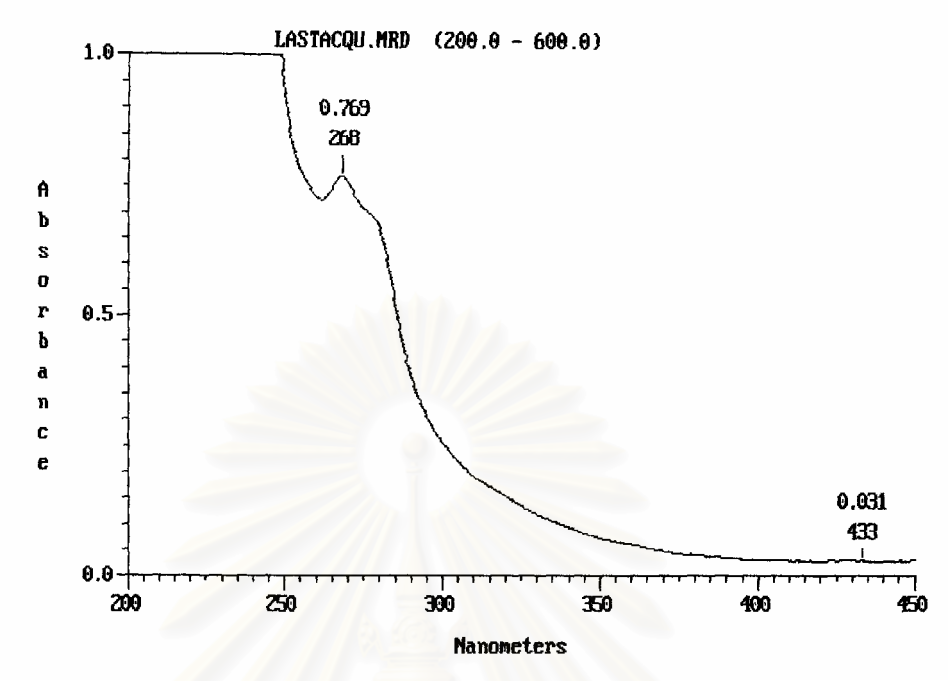

Figure 15. UV Spectrum of peracetyl CSF 2 (MeOH)

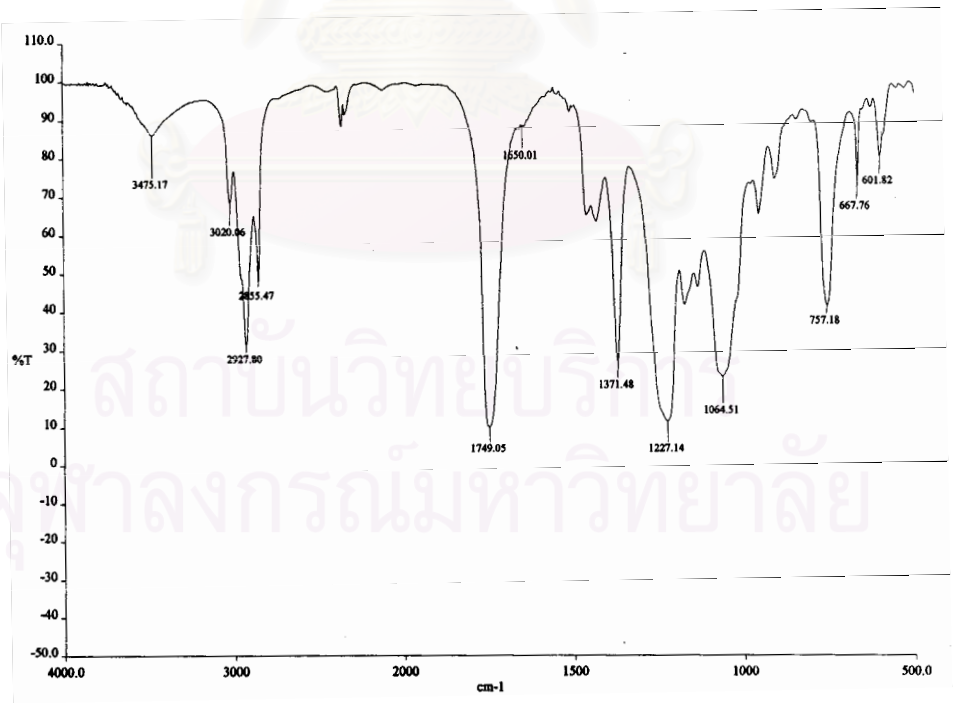

Figure 16. IR Spectrum of peracetyl CSF 2 (film)

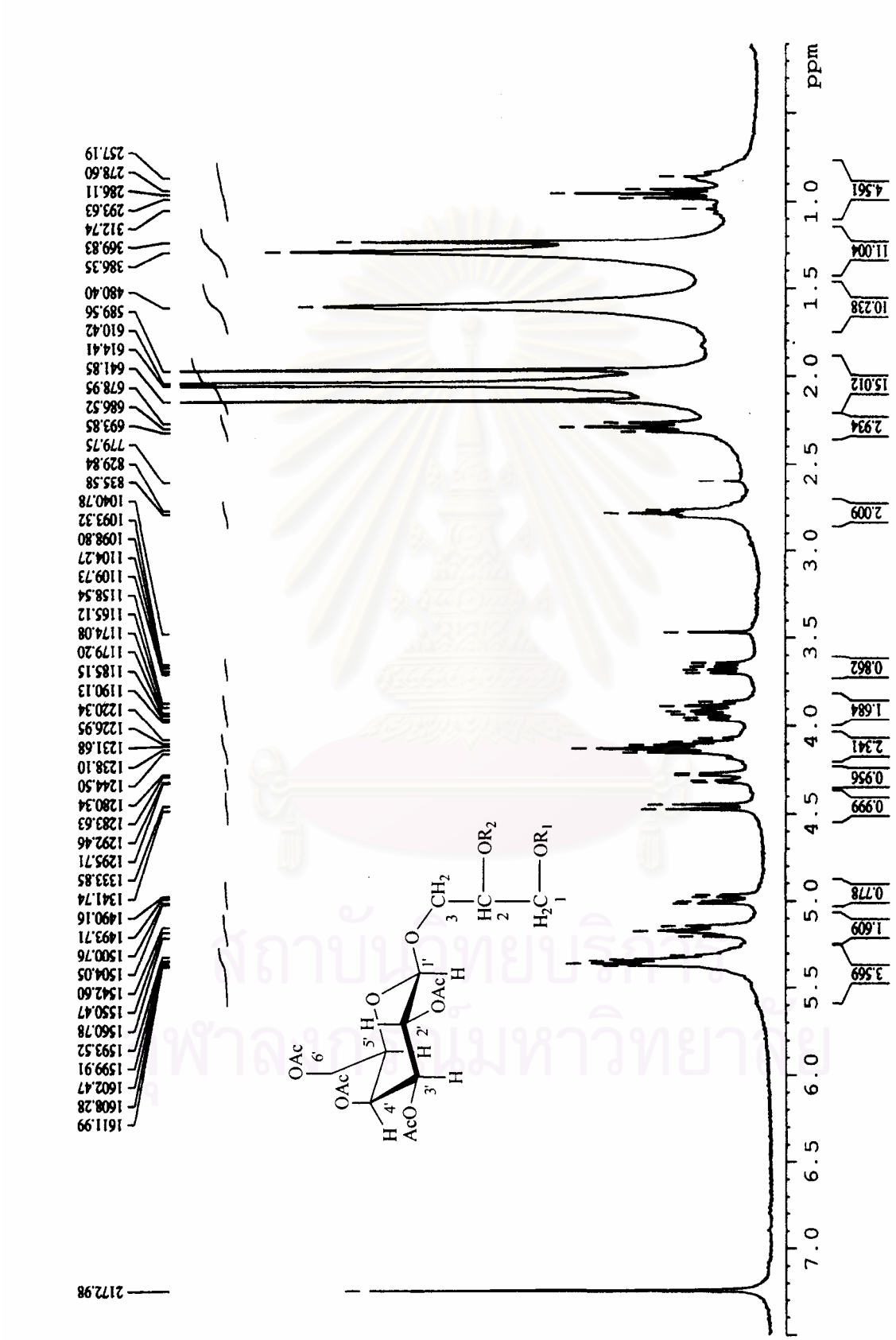

Figure 17. ^{1}H NMR (300 MHz) Spectrum of compound peracetyl CSF 2 ($CDCL_3$)

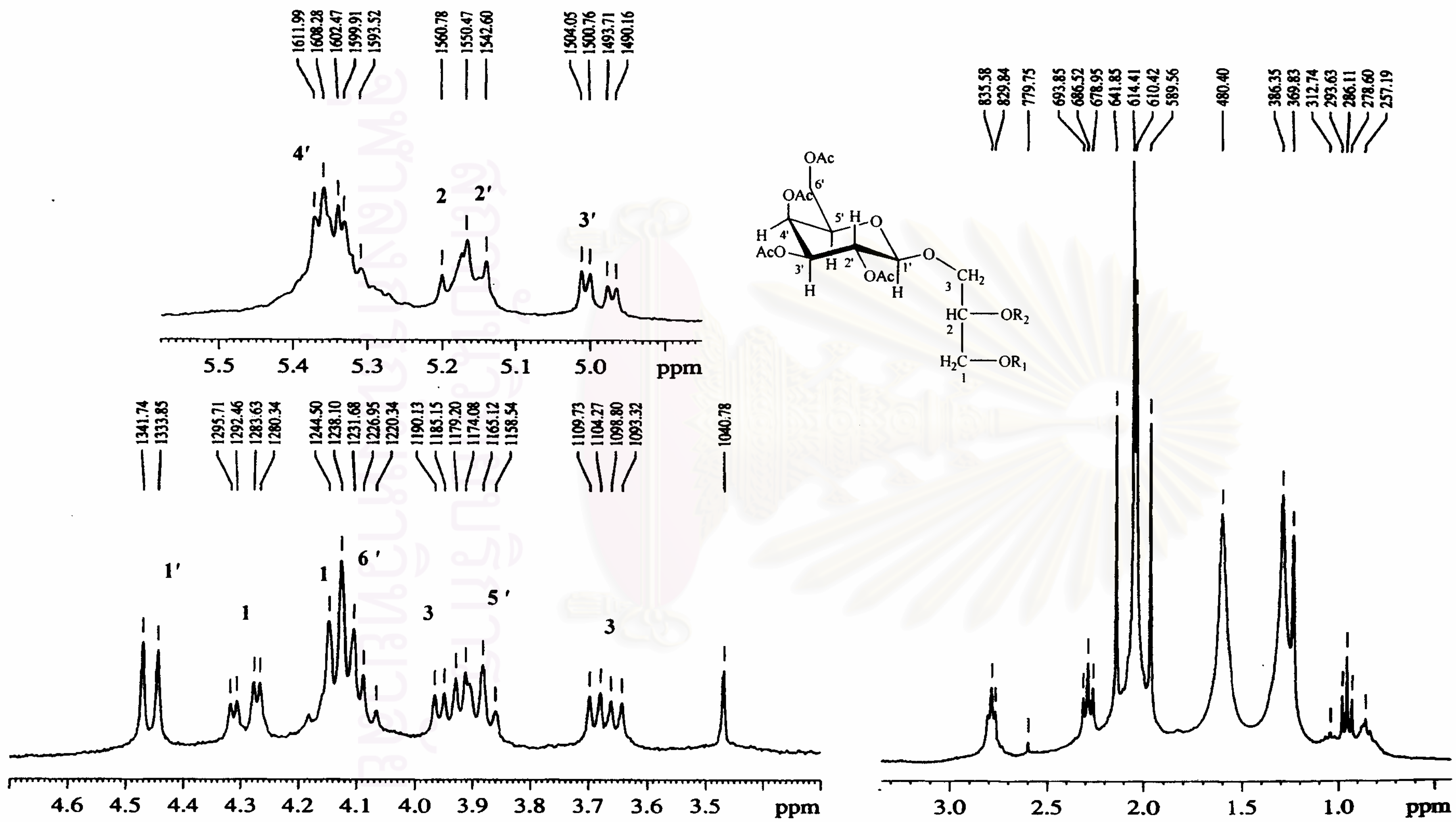

Figure 18. Expanded ^{1}H NMR (300 MHz) Spectrum of compound peracetyl CSF 2 ($CDCL_3$)

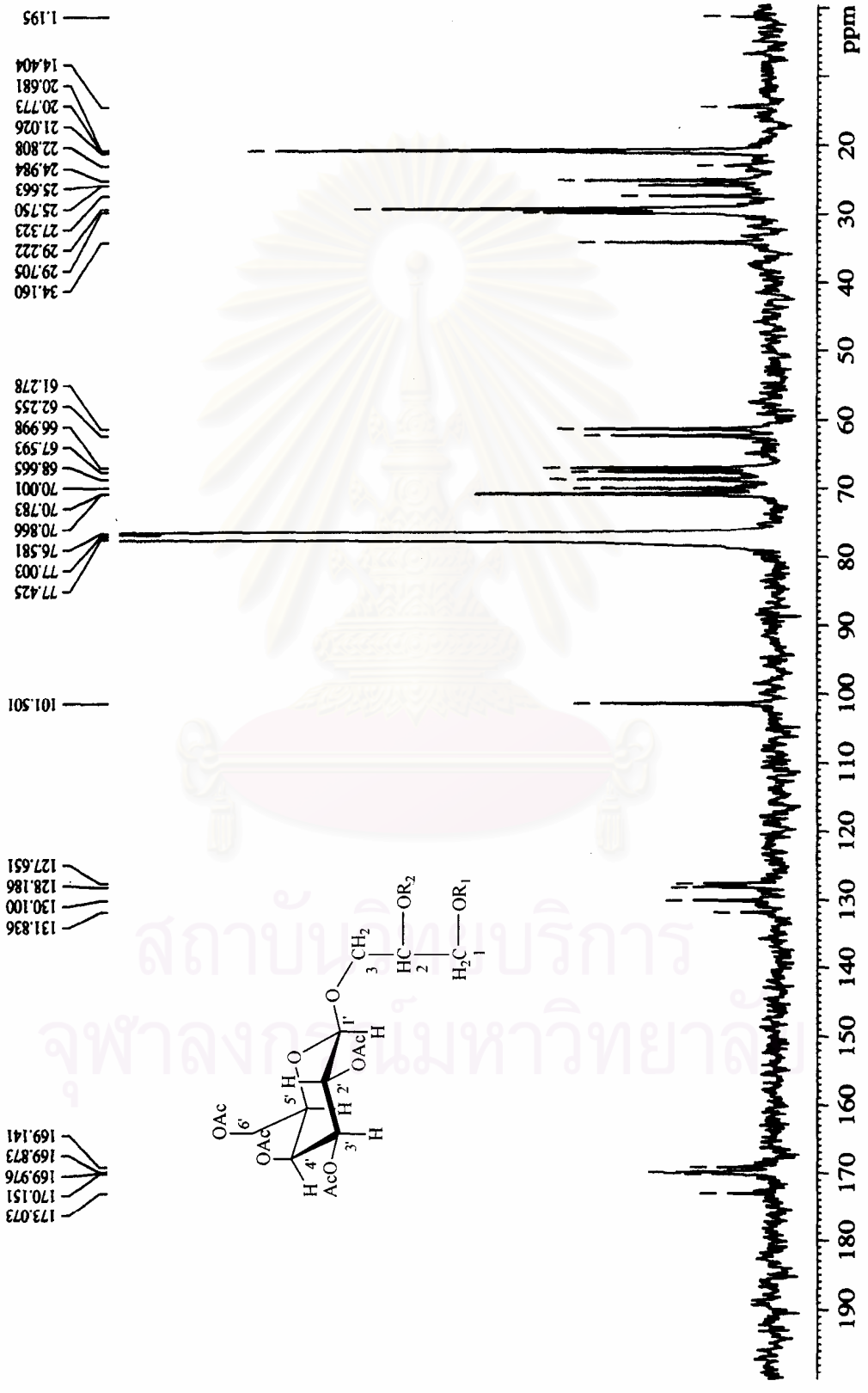

Figure 19. ^{13}C NMR (75 MHz) Spectrum of compound peracetyl CSF 2 ($CDCL_3$)

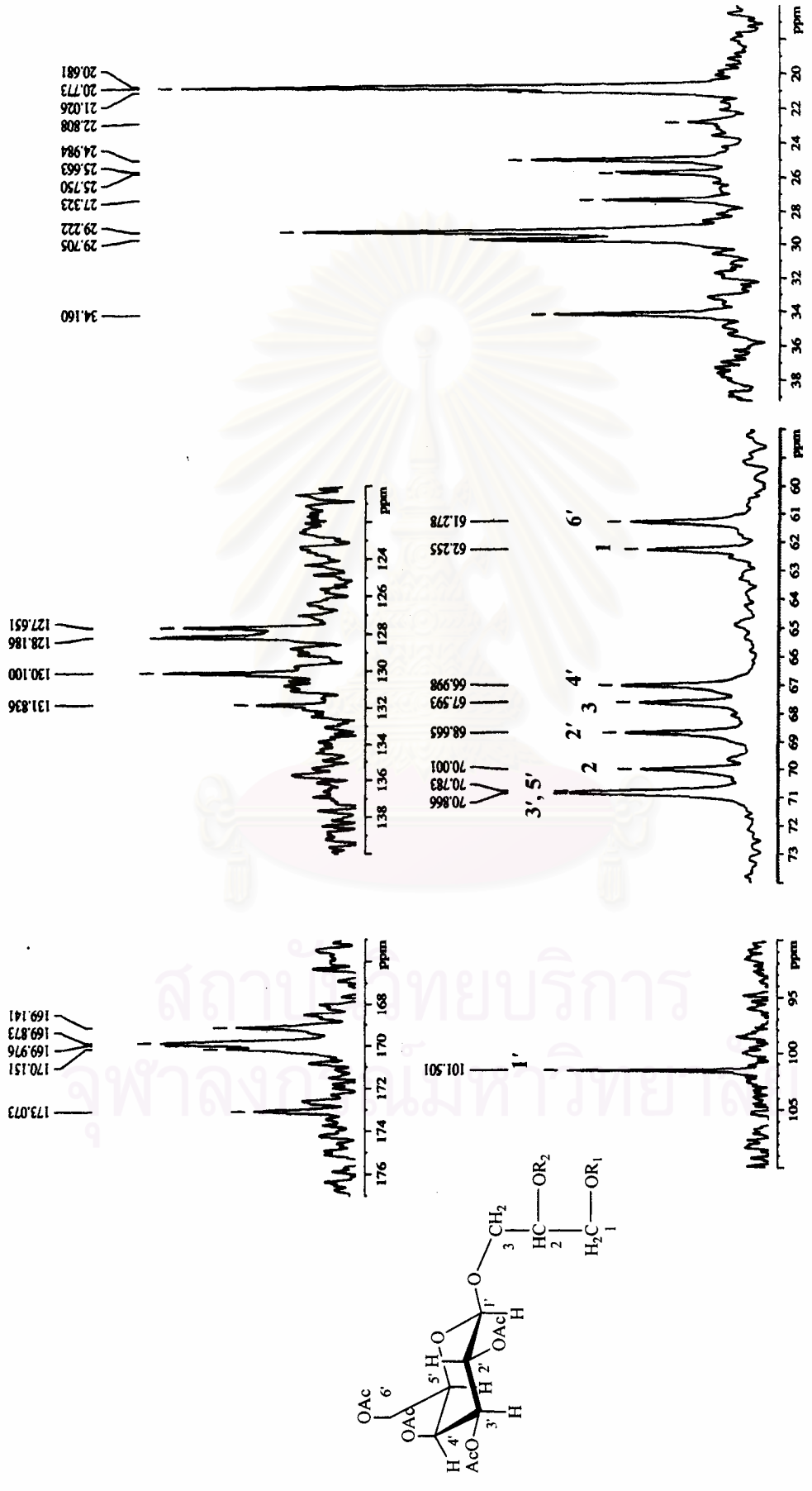

Figure 20. Expanded ^{13}C NMR (75 MHz) Spectrum of compound peracetyl CSF 2 ($CDCL_3$)

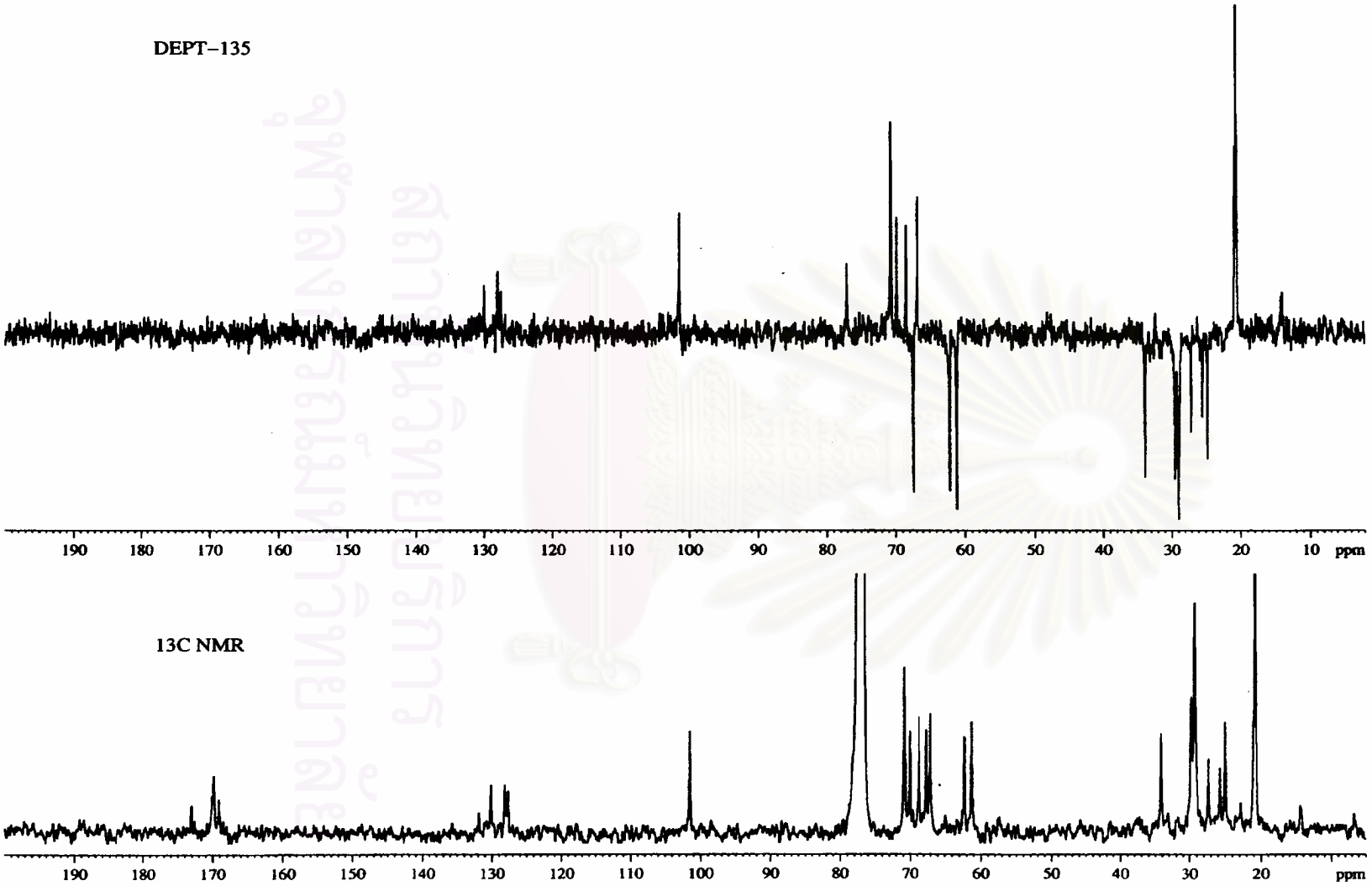

Figure 21. **^{13}C NMR (75 MHz) and DEPT-135 Spectrum of compound peracetyl CSF 2 ($CDCL_3$)**

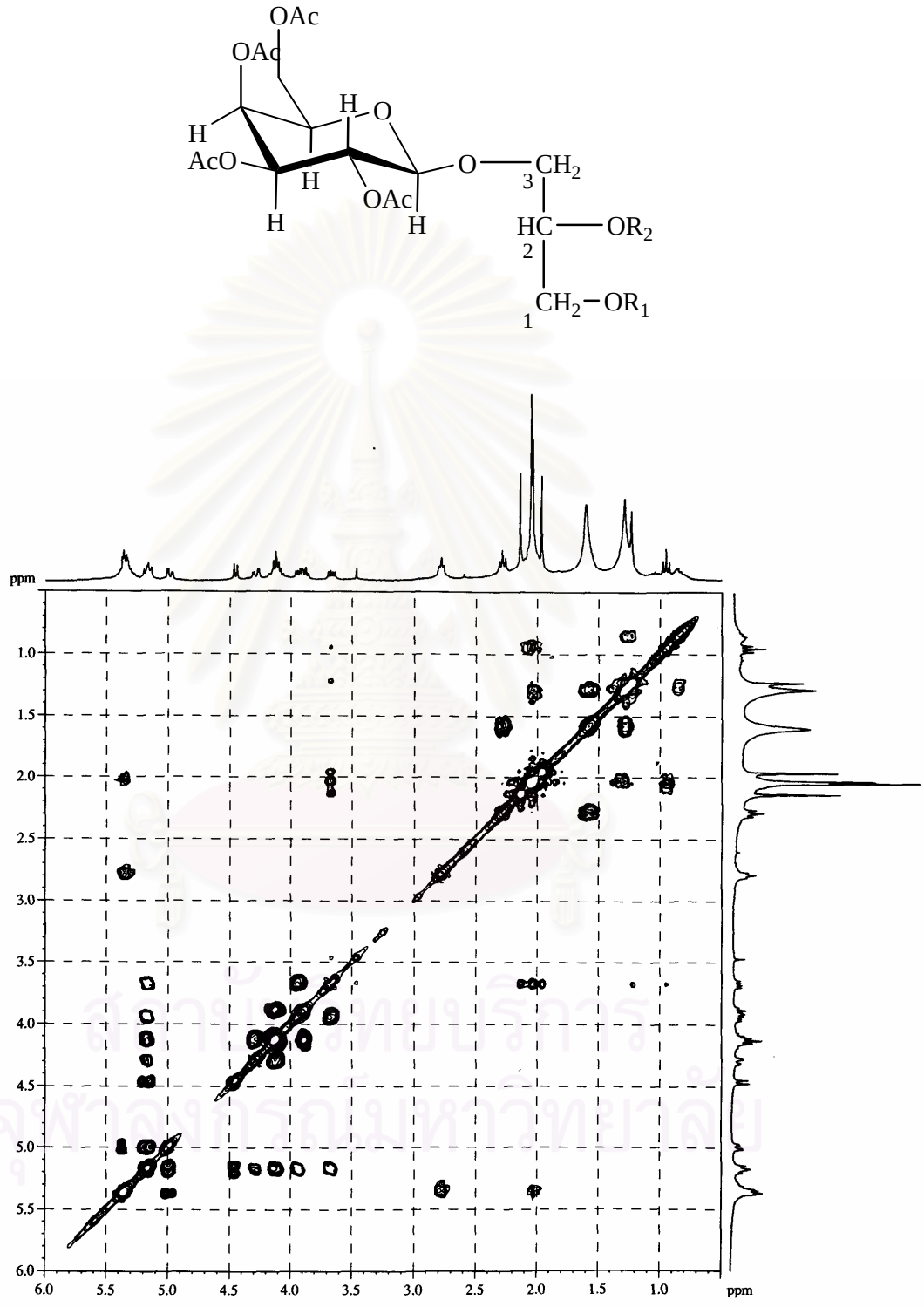

Figure 22. ^{1}H-^{1}H COSY Spectrum of peracetyl CSF 2 ($CDCl_3$)

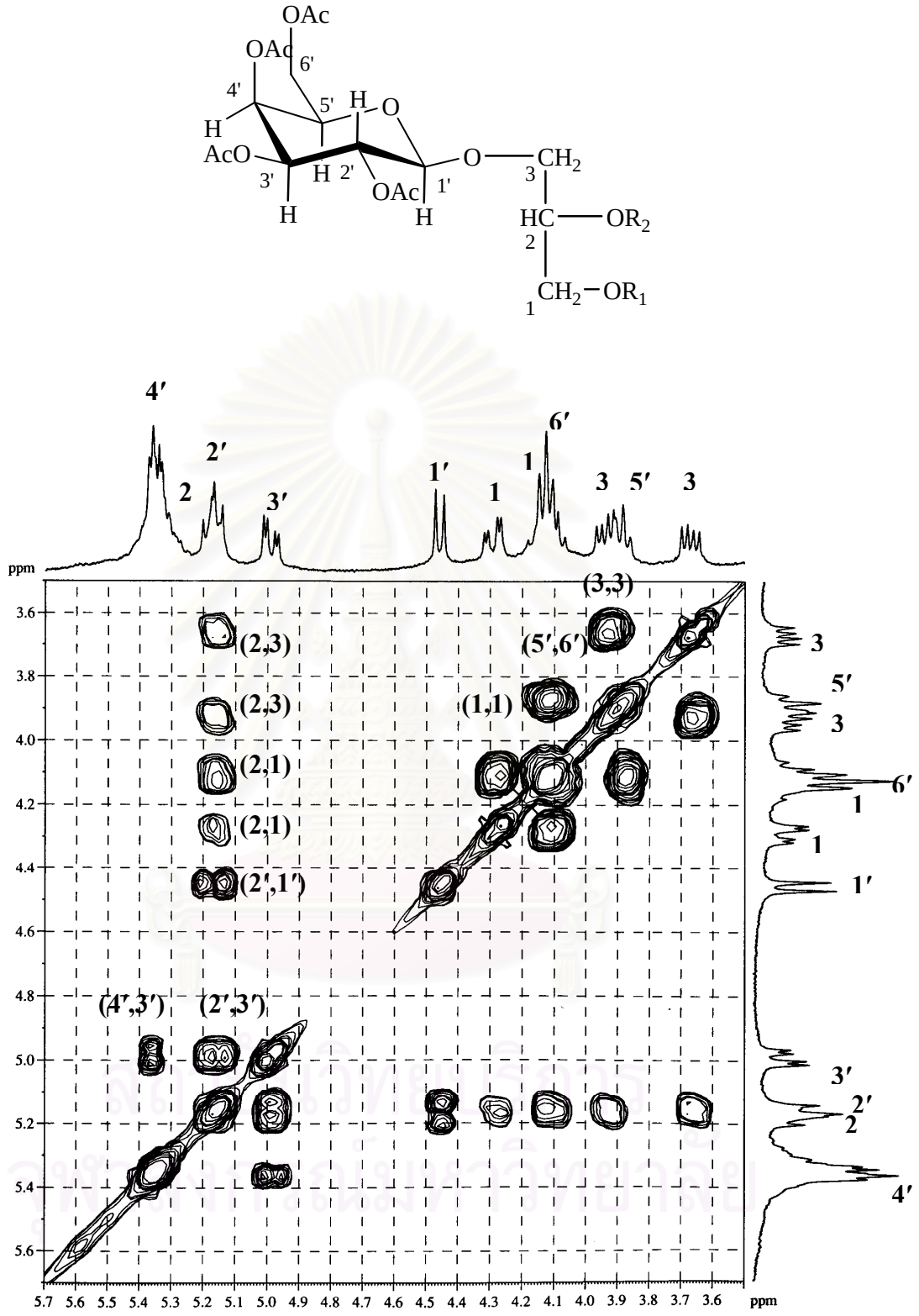

Figure 23. Expanded ^{1}H-^{1}H COSY Spectrum of peracetyl CSF 2 ($CDCl_3$)

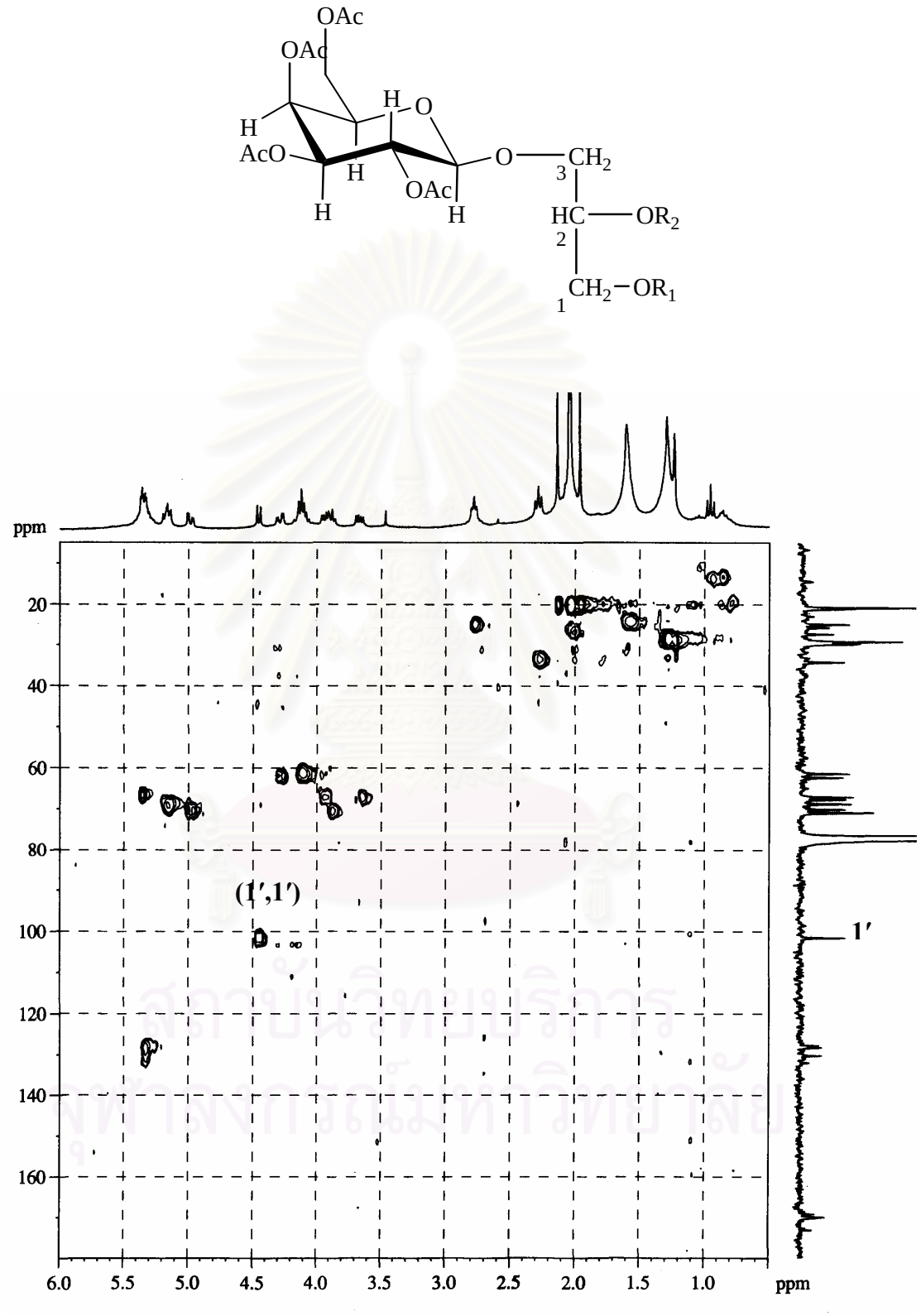

Figure 24. HMQC Spectrum of peracetyl CSF 2 ($CDCl_3$)

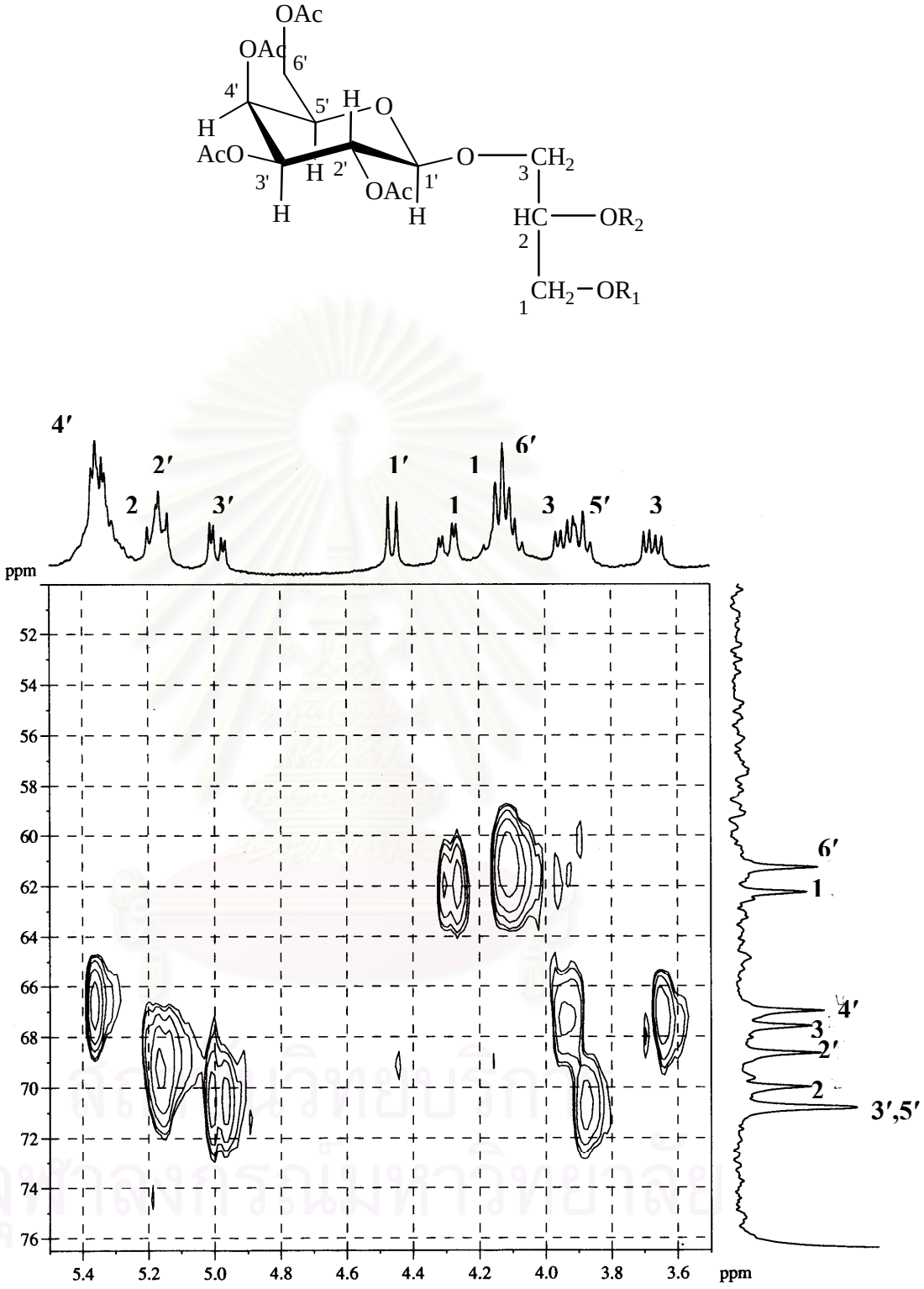

Figure 25. Expanded HMQC Spectrum of peracetyl CSF 2 ($CDCl_3$)

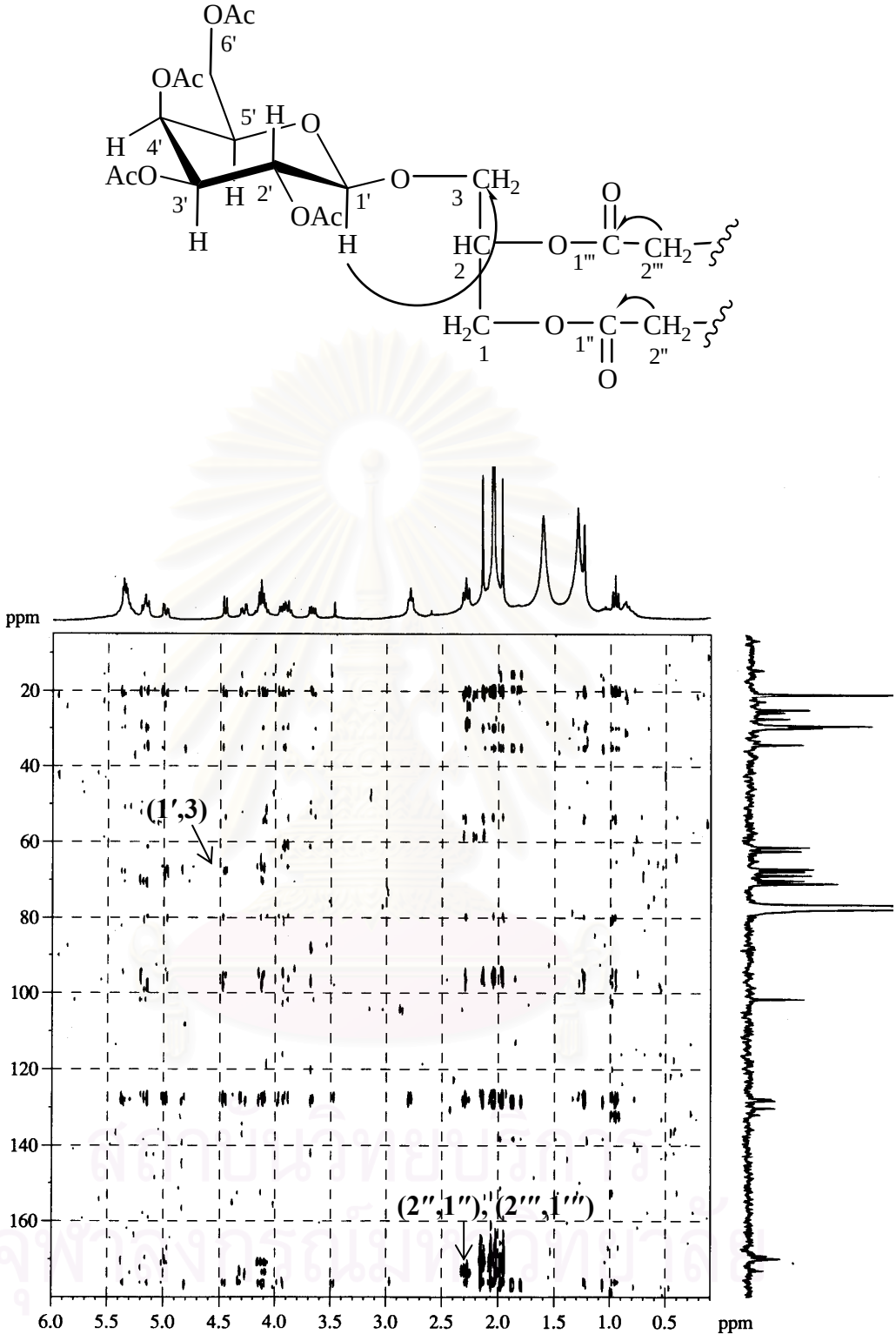

Figure 26. HMBC ($^{n}J_{C\text{-}H}$ = 8 Hz) Spectrum of peracetyl CSF 2 ($CDCl_3$)

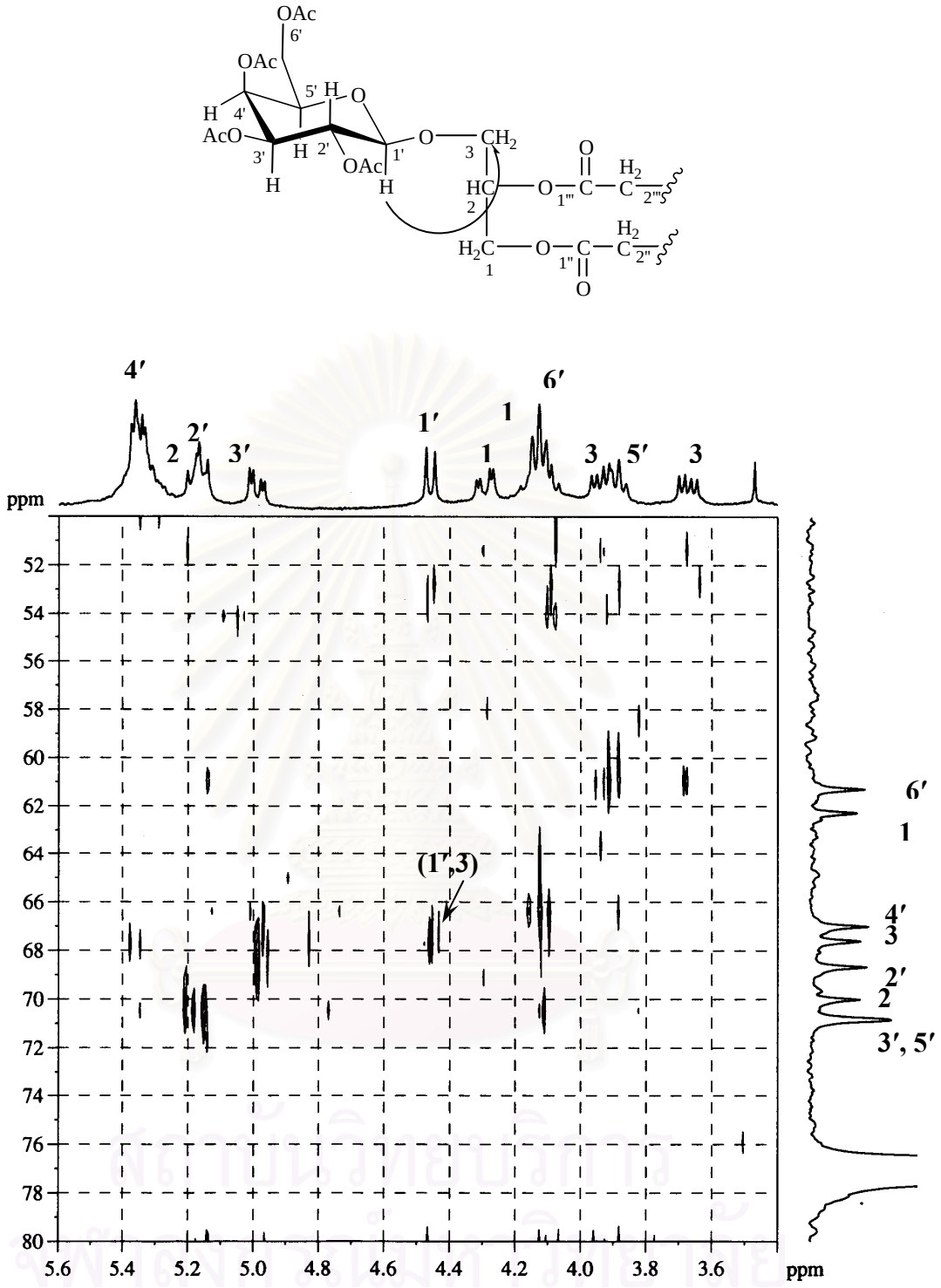

Figure 27. Expanded HMBC ($^{n}J_{C\text{-}H}$ = 8 Hz) Spectrum of peracetyl CSF 2 ($CDCl_3$)

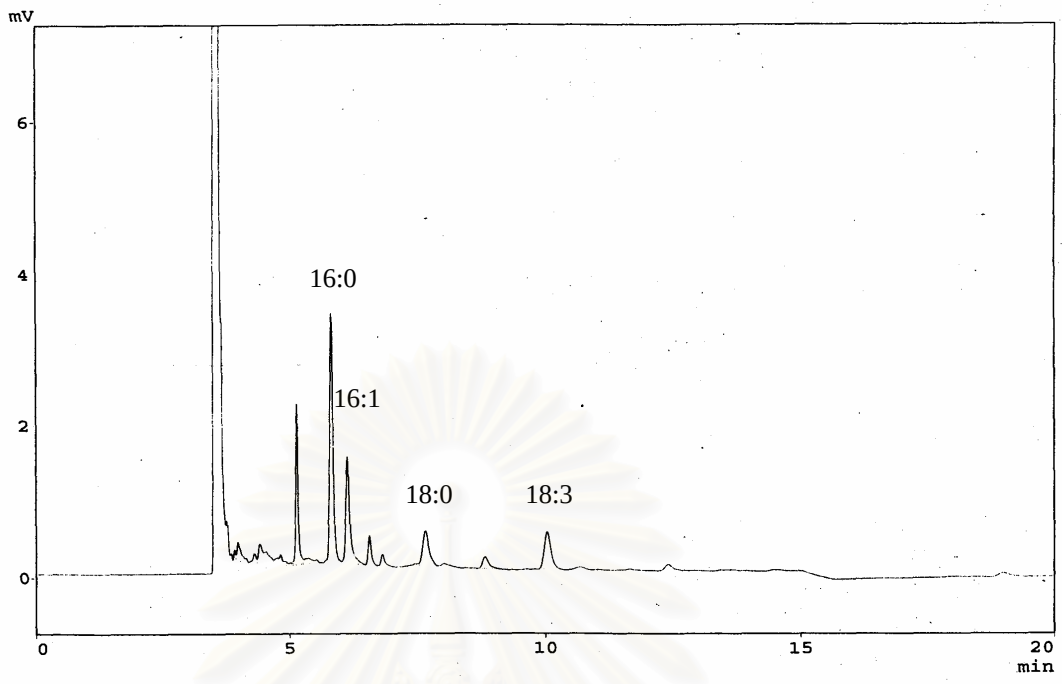

Figure 28. GC chromatogram of peracetyl CSF 2 acid hydrolysate

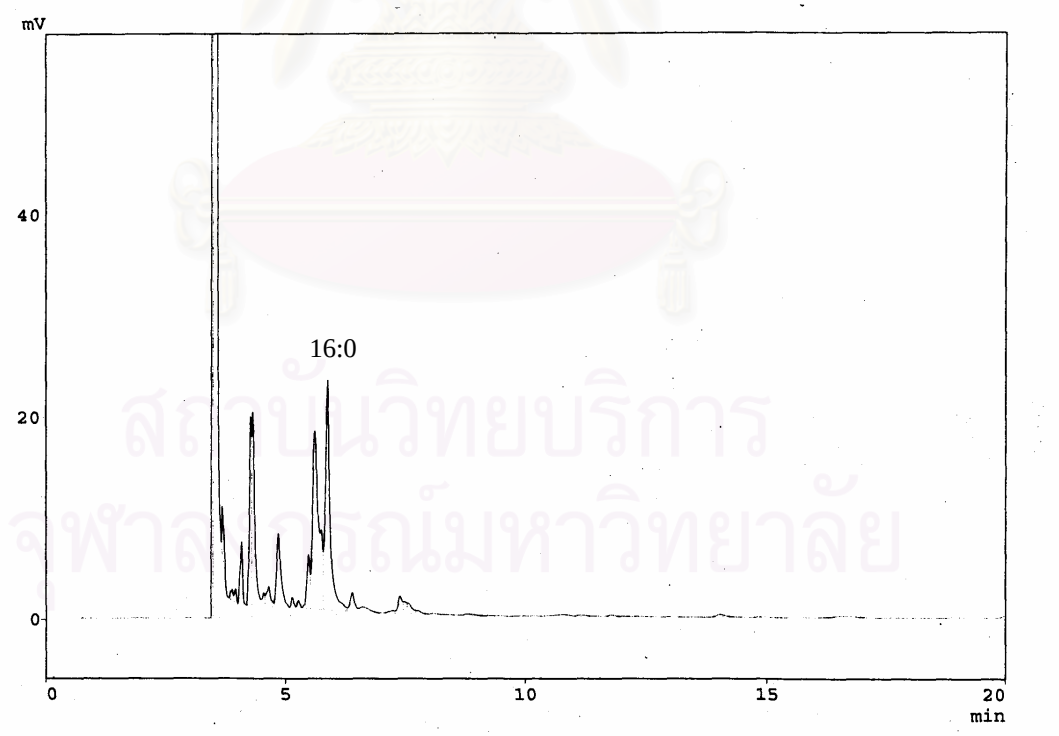

Figure 29. GC chromatogram of peracetyl CSF 2 enzymatic hydrolysate

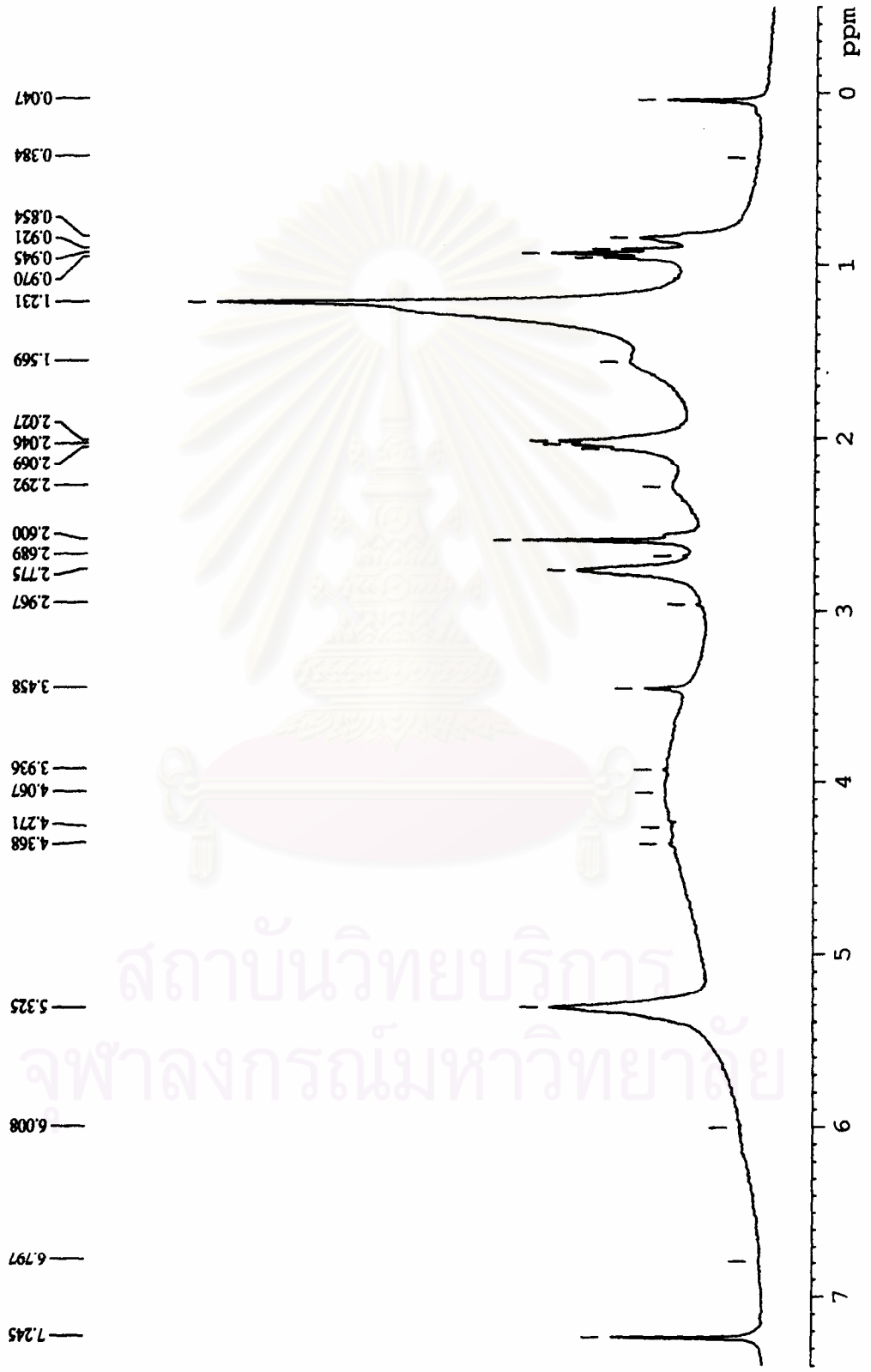

Figure 30. ^{1}**H NMR (300 MHz) Spectrum of compound CSF 3 ($CDCL_3$)**

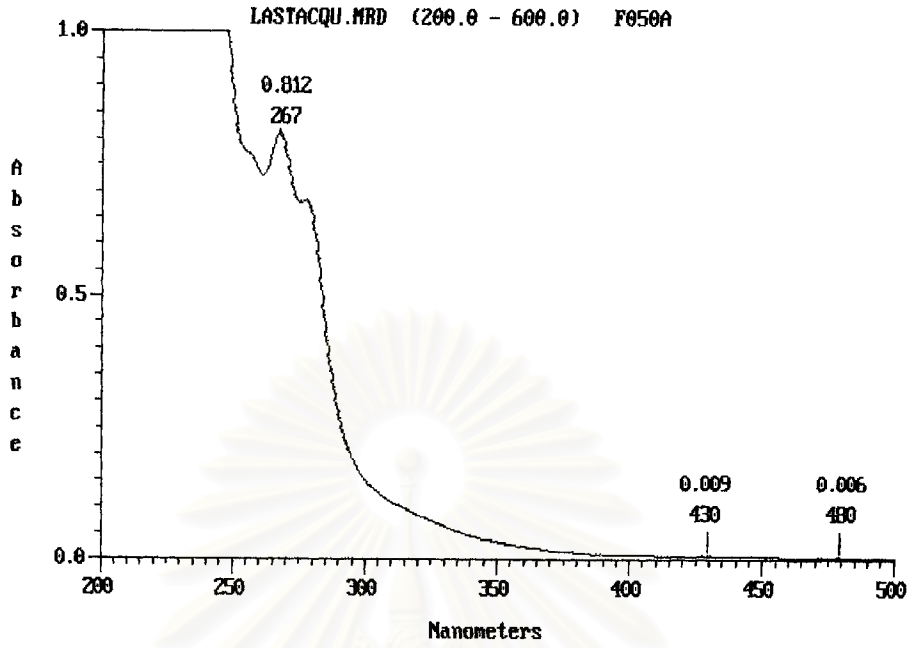

Figure 31. UV Spectrum of peracetyl CSF 3 (MeOH)

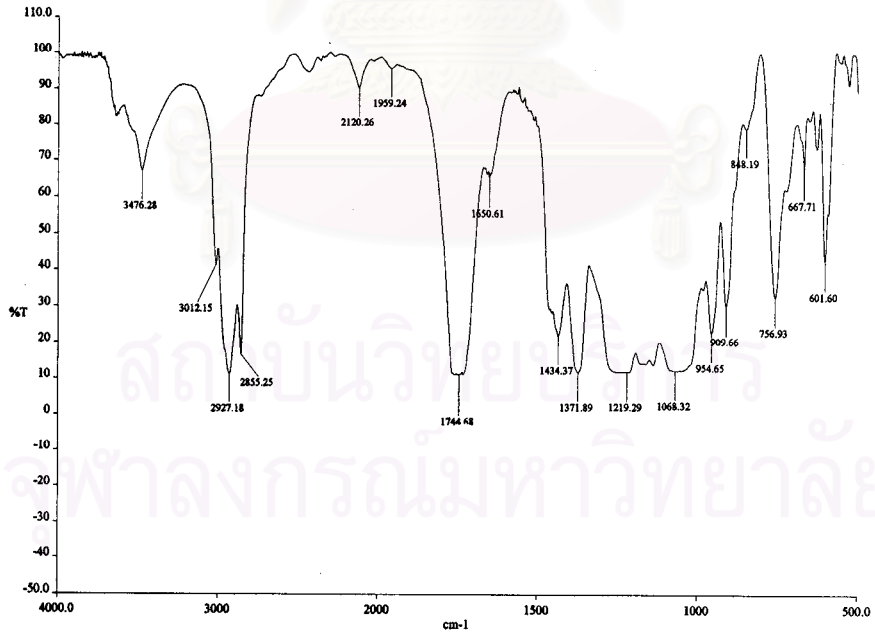

Figure 32. IR Spectrum of peracetyl CSF 3 (film)

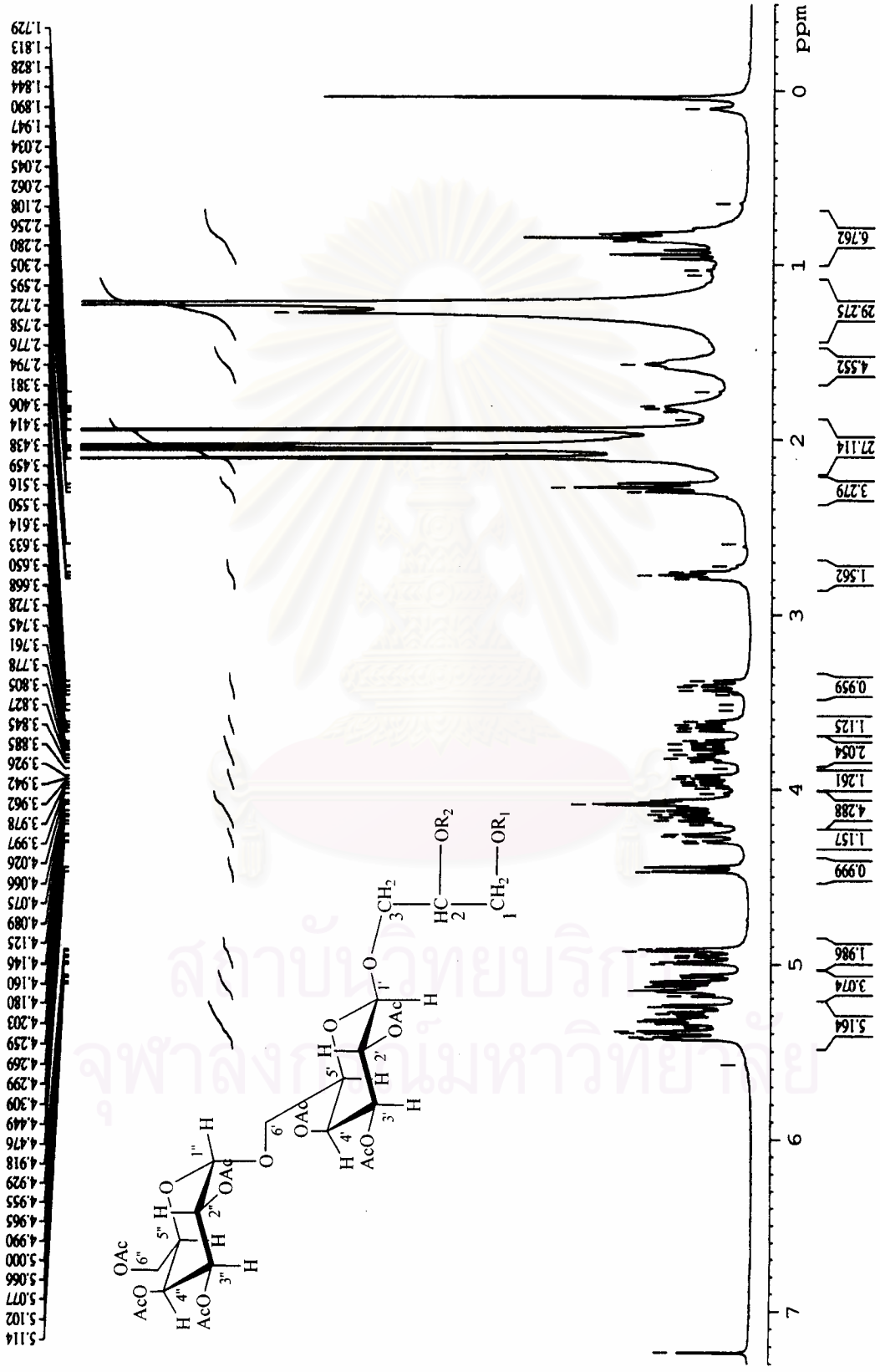

Figure 33. ^{1}H NMR (300 MHz) Spectrum of compound peracetyl CSF 3 ($CDCL_3$)

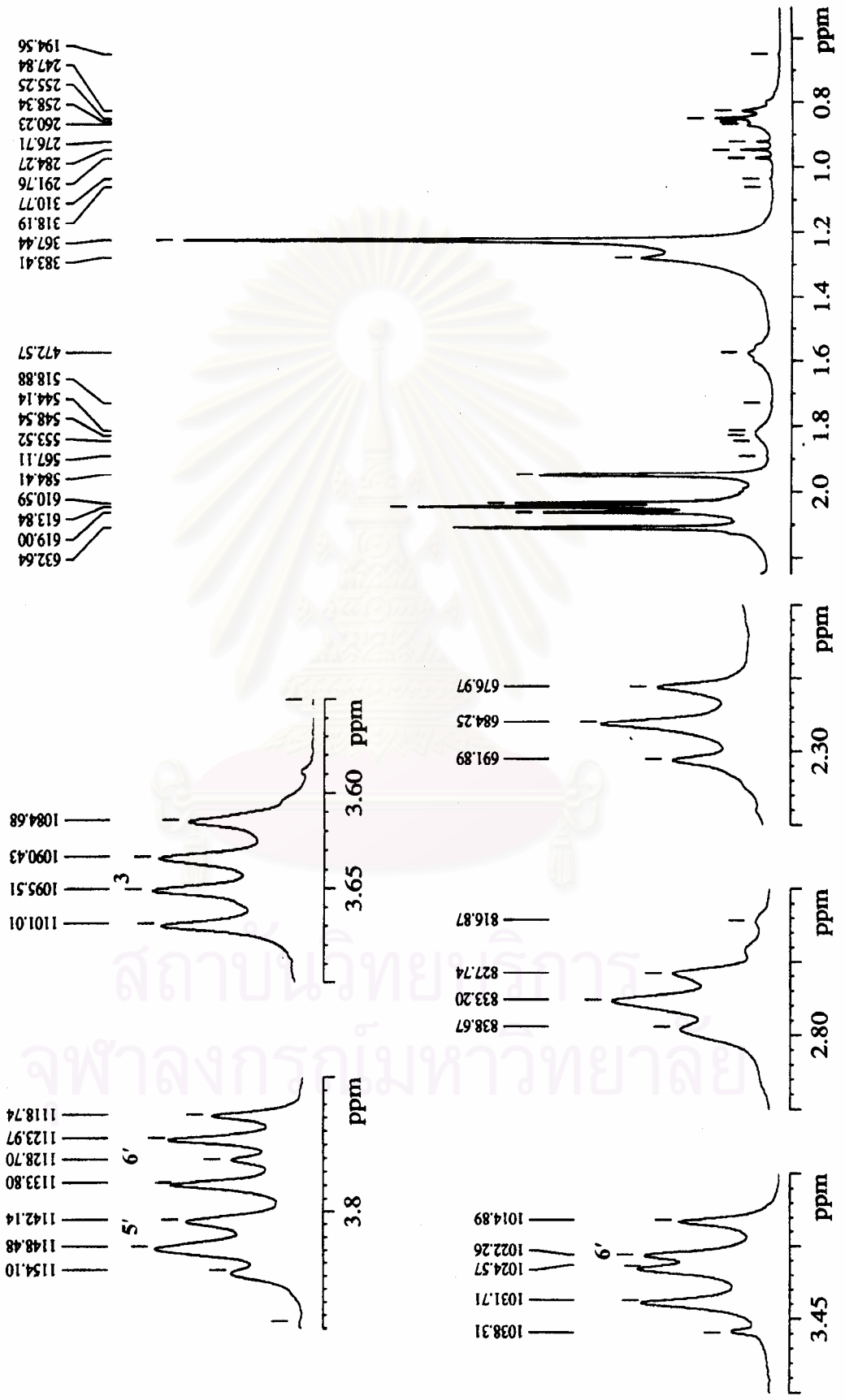

Figure 34. Expanded ^{1}H NMR (300 MHz) Spectrum of compound peracetyl CSF 3 ($CDCL_3$)

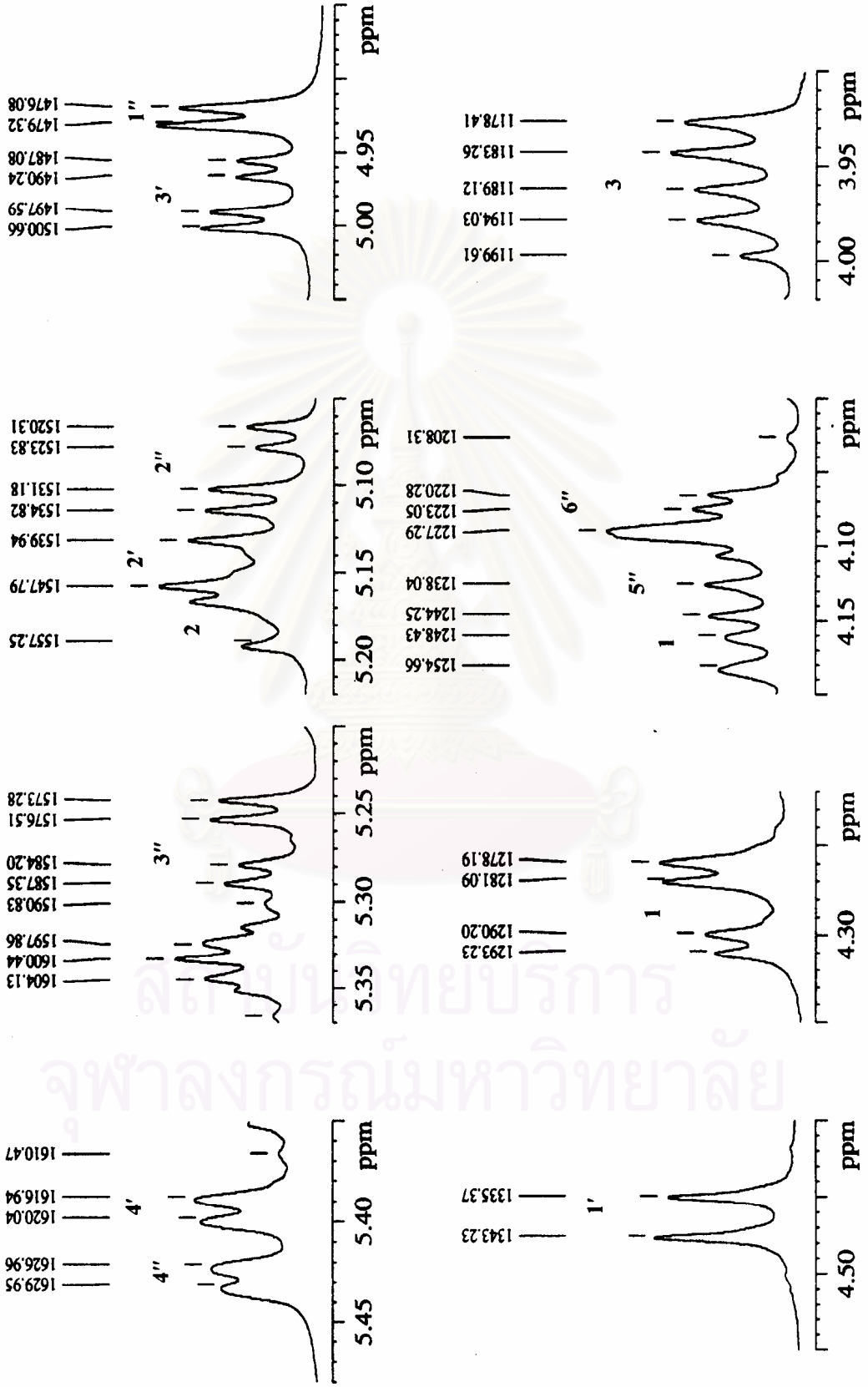

Figure 34. Expanded ^{1}H NMR (300 MHz) Spectrum of compound peracetyl CSF 3 ($CDCL_3$) (continued)

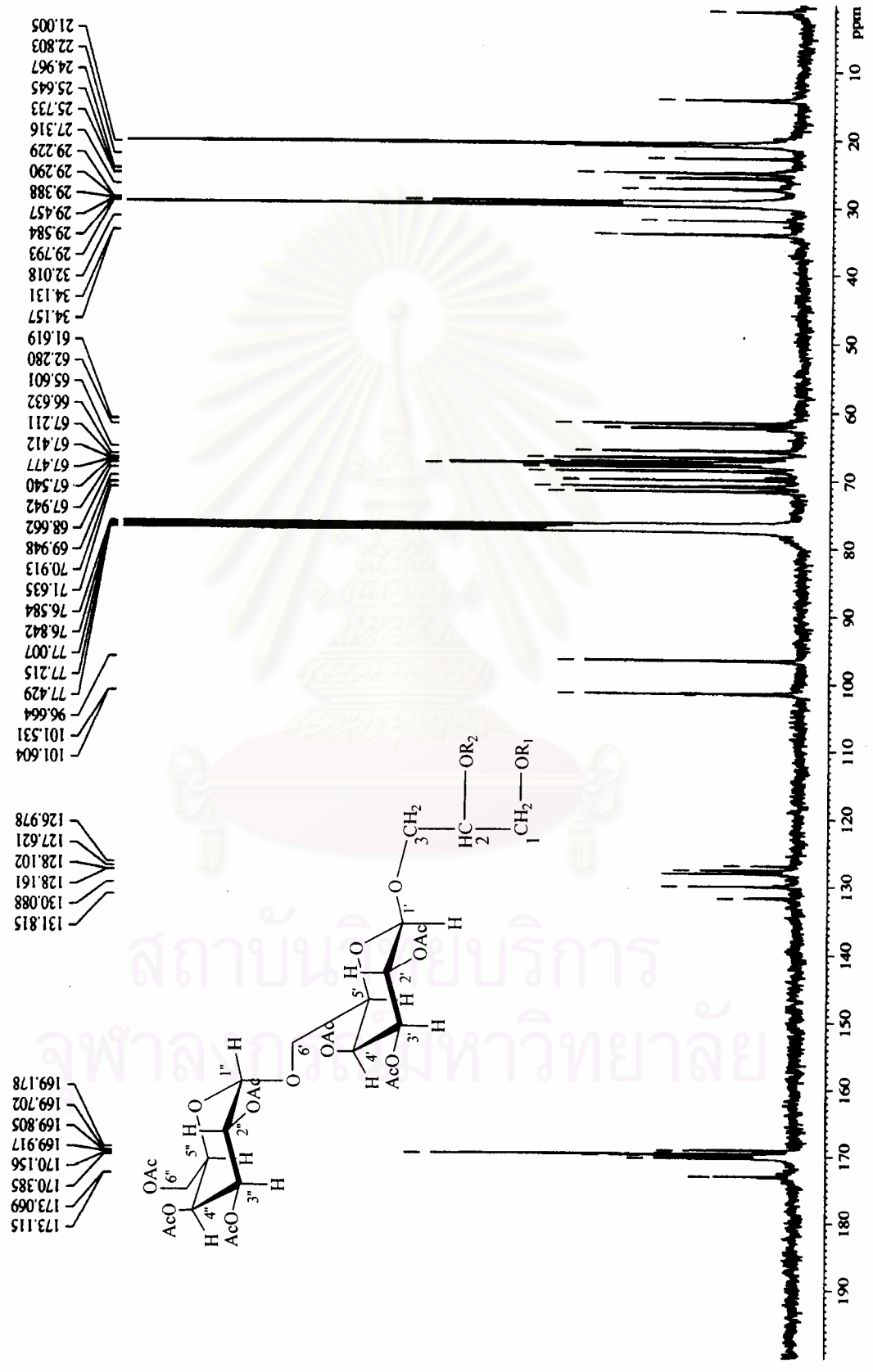

Figure 35. ^{13}C NMR (75 MHz) Spectrum of compound peracetyl CSF 3 ($CDCL_3$)

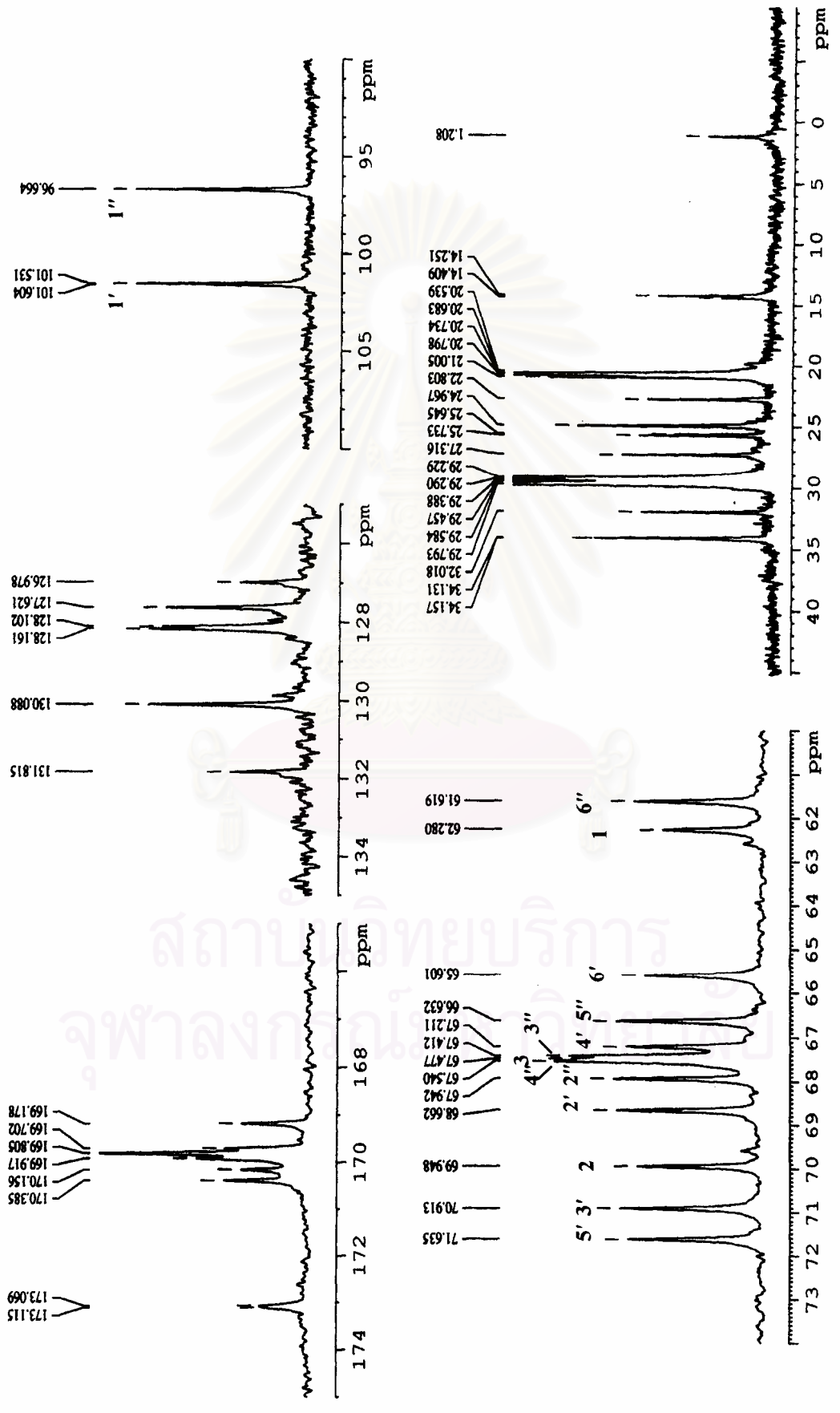

Figure 36. Expanded ^{13}C NMR (75 MHz) Spectrum of compound peracetyl CSF 3 ($CDCL_3$)

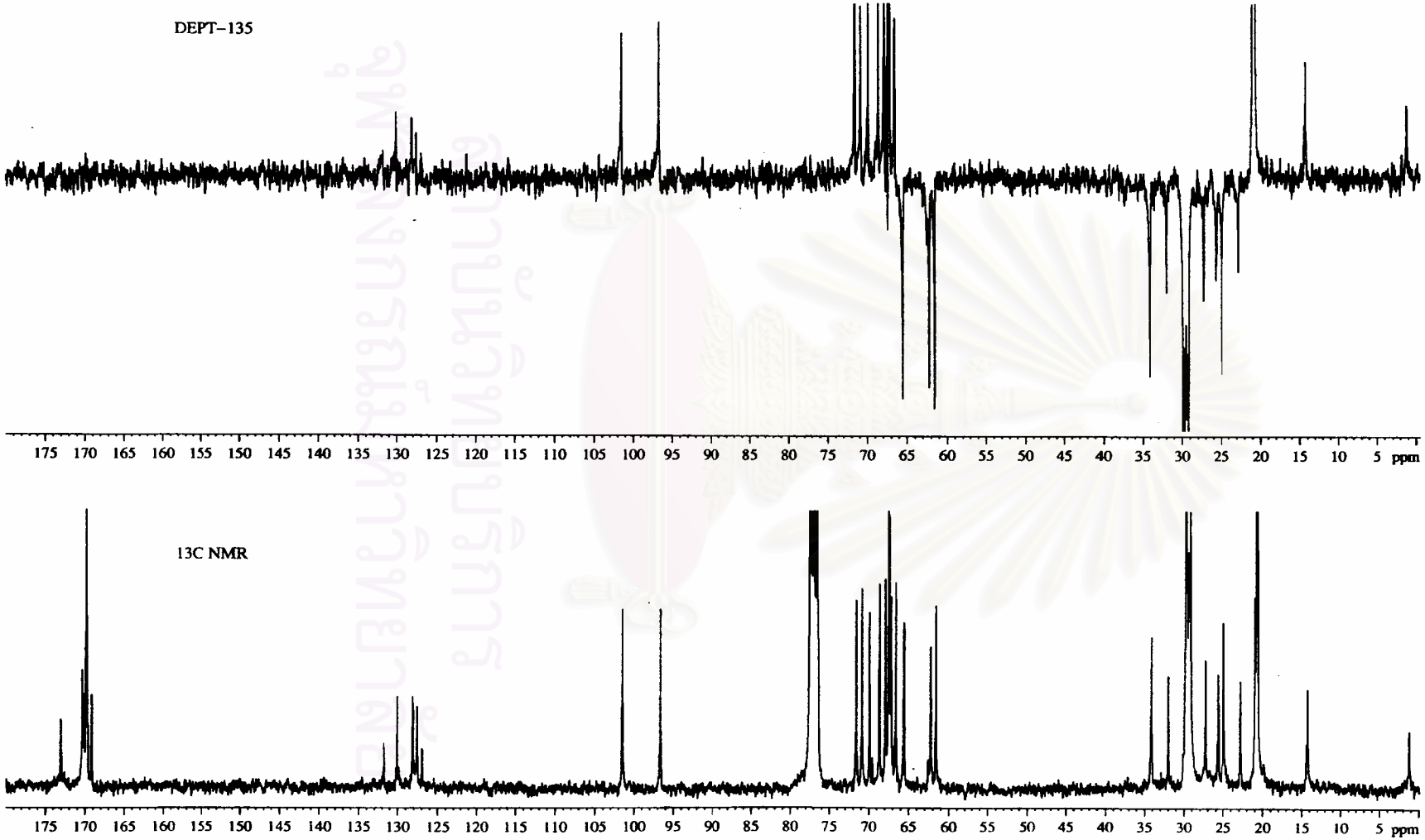

Figure 37. **^{13}C NMR (75 MHz) and DEPT-135 Spectrum of compound peracetyl CSF 3 ($CDCL_3$)**

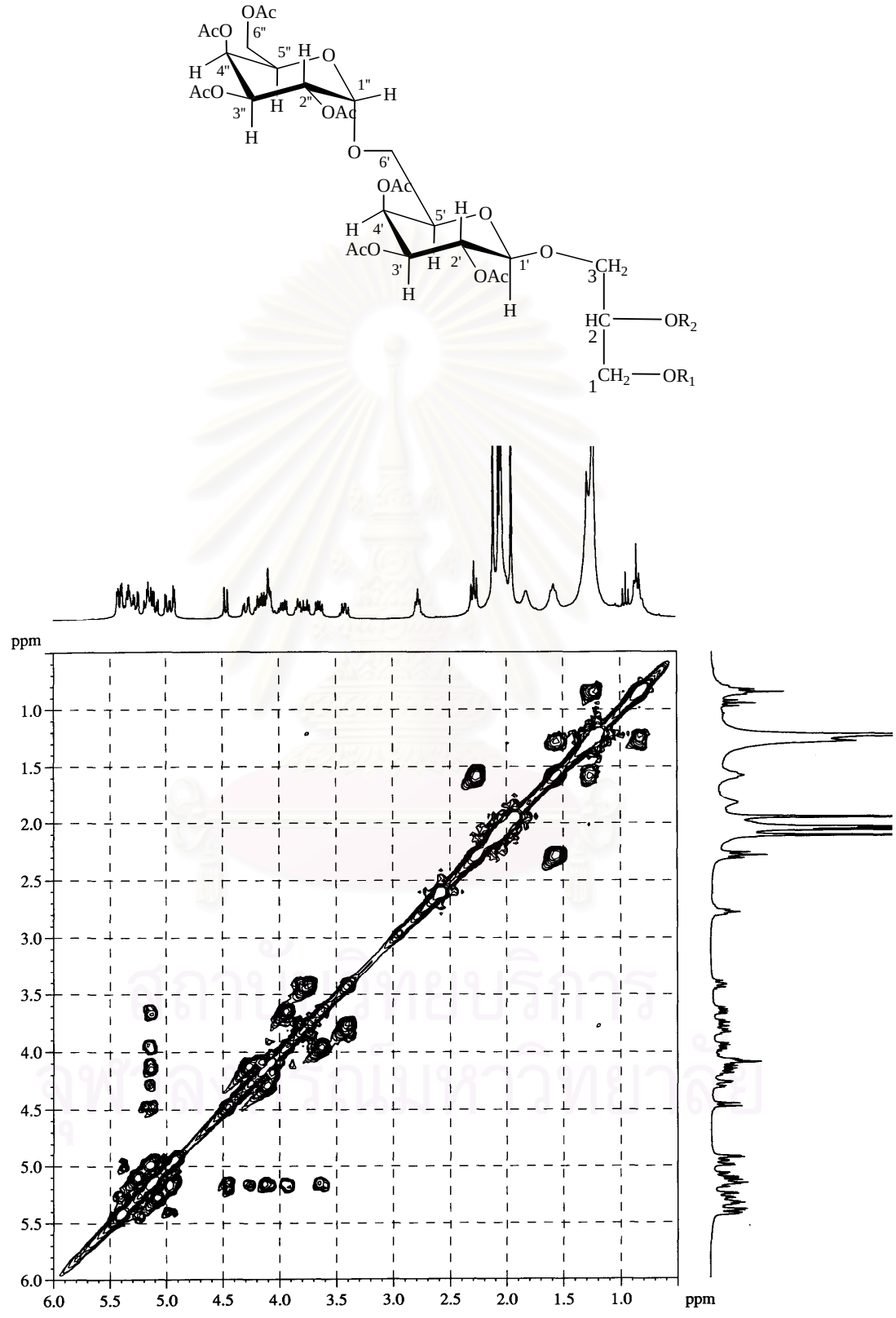

Figure 38. ^{1}H-^{1}H COSY Spectrum of peracetyl CSF 3 ($CDCl_3$)

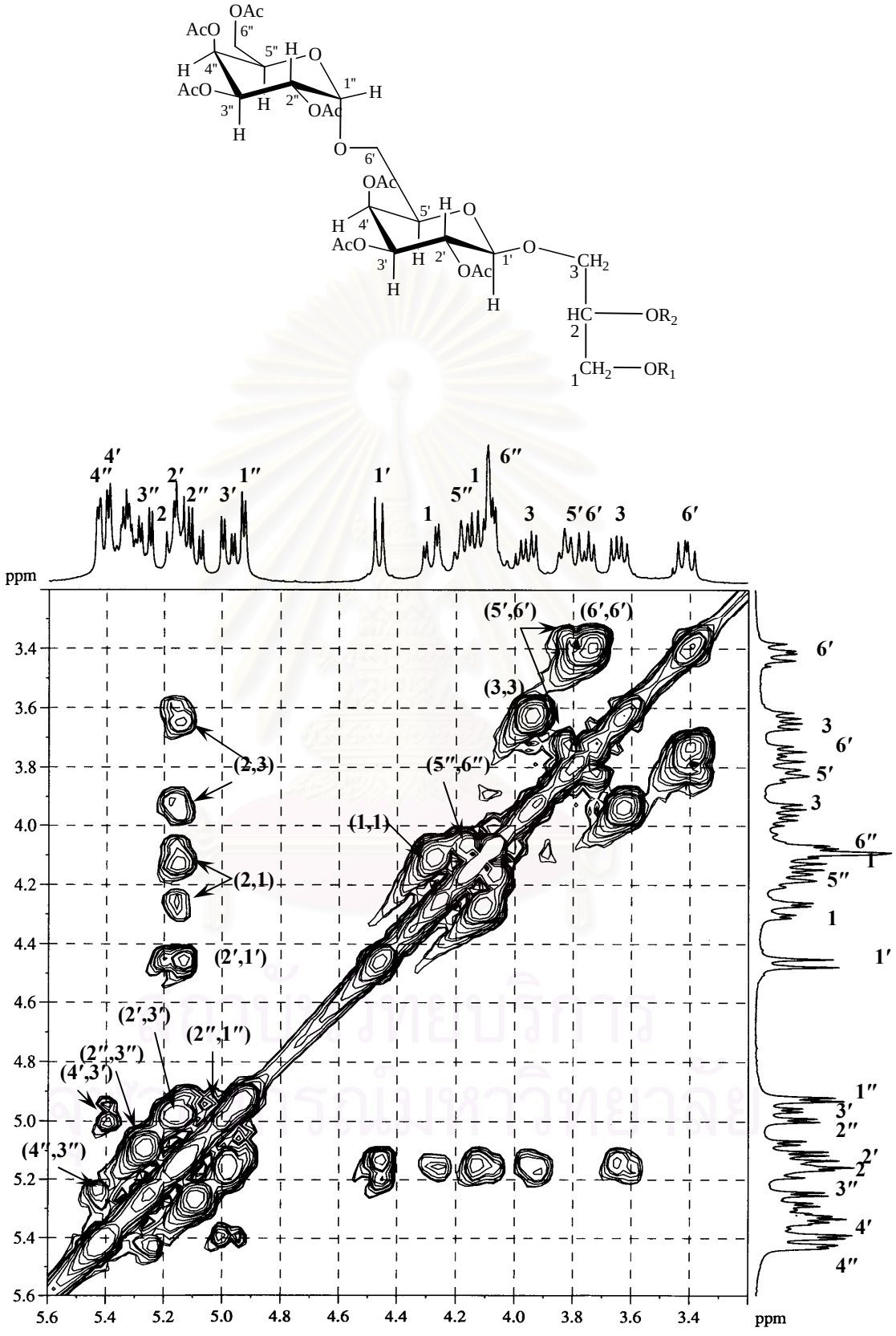

Figure 39. Expanded ^{1}H-^{1}H COSY Spectrum of peracetyl CSF3 ($CDCl_3$)

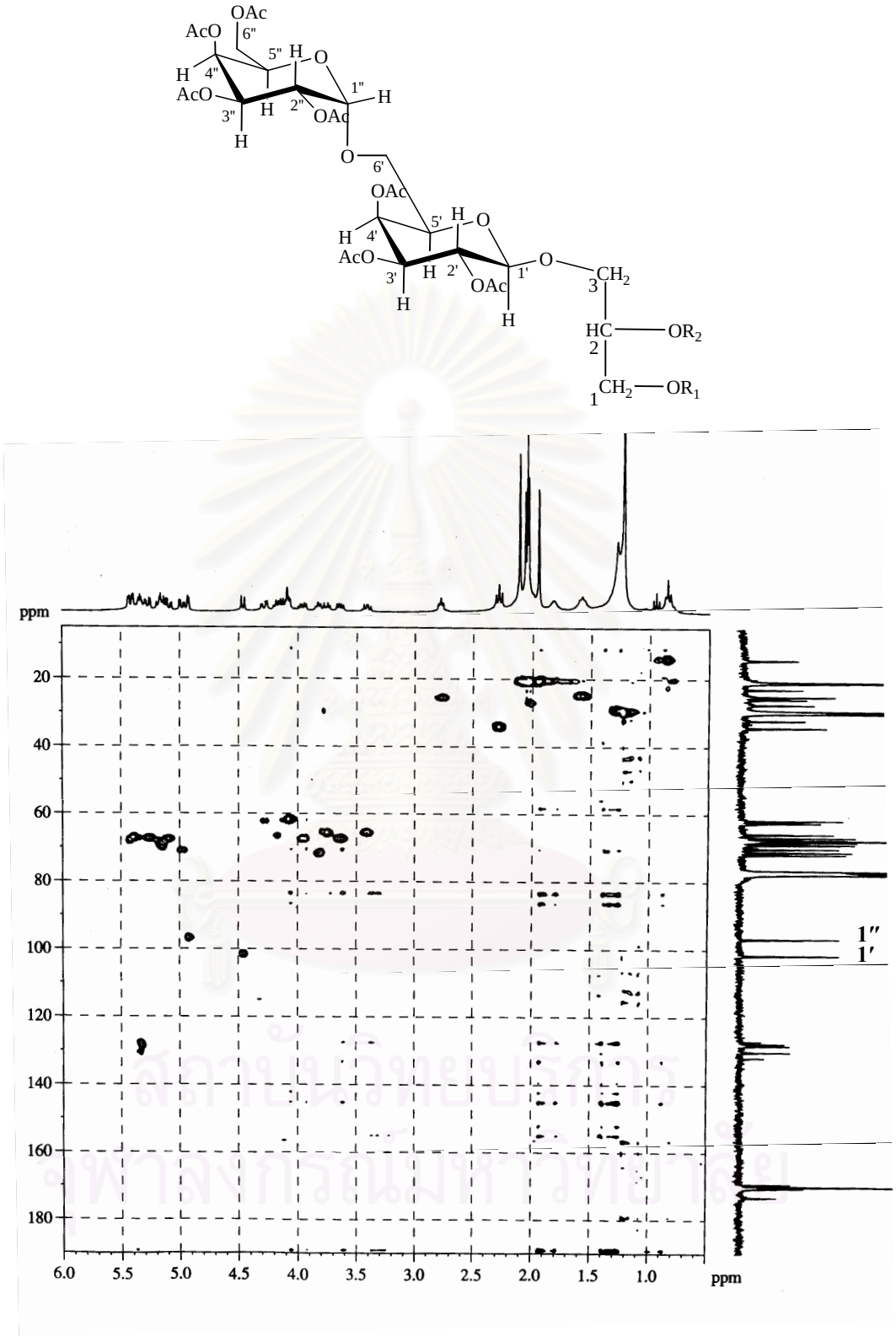

Figure 40. HMQC Spectrum of peracetyl CSF 3 ($CDCl_3$)

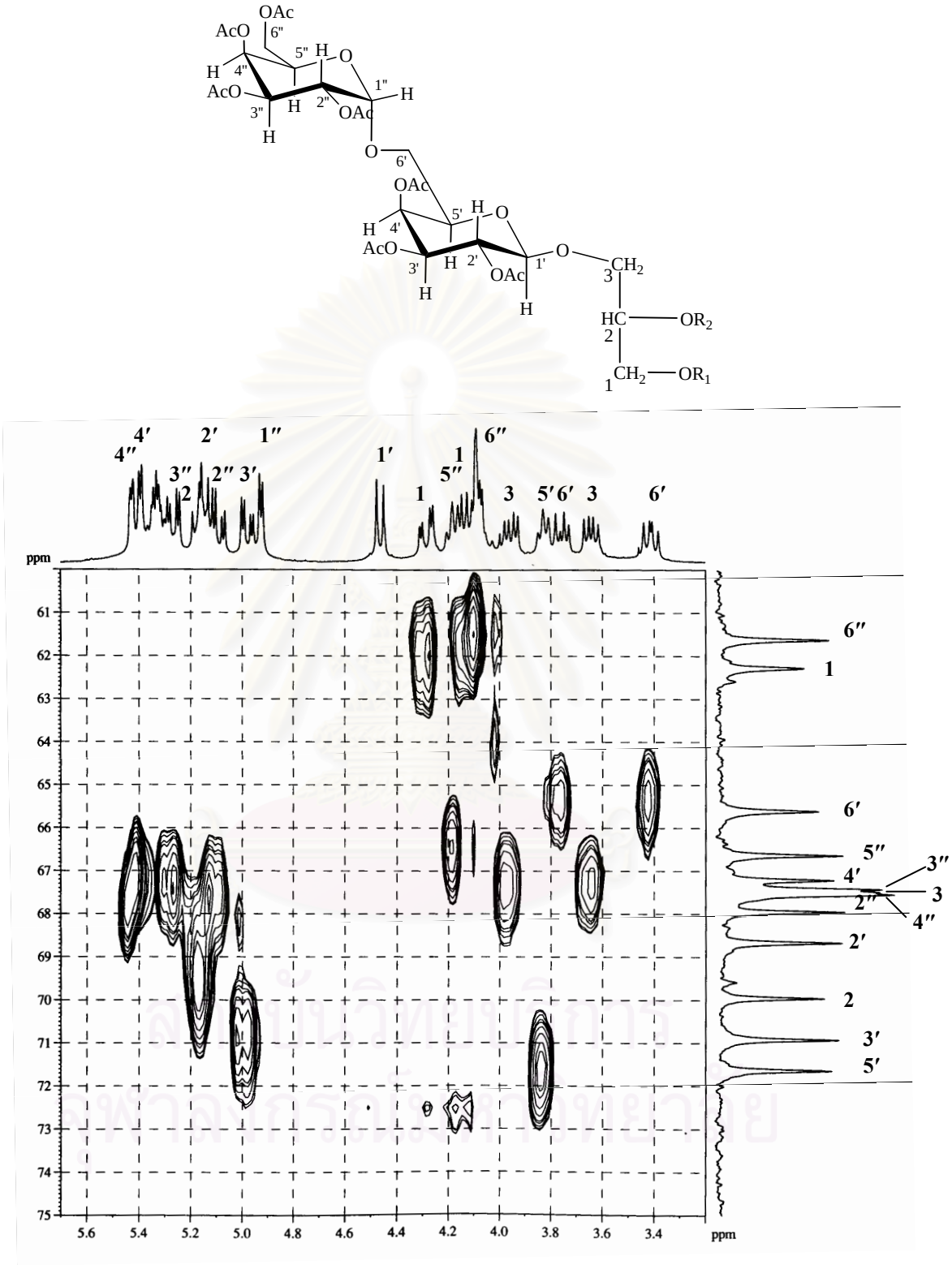

Figure 41. Expanded HMQC Spectrum of peracetyl CSF 3 ($CDCl_3$)

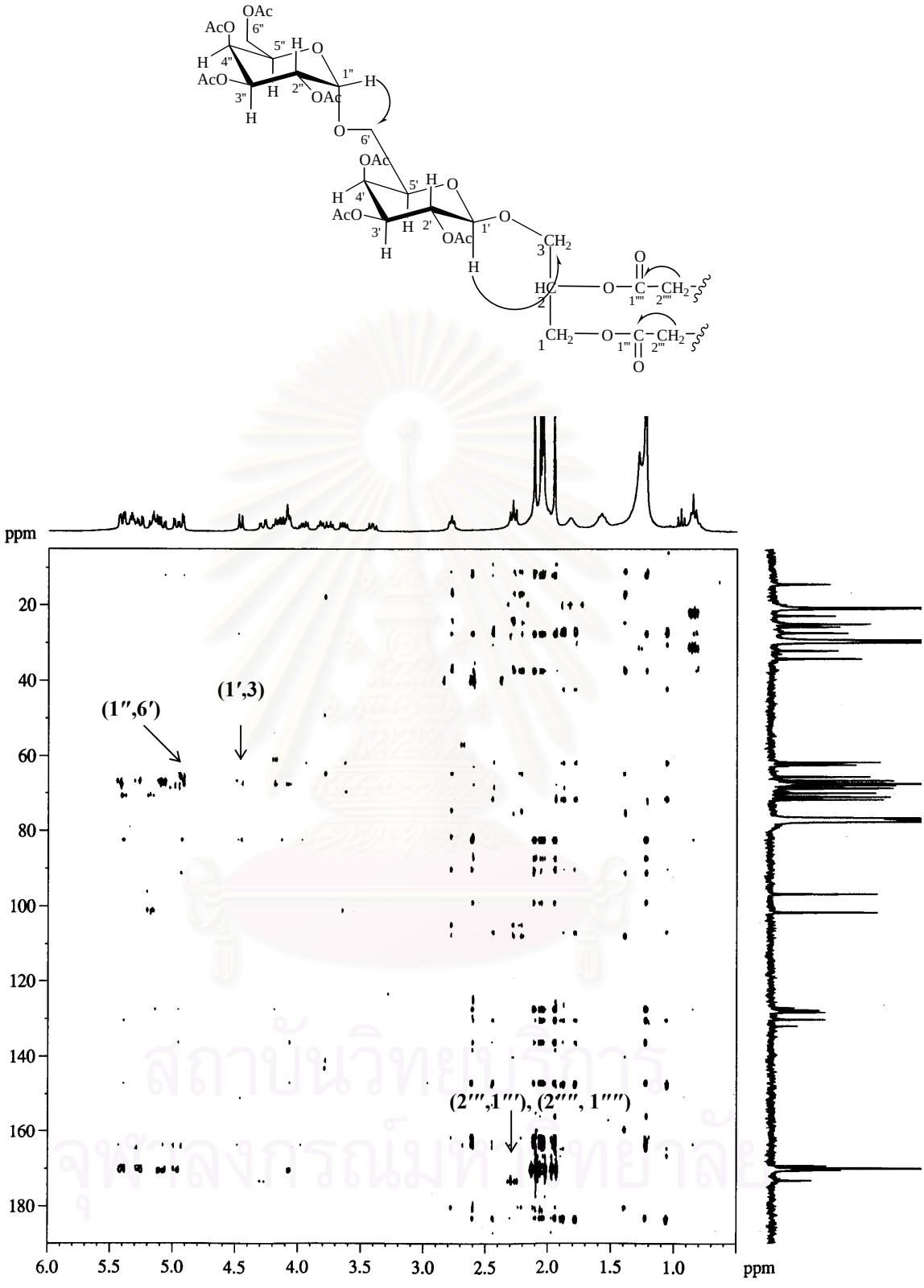

Figure 42. HMBC ($^{n}J_{C\text{-}H}$ = 8 Hz) Spectrum of peracetyl CSF 3 ($CDCl_3$)

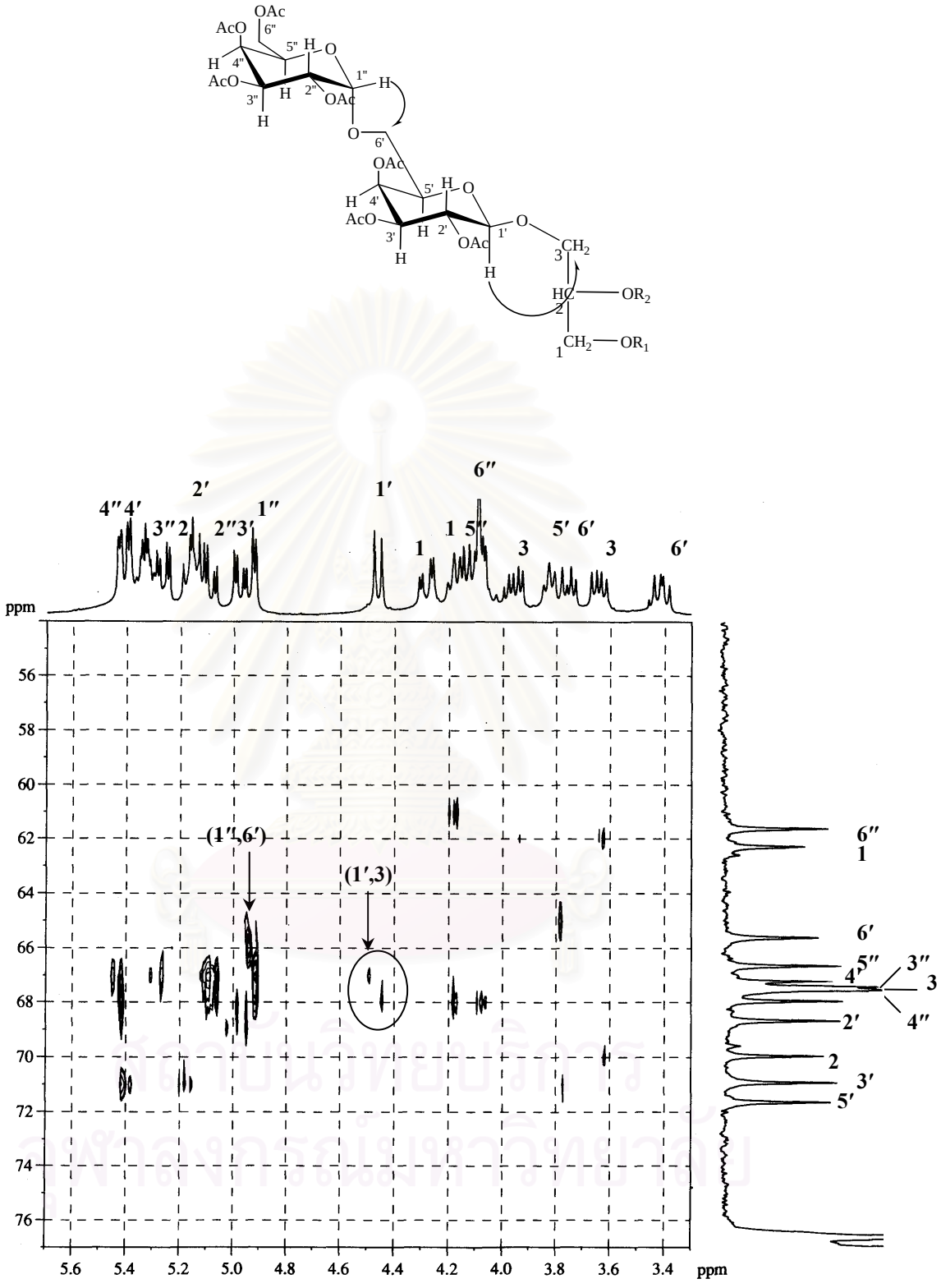

Figure 43. Expanded HMBC ($^{n}J_{C\text{-}H}$ = 8 Hz) Spectrum of peracetyl CSF 3 ($CDCl_3$)

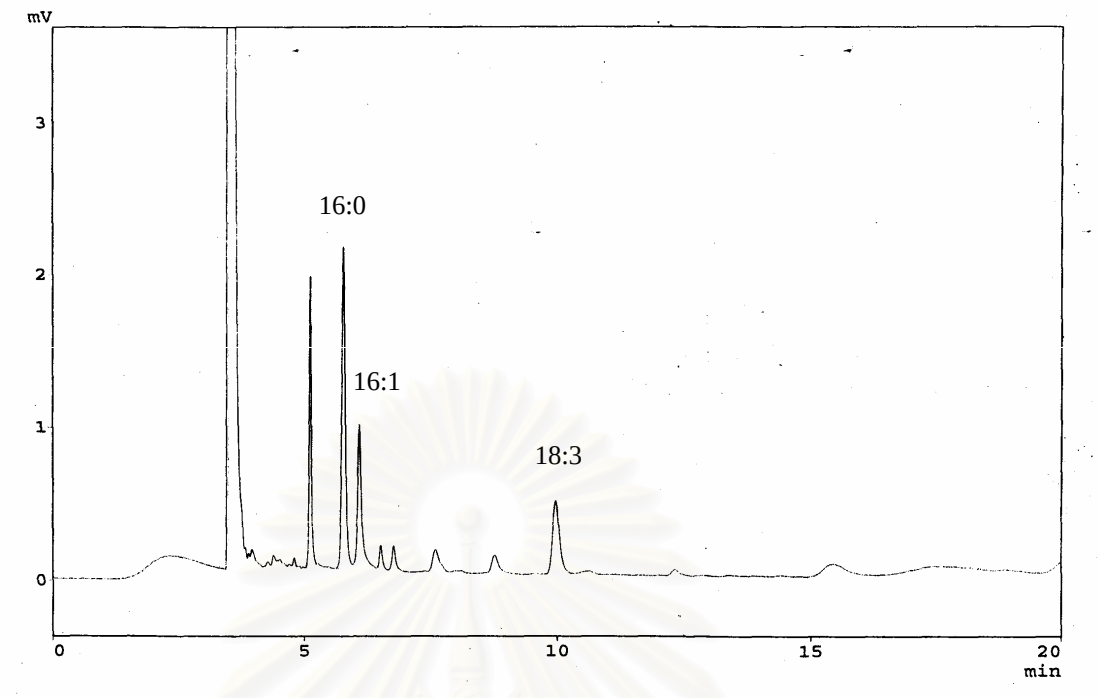

Figure 44. GC chromatogram of peracetyl CSF 3 acid hydrolysate

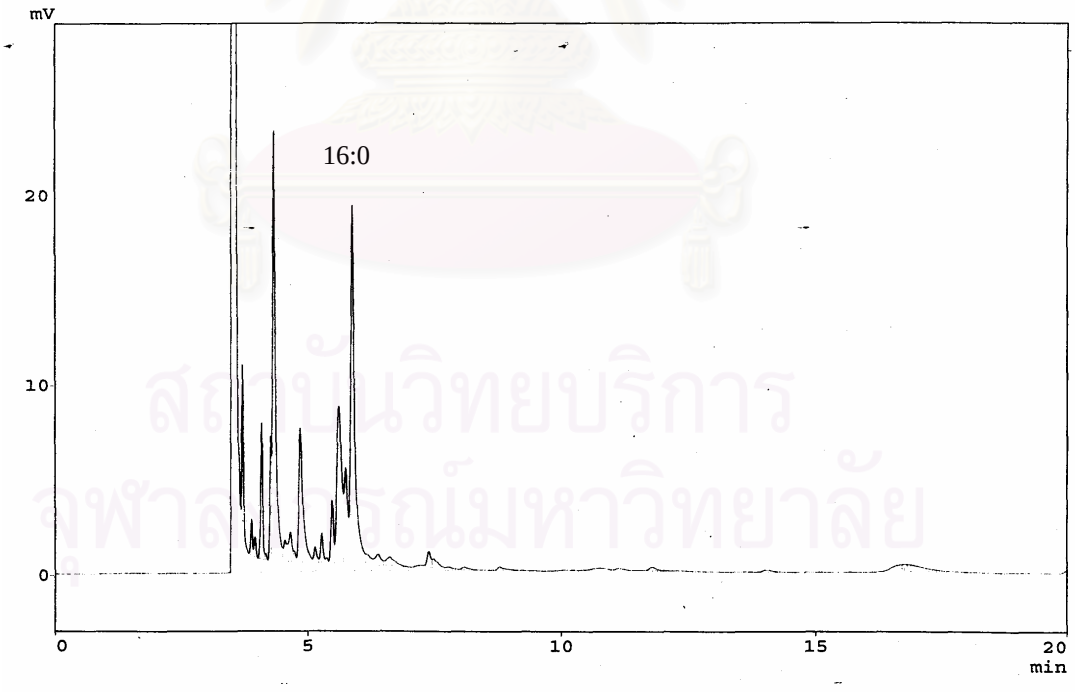

Figure 45. GC chromatogram of peracetyl CSF 3 enzymatic hydrolysate

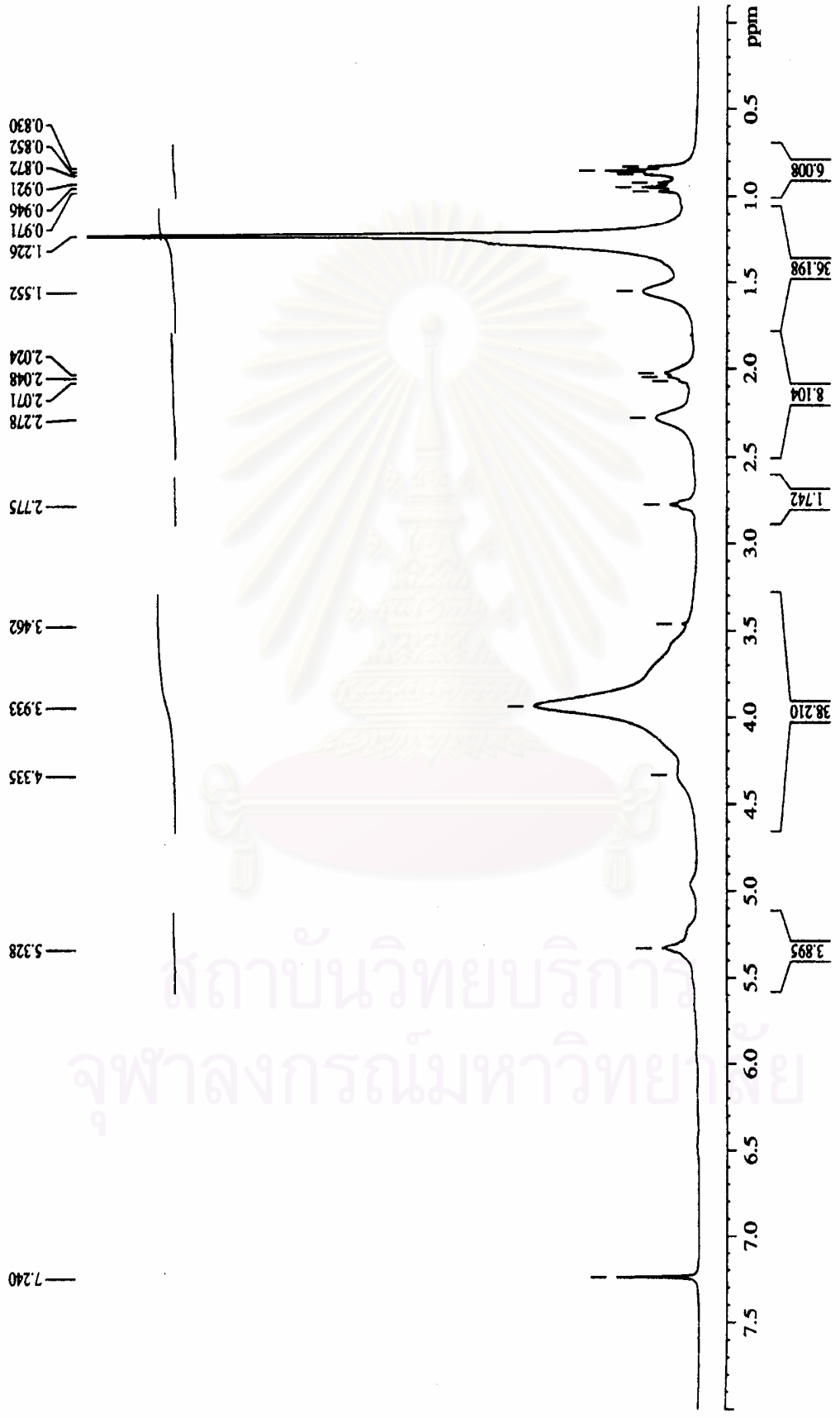

Figure 46. ^{1}H NMR (300 MHz) Spectrum of compound CSF 4 ($CDCL_3$)

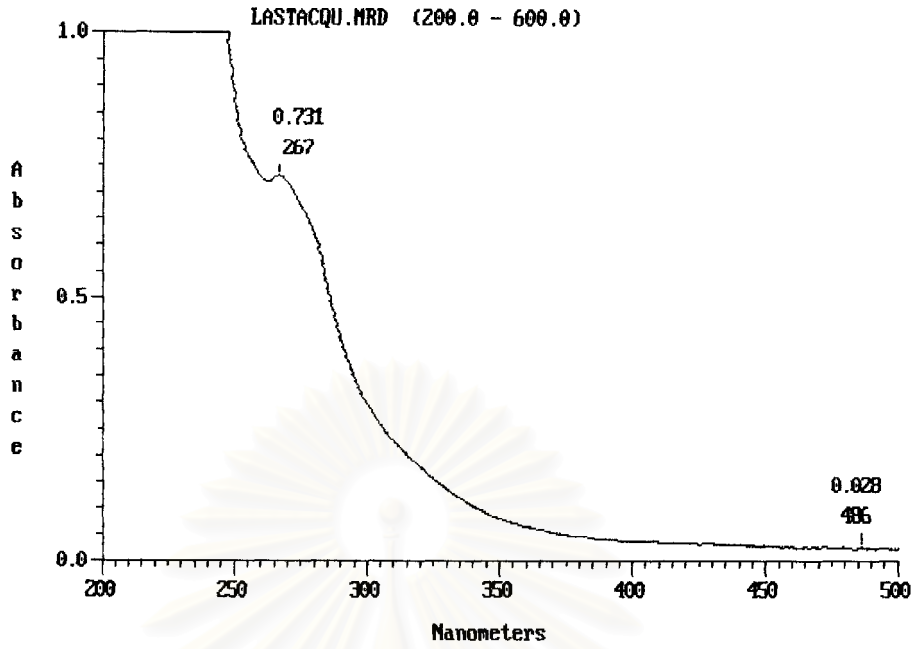

Figure 47. UV Spectrum of peracetyl CSF 4 (MeOH)

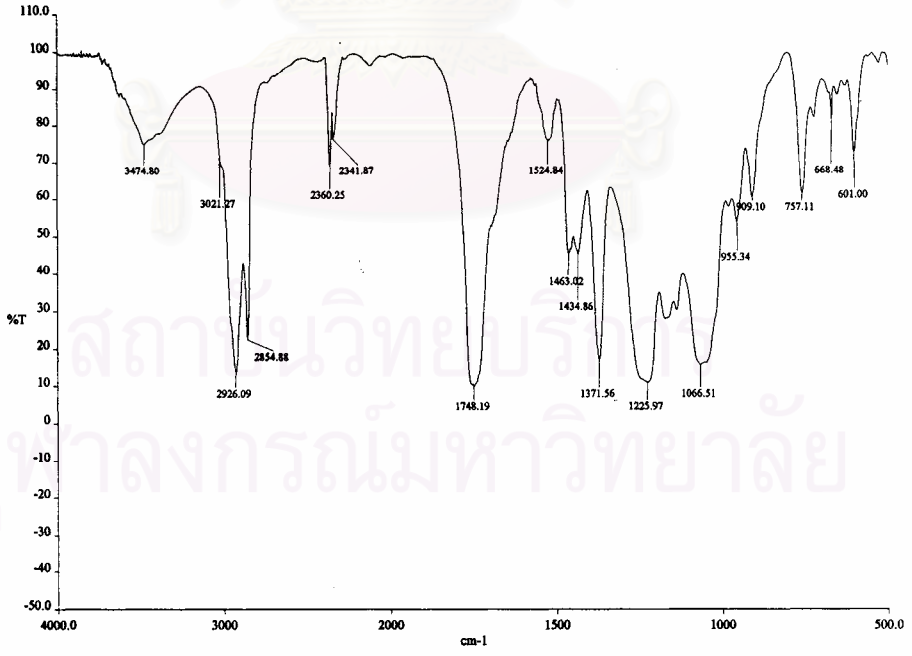

Figure 48. IR Spectrum of peracetyl CSF 4 (film)

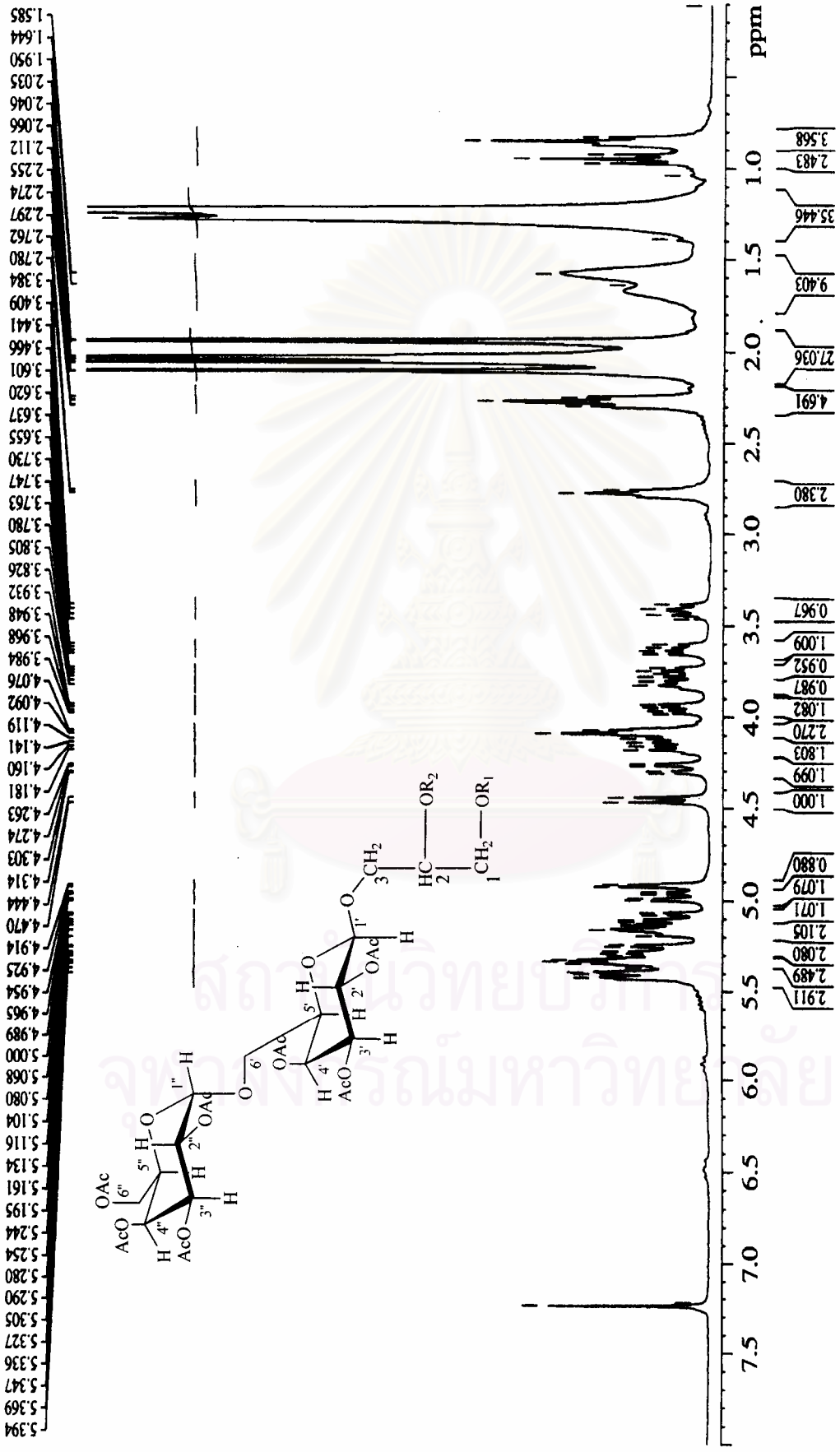

Figure 49. ^{1}H NMR (300 MHz) Spectrum of compound peracetyl CSF 4 ($CDCL_3$)

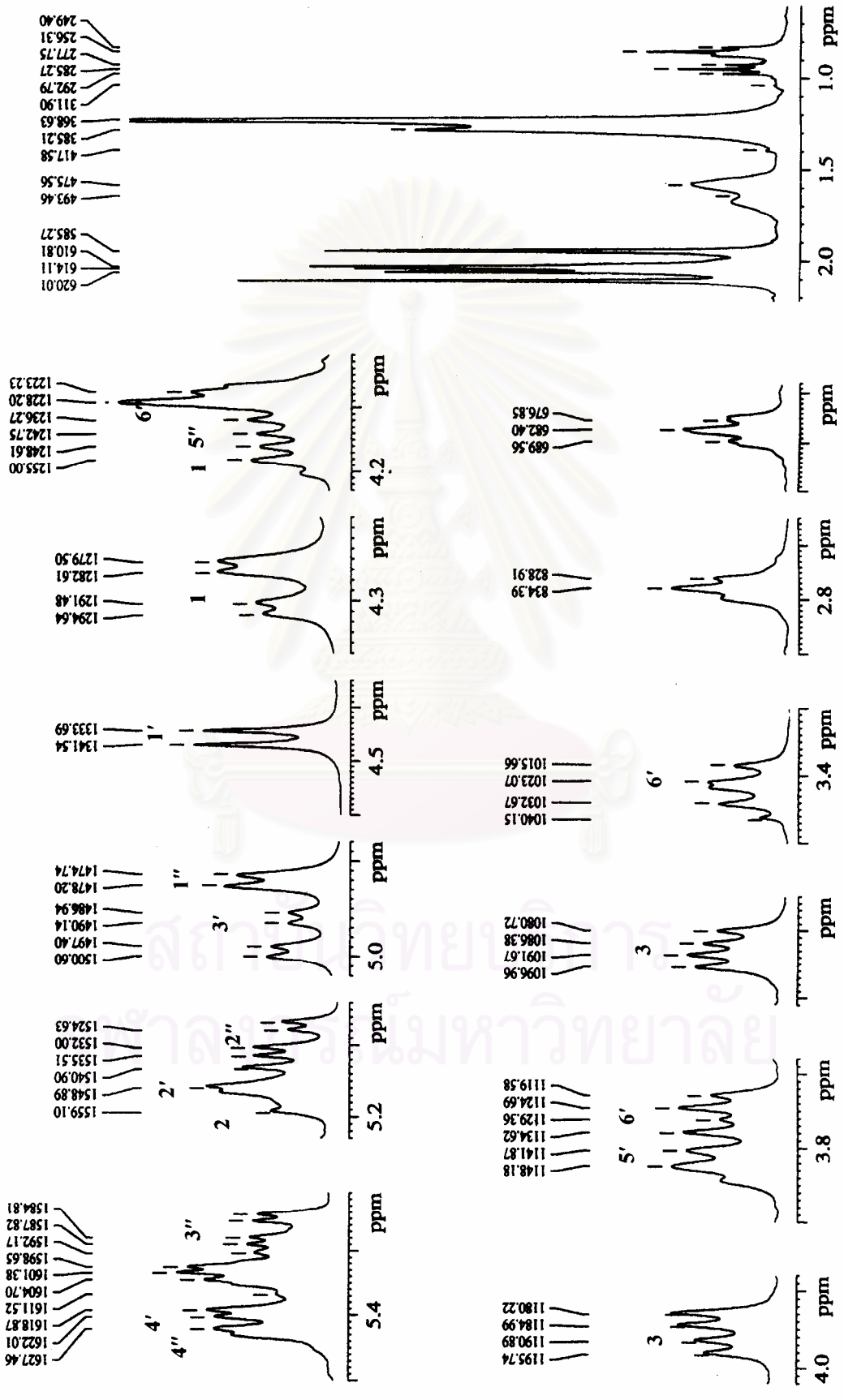

Figure 50. Expanded ^{1}H NMR (300 MHz) Spectrum of compound peracetyl CSF 4 ($CDCL_3$)

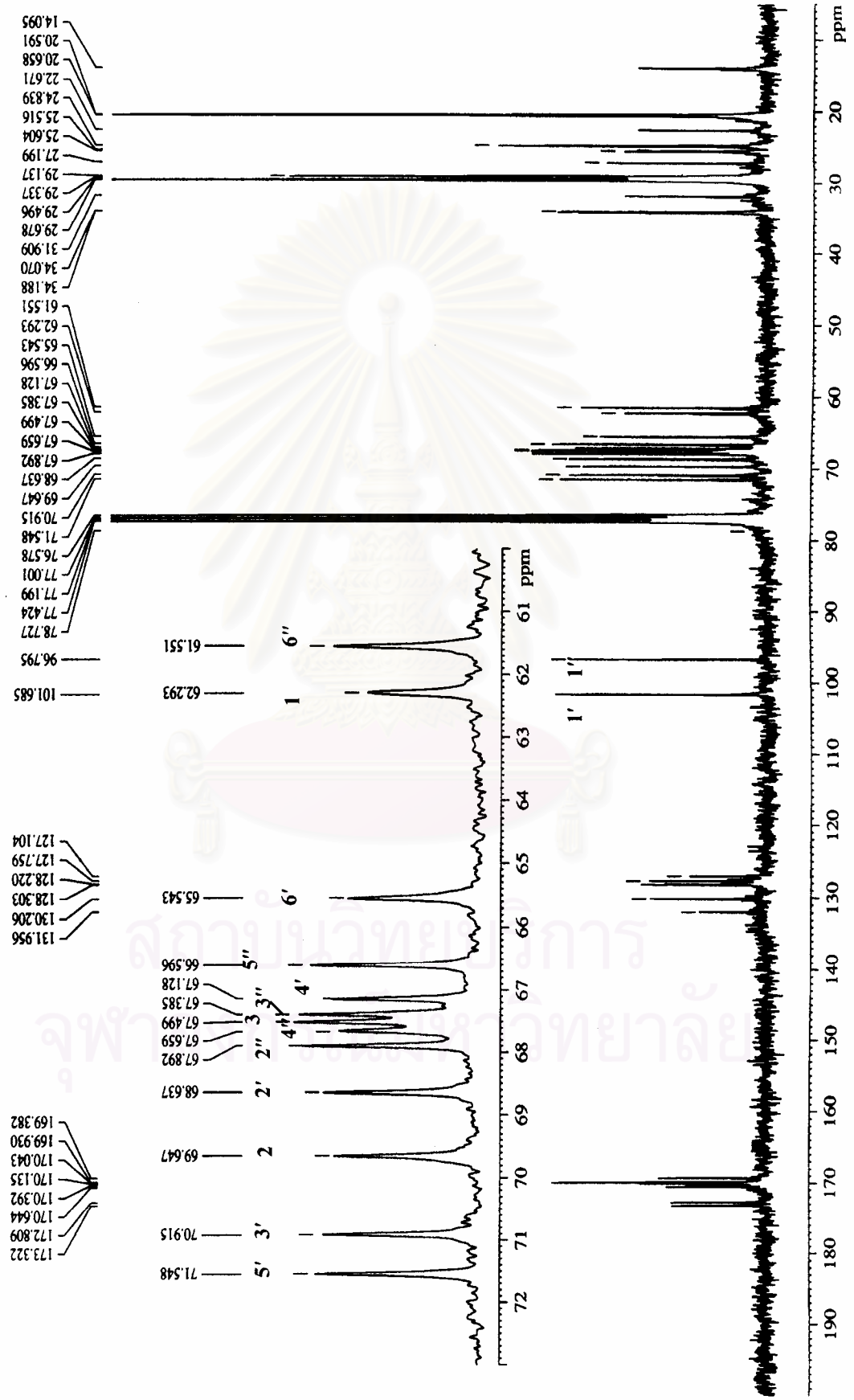

Figure 51. ^{13}C NMR (75 MHz) Spectrum of compound peracetyl CSF 4 ($CDCL_3$)

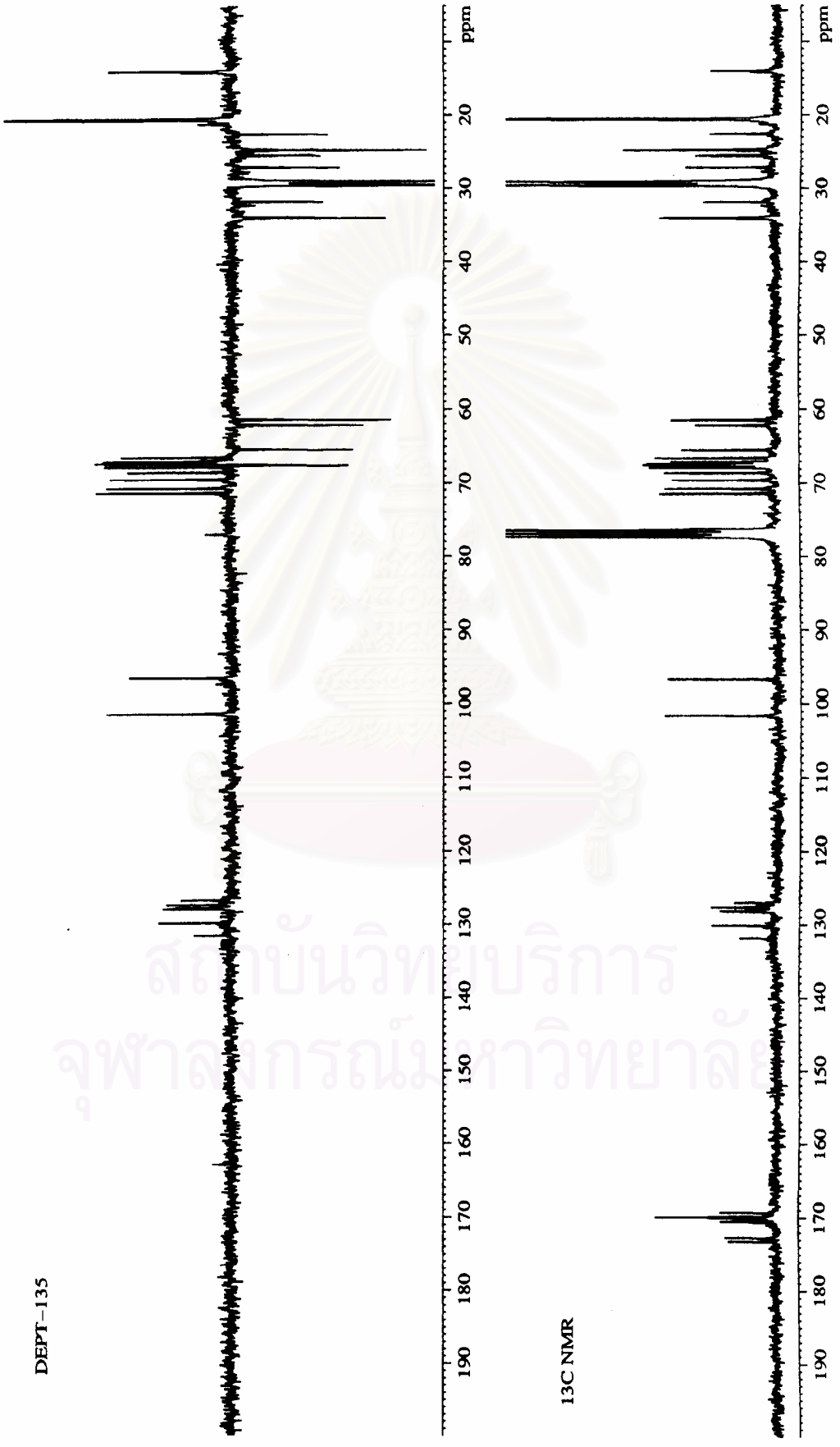

Figure 52. ^{13}C NMR (75 MHz) and DEPT-135 Spectrum of compound peracetyl CSF 4 ($CDCL_3$)

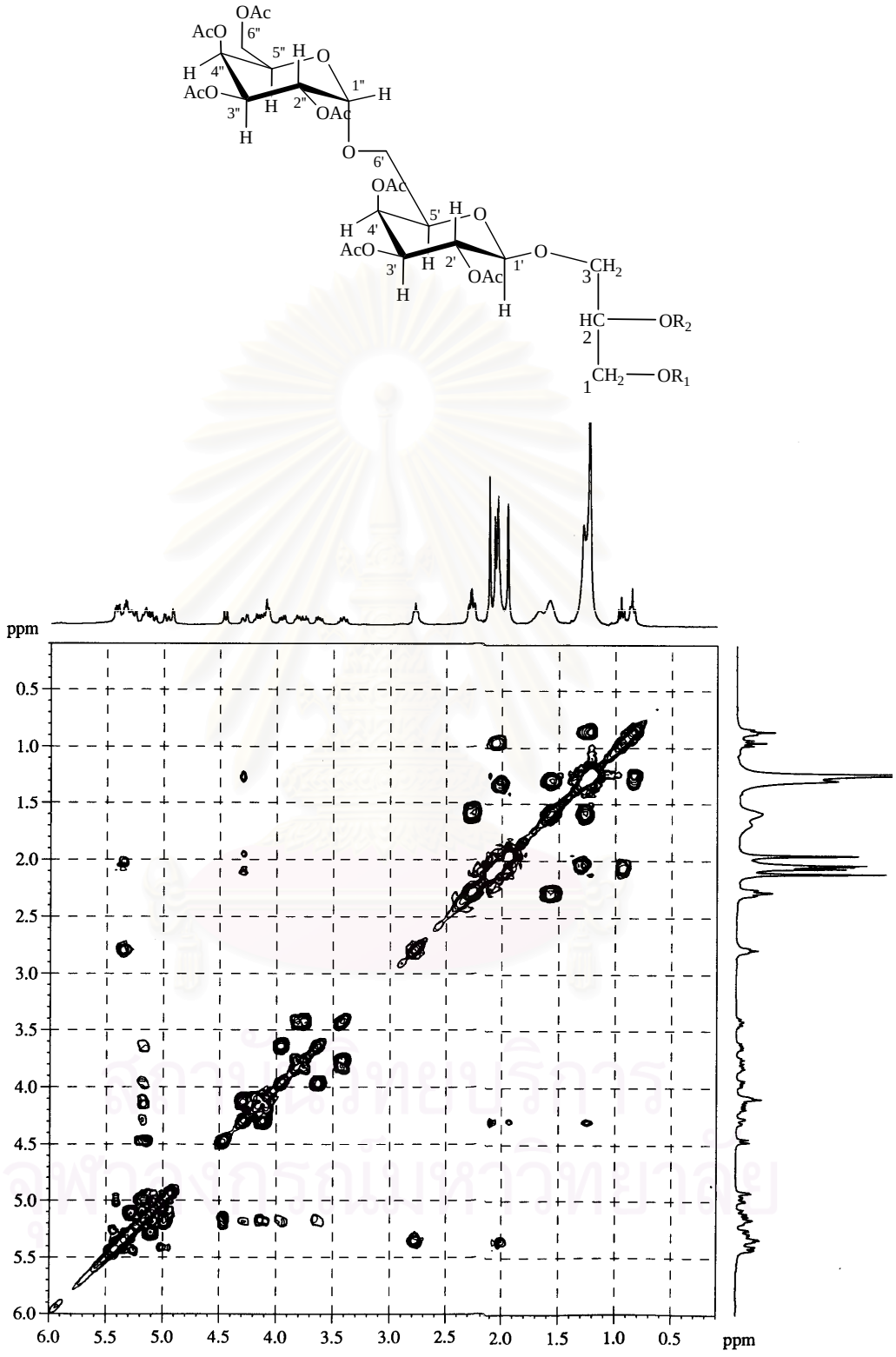

Figure 53. ^{1}H-^{1}H COSY Spectrum of peracetyl CSF 4 ($CDCl_3$)

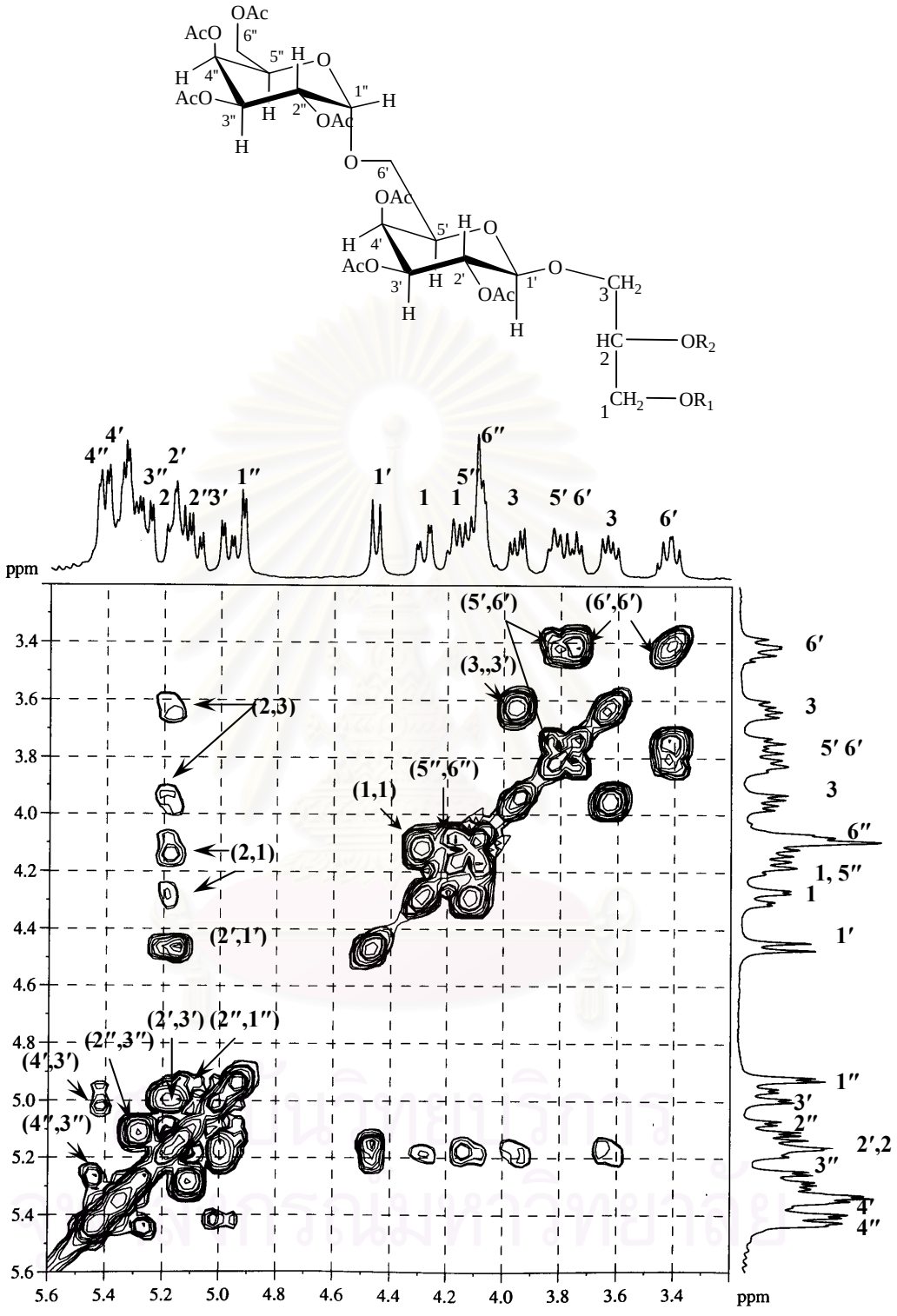

Figure 54. Expanded ^{1}H-^{1}H COSY Spectrum of peracetyl CSF 4 ($CDCl_3$)

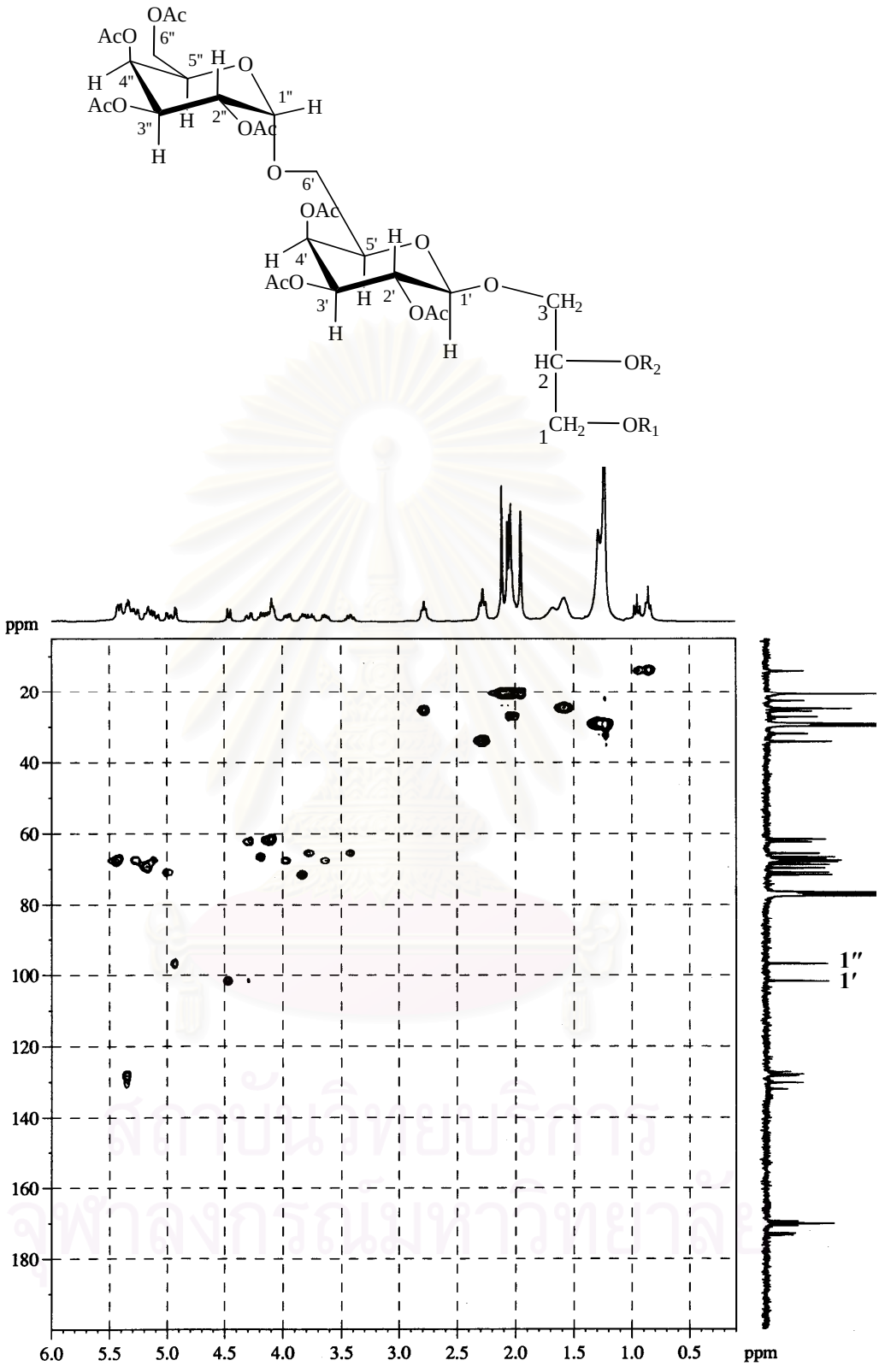

Figure 55. HMQC Spectrum of peracetyl CSF 4 ($CDCl_3$)

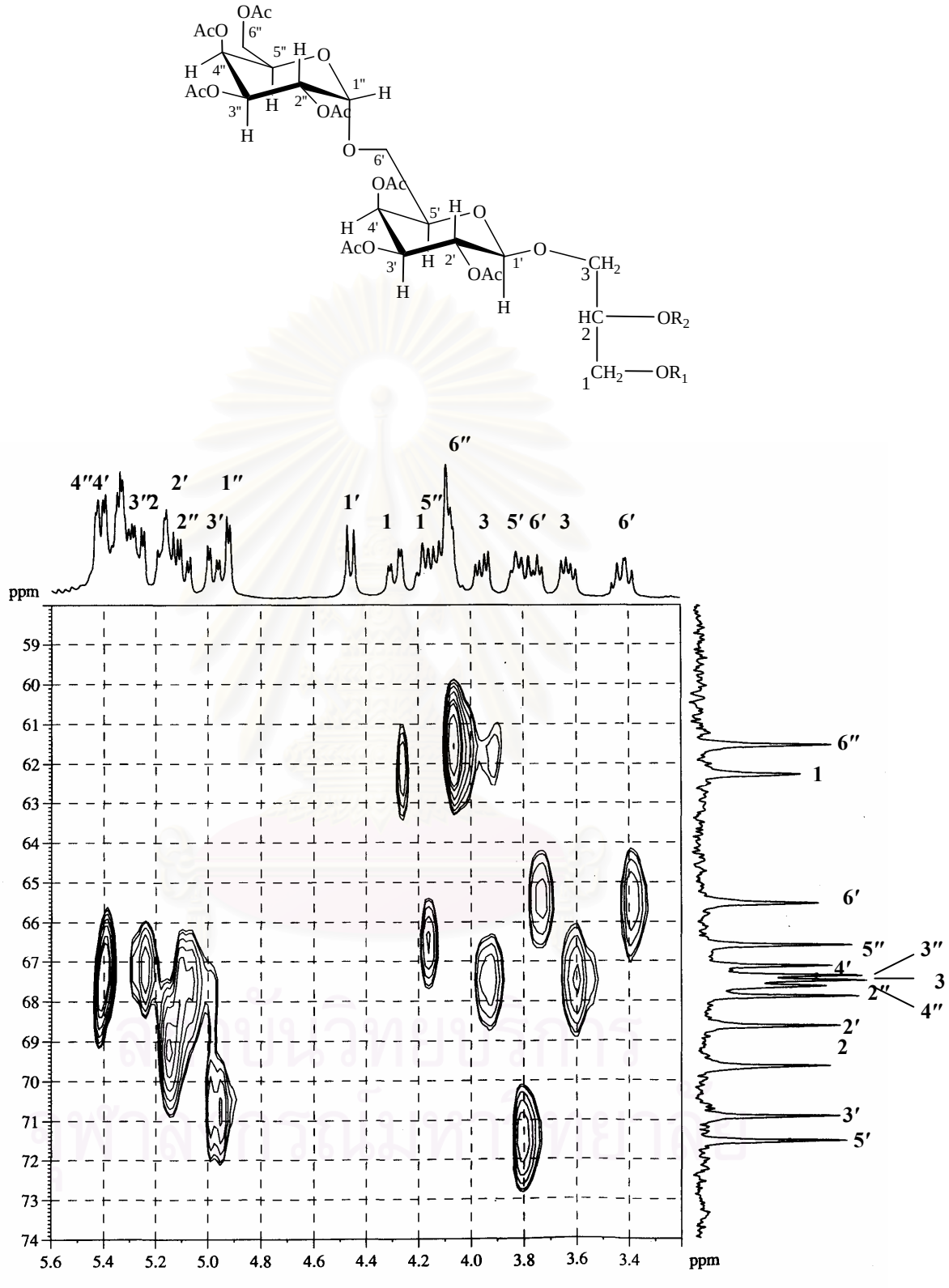

Figure 56. Expanded HMQC Spectrum of peracetyl CSF 4 ($CDCl_3$)

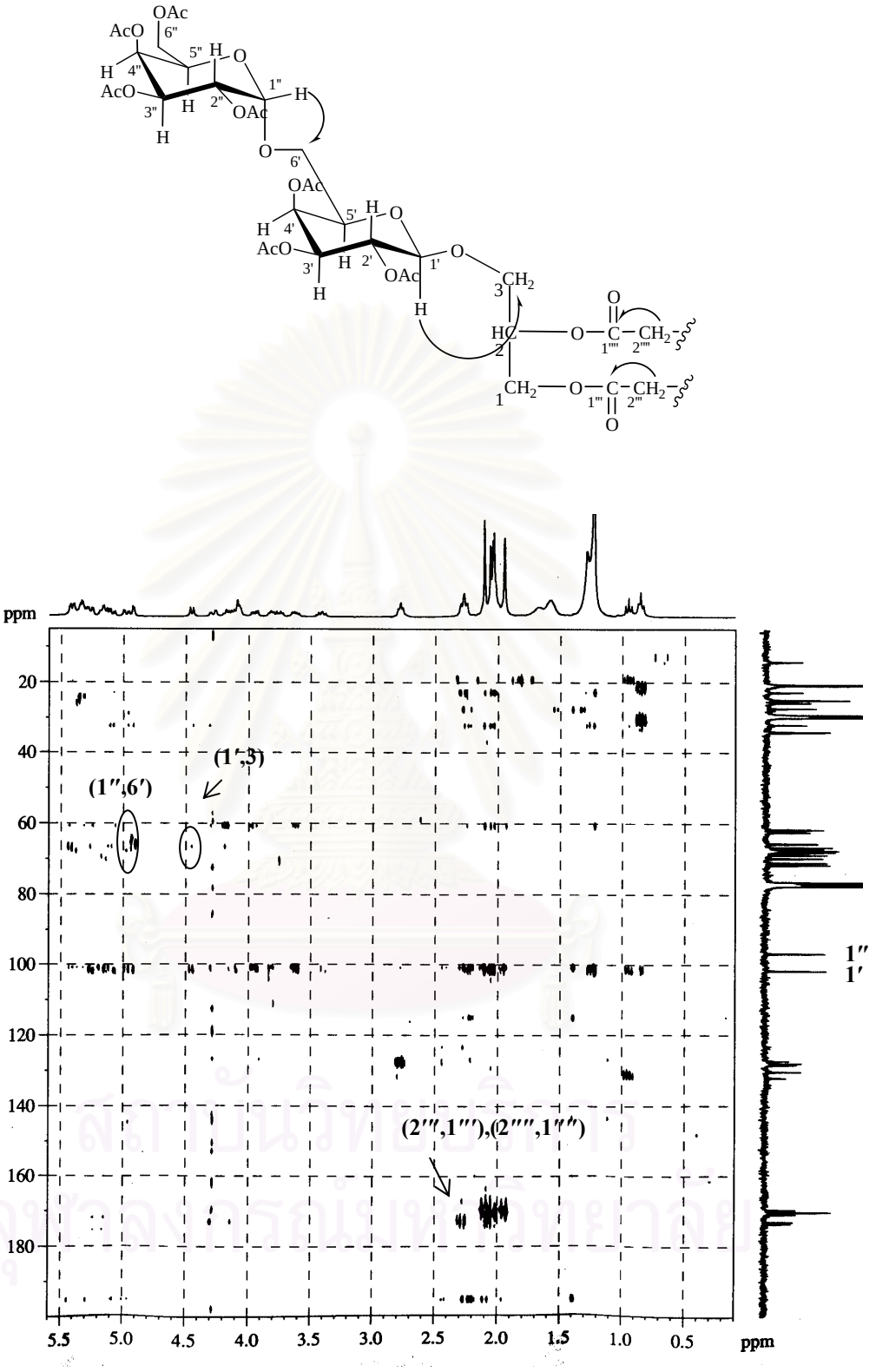

Figure 57. HMBC ($^{n}J_{C\text{-}H}$ = 8 Hz) Spectrum of peracetyl CSF 4 ($CDCl_3$)

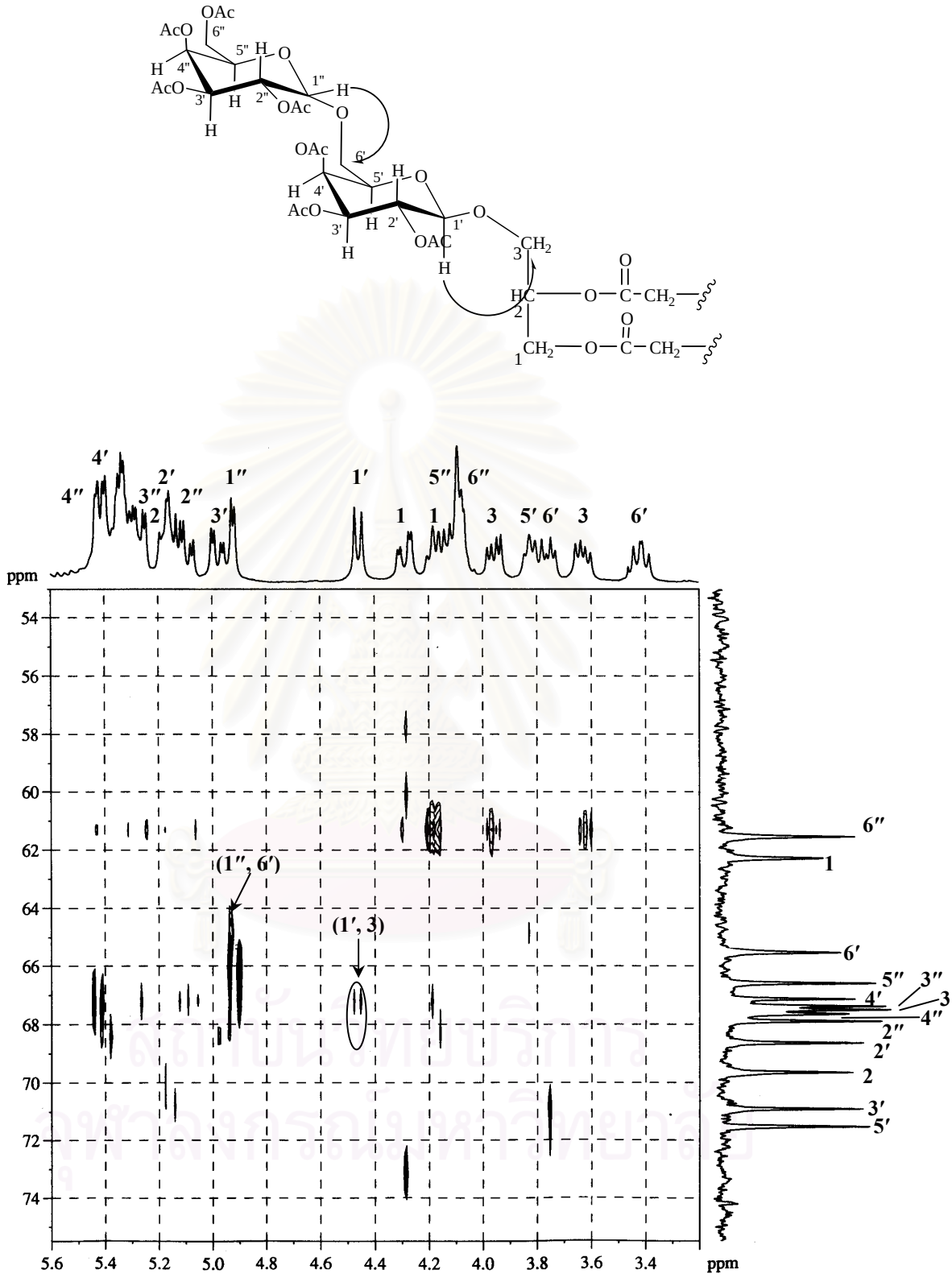

Figure 58. HMBC ($^{n}J_{C\text{-}H}$ = 8 Hz) Spectrum of peracetyl CSF 4 ($CDCl_3$)

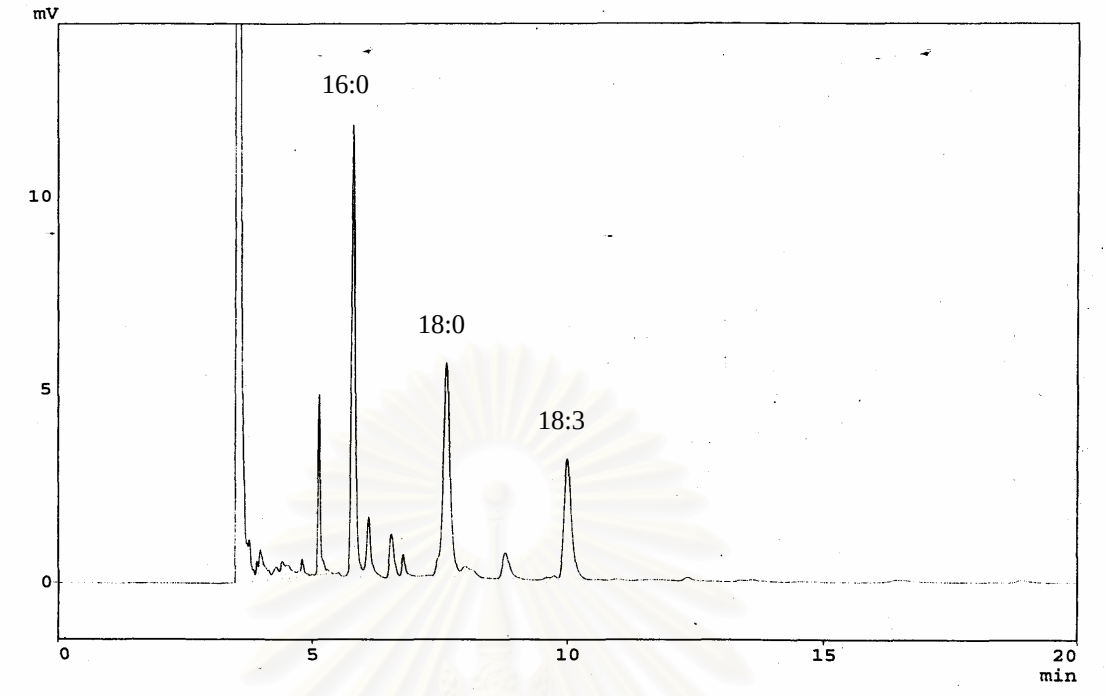

Figure 59. GC chromatogram of peracetyl CSF 4 acid hydrolysate

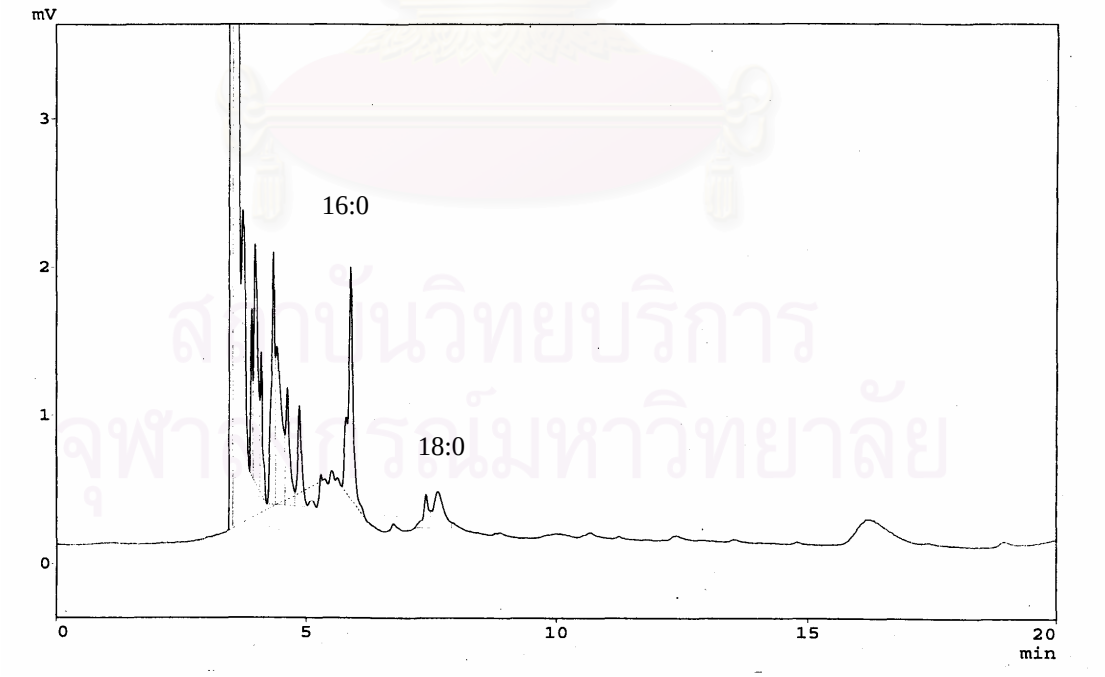

Figure 60. GC chromatogram of peracetyl CSF 4 enzymatic hydrolysate

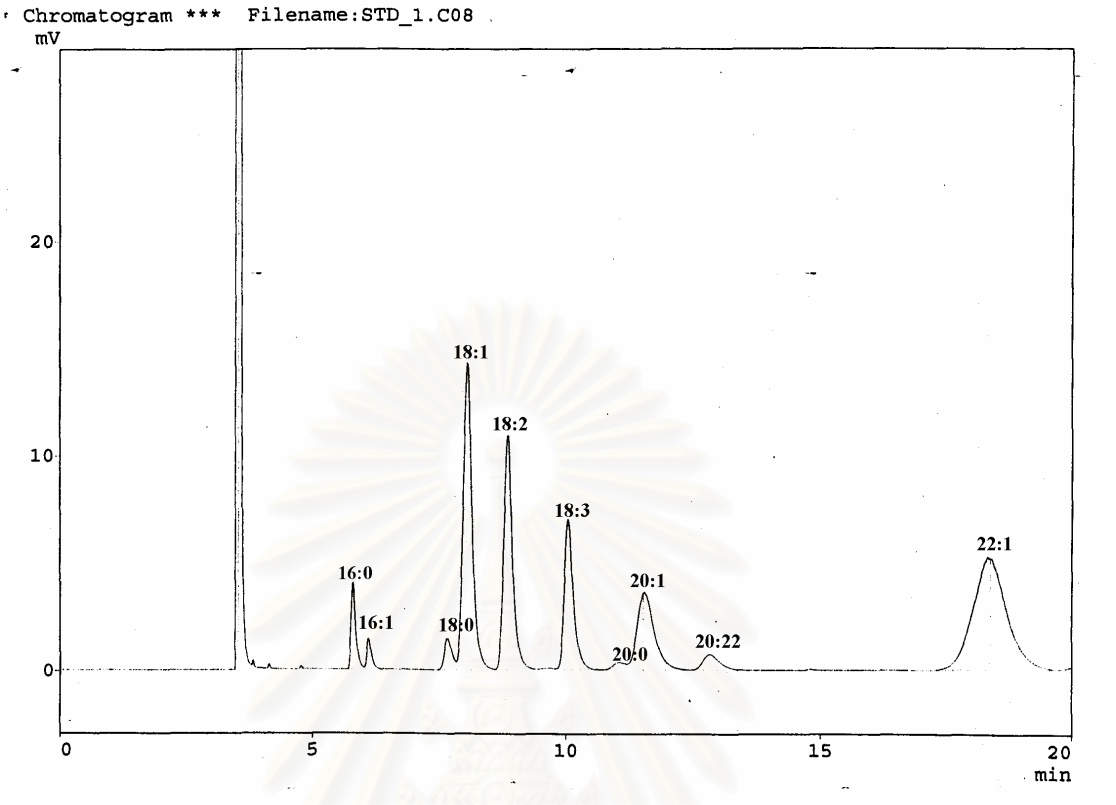

Figure 61. GC chromatogram of fatty acid methyl ester mixture #13

Compositions	% w/w
Methyl palmitate (16:0)	3.0
Methyl palmitoleate (16:1)	1.0
Methyl stearate (18:0)	2.0
Methyl oleate (18:1)	20.0
Methyl linoleate (18:2)	15.0
Methyl linolenate (18:3)	10.0
Methyl eicosanoate (20:0)	1.0
Methyl eicosenoate (20:1)	2.0
Methyl eicosadienoate (20:2)	2.0
Methyl behenate (22:0)	1.0
Methyl erucate (22:1)	30.0

VITA

Miss Warangkana Lertsupwichit was born on August 31st, 1972 in Khonkaen, Thailand. She graduated with Bachelor's Degree of Science in Pharmacy in 1995 from the Faculty of Pharmacy, Mahidol University, Thailand. She is presently working as a pharmacist in Department of Community Pharmacy, Phukeiow Hospital, Chaiyaphum Province.