สารทุติยภูมิของสเตรปโตมัยซิส TRA 9839-2 จากป่าซายเลน

นางสาว กุสุมา จิตแสง

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SECONDARY METABOLITES OF MANGROVE *STREPTOMYCES* SP. TRA 9839-2

Miss Kusuma Jitsaeng

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By	Miss Kusuma Jitsaeng
Department	Pharmacognosy
Thesis advisor	Mr. Khanit Suwanborirux, Ph.D.
Thesis co-advisor	Associate Professor Somboon Tanasupawat, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of
Pharmaceutical Sciences
(Associate Professor Sunibhond Pummangura, Ph.D.)

Thesis committee

.....Chairman
(Associate Professor Kittisak Likhitwitayawuid, Ph.D.)

.....Thesis advisor
(Mr. Khanit Suwanborirux, Ph.D.)

.....Thesis co-advisor
(Associate Professor Somboon Tanasupawat, Ph.D.)

.....Member
(Mr. Prasat Kittakoop, Ph.D.)

.....Member
(Associate Professor Kalaya Pharadai, M.Eng.)

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KUSUMA JITSAENG: SECONDARY METABOLITES OF MANGROVE *STREPTOMYCES* SP. TRA 9839-2
THESIS ADVISOR: MR. KHANIT SUWANBORIRUX. Ph.D.,
THESIS CO-ADVISOR: ASSOCIATE PROFESSOR SOMBOON TANASUPAWAT, Ph.D. 144 pp. ISBN 9743466061

A mangrove actinomycete strain TRA 9839-2, isolated from the mangrove forest along the Andaman coast, Trang province was identified as *Streptomyces* sp. based on morphological, cultural, physiological, and biocemical including the cell wall component studies. The EtOAc extract of fermentation broth of TRA 9839-2 exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633 and antiviral activity against *Herpes simplex* virus types I and II. Isolation of the EtOAc extract by means of chromatographic techniques yielded four known compounds including, pyrrole-2-carboxylic acid, *trans*-cinnamic acid, enterocin, and 5-deoxyenterocin. Structure elucidation of the isolated compounds were performed by extensive analyses of spectral data and comparison with literature data. Examination of biological activities revealed that pyrrole-2-carboxylic acid displayed weak cytotoxicity against KB cell at ED_{50} = 9.9 μg/ml. Enterocin exhibited weak antiviral activity against *Herpes simplex* virus type I at the concentration 50 μg/ml, antimalarial activity at EC_{50} = 9.4 μg/ml, and weak cytotoxicity against KB cell at ED_{50} = 20 μg/ml. *trans*-cinnamic acid showed antitubercular activity at MIC = 200 μg/ml.

Department of Pharmacognosy Student's signature.....
Field of study Pharmacognosy Advisor's signature.....
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จากการศึกษาลักษณะทางสัณฐานวิทยา การเจริญ สรีรวิทยาและชีวเคมีรวมทั้งองค์ประกอบของผนังเซลล์ของแบคทีเรียกลุ่มแอคติโนมัยซิสสายพันธ์ TRA 9839-2 ที่แยกได้จากป่าชายเลน ชายฝั่งทะเลอันดามัน จังหวัด ตรัง สามารถบ่งชี้ได้ว่าเป็นแบคทีเรียในสกุล สเตรปโตมัยซิส สารสกัดหยาบด้วยเอธิลอะซีเตทของน้ำหมักเชื้อ TRA 9839-2 แสดงฤทธิ์ต้านเชื้อแบคทีเรีย *Bacillus subtilis* ATCC 6633 และฤทธิ์ต้านไวรัสเริม *Herpes simplex* virus types I และ II เมื่อทำการแยกสกัดด้วยวิธีทางรงคเลขสามารถแยกสารที่มีรายงานการค้นพบแล้ว 4 ชนิด คือ สาร pyrrole-2-carboxylic acid สาร *trans*-cinnamic acid สาร enterocin และ สาร 5-deoxyenterocin การพิสูจน์เอกลักษณ์และโครงสร้างของสารทั้ง 4 ชนิดทำได้โดยการวิเคราะห์ข้อมูลคุณสมบัติทางกายภาพ, ทางเคมี และข้อมูลทางสเปคโตรสโคปี การทดสอบฤทธิ์ทางชีวภาพของสารบริสุทธิ์ที่แยกได้พบว่า สาร pyrrole-2-carboxylic acid แสดงฤทธิ์เป็นพิษต่อเซลล์ที่ ED_{50} = 9.9 μg/ml สาร enterocin แสดงฤทธิ์ต้านเชื้อไวรัสเริม *Herpes simplex* virus type I ที่ความเข้มข้น 50 μg/ml ฤทธิ์ต้านเชื้อมาลาเรียที่ EC_{50} = 9.4 μg/ml และแสดงความเป็นพิษต่อเซลล์อย่างอ่อนที่ ED_{50} = 20 μg/ml สาร *trans*-cinnamic acid แสดงฤทธิ์ต้านเชื้อวัณโรคที่ MIC = 200 μg/ml

ภาควิชาเภสัชเวท ลายมือชื่อนิสิต.....

สาขาเภสัชเวท ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2543 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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LIST OF ABREVIATIONS

$[\alpha]^{20}_{D}$	=	specific rotation at 20° and Sodium D line (589 nm)
ATCC	=	American Type Culture Collection, Maryland, U.S.A.
ax	=	axial
BC	=	breast cancer cell
br s	=	broad singlet (for NMR spectral data)
°C	=	degree Celsius
^{13}C NMR	=	carbon-13 nuclear magnetic resonance
$CDCl_3$	=	deuterated chloroform
$CHCl_3$	=	chloroform
CD_3OD	=	deuterated methanol
cm	=	centimeter
COSY	=	^{1}H-^{1}H correlation spectroscopy
δ	=	chemical shift
DEPT	=	distortion enhancement by polarization transfer
d	=	doublet (for NMR spectral data)
dd	=	doublet of doublets (for NMR spectral data)
DMSO-d_6	=	deuterated dimethylsulphoxide
dt	=	doublet of triplets (for NMR spectral data)
EC_{50}	=	50% effective concentration
ED_{50}	=	50% effective dose
ε	=	molar absorptivity
EIMS	=	electron impact mass spectroscopy
eq	=	equatorial
EtOAc	=	ethyacetate
FABMS	=	fast atom bombardment mass spectroscopy
g	=	gram
GBP	=	glucose-beef extract peptone

GPM = glycerol-peptone medium

HCT116 = human colorectal carcinoma cell line

HMBC = ^{1}H-^{13}C heteronuclear correlation

HMQC = ^{1}H-detected heteronuclear multiple quantum coherence

^{1}H NMR = proton nuclear magnetic resonance

Hz = hertz

IC_{50} = 50% inhibition concentration

IR = infared

KB = human epidermoid carcinoma cell of the nasopharynx

l = litter

μl = microlitter

λ_{max} = wavelength of maximum absorption

$[M+H]^{+}$ = protonated molecular ion

m = multiplet (for NMR spectral data)

MeOH = metanol

mg = milligram

μg = microgram

MHz = megahertz

MIC = minimum inhibitory concentration

ml = milliliter

mm = millimeter

ν_{max} = wave number at maximum absorption

nm = nanometer

NMR = nuclear magnetic resonance

NO. = Number

PCA = potato carrot agar

ppm = part per million

s = singlet (for NMR spectral data)

SCA = sodium caseinate agar

SDA = sabuoraud dextrose agar

SEM = scanning electron microscope

t = triplet (for NMR spectral data)

TFA = trifluoroacetic acid

THF = tetrahydrofuran

TLC = thin layer chromatography

TSA = trypticase soy agar

UV = ultraviolet

Vero cell line = African green monkey kidney cell line

YMA = yeast-malt extract agar

CHAPTER I

INTRODUCTION

Man has been living his life on nature since pre-historical age, thousand years ago. Natural products have been used in basic needs of human-being such as house hold, food, cloth and the last but not least important, medicine. Great deals of natural origins were used in traditional medication since early of age, both plant and animal materials including microorganisms. After the discovery of Penicillin antibiotic by Sir Alexander Fleming in 1929 (Evans, 1996), which led the world to modern remedy, microorganisms have become even more important as the abundant sources of bioactive compounds which are proved to be secondary metabolites. Numerous compounds were isolated and showed various biological activities, antibiotic, cytotoxicity, anti-inflammatory, *etc*. Several of them were developed in pharmaceutical and medicinal use. For example, the fungi *Penicillium* and *Cephalosporium* produced β-lactam antibiotics. The gram-positive rod bacteria, *Bacillus* produced peptides antibiotics and the actinomycetes, hyphae bearing gram-positive bacteria produced various structures of antibiotics, aminoglycosides, macrolides, tetracycline and chloramphenical. Thus, the microorganisms are a brilliant source of medicines (Tyler, Brand and Robbers, 1988).

Among the actinomycetes, *Streptomyces* are very attractive as they can amazingly produce large numbers of antibiotics about 95% of a total about 2000 antibiotics (Goodfellow, William and Mordarski, 1988). Therefore, researchers pay large attention to seek for novel bioactive metabolites from *Streptomyces*. As *Streptomyces* are soil bacteria, most researches intensively focused on terrestrial *Streptomyces*. However the increase of antibiotic resistant pathogenic bacteria has become a serious problem in infectious disease therapy nowadays. The discovery of new effective antibiotics and new secondary metabolites as lead compounds are required. *Streptomyces* began to be

targeted of the investigation. New species and/or same species from different origins are expected to be the sources of new structures and/or new activities of bioactive substances.

Marine *Streptomyces*, which can survive in extremely environment with high salinity, osmotic pressure and lack of nutrient were considered (Attaway and Zoborosky, 1993). In such stress conditions, marine *Streptomyces* speculatively produced many interesting bioactive compounds. Reports of the discovery are as follows.

1. Octalactin A isolated from octacoral, *Pacificgorgia* sp. associated *Streptomyces* sp. PG-19 exhibited cytotoxic activity against B-16-F10 murine melanoma cell line (IC_{50} = $0.72x10^{-3}$ μg/ml) and cytotoxic activity against HCT-116 human colon tumor cell line (IC_{50} = 0.5 μg/ml) (Tapiolas *et al*., 1991).
2. Salinamides A and B from *Streptomyces* sp. CNB-091 isolated from the surface of gelly fish, *Cassiopeia xamachana* have anti-inflammatory activity in phorbal ester induced mouse ear edema assay. At the testing doses 50 μ g/ear, these compounds showed 84% and 83% inhibition respectively. More over both of them also exhibited antibacterial activity against *Streptococcus pyogenes* and *S. pneumoniae* (Trischman *et al*., 1994).
3. Halichomycin, the macrolide cytotoxic agent isolated from *Streptomyces hygroscopicus* living in gastrointestinal tract of marine fish, *Halichoeres bleekeri* revealed cytotoxic activity against P-388 lymphocytic leukemia (ED_{50} = 0.13 μg/ml) (Takahashi *et al*., 1994).
4. Altemicidin from marine sediment *Streptomyces sioyaensis* SA1758 showed brine shrimp lethality activity (IC_{50} = 30 μg /ml) and inhibited growth of tumor cell L12100 (Takahasi *et al*., 1989).

Apart from marine environment, mangrove forest and estuarine environments are also interesting sources of new secondary metabolites and bioactive compounds because of biodiversity and sophisticated ecological system. The species living in this surrounding are endurable to the changes of salinity and current between low and high tides. Furthermore, mangrove sediment is rich of organic matters consistent with high sulfur and nitrogen (ประเสริฐ โพธิปักษ์ 2540), which can be used in secondary metabolite production of microorganisms. Therefore the discovery of new effective bioactive substances and new structural compounds is expected. Reports of investigation for such compounds are as follows.

1. Auranticins A and B from the fungus *Preussia aurantica* (Poch *et al*., 1991).
2. Luisols A and B, new aromatictetraols isolated from the estuarine actinomycete *Streptomyces* sp. CNH-370 (Cheng *et al*., 1999).
3. Arenaric acid, a new pentacyclic polyether isolated from estuarine *Streptomyces* sp. CNH-248(Cheng *et al*., 1999)
4. Lagunapyrones A-C, new skeleton class of cytotoxic acetogenins isolated from estuarine actinomycete (Lindel, Jensen and Fenical, 1996).

The above reports proved that mangrove microorganisms are one of the most interesting sources of new secondary metabolites. In Thailand, mangrove microorganisms have not been chemically explored. During the course of biologically active screening, EtOAc extract of the actinomycete TRA 9839-2 isolated from mangrove forest along Andaman coast, Trang province exhibited antibacterial activity against *Bacillus subtilis* ATCC 6633 and antiviral activity against *Herpes simplex* virus types I and II. The strain TRA 9839-2 could therefore be the source of active metabolites. Thus, it was selected for further studies on the isolation and structure elucidation of bioactive substances. The main objectives of this investigation are:

1. To identify and characterize the actinomycete TRA 9839-2.
2. To purify the secondary metabolites produced by the mangrove strain, TRA 9839-2
3. To elucidate chemical structures of the isolated compounds.
4. To examine biological activities of pure compounds isolated from the mangrove strain TRA 9839-2.

CHAPTER II

HISTORICAL

About three-fourths of *Streptomyces* isolates may produce antibiotics (Alexander, 1961). Therefore *Streptomyces* have received great attention from research groups all over the world as the sources of bioactive compounds. Although many of terrestrial *Streptomyces* metabolites were developed in pharmaceutical and medical sciences, the requirement of new effective compounds is still. As the high opportunity to obtain bioactive substances from *Streptomyces*, some researchers expected to discover new bioactive metabolites from *Streptomyces* isolated from different environments. Marine and estuarine (mangrove and littoral) environments which are different from terrestrial environment in their salinity and components, have become new targets of the investigation. *Streptomyces* isolated from these surrounding produce several bioactive metabolites belonging to rare structures (Jensen and Fenical, 1994). The successes in discovery of secondary metabolites from such environments are shown as follows.

1. Secondary metabolites from estuarine *Streptomyces*

Saphenic acid **[1]**, 6-acetylphenazine-1-carboxylic acid **[2]** and Phenazine L-quinovose esters **[3-6]** were isolated from *Streptomyces* sp. CNB-253 from a shallow sediment collected in Bodega Bay. The phenazine alkaloids contained L-quinovose, which is a rare sugar found in nature. 3'-*O*-quinovosyl saphenate **[3]** showed antibacterial activity against *Hemophilus influenzae* (MIC = 1 μg/ml) and *Clostridium perfringens* (MIC = 4 μg/ml). 2'-*O*-quinovosyl saphenate **[4]** showed antibacterial activity against *Escherichia coli* (MIC = 4 μg/ml), *Samonella enteritidis* (MIC = 4 μg/ml) and *Clostridium perfringens* (MIC = 1 μg/ml) (Pathirana *et al*., 1992).

Bioxalomycins α1, α2, β1, and β2 **[7-10]** (Zaccardi *et al*., 1994) were isolated from *Streptomyces viridodiastaticus* subsp. *litoralis* LL-31F508 from an intertidal soil sample collected in Key west. Bioxalomycin α2 **[10]** exhibited antibacterial activity at the MICs ranging from ≤ 0.002 to 0.008 μg/ml for gram-positive microorganisms (*Staphylococcus, Streptococcus and Bacillus*) and from 0.05 to 5 μg/ml for gram-negative microorganisms (*Enterococcus*) (Singh *et al*., 1994).

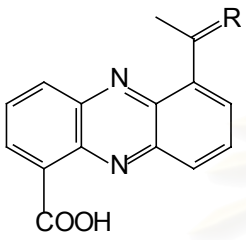
Lagunapyrones A-C **[11-13]** were isolated from unidentified marine actinomyces from a sediment sample collected in Agau Hedionda Lagoon in Carsbad, California. Lagunapyrone A **[11]** showed modest *in vitro* cytotoxicity against human colon cancer cell line HCT-116 (ED_{50} = 3.5 μg/ml) (Lindel, Jensen, and Fenical, 1996).

Actinoflavoside **[14]**, a glycoside composed of a rare amino sugar was isolated from an estuarine isolate *Streptomyces* sp. CBN-689. Compound **[14]** displayed weak antibacterial activity against gram-positive bacteria, *Staphylococcus pneumoniae*, *S. pyogenes*, *S. aureus* and *Micrococcus luteus* (MIC = 64 μg/ml) (Jiang *et al*., 1997).

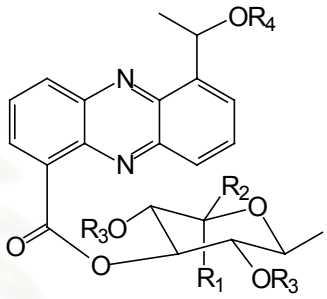
Halawanones A-D **[15-18]**, polycyclic quinones were isolated from *Streptomyces halstedii olivaceous* BD-18T from a sediment sample collected from an estuarine environment at the mouth of Halawa stream, Oahu (Ford *et al*., 1998).

Luisols A-B **[19-20]** were isolated from *Streptomyces violaceusniger* CNH-370 from sandy sediment collected near San Diego in San Luis estuary. These compounds contain the rare epoxynaphtho[2,3c]furan, the structure feature found in only fungal anthraquinones. Luisol A **[19]** was related to anthraquinone antibiotics of granaticin class (Cheng *et al*., 1999).

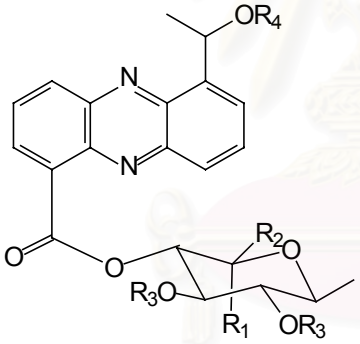
Arenaric acid **[21]** was isolated from *Streptomyces* sp. CNH-248 from a sediment sample collected in the North San Diego from an estuary near Doheny Beach. Its structure is related to the antibiotic K-41A and oxolonomycin (Cheng *et al*., 1999).



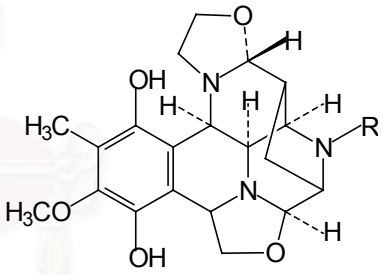
[1] Saphenic acid : R= =O
[2] 6-acetylphenazine-1-carboxylic acid : R = H, OH



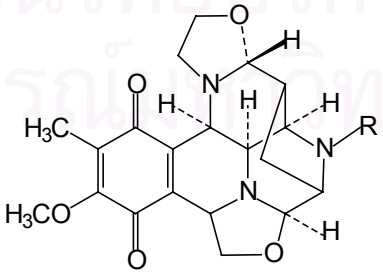
[3] 3'-*O*-quinovosyl saphenate : R_1=OH, R_2=R_3=R_4=H
[6] R_1=R_3=R_4=H, R_2=OH



[4] 2'-*O*-quinovosyl saphenate : R_1=OH, R_2=R_3=R_4=H
[5] R_1=R_3=R_4=H, R_2=OH

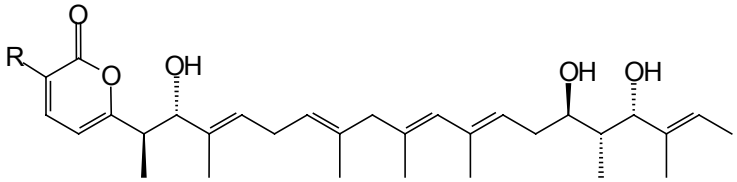


[7] Bioxalomycin α 1 : R = H
[8] Bioxalomycin α 2 : R = CH_3

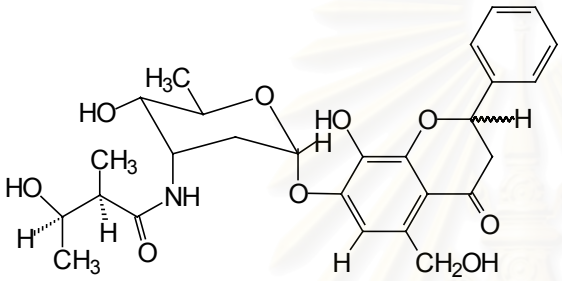


[9] Bioxalomycin β 1 : R = H
[10] Bioxalomycin β 2 : R = CH_3

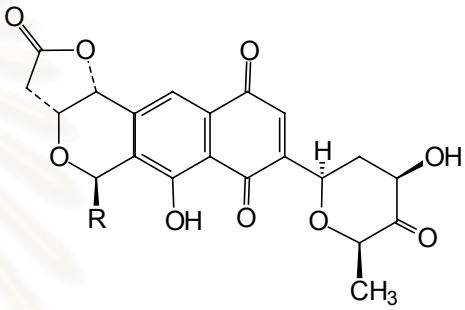
Figure 1. Structures of secondary metabolites from estuarine *Streptomyces*



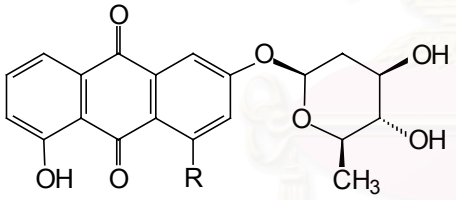
[11] Lagunapyrone A : R = CH_3
[12] Lagunapyrone B : R = $CH_2CH_2CH_3$
[13] Lagunapyrone C : R = $CH_2CH_2CH_2CH_3$



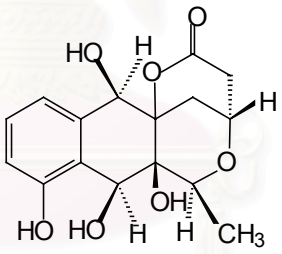
[14] Actinoflavoside



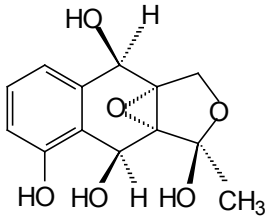
[15] Halawanone A : R = CH_2CH_3
[16] Halawanone B : R = CH_3



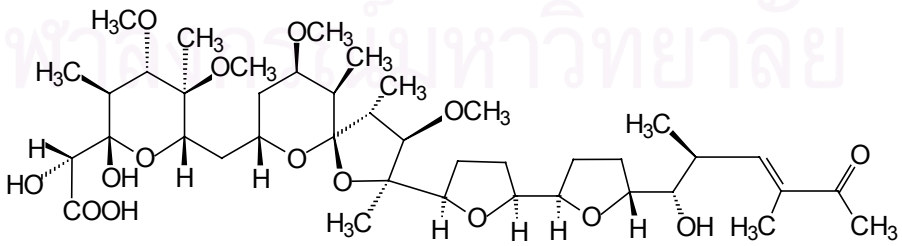
[17] Halawanone C : R = CH_3
[18] Halawanone D : R = CH_2CH_3



[19] Luisol A



[20] Luisol B



[21] Arenalic acid

Figure 1. Structures of secondary metabolites from estuarine *Streptomyces* (continued)

2. **Secondary metabolites of marine *Streptomyces***

Aplasmomycin **[22]** and Aplasmomycins B-C **[23-24]** (Seto *et al*., 1978) are ionophoric antibiotics, which were isolated from *Streptomyces griseus* SS-20 from marine sediment collected in Sagami Bay. At the concentration of 500 μg/ml, these compounds exhibited antibacterial activity against *Bacillus subtilis* PCT219, *Staphylococcus aureus* FDA209P and *S. aureus* Smith (Okami *et al*., 1976).

Istamycins A-B **[25-26]** are aminoglycoside antibiotics, which were isolated from *Streptomyces tenjamariensis* SS-939 from a sea mud sample collected in the shallow sea mud around Tenjin-island in Sagami Bay. Both compounds have strong activity against gram-positive and gram-negative bacteria, especially aminoglycoside resistant strains (Okami *et al*., 1979) (Hotta *et al*., 1980).

Altemicidin **[27]**, a monoterpene alkaloid, was isolated from *Streptomyces sioyaensis* SS-1758 from a sea mud sample. Altemicidin was the first isolated monoterpene alkaloid having a 6-azaindene skeleton as a metabolite of microorganisms. This compound showed brine shrimp lethality activity (IC_{50} = 3.0 μg/ml), inhibited growth of tumor cell L12100 (IC_{50} = 0.84 μg/ml), MIC carcinoma (IC_{50} = 0.82 μg/ml) and exhibited antibacterial activity against *Xanthomonas oryzae* (MIC = 6.25 μg/ml) (Takahashi *et al*., 1989).

Maduralide **[28]**, a 24-membered ring macrolide glycoside isolated from an unidentified marine bacterium of the order Actinomycetales showed weak antibacterial activity against *Bacillus subtilis* (Pathirana *et al*., 1991).

Marinone **[29]** and Debromomarinone **[30]** were sesquiterpenoid napthoquinone antibiotics isolated from *Streptomyces* sp. CNB-632 (Pathirana, Jensen and Fenical, 1992).

Octalactins A-B **[31-32]** were eight membered ring lactones isolated from *Streptomyces* sp. PG-19 from the surface of cortez gorgonian octacoral *Pacificgorgia* sp. Octalactin A **[31]** showed cytotoxicity activity against B-16-F10 (IC_{50} = 7.2x10^{-3} μg/ml) and HCT-116 (IC_{50} = 0.5 μg/ml) (Tapiolas *et al*., 1991).

Salinamides A-E **[33-37]** (Moore *et al*., 1999) were depsipeptides were isolated from *Streptomyces* sp. CNB-091 from the surface of jelly fish *Cassiopiea xamachana* collected in Florida keys. Salinamides A and B displayed antibacterial activity against *Streptococcus pneumonie* and *Staphylococcus pyrogenes* (MIC = 4 μg/ml for **[33]** and 2 μg/ml for **[34]**). These two compounds also have anti-inflammatory activity testing by mouse ear edema assay using phorbol ester 50 μg/ear to induce mouse ear edema. Compound **[33]** showed 84% inhibition while **[34]** showed 83 % inhibition at the dose 50 μg/ear (Trichman *et al*., 1994).

Halichomycin **[38]** constituted a new class macrolide, which was isolated from *Streptomyces hygroscopicus* from the gastrointestinal tract of a marine fish *Halichoeres bleekeri*. The compound exhibited potent cytotoxicity against P-388 lymphocytic leukemia (ED_{50} = 0.13 μg/ml) (Takahashi *et al*., 1994).

γ-Indomycinone **[39]**, rubiflavinone C-1 **[40]**, β-indomycinone **[41]** and rubiflavinone C-2 **[42]** are the antraquinone-γ-pyrone nucleus compounds, which were isolated from *Streptomyces* sp. PC001 from the deep-sea sediment. Mixture of **[40]** and **[42],** which are isomers, showed cytotoxicity against CHO cell line UV20 (Schumacher *et al*., 1995).

Aburatubolactam A **[43]** which was isolated from a *Streptomyces* sp. SCRC-A20 found on an unidentified marine mollusk was an inhibitor of superoxide ion generation (Faulkner, 1996).

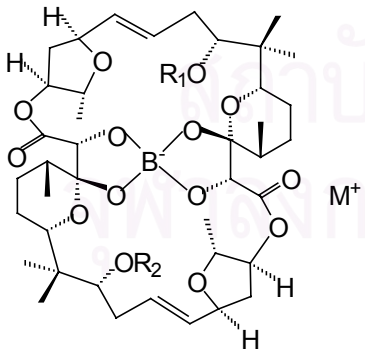
Urauchimycins A **[44]** and B **[45]** are the antimycin antibiotics found in *Sreptomyces* sp. Ni-80, which was isolated from an unidentified sponge (Faulkner, 1995).

Anthranilamide **[46]**, which was isolated from a marine *Streptomyces* sp. B7747 derived from sediment from the Gulf of Mexico showed the phytotoxic and antimicroalgal activities (Faulkner, 1999).

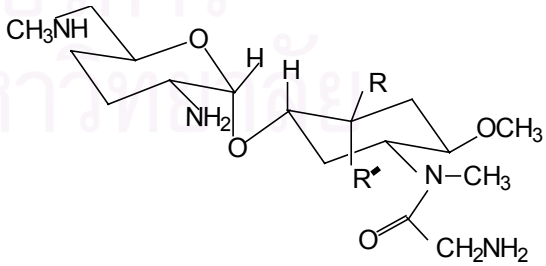
Cyclomarins A-C **[47-49]** are cyclic peptides, which were isolated from *Streptomyces* sp. CNB-982 from a sediment sample collected in Mission Bay, CA. Cyclomarin A **[47]** displayed potent anti-inflammatory activity in phorbol ester induced mouse ear edema assay at the testing dose 50 μg/ear showing 92% inhibition (Renner *et al*., 1999).

δ-indomycinone **[50]**, a pluramycin class antibiotic, was isolated from a marine *Streptomyces* sp. B8300 from a lagoon on the Gulf of Mexico (Faulkner, 1999).

Dihydrophencomycin methyl ester **[51]**, a weakly antimicrobial alkaloid, was isolated from *Streptomyces* sp. B825, which was isolated from a marine sediment sample from the Gulf of Mexico (Faulkner, 1995).

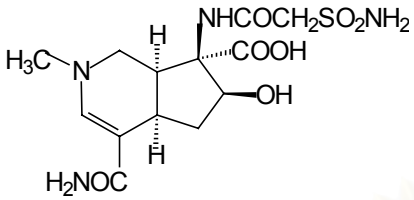


[22] Aplasmomycin : $R_1 = R_2 = H$
[23] Aplasmomycin B : $R_1 = Ac$, $R_2 = H$
[24] Aplasmomycin C : $R_1 = R_2 = Ac$

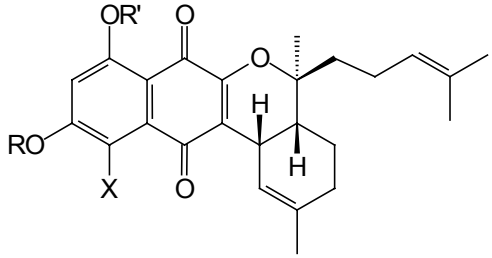


[25] Istamycin A : $R = NH_2$, $R' = H$
[26] Istamycin B : $R = H$, $R' = NH_2$

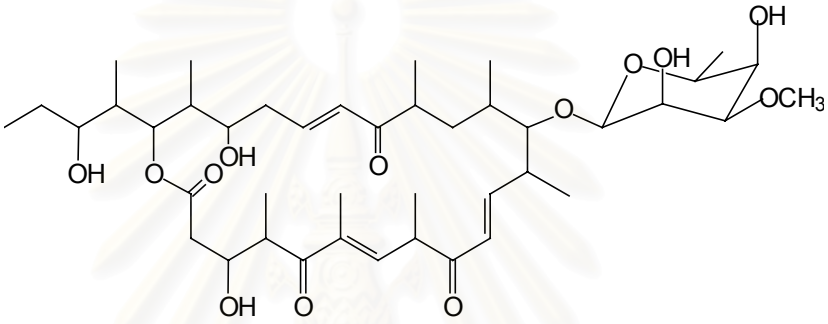
Figure 2. Structures of secondary metabolites from marine *Streptomyces*



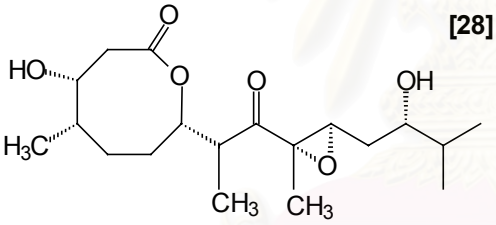
[27] Altemicidin



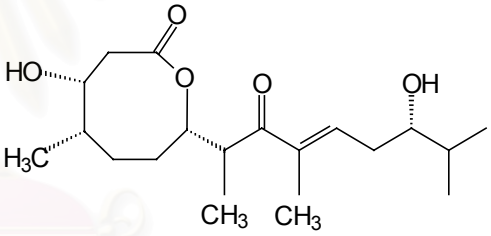
[29] Marinone : R = R' = H, X = Br
[30] Debromomarinone : R = R' = X = H



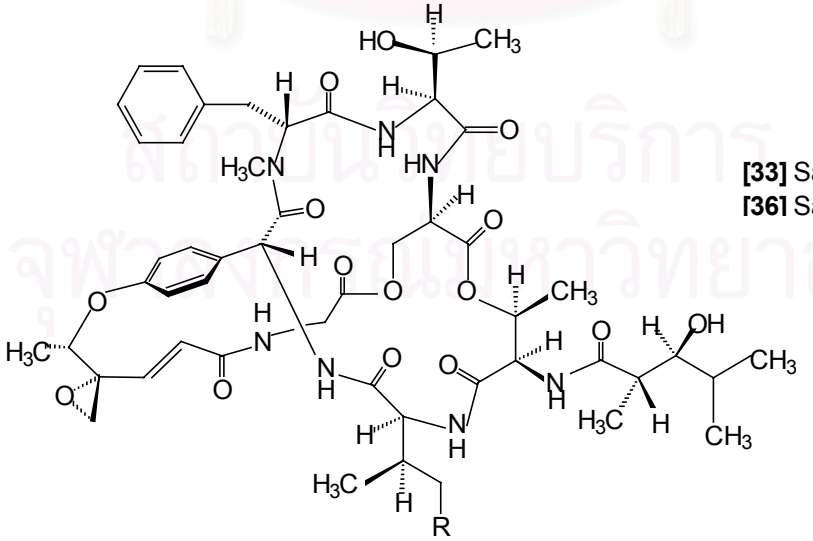
[28] Maduralide



[31] Octalactin A

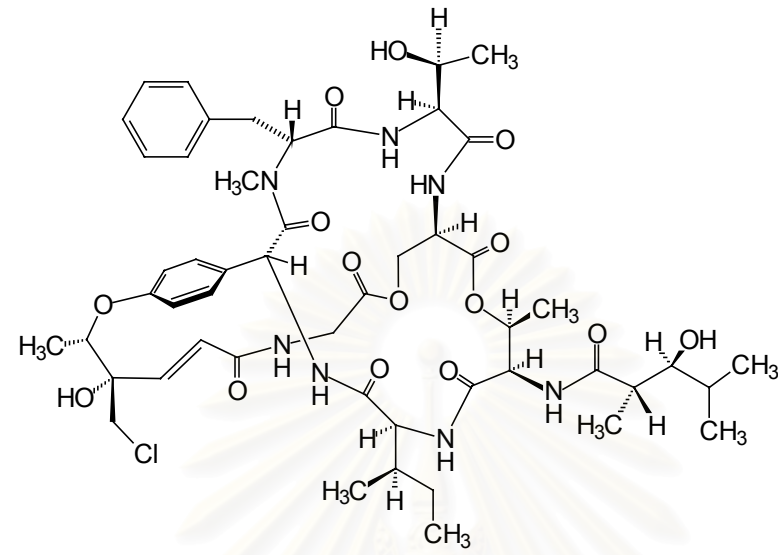


[32] Octalactin B

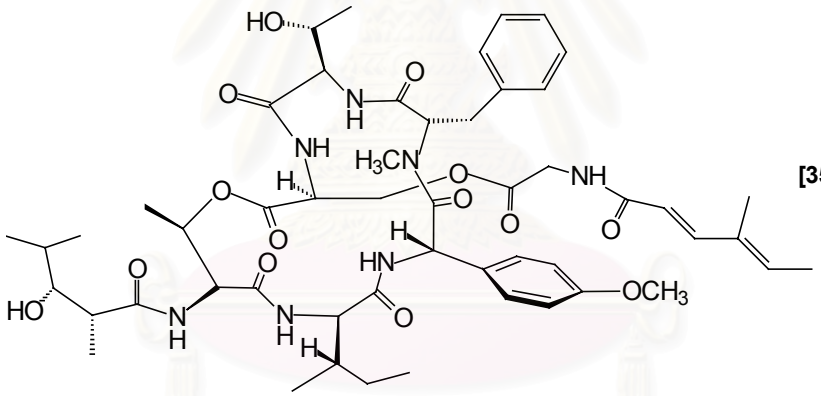


[33] Salinamide A : R = CH_3
[36] Salinamide D : R = H

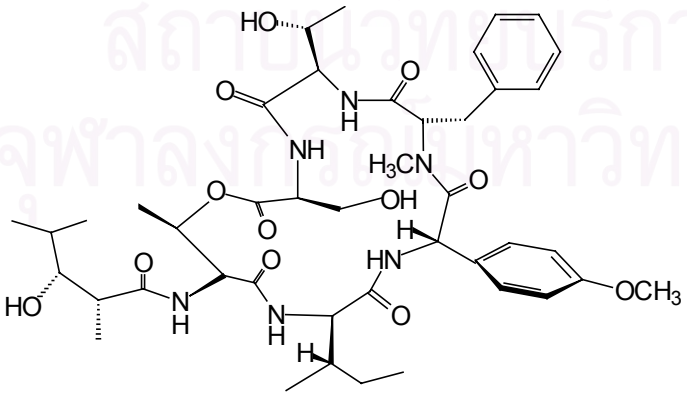
Figure 2. Structures of secondary metabolites from marine *Streptomyces* (continued)



[34] Salinamide B

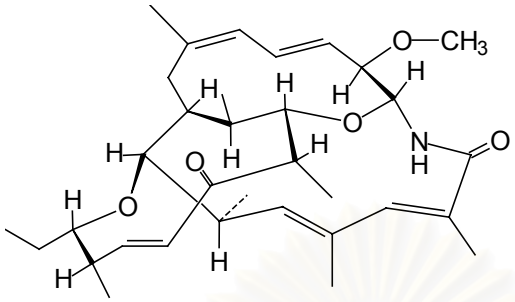


[35] Salinamide C

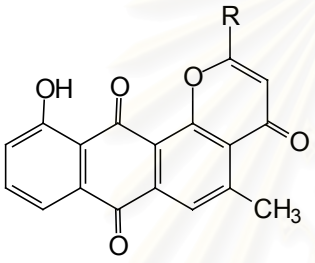


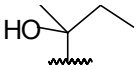
[37] Salinamide E

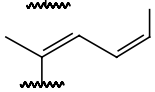
Figure 2. Structures of secondary metabolites from marine *Streptomyces* (continued)

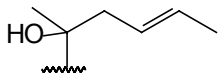


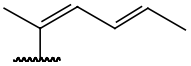
[38] Halichomycin

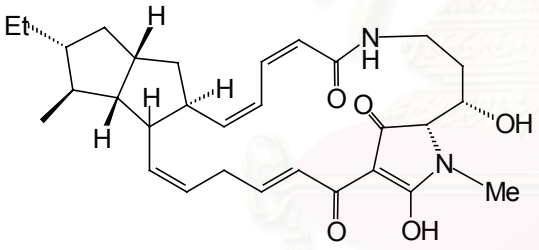


[39] γ-indomycinone : **R =** 

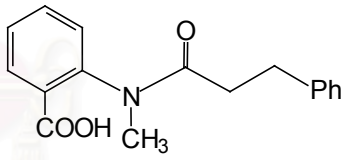
[40] Rubiflavinone C-1 : **R =** 

[41] β-indomycinone : **R =** 

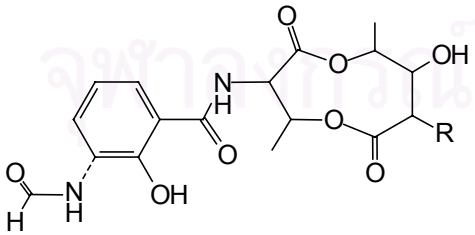
[42] Rubiflavinone C-2 : **R =** 



[43] Aburatubolactam A



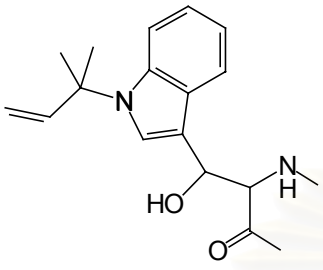
[46] Anthranilamide



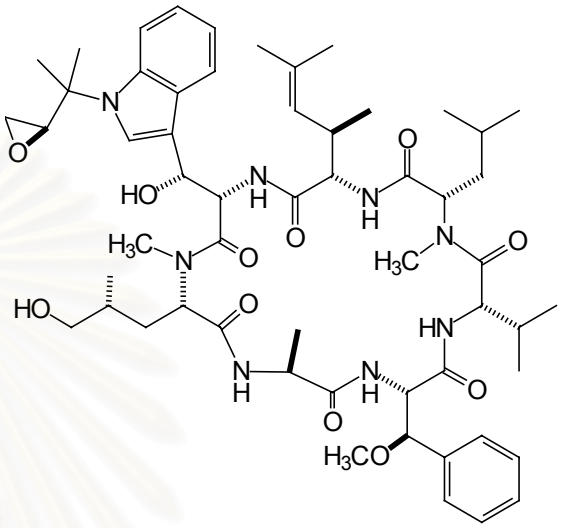
[44] Urauchimycin A : R = $CH_2CH(CH_3)C_2H_5$

[45] Urauchimycin B : R = $C_2H_4CH(CH_3)_2$

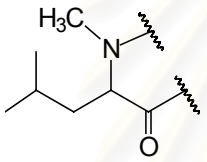
Figure 2. Structures of secondary metabolites from marine *Streptomyces* (continued)



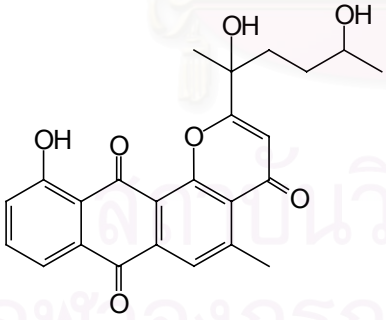
[49] Cyclomarin C



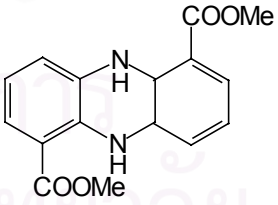
[47] Cyclomarin A



[48] Cyclomarin B



[50] δ-indomycinone



[51] Dihydrophencomycin methyl ester

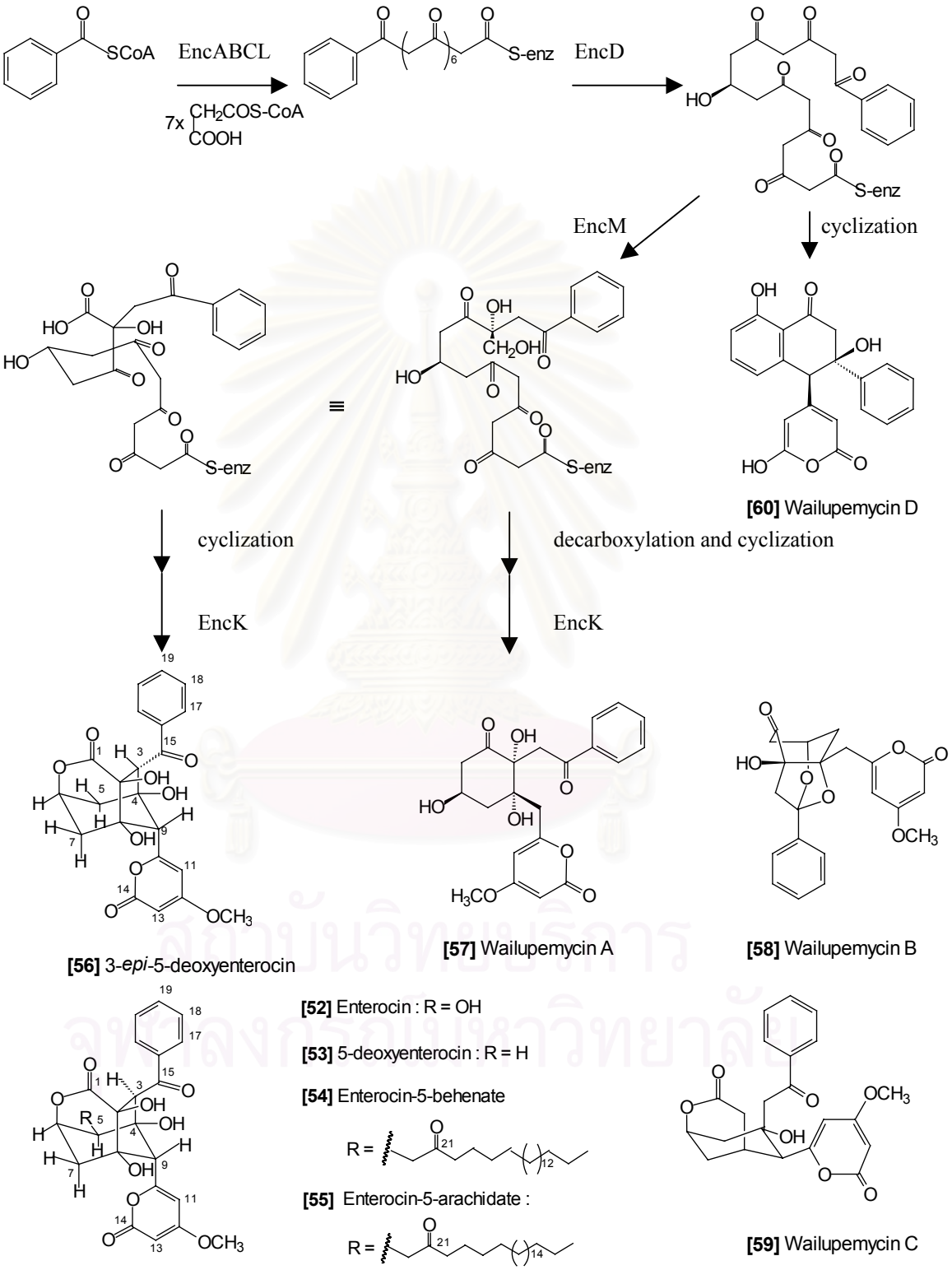
Figure 2. Structures of secondary metabolites from marine *Streptomyces* (continued)

3. The discovery of enterocin and its related compounds

Enterocin **[52]** was first discovered from the culture broth of two strains Streptomyces, *Streptomyces candidus* var. *enterostaticus* WS-8096 and *S. viridochromogenes* M-127. Examination of its biological activity showed that enterocin was a bacteriostatic antibiotic against gram-negative and gram-positive bacteria. Testing by a disc diffusion method at the concentration 4 mg/ml, enterocin inhibited growth of *Escherichia coli* NIHJ JC-2 (18 mm inhibition zone), *Proteus vulgaris* IAM-1095 (30 mm inhibition zone), *Sarcina lutea* PCI-1001 (26 mm inhibition zone) and *Staphylococcus aureus* 209P JC-1 (17 mm inhibition zone) (Miyairi *et al*., 1976). Structure determination of enterocin **[52]** was performed by X-ray crystallographic analyses presenting that enterocin **[52]** possesses a unique tricyclic structure, α-pyrone and benzyl ring (Tokuma *et al*., 1976). In the same year, Seto *et al*. reported biosynthetic studies of vulgamycin, an antibiotic produced by *S. hygroscopicus* No. A-5294. Vulgamycin, which has been proved to be identical with enterocin was synthesized from methionine, seven acetate units and benzoate as the starter (Seto *et al*., 1976).

In 1987 a enterocin derivative, 5-deoxyenterocin **[53]** was found from the culture of *Streptomyces* sp. L11-1, and it possessed antibacterial activity against *Staphylococcus aureus*, *Sarcina lutea*, *Klebsiella pneumoniae* and *Vibrio percolans* at the concentration 0.5 mg/ml (Yakimura *et al*., 1987). Enterocin-5-arachidate **[54]** and enterocin-5-behenate **[55]** were isolated from an unidentified western Australian ascidian of the genus *Didemnum* together with enterocin **[52]** and 5-deoxyenterocin **[53]** (Kang *et al*., 1996). Furthermore, 3-*epi*-5-deoxyenterocin **[56],** wailupemycins A-C **[57-59]** were also discovered in the same year from *Streptomyces* sp. BD-26T isolated from shallow water marine sediment. 3-*epi*-5-deoxyenterocin **[56]** showed antibacterial activity against *Staphylococcus aureus* at the concentration 1 mg/disc (18 mm inhibition zone) and wailupemycin A **[57]** showed antibacterial activity against *Escherichia coli* at the concentration 0.1 mg/disc (18 mm inhibition zone) (Sitachitta *et al*., 1996). This strain

was later proved to be a new species *Streptomyces maritimus*. As the enterocins and wailupemycins belonged to extremely strange structures, the biosynthetic pathway of these bacteriostatic polyketides was studied by encoding biosynthetic gene cluster. The study revealed that enterocin **[52]** is derived from an uncommon benzoate starter unit and seven malonate molecules and undergoes the carbon rearrangement by a type II polyketide synthase (PKS), which was controlled by the PKS gene set (*enc*) clustered with relevant gene involved in formation of the benzoic starter unit. The type II PKS generated linear-poly-β-ketide and then α-oxidation and Favorskii-like rearrangement takes place on the linear octaketide to form the enterocins. Isolation of the wild type *S. maritimus* led to the discovery of wailupemycin D **[60],** which is probably derived from a linear polyketide intermediate. The rearrangement probably takes place after the C9 ketoreduction. On the other hand, wailupemycins A-C **[57-59]**, cyclization takes place after the decarboxylation of the intermediate (Scheme 1) (Piel *et al*., 2000).



Scheme 1. Structures and proposed biosynthesis of enterocins and wailupemycins

4. Characteristics of the genus *Streptomyces*

The genus *Streptomyces* is a member of the Streptomycetaceae family in the order Actinomycetales. *Streptomyces* are gram-positive aerobic bacteria possessing vegetative hyphae (0.5-2.0 μm in diameter), which produce an extensive branched mycelia that rarely fragment. The mature aerial mycelium forms three to many spore chains. A few species bear short chain of spore on the substrate mycelium. Sclerotia-, pycnidial-, sporangia- and synnemata-like structure may be formed by some species. Spores are non-motile. The genus form discrete and lichenoid, leathery or butyros colonies. Initially, colonies are relatively smooth surface, but later they develop a weft aerial mycelium that may appear floccose, granular, powdery or velvety. *Streptomyces* produce a wide variety of pigments responsible for the vegetative and aerial mycelia. Colored diffusible pigment may also be formed. Each strain may produce one or more antibiotics. *Streptomyces* are gram-positive but not acid-alcohol-fast. The cell wall peptidoglycan contains major amount of L-diaminopimelic acid (L-DAP). They lack of mycolic acid but contain major amounts of saturated, *iso*-, and *anteiso*-fatty acids; possess either hexa- or octahydrogenated menaquinones with nine isoprene units as the predominant isoprenolog; and have complex polar lipid patterns that typically contain diphosphatidylinositol, and phosphatidylinositol manosides. *Streptomyces* have oxidative type of metabolism and are catalase positive. They generally reduce nitrates to nitrites and degrade adenine, esculin, casein, gelatin, hypoxanthine starch and L-tyrosine. They can use a wide range of organic compounds as sole sources of carbon for energy and growth. The optimum temperature for growth is 25-37 °C; some species grow at psychrophilic and thermophilic range of temperature; the optimum pH range for growth is 6.5-8. They are widely distributed and abundant in soil including composts. A few species are pathogenic for animals and humans; others are phytopatogens (Holt *et al*., 1994).

CHAPTER III

EXPERIMENTAL

1. Sample collection and isolation

The bacterial sample TRA 9839-2 was isolated from rotten bark collected in mangrove forest along the west coast of the Andaman sea, Trang province in October 1998. Spread plate technique (Brock *et al*., 1993) was used to isolate the strain. 0.5 g of a rotten bark sample was suspended into 4.5 ml of sterile seawater, which was then diluted to 1:10 dilution and heated in water bath at 60^{o}C temperature for 5 minutes. 0.1 ml of the dilution was spread on Sodium Caseinate Agar (SCA, Appendix B 1.3) and Potato Carrot Agar (PCA, Appendix B 1.4) added with antibiotics. Plates were incubated at room temperature for 7-14 days until the powdery colonies appeared. The white powdery colony was picked up and streaked on Yeast-Malt extract Agar (YMA, Appendix B 1.2) to obtain single colonies. Each colony was transferred into YMA slant and incubated at room temperature for 7 days. The stock culture was kept at 15^{o}C temperature.

2. Identification and characterization of the strain

The isolated TRA 9839-2 was identified and characterized by the methods as described in Bergey's manual of determinative bacteriology (Holt *et al*., 1994) and in the International *Streptomyces* project (ISP) (Shirling and Gottlieb, 1966).

Morphological characteristics, gram staining, spore bearing hyphae, spore chain, number of spores, spore size, spore shape and spore surface were determined on the

culture grown on YMA (14 days), observing under scanning electron microscope (SEM) using De man, De man and Gupta method (1986).

Cultural characteristics were studied on the colors of mature aerial mycelium, substrate mycelium, spore and diffusible soluble pigment. To determine the cultural characteristics, the strain was cultured on five different agar media (yeast-malt extract agar (Appendix B 1.2), oatmeal agar (Appendix B 1.5), inorganic salt-starch agar (Appendix B 1.6), glycerin asparagine agar (Appendix B 1.7), and tyrosine agar (Appendix B 1.8)).

Physiological and biochemical characteristics were studied, which include melanin production and carbon utilization, reduction of nitrate, liquefaction of gelatin, coagulation of milk, hydrolysis of starch, and cellulose decomposition. Effects of pH, temperature, and concentration of NaCl on growth were also studied by using YM agar.

Cell wall component study was performed using the methods of Komagata and Suzuki (1987).

3. Fermentation methods

3.1 Seed culture production

The strain TRA 9839-2 was grown on a YM slant at room temperature for 3 days. It was then inoculated into 250 ml of glucose beef extract peptone (GBP, Appendix B 1.15) broth in 500 ml erlenmeyer flasks, and incubated on a rotary shaker (200 rpm) at room temperature for 3 days.

3.2 Fermentation for secondary metabolites

One percent v/v of seed culture was inoculated into 250 ml glycerol-peptone medium (GPM, Appendix B 1.1) in 500 ml erlenmeyer flasks. Then they were incubated on a rotary shaker (200 rpm) at room temperature for 7 days. Several flasks of culture were prepared to obtain 35 L of GMP broth.

4. Chromatographic techniques

4.1 Analytical thin-layer chromatography (TLC)

Technique : one dimensional ascending

Adsorbent : Silica gel F254 or C-18 reversed phase pre-coated plate (E.Merck)

Layer thickness : 250 μm

Distance : 5 cm

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

Detection : 1. Visual detection under daylight

2. Visual detection under ultraviolet light at wavelength 254 and 365 nm

3. Visual detection in Iodine vapor

4. Visual detection under daylight after spraying anisaldehyde (0.5% anisaldehyde reagent, 5% sulfuric acid, and 10% glacial acetic acid in MeOH) reagent and heating until the colors developed

4.2 Column chromatography

4.2.1 Gel filtration chromatography

Gel filter : Sephadex LH-20

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

Sample loading : The sample was dissolved in a small volume of an eluant and applied gently on the top of the column.

Detection : Fractions were examined by TLC technique in the same manner as describe in section 4.1.

4.2.2 Flash column chromatography

Adsorbent : Silica gel 60 (No. 7734) particle size 63-200 μm (70-230 Mesh ASTM) (E.Merck), C-18 reversed phase gel particle size 40-63 μm (230-400 Mesh ASTM) (E.Merck)

Packing method : 1. Wet packing: The adsorbent was suspended in an eluant and then pour into a column, set it tight by using air pump before use.

2. Dry packing: The adsorbent was put in to the column, set it tight by mechanical force before use.

Loading method : 1. Wet loading: The sample was dissolved in a small amount of an eluant and then applied gently on the top of the column.

2. Dry loading: The sample was dissolved in a small amount of an eluant or organic solvent. Then the

solution was adsorbed by adsorbent, triturated, dried and placed smoothly on the top of the column.

Detection : Fractions were examined in the same manner as described in section 4.1

4.3 Crystallization

Compound Por0226 was crystallized from a mixture of $CHCl_3$ and MeOH (1:1) at room temperature.

Compound Por0225223 was crystallized from a mixture of hexane and $CHCl_3$. It was dissolved in $CHCl_3$ until saturation, then hexane was added. The solution was left standing in a freezer.

5. Spectroscopy

5.1 Ultraviolet (UV) absorption spectra

UV (in MeOH) spectra were obtained from a Milton Roy Spectronic 3000 Array spectrophotometer

5.2 Infrared (IR) absorption spectra

IR spectra were obtained from a Perkin-Elmer FT-IR 1760X spectrophotometer. The compounds were examined as dry film on NaCl cell or KBr disc.

5.3 Mass spectra

FABMS spectra were obtained from a JOEL JMS-HX 110 double focussing mass spectrometer of EBE arrangement with JMS-DA 7000 data system, 10 kV acceleration voltage, fast-atom xenon gas accelerated at voltage of 3 kV. Glycerol and m-nitrobenzyl alcohol (mNBA) were used as the matrix, and NaCl was used as alkali metal cation source. Electrospray ionization time of flight mass spectrum (ESI-TOF MS) were recorded with a Micromass (ESI-TOF MS) spectrometer with ionization energy at 31 eV.

5.4 Proton and carbon nuclear magnetic resonance (NMR) spectra

^{1}H and ^{13}C NMR, DEPT 135, DEPT 90, HMQC, HMBC and COSY spectra were obtained from a Bruker AVANCE DPX-300 FT-NMR spectrometer, operating 300 MHz for proton and 75 MHz for carbon. Deuterated solvents, dimethylsulfoxane (DMSO-d_6), chloroform ($CDCl_3$), and methanol (CD_3OD) were used in NMR experiments. Referent signals were the signals of residual undeuterated solvents at δ 7.24(^{1}H) and 77.0 (^{13}C) ppm for $CDCl_3$; δ 2.49 (^{1}H) and 39.7 (^{13}C) ppm for DMSO-d_6 and δ 3.30 (^{1}H) and 49.0 (^{13}C) ppm for CD_3OD.

6. Optical rotation

Optical rotations were measured on a Perkin Elmer 341 polarimeter, using a sodium lamp at wavelength 589 nm.

7. Melting point

Melting points were examined on a Gallenkamp melting point apparatus.

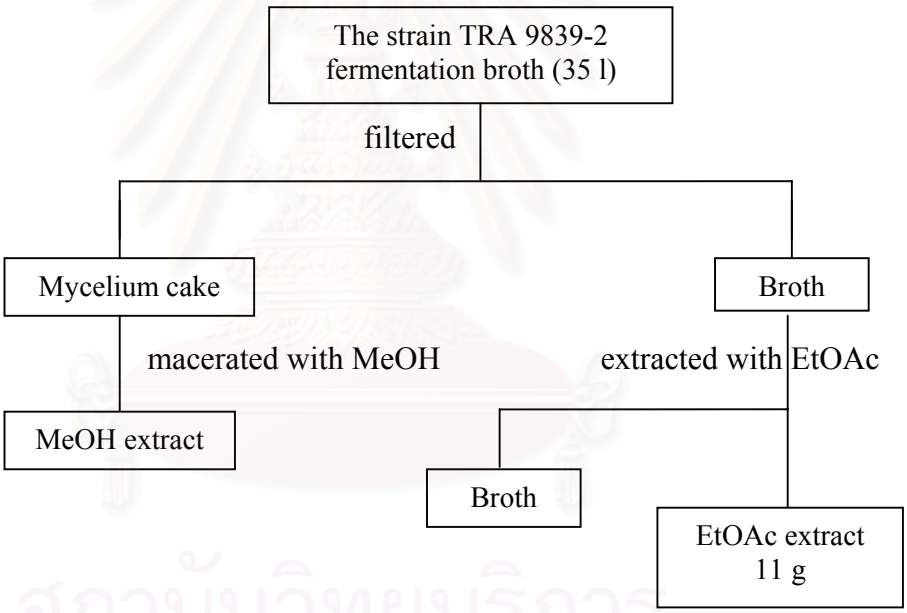
8. Solvent

All commercial solvents were redistilled prior to use.

The measurements of MS, UV, IR, NMR and Optical rotation properties were performed at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University; The Scientific and Technological Research Equipment Center (STREC), Chulalongkorn University; The National Center for Genetic Engineering and Biotechnology (BIOTEC); National Science and Technology Development Agency (NSTDA); and The Institute of Molecular and Cellular Biosciences, The University of Tokyo.

9. Extraction

The fermentation broth of TRA 9839-2 (35 l) was filtered through a filter paper (Whatman NO.1). Kieselguhr was used as filter aid. Mycelium cake was macerated in MeOH. The filtrate was repeatedly extracted with EtOAc (1:1) three times. The EtOAc fractions were pooled and evaporated under reduced pressure at the temperature not exceed 40°C, to yield 11 g of EtOAc extract.



Scheme 2. Extraction of fermentation broth of TRA 9839-2

10. Isolation and purification of the EtOAc extract

The EtOAc extract of TRA 9839-2 from fermentation broth exhibited antibacterial activity against *Bacillus subtilis* ATCC 6633 at the concentration of 1 mg/disc with inhibition zone 18 mm (disc diffusion method) (Lorian *et al*., 1980), and also exhibited antiviral activity against *Herpes simplex* virus types I and II. The EtOAc extract was subsequently purified by chromatographic techniques.

The EtOAc extract was divided into 4 portions (2.5 g each). Each portion was dissolved in a small volume of MeOH and then fractionated on a Sephadex LH-20 column (3x60 cm). MeOH was used to elute the column. Fractions (25 ml each) were collected, and examined. Fraction combination was by the TLC on Silica gel plate with $CHCl_3$ and MeOH (8:2) as a developing solvent. Fractions with the same TLC pattern were pooled and dried. Four fractions (F01-04) were obtained (Table III-1).

Table III-1. Isolation of the EtOAc extract by a Sephadex LH-20 column

Fraction code	Number of fraction	Volume (ml) MeOH	Weight (g)
F01	1-9	225	2.9
F02	10-14	125	6.5
F03	15-21	175	0.23
F04	21-23	315	0.10

The fractions F01 and F02 were able to inhibit growth of *Bacillus subtilis* ATCC 6633 with respective13 and 15 mm inhibition zones at the testing concentration of 1 mg/disc. Therefore, F02 was further purified. F02 was divided into 6 portions (ca. 1 g each) and chromatographed on a Sephadex LH-20 (3x60 cm) column. Mixture of $CHCl_3$ and MeOH was used as an eluant. Fractions of 25 ml were collected. They were then

combined according to their TLC behavior (Silica gel plate with $CHCl_3$ and MeOH (8:2) as a developing solvent) to give four fractions (F021-24) (Table III-2).

Table III-2. Isolation of the fraction F02 by a Sephadex LH-20 column

Fraction code	Number of fraction	Volume (ml) $CHCl_3$:MeOH (1:1)	Weight (g)
F021	1-5	75	0.74
F022	6-7	50	3.5
F023	8-9	50	1.5
F024	10-13	100	0.05

Fraction F024 gave brown crystals of compound Por024 (45 mg, 0.41% yield of the EtOAc extract). The biological activities and structure elucidation of this compound are described in Chapter IV.

The fraction F022 was further purified on a Silica gel column (4.5x15 cm), using dry packing and dry loading method. Gradient elution with $CHCl_3$ and EtOAc mixtures (from 60% $CHCl_3$ in EtOAc to 100% EtOAc) were used. Seven fractions (F0221-7) were obtained (Table III-3).

Table III-3. Isolation of F022 by a Silica gel column

Fraction code	Number of fraction	Ratio of $CHCl_3$:EtOAc	Volume (ml)	Weight (g)
F0221	1-2	2:3	50	0.020
F0222	3-10	2:3	200	0.200
F0223	11-14 15-16	2:3 1:2	100	0.097
F0224	17-22 23-24	1:2 1:4	50	0.253
F0225	25-29 30-32	1:4 0:1	160	0.900
F0226	33-40	0:1	50	0.540
F0227	41-50	0:1	125	1.200

Fraction F0226 was crystallized from 50% $CHCl_3$ in MeOH to furnish a white amorphous powder of compound Por0226 (90 mg, 0.82% yield of the EtOAc extract). The biological activities and structure elucidation of this compound are described in Chapter IV.

Fractions F0224 and F0225, and mother liquor of F0226 showed antibacterial activity against *Bacilus subtilis* ATCC 6633 with 23, 20, 20 mm of inhibition zones, respectively at the concentration of 1mg/disc. Therefore fraction F0225 was further purified on a Sephadex LH-20 column (1x40 cm). MeOH was used to elute the column. Three fractions (F02251-3) were obtained (Table III-4).

Table III-4. Isolation of F0225 by a Sephadex LH-20 column

Fraction code	Number of fraction	Volume of MeOH (ml)	Weight (g)
F02251	1-4	80	0.010
F02252	5-9	100	0.400
F02253	10-17	160	0.003

The fraction F02252 was further purified on a Silica gel column (2x14 cm). Gradient elution from 50% $CHCl_3$ in EtOAc to 50% EtOAc in MeOH was performed. Three fractions (F022521-3) were obtained (Table III-5).

Table III-5. Isolation of F02252 by a Silica gel column

Fraction code	Number of fraction	Ratio of $CHCl_3$:EtOAc:MeOH	Volume (ml)	Weight (g)
F022521	1-8	1:2:0	200	0.015
F022522	9-14 15-17 18-20	1:2:0 0:9.5:0.5 0:9:1	150 75 75	0.160
F022523	21-22 23-26 17-30	0:7:3 0:7:3 0:1:1	50 100 100	0.047

The fraction F022522 was purified by a RP-18 reversed phase gel column (2x14 cm), and a mixture of MeOH and H_2O (1:1) was used as an eluant. Four fractions (F0225221-4) were obtained (Table III-6).

Table III-6. Isolation of F022522 by a RP-18 reversed phase gel column

Fraction code	Number of fraction	Volume (ml)	Weight (g)
F0225221	1-14	28	0.098
F0225222	15-20	12	0.006
F0225223	22-30	14	0.003
F0225224	31-50	38	0.002

F0225223 was recrystallized from a mixture of $CHCl_3$ and hexane, to yield a white precipitate, compound Por0225223 (3 mg, 0.027 % yield of the EtOAc extract). The biological activities and structure elucidation of this compound were described in Chapter IV.

In addition, fraction F023 (Table III-2) was further separated on a Sephadex LH-20 column (1.5x20 cm). The mixture of hexane, $CHCl_3$ and MeOH (35:50:10) was used as an eluant. They were combined according to TLC pattern on Silica gel plate using a mixture $CHCl_3$ and MeOH (8:2) as developing solvent. Three fractions (F021-3) were obtained (Table III-7).

Table III-7. Isolation of F023 by a Sephadex LH-20 column

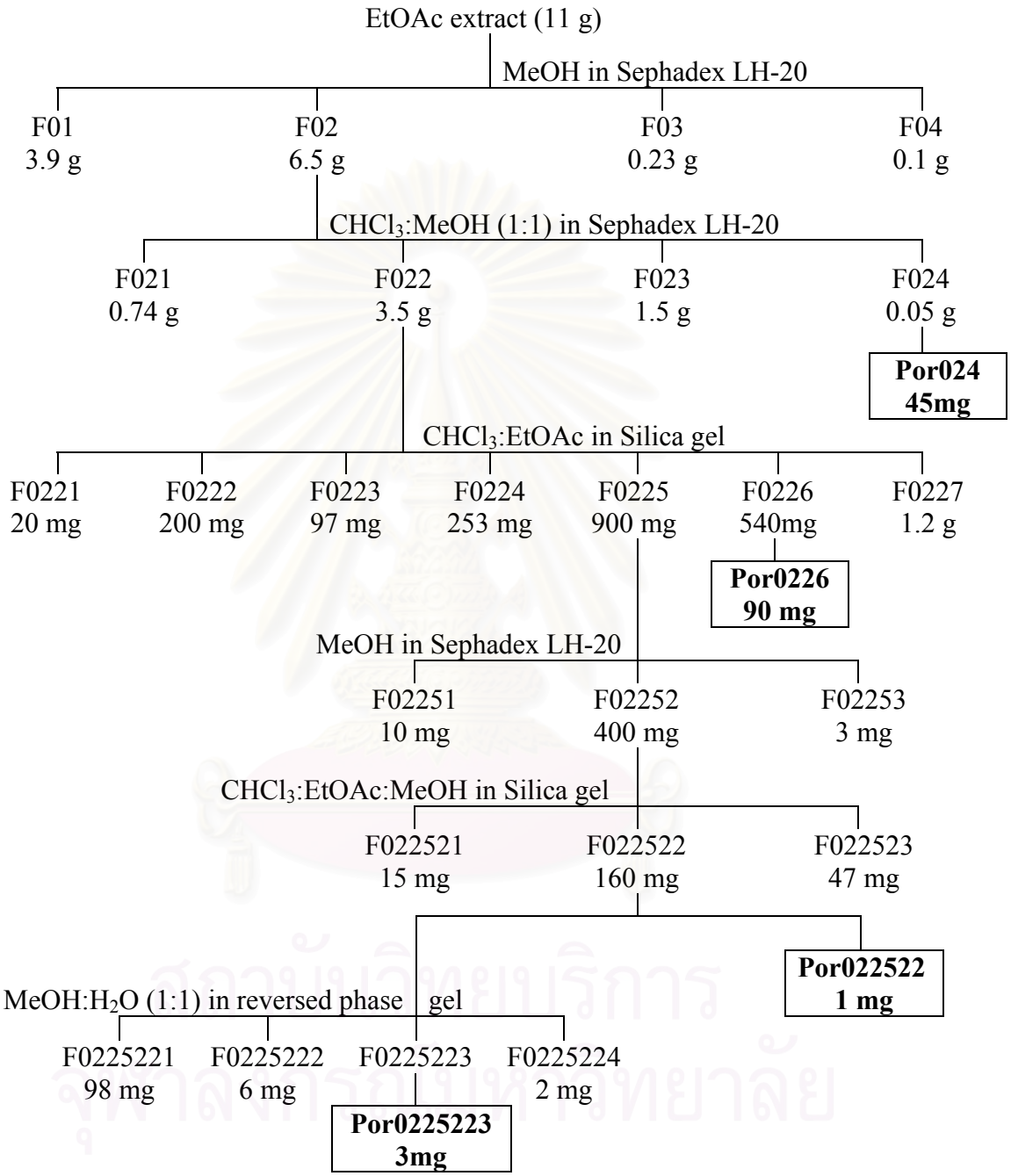
Fraction code	Number of fraction	Volume (ml) Hexane:$CHCl_3$:MeOH (35:50:10)	Weight (g)
F0231	1-6	120	0.008
F0232	7-14	160	1.100
F0233	15-20	120	0.161

Fraction F0232 was further purified on a Silica gel column (2x15 cm). Gradient elution (from 60% $CHCl_3$ in EtOAc to 20% MeOH in EtOAc) was carried out. The fraction combination was guided by TLC pattern on Silica gel plate using 60% $CHCl_3$ in EtOAc as developing solvent. Three fractions (F02321-3) were obtained. (Table III-8)

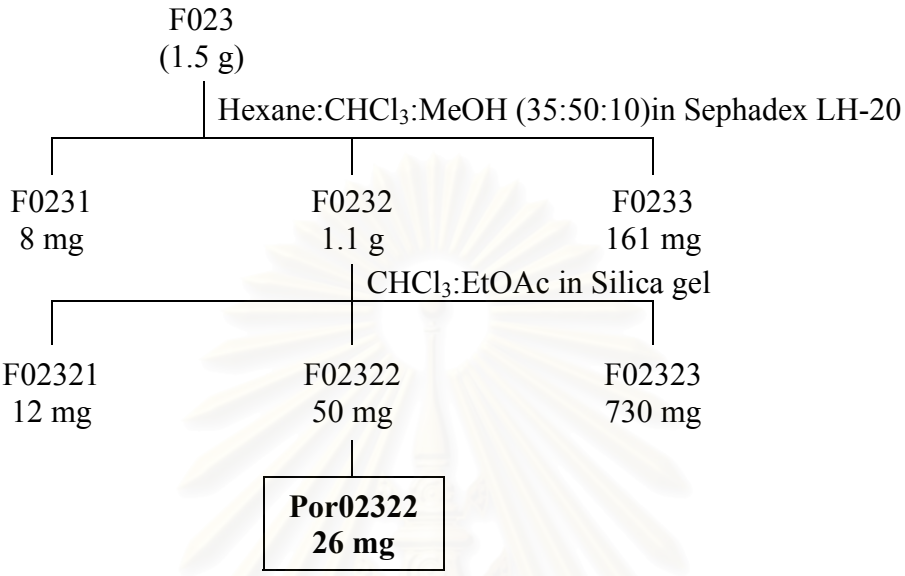
Table III-8. Isolation of F0232 by a Silica gel column

Fraction code	Number of fraction	Ratio of $CHCl_3$:EtOAc:MeOH	Volume (ml)	Weight (g)
F02321	1-3	2:1:0	75	0.012
F02322	4-8 9-12 13-15	3:2:0 1:1:0 1:2:0	125 100 75	0.050
F02323	16-28 29-32 33-36	1:2:0 0:9:1 0:8:2	325 100 100	0.730

F02322 gave white crystals of compound Por02322 (26 mg, 0.24% yield of the EtOAc extract). Biological activities and structure elucidation of this compound were described in Chapter IV.



Scheme 3. Isolation of the EtOAc extract of TRA 9839-2



Scheme 3. Isolation of the EtOAc extract of TRA 9839-2 (continued)

11. Physical and spectral data of the isolated compounds

11.1 Compound Por024

FAB MS : m/z 112 ($[M+H]^+$) : Figure 10

UV : λ_{max} (log ε) nm in MeOH : Figure 11
262 (4.19) nm

IR (KBr disc) : ν_{max} cm^{-1} : Figure 12
3400-3200, 3361, 3031, 1672, 1557,1444, 1333, 1268 cm^{-1}

^{1}H NMR (300 MHz in DMSO-d_6) : δ in ppm, *J* in Hz : Figure 13
12.2 (H, br s; OH), 11.7 (H, br s; NH), 6.9 (H, br s; H-5), 6.7 (H, br s Hz; H-3), 6.1 (H, br s; H-4) ppm

^{13}C NMR (75 MHz in DMSO-d_6) : δ in ppm : Figure 14
162.3 (C-1'), 123.8 (C-5), 123.2 (C-2), 115.1 (C-3), 109.7 (C-4) ppm

11.2 Compound Por02322

FAB MS : m/z 149 ($[M+H]^+$) : Figure 17

UV : λ_{max} (log ε) nm in MeOH : Figure 18
271 (4.13) nm

IR (KBr disc) : ν_{max} cm^{-1} : Figure 19
3600-3300, 1696, 1634, 1227, 983, 771, 705 cm^{-1}

^{1}H NMR (300 MHz in $CDCl_3$) : δ in ppm, *J* in Hz : Figure 20
7.7 (H, d, *J* = 16 Hz; H-1'), 7.5 (2H, m; H-2, H-6), 7.3 (3H, m; H-3, H-4, H-5), 6.4 (H, d, *J* = 16 Hz; H-2') ppm

^{13}C NMR (75 MHz in $CDCl_3$) : δ in ppm : Figure 21
172.0 (C-3'), 146.9 (C-1'), 133.9 (C-1), 130.6 (C-4), 128.8 (C-3, C-5), 128.2 (C-2, C-6), 117.2 (C-2') ppm

11.3 Compound Por0226

FAB MS : m/z 445 ($[M+H]^+$) : Figure 25

Optical rotation : $[\alpha]^{20}_D$, in MeOH
-8.94° (1 mg/ml)

UV : λ_{max} (log ε) nm in MeOH : Figure 26
250 (4.18), 283 (4.02) nm

IR (KBr disc) : ν_{max} cm^{-1} : Figure 27
3500-3400, 1685, 1638, 1566 cm^{-1}

^{1}H NMR (300 MHz in CD_3OD) : δ in ppm, *J* in Hz : Figures 28a-b
: Tables IV-4/1, IV-4/2

^{13}C NMR (75 MHz in CD_3OD) : δ in ppm : Figures 29-30
: Tables IV-4/1, IV-4/2

11.4 Compound Por0225223

ESI-TOF MS : m/z 429 ($[M+H]^+$) : Figure 39

Optical rotation : $[\alpha]^{20}_D$, in MeOH
- 41.2° (0.05 mg/ml)

UV : λ_{max} (log ε) nm in MeOH : Figure 40
249 (4.07), 283 (3.90) nm

IR (NaCl cell) : ν_{max} cm^{-1} : Figure 41
3500-3300, 2924, 1693, 1567 cm^{-1}

^{1}H NMR (300 MHz in $CDCl_3$) : δ in ppm, *J* in Hz : Figures 42a-b
: Table IV-7/1

^{13}C NMR (75 MHz in $CDCl_3$) : δ in ppm : Figures 43-44
: Table IV-7/2

12. Biological activity

12.1 Antimicrobial activity

Evaluation of the antimicrobial activity of the fractions and pure compounds was conducted using the agar disc diffusion method (Lorian, 1980). Antimicrobial activity was performed against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and the yeast strain *Candida albicans* ATCC 10231.

All tested bacteria were cultivated for 24 hours on Tryptic Soy Agar (TSA) (Difco®), whereas the yeast strain was cultivated on Sabouraud's dextrose agar (SDA) (Difco®) for 48 hours at 37°C temperature. Afterward, the cultures were subsequently washed from agar by normal saline solution (NSS). The cell suspensions were adjusted to a 0.5 turbidity, standard of McFarland NO.1 to provide approximately $1x10^8$ CFU (colony forming unit/ml).

Each of molten (20 ml) TSA and SDA was separately poured into 9-cm diameter petri dishes and allowed to solidify to form base agar. A loopful of each tested microorganism was spread on the surface of TSA and SDA plates. Fractions were dissolved in MeOH and then applied on a sterile paper disc or Silica gel TLC aluminium sheet (0.5x6.5 cm) adjusted to the concentration of 1 mg/disc or plate. Then disc or plate was placed on the surface of the incubated agar plate already spread with tested microorganisms. Plates were incubated at 37°C, 18 hours for bacteria and 48 hours for yeast. The diameters and R*f* values of inhibition zones were measured. Fractions showing antimicrobial activity were subsequently selected for further study.

12.2 Anti-herpes simplex virus

12.2.1 Sulforhodamine B colorimetric method

Examination of anti-HSV activity was performed according to the modified colorimetric method (Skehan *et al*., 1990). The vero cell line suspension was added to 96-well microtiter plate (190 µl/well) and incubated in a CO_2 incubator at 37°C for 30 minutes. The sample was dissolved in 10 µl of 100% dimethylsulfoxide (DMSO) and *Herpes simplex* viruses (30 plaque forming unit, PFU/well) were added to 96-well microtiter plate before incubating in CO_2 incubator at the same condition for 3 days. After incubation 100 µl/well of cold 50% aqueous trichloroacetic acid (TCA) was added to fix the cultures. Then they were further incubated at 4°C for 30 minutes. The fixed

cultures were stained with 0.4% (w/v) Sulforhodamine B (SRB) dissolved in 1% acetic acid for 30 minutes. Then, they were washed by tap water four times and dried at room temperature. Unbound dye was removed by washing with 1% acetic acid and protein-bound dye was extracted with 10 mM unbufferred Tris base (pH 10) to determine optical density at 515 nm using ELISA plate reader. ED_{50} values (μg/ml) were calculated by non-linear regression analysis (percent survival versus concentration). Acyclovir was used as a positive control.

12.2.2 Plaque reduction method

Cell-free HSV (30 plaque forming unit, PFU/well) was mixed with the maintenance medium containing various concentrations of sample and incubated at 37°C for 1 hour. After incubation, the mixtures were inoculated into monolayer vero cell culture on 96-well microtiter plates and incubated at 37°C for 2 days in CO_2 incubator. The cells were stained with 1% crystal violet in 10% formalin for 1 hour. The plaques were counted under an inverted microscope.

12.3 Antimalarial activity

Antimalarial assay was examined according to the method of Trager and Jensen (1976) using continuous cell cultures (*in vitro*) of asexual erythrocytic stages of *Plasmodium falciparum* (K1, multidrug resistant strain). Quantitative assessment of antimalarial activity *in vitro* was determined by means of the microculture radioisotope technique based on method described by Dejardins *et al*., 1979. Effective concentration (EC_{50}) represents the concentration, which reduced 50% of parasite growth as indicated by the *in vitro* uptake of [3H]-hypoxanthine by *P. falciparum*. An EC_{50} value of 0.16 μg/ml (3.1 μM) was observed for the standard sample, chloroquine in the same condition.

12.4 Cytotoxic activity

Cytotoxic activity against a breast cancer (BC), human epidermoid carcinoma of nasopharynx (KB) and vero cell lines was performed by sulforhodamine B (SRB) method (Ahn *et al*., 1999).

12.5 Antitubercular activity

Antitubercular activity was performed by a microplate alamar blue assay (MABA). Mycobacterium tuberculosis $H_{37}Ra$ was used as a tested microorganism. The minimum inhibitory concentrations (MICs) of the tested compounds were measured in μg/ml (Collins and Franzblau, 1997).

CHAPTER IV

RESULTS AND DISCUSSION

1. Isolation, identification, and characterization of the strain

The strain TRA 9839-2 was isolated from a rotten bark sample collected in mangrove forest along the Andaman coast of Trang province in October 1998. This filamentous bacterium is gram-positive, producing aerial mycelia and spores. Study of morphological characteristics under electron microscope revealed the spiral spore chains at the end of aerial mycelia. Each chain contained at least 20 oval spores with smooth surface (Figure 3). This strain contained L-diaminopimelic acid in the cell wall. The strain formed powdery colonies with pale beige spores and brown soluble pigments on YM agar after incubating for 14 days (Figure 4). Cultural characteristics of the strain are shown in Table IV-1. Physiological and biochemical characteristics are shown in Tables IV-2, IV-3.

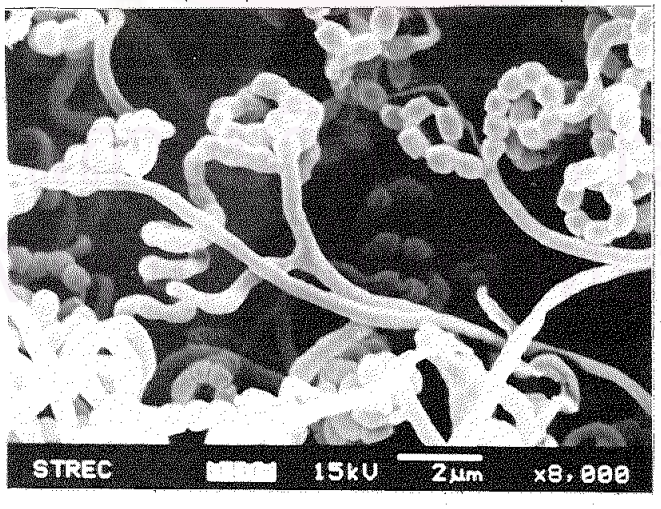


Figure 3. Scanning electron micrograph of TRA 9839-2

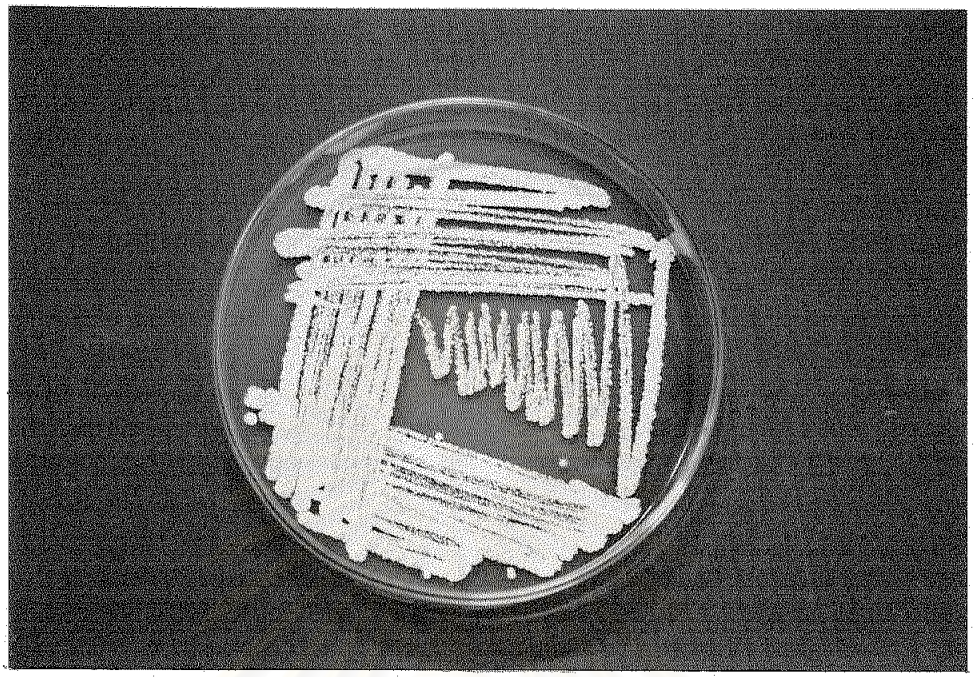


Figure 4. The colonial appearance of TRA 9839-2 on YM agar plate at room temperature after incubating for 14 days

Table IV-1. Cultural characteristics of TRA 9839-2

Media	Growth appearance	Colors on reversed plate	Aerial mycelium	spore	Soluble pigment
Yeast-malt extract agar	good powdery	browish gold	pale raddish	pale beige	yellowish brown
Oatmeal agar	good powdery	-	grayish white	pale beige	-
Inorganic salt-starch agar	good powdery	pale raddish	grayish white	pale beige	-
Glycerol-asparagine agar	good powdery	grayish white	pale raddish	pale beige	-
Tyrosine agar	good powdery	grayish yellow	bluish white	pale beige	-

*Color determination of the strain according to Jacal color card L2200, Japan Color Research Institute

Table IV-2. Physiological and biochemical characteristics of TRA 9839-2

Physiological and biochemical characteristic	Result
Optimum temperature for growth	25-30^{o}C
Nitrate reduction	negative
Liquefaction of gelatin	negative
Starch hydrolysis	negative
Coagulation of milk	negative
Peptonization of milk	positive*
Melanin formation	negative
Tyrosinase reaction	positive
Cellulolytic activity	negative
NaCl tolerance	16%
pH tolerance	5-11

* positive at day 21

Table IV-3. Carbon utilization pattern of TRA 9839-2

Growth	Source of carbon
negative	L-rhamnose, ruffinose
moderate	L-arabinose, inositol
positive	D-xylose, manitol, D-fructose, sucrose

* The growth of TRA 9839-2 compared with negative control.

The strain TRA 9839-2 was identified as *Streptomyces* sp. by comparison with the characteristics of the genus *Streptomyces* in Bergey's manual of determinative bacteriology (Holt *et al*., 1994).

2. Fermention and extraction

The strain TRA 9839-2 was cultivated in glycerol peptone medium (GPM, Appendix B 1.1) totally 35 l to yield 11 g of EtOAc extract.

3. Isolation and purification of the EtOAc extract

Four compounds were isolated from the culture broth of the mangrove *Streptomyces* sp. TRA 9839-2. Compound Por024 (45mg, 0.41 % yield of the EtOAc extract) were obtained from fraction F024 as brown crystals. Compound Por02322, (26 mg, 0.24 % yield of the EtOAc extract) were obtained from fraction F02322 as colorless needle crystals. Compound Por0226, (90 mg, 0.82 % yield of the EtOAc extract) was obtained as a white amorphous solid after recrystallization of the fraction F0226 from a mixture of $CHCl_3$ and MeOH (1:1). Compound Por0225223 (3 mg, 0.027 %yield of the EtOAc extract) was obtained as a white amorphous solid after crystallization of the fraction F0225223 from the mixture of $CHCl_3$ and hexane. Chemical structures of these compounds were determined by analyses of spectroscopic data, including IR, UV, NMR and Mass spectra, as well as by comparison their spectral data with those of published values.

4. Structure elucidation of the isolated compounds

4.1 Compound Por024

The mass spectrum of Por024 (Figure 10) showed a protonated molecular ion $[M+H]^+$ at m/z 112 suggesting the molecular formula $C_5H_5NO_2$. The UV spectrum of Por024 (Figure 11) displayed maximum absorption at 262 (log ε 4.19) nm. The IR spectrum of Por024 (Figure 12) revealed absorption bands at 3400-3200 (O-H stretching of carboxylic acid), 3361 (N-H stretching of secondary amine), 3038 (C-H stretching of aromatic), 1672 (C=O stretching), 1557 (N-H bending), 1444 (C-O stretching), 1333 (O-H bending) and 1268 (C-N stretching) cm^{-1}. The degree of unsaturation counted from this molecular formula is four, which is required for the molecular formula $C_5H_5NO_2$.

The ^1H NMR spectrum of Por024 in DMSO-d_6 (Figure 13) showed five proton signals including three aromatic protons at δ 6.9 (1H, br s; H-5), 6.7 (1H, br s; H-3), 6.1 (1H, br s Hz; H-4), one amine proton at δ 11.7 (NH, br s; H-1), and one hydroxy proton at δ 12.2 (OH, br s) ppm. The ^{13}C NMR spectrum of Por024 (Figure 14) presented five carbon signals, which could be classified by DEPT 135 (Figure 15) as three methine carbons at δ 123.8 (C-5), 115.1 (C-3), 109.7 (C-4), one sp^2 quaternary carbon at δ 123.2 (C-2), and one carbonyl carbon at δ 162.3 (C-1') ppm.

The ^1H, ^1H COSY spectrum of Por024 (Figure 16) showed the correlations of the coupling protons as follows: H-3 (δ 6.7 ppm)-H-4 (δ 6.1 ppm)-H-5 (δ 6.9 ppm); NH-1 (δ 11.7 ppm)-H-5 (δ 6.9 ppm), and long-range correlations of NH-1 (δ 11.7 ppm) to H-4 (δ 6.1 ppm) and H-3 (δ 6.7 ppm). These correlations suggested the presence of a pyrrole ring in compound Por024.

From these available spectral data, Por024 was thus identified as a known compound, pyrrole-2-carboxylic acid. It has been previously isolated from the fermentation broth of *Streptomyces* sp. 82-85, and this compound showed anti-platelet aggregation induced by ADP-arachidonic acid, collagen and tumor cell in rats and rabbits (Komiyama *et al*., 1986). At the dose 1g/kg orally, the compound showed 34% inhibition of ADP-induced blood platelet aggregation in rats, 1 hour after treatment (Umezawa *et al*., 1986). In addition, pyrrole-2-carboxylic acid showed blood-sugar lowering activity in rats at 4 mM/kg with no toxic symptoms (Fang *et al*., 1969).

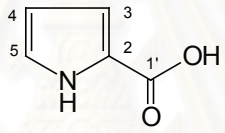


Figure 5. Structure of pyrrole-2-carboxylic acid

4.2 Compound Por02322

The mass spectrum of Por02322 (Figure 17) showed a protonated molecular ion $[M+H]^+$ at m/z 149, indicating the molecular formula $C_9H_8O_2$. The UV spectrum of Por02322 (Figure 18) displayed maximum absorption at 271 (log ε 3.13) nm. The IR spectrum of Por02322 (Figure 19) revealed absorption bands at 3600-3300 (O-H stretching), 3061 (=C-H stretching of aromatic), 1696 (C=O stretching), 1634 (C=C stretching), 1227 (C-O stretching), 983 (=C-H bending of two substituted *trans* alkene), and 771, 705 (=C-H bending of mono-substituted aromatic) cm^{-1}. The degree of unsaturation counted from this molecular formula is six, which is required for the molecular formula $C_9H_8O_2$.

The ^{1}H NMR spectrum of Por02322 (Figure 20) in $CDCl_3$ exhibited four proton signals including five aromatic protons at δ 7.3 (3H, m; H-3, H-4, H-5), 7.5 (2H, m; H-2, H-6) ppm and two *trans* coupled olefinic protons at δ 7.7 (1H, d, J = 16 Hz; H-1') and 6.4 (1H, d, J = 16 Hz; H-2') ppm. The splitting pattern of aromatic proton signals in the ^{1}H NMR spectrum suggested the mono-substituted benzene.

The ^{13}C NMR spectrum of Por02322 in $CDCl_3$ (Figure 21) displayed seven carbon signals. The DEPT 135 (Figure 22) and the HMQC (Figure 23) spectral data of Por02322 indicated that these seven carbon signals representing nine carbons, which include two *trans* alkene carbon signals at δ 146.9 (C-1') and 117.2 (C-2') ppm, and five aromatic methine carbons at δ 130.6 (C-4), 128.8 (2C; C-3 and C-5), and 128.2 (2C; C-2 and C-6) ppm. The carbons at δ 128.8 (2C; C-3 and C-5) ppm are *meta*-carbons of substituted group, and the most downfield aromatic methine carbon at δ 130.6 (C-4) ppm is *para*-carbon of substituted group. There are two quaternary carbons signals at δ 172.0 and 133.9 ppm, one of which at δ 172.0 (C-3') ppm is the carboxylic carbonyl carbon signal, showing the upfield shift from normal ketone carbonyl. The quaternary carbon at δ 133.9 (C-1) ppm is the aromatic carbon attached with a substituted group. The *trans* alkene carbon at δ 117.2 ppm could be assigned as C-2', based on the upfield shift of α-carbon of α, β unsaturated carboxylic carbonyl, whereas carbon signal at δ 146.9 ppm was assigned to C-1', based on its downfield shift of α-carbon of α, β unsaturated carboxylic carbonyl.

The HMBC ($^{n}J_{CH}$ = 8 Hz) spectrum of Por02322 (Figure 24) showed the correlations of carbons and protons as follows: H-3 and H-5 (δ 7.3 ppm) to C-2 and C-6 (δ 128.2 ppm) and C-1 (δ 133.9 ppm), H-4 (δ 7.3 ppm) to C-2, C-6 (δ 128.2 ppm), and H-2 and H-6 (δ 7.5 ppm) to C-3 and C-5 (δ 128.8 ppm), indicating the presence of mono-substituted benzene ring; H-1' (δ 7.7 ppm) to C-2' (δ 117.2 ppm) and C-3' (δ 172.0 ppm), suggesting the presence of *trans* alkene carboxylic part. The HMBC spectral data of

compound Por02322 also demonstrated the correlation of H-1' (δ 7.7 ppm) to C-1 (δ 133.9 ppm) and C-2 and C-6 (δ 128.8 ppm), and H-2' (δ 6.4 ppm) to C-1 (δ 133.9 ppm).

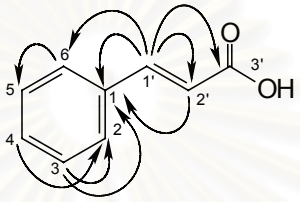


Figure 6. Long-range correlations from HMBC spectrum ($^{n}J_{CH}$ = 8 Hz) of Por02322 in $CDCl_3$

On the basis of these spectral data, Por02322 was identified as *trans*-cinnamic acid, which has been known as the starter unit of natural products produced through the Shikimmic acid pathway. Commonly, *trans*-cinnamic acid has been reported as a constituent of various species of plants. Recently, *trans*-cinnamic acid was isolated from microorganism, the white rot fungus, *Bjerkandera adusta*. The biosynthetic study of *trans*-cinnamic acid revealed that *trans*-cinnamic acid was derived from the deamination of L-phenylalanine by phenylalanine ammonia lyase (Lapadatescu *et al*,. 2000). Currently, *trans*-cinnamic acid was found to possess moderate insecticidal activity at the concentration 2.5 mg/paper using a filter paper diffusion method (Park *et al*., 2000).

2.3 Compound Por0226

The mass spectrum of Por0226 (Figure 25) showed a protonated molecular ion $[M+H]^+$ at m/z 445 consistent with the molecular formula $C_{22}H_{20}O_{10}$. The UV spectrum of Por02226 (Figure 26) displayed maximum absorption peaks at 250 (log ε 4.18) and 283 (log ε 4.02) nm. The IR spectrum of Por0226 (Figure 27) showed absorption bands at 3500-3400 (O-H stretching), 1685 (C=O stretching), 1638 (C=C stretching and 1566 (C-O stretching) cm^{-1}. The degree of unsaturation counted from the structure is thirteen, which is required for the molecular formula $C_{22}H_{20}O_{10}$.

The ^1H NMR spectrum of Por0226 in CD_3OD (Figures 28a-b) exhibited the presence of one methoxy group at δ 3.88 ppm; a pair of non-equivalent methylene protons at δ 1.86 (1H, dt, *J* = 14.6, 2.7 Hz; H-7_{eq}) and 2.63 (1H, dd, *J* = 14.6, 2.7 Hz; H-7_{ax}) ppm; five aromatic protons at δ 7.92 (2H, d, *J* = 7.4 Hz; H-17, 17'), 7.57 (1H, t, *J* = 7.4 Hz; H-18, 18'), 7.46 (2H, t, *J* = 7.4 Hz; H-19) ppm; two olefinic meta-coupled protons at δ 6.37 (1H, d, *J* = 2 Hz; H-11) and 5.64 (1H, d, *J* = 2 Hz; H-13) ppm; and four aliphatic methine protons at δ 4.78 (1H, dt, *J* = 4.5, 2.7 Hz; H-6), 4.72 (1H, d, *J* = 2.7 Hz; H-9), 4.67 (1H, s; H-3), and 4.64 (1H, d, *J* = 4.5 Hz; H-5) ppm. On the ^1H NMR spectrum of compound Por0226, two pairs of equivalent protons and one proton suggesting that the aromatic ring was a mono-substituted benzene.

The ^1H, ^1H COSY spectrum of compound Por0226 in $CDCl_3$ (Figure 31) revealed the correlations of the following protons: H-5 (δ 4.64 ppm)-H-6 (δ 4.78 ppm)-H-$7_{eq, ax}$ (δ 1.86, 2.63 ppm); H-7_{eq} (δ 1.86 ppm)-H-9 (δ 4.72 ppm); H-7_{eq} (δ 1.86 ppm)-H-7_{ax} (δ 2.63 ppm); H-11 (δ 6.37 ppm)-H-13 (δ 5.64 ppm) and 2H-17 and 17' (δ 7.92 ppm)-2H-18 and 18' (δ 7.46 ppm)-H-19 (δ 7.57 ppm). From these ^1H, ^1H COSY correlations, H-5 (δ 4.64 ppm) was coupled with H-6 (δ 4.78 ppm) with *J* = 4.5 Hz of vicinal coupling and doublet splitting pattern. H-6 (δ 4.78 ppm) was coupled with H-5 (δ 4.64 ppm) and H-7_{eq} (δ 1.86 ppm) with *J* = 4.5, 2.7 Hz respectively, therefore, the splitting pattern of H-

6 (δ 4.78 ppm) was doublet of triplets. H-7_{eq} (δ 1.86 ppm) was coupled with H-7_{ax} (δ 2.63 ppm) with J = 14.6 Hz of geminal coupling, and with H-6 (δ 4.78 ppm) and H-9 (δ 4.72 ppm) (W long-range coupling with J = 2.7 Hz, thus H-7_{eq} (δ 1.86 ppm) was doublet of triplets, while H-7_{ax} (δ 2.63 ppm) doublet of doublets). H-11 (δ 6.37 ppm) was coupled with H-13 (δ 5.64 ppm) with J = 2 Hz. 2H-17 and 17' (δ 7.92 ppm) were coupled with 2H-18 and 18' (δ 7.46 ppm) with J = 7.4 Hz. 2H-18 and 18' (δ 7.46 ppm) were also coupled with H-19 (δ 7.57 ppm) with J = 7.4 Hz. Both 2H-18 and 18' (δ 7.46 ppm) and H-19 (δ 7.57 ppm) were triplets. The coupling system of 2H-17 and 17' (δ 7.92 ppm)-2H-18 and 18' (δ 7.46 ppm)-H-19 (δ 7.57 ppm) revealed the existense of a mono-substituted benzene.

The ^{13}C NMR spectrum of Por0226 in CD_3OD (Figure 29) displayed twenty carbon signals. Classification of these carbon signals by DEPT 135 and DEPT 90 (Figure 30) experiments indicated that there were one methoxy (δ 57.0 ppm), one methylene (δ 36.7 ppm), nine methine (δ 133.9, 129.4, 129.3, 107.1, 88.9, 77.3, 71.1, 56.6, and 54.6 ppm), and nine quaternary (δ 197.4, 175.3, 173.2, 166.8, 162.0, 140.6, 80.7, 79.9, and 77.5 ppm) carbons. According to the HMQC spectrum (Figure 33), three (δ 133.9, 129.4, and 129.3 ppm) of nine methine carbon signals were aromatic carbons of a mono-substituted benzene, two of which represented two pairs of equivalent carbons at *ortho* and *meta* position of substituted group at δ 129.4 (C-17 and 17') and 129.3 (C-18 and 18') ppm, respectively. The most downfield carbon signal at δ 133.9 (C-19) ppm is the *para*-carbon. Other six methine carbon signals were classified (based on the chemical shifts) to two sp^2 carbons attributable to C-11 (δ 107.1 ppm) and C-13 (δ 88.9 ppm); four sp^3 methine carbons, two of which appeared to be oxygenated carbon as shown in downfield shift assignable as C-6 (δ 77.3 ppm) and C-5 (δ 71.1 ppm). The last two sp^3 carbons were assigned as C-9 (δ 56.6 ppm) and C-3 (δ 54.6 ppm). Analysis of nine quaternary carbon signals revealed one α, β unsaturated ketone carbonyl shown in upfield shift from normal ketone carbonyl assigned as C-15 (δ 197.4 ppm); two ester

carbonyls appeared in upfield shift from ketone carbonyl, assigned as C-1 (δ 175.3ppm) and C-14 (δ 162.0 ppm); three oxygenated sp^2 quaternary carbons shown in downfield shift assigned as C-10 (δ 166.8 ppm), C-12 (δ 173.2 ppm), and C-16 (δ 140.6 ppm) and three hydroxylated sp^3 quaternary carbons presented in downfield shift from normal sp^3, assigned as C-2 (δ 80.7 ppm), C-4 (δ 79.9 ppm), and C-8 (δ 77.5 ppm). The methoxy and methylene carbons were assigned as 12-OCH_3 (δ 57.0 ppm) and C-7 (δ 36.7 ppm), respectively. The assignment of carbons and protons are summarized in Table IV-4.

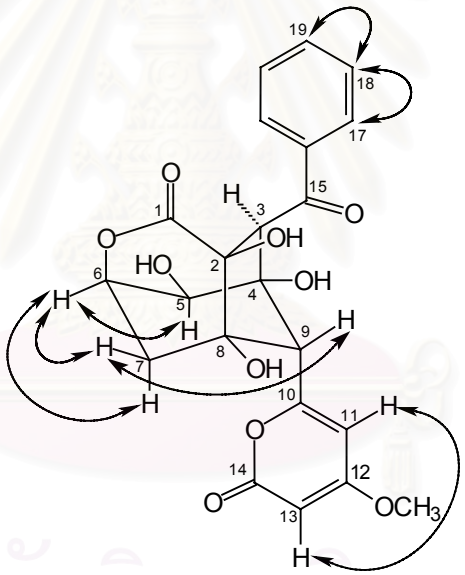
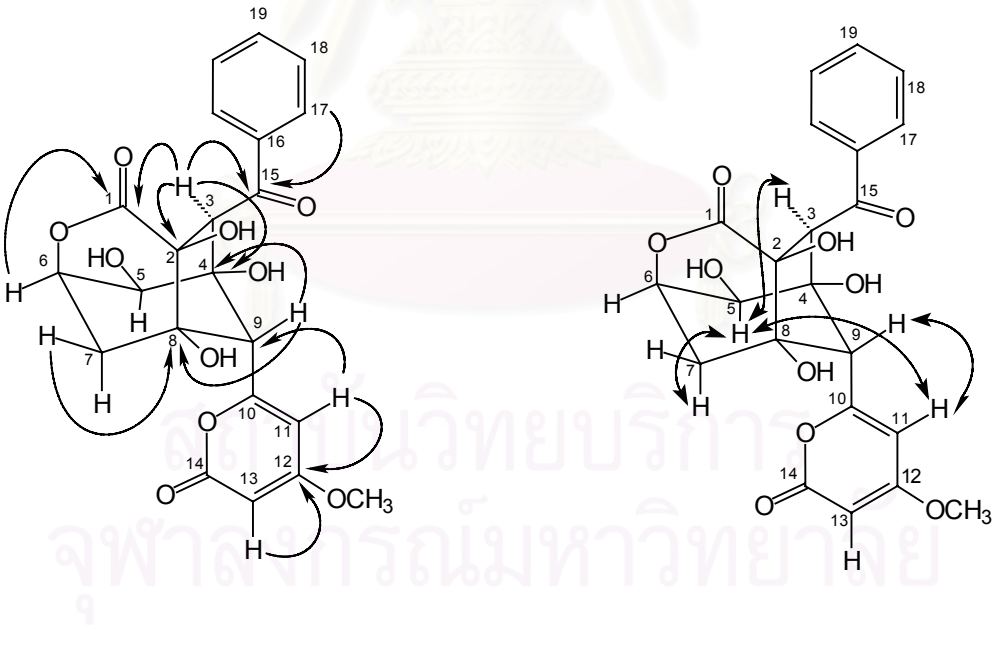


Figure 7. The ^1H, ^1H COSY correlations of Por0226 in $CDCl_3$

Structure determination of compound Por0226 was accomplished by the HMBC spectra ($^{n}J_{CH}$ = 8, 4 Hz) (Figures 34a-c, 35a-c). The long-range correlations are summarized in Table IV-4. The correlations of H-3 (δ 4.67 ppm) and H-6 (δ 4.78 ppm) to C-1 (δ 197.4 ppm); H-7_{ax} (δ 2.63 ppm) and H-3 (δ 4.67 ppm) to C-2 (δ 80.7 ppm); H-5 (δ 4.64 ppm) to C-3 (δ 54.6 ppm); H-3 (δ 4.67 ppm), H-5 (δ 4.64 ppm), and H-6 (δ 4.78 ppm) to C-4 (δ 79.9 ppm); H-3 (δ 4.67 ppm) and H-7_{eq} (δ 1.85 ppm) to C-9 (δ 56.6 ppm), leading to a skeleton of the tricyclic ring. The correlations in HMBC spectrum ($^{n}J_{CH}$ = 8 Hz) (Figures 34a-c) of H-13 (δ 5.64 ppm) to C-11 (δ 107.1 ppm); H-11 (δ 6.37 ppm), H-13 (δ 5.64 ppm), and 12-OCH_3 (δ 3.88 ppm) to C-12 (δ 173.2 ppm) revealed the presence of the 4-methoxy-2-pyrone part. The correlation of H-17 and 17' (δ 7.92 ppm) to C-15 (δ 197.4 ppm) displayed the presence of the phenyl ketone part.

The complete stucture of compound Por0226 was established by the HMBC correlation of H-11 (δ 6.37 ppm) to C-9 (δ 56.6 ppm) indicated the attachment of the tricyclic ring and the 4-methoxy-2-pyrone ring through C-9 (δ 56.6 ppm) and C-10 (δ 166.8 ppm), and the correlation of H-3 (δ 4.67 ppm) to C-15 (δ 197.4 ppm) exhibited the connectivity of the tricyclic ring and the phenyl ketone through C-3 (δ 54.7 ppm) and C-15 (δ 197.4 ppm). The NOESY spectrum of Por0226 in $CDCl_3$ (Figure 36) revealed the correlations of proximity protons as summarized in Table IV-4/1. The NOESY correlations confirmed the relative configuration of Por0226 (Figure 8(b)). The NMR spectral data of compound Por0226 are summarized in Tables IV-4/1, IV-4/2.

By analyses of these available spectral data and comparison of the NMR spectral data of Por0226 in DMSO-d_6 with those of enterocin (Table IV-5) (Seto *et al*., 1976), compound Por0226 was identified as the known compound, enterocin or 7-benzylhexahydro-4a,b,7a,8-tetrahydroxy-5-(4-methoxy-2-oxo-2*H*-pyran-6-yl)-[3S-(3α, 4aβ,5α,6β,7α,7aβ]-3,6-methanocyclopenta[c]pyran-1(3*H*)-one, which was previously isolated from terrestrial *Streptomyces*, *S. candidus* var. *enterostaticus* WS-8096 and *S. viridochromogenes* M-127 (Miyairi *et al*., 1976) and marine *Streptomyces, S. maritimus* (Piel *et al*., 2000) and Australian ascidian of the genus *Didemnum* (Kang *et al*., 1996). Enterocin is commonly known as a bacteriostatic antibiotic against *Escherichia coli*, *Proteus vulgaris*, *Sarcina lutea* and *Staphylococcus aureus* at the concentration of 4 mg/ml, testing by a disc diffusion method (Miyairi *et al*., 1976).



(a) (b)

Figure 8. (a) Long-range correlations from HMBC spectral data of Por0226 in CD_3OD
(b) The NOESY correlations of Por0226 in $CDCl_3$

Table IV-4/1. The ^{1}H, ^{13}C, ^{1}H, ^{1}H COSY, and NOESY spectral data of Por0226

Position	δ ^{13}C (ppm) (CD_3OD)	δ ^{1}H (ppm) (CD_3OD)	COSY ($CDCl_3$)	NOESY ($CDCl_3$)
1	175.3	-	-	-
2	80.7	-	-	-
3	54.6	4.67 (H, s)	-	H5, H17, 17'
4	79.9	-	-	-
5	71.1	4.64 (H, d, J = 4.4 Hz)	H6	H3, H6, $H7_{ax}$, H11
6	77.3	4.78 (H, dt, J = 4.5, 2.7 Hz)	H5	H5, $H7_{eq, ax}$
7	36.7	H_{eq} 1.86 (H, dt, J = 14.6, 2.7 Hz) H_{ax} 2.63 (H, dd , J = 14.6, 2.7 Hz)	$H7_{ax}$ $H7_{aq}$	H5, H6, $H7_{ax}$ H6, $H7_{eq}$
8	77.5	-	-	-
9	56.6	4.72 (H, d, J = 2.7 Hz)	$H7_{eq}$	H11
10	166.8	-	-	-
11	107.1	6.37 (H, d, J = 2 Hz)	H13	H5, H9
12	173.2	-	-	-
12-OCH_3	57.0	3.88 (3H, s)	-	H13
13	88.9	5.64 (H, d, J = 2 Hz)	H11	12-OCH_3
14	162.0	-	-	-
15	197.4	-	-	-
16	140.6	-	-	-
17,17’	129.4	7.92 (2H, d, J = 7.4 Hz)	H18	H3, H18, 18'
18,18’	129.3	7.46 (2H, t, J = 7.4 Hz)	H17, H19	H17, 17', H19
19	133.9	7.57 (H, t, J = 7.4 Hz)	H18	H18, 18'

Table IV-4/2. The ^{1}H, ^{13}C, and HMBC spectral data of Por0226 in CD_3OD

Position	δ ^{13}C (ppm)	δ ^{1}H (ppm)	HMBC ($^{n}J_{CH}$ = 8 Hz)	HMBC ($^{n}J_{CH}$ = 4 Hz)
1	175.3	-	-	-
2	80.7	-	-	-
3	54.6	4.67 (H, s)	C1, C2, C4, C5, C6, C9, C15	C1, C2, C4, C5, C6,C9, C15
4	79.9	-	-	-
5	71.1	4.64 (H, d, *J* = 4.4 Hz)	C3, C4	C3, C4
6	77.3	4.78 (H, dt, *J* = 4.5, 2.7 Hz)	C1, C4, C5	C1, C4, C5
7	36.7	H_{eq} 1.86 (H, dt, *J* = 14.6, 2.7 Hz) H_{ax} 2.63 (dd, H, *J* = 14.6, 2.7 Hz)	C5, C6, C8, C9 C2, C4, C5, C6	C3, C4, C5, C6 C3, C5, C6
8	77.5	-	-	-
9	56.6	4.72 (H, d, *J* = 2.7 Hz)	C4, C8	C3, C4, C11
10	166.8	-	-	-
11	107.1	6.37 (H, d, *J* = 2 Hz)	C9, C12, C13, C14	C9, C12, C13, C14
12	173.2	-	-	-
12-OCH_3	57.0	3.88 (3H, s)	C12	C3, C11, C12
13	88.9	5.64 (H, d, *J* = 2 Hz)	C11, C12	C10, C11, C12
14	162.0	-	-	-
15	197.4	-	-	-
16	140.6	-	-	-
17,17’	129.4	7.92 (2H, d, *J* = 7.4 Hz)	C18, 18', C19	C15
18,18’	129.3	7.46 (2H, t, *J* = 7.4 Hz)	C17, 17'	C16, C17,17'
19	133.9	7.57 (H, t, *J* = 7.4 Hz)	C18, 18'	C18, 18'

Table IV-5. The ^{1}H, ^{13}C NMR spectral data of Por0226 and enterocin in DMSO-d_6

Position	Por0226		Enterocin	
	δ ^{13}C (ppm)	δ^{1}H (ppm)	δ ^{13}C (ppm)	δ^{1}H (ppm)
1	173.5	-	173.4	-
2	79.9	-	79.6	-
2-OH	-	5.90 (OH, s)	-	5.90 (OH, s)
3	53.2	4.46 (H, br s)	52.9	4.47 (H, br s)
4	78.3	-	78.1	-
4-OH	-	5.86 (OH, s)	-	5.85 (OH, s)
5	69.4	4.46 (1H, br s)	69.2	4.47 (H, br s)
5-OH	-	5.65 (5-OH, d, J = 5.5 Hz)	-	5.65 (5-OH, d, J = 5.5 Hz)
6	75.6	4.65 (H, br s)	75.3	4.66 (H, br s)
7	35.8	H_{eq} 1.55 (H, bd, J = 14.4 Hz) H_{ax} 2.27 (H, bd, J = 14.4 Hz)	35.5	H_{eq} 1.68 (H, bd, J = 14.4 Hz) H_{ax} 2.27 (H, bd, J = 14.4 Hz)
8	76.5	-	76.2	-
8-OH	-	5.48 (OH, s)	-	5.46 (OH, s)
9	54.8	4.65 (H, br s)	54.6	4.66 (H, br s)
10	163.3	-	163.1	-
11	104.8	6.28 (H, d, J = 1.8 Hz)	104.6	6.29 (H, d, J = 2.3 Hz)
12	170.6	-	170.4	-
12-OCH_3	56.5	3.82 (3H, s)	56.1	3.87 (3H, s)
13	87.9	5.60 (H, d, J = 1.8 Hz)	87.6	5.60 (H, d, J = 2.3 Hz)
14	161.2	-	161.2	-
15	194.8	-	194.7	-
16	139.6	-	139.3	-
17, 17'	128.8	7.76 (2H, d, J = 7.4 Hz)	128.0	7.45-7.85 (5H, m)
18, 18'	128.4	7.50 (2H, t, J = 7.4 Hz)	127.7	
19	132.6	7.61 (H, t, J = 7.4 Hz)	132.4	

*^{1}H, ^{13}C NMR spectra of Por0226 are shown in Figures 37-38.

2.4 Compound Por0225223

The mass spectrum of Por0225223 (Figure 39) showed a protonated molecular ion $[M+H]^+$ at m/z 429 consistent with the molecular formula $C_{22}H_{20}O_9$. The UV spectrum of Por0225223 (Figure 40) displayed maximum absorption peaks at 249 (log ε 4.07) and 283 (log ε 3.90) nm. The IR spectrum of Por0225223 (Figure 41) revealed the absorption bands at 3400-3200 (O-H stretching), 2934 (C-H stretching), 1639 (C=O stretching) and 1567 (C-O stretching) cm^{-1}. The degree of unsaturation counted from this molecular formula is thirteen, which is required for the molecular formula $C_{22}H_{20}O_9$.

The ^1H NMR spectrum of compound Por0225223 in $CDCl_3$ (Figures 42a-b) showed the presence of one methoxy protons at δ 3.81 (3H, s; 12-OCH_3) ppm, two methylene protons at δ 1.86 (H, dq, *J* = 14.5, 1.8 Hz; H-7_{eq}) and 2.68 (H, dd, *J* =14.5 Hz; H-7_{ax}) δ 2.19 (H, br d, *J* = 15.2 Hz; H-5_{aq}) and 2.80 (H, dd, *J* = 15.2 Hz; H-5_{ax}) ppm; one mono-substituted benzene at δ 7.84 (2H, d, *J* = 7.4 Hz; H-17 and 17'), 7.60 (1H, t, *J* =7.4 Hz; H-19), 7.48 (2H, t, *J* = 7.4 Hz; H-18 and 18') ppm; and five methine at δ 6.31 (H, d, *J* = 2 Hz; H-11), 5.48 (H, d, *J* = 2 Hz; H-13), 4.98 (H, br s; H-6), 4.23 (H, br s; H-9), and 3.99 (H, s; H-3) ppm.

The ^1H, ^1H COSY spectrum of Por0225223 in $CDCl_3$ (Figure 46) revealed the correlations of coupling protons as follows; H-$5_{eq,\ ax}$ (δ 2.19, 2.80 ppm)-H-6 (δ 4.98 ppm)-H-$7_{eq,\ ax}$ (δ 1.86, 2.68 ppm); H-5_{eq} (δ 2.19 ppm)-H-7eq (δ 1.86 ppm); H-5_{eq} (δ 2.19 ppm)-H-9 (δ 4.23 ppm); H-7_{eq} (δ 1.86 ppm)-H-9 (δ 4.23 ppm); H-11 (δ 6.31ppm)-H-13 (δ 5.48 ppm); H-17 and 17' (δ 7.84 ppm)-H-18 and 18' (δ 7.48 ppm)-H-19 (δ 7.60 ppm). The ^1H, ^1H COSY spectral data are summarized in Table IV-6. Comparison of ^1H NMR spectra of compound Por0225223 and Por0226 (Figure 45) revealed the similarity of these two compounds, both consisting of the tricyclic, mono-substituted benzene and 4-methoxy-2-pyrone rings. The difference of these two compounds was the presence of

methylene protons signals at δ 2.19 and 2.80 ppm (H-$5_{eq, ax}$) in Por0225223 instead of the presence of methine proton signal in Por0226. The ESI-TOF MS spectral data confirmed that Por0225223 was the deoxy derivative of compound Por0226.

The ^{13}C NMR spectrum of compound Por0225223 in $CDCl_3$ (Figure 43) exhibited twenty carbon signals; there was one carbon signal overlapping with the signal of residual undeuterated $CDCl_3$ (δ 77.7 ppm). Classification of carbons by DEPT 135 experiment (Figure 44) showed one methoxy at δ 56.0 (12-OCH_3) ppm, two methylene at δ 38.0 (C-7) and 37.2 (C-5) ppm, eight methine at δ 134.1, 128.3, 128.8, 105.3, 88.6, 60.6, and 56.6 ppm, and nine quaternary carbons at δ 196.9, 174.0, 170.8, 163.8, 159.7, 137.5, 77.7, 76.8, and 76.1 ppm. Analyses of methine carbon signals revealed that there are two pairs of equivalence aromatic carbons at δ 128.8 (C-17 and 17') ppm and 128.3 (C-18 and 18') ppm, and one aromatic methine carbon at δ 134.1 (C-19) ppm, indicating the presence of aromatic ring is the mono-substituted benzene ring. Two methine carbons are olefinic carbons appeared at δ 105.3 (C-11) and 88.6 (C-11) ppm, and three sp^3 carbons at δ 74.5 (C-6), 60.6 (C-3), and 56.6 (C-9) ppm, one of which is an oxygenated carbon δ 74.5 (C-6) ppm. Nine quaternary carbons could be classified as three carbonyl carbons (one of which is α, β unsaturated ketone carbonyl at δ 196.9 (C-15) ppm, and the other two are ester carbonyl carbons at δ 174.0 (C-1) and 163.8 (C-14) ppm), three olefinic quaternary carbons (two of which are oxygenated carbons at δ 159.7 (C-10) and 170.8 (C-12) ppm, and the other one is a carbon attached to substituted group in mono-substitute benzene ring), and three sp^3 quaternary carbons (hydroxylated carbon at δ 77.7(C-2), 76.8 (C-4) and 76.1 (C-8) ppm). Analyses of spectral data described and comparison with the literature data, Por02253223 was identified as 5-deoxyenterocin. From the previous report (Sitachitta *et al*., 1996), there are two deoxy derivatives of enterocin including, 5-deoxyenterocin and 3-*epi*-5-deoxyenterocin. The NOESY spectrum of Por0225223 in $CDCl_3$ (Figure 47) revealed the correlations of proximity protons as summarized in Table IV-6. The NOESY correlations confirmed the relative

configuration of Por0225223 (Figure 9 (b)). Comparison of ^{1}H and ^{13}C NMR data of compound Por0225223 with reported data (Tables IV-7/1, IV-7/2) of those two derivatives (Sitachitta *et al*., 1996) conclusively confirmed that Por0225223 was a known compound, 5-deoxyenterocin or 7-benzylhexahydro-4a,b,7a,-trihydroxy-5-(4-methoxy-2-oxo-2*H*-pyran-6-yl)-[3S-(3α,4aβ,5α,6β,7α,7aβ]-3,6-Methanocyclopenta[c]pyran-1(3*H*)-one, which was previously isolated from the marine *Streptomyces*, *S. maritimus* (Piel *et al*., 2000) and Australian ascidian of the genus *Didemnum* (Kang *et al*., 1996). The compound 5-deoxyenterocin was previously reported to possess antimicrobial activity against *Staphylococcus aureus*, *Sarcina lutea*, *Klebsiella pneumoniae* and *Vibrio percolans* at the concentration of 0.5 mg/ml using a disc diffusion method (Yakimura *et al*., 1987).

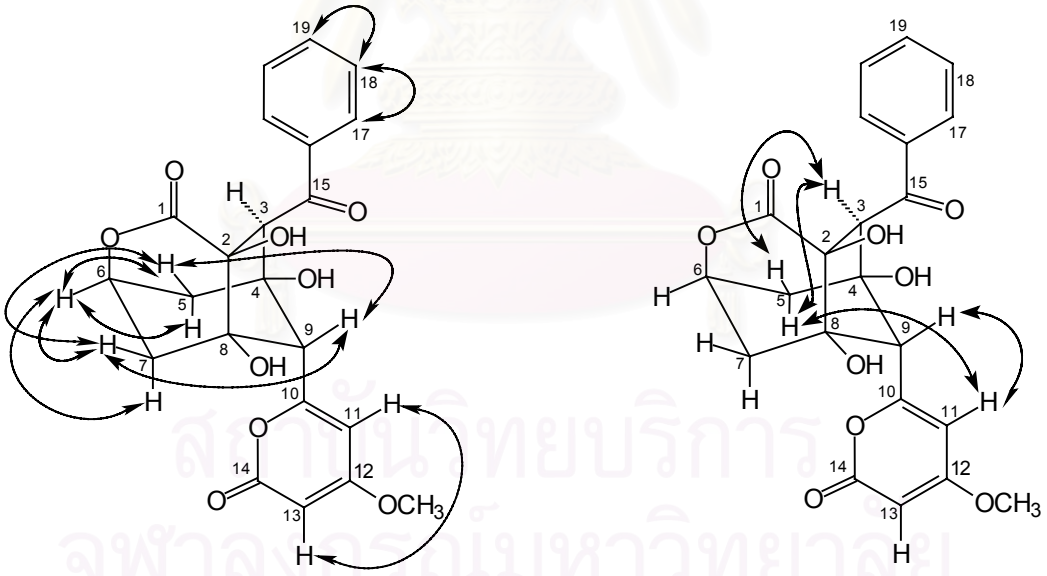


Figure 9. (a) The ^{1}H, ^{1}H COSY correlations of Por0225223 in $CDCl_3$

(b) The NOESY correlations of Por0225223 in $CDCl_3$

Table IV-6. The ^{1}H, ^{13}C, ^{1}H, ^{1}H COSY, and NOESY spectral data of Por0225223 in $CDCl_3$

Position	Por0225223			
	δ ^{13}C (ppm)	δ ^{1}H (ppm)	^{1}H, ^{1}H COSY	NOESY
1	174.0	-	-	-
2	77.7	-	-	-
3	60.6	3.99 (H, s)	-	$H5_{aq,ax}$, H17, 17' H18, 18'
4	76.8	-	-	-
5	38.0	H_{eq} 2.19 (H, d, J = 15.2 Hz) H_{ax} 2.80 (H, dd, J = 15.2, 4.2 Hz)	$H5_{ax}$, H6, $H7_{eq}$, H9 $H5_{eq}$, H6	H3, $H5_{ax}$, H6 H3, $H5_{eq}$, H6, H11
6	74.5	4.98 (H, br s)	$H5_{eq, ax}$, $H7_{eq, ax}$	$H5_{eq, ax}$, $H7_{eq, ax}$
7	37.2	H_{eq} 1.86 (H, dq, J = 14.5, 1.8 Hz) H_{ax} 2.68 (H, dd, J = 14.5, 3 Hz)	$H7_{ax}$, $H5_{eq}$, H6, H9 $H7_{eq}$, H6	H6, $H7_{ax}$ H6, $H7_{eq}$
8	76.1	-	-	-
9	56.6	4.23 (H, br s)	$H5_{eq}$, $H7_{eq}$	H11
10	159.7	-	-	-
11	105.3	6.31 (H, d, J = 2 Hz)	H13	$H5_{ax}$, H9, 12-OCH_3
12	170.8	-	-	-
12-OCH_3	56.0	3.81 (3H, s)	-	-
13	88.6	5.48 (H, d, J = 2 Hz)	H11	12-OCH_3
14	163.8	-	-	-
15	196.9	-	-	-
16	137.5	-	-	-
17, 17'	128.8	7.84 (2H, d, J = 7.4 Hz)	H18, 18'	H3, H18, 18'
18, 18'	128.3	7.48 (2H, t, J = 7.4 Hz)	H17, 17', H19	H3, H17, 17'
19	134.1	7.60 (H, t, J = 7.4 Hz)	H18, 18'	H17, 17', H18,18'

Table IV-7/1. The ^{13}C spectral data of Por0225223, 5-deoxyenterocin, and 3-*epi*-5-deoxyenterocin in $CDCl_3$

Position	δ ^{13}C (ppm)		
	Por0225223	5-deoxyenterocin	3-*epi*-5-deoxyenterocin
1	174.0	174.6	172.9
2	77.7	77.7	77.4
3	**60.6**	**60.0**	**64.9**
4	76.8	76.7	75.7
5	**38.0**	**38.3**	**33.3**
6	74.5	74.9	73.6
7	37.2	37.5	36.5
8	76.1	76.5	75.5
9	**56.6**	**56.8**	**59.3**
10	159.7	160.3	160.0
11	105.3	105.8	104.6
12	170.8	171.3	171.1
12-OCH_3	56.0	56.3	56.0
13	88.6	89.0	88.4
14	163.8	164.4	164.1
15	196.9	197.6	197.7
16	137.5	138.6	137.6
17, 17'	128.8	129.1	128.9
18, 18'	128.3	128.6	128.4
19	134.1	134.6	133.8

Table IV-7/2. The ^{1}H spectral data of Por0225223, 5-deoxyenterocin, and 3-*epi*-5-deoxyenterocin in $CDCl_3$

Position	δ ^{1}H (ppm)		
	Por0225223	5-deoxyenterocin	3-*epi*-5-deoxyenterocin
1	-	-	-
2	-	-	-
3	**3.99 (H, s)**	**4.01 (H, s)**	**4.13 (H, s)**
4	-	-	-
5	**H_{eq} 2.19(H, d, *J* = 15.2 Hz)** **H_{ax} 2.80(H, dd, *J* = 15.2, 4.2Hz)**	**H_{eq} 2.19(H, dd, *J* = 15.5, 1.9 Hz)** **H_{ax} 2.86(H, dd, *J* = 15.5, 4.4 Hz)**	**H_{eq} 2.95(H, br d, *J* = 15.4, 1.9 Hz)** **H_{ax} 2.56(H, dd, *J* = 15.4, 3.2 Hz)**
6	4.98 (H, br s)	4.99 (H, m)	4.84 (H, m)
7	H_{eq} 1.86 (H, dq, *J* = 14.5, 1.8 Hz) H_{ax} 2.68 (H, dd, *J* = 14.5, 3 Hz)	H_{eq} 1.86 (H, dd, *J* = 14.7, 2.4 Hz) H_{ax} 2.73 (H, dd, *J* = 14.7, 2.9 Hz)	H_{eq} 1.76 (H, br d, *J* = 14.7 Hz) H_{ax} 2.48 (H, dd, *J* = 14.7, 3.2 Hz)
8	-	-	-
9	**4.23 (H, br s)**	**4.24 (H, br s)**	**3.40 (H, br s)**
10	-	-	-
11	6.31 (H, d, *J* = 2 Hz)	6.31 (H, d, *J* = 1.9 Hz)	6.30 (H, d, *J* = 1.8 Hz)
12	-	-	-
12-OCH_3	3.81 (3H, s)	3.84 (3H, s)	3.8 (3H, s)
13	5.48 (H, d, *J* = 2 Hz)	5.49 (H, d, *J* = 1.9 Hz)	5.49 (d, *J* = 1.8 Hz)
14	-	-	-
15	-	-	-
16	-	-	-
17, 17'	7.84 (2H, d, *J* = 7.4 Hz)	7.87 (2H, d, *J* = 7.5 Hz)	7.96 (2H, d, *J* = 7.5 Hz)
18, 18'	7.48 (2H, t, *J* = 7.4 Hz)	7.16 (2H, t, *J* = 7.5 Hz)	7.43 (2H, t, *J* = 7.5 Hz)
19	7.60 (H, t, *J* = 7.4 Hz)	7.49 (H, t, *J* = 7.5 Hz)	.58 (H, t, *J* = 7.5 Hz)

5. Biological activity of the isolated compounds

The EtOAc extract of the fermentation broth of *Streptomyces* sp. TRA 9839-2 exhibited antibacterial activity against *Bacillus subtilis* ATCC 6633 and antiviral activity against *Herpes simplex* virus types I and II. It was further purified by several chromatographic techniques with bio-activity guided fractionation. The isolated compounds were tested for their biological activities including antimicrobial, antiviral, antimalarial and cytotoxic activities.

5.1 Antimicrobial activity

The antimicrobial activity of the EtOAc extract, fractions and isolated compounds were evaluated by a disc diffusion method (Lorian, 1989). The EtOAc extract and fractions were examined at the concentration of 1 mg/disc (0.6 mm diameter) while the pure compounds were tested at the concentration of 500 μg/disc (0.6 mm diameter). The antimicrobial activity was measured from the inhibition zones (mm) of testing microorganisms, including the bacterial strains *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and the yeast strain *Candida albicans* ATCC 10231. Antimicrobial activity is shown in Table IV-8.

Table IV-8. Antimicrobial activity of the EtOAc extract, fractions and isolated compounds from TRA 9839-2

Compound/ fraction	Inhibition zone (mm)			
	B. subtilis ATCC 6633	*S. aureus* ATCC 25923	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
EtOAc extract	18 mm	-	-	-
F01	13 mm	ND	ND	ND
F02	15 mm	ND	ND	ND
F03	-	ND	ND	ND
F04	-	ND	ND	ND
F021	18 mm	ND	ND	ND
F0221	-	ND	ND	ND
F0222	-	ND	ND	ND
F0223	-	ND	ND	ND
F0224	23 mm	ND	ND	ND
F0225	20 mm	ND	ND	ND
F0226	20 mm	ND	ND	ND
F0227	-	ND	ND	ND
F02321	-	ND	ND	ND
F02322	-	ND	ND	ND
F02323	18 mm	ND	ND	ND
F022522	± 23 mm	ND	ND	ND
Por024	8 mm	-	-	-
Por02322	-	-	-	-
Por0226	-	-	-	-

-, inactive

ND, Not determined

±, Bacteriostatic

From Table IV-8, the EtOAc extract showed significant antibacterial activity against *Bacillus subtilis*, and therefor this activitywas used as a guide for further investigation. Fraction which displayed antibacterial activity (F0224, F0225, and F0226) were purified to obtain pure compounds, Por0226 and Por0225223, however these pure compounds did not exhibit the antibacterial activity. The active compound could not be isolated because of the small amount of the fractions.

5.2 Antiviral activity

The EtOAc extract and isolated compounds were tested for antiviral activity against *Herpes simplex* virus types I and II. Acyclovir was used as a positive control of 100% inhibition. The compounds were tested at the concentration 50 μg/ml. The results of anti- *Herpes simplex* viral activity are shown in Table IV-9. The EtOAc showed strong activity against *Herpes simplex* virus types I and II but the compound Por0226 showed only weak activity against *Herpes simplex* virus type I at the concentration of 50 μg/ml.

Table IV-9. Antiviral activity against *Herpes simplex* virus types I and II of the isolated compounds from TRA 9839-2

Fraction/Compound	Cytotoxicity against vero cell	Anti HSV-I activity	Anti HSV-II activity
EtOAc extract	> 20 μg/ml	++	++++
Por0226	ND	+	-
Por0225223	ND	-	-

++++, 100% inhibition +++, 75% inhibition

++, 50% inhibition +, 25% inhibition

-, inactive ND, Not determined

5.3 Antimalarial activity

The isolated compounds were tested for antimalarial activity against *Plasmodium falciparum* K1 multi drugs resistant strain by hypoxanthine uptake method (Trager and Jensen, 1976). Chloroquine phosphate was used as positive control. The antimalarial results are shown in Table IV-10. Por0226 exhibited weak antimalarial activity with the EC_{50} = 9.4 μg/ml (21.2 μM).

Table IV-10. Antimalarial activity of the isolated compounds from the TRA 9839-2

Compound	Animalarial activity	
	EC_{50} (μg/ml)	μM
Por024	-	-
Por02322	ND	ND
Por0226	9.4	21.2
Por0225223	ND	ND
Chloroquine phosphate	0.16	3.1

-, inactive

ND, Not determined

5.4 Cytotoxic activity

The isolated compounds were tested for cytotoxic activity against KB and BC cells, and the results are shown in Table IV-11. Por024 and Por0226 exhibited weak cytotoxicity against KB cell with the ED_{50} = 9.9 μg/ml and 20 μg/ml, respectively.

Table IV-11. Cytotoxic activity of the isolated compounds from TRA 9839-2

Compound	KB cell ED_{50} (μg/ml)	BC cell ED_{50} (μg/ml)
Por024	9.9	-
Por02322	ND	ND
Por0226	20	-
Por0225223	ND	ND

-, inactive

ND, Not determined

5.5 Antitubercular activity

The isolated compounds were tested for antitubercular activity against *Mycobacterium tuberculosis* $H_{37}Ra$ by a microplate alamar blue assay (MABA), and the results are shown in Table IV-12. Por02322 inhibited *Mycobacterium tuberculosis* $H_{37}Ra$ with the minimum inhibitory concentration (MIC) 200 μg/ml.

Table IV-12. Antitubercular activity of the isolated compounds from TRA 9839-2

Compound	Antitubercular activity (MIC = μg/ml)
Por024	ND
Por02322	200
Por0226	ND
Por0225223	-

-, inactive

ND, Not determined

CHAPTER V

CONCLUSION

The actinomycete, TRA 9839-2 isolated from a mangrove forest along Andaman coast of Trang province was identified as *Streptomyces* based on the morphological, cultural, physiological, and biochemical characteristics, including the cell wall component study. The mangrove *Streptomyces* sp. TRA 9839-2 was cultivated in seawater basal medium (35 litters) to give 11 g of the EtOAc extract, which exhibited antibacterial activity against *Bacillus subtilis* ATCC 6633 and antiviral activity against *Herpes simplex* virus types I and II. Isolation and purification of the EtOAc extract were carried out, using several chromatographic techniques with bioactivity-guided frationation, to give four compounds, including pyrrole-2-carboxylic acid, *trans*-cinnamic acid, enterocin, and 5-deoxyenterocin. The structure elucidation of these compounds was accomplished by analyses of their spectroscopic data and by comparison of their spectral data with those of known compounds. Pyrrole-2-carboxylic acid possessed weak cytotoxic activity against KB cell with the ED_{50} = 9.9 μg/ml, whereas enterocin showed antiviral activity against *Herpes simplex* type I (25% inibition at the concentration of 50 μg/ml), antimalarial activity with the EC_{50} = 9.4 μg/ml and weak cytotoxic activity against KB cell with the ED_{50} = 20 μg/ml. In addition, *trans*-cinnamic acid inhibited *Mycobacterium tuberculosis* H_{37}Ra with the MIC = 200 μg/ml.

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APPENDIX A

(Figures)

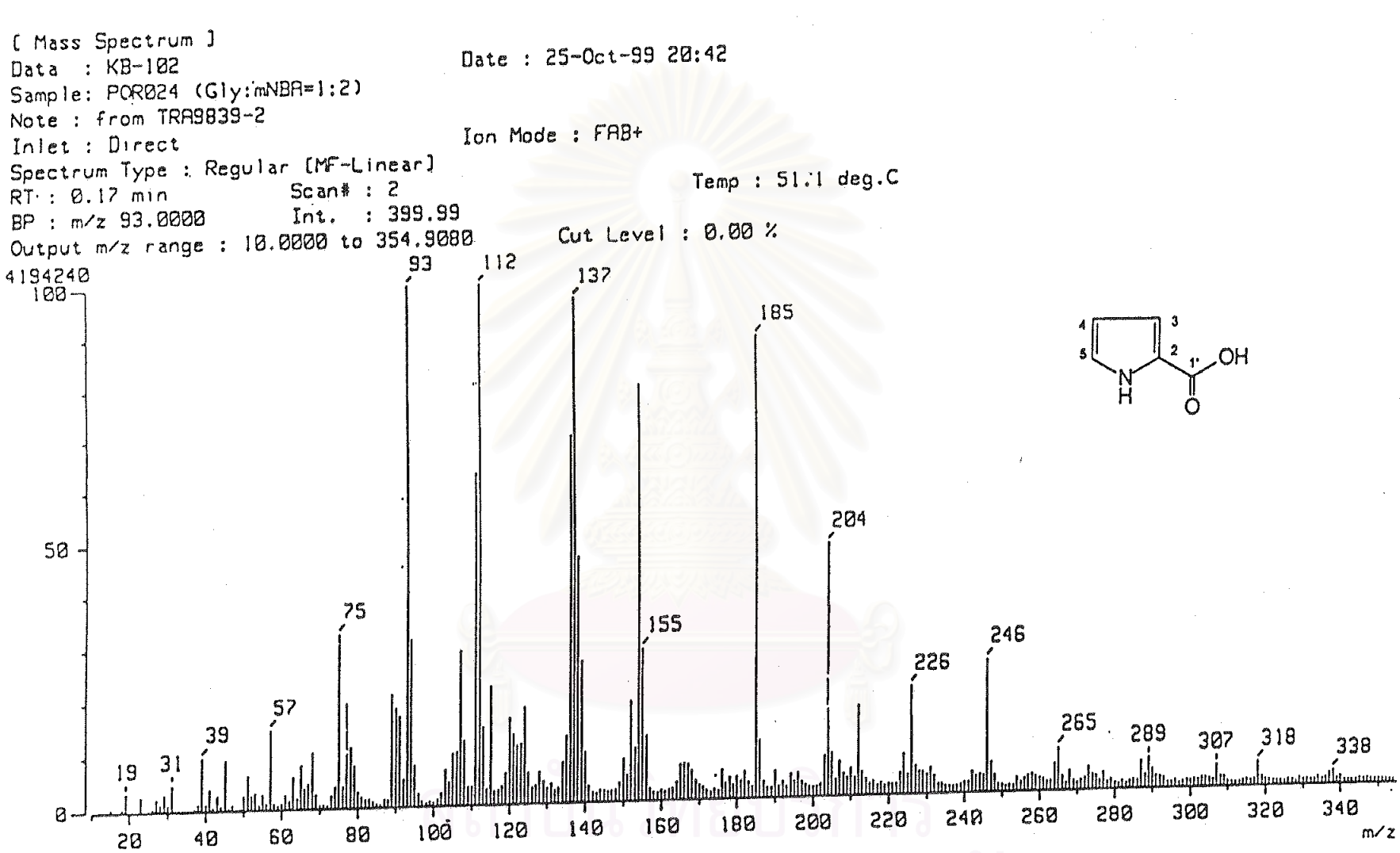


Figure 10. The FABMS spectrum of Por024

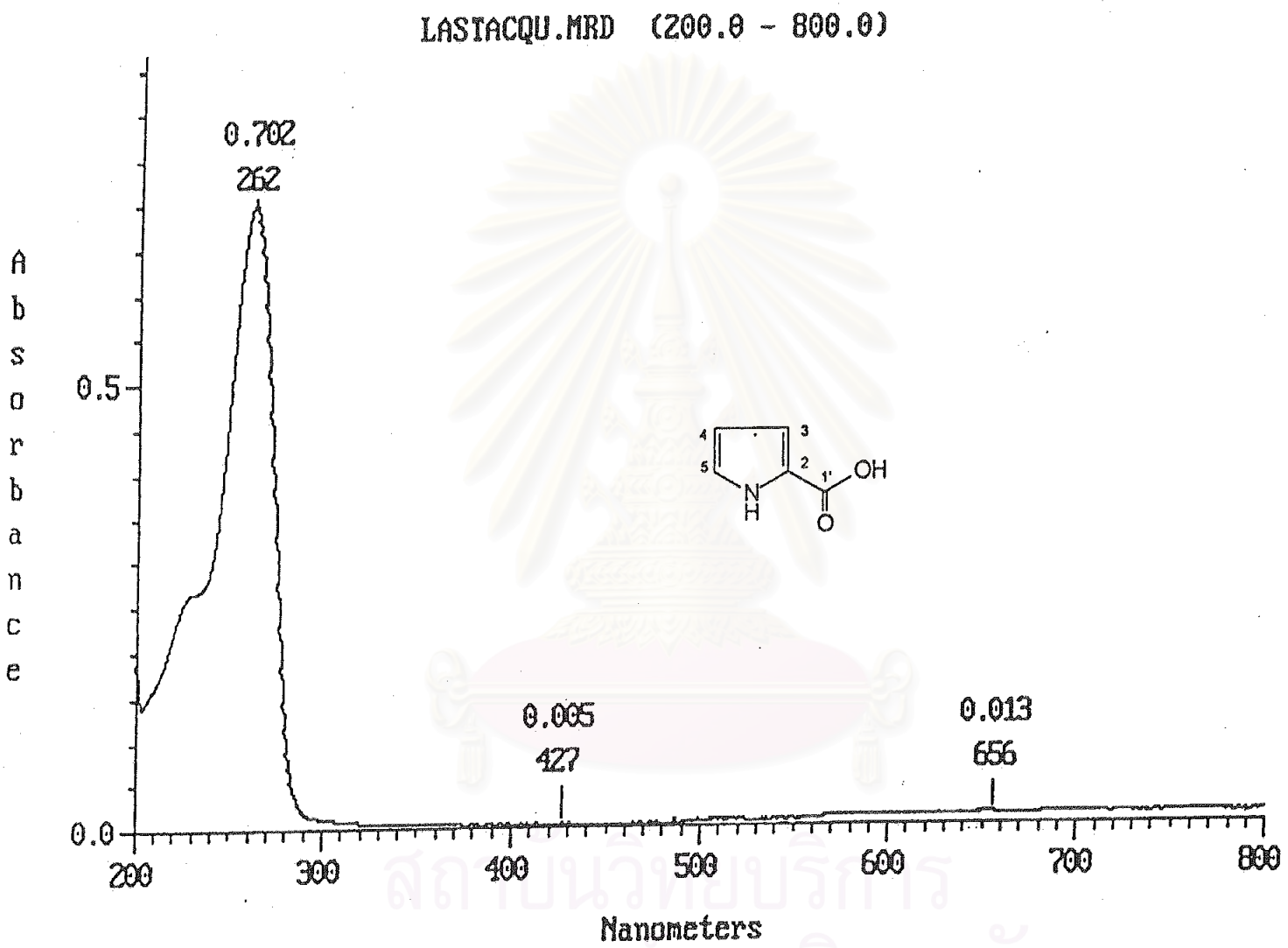


Figure 11. The UV spectrum of Por024 (in MeOH)

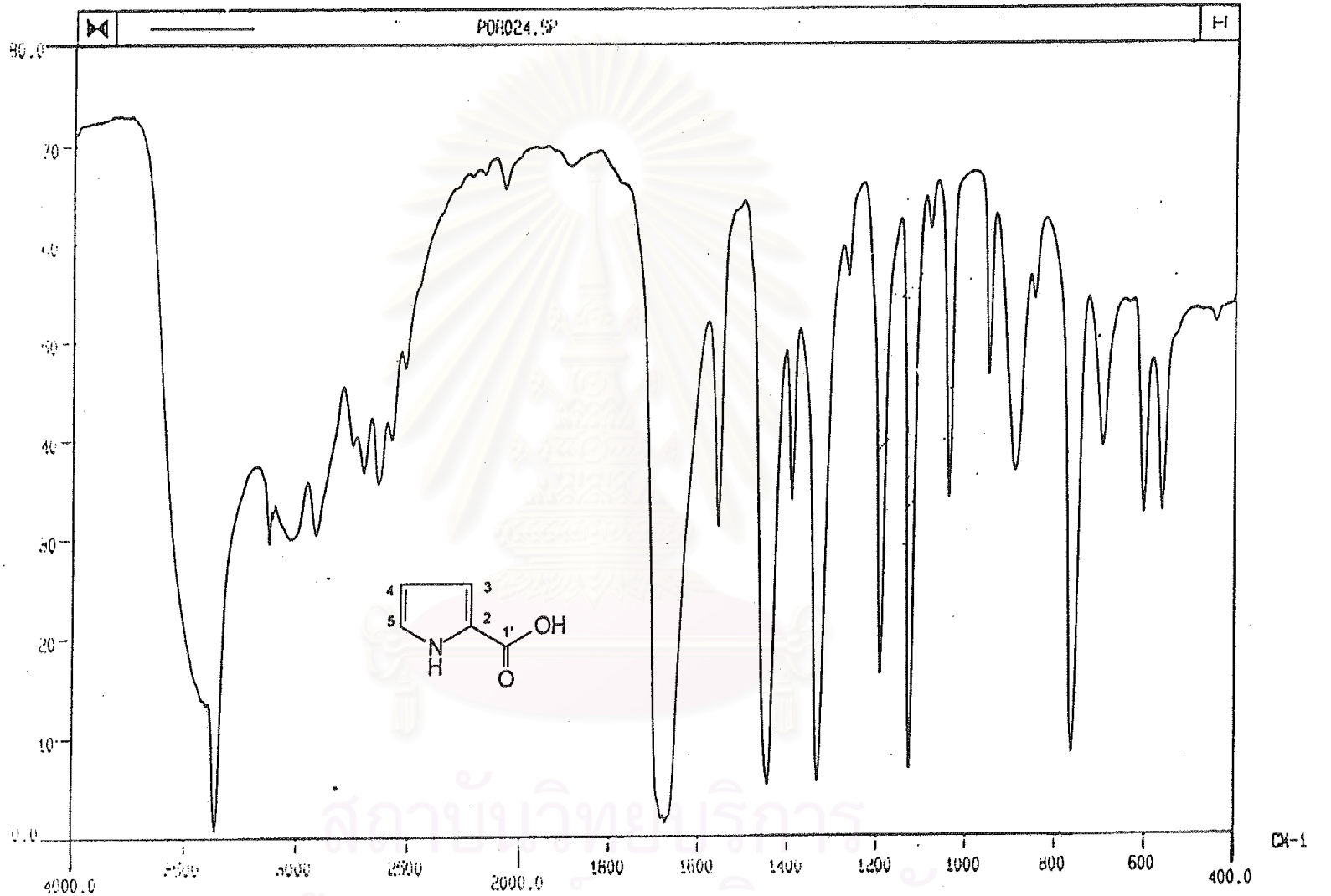


Figure 12. The IR spectrum of Por024 (KBr disc)

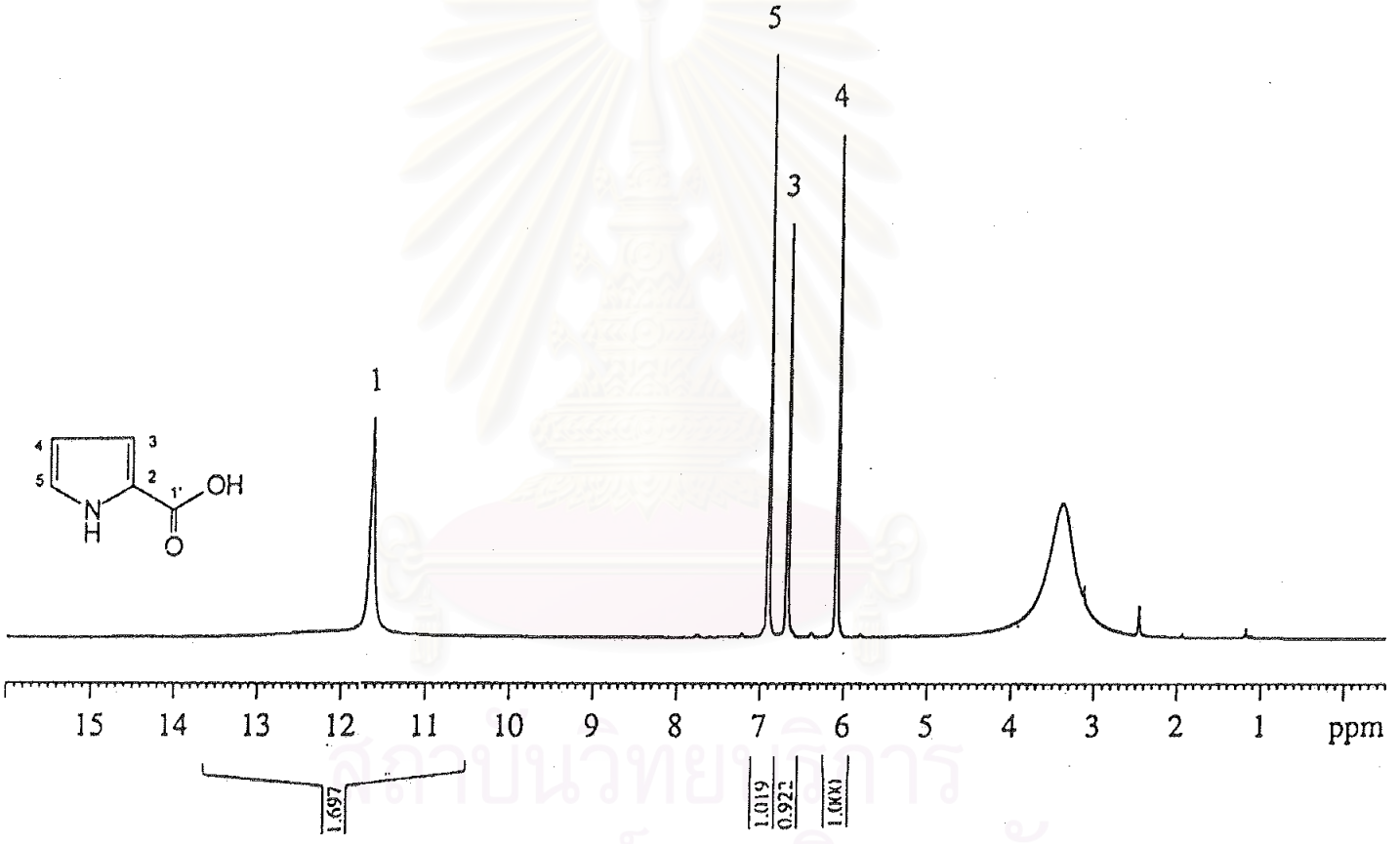


Figure 13. The 300 MHz ^{1}H NMR spectrum of Por024 (in DMSO-d_6)

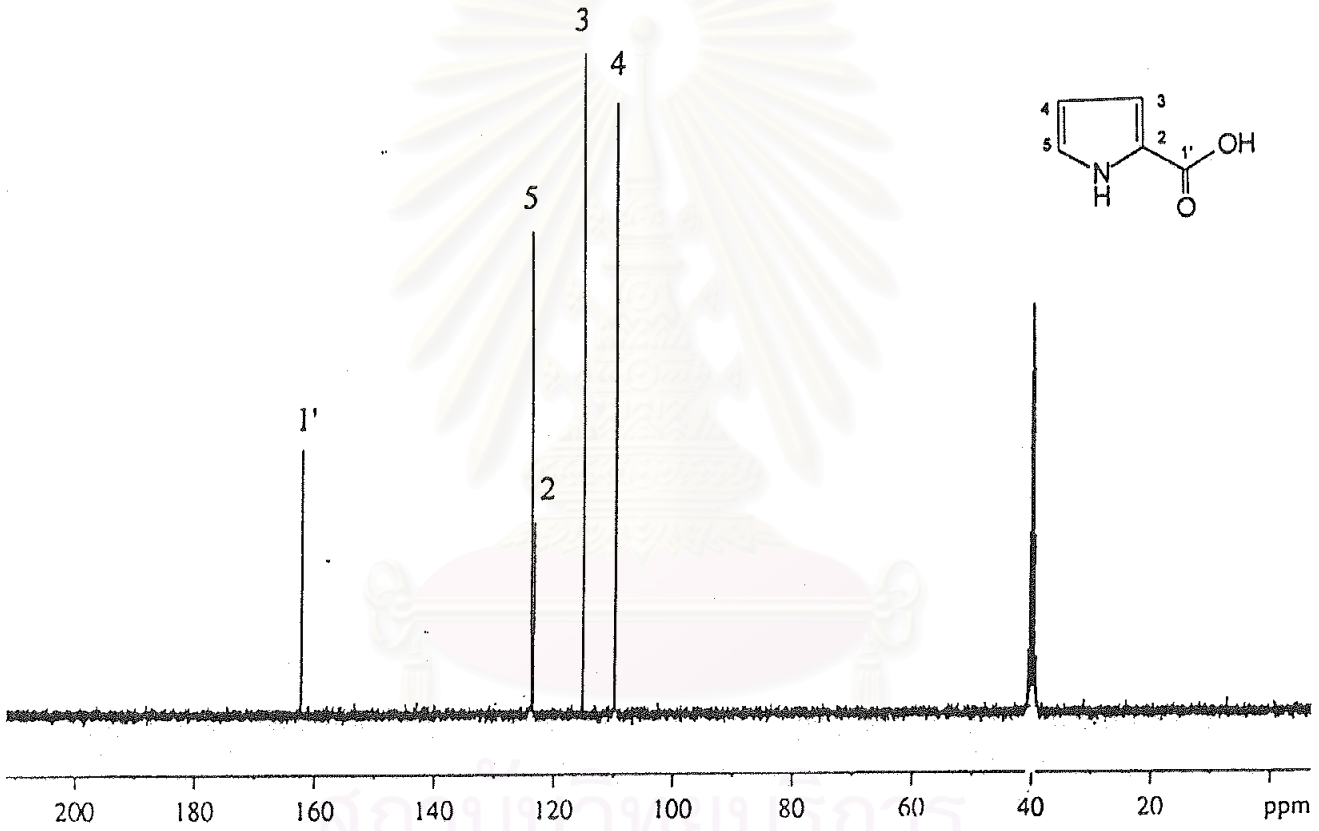


Figure 14. The 75 MHz ^{13}C NMR spectrum of Por024 (in DMSO-d_6)

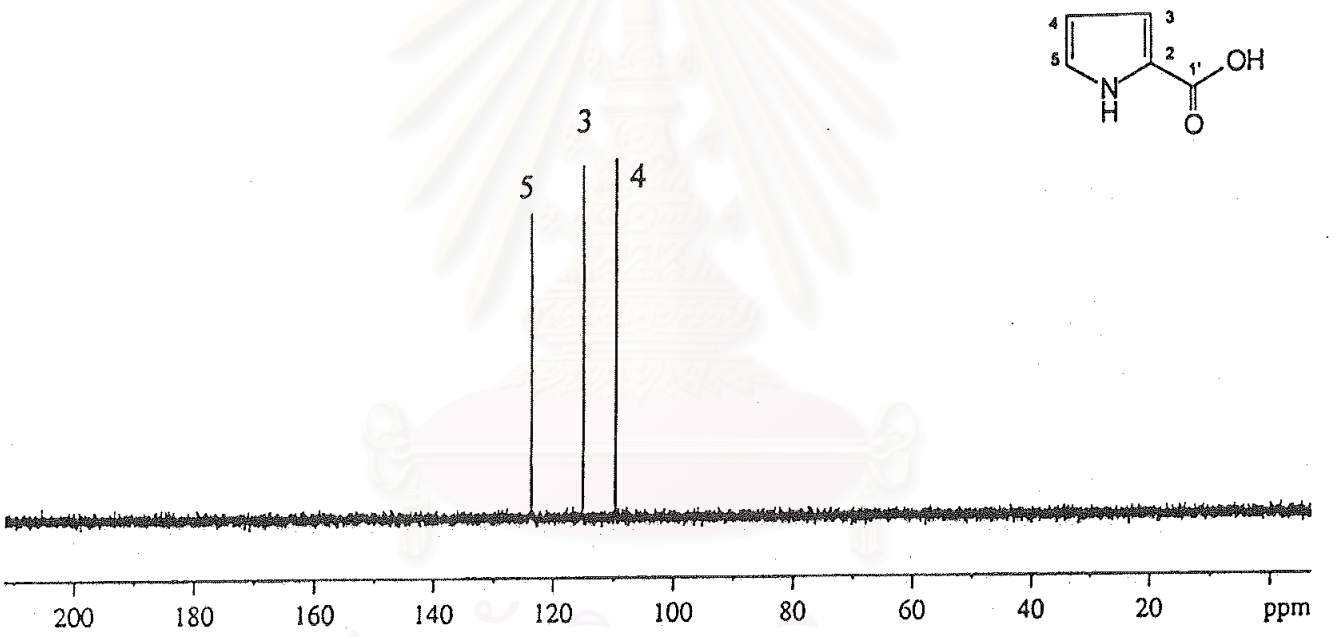


Figure 15. The 75 MHz DEPT 135 spectrum of Por024 (in DMSO-d_6)

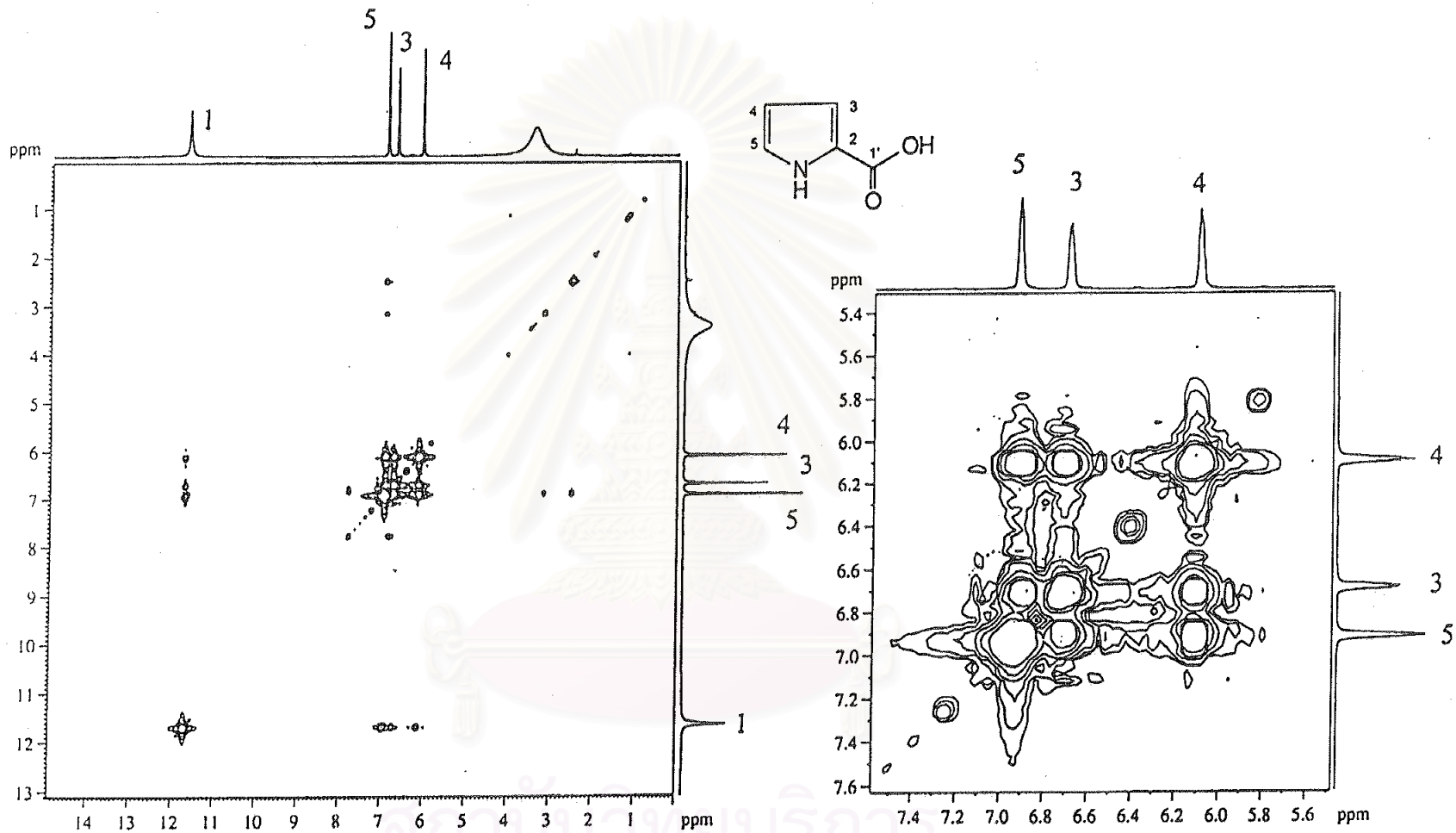


Figure 16. The 300 MHz H, H COSY spectrum of Por024 (in DMSO-d_6)

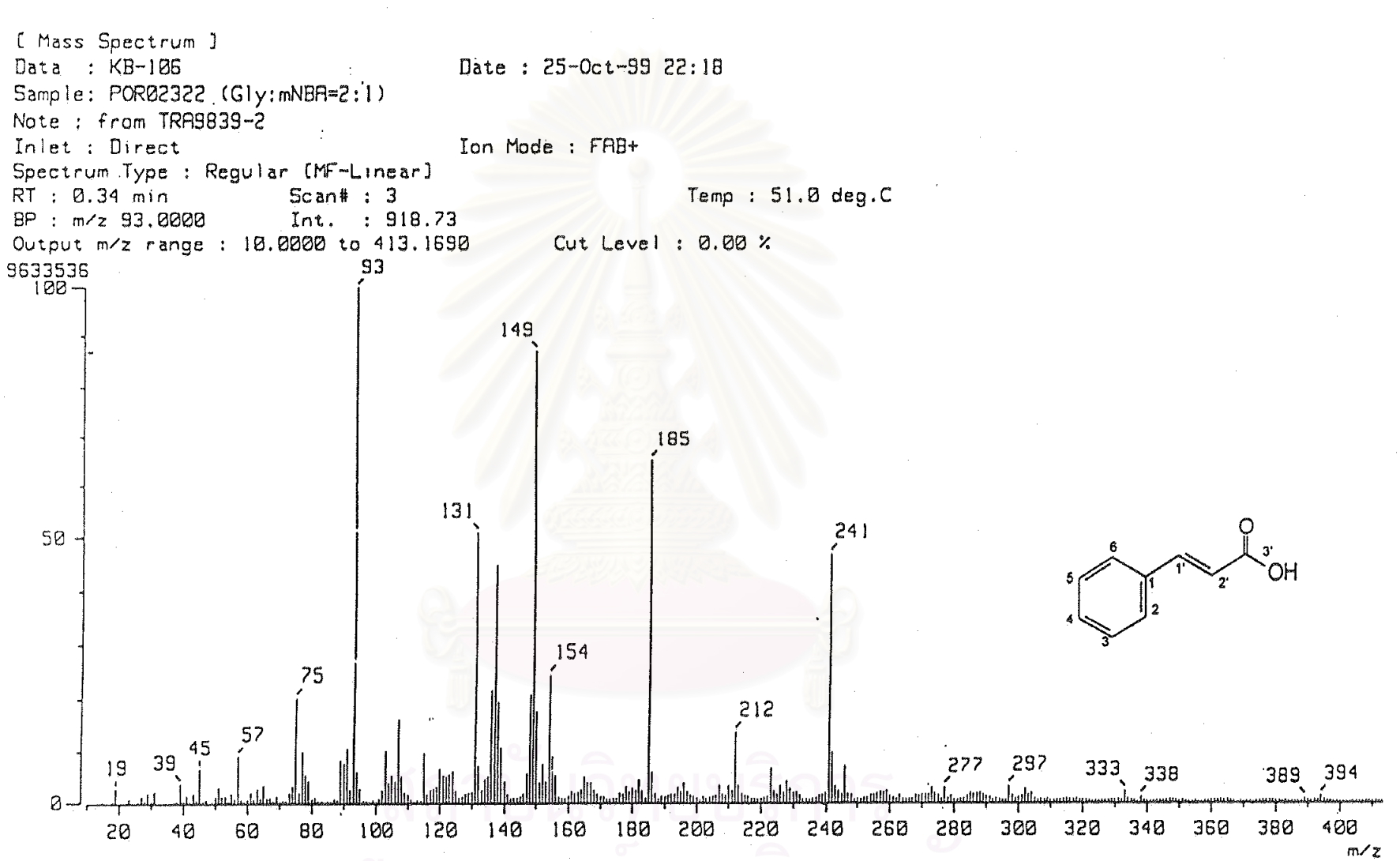


Figure 17. The FABMS spectrum of Por02322

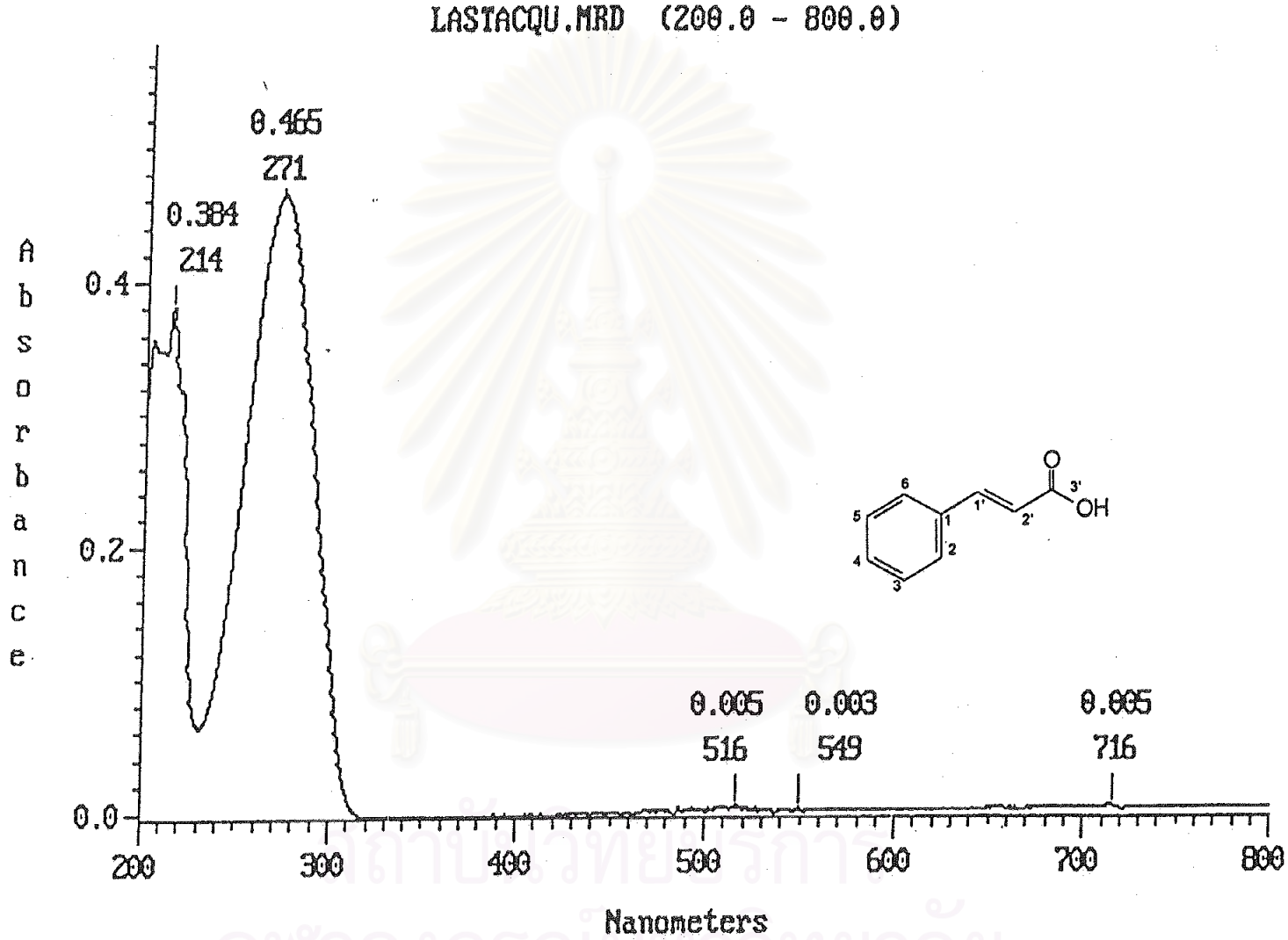


Figure 18. The UV spectrum of Por02322 (in MeOH)

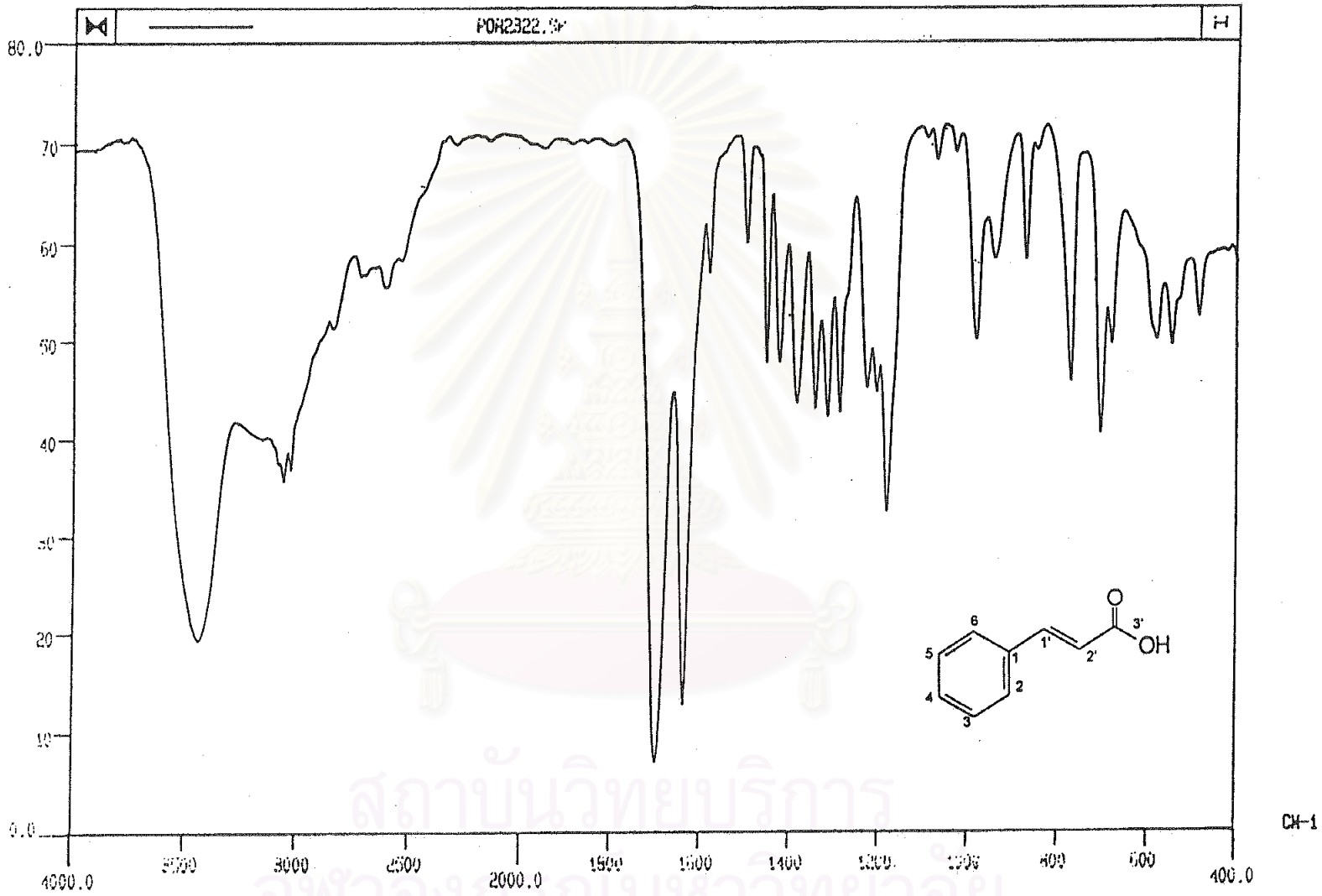


Figure 19. The IR spectrum of Por02322 (KBr disc)

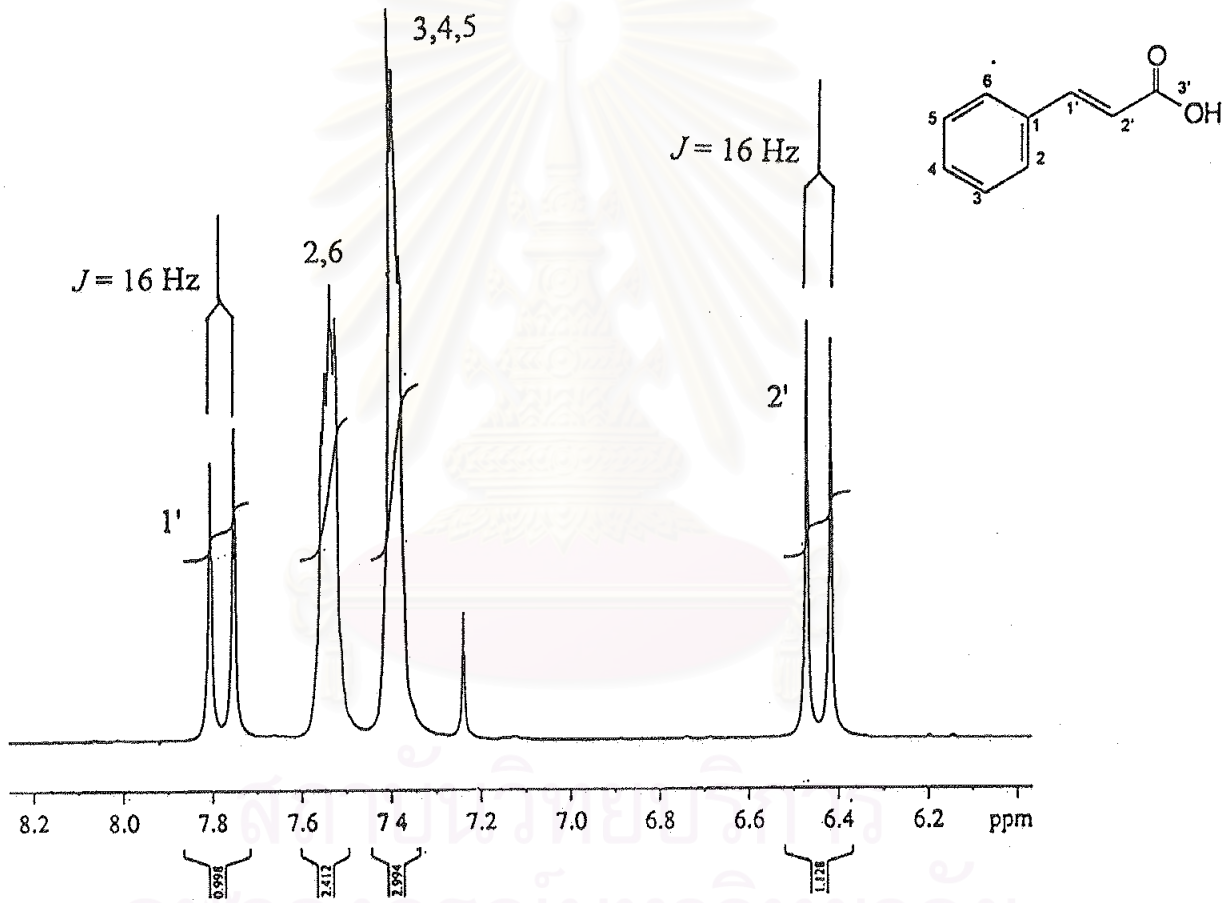


Figure 20. The 300 MHz ^{1}H NMR spectrum of Por02322 (in $CDCl_3$)

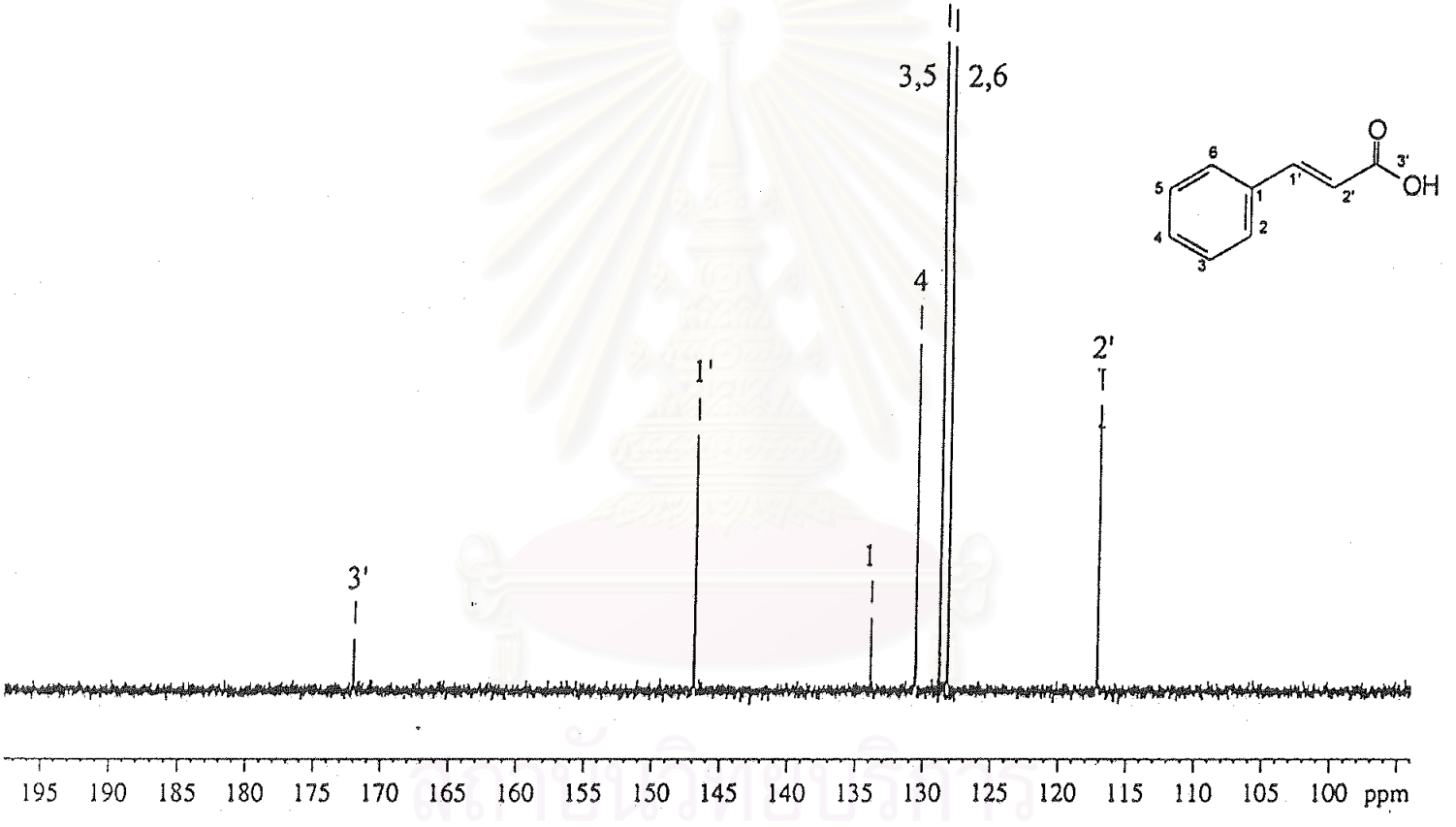


Figure 21. The 75 MHz ^{13}C NMR spectrum of Por02322 (in $CDCl_3$)

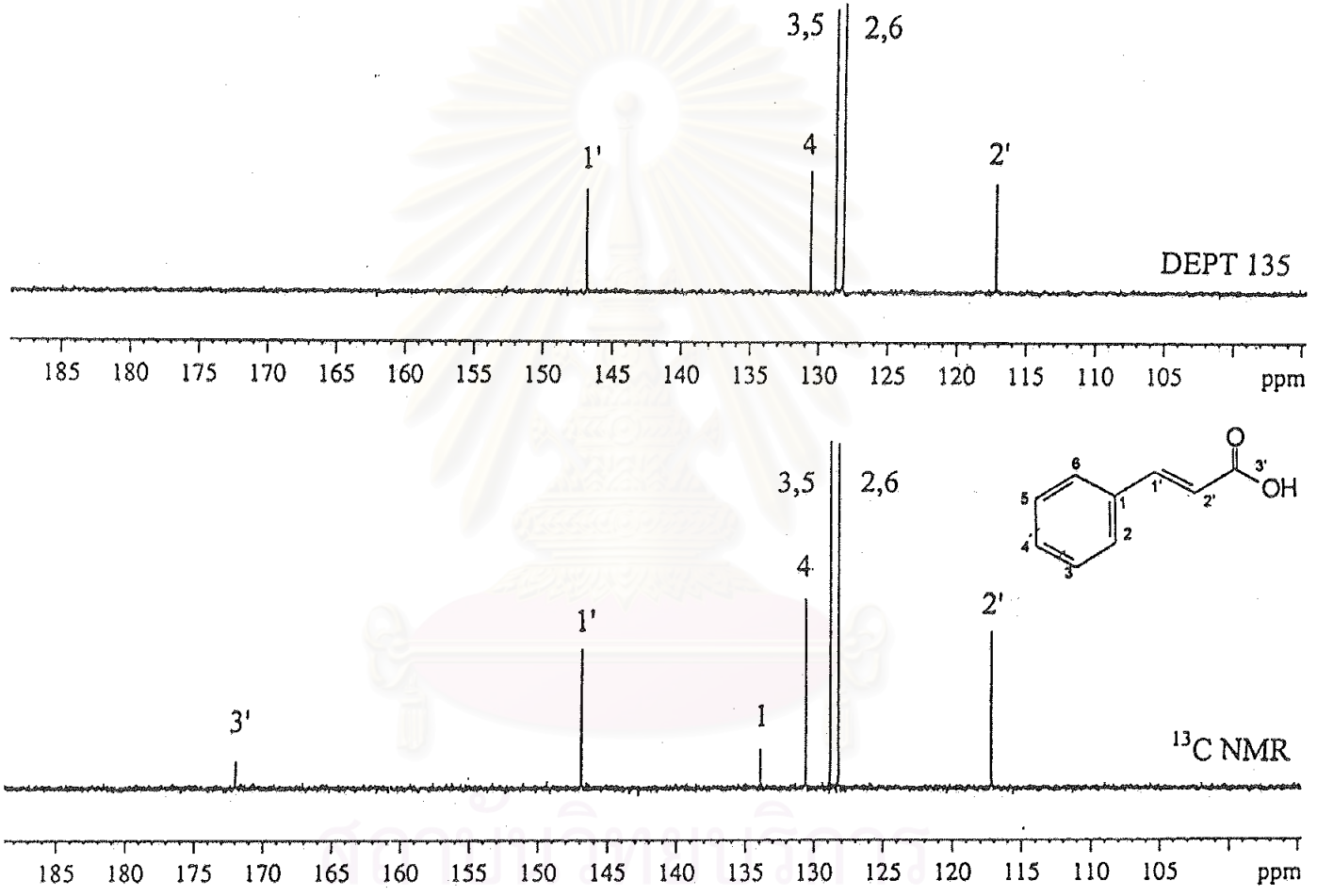


Figure 22. The 75 MHz ^{13}C NMR and DEPT 135 spectra of Por02322 (in $CDCl_3$)

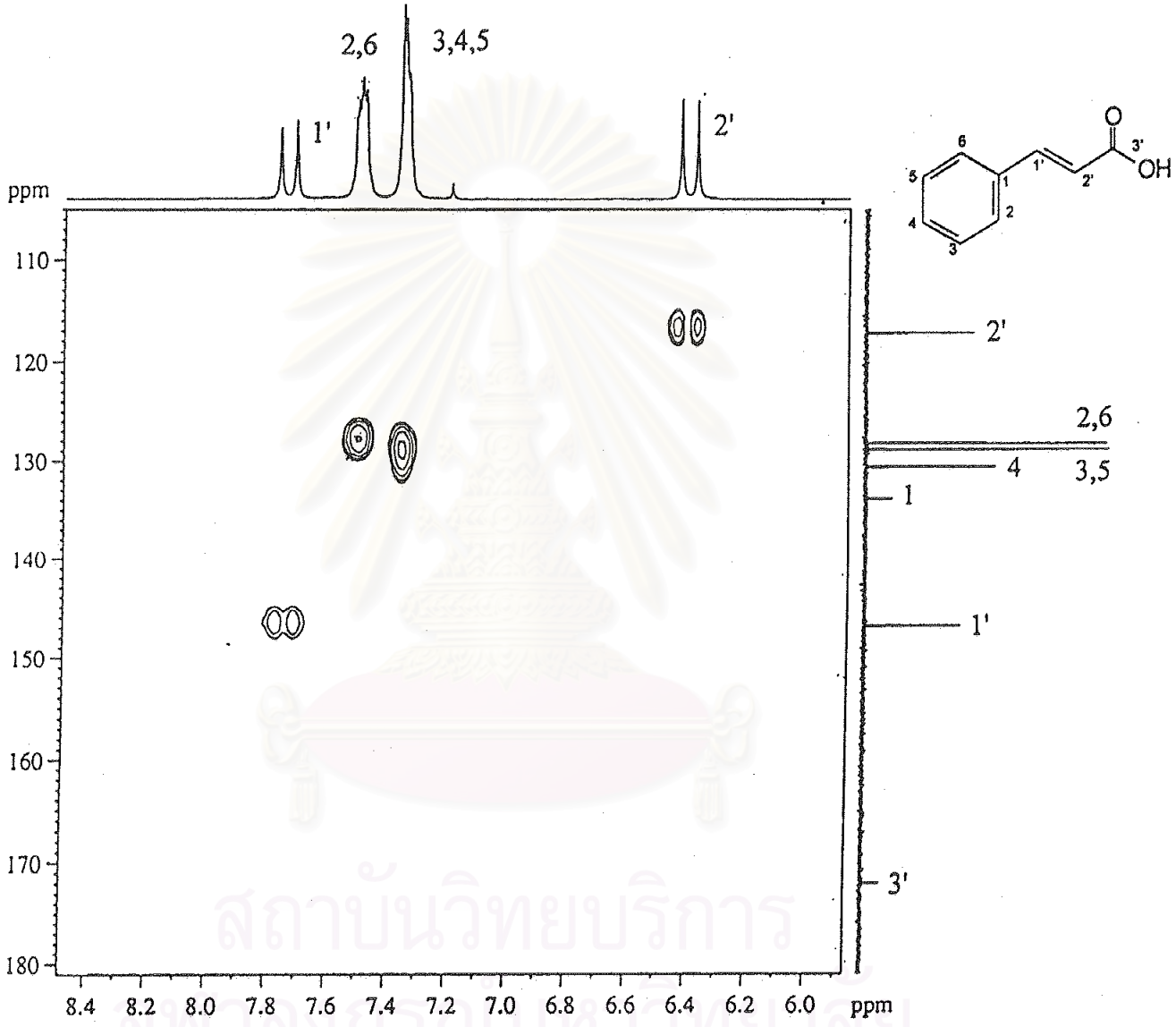


Figure 23. The 300 MHz HMQC spectrum of Por02322 (in $CDCl_3$)

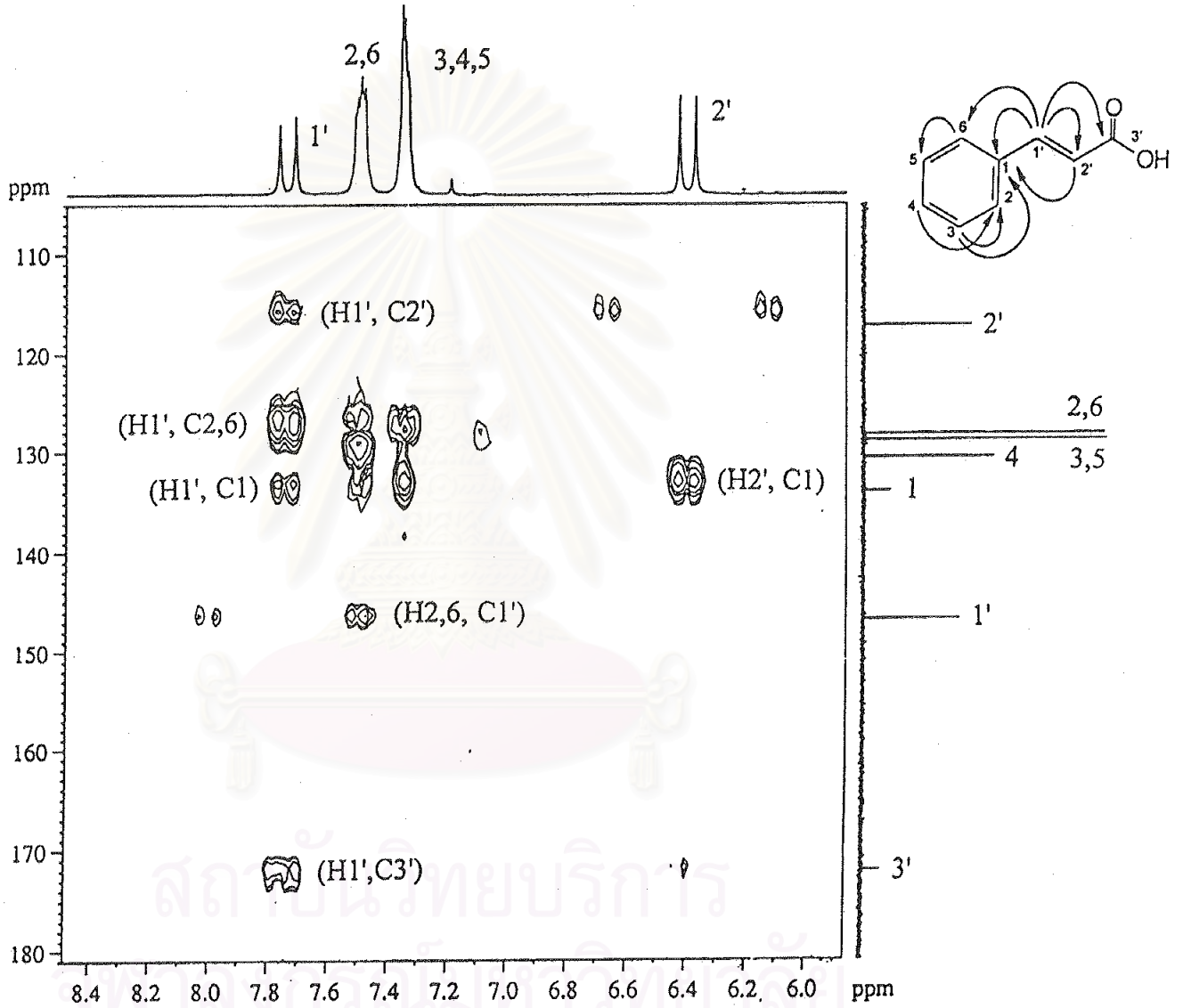


Figure 24. The 300 MHz HMBC ($^{n}J_{CH}$ = 8 Hz) of Por02322 (in $CDCl_3$)

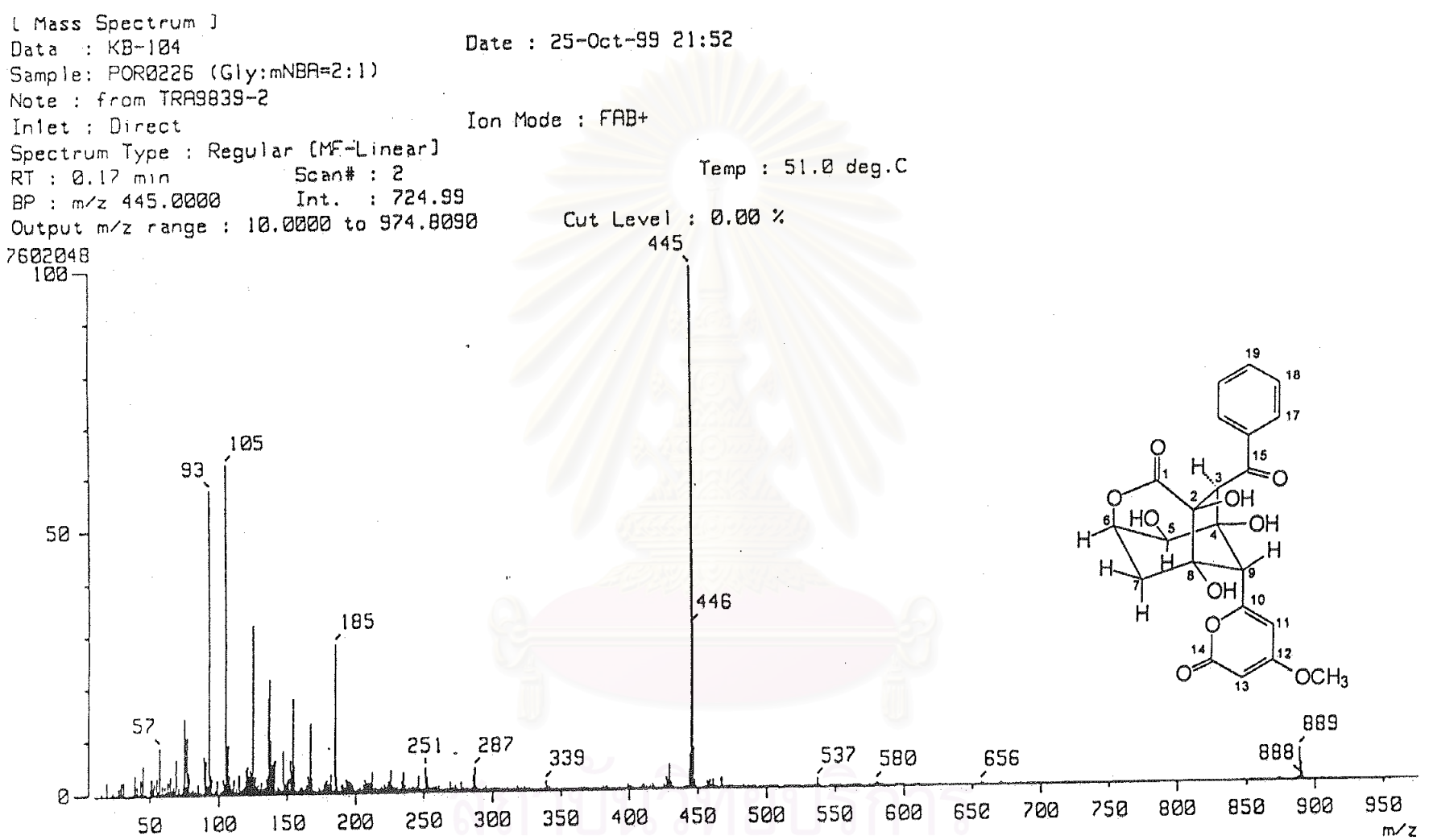


Figure 25. The FABMS spectrum of Por0226

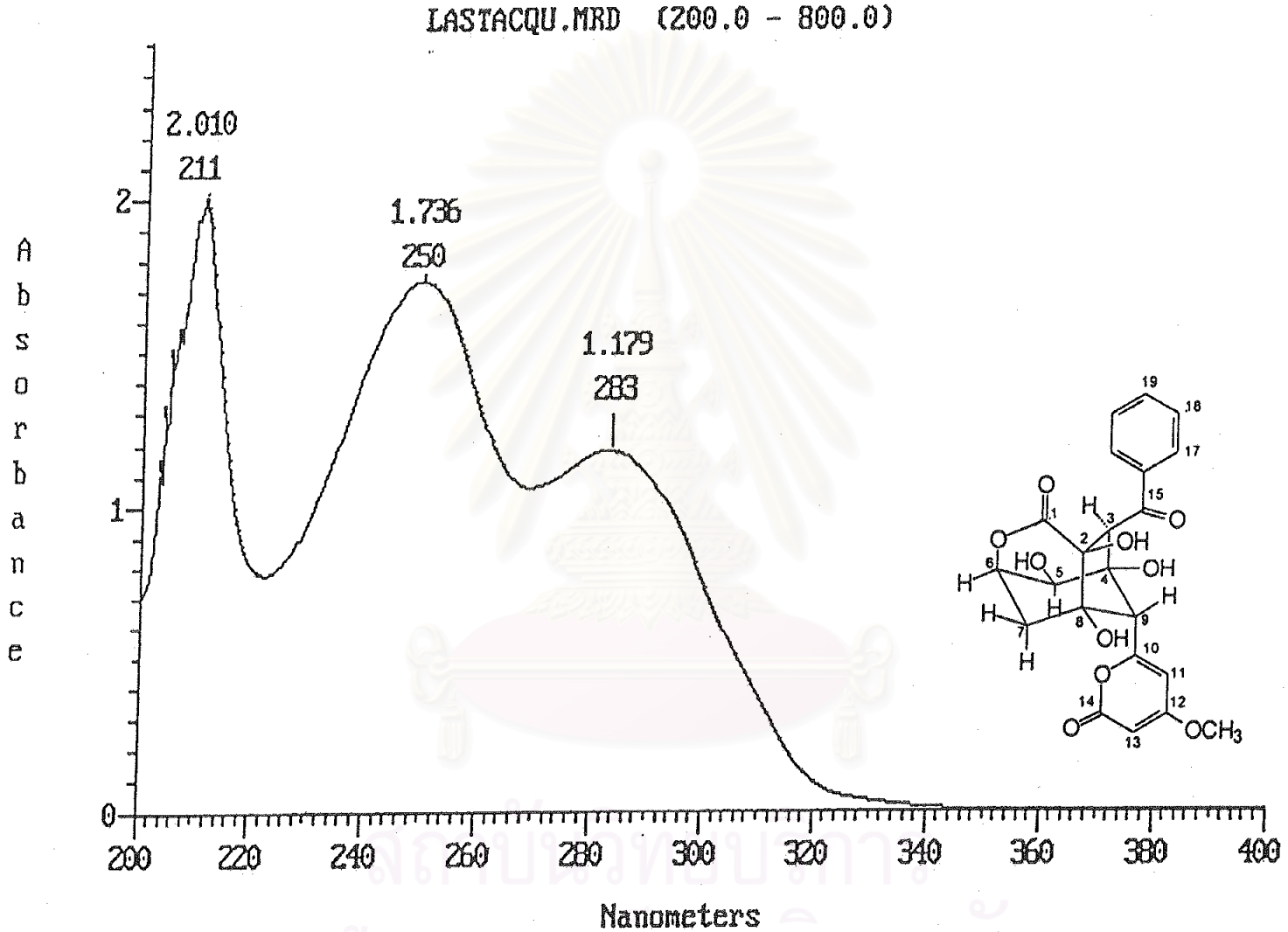


Figure 26. The UV spectrum of Por0226 (in MeOH)

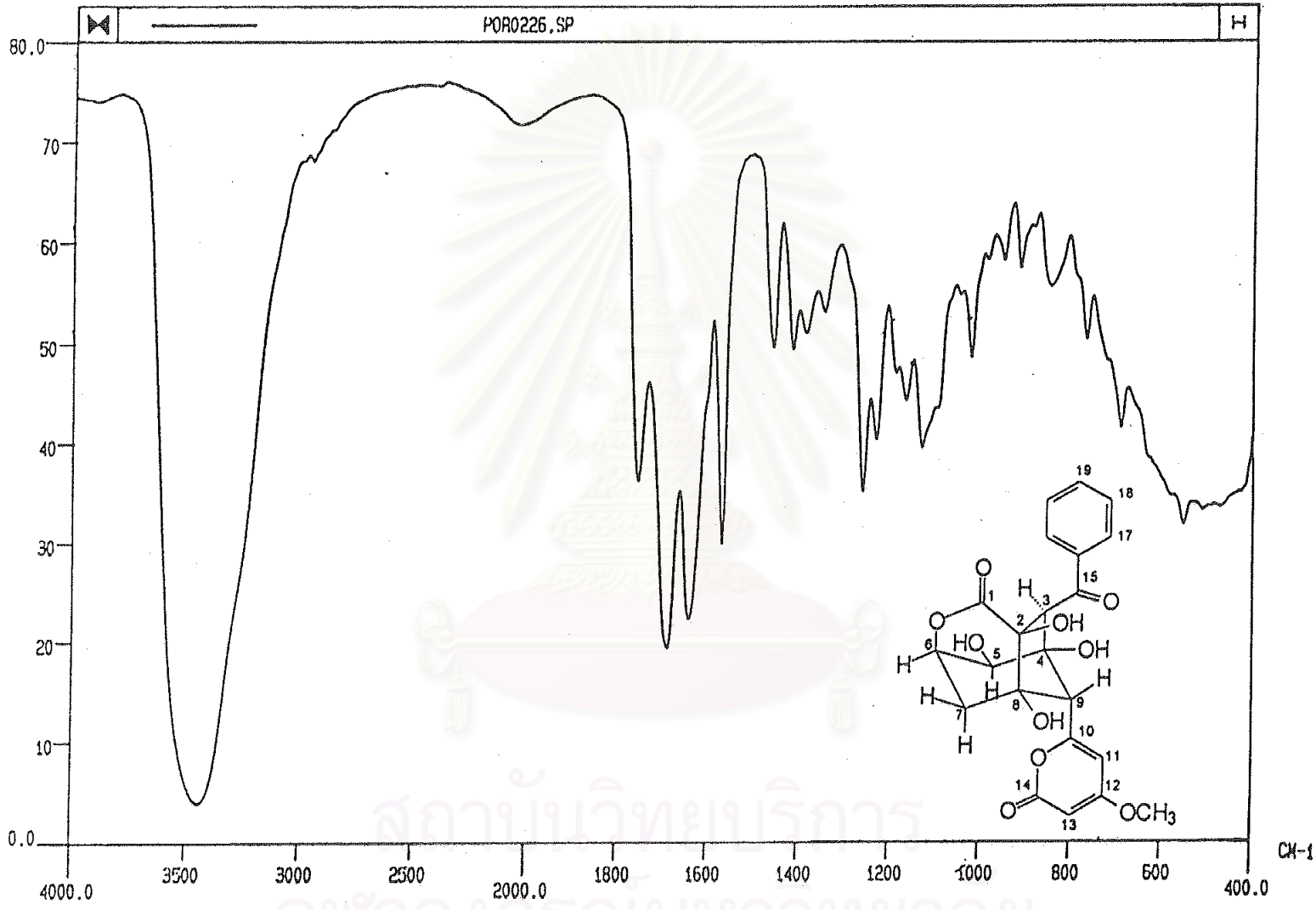


Figure 27. The IR spectrum of Por0226 (KBr disc)

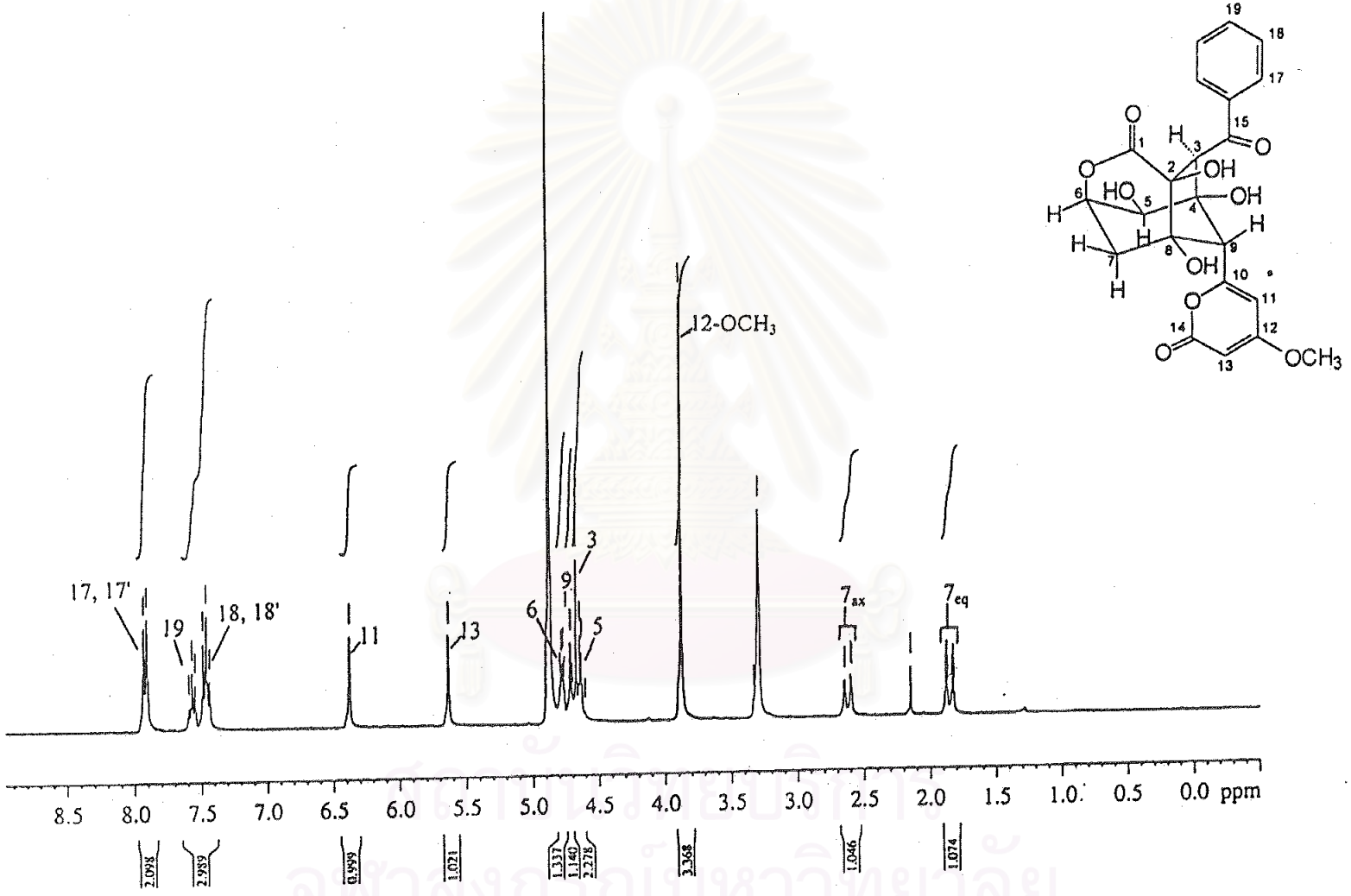


Figure 28a. The 300 MHz ^{1}H NMR spectrum of Por0226 (in CD_3OD)

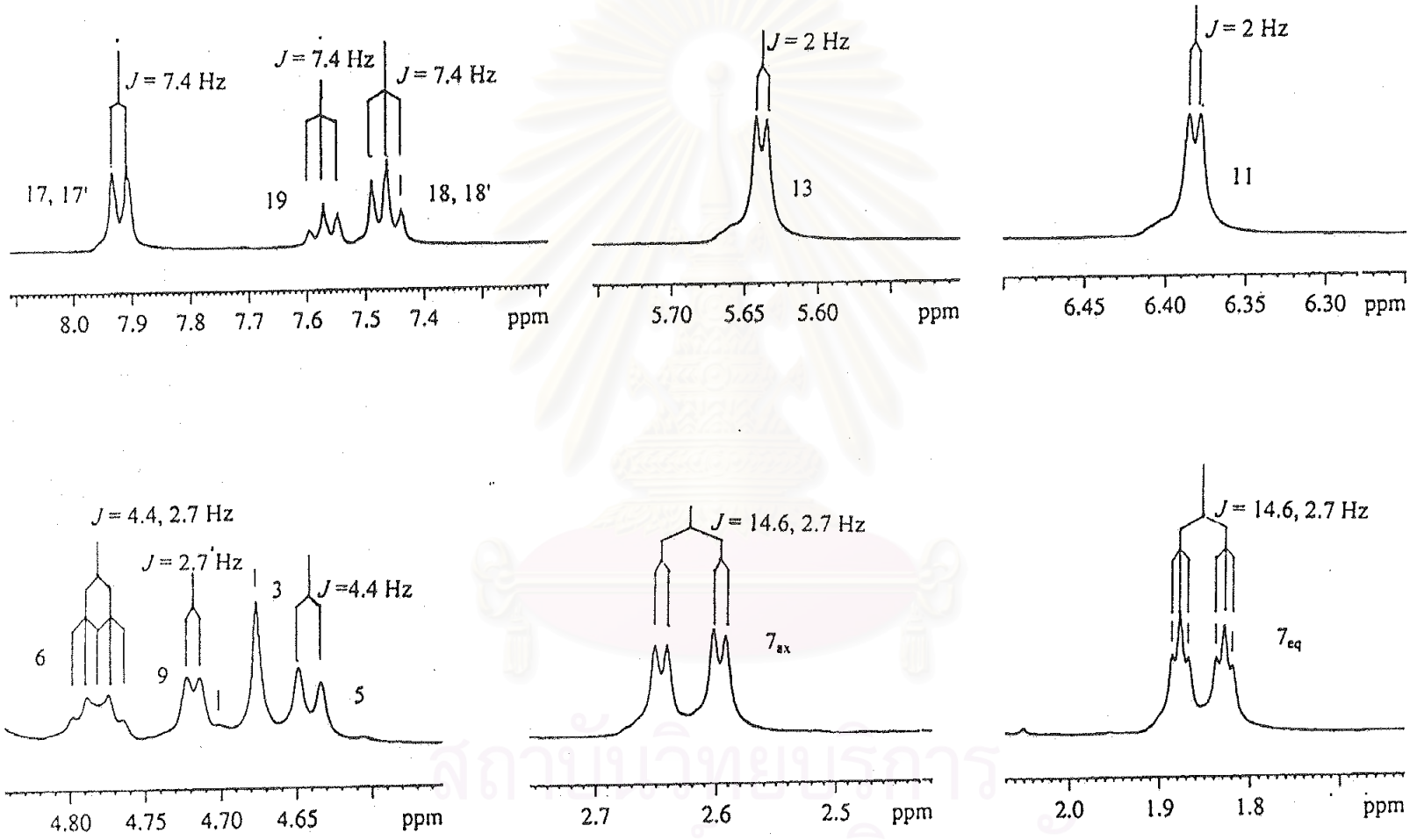


Figure 28b. The 300 MHz ^{1}H NMR expanded spectrum of Por0226 (in CD_3OD)

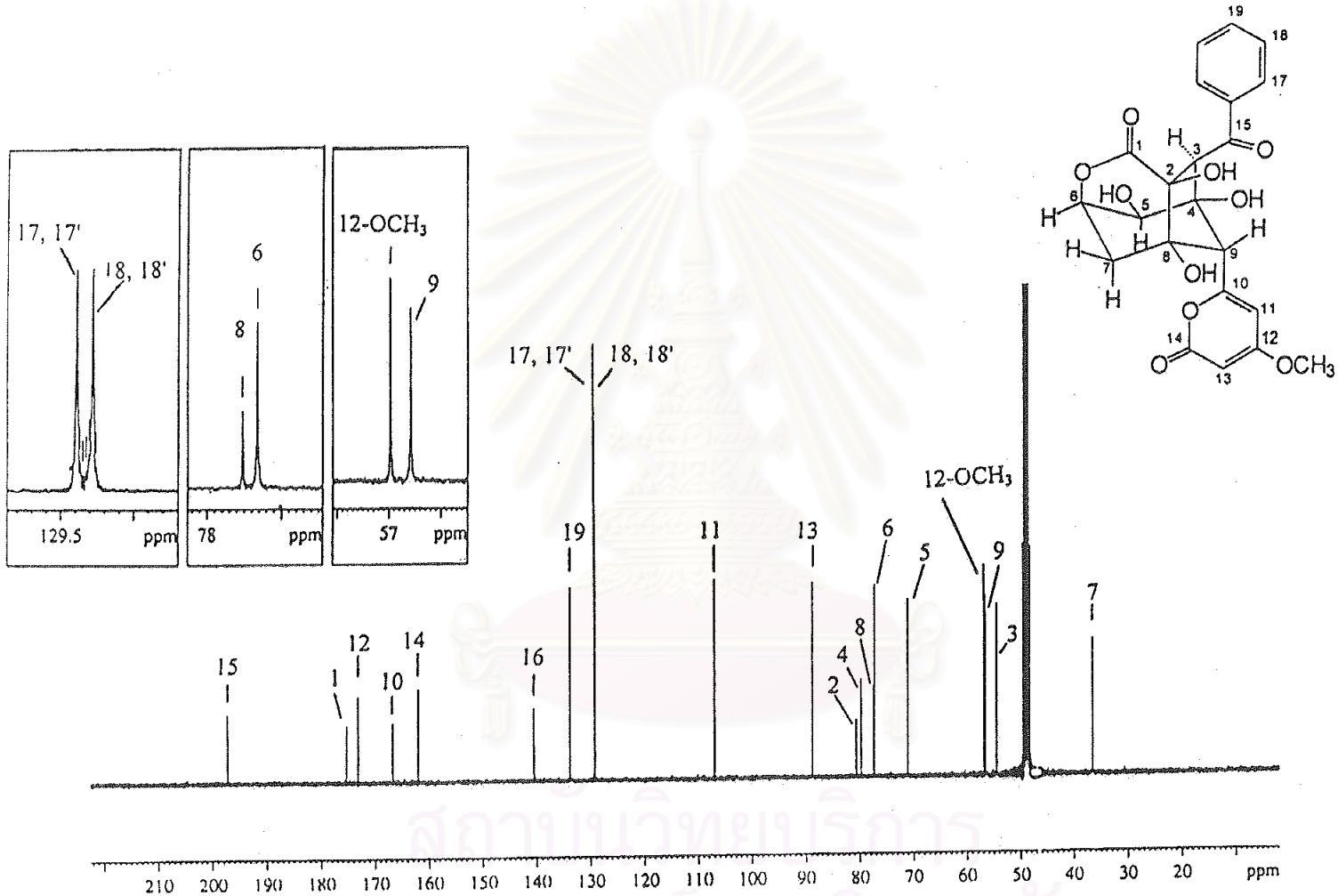


Figure 29. The 75 MHz ^{13}C NMR spectrum of Por0226 (in CD_3OD)

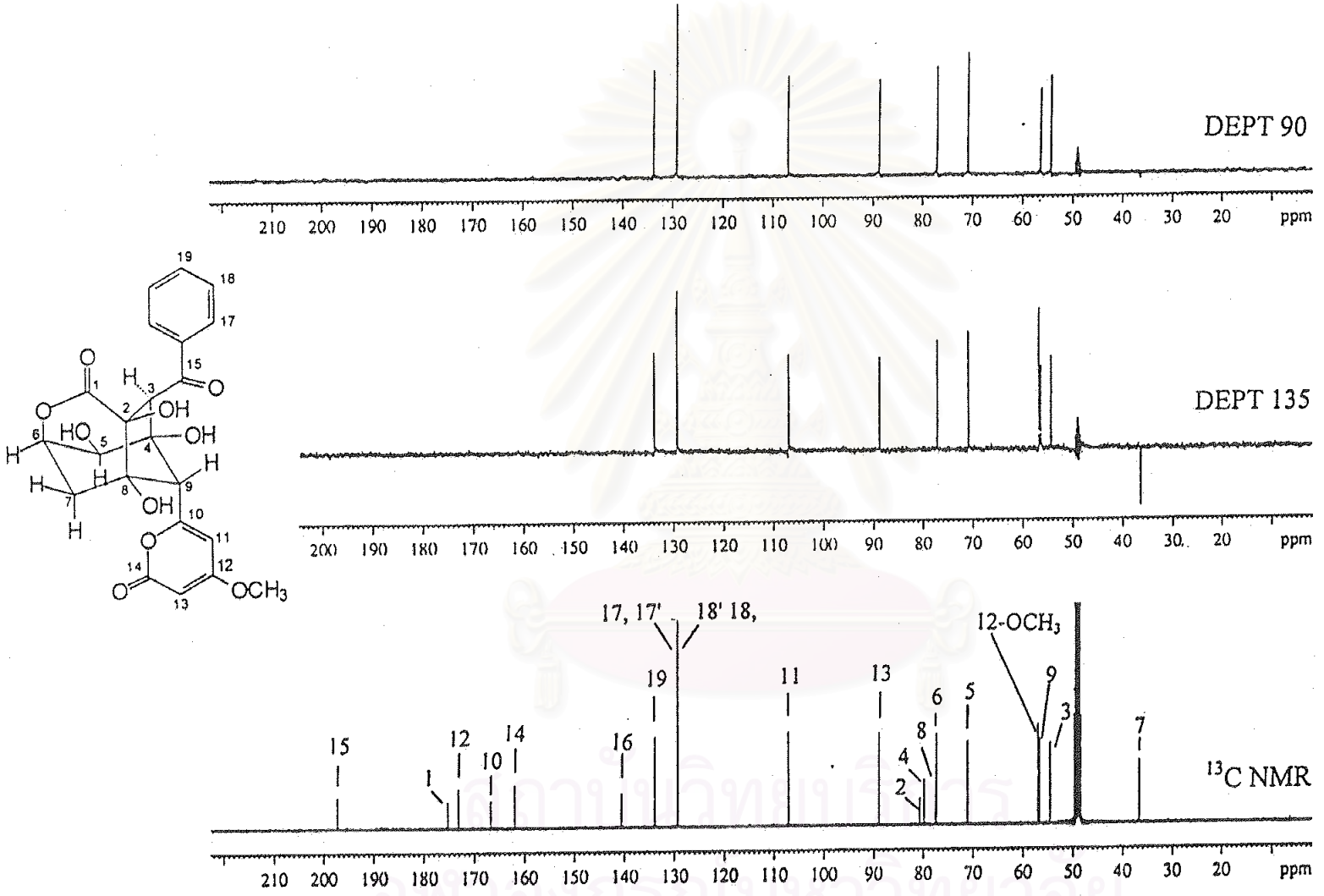


Figure 30. The 75 MHz ^{13}C NMR, DEPT 135 and DEPT 90 spectra of Por0226 (in CD_3OD)

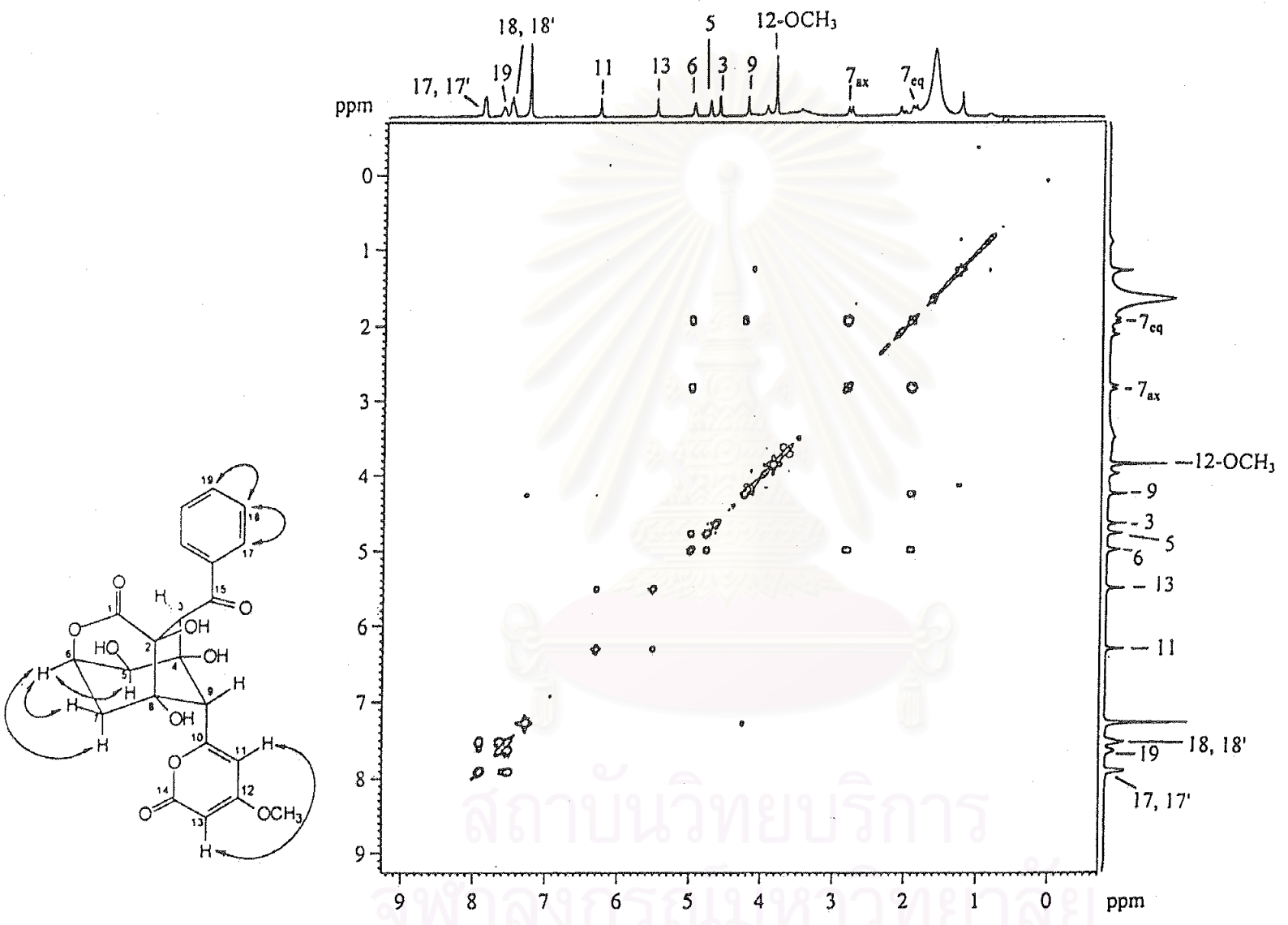


Figure 31. The 300 MHz ^{1}H, ^{1}H COSY spectrum of Por0226 (in $CDCl_3$)

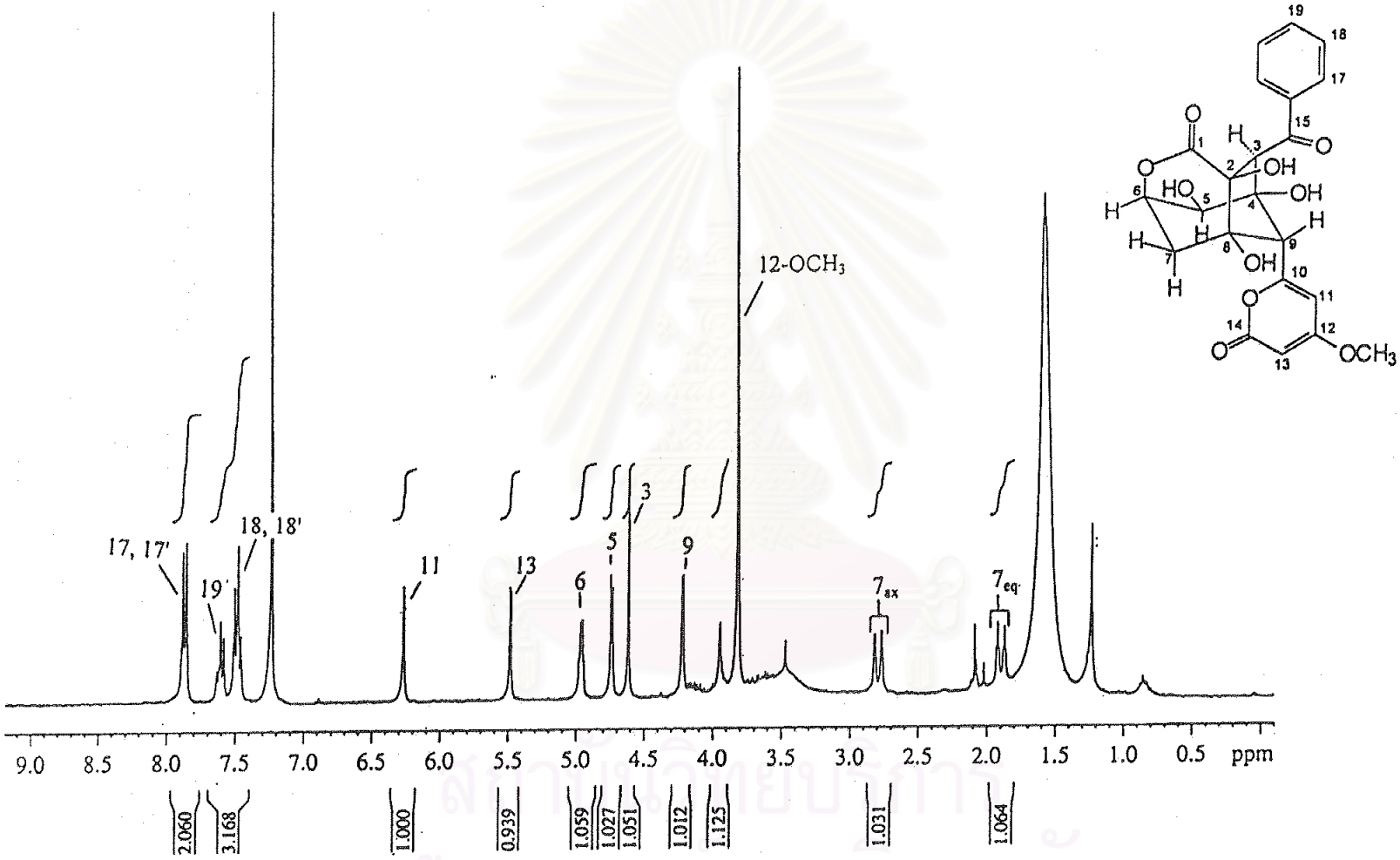
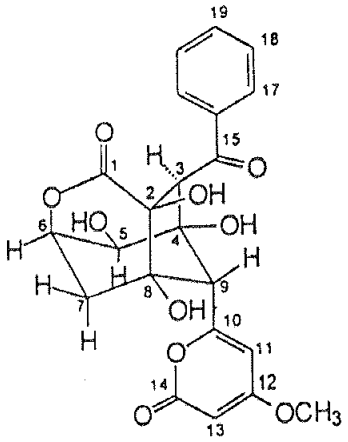


Figure 32. The 300 MHz ^{1}H NMR spectrum of Por0226 (in $CDCl_3$)



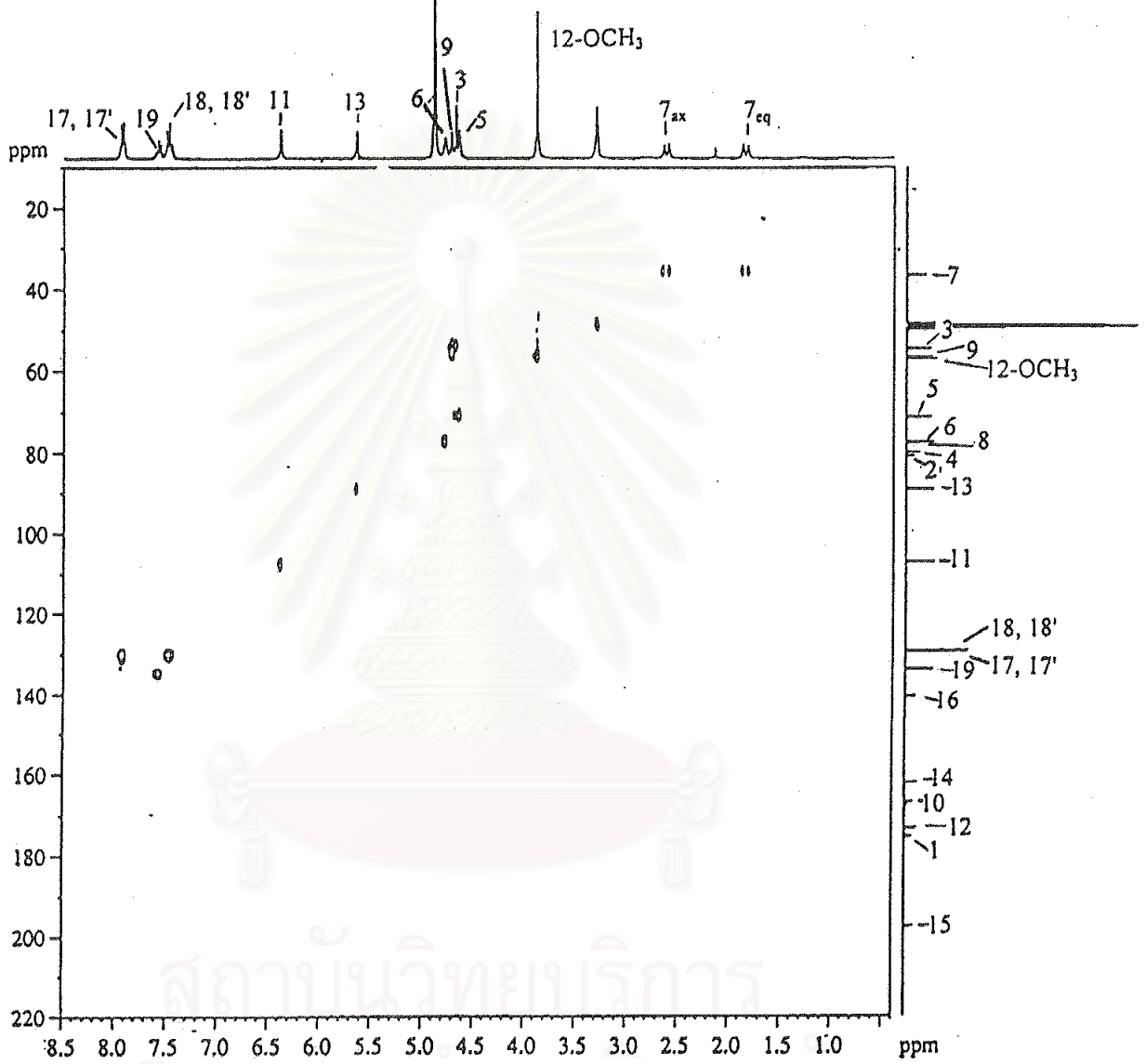


Figure 33a. The 300 MHz HMQC spectrum of Por0226 (in CD_3OD)

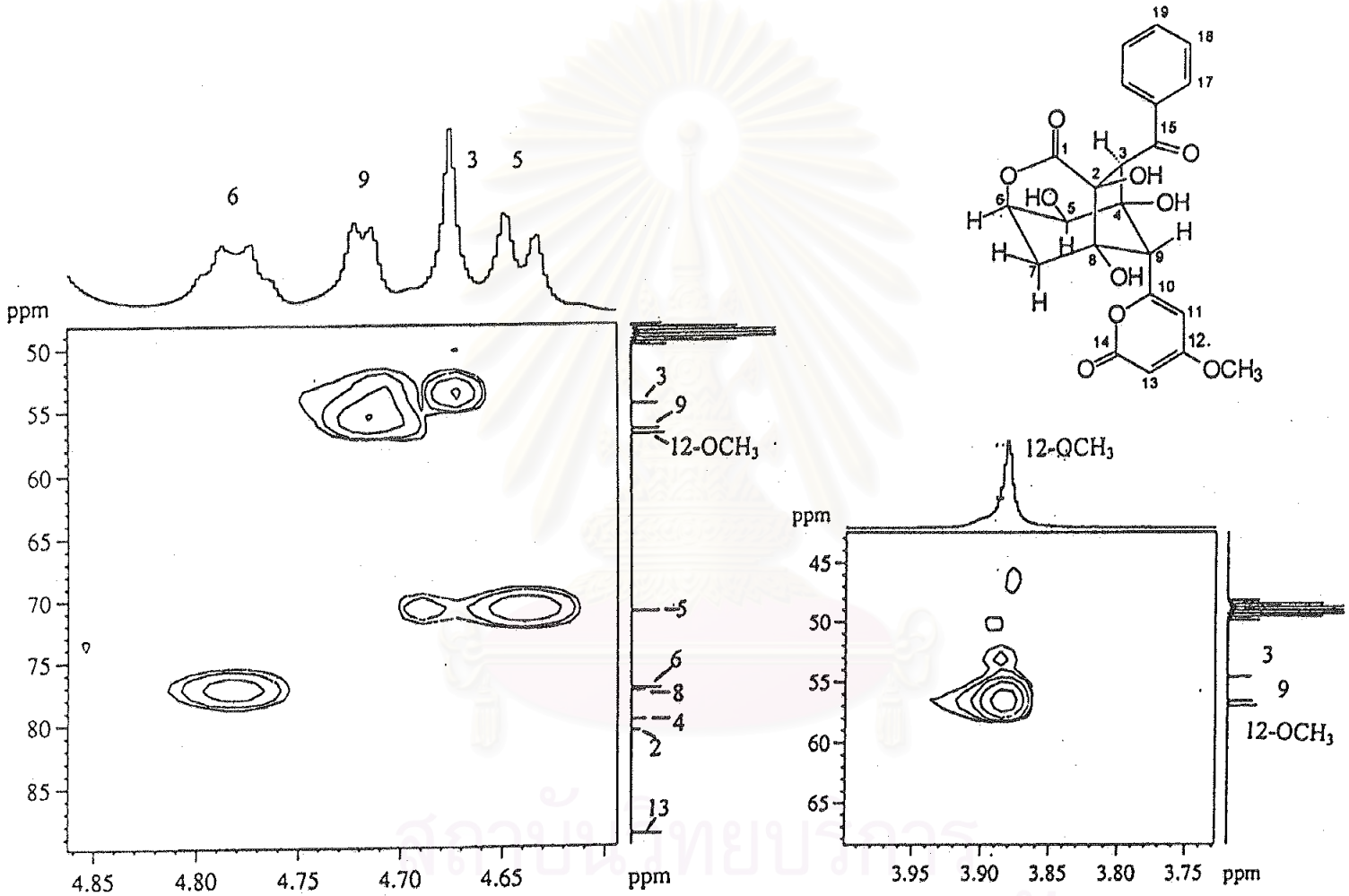


Figure 33b. The expanded 300 MHz HMQC spectrum of Por0226 (in CD_3OD)

(δ_H 4.60-4.86, δ_C 49-89 ppm and δ_H 3.73-4.0, δ_C 43-68 ppm)

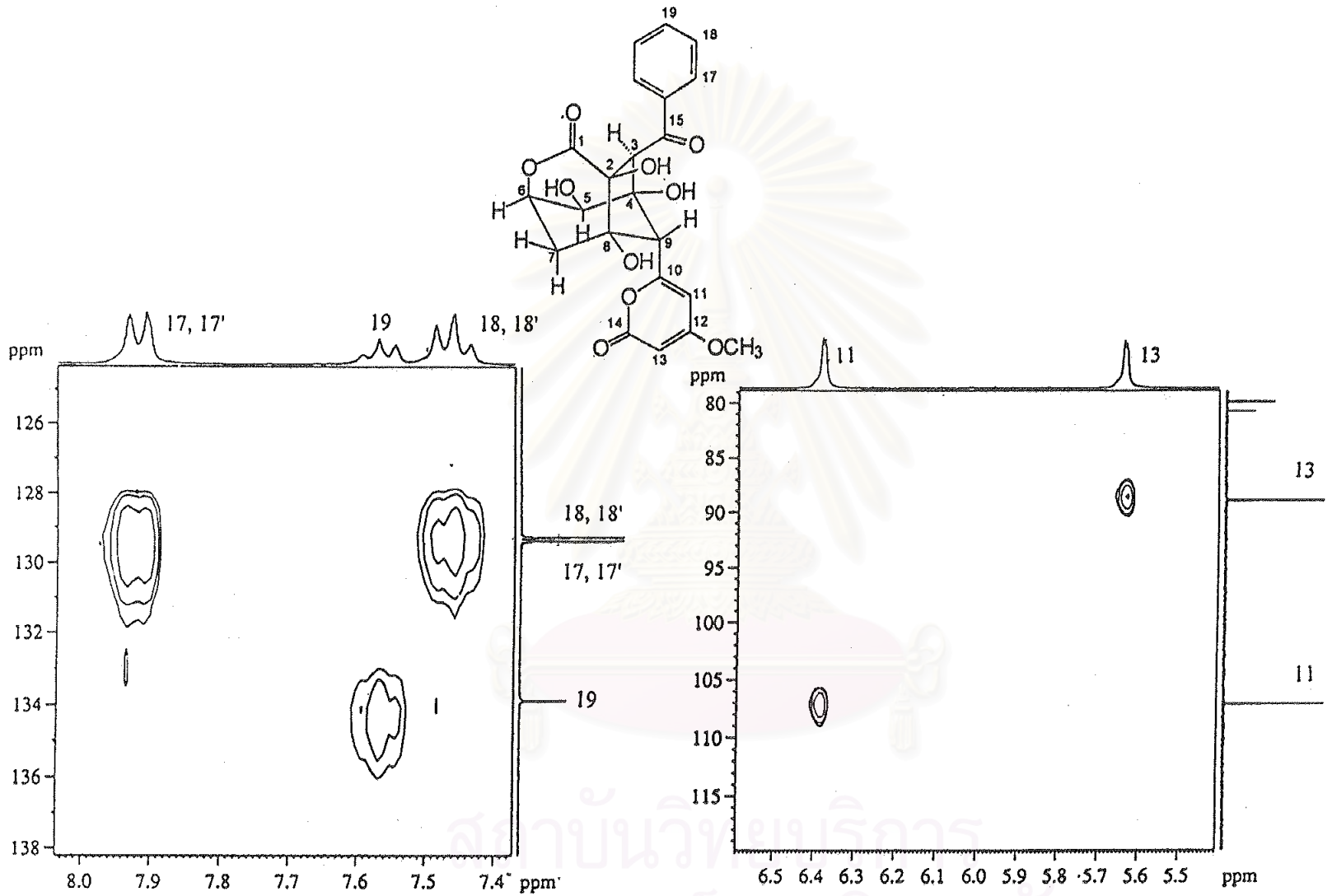


Figure 33c. The expanded 300 MHz HMQC spectrum of Por0226 (in CD_3OD)

(δ_H 7.38-8.03, δ_C 127-138 ppm and δ_H 5.4-6.6, δ_C 79-119 ppm)

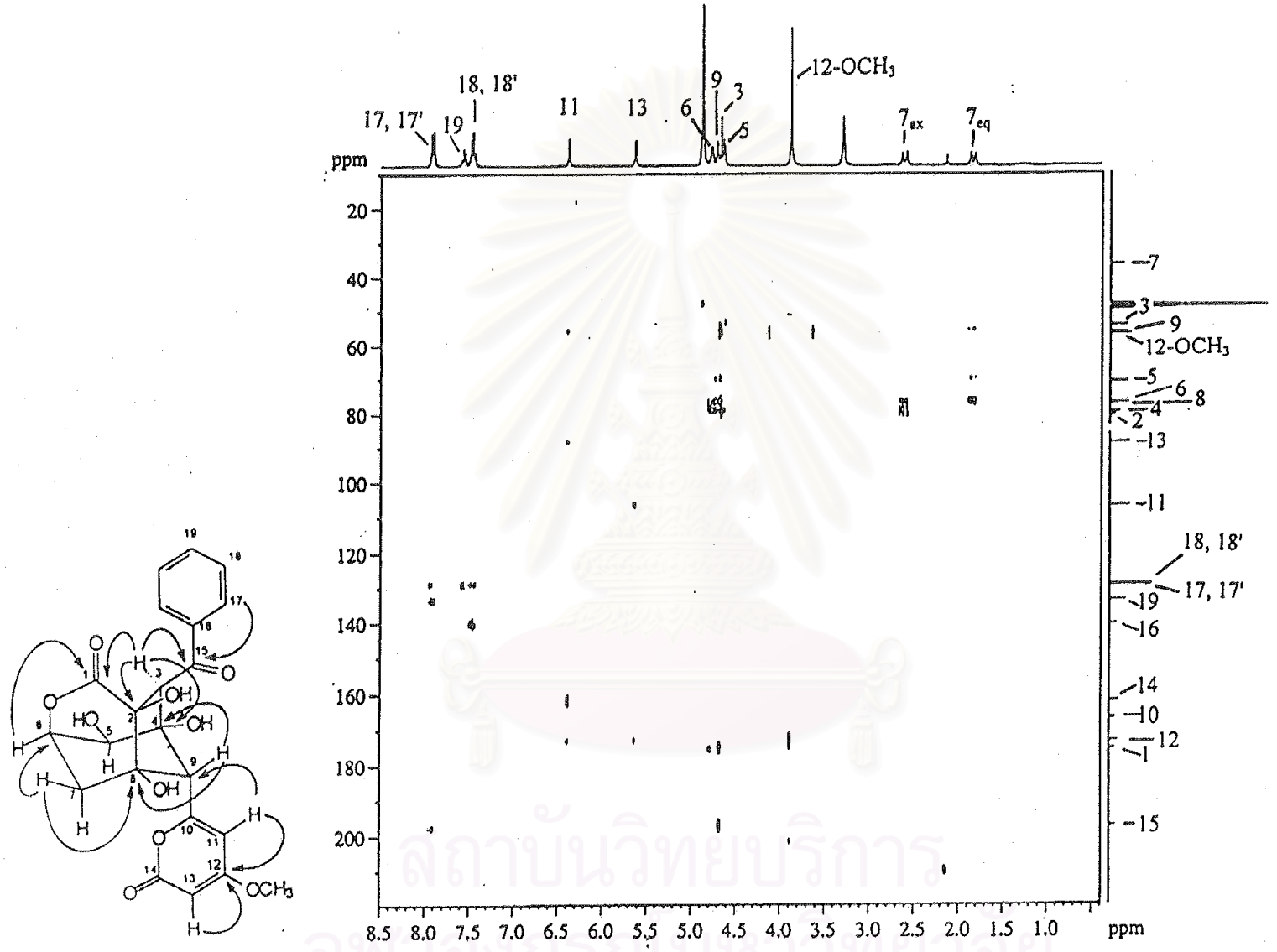


Figure 34a. The 300 MHz HMBC ($^{n}J_{CH}$ = 8 Hz) spectrum of Por0226 (in CD_3OD)

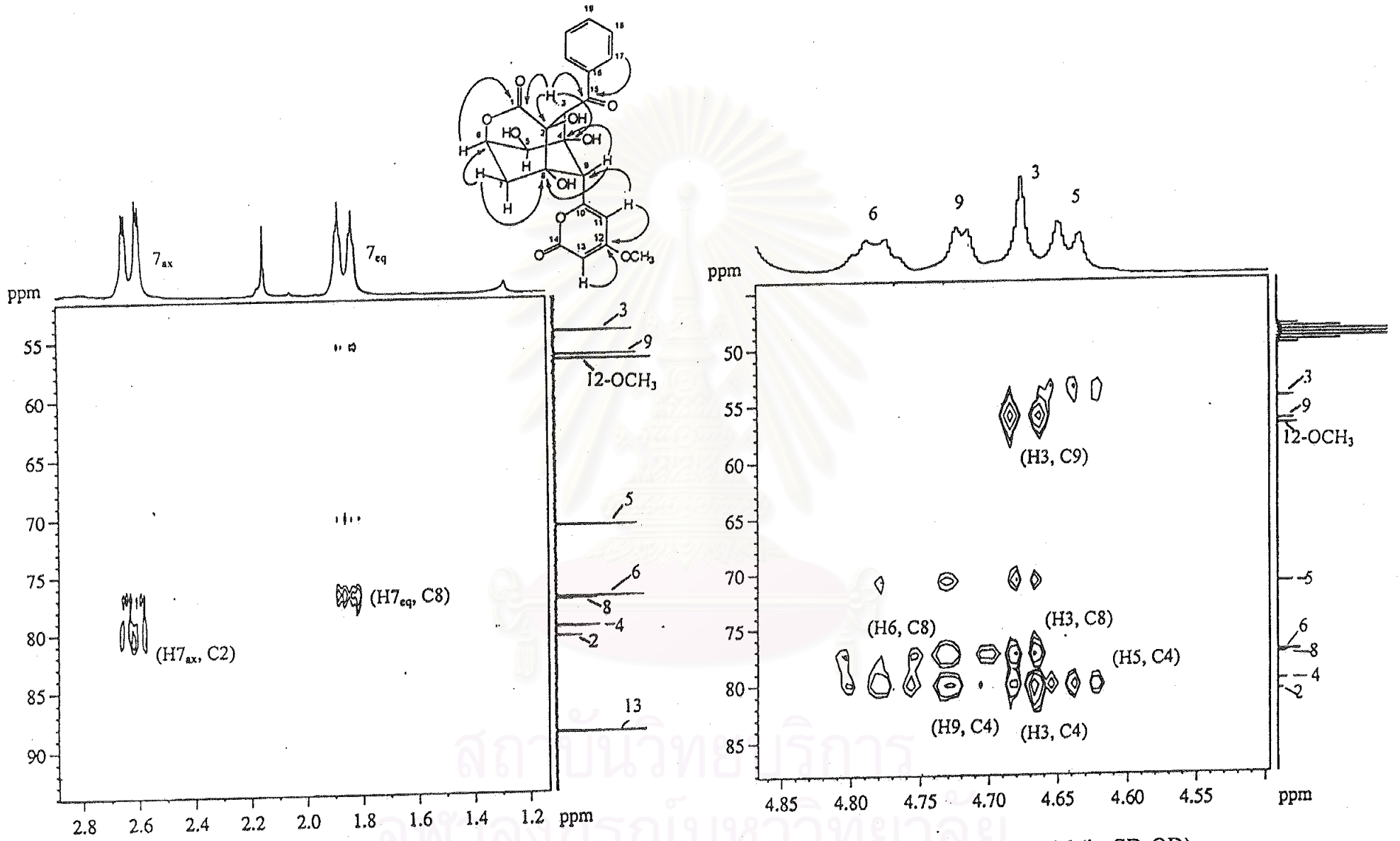


Figure 34b. The expanded 300 MHz HMBC ($^{n}J_{CH}$ = 8 Hz) spectrum of Por0226 (in CD_3OD)

(δ_H 1.1-2.9, δ_C 53-94 ppm and δ_H 4.50-4.87, δ_C 44-88 ppm)

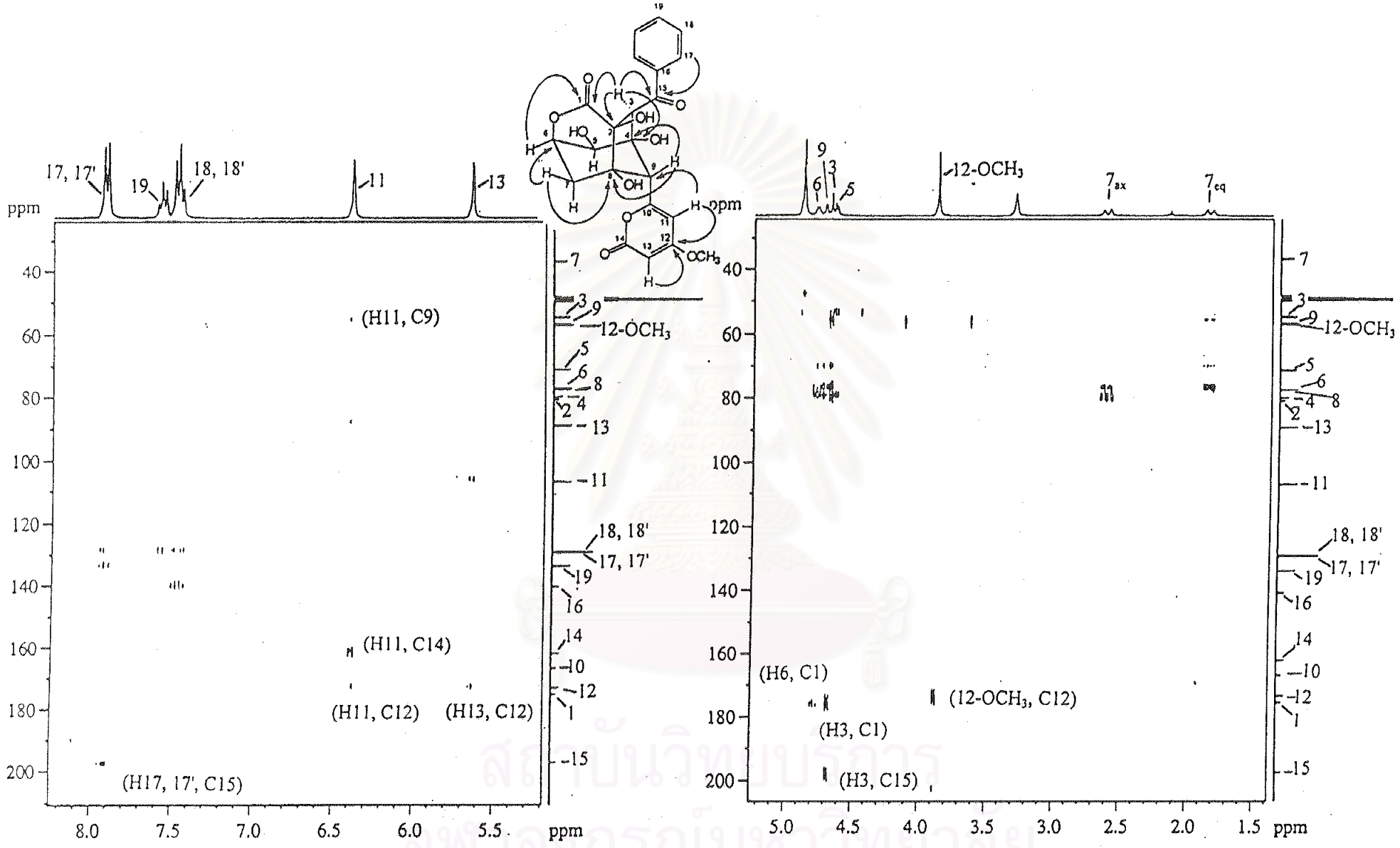


Figure 34c. The expanded 300 MHz HMBC ($^{n}J_{CH}$ = 8 Hz) spectrum of Por0226 (in CD_3OD)

(δ_H 5.2-8.2, δ_C 22-210 ppm and δ_H 1.4-5.2, δ_C 22-210 ppm)

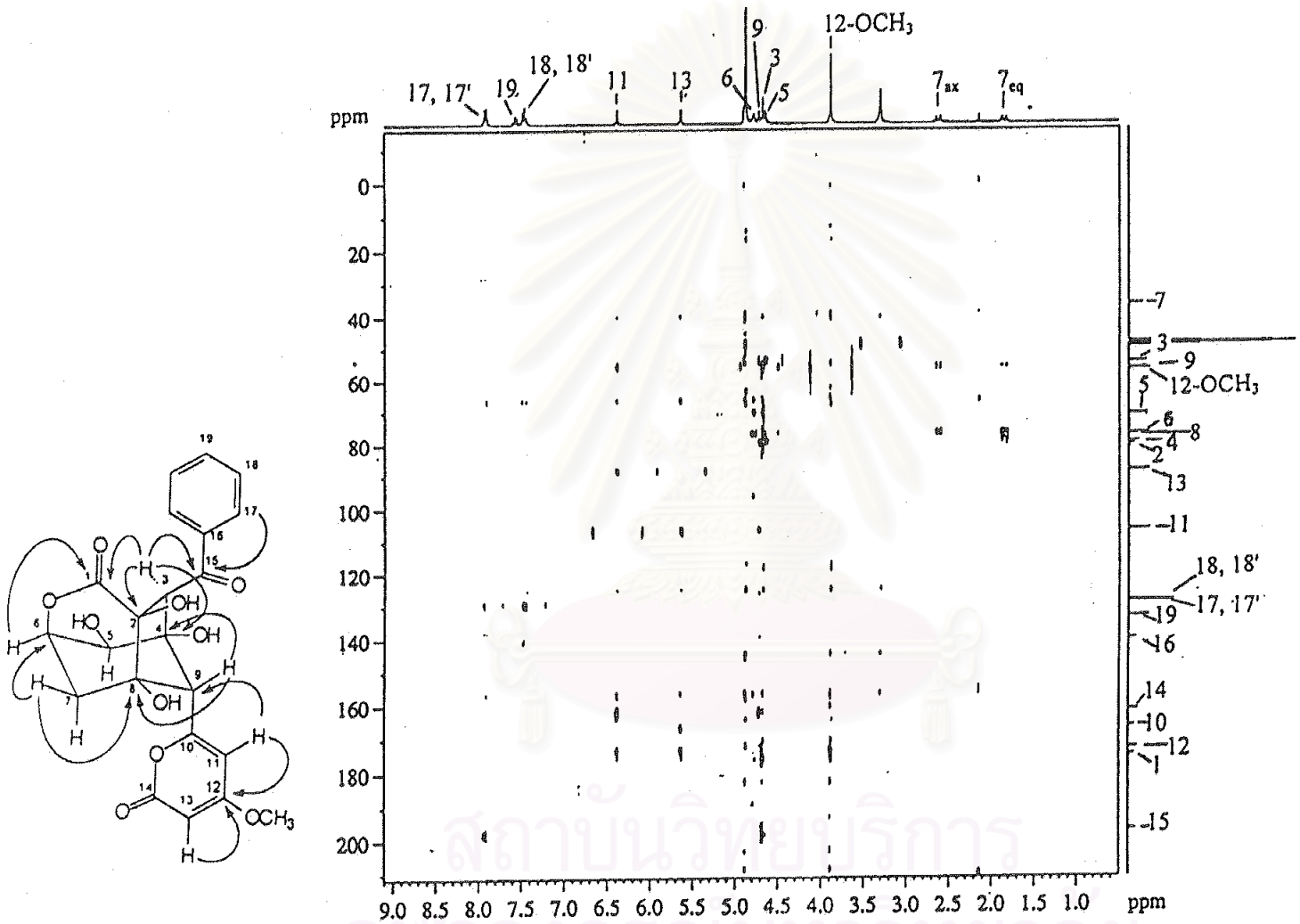


Figure 35a. The 300 MHz HMBC ($^{n}J_{CH}$ = 4 Hz) spectrum of Por0226 (in CD_3OD)

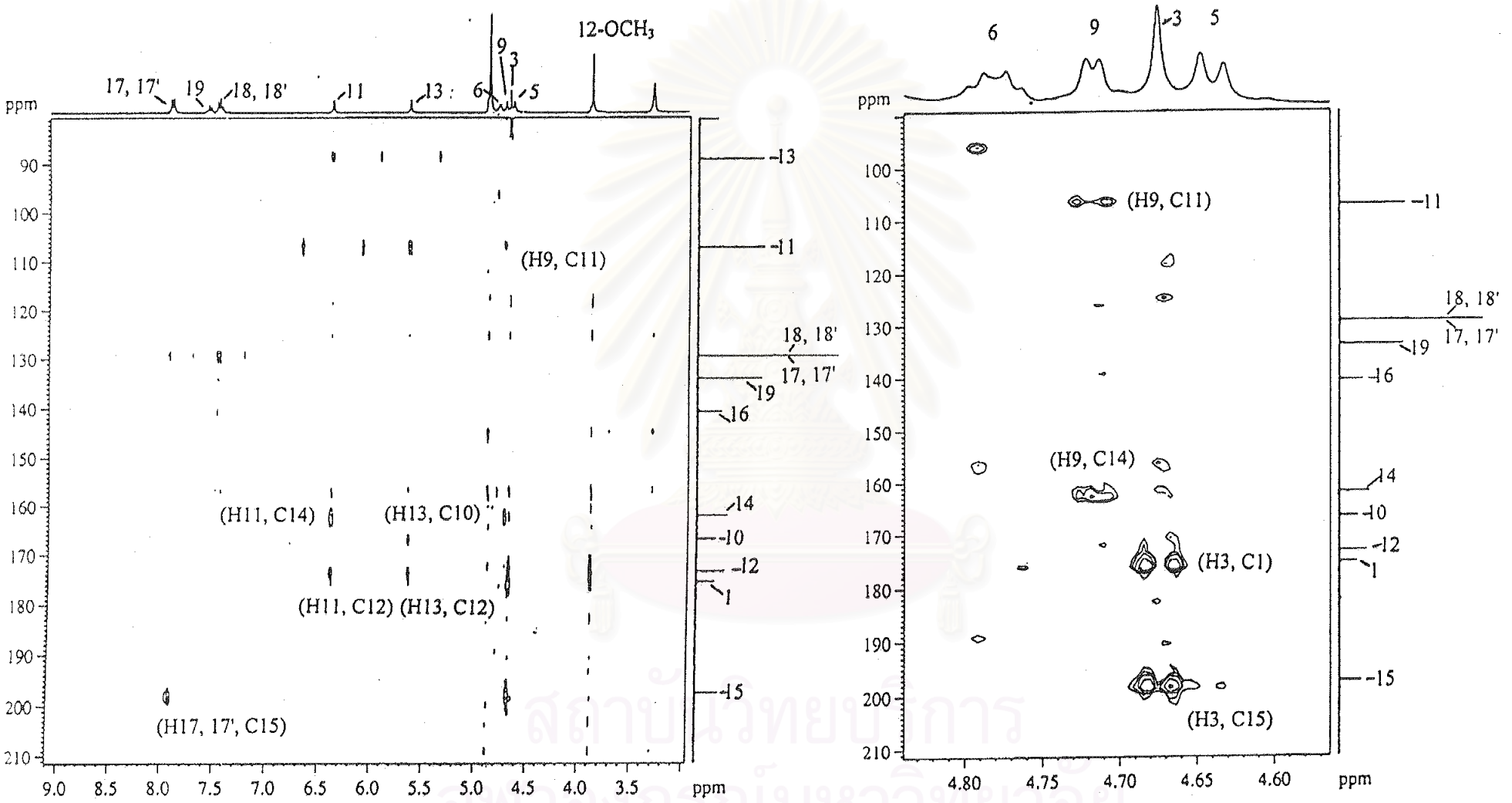


Figure 35b. The expanded 300 MHz HMBC ($^{n}J_{CH}$ = 4 Hz) spectrum of Por0226 (in CD_3OD)

(δ_H 3.0-9.1, δ_C 80-210 ppm and δ_H 4.57-4.84, δ_C 90-210 ppm)

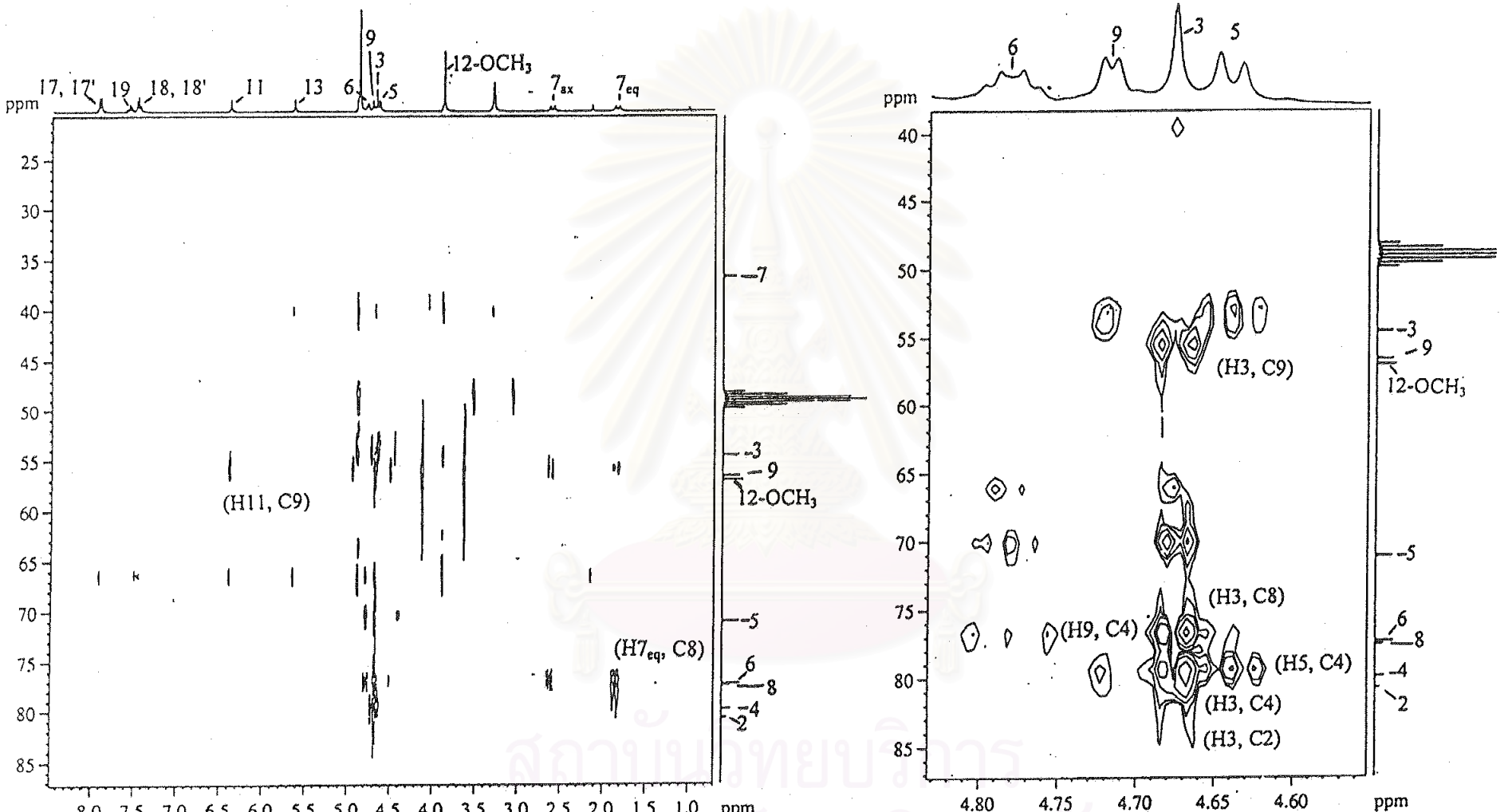


Figure 35c. The expanded 300 MHz HMBC ($^{n}J_{CH}$ = 4 Hz) spectrum of Por0226 (in CD_3OD)

(δ_H 0.8-8.4, δ_C 20-87 ppm and δ_H 4.55-4.83, δ_C 39-87 ppm)

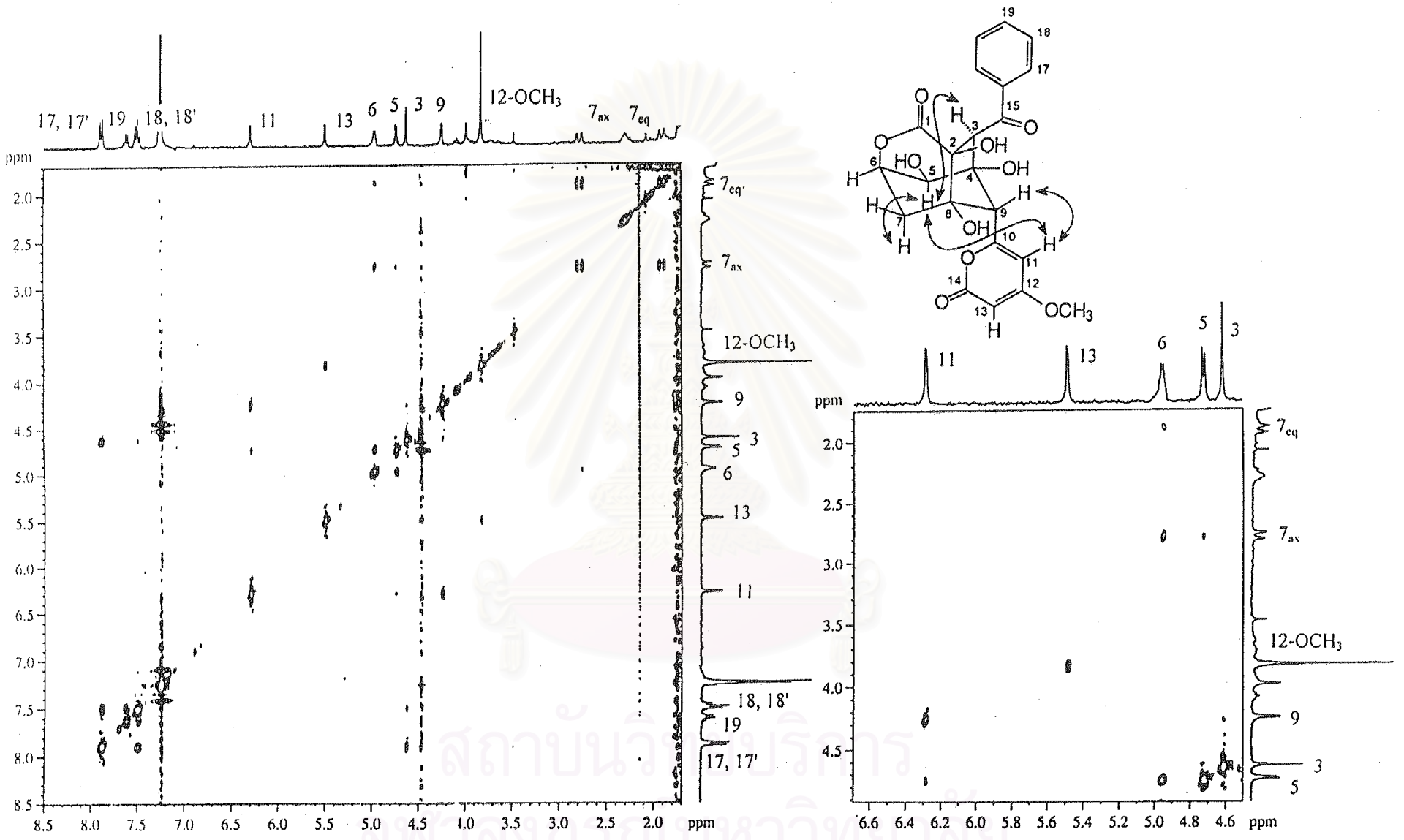


Figure 36. The 300 MHz NOESY spectrum of Por0226 (in $CDCl_3$)

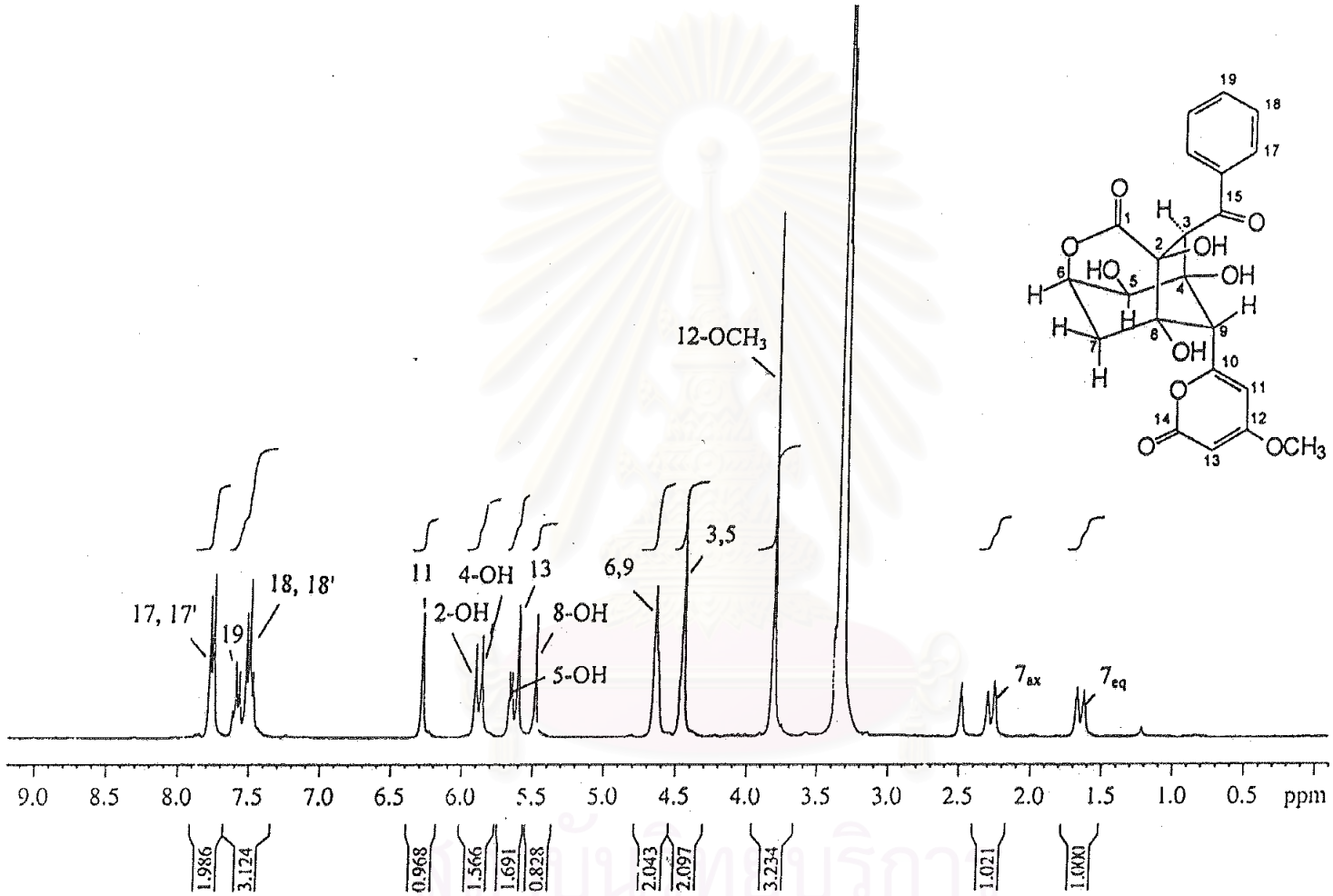


Figure 37. The 300 MHz ^{1}H NMR spectrum of Por0226 (in DMSO-d_6)

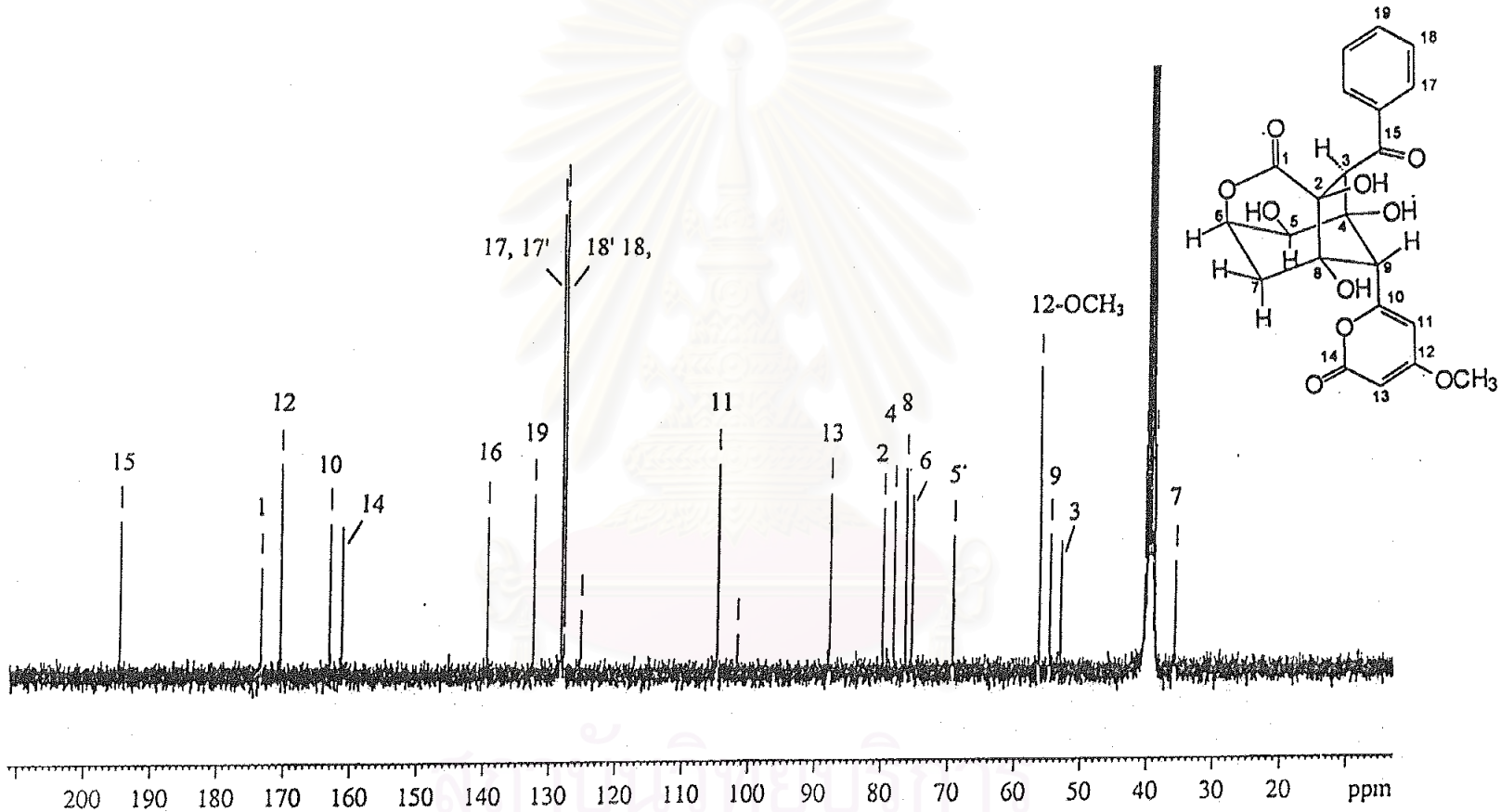


Figure 38. The 75 MHz ^{13}C NMR spectrum of Por0226 (in DMSO-d_6)

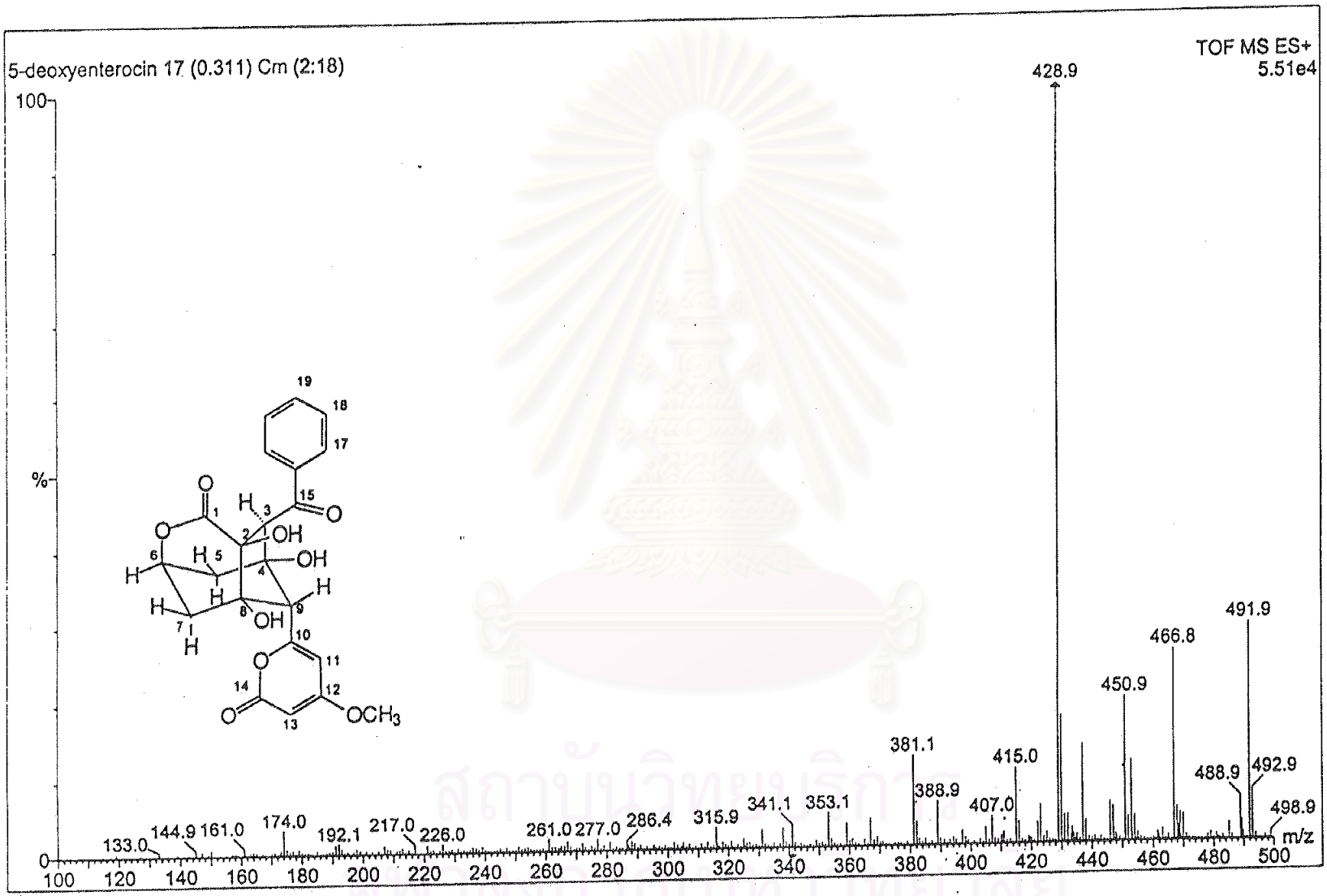


Figure 39. The ESI-TOF MS spectrum of Por0225223

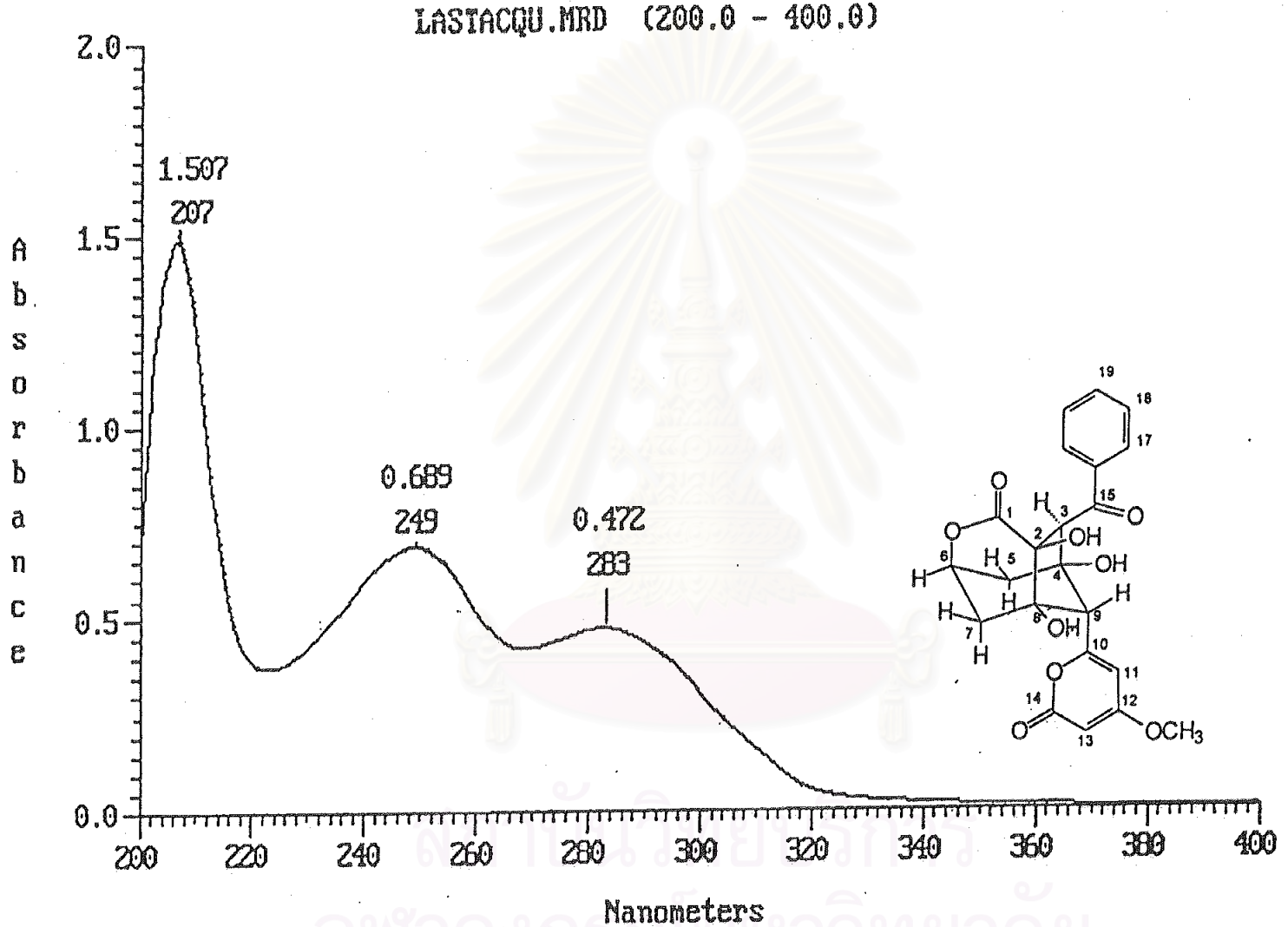


Figure 40. The UV spectrum of Por0225223 (in MeOH)

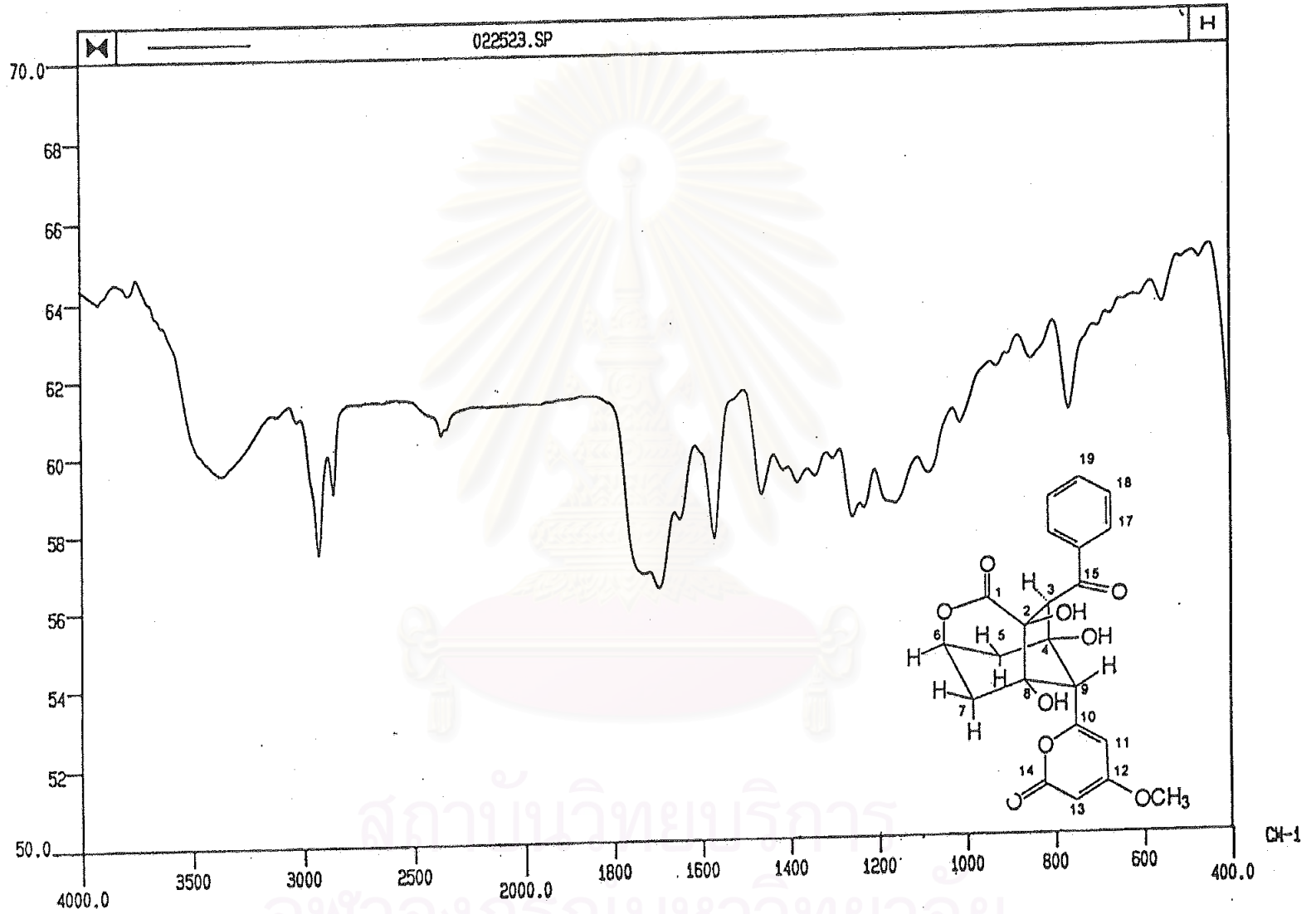


Figure 41. The IR spectrum of Por0225223 (NaCl cell)

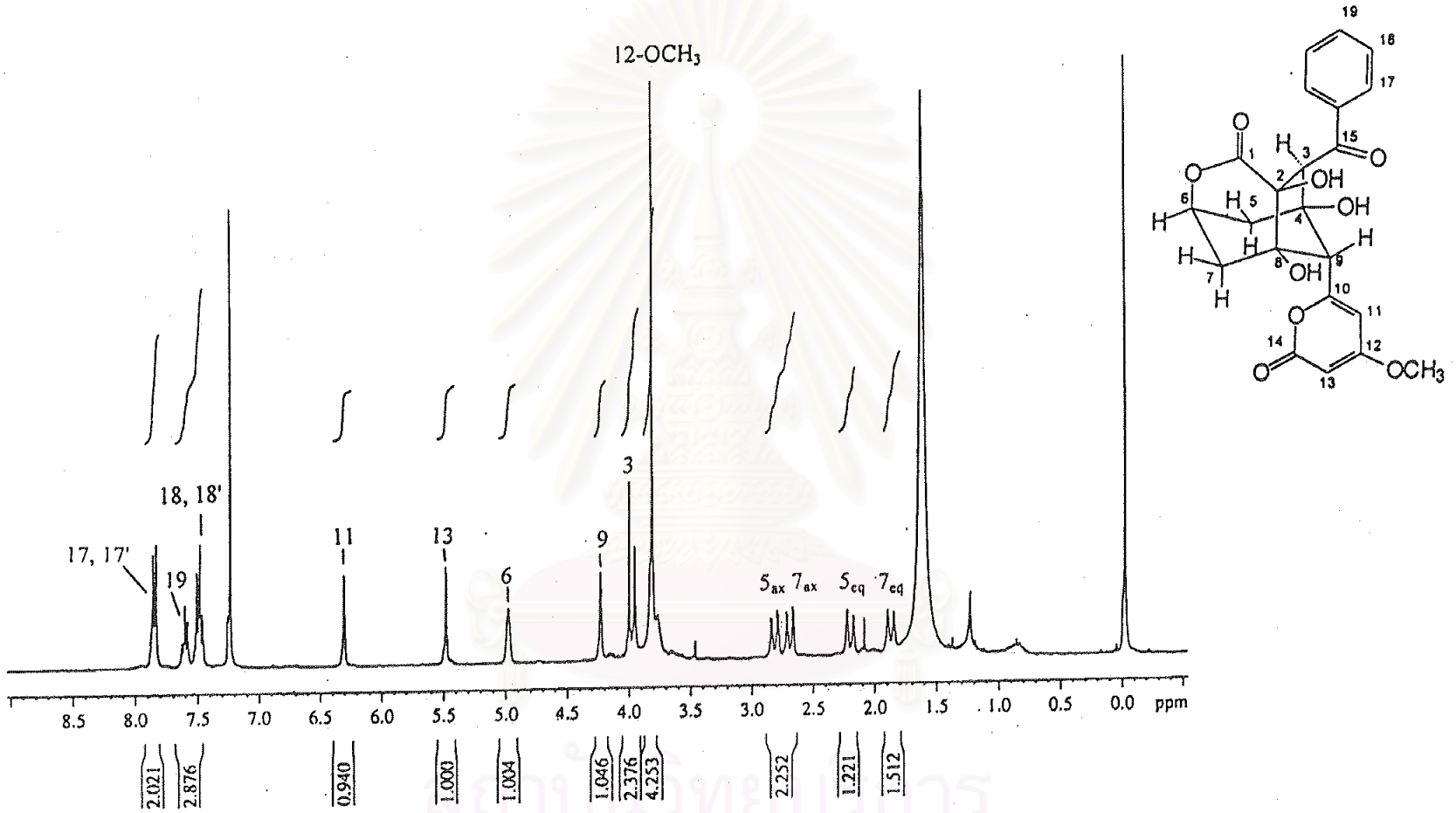


Figure 42a. The 300 MHz ^{1}H NMR spectrum of Por0225223 (in $CDCl_3$)

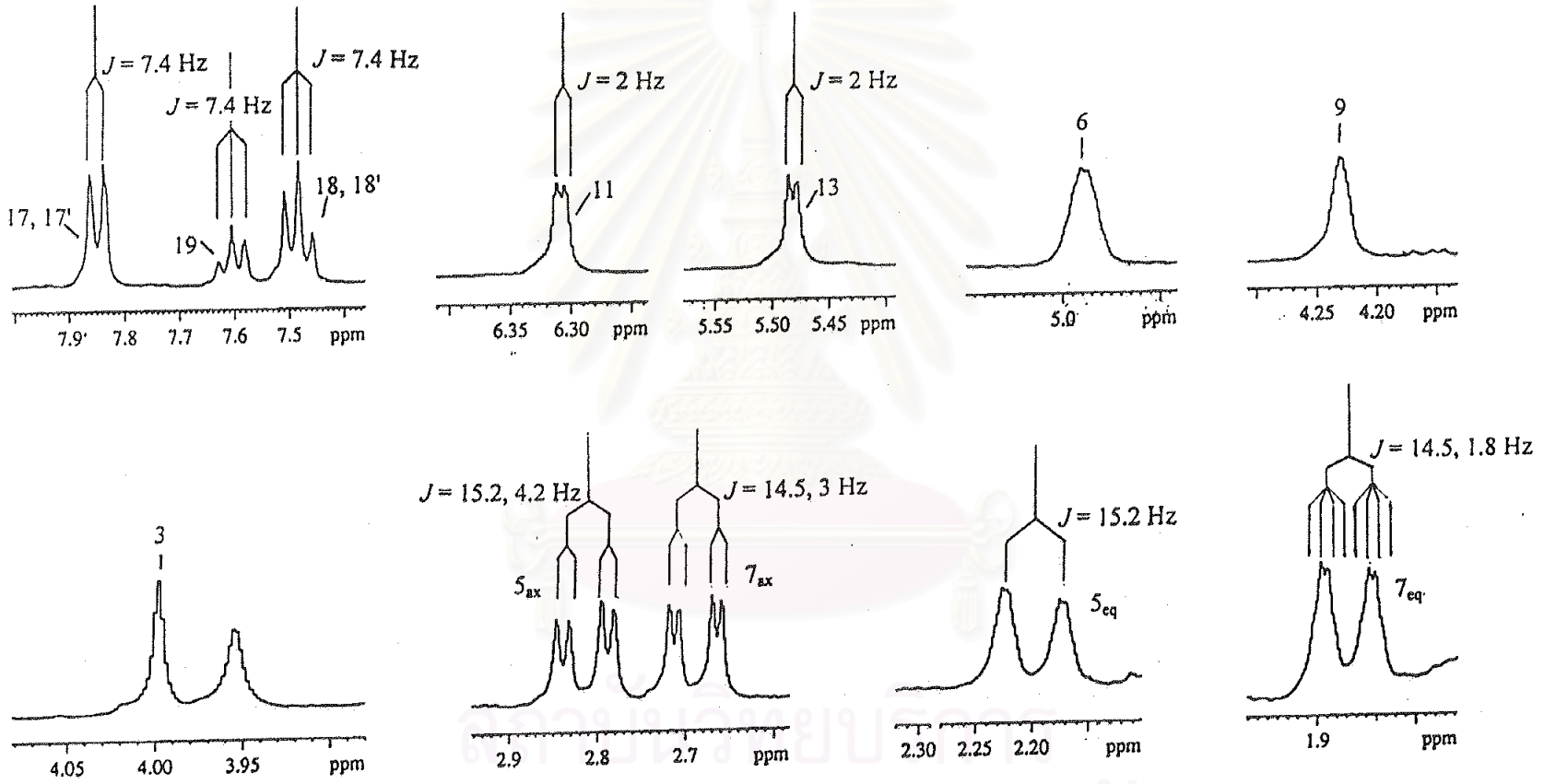


Figure 42b. The 300 MHz ^{1}H NMR (expanded) spectrum of Por0225223 in ($CDCl_3$)

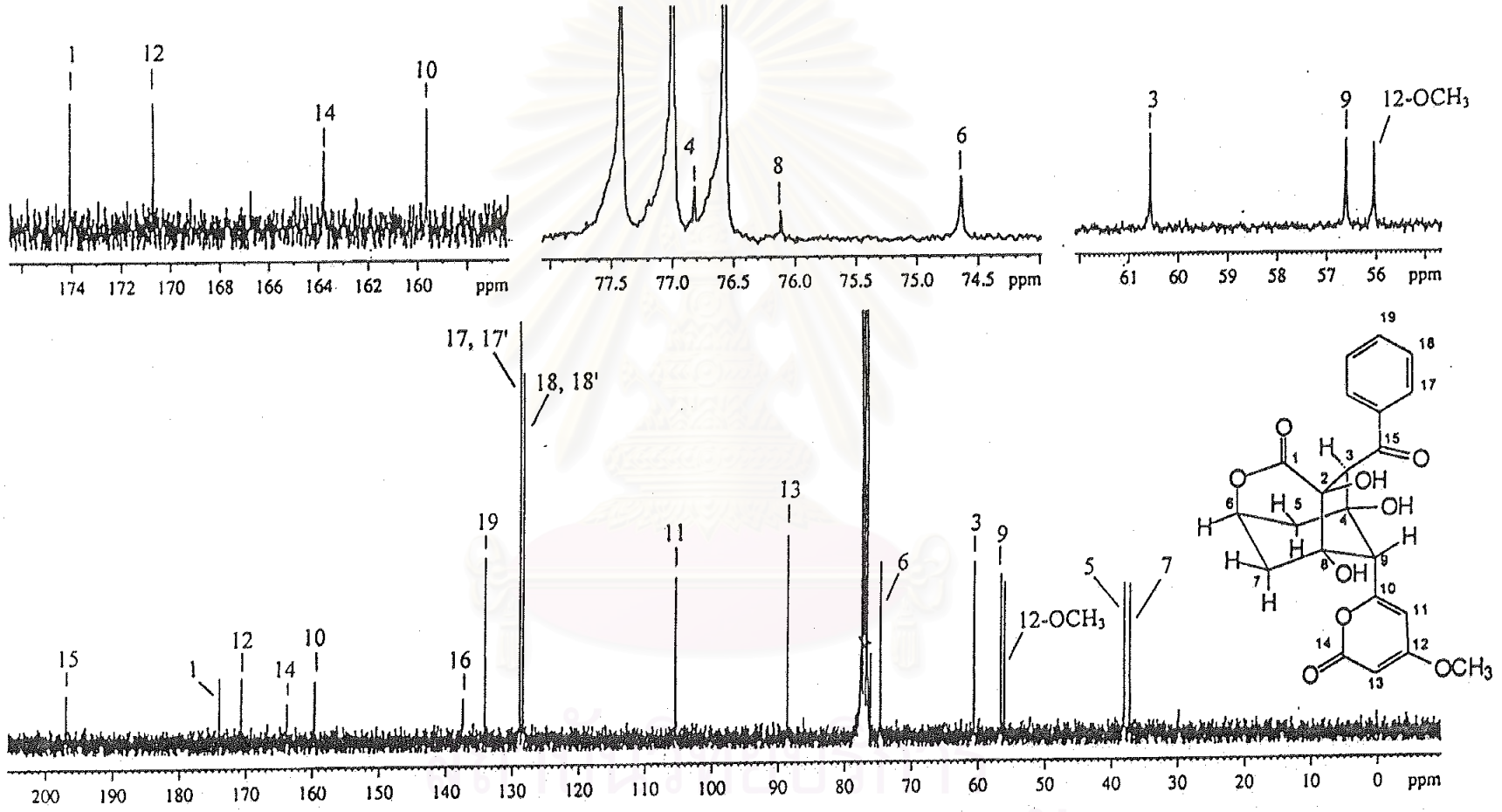


Figure 43. The 75 MHz ^{13}C NMR spectrum of Por0225223 (in $CDCl_3$)

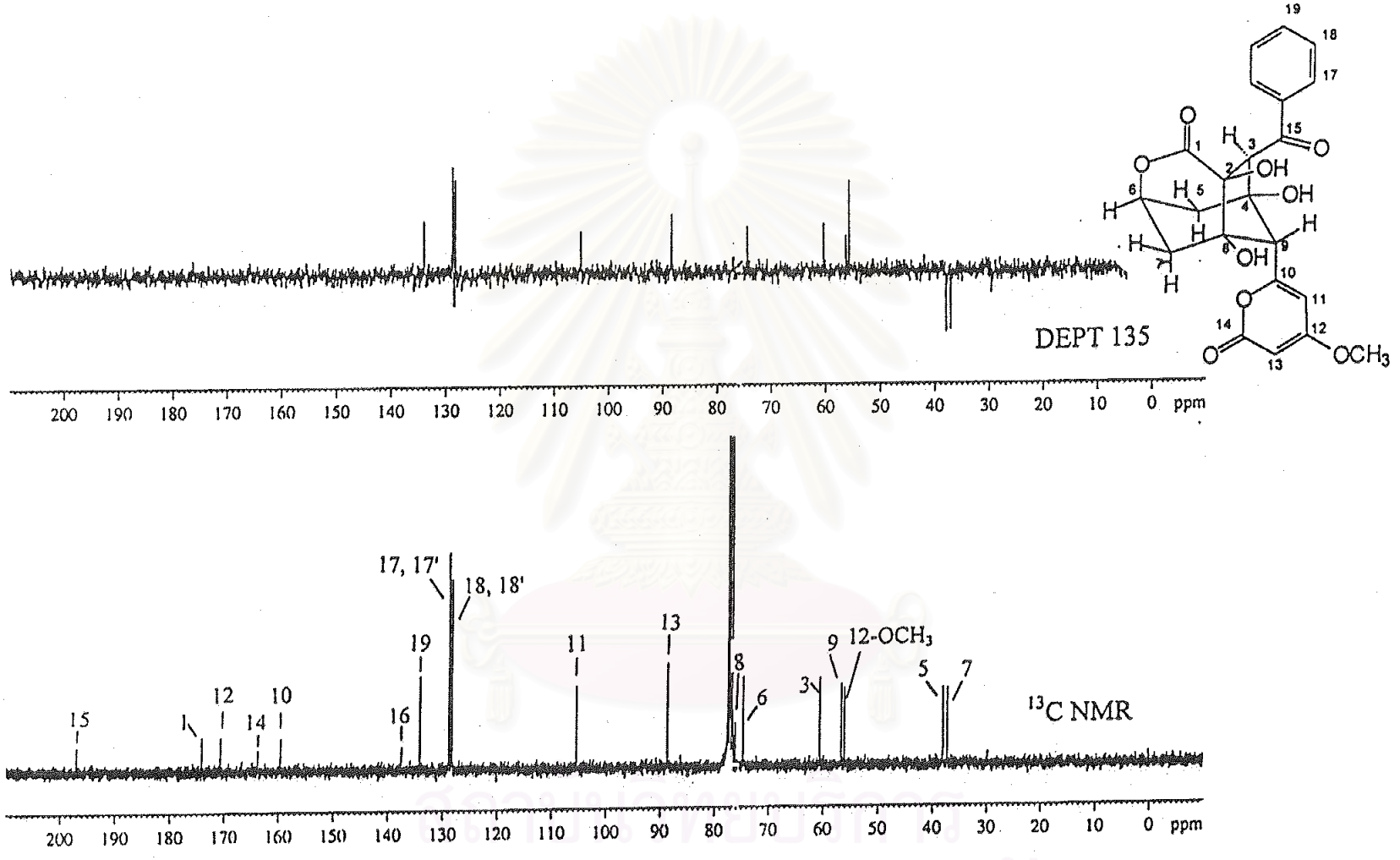


Figure 44. The 75 MHz ^{13}C NMR, DEPT 135 spectra of Por0225223 (in $CDCl_3$)

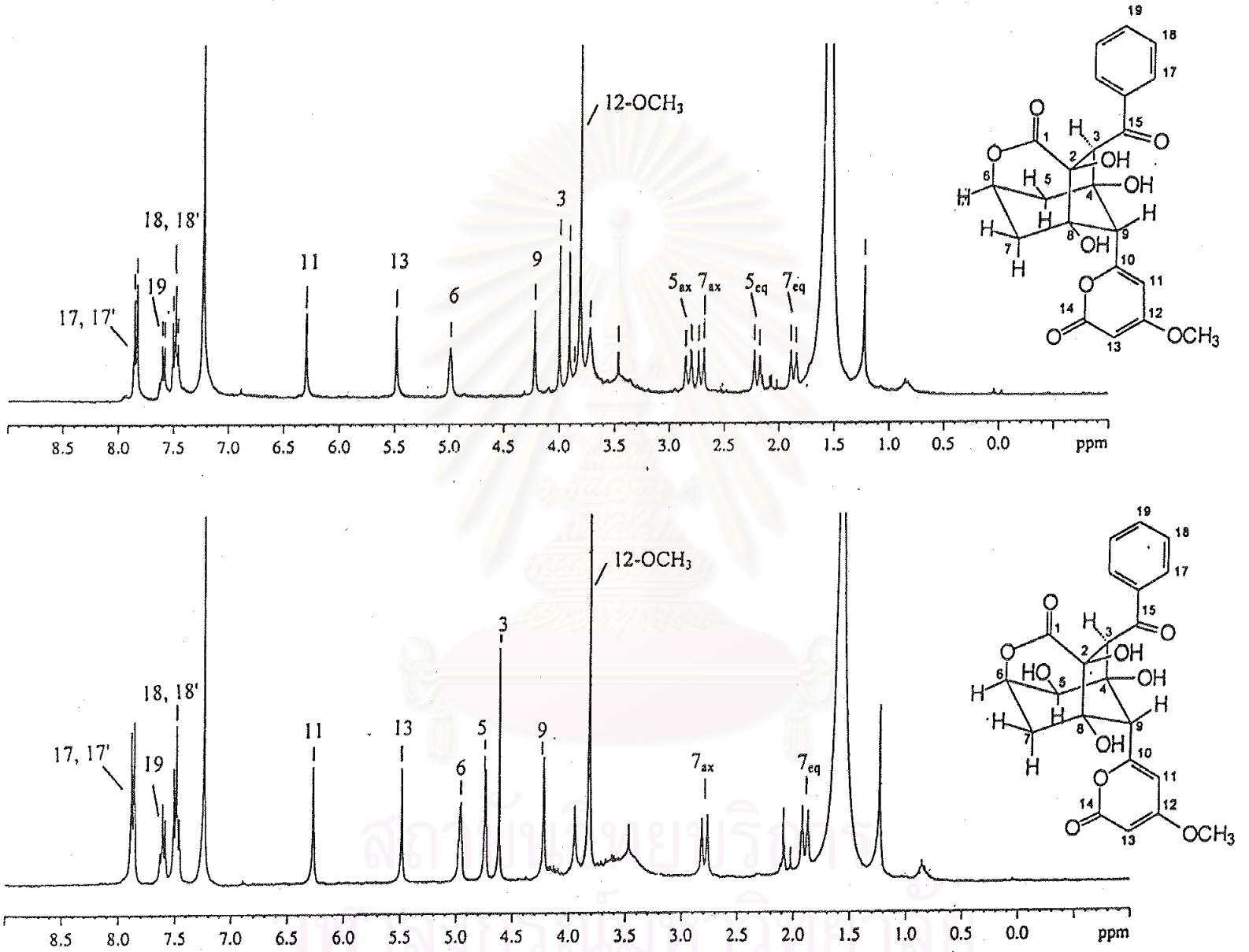


Figure 45. The 300 MHz ^{1}H NMR spectra of Por0225223 and Por0226 (in $CDCl_3$)

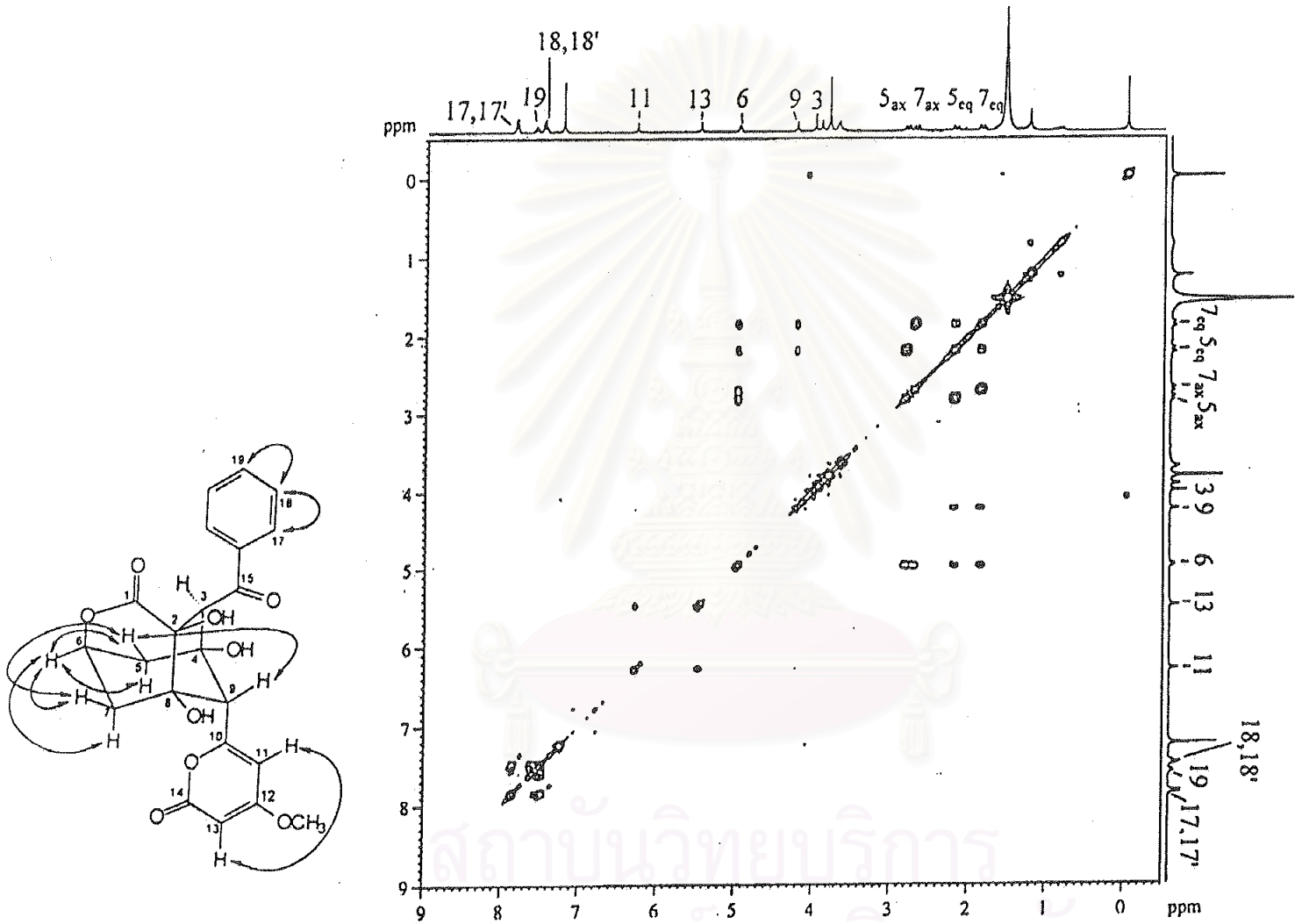


Figure 46. The 300 MHz ^{1}H, ^{1}H COSY spectrum of Por0225223 (in $CDCl_3$)

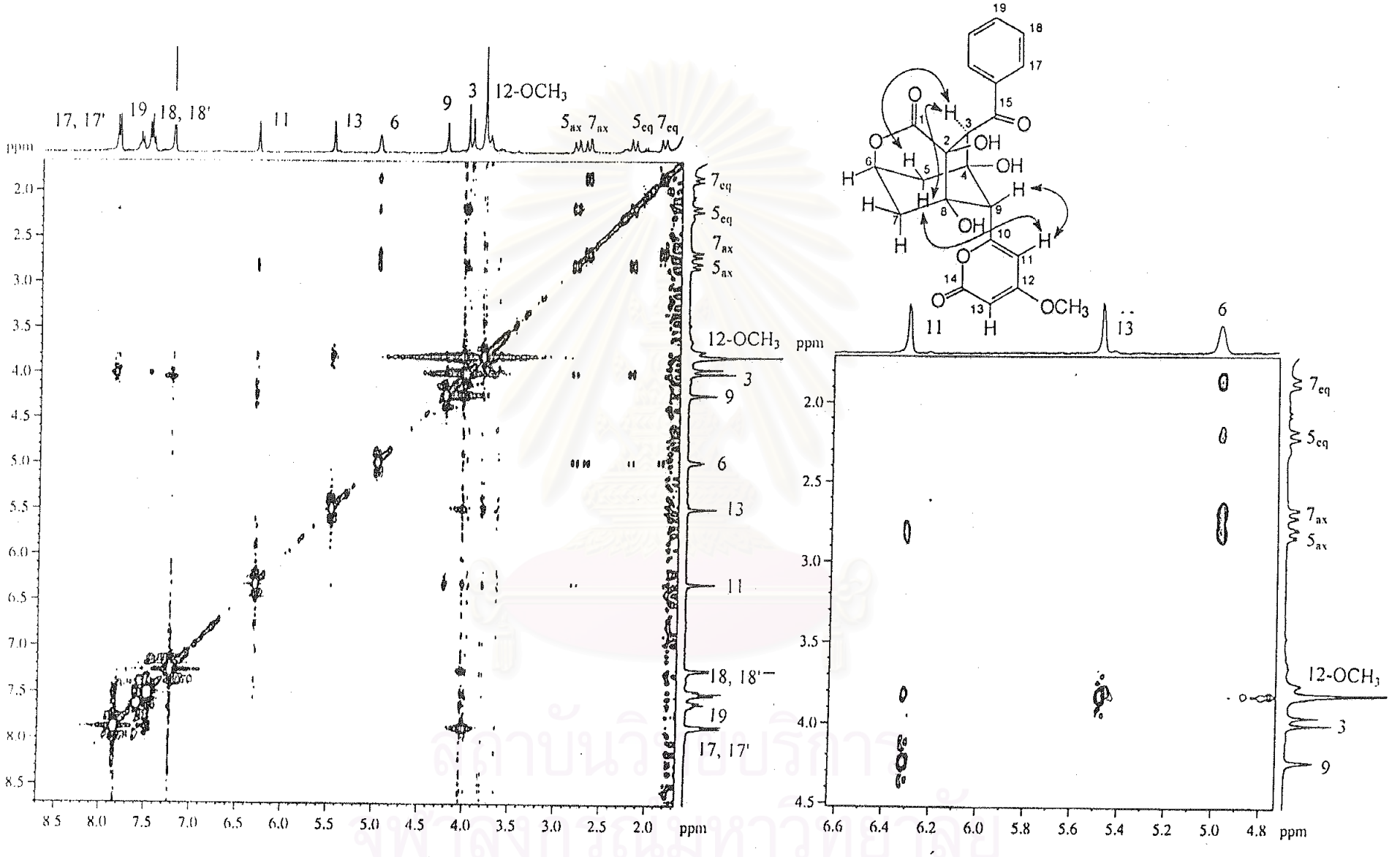


Figure 47. The 300 MHz NOESY spectrum of Por0225223 (in $CDCl_3$)

APPENDIX B

(Media)

1. Media

The media were prepared by sterilization in the autoclave at 121 °C for 20 minutes. pH was adjusted wit NaOH or HCl before addition of agar and before sterilization.

1.1 Glycerol peptone media (GPM)

Glycerol	20.0	g
Molasses	10.0	g
Beef extract	5.0	g
Peptone	5.0	g
$CaCO_3$	4.0	g
Sea water 35 ppt	1000	ml

1.2 Yeast-malt extract agar (YMA)

Glucose	10.0	g
Peptone	5.0	g
Yeast extract	3.0	g
Malt extract	3.0	g
Distilled water	1000	ml
Agar	15-20	g

1.3 Sodium casienate agar (SCA)

Sodium casienate	20.0	g
Glucose	10.0	g
K_2PO_4 (anhydrous)	0.2	g
$MgSO_4.7H_2O$	0.2	g
$FeSO_4.7H_2O$	trace	
Distilled water	1000	ml
Agar	15-20	g

1.4 Potato carrot agar (PCA)

Potato	30.0	g
Carrot	25.0	g
Tap water	1000	ml
Agar	15-20	g
pH 7.0		

1.5 Oatmeal agar

Oatmeal agar	18.0	g
Distilled water	1000	ml
pH 7.2		

1.6 Inorganic salt- starch agar

Difco soluble starch	10.0	g
K_2HPO_4 (anhydrous)	1.0	g
$MgSO_4.7H_2O$	1.0	g
NaCl	1.0	g
$(NH_4)_2SO_4$	2.0	g
$CaCO_3$	2.0	g
Distilled water	1000	ml
Trace salt solution	1	ml
Agar	15-20	g
pH 7.0-7.4		

1.7 Glycerol asparagine agar

L- asparagine (anhydrous)	1.0	g
K_2HPO_4	1.0	g
Glycerol	10.0	g
Distilled water	1000	ml

Trace salt solution	1	ml
Agar	15-20	g
pH 7.0-7.4		

1.8 Tyrosine agar

Glycerol	15.0	g
L-tyrosine	0.5	g
L-asparagine	1.0	g
K_2HPO_4 (anhydrous)	0.5	g
$MgSO_4$. $7H_2O$	0.5	g
NaCl	0.5	g
$FeSO_4.7H_2O$	0.01	g
Distilled water	1000	ml
Trace salt solution	1	ml
Agar	15-20	g
pH 7.2 -7.4		

1.9 Carbon utilization medium

Steriled carbon source

No carbon source (negative control)

D-glucose (positive control)

L-arabinose

Sucrose	D-fructose
D-xylose	Rhamnose
Inositol	Raffinose
D-manitol	Cellulose

Pridham and Gottlieb trace salt

$CuSO_4.5H_2O$	0.64	g
$FeSO_4.7H_2O$	0.11	g

$MnCl_2.4H_2O$	0.79	g
$ZnSO_4.7H_2O$	0.15	g
Distilled water	1000	ml

Basal mineral salt agar

$(NH_4)_2SO_4$	2.64	g
K_2HPO_4 (anhydrous)	2.38	g
$K_2HPO_4.3H_2O$	5.65	g
$MgSO_4.7H_2O$	1.00	g
Pridham and Gottlieb trace salt	1.0	ml
Distilled water	1000	ml
Agar	15-20	g

1.10 Skim milk

Bacto skim milk, dehydrated (Difco)	100	g
Distilled water	1000	ml

1.11 Peptone nitrate broth

Peptone	10.0	g
KNO_3	1.0	g
NaCl	5.0	g
Distilled water	1000	ml

pH 7.0

1.12 Tyrosinase reaction agar

Peptone	5.0	g
Meat extract	3.0	g
L-tyrosine	5.0	g
Distilled water	1000	ml
Agar	15-20	g

1.13 Tryptic soy agar (TSA) (Difco®Tryptic soy agar)

Tryptone peptone (pancreatic digest of casien)	15.0	g
Soytone peptone (papaic gigest of soybean meal)	5.0	g
Sodium chloride	5.0	g
Agar	15	g
Purified water	1000	ml
pH 7.30± 0.2		

1.14 Sabouraud's dextrose agar (SDA) (Difco®Sabouraud dextrose agar)

Neopeptone	10.0	g
Dextrose	40.0	g
Agar	15	g
Purified water	1000	ml
pH 5.60± 0.2		

1.15 Glucose-beef extract peptone media (GBP)

Glucose	15.0	g
Peptone	6.0	g
Beef extract	3.0	g
Yeast extract	3.0	g
Sea water (35 ppt)	1000	ml
pH 7.0		

VITA

Miss Kusuma Jitsaeng was born on 16th July 1976, Khonkaen, Thailand. She received her Bachelor's Degree of Sciences in Pharmacy in 1998 from the Faculty of Pharmaceutical Sciences, Khonkaen University, Thailand. She was awarded a scholarship from the University Development Commission (UDC). At present, she is a faculty member of the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand.