สารทุติยภูมิของสเตรปโตมัยซิสสายพันธุ์ AAR 1-1 และ AAR 14 ที่อยู่ร่วมกับฟองน้ำทะเล

นางสาวชุติมา เพ็ชรประยูร

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SECONDARY METABOLITES OF *STREPTOMYCES* STRAINS AAR 1-1 AND AAR 14 ASSOCIATED WITH MARINE SPONGES

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Thesis Title	SECONDARY METABOLITES OF *STREPTOMYCES* STRAINS AAR 1-1 AND AAR 14 ASSOCIATED WITH MARINE SPONGES
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ชุติมา เพ็ชรประยูร : สารทุติยภูมิของสเตรปโตมัยซิสสายพันธุ์ AAR 1-1 และ AAR 14 ที่อยู่ร่วมกับฟองน้ำทะเล (SECONDARY METABOLITES OF *STREPTOMYCES* STRAINS AAR 1-1 AND AAR 14 ASSOCIATED WITH MARINE SPONGES) อ.ที่ปรึกษา : อ.ดร. คณิต สุวรรณบริรักษ์ อ.ที่ปรึกษาร่วม : รศ.ดร. สมบูรณ์ ธนาศุภวัฒน์ 175 หน้า ISBN 974-13-1255-5

ในการตรวจสอบหาสารที่มีฤทธิ์ทางชีวภาพจากจุลชีพทางทะเล พบว่าเชื้อแอคติโนมัยซีทส์สายพันธุ์ AAR 1-1 และ AAR 14 ที่อาศัยอยู่ร่วมกับฟองน้ำทะเลสีม่วงอมน้ำเงิน และสีขาวอมม่วง ตามลำดับ จากหมู่เกาะอาดังราวี แสดงฤทธิ์ทางต้านจุลชีพที่น่าสนใจ จากลักษณะทางสัณฐานวิทยา การเจริญ สรีรวิทยา และชีวเคมี สามารถพิสูจน์เอกลักษณ์ของสายพันธุ์ AAR 1-1 และ AAR 14 ได้เป็นแบคทีเรียในสกุลสเตรปโตมัยซิส จากการสกัดแยกสารควบคู่ไปกับการทดสอบฤทธิ์ต้านจุลชีพต่อเชื้อ *Staphylococcus aureus* ATCC 25923 ของสิ่งสกัดในชั้นเอธิลอะซิเตทจากน้ำหมักของเชื้อสายพันธุ์ AAR 1-1 แยกได้สารที่เคยพบแล้ว 1 ชนิด คือ actinomycin D จากการสกัดแยกสารควบคู่ไปกับการทดสอบฤทธิ์ต้านจุลชีพต่อเชื้อ *Candida albicans* ATCC 10231 ของสิ่งสกัดในชั้นเมธานอลจากน้ำหมักของเชื้อสายพันธุ์ AAR 14 แยกได้สารจำพวก diketopiperazines ที่เคยพบมาแล้ว 2 ชนิด คือ *cyclo*-(L-prolyl-D-leucyl) และ *cyclo*-(L-prolyl-D-valyl) อนุพันธ์ของ acetamide 1 ชนิด คือ *N*-[2′-(4″-hydroxyphenyl)ethyl]acetamide และส่วนผสมของสารชนิดใหม่พวก antimycins 3 ชนิด โดยองค์ประกอบส่วนใหญ่เป็น antimycins B_1 และ B_2 และส่วนน้อยเป็น antimycin B_3 การพิสูจน์สูตรโครงสร้างทางเคมีของสารเหล่านี้ ทำได้โดยการวิเคราะห์ข้อมูลทางสเปกโตรสโคปีจาก UV IR MS ^{1}H และ ^{13}C NMR ร่วมกับการเปรียบเทียบข้อมูลกับเอกสารต่างๆ สาร actinomycin D แสดงฤทธิ์ต้านจุลชีพต่อเชื้อ *S. aureus* ATCC 25923 และ *Bacillus subtilis* ATCC 6633 ฤทธิ์ต้านเชื้อมาเลเรีย *Plasmodium falciparum* (K1 multi-drug resistant strain) ฤทธิ์ความเป็นพิษต่อเซลล์ oral human epidermoid carcinoma และ breast cancer และฤทธิ์ต้านเชื้อวัณโรค *Mycobacterium tuberculosis* H37Ra ส่วนสารจำพวก diketopiperazines แสดงฤทธิ์ยับยั้งการเจริญของเชื้อรา *C. albicans* ATCC 10231

ภาควิชา	เภสัชเวท	ลายมือชื่อนิสิต.....
สาขาวิชา	เภสัชเวท	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา	2543	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEY WORD : MARINE *STREPTOMYCES* / ANTIMICROBIAL ACTIVITY /ANTIMALARIAL ACTIVITY / CYTOTOXIC ACTIVITY/ ANTITUBERCULOUS ACTIVITY / ACTINOMYCIN D/ DIKETOPIPERAZINE / ACETAMIDE / ANTIMYCIN
CHUTIMA PETCHPRAYOON : SECONDARY METABOLITES OF *STREPTOMYCES* STRAINS AAR 1-1 AND AAR 14 ASSOCIATED WITH MARINE SPONGES. THESIS ADVISOR : MR. KHANIT SUWANBORIRUX, Ph.D. THESIS CO-ADVISOR : ASSOC. PROF. SOMBOON TANASUPAWAT, Ph.D. 175 pp. ISBN 974-13-1255-5.

In the course of our investigation on bioactive compounds from marine microorganisms, actinomycetes strains AAR 1-1 and AAR 14 associated with a bluish purple and a purplish white marine sponges, respectively, from Adang-ravee Island showed interesting antimicrobial activity. Based on morphological, cultural, physiological, and biochemical characteristic studies, the strains AAR 1-1 and AAR 14 were identified as the genus *Streptomyces*. The bioassay-directed fractionation, using antimicrobial activity against *Staphylococcus aureus* ATCC 25923, of the ethyl acetate extract from the fermentation broth of the strain AAR 1-1 led to the isolation of a known compound, actinomycin D. Directed by antimicrobial activity against *Candida albicans* ATCC 10231, fractionation of the methanol extract from the fermentation broth of the strain AAR 14 yielded two known diketopiperazines, *cyclo*-(L-prolyl-D-leucyl) and *cyclo*-(L-prolyl-D-valyl), one acetamide derivative, *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide, and a mixture of three new members of antimycins containing two major components, antimycins B_1 and B_2, together with a minor component, antimycin B_3. The structure elucidation of these compounds was achieved by analyses of UV, IR, MS, ^{1}H, and ^{13}C NMR spectral data as well as comparison with the literatures. The isolated actinomycin D showed antimicrobial activity against *S. aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633, antimalarial activity against *Plasmodium falciparum* (K1 multi-drug resistant strain), cytotoxic activity against oral human epidermoid carcinoma cell lines and breast cancer cell lines, and antituberculous activity against *Mycobacterium tuberculosis* H37Ra. Two diketopiperazines showed fungistatic activity against *C. albicans* ATCC 10231.

Department	Pharmacognosy	Student's signature……...
Field of study	Pharmacognosy	Advisor's signature……...
Academic year	2000	Co-advisor's signature……..

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ABBREVIATIONS

%	=	percent or part per hundred
^{o}C	=	degree Celsius
δ	=	chemical shift
ε	=	molar absorptivity
μg	=	microgram
μl	=	microliter
λ_{max}	=	wave length at maximum absorption
ν_{max}	=	wave number at maximum absorption
$[\alpha]_D^{25}$	=	specific rotation at 25^{o}C and sodium D line (589 nm)
^{13}C NMR	=	carbon-13 nuclear magnetic resonance
^{1}H NMR	=	proton nuclear magnetic resonance
^{1}H-^{1}H COSY	=	^{1}H-^{1}H correlation spectroscopy
a-Ile	=	*allo*-isoleucine
ATCC	=	American Type Culture Collection, Maryland, USA
BC	=	breast cancer cells
br s	=	broad singlet
c	=	concentration
$CDCl_3$	=	deuterated cholroform
$CHCl_3$	=	chloroform
chrom	=	chromophore
cm	=	centimeter
d	=	doublet
DAP	=	diaminopimelic acid
dd	=	doublet of doublets
DEPT	=	distortionless enhancement by polarization transfer
DMSO-d_6	=	deuterated dimethyl sulphoxide
dq	=	doublet of quartets
dt	=	doublet of triplets
EC_{50}	=	50% effective concentration

ED_{50}	=	50% effective dose
ESI-TOF	=	electrospray ionization-time of flight
EtOAc	=	ethyl acetate
EtOH	=	ethanol
g	=	gram
GPBY	=	Glucose peptone beef extract yeast extract medium
GPM	=	Glycerol peptone medium
H_2O	=	water
HCT-116	=	human colon cancer cell line
HIV	=	human immunodeficiency virus
HMBC	=	^{1}H-detected heteronuclear multiple bond correlation
HMQC	=	^{1}H-detected heteronuclear multiple quantum coherence
HSV	=	herpes simplex virus
HyPro	=	hydroxyproline
Hz	=	hertz
IR	=	infrared
J	=	*coupling constant*
KB	=	human epidermoid carcinoma cells of the nasopharynx
KBr	=	potassium bromide
km	=	kilometer
KNO_2	=	potassium nitrate
l	=	liter
M	=	molar
m	=	multiplet
m/z	=	mass to charge
M^+	=	molecular ion
MA	=	marine agar
MABA	=	Microplate Alamar Blue Assay
MeAla	=	methylalanine
MeCN	=	acetonitrile
MeIle	=	methylisoleucine
MeOH	=	methanol

MeV	=	methylvaline
MeVal	=	methylvaline
mg	=	milligram
MHz	=	megahertz
MIC	=	minimum inhibition concentration
min	=	minute
ml	=	milliliter
mm	=	millimeter
ms	=	mass spectroscopy
N	=	normal
NaCl	=	sodium chloride
ng	=	nanogram
nm	=	nanometer
NMR	=	nuclear magnetic resonance
No	=	number
NSS	=	normal saline solution
OxoPip	=	4-oxopipecolic acid
OxoPro	=	γ-oxoproline
P	=	proline
P388	=	murine leukemia cells
PCA	=	potato carrot agar
Pip	=	pipercolic
ppm	=	part per million
ppt	=	part per thousand
Pro	=	proline
q	=	quartet
quin	=	quintet
rpm	=	round per minute
S	=	sarcosine
s	=	singlet
Sar	=	sarcosine
SCA	=	sodium caseinate agar

SDA	=	Sabouraud dextrose agar
SEM	=	scanning electron microscopy
sp.	=	species
SRB	=	sulforhodamine B
T	=	threonine
t	=	triplet
Thr	=	threonine
TLC	=	thin layer chromatography
TOCSY	=	totally correlation spectroscopy
TSA	=	Tryptic soy agar
UV	=	ultraviolet
V	=	valine
Val	=	valine
YM	=	Yeast extract-malt extract

CHAPTER I

INTRODUCTION

In the discovery of biologically active substances, one important source is microorganisms. Most antibiotics of microbial origin come from terrestrial bacteria in the order Actinomycetales. Actinomycetes have been described as the greastest source of antibiotics since Waksman introduced Streptomycetes into his systematic screening program for new antibiotics in the early 1940s. They have provided more than 4,000 antibiotics , including many of those important in medicine, such as aminoglycosides, anthracyclines, chloramphenicol, β-lactams, macrolides and tetracyclines (Okami and Hotta, 1988). However, in the late 1960s, the search for novel metabolites took a new direction as the realm of exploration expanded to include organisms and microorganisms in the sea.

Marine microorganisms have become an important point of study in the search for novel microbial products. Today, academic interest in marine microorganisms is on the rise because of the growing number of unique, biologically active secondary metabolites reported from marine bacteria. The ability of marine bacteria to produce biologically active substances was documented for more than 45 years ago (Jensen and Fenical, 1994). The biological activities reported for secondary metabolites include not only antibiotic but also antitumor and antiviral activities.

It is not easy to define “marine” bacteria because many isolates will tolerate quite a wide range of salinities. Bacteria found in coastal waters may have been washed into the oceans from rivers, estuaries and sewage outfalls. For these reasons, it is probably best to adop a pragmatic definition of marine bacteria as those isolated on seawater media, from marine organisms and sediments (Faulkner, 1999). Examples for metabolites from marine actinomycetes are wailupemycins A-C, 3-epi-5-deoxyenterocin, 5-deoxyenterocin, and enterocin. These compounds were isolated from Actinomycetes designated BD-26T (20) which was determined to belong to the genus *Streptomyces* and isolated from sediment collected at Wailupe beach park on

the south-east shore of OaHu, Hawaii. Wailupemycin A exhibited antimicrobial activity towards *Escherichia coli,* while 3-epi-5-deoxyenterocin inhibited the growth of *Staphylococcus aureus*. Enterocin was reported to be bacteriostatic against gram-positive and gram-negative bacteria (Sitachitta *et al.,* 1996). Lagunapyrones A-C were produced by an unidentified marine actinomycete (culture CNB-984) isolated from sediment collected in the Agua Hedionda Lagoon in Carlsbad, California. Lagunapyrone B showed cytotoxicity against the human colon cancer cell line HCT-116 at ED_{50} = 3.5 μg/ml (Lindel *et al.*, 1996). Salinamides A and B, produced by an actinomycete that was isolated from the surface of the jellyfish *Cassiopeia xamachana* collected in the Florida Keys, exhibited antibacterial activity against *Streptococcus pneumoniae* and *Staphylococcus pyrogenes* with MIC 4 μg/ml for salinamide A and 4 and 2 μg/ml for B. Salinamides A and B showed potent topical antiinflammatory activity using the phorbol ester induced mouse ear edema assay (Trischman *et al*., 1994).

In the course of our investigation on bioactive compounds from marine bacteria, the actinomycetes strains AAR 1-1 and AAR 14 were isolated from a bluish purple sponge AR 990325-20H and a purplish white sponge AR 99024-09K, respectively, from Adang-ravee Island, Satool Province, Thailand. The crude extracts from those bacteria showed antimicrobial activity against *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25923 for the strain AAR 1-1 and *Candida albicans* ATCC 10231 for the strain AAR 14. Therefore, the main objectives of this investigations are as follows:

1. To isolate and identify the marine microorganism strains AAR 1-1 and AAR 14.
2. To isolate and purify secondary metabolites from the strains AAR 1-1 and AAR 14.
3. To elucidate the chemical structures of the isolated compounds.
4. To evalute the antimicrobial, antimalarial, cytotoxic, and antituberculous activities of the isolated compounds.

CHAPTER II

REVIEW OF LITERATURE

Microorganisms have had a profound effect on the development of chemistry and upon medical science. They are not only the cause of infection, but the producer of organic substances those can cure infection. Over the past 60 years, between 30,000 and 50,000 natural products have been discovered from microorganisms. More than 10,000 of these compounds are biologically actives and more than 8,000 are antibiotic and antitumor agents (Attaway and Zaborsky, 1993). The microbial products are in use today as antibiotics, antitumor agents, and agrichemicals. Most metabolites of microbial origin come from terrestrial bacteria belonging to one taxonomic group, the order Actinomycetales. Although these bacteria continue to be studied extensively, it is clear that the rate of discovering novel metabolites from terrestrial actinomycetes is decreasing, and infectious diseases are rapidly developing resistance toward traditional antibiotics. For these reasons, it is imperative that new sources of bioactive natural products must be explored.

Marine microorganisms are of considerable current interest as a new promising source of bioactive substances. Marine microorganisms encompass a complex and diverse assemblage of microscopic life forms and occur throughout the ocean, including environments of extreme variations in pressure, salinity, and temperature. Marine microorganisms have developed unique metabolic and physiological capabilities that not only ensure survival in extreme habitats, but also offer the potential for the production of metabolites which would not be observed from terrestrial microorganisms (Fenical, 1993)

1. Characteristics of the oceans

The oceans occupy 71% or 350 x 10^6 km^3 of the earth's surface. The environmental conditions in the marine ecosphere are remarkably uniform. The great uniformity is brought about by various mixing mechanisms that include tidal

movements, currents, and thermohaline circulation. Tides are produced by the pull of the moon and the sun. Currents arise from the frictional drag of wind blowing across the surface of the oceans and rotation of the earth. The rotational force of the earth and land obstacles result in largely circular current systems. Deep currents arise from variations in temperature and salinity which create differences in water mass densities. These water mass densities can cause thermohaline currents which mix the water mass vertically.

The oceans are the ultimate sink for all water soluble minerals and are saline because of the water cycle. When the evaporated water precipitates as rain or snow, it may enter the oceans directly or indirectly after passing over or through the terrestrial environment as runoff, for these reasons, minerals are leached into the oceans at much higher rate than they can be returned to land. The oceans contain almost every naturally occurring chemical element, but most elements are in low concentrations (Table 1). The major elements are sodium, chlorine, magnesium, sulfate, calcium and potassium. Minor components are including of bromine, carbon, strontium, boron, silica and fluorine. Besides, nitrogen, phosphorus and iron, which are essential for microbial growth are trace elements in sea water.

The environmental conditions of the oceans are highly consistent. They are found to vary only slightly from place to place. Salinities are normally in the range of 33-37 ppt with an average of 35 ppt. The pH of seawater is generally 8.3-8.5. Seasonal temperature fluctuations at any location are usually no more than 20 ^{o}C and variations of temperature over all of the oceans are within 35 ^{o}C.

There are definite zones that can be recognized for oceans. The littoral or intertidal zone, flooded and dried at high and low tides, respectively, is the interface between the marine ecosphere and the lithosphere that occurs at the seashore. The sublitoral zone, neritic, extends from the low tide mark to the edge of the continental shelf. Pelagic is used for designation of open water, the high sea, and includes portions of the neritic and the entirety of the oceanic province. The benthic region begins at the intertidal zone and extends downward, including continental

shelf, continental slope and sea floor. There are known as bathyl region, abyssal plain and hadal region.

Table 1 The elements in seawater

Element	Concentration (ppm)	Element	Concentration (ppm)
H	1.1×10^{5}	B	4.6
O	8.6×10^{5}	Si	3.0
Cl	1.9×10^{4}	F	1.0
Na	1.1×10^{4}	N	5.0×10^{-1}
Mg	1.4×10^{3}	Li	1.7×10^{-1}
SO_4^{2-}	8.9×10^{2}	Ru	1.2×10^{-1}
Ca	4.0×10^{2}	P	7.0×10^{-2}
K	3.8×10^{2}	I	6.0×10^{-2}
Br	65.0	Fe	1.0×10^{-2}
C	28.0	Zn	1.0×10^{-2}
Sr	8.0	Mo	1.0×10^{-2}

Ross, 1970

The pelagic marine habitat is a unique environment for macro- as well as microorganisms. All primary production is microscopic algae and bacteria. The highest biomass of microorganisms in seawater is normally near the surface and decrease with depth because of light energy and mineral nutrients. Marine microorganisms should exhibit growth at salinities between 20-40 ppt and can grow at the low nutrient concentrations found in the oceans. Except in tropical surface water, most marine bacteria must be capable of growth at low temperature. There are many kinds of microorganisms found in marine environments such as bacteria, fungi, yeasts, viruses and such microorganisms do not occur in freshwater or in soil (Atlas and Bartha, 1981).

In recent years, marine microorganisms are receiving increased attention. Some newer chemicals that have been discovered are microorganism metabolites. There also is an interest in the culture of symbiotic marine microorganisms, which are associated with macroscopic hosts. A variety of interesting compounds are microbial origin and sometimes do not occur in terrestrial microorganisms. There is a worldwide effort to better characterize the marine microbial community and to study microorganisms for modern biomedicines (Prescott *et al.*, 1999).

2. Characterizations of filamentous bacteria, Streptomyces.

In the classification of bacteria, there is one major group of bacteria that have filaments like fungi and this group is called "actinomycetes". The term "actinomycetes" is common name of bacteria in the order Actinomycetales which are aerobic gram-positive bacteria that form branching filaments or hyphae that may persist as a stable mycelium or may break up into rod shaped or coccoid elements. According to Bergey's manual of systematic bacteriology (Williums, Sharpe and Holt, 1989), the genera of actinomycetes are devided into eight sections. (Table 2)

Streptomyces is a genus in the family *Streptomycetaceae* (Streptomycetes and related genera group). Bacteria in this genus have vegetative hyphae, 0.5-2.0 μm diameter, which produce and extensively branched mycelium that rarely fragments. The aerial mycelium at maturity forms chains of three to many nonmotile conidiospores with surface texture can be smooth, hairy or spiny. Form discrete and lichenoid, leathery or butyrous colonies and produce a wide variety of pigments responsible for the color of the vegetative and aerial mycelium. All have a type I cell wall, L-diaminopimelic acid (L-DAP), and a guanine plus cytosine (G+C) content 69-78%. *Streptomyces* can use a wide range of organic compounds as sole sources of carbon for energy and growth (Williums, Sharpe and Holt, 1989; Prescott *et al*., 1999).

Table 2 Characteristics of actinomycetes groups in Bergey's manual of systematic bacteriology.

Group	Cell Wall Type	Spore arrangement
Nocardioform actinomycetes	I, IV, VI	Varies
Genera with multilocular sporangia	III	Clusters of spores
Actinoplanetes	II	Varies
Streptomycetes and related genera	I	Chains of 5 to more than 50 spores
Maduromycetes	III	Varies
Thermomonospora and related genera	III	Varies
Thermoactinomycetes	III	Single spores
Other genera[a]	-	-

[a] cannot assigned to other genera

Streptomyces is an enormous genus, with the member around 500 species. They are determined by means of a combination of morphological and physiological characteristics, including the following: the color of the aerial and substrate mycelia, spore arrangement, surface features of individual spores, carbohydrate use, antibiotic production, melanin synthesis, nitrate reduction, and hydrolysis of urea and hippuric acid. *Streptomyces* are very important, both ecologically and medically. The natural habitat of most *Streptomyces* is the soil and they are best known for producing many antibiotics which are useful in medical and biological research (Williams, Sharpe and Holt, 1989; Prescott *et al*., 1999).

3. Chemistry of actinomycins

3.1 Historical development

The actinomycins are orange to red crystalline antibiotic metabolites from various species of *Streptomyces*. These compounds are highly toxic and cannot be used in the treatment of infectious diseases. However, they are interesting because in small and nontoxic dose they have an antineoplastic effect. Actinomycins D and C_3 are highly effective chemotherapeutics in the treatment of Wilms'tumor, trophoblastic tumors, and rhabdomyosarcoma (Brockmann, 1961 and Hollstein, 1974).

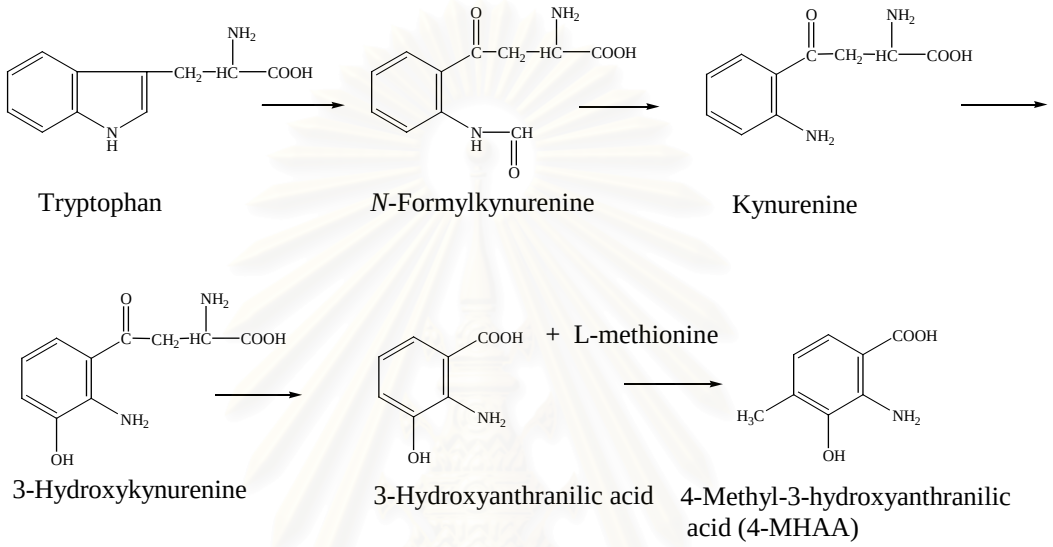
The group named actinomycins was coined by Waksman, who discovered these antibiotics in cultures of *Actinomyces antibioticus* in 1940 (Waksman and Woodruff, 1940). The molecule of the actinomycins consists of two pentapeptide residues and a chromophore named 2-amino-4,6-dimethyl-3-phenoxazone-1,9-dicarboxylic acid or 3-amino-1,8-dimethyl-2-phenoxazone-4,5-dicarboxylic acid or actinocin. Actinomycins with the same two pentapeptide residues are called isoactinomycins, while those with two different residues are referred to as anisoactinomycins. The pentapeptide chain which connected to benzenoid ring is called α chain and the other chain that attached to quinoid ring is called β chain. The chromophore moiety is responsible for orange to red color of the compounds. Thus the actinomycins are representatives of a class of natural products which can be named chromopeptides (Hollstein, 1974).

3.2 Synthesis

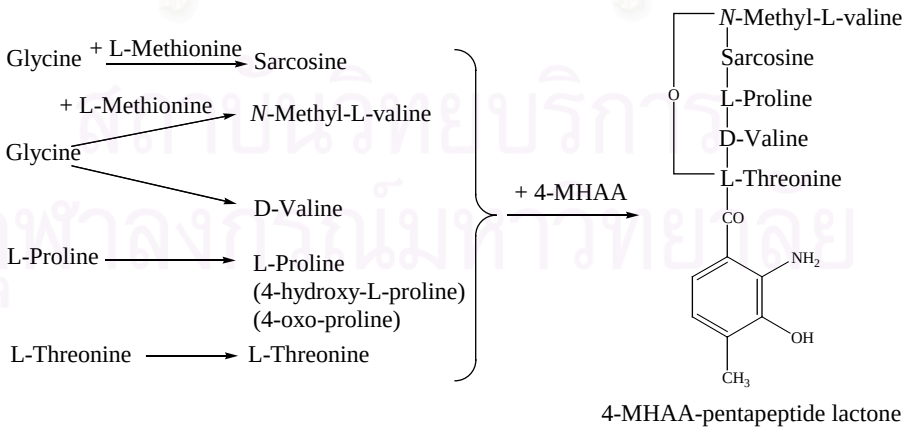
3.2.1 Biosynthesis

In the biosynthesis of actionmycins, threonine is synthesized from aspartic acid and threonine is precursor of isoleucine. Proline is synthesized from glutamic acid. Glycine is precursor of sarcosine. The main source of the *N*-methyl in

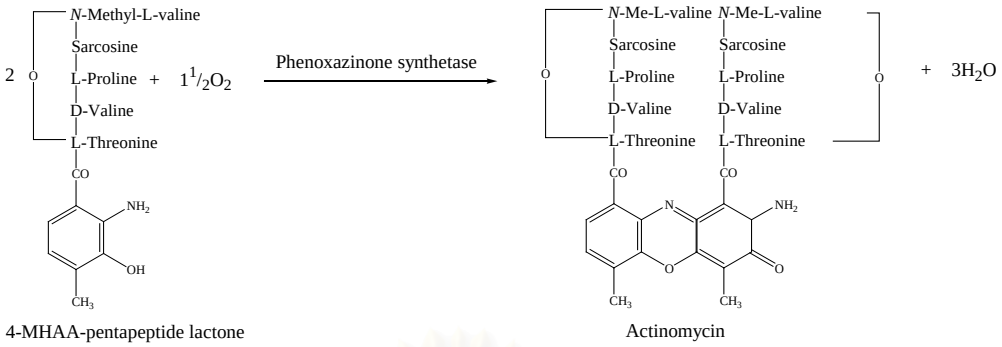
sarcosine and methylvaline is methionine. The biosynthesis of the chromophore unit proceeds from tryptophan *via* kynurenine, 3-hydroxykynurenine to 3-hydroxyanthranilic acid. The latter is methylated by methionine at the 4 position to give 4-methyl-3-hydroxyanthranilic acid, Schemes 1-3 show the entire biosynthetic pathway of the actinomycins (Hollstein, 1974).



Scheme 1 Biosynthesis of 4-methyl-3-hydroxyanthranilic acid from tryptophan and methionine (Hollstein, 1974).



Scheme 2 Biosynthesis of amino acid precursors from intracellular amino acid pool (Hollstein, 1974).

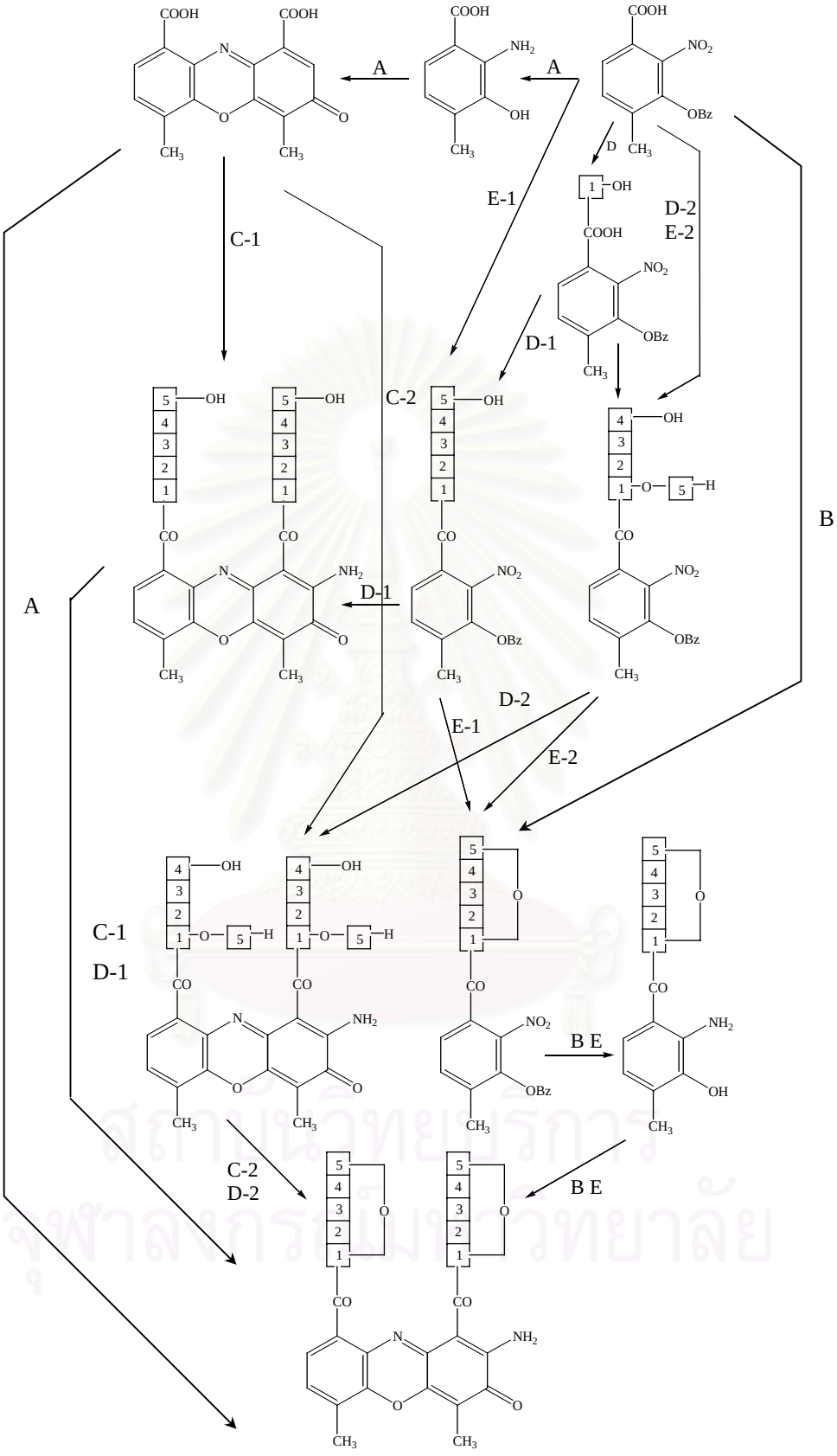


Scheme 3 Oxidative condensation of two molecules of 4-MHAA pentapeptide lactone to form one molecule of actinomycin (Hollstein, 1974).

3.2.2 Synthesis of actinomycins

There are several pathways in which actinomycins can be synthesized, as shown in Scheme 4. (Hollstein, 1974)

Path A: Connection of two intact lactone rings with 2-amino-4,6-dimethyl-3-phenoxazone-1,9-dicarboxylic acid, prepared by oxidative dimerization. Path B: Connection of an intact lactone ring with the monomeric unit, followed by oxidative dimerization. Path C: Connection of two peptide residues either in the open pentapeptide chain (C-1) or with amino acid 5 esterified with 1 (C-2) to 2-amino-4,6-dimethyl-3-phenoxazone-1,9-dicarboxylic acid followed by cyclization to the lactone rings. Path D: Building up of one pentapeptide chain (D-1) or one tetrapeptide esterified at 1 with 5 (D-2) on the monomeric unit, followed sucessively by oxidative dimerization and lactonization. Path E: Building up of one pentapeptide chain (E-1) or one tetrapeptide esterified at 1 with 5 (E-2) on the monomeric unit, followed sucessively by lactonization and oxidative dimerization.



Scheme 4 Pathways for the synthesis of actinomycins (Hollstein, 1974).

3.3 Actinomycin antibiotics

The actinomycins are a family of chromopeptide antibiotics isolated from various *Streptomyces* strains, of which twenty-five native and many synthetic variants are known. The natural actinomycins are shown as follows.

Actinomycin C_1 (D) **[1],** red crystals, was produced by *Streptomyces antibioticus* and *S. chrysomallus*. Actinomycin C_1 was active against gram-positive bacteria but had only a limited activity against gram-negative organisms. The minimum inhibitory concentration against *Bacillus subtilis* had been given as 0.02 μg/ml. This antibiotic was highly toxic, the lethal dose in mice was 5 mg/kg intraperitoneal or 50 mg/kg oral. It had antineoplastic effects in treatment of carcinoma. The cytostatic properties were due to its complexing with DNA and the consequential inhibition of RNA synthesis (Glasby, 1993).

Actinomycin C_2 **[2]**, red needles or bipyramids, was isolated from *antibioticus* and *S. chrysomallus*. The biological activity and toxicity are virtually identical to those given for actinomycin D. The concentration required to inhibit the growth of *Bacillus subtilis* was 0.25 μg/ml (Glasby, 1993).

Actinomycin C_{2a} **[3]**, the isomer of actinomycin C_2, was red needles antibiotic and was produced by *S. chrysomallus*. Its toxicity and biological activity were similar to those actinomycin D (Glasby, 1993).

Actinomycin C_3 **[4]** formed reddish crystals and was produced by *S. antibioticus* and *S. chrysomallus*. This compound was active against gram-positive bacteria and inhibited the growth of *B. subtilis* at a concentration of 0.25 μg/ml. Lethal dose in mice was 50 mg/kg when given orally. The antibiotic complexed with DNA and inhibited the synthesis of RNA (Glasby, 1993)

Actinomycins E_1-E_2 **[5,6]** were produced by *S. antibioticus* in a medium containing DL-isoleucine. Both formed red crystals. The biological activity was similar to actinomycin C_3 (Glasby, 1993).

Actinomycins F_1-F_4 **[7-10]**, reddish crystals, were isolated from *S. chrysomallus* and *Streptomyces* sp. BOP476 (NRRL 2580). They had a biological activity similar to that of actinomycin C_3 (Glasby, 1993).

Actinomycin F_8 **[11]** was produced by *S. antibioticus* as red plates. This compound was active against gram-positive organisms and possessed antineoplastic property (Glasby, 1993).

Actinomycin $X_{0\alpha}$ **[12]** was produced by *S. antibioticus* as reddish crystals. It had not yet been established in which chain the hydroxyproline moiety was located. This antibiotic inhibited the growth of *B. subtilis* at a concentration of only 0.15 times that of actinomycin C_3 (Glasby, 1993).

Actinomycin $X_{0\beta}$ **[13]**, reddish crystals, was isolated from a number of *Streptomyces* species. The minimum inhibitory concentration against *B. subtilis* was 0.25 times that of actinomycin C_3 (Glasby, 1993).

Actinomycin $X_{0\gamma}$ **[14]** furnished red prisms and was produced by *S. antibioticus*. It was active against gram-positive organisms and had antineoplastic effect (Glasby, 1993).

Actinomycin $X_{0\delta}$ **[15]** was produced by the reduction of actinomycin X_1 with aluminium isopropoxide. It formed red prisms. The minimum inhibitory concentration against *B. subtilis* was 0.4 times that of actinomycin C_3 (Glasby, 1993).

Actinomycin X_{1a} **[16]** was obtained from *Streptomyces* species. This compound was red crystals and the minnimum inhibitory concentration against *B. subtiles* was 0.7 times that of actinomycin C_3 (Glasby, 1993).

Actinomycin X_2 **[17]**, red crytals, was obtained from several *Streptomyces* species. The minimum inhibitory concentration again *B. subtilis* was 1.5 times that of actinomycin C_3 (Glasby, 1993).

Actinomycins Z_1-Z_5 **[18-22]** were isolated from *S. fradiae* and *S. antibioticus*. Actinomycins Z_3 and Z_5 were more potent than actinomycin D in cytotoxicity assays against three human tumor cell lines; stomach, liver, and breast (Glasby, 1993 and Lackner *et al*., 2000).

Actinomycins Pip 1α, Pip 1β and Pip 2 **[23-25]** was produced by *antibioticus* when DL-pipecolic acid was provided in the culture medium. All were red crystals and active against gram-positive bacteria (Glasby, 1993; Formica *et al*, 1968 and Formica and Katz *et al*, 1973).

4. Chemistry of antimycins

4.1 Historical development

Antimycins are a group of natural antibiotics that produced from various species of *Streptomyces* (Kim *et al*., 1999). These antibiotics are responsible for the antifungal activity and considerable biochemical use in connection with electron-transport processes because of its specific inhibitory action on certain of the enzymes involved (Birch *et al.*, 1991).

The structure of antimycins revealed two ring moieties, 3-formamidosalicylamide and 9-membered dilactone, which are connected through an amide bond bridge (Kim *et al*., 1999). The dilactone ring has alkyl and acyl side chains that were different in each antimycin.

4.2 Antimycin antibiotics

The antimycins are the natural antibiotics produced from various species of *Streptomyces*. These compounds have long been considered to be a group of very closely related compounds, differing only in the nature of acyl and alkyl substituents in the dilactone ring (Ha and Wilkins, 1989). The natural antimycins are shown as follow.

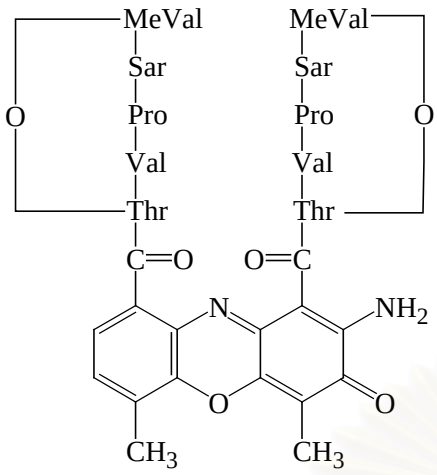
Antimycin A_{1a} **[26]** was isolated from *Streptomyces* sp. (Ha and Wilkins, 1989) and its isomer, antimycin A_{1b} **[27],** was produced by *Streptomyces* NRRL 2288, *S. kitazawaensis* and various species of *Streptomyces* (Birch *et al*., 1961; Ha and Wilkins, 1989; Selwood *et al*., 1990; and Glasby, 1993). Antimycin A_{1b} was a potent specific inhibitor of mammalian electron-transport systems (ubiquinol-cytochrome *c* oxidoreductase); in vitro moderately active against adult filarial *Dipetalonema viteae*; and exhibited insecticide, fungicide, and miticide activities. Because of high specificity and strong affinity, antimycin A_{1b} has been widely used in functional studies of the enzyme.

Antimycins A_{2a} and A_{2b} **[28-29]** were isomeric compounds that were produced by *Streptomyces* sp. (Ha and Wilkins, 1989).

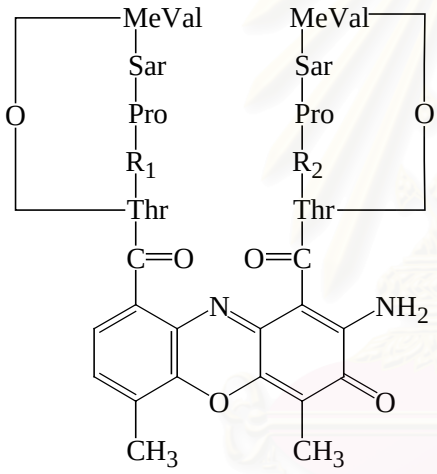
Antimycin A_{3a} **[30]** was isolated from *Streptomyces* sp. (Ha and Wilkins, 1989) and its isomer antimycin A_{3b} **[31],** was isolated from *S. blastmyceticus* as colorless needles (Ha and Wilkins, 1989 and Glasby, 1993). Antimycin A_{3b} was a potent specific inhibitor of the cytochrome *bc*1 complex (ubihydroquinone: cytochrome *c* oxidoreductase) (Miyoshi *et al.*, 1991) and had an antibiotic activity similar to that of antimycin A_{1b} (Glasby, 1993).

Two pair of isomers, antimycins A_{4a}, A_{4b} **[32-33]** and antimycins A_{5a}, A_{5b} **[34-35]** were isolated from *Streptomyces* sp. (Ha and Wilkins, 1989).

Urauchimycins A and B **[36-37]** were produced by *Streptomyces* sp. Ni-80, which was isolated from an unidentified sponge collected in Japan. They exhibited inhibitory activity against morphological differentiation of *Candida albicans* at the concentration of 10 μg/ml (Imamura *et al*, 1993).



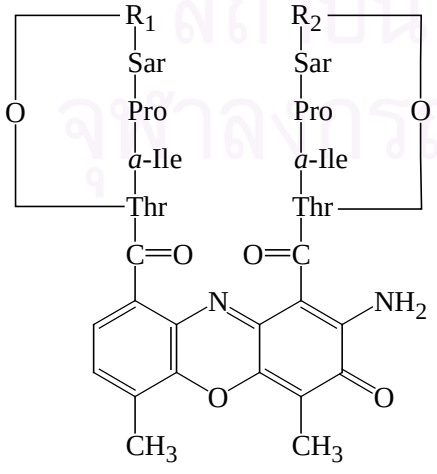
[1] Actinomycin D



[2] Actinomycin C_2 R_1 = Val, R_2 = *a*-Ile

[3] Actinomycin C_{2a} R_1 = *a*-Ile, R_2 = Val

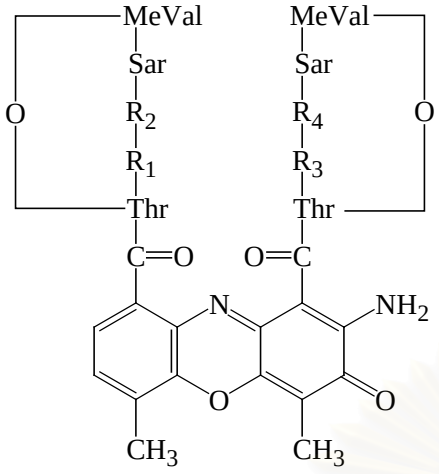
[4] Actinomycin C_3 $R_1 = R_2 =$ *a*-Ile



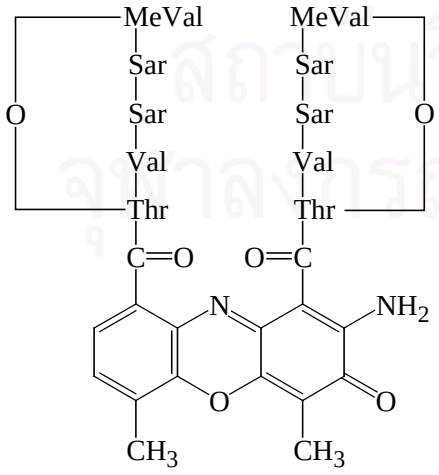
[5] Actinomycin E_1 R_1 = MeVal, R_2 = MeIle

or R_1 = MeIle, R_2 = MeVal

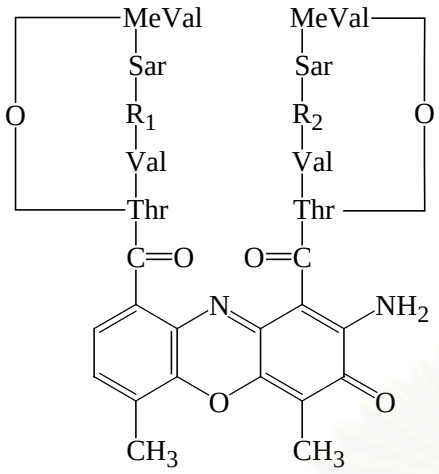
[6] Actinomycin E_2 $R_1 = R_2$ = MeIle



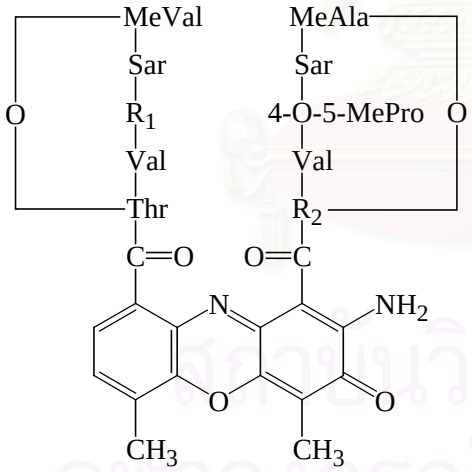
	R_1	R_2	R_3	R_4
[7] Actinomycin F_1	Val	Sar	*a*-Ile	Sar
or	*a*-Ile	Sar	Val	Sar
[8] Actinomycin F_2	Val	Pro	*a*-Ile	Sar
or	Val	Sar	*a*-Ile	Pro
or	*a*-Ile	Pro	Val	Sar
or	*a*-Ile	Sar	Val	Sar
[9] Actinomycin F_3	*a*-Ile	Sar	*a*-Ile	Sar
[10] Actinomycin F_4	*a*-Ile	Pro	*a*-Ile	Sar
or	*a*-Ile	Sar	*a*-Ile	Pro



[11] Actinomycin F_8



	R_1	R_2
[12] Actinomycin $X_{0\alpha}$	HyPro	Sar
or	Sar	HyPro
[13] Actinomycin $X_{0\beta}$	HyPro	Pro
or	Pro	HyPro
[14] Actinomycin $X_{0\gamma}$	Sar	Pro
or	Pro	Sar
[15] Actinomycin $X_{0\delta}$	*a*-HyPro	Pro
or	Pro	*a*-HyPro
[16] Actinomycin X_{1a}	Sar	OxoPro
or	OxoPro	Sar
[17] Actinomycin X_2	Pro	OxoPro
or	OxoPro	Pro



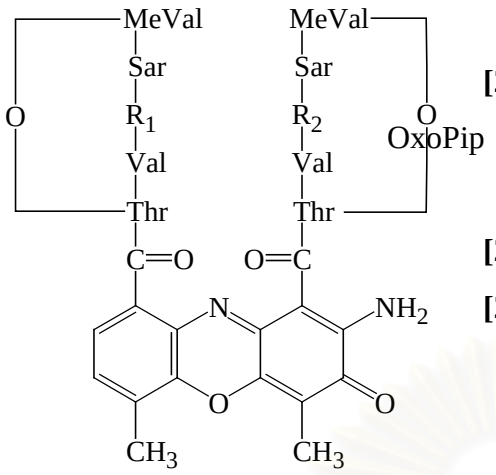
[18] Actinomycin Z_1 R_1 = 3-OH-5-MePro, R_2 = 4-OH-Thr

[19] Actinomycin Z_2 R_1 = 3-OH-5-MePro, R_2 = Thr

[20] Actinomycin Z_3 R_1 = 3-OH-5-MePro, R_2 = 4-Cl-Thr

[21] Actinomycin Z_4 R_1 = 5-MePro, R_2 = Thr

[22] Actinomycin Z_5 R_1 = 5-MePro, R_2 = 4-Cl-Thr

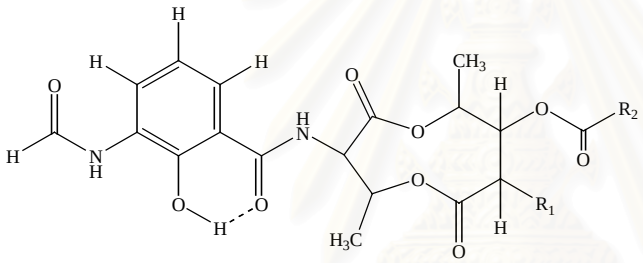


[23] Actinomycin Pip 1α R_1 = Pip, R_2 =

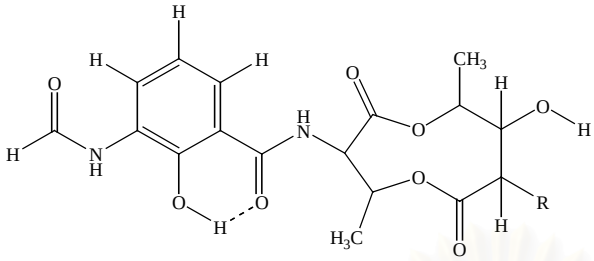
or R_1 = OxoPip, R_2 = Pip

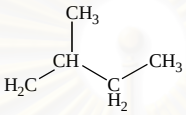
[24] Actinomycin Pip 1β R_1 = Pip, R_2 = Pro

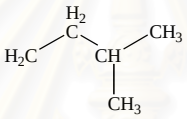
[25] Actinomycin Pip 2 $R_1 = R_2$ = Pip



	R_1	R_2
[26] Antimycin A_{1a}	n-C_6H_{13}	
[27] Antimycin A_{1b}	n-C_6H_{13}	
[28] Antimycin A_{2a}	n-C_5H_{11}	
[29] Antimycin A_{2b}	n-C_5H_{11}	
[30] Antimycin A_{3a}	n-C_4H_9	
[31] Antimycin A_{3b}	n-C_4H_9	
[32] Antimycin A_{4a}	n-C_4H_9	
[33] Antimycin A_{4b}	n-C_4H_9	
[34] Antimycin A_{5a}	n-C_2H_5	
[35] Antimycin A_{5b}	n-C_2H_5	



[36] Urauchimycin A R = 

[37] Urauchimycin B R = 

CHAPTER III

EXPERIMENTAL

1. Sample collection and isolation of actinomycetes

Two marine actinomycetes strains, AAR 1-1 and AAR 14, were isolated from two unidentified marine sponges, a bluish purple sponge AR 990325-20H and a purplish white sponge AR 990324-09K, respectively. The two sponges were collected at the depth of 35-40 feet from Adang-ravee Island, Satool Province, Thailand, in March 1999. In order to isolate the marine bacteria, 1 g of each sponge was cut into small pieces and suspended in 5.0 ml sterile sea water. The suspension was subsequently diluted to 1:10, 1:100, 1:1,000, 1:10,000 and 1:100,000 with sterile sea water. The forth and fifth dilutions (0.1 ml) were spreaded on the surface of marine agar (MA) plates (Brock *et al*., 1993) and then incubated at room temperature for 7-14 days. The powdery colonies of actinomycetes were picked up and streaked for purification on yeast extract-malt extract (YM) agar. Each single colony was tranferred to YM slants and incubated at room temperature for 7-14 days. The cultures were preserved at 4^{o}C and deposited at the Department of Pharmacognosy and the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

2. Identification of actinomycetes

The characterization of the actinomycetes strains AAR 1-1 and AAR 14 were carried out as dercribed in the methods for characterization of *Streptomyces* species (Shirling and Goltieb, 1966) and in Bergey's manual of systematic bacteriology (Williams, Sharpe and Holt, 1989).

2.1 Morphological and cultural characteristics

2.1.1 Determination of morphological characteristics

The morphological characteristics and spores bearing structures were studied by using simple inclined coverslip technique (Williams and Cross, 1971). The isolates were streaked in a cross-hatched pattern on the surface of YM agar plates then inserted coverslips at an angle. The agar plates with inclined coverslips were incubated at room temperature for 10-14 days until mature spores bearing hyphae were shown. The spores and mycelium were observed with scanning electron microscope (SEM) using the method of De man *et al.* (1986)

2.1.2 Cultural characteristics

Cultural characteristics were studied on the colors of mature aerial mycelium, substrate mycelium, spores, and diffusible soluble pigment when growing on different agar media, such as yeast extract-malt extract agar, inorganic salt-starch agar, oatmeal agar, tyrosine agar, and glycerol asparagine agar. The streaked plates were incubated at room temperature for 10-14 days, then examined the colors of aerial mycelium, substrate mycelium, spores, and their growth characteristics.

2.2 Physiological and biochemical characteristics

2.2.1 pH tolerance

The ability of growth was observed on YM agar plates with different pHs (pH 5, 6, 7, 8, 9, 10 and 11). The isolates were inoculated, and they were subsequently incubated at room temperature for 10-14 days.

2.2.2 Salt tolerance

The ability of microorganisms to grow on YM agar plates with different salinity was tested. The YM agar plates were supplemented with 1, 2, 3, 4, 5, 6, 7, 8, 9,10, 11, and 12% w/v of NaCl. The isolates were inoculated, and they were subsequently incubated at room temperature for 10-14 days.

2.2.3 Carbon utilization

Carbon utilization media were prepared and carbon sources were added to give concentration of approximately 1% w/v. The carbons required for the test were no carbon source (negative control), D-glucose (positive control), L-arabinose, sucrose, D-xylose, L-inositol, D-fructose, rhamnose, mannose, and raffinose. Streaked plates were observed after incubating at room temperature for 10-14 days. The growth on each carbon sourse was compared with the two controls, negative and positive.

2.2.4 Melanin formation

The isolates were cultivated on tyrosine agar plates and incubated at room temperature for 10-14 days. Melanoid pigments of inoculated plates and uninoculated control were compared. Culture forming a greenish brown to brown to dark diffusible pigment or a distinct brown pigment modified by other color should be recorded as positive (+). Absence of brown to black color, or total absence of diffusible pigment, should be recorded as negative (-) for melanoid pigment production.

2.2.5 Nitrate reduction

The isolates were incubated in tubes of broth containing 0.01% KNO_3 (at room temperature for 4-6 days). On the forth day, 1 ml of culture broth was transfered into a test tube and added three drops each of 0.33% sulphanilic acid in 5

N-acetic acid solution and 0.6% dimethyl - α - naphthylamine in 5 N-acetic acid solution, respectively. A red color showing the reduction of nitrate to nitrite indicated a positive reaction.

2.2.6 Starch hydrolysis

The isolates were inoculated on the surface of inorganic salt-starch agar plate and incubated at room temperature for 10-14 days. After incubation, Lugol's iodine solution was flooded on the plate. The clear colorless zones indicated that starch was hydrolysed.

2.2.7 Gelatin hydrolysis

The isolates were inoculated in tubes of nutrient gelatin broth and incubated at room temperature for 10-14 days. Liquefaction was examined after incubating 2-3 days by keeping in a refrigerator for 2 hours.

2.2.8 Milk coagulation and peptonization

The isolates were inoculated in tubes containing skim milk broth, and incubated at room temperature for 10-14 days. If milk was peptonized, it would be converted to clear solution and if it was coagulated, it would be precipitated.

2.2.9 Cellulose decomposition

The isolates were inoculated in tubes of cellulose decomposition broth and incubated at room temperature for 30 days. If the strain was considered to produce cellulase, the filter paper (Whatman No.1) would be digested.

2.3 Cell wall analysis

The chemical analysis of cell wall 2,6-diaminopimelic acid (DAP) isomers were carried out by the method of Komagata and Suzuki (1987). Approximately 3 mg of dried cells were hydrolysed with 1 ml of 6 N hydrochloric acid in a screw-capped tube at 100^{o}C for 18 hours. After cooling, each hydrolysate was filtered and then applied on the base line of a cellulose TLC plate (20 cm x 20 cm, E. Merck No.5577). The standard of DL-DAP was applied for reference purposes. The TLC plate was developed with the mixture of methanol, water, 6N hydrochloric acid and pyridine (80:26:4:10, v/v). The spots were visualized by spraying with 0.2% ninhydrin solution in water-saturated n-butanol followed by heating at 100^{o}C for 5 minutes. DAP isomers appeared as dark-green spots with R_f 0.29 (LL-isomer) and 0.24 (meso-and DD-isomer). Spots will gradually disappear in few minutes.

3. Chromatographic techniques

3.1 Analytical thin-layer chromatography (TLC)

Technique	:	One dimension ascending
Adsorbent	:	Silica gel F_{254} (E.Merck)
Layer thickness	:	250 μm
Distance	:	5 cm
Temperature	:	Room temperature (25-35^{o}C)
Detection	:	1. Visual detection under daylight. 2. Ultraviolet light at wavelengths of 254 and 365 nm. 3. Spraying with anisaldehyde reagent and heated until colors developed. 4. Visual detection in iodine vapor.

3.2 Column chromatography

3.2.1 Gel filtration chromatography

Gel filter	:	Sephadex LH-20 (Pharmacia Biotech AB)
Packing method	:	Sephadex gel was suspended in the eluant and left standing overnight to swell prior to use then poured into the column and allowed to sattle.
Sample loading	:	The sample was dissolved in a small volume of the eluant and loaded on top of the column.
Detection	:	Fractions were examined by TLC technique in the same manner as described in Section 4.1.

3.2.2 Flash column chromatography

Adsorbent	:	Silica gel 60 (No. 7734), particle size 0.063-0.200 mm (70-230 mesh ASTM) (E. Merck)
Packing method	:	The adsorbent was suspended in the eluant. The adsorbent slurry was poured into the column and then allowed to settle overnight.
Sample loading	:	The sample was dissolved in a small volume of the eluant and loaded on top of the column.
Detection	:	Fractions were examined by TLC technique in the same manner as described in Section 4.1.

4. Crystallization technique

Compound FK009 was crystallized by dissolving in chloroform until saturation and left standing at room temperature until orange-needle crystals were formed.

Compounds TK030, TK034 and TK034-2 were crystallized from a mixture of chloroform and methanol. Each compound was dissolved in methanol until saturation and 1-2 drops of chloroform were subsequently added. The solution was left standing at -29^{o}C until crystals were formed.

5. Spectroscopy

5.1 Ultraviolet (UV) absorption spectroscopy

UV spectra were obtained from a Milton Roy Spectronic 3000 Array spectrometer.

5.2 Infrared (IR) absorption spectroscopy

IR spectra were obtained from a Perkin Elmer 2000 FT-IR spectrometer. The compounds were examined with KBr disc.

5.3 Mass spectroscopy (MS)

Electrospray Ionization-Time of Flight mass spectra (ESI-TOF MS) were recorded on a Micromass LCT mass spectrometer. MeCN : H_2O (50:50) containing 0.02% of formic acid was used as a solvent.

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

^{1}H and ^{13}C NMR, DEPT 90 and 135, HMQC, HMBC and ^{1}H-^{1}H COSY spectra were obtained from a Bruker AVANCE DPX-300 FT-NMR spectrometer, operating at 300 MHz for protons and 75 MHz for carbons and chemical shifts (ppm) of the residual undeuterated solvents ($CDCl_3$, DMSO-d_6) were used as reference. Proton detected heteronuclear correlations were measured using

HMQC (optimized for $^{1}J_{HC}$ = 145 Hz) and HMBC (optimized for $^{n}J_{HC}$ = 3, 4, and 8 Hz) pulse sequences.

5.5 Optical rotation

Optical rotations were obtained from a Perkin-Elmer 341 polarimeter using a sodium lamp operating at 589 nm.

The measurements of UV, IR, NMR and optical rotation properties were performed at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The MS property was performed at the National Center for Genetic Engineering and Biotechnology (BIOTEC).

6. Solvents

All commercial grade solvents were redistilled prior to use.

7. Biological activity

7.1 Antimicrobial activity

The antimicrobial activitiy of the isolated fractions and pure compounds was examined by the agar disc diffusion method (Lorian, 1980) against *Staphylococcus aureus* ATCC 25933, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 10231. All tested bacteria were cultivated on tryptic soy agar (TSA, Difco®) slants at 37°C temperature for 24 hours, but the yeast strain was cultivated on Sabouraud's dextrose agar (SDA, Difco®) slant at 30°C temperature for 24 hours. After that the cultures were suspended in sterile normal saline solution (NSS). Each 20 ml of molten culture media was poured into 9-cm diameter petri dishes and allowed to gel. To prepare seed plates, a sterile cotton applicator swab was dipt into the suspensions of the test organisms, expressed excess

liquid and streaked a sterile petri dish containing gelatineous culture media in all directions. Test samples were dissolved in suitable solvent and then applied on sterile paper discs (6 mm diameter) or silica gel TLC aluminum sheets, and blank paper discs were impregnated with 20 μl of using solvent. After the disc or TLC plates were dried, they were applied on to the seed plates and allowed to prediffuse at 4^{o}C for 2 hours. The plates and discs were placed in an incubator for 24 hours at 30^{o}C or 37^{o}C for yeast and bacteria, respectively. The diameter and R_f values of inhibition zones were measured. The fractions those exhibited antimicrobial activity were subsequently selected for further study.

7.2 Antimalarial activity

Plasmodium faciparum, K1 multidrug resistant strain was cultured according to the method of Trager and Jensen (1976) using continuous cultures (in vitro) of a sexual erythrocytic stage. Quantitative assessment of antimalarial activity (*in vitro*) was determined by means of the microculture radioisotope technique based upon the method described by Desjardins *et al*., 1979. Effective concentration (EC_{50}) represents the concentration which causes 50% reduction in parasite growth as indicate by the *in vitro* uptake of [^{3}H]-hypoxanthine by *Plasmodium faciparum.* An EC_{50} value of 0.16 μg/ml (3.1 μM) was observed for the standard sample, chloroquine diphosphate, in the same test condition.

7.3 Cytotoxic activity

Cytotoxic activity against a breast cancer cell line (BC), a human epidermoid carcinoma cell line of the nasopharynx (KB), and a Vero cell line (African green monkey kidney cell line) was performed by sulforhodamine B (SRB) colorimetric method (Skehan *et al*., 1990).

7.4 Antituberculous activity

Antituberculous activity against *Mycobacterium tuberculosis* H37Ra was done using the Microplate Alamar Blue Assay (MABA) (Collins and Franzblau, 1997). The candidate compounds were tested against *Mycobacterium tuberculosis* H37Ra strain in the volume of 200 μl in 96 well microplate. In each well, a standard inoculum of the bacteria (of about 10^5 cell/ml) was incubated with the candidate compounds for about one week. Then Alamar Blue dye was added to a control well every day. If the color of the control well turns pink, which means that the bacteria has grown enough, then the dye is added to all wells in the plate and the color is read a day after.

8. Fermentation method

Both cultures, AAR 1-1 and AAR 14, were streaked on YM agar slants and incubated at room temperature for 7-10 days. Then each of culture was transferred into a 500 ml Erlenmeyer flask containing 250 ml of seed medium (GPBY) and incubated on a rotatory shaker (200 rpm) at room temperature for 3 days. Five milliliter of each seed culture was inoculated into 250 ml of production media (GPM) in 500 ml Erlenmeyer fiask and incubated on a rotatory shaker (200 rpm) at room temperature for 6 days.

9. Extraction and isolation

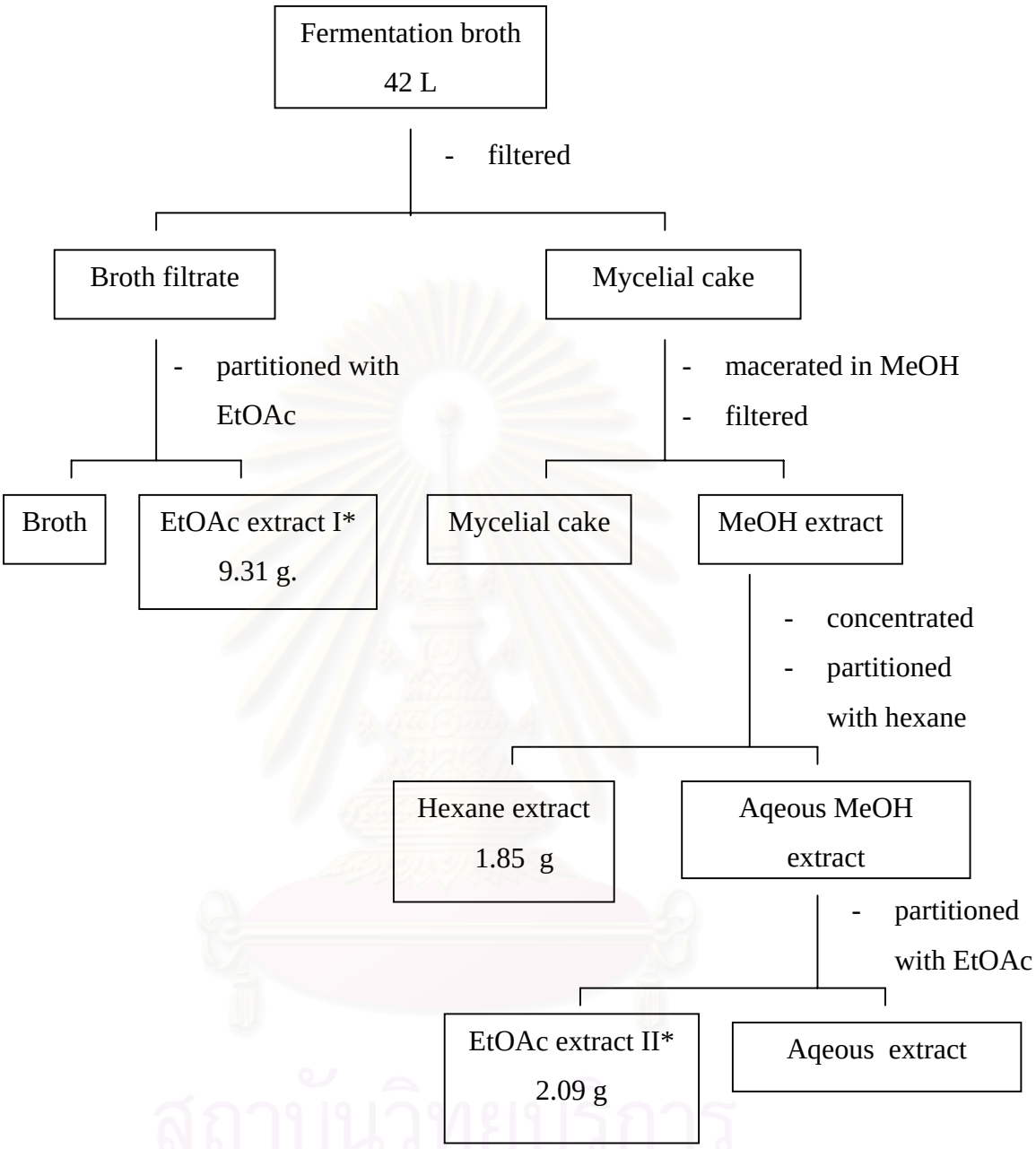
9.1 Extraction and isolation of marine actinomycete AAR 1-1 fermentation broth.

The fermentation broth (42 L) of marine actinomycete AAR 1-1 was filtered through a Buchner funnel packed with kieselguhr. The mycelial cake was washed with a minimum amount of water which was subsequently combined with the original broth filtrate. The broth filtrate was partitioned with ethyl acetate three times. The ethyl acetate parts were combined and then evaporated under reduced pressure at

temperature not exceeding 50^{o}C to yield 9.31 g of the ethyl acetate extract I. The mycelial cake was macerated in methanol for 3 days and then filtered. The filtrate was evaporated to remove methanol and was further partitioned with hexane and ethyl acetate, respectively. Each extract was evaporated to give 1.85 g for the hexane extract and 2.09 g for the ethyl acetate extract II (Scheme 5). All extracts were examined for antimicrobial activity using an agar diffusion method (Section 8.1)

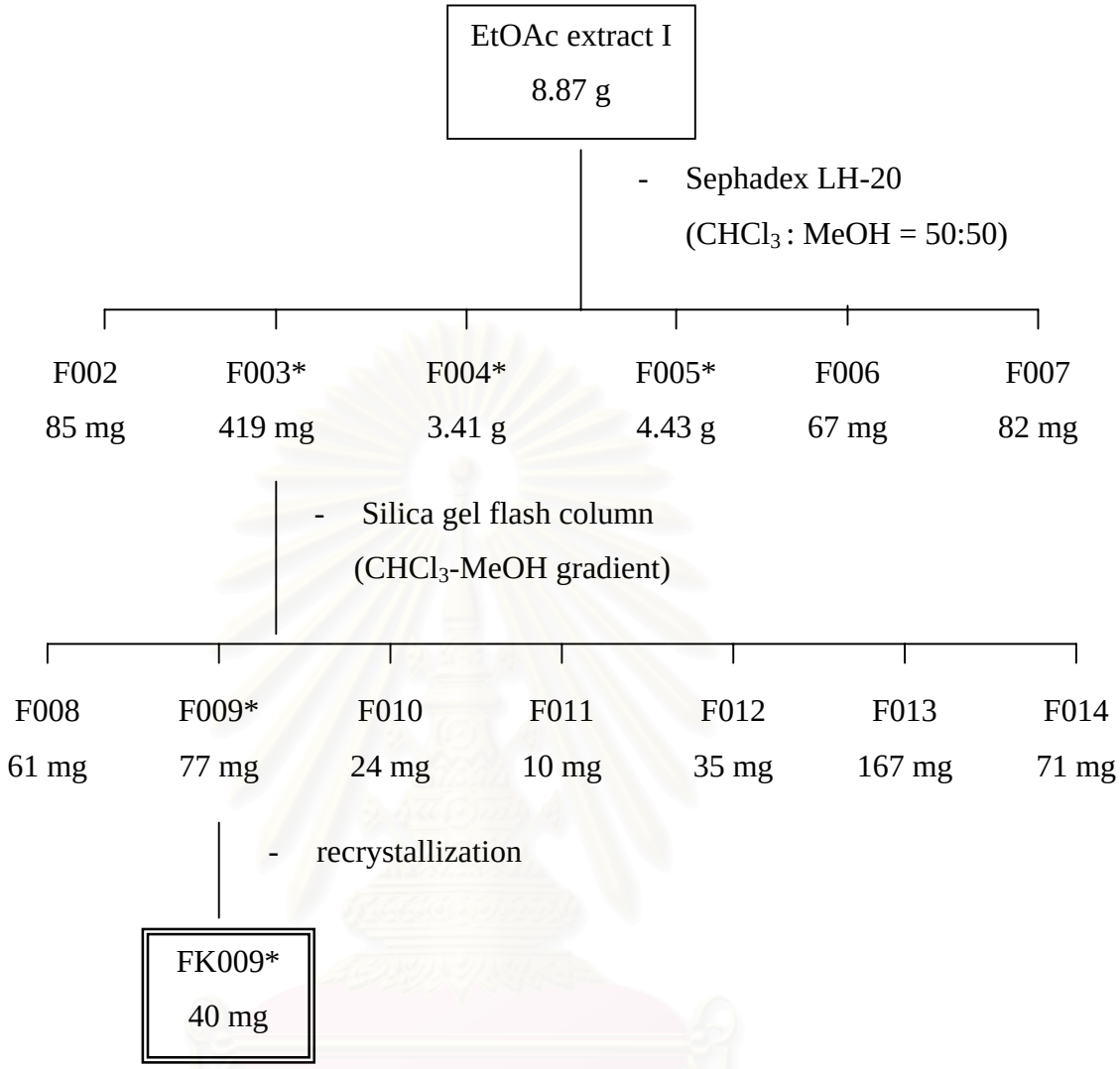
The ethyl acetate extract I (9.31 g) showed antimicrobial activity against *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633 with the inhibition zones of 16 and 19 mm (1 mg/disc), respectively.

The ethyl acetate extract I (8.87 g) was dissolved in a small amount of a mixture of $CHCl_3$: MeOH (50:50) and then fractionated by a Sephadex LH-20 gel filtration column. The column (2.5 cm inner diameter and 70 cm long) was eluted with isocratic elution of $CHCl_3$: MeOH (50:50) and 20-ml fractions were collected. The combined fractions were guided by TLC technique (Section 4.1, $CHCl_3$: MeOH, 9:1) to give six fractions (F002-F007). Directed by bioassay using antimicrobial activity against *Staphylococcus aureus* ATCC 25923, fraction F003 (419 mg) was further purified by a silica gel flash column (2.5 cm inner diameter and 16 cm long), using a gradient elution of $CHCl_3$ and MeOH from 100% $CHCl_3$ to 100% MeOH to yield seven fractions (F008-F014).



*Positive test for antimicrobial activity against *Staphylococcus aureus* ATCC 25923.

Scheme 5 Extraction of AAR 1-1 fermentation broth.



*Positive test for antimicrobial activity against *Staphylococcus aureus* ATCC 25923.

Scheme 6 Fractionation of the ethyl acetate extract I obtained from AAR 1-1.

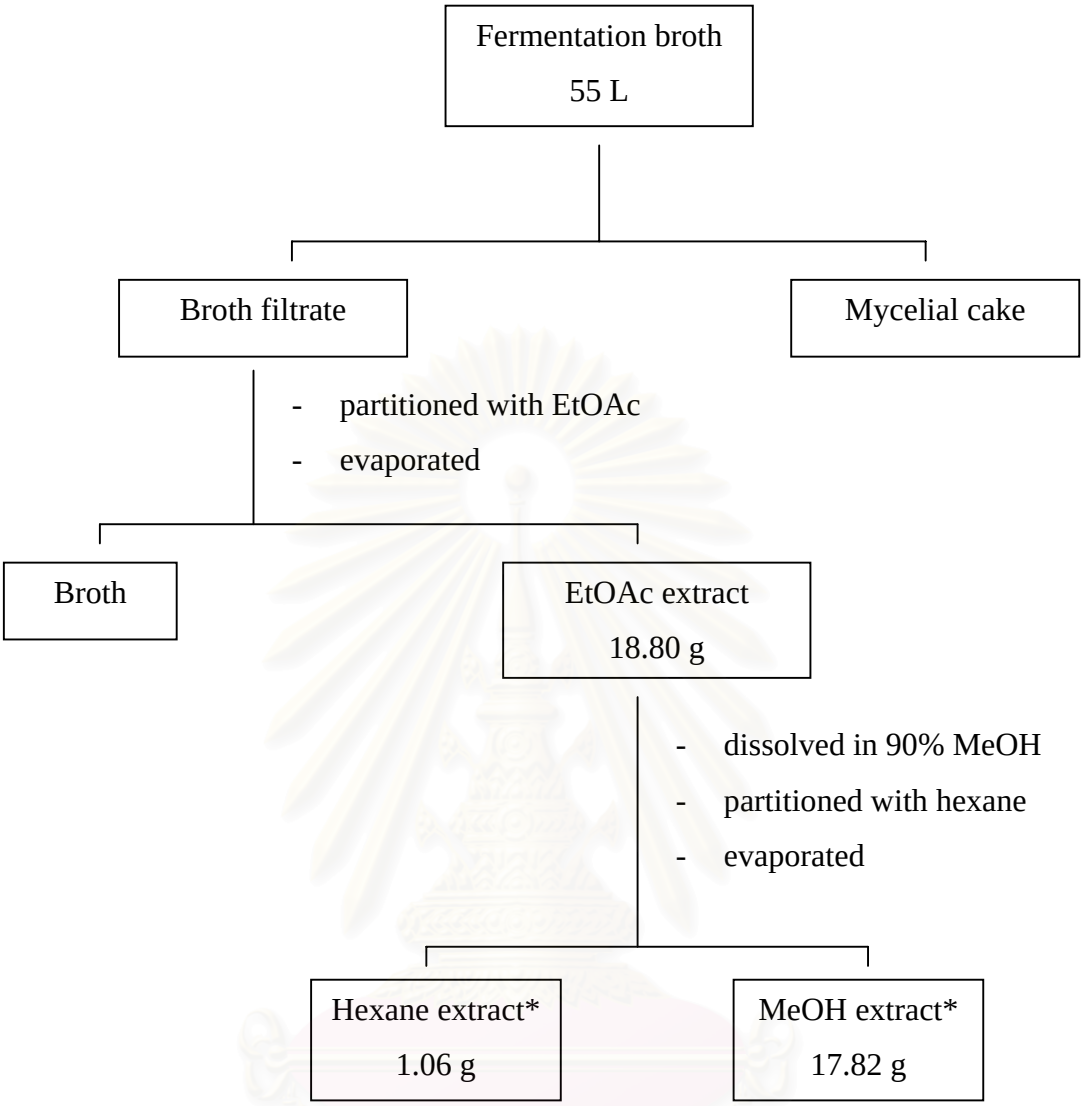
Fraction F009 (77 mg) gave orange needle crystals (FK009 40 mg, 9.5×10^{-4} % w/v of fermentation broth) which were washed with cool methanol and recrystallized from chloroform (Scheme 6). The TLC of purified crystals showed only one spot at R_f 5.2 ($CHCl_3$: MeOH = 9:1) under detection as described in Section 4.1. Compound FK009 showed several bioactivities, such as antimicrobial activity against *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633, antimalarial activity against *Plasmodium falciparum*, K1 multiple-drug resistant

strain, at EC_{50} 2.6 ng/ml, antituberculous activity against *Mycobacterium tuberculosis* H37Ra at MIC 0.125 μg/ml, and cytotoxic activity against KB cells (oral human epidermoid carcinoma) at ED_{50} 0.078 μg/ml and BC cells (breast cancer) at ED_{50} 0.15 μg/ml. The isolated compound FK009 was identified as actinomycin D which was a known compound previously isolated from terrestial *Streptomyces* sp. (Waksman and Woodruff, 1940).

9.2 Extraction and isolation of marine actinomycete AAR 14 fermentation broth.

The fermentation broth (55 L) of marine actinomycete AAR 14 was filtered through a Buchner funnel packed with kieselguhr. The mycelial cake was washed with a minimum amount of water which was subsequently combined with the original broth filtrate. The broth filtrate was repeatedly partitioned three times with ethyl acetate. The ethyl acetate parts were combined and evaporated under reduced pressure at temperature not exceeding 50^{o}C to yield 18.80 g. The crude ethyl acetate extract was dissolved in methanol solution and then partitioned with hexane. Each part was evaporated to give the hexane extract 1.06 g and the methanol extract 17.82 g (Scheme 7).

The methanol extract exhibited fungistatic activity against *Candida albicans* ATCC 10231 at the concentration of 1 mg/disc with inhibition zone 35 mm in agar disc diffusion method (Lorian, 1980). Therefore, the methanol extract was subsequently purified by chromatographic technique.

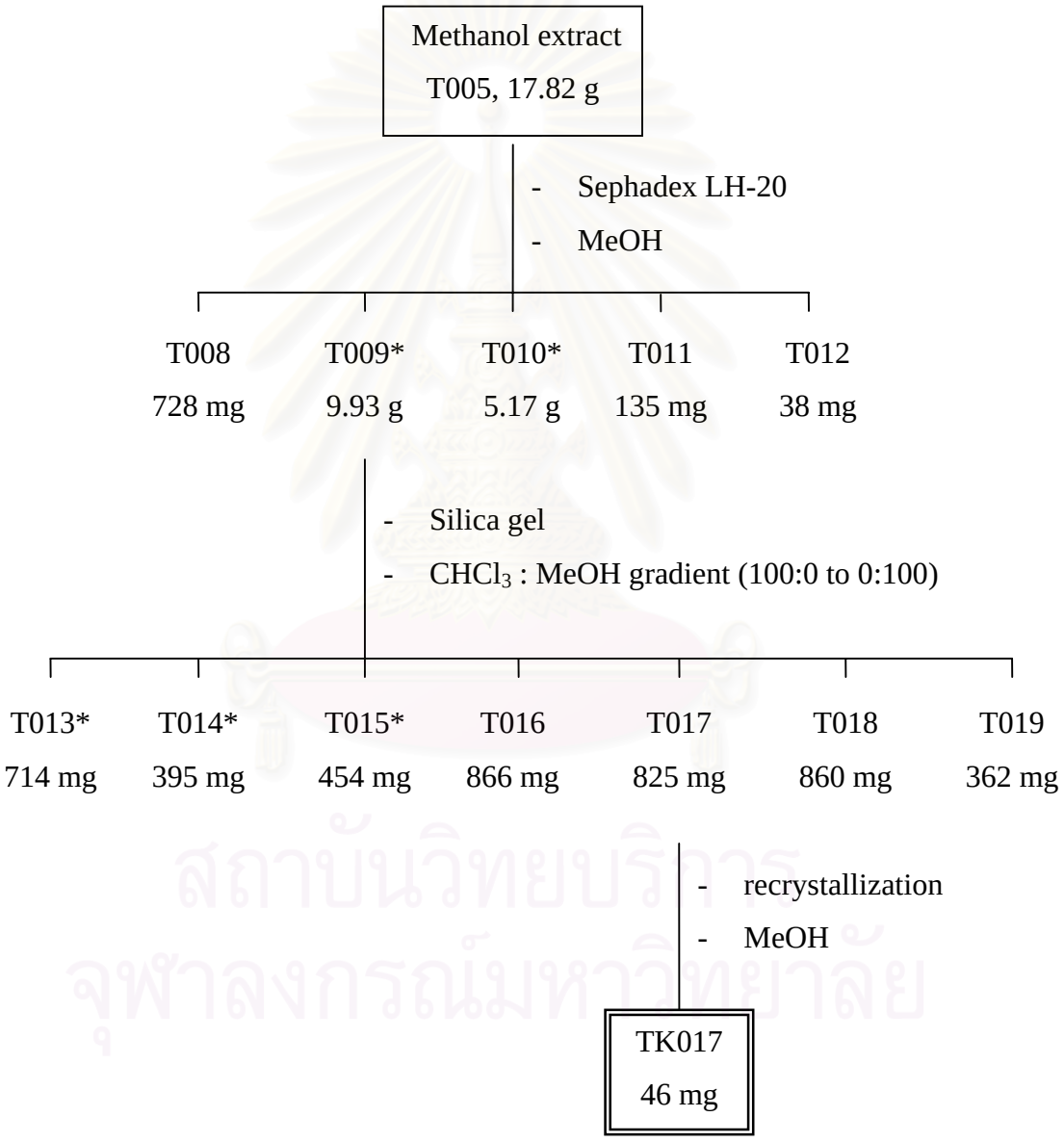


*Positive test for antimicrobial activity against *Candida albicans* ATCC 10231.

Scheme 7 Extraction of AAR 14 fermentation broth.

The methanol extract was dissolved in a small volume of methanol and then fractionated by a Sephadex LH-20 gel filtration column. Methanol was used to elute the column and 20-ml fractions were collected. Fractions were combined by TLC technique (Section 4.1, $CHCl_3$: MeOH = 9:1) and gave five fractions (T008-T012). The fractions T009 and T010 showed fungistatic against *Candida albicans* ATCC 10231 with 32 and 36 mm of inhibition zones at concentration 1 mg/disc, respectively. Fraction T009 was then purified by flash column chromatography over

silica gel (5 cm inner diameter and 15 cm long) with gradient elution of 100% $CHCl_3$ to 100% MeOH. This procedure provided seven fractions (T013-T019) and fraction T017 gave colorless crystals (TK017 46 mg, 8.4×10^{-5} % w/v of fermentation broth) which were washed with cool methanol. The TLC of purified crystals showed only one spot at R_f 0.22 ($CHCl_3$: MeOH, 9:1) under detection as described in Section 4.1 (Scheme 8).



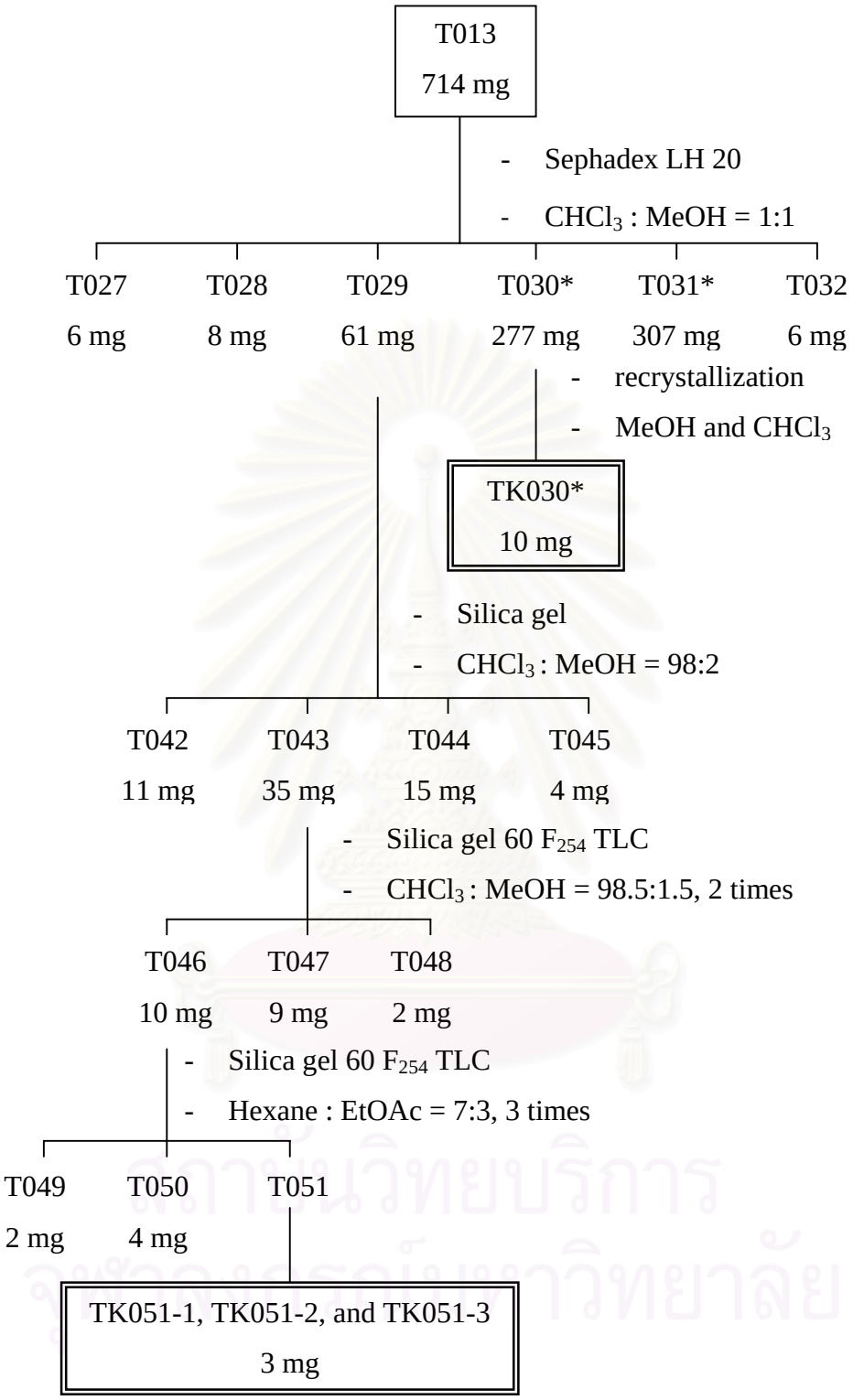
*Positive test for antimicrobial activity against *Candida albicans* ATCC 10231.

Scheme 8 Fractionation of the methanol extract obtained from AAR 14.

Fractions T013, T014, and T015 showed fungistatic activity against *Candida albicans* ATCC 10231 with 40, 36, and 32 mm of inhibition zones at concentration 1 mg/disc, respectively. Therefore, fractions T013 and T014 were further purified.

Fraction T013 (714 mg) was purified on a Sephadex LH-20 column (2.5 cm inner diameter and 90 cm long) and eluted with $CHCl_3$: MeOH (50:50), providing six fractions (T027-T032). Fraction T030 (277 mg) gave amorphous solid that was recrystallized from a mixture of $CHCl_3$ and MeOH to yield 10 mg of TK030 (1.8×10^{-5} % w/v of fermentation broth) (Scheme 8a). The TLC chromatogram of the purified compound showed only one spot at R_f 0.13 in $CHCl_3$: EtOAc (6:4). This compound showed fungistatic activity against *Candida albicans* ATCC 10231 with inhibition zone 24 mm at concentration 500 μg/disc.

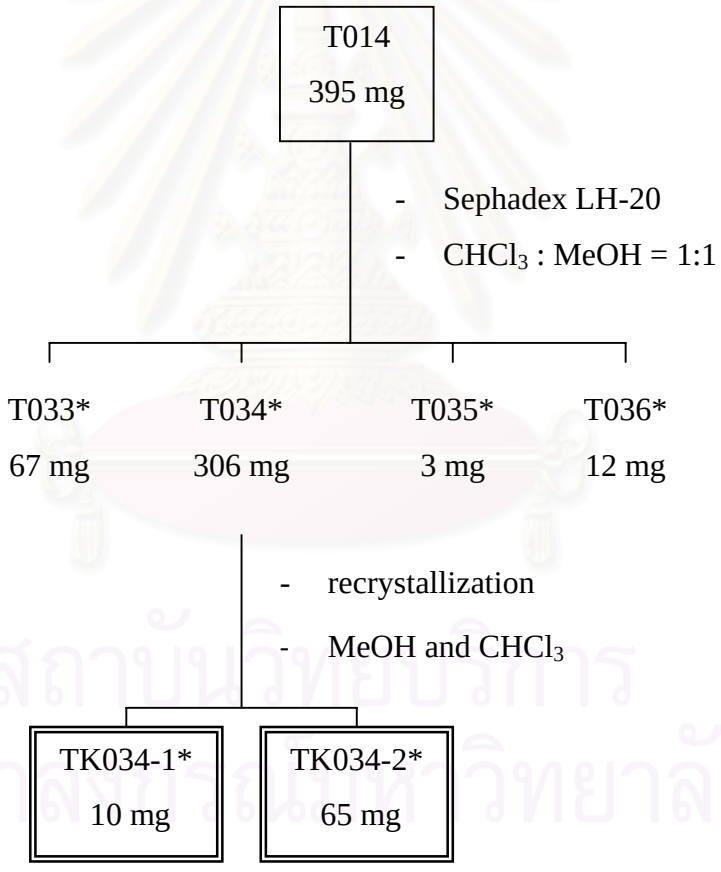
Fraction T029 showed interesting spots under the detection of UV_{254} so it was further chromatographed over a silica gel flash column (2.5 cm inner diameter and 13 cm long), using a mixture of $CHCl_3$: MeOH (98:2), to give four fractions (T042-T045). Fraction T043 was further purified by silica gel 60 F_{254} TLC plate with a solvent system of $CHCl_3$: MeOH (98.5:1.5, double developing) to yield three fractions (T046-T048). Then fraction T046 was purified by silica gel 60 F_{254} TLC plate, eluted with hexane : EtOAc (7:3) as a developing solvent, triple developing, to give three fractions (T049-T051). Fraction T051 gave white amorphous solid (TK051-1 and TK051-2 3 mg, 5×10^{-6} % w/v of fermentation broth) that showed a single spot (R_f 0.5, $CHCl_3$: EtOAc = 6:4) under detection as described in Section 4.1 (Scheme 8a).



*Positive test for antimicrobial activity against *Candida albicans* ATCC 10231.

Scheme 8a Fractionation of fraction T013 obtained from AAR 14.

Fraction T014 was further seperated on a Sephadex LH-20 column (2.5 cm inner diameter and 90 cm long) using $CHCl_3$: MeOH (50:50) as an eluting solvent to yield four fractions (T033-T036), as shown in Scheme 8b. All fractions showed fungistatic activity against *Candida albicans* ATCC 10231. Fraction T034 gave two pure compounds, TK034-1 (10 mg, 1.8×10^{-5} % w/v of fermentation broth) and TK034-2 (65 mg, 1.2×10^{-4} % w/v), at R_f 0.20 and 0.13 ($CHCl_3$: EtOAc = 6:4) which exhibited fungistatic activity against *Candida albicans* ATCC 10231 with inhibition zones 25 and 24 mm at concentration 500 μg/disc, respectively. The compound TK034-2 was similar to compound TK030.



*Positive test for antimicrobial activity against *Candida albicans* ATCC 10231.

Scheme 8b Fractionation of fraction T014 obtained from AAR 14.

CHAPTER IV

RESULTS AND DISCUSSION

1. Sample collection and isolation of actinomycetes.

Two unidentified marine sponges were collected at 35-40 feet deep, from Adang-ravee Island, Satool Province, Thailand, in March 1999. The actinomycete strain AAR 1-1 was isolated by a spread plate technique on marine agar (MA) plate (Brock *et al*., 1993) from a bluish purple marine sponge AR 990325-20H (Figure 1) and the strain AAR 14 was isolated from a purplish white marine sponge AR 990324-09K (Figure 2).

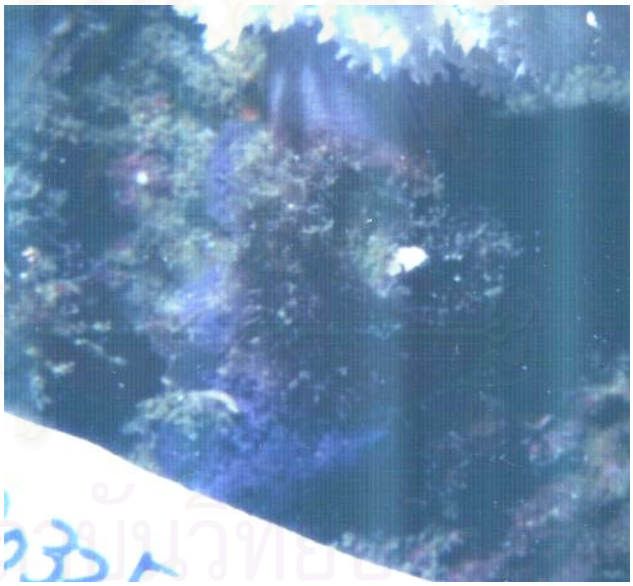


Figure 1 The unidentified bluish purple marine sponge AR 990325-20H.

Figure 2 The unidentified purplish white marine sponge AR 990324-09K.

2. Identification of actinomycetes.

The characteristics described in Bergey's manual of systematic bacteriology (Williams, Sharpe and Holt, 1989) and the methods for characterization of *Streptomyces* species (Shirling and Gottlieb, 1966) were employed for the identification and characterization of the strains.

2.1 Morphological and cultural characteristics.

2.1.1 Morphological characteristics

The morphological characteristics were observed after cultivation on yeast extract-malt extract (YM) agar plate at room temperature for 14 days. The strain AAR 1-1 was a gram positive bacterium. Vegetative growth was powdery colonies with irregular edge and yellow pigment. Aerial mycelium growing abundantly on YM agar medium was initially white and then changed to yellowish gray during the incubation period as shown in Figure 3. The hyphae were 0.6-0.9 μm in diameter with cylindrical spores in coiled chains on lateral branches of the aerial

hyphae. The cylindrical spores were smooth and 0.5 to 0.6 by 0.7 to 0.9 μm in size as shown in Figure 4.

Figure 3 The colonial appearance of *Streptomyces* sp. AAR 1-1 on YM agar incubated for 14 days.

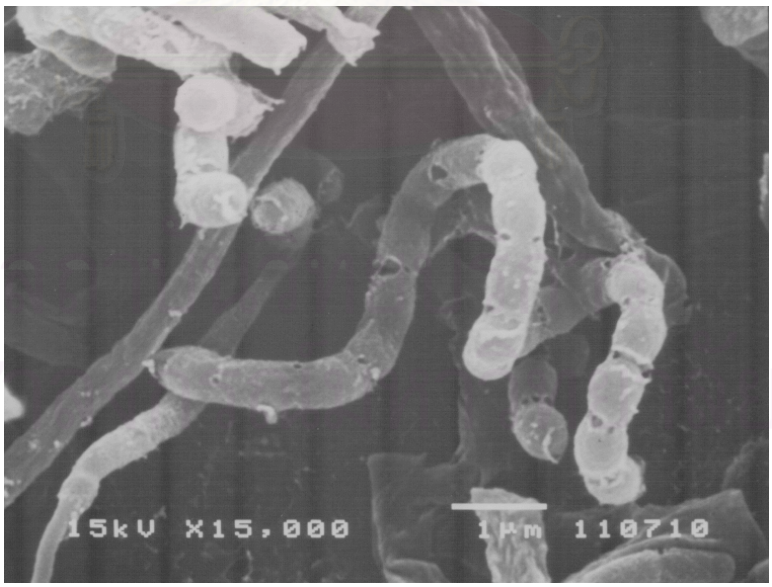


Figure 4 Scanning electron micrograph of *Streptomyces* sp. AAR 1-1 on YM agar incubated for 14 days.

The strain AAR 14 was a gram positive bacterium. Vegetative growth on YM agar plate at 14 days old was powdery colonies with irregular edge as shown in Figure 5. Aerial mycelium was powdery brownish yellow, 0.5-0.6 μm in diameter. The hyphae were long, straight and branching. The spores were smooth and cylindrical in shape, 0.5 by 0.6 to 1.0 μm, as shown in Figure 6.

Figure 5 The colonial appearance of *Streptomyces* sp. AAR 14 on YM agar incubated for 14 days.

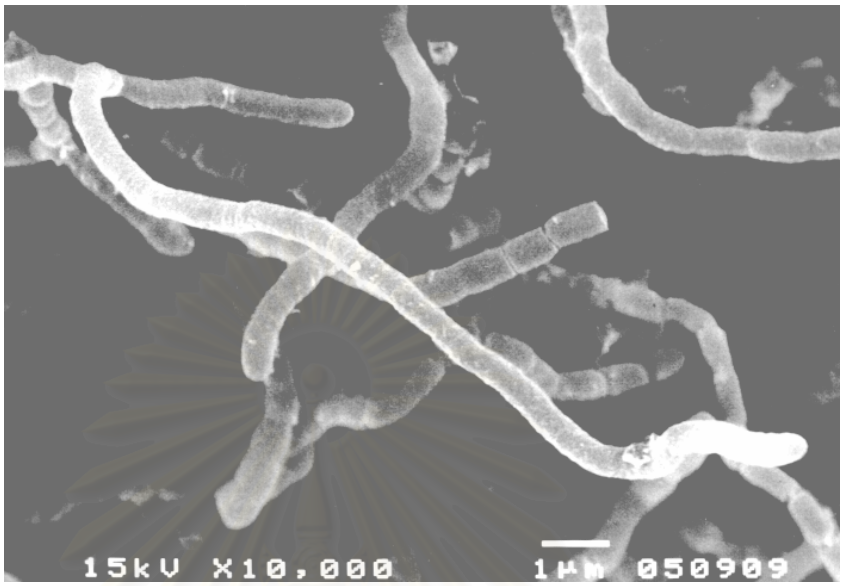


Figure 6 Scanning electron micrograph of *Streptomyces* sp. AAR 14 on YM agar incubated for 14 days.

2.1.2 Cultural characteristics

Cultural characteristics of the strains AAR 1-1 and AAR 14 cultivated on various media at room temperature for 14 days are shown in Tables 3 and 4, respectively.

The strains AAR 1-1 and AAR 14 grew well on yeast extract-malt extract agar, tyrosine agar, glycerol asparagine agar, and inorganic salt-starch agar and moderately on oatmeal agar. For the strain AAR 1-1, the color of aerial mycelium was yellowish gray on all media but the color of substrate mycelium was different, gold or brownish olive, on particular medium. However, the strain AAR 1-1 produced soluble yellow pigment on all media. For strain AAR 14, the color of substrate mycelium and aerial mycelium were different in each medium. The strain AAR 14 did not produce soluble pigment in any media.

Table 3 Cultural characteristics of AAR 1-1.

Medium	Growth	Color tone of[a]		
		Substrate mycelium	Aerial mycelium	Soluble pigment
Yeast extract- malt extract agar	Good	Gold	Yellowish gray	Yellow
Oatmeal agar	Moderate	-	Yellowish gray	Yellow
Tyrosine agar	Good	Brownish olive	Yellowish gray	Yellow
Glycerol asparagine agar	Good	Brownish olive	Yellowish gray	Yellow
Inorganic salt-starch agar	Good	Gold	Yellowish gray	Yellow

[a]Color names used in this table were based on the Jacal Color Card L 2200 (Japan Color Research Institute).

Table 4 Cultural characteristics of AAR 14.

Medium	Growth	Color tone of[a]		
		Substrate mycelium	Aerial mycelium	Soluble pigment
Yeast extract- malt extract agar	Good	Yellowish brown	Grayish yellow	-
Oatmeal agar	Moderate	-	Yellowish white	-
Tyrosine agar	Good	Reddish brown	Grayish pink	-
Glycerol asparagine agar	Good	Light yellowish brown	Grayish olive	-
Inorganic salt-starch agar	Good	Dark yellowish brown	Grayish pink	-

[a]Color names used in this table were based on the Jacal Color Card L 2200 (Japan Color Research Institute).

2.2 Physiological and biochemical characteristics.

The strains AAR 1-1 and AAR 14 were able to use D-glucose, L-arabinose, D-xylose, D-fructose, and mannose as carbon sources and showed different activities as shown in Table 5.

Table 5 Carbon utilization of AAR1-1 and AAR14.

Carbon	AAR 1-1	AAR 14
No carbon (negative control)	-	-
D-glucose (positive control)	+ + + +	+ + + +
L-arabinose	+ + + +	+ + + +
Sucrose	+	+
D-xylose	+ + +	+ + + +
L-inositol	+ + +	+
D-fructose	+ + + +	+ + + +
Rhamnose	+ + + +	+
Mannose	+ + + +	+ + + +
Raffinose	-	+

Carbon utilization; ++++, very good growth; +++, good growth; ++, moderate growth; +, weak growth; -, no growth

Table 6 Physiological and biochemical characteristics of AAR 1-1 and AAR 14.

Characteristics	AAR 1-1	AAR 14
Melanin formation	-	-
Nitrate reduction	+	+
Starch hydrolysis	+	+
Gelatin hydrolysis	-	+
Coagulation of milk	-	-
Peptonization of milk	+	+
Cellulose decomposition	-	-
Growth at pH :		
5	+	+
6	+	+
7	+	+
8	+	+
9	+	+
10	+	+
11	+	+
Growth in NaCl :		
0-10%	+	+
11%	-	+
12%	-	+

+, positive ; -, negative

The strains AAR 1-1 and AAR 14 showed positive reaction for nitrate reduction, starch hydrolysis, and milk peptonization. They could grow at pH 5-11 and in 0-10% w/v of NaCl. In addition, the strain AAR 14 probably grew in 11-12% w/v of NaCl. Variable characteristics are shown in Table 6.

2.3 Cell wall analysis

The chemical analysis of cell wall component of the two strains exhibited that they contain LL-isomer of diaminopimelic acid, cell wall type I.

On the basis of morphological, cultural, physiological, and biochemical characteristics and cell wall component, the strains AAR 1-1 and AAR 14 were identified as of the genus *Streptomyces*. However their species could not be identified on the basis of available data.

3. Structure elucidation of the isolated compounds.

3.1 Structure elucidation of atinomycin D (FK009)

Compound FK009 was isolated as orange-needle crystals. The ESI-TOF mass spectrum (Figure 26) established the pseudomolecular ion peak at *m/z* 1255$(M+H)^+$ implying the molecular formula of $C_{62}H_{86}N_{12}O_{16}$. The UV spectrum (in MeOH) (Figure 27) exhibited λ_{max} (log ε) at 240 (4.35) and 442 (4.20) nm. The IR spectrum (Figure 28) confirmed the presence of amide carbonyl (ν_{max} 1636 cm^{-1}) and lactone carbonyl (ν_{max} 1751 cm^{-1}). This compound showed optical rotation $[\alpha]_D^{25}$ -181.72^o (c = 0.132 in MeOH).

The 300 MHz ^1H NMR spectrum (in $CDCl_3$) of compound FK009 (Figures 29-30) exhibited eighty four protons which were refered to thirteen methyl proton signals [δ 0.71 (6H), 0.86, 0.88, 0.92, 0.94, 1.09, 1.10, 1.22 (6H), 2.22, 2.53, 2.85 (6H), 2.87, and 2.91 ppm], thirteen methylene proton signals (δ 1.79, 1.84, 2.05 (2H), 2.26 (2H), 2.67, 2.95, 3.59, 3.61, 3.70 (2H), 3.80, 3.94, 4.69, and 4.76 ppm), ten methine proton signals [δ 2.14 (4H), 2.64 (2H), 3.49 (2H), 4.46, 4.57, 5.15 (2H), 5.94, 6.00, 7.34, and 7.62 ppm] and four amide proton signals (δ 7.15, 7.76, 7.94, and 8.09 ppm). The 75 MHz ^{13}C NMR spectrum (in $CDCl_3$) of this compound (Figure 31) showed sixty two carbons which could be classified by the DEPT 90 and DEPT 135 spectra (Figures 32-33) as sixteen methyl carbon signals (δ 7.93, 15.17, 17.53, 17.93,

19.12, 19.18, 19.22, 19.24, 19.37, 19.42, 21.70, 21.80, 34.92, 34.98, 39.27, and 39.38 ppm), eight methylene carbon signals (δ 22.98, 23.14, 31.05, 31.37, 47.38, 47.64, 51.40, and 51.44 ppm), fifteen methine carbon signals [δ 26.96 (2C), 31.63, 31.91, 54.93, 55.30, 56.22, 56.38, 58.74, 58.91, 71.27, 71.43, 74.97, 75.04, 125.72, and 130.10 ppm] and twenty one quaternary carbon signals [δ 101.59, 111.38, 127.42, 128.95, 132.48, 140.29, 144.92, 145.69, 147.47, 165.83, 166.07, 166.24, 166.28, 167.32, 167.41, 168.20, 168.68, 173.02 (2C), 173.10, 173.48, and 178.85 ppm].

Compound FK009 has two major parts, one phenoxazone ring and two pentapeptide cyclic rings (α and β rings). The ^{1}H,-^{1}H COSY spectrum (Figures 37-38) exhibited the correlations in each amino acid as follow: threonine (α); N-H (δ 7.15 ppm) to H-2 (δ 4.46 ppm) and H-3 (δ 5.15 ppm) to H-4 (δ 1.22 ppm): threonine (β); N-H (δ 7.76 ppm) to H-2 (δ 4.57 ppm) and H-3 (δ 5.15 ppm) to H-4 (δ 1.22 ppm): valine (α); N-H (δ 8.09 ppm) to H-2 (δ 3.49 ppm) and H-4 (δ 1.09 or 1.10 ppm) to H-3 (δ 2.14 ppm) and the 3-Me protons (δ 0.86 or 0.88 ppm): valine (β); N-H (δ 7.94 ppm) to H-2 (δ 3.49 ppm) and H-4 (δ 1.09 or 1.10 ppm) to H-3 (δ 2.14 ppm) and the 3-Me protons (δ 0.86 or 0.88 ppm): proline (α); H-2 (δ 6.00 ppm) to H_a-3 (δ 2.67 ppm) and H_b-3 (δ 1.79 ppm) and and H_a-4 (δ 2.05 ppm) to H_b-4 (δ 2.26 ppm), H_a-5 (δ 3.94 ppm) and H_b-5 (δ 3.70 ppm): proline (β) ; H-2 (δ 5.94 ppm) to H_a-3 (δ 2.95 ppm) and H_b-3 (δ 1.84 ppm) and H_a-4 (δ 2.05 ppm) to H_b-4 (δ 2.26 ppm), H_a-5 (δ 3.80 ppm) and H_b-5 (δ 3.70 ppm): sarcosine (α); H_a-2 (δ 3.61 ppm) to H_b-2 (δ 4.69 ppm): sarcosine (β); H_a-2 (3.59 ppm) to H_b-2 (δ 4.76 ppm); methylvaline (α); 3-Me protons (δ 0.92 or 0.94 ppm) to H-2 (δ 2.64 ppm), H-3 (δ 2.64 ppm) and H_3-4 (δ 0.71 ppm): methylvaline (β); 3-Me protons (δ 0.92 or 0.94 ppm) to H-2 (δ 2.64 ppm), H-3 (δ 2.64 ppm) and H_3-4 (δ 0.71 ppm). The ^{1}H-^{1}H COSY spectrum of FK009 also showed the ortho coupling correlation in the phenoxazone ring of H-7 (δ 7.34 ppm) to H-8 (δ 7.62 ppm).

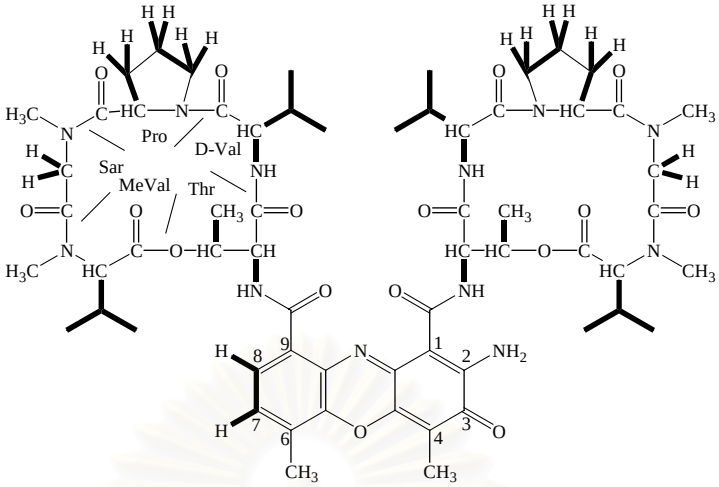


Figure 7 The ^{1}H-^{1}H correlations (bold line) in the ^{1}H-^{1}H COSY spectrum of actinomycin D (FK009).

According to the HMQC spectrum (Figures 34-36), the one-bond connected protons and carbons could be assigned as summarized in Table 7. The complete ^{13}C assignments of FK009 were achieved by analysis of the HMBC ($^{n}J_{HC}$ = 4 and 8 Hz) spectra (Figures 39-45). The HMBC spectra exhibited the ^{1}H-^{13}C long-range correlations in the phenoxazone ring as follows: H-7 at (7.34 ppm) to C-9 (δ 132.48 ppm), C-5a (δ 140.29 ppm), C-6-Me (δ 15.17 ppm), and C-8 (δ 125.72 ppm); H-8 (δ 7.62 ppm) to C-6 (δ 127.42 ppm), C-5a (140.29 ppm), C-7 (δ 130.10 ppm), and C-9-CO (δ 166.24 ppm); the 4-Me protons (δ 2.22 ppm) to C-3 (δ 178.85 ppm), C-4 (δ 111.34 ppm), and C-4a (δ 145.69 ppm); and the 6-Me protons (δ 15.17 ppm) to C-5a (δ 140.29 ppm), C-6 (δ 127.42 ppm), C-7 (δ 130.10 ppm), and C-8 (δ 125.72 ppm). For the two pentapeptide cyclic rings, the ^{1}H-^{13}C the long-range correlations obtained from the HMBC spectra were shown as follows. Threonine of the α chain showed long-range correlations of the amide proton (δ 7.15 ppm) to the amide carbonyl carbon (C-1, δ 168.20 ppm) and C-9-CO of the phenoxazone ring (δ 166.24 ppm); H-2 at 4.46 ppm to C-9-CO of the phenoxazone ring (δ 166.24 ppm); H-3 (5.15 ppm) to amide carbonyl carbon (C-1, δ 168.20 ppm); and H-4 at δ 1.22 ppm to C-2 (δ 55.30 ppm) and C-3 (δ 74.97 or 75.04 ppm). Threonine of the β chain showed long-range correlations of amide proton (δ 7.76 ppm) to amide carbonyl carbon (δ 168.68

ppm) and C-1-CO of the phenoxazone ring (δ 166.28 ppm); H-2 (δ 4.57 ppm) to C-1-CO of the phenoxazone ring (δ 166.28 ppm); H-3 (5.15 ppm) to amide carbonyl carbon (C-1, δ 168.68 ppm); and H-4 (δ 1.22 ppm) to C-2 (δ 54.93 ppm) and C-3 (δ 74.97 or 75.04 ppm). Valines of the α and β chains showed long-range correlations of the 3-Me protons (δ 0.86 and 0.88 ppm) to C-2 (δ 58.74 or 58.91 ppm), C-3 (δ 31.63 or 31.91 ppm), and C-4 (19.12 or 19.18 ppm); and H-4 (δ 1.09 and 1.10 ppm) to C-2 (δ 58.74 or 58.91 ppm), C-3 (δ 31.63 or 31.91 ppm), and C-3-Me (δ 19.22 or 19.24 ppm). Sarcosine of the α chain showed long-range correlations of methyl amide protons (δ 2.85 ppm) to amide carbonyl carbon (C-1, δ 165.83 or 166.07 ppm), C-2 (δ 51.40 or 51.44 ppm), and amide carbonyl carbon (C-1) of proline (δ 173.02 ppm); H_a-2 (δ 3.61 ppm) to amide carbonyl carbon (C-1, δ 165.83 or 166.07 ppm) and amide carbonyl carbon (C-1) of proline (δ 173.02 ppm). Sarcosine of the β chain showed long-range correlations of methyl amide protons (δ 2.85 ppm) to amide carbonyl carbon (C-1, δ 165.83 or 166.07 ppm), C-2 (δ 51.40 or 51.44 ppm), and amide carbonyl carbon (C-1) of proline (δ 173.02 ppm); H_a-2 (δ 3.59 ppm) to amide carbonyl carbon (C-1, δ 165.83 or 166.07 ppm) and amide carbonyl carbon (C-1) of proline (δ 173.02 ppm). Methylvalines of the α and β chains showed long-range correlations of methyl amide protons (δ 2.87 and 2.91 ppm) to amide carbonyl carbon (C-1) of sarcosine (δ 165.83 or 166.07 ppm), and C-2 (δ 71.27 or 71.43 ppm); H-2 and H-3 (δ 2.64 ppm) to C-3 (δ 26.96 ppm), C-3-Me (δ 21.70 or 21.80), and amide carbonyl carbon (C-1) of sarcosine (δ 165.83 or 166.07 ppm); the 3-Me protons (δ 0.92 and 0.94 ppm) to C-2 (δ 71.27 or 71.43 ppm), C-3 (δ 26.96 ppm), and C-4 (δ 19.37 or 19.42 ppm); and H-4 (δ 0.71 ppm) to C-2 (δ 71.27 or 71.43 ppm), C-3 (δ 26.96 ppm), and C-3-Me (δ 21.70 or 21.80 ppm). The connection between the phenoxazone ring and two pentapeptide cyclic was achieved by the analysis of HMBC spectra. The amide proton of threonine of the α chain (δ 7.15 ppm) was attached to C-9-CO of the phenoxazone ring and the amide proton of threonine of the β chain (δ 7.76 ppm) was attached to C-1-CO of the phenoxazone ring. The HMBC correlations of FK009 are shown in Figures 8-13 and summarized in Table 7.

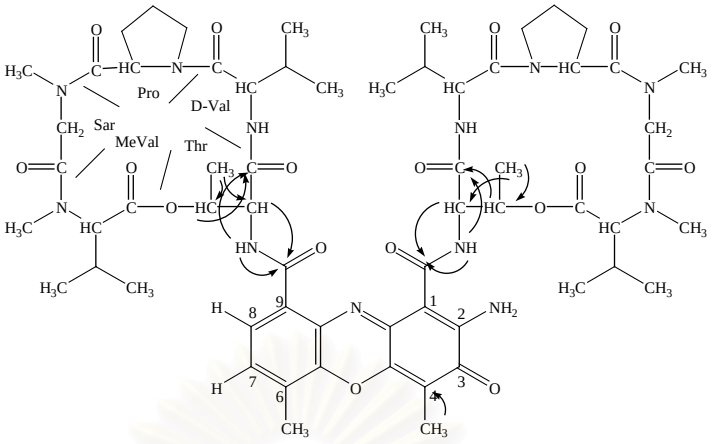


Figure 8 The ^{1}H-^{13}C long-range correlations in the HMBC spectrum of threonines of actinomycin D (FK009).

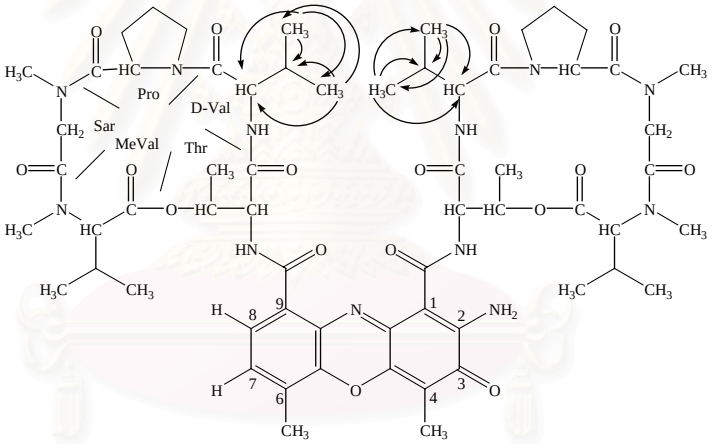


Figure 9 The ^{1}H-^{13}C long-range correlations in the HMBC spectrum of valines of actinomycin D (FK009).

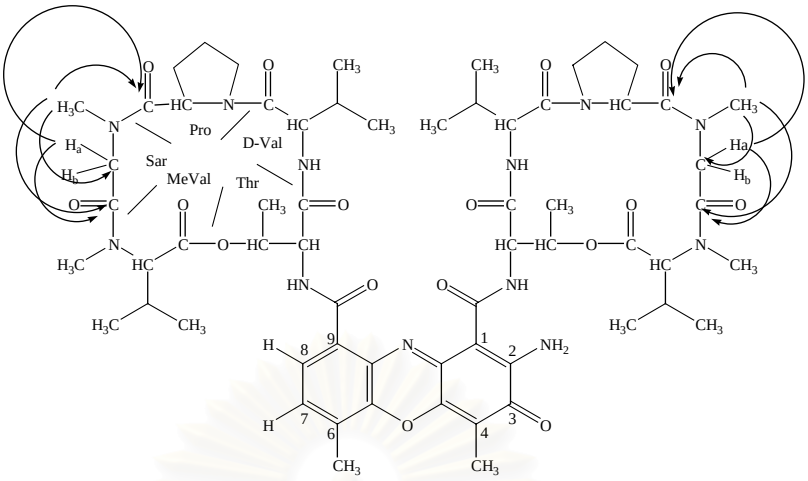


Figure 10 The ^{1}H-^{13}C long-range correlations in the HMBC spectrum of sarcosines of actinomycin D (FK009).

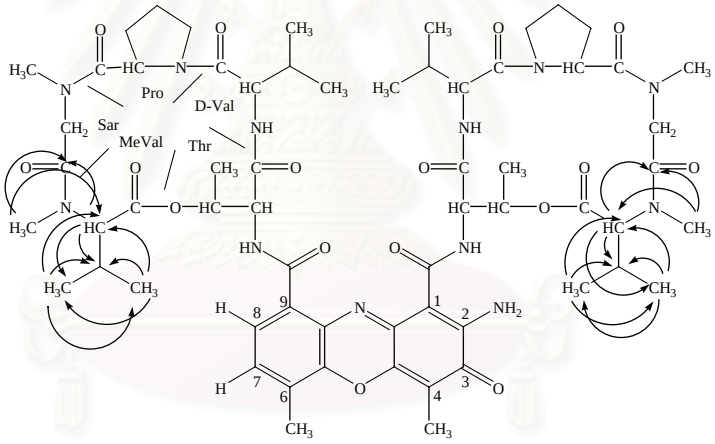


Figure 11 The ^{1}H-^{13}C long-range correlations in the HMBC spectrum of methylvalines of actinomycin D (FK009).

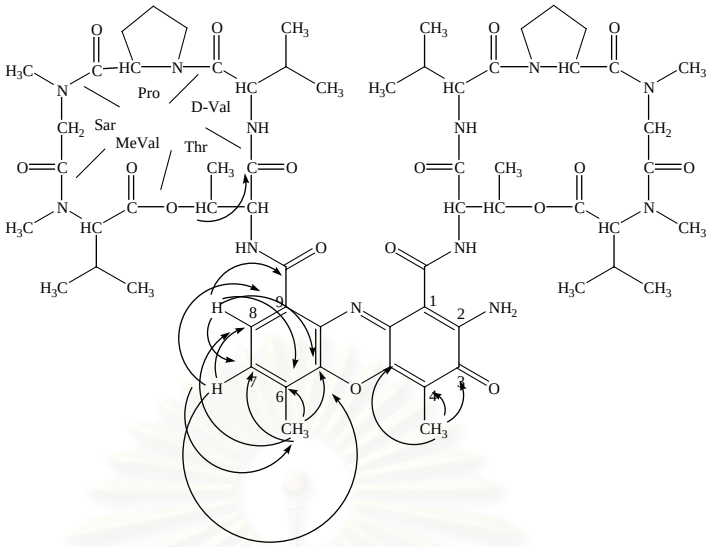


Figure 12 The ^{1}H-^{13}C long-range correlations in the HMBC spectrum of the phenoxazole ring of actinomycin D (FK009).

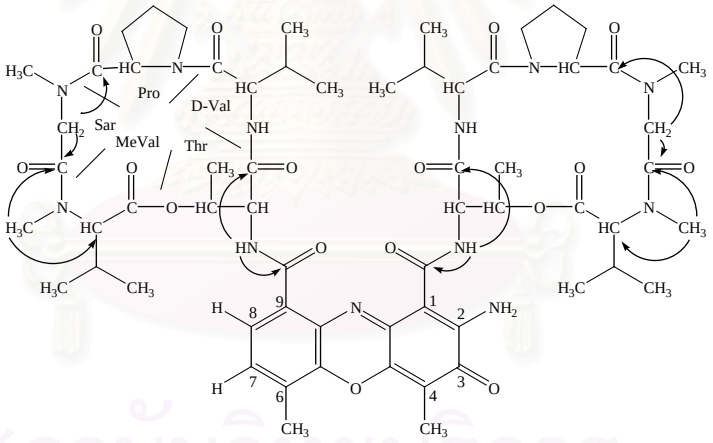


Figure 13 The important ^{1}H-^{13}C long-range correlations in the HMBC spectrum of actinomycin D (FK009).

Table 7 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) and the ^{1}H, ^{13}C long-range correlations in the HMBC spectrum of actinomycin D (FK009).

FK009 (actinomycin D)					
	Position	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	^{1}H-^{13}C long-range correlations in	
				HMBC ($^{n}J_{HC}$ = 8 Hz)	HMBC ($^{n}J_{HC}$ = 4 Hz)
Chrom	1	101.59	-		
	2	147.47	-		
	3	178.85	-		
	4	111.38	-		
	4a	145.69	-		
	5a	140.29	-		
	6	127.42	-		
	7	130.10	7.34, 1H, d (7.77)	9	5a, 6-Me, 8
	8	125.72	7.62, 1H, d (7.77)	6	5a, 7, 9-CO
	9	132.48	-		
	9a	128.95	-		
	10a	144.92	-		
	2-NH_2	-	-	-	-
	4-CH_3	7.93	2.22, 3H	3, 4, 4a	3, 4, 4a
	6-CH_3	15.17	2.53, 3H	5a, 6, 7	5a, 6, 7, 8
	9-CO	166.24	-		
	1-CO	166.28	-		
Thr(1, 2)[a]	1	168.20/168.68	-		
	2	55.30 (1)	4.46, 1H, dd (6.68, 1.90)		9-CO
		54.93 (2)	4.57, 1H, dd (6.34, 1.90)		1-CO
	3	74.97/75.04	5.15, 2H, dq (5.04, 1.90)		1 (T)
	4	17.53/17.93	1.22, 6H, d (5.04)	2 (T), 3(T)	2 (T), 3(T)
	NH	-	7.15, 1H, d (6.68) (1)[a]		1 (T), 1-CO, 9-CO
		-	7.76, 1H, d (6.34) (2)[a]		1 (T), 1-CO, 9-CO
D-Val(1, 2)[a]	1	173.10/173.48	-		
	2	58.74/58.91	3.49, 2H, m		
	3	31.63/31.91	2.14, 2H, m		
	3-Me	19.22/19.24	0.86, 3H, d (6.79)	2 (V), 3 (V), 4 (V)	3 (V), 4 (V)
			0.88, 3H, d (6.87)	2 (V), 3 (V), 4 (V)	3 (V), 4 (V)

Table 7 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) and the ^{1}H, ^{13}C long-range correlations in the HMBC spectrum of actinomycin D (FK009) (continued).

FK009 (actinomycin D)					
	Position	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	^{1}H-^{13}C long-range correlations in	
				HMBC ($^{n}J_{HC}$ = 8 Hz)	HMBC ($^{n}J_{HC}$ = 4 Hz)
	4	19.12/19.18	1.10, 3H, d (6.34)	2 (V), 3 (V), 3-Me (V)	2 (V), 3 (V), 3-Me (V)
			1.09, 3H, d (6.38)	2 (V), 3 (V), 3-Me (V)	2 (V), 3 (V), 3-Me (V)
	NH	-	8.09, 1H, d (5.85) (1)[a]		
		-	7.94, 1H, d (6.19) (2)[a]		
Pro(1,2)[a]	1	173.02,2CO	-		
	2	56.22/56.38	6.00, 1H, d (9.05) (1)[a]		
			5.94, 1H, d (9.08) (2)[a]		
	3	31.05/31.37	1.79, 1H, m / 2.67, 1H, m (1)[a]		
			1.84, 1H, m / 2.95, 1H, m (2)[a]		
	4	22.98/23.14	2.05, 2H, m		
			2.26, 2H, m		
	5	47.38 (1)[a]	3.70, 1H, m / 3.94, 1H, m (1)[a]		
		47.64 (2)[a]	3.70, 1H, m / 3.80, 1H, m (2)[a]		
Sar(1,2)[a]	1	165.83/166.07	-		
	2	51.40/51.44	3.61, 1H, d (18.31) / 4.69, 1H, d (18.31) (1)[a]		1 (S), 1(P)
			3.59, 1H, d (18.13) / 4.76, 1H, d (18.13) (2)[a]		1 (S), 1(P)
	N-CH_3	34.92/34.98	2.85, 6H, s	2 (S), 1(P)	1 (S), 2 (S), 1(P)
MeVal (1,2)[a]	1	167.32/167.41	-		
	2	71.27/71.43	2.64, 2H, m	3(MeV)	3(MeV), 3-Me(MeV), 1 (S)
	3	26.96	2.64, 2H, m	3(MeV)	3(MeV), 3-Me(MeV), 1 (S)

Table 7 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) and the ^{1}H, ^{13}C long-range correlations in the HMBC spectrum of actinomycin D (FK009) (continued).

FK009 (actinomycin D)					
	Position	δ_C (ppm)	δ_H (ppm), mult. (J in Hz.)	^{1}H-^{13}C long-range correlations in	
				HMBC ($^{n}J_{HC}$ = 8 Hz)	HMBC ($^{n}J_{HC}$ = 4 Hz)
	3-Me	21.70/21.80	0.92, 3H, d (5.64)	2(MeV), 3(MeV), 4(MeV)	2(MeV), 3(MeV)
			0.94, 3H, d (5.25)	2(MeV), 3(MeV), 4(MeV)	2(MeV), 3(MeV)
	4	19.37/19.42	0.71, 6H, d (5.06)	2(MeV), 3(MeV), 3-Me(MeV)	2(MeV), 3(MeV), 3-Me(MeV)
	N-CH_3	39.27 (1)[a]	2.87, 3H, s	1 (S), 2 (MeV)	1 (S), 2 (MeV)
		39.38 (2)[a]	2.91, 3H, s	1 (S), 2 (MeV)	1 (S), 2 (MeV)

Chrom, chromophore

Thr, T, threonine

Val, V, valine

Sar, S, sarcosine

Pro, P, proline

MeVal, MeV, methylvaline

[a](1), amino acid in α chain; (2), amino acid in β chain

Table 8 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) of FK009 and actinomycin D (in DMSO-d_6 and $CDCl_3$).

	Position	FK009 (actimomycin D)		Actinomycin D[a] (in DMSO)		Actinomycin D (in $CDCl_3$)	
		δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	δ_H (ppm), mult. (*J* in Hz.)[b]	δ_H (ppm), mult. (*J* in Hz.)[c]
Chrom	1	101.59	-	ND	-	-	-
	2	147.47	-	ND	-	-	-
	3	178.85	-	ND	-	-	-
	4	111.38	-	ND	-	-	-
	4a	145.69	-	ND	-	-	-
	5a	140.29	-	ND	-	-	-
	6	127.42	-	ND	-	-	-
	7	130.10	7.34, 1H, d (7.77)	ND	7.42	7.37	7.37 (8.0)
	8	125.72	7.62, 1H, d (7.77)	ND	7.44	7.64	7.64 (8.0)
	9	132.48	-	ND	-	-	-
	9a	128.95	-	ND	-	-	-
	10a	144.92	-	ND	-	-	-
	4-CH_3	7.93	2.22, 3H	9.2	2.15	2.24	2.27
	6-CH_3	15.17	2.53, 3H	16.2	2.52	2.56	2.55
	1-CO/ 9-CO	166.24/166.28		ND	-	-	-
	2-NH	-	-	-	ND	7.3-7.4	7.0-7.5
Thr (1,2)	1	166.24/166.28	-	ND	-	-	-
	2	54.93	4.46, 1H, dd (6.68, 2.10)	56.0	4.53	4.51 (2.0-2.5)	4.50, (6.8/2)
		55.30	4.57, 1H, dd (6.34, 1.80)			4.62 (6.5)	4.60, (6.6)
	3	74.97/75.04	5.15, 2H, dq	75.1	5.11	5.21 (2.0-2.5)/5.15	5.21 (6.1/2), 5.17 (6.1/2)
	4	17.53/17.93	1.22, 6H, d (5.04)	18.5	1.20, d	1.27 (6.0)	1.26, (6.2)
	NH	-	7.15, 1H, d (6.68)	-	7.49, br s	7.20 (6.8)	7.21, (6.8)
		-	7.76, 1H, d (6.34)			7.82 (6.2)	7.84, (6.6)

Table 8 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) of FK009 and actinomycin D (in DMSO-d_6 and $CDCl_3$) (continued).

	Position	FK009 (actimomycin D)		Actinomycin D[a] (in DMSO)		Actinomycin D (in $CDCl_3$)	
		δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	δ_H (ppm), mult. (*J* in Hz.)[b]	δ_H (ppm), mult. (*J* in Hz.)[c]
Val(1,2)	1	173.10/173.48	-	ND	-	-	-
	2	58.74/58.91	3.49, 2H, m	59.2/59.3	3.57, q	3.62	3.53 (5.6), 3.55
	3	31.63/31.91	2.14, 2H, br s	32.3	2.00, m	~2.2	2.17 (M)
	3-Me	19.22/19.24	0.86, 3H, d (6.79)	20.2	0.76, d	0.91 (6.5)	0.89 (6.5)
			0.88, 3H, d (6.87)				0.90 (6.5)
	4	19.12-19.18	1.10, 3H, d (6.34)	20.7	1.00, d	1.13 (6.5)	1.11 (6.4)
			1.09, 3H, d (6.38)				
	NH	-	8.09, 1H, d (5.85)	-	ND	8.19 (5.7)	8.10 (5.6)
		-	7.94, 1H, d (6.19)	-		7.94 (6.0)	7.94 (5.6)
Pro(1,2)	1	173.02,2CO	-	ND	-	-	-
	2	56.22/56.38	5.94, 1H, d (9.08)	57.4	6.09, m	~3.9	5.98 (8.0)
			6.00, 1H, d (9.05)			~4.0	6.03 (8.0)
	3	31.05/31.37	1.79, 1H,m	32.1	1.74, m	2.1-2.2	1.7-2.4/2.70, m
			2.67, 1H, m				
			1.84, 1H,m				
			2.95, 1H, m				1.7-2.4/2.90, m
	4	22.98/23.14	2.05, 2H, m	24.1	2.02, m	1.85	1.7-2.4
			2.26, 2H, m				
	5	47.38	3.70, 1H, m	48.4	3.49, m	3.70-3.75	3.4-4.2
			3.94, 1H, m			2.67	
		47.64	3.70, 1H, m				
			3.80, 1H, m				

Table 8 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) of FK009 and actinomycin D (in DMSO-d_6 and $CDCl_3$) (continued).

	Position	FK009 (actimomycin D)		Actinomycin D[a] (in DMSO)		Actinomycin D (in $CDCl_3$)	
		δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	δ_H (ppm), mult. (*J* in Hz.)[b]	δ_H (ppm), mult. (*J* in Hz.)[c]
Sar (1,2)	1	165.83/166.07	-	ND	-	-	-
	2	51.40/51.44	3.61, 1H, d (18.31)	52.5	4.08, d	3.63 (17.8)	3.61 (18)
			4.69, 1H, d (18.31)		4.53, d	4.79 (17.8)	4.73 (18)
			3.59, 1H, d (18.13)			3.63 (17.8)	3.62 (18)
			4.76, 1H, d (18.13)			4.72 (17.8)	4.79 (18)
	$N\text{-}CH_3$	34.92/34.98	2.85, 6H, s	35.9	2.75, s	2.89/2.94	2.87
MeVal(1,2)	1	167.32/167.41	-	ND	-	-	
	2	71.27/71.43	2.64, 2H, m	71.0	3.10, d/3.06, d	6.03 (7.5)/5.95 (7.5)	2.67, m
	3	26.96	2.64, 2H, m	28.2	2.47, m	2.60-2.75	2.67, m
	3-Me	21.70/21.80	0.92, 3H, d (5.64)	20.3	0.95, d	0.96	0.95 (5.3)
			0.94, 3H, d (5.25)				
	4	19.37/19.42	0.71, 6H, d (5.06)	22.7	0.64, d	0.76	0.74 (5.2)
	$N\text{-}CH_3$	39.27	2.87, 3H, s	40.1	2.64, s	2.89	2.88
		39.38	2.91, 3H, s		2.96, s		2.93

[a] From YU and Tseng, 1992

[b] From Arison and Hoogsteen., 1970

[c] From Lackner, 1971

ND, Not determined

The spectral data of compound FK009 were identical to those of the known compound, actinomycin D, which was previously isolated from *Streptomyces antibioticus* (Waksman and Wooddruff, 1940) and *Streptomyces chrysomallus* (Glasby, 1993).

3.2 Structure elucidation of *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017)

Compound TK017 was isolated as colorless crystals and identified as *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide. The ESI-TOF mass spectrum of compound TK017 (Figure 46) showed the pseudomolecular ion peak at m/z 180 $(M+H)^+$ implying a molecular formula of $C_{10}H_{13}NO_2$. The UV spectrum (in MeOH)(Figure 47) exhibited λ_{max} (log ε) at 223 (3.87) and 278 (3.22) nm. The IR spectrum (Figure 48) showed absorption bands for hydroxy group (ν_{max} 3333 cm^{-1}) and amide carbonyl (ν_{max} 1631 cm^{-1}).

The 300 MHz ^1H NMR spectrum (in DMSO-d_6) of TK017 (Figure 49) showed signals due to hydroxy proton (δ 9.17 ppm), and amide proton (δ 7.86 ppm), two set of aromatic equivalent protons [δ 6.66 ppm (H-3′′, H-5′′) and δ 6.97 ppm (H-2′′, H-6′′)], methyl protons [δ 1.76 ppm, 3H (H-2)], and two methylene groups [δ 3.15 ppm, (H_2-1′) and δ 2.55 ppm, (H_2-2′)]

The 75 MHz ^{13}C NMR, DEPT 90, and DEPT 135 spectra (in DMSO-d_6) (Figures 50-52) showed signals that have been assigned to one methyl carbon, two methylene carbons, four methine carbons, and three quaternary carbons. Further assignments of correlations between the directly bonded protons and carbons by analyses of the HMQC spectrum (Figure 53) are shown in Table 9. The ^1H-^1H correlations in the ^1H-^1H COSY spectrum (Figure 54) exhibited the proton connectivities as follows; H-2"or H-6′′ / H-3′′ or H-5′′; H-N / H_2-1′ / H_2-2′.

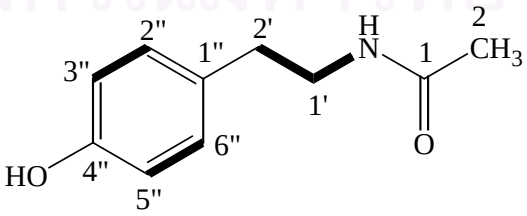


Figure 14 The ^1H-^1H correlations (bold line) in the ^1H-^1H COSY spectrum of *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017).

All spectral data indicated that this compound contained a phenyl ring which has been substituted at para-position, one is a hydroxy group and another is the *N*-ethylacetamide group. The complete assignment of compound TK017 was achieved by the analysis of splitting pattern of protons and chemical shifts of carbon. The methyl carbon at δ 22.15 ppm (C-2) and the carbonyl carbon at δ 168.23 ppm were assigned to the acetamide group. The quaternary aromatic carbon at δ 154.80 ppm (C-4″) confirmed the attachment of a hydroxy group to the phenyl ring at C-4″.

Table 9 The ^{1}H and ^{13}C NMR spectral data (in DMSO-d_6) of *N*-[2′-(4″-hydroxyphenyl)ethyl]acetamide (TK017).

Position	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)
1	168.23	-
2	22.15	1.76, 3H, s
1′	40.07	3.15, 2H
2′	33.92	2.55, 2H, t (7.40)
1″	128.83	-
2″	128.70	6.97, d (8.17)
3″	114.41	6.66, d (8.17)
4″	154.80	-
5″	114.41	6.66, d (8.17)
6″	128.70	6.97, d (8.17)
1-NH	-	7.86, br s
4″-OH	-	9.17, br s

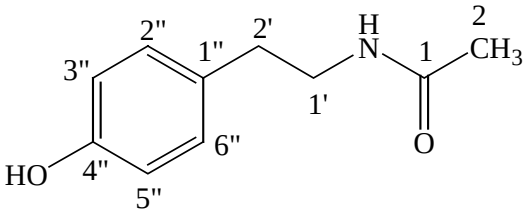


Figure 15 Chemical structure of *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017).

3.3 Structure elucidation of *cyclo*-(L-propyl-D-leucyl) (TK030 and TK034-2)

Compound TK030 was isolated as colorless amorphous solid. The ESI-TOF mass spectrum (Figure 55) showed the pseudomolecular ion peak at *m/z* 211 $(M+H)^+$ suggesting a molecular formula of $C_{11}H_{18}N_2O_2$, and this compound showed optical rotation ($[\alpha]_D^{25}$ -80.36°, c = 0.125 in MeOH). The UV spectrum (in MeOH) (Figure 56) exhibited λ_{max} (log ε) at 210 (3.53) nm. The IR spectrum (Figure 57) presented several bands of amide NH group (ν_{max} 3215 cm^{-1}) and amide carbonyl group (ν_{max} 1674 cm^{-1}).

The 300MHz ^1H NMR spectrum (in $CDCl_3$) of compound TK030 (Figure 58) was integrated to eighteen protons and one proton at δ 5.96 ppm was an amide proton, designable to 4-NH. The 75 MHz ^{13}C NMR, DEPT 135, and DEPT 90 spectra (in $CDCl_3$) (Figures 59-61) exhibited that this compound contains eleven carbons; two methyl carbons, four methylene carbons, three methine carbons, and two quaternary carbons. The HMQC spectrum (Figures 62-63) allowed the assignments of protons and their respective carbons, as shown in Table 10. The ^1H-^1H correlations in the ^1H-^1H COSY spectrum (Figures 64-65) established the proton connectivities as follows: H-11, 11-Me protons and H_3-12; H_2-9 and H_2-8; H-3 and H_2-10; H-6 and H_2-7.

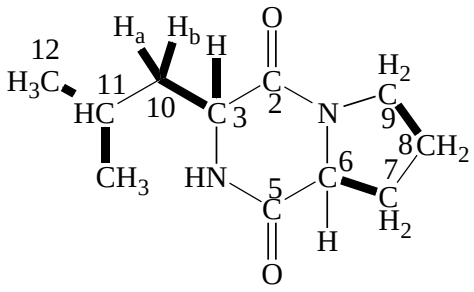


Figure 16 The ^{1}H-^{1}H correlations (bold line) in the ^{1}H-^{1}H COSY spectrum of *cyclo*-(L-propyl-D-leucyl) (TK030).

The complete assignment of TK030 was achieved by the analysis of the HMBC ($^{n}J_{HC}$ = 8 Hz) spectrum (Figure 66). The HMBC spectrum showed correlations of 11-Me protons (δ 0.93 ppm) and H_3-12 (δ 0.98 ppm) to C-11 (δ 24.84 ppm) and C-10 (δ 38.72 ppm); H-10a (δ 1.50 ppm) to C-11 (δ 24.84 ppm) and C-2 (δ 165.93 ppm); H-10b (δ 2.04 ppm) to C-11 (δ 24.84 ppm) and C-3 (δ 53.45 ppm); H_2-9 (δ 3.55 ppm) to C-7 (δ 28.22 ppm).

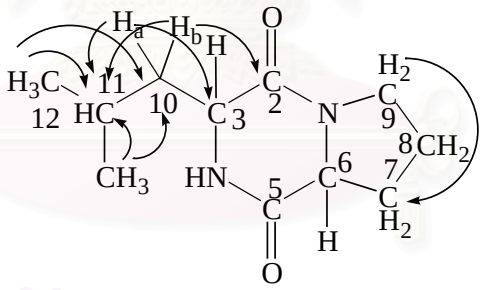


Figure 17 The ^{1}H-^{13}C long-range correlations in the HMBC spectrum of *cyclo*-(L-propyl-D-leucyl) (TK030).

Table 10 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) and the ^{1}H, ^{13}C long-range correlations in the HMBC spectrum of *cyclo*-(L-propyl-D-leucyl) (TK030).

position	*Cyclo*-(L-propyl-D-leucyl) (TK030)		
	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	^{1}H-^{13}C long-range correlations in HMBC ($^{n}J_{CH}$ = 8 Hz)
2	165.93	-	
3	53.45	3.99, dd (9.41,3.18)	
4	-	5.96, br s	
5	169.91	-	
6	59.02	4.09, t (8.06)	
7a	28.22	2.11, m	
7b		2.32, m	
8a	22.86	1.89, m	
8b		2.00, m	
9a/9b	45.57	3.55, 2H, m	C-7
10a	38.72	1.50, m	C-3, C-11
10b		2.04, m	C-2, C-11
11	24.84	1.71, m	
11-Me	21.33	0.93, 3H, d (6.49)	C-10, C-11
12	23.40	0.98, 3H, d (6.56)	C-10, C-11

Compound TK030 was proposed as a diketopiperazine, *cyclo*-(prolyl-leucyl). The series of *cyclo*-(prolyl-leucyl) diketopiperazine were previously reported as shown in Table 11.

Table 11 Sources of diketopiperazine, *cyclo*-(prolyl-leucyl).

Compounds	Source	References
cyclo-(prolyl-leucyl)	a marine *Micrococcus* sp. associated with a marine sponge, *Tedania ignis*	Stierle *et al*., 1988
	Streptomyces sp. TRA9851-2	Suntornchashwej, 2000
cyclo-(L-prolyl-L-leucyl)	*Streptomyces lavendulae* No.314	Kubo *et al*., 1977
	a Caribbean sponge ***Tedania ignis***	Schmitz *et al*., 1983
	an Antarctic sponge-associated bacterium, *Pseudomonas aeruginosa*	Jayatilake *et al*., 1996
cyclo-(L-prolyl-D-leucyl) *cyclo*-(D-prolyl-D-leucyl)	a caribbean sponge *Calyx* cf. *podatypa*	Adamczeski *et al*., 1995
cyclo-(D-prolyl-leucyl)	a marine *Bacillus* sp. Sc018	Jaruchoktaweechai, 1999
cyclo-(L-prolyl-D-leucyl)	a marine *Bacillus* sp. Sc026	

It is well known that optical rotations ($[\alpha]_D$) for proline-containing diketopiperazines are positive if proline of diketopiperazines is D-proline, while showing negative if the proline is L-proline (Adamczeski *et al*, 1995). Optical rotation of TK030 was -80.36°, therefore this compound contained L-proline in the structure. TK030 was then identified as *cyclo*-(L-prolyl-D-leucyl) by comparison its ^{1}H, ^{13}C NMR spectral data and optical rotation with the previous report (Jaruchoktaweechai, 1999) as shown in Tables 12-13.

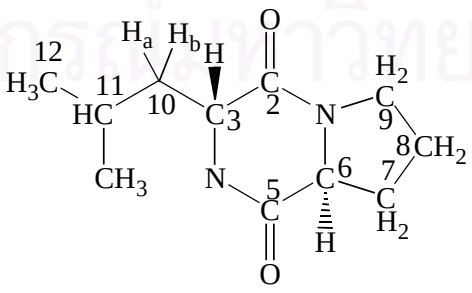


Figure 18 Chemical structure of *cyclo*-(L-prolyl-D-leucyl) (TK030).

Table 12 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) of *cyclo*-(L-prolyl-D-leucyl), *cyclo*-(L-prolyl-L-leucyl), and *cyclo*-(D-prolyl-D-leucyl).

Position	*Cyclo*-(L-propyl-D-leucyl) (TK030)		*Cyclo*-(L-prolyl-D-leucyl)[a]		*Cyclo*-(L-prolyl-L-leucyl)[b]		*Cyclo*-(D-prolyl-D-leucyl)[c]	
	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)
2	165.93	-	166.0	-	-	-	169.6	-
3	53.45	3.99, dd(9.41,3.18)	53.4	3.95, dd(9.3, 3.3)	53.60	3.9-4.24, m	56.4	3.92, dd(9.9, 5.4, 4.5)
4	-	5.96, br s	-	6.18, br s	-		-	6.18, br s
5	169.91	-	170.0	-	-		166.4	-
6	59.02	4.09, t(8.06)	59.0	4.11, t(8.1)	59.20	3.9-4.24, m	58.1	4.07, dd(6.9, 1.5)
7a	28.22	2.11, m	28.2	2.10, m	28.33	1.4-2.5	29.1	2.37, ddd (8.7, 6.4, 2.4)
7b		2.32, m		2.32, m				
8a	22.86	1.89, m	22.8	1.89, 2H, m	23.50	1.4-2.5	23.1	1.96, m
8b		2.00, m						1.88, m
9a/9b	45.57	3.55, 2H, m	45.5	3.52, 2H, m	45.72	3.6, m	45.7	3.52, dt, (9.8, 2.7)/3.62, dt (9.0, 4.5)
10a	38.72	1.50, m	38.6	1.50, m	38.87	1.4-2.5	42.6	1.75, 9 (6.3)
10b		2.04, m		1.99, m				1.63, ddd (11.1, 6.5, 1.8)
11	24.84	1.71, m	24.7	1.79, m	24.96	1.4-2.5	24.5	1.60-1.66, m
11-Me	21.33	0.93, 3H, d(6.49)	21.3	0.90, 3H, d (6.3)	21.40	0.96, d	21.4	0.97, 3H, d (6.3)
12	23.40	0.98, 3H, d(6.56)	23.4	0.95, 3H, d (6.3)	22.95	1.02, d	22.3	0.94, 3H, d (6.3)

[a] From Jaruchoktaweechai, 1999.

[b] From Jayatilake *et al.*, 1996.

[c] From Adamczeski *et al.*, 1995.

Table 13 Optical rotations of *cyclo*-(L-prolyl-D-leucyl) (TK030), *cyclo*-(L-prolyl-D-leucyl), *cyclo*-(L-prolyl-L-leucyl), and *cyclo*-(D-prolyl-D-leucyl).

Compounds	$([\alpha]_D$ (^{o})	Concentration (g/100 ml)
cyclo-(L-prolyl-D-leucyl) (TK030)	-80.36	0.125[d]
cyclo-(L-prolyl-D-leucyl)[a]	-91.3	0.140[d]
cyclo-(L-prolyl-D-leucyl)[b]	-90.6	0.140[e]
cyclo-(L-prolyl-L-leucyl)[c]	-136.0	0.120[e]
cyclo-(D-prolyl-D-leucyl)[b]	+142.14	0.280[e]

[a] From Jaruchoktaweechai, 1999

[b] From Adamczeski *et al*., 1995

[c] From Jayatilake *et al*., 1996

[d] Data were recorded in MeOH

[e] Data were recorded in EtOH

The TK030 was proposed as a diketopiperazine, *cyclo*-(L-prolyl-D-leucyl), which was previously isolated from the Caribean sponge, *Calyx* cf. *Podatypa* (Adamczeski *et al*, 1945) and a marine bacterium, *Bacillus* sp. SC026 (Jaruchoktaweechai, 1999).

The ^1H and ^{13}C NMR spectral data of compound TK034-2 was identical to those of compound TK030 and both compounds showed similar optical rotation, ($[\alpha]_D$) -80.36° at c = 0.125 in MeOH for compound TK030 and -97.85° at c = 0.175 in MeOH for compound TK034-2. Therefore, TK034-2 was also identified as *cyclo*-(L-prolyl-D-leucyl).

3.4 Structure elucidation of *cyclo*-(L-propyl-D-valyl) (TK034-1)

Compound TK034-1 was isolated as colorless amorphous solid. The ESI-TOF mass spectrum (Figure 67) gave the pseudomolecular ion peak at *m/z* 197 $(M+H)^+$, indicating a molecular formula $C_{10}H_{16}N_2O_2$. The UV spectrum (Figure 68) showed λ_{max} (log ε) at 212 (3.58) and 724 (1.38) nm. The IR spectrum (Figure 69) exhibited amide NH at ν_{max} 3259 cm^{-1}, and amide carbonyl at ν_{max} 1634 cm^{-1}. This compound showed optical rotation ($[\alpha]_D^{25}$ -121.91°, c = 0.099 in MeOH).

The 300 MHz ^1H NMR spectrum (in $CDCl_3$) of TK034 (Figure 70) showed two methyl proton signals at δ 0.88 and 1.04 ppm, six nonequivalent methylene proton signals at δ 1.87(1H), 2.00(1H), 2.02(1H), 2.33(1H), 3.51(1H), and 3.56(1H) ppm, three methine proton signals at δ 2.60(1H), 3.91(1H), 4.05(1H) ppm, and one amide proton signal at δ 6.20 ppm. The 75 MHz ^{13}C NMR, DEPT 90, and DEPT 135 spectra (in $CDCl_3$) (Figures 71-73) showed ten carbon signals attributable to two methyl carbons (δ 16.17 and 19.31 ppm), three methylene carbons (δ 22.46, 28.61, and 45.18 ppm), three methine carbons (δ 28.47, 58.83 and 60.41 ppm), and two quaternary carbons (δ 164.69 and 169.84 ppm). The ^1H-^1H COSY spectrum (Figures 76-77) exhibited the connectivities as follows: H-10 / H_3-11 / 10-Me protons; H-6 / H-7a / H-7b; H-8a / H-8b / H-9a / H-9b.

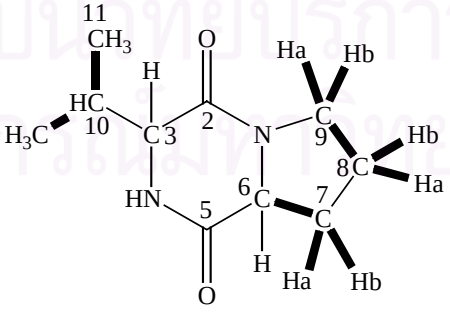


Figure 19 The ^1H-^1H correlations (bold line) in the ^1H-^1H COSY spectrum of *cyclo*-(L-propyl-D-valyl) (TK034-1).

The correlations between protons and their respective carbons were assigned by analyses of the HMQC spectrum (Figures 74-75) as shown in Table 14. The complete assignment of compound TK034 was achieved by the analysis of the HMBC ($^{n}J_{HC}$ = 8 Hz) spectrum (Figure 78). The amide carbonyl carbon at δ 164.69 ppm was assigned as C-2 by its long-range correlations with H-11 (δ 0.88 ppm), 10-Me protons (δ 1.04 ppm), and H-10 (δ 2.60 ppm). The HMBC spectrum also showed the correlations of H-11 (δ 0.88 ppm) and 10-Me protons (δ 1.04 ppm) to C-3 (δ 60.41 ppm), H-7b (δ 2.33 ppm) to C-9 (δ 45.18 ppm), H-7a (δ 2.02 ppm) to C-6 (58.83 ppm) and H-8b (δ 2.00 ppm) to C-6 (δ 58.83 ppm) (Figure 20).

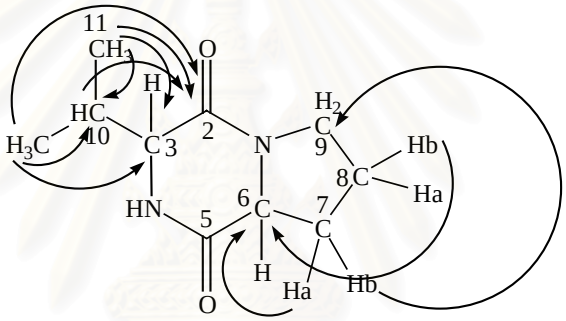


Figure 20 The ^{1}H-^{13}C long-range correlations in the HMBC spectrum of *cyclo*-(L-propyl-D-valyl) (TK034-1).

Table 14 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) and the ^{1}H, ^{13}C long-range correlations in the HMBC spectrum of *cyclo*-(L-prolyl-D-valyl) (TK034-1).

Position	TK034 [*cyclo*-(L-prolyl-D-valyl)		
	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	^{1}H-^{13}C long-range correlations in HMBC ($^{n}J_{CH}$ = 8 Hz)
2	164.69	-	
3	60.41	3.91, br s	
4	-	6.20, br s	
5	169.84	-	
6	58.83	4.05, m	
7a	28.61	2.02, m	C-6
7b		2.33, m	C-9
8a	22.46	1.87, m	
8b		2.00, m	C-6
9a/9b	45.18	3.51, 2H, m	
10	28.47	2.60, 2H, m	C-2
10-Me	19.31	1.04, 3H, d (7.18)	C-11, C-10, C-3, C-2
11	16.17	0.88, 3H, d (6.80)	C-10-Me, C-10, C-3, C-2

Compound TK034-1 was then proposed as a diketopiperazine, *cyclo*-(prolyl-valyl). A series of *cyclo*-(prolyl-valyl) diketopiperazines was previously reported as follows: *cyclo*-(L-prolyl-L-valyl) from *Streptomyces lavendulae* No.314 (Kubo *et al.*, 1977), a marine sponge *Tedania ignis* (Schmitz *et al*., 1983), and an Antarctic sponge-associated bacterium, *Pseudomonas aeruginosa* (Jayatilake *et al*., 1996); and *cyclo*-(L-prolyl-D-valyl) from a Caribbean sponge, *Calyx* cf. *podatypa* (Adamczeski *et al*., 1995).

It is know that optical rotations of proline-containing diketopiperazines depended on the configuration of proline; diketopiperazines containing D-proline present positive but those with L-proline present negative (Adamczeski *et al*, 1995).

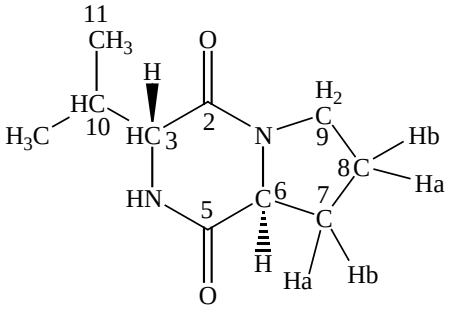


Figure 21 Chemical structuresof *cyclo*-(L-prolyl-D-valyl) (TK034-1).

Table 15 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) of *cyclo*-(L-prolyl-D-valyl) (TK034-1), *cyclo*-(L-prolyl-L-valyl), and *cyclo*-(L-prolyl-D-valyl).

Position	TK034 [*cyclo*-(L-prolyl-L-valyl)		*cyclo*-(L-prolyl-L-valyl)[a]		*cyclo*-(L-prolyl-L-valyl)[b]		*cyclo*-(L-prolyl-D-valyl)[c]	
	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz.)
2	164.69	-	ND	-	165.1	ND	ND	-
3	60.41	3.91, br s	ND	3.96-4.1, m	60.58	ND	60.4	3.94, br s
4	-	6.20, br s	ND	ND	-	ND	-	5.72, dd (1.5, 1.2)
5	169.84	-	ND	-	170.19	ND	ND	-
6	58.83	4.05, m	ND	3.69-4.1,m	59.03	ND	58.9	4.08, dt (7.8, 1.8)
7a	28.61	2.02, m	ND	ND	28.74	ND	28.6	2.3-2.4, m
7b		2.33, m	ND	ND		ND		2.06-2.1, m
8a	22.46	1.87, m	ND	ND	22.57	ND	22.5	1.99-2.02, m
8b		2.00, m	ND	ND		ND		1.88-1.93, m
9a/9b	45.18	3.51, 2H, m	ND	3.6, 2H, m	45.36	ND	45.2	3.55, dt (9.1, 2.8) / 3.63, m
10	28.47	2.60, 2H, m	ND	ND	28.58	ND	28.4	2.64, m
10-Me	19.31	1.04, 3H, d(7.18)	ND	1.10, 3H, d	16.26	ND	16.1	1.06, 3H, d (7.2)
11	16.17	0.88, 3H, d(6.80)	ND	0.92, 3H, d	16.26	ND	19.4	0.91, 3H, d (7.2)

[a]From Schmitz *et al*., 1983

[b]From Jayatilake *et al*.,1996

[c]From Adamczeski *et al*., 1995

ND, Not determined

Table 16 Optical rotations of *cyclo*-(L-prolyl-D-valyl) (TK034-1), *cyclo*-(L-prolyl-L-valyl), and *cyclo*-(L-prolyl-D-valyl).

Compounds	$([\alpha]_D$ (^{o})	Concentration (g/100 ml)
cyclo-(L-prolyl-D-valyl) (TK034-1)	-121.91	0.099[f]
cyclo-(L-prolyl-L-valyl)[a]	-134.0	0.160[g]
cyclo-(L-prolyl-L-valyl)[b]	-139.4	0.160[g]
cyclo-(L-prolyl-L-valyl)[c]	-180	ND[g]
cyclo-(L-prolyl-L-valyl)[d]	-161	ND[g]
cyclo-(L-prolyl-D-valyl)[e]	-74.15	0.260[g]
cyclo-(L-prolyl-D-valyl)[c]	-120	ND[g]

[a] From Schmit *et al.*, 1983

[b] From Jayatilake *et al.*, 1996

[c]From Fischer *et al.*, 1908

[d]From Siemion, 1971

[e] From Adamczeski *et al.*, 1995

[f] Data were recorded in MeOH

[g] Data were recorded in EtOH

ND, Not determined

By the comparison of ^{1}H, ^{13}C NMR spectral data and optical rotation with the literatures as shown in Tables 15-16 (Fischer *et al.*, 1908 and Adamczeski *et al*., 1995), the compound TK034-1 was identified as *cyclo*-(L-prolyl-D-valyl).

3.5 Structure elucidation of antimycins B_1 and B_2 (TK051-1 and TK051-2)

Compound TK051 was isolate as colorless solid. The UV spectrum (in MeOH) (Figure 80) showed λ_{max} (log ε) at 221 (3.82) and 314 (3.04) nm. The IR spectrum (Figure 81) showed absorption bands of carbonyl (ν_{max} 1736 cm^{-1}) and NH group (ν_{max} 3421 cm^{-1}). The ESI-TOF mass spectrum (Figure 79) of compound TK051 showing two sets of the large pseudomolecular ion peaks at *m/z* 493 $(M_1+H)^+$, 515 $(M_1+Na)^+$ and 507 $(M_2+H)^+$, 529 $(M_2+Na)^+$ indicated that TK051 was an approximate 1:1 mixture of two major closely related compounds, one (TK051-1)

having molecular weight 492 ($C_{24}H_{32}N_2O_2$) and the other (TK051-2) having molecular weight 506 ($C_{25}H_{34}N_2O_9$).

Based on extensive analyses of NMR spectral data including 1-D ^1H, ^{13}C, DEPT 90, and DEPT 135 and 2-D ^1H, ^1H COSY, TOCSY, HMQC, and HMBC spectra (Figures 82-98), the compounds contained the common basic structure of an 3-acetamidosalisylamide connecting with a 9-membered dilactone ring, accounting for $C_{19}H_{23}N_2O_9$.

The 300 MHz ^1H NMR and the 75 MHz ^{13}C NMR, DEPT 90, and DEPT 135 spectra were shown in Figures 82-85. The HMQC spectrum (Figures 86-88) allowed the assignments of protons and their respective carbons, as shown in Table 17. The ^1H-^1H correlations in the ^1H-^1H COSY and the TOCSY spectra (in $CDCl_3$) (Figures 89-92) presented the consecutive proton connectivities in the aromatic ring as follows; H-4′ / H-5′ / H-6′ and in the dilactone ring as follows: 3-NH / H-3 / H-4 / 4-Me protons and 9-Me protons / H-9 / H-8 / H-7 (Figure 22).

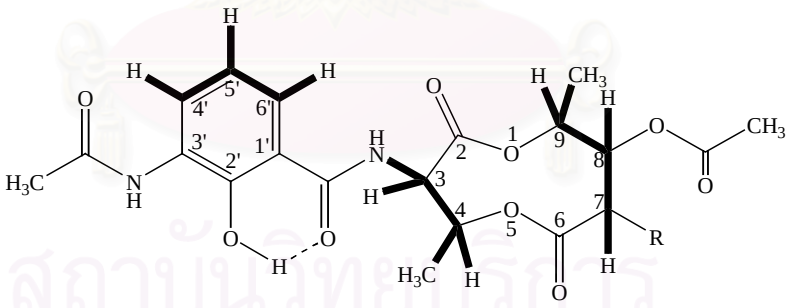


Figure 22 The ^1H-^1H correlations (bold line) in the ^1H, ^1H COSY spectrum of the basic structures of antimycins B_1 and B_2 **(**TK051-1 and TK051-2).

The difference of the two compounds was the alkyl side chains located at C-7 which were C_4H_9 and C_5H_{11} for TK051-1 and TK051-2, respectively. The C_4H_9 was identified as an n-butyl group by the following connectivities in the ^1H, ^1H COSY spectrum H_2-1′′ / H_2-2′′ / H_2-3′′ / CH_3-4′′ (Figure 23) and the C_5H_{11} as an isopentyl group by H_2-1′′ / H_2-2′′ / H_2-3′′ / CH_3-4′′ and CH_3-5′′ (Figure 24). Then the dilactone ring was connected to the side chain by the correlation from H-7 to H-1′′.

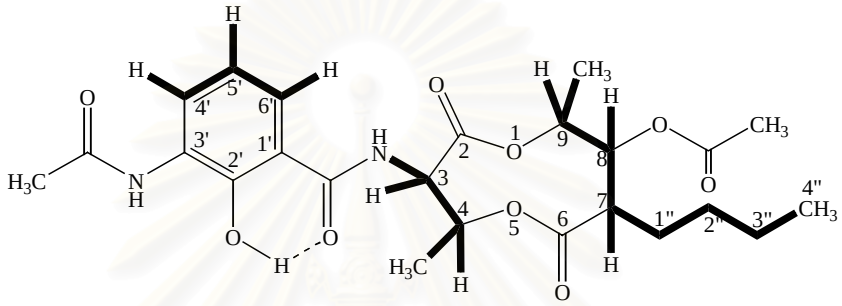


Figure 23 The ^1H-^1H correlations (bold line) in the ^1H, ^1H COSY spectrum of antimycin B_1 (TK051-1).

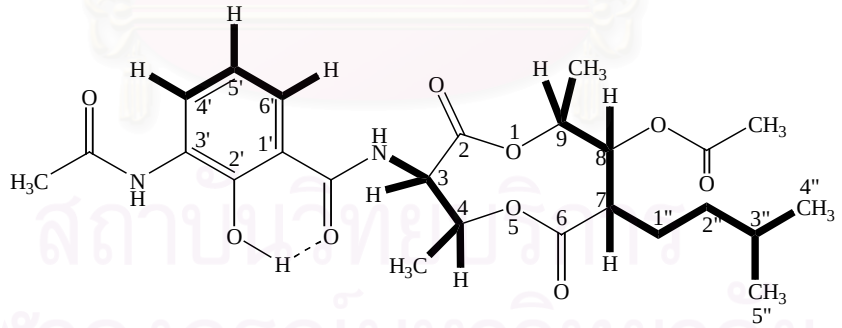


Figure 24 The ^1H-^1H correlations (bold line) in the ^1H, ^1H COSY spectrum of antimycin B_2 **(**TK051-2).

Table 17 The ^{1}H and ^{13}C NMR spectral data (in $CDCl_3$) and the ^{1}H, ^{13}C long-range correlations in the HMBC spectrum of the basic structure of antimycins B_1 and B_2 **(**TK051-1 and TK051-2).

position	Antimycins B_1 and B_2 **(**TK051-1 and TK051-2) ($CDCl_3$)			
	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	^{1}H-^{13}C long-range correlations in	
			HMBC ($^{n}J_{CH}$ = 8 Hz)	HMBC ($^{n}J_{CH}$ = 4 Hz)
2	169.34	-		
3	53.75	5.26, t (7.5)	1′-CO, C-4	
4	71.03	5.70, quin (7.5)		1′-CO, C-6
6	172.66[a]/172.63[b]	-		
7	50.18[a]/50.38[b]	2.48, m		
8	75.81[a]/75.85[b]	5.03, m	C-9, 8-O<u>CO</u>CH_3	C-7, 8-O<u>CO</u>CH_3
9	74.80	4.97, m	C-2, C-8	C-2, C-7
3-NH	-	7.08, d (7.5)	1′-CO	1′-CO
4-Me	15.14	1.30, 3H, d (7.5)	C-3, C-4	C-3, C-4
8-<u>CO</u>CH_3	169.27	-		
8-CO<u>CH_3</u>	20.86	2.11, 3H, s	8-<u>CO</u>CH_3	8-<u>CO</u>CH_3
9-Me	17.96	1.28, 3H, d (5.3)	C-9	C-9
1′	112.42	-		
2′	150.31	-		
3′	128.29	-		
4′	124.04	8.50, d (7.9)	C-2′, C-6′	
5′	118.91	6.89, t (7.9)	C-1′, C-3′, C-6′	C-3′, C-4′
6′	119.46	7.19, d (7.9)	C-2′, C-4′	C-4′, 1′-CO
1′-CO	169.86	-		
3′-NH<u>CO</u>CH_3	168.36	-		
3′-NHCO<u>CH_3</u>	24.97	2.21, 3H, s	3′-NH<u>CO</u>CH_3	3′-NH<u>CO</u>CH_3
3′- NH	-	7.85, bs s		
OH	-	12.55, br s	C-1′, C-2′, C-3′	C-1′, C-2′, C-3′

a = antimycin B_1
b = antimycin B_2

Table 18 The ^{1}H and ^{13}C assignment of the alkyl side chains of antimycins B_1 and B_2 **(**TK051-1 and TK051-2).

position	Antimycins B_1 (TK051-1) ($CDCl_3$)		Antimycins B_2 **(**TK051-2) ($CDCl_3$)	
	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)
1″	29.36	1.20, 2H, m	26.50	1.70, 1H, m / 1.30, 1H
2″	28.29	1.31, 2H, m	36.21	1.11, 2H, m
3″	22.56	1.23, 2H, m	27.95	1.51, m
4″	13.95	0.85, 3H, t (6.9)	22.19	0.84, 3H, d (6.7)
5″	-	-	22.77	0.84, 3H, d (6.7)

The complete assignment of the basic structure was achieved by the aids of the HMBC ($^{n}J_{HC}$ = 4, 8 Hz) spectra (in $CDCl_3$) (Figures 93-98) as shown in Table 17. The connectivities of the quaternary carbons assigned by the HMBC spectra as follows: H-6′, 3-NH, and H-3 to 1′-CO; 3-NH and H-9 to C-2; H-4 to C-6 suggested the connectivities of each fragment in the molecule. The hydroxyl proton at δ 12.55 ppm was substituted at C-2′ of the aromatic ring based on its downfield chemical shift due to the chelation to the amide carbonyl at 1′-CO and the ^{1}H-^{13}C long-range correlations of 2′-OH to C-1′, C-2′, and C-3′. The O-acetyl group was placed at C-8 by the observation of ^{1}H-^{13}C long-range correlations of H-8 and the methyl protons to carbonyl carbon at δ 169.27 ppm. Therefore, the remaining NH-acetyl group was located at C-3′ of the aromatic ring.

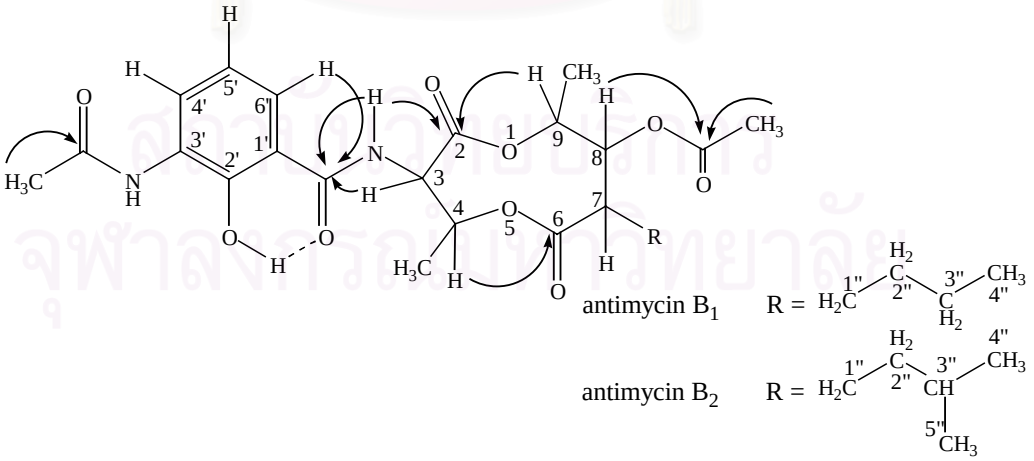


Figure 25 The important ^{1}H-^{13}C long-range correlations in the HMBC spectrum of antimycins B_1 and B_2 **(**TK051-1 and TK051-2).

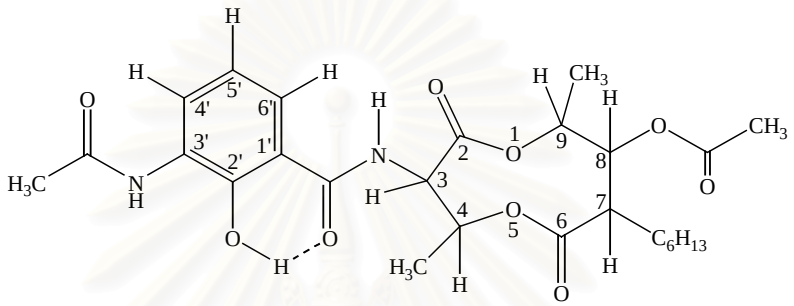
The basic structures of antimycins B_1 and B_2 (TK051-1 and TK051-2) was confirmed by comparison of the ^1H and ^{13}C NMR spectral data with previous reported two antimycin antibiotics, urauchimycins A and B (Imamura *et al*., 1993), as shown in Table 19.

Table 19 The ^1H and ^{13}C NMR spectral data (in $CDCl_3$) of the basic structures of antimycins B_1 and B_2 **(**TK051-1 and TK051-2) and urauchimycins A and B.

position	Antimycins B_1 and B_2 **(**TK051-1 and TK051-2) ($CDCl_3$)		Urauchimycina A ($CDCl_3$)		Urauchimycina A ($CDCl_3$)	
	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)	δ_C (ppm)	δ_H (ppm), mult. (*J* in Hz)
2	169.34	-	170.1	-	170.1	-
3	53.75	5.26, t (7.5)	53.8	5.24	53.7	5.32
4	71.03	5.70, quin (7.5)	70.7	5.69	70.8	5.69
6	172.66[a]/172.63[b]	-	173.8	-	173.8	-
7	50.18[a]/50.38[b]	2.48, m	50.0	2.49	52.3	2.32
8	75.81[a]/75.85[b]	5.03, m	77.1	2.59	77.1	3.60
9	74.80	4.97, m	76.3	4.88	76.3	4.87
3-NH	-	7.08, d (7.5)	-	7.09	-	7.07
4-Me	15.14	1.30, 3H, d (7.5)	15.0	1.30	15.0	1.30
8-$\underline{CO}CH_3$	169.27	-	-	-	-	-
8-$CO\underline{CH_3}$	20.86	2.11, 3H, s	-	-	-	-
9-Me	17.96	1.28, 3H, d (5.3)	18.5	1.45	18.4	1.46
1′	112.42	-	112.6	-	112.6	-
2′	150.31	-	150.6	-	150.6	-
3′	128.29	-	127.4	-	127.4	-
4′	124.04	8.50, d (7.9)	124.8	8.55	124.8	8.55
5′	118.91	6.89, t (7.9)	119.0	6.92	119.0	6.92
6′	119.46	7.19, d (7.9)	120.1	7.24	120.1	7.26
1′-CO	169.86	-	169.4	-	169.4	-
3′-NH$\underline{CO}CH_3$	168.36	-	158.9	-	158.9	-
3′-NHCO$\underline{CH_3}$	24.97	2.21, 3H, s	-	-	-	-
3′- NH	-	7.85, bs s	-	7.90	-	7.88
OH	-	12.55, br s		12.63	-	12.40

a = antimycin B_1
b = antimycin B_2

Additionally, the ESI-TOF mass spectrum of the mixture antimycin B_3 (Figures 79) also presented the small pseudomolecular ion peaks at *m/z* 521 $(M+H)^+$ and 543 $(M+Na)^+$ implying a molecular formula $C_{26}H_{36}N_2O_9$. This minor compound should have the same basic structure as antimycins B_1 and B_2, but contained different alkyl side chain. Its side chain was then proposed as C_6H_{13}. The minor compound was named as antimycin B_3 and its tentative structure is shown below.



antimycin B_3

In summary, TK051 was a mixture of three components containing two major compounds, antimycins B_1 and B_2, and a minor compound, antimycin B_3, in the approximate ratio 5:4:1, respectively, on the basis of its pseudomolecular ion peak intensity in the mass spectrum.

In the previous reports, antimycin A was a mixture of closely related compounds, antimycins A_1, A_2, A_3, A_4 and A_5, that were produced by various species of *Streptomyces*. Antimycins A_1 and A_3 were a potent inhibitors of the cytochrome *bc*1 complex and exhibited insecticidal, fungicidal, and miticidal activities (Selwood *et al*., 1990; Miyoshi *et al*., 1991; and Glasby, 1993).

4. Biological activity

The results of biological activities including antimicrobial, antimalarial, cytotoxic, and antituberculous activities of the crude extracts and pure compounds obtained from *Streptomyces* sp. AAR 1-1 and AAR 14 are shown in Tables 19-23.

4.1 Biological activity of the extracts of the fermentation broth from *Streptomyces* sp. AAR 1-1

From the screening tests of antimicrobial activity, the ethyl acetate extracts I and II from the fermentation broth of *Streptomyces* sp. AAR 1-1 was active against *Staphylococcus aureus* ATCC 25933 and *Bacillus subtilis* ATCC 6633. Based on bioassay-directed fractionation, one active compound, FK009 (actinomycin D), was purified from the active fraction F003 which was fractionated from the active ethyl acetate extract I. The biological activities of the crude extracts and the pure compound including antimicrobial, antimalarial, cytotoxic, and antituberculous activities are presented in Tables 20 and 21.

Table 20 Antimicrobial activity of the crude extracts, fractions, and actinomycin D obtained from *Streptomyces* sp. AAR 1-1.

Fractions or Compounds	Concentration (μg/disc)	Inhibition zones (mm)			
		S. aureus ATCC 25923	*B. subtilis* ATCC 6633	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
EtOAc extract I	1,000	16	20	-	-
EtOAc extract II	1,000	15	ND	-	-
Hexane extract	1,000	-	ND	-	-
F002	1,000	-	-	-	-
F003	1,000	17	ND	ND	ND
F004	1,000	7	ND	ND	ND
F005	1,000	7	ND	ND	ND
F006	1,000	-	-	-	-
F007	1,000	-	-	-	-
Actinomycin D (FK009)	500	22	11	-	-

-, Inactive

ND, Not determined

Table 21 Antimalarial, cytotoxic, and antituberculous activities of the crude extract and actinomycin D obtained from *Streptomyces* sp. AAR 1-1.

Fractions or Compounds	Antimalarial activity[a] EC_{50} (μg/ml)	Cytotoxic activity ED_{50} (μg/ml)		Antituberculous activity[d] MIC (μg/ml)
		KB[b]	BC[c]	
EtOAc extract I	0.34	ND	ND	ND
Actinomycin D (FK009)	0.0026	0.078	0.15	0.125

[a]Antimalarial activity against *Plasmodium falciparum*, K1 multi-drug resistant strain.

[b]KB, Human epidermoid carcinoma cell lines of nasopharynx.

[c]BC, Breast cancer cell lines.

[d]Antituberculous activity against *Mycobacterium tuberculosis* H37Ra

ND, not determined

In the previous report, actinomycin D was used as antineoplastic drug Reynolds, 1996). Additionally, it was found to be a potent inhibitor of human immunodeficiency virus (HIV) type I reverse transcriptase (Davis *et al.*, 1998 and Rill and Hecker, 1996).

4.2 Biological activity of the extracts of the fermentation broth from *Streptomyces* sp. AAR 14

In the preliminary bioactivity screening test, the methanol extract and the hexane extract from the fermentation broth of *Streptomyces* sp. AAR 14 showed fungistatic activity against *Candida albicans* ATCC 10231. Based on bioassay-guided isolation, two active compounds were isolated, TK030 [*cyclo*-(L-prolyl-D-leucyl)] and TK034-1 [*cyclo*-(L-prolyl-D-valyl)] from the active methanol extract. The pure compound, TK017 [*N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide], was isolated from inactive fraction, T017. The results of biological activities including antimicrobial and antimalarial activities are shown in Tables 22 and 23.

Table 22 Antimicrobial activity of the crude extracts, fractions, and pure compounds obtained from *Streptomyces* sp. AAR 14.

Fractions or Compounds	Concentration (μg/disc)	Inhibition zones (mm)			
		S. aureus ATCC 25923	*B. subtilis* ATCC 6633	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
MeOH extract	1,000	-	-	-	35
Hexane extract	1,000	-	-	-	11
T008	1,000	ND	ND	ND	-
T009	1,000	ND	ND	ND	33
T010	1,000	ND	ND	ND	36
T011	1,000	ND	ND	ND	-
T012	1,000	ND	ND	ND	-
T013	1,000	ND	ND	ND	41
T014	1,000	ND	ND	ND	37
T015	1,000	ND	ND	ND	32
T016	1,000	ND	ND	ND	14
T017	1,000	ND	ND	ND	-
T018	1,000	ND	ND	ND	-
T019	1,000	ND	ND	ND	-
T027	1,000	ND	ND	ND	10
T028	1,000	ND	ND	ND	13
T029	1,000	ND	ND	ND	ND
T030	1,000	ND	ND	ND	35
T031	1,000	ND	ND	ND	32
T032	1,000	ND	ND	ND	-
T033	1,000	ND	ND	ND	12
T034	1,000	ND	ND	ND	45
T035	1,000	ND	ND	ND	35
T036	1,000	ND	ND	ND	24
N-[2′-(4″-hydroxyphenyl)ethyl] acetamide (TK017[a])	1,000	ND	ND	ND	-
cyclo-(L-prolyl-D-leucyl) (TK030[a])	500	ND	ND	ND	24

Table 22 Antimicrobial activity of the crude extracts, fractions, and pure compounds obtained from *Streptomyces* sp. AAR 14 (continued).

Fractions or Compounds	Concentration (μg/disc)	Inhibition zones (mm)			
		S. aureus ATCC 25923	*B. subtilis* ATCC 6633	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
cyclo-(L-prolyl-D-valyl) (TK034-1[a])	500	ND	ND	ND	25
cyclo-(L-prolyl-D-leucyl) (TK034-2)[a]	500	ND	ND	ND	24

[a]Pure compound

-, inactive

ND, Not determined

Table 23 Antimalarial activity of the MeOH extract obtained from *Streptomyces* sp. AAR 14.

Fractions or compounds	Antimalarial activity[a] EC_{50} (μg/ml)
MeOH extract	-

[a]Antimalarial activity against *Plasmodium falciparum*, K1 multi-drug resistant strain.

-, inactive

It was previously reported that *cyclo*-(L-prolyl-D-leucyl) showed anti-herpes simplex viruses type I and type II activity (Jaruchoktaweechai, 1999).

CHAPTER V

CONCLUSION

Two actinomycetes strains, AAR 1-1 and AAR 14, were separately isolated from two unidentified marine sponges, bluish purple sponge AR 990325-20H and purplish white sponge AR 990324-09K, respectively, from Adang-ravee Island. The organic solvent solubles of these strains showed interesting antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, and *Candida albicans* ATCC 10231. Based on morphological, cultural, physiological, and biochemical characteristic and cell wall component studies, the strains AAR 1-1 and AAR 14 were identified as *Streptomyces*.

Directed by antimicrobial activity against *S. aureus* ATCC 25923, a known compound, actinomycin D (FK009), was obtained from the ethyl acetate extract of the fermentation broth of *Streptomyces* sp. AAR 1-1. The bioassay-guided fractionation, using antimicrobial against *C. albicans* ATCC 10231 of the methanol extract from the fermentation broth of *Streptomyces* sp. AAR 14 gave two known diketopiperazines including *cyclo-(*L-prolyl-D-leucyl) (TK030 and TK034-2) and *cyclo-(*L-prolyl-D-valyl) (TK034-1), one acetamide derivative, *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017), and a mixture of three new derivatives of antimycins, namely antimycins B_1, B_2, and B_3.

The isolated actinomycin D (FK009) exhibited antimicrobial activity against *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 with inhibition zones of 22 and 11 mm, respectively at concentration 500 μg/disc, antimalarial activity against *Plasmodium falciparum*, K1 multiple-drug resistant strain, at EC_{50} 2.07×10^{-3} μM, cytotoxic activity against KB cells (oral human epidermoid carcinoma) at EC_{50} 6.22×10^{-2} μM and BC cells (breast cancer) at ED_{50} 1.20×10^{-1} μM, and antituberculous activity against *Mycobacterium tuberculosis* H37Ra at MIC 9.97×10^{-2} μM. Both diketopiperazines, *cyclo*-(L-prolyl-D-leucyl) (TK030 and TK034-2) and *cyclo-(*L-prolyl-D-valyl) (TK034-1), showed fungistatic activity against *C. albicans* ATCC

10231 with inhibition zones of 25 and 24 mm, respectively at concentration 500 μg/disc. The isolated *N*-[2′-(4′′-hydroxy phenyl) ethyl] acetamide was tested for antimicrobial and antimalarial activities, but it did not show any activities. The mixture of new antimycins derivatives, antimycins B_1, B_2, and B_3 has not been tested for any biological activities due to the limited amount of sample.

This study is the evidence that the marine *Streptomyces* strains AAR 1-1 and AAR 14 associated with marine sponges can produce biologically active substances such as actinomycin D and diketopiperazines. Additionally, the acetamide derivative, *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide and the new antimycins derivatives, antimycins B_1, B_2, and B_3, are produced by the strain AAR 14.

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APPENDIX

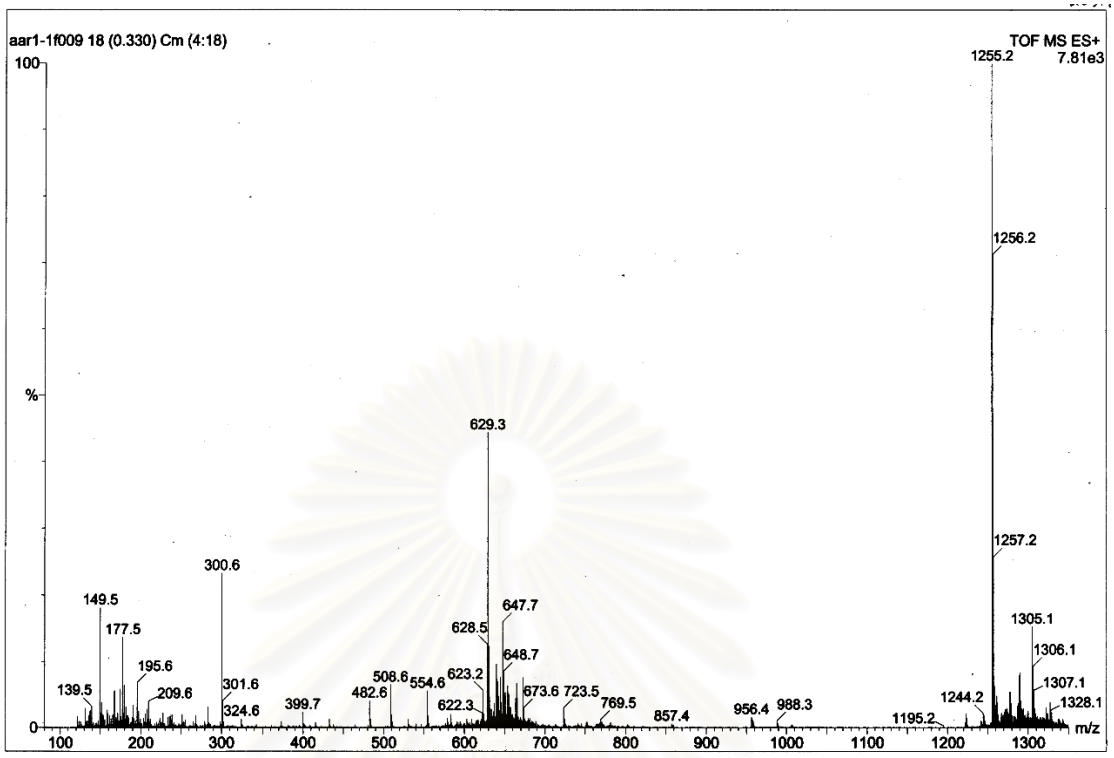


Figure 26 The ESI-TOF mass spectrum of compound FK009.

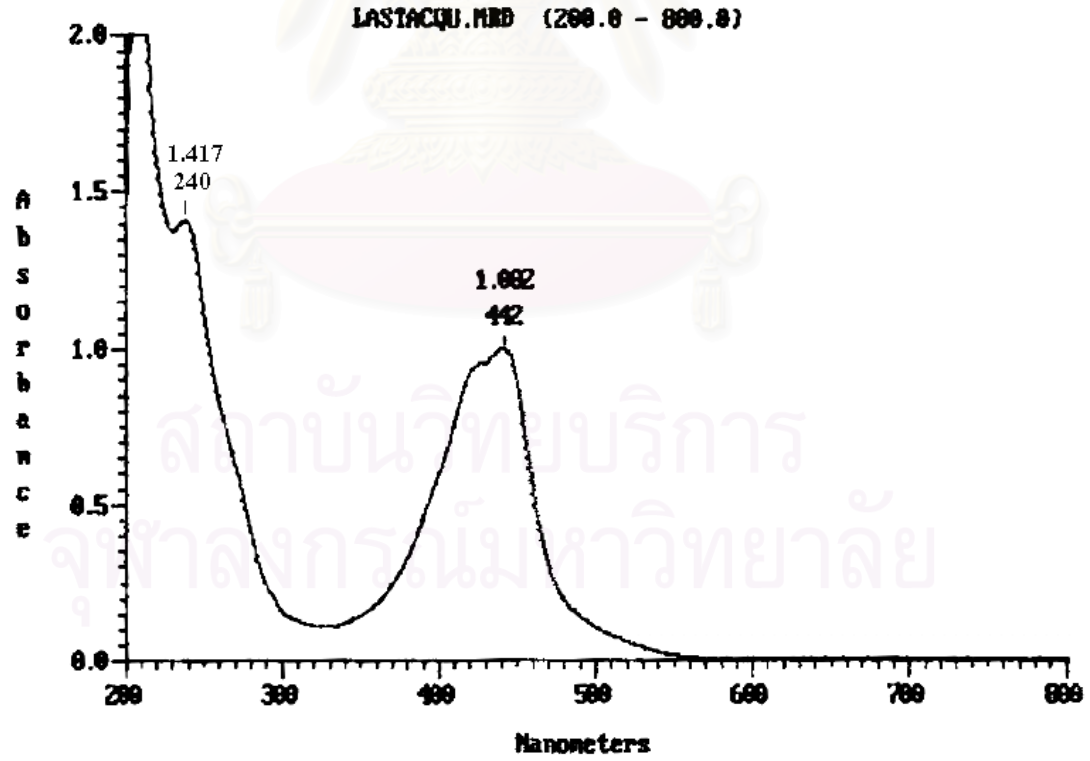


Figure 27 The UV spectrum (in MeOH) of actinomycin D (FK009).

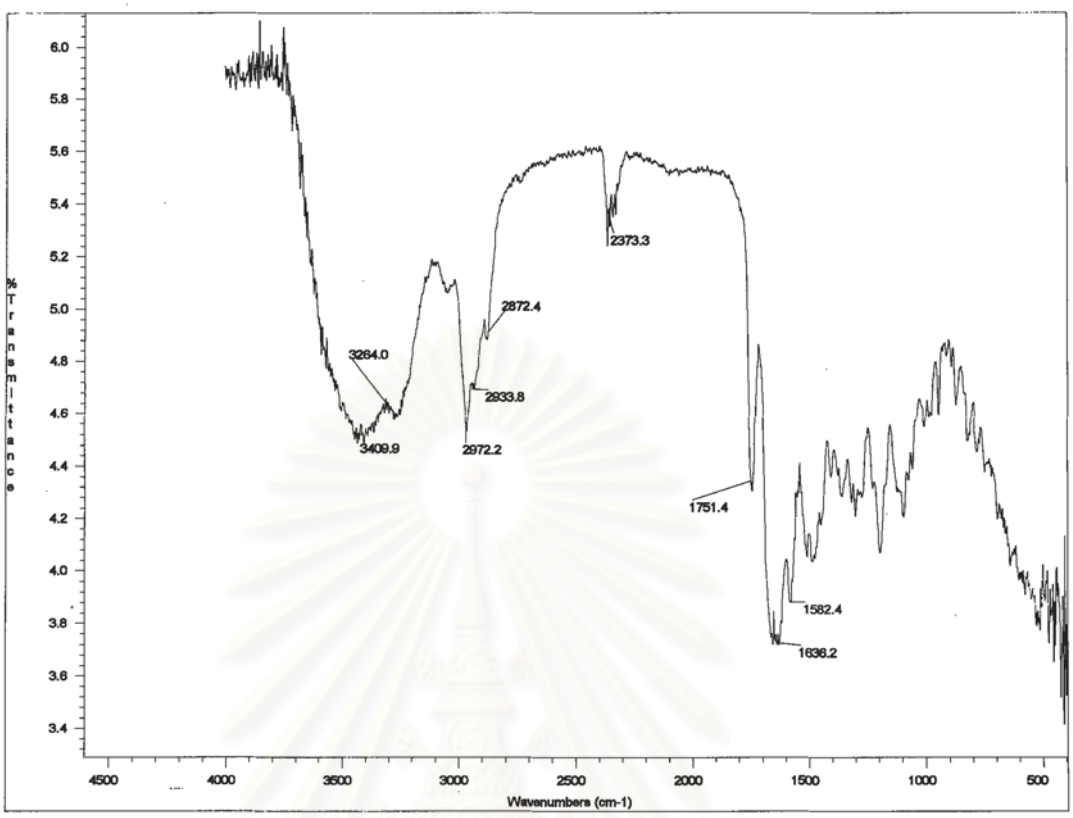
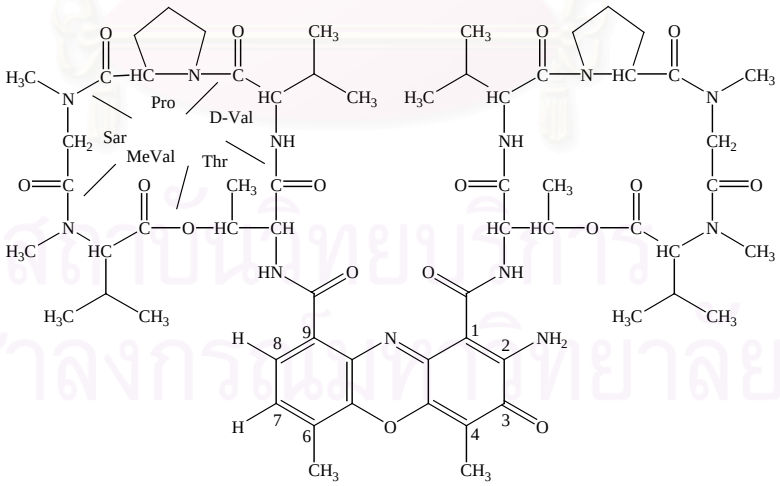


Figure 28 The IR spectrum of actinomycin D (FK009) (KBr disc).



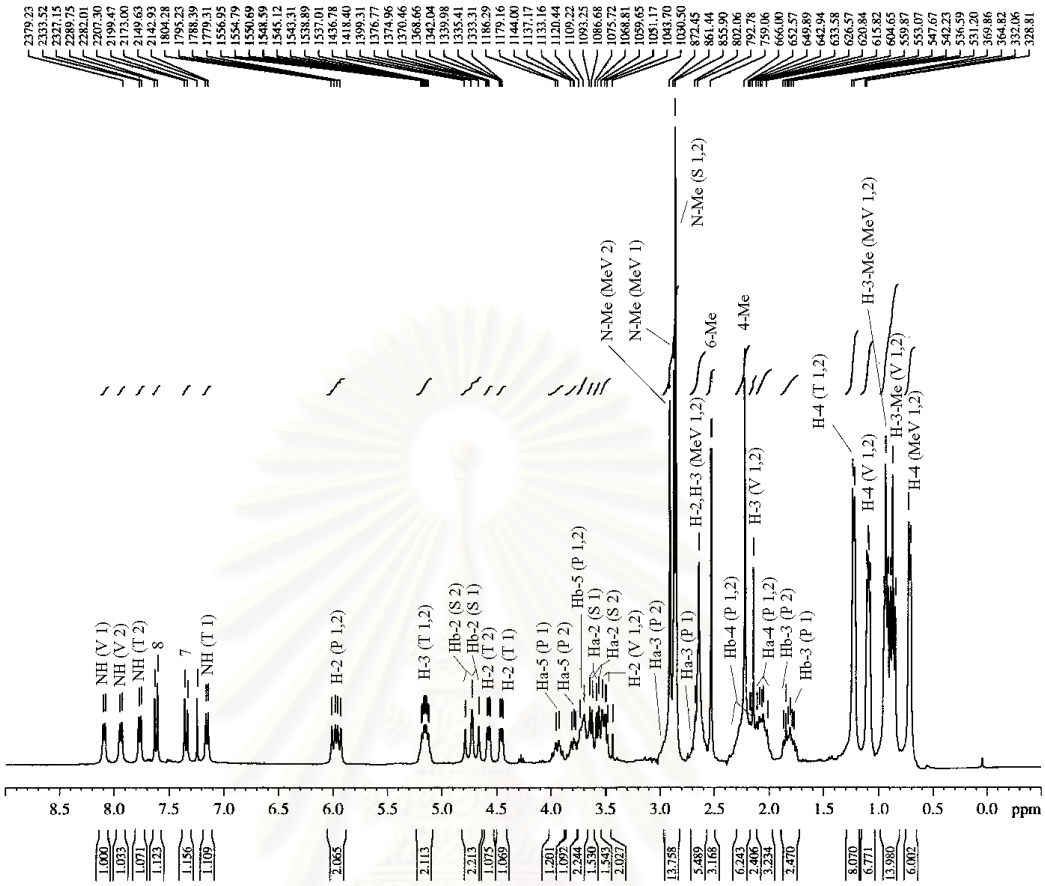
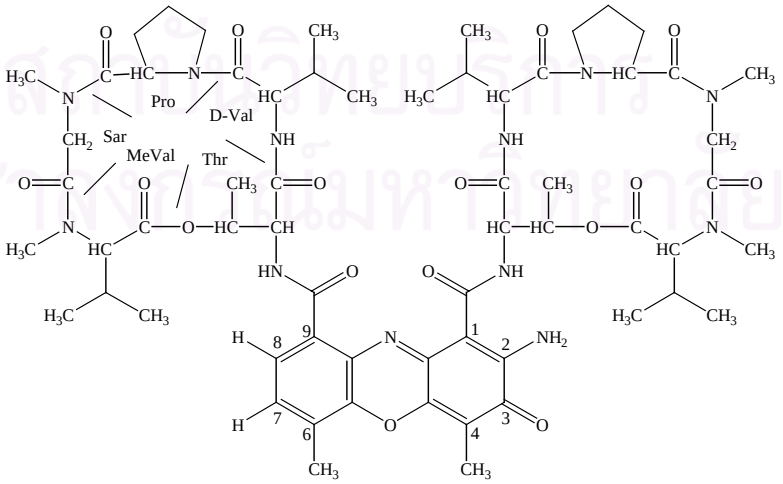


Figure 29 The 300 MHz ^{1}H NMR spectrum (in $CDCl_3$) of actinomycin D (FK009). T = threonine, V = valine , P = proline, S = sarcosine, MeV = methylvaline (1) = amino acid in α chain, (2) = amino acid in β chain



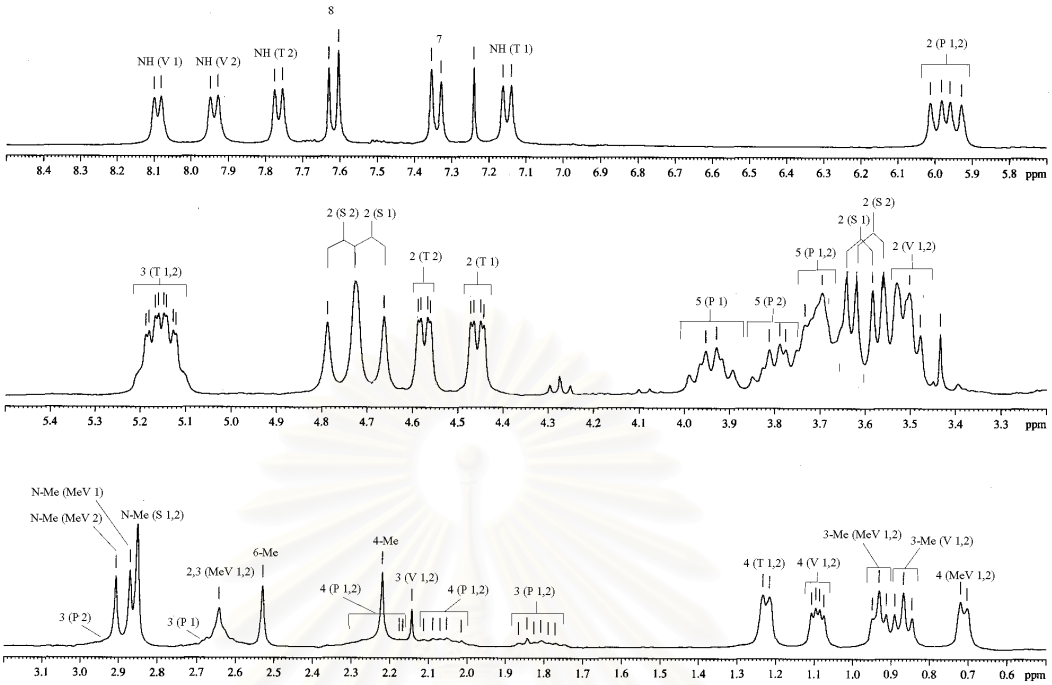
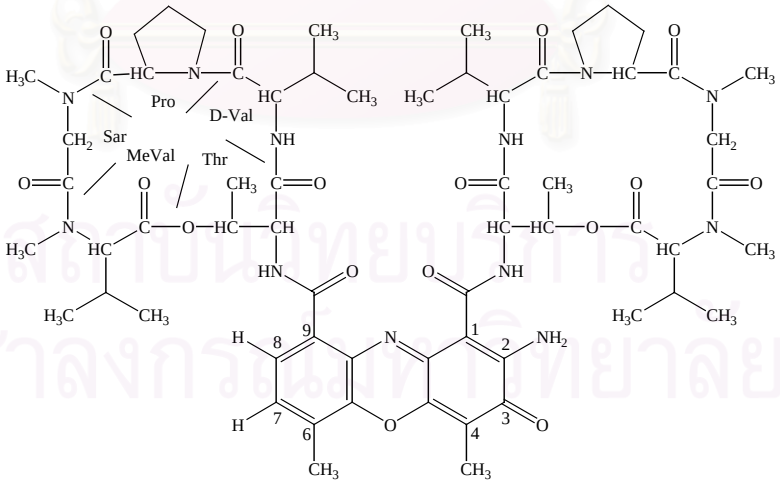


Figure 30 The 300 MHz ^{1}H NMR spectrum (in $CDCl_3$) of actinomycin D (FK009) (expanded from δ_H 0.50-8.50 ppm).



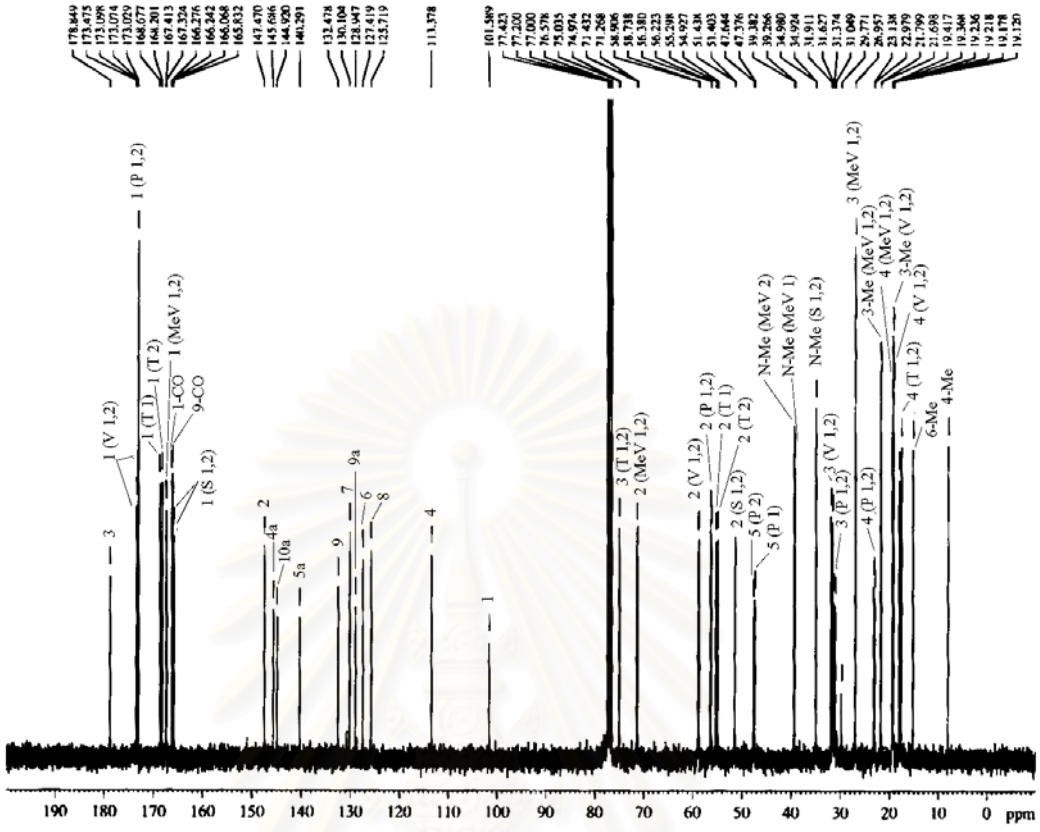
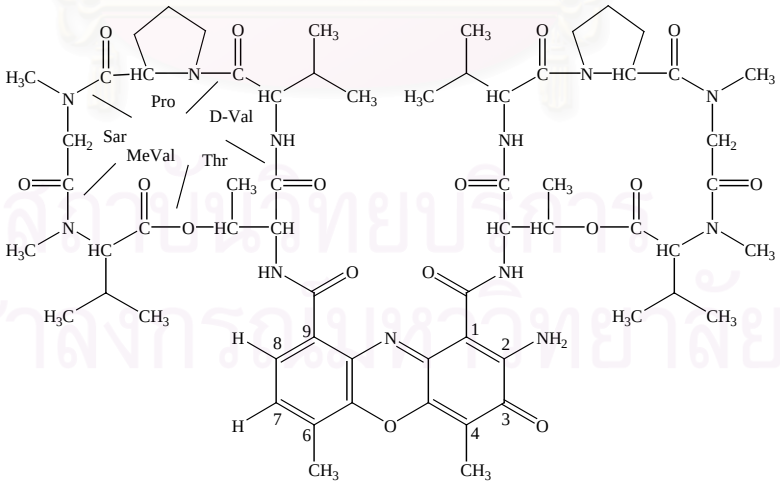


Figure 31 The 75 MHz ^{13}C NMR spectrum (in $CDCl_3$) of actinomycin D (FK009).



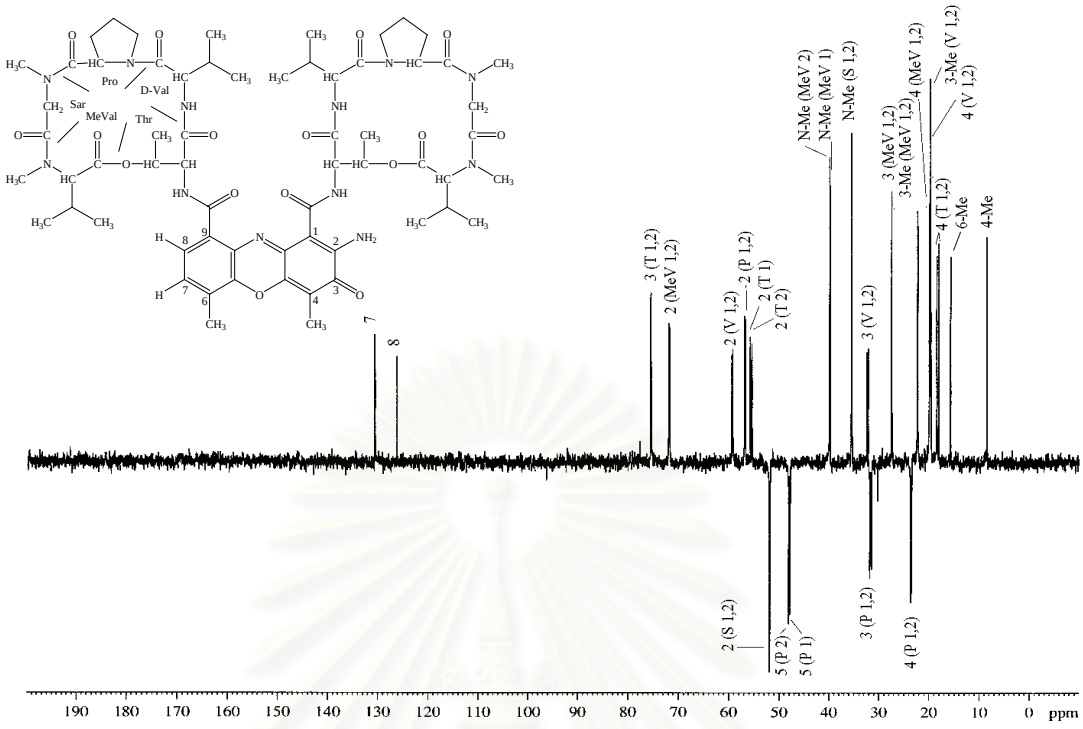


Figure 32 The 75 MHz DEPT 135 spectrum (in $CDCl_3$) of actinomycin D (FK009).

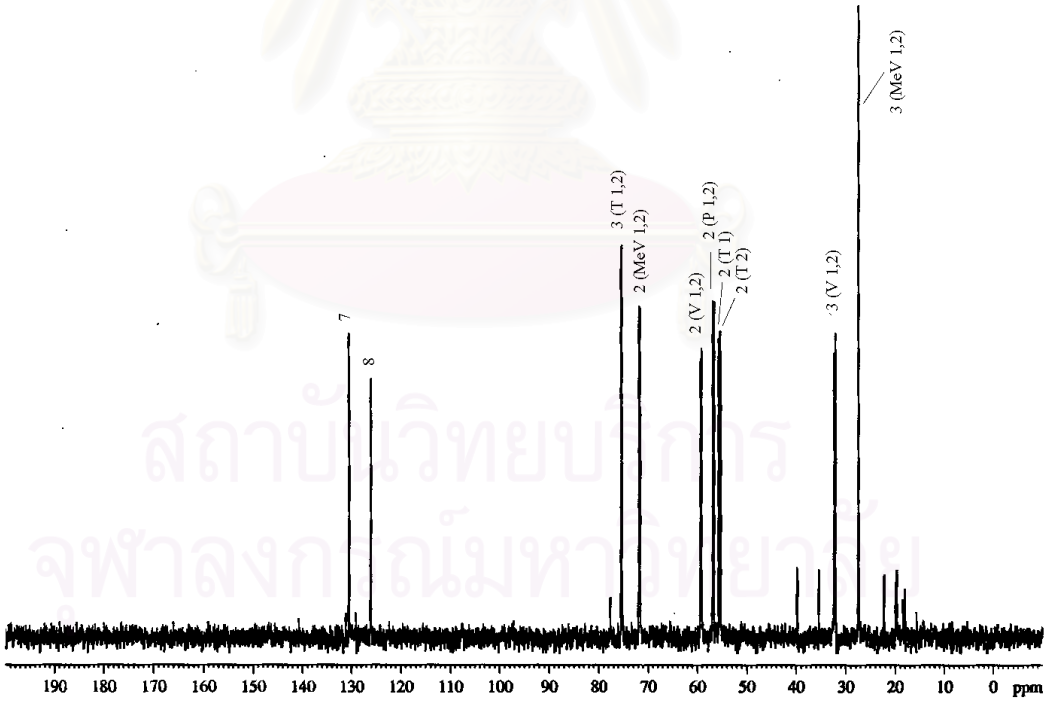


Figure 33 The 75 MHz DEPT 90 spectrum (in $CDCl_3$) of actinomycin D (FK009).

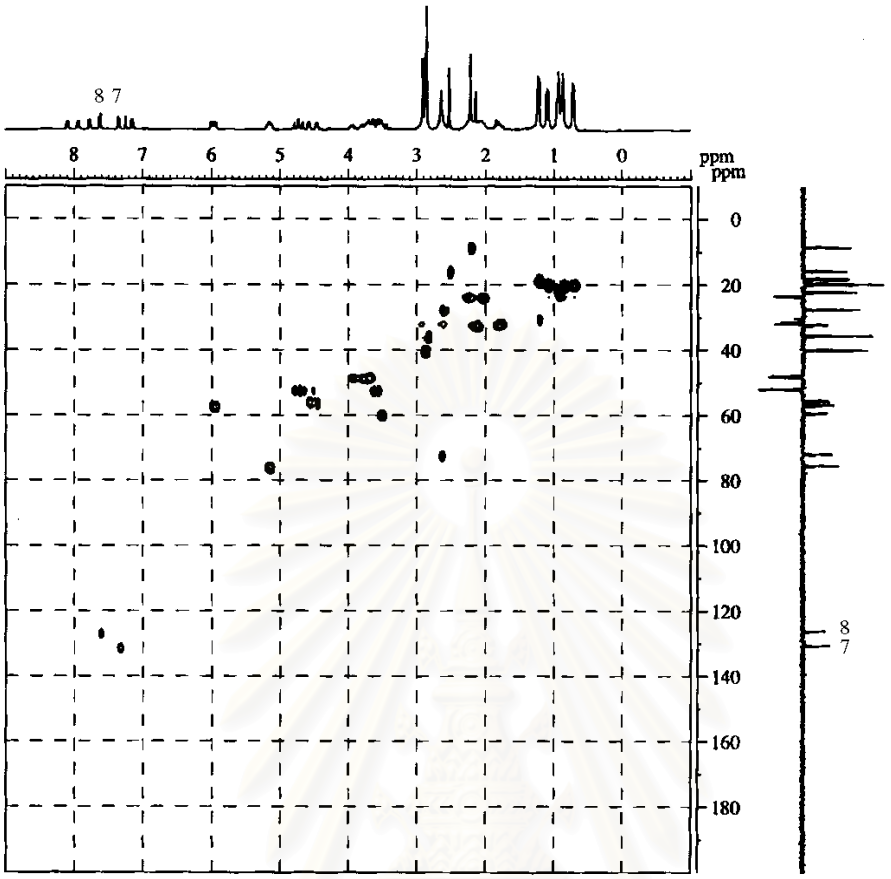
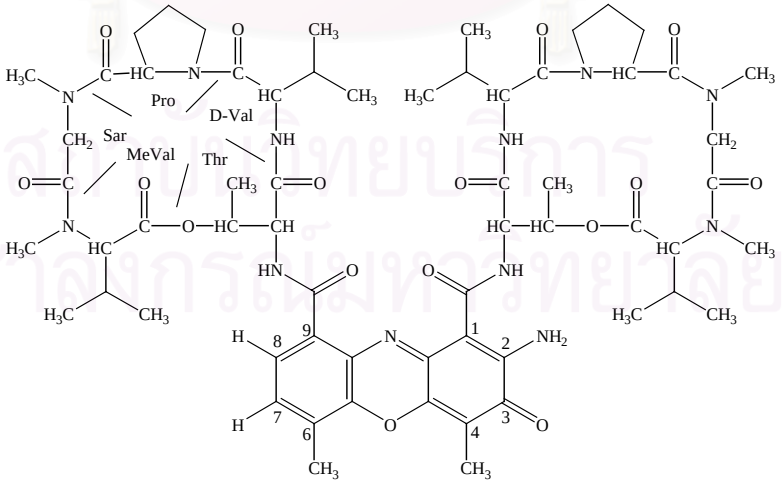


Figure 34 The 300 MHz HMQC spectrum (in $CDCl_3$) of actinomycin D (FK009).



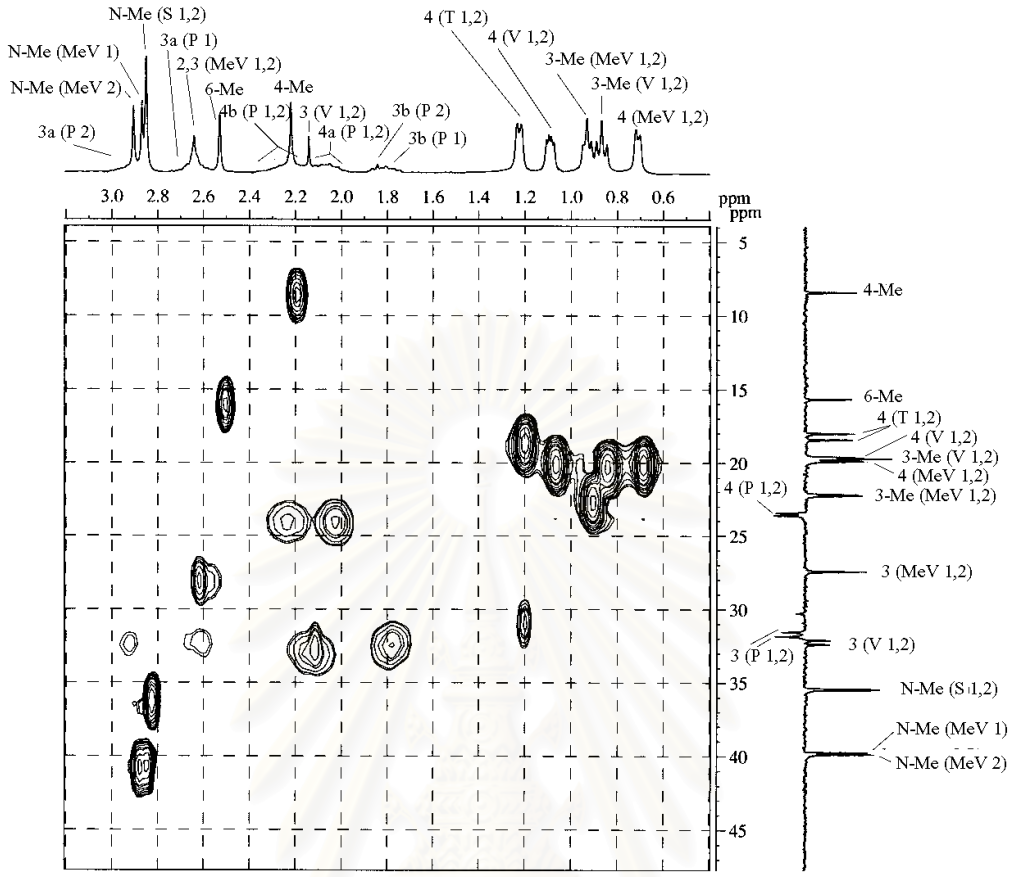
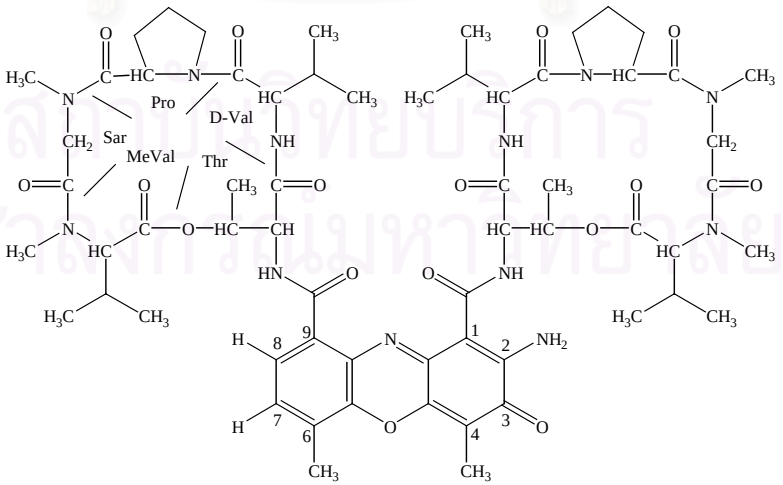


Figure 35 The 300 MHz HMQC spectrum (in $CDCl_3$) of actinomycin D (FK009) (expanded from δ_H 0.40-3.20 ppm and δ_C 4.00-48.00 ppm).



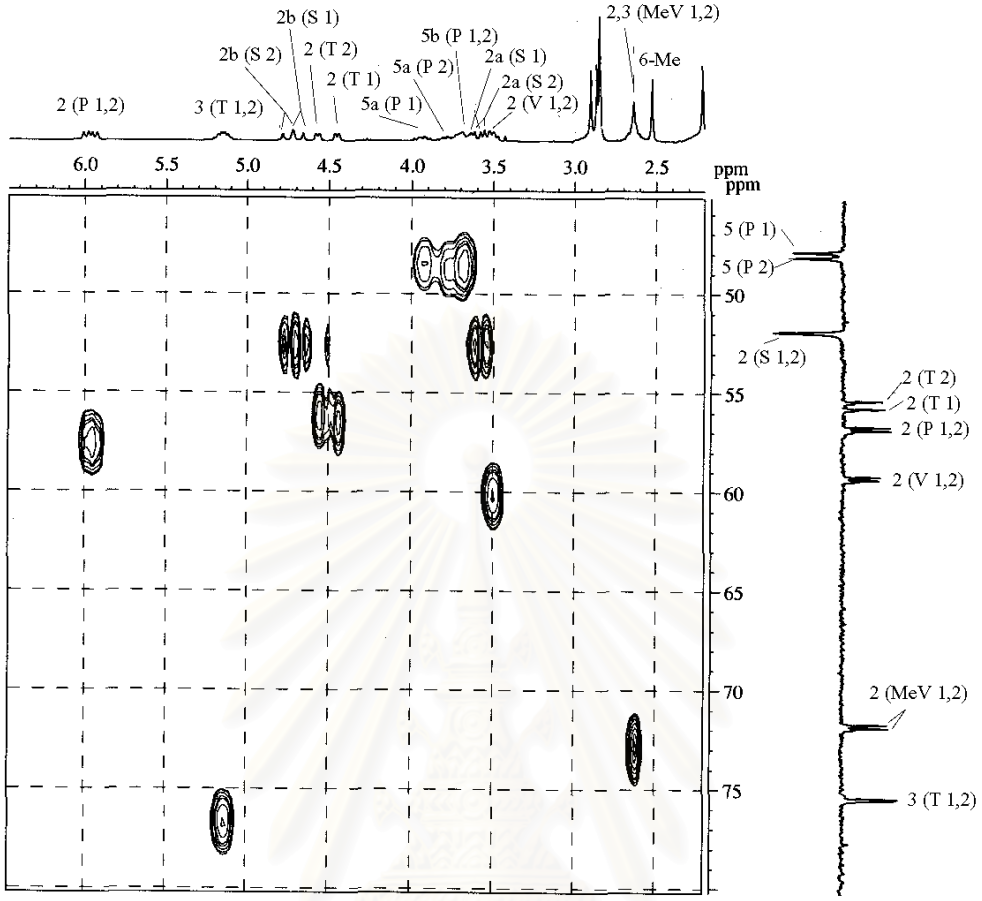
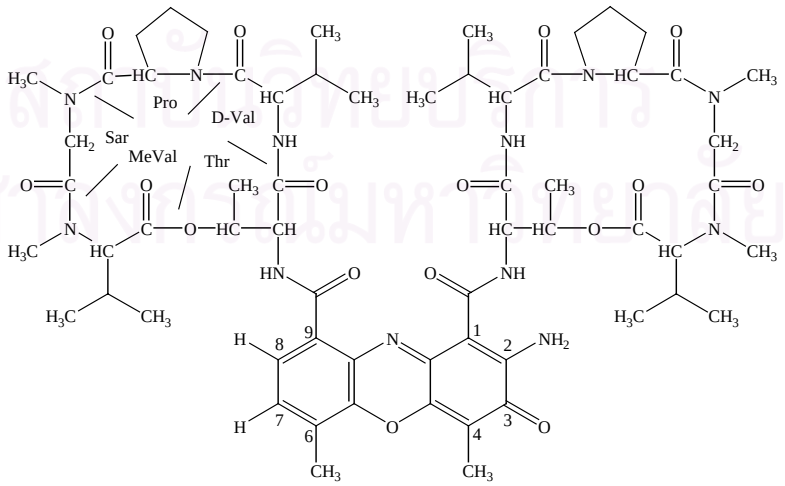


Figure 36 The 300 MHz HMQC spectrum (in $CDCl_3$) of actinomycin D (FK009) (expanded from δ_H 2.20-6.50 ppm and δ_C 45.00-80.00 ppm).



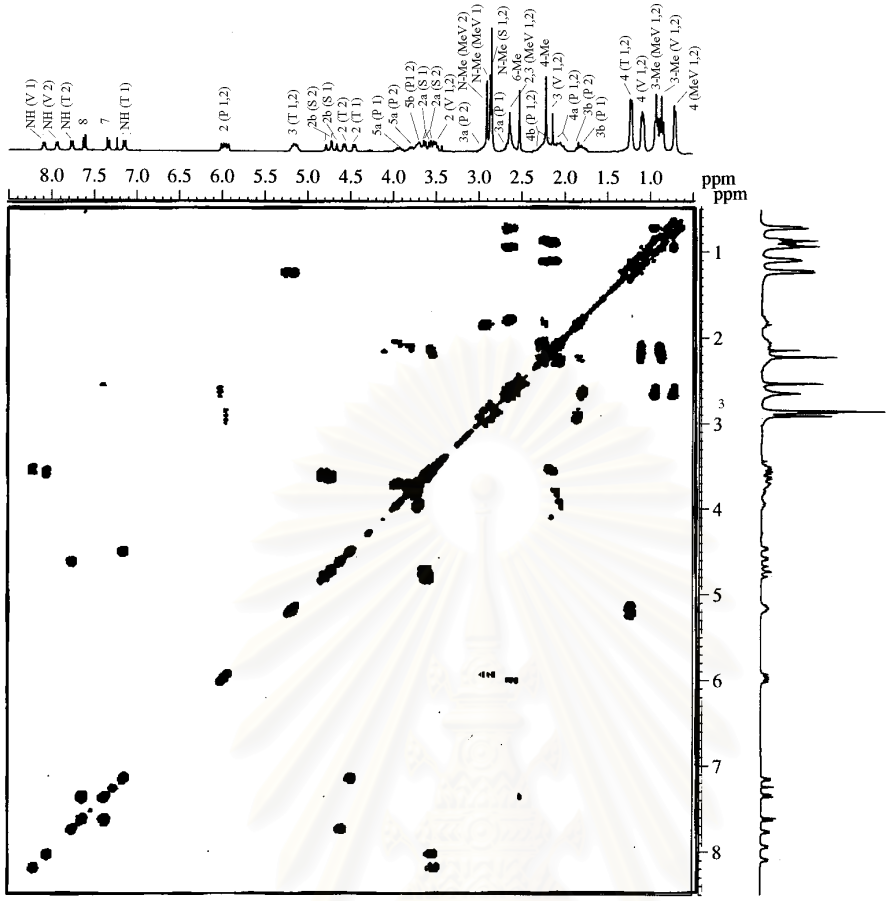
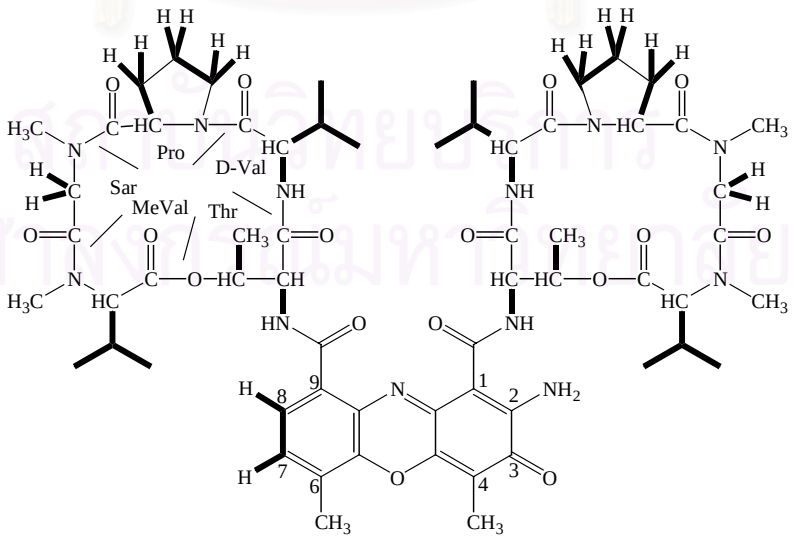


Figure 37 The 300 MHz ^{1}H-^{1}H COSY spectrum (in $CDCl_3$) of actinomycin D (FK009).



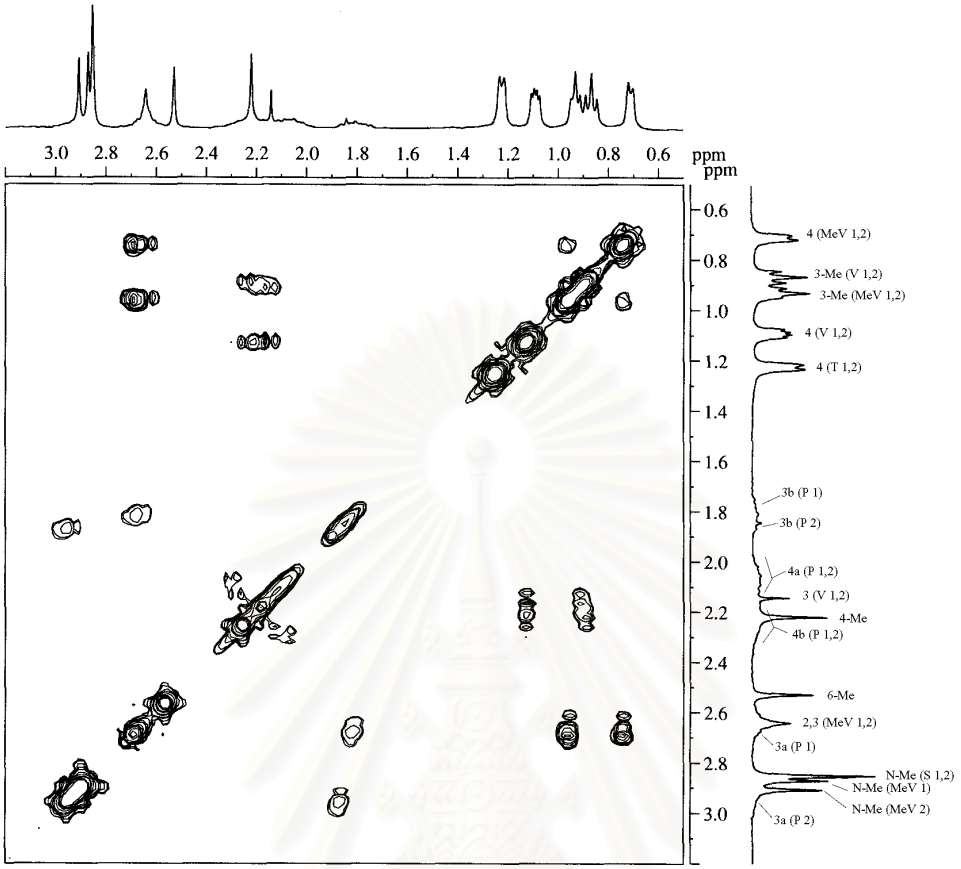
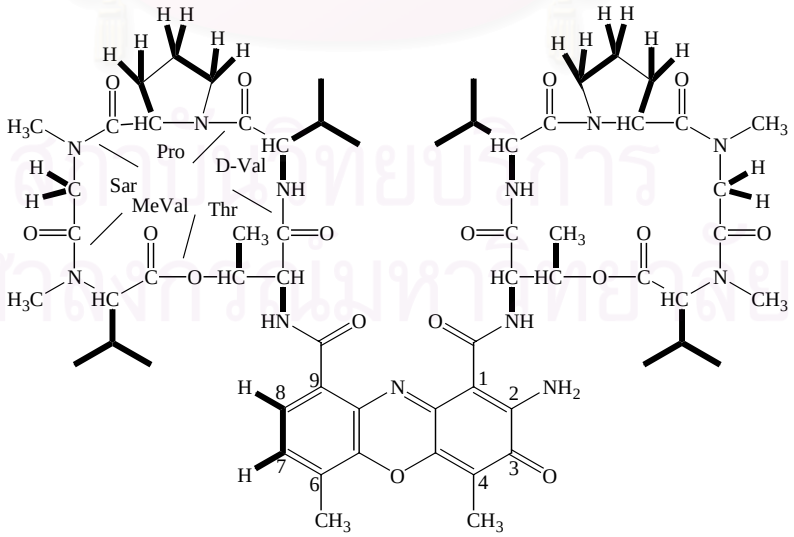


Figure 38 The 300 MHz ^{1}H-^{1}H COSY spectrum (in $CDCl_3$) of actinomycin D (FK009) (expanded from δ_H 0.50-3.20 ppm).



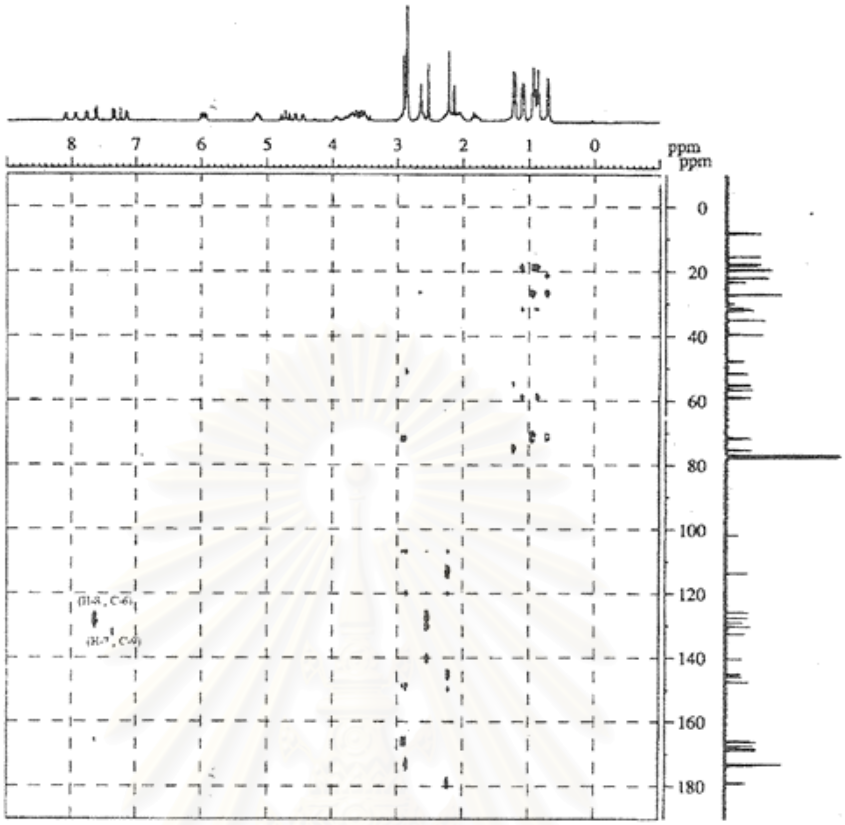
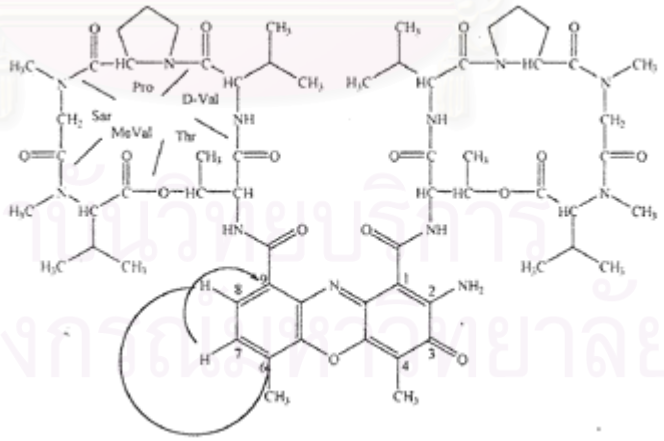


Figure 39 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) (in $CDCl_3$) of actionmycin D (FK009).



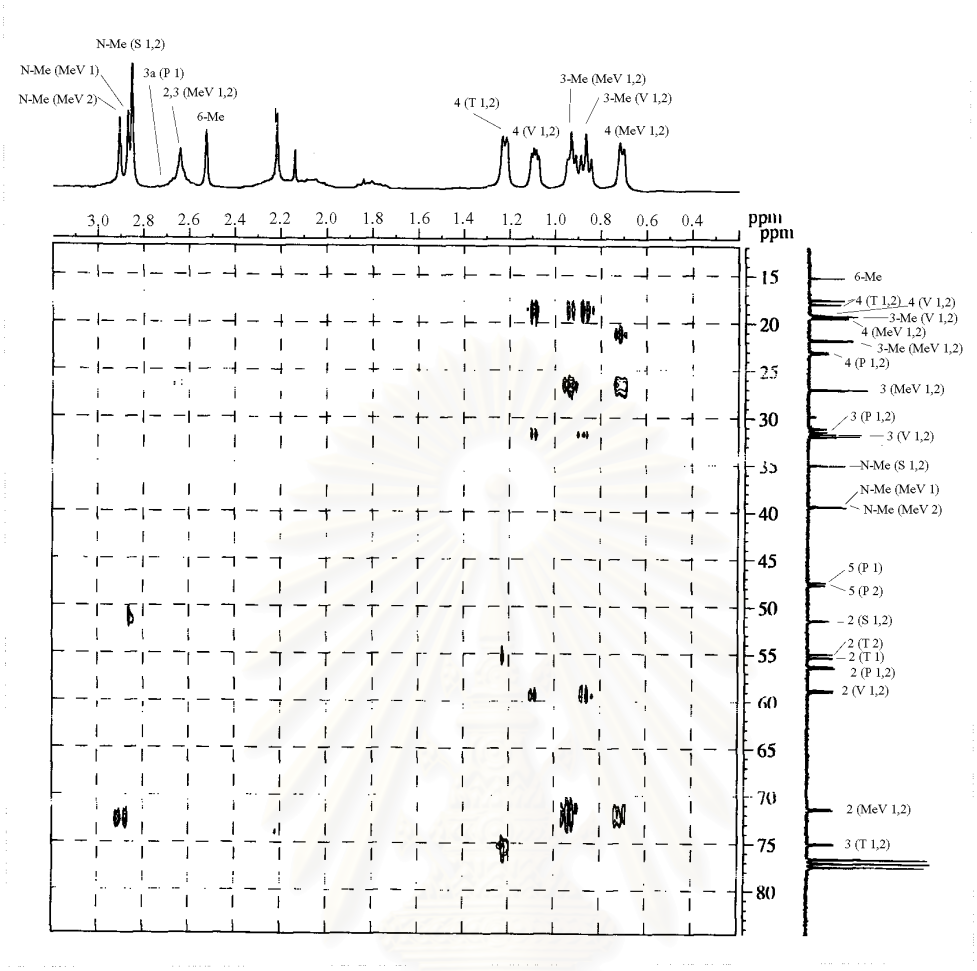
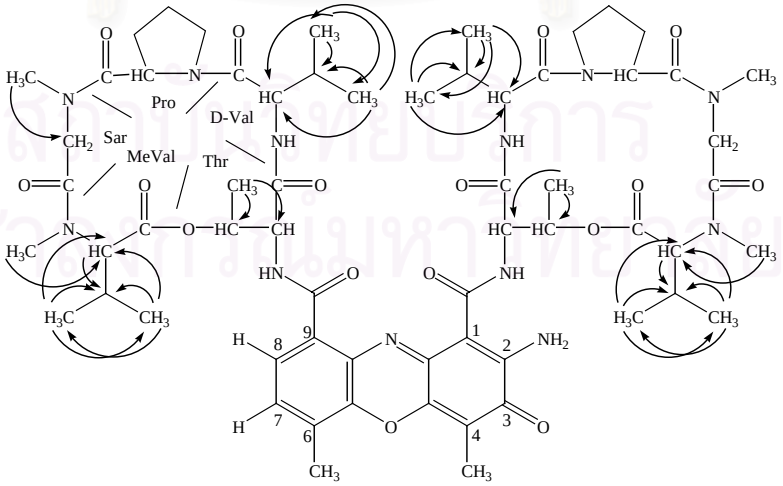


Figure 40 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) (in $CDCl_3$) of actinomycin D (FK009) (expanded from δ_H 0.20-3.20 ppm and δ_C 12.00-85.00 ppm).



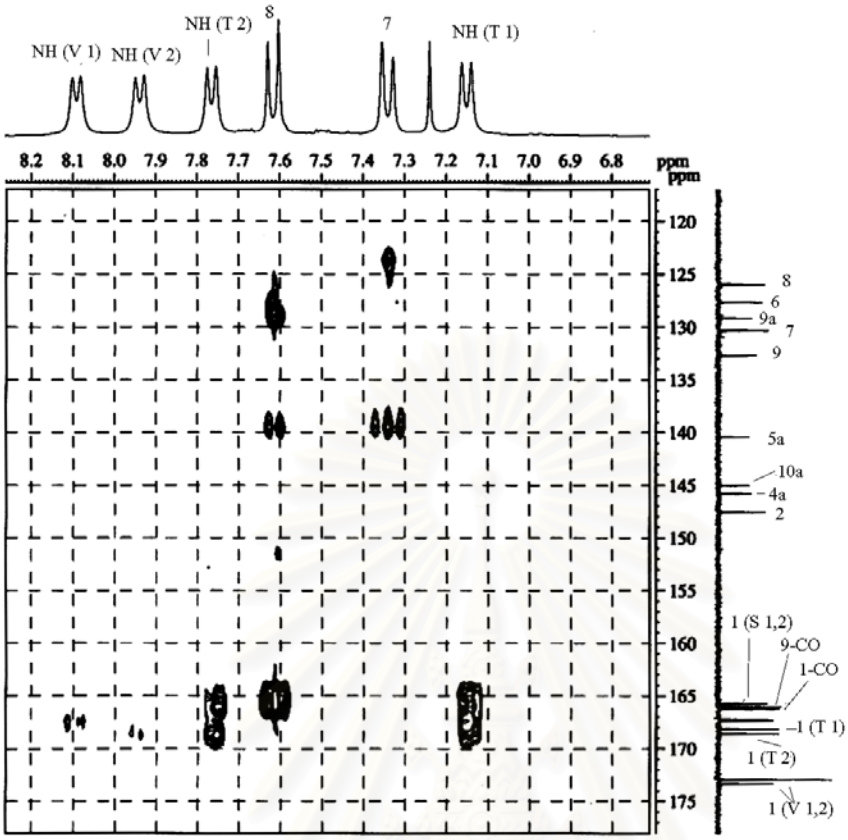
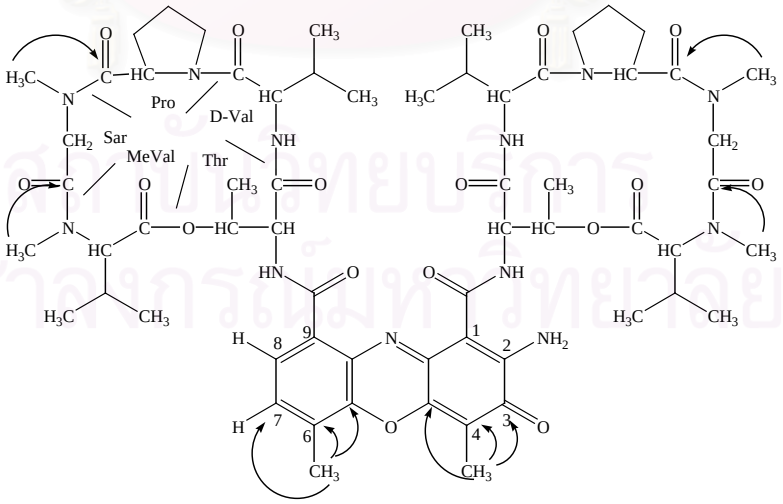


Figure 41 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) (in $CDCl_3$) of actinomycin D (FK009) (expanded from δ_H 2.00-3.20 ppm and δ_C 90.00-190.00 ppm).



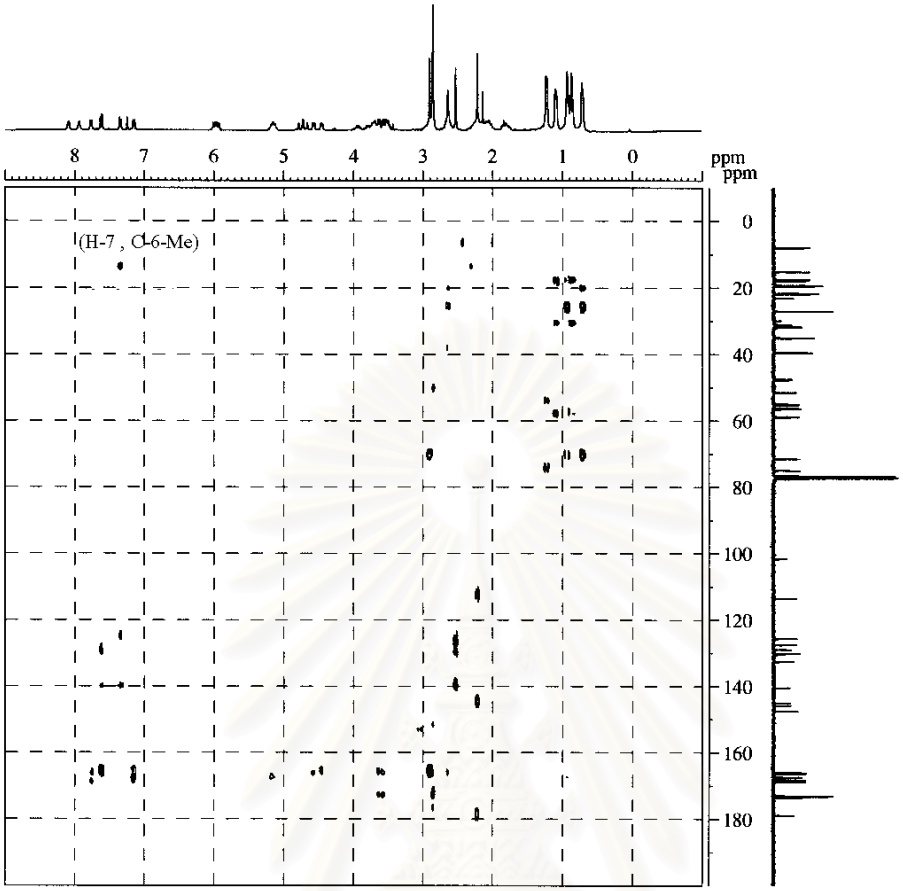
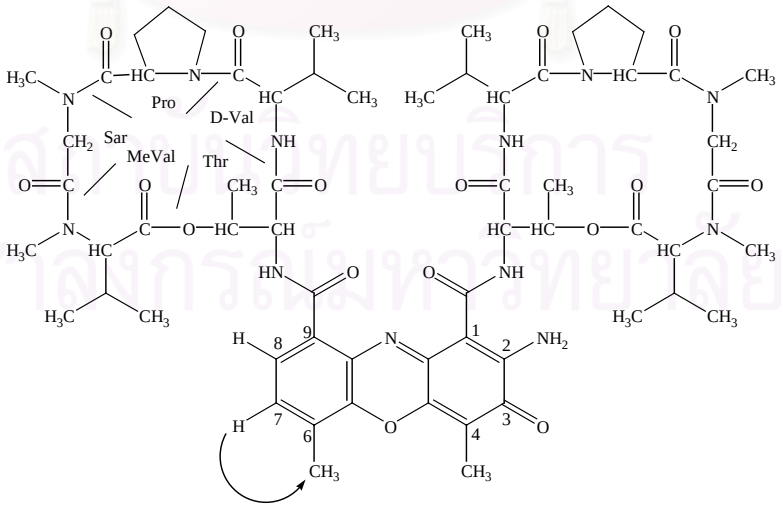


Figure 42 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) (in $CDCl_3$) of actinomycin D (FK009).



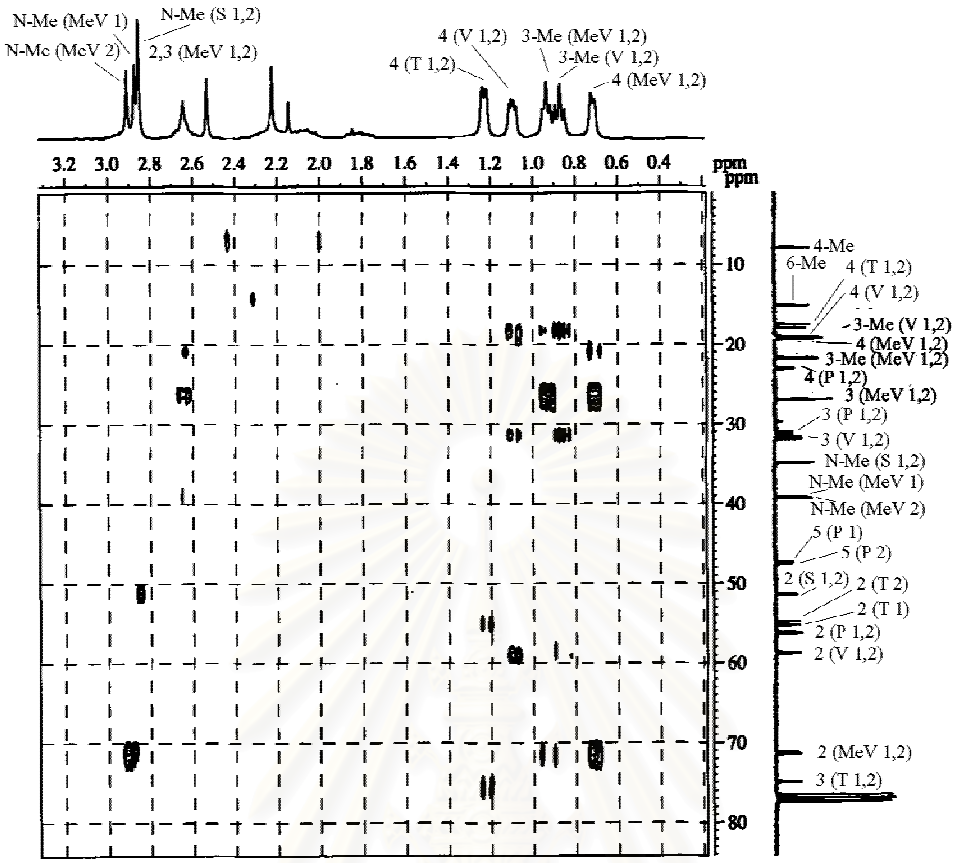
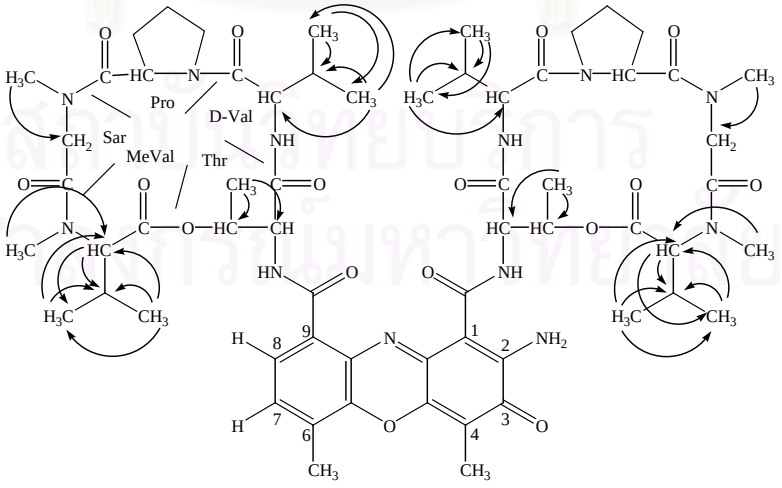


Figure 43 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) (in $CDCl_3$) of actinomycin D (FK009) (expanded from δ_H 0.20-3.30 ppm and δ_C 1.00-84.00 ppm).



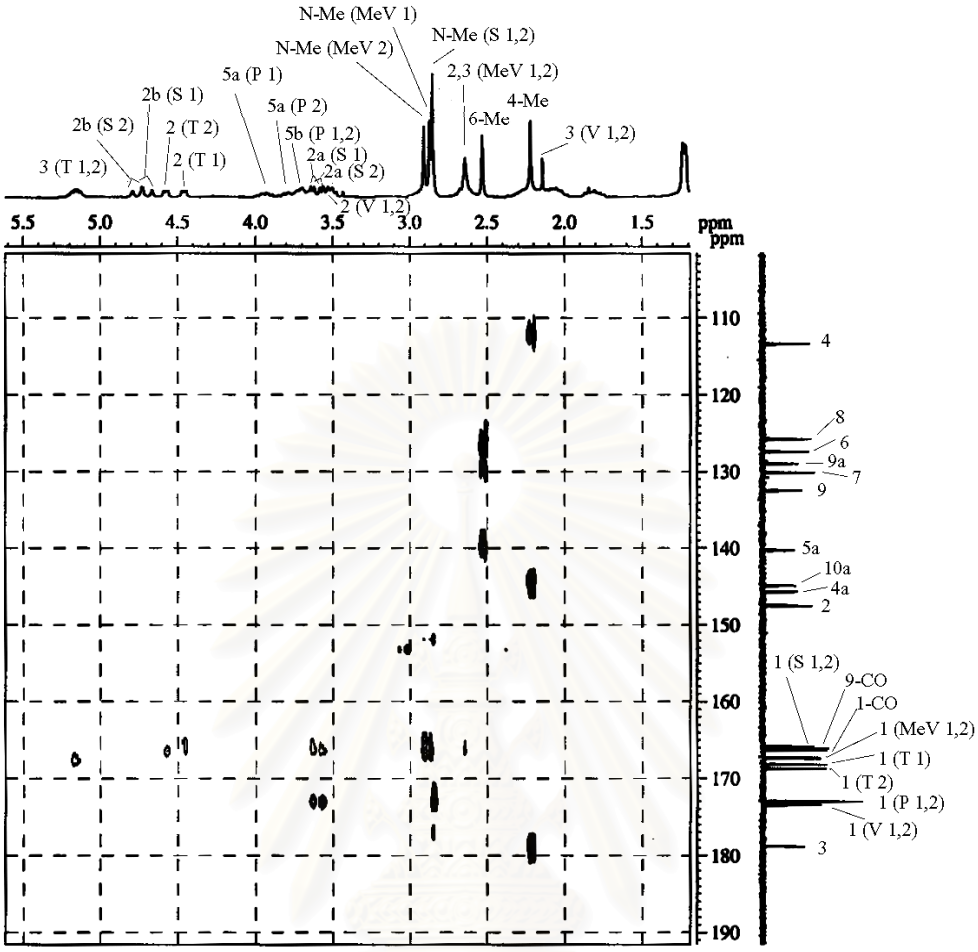
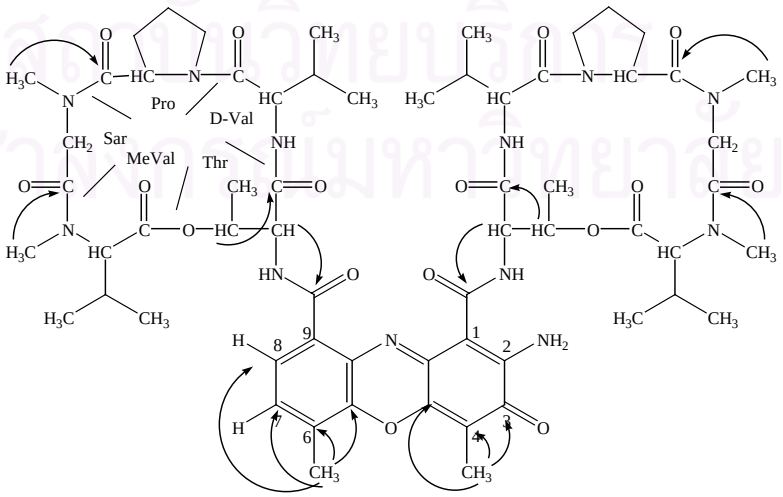


Figure 44 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) (in $CDCl_3$) of actinomycin D (FK009) (expanded from δ_H 1.20-5.60 ppm and δ_C 102.00-191.00 ppm).



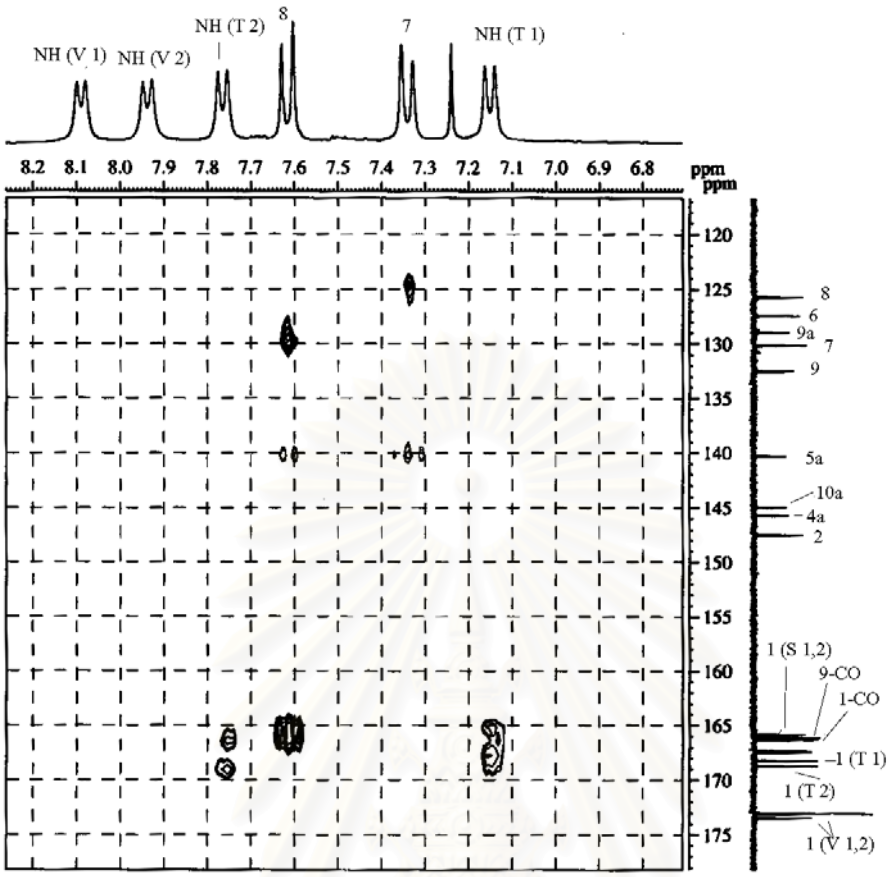
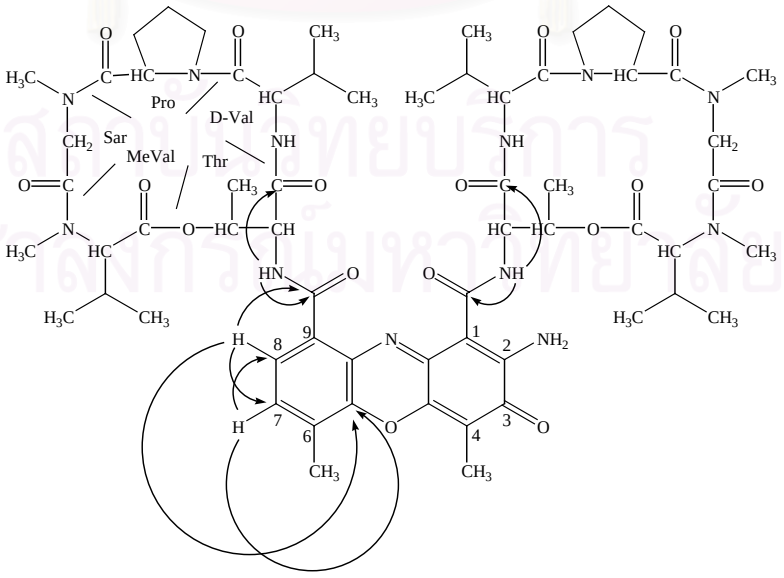


Figure 45 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) (in $CDCl_3$) of actinomycin D (FK009) (expanded from δ_H 6.70-8.26 ppm and δ_C 117.00-178.00 ppm).



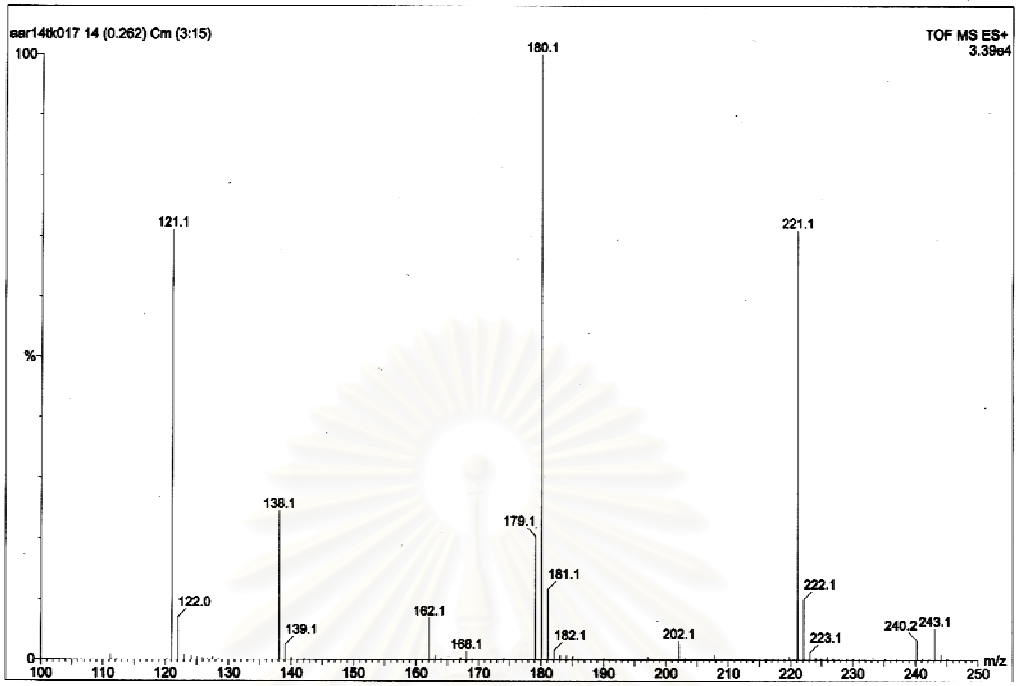


Figure 46 The ESI-TOF mass spectrum of [*N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017).

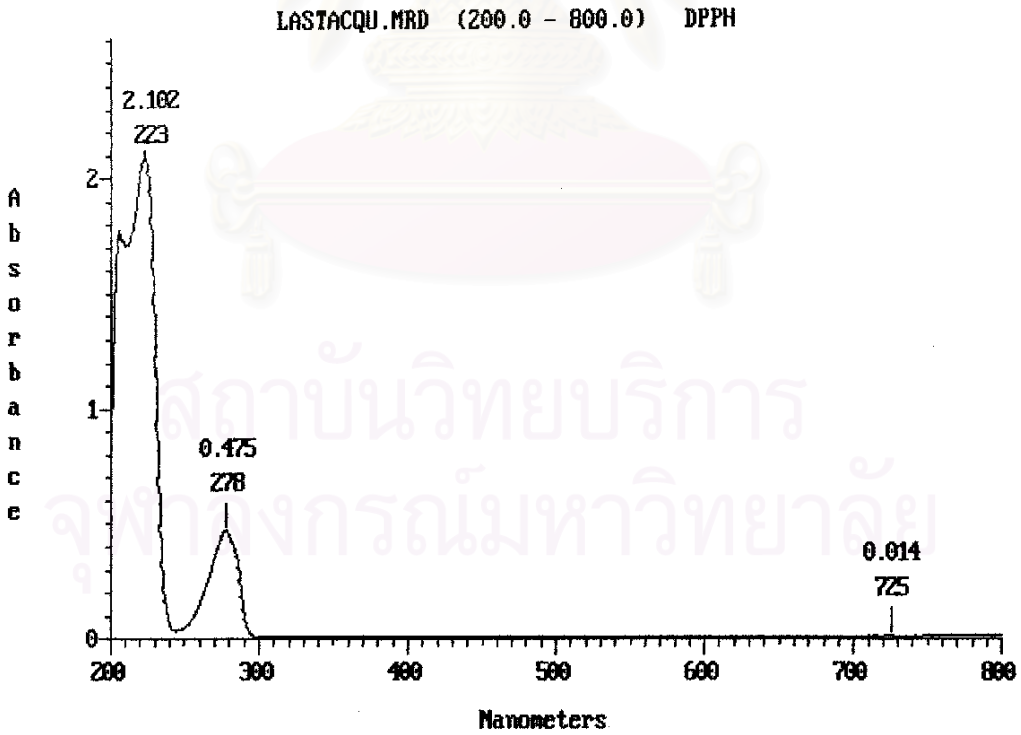


Figure 47 The UV spectrum (in MeOH) of [*N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017).

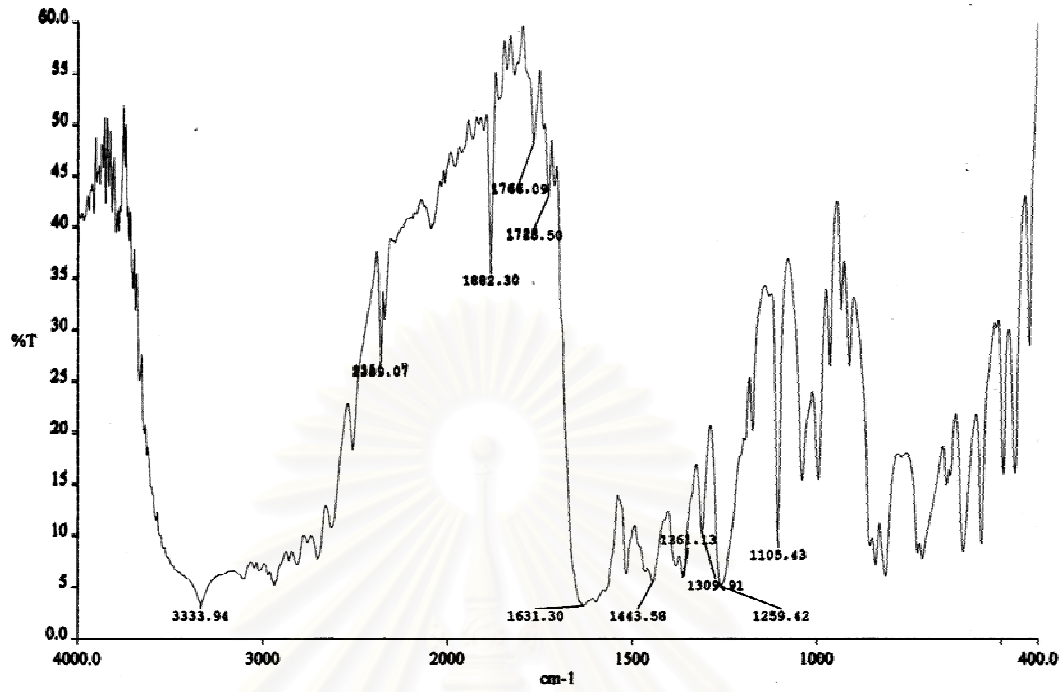
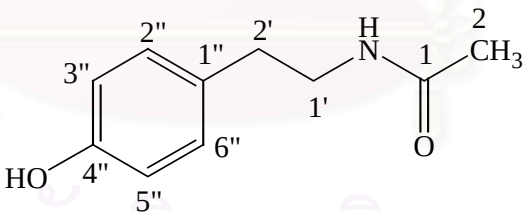


Figure 48 The IR spectrum of [*N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017) (KBr disc).



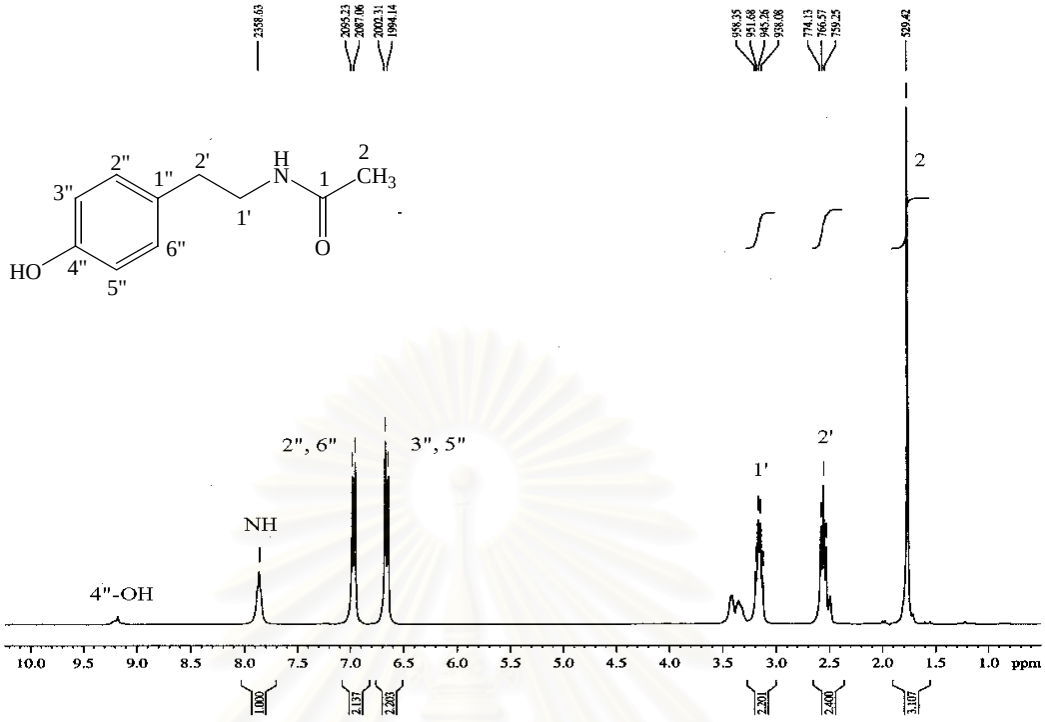


Figure 49 The 300 MHz ^{1}H NMR spectrum (in DMSO-d_6) of *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017), water suppression.

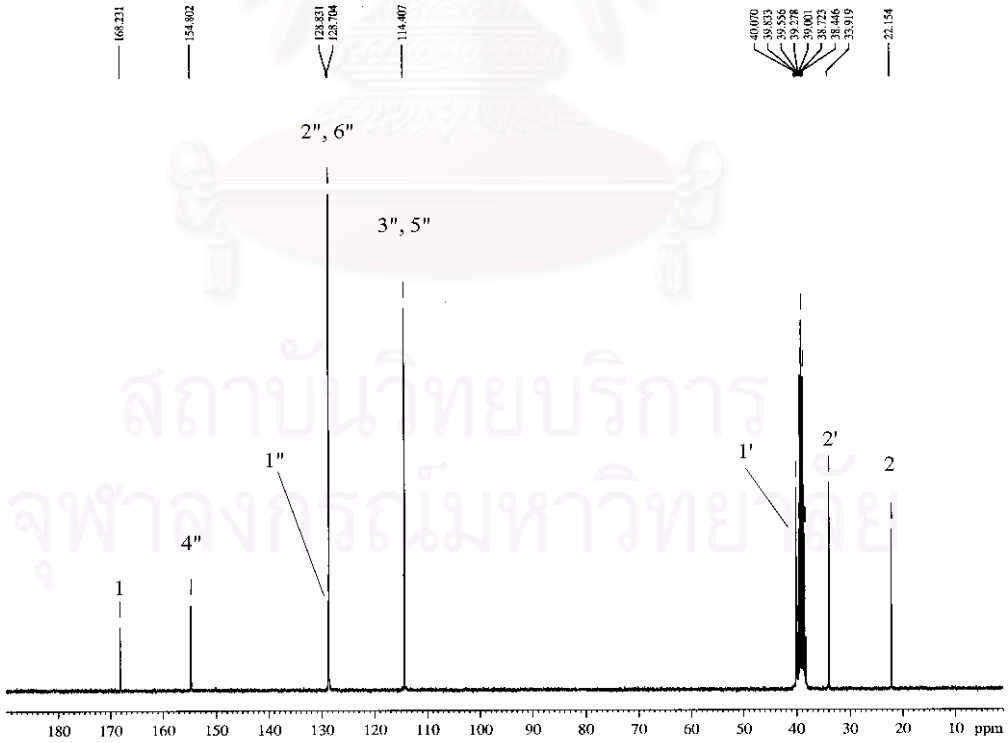


Figure 50 The 75 MHz ^{13}C NMR spectrum (in DMSO-d_6) of *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017).

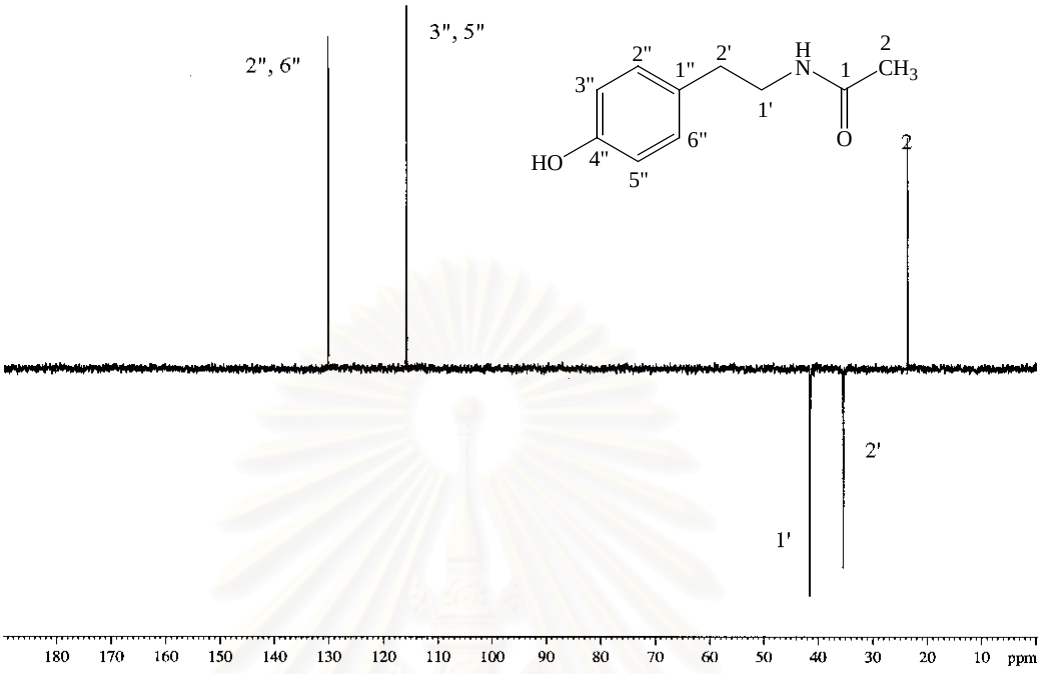


Figure 51 The 75 MHz DEPT 135 spectrum (in DMSO-d_6) of *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017).

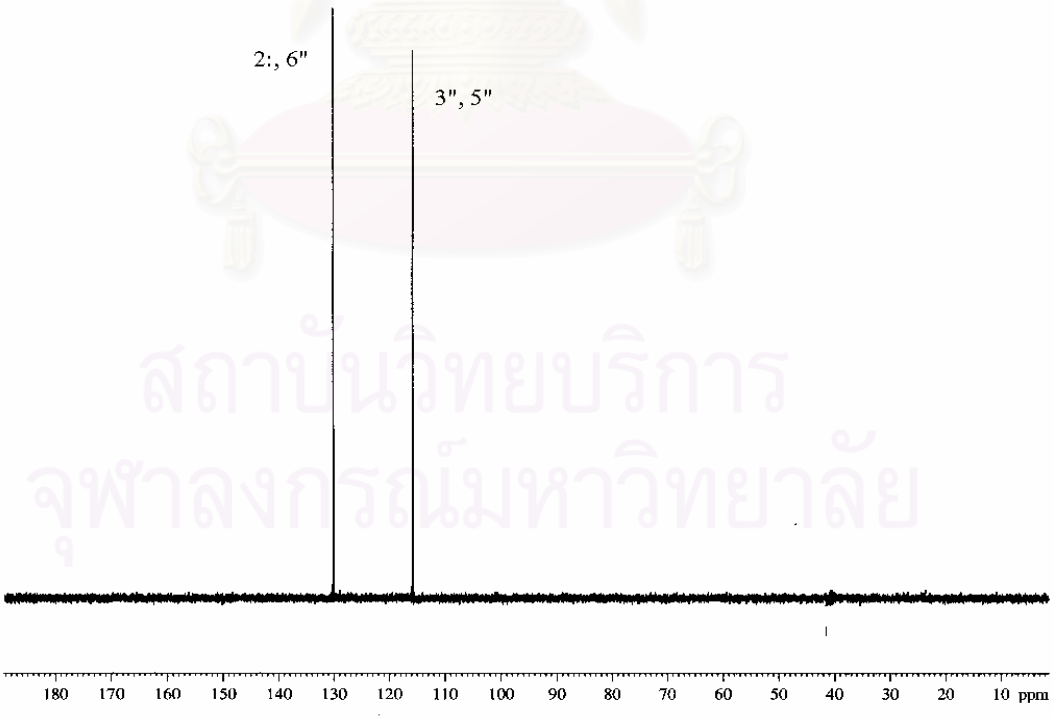


Figure 52 The 75 MHz DEPT 90 spectrum (in DMSO-d_6) of *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017).

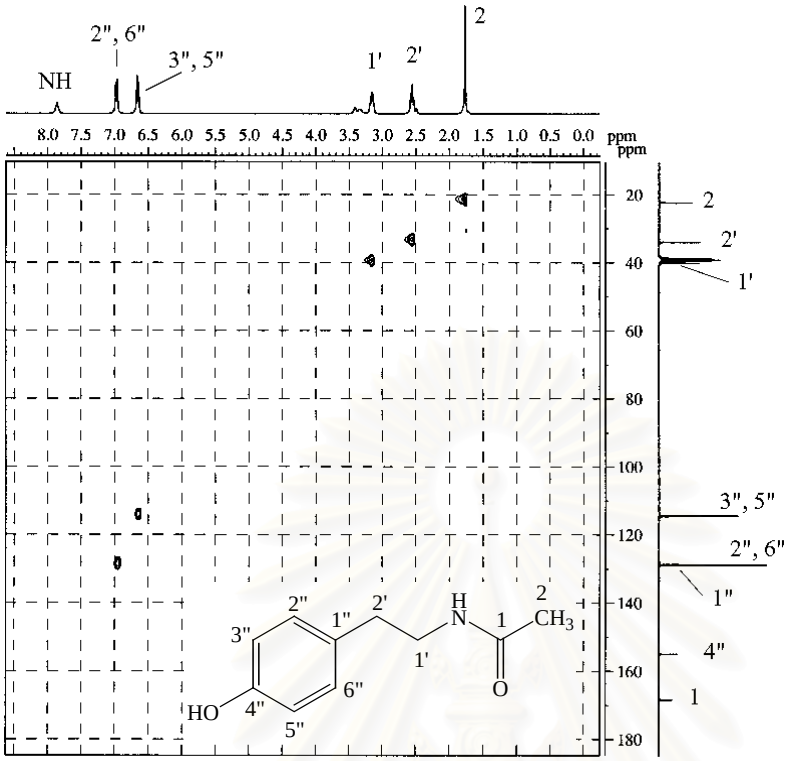


Figure 53 The 300 MHz HMQC spectrum (in DMSO-d_6) of *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017).

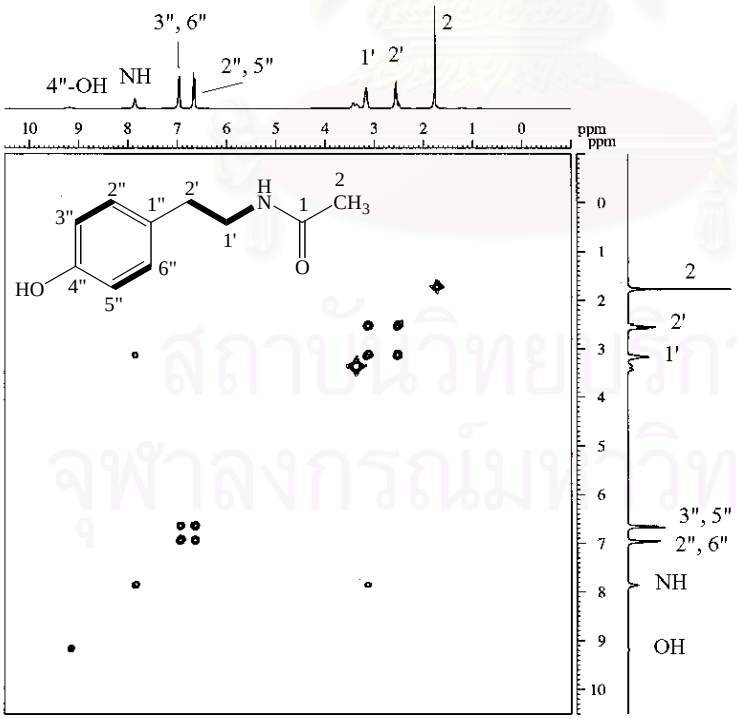


Figure 54 The 300 MHz ^{1}H-^{1}H COSY spectrum (in DMSO-d_6) of *N*-[2′-(4′′-hydroxyphenyl)ethyl]acetamide (TK017).

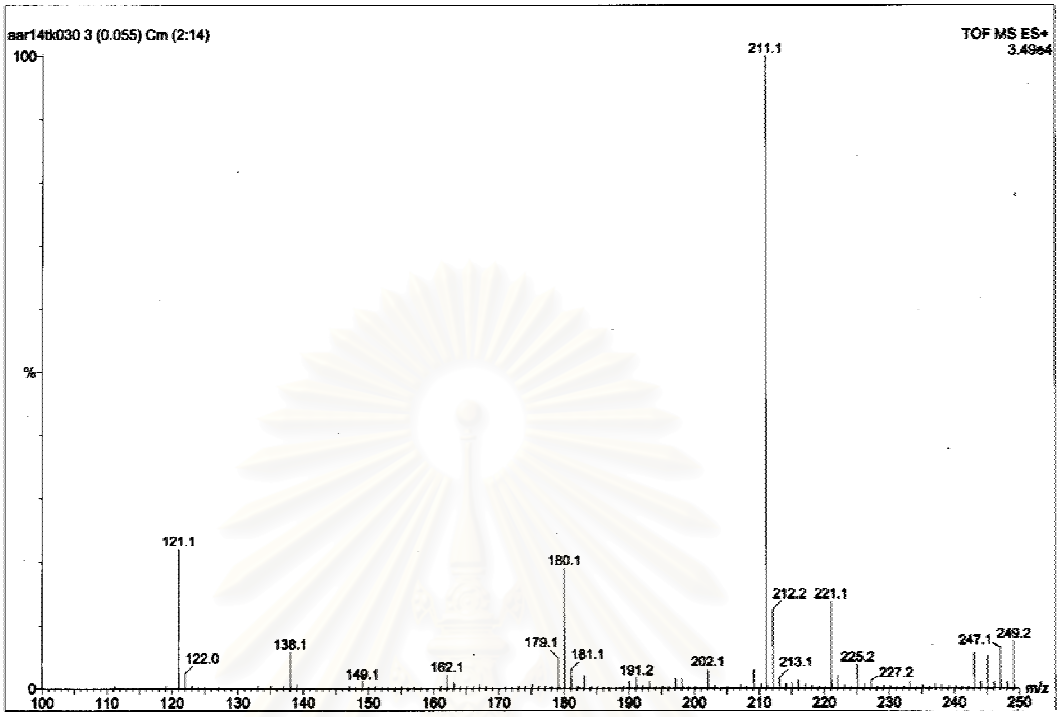


Figure 55 The ESI-TOF mass spectrum of *cyclo*-(L-prolyl-D-leucyl) (TK030).

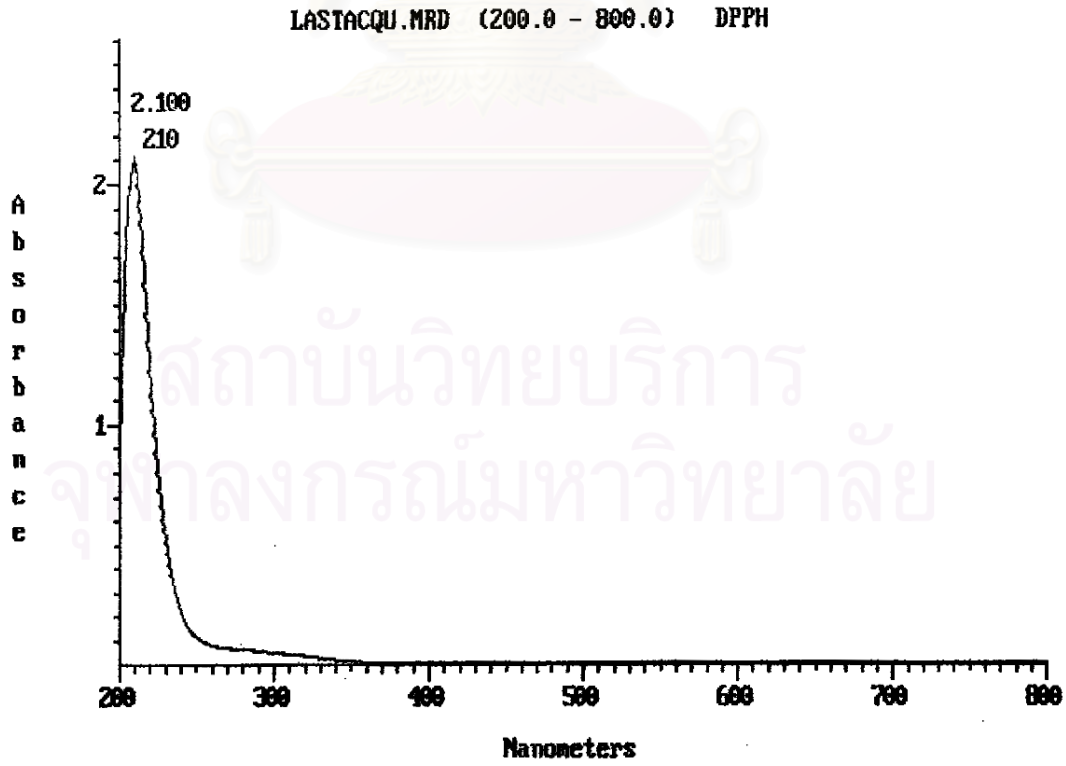


Figure 56 The UV spectrum (in MeOH) of *cyclo*-(L-prolyl-D-leucyl) (TK030).

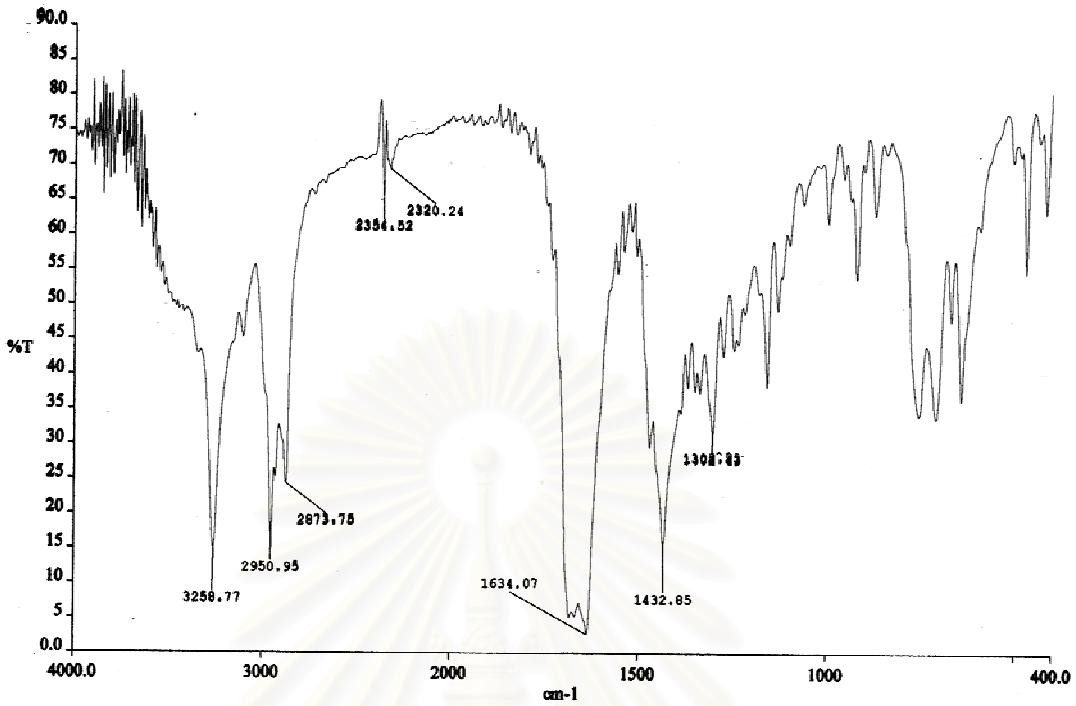
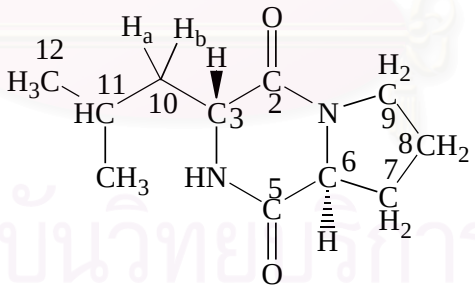


Figure 57 The IR spectrum of *cyclo*-(L-prolyl-D-leucyl) (TK030) (KBr disc).



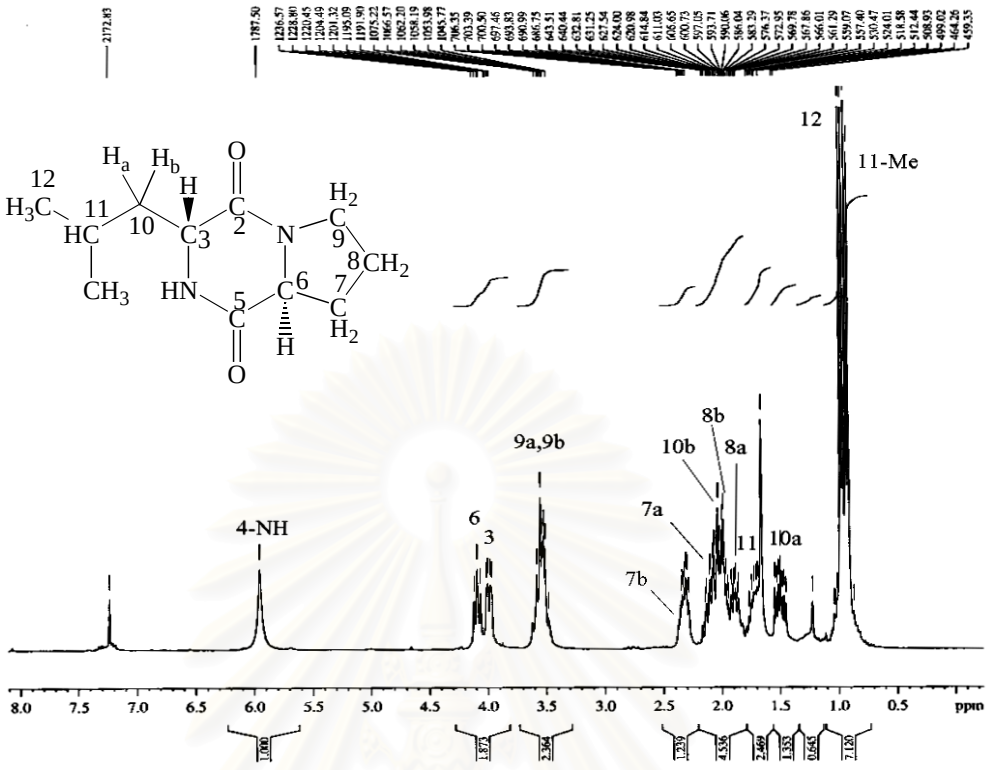


Figure 58 The 300 MHz ^{1}H NMR spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-leucyl) (TK030).

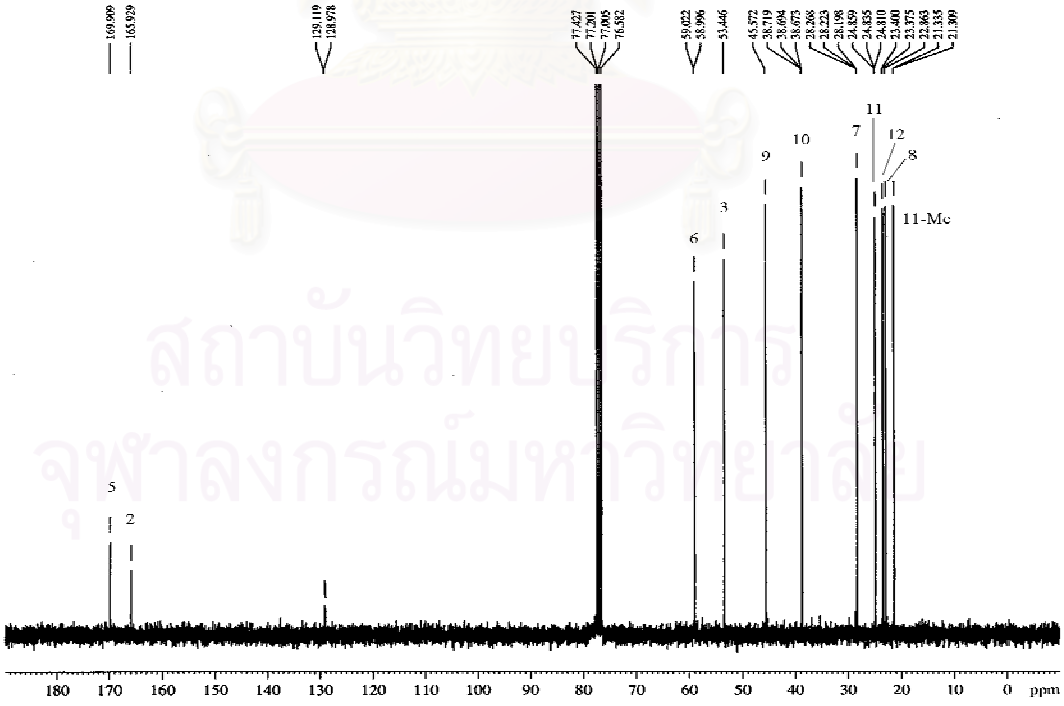


Figure 59 The 75 MHz ^{13}C NMR spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-leucyl) (TK030).

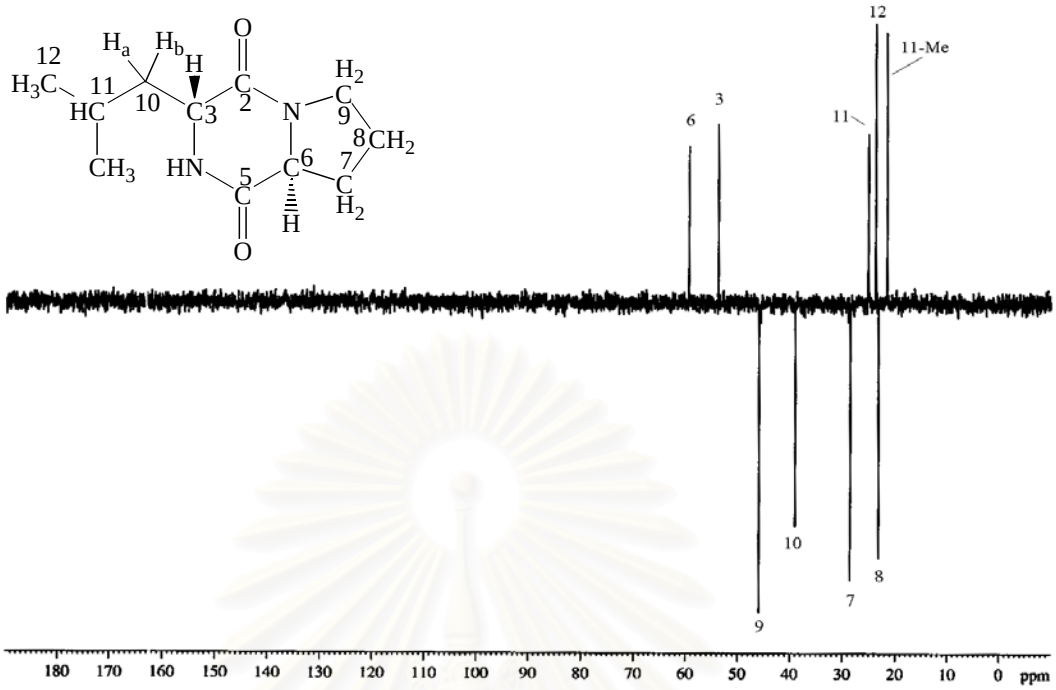


Figure 60 The 75 MHz DEPT 135 spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-leucyl) (TK030).

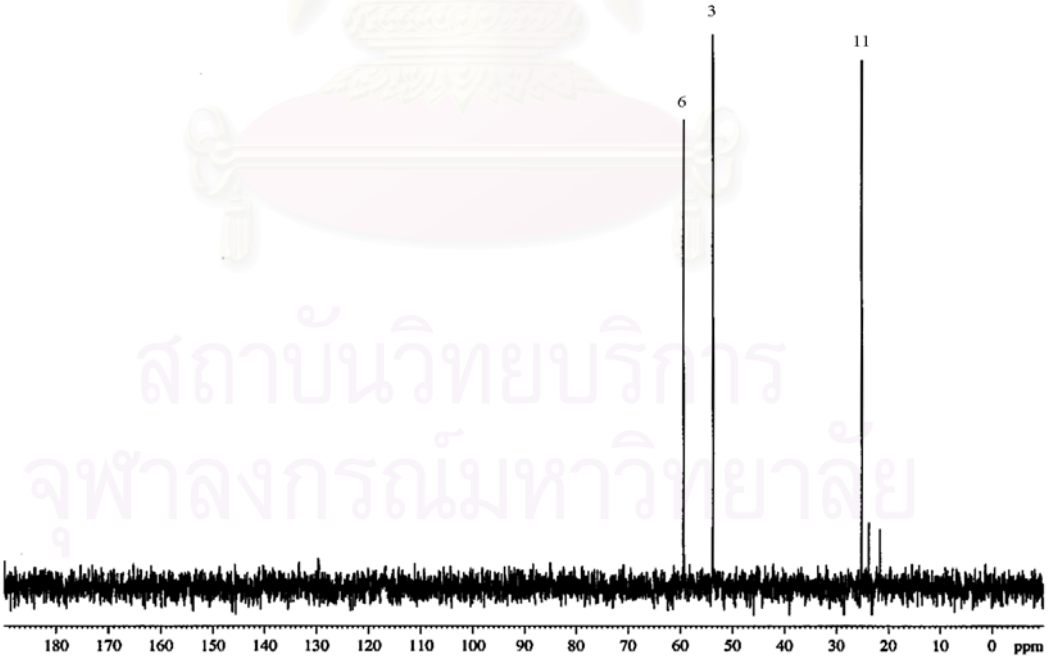


Figure 61 The 75 MHz DEPT 90 spectrum of (in $CDCl_3$) *cyclo*-(L-prolyl-D-leucyl) (TK030).

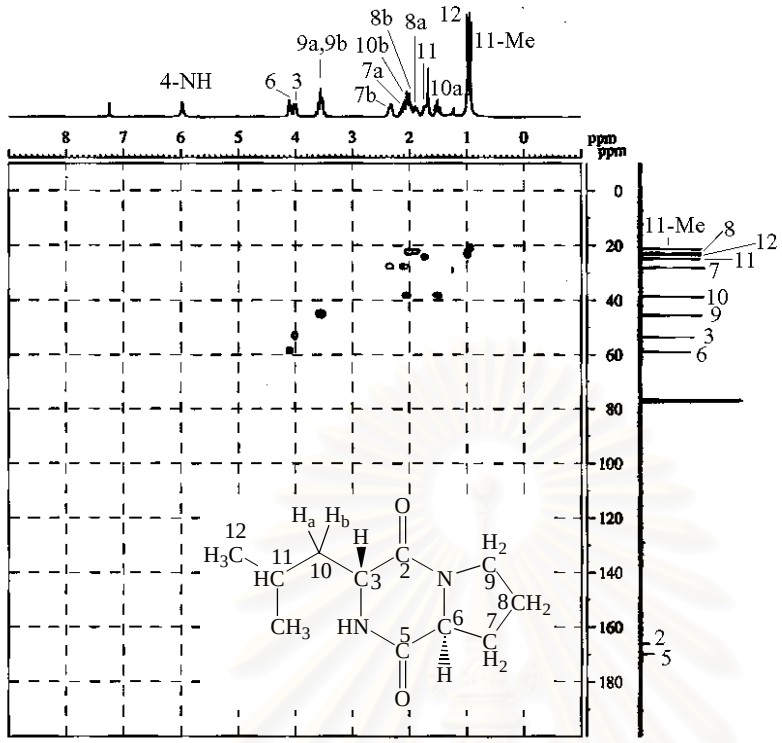


Figure 62 The 300 MHz HMQC spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-leucyl) (TK030).

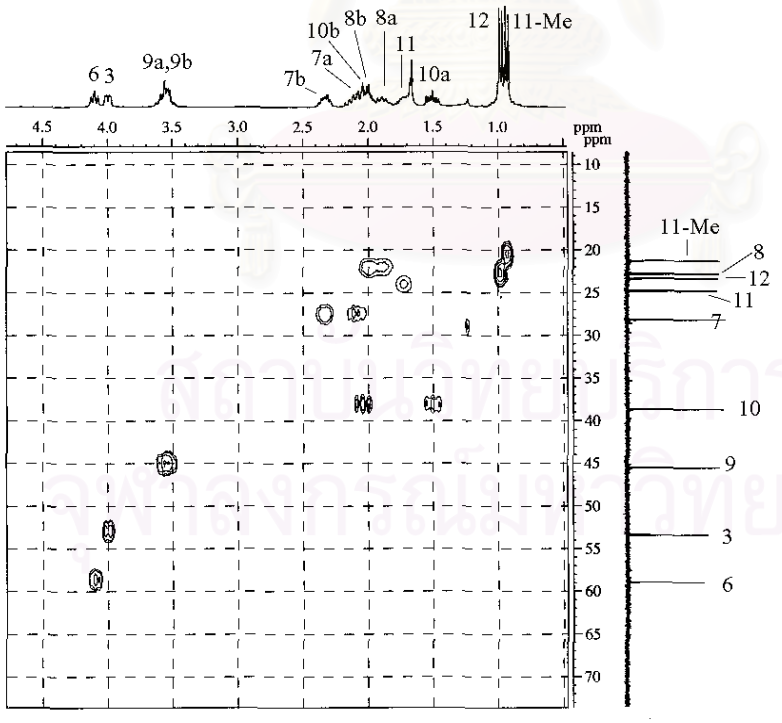


Figure 63 The 300 MHz HMQC spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-leucyl) (TK030) (expanded from δ_H 0.50-4.70 ppm and δ_C 9.00-74.00 ppm).

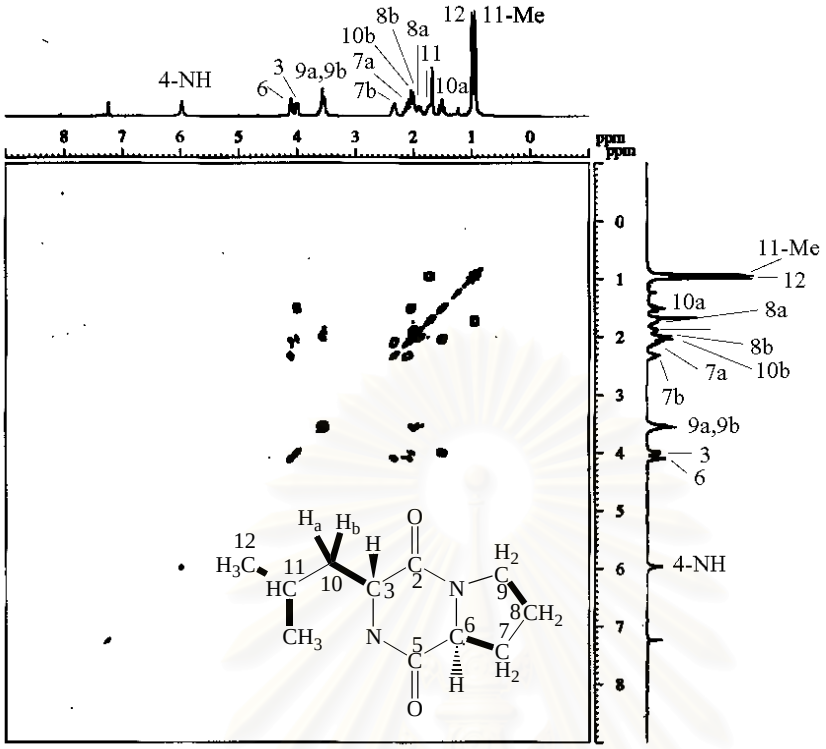


Figure 64 The 300 MHz ^{1}H-^{1}H COSY spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-leucyl) (TK030).

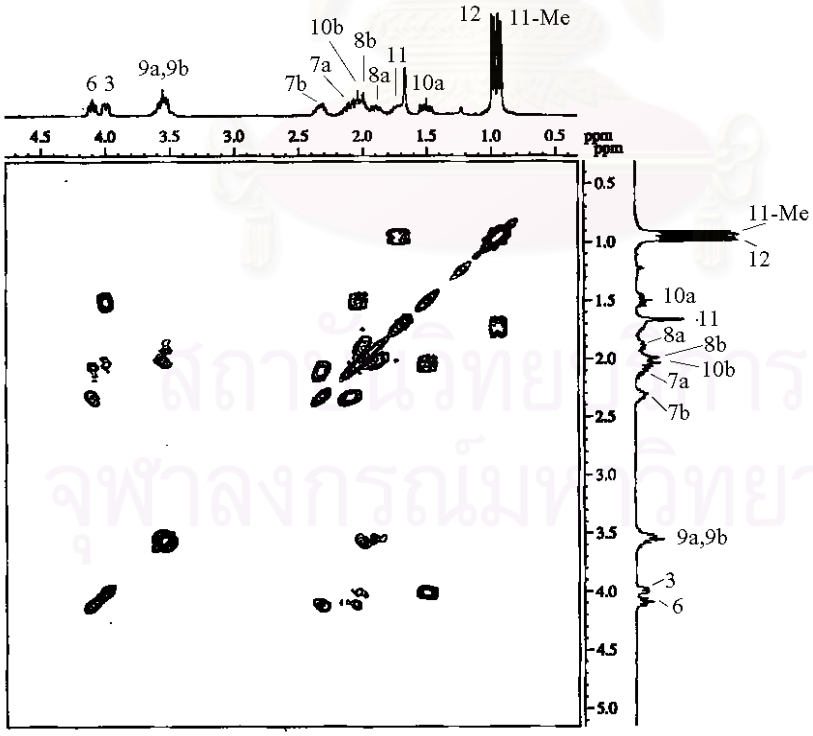


Figure 65 The 300 MHz ^{1}H-^{1}H COSY spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-leucyl) (TK030) (expanded from 0.40-4.70 ppm).

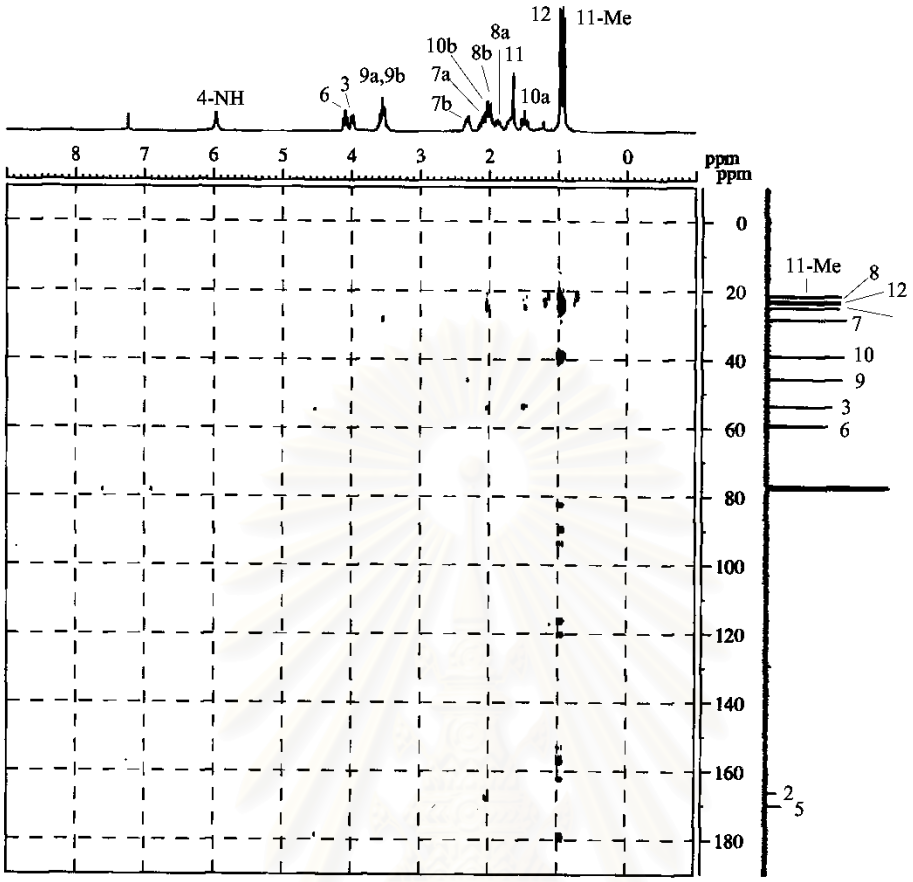
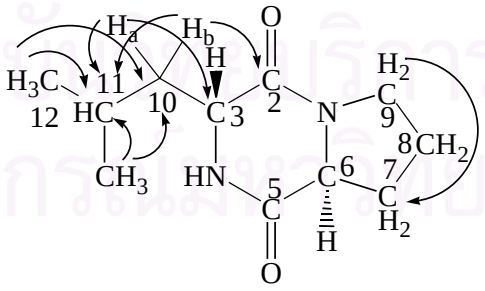


Figure 66 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) (in $CDCl_3$) of *cyclo*-(L-prolyl-D-leucyl) (TK030).



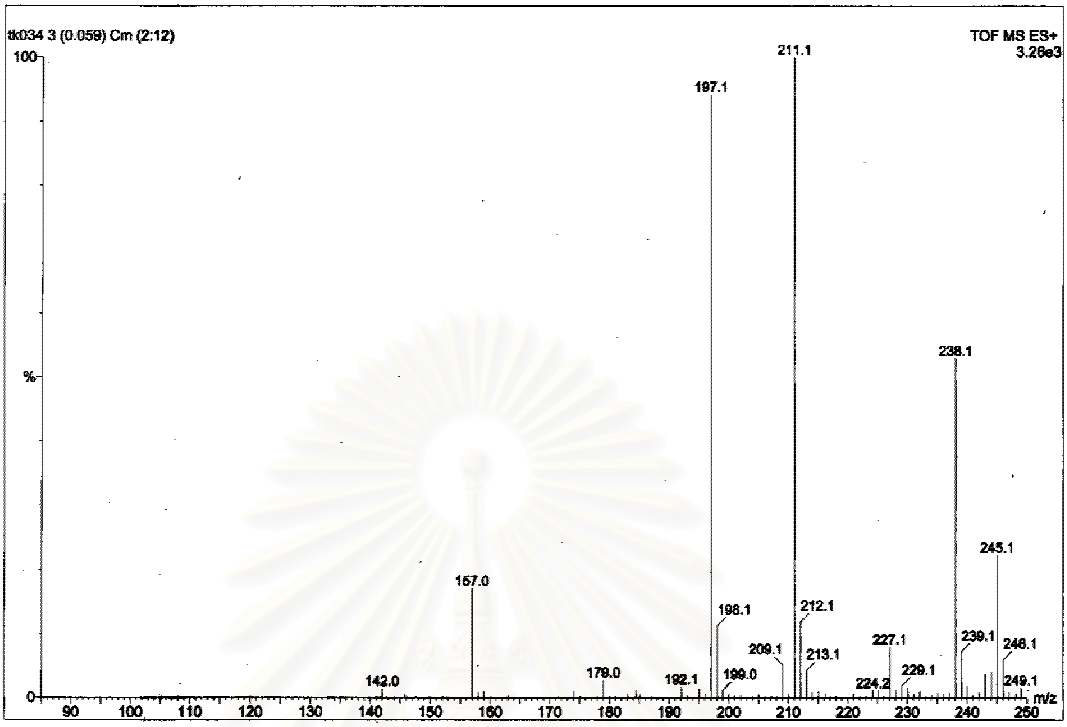


Figure 67 The ESI-TOF mass spectrum of *cyclo*-(L-prolyl-D-valyl) (TK034).

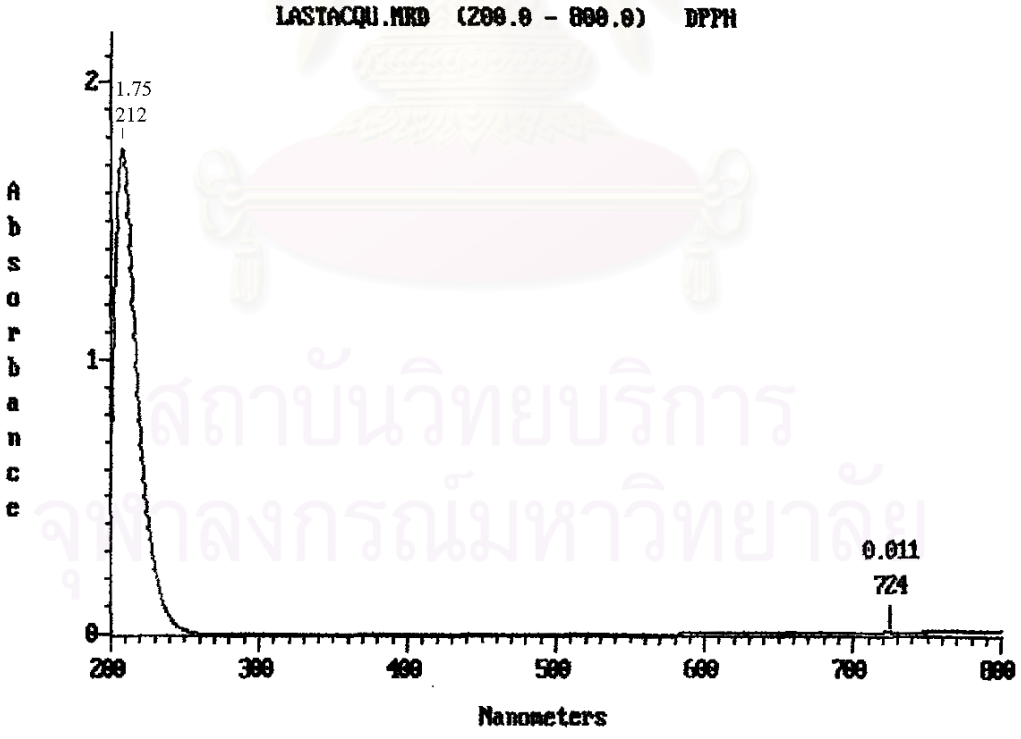


Figure 68 The UV spectrum (in MeOH) of *cyclo*-(L-prolyl-D-valyl) (TK034).

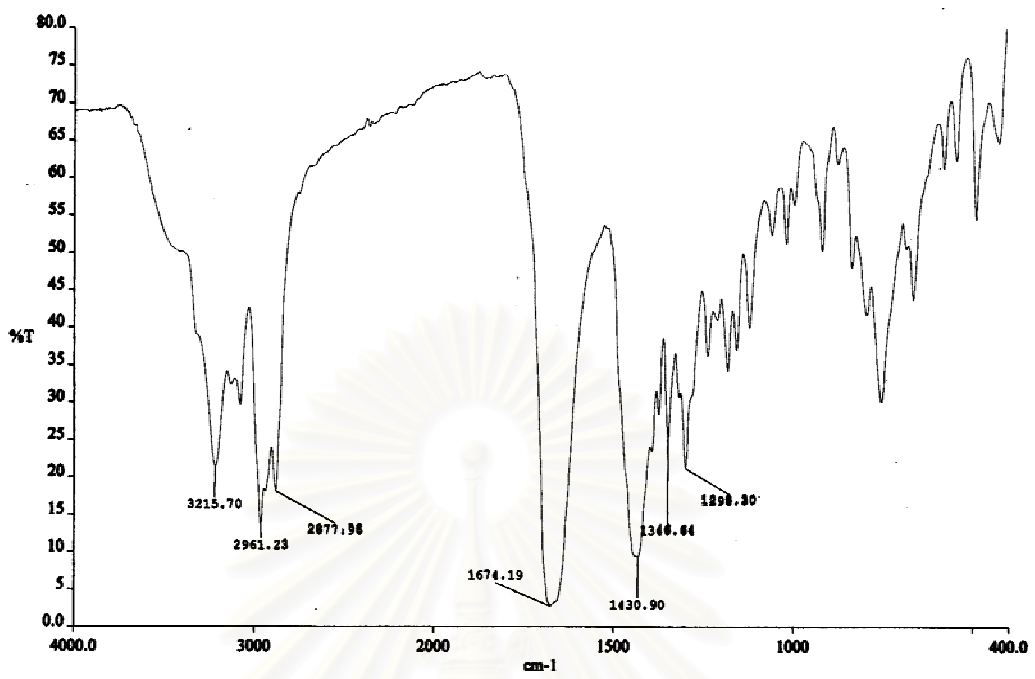
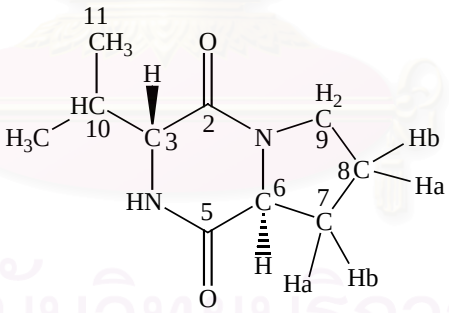


Figure 69 The IR spectrum of *cyclo*-(L-prolyl-D-valyl) (TK034) (KBr disc).



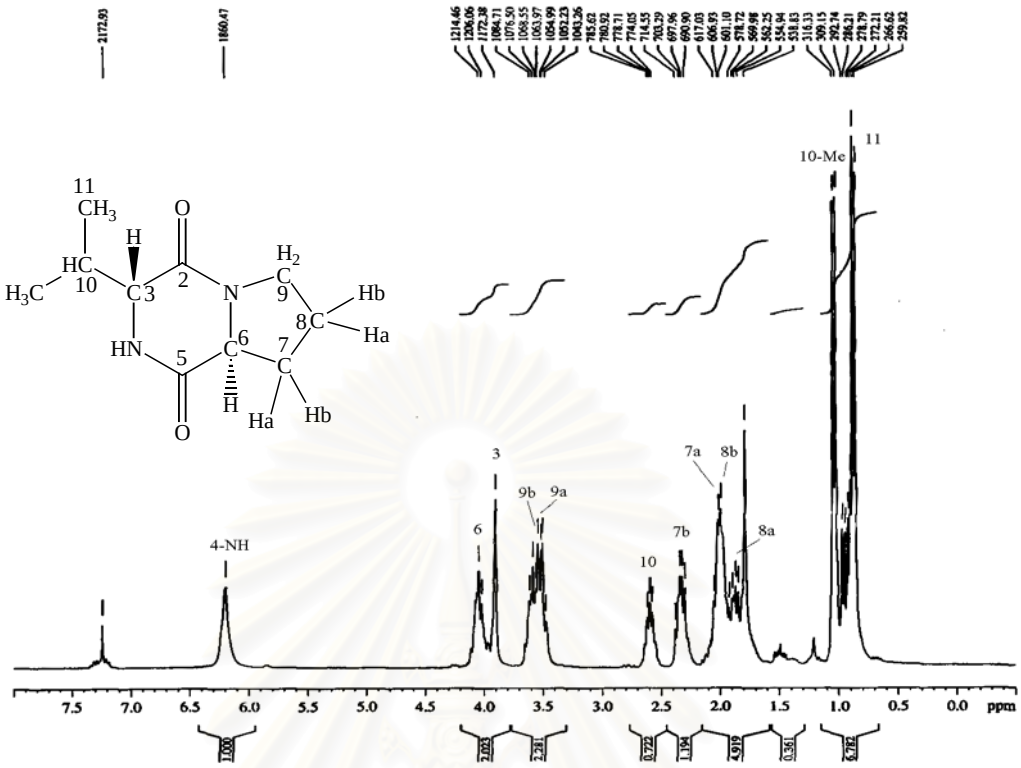


Figure 70 The 300 MHz ^{1}H NMR spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-valyl) (TK034).

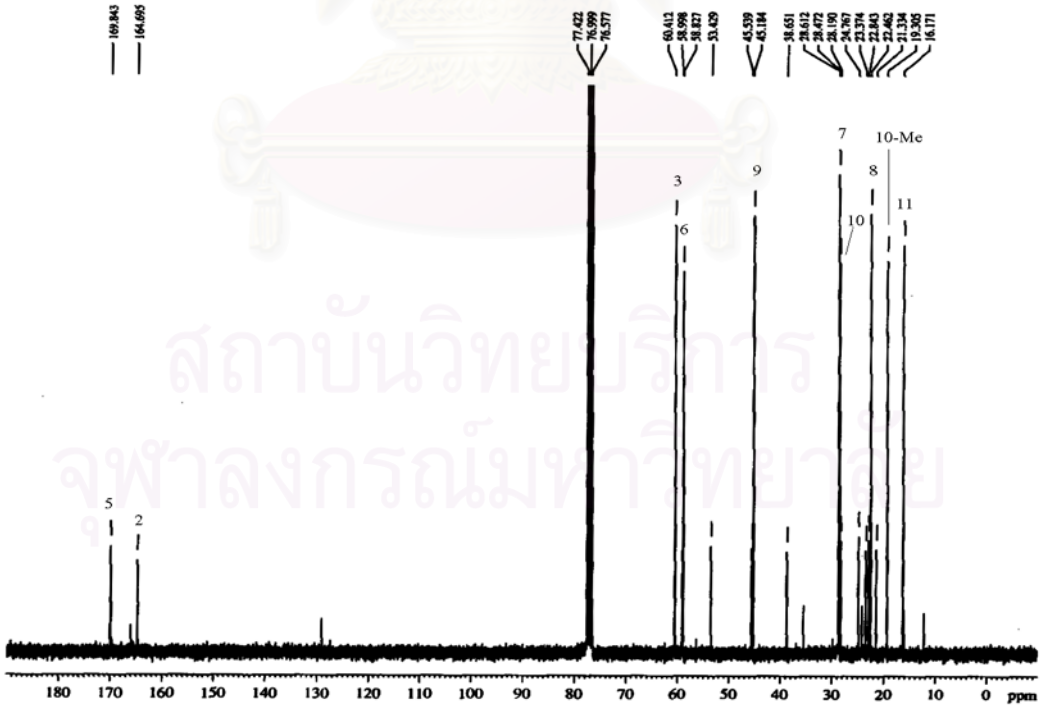


Figure 71 The 75 MHz ^{13}C NMR spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-valyl) (TK034)

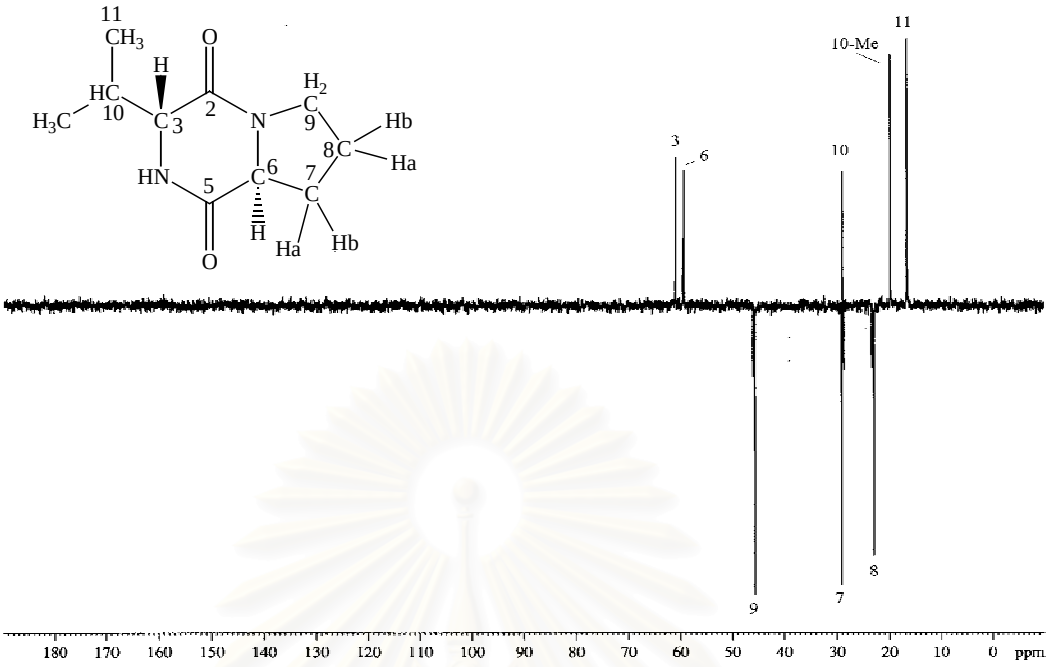


Figure 72 The 75 MHz DEPT 135 spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-valyl) (TK034).

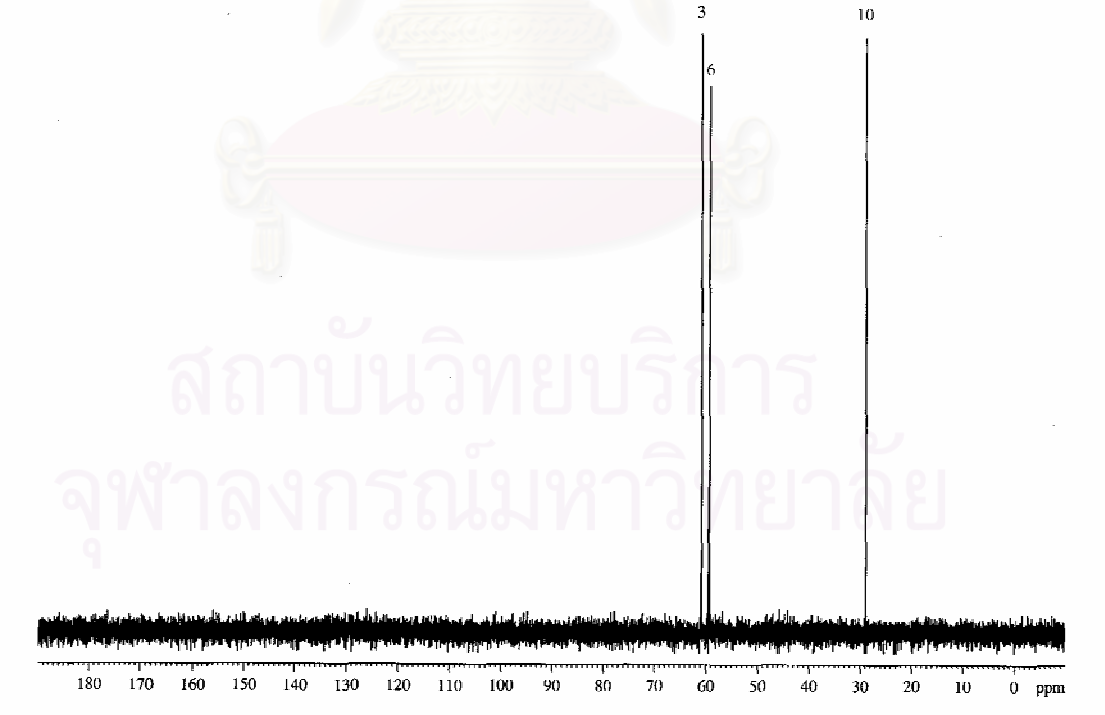


Figure 73 The 75 MHz DEPT 90 spectrum (in $CDCl_3$)of *cyclo*-(L-prolyl-D-valyl) (TK034).

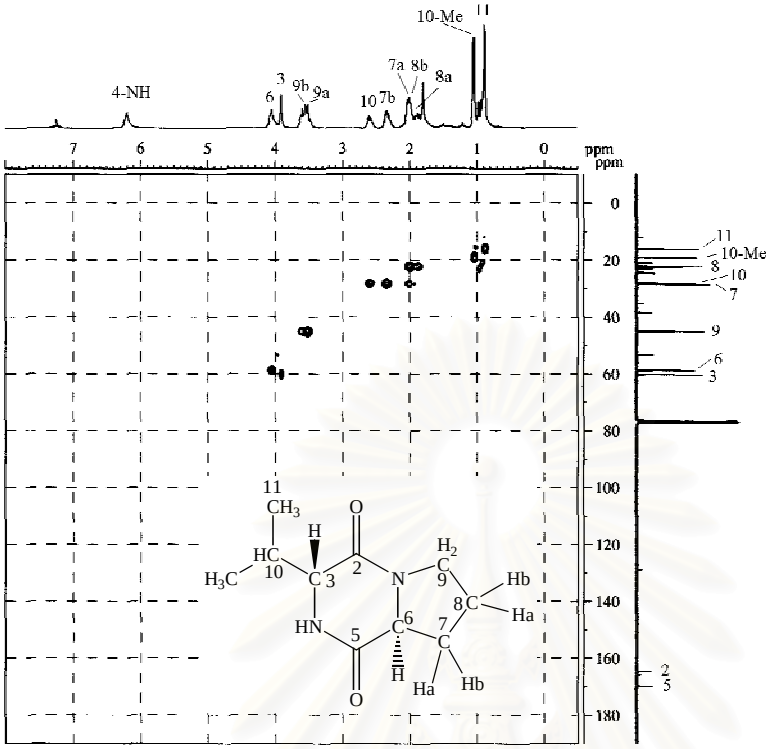


Figure 74 The 300 MHz HMQC spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-valyl) (TK034).

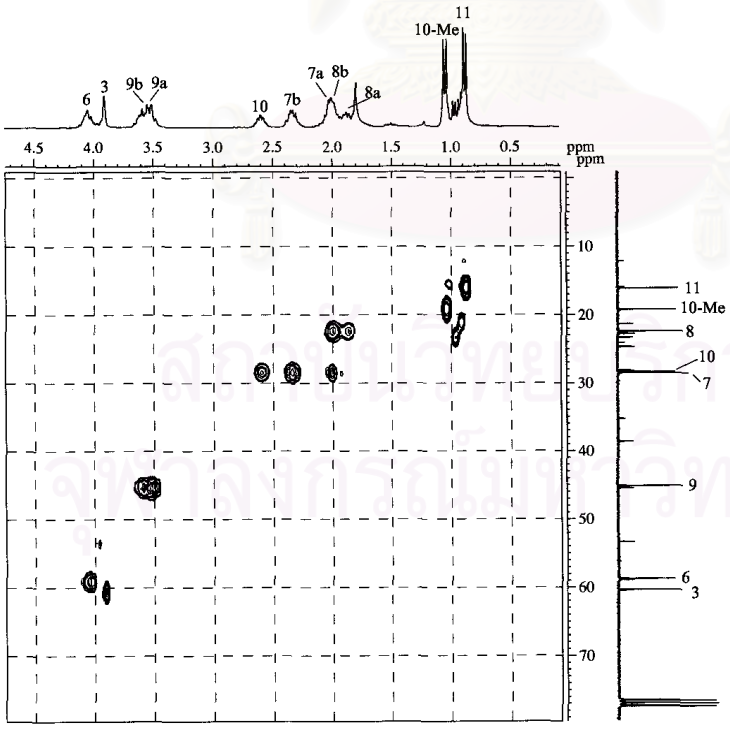


Figure 75 The 300 MHz HMQC spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-valyl) (TK034) (expanded from δ_H 0.10-4.70 ppm and δ_C 0-80.00 ppm).

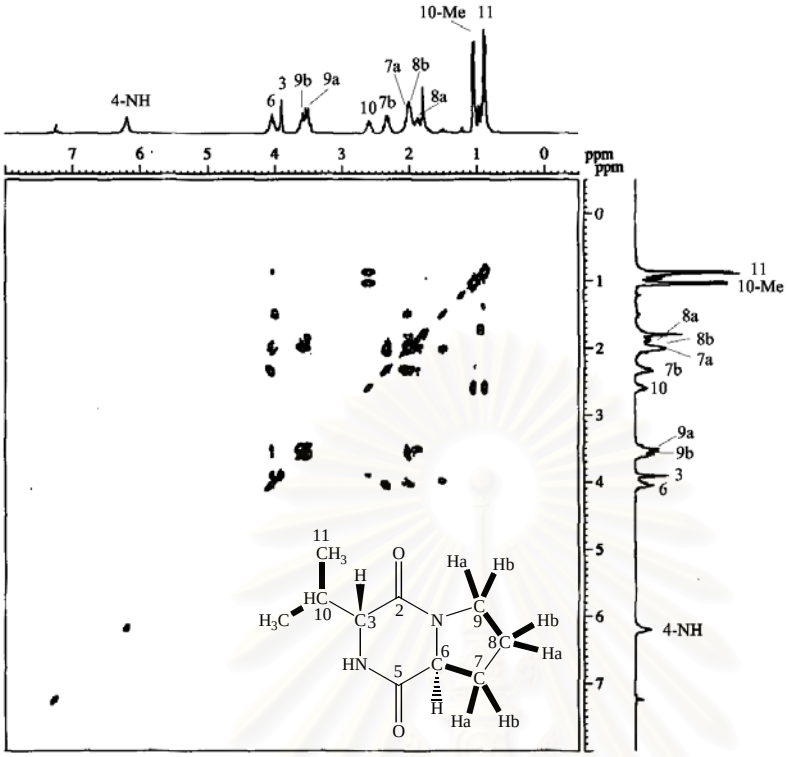


Figure 76 The 300 MHz ^{1}H-^{1}H COSY spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-valyl) (TK034).

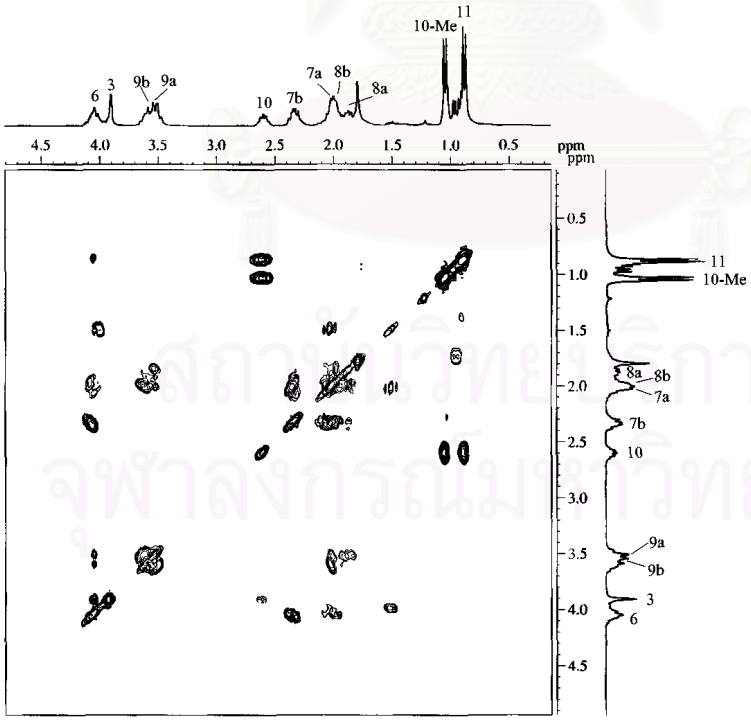


Figure 77 The 300 MHz ^{1}H-^{1}H COSY spectrum (in $CDCl_3$) of *cyclo*-(L-prolyl-D-valyl) (TK034) (expanded from δ_H 0.20-4.80 ppm).

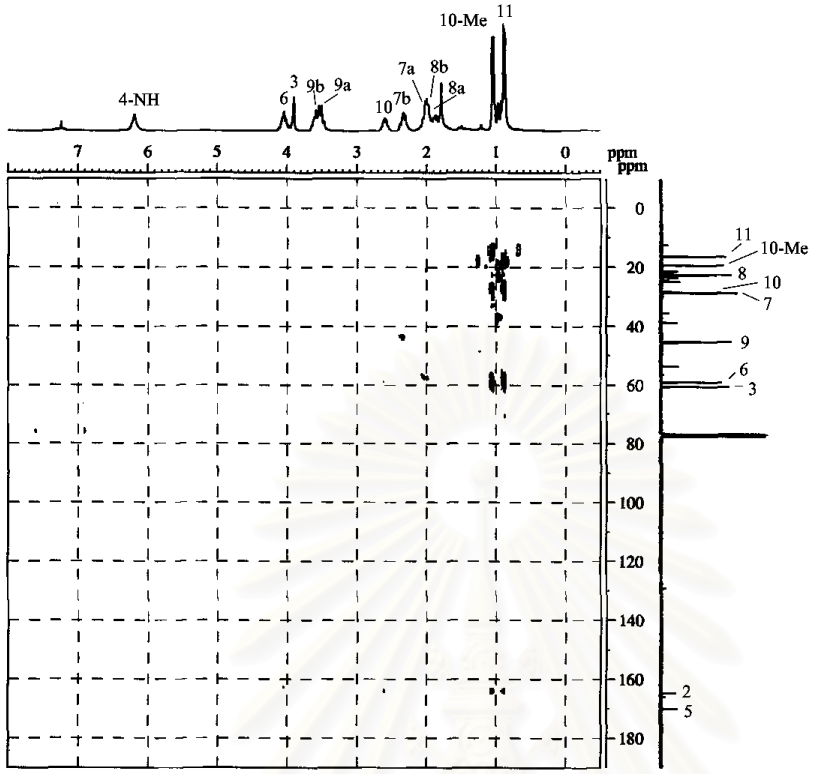
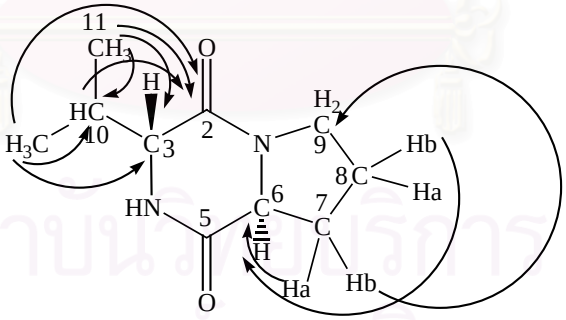


Figure 78 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) (in $CDCl_3$) of *cyclo*-(L-prolyl-D-valyl) (TK034).



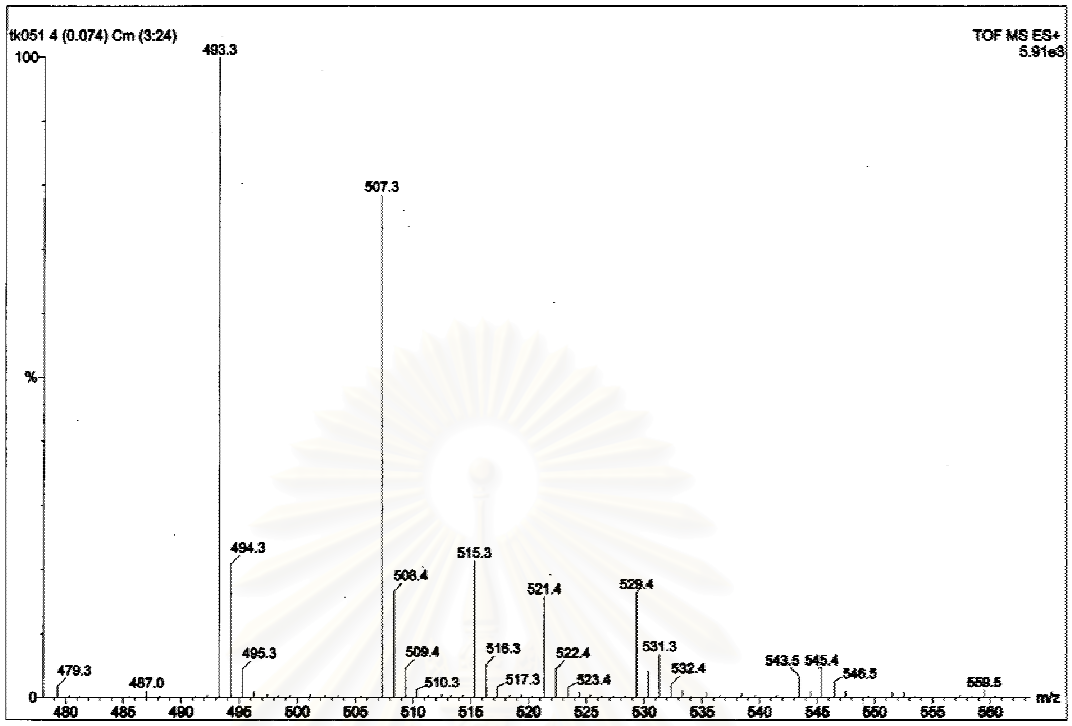
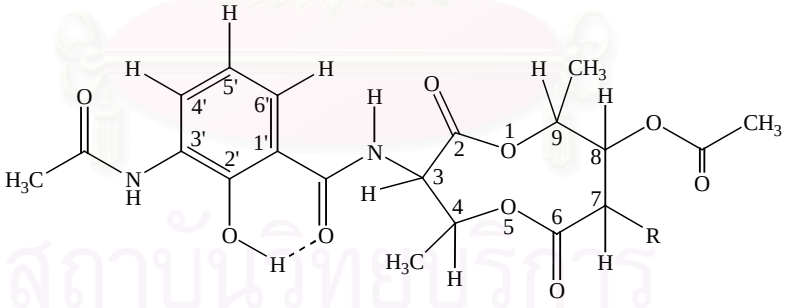
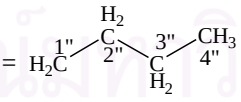
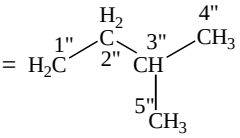


Figure 79 The ESI-TOF mass spectrum of antimycins B_1 and B_2 (TK051-1 and TK051-2).



antimycin B_1 R =  M.W. = 492

antimycin B_2 R =  M.W. = 506

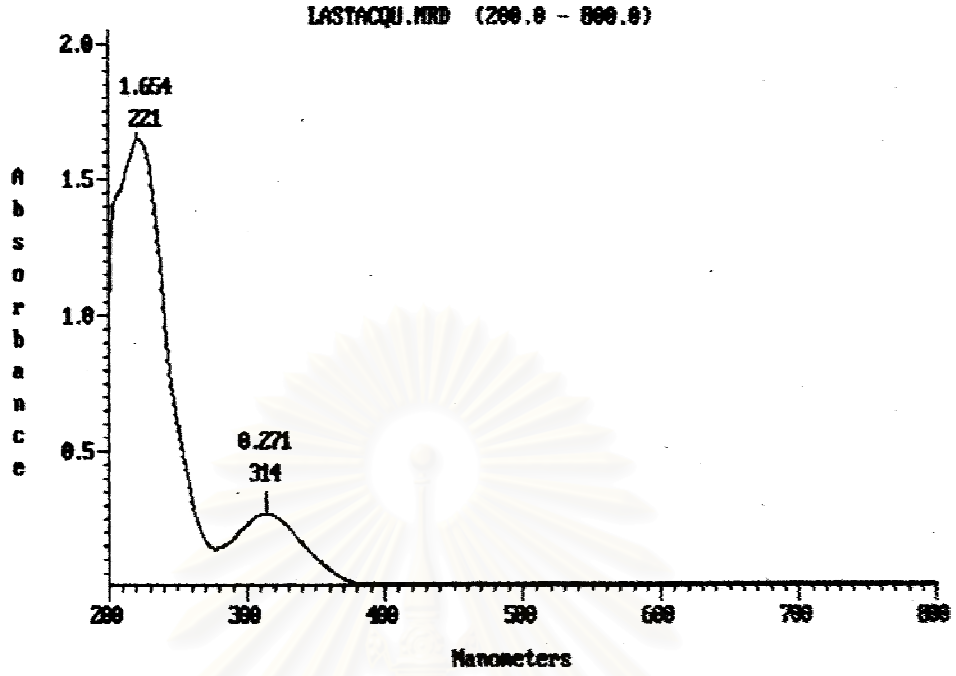


Figure 80 The UV spectrum (in MeOH) of antimycins B_1 and B_2 (TK051-1 and TK051-2).

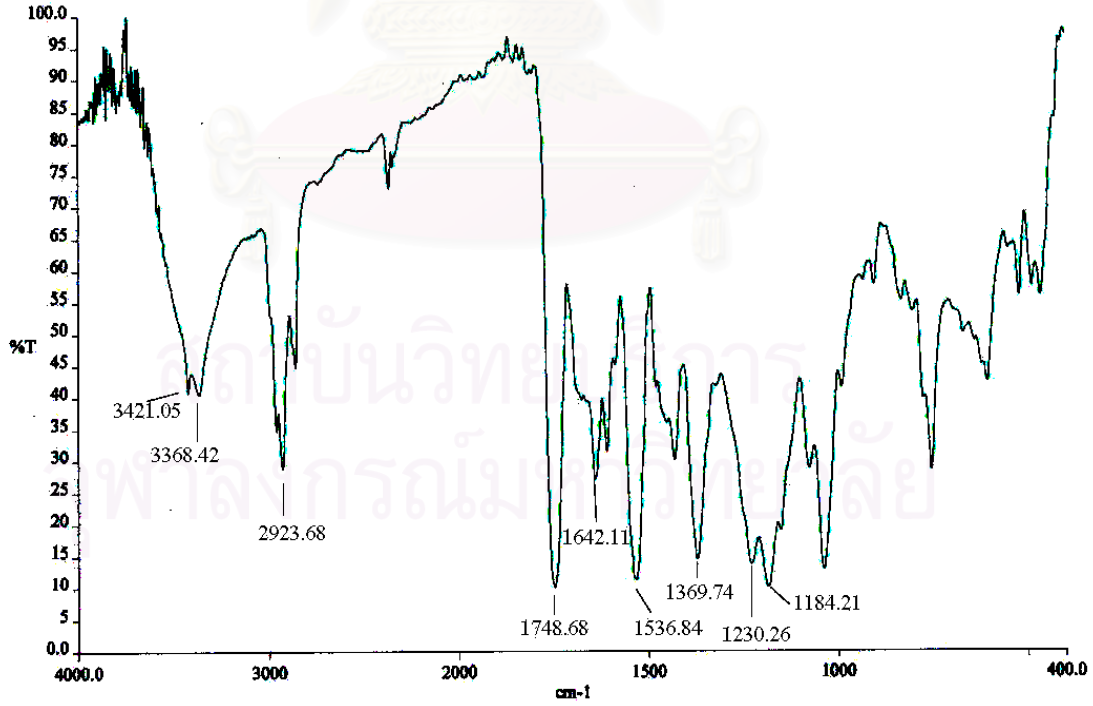


Figure 81 The IR spectrum of antimycins B_1 and B_2 (TK051-1 and TK051-2) (KBr disc).

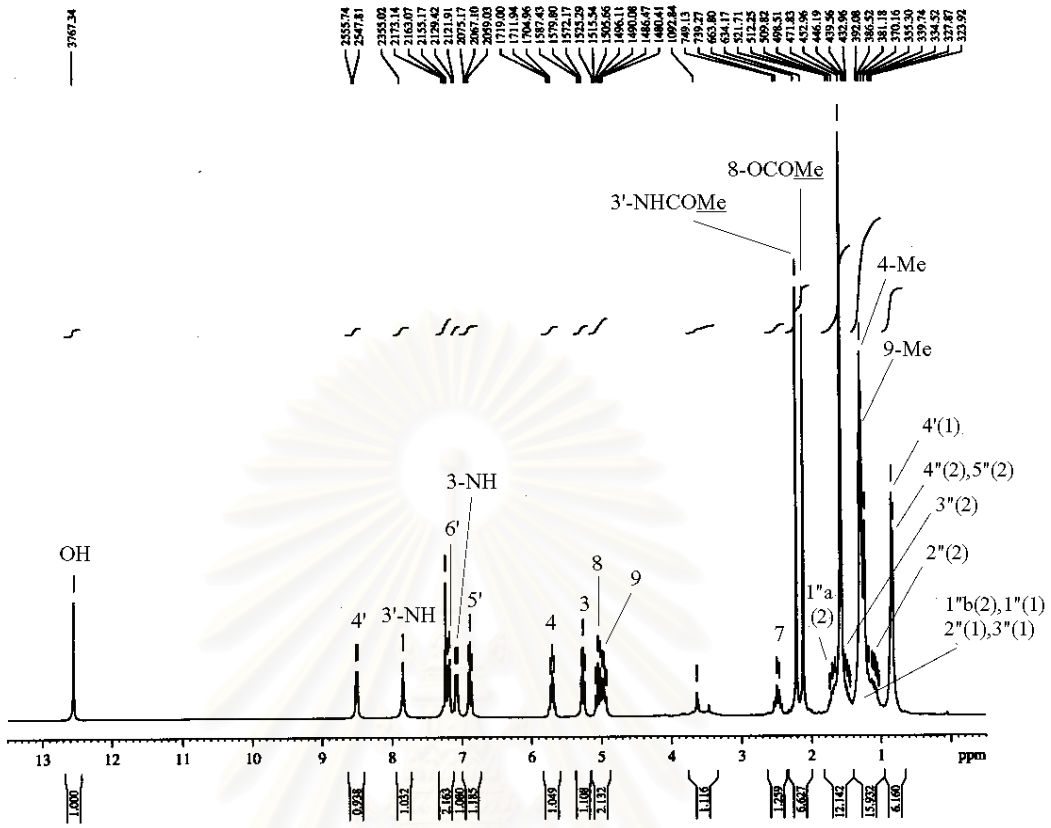
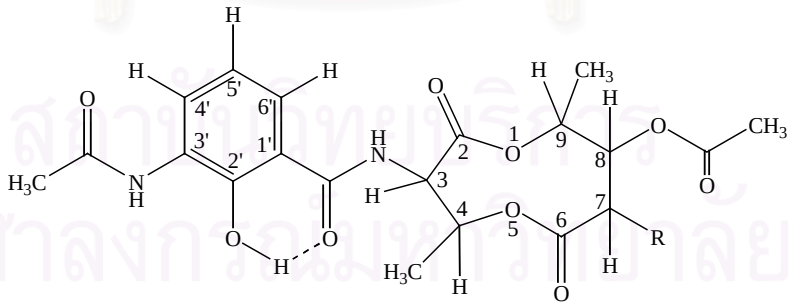
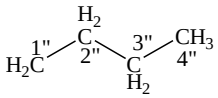


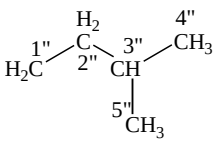
Figure 82 The 300 MHz ^{1}H NMR spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2).

(1) = proton signals for antimycin B_1 (TK051-1),

(2) = proton signals for antimycin B_2 (TK051-2)



antimycin B_1 R =  M.W. = 492

antimycin B_2 R =  M.W. = 506

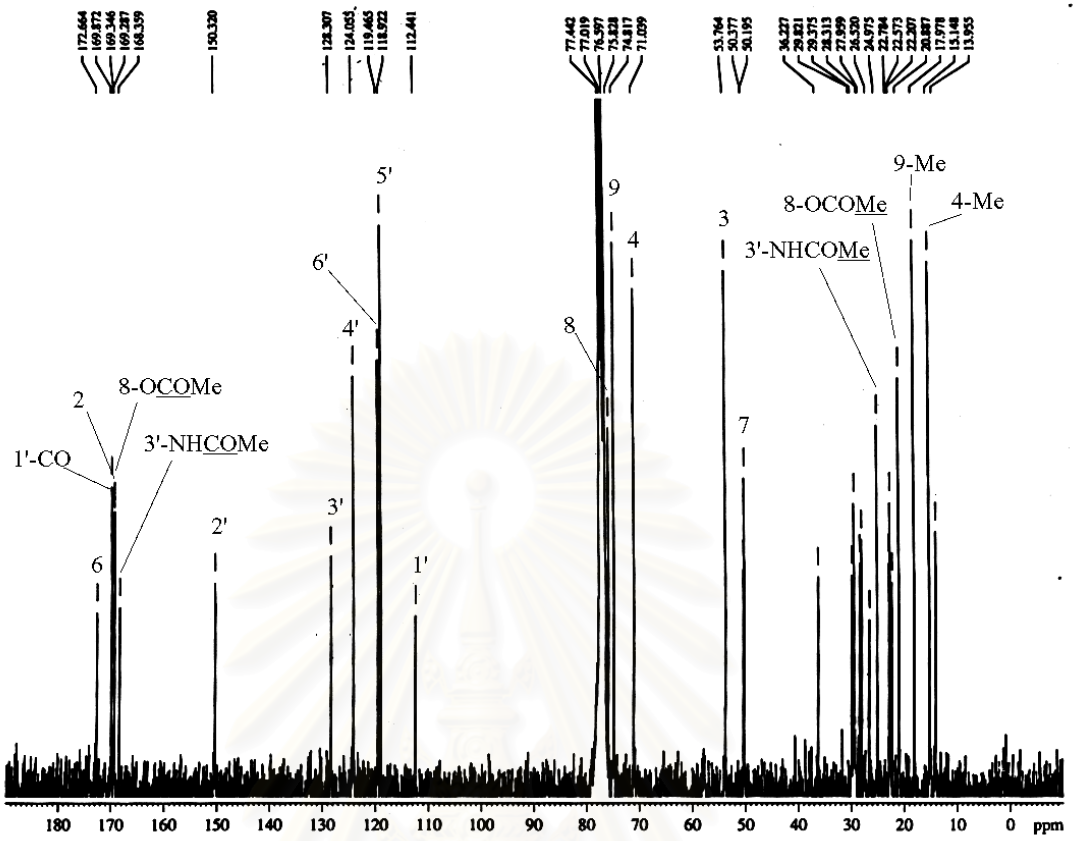
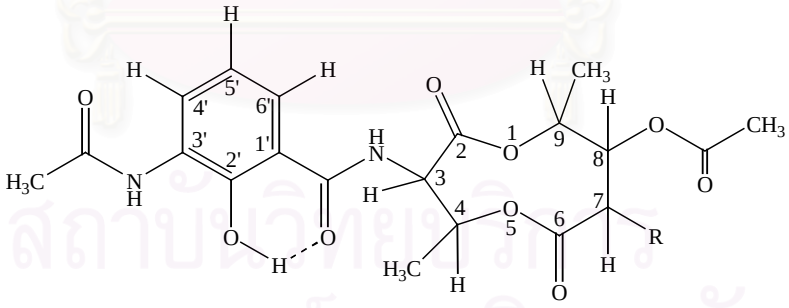
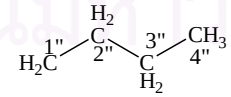
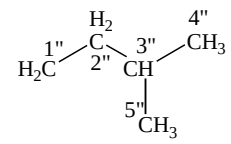


Figure 83 The 75 MHz ^{13}C NMR spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2).



antimycin B_1 R =  M.W. = 492

antimycin B_2 R =  M.W. = 506

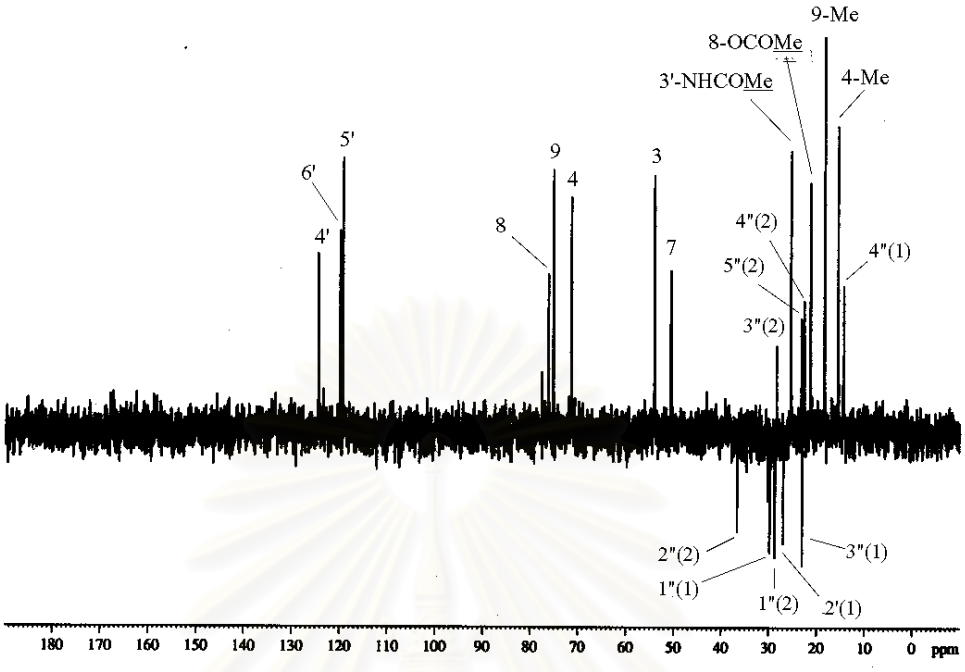


Figure 84 The 75 MHz DEPT 135 spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2).

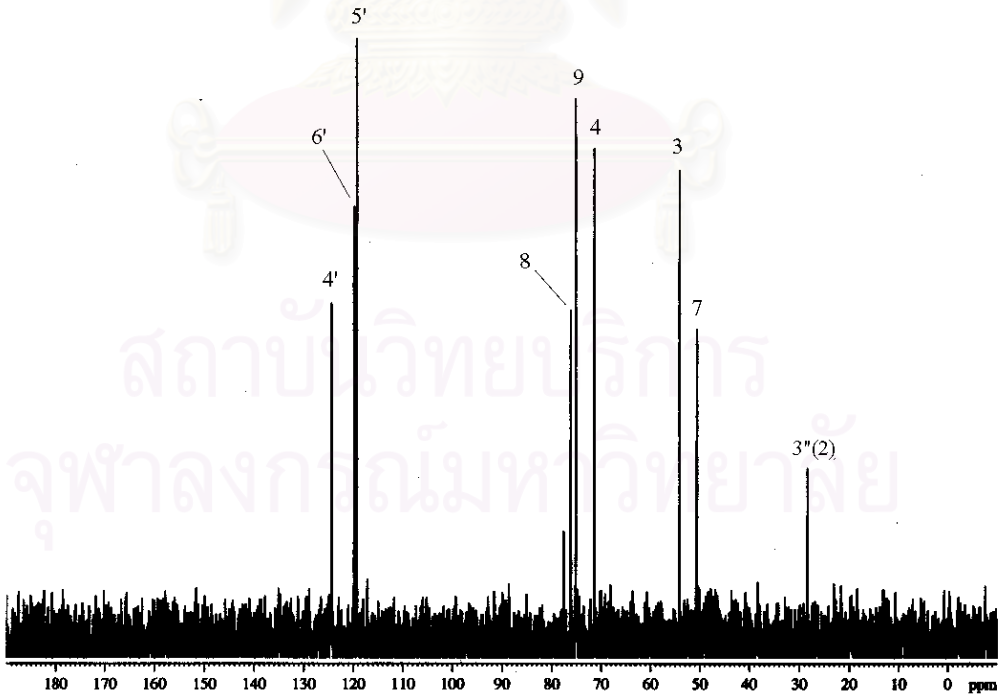


Figure 85 The 75 MHz DEPT 90 spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2).

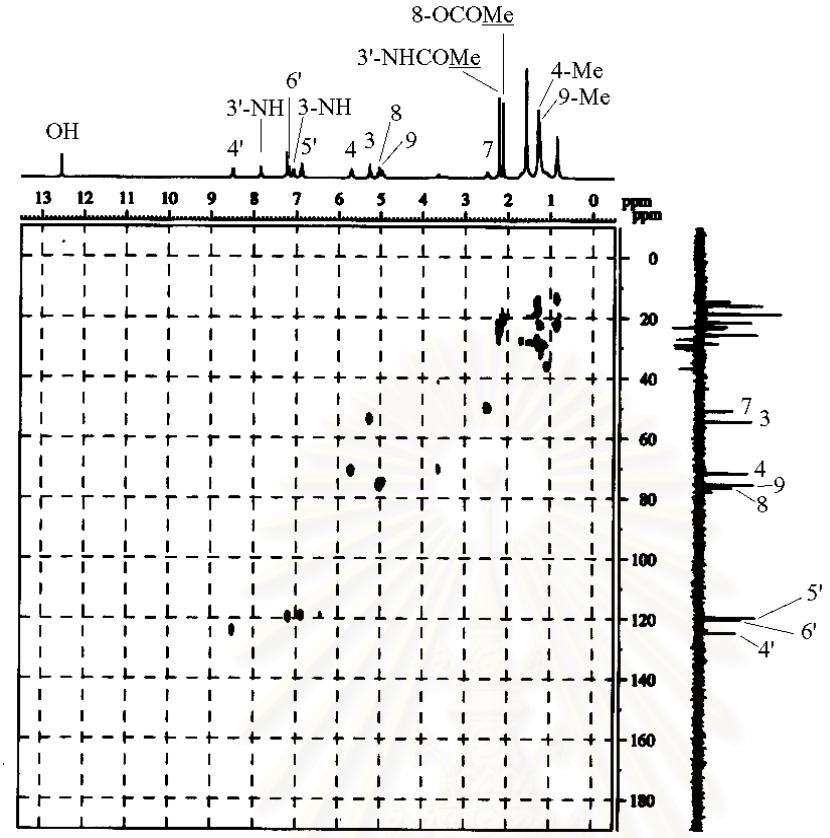
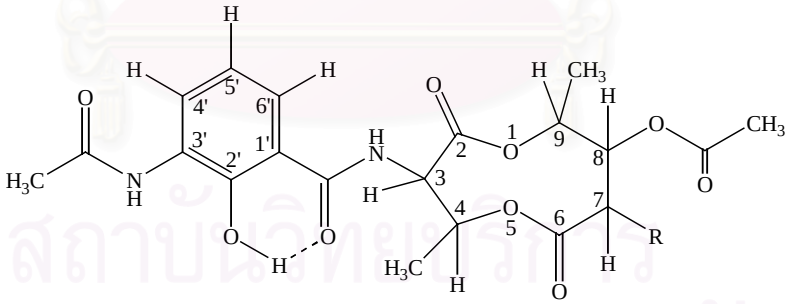
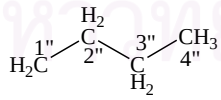


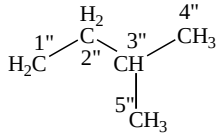
Figure 86 The 300 MHz HMQC spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2).



antimycin B_1 R =



antimycin B_2 R =



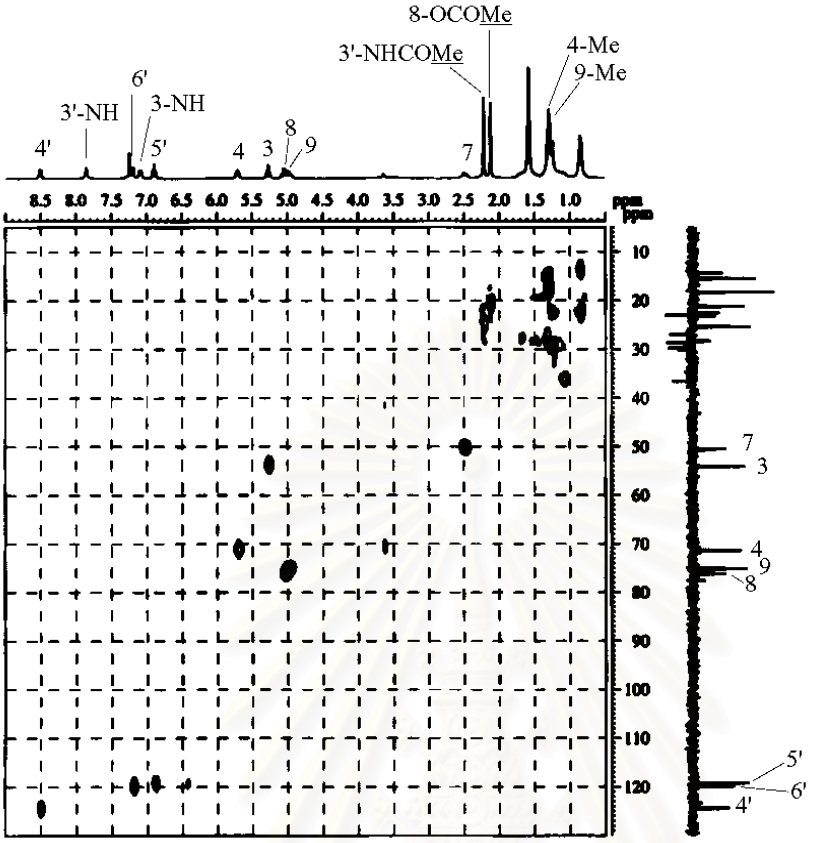
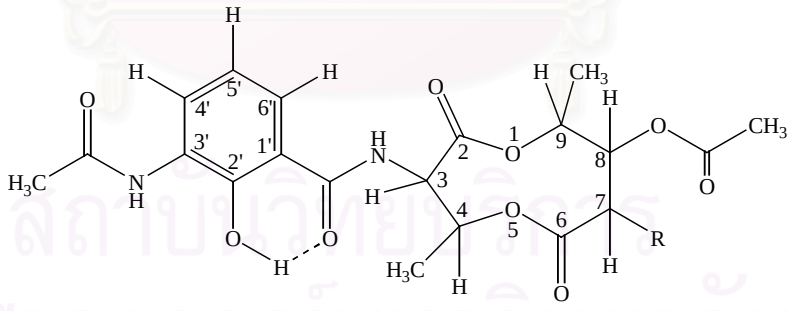
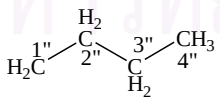


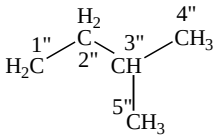
Figure 87 The 300 MHz HMQC spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2) (expanded from δ_H 0.50-9.00 ppm and δ_C 5-130 ppm).



antimycin B_1 R =



antimycin B_2 R =



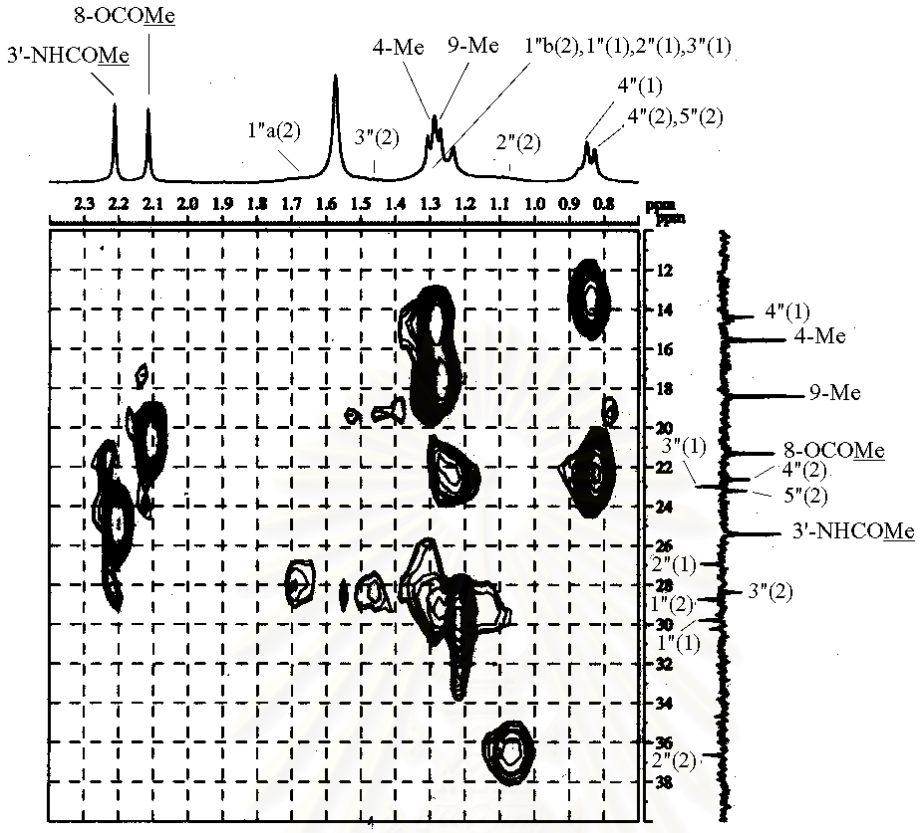
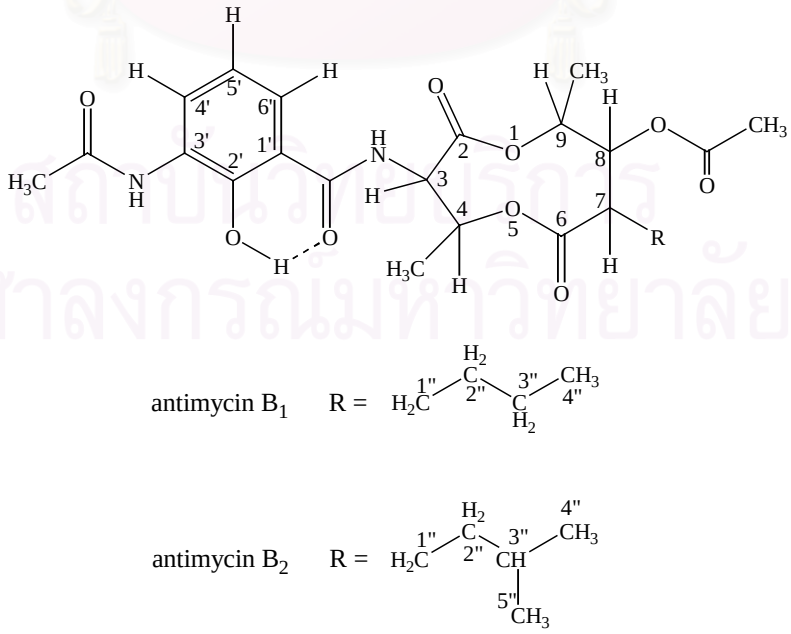


Figure 88 The 300 MHz HMQC spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2) (expanded from δ_H 0.70-2.40 ppm and δ_C 10-40 ppm).



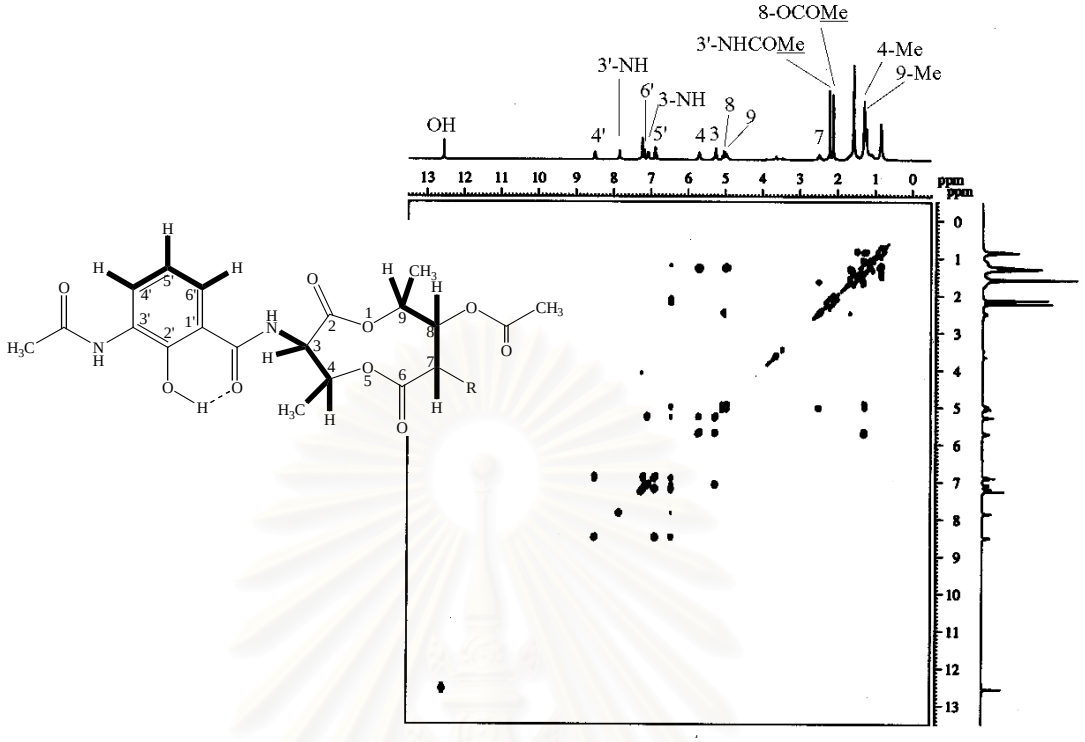


Figure 89 The 300 MHz ^{1}H-^{1}H COSY spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2).

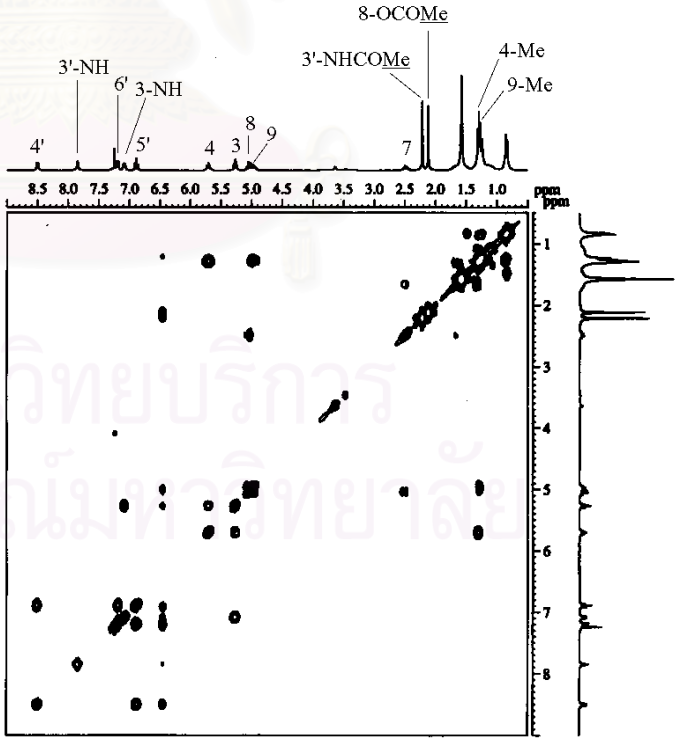


Figure 90 The 300 MHz ^{1}H-^{1}H COSY spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2) (expanded from δ_H 0.50-9.00 ppm).

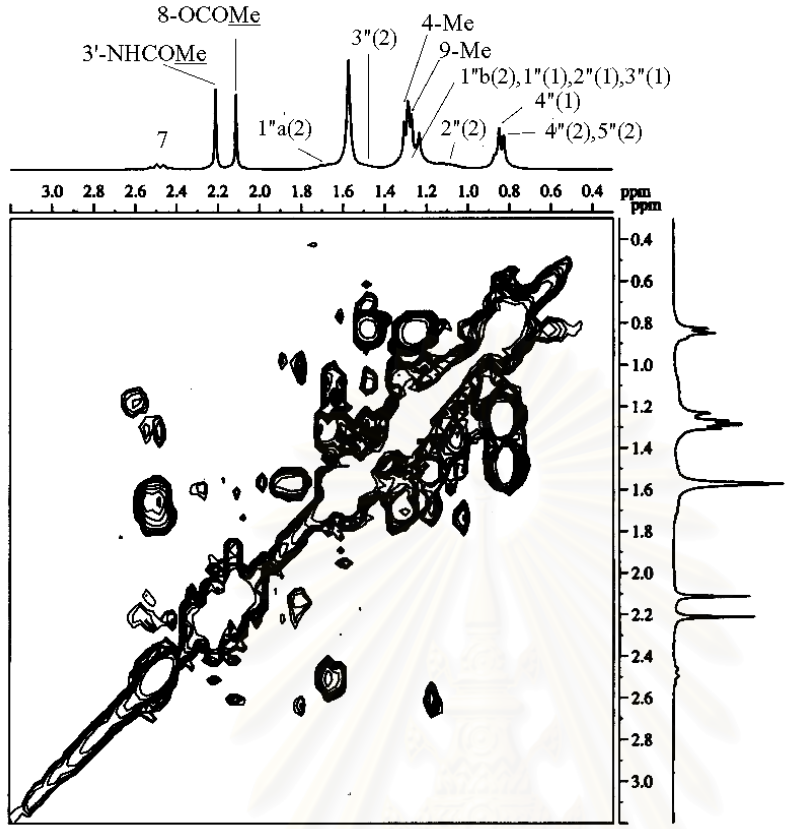
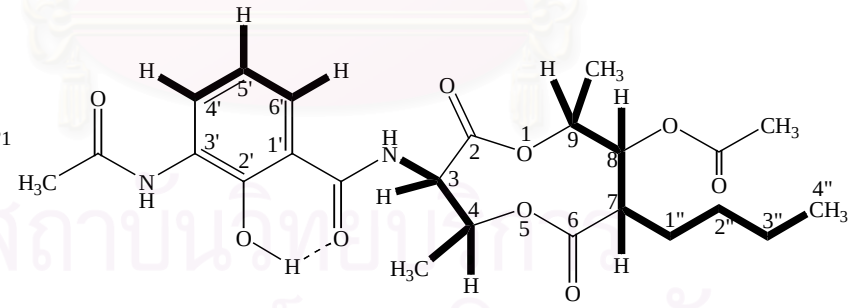
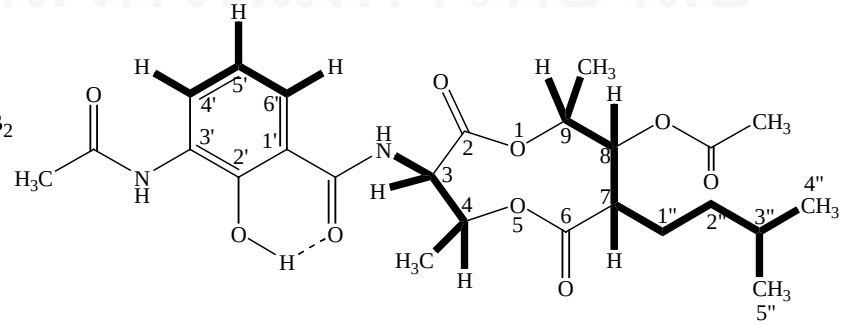


Figure 91 The 300 MHz ^{1}H-^{1}H COSY spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2) (expanded from δ_H 0.30-3.20 ppm).

antimycin B_1



antimycin B_2



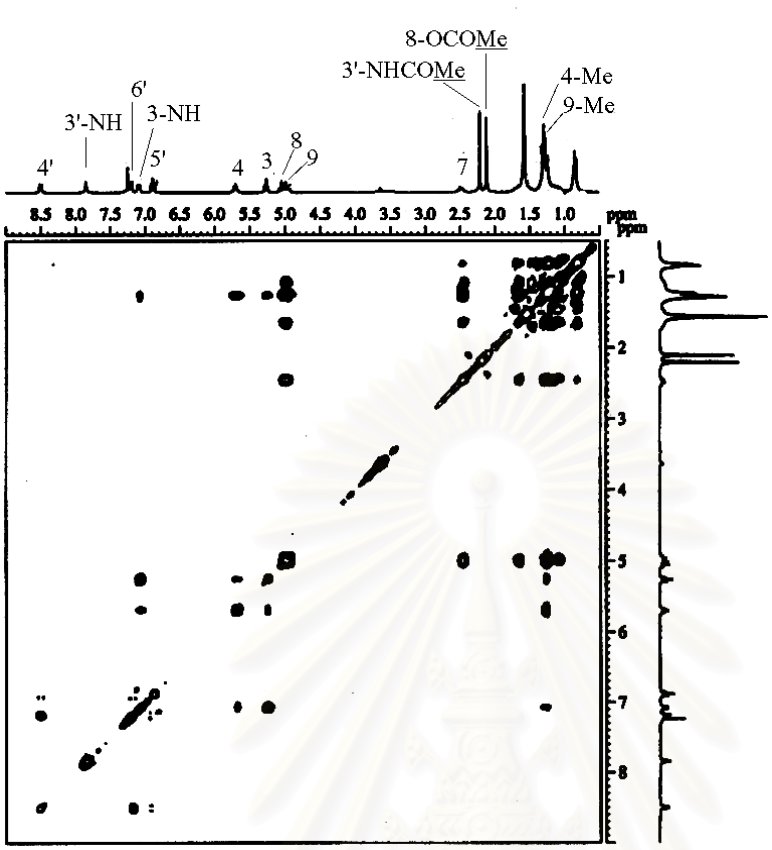
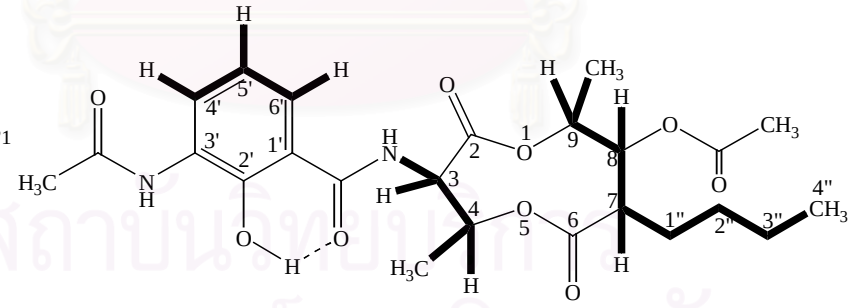
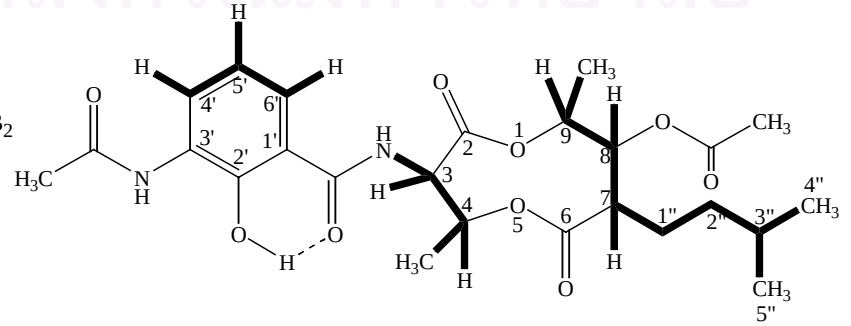


Figure 92 The 300 MHz TOCSY spectrum (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2) (expanded from δ_H 0.50-9.00 ppm).

antimycin B_1



antimycin B_2



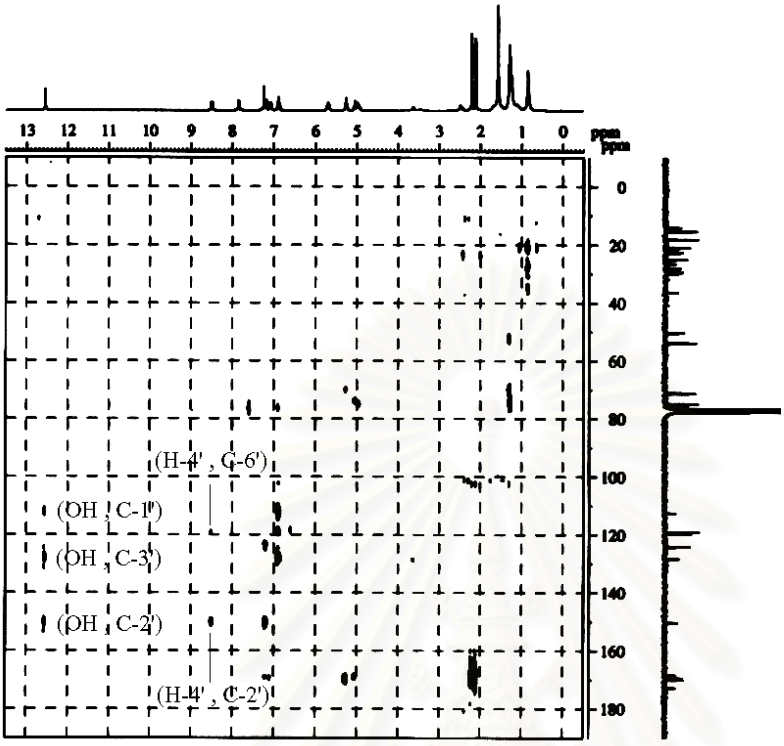
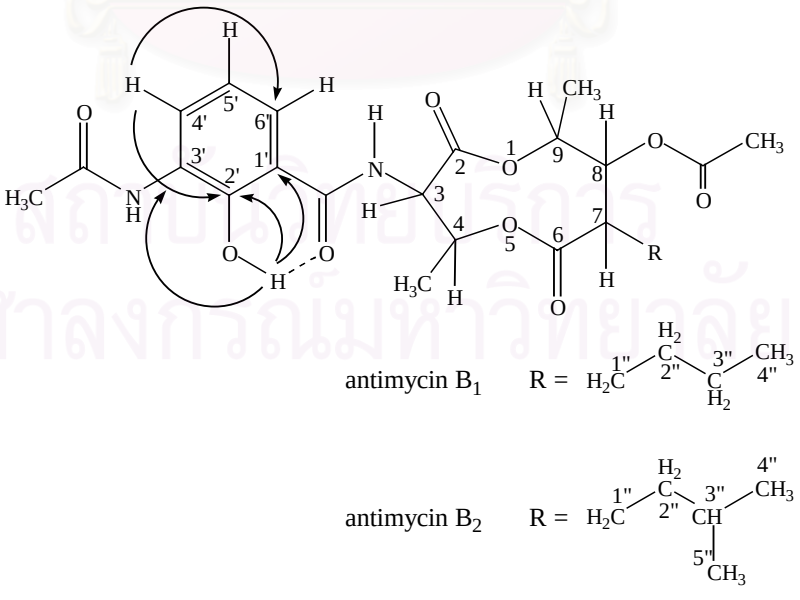


Figure 93 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2).



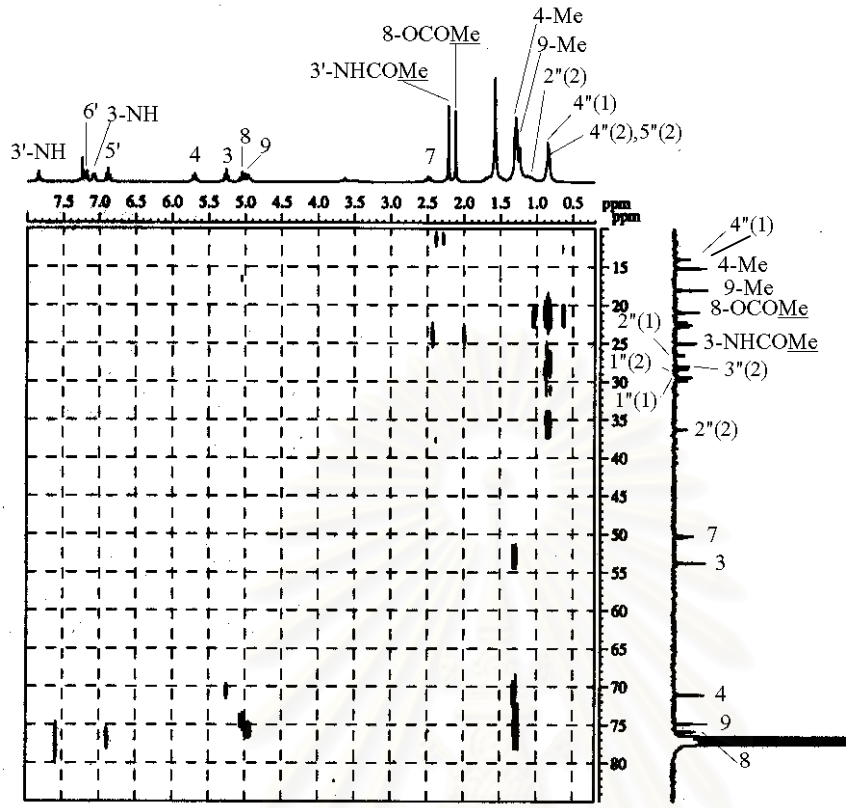
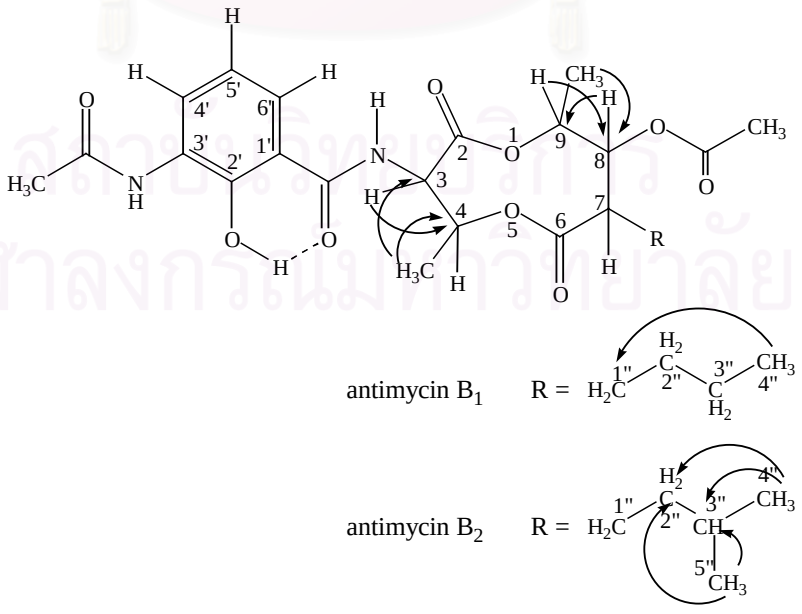


Figure 94 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2) (expanded from δ_H 0.10-8.00 ppm and δ_C 10-85 ppm).



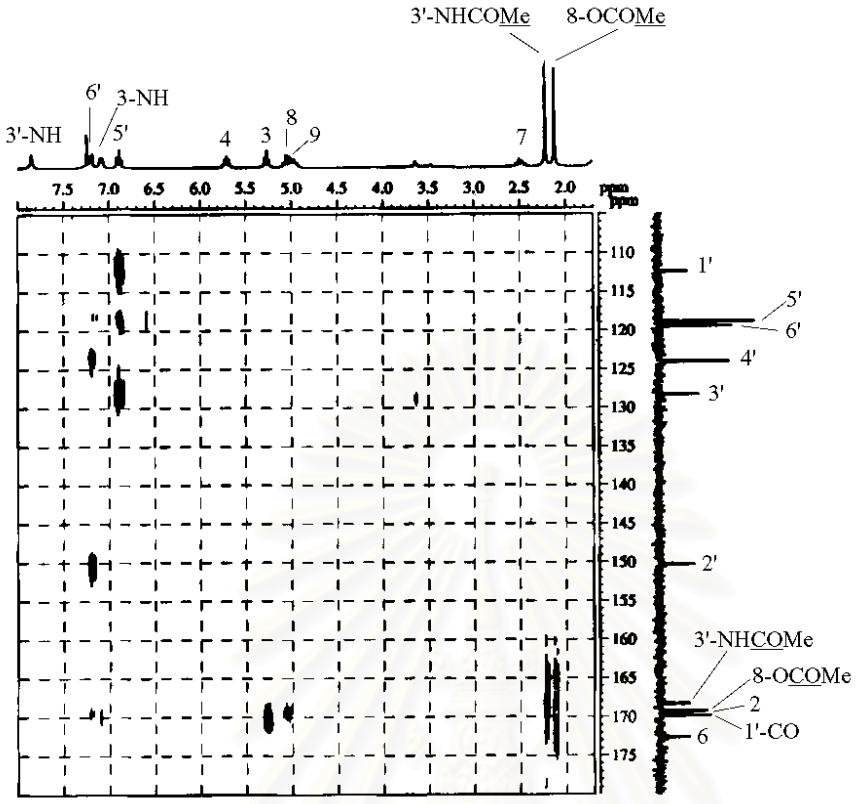
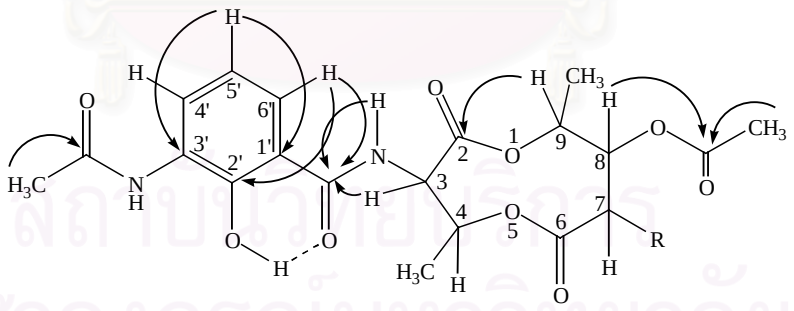
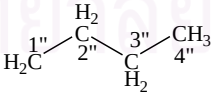


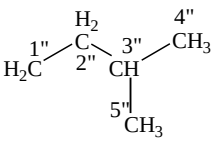
Figure 95 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 8 Hz) (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2) (expanded from δ_H 1.70-8.00 ppm and δ_C 100-180 ppm).



antimycin B_1 R =



antimycin B_2 R =



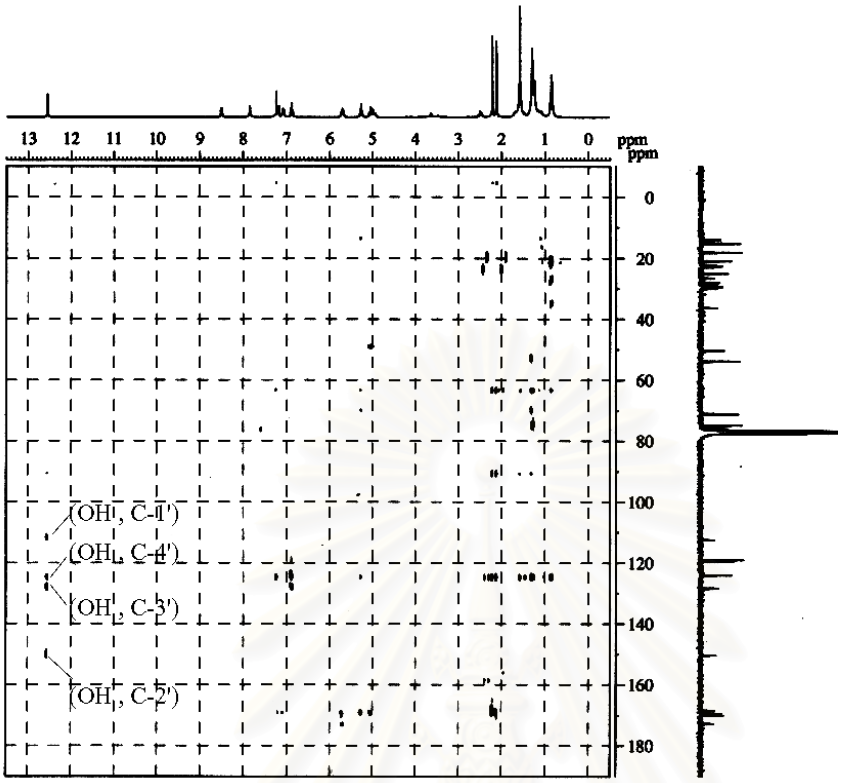
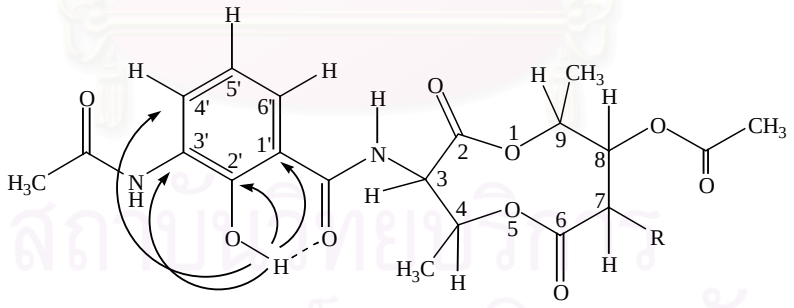
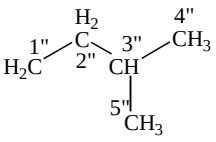


Figure 96 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2).



antimycin B_1 R = $-CH_2-CH_2-CH(CH_3)-CH_2-CH_3$



antimycin B_2 R = $-CH_2-CH_2-CH(CH_2-CH_3)-CH_3$

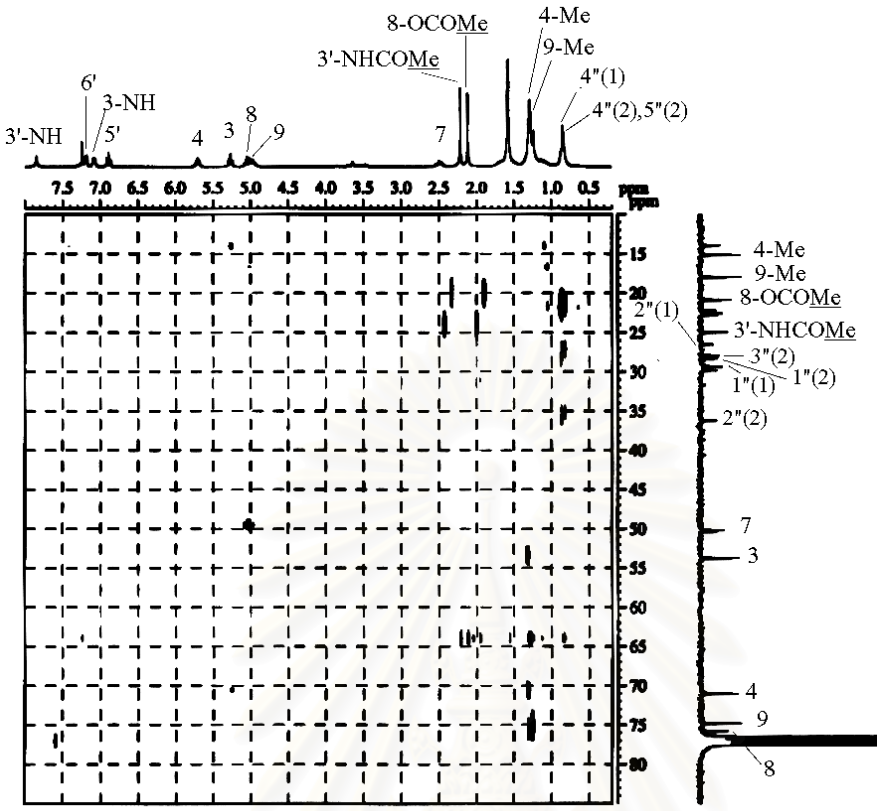
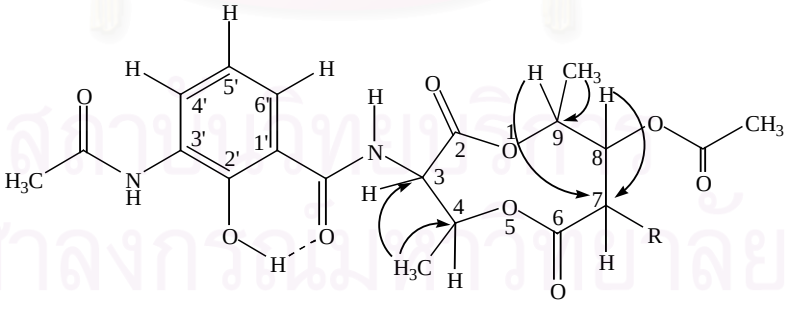
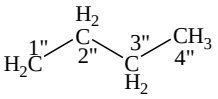
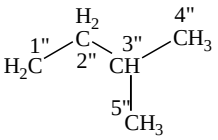


Figure 97 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2) (expanded from δ_H 0.5-8.00 ppm and δ_C 10-85 ppm).



antimycin B_1 R = 

antimycin B_2 R = 

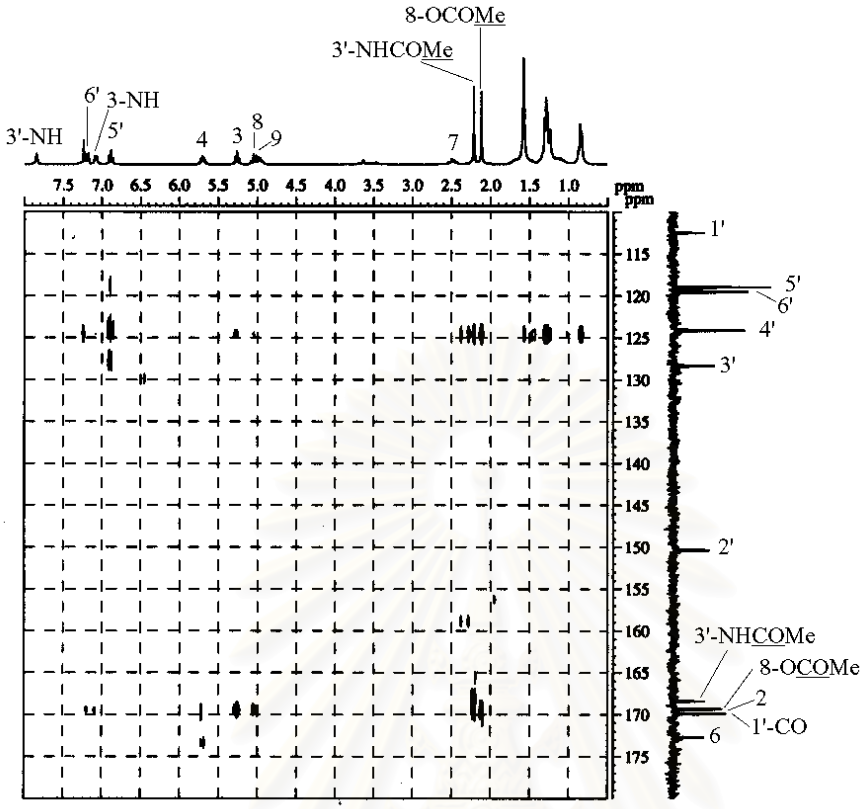
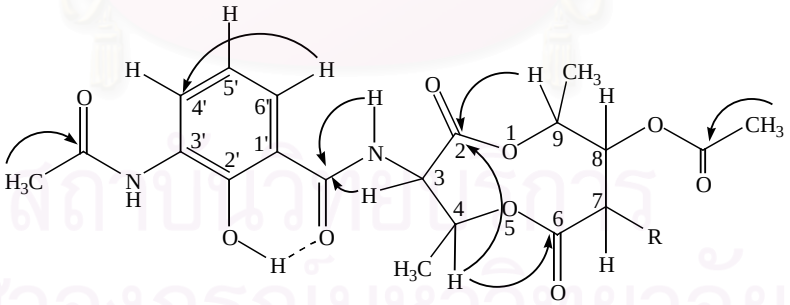
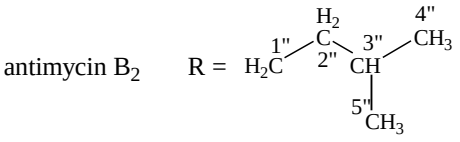


Figure 98 The 300 MHz HMBC spectrum ($^{n}J_{HC}$ = 4 Hz) (in $CDCl_3$) of antimycins B_1 and B_2 (TK051-1 and TK051-2) (expanded from δ_H 0.5-8.00 ppm and δ_C 110-180 ppm).



antimycin B_1 R = $-CH_2-CH(CH_3)-CH_2-CH_2-CH_3$



antimycin B_2 R = $-CH_2-CH(CH_3)-CH(CH_3)-CH_2-CH_3$

MEDIA

Each media was dispensed in 100 ml distilled water or sea water and sterilized at 121°C under 15 pound pressure for 15 minutes, except for the carbon utilization media which were sterilized at 100°C

1. Carbon utilization medium (ISP-9)

Carbohydrate	1.0	g
$(NH_4)_2 SO_4$	0.264	g
$KH_2 PO_4$. anhydrous	0.238	g
K_2HPO_4. $3H_2O$	0.565	g
$MgSO_4$. $7H_2O$	0.1	g
Pridham and Gottlieb trace salts (B)	0.1	ml
Agar	1.5	g

pH 6.8-7.0

Pridham and Gottlieb trace salts (B)

$CuSO_4$. $5H_2O$	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	100.0	ml

2. Cellulose decomposition medium

Filter paper saturated with Czapek's solution, free from sucrose.

Czapek's solution

K_2HPO_4	0.1	g
$MgSO_4$	0.05	g

NH_4Cl	0.2	g
KCl	0.05	g
$FeSO_4$	0.001	g
Distilled water	100.0	ml

pH 7.0-7.2

3. Glucose peptone beef extract yeast extract medium (GPBY)

Glucose	1.5	g
Peptone	0.6	g
Beef extract	0.3	g
Yeast extract	0.3	g
$MgSO_4. 7H_2O$	0.25	g

pH 7.0-7.4

4. Glycerol asparagine agar

L-asparagine (anhydrous basis)	0.1	g
Glycerol	1.0	g
K_2HPO_4 (anhydrous basis)	0.1	g
Trace salts solution (A)	0.1	ml
Agar	2.0	g

Trace salts solution (A)

$FeSO_4. 7H_2O$	0.1	g
$MnCl_2. 4H_2O$	0.1	g
$ZnSO_4. 7H_2O$	0.1	g
Distilled water	100.0	ml

5. Glycerol peptone medium (GPM)

Glycerol	2.0	g
Mollase	1.0	g
Beef extract	0.5	g
Peptone	0.5	g
$CaCO_3$	0.4	g
Sea water 35 $^{o}/_{oo}$	50	ml
Distilled water	50	ml

pH 7.2

6. Inorganic salt-starch agar

Soluble starch	1.0	g
K_2HPO_4 (anhydrous basis)	0.1	g
$MgSO_4$. $7H_2O$	0.1	g
NaCl	0.1	g
$(NH_4)_2$ SO_4	0.2	g
$CaCO_3$	0.2	g
Trace salts solution (A)	0.1	ml
Agar	2.0	g

pH 7.0-7.4

7. Modified starch casein nitrate agar (sodium caseinate)

Casein (sodium caseinate)	0.03	g
KNO_3	0.2	g
Starch	1.0	g
Agar	1.5-1.8	g

8. Nutrient gelatin broth

Beef extract	1.0	g
Peptone	1.0	g
NaCl	0.1	g
Gelatin	10.0	g

9. Oatmeal agar (Difco®)

Oatmeal agar	7.25	g

10. Peptone nitrate broth

Peptone	1.0	g
KNO_3	0.1	g
NaCl	0.5	g

pH 7.0

11. Potato carrot agar (PCA)

Potato	3.0	g
Carrot	0.25	g
Agar	1.5	g

Potato and carrot were cut in to small pieces and then boiled them at 100°C for 30 minutes. The extract was filtered and the filtrate was used to prepare the medium.

12. PY medium

Glucose	2.0	g
Soluble starch	1.0	g

Yeast extract	0.3	g
Peptone	0.5	g
Beef extract	0.5	g
$CaCO_3$	0.3	g

13. Sabouraud dextrose agar (SDA, Difco®)

Sabouraud dextrose agar (Difco®)	6.5	g

14. Skim milk broth

Skim milk (Difco®)	10.0	g

15. Tryptic soy agar (TSA, Difco®)

Tryptic soy agar (Difco®)	4.0	g

16. Tyrosine agar

Glycerol	1.5	g
L-tyrosine (Difco)	0.05	g
L-asparagine (Difco)	0.1	g
K_2HPO_4 (anhydrous basis)	0.05	g
$MgSO_4$. $7H_2O$	0.05	g
NaCl	0.05	g
$FeSO_4$. $7H_2O$	0.01	g
Trace salts solution (A)	0.1	ml
Agar	2.0	g

pH 7.2-7.4

17. Yeast extract-malt extract agar

Glucose	1.0	g
Peptone	0.5	g
Yeast extract	0.3	g
Malt extract	0.3	g
Agar	2.0	g

pH 6.2

18. Yeast extract-malt extract agar (ISP-2)

Glucose	0.4	g
Yeast extract	0.4	g
Malt extract	1.0	g
Agar	2.0	g

pH 7.3

VITA

Miss Chutima Petchprayoon was born on October 5, 1973 in Nakhornnayok, Thailand. She received her Bachelor Degree of Science in Pharmacy in 1996 from the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. During 1996-1999, she was a pharmacist in Bundharik Hospital, Ubon Ratchathani, Thailand.