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CHEMICAL CONSTITUENTS OF THE STEM BARK OF *CROTON ROXBURGHII* FROM CHAIYAPHUM PROVINCE

Miss Praworada Cholsuk

A Thesis Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in Pharmacy

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By	Miss Praworada Cholsuk
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Thesis Co-advisor	Associate Professor Amorn Petsom, Ph. D.

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……...Dean of the Faculty of Pharmaceutical Sciences
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(Associate Professor Sumphan Wongseripipatana, Ph. D.)

…….. Thesis Advisor
(Associate Professor Chaiyo Chaichantipyuth, M. Sc. in Pharm)

…….. Thesis Co-advisor
(Associate Professor Amorn Petsom, Ph.D.)

…….. Member
(Associate Professor Thatree Phadungcharoen, M. Sc. in Pharm)

…….. Member
(Assistant Professor Rutt Suttisri, Ph. D.)

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จากการศึกษาองค์ประกอบทางเคมีของเปลือกต้นเปล้าใหญ่ (*Croton roxburghii* N.P. Balakr.) จากอำเภอหนองบัวระเหว จังหวัดชัยภูมิ สามารถสกัดแยกสารบริสุทธิ์ จากสิ่งสกัดเอธิลอะซีเตท ได้สองชนิด ซึ่งเป็นสารใหม่ในกลุ่มเคลอโรเดน ไดเทอร์ปีน คือ 3*α*, 4*β*-dihydroxy-5*α*, 10*β*-*trans*-17α, 20*α*-cleroda-13 (14)-en-15, 16-olide และ 11-acetoxy-3*α*, 4*β*-dihydroxy-5*α*, 10*β*-*trans*-17α, 20*α*-cleroda-13 (14)-en-15, 16-olide การพิสูจน์เอกลักษณ์ และสูตรโครงสร้างทางเคมีของสารทั้งสอง กระทำโดยการวิเคราะห์ข้อมูลทางสเปคโตรสโคปี จาก UV, IR, MS, 1-D NMR, 2-D NMR และ X-ray ร่วมกับการเปรียบเทียบข้อมูลที่ได้กับสารที่มีการรายงานในอดีต สารประกอบที่แยกได้ทั้งสอง เมื่อนำมาทดสอบการยับยั้งเซลล์มะเร็งเต้านม (BT 474) ตับ (HEP-G2), ลำไส้ (SW 620), ปอด (CHAGO) และกระเพาะอาหาร (KATO-3) พบว่าสารทั้งสองไม่มีฤทธิ์ในการยับยั้งเซลล์มะเร็งทั้งหมดที่ทดสอบ

ภาควิชา .....เภสัชเวท.....	ลายมือชื่อนิสิต.....
สาขาวิชา .....เภสัชเวท.....	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา .....2546.....	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

##4476584233 PHARMACOGNOSY
KEY WORD: *CROTON ROXBURGHII*/ DITERPENE/ CLERODANE
PRAWORADA CHOLSUK : CHEMICAL CONSTITUENTS OF THE STEM BARK OF *CROTON ROXBURGHII* FROM CHAIYAPHUM PROVINCE. THESIS ADVISOR: ASSOC. PROF. CHAIYO CHAICHANTIPYUTH, M.Sc. in Pharm. THESIS CO-ADVISOR: ASSOC. PROF. AMORN PETSOM, Ph. D., 121 pp. ISBN 974-17-5127-3

In the course of the investigation for chemical constituents of the stem bark of *Croton roxburghii* N.P.Balakr., two new clerodane-type diterpenoids, 3*α*, 4*β*-dihydroxy-5*α*, 10*β*-*trans*-17α, 20*α*-cleroda-13 (14)-en-15, 16-olide and 11-acetoxy-3*α*, 4*β*-dihydroxy-5*α*, 10*β*-*trans*-17α, 20*α*-cleroda-13 (14)-en-15, 16-olide have been isolated from crude ethyl acetate extract. The structures of these compounds were established by analysis of their spectroscopic data (UV, IR, MS, 1-D NMR, 2-D NMR, and X-ray diffraction analysis) as well as comparision with previously reported values. Each compound was tested for cytotoxicity against various human tumor cell lines: BT 474 (breast cancer), HEP-G2 (hepatoma), SW 620 (colon cancer), CHAGO (lung cancer), and KATO-3 (gastric cancer). Both compounds showed no cytotoxic activity against all tested cancer cell lines.

Department .. Pharmacognosy.. Student's signature…….
Field of study.. Pharmacognosy.. Advisor's signature……
Academic year.. 2003.. Co-advisor's signature……

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LIST OF ABBREVIATIONS

br	= Broad (for NMR spectral data)
c	= Concentration (g/ml)
°C	= Degree Celcius
$CDCl_3$	= Deuterated chloroform
$CHCl_3$	= Chloroform
cm	= Centimeter
cm^{-1}	= reciprocal centimeter (unit of wave number)
^{13}C-NMR	= Carbon-13 Nuclear Magnetic Resonance
d	= doublet (for NMR spectral data)
dd	= doublet of doublets (for NMR spectral data)
ddd	= doublet of doublets of doublets (for NMR spectral data)
dddd	= doublet of doublets of doublets of doublets (for NMR spectral data)
dia.	= Diameter
2D	= Two Dimensional
DEPT	= Distortionless Enhancement by Polarization Transfer
EIMS	= Electron Impact Mass Spectroscopy
EtOAc	= Ethyl Acetate
g	= Gram
^{1}H-^{1}H COSY	= Homonuclear (Proton-Proton) Correlation Spectroscopy
HMBC	= 1H-detected Heteronuclear Multiple Bond Coherence
HMQC	= 1H-detected Heteronuclear Multiple Quantum Coherence
^{1}H-NMR	= Proton Nuclear Magnetic Resonance
Hz	= Hertz
IR	= Infrared
J	= Coupling Constant
KBr	= Potassium bromide
Kg	= Kilogram
L	= Liter
m	= Multiplet (for NMR spectral data)
mg	= Milligram
ml	= Milliliter

LIST OF ABBREVIATIONS (Cont.)

mm	=	Millimeter
MeOH	=	Methanol
MS	=	Mass Spectroscopy
m/z	=	mass-to-charge ratio
M^+	=	Molecular Ion
No.	=	Number
NMR	=	Nuclear Magnetic Resonance
NOESY	=	Nuclear Overhauser Enhancement Spectroscopy
ppm	=	part per million
q	=	Quartet (for NMR spectral data)
s	=	Singlet (for NMR spectral data)
t	=	Triplet (for NMR spectral data)
TLC	=	Thin Layer Chromatography
UV-VIS	=	Ultraviolet and Visible Spectrophotometry
υ_{max}	=	Wave number at maximum absorption
λ_{max}	=	Wavelength at maximum absorption
δ	=	Chemical Shift
ε	=	Molar Absorptivity
$[\alpha]^{25}_{D}$	=	Specific Rotation at 25°C and Sodium D line (589 nm)

CHAPTER I

INTRODUCTION

Croton roxburghii N.P Balakr. (*Croton oblongifolius* Roxb.) (Figure 1.) is classified in the family of Euphorbiaceae. This plant is an indigenous plant known in Thailand as เปล้าใหญ่ Plao yai (Central),เปล้าหลวง Plao luang (Northern), ควะวู Khwa-wu (Karen-Kanchanaburi), เซ่งเค่คัง Seng-khe-khang, สะกาวา Sa-ka-wa, ส่ากูวะ Sa-ku-wa (Karen-Mae Hong Son), เปาะ Po (Kamphaeng Phet), ห้าเยิ่ง Ha-yoeng (Shan-Mae Hong Son) (เต็ม สมิตินันท์, 2544).

This plant is very interesting as a medicinal plant, because it is believed that all parts of the plant can be used as drugs. The seeds and fruits are known to have purgative effect. The flowers are believed to be parasiticide. The bark is used in India as a remedy for chronic liver enlargement and remittent fever, whereas in Thailand it is used to cure biliary diseases and to reduce phlegm. The root bark is given in small doses as a purgation; whereas larger quantity is poisonous. The sapwood is used for dyspepsia, while the heartwood is recommended for flatulence. The leaves are used in Cambodia for liver complaints and scabies (Blatter, Caius and Mhaskar, 1975; สาย สนม กิตติขจร, 2526). Moreover, this plant has been used as folk-medicine in conjunction with *Croton stellatopilosus* Ohba. (*C. sublyratus* Kurz) for treating gastric ulcer and gastric cancer (Roengsumran *et al*., 2002).

1. Characteristics of the genus *Croton*

The genus *Croton* belongs to the family Euphorbiaceae. They are trees or shrubs (rarely herbs). Leaves usually alternate, usually 2-glandular at the base. Flowers monoecious (in the Indian species), solitary or clustered on the rhachis of a terminal raceme; bracts small. Male flowers: Calyx 5-(rarely 4-6-) partite; segments imbricate or subvalvate. Petals 5 (rarely 4-6), never exceeding but sometimes shorter than the calyx. Disk of 4-6 glands opposite the sepals. Stamens many, inserted on a hairy receptacle; filaments free, inflexed in bud, at length straight; anthers adnate, with parallel cells. Pistillode 0. Female flowers: Sepals usually more ovate than in the male, rarely accrescent in fruit. Petals smaller than the sepals or obsolete. Disk annular, or of 4-6 glands opposite the sepals. Ovary 3- (rarely 2-4-) celled; ovule

solitary in each cell; style usually long and slender, 2-4-cleft. Capsule sub-equally 6-valved, or of 3 separating 2-valved cocci. Seeds smooth; caruncle small; testa crustaceous; albumen copious; cotyledons broad. Tropics and subtropics. (Blatter, Caius and Mhaskar, 1975)

This genus exhibits various well-marked medicinal properties: bitter, tonic, and stimulant; vulnerary and astringent; diuretic and cathartic; antisyphilitic. (Blatter, Caius and Mhaskar, 1975)

According to (เต็ม สมิตินันท์, 2544), there are 27 species of the genus *Croton* in Thailand as follow.

1. *C. acutifolius* Esser — เปล้า Plao, **เปล้าแพะ** Plao phae, มะดอไก่ Mado kai (Northern).
2. *C. argyratus* Blume — **เปล้า** Plao (Prachuap Khiri Khan); เปล้าเงิน Plao ngoen (Nong Khai).

   C. birmanicus Mull.Arg. = *C. tiglium* L.
3. *C. bonplandianus* Daillon — **เปล้าทุ่ง** Plao thung (General).
4. *C. cascarilloides* Raeusch. — เปล้าเงิน Plao ngoen (Songkhla); **เปล้าน้ำเงิน** Plao nam ngoen (Prachuap Khiri Khan).
5. *C. caudatus* Geiseler — **กระดอหดใบขน** Krado hot bai khon (Chanthaburi); โคคลาน Kho khlan (Nakhon Ratchasima); ปริก Prik (Trang); โคคลานใบขน Kho khlan bai khon (General); กูเราะปริยะ Ku-ro-pri-ya (Malay-Narathiwat).
6. *C. columnaris* Airy Shaw — **เปล้าคำ** Plao kham (Sukhothai).
7. *C. crassifolius* Geiseler — ปังคี Pang khi, **พังคี** Phang khi (Chiang Mai).

C. cumingii Mull.Arg. = *C. cascarilloides* Raeusch.

8. *C. delpyi* Gagnep. **เปล้า** Plao, เปล้าน้อย Plao noi, นมน้ำเขียว Nom nam khiao (Southeastern).
9. *C. griffithii* Hook.f. จิก Chik, **เปล้า** Plao (Peninsular).
10. *C. hirtus* L. Her. **เปล้าล้มลุก** Plao lom luk (Peninsular).
11. *C. hutchinsonianus* Hosseus เปล้า Plao, **เปล้าแพะ** Plao phae, เปล้าเลือดPlao lueat, แม่ลาเลือด Mae la lueat, เหมือดฮ้อน Mueat hon (Northern).
12. *C. kerrii* Airy Shaw **เปล้า** Plao (General).
13. *C. kongensis* Gagnep. **เปล้าเงิน** Plao ngoen, เปล้าน้อย Plao noi (Northeastern); เปล้าน้ำเงิน Plao nam ngoen (Eastern); เสปอตุ Se-po-tu (Karen-Chiang Mai).
14. *C. krabas* Gagnep. **ทรายขาว** Sai khao (Northern); พริกนา Prik na (Central); ฝ้ายน้ำ Fai nam (Eastern).
15. *C. lachnocarpus* Benth. **ขี้อ้น** Khi on (Southwestern).
16. *C. longissimus* Airy Shaw **เปล้าน้อย** Plao noi (Lampang).
17. *C. mekongensis* Gagnep. **เปล้าน้ำเงิน** Plao nam ngoen, พริกนา Prik na (Northern).

C. oblongifolius Roxb. = *C. roxburghii* N.P.Balakr.

18. *C. poilanei* Gagnep. เปล้า Plao, **เปล้าใหญ่** Plao yai (Southeastern); เปล้าหลวง Plao luang, เปล้าเลือด Plao lueat (Northern).

C. pierrei Gagnap. = *C. cascarilloides* Raeusch.

19. *C. robustus* Kurz — **เปล้าเลือด** Plao lueat (Lampang).

C. rottleri Geiseler = *Chrozophora rottleri* (Geiseler) A.Juss. ex Spreng.

20. *C. roxburghii* N.P.Balakr. — ควะวู Khwa-wu (Karen-Kanchanaburi); เซ่งเค่คัง Seng-khe-khang, สะกาวา Sa-ka-wa, ส่ากูวะ Sa-ku-wa (Karen-Mae Hong Son), เปาะ Po (Kamphaeng Phet);เปล้าหลวง Plao luang (Northern); **เปล้าใหญ่** Plao yai (Central); ห้าเยิ่ง Ha-yoeng (Shan-Mae Hong Son).

21. *C. santisukii* Airy Shaw — **เปล้าสันติสุข** Plao santisuk (Southwestern).

22. *C. sepalinus* Airy Shaw — **เปล้าเงิน** Plao ngoen (Peninsular).

C. siamensis Craib = *C. robustus* Kurz

23. *C. stellatopilosus* Ohba — **เปล้าน้อย** Plao noi (Prachin Buri, Prachuap Khiri Khan); เปล้าท่าโพ Plao tha po (Southeastern).

24. *C. thorelii* Gagnep. — **เปล้าตะวัน** Plao tawan (Southeastern).

25. *C. tiglium* L. — บะกั้ง Ba kang (Phrae); มะข่าง Ma khang, มะคัง Ma khang, มะตอด Matot, หมากทาง Mak thang, หัสคืน Has sa khuen (Northern); ลูกผลาญศัตรู Luk phlan sattru, **สลอด** Salot, สลอดต้น Salot ton, หมากหลอด Mak lot (Central); หมากยอง

Mak-yong (Shan-Mae Hong Son); Croton oil plant.

C. tomentosus Mull.Arg. = *C. crassifolius* Geiseler

26. *C. trachycaulis* Airy Shaw — กวาวะ Kwa-wa, กวาโอะวะ Kwa-o-wa (Karen-Kanchanaburi); **ขี้อัน** Khi on (Prachuap Khiri Khan).

27. *C. wallichii* Mull. Arg. — เปล้า Ploa, **เปล้านา** Plao na (General).

2. Characteristics of *Croton roxburghii* N.P. Balakr.

Deciduous shrub or small tree to 12 m, branching in whorls. **BARK** grey-brown, thin, smooth or slightly cracking, inner bark reddish. **LEAF** 10-30x4-10 cm, often clustered near end of twigs & appearing whorled, oblong or oblanceolate, pointed or blunt at both ends, **closely but irregularly toothed**. Young leaves pinkish-brown with **yellowish scales**, mature leaves dull green & smooth above, **smooth or nearly so below.** 13-19 pairs of side veins. Stalks 1-7 cm, swollen at top with a pair of rounded glands. **FLOWER** ± 0.7 cm, greenish-white, slightly fragrant, in narrow clusters to 36 cm, all males or with females below males. Main stalks densely scaly at first, later smooth. Individual stalks slender, 2-5 mm, densely scaly. Males with ovate sepals ±3 mm, hairly at first, petals ±3 mm, densely white-hairy outside & along margin. 10-12 stamens with yellow anthers. Females with sepals, densely scaly-hairy all over, petals ±2 mm (sometimes absent), 3 free stigmas 3-4 mm, forked near top. **FRUIT** 0.6-0.8 cm, globose, grooved or slightly 3(2) lobed, sparsely scaly, leathery. **Stalks** 3-5 mm. (Figure 1). This plant commonly found throughout Northern in open areas & secondary growth, also semi-open forests to 650 m. of Thailand (Gardner, Sidisunthorn and Anusarnsunthorn, 2000)

3. The purposes of this research

This study was a continuation of the investigation of chemical constituents from *Croton roxburghii* N.P.Balakr. The plant specimen collected from Chaiyaphum province at N 16° 38′ 47.8″ and E 101° 47′ 45.1″ has been examined.

The main purposes of this present study were to isolate and elucidate the chemical structure of each isolated compounds, and to screen for the cytotoxic activity of the crude extracts and isolated compounds from the stem bark of *Croton roxburghii* N.P. Balakr. collected from Chaiyaphum province, Thailand.

Figure 1: *Croton roxburghii* N.P. Balakr.

CHAPTER II

HISTORICAL

1. Chemical constituents of *Croton roxburghii* N.P.Balakr.

From several previous phytochemical studies, *Croton roxburghii* N.P. Balakr. has been shown to be a rich source of diterpenoid compounds. Up to date, eight different types of the main diterpene skeletons have been found in this plant, namely Labdane, Clerodane, Pimarane, Kaurane, Cembrane, Cleistanthane, Trachylobane, and Abeitane. The basic structure of each diterpene skeleton and the structure of each compound as showed in **Figure 2**, and **Figure 3** respectively. In addition to those eight diterpenes, triterpenes, steroids, steroids glucosides and several other chemical compounds are also presented, as summarized in the **Table 1**.

Table 1. Chemical constituents of *Croton roxburghii* N.P.Balakr.

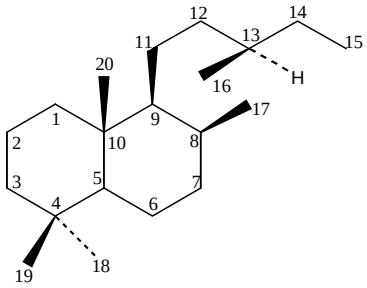
Compounds	Plants parts	References
Diterpenes		
1. Labdane Diterpenes		
• labda-7,12 (*E*),14-triene **[1]**	stem bark	Roengsumran *et al*., 1999a
• labda-7,12 (*E*),14-triene-17-al **[2]**	stem bark	Roengsumran *et al*., 1999a
• labda-7,12 (*E*),14-triene-17-ol **[3]**	stem bark	Roengsumran *et al*., 1999a
• labda-7,12 (*E*),14-triene-17-oic acid **[4]**	stem bark	Roengsumran *et al*., 1999a
• *ent*-8 (17),12*E*,14-labdatrien-18-oic acid **[5]**	stem bark	Pattamadilok, 1998
• 12,15-epoxy-8 (17),12,14-labdatriene **[6]**	stem bark	Pattamadilok, 1998
• labda-7, 13 (*Z*)-diene-17,12-olide **[7]**	stem bark	Baiagern, 1999
• labda-7, 13 (*Z*)-diene-17,12-olide-16-ol **[8]**	stem bark	Baiagern, 1999
• 2-acetoxy-labda-8(17),12 (*E*),14-triene-3-ol **[9]**	stem bark	Kuptiyanuwat, 1999; Roengsumran *et al*., 2001
• 3-acetoxy-labda-8(17),12 (*E*),14-triene-2-ol **[10]**	stem bark	Kuptiyanuwat, 1999; Roengsumran *et al*., 2001

Compounds	Plants parts	References
• labda-8 (17),12(*E*),14- triene-2,3-diol **[11]**	stem bark	Kuptiyanuwat, 1999; Roengsumran *et al*., 2001
• 12 (*E*), 14-labdadiene-7,8-diol **[12]**	stem bark	Boontha, 2000
• 6-acetoxy-12(*E*),14-labdadiene-7,8-diol **[13]**	stem bark	Boontha, 2000
• 12 (*E*), 14-labdadiene-6,7,8-triol **[14]**	stem bark	Boontha, 2000
• nidorellol **[15]**	stem bark	Roengsumran *et al*., 2002
• (5*S*, 8*S*, 9*S*, 10*R*, 13*S*)-8, 13-epoxylabda-1, 14-dien-3-one **[16]**	stem bark	Permpanya, 2003
• (5*S*, 8*S*, 9*S*, 10*R*, 12*S*, 13*S*)-8, 13-epoxy-12-hydroxy-labda-1, 14-dien-3-one **[17]**	stem bark	Permpanya, 2003
2. Clerodane Diterpenes (-)-hardwickiic acid **[18]**	root bark, wood	Aiyar and Seshadri, 1972b
	stem bark	Aiyar and Seshadri, 1972a; Surachethapan, 1996; Baiagern, 1999; Sirimongkhon, 2000; Sriyangnok, 2000
• 11-dehydro-(-)-hardwickiic acid **[19]**	stem bark	Aiyar and Seshadri, 1972a
	root bark, wood	Aiyar and Seshadri, 1972b
• (-)-20-benxyloxyhardwickiic acid **[20]**	stem bark	Baiagern, 1999
• methyl-15,16-epoxy-12-oxo-3,13 (16),14-clerodatriene-20,19-olide-17-oate **[21]**	stem bark	Tanwattanakun, 1999
• crovatin **[22]**	stem bark	Siriwat, 1999
• croblongifolin **[23]**	stem bark	Roengsumran *et al*., 2002
3. Pimarane Diterpenes • oblongifoliol **[24]**	stem bark	Rao *et al*., 1968
	root bark, wood	Aiyar and Seshadri, 1972b
• 19-deoxyoblongifoliol **[25]**	stem bark	Rao *et al*., 1968
	root bark, wood	Aiyar and Seshadri, 1972b;

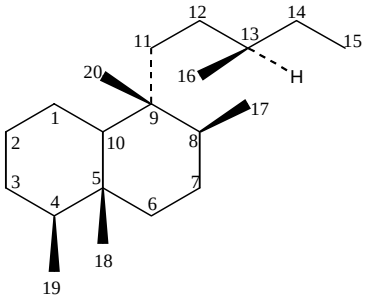
Compounds	Plants parts	References
• 3-deoxyoblongifoliol **[26]**	stem bark	Aiyar and Seshadri, 1971a
	root bark, wood	Aiyar and Seshadri, 1972b
• oblongifolic acid **[27]**	stem bark	Aiyar and Seshadri, 1970
	root bark, wood	Aiyar and Seshadri, 1972b
• *ent*-isopimara-7,15-dien **[28]**	stem bark	Aiyar and Seshadri, 1971b
	root bark, wood	Aiyar and Seshadri, 1972b
• *ent*-isopimara-7,15-dien-19-aldehyde **[29]**	stem bark	Aiyar and Seshadri, 1971b
	root bark, wood	Aiyar and Seshadri, 1972b
• 19-hydroxy-*ent*-isopimara-7,15-dien **[30]**	stem bark	Aiyar and Seshadri, 1971b
• (-)-pimara-9 (11), 15-dien-19-oic acid (acanthoic acid) **[31]**	stem bark	Tanwattanakun, 1999
• (-)-pimara-9 (11), 15-dien-19-ol **[32]**	stem bark	Tanwattanakun, 1999
4. Kaurane Diterpene • *ent*-kaur-16-en-19-oic acid **[33]**	stem bark	Pattamadilok, 1998; Sirimongkhon, 2000
5. Cembrane Diterpenes • crotocembraneic acid **[34]**	stem bark	Surachethapan, 1996; Roengsumran *et al.*, 1998
• neocrotocembraneic acid **[35]**	leaves	Achayindee, 1996;
	stem bark	Roengsumran *et al.*, 1998
• neocrotocembranal **[36]**	stem bark	Roengsumran *et al.*, 1999b
• poilaneic acid **[37]**	stem bark	Boontha, 2000
• (2*E*,7*E*,11*E*) 1-isopropyl-1,4-dihydroxy-4,8-dimethycyclotetradeca-2,7,11-triene-12-carboxylic acid **[38]**	stem bark	Tanwattanakun, 1999
6. Cleistanthane Diterpene • 3,4-seco-cleistantha-4 (18),13 (17),15-trien-3-oic acid **[39]**	stem bark	Siriwat, 1999; Sriyangnok, 2000
7. Trachylobane Diterpene • trachyloban-19-oic-acid **[40]**	stem bark	Boontha, 2000

Compounds	Plants parts	References
8. Abeitane Diterpene • abeita-7,13-dien-3-one **[41]**	stem bark	Sriyangnok, 2000
Triterpenes • acetyl aleuritolic acid **[42]**	stem bark	Aiyar, *et al*., 1971c
Steroids • β-sitosterol **[43]**	stem bark	Roa, *et al*., 1968
	wood	Chaicharoenpong, 1996
	leaves	Achayindee, 1996
• campesterol **[44]**	wood	Chaicharoenpong, 1996
	stem bark	Pattamadilok, 1998
• stigmasterol **[45]**	wood	Chaicharoenpong, 1996
	leaves	Achayindee, 1996
	stem bark	Pattamadilok, 1998
Steroid Glucosides • *β*-sitosteryl-3-O- *β* -D-glucopyranoside **[46]**	wood	Chaicharoenpong, 1996
	stem bark	Surachethapan, 1996
• campesteryl 3-O- *β* -D-glucopyranoside **[47]**	wood	Chaicharoenpong, 1996
	stem bark	Surachethapan, 1996
• stigmasteryl-3-O- *β* -D-glucopyranoside **[48]**	wood	Chaicharoenpong, 1996
	stem bark	Surachethapan, 1996
Coumarin • 7-hydroxy-6-methoxycoumarin (Scopoletin) **[49]**	wood	Chaicharoenpong, 1996
Miscellaneous • mixture of long chain aliphatic hydrocarbons (C_{27}-C_{33})	wood	Chaicharoenpong, 1996
	leaves	Achayindee, 1996
• mixture of long chain aliphatic carboxylic acids (C_{18}, C_{22}-C_{34})	wood	Chaicharoenpong, 1996

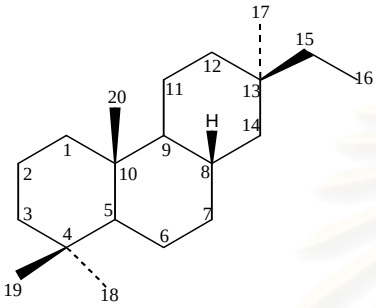
Compounds	Plants parts	References
• mixture of long chain alcohols (C_{28}-C_{29}, C_{31}-C_{32}, C_{34})	leaves	Achayindee, 1996
• 6, 10, 14-trimethyl-2-pentadecanone **[50]**	leaves	Achayindee, 1996
• potassium chloride	leaves	Achayindee, 1996



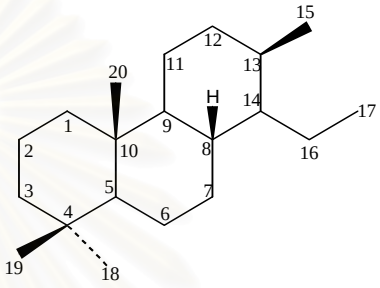
Labdane



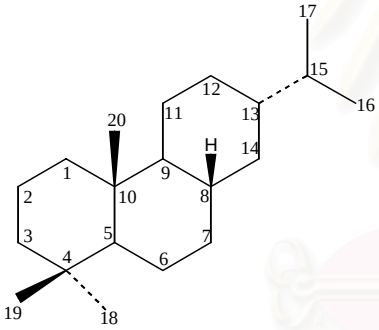
Clerodane



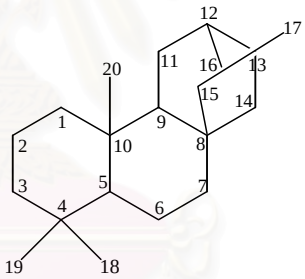
Pimarane



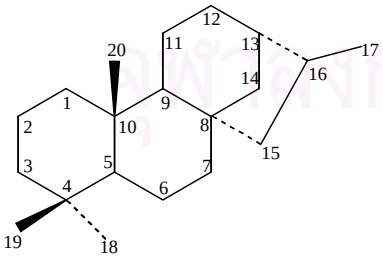
Cleistanthane



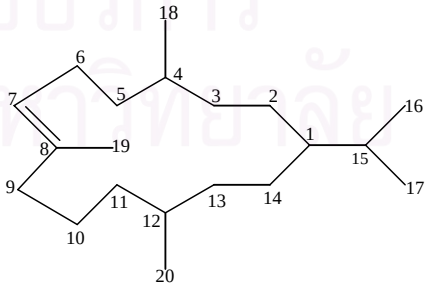
Abietane



Trachylobane

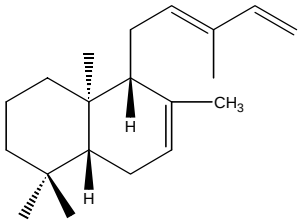


Kaurane

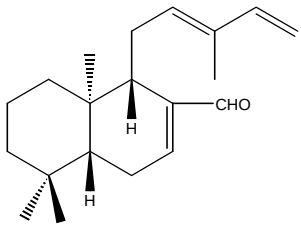


Cembrane

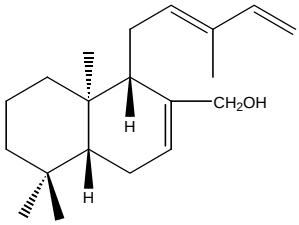
Figure 2: Basic Structure of Diterpenoids in *Croton roxburghii* N.P.Balakr.



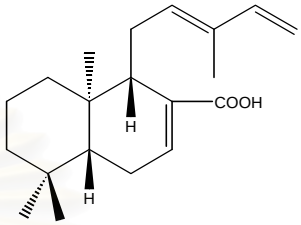
labda-7,12 (*E*),14-triene **[1]**



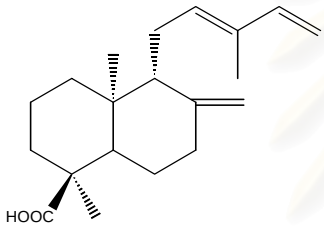
labda-7,12 (*E*),14-triene-17-al **[2]**



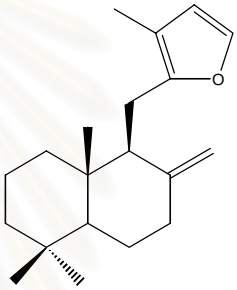
labda-7,12 (*E*),14-triene-17-ol **[3]**



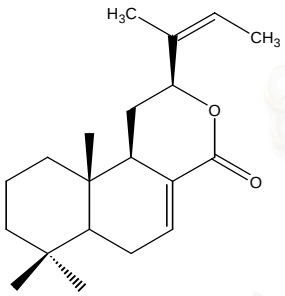
labda-7,12 (*E*),14-triene-17-oic acid **[4]**



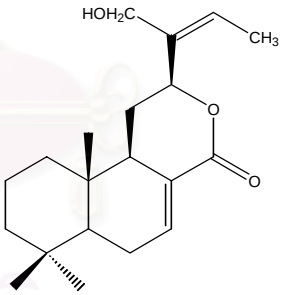
ent-8 (17),12*E*,14-labdatrien-18-oic acid **[5]**



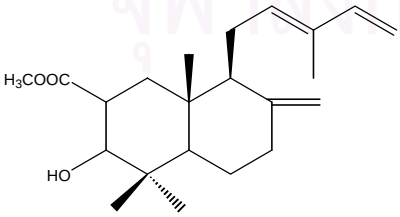
12, 15-epoxy-8(17), 12, 14-labdatriene **[6]**



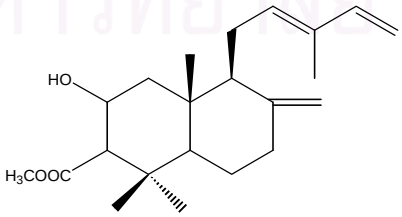
labda-7, 13 (*Z*)-diene-17,12-olide **[7]**



labda-7, 13 (*Z*)-diene-17,12-olide-16-ol **[8]**

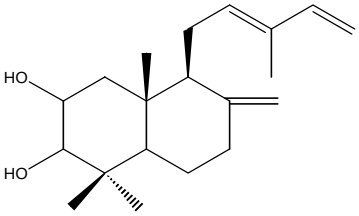


2-acetoxy-labda-8(17), 12(*E*)-14 -triene-3-ol **[9]**

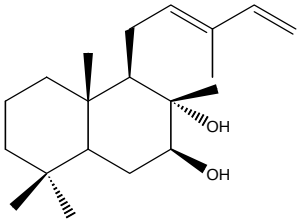


3-acetoxy-labda-8(17), 12(*E*)-14-triene-2-ol **[10]**

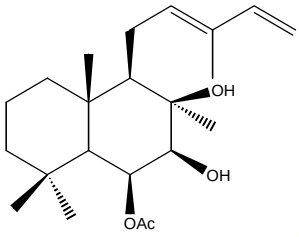
Figure 3: Chemical constituents of *Croton roxburghii*



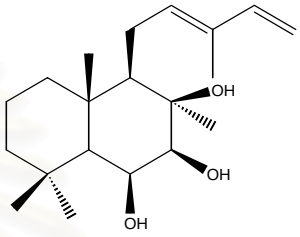
labda - 8(17), 12(*E*), 14-triene-2,3-diol **[11]**



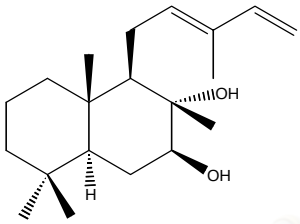
12 (*E*), 14-labdadiene-7, 8-diol **[12]**



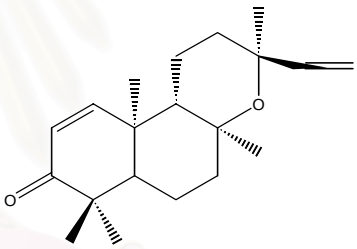
6-acetoxy-12(*E*), 14-labdadiene-7, 8-diol **[13]**



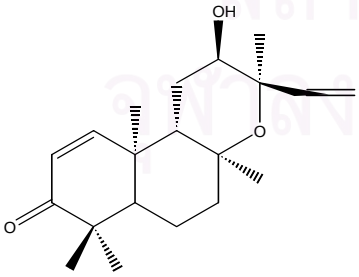
12 (*E*), 14-labdadiene-6, 7, 8-triol **[14]**



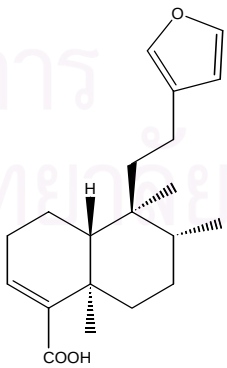
nidorellol **[15]**



5*S*, 8*S*, 9*S*, 10*R*, 13*S*)-8, 13-epoxylabda-1, 14-diene-3-one **[16]**

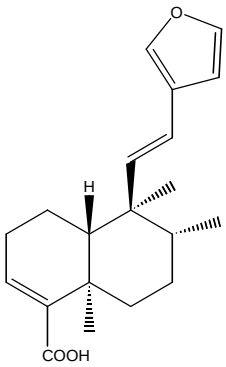


5*S*, 8*S*, 9*S*, 10*R*, 12*S*, 13*S*)-8, 13-epoxy-12-hydroxy-labda-1, 14-diene-3-one **[17]**

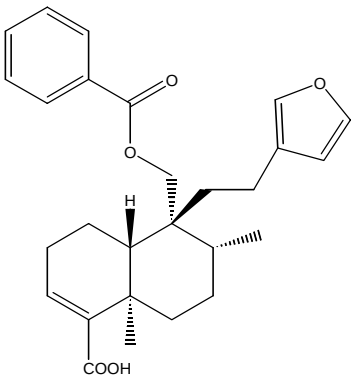


(-)-hardwickiic acid **[18]**

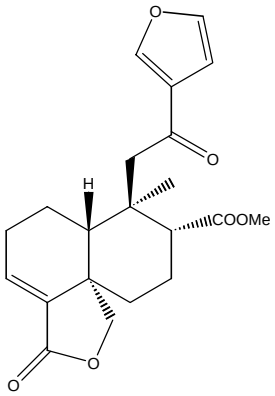
Figure 3: Chemical constituents of *Croton roxburghii* (continued)



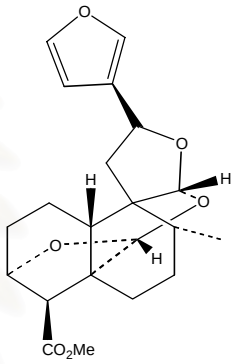
11-dehydro-(-)-hardwickiic acid **[19]**



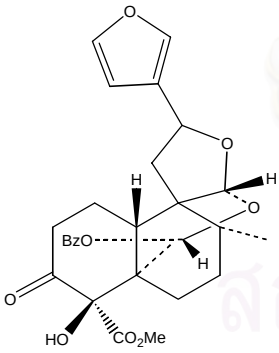
(-)-20-benzyloxyhardwickiic acid **[20]**



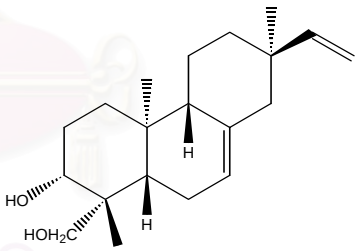
methyl -15, 16-epoxy-12-oxo-3,13 (16), 14-clerodatriene-20, 19-olide-17-oate **[21]**



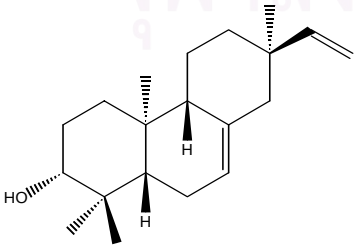
crovatin **[22]**



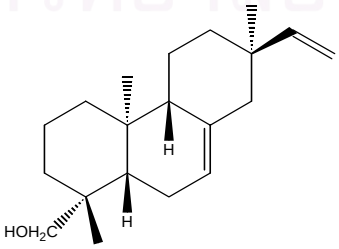
croblongifolin **[23]**



oblongifoliol **[24]**

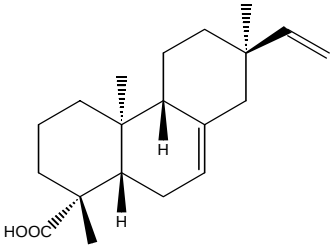


19-deoxyoblongifoliol **[25]**

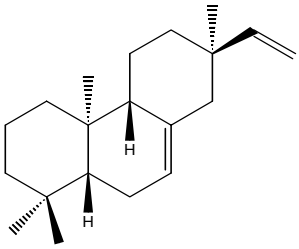


3-deoxyoblongifoliol **[26]**

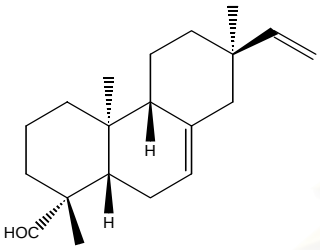
Figure 3: Chemical constituents of *Croton roxburghii* (continued)



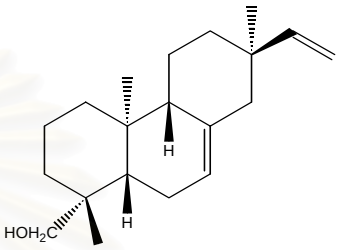
oblongifolic acid **[27]**



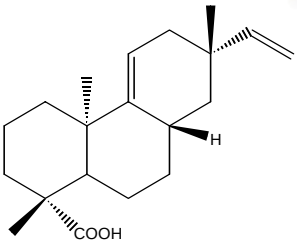
ent-isopimara-7,15-diene **[28]**



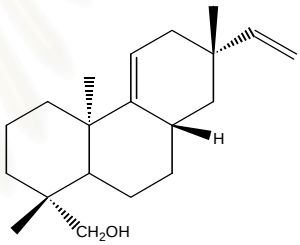
ent-isopimara-7,15-dien-19-aldehyde **[29]**



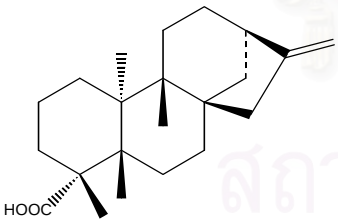
19-hydroxy-*ent*-isopimara-7, 15-diene **[30]**



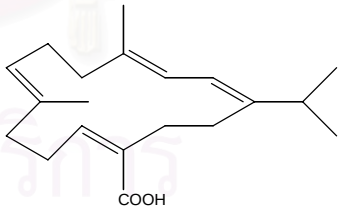
(-)-pimara-9 (11), 15-diene-19-oic acid (acanthoic acid) **[31]**



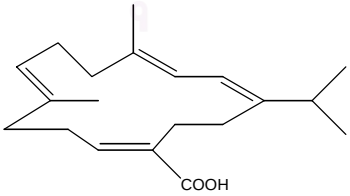
(-)-pimara-9 (11), 15-dien-19-ol **[32]**



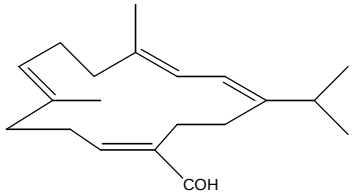
ent-kaur-16-en-19-oic acid **[33]**



crotocembraneic acid **[34]**

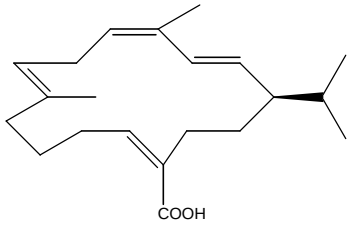


neocrotocembraneic acid **[35]**

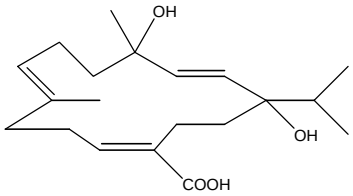


neocrotocembranal **[36]**

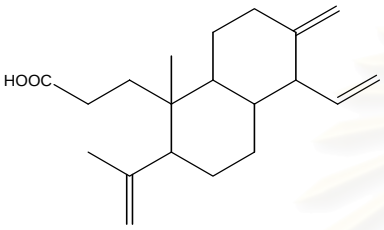
Figure 3: Chemical constituents of *Croton roxburghii* (continued)



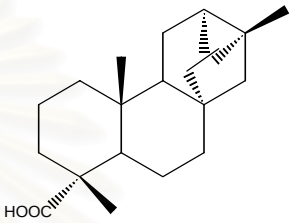
poilaneic acid **[37]**



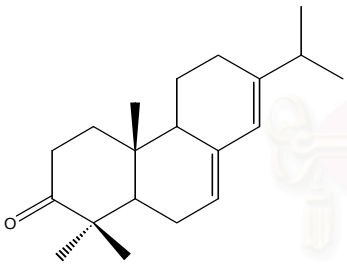
(2*E*,7*E*,11*E*) 1-isopropyl-1,4-dihydroxy-4,8-dimethylcyclotetradeca-2, 7, 11-triene-12-carboxylic acid **[38]**



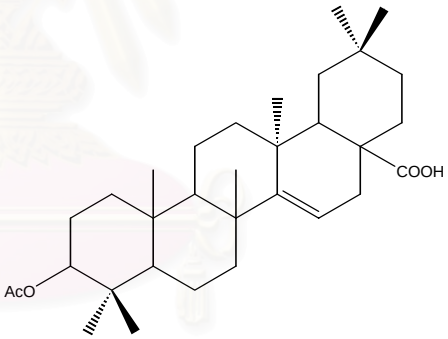
3, 4-seco-cleistantha-4(18), 13(17) 15-trien-3-oic acid **[39]**



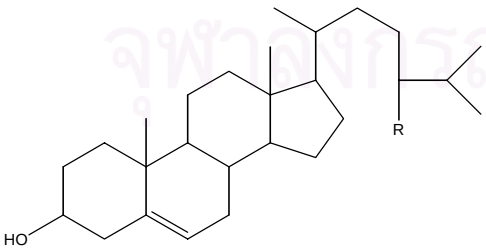
trachyloban-19-oic-acid **[40]**



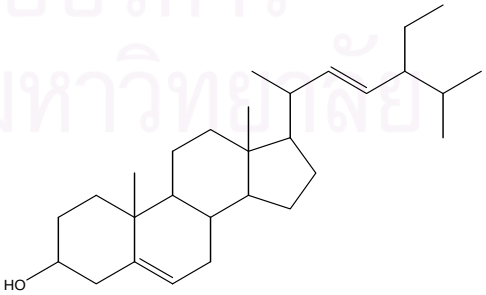
abeita-7,13-dien-3-one **[41]**



acetyl aleuritolic acid **[42]**

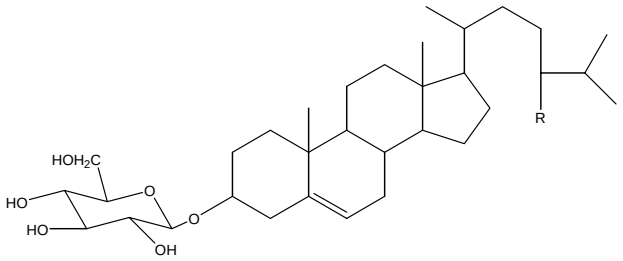


β-sitosterol : R= C_2H_5 **[43]**
campesterol : R= CH_3 **[44]**

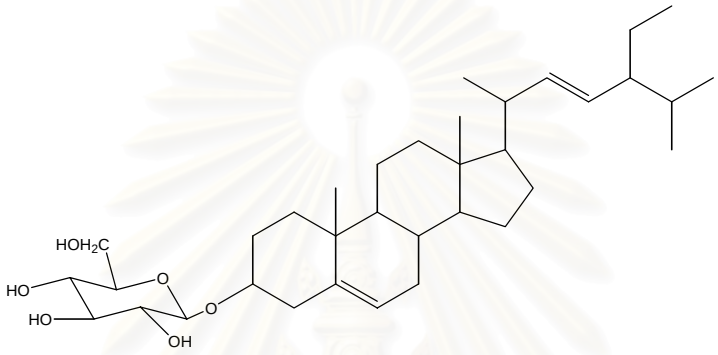


stigmasterol **[45]**

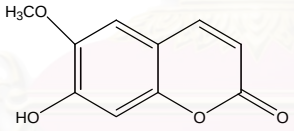
Figure 3: Chemical constituents of *Croton roxburghii* (continued)



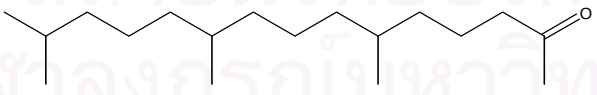
β-sitosteryl-3-O- β -D-glucopyranoside: R= C_2H_5 **[46]**
campesteryl 3-O- β -D-glucopyranoside: R= CH_3 **[47]**



stigmasteryl-3-O- β -D-glucopyranoside **[48]**



7-hydroxy-6-methoxycoumarin (Scopoletin) **[49]**



6, 10, 14-trimethyl-2-pentadecanone **[50]**

Figure 3: Chemical constituents of *Croton roxburghii* (continued)

2. Biological activities of diterpene compounds from *Croton roxburghii* N.P.Balakr.

Diterpenoids isolated from *Croton roxburghii* had been investigated for many biological activities such as cytotoxicity, antimicrobial, antiplatelet aggregation, cAMP phosphodiesterase inhibition, antioxidant and antibacterial. The biological activities which have been reported as potent are cytotoxicity, antiplatelet aggregration, antimicrobial and insecticidal.

2.1 Cytotoxicity:

Some of the diterpene compounds listed in Table 2 have been shown to exhibit *in vitro* cytotoxicity against many human tumor cell lines, as summarized below.

Table 2. Cytotoxic activity against cancer cell line of some diterpene compounds from *Croton roxburghii*

Compounds	**IC_{50} (µg/ml)**					**References**
	KATO-3	**SW620**	**BT474**	**HEP-G2**	**CHAGO**	
[8]	7.1	6.5	> 10	5	6.4	Baiagern, 1999
[9]	5.7	7.1	> 10	> 10	> 10	Roengsumran *et al*., 2001
[10]	3.3	> 10	5.9	> 10	> 10	Roengsumran *et al*., 2001
[11]	2.2	2.7	4.6	3.7	3.3	Roengsumran *et al*., 2001
[23]	0.35	0.47	0.12	0.35	0.24	Roengsumran *et al*., 2002
[32]	6.5	5.9	> 10	6.7	6.1	Tanwattanakun, 1999

[8] = labda-7, 13 (*Z*)-diene-17,12-olide-16-ol

[9] = 2-acetoxy-3-hydroxy-labda-8 (17), 12 (*E*)-14-triene

[10] = 3-acetoxy-2-hydroxy-labda-8 (17), 12 (*E*)14-triene

[11] = 2, 3-dihydroxy-labda-8 (17), 12 (*E*)-14-triene

[23] = croblongifolin

[32] = (-)-pimara-9 (11), 15-diene-19-ol

Tumor Cell Lines:

KATO-3 = human gastric carcinoma

SW620 = human colon adenocarcinoma

BT474 = human breast ductal carcinoma

HEP-G2 = human liver hepatoblastoma

CHAGO = human undifferentiated lung carcinoma

From the data in Table 2 it is very interesting to note that, among the three structurally related labdane diterpenes **[9-11]**, **[9]** and **[10]** were less active but more selective than **[11]**. The presence of the acetyl group is believed to be the cause of this, since it is likely that an acetylation of these compounds could decrease their ability to form hydrogen bond with certain receptor on tumor cells and made them more selective but less active (Roengsumran *et al*., 2001)

Furthermore, neocrotocembranal **[36]** exhibited cytotoxicity against P-388 cells (lymphoid neoplasm) *in vitro* with an IC_{50} value of 6.48 (µg/ml)

2.2 Antiplatelet aggregration:

Another notable compound derived from this plant, is neocrotocembranal **[36]**. This compound inhibited platelet aggregation induced by thrombin with an IC_{50} value of 47.21(µg/ml). However, two other cembranoid diterpenes, crotocembraneic acid **[34]** and neocrotocembraneic acid **[35]**, showed no inhibitory effect on platelet aggregation. Thus a hypothesis that the reactive aldehyde functionality plays an important part in this effect is proposed (Roengsumran *et al.*, 1999b).

2.3 Insecticidal:

(-)-Hardwickiic acid **[18]**, a well-known clerodane diterpene, has been reported as having insecticidal activity against *Aphis craccivora* (Aphididae). The

compound, at a dose of 5 ppm/insect, caused 62% mortality of adult female aphids after 24 hours (Bandara *et al*., 1987).

2.4 Antimicrobial activities:

The clerodane diterpene compound, (-)-hardwickiic acid **[18]** exhibited antimicrobial activity (Baigern, 1999).

2.5 Inhibition of cAMP phosphodiesterase activity:

Cembranoid compounds, crotocembraneic acid **[34]** and neocrotocembraneic acid **[35]** have been reported to act as inhibitions of cAMP phosphodiesterase activity (Singtothong, 1999).

3. Biogenetic pathway of diterpenoids compounds

3.1 Introduction

Diterpenoids are the member of terpenoids group, which conform to the "biogenetic isoprene rule" firstly developed by Wallach (1914) and refined by Ruzicka *et al*.(1953), a rule which states that a terpene is constituted by union of two or more isoprene units in a "head-to-tail" manner (Nicholas, 1973)

The combination of two isoprene units arranged "head-to-tail" is shown below:

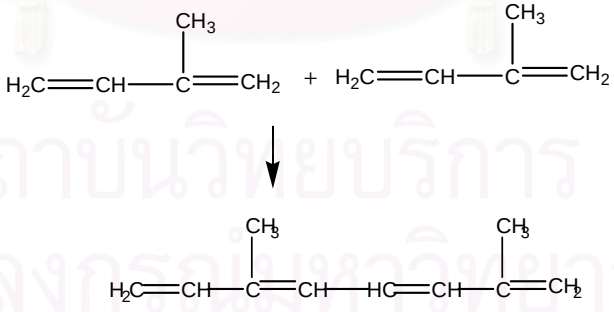


Figure 4: The formation of isoprene units: head-to-tail manner

Terpenoids have wide varieties of compounds according to the number of isoprene units contained in their molecules as shown in Table 3

Table 3. Classification of terpenoids

Class	Number of isoprene unit	Number of carbons	General formula
Hemiterpenoids	1	5	C_5H_8
Monoterpenoids	2	10	$C_{10}H_{16}$
Sesquiterpenoids	3	15	$C_{15}H_{24}$
Diterpenoids	4	20	$C_{20}H_{32}$
Sesterterpenoids (Ophiobalanes)	5	25	$C_{25}H_{40}$
Triterpenoids	6	30	$C_{30}H_{48}$
Tetraterpenoids	8	40	$C_{40}H_{64}$
Polyterpenoids	n	5n (n>8)	$(C_5H_8)_n$

3.2 Biosynthesis of Diterpenoids

The biosynthesis of diterpenoids involves the following mechanisms:

3.2.1 Formation of isopentenyl pyrophosphate.

3.2.2 Polymerization of isopentenyl pyrophosphate.

3.2.3 Formation of cyclic diterpenoids.

3.2.1 Formation of isopentenyl pyrophosphate.

All terpenoid compounds originate from isopentenyl pyrophosphate, which is also known as “activated isoprene”. Isopentenyl pyrophosphate is synthesized from acetyl CoA in the same manner by both plants and animals as the following steps (Bu’Lock, 1965; Luckner, 1972):

a) Acetoacetyl CoA is first formed from two molecules of acetyl CoA by “head-to-tail” condensation. This reaction is catalyzed by the enzyme thiolase.

b) A third molecule of acetyl CoA adds to the carbonyl group at position three of acetoacetyl CoA to form 3-hydroxy-3-methylglutaryl CoA. The steps (a) and (b) are normally interconvertible. 3-hydroxy-3-methylglutaryl CoA may be derived from leucine as Figure 5.

c) 3-Hydroxy-3-methylglutaryl CoA is then reduced to an intermediate product mevaldic acid. This reaction is practically irreversible.

d) The enzyme mevaldate reductase transfers the hydrogen stereo-specifically from NADPH or NADH, mevalonic acid is thus formed in Figure 5.

e) Mevalonic acid is then phosphorylated at the primary alcoholic group to form mevalonic acid monophosphate and then in a second reaction step mevalonic acid pyrophosphate is formed.

f) The product of a third phosphorylation at tertiary alcoholic group via ATP undergoes concerted elimination of a molecule of water and decarboxylation yield Δ^3 isopentenyl pyrophosphate.

All of these steps are illustrated in Figure 5.

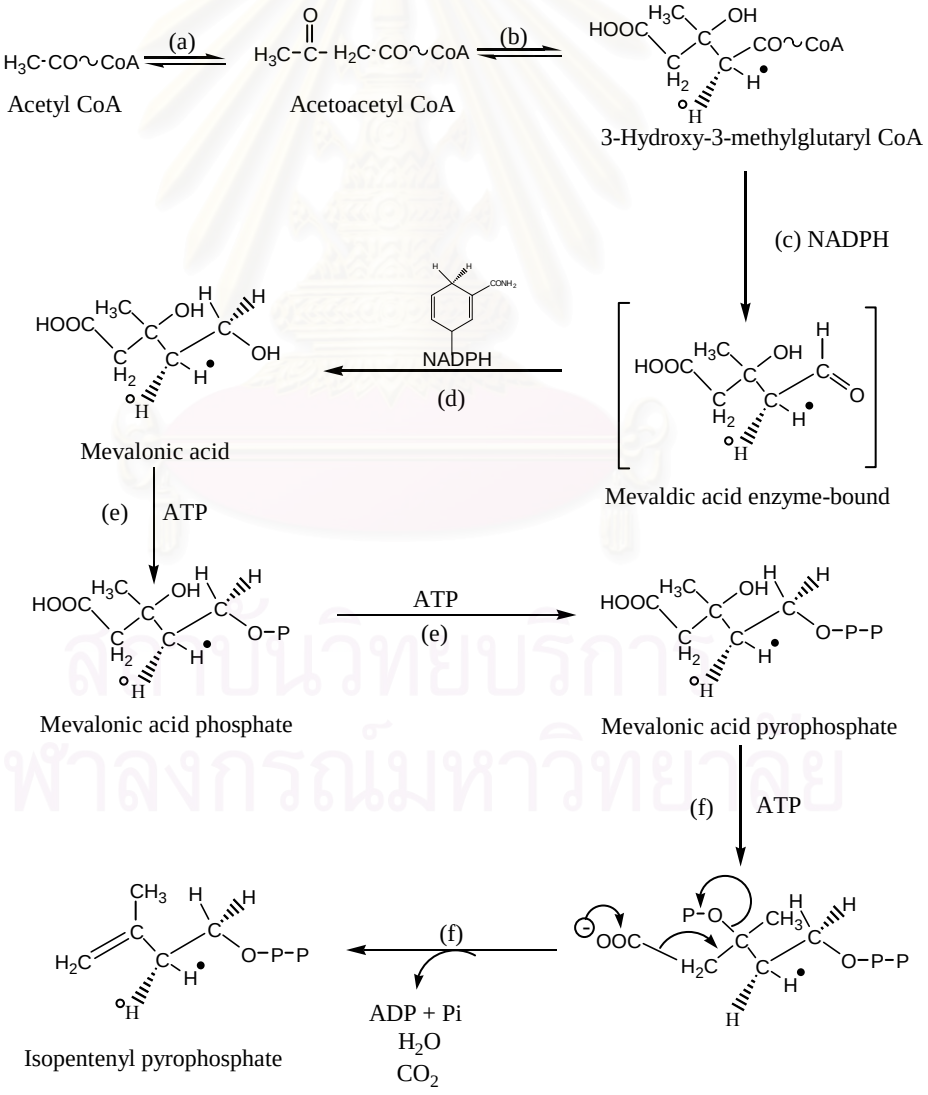


Figure 5: Biosynthesis of isopentenyl pyrophosphate from acetyl CoA

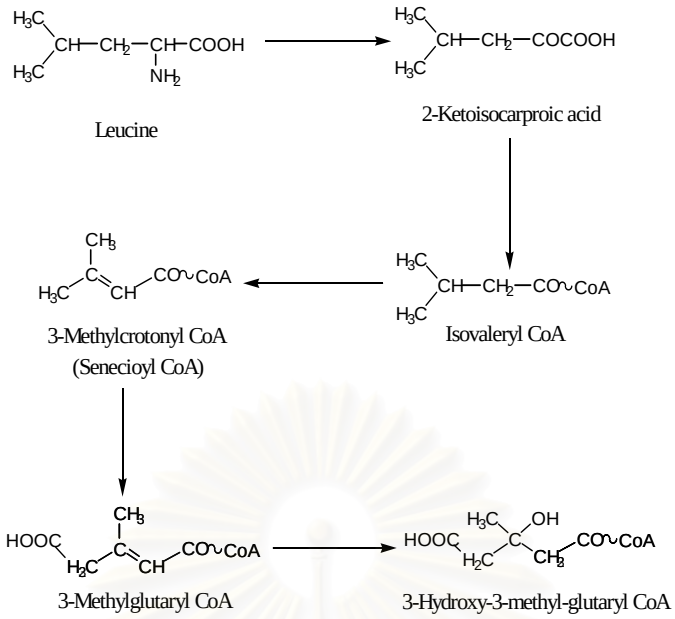


Figure 6: Conversion of leucine to 3-hydroxy-3methylglutaryl CoA.

3.2.2 Polymerization of Isopentenyl pyrophosphate

The formation of diterpenoids takes place by the polymerization of several molecules of isopentenyl pyrophosphate. These reactions of polymerization are shown in Figure 7 and described below (Luckner, 1972).

a) By the shift of the double bond of isopentenyl pyrophosphate catalyzed by isopentenyl pyrophosphate isomerase, this yields 3, 3-dimethyl-allyl pyrophosphate which serves as a starter molecule for this polymerization both in plants and animals. As an allylic ester, 3, 3-dimethylallyl pyrophosphate or the derived cation is an effective electrophilic alkylating agent. The elimination of a hydrogen atom in this reaction at C-2 is strictly stereospecific. The α – hydrogen atom (°H) is always eliminated.

b) One molecule of dimethylallyl pyrophosphate then serves as an acceptor for one molecule of isopentenyl pyrophosphate. The pyrophosphate group is then lost from the starter molecule. The condensation may be considered as a nucleophilic substitution by the CH_2 group of isopentenyl pyrophosphate. The substitution causes an inversion of configuration at C-1 of the starter molecule since the CH_2 group of isopentenyl pyrophosphate opposite the pyrophosphate group enters the molecule from the side in a concerted reaction. The new C-C geranylpyrophosphate, C_{10} occurs simultaneously during the resulting shift of the double bond.

c) Since geranyl pyrophosphate is an allylic ester, the process can be repeated by a similar mechanism, generating farnesyl pyrophosphate and this is then converted to geranylgeranyl pyrophosphate, C_{20}. Configurations around all the double bonds in these compounds are *trans*.

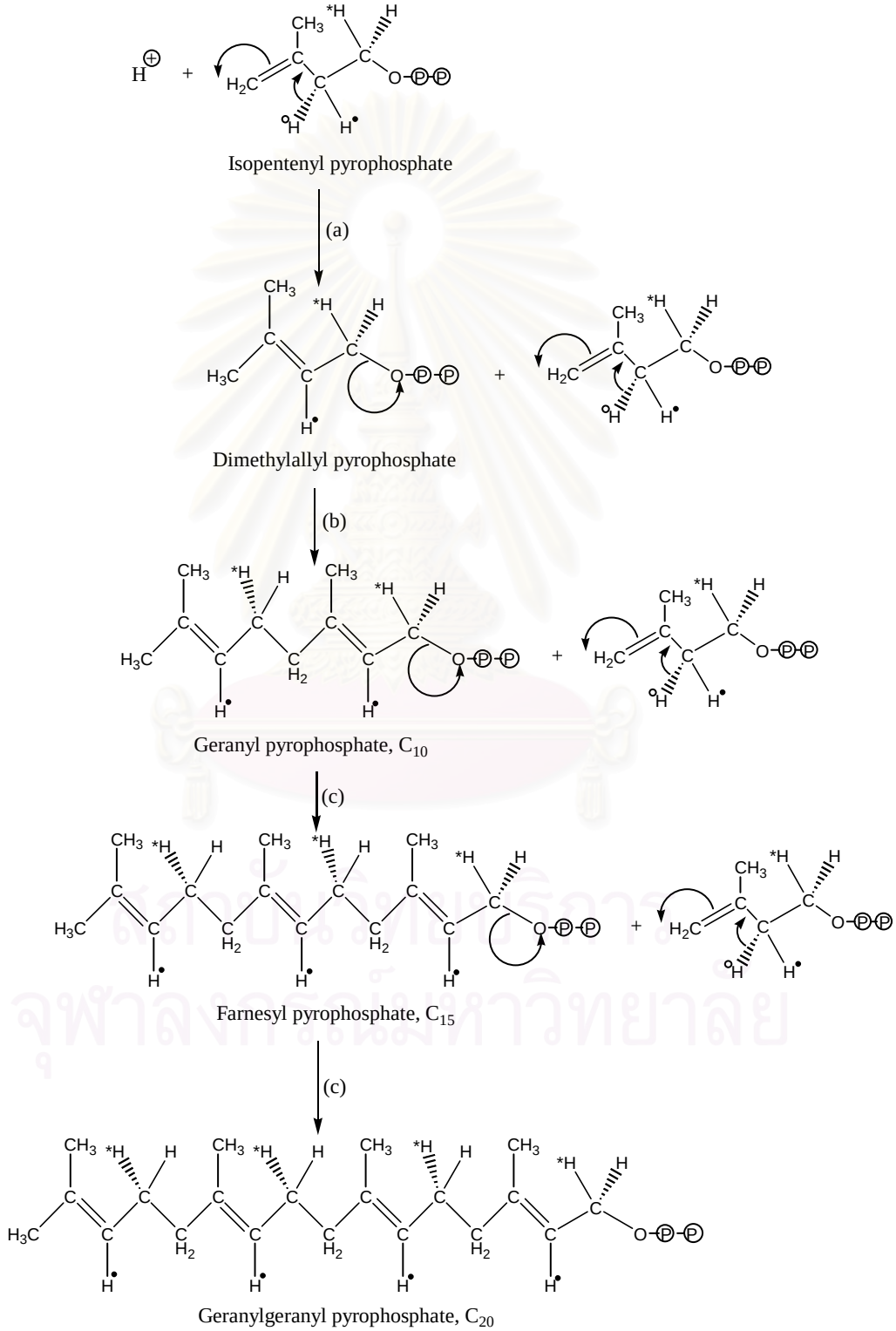
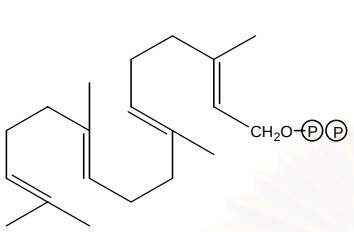


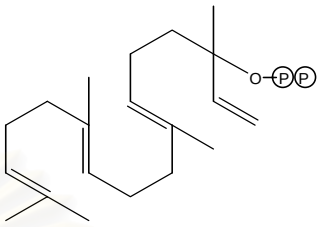
Figure 7: Polymerization of isopentenyl pyrophosphate

3.2.3 Formation of Cyclic Diterpenoids

All of the presently known cyclic diterpenoids are considered to be derived, as a result of the Ruzicka biogenetic isoprenoid rule, from geranylgeranyl pyrophosphate, by direct cyclization or by secondary rearrangements (Nicholas, 1973).

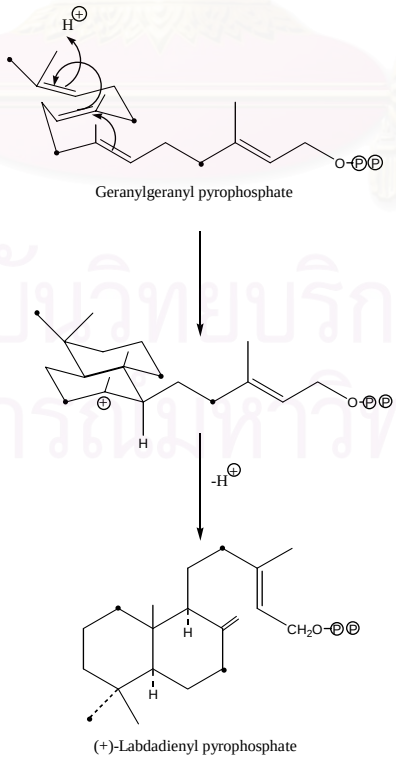


Geranylgeranyl pyrophosphate



Geranyllinaloyl pyrophosphate

Geranylgeranyl pyrophosphate is converted to bicyclic and tricyclic derivatives. Cyclization is catalyzed by enzymes and initiated by protonation. The cyclic precursor shown is the bicyclic (+) - labdadienyl pyrophosphate. From its mode of formation with the first three isoprene units folded in “chair-like” conformation, this necessarily has the typical trans-anti-trans stereochemistry, but not for all diterpenoids (Richards and Hendrickson, 1964; Bu’Lock, 1965; Luckner, 1972. Biosynthesis of a bicyclic precursor of diterpenoids is shown below in Figure 8.



Geranylgeranyl pyrophosphate

(+)-Labdadienyl pyrophosphate

Figure 8: Biosynthesis of a bicyclic precursor of diterpenoids.

4. Biogenetic pathway of diterpenoids in *Croton roxburghii* N.P.Balakr.

The diterpenes are C_{20} compounds biogenetically derived from geranylgeranyl pyrophosphate. The notable feature of diterpene structures is the fascinating variation encountered in their skeletons, which accounts for the division of these compounds into several types. The following correlation chart shows the main diterpene skeletons found in *Croton roxburghii* N.P.Balakr. (Devon and Scott, 1972)

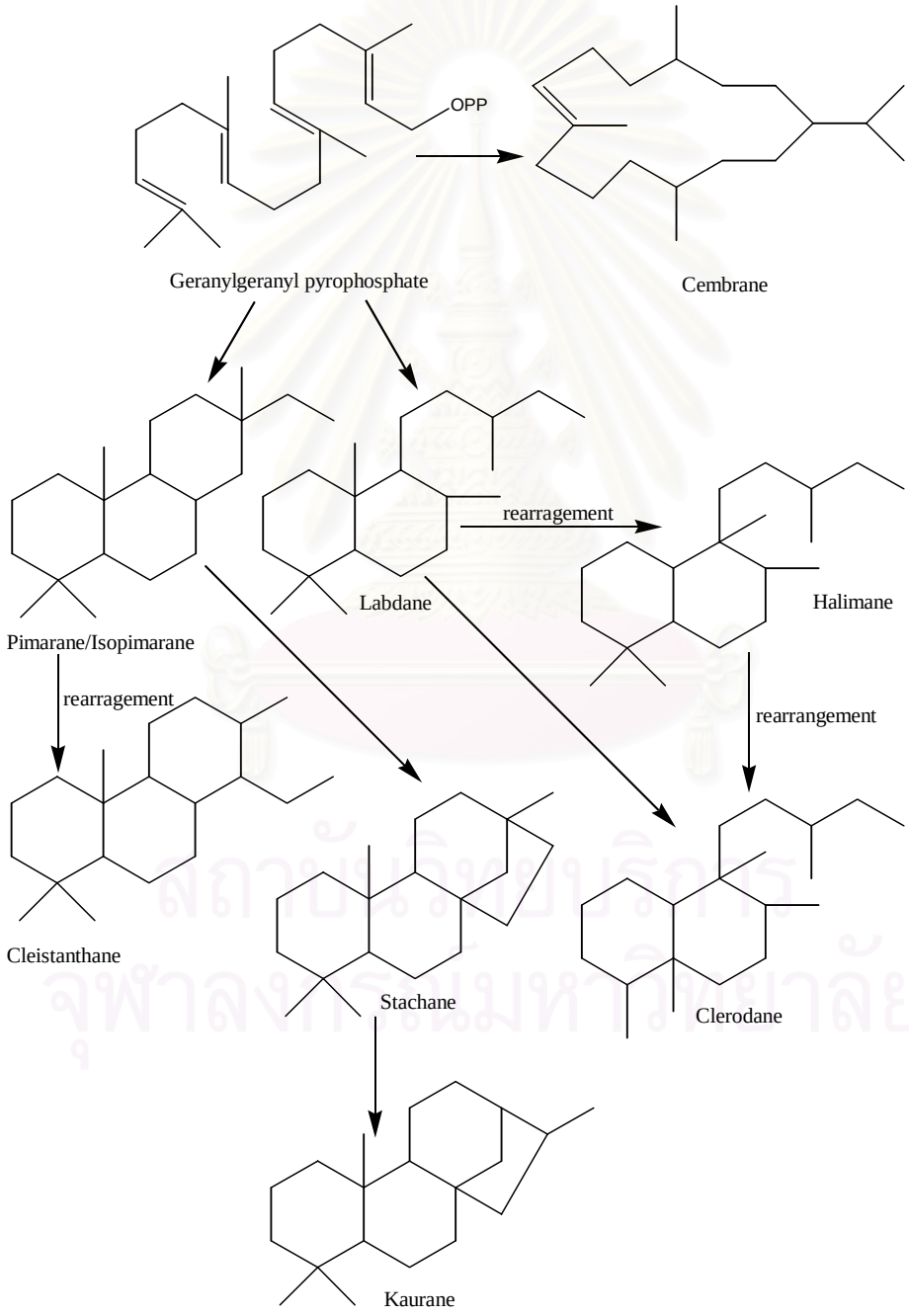


Figure 9: Biogenetic pathway of diterpenoids in *Croton roxburghii* N.P.Balakr.

5. Clerodane diterpenoids

5.1 Introduction

Clerodane diterpenoids represent a large group of secondary metabolites from plants. The vast majority of clerodanes have been isolated from dicotyledonous plants, only a limited range of these compounds can be produced from monocotyledonous species, fungi, and bacteria (Merritt and Ley, 1992). They often show a variety of interesting biological activities: antifeedant, antitumor, antifungal, antibiotic, anti-peptic ulcer and so on (Tokoroyama, 2000). Their structures consist of four isoprene units linked in a head to tail manner (Robbers, Speedie and Tyler, 1996). Their class name derived from "clerodin", the first member of clerodane series isolated from *Clerodendron infortunatum* Linn, family Verbenaceae (Barton *et al.*, 1961), and clerodin also showed antifeedant activity (Merritt and Ley, 1992; Tokoroyama, 2000). Clerodanes (Figure 10) arise from labdanes by two methyl migrations from C-4 methyl group migrate to C-5 position, and C-10 methyl group migrate to C-9 position.

5.2 Variations in the stereostructures of clerodane diterpenoids

5.2.1 *trans* and *cis*-clerodane:

First variation, clerodane diterpenoids are classified into two major groups, *trans* and *cis*, with respect to the configuration of the decalin ring fusion. The *trans* clerodanes can arise via a concerted migration process to intermediate (3) Figure 13, whilst the *cis* compounds require a stepwise process, with a 'pause' at intermediate (4) Figure 13. This can then lead to either *cis* or *trans* compounds, depending on which of the C-4 methyl groups migrate. This is true for around three quarters of all naturally occurring clerodane diterpenoids which has a *trans*-configuraton, but the remaining quarter has a *cis*-configuration (Merritt and Ley, 1992; Tokoroyama, 2000).

5.2.2 *cis*-clerodane with *cis*-substitution (CC) and *cis*-clerodane with *trans*-substitution (CT) / *trans*-clerodane with *cis*-substitution (TC) and *trans*-clerodane with *trans*- substitution (TT):

The second variation is associated with the relative configuration of one carbon unit group substitution at C-8 and C-9 position, which is mostly *cis* as predicted from the biosynthetic process. If the substitution groups at C-8 and C-9 are *cis* position,

there are types CC (*cis*-clerodane with *cis*-substitution) (Figure14), while the substitution groups at C-8 and C-9 are *trans* position, there are types CT (*cis*-clerodane with *trans*-substitution) (Figure 14). However, it is *trans* in a few percent of naturally occurring clerodane diterpenoids types TC (*trans*-clerodane with *cis*-substitution) and types TT (*trans*-clerodane with *trans*-substitution), which are frequently bioactive (Tokoroyama, 2000).

5.2.3 *neo*-clerodanes and *ent-neo*-clerodanes:

The third variations are compared to the absolute configuration of clerodin (Figure 11). The compounds with the same absolute configuration as clerodin being termed *neo*-clerodanes and those compounds enantiomeric to clerodin being termed *ent-neo*-clerodanes (Merritt and Ley, 1992, Tokoroyama, 2000). Casearborins A from the roots of *Casearia arborescens* and intrapetacins from the roots of *Licania intrapetiolaris* are the examples of *ent-neo*-clerodanes as showed in Figure 12.

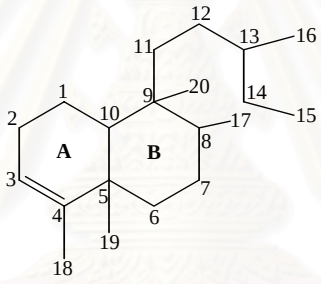
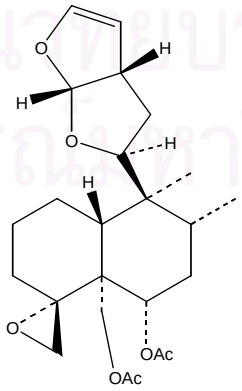
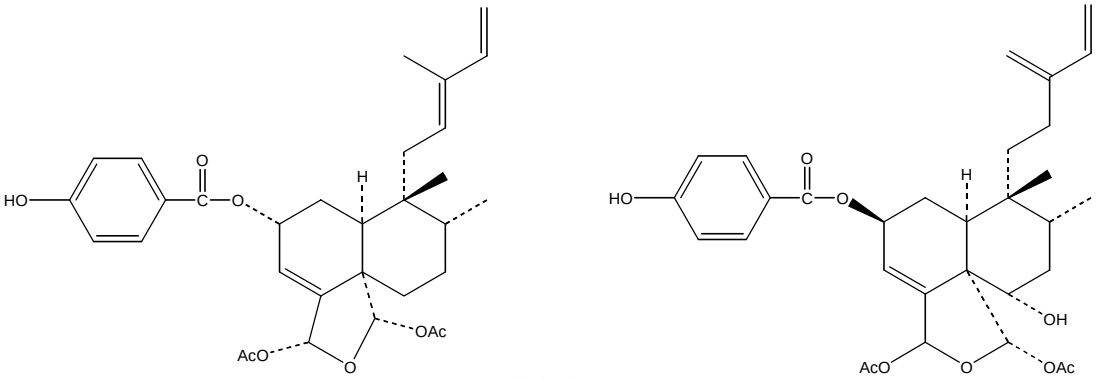


Figure 10: Clerodane Diterpenoids Main Carbon Skeleton



clerodin

Figure 11: Clerodin-the first member of clerodane series



casearborins A intrapetacins

Figure 12: Example of *ent-neo*-clerodanes

5.3 Biosynthesis of Clerodane Diterpenoids

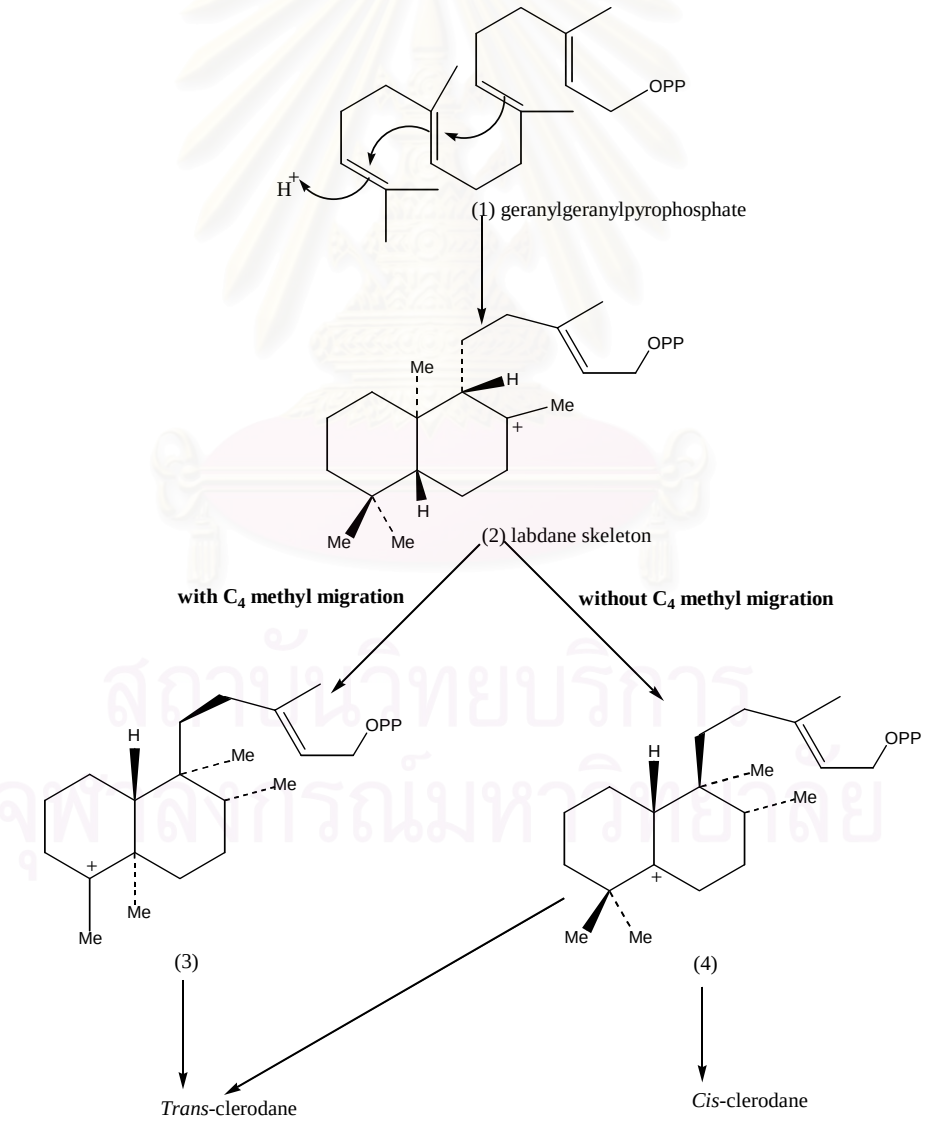


Figure 13: Biosynthesis of clerodane diterpenoids

5.4 Variations in the stereostructure of clerodane diterpenoids

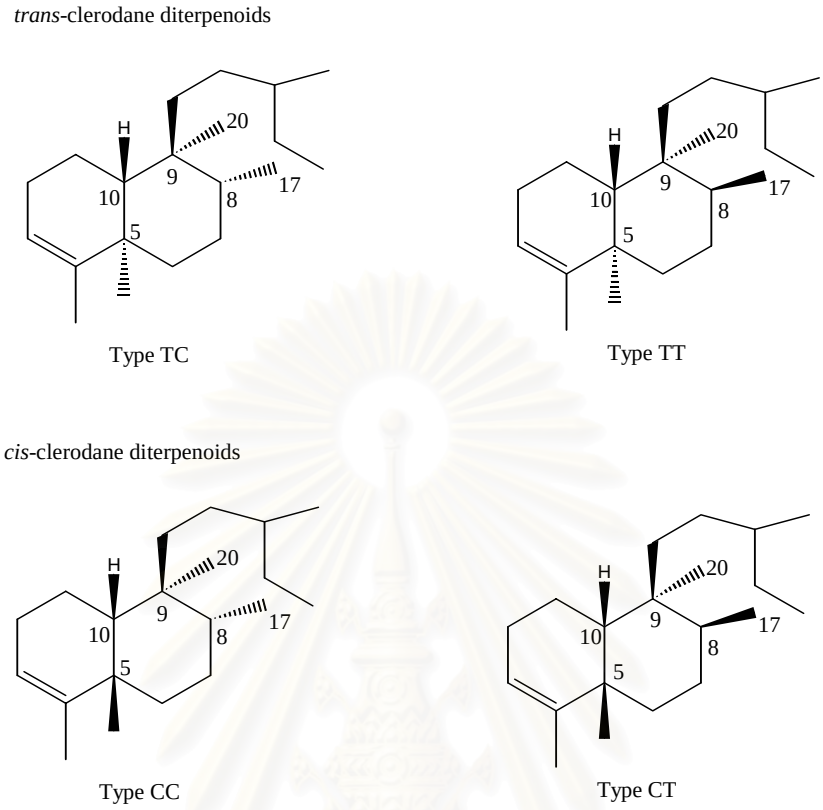


Figure 14: Stereochemical variations in clerodane diterpenoids

5.5 Biological activities of clerodane diterpenoids

From previous studies, it revealed that clerodane diterpenoids often show a variety of interesting biological activities as described below.

5.5.1 Clerodane diterpenes with anti-peptic ulcer activity

From stems, barks and leaves of a well-known Thai medicinal plant, *Croton stellatopilosus* Ohba. (*Croton sublyratus* Kurz family Euphorbiaceae) many clerodane diterpenes have been isolated, some of which have shown significant anti-peptic ulcer activity. Kitazawa *et al.* (1980) reported plaunols B-E **[55-58]** isolated from this plant as exhibiting significant inhibitory activity against Shay ulcers in rats with 55, 36, 44, 52% inhibition respectively at a dose of 3 mg/kg; intraperitoneal (i.p.) and 85, 88, 61, 82% inhibition respectively at a dose of 10 mg/kg;i.p. The mechanism of this action is the depression of gastric secretion.

Several studies (Brito *et al*., 1998; Maciel *et al*., 2000; Hiruma-Lima *et al*., 2002) showed that *trans*-dehydrocrotonin **[59]** from barks of *Croton cajucara* Benth. was as effective as cimetidine in inhibitory activity against HCl/ethanol-induced ulcer in mice with 48.1% inhibition of *trans*-dehydrocrotonin, and 48.6% inhibition of cimetidine at a dose of 100 mg/kg; orally.

Recent study by Hiruma-Lima *et al*. (2002) has indicated that *trans*-crotonin **[60]** from barks of *Croton cajucara* Benth. was as effective as cimetidine in inhibiting indomethacin/bethanechol-induced ulcer in animals with 78% inhibition of *trans*-crotonin , and 77% inhibition of cimeditine at a dose of 100 mg/kg; orally.

5.5.2 Clerodane diterpenes with anti-inflammatory activity

Ichihara *et al*., 1992 revealed that cajucarinolide **[61]** and isocajucarinolide **[62]** isolated from the cortices of *Croton cajucara* possess anti-inflammatory activity and inhibit bee venom phospholipase A_2 *in vitro*. Cajucarinolide **[61]** and isocajucarinolide **[62]** showed anti-inflammatory activity against topical inflammation in the mouse ear induced by teleocidin with IC_{50} of 5.6, and 3.0 µg, respectively and were potent inhibitors of PLA_2 *in vitro* with IC_{50} of 5.8, and 2.3 µg, respectively. They also found that γ-hydroxybutenolide was crucial to inhibit the inflammation.

Later, Carvalho *et al*., 1996 proposed that *trans*-dehydrocrotonin **[59]** from barks of *Croton cajucara* Benth showed a significant inhibition of carrageenan-induced paw edema and cotton pellet granuloma in rats. It also inhibited the writhings in mice induced by acetic acid. The anti-inflammatory effect was observed in the acute and chronic phase of the inflammatory process.

5.5.3 Clerodane diterpenes with anti-tumor activity

Grynberg *et al*., 1999 reported that *trans*-dehydrocrotonin **[59]** from barks of *Croton cajucara* Benth was as effective as 5-FU in anti-tumor effect against ascitic S180 and Ehrlich tumor growth *in vivo* (%T/C 137 of *trans*-dehydrocrotonin, and 140 of 5-FU for S180 and %T/C 128 of *trans*-dehydrocrotonin, and 144 of 5-FU for Ehrlich respectively), when T is the increase in the survival time of treated mice, and C is the increase in the survival time of control group. The anti-tumor activity is dose dependent for both tumors. Furthermore, they suggested that α, β-unsaturated carbonyl moiety is significant for anti-tumor activity. This moiety has been shown to

bind to receptors that induce increased activities of enzyme for metabolizing xenobiotic agents.

5.5.4 Clerodane diterpenes with cytotoxic activity

Casearins A-R **[64-81]**, isolated from the leaves of *Casearia sylvestris* Sw. (Flacourtiaceae), have shown cytotoxic activity against cloned chinese hamster V-79 cells *in vitro* (Morita *et al*., 1991)

In addition, articulin acetate **[63]**, isolated from aerial parts of *Baccharis gaudichaudiana* DC. (Compositae), exhibited significant cytotoxicity against P-338 (murine lymphoid neoplasm) cell, with and ED_{50} value of 1.7 µg/ml (Fullas *et al*., 1994). Roengsumran *et al*. (2002) also found that croblongifolin **[23]**, isolated from stem barks of *Croton oblongifolius* Roxb., showed significant cytotoxicity against human tumor cell lines, when compared with doxorubicin hydrochloride with IC_{50} value as shown in Table 4.

Table 4. Cytotoxicity IC_{50} (µM) data of croblongifolin

Cell lines					
Compounds	**KATO 3**	**SW 620**	**BT 474**	**HEP-G2**	**CHAGO**
croblongifolin [23]	0.35	0.47	0.12	0.35	0.24
doxorubicin HCl	3.00	1.94	0.18	1.59	0.53

5.5.5 Clerodane diterpenes with insect antifeedant activity

The clerodane diterpenes, which have insect antifeedant properties are listed in Table 5.

Table 5. Distribution of natural insect antifeeding clerodane diterpenoids

Compounds	Family	Plant	Plant parts	References
ajugacumbin A, B, and C **[82-84]**	Labiatae	• *Ajuga decumbens* Thunb.	whole plants	Zhi-da *et al*., 1989
jodrellin A **[85]**	Labiatae	• *Scutellaria woronowii* Juz. • *S. violacea* Heyne ex Wall.	whole plants whole plants	Anderson *et al*., 1989 Cole *et al*., 1991
jodrellin B **[86]**	Labiatae	• *Scutellaria woronowii* Juz.	whole plants	Anderson *et al*., 1989
kolavenol **[87]**	Caesalpiniaceae Aristolochiaceae Compositae	• *Hardwickia pinnata* Roxb. • *Aristolochia galeata* Mart. et Zucc. • *Solidago altissima* L. • *Melampodium divaricatum* DC. • *Plazia daphnoides* Wedd.	Oleoresin roots rhizomes leaves aerial parts	Misra *et al*., 1979 Lopes and Bolzani, 1988 Merritt and Ley, 1992 Hubert and Wiemer, 1985 Zdero *et al*., 1988

Compounds	Family	Plant	Plant parts	References
(-)-hardwickiic acid **[18]**	Caesalpiniaceae	• *Hardwickia pinnata* Roxb.	oleoresin	Misra *et al*., 1979
	Compositae	• *Baccharis macraei* Hook.et Arn.	aerial parts	Gambaro *et al*., 1986
		• *Grangea maderaspatana* Poir.	aerial parts	Singh *et al*., 1988
	Euphorbiaceae	• *Croton californicus* Muell. Arg.	whole plants	Merritt and Ley, 1992
		• *C. oblongifolius* Roxb.	stem bark, wood	Aiyar and Seshadri, 1972a
		• *C. aromaticus* L.	roots	Bandara *et al*., 1987
11-dehydro-(-)-hardwickiic acid **[19]**	Euphorbiaceae	• *Croton oblongifolius* Roxb.	stem bark, wood	Aiyar and Seshadri, 1972b
(-)-20-benzyloxy hardwickiic acid **[20]**	Euphorbiaceae	• *Croton oblongifolius* Roxb.	stem bark, wood	Baiagern, 1999
methyl-15,16-epoxy- 12-oxo-3,13(16),14-clerodatriene-20,19-olide-17-oate **[21]**	Euphorbiaceae	• *Croton oblongifolius* Roxb.	stem bark, wood	Tanwattanakun, 1999

Compounds	Family	Plant	Plant parts	References
16α-hydroxy-3,13(14)*Z*-dien-15,16-olide **[88]**	Annonaceae	• *Polyalthia longifolia* Benth. et Hook. f. ex Hook. f.	leaves	Phadnis *et al*., 1988
16-oxocleroda-3,13(14)*E*-dien-15-oic acid **[89]**	Annonaceae	• *Polyalthia longifolia* Benth. et Hook. f. ex Hook. f.	leaves	Phadnis *et al*., 1988

5.5.6 Clerodane diterpenes with insect growth inhibitory effect

Kubo *et al*. (1991) found that *trans*-dehydrocrotonin **[59]** showed an ED_{50} value of 30 ppm against the lepidopteran pest insects, *Pectinophora gossypiella* (pink ball worm) and *Heliothis virescens* (tobacco bud worm).

5.5.7 Clerodane diterpenes with antibacterial activity

(-)- Hardwickiic acid **[18]**, crolechinic acid **[90]**, korberin A **[91]** and korberin B **[92]** were reported as having antibacterial activity. (-)-Hardwickiic acid **[18]** showed activity against gram-positive bacteria (*Bacillus subtilis*) when compare to streptomycin, with a MIC value of 0.78, and 3.12 for 24 hrs, 1.56, and 3.12 for 48 hrs. (McChesney *et al*., 1991). Crolechinic acid **[90]**, korberin A **[91]** and korberin B **[92]** also showed activity against *Bacillus subtilis*, when compare to penicillin V and Chloramphenicol with a MIC value of 0.2, 0.04, 0.05, 0.008, and 0.008 respectively (Chen *et al*., 1994). It is interesting that both korberin A **[91]** and korberin B **[92]** have lactone functionality in their structure which show an activity against *B. subtilis*.

5.5.8 Clerodane diterpenes with anti-hyperglycemic activity

trans-Dehydrocrotonin **[59]** has been reported as having significant anti-hyperglycemic activity in alloxan-induced diabetic rats, at oral dose of 50 mg/kg body weight compare to diabetic controls with a fasting blood glucose levels (mg/dl) value of 131.99 ± 9.17 and 458.71 ± 8.79 respectively. In addition, *trans*-dehydrocrotonin **[59]** (50mg/kg) also effectively lowered the blood sugar levels in glucose fed normal

rats similar to glibenclamide (2mg/kg) but produce more reduction in blood sugar level than glibenclamide (Farias *et al*., 1997; Maciel *et al*. 2000).

5.5.9 Clerodane diterpenes with anti-hyperlipaemia activity

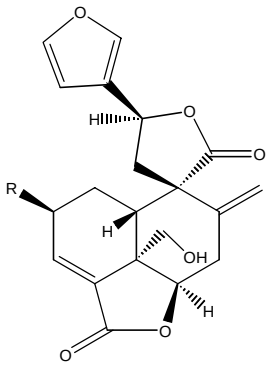
trans-Dehydrocrotonin **[59]** has been reported as having anti-hyperlipaemia activity, by significantly decreased blood levels of total cholesterol and triglycerides in mice treated orally with 25 and 50 mg/kg *trans*-dehydrocrotonin on Triton WR 1339 (tyloxapol)-induced hyperlipaemia compare to gemfibrozil (Silva *et al*., 2001).

5.5.10 Clerodane diterpenes with anti-estrogenic activity

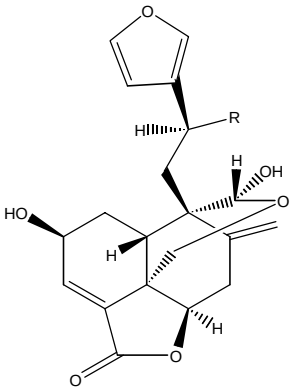
trans-Dehydrocrotonin **[59]** produced significant decrease in mice uterine weight, 49 ± 3, and 54 ± 4 mg/30g body weight of *trans*-dehydrocrotonin and estrogen respectively. It also decreased % of vagina opening, 83% vagina opening when compared to estrogen (100%) (Maciel *et al*., 2000).

5.5.11 Clerodane diterpenes with anti-mutagenic activity

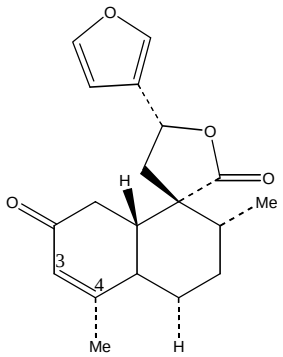
Not only *trans*-dehydrocrotonin **[59]** has activities as list aboved, it has also been reported that it reduced the frequency of induced chromosomal aberrations and micronuclei by cyclophosphamide in the bone marrow cell of mice. It also showed no cytotoxic effects in the bone marrow cells regardless of the route of exposure. This activity may result from inhibition of the enzymatic activation and chemical inactivation of cyclophosphamide that could be involved in the chemoprotective effect of *trans*-dehydrocrotonin **[59]**. Chemical structure of diterpenic lactone provides protective effects on the bone marrow cells instead of harmful effects that could diminish the risk of cancer or other diseases in humans (Agner *et al*., 2001).



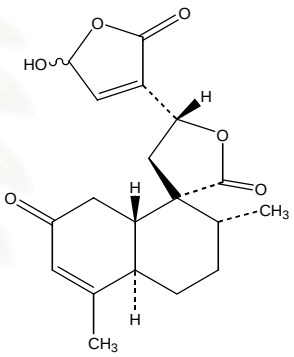
plaunol B **[55]**: R= H
plaunol C **[56]**: R= OH



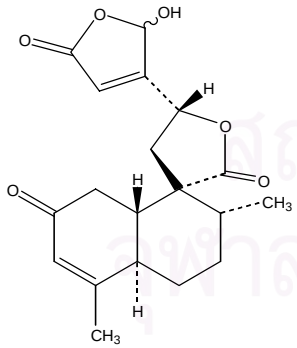
plaunol D **[57]**: R= OH
plaunol E **[58]**: R= OAc



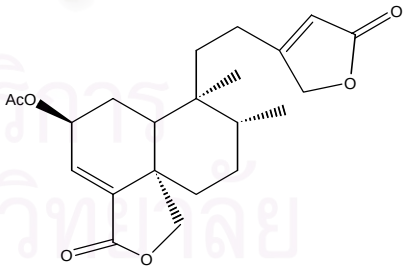
3-4 = double bond: *trans*-dehydrocrotonin **[59]**
3-4 = single bond: *trans*-crotonin **[60]**



cajucarinolide **[61]**

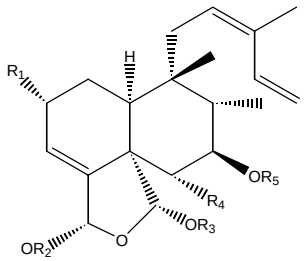


isocajucarinolide **[62]**

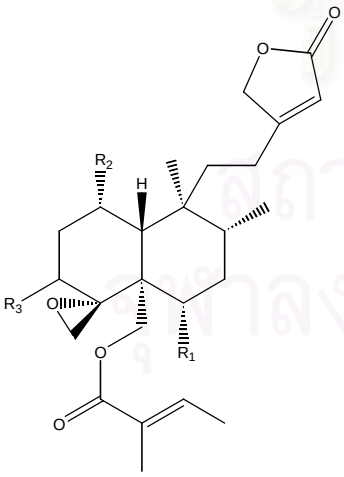


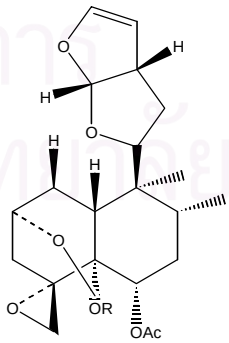
articulin acetate **[63]**

Figure 15: Bioactive clerodane diterpenes



casearin A **[64]**: R_1 = OMe, R_2 = Ac, R_3 = Ac, R_4 = OH, R_5 = Bu
casearin B **[65]**: R_1 = OMe, R_2 = Ac, R_3 = Ac, R_4 = OAc, R_5 = Bu
casearin C **[66]**: R_1 = OH, R_2 = Ac, R_3 = Ac, R_4 = OAc, R_5 = Dc
casearin D **[67]**: R_1 = OH, R_2 = Bu, R_3 = Ac, R_4 = OH, R_5 = Bu
casearin E **[68]**: R_1 = OH, R_2 = Et, R_3 = Ac, R_4 = OH, R_5 = Dc
casearin F **[69]**: R_1 = OH, R_2 = Et, R_3 = Ac, R_4 = OH, R_5 = Bu
casearin G **[70]**: R_1 = OMe, R_2 = Ac, R_3 = Ac, R_4 = H, R_5 = Bu
casearin H **[71]**: R_1 = OH, R_2 = Ac, R_3 = Ac, R_4 = H, R_5 = Bu
casearin I **[72]**: R_1 = OH, R_2 = Ac, R_3 = Bu, R_4 = H, R_5 = Bu
casearin J **[73]**: R_1 = OMe, R_2 = Bu, R_3 = Ac, R_4 = OH, R_5 = Bu
casearin K **[74]**: R_1 = OAc, R_2 = Ac, R_3 = Ac, R_4 = OH, R_5 = Bu
casearin L **[75]**: R_1 = OMe, R_2 = Bu, R_3 = Ac, R_4 = OAc, R_5 = H
casearin M **[76]**: R_1 = OH, R_2 = Bu, R_3 = Bu, R_4 = OAc, R_5 = H
casearin N **[77]**: R_1 = OMe, R_2 = Ac, R_3 = Bu, R_4 = OAc, R_5 = Bu
casearin O **[78]**: R_1 = OMe, R_2 = Bu, R_3 = Ac, R_4 = OAc, R_5 = Bu
casearin P **[79]**: R_1 = OMe, R_2 = Ac, R_3 = Ac, R_4 = OAc, R_5 = Ac
casearin Q **[80]**: R_1 = OH, R_2 = Ac, R_3 = Ac, R_4 = OAc, R_5 = Bu
casearin R **[81]**: R_1 = =O, R_2 = Ac, R_3 = Ac, R_4 = OH, R_5 = Bu
(Bu = butylate, Dc = decanoate)

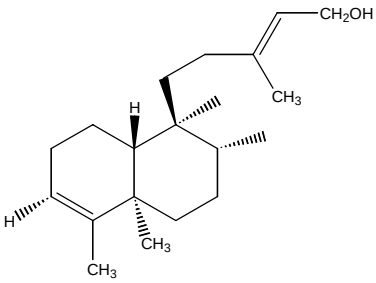




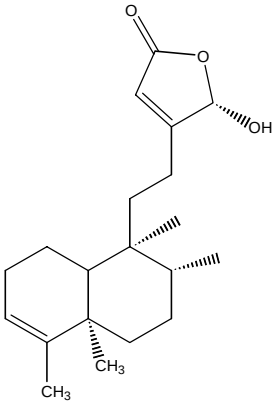
ajugacumbin A **[82]** : R_1 = OAc, R_2 = R_3 = H
ajugacumbin B **[83]** : R_1 = OH, R_2 = R_3 = H
ajugacumbin C **[84]** : R_1 = R_2 = R_3 = OAc

jodrellin A **[85]** : R = Ac
jodrellin B **[86]** : R = $CO^{i}Pr$
($CO^{i}Pr$ = isobutyrate ester)

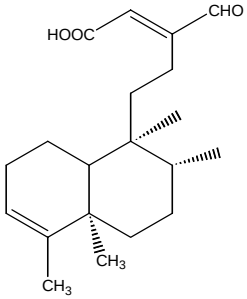
Figure 15: Bioactive clerodane diterpenes (continued)



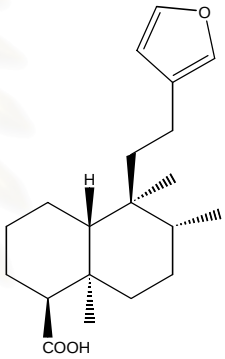
kolavenol **[87]**



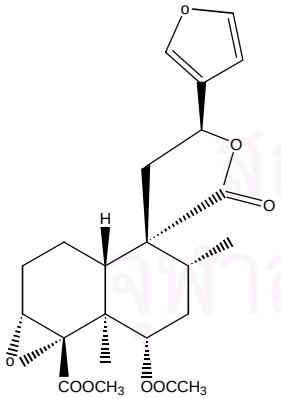
16α-hydroxy-3, 13(14) *Z*-dien-15, 16-olide **[88]**



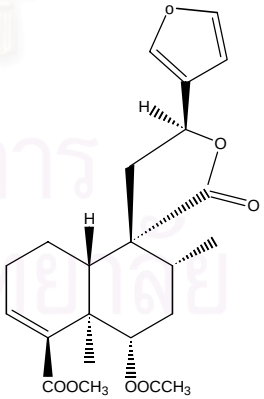
16-oxo-cleroda-3, 13 (14) *E* - diene-15-oic acid **[89]**



crolechinic acid **[90]**



korberin A **[91]**



korberin B **[92]**

Figure 15: Bioactive clerodane diterpenes (continued)

CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The stem barks of *Croton roxburghii*. used in this study were collected from Nong Bua Rawhae district, Chaiyaphum province, Thailand at N 16° 38′ 47.8″ and E 101° 47′ 45.1″ in March 2002. The plant material was authenticated by comparison with the voucher specimen no. BKF 084729, deposited in the herbarium of the Royal Forest Department, Ministry of Agriculture and Co-operatives of Thailand. The dried stem barks (5.5 kg.) were obtained after drying at the temperature of about 50°C and ground for extraction.

2. General Techniques

2.1 Analytical Thin Layer Chromatography (TLC)

Technique : One dimension, ascending

Adsorbent : Silica gel 60 F_{254} precoated plate (E.Merck)

Layer thickness : 0.2 mm.

Developing distance : 5.0 cm.

Temperature : Laboratory room temperature (30-35°C)

Detection : 1. Ultraviolet light at wavelength of 254 and 356 nm

2. Visual detection in iodine vapor.

3. Anisaldehyde-H_2SO_4 reagent and heat at 100-105°C for a few minutes

2.2 Column Chromatography

2.2.1 Conventional Column Chromatography

Adsorbent : 1. Silica gel 60 (No. 7734, E. Merck)
Particle size 0.063-0.200 nm (70-230 mesh ASTM)

2. Silica gel 60 (No. 9385, E. Merck)
Particle size 0.040-0.063 nm (230-400 mesh ASTM)

Packing method : Wet packing

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

Detection : Fractions were examined using TLC technique. In order to detect the compounds in each of the fractions, the TLC plate was observed under UV light at wavelength of 254 nm, and then exposed to iodine vapor and anisaldehyde-H_2SO_4 reagent, respectively. Fractions of similar chromatographic pattern were combined

2.2.2 Flash Column Chromatography

Adsorbent : 1. Silica gel 60 (No. 7734, E. Merck)
Particle size 0.063-0.200 nm (70-230 mesh ASTM)
2. Silica gel 60 (No. 9385, E. Merck)
Particle size 0.040-0.063 nm (230-400 mesh ASTM)

Packing method : The adsorbent was wet-packed after being suspended in eluent. The slurry of adsorbent was poured into the column, tapped and pressed down under air pump, then allowed to settle.

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

Detection : Fractions were examined in the same manner as described in section 2.2.1.

2.2.3 Vacuum Liquid Column Chromatography

Adsorbent : 1. Silica gel 60 (No. 7734, E. Merck)
Particle size 0.063-0.200 nm (70-230 mesh ASTM)
2. Silica gel 60 (No. 9385, E. Merck)
Particle size 0.040-0.063 nm (230-400 mesh ASTM)

Packing method : Dry packing. The absorbent was poured into the column and then the top surface was adjusted by spreading and pressing. Eluent was eluted into column and sucked with vacuum pump to allow homogeneous setting of silica gel particles and then smoothened the top surface again.

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

Detection : Fractions were examined in the same manner as described in section 2.2.1.

2.3 Spectroscopic Techniques

2.3.1 Ultraviolet (UV) Absorption Spectra

UV spectra were obtained from a Shimadzu UV-160A UV/VIS spectrophotometer and a Milton Roy spectronic 3000 Array spectrophotometer at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.3.2 Infrared (IR) Absorption Spectra

IR spectra were recorded on a Perkin-Elmer 2000 FT-IR 1760X spectrometer at the Scientific and Technological Research Equipment Center, Chulalongkorn University.

2.3.3 Mass Spectra (MS)

FAB (+) MS of compound A-1 and A-2 was obtained at the Department of Medicinal Organic Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan.

2.3.4 Nuclear Magnetic Resonance (NMR) Spectra

^{1}H NMR, ^{13}C NMR, DEPT 135, 2D-NMR (HMQC and HMBC) spectra of isolated compounds were record at 400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR, and 500 MHz for HMQC and HMBC , on a JEOL JMN (Alpha series) Spectrometer at the Department of Medicinal Organic Chemistry, Faculty of Pharmaceutical Sciences, Chiba University, Chiba, Japan.

^{1}H and ^{1}H COSY, ^{1}H and ^{1}H NOESY, spectra of isolated compounds were recorded at 300 MHz, on a Bruker ADVANCE DPX-300 FT-NMR spectrometer at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Deuterated choloroform (chloroform-*d*) was used as the NMR solvent throughout this study. Spectral data were reported in ppm scale using the solvent chemical shift as the reference frequency. Proton detected heteronuclear correlations were measured using HMQC (optimized for $^{n}J_{HC}$ = 145 Hz) and HMBC (optimized for $^{n}J_{HC}$ = 4 and 8 Hz)

2.4 Physical Property Measurement

2.4.1 Melting Points

Melting points were determined on a Gallenkamp Melting Point Apparatus at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.4.2 Optical Rotations

Optical rotations were measured on a Perkin-Elmer Polarimeter model 341 using a sodium lamp operation at 589 nm at the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2.5 Solvents

Organic solvents used in extraction were of commercial grade. In column chromatography, solvents were redistilled prior to use.

3. Extraction and Isolation

3.1 Extraction of the stem bark of *Croton roxburghii* N.P. Balakr.

The dried-powdered stem bark of *Croton roxburghii* (5.5 kg.) was macerated three times, each time for three days with hexane (3x10L.), ethyl acetate (3x10L.) and then acetone (3x10L.), successively. The obtained extracts were evaporated under reduced pressure at a temperature of approximately 40°C to give 171 g of hexane extract (3.11% of dry weight of the stem bark), 459 g of ethyl acetate extract (8.34% of dry weight of the stem bark) and 800 g of acetone extract (14.55% of dry weight of the stem bark).

3.2 Isolation

The crude ethyl acetate extract (60.40g) was chromatographed on a vacuum silica gel liquid column: using quick column technique (silica gel 60,

No. 7734, 200g), eluted with hexane, ethyl acetate, chloroform and acetone to yield 35 fractions of 200 ml each. The total of 4 fractions obtained according to each of the eluents used as shown in **Table 6**.

Table 6. Combination of fractions from vacuum liquid column chromatography of the crude ethyl acetate extract (60.4 g)

Eluents	Fraction code	Number of fraction	Weight (g)
Hexane	F001	1-8	1.13
EtOAc	F002	9-22	56.70
$CHCl_3$	F003	23-26	0.22
Acetone	F004	27-35	2.20

Fraction F002 (56.70g) was further chromatographed on a conventional silica gel column (silica gel 60, No. 7734), eluted with chloroform-methanol mixtures of increasing polarity (1-40% MeOH in $CHCl_3$) to yield 75 fractions of approximately 30 ml each. Fractions showing similar TLC patterns in 8% methanol in $CHCl_3$ were combined to give a total eleven fractions, as shown in **Table 7**.

Table 7. Further fractionation of fraction F002 (56.70 g) by column chromatography

Eluents	Fraction code	Number of fraction	Weight (g)
100% $CHCl_3$	F005	1-3	3.56
1% MeOH in $CHCl_3$	F006	4-7	2.45
2% MeOH in $CHCl_3$	F007	8-10	4.28
3% MeOH in $CHCl_3$	F008	11-13	5.43
5% MeOH in $CHCl_3$	F009	14-17	5.23
8% MeOH in $CHCl_3$	F010	18-36	12.38
12% MeOH in $CHCl_3$	F011	37-39	0.32
16% MeOH in $CHCl_3$	F012	40-42	0.82
20% MeOH in $CHCl_3$	F013	43-49	2.50
40% MeOH in $CHCl_3$	F014	50-62	7.17
100% MeOH	F015	63-75	6.83

Fraction F010 (12.38 g) was subjected to flash column chromatography over silica gel 60 (No. 9385) using chloroform-methanol mixtures of increasing polarity (1-15 % MeOH in $CHCl_3$) as the mobile phase. Eluate was collected at approximately 30 ml per fraction and then examined by TLC using 8% methanol in $CHCl_3$ as the developing solvent. Fractions showing similar TLC patterns were combined to give a total of nine fractions, as shown in **Table 8**.

Table 8. Further fractionation of F010 (12.38 g) by flash column chromatography

Eluents	Fraction code	Number of fraction	Weight (g)
100% $CHCl_3$	F016	1-5	0.45
1% MeOH in $CHCl_3$	F017	6-12	1.34
2% MeOH in $CHCl_3$	F018	13-17	1.28
3% MeOH in $CHCl_3$	F019	18-20	1.03
5% MeOH in $CHCl_3$	F020	21-28	3.38
8% MeOH in $CHCl_3$	F021	29-33	2.06
10% MeOH in $CHCl_3$	F022	34-39	0.92
15% MeOH in $CHCl_3$	F023	40-44	0.82
100% MeOH	F024	45-52	1.98

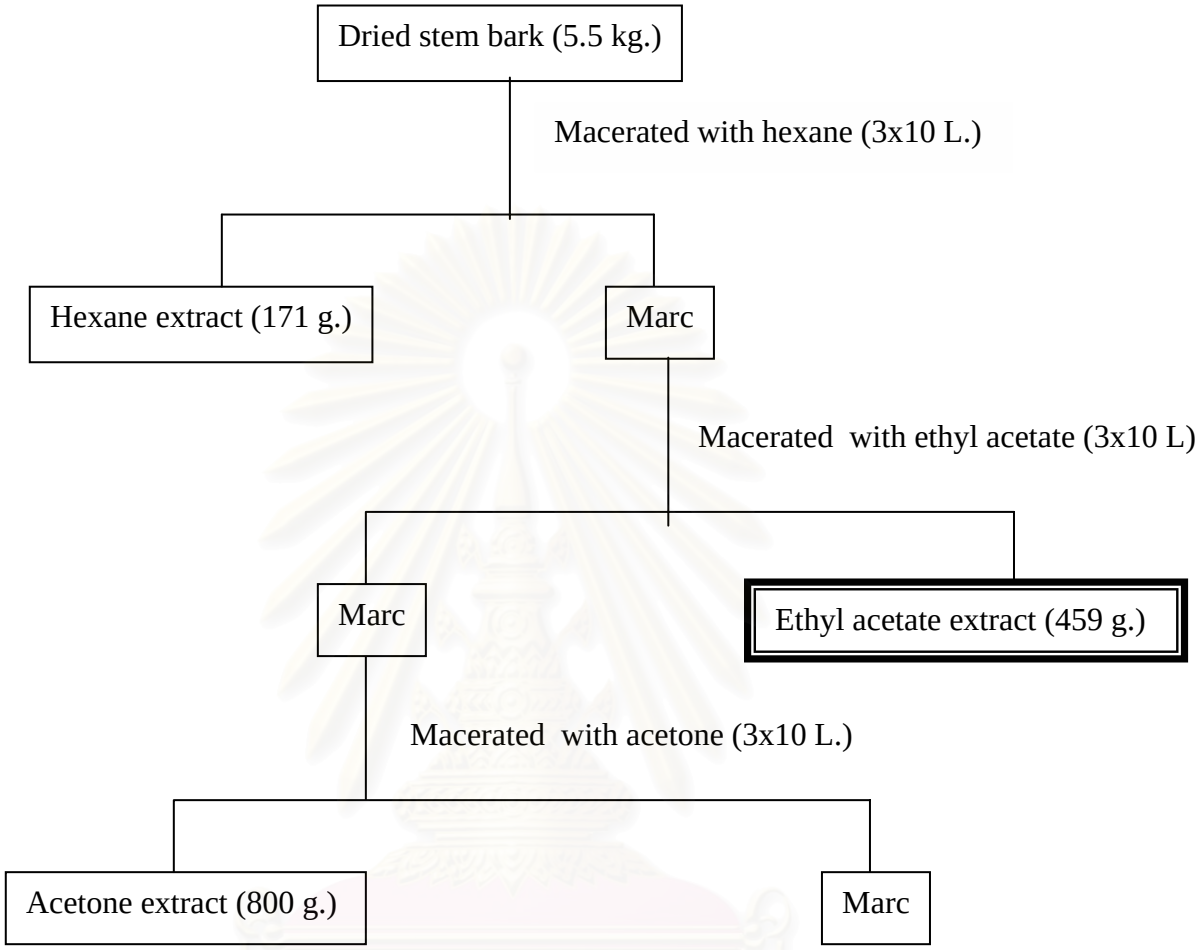
Fraction F020 (3.38 g) was again rechromatographed on a flash silica gel column (silica gel 60, No.9385) and eluted with 5% MeOH in $CHCl_3$. Fractions (approximately 30 ml each) were combined according to their TLC pattern to give a total of six fractions as described in **Table 9**. Fractions F027 and F028 was recrystallized by adding approximately equal volume of F027, F028 and diethyl ether, then sonicated for 5 minutes for dissolving. The fine white powder of compound A-1 (0.3548 g) and compound A-2 (0.1789 g) occurred respectively. Compound A-1 and A-2 were then recrystallized again with ethyl acetate, prism crystal of compound A-1, and needle crystal of compound A-2 were obtained.

Table 9. Further fractionation of F020 (3.38 g) by flash column chromatography using isocratic eluents (5% MeOH in chloroform).

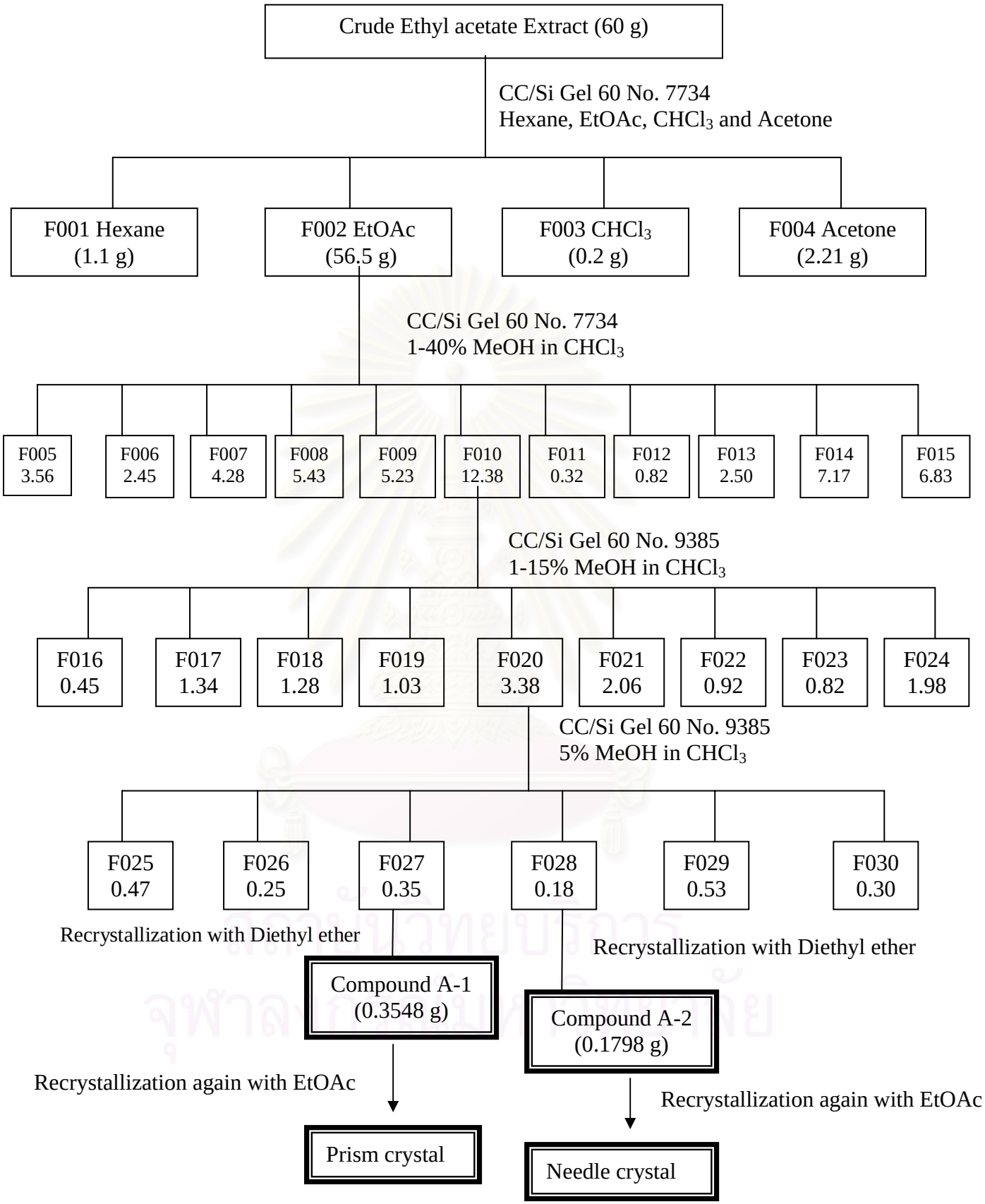
Fraction code	Number of fraction	Weight (mg)
F025	1-10	466.80
F026	11-15	246.80
F027	16-23	354.80*
F028	24-27	178.90**
F029	28-33	530.70
F030	34-40	298.40

Remark: * = Compound A-1

** = Compound A-2



Scheme 1. Extraction scheme of the stem bark of *Croton roxburghii* N.P.Balakr.



Scheme 2. Isolation scheme of the ethyl acetate extract of *Croton roxburghii* N.P.Balakr.

4. Physical and Spectral Data of the Isolated Compounds

4.1 Compound A-1

Compound A-1 was obtained as prism crystal

Melting point : 152-153ºC

$[\alpha]^{25}_{D}$: -10º ($CHCl_3$; *c* 0.01 g/ml)

UV : λ_{max} nm (log ε), in MeOH; **Figure 20**

210 (4.09)

IR : υ_{max} cm^{-1}, KBr disc; **Figure 21**

3436, 2965, 2903, 1809, 1728, 1629

EIMS : *m/z* (% relative intensity); **Figure 22**

336 [M^+] (4), 319 (28), 301 (34), 289 (11), 154 (100)

^{1}H-NMR : δ ppm, 400 MHz, in $CDCl_3$; **Figure 23**

5.84 (1H, dd, *J* = 3.5, 6.0 Hz), 4.75 (2H, d, *J* = 1.8 Hz), 3.58 (1H, d, *J* = 2.5 Hz), 2.33 (1H, ddd, *J* = 3.7, 14.6, 14.7 Hz), 2.24 (1H, ddd, *J* = 4.8, 14.3, 14.5 Hz), 2.00 (1H, tdd, *J* = 4.0, 13.7, 13.7 Hz), 1.76 (1H, dd, *J* = 1.8, 12.3 Hz), 1.69 (1H, m), 1.66 (1H, s), 1.58 (1H, d, *J* = 3.5 Hz), 1.50 (1H, dd, *J* = 2.8, 2.8 Hz), 1.48 (1H, m), 1.41 (1H, s), 1.40 (1H, s), 1.39 (1H, m), 1.26 (3H, s), 1.24 (1H, br s), 1.14 (3H, s), 0.80 (3H, s), 0.79 (3H, s)

^{13}C-NMR : δ ppm, 100 MHz, in $CDCl_3$; **Figure 24**

174.15 (s), 171.29 (s), 114.93 (d), 76.28 (d), 76.26 (s), 73.14 (t), 41.32 (s), 40.73 (d), 38.65 (d), 36.19 (d), 35.59 (t), 32.28 (t), 30.44 (t), 26.38 (t), 22.44 (t), 21.62 (q), 18.15 (q), 17.16 (q), 16.45 (t), 15.98 (q)

4.2 Compound A-2

Compound A-2 was obtained as needle crystal

Melting point : 231-232ºC

$[\alpha]^{25}_{D}$: -57º ($CHCl_3$; *c* 0.01 g/ml)

UV : λ_{max} nm (log ε), in MeOH; **Figure 30**

209 (4.29)

IR : υ_{max} cm^{-1}, KBr disc; **Figure 31**

3552, 2952, 2882, 1786, 1732, 1637

EIMS : *m/z* (% relative intensity); **Figure 32**

395 $[M^+ + H^+]$ (2), 377 (7), 317 (13), 307 (19), 192 (29), 154 (100)

^{1}H-NMR : δ ppm, 400 MHz, in $CDCl_3$; **Figure 33**

5.92 (1H, br s), 5.35 (1H, dd, *J* = 1.5, 11.0 Hz), 4.89 (1H, dd, *J* = 1.8, 17.3 Hz), 4.76 (1H, dd, *J* = 1.5, 17.5 Hz), 3.60 (1H, s), 2.75 (1H, d, *J* = 15.5 Hz), 2.64 (1H, dd, *J* = 11.0, 15.5 Hz), 2.02 (3H, s), 2.08 (1H, m), 1.93 (1H, dd, *J* = 2.0, 12.5 Hz), 1.80 (1H, m), 1.69 (1H, m), 1.68 (1H, m), 1.56 (1H, dd, *J* = 3.8, 7.8 Hz), 1.51 (1H, m), 1.45 (1H, m), 1.39 (1H, dd, *J* = 3.0, 11.5 Hz), 1.33 (1H, m), 1.13 (3H, s), 1.26 (3H, s), 0.92 (3H, d, *J* = 6.5 Hz), 0.79 (3H, s)

^{13}C-NMR : δ ppm, 100 MHz, in $CDCl_3$; **Figure 34**

173.62 (s), 170.83 (s), 167.20 (s), 117.46 (d), 76.75 (d), 76.30 (d), 76.16 (s), 73.03 (t), 43.95 (s), 41.60 (s), 40.35 (d), 37.31 (d), 31.95 (t), 30.10 (t), 29.82 (t), 26.95 (t), 21.81 (q), 20.92 (q), 17.93 (t), 16.96 (q), 16.61 (q), 13.08 (q)

5. **Cytotoxicity test**

Cytotoxicity test was carried out at the Institute of Biotechnology and Genetic Engineering. Bioassay of cytotoxic activity against human tumor cell culture *in vitro* was performed by the MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method (Carmichael *et al*., 1987; Twentyman and Luscombe, 1987). In principle, the viable cell number/well is directly proportional to the production of formazan which, following solubilization, can be measured spectrophotometrically.

The human tumor cell line was harvested from exponential-phase maintenance culture (T-75 cm^2 flask), counted by tryptan blue exclusion, and dispensed within replicate 96-well culture plates in 100 µl volumes using a repeating pipette, following at 24-h incubation at 37°C, with 5% CO_2, 100% relative humidity and 100 µl of culture medium. Culture medium containing sample was dispensed within appropriate wells (control group, N=6; each sample treatment group, N=3). Peripheral wells of each plate (lacking cells) were utilized for sample blank (N=2) and medium/tetrazolium reagent blank (N=6) "background" determinations. Culture plates were then incubated for 4 days prior to the addition of tetrazolium reagent. MTT stock solution was prepared as follows: 5 mg MTT/ml PBS was sterile and filtered with 0.45 µm filtered units. MTT working solution was prepared just prior to culture application by diluting MTT stock solution 1:5 (v/v) in pre-warmed standard culture medium. MTT working solution (50 µl) was added to each culture well resulting in 50 µg MTT/250 µl total medium volume and cultures were incubated at 37°C for 4 to 24h depending upon individual cell line requirements. Following incubation, cell monolayer and formazan were inspected microscopically: culture plates containing suspension lines or any detached cells were centrifuged at low speed for 5 min. All 10-20 µl of culture medium supernatant was removed from wells by slow aspiration through a blunt 18-guage needle and replaced with 150 µl of DMSO using a pipette. Following through formazan solubilization, the absorbance of each well was measured using a microculture plate reader at 540 nm (single wavelength, calibration factor = 1.00)

Samples were tested for cytotoxic activity towards 5 cancer cell lines, including HEP-G2 (hepatoma), SW 620 (colon), Chago (lung), Kato-3 (gastric) and BT 474 (breast), following the experimental method for bioassay of cytotoxic activity.

CHAPTER IV

RESULTS AND DISCUSSION

By means of several chromatographic techniques, two compounds: A-1 and A-2, were isolated from crude ethyl acetate extract of the stem bark of *Croton roxburghii* N.P. Balakr.

Spectroscopic data (UV, IR, MS and NMR) were used to determine the chemical structures of the two compounds. The structures were also confirmed by comparative analysis using previous reports as references.

Structure Determination of the Isolated Compounds

1. Structure determination of compound A-1

Compound A-1 was obtained as prism crystal (0.3548 g) with a melting point of 152-153 ºC.

The FT-IR spectrum of compound A-1 (Figure 21) displayed bands indicating olefinic group (1629 cm^{-1}), carbonyl group (1728 cm^{-1}), and hydroxyl group (3436 cm^{-1})

Table 10. The IR absorption band assignments of compound A-1

Wave number (cm^{-1})	**Tentative assignments**
3436	O-H stretching
2965, 2903	alkane C-H stretching (CH_2, CH_3)
1728	C=O stretching
1629	C=C streching

The ^{1}H-NMR spectrum (Figure 23) of compound A-1 showed four methyl groups at δ_H 1.26 (3H, *s*; H-20), 1.14 (3H, *s*; H-18), 0.80 (3H, *s*; H-17), and 0.79 (3H, *s*; H-19) respectively. Three of methyl groups, at δ_H 1.26, 1.14, and 0.79 attached to quaternary carbons. The ^{1}H-NMR spectrum also showed one olefinic proton at δ_H 5.84 (1H, *dd*, *J* = 3.5, 6.0 Hz; H-14), and two downfield signals at δ_H 4.75 (2H, *d*, *J* =1.8 Hz; H-16), and δ_H 3.58 (1H, *d*, *J* =2.5; H-3).

The ^{13}C-NMR spectrum (Figure 24) of compound A-1 showed twenty carbon resonances, two of which are olefinic carbons (δ_C 114.93, and 171.29) and one ester carbonyl carbon (δ_C 174.15) was also observed.

In DEPT experiment (Figure 25), one sp^2 methine carbon signals (δ_C 114.93) was observed together with three saturated methine carbon signals (δ_C 36.19, 40.73, and 76.28), the downfield δ_C 76.28 signal should be attached to an oxygen atom in the molecule. Seven methylene carbon signals at δ_C 16.45, 22.44, 26.38, 30.44, 32.28, 35.59, and 73.14 were shown, the downfield δ_C 73.14 signal also indicated proximity to an oxygen atom in the molecule. Four methyl signals resonated at δ_C 15.98, 17.16, 18.15, and 21.62 respectively. According to the ^{13}C-NMR and DEPT experiment, it could be concluded that there were five quaternary carbons (δ_C 38.65, 41.32, 76.26, 171.29, and 174.15) in this structure.

The downfield signal at δ_C 76.26, and 76.28 should be the resonances of the carbons which are attached to oxygen atoms, and it may confirm the presence of hydroxyl groups in the molecule.

In the FAB (+) MS spectrum (Figure 22), compound A-1 gave a molecular ion peak $[M+Na]^+$ at *m/z* 359, consistent with the molecular formular $C_{20}H_{32}O_4$.

The molecular formula of compound A-1 was assigned as $C_{20}H_{32}O_4$ based on ^{1}H, ^{13}C NMR spectra (Table 11) and FAB (+) MS. The IR stretching indicated conjugated carbonyl group at 1728 cm^{-1}, olefenic groups at 1629 cm^{-1}, and hydroxyl group at 3436 cm^{-1}. The ^{13}C NMR spectrum and DEPT experiments reveal the presence of 20 non-equivalent carbons, of which 19 are sp^3 (four methyl, seven methylene, three methine and five quarternary carbons) and one sp^2 methine carbon), hybridized carbons, together with a carbonyl carbon (δ_C 174.15), one double bond group (δ_C 114.93) and three oxygenated carbons (δ_C 76.28, 76.26, and 73.14). The molecular formula $C_{20}H_{32}O_4$ of compound A-1 defined a degree of unsaturation of five; therefore, compound A-1 should consist of three rings in addition to one double bond and one carbonyl group. Several 2D-NMR techniques were then used to assist in the interpretation of the structure of this compound. All of the proton-proton spin systems were traced by using data from a COSY experiment (Figure 28).

Heteronuclear correlation experiments, HMQC (Figure 26) and HMBC (Figure 27) allowed unambiguous assignment of all ^{1}H-NMR and ^{13}C-NMR resonances in compound A-1.

From HMBC spectrum, methyl group at 1.14 ppm (H-18) correlated with saturated methine carbon at 76.28 ppm (C-3), and quaternary carbon at 76.26 ppm (C-4). So it can implies that this methyl group (H-18) should connect to C-4. The carbon atom which attached with hydroxyl group (δ_C 76.28, 76.26 ppm) should be C-3, and C-4. The methylene carbon at 32.28 ppm should be C-2 with confirmed by the COSY spectrum that showed correlated between H-2 and H-3. The HMBC spectrum also showed that methyl group at 0.79 ppm (H-19) correlated with saturated methine carbon at 40.73 ppm (C-10). Moreover, the NOESY spectrum (Figure 29) showed that this methyl group correlated with methyl group at 1.14 ppm (H-18). Thus the C-19 methyl group must connect to C-5. Moreover, H-17 also correlated with H-8, it can conclude that methyl group at C-17 must be connected with C-8.

Furthermore, proton at 5.84 ppm (H-14) correlated with quaternary carbons at 171.29 ppm (C-13) and 174.15 ppm (C-15), thus sp^2 methine carbon at 114.93 ppm (C-14) must be located between C-13 and C-15. In addition, proton at 4.75 ppm (H-16) also correlated with quaternary carbons at 171.29 ppm (C-13) and 174.15 ppm (C-15), it means that methylene carbon at 73.14 ppm (C-16) which is the carbon bearing an oxygen atom should connect to C-13, and this ring known as “butenolide ”.

The HMBC spectrum also showed that proton at 2.24 ppm (H-12) correlated with quaternary carbons at 171.29 ppm (C-13) and methylene carbon at 35.59 ppm (C-11). Thus, it can confirm the position of C-12.

The confirmation of C-11 position can be supported by the HMBC spectrum data, the correlation are described as follows; proton at 1.50 ppm, and 1.66 ppm (H-11) correlated with saturated methine carbons at 40.73 ppm (C-10), and 36.19 ppm (C-8), quaternary carbon at 38.65 ppm (C-9), methylene carbon at 22.44 ppm (C-12), and quarternary carbon at 171.29 ppm (C-13). Therefore C-11 must locate between C-9 and C-12, and C-11 must be connected to C-12 with confirmed by the COSY spectrum that showed correlated between H-11 and H-12.

From the comparison of the ^{13}C, and ^{1}H NMR data of compound A-1 with those of the previously reported structure of 3*α*, 4*β*-dihydroxy-5*β*, 10*β* *cis*-17*α*, 20*α*-cleroda-13(14)-en-15, 16-olide (Fang, N. *et al*., 1988) (Table 12) and the relative stereochemistry of compound A-1 was established by X-ray crystallography (Figure 17). It was deduced that the methyl group at C-19 which attached to C-5 is *α* in stead of *β*.

Therefore, the compound A-1 was determined to be as 3*α*, 4*β*-dihydroxy-5*α*, 10*β* *trans*-17*α*, 20*α*-cleroda-13(14)-en-15, 16-olide (Figure 16)

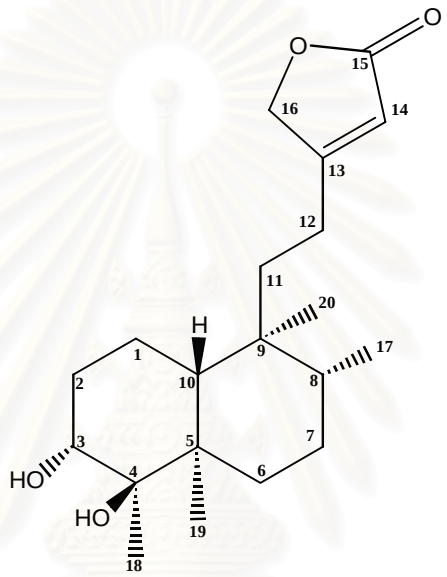


Figure 16. Structure of compound A-1

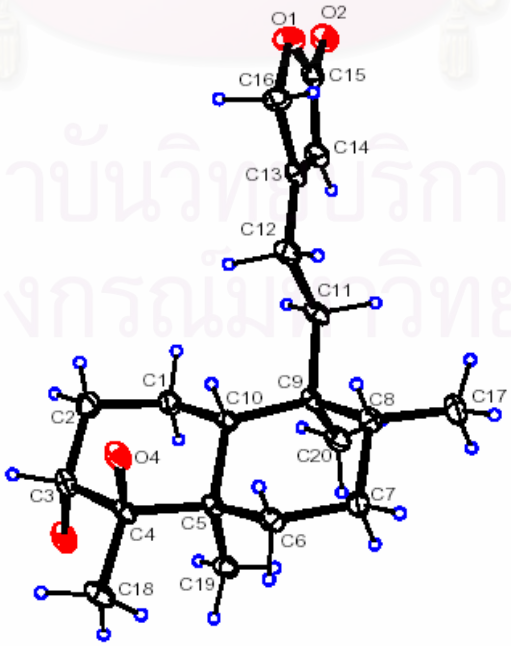


Figure 17a. ORTEP structure of compound A-1

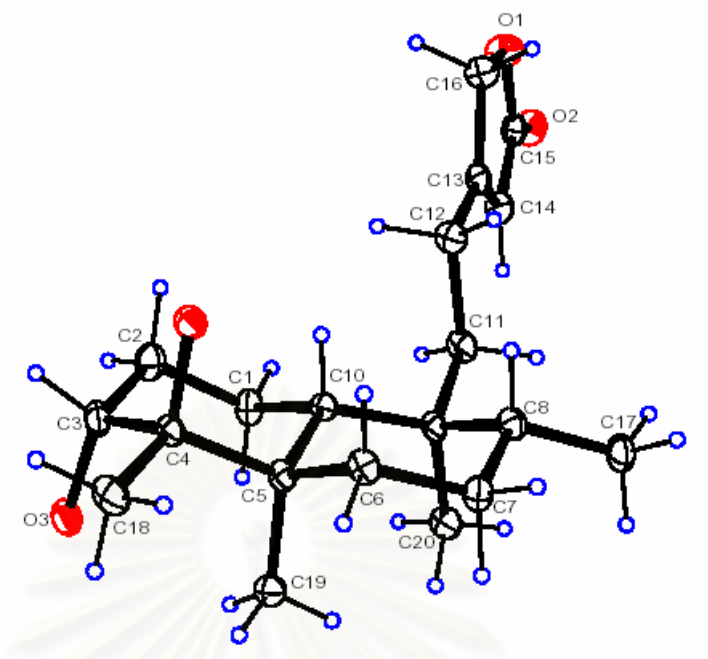


Figure 17b. ORTEP structure of compound A-1 (chair form)

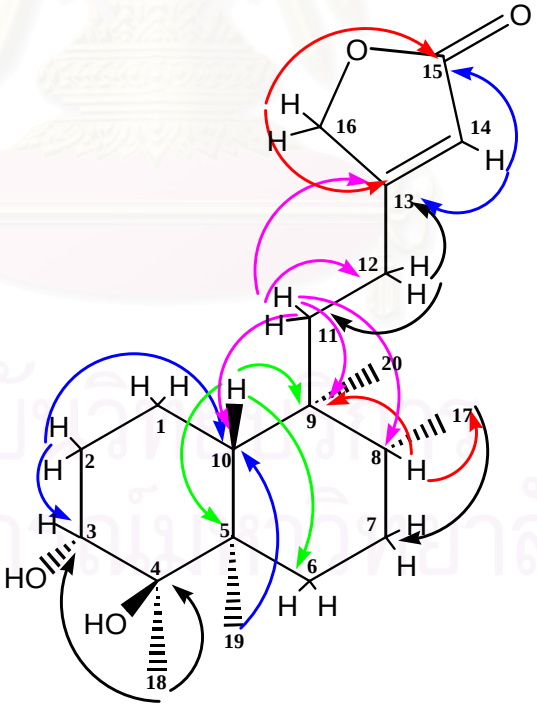


Figure 18. Long-range correlation from HMBC spectrum of compound A-1

Table 11. ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, NOESY and HMBC spectral data of compound A-1

		δ_C (ppm)	δ_H (ppm) (multiplicity, *J* in Hz)	^{1}H-^{1}H COSY	NOESY	HMBC
1	CH_2	16.45	1.24, 1H, *m* 1.62, 1H, *m*	-	-	-
2	CH_2	32.28	1.40, 1H, *m* 1.58, 1H, *d* (3.5)	H-3,H-1	H-3	C-3, C-10
3	CH	76.28	3.58, 1H, *d* (2.5)	H-2	H-2	-
4	>C<	76.26	-	-	-	-
5	>C<	41.32	-	-	-	-
6	CH_2	30.44	1.69, 1H, *m* 2.00, 1H, *tdd* (4.0, 13.7, 13.7)	-	-	-
7	CH_2	26.38	1.39, 1H, *m* 1.48, 1H, *m*	-	-	-
8	CH	36.19	1.41, 1H, *m*	H-17	-	C-9, C-17
9	>C<	38.65	-	-	-	-
10	CH	40.73	1.76, 1H, *dd* (1.8, 12.3)	-	-	C-5, C-6, C-9
11	CH_2	35.59	1.50, 1H, *dd* (2.8, 2.8) 1.66, 1H, *m*	H-12	-	C-8, C-9, C-10, C-12, C-13
12	CH_2	22.44	2.24, 1H, *ddd* (4.8, 14.3, 14.5) 2.33, 1H, *ddd* (3.7, 14.6, 14.7)	H-11	-	C-11, C-13, C-14
13	>C=	171.29	-	-	-	-
14	CH	114.93	5.84, 1H, *dd* (3.5, 6.0)	-	-	C-13, C-15
15	C=O	174.15	-	-	-	-
16	CH_2	73.14	4.75, 2H, *d* (1.8)	-	-	C-13, C-15
17	CH_3	15.98	0.80, 3H, *s*	H-8	-	C-7
18	CH_3	17.16	1.14, 3H, *s*	-	H-19	C-3, C-4
19	CH_3	18.15	0.79, 3H, *s*	-	H-18	C-10
20	CH_3	21.62	1.26, 3H, *s*	-	-	-

Table 12. ^{13}C NMR data of compound A-1 and 3*α*, 4*β*-dihydroxy-5*β*, 10*β cis*-17*α*, 20*α*-cleroda-13(14)-en-15, 16-olide

Position	δ_C Compound A-1 (ppm)	δ_C 3*α*, 4*β*-dihydroxy-5*β*, 10*β cis*-17*α*, 20*α* -cleroda-13(14)-en-15, 16-olide (ppm)
1	16.45	17.9
2	32.28	28.9
3	76.28	76.0
4	76.26	76.8
5	41.32	42.4
6	30.44	28.7
7	26.38	27.7
8	36.19	36.7
9	38.65	38.9
10	40.73	42.2
11	35.59	33.7
12	22.44	23.6
13	171.29	171.9
14	114.93	114.7
15	174.15	174.2
16	73.14	73.1
17	15.98	15.7
18	17.16	28.7
19	18.15	21.0
20	21.62	21.9

2. Structure determination of compound A-2

Compound A-2 was obtained as needle crystal (0.1789 g.) with a melting point of 231-232ºC

The FT-IR spectrum of compound A-2 (Figure 31) displayed bands indicating an olefinic group (1637 cm^{-1}), carbonyl group (1732 cm^{-1}), and hydroxyl group (3552 cm^{-1}).

Table 13. The IR absorption band assignments of compound A-2

Wave number (cm^{-1})	**Tentative assignments**
3552	O-H stretch
2952, 2882	alkene C-H stretch (CH_2, CH_3)
1732	C=O stretch
1637	C=C stretching

The ^{1}H-NMR spectrum (Figure 33) of compound A-2 showed five methyl groups at δ_H 0.79 (3H, *s*; H-19), 0.92 (3H, *d*, *J*=6.5; H-17), 1.13 (3H, *s*; H-18), 1.26 (3H, *s*; H-20), 2.02 (3H, *s*; H-22), and one olefinic proton at δ_H 5.92 (1H, *br s*; H-14). Three of methyl groups at δ_H 1.13 (3H, *s*; H-18), 1.26 (3H, *s*; H-20), and 0.79 (3H, *s*; H-19) attached to a quaternary carbon, and one methyl group attached to methine carbon at δ_H 0.92 (3H, *d*, *J*=6.5; H-17), another one methyl group belonged to acetyl group at δ_H 2.02 (3H, *s*; H-22).

The ^{13}C-NMR spectrum (Figure 34) of compound A-2 showed twenty-two carbon resonances, two of which are olefinic carbons (δ_C 117.46, and 167.20). The presence of two ester carbonyls (δ_C 173.62, and 170.83) were also observed.

In a DEPT experiment (Figure 35) one sp^2 methine carbon signals (δ_C 117.46) together with four methine carbon signal (δ_C 37.31, 40.35, 76.30, and 76.75). The two most downfield methine signals is the result of their proximity to an oxygen atom in the molecule. Six methylene carbon signals at δ_C 17.93, 26.95, 29.82, 30.10, 31.95,

and 73.03 were shown. The downfield δ_C 73.03 signal implied that this methylene carbon should be attached to an oxygen atom. Five methyl signals resonated at 13.08, 16.61, 16.96, 20.92, and 21.81. According to the ^{13}C-NMR, DEPT experiment it could be concluded that there were four quaternary carbons (δ_C 41.60, 43.95, 76.16, and 167.20 ppm) in this structure.

In the FAB (+) MS spectrum (Figure 32), compound A-2 gave a molecular ion peak $[M+H]^+$ at *m/z* 395, and $[M+K]^+$ at *m/z* 433 consistent with the molecular formula $C_{22}H_{34}O_6$. Its mass spectrum exhibited a peak at *m/z* 377 corresponding to $[M^+ - OH^-]$ indicated the loss of a hydroxyl group from the molecule.

The molecular formula of compound A-2 was assigned as $C_{22}H_{34}O_6$ base on ^{1}H, ^{13}C NMR spectra (Table 14) and FAB (+) MS. The IR stretching indicated conjugated carbonyl group at 1786 cm^{-1}, and 1732 cm^{-1}, olefenic groups at 1637 cm^{-1}, and hydroxyl group at 3552 cm^{-1}. The ^{13}C NMR spectrum and DEPT experiments reveal the presence of 22 non-equivalent carbons, of which 19 are sp^3 (five methyl, six methylene, four methine and four quarternary carbons) and one sp^2 (one methine carbon), hybridized carbons, together with two carbonyl carbons (δ_C 173.62 and 170.83), one double bond group (δ_C 117.46) and four oxygenated carbons (δ_C 76.75, 76.30, 76.16 and 73.03). The molecular formula $C_{22}H_{34}O_6$ of compound A-2 defined a degree of unsaturation of six; therefore, compound A-2 should consist of three rings in addition to one double bond and two carbonyl groups. Several 2D-NMR techniques were then used to assist in the interpretation of the structure of this compound. All of the proton-proton spin systems were traced by using data from a COSY experiment (Figure 38). Heteronuclear correlation experiments, HMQC (Figure 36) and HMBC (Figure 37) allowed unambiguous assignment of all ^{1}H-NMR and ^{13}C-NMR resonances in compound A-2.

The ^{1}H-NMR and ^{13}C-NMR spectra of compound A-2 were similar to those of compound A-1 (Table 15), except at methine proton on the carbon bearing acetoxy group of H-11 [δ_H = 5.35 ppm (*dd*, *J* = 1.5, 11.0 Hz)] and at oxygenated carbon of C-11 (δ_C = 76.75 ppm). Moreover there were two additional carbons signals: one carbonyl carbon signal at 170.83 ppm (C-21), and one methyl carbon signal at 20.92 ppm (C-22).

From the HMBC spectrum, the saturated methine proton at 5.35 ppm (H-11) correlated with carbonyl carbon at 170.83 ppm (C-21), and the methyl group at 2.02 ppm (H-22) also correlated with this carbonyl carbon (C-21) at 170.83 ppm. Therefore the acetoxy group must connect to an oxygenated carbon at C-11. The compound A-2 was assigned as 11-acetoxy-3*α*, 4*β*-dihydroxy-5*α*, 10*β*-*trans*-17α, 20*α*-cleroda-13 (14)-en-15, 16-olide (Figure 19)

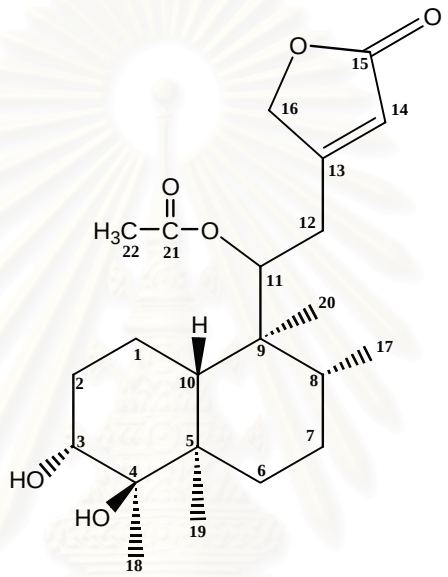


Figure 19. Structure of compound A-2

Table 14. ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, NOESY, and HMBC spectral data of compound A-2

		δ_C (ppm)	δ_H (ppm), *J* in Hz (multiplicity, *J* in Hz)	^{1}H-^{1}H COSY	NOESY	HMBC
1	CH_2	17.93	1.69, 1H, *m* 1.80, 1H, *m*	-	H-8	C-10
2	CH_2	31.95	1.39, 1H, *dd* (3.0, 11.5) 1.56, 1H, *dd* (3.8, 7.8)	-	-	-
3	CH	76.30	3.60, 1H, *s*	H-7, H-21	H-7, H-19, H-21	-
4	>C<	76.16	-	-	-	-
5	>C<	41.60	-	-	-	-
6	CH_2	30.10	1.68, 1H, *m* 2.08, 1H, *m*	-	H-7	-
7	CH_2	26.95	1.45, 1H, *m* 1.51, 1H, *m*	-	-	-
8	CH	37.31	1.33, 1H, *m*	H-20	-	-
9	>C<	43.95	-	-	-	-
10	CH	40.35	1.93, 1H, *dd* (2.0, 12.5)	-	-	C-5, C-6, C-9, C-11
11	CH	76.75	5.35, 1H, *dd* (1.5, 11.0)	H-12	H-12, H-17, H-20	C-8, C-9, C-10, C-12, C-13, C-21
12	CH_2	29.82	2.64, 1H, *dd* (11.0, 15.5) 2.75, 1H, *d* (15.5)	-	H-8, H-10	C-11,C-13, C-14, C-16
13	>C=	167.20	-	-	-	-
14	CH	117.46	5.92, 1H, *br s*	H-12a, b, H-16a, b	-	C-13, C-15
15	C=O	173.62	-	-	-	-
16	CH_2	73.03	4.67, 1H, *dd* (1.5, 17.5) 4.89, 1H, *dd* (1.8, 17.3)	-	-	C-12, C-13, C-14, C-15
17	CH_3	16.61	0.92, 3H, *d* (6.5)	-	-	C-8, C-9
18	CH_3	16.96	1.13, 3H, *s*	-	-	C-4, C-5,

Table 14. ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY, NOESY, and HMBC spectral data of compound A-2 (continued)

		δ_C (ppm)	δ_H (ppm), *J* in Hz (multiplicity, *J* in Hz)	^{1}H-^{1}H COSY	NOESY	HMBC
19	CH_3	13.08	0.79, 3H, *s*	-	-	C-10
20	CH_3	21.81	1.26, 3H, *s*	-	-	-
21	C=O	170.83	-	-	-	-
22	CH_3	20.92	2.02, 3H, *s*	-	-	C-21

Table 15. ^{1}H-NMR and ^{13}C-NMR spectral data of compound A-1 and compound A-2

Position	δ_C (ppm)		δ_H (ppm)	
	compound A-1	compound A-2	compound A-1	compound A-2
1	16.45	17.93	1.24 1.62	1.69 1.80
2	32.28	31.95	1.40 1.58	1.39 1.56
3	76.28	76.30	3.58	3.60
4	76.26	76.16	-	-
5	41.32	41.60	-	-
6	30.44	30.10	1.69 2.00	1.68 2.08
7	26.38	26.95	1.39 1.48	1.45 1.51
8	36.19	37.31	1.41	1.33
9	38.65	43.95	-	-
10	40.73	40.35	1.76	1.93
11	35.59	76.75	1.50 1.66	5.35
12	22.44	29.82	2.24 2.33	2.64 2.75
13	171.29	167.20	-	-
14	114.93	117.46	5.84	5.92
15	174.15	173.62	-	-
16	73.14	73.03	4.75	4.67 4.89
17	15.98	16.61	0.80	0.92
18	17.16	16.96	1.14	1.13
19	18.15	13.08	0.79	0.79
20	21.62	21.81	1.26	1.26
21	-	170.83	-	-
22	-	20.92	-	2.02

3. Results of Cytotoxic activity

The *in vitro* activity of some compounds (10 µg/ml) from *Croton roxburghii* against 5 cell lines, for example, KATO-3 (gastric cancer), SW 620 (colon cancer), BT 474 (breast cancer), HEP-G2 (hepatoma) and CHAGO (lung cancer) are reported in Table 16.

Table 16. Cytotoxicity data of the diterpenes from *Croton roxburghii*

Compounds (10 µg/ml)	% Survival				
	KATO-3	SW 620	BT 474	HEP-G2	CHAGO
[1]	52	68	97	65	77
[2]	51	81	102	71	79
Hexane extract	17	7	52	21	8
EtOAc extract	27	42	76	49	81
Acetone extract	25	11	56	29	41

The results from Table 16 showed that, compound A-1 [1] and compound A-2 [2] exhibited no cytotoxic activity against all cancer cell lines, whereas crude extracts (hexane extract, ethyl acetate extract and acetone extract) of this plant, showed more cytotoxic activity than isolated compounds (compound A-1 and compound A-2). Thus, crude extracts should be has other constituents which expressed higher cytotoxic activity than compound A-1 and compound A-2. This, therefore the study should be further investigation in the future.

CHAPTER V

CONCLUSION

From the stem bark of *Croton roxburghii* N.P. Balakr. (Euphorbiaceae) collected from Nong Bua Rawhae district, Chaiyaphum province, Thailand, two new clerodane-type diterpenoids compounds have been isolated. Their chemical structures were elucidated and identified by several spectroscopic techniques and comparison with previous reports. Compound A-1 was identified as 3*α*, 4*β*-dihydroxy-5*α*, 10*β*-*trans*-17*α*, 20*α*-cleroda-13 (14)-en-15, 16-olide which compare with 3*α*, 4*β*-dihydroxy-5*β*, 10*β*-*cis*-17α, 20*α*-cleroda-13 (14)-en-15, 16-olide (a clerodane type diterpene previously found in the dried leaves of *Ageratina saltillensis*; Compositae). The difference is only the stereochemistry of the methyl group at position C-5 of compound A-1 is *α* instead of *β* as previously reported. In this study the X-ray structure of 4*β*-dihydroxy-5*α*, 10*β*-*trans*-17α, 20*α*-cleroda-13 (14)-en-15, 16-olide has been provided.

Compound A-2 was identified as 11-acetoxy-3*α*, 4*β*-dihydroxy-5*α*, 10*β*-*trans*-17α, 20*α*-cleroda-13 (14)-en-15, 16-olide.

The isolated compounds showed no cytotoxic activity against 5 cell lines, while the crude extracts of this plant showed higher cytotoxic activity than that of isolated compounds (compound A-1 and compound A-2). As these results, it can assume that crude extracts should have other compounds that show more potency in cytotoxic activity than compound A-1 and compound A-2. Thus, the investigation of the bioactive substances from this plant specimen should be continued. Furthermore, this study has also provided the additional chemotaxonomic information of *Croton roxburghii* N.P. Balakr.

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APPENDICES

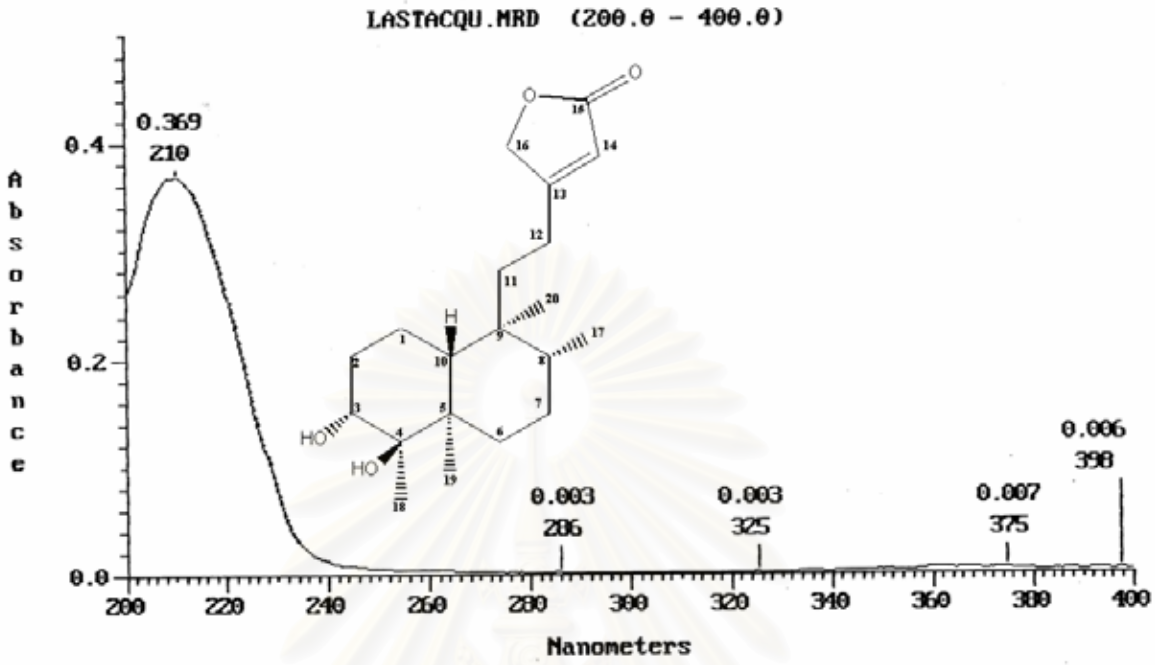

Figure 20: The UV spectrum of compound A-1 (in MeOH)

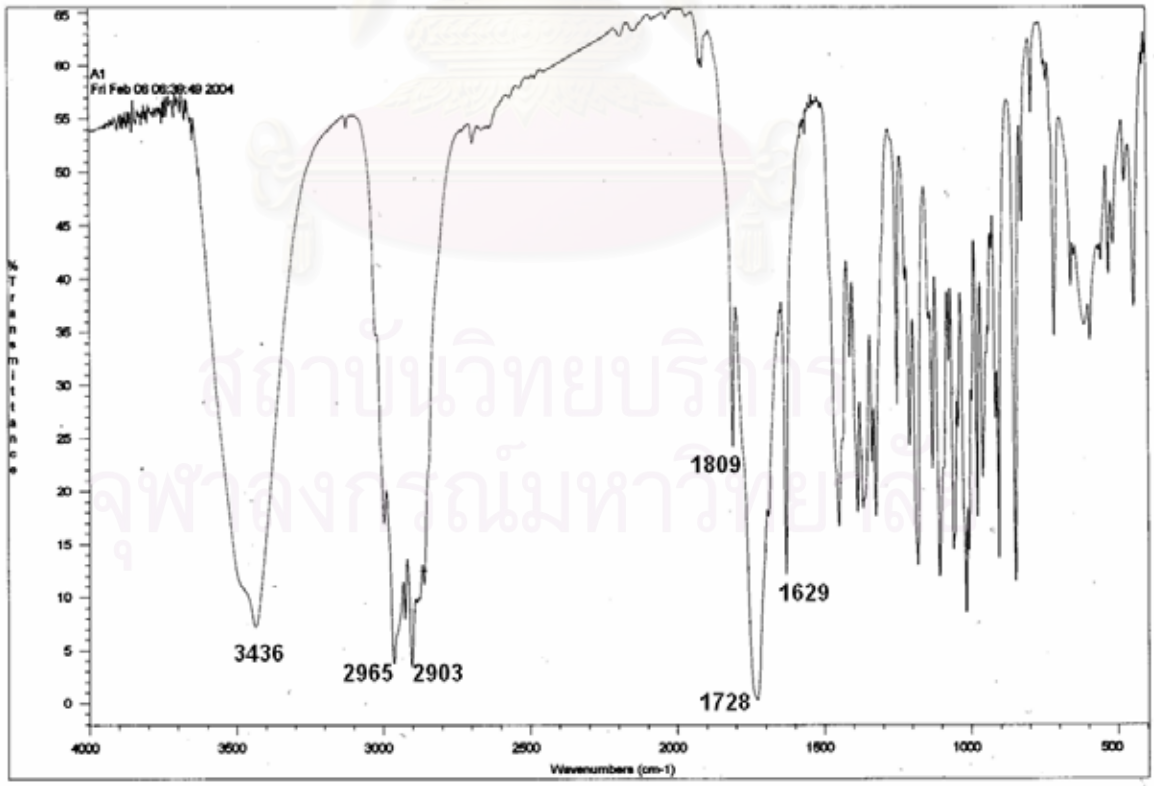

Figure 21: The IR spectrum of compound A-1 (KBr disc)

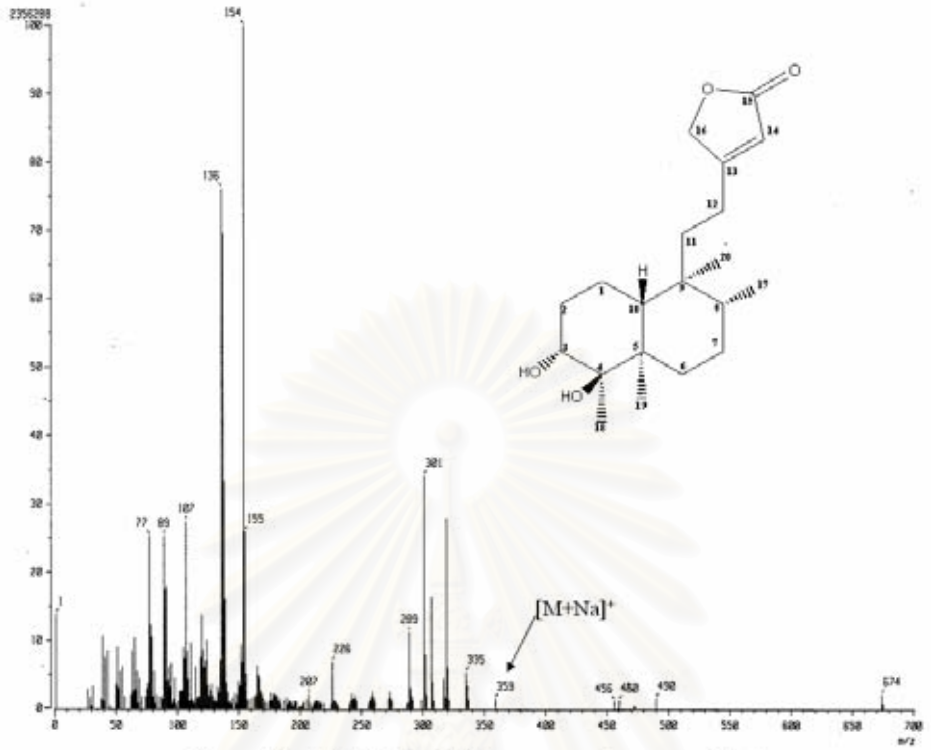

Figure 22: The FAB (+) MS spectrum of compound A-1

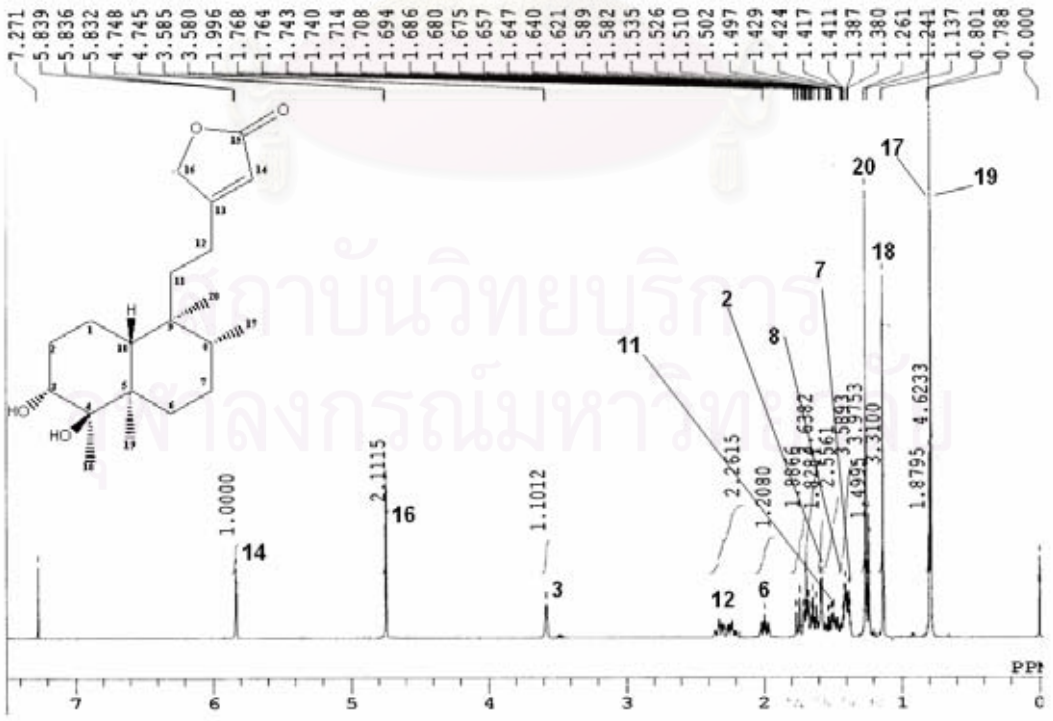

Figure 23a: The 400 MHz ^{1}H-NMR spectrum of compound A-1 (in $CDCl_3$)

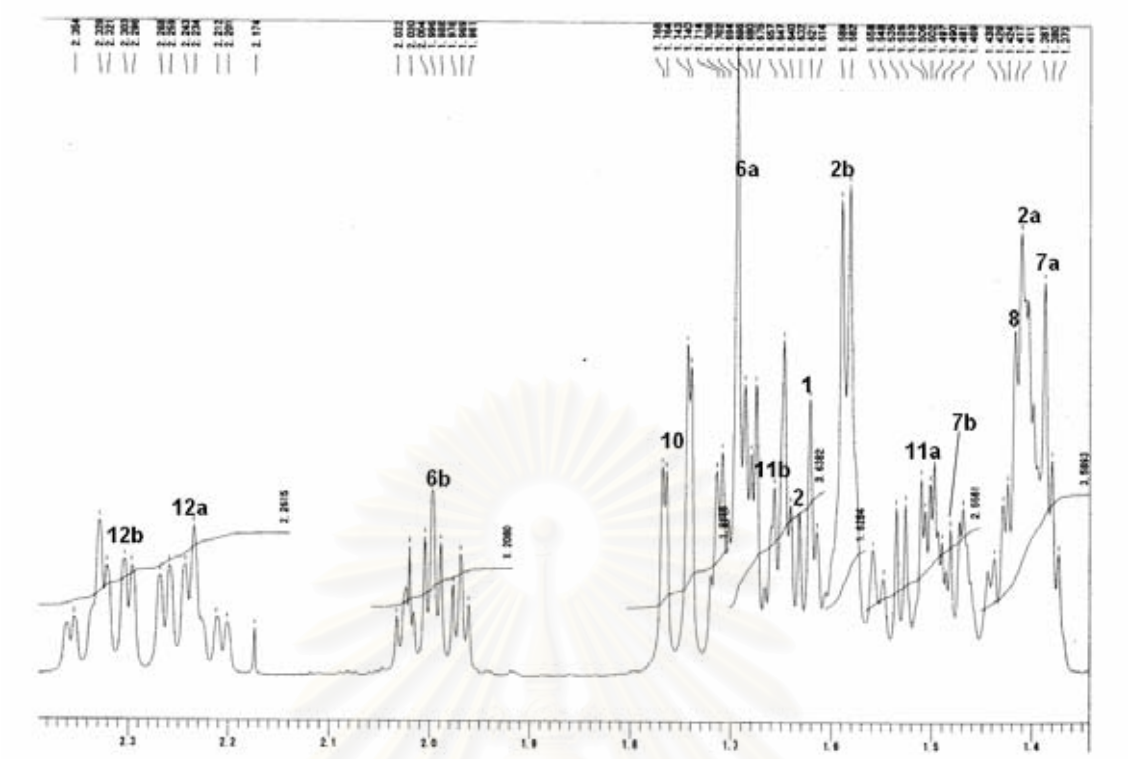

Figure 23b: The expanded 400 MHz ^{1}H-NMR spectrum of compound A-1 (in $CDCl_3$)

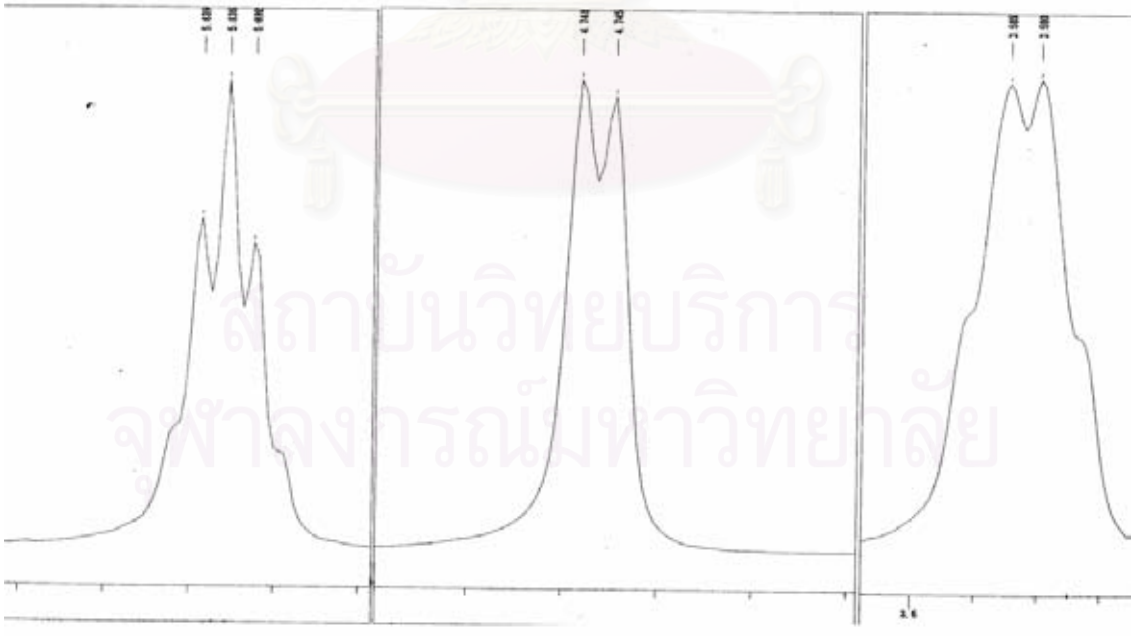

Figure 23c: The expanded 400 MHz ^{1}H-NMR spectrum of compound A-1 (in $CDCl_3$)

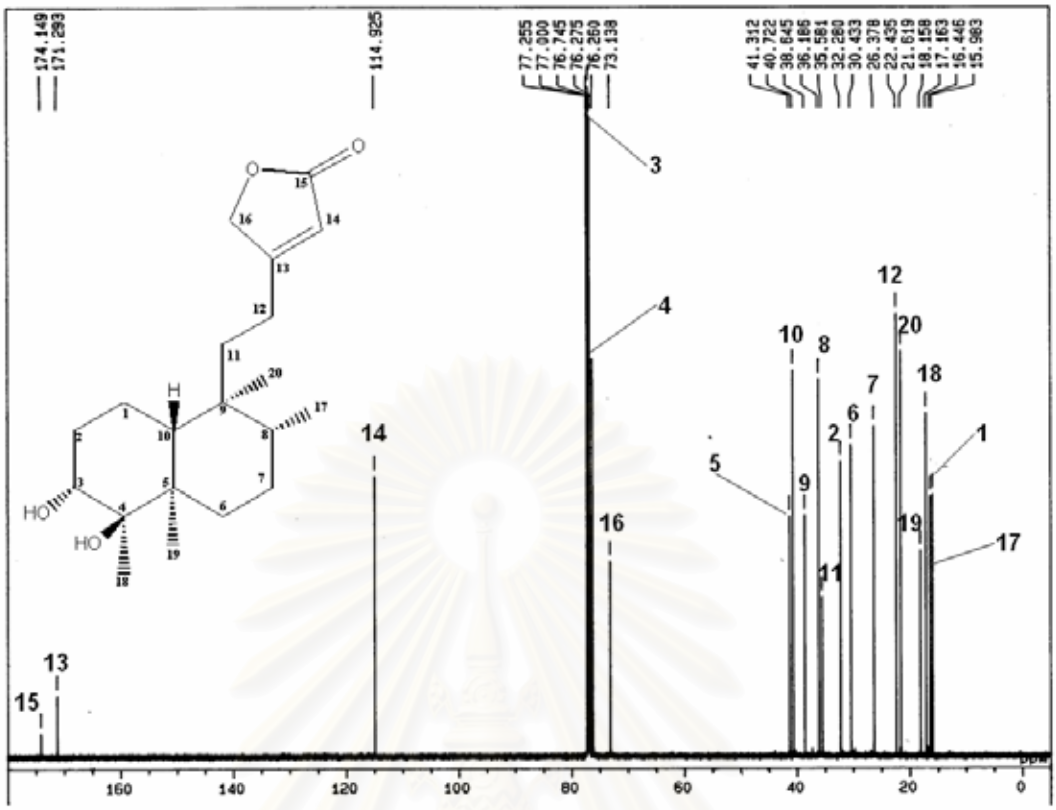

Figure 24: The 100 MHz ^{13}C-NMR spectrum of compound A-1 (in $CDCl_3$)

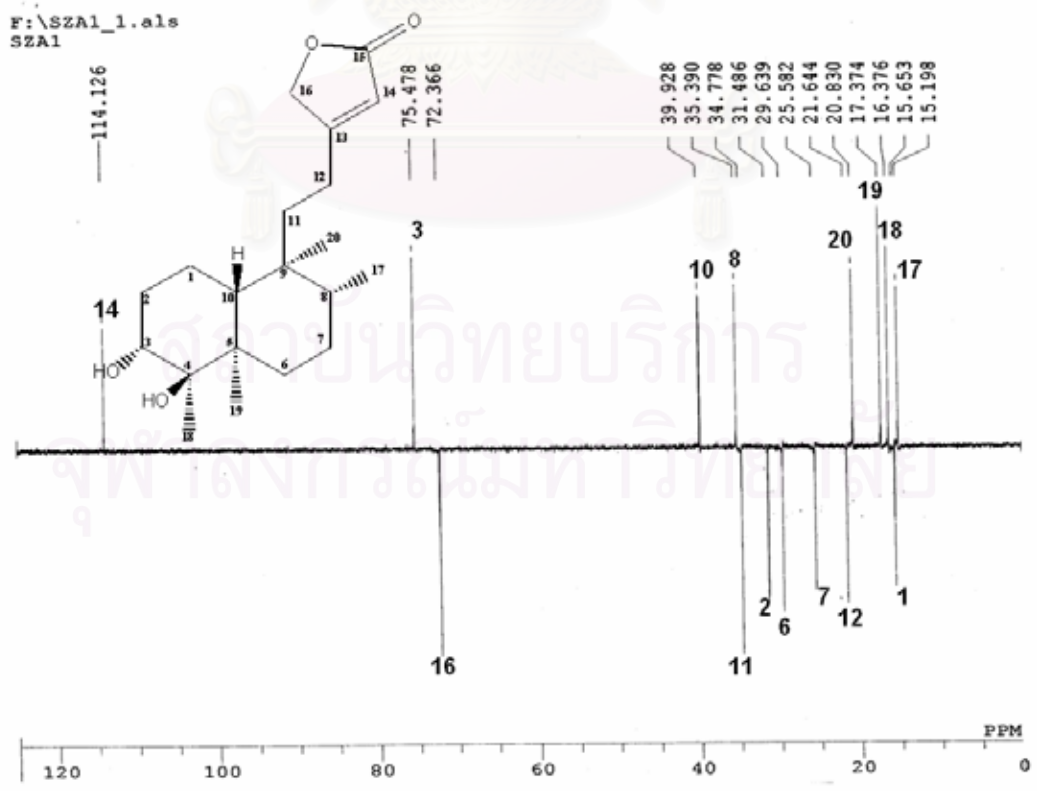

Figure 25: The DEPT-135 spectrum of compound A-1 (in $CDCl_3$)

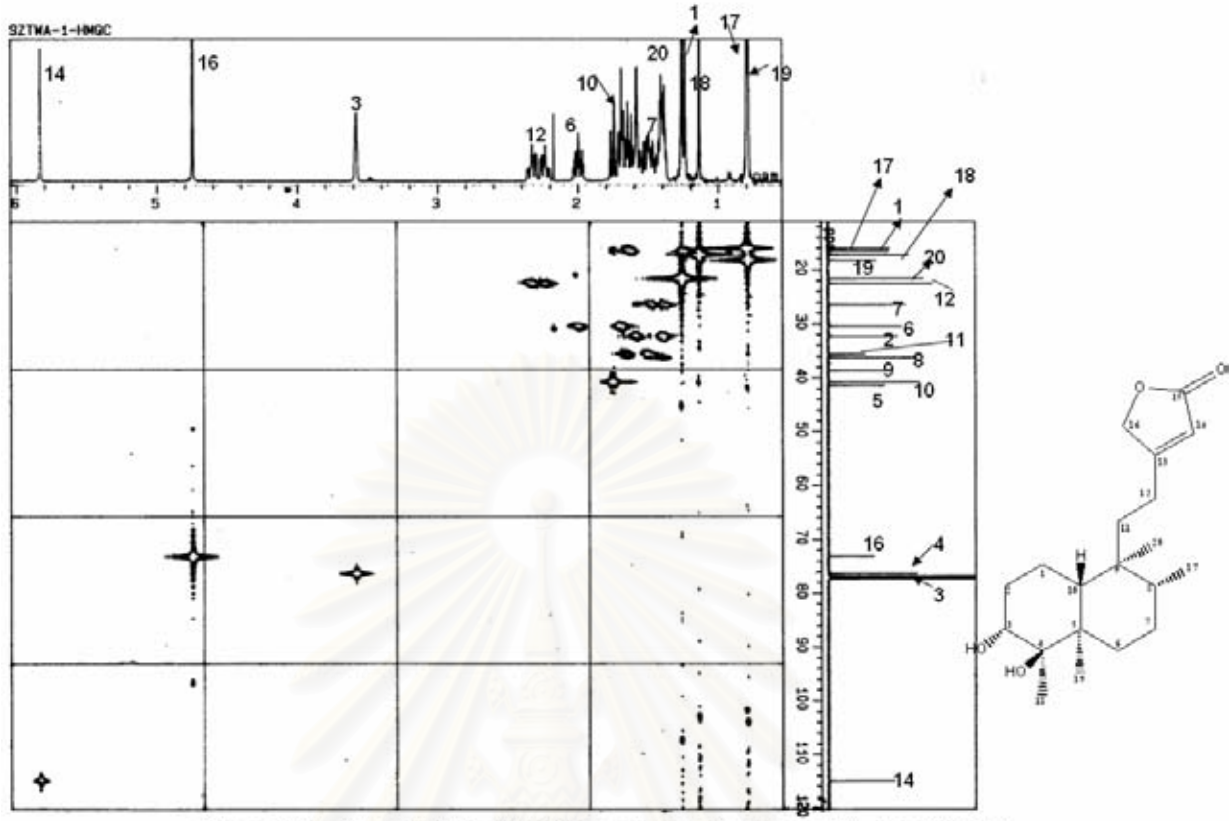

Figure 26a: The 500 MHz HMQC spectrum of compound A-1 (in $CDCl_3$)

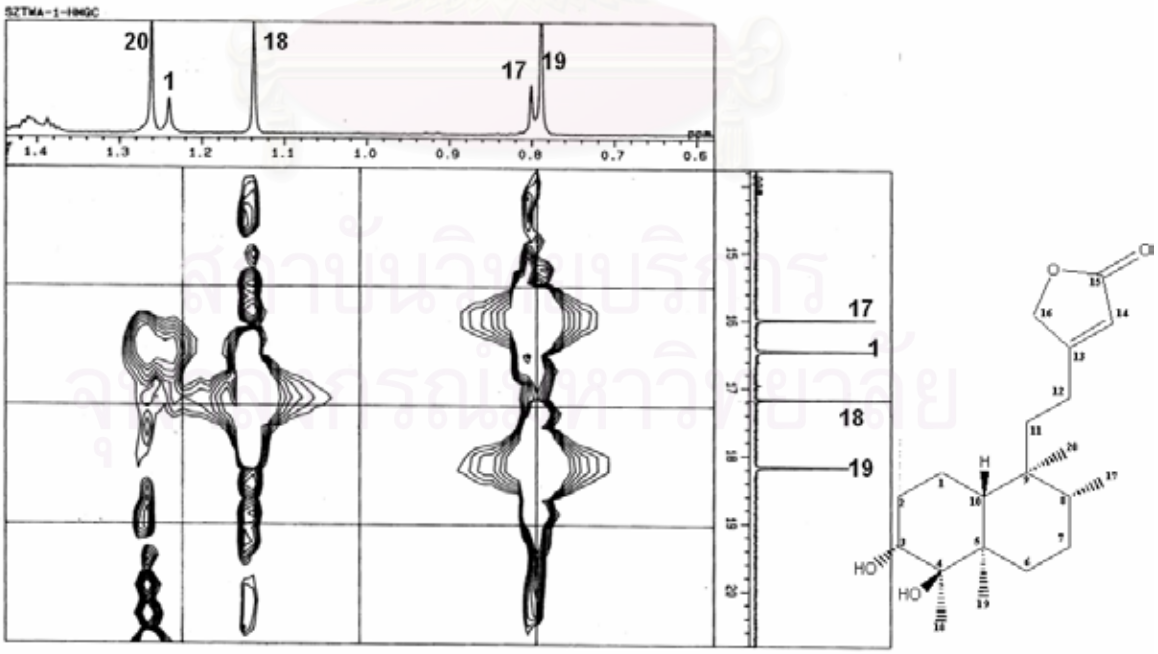

Figure 26b: The expanded 500 MHz HMQC spectrum of compound A-1 (in $CDCl_3$)
(δ_H 0.6-1.3 ppm, δ_C 15.0-20.0 ppm)

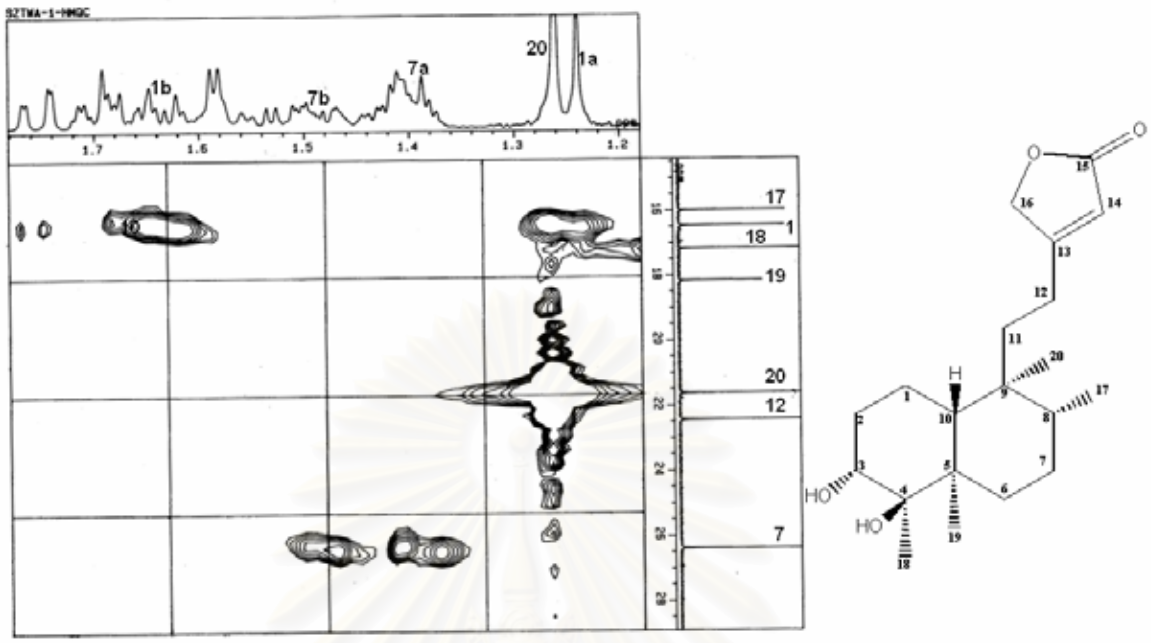

Figure 26c: The expanded 500 MHz HMQC spectrum of compound A-1 (in $CDCl_3$) (δ_H 1.2-1.8 ppm, δ_C 15.0-29.0 ppm)

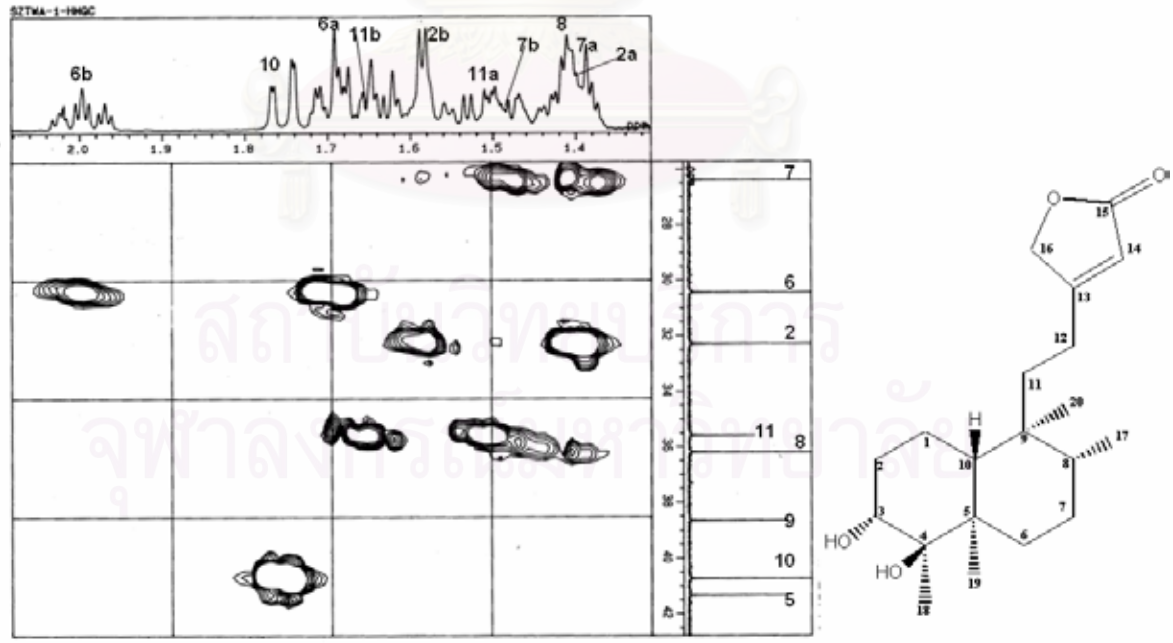

Figure 26d: The expanded 500 MHz HMQC spectrum of compound A-1 (in $CDCl_3$) (δ_H 1.3-2.1 ppm, δ_C 27.0-43.0 ppm)

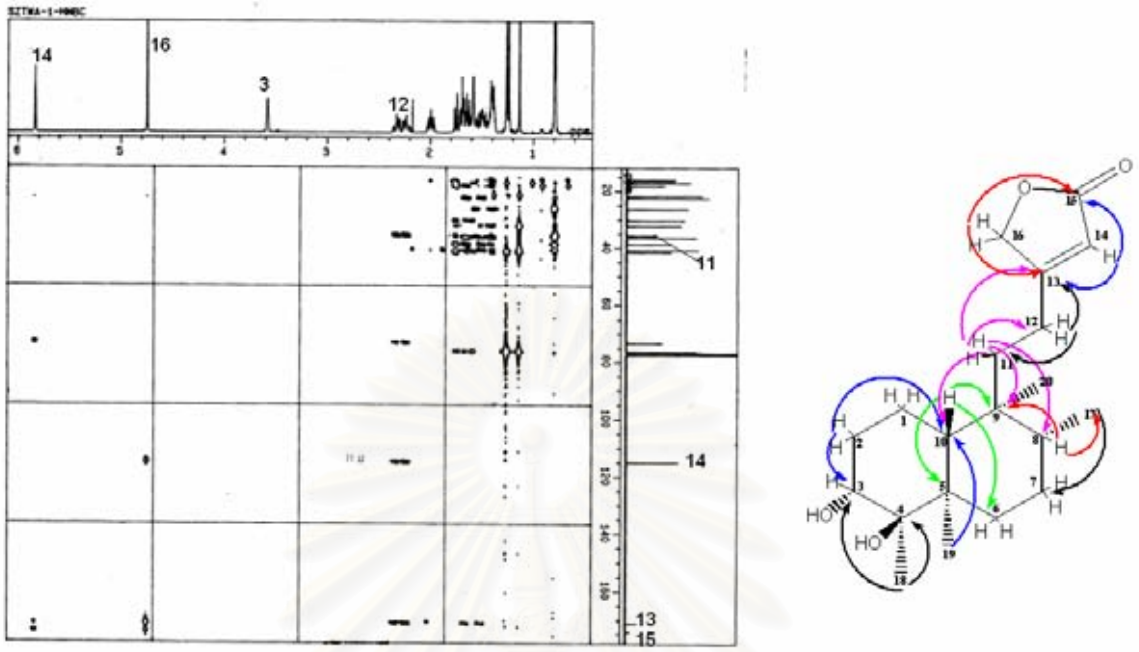

Figure 27a: The 500 MHz HMBC spectrum of compound A-1 (in $CDCl_3$)

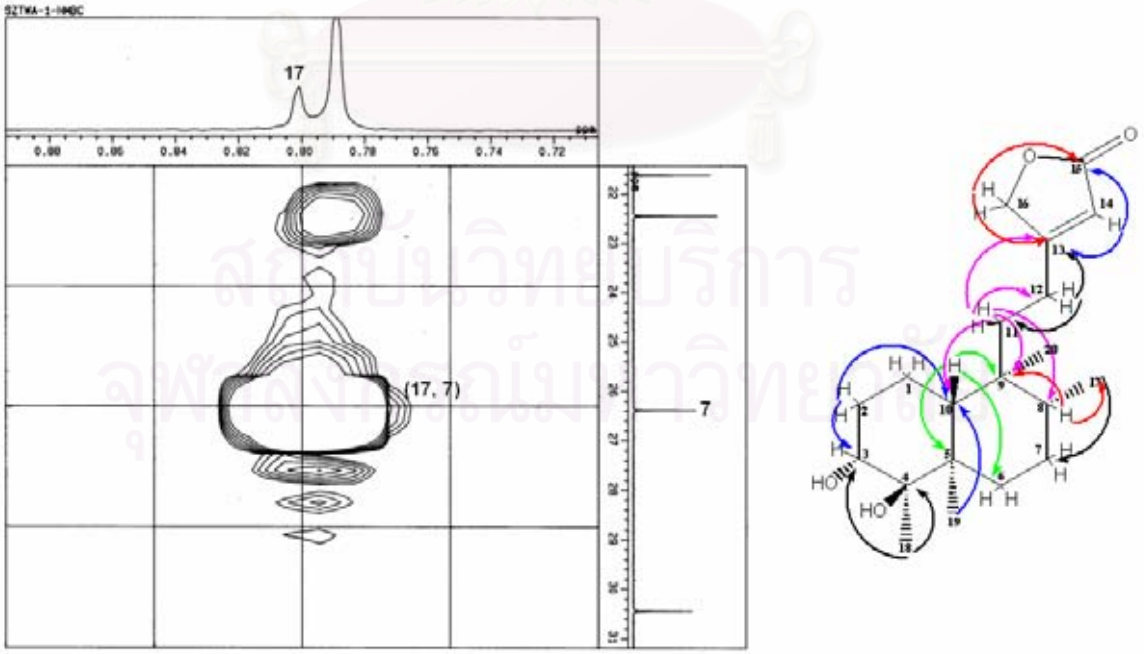

Figure 27b: The expanded 500 MHz HMBC spectrum of compound A-1 (in $CDCl_3$)
(δ_H 0.70-0.90 ppm, δ_C 20.0-31.0 ppm)

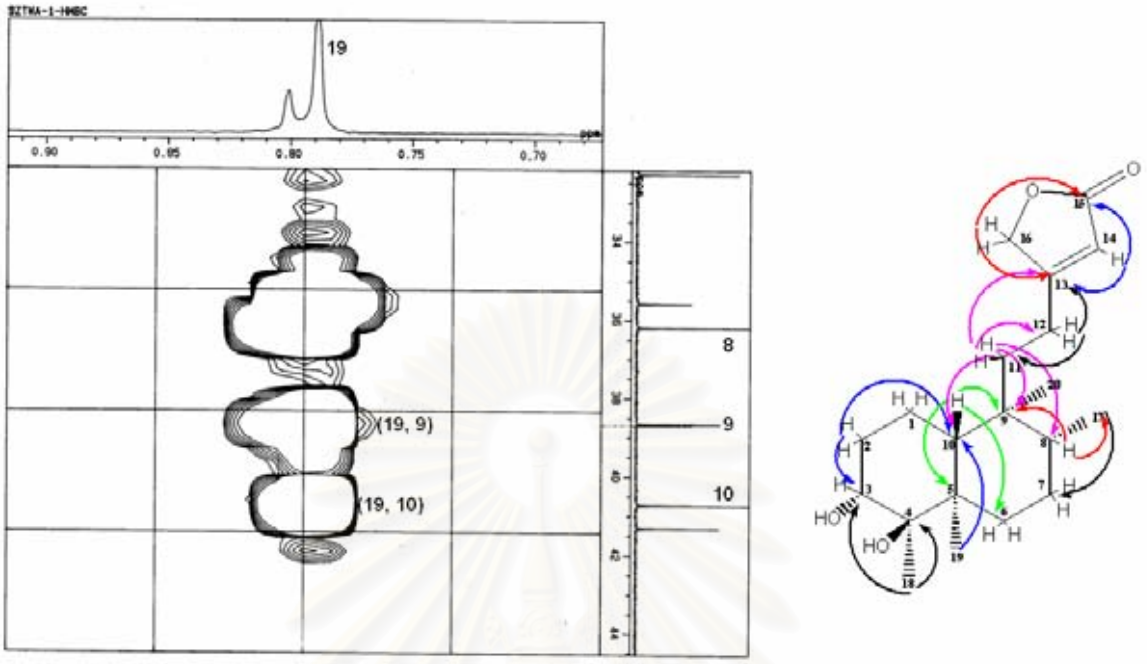

Figure 27c: The expanded 500 MHz HMBC spectrum of compound A-1 (in $CDCl_3$)
(δ_H 0.70-0.90 ppm, δ_C 32.0-44.0 ppm)

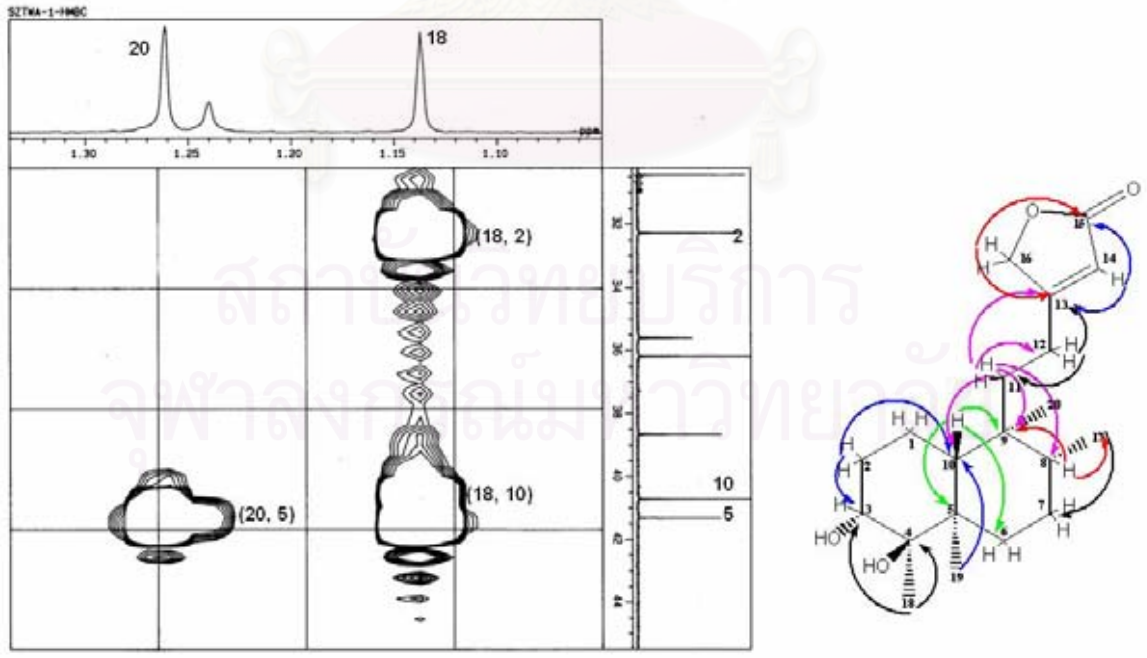

Figure 27d: The expanded 500 MHz HMBC spectrum of compound A-1 (in $CDCl_3$)
(δ_H 1.05-1.35 ppm, δ_C 30.0-44.0 ppm)

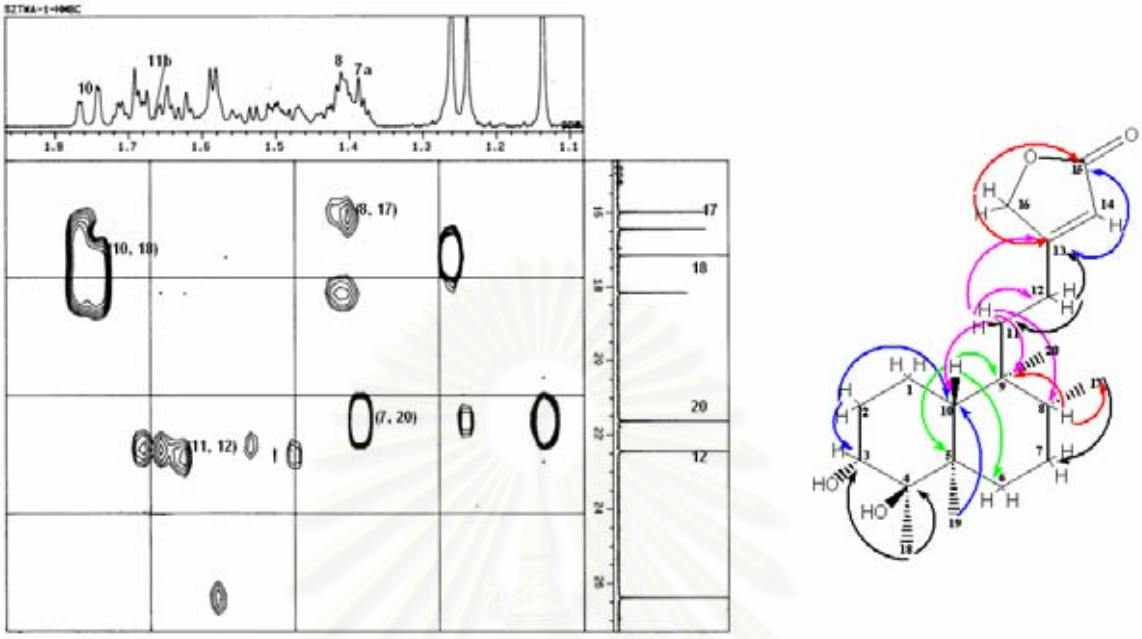

Figure 27e: The expanded 500 MHz HMBC spectrum of compound A-1 (in $CDCl_3$) (δ_H 1.08-1.85 ppm, δ_C 15.0-27.0 ppm)

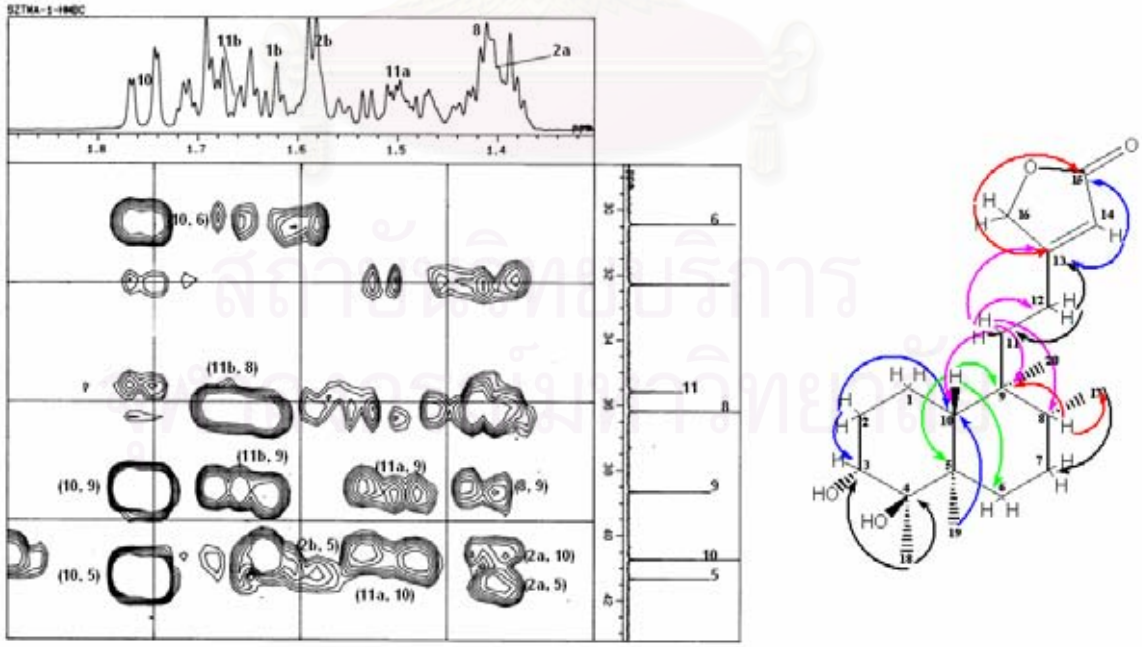

Figure 27f: The expanded 500 MHz HMBC spectrum of compound A-1 (in $CDCl_3$) (δ_H 1.30-1.90 ppm, δ_C 29.0-43.0 ppm)

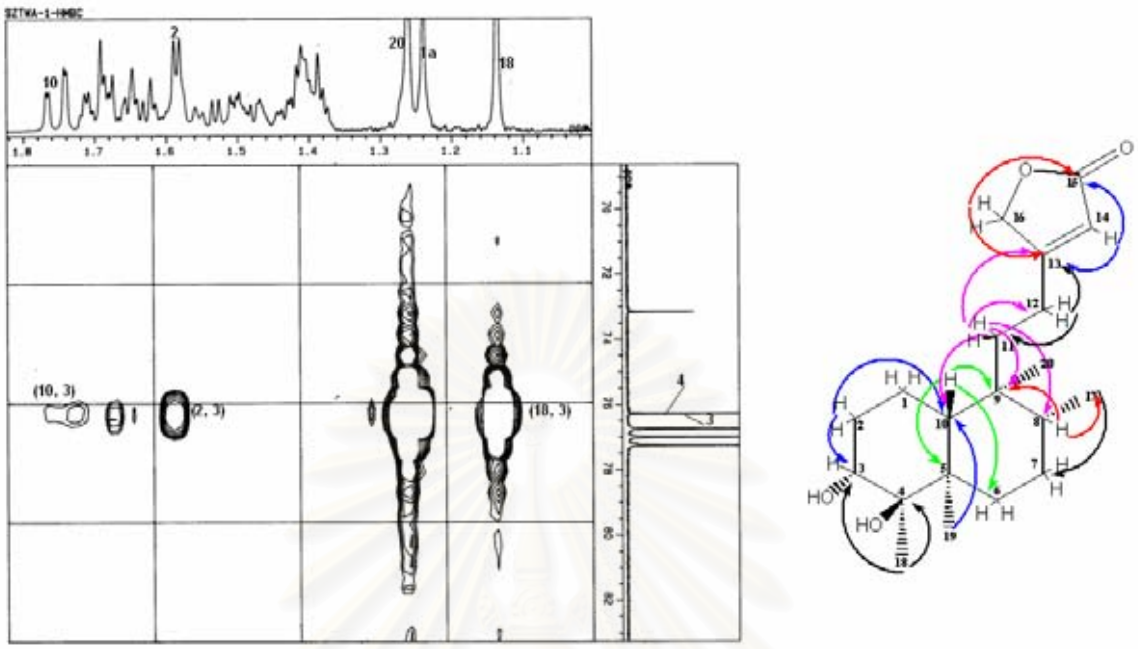

Figure 27g: The expanded 500 MHz HMBC spectrum of compound A-1 (in $CDCl_3$) (δ_H1.0-1.8 ppm, δ_C 69.0-83.0 ppm)

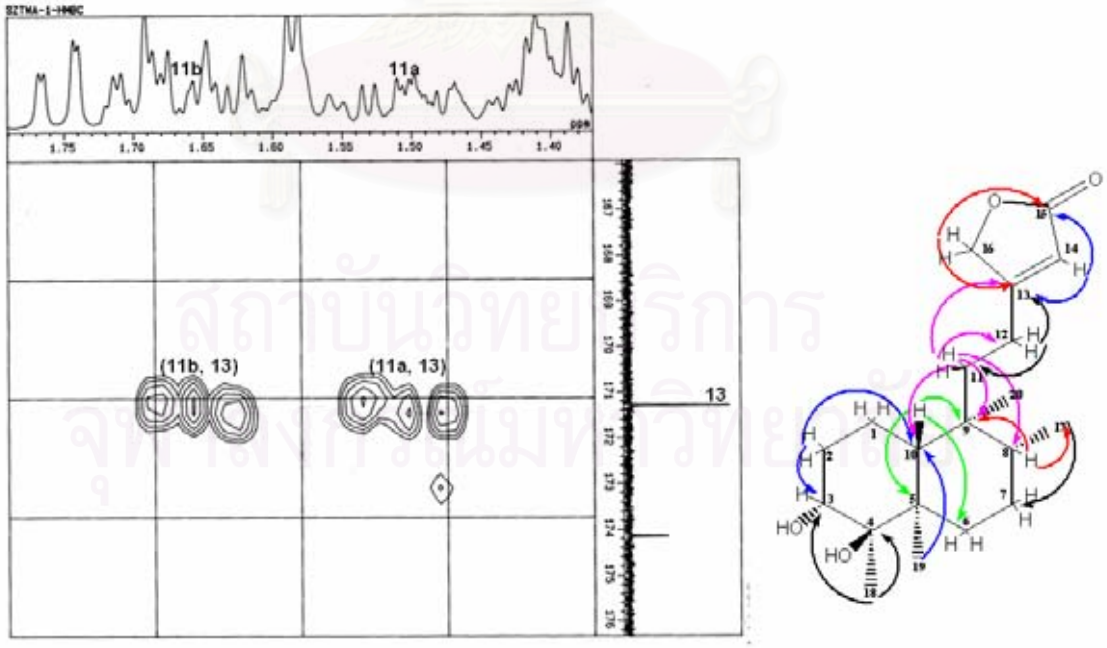

Figure 27h: The expanded 500 MHz HMBC spectrum of compound A-1 (in $CDCl_3$) (δ_H 1.34-2.42 ppm, δ_C 166.0-176.0 ppm)

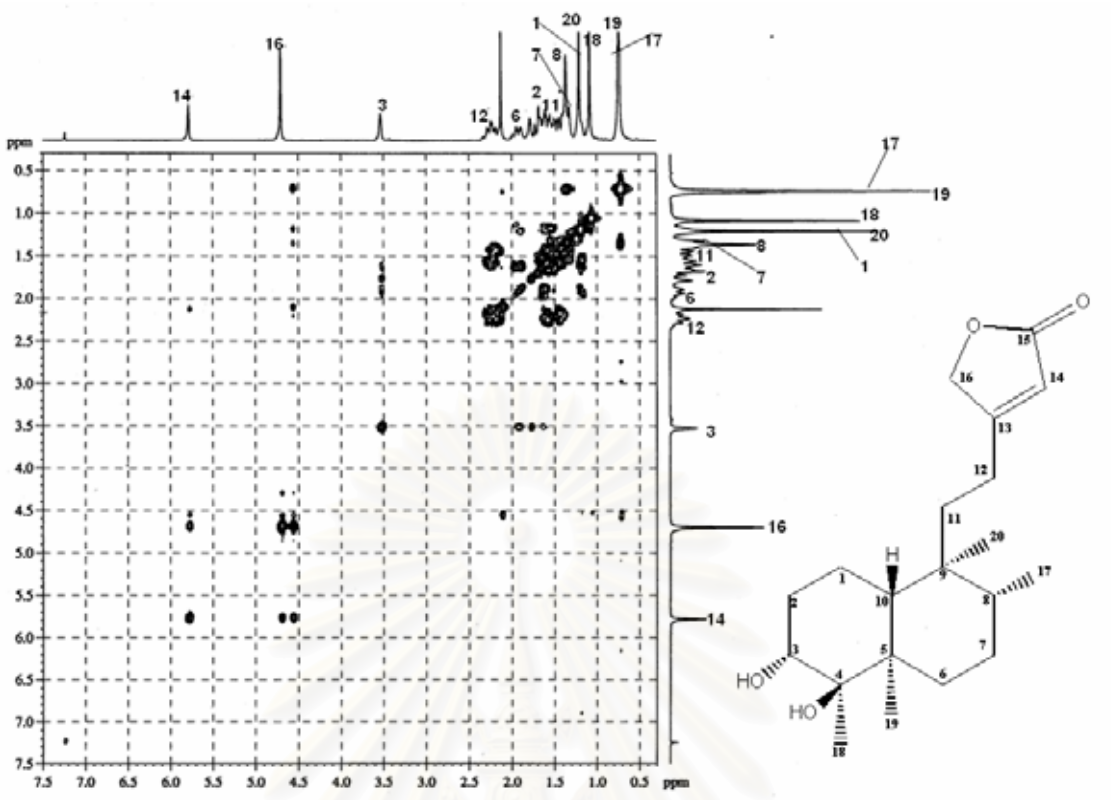

Figure 28a: The 300 MHz ^{1}H-^{1}H COSY spectrum of compound A-1 (in $CDCl_3$)

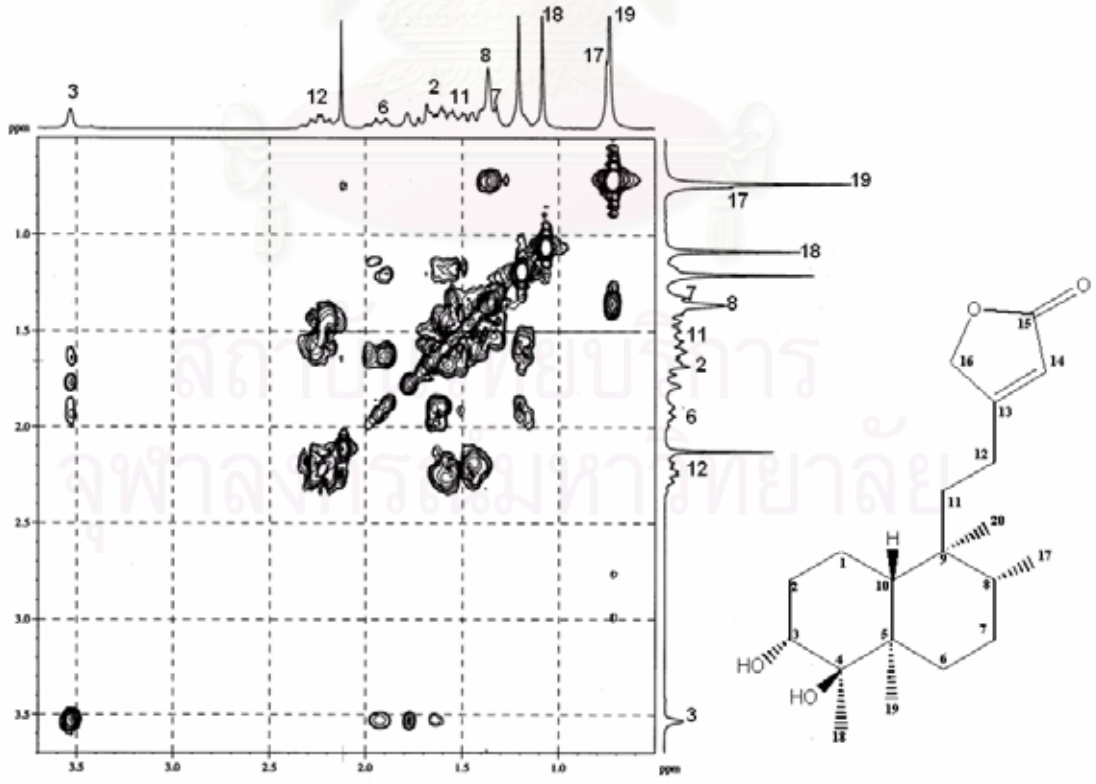

Figure 28b: The expanded 300 MHz ^{1}H-^{1}H COSY spectrum of compound A-1 (in $CDCl_3$) (δH 0.50-3.70 ppm)

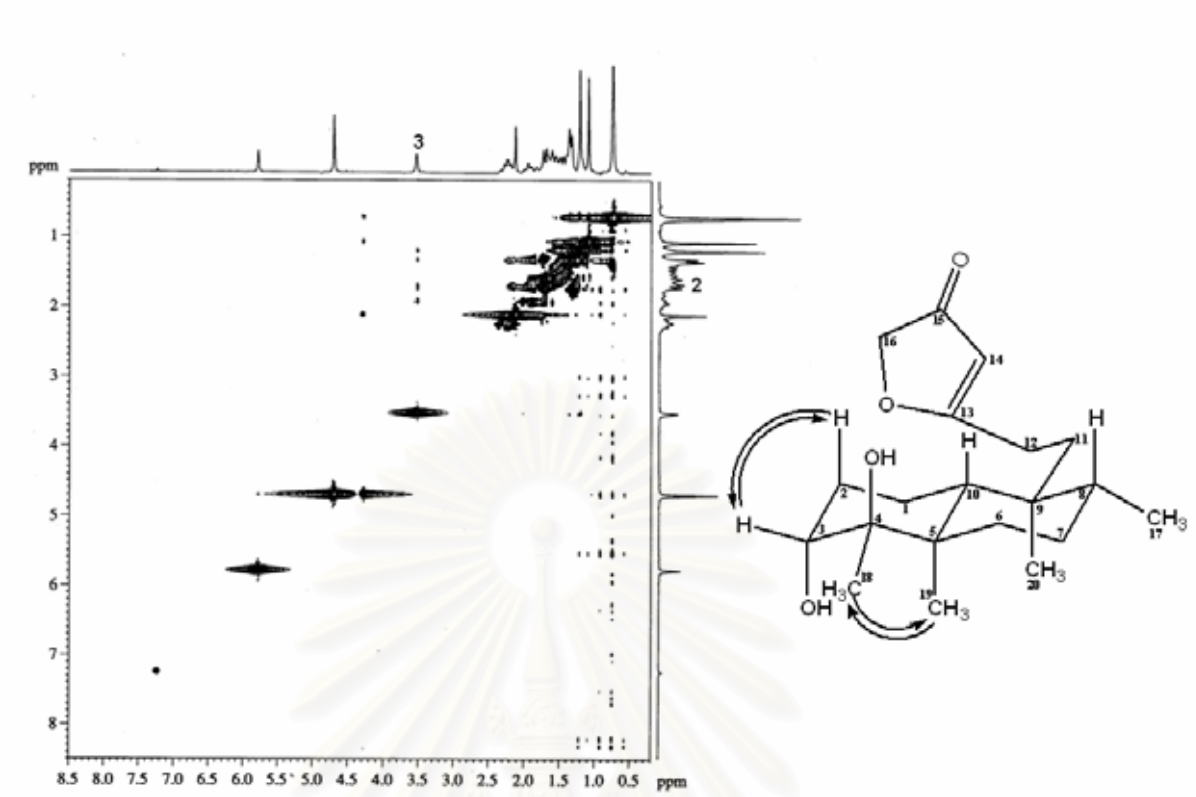

Figure 29a. The 300 MHz NOESY spectrum of compound A-1 (in $CDCl_3$)

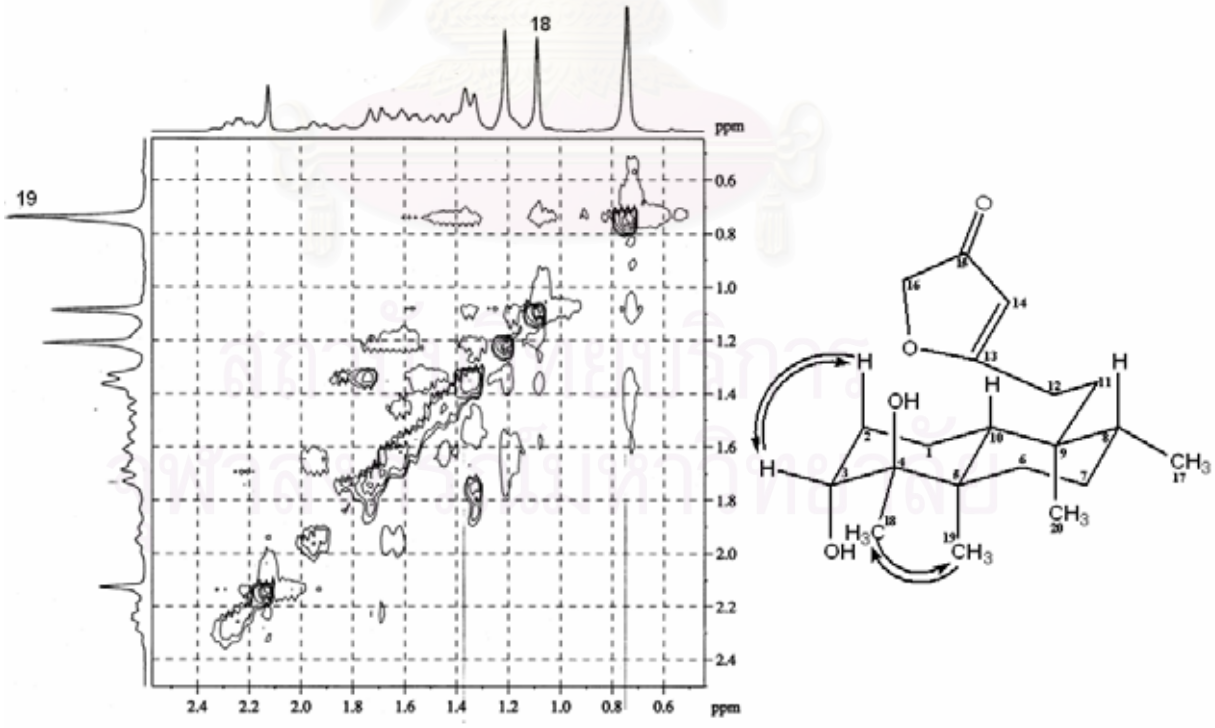

Figure 29b. The expanded 300 MHz NOESY spectrum of compound A-1 (in $CDCl_3$)

(δ_H 0.0-2.5 ppm)

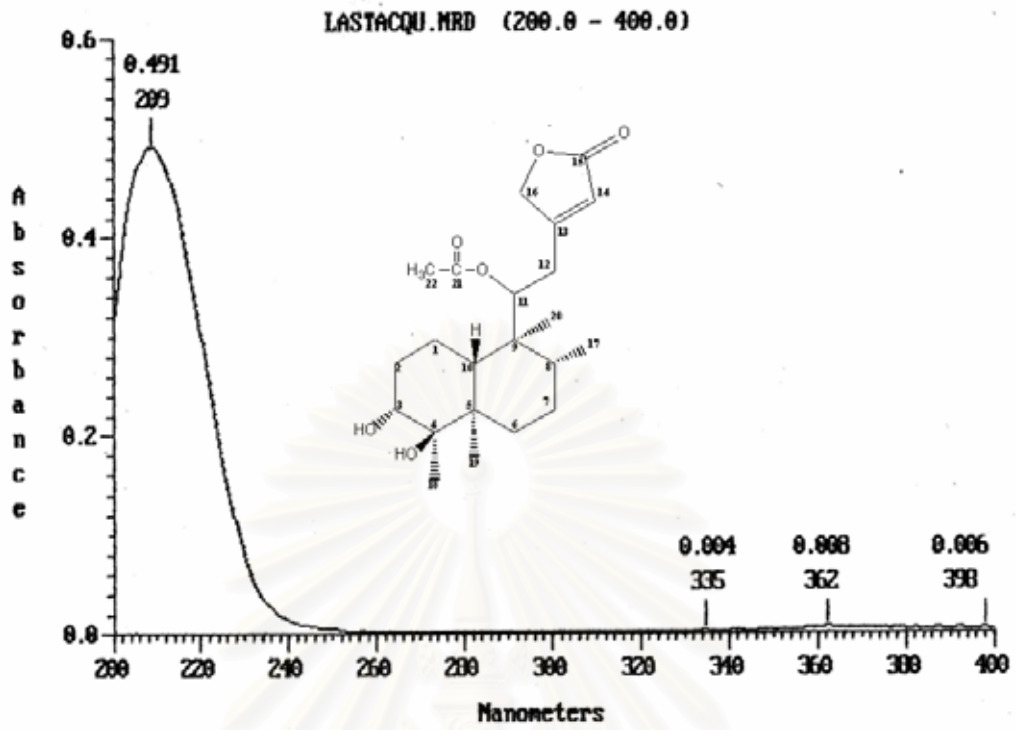

Figure 30: The UV spectrum of compound A-2 in MeOH

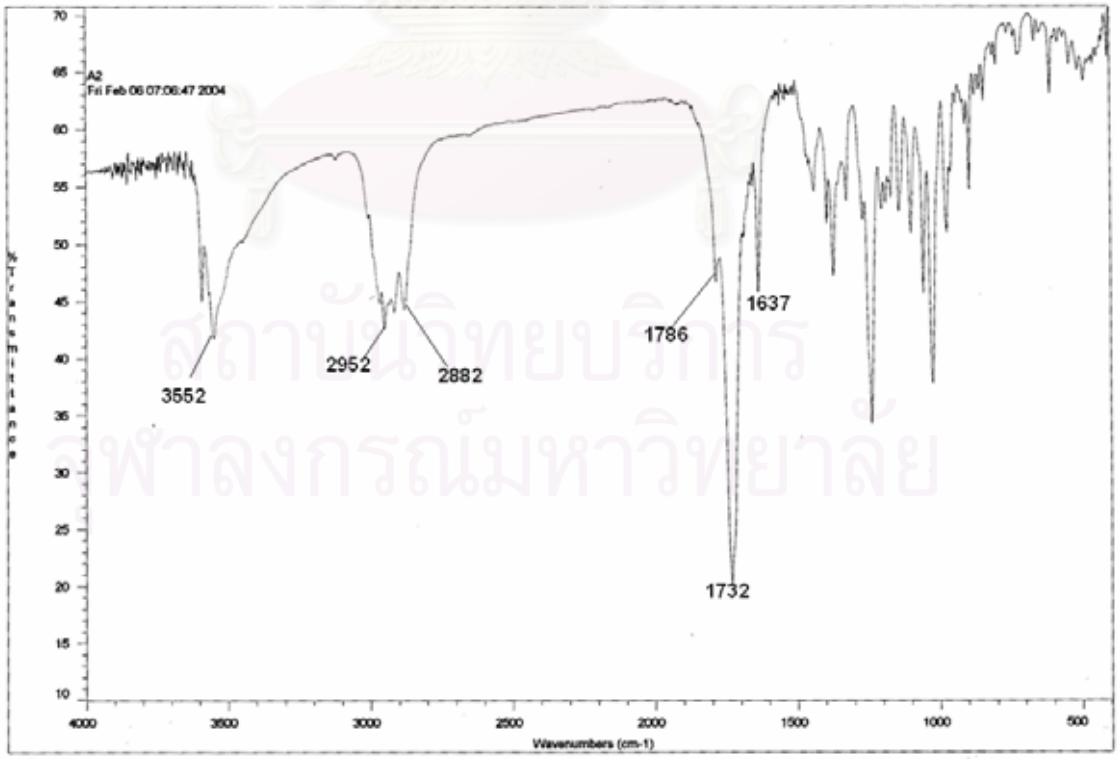

Figure 31: The IR spectrum of compound A-2 (KBr disc)

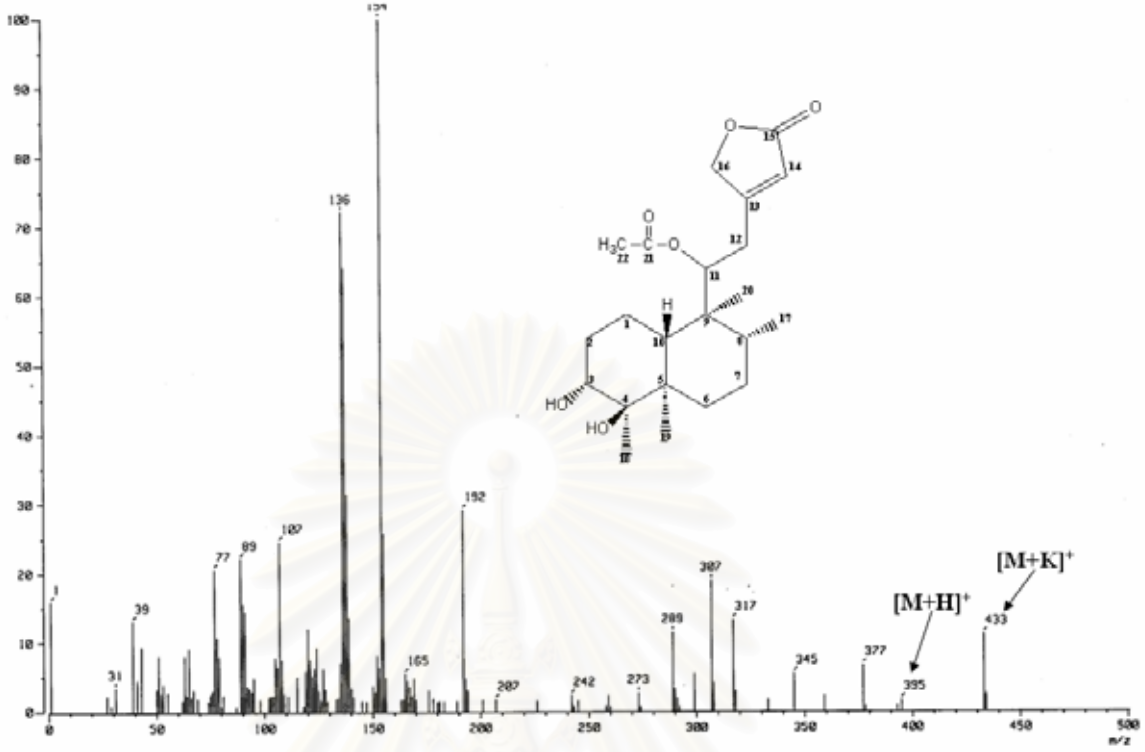

Figure 32: The FAB (+) MS spectrum of compound A-2

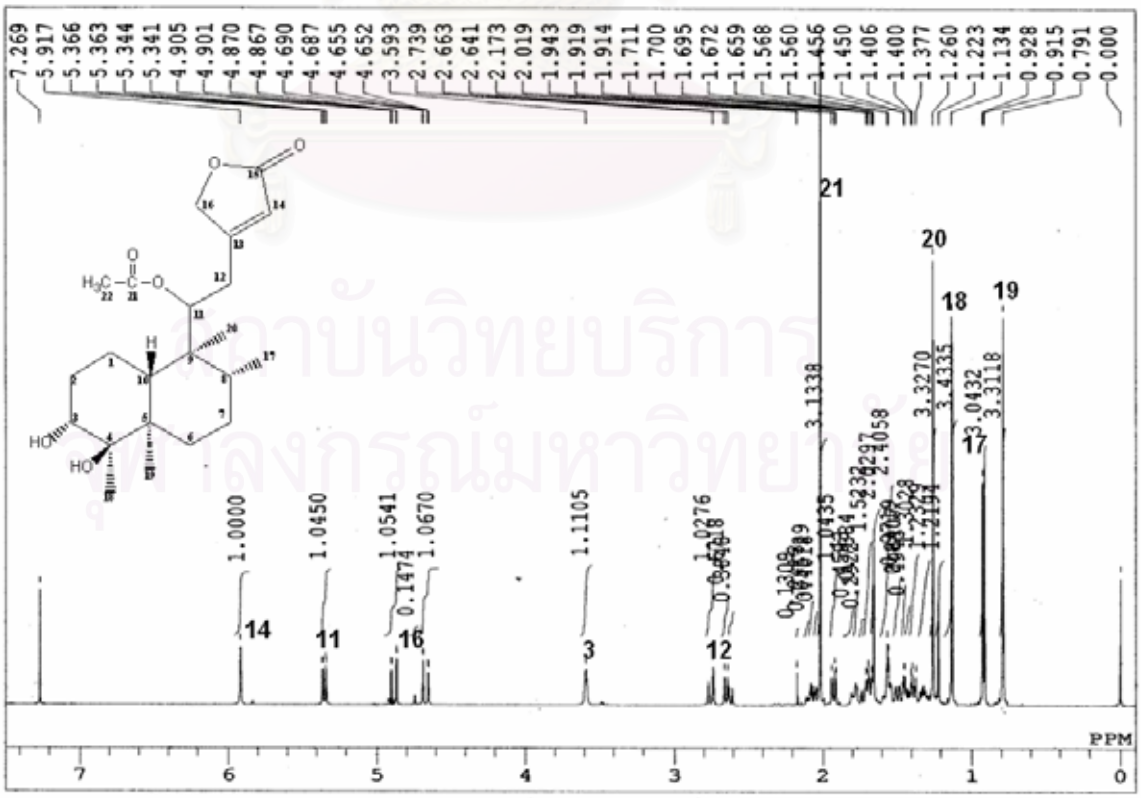

Figure 33a: The 400 MHz ^{1}H-NMR spectrum of compound A-2 (in $CDCl_3$)

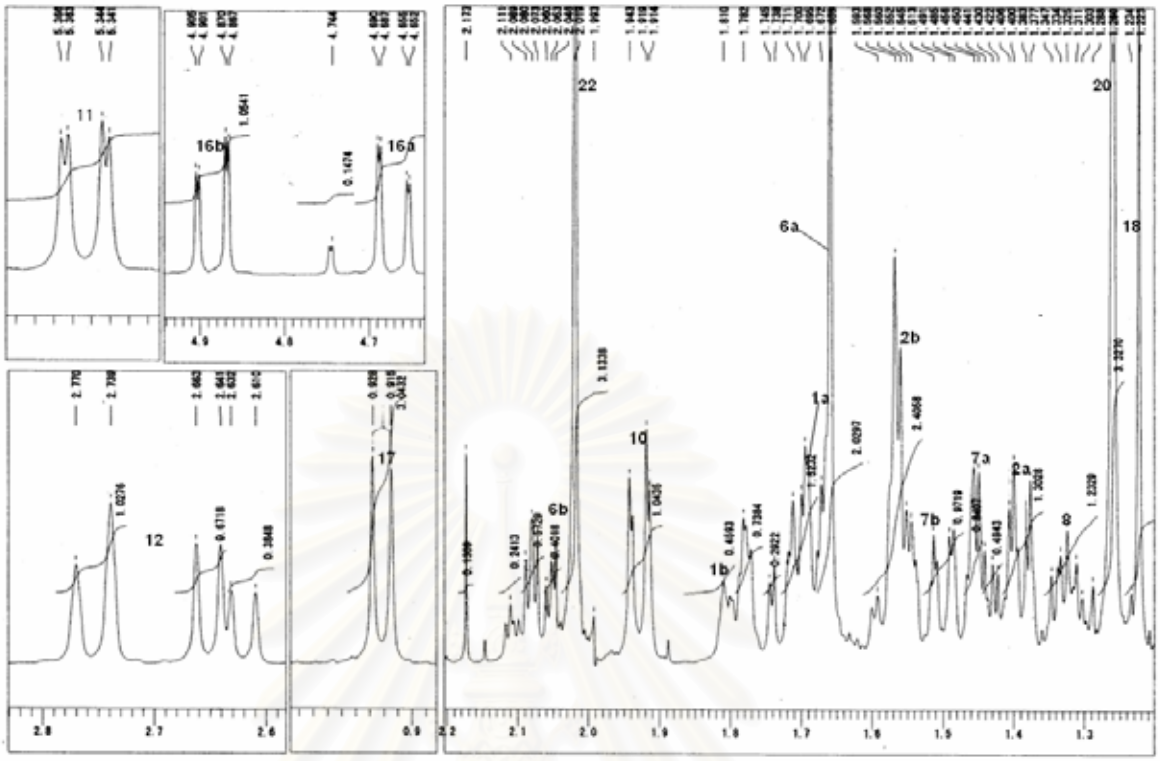

Figure 33b: The expanded 400 MHz ^{1}H-NMR spectrum of compound A-2 (in $CDCl_3$)

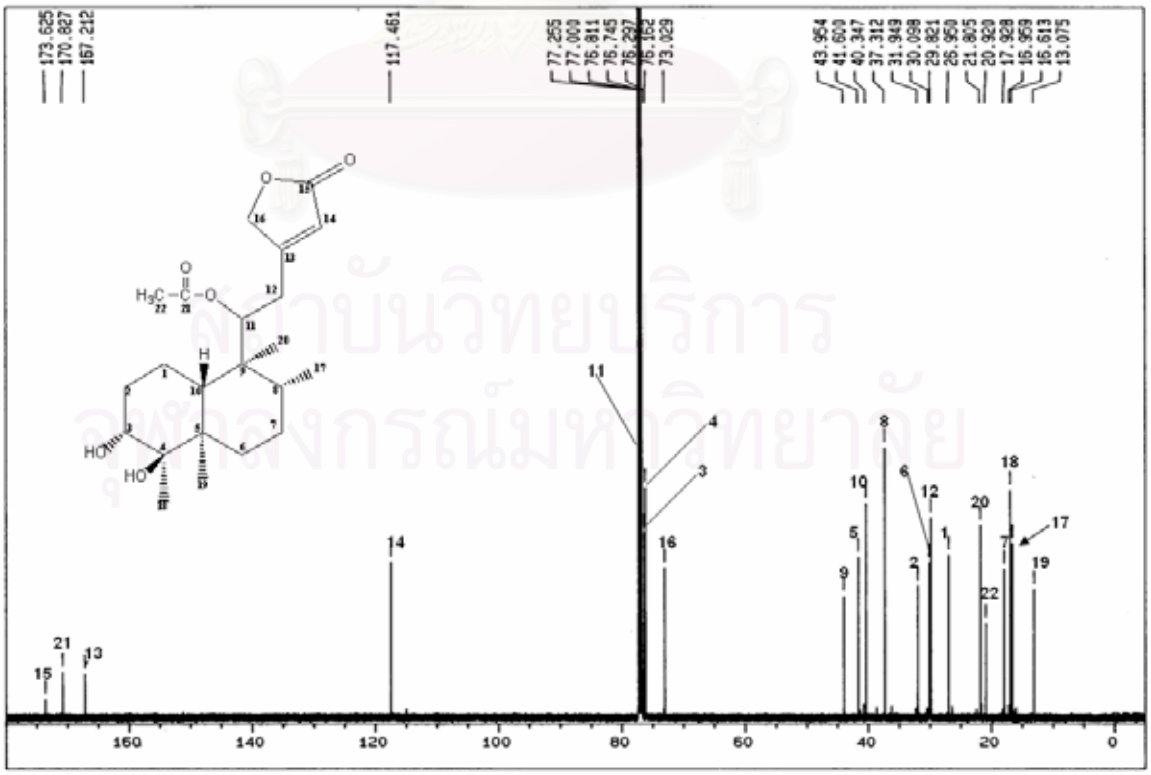

Figure 34a: The 100 MHz ^{13}C-NMR spectrum of compound A-2 (in $CDCl_3$)

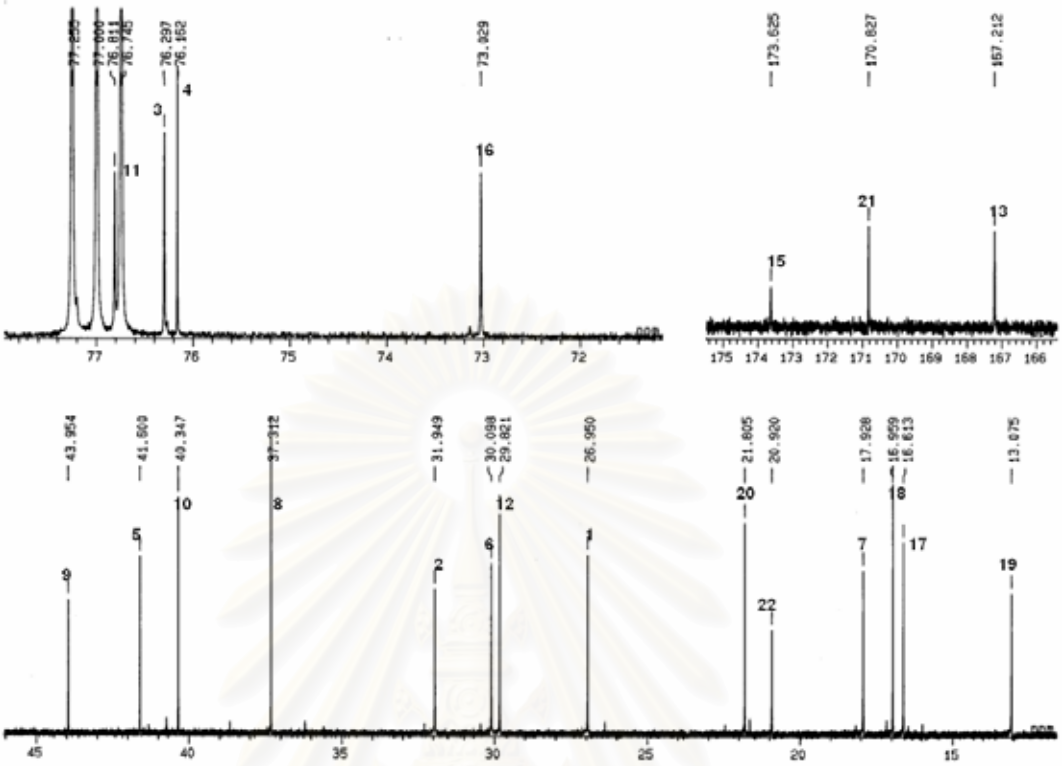

Figure 34b: The expanded 100 MHz ^{13}C-NMR spectrum of compound A-2 (in $CDCl_3$)

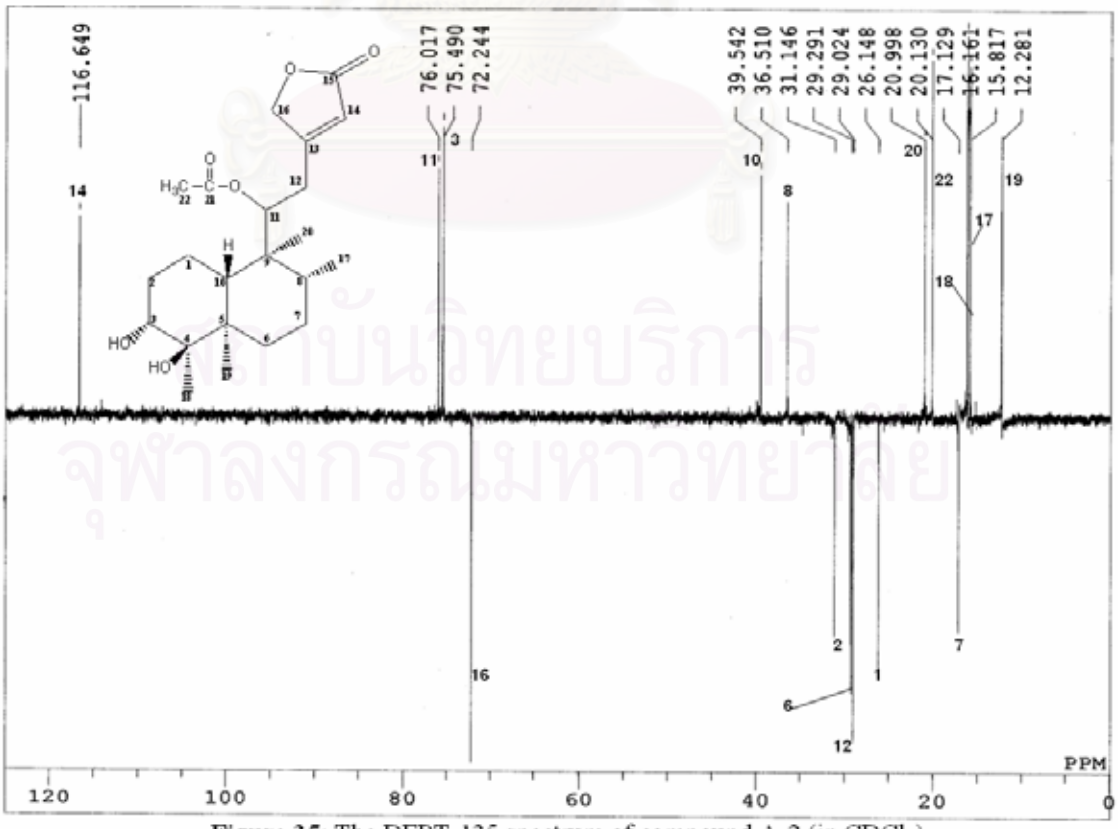

Figure 35: The DEPT-135 spectrum of compound A-2 (in $CDCl_3$)

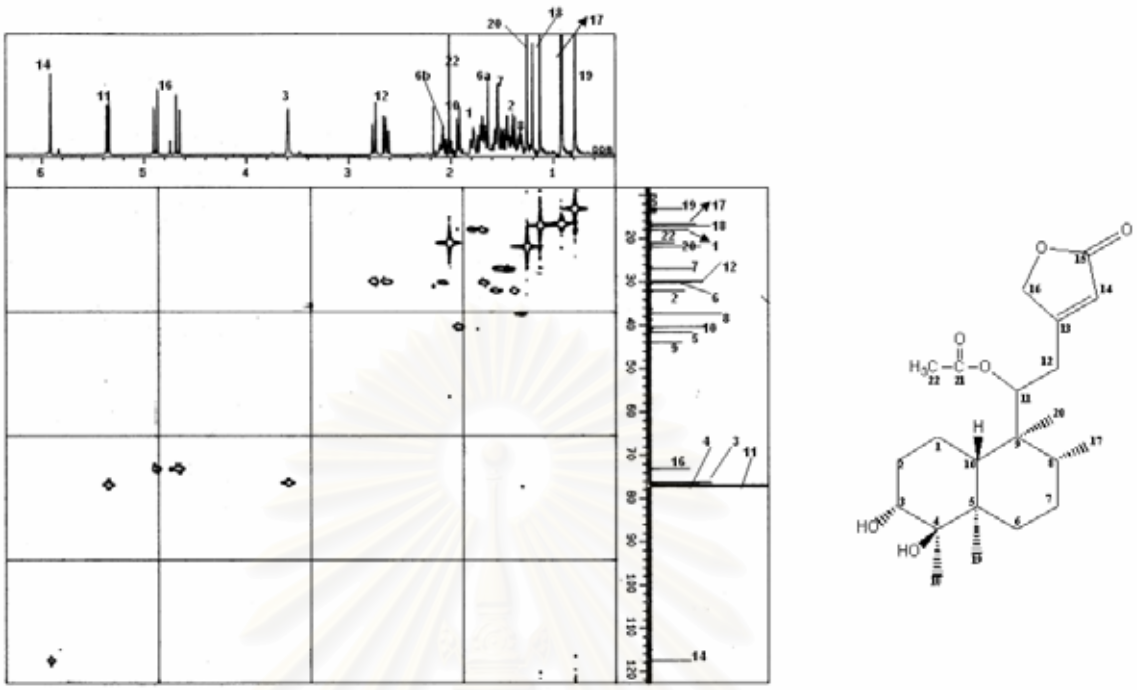

Figure 36a: The 500 MHz HMQC spectrum of compound A-2 (in $CDCl_3$)

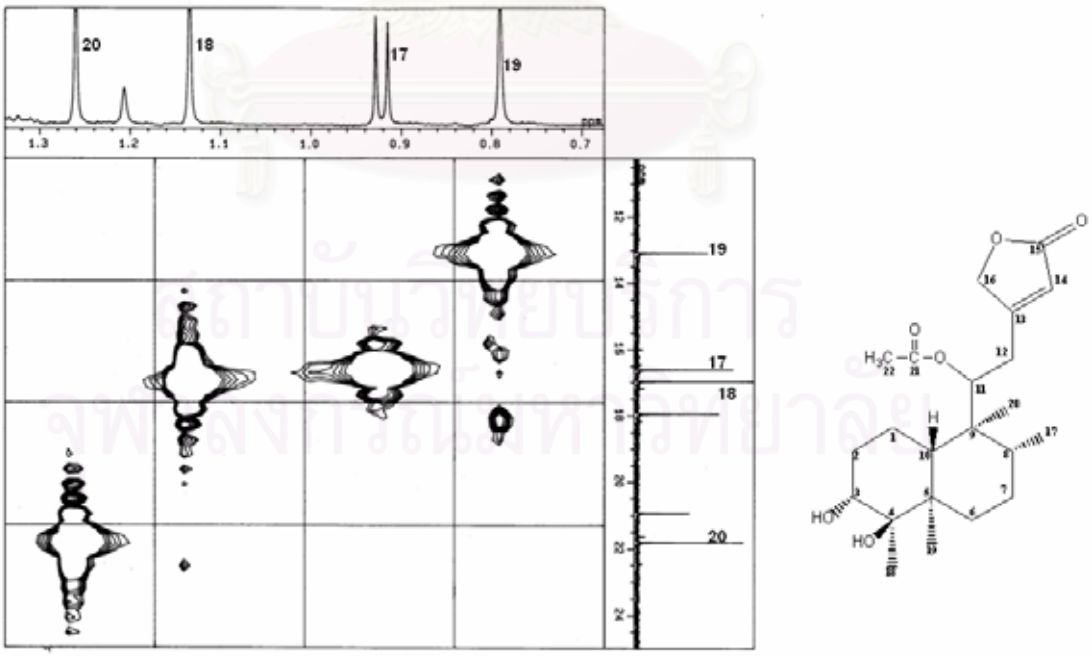

Figure 36b: The expanded 500 MHz HMQC spectrum of compound A-2 (in $CDCl_3$)
(δ_H 0.7-1.3 ppm, δ_C 10.0-24.0 ppm)

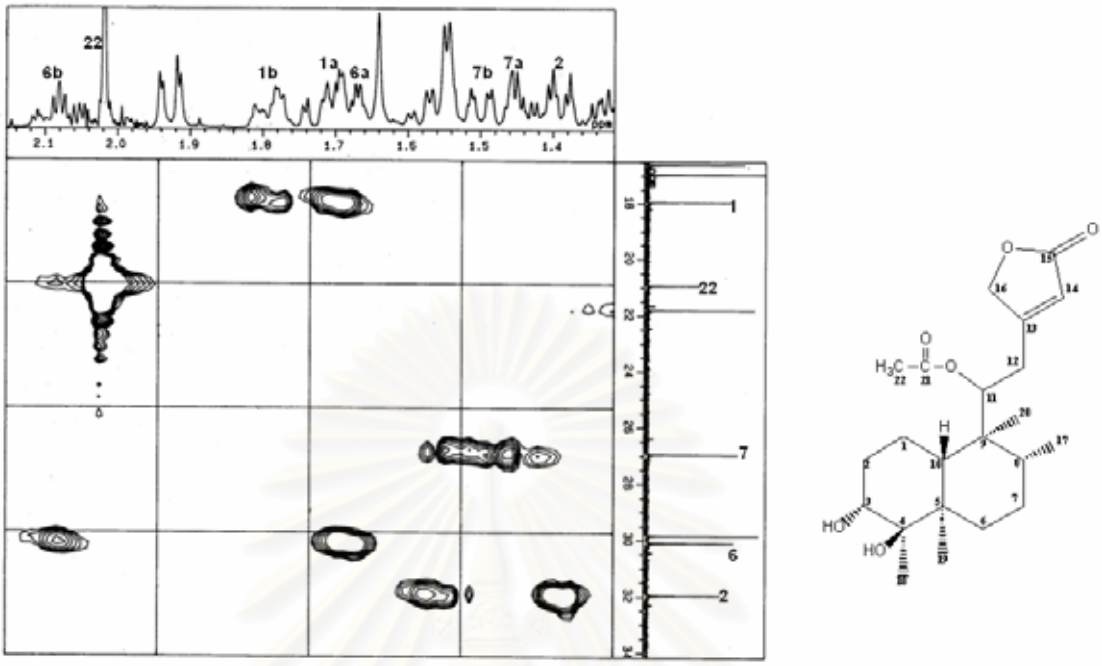

Figure 36c: The expanded 500 MHz HMQC spectrum of compound A-2 (in $CDCl_3$) (δ_H 1.3-2.2 ppm, δ_C 17.0-34.0 ppm)

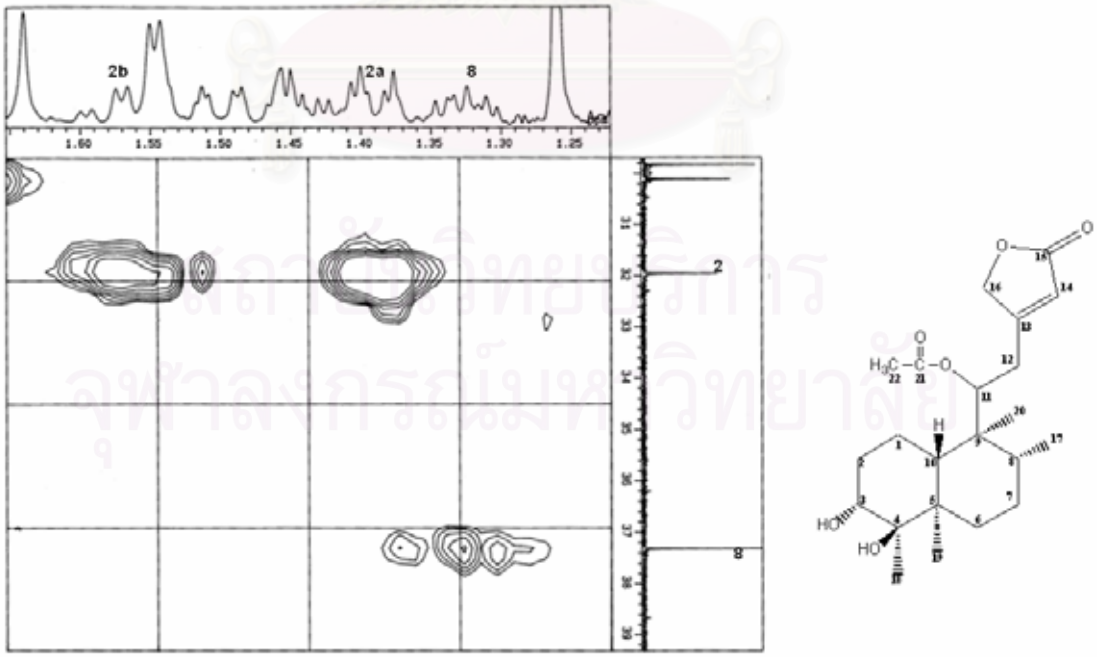

Figure 36d: The expanded 500 MHz HMQC spectrum of compound A-2 (in $CDCl_3$) (δ_H 1.2-1.7 ppm, δ_C 30.0-39.0 ppm)

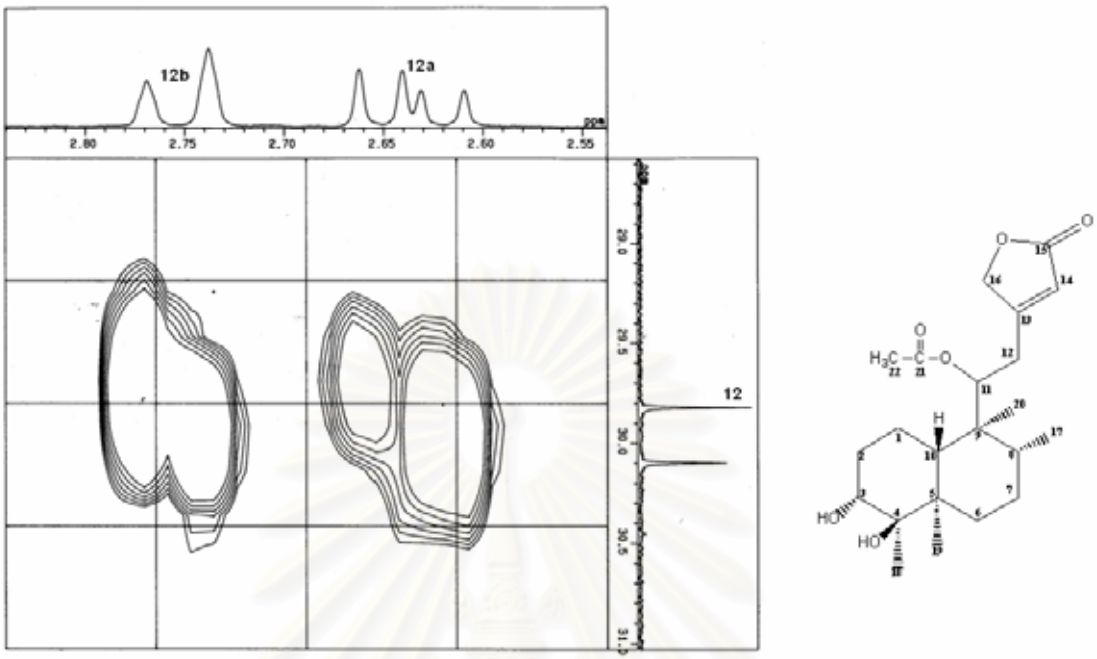

Figure 36e: The expanded 500 MHz HMQC spectrum of compound A-2 (in $CDCl_3$) (δ_H 2.55-2.85 ppm, δ_C 28.5-31.0 ppm)

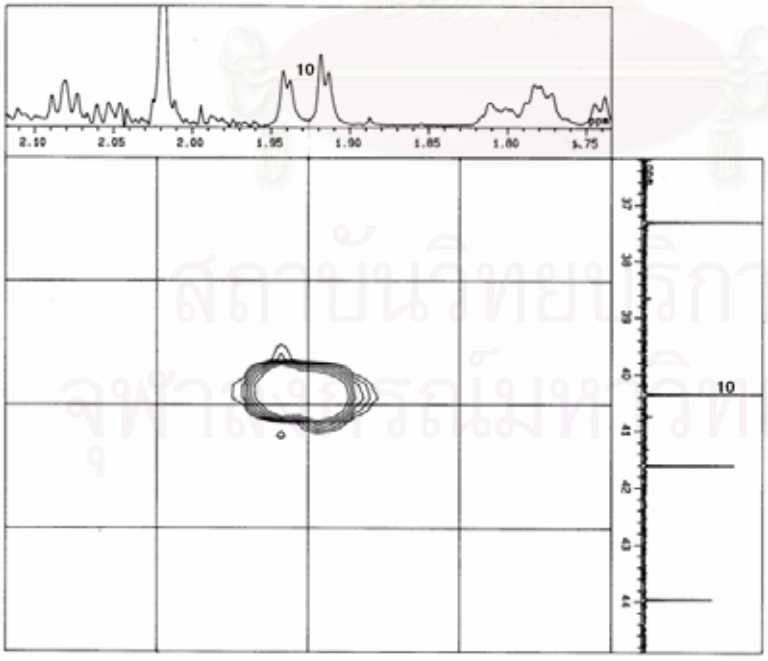

Figure 36f: The expanded 500 MHz HMQC spectrum of compound A-2 (in $CDCl_3$) (δ_H 1.75-2.10 ppm, δ_C 36.0-45.0 ppm)

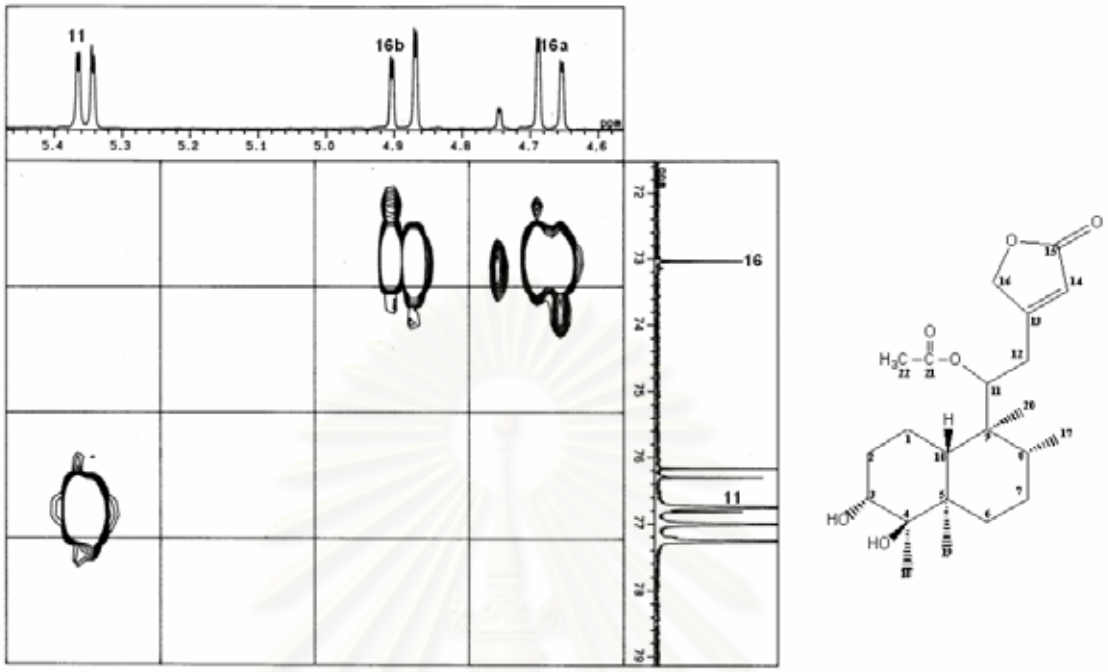

Figure 36g: The expanded 500 MHz HMQC spectrum of compound A-2 (in $CDCl_3$) (δ_H 4.6-5.4 ppm, δ_C 72.0-79.0 ppm)

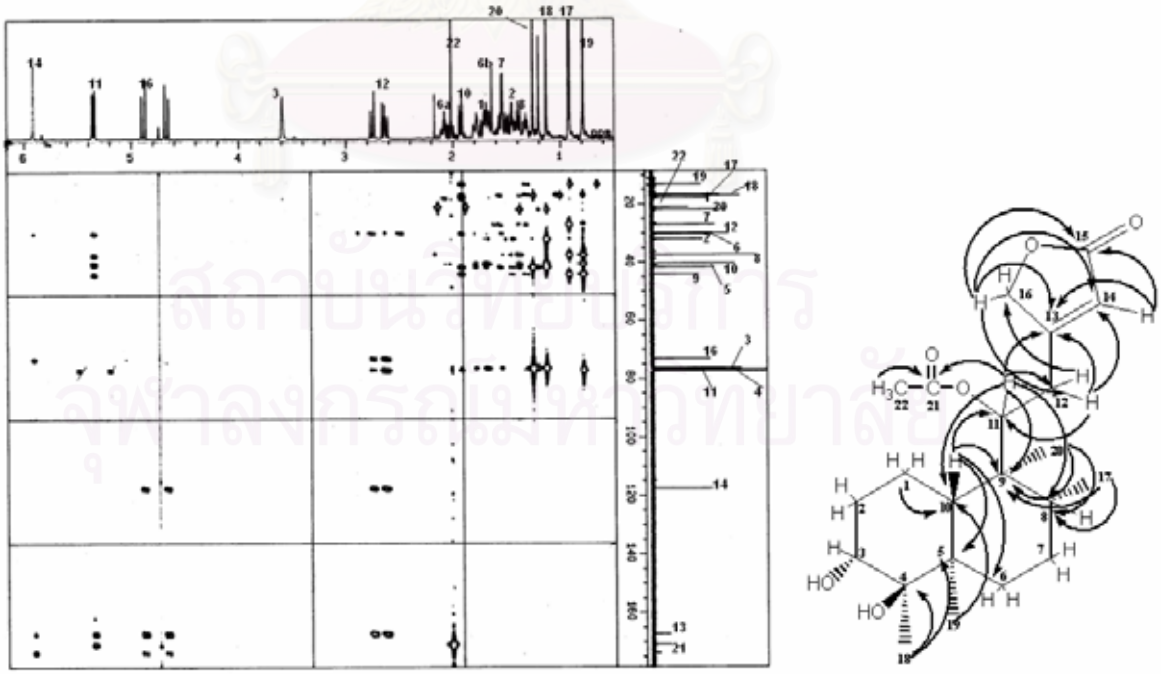

Figure 37a: The 500 MHz HMBC spectrum of compound A-2 (in $CDCl_3$)

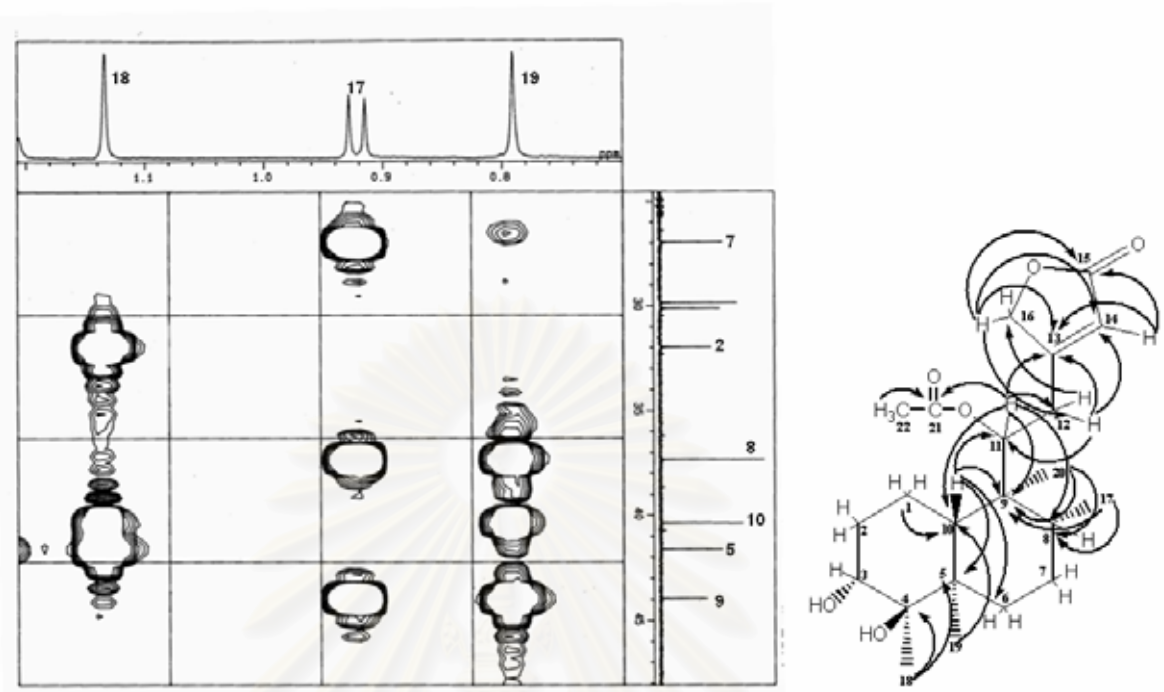

Figure 37b: The expanded 500 MHz HMBC spectrum of compound A-2 (in $CDCl_3$) (δ_H 0.7-1.2 ppm, δ_C 25.0- 48.0 ppm)

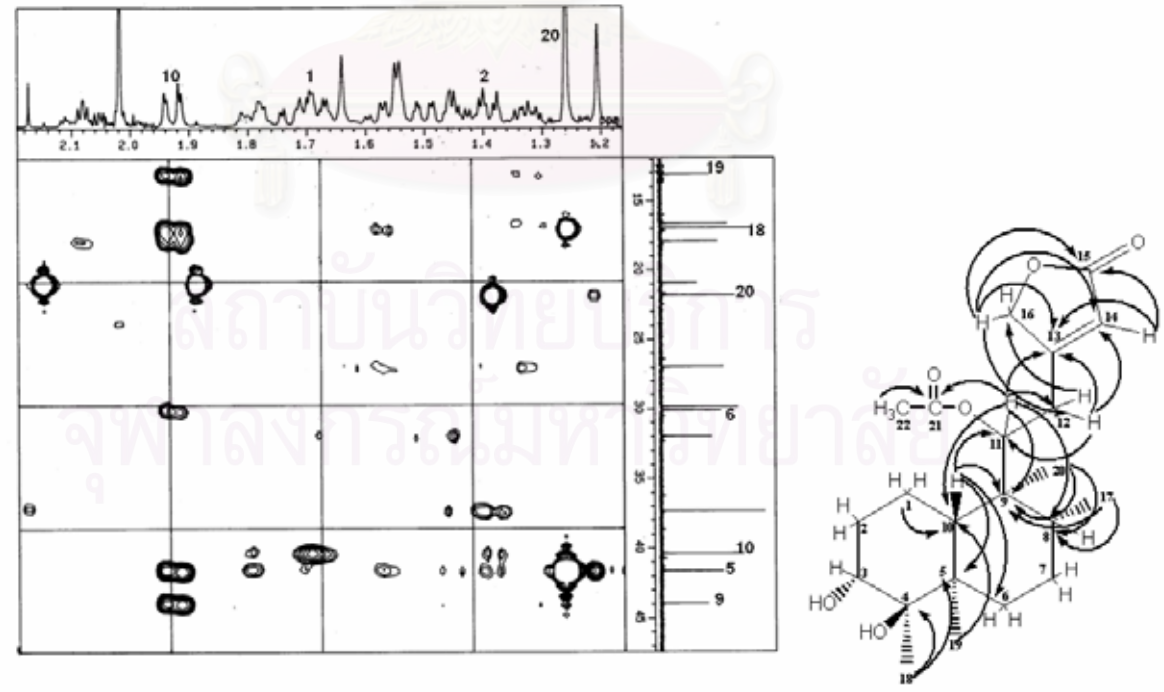

Figure 37c: The expanded 500 MHz HMBC spectrum of compound A-2 (in $CDCl_3$) (δ_H 1.2-2.2 ppm, δ_C 12.0- 47.0 ppm)

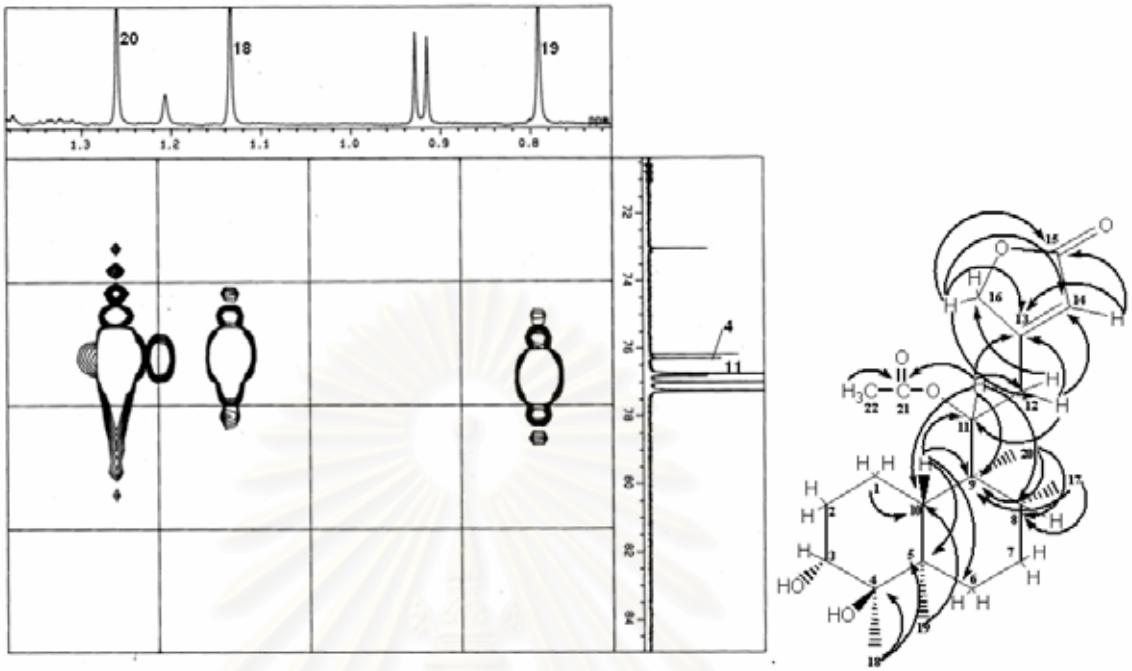

Figure 37d: The expanded 500 MHz HMBC spectrum of compound A-2 (in $CDCl_3$) (δ_H 0.7-1.4 ppm, δ_C 70.0- 85.0 ppm)

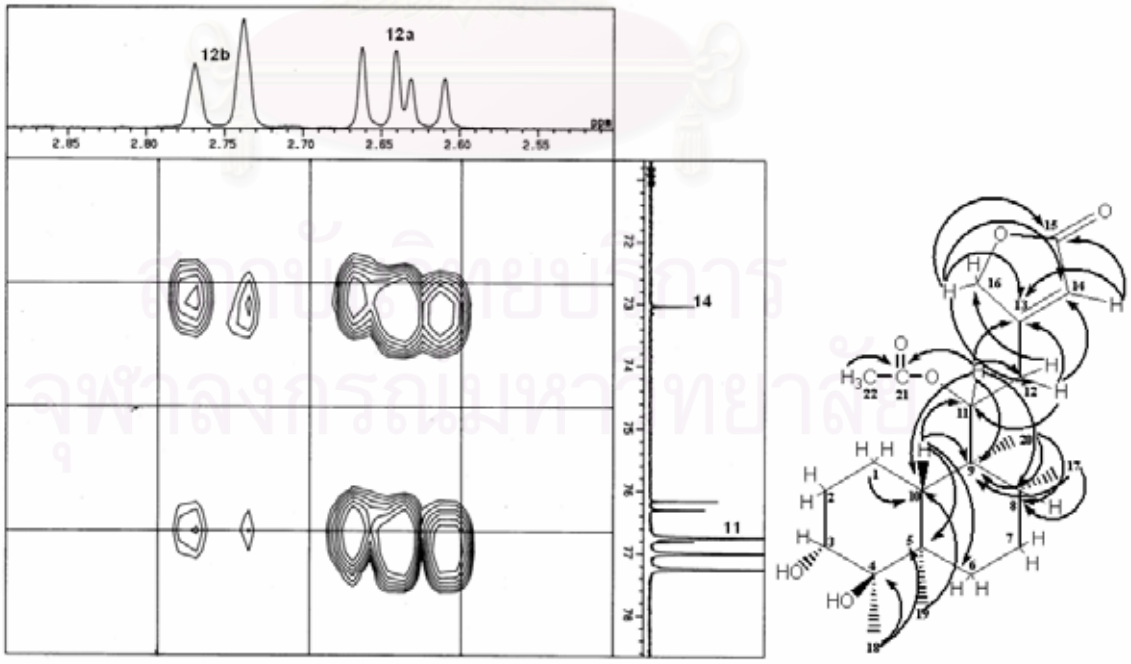

Figure 37e: The expanded 500 MHz HMBC spectrum of compound A-2 (in $CDCl_3$) (δ_H 2.5 - 2.9 ppm, δ_C 71.0- 79.0 ppm)

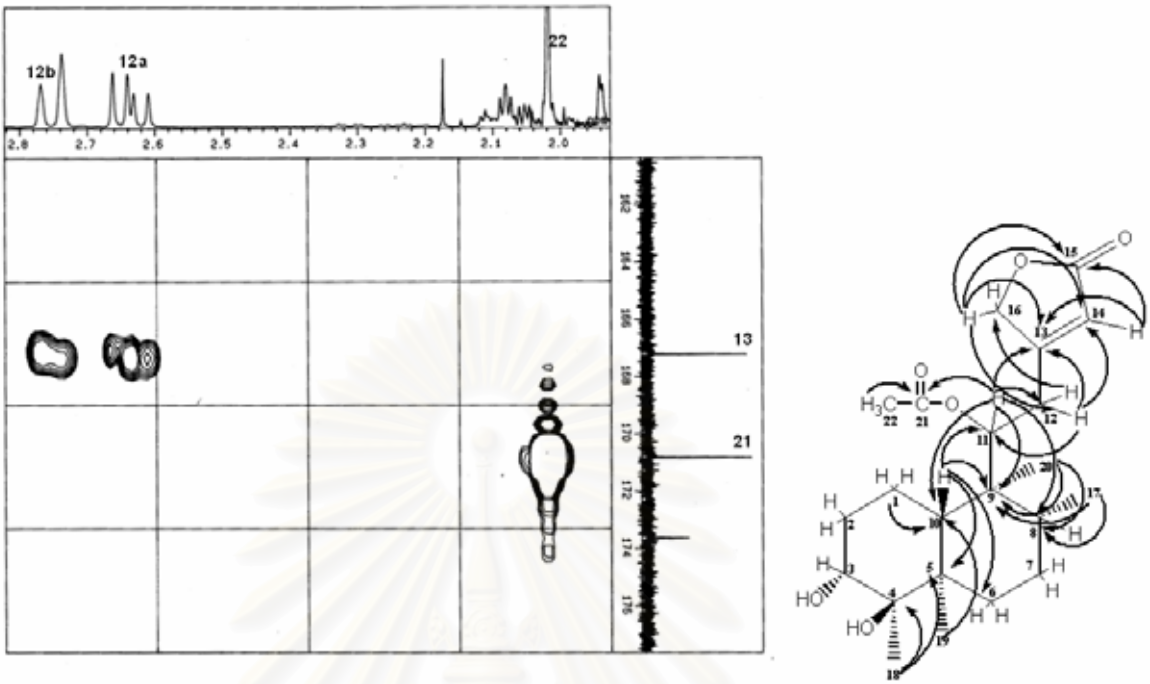

Figure 37f: The expanded 500 MHz HMBC spectrum of compound A-2 (in $CDCl_3$)
(δ_H 2.5 - 2.9 ppm, δ_C 71.0- 79.0 ppm)

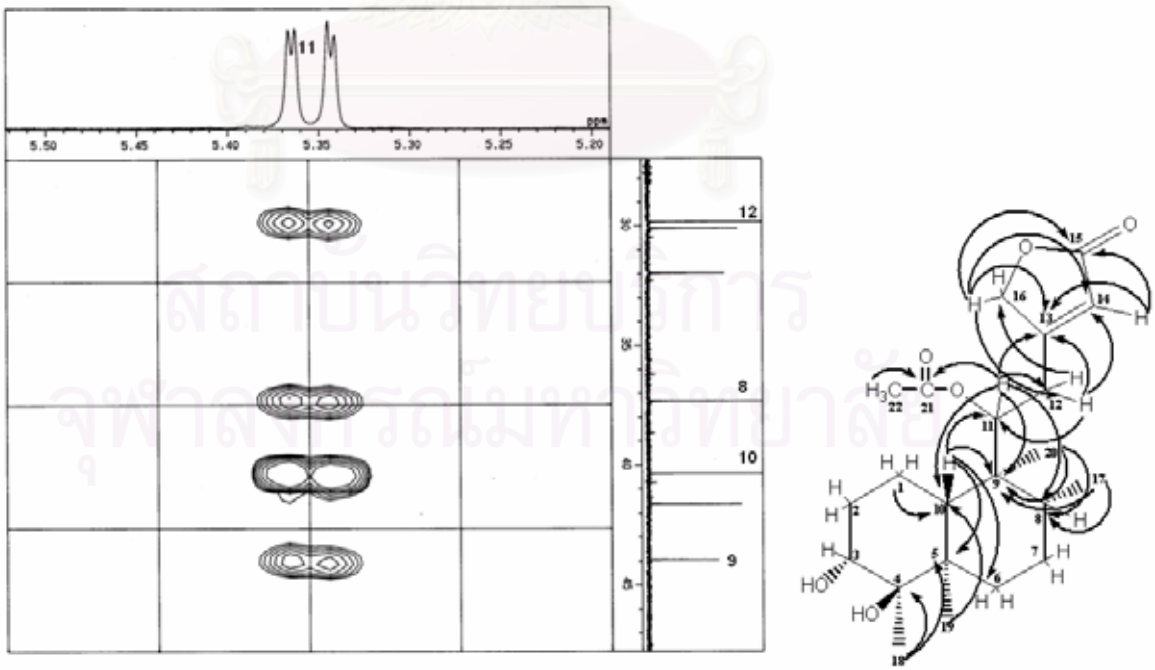

Figure 37g: The expanded 500 MHz HMBC spectrum of compound A-2 (in $CDCl_3$)
(δ_H 5.2 – 5.5 ppm, δ_C 25.0- 50.0 ppm)

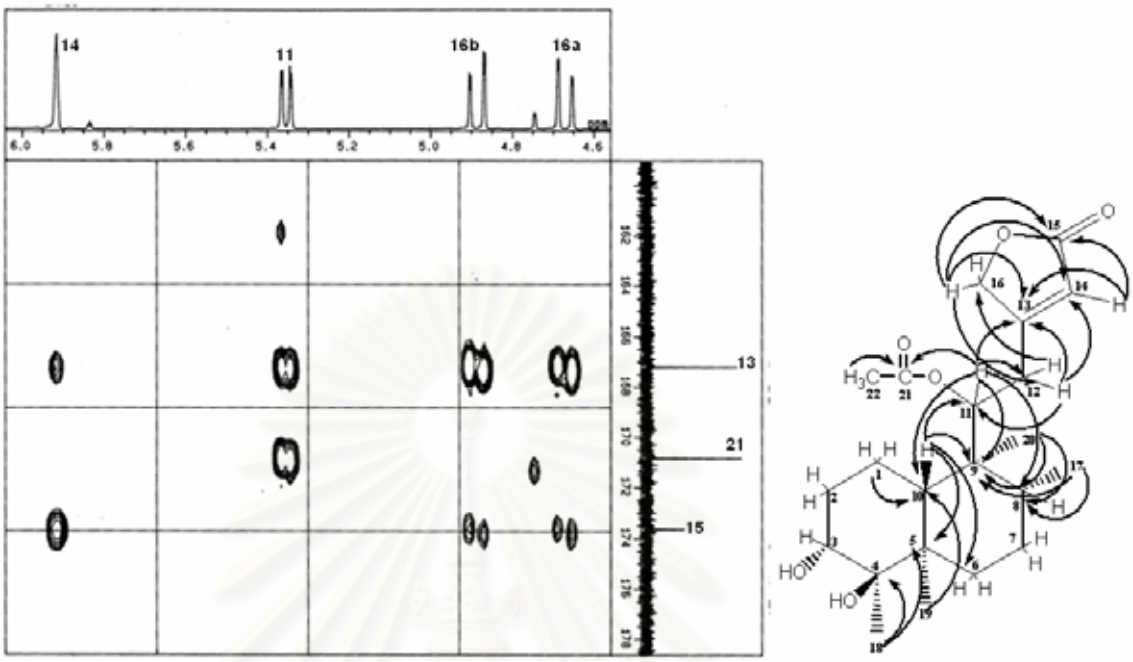

Figure 37h: The expanded 500 MHz HMBC spectrum of compound A-2 (in $CDCl_3$) (δ_H 4.6 – 6.0 ppm, δ_C 160.0- 178.0 ppm)

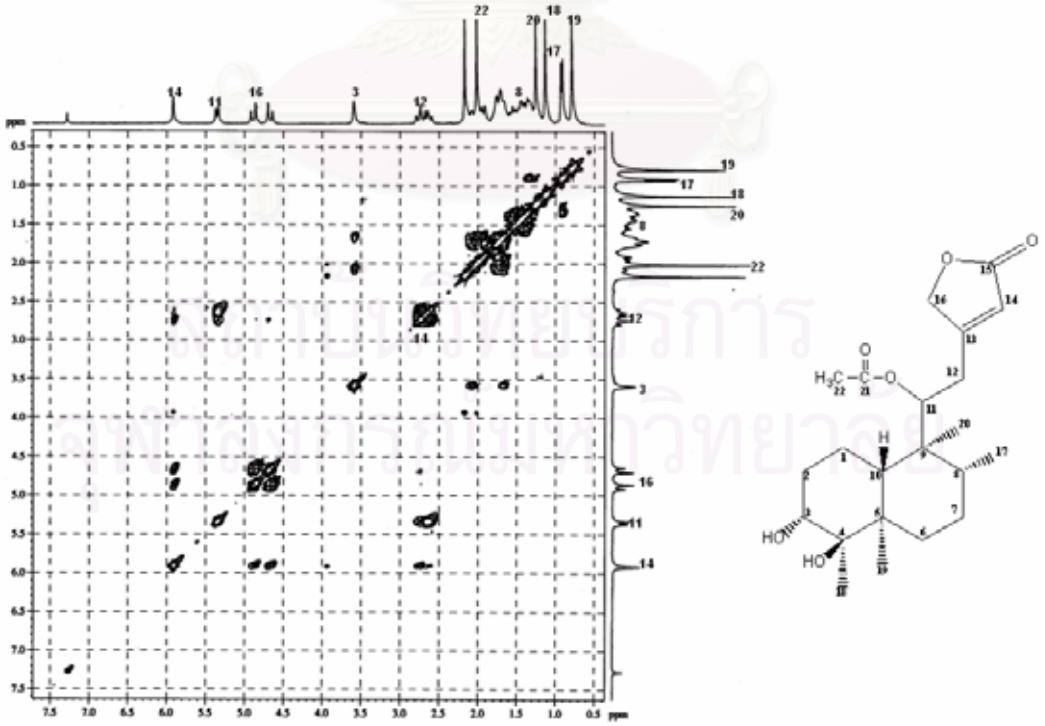

Figure 38a: The 300 MHz ^1H-^1H COSY spectrum of compound A-2 (in $CDCl_3$)

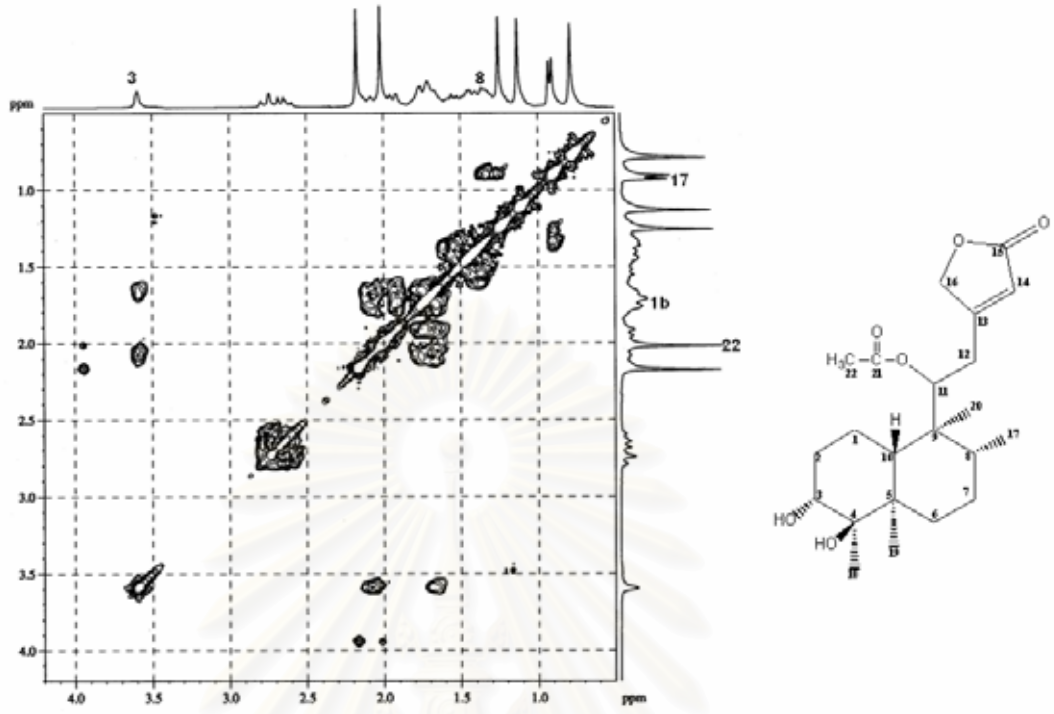

Figure 38b: The expanded 300 MHz ^{1}H-^{1}H COSY spectrum of compound A-2 (in $CDCl_3$) (δ_H 0 – 4.0 ppm)

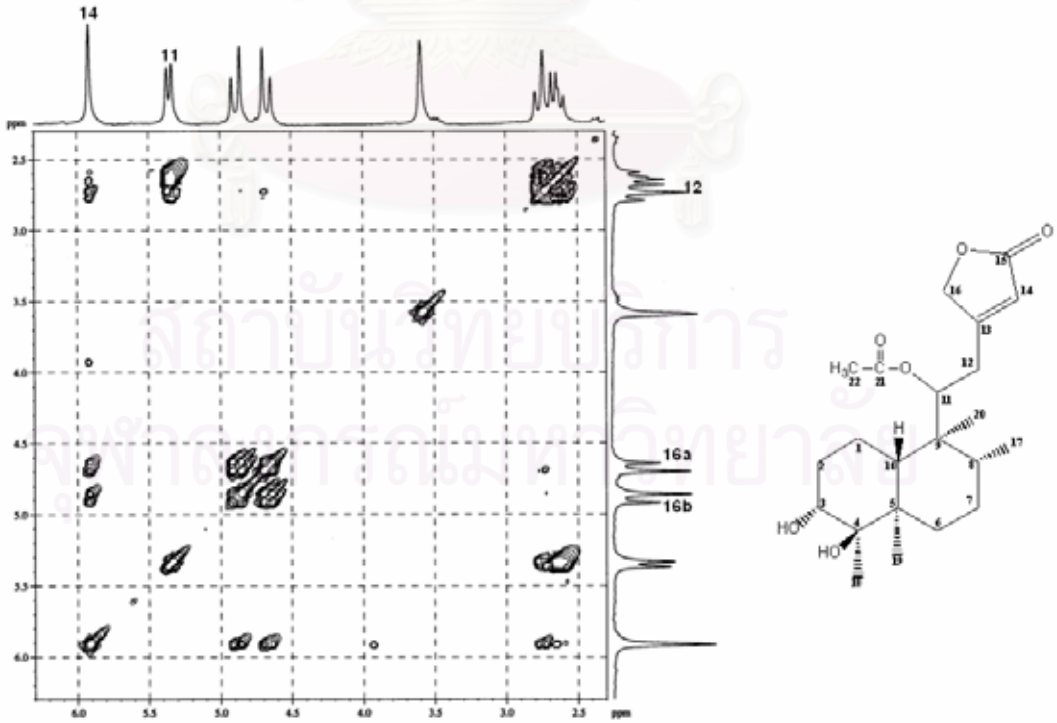

Figure 38c: The expanded 300 MHz ^{1}H-^{1}H COSY spectrum of compound A-2 (in $CDCl_3$) (δ_H 2.5 – 6.5 ppm)

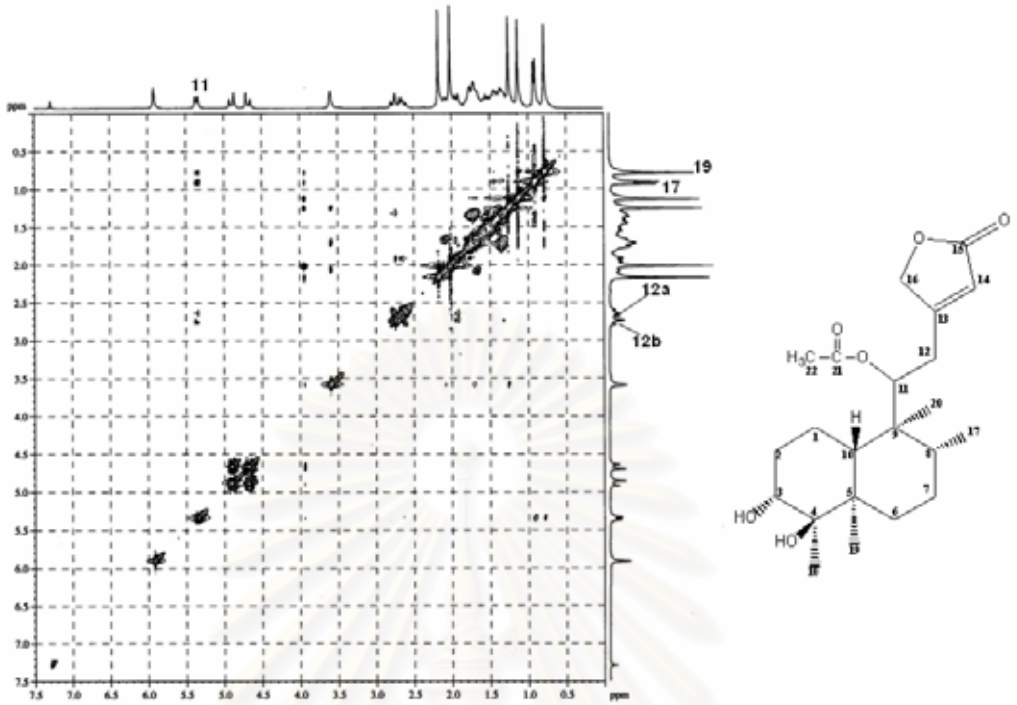

Figure 39a: The 300 MHz NOESY spectrum of compound A-2 (in $CDCl_3$)

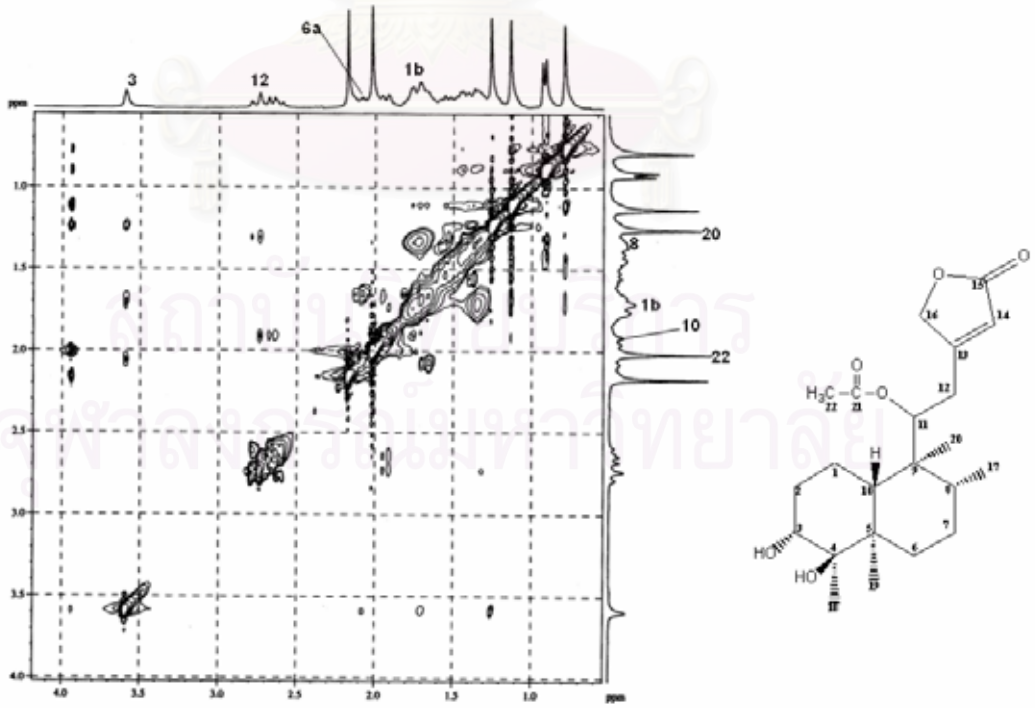

Figure 39b: The expanded 300 MHz NOESY spectrum of compound A-2 (in $CDCl_3$)
(δ_H 0.0 – 4.0 ppm)

Crystal data and structure refinement for Compound A-1

A. Crystal Data

Empirical Formula	$C_{20}H_{32}O_4$
Formula Weight	336.47
Crystal Color, Habit	colorless, prism
Crystal Dimensions	0.47 X 0.35 X 0.32 mm
Crystal System	orthorhombic
Lattice Type	Primitive
No. of Reflections Used for Unit Cell Determination (2θ range)	250 (0.0 - 25.0°)
Lattice Parameters	a = 11.210(2) Å b = 11.456(2) Å c = 14.041(3) Å V = 1803.2(6) Å^3
Space Group	$P2_12_12_1$ (#19)
Z value	4
D_{calc}	1.239 g/cm^3
F_{000}	736.00
μ(MoKα)	0.84 cm^{-1}

B. Intensity Measurements

Diffractometer	Bruker/SMART 1000 CCD
Radiation	MoKα (λ = 0.71069 Å) graphite monochromated
Temperature	-173.0 °C
Voltage, Current	45 kV, 30 mA
Collimator Size	0.5 mm
Detector Aperture	70 mm x 70 mm
Data Images	0 exposures

2θ max	56.8^{o}
No. of Reflections Measured	Total: 10857 Unique: 2418 (R_{int} = 0.066)
Corrections	Lorentz-polarization Secondary Extinction (coefficient: 7.54700e-08)

C. Structure Solution and Refinement

Structure Solution	Direct Methods (SIR97)
Refinement	Full-matrix least-squares
Function Minimized	$\sum w (\|Fo\| - \|Fc\|)^2$
Least Squares Weights	$1/\sigma^2 (Fo) = 4Fo^2/ \sigma^2 (Fo^2)$
p- factor	0.0800
Anomalous Dispersion	All non-hydrogen atoms
No. of Observations ($I>0.00\sigma$ (I), $2\theta < 56.83^{o}$)	2334
No. Variables	219
Reflection/Parameter Ratio	10.66
Residuals: R; Rw	0.056; 0.064
Residuals: R1	0.048
No. of Reflections to calc R1	2027
Goodness of Fit Indicator	0.98
Max Shift/Error in Final Cycle	0.007
Maximum peak in Final Diff. Map	0.52 $e^{-}/Å^3$
Minimum peak in Final Diff. Map	-0.38 $e^{-}/Å^3$

Table 17. Atomic coordinates and equivalent isotropic displacement parameters (B_{iso}/B_{eq}) for compound A-1

atom	x	y	z	B_{eq}
O(1)	1.2261(2)	0.2007(2)	0.4759(1)	1.68(4)
O(2)	1.3834(2)	0.1085(2)	0.5404(1)	1.72(4)
O(3)	0.5810(2)	-0.1707(2)	0.8086(1)	1.70(4)
O(4)	0.5572(2)	0.1120(2)	0.6870(1)	1.40(4)
C(1)	0.8105(2)	-0.0470(2)	0.7575(2)	1.38(5)
C(2)	0.7153(3)	-0.0924(2)	0.6892(2)	1.56(5)
C(3)	0.5910(2)	-0.0877(2)	0.7323(2)	1.38(4)
C(4)	0.5603(2)	0.0360(2)	0.7696(2)	1.13(4)
C(5)	0.6593(2)	0.0871(2)	0.8373(2)	1.07(4)
C(6)	0.6333(2)	0.2174(2)	0.8555(2)	1.21(4)
C(7)	0.7333(2)	0.2797(2)	0.9084(2)	1.34(4)
C(8)	0.8529(2)	0.2730(2)	0.8562(2)	1.24(4)
C(9)	0.8898(2)	0.1451(2)	0.8327(2)	1.06(4)
C(10)	0.7825(2)	0.0798(2)	0.7845(2)	0.99(4)
C(11)	0.9987(2)	0.1459(2)	0.7631(2)	1.27(4)
C(12)	0.9817(2)	0.1913(2)	0.6613(2)	1.41(5)
C(13)	1.0946(2)	0.1839(2)	0.6050(2)	1.17(4)
C(14)	1.1990(2)	0.1354(2)	0.6288(2)	1.38(5)
C(15)	1.2809(2)	0.1444(2)	0.5489(2)	1.32(4)
C(16)	1.1055(2)	0.2307(2)	0.5058(2)	1.38(5)
C(17)	0.9454(3)	0.3407(2)	0.9127(2)	1.69(5)
C(18)	0.4353(2)	0.0350(3)	0.8125(2)	1.64(5)
C(19)	0.6574(2)	0.0212(2)	0.9328(2)	1.26(5)
C(20)	0.9375(2)	0.0839(2)	0.9231(2)	1.45(5)
O(1)	1.2261(2)	0.2007(2)	0.4759(1)	1.68(4)
O(2)	1.3834(2)	0.1085(2)	0.5404(1)	1.72(4)

Table 17. Atomic coordinates and equivalent isotropic displacement parameters (B_{iso}/B_{eq}) for compound A-1 (continued)

atom	x	y	z	B_{eq}
H(1)	0.8911	-0.0586	0.7231	1.4
H(2)	0.8175	-0.0959	0.8187	0.0
H(3)	0.7163	-0.0439	0.6269	1.1
H(4)	0.7302	-0.1668	0.6707	2.3
H(5)	0.5373	-0.1081	0.6773	2.3
H(6)	0.6193	0.2578	0.7965	2.7
H(7)	0.5607	0.2224	0.8975	1.7
H(8)	0.7438	0.2430	0.9787	2.1
H(9)	0.7150	0.3667	0.9190	3.1
H(10)	0.8358	0.3138	0.7935	1.2
H(11)	0.7711	0.1172	0.7213	1.0
H(12)	1.0277	0.0667	0.7547	0.3
H(13)	1.0621	0.2016	0.7957	2.5
H(14)	0.9553	0.2726	0.6637	0.7
H(15)	0.9177	0.1384	0.6302	1.9
H(16)	1.2167	0.0990	0.6893	2.1
H(17)	1.0945	0.3151	0.5070	1.8
H(18)	1.0438	0.1927	0.4605	1.1
H(19)	1.0224	0.3479	0.8852	1.3
H(20)	0.9229	0.4284	0.9171	1.9
H(21)	0.9531	0.3130	0.9775	2.0
H(22)	0.3807	-0.0010	0.7647	1.1
H(23)	0.4293	-0.0143	0.8697	2.1
H(24)	0.4168	0.1170	0.8369	1.9
H(25)	0.6808	-0.0606	0.9335	3.0
H(26)	0.7104	0.0506	0.9766	1.4
H(27)	0.5795	0.0233	0.9618	2.1
H(28)	0.8842	0.0786	0.9775	2.3
H(29)	0.9572	0.0037	0.9075	2.7
H(30)	1.0102	0.1129	0.9464	1.6

Table 18. Bond lengths [ºA] for compound A-1

atom	atom	Bond lengths [ºA]
O(1)	C(15)	1.358(3)
O(1)	C(16)	1.457(3)
O(2)	C(15)	1.226(3)
O(3)	C(3)	1.437(3)
O(4)	C(4)	1.451(3)
C(1)	C(10)	1.534(4)
C(2)	C(3)	1.520(4)
C(3)	C(4)	1.549(4)
C(4)	C(5)	1.575(3)
C(4)	C(18)	1.525(3)
C(5)	C(6)	1.543(4)
C(5)	C(10)	1.569(3)
C(5)	C(19)	1.539(3)
C(6)	C(7)	1.523(4)
C(7)	C(8)	1.530(4)
C(8)	C(9)	1.558(3)
C(8)	C(17)	1.519(3)
C(9)	C(10)	1.571(3)
C(9)	C(11)	1.563(4)
C(9)	C(20)	1.544(3)
C(11)	C(12)	1.533(3)
C(12)	C(13)	1.494(3)
C(13)	C(14)	1.338(4)
C(13)	C(16)	1.498(3)
C(14)	C(15)	1.454(4)

Table 18. Bond lengths [°A] for compound A-1 (continued)

atom	atom	Bond lengths [°A]
C(1)	H(1)	1.03
C(1)	H(2)	1.03
C(2)	H(3)	1.04
C(2)	H(4)	0.91
C(3)	H(5)	1.01
C(6)	H(6)	0.96
C(6)	H(7)	1.01
C(7)	H(8)	1.08
C(7)	H(9)	1.03
C(8)	H(10)	1.01
C(10)	H(11)	0.99
C(11)	H(12)	0.97
C(11)	H(13)	1.06
C(12)	H(14)	0.98
C(12)	H(15)	1.04
C(14)	H(16)	0.97
C(16)	H(17)	0.97
C(16)	H(18)	1.03
C(17)	H(19)	0.95
C(17)	H(20)	1.04
C(17)	H(21)	0.97
C(18)	H(22)	1.00
C(18)	H(23)	0.98
C(18)	H(24)	1.02
C(19)	H(25)	0.97
C(19)	H(26)	0.92
C(19)	H(27)	0.96
C(20)	H(28)	0.97
C(20)	H(29)	0.97
C(20)	H(30)	0.94

VITA

Miss Praworada Cholsuk was born on March 4th, 1972 in Bangkok, Thailand. She received her Bachelor's degree of Science in Pharmacy in 1995 from the faculty of Pharmacy, Mahidol University, Thailand and received her Graduate Diploma in English for Careers in 1999 from the Language Institute, Thammasat University, Thailand. She is presently working as the position of Regulatory Affairs Associate at Pfizer (Thailand) Limited Company.