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ปีการศึกษา 2554

PHYTOCHEMICAL STUDY OF *DIOSPYROS COLLINSAE* STEMS

Miss Buntita Bumroong

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Pharmacy Program in Pharmaceutical Botany

Department of Pharmacognosy and Pharmaceutical Botany

Faculty of Pharmaceutical Sciences

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Thesis Title	PHYTOCHEMICAL STUDY OF *DIOSPYROS COLLINSAE* STEMS
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Field of Study	Pharmaceutical Botany
Thesis Advisor	Witchuda Thanakijcharoenpath, Ph.D.

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อ. ที่ปรึกษาวิทยานิพนธ์หลัก : อ.ดร.วิชชุดา ธนกิจเจริญพัฒน์, 162 หน้า.

จากการศึกษาทางพฤกษเคมีของลำต้นพลับยอดดำ (วงศ์ Ebenaceae) สามารถแยกได้สารในกลุ่มไตรเทอร์ปีนอยด์ 4 ชนิด คือ friedelin, lupeol, betulin และ betulinic acid และสารในกลุ่มสติลบินอยด์ 2 ชนิด คือ diptoindonesin D และ diptoindonesin G รวมทั้งสารผสมของ β-sitosterol กับ stigmasterol การพิสูจน์โครงสร้างทางเคมีของสารที่แยกได้ทำโดยอาศัยการวิเคราะห์เชิงสเปกตรัมด้วย UV, IR, MS และ NMR ร่วมกับการเปรียบเทียบกับข้อมูลที่เคยมีรายงานมาก่อนแล้ว สาร diptoindonesin G แสดงฤทธิ์ต้านเชื้อมาลาเรีย *Plasmodium falciparum* โดยมีค่า IC_{50} เท่ากับ 6.88 ไมโครกรัมต่อมิลลิลิตร และแสดงฤทธิ์เป็นพิษต่อเซลล์มะเร็งปอดของมนุษย์ชนิด NCI-H187 และเซลล์มะเร็งเต้านมของมนุษย์ชนิด MCF-7 โดยมีค่า IC_{50} เท่ากับ 1.94 และ 43.28 ไมโครกรัมต่อมิลลิลิตรตามลำดับ การศึกษานี้ได้ให้หลักฐานที่แสดงถึงการสร้างสารในกลุ่มสติลบินอยด์ของพืชสกุล *Diospyros* เป็นครั้งแรก

ภาควิชา เภสัชเวทและเภสัชพฤกษศาสตร์ ลายมือชื่อนิสิต.....
สาขาวิชา เภสัชพฤกษศาสตร์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....
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KEYWORDS : *DIOSPYROS COLLINSAE* / TRITERPENOIDS / STEROIDS / STILBENOIDS

BUNTITA BUMROONG : PHYTOCHEMICAL STUDY OF *DIOSPYROS COLLINSAE* STEMS. ADVISOR : WITCHUDA THANAKIJCHAROENPATH, Ph.D., 162 pp.

Phytochemical study of the stems of *Diosyros collinsae* Craib (family Ebenaceae) led to the isolation of four triterpenoids, namely friedelin, lupeol, betulin and betulinic acid, two stilbenoids, diptoindonesin D and diptoindonesin G, together with a mixture of β-sitosterol and stigmasterol. Identification of the isolated compounds has been accomplished by spectroscopic analyses, including UV, IR, MS and NMR, and comparison with previously reported data. Diptoindonesin G exhibited antimalarial activity against *Plasmodium falciparum* with an IC_{50} value of 6.88 µg/ml as well as cytotoxic activity against human small-cell lung cancer (NCI-H187) and breast adenocarcinoma (MCF-7) cell lines with IC_{50} values of 1.94 and 43.28 µg/ml, respectively. The present study provided the first evidence for the production of stilbenoids in *Diospyros* Species.

Department : Pharmacognosy and Pharmaceutical Botany Student's Signature Buntita B.

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LIST OF ABBREVIATIONS

α	=	Alpha
β	=	Beta
br s	=	Broad singlet (for NMR spectra)
°C	=	Degree Celsius
Calcd	=	Calculated
CC	=	Column chromatography
$CDCl_3$	=	Deuterated chloroform
CH_2Cl_2	=	Dichloromethane
cm	=	Centimeter
cm^{-1}	=	Reciprocal centimeter (unit of wave number)
^{13}C NMR	=	Carbon-13 Nuclear Magnetic Resonance
CPM_T	=	Count per minute of treated
CPM_U	=	Count per minute of untreated
2D NMR	=	Two dimensional Nuclear Magnetic Resonance
d	=	Doublet (for NMR spectra)
dd	=	Doublet of doublets (for NMR spectra)
ddd	=	Doublet of doublets of doublets (for NMR spectra)
DMSO-d_6	=	Deuterated dimethyl sulfoxide
δ	=	Chemical shift
ε	=	Molar absorptivity
ESI-MS	=	Electrospray Ionization Mass Spectrometry
EtOAc	=	Ethyl acetate
FU_C	=	Fluorescent unit from treated
FU_T	=	Fluorescent unit from untreated
g	=	Gram
h	=	Hour
^1H NMR	=	Proton Nuclear Magnetic Resonance

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

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

CHAPTER I

INTRODUCTION

The genus *Diospyros*, which includes several plants of medicinal importance, is the largest genus of the family Ebenaceae. The genus contains about 450-500 species of deciduous and evergreen shrubs and trees. Plants of this genus are mainly found in the tropics with a few in the subtropics. The characteristic features of *Diospyros* plants, described by Phengklai (1981), are as follows.

"Trees or shrubs, dioecious, sometimes monoecious or polygamous; mostly unarmed; all parts often turning blackish when dry. *Leaves* distichous, and mostly reflexed, penninerved. *Inflorescences* cymose or fasciculate, axillary or ramiflorous, rarely cauliflorous, or flowers solitary. *Flowers* actinomorphic. *Calyx* more or less deeply lobed, persistent and usually accrescent in fruit; lobes valvate or imbricate in bud. *Corolla* gamopetalous, caduceus; segments patent, contorted in bud. *Stamens* 6-∞, free, or in pairs, on the base of corolla-tube, or in bundles on receptacle; anthers basifixed, 2-locular, longituditionally dehiscent. *Rudimentary ovary* usually present in male flowers. *Staminodes* usually present in female flowers. *Ovary* superior, (3-)4(-16)-locular; ovules 1(-2) in each locule, pendulous; styles 1-5. *Fruit* indehiscent, fleshy, dry or woody, 1-many-seeded; endosperm ruminate or smooth."

Sixty eight *Diospyros* species found in Thailand have been recorded as follows. (กรมป่าไม้, สำนักงานวิชาการป่าไม้, ส่วนพฤกษศาสตร์ป่าไม้, พ.ศ. 2544; Phengklai, 2005).

1. *Diospyros andamanica* (Kurz) Bakh. var. *aequabilis* Bakh.
2. *D. apiculata* Hiern
3. *D. areolata* King & Gamble
4. *D. bambuseti* Fletcher
5. *D. bejaudii* Lec.
6. *D. borneensis* Hiern
7. *D. brandisiana* Kurz

8. *D. buxifolia* (Bl.) Hiern
9. *D. castanea* Fletcher
10. *D. cauliflora* Bl.
11. *D. coaetanea* (Craib) Fletcher
12. *D. collinsae* Craib
13. *D. confertiflora* (Hiern) Bakh.
14. *D. curranii* Merr.
15. *D. curraniopsis* Bakh.
16. *D. dasyphylla* Kurz
17. *D. decandra* Lour.
18. *D. dictyoneura* Hiern
19. *D. diepenhorstii* Miq.
20. *D. dumetorum* W. W. Sm.
21. *D. ehretioides* Wall. ex G. Don
22. *D. ferrea* (Willd.) Bakh. var. *ferrea* (Willd.) Bakh.
23. *D. ferrea* (Willd.) Bakh. var. *littorea* (R.Br.) Bakh.
24. *D. filipendula* Pierre ex Lec.
25. *D. frutescens* Bl.
26. *D. fulvopilosa* Fletcher
27. *D. glandulosa* Lace
28. *D. gracillis* Fletcher
29. *D. hasseltii* Zoll.
30. *D. insidiosa* Bakh.
31. *D. kaki* L. (exotic plant)
32. *D. kerrii* Craib
33. *D. kurzii* Hiern
34. *D. lanceifolia* Roxb.
35. *D. latisepala* Ridl.
36. *D. longipilosa* Phengklai

37. *D. malabarica* (Desr.) Kostel. var. *malabarica* Kostel.
38. *D. malabarica* (Desr.) Kostel. var. *siamensis* (Hochr.) Phengklai
39. *D. martabanica* C.B. Clarke
40. *D. mollis* Griff.
41. *D. montana* Roxb.
42. *D. oblonga* Wall. ex G. Don
43. *D. pendula* Hasselt ex Hassk.
44. *D. philippensis* A. DC. (exotic plant)
45. *D. phuketensis* Phengklai
46. *D. pilosanthera* Blanco
47. *D. pilosula* (A. DC.) Hiern
48. *D. pubicalyx* Bakh.
49. *D. pyrrhocarpa* Miq.
50. *D. ranongensis* Phengklai
51. *D. rhodocalyx* Kurz
52. *D. rubra* Lec.
53. *D. scalariformis* Fletcher
54. *D. scortechinii* King & Gamble
55. *D. sumatrana* Miq.
56. *D. tahanensis* Bakh.
57. *D. thaiensis* Phengklai
58. *D. toposia* Buch-Ham. var. *toposia* Buch-Ham.
59. *D. toposia* Buch-Ham. var. *toposioides* (King & Gamble) Phengklai
60. *D. transitoria* Bakh.
61. *D. trianthos* Phengklai
62. *D. truncata* Zoll. ex Moritzi
63. *D. undulata* Wall. ex G. Don var. *cratericalyx* (Craib) Bakh.
64. *D. undulata* Wall. ex G. Don var. *undulata* Wall.
65. *D. variegata* Kurz

66. *D. venosa* Wall. ex A. DC.

67. *D. wallichii* King & Gamble

68. *D. winitii* Fletcher

Diospyros plants have long been known for their medicinal properties. Many of them have been employed for the treatment of various disorders and diseases in several countries. In Thailand, the most well-known example is *D. mollis*, known in Thai as "Ma kluea" (มะเกลือ), the fruit of which is used as an anthelmintic. Several isolated compounds as well as extracts from *Diospyros* species have been found to exhibit interesting biological activities. Therefore, *Diospyros* can be considered as one of the interesting plant genera for phytochemical investigation.

The subject of this study, *Diospyros collinsae* Craib, is a *Diospyros* plant endemic to Thailand. Its Thai vernacular name is "Phlap yot dam" (พลับยอดดำ). Morphological description of this plant, according to Phengklai (1981), is as follows.

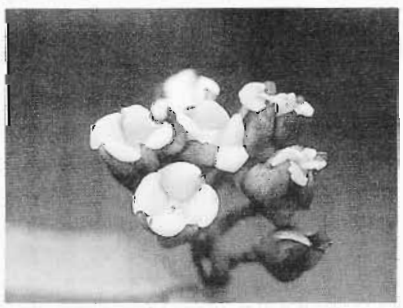
"Evergreen tree, up to 6 m high; terminal bud with black hairs. *Leaves* oblong, oblanceolate, 15-23 by 4-6 cm, base obtuse or slightly acute, apex acute to acuminate, rarely rounded, coriaceous, glabrous except for sparsely blackish hairs along midrib and nerves on lower surface; lateral veins 9-12 pairs, arched and anastomosing well away from the margin, conspicuous on both sides; reticulation fine, prominent on upper surface, conspicuous on lower surface; petiole 1-1.4 cm long, sparsely pilose later glabrescent. *Male flowers* cymose, 4-merous; pedicel ± 4 mm long, tomentose. *Calyx* ovoid, 4-6 mm long, divided to one sixth, tomentose outside, glabrous inside. *Corolla* urceolate or ovoid, 6-8 mm long, divided to a quarter, pubescent outside, glabrous inside. *Stamens* 14-20, glabrous. *Rudimentary ovary* sericeous. *Female fowers* and *fruit* unknown."

D. collinsae Craib is one of Thai *Diospyros* species with no previous phytochemical report. This study was therefore conducted in order to investigate the

chemical constituents of the stems of this plant. The result obtained would contribute to the knowledge on chemical nature of the genus *Diospyros*, thus providing useful information in the fields of phytochemistry and chemotaxonomy.

A

B



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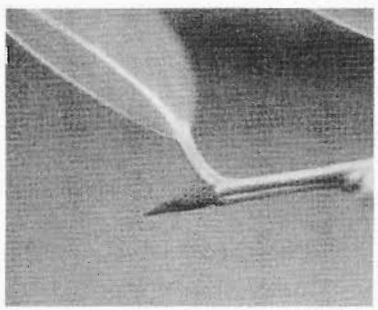

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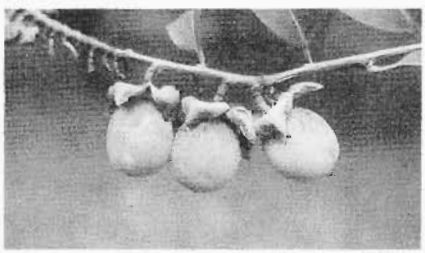


E

Figure 1. *Diospyros collinsae* Craib

A) Tree, B) Leaves, C) Flowers, D) Terminal buds, E) Fruits

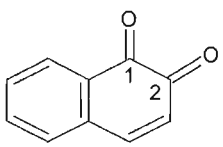
CHAPTER II
HISTORICAL

1. Chemical Constituents of *Diospyros* Species

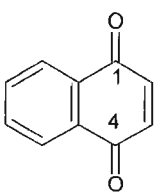
A number of *Diospyros* species have been phytochemically investigated, revealing a wide range of phytochemicals as their chemical constituents. These phytochemicals include naphthoquinones and naphthalene derivatives, triterpenoids, steroids, flavonoids, coumarins, tannins, and others. Of these, naphthoquinones and triterpenoids are widely found and considered as major constituents of *Diospyros* plants. The presence of these two groups of compounds in the genus *Diospyros* is summarized as below.

1.1 Naphthoquinones

The genus *Diospyros* is a rich source of 1,4-naphthoquinones, while only a few 1,2-naphthoquinones have been found. The former type of quinones can be used as chemotaxonomic markers of the genus. Almost all 1,4-naphthoquinones found in *Diospyros* plants are derivatives of plumbagin (1) and 7-methyljuglone (2), both of which are widely distributed in the genus. Partially reduced quinone derivatives have also been isolated. Both monomeric and oligomeric forms of 1,4-naphthoquinones can also be found. The majority of the compounds are of the oligomeric group which includes dimers, trimers, and tetramers.



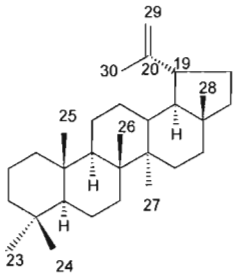
1,2-naphthoquinones



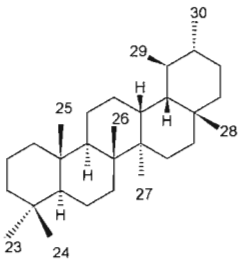
1,4-naphthoquinones

1.2 Triterpenoids

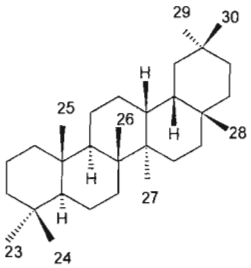
Triterpenoids are widespread in the genus *Diospyros*. Almost all of the compounds found in this genus contain the pentacyclic skeleton. Pentacyclic triterpenoids frequently found in *Diospyros* plants belong to three types: lupanes, ursanes and oleananes. The most common type is the lupanes, of which the major derivatives are lupeol, betulin and betulinic acid. The ursanes are represented by α-amyrin and ursolic acid, while the oleananes is represented by oleanolic acid. Few derivatives of other pentacyclic types have been isolated from *Diospyros* plants. Tetracyclic triterpenoids are relatively rare in the genus *Diospyros*. So far, all isolated compounds of this group belong to the lanostane type.



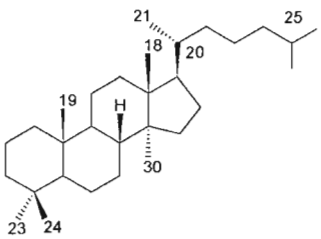
lupane type



ursane type



oleanane type



lanostane type

Chemical compounds found in *Diospyros* species has been compiled (Mallavadhani *et al.*, 1998). The present work provides further compilation of data reported in the literature since 1998, dealing with naphthalene-based aromatics, including naphthoquinones and naphthalene derivatives, and triterpenoids, as shown in Tables 1 and 2, respectively.

Table 1. Distribution of naphthoquinones and naphthalene derivatives in the genus *Diospyros*

Compound	Species	Reference
1.1 Naphthoquinones		
plumbagin (1)	*D. anisandra*	Borges-Argáez *et al.*, 2007
	D. assimilis	Ganapaty *et al.*, 2006
	D. canaliculata	Kuete *et al.*, 2009
	D. crassiflora	Tangmouo *et al.*, 2006
	D. greeniwayi	Khan and Rwekika, 1998
	D. maritima	Higa *et al.*, 2002
	D. novoguinensis	Khan and Timi, 1999c
	D. olen	Evans *et al.*, 1999
	D. sylvatica	Ganapaty *et al.*, 2004
7-methyljuglone (2)	*D. greeniwayi*	Khan and Rwekika, 1998
	D. hallierii	Khan and Timi, 1999a
	D. lycioides	Cai *et al.*, 2000
	D. mafiensis	Khan and Rwekika, 1999
	D. novoguianensis	Khan and Timi, 1999c
	D. paniculata	Sinha, Bansal and Pattnaik, 2009

Table 1. Distribution of naphthoquinones and naphthalene derivatives in the genus *Diospyros* (continued)

Compound	Species	Reference
7-methyljuglone (2)	*D. virginiana*	Wang *et al.*, 2011
2,3-epoxyplumbagin (3)	*D. maritima*	Higa *et al.*, 2002
6-hydroxy-5-methoxy-2-methyl-1,4-naphthoquinone (4)	*D. maritima*	Chang, Huan and Kuo, 2007
shinanolone (5)	*D. virginiana*	Wang *et al.*,2011
epi-isoshinanolone (6)	*D. maritima*	Chang *et al.*, 2009
canaliculatin (7)	*D. canaliculata*	Tangmouo *et al.*, 2005
cyclocanaliculatin (8)	*D. crassiflora*	Tangmouo *et al.*, 2006
crassiflorone (9)	*D. crassiflora*	Tangmouo *et al.*, 2006
mamegakinone (10)	*D. chamaethamnus*	Costa *et al.*,1998
	D. lycioides	Li, Van der Bijl and Wu, 1998
biramentaceone (11)	*D. chamaethamnus*	Costa *et al.*,1998
	D. novoguianensis	Khan and Timi, 1999c
3,3′-biplumbagin (12)	*D. maritima*	Higa *et al.*, 2002
methylene-3,3′-biplumbagin (13)	*D. maritima*	Higa *et al.*, 2002
3,8′-biplumbagin (14)	*D. maritima*	Higa *et al.*, 2002
diospyrin (15)	*D. assimilis*	Ganapaty *et al.*, 2006
	D. glandulosa	Theerachayanan *et al.*, 2007

Table 1. Distribution of naphthoquinones and naphthalene derivatives in the genus *Diospyros* (continued)

Compound	Species	Reference
diospyrin (15)	*D. montana*	Ravishankara *et al.*, 2000
	D. piscatoria	Adeniyi *et al.*, 2000
	D. rhodocalyx	Theerachayanan *et al.*, 2007
	D. sylvatica	Ganapaty *et al.*, 2004
	D. virginiana	Wang *et al.*, 2011
isodiospyrin (16)	*D. chamaethammus*	Costa *et al.*, 1998
	D. ehretioides	Prajoubklang *et al.*, 2005
	D. greeniwayi	Khan and Rwekika, 1998
	D. mafiensis	Khan and Rwekika, 1999
	D. piscatoria	Adeniyi *et al.*, 2000
	D. sylvatica	Ganapaty *et al.*, 2004
	D. virginiana	Wang *et al.*, 2011
8′-hydroxyisodiospyrin (17)	*D. assimilis*	Ganapaty *et al.*, 2006
2-ethoxy-8′-hydroxyisodiospyrin (18)	*D. maritima*	Chang *et al.*, 2007
3-ethoxy-8′-hydroxyisodiospyrin (19)	*D. maritima*	Chang *et al.*, 2007
habinone (20)	*D. greeniwayi*	Khan and Rwekika, 1998
lemuninol A (21)	*Diospyros sp.*	Okuyama *et al.*,1999

Table 1. Distribution of naphthoquinones and naphthalene derivatives in the genus *Diospyros* (continued)

Compound	Species	Reference
diosquinone (22)	*D. chamaethamnus*	Costa *et al.*,1998
undulatanone (23)	*D. undulata* var. *cratericalyx*	Aoonpakh, 2001
	D. wallichii	Abdul-Wahab *et al.*, 2010
isodiospyrol A (24)	*D. ehretioides*	Prajoubklang *et al.*, 2005
diosindigo A (25)	*D. canaliculata*	Kuete *et al.*, 2009
	D. crassiflora	Kuete et al., 2009
	D. greeniwayi	Khan and Rwekika, 1998
	D. mafiensis	Khan and Rwekika, 1999
	D. sylvatica	Ganapaty *et al.*, 2004
	D. villosiuscula	Khan and Timi, 1999b
diosindigo B (26)	*D. villosiuscula*	Khan and Timi, 1999b
xylospyrin (27)	*D. chamaethammus*	Costa *et al.*, 1998
bisisodiospyrin (28)	*D. maritima*	Chang *et al.*, 2007
	D. piscatoria	Adeniyi *et al.*, 2000
diospyrone (29)	*D. canaliculata*	Tangmouo *et al.*, 2005
	D. chamaethammus	Costa *et al.*, 1998
6″,8′-bisdiosquinone (30)	*D. mafiensis*	Khan and Rwekika, 1999

Table 1. Distribution of naphthoquinones and naphthalene derivatives in the genus *Diospyros* (continued)

Compound	Species	Reference
6-[2-(7-methyljuglonyl)]isoxylospyrin (31)	*D. chamaethamnus*	Costa *et al.*, 1999
1.2 Naphthalene derivatives		
5,6,8-trimethoxy-3-methyl-1-naphthol (32)	*D. kaki*	Matsushita *et al.*, 2010
4-hydroxy-5-methoxy-2-naphthaldehyde (33)	*D. assimilis*	Ganapaty *et al.*, 2006
	D. kaki	Matsushita *et al.*, 2010
4-hydroxy-3,5-dimethoxy-2-naphthaldehyde (34)	*D. assimilis*	Ganapaty *et al.*, 2006
4-hydroxy-5,6-dimethoxy-2-naphthaldehyde (35)	*D. kaki*	Matsushita *et al.*, 2010
(4-hydroxy-5,6-dimethoxynaphthalene-2-carbaldehyde)	*D. virginiana*	Wang *et al.*, 2011
4-hydroxy-5,8-dimethoxy-2-naphthaldehyde (36)	*D. kaki*	Matsushita *et al.*, 2010
4,8-dihydroxy-5-methoxy-2-naphthaldehyde (37)	*D. kaki*	Matsushita *et al.*, 2010
5-hydroxy-4-methoxy-2-naphthaldehyde (38)	*D. assimilis*	Ganapaty *et al.*, 2006
2-hydroxymethyl-1,5-dimethoxynaphthalen-4-ol (39)	*D. wallichii*	Abdul-Wahab *et al.*, 2010
diospyrosonaphthoside (40)	*D. angustifolia*	Pathak, Kulshreshtha and Maurya, 2004
2,2′-bis-hydroxymethyl-1,1′,5,5′-tetramethoxy-3,3′-binaphthalen-4,4′-diol (41)	*D. wallichii*	Abdul-Wahab *et al.*, 2010

Table 2. Distribution of triterpenoids in the genus *Diospyros*

Compound	Species	Reference
1. Lupane type		
lupeol (42)	*D. canaliculata*	Dzoyem *et al.*, 2011
	D. crassiflora	Tangmouo *et al.*, 2006
	D. glandulosa	Thanakijcharoenpath and Theanphong, 2007
	D. greeniwayi	Khan and Rwekika, 1998
	D. hallierii	Khan and Timi, 1999a
	D. maritima	Higa *et al.*, 2002
	D. mespiliformis	Mohamed *et al.*, 2009
	D. ranongensis	Reutrakul *et al.*, 2010
	D. rhodocalyx	Theerachayanan *et al.*, 2007
	D. rubra	Prachayasittikul *et al.*, 2009
	D. undulata var. cratericalyx	Aoonpakh, 2001
	D. villosiuscula	Khan and Timi, 1999b
	D. virginiana	Wang *et al.*, 2011
lupeol acetate (43)	*D. rubra*	Prachayasittikul *et al.*, 2009
lupeol caffeate (44)	*D. maritima*	Chang and Kuo, 1999

Table 2. Distribution of triterpenoids in the genus *Diospyros* (continued)

Compound	Species	Reference
3-(*Z*)-coumaroyllupeol (45)	*D. maritima*	Chang and Kuo, 1998
lupenone (46)	*D. canaliculata*	Tangmouo *et al.*, 2005
	D. crassiflora	Tangmouo *et al.*, 2006
	D. mespiliformis	Mohamed *et al.*, 2009
	D. rubra	Prachayasittikul *et al.*, 2009
betulin (47)	*D. mespiliformis*	Mohamed *et al.*, 2009
	D. paniculata	Sinha *et al.*, 2009
	D. rubra	Prachayasittikul *et al.*, 2009
	D. undulata var. cratericalyx	Aoonpakh, 2001
	D. virginiana	Wang *et al.*, 2011
28-*O*-acetylbetulin (48)	*D. maritima*	Chang *et al.*, 2009
	D. rubra	Prachayasittikul *et al.*, 2009
(*E*)-betulin-3β-*p*-coumarate (49)	*D. maritima*	Chang *et al.*, 2009
(*Z*)-betulin-3β-*p*-coumarate (50)	*D. maritima*	Chang *et al.*, 2009
3-(*E*)-coumaroyl-28-palmitoylbetulin (51)	*D. maritima*	Chang and Kuo, 1999
3-(*Z*)-coumaroyl-28-palmitoylbetulin (52)	*D. maritima*	Chang and Kuo, 1998

Table 2. Distribution of triterpenoids in the genus *Diospyros* (continued)

Compound	Species	Reference
3-(*E*)-feruloyl-28-palmitoylbetulin (53)	*D. maritima*	Chang and Kuo, 1998
3-(*E*)-coumaroylbetulin-28-yl ethylnonanedioate (54)	*D. maritima*	Kuo and Chang, 2000
3-(*E*)-coumaroylbetulin-28-yl ethylsuccinate (55)	*D. maritima*	Kuo and Chang, 2000
3-(*E*)-coumaroylbetulin-28-yl ethyl (2R)-2-hydroxysuccinate (56)	*D. maritima*	Kuo and Chang, 2000
12,13-didehydro-20,29-dihydrobetulin (57)	*D. virginiana*	Wang *et al.*, 2011
betulinaldehyde (58)	*D. discolor*	Chen *et al.*, 2007
	D. maritima	Chang *et al.*, 2009
	D. rhodocalyx	Theerachayanan *et al.*, 2007
	D. virginiana	Wang *et al.*, 2011
3-(*E*)-coumaroylbetulinaldehyde (59)	*D. maritima*	Chang and Kuo, 1999
betulinic acid (60)	*D. angustifolia*	Pathak *et al.*, 2004
	D. canaliculata	Tangmouo *et al.*, 2005
	D. crassiflora	Tangmouo *et al.*, 2006
	D. decandra	Nareeboon *et al.*, 2006

Table 2. Distribution of triterpenoids in the genus *Diospyros* (continued)

Compound	Species	Reference
betulinic acid (60)	*D. discolor*	Chen *et al.*, 2007
	D. greeniwayi	Khan and Rwekika, 1998
	D. mafiensis	Khan and Rwekika, 1999
	D. maritima	Chang *et al.*, 2009
	D. mespiliformis	Mohamed *et al.*, 2009
	D. virginiana	Wang *et al.*, 2011
betulinic acid acetate (61)	*D. maritima*	Chang *et al.*, 2009
3-*O*-betulinic acid *p*-coumarate (62)	*D. maritima*	Chang *et al.*, 2009
3-oxo-20(29)-lupen-28-oic acid (63)	*D. maritima*	Chang *et al.*, 2009
2. Ursane type		
α-amyrin (64)	*D. kaki*	Chen *et al.*, 2002
	D. melanoxylon	Mallavadhani, Panda and Rao, 2001
α-amyrin palmitate (65)	*D. blancoi*	Ragasa *et al.*, 2009
α-amyrin palmitoleate (66)	*D. blancoi*	Ragasa *et al.*, 2009

Table 2. Distribution of triterpenoids in the genus *Diospyros* (continued)

Compound	Species	Reference
uvaol (67)	*D. kaki*	Chen *et al.*, 2002
	D. melanoxylon	Mallavadhani *et al.*, 2001
ursaldehyde (68)	*D. discolor*	Chen *et al.*, 2007
ursolic acid (69)	*D. glandulosa*	Thanakijcharoenpath and Theanphong, 2007
	D. kaki	Chen *et al.*, 2002
	D. melanoxylon	Mallavadhani *et al.*, 2001
	D. ranongensis	Reutrakul *et al.*, 2010
	D. virginiana	Wang *et al.*, 2011
19 α-hydroxy ursolic acid (70) (pomolic acid)	*D. kaki*	Chen *et al.*, 2002 Thuong *et al.*, 2008
pomolic acid methyl ester (71)	*D. melanoxylon*	Mallavadhani *et al.*, 2001
24-hydroxy ursolic acid (72)	*D. kaki*	Fan and He, 2006
24-hydroxy-3-*epi*-ursolic acid (73)	*D. kaki*	Thuong *et al.*, 2008
19 α,24-dihydroxy ursolic acid (74)	*D. kaki*	Chen *et al.*, 2002
19α,24-dihydroxyurs-12-en-3-on-28-oic acid (75)	*D. kaki*	Thuong *et al.*, 2008
rotungenic acid (76)	*D. kaki*	Thuong *et al.*, 2008

Table 2. Distribution of triterpenoids in the genus *Diospyros* (continued)

Compound	Species	Reference
barbinervic acid (77)	*D. kaki*	Thuong *et al.*, 2008
coussaric acid (78)	*D. kaki*	Thuong *et al.*, 2008
3α,19α-dihydroxyurs-12-en-24,28-dioic acid (79)	*D. kaki*	Thuong *et al.*, 2008
3α,19α-dihydroxyurs-12,20(30)-dien-24,28-dioic acid (80)	*D. kaki*	Thuong *et al.*, 2008
corsolic acid (81)	*D. melanoxylon*	Mallavadhani *et al.*, 2001
2α,3β,19α-trihydroxy-11-oxours-12-en-24,28-dioic acid (82)	*D. decandra*	Sutthivaiyakit *et al.*, 2012
2α,3β,19α-trihydroxy-28-1′-β-D-[glucopyranosyl-(1″→6′)-glucopyranosyl]-urs-12-en-24,28-dioic acid (83)	*D. decandra*	Sutthivaiyakit *et al.*, 2012
kakidiol (84)	*D. kaki*	Chen, Wang and Jia, 2009
kakisaponin A (85)	*D. kaki*	Chen *et al.*, 2009
kakisaponin B (86)	*D. kaki*	Chen *et al.*, 2009
kakisaponin C (87)	*D. kaki*	Chen *et al.*, 2009
18,19-*seco*-3β-hydroxy-urs-12-en-18-one (88)	*D. kaki*	Chen, Ren and Yu, 2012
2α,3β-dihydroxy-18,19-seco-19-oxours-11,13(18)-dien-24,28-dioic acid (89)	*D. decandra*	Sutthivaiyakit *et al.*, 2012

Table 2. Distribution of triterpenoids in the genus *Diospyros* (continued)

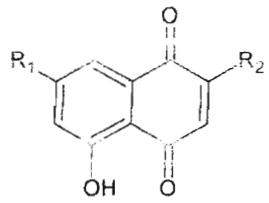
Compound	Species	Reference
2α,3β-dihydroxy-19-nor-11-oxo-20-dimethylurs-12-en-24,28-dioic acid (2α,3β-dihydroxy-19-nor-11-oxoolean-12-en-24,28-dioic acid) (90)	*D. decandra*	Sutthivaiyakit *et al.*, 2012
2-Oxo-3β,19α-dihydroxy-24-nor-urs-12-en-28-oic acid (91)	*D. decandra*	Nareeboon *et al.*, 2006
2-oxo-3β,19α,22α-trihydroxy-24-nor-urs-12-en-28-oic acid (92)	*D. decandra*	Nareeboon *et al.*, 2006
3-oxo-2,19α, 22α-trihydroxy-24-nor-urs1,4,12-trien-28-oic acid (93)	*D. decandra*	Nareeboon *et al.*, 2006
4-oxo-19α,22α-dihydroxy-3,24-dinor-2,4-secours-12-en-2,28-dioic acid (94)	*D. decandra*	Nareeboon *et al.*, 2006
19α,22α-dihydroxy-24-nor-2,3-seco-urs-12-en-2,3,28-trioic acid trimethyl ester (95)	*D. decandra*	Nareeboon *et al.*, 2006
3. Oleanane type		
β-amyrin (96)	*D. angustifolia*	Pathak *et al.*, 2004
	D. glandulosa	Thanakijcharoenpath and Theanphong, 2007
	D. maritima	Higa *et al.*, 2002
	D. melanoxylon	Mallavadhani *et al.*,2001

Table 2. Distribution of triterpenoids in the genus *Diospyros* (continued)

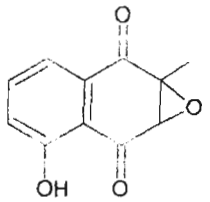
Compound	Species	Reference
β-amyrin palmitate (97)	*D. blancoi*	Ragasa *et al.*, 2009
β-amyrin palmitoleate (98)	*D. blancoi*	Ragasa *et al.*, 2009
oleanolic acid (99)	*D. glandulosa*	Thanakijcharoenpath and Theanphong, 2007
	D. kaki	Thuong *et al.*, 2008
	D. melanoxylon	Mallavadhani *et al.*,2001
24-hydroxy-3-*epi*-oleanolic acid (100)	*D. kaki*	Thuong *et al.*, 2008
3β-acetoxyolean-12-en-28-oic acid (101)	*D. maritima*	Chang *et al.*, 2009
spathodic acid (102)	*D. kaki*	Thuong *et al.*, 2008
maslinic acid methyl ester (103)	*D. melanoxylon*	Mallavadhani *et al.*,2001
3-β-hydroxy-28,19β-oleanolide (104)	*D. angustifolia*	Pathak *et al.*, 2004
diospyrosooleanolide (105)	*D. angustifolia*	Pathak, Kulshreshtha and Maurya, 2004

Table 2. Distribution of triterpenoids in the genus *Diospyros* (continued)

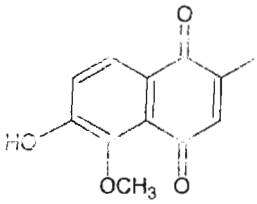
Compound	Species	Reference
4. Taraxerane type		
3β-hydroxytaraxastan-28, 20β-olide (106)	*D. maritima*	Chang *et al.*, 2009
5. Fridelane type		
Friedelin (107)	*D. glandulosa*	Thanakijcharoenpath and Theanphong, 2007
	D. maritima	Higa *et al.*, 2002
	D. angustifolia	Pathak, Kulshreshtha and Maurya, 2004
4. Lanostane type		
24-ethyl-3β-methoxylanost-9(11)-en-25-ol (108)	*D. discolor*	Chen *et al.*, 2007
3β-methoxy-24-methylenelanost-9(11)-en-25-ol (109)	*D. discolor*	Chen *et al.*, 2007
3β-methoxy-25-methyl-24-methylenelanost-9(11)-en-21-ol (110)	*D. discolor*	Chen *et al.*, 2007
3β-methoxy-24-methyllanosta-9(11),25-dien-24-ol (111)	*D. discolor*	Chen *et al.*, 2007



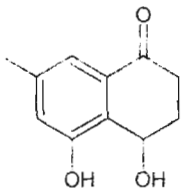
	R_1	R_2
plumbagin (1)	H	CH_3
7-methyl juglone (2)	CH_3	H



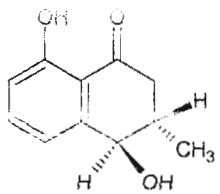
2,3-epoxyplumbagin (3)



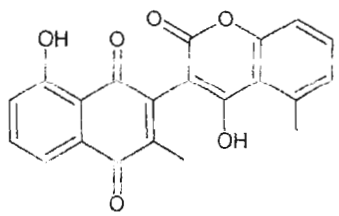
6-hydroxy-5-methoxy-2-methyl-1,4-naphthoquinone (4)



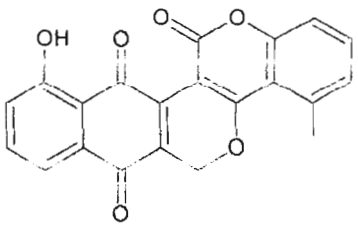
shinanolone (5)



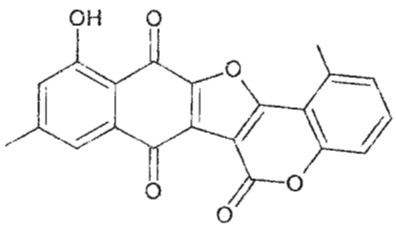
epi-isoshinanolone (6)



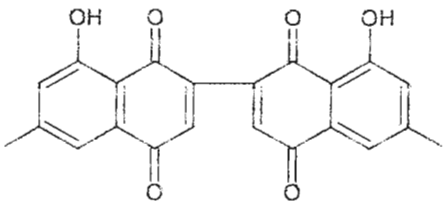
canaliculatin (7)



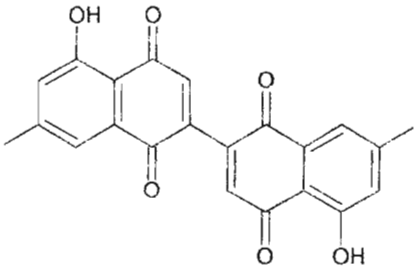
cyclocanaliculatin (8)



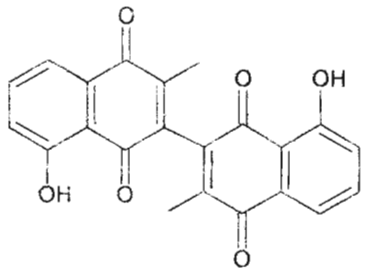
crassiflorone (9)



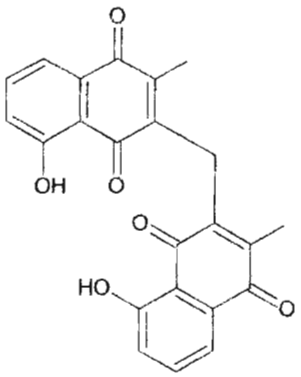
mamegakinone (10)



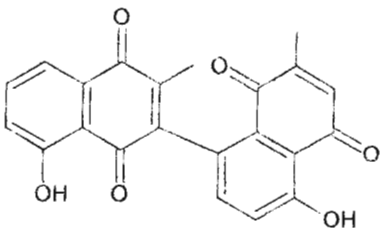
biramentaceone (11)



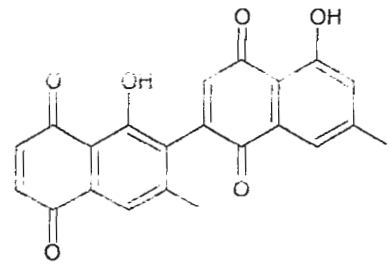
3,3′-biplumbagin (12)



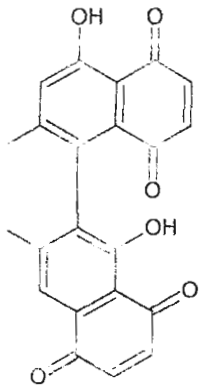
methylene-3,3′-biplumbagin (13)



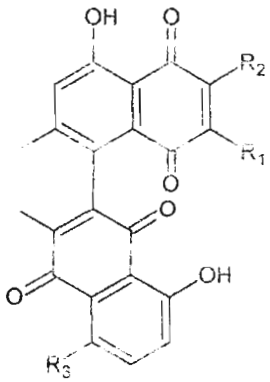
3,8′-biplumbagin (14)



diospyrin (15)



isodiospyrin (16)

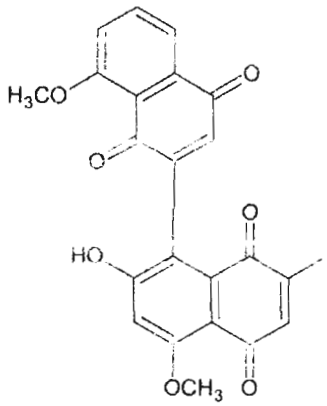


8′-hydroxyisodiospyrin (17)

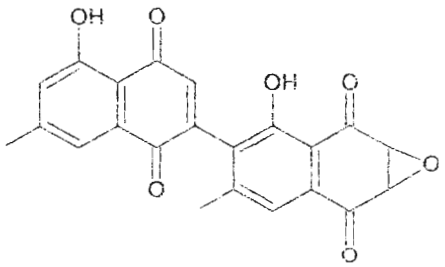
2-ethoxy-8′-hydroxyisodiospyrin (18)

3-ethoxy-8′-hydroxyisodiospyrin (19)

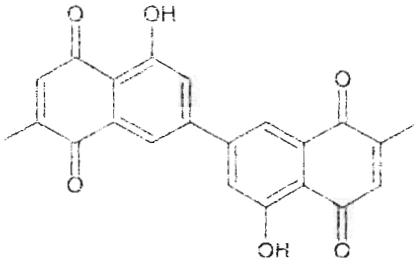
habinone (20)



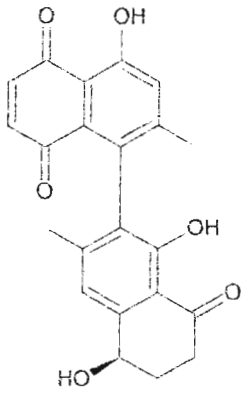
lemuninol A (21)



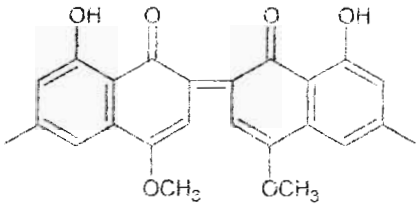
diosquinone (22)



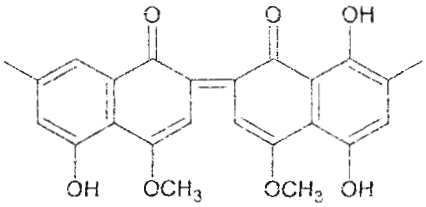
undulatanone (23)



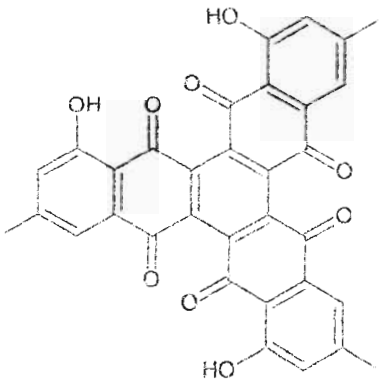
isodiospyrol A (24)



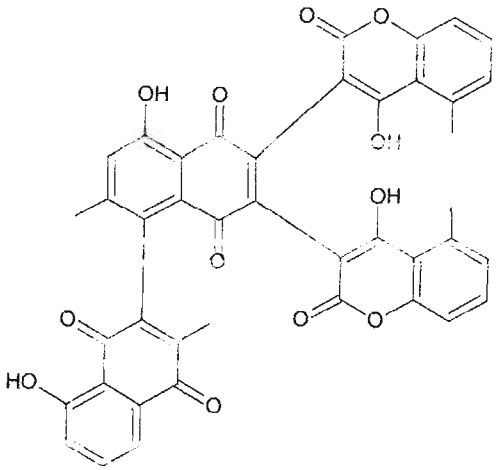
diosindigo A (25)



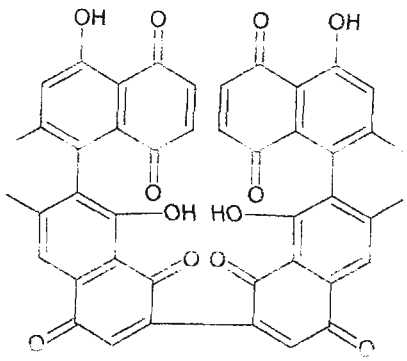
diosindigo B (26)



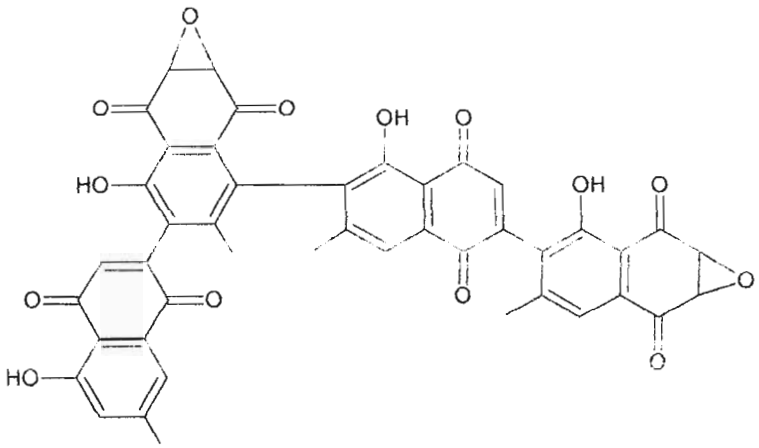
xylospyrin (27)



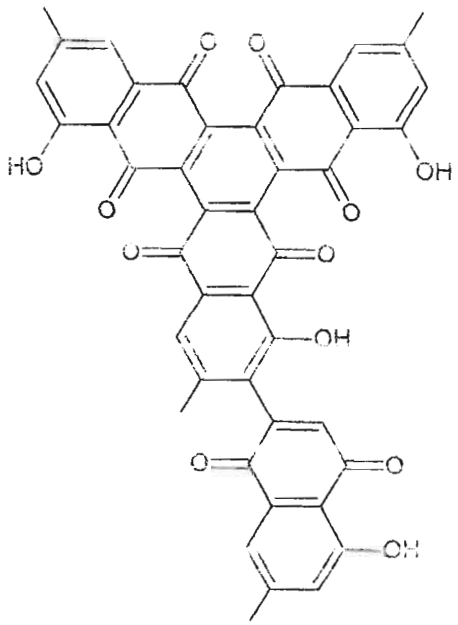
bisisodiospyrin (28)



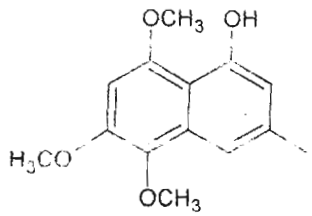
diospyrone (29)



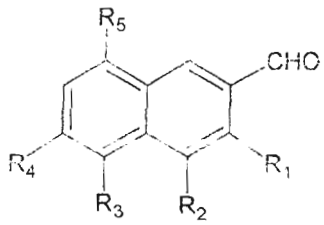
6″,8′-bisdiosquinone (30)



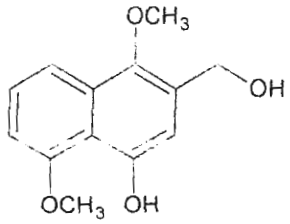
6-[2-(7- methyljuglonyl)]isoxylospyrin (31)



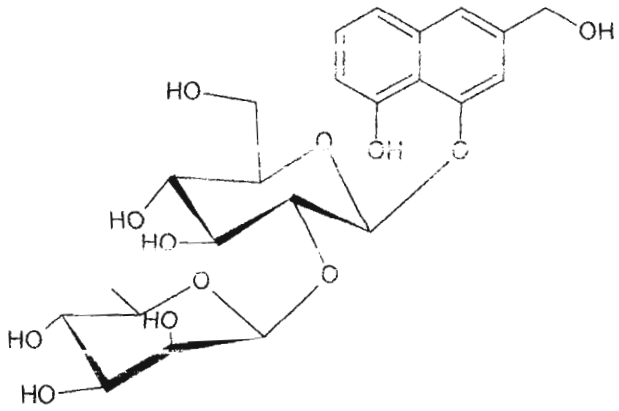
5,6,8-trimethoxy-3-methyl-1-naphthol (32)



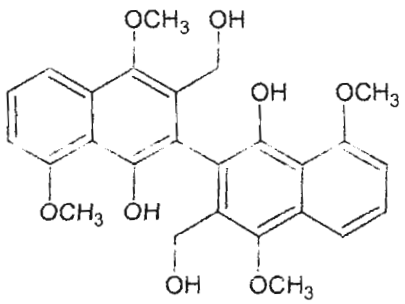
	R_1	R_2	R_3	R_4	R_5
4-hydroxy-5-methoxy-2-naphthaldehyde (33)	H	OH	OCH_3	H	H
4-hydroxy-3,5-dimethoxy-2-naphthaldehyde (34)	OCH_3	OH	OCH_3	H	H
4-hydroxy-5,6-dimethoxy-2-naphthaldehyde (35)	H	OH	OCH_3	OCH_3	H
4-hydroxy-5,8-dimethoxy-2-naphthaldehyde (36)	H	OH	OCH_3	H	OCH_3
4,8-dihydroxy-5-methoxy-2-naphthaldehyde (37)	H	OH	OCH_3	H	OH
5-hydroxy-4-methoxy-2-naphthaldehyde (38)	H	OCH_3	OH	H	H



2-hydroxymethyl-1,5-dimethoxynaphthalen-4-ol (39)

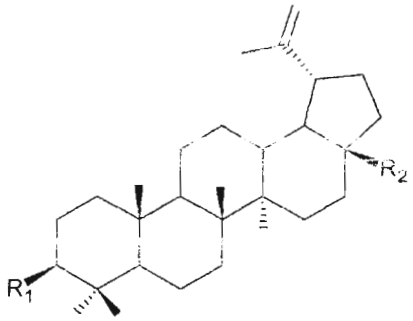


diospyrosonaphthoside (40)

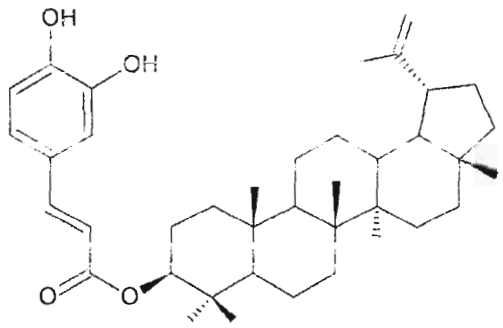


2,2′-bis-hydroxymethyl-1,1′,5,5′-tetramethoxy-3,3′-binaphthalen-4,4′-diol

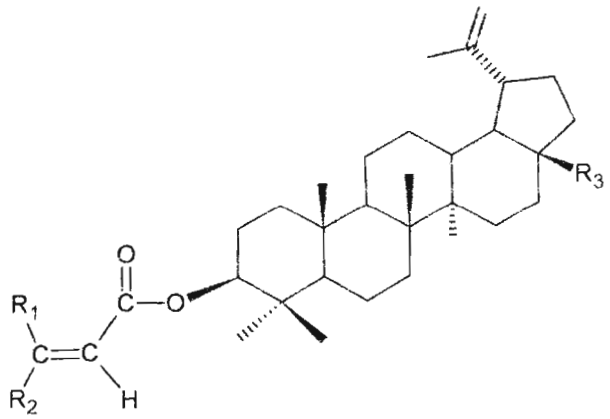
(41)

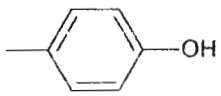
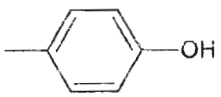
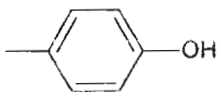
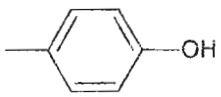


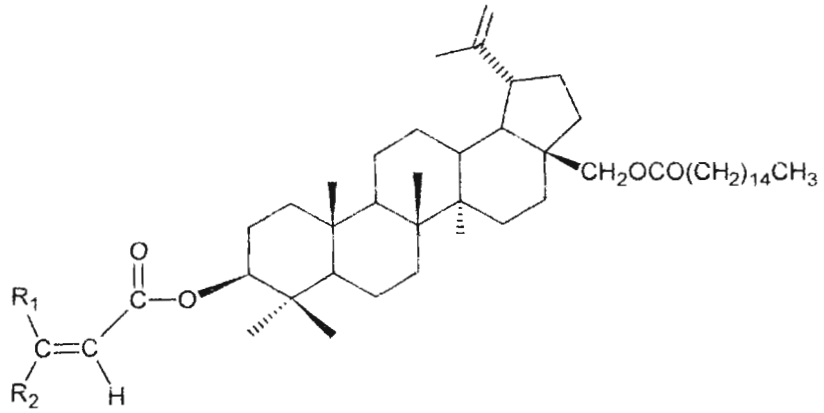
	R_1	R_2
lupeol (42)	OH	CH_3
lupeol acetate (43)	$OCOCH_3$	CH_3
lupenone (46)	=O	CH_3
betulin (47)	OH	CH_2OH
28-*O*-acetylbetulin (48)	OH	CH_2OCOCH_3
betulinaldehyde (58)	OH	CHO
betulinic acid (60)	OH	COOH
betulinic acid acetate (61)	$OCOCH_3$	COOH
3-oxo-20(29)-lupen-28-oic acid (63)	=O	COOH

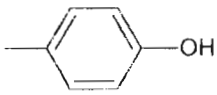
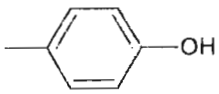
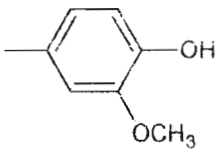


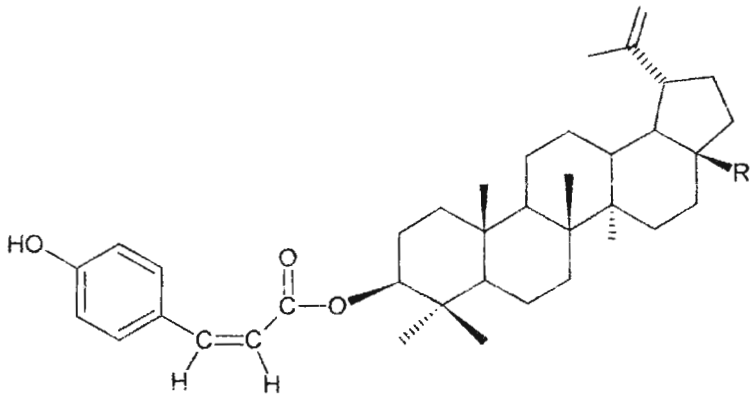
lupeol caffeate (44)



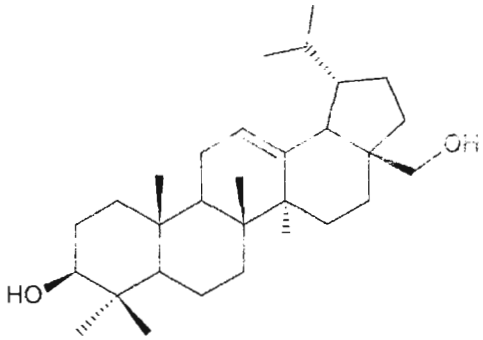
	R_1	R_2	R_3
3-(*Z*)-coumaroyllupeol (45)		H	CH_3
(*E*)-betulin-3β-*p*-coumarate (49)	H		CH_2OH
(*Z*)-betulin-3β-*p*-coumarate (50)		H	CH_2OH
3-(*E*)-coumaroylbetulinaldehyde (59)	H		CHO



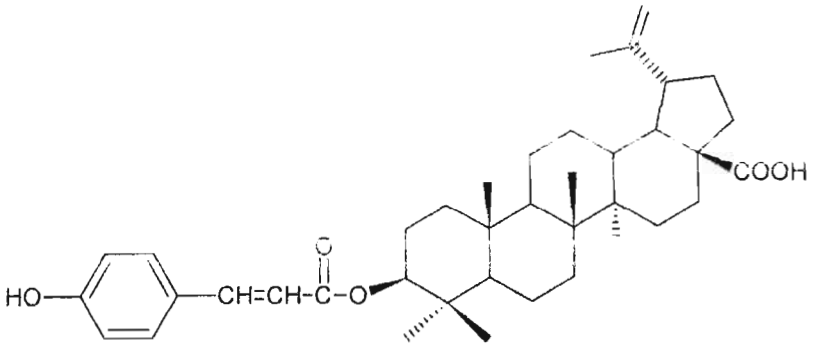
	R_1	R_2
3-(*E*)-coumaroyl-28-palmitoylbetulin (51)	H	
3-(*Z*)-coumaroyl-28-palmitoylbetulin (52)		H
3-(*E*)-feruloyl-28-palmitoylbetulin (53)	H	



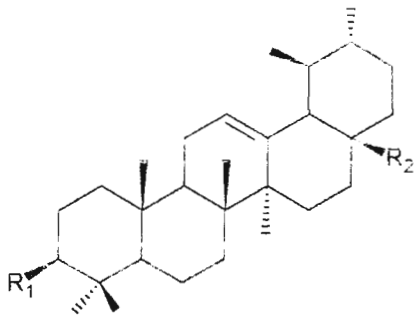
3-(*E*)-coumaroylbetulin-28-yl ethylnonanedioate (54)	R = $CH_2OCOCH_2(CH_2)_5CH_2CO_2CH_2CH_3$
3-(*E*)-coumaroylbetulin-28-yl ethylsuccinate (55)	R = $CH_2OCOCH_2CH_2CO_2CH_2CH_3$
3-(*E*)-coumaroylbetulin-28-yl ethyl (2R)-2-hydroxysuccinate (56)	R = $CH_2OCOCHOHCH_2CO_2CH_2CH_3$



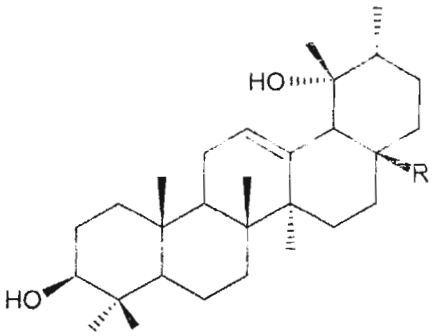
12,13-didehydro-20,29-dihydrobetulin (57)



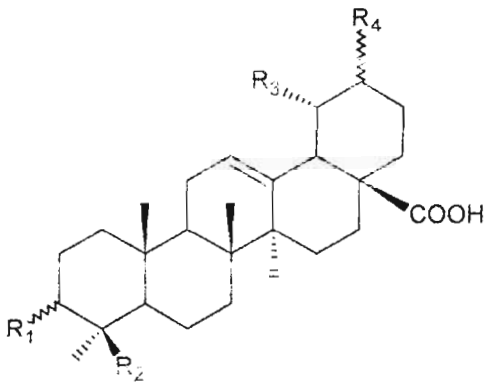
3-*O*-betulinic acid *p*-coumarate (62)



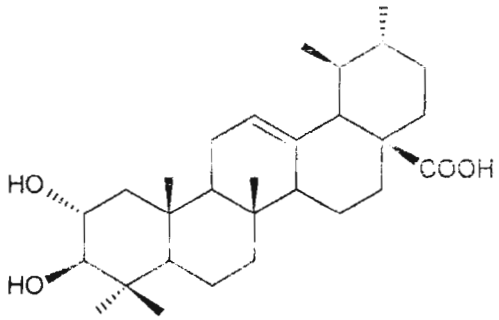
	R_1	R_2
α-amyrin (64)	OH	CH_3
α-amyrin palmitate (65)	$OCO(CH_2)_{14}CH_3$	CH_3
α-amyrin palmitoleate (66)	$OCO(CH_2)_7$ $CH=CH(CH_2)_5CH_3$	CH_3
uvaol (67)	OH	CH_2OH
ursaldehyde (68)	OH	CHO
ursolic acid (69)	OH	COOH



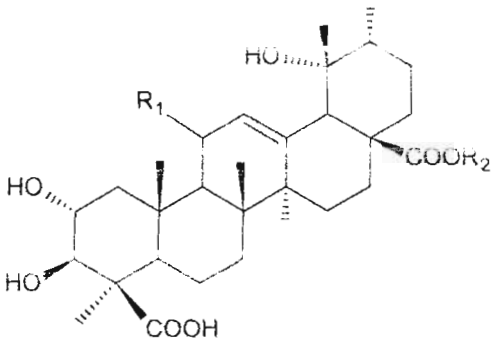
19 α-hydroxy ursolic acid (70)	R = COOH
(pomolic acid)	
pomolic acid methyl ester (71)	R = $COOCH_3$



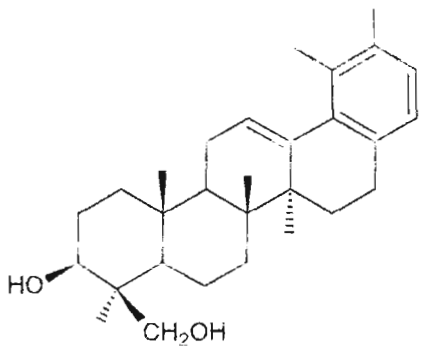
	R_1	R_2	R_3	R_4
24-hydroxy ursolic acid (72)	β-OH	CH_2OH	H	α-CH_3
24-hydroxy-3-*epi*-ursolic acid (73)	α-OH	CH_2OH	H	α-CH_3
19α,24-dihydroxy ursolic acid (74)	β-OH	CH_2OH	OH	α-CH_3
19α,24-dihydroxyurs-12-en-3-on-28-oic acid (75)	= O	CH_2OH	OH	α-CH_3
rotungenic acid (76)	β-OH	CH_2OH	OH	α-CH_3
barbinervic acid (77)	α-OH	CH_2OH	OH	α-CH_3
coussaric acid (78)	α-OH	CH_2OH	OH	= CH_2
3α,19α-dihydroxyurs-12-en-24,28-dioic acid (79)	α-OH	COOH	OH	α-COOH
3α,19α-dihydroxyurs-12,20(30)-dien-24,28-dioic acid (80)	α-OH	COOH	OH	= CH_2



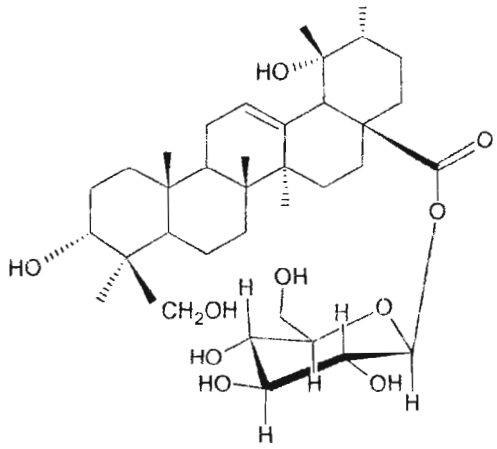
corsolic acid (81)



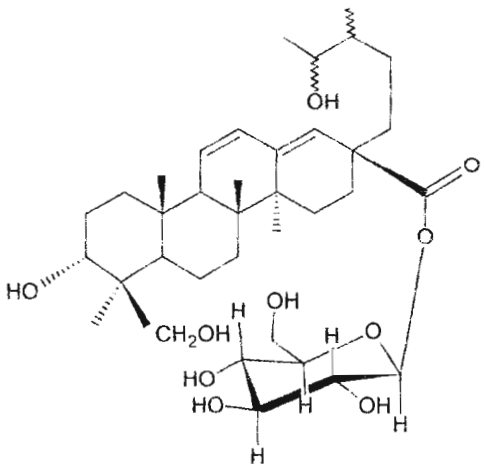
	R_1	R_2
2α,3β,19α-trihydroxy-11-oxours-12-en-24,28-dioic acid (82)	= O	H
2α,3β,19α-trihydroxy-28-1′-β-D -[glucopyranosyl-(1″→6′)-glucopyranosyl]-urs-12-en-24,28-dioic acid (83)	H	Glc-Glc



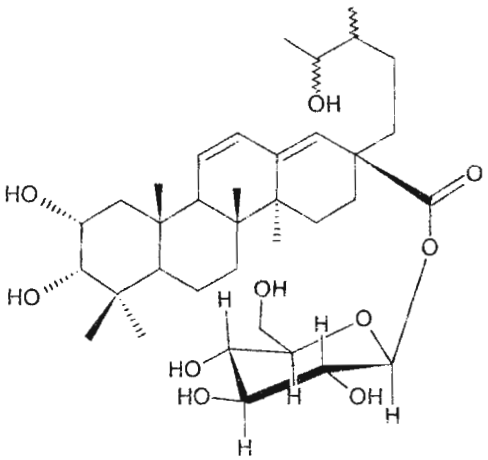
kakidiol (84)



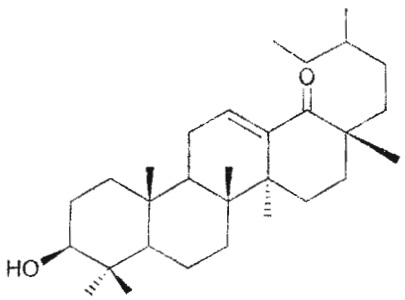
kakisaponin A (85)



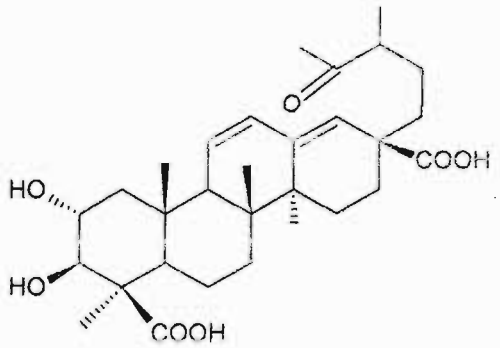
kakisaponin B (86)



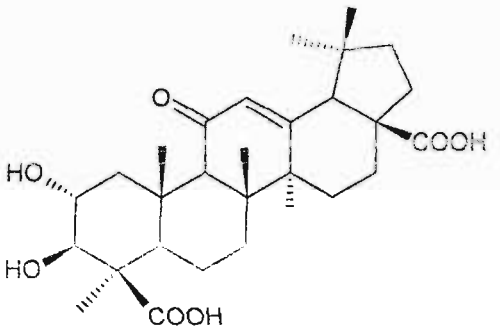
kakisaponin C (87)



18,19-*seco*-3β-hydroxy-urs-12-en-18-one (88)

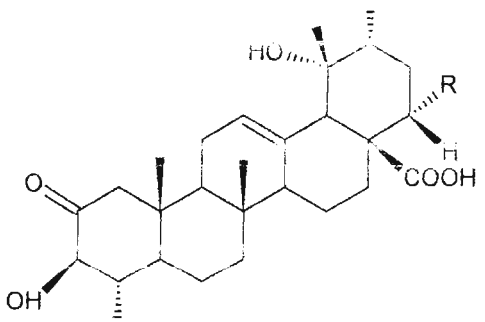


2α,3β-dihydroxy-18,19-seco-19-oxours-11,13(18)-dien-24,28-dioic acid (89)



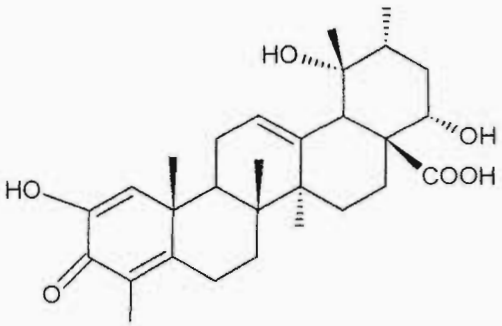
2α,3β-dihydroxy-19-nor-11-oxo-20-dimethylurs-12-en-24,28-dioic acid

(2α,3β-dihydroxy-19-nor-11-oxoolean-12-en-24,28-dioic acid) (90)

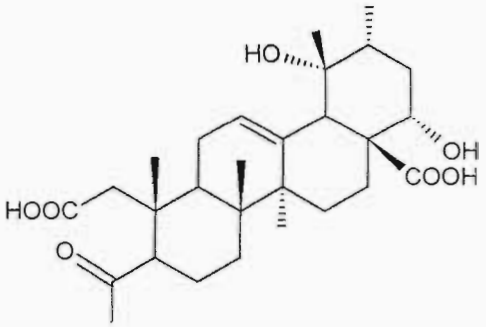


2-oxo-3β,19α-dihydroxy-24-nor-urs-12-en-28-oic acid (91) R = H

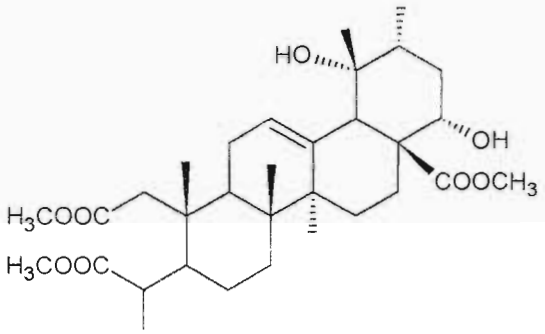
2-oxo-3β,19α,22α-trihydroxy-24-nor-urs-12-en-28-oic acid (92) R = OH



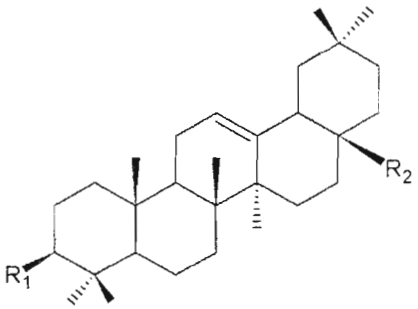
3-oxo-2,19α, 22α-trihydroxy-24-nor-urs-1,4,12-trien-28-oic acid (93)



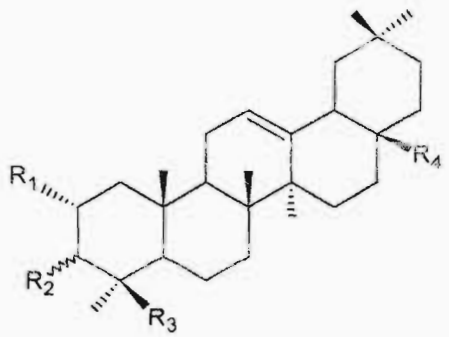
4-oxo-19α,22α-dihydroxy-3,24-dinor-2,4-secours-12-en-2,28-dioic acid (94)



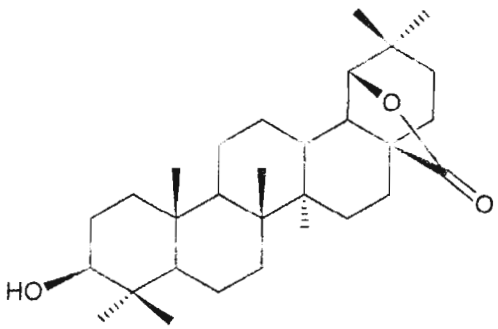
19α,22α-dihydroxy-24-nor-2,3-seco-urs-12-en-2,3,28-trioic acid trimethyl ester (95)



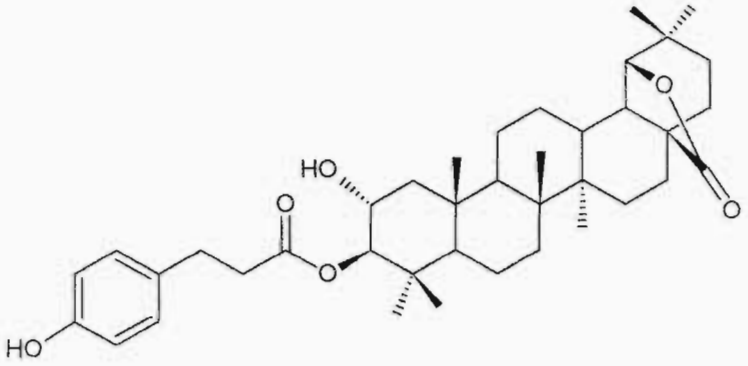
	R_1	R_2
β-amyrin (96)	OH	CH_3
β-amyrin palmitate (97)	$OCO(CH_2)_{14}CH_3$	CH_3
β-amyrin palmitoleate (98)	$OCO(CH_2)_7CH=CH(CH_2)_5CH_3$	CH_3
oleanolic acid (99)	OH	COOH
3β-acetoxyolean-12-en-28-oic acid (101)	$OCOCH_3$	COOH



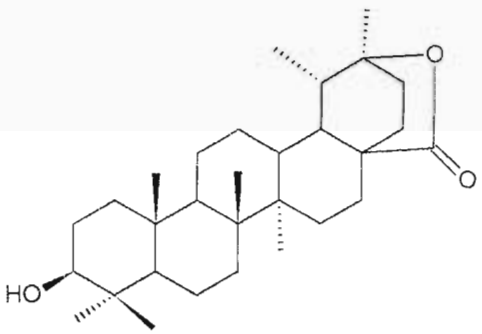
	R_1	R_2	R_3	R_4
24-hydroxy-3-*epi*-oleanolic acid (100)	H	α-OH	CH_2OH	COOH
spathodic acid (102)	H	β-OH	CH_2OH	COOH
maslinic acid methyl ester (103)	OH	β-OH	CH_3	$COOCH_3$



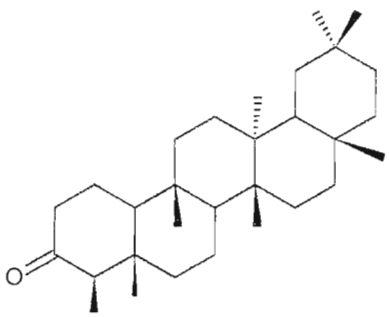
3-β-hydroxy-28,19β-oleanolide (104)



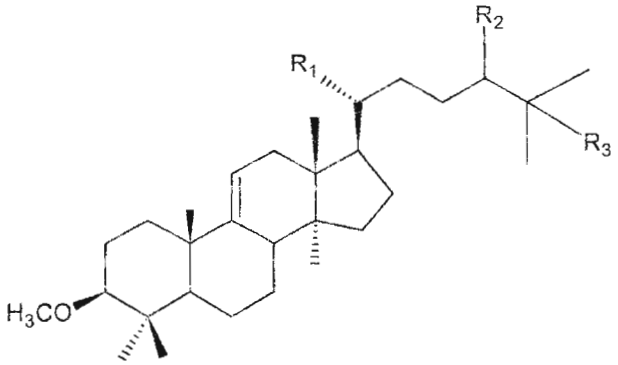
diospyrosooleanolide (105)



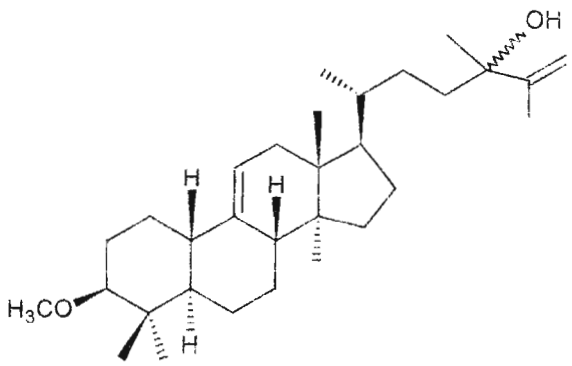
3β-hydroxytaraxastan-28, 20β-olide (106)



friedelin (107)



	R_1	R_2	R_3
24-ethyl-3β-methoxylanost-9(11)-en-25-ol (108)	CH_3	CH_2CH_3	OH
3β-methoxy-24-methylenelanost-9(11)-en-25-ol (109)	CH_3	$=CH_2$	OH
3β-methoxy-25-methyl-24-methylenelanost-9(11)-en-21-ol (110)	CH_2OH	$=CH_2$	CH_3



3β-methoxy-24-methyllanosta-9(11),25-dien-24-ol (111)

2. Biological and Pharmacological Activities of *Diospyros* Species

Medicinal uses of certain *Diospyros* plants have been known for a long time. A number of them have been investigated for their biological and pharmacological activities. Several extracts and isolated compounds from the plants have been found to exhibit interesting activities. Mallavadhani *et al.* (1998) have reviewed pharmacological activities of *Diospyros* species, publishing in 1998. Biological and pharmacological activities of extracts from the plants, reported in the literature since 1998, are summarized here in Table 3.

Table 3. Biological and pharmacological activities of extracts from *Diospyros* species

Species	Plant part	Biological activity	Reference
D. abyssinica	n.i.	Antioxidant	Ma ga *et al.*, 2006
		15-Lipoxygenase inhibitory	Ma ga *et al.*, 2006
D. anisandra	Leaves, Roots, Stem bark	Antimicrobial	Borges-Argáez *et al.*, 2007
D. barteri	All parts	Antiviral	Moody, Robert and Hughes, 2002
	Leaves	Antimicrobial	Oluremi, Osungunna and Ogbole, 2010
D. canaliculata	n.i.	Antifungal	Dzoyem *et al.*, 2011
D. cordifolia	Bark	Antitumor	Das *et al.*, 2011
	Stem bark	Analgesic	Das *et al.* 2010
		Anti-inflammatory	Das *et al.* 2010
D. digyna	Fruits	Antioxidant	Yania, Gutierrez-Orozco and Leon, 2011
D. discolor	Bark, Fruits, Leaves	Antioxidant	Kumar *et al.*, 2006
D. ebenum	Leaves	Antibacterial	Baravalia *et al.*, 2009
		Antioxidant	Baravalia *et al.*, 2009

n.i. = not indicated

Table 3. Biological and pharmacological activities of extracts from *Diospyros* species (continued)

Species	Plant part	Biological activity	Reference
D. fischeri	Root	Anticonvulsant	Moshi *et al.*, 2007b
	Stem bark	Anticonvulsant	Moshi *et al.*, 2007a
D. kaki	Calyx	Anticancer	Jo *et al.*, 2011
	Fruits	Antigenotoxic	Jang *et al.*, 2010
		Antioxidant	Jang *et al.*, 2010
	Leaves	Anticoagulant	Sa, Kim and Choi, 2005
		Antithrombotic	Sa *et al.*, 2005
		Neuroprotective	Bei *et al.*, 2007
	Peels	Cytotoxic	Kawase *et al.*, 2003
	n.i.	Antidiabetic	Lee, Chung and Lee, 2006.
	n.i.	Antioxidant	Kim *et al.*, 2011
		Anti-inflammatory	Kim *et al.*, 2011

n.i. = not indicated

Table 3. Biological and pharmacological activities of extracts from *Diospyros* species (continued)

Species	Plant part	Biological activity	Reference
D. lotus	Fruits	Antioxidant	Loizzo *et al.*, 2009
		Antiproliferative	Loizzo *et al.*, 2009
	Fruits	Antidiabetic	Azadbakhta *et al.*, 2010
D. malabarica	Bark	Antioxidant	Kumar *et al.*, 2006
D. melanoxylon	Bark	Anticandidal	Rath *et al.*, 2009
		Antihyperglycemic	Jadhav, Masirkar and Deshmukh, 2009
	Leaves	Analgesic	Devi *et al.*, 2010
		Antipyretic	Devi *et al.*, 2010
		Diuretic	Devi *et al.*, 2010
	Roots, Stem heartwood, Stem bark	Antiplasmodial	Kantamreddi and Wright, 2008
D. mespiliformis	Fruits	Antioxidant	Ndhlala *et al.*, 2008
	Leaves	Antibacterial	Dangoggo *et al.*, 2012
	Stem bark	Analgesic	Adzu *et al.*, 2002a

Table 3. Biological and pharmacological activities of extracts from *Diospyros* species (continued)

Species	Plant part	Biological activity	Reference
D. mespiliformis	Stem bark	Anti-inflammatory	Adzu *et al.*, 2002a
		Antimalarial	Adzu *et al.*, 2002a
		Antipyretic	Adzu *et al.*, 2002a
		Neuroactive	Adzu *et al.*, 2002b
D. monbutensis	All parts	Antiviral	Moody, Robert and Hughes, 2002
D. peregrina	Fruits, Root,	Antimicrobial	Dewanjee *et al.*, 2007a
	Stem bark		
	Bark	Antitumor	Venu *et al.*, 2011
	Fruits	Anthelmintic	Dewanjee *et al.*, 2007b
	Stem bark	Antiplasmodial	Kantamreddi and Wright, 2008
D. preussi	Seed oil	Antioxidant	Okonkwo and Okonkwo, 2009
D. rubra	Stem	Antimalarial	Prachayasittikul *et al.*, 2009
D. sylvatica	Roots	Antiplasmodial	Ganapaty *et al.*, 2004
		Antitermitic	Kantamreddi and Wright, 2008
	Stem bark, Stem heartwood	Antiplasmodial	Kantamreddi and Wright, 2008

Table 3. Biological and pharmacological activities of extracts from *Diospyros* species (continued)

Species	Plant part	Biological activity	Reference
D. tomentosa	Stem bark	Antiplasmodial	Kantamreddi and Wright, 2008
D. variegata	Stem	Analgesic	Trongsakul *et al.*, 2003
		Antipyretic	Trongsakul *et al.*, 2003
		Anti-inflammatory	Trongsakul *et al.*, 2003
D. wallichii	Fruits, Leaves	Antibacterial	Nematollahi, Aminimoghadamfarouj and Wiart, 2011
		Antioxidant	Nematollahi *et al.*, 2011

CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The stems of *Diospyros collinsae* Craib (Ebenaceae) were collected from Suan Luang Rama IX Public Park, Bangkok, Thailand, in June 2009. The plant material was identified by Dr. Chirayupin Chandraprasong of Suan Luang Rama IX Public Park.

2. General Techniques

2.1 Chromatographic Technique

2.1.1 Thin-Layer Chromatography (TLC)

Technique	One dimension, ascending
Adsorbent	Silica gel 60 F_{254} (E. Merck) precoated plates
Layer thickness	0.2 mm
Solvent system	Various solvent systems depending on materials to be separated
Distance	7 cm
Temperature	25-35 °C (Laboratory temperature)
Detection	1) Visual detection under daylight 2) Ultraviolet light (254 and 365 nm) 3) Spraying with 10% sulfuric acid in ethanol and heating at 110 °C for 5-10 minutes 4) Spraying with anisaldehyde-sulfuric acid reagent and heating at 110 °C for 5-10 minutes 5) Liebermann-Burchard reagent

2.1.2 Column Chromatography (CC)

Column	Flat bottom glass column (various diameters)
Adsorbent	Silica gel 60 (No. 9385, E. Merck) particle size 0.040-0.063 nm (230 - 400 mesh ASTM)
Packing method	Wet packing
Solvent system	Various solvent systems depending on materials to be separated
Sample loading	1) The sample was dissolved in a small amount of organic solvent, mixed with a small quantity of adsorbent, triturated, dried and then applied on the top of the column. 2) The sample was dissolved in a small volume of the eluent, then applied on the top of the column.
Detection	Fractions were examined by TLC observed under daylight and UV light at the wavelengths of 254 and 365 nm. The TLC plate was then sprayed with 10% sulfuric acid in ethanol and/or anisaldehyde-sulfuric acid reagent and heated at 110 °C for 5-10 minutes. Fraction of similar chromatographic patterns were combined

2.2 Spectroscopy

2.2.1 Ultraviolet (UV) Spectra

UV spectra were recorded on a Shimadzu UV-160A spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.2.2 Infrared (IR) Absorption Spectra

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

2.2.3 Mass Spectra

Electrospray Ionization (ESI) mass spectra were recorded on a Micromass LCT mass spectrometer (National Center for Genetic Engineering and Biotechnology, BIOTEC, Thailand).

2.2.4 Proton and Carbon 13 Nuclear Magnetic Resonance (^{1}H- and ^{13}C-NMR) Spectra

The ^{1}H NMR (500 Hz) and ^{13}C NMR (125 Hz) spectra were recorded on a JEOL JNM-A500, Varian Unity INOVA (Scientific and Technological Research Equipment Center, Chulalongkorn University).

The NMR solvents used in this study included DMSO-d_6, $CDCl_3$ and acetone-d_6. Chemical shifts were presented in ppm scale, using the chemical shift of the solvent as the reference signal.

2.3 Melting Points

Melting points were recorded on a Fisher-Johns melting point apparatus (Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

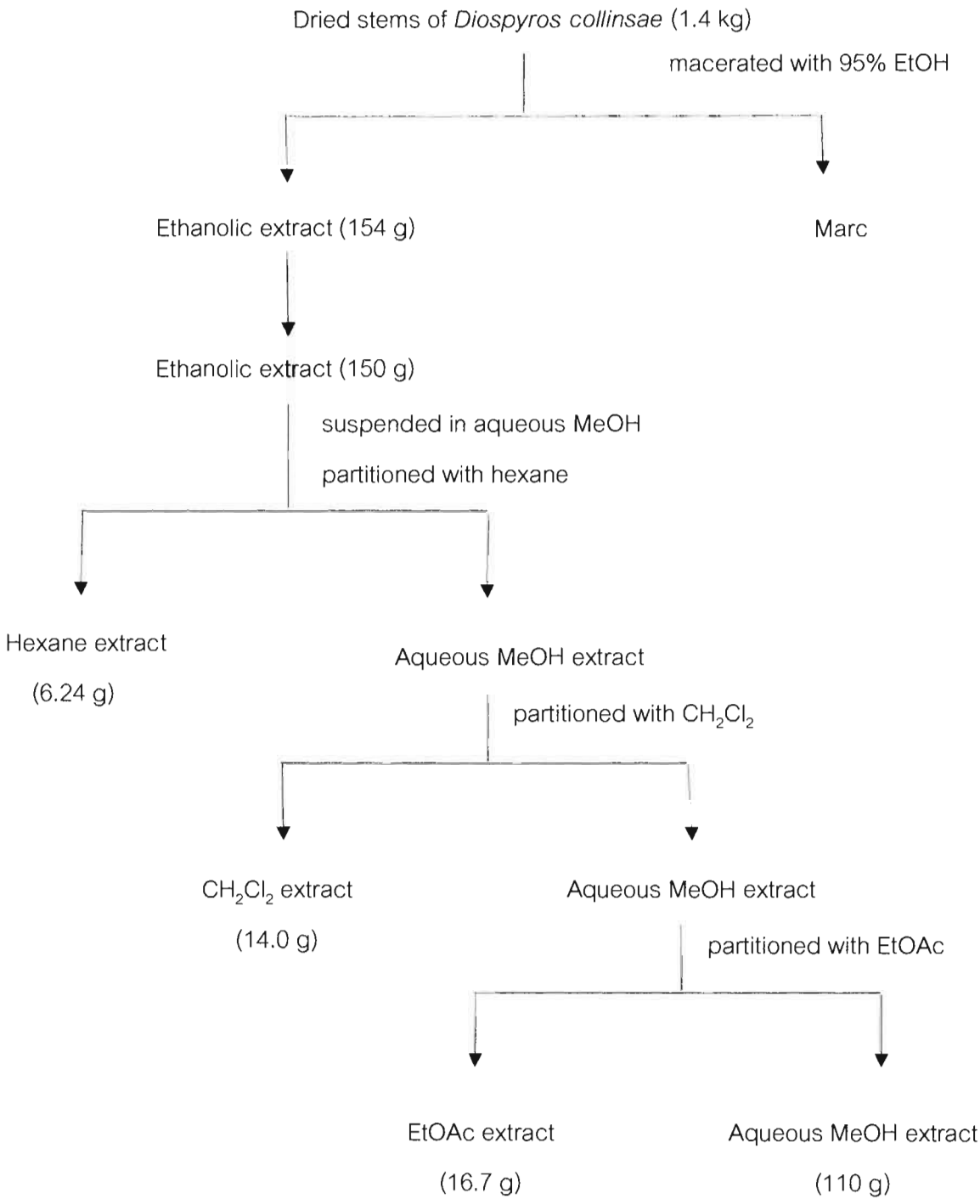
2.4 Solvent

Throughout this work, all organic solvents were of commercial grade and were redistilled prior to use.

3. Extraction and Isolation

3.1 Extraction

Dried, powdered stems of *D. collinsae* (1.4 kg) were extracted with 95% EtOH by maceration (5 x 30 L, 3 days each) and then filtered. The filtrates were combined and evaporated under reduced pressure to give 154 g of crude ethanolic extract (11% of dry weight). The crude extract (150 g) was suspended in aqueous MeOH and partitioned with hexane (5 x 3 L) and then evaporated to dryness under reduced pressure to give a hexane extract (6.24 g, 0.46% of dry weight). The aqueous MeOH layer was further partitioned with CH_2Cl_2 (5 x 3 L) and then EtOAc (5 x 3 L) to give a CH_2Cl_2 extract (14.0 g, 1.03% of dry weight) and an EtOAc extract (16.7 g, 1.22% of dry weight) together with an aqueous MeOH extract (110 g, 8.07% of dry weight).



Scheme 1. Extraction of *D. collinsae* stems

3.2 Isolation

3.2.1 Isolation of Compounds from the Hexane Extract

A portion of the hexane extract (3.0 g) was separated on a silica gel column (100 g, 5 x 13 cm), eluted with solvent mixtures of increasing polarity from hexane – CH_2Cl_2 (9:1) to CH_2Cl_2 – MeOH (9:1). One hundred 50-ml fractions were collected and combined according to their TLC patterns into four major fractions (DH01-DH04) as shown in Table 4. The column was then washed down with MeOH.

Table 4. Combined fractions from the hexane extract

Fraction	Number of eluates	Weight (mg)
DH01	1-24	159.7
DH02	25-40	141.6
DH03	41-70	370.0
DH04	71-100	795.6
MeOH eluate		1520

3.2.1.1 Isolation of Compound DC-1

Fraction DH02, which gave a major yellow spot on TLC under detection with anisaldehyde-sulfuric acid reagent, was selected for further investigation. The fraction, when dissolved in hexane, was separated into soluble and insoluble parts. The insoluble part was purified by recrystallization in MeOH to give compound DC1 as colorless needles (13.1 mg). The soluble part (128.5 mg) was submitted to further purification on a silica gel column (5 g, 2 cm x 13 cm), eluted with hexane-CH_2Cl_2 (1:1). Seventy 10-ml fractions were collected and then combined according to their TLC patterns into four major fractions (DH021-DH024) as shown in Table 5. The column was then washed down with MeOH.

Table 5. Combined fractions from DH02

Fraction	Number of eluates	Weight (mg)
DH021	1-15	16.4
DH022	16-40	28.6
DH023	42-56	15.7
DH024	57-70	29.3
MeOH eluate		27.5

Compound DC-1 was obtained from fraction DHO22 as colorless needless (5.4 mg) by recrystallization in MeOH. The compound gave a purple color upon detection with Liebermann-Burchard reagent.

3.2.1.2 Isolation of Compounds DC-2 and DC-3

Fraction DH03 (370 mg) was separated on a silica gel column (75 g, 2.5 × 23 cm) using hexane – CH_2Cl_2 (1:1) as the eluent. Sixty 20-ml fractions were collected and then combined according to their TLC patterns into three fractions (DH031 – DH033) as shown in Table 6. The column was then washed down with MeOH.

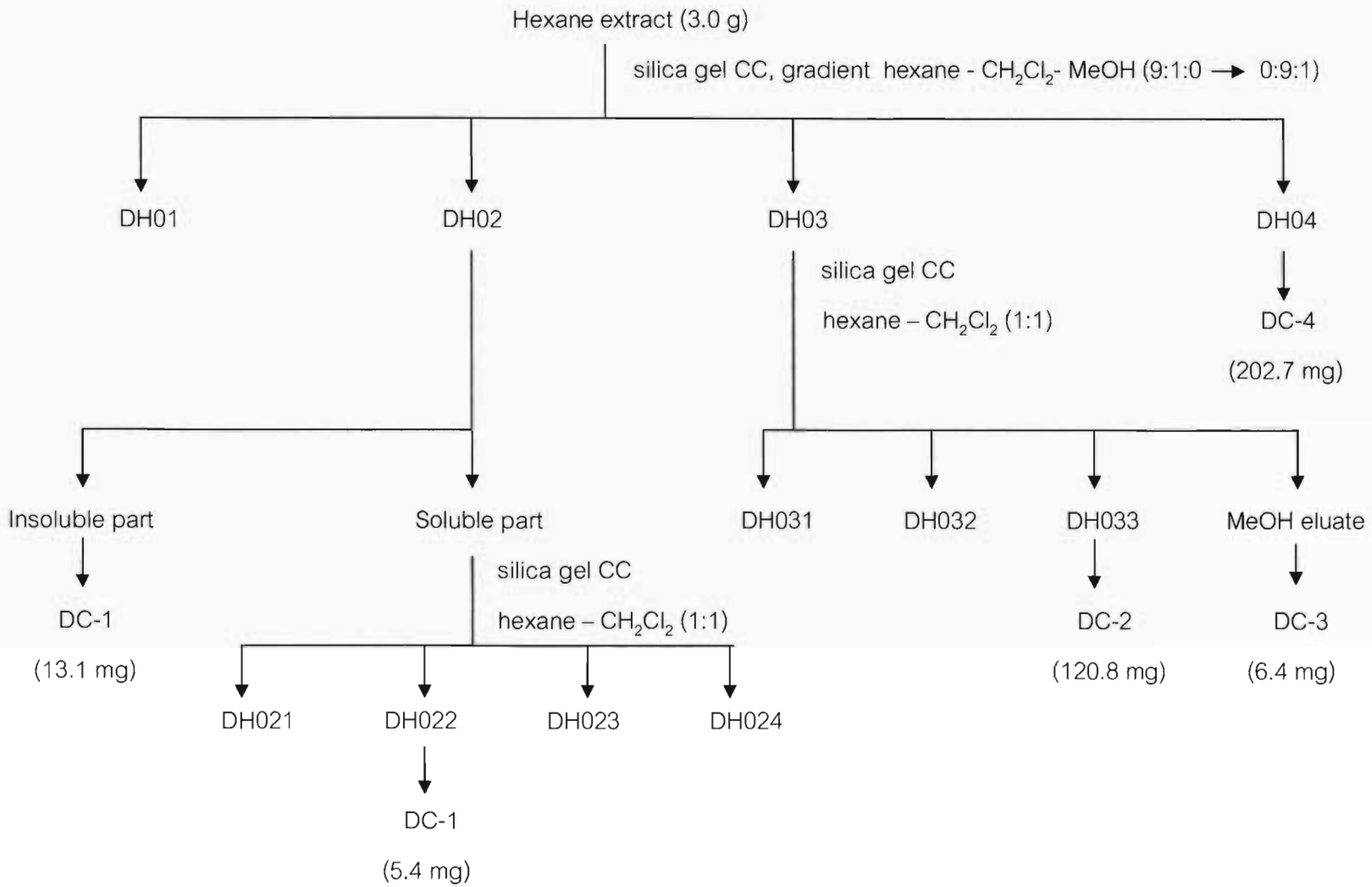
Table 6. Combined fractions from DH03

Fraction	Number of eluates	Weight (mg)
DH031	1-30	34.3
DH032	31-45	30.1
DH033	46-60	120.8
MeOH eluate		173.2

Fraction DH033, which gave a single red-violet spot on TLC under detection with 10% sulfuric acid in ethanol, yielded compound DC-2 as colorless needles (120.8 mg) after removal of the eluent. The compound gave a purple color upon detection with Liebermann-Burchard reagent The MeOH eluate, which gave a large pink-violet spot upon TLC investigation with 10% sulfuric acid in ethanol, was purified by recrystallization in MeOH to give compound DC-3 as colorless needles (6.4 mg). The compound gave a green color upon detection with Liebermann-Burchard reagent

3.2.1.3 Isolation of Compound DC-4

Fraction DH04 (795.6 mg) gave a major red-violet spot on TLC upon detection with 10% sulfuric acid in ethanol. This fraction was recrystallized in MeOH to give compound DC-4 as white amorphous powder (202.7 mg).The compound gave a purple color upon detection with Liebermann-Burchard reagent.



Scheme 2. Isolation of compounds from the hexane extract of *D. collinsae* stems

3.2.2 Isolation of Compounds from the CH_2Cl_2 Extract

A portion of the CH_2Cl_2 extract (4.0 g) was separated on a silica gel column (120 g, 5 x 15.4 cm), eluted with CH_2Cl_2 – MeOH mixtures of increasing polarity from CH_2Cl_2 – MeOH (1:0) to CH_2Cl_2 – MeOH (87:13). Seventy 50-ml fractions were collected and then combined according to their TLC patterns into six major fractions (DD01-DD06) as shown in Table 7. The column was then washed down with MeOH.

Table 7. Combined fractions from CH_2Cl_2 extract

Fraction	Number of eluates	Weight (mg)
DD01	1-20	150.1
DD02	21-24	389.1
DD03	25-39	1501.3
DD04	40-49	45.8
DD05	50-60	67.2
DD06	61-70	59.1
MeOH eluate		1780

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3.2.2.1 Isolation of Compound DC-5

Fraction DD02 (389.1 mg) was submitted to further purification on a silica gel column (30 g, 2 x 22 cm), using CH_2Cl_2 – MeOH (99:1) as the eluent. Seventy-one 20-ml fractions were collected and combined according to their TLC patterns into four major fractions (DD021 – DD024), as shown in Table 8. The column was then washed down with MeOH.

Table 8. Combined fractions from DD02

Fraction	Number of eluates	Weight (mg)
DD021	1-15	25.8
DD022	16-19	30.2
DD023	30-40	83.2
DD024	41-71	126.4
MeOH eluate		108.9

Fraction DD023, which gave a single red-violet spot on TLC upon detection with 10% sulfuric acid in ethanol, yielded compound DC-5 as white powder (83.2 mg) after removal of the eluent. The compound gave a purple color upon detection with Liebermann-Burchard reagent.

3.2.2.2 Isolation of Compound DC-4

Fractions DD03 and DD024, which gave identical, major red-violet spot on TLC upon detection with 10% sulfuric acid in ethanol, were combined together. The pooled fraction was then purified by recrystallization in methanol to give compound DC-4 as white amorphous powder (457.4 mg).

3.2.3 Isolation of Compounds from the EtOAc Extract

A portion of the EtOAc extract (10.0 g) was subjected to a silica gel column (400 g, 10 x 14 cm), eluted with CH_2Cl_2 – MeOH mixtures of increasing polarity from CH_2Cl_2 – MeOH (95:5) to CH_2Cl_2 – MeOH (7:3). One hundred and twenty eight 50-ml fractions were collected and then combined according to their TLC patterns into six major

fractions (DE01-DE06) as shown in Table 9. The column was then washed down with methanol.

Table 9. Combined fractions from EtOAc extract

Fraction	Number of eluates	Weight (mg)
DE01	1-34	1300
DE02	35-55	247.7
DE03	56-80	389.3
DE04	81-95	1200
DE05	96-109	2569.3
DE06	110-128	2192.7
MeOH eluate		2080

3.2.3.1 Isolation of Compound DC-4

Fraction DE01 (1.3 g), which gave a major red-violet spot on TLC upon detection with 10% sulfuric acid in ethanol, was purified by recrystallization in MeOH to give an additional amount of compound DC-4 (242.4 mg).

3.2.3.2 Isolation of Compound DC-6

Fraction DE02 (247.7 mg), was further separated on a silica gel column (15 g, 2 x 11 cm) eluted with CH_2Cl_2 – MeOH (95:5). Sixty two 20-ml fractions were collected and then combined into four major fractions (DE021-DE024) as shown in Table 10. The column was then washed down with methanol.

Table 10. Combined fractions from DE02

Fraction	Number of eluates	Weight (mg)
DE021	1-7	12.7
DE022	8-24	14.4
DE023	25-30	17.1
DE024	31-62	32.6
MeOH eluate		168.5

Fraction DE022, which gave a single pink-violet spot on TLC upon detection with anisaldehyde-sulfuric acid reagent, was removed of the eluent to yield compound DC-6 as orange powder (14.4 mg).

3.2.1.2 Isolation of Compound DC-7

Fraction DE04 was chromatographed on a silica gel column (50 g, 2.5x14 cm), using CH_2Cl_2 – MeOH (92:8) as the eluent. Fifty-four 25 ml-fractions were collected and then combined according to their TLC patterns into five major fractions (DE041 - DE045) as shown in Table 11. The column was then washed down with methanol.

Table 11. Combined fractions from DE04

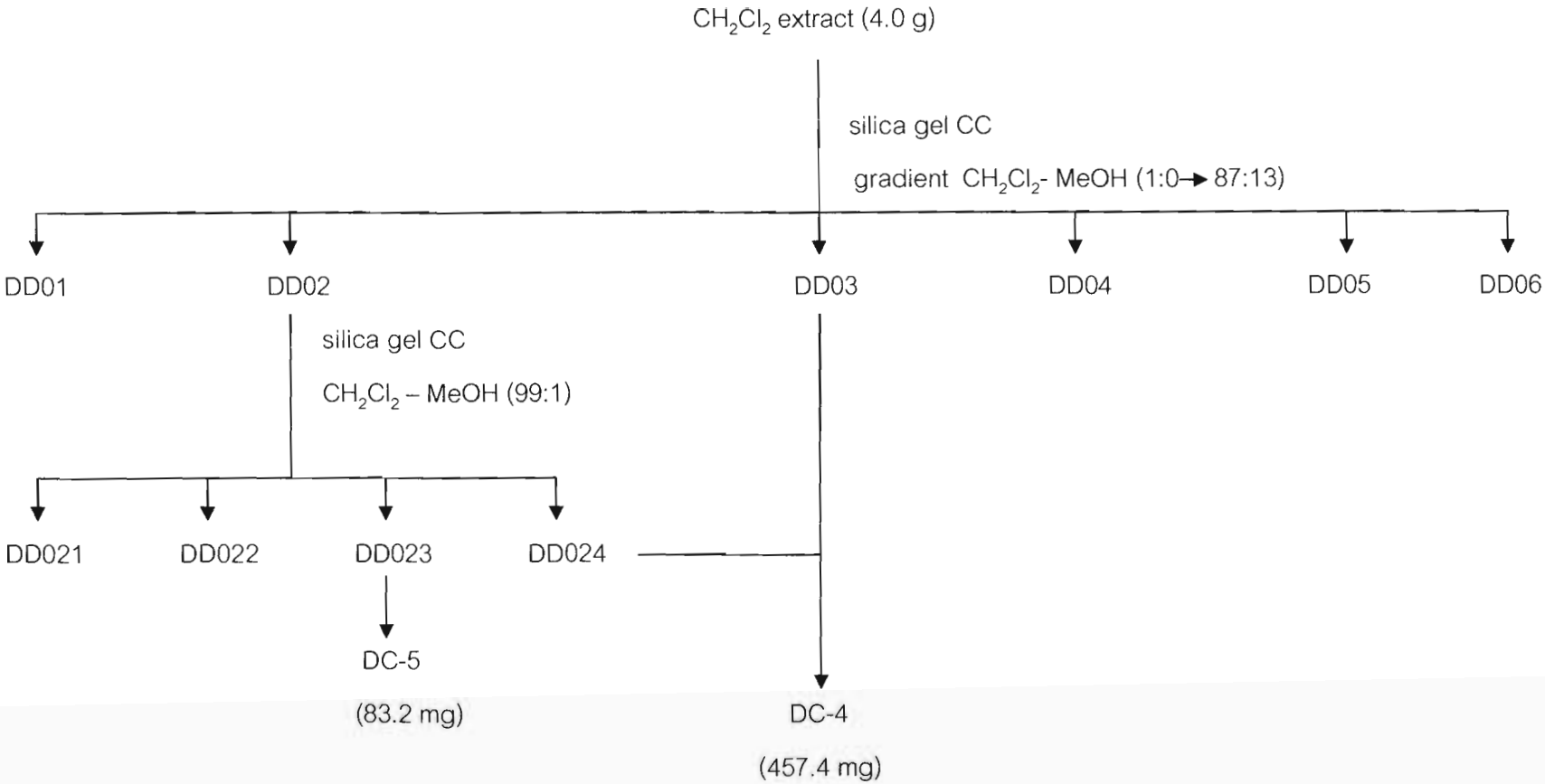
Fraction	Number of eluates	Weight (mg)
DE041	1-14	9.3
DE042	15-19	14.2
DE043	20-25	165.2
DE044	26-37	245
DE045	38-54	138.7
MeOH eluate		608.5

Fraction DE042 (14.2 mg) was further separated on a silica gel column (15 g, 2 × 11 cm) eluted with CH_2Cl_2 – MeOH (9:1). Fifty 5 ml-fractions were collected and then combined according to their TLC patterns into three major fractions (DE0421-DE0423) as shown in Table 12.

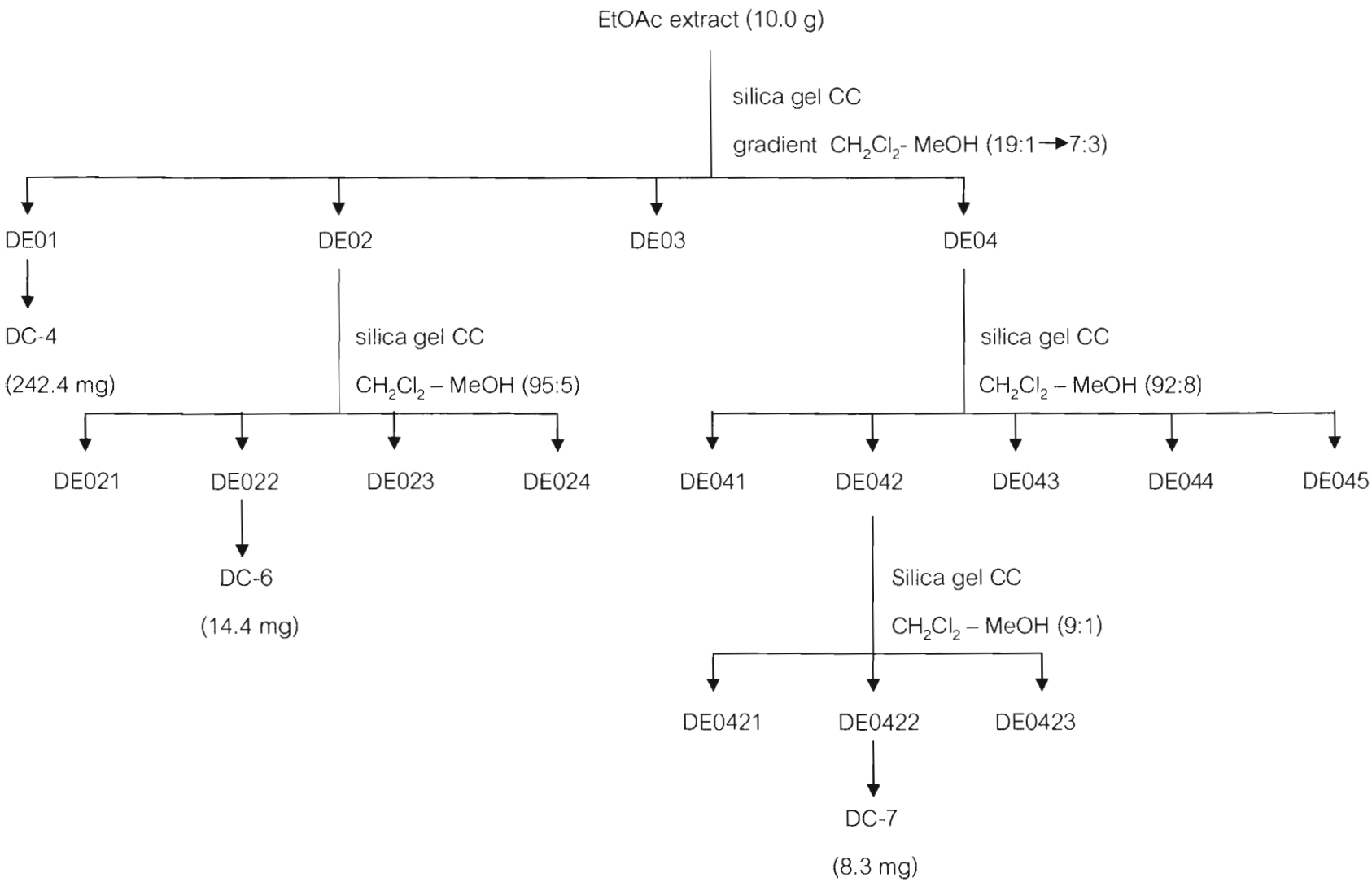
Table 12. Combined fractions from DE042

Fraction	Number of eluates	Weight (mg)
DE0421	1-25	2.7
DE0422	26-36	8.3
DE0423	37-50	2.1

Fraction DE0422, which gave a single pink-violet spot on TLC upon detection with anisaldehyde-sulfuric acid reagent, yielded compound DC-7 as a brown amorphous solid (8.3 mg) after removal of the eluent.



Scheme 3. isolation of compounds from the CH_2Cl_2 extract of *D. collinsae* stems



Scheme 4. Isolation of compounds from the EtOAc extract of *D. collinsae* stems

4. Physical and Spectral Data of Isolated Compounds

4.1 Compound DC-1 (Friedelin)

Appearance	Colorless needles (methanol)
Solubility	Soluble in hexane and chloroform
Melting point	262-263 °C
IR ν_{max} cm^{-1} (KBr)	3431, 2928, 2869, 1716, 1457 and 1389 (Figure 2).
^{1}H- NMR (δ ppm, 500 MHz, $CDCl_3$)	0.70 (3H, *s*), 0.85 (3H, *s*), 0.85 (3H, *d*, *J* = 6.6 Hz), 0.93 (3H, *s*), 0.98 (3H, *s*), 0.99 (3H, *s*), 1.03 (3H, *s*), 1.16 (3H, *s*), 1.66 (1H, *m*), 1.94 (1H, *m*), 2.25 (2H, *m*), 2.25 (2H, *m*), 2.37 (1H, *d*, *J* = 2.0, 5.0, 12.0 Hz) (Table 13 and Figure 3).
^{13}C- NMR (δ ppm, 125 MHz, $CDCl_3$)	6.8, 14.6, 17.9, 18.2, 18.7, 20.2, 22.3, 28.2, 30.0, 30.5, 31.8, 32.1, 32.4, 32.8, 35.0, 35.3, 35.6, 36.0, 37.4, 38.3, 39.2, 39.7, 41.3, 41.5, 42.1, 42.8, 53.1, 58.2, 59.5 and 213.3 (Table 13 and Figures 4a-4b).
ESI-MS *m/z*	427.39 $[M+H]^+$ (Figure 5).

4.2 Compound DC-2 (Lupeol)

Appearance	Colorless needles (methanol)
Solubility	Soluble in chloroform
Melting point	214-215 °C
IR ν_{max} cm^{-1} (KBr)	3411, 2928, 1464, 1455, 1381, 1014 and 882 (Figure 6).
^{1}H- NMR (δ ppm, 500 MHz, $CDCl_3$)	0.74 (3H, *s*), 0.77 (3H, *s*), 0.81 (3H, *s*), 0.92 (3H, *s*), 0.95 (3H, *s*), 1.01 (3H, *s*), 1.66 (3H, *s*), 2.36

	(1H, *m*), 3.17 (1H, *dd*, *J* = 11.4, 4.7 Hz), 4.55 (1H, *dd*, *J* = 2.4, 1.4 Hz) and 4.67 (1H, *d*, *J* = 2.4 Hz) (Table 14 and Figures 7a-7b).
^{13}C- NMR (δ ppm, 125 MHz, $CDCl_3$)	14.5, 15.4, 16.0, 16.1, 18.0, 18.3, 19.3, 20.9, 25.1, 27.4, 27.4, 28.0, 29.8, 34.3, 35.6, 37.2, 38.0, 38.7, 38.9, 40.0, 40.8, 42.8, 43.0, 48.0, 48.3, 50.4, 55.3, 79.0, 109.3 and 151.0 (Table 14 and Figures 8a-8b).

4.3 Compound DC-3 (a Mixture β-Sitosterol and Stigmasterol)

Appearance	Colorless needles (methanol)
Solubility	Soluble in chloroform
IR ν_{max} cm^{-1} (KBr)	3430, 2959, 2869, 1728, 1465, 1321 and 1061 (Figure 9).
^{1}H- NMR (δ ppm, 500 MHz, $CDCl_3$)	0.66 (3H, *s*), 0.76-0.86 (9H), 0.90 (3H, *d*, *J* = 6.6 Hz), 0.99 (3H, *s*), 3.50 (1H, *m*) , 5.01 (1H, *dd*, *J* = 15.1, 8.7 Hz), 5.13 (1H, *dd*, *J* = 15.1, 8.7 Hz) and 5.33 (1 H, *d*, *J* = 5.2 Hz) (Figure 10).
^{13}C- NMR (δ ppm, 125 MHz, $CDCl_3$)	11.9, 12.0, 18.8, 19.0, 19.4, 19.8, 21.1, 23.1, 24.3, 26.1, 28.2, 29.2, 31.7, 31.9, 31.9, 33.9, 36.1, 36.5, 37.3, 39.8, 42.3, 42.3, 45.8, 50.1, 56.1, 56.8, 71.8, 121.7, 129.3, 138.3 and 140.8 (Table 15 and Figures 11a-11b).

4.4 Compound DC-4 (Betulinic acid)

Appearance	White amorphous powder (methanol)
Solubility	Soluble in chloroform and methanol
Melting point	295-296 °C

IR ν_{max} cm^{-1} (KBr)	3436, 2943, 1689, 1453, 1376, 1032 and 883 (Figure 12).
^{1}H- NMR (δ ppm, 500 MHz, $CDCl_3$)	0.73 (3H, *s*), 0.80 (3H, *s*), 0.92 (3H, *s*), 0.94 (3H, *s*), 0.96 (3H, *s*), 1.67 (3H, *s*), 2.97 (1H, *m*) 3.17 (1H, *dd*, *J* = 11.4, 4.8 Hz), 4.59 (1H, *dd*, *J* = 2.0, 1.5 Hz) and 4.72 (1H, *d*, *J* = 2.0 Hz) (Table 16 and Figures 13a-13b).
^{13}C- NMR (δ ppm, 125 MHz, $CDCl_3$)	14.7, 15.3, 16.0, 16.1, 18.3, 19.4, 20.8, 25.5, 27.4, 28.0, 29.7, 30.5, 32.1, 34.3, 37.0, 37.2, 38.4, 38.7, 38.9, 40.7, 42.4, 46.9, 49.3, 50.5, 55.3, 56.3, 79.0, 109.7, 150.4 and 179 (Table 16 and Figures 14a-14b).

4.5 Compound DC-5 (Betulin)

Appearance	Colorless needles (methanol)
Solubility	Soluble in chloroform and methanol
Melting point	251-252 °C
^{1}H- NMR (δ ppm, 500 MHz, $CDCl_3$)	0.74 (3H, *s*), 0.80 (3H, *s*), 0.95 (3H, *s*), 0.96 (3H, *s*),1.0 (3H, *s*), 1.66 (3H, *s*), 2.36 (1H, *m*), 3.16 (1H, *dd*, *J* = 11.4, 4.8 Hz), 3.31 (1H, *d*, *J* =10.9 Hz) 3.78 (1H, *d*, *J* = 10.9 Hz) 4.56 (1H, *dd*, *J* = 2.2, 1.5 Hz) and 4.66 (1H, *d*, *J* = 2.2 Hz) (Table 17 and Figures 15a-15b).
^{13}C- NMR (δ ppm, 125 MHz, $CDCl_3$)	14.7, 15.3, 16.0, 16.1, 18.3, 19.1, 20.8, 25.2, 27.0, 27.4, 28.0, 29.7, 34.0, 34.2, 37.1, 37.3, 38.7, 38.8, 39.1, 40.9, 42.7, 47.8, 47.8, 48.7, 50.4, 55.3, 60.5, 79.0, 109.7, and 150.5 (Table 17 and Figures 16a-16b).

4.6 Compound DC-6 (Diptoindonesin G)

Appearance	Orange powder
Solubility	Soluble in methanol
Melting point	186-187 °C
UV λ_{max} (MeOH) nm	210, 248, 285 and 360 (Figure 17).
IR ν_{max} cm^{-1} (KBr)	3391, 2922, 1614, 1585, 1466, 1406, 1230, 1041 and 825 (Figure 18).
^{1}H- NMR (δ ppm, 500 MHz, DMSO-d_6)	6.27 (1H, *d*, *J* = 2.2 Hz), 7.0 (1H, *d*, *J* = 8.7 Hz), 7.08 (1H, *d*, *J* = 2.2 Hz), 7.34 (1H, *d*, *J* = 1.7 Hz), 7.37 (1H, *d*, *J* = 1.7 Hz), 7.73 (1H, *d*, *J* = 8.7 Hz) and 14.13 (1H, *s*) (Table 18 and Figures 19a-19b).
^{13}C- NMR (δ ppm, 125 MHz, DMSO-d_6)	102.9, 103.6, 104.9, 107.9, 108.2, 111.0, 116.8, 121.0, 124.1, 124.8, 131.2, 134.8, 153.2, 157.1, 157.9, 160.2, 164.9, 167.2 and 186.8 (Table 18 and Figure 21).
HRESI -MS *m/z*	359.0563 $[M-H]^{+}$ (Figure 24).

4.7 Compound DC-7 (Diptoindonesin D)

Appearance	Brown amorphous solid
Solubility	Soluble in methanol
Melting point	186-187 °C
$[\alpha]^{20}_{D}$	- 130° (*c* 0.015; MeOH)
UV λ_{max} (MeOH) nm	207, 224, 245, 275 and 371 (Figure 25).
IR ν_{max} cm^{-1} (KBr)	3433, 2922, 1608, 1514, 1384, 1273 and 468 (Figure 26).
^{1}H- NMR (δ ppm, 500 MHz, acetone-d_6)	5.89 (1H, *s*), 6.38 (1H, *d*, *J* = 2.4 Hz), 6.63 (2H, *d*, *J* = 8.9 Hz), 6.68 (2H, *d*, *J* = 8.9

	Hz), 6.80 (1H, *d*, *J* = 2.5 Hz), 6.90 (1H, *d*, *J* = 2.5 Hz), 6.92 (1H, *d*, *J* = 2.4 Hz) and 13.70 (1H, *s*) (Table 19 and Figures 27a-27b).
^{13}C- NMR (δ ppm, 125 MHz, acetone-d_6)	55.2, 107.1, 107.5, 110.2, 111.2, 111.6, 113.2, 116.2, 128.9, 130.5, 139.6, 142.8, 156.8, 157.0, 158.8, 165.4, 167.4, 196.8 and 197.3 (Table 19 and Figures 29a-29b).
HRESI -MS *m/z*	377.0662 $[M-H]^+$ (Figure 32).

5. Determination of Biological Activities

Assays for cytotoxic and antimalarial activities were performed at the National Center for Genetic Engineering and Biotechnology, BIOTEC, Pathumthani, Thailand.

5.1 Cytotoxic Activity

5.1.1 Cytotoxic activity Against NCI-H187, KB and MCF-7 Cell Lines

The cytotoxic activity of the isolated compound (DC-7) against human small cell lung carcinoma (NCI-H187, ATCC CRL-5804), epidermoid carcinoma of oral cavity (KB, ATCC CCL-17) and breast adenocarcinoma (MCF-7, ATCC HTB-22) cell lines was determined by Resazurin microplate assay (Brien *et al.*, 2000). Ellipticine, doxorubicin and tamoxifen were used as the positive control, and 0.5 % DMSO as the negative control. Briefly, NCI-H187 and MCF-7 cells were diluted to 9×10^4 cells/ml and KB cells were diluted to 7×10^4 cells/ml in fresh medium. Successively, 5 µl of the test sample diluted in 5 % DMSO and 45 µl of the cell suspension were added to microwell plates. The plates were incubated at 37°C in 5% CO_2 for 5 days (for NCI-H187) or 3

days (for KB and MCF-7). Then, 12.5 μl of 62.5 μl/ml resazurin solution were added to each well. The plates were incubated at 37°C for 4 hours. Fluorescence signals were measured at the excitation and emission wavelengths of 530 nm and 590 nm, respectively. The percent inhibition of cell growth was calculated by the following equation:

$$\% \text{ Inhibition} = [1- (FU_T/ FU_C)] \times 100$$

whereas FU_T and FU_C were the mean fluorescent unit from treated and untreated conditions, respectively.

5.1.2 Cytotoxic Activity against Vero Cell Line

The cytotoxic activity of the isolated compound (DC-7) against African green monkey kidney cell line (Vero, ATCC CCL-81) was determined by Green fluorescent protein (GFP) detection (Hunt *et. al.*, 1999). Ellipticine and 0.5% DMSO were used as the positive and negative controls, respectively. In brief, 45 μl of the cell suspension (3.3×10^4 cells/ml) were added to each well of microwell plates containing 5 μl of the test compound previously diluted in 0.5% DMSO. The plate was then incubated at 37°C in 5% CO_2 for 4 days. Fluorescence signals were measured, using Spectra Max M5 microplate reader (Molecular Devices, USA) in the bottom-reading mode, at the excitation and emission wavelengths of 485 nm and 535 nm, respectively. The fluorescence signal at day 4 was subtracted with the background fluorescence at day zero. The percent inhibition of cell growth was calculated by the same equation as in **5.1.1**. If cell viability was more than 50%, the IC_{50} value was reported as inactive. If cell viability was less than 50%, the IC_{50} value was reported from two-fold serial dilution.

5.2 Antimalarial Activity

Plasmodium falciparum (K1, multidrug resistant strain) was cultivated *in vitro*, according to Trager and Jensen (1976), in RPMI 1640 medium containing 20 mM

HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 32 mM $NaHCO_3$ and 10% heat activated human serum with 3% erythrocytes, in humidified 37°C incubator with 3% CO_2. Cultures were passaged with fresh mixture of erythrocytes and medium for every day to maintain cell growth.

Quantitative assessment of *in vitro* antimalarial activity of the isolated compound (DC-7) was determined by microculture radioisotope techniques based upon the methods described by Desjardins *et al.* (1979). Dihydroartemisinine and mefloquine were used as the positive control, and 0.1 % DMSO as the negative control. Briefly, a mixture of 200 µl of 1.5% erythrocytes with 1% parasitemia at the early ring stage was pre-exposed to 25 µl of the medium containing the test sample dissolved in 1% DMSO (0.1% final concentration) for 24 hours. Subsequently, 25 µl of [^{3}H] hypoxanthine (Amersham, USA) in culture medium (0.5 µCi) was added to each well and the plates were incubated for an additional 24 hours. Levels of incorporated radioactive labeled hypoxanthine, indicating parasite growth, were determined using the Top Count microplate scintillation counter (Packard, USA). The percentage of parasite growth was calculated using the signal count per minute of treated (CPM_T) and untreated conditions (CPM_U), by the following equation.

$$\% \text{ parasite growth} = CPM_T/CPM_U \times 100$$

CHAPTER IV
RESULTS AND DISCUSSION

Phytochemical investigation of the hexane, CH_2Cl_2 and EtOAc extracts of the dried stems of *D. collinsae* led to the isolation of seven chemical constituents. The identification of the isolated compounds was achieved through spectroscopic analysis, and confirmed by comparison with literature values. The details are as follows.

1. Identification of Compound DC-1 (Friedelin)

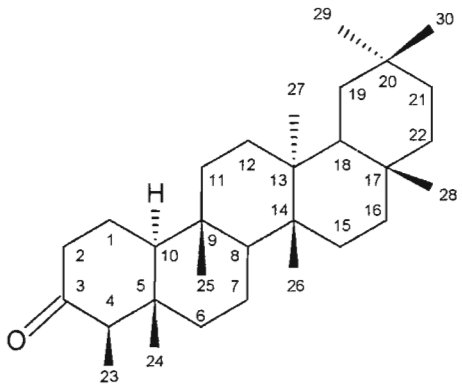
Compound DC-1 was obtained as colorless needles. This compound gave purple color to Liebermann-Burchard reagent, suggesting that it was a triterpenoid. Its IR spectrum (Figure 2) exhibited a strong absorption at 1716 cm^{-1}, indicating the presence of a carbonyl functionality in its molecule.

The ^1H NMR spectrum of DC-1 (Figure 3) showed eight methyl signals which were indicative of the friedelane skeleton. These signals included a doublet due to one secondary methyl at δ 0.85 ppm (J = 6.6 Hz, H-23) and singlets due to seven tertiary methyls at δ 0.70 (H-24), 0.85 (H-25), 0.93 (H-29), 0.98 (H-26), 0.99 (H-30), 1.03 (H-27) and 1.16 (H-28) ppm. Other signals appeared in the range of δ 1.00 - 2.50 ppm.

The ^{13}C NMR spectrum (Figures 4a-4b) displayed 30 carbon signals, supportive of the triterpenoid nature. The most downfield signal at δ 213.3 ppm suggested the presence of a keto functionality in the molecule of DC-1. ^{13}C NMR data of the compound were then compared with those of friedelin, a typical friedelane derivative with 3-keto substituent (Akihisa *et al.*, 1992), and found to be in full agreement. The comparison allowed the 30 carbon signals of DC-1 to be differentiated into eight methyl signals at δ 6.8 (C-23), 14.6 (C-24), 17.9 (C-25), 18.7 (C-27), 20.2 (C-26), 31.8 (C-30), 32.1 (C-28), and 35.0 (C-29) ppm; eleven methylene signaals at δ 18.2 (C-7), 22.3 (C-1), 30.5 (C-12),

32.4 (C-15), 32.8 (C-21), 35.3 (C-19), 35.6 (C-11), 36.0 (C-16), 39.2 (C-22) , 41.3 (C-6) and 41.5 (C-2) ppm; four methine signals at δ 42.8 (C-18), 53.1 (C-8), 58.2 (C-4) and 59.5 (C-10) ppm; and seven quaternary signals at δ 28.2 (C-20), 30.0 (C-17), 37.4 (C-9), 38.3 (C-14), 39.7 (C-13), 42.1 (C-5) and 213.3 (C-3) ppm. Comparison of ^{13}C-NMR assignments of DC-1 and friedelin is shown in Table 13. The assignment of some proton signals of DC-1 is also shown in the same table.

The information mentioned above indicated that DC-1 was friedelin. The identification was also confirmed by the ESI mass spectrum (Figure 5), where a pseudomolecular peak $[M+H]^+$ at *m/z* 427.39, corresponding to the molecular formula of $C_{30}H_{50}O$, was observed. DC-1 was therefore identified as friedelin. The structure of the compound is shown below.



friedelin (107)

The presence of friedelin in *Diospyros* species has been previously documented. The compound has been found to exhibit various biological activities eg. cytotoxic (Zheng, 1994), antifungal (Duraipandiyan, Gnanasekar and Ignacimuthu, 2010), anti-inflammatory, analgesic and antipyretic activities (Antonisamy, Duraipandiyan and Ignacimuthu, 2011).

Table 13. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignments of compound DC-1 (in $CDCl_3$) and the reported ^{13}C NMR assignment of friedelin (in $CDCl_3$)

position	DC-1		Friedelin*
	δ_H (ppm) (mult.,*J* in Hz)	δ_C (ppm)	δ_C (ppm)
1	1.66 (*m*), 1.94 (*m*)	22.3	22.3
2	2.37 (*ddd*, 2.0, 5.0, 12.0), 2.25 (*m*)	41.5	41.5
3	-	213.3	213.2
4	2.25 (*m*)	58.2	58.2
5	-	42.1	42.1
6	-	41.3	41.3
7	-	18.2	18.2
8	-	53.1	53.1
9	-	37.4	37.4
10	-	59.5	59.4
11	-	35.6	35.6
12	-	30.5	30.5
13	-	39.7	39.7
14	-	38.3	38.3
15	-	32.4	32.4
16	-	36.0	36.0
17	-	30.0	30.0
18	1.52 (*m*)	42.8	42.8
19	-	35.3	35.3
20	-	28.2	28.1
21	-	32.8	32.7
22	-	39.2	39.2
23	0.85 (*d*, 6.6)	6.8	6.8
24	0.70 (*s*)	14.6	14.6
25	0.85 (*s*)	17.9	17.9
26	0.98 (*s*)	20.2	20.2
27	1.03 (*s*)	18.7	18.6
28	1.16 (*s*)	32.1	32.1
29	0.93 (*s*)	35.0	35.0
30	0.99 (*s*)	31.8	31.8

* Akihisa *et al.*, 1992

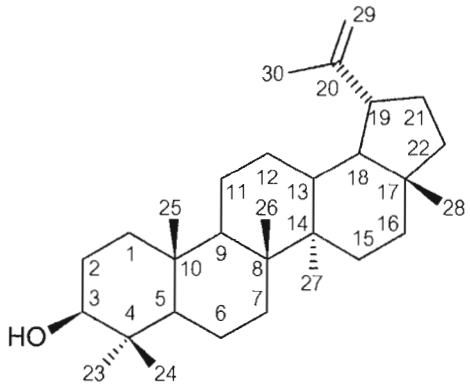
2. Identification of Compound DC-2 (Lupeol)

Compound DC-2 was obtained as colorless needles which gave purple color to Liebermann-Burchard reagent, suggesting its triterpenoid nature. The presence of the hydroxyl substituent in the molecule of DC-2 was suggested by an absorption band at 3410 cm^{-1}, displayed in the IR spectrum (Figure 6).

The ^{1}H NMR spectrum of DC-2 (Figures 7a-7b) showed a pair of broad singlet-like signals (1 H each) at δ 4.55 (*dd*, *J* = 2.4, 1.4 Hz) and 4.67 (*d*, *J* = 2.4 Hz) ppm. Such resonances are characteristic of a lupane-type triterpenoid, corresponding to exomethylene protons (H-29) in the isopropenyl side chain on ring D of the lupane skeleton. Seven tertiary methyls of the compound were observed as three-proton singlets at δ 0.74 (H-24), 0.77 (H-28), 0.81 (H-25), 0.92 (H-27), 0.95 (H-23), 1.01 (H-26), and 1.66 (H-30) ppm. A doublet of doublets at δ 3.17 ppm (1H, *J* = 11.4, 4.7 Hz), assignable to the hydroxymethine proton (H-3), pointed out that DC-2 was a triterpenoid alcohol.

The ^{13}C NMR spectrum (Figures 8a-8b) exhibited 30 carbon signals, supporting that DC-2 was a triterpenoid. The presence of the exomethylene moiety in the molecule was represented by a pair of downfield signals at δ 109.3 (C-29) and 151.0 (C-20) ppm, while a signal at δ 79.0 ppm was assignable to the hydroxyl-substituted methine carbon (C-3). ^{13}C-NMR data of DC-2 were found to be in full agreement with the reported values for lupeol (Reynolds *et al.*, 1986), a common triterpenoid alcohol of the lupane type. Comparison of ^{13}C-NMR assignments of DC-2 and lupeol, together with ^{1}H NMR assignment of DC-2, is shown in Table 14.

The information mentioned above are in accordance with the chemical structure of lupeol. Therefore, DC-2 was identified as lupeol. The structure of the compound is shown below.



lupeol (42)

Lupeol has been found in various plants, including *Diospyros* species. Some interesting bioactivities of the compound have been reported eg. anti-inflammatory (Fernandez *et al.*, 2001), anti-arthritic (Geetha and Varalakshmi, 2001), cytotoxic (Wada, Iida and Tanaka, 2001), chemopreventive and anti-neoplastic activities (Chaturvedi, Bhui and Shukla, 2008).

Table 14. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignments of compound DC-2 (in $CDCl_3$) and the reported ^{13}C NMR assignment of lupeol (in $CDCl_3$)

position	DC-2		Lupeol*
	δ_H (ppm) (mult.,*J* in Hz)	δ_C (ppm)	δ_C (ppm)
1	-	38.7	38.7
2	-	27.4	27.4
3	3.17 (*dd*, 11.4, 4.7)	79.0	78.9
4	-	38.9	38.8
5	-	55.3	55.3
6	-	18.3	18.3
7	-	34.3	34.2
8	-	40.8	40.8
9	-	50.4	50.4
10	-	37.2	37.1
11	-	20.9	20.9
12	-	25.1	25.1
13	-	38.0	38.0
14	-	42.8	42.8
15	-	27.4	27.4
16	-	35.6	35.5
17	-	43.0	43.0
18	-	48.3	48.2
19	2.36 (*m*)	48.0	47.9
20	-	151.0	150.9
21	-	29.8	29.8
22	-	40.0	40.0
23	0.95 (*s*)	28.0	28.0
24	0.74 (*s*)	15.4	15.4
25	0.81 (*s*)	16.1	16.1
26	1.01 (*s*)	16.0	15.9
27	0.92 (*s*)	14.5	14.5
28	0.77 (*s*)	18.0	18.0
29	4.55 (*dd*, 2.4, 1.4) 4.67 (*d*, 2.4)	109.3	109.3
30	1.66 (*s*)	19.3	19.3

* Reynolds *et al.*, 1986

3. Identification of Compound DC-3 (a Mixture of β-Sitosterol and Stigmasterol)

Compound DC-3 was obtained as colorless needles. It gave positive green color with Liebermann-Burchard reagent, indicative of the steroid skeleton. The IR spectrum (Figure 9) showed a hydroxyl absorption band at 3430 cm^{-1}.

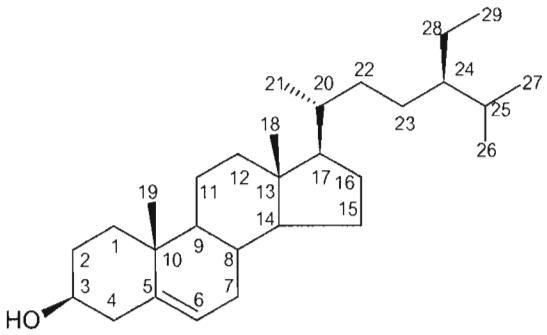
The ^{1}H and ^{13}C NMR spectra of DC-3 (Figures 10, 11a-11b) provided evidences which indicated that DC-3 was a mixture of β-sitosterol and stigmasterol. In both spectra, signals for β-sitosterol were prominent while those for stigmasterol were hardly observed. The signals not labeled with their exact for stigma steroid were chemical shifts. In addition, the integration values for certain proton signals due to stigmasterol, which were distinguishable from signals due to β-sitosterol, were not given. Therefore, it could be deduced that β-sitosterol was a major component of DC-3 and the relative amount of stigmasterol in the compound was very small.

In the ^{1}H NMR spectrum (Figure 10), methyl signals of the two steroids were observed in the range of δ 0.60 - 1.20 ppm. An olefinic signal of the tri-substituted double bond at δ 5.33 ppm (1 H, *d*, *J* = 5.2 Hz) and a methine proton multiplet at δ 3.50 ppm were attributable to H-6 and H-3 of both β-sitosterol and stigmasterol. Two double doublets at δ 5.01 (*J* = 15.1, 8.7 Hz, H-22) and 5.13 (*J* = 15.1, 8.7 Hz, H-23) ppm were indicative of stigmasterol.

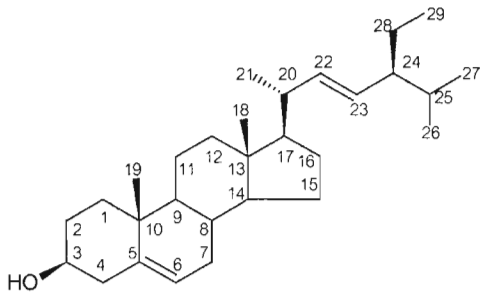
In the ^{13}C NMR spectrum (Figures 11a-11b), the carbon signal due to the oxygenated C-3 of both β-sitosterol and stigmasterol was observed at δ 71.8 ppm. The signals at δ 140.8 and 121.7 ppm could be assigned to olefinic C-5 and C-6 of the two compounds. Two less intense olefinic signals appearing around δ 138 and 129 ppm were assignable to C-22 and C-23 of stigmasterol.

Therefore, DC-3 was determined to be a mixture of β-sitosterol and stigmasterol, of which the major component was β-sitosterol. The two steroids are common

phytosterols widely distributed in the plant kingdom and frequently occur together. Comparison of the ^{13}C NMR data of DC-3 with the reported data of β-sitosterol and stigmasterol (De-Eknamkul and Potduang, 2003) is shown in Table 15.



β-sitosterol



stigmasterol

Table 15. ^{13}C (125 MHz) NMR assignments of compound DC-3 (in $CDCl_3$), β-sitosterol (in $CDCl_3$) and stigmasterol (in $CDCl_3$)

Position	δ_C (ppm)		
	DC-3*	β-sitosterol**	stigmasterol**
1	37.3	37.2	37.2
2	31.7	31.6	31.6
3	71.8	71.8	71.8
4	42.3	42.2	42.3
5	140.8	140.7	140.7
6	121.7	121.7	121.7
7	31.9	31.9	31.9
8	31.9	31.9	31.9
9	50.1	50.1	50.1
10	36.5	36.5	36.6
11	21.1	21.1	21.1
12	39.8	39.7	39.7

Table 15. ^{13}C (125 MHz) NMR assignments of compound DC-3 (in $CDCl_3$), β-sitosterol (in $CDCl_3$) and stigmasterol (in $CDCl_3$) (continued)

Position	δ_C (ppm)		
	DC-3*	β-sitosterol**	stigmasterol**
13	42.3	42.3	42.3
14	56.8	56.7	56.8
15	24.3	24.3	24.3
16	28.2, (29.0)	28.2	28.9
17	56.1	56.0	55.9
18	12.0	11.8	12.0
19	19.4	19.4	19.4
20	36.1, (40.5)	36.1	40.5
21	18.8, (21.1)	18.8	21.1
22	33.9, (138.3)	33.9	138.3
23	26.1, (129.3)	26.0	129.2
24	45.8, (51.2)	45.8	51.2
25	29.2, (31.9)	29.1	31.9
26	19.8, (21.1)	19.8	21.2
27	19.0	19.0	19.0
28	23.1, (25.5)	23.0	25.4
29	11.9	12.0	12.2

*Chemical shifts in parenthesis are estimated values for certain carbons of stigmasterol, the signals of which were obviously observed at different chemical shifts from those for β-sitosterol.

**De-Eknamkul and Potduang, 2003

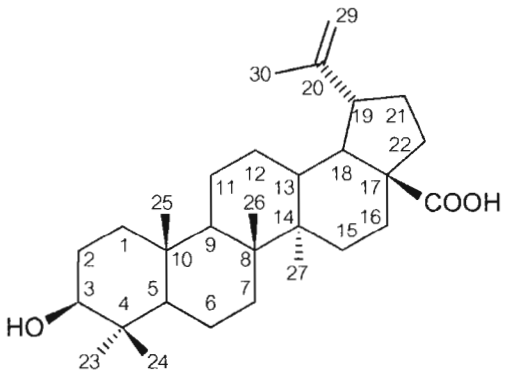
4. Identification of Compound DC-4 (Betulinic acid)

Compound DC-4 was obtained as white amorphous powder. The compound gave purple color when sprayed with Liebermann-Burchard reagent, suggestive of the triterpenoid nucleus. The presence of hydroxyl and carbonyl groups in the molecule of DC-4 was suggested by absorption bands at 3436 and 1689 cm^{-1}, respectively, in the IR spectrum (Figure 12).

In the ^{1}H NMR spectrum (Figures 13a-13b), characteristic proton signals for a lupane-type triterpenoid with the isopropenyl group were observed at δ 4.59 (*dd*, *J* = 2.0, 1.5 Hz, H-29) and 4.72 (*d*, *J* = 2.0 Hz, H-29) ppm. The signal of the hydroxymethine proton (H-3) was discernible as a doublet of doublet at δ 3.17 (*J* =11.4, 4.8 Hz) ppm. The ^{1}H NMR spectrum of DC-4 was similar to that of DC-2 except for the absence of one methyl singlet, suggesting that DC-4 was a derivative of DC-2, of which one methyl group on the basic skeleton was replaced by another functional group.

The ^{13}C NMR spectrum (Figures 14a-14b) exhibited 30 carbon signals. Comparison of this spectrum with that of DC-2 indicated that one methyl signal of DC-2 was replaced by a carbonyl signal of DC-4 which was observed at δ 179.7 ppm (C-28). ^{13}C NMR data of DC-4 was then compared with those of betulinic acid (Siddiqui *et al.*, 1988) and found to be in full agreement. ^{13}C NMR assignments of DC-4 and betulinic acid, together with ^{1}H NMR assignment of DC-4, are shown in Table 16.

DC-4 was, therefore, identified as betulinic acid. The structure of the compound is shown below.



betulinic acid (60)

Betulinic acid can be found widely in the genus *Diospyros.* Several biological activities of the compounds have been reported, eg. antibacterial (Chandramu *et al.*, 2003), anti-inflammatory (Del. Recio *et al.*, 1995), anti-HIV (Hashimoto *et al.*, 1997), and anticancer (Fulda, 2008) activities. The compound has been found to exert remarkable anticancer effects and considered to be promising in cancer drug development.

Table 16. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignments of compound DC-4 (in $CDCl_3$) and the reported ^{13}C NMR assignment of betulinic acid (in $CDCl_3$)

position	DC-4		Betulinic acid*
	δ_H (ppm) (mult.,*J* in Hz)	δ_C (ppm)	δ_C (ppm)
1	-	38.7	38.7
2	-	27.4	27.4
3	3.17 (*dd*, 11.4, 4.8)	79.0	78.9
4	-	38.9	38.8
5	-	55.3	55.3
6	-	18.3	18.3
7	-	34.3	34.3
8	-	40.7	40.7
9	-	50.5	50.5
10	-	37.2	37.2
11	-	20.8	20.8
12	-	25.5	25.5
13	-	38.4	38.4
14	-	42.4	42.4
15	-	30.5	30.5
16	-	32.1	32.1
17	-	56.3	56.3
18	-	46.9	46.8
19	2.97 (*m*)	49.3	49.2
20	-	150.4	150.3
21	-	39.7	39.7
22	-	37.0	37.0
23	0.92 (*s*)	28.0	27.9
24	0.73 (*s*)	15.3	15.3
25	0.80 (*s*)	16.0	16.0
26	0.94 (*s*)	16.1	16.1
27	0.96 (*s*)	14.7	14.7
28	-	179.7	180.5
29	4.59 (*dd*, 2.0, 1.5) 4.72 (*d*, 2.0)	109.7	109.6
30	1.67 (*s*)	19.4	19.4

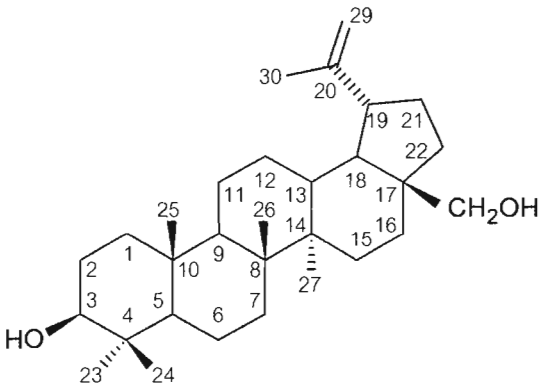
*Siddiqui *et al.*, 1988

5. Identification of Compound DC-5 (Betulin)

Compound DC-5 was obtained as colorless needles. Its triterpenoid nature was suggested by the positive result (purple color) with Liebermann-Burchard reagent. The ^{1}H-NMR spectrum (Figures 15a-15b) exhibited characteristic proton signals of a lupane-type triterpenoid with the isopropenyl group at δ 4.56 (*dd*, *J* = 2.2, 1.5 Hz, H-29) and 4.66 (*d*, *J* = 2.2 Hz, H-29) ppm. A signal of the hydroxymethine proton (H-3) at δ 3.16 (*dd*, *J* = 11.4, 4.8 Hz, H-3) ppm was also observed. The ^{1}H-NMR spectrum of DC-5, like that of DC-4, is similar to that of DC-2. Two prominent differences, observed in the spectrum of DC-5, are the absence of one methyl singlet and the addition of a pair of doublets (*J* = 10.9 Hz) at δ 3.31 and 3.78 ppm.

The ^{13}C-NMR spectrum of DC-5 (Figures 16a-16b), when compared with that of DC-2, indicated the absence of one methyl signal and an additional hydroxymethylene signal at δ 60.5 ppm (C-28). The above information suggested that DC-5 was a derivative of DC-2, of which one methyl group was replaced by a primary alcoholic group. Comparison of ^{13}C-NMR data of DC-5 with those of betulin (Tinto, Blair and Alli, 1992) indicated full agreement of their data. ^{1}H and ^{13}C NMR assignments of DC-5 and betulin, together with ^{1}H-NMR assignment of DC-5, are shown in Table 17.

Therefore, DC-5 was identified as betulin. The structure of the compound is shown below.



betulin (47)

Betulin is one of the lupane-type triterpenoids frequently found in *Diospyros* species. The compound has been shown to exhibit various biological activities, eg. antimycobacterial (Cantrell, Franzblau and Fisher *et al.*, 2001), anti-inflammatory (Del. Recio *et al.*, 1995) and antitumor (Rzeski *et al.*, 2009) activities.

Table 17. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignments of compound DC-5 (in $CDCl_3$) and the reported ^{13}C NMR assignment of betulin (in $CDCl_3$)

position	DC-5		Betulin*
	δ_H (ppm) (mult.,*J* in Hz)	δ_C (ppm)	δ_C (ppm)
1	-	38.7	38.8
2	-	27.4	27.2
3	3.16 (*dd*, 11.4, 4.8)	79.0	78.9
4	-	38.8	38.9
5	-	55.3	55.3
6	-	18.3	18.3
7	-	34.2	34.3
8	-	40.9	40.9
9	-	50.4	50.4
10	-	37.1	37.2
11	-	20.8	20.9
12	-	25.2	25.3
13	-	37.3	37.3
14	-	42.7	42.7
15	-	27.0	27.0
16	-	29.1	29.2
17	-	47.8	47.8
18	-	48.7	48.8
19	2.36 (*m*)	47.8	47.8
20	-	150.5	150.6
21	-	29.7	29.8
22	-	34.0	34.0
23	0.95 (*s*)	28.0	28.0
24	0.74 (*s*)	15.3	15.4
25	0.80 (*s*)	16.1	16.1
26	1.00 (*s*)	16.0	16.0
27	0.96 (*s*)	14.7	14.8
28	3.31 (*d*, 10.9), 3.78 (*d*, 10.9)	60.5	60.2
29	4.56 (*dd*, 2.2, 1.5), 4.66 (*d*, 2.2)	109.7	109.6
30	1.66 (*s*)	19.1	19.1

*Tinto,Blair and Alli, 1992

6. Identification of Compound DC-6 (Diptoindonesin G)

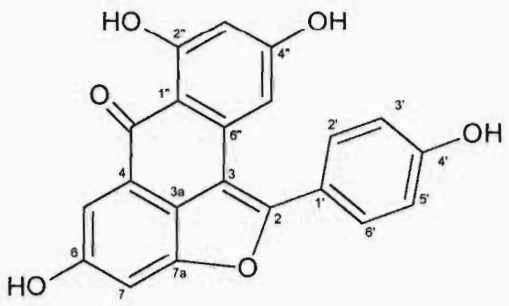
Compound DC-6 was obtained as orange powder. Its UV spectrum (Figure 17) exhibited absorption maxima at 210, 248, 285 and 360 nm. The IR spectrum (Figure 18) showed an OH band at 3391 cm^{-1}, together with absorption bands for the aromatic group at 1614, 1585 and 1466 cm^{-1}.

In the ^{1}H NMR spectrum (Figures 19a-19b) six doublets, due to a total of eight protons, were observed in the aromatic region, together with a singlet at δ 14.13 ppm. This information suggested that DC-6 was a phenyl derivative with a chelated hydroxyl group, containing eight aromatic protons. The ^{1}H-^{1}H COSY spectrum (Figure 20) was helpful in clarifying the couplings of the aromatic signals. A pair of reciprocally coupled signals at δ 6.27 (*d*, *J* = 2.2 Hz, H-3″) and 7.08 (*d*, *J* = 2.2 Hz, H-5″), as well as that at δ 7.34 (*d*, *J* = 1.7 Hz, H-7) and 7.37 (*d*, *J* = 1.7 Hz, H-5), suggested that DC-6 contained two 1, 2, 3, 5- tetrasubstituted phenyl moieties in the molecule. The rest of the aromatic signals, two doublets at δ 7.00 (2 H each, *J* = 8.7 Hz, H-3′/5′) and 7.73 ppm (2 H each, *J* = 8.7 Hz, H-2′/6′), indicated the presence of a *p*-substituted phenyl moiety.

The ^{13}C NMR spectrum (Figure 21) exhibited 19 carbon signals, two of which (δ116.8, 131.2 ppm) were distinctly higher than the others. These two signals were thus assignable to two pairs of equivalent carbons in the *p*-substituted phenyl ring (C-2′/6′ and C-3′/5′). Through analysis of the ^{13}C NMR data with the aid of HSQC experiment (Figure 22), DC-6 was revealed to contain 21 carbons including 8 methine and 13 quaternary ones. The most downfield signals at δ 186.8 ppm indicated the presence of a keto function.

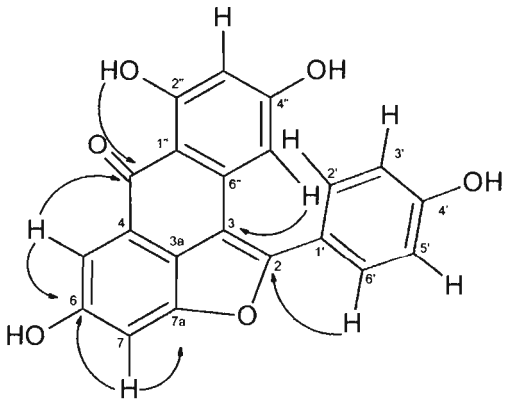
Data obtained from the HMBC experiment led to the connection of the substructures of DC-6, suggesting the structure of the 2-arylbenzofuran

diptoindonesin G for the compound. The structure of diptoindonesin G is shown below.



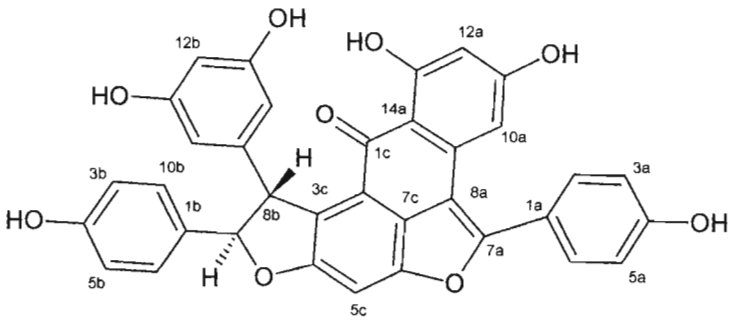
diptoindonesin G

In the HMBC spectrum (Figures 23a-23e), a correlation between the proton signal at δ 14.13 (2″-OH) and the carbonyl signal at δ 186.8 ppm (Figure 23e) was observed, indicating the presence of the benzoyl moiety. The attachment of this moiety to the benzofuran skeleton at C-3 and C-4 was confirmed by a correlation between the proton signal at δ 7.08 (H-5″) and the carbon signal at δ 108.2 (C-3) ppm (Figure 23a), together with a correlation between the proton signal at δ 7.37 (H-5) and the carbonyl signal at δ 186.8 ppm (Figure 23b). The attachment of the *p*-hydroxyphenyl group at C-2 was confirmed by a correlation between the proton signal at δ 7.73 (H-2′/6′) and the carbon signal at δ 157.1 (C-2) ppm (Figure 23b).



key HMBC correlations of DC-6

Comparison of ^{1}H and ^{13}C NMR data of DC-6 with those reported for diptoindonesin G (Juliawaty *et al.*, 2009) indicated that the data of the two compounds were mainly in agreement, except for the assignments for C-4, C-6, C-7a and C-6″. The difference due to C-6 and C-7a was their reversed chemical shift assignments (DC-6 : $\delta_{C\text{-}6}$ 157.9, $\delta_{C\text{-}7a}$ 153.2 ; reported values : $\delta_{C\text{-}6}$ 154.0, $\delta_{C\text{-}7a}$ 158.3). The assignments for C-6 and C-7a of DC-6 were based on data obtained from the HMBC spectrum where the correlations of the carbon signal at δ 157.9 (C-6) ppm with the proton signals at δ 7.37 (H-5) and 7.34 (H-7) ppm, as well as the correlation of the carbon signal at δ 153.2 (C-7a) ppm with the proton signal at δ 7.34 (H-7) ppm, were clearly observed (Figure 23b). (In the published paper of Juliawaty *et al.*, the correlations of H-7 with C-6 and C-7a were reported but the correlation of H-5 with any of these two carbons was not documented.) The assignments for C-4 and C-6″, which could not be accomplished through HMBC analysis, were based on ^{13}C NMR data of hopeachinol A, a 2-arylbenzofuran derivative structurally related to diptoindonesin G. C-4 (δ 124.8 ppm) and C-6″ (δ 134.8 ppm) were assigned according to the chemical shift assignments for their corresponding carbons which were C-2c (δ 126.3 ppm) and C-9a (δ 135.6 ppm) of hopeachinol A, respectively (Ge *et al.*, 2010).



hopeachinol A

The identity of DC-6 was also confirmed by the HRESIMS spectrum (Figure 24) where a pseudomolecular ion $[M-H]^+$ at m/z 359.0563 were observed, indicative of the molecular formula of $C_{21}H_{12}O_6$.

All above information is in accordance with that of diptoindonesin G. Therefore, DC-6 was identified as diptoindonesin G, a stilbenoid previously isolated from the stem bark of *Hopea mengarawan* (Tanaka, *et al.*, 2000) and the stems and twings of *H. chinensis* (Ge *et al.*, 2010) of the family Dipterocarpaceae.

Many stilbenoids have been found in plants of certain families such as Vitaceae, Leguminosae and Dipterocarpaceae (Tanaka *et al.*, 2000). However, their presence in *Diospyros* plant, has never been previously reported. Some stilbenoids have been found to exhibit interesting bioactivities including chemopreventive (Jang *et al.*, 1997) and hepatoprotective (Oshima *et al.,* 1995) activities. Diptoindonesin G has been demonstrated to possess immunosuppressive activity in the assay using a con A induced proliferation of mouse splenic lymphocytes (T cells) (Ge *et al.*, 2010).

Table 18. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignments of compound DC-6 (DMSO-d_6) and diptoindonesin G (acetone-d_6)

Position	DC-6		Diptoindonesin G*	
	δ_H (ppm) (mult.,*J* in Hz)	δ_C (ppm)	δ_H (ppm) (mult.,*J* in Hz)	δ_C (ppm)
2	-	157.1	-	157.5
3	-	108.2	-	109.1
3a	-	124.1	-	125.9
4	-	124.8	-	135.6
5	7.37 (*d*, 1.7)	107.9	7.53 (*d*, 1.5)	108.1
6	-	157.9	-	154.0
7	7.34 (*d*, 1.7)	104.9	7.34 (*d*, 1.5)	104.6
7a	-	153.2	-	158.3
1′	-	121.0	-	122.4
2′/6′	7.73 (*d*, 8.7)	131.2	7.83 (*d*, 8.8)	131.5
3′/5′	7.00 (*d*, 8.7)	116.8	7.10 (*d*, 8.8)	116.9
4′	-	160.2	-	160.5
C=O	-	186.8	-	187.8
1″	-	111.0	-	112.1
2″	-	167.2	-	168.4
3″	6.27 (*d*, 2.2)	102.9	6.36 (*d*, 2.2)	103.2
4″	-	164.9	-	164.7
5″	7.08 (*d*, 2.2)	103.6	7.25 (*d*, 2.2)	103.6
6″	-	134.8	-	139.3
6-OH	-	-	9.30 (*br s*)	-
4′-OH	-	-	9.30 (*br s*)	-
4″OH	-	-	9.30 (*br s*)	-
2″-OH	14.13 (s)	-	14.18 (*s*)	-

* Juliawaty *et al.*, 2009

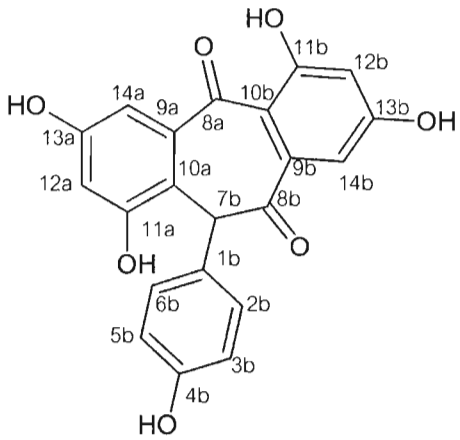
7. Identification of Compound DC-7 (Diptoindonesin D)

Compound DC-7 was obtained as a brown amorphous solid. Its UV spectrum (Figure 25) exhibited absorption maxima at 207, 224, 245, 275 and 371 nm. The IR spectrum (Figure 26) showed absorption bands at 33433, 2922, 1608, 1514 cm^{-1}, suggesting the presence of hydroxyl and aryl groups in the molecule.

The ^{1}H NMR spectrum of DC-7 (Figures 27a-27b) displayed a set of six aromatic signals in the region of δ 6.30 - 7.00 ppm. A pair of doublets at δ 6.38 (J = 2.4 Hz, H-12b) and 6.92 (J = 2.4 Hz H-14b), as well as another pair at δ 6.80 (J = 2.5 Hz, H-12a) and 6.90 (J = 2.5 Hz, H-14a) ppm, represented a pair of proton signals *meta*-coupled to each other. This was supported by their correlation in the ^{1}H-^{1}H COSY spectrum (Figure 28). These signals suggested the presence of two 1,2,3,5 - tetrasubstituted phenyl moieties in the molecule of DC-7. The presence of another moiety, a *p* – substituted phenyl ring, could be deduced from the two other doublets (2 H each, J = 8.9 Hz) at δ 6.63 (H-3b/5b) and 6.68 (H-2b/6b) ppm. In addition to aromatic signals, two singlets at δ 5.89 and 13.70 ppm were observed. The latter indicated the presence of a chelated hydroxyl group.

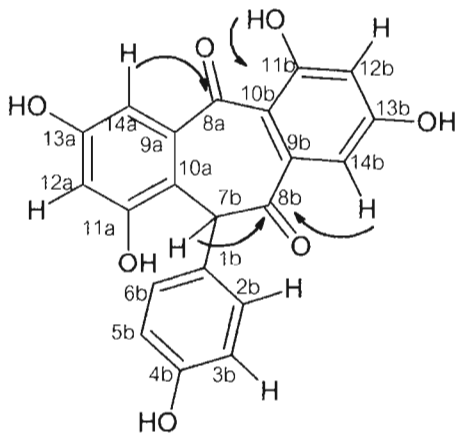
In the ^{13}C NMR spectrum (Figure 29) nineteen carbon signals were recorded. According to the HSQC spectrum (Figure 30a-30c), these could be classified into seven signals of methane carbon (δ107.1, 107.5, 110.2, 113.2, 116.2 and 128.9 ppm.) and twelve signals of quaternary carbons (δ111.2, 111.6, 130.5, 139.6, 142.8, 156.8, 157.0, 158.8, 165.4, 167.4, 196.8 and 197.3 ppm.). Based on their intensity, two methine carbons signals at δ 116.2 and 128.9 ppm were attributable to two pairs of equivalent carbons in the *p*–substituted phenyl ring. DC-7 was thus expected to contain 21 carbons. The two most downfield signals at δ 196.8 and 197.3 ppm. Indicated the presence of two keto groups.

The ^{1}H and ^{13}C NMR data of DC-7 were compared and found to be in full agreement with the reported values for the stilbenoid diptoindonesin D (Chatsumpun, Sritularak and Likhitwitayawuid, 2010) as shown in Table 19, suggesting that DC-7 was diptoindonesin D.



diptoindonesin D

Data obtained from the HMBC experiment (Figures 31a-31e) supported such proposed structure. The location of one of the carbonyl groups between the two tetrasubstituted phenyl rings was confirmed by a correlation between the proton signal at δ 6.90 ppm (H-14a) and the carbonyl signal at 197.3 ppm (C-8a) (Figure 31b), and by the presence of the chelated hydroxyl group attached to C-11b, which indicated by a correlation between the proton signal at δ 13.70 ppm (11b-OH) and the carbon signal at δ 167.4 ppm (C-11b) (Figure 31e). The location of the other carbonyl group at C-8b was confirmed by correlations of the both proton signals at δ 5.89 (H-7b) and 6.92 (H-14b) ppm with the carbonyl signal at δ 196.8 ppm (C-8b) (Figure 31b).



key HMBC correlations of DC-7

Information obtained from the HRESIMS spectrum (Figure 32) supported the established identity of DC-7 as diptoindonesin D. The spectrum displayed a pseudomolecular ion $[M-H]^+$ at m/z 377.0662, indicative of the molecular formula of $C_{21}H_{14}O_7$. According to all above information, DC-7 was identified as diptoindonesin D.

Diptoindonesin D possesses two stereoisomeric forms due to the chiral carbon at position 7b. DC-7 has been proven to be (-) form, according to its specific rotation ($[\alpha]_D$ = -130°). (+) Diptoindonesin D has been reported as being isolated from the stem bark of *Hopea dryocalanoides* (Dipterocarpaceae) (Sahidin, *et al.*, 2005) and the stem wood of *Millettia leucantha* (Leguminosae) (Chatsumpun, et al., 2010).

Table 19. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR assignments of compound DC-7 (acetone-d_6) and diptoindonesin D (acetone-d_6)

position	DC-7		Diptoindonesin D*	
	δ_H (ppm) (mult.,*J* in Hz)	δ_C (ppm)	δ_H (ppm) (mult.,*J* in Hz)	δ_C (ppm)
8a	-	197.3	-	197.4
9a	-	142.8	-	142.8
10a	-	111.2	-	111.2
11a	-	156.8	-	156.8
12a	6.80 (*d*, 2.5)	107.5	6.79 (*d*, 2.5)	107.5
13a	-	158.8	-	158.8
14a	6.90 (*d*, 2.5)	110.2	6.90 (*d*, 2.5)	110.2
1b	-	130.5	-	130.5
2b,6b	6.68 (*d*, 8.9)	128.9	6.68 (*d*, 9)	128.9
3b,5b	6.63 (*d*, 8.9)	116.2	6.63 (*d*, 9)	116.2
4b	-	157.0	-	157.0
7b	5.89 (*s*)	55.2	5.88 (*s*)	55.2
8b	-	196.8	-	196.7
9b	-	139.6	-	139.6
10b	-	111.6	-	111.7
11b	-	167.4	-	167.3
12b	6.38 (*d*, 2.4)	107.1	6.38 (*d*, 2.5)	107.0
13b	-	165.4	-	165.2
14b	6.92 (*d*, 2.4)	113.2	6.92 (*d*, 2.5)	113.1
11b-OH	13.70 (*s*)		13.69 (*s*)	

*Chatsumpun, et al., 2010

8. Cytotoxic and Antimalarial Activities of Compound DC-6

The cytotoxic and antimalarial activities of the isolated compound DC-6 (diptoindonesin G) were investigated. The results obtained are shown in Tables 20 and Table 21, respectively.

Table 20. Cytotoxic activity of the test compounds

Compound	IC_{50} (μg /ml)			
	NCI-H187	KB	MCF-7	Vero Cell
DC-6	1.94	> 50	43.28	> 50
Ellipticine	0.71	1.14	-	1.98
Doxorubicin	0.54	0.35	8.82	-
Tamoxifen	-	-	9.47	-

Table 21. Antimalarial activity of the test compounds

Compound	IC_{50} (μM)
DC-6	19.11 (6.88 μg/ml)
Dihydroartemisinin	1.25×10^{-3}
Mefloquine	24.5×10^{-3}

As shown in Table 20, DC-6 exhibited cytotoxic activity against human small-cell lung cancer (NCI-H187) and breast adenocarcinoma (MCF-7) cell lines, but was inactive against epidermoid carcinoma (KB) cell line. The compound was considered strongly active against the NCI-H187 cell line (IC_{50} = 1.94 μg /ml), though its activity was not as strong as those of ellipticine and doxorubicin. Its activity against the MCF-7 cell line (IC_{50} = 43.28 μg /ml) was considered weak. The compound appeared to be inactive against Vero cells, suggesting its selective cytotoxicity on the cancerous cells as compared to normal ones.

The result shown in Table 21 indicated that DC-6 exhibited antimalarial activity against *Plasmodium falciparum* K1 strain. Although its activity was not distinguished, especially when compared with those of dihydroartemisinin and mefloquine, the selectivity of the compound, as indicated by the lack of cytotoxicity against Vero cells, may be considered as point of interest for further detailed investigation on antimalarial activity of the compound.

CHAPTER V
CONCLUSION

Phytochemical investigation of the stems of *Diospyros collinsae* Craib led to the isolation of seven compounds. These compounds include four triterpenoids, namely friedelin, lupeol, betulin and betulinic acid, and two stilbenoids, diptoindonesin D and diptoindonesin G, and a mixture of β-sitosterol and stigmasterol. The isolation of diptoindonesins D and G in the present work gave the first evidence for the occurrence of stilbenoids in the genus *Diospyros*.

The stilbenoid diptoindonesin G has been found to exhibit antimalarial activity and cytotoxic activities against NCI-H187 and MCF cancer cell lines, while the compound appeared to be non-cytotoxic to normal cells.

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APPENDIX

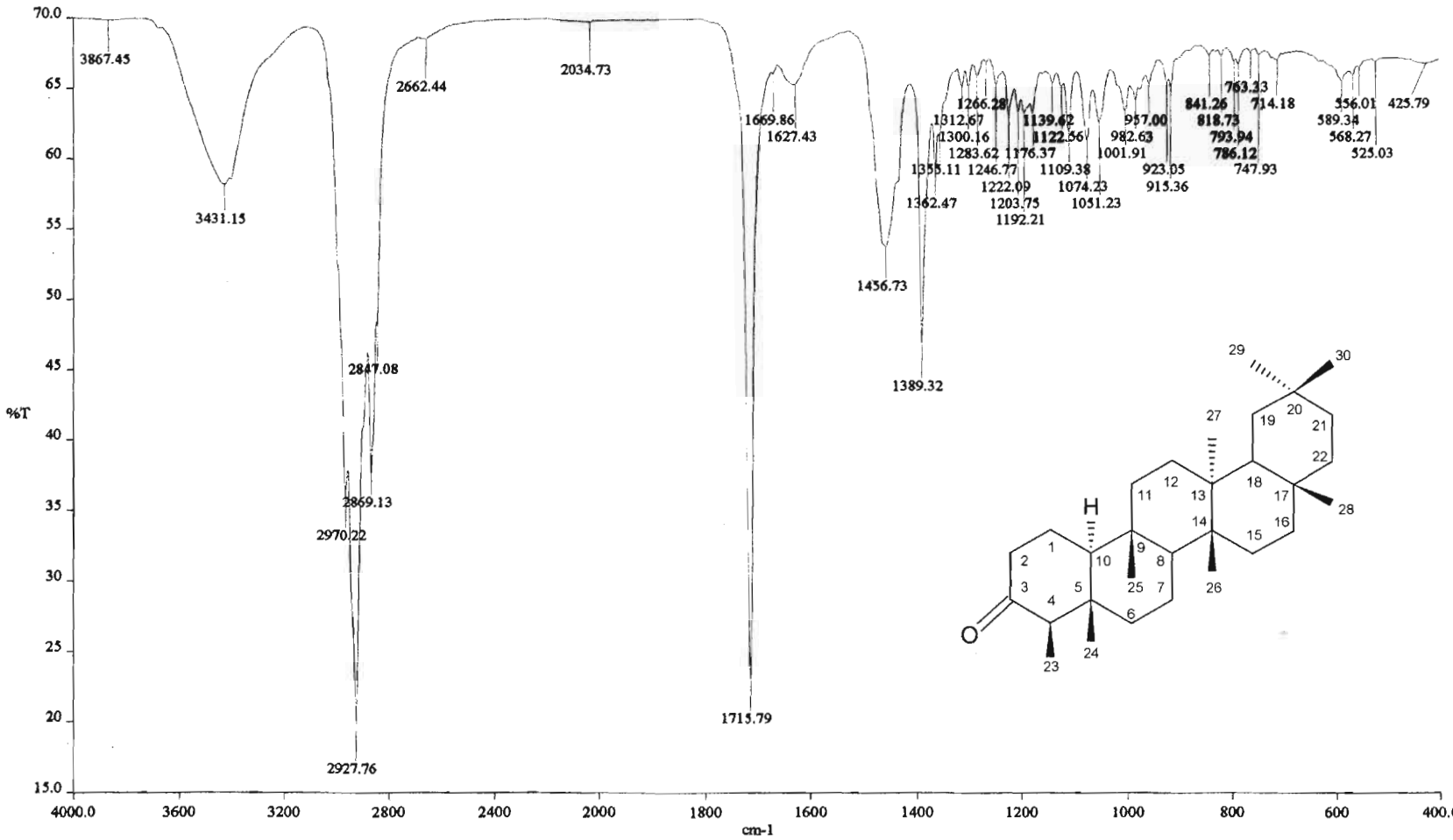


Figure 2. IR spectrum of compound DC-1 (KBr)

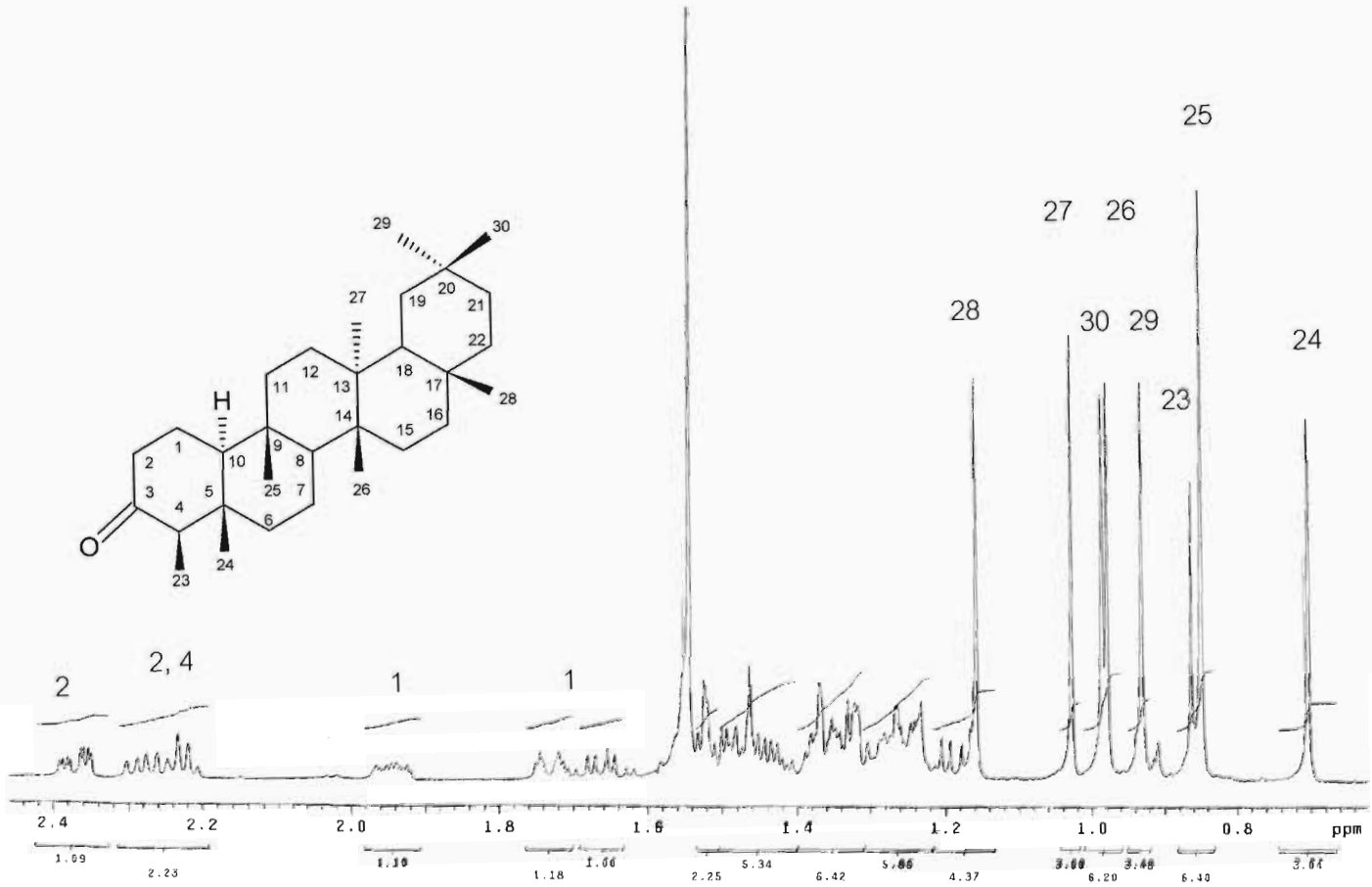


Figure 3. ^{1}H NMR (500 MHz) spectrum of compound DC-1 (in $CDCl_3$)

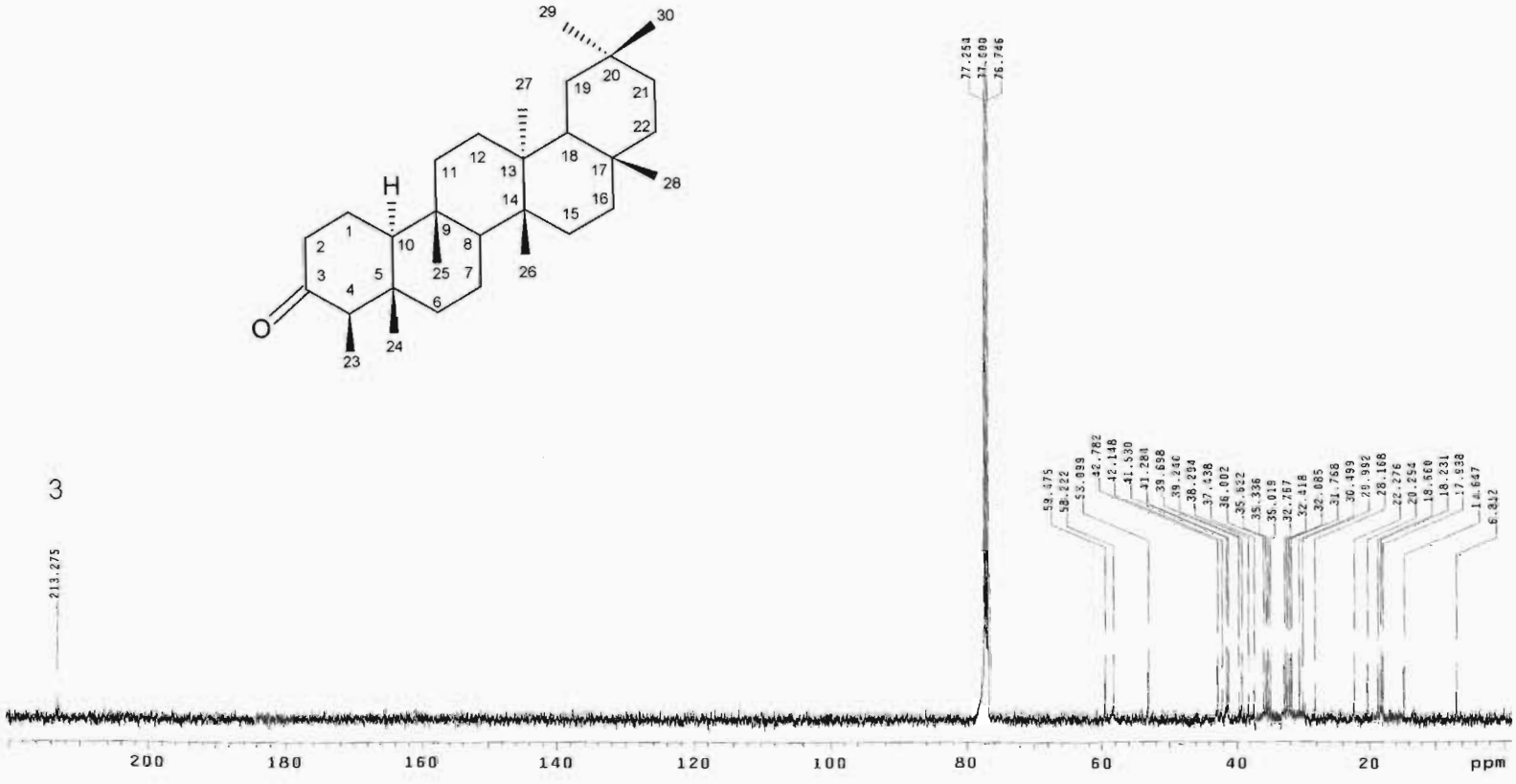


Figure 4a. ^{13}C NMR (125 MHz) spectrum of compound DC-1 (in $CDCl_3$)

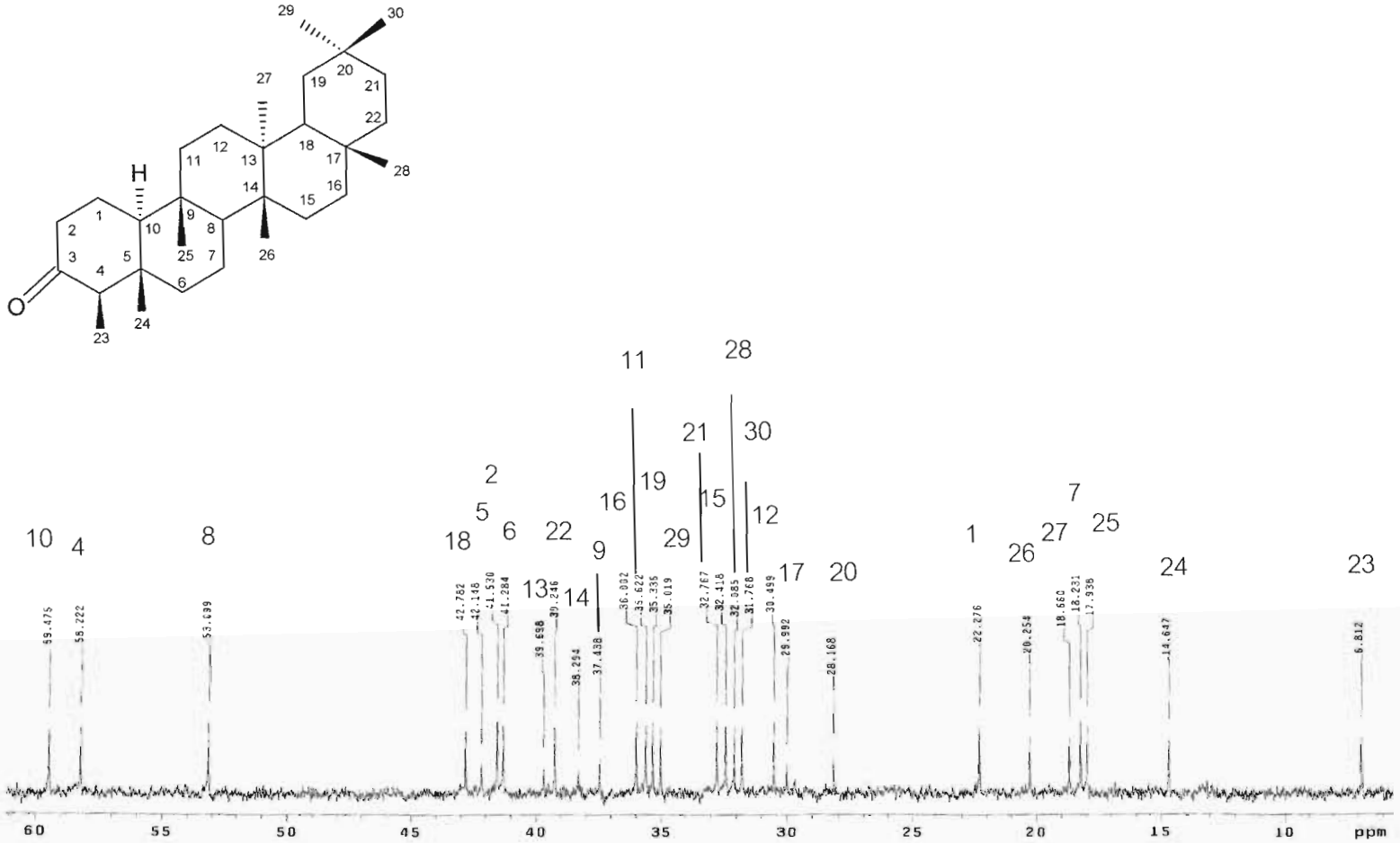


Figure 4b. ^{13}C NMR (125 MHz) spectrum of compound DC-1 (in $CDCl_3$) (expanded)

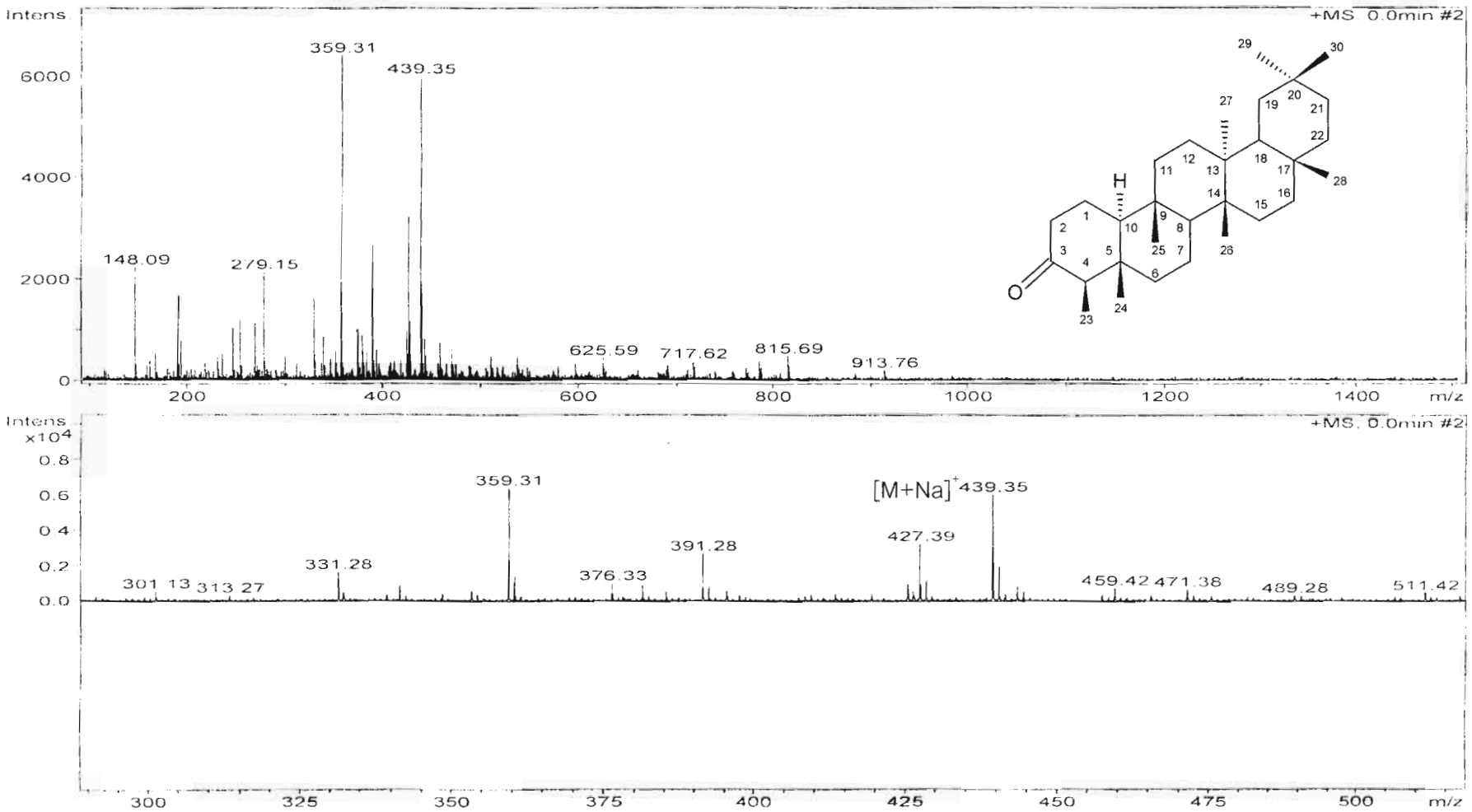


Figure 5. ESI Mass spectrum of compound DC-1

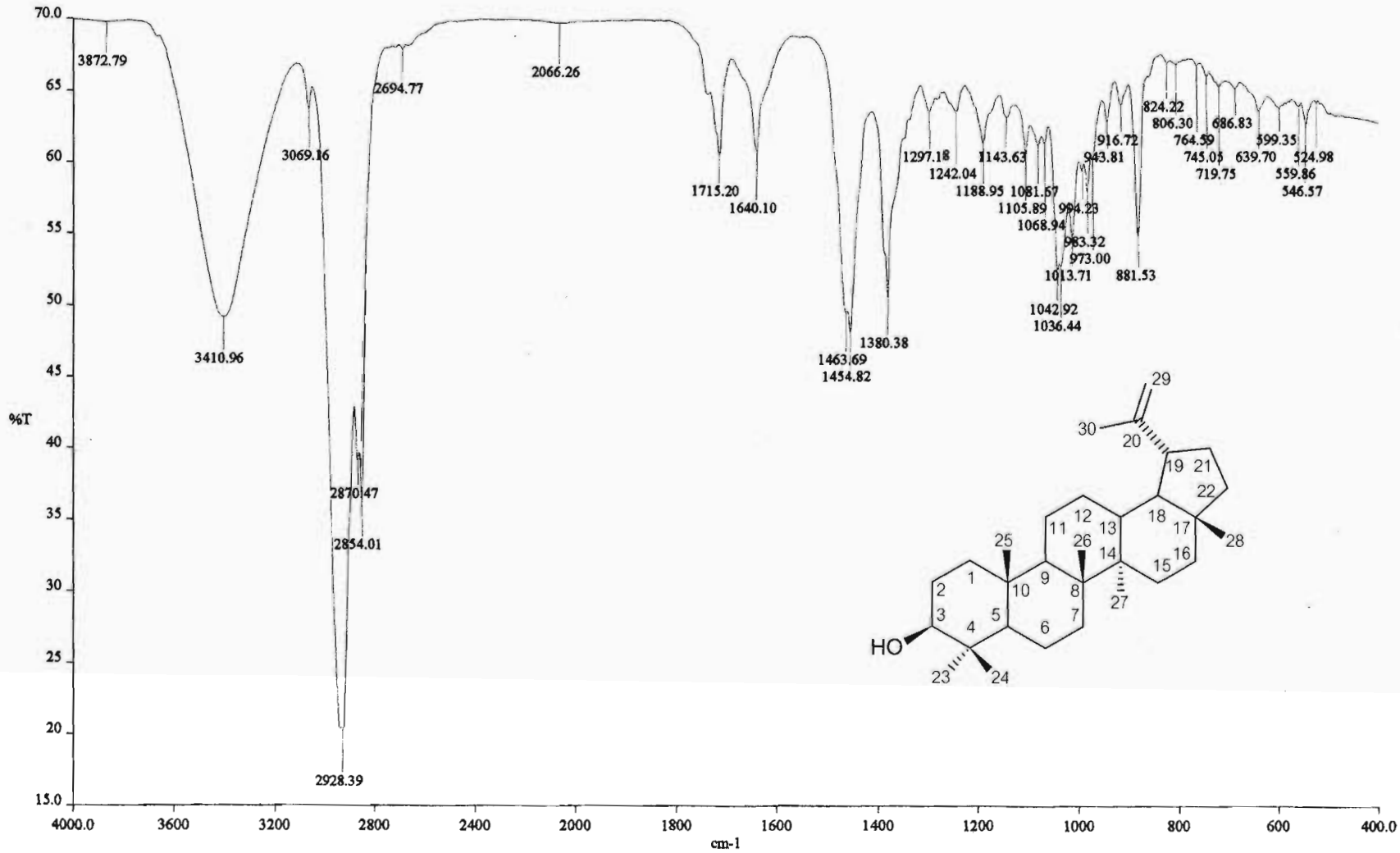


Figure 6. IR spectrum of compound DC-2 (KBr)

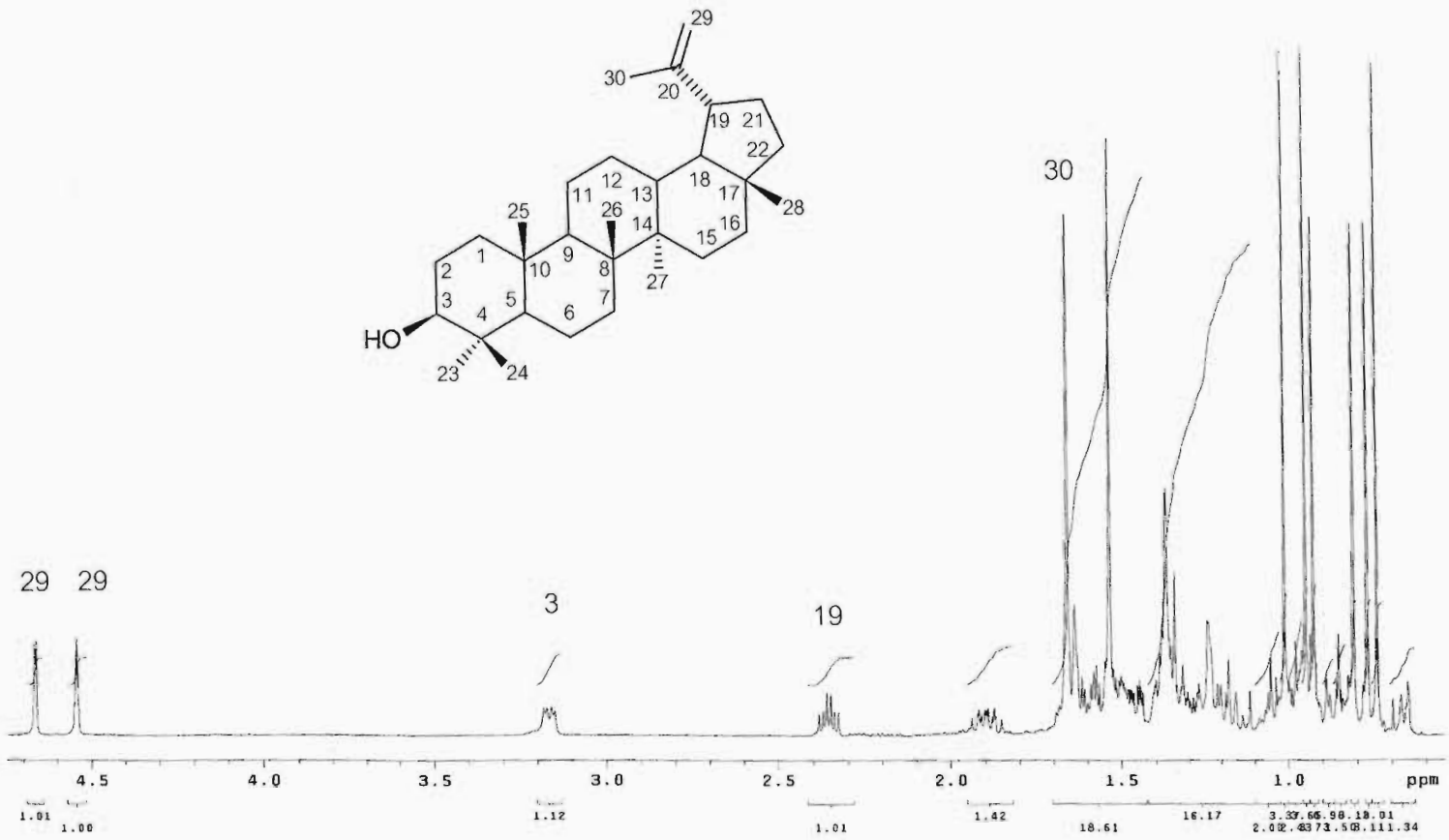


Figure 7a. ^{1}H NMR (500 MHz) spectrum of compound DC-2 (in $CDCl_3$)

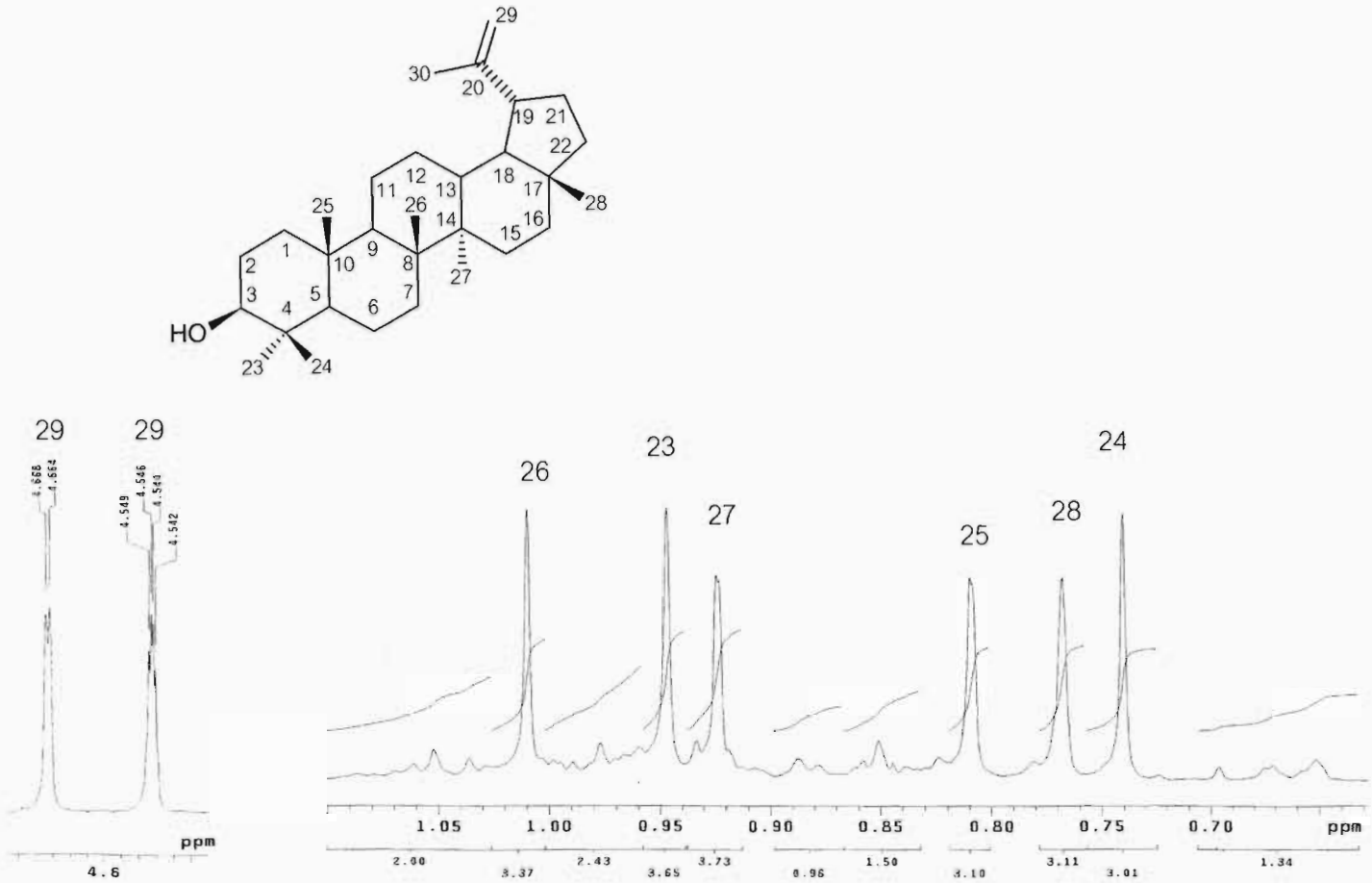


Figure 7b. ^{1}H NMR (500 MHz) spectrum of compound DC-2 (in $CDCl_3$) (expanded)

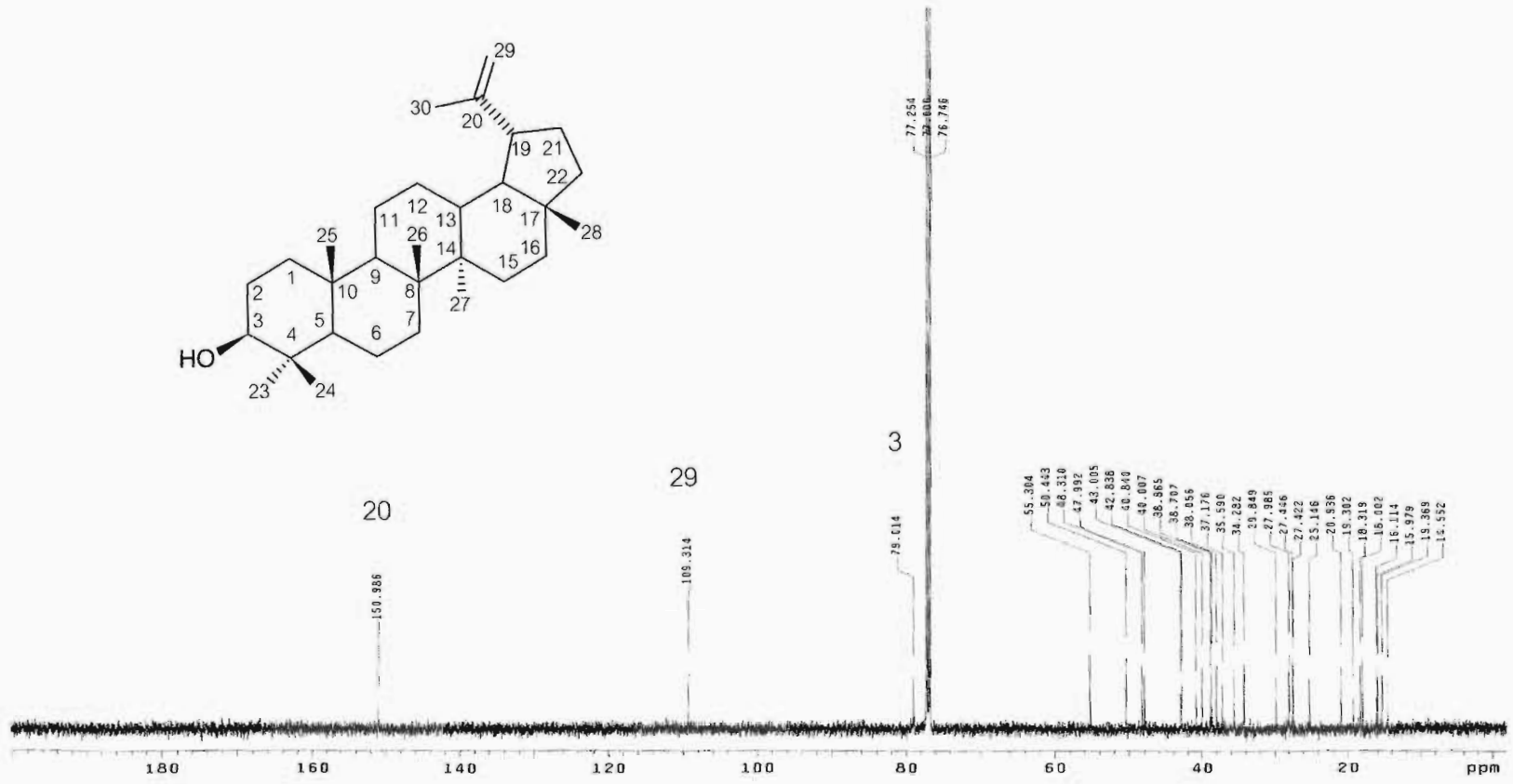


Figure 8a. ^{13}C NMR (125 MHz) spectrum of compound DC-2 (in $CDCl_3$)

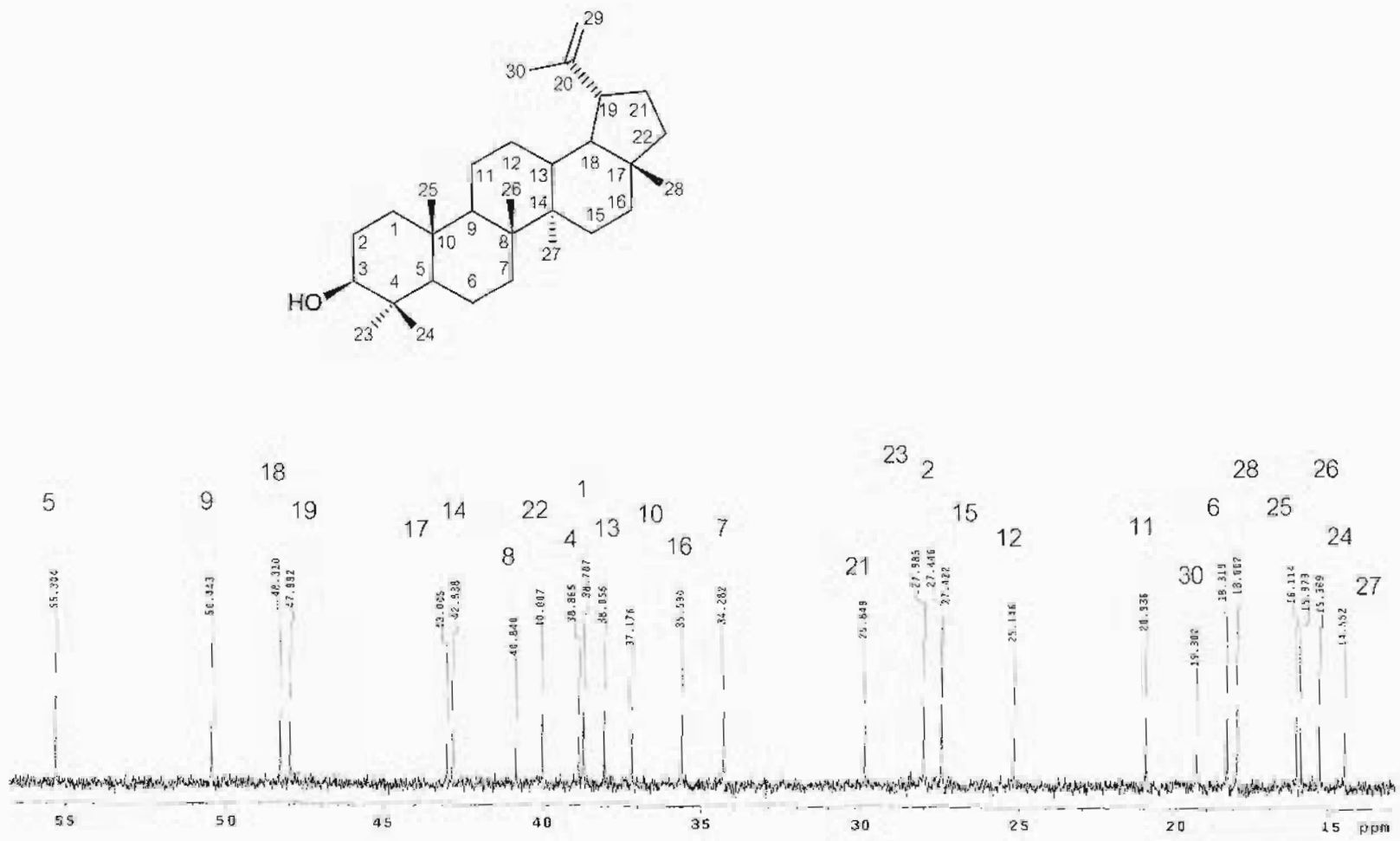


Figure 8b. ^{13}C NMR (125 MHz) spectrum of compound DC-2 (in $CDCl_3$) (expanded)

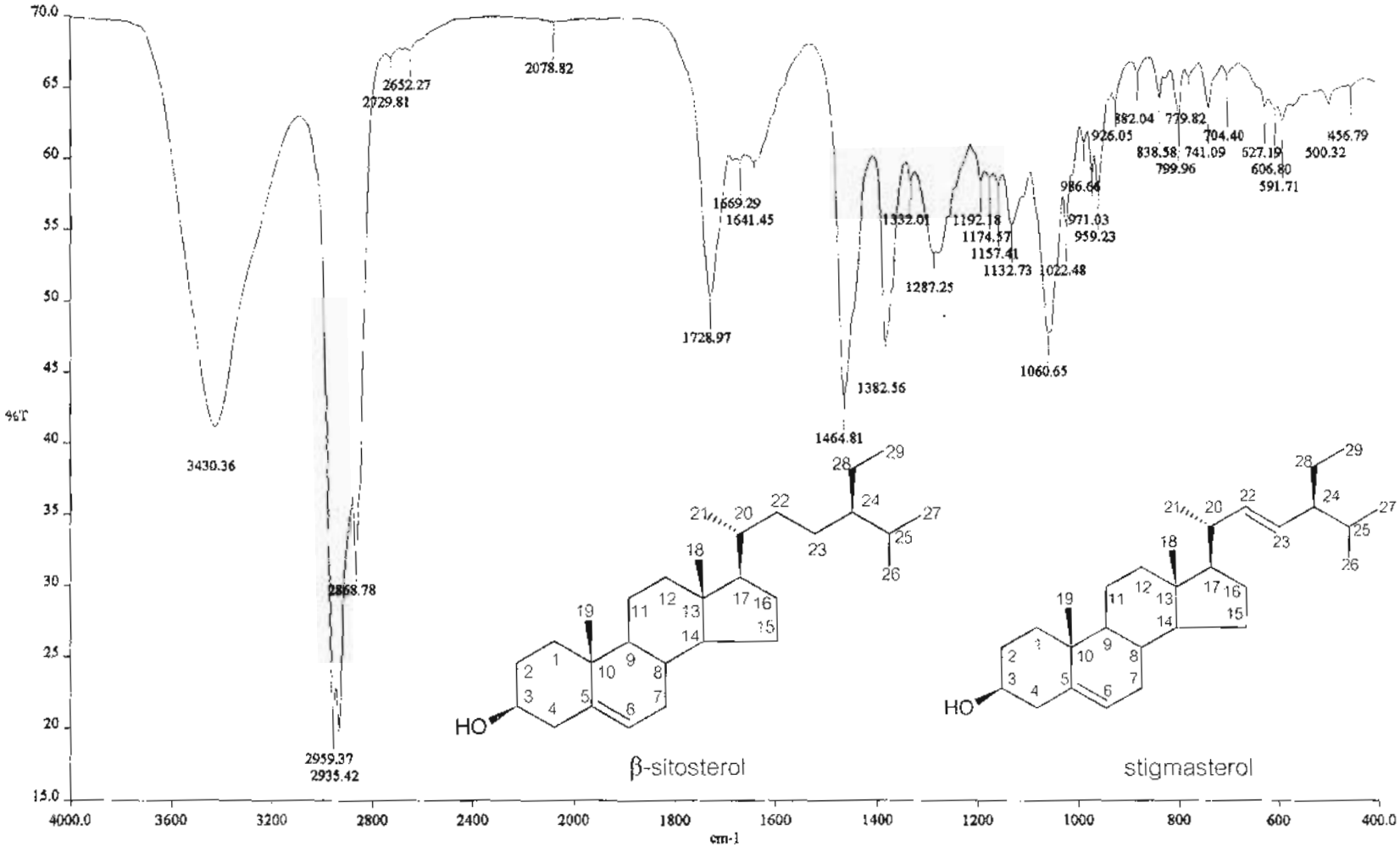


Figure 9. IR spectrum of compound DC-3 (KBr)

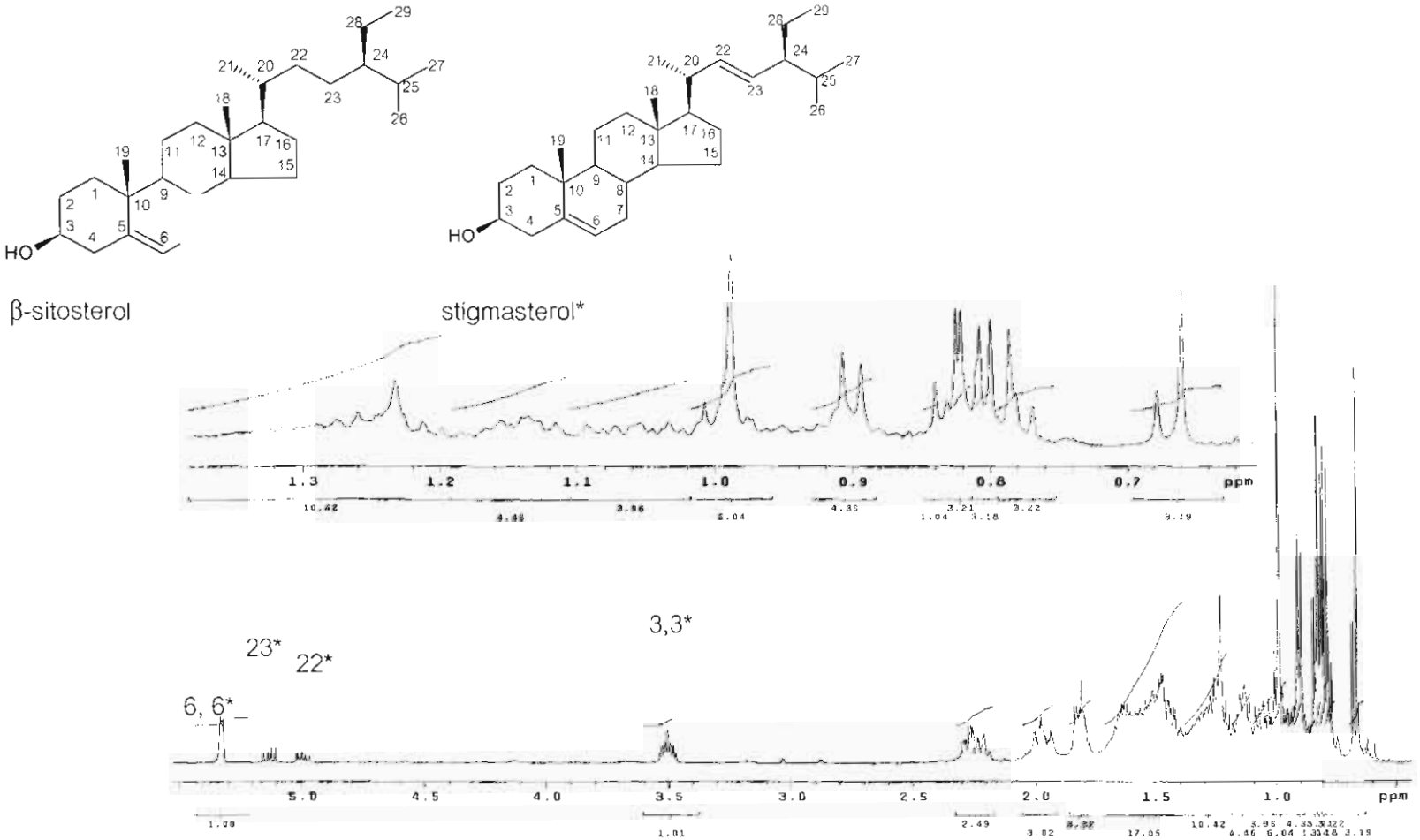


Figure 10. ^{1}H NMR (500 MHz) spectrum of compound DC-3 (in $CDCl_3$)

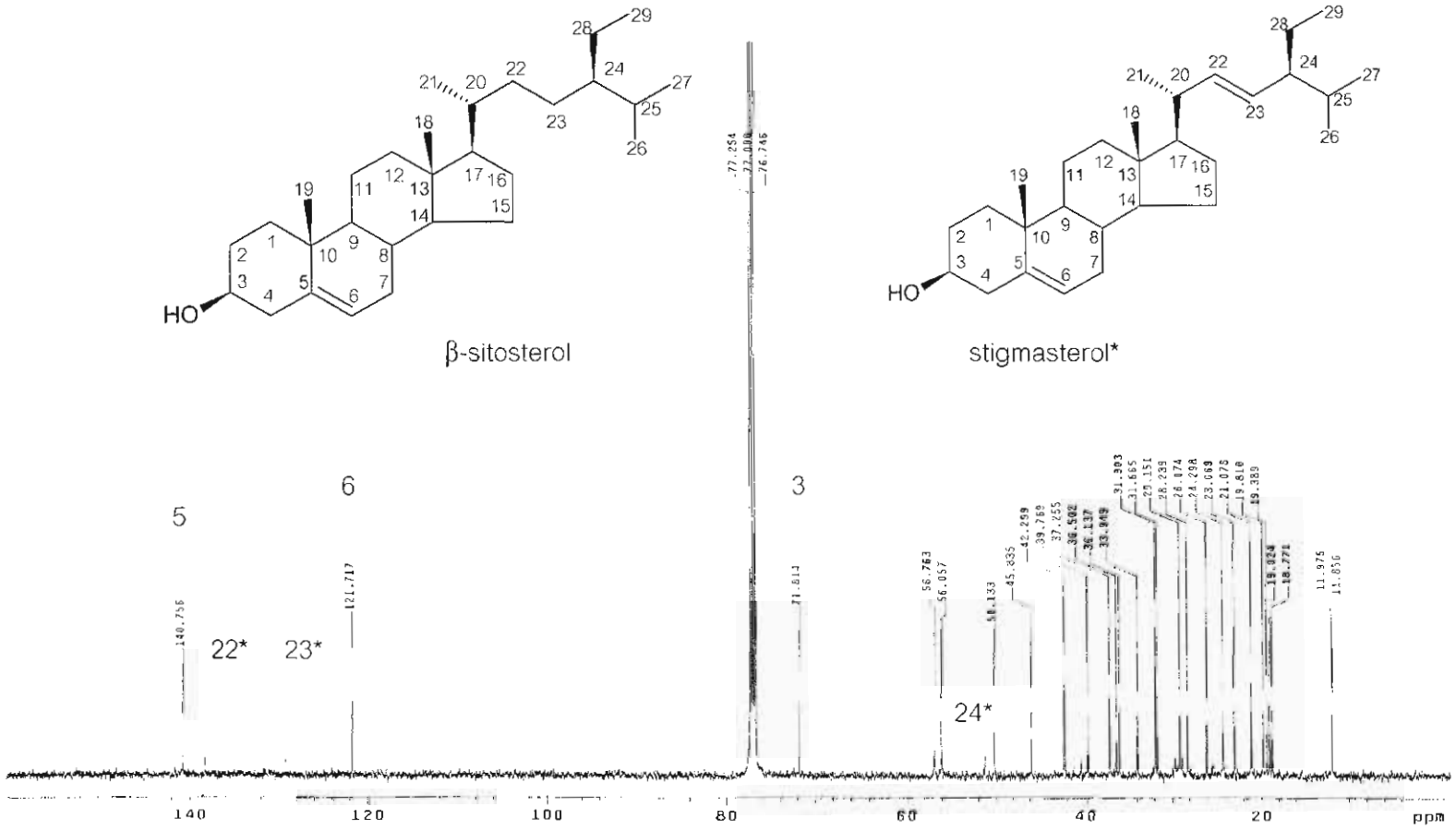
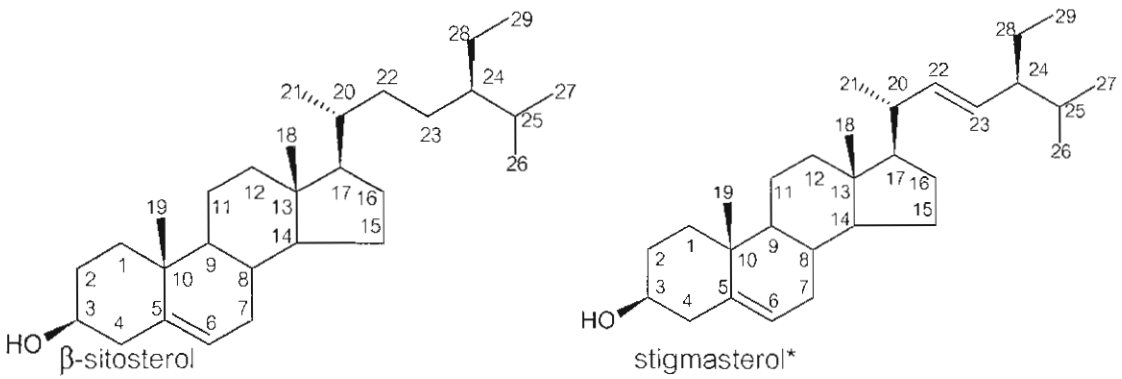


Figure 11a. ^{13}C NMR (125 MHz) spectrum of compound DC-3 (in $CDCl_3$)



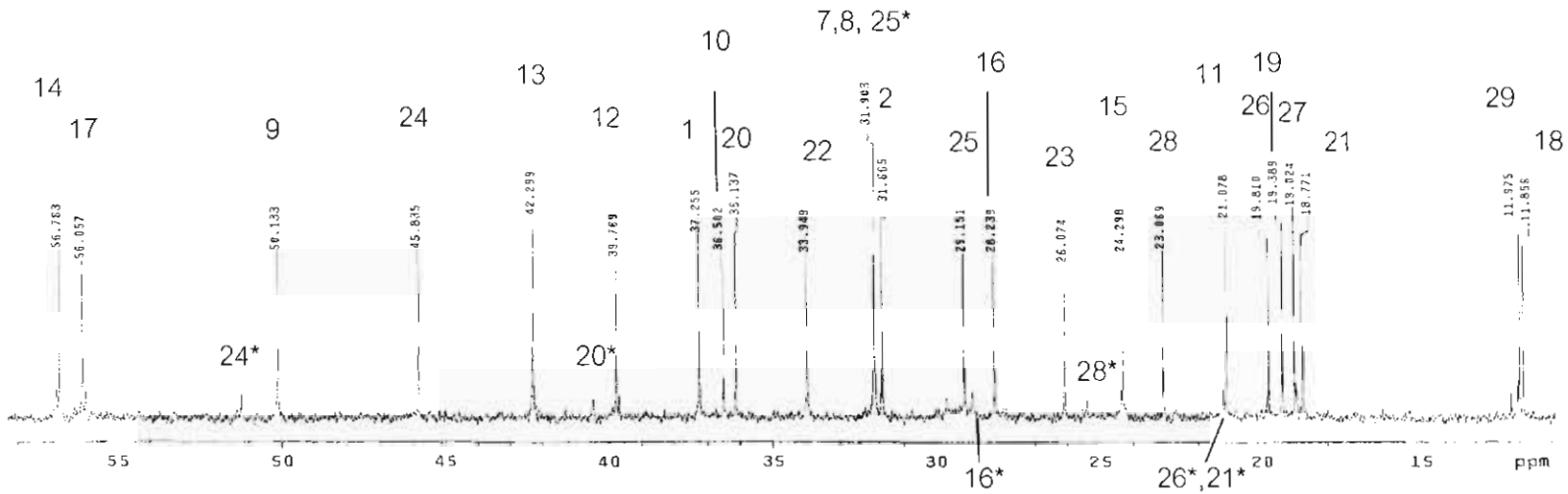


Figure 11b. ^{13}C NMR (125 MHz) spectrum of compound DC-3 (in $CDCl_3$) (expanded)

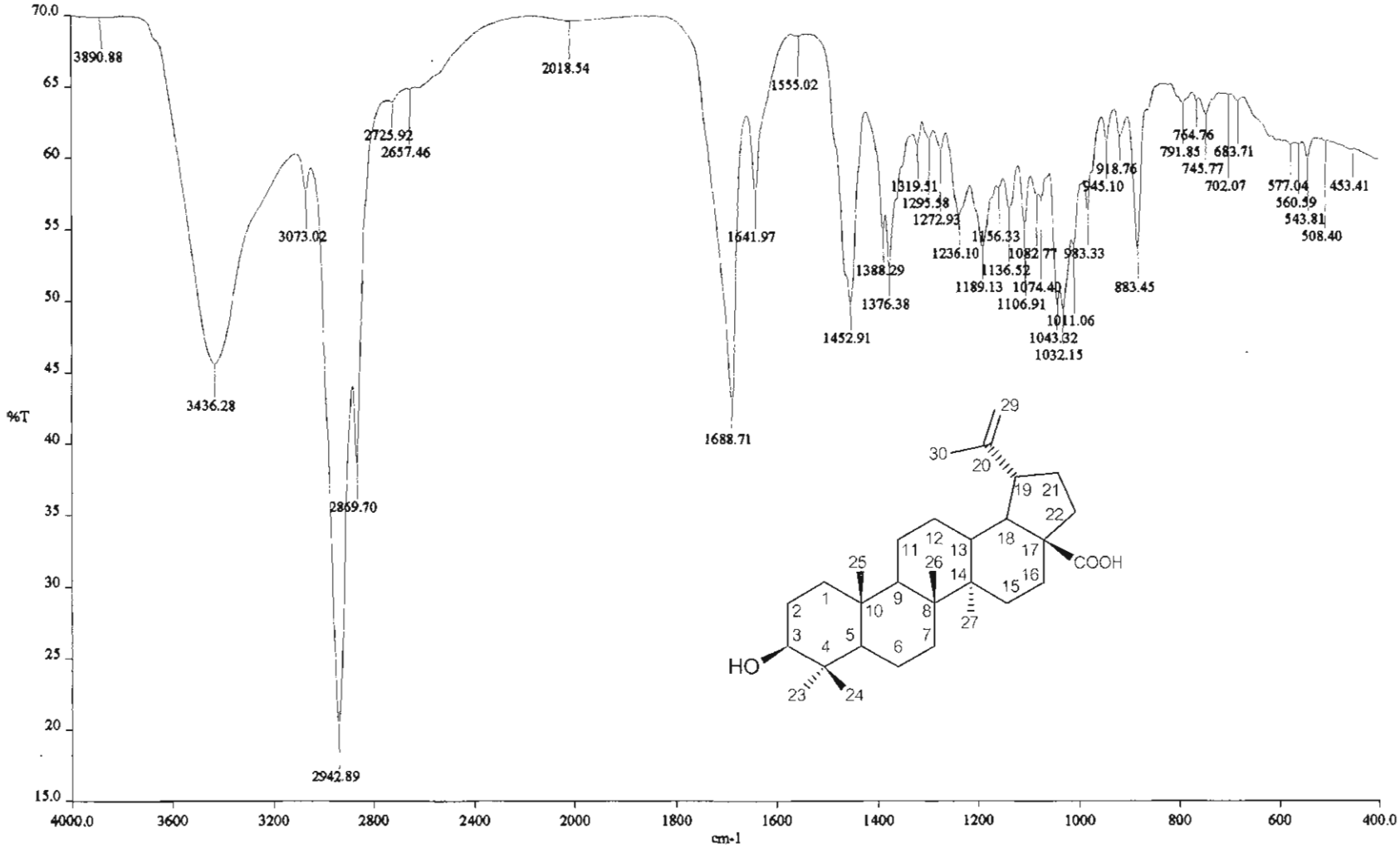


Figure 12. IR spectrum of compound DC-4 (KBr)

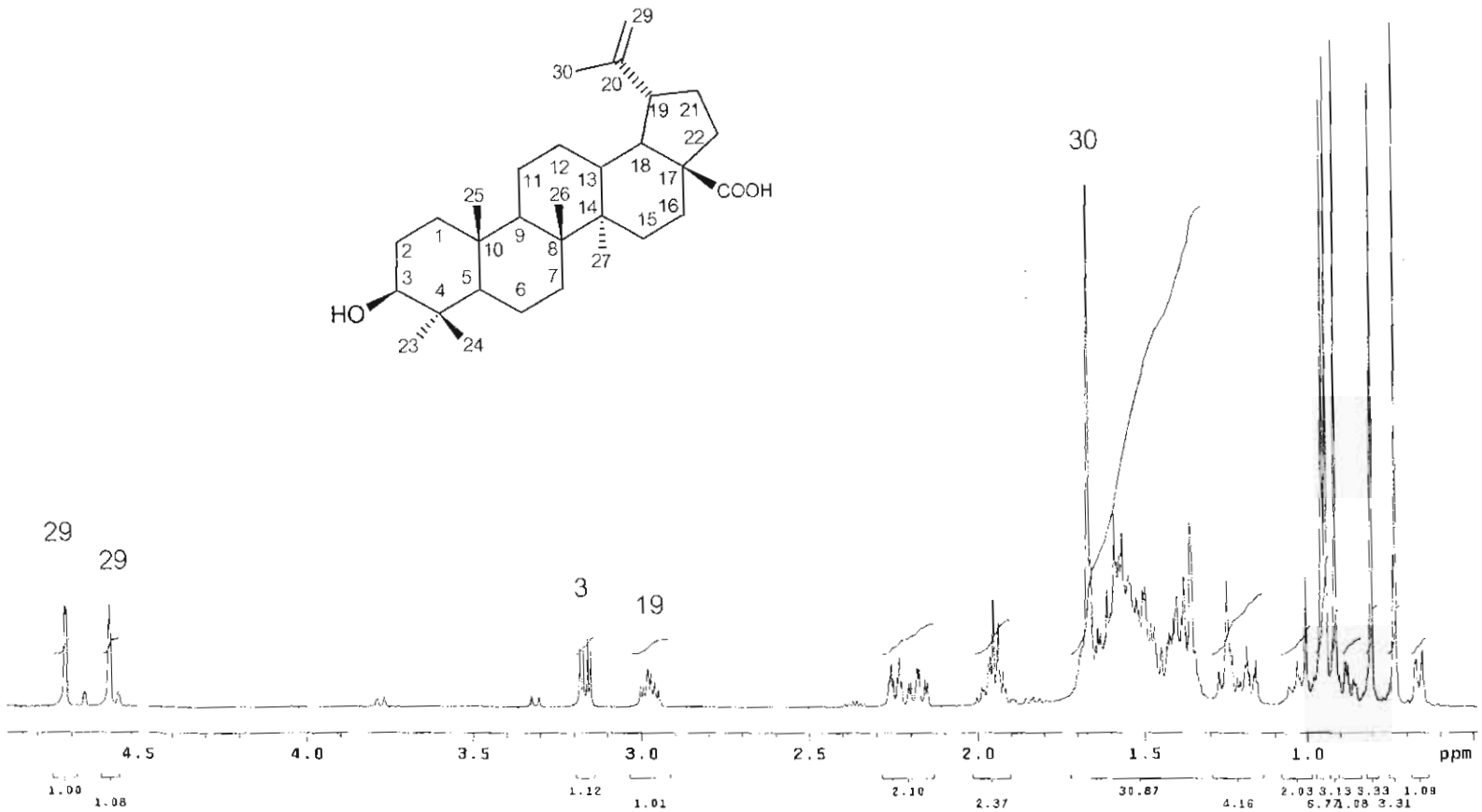


Figure 13a. ^{1}H NMR (500 MHz) spectrum of compound DC-4 (in $CDCl_3$)

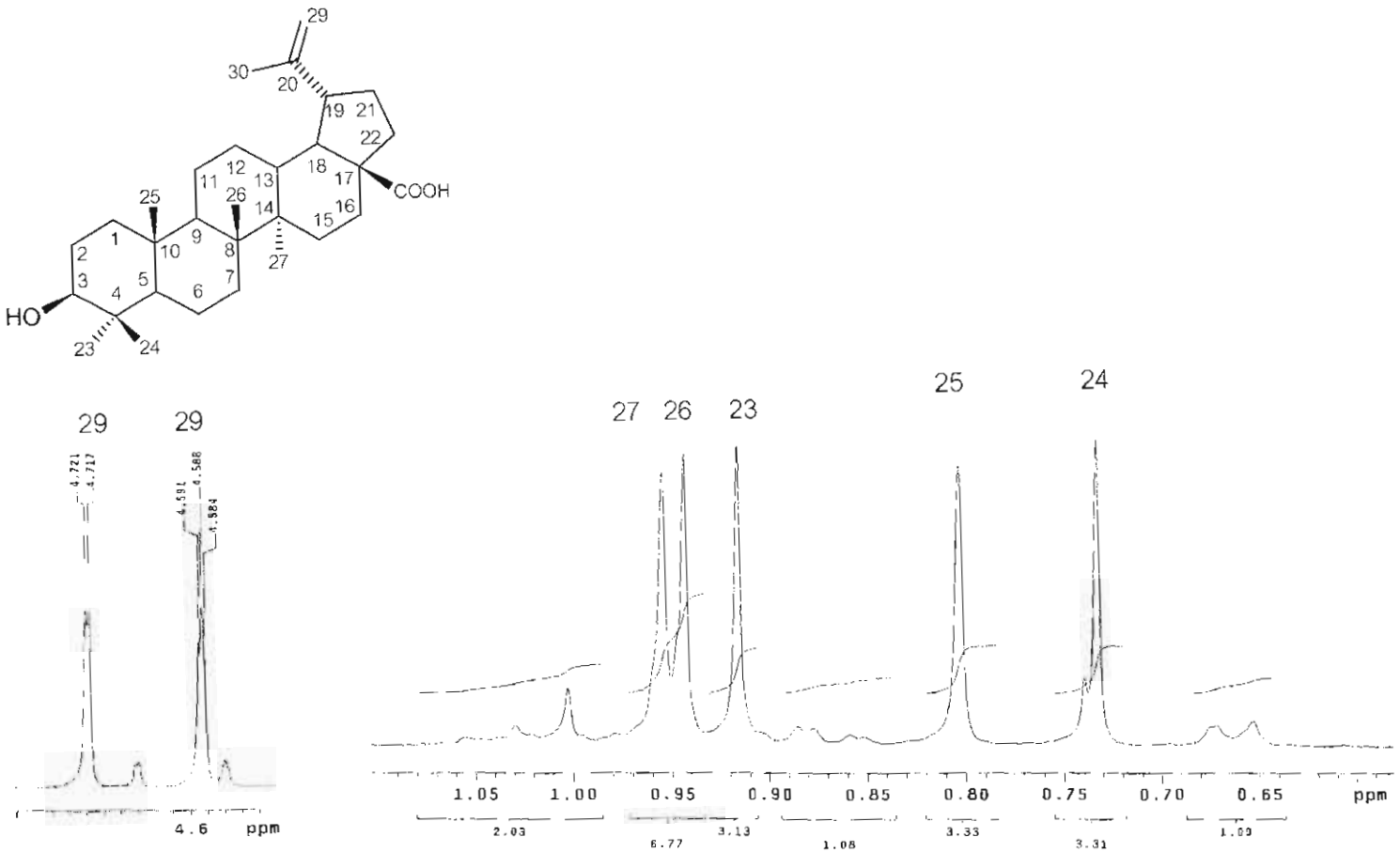


Figure 13b. ^{1}H NMR (500 MHz) spectrum of compound DC-4 (in $CDCl_3$) (expanded)

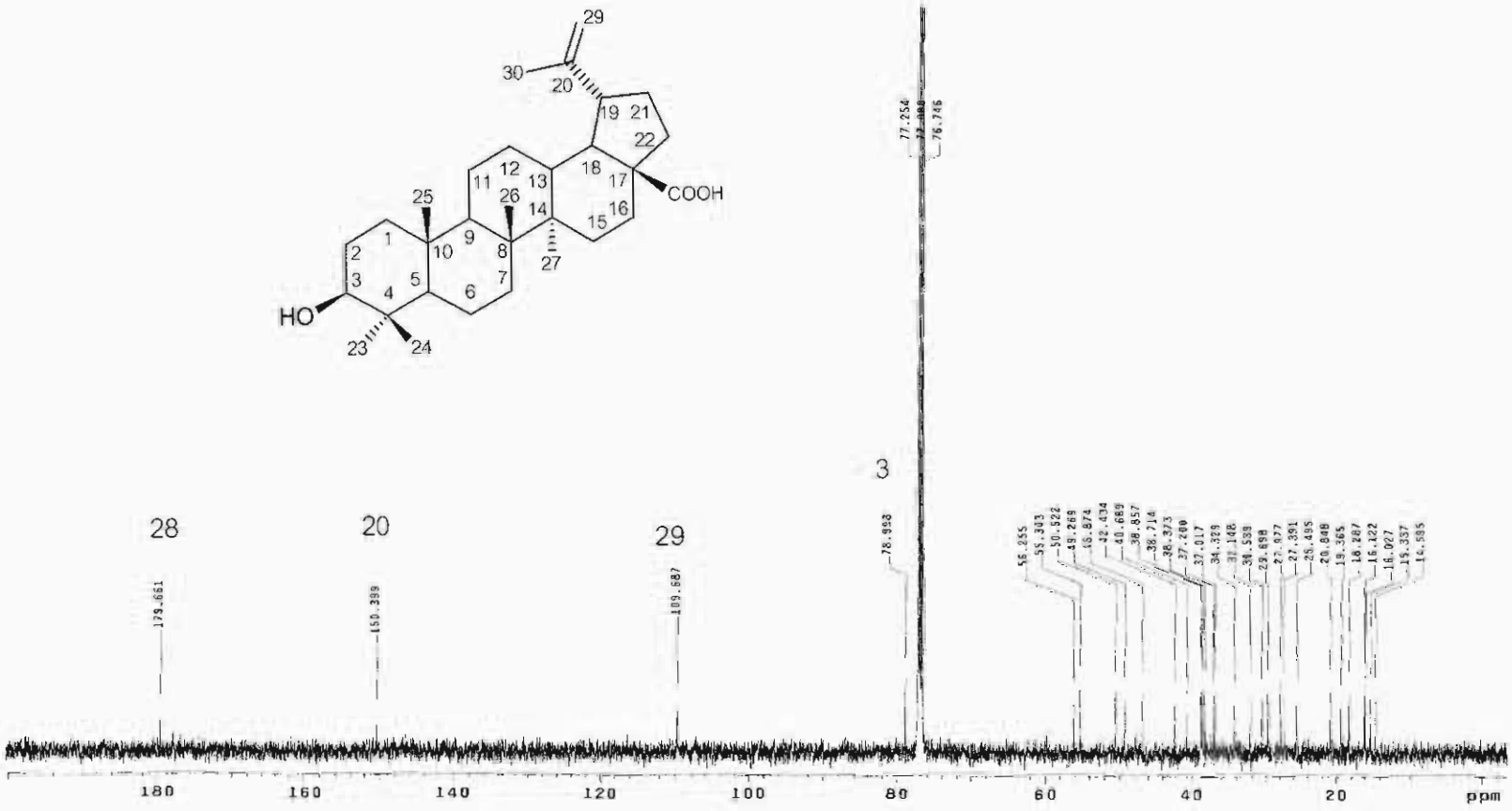
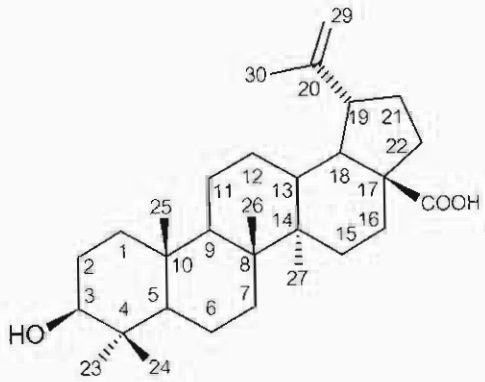


Figure 14a. ^{13}C NMR (125 MHz) spectrum of compound DC-4 (in $CDCl_3$)



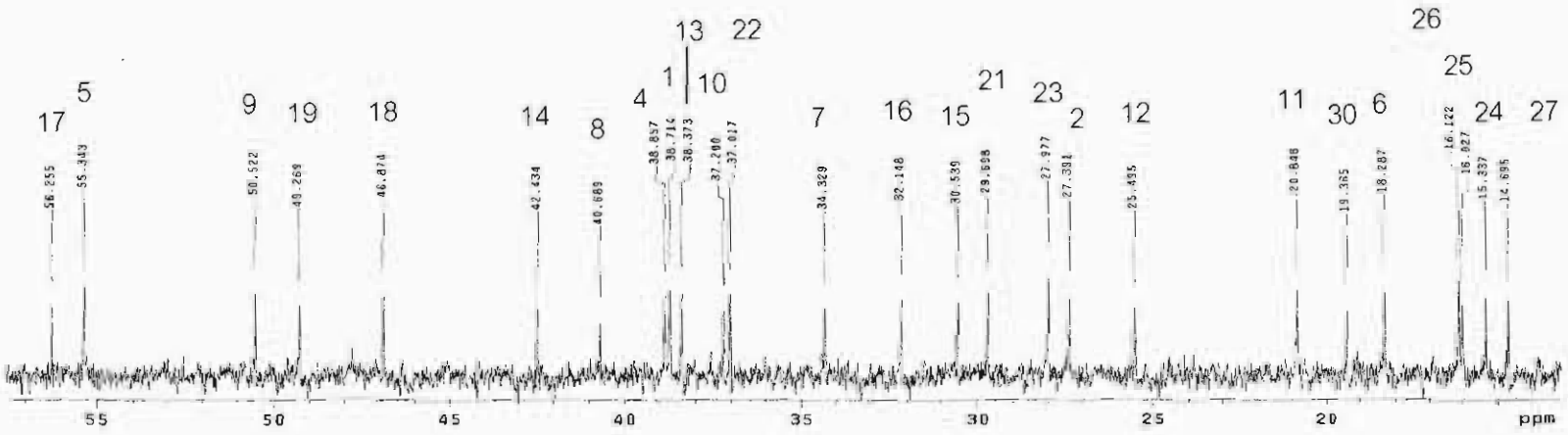


Figure 14b. ^{13}C NMR (125 MHz) spectrum of compound DC-4 (in $CDCl_3$) (expanded)

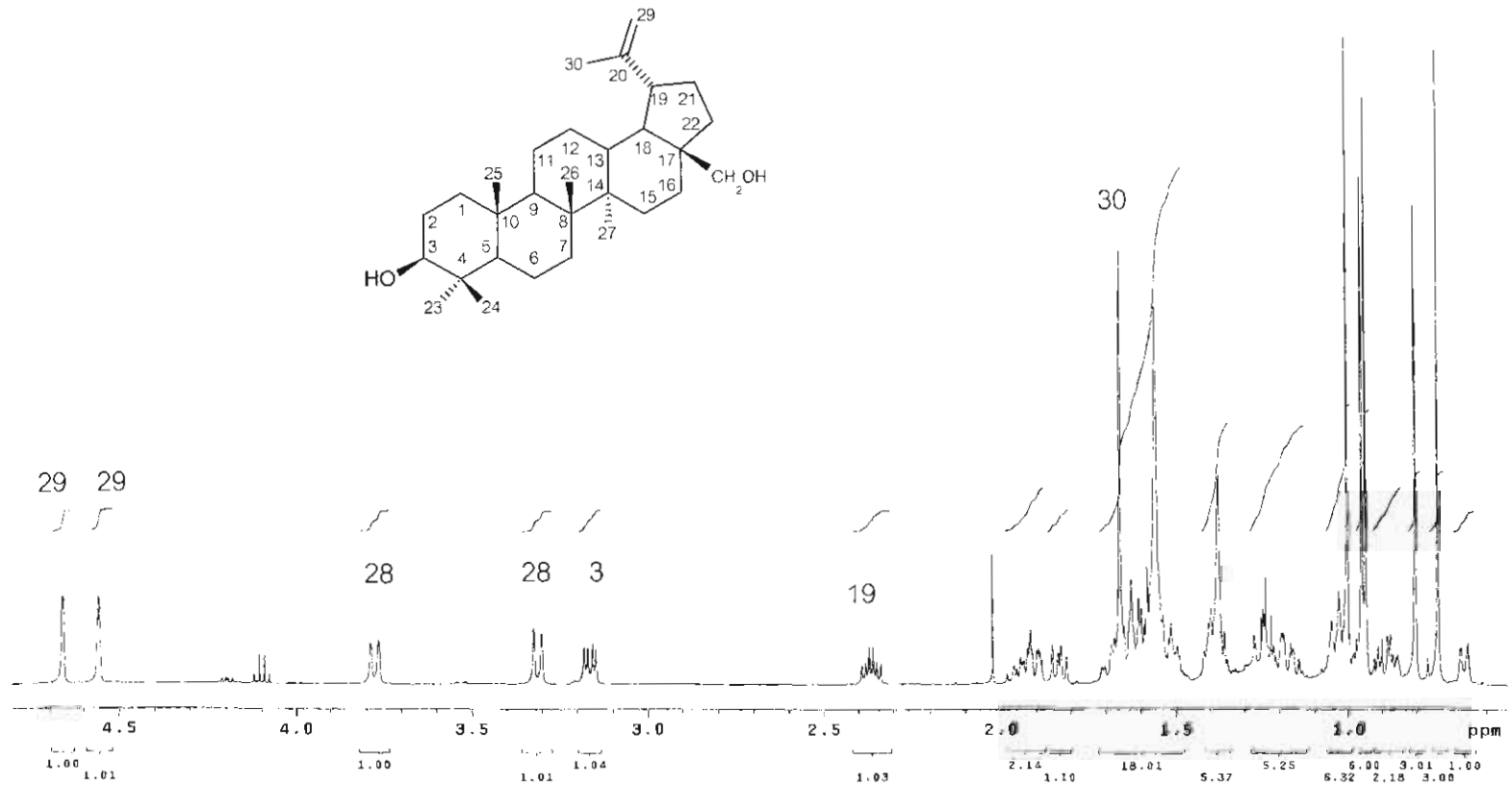


Figure 15a. ^{1}H NMR (500 MHz) spectrum of compound DC-5 (in $CDCl_3$)

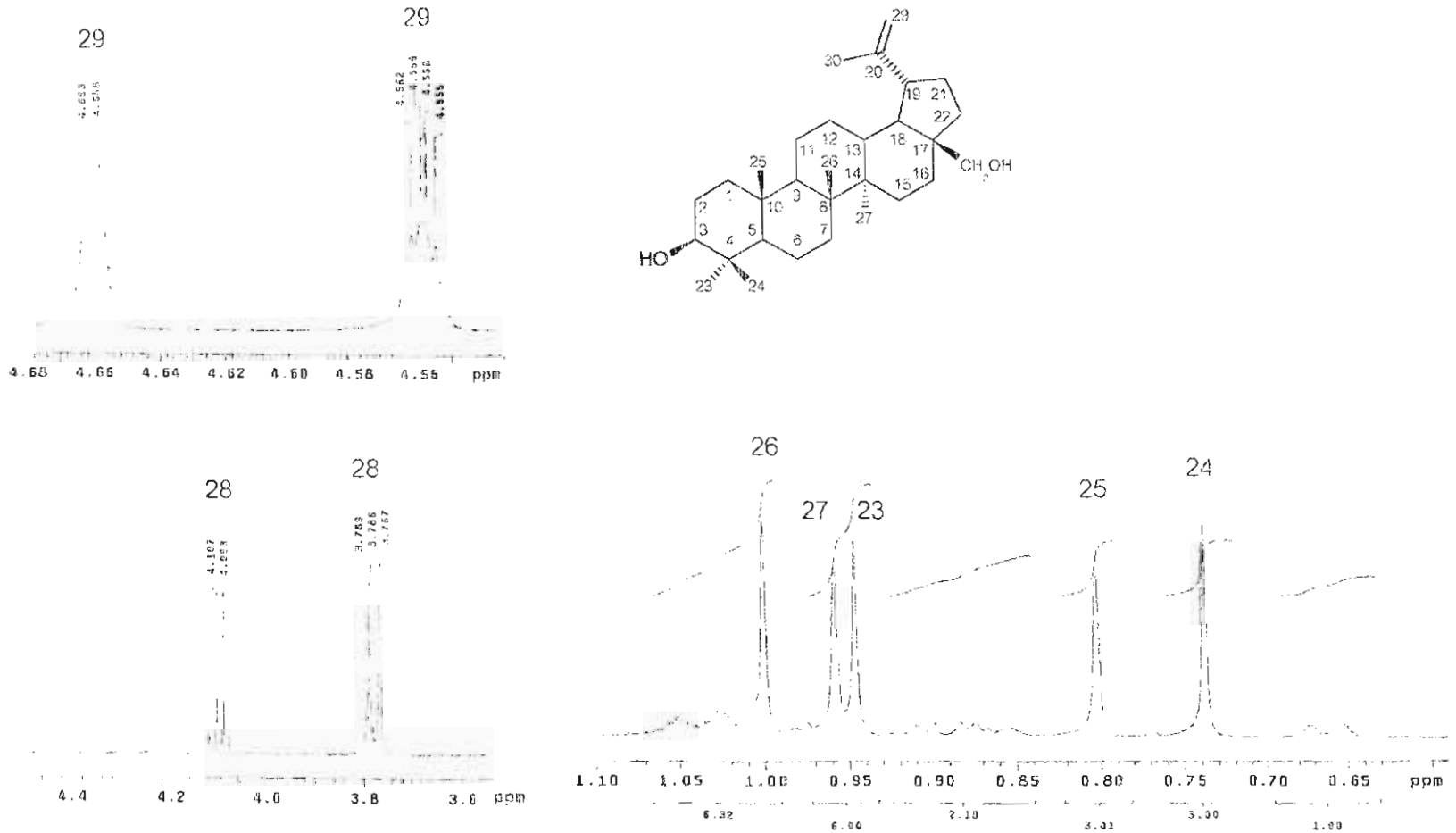


Figure 15b. ^{1}H NMR (500 MHz) spectrum of compound DC-5 (in $CDCl_3$) (expanded)

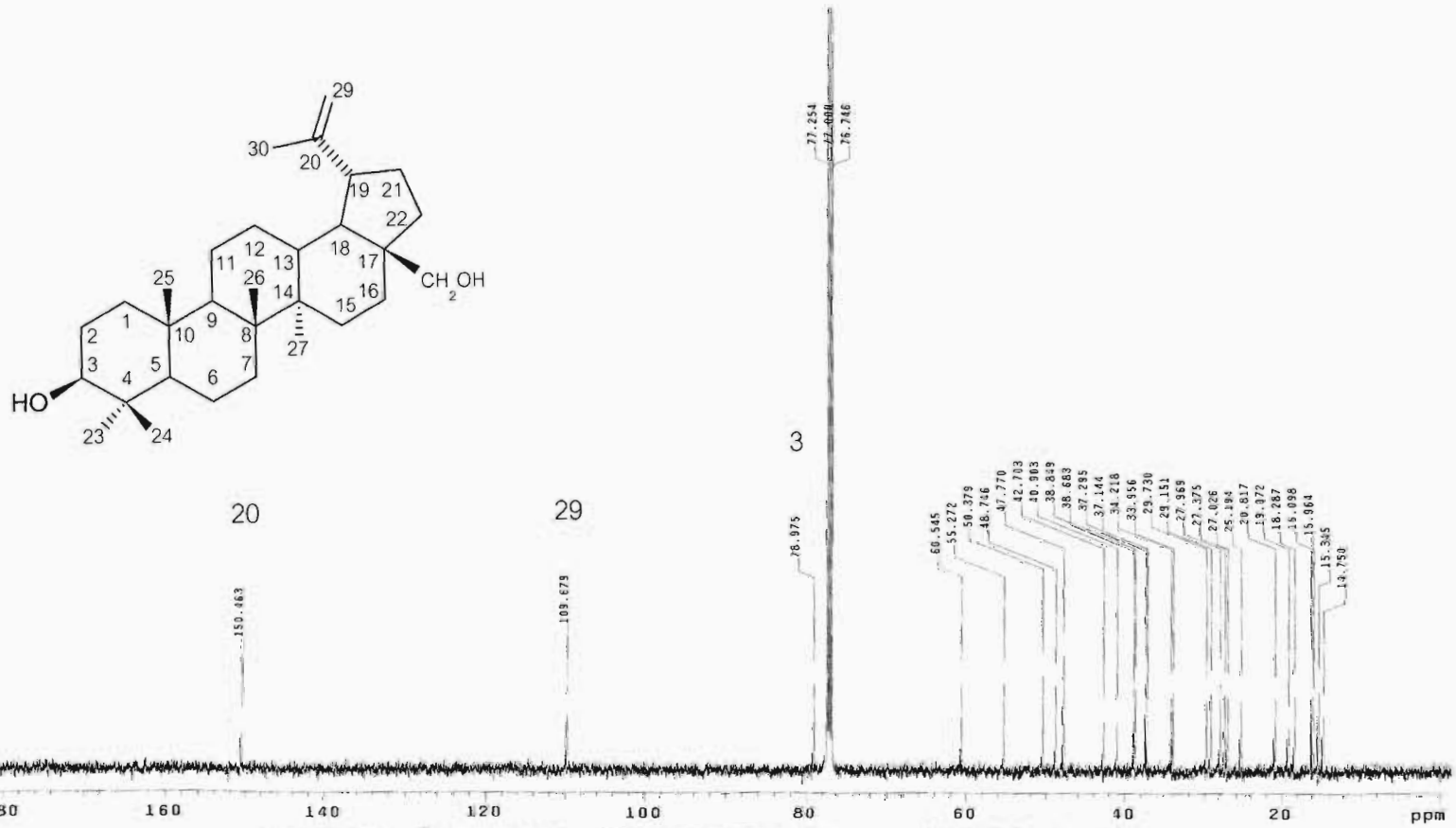


Figure 16a. ^{13}C NMR (125 MHz) spectrum of compound DC-5 (in $CDCl_3$)

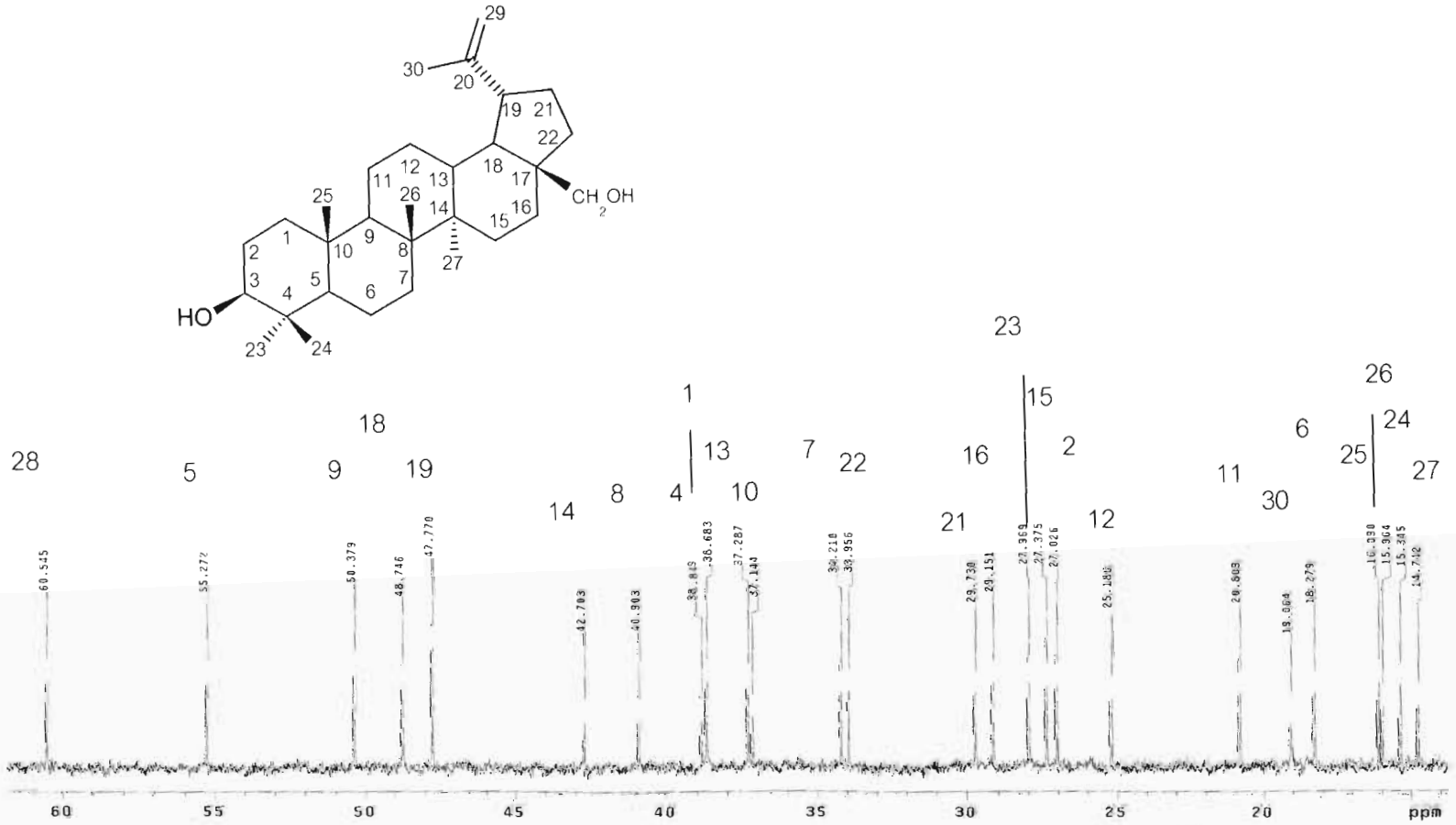


Figure 16b. ^{13}C NMR (125 MHz) spectrum of compound DC-5 (in $CDCl_3$) (expanded)

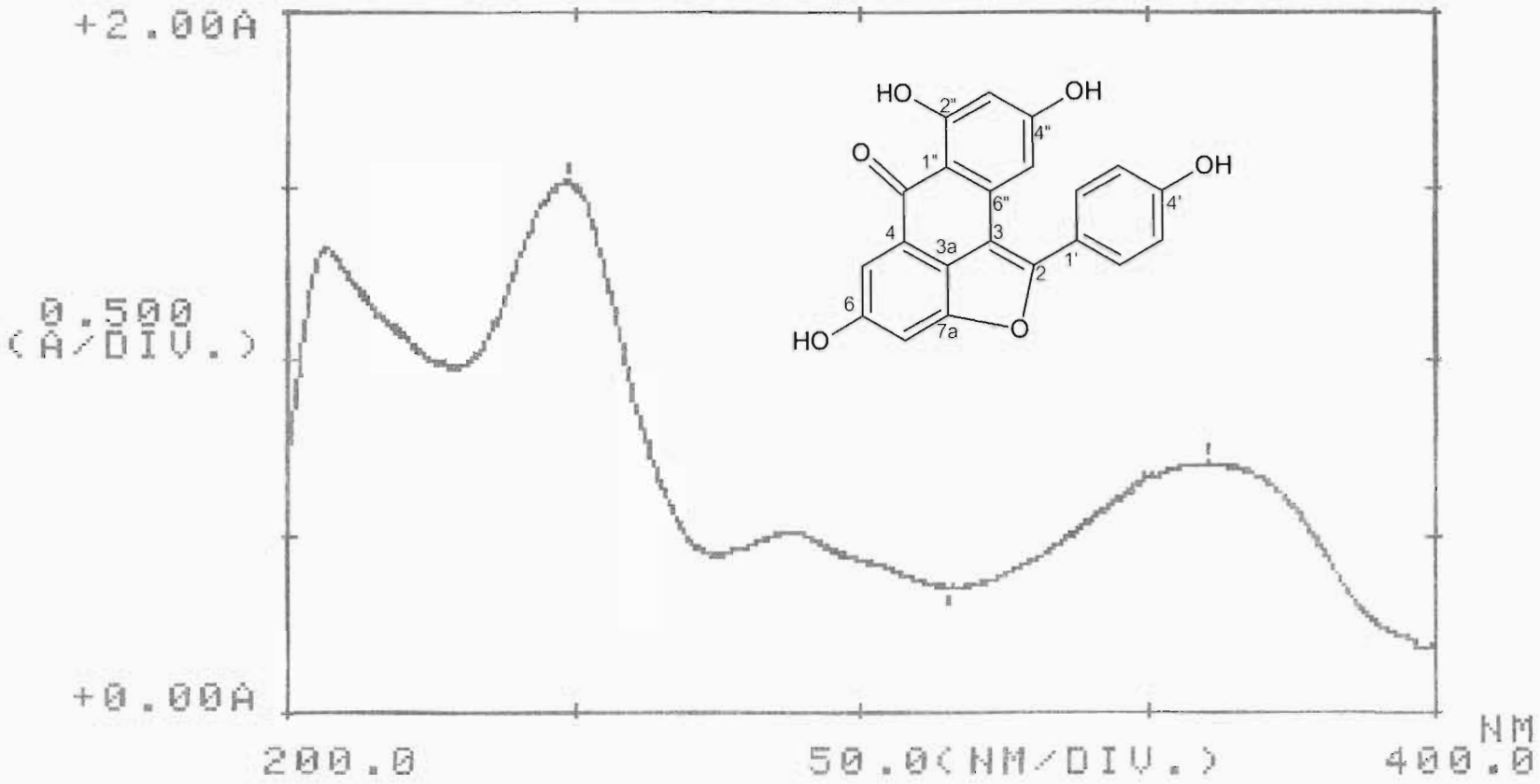


Figure 17. UV spectrum of compound DC-6

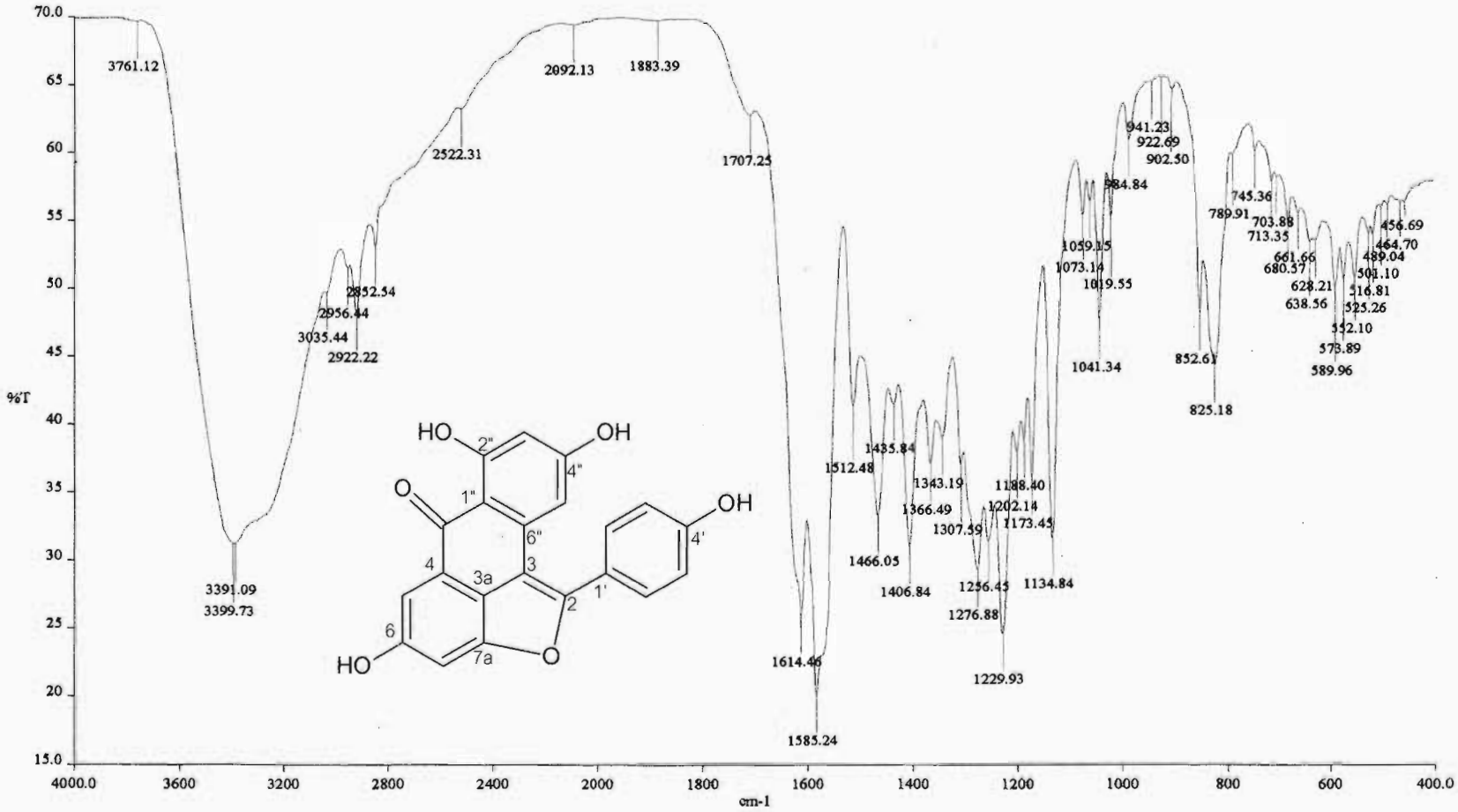


Figure 18. IR spectrum of compound DC-6 (KBr)

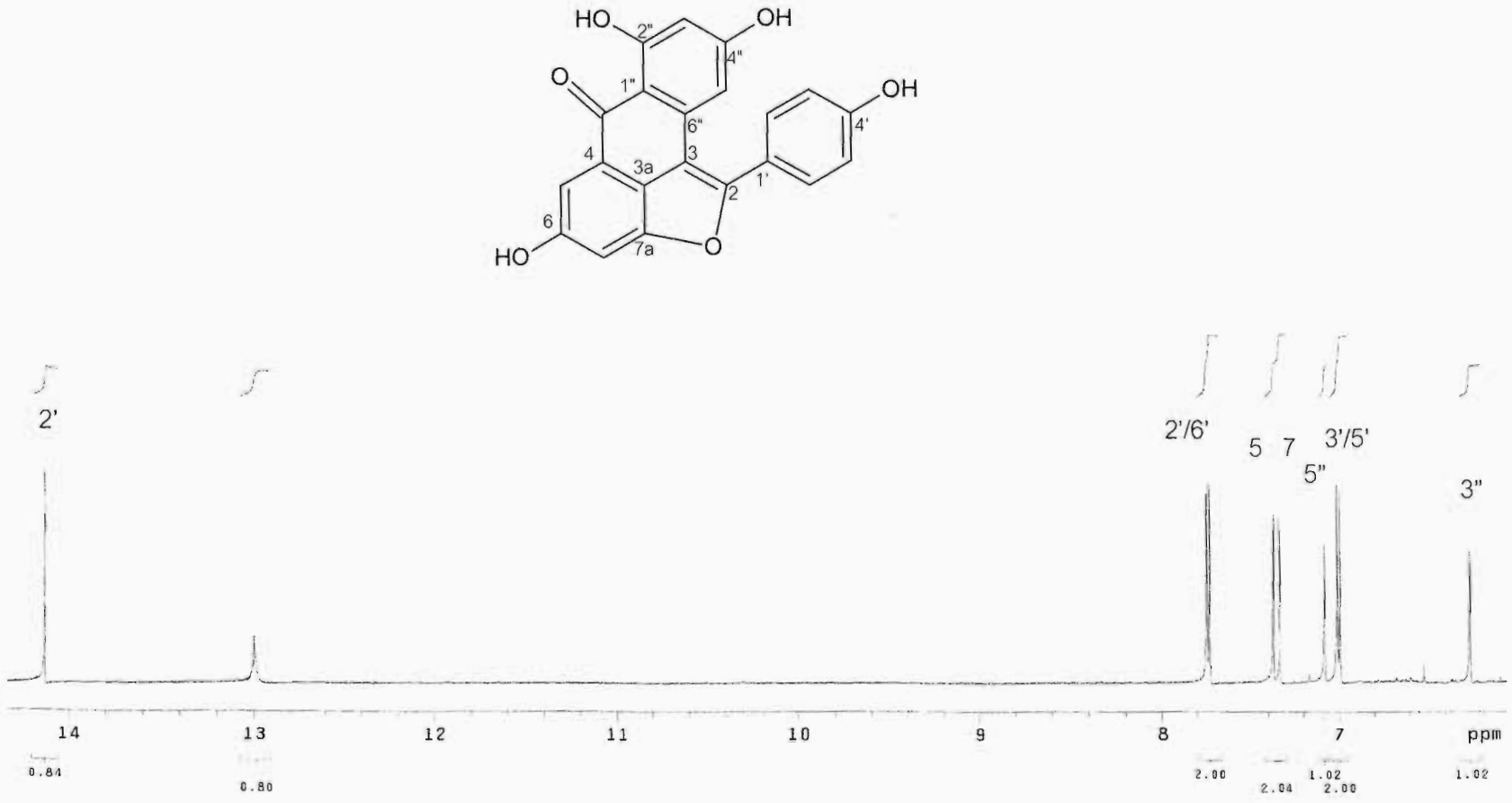


Figure 19a. ^{1}H NMR (500 MHz) spectrum of compound DC-6 (in DMSO-d_6)

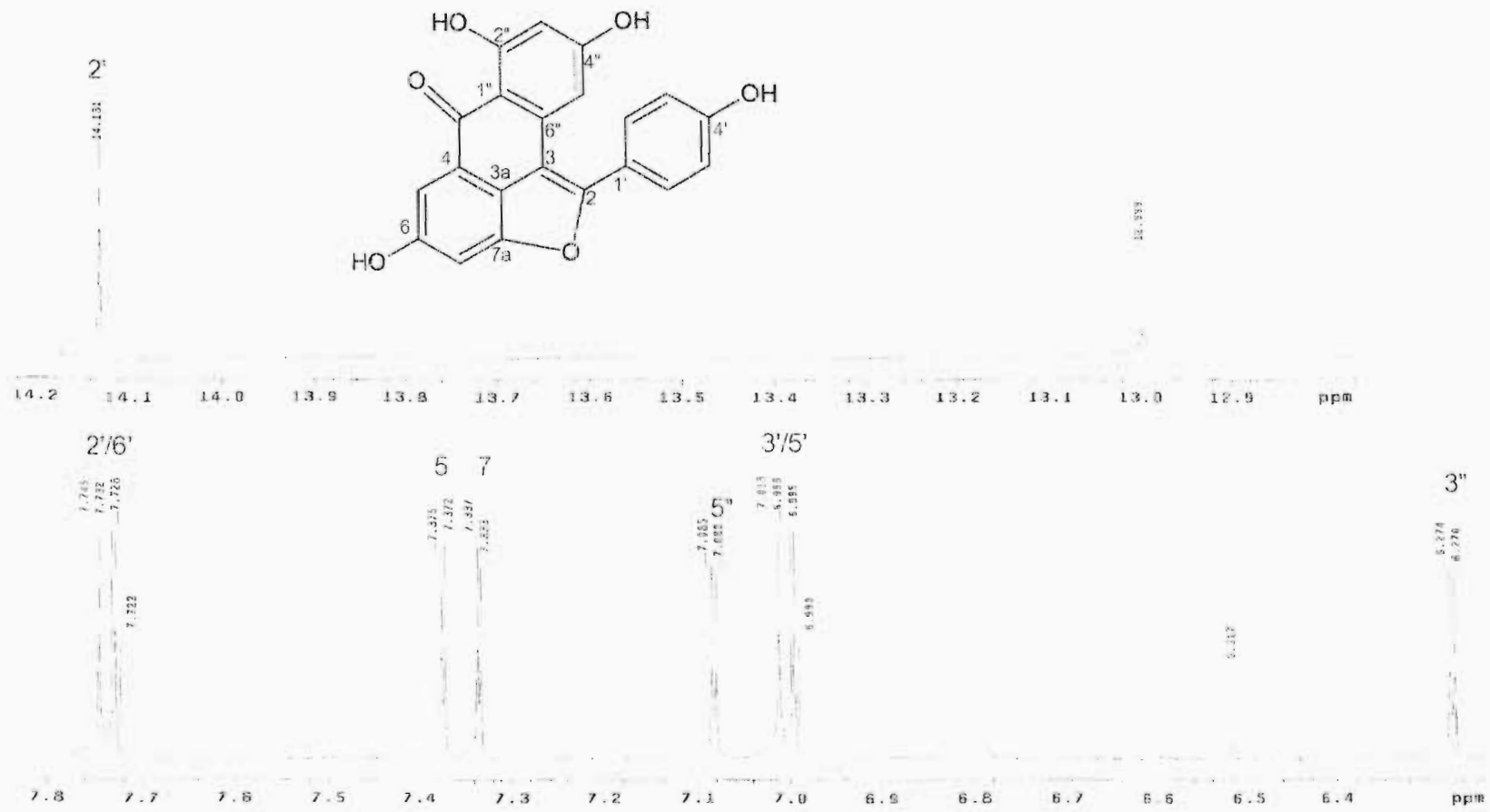


Figure 19b. ^{1}H NMR (500 MHz) Spectrum of compound DC-7(in DMSO-d_6) (expanded)

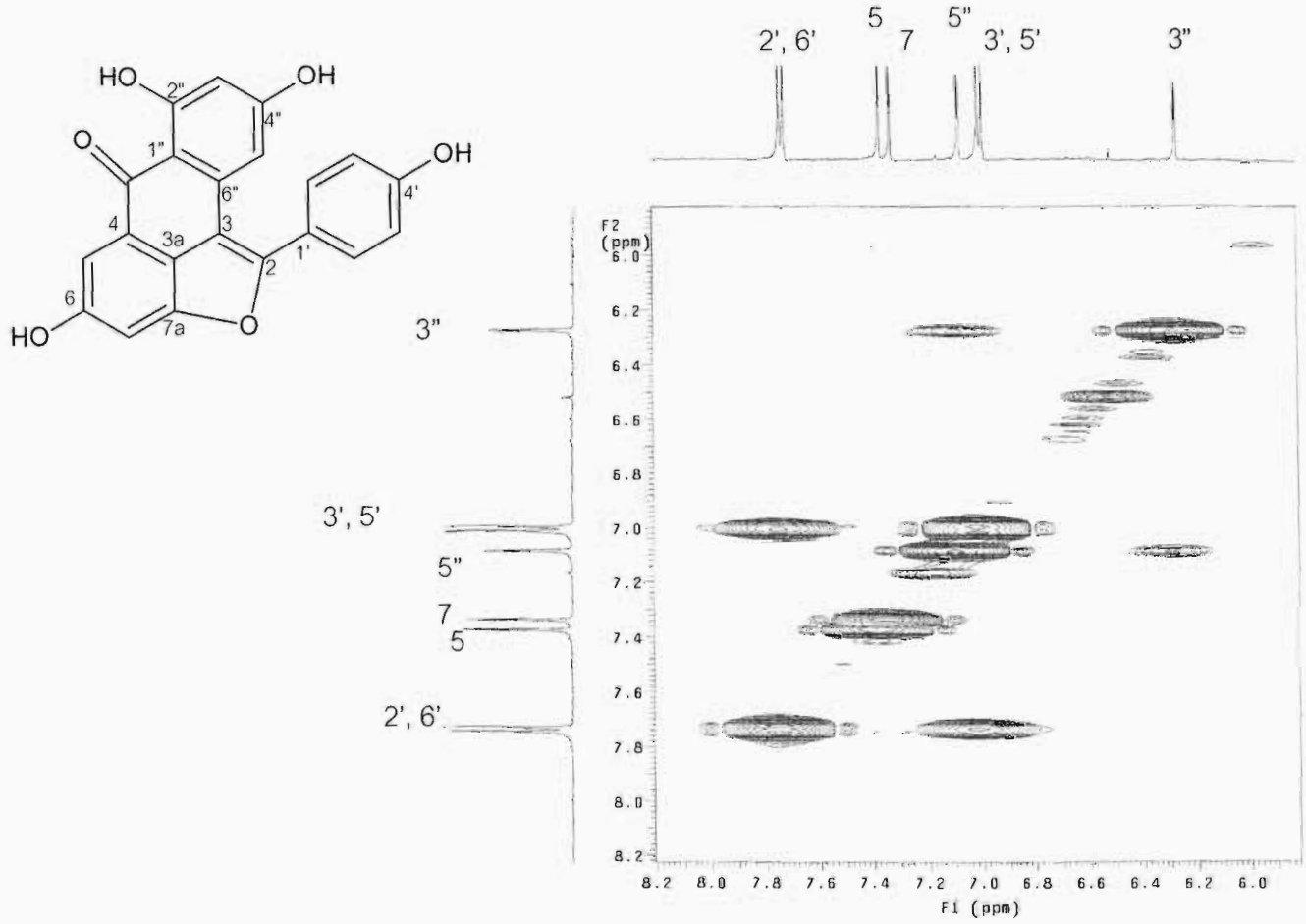


Figure 20. ^{1}H-^{1}H COSY spectrum of compound DC-6 (in DMSO-d_6) (expanded)

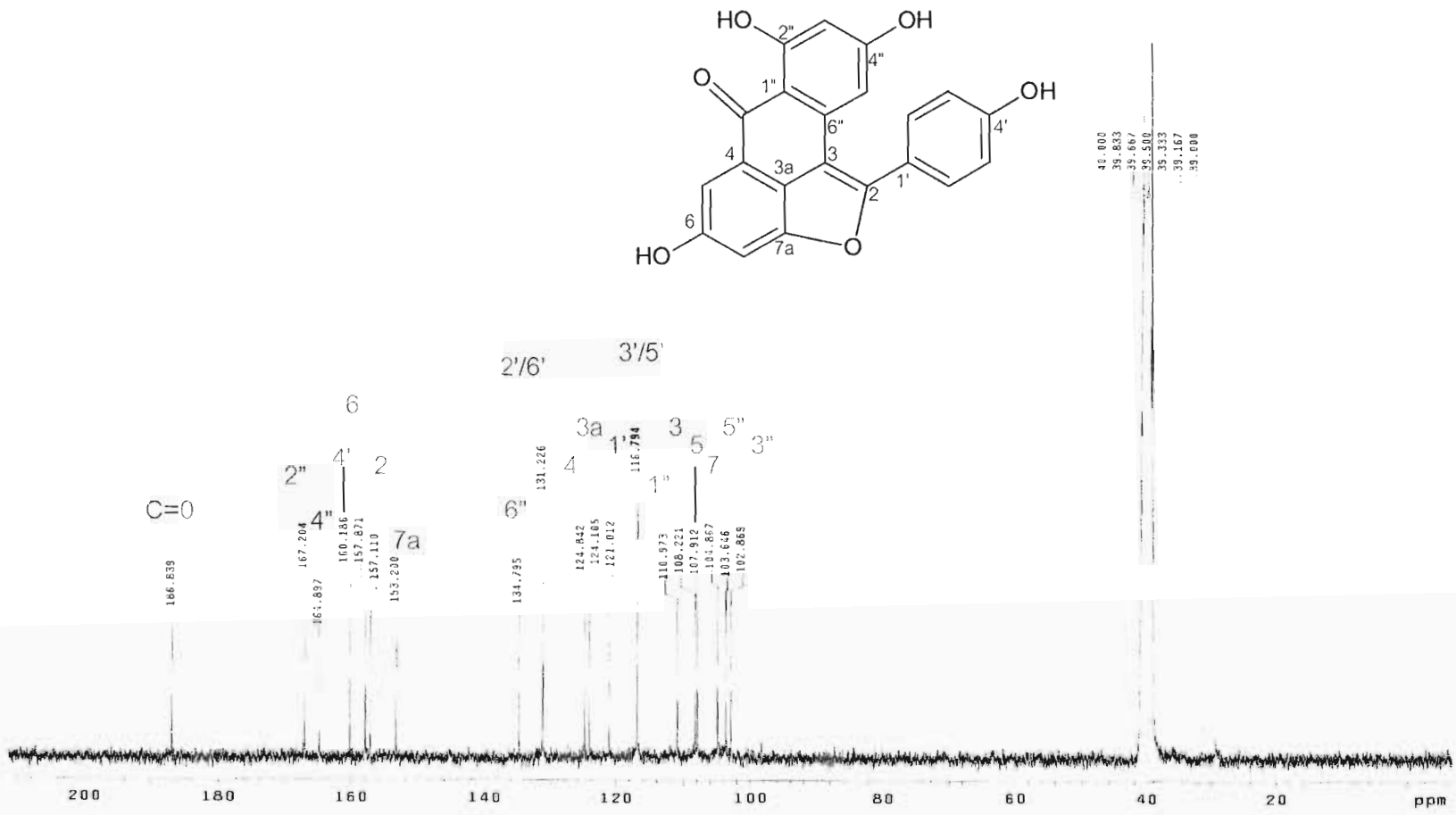


Figure 21. ^{13}C NMR (125 MHz) spectrum of compound DC-6 (in DMSO-d_6)

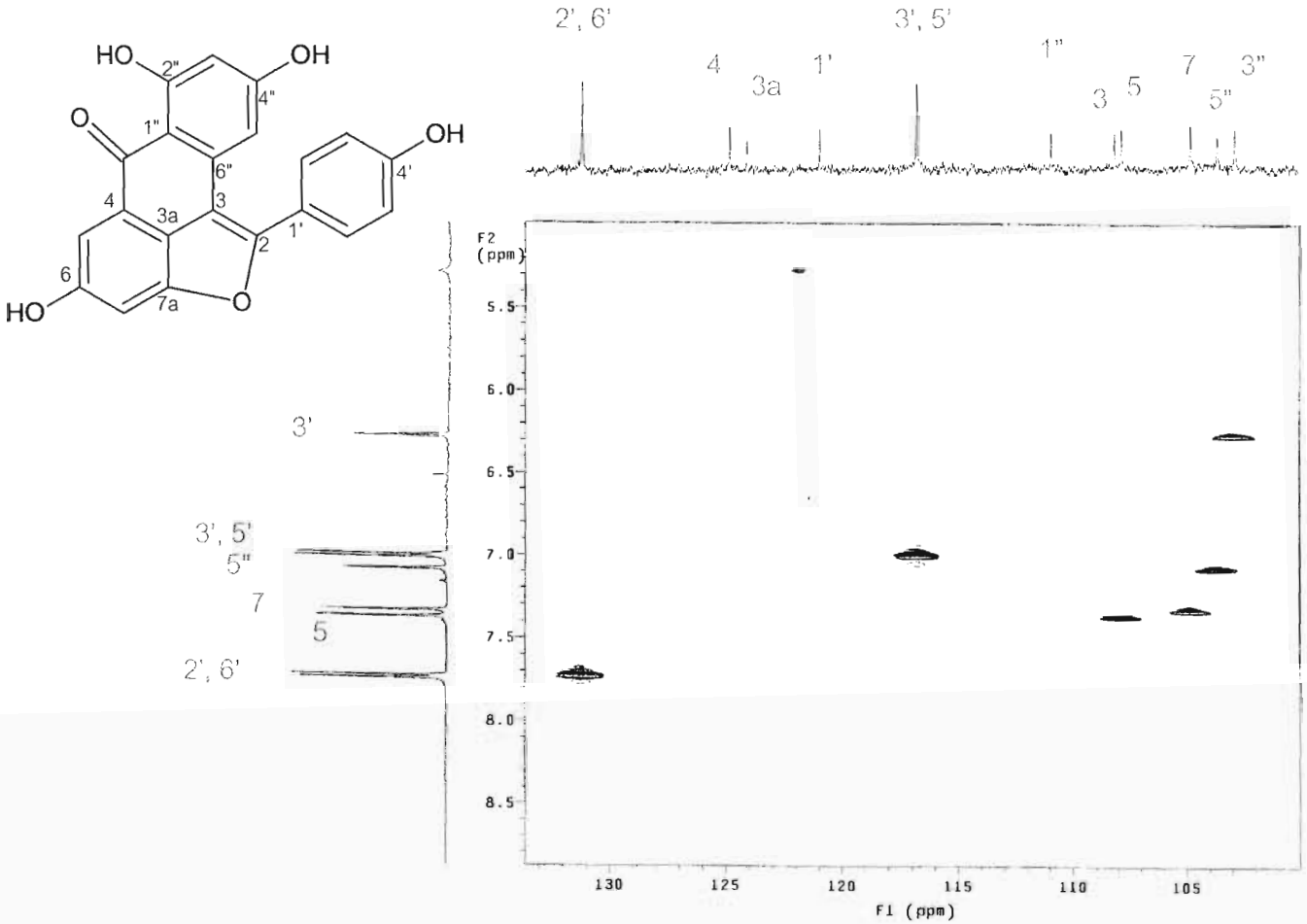


Figure 22. HSQC spectrum of compound DC-6 (in DMSO-d_6) (expanded)

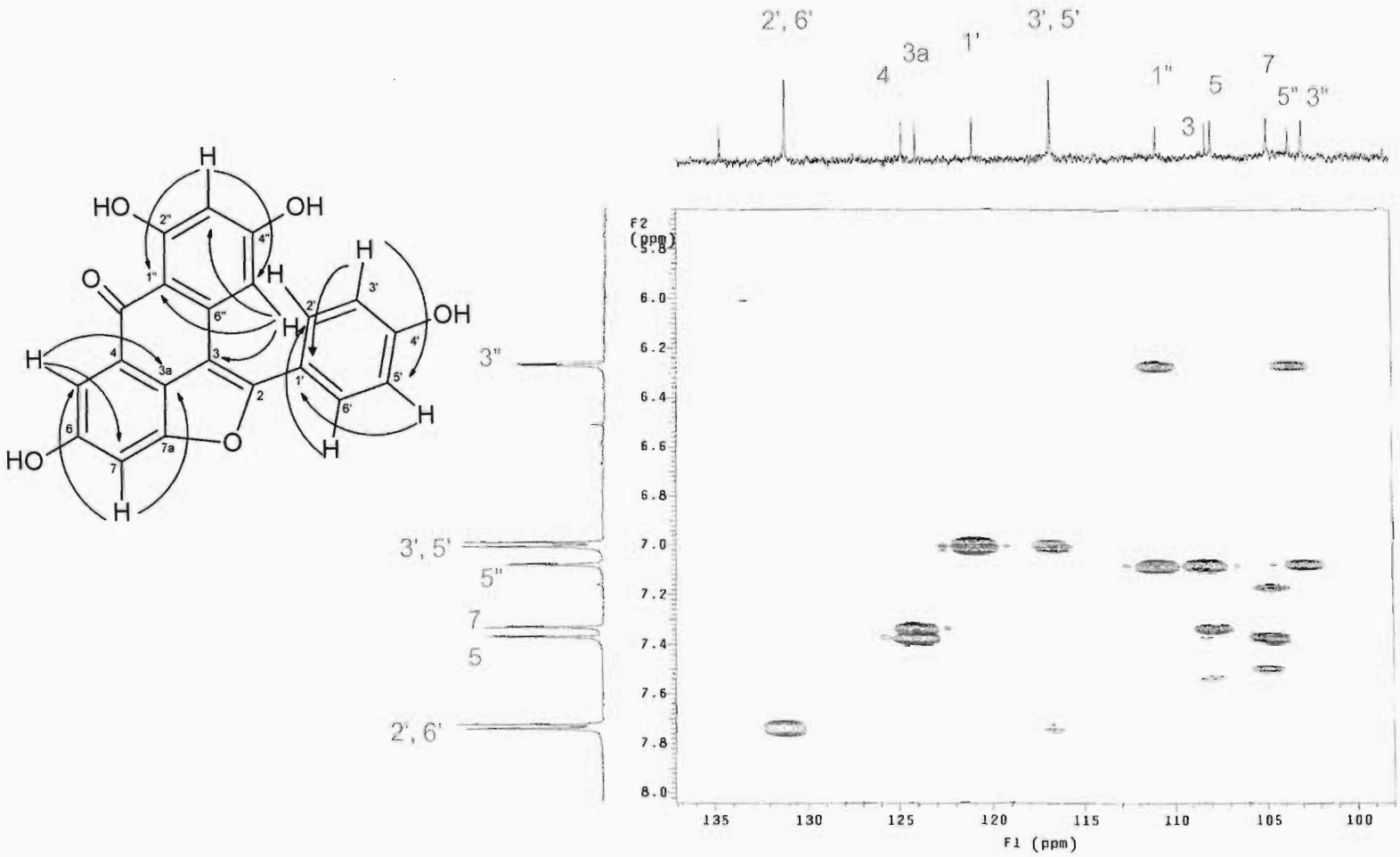


Figure 23a. HMBC spectrum of compound DC-6 (in DMSO-d_6) (expanded)

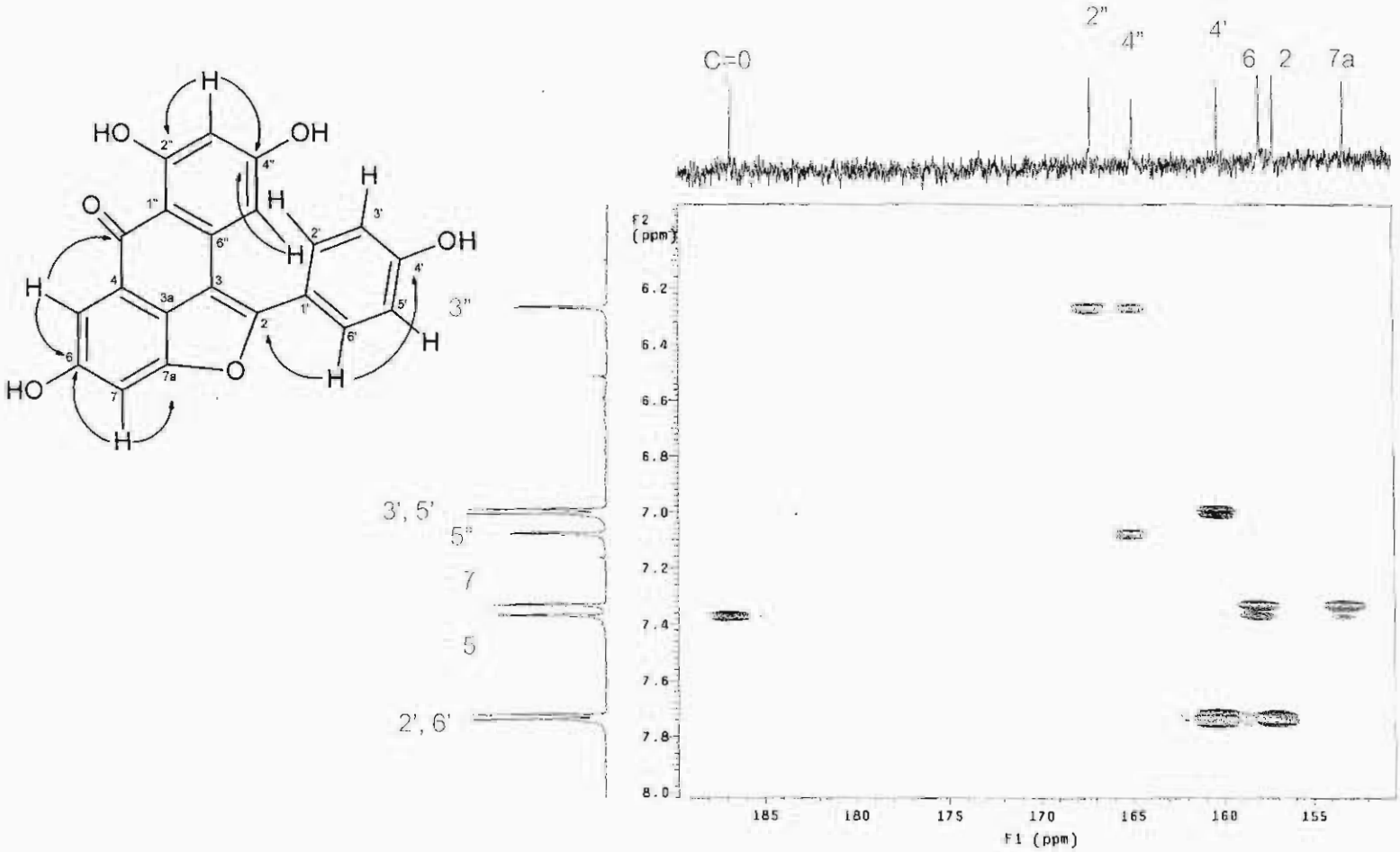


Figure 23b. HMBC spectrum of compound DC-6 (in DMSO-d_6) (expanded)

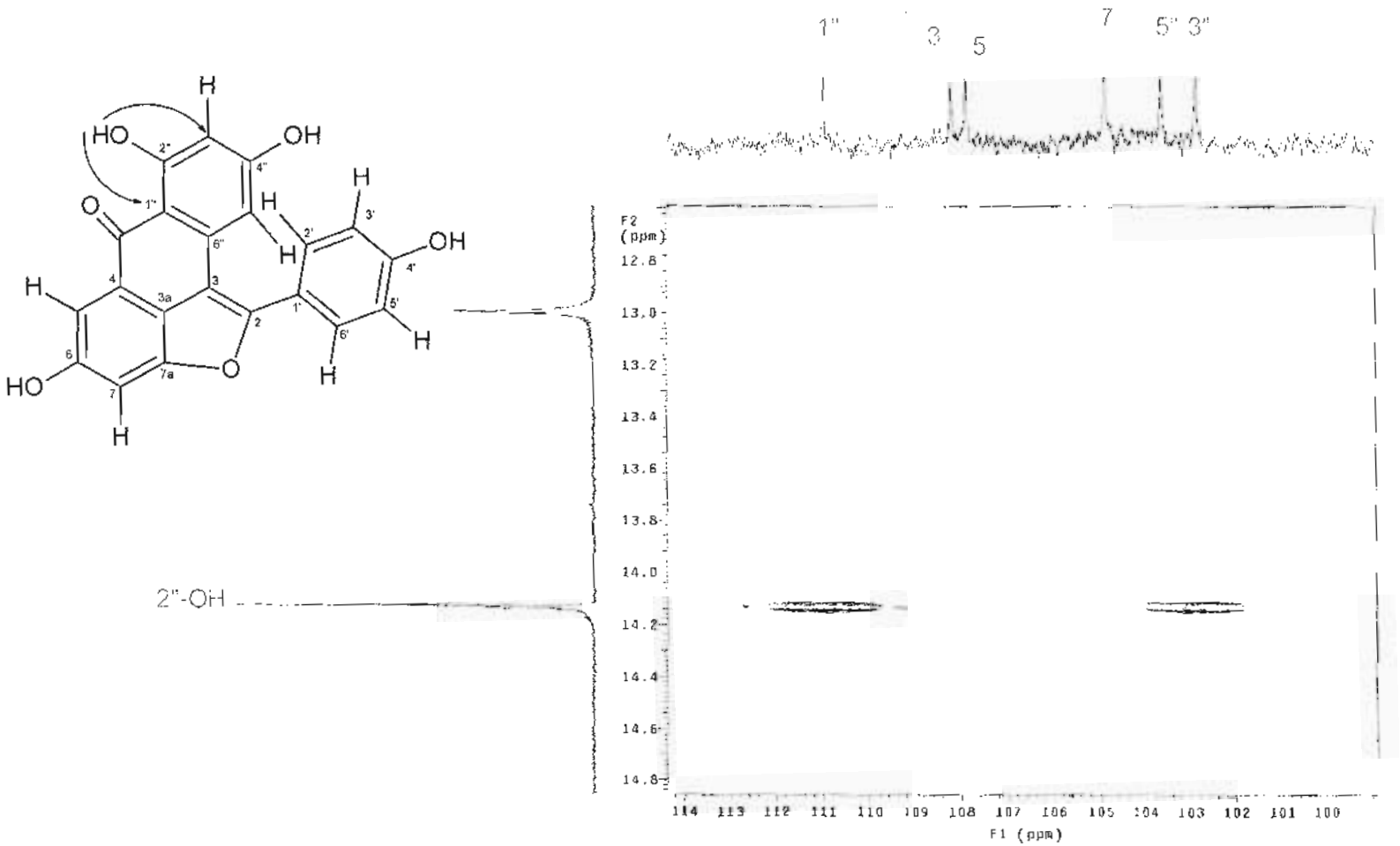


Figure 23c. HMBC spectrum of compound DC-6 (in DMSO-d_6) (expanded)

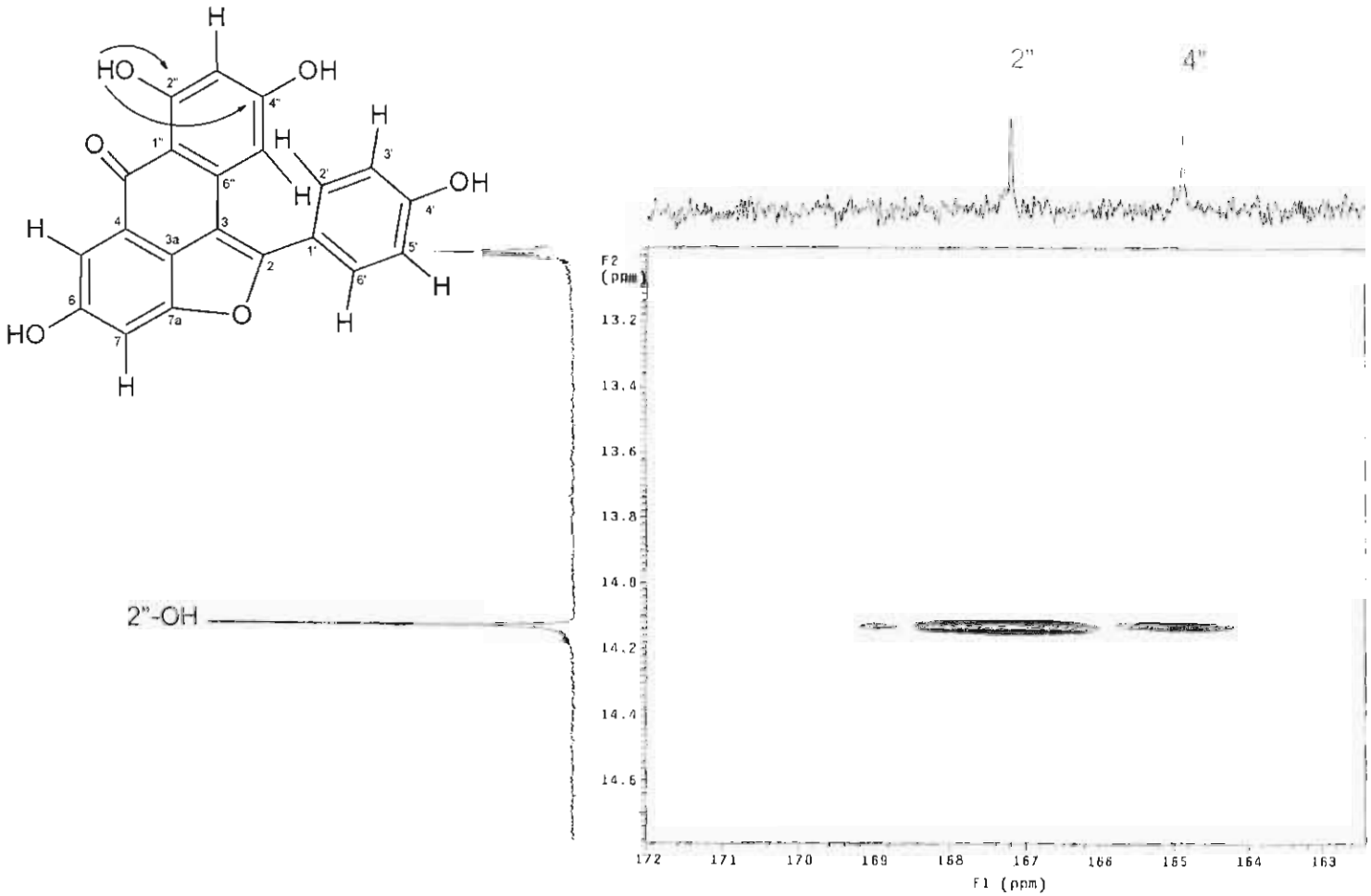


Figure 23d. HMBC spectrum of compound DC-6 (in DMSO-d_6) (expanded)

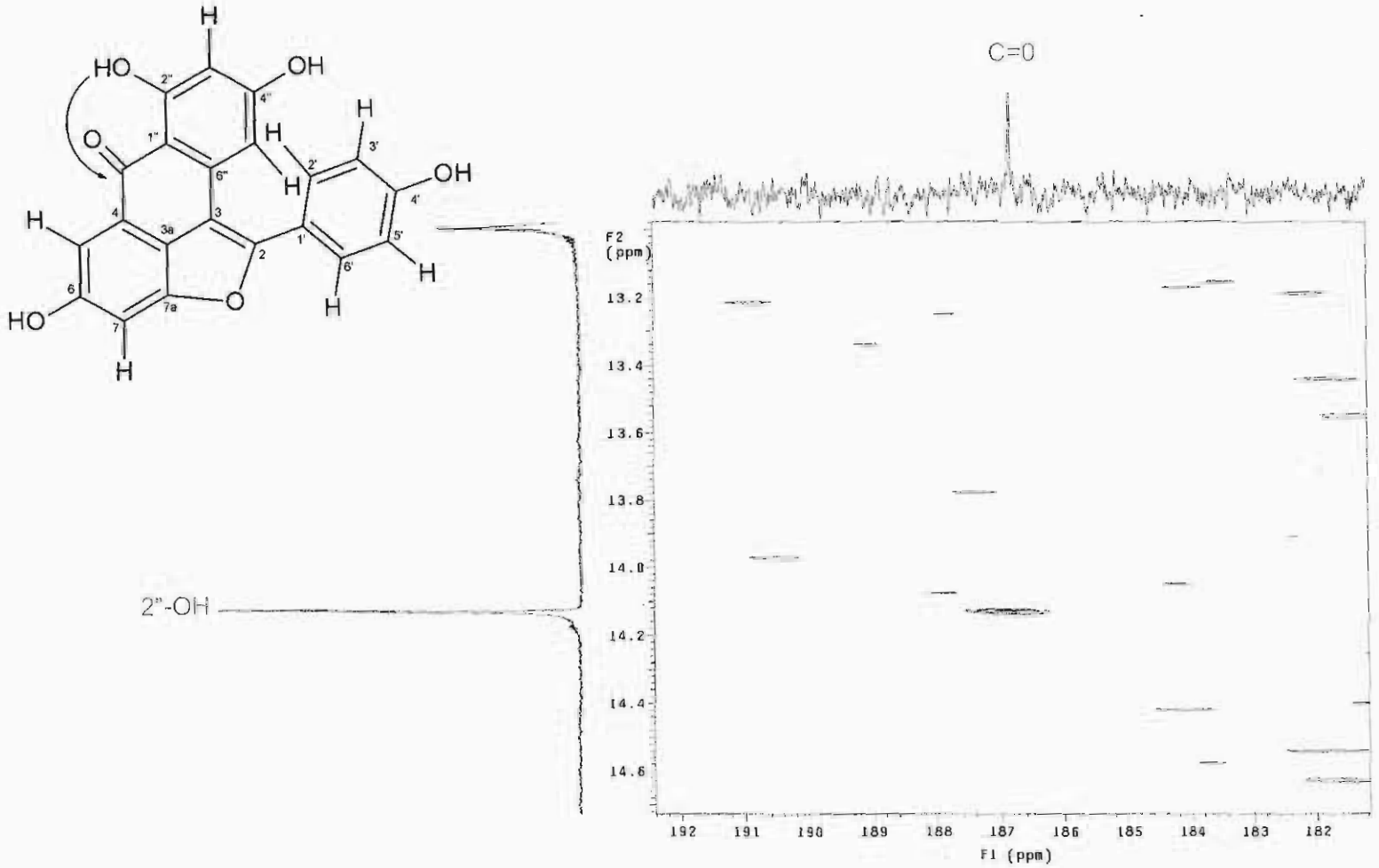


Figure 23e. HMBC spectrum of compound DC-6 (in DMSO-d_6) (expanded)

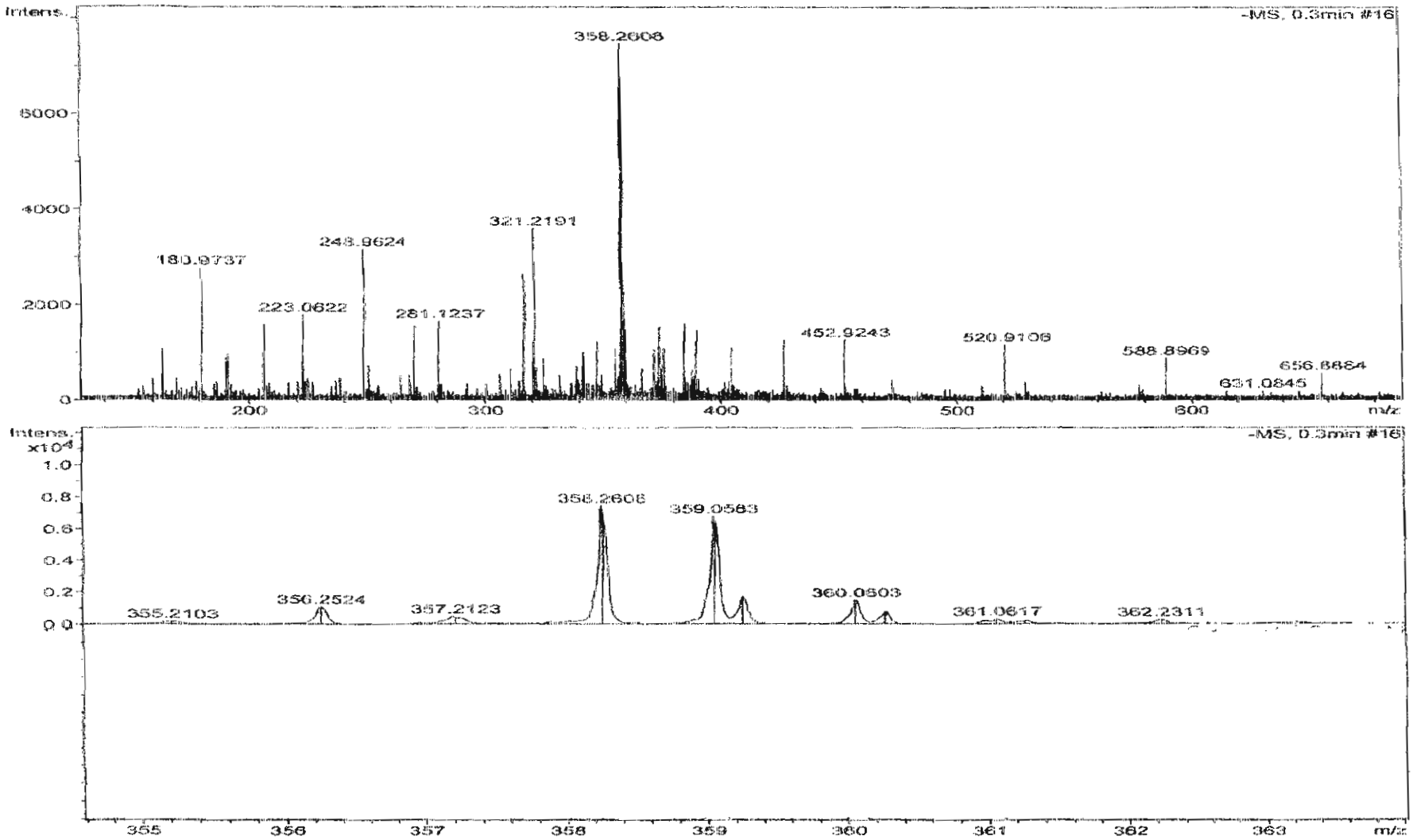


Figure 24. HRESI Mass spectrum of compound DC-6

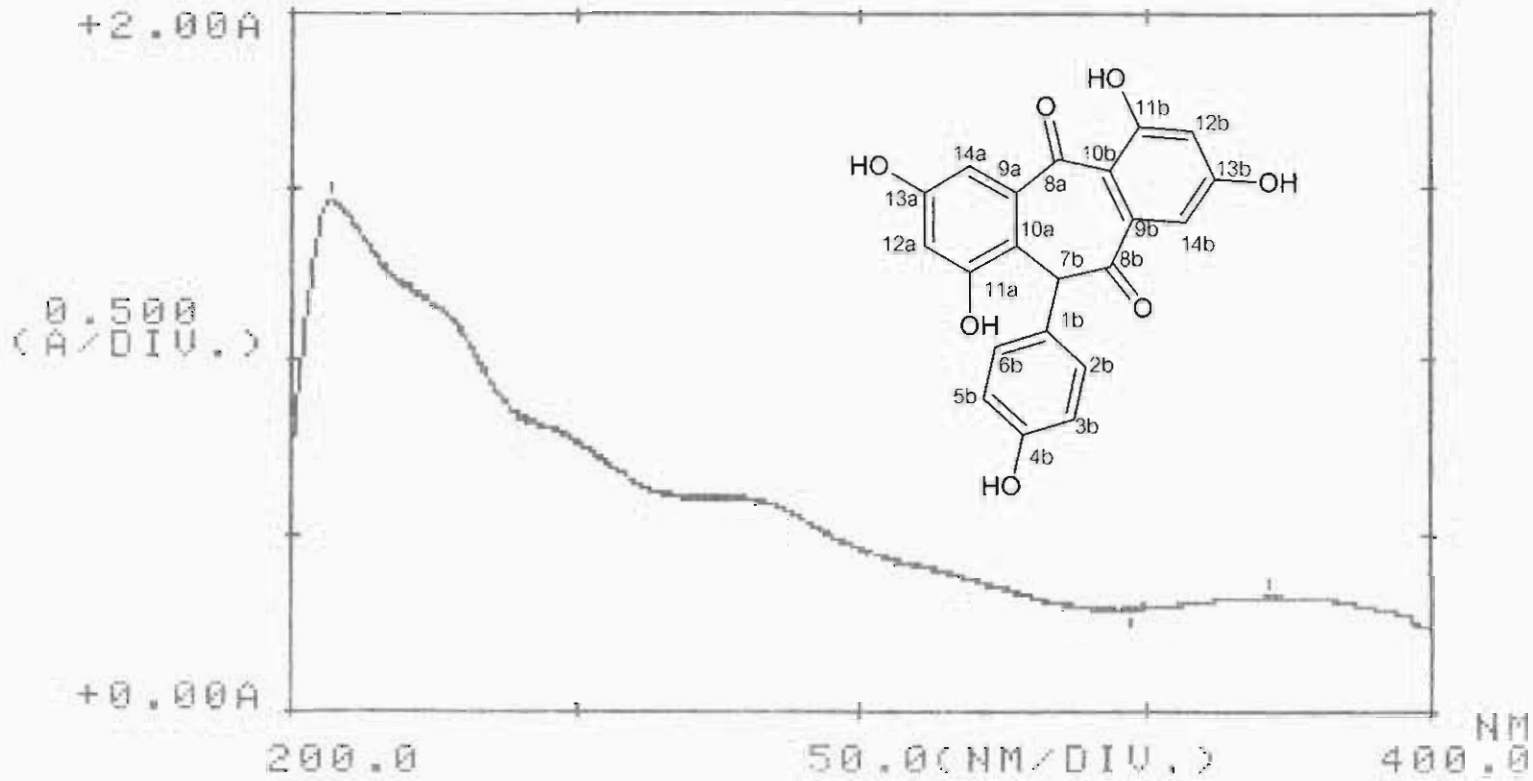


Figure 25. UV spectrum of compound DC-7

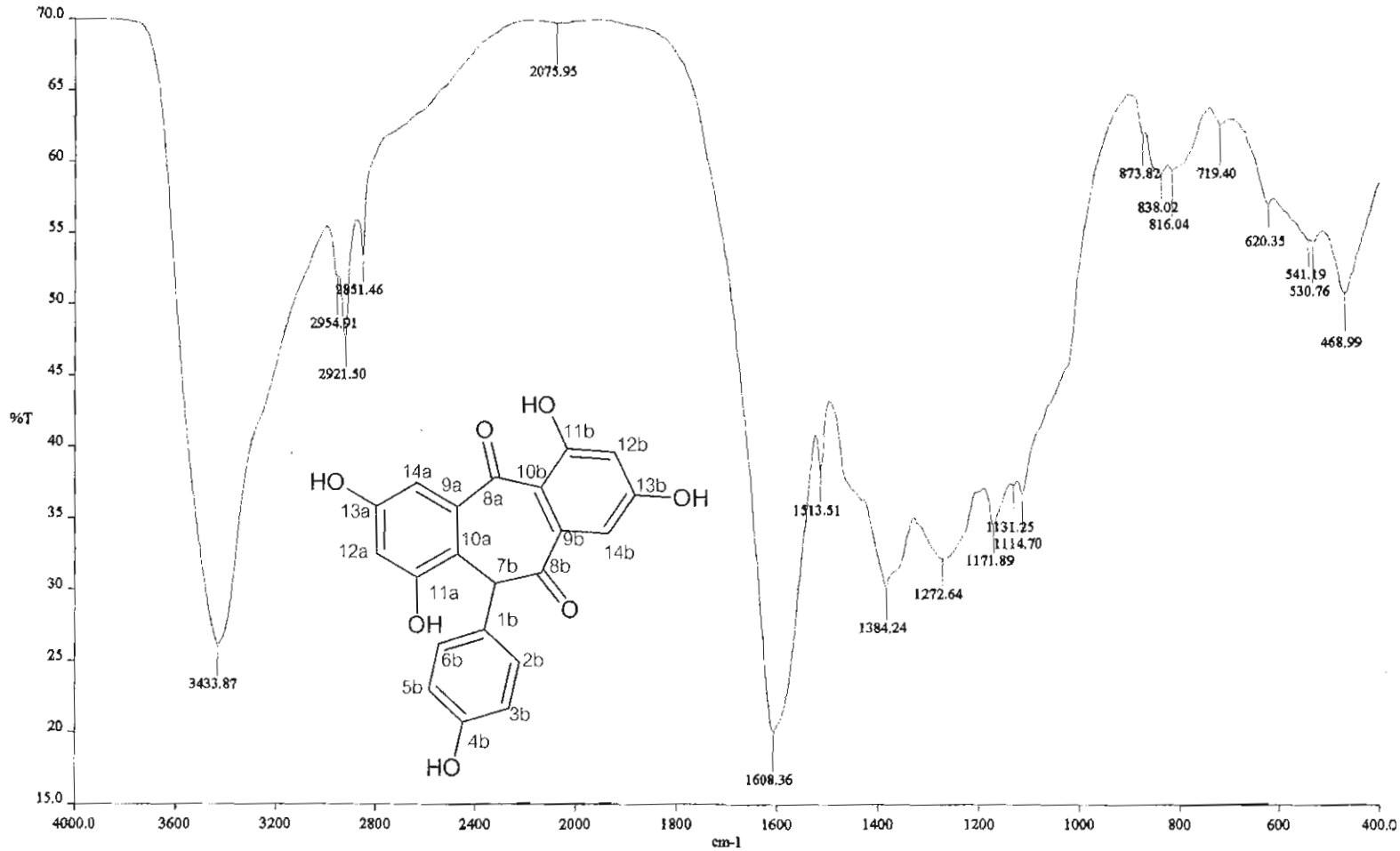


Figure 26. IR spectrum of compound DC-7 (KBr)

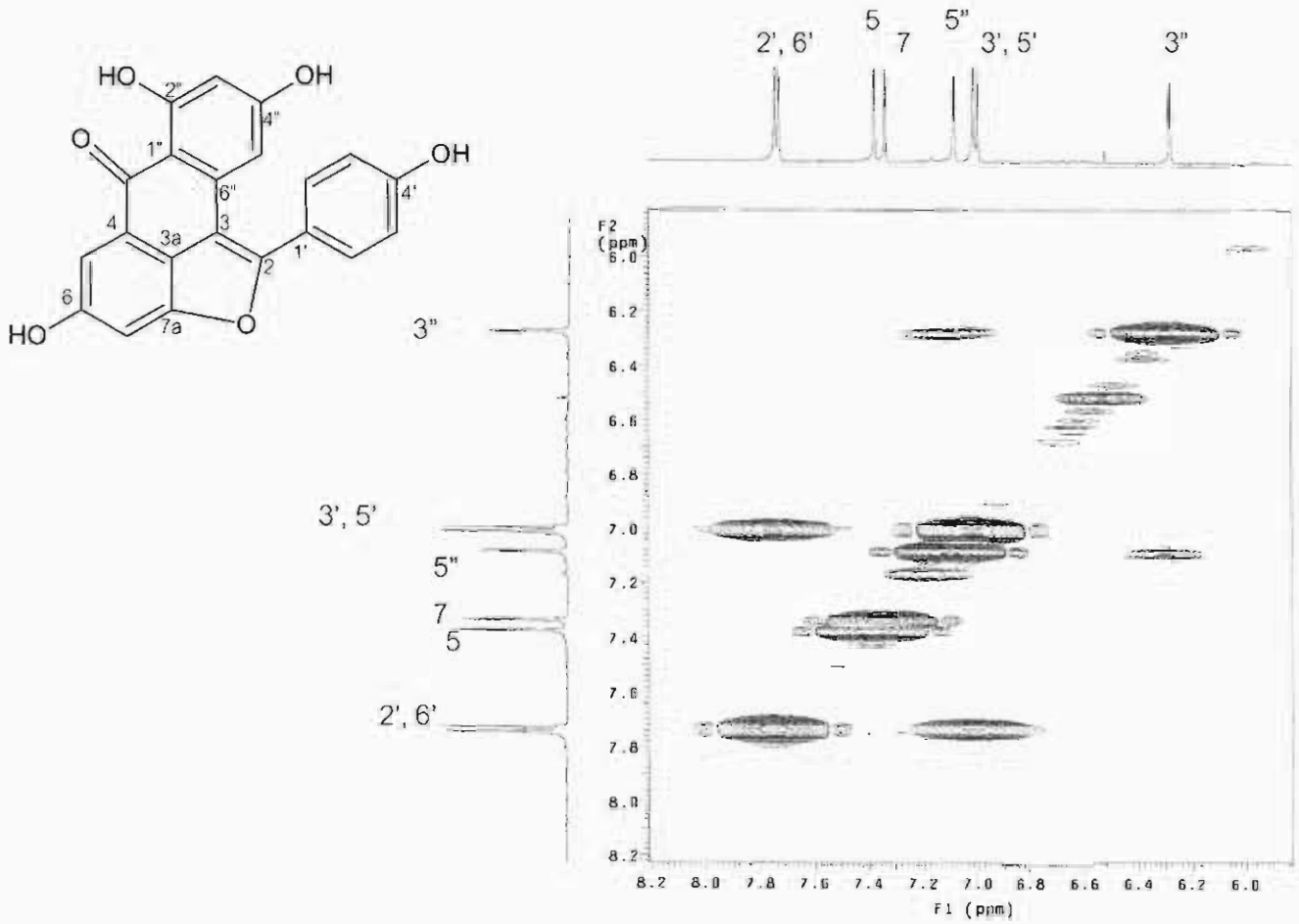


Figure 20. ^{1}H-^{1}H COSY spectrum of compound DC-6 (in DMSO-d_6) (expanded)

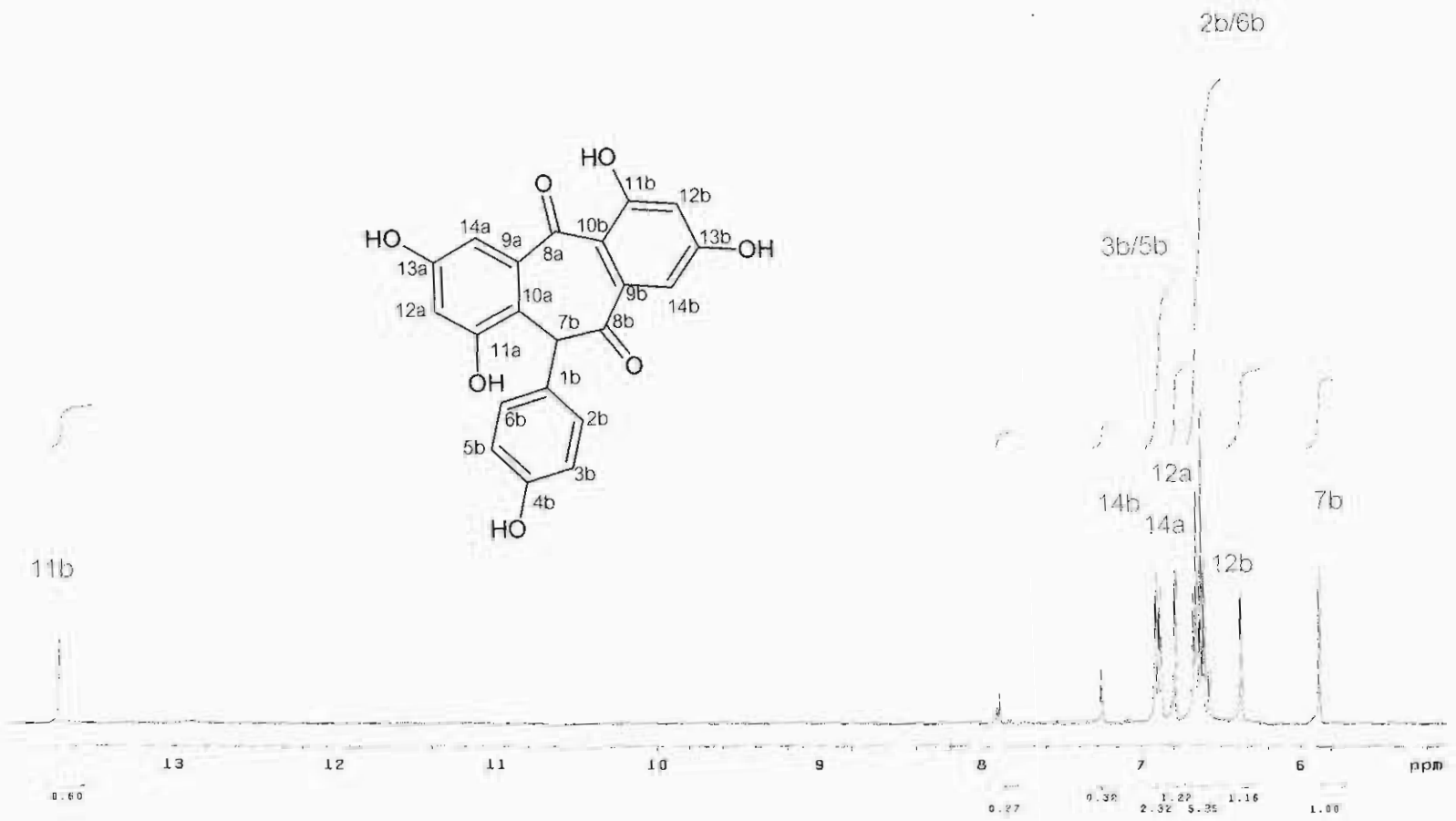


Figure 27a. ^{1}H NMR (500 MHz) spectrum of compound DC-7 (in acetone-d_6)

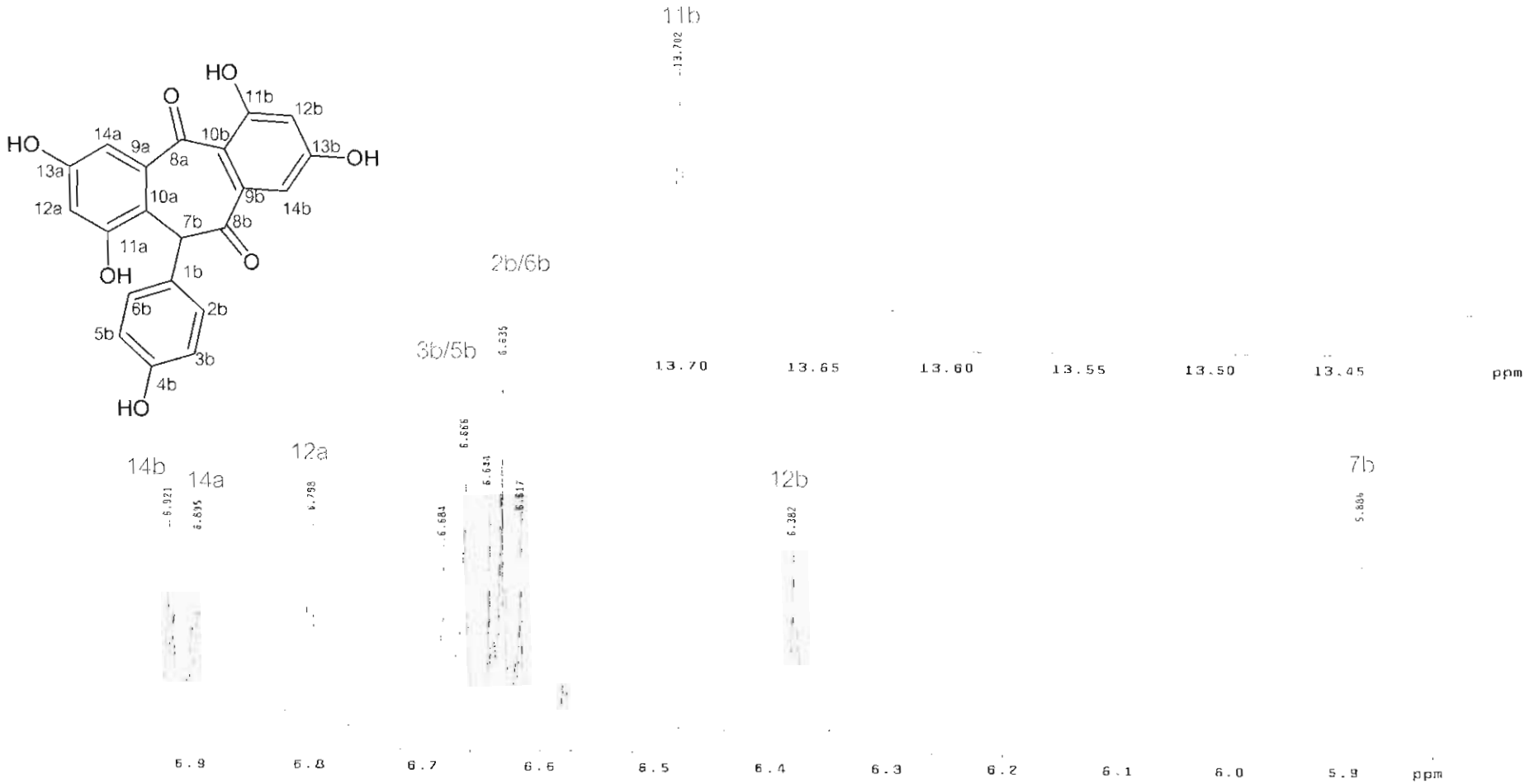


Figure 27b. ^{1}H NMR (500 MHz) spectrum of compound DC-7 (in acetone-d_6) (expanded)

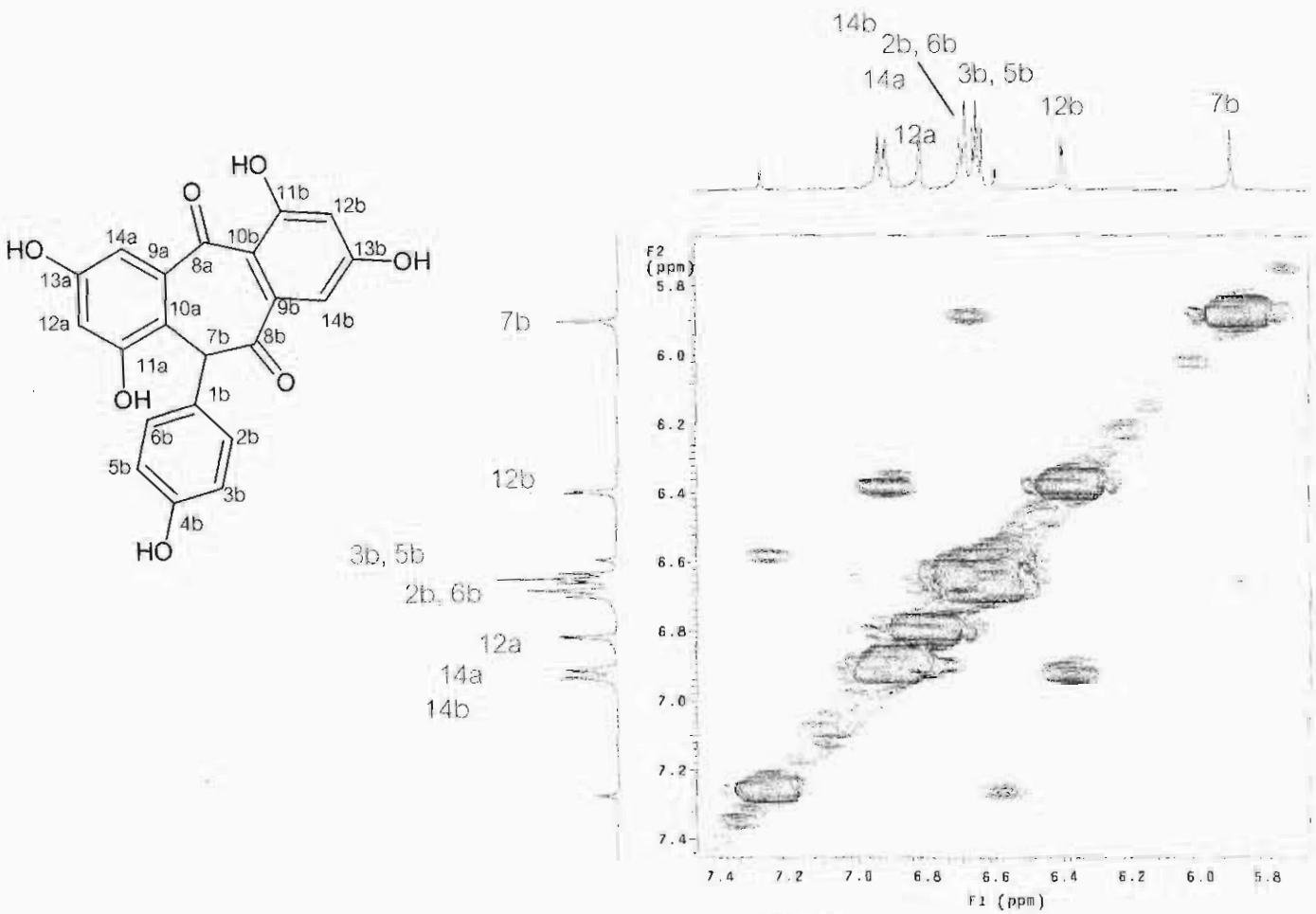


Figure 28. ^{1}H-^{1}H COSY spectrum of compound DC-7 (in acetone-d_6) (expanded)

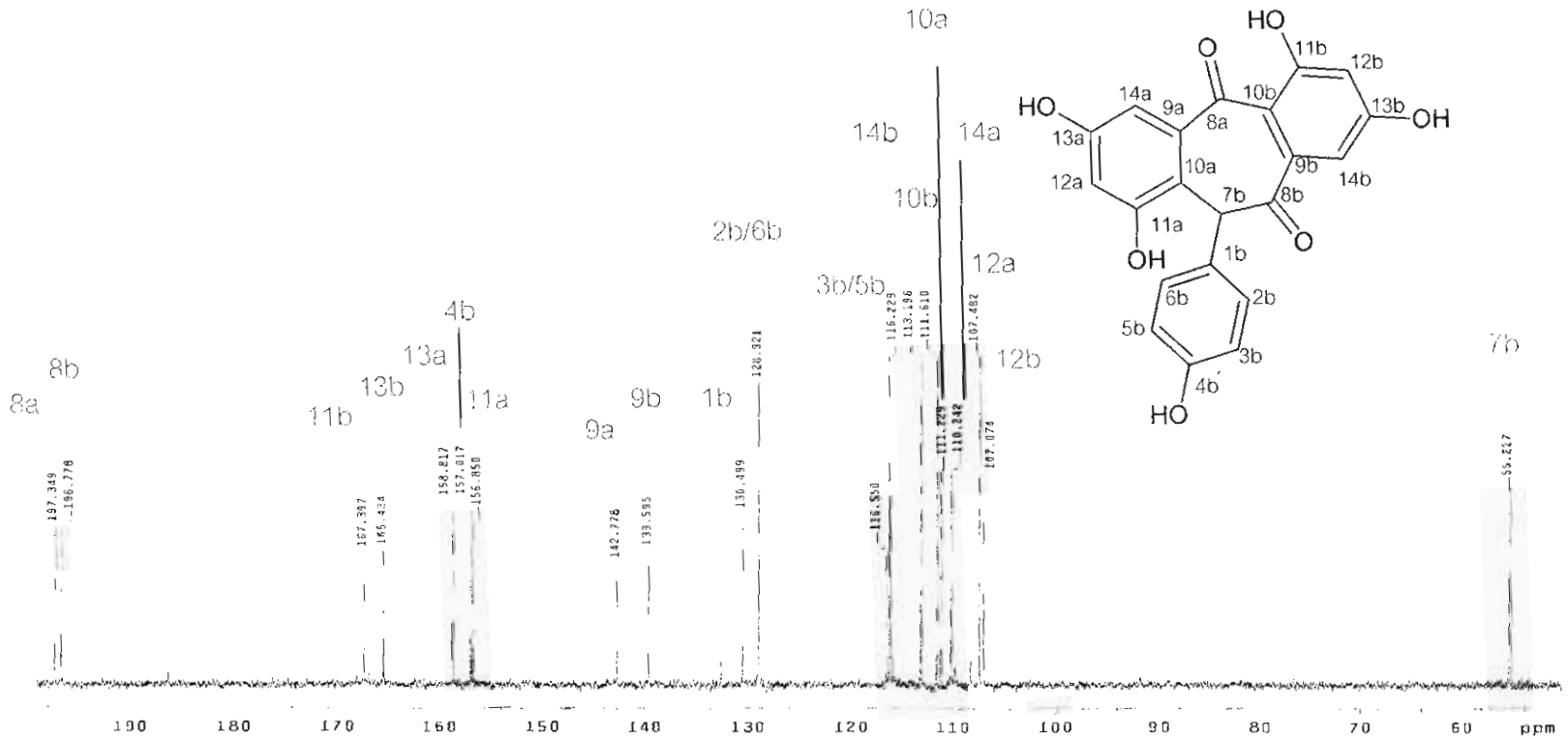


Figure 29. ^{13}C NMR (125 MHz) spectrum of compound DC-7 (in acetone-d_6)

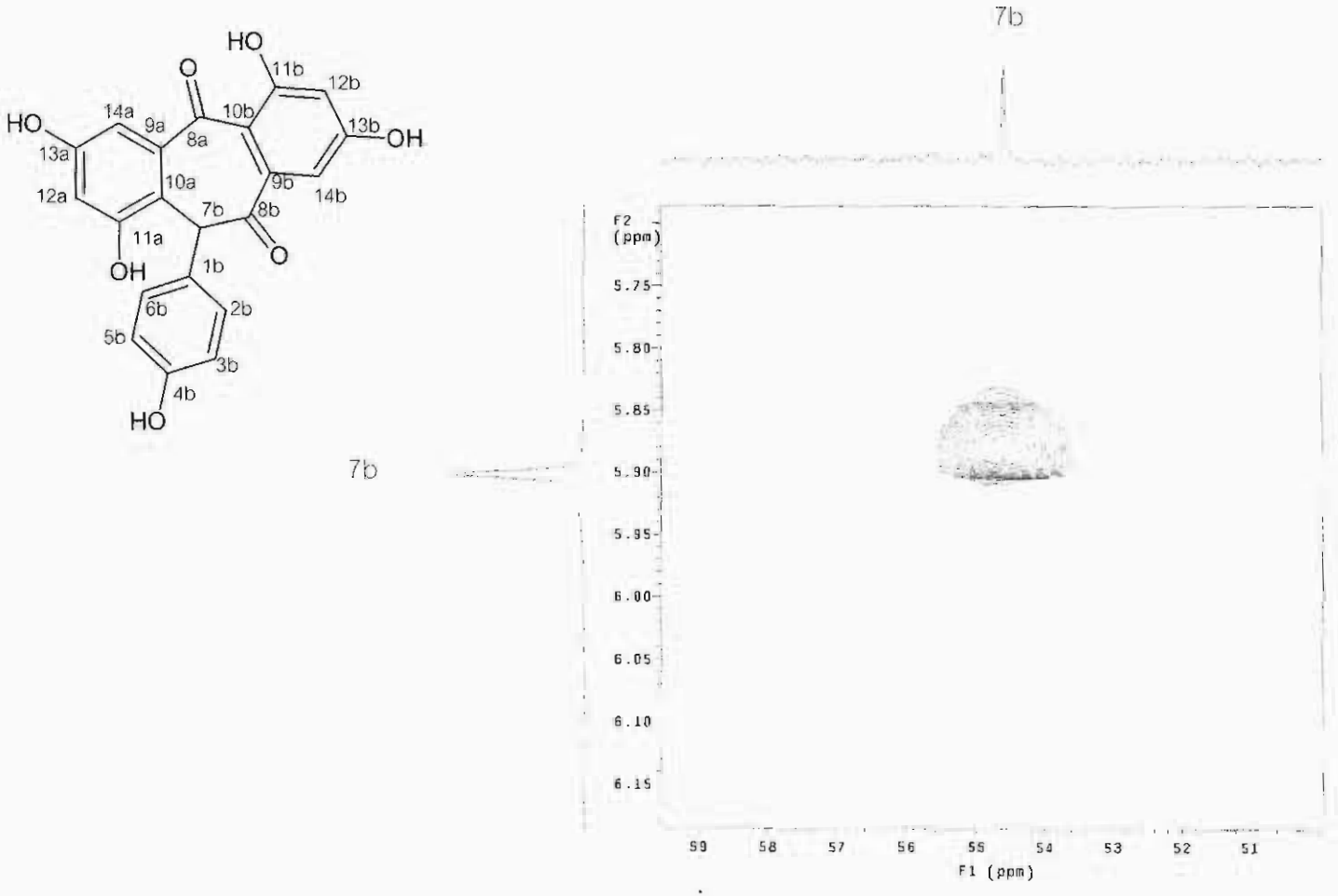


Figure 30a. HSQC spectrum of compound DC-7 (in acetone-d_6) (expanded)

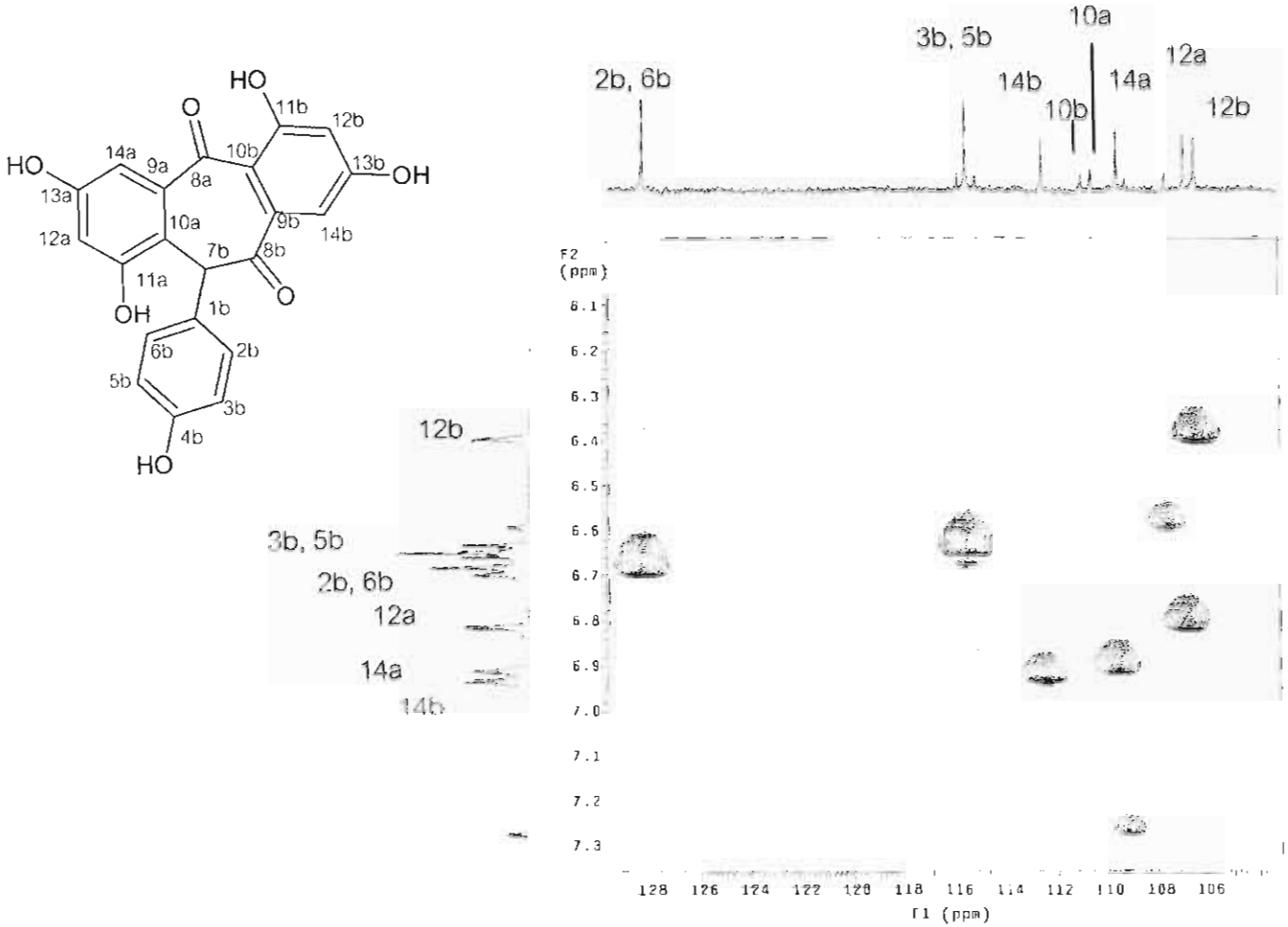


Figure 30b. HSQC spectrum of compound DC-7 (in acetone-d_6) (expanded)

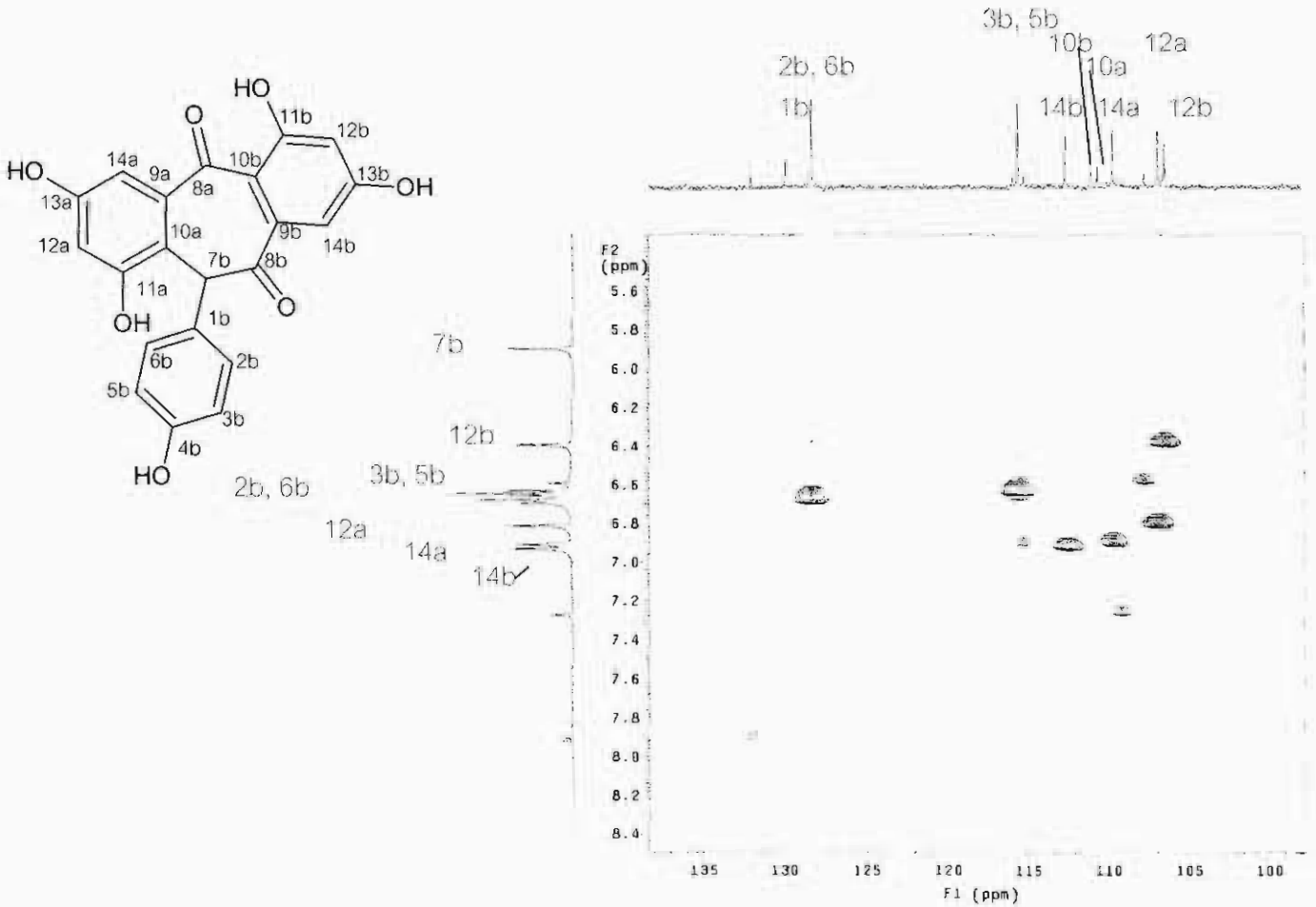


Figure 30c. HSQC spectrum of compound DC-7 (in acetone-d_6) (expanded)

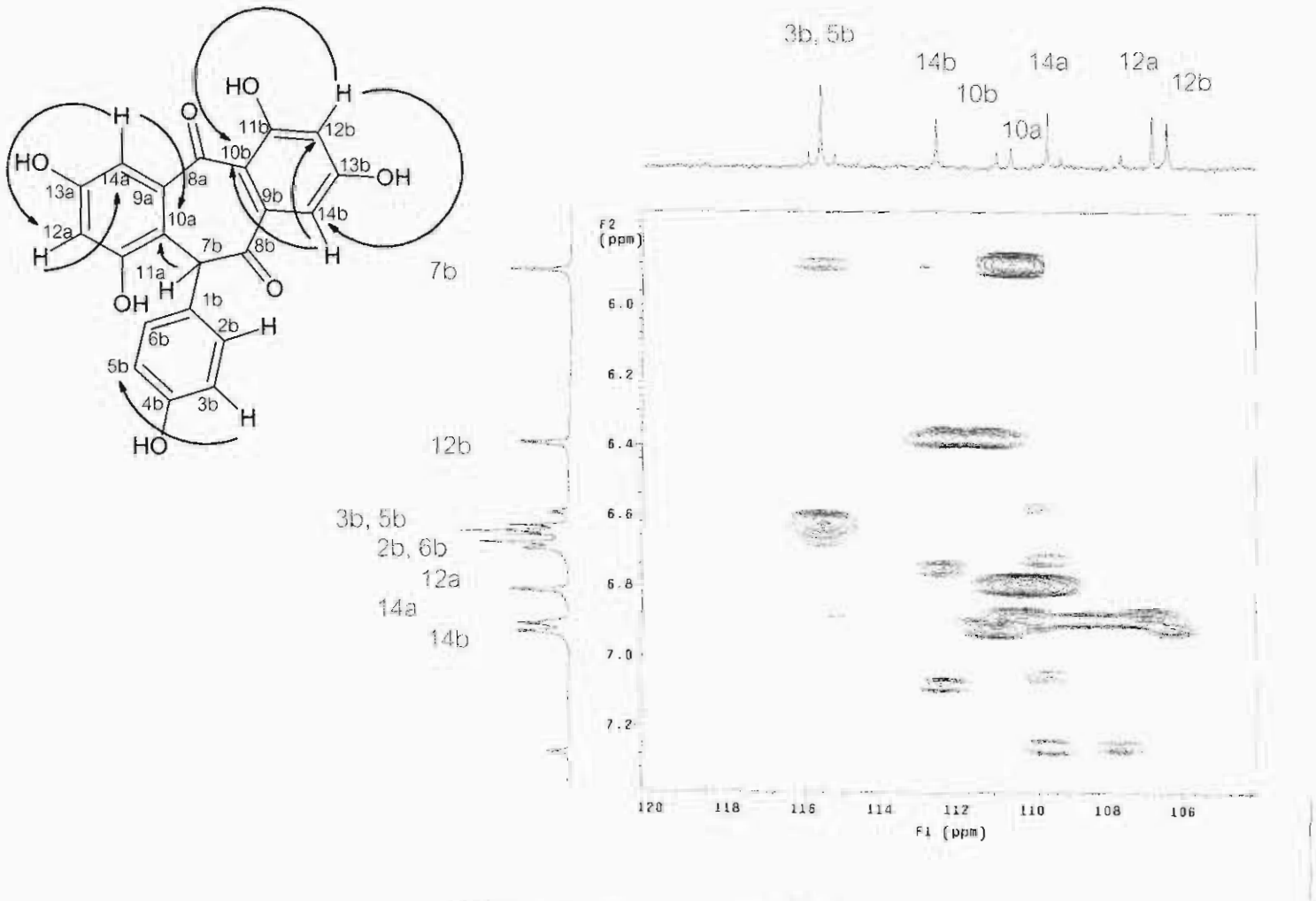


Figure 31a. HMBC spectrum of compound DC-7 (in acetone-d_6) (expanded)

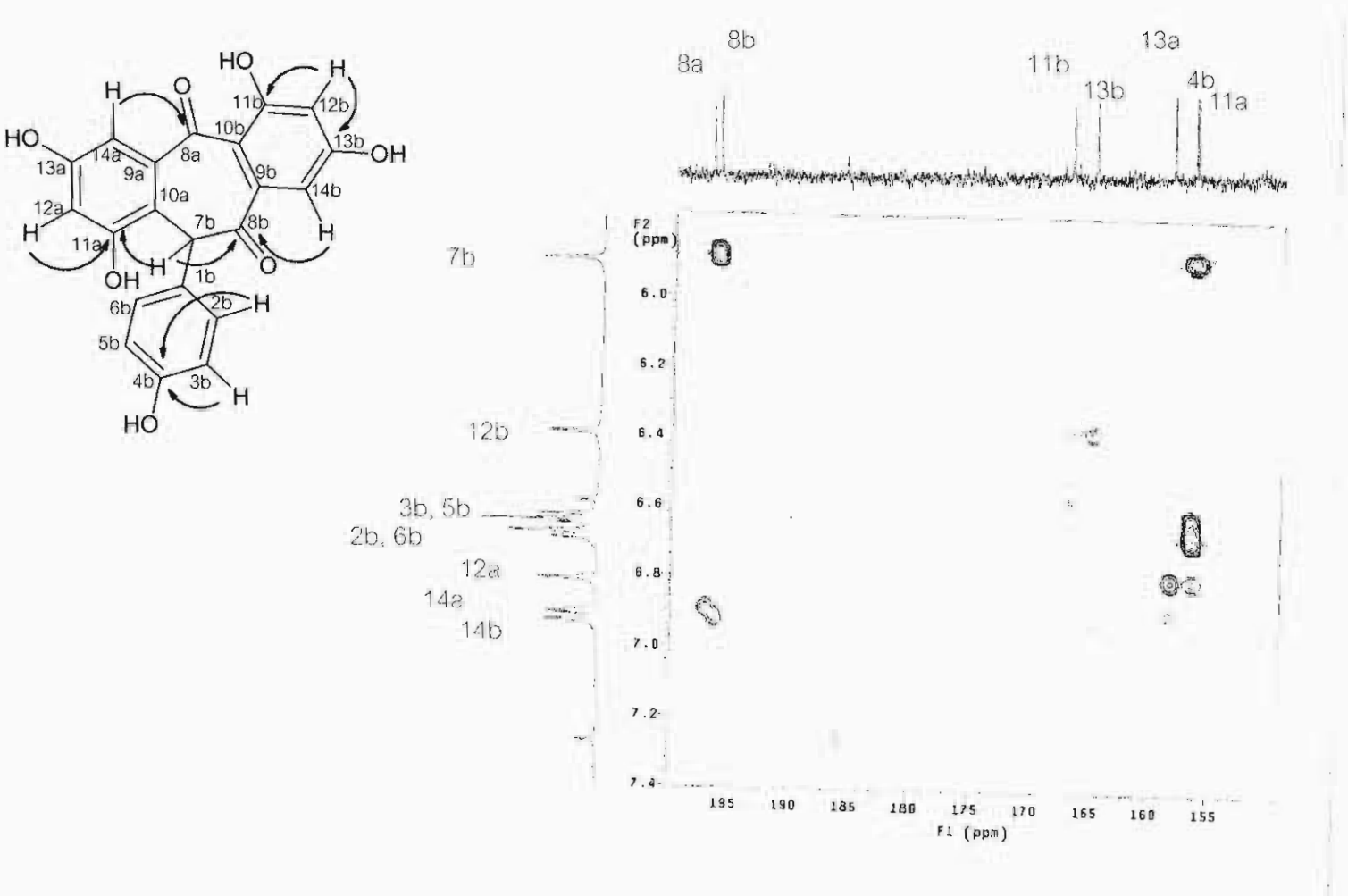


Figure 31b. HMBC spectrum of compound DC-7 (in acetone-d_6) (expanded)

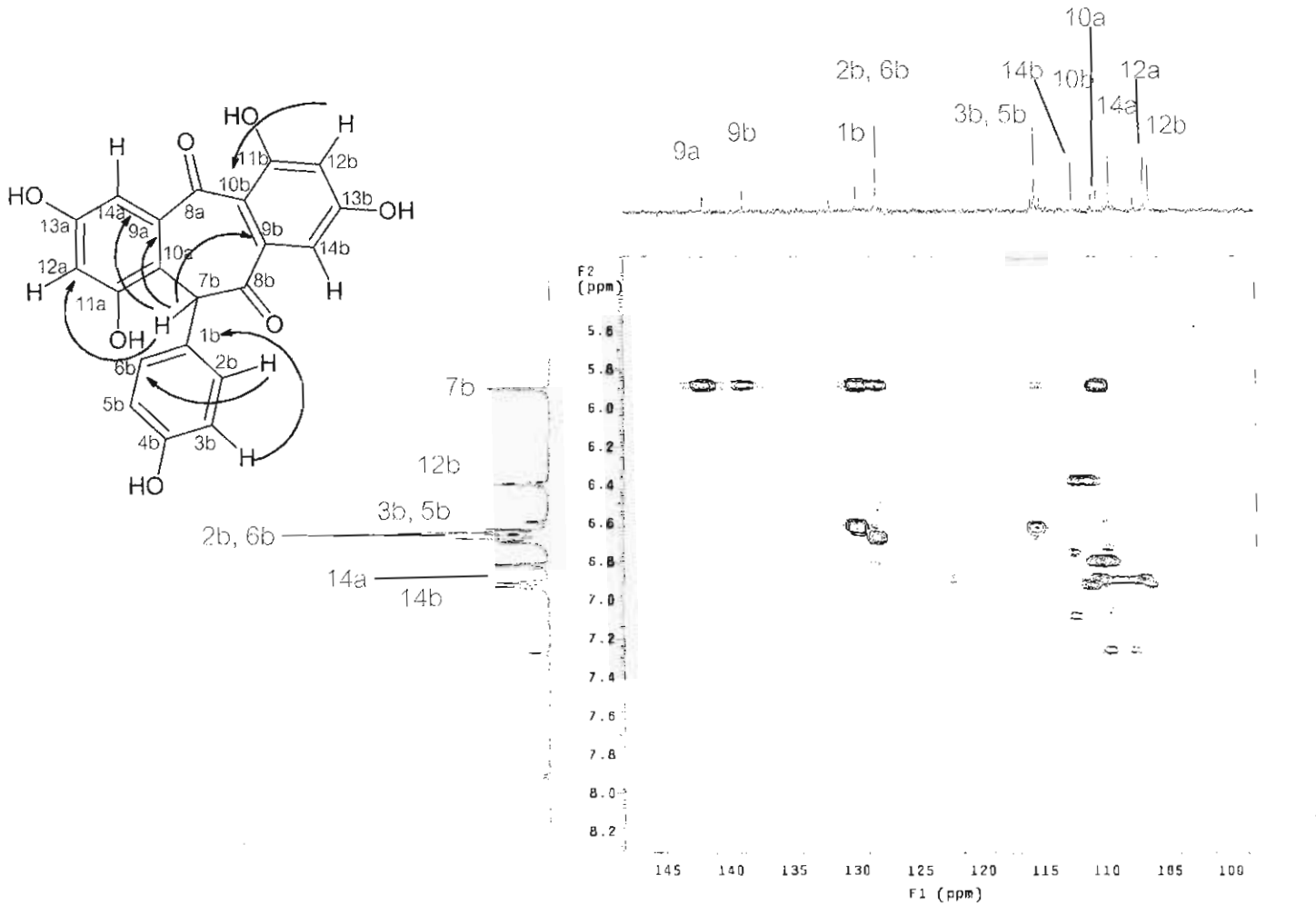


Figure 31c. HMBC spectrum of compound DC-7 (in acetone-d_6) (expanded)

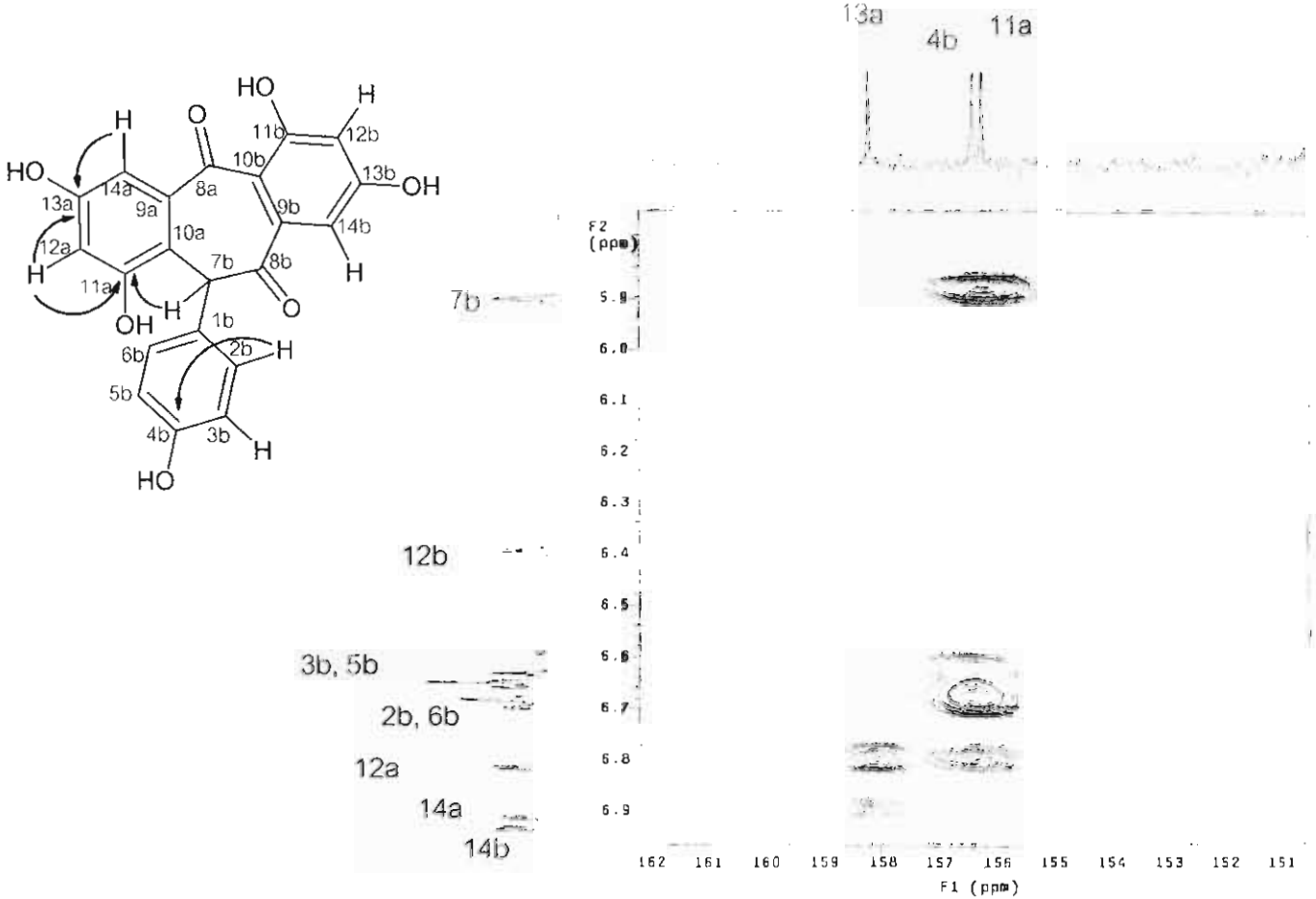


Figure 31d. HMBC spectrum of compound DC-7 (in acetone-d_6) (expanded)

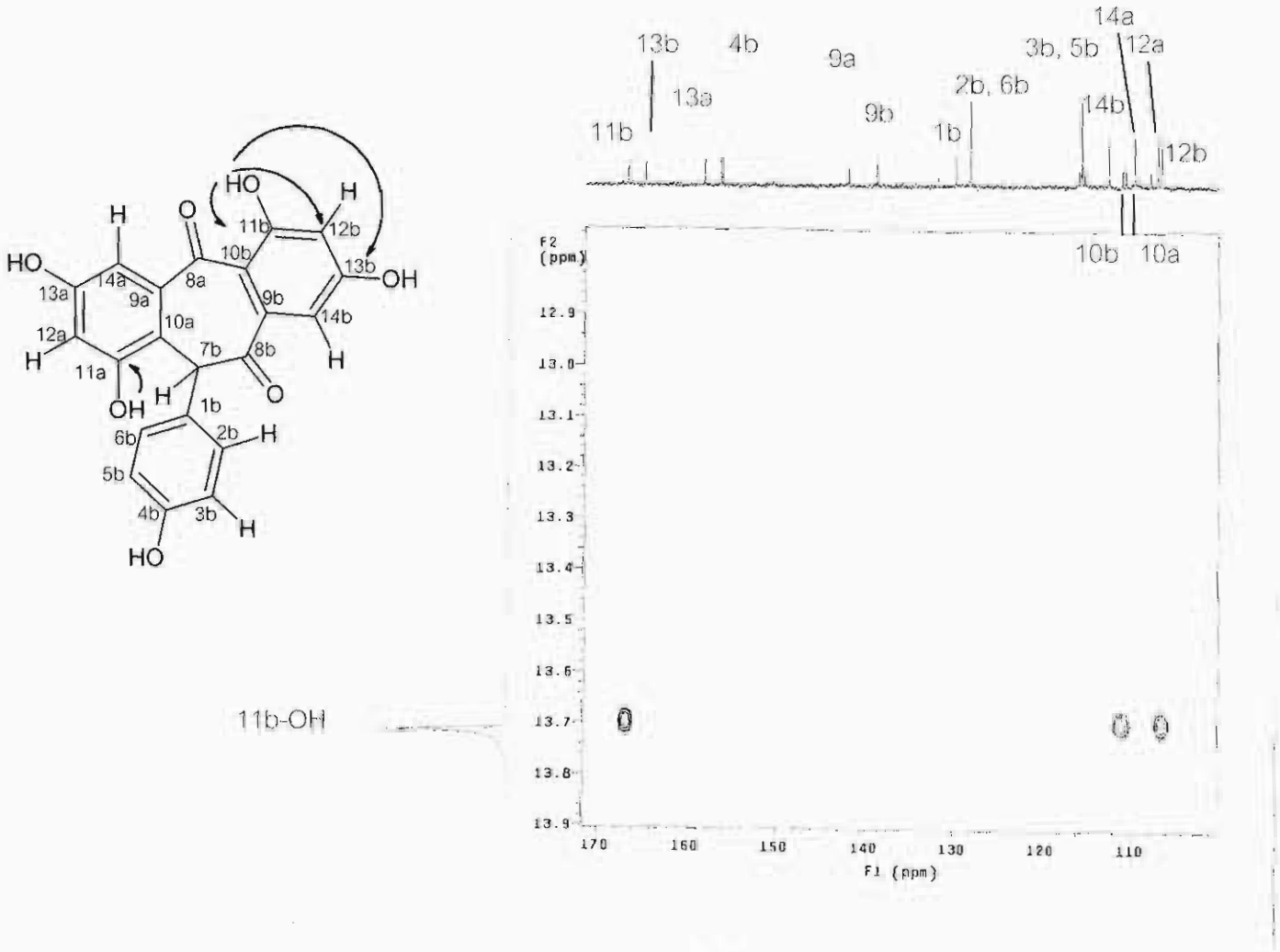


Figure 31e. HMBC spectrum of compound DC-7 (in acetone-d_6) (expanded)

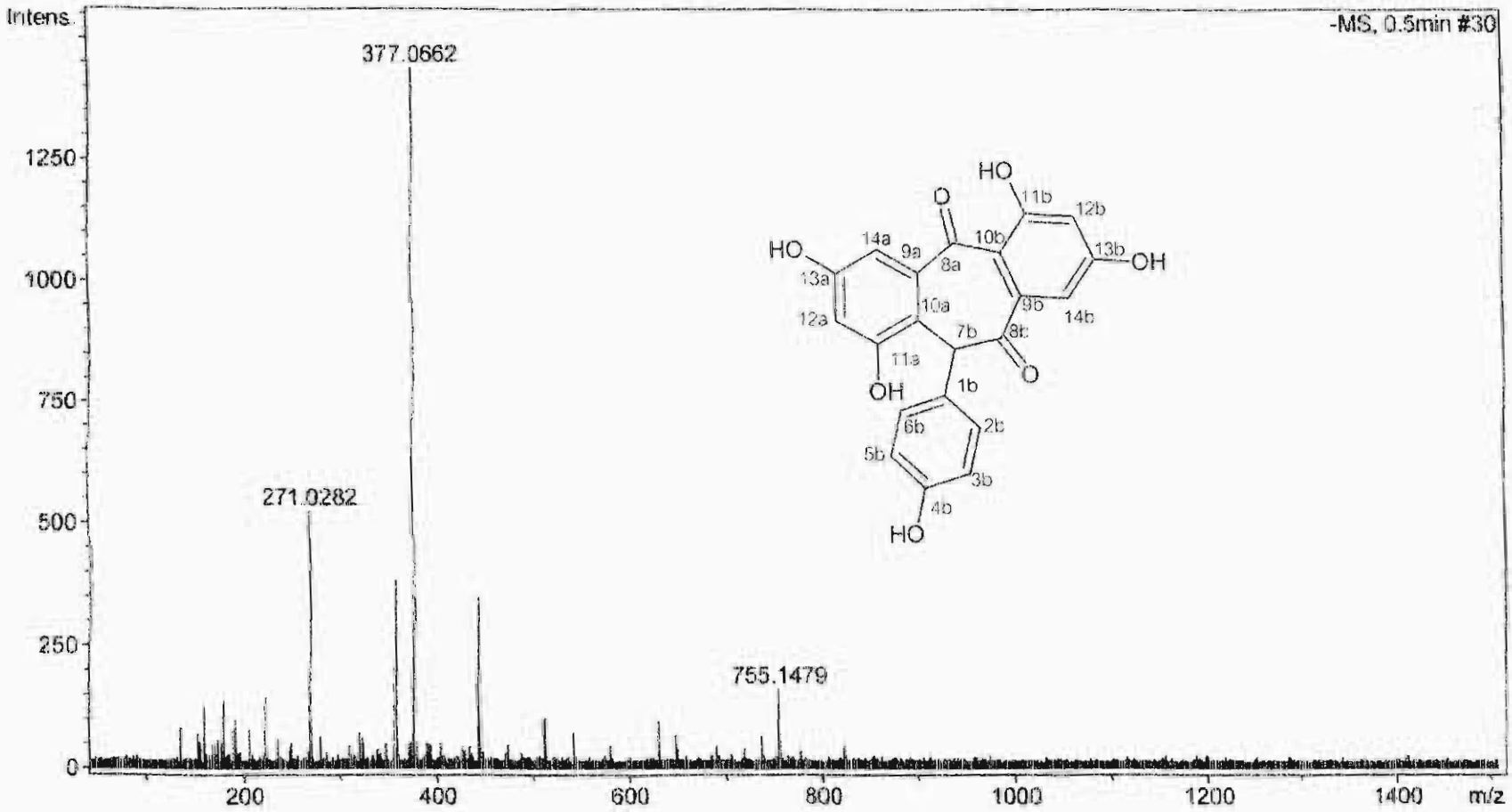


Figure 32. HRESI Mass spectrum of compound DC-7

VITA

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Poster presentation

Bumroong, B. and Thanakijcharoenpath, W. Triterpenoids from *Diospyros collinsae*. Presented at the 28th Annual Research Conference in Pharmaceutical Sciences. January 20, 2012, Bangkok, Thailand.