CHAPTER IV

RESULTS AND DISCUSSION

In this phytochemical investigation, the constituents of the leaves of *Diospyros undulata* var. *cratericalyx* (Craib) Bakh. was extensively studied. Purification of the hexane extract of the leaves by column chromatography afforded six compounds, whereas another compound was isolated from the chloroform extract. The identification and structure elucidation of these compounds were based on the interpretation of their spectral data, and further confirmed by comparison with those described in the literature. They were proposed to be either naphthoquinones or triterpenoids, both of which are abundantly found in this plant family (Ebenaceae).

Identification and Structure Elucidation

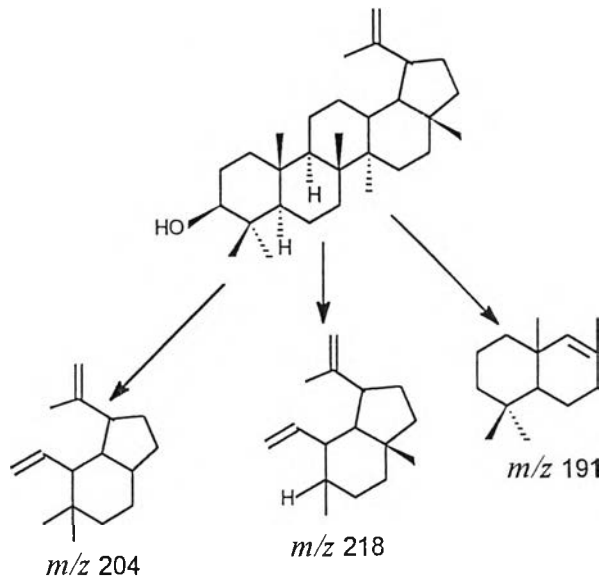
1. Identification of Compound DU1

Compound DU1 was recrystallized as white powder from methanol and gave violet color to vanillin-sulphuric acid reagent, suggesting the presence of a triterpenoid nucleus. The IR spectrum of DU1 (Figure 3) exhibited a broad band at 3464 cm^{-1}(O-H stretching) indicating the presence of hydroxyl groups in the molecule.

The ^{1}H NMR spectrum of DU1 (Figure 4a-4b) is too complex to allow direct assignment of most methylene and methine chemical shifts in the structure. However, this experiment displayed the signals of 7 methyl protons as singlets at δ 0.73, 0.76, 0.80, 0.92, 0.94, 1.00 and 1.65 ppm. The presence of two downfield singlets of exomethylene protons could be observed at δ 4.50 and 4.70 ppm.

The ^{13}C-NMR spectrum (Figure 5a, 5b) showed the signals of 30 carbon atoms, supporting the assignment of DU1 as a triterpenoid derivative. DEPT-90 and DEPT-135 (Figure 6a, 6b) experiments helped in identifying the signals of 7 methyl carbons at δ 14.7,15.5, 16.1, 16.2, 18.1, 19.4, and 28.1 ppm, 11 methylene carbons at δ 18.4, 21.0, 25.2, 27.5, 27.5, 29.9, 34.4, 35.7, 38.8, 40.1, and 109.2 ppm, 6 methine carbons at δ 38.1, 48.0, 48.3, 50.5, 55.3, and 78.9 ppm, and 6 quaternary carbons at δ 37.2, 38.9, 40.9, 42.8, 43.1 and 150.7 ppm.

The EIMS of DU1 (Figure 2) showed the molecular ion [M^+] peak at *m/z* 426 ($C_{30}H_{50}O$). Intense EIMS fragment peaks at *m/z* 189, 191, and 218 were important in showing compound DU1 as having a skeleton structure of the lupane-type triterpenoid (Ogunkoya, 1981).



Scheme 2 Mass Spectral Fragmentation of compound DU1

Therefore, the identity of DU1 was deduced as lupeol (**61**), which is a lupane-type triterpenoid commonly found in several *Diospyros* species, mainly by comparison of its carbon chemical shifts with those reported in the literature (Sholichin *et al.*, 1980).

The 7 methyl carbon signals at 28.1, 15.5, 16.3, 16.1, 14.7, 18.1 and 6.0 ppm could then be assigned as those of C-23, C-24, C-25, C-26, C-27, C-28, and C-29, respectively, whereas the 11 methylene carbon signals at δ 38.8, 27.6, 18.5, 34.4, 21.0, 25.2, 27.5, 35.6, 29.9 40.0, and 109.2 ppm were assigned as those of C-1, C-2, C-6, C-7, C-11, C-12, C-16, C-21, C-22 and C-30, respectively. Similarly, the 6 methine carbon signals at δ 79.0, 55.3, 50.5, 38.1, 48.1 and 48.4 ppm were assignable as those of C-3, C-5, C-9, C-13, C-18 and C-19, respectively, and the 6 quaternary carbon signals at δ38.9, 40.9, 37.3, 42.9, 43.1 and 150.8 ppm could then be assigned as those of C-4, C-8, C-10, C-14, C-17 and C-20, respectively.

Therefore, the methyl signals in the ^{1}H-NMR spectrum (Figure 4a-4b) could be assigned as follows : the signals at δ 0.94 (s) , 0.73 (s), 0.80 (s), 1.00 (s), 0.92 (s), 0.76 (s) and 1.65 (s) ppm could be assigned to H_3-23, H_3-24, H_3-25, H_3-26, H_3-27,

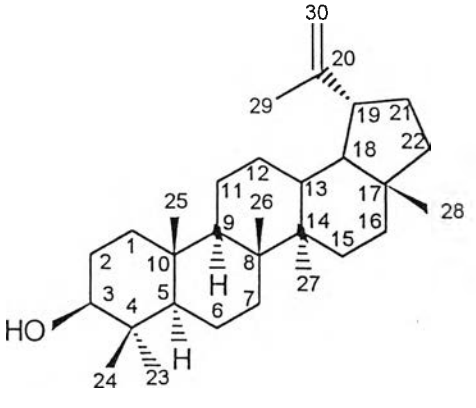
H_3-28 and H_2-29, respectively, whereas the two exomethylene singlets at δ 4.50 and 4.70 ppm were assigned as those of H_2-30.

The complete carbon chemical shift assignments of DU1 was found to be fully in agreement with those of previously reported lupeol (Table 10).

Table 10 Comparison of ^{13}C-NMR chemical shifts in $CDCl_3$ of DU1 and lupeol (Sholichin *et al*, 1980)

position	DU1 (ppm)	lupeol (ppm)	position	DU1 (ppm)	lupeol (ppm)
1	38.8	38.7	16	35.7	35.6
2	27.5	27.5	17	43.1	43.0
3	78.9	79.0	18	48.0	48.0
4	38.9	38.9	19	48.3	48.3
5	55.3	55.3	20	150.7	150.9
6	18.4	18.3	21	29.9	29.9
7	34.4	34.3	22	40.1	40.0
8	40.9	40.9	23	28.1	28.0
9	50.5	50.5	24	15.5	15.3
10	37.2	37.2	25	16.2	16.1
11	21.0	21.0	26	16.1	16.0
12	25.2	25.2	27	14.7	14.6
13	38.1	38.1	28	18.1	18.0
14	42.9	42.9	29	19.4	19.3
15	27.5	27.5	30	109.2	109.3

Therefore, it was concluded that compound DU1 is the triterpenoid lupeol, the structure of which is shown below.



Lupeol (DU1)

Lupeol was previously isolated from several species of ebenaceous plants, i.e. *Diospyros greeniwayi* (Khan and Rwekika, 1998), *D. cornii* (Khan, Nkunya and Wevers, 1973) and *Diospyros maritima* (Kuo *et al.*, 1997). Previous pharmaceutical studies of lupeol revealed antifungal activity and germination inhibitory activity (Higa *et al.*, 1998). The large quantity of lupeol found in the leaves of *D. undulata* var. *cratericalyx* might prove to be useful in the development of medicinal agents.

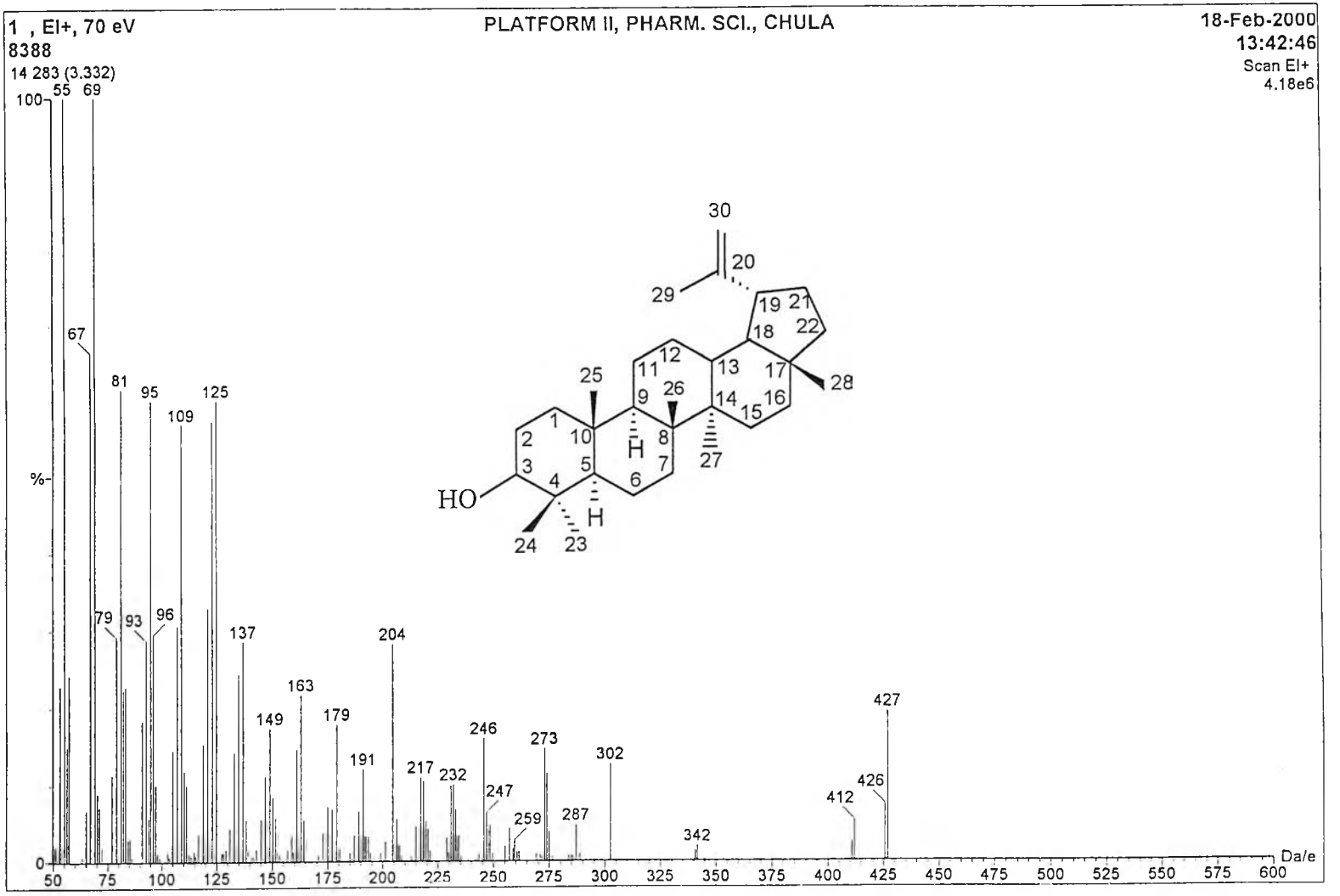


Figure 2 EI mass spectrum of compound **DU1**

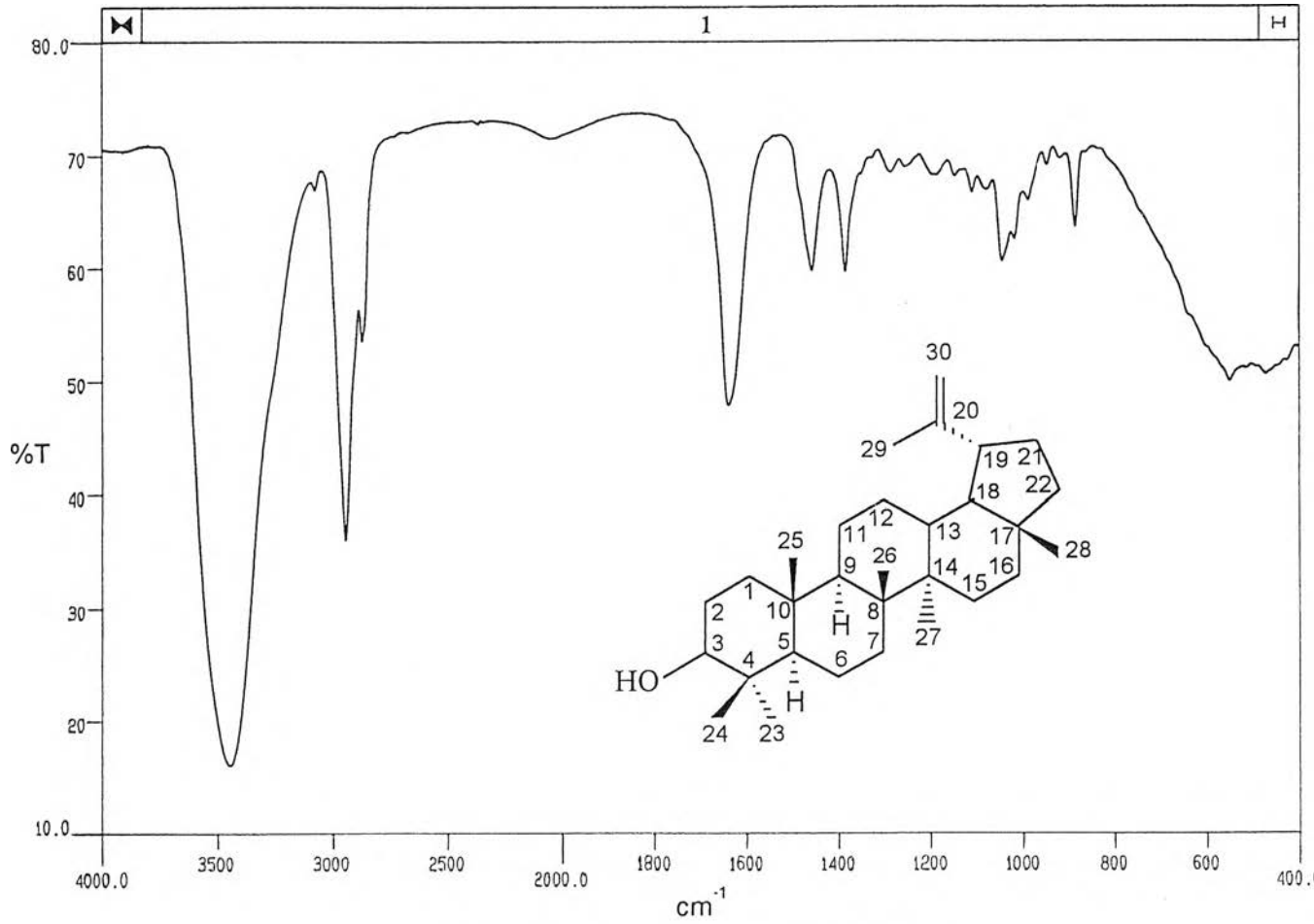


Figure 3 IR spectrum of compound **DU1**

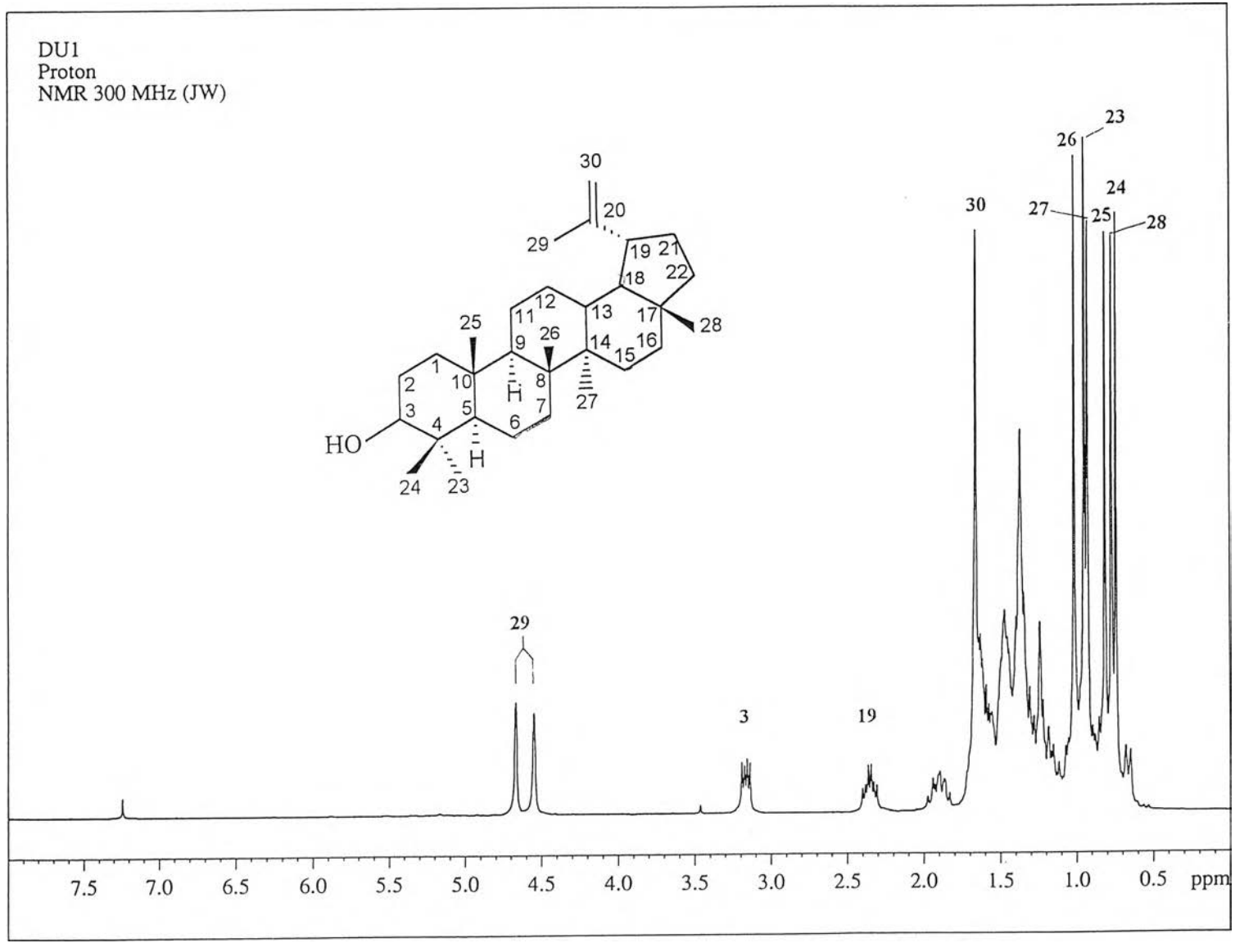


Figure 4a The 300 MHz ^{1}H NMR spectrum of compound DU1

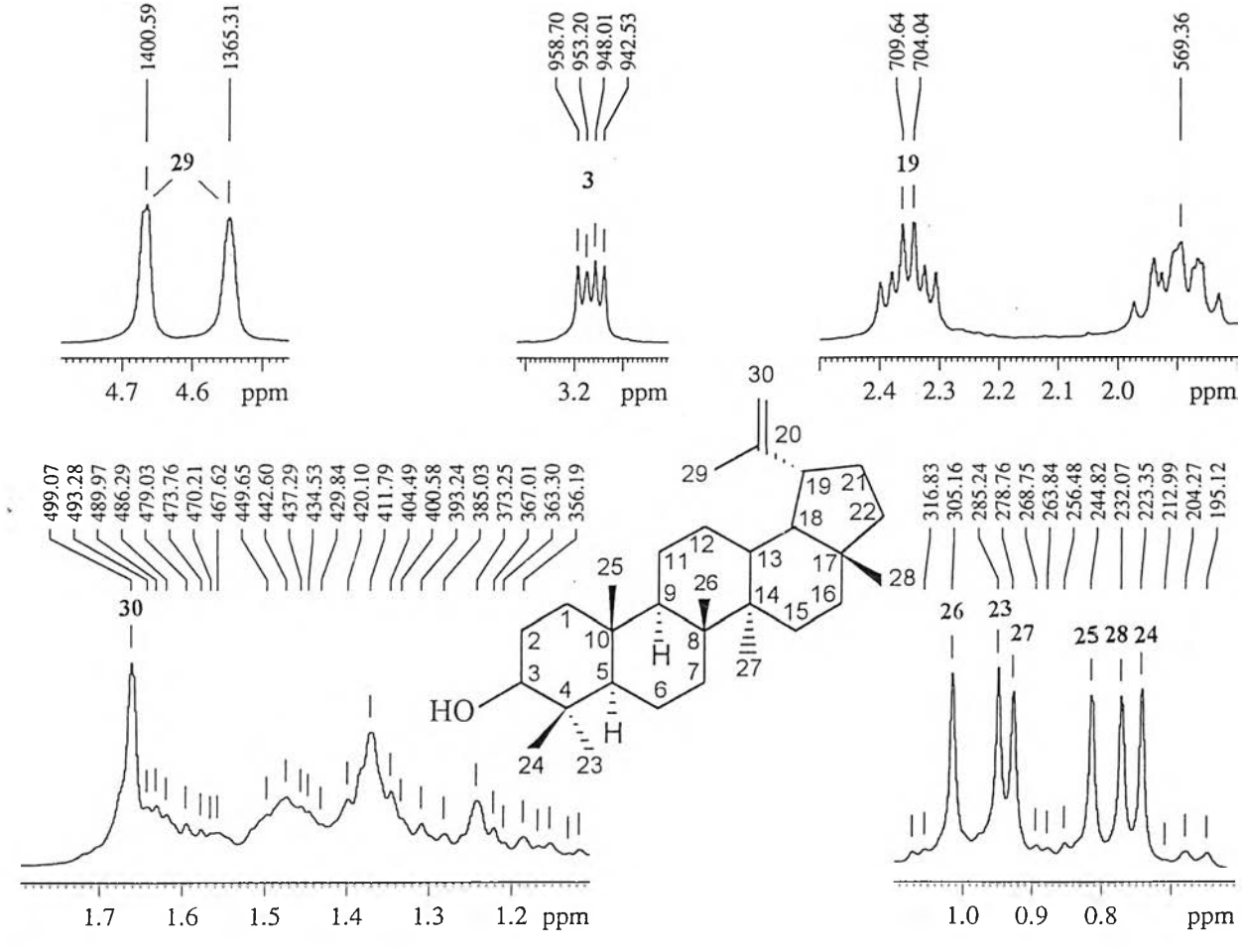


Figure 4b The 300 MHz ^{1}H NMR spectrum of compound DU1 (expanded from d 0.60–4.78 ppm)

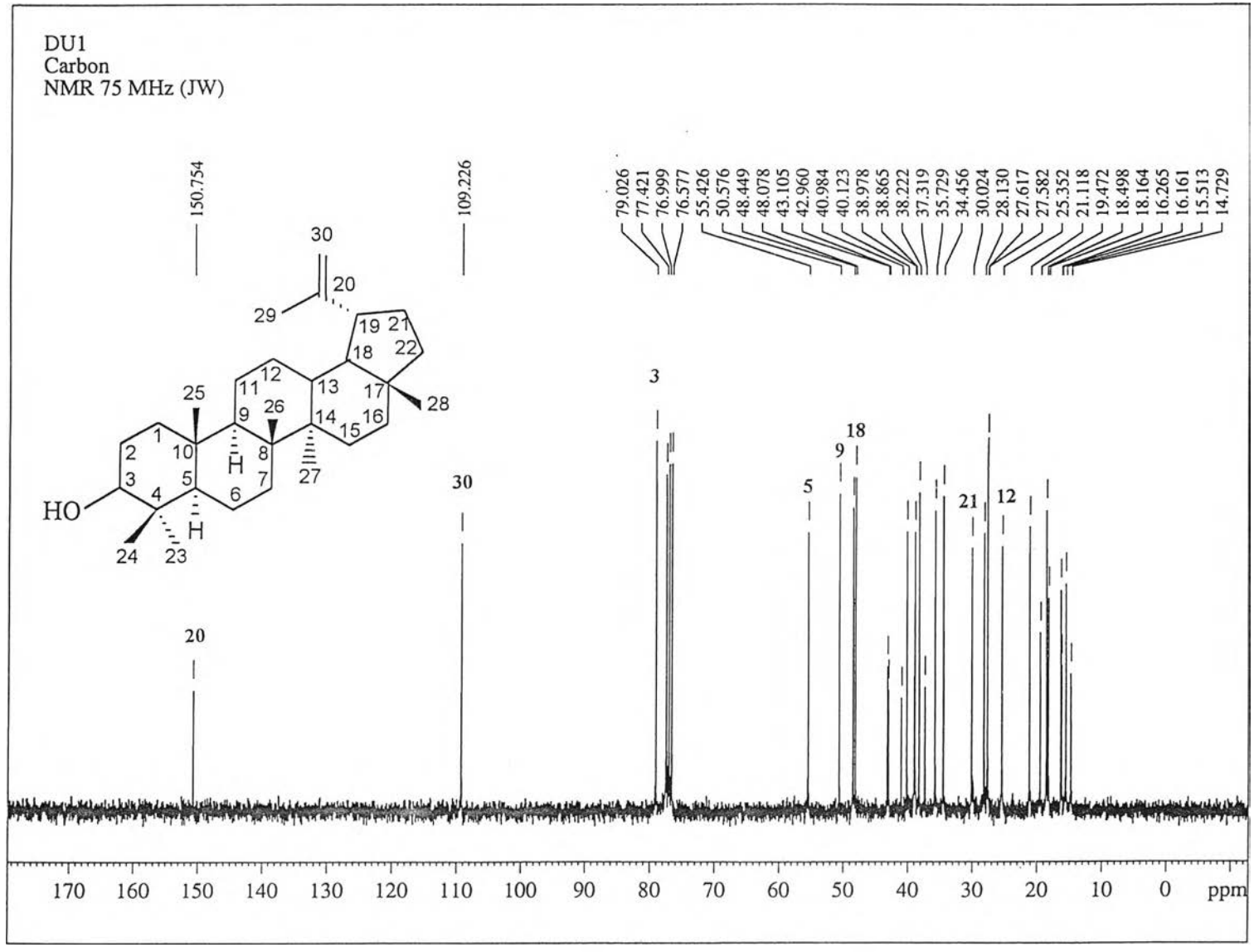


Figure 5a The 75 MHz ^{13}C NMR spectrum of compound DU1

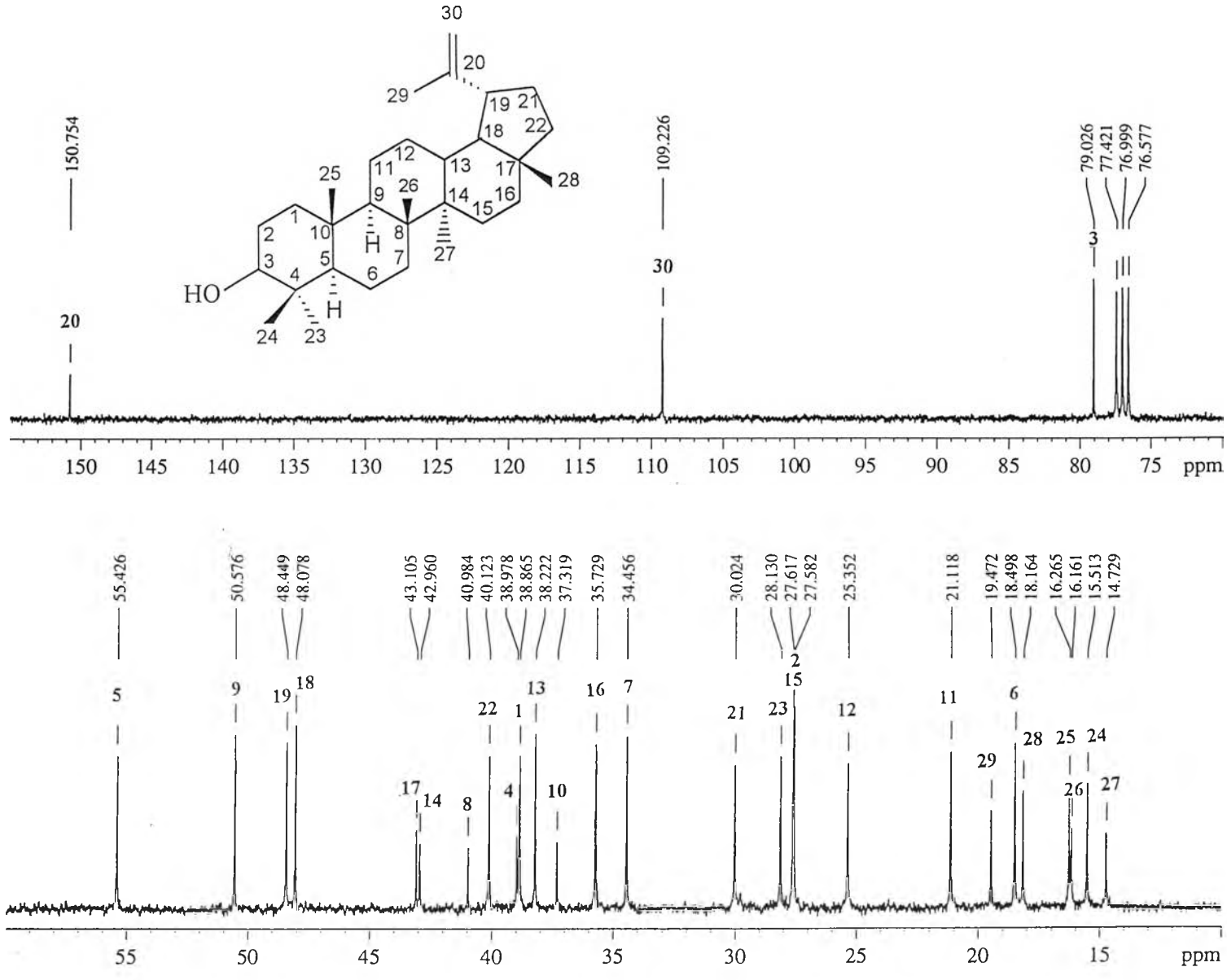


Figure 5b The 75 MHz ^{13}C NMR spectrum of compound DU1 (expanded from d 155.0–10.0 ppm)

DU1
DEPT-90
(JW)

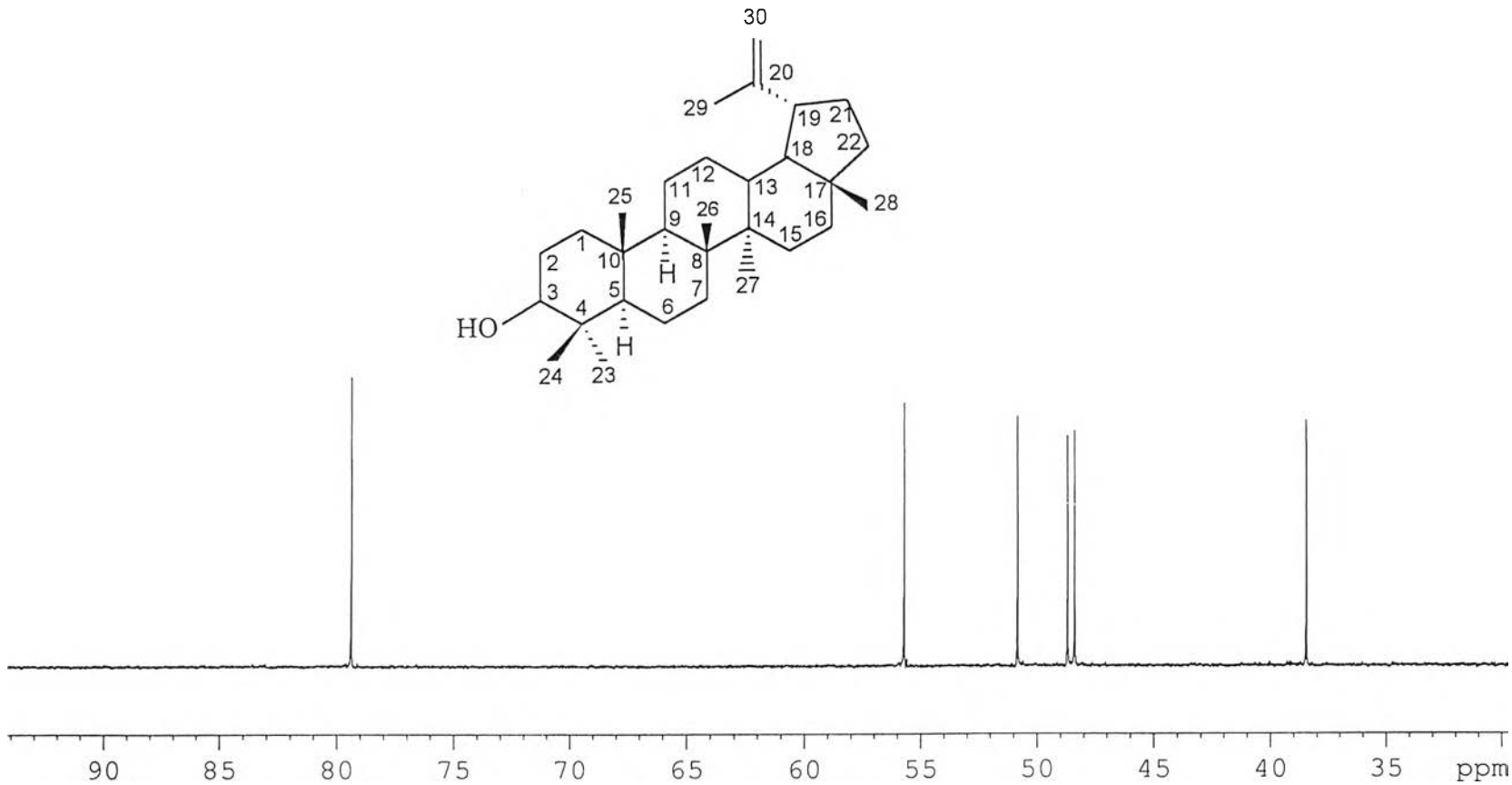


Figure 6a The DEPT-90 spectrum of compound DU1

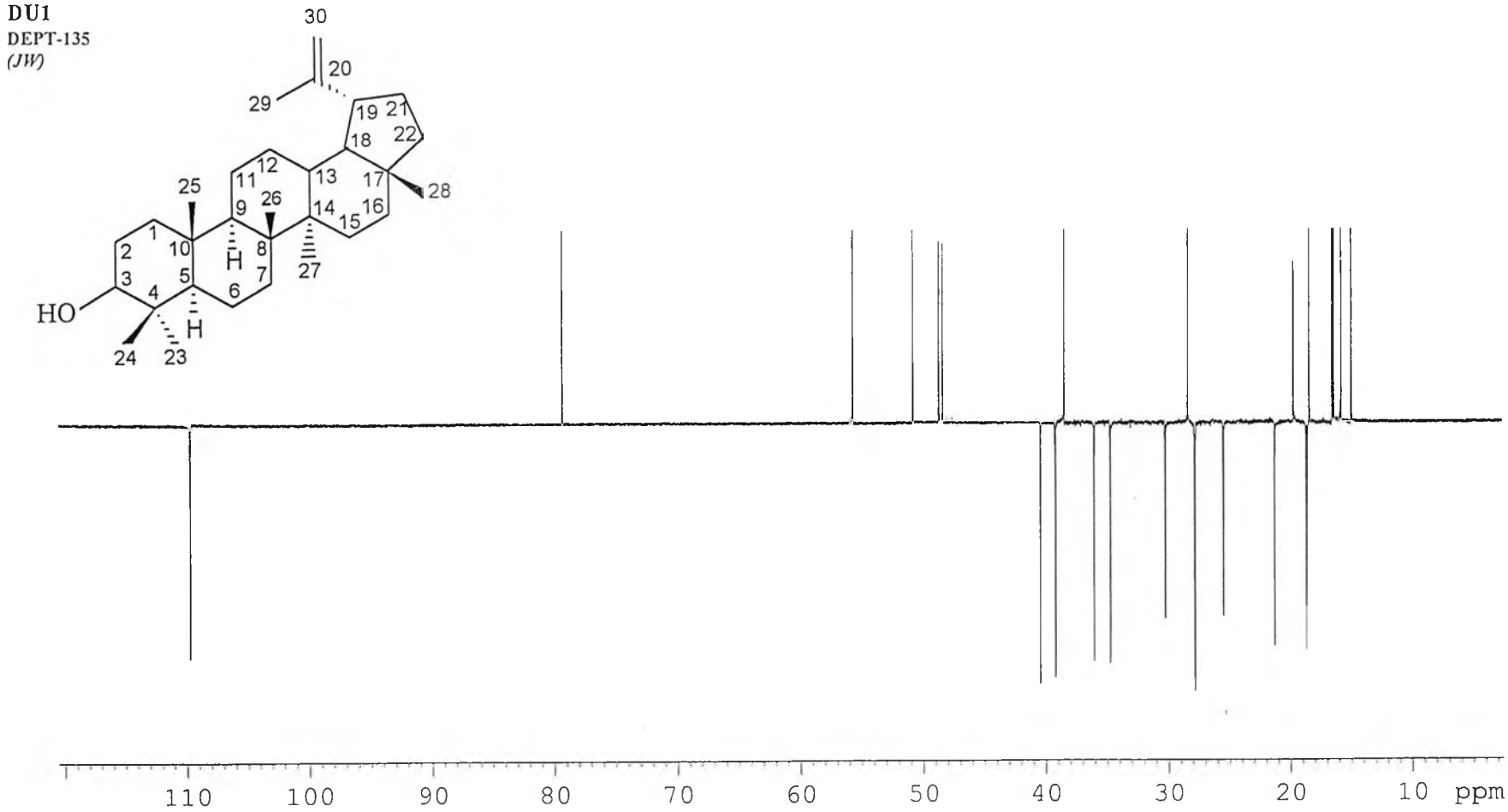


Figure6b The DEPT-135 spectrum of compound DU1

2. Identification of Compound DU2

Compound DU2 was crystallized as colorless needles from acetone-chloroform (2 : 1) and gave a yellow-brown color to vanillin-sulphuric acid reagent, suggesting the presence of a triterpenoid nucleus. Its IR spectrum (Figure 8) showed absorption bands at 2933 and 2869 cm^{-1}(C–H stretching), 1715 and 1632 cm^{-1}(C–H stretching), 1457 and 1390 cm^{-1}(C–H bending)

The EIMS of DU2 (Figure 7) displayed a prominent molecular ion peak at *m/z* 426 ($C_{30}H_{50}O$). Intense EIMS fragment peaks at *m/z* 95, 123, 163, 205, 246, 273 and 426 were important in showing compound DU2

The ^{1}H NMR (Figure 9a-9b) spectrum of DU2 displayed signals due to one secondary and seven tertiary methyls suggesting that DU2 has the friedelane skeleton (Klass *et al.*, 1992). The proton peaks were observed for H-23 (3H, s ; δ = 0.86), H-24 (3H, s ; δ = 0.70), H-25 (3H, s ; δ = 0.84), H-26 (3H, d ; δ = 1.00), H-27 (3H,s ; δ = 1.02), H-28 (3H,s ; δ = 1.15), H-29 (3H,d ; δ = 1.00) and H-30 (3H,s ; δ = 0.92)

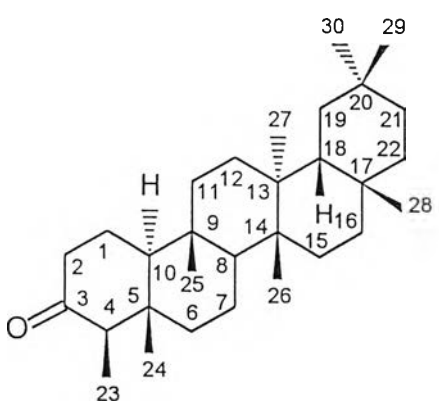
The ^{13}C NMR spectrum (Figure 10a-10b) showed the signals of 30 carbons atom, supporting the assignment of DU2 as a triterpenoid derivative. The identity of compound DU2 was then mainly proven by comparison of its carbon chemical shifts with those of a friedelane-type triterpene with 3-keto substituent, friedelin (Klass *et al.*, 1992). DEPT-45, DEPT-90 and DEPT-135 (Figure 11a, 11b) experiments indicated the signals of 8 methyl carbons at 6.8, 14.6, 18.0, 20.2, 18.6, 32.1, 31.8 and 35.0 ppm. These signals could then be assigned as those of C-23, C-24, C-25, C-26, C-27, C-28, C-29 and C-30, respectively. whereas the 11 methylene carbon signals at δ 22.3, 41.5, 41.3, 18.2, 35.6, 30.5, 32.7, 36.0, 35.3, 32.4 and 39.2 ppm were assigned as those of C-1, C-2, C-6, C-7, C-11, C-12, C-15, C-16, C-19, C-21 and C-22, respectively.

Similarly, 4 methine carbon signals at δ 58.2, 53.1, 59.4 and 42.8 ppm were assigned as those of C-4, C-8, C-10 and C-18, respectively, and the 7 quaternary carbon signals at δ 213.2, 42.1, 37.4, 35.3, 39.7, 30.0 and 28.1 ppm could then be assigned as those of C-3, C-5, C-9, C-13, C-14, C-17 and C-20, respectively. The HETCOR experiment was used to establish the correlations between each carbon signal and its directly attached proton signals the correct assignment of H-1, H-2, H-4, H-6, H-7, H-10, H-11, H-12, H-15, H-16, H-21 and H-22 respectively.

Table 11 Comparison of ^{13}C-NMR chemical shifts in $CDCl_3$ of DU2 and friedelin (Ageta *et al.*, 1995)

position	DU2	friedelin	position	DU2	friedelin
1	22.3	22.3	16	36.0	36.0
2	41.5	41.5	17	30.0	30.0
3	213.2	213.2	18	42.8	42.8
4	58.2	58.2	19	35.3	35.3
5	42.1	42.1	20	28.1	28.2
6	41.3	41.3	21	32.7	32.8
7	18.2	18.2	22	39.2	39.2
8	53.1	53.1	23	6.8	6.8
9	37.4	37.4	24	14.6	14.7
10	59.4	59.5	25	18.0	18.0
11	35.6	35.6	26	20.2	20.3
12	30.5	30.5	27	18.6	18.7
13	38.3	39.3	28	32.1	32.1
14	39.7	39.7	29	35.0	35.0
15	32.4	32.4	30	31.8	31.8

Therefore, it was concluded that DU2 is friedelin, the structure of which is shown below.



Friedelin (DU2)

Friedelin is a known triterpenoid previously isolated from several species of ebenaceous plants, i.e. *Diospyros maritima* (Higa *et al.*, 1998), *D. buxifolia* and *D. kaki* (Chandler and Hooper),1979.

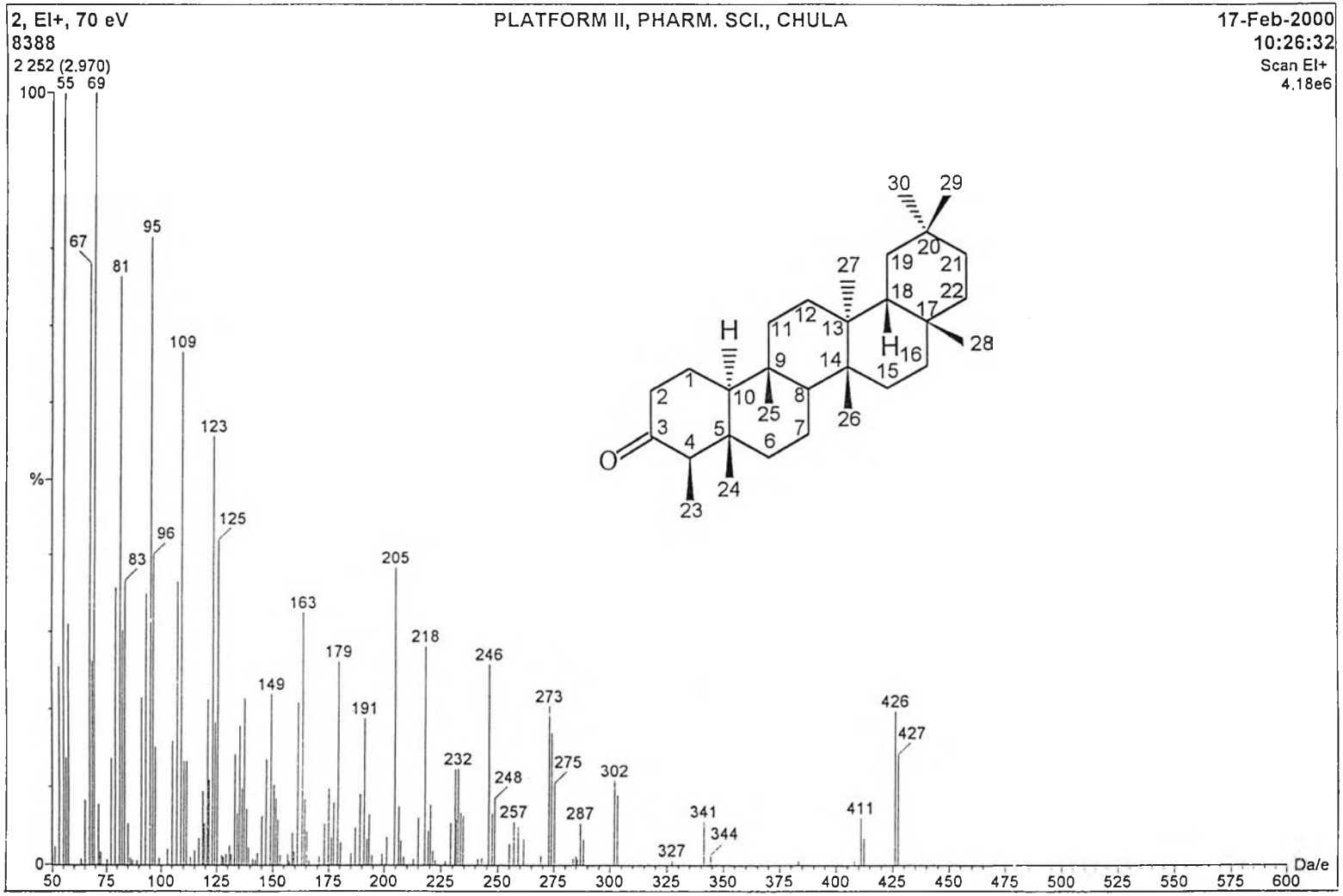


Figure 7 EI mass spectrum of compound DU2

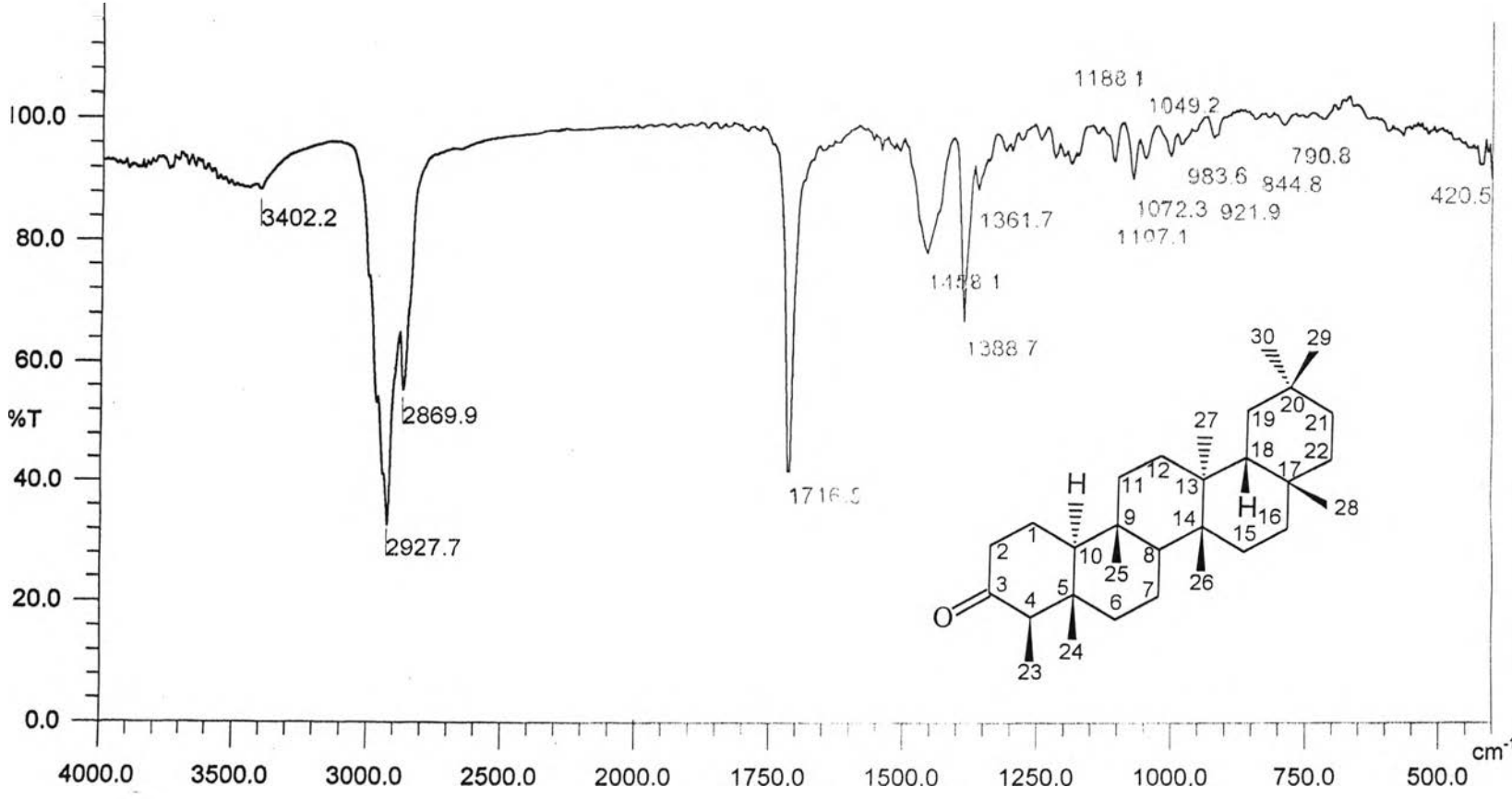


Figure 8 IR spectrum of compound DU2

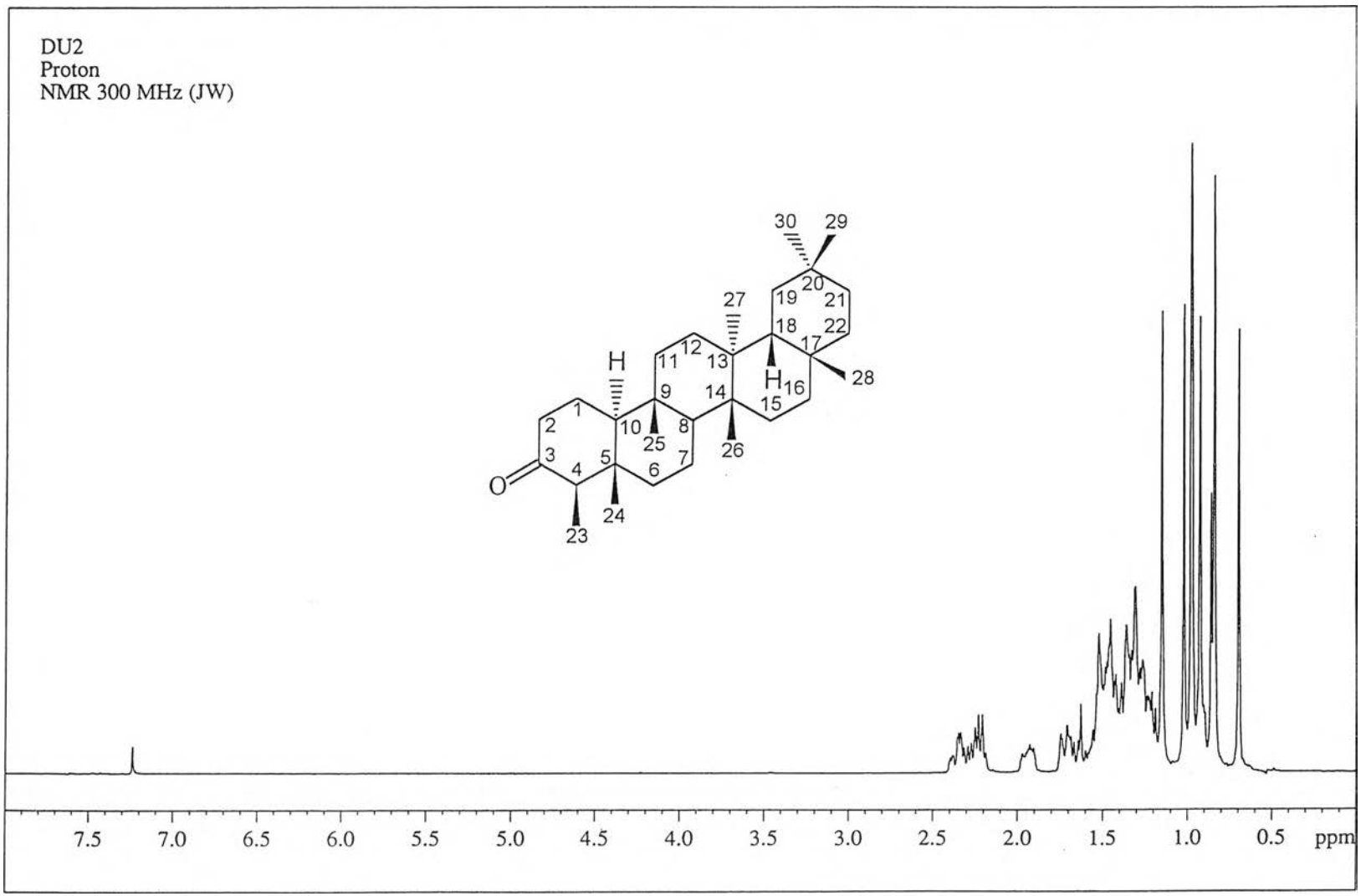


Figure 9a The 300 MHz ^{1}H NMR spectrum of compound DU2

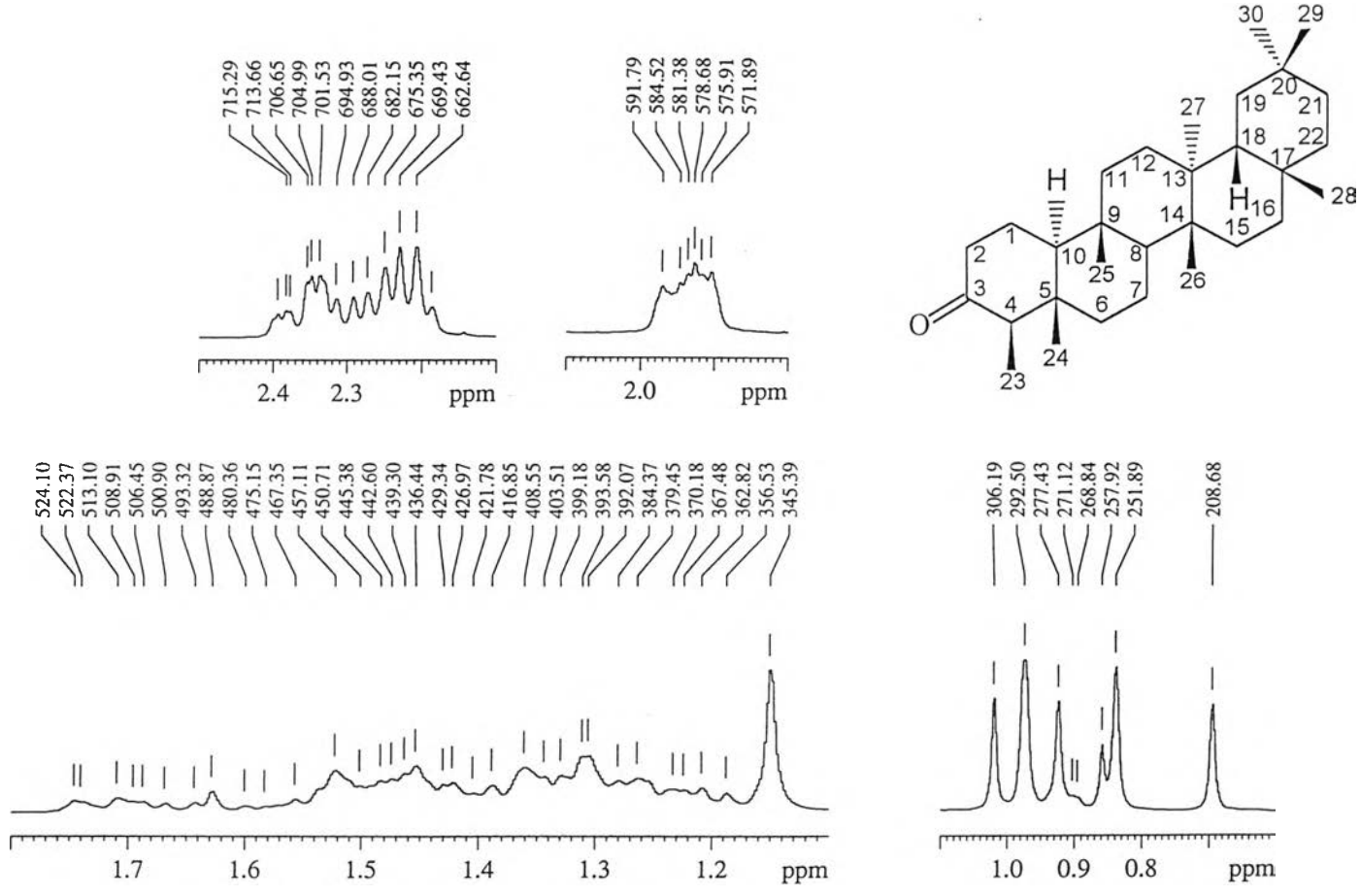


Figure 9b The 300 MHz ^{1}H NMR spectrum of compound DU2 (expanded 0.60–2.50 ppm)

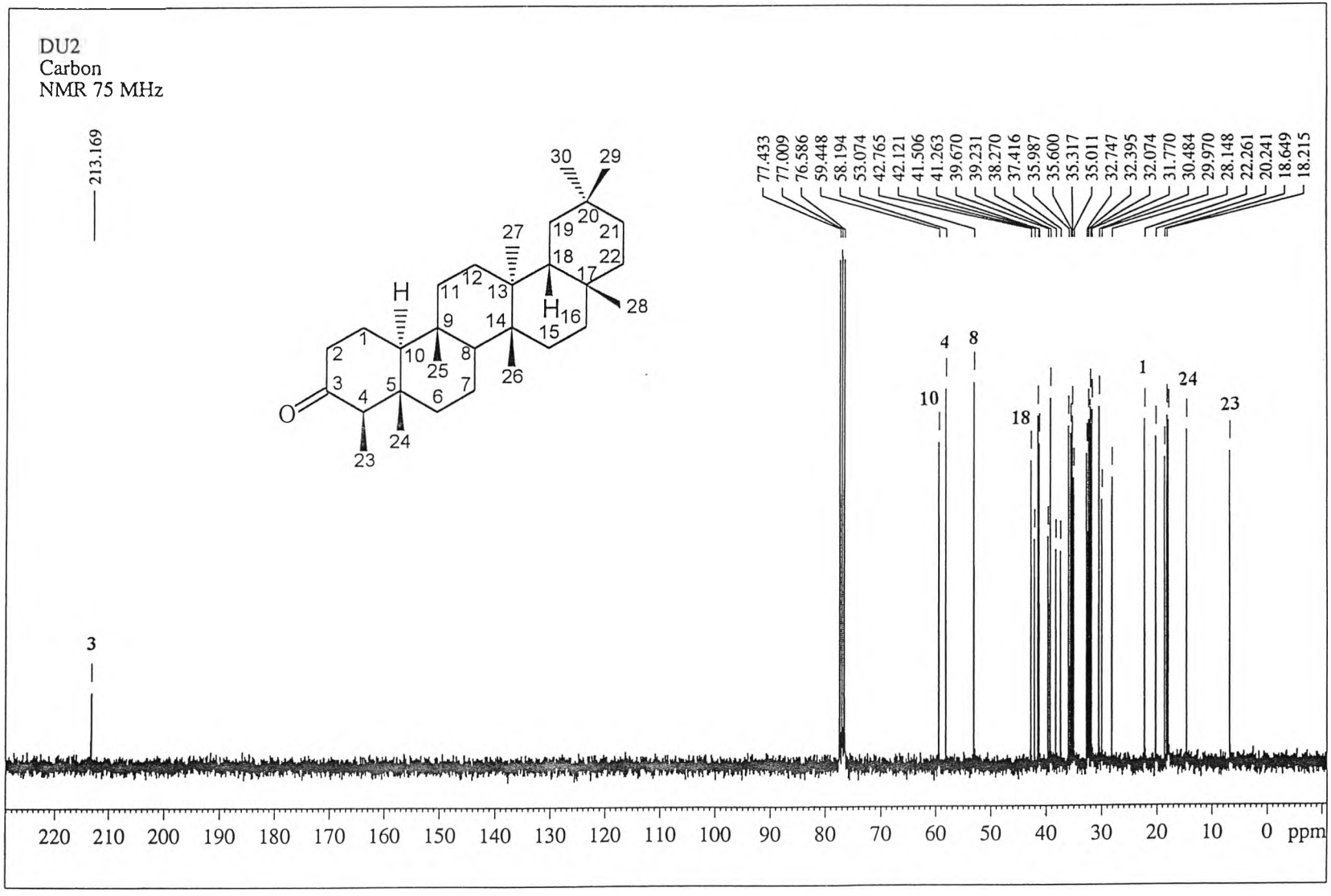


Figure 10a The 75 MHz ^{13}C NMR spectrum of compound DU2

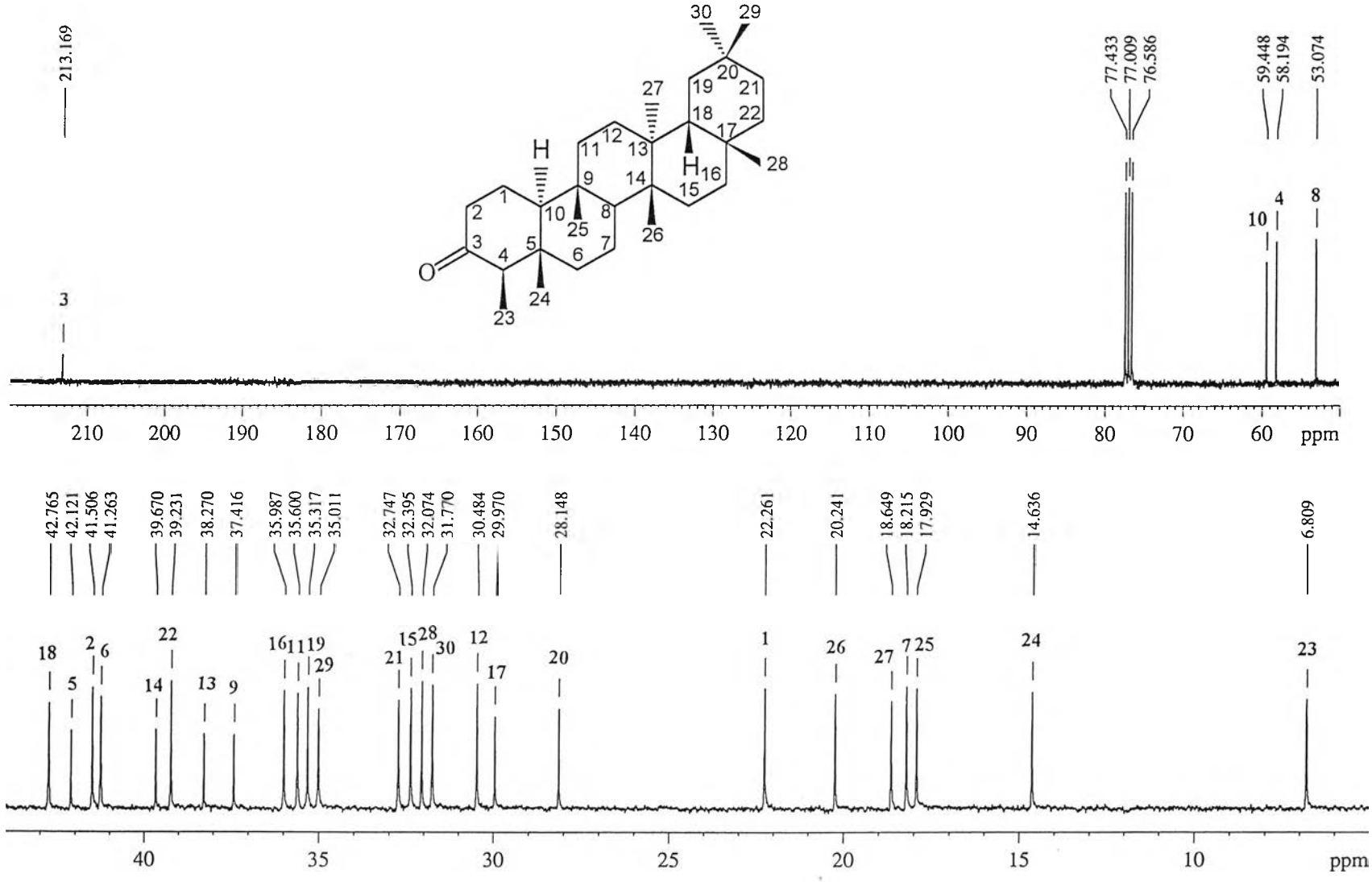


Figure 10b The 75 MHz ^{13}C NMR spectrum of compound DU2 (expanded from 220.0–5.0 ppm)

DU2
DEPT-90
(JW)

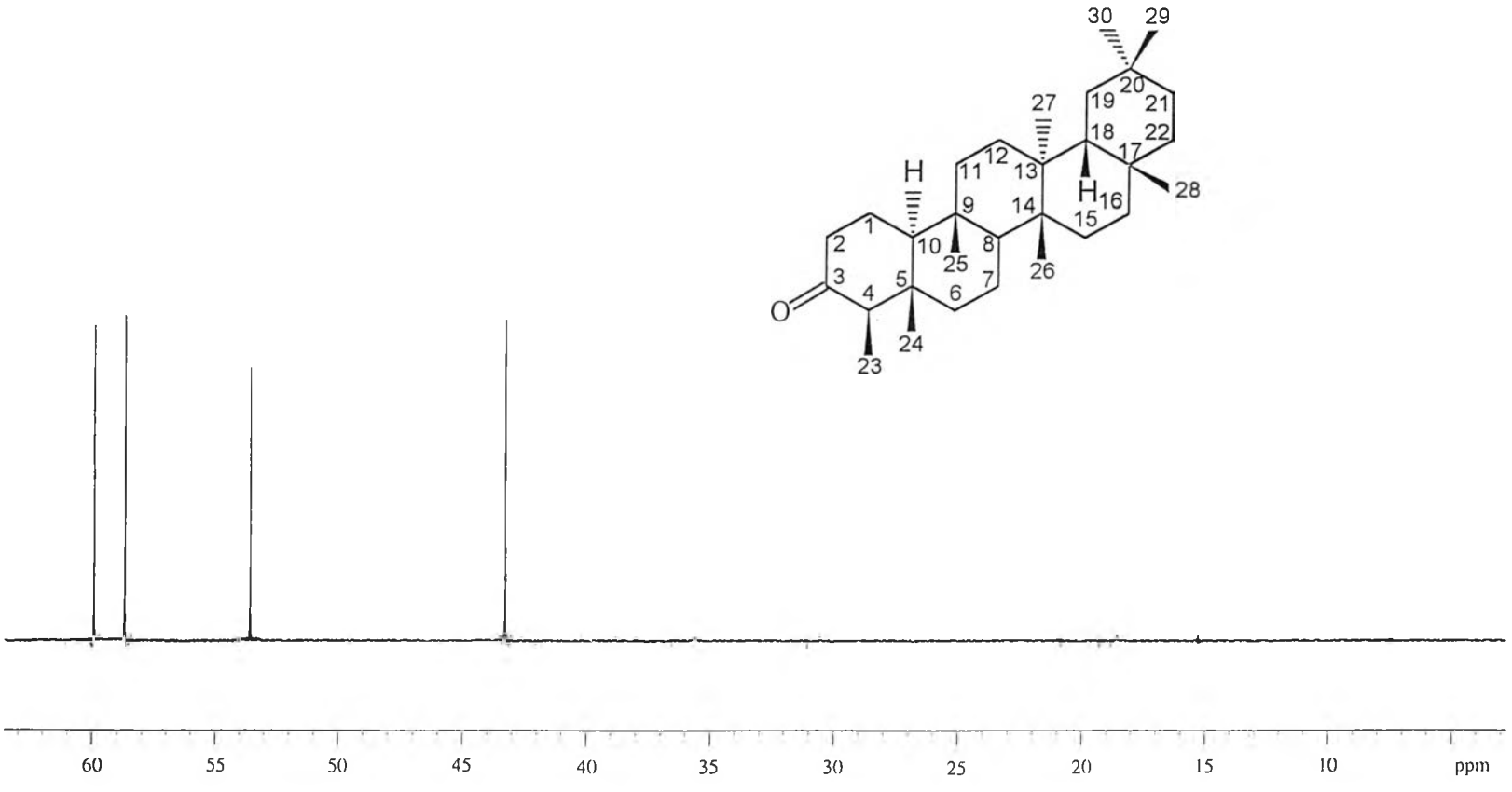


Figure 11a The DEPT-90 spectrum of compound DU2

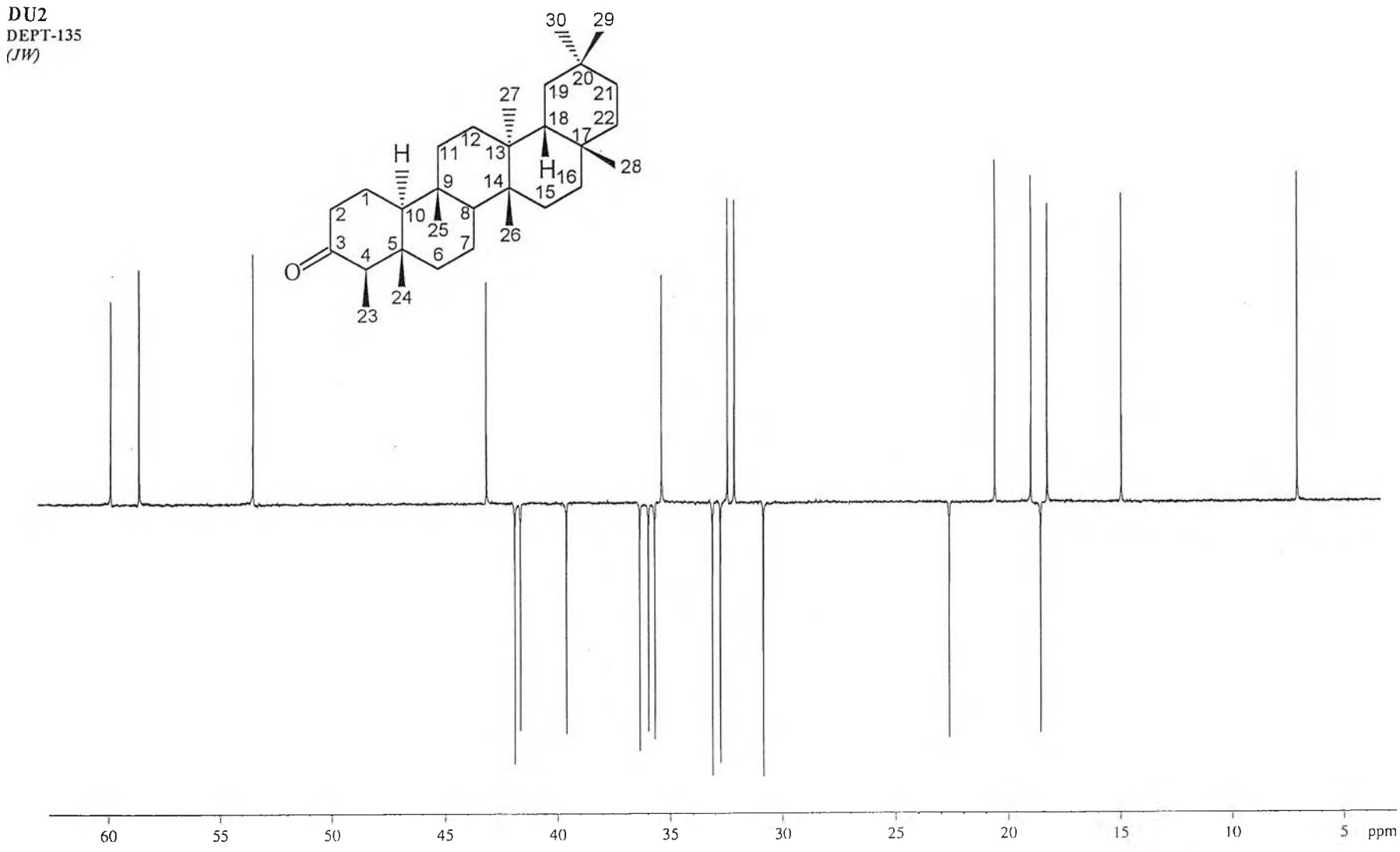


Figure 11b The DEPT-135 spectrum of compound DU2

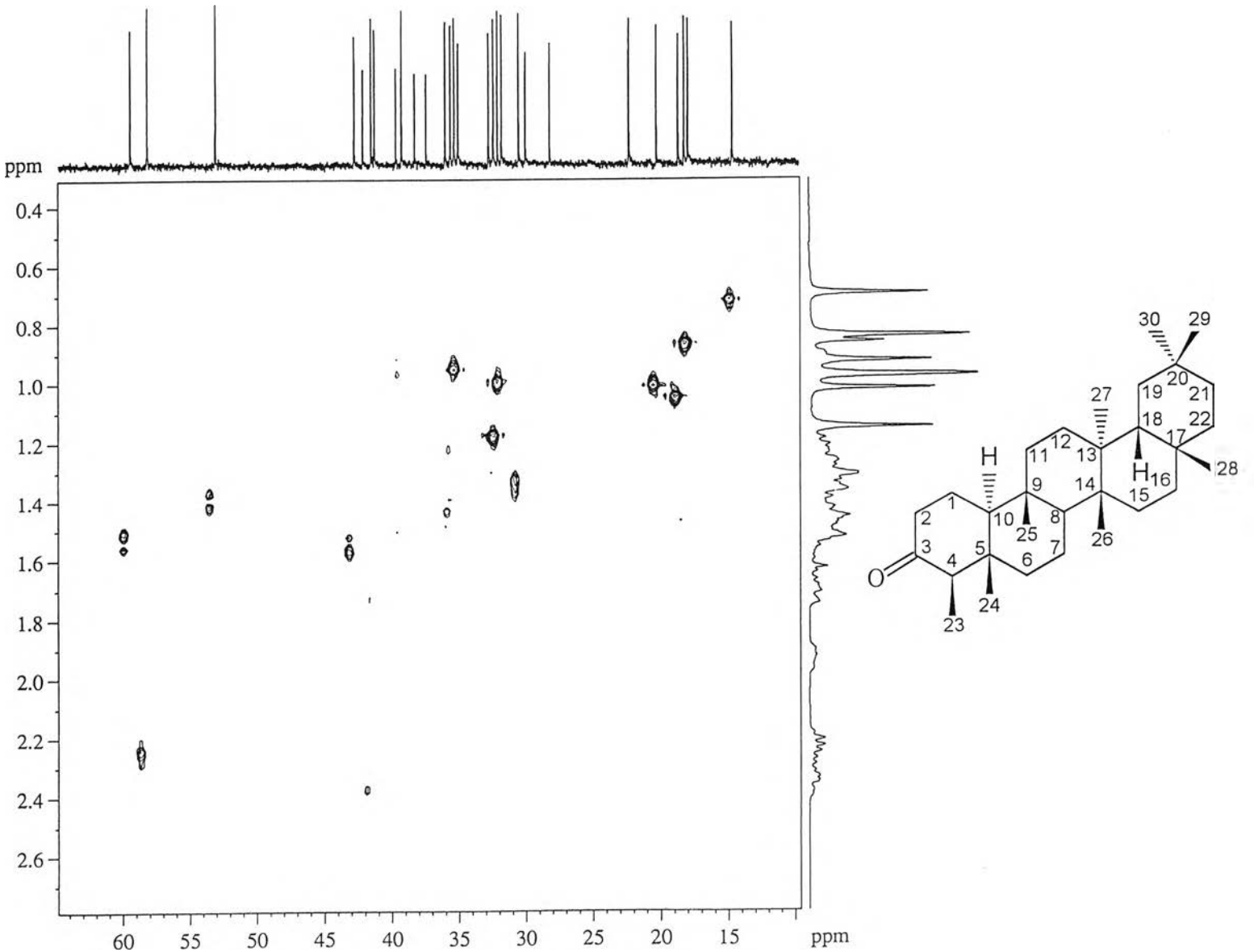


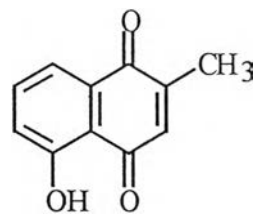
Figure 12 The HETCOR spectrum of compound DU2

3. Identification of Compound DU3

Compound DU3, crystallized as red needles from hexane, showed its molecular ion [M^+] at *m/z* 374 in the EIMS (Figure 14). However, its ^{1}H-NMR spectrum showed integration for only 7 protons, whereas the ^{13}C-NMR spectrum displayed only 11 carbon signals. Therefore, it is possible that this compound is a symmetrical dimer and a tentative molecular formula of $C_{22}H_{14}O_6$ was suggested. The UV spectrum (Figure 15) exhibited absorption maxima at λ_{max} 264 (log ε 4.42) and 436 (log ε 3.87) nm, while the IR spectrum (Figure 16) showed two peaks at 1656 and 1647 cm^{-1} corresponding to the unchelated and chelated quinone carbonyls, respectively, and the hydroxy absorption band at 3450 cm^{-1}. The existence of a hydroxy group hydrogen-bonded to a carbonyl was also indicated by the most downfield signal in the proton NMR spectrum at δ 12.57 ppm. Compound DU3 is thus proposed as a symmetrical dimer of juglone derivative.

The ^{1}H NMR spectrum of DU3 (Figure 17a-17b) also showed the presence of two *ortho*-coupled aromatic proton signals at δ 7.27 and 7.18 ppm (each 2H, d, *J*=8.7 Hz, H-6/6′ and H-7/7′, respectively), together with a quinonoid proton signal at δ 6.79 ppm (2H, d, *J*=1.4 Hz, H-3/3′) allylically coupled to a methyl signal at δ 2.00 (6H, d, *J*=1.4 Hz, CH_3-11/11′).

The ^{13}C NMR spectrum (Figure 18) and DEPT-90 and DEPT-135 spectra (Figure 19a-19b) displayed two carbonyl signals at δ 190.5 (C-4/4′) and 185.2 (C-1/1′) ppm, a signal for methyl carbon at δ 16.6 ppm (C-11/11′), three methine carbon signals at δ 137.9 (C-7/7′), 134.9 (C-3/3′), and 124.3 (C-6/6′) ppm, and five quaternary carbon signals at δ 161.3 (C-5/5′), 150.0 (C-2/2′), 135.5 (C-8/8′), 128.1 (C-9/9′) and 115.4 (C-10/10′) ppm.



Plumbagin (**109**)

These spectral data closely resembled those of plumbagin (**109**) (Tezuka *et al.*, 1973), suggesting that the compound is a symmetrical dimer of plumbagin. The *ortho*-coupling pattern of the two benzenoid proton signals implied that DU3 could be either 6,6′- or 8,8′-dimer. However, inspection of its HMBC spectrum (Figure 22a-

22c) revealed the cross-peak between the signals of H-7 (δ 7.18 ppm) and C-8′ (δ 135.5 ppm), hence establishing the linkage as 8-8′. Therefore, compound DU3 was identified as the known naphthoquinone dimer, maritinone (Tezuka, *et al.*, 1973). The ^{1}H-^{1}H COSY (Figure 21) and HETCOR (Figure 20) experiments were also used in the complete assignments of this compound (Table 12). The spectral data of DU3 were in agreement with those reported in the literature.

Table 12 ^{1}H, ^{13}C-assignments and HMBC correlations of compound DU3

position	^{1}H	^{13}C	HMBC correlated with carbon
	δ_H (ppm)	δc (ppm)	signals at δc (ppm)
1, 1′	-	185.2	-
2, 2′	-	150.0	-
3, 3′	6.79 (d, *J* = 1.4 Hz)	134.9	16.6, 115.4, 185.2
4, 4′	-	190.5	-
5, 5′	-	161.3	-
6, 6′	7.27 (d, *J*=8.7 Hz)	124.3	115.4, 135.5, 161.3
7, 7′	7.18 (d, *J*=8.7 Hz)	137.9	128.1, 135.5, 161.3
8, 8′	-	135.5	-
9, 9′	-	128.1	-
10, 10′	-	115.4	-
11, 11′	2.00 (d, *J*=1.4 Hz)	16.6	134.9, 150.0, 185.2
5-OH, 5′-OH	12.57 (s)	-	115.4, 124.3, 161.3

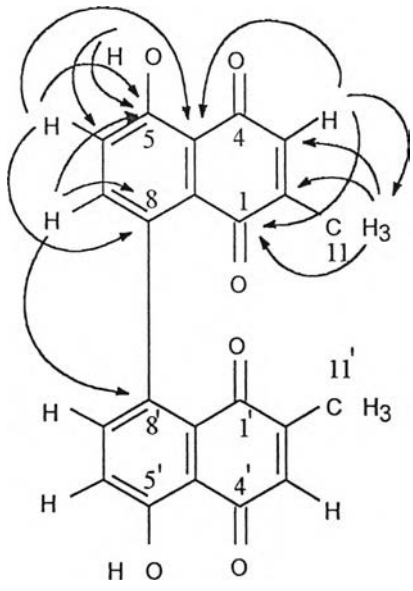


Figure 13 Selected long-range C-H correlations of compound DU3 observed in the

Maritinone is a naphthoquinone derivative previously isolated from several *Diospyros* species, i.e. *Diospyros maritima* (Tezuka *et al.*, 1973 ; Higa *et al.*, 1998) and *D. samoensis* (Richomme *et al.*, 1991) and *Diospyros maritima* (Higa *et al.*, 1998). The compound was examined for ichthyotoxicity activity, germination inhibitory activity, and antifungal activities (Higa *et al.*, 1998).

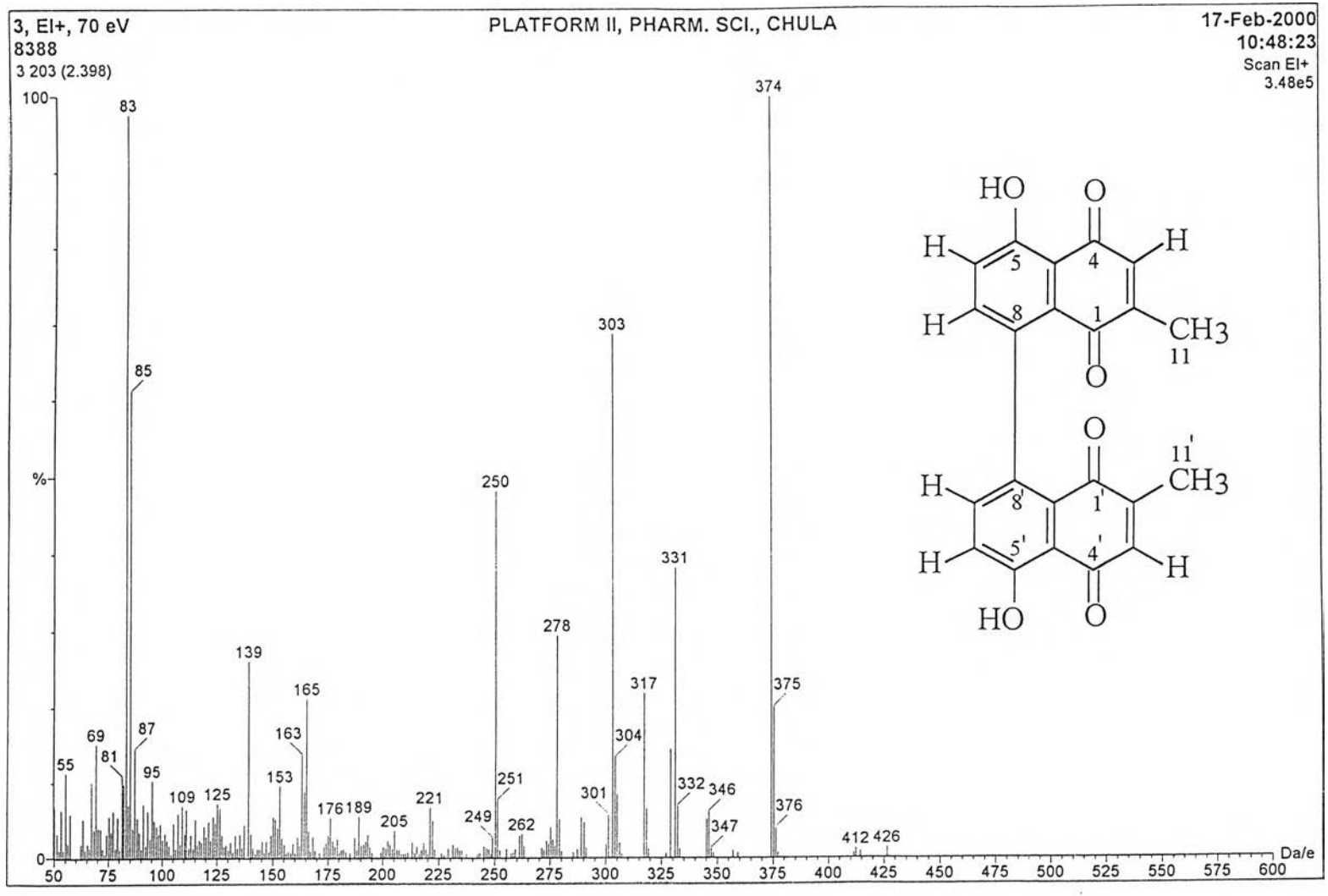


Figure 14 EI mass spectrum of compound DU3

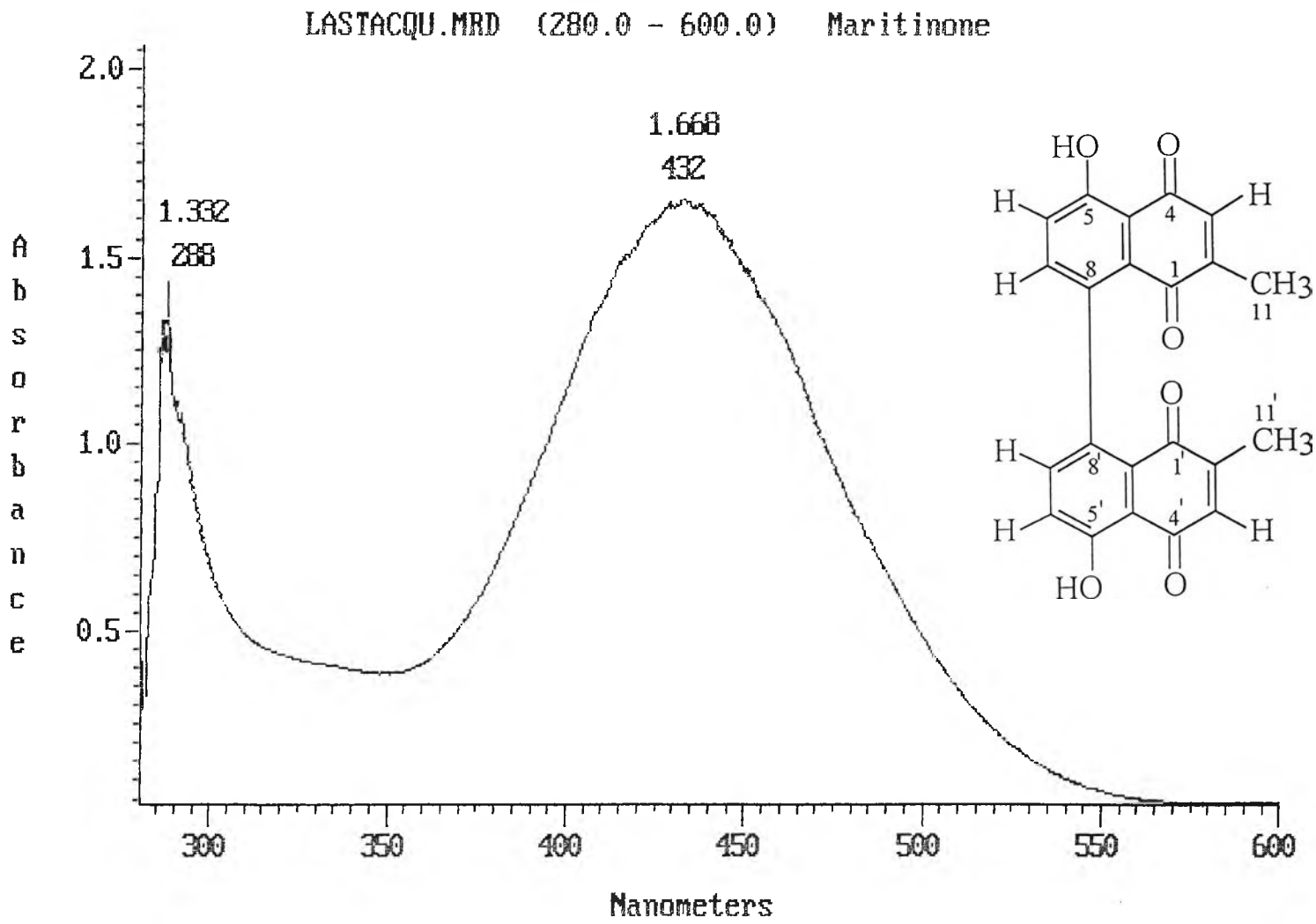


Figure 15 UV spectrum of compound DU3

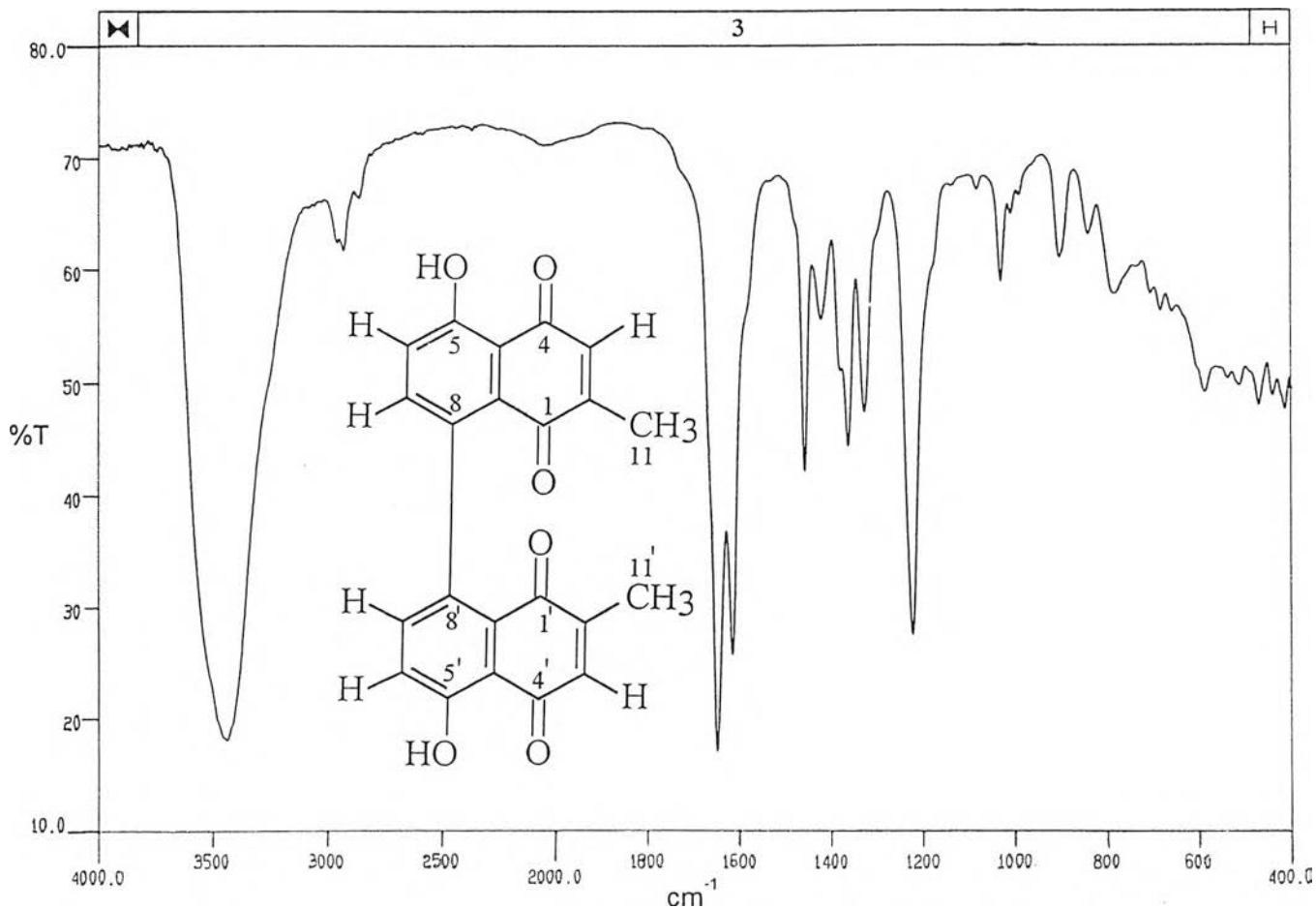


Figure 16 IR spectrum of compound DU3

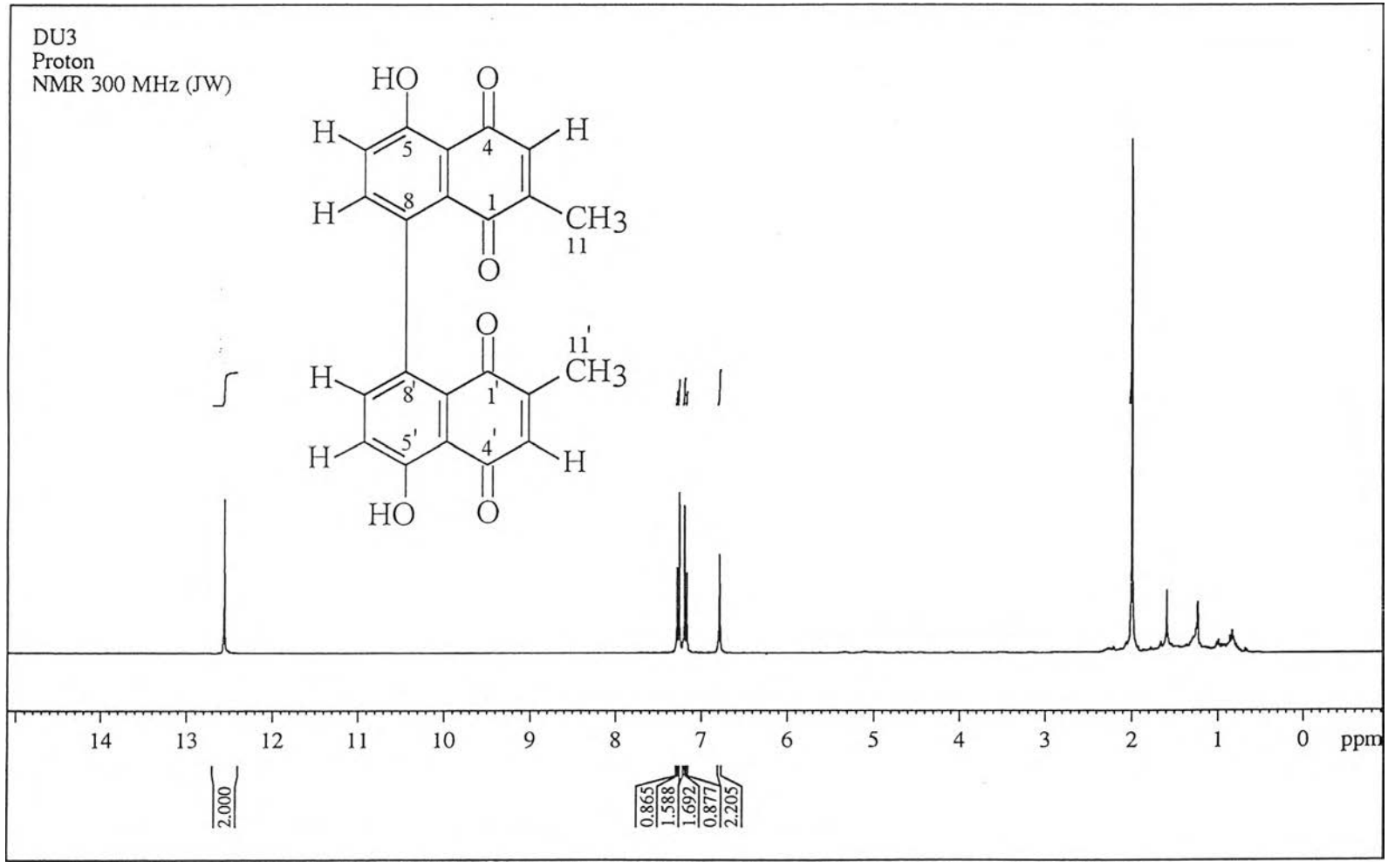


Figure 17a The 300 MHz H NMR spectrum of compound DU3

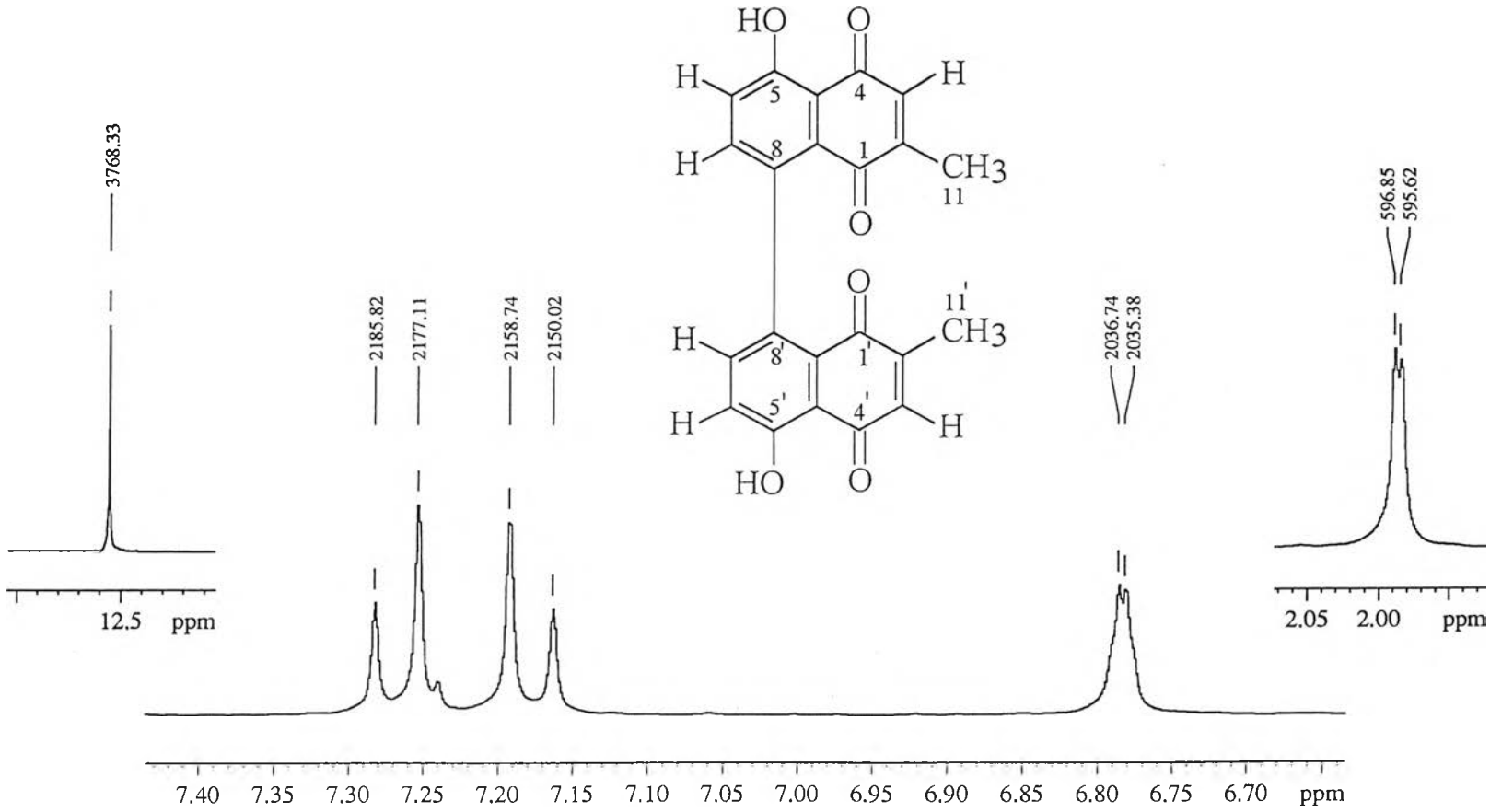


Figure 17b The 300 MHz ^{1}H NMR spectrum of compound DU3 (expanded from d 13.04–1.92 ppm)

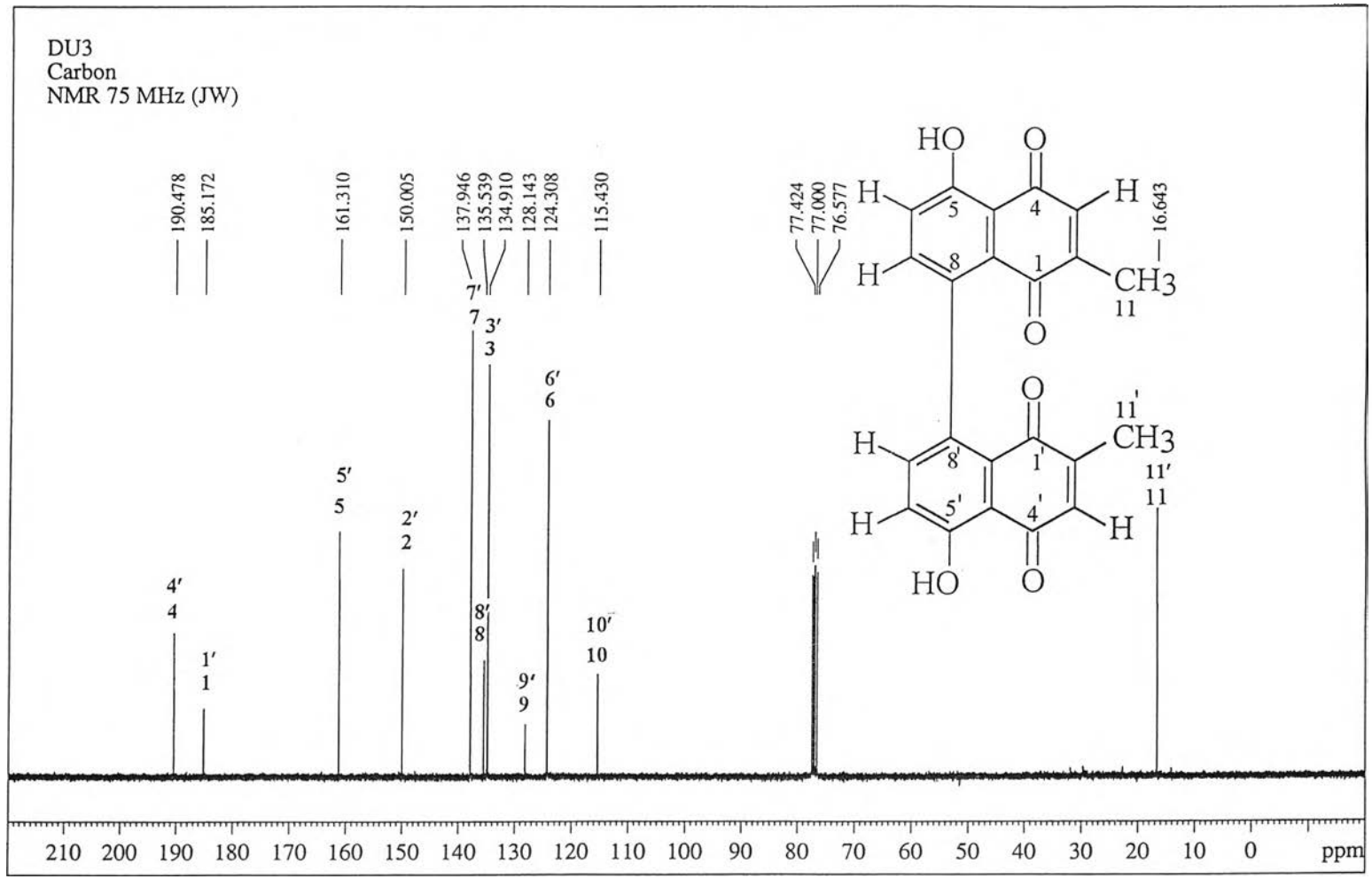


Figure 18 The 75 MHz C NMR spectrum of ccompound DU3

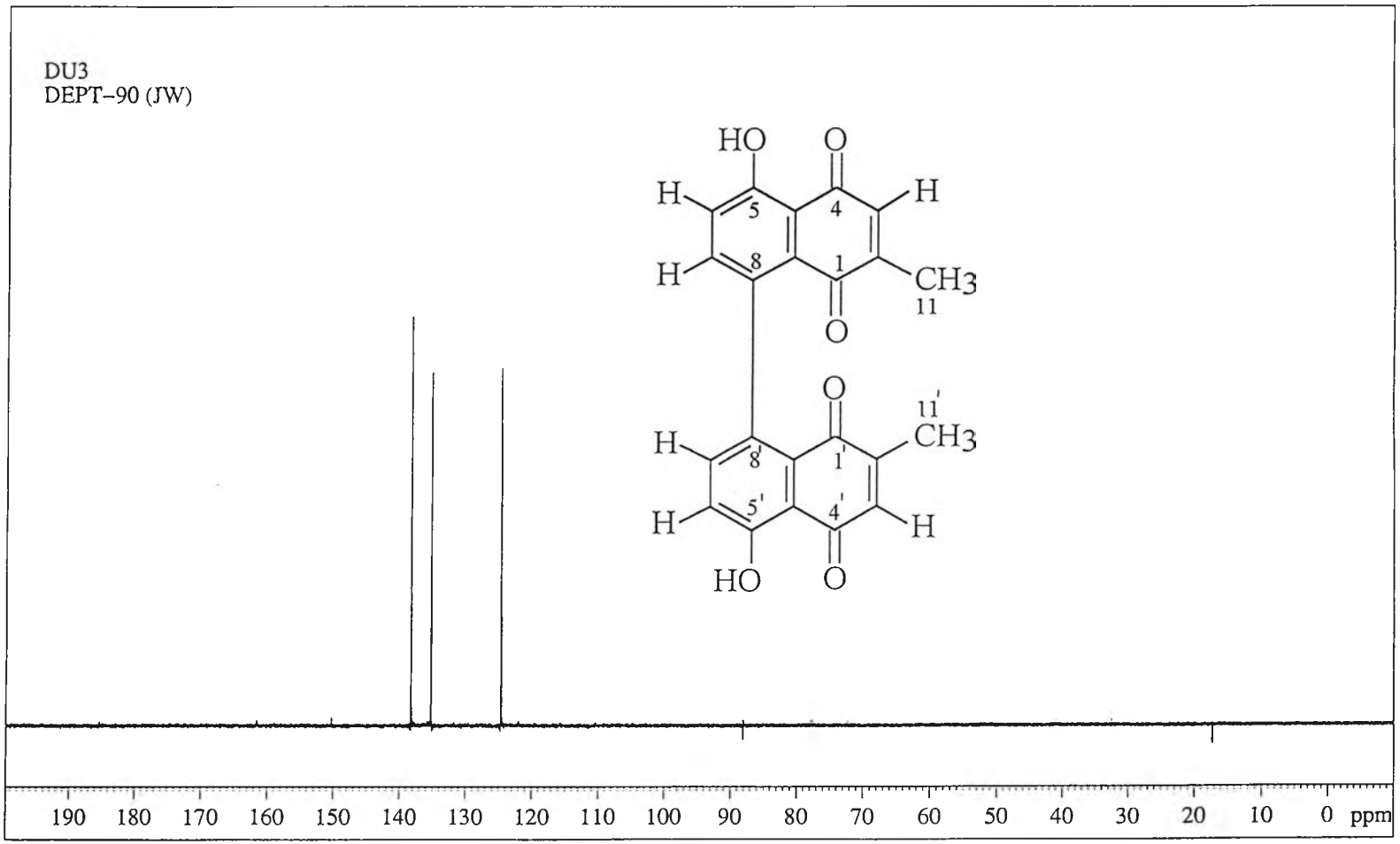


Figure 19a The DEPT–90 spectrum of compound DU3

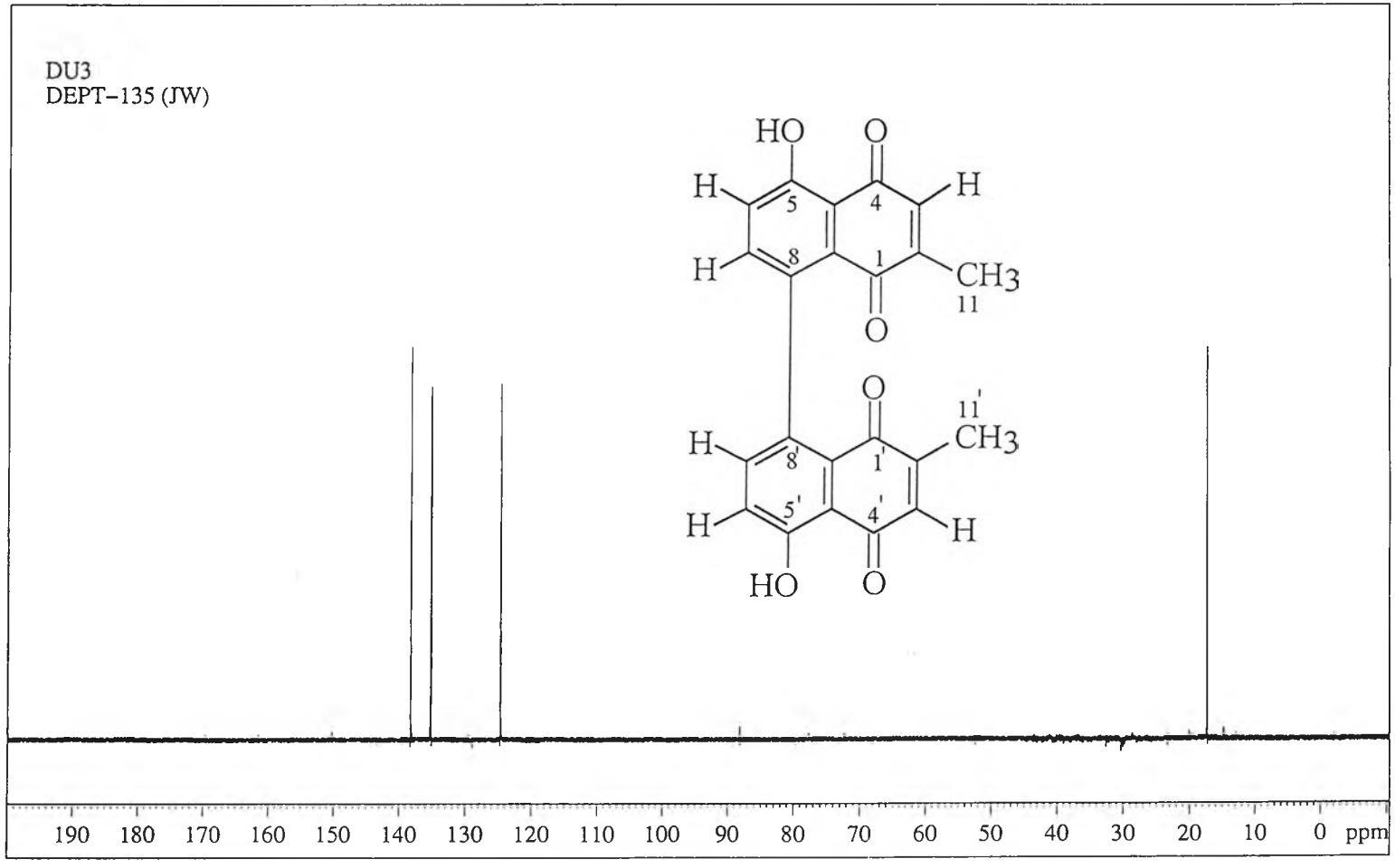
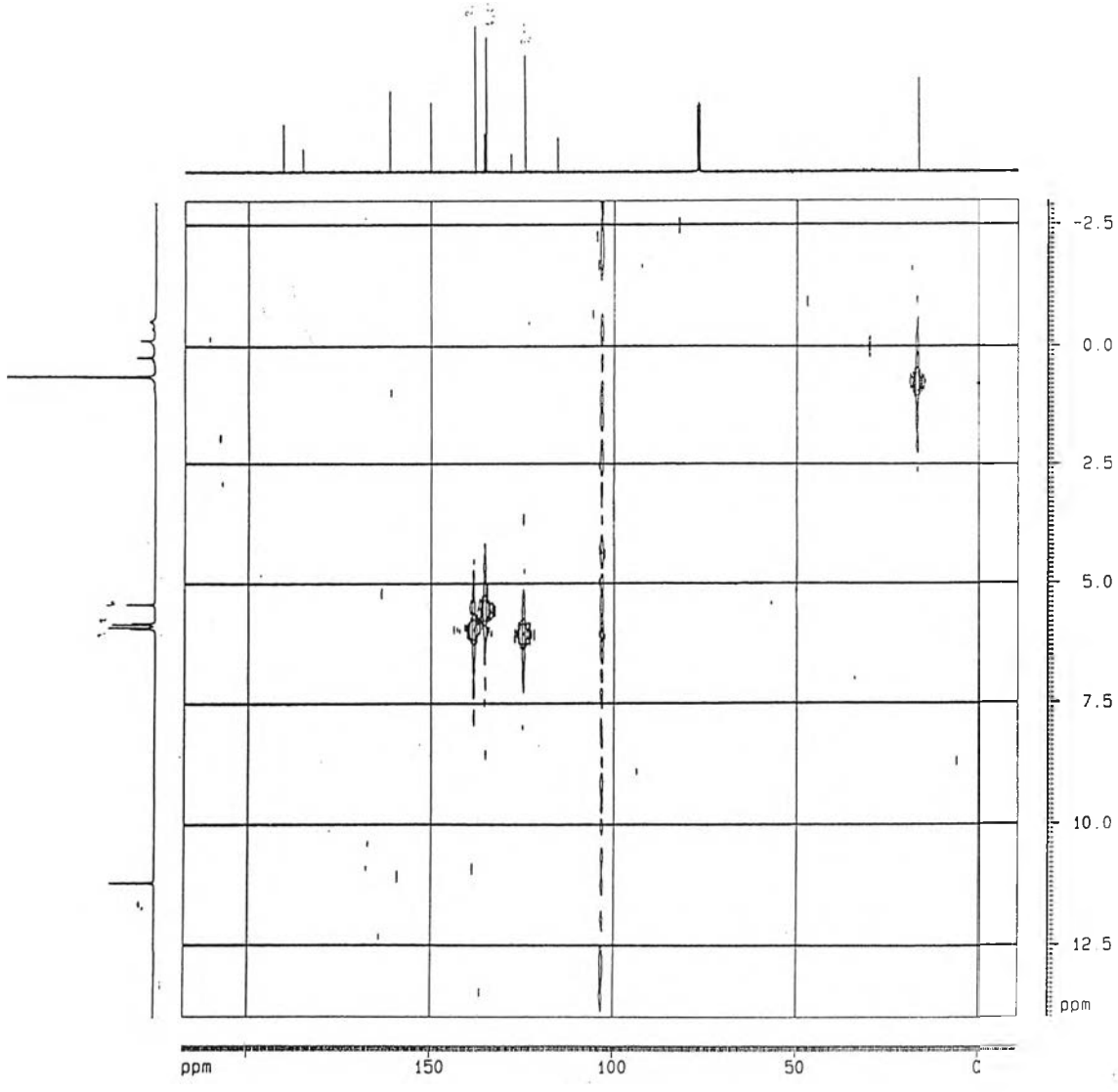


Figure 19b The DEPT-135 spectrum of compound DU3



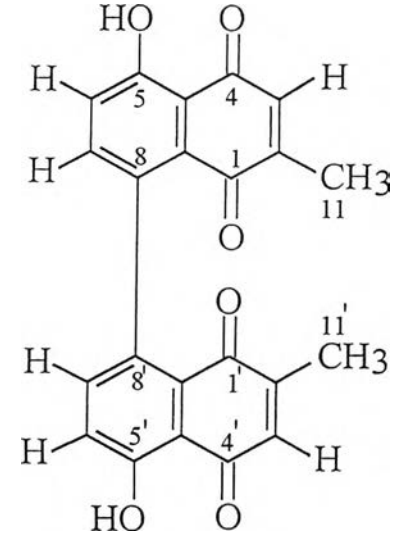
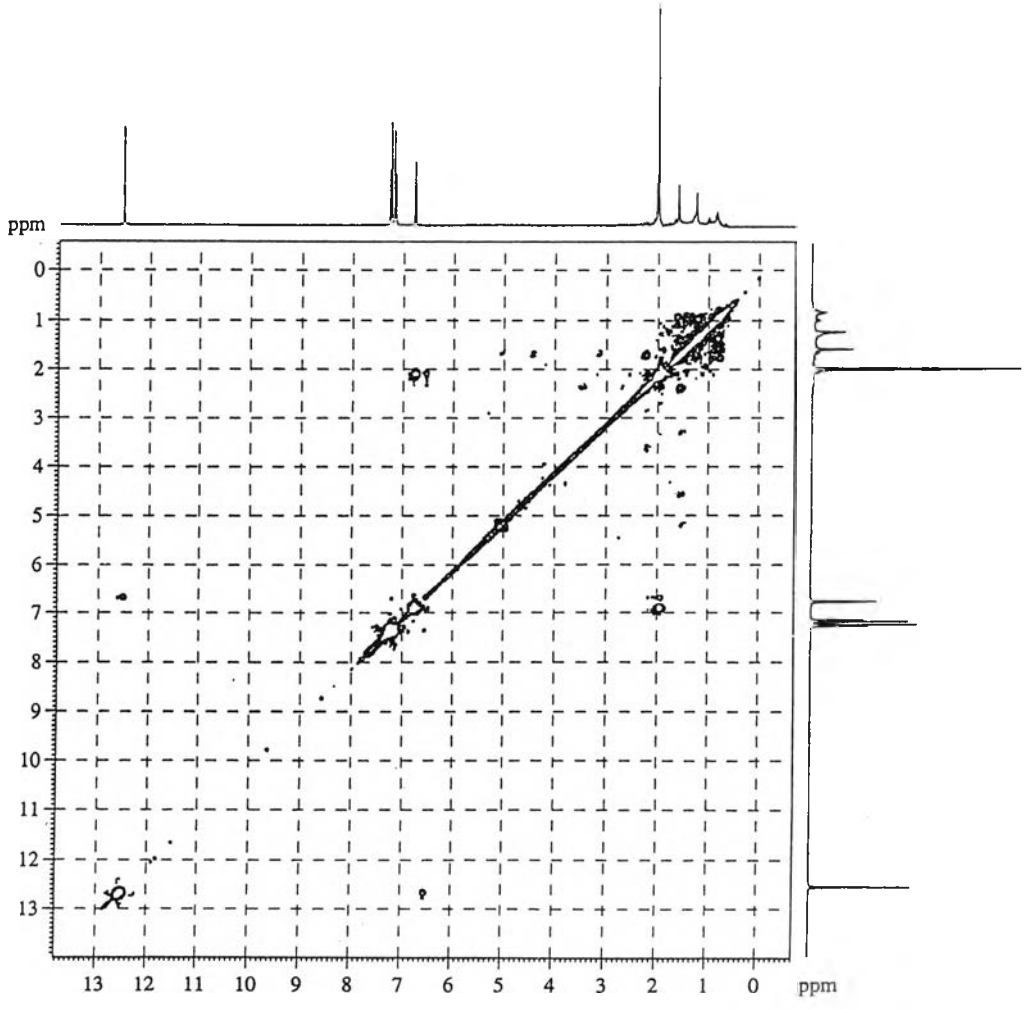


Figure 20 The HETCOR spectrum of compound DU3



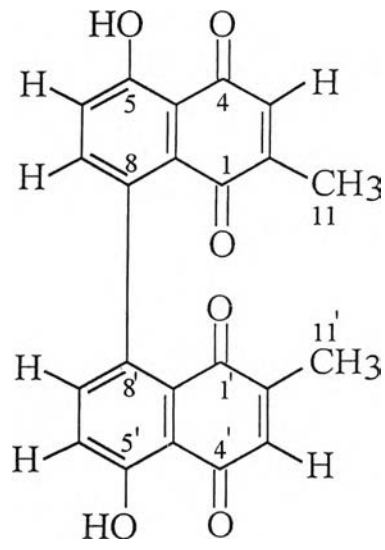
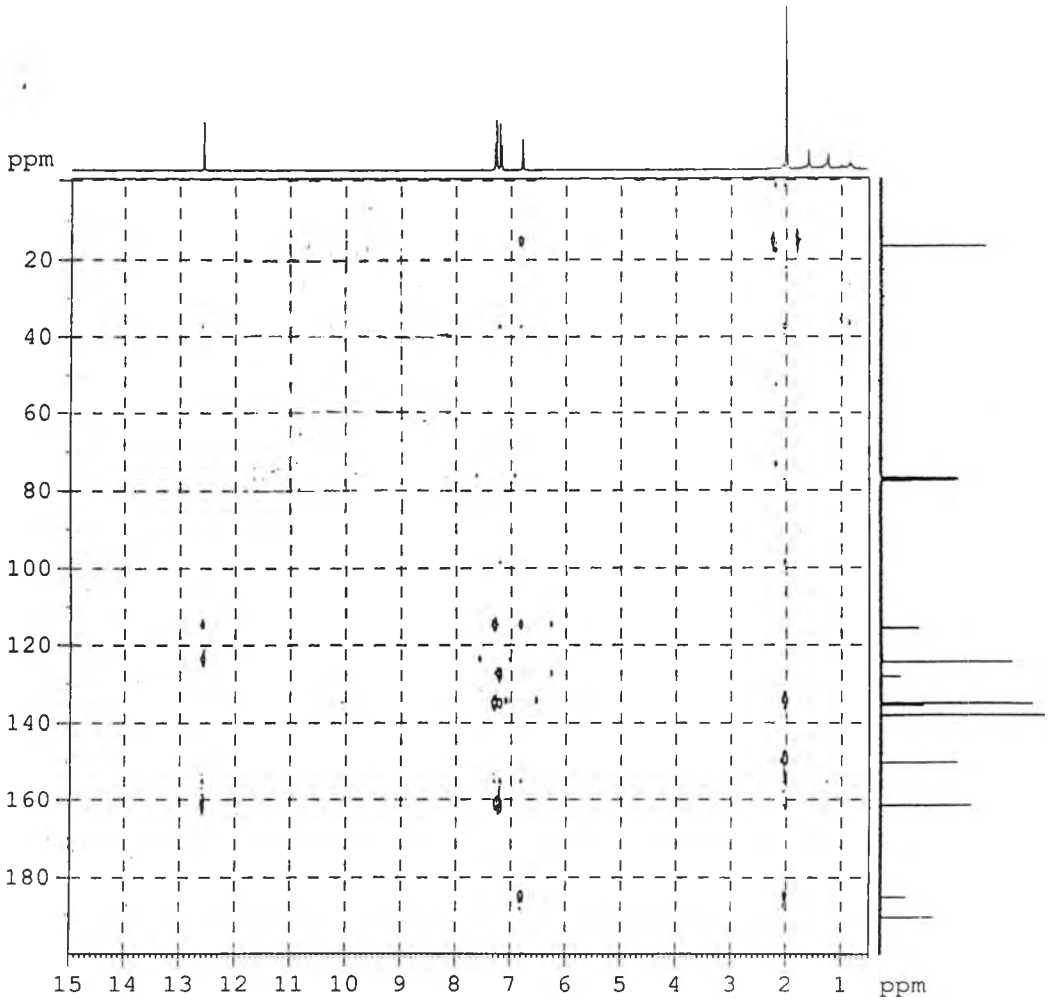


Figure 21 ^{1}H-^{1}H COSY spectrum of compound DU3



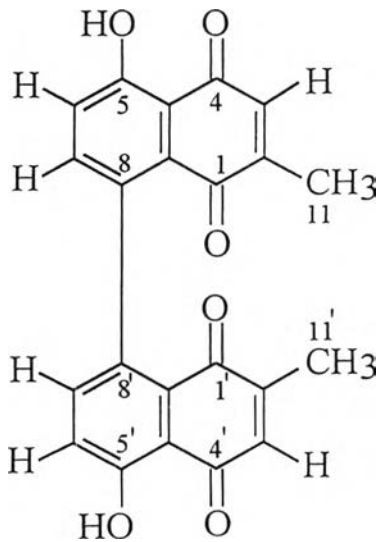
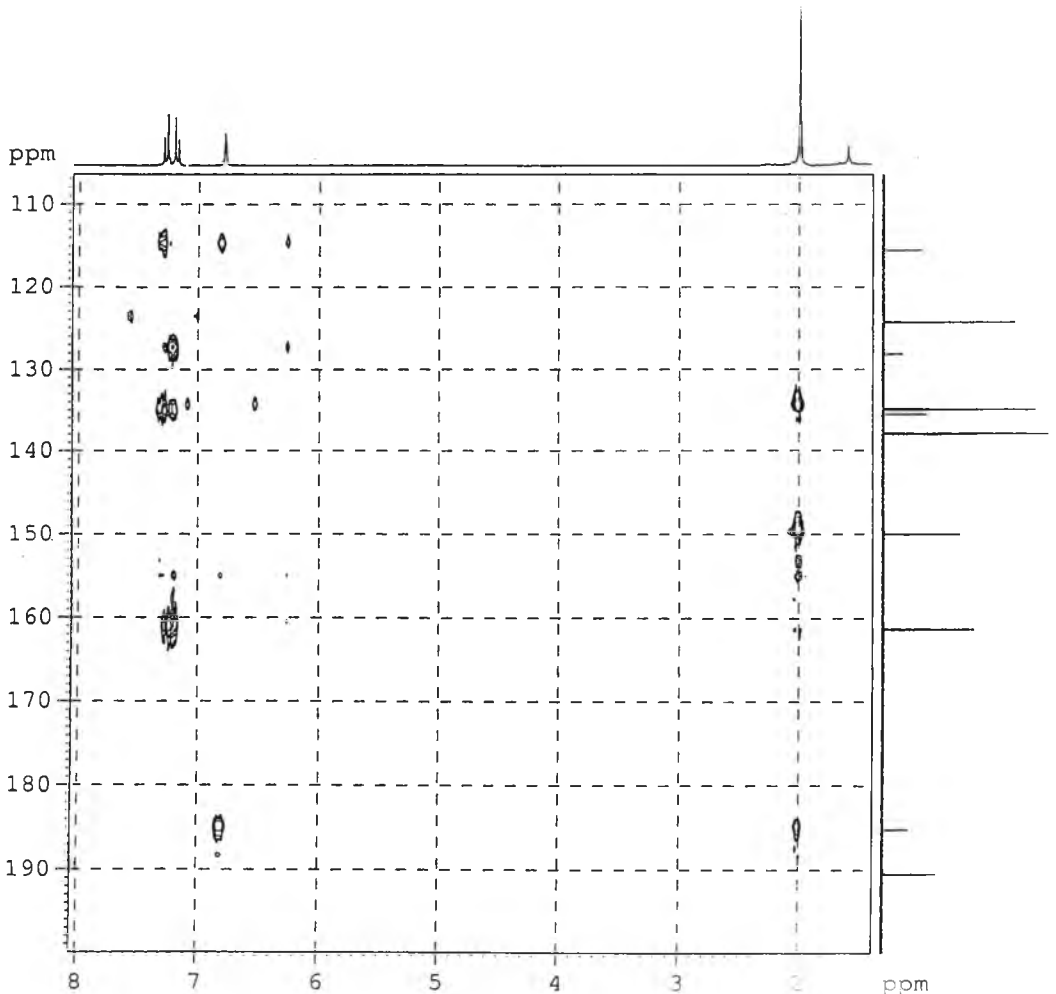


Figure 22a ^{1}H-^{13}C HMBC spectrum of compound DU3



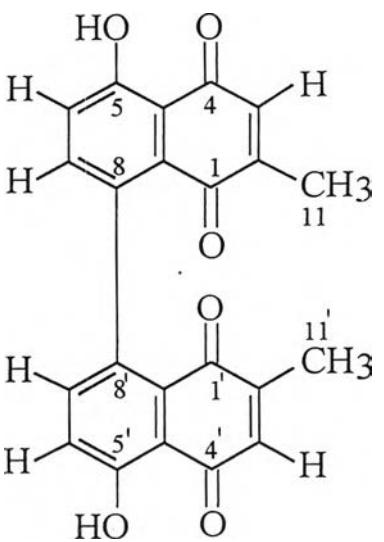


Figure 22b ^{1}H-^{13}C HMBC spectrum of compound DU3 (expanded)

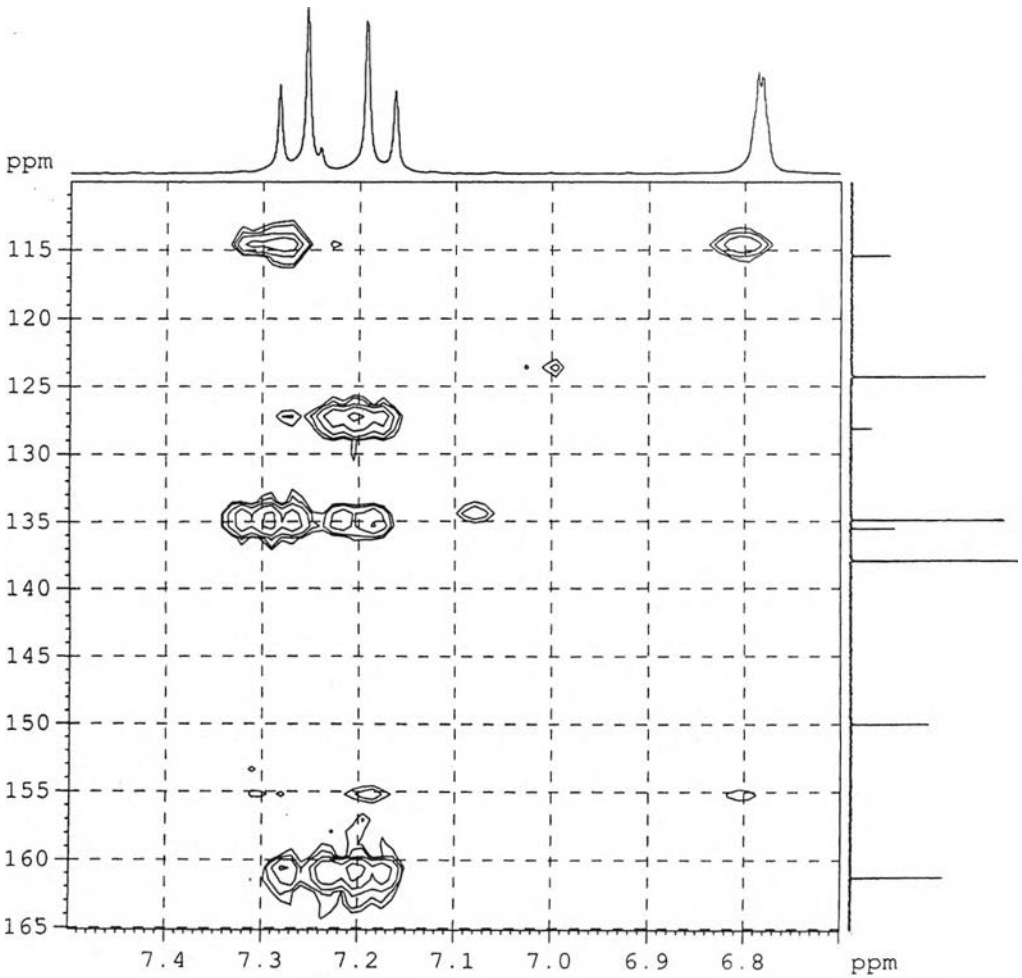
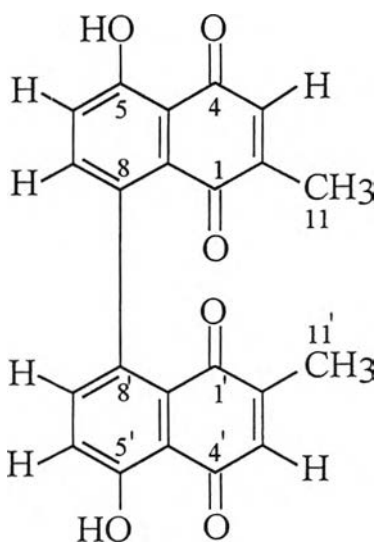


Figure 22c ^{1}H-^{13}C HMBC spectrum of compound DU3 (expanded)



4. Structure Elucidation of Compound DU4

Compound DU4 was recrystallized as red needles from benzene-hexane (1:1). Its molecular ion peak appeared at *m/z* 374, identical to that of maritinone (compound DU3). The ^{1}H (Figure 27a-27b) and ^{13}C NMR spectra (Figure 28) of this compound also appeared to represent only half the number of protons and carbon that could be deduced from the molecular weight. Therefore, DU4 appeared to be another symmetrical naphthoquinone dimer, of which a molecular formula of $C_{22}H_{14}O_6$ was again suggested.

The UV spectrum (Figure 25) displayed absorption maxima at λ_{max} 212 (log ε 3.97), 250 (log ε 3.69), 285 (log ε 3.32) and 420 nm (log ε 3.09). The IR spectrum (Figure 26) gave two distinct carbonyl bands at 1643 and 1609 cm^{-1}, indicating the presence of both free and hydrogen-bonded carbonyl groups of the naphthoquinone. The mass spectrum displayed significant mass fragment peaks at *m/z* 357 ($[M-OH]^+$), 346 ($[M-CO]^+$), 331 ($[M-CO-Me]^+$), 318 ($[M-2CO]^+$) and at m/z 187, which represented the fission of the molecule into two identical subunits ($C_{11}H_7O_3$).

The ^{1}H NMR spectrum of DU4 (Figure 27a-27b) showed the presence of a quinonoid proton signal at δ 6.83 (2H, d, *J*=1.1 Hz, H-3/3′) long-range coupled to the methyl group attached to position 2/2′ (δ 2.21 ppm, 6H, d, *J*=1.1 Hz, H_3-11/11′). A signal of hydrogen-bonded hydroxyl group appeared as the most downfield one at δ 12.48 ppm (2H, s, OH-5/5′). A couple of broad singlets at δ 7.71 and 7.24 ppm could be assigned to those of *meta*-coupled H-6/6′ and H-8/8′, respectively.

The ^{13}C NMR spectrum (Figure 28) and DEPT-90 and DEPT-145 experiment (Figure 33) helped in identifying the carbon signals of two carbonyls at δ 190.5 (C-4/4′) and 184.5 ppm (C-1/1′), one methyl carbon at δ 16.5 ppm (C-11/11′), three methine carbons at δ 135.5 ppm (C-3/3′), 118.7 ppm (C-6/6′) and 137.7 ppm (C-8/8′) and five quaternary carbons at δ 149.8 (C-2/2′), 158.9 (C-5/5′), 131.2 (C-7/7′), 131.9 (C-9/9′) and 115.3 ppm (C-10/10′).

Analysis of HMBC experiments (Figure 31a-31d) confirmed the quinonoid part of DU4 to be identical to that of maritinone, judging from the cross-peaks observed between H_3-11/11′ and C-1/1′, C-2/2′ and C-3/3′. As for the benzenoid part, the hydroxy group could be located at position C-5/5′ according to its downfield proton signal and the HMBC cross-peaks between this hydroxy proton and C-5/5′, C-7/7′ and C-10/10′. Therefore, in order for this molecule to be composed of two

identical naphthoquinone monomers, the linkage between the benzenoid parts could be between positions 6-6′ (elliptinone), 7-7′ or 8-8′ (maritinone). But since in the cases of both elliptinone and maritinone the two benzenoid protons had to be ortho-coupling, compound DU4 should be connected at positions 7-7′. This connectivity was also confirmed from the long-range correlation observed between H-6 at δ 7.71 ppm and C-8′ at δ 137.7 ppm. Compound DU4 was thus elucidated as a new naphthoquinone dimer, 7,7′-biplumbagin, and was given the trivial name, undulatanone (**110**). The ^{1}H and ^{13}C NMR assignments and long-range correlation of compound DU4 are summarized in Table. 13

Table 13 ^{1}H, ^{13}C-assignments and HMBC correlations of compound DU4

position	^{1}H	^{13}C	**HMBC correlated with carbon**
	δ_H **(ppm)**	**δc (ppm)**	**signals at δc (ppm)**
1, 1′	-	184.5	-
2, 2′	-	149.8	-
3, 3′	6.83 (d, *J*=1.1 Hz)	135.5	16.5, 184.5
4, 4′	-	190.5	-
5, 5′	-	158.9	-
6, 6′	7.71 (2H, br s)	118.7	131.2
7, 7′		131.2	-
8, 8′	7.24 (2H,br s)	137.7	-
9, 9′	-	131.9	-
10, 10′	-	115.3	-
11, 11′	2.21 (6H, d, *J*= 1.1Hz)	16.5	135.5, 149.8, 184.5
5-OH, 5′-OH	12.48 (2H, s)	-	118.7, 158.9, 115.3

The results obtained from HMBC spectral examination suggested that the assignments of DU4

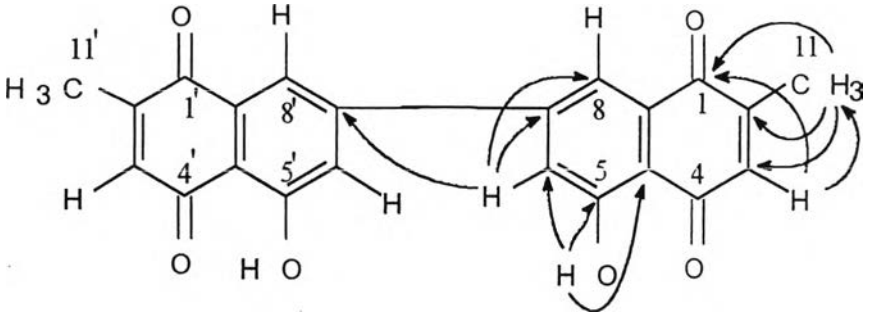


Figure 23 Selected long-rang C-H correlations of compound DU4 observed in HMBC spectru

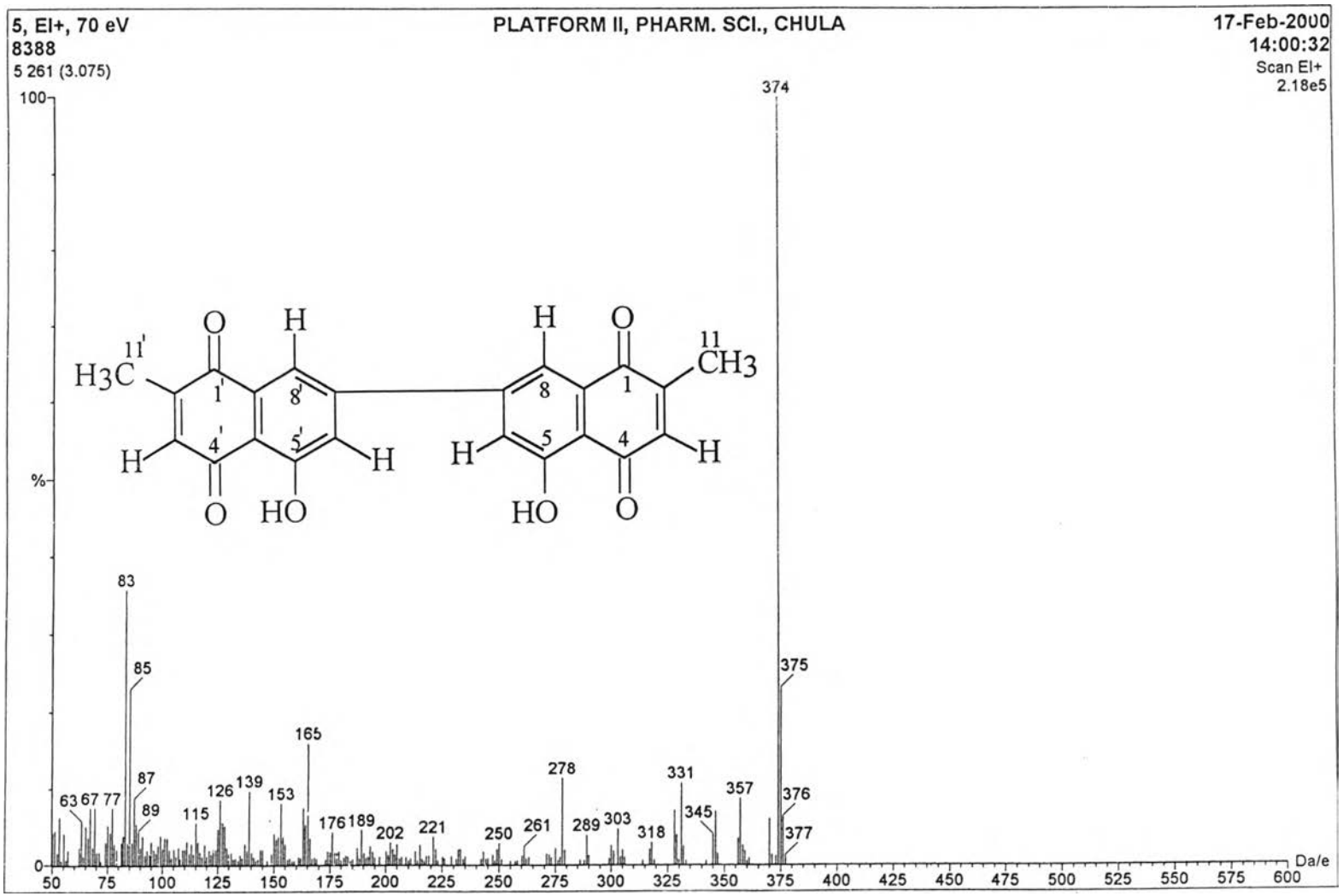


Figure 24 EI mass spectrum of compound DU4

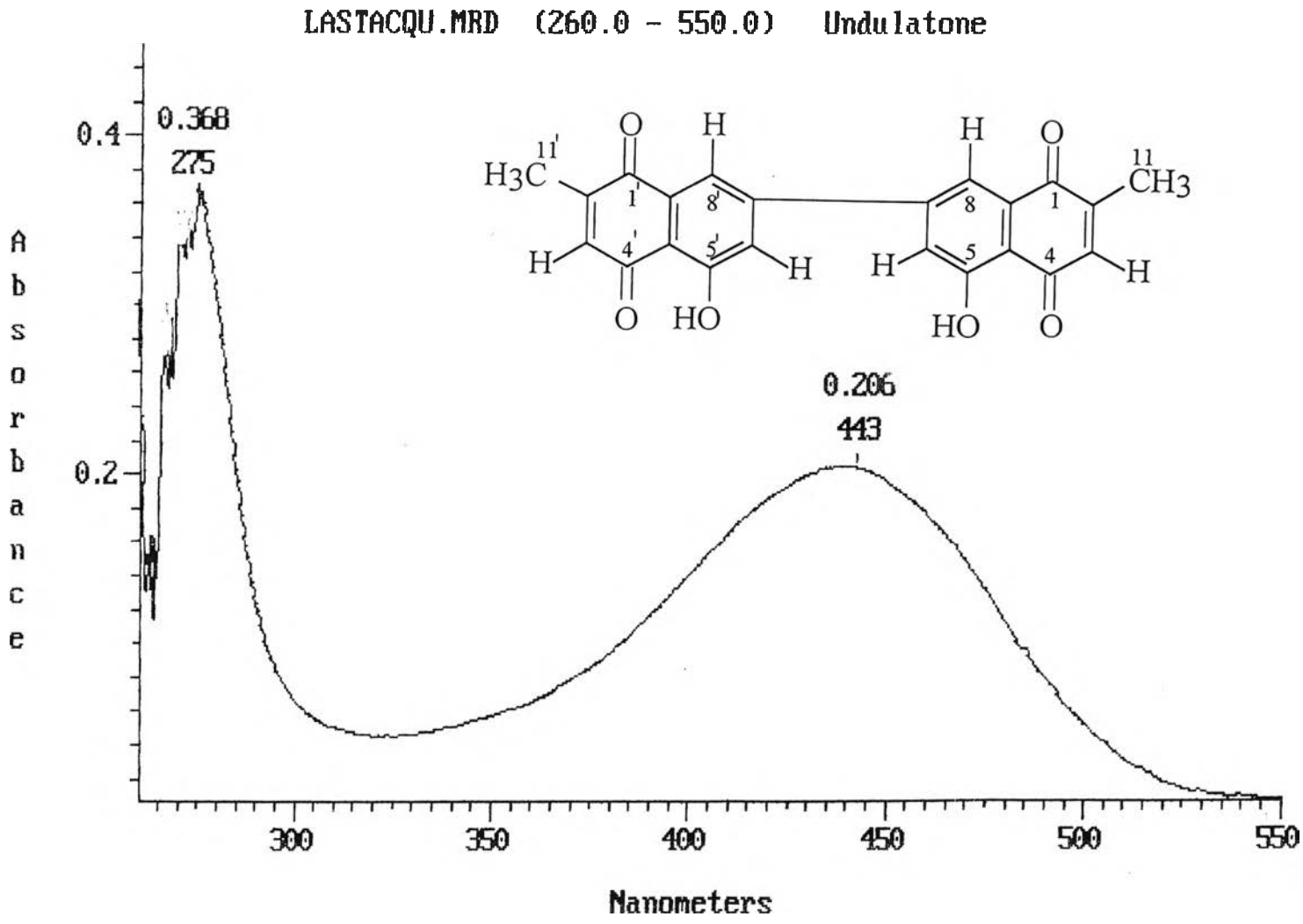


Figure 25 UV spectrum of compound DU4

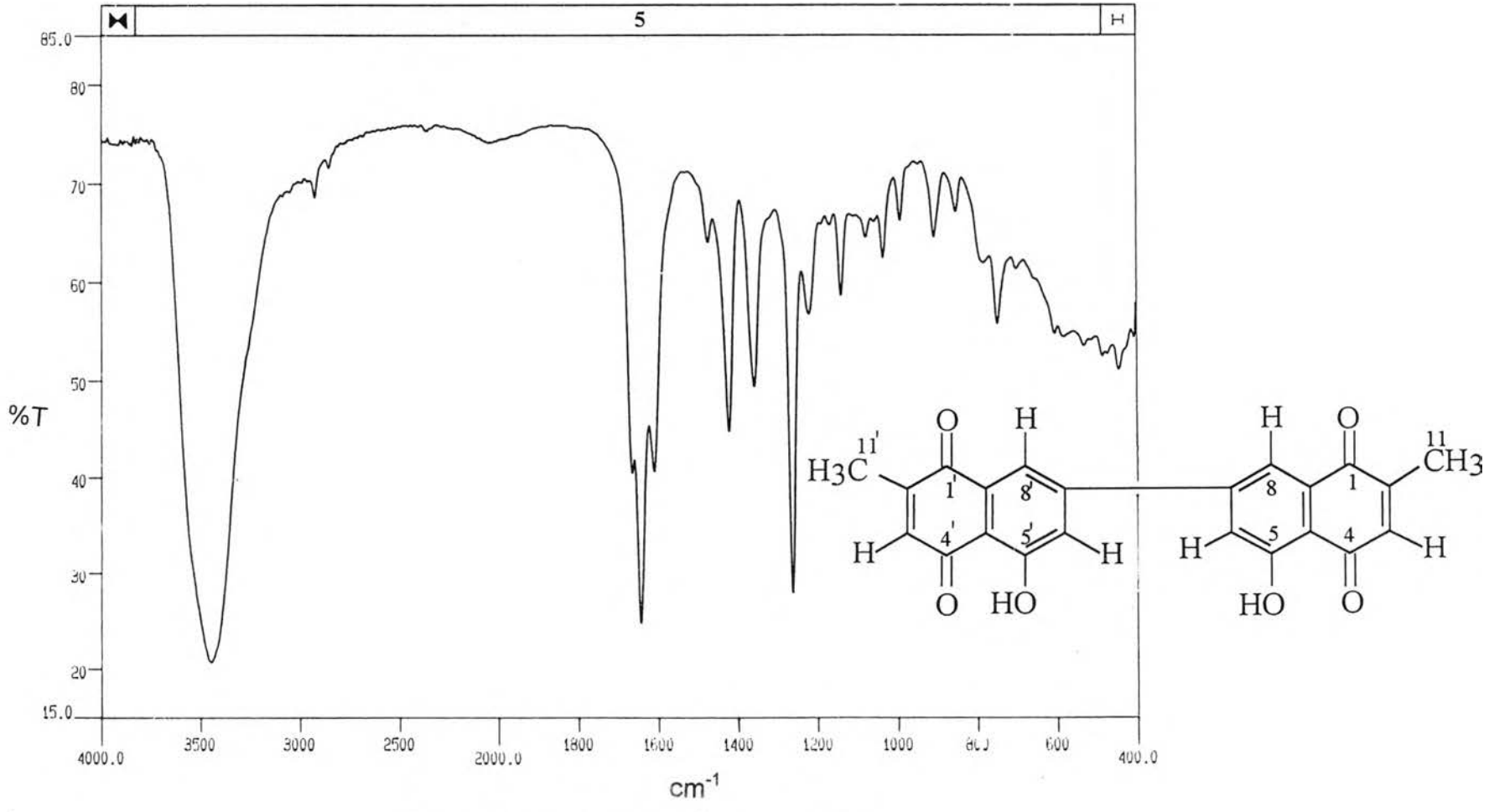


Figure 26 IR spectrum of compound DU4

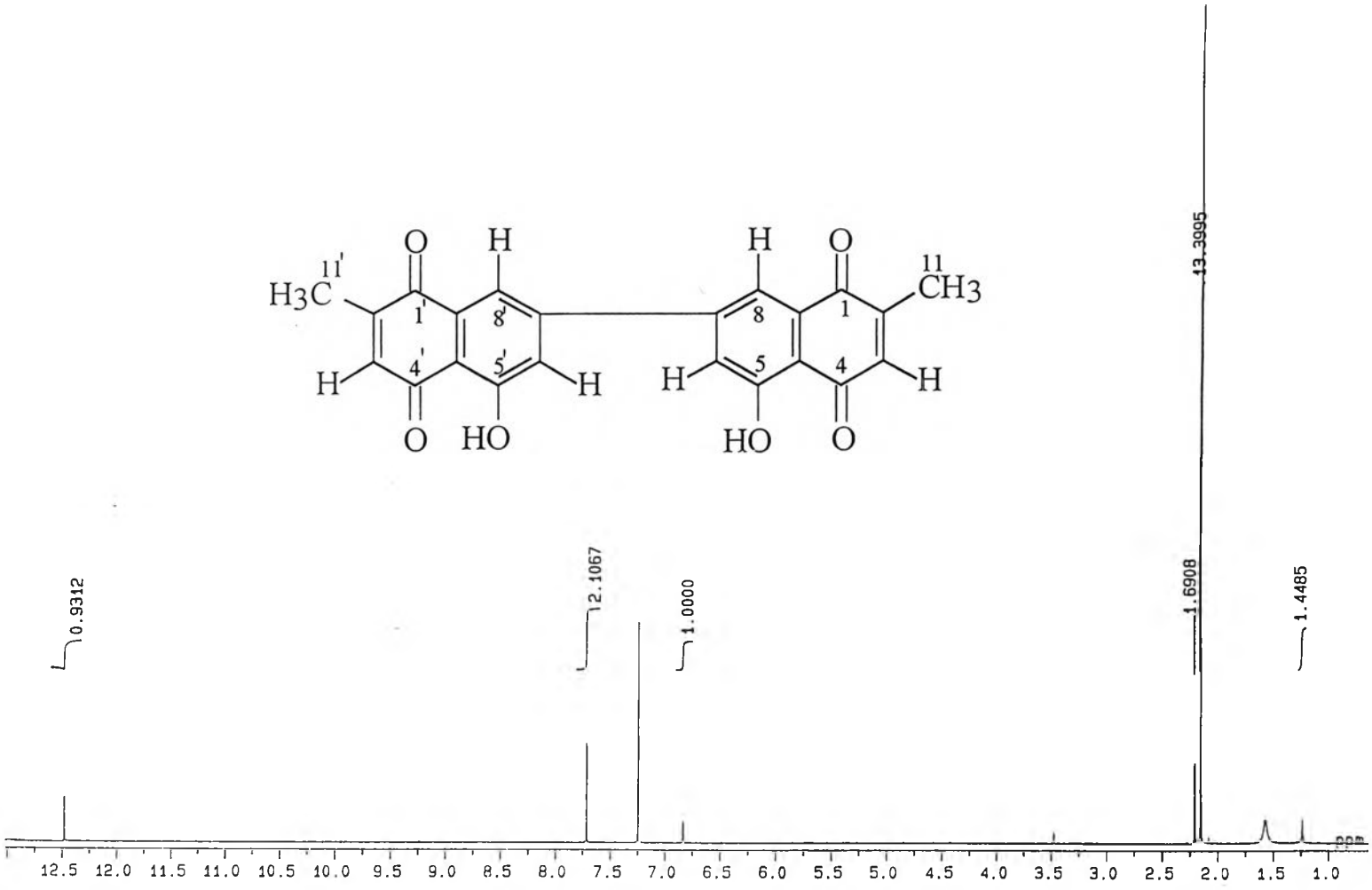


Figure 27a The 300 MHz ^{1}H NMR spectrum of compound DU4

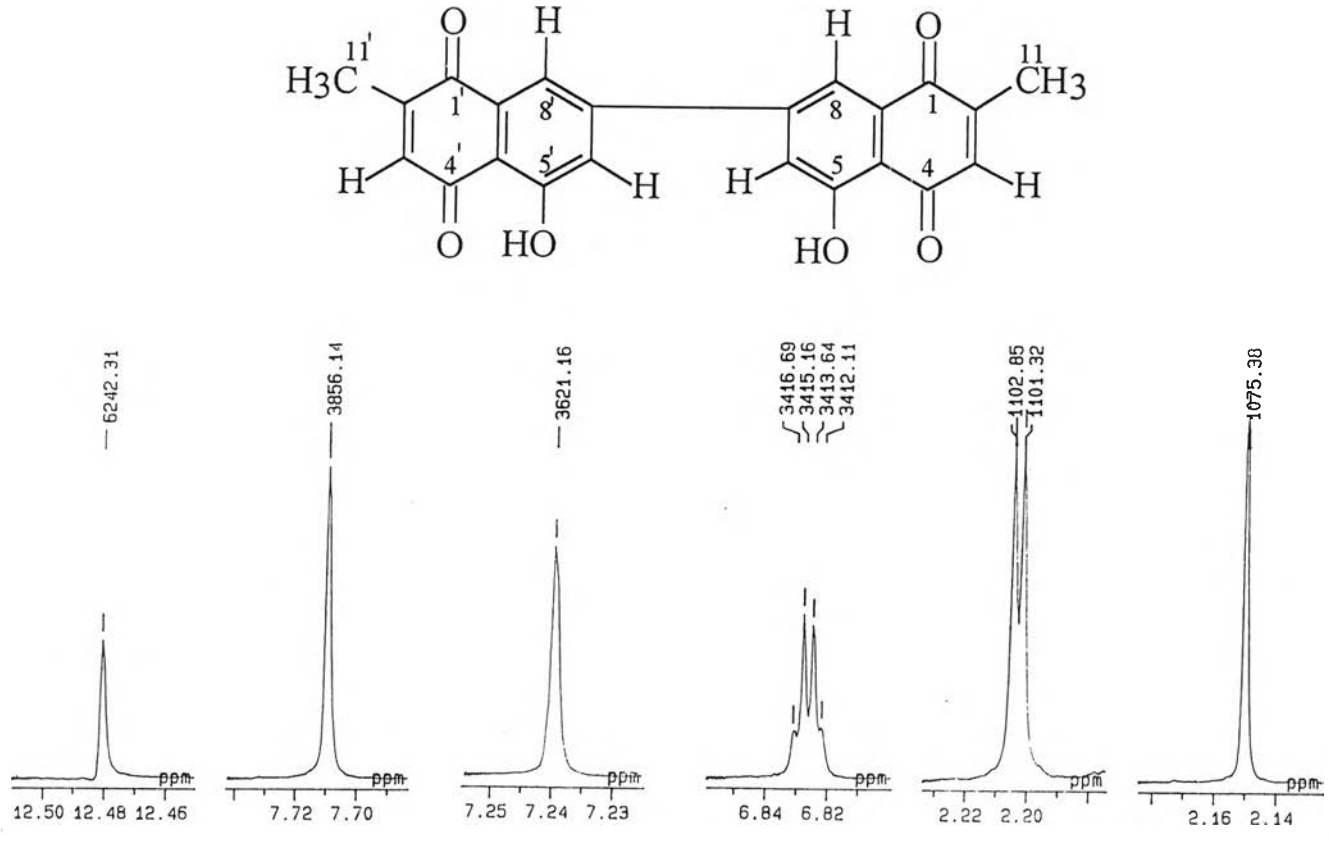


Figure 27b The 300 MHz ^{1}H NMR spectrum of compound DU4(expanded)

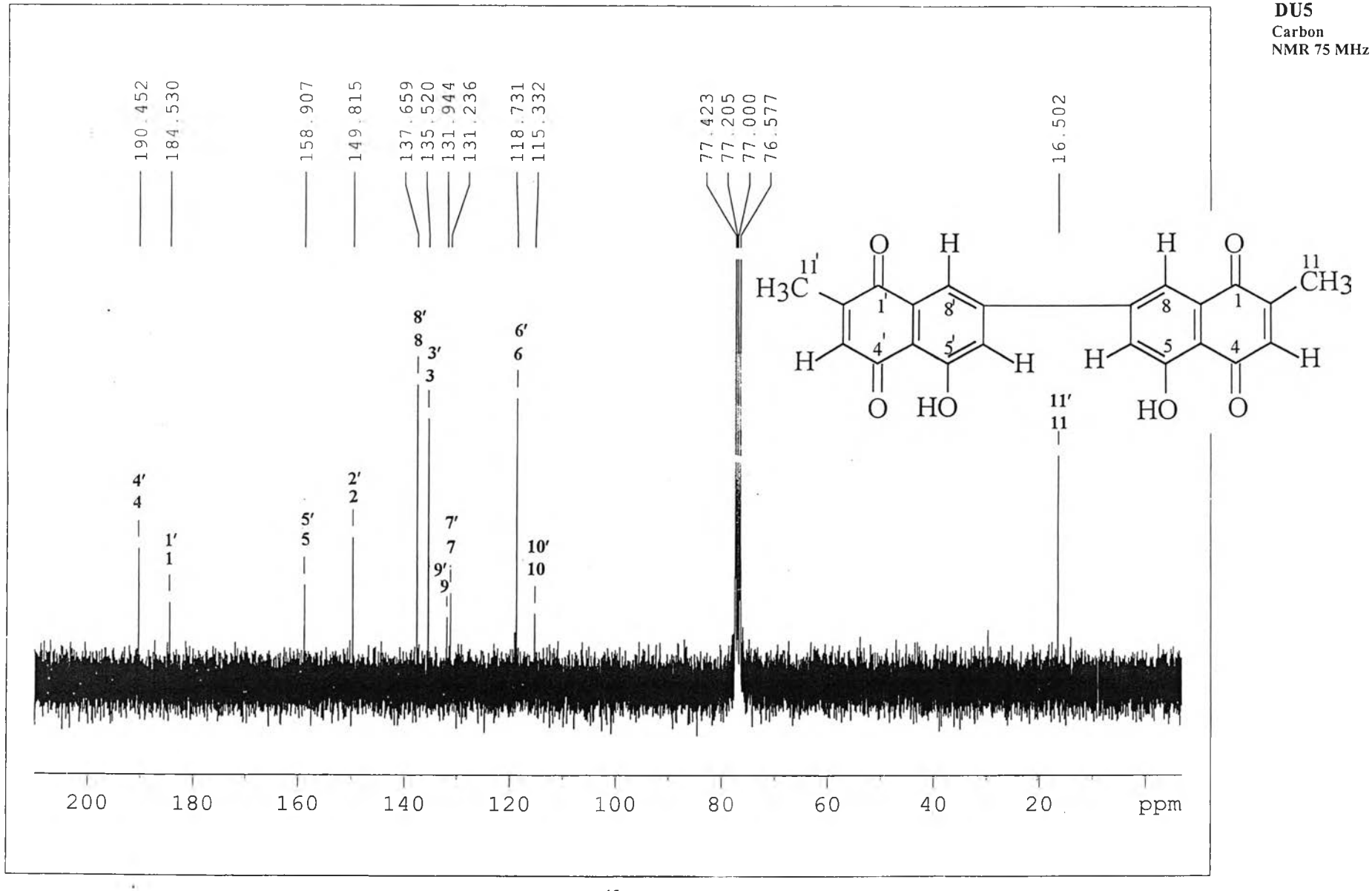


Figure 28 The 75 MHz ^{13}C NMR spectrum of compound DU4

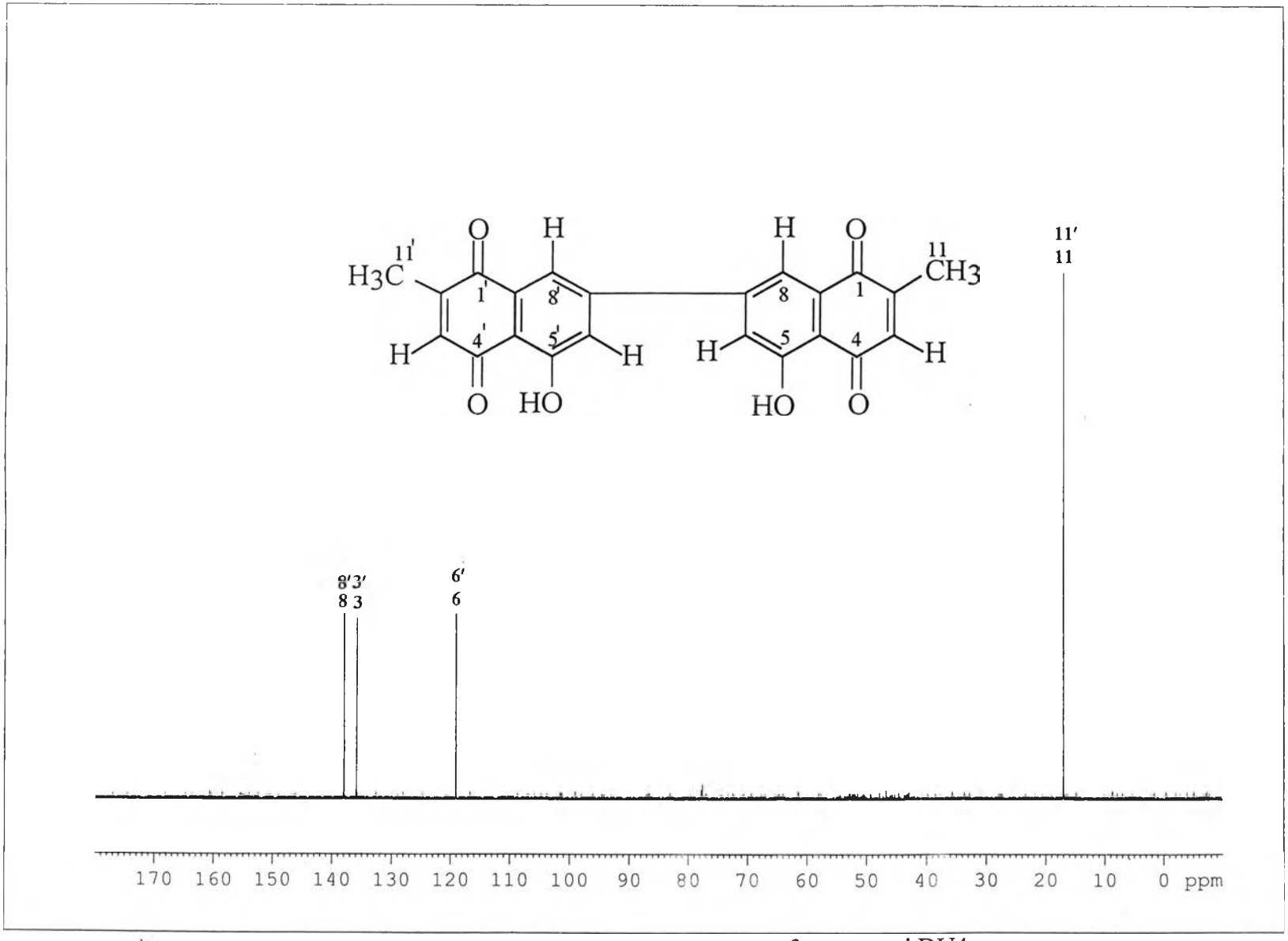


Figure 29 The DEPT-135 spectrum of compound DU4

Dios
DEPT-135
(JW)

```
Current Data Parameters
NAME                  dios
EXPNO                    6
PROCNO                   1

F2 - Acquisition Parameters
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Time                 23.32
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PROBHD     5 mm Dual 13
PULPROG                dept
TD                    32768
SOLVENT               CDCl3
NS                     5120
DS                        8
SWH              17361.111 Hz
FIDRES           0.529619 Hz
AQ               0.9437684 sec
RG                     4096
DW                   28.800 usec
DE                   43.11 usec
TE                   300.0 K
P1                    5.50 usec
p2                   11.0 usec
P3                    9.50 usec
p4                   19.0 usec
CNST2          145.0000000
d2               0.0034483 sec
d12              0.0000200 sec
DELTA            0.0000070 sec
D1               2.00000000 sec
PL2                 -3.00 dB
SFO2           300.1315000 MHz
NUC2                   1H
SFO1            75.4755556 MHz
NUC1                   13C
PL1                 -6.00 dB
P0                  14.25 usec
PL12                19.00 dB
DE                  43.11 usec
CPDPRG2           waltz16
PCPD2              100.00 usec

F2 - Processing parameters
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SF              75.4677190 MHz
WDW                    EM
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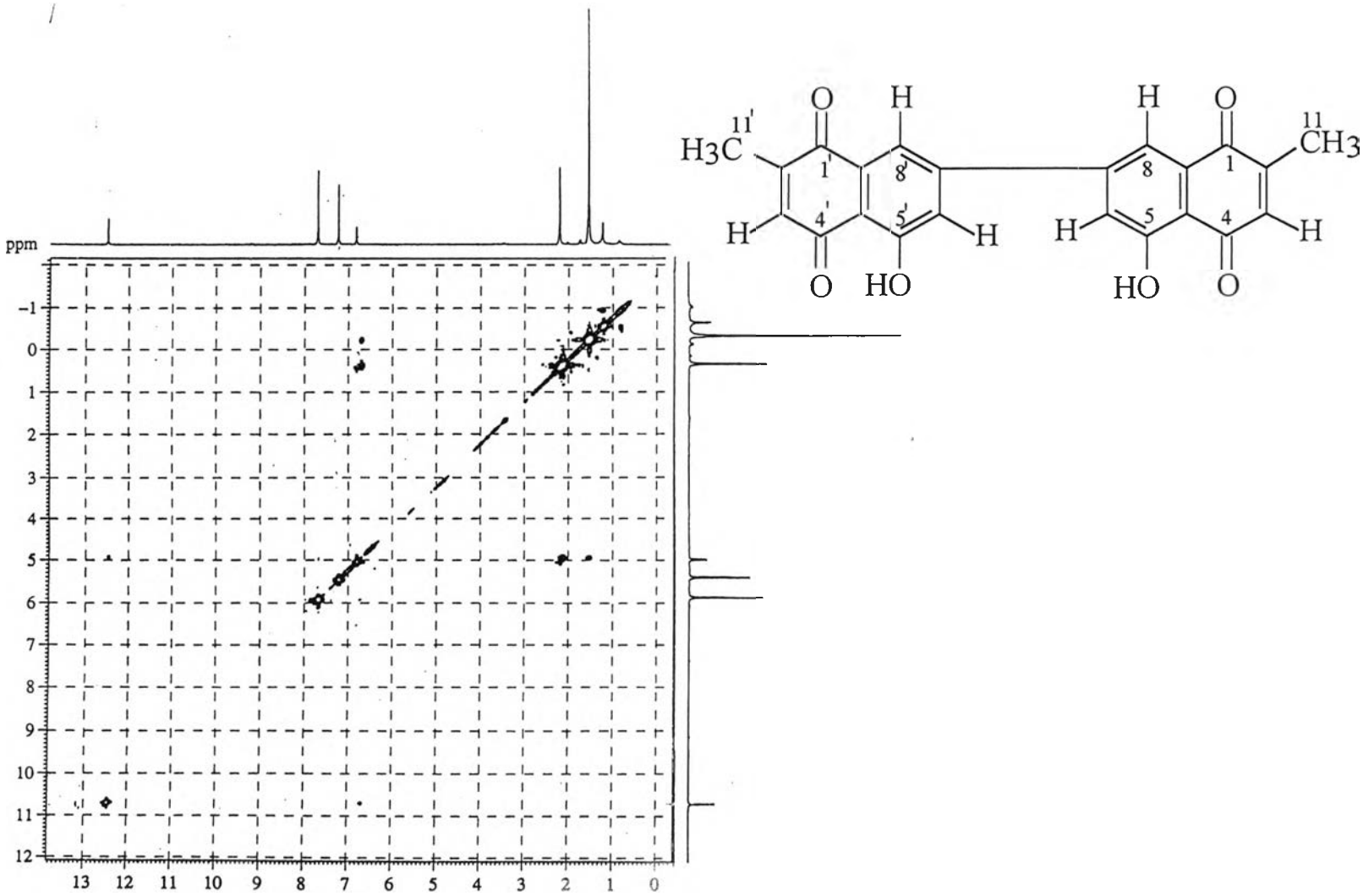


Figure 30 ^{1}H-^{1}H COSY spectrum of compound DU4

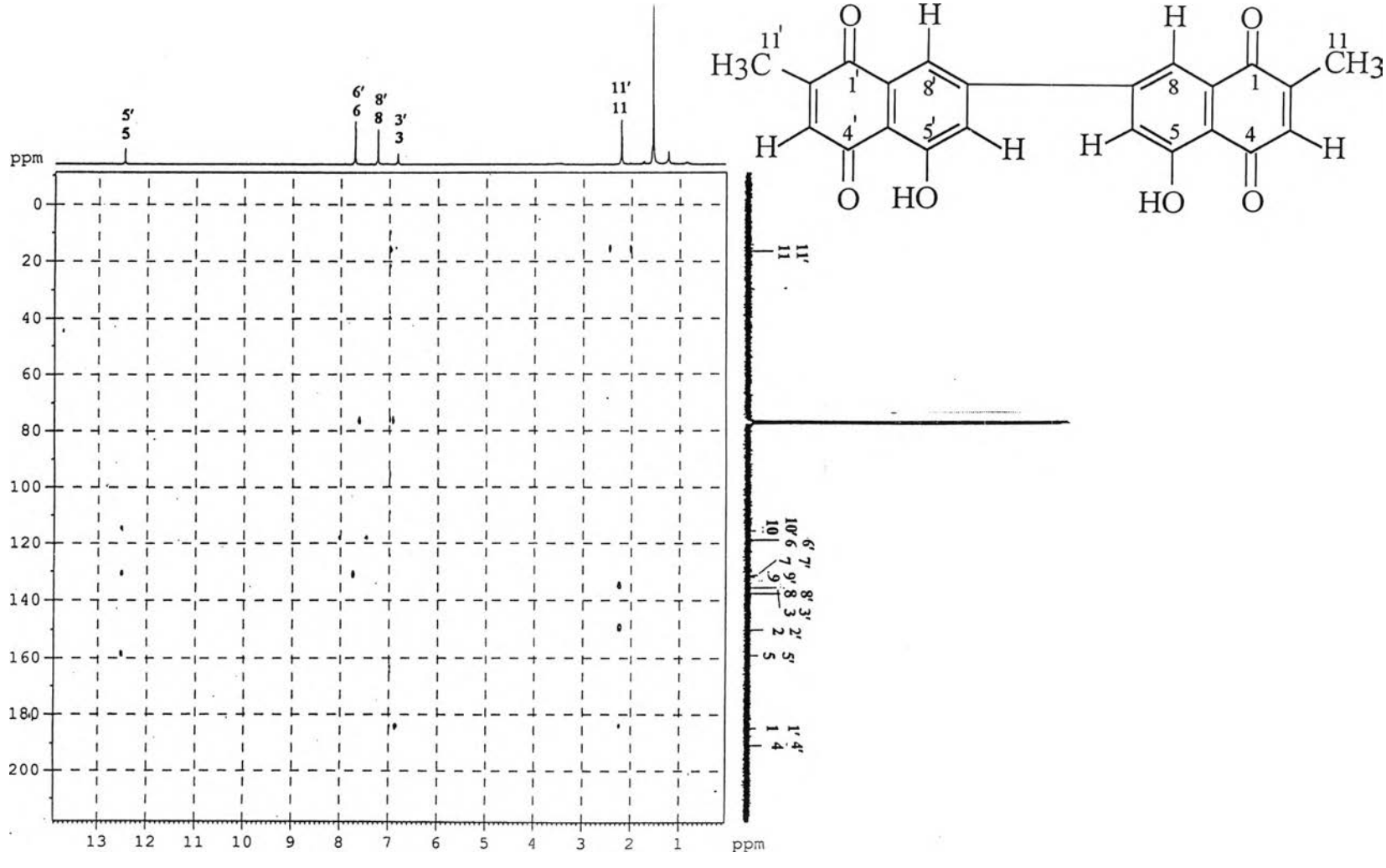


Figure 31a ^{1}H-^{13}C HMBC spectrum of compound DU4

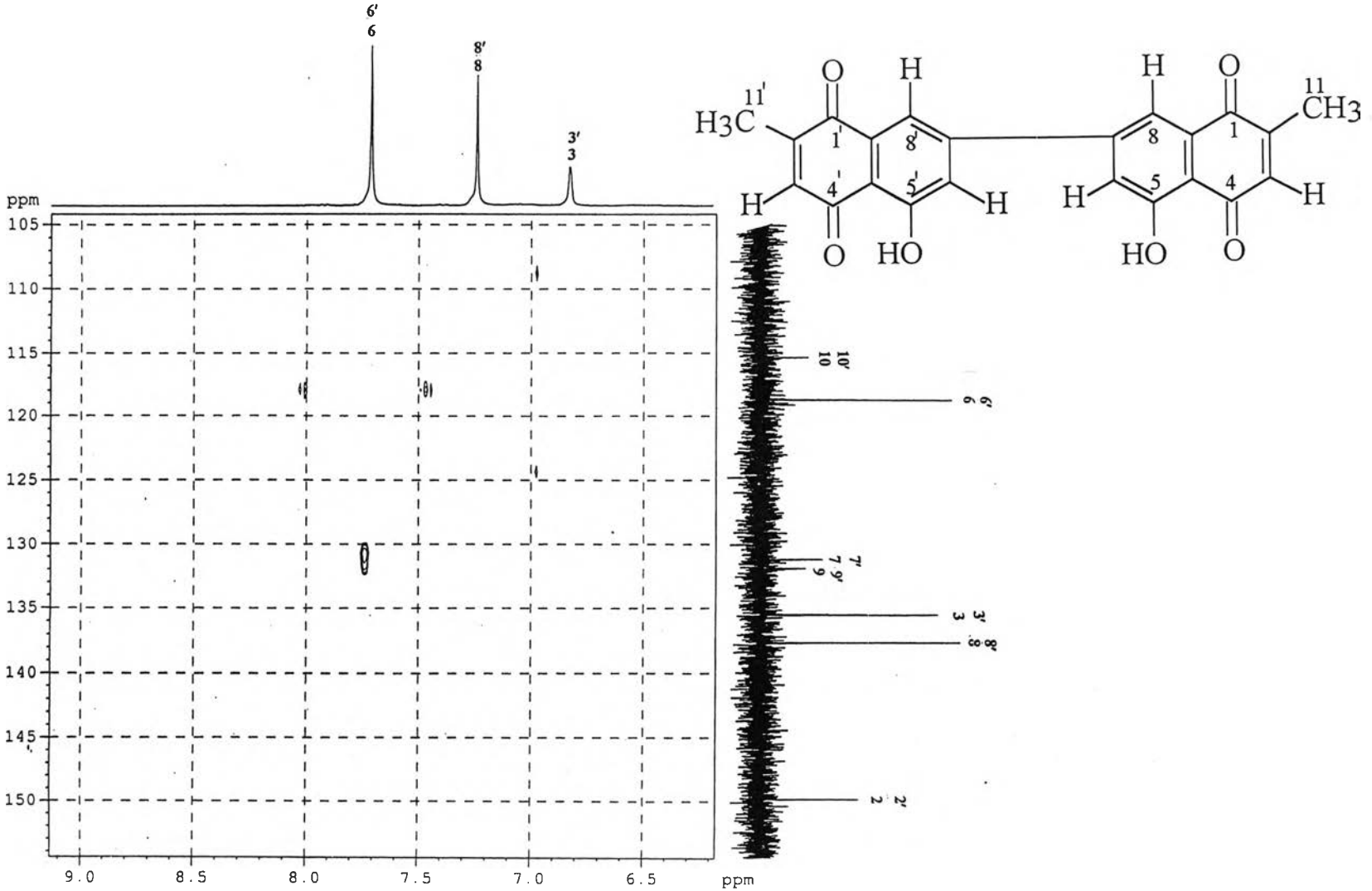


Figure 31b ^{1}H-^{13}C HMBC spectrum of compound DU4(expanded)

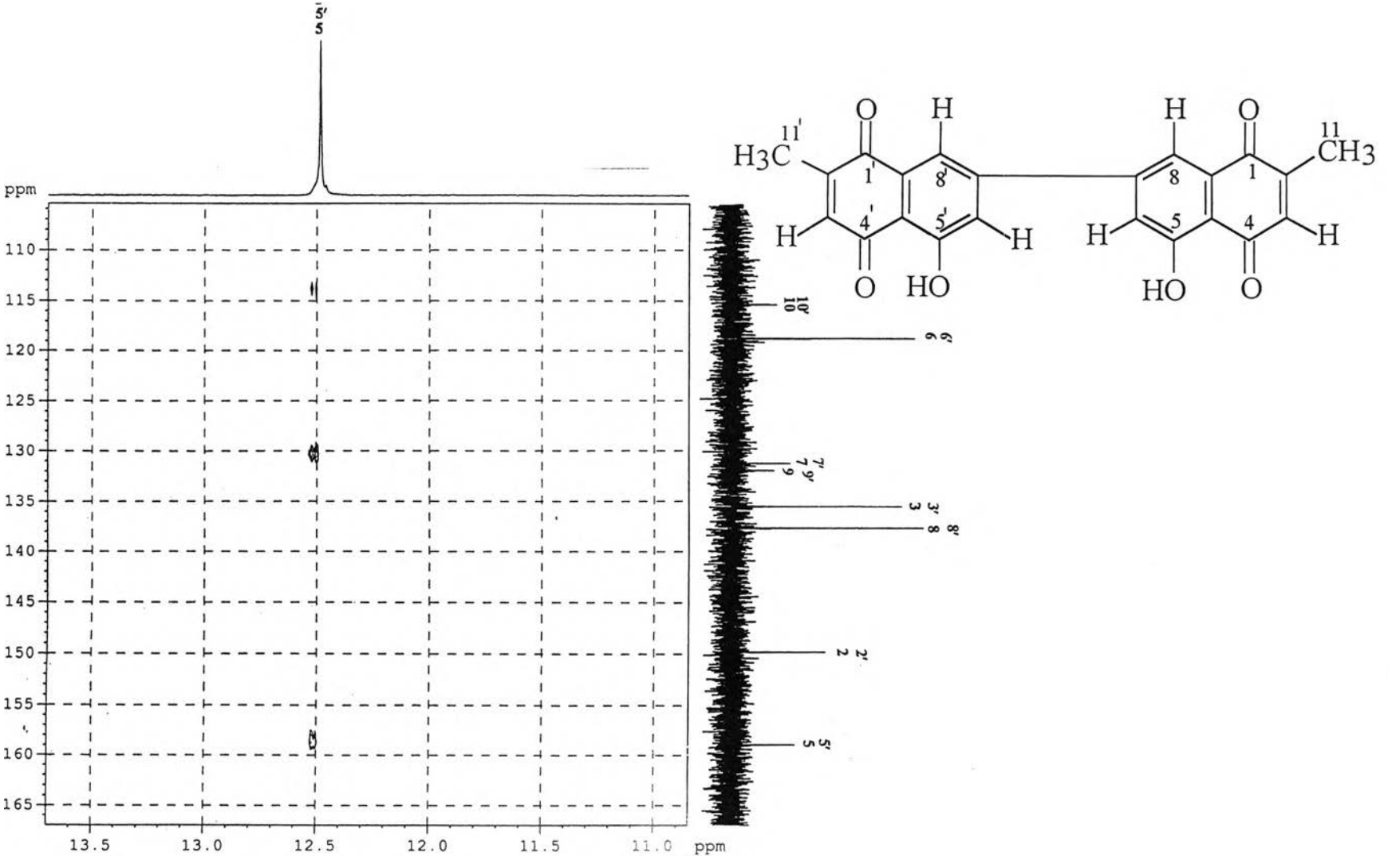


Figure 31c ^{1}H-^{13}C HMBC spectrum of compound DU4 (expanded)

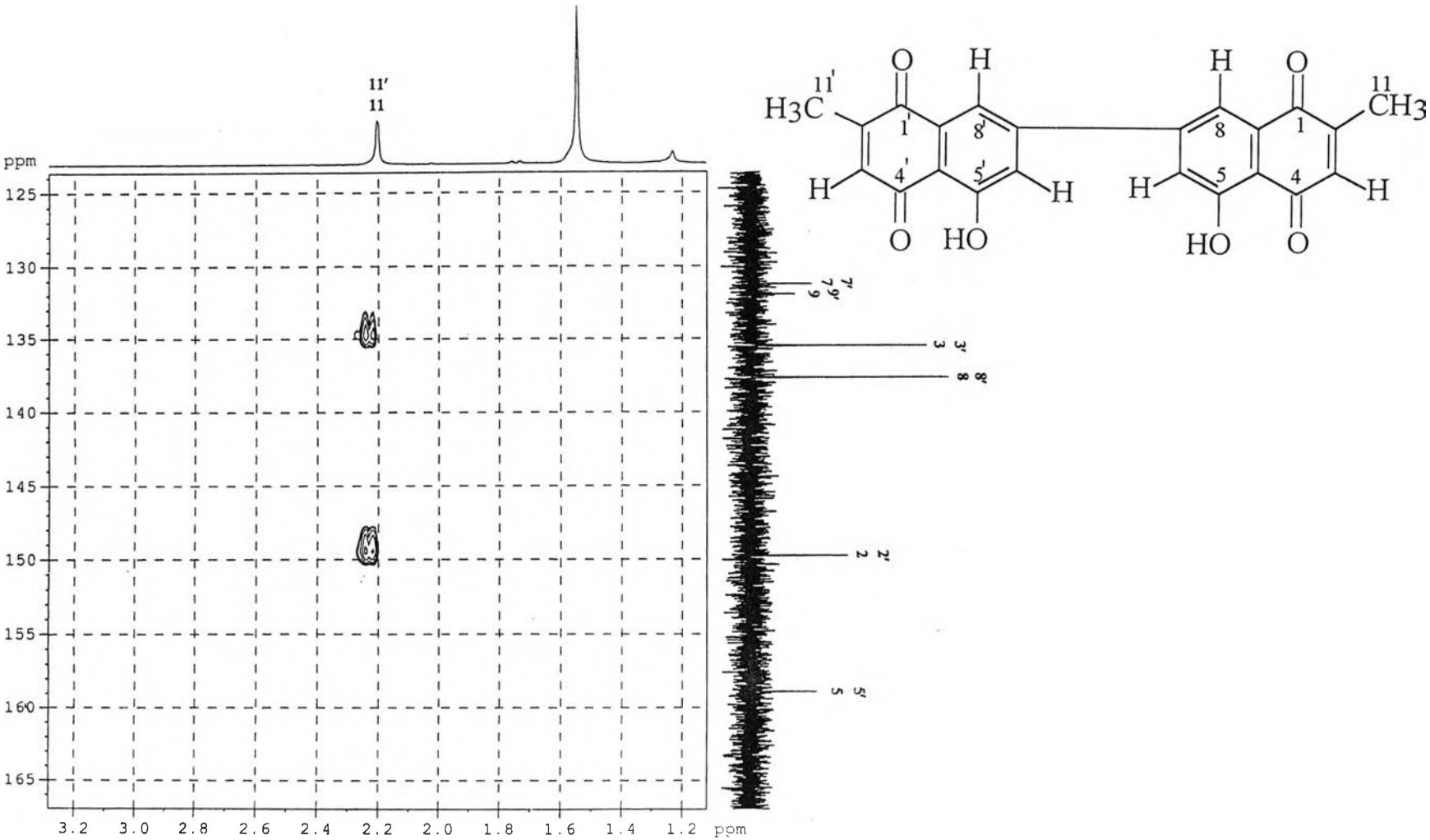


Figure 31d ^{1}H-^{13}C HMBC spectrum of compound DU4 (expanded)

6. Identification of Compound DU5

Compound DU5 was obtained as orange needles from hexane. The IR spectrum (Figure 35) exhibited maximum absorption bands at 3450 cm^{-1}, indicating the presence of hydroxyl group in the molecule and showed two distinct carbonyl bands at 1647 and 1612, cm^{-1} thus demonstrating both free and hydrogen-bonded carbonyls and, finally, stretching of aromatic ring at 1457 cm^{-1} (Waterman and Mbi, 1979). The complex UV spectrum with maxima at 210, 255, 267 and 424 nm was typical of a monomeric quinone (Tezuka *et al.*, 1973). The EIMS spectrum (Figure 36) exhibited a molecular ion peak at *m/z* 188 then showed the loss of CH_3, CO, CH_3+CO and 2CO in the monomeric quinone as fragment peaks at *m/z* 173, 160, 145 and 132, respectively.

The ^{1}H NMR spectrum of DU5 (Figure 36a-36b) showed a downfield singlet signal of hydrogen-bonded hydroxy group at δ 12.01 ppm. Three aromatic protons were observed as a doublet of doublets at d 7.62 ppm (*J*=7.4, 2.1 Hz, H-8), a triplet at δ 7.61 ppm (*J*=7.4 Hz, H-7) and a doublet of doublet at δ 7.63 ppm (*J*=7.4, 2.1 Hz, H-6) while one quinonoid proton was presented at δ 6.82 ppm (d, *J*= 1.0 Hz, H-3). The remaining signal is that of the methyl resonance at δ 2.20 ppm (d, *J*=1.0 Hz, H_3-11).

The ^{13}C NMR spectrum (Figure 37) showed eleven carbon signals, of which the DEPT-135 experiment (Figure 38) indicated these as the signals of one methyl carbons at δ 16.5 ppm, four methine carbons at δ 119.3, 124.1, 135.4 and 136.1 ppm, and six quaternary carbons at δ 190.2, 184.8, 161.1, 149.6, 132.0 and 115.1 ppm. Two of the most downfield carbon signals were assigned as those of the quinonoid carbonyl groups.

The structure of DU5 was therefore identified as that of the naphthoquinone plumbagin (**109**) by comparison with reported data (Khan and Rwekika, 1998) and also confirmed by HETCOR experiment (Figure 39a-39b) which provided the information for the assignments of the methine carbons at C-3 (δ 135.4 ppm), C-6 (δ 124.1 ppm), C-7 (δ 136.1 ppm), C-8 (δ 119.3 ppm) and the methyl carbon at C-11 (δ 16.5 ppm), and HMBC experiment (Figure 43a-43c) which displayed correlations of the H_3-11 proton signal at δ 2.20 ppm with C-1 (δ 184.7 ppm), C-2 (δ 149.6 ppm), and C-3 (δ 135.4 ppm), confirming its position as attached at C-2.

The hydroxy proton at δ 12.01 ppm showed long-range coupling with C-5 (δ 161.1 ppm), C-6 (δ 124.1 ppm) and C-10 (δ 115.1 ppm), as depicted in scheme 4. The ^{1}H and ^{13}C NMR assignments and long-range correlation of compound DU5 are summarized in Table. 14

Table 14 ^{1}H, ^{13}C-assignments and HMBC correlations of compound DU5

position	^{1}H	^{13}C	HMBC correlated with carbon
	δ_H (ppm)	δc (ppm)	signals at δc (ppm)
1	-	184.7	-
2	-	149.6	-
3	6.82 (d, *J*=1.0 Hz)	135.4	184.7, 115.1, 16.5
4	-	190.2	-
5	-	161.1	-
6	7.63 (1H, *J*=7.4, 2.1 Hz)	124.1	161.1, 115.1
7	7.61 (1H, *J*=7.4 Hz)	136.1	161.1, 124.1, 132.0
8	7.24 (1H, *J*=7.4, 2.1 Hz)	119.3	184.7, 124.1, 115.1
9	-	132.0	-
10	-	115.1	-
11	2.20 (3H, d, *J*=1.0 Hz)	16.5	184.7, 149.6, 135.4
5-OH	12.01 (1H, s)	-	161.1, 124.1, 115.1

The results obtained from HMBC spectral examination suggested that the assignments of DU5

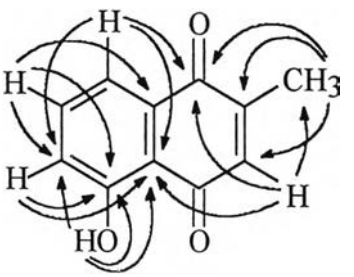


Figure 32 Structure of DU5 as confirmed by the HMBC experiment

Therefore, it was concluded that DU5 is the naphthoquinone plumbagin. The compound was previously isolated from several species of the family Ebenaceae, for example, *Diospyros kaki* (Tezuka *et al.*, 1972), *D. samoensis* (Richomme *et al.*, 1991), *D. chamaethamnus* (Costa *et al.*, 1998), *D. greeniwayi* (Khan and Rwekika, 1998). Plumbagin is the first naphthoquinone in higher plants shown to be formed through the acetate pathway which has long been known for the formation of naphthoquinone in fungi (Thomson, 1971). It is interesting to note that, plumbagin, found in significant quantity in *D. mespiliformis*, was shown to have antibacterial activity against a wide rang of organisms (Lajubutu *et al.*, 1995). Plumbagin found in the stems of *D. maritima* also exhibited strong cytotoxicity against HEPA-3B (hepatoma), KB (nasopharynx carcinoma cells), COLO-205 (colon carcinoma) and HELA (cervix carcinoma) cells with ED_{50} of 0.25, 1.81, 0.13 and 0.27 μg/mL, respectively (Kuo *et al.*, 1997). The compound has been studied using a blood platelet aggregation assay (Kuke *et al.*, 1998) and was found to show maximum activity. Furthermore, plumbagin was reported as showing ecdysis inhibition activity against the larva of pink bollworm (*Pectinophora gossypiella*) and exhibiting nematocidal activity against the larva of dog roundworm (*Toxocara canis*) (Higa *et al.*, 1998). The quinone is therefore expected to be utilized both in the medicinal and agricultural fields.

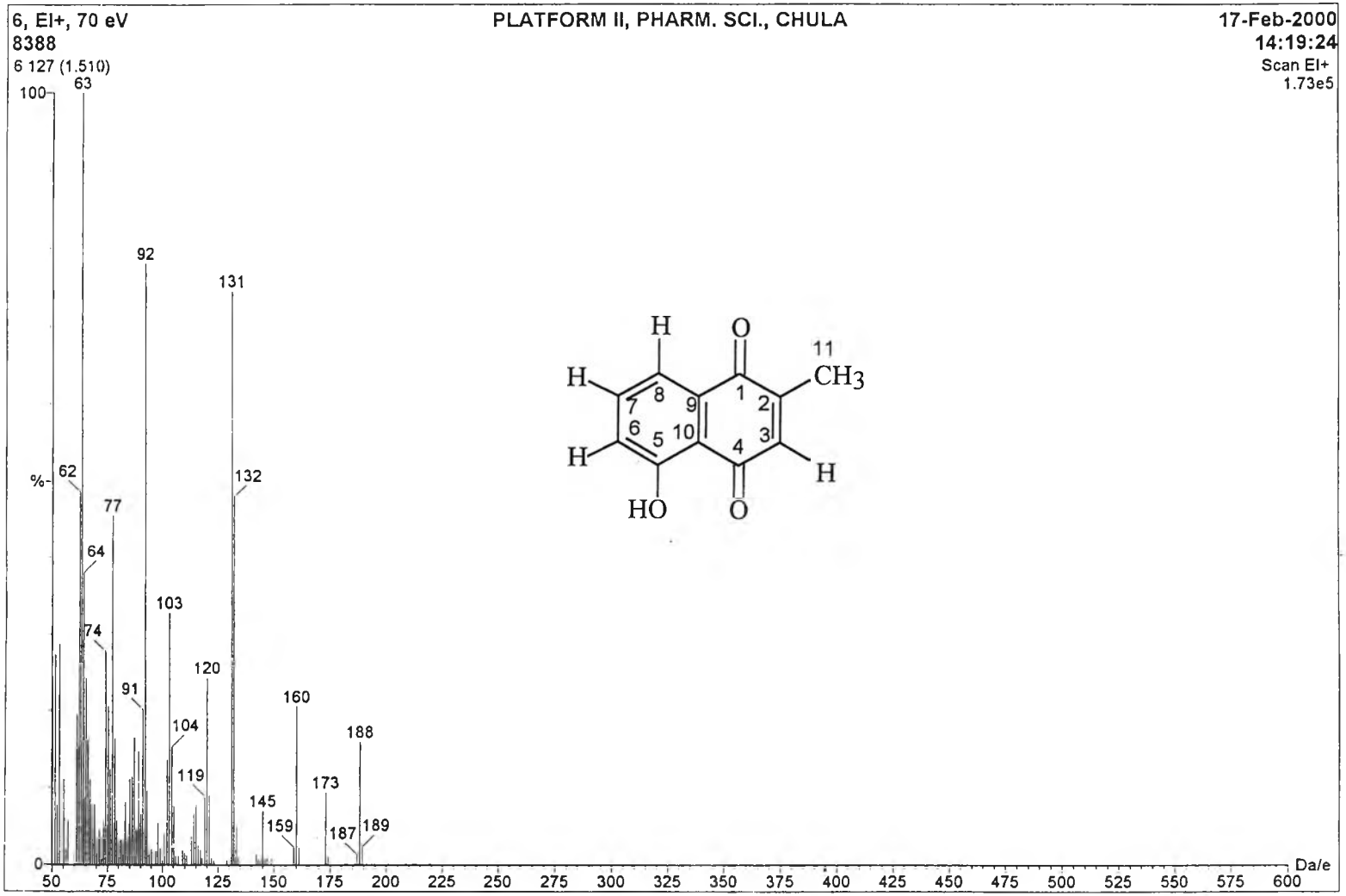


Figure 33 EI mass spectrum of compound DU5

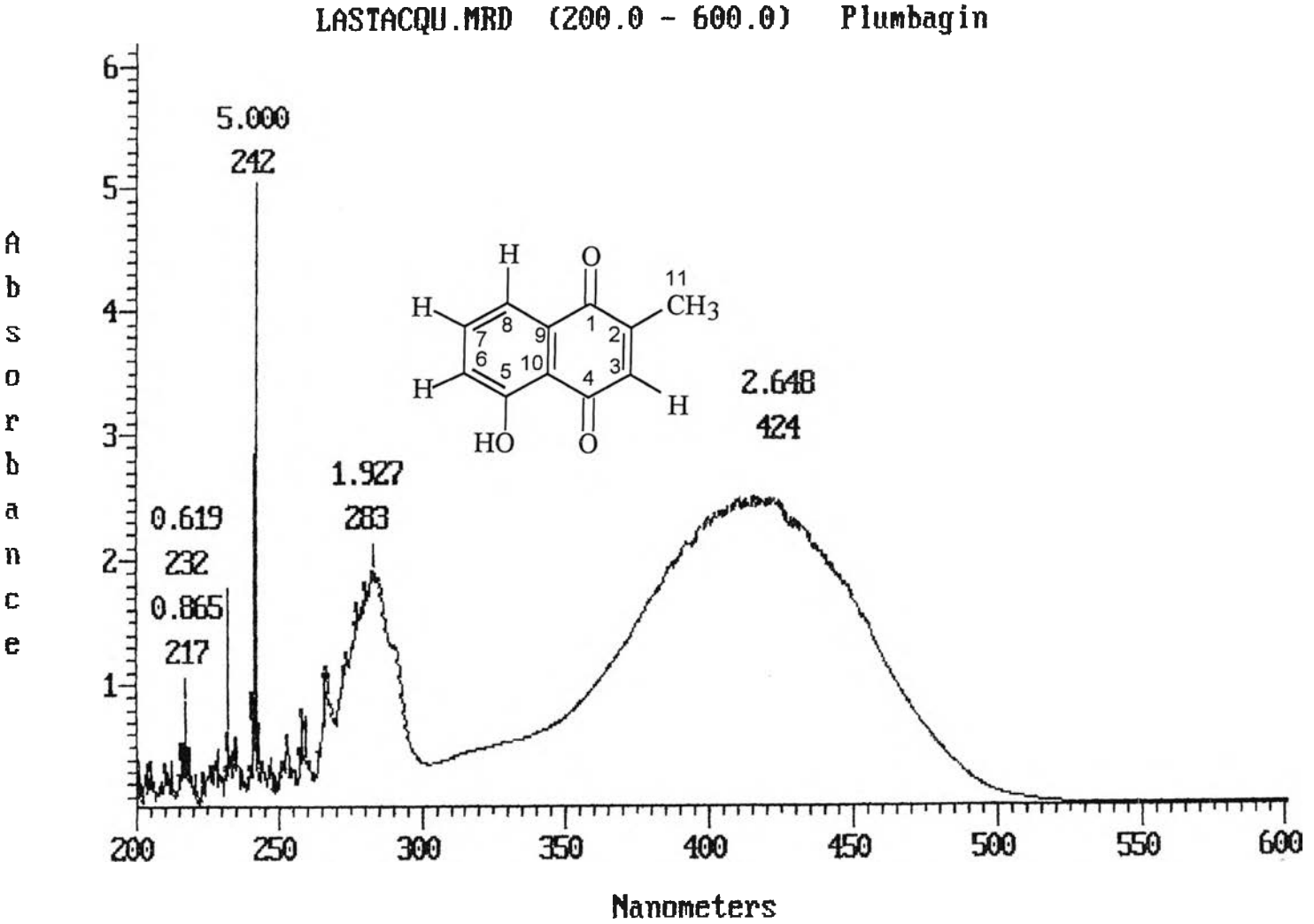


Figure 34 UV spectrum of compound DU5

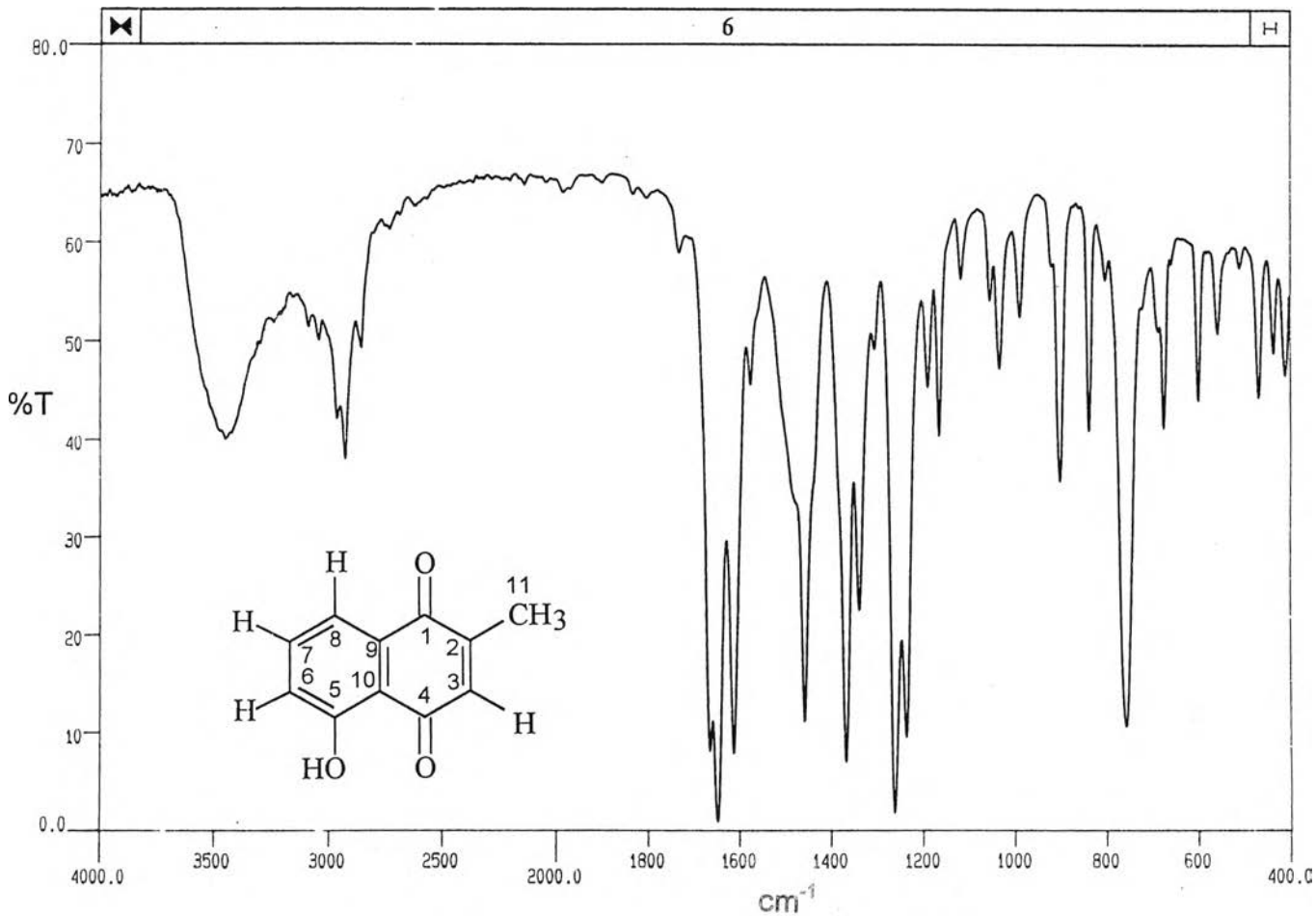


Figure 35 IR spectrum of compound DU5

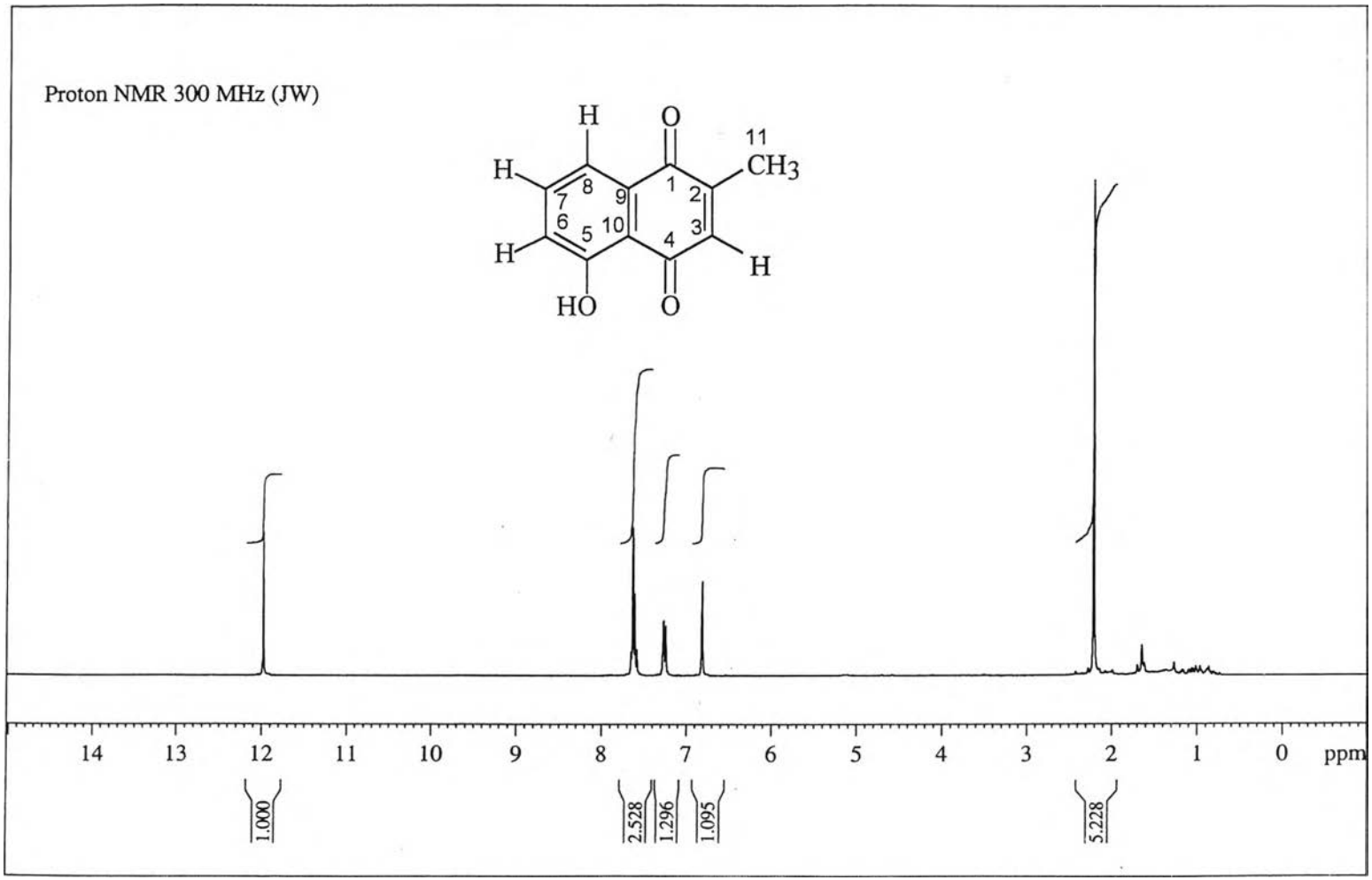


Figure 36a The 300 MHz ^{1}H NMR spectrum of compound DU5

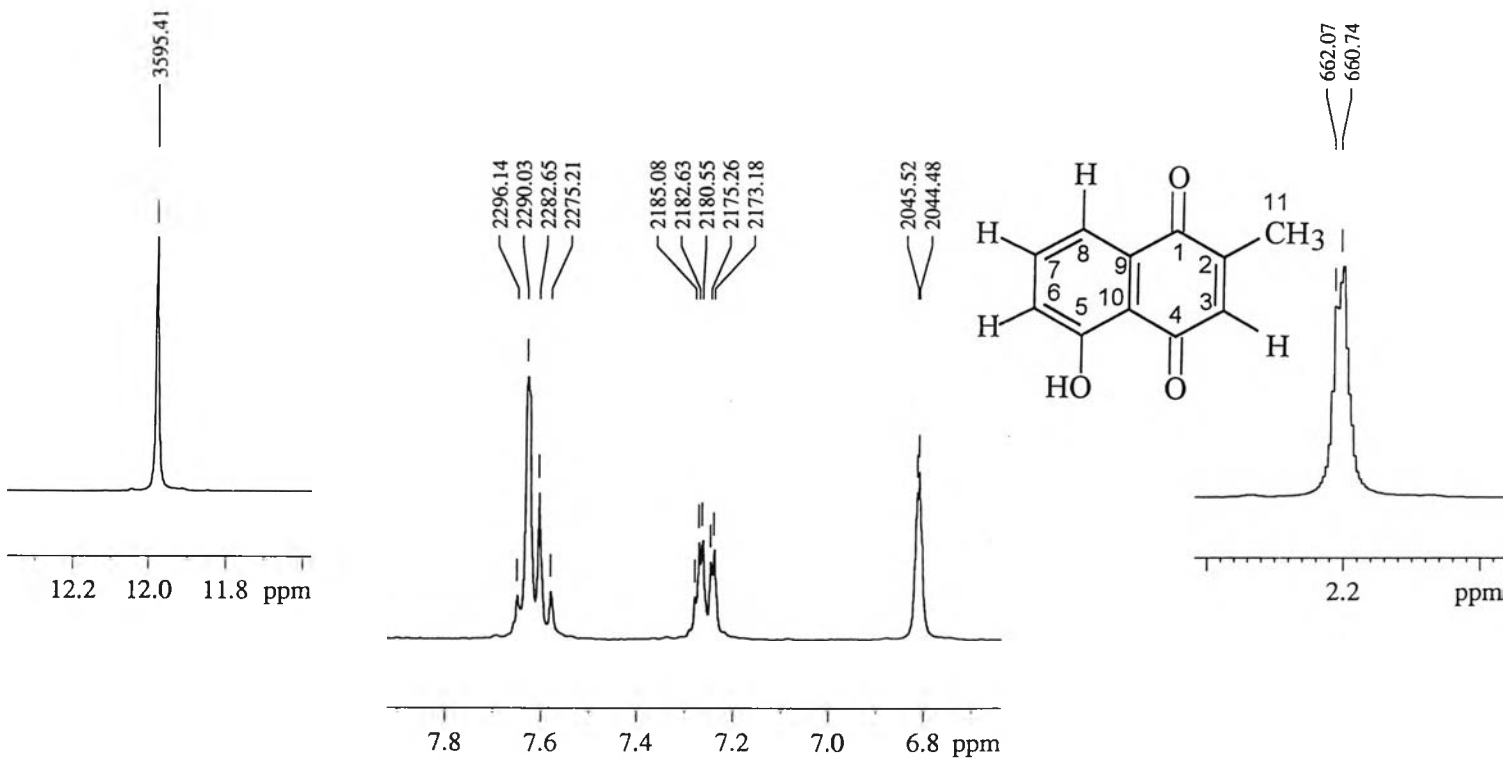


Figure 36bThe 300 MHz ^{1}H NMR spectrum of compound DU5 (expanded 12.37–2.02 ppm)

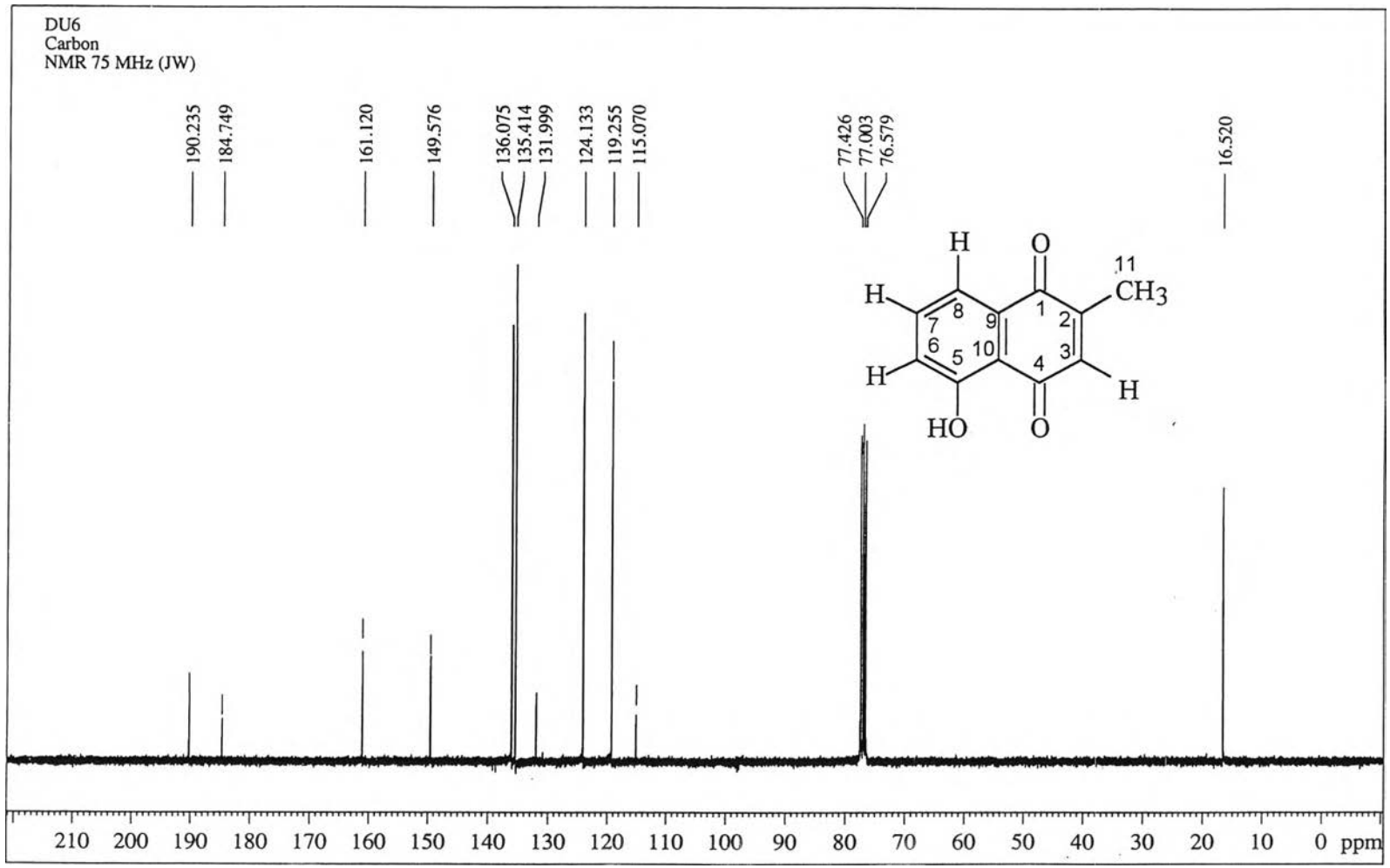


Figure 37 The 75 MHz ^{13}C NMR spectrum of compound DU5

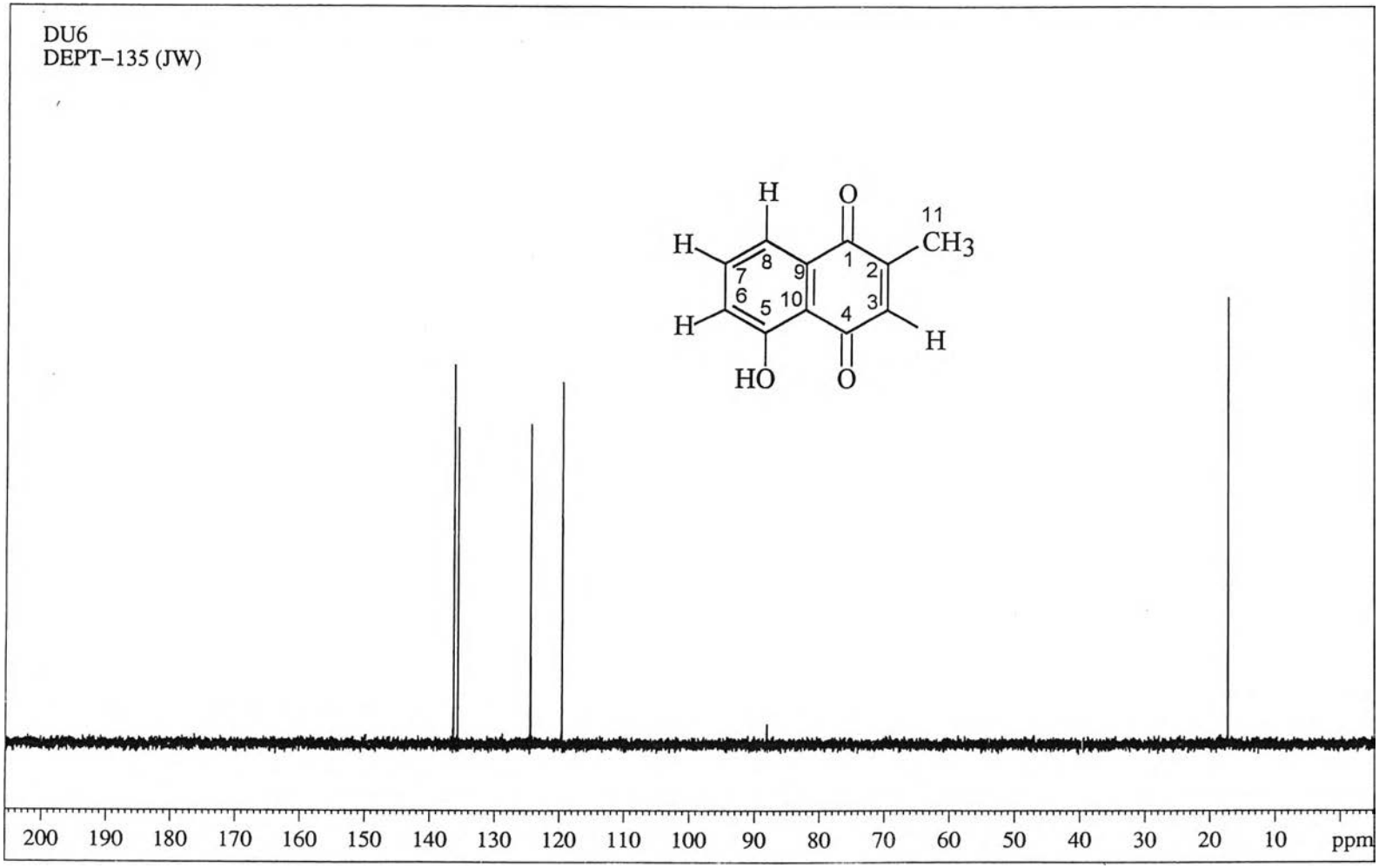
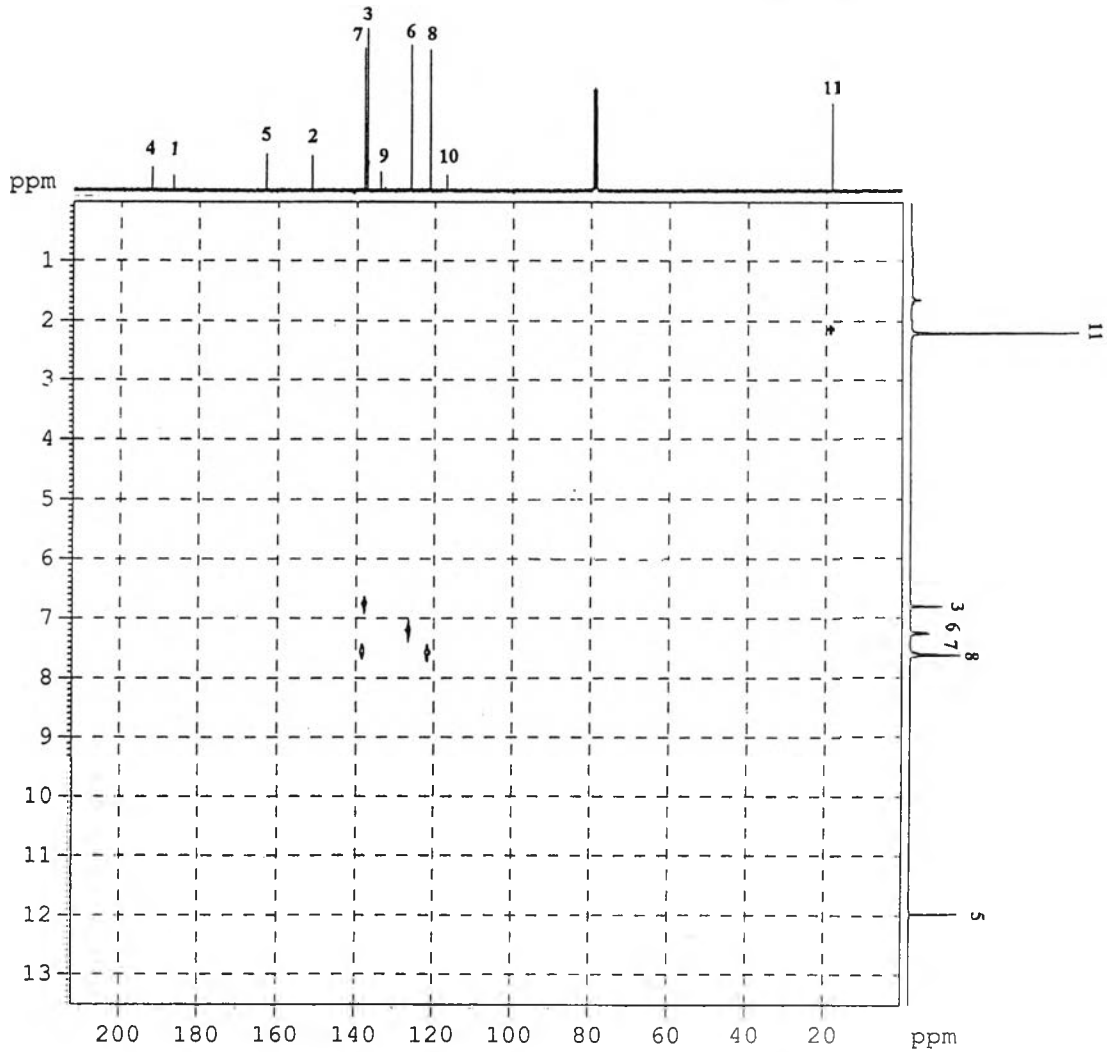


Figure 38 The DEPT-135 spectrum of compound DU5



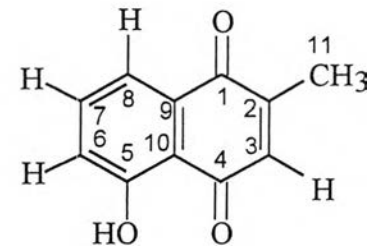
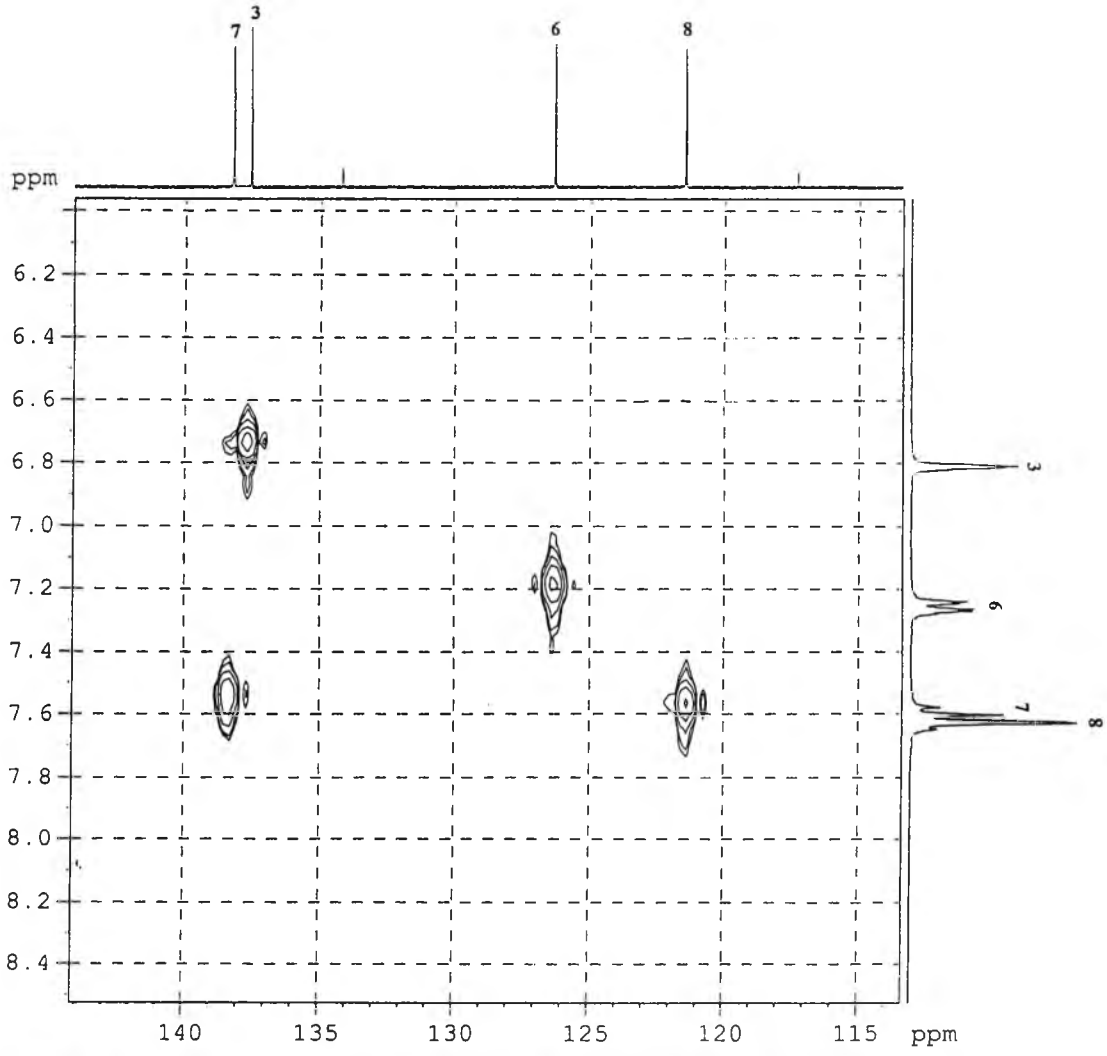


Figure 39a The HETCOR spectrum of compound DU5



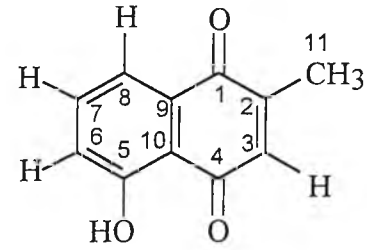
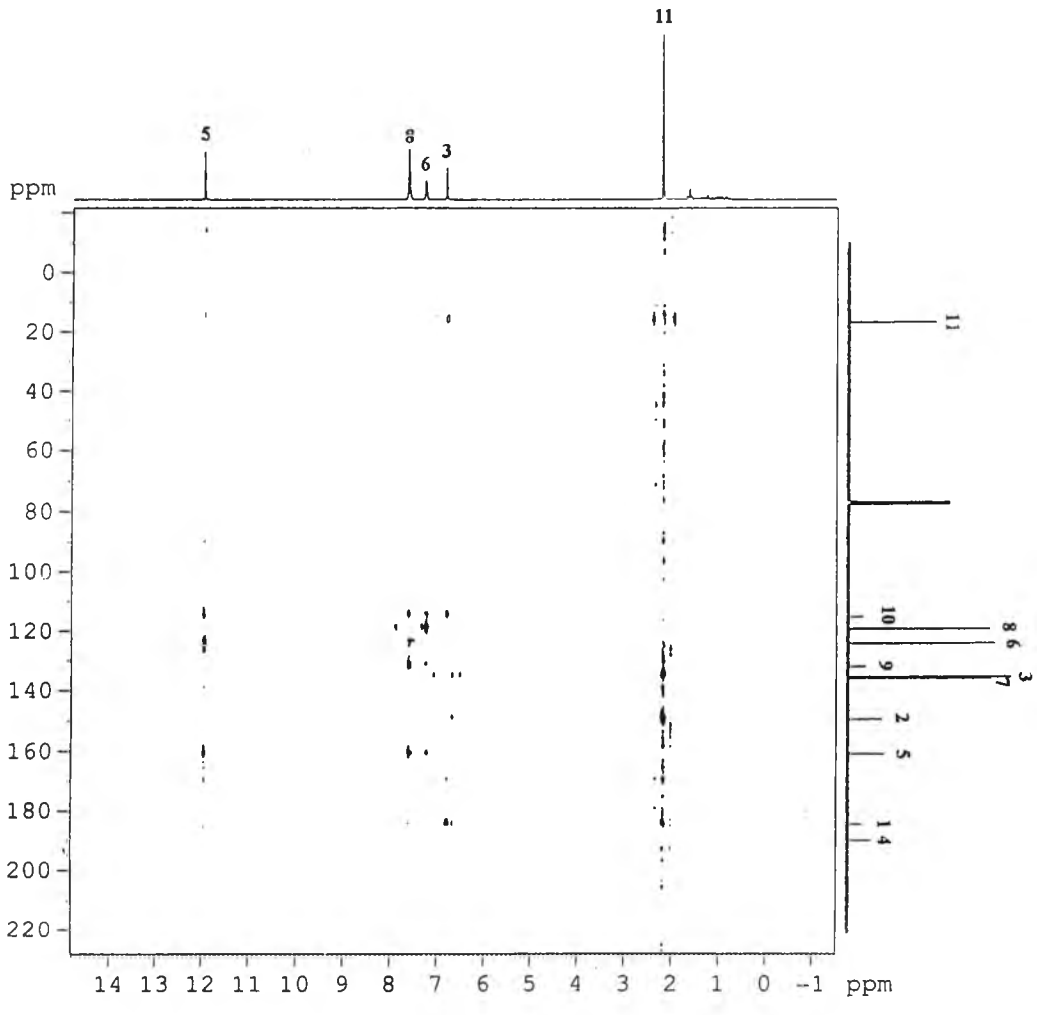


Figure 39b The HETCOR spectrum of compound DU5 (expanded)



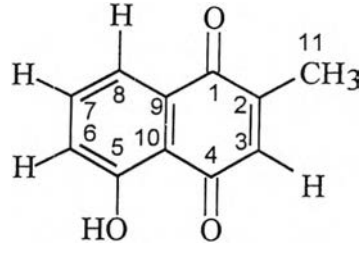
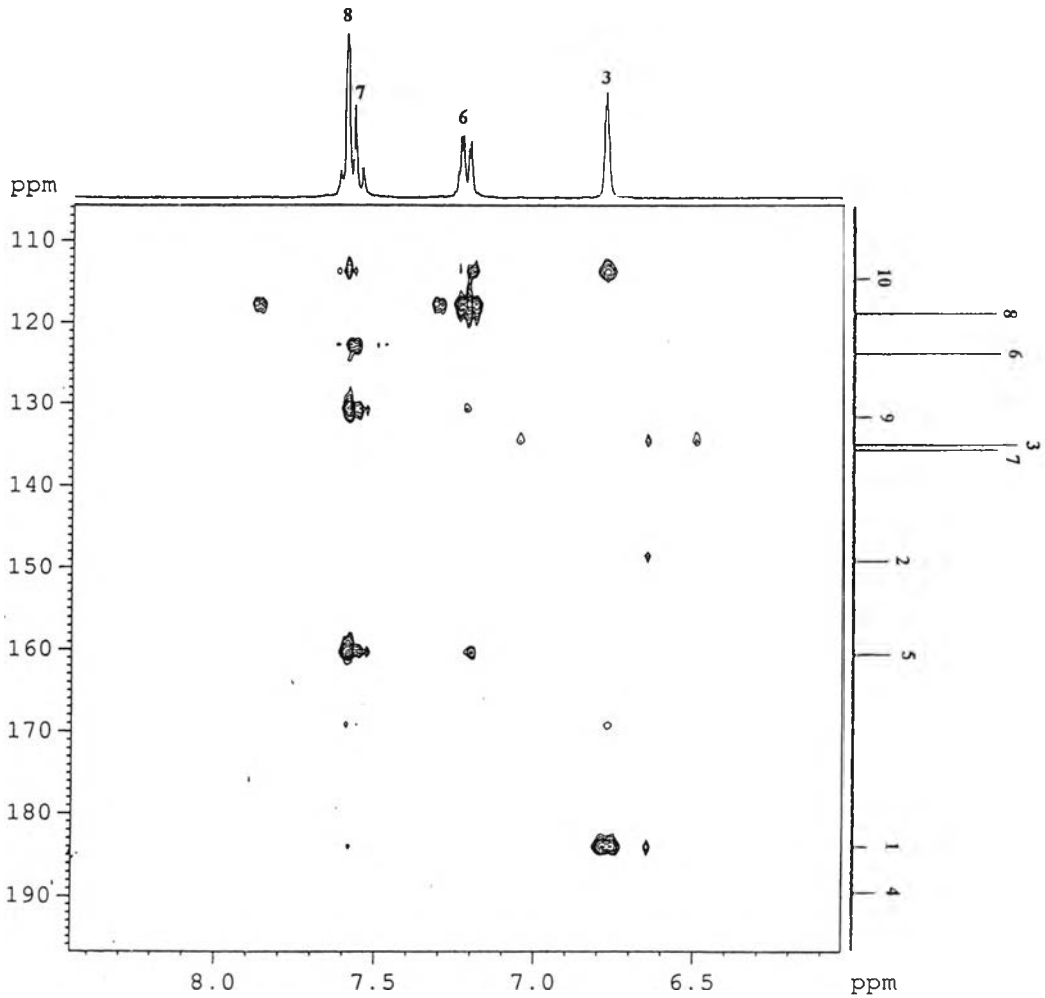


Figure 40a ^{1}H-^{13}C HMBC spectrum of compound DU5



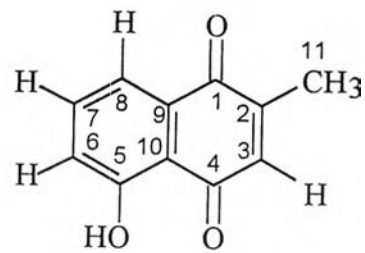
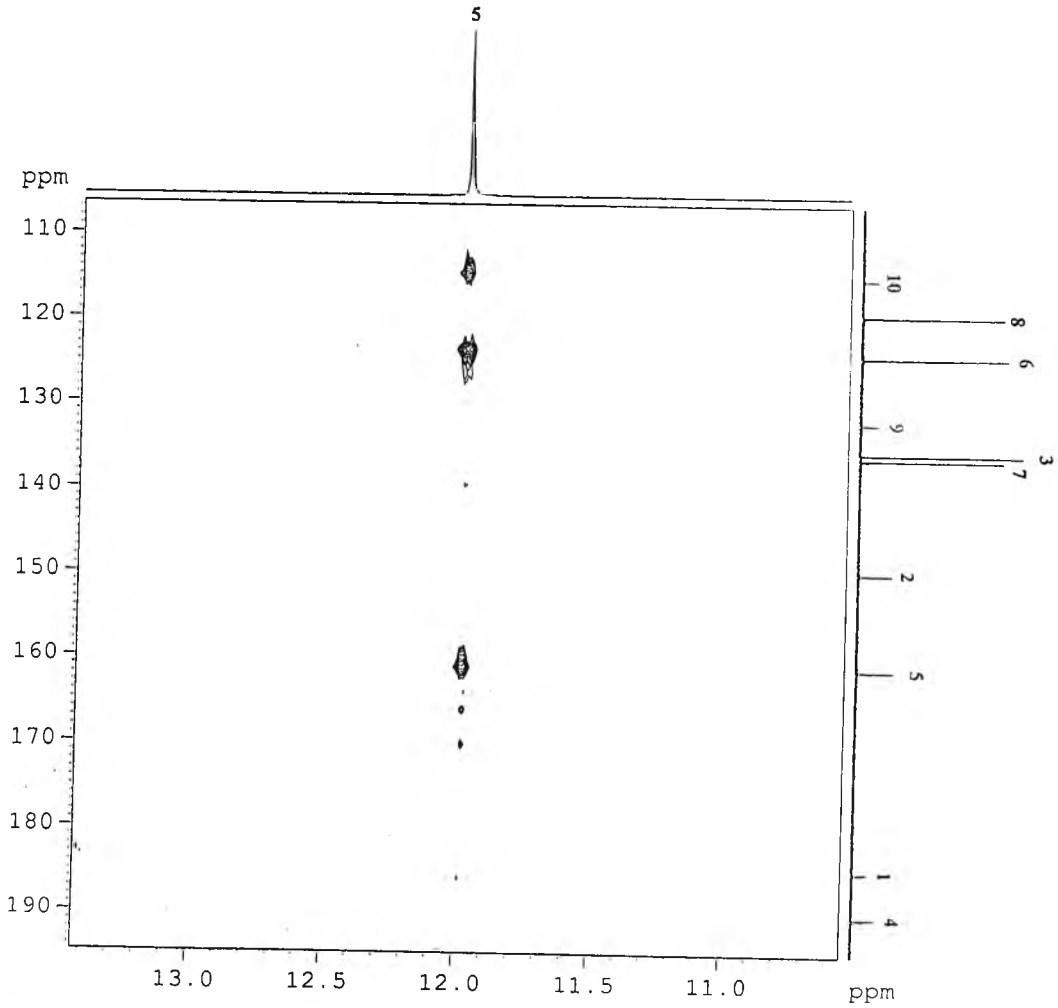


Figure 40b ^{1}H-^{13}C HMBC spectrum of compound DU5 (expanded)



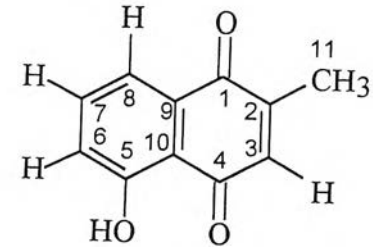


Figure 40c ^{1}H-^{13}C HMBC spectrum of compound DU5 (expanded)

6. Identification of Compound DU6

Compound DU6 was obtained as white needles from acetone and gave violet color to vanillin-sulphuric acid reagent, suggesting its triterpenic nature. The IR spectrum (Figure 42) revealed absorption bands for hydroxy group at 3449 cm^{-1} (O-H stretching) and (C-H stretching) at 2932 and 2869 cm^{-1}

The ^{1}H NMR spectrum of DU6 (Figure 43a-43b) displayed the signals for six methyl protons as singlets at δ 0.71, 0.80, 0.95, 0.98, 1.38, and 1.68 ppm (3H each). Furthermore, exomethylene protons appeared as a pair of broad singlets at δ 4.52 and 4.65 ppm (1H each), while two doublets at δ 3.80 and δ 3.37 ppm (1H each, *J*=2.3 Hz) could be attributed to hydroxy methylene protons. Another one proton double doublet at δ 3.15 ppm (*J*=4.4 and 2.1 Hz) could be assigned to carbinylic proton. Evidence from the ^{1}H NMR suggested the presence of a lupane skeleton in the molecule.

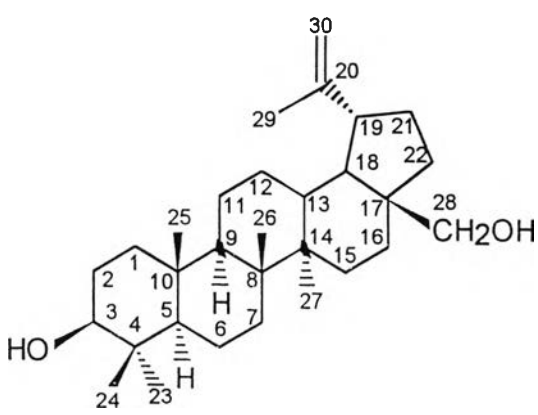
The ^{13}C-NMR spectrum (Figure 44a-44b) showed the signals of 30 carbon atoms, supporting the assignment of DU6 as a triterpenoid derivative. DEPT-90 and DEPT-135 experiments (Figure 45a-45b) helped in identifying the of signals six methyl carbons at δ 14.9,15.5, 16.1, 16.2, 19.2, and 28.1 ppm, twelve methylene carbons at δ 18.4, 20.9, 25.3, 27.1, 27.5, 29.3, 29.8, 34.1, 34.3 38.8, 60.6, and 109.6 ppm, six methine carbons at δ 37.4, 47.8, 48.8, 50.4, 55.3, and 79.0 ppm, and five quaternary carbons at δ 37.2, 38.9, 41.0, 42.8, and 150.3 ppm.

Mass spectrum of DU6 (Figure 41) showed a molecular ion peak at *m/z* 442 corresponding to the molecular formula $C_{30}H_{50}O_2$. The mass fragment peaks at *m/z* 135, 207, 220 and 234 were the results of cleavage at different positions across the C ring of lupane skeleton. This also indicated that, in addition to a hydroxy group normally found at C-3 on ring A, there is another hydroxy function located on either ring D or E.

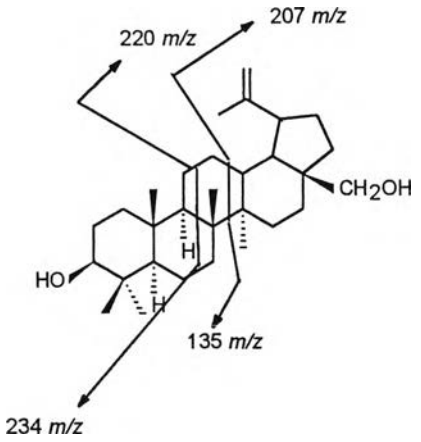
Therefore, it was concluded that compound DU6 is the triterpenoid betulin (**67**) by comparison of its ^{13}C NMR chemical shift data with reported values (Table 15)

Table 15 Comparison of ^{13}C-NMR chemical shifts in $CDCl_3$ of DU6 and betulin (Sholichin *et al*, 1980)

position	DU7 (ppm)	betulin (ppm)	position	DU7 (ppm)	betulin (ppm)
1	38.8	38.7	16	29.2	29.2
2	27.5	27.4	17	47.8	47.9
3	79.0	79.1	18	47.8	47.9
4	38.9	38.8	19	48.8	48.8
5	55.3	55.3	20	150.3	150.5
6	18.4	18.3	21	29.9	29.8
7	34.3	34.3	22	34.1	34.0
8	41.0	41.0	23	28.1	28.1
9	50.4	50.5	24	15.5	15.4
10	37.4	37.3	25	16.1	16.1
11	21.0	20.9	26	16.2	16.1
12	25.3	25.2	27	14.9	14.8
13	37.2	37.2	28	60.6	60.6
14	42.8	42.8	29	19.2	19.1
15	27.2	27.1	30	109.6	109.7



Betulin (DU6)



Scheme 3 Mass Spectral Fragmentation of compound DU6

The occurrence of betulin was reported mainly from the order Buxales, Dilleniales, Ebenales and Lamiales (Dahlgren *et al.*, 1981). It has been isolated from several ebenaceous species, i.e. *Diospyros walkeri* (Herath *et al.*,1978), *D. wallichii* (Zakaria *et al.*, 1984), *D. zenkeri* (Zhong *et al.*, 1984) and *D. maritima* (Kuo *et al*, 1997c). Betulin has been used as an antiseptic (Batta and Rangaswami, 1973), and also showed inhibitory effect against Epstein-Barr virus activation (Konoshima *et al.*, 1987). The compound also exhibited antitumor activity against human epidermoid carcinoma of the nasopharynx *in vitro* (Miles *et al.*, 1974) and Walker 256 tumour system (Sheth *et al.*, 1973). Betulin from the leaves of *D. leucomelas* showed anti-inflammatory activity in the carrageenan and serotonin paw edema test and TPA and EPP ear edema test (Recio *et al.*, 1995).

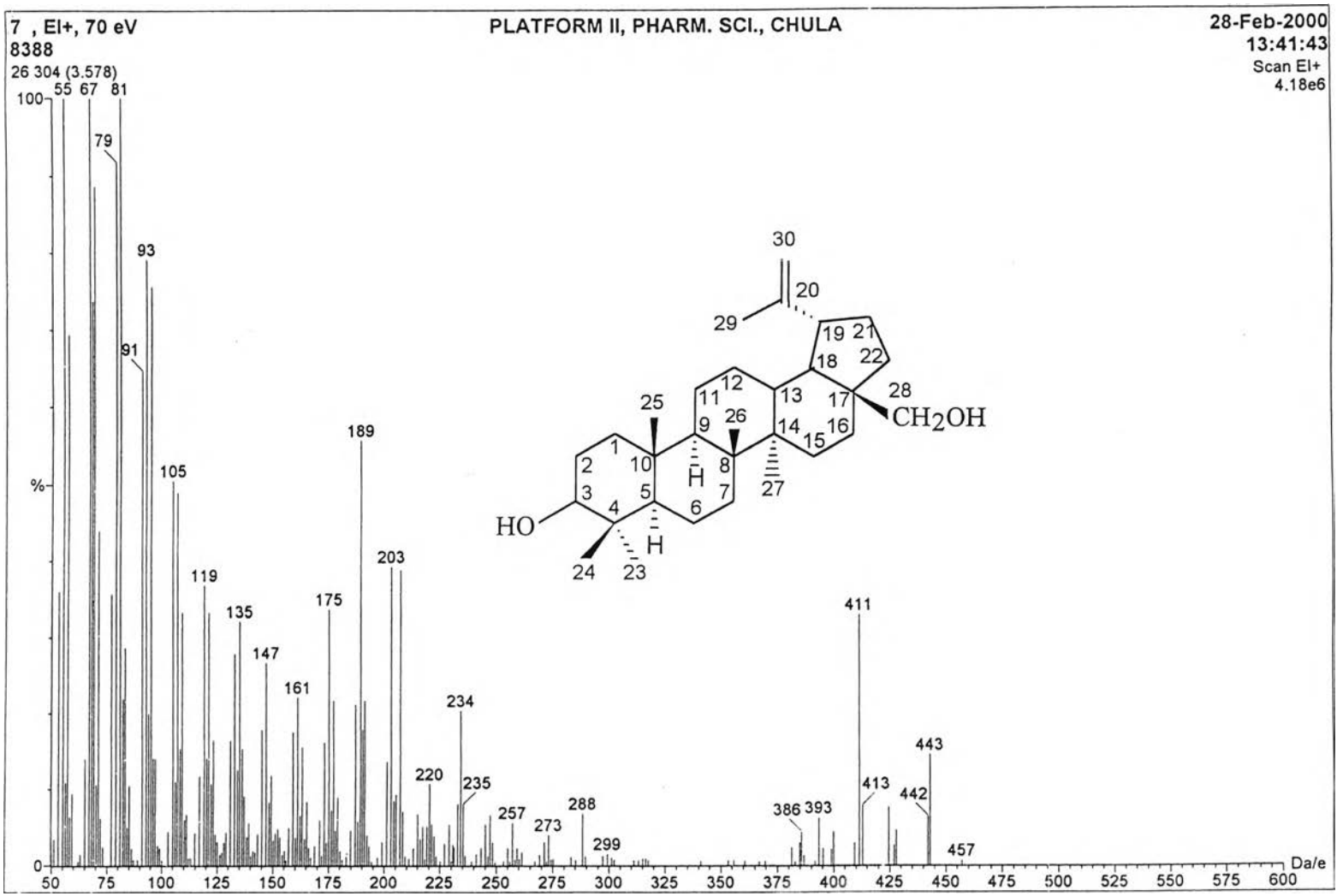


Figure 41 EI mass spectrum of compound DU6

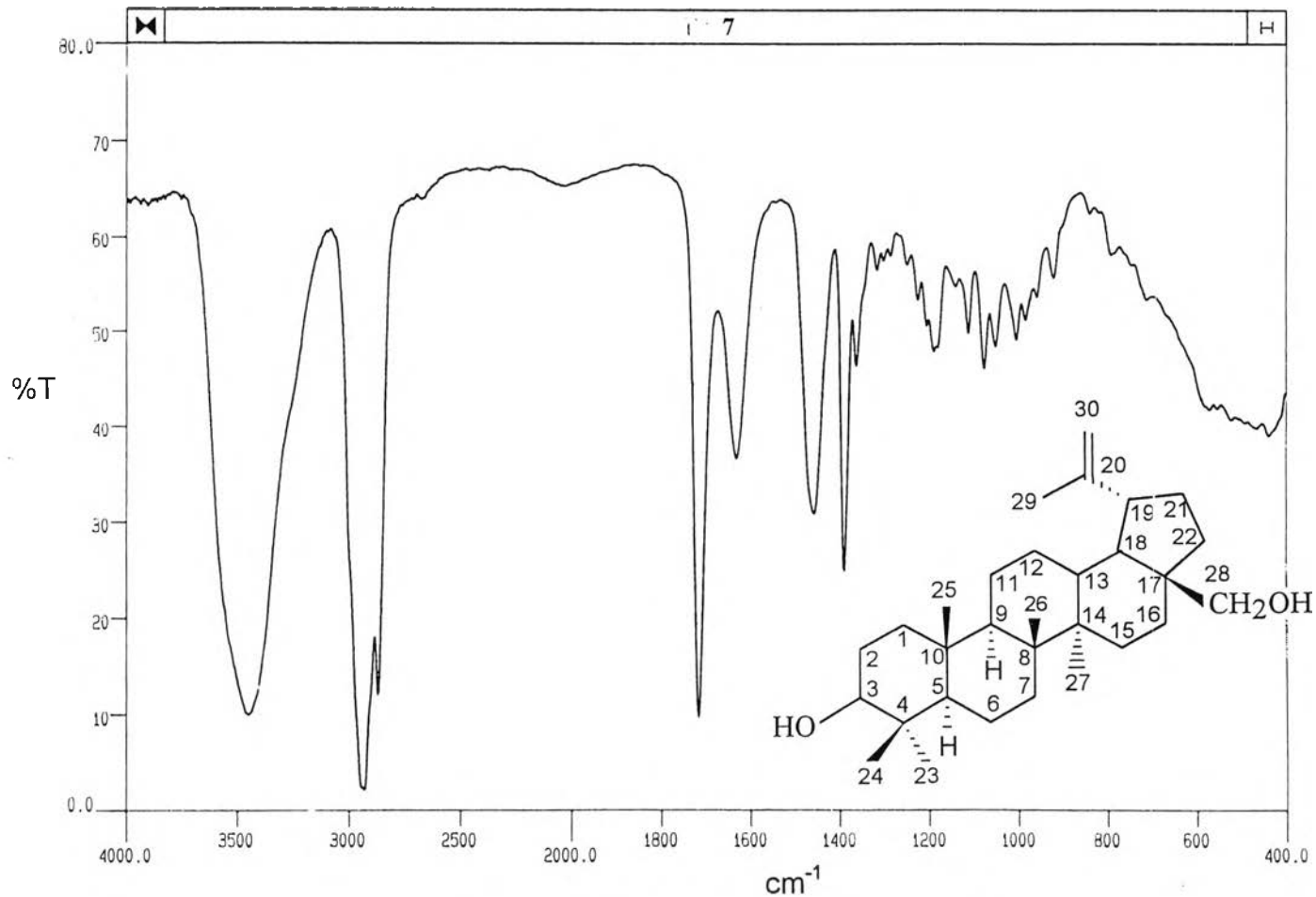


Figure 42 IR spectrum of compound DU6

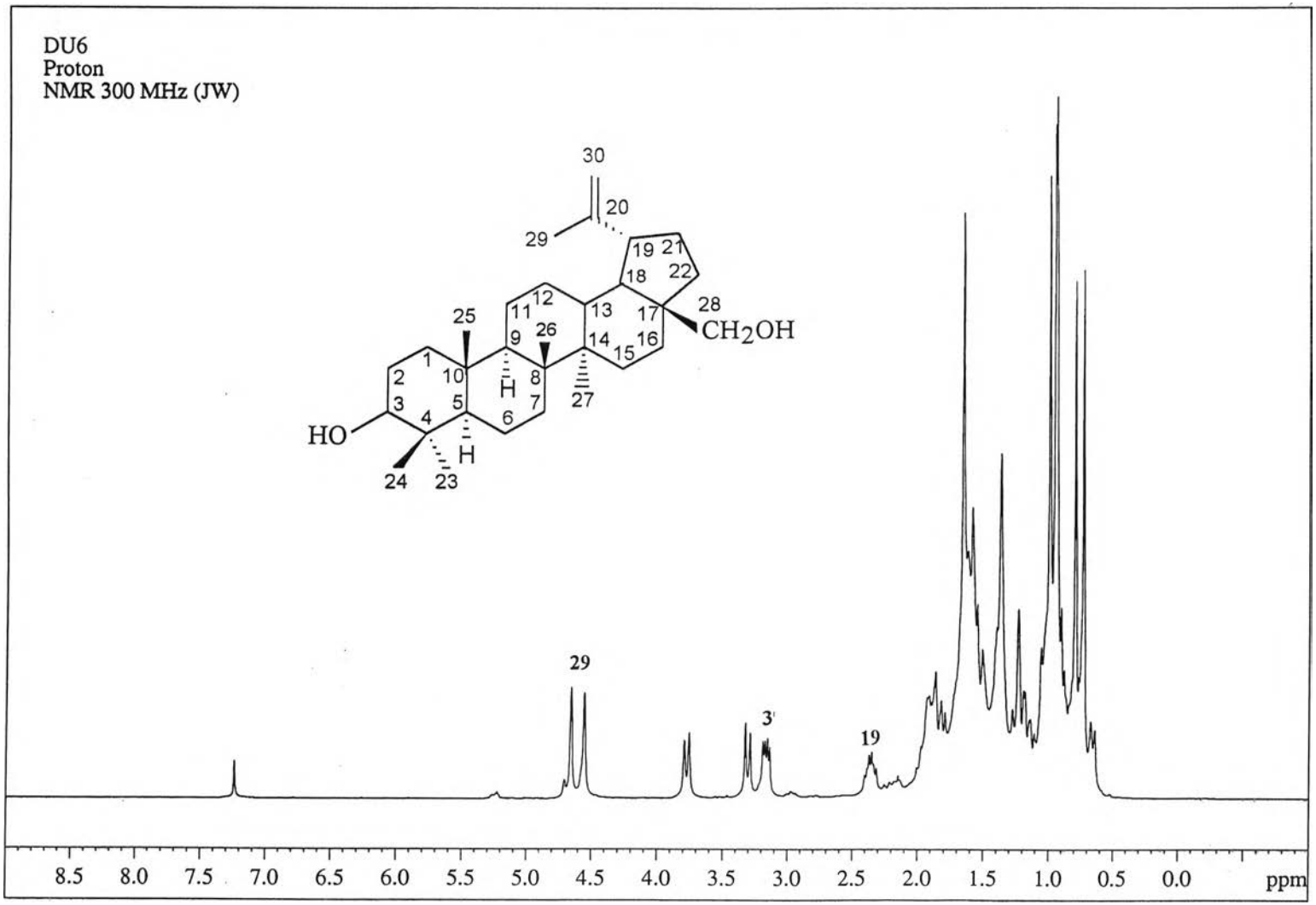


Figure 43a The 300 MHz ^{1}H NMR spectrum of compound DU6

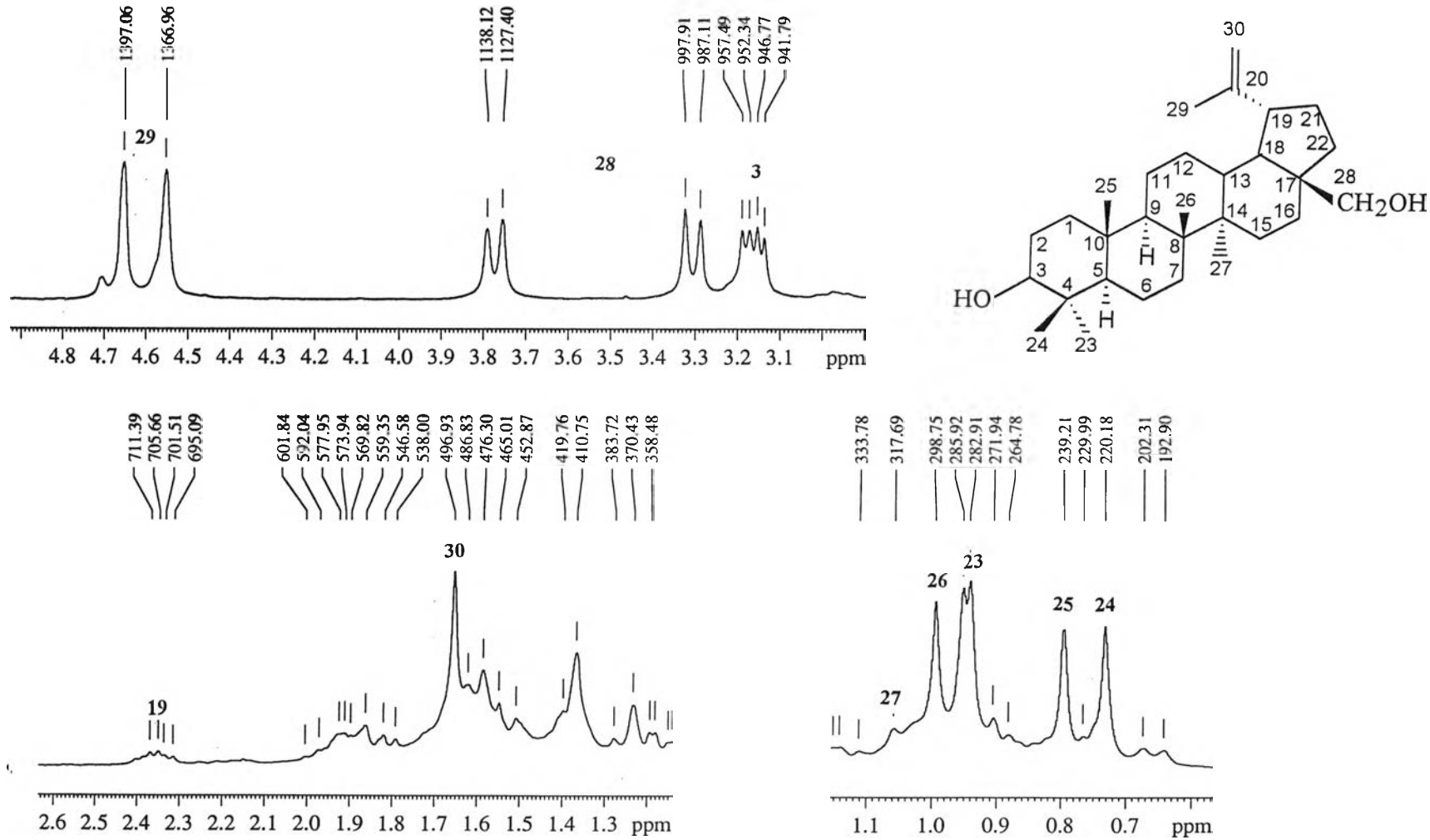


Figure 43b The 300 MHz ^{1}H NMR spectrum of compound DU6 (expanded from 4.92–0.97 ppm)

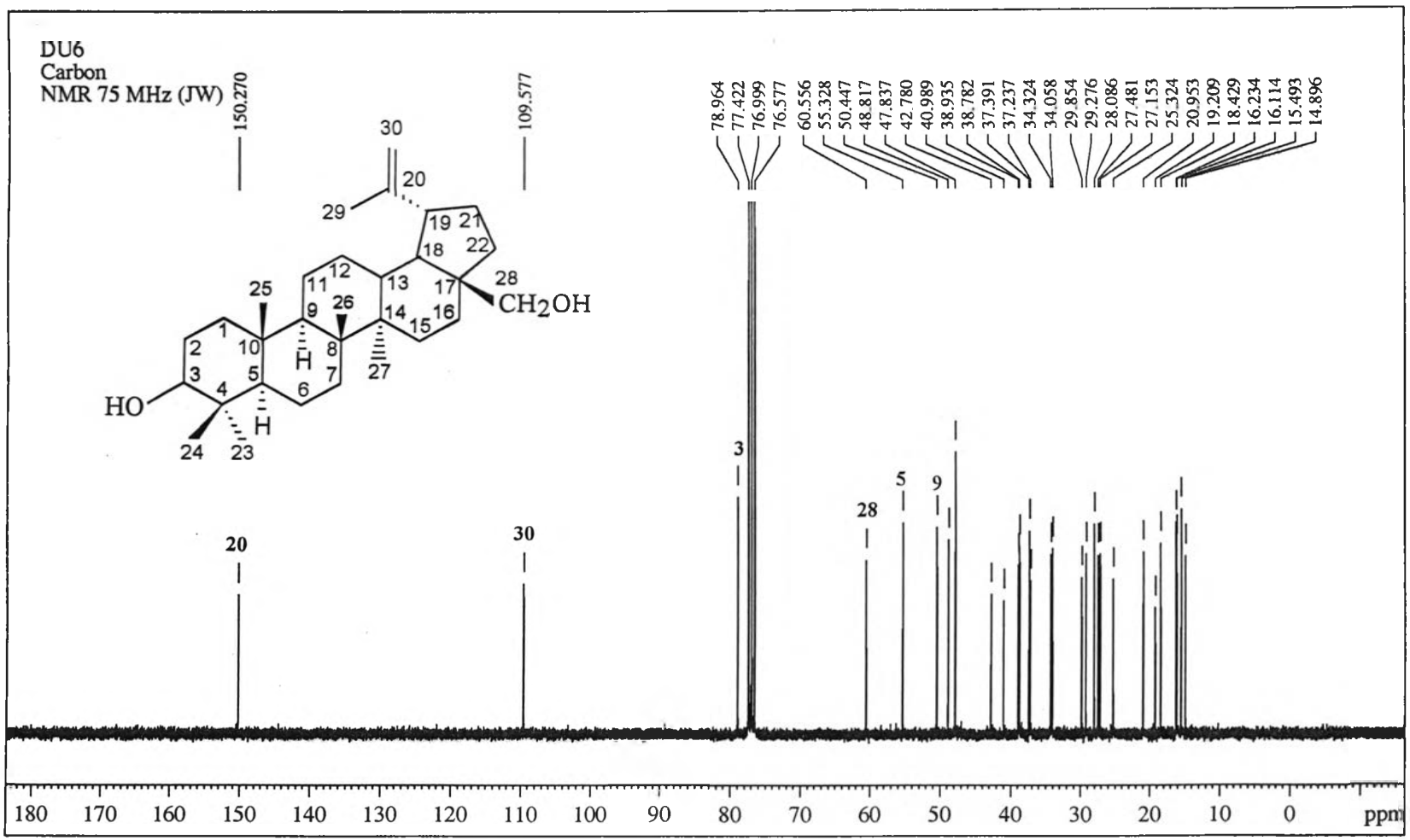


Figure **44a** The 75 MHz ^{13}C NMR spectrum of compound DU6

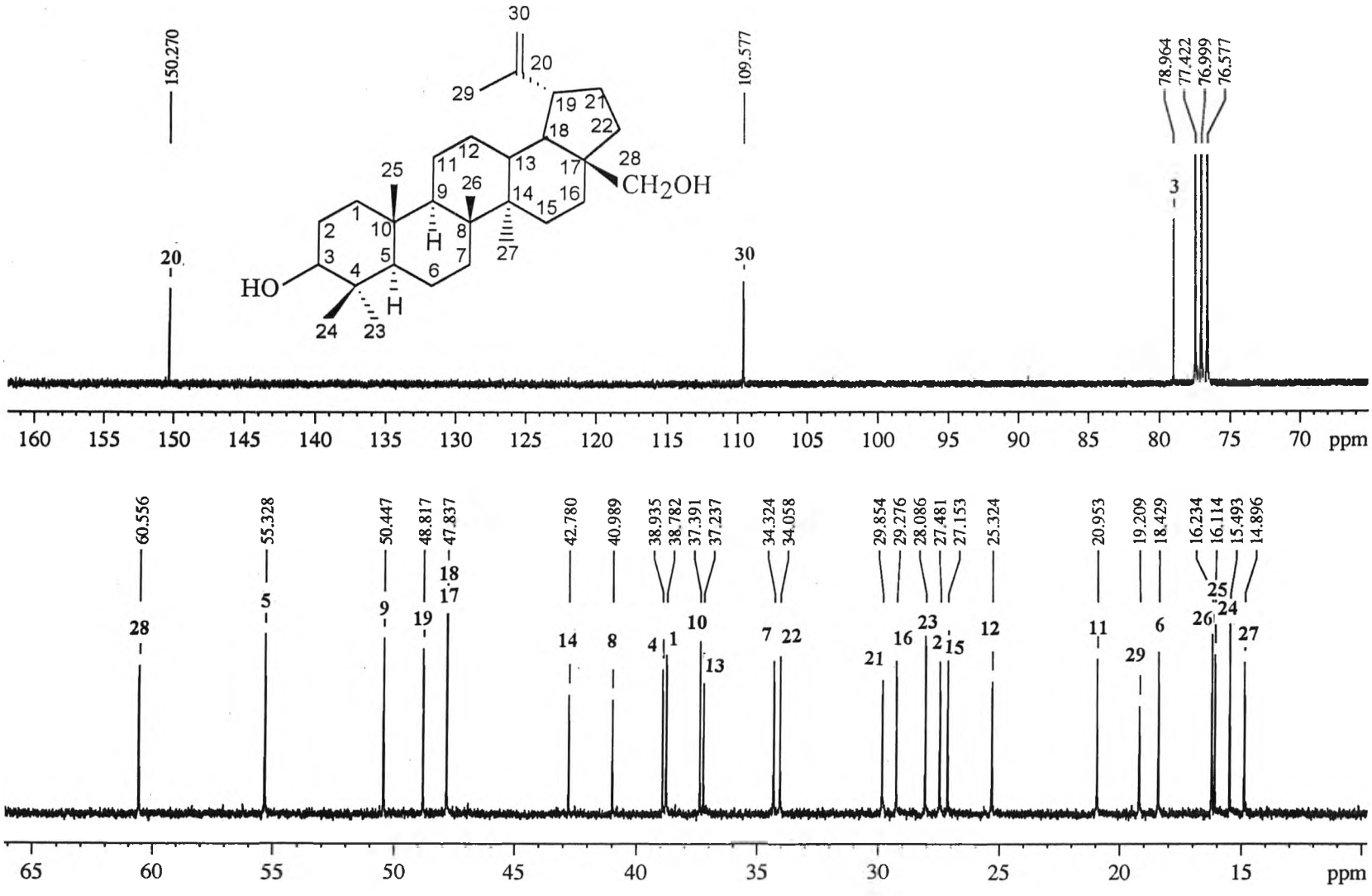


Figure **44b** The 75 MHz ^{13}C NMR spectrum of compound DU6 (expended from 161.9–9.8 ppm)

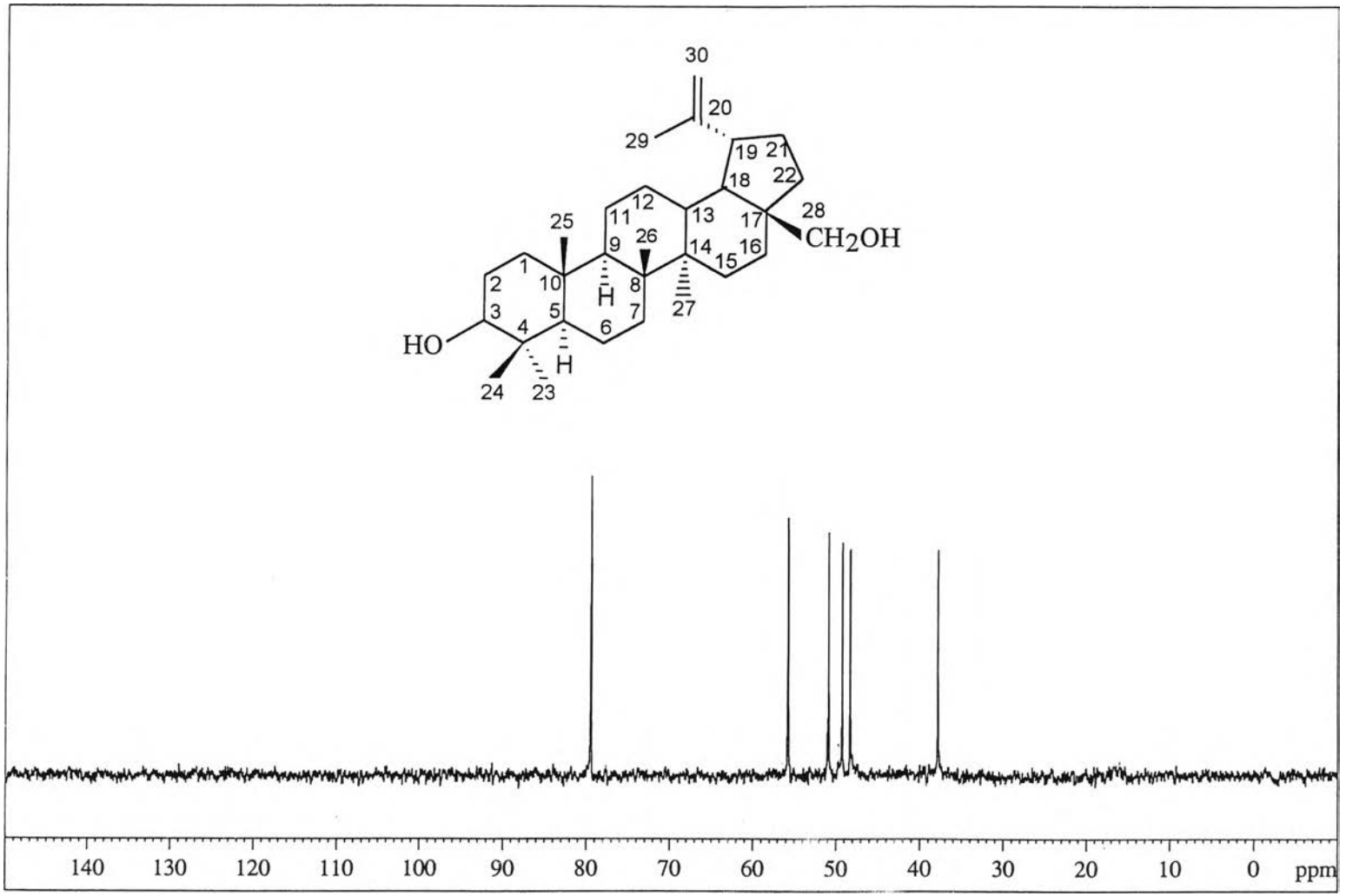


Figure 45a The DEPT–90 spectrum of compound DU6

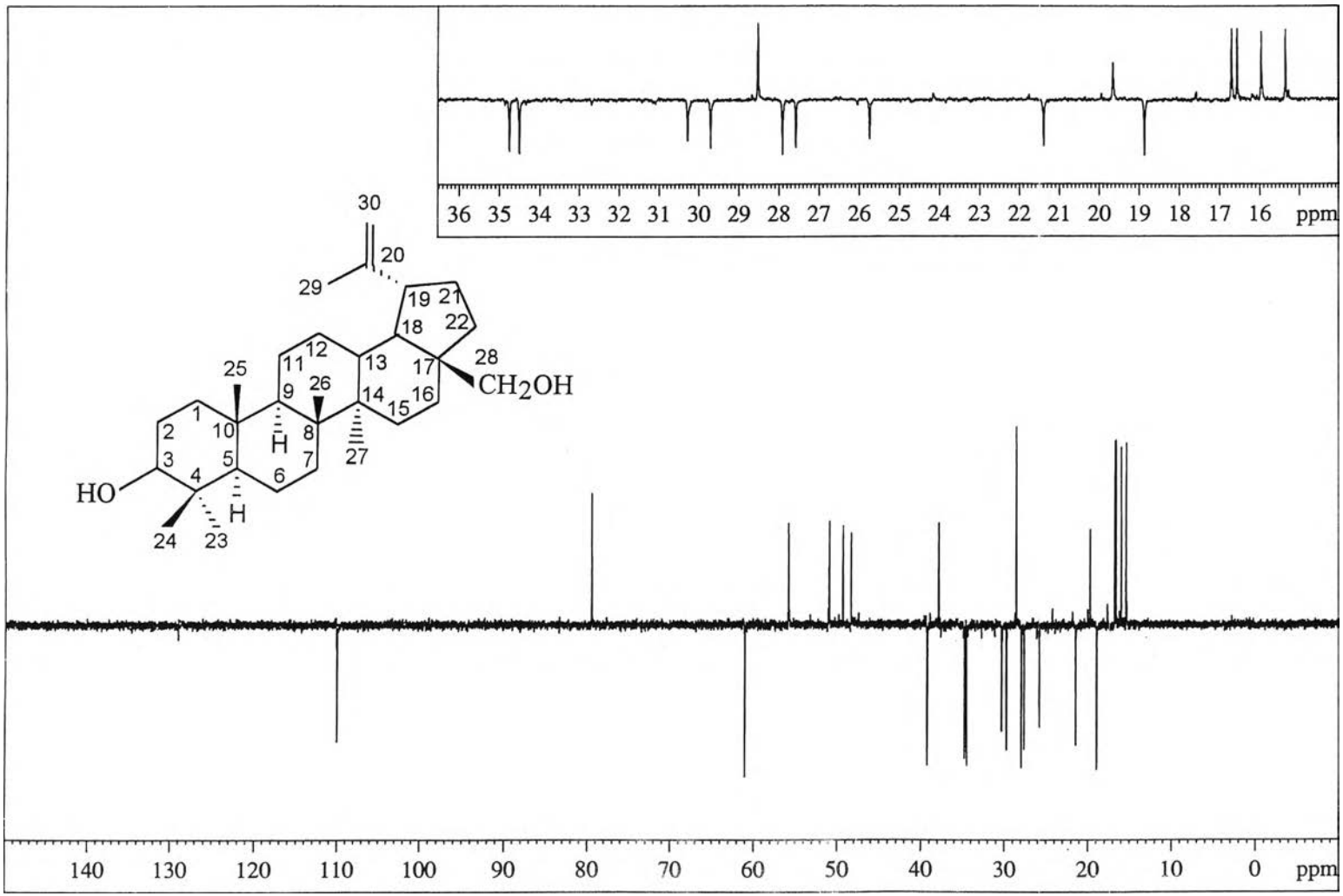


Figure 45b The DEPT–135 spectrum of compound DU6