CHAPTER 3

Chapter 3

ISOLATION AND CHARACTERISATION

Introduction

While studying the different solvent extract of *Dodoneae viscosa* it was found that the MIC of the ether extracts was lesser as compared to all other solvent extracts. Thus the ether extract, found most effective against S. aureus, was selected for my further study. In this chapter the compound responsible for the anti-bacterial activity was isolated for the characterisation of its structure. The ether extract was first of all fractionated into different fractions according to the decreasing polarity and then followed by isolation at a particular polarity and checked for its affectivity by disc diffusion method. Finally most active and highly pure fraction was subjected to the characterisation.

Materials and methods

Processing of raw materials:

Fresh leaves of *Dodoneae viscosa* were cleaned as described earlier and air dried. Then were crushed with fresh and distilled water and the extract was filtered with the help of a muslin cloth. Clear water extract was then transferred to separating funnel. Ether was carefully added and each time one third of the total volume of extract, ether was added. This was vigorously shaken and kept for one hour till two clear layers were seen, one of water and other dark green in color which was the ether extract. Slowly the lower layer of water was

removed and the upper layer of ether was collected in a well washed and pre-autoclaved glass bottle. This process was repeated for six times till the color of the ether extract fades and turns pale green to colorless which indicated that all compounds had been extracted by this method of liquid-liquid extraction process. It was being seen that this type of extraction process was better than the soxlet process as we got a better result with the ether extract might be because the compounds be present in the inner tissues of the leaves. This process was found to be more useful, provided that there was a vast difference between the densities of the two extracting solvents. The ether extract was dark green solution. The compound was recovered by concentrating it by air drying and then by rota-evaporator at 40°C followed by high vacuum. Please note that maximum care was taken while handling ether and to avoid direct exposure to myself and laboratory persons. Thus the total compounds extracted were tested for its biological activity.

Fractionation and isolation process:

The fractions were identified with the help of thin layer chromatography using the 60 F-254 TLC plates from Merck. The mobile phases were selected with the help of trial and error method. The solvent combination for the mobile phase 40% Ethyl acetate: 60% Hexane found to be more suitable. This combination separates maximum of 9 compounds from the main ether extract on TLC (Table 3.1).

Fractionation and isolation were done with the help of serial column chromatography.

Column1:

The first of the series was having an inner diameter of 10cm and the loaded bed of 30cm in length.

Type: Adsorption column chromatography.

Column grade Silica gel 60/120 MESH.

Loading slurry prepared in 100% distilled hexane.

Elution phase: Combination of ethyl acetate and hexane.

Elute volume: 10 ml/fraction.

With a proper rinse of distilled hexane the column was loaded with column grade silica gel. The silica gel slurry was filled to 40 ml. The dried ether extract was then weighed and 4gm of the extract were mixed with 2gm of same silica gel. The sample prepared was then dried and then carefully loaded on the column. The sample volume stands to be 2% of the total bed volume of the column.

Initially the elution was done by 100% hexane for 4 washes and then polarity was reduced to 50% ie. 50% hexane: 50% ethyl acetate (1:1). At 50% polarity no compound remains on the column. The first 1^{st} (Rf= 0.91) and 2^{nd} (Rf= 0.82) compounds out of the 9 compounds were collected in fractions of 1% of ethyl acetate and 98% of hexane. The 3^{rd} (Rf= 0.68) and 4^{th} (Rf= 0.57) compounds were collected in the fraction of 2-4% of ethyl acetate. The 5^{th} (Rf= 0.45), 6^{th} (Rf= 0.42) and 7^{th} (Rf= 0.37) compounds were eluted by the 6% to 8% of ethyl acetate. The 8^{th} (Rf= 0.2) compound was eluted by 14% of ethyl acetate. The 9^{th} (Rf= 0.08) compound was extracted by 50% ethyl acetate. 96 fractions were collected out of which 9 purest forms were selected to study antibacterial test against *Staphylococcus aureus* by disc diffusion method. The most active compound was found to be 6^{th} compound having Rf value of 0.42. But as the Rf of the 5^{th}, 6^{th} and 7^{th}

compound were so close to each other that it was necessary to load on the next column. The fractions eluted with 6% to 8% were pooled together. This fraction contained mixture of a small fraction of 4^{th} compound along with 5^{th}, 6^{th} and 7^{th} compound. This pooled fraction was loaded on the next column (column2).

Column2:

The second of the series was having an inner diameter of 2cm and the loaded bed of 30cm in length.

Type: Adsorption column chromatography.

Column grade Silica gel 60/120 MESH.

Loading slurry prepared in 3% ethyl acetate: 97% distilled hexane.

Elution phase: Combination of ethyl acetate and hexane.

Elute volume: 10 ml/fraction.

The initial elution with 4% ethyl acetate removes the remnants of 4^{th} compound completely. With 5% and then followed by 6% ethyl acetate elution, with smaller steps such as 5.2%, 5.4%, etc up to 6%, there were the separation of 5^{th} and 6^{th} compounds. The elutions were showing the mixture till 5.6% ethyl acetate concentration. The 7^{th} compound started appearing at 6.5% itself and was collected at 7.8% followed by next at 8.4% ethyl acetate. So there was a mixture. The TLC showed a clear spots at Rf 0.42 and 0.37. The fractions containing the 6^{th}, 7^{th} and the rest were pooled together for next column (column3).

Column3:

The third of the series was having an inner diameter of 1cm and the loaded bed of 15cm in length.

Type: Adsorption column chromatography.

Column grade Silica gel 100/120 MESH.

Loading slurry prepared in 4% ethyl acetate: 98% distilled hexane.

Elution phase: Combination of ethyl acetate and hexane.

Elute volume: 10 ml.

As the separation was very critical because the Rf values were very close to each other, I decided to use the finer column and also finer grade silica gel. The elution were done very carefully as I started to elute with 4.8% ethyl acetate. By elution of 5.3% ethyl acetate there was no trace of the 5^{th} compound. The 6^{th} compound first appeared in the fractions eluted by 5.9% and the compound completely got eluted by 6.2% ethyl acetate. So the compound was considered to be completely eluted by 6% ethyl acetate: 94% hexane, which was standardized. This compound was 22mg, which was extracted from 1kg of air-dried leaves. So the yield was 0.0022%. The compound was then subjected to antibacterial test. Then the MIC was studied of this pure compound.

Analysis for characterization:

The characterization was done with the help of Nuclear Magnetic Resonance (NMR) of Varian make, Germany. The model was Varian Mercury YH300, 300MHz with superconducting magnet and TMS as internal standard. The $CdCl_3$ used as a solvent was from Aldrich. The IR was studied with the help of Fourier Transform Infrared Spectrophotometer, model- FTIR 8400 and make- Shimatzu. The KBr plate method was used and diluent used was $CdCl_3$ from Aldrich. The elemental analysis was done on Flash EA1112 series of Thermo Electron make, UK.

The gas chromatography and mass spectra was studied with the help of GCMS-QP5050. The injection temperature was 200°C and the interface temperature was 290°C.The oven temperature program time

was for 19.33 min and the column flow was 1ml/min and total flow was 16.1ml/min.

Elucidation of structure

The structure elucidation was done with the help of 1H NMR, 13C NMR, FTIR, Mass spectroscopy and Elemental analysis.

The results are as follows:

IR ($CDCl_3$); Broad O-H stretch 3400- 2400cm^{-1} ; Aromatic lactone carbonyl stretching 1745cm^{-1}, 1172cm^{-1}; medium 804cm^{-1} , 756 cm^{-1} Ar-H bending vibration; medium aromatic stretch (C=C) 1654 cm^{-1},1602 cm^{-1}, weak aromatic stretch 1560 cm^{-1}, medium 1461 cm^{-1} ; 3000 cm^{-1} Ar-H stretching frequency; medium C-H stretch of alkane 2854 cm^{-1}, strong C-H stretch of CH_3 2925 cm^{-1}, weak 756 cm^{-1}; strong C=O of saturated delta lactone 1745cm^{-1}; 1361 cm^{-1}, 1280 cm^{-1}, 1215 cm^{-1} –Breathing vibrations of aromatic ring; 1020 cm^{-1} and 1095 cm^{-1} (-C-O-C-) ether linkage.

^{1}H NMR (300 MHz, CDCl3) δ 1.25 (3H, *s, Ch3*); 1.50- 1.80 (1H, *broad singlet, OH*, D_2O exchangeable); 3.86 (1H, *d, J* = 12.6 Hz, *H1a*); 3.90 (1H, *d, J* = 12.3 Hz, *H1b*); 4.03 (1H, *s, CH*-OH); 6.52 (1H, *d, J* = 8 Hz, Ar-*H*); 6.96 (1H, *d, J* =3Hz,Ar-*H*); 8.02(1H, *dd, J* = 8.0, 3.0 Hz, Ar-H).

^{13}C NMR (75 mHz, CDCl3) δ 29.77 Hz, (*CH3*); 60.5 Hz (*CH2*); 60.92 Hz (*CH*-OH); 115.56 (C, Ar-*C*); 118.31 (C, Ar-*C*); 118.85 (C, Ar-*C*); 130.29 (C, Ar-*C*); 130.61 (C, Ar-*C*); 136.38 (C, Ar-*C*); 178.98 (C=O).

GCMS m/z (m+/-1) (rel. Int) 177 (22, M); 149 (100, M-29); 121 (10, M-29-CO); 105 (15, M-29-CO-HMe); 93 (12, M-29-CO-HMe-CH); 76

(15, M-29-CO-HMe-CH-O); 65 (18, M-29-CO-HMe-CH- O- CH); 15 (10, M-29-CO-HMe-CH- O- CH-CH)

Elemental analysis:

Carbon- 66.84%, Hydrogen- 5.43% , Oxygen- 27.73%, Nitrogen- nil Sulphur- nil.

The physical characteristics of the compound was as follows:

It was pale greenish yellow in color, non-anhydrous powder non diffracting cylindrical needle like crystal, soluble in oil, DMSO, ethanol, ether, Ethylaccetate and stable till 100°c. It had a melting point: 198°c and atomic mass:178 atomic mass unit.

The elemental analysis reports Carbon 66.84%; Hydrogen 5.43%; Nitrogen and Sulfur was absent. The mass spectrum demonstrates molecular ion peak at 177m/z (n= +/-1). The molecular weight computed to be 178 and thus the formula calculated as C10 H10 O3. Carbon: 67.41 (calculated), 66.84% (observed); Hydrogen: 5.61(calculated), 5.43% (observed).

Please refer to Figures 3.3a, 3.3b, 3.3c, 3.4, 3.5, 3.6 and 3.7 for discussion of the structure elucidation.

The IR **1361 cm^{-1}, 1280 cm^{-1}, 1215 cm^{-1}** -Breathing vibrations of aromatic ring indicated the presence of the aromatic structure which was confirmed by the peak at medium aromatic stretch **1654 cm^{-1},1602 cm^{-1}**, weak aromatic stretch **1560 cm^{-1}**, medium **1461 cm^{-1}** because of the stretching of the aromatic structure and **1654 cm^{-1}** corresponded to the C=C stretching.

The ^{1}H NMR showed that the peak at δ **6.52 (1H, *d, J* = 8 Hz, Ar- *H*)** was a doublet, which correlated to proton at C8.

The ^{1}H NMR peak at δ **8.02 (1H, *dd, J* = 8.0, 3.0 Hz, Ar-*H*)** showed a doublet of doublet, which correlated to proton at C7. The C7 and C5 are having a meta coupling constant of ***J* = 3 Hz.**

The doublet seen at δ **6.96 (1H, *d, J* = 3Hz, Ar-*H*)** correlated to the proton at C5.

The proton on the C7 of the aromatic ring showed an ortho coupling constant with protons on the C8 with a coupling constant of, ***J*= 8Hz**, which was less than the constant of meta coupling.

The **medium** IR peaks at **804cm^{-1}** and **756 cm^{-1}** indicated the Ar-H bending vibration. The ^{13}C NMR peaks at δ **115.56 (C, Ar-*C*);** δ **118.31 (C, Ar-*C*);** δ **118.85 (C, Ar-*C*);** δ **130.29 (C, Ar-*C*);** δ **130.61 (C, Ar-*C*);** δ **136.38 (C, Ar-*C*)** confirmed the aromatic ring.

The ^{1}H NMR showed enough evidence that the position 6 substituted with a CH_3 group which showed a peak integrating the 3 protons of CH_3 **δ 1.25 (3H, *s, CH3*)**. IR peak for CH_3 was reflected as **medium C-H stretch of alkane 2854 cm^{-1}, strong C-H stretch of CH_3 2925 cm^{-1}** proves the functional group.

The ^{1}H NMR showed two doublet at δ **3.86 (1H, *d, J* = 12.6 Hz, *H1a*);** δ **3.90 (1H, *d, J* = 12.3 Hz, *H1b*);** which had a coupling constant of 12Hz, corresponding to the protons at C1. The shielding effect was seen due to the presence of two oxygen, one at 2nd position and other at the C3. The IR showed the presence of aromatic lactone carbonyl stretching **1745cm^{-1}, 1172cm^{-1}.** Also a ether linkage (-C-O-C-) is seen at **1020 cm^{-1}** and **1095 cm^{-1}** ether linkage The position of the C was confirmed by the peak at δ **60.5 Hz (*CH2*)** which was a little down field due to shielding effect in the ^{13}C NMR.

The broad singlet at δ **1.50- 1.80 (1H, *broad singlet, OH*, D_2O exchangeable)** in ^{1}H NMR corresponds to the H of the hydroxyl group at C4, which was D_2O exchangeable.

The OH peak was seen clearly as a broad strong peak at **Broad O-H stretch 3400- 2400cm^{-1}** in the IR spectra. The H on C4 gives a singlet at δ **4.03 (1H, *s*, *CH*-OH)** with a far shielding effect of the electron density. The C4 peak of the CHOH was seen at δ **60.92 Hz (*CH*-OH)** in the ^{13}C NMR.

The C=O which was a carboxylic group was seen as a single peak at δ **178.98 (C=O).** in the ^{13}C NMR. The C=O saturated delta lactone peak was well visible as and a strong C=O of saturated delta lactone **1745cm^{-1}** in the IR spectra.

The structure was confirmed from the evidences of GCMS. The mass break up is as follows:

GCMSm/z (n+/-1) (rel. Int) 177 (22, M); 149 (100, M-29); 121 (10, M-29-*CO*); 105 (15, M-29-*CO-HMe*); 93 (12, M-29-*CO-HMe-CH*); 76 (15, M-29-*CO-HMe-CH-O*); 65 (18, M-29-*CO-HMe-CH-O- CH*); 15 (10, M-29-*CO-HMe-CH- O- CH-CH*).

The compound was named and called as Dodochromen hence forth in the thesis. The whole interpretation was summarized in table 3.2

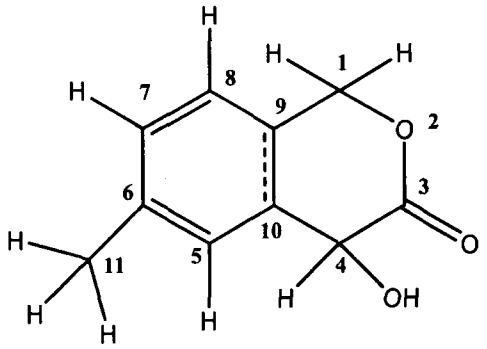

Figure3.1 Structure of Dodochromen

(4 Hydroxy 6 methyl isochromen 3 one)

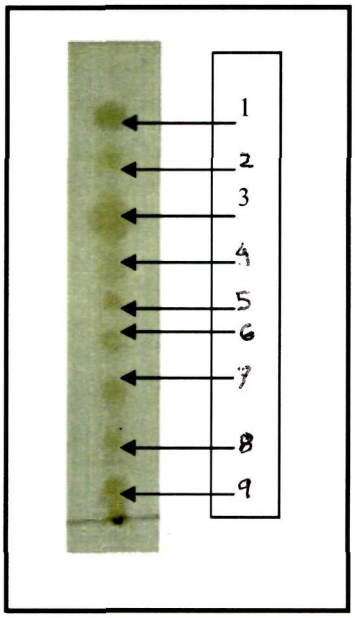

Figure 3.2: TLC showing all the 9 compounds of ether extract.

The mobile phase used was 40% ethyl acetate: 60% hexane

Compounds	1	2	3	4	5	6	7	8	9
Rf values	0.91	0.82	0.68	0.57	0.45	0.42	0.37	0.2	0.08

Table 3.1: Rf values of all compounds shown in the figure 3.1

Table 3.2: Summary of Elucidation

Analysis	Peak	Type	Corresponding H/C or functional group.	Explanation
IR	1361cm^{-1}	medium stretch	aromatic stretch	confirmed by next peaks
	1280cm^{-1}	medium stretch		
	1215cm^{-1}	medium stretch		Breathing vibration of aromatic ring
	1654cm^{-1}	medium stretch		Due to the stretching of the aromatic structure.
	1602cm^{-1}	medium stretch		
	1560cm^{-1}	weak stretch		
	1461cm^{-1}	weak stretch		
	3000cm^{-1}	weak stretch		Ar-H stretching frequency
	756 cm^{-1}			
	804 cm^{-1}			Ar-H bending vibrations
H-NMR	δ 6.52	doublet; J=8.0Hz	1H; Ar-H	Correlated to proton at 8th carbon
	δ 6.96	doublet; J=3.0Hz	1H; Ar-H	Correlated to proton at 5th carbon and had a meta coupling constant of J=3.0Hz with carbon 7
	δ 8.02	doublet of doublet; J= 8.0, J= 3.0 Hz	1H; Ar-H	Correlated to the proton at 7th carbon and showed an ortho coupling J= 8Hz with proton of 9th carbon
CNMR	δ 115.56	Singlet	C, Ar-C	Correlated to C10
	δ 118.31	Singlet	C, Ar-C	Correlated to C9
	δ 118.85	Singlet	C, Ar-C	Correlated to C8
	δ 130.29	Singlet	C, Ar-C	Correlated to C7
	δ 130.61	Singlet	C, Ar-C	Correlated to C6
	δ 136.38	Singlet	C, Ar-C	Correlated to C5
			Confirmed **the aromatic structure**	
H-NMR	δ 1.25	Singlet	3H, CH3	Correlated to the substitution of the 6th C Peak integrates 3 protons
IR	2854cm^{-1}	medium stretch	CH	
	2925 cm^{-1}	strong stetch	CH of CH3	Aliphatic CH stretch Confirmed the alkane group at 6th C position
CNMR	δ 60.5 Hz	Singlet	C of CH3	Correlated to the methyl group substituted on the 6th C little down field due to shielding effect
			Confirmed **the CH3 group at 6th C on the aromatic group**	

Analysis	Peak	Type	Corresponding H/C or functional group.	Explanation
H-NMR	δ 3.86	doublet, J= 12.6 Hz	1H, H1a	Down shielded . Due to the presence of 2 Oxygen one at C2 and C3 positions
	δ 3.9	doublet, J = 12.3 Hz	1H, H1b	
IR	1745cm^{-1}, 1172 cm-1 1095 cm-1 1020 cm-1	strong	aromatic lactonic group	
IR	1745cm-1	strong	C=O	Saturated delta lactone peak
	Confirmed **the aromatic ester which** was **a saturated delta lactone**			
H-NMR	δ 1.50- 1.80	broad singlet	1H, OH	corresponds to the H of the hydroxyl group at C4 which was D2O exchangeable
IR	3400-2400cm-1	broad strong peak	Broad O-H stretch	OH group was confirmed
H-NMR	δ 4.03	Singlet	1H, CH-OH	H on C4 had far shielding effect of the electron density
CNMR	δ 60.94Hz	Singlet	CH-OH	C4 peak of the CHOH
	Confirmed **the substitution of C4 with OH and also the C=O at the C3 position**			
Mass Spectra	177 m/z+/-1	molecular ion peak	C10H10O3	177 (22, M)
	149m/z+/-1	Base peak	C9H9O2	149 (100, M-29)
	121m/z+/-1		C7H7O	121 (10, M-29-CO)
	105m/z+/-1		C7H5O	105 (15, M-29-CO-HMe)
	93m/z+/-1		C6H4O	93 (12, M-29-CO-HMe-CH)
	76m/z+/-1		C6H4	76 (15, M-29-CO-HMe-CH-O)
	65m/z+/-1		C5H4	65 (18, M-29-CO-HMe-CH- O- CH);
	50m/z+/-1		C4H4	15 (10, M-29-CO-HMe-CH- O- CH-CH)
	Confirmed **the structure as '*4 Hydroxy 6 methyl isochromen 3 one*'**			

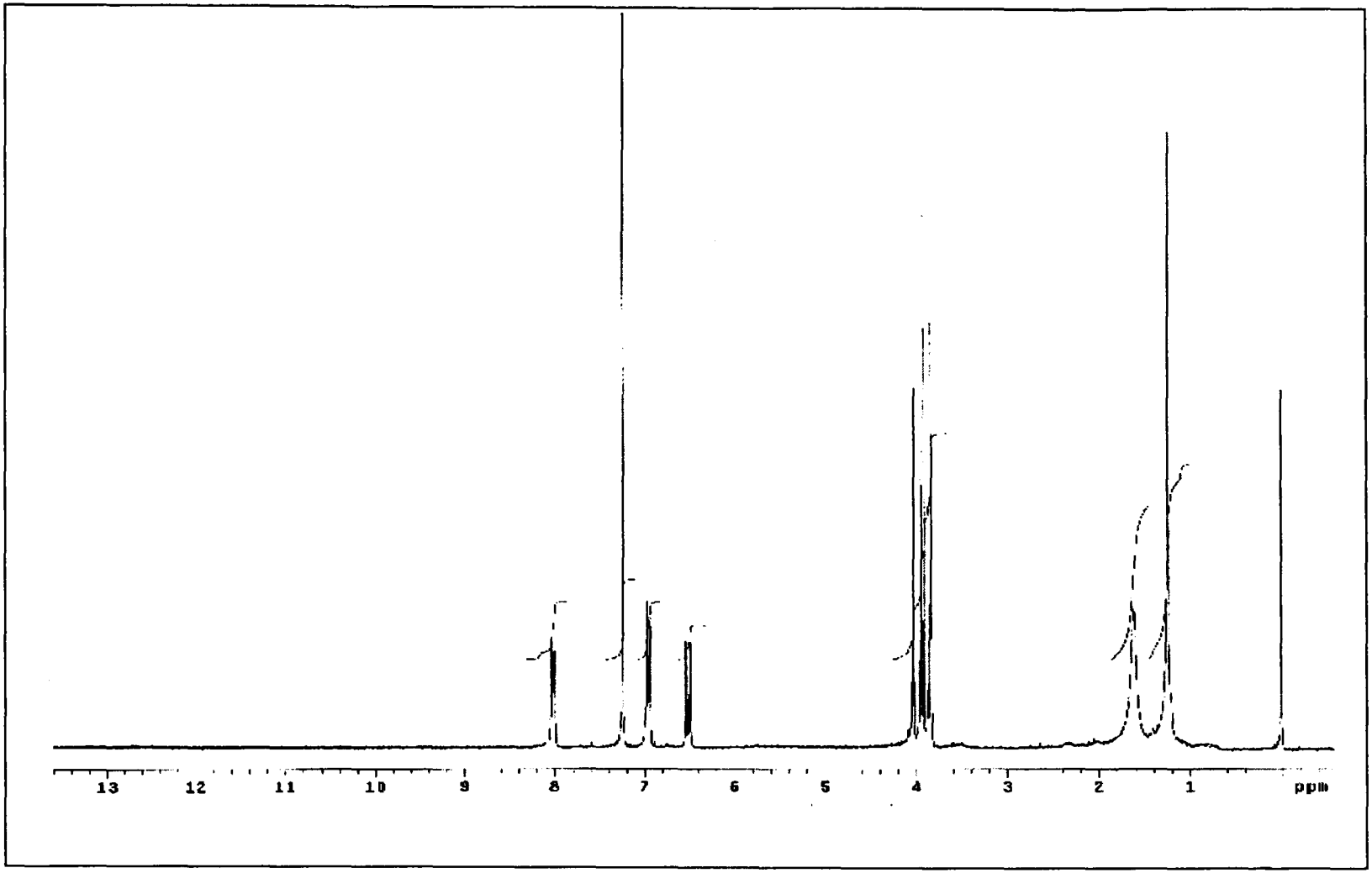

Figure 3.3a: H^1 NMR of Dodochromen in $CDCl_3$; Mol wt:178; $C_{10}H_{10}O_3$ (X axis: δ ppm; Y axis: peak height)

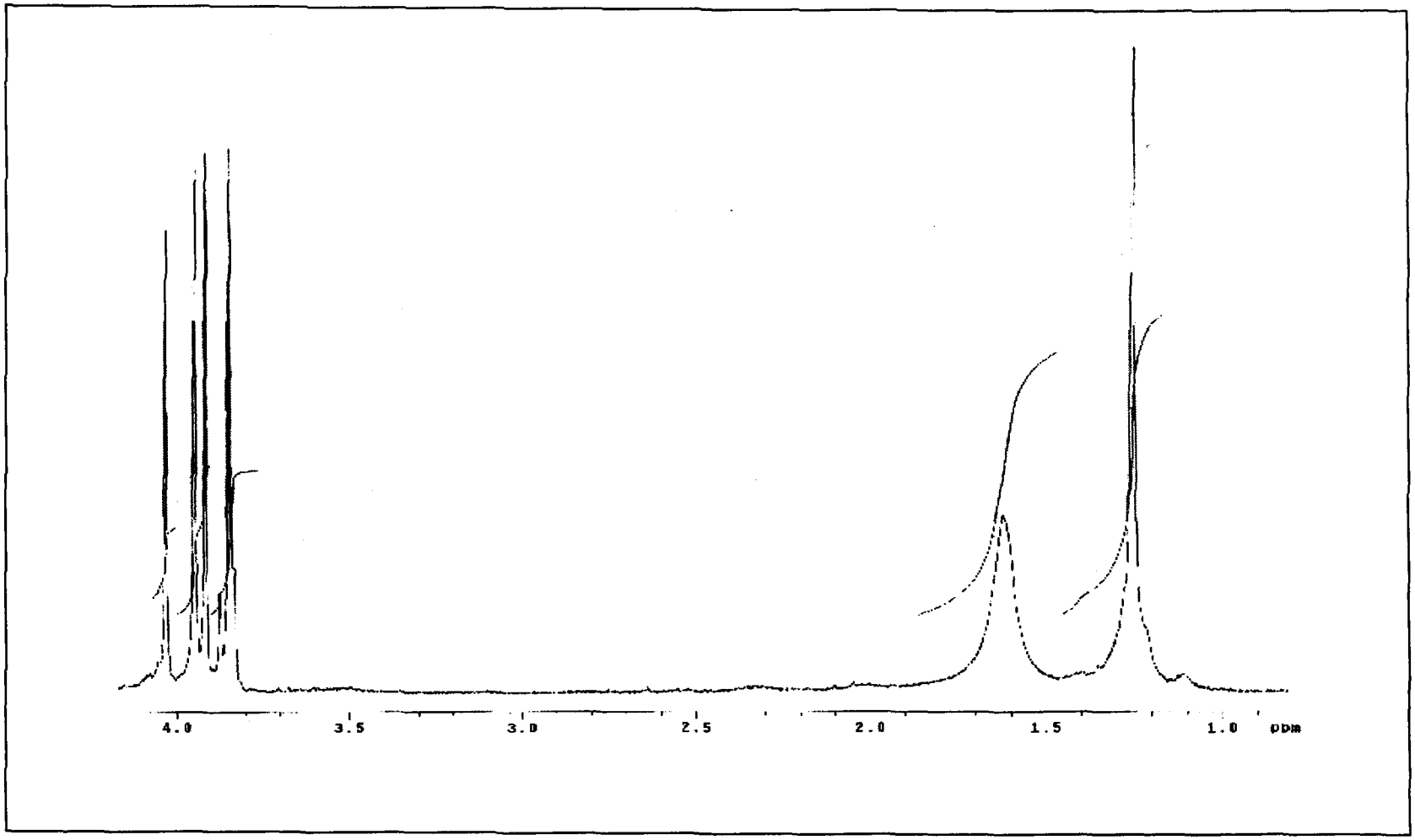

Figure 3.3b: H^1 NMR of Dodochromen in $CDCl_3$; Mol wt:178; $C_{10}H_{10}O_3$(X axis: δ ppm; Y axis: peak height)

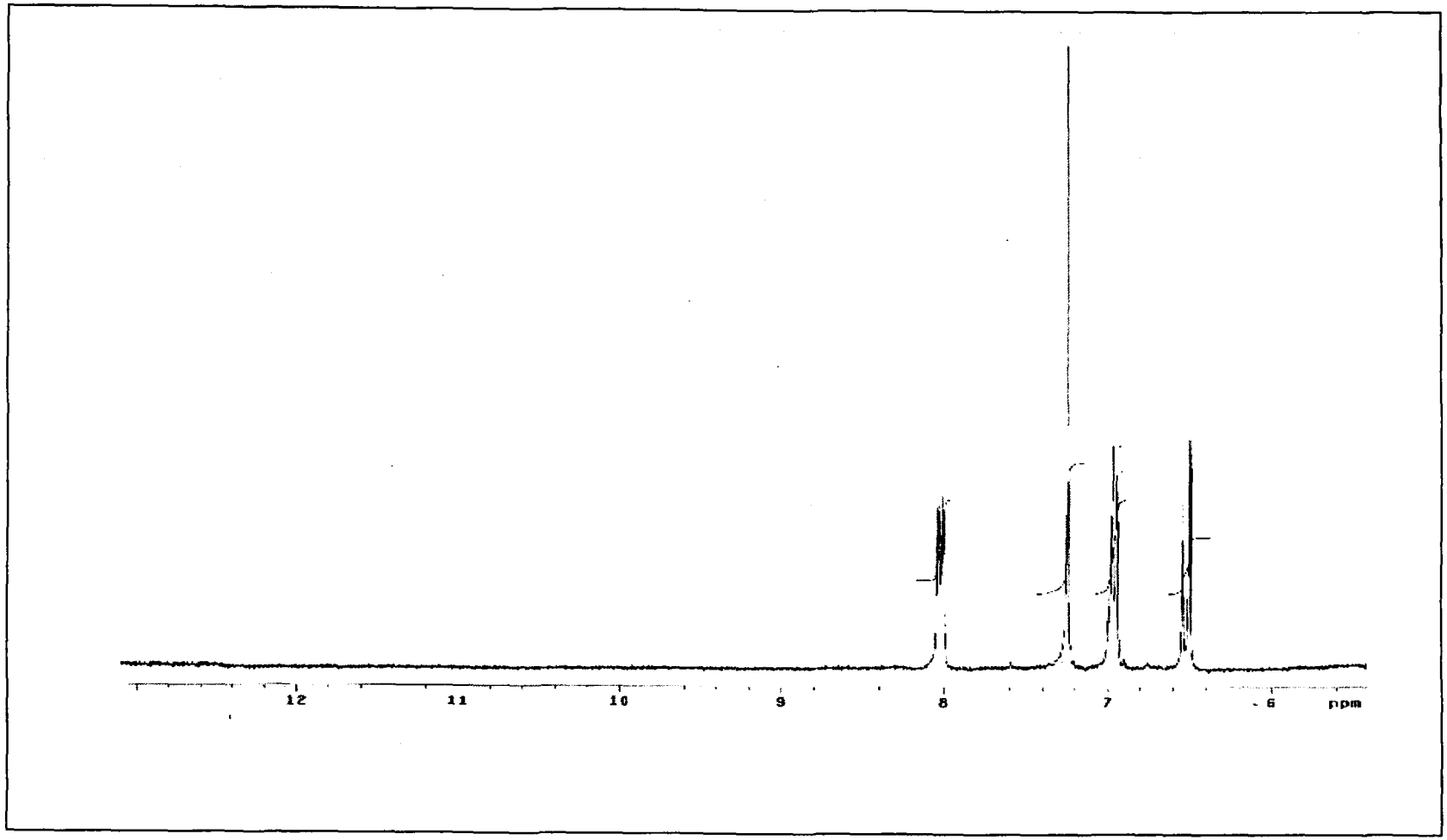

Figure 3.3c: H^1 NMR of Dodochromen in $CDCl_3$; Mol wt:178; $C_{10}H_{10}O_3$(X axis: δ ppm; Y axis: peak height)

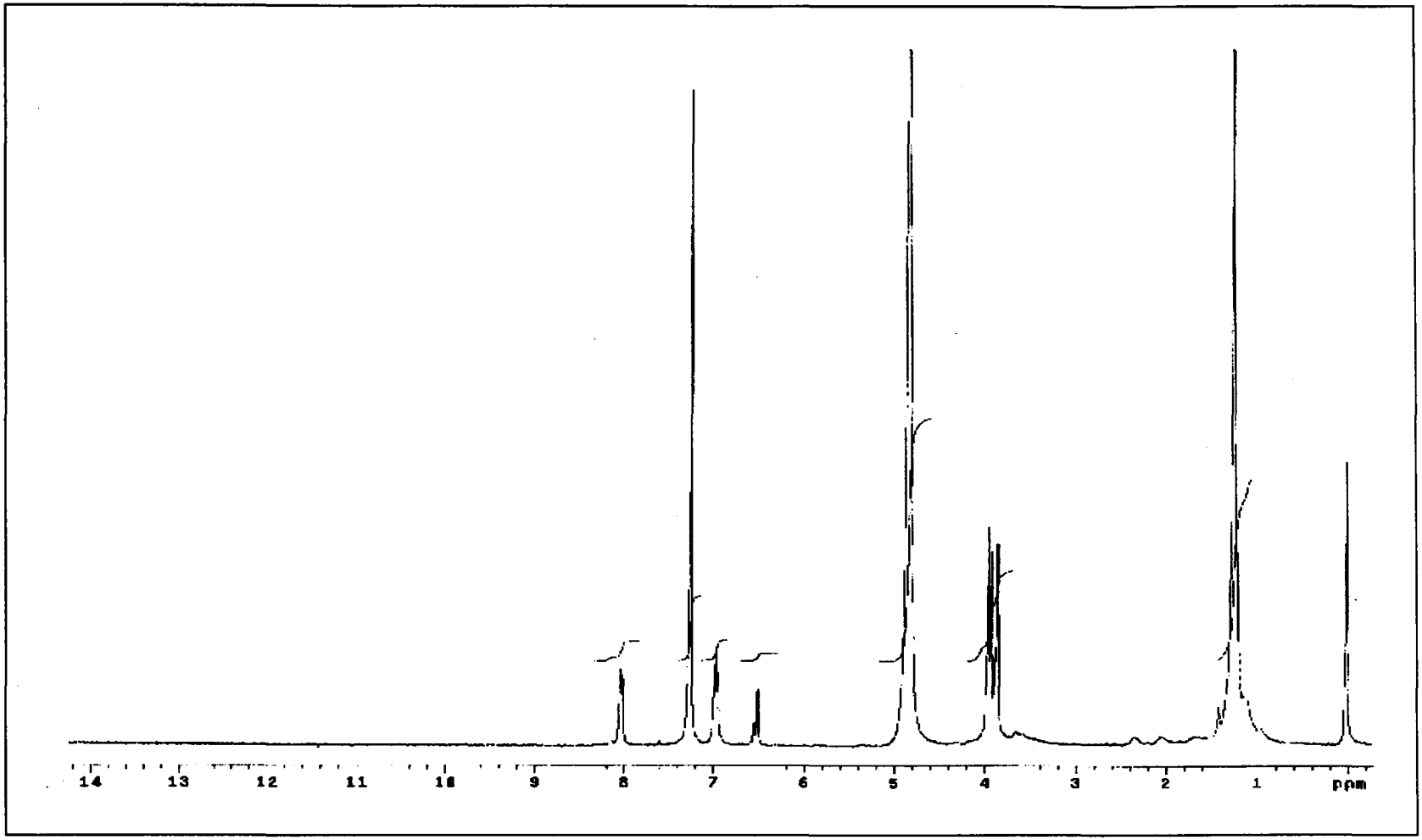

Figure 3.4: H^1 NMR of Dodochromen in $CDCl_3$ with D2O exchange; Mol wt:178; $C_{10}H_{10}O_3$

(X axis: δppm; Y axis: peak height)

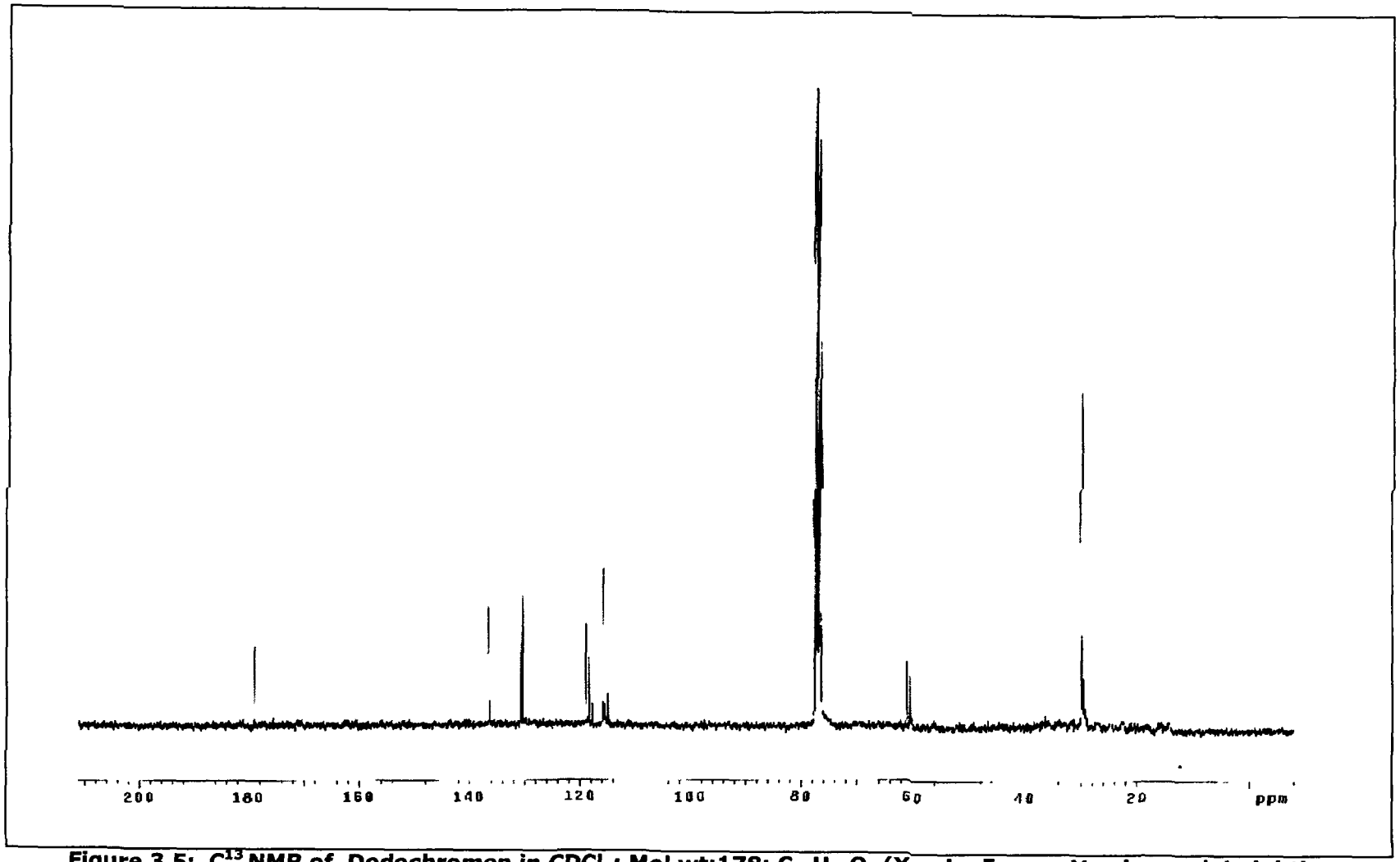

Figure 3.5: C^{13} NMR of Dodochromen in $CDCl_3$; Mol wt:178; $C_{10}H_{10}O_3$ (X axis: δ ppm; Y axis: peak height)

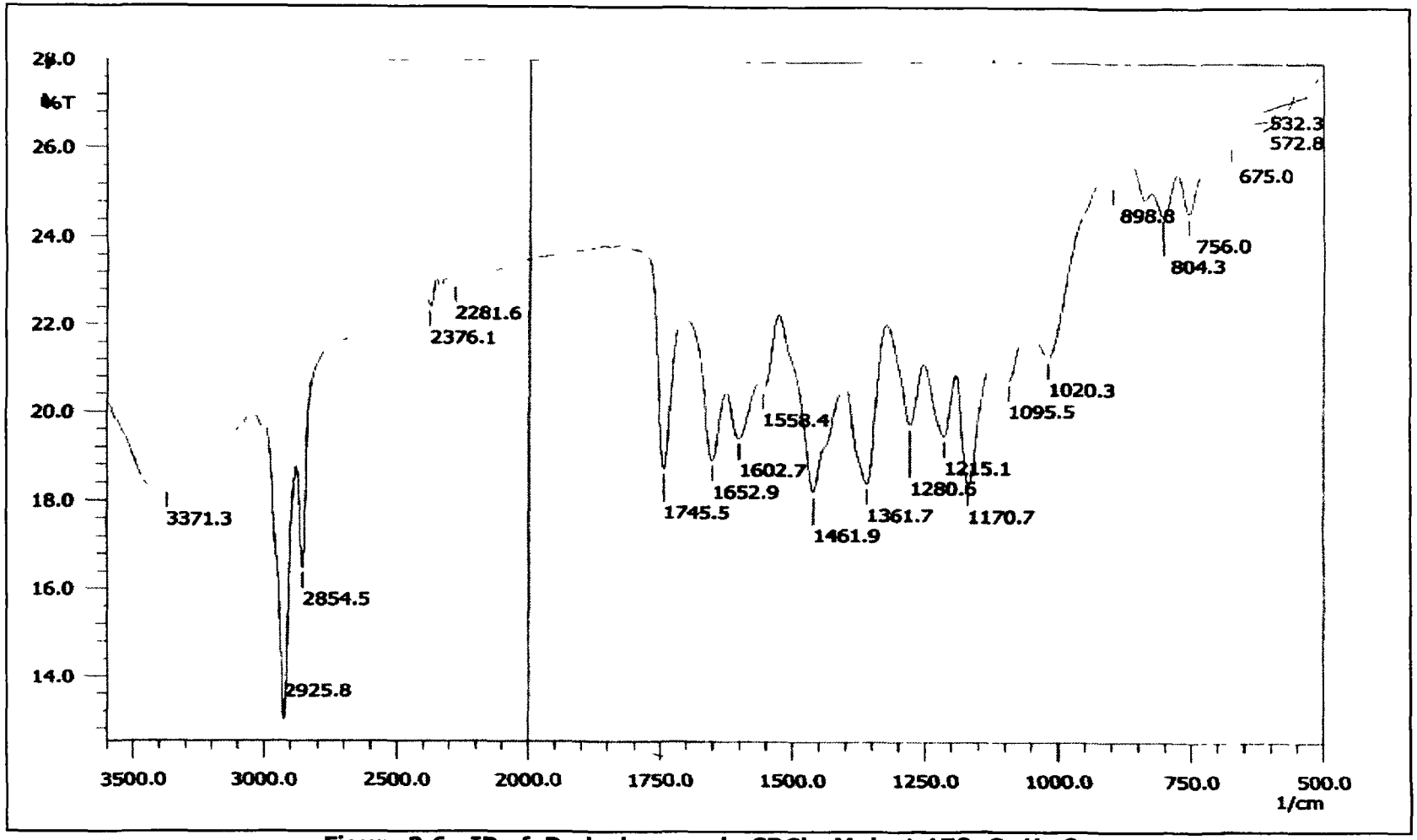

Figure 3.6: IR of Dodochromen in $CDCl_3$; Mol wt:178; $C_{10}H_{10}O_3$

(X axis: Wave number; Y axis: % transmittance)

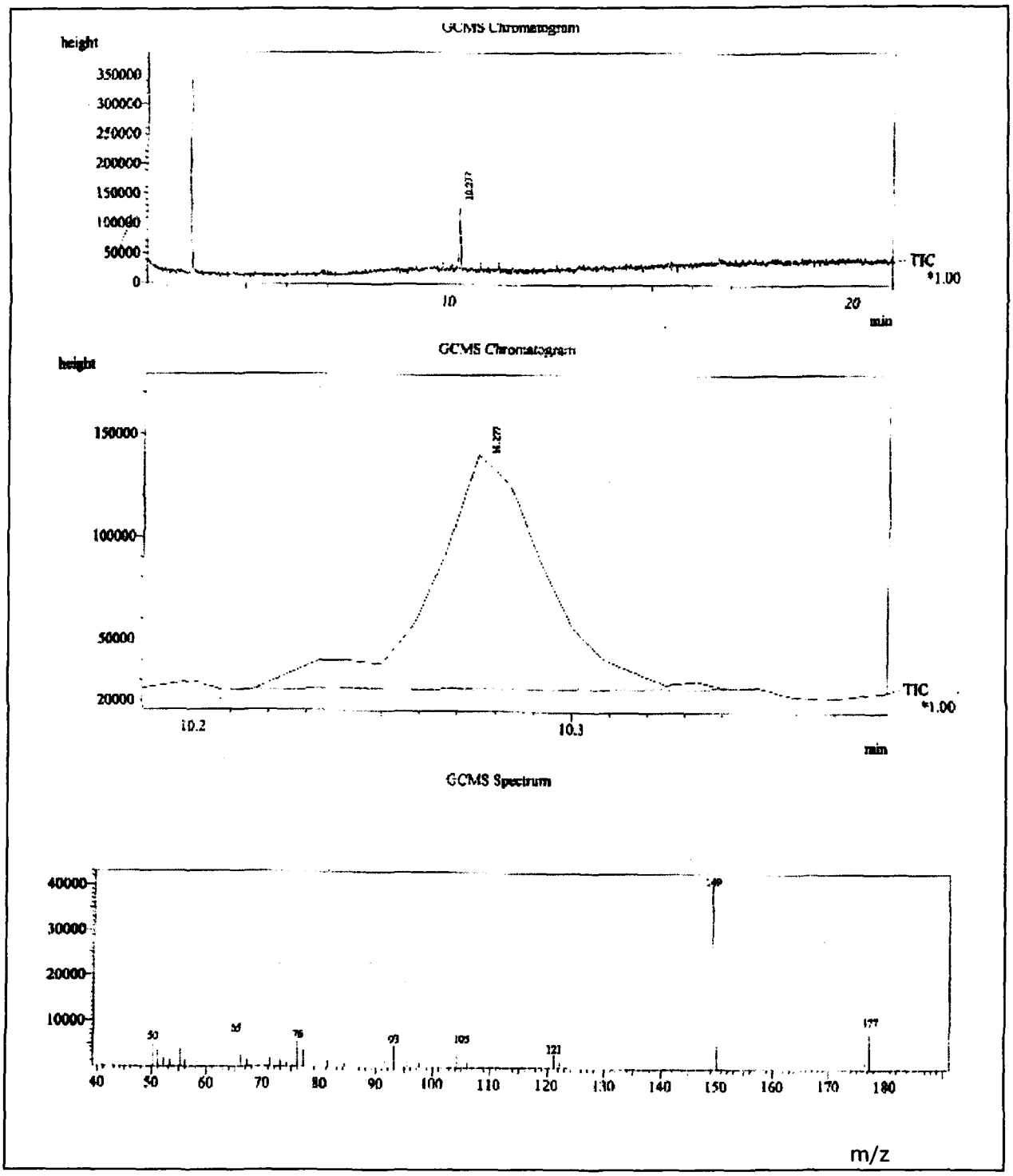

Figure 3.7: GCMS of Dodochromen; Mol wt:178; $C_{10}H_{10}O_3$

(Mass Spectra: R Time: 10.27; Mass peaks: 38; Base peak: 149.15)

(X axis: mass to charge (m/Z); Y axis: % of base peak)