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Bioassay-guided isolation of the antimicrobial compounds of *Coleus amboinicus* Loureiro (Lamiaceae)

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ABSTRACT

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The essential oil of the aerial part of *Coleus amboinicus* showed antibacterial activity against *Bacillus subtilis* (gram +) and *Pseudomonas fluorescens* (gram -) and antifungal activity against *Cladosporium cucumerinum*. Bioassay-guided fractionation revealed that carvacrol and β-caryophyllene-4,5-oxide were the major contributors to the antimicrobial activity of the essential oil. The sesquiterpene α-humulene and monoterpenes α-pinene, α-terpinene and cymene present in significant amounts were inactive.

Keywords: *Coleus amboinicus*, essential oil, antibacterial, antifungal, carvacrol, β-caryophyllene-4,5-oxide

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INTRODUCTION

Coleus amboinicus Loureiro (Lamiaceae) is a perennial succulent erect or spreading aromatic herb, cultivated in the geographical "belt" extending from India to Malaya, the plant is also grown in other tropical countries (Merril, 1912). The plant is occasionally cultivated for its aromatic leaves as condiment and for its medicinal values. A number of medicinal claims about the plant had been reported. Guerrerro (1921) stated that the leaves in infusion or in syrup are used as aromatic carminative, administered in cases of dyspepsia and also as a cure for asthma. The plant is also used in treating coryza, influenza, hyperthermia, adiaphoretic pyrexia, cough, asthma, bronchitis, haemoptysia, sore throat,laryngitis, hoarseness, haematemesia, gas pains, and apistaxis (Nguyen and Doan, 1990; Quisumbing, 1975).

Fresh leaves of the plant yield 0.555 percent volatile oil (Wehmer, 1929). Its volatile or essential oil contains largely carvacrol (Weehuizen, 1918). Apart from carvacrol, Pino *et al.* (1990) reported that other monoterpenes such as thymol, *p*-cymene, 1,8 cineol, terpinen-4ol, α-terpineol, sabinene, mycene, α-pinene, α-phellandrene, α-terpinene, limonene, linalool, γ-terpinene and terpinolene and sesquiterpenes such as α-humulene, humulene epoxide, β-bisabolene, α-caryophellene, caryophellene epoxide and farnesene. Because of the various pharmacological activities described on essential oil (Janssen *et al.*, 1986) in general as well as the medicinal claims about this plant,this study was therefore carried out to isolate and identify the antimicrobial constituents of *C. amboinicus* through bioassay-guided fractionation.

MATERIALS AND METHODS

Extraction of the Essential Oil

Fresh leaves and stems of *C. amboinicus* were subjected to steam distillation using Calvenger apparatus for 8 hours to yield 0.05 to 0.075% (w/v) crude yellow lemon-scented essential oil. The essential oil obtained was used in the bioassay-guided fractionation.

Fractionation and Isolation of the Active Compounds

The essential oil obtained through steam distillation was injected into a GC apparatus (Perkin Elmer) at temperature gradient of 50°C increment to reach 280°C in 30 minutes to determine the possible number of compounds present in the oil. Fractionation of the essential oil was carried out using open-column and eluted with petrol ether, petrol ether in diethyl ether gradient starting at 2.5% increasing up to 10% in increments 2.5% and up to 50% in increments of 5%. The column was eluted with 150 ml of each increment. The column was further eluted with a mixture of 150 ml diethylether and ethyl acetate (50:50), followed by 150 ml ethyl acetate and finally 150 ml methanol. The different fractions were controlled by Thin Layer chromatography (TLC) and gas chromatography (GC). Fractions with the same TLC and GC profiles were pooled to yield 14 fractions. The collected fractions were concentrated in vacuo. All the 14 fractions were subjected to antimicrobial (antifungal and antibacterial) assay. Fractions showing antimicrobial activity purified using open column chromatography and TLC.

Identification and Structural Elucidation of the Pure Isolated Compounds

The isolated pure compounds were subjected to spectroscopic analysis such as GC-MS, EIMS and NMR (^{1}H and ^{13}C). The spectra obtained were compared and checked with the library of spectra to validate the identity of each compound. The isolated compounds already known were no longer subjected to other spectroscopic analysis such as IR, UV and X-ray. The information derived from MS, ^{1}H-NMR and ^{13}C-NMR served as basis for structural elucidation.

Antibacterial and Antifungal Assay

The direct bioautographic TLC assays using *Bacillus subtilis* (gram +) and *Pseudomonas fluorescens* (gram-) for antibacterial (Homan and Fuchs, 1987 - a modification of Lund and Lyon, 1975) and *Claudosporium cucumerinum* for antifungal (Homan and Fuchs, 1987) activities were adapted. For compounds detected through GC and TLC but were not isolated and

compound isolated in small amount i.e. thymol, their synthetic equivalents were evaluated for antimicrobial analysis.

Antibacterial Assay. A known amount of test substance was spotted onto the TLC plate which was developed using a suitable solvent system and air dried. A suspension of the bacteria in nutrient broth was sprayed onto the developed TLC plate. The plates were incubated in a humidifier at 33°C for 24 hour. Detection of the antibacterial activity was carried out by spraying with *p*-iodotetrazolium violet (INT) onto the bacteria-impregnated TLC plates. The active compounds appeared as clear spot against a pinkish to light purple background. All tests were conducted in triplicates.

Antifungal Assay. A known amount of test substance was spotted onto the TLC and developed in a suitable solvent system. After air drying, the TLC was sprayed with a suspension of *C. cucumenrinum* in a glucose-salt solution. The sprayed plates were incubated in the dark in a moist chamber at 25°C for three days. The fungus was allowed to grow and sporulate. Antifungal substances showed sporulation inhibition which was seen as a white spot on a gray-black background.

RESULTS AND DISCUSSION

Identification and Structure Elucidation of the Active Isolated Compounds

Gas chromatography of the essential oil of *C. amboinicus* yielded a mixture of 57 compounds consisting of hydrocarbons, sesquiterpenes, monoterpenes and phenolic alcohol. Bioassay-guided isolation of the antimicrobrial compounds (Figure 1) led to the isolation of two main active compounds. Of the 14 pooled fractions derived from column chromatography, Fractions 9-10 along with Fraction 3 showed high antimicrobial activity while the other fractions exhibited low to no activity. Rechromatography of Fraction 3 using PE:DEE (25:75) yielded β-caryophyllene-4,5-oxide (24 mg). Purification of Fraction 8 yielded carvacrol (590 mg). Fraction 10 was obtained as pure carvacrol (995 mg) while rechromatography of Fraction 5 using PE:DEE (85:15) on Si60 yielded the inactive α-humulene (29 mg). Fraction

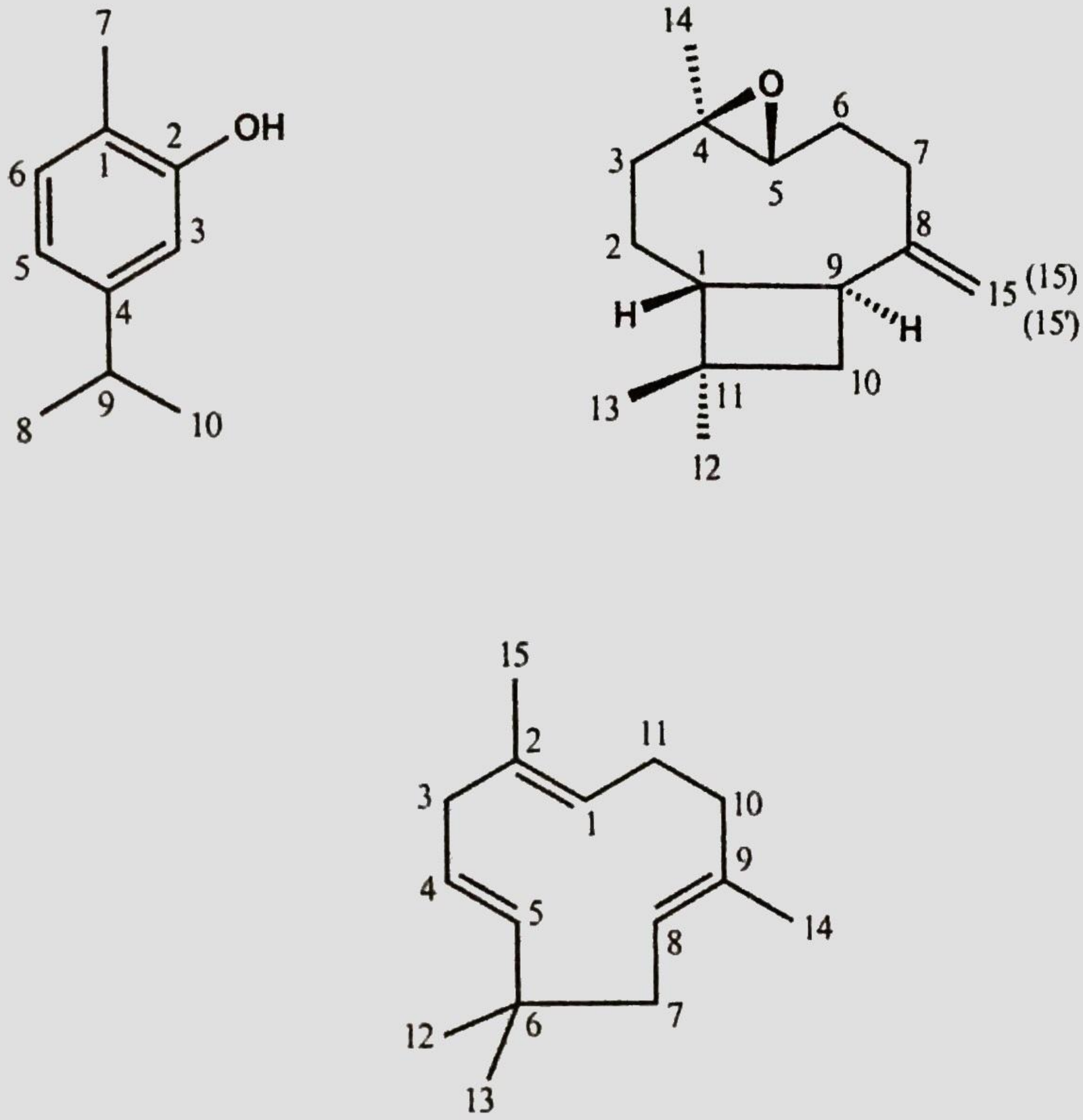


Figure 1. Structures of isolated compounds from the esential oil of *Coleus amboinicus*: (**1**) – Carvacrol, (**2**) β-Caryophyllene-4,5-oxide, (**3**)α-Humulene

1, a mixture of α-pinene, α-terpinene and cymene, collected in fairly large amount (710 mg) showed no antimicrobial activity.

Identification and structure elucidation were concentrated on compounds which were isolated and purified in fairly large amount. These were carvacrol, α-humulene and β-caryophyllene 4,5 oxide (Fig. 1).The identity of these compoundswere established on the basis of their spectroscopic data were obtained from MS, ^{1}H NMR and ^{13}C NMR.

Carvacrol (1)-Yellow Oil

Molecular Formula. $C_{10}H_{14}O$. EIMS *m/z* (rel. int.): 150 $[M]^{+}$ (34), 135 (100), 133 (3), 115 (10), 107 (15.54), 103 (2), 91 (20), 77 (12), 69.1 (6), 59 (9), 55 (12), 43.0 (36), 32 (4), 27 (16) (Figure 2a).

^{1}H-NMR (300 MHz, $CDCl_3$): δ, 7.05 (1H, d, *J* 7.7 Hz, H-6), 6.75 (1H, dd, *J*1.6, 7.7 Hz, H-5), 6.66 (1H, d, *J* 1.6 Hz, H-3), 4.69 (OH),), 2, 85 (1H, septet, *J* 7.0, H-9), 2.23 (3H, s, Me-7), 1.25 (6H, d, *J* 7.0 Hz, me-8, Me-10) (Figure 2b). The data were in agreement with those by Dev *et al.* (1982).

^{13}C-NMR (75 MHz, $CDCl_3$): δ 153.59 (C-2), 148.45 (C-4), 130.79 (C-6), 120.78 (C-1), 118.76 (C-5), 112.98 (C-3), 33.66 (C-9), 23.99 (C-8, C-10) (Figure 2c-2d). The data conformed to those reported by Bohlman *et al.* (1975).

β -Caryophyllene-4,5-oxide (3)-White Solid

Molecular formula. EIMS *m/z* (REL. INT.): 220$[M]^{+}$ (4), 79 (100), 205 (7), 191(2), 187 (13), 161 (32), 149 (29), 133 (16), 121.0 (40), 109 (64), 93 (81), 67 (44), 55.0 (40) (Figure 3a).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 4.95 (1H, d, *J* 1.71 Hz, H-15), 4.84 (1H, d, *J* 1.71 Hz, H-15), 2.92 (1H, dd, *J* 4.0, 9.78 Hz, H-5), 2,66 (1H, d, q, *J* 9.78, H-9), 2.34 (2H, dt, *J* 4.33, 9.78 Hz, H-7), 1.74 (2H, t, *J* 10.67 Hz, H-5), 1.70 (2H, dd, *J* 4.06, 6.18 Hz, H-6), 1.66 (2H, dd, 10.67, 8.46 Hz, H-2), 1.64 (3H, s, Me-14), 1.42 (1H, dt, 4.33, 10.67 Hz, H-1), 0.99 (3H, s, Me-12), 0.95 (3H, s, Me-13) (Figure 3b). The values obtained are identical to those reported for synthetically-derived **2** (Barrero *et al.*, 1995).

^{13}C-NMR (75 MHz, $CDCl_3$): δ 152.11 (C-8), 113.01 (C-15), 51.05

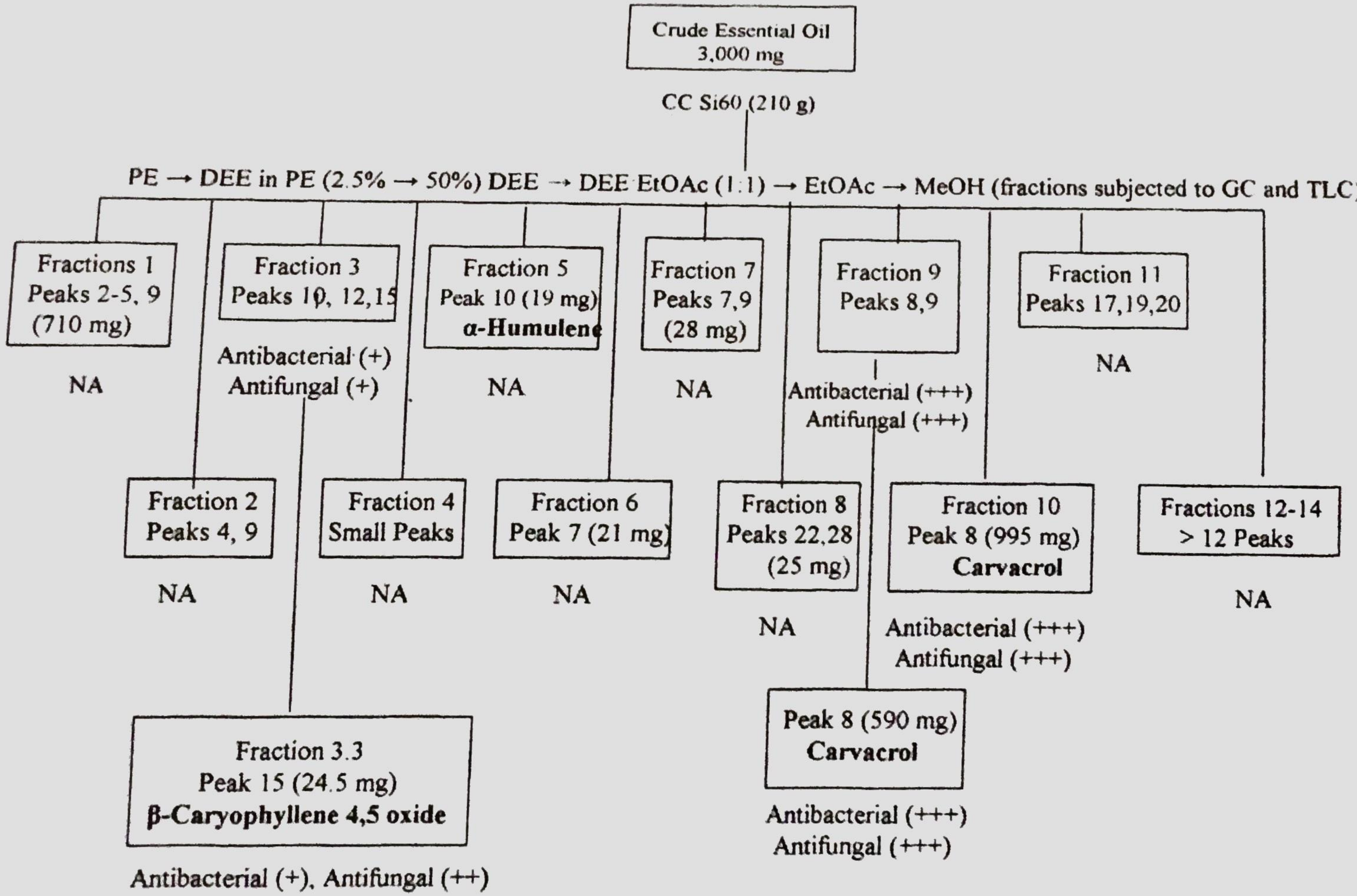


Figure 2. Schematic diagram of the bioassay-guided isolation of the active compounds from the essential oil of Coleus amboinicus. (NA - no activity; Activity rating: + - low, ++ - moderate, +++ - high).

Figure 2a. EI-MS of carvacrol

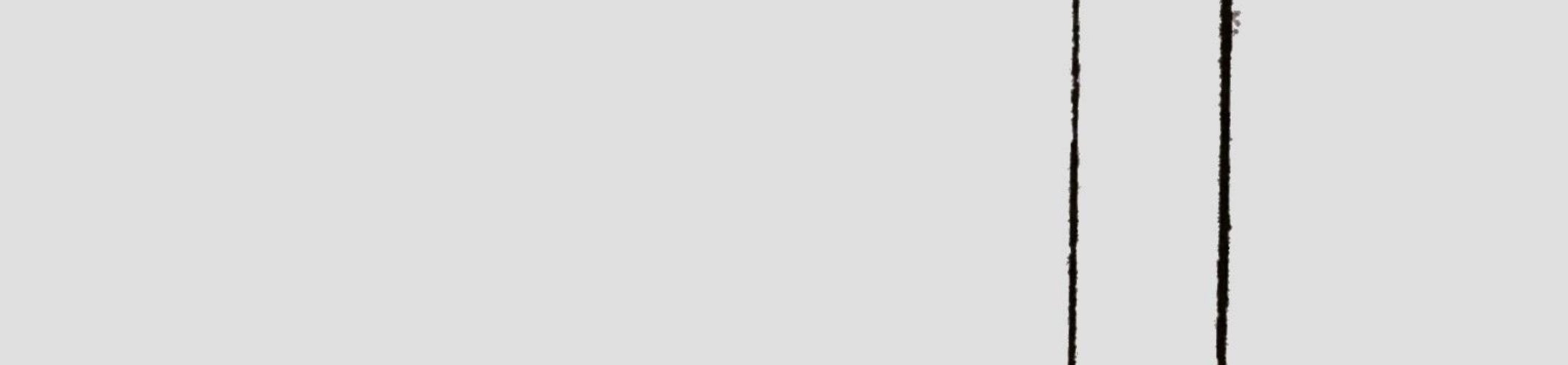
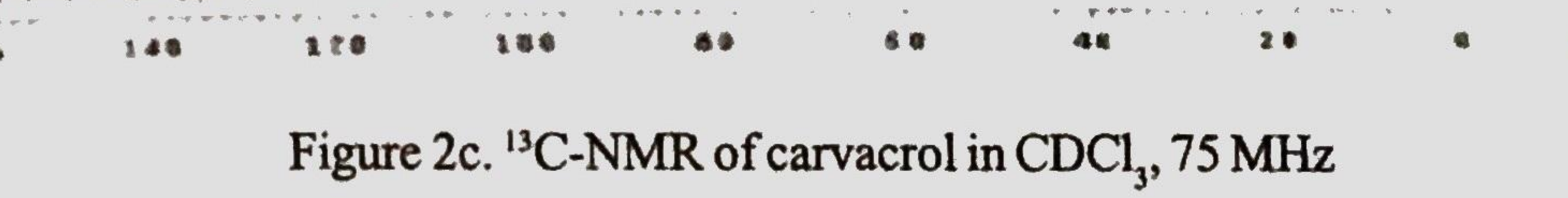


Figure 2b. ^{1}H-NMR of carvacrol in $CDCl_3$, 300 MHz

Figure 2c. ^{13}C-NMR of carvacrol in $CDCl_3$, 75 MHz

CH_2 down, CH/CH_3 up



CH carbons

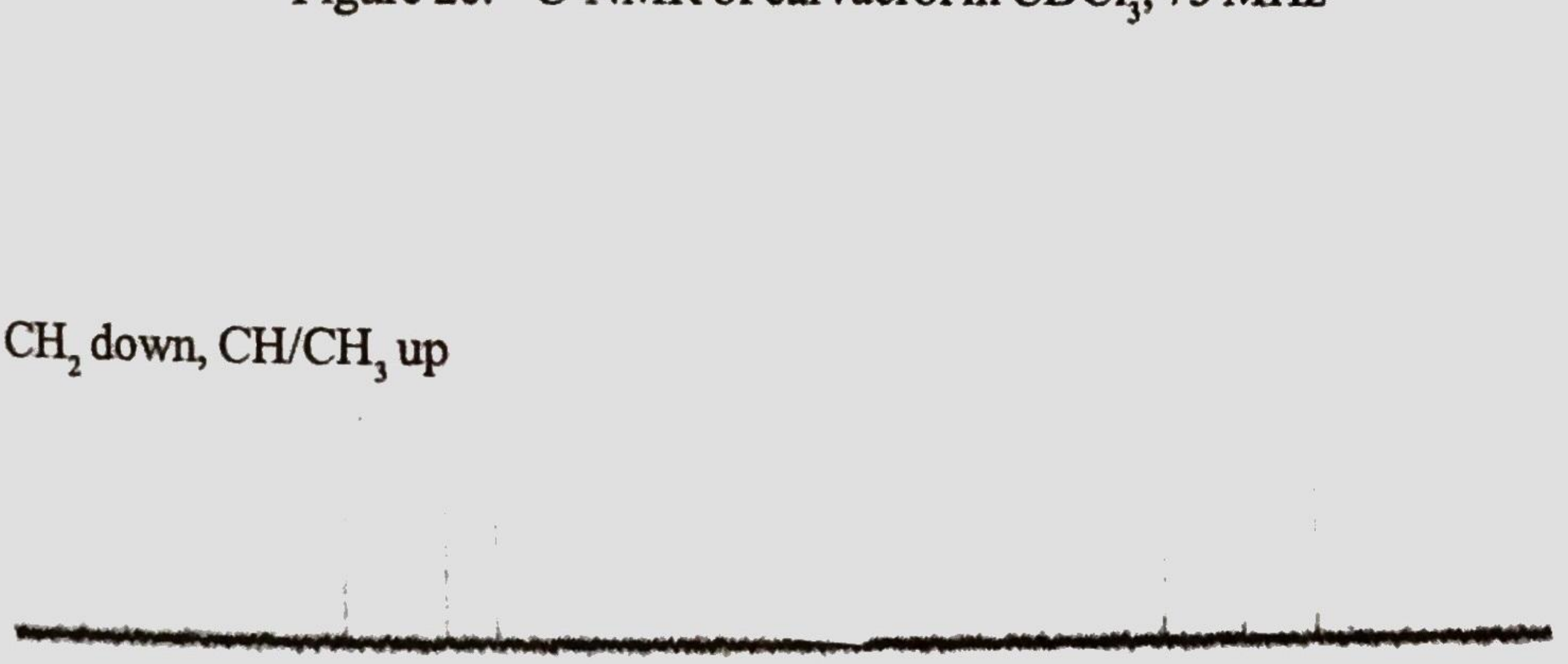


Figure 2d. DEPT of carvacrol in CDC3, 75 MHz

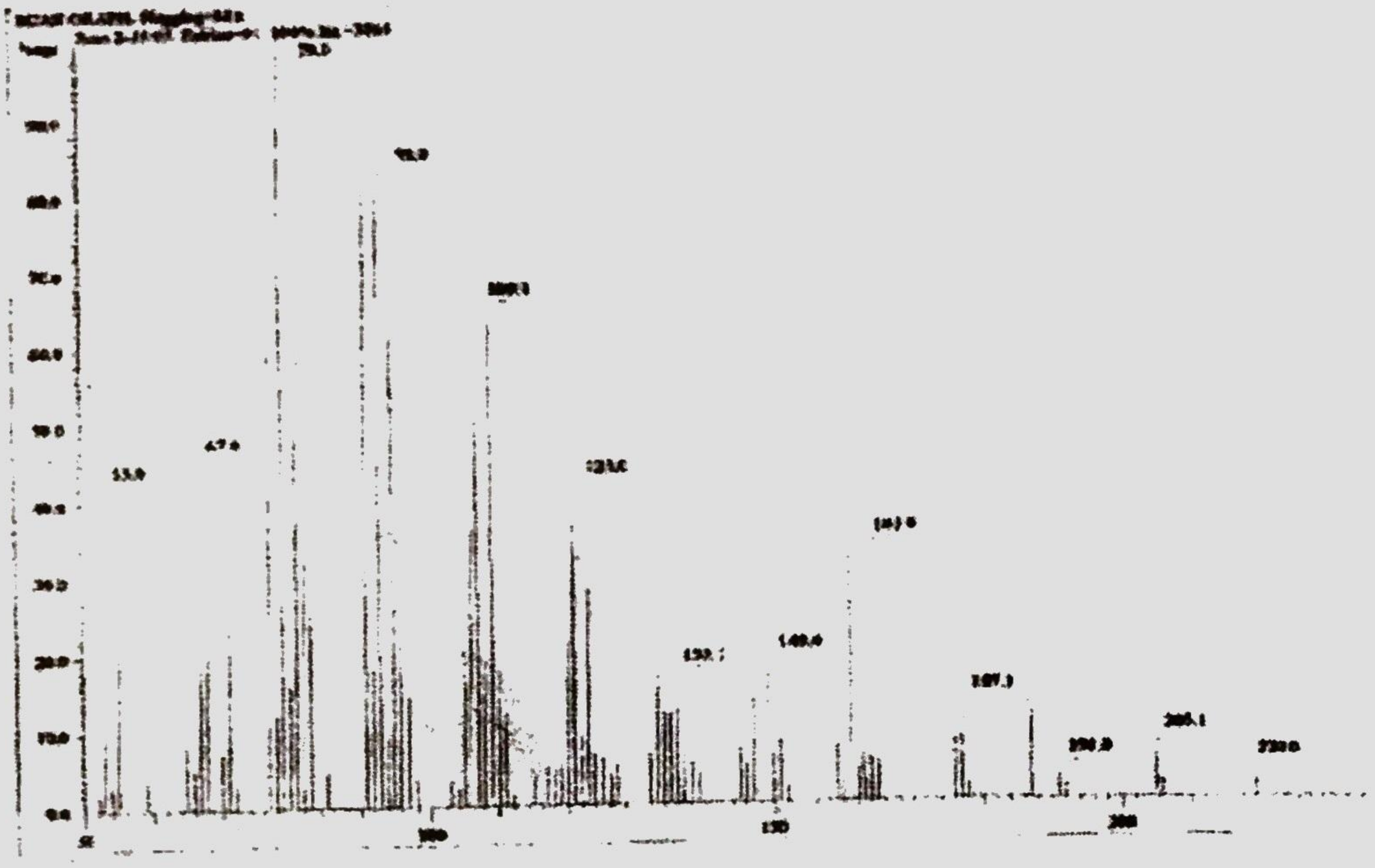


Figure 3a. EI-MS of β–caryophyllene 4,5-oxide

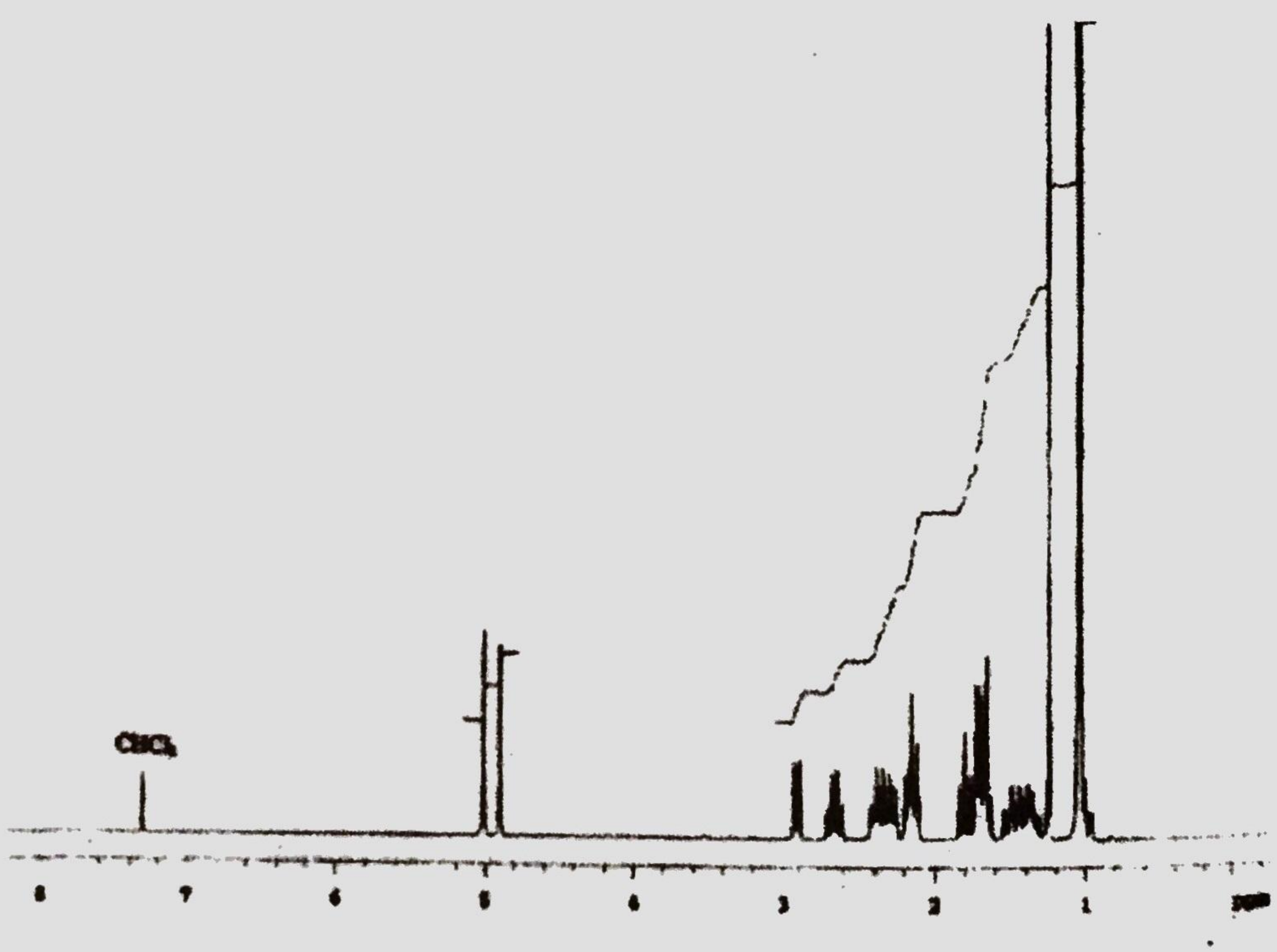


Figure 3b. 1H-NMR of β–caryophyllene 4,5-oxide in $CDCl_3$, 300 MHz

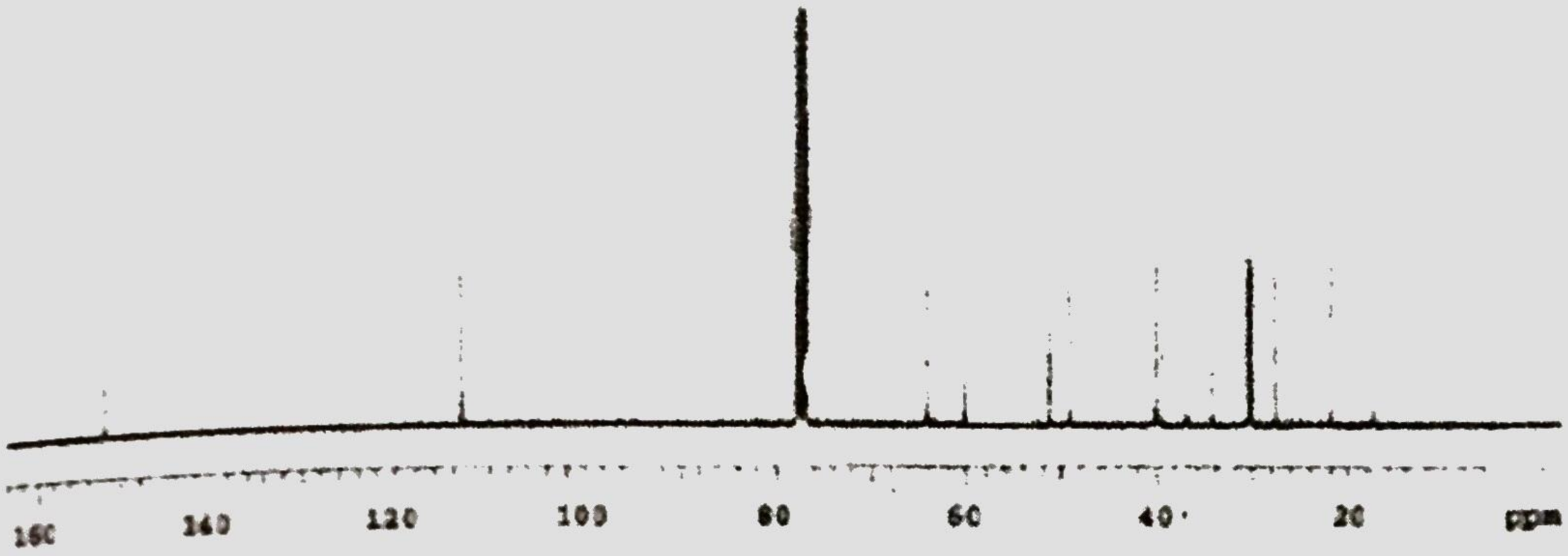


Figure 3c. ^{13}C-NMR of β–caryophyllene 4,5-oxide in $CDCl_3$, 75 MHz

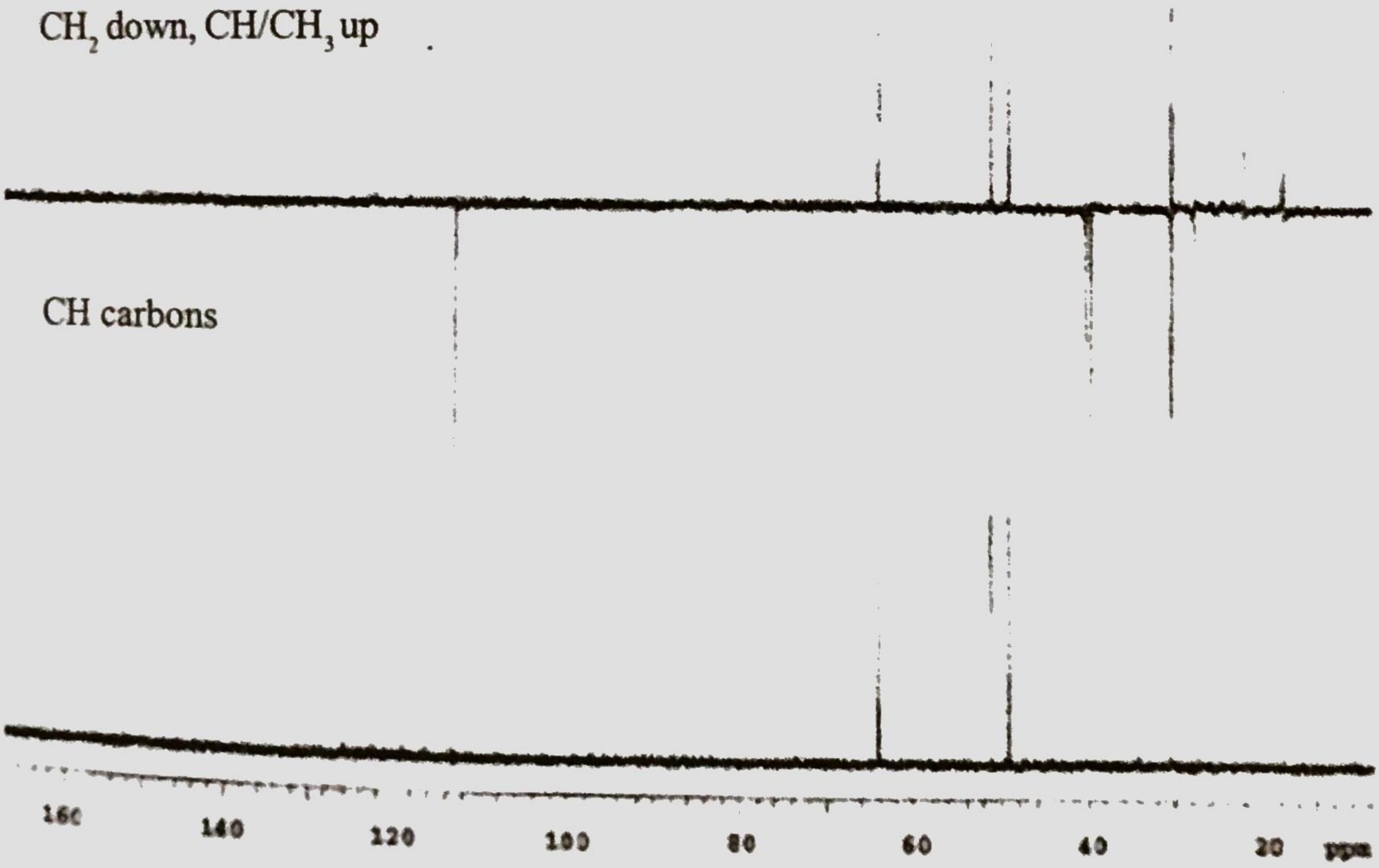


Figure 3d. DEPT of β–caryophyllene 4,5-oxide in $CDCl_3$, 75 MHz

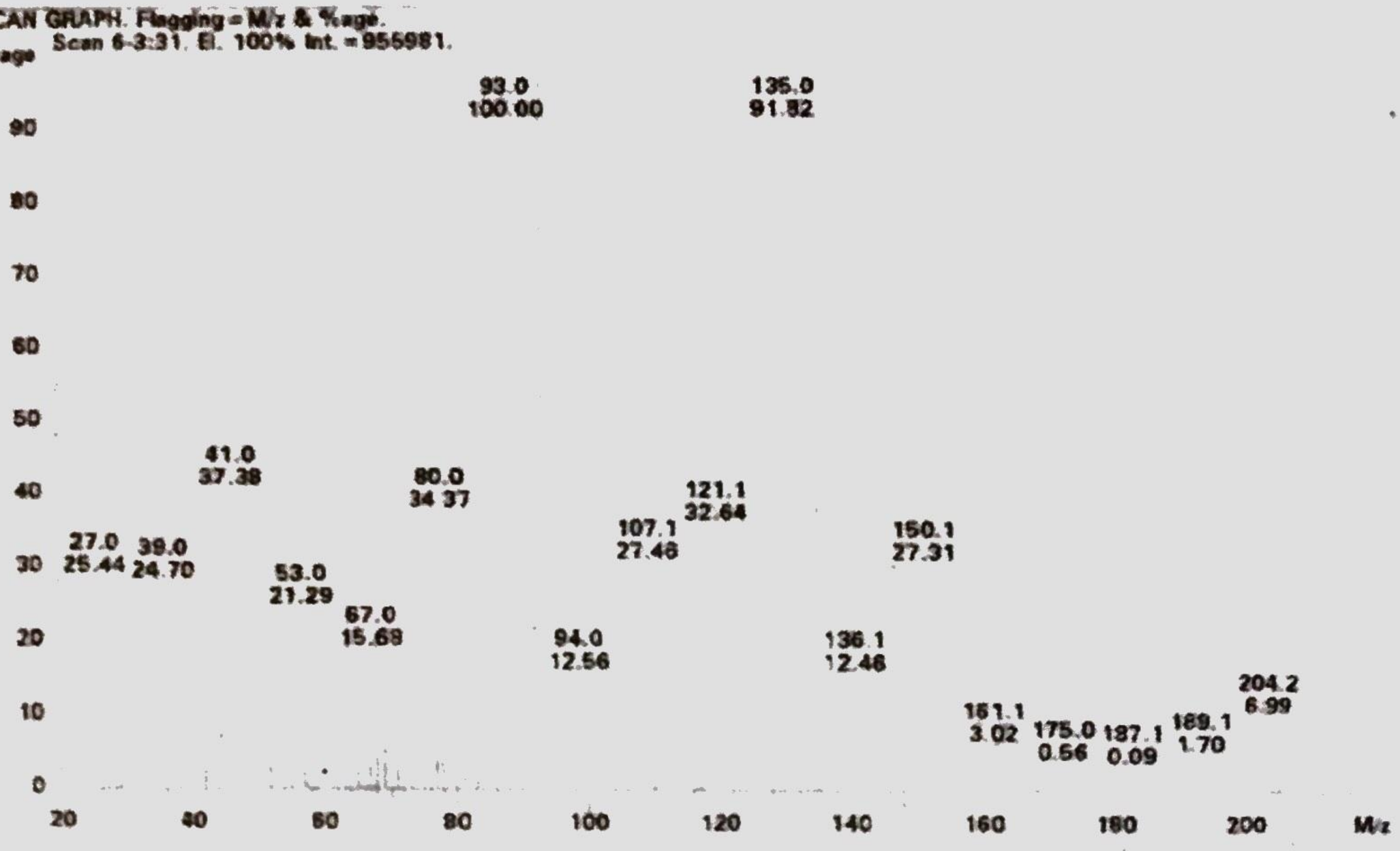


Figure 4a. EI-MS of α-humulene

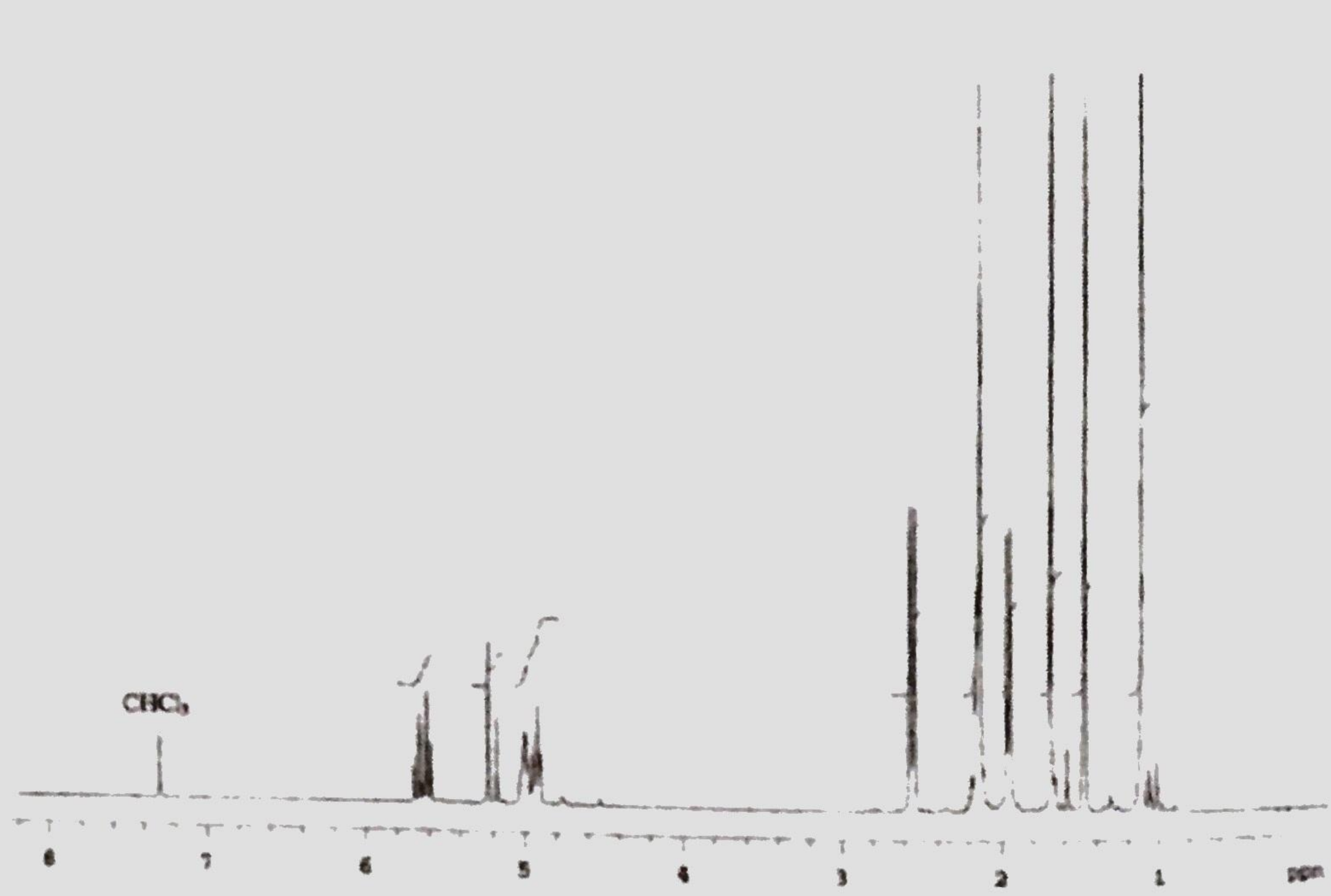


Figure 4b. 1H-NMR of α-humulene in $CDCl_3$, 300 MHz

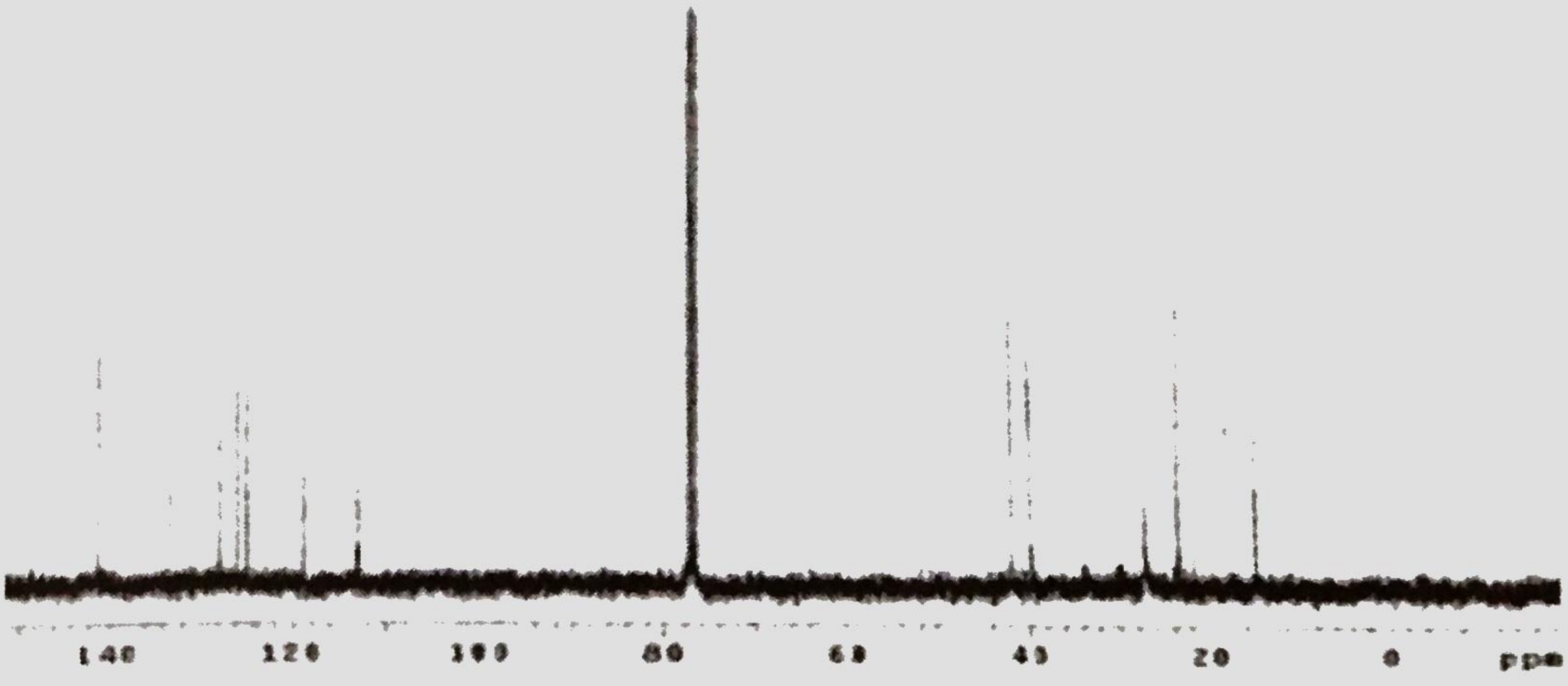
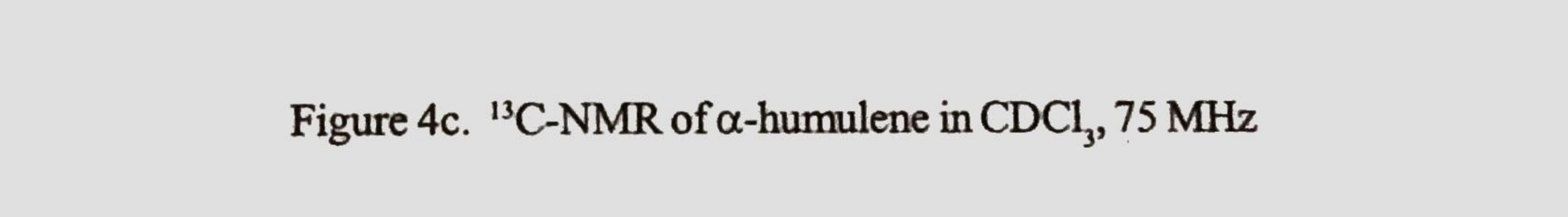


Figure 4c. ^{13}C-NMR of α-humulene in $CDCl_3$, 75 MHz

CH_2 down, CH/CH_3 up



CH carbons

Figure 4d. DEPT of α-humulene in $CDCl_3$, 75 MHz

(C-1), 64.01 (C-5), 60.09 (C-4), 49.00 (C-9), 40.04 (C-10), 39.43 (C-3), 34.28 (C-11), 30.46 (C-6), 30.16 (C-13), 30.08 (C-7), 27.48 (C-2), 21.89 (C-12), 17.26 (C-14) (Figure 3c-d).

α-Humulene (3) - Yellow Oil

Molecular Formula: $C_{15}H_{24}$. EIMS *m/z* (rel. int.): 204.2 $[M]^+$ (7), 93 (100), 189 (2), 136 (12), 121 (33), 94.0 (12), 80 (34), 67 (16), 53 (21), 41 (37), 39 (24), 27 (25) (Figure 4a).

^{1}H-NMR (300 MHz, $CDCl_3$): δ 5.60 (1H, dt, 7.6, 15.7 Hz, H-4), 5.23 (1H, bd, 15.7 Hz, H-5), 5.00 (1H, bt, 7.8 Hz, H-1), 4.92 (1H, t, 7.6 Hz, H-8), 2.53 (2H,d, 7.6 Hz, H-3), 2.10 (4H, m, , H-7, H-10), 1.96 (2H, d, 7.6 Hz, H-11), 1,69 (3H, s, Me-14), 1.48 (3H, s, H-15), 1.12 (6H,s, H-12, H-13) (Figure 4b). The values presented were in agreement to those reported by McMurray *et al.*, 1987.

^{13}C-NMR (75 MHz, $CDCl_3$): δ 140.99 (C-5), 139.20 (C-9), 133.14 9C-2), 127.70 (C-14), 125.82 (C-8), 124.5 (C-1), 41.96 (C-7), 39.72 (C-10), 37.35 (C-6), 27.20 (C-12, C-13), 23.33 (C-11), 17.92 (C-14), 15.06 (C-15) (Figure 4c-d). The ^{13}C values are identical to α-humulene isolated from *Cananga odorata* (Randriamiharisoa *et al.*, 1986) and to those reported for synthetically-derived ones (Barrero *et al.*, 1995).

Antimicrobial Activity

The antimicrobial activity of the essential oil of *C. amboinicus* is attributed mainly to carvacrol (Table 1). Carvacrol was found active to the gram-positive bacteria, *B. subtilis*, and-negative one, *P. flurescens*. Its activity was even slightly higher than the commercial antibiotics ampicillin and isoniazid especially on *B. subtilis*. For *P. fluorescens*, the minimum effective dose of carvacrol in the in vitro study was 10 and 100 times higher than ampicillin and isoniazid, respectively. The activity of carvacrol was consistent with that reported by Janssen *et al.* (1986). β-caryophyllene-4,5-oxide on the other hand, was weakly active on *B. subtilis* and inactive on *P. fluorescens*. However,it has a moderate activity towards *C. cucumerinum*. Aside from being antibacterial, carvacrol also exhibited high antifungal activity but was comparatively less effective than the commercial fungicides ketokonazole and nystatin as checks.

Table 1. Antimicrobial activity of the different compounds present inthe essential oil of *C. amboinicus*

Compound	Minimum effective concentration (ug/spot)*		
	Antibacterial		Antifungal
	B. subtilis	*P. fluorescens*	*C. cucumerinum*
Carvacrol	1	50	25
Thymol**	75	50	50
β-caryophyllene-4,5-oxide	>100	NA	100
α- Humulene	NA	NA	NA
p-Cymene***	NA	NA	NA
α-Pinene***	NA	NA	NA
α-Terpinene***	NA	NA	NA
Isoniazid (Control)	2.5	0.05	
Ampicillin (Control)	2.5	5.0	
Ketokonazole			1.25
Nystatin			0.165

* Direct autobiographic TLC antifungala and antibacterial assay.

** Isomer of carvacrol detected in GC in trace amount. Synthetic material was used in the bioassay for comparison purposes.

NA - No activity at 100 μg/spot

The other monoterpenes present in large amount in the essential oil like α-pinene, α-terpinene and cymene (Fraction 1) and the sesquiterpene α-humulene (Fraction 5) had neither antifungal nor antibacterial activities.

The high antimicrobial activity of carvacrol could be associated with the hydroxyl functionality. *p*-Cymene which has the same basic chemical structure of carvacrol but differs in the absence of OH was inactive. To further support the importance of the hydroxyl moiety in the activity of these molecules, the glucoside derivatives of carvacrol and its isomer, thymol, were prepared and bioassayed. The two derivatives bearing a glucose unit instead of hydroxyl group were no longer active, thus supporting the above-mentioned hypothesis. The position of the functional OH may also play an important role in the structure-activity relationship. Carvacrol possessing a free hydroxyl moiety was more active than thymol having a more hindered OH.

ACKNOWLEDGEMENT

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