Supporting Information

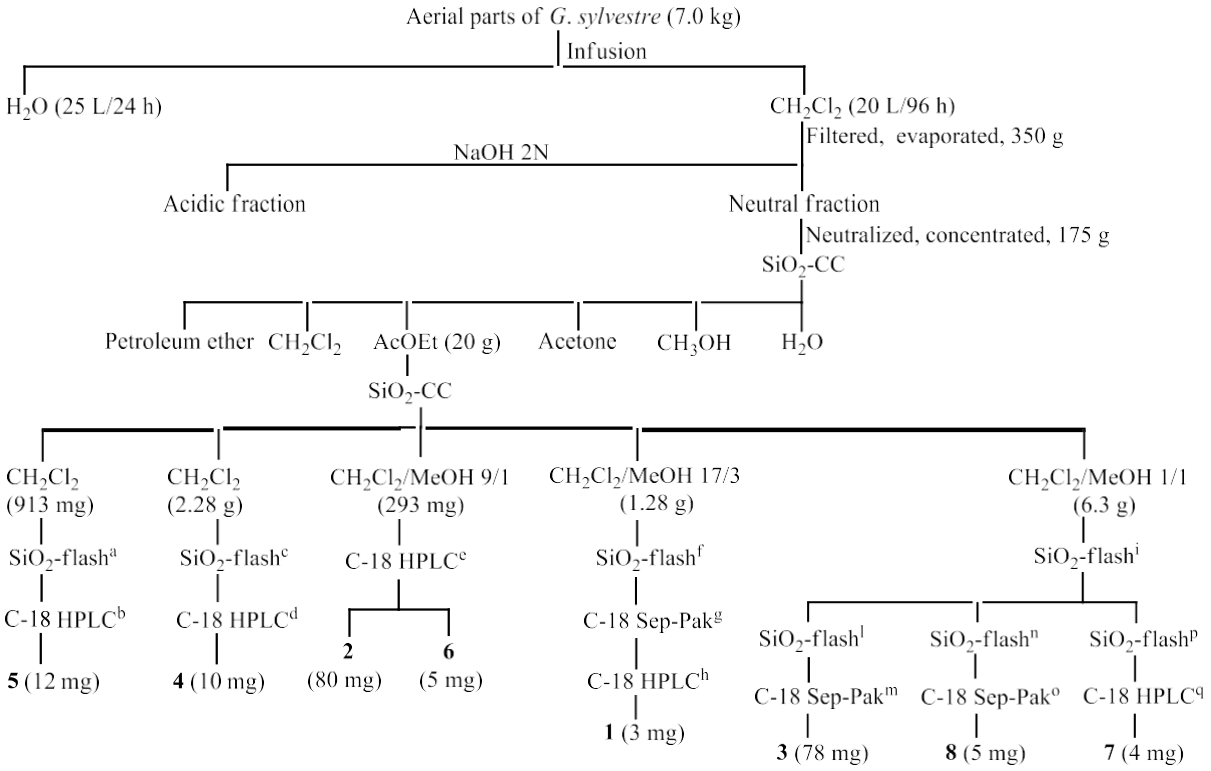
***Rec. Nat. Prod*. 14:3 (2020) 213-218**

Antimicrobial Effect and Antioxidant Activity of Triterpenes Isolated from *Gymnema sylvestre* R. Br.

Valeria Romanucci [1*], Maria Giordano [2], Sergio Davinelli [3], Cinzia Di Marino [1], Afef Ladhari [4] and Anna De Marco [5]

[1]*Department of Chemical Sciences, University Federico II, Via Cinthia 4, 80126 Napoli, Italy*
[2]*Department of Agricultural Sciences, University Federico II, 80055 Portici, Italy*
[3]*Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA* 02115, *United States*
[4]*Université de Carthage, Institut National Agronomique de Tunisie (INAT), Laboratoire GREEN-TEAM (LR17AGR01), 43 avenue Charles Nicolle, 1082 Tunis, Tunisie*
[5] *Department of Pharmacy, University Federico II, Via Montesano 49, 80131 Napoli, Italy*

Table of Contents **Page**



[a] (a) CH_2Cl_2/MeOH (24:1); (b) MeOH/CH_3CN/H_2O (3:4:3); (c) CH_2Cl_2/MeOH (47:3); (d) MeOH/CH_3CN/H_2O (1:7:2); (e) MeOH/CH_3CN/H_2O (2:2:1); (f) CH_2Cl_2/MeOH (9:1); (g) MeOH/CH_3CN/H_2O (1:2:3); (h) MeOH/CH_3CN/H_2O (2:3:5); (i) CH_2Cl_2/MeOH (19:1); (l) Petroleum ether/AcOEt (22:3); (m) MeOH/CH_3CN/H_2O (2:1:2); (n) Petroleum ether/AcOEt (85:15); (o) MeOH/CH_3CN/H_2O (3:1:3); (p) Petroleum ether/AcOEt (1:1); (q) MeOH/CH_3CN (1:1).

Figure S1: Separation Procedures of Compounds **1-8** from *G. sylvestre*[a]

S2: *Strains and Culture Conditions:* The Gram-positive strains *Bacillus subtilis*, *Enterococcus faecalis* NCTC 775 and *Staphylococcus aureus* W46 and the Gram-negative strains *Pseudomonas aeruginosa* ATCC 10145, *Escherichia coli* K12 and *Enterobacter aerogenes* NCTC 10006 were provided by Sigma Aldrich (Milan, Italy). The growth of all strains started from a stock culture maintained at - 80°C in Brain Heart Infusion broth (BHI, by Sigma Aldrich-Milan, Italy) with 20% glycerol. Each microorganism was inoculated in 10 mL of fresh sterile BHI broth and incubated for 1 day at 30°C with or without 10% CO_2 for Gram-positive and Gram-negative, respectively. After the initial activation, the culture was renewed by transferring 100 μL of inoculum into 10 mL of new sterile BHI broth and grown under the same conditions as previously reported.

S3: *Antibacterial Activity:* The plant extract (200 mg) was solubilized in 1.5 mL of dimethyl sulfoxide (DMSO) and after diluted with BHI broth to obtain a concentration varying from 10.0 to 500 mg/mL. As control it was used the broth containing only DMSO diluted in the same way. The antibacterial effects of extracts and triterpenes were evaluated through a microdilution test in the 96-well polystyrene plates, whose wells were filled with 125 μL of the bacterial suspension at $1x10^7$ CFU/mL. Then, 125 μL of each extract or triterpene, very soluble in DMSO, was added at previous reported concentrations. The plates were incubated for 1 day at 30°C with 10% CO_2 for Gram-positive and without for Gram-negative. The minimum inhibitory concentration (MIC) for each microorganism was determined to be the lowest concentration in order to have a complete inhibition of visible bacterial growth for each sample. The minimal bactericidal concentration (MBC), was defined as the lowest

concentration of the extract or of the samples which completely inhibited the microbial growth of the test strains on solid media in Petri dishes that were incubated at 30°C for 2 days [17].

S4: *Cell Culture:* The RAT-1 immortalised rat fibroblasts were obtained from the American Type Tissue Culture Collection and were cultured in Minimum Essential Medium supplemented with 10% foetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

S5: *Cytotoxicity and Cell Proliferation Assays:* Cytotoxicity was evaluated using the MTT assay as an indicator of the metabolic competence of the cells. Samples were dissolved in DMSO and subsequently diluted in medium to the final concentration of 0 mM to 1 mM (concentration of DMSO 0.5%). Briefly, $3x10^4$ cells/well were seeded in 24-well culture plates, grown for an additional 24 h and then incubated in medium containing increasing amounts of each compound (from 0 to 1 mM). At the end of the incubation period (48 h), the medium was removed, and the cultures were incubated with medium containing 1 mg/mL MTT for 2 h at 37°C. The medium was then discarded and 250 µL of acid-isopropanol (0.04 N HCl in isopropanol) was added to each well to stop the cleavage of the tetrazolium ring by dehydrogenase enzymes that convert MTT to an insoluble purple formazan in living cells. The plates were then kept at room temperature and shaken for approximately 15–20 min, and the level of the coloured formazan derivative was determined on a multi-scan reader at a wavelength of 540 nm (reference wavelength 630 nm). Inhibition activity was expressed as percentages of control with DMSO.

S6: *Determination of Cellular ROS:* The ROS-fluorescent probe 2ˋ,7ˋ-dichlorofluorescein diacetate (DCFH-DA) was used to detect endogenous ROS levels. The cells (2×10^4) were plated in 96-well plates, and after 36 h, the medium was replaced with fresh medium supplemented with the tested compounds. After 1, 16 and 48 h, the cells were washed once with Hanks' Balanced Salt Solution and incubated in the same buffer containing 10 µM DCFH-DA for 45 min at 37°C. The intracellular fluorescence was detected using a SPECTRAmax GEMINI spectrofluorometer (San Diego, California). H_2O_2 was used at 100 µM in the last 15 min of DCFH-DA incubation to evaluate the effect of pre-treatment with the tested compounds for the prevention of intracellular ROS generation. The data shown are the mean of three independent experiments performed on triplicate samples. The SD values were <20% for each of the tested conditions and compounds.

S7: *Statistical Analysis:* All determination were done in triplicate for each sample to be analyzed and IC_{50} values were calculated by using the equation of line. The results are given as mean Standard Deviation (SD). Student's t-test was used for comparison between two means and a one-way analysis of variance (ANOVA) was used for comparison of more than two means. A difference was considered statistically significant when $p \leq 0.05$.

Table S1. Values of MIC (mg/L) of different microorganisms treated with different extracts

Microorganism	PE	DCM	AE	A	M	W	Ciprofloxacin
B. subtilis	156.9±1.9	111.2±4.1	75.4±2.1	145.3±4.3	122.6±2.3	139.9±4.9	59.6±1.8
E. faecalis	168.3±2.6	99.6±2.2	80.2±2.4	141.2±5.9	126.9±3.9	147.3±5.6	71.2±2.5
S. aureus	152.3±3.6	121.3±1.9	80.5±3.2	133.6±5.6	142.3±4.1	145.6±4.5	69.3±2.0
P. aeruginosa	142.3±2.1	102.3±3.2	82.5±4.1	131.2±6.6	112.6±2.5	148.8±5.4	66.6±2.3
E. coli	150.0±4.5	115.6±3.2	87.2±2.0	123.3±7.1	145.9±3.7	159.6±6.6	75.5±1.9
E. aerogenes	140.5±2.3	130.9±7.5	91.3±2.3	142.4±5.5	134.9±3.5	150.4±7.5	65.0±1.2

Table S2. Values of MBC (mg/L) of different microorganisms treated with different extracts

Microorganism	PE	DCM	AE	A	M	W	Ciprofloxacin
B. subtilis	270.5±6.3	250.9±8.5	201.3±7.1	292.4±8.4	234.9±5.4	310.4±9.5	135.0±4.3
E. faecalis	292.3±6.6	221.3±5.9	178.5±8.3	283.4±8.6	242.3±5.3	295.6±9.2	179.3±5.4
S. aureus	336.9±5.9	211.2±5.1	191.4±6.2	305.3±7.5	252.6±6.3	279.5±8.3	189.6±6.4
P. aeruginosa	282.3±4.1	212.3±6.2	184.5±7.3	241.1±7.6	232.4±5.2	268.8±6.4	146.6±6.2
E. coli	348.3±5.6	201.6±8.2	156.2±6.3	311.1±8.5	226.5±6.2	297.2±8.3	181.2±5.3
E. aerogenes	310.0±4.5	235.6±5.2	187.2±5.1	253.4±7.1	275.5±7.3	329.2±9.3	175.5±5.1

Table S3. Values of MIC (mg/L) of different microorganisms treated with isolated triterpenes

Microorganism	1	2	3	4	5	6	7	8	Control[a]
B. subtilis	21.8±0.5	13.2±01	7.2±0.2	7.3±0.3	13.6±0.3	19.2±1.1	9.2±0.1	19.2±2.1	6.5±0.3[a]
E. faecalis	18.0±0.1	13.1±0.1	8.2±0.3	9.5±0.4	13.2±0.3	18.0±2.2	8.0±0.2	18.0±1.2	7.8 [a]±0.2
S. aureus	16.9±0.2	12.8±0.1	7.5±0.3	8.2±0.2	12.0±0.2	18.6±1.3	8.6±0.3	18.6±1.3	6.9 [a]±0.3
P. aeruginosa	19.0±0.2	12.5±0.1	7.3±0.2	10.9±0.3	12.8±0.4	19.6±2.1	9.6±0.1	19.6±1.1	8.0[b]±0.2
E. coli	11.6±0.1	11.6±0.2	7.5±0.1	7.4±0.2	11.1±0.2	17.2±1.2	7.2±0.2	17.2±1.2	7.0[b]±0.2
E. aerogenes	10.8±0.2	12.0±0.2	9.5±0.3	11.2±0.1	11.5±0.2	16.2±2.2	10.2±0.2	20.2±1.2	7.8[b]±0.2

[a]Ampicillin for Gram-positive bacteria. [b]Ciprofloxacin for Gram-negative bacteria.

Table S4. Values of MBC (mg/L) of different microorganisms treated with isolated triterpenes

Microorganism	1	2	3	4	5	6	7	8	Control[a]
B. subtilis	43.8±3.5	26.2±3.1	17.2±0.7	15.5±1.3	27.6±2.3	39.2±1.1	19.2±0.1	40.2±3.1	14.5[a]±1.4
E. faecalis	34.0±2.1	27.1±2.1	18.2±1.6	21.5±1.4	28.2±2.1	39.0±1.3	18.0±0.7	40.0±2.5	15.8[a]±1.3
S. aureus	38.9±3.2	25.8±3.1	17.5±1.5	18.2±1.4	25.0±1.9	40.6±2.3	17.6±1.3	39.6±2.4	14.9[a]±0.9
P. aeruginosa	23.0±2.2	25.5±3.1	17.1±1.7	22.9±1.3	25.8±1.8	41.6±3.1	21.6±1.9	41.6±2.1	17.0[b]±1.3
E. coli	22.6±2.1	21.6±3.2	15.5±1.4	15.8±2.4	22.1±2.2	35.2±2.2	17.2±1.9	36.2±2.2	15.0[b]±1.1
E. aerogenes	10.8±0.8	12.0±0.9	19.5±1.3	21.2±1.1	23.5±1.2	33.2±2.2	21.2±2.2	42.2±2.2	16.8[b]±1.2

[a]Ampicillin for Gram-positive bacteria and Ciprofloxacin for Gram-negative bacteria.

Table S5. IC_{50} values for the triterpenes **1**–**8** using MTT assay.

Triterpene	1	2	3	4	5	6	7	8
IC_{50} (μM)[a]	96±10	450±51	144±23	28±4	269±38	58±8	397±43	839±91

[a]Concentration inhibiting cell growth by 50%.

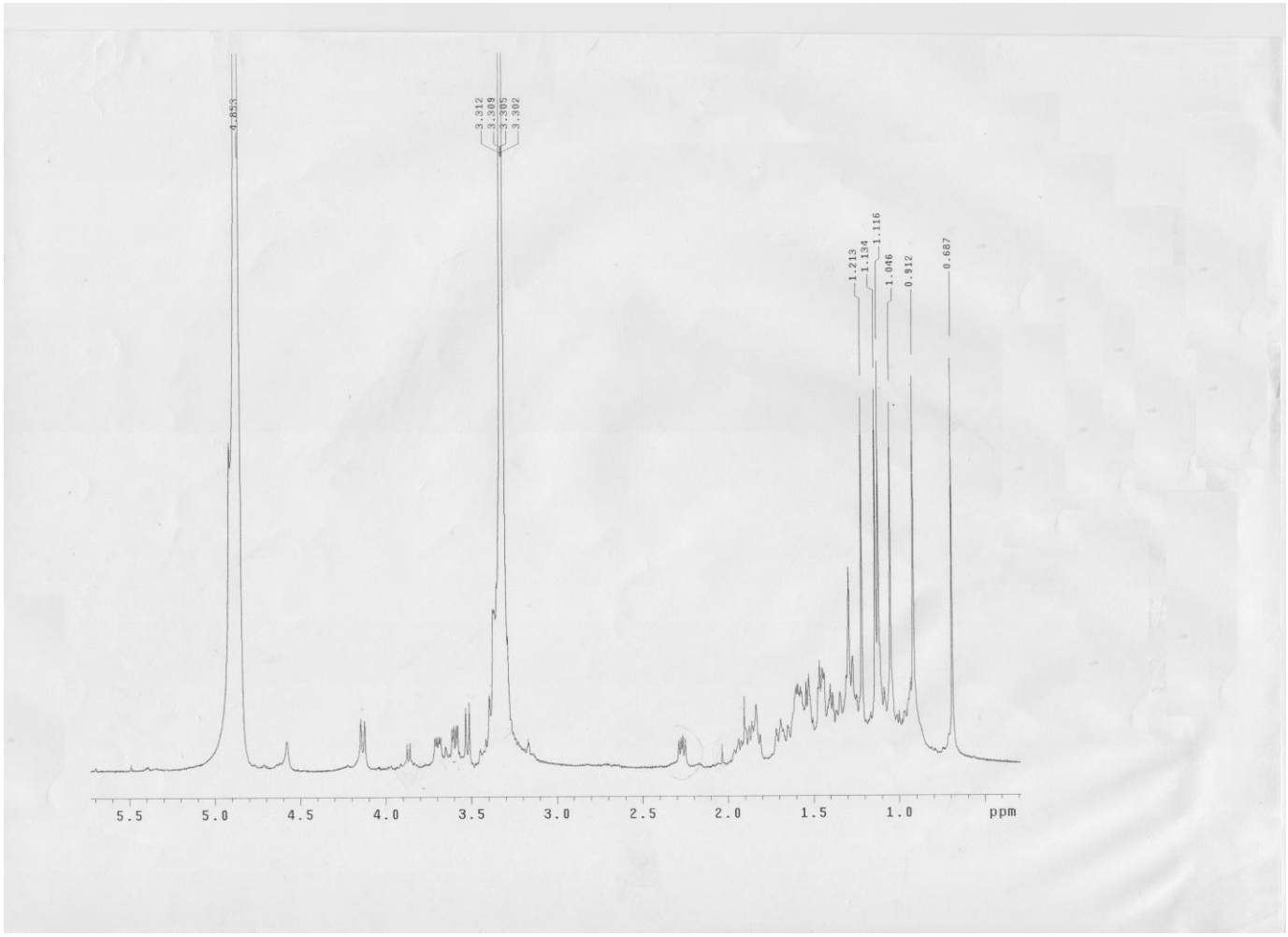


Figure S2: ^{1}H NMR spectrum of compound **1** in CD_3OD

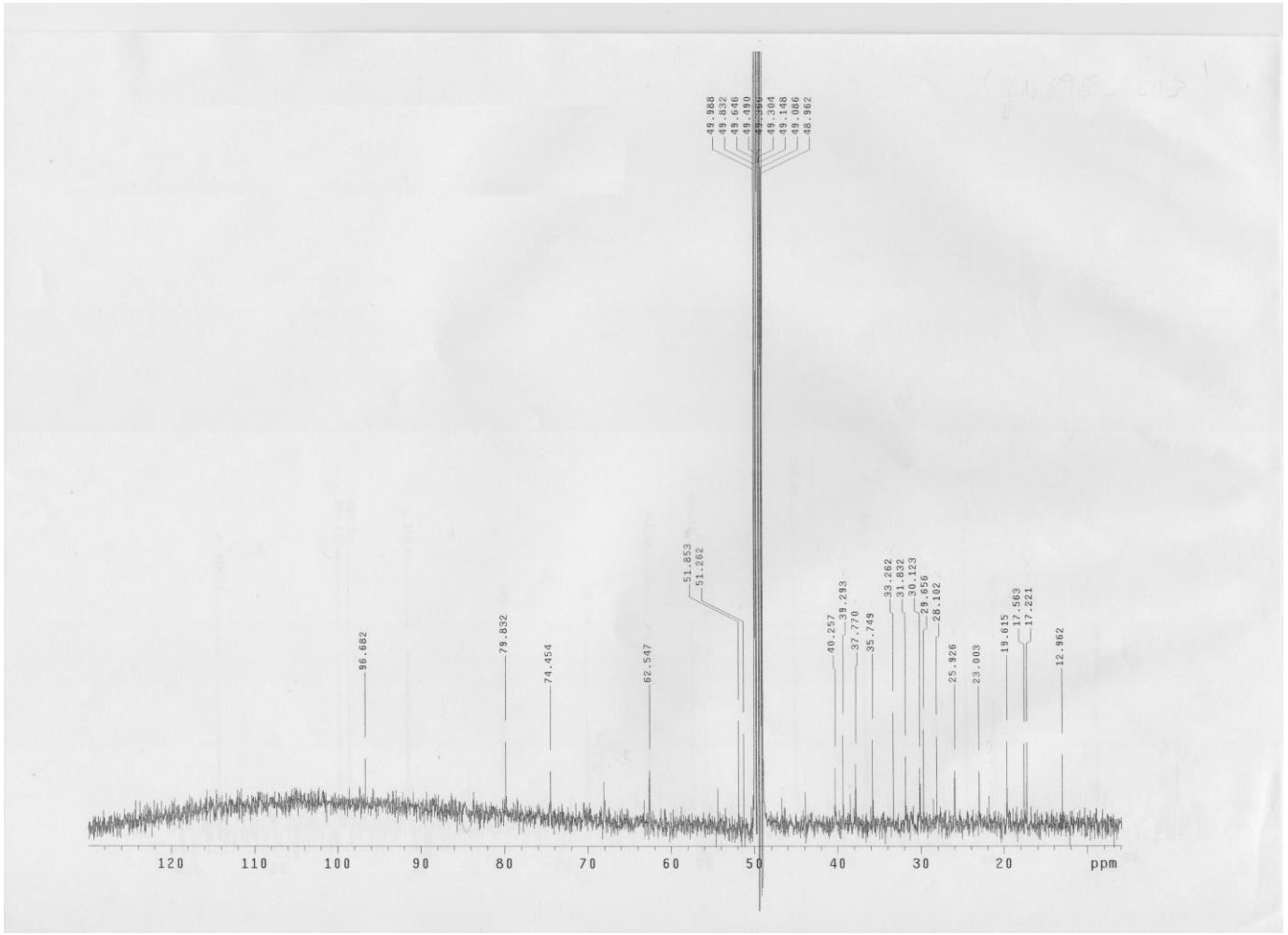


Figure S3: ^{13}C NMR spectrum of compound **1** in CD_3OD

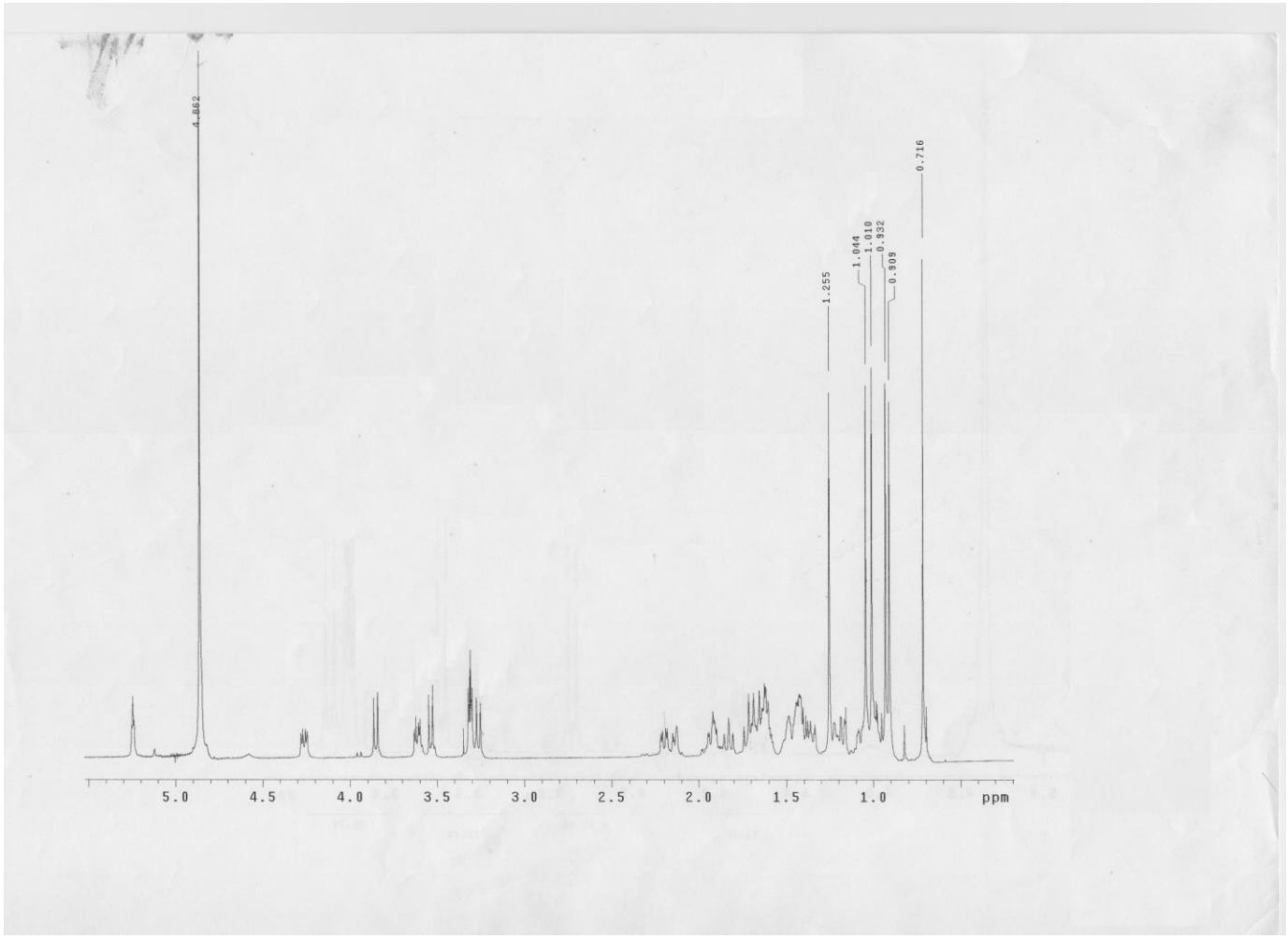


Figure S4: ^{1}H NMR spectrum of compound **2** in CD_3OD

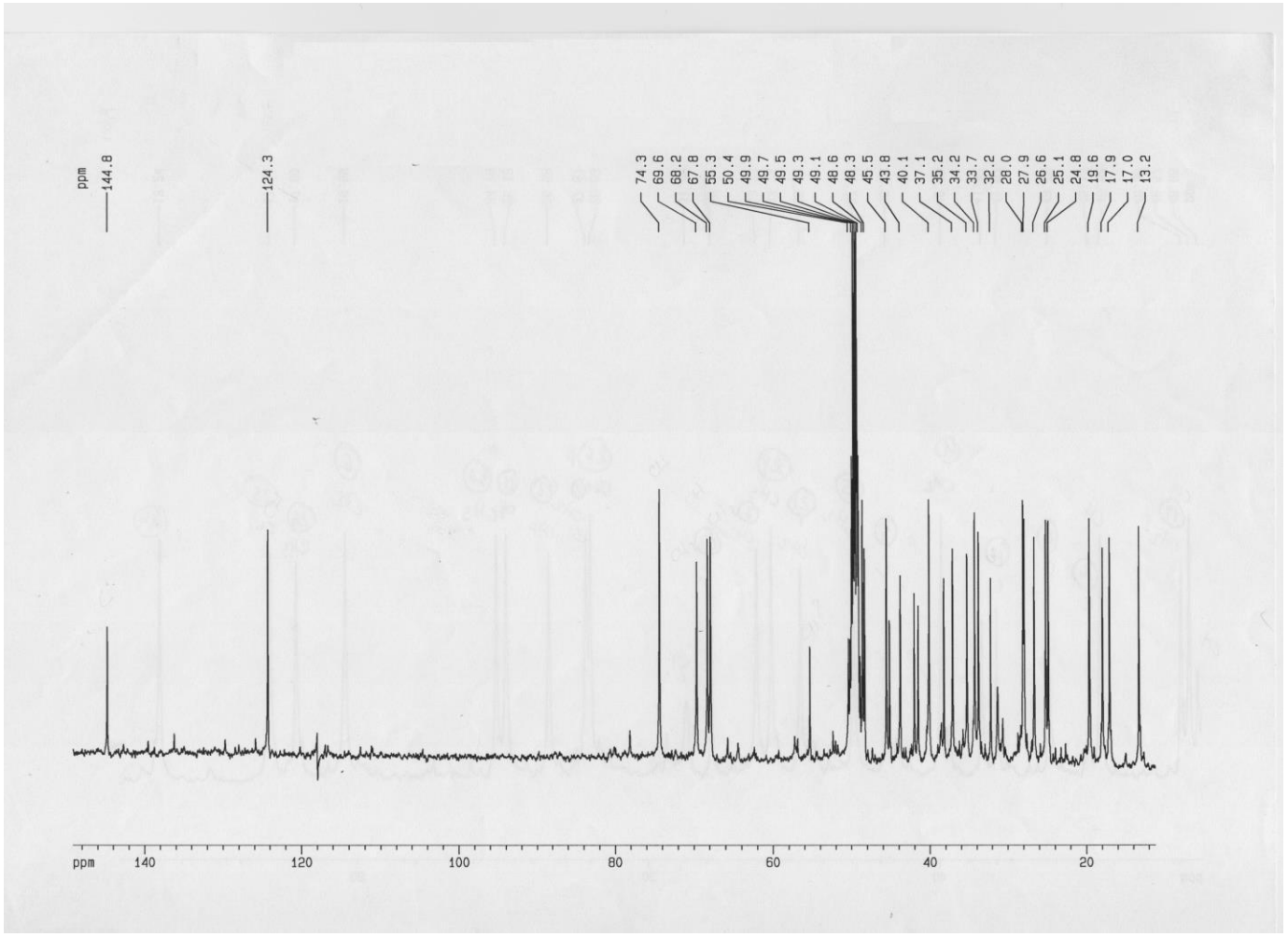


Figure S5: ^{13}C NMR spectrum of compound **2** in CD_3OD

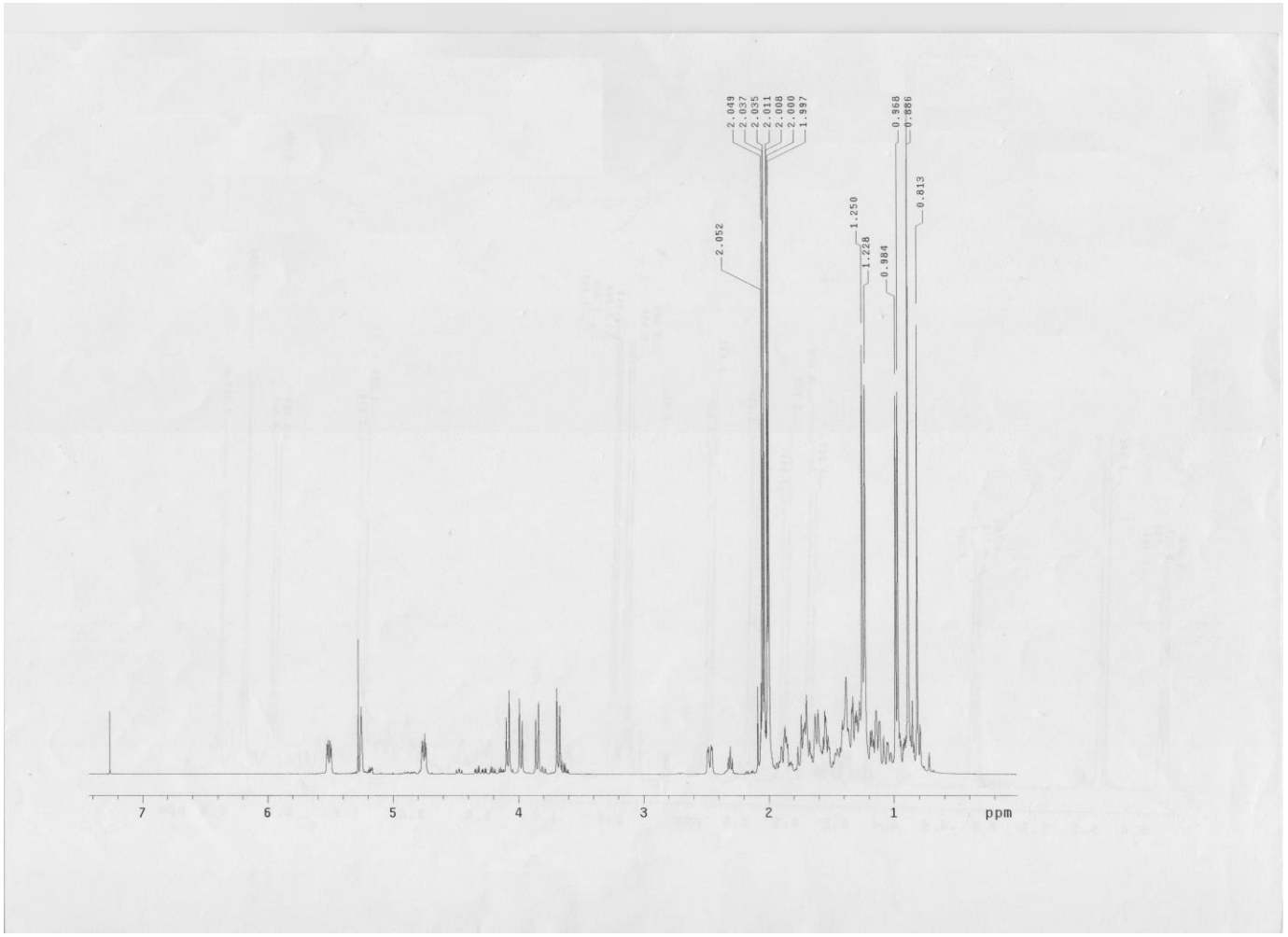


Figure S6: ^{1}H NMR spectrum of compound **3** in $CDCl_3$

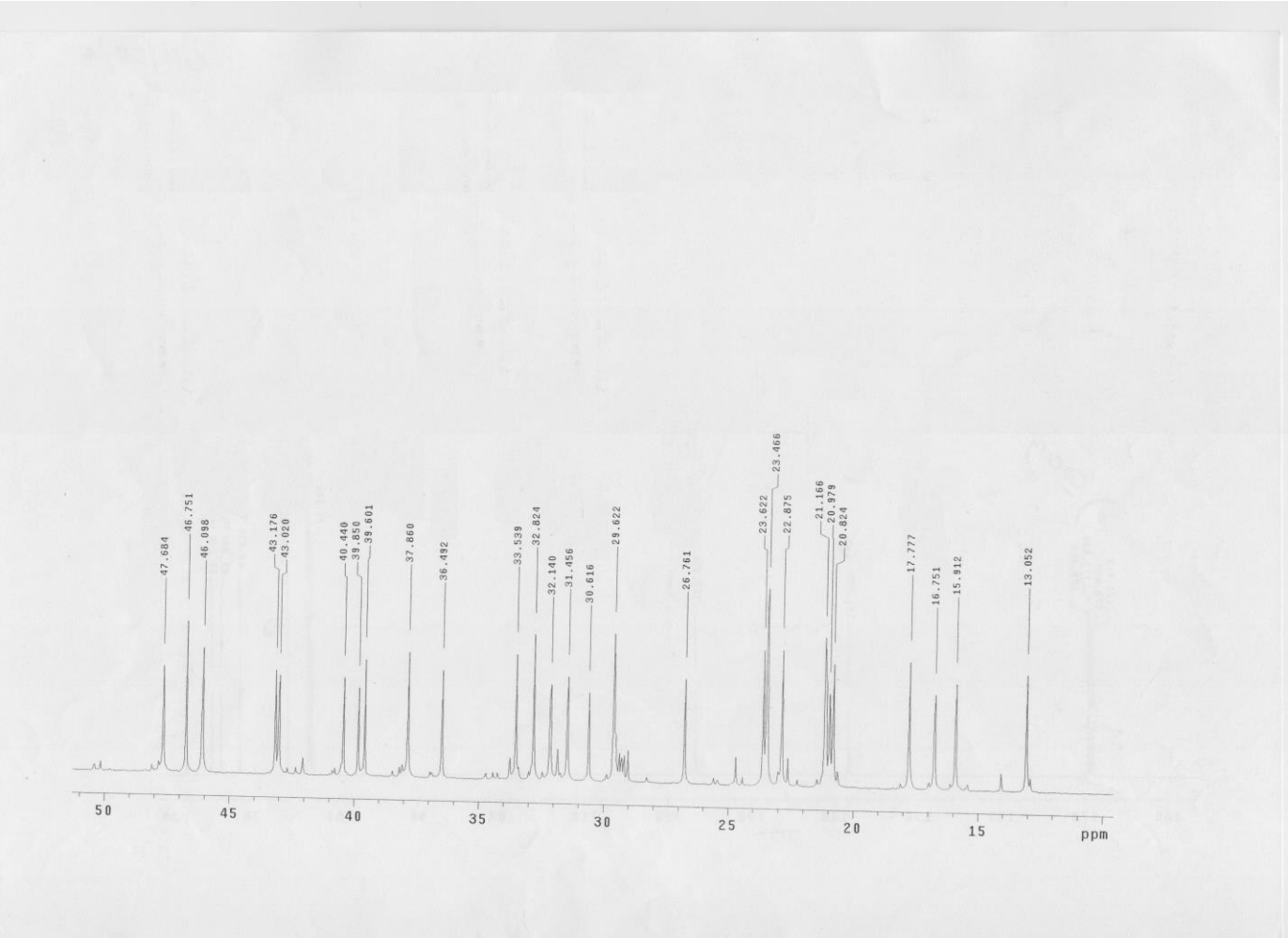


Figure S7: ^{13}C NMR spectrum of compound **3** in $CDCl_3$

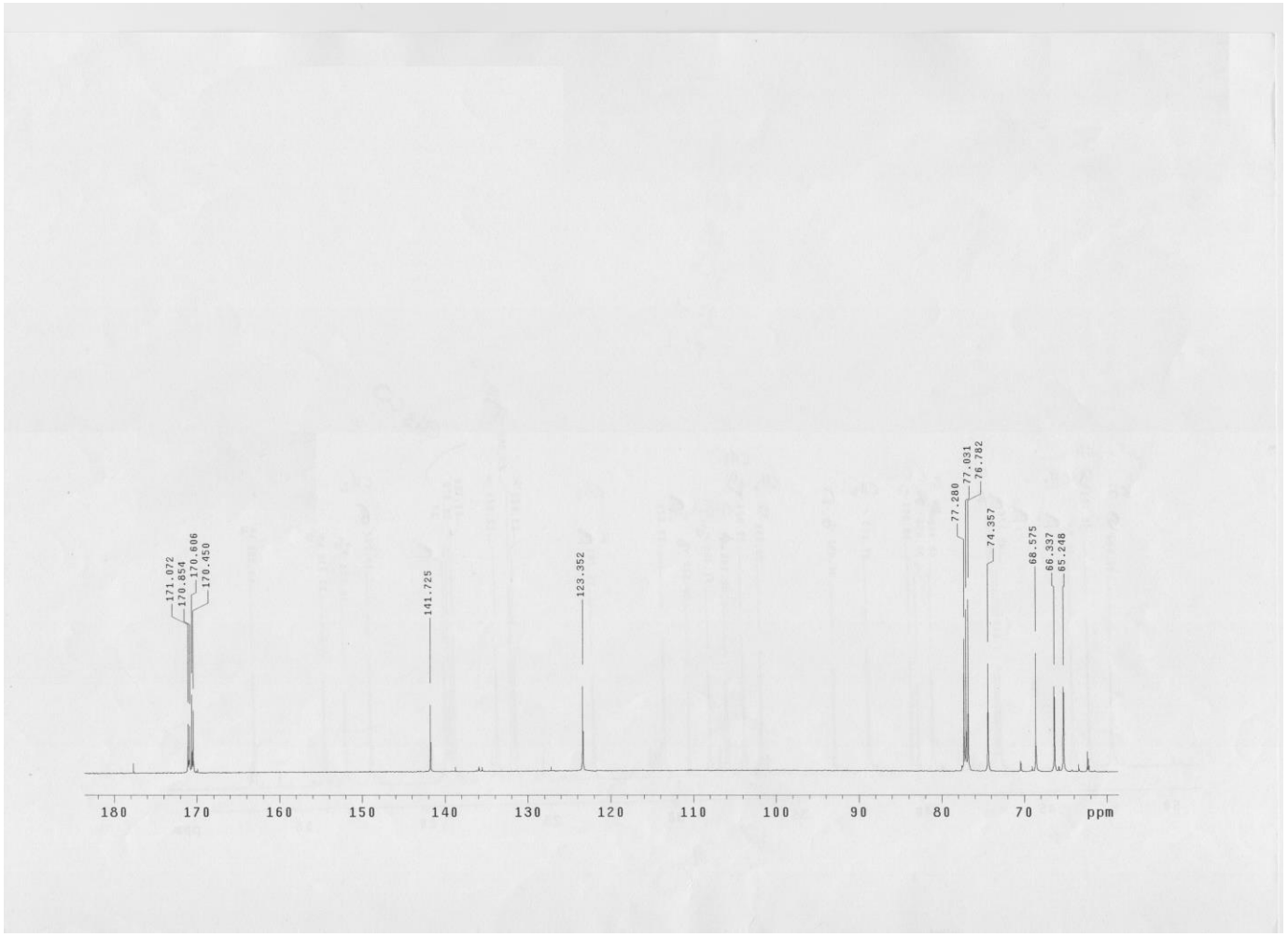


Figure S8bis: ^{13}C NMR spectrum of compound **3** in $CDCl_3$

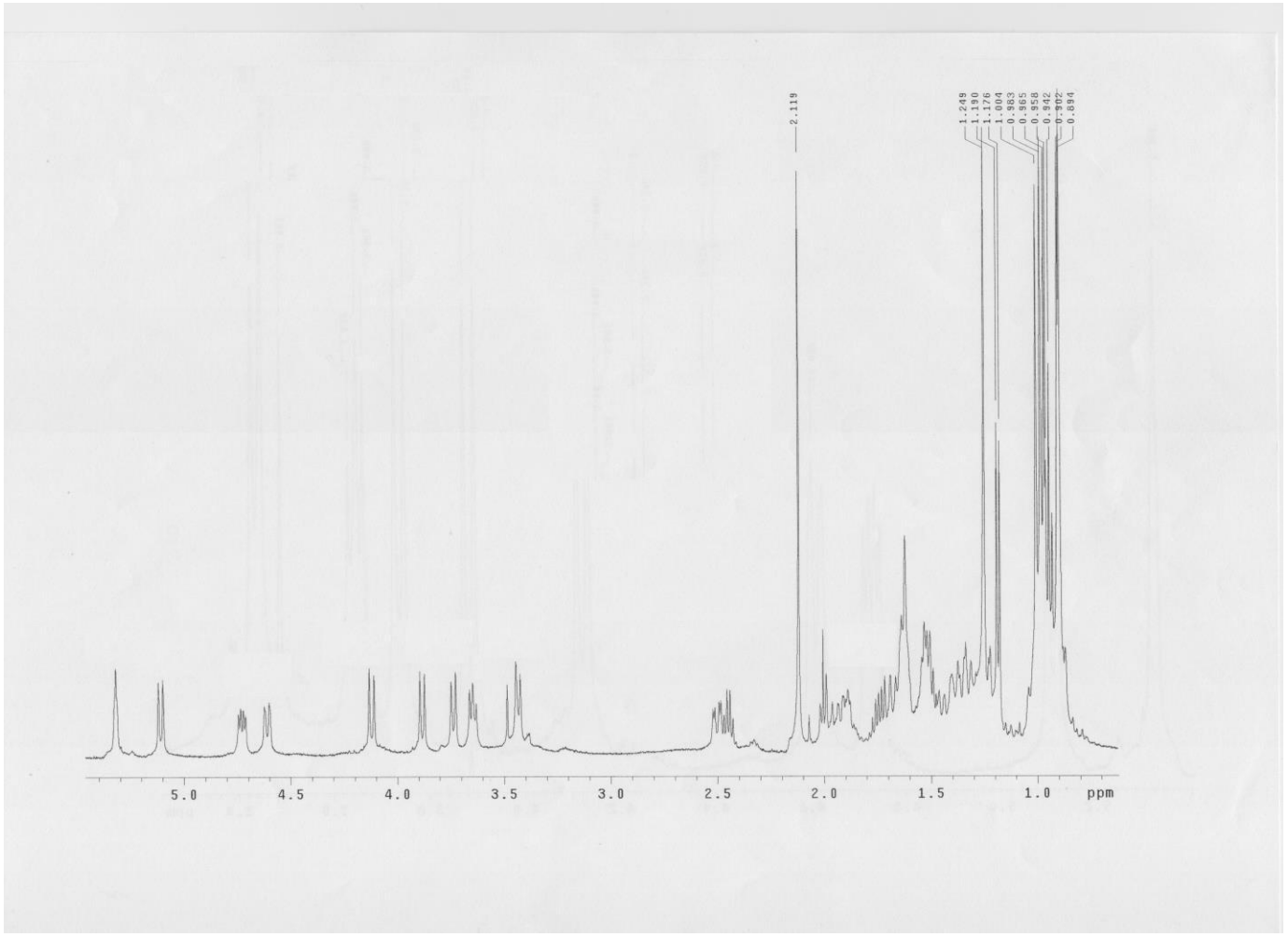


Figure S9: ^{1}H NMR spectrum of compound **4** in $CDCl_3$

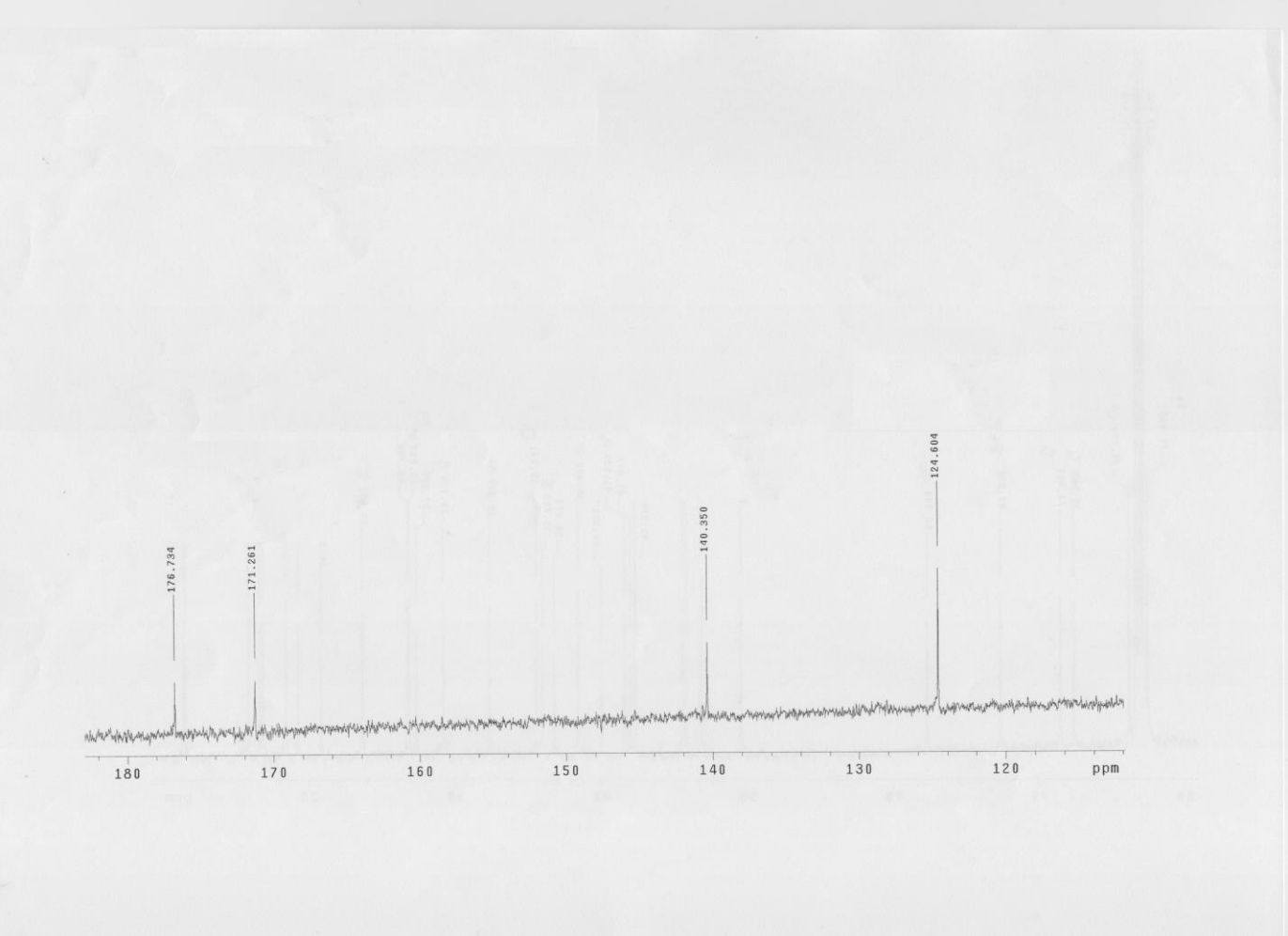


Figure S10: ^{13}C NMR spectrum of compound **4** in $CDCl_3$

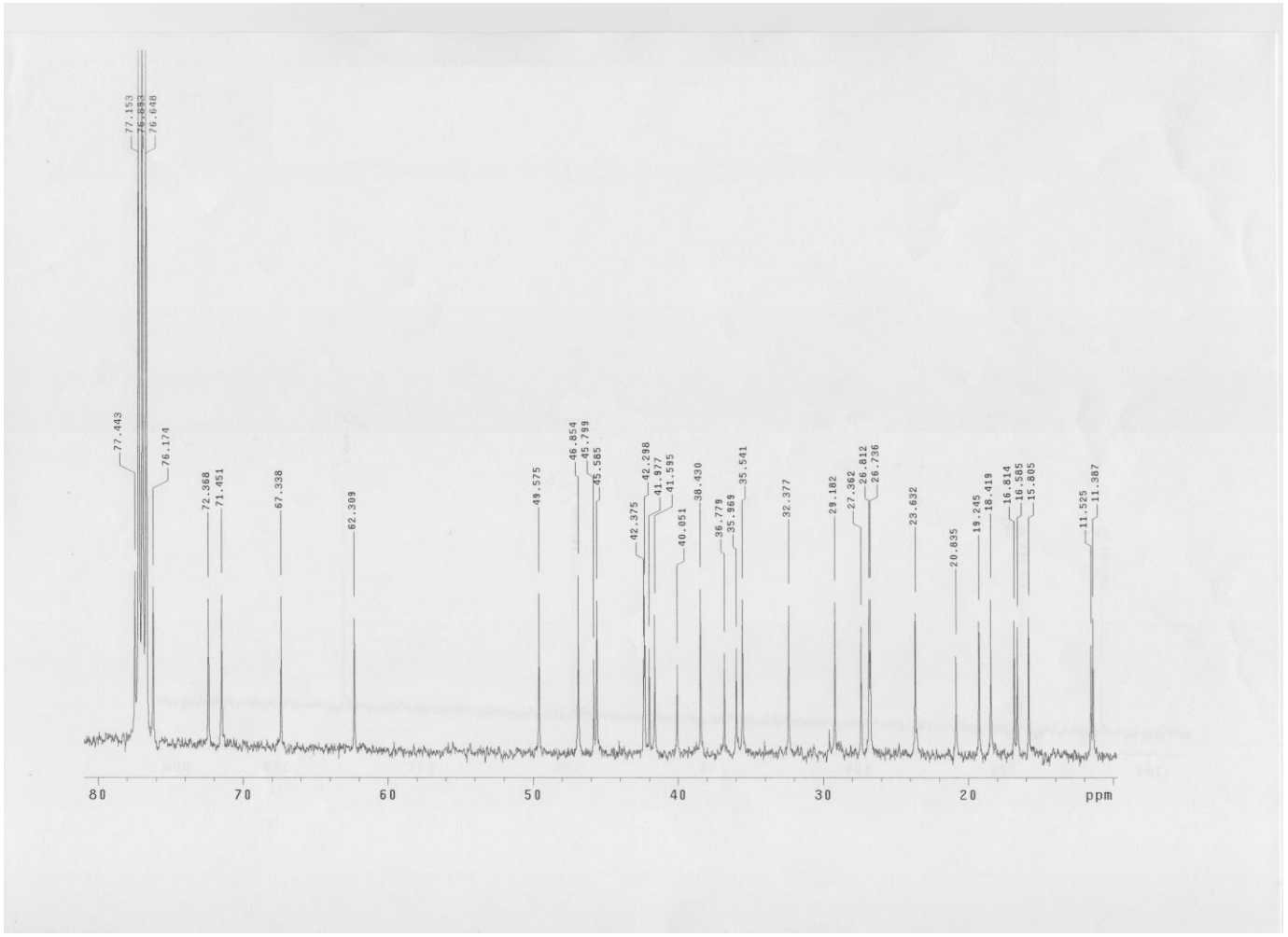


Figure S10 bis:^{13}C NMR spectrum of compound **4** in $CDCl_3$

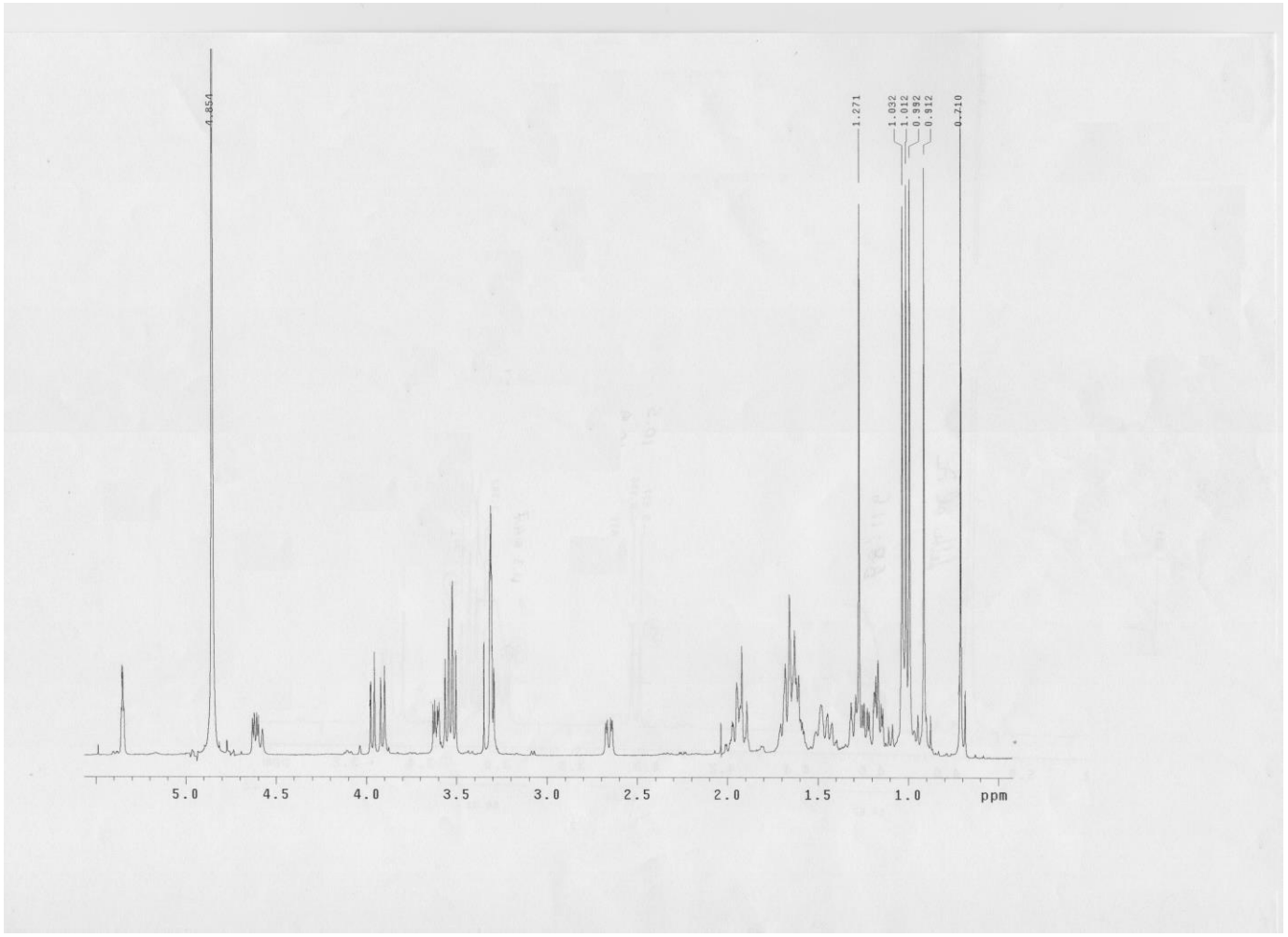


Figure S11: ^{1}H NMR spectrum of compound **5** in $CDCl_3$

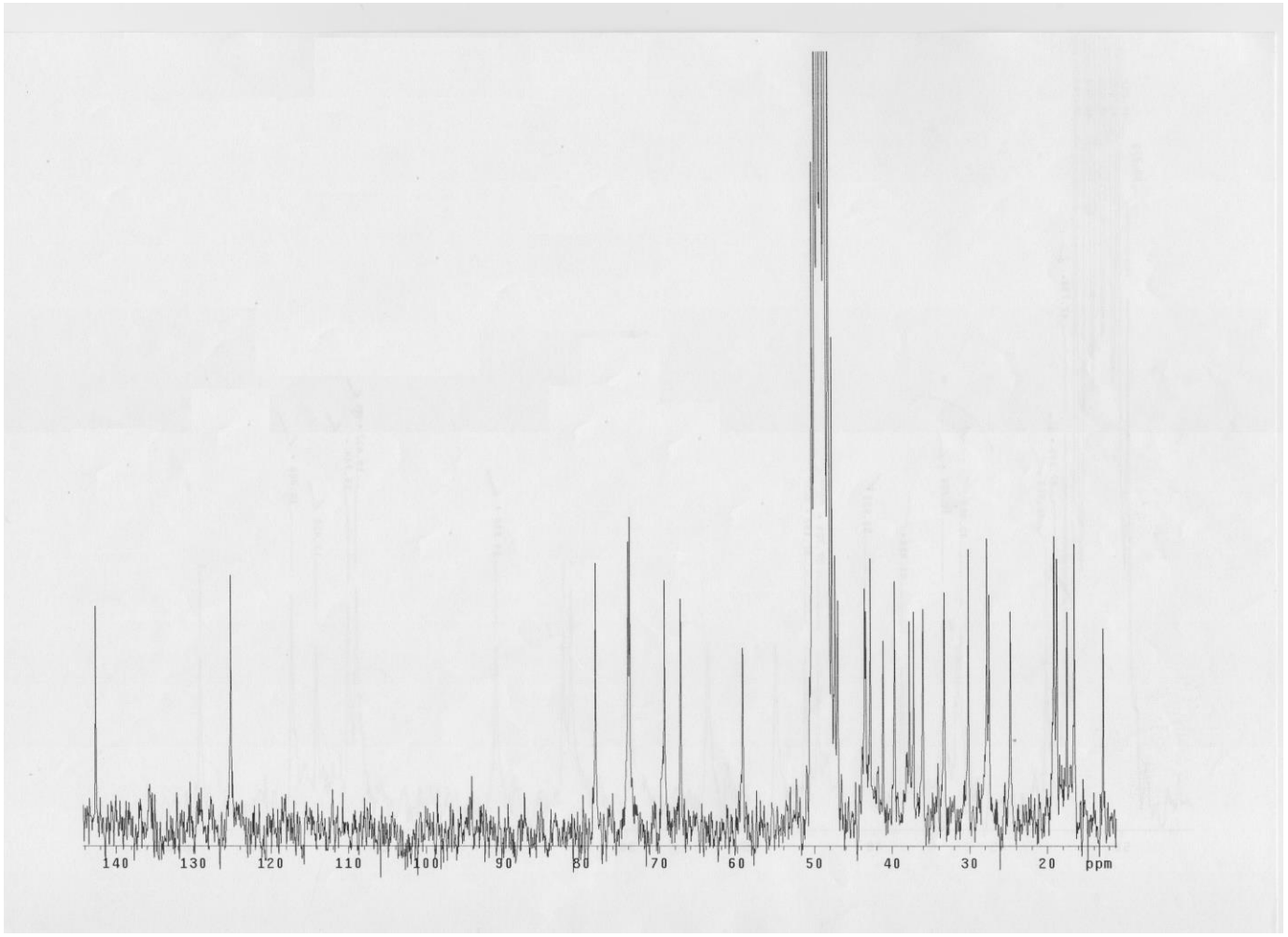


Figure S12:^{13}C NMR spectrum of compound **5** in $CDCl_3$

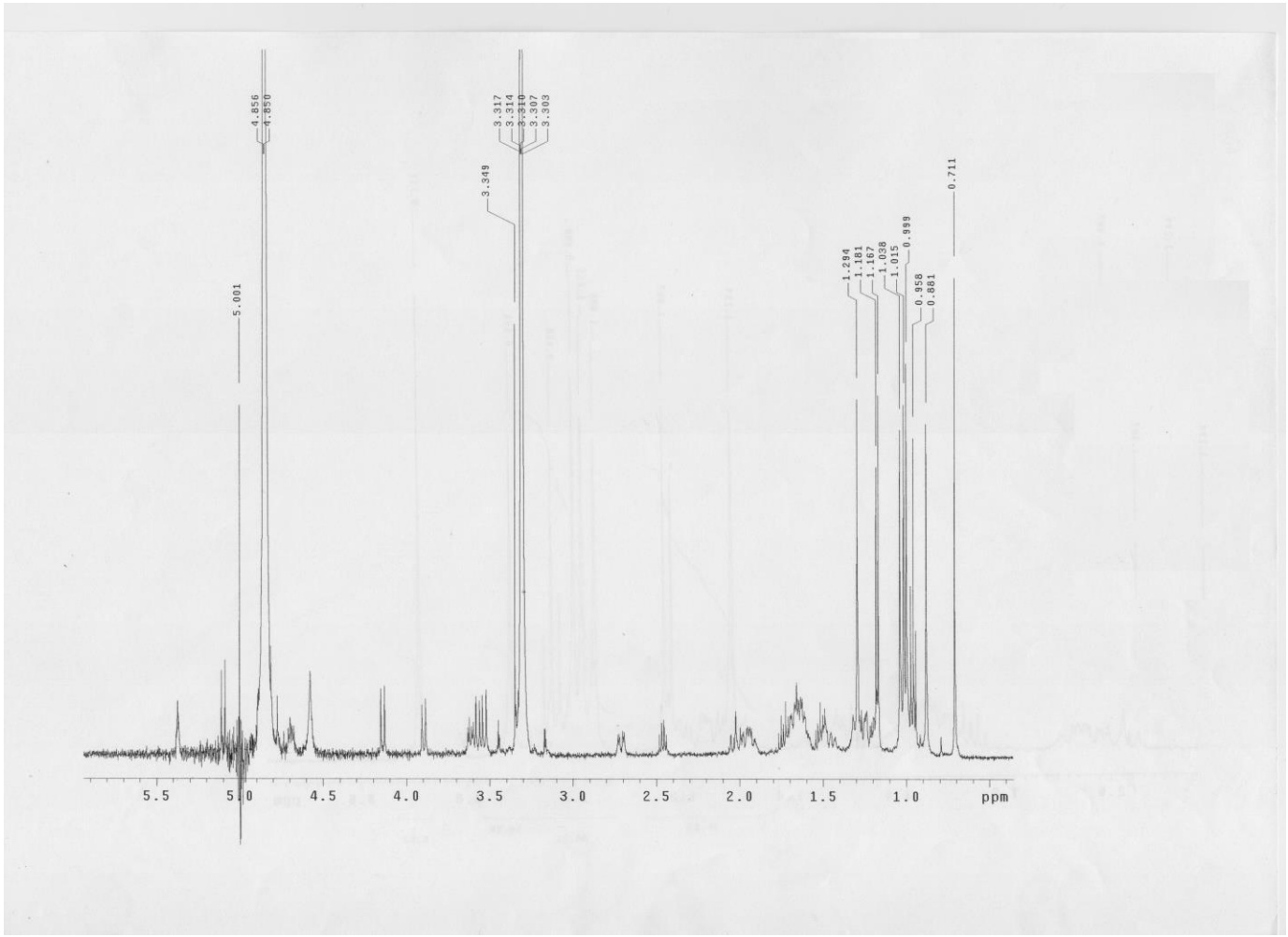


Figure S13: ^{1}H NMR spectrum of compound **6** in $CDCl_3$

Figure S14:^{13}C NMR spectrum of compound **6** in $CDCl_3$

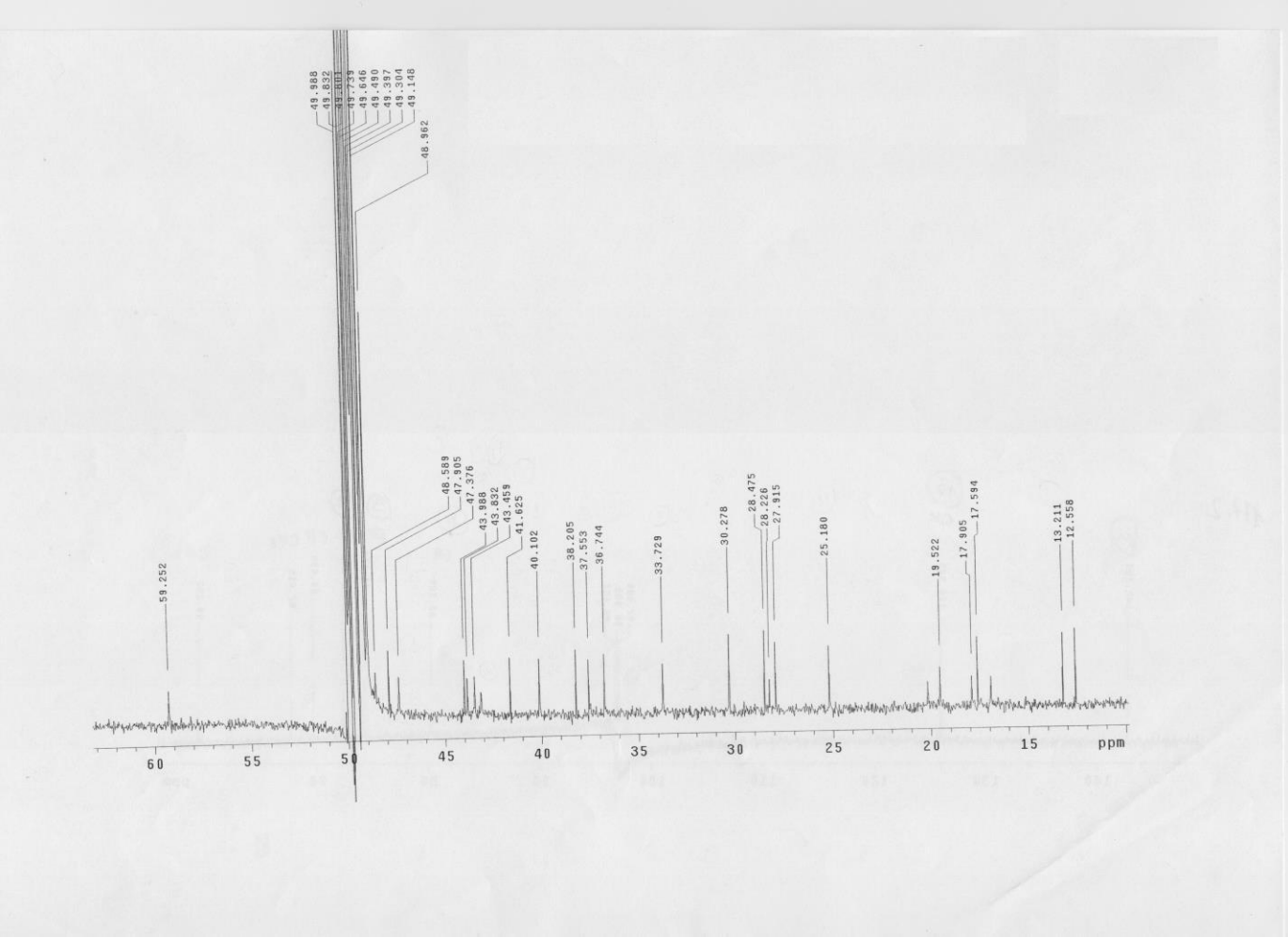


Figure S14 bis: ^{13}C NMR spectrum of compound **6** in $CDCl_3$

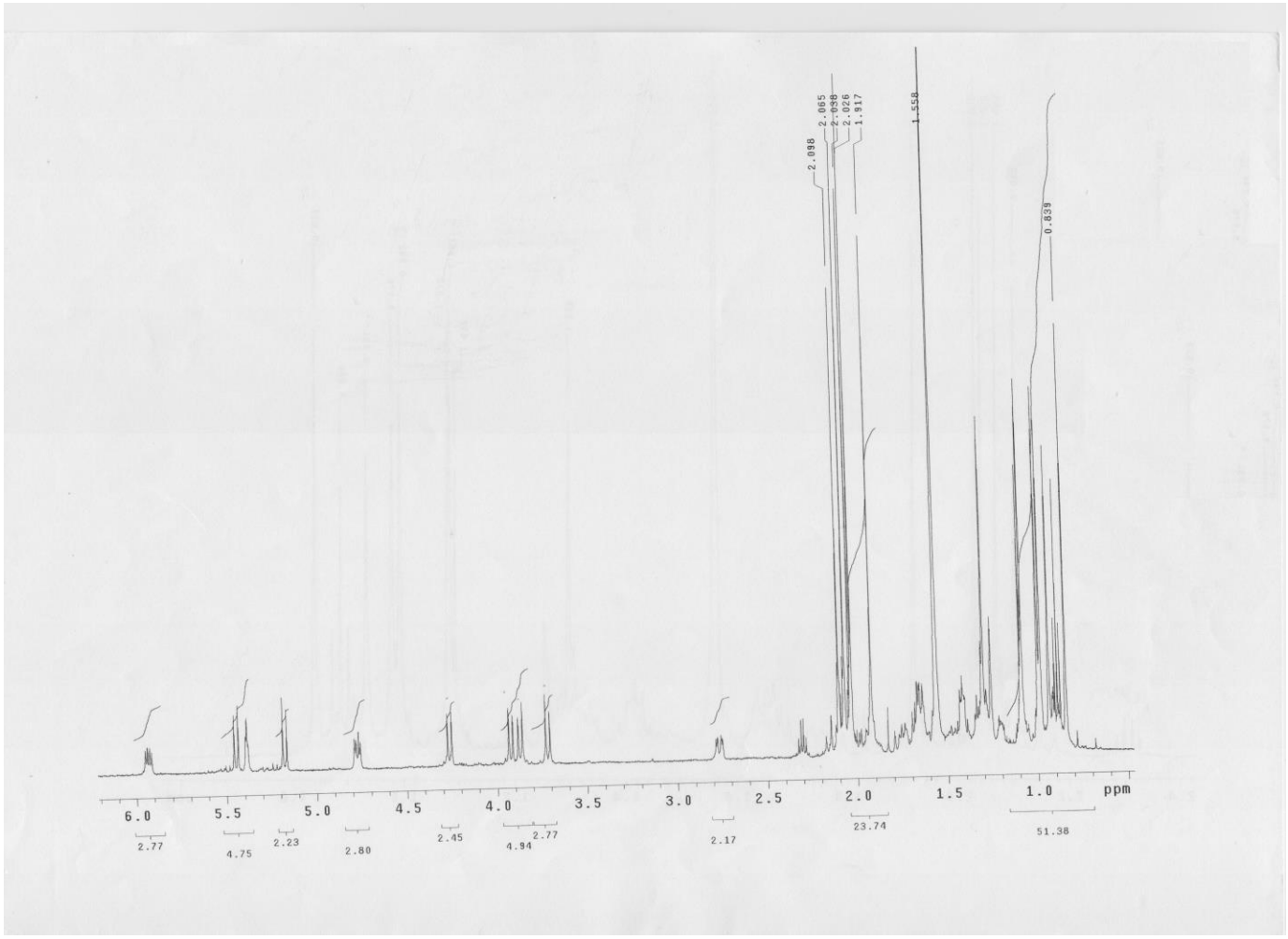


Figure S15:[1]H NMR spectrum of compound **7** in $CDCl_3$

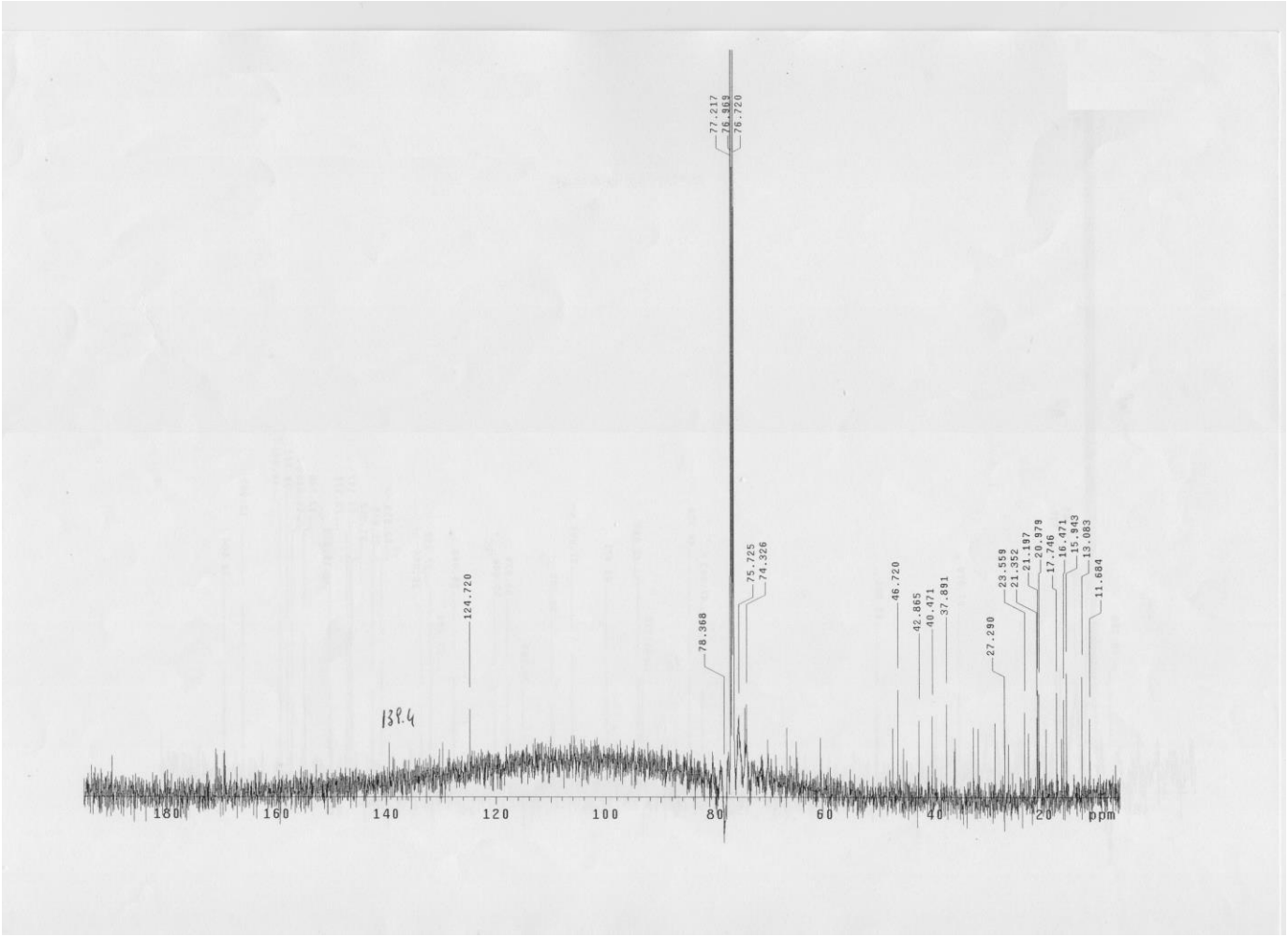


Figure S16:^{13}C NMR spectrum of compound **7** in $CDCl_3$

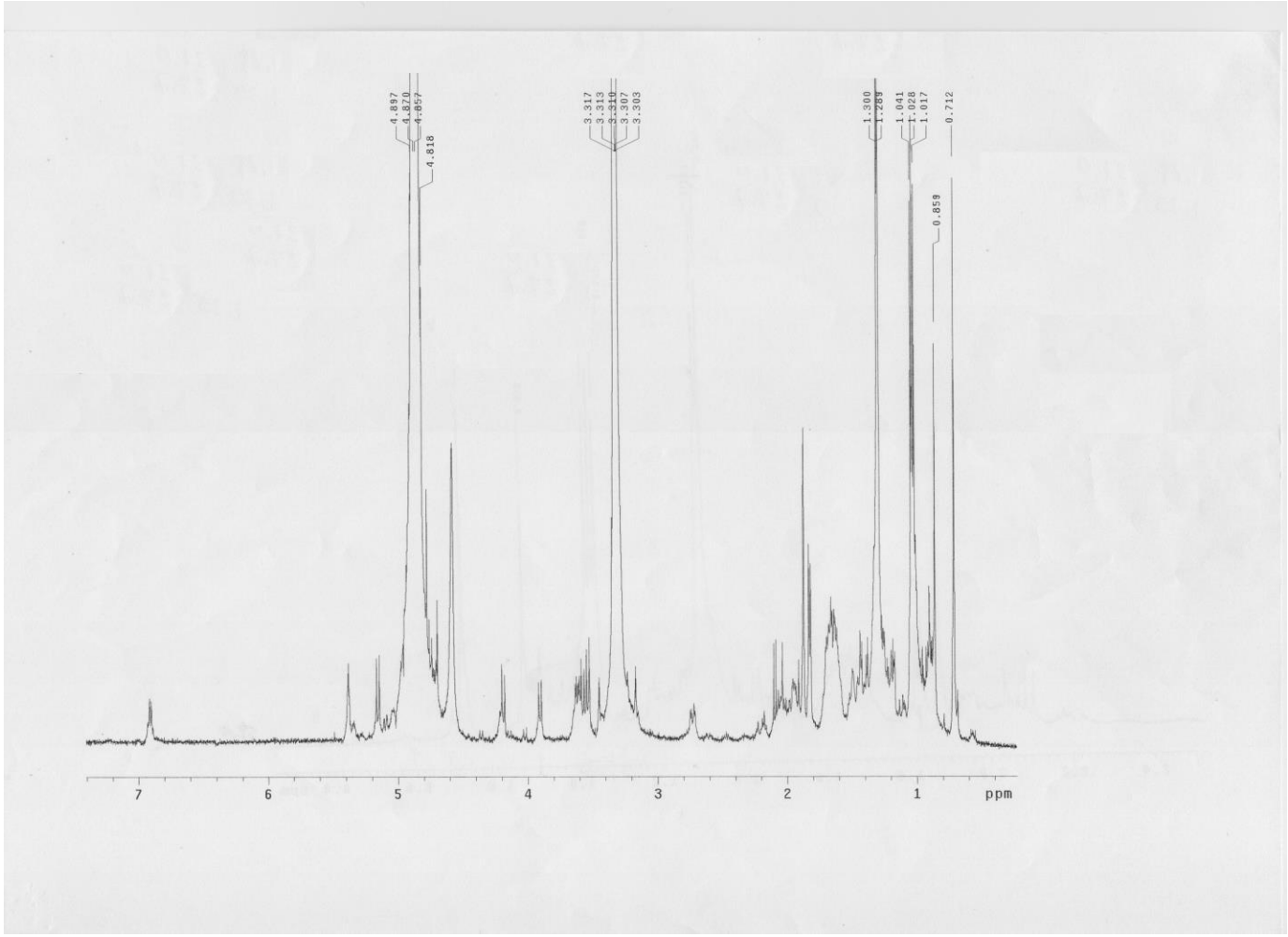


Figure S17:^{1}H NMR spectrum of compound **8** in CD_3OD

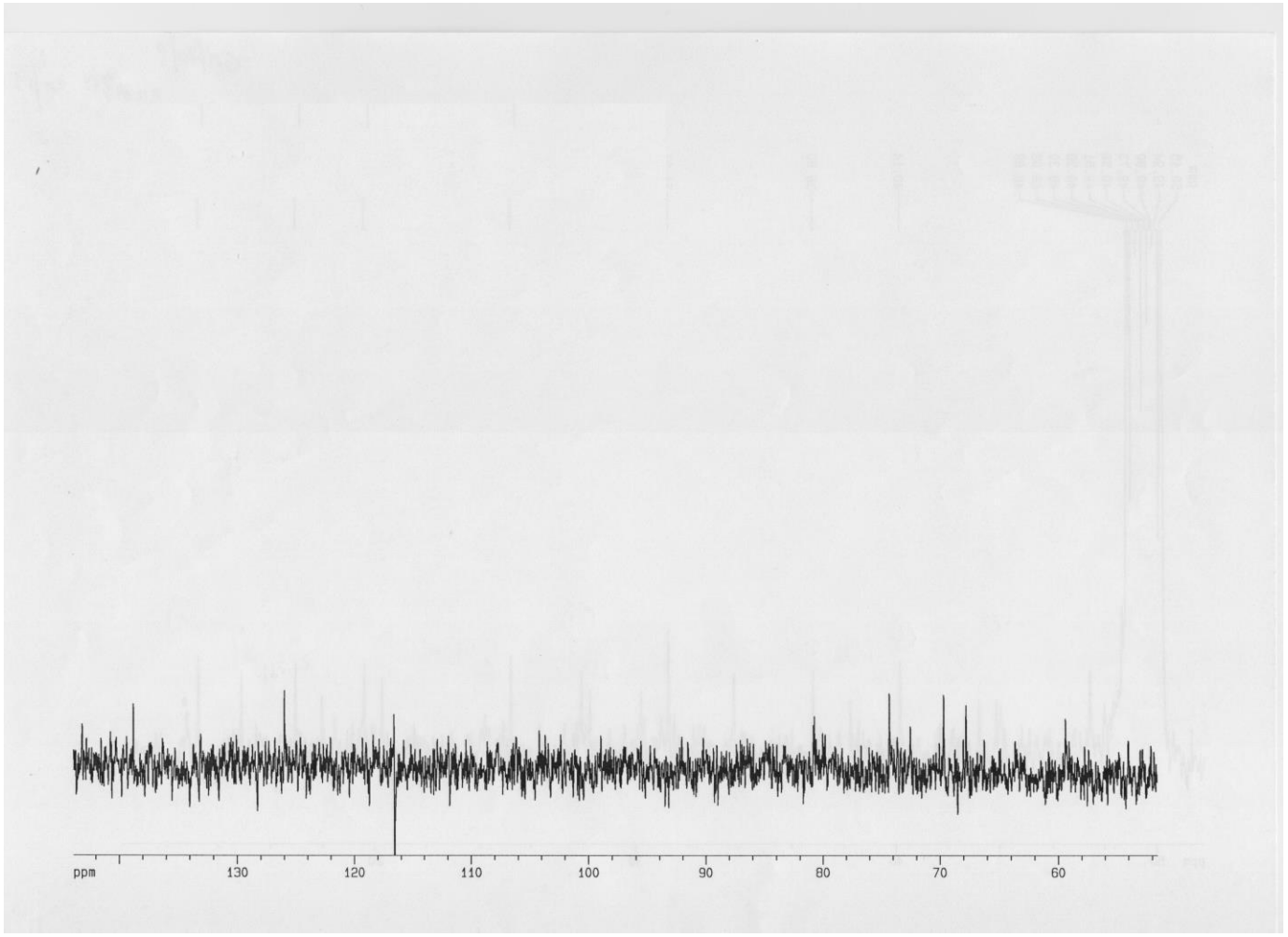


Figure S18: ^{13}C NMR spectrum of compound **8** in CD_3OD

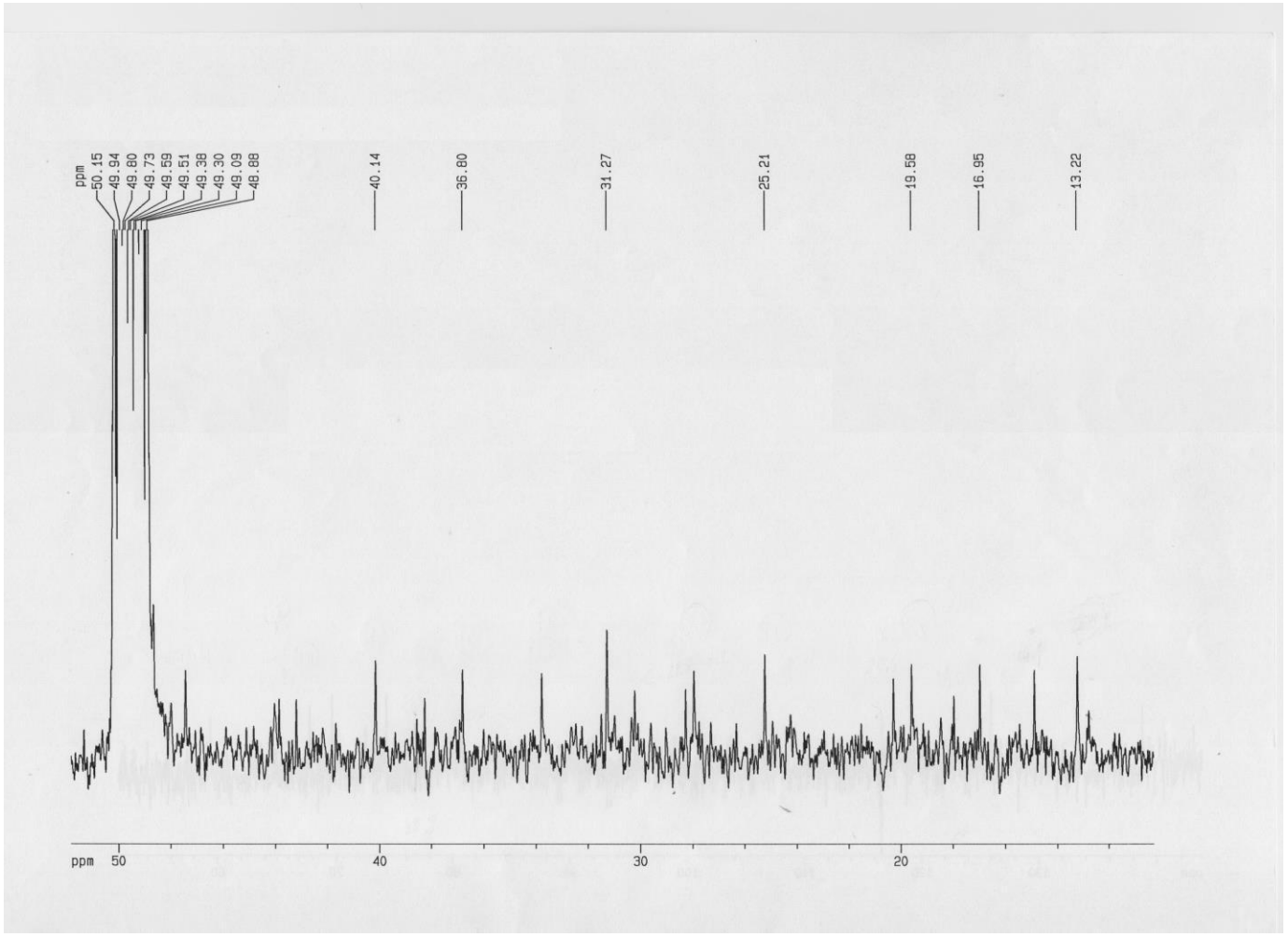


Figure S18 bis: ^{13}C NMR spectrum of compound **8** in CD_3OD