Supporting Information

Isolation and identification of L/D-lactate-conjugated bufadienoldies from toad eggs revealing lactate racemization in amphibians

Shiwen Zhou,[a,‡] Qingfei Zheng,[b,‡] Xiuyong Huang,[a] Yong Wang,[c] Sifan Luo,[a] Renwang Jiang,[a] Lei Wang,*[a] Wencai Ye,*[a] and Haiyan Tian*[a]

[a] Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, Guangzhou 510632, China

[b] State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai 200032, China

[c] School of stomatology and medicine, Foshan University, Foshan 528000, China

‡ These authors contributed equally.

Corresponding authors:

E-mail: tianhaiyan1982@163.com

E-mail: chyewc@gmail.com.

E-mail: cpuwanglei@126.com

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1. Experimental Sections

1.1 General Experimental Procedures

Melting points were measured on an X-5 micro-MP apparatus (Huayan Corporation, Shanghai, China) and apparatus was not corrected. The optical rotations were carried out with a JASCO digital polarimeter (JASCO Corporation, Tokyo, Japan) using a thermostable optical glass cell (0.1 dm path length and *c* in g/100 mL). HR-ESI-MS were acquired on an Agilent 6210 HPLC/MS TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). UV spectra were recorded on a JASCO V-550 UV/VIS spectrometer (JASCO Corporation, Tokyo, Japan). IR spectra were obtained on a JASCO FT-IR-4600 infrared spectrometer (JASCO Corporation, Tokyo, Japan) in KBr pellets. All NMR spectra were recorded on Bruker AV-300 spectrometer (Bruker Instrument, Inc., Zurich, Switzerland) using the residual signals (CD_3OD: $\boldsymbol{\delta}_H$ 3.31/ $\boldsymbol{\delta}_c$ 49.0) as the internal standard. HPLC was performed on an Agilent 1200 HPLC system equipped with a diode array detector, using a biphenyl column (Kinetex Biphenyl, 5 μm, 4.6×250 mm, Phenomenex, USA) for analysis. Pre-HPLC was performed on a Wufeng L-100 HPLC system (Wufeng Corporation, Shanghai, China), using a biphenyl preparative column (Kinetex Biphenyl, 10 μm, 10×250 mm, Phenomenex, USA) for preparative purification. Open column chromatography (CC) was performed on silica gel (200-300 mesh, Haiyang Chemical Group Corporation, Qingdao, China) and reverse-phase C18 silica gel (YMC Corporation, Japan). X-ray single-crystal diffraction data for compounds were collected on Rigaku X-ray diffractometer (Rigaku Corporation, Japan) at 100(10) K.

1.2 Material

The eggs of *Bufo bufo gargarizans* were purchased from Dongtai Toad Breeding Base in Jiangsu province of china between March and May 2015, and authenticated (No.201503031) was deposited in the Institute of Traditional Chinese Medicine and Natural Products, College of Pharmacy, Jinan University, China.

Human cell lines A549 and BGC823 were purchased from ATCC and were cultured in RMPI 1640 Medium containing 10% fetal bovine serum, 100 IU/ml

penicillin and 100 mg/ml streptomycin in humidified 5% CO_2 atmosphere at 37 ˚C as previously described.

1.3 Extraction and isolation

The dried and roughly powdered eggs of *Bufo bufo gargarizans* (25 Kg) were extracted with 95% EtOH under ultrasonic condition three times. After removal of the solvent extract under reduced pressure, the crude extract (2.5 Kg) was suspended in 20% EtOH (20 L) and subsequently partitioned by hexamethylene (3×20 L) and EtOAc (3×20 L). The concentrated ethyl acetate layer (253 g) was subjected to silica gel (200-300 mesh), eluted with dichloromethane-methanol (100:1, 50:1, 20:1 and 10:1) to give 16 fraction (**Fr.1-16**). **Fr.5** (3.2 g) **Fr.8** (2.4 g) **Fr.9** (800 mg) was further separated by reverse-phase C18 silica using methanol-water gradients and Pre-HPLC.

The mixtures of compounds **1** and **2** were isolated from **Fr.5** and analyzed to be a single chromatographic peak by HPLC using a biphenyl column (Kinetex Biphenyl, 5 μm, 4.6×250 mm, Phenomenex, USA). However they were verified to be mixtures for its H-3′ being splitting in the ^{1}H NMR (Figure S5), then the mixtures were separated by chiral resolution using C-4 column (Kinetex Cellulose-4, 10 μm, 10×250 mm, Phenomenex, USA). Thus, the compounds **1** (20.1 mg) and **2** (18.8 mg) were obtained by chiral resolution under the condition of a gradient elution ranging from 75%-90% methanol-water within 60 min from the mixtures.

The mixtures of compounds **3** and **4** were isolated from **Fr.8** and also analyzed to be a single chromatographic peak by HPLC using a biphenyl column (Kinetex Biphenyl, 5 μm, 4.6×250 mm, Phenomenex, USA). They were also verified to be mixtures for its H-3′ being splitting in the ^{1}H NMR (Figure S6), then the mixtures were separated by chiral resolution using C-4 column (Kinetex Cellulose-4, 10 μm, 10×250 mm, Phenomenex, USA) and the compounds **3** (5.1 mg) and **4** (5.3 mg) were obtained by chiral resolution by chiral resolution under the condition of a gradient elution ranging from 75%-100% methanol-water within 50 min from the mixtures.

Similarly, compounds **5** (1.2 mg) and **6** (1.3 mg) were obtained by chiral resolution by chiral resolution under the condition of a gradient elution ranging from 75%-100%

methanol-water within 50 min from the mixtures of compounds **5** and **6** which isolated from **Fr.9**.

1.4 Cell Titer-Glo Luminescent Cell Viability Assay

Cell Viability Assay was done according to the protocol of Cell Titer-Glo Luminescent kit (Promega). In brief, 5000 cell was seeded on 96-well plates in culture medium, 100μl per well for 24 hours. Prepare control wells containing medium without cells to obtain a value for background luminescence. The test compound was added to experimental wells, and incubated for additional 48 hours. Add a volume of CellTiter-Glo Reagent equal to the volume of cell culture medium present in each well (e.g., add 100μl of reagent to 100μl of medium containing cells for a 96-well plate), Mix contents for 2 minutes on an orbital shaker to induce cell lysis. Allow the plate to incubate at room temperature for 10 minutes to stabilize luminescent signal. Luminescence signal was recorded by BIOTEK FLX800. Data was analyzed by Graphpad prism 5.

2. HPLC-HRMS spectra of Fr.5, Fr.8 and Fr.9 from toad *Bufo bufo gargarizans*

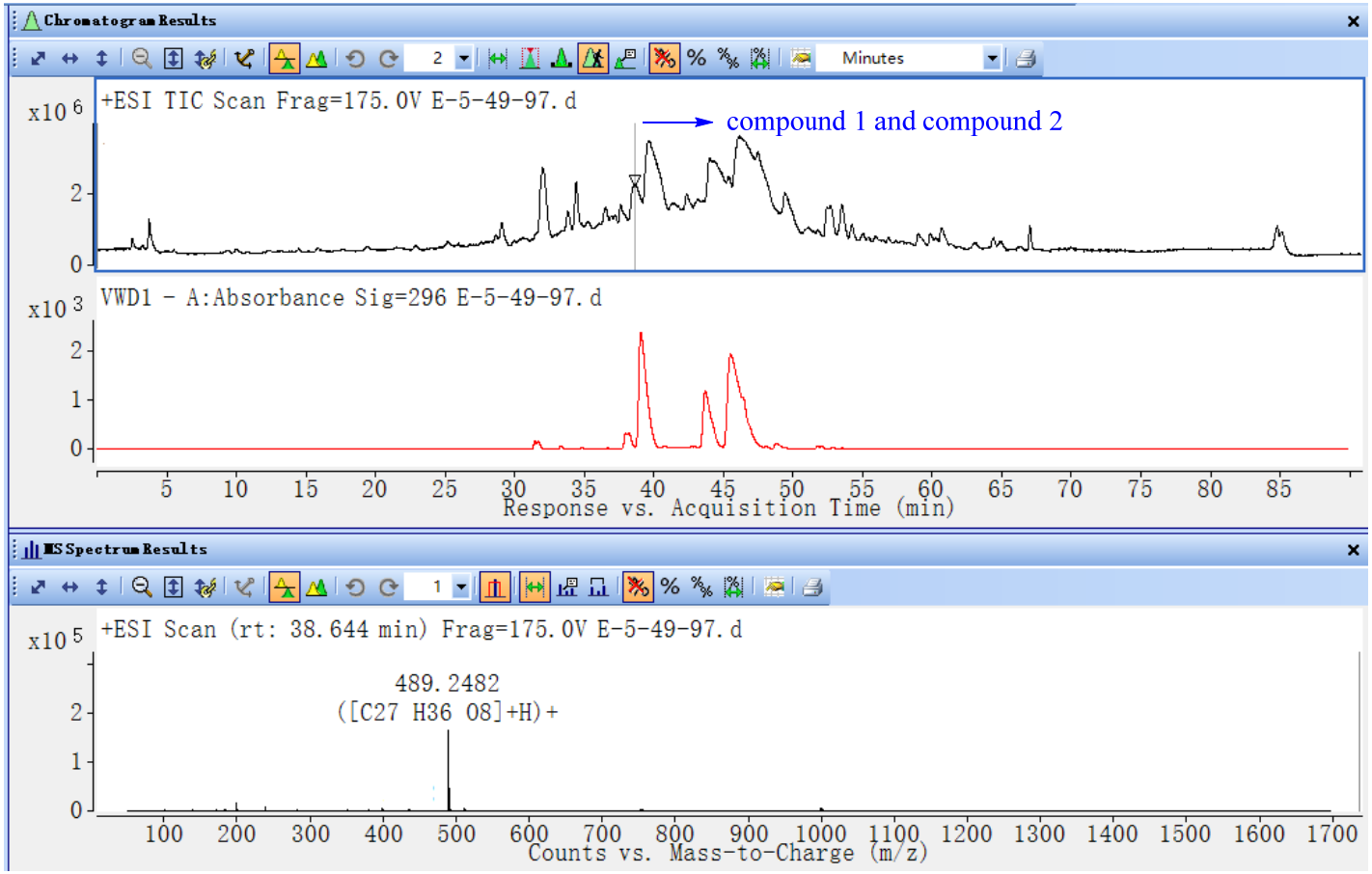

Figure S1. HPLC-HRMS of **Fr.5**

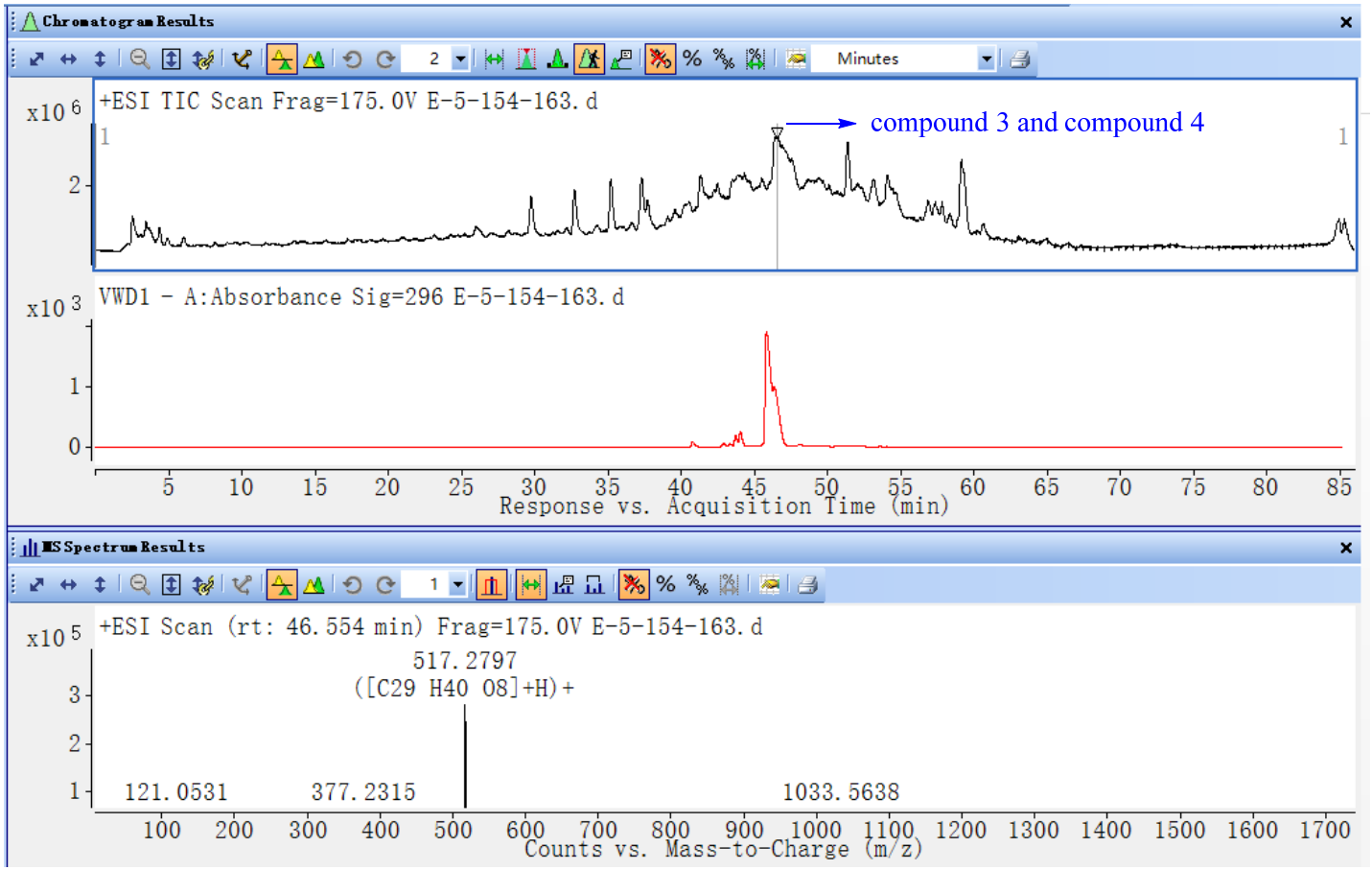

Figure S2. HPLC-HRMS of **Fr.8**

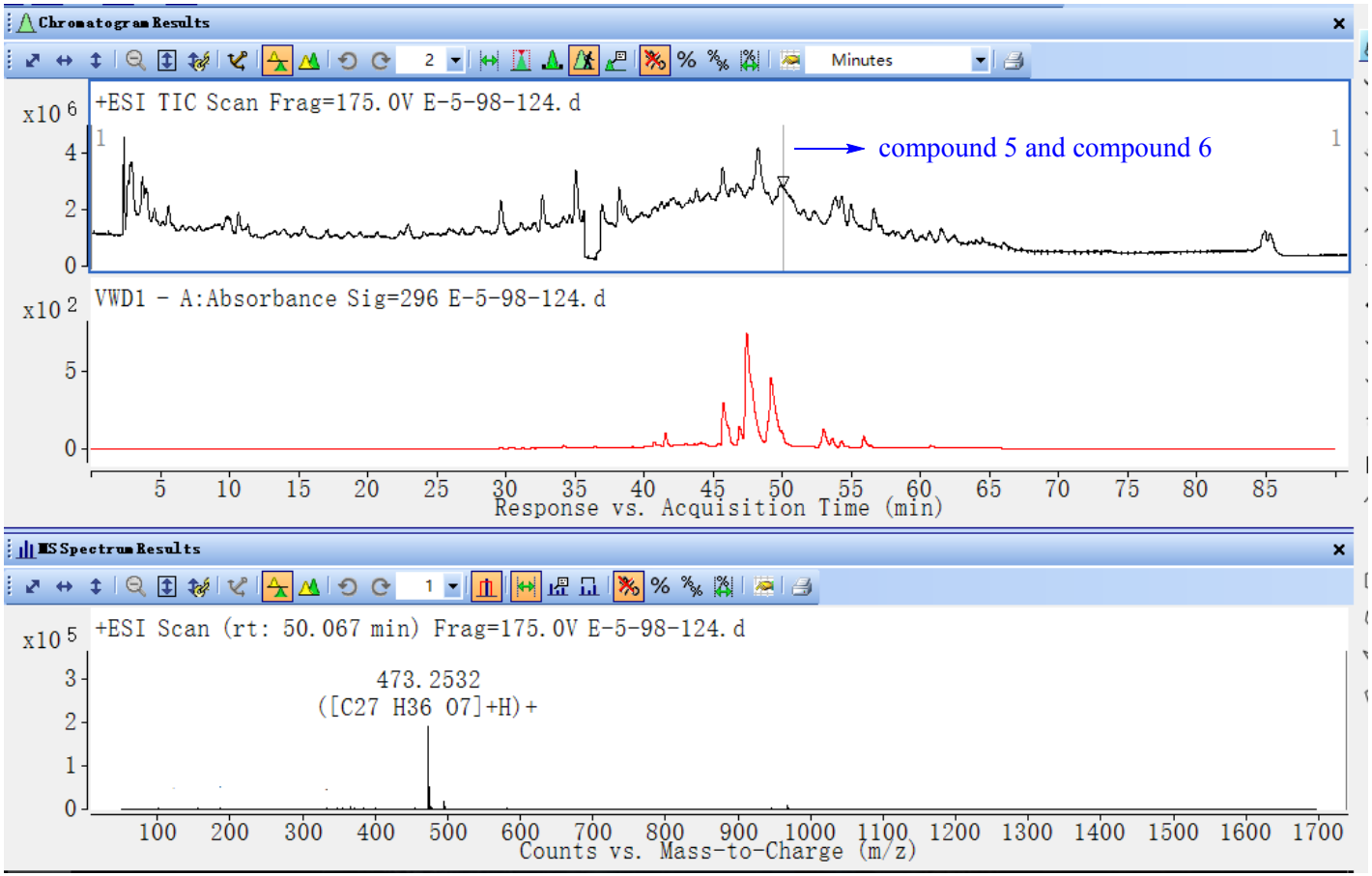

Figure S3. HPLC-HRMS of **Fr.9**

3. Modified Mosher′s Method for Absolute Configuration of compound 2

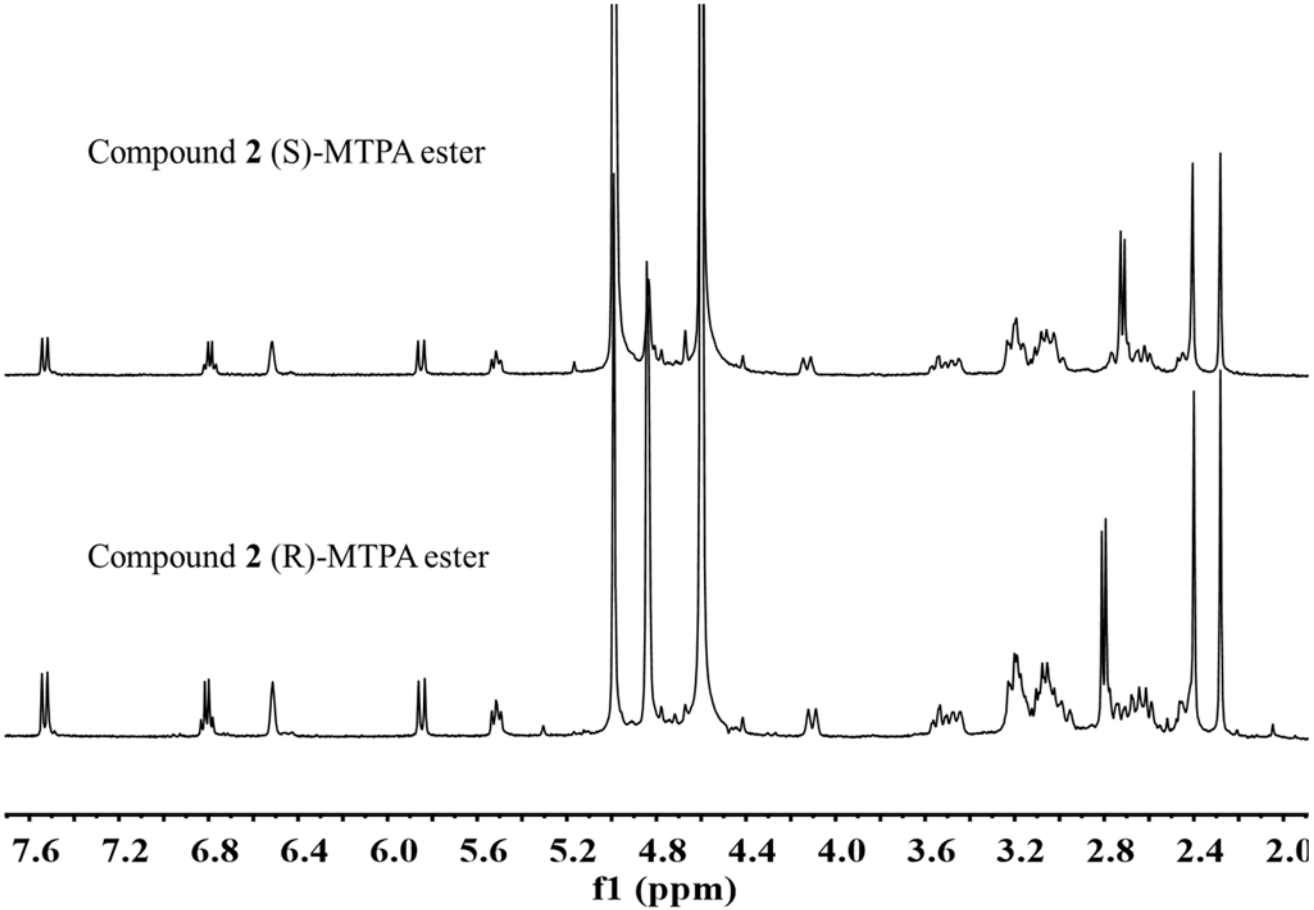

Figure S4 Comparison of the ^{1}H NMR spectra for (S)-MTPA ester and (R)-MTPA ester of compound **2**

Compound **2** 0.89mg and 0.81mg were dissolved in 500 μL deuterated pyridine which was treated with (S)-MTPACl (15μL) and (R)-MTPACl (15μL) respectively under an atmosphere of nitrogen at room temperature. NMR spectra were measured after 24 h′s reaction under sealed environment. Comparision of the ^{1}H NMR spectra of (*S*)-MTPA ester and (*R*)-MTPA ester (Figure **S1**) suggested the absolute configuration of C-2′ was *R*.

4. X-ray crystallography

Table 1. Crystal data and structure refinement for compound **1**

Identification code	compound **1**
Empirical formula	$C_{27}H_{36}O_8$
Formula weight	488.56
Temperature	100.00(10) K
Wavelength	1.54184 Å
Crystal system, space group	Monoclinic, P2(1)
Unit cell dimensions	a = 6.26417(6) Å alpha = 90 deg
	b = 18.04765(14) Å beta = 91.2643(9) deg
	c = 10.53779(10) Å gamma = 90 deg
Volume	1191.043(19) Å^3
Z, Calculated density	2, 1.362 Mg/m^3
Absorption coefficient	0.820 mm^-1
F(000)	524
Crystal size	0.41 x 0.32 x 0.20 mm
Theta range for data collection	4.196 to 73.630 deg
Limiting indices	-7<=h<=5, -22<=k<=22, -12<=l<=12
Reflections collected / unique	10324 / 4548 [R(int) = 0.0191]
Completeness to theta = 67.684	99.8 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	1.00000 and 0.91008
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	4548 / 1 / 323
Goodness-of-fit on F^2	1.028
Final R indices [I>2sigma (I)]	R1 = 0.0255, wR2 = 0.0647
R indices (all data)	R1 = 0.0260, wR2 = 0.0652
Absolute structure parameter	0.01(5)
Extinction coefficient	0.0015(4)
Largest diff. peak and hole	0.198 and -0.141 e.Å^-3

Table 2. Crystal data and structure refinement for compound **2**

Identification code	Compound **2**

Empirical formula	$C_{27}H_{36}O_8$
Formula weight	488.56
Temperature	100.00(10) K
Wavelength	1.54178 Å
Crystal system, space group	Monoclinic, C2
Unit cell dimensions	a = 32.2353(3) Å alpha = 90.0 deg
	b = 7.66968(8) Å beta = 99.2221(11) deg
	c = 10.48751(13) Å gamma = 90.0 deg
Volume	2559.37(5) Å^3
Z, Calculated density	4, 1.315 Mg/m^3
Absorption coefficient	0.811 mm^-1
F(000)	1088
Crystal size	0.40 x 0.26 x 0.21 mm
Theta range for data collection	4.2708 to 73.6524 deg
Limiting indices	-37<=h<=39, -9<=k<=9, -13<=l<=12
Reflections collected / unique	18236 / 5075 [R(int) = 0.0245]
Completeness to theta = 66.9682	99.96 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	1.00000 and 0.80644
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	5075 / 2 / 335
Goodness-of-fit on F^2	1.052
Final R indices [I>2sigma (I)]	R1 = 0.0380, wR2 = 0.1093
R indices (all data)	R1 = 0.0382, wR2 = 0.1097
Absolute structure parameter	0.02(4)
Extinction coefficient	0.0012(2)
Largest diff. peak and hole	0.790 and -0.249 e.Å^-3

Table 3. Crystal data and structure refinement for compound **3**

Identification code	compound **3**
Empirical formula	C_{29} H_{42} O_9
Formula weight	534.62
Temperature	100.00(10) K
Wavelength	1.54184 Å
Crystal system, space group	Monoclinic, P2(1)
Unit cell dimensions	a = 10.27418(11) Å alpha = 90.0 deg
	b = 12.53092(12) Å beta = 107.8950(10) deg
	c = 10.87663(16) Å gamma = 90.0 deg
Volume	1332.57(3) Å^3
Z, Calculated density	2, 1.332 Mg/m^3
Absorption coefficient	0.806 mm^-1
F(000)	576

Crystal size	0.39 x 0.25 x 0.20 mm
Theta range for data collection	3.5271 to 73.5276 deg
Limiting indices	-12<=h<=10, -15<=k<=15, -13<=l<=13
Reflections collected / unique	12315 / 5169 [R(int) = 0.0395]
Completeness to theta = 66.9682	99.96 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	1.00000 and 0.82785
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	5075 / 2 / 335
Goodness-of-fit on F^2	1.042
Final R indices [I>2sigma (I)]	R1 = 0.0559, wR2 = 0.1467
R indices (all data)	R1 = 0.0569, wR2 = 0.1483
Absolute structure parameter	0.01(10)
Extinction coefficient	0.0018(7)
Largest diff. peak and hole	1.520 and -0.318 e.Å^-3

Table 4. Crystal data and structure refinement for compound **5**

Identification code	compound **5**
Empirical formula	C_{27} H_{38} O_8
Formula weight	490.57
Temperature	173(2) K
Wavelength	1.54184 Å
Crystal system, space group	Monoclinic, P2(1)
Unit cell dimensions	a = 9.4990(2) Å alpha = 90 deg
	b = 10.4196(3) Å beta = 96.497(3) deg
	c = 12.8765(5) Å gamma = 90 deg
Volume	1266.28(7) Å^3
Z, Calculated density	2, 1.287 Mg/m^3
Absorption coefficient	0.771 mm^-1
F(000)	528
Crystal size	0.38 x 0.29 x 0.25 mm
Theta range for data collection	3.4546 to 62.7167 deg
Limiting indices	-9<=h<=10, -11<=k<=11, -14<=l<=14
Reflections collected / unique	5345 / 3166 [R(int) = 0.0249]
Completeness to theta = 66.9682	99.79 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	1.00000 and 0.82302
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	3166 / 1 / 324
Goodness-of-fit on F^2	1.006
Final R indices [I>2sigma (I)]	R1 = 0.0452, wR2 = 0.1215
R indices (all data)	R1 = 0.0565, wR2 = 0.1300

Absolute structure parameter	-0.02(14)
Extinction coefficient	n/a
Largest diff. peak and hole	0.441 d -0.215 e.Å^-3

5. ^{1}H NMR Spectra of Unseparated Mixture of compounds 1and 2, compounds 3and 4, compounds 5 and 6

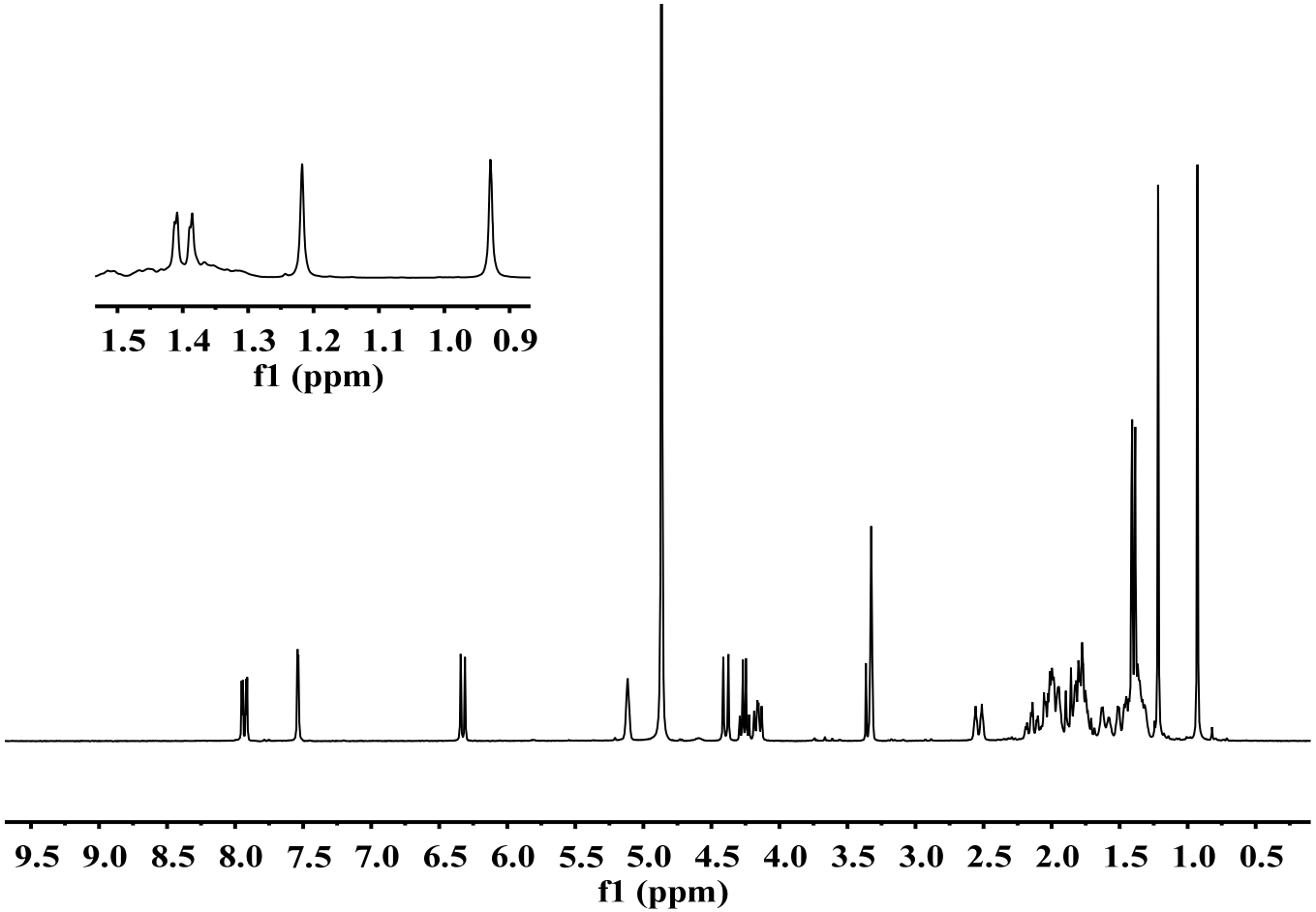

Figure S5. ^{1}H NMR Spectra of Unseparated Mixture of compounds **1**and **2**

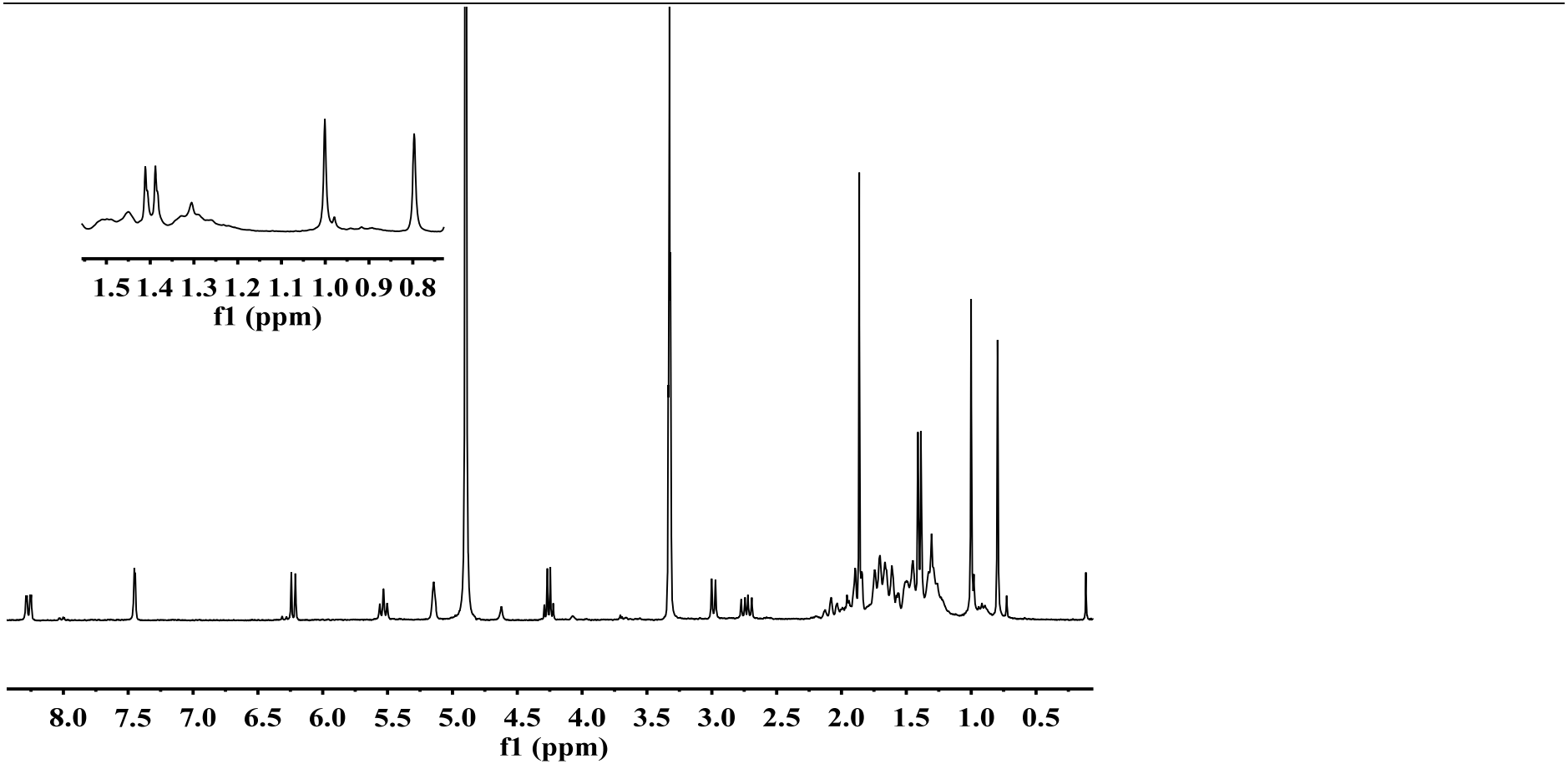

Figure S6. ^{1}H NMR Spectra of Unseparated Mixture of compounds **3** and **4**

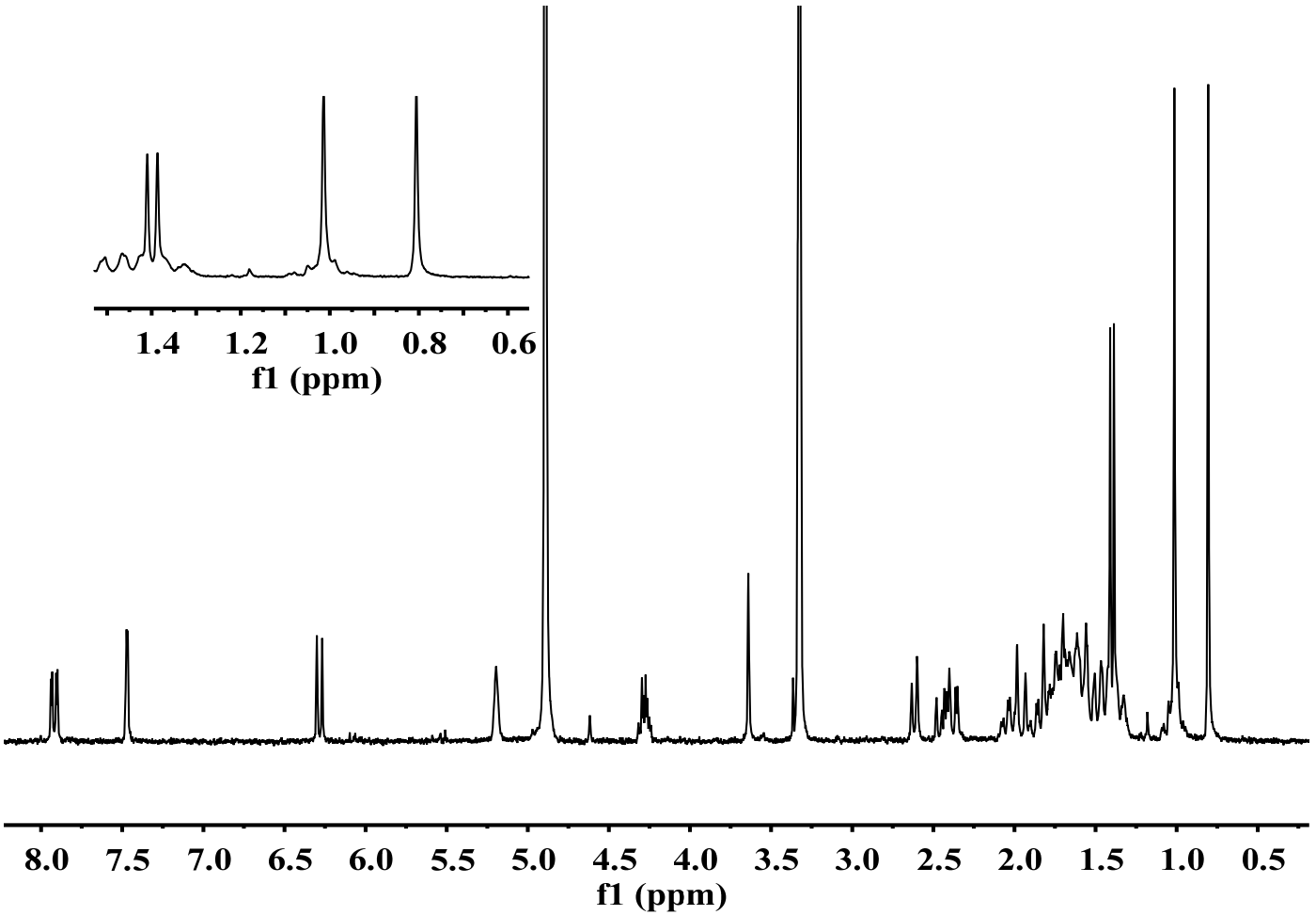

Figure S7. ^{1}H NMR Spectra of Unseparated Mixture of compounds **5** and **6**

6. Spectra of compounds 1-6

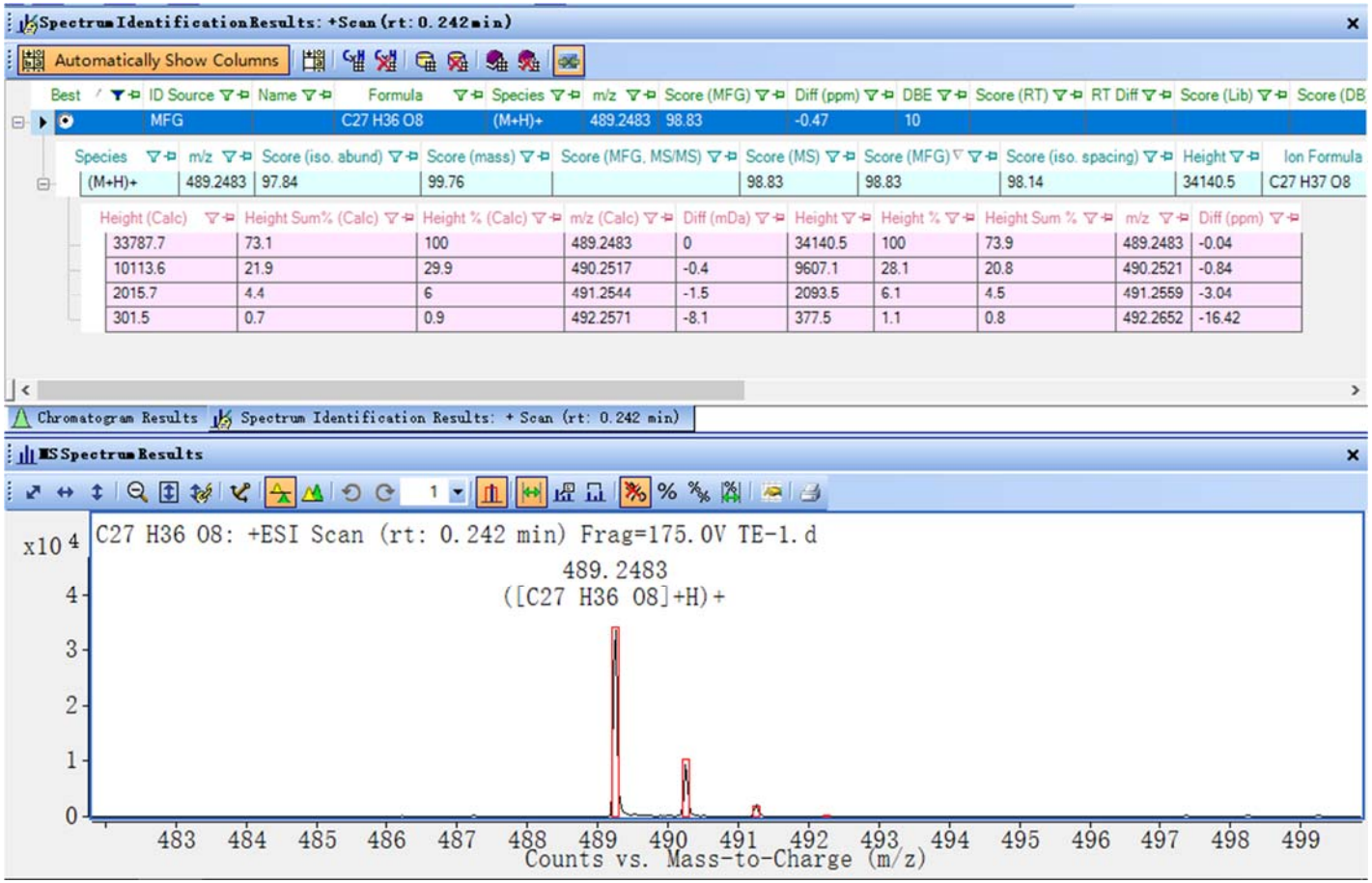

Figure S8. HR-ESI-MS of compound **1**

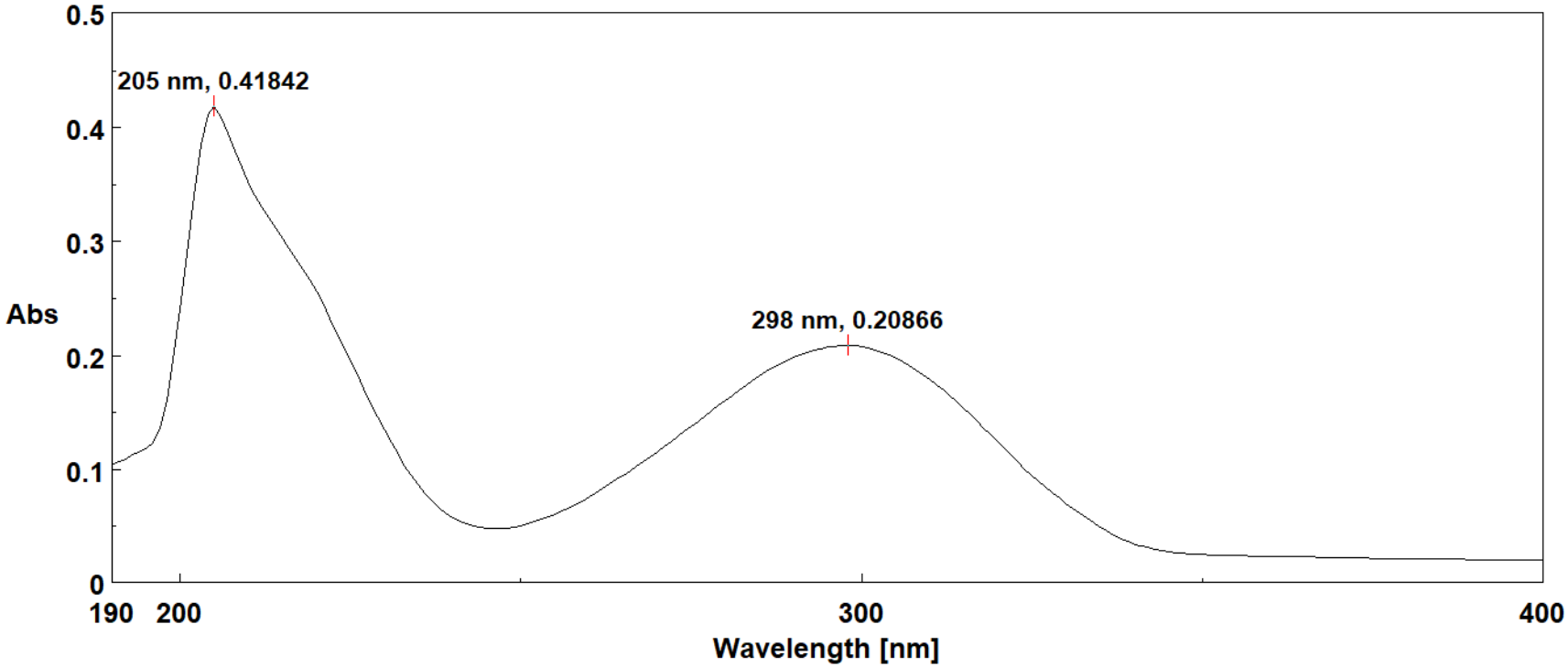

Figure S9. UV spectrum of compound **1**

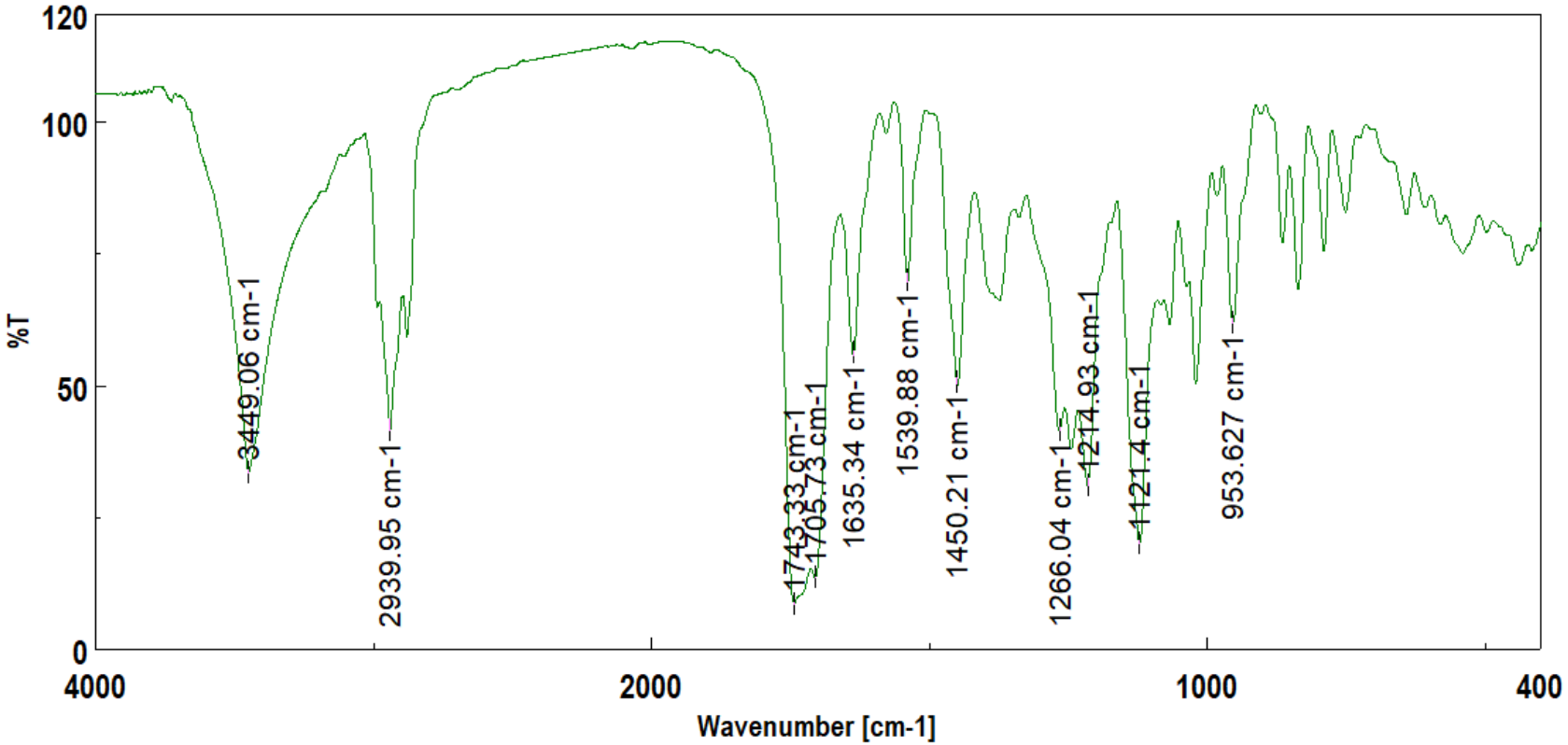

Figure S10. IR spectrum of compound **1** (KBr)

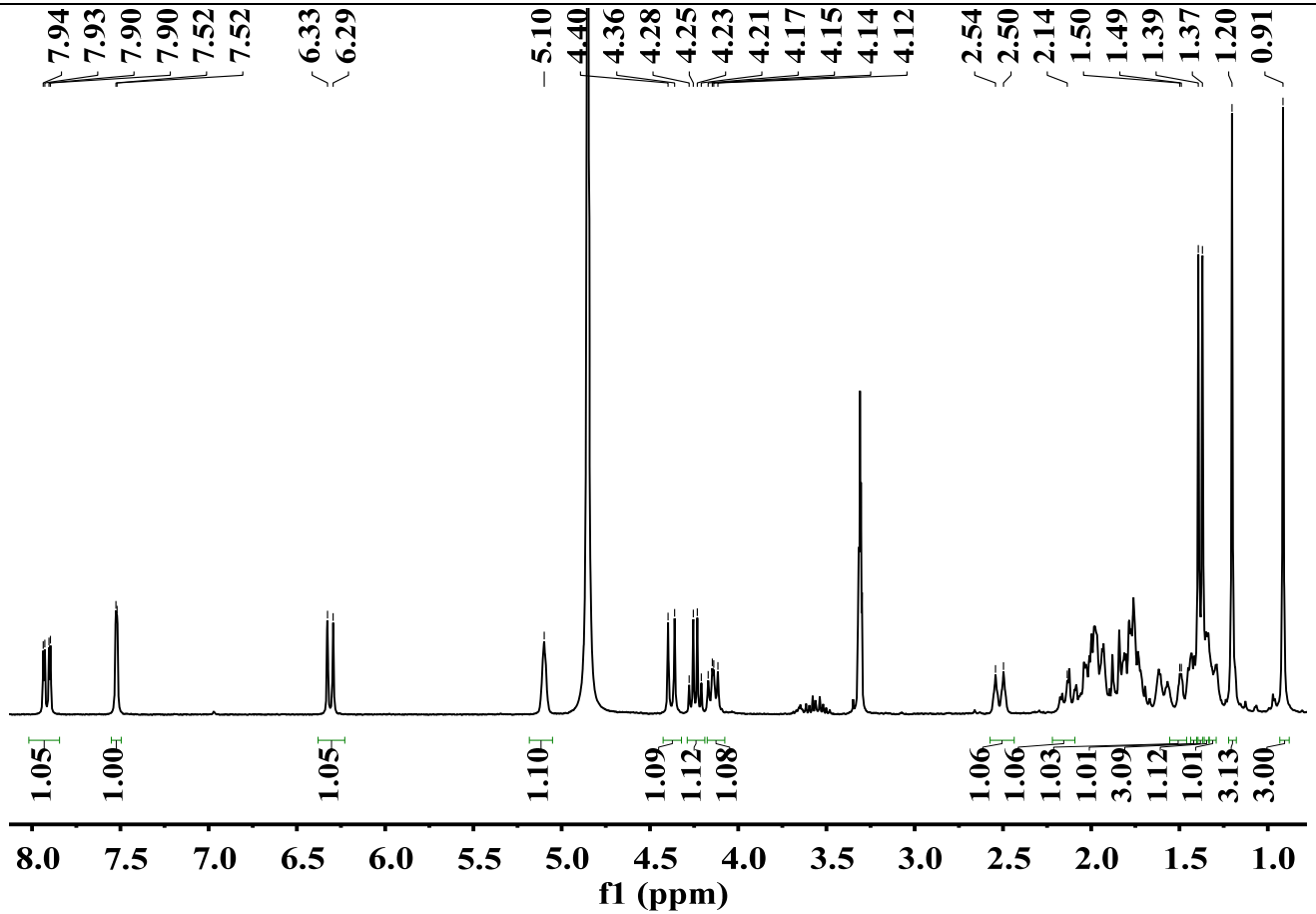

Figure S11. ^{1}H NMR spectrum of compound **1** (in CD_3OD, 300 MHz)

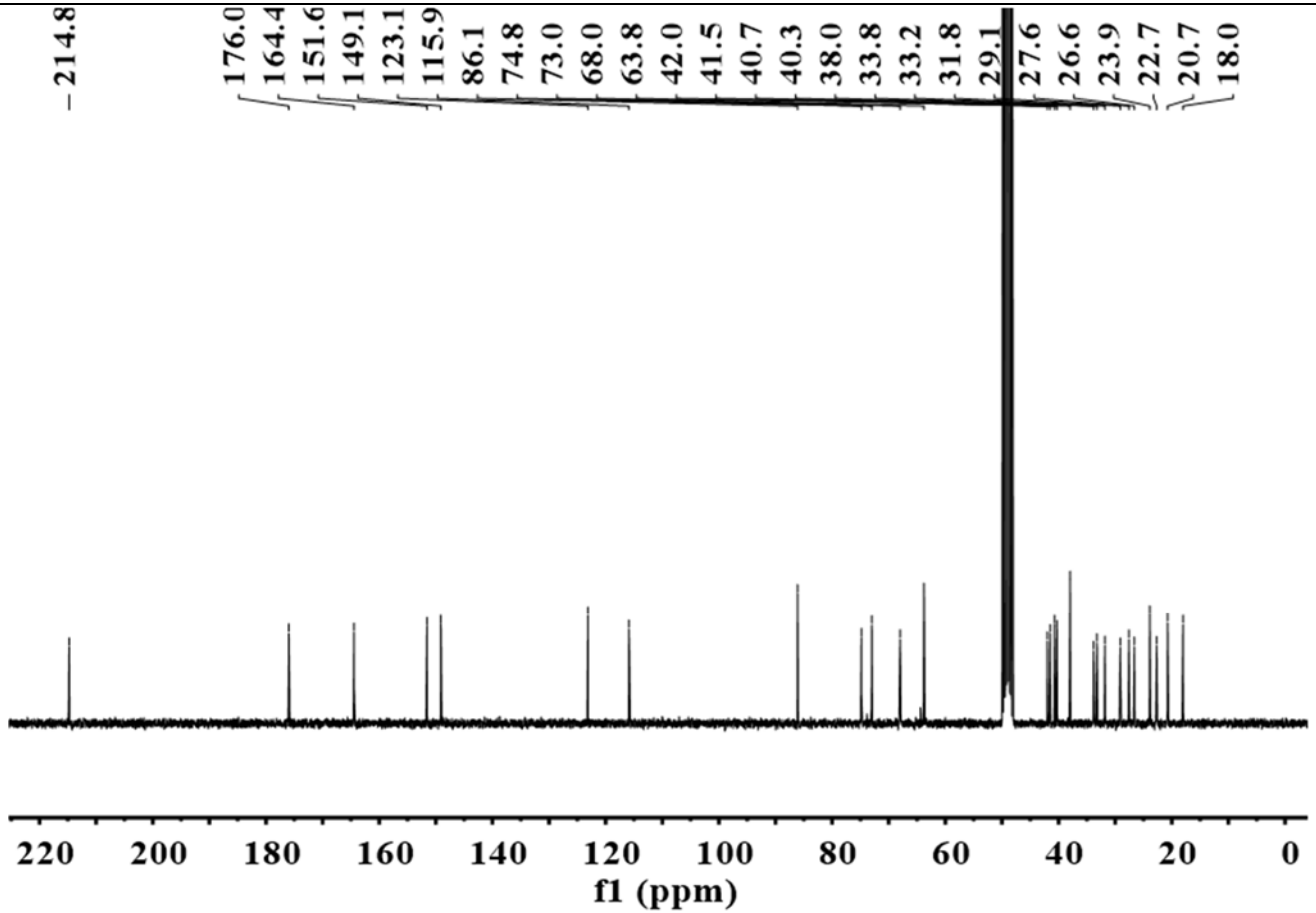

Figure S12. ^{13}C NMR spectrum of compound **1** (in CD_3OD, 75 MHz)

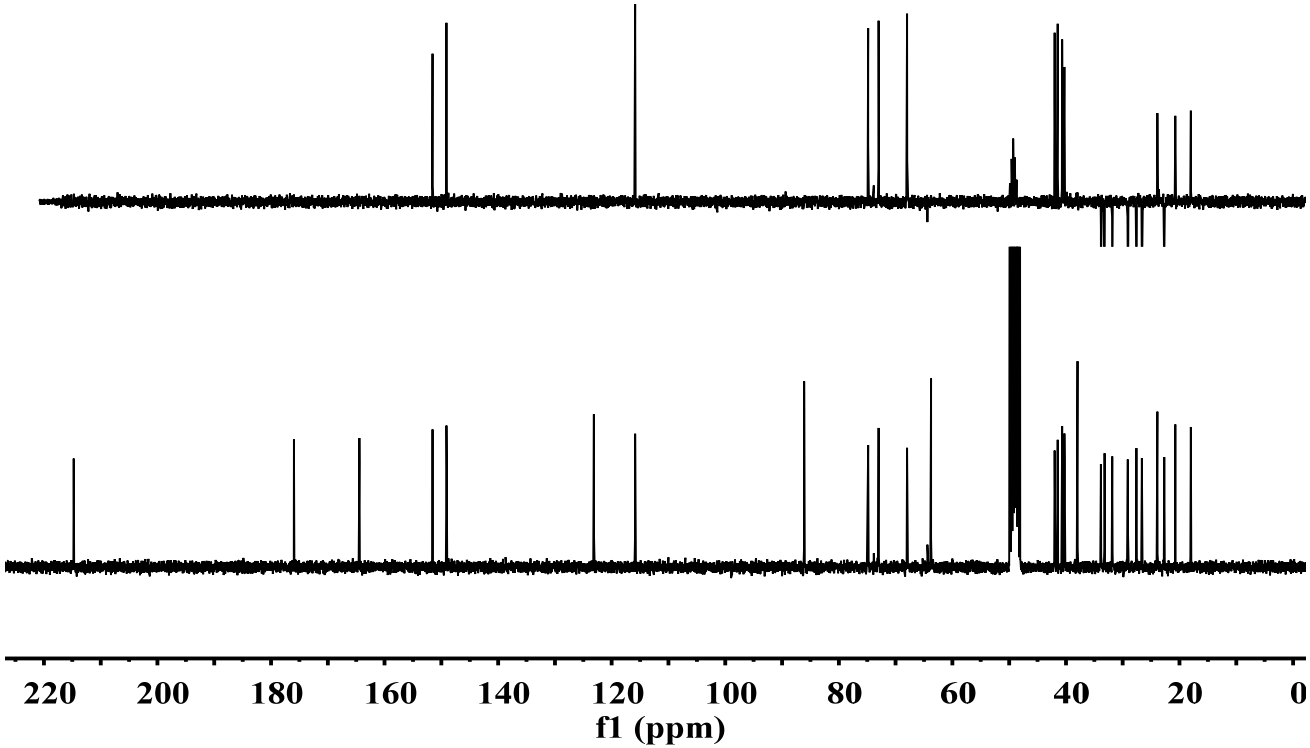

Figure S13. DEPT-135 and ^{13}C NMR spectrum of compound **1** (in CD_3OD, 75 MHz)

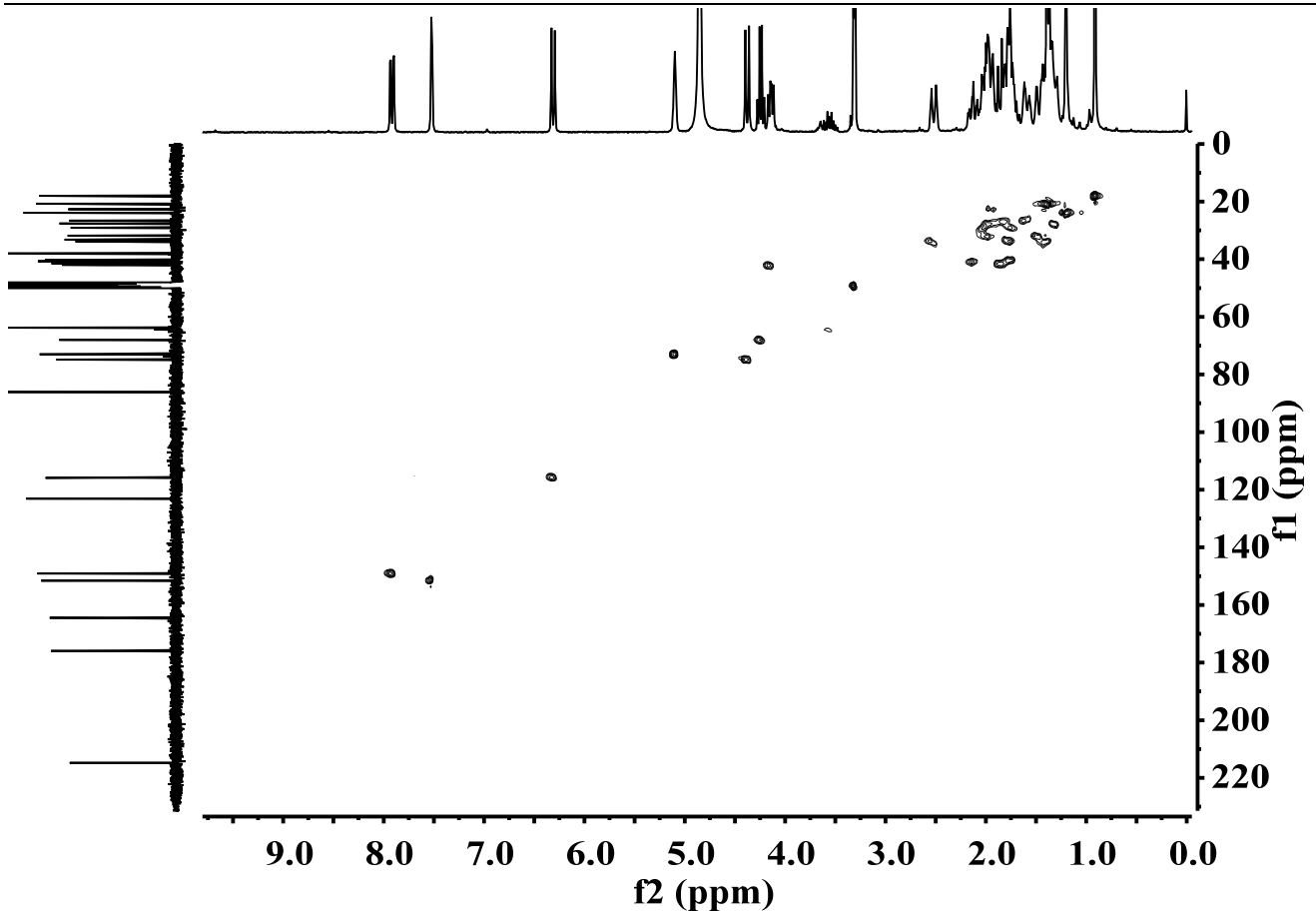

Figure S14. HMQC spectrum of compound **1** (in CD_3OD)

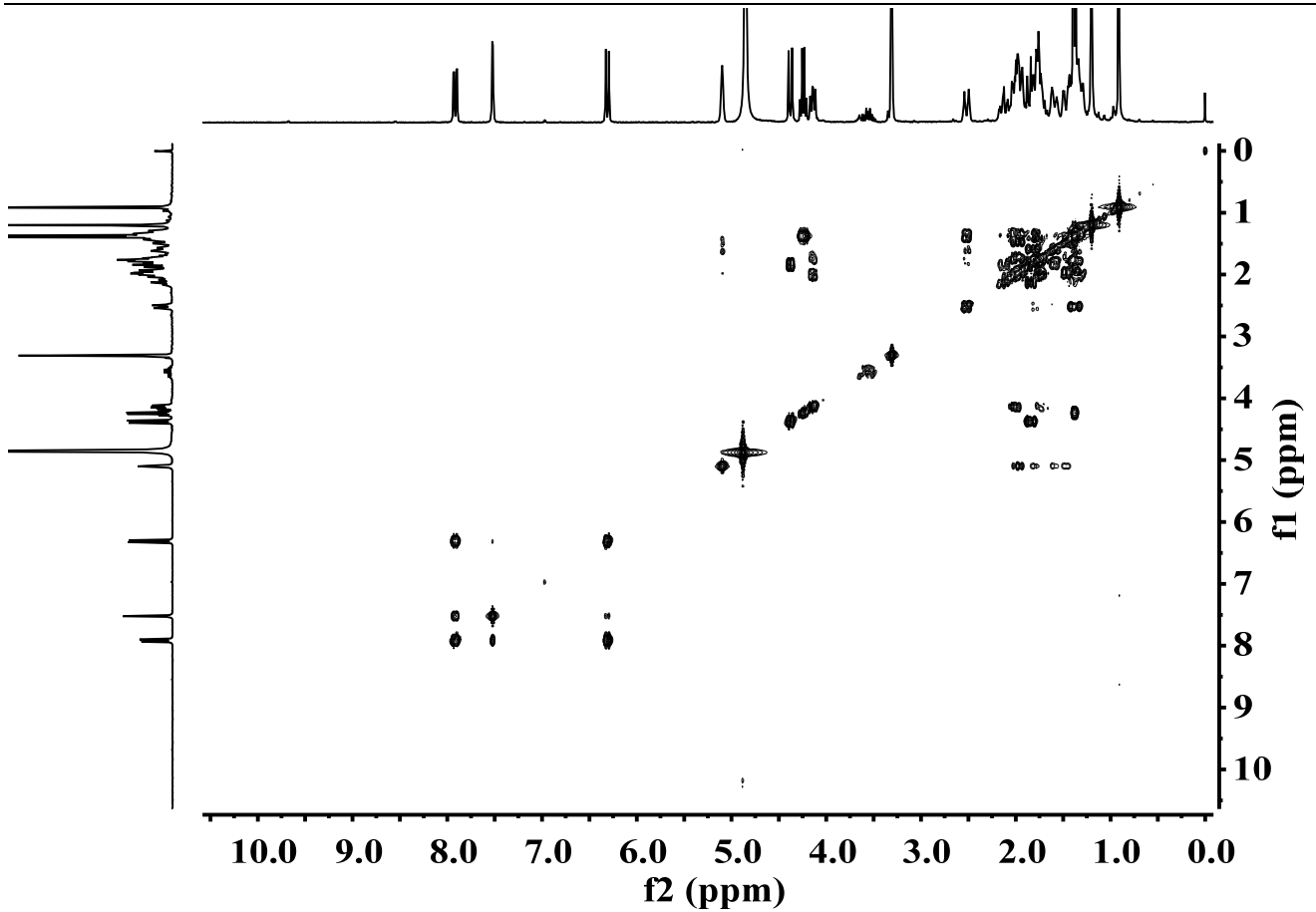

Figure S15. ^{1}H-^{1}H COSY spectrum of compound **1** (in CD_3OD)

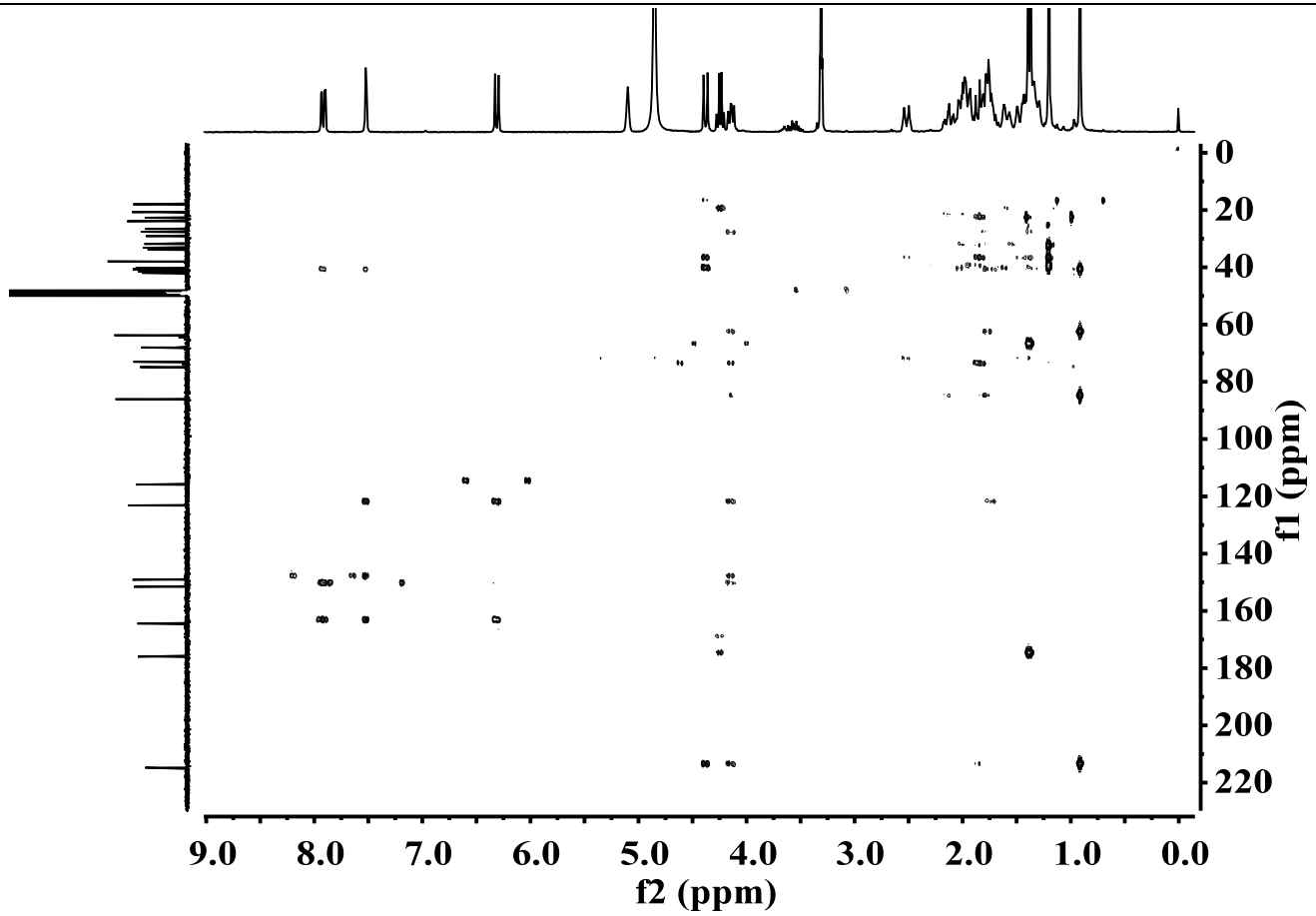

Figure S16. HMBC spectrum of compound **1** (in CD_3OD)

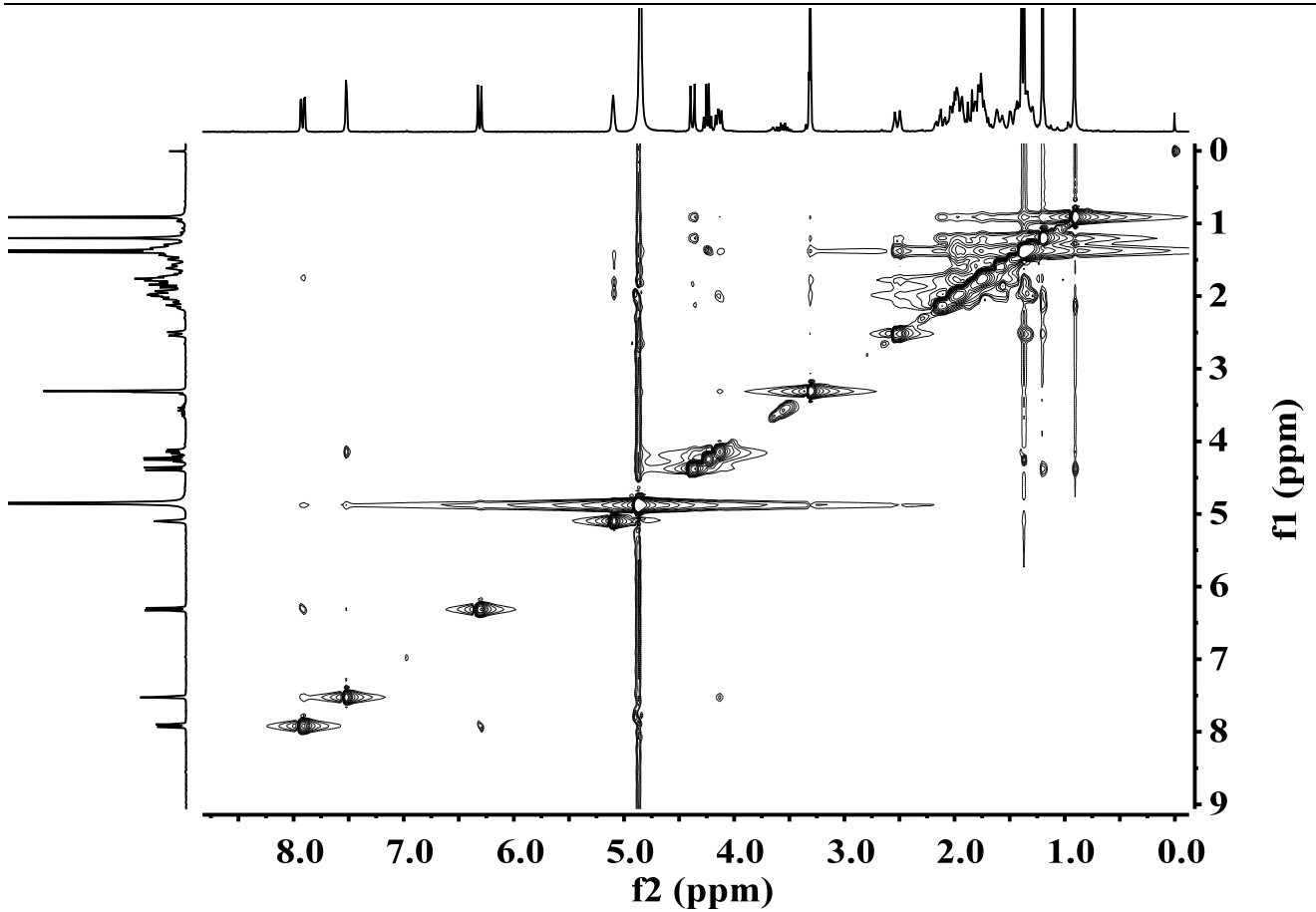

Figure S17. NOESY spectrum of compound **1** (in CD_3OD)

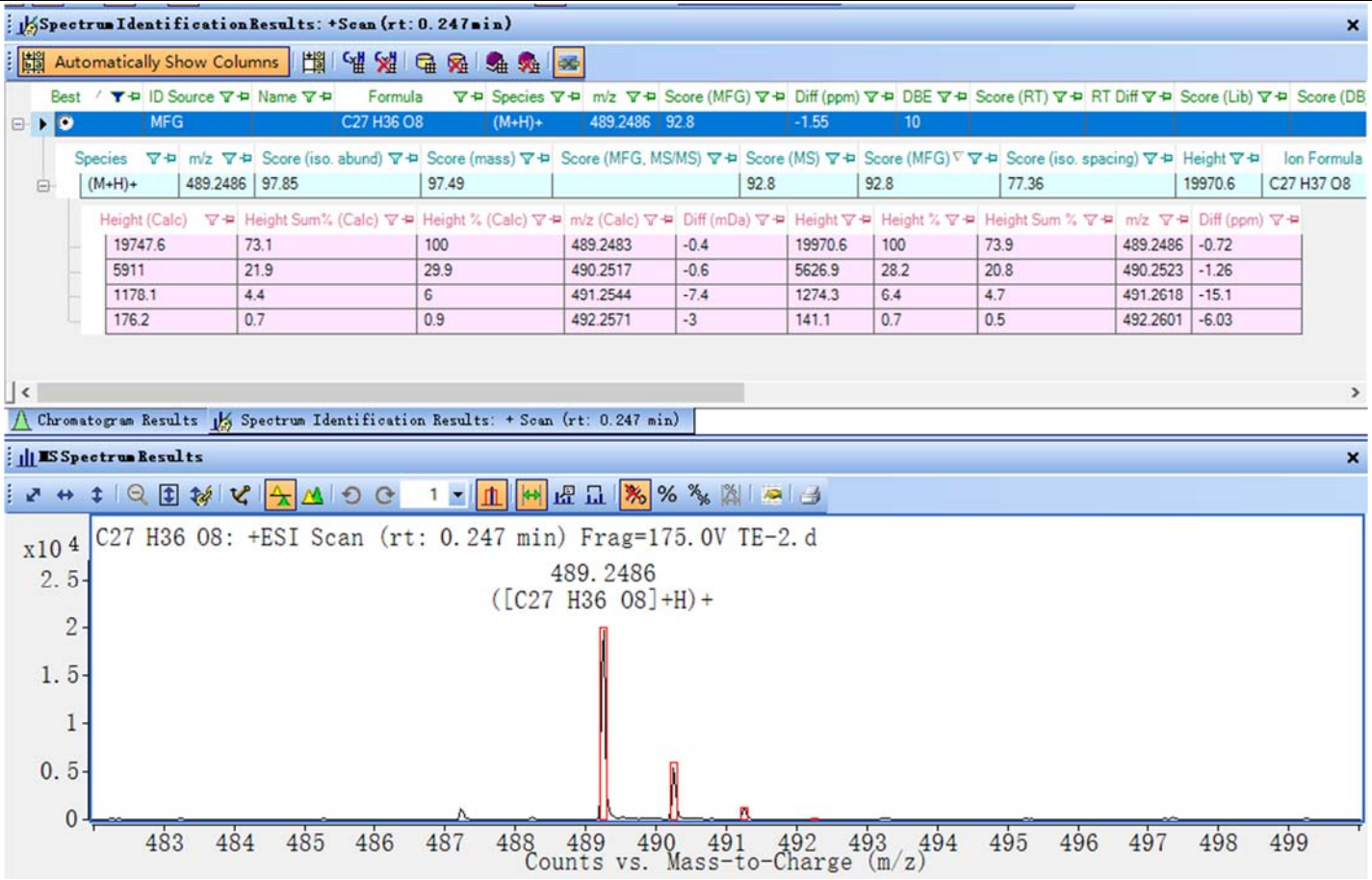

Figure S18. HR-ESI-MS of compound **2**

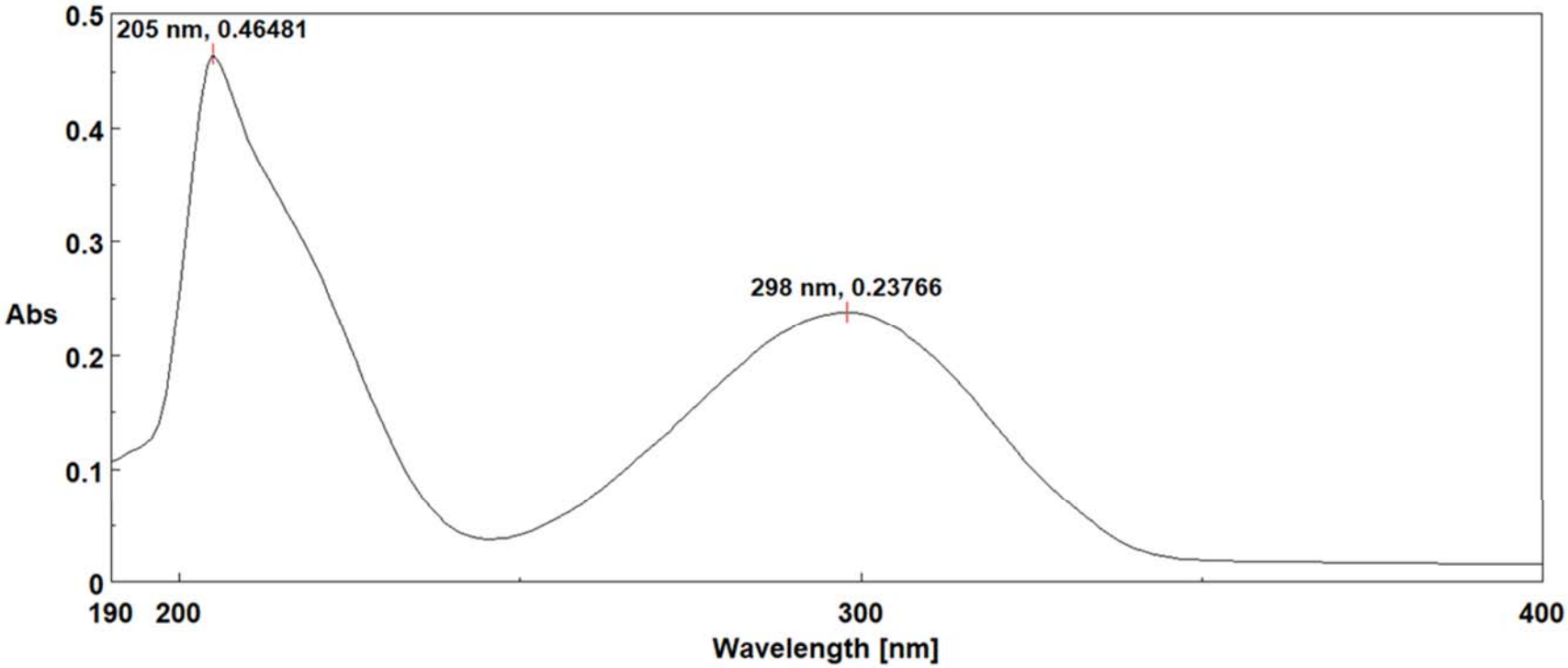

Figure S19. UV spectrum of compound **2**

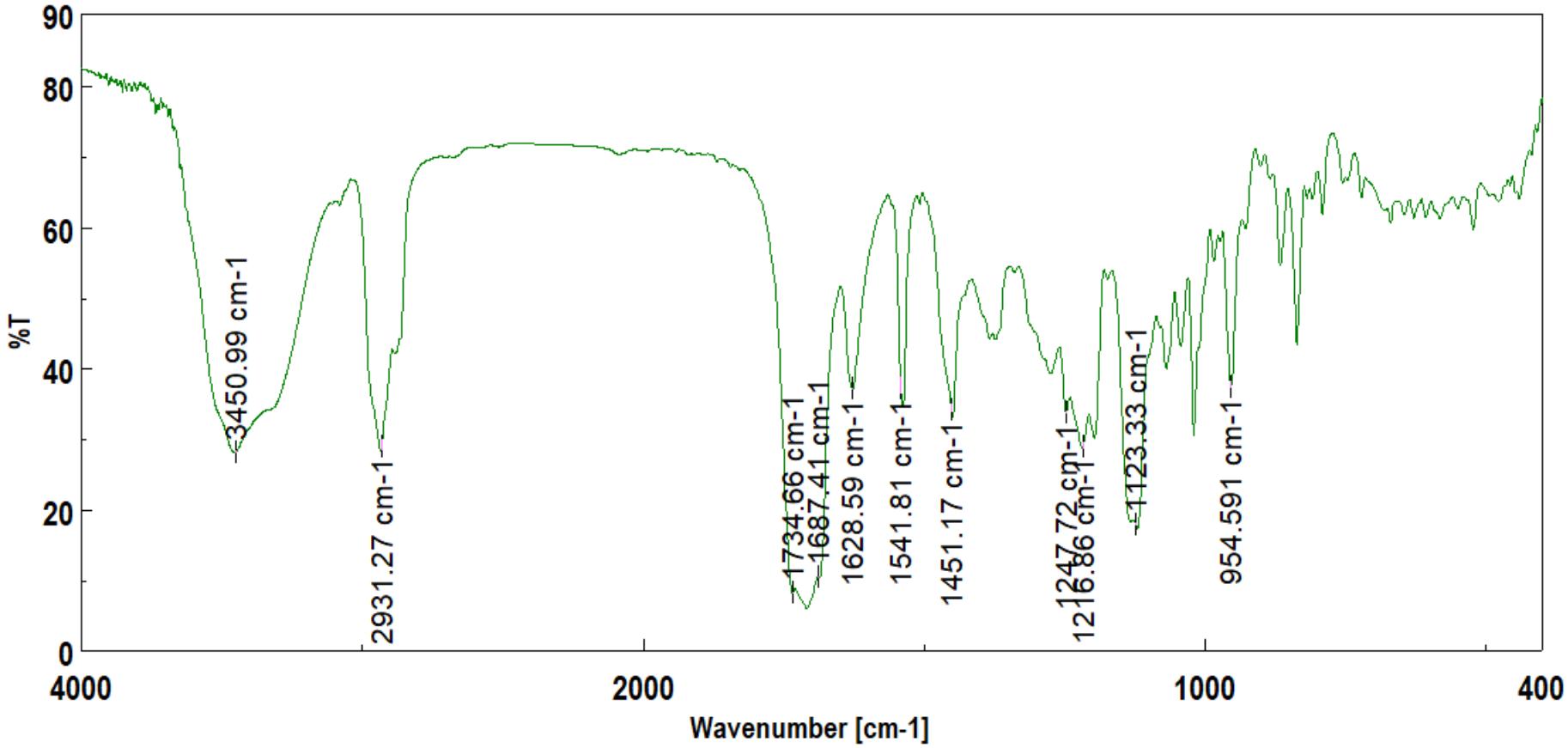

Figure S20. IR spectrum of compound **2** (KBr)

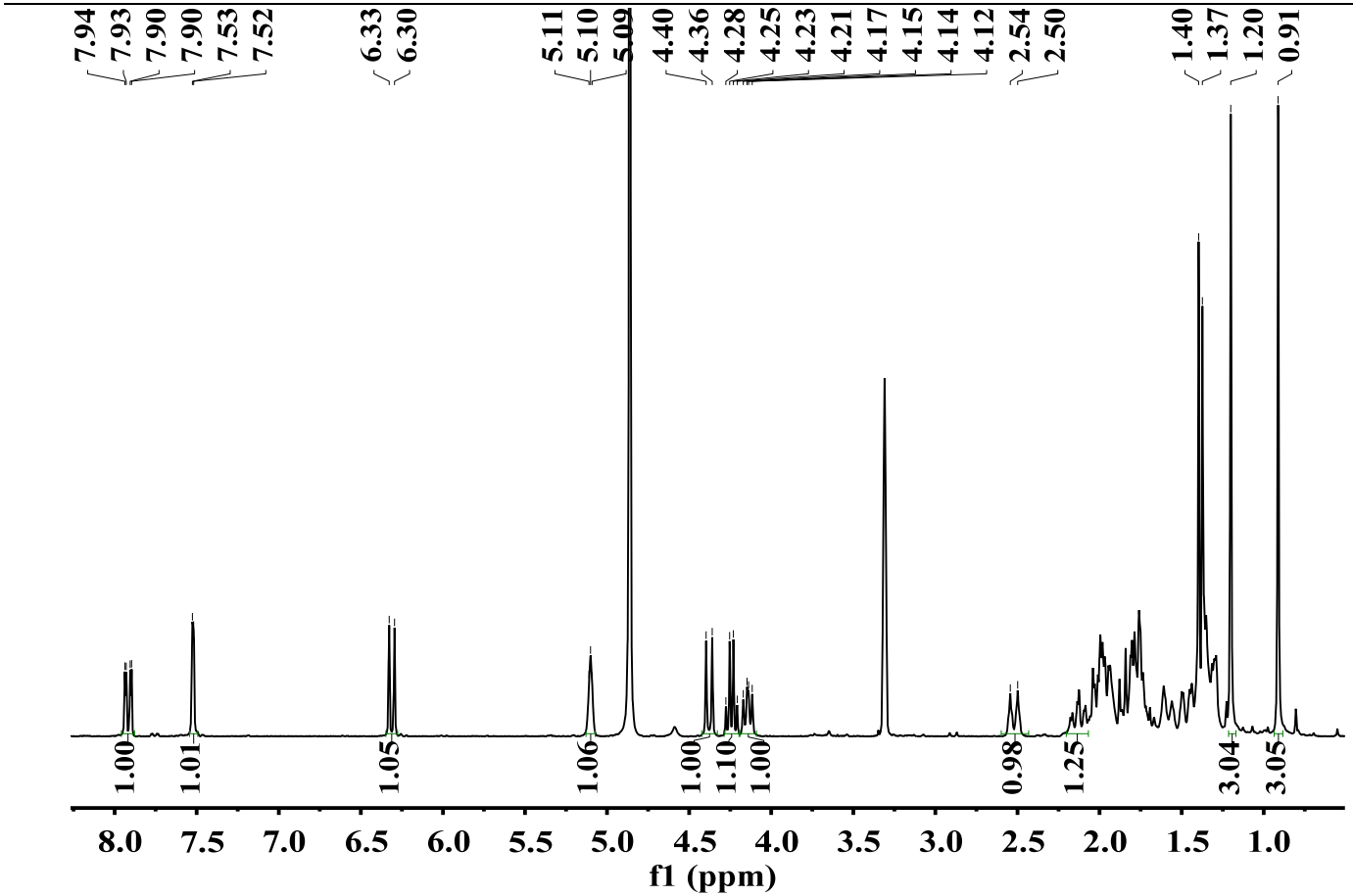

Figure S21. 1H NMR spectrum of compound **2** (in CD3OD, 300 MHz)

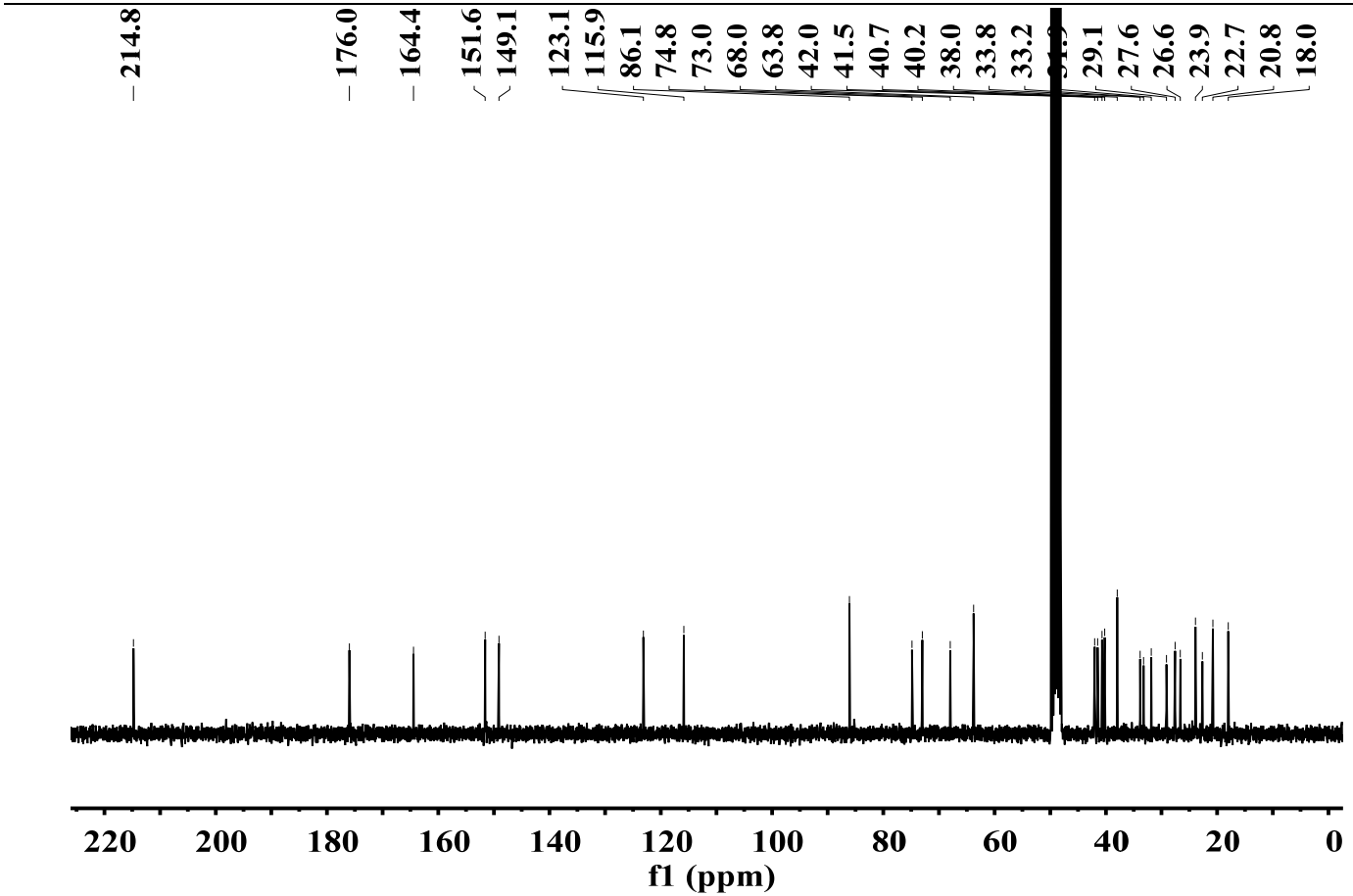

Figure S22. ^{13}C NMR spectrum of compound **2** (in CD_3OD, 75 MHz)

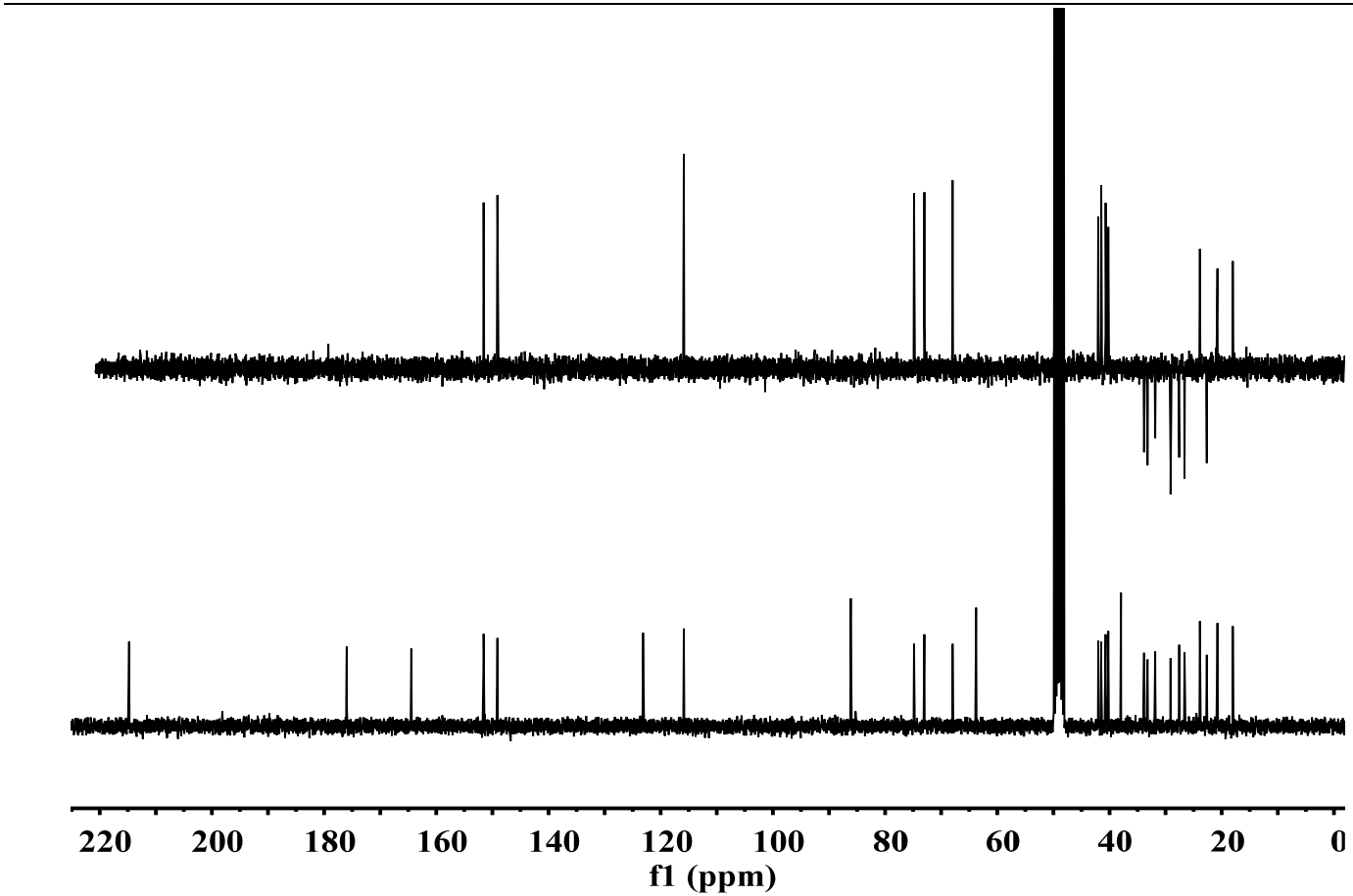

Figure S23. DEPT-135 and ^{13}C NMR spectrum of compound **2** (in CD_3OD, 75 MHz)

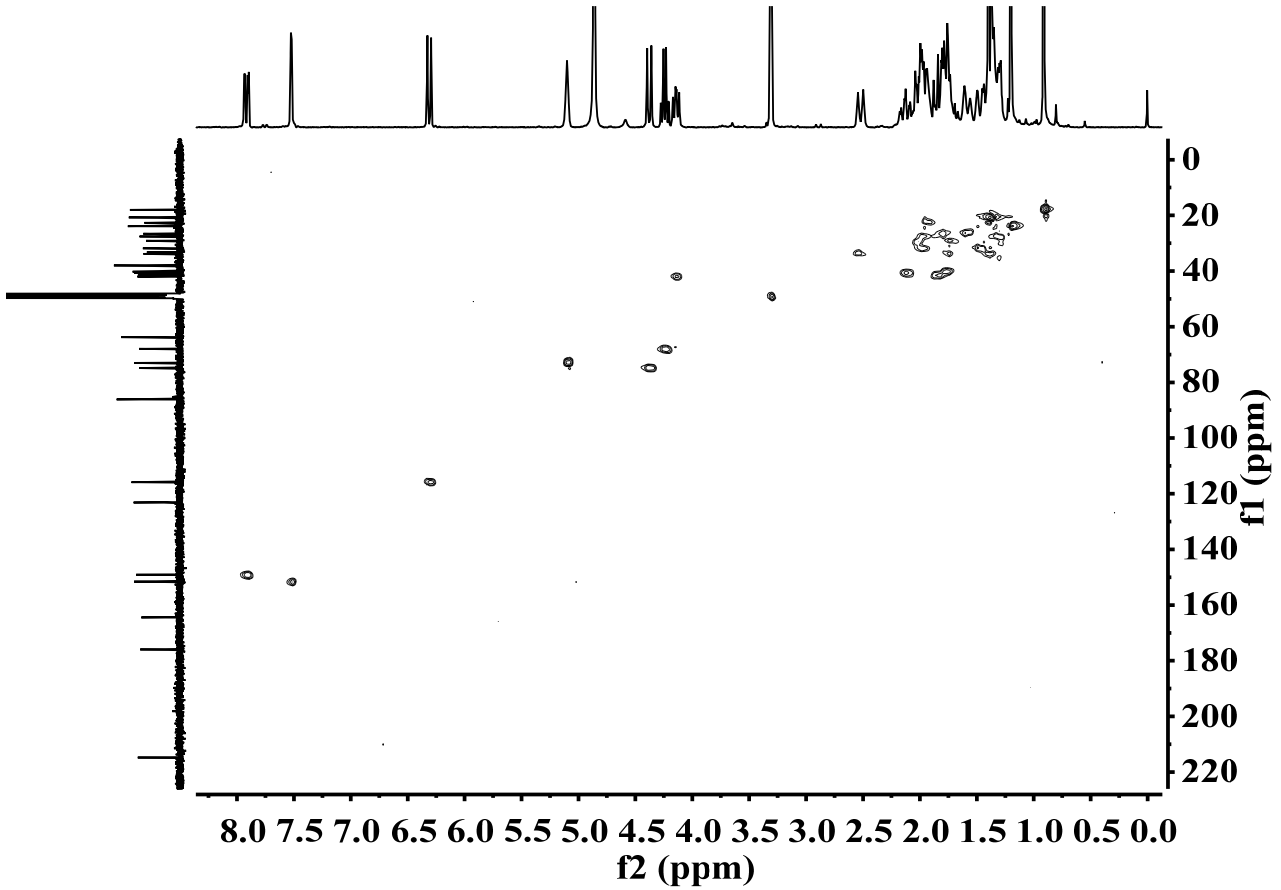

Figure S24. HMQC spectrum of compound **2** (in CD_3OD)

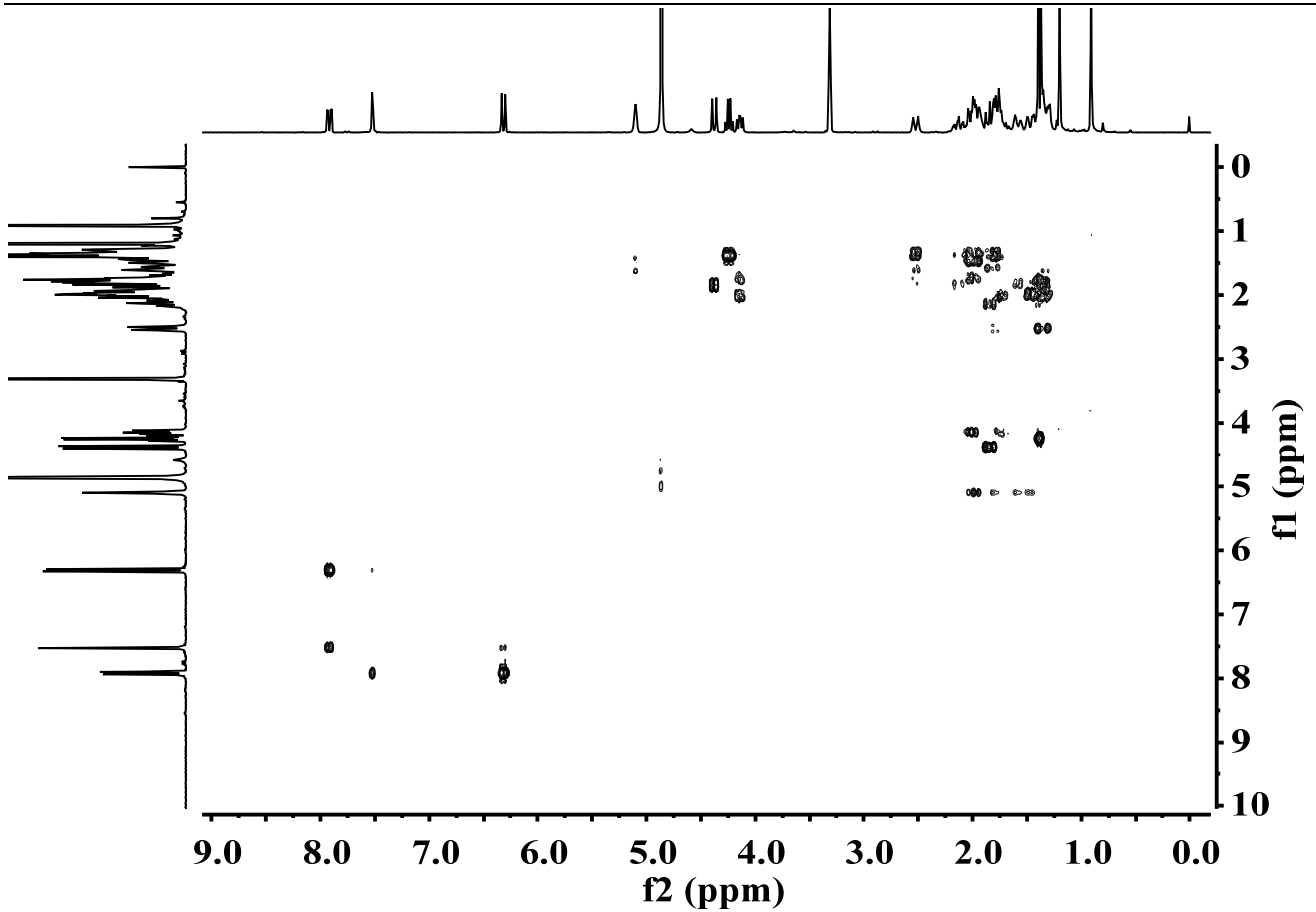

Figure S25. ^{1}H-^{1}H COSY spectrum of compound **2** (in CD_3OD)

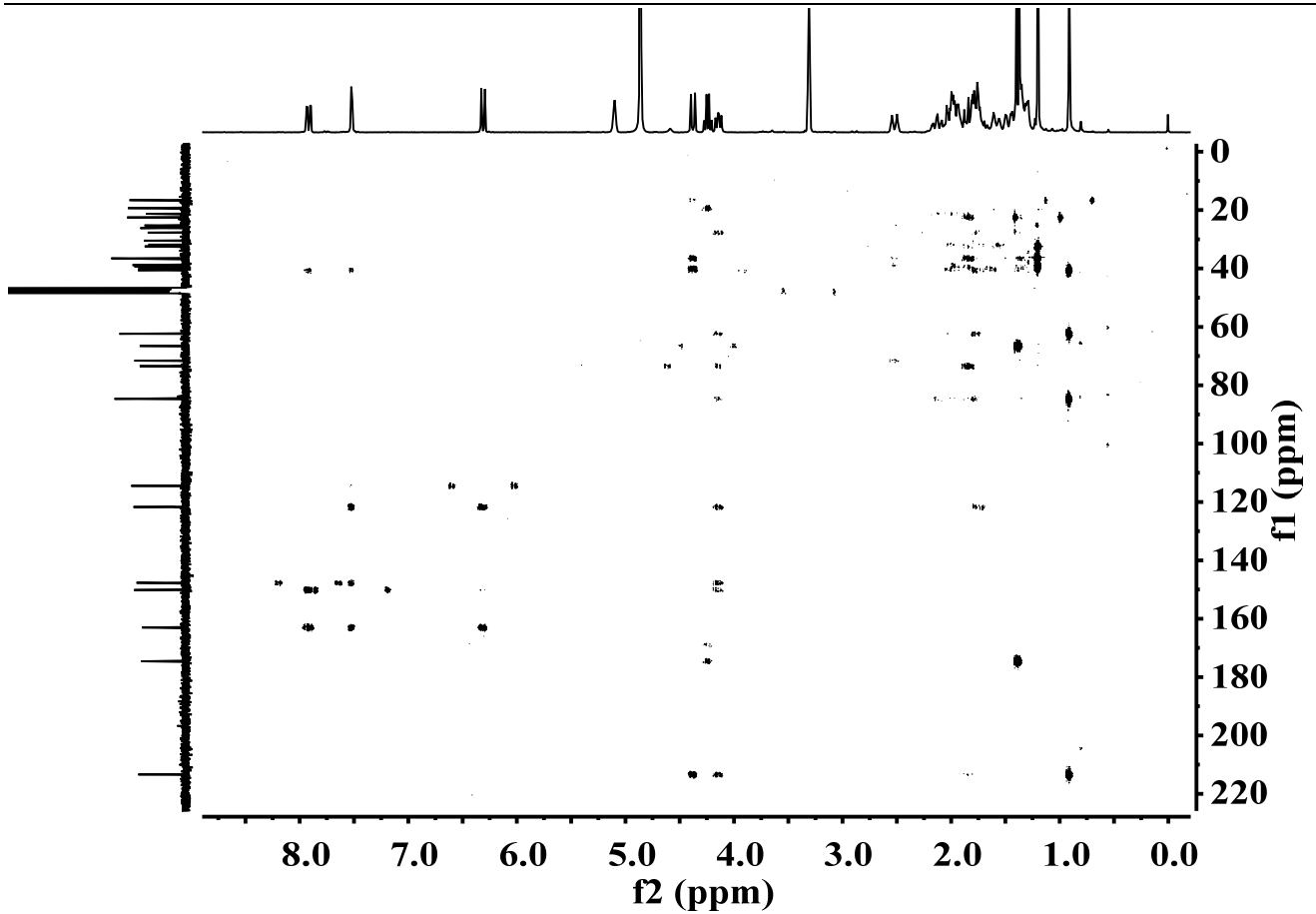

Figure S26. HMBC spectrum of compound **2** (in CD_3OD)

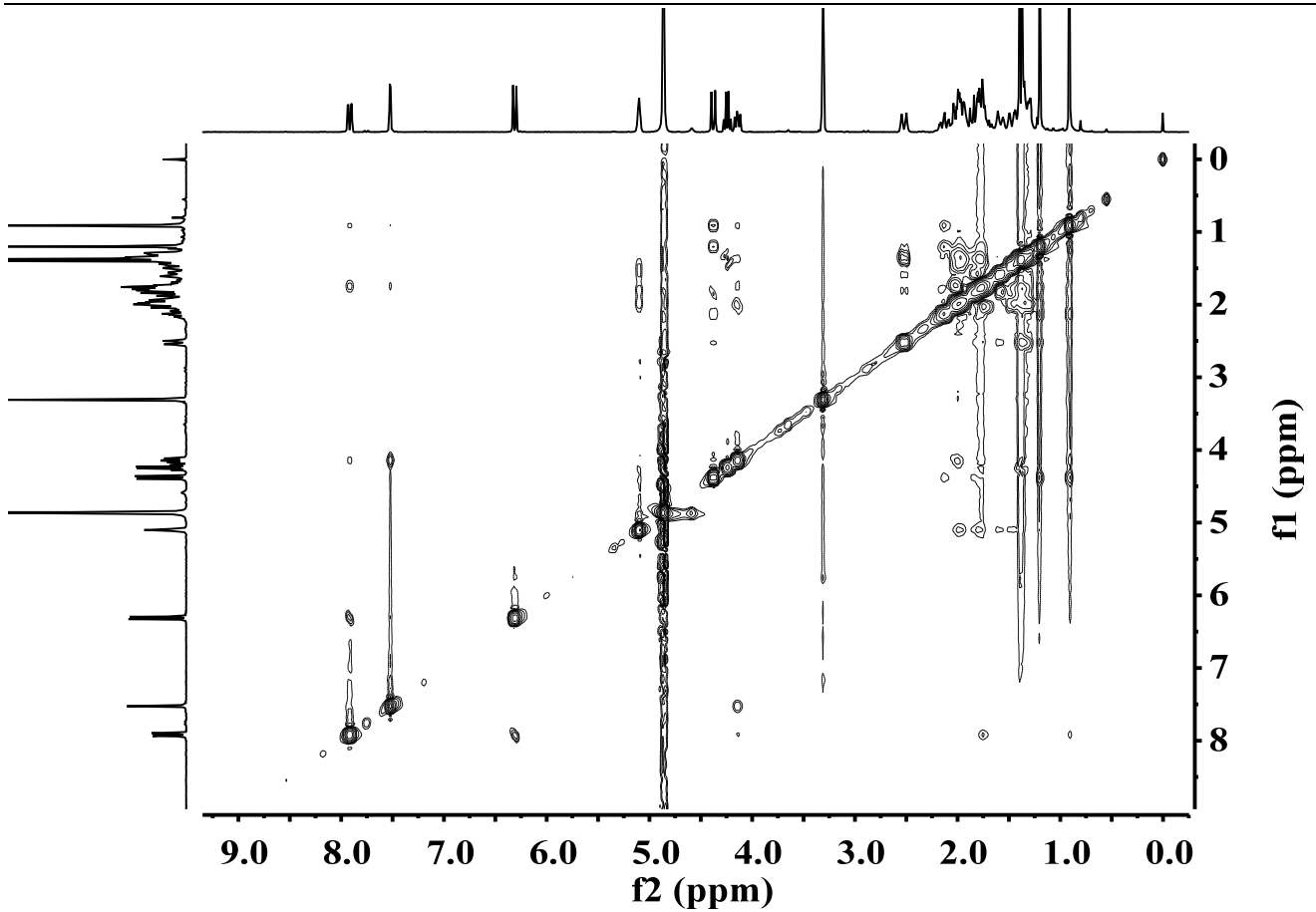

Figure S27. NOESY spectrum of compound **2** (in CD_3OD)

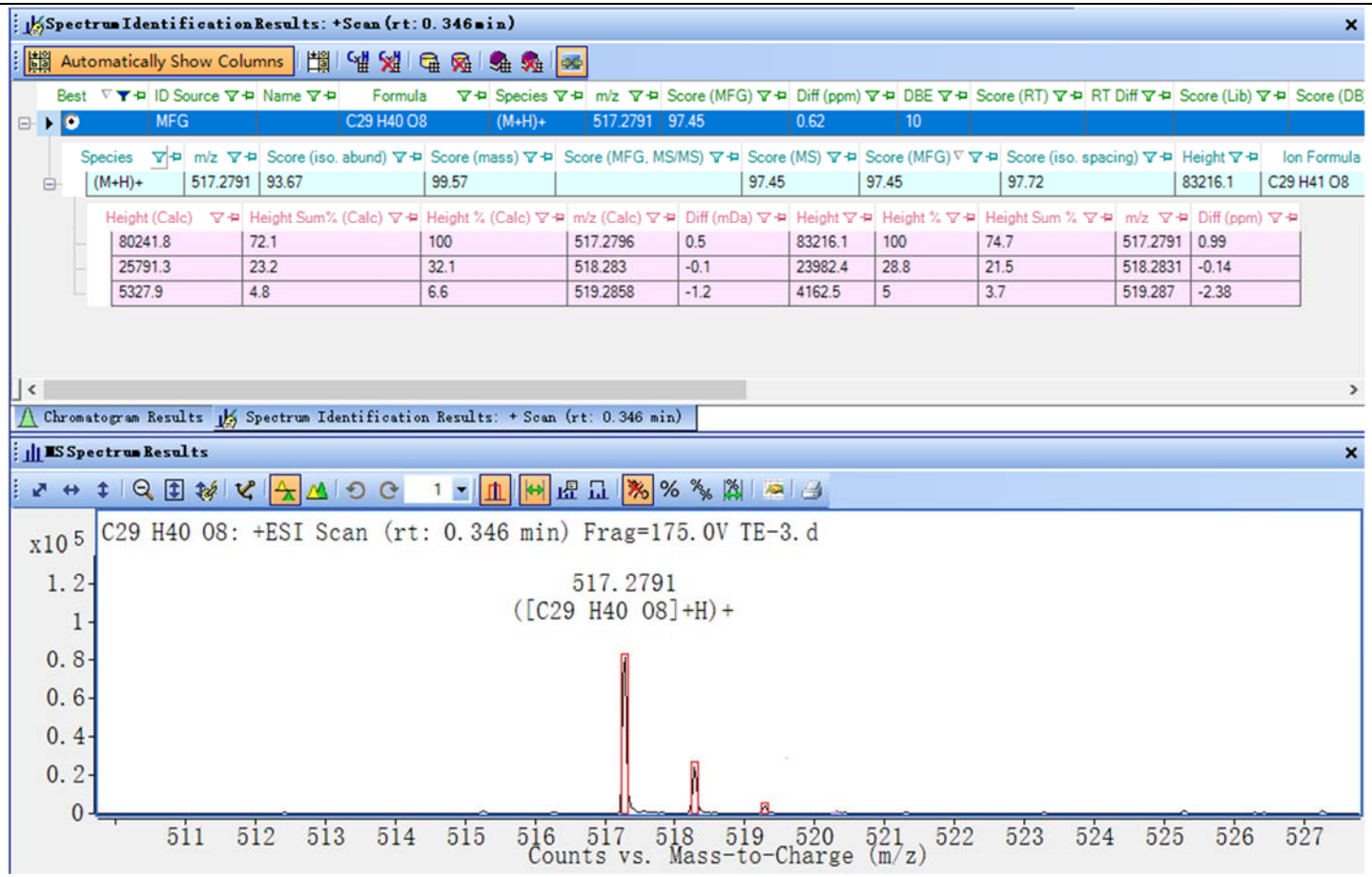

Figure S28. HR-ESI-MS of compound **3**

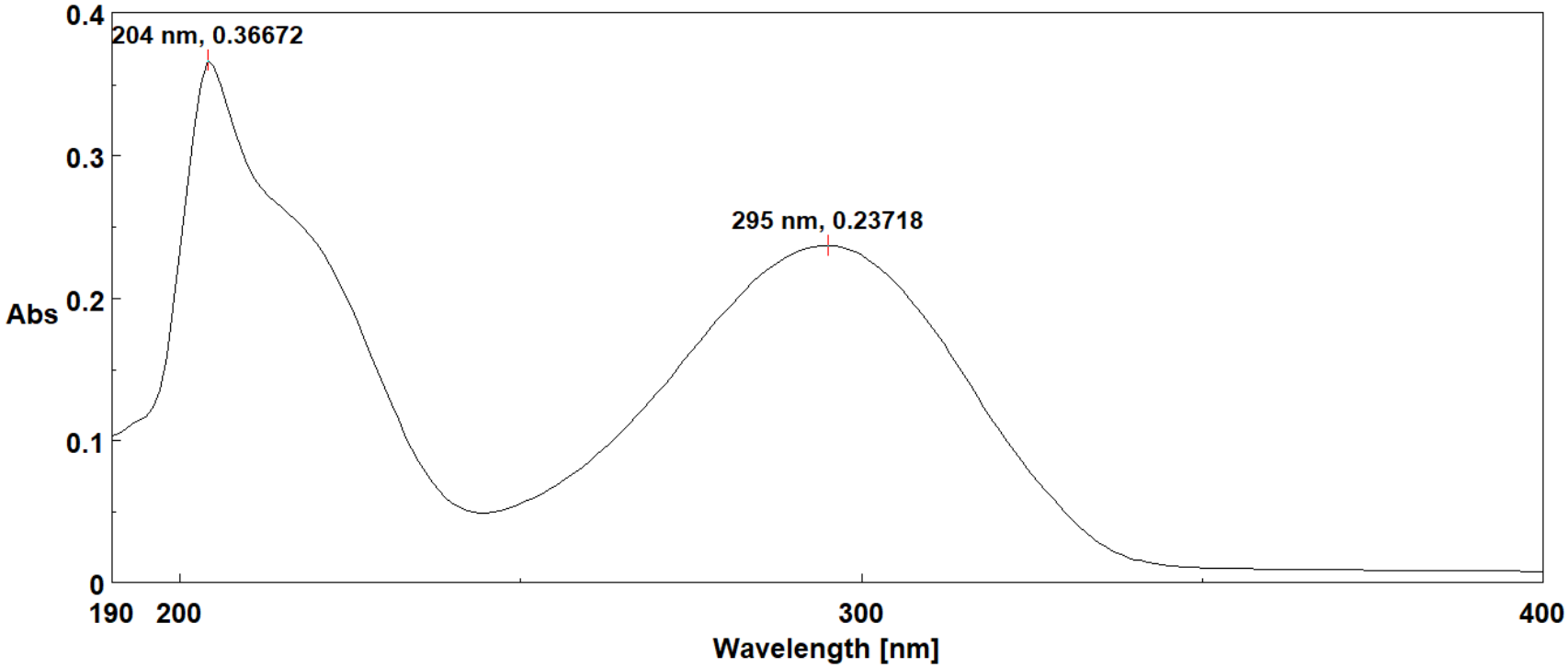

Figure S29. UV spectrum of compound **3**

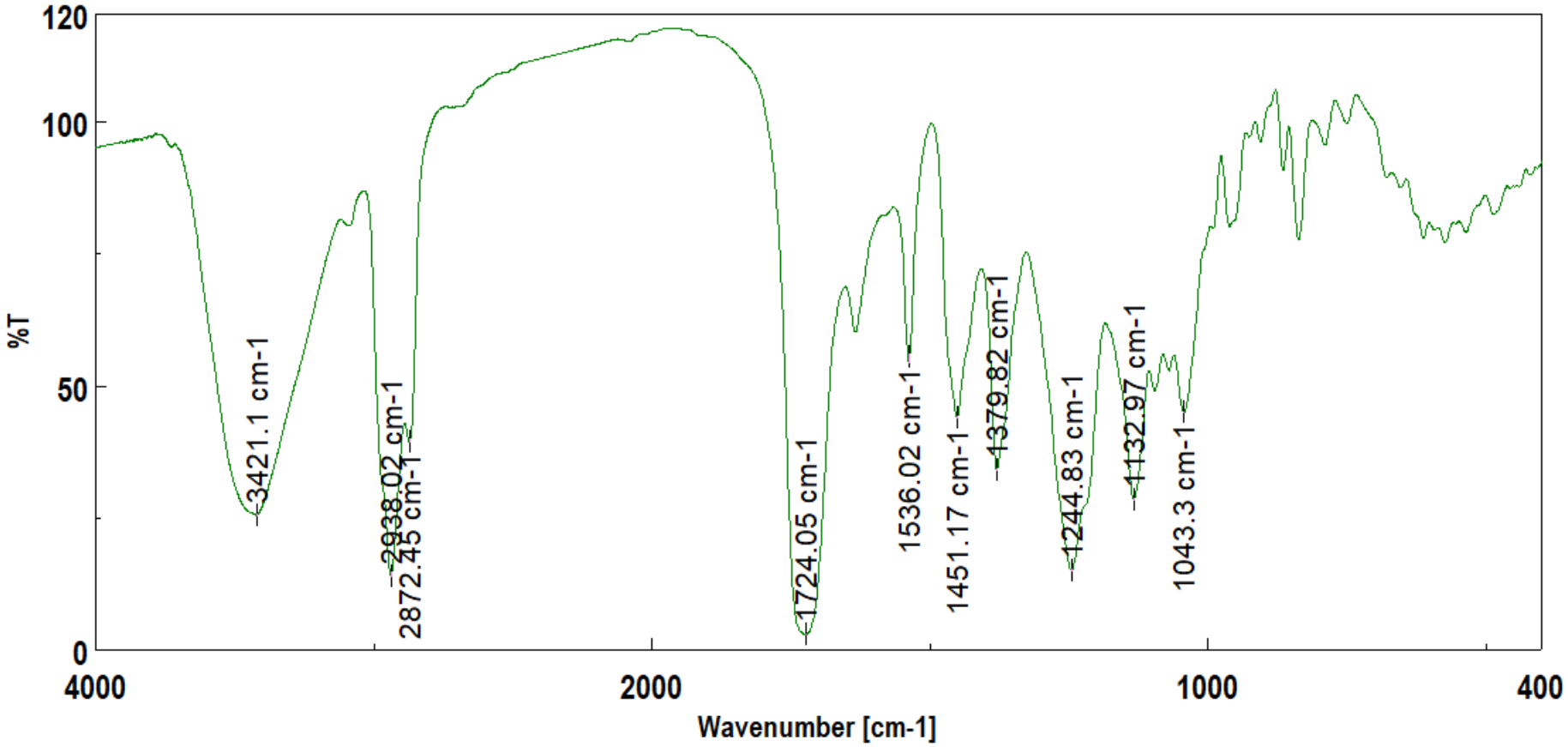

Figure S30. IR spectrum of compound **3** (KBr)

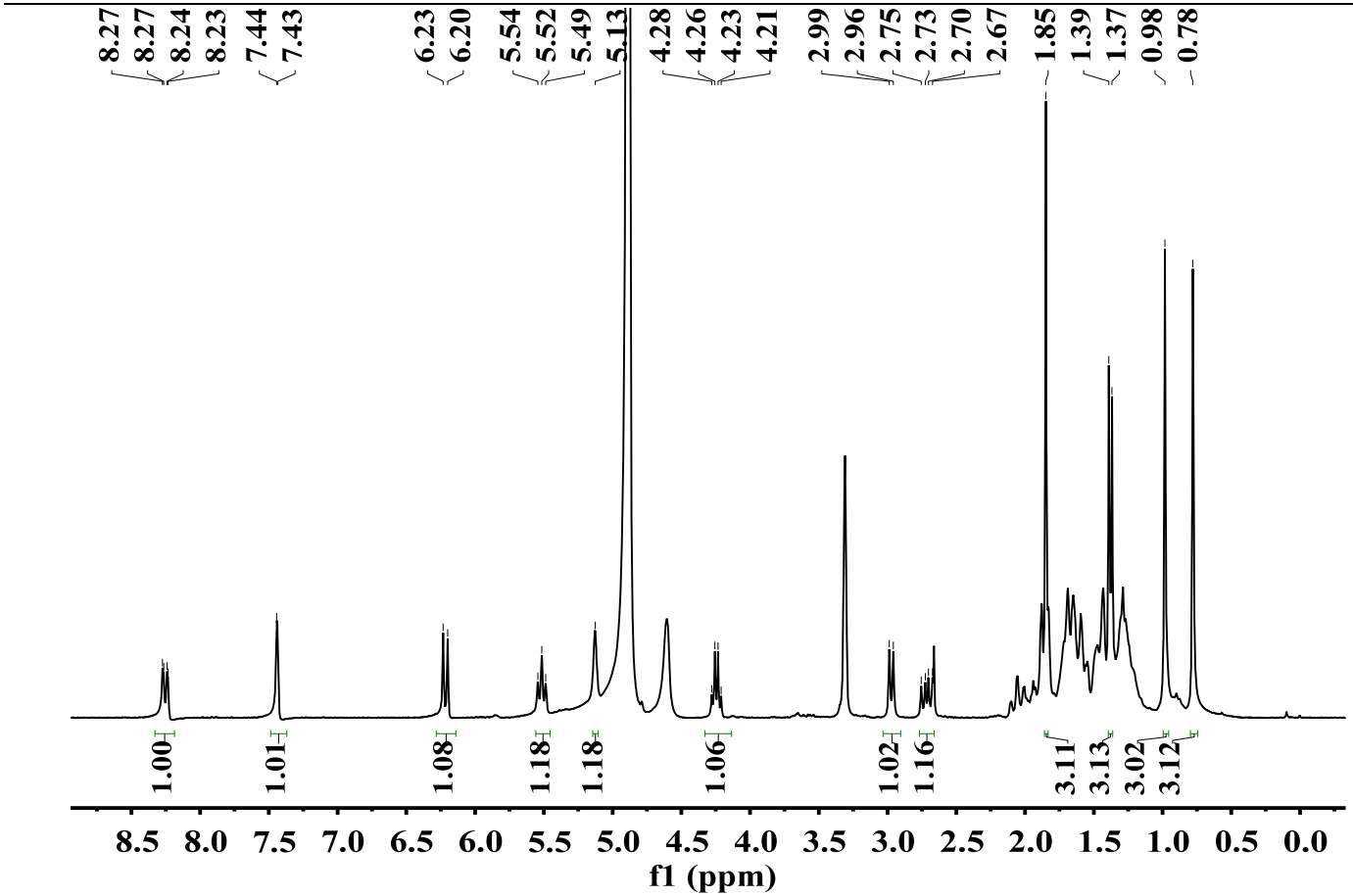

Figure S31. ^{1}H NMR spectrum of compound **3** (in CD_3OD, 300 MHz)

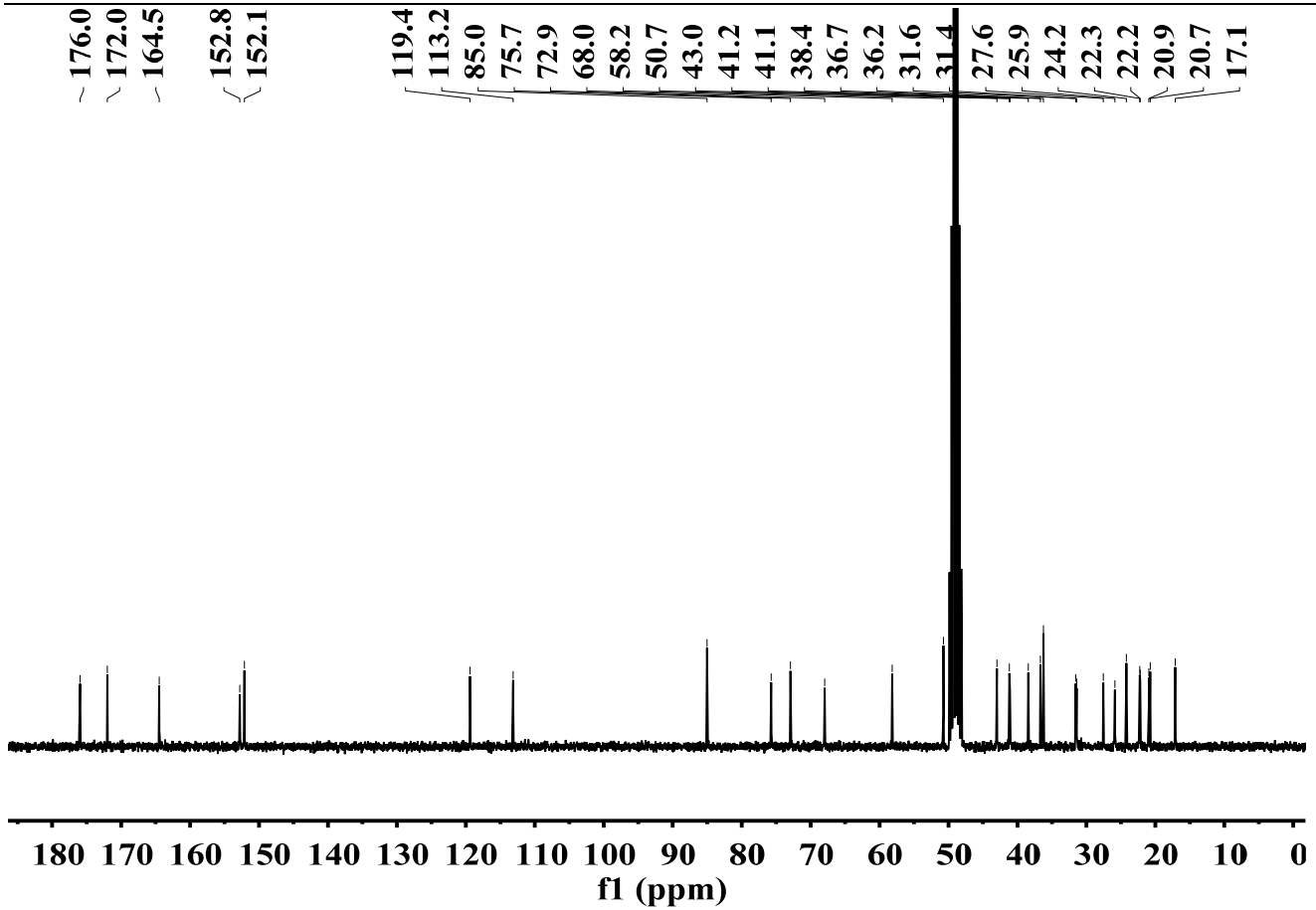

Figure S32. ^{13}C NMR spectrum of compound **3** (in CD_3OD, 75 MHz)

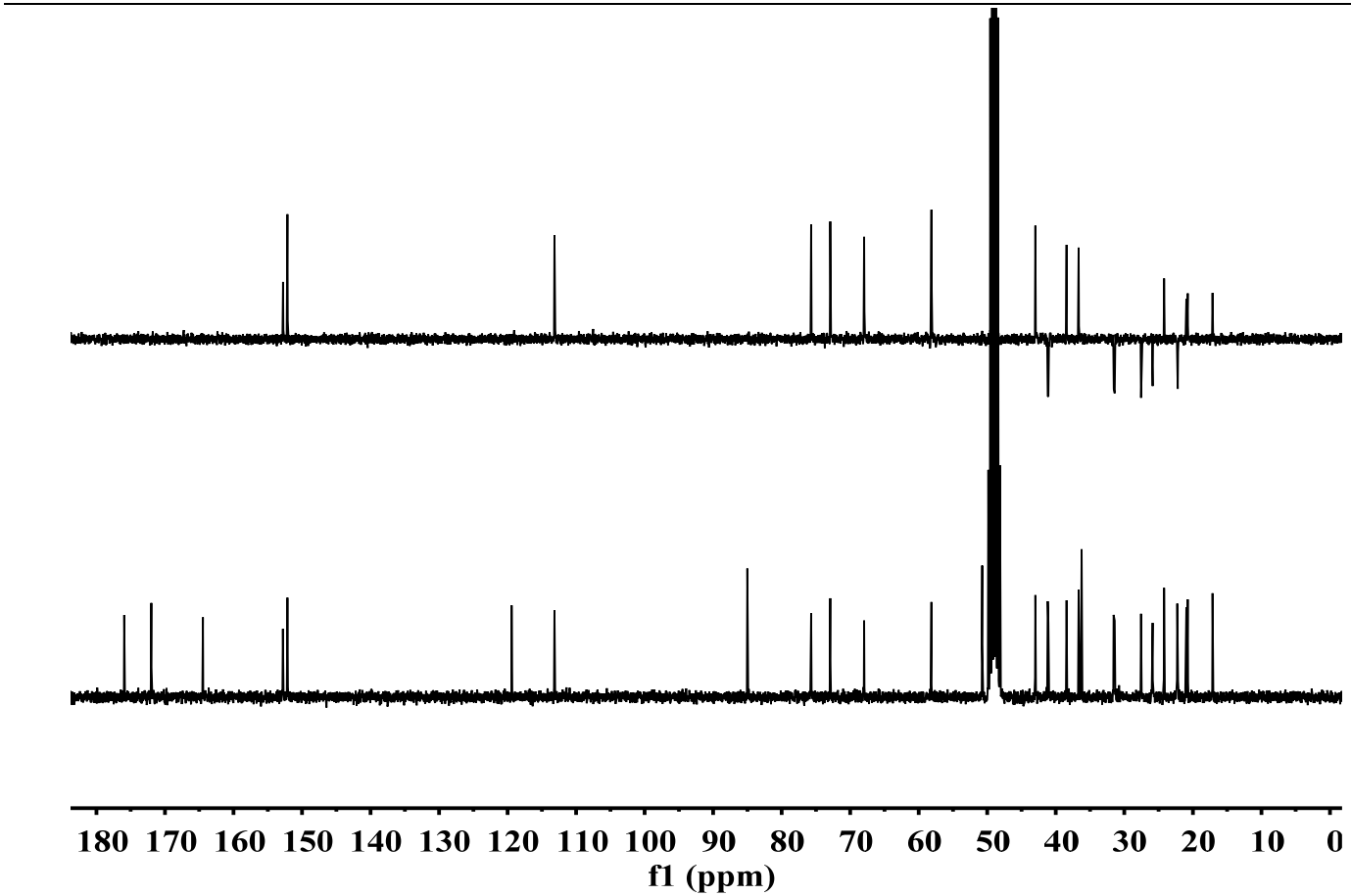

Figure S33. DEPT-135 and ^{13}C NMR spectrum of compound **3** (in CD_3OD, 75 MHz)

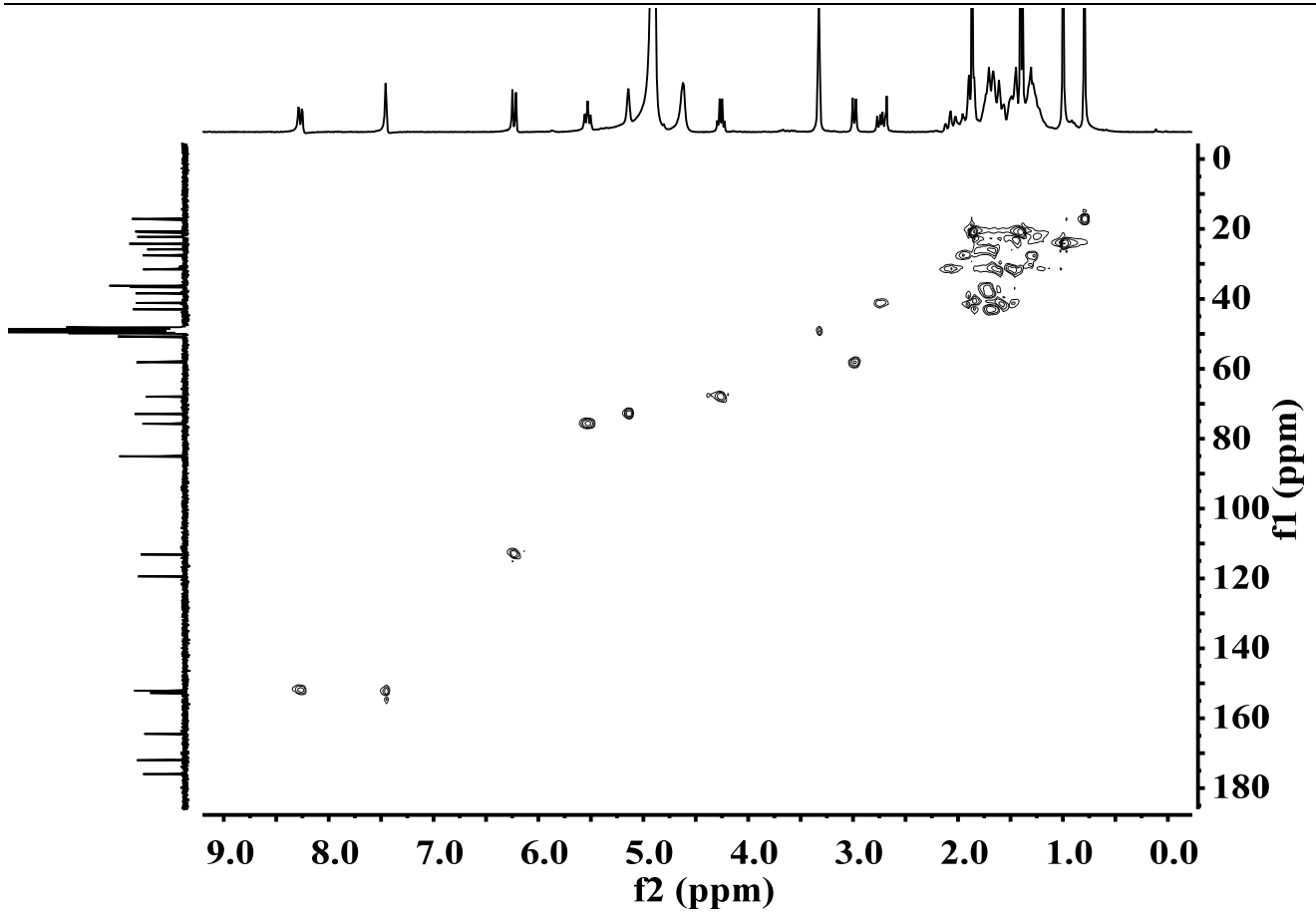

Figure S34. HSQC spectrum of compound **3** (in CD_3OD)

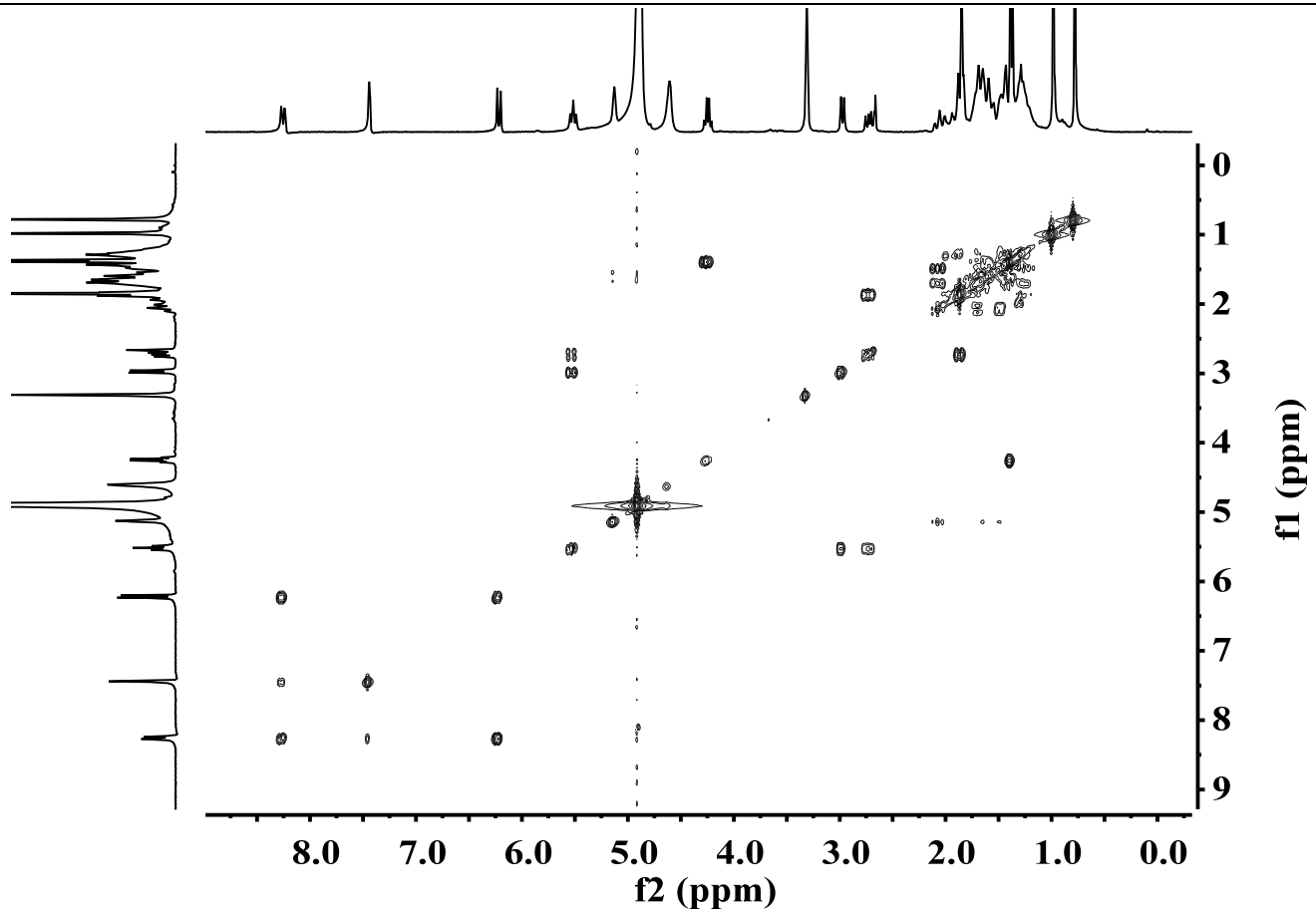

Figure S35. ^{1}H-^{1}H COSY spectrum of compound **3** (in CD_3OD)

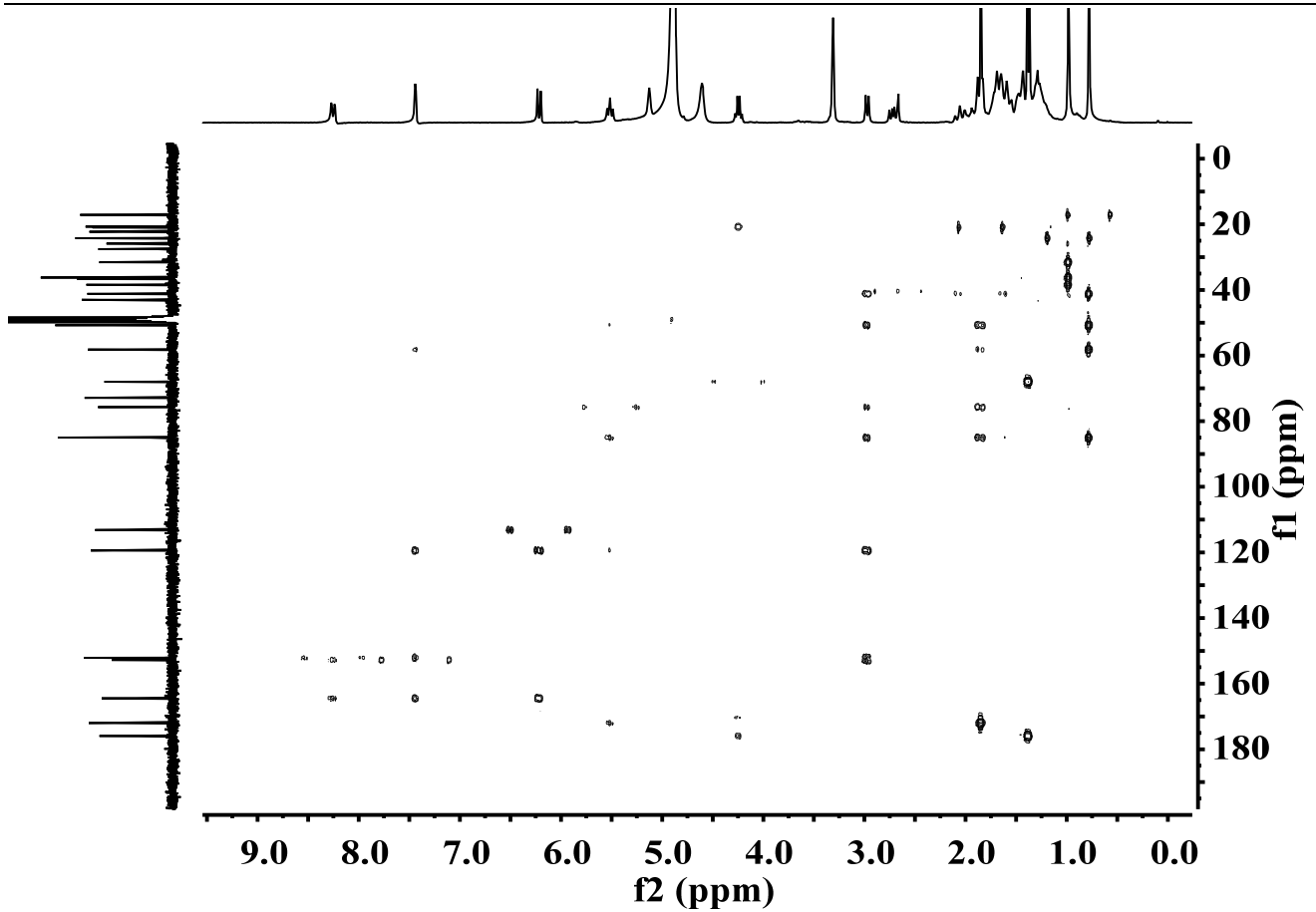

Figure S36. HMBC spectrum of compound **3** (in CD_3OD)

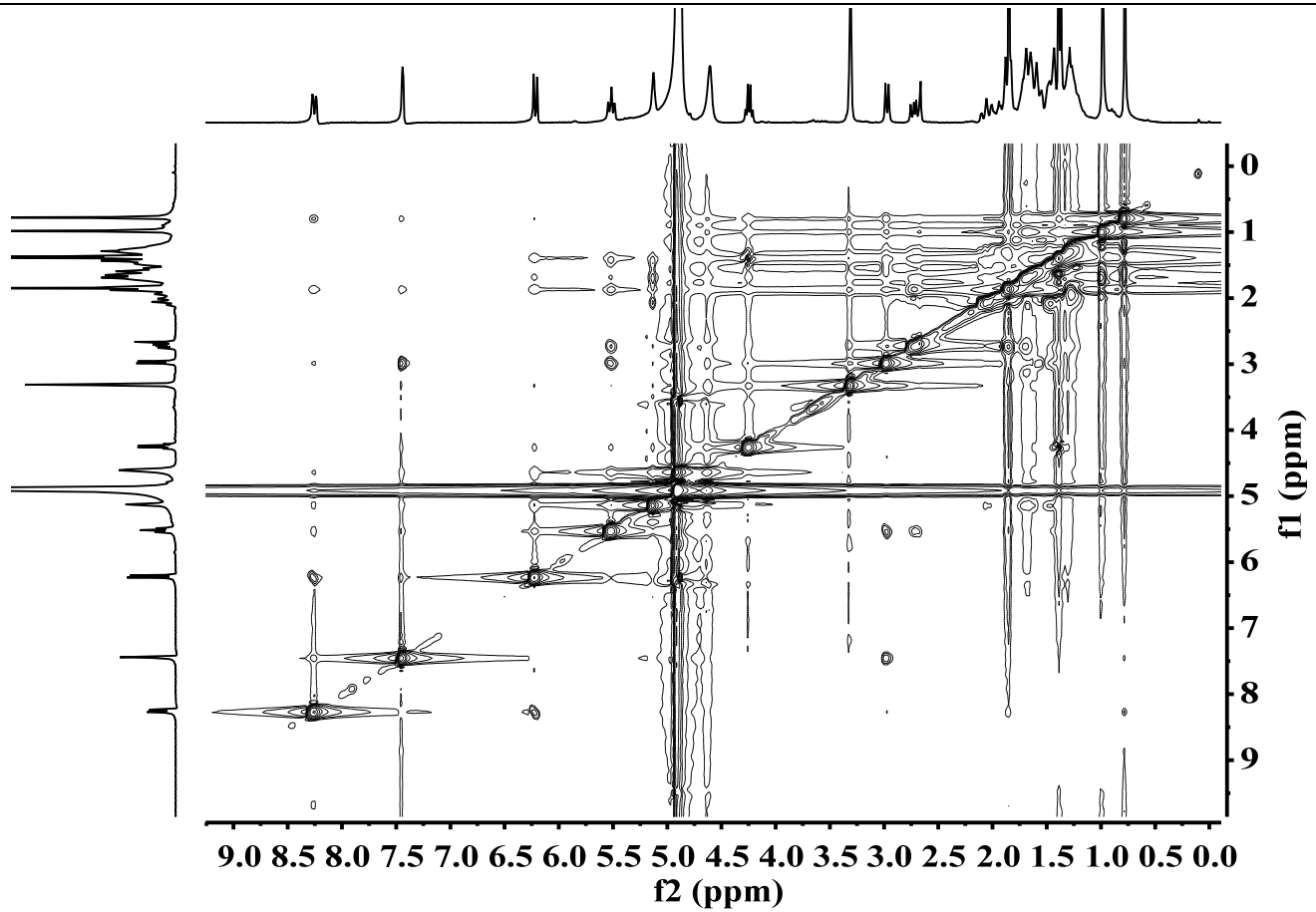

Figure S37. NOESY spectrum of compound **3** (in CD_3OD)

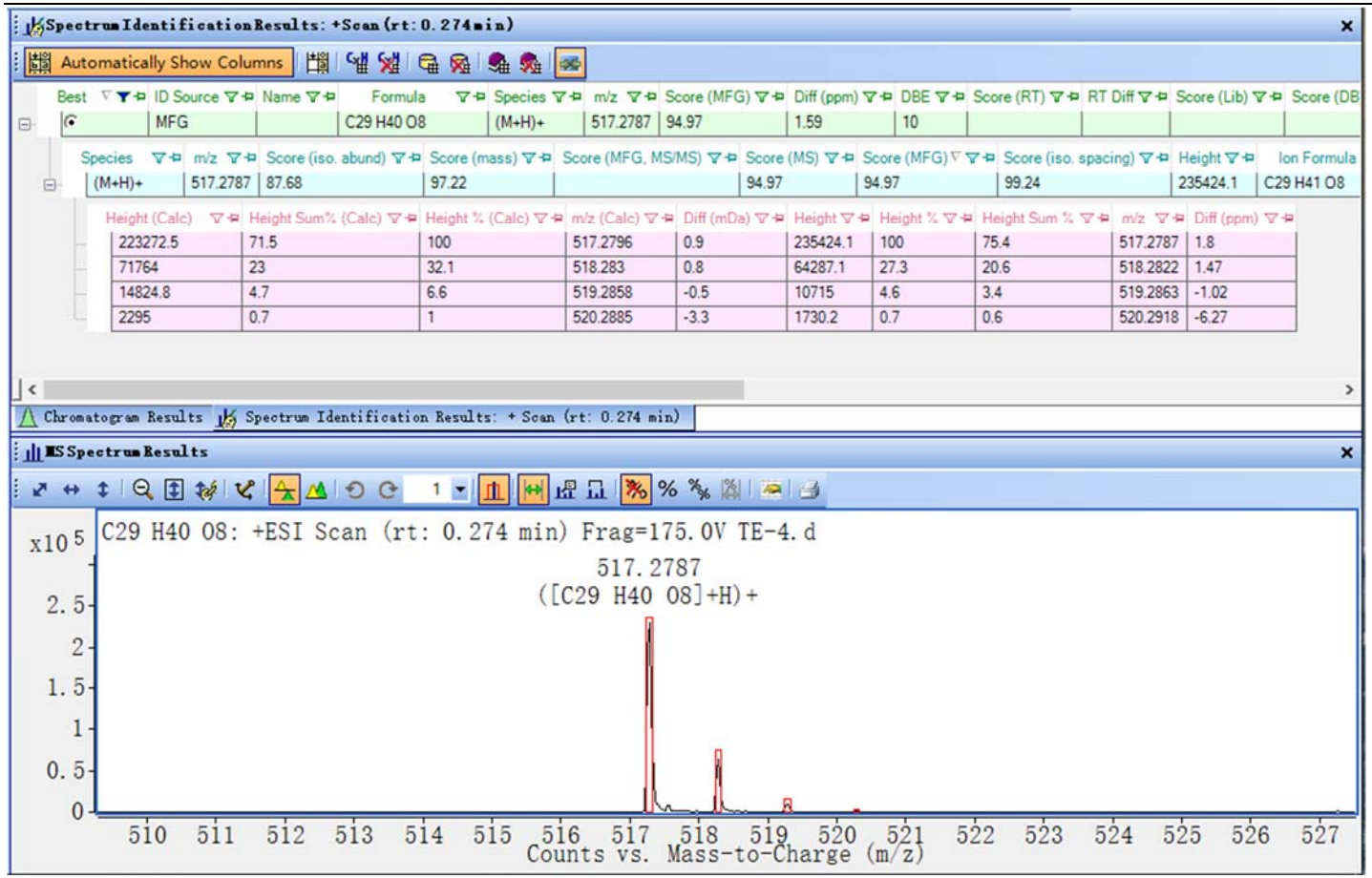

Figure S38. HR-ESI-MS of compound **4**

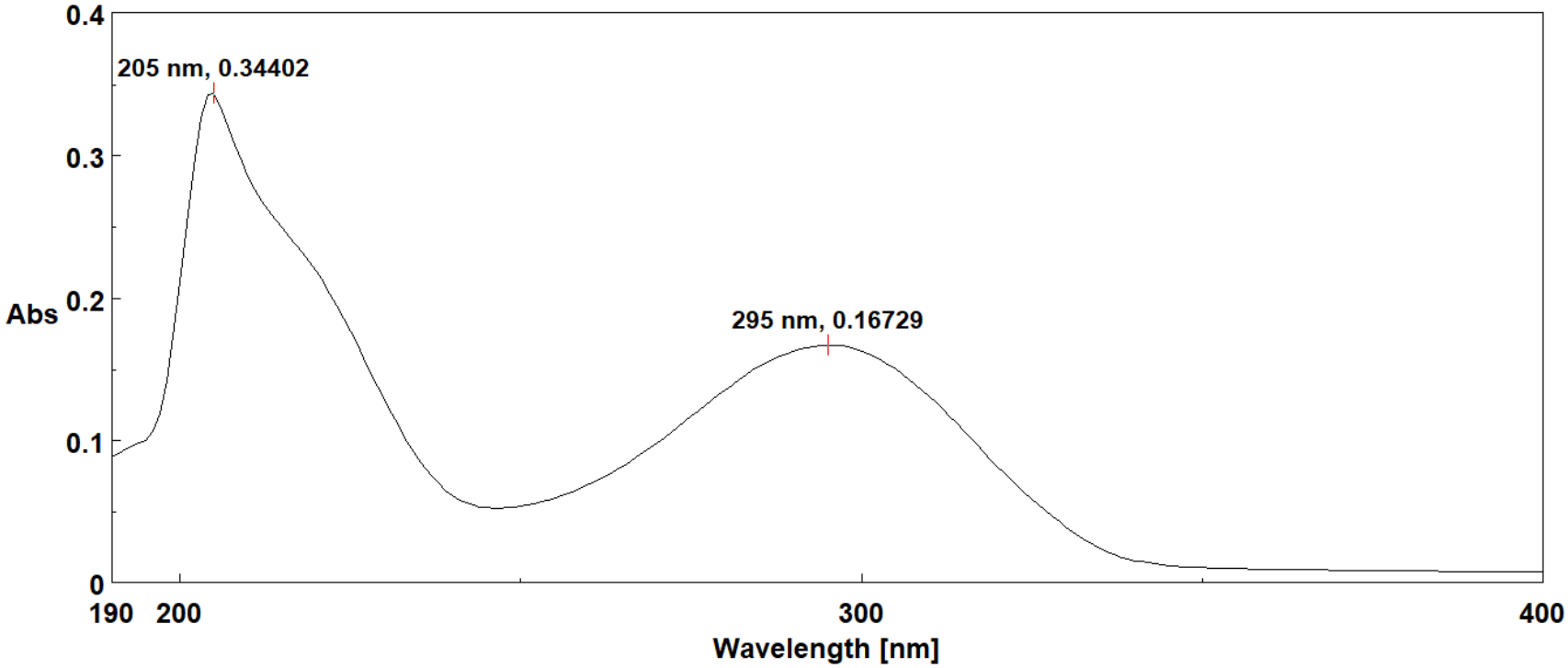

Figure S39. UV spectrum of compound **4**

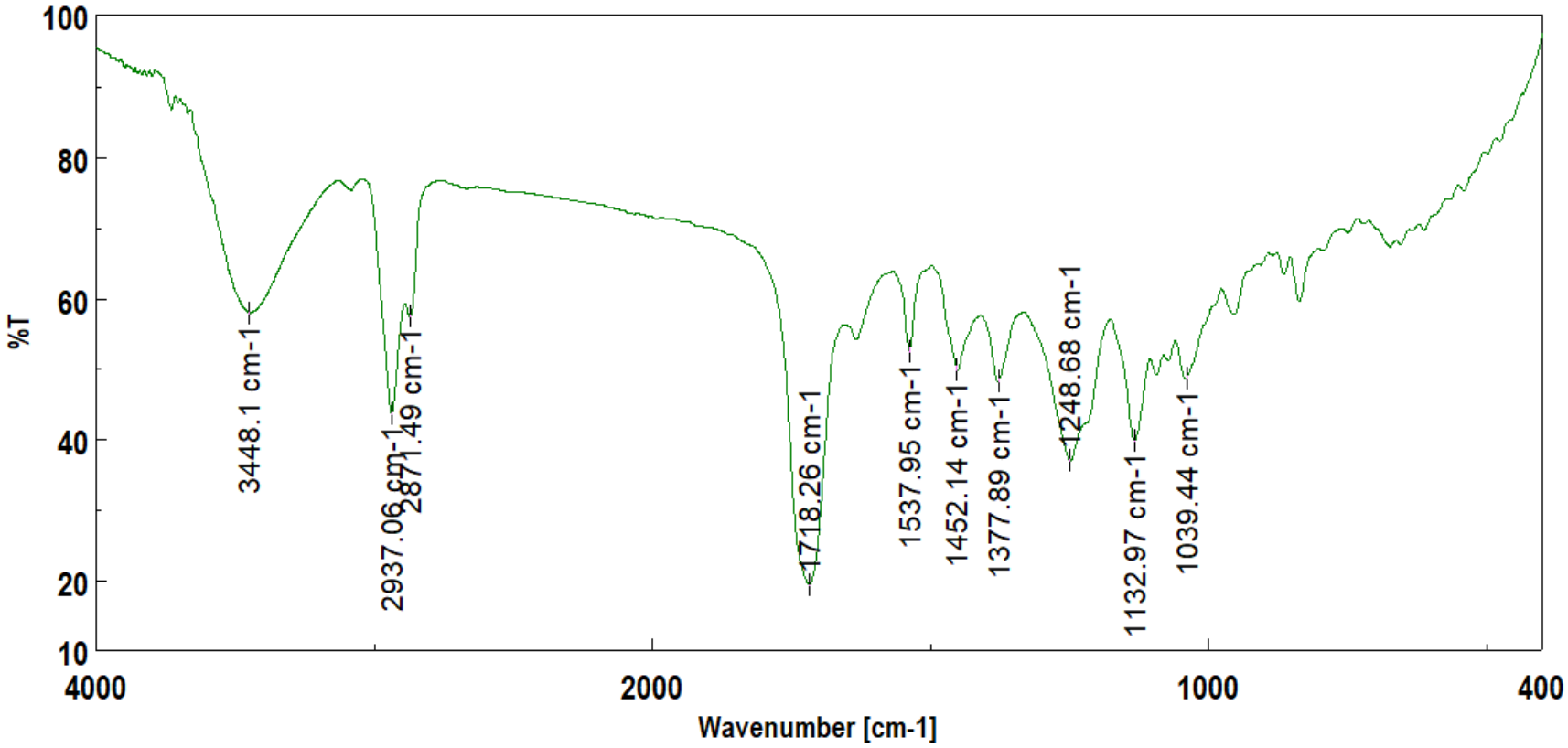

Figure S40. IR spectrum of compound **4** (KBr)

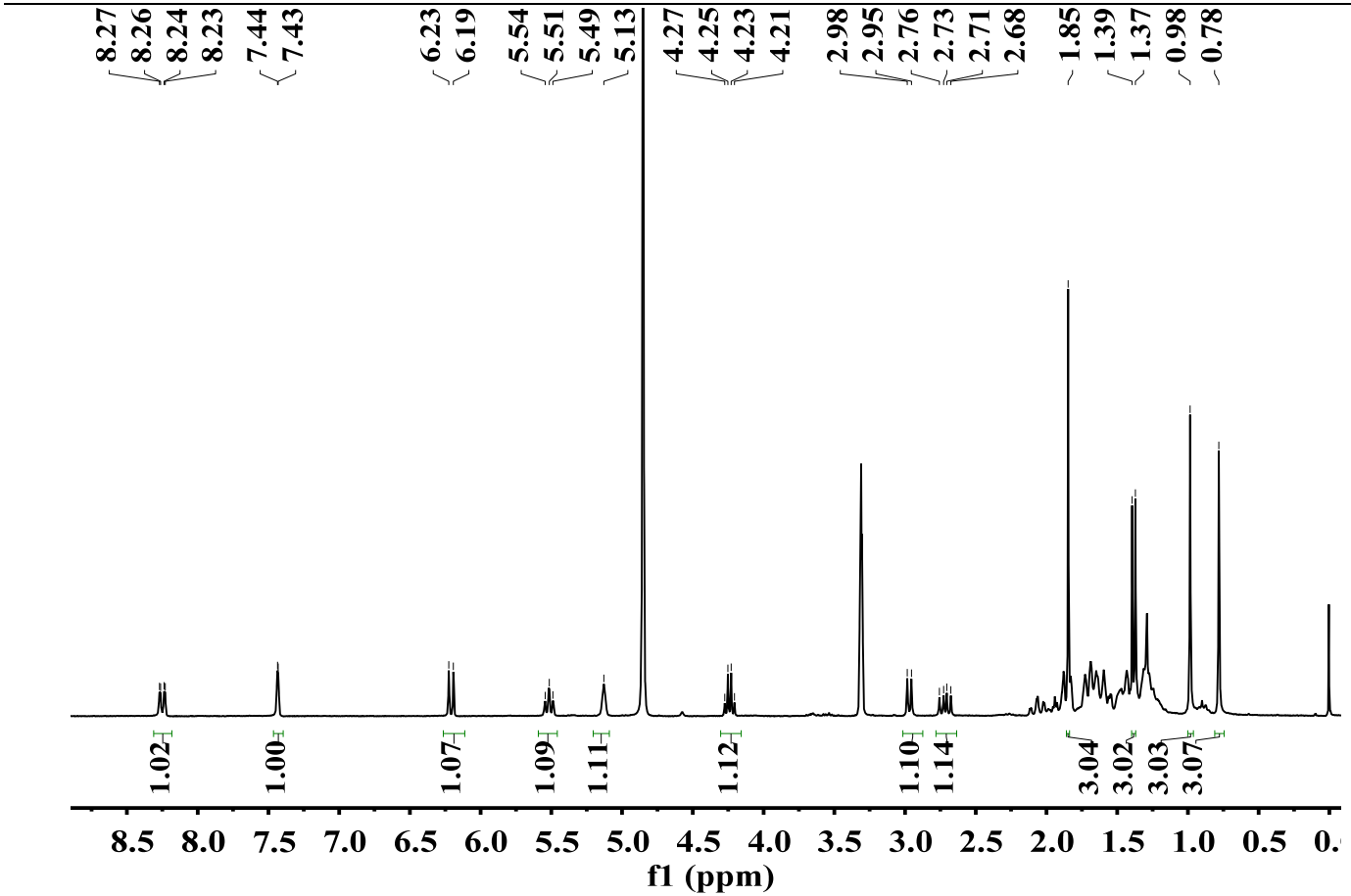

Figure S41. ^{1}H NMR spectrum of compound **4** (in CD_3OD, 300 MHz)

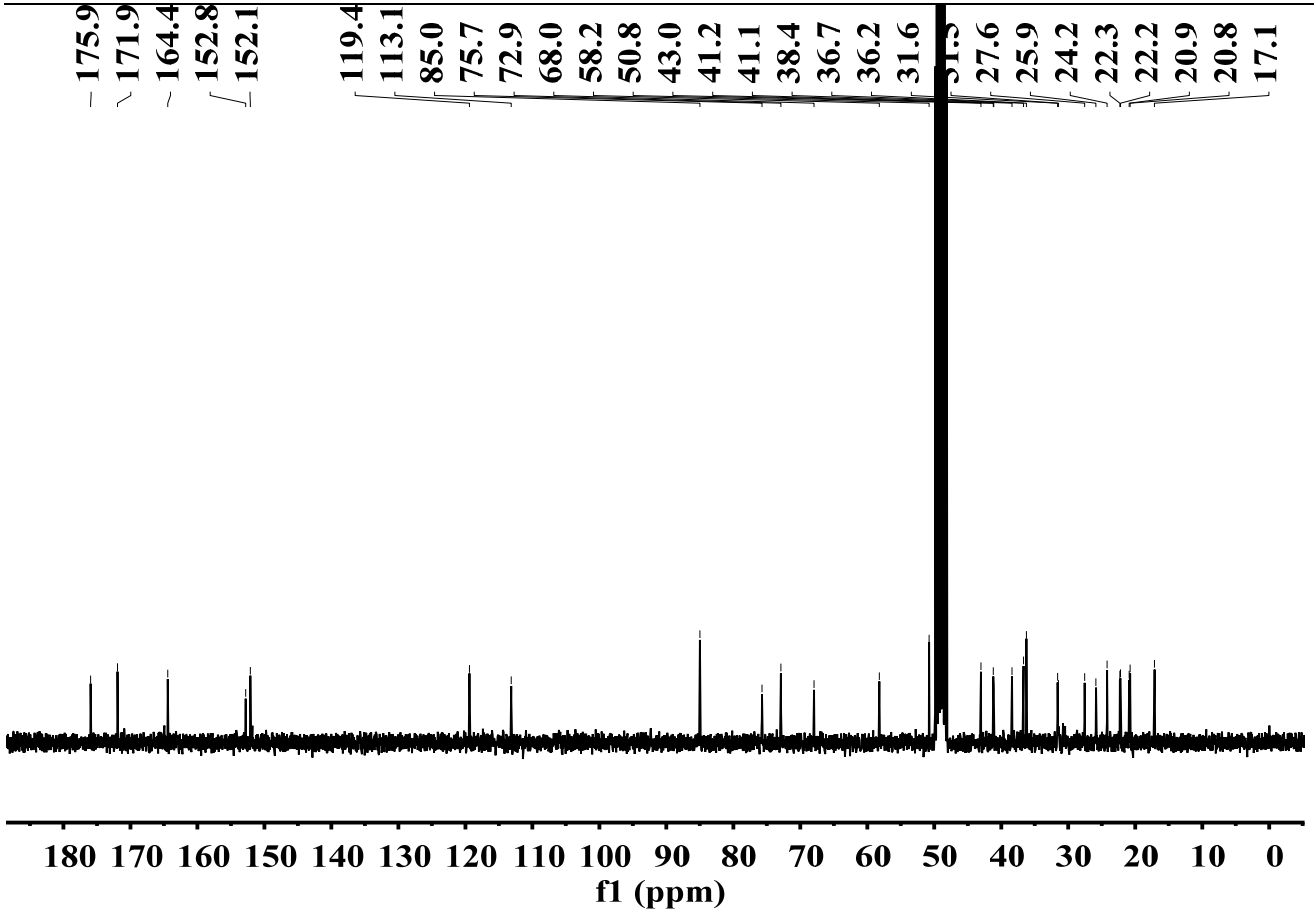

Figure S42. ^{13}C NMR spectrum of compound **4** (in CD_3OD, 75 MHz)

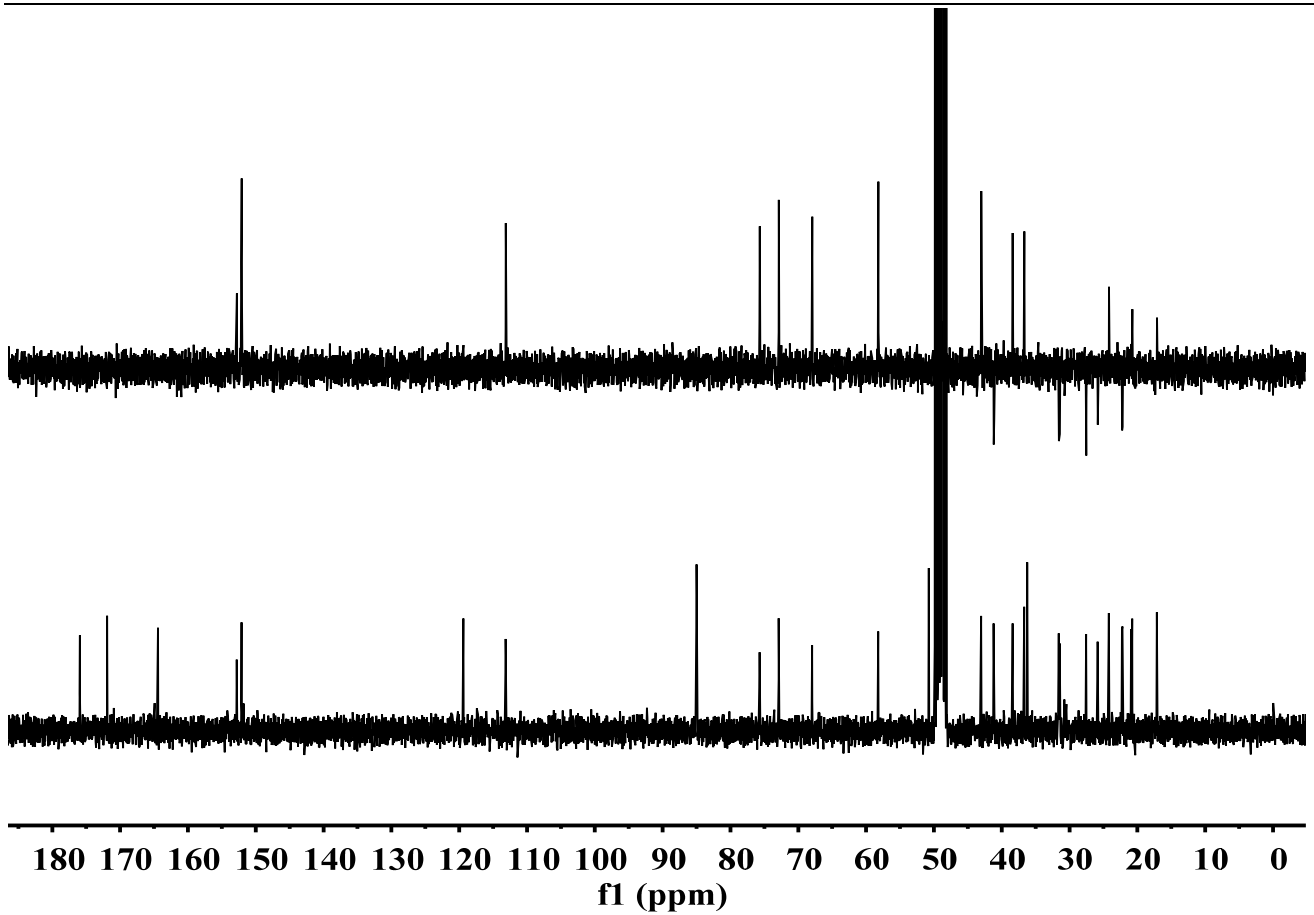

Figure S43. DEPT-135 and ^{13}C NMR spectrum of compound **4** (in CD_3OD, 75 MHz)

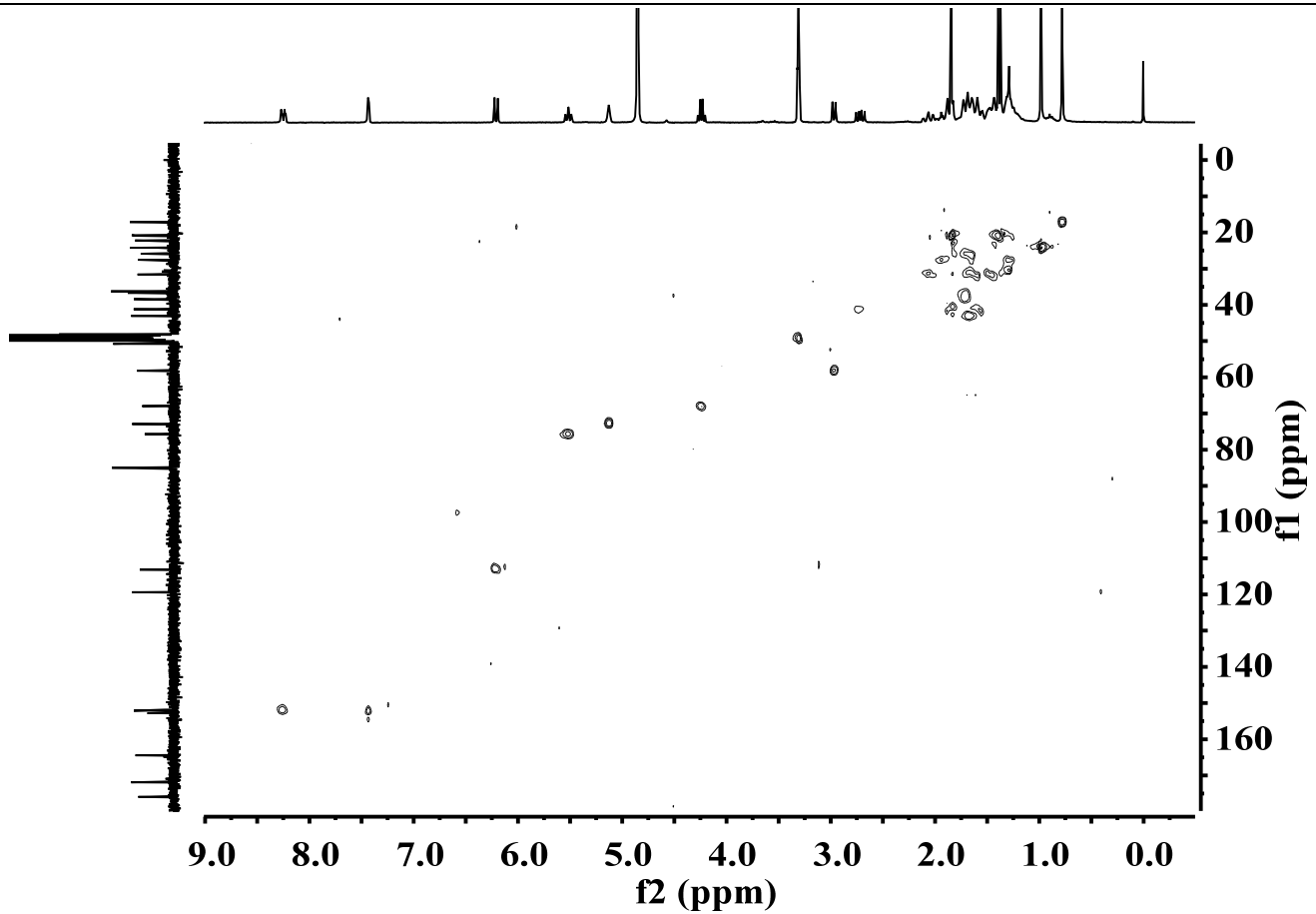

Figure S44. HMQC spectrum of compound **4** (in CD_3OD)

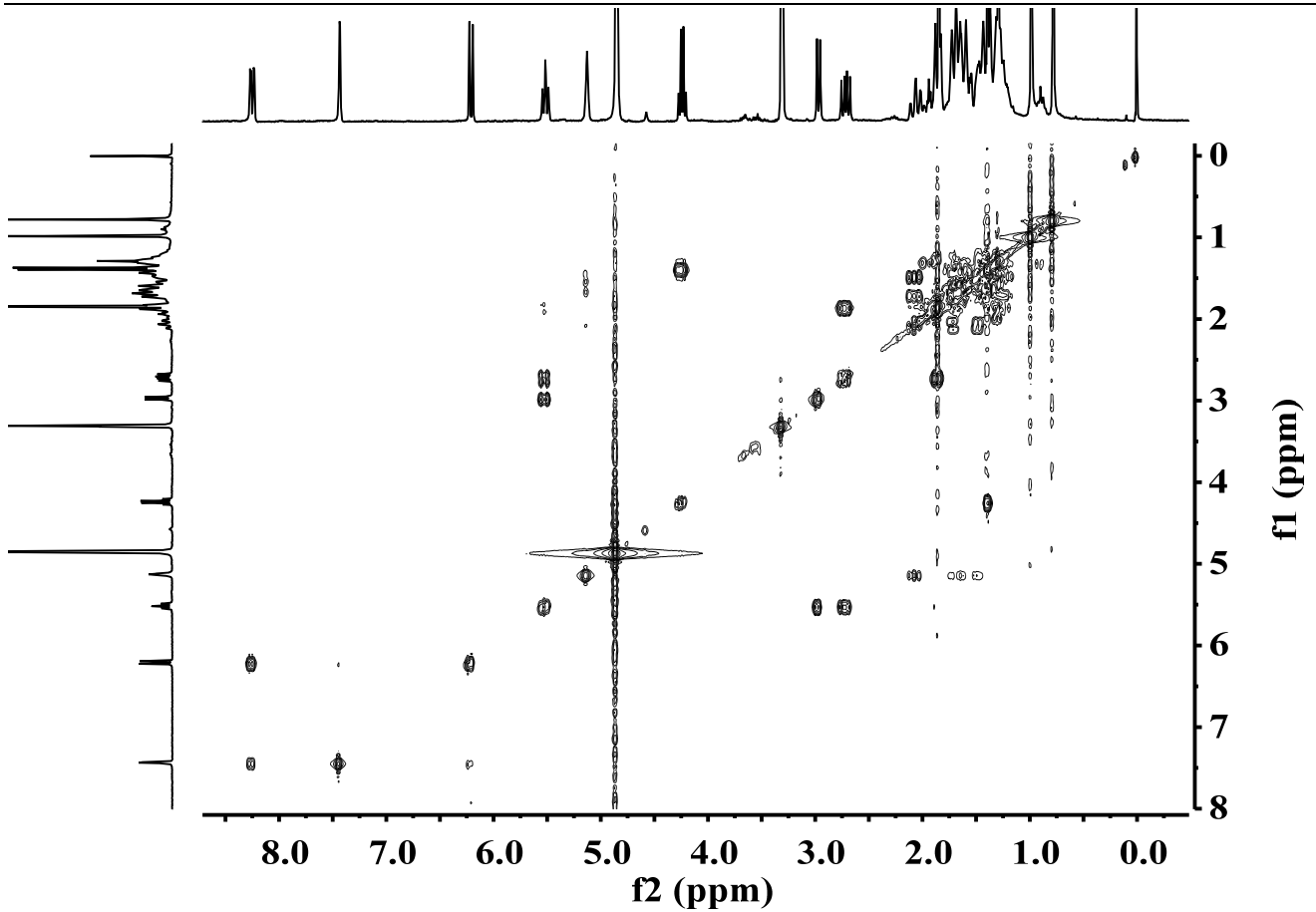

Figure S45. ^{1}H-^{1}H COSY spectrum of compound **4** (in CD_3OD)

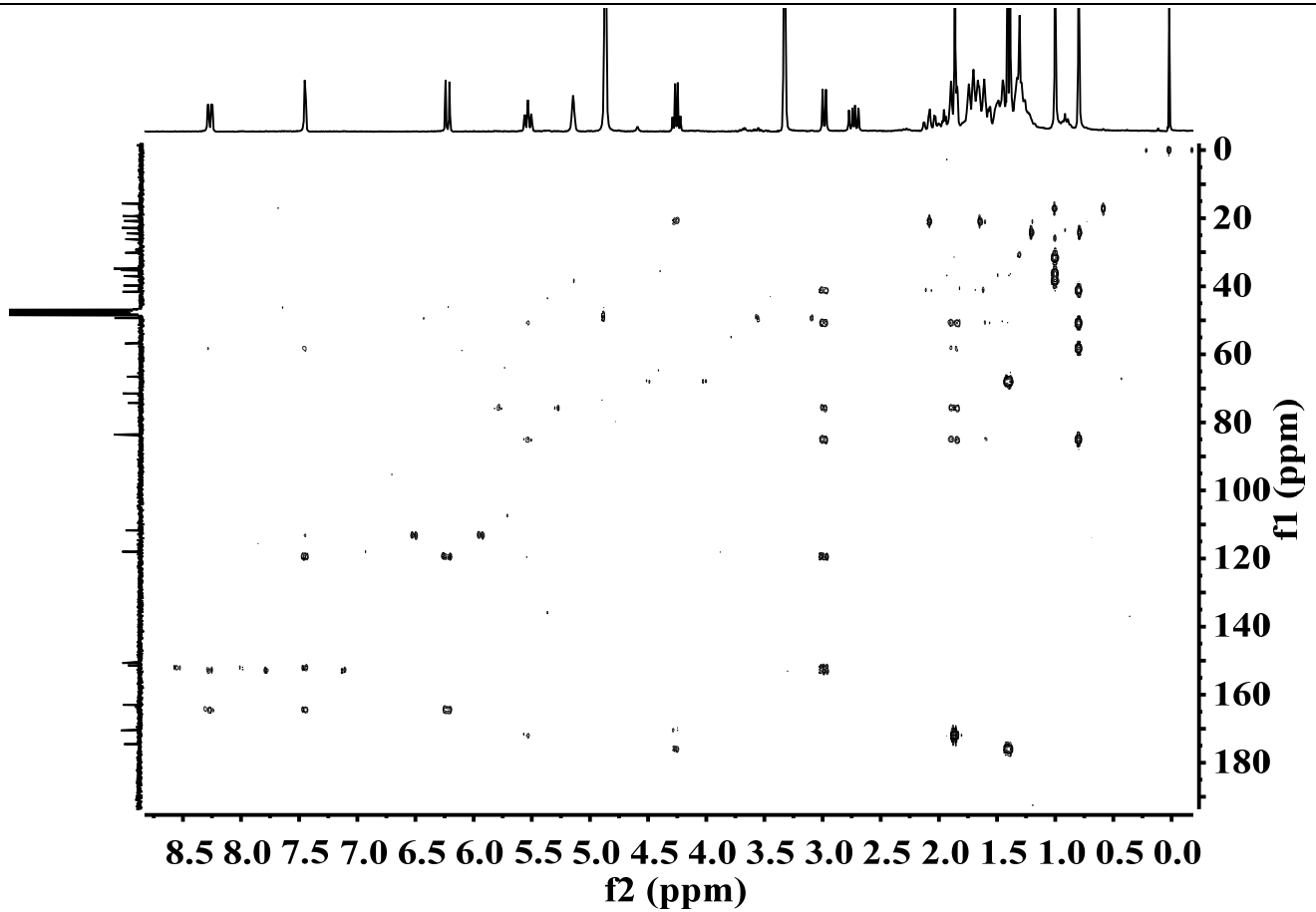

Figure S46. HMBC spectrum of compound **4** (in CD_3OD)

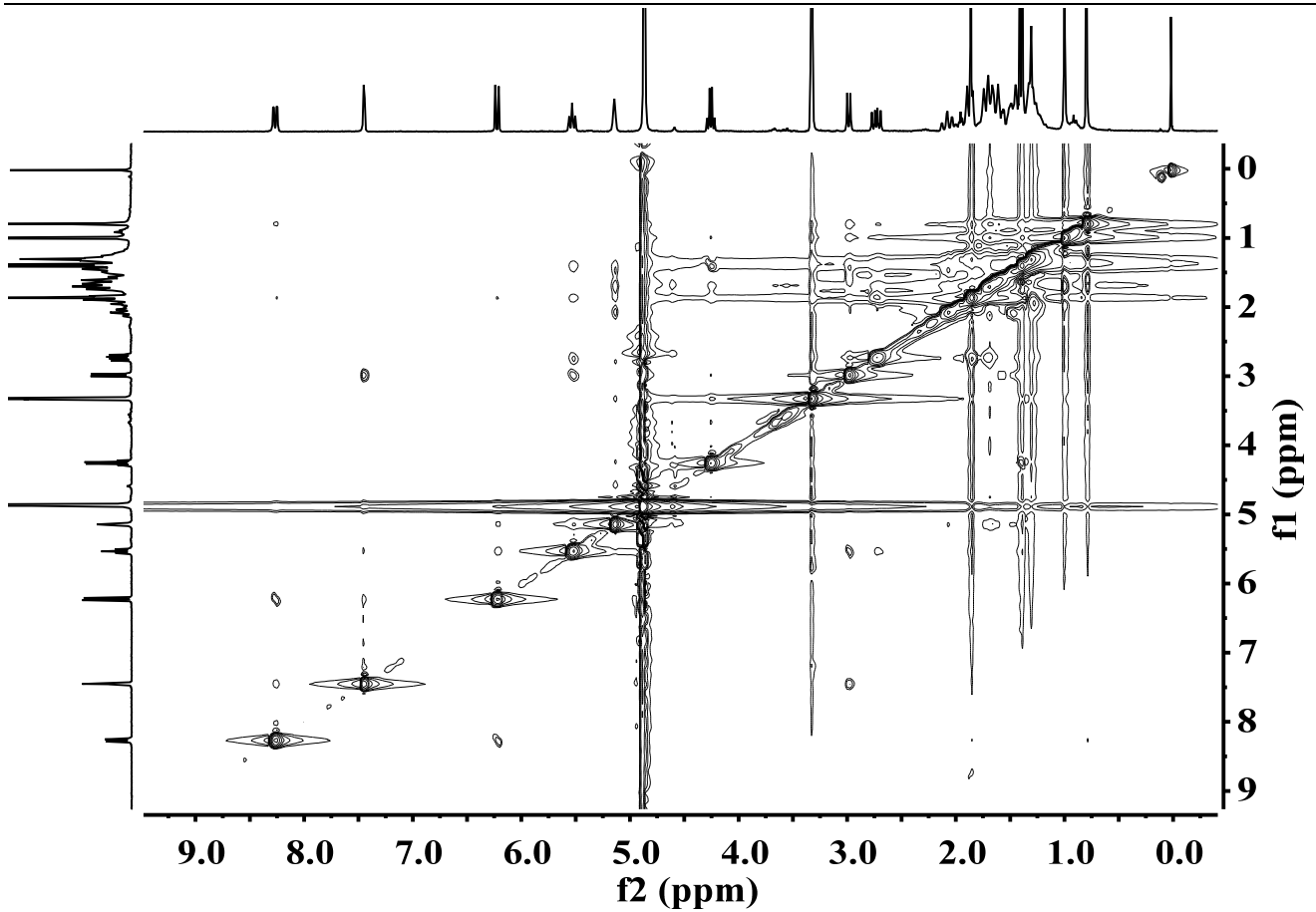

Figure S47. NOESY spectrum of compound **4** (in CD_3OD)

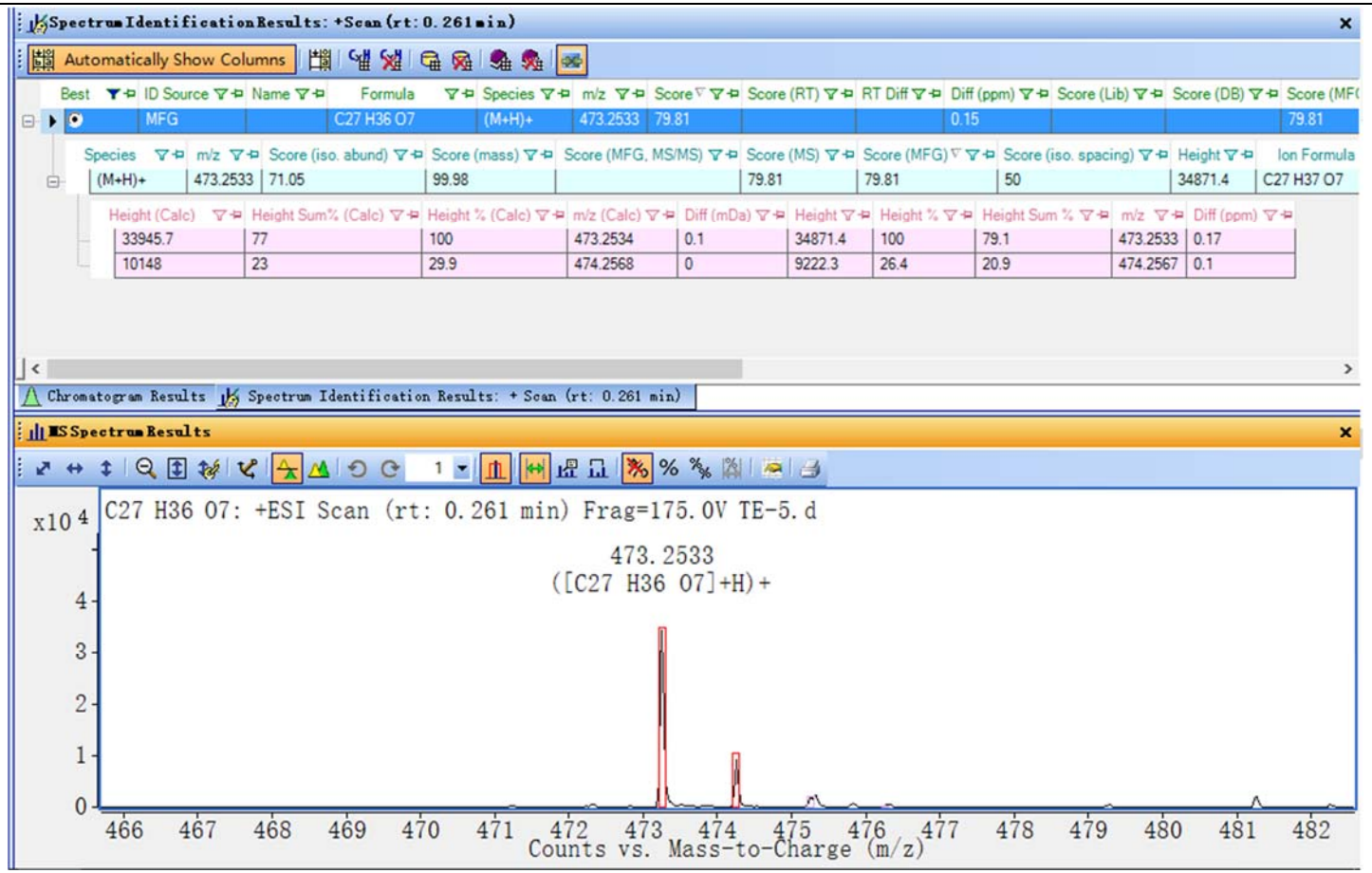

Figure S48. HR-ESI-MS of compound **5**

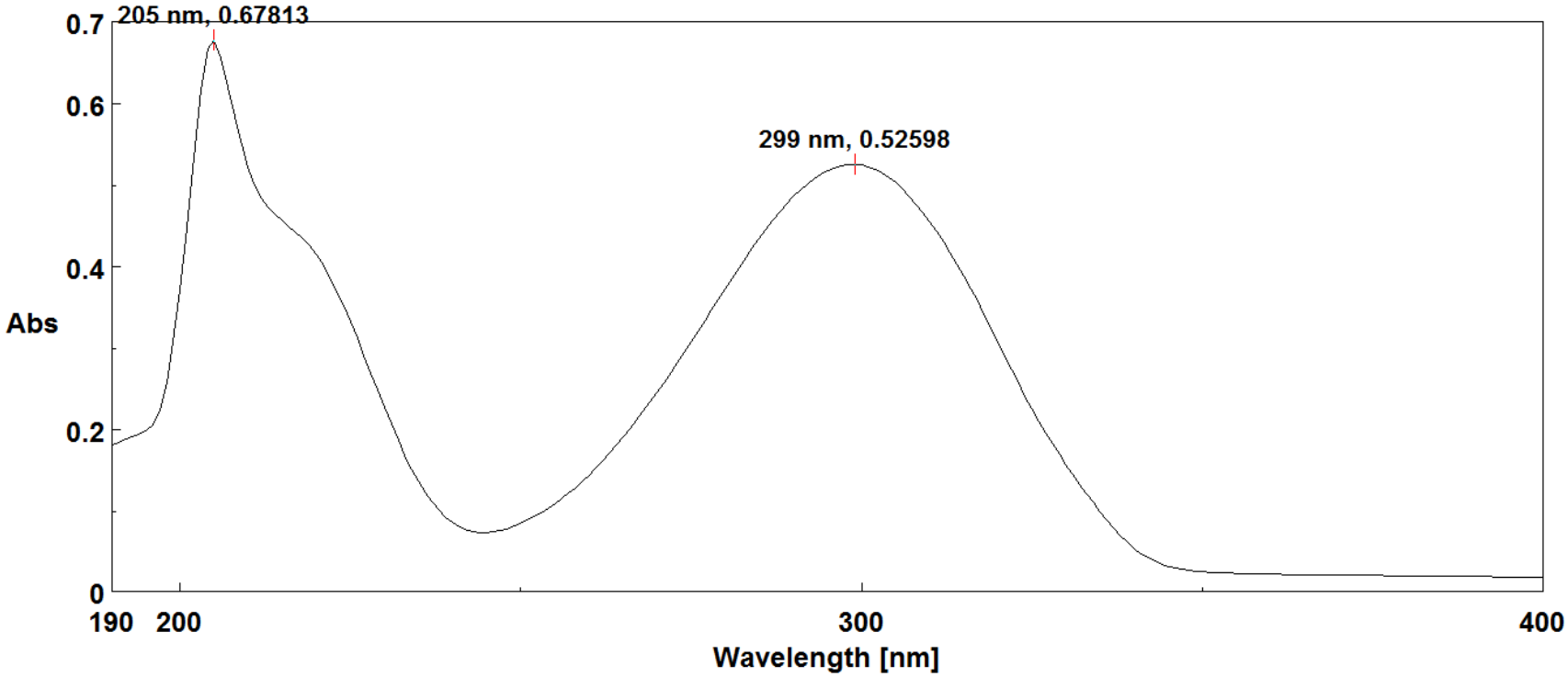

Figure S49. UV spectrum of compound **5**

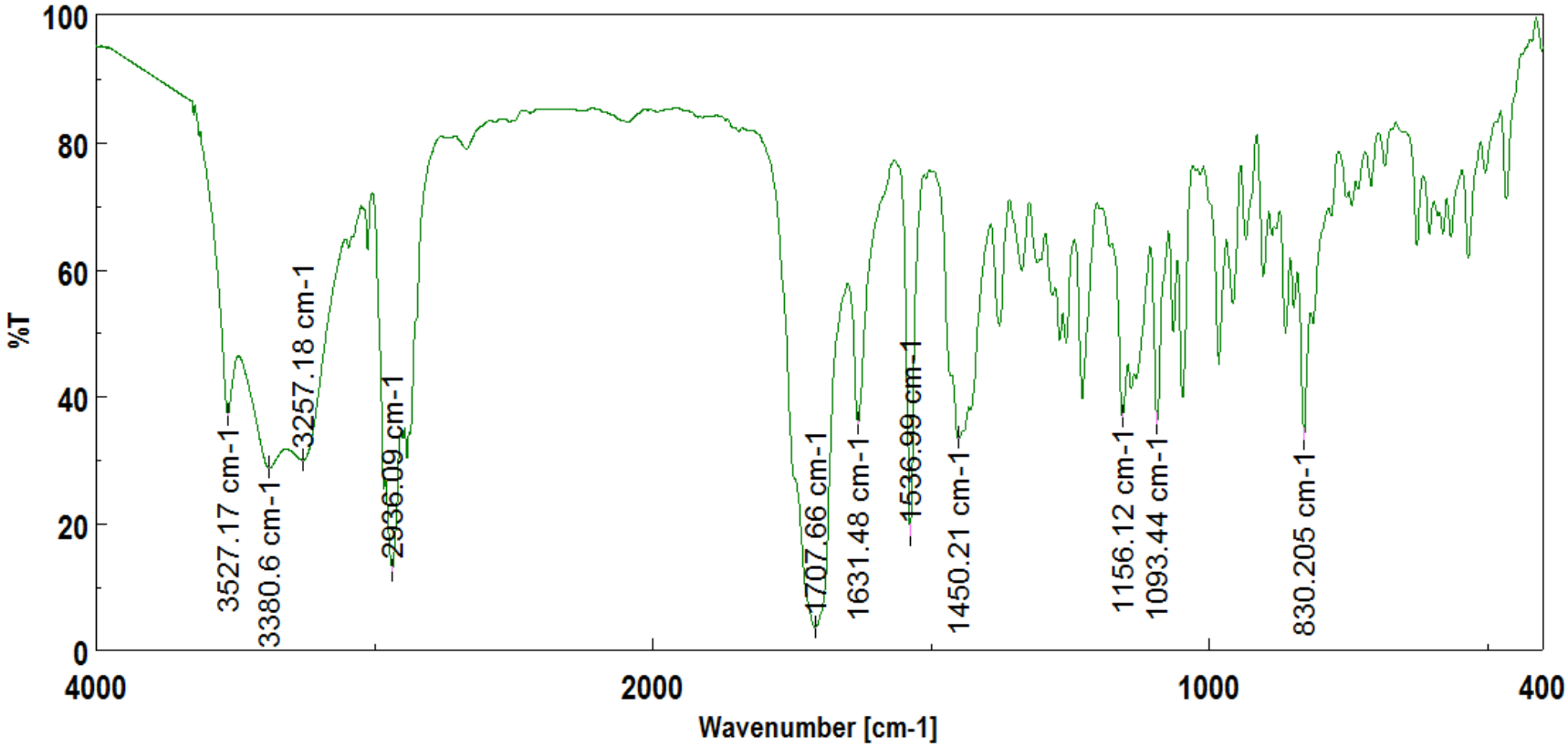

Figure S50. IR spectrum of compound **5** (KBr)

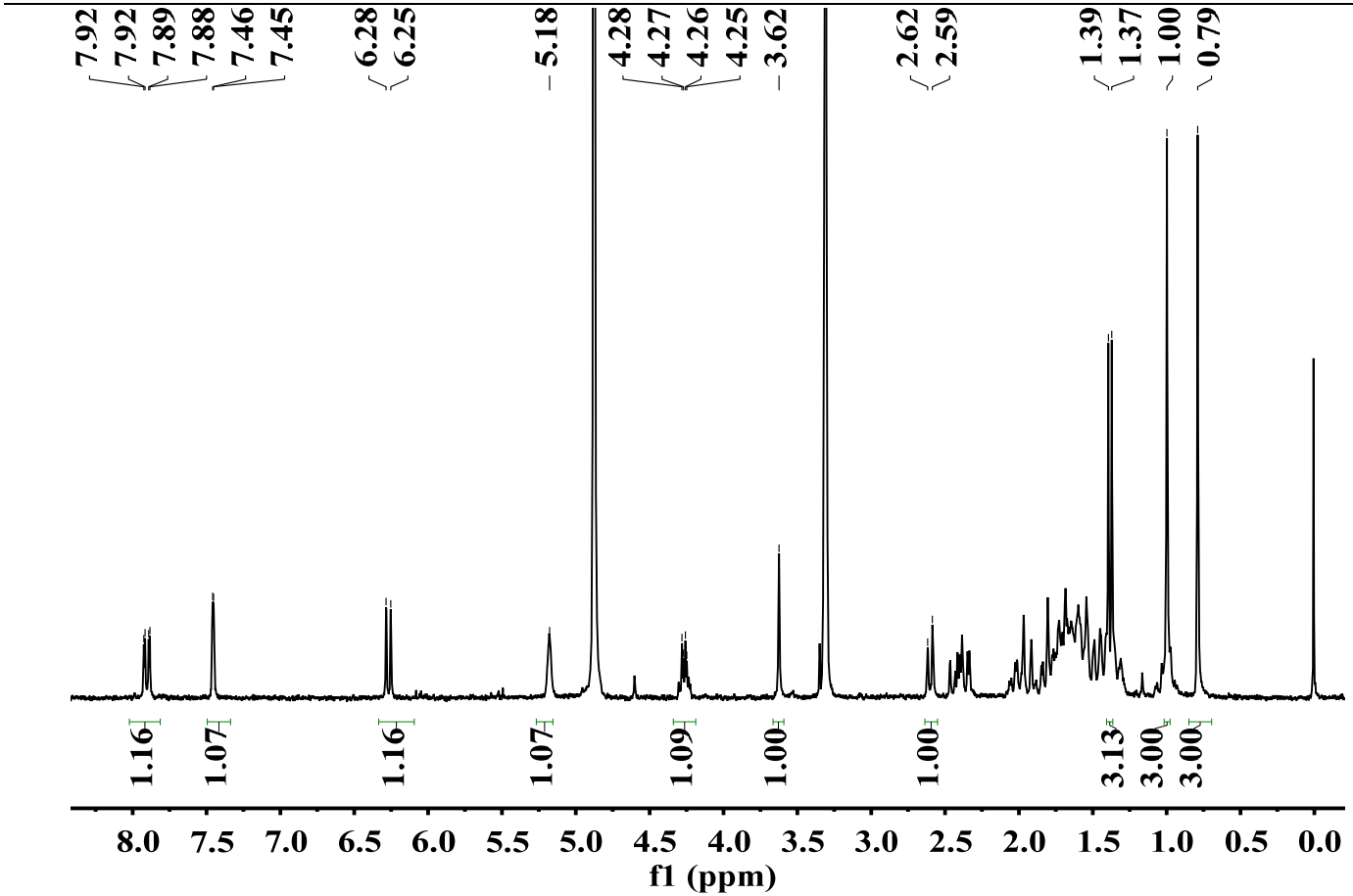

Figure S51. ^{1}H NMR spectrum of compound **5** (in CD_3OD, 300 MHz)

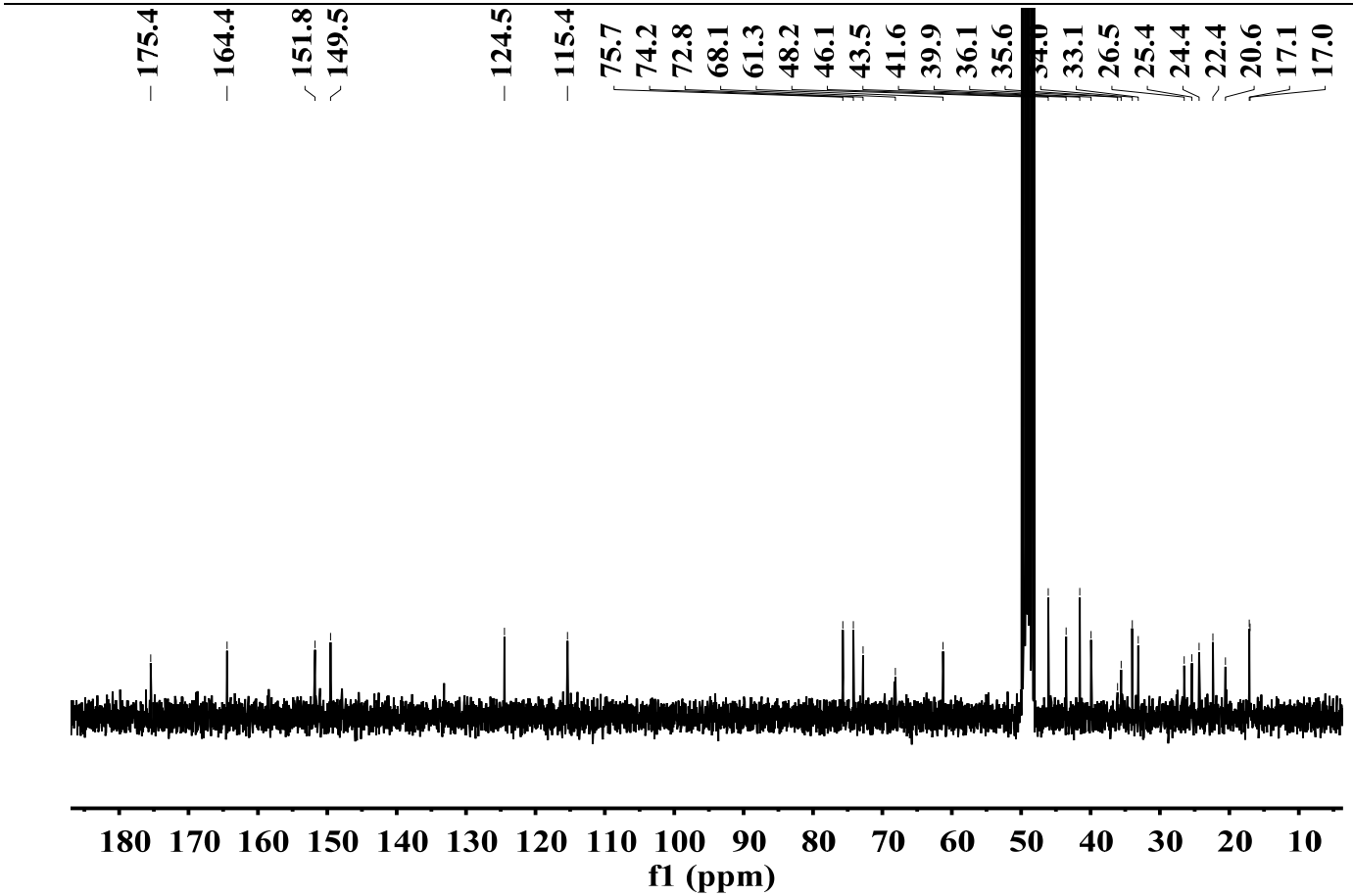

Figure S52. ^{13}C NMR spectrum of compound **5** (in CD_3OD, 75 MHz)

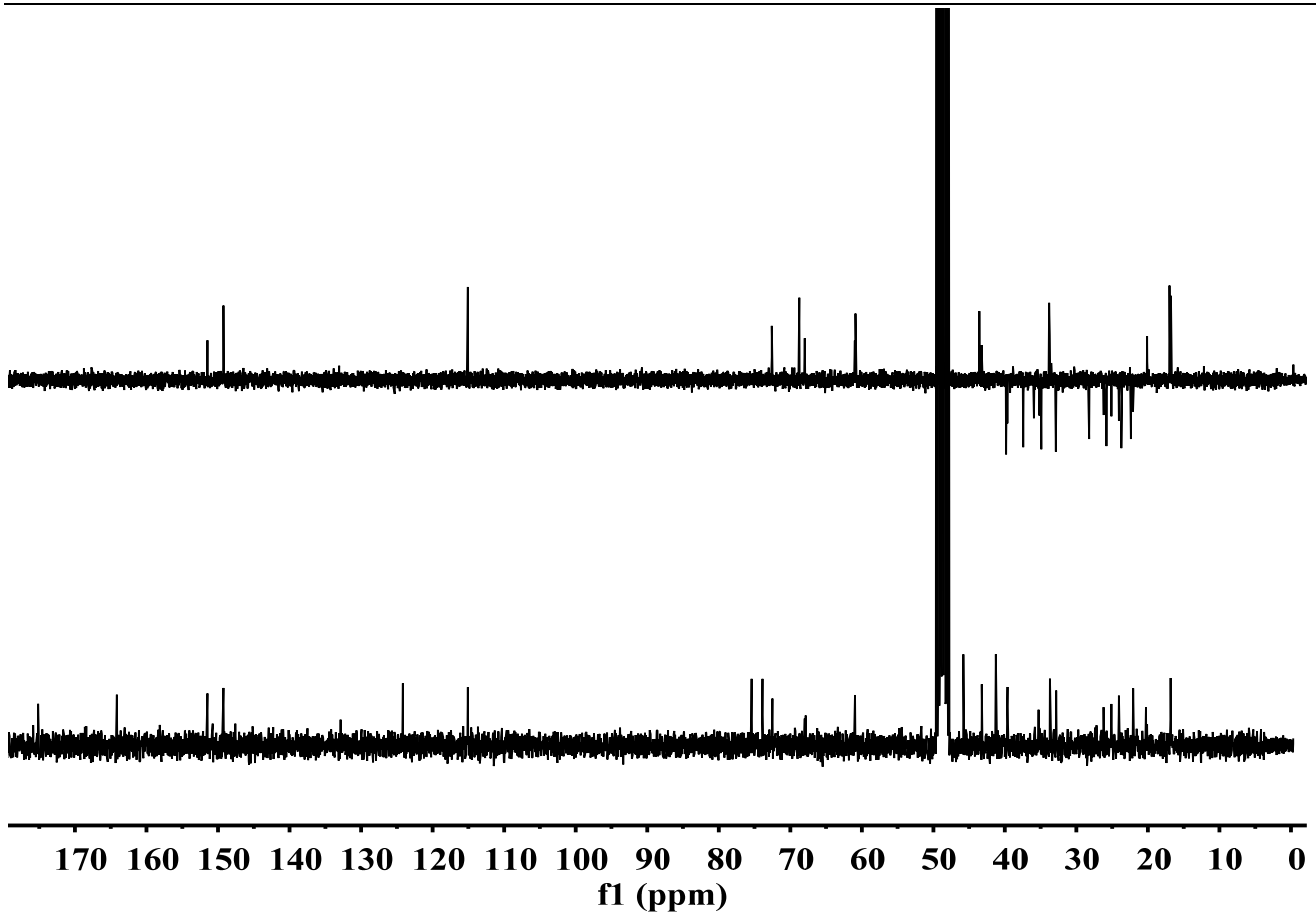

Figure S53. DEPT-135 and ^{13}C NMR spectrum of compound **5** (in CD_3OD, 75 MHz)

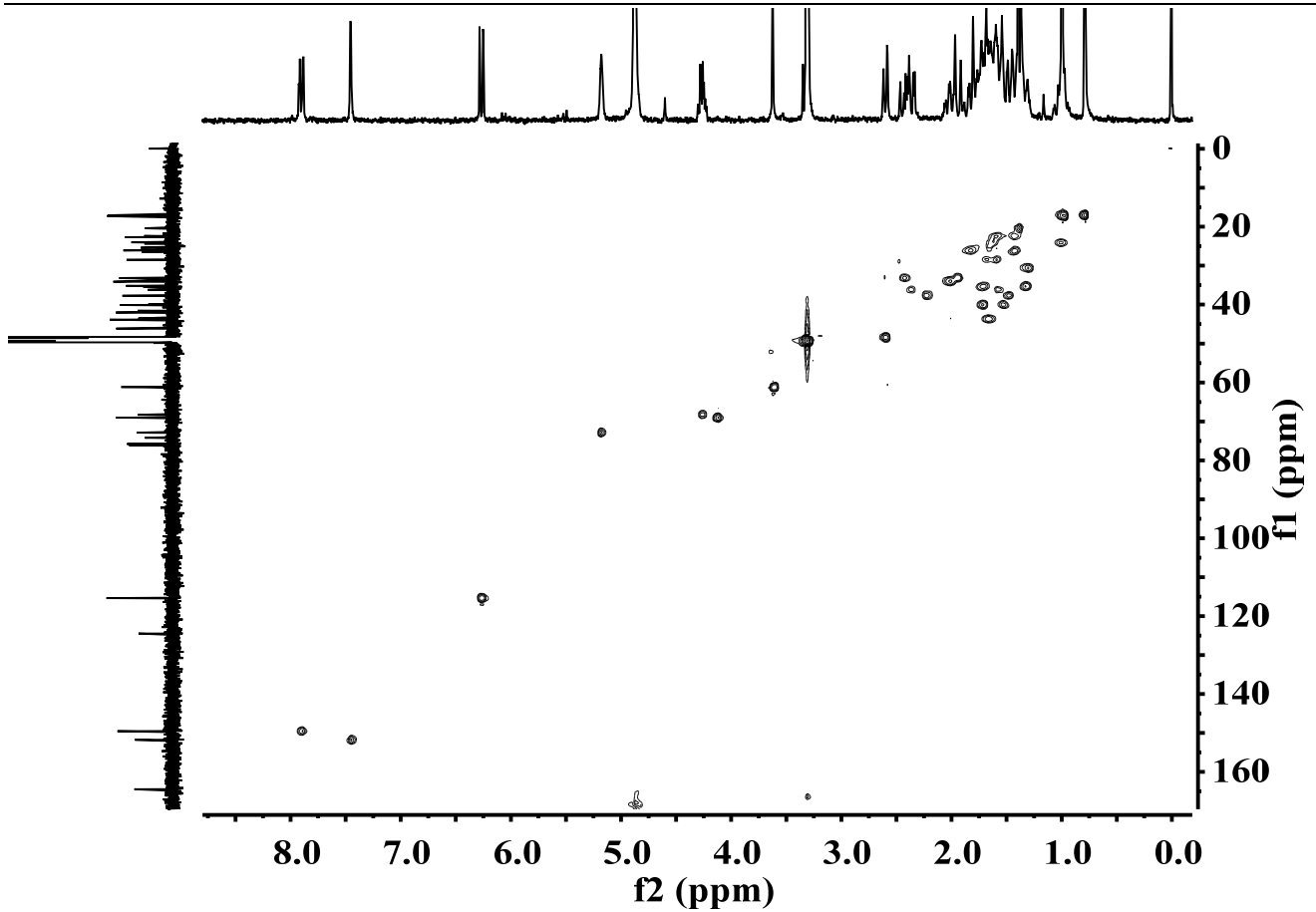

Figure S54. HMQC spectrum of compound **5** (in CD_3OD)

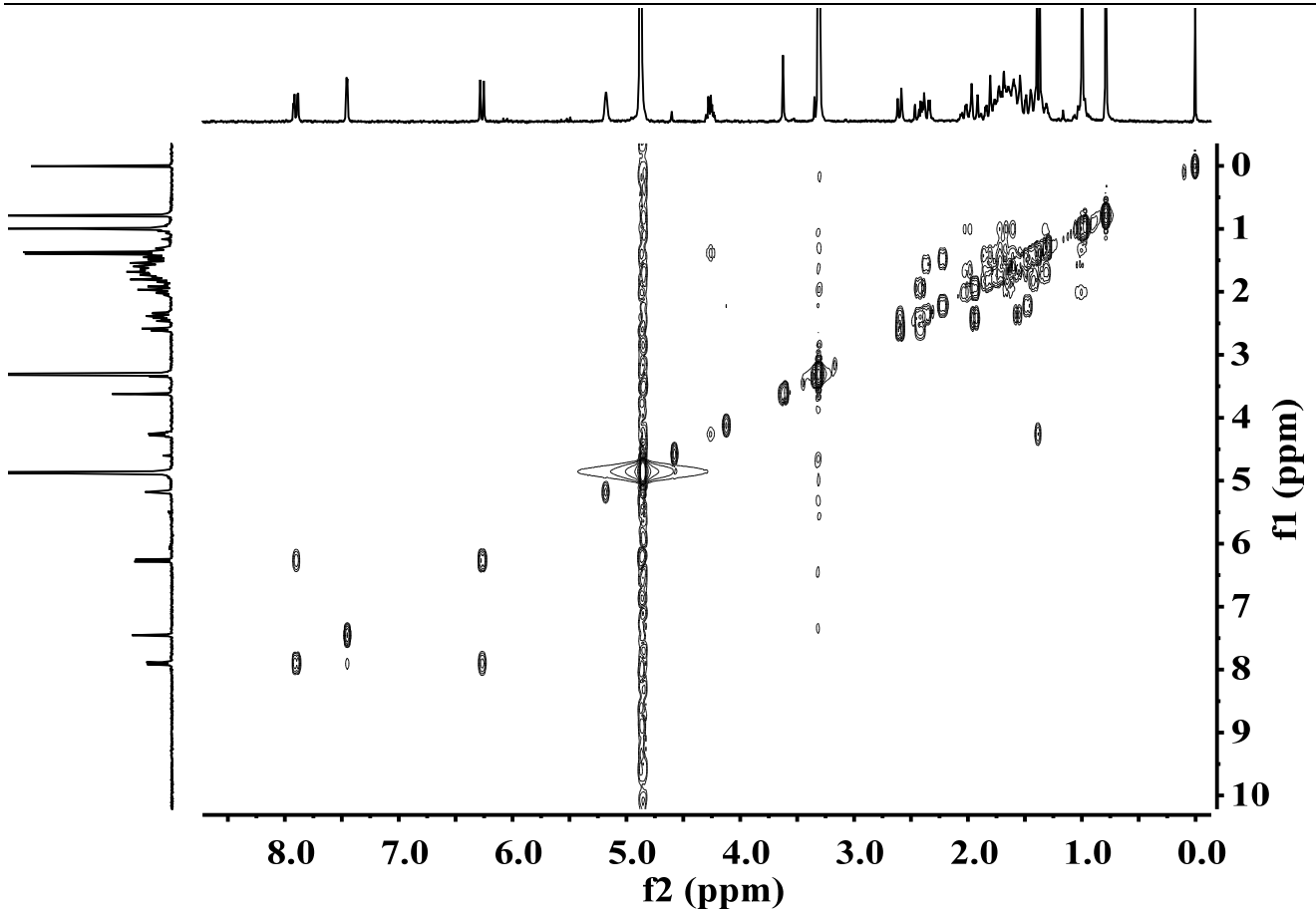

Figure S55. ^{1}H-^{1}H COSY spectrum of compound **5** (in CD_3OD)

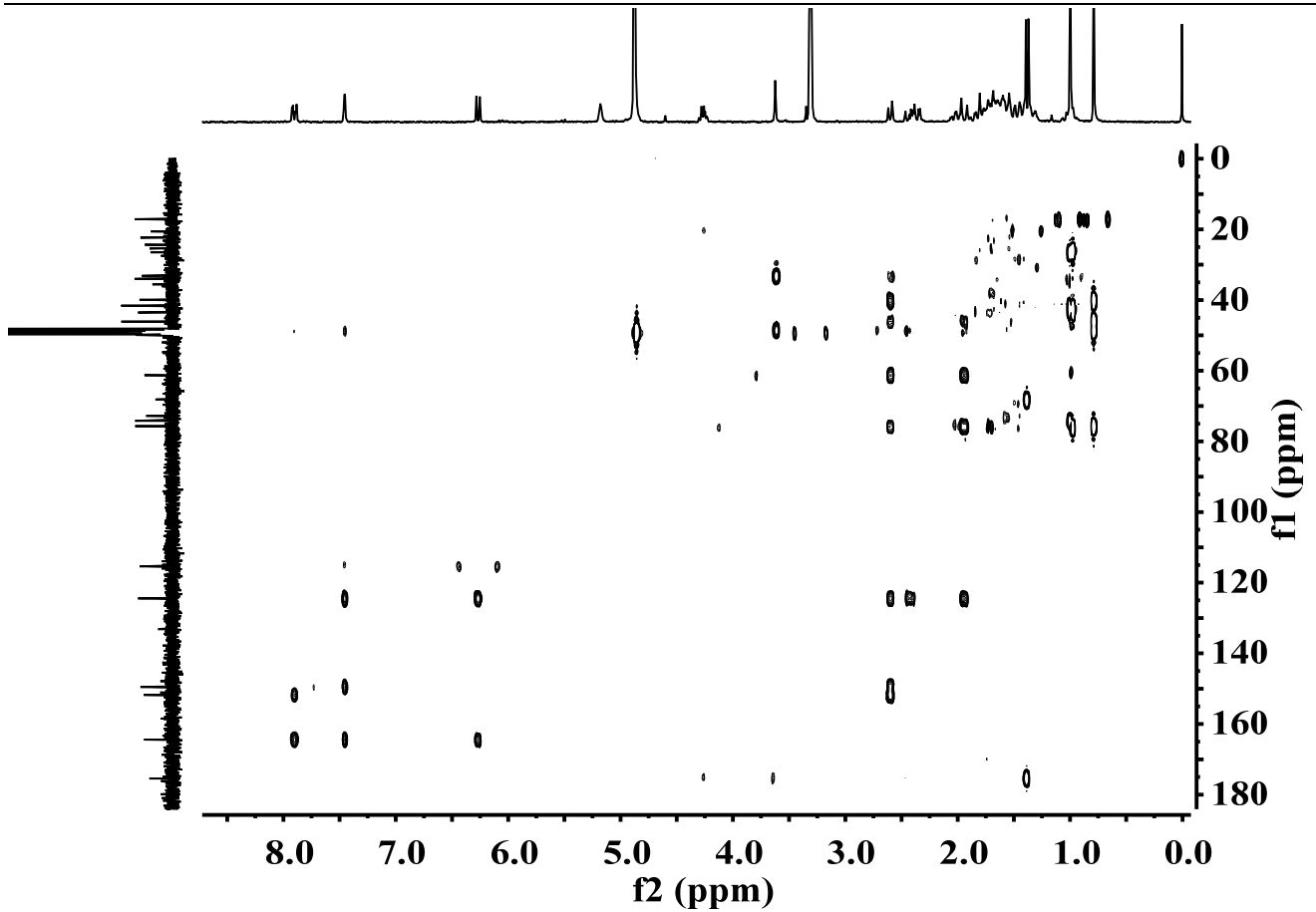

Figure S56. HMBC spectrum of compound **5** (in CD_3OD)

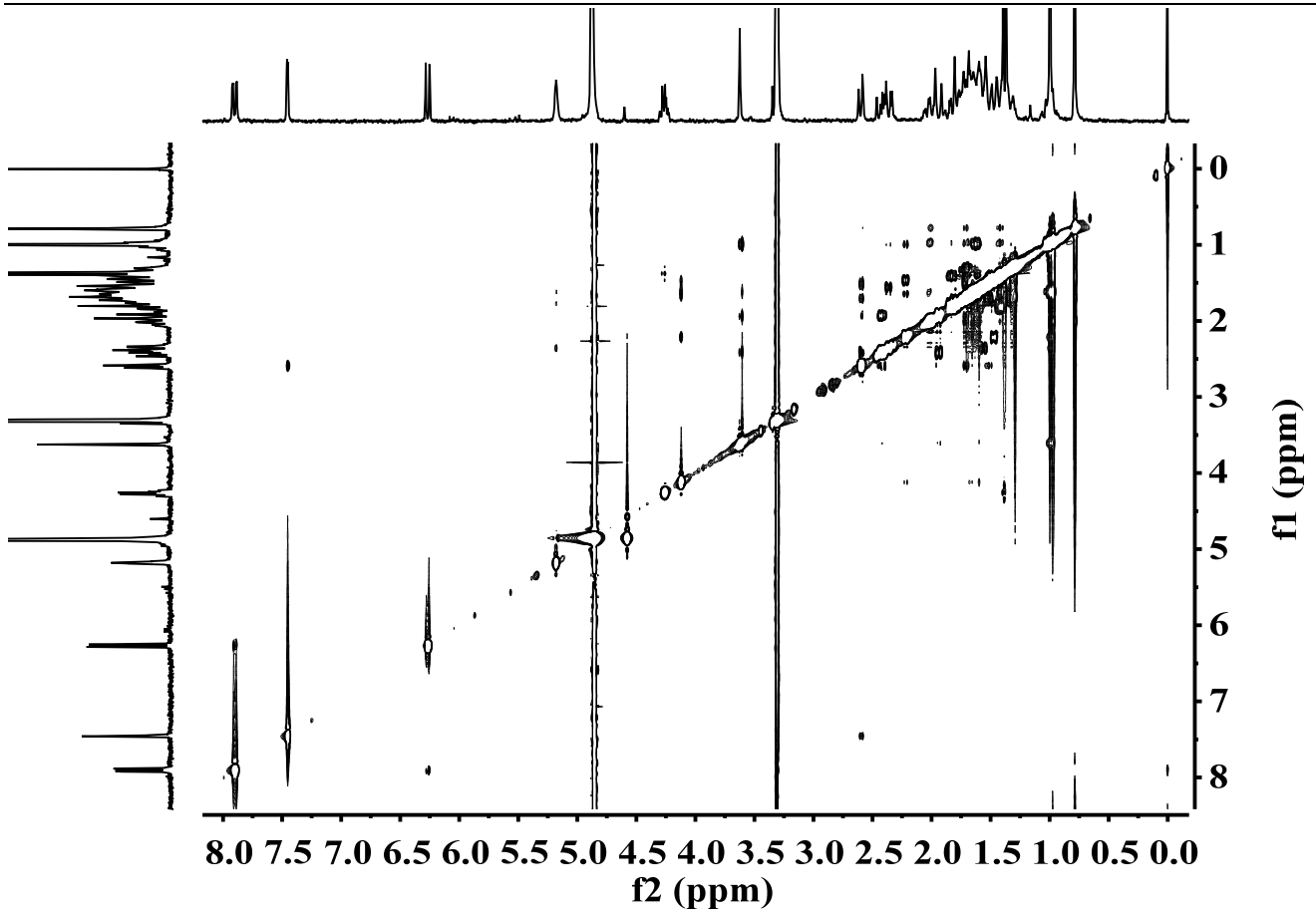

Figure S57. NOESY spectrum of compound **5** (in CD_3OD)

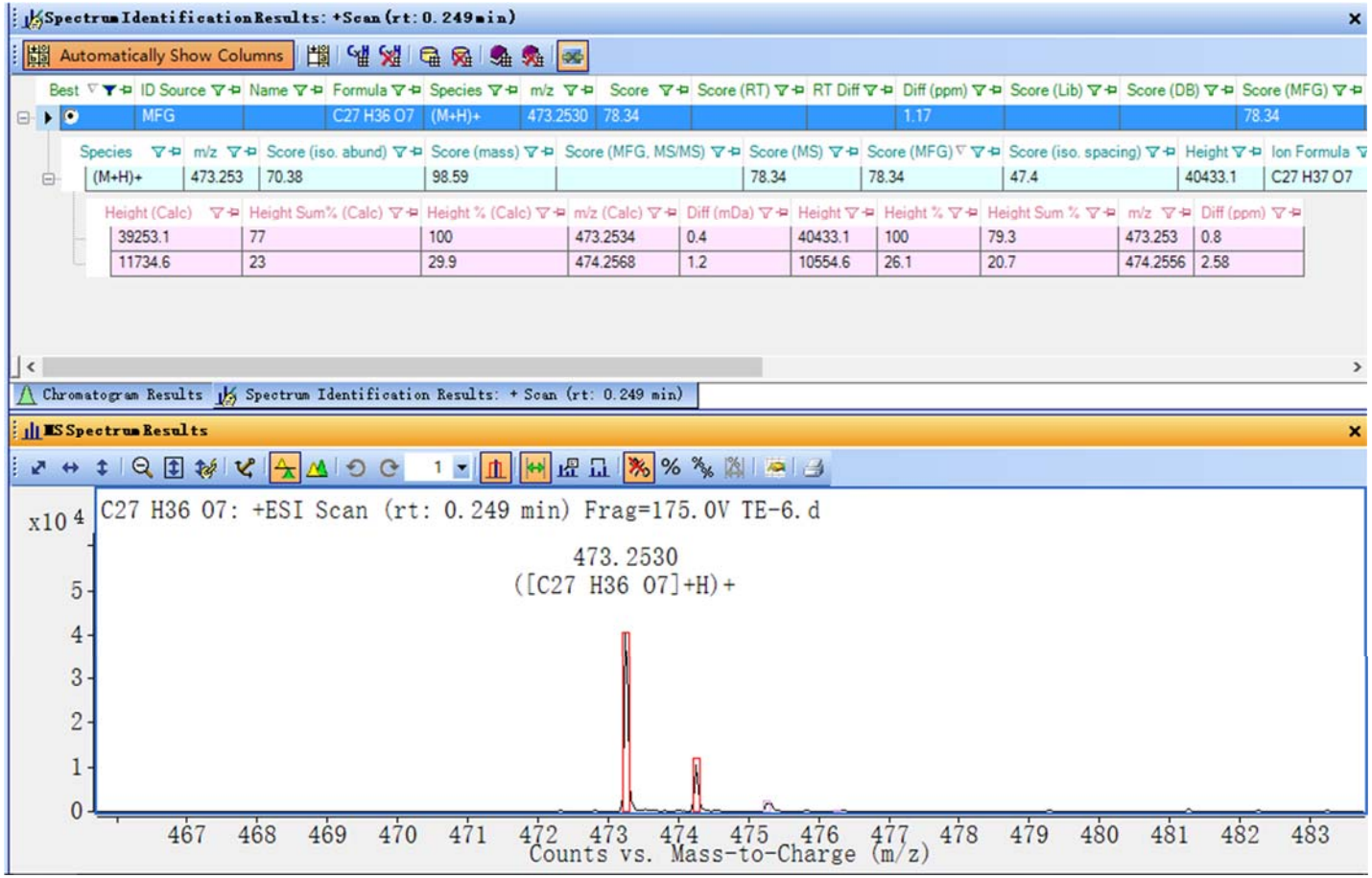

Figure S58. HR-ESI-MS of compound **6**

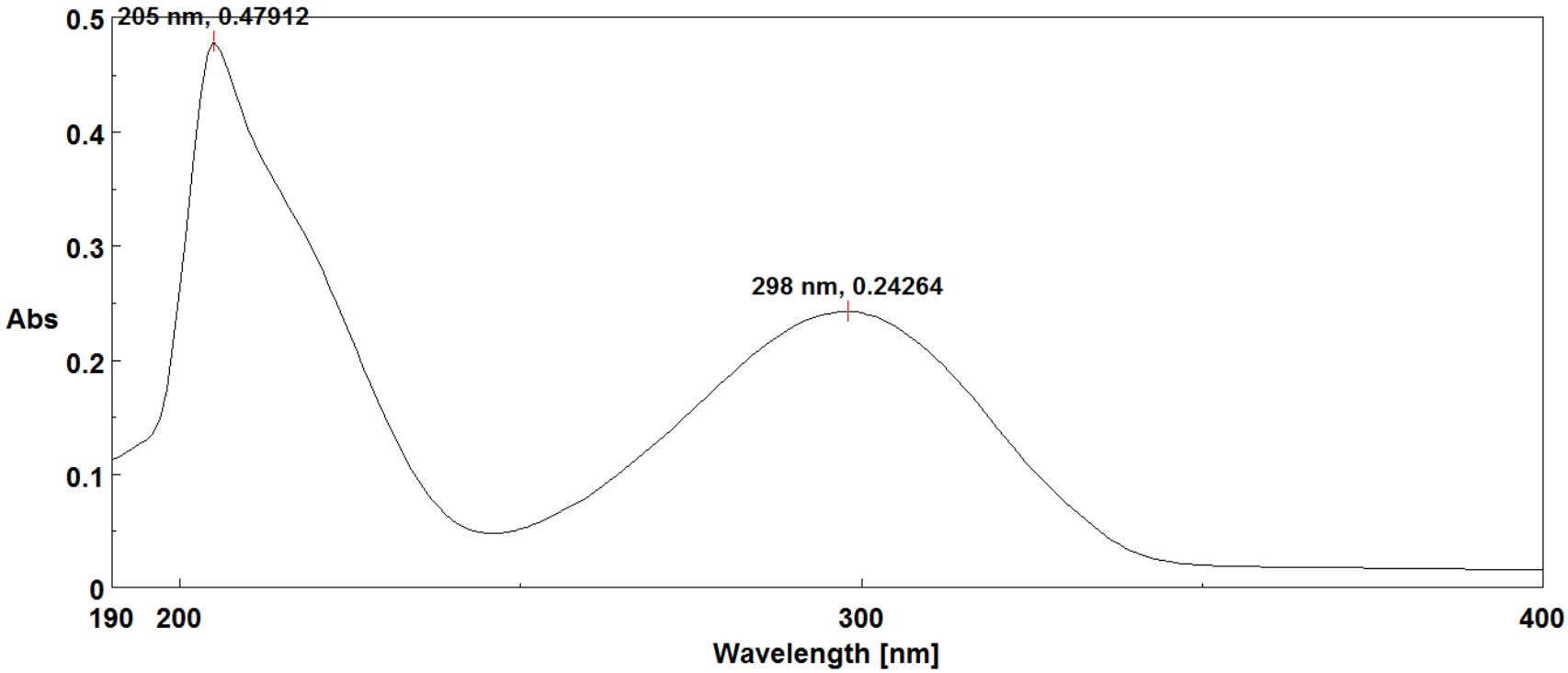

Figure S59. UV spectrum of compound **6**

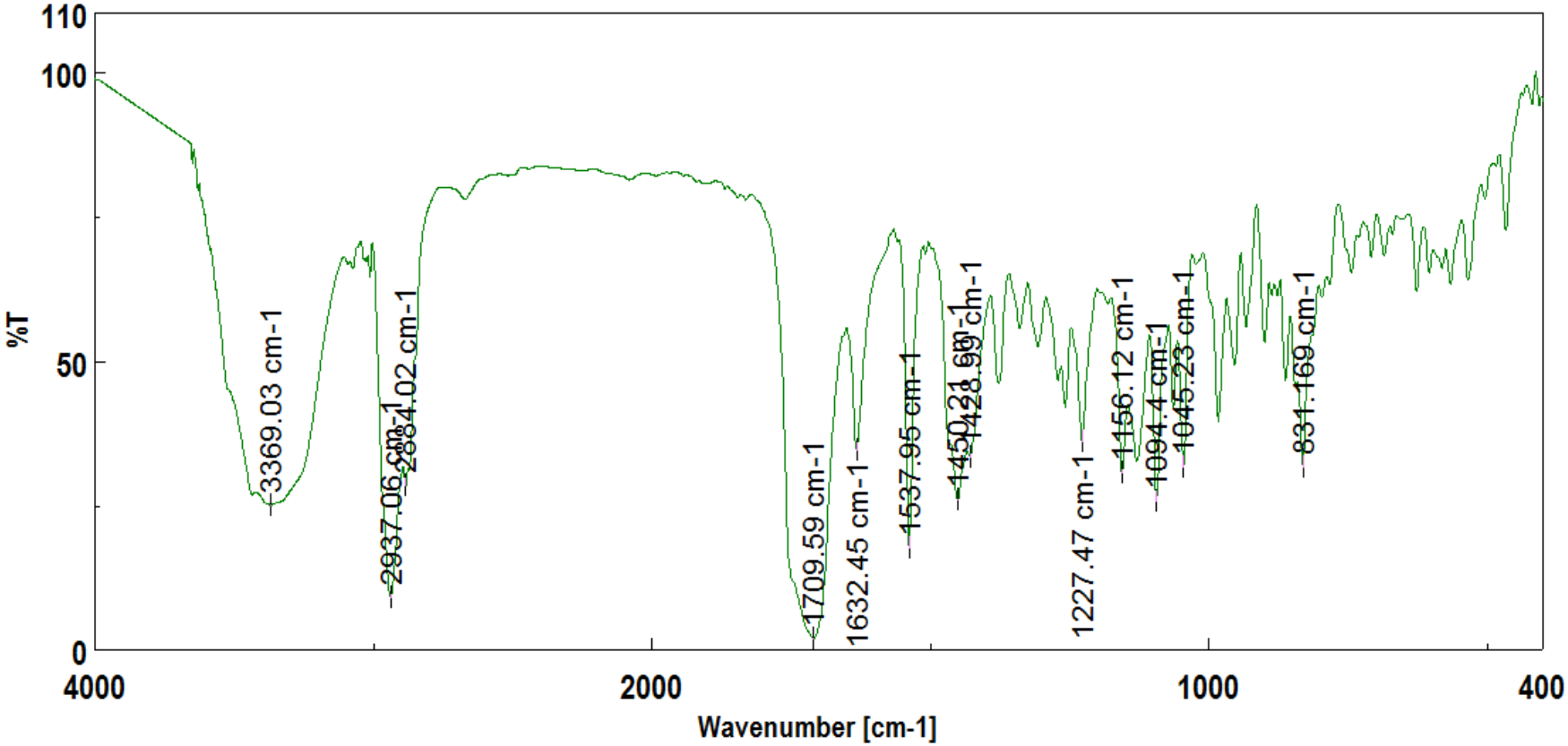

Figure S60. IR spectrum of compound **6** (KBr)

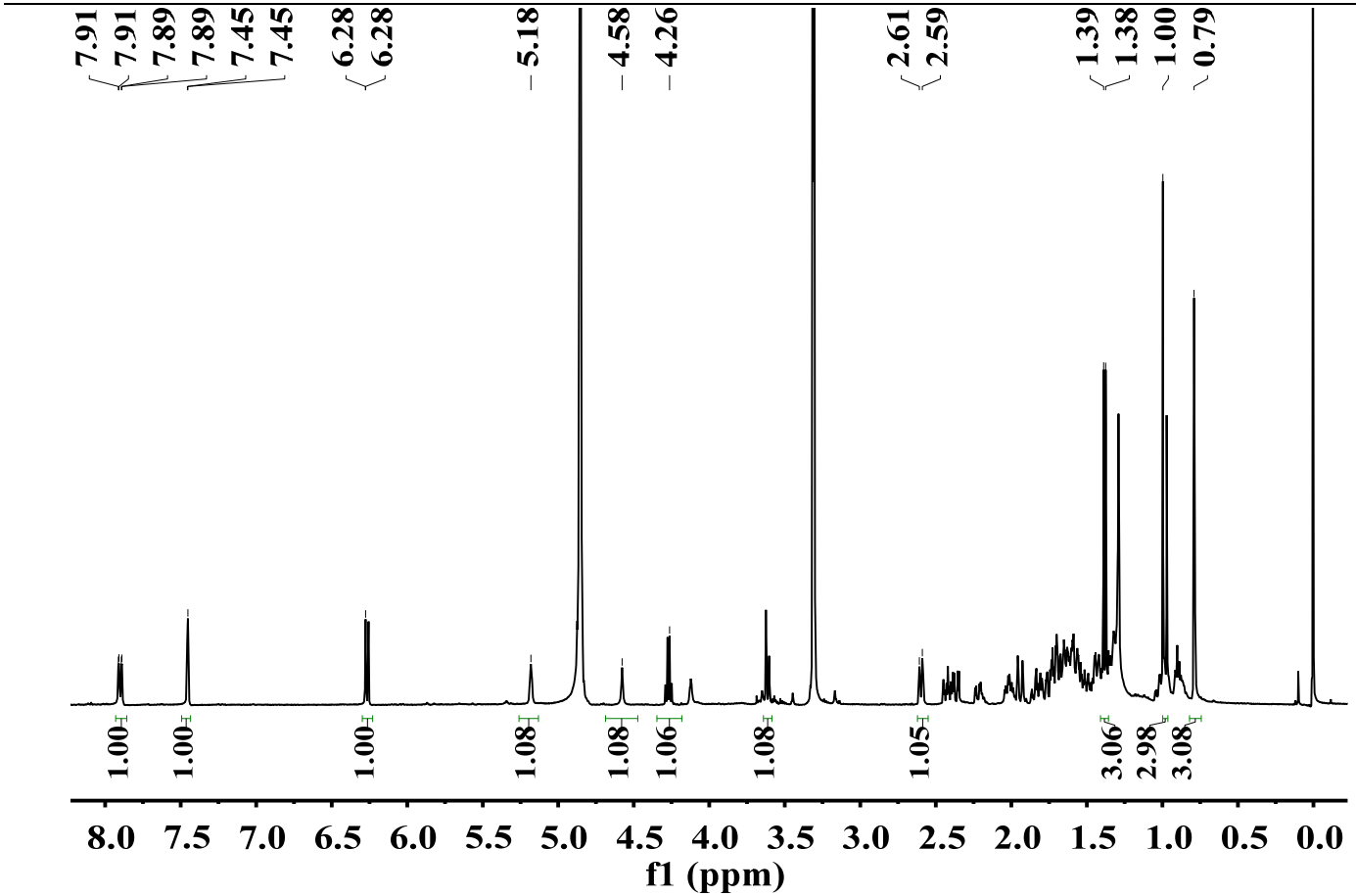

Figure S61. ^{1}H NMR spectrum of compound **6** (in CD_3OD, 300 MHz)

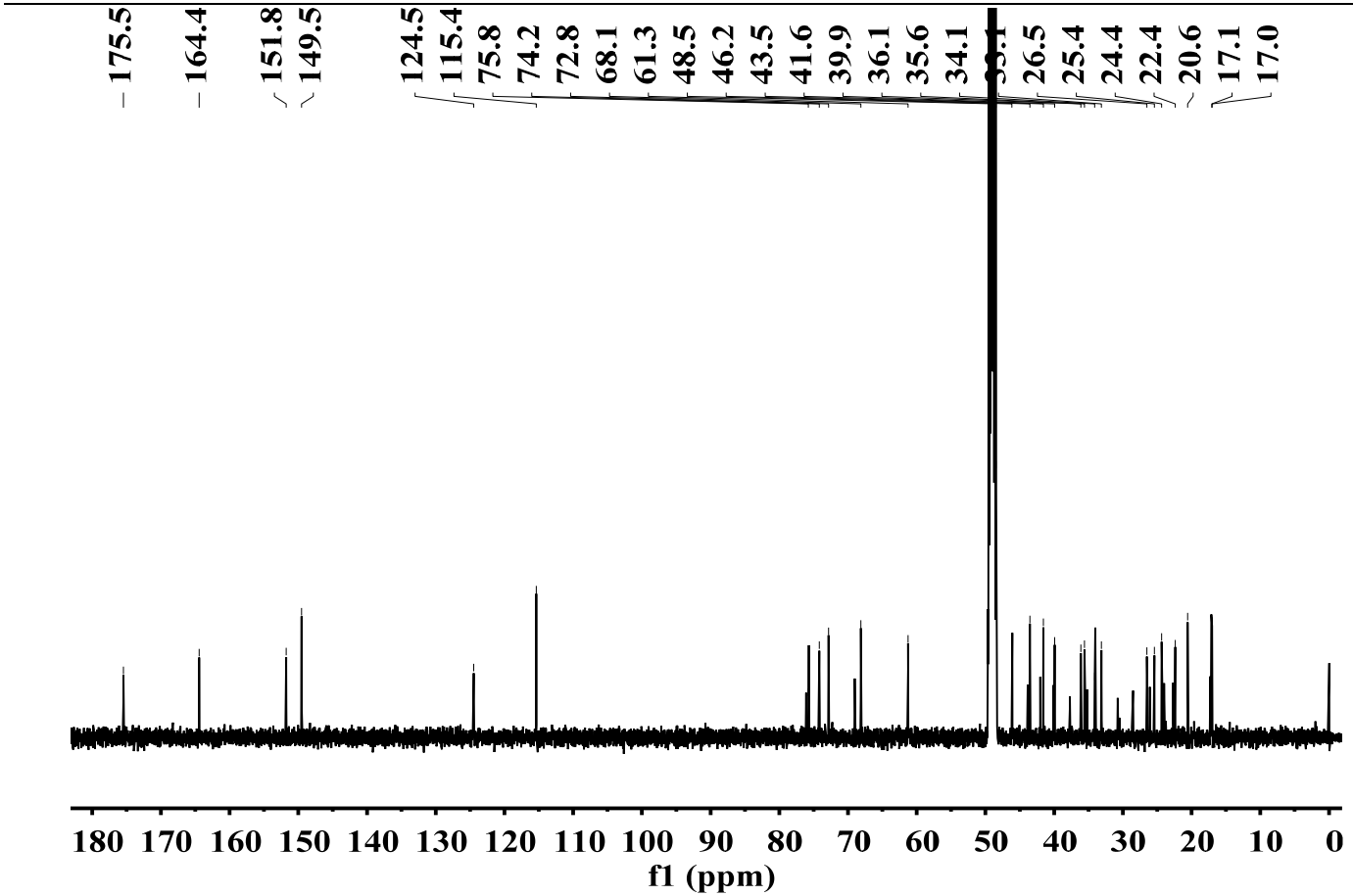

Figure S62. ^{13}C NMR spectrum of compound **6** (in CD_3OD, 75 MHz)

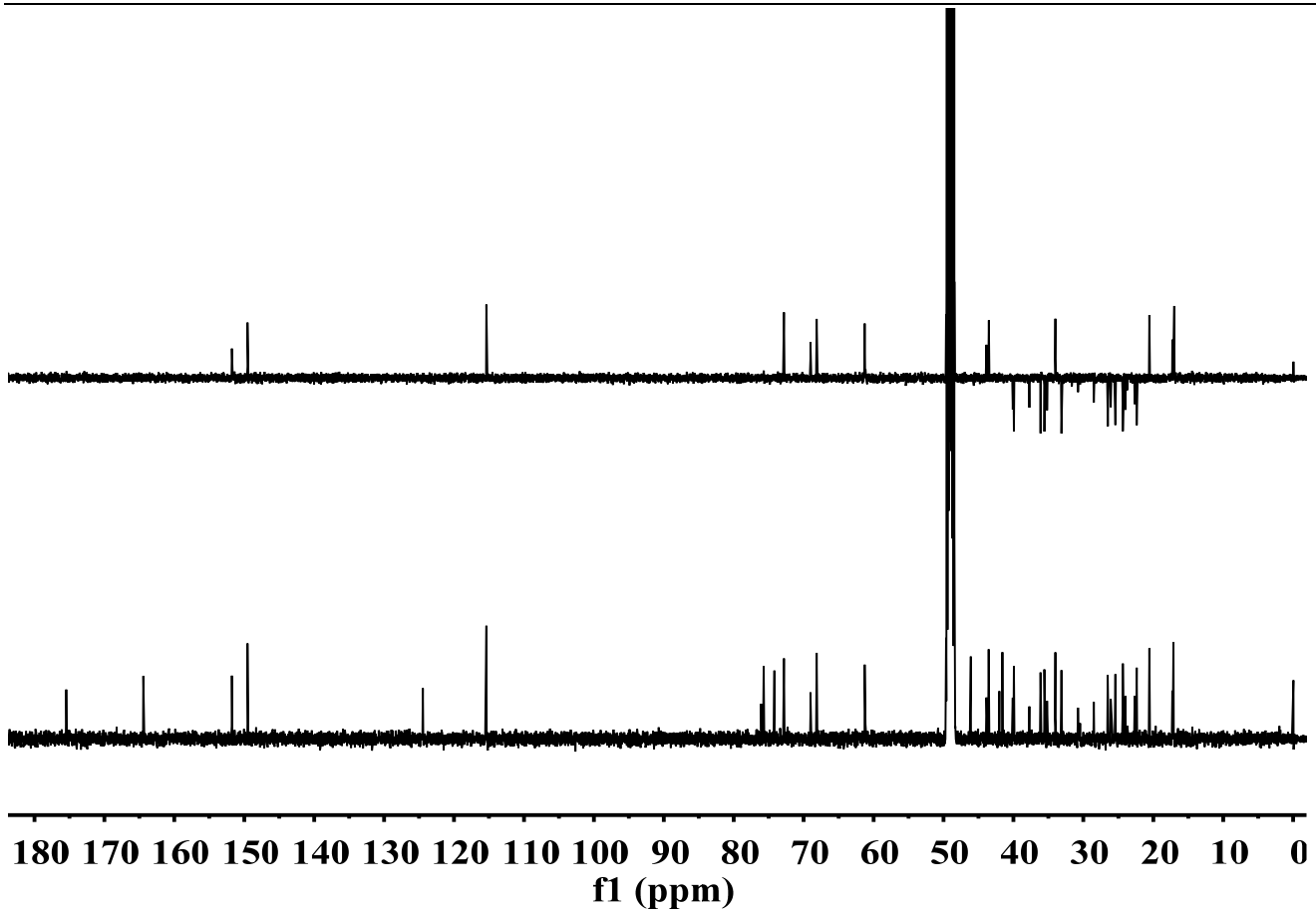

Figure S63. DEPT-135 and ^{13}C NMR spectrum of compound **6** (in CD_3OD, 75 MHz)

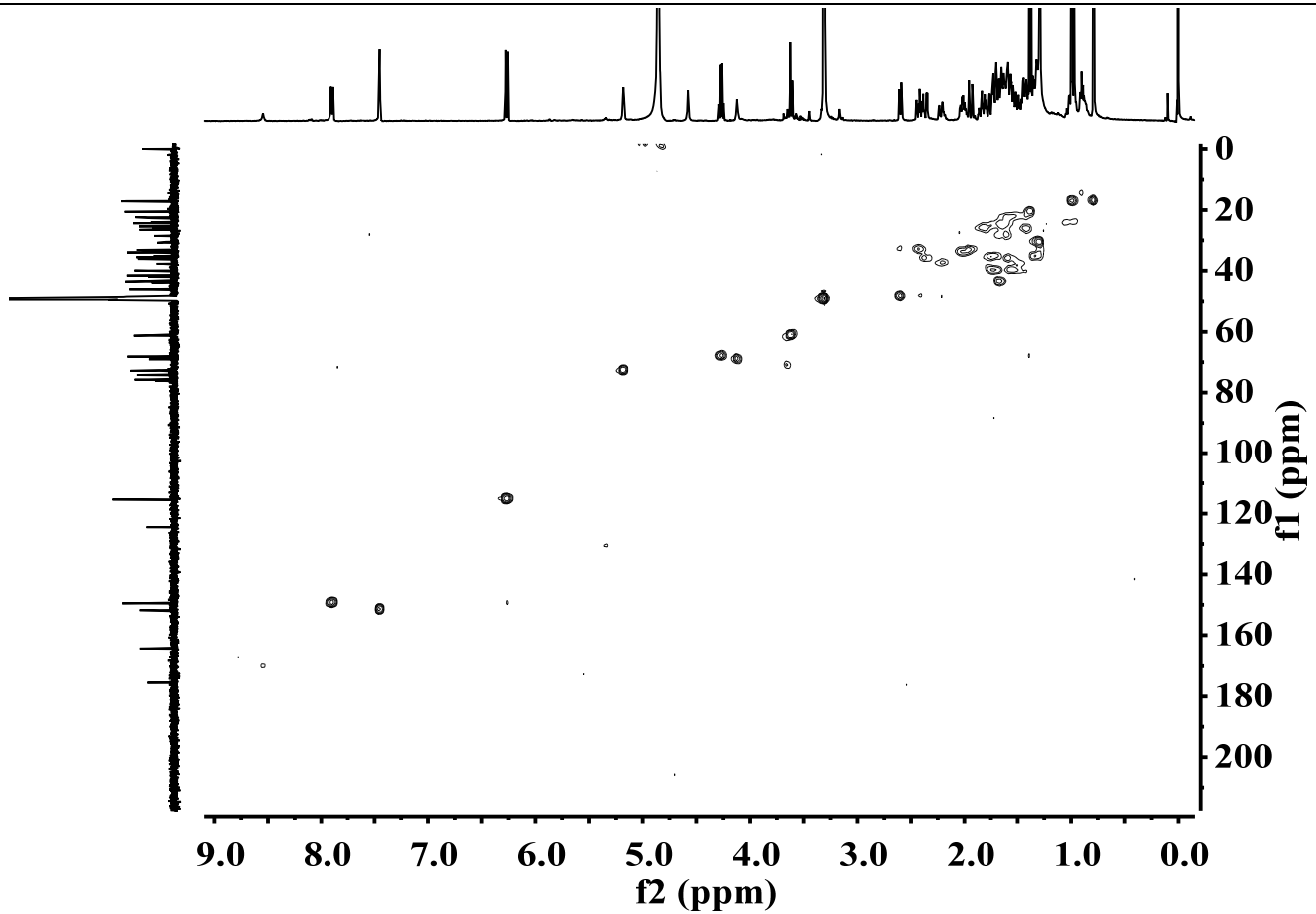

Figure S64. HMQC spectrum of compound **6** (in CD_3OD)

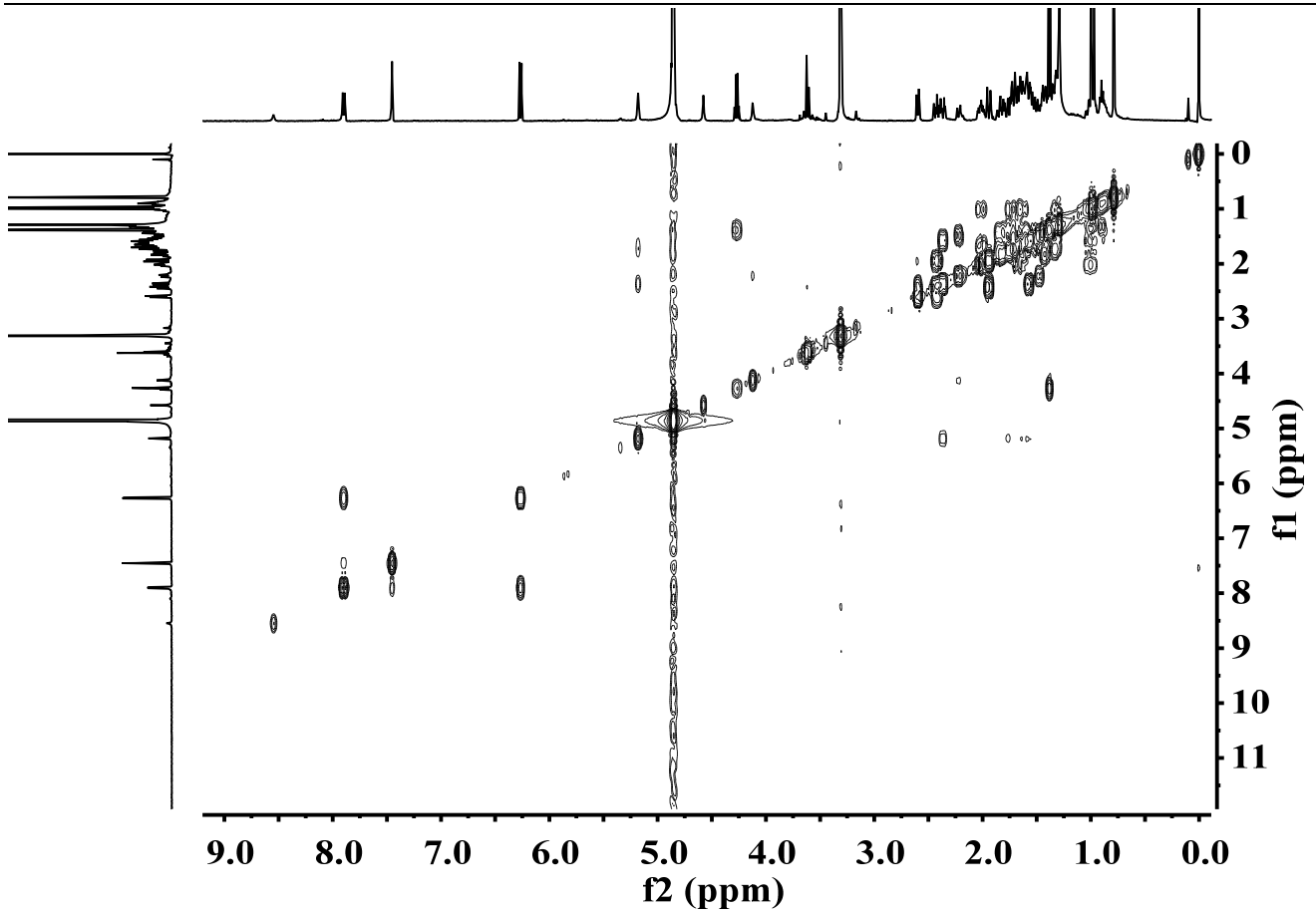

Figure S65. ^{1}H-^{1}H COSY spectrum of compound **6** (in CD_3OD)

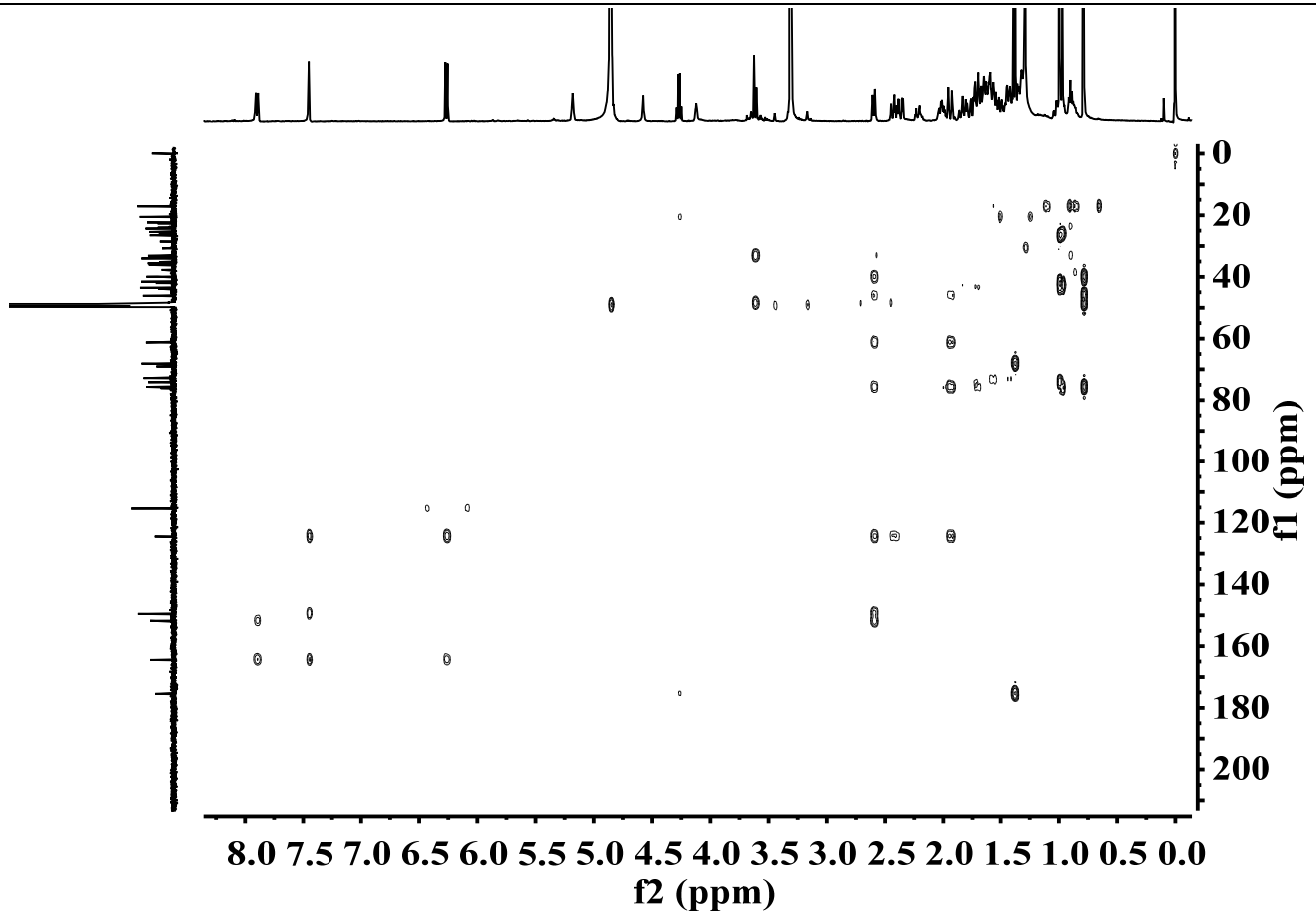

Figure S66. HMBC spectrum of compound **6** (in CD_3OD)

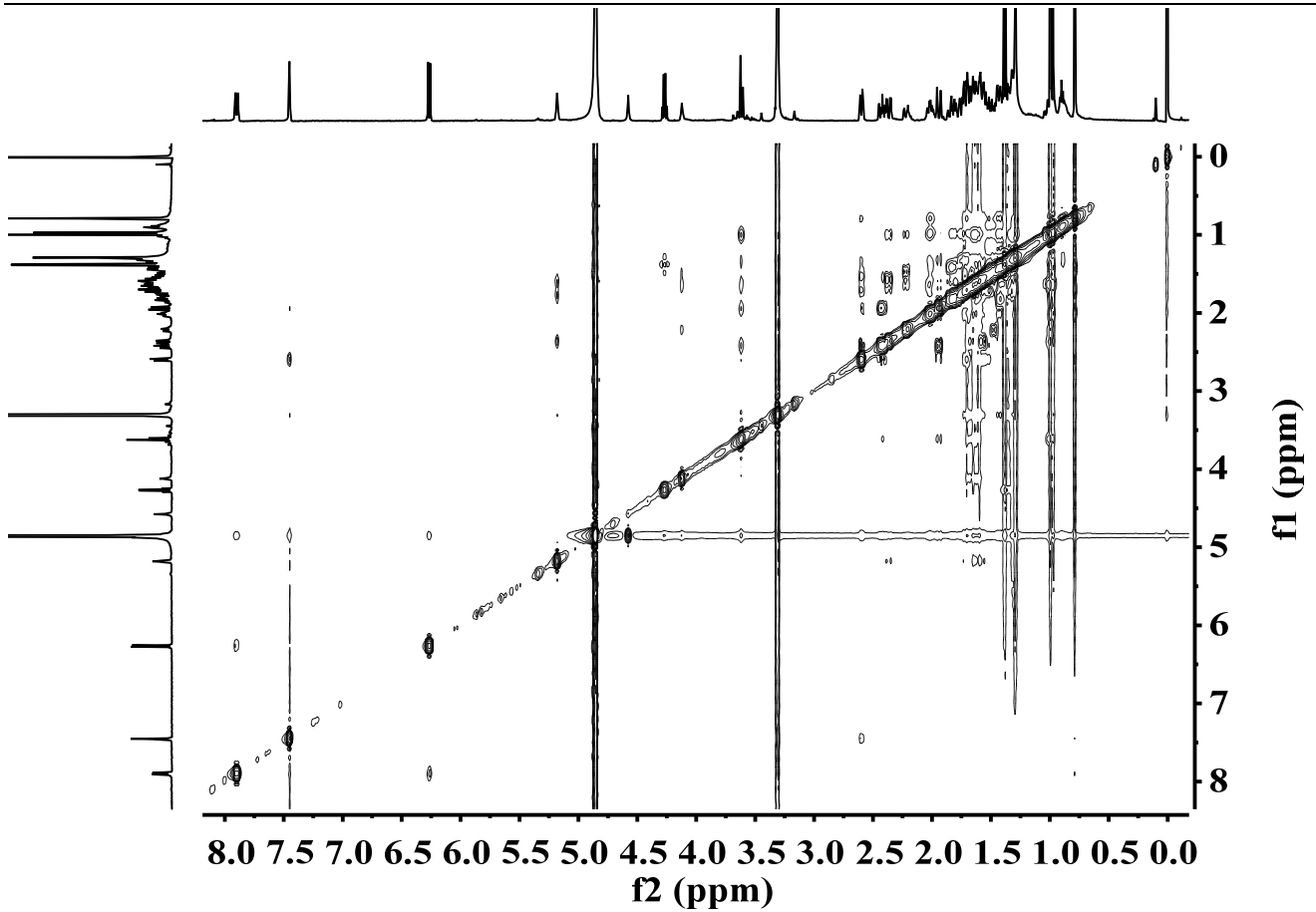

Figure S67. NOESY spectrum of compound **6** (in CD_3OD)

7. The identification of compounds 1-6 in crude extract of toad eggs.

The analyses were performed on Agilent 6210 ESI/TOF mass spectrometer using. Kinetex Cellulose-4 column (10 μm, 10×250 mm, Phenomenex, USA). A gradient program with mobile phase consisting of methanol-water (75-90%, 60 min) containing 0.01% (v/v) formic acid was used The chromatographic peaks with the same molecular weight of compounds **1-6** (peak 1-6) were present on the extracted ion chromatograph (EIC) of the total ethyl acetate extract of toad eggs (**Fig.S68**).

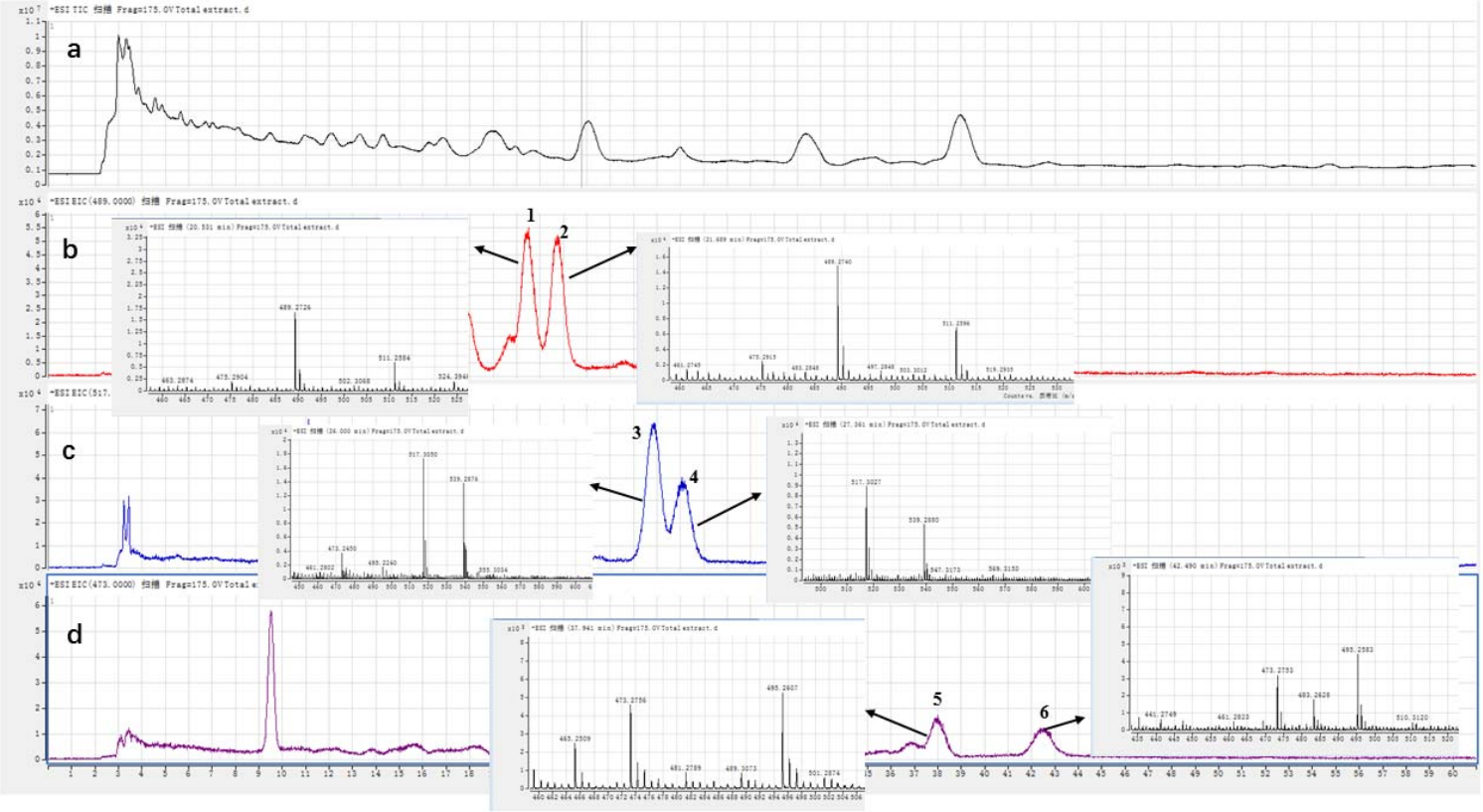

Fig. S68 a): the total ion chromatograph of ethyl acetate extract of toad eggs; **b)**: the extract ion chromatograph for *m/z* 489.0±0.5. Peak 1: retention time: 20.531 min, *m/z* 489.27 [M+H], 511.26 [M+Na]. Peak 2: retention time: 21.689 min, *m/z* 489.27 [M+H], 511.26 [M+Na]; **c)**: the extract ion chromatograph for *m/z* 517.0±0.5, Peak 3: retention time: 26.000 min, *m/z* 517.30 [M+H], 539.29 [M+Na]. Peak 4: retention time: 27.361 min, *m/z* 517.30 [M+H], 539.29 [M+Na]; **d)**: the extract ion chromatograph for *m/z* 517.0±0.5, Peak 5: retention time: 37.941 min, *m/z* 473.28 [M+H], 495.26 [M+Na]. Peak 6: retention time: 42.490 min, *m/z* 473.28 [M+H], 495.26 [M+Na].