SUPPLEMENTARY MATERIAL

Anatoluin A and B isolated from medicinal *Tricholoma anatolicum* are new cytotoxic ergostanoids against the most common cancers

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Abstract

Tricholoma anatolicum is an edible mushroom from matsutake group growing under Cedar trees. Bioactivity-guided fractionation of *Tricholoma anatolicum* was afforded two new (**1** and **2**), three known ergosterols (**3**-**6**), and four known (**6**-**9**) compounds. Structures were identified as anatoluin A (**1**), anatoluin B (**2**), 5*α*,6*α*-epoxy-ergosta-7,22-dien,3*β*-ol (**3**), ergosterol-endoperoxide (**4**), ergosterol,3*β*-ol (**5**), 3,5-dihydroxyfuran-2(5*H*)-one (**6**), mannitol (**7**), turanose (**8**), fumaric acid (**9**) using spectroscopic techniques. The cytotoxic activity of extract and isolated compounds was performed using MTT assay against MCF7, HT29, H1299, and HeLa cancerous cell lines while toxicity against PDF and L929 fibroblast healthy cell lines. The lipid peroxidation inhibitory and $ABTS^{\bullet+}$ scavenging activities were used to determine antioxidant activity. The polar extracts exhibited significant cytotoxic activity. The more perfect is that the extracts and isolated compounds (**1**-**5**) were inactive against PDF and L929 healthy cell lines. Compounds **1**-**3** and **4** exhibited noticeable cytotoxic activity, while **1**-**5** moderately inhibited lipid peroxidation.

Keywords: *Tricholoma anatolicum*; Isolation and elucidation; Cytotoxic activity; Cancer cell lines; Fibroblast cell lines; Lanostane steroids (ergosterols)

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Table S1. The Cytotoxic and antioxidant activities of compounds (**1**-**5**) and the extracts of *Tricholoma anatolicum*.[a]

	Antioxidant activity		**Cytotoxic activity**				**Toxicity**	
	Lipid peroxidation inhibitory assay	**ABTS assay**	**Against MCF7**	**Against HT29**	**Against H1299**	**Against HeLa**	**Against PDF**	**Against L929**
extracts & compounds	IC_{50} (µg/mL)	IC_{50} (µg/mL)	LC_{50} (µg/mL)	LC_{50} (µg/mL)	LC_{50} (µg/mL)	LC_{50} (µg/mL)	LC_{50} (µg/mL)	LC_{50} (µg/mL)
Petroleum ether	330 ± 3.3	>500	48.2 ± 0.9	69.5 ± 1.2	62.2 ± 1.4	>100	76.8 ± 0.6	62.8 ± 0.6
Acetone	68.1 ± 1.2	460 ± 2.2	22.9 ± 0.2	31.6 ± 0.2	37.2 ± 0.8	11.8± 0.1	105 ± 0.2	90.5 ± 0.2
Methanol	82.5 ± 1.8	98.6 ± 1.9	37.5 ± 0.8	41.2 ± 0.9	19.3 ± 0.1	>100	95.1 ± 0.8	79.5 ± 0.8
1	62.1 ± 1.1	>100	20.5 ± 1.5	28.3 ± 1.5	34.3 ± 1.1	14.3 ± 1.7	>100	93.2 ± 2.5
2	71.9 ± 1.2	>100	21.1 ± 1.9	34.5 ± 1.7	34.6 ± 1.1	22.2 ± 1.3	>100	88.5 ± 2.3
3	72.6 ± 1.3	>100	31.5 ± 2.0	25.1 ± 1.3	35.1 ± 1.0	*NT*	>100	84.7 ± 2.2
4	96.6 ± 1.9	>100	>100	27.8 ± 1.1	*NT*	*NT*	52.8 ± 0.5	65.6 ± 2.2
5	75.4 ± 1.4	>100	38.8 ± 2.1	*NA*	39.4 ± 1.0	*NT*	>100	85.4 ± 1.7
Doxorubicin	*NT*	*NT*	10.5 ± 0.06	*NT*	9.11 ± 1.8	5.83 ± 0.2	*NT*	*NT*

[a] Values expressed herein are mean ± SEM of three parallel measurements. $p<0.05$. IC_{50}: Median inhibition concentration that scavenges the radicals in medium; LC_{50}: Median lethal concentration that kills 50% of the cancerous or healthy cells.

NT: not tested. *NA*: not active. H1299: human non-small cell lung carcinoma cell line; MCF-7: breast cancer cell line; HT-29: human colon adenocarcinoma cell line; HeLa: cervical cancer cell line; PDF: dermal fibroblast cell line; L929: mouse fibroblast

Table S2. ^{1}H-NMR and ^{13}C-NMR spectral data of compounds **1** and **2**[a].

Position	**1** (6α-OH)		**2** (6β-OH)		**L1** (6α-OH)	**L2** (6β-OH)
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_C
1	26.8	2.10; 1.28 (2H, *m*)	27.8	2.22; 1.39 (2H, *t*, *J*=5.4 Hz)	28.9	28.7
2	30.3	1.83; 1.43 (2H, *m*)	31.3	1.85;1.47 (2H, *m*)	30.8	31.7
3	67.0	3.95 (1H, *m*)	67.9	3.90 (1H, *m*)	66.6	66.8
4	39.4	1.54; 2.02 (2H, *m*)	40.6	2.12; 1.46 (2H, *m*)	34.1	34.8
5	77.5	-	78.6	-	88.3	86.6
6	72.8	3.59 (1H, *d*, *J*=5.4 Hz)	71.2	3.87 (1H, br*s*)	73.0	72.2
7	119.3	5.30 (1H, *d*, *J*=5.3 Hz)	122.0	5.04 (1H, br*m*)	123.2	122.5
8	143.1	-	143.2	-	141.6	141.8
9	74.6	-	75.8	-	84.3	84.7
10	40.1	-	42.2	-	54.2	51.0
11	28.3	1.52 (2H, *m*)	28.2	1.81; 1.56 (2H, *m*)	22.8	23.1
12	35.0	1.76; 1.53 (2H, *m*)	36.5	1.81; 1.59 (2H, *m*)	36.6	36.6
13	43.6	-	44.8	-	42.1	41.9
14	50.6	2.41 (1H, *m*)	51.6	2.50 (1H, *m*)	52.1	52.1
15	22.8	1.49;1.20 (2H, *m*)	23.8	1.43; 1.53 (2H, *m*)	23.1	23.2
16	27.9	1.80;1.68 (2H, *m*)	29.4	1.78; 1.33 (2H, *m*)	28.2	28.1
17	55.8	1.29 (1H, *m*)	57.2	1.34 (1H, *m*)	55.5	55.5
18	11.6	0.54 (3H, *s*)	12.1	0.61 (3H, *s*)	11.6	11.7
19	21.6	1.04 (3H, *s*)	20.8	1.04 (3H, *s*)	16.2	17.3
20	40.4	1.95 (1H, *m*)	41.9	2.03 (1H, *q*, *J*=7.3 Hz)	40.3	40.4
21	21.0	0.95 (3H, *d*, *J*= 6.6 Hz)	21.6	1.03 (3H, *d*, *J*=6.6 Hz)	21.1	21.1
22	135.4	5.11 (1H, *dd*, *J*= 8.2; 15.3 Hz)	137.0	5.18 (1H, *dd*, *J*=7.8; 15.1 Hz)	135.2	135.2
23	133.0	5.14 (1H, *dd*, *J*= 7.4; 15.3 Hz)	133.3	5.24 (1H, *dd*, *J*=7.3; 15.1 Hz)	132.4	132.3
24	42.7	1.79 (1H, *m*)	44.4	1.84 (1H, *m*)	42.8	42.8
25	33.0	1.40 (1H, *m*)	34.4	1.46 (1H, *m*)	33.1	33.1
26	19.8	0.77 (3H, *d*, *J*=6.8 Hz)	20.5	0.86 (3H, *d*, *J* =6.8 Hz)	19.6	19.7
27	19.5	0.75 (3H, *d*, *J*=7.0 Hz)	20.1	0.84 (3H, *d*, *J* =6.8 Hz)	20.0	20.0
28	17.5	0.85 (3H, *d*, *J*=6.8 Hz)	18.2	0.93 (3H, *d*, *J* =6.9 Hz)	17.6	17.6

[a] Compound anatoluin A (**1**) was run in $CDCl_3$ + CD_3OD using 600 MHz NMR while anatoluin B (**2**) in CD_3OD using 500 MHz NMR. *L1*: 5α,9α-epidioxy-(22*E*)-ergosta-7,22-diene-3β, 6α-diol (**L1**), *L2*: 5α,9α-epidioxy-(22*E*)-ergosta-7,22-diene-3β, 6β-diol (**L2**) which were run in $CDCl_3$ at 100 Mhz (Yaoita et al., 2001).

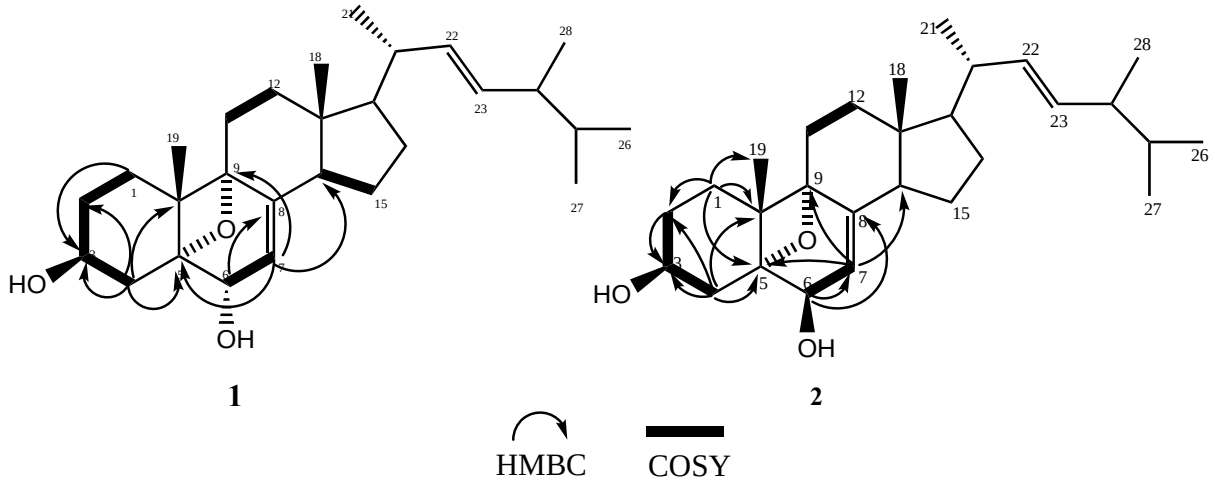


Figure S1. The important key HMBC and COSY correlations of **1** and **2**

JEOL HX 110 MASS SPECTROMETER (EI-HR)

STUDENT NAME	Erhan	SAMPLE CODE	DATED
SUPERVISOR NAME	Dr Mehmet	TAI	20/1/17

Mass	Theoretical Mass	Delta [ppm]	Delta [mmu]	RDB	Composition
428.3299	428.3290	2.0	0.9	7.0	$C_{29}H_{44}O_3$
410.3177	410.3185	-1.9	-0.8	8.0	$C_{29}H_{42}O_2$

Figure S2. HRMS of compound **1**

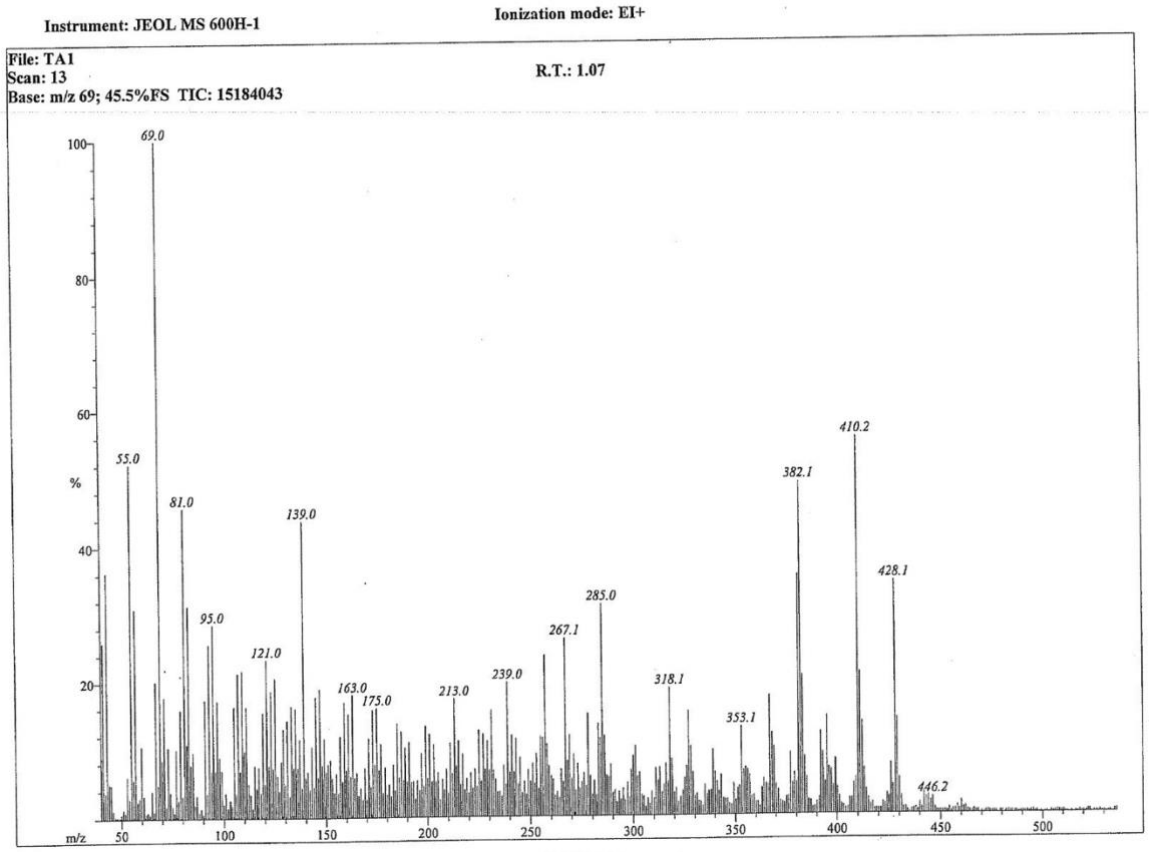


Figure S3. EI-MS of compound **1**

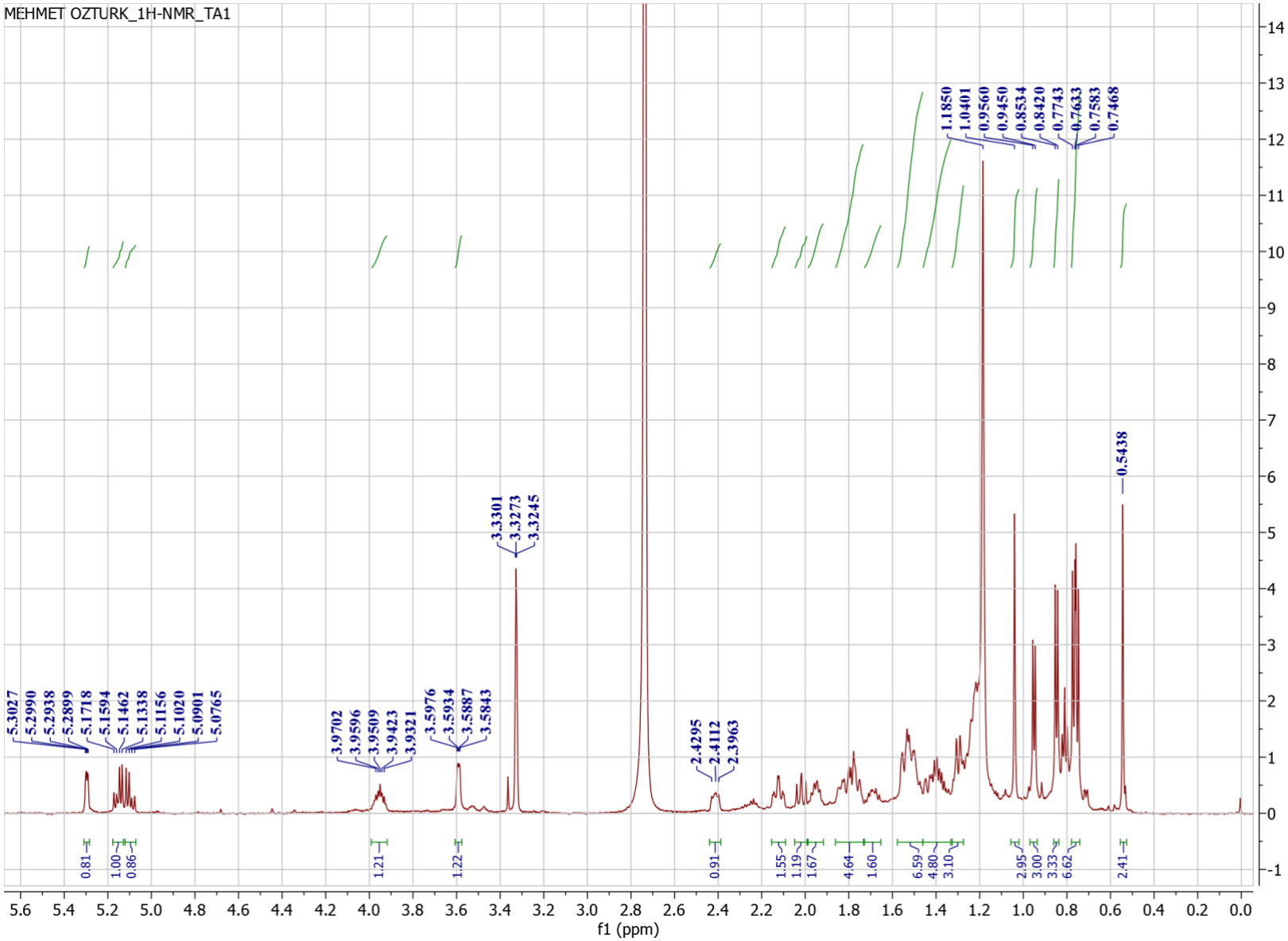


Figure S4. ^{1}H-NMR (600 MHz, $CDCl_3$ + CD_3OD) of compound **1**

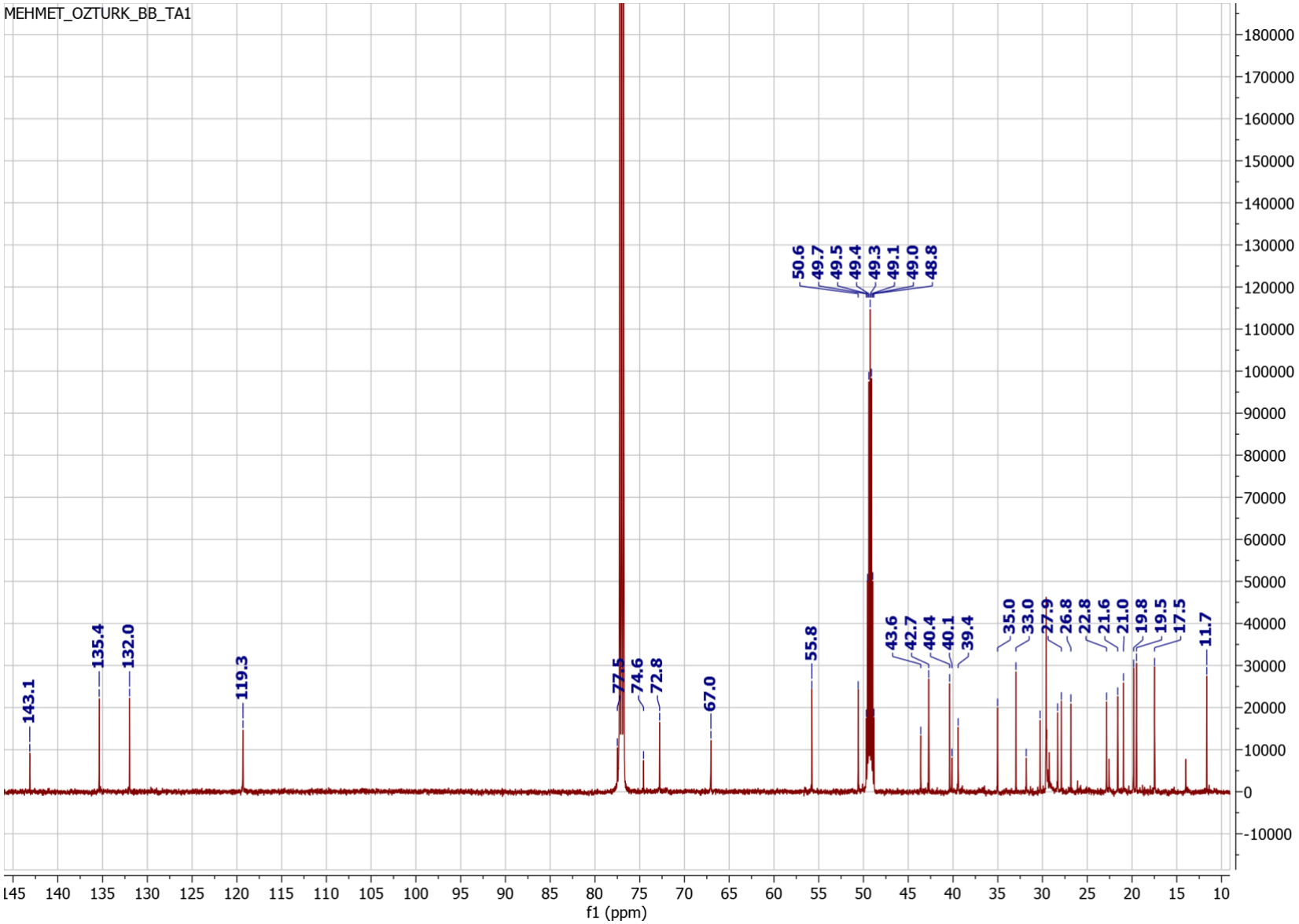


Figure S5. ^{13}C NMR (150 MHz, $CDCl_3$ + CD_3OD) of compound **1**

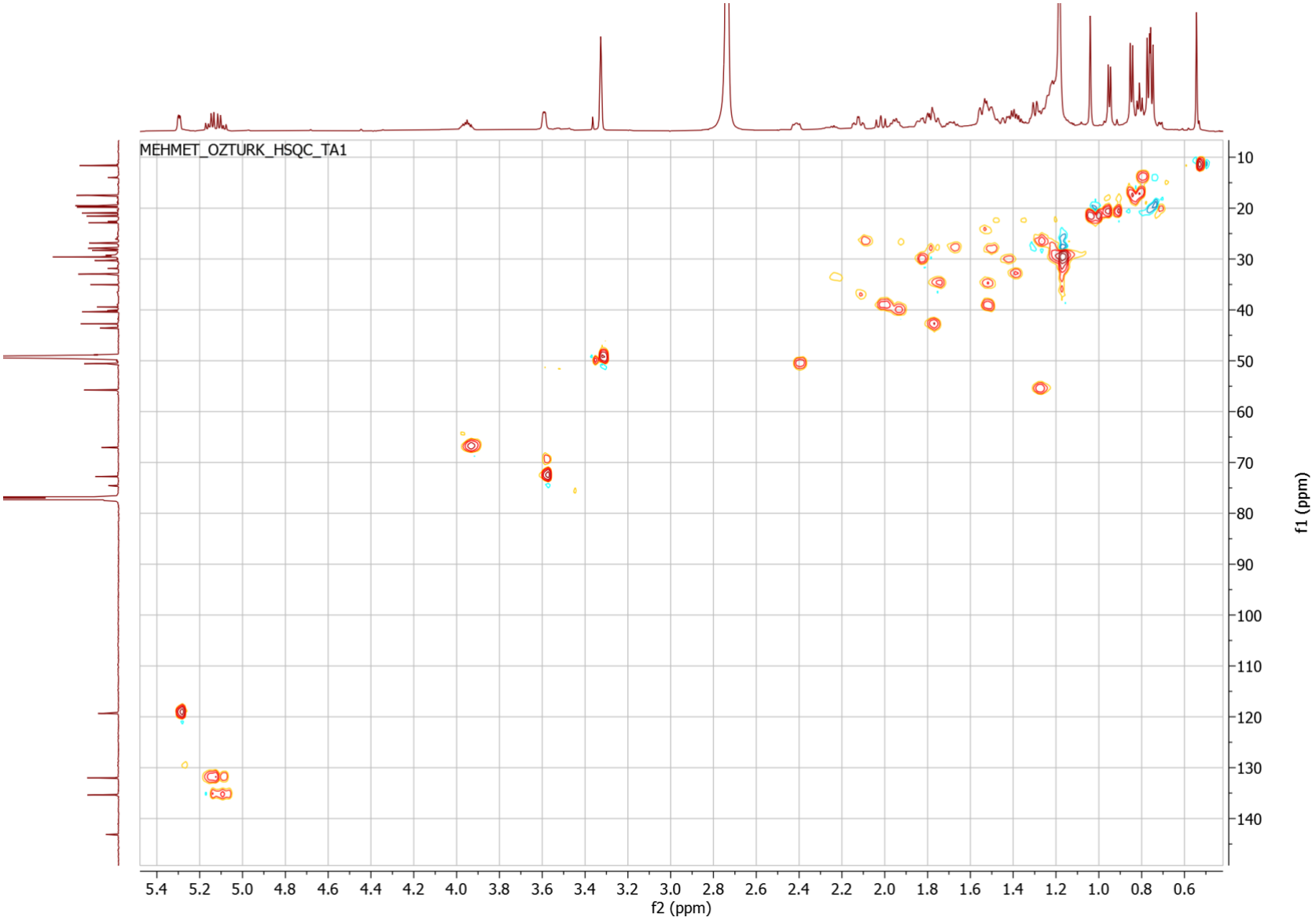


Figure S6. HSQC spectra of compound **1**

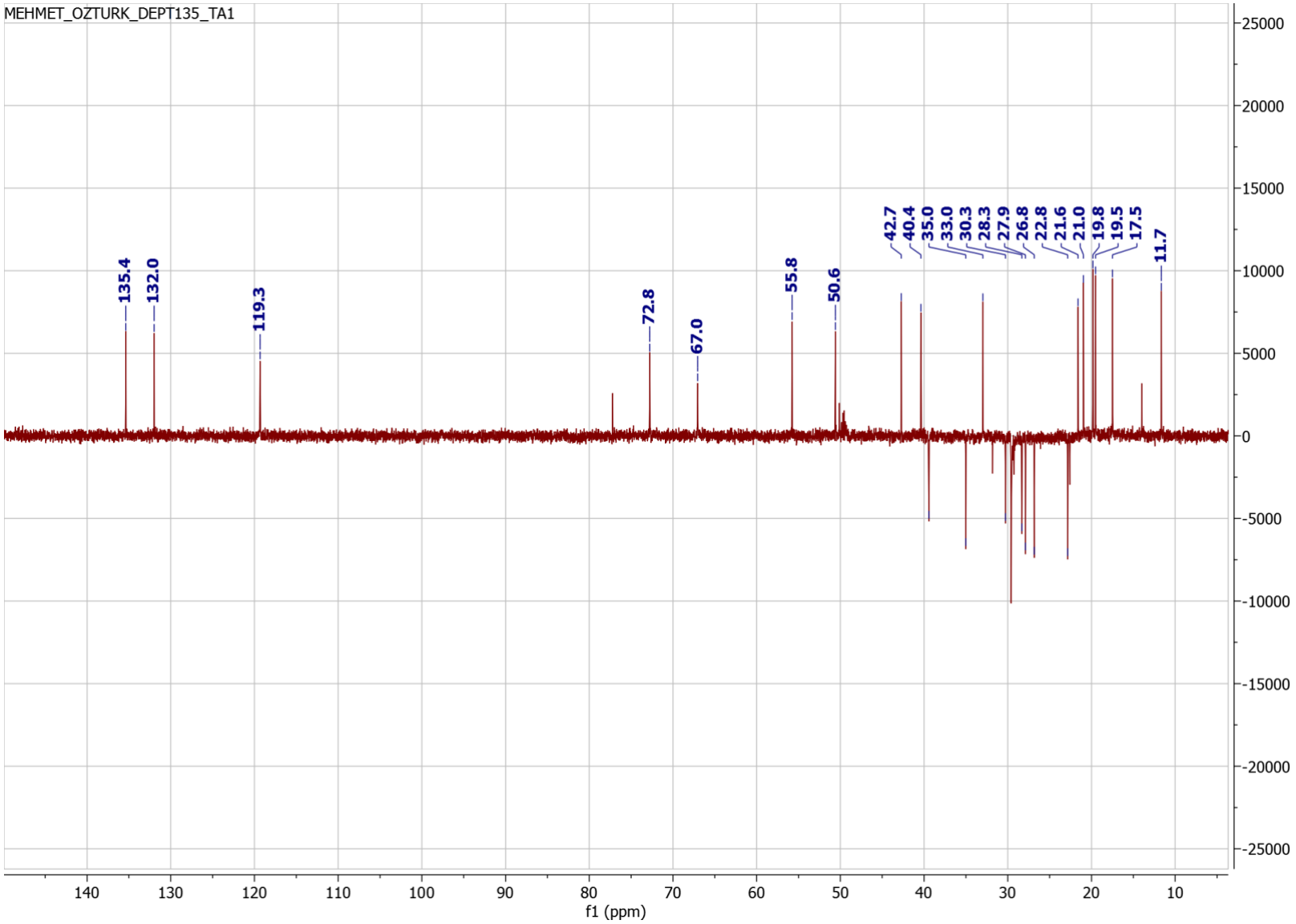


Figure S7. DEPT 135 spectra of compound **1**

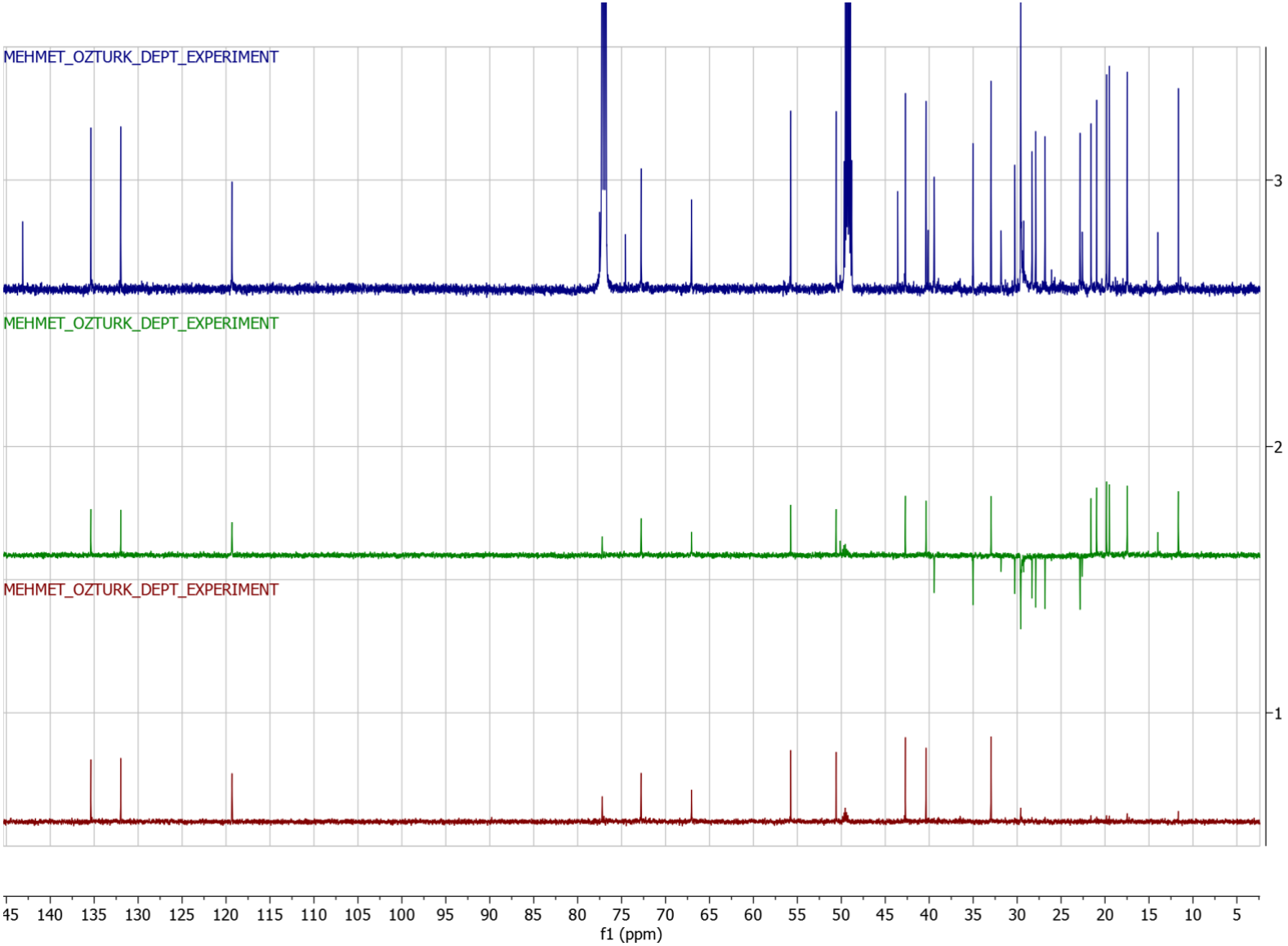


Figure S8. DEPT experiment of compound **1**

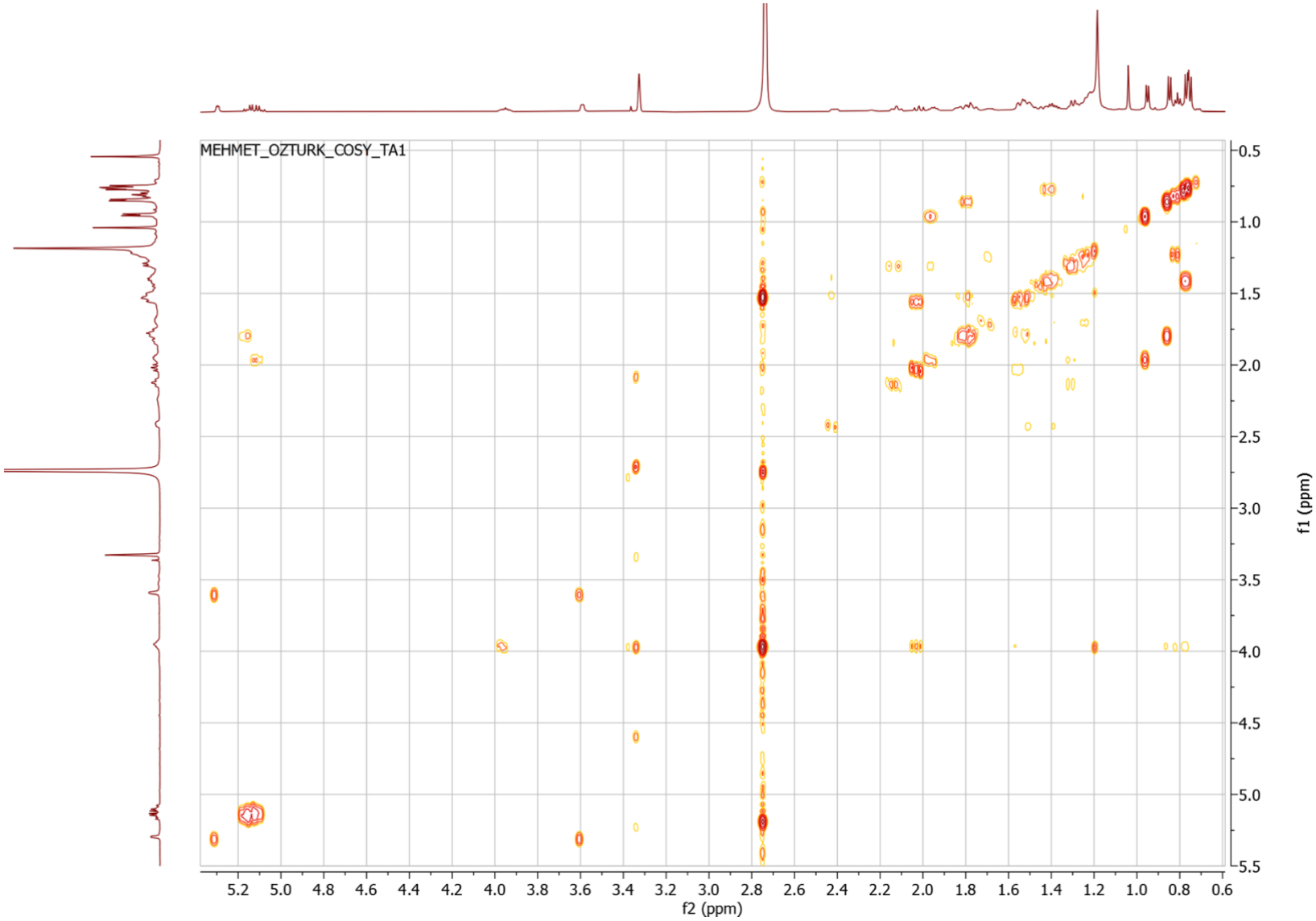


Figure S9. COSY spectra of compound **1**

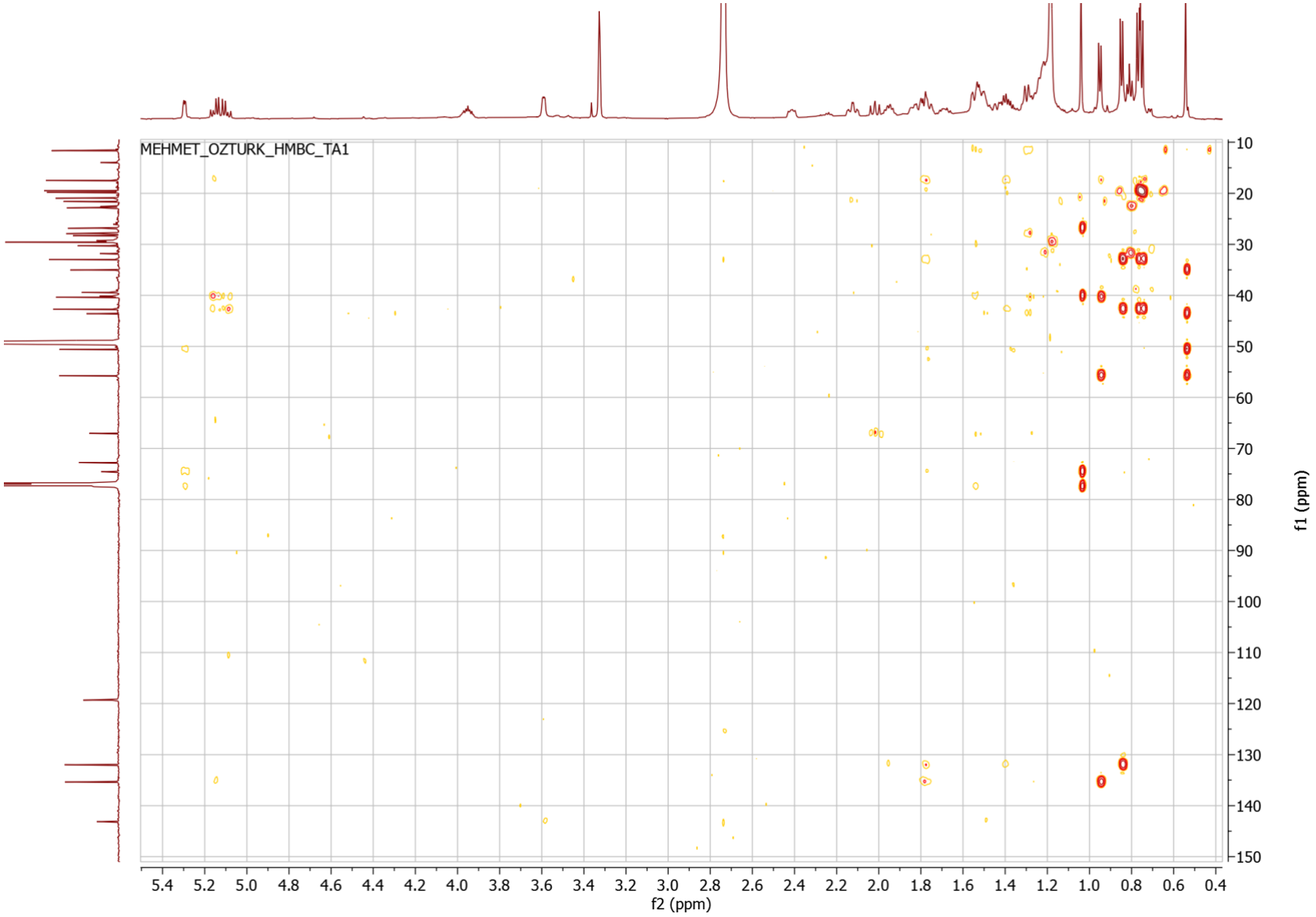


Figure S10. HMBC spectra of compound **1**

JEOL HX 110 MASS SPECTROMETER (EI-HR)

STUDENT NAME	Erhan	SAMPLE CODE	DATED
SUPERVISOR NAME	Dr Mehmet	TA 2	19/1/17

Mass	Theoretical Mass	Delta [ppm]	Delta [mmu]	RDB	Composition
428.3280	428.3290	-2.4	-1.0	7.0	$C_{28}H_{44}O_4$
410.3168	410.3185	-4.1	-1.7	8.0	$C_{28}H_{42}O_3$
382.3210	382.3236	-6.7	-2.6	7.0	$C_{26}H_{42}O_3$

Figure S11. HRMS of compound **2**

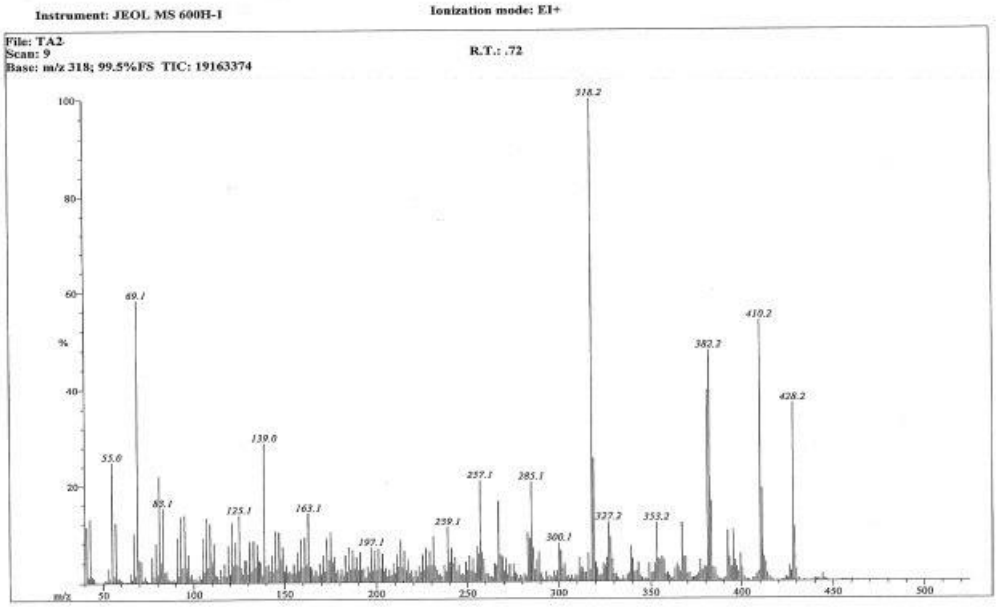


Figure S12. EI-MS of compound **2**

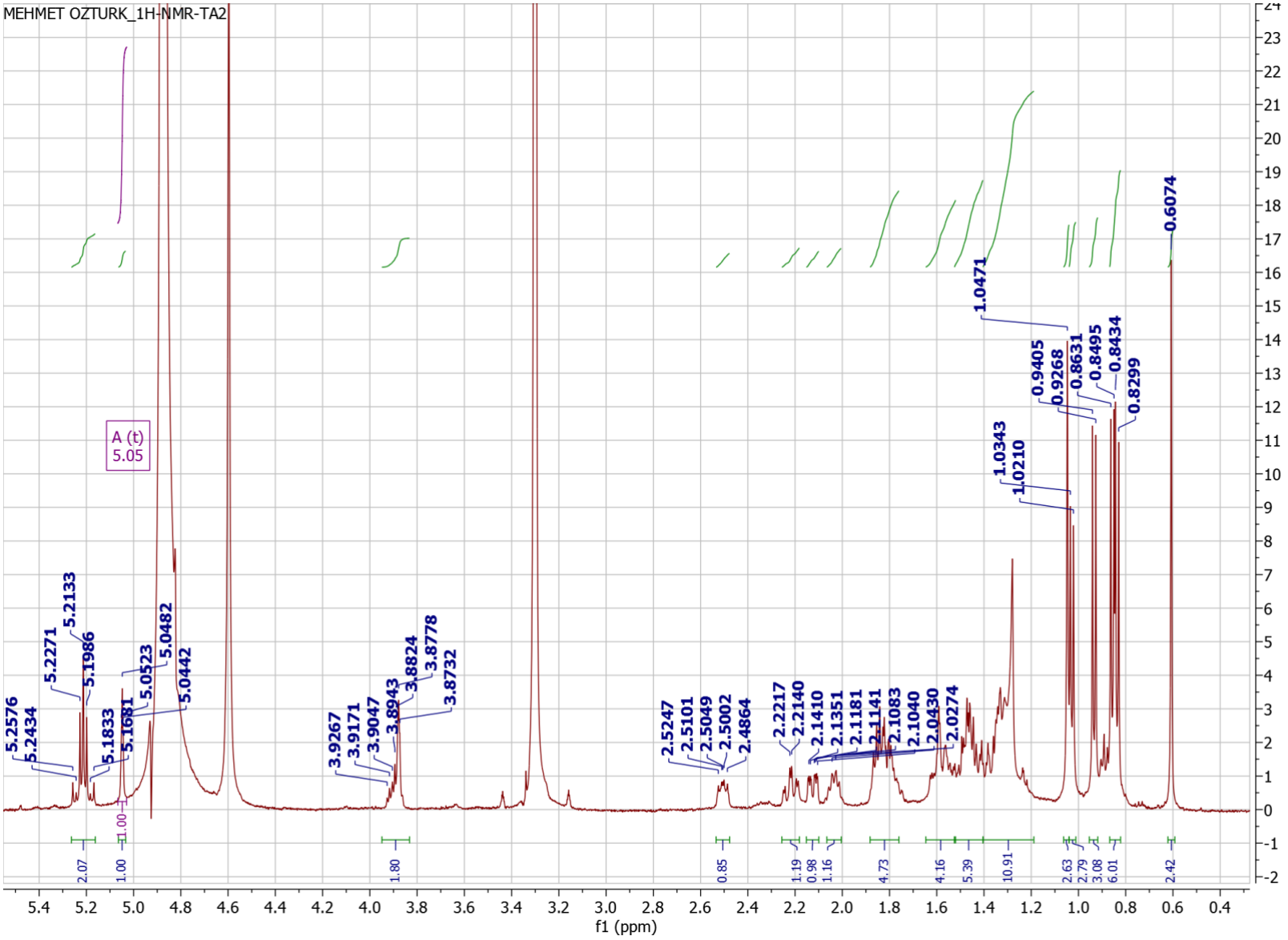


Figure S13. ^{1}H NMR (500 MHz, CD_3OD) of compound **2**

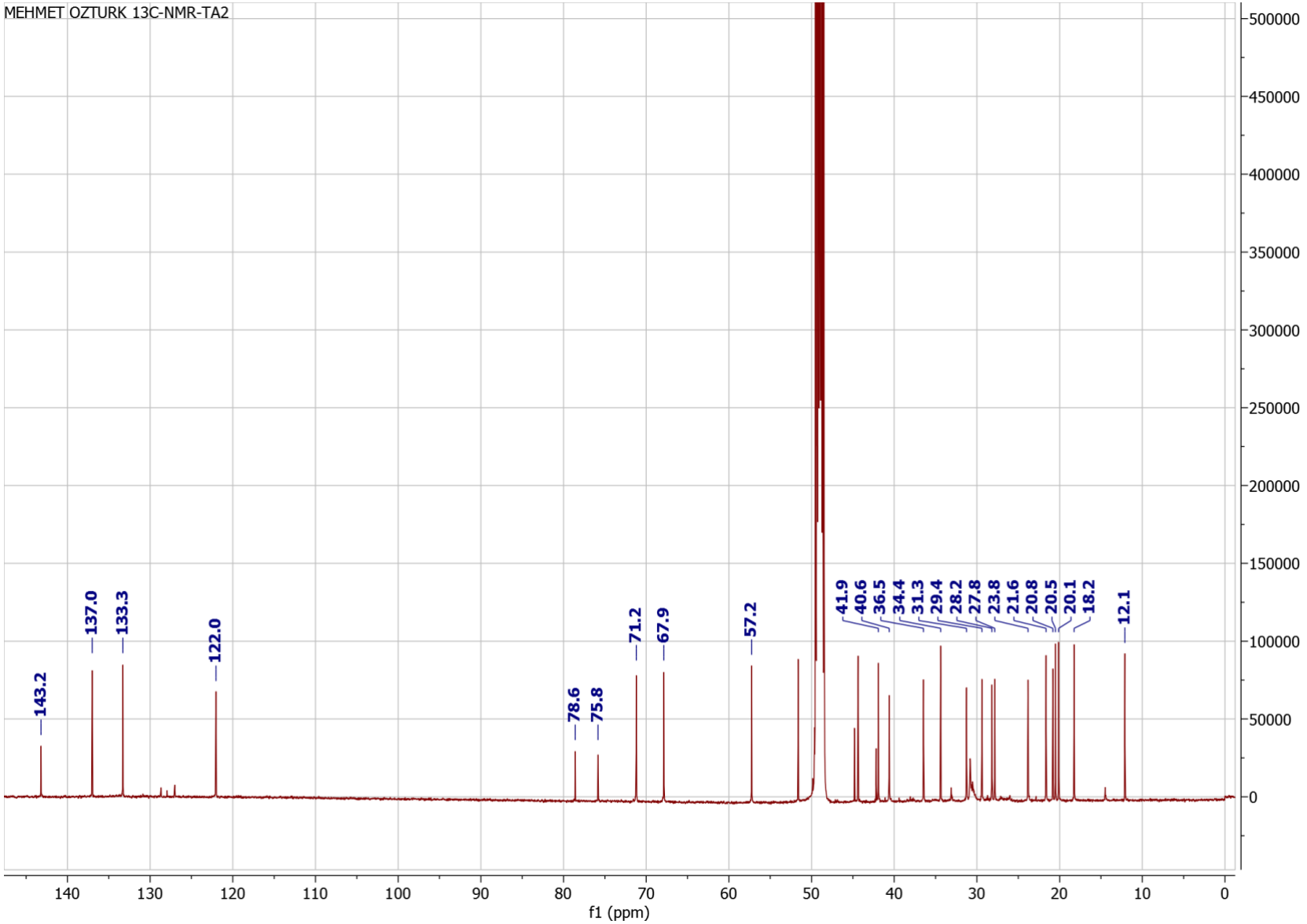


Figure S14. ^{13}C-NMR (125 MHz, CD_3OD) of compound **2**

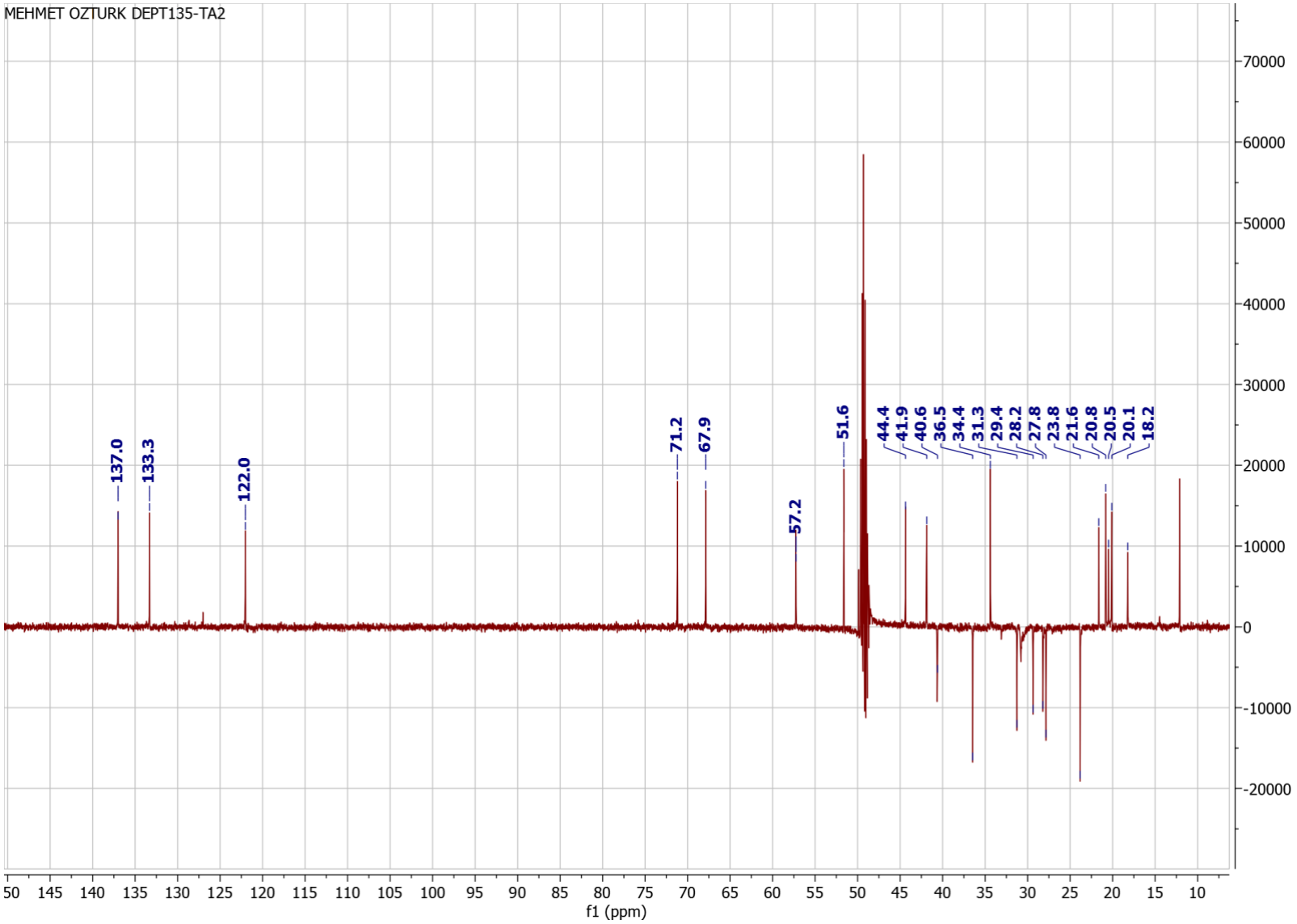


Figure S15. DEPT 135 spectra of compound **2**

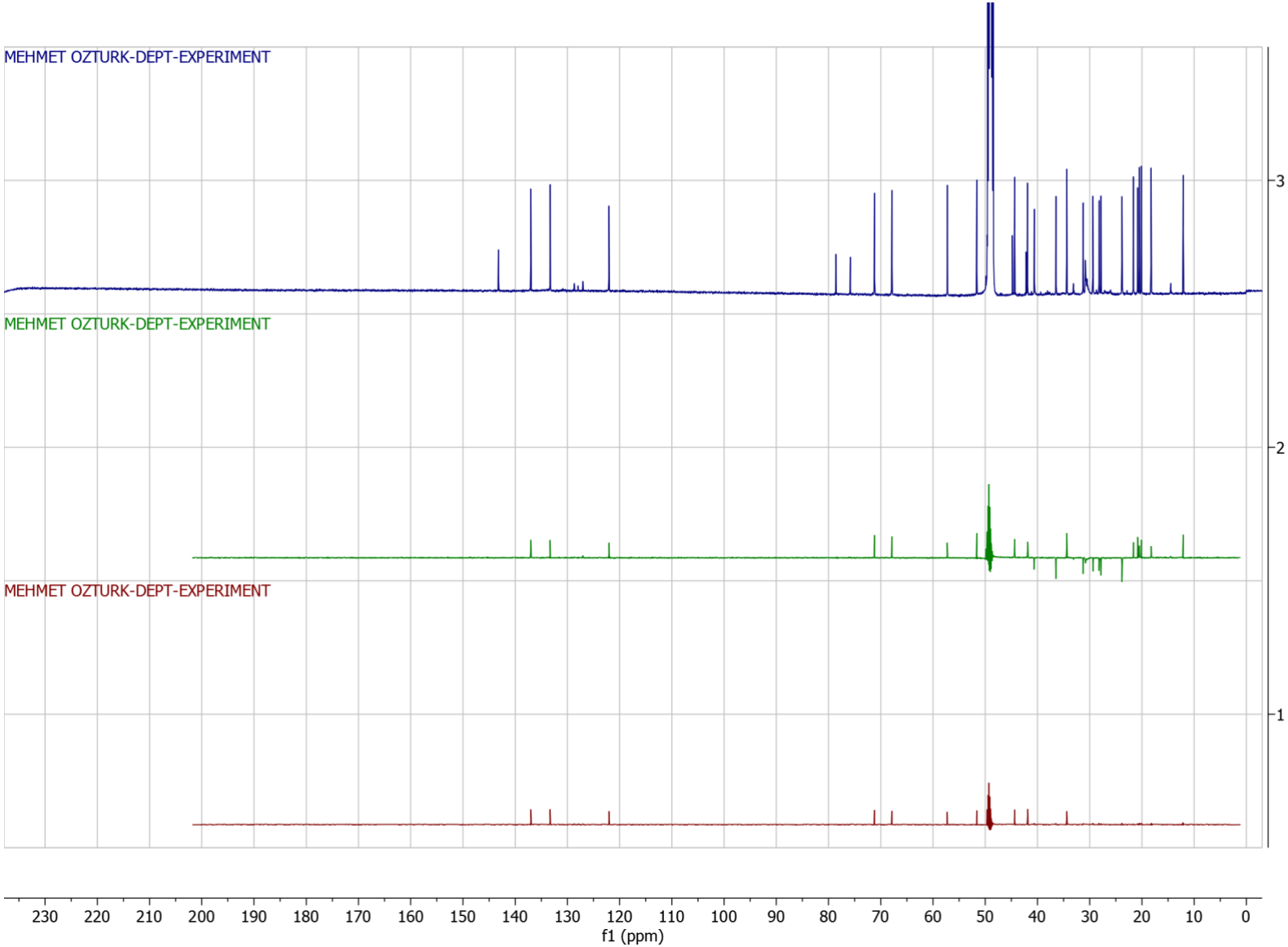


Figure S16. DEPT experiment of compound **2**

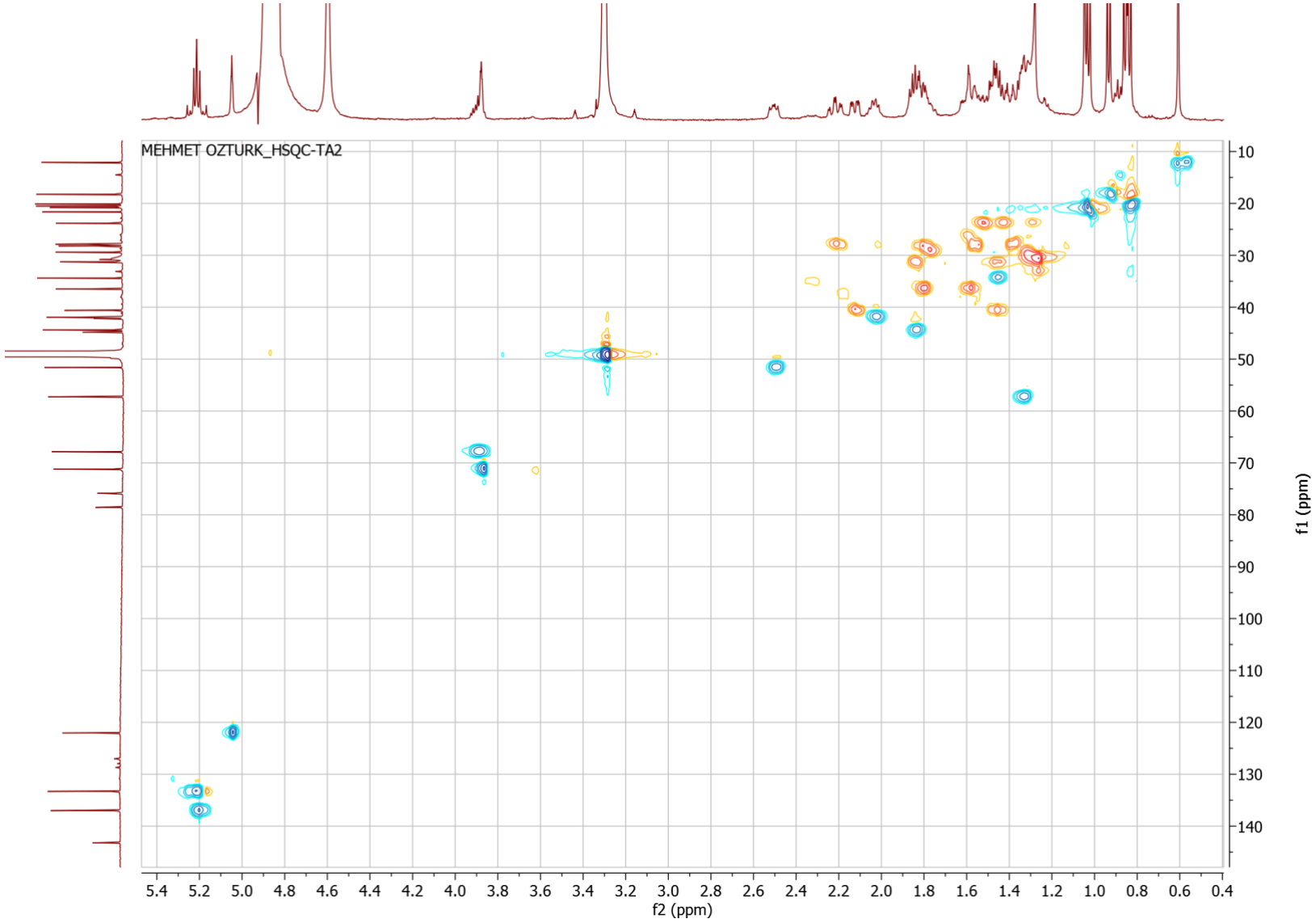


Figure S17. HSQC spectra (CD_3OD) of compound **2**

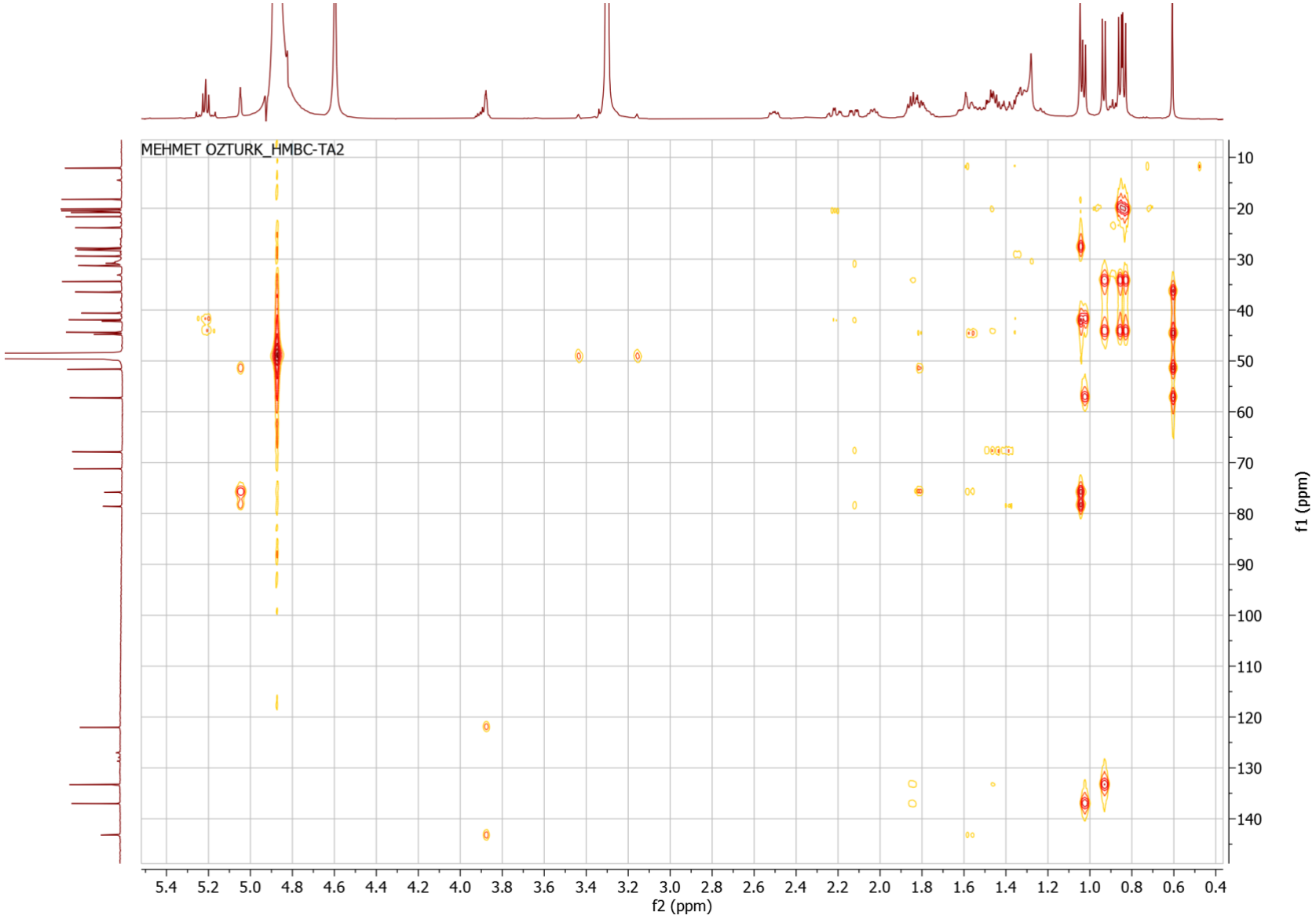


Figure S18. HMBC spectra of compound **2**

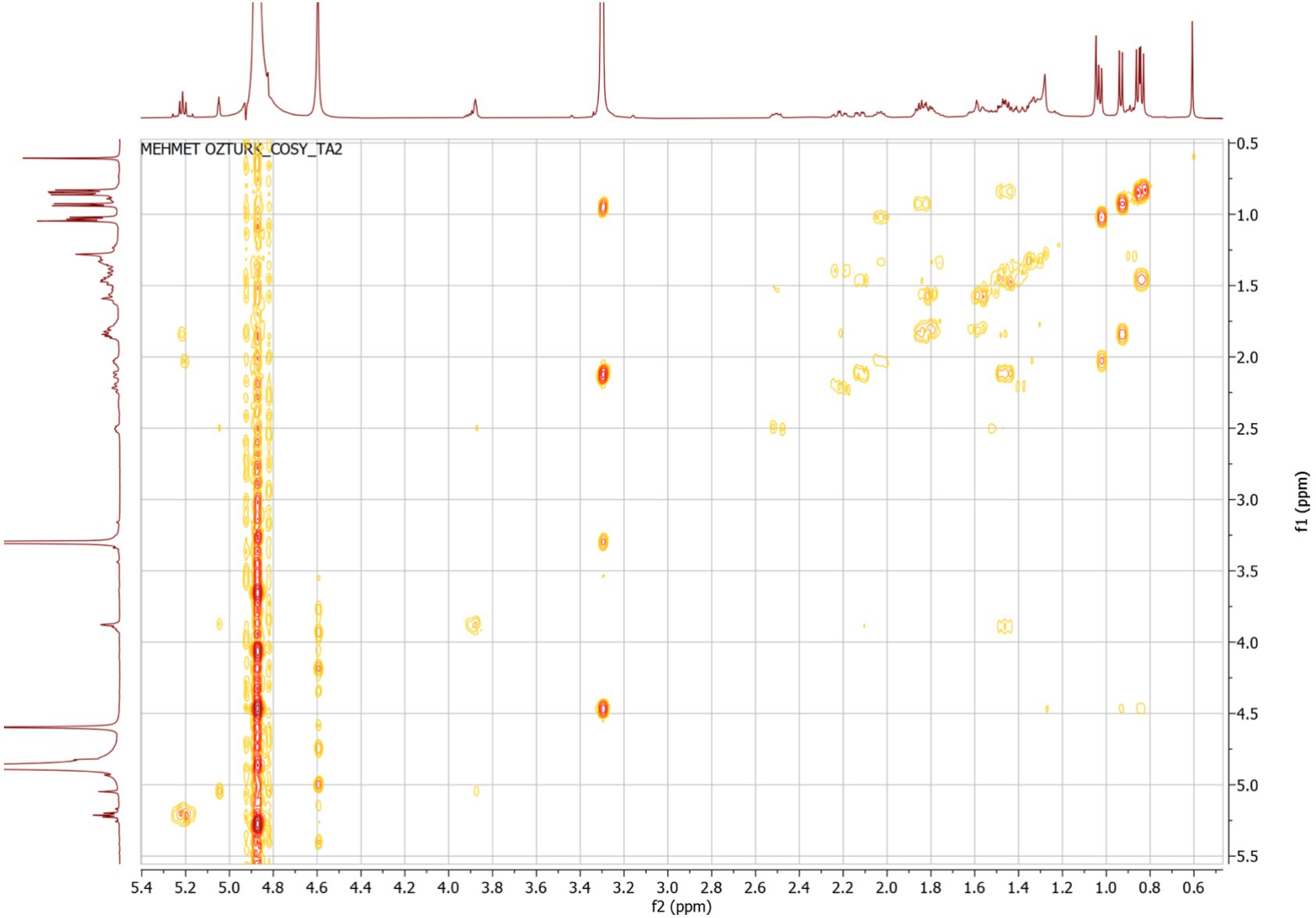


Figure S19. COSY spectra of compound **2**

Table S3. The NMR data of new (1 and 2) and known (3-9) compounds

3.4.1. 5α,9α-epoksiergosta-7,22-dien, 3β,6α-diol **(1)** ^{1}H-NMR ($CDCl_3$ +CD_3OD, 600 MHz); 2.10; 1.28 (2H, *m*, H-1), 1.83; 1.43 (2H, *m*, H-2), 3.95 (1H, *m*, H-3), 1.54; 2.02 (2H, *m*, H-4), 3.59 (1H, *d*, *J*=5.4 Hz, H-6), 5.30 (1H, *d*, *J*=5.3 Hz, H-7), 1.52 (2H, *m*, H-11), 1.76; 1.53 (2H, *m*, H-12), 2.41 (1H, *m*, H-14), 1.49;1.20 (2H, *m*, H-15), 1.80;1.68 (2H, *m*, H-16), 1.29 (1H, *m*, H-17), 0.54 (3H, *s*, H-18), 1.04 (3H, *s*, H-19), 1.95 (1H, *m*, H-20), 0.95 (3H, *d*, *J*= 6.6 Hz, H-21), 5.11 (1H, *dd*, *J*= 8.2; 15.3 Hz, H-22), 5.14 (1H, *dd*, *J*= 7.4; 15.3 Hz, H-23), 1.79 (1H, *m*, H-24), 1.40 (1H, *m*, H-25), 0.77 (3H, *d*, *J*=6.8 Hz, H-26), 0.75 (3H, *d*, *J*=7.0 Hz, H-27), 0.85 (3H, *d*, *J*=6.8 Hz, H-28). ^{13}C-NMR ($CDCl_3$ +CD_3OD, 150 MHz); 26.8 (C-1), 30.3 (C-2), 67.0 (C-3), 39.4 (C-4), 77.5 (C-5), 72.8 (C-6), 119.3 (C-7), 143.1 (C-8), 74.6 (C-9), 40.1 (C-10), 28.3 (C-11), 35.0 (C-12), 43.6 (C-13), 50.6 (C-14), 22.8 (C-15), 27.9 (C-16), 55.8 (C-17), 11.6 (C-18), 21.6 (C-19), 40.4 (C-20), 21.0 (C-21), 135.4 (C-22), 133.0 (C-23), 42.7 (C-24), 33.0 (C-25), 19.8 (C-26), 19.5 (C-27), 17.5 (C-28)

3.4.2. 5α,9α-epoksiergosta-7,22-dien, 3β,6β-diol **(2)** ^{1}H-NMR (CD_3OD, 500 MHz); 2.22; 1.39 (2H, *t*, *J*= 5.4 Hz, H-1), 1.85;1.47 (2H, *m*, H-2), 3.90 (1H, *m*, H-3), 2.12; 1.46 (2H, *m*, H-4), 3.87 (1H, brs, H-6), 5.04 (1H, br*m*, H-7), 1.81; 1.56 (2H, *m*, H-11), 1.81; 1.59 (2H, *m*, H-12), 2.50 (1H, *m*, H-14), 1.43; 1.53 (2H, *m*, H-15), 1.78; 1.33 (2H, *m*, H-16), 1.34 (1H, *m*, H-17), 0.61 (3H, *s*, H-18), 1.04 (3H, *s*, H-19), 2.03 (1H, *q*, *J*=7.3 Hz, H-20), 1.03 (3H, *d*, *J*=6.6Hz, H-21), 5.18 (1H, *dd*, *j*=7.8;15.1 Hz, H-22), 5.24 (1H, *dd*, *J*=7.3; 15.1 Hz, H-23), 1.84 (1H, *m*, H-24), 1.46 (1H, *m*, H-25), 0.86 (3H, *d*, *J* =6.8 Hz, H-26), 0.84 (3H, *d*, *J* =6.8 Hz, H-27), 0.93 (3H, *d*, *J* =6.9 Hz, H-28). ^{13}C-NMR (CD_3OD, 125 MHz); 27.7 (C-1), 31.2 (C-2), 67.8 (C-3), 40.6 (C-4), 78.6 (C-5), 71.2 (C-6), 122.0 (C-7), 143.2 (C-8), 75.8 (C-9), 42.2 (C-10), 28.2 (C-10), 36.5 (C-11), 44.8 (C-12), 51.6 (C-13), 23.8 (C-14), 29.4 (C-15), 57.2 (C-16), 12.1 (C-18), 20.8 (C-19), 41.9 (C-20), 21.6 (C-21), 137.0 (C-22), 133.3 (C-23), 44.4 (C-24), 34.4 (C-25), 20.5 (C-26), 20.1 (C-27), 18.2 (C-28).

3.4.3. 5α,6α-Epoxy-ergosta-7,22-dien, 3β-ol (**3**) ^{1}H-NMR ($CDCl_3$ +CD_3OD, 500 MHz); 1.39; 1.37 (2H, *m*, H-1), 1.68;1.29 (2H, *m*, H-2), 3.85 (1H, *m*, H-3), 1.94;1.92 (2H, *m*, H-4), 3.44 (1H, *m*, H-5), 5.18 (1H, *m*, H-7), 1.82 (1H, *m*, H-9), 1.40; 1.44 (2H, *m*, H-11), 1.17; 1.53 (2H, *m*, H-12), 1.78 (1H, *m*, H-14), 1.40; 1.50 (2H, *m*, H-15), 1.61; 1.14 (2H, *m*, H-16), 1.55 (1H, *m*, H-17), 0.47 (3H, *s*, H-18), 0.92 (3H, *s*, H-19), 1.92 (1H, *m*, H-20), 0.89 (3H, *d, J*=6.5Hz, H-21), 5.03(1H, *dd, J*=8.0; 15.0 Hz, H-22), 5.09(1H, *dd, J*=7.5; 15.0 Hz, H-23), 1.70 (1H, *m*, H-24), 1.33 (1H, *m*, H-25), 0.69 (3H, *d, J*=7.5Hz, H-26), 0.70 (3H, *d, J*=7Hz, H-27), 0.78 (3H, *d, J* =6.5 Hz, H-28). ^{13}C-NMR ($CDCl_3$ +CD_3OD, 125 MHz); 32.5 (C-1), 30.2 (C-2), 67.1 (C-3), 38.7 (C-4), 75.7 (C-5), 72.9 (C-6), 117.3 (C-7), 143.2 (C-8), 42.9 (C-9), 36.8 (C-10), 21.7 (C-11), 39.1 (C-12), 43.4 (C-13), 54.5 (C-14), 22.7 (C-15), 27.7 (C-16), 55.8 (C-17), 11.9 (C-18), 18.0 (C-19), 40.2 (C-20), 20.8 (C-21), 135.3 (C-22), 131.8 (C-23), 42.6 (C-24), 32.8 (C-25), 19.6 (C-26), 19.3 (C-27), 17.3 (C-28).

3.4.4. Ergosterol endoperoxide (**4**) ^{1}H-NMR ($CDCl_3$, 500 MHz); 2.07; 1.91 (2H, *bm*, H-1), 1.75; 1.33 (2H, *m*, H-2), 3.95 (1H, *m*, H-3), 1.84; 1.53 (2H, *m*, H-4), 6.22 (1H, *d, j*=8.5 Hz, H-6), 6.49 (1H, *d, j*=8.5 Hz, H-7), 2.01 (1H, *brm*, H-9) 1.50; 1.22 (2H, *m*, H-11), 1.94; 1.24 (2H, *m*, H-12), 1.55 (1H, *bm*, H-14), 1.69; 1.93 (2H, *m*, H-15), 1.44; 1.21 (2H, *bm*, H-16), 1.21 (1H, *bm*, H-17), 0.79 (3H, *s*, H-18), 0.86 (3H, *s*, H-19), 2.01 (1H, *m*, H-20), 0.97 (3H, *d, j*=6.5 Hz, H-21), 5.12 (1H, *dd, j*=7.5; 15 Hz, H-22), 5.20 (1H, *dd, j*=7.5; 15 Hz, H-23), 1.84 (1H, *m*, H-24), 1.47 (1H, *bm*, H-25), 0.81 (3H, *d, j*=7.0 Hz, H-26), 0.79 (3H, *d, j*=6.5 Hz, H-27), 0.88 (3H, *d, j*=6.5 Hz, H-28) ^{13}C-NMR ($CDCl_3$, 125 MHz); 36.9 (C-1), 28.6 (C-2), 66.4 (C-3), 30.1 (C-4), 82.1 (C-5), 135.4 (C-6), 130.7 (C-7), 79.4 (C-8), 39.7 (C-9), 36.9 (C-10), 23.3 (C-11), 39.3 (C-12), 44.5 (C-13), 51.1 (C-14), 34.7 (C-15), 23.6 (C-16), 56.2 (C-17), 12.8 (C-18), 18.2 (C-19), 39.8 (C-20), 20.9 (C-21), 135.2 (C-22), 132.3 (C-23), 42.7 (C-24), 33.1 (C-25), 19.9 (C-26), 19.7 (C-27), 17.5 (C-28).

3.4.5. Ergosterol, 3β-ol (**5**) ^{1}H-NMR ($CDCl_3$, 300 MHz)1.23; 1.28 (2H, *m*, H-1), 1.85; 1.43 (2H, *m*, H-2), 3.63 (1H, *m*, H-3), 2.46; 2.26 (2H, *m*, H-4), 5.55 (*dd, J*=2.1; 5.4 Hz, H-6), 5.36. (*dd, J*=2.9; 5.4 Hz, H-7), 1.95 (1H, *m*, H-9), 1.57 (2H, *m*, H-11), 1.88; 1.84 (2H, *m*, H-12), 1.86 (1H. *m*. H-14), 1.64;

1.62 (2H. *m*. H-15), 1.76; 1.72 (2H, *m*, H-16), 1.25 (1H, *m*, H-17), 0.61 (3H, *s*, H-18), 0.92 (3H, *s*, H-19), 2.02 (1H, *m*, H-20), 1.00 (3H, *d*, *J*=6.0 Hz, H-21), 5.18 (1H, *dd*, *J*=15.3; 6.9 Hz, H-22), 5.17 (1H, *dd*, *J*=15.3; 7.5 Hz, H-1, H-23), 1.82 (1H, *m*, H-24), 1.45 (1H, *m*, H-25), 0.80 (3H, *d*, *J*= 6.6 Hz, H-26), 0.82 (3H, *d*, *J*= 6.6 Hz, H-27), 0.89 (3H, *d*, *J*=6.0 Hz, H-28). ^{13}C-NMR ($CDCl_3$, 100 MHz); 38.3 (C-1), 31.9 (C-2), 70.2 (C-3), 40.7 (C-4), 139.7 (C-5), 119.6 (C-6), 116.3 (C-7), 141.3 (C-8), 46.2 (C-9), 37.1 (C-10), 21.1 (C-11), 39.1 (C-12), 42.9 (C-13), 54.6 (C-14), 22.9 (C-15), 28.2 (C-16), 55.7 (C-17), 12.0 (C-18), 16.2 (C-19), 40.4 (C-20), 21.2 (C-21), 135.5 (C-22), 131.9 (C-23), 42.8 (C-24), 33.1 (C-25), 19.6 (C-26), 19.9 (C-27), 17.6 (C-28).

3.4.6. 3,5-dihydroxyfuran-2(5H)-one (**6**) ^{1}H-NMR (D_2O, 500 MHz); 7.41 (1H, *d*, *J*=8.0 Hz, H-4), 5.69 (1H, *d*, *J*=8.0 Hz, H-5). ^{13}C-NMR (D_2O, 125 MHz); 170.1 (C-2), 155.7 (C-3), 146.1 (C-4), 103.7 (C-5).

3.4.7. Mannitol (**7**) ^{1}H-NMR (D_2O, 600 MHz); 3.74 (1H, *dd*, *J* = 9.1, 2.5 Hz, H-1), 3.54 (1H, *dd*, *J* = 11.8, 6.2 Hz, H-1$^{'}$), 3.66 (1H, *dt*, *J* = 6.0, 2.5 Hz, H-2-2$^{'}$), 3.69 (1H, *d*, *J* = 6.0 Hz, H-3-3$^{'}$). ^{13}C-NMR (D_2O, 150 MHz)63.1 (C-1-1$^{'}$), 70.8 (C-2-2$^{'}$), 69.2 (C-3-3$^{'}$).

3.4.8. Turanose (**8**) ^{1}H-NMR (D_2O, 500 MHz); 5.08, (1H, *d*, *J*=1.8 H, H-1), 3,54 (1H, *dd*, *J*=10,2; 4,0 Hz, H-2), 3,33 (1H, *t*, *J*=9,6Hz, H-3), 3.75 (1H, *m*, H-4), 3.70 (1H, *dt*, *J*=1,8; 5,4; 5,4 Hz, H-5), 3.65 (2H, *dd*, *J*= 5,4; 5,4 Hz, H-6). ^{13}C-NMR (D_2O, 125 MHz); 95.9 (C-1), 73.7 (C-2), 72.3 (C-3), 75.2 (C-4) 74.8 (C-5), 63.2 (C-6).

3.4.9. Fumaric acid (**9**) ^{1}H-NMR (D_2O, 500 MHz); 6.49 (1H, s, H-2-2$^{'}$). (D_2O, 125 MHz); 175.8, (C-1-1$^{'}$).