SUPPLEMENTARY MATERIAL

Cycloartanol and Sutherlandioside C peracetate from *Sutherlandia frutescens*

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ABSTRACT: A novel cycloartanol and an acylated Sutherlandioside C together with two known cycloartane derivatives, Sutherlandioside A and Sutherlandioside B, were isolated from the aerial parts of *Sutherlandia frutescens*. The structures of these compounds were established by a combination of 1- and 2-D NMR techniques and further confirmed by high resolution ToF mass spectrometry (HRToFMS). Preliminary biological studies were also conducted to assess the activity of different plant extracts, fractions and compounds on cytokine expression. The results of the assays suggested that some components in the plant extract influence the immune system by suppressing the expression of IL-6, IL-10 and TNFα.

Keywords: *Sutherlandia frutescens*, cancer bush, cycloartenol, acetylated Sutherlandioside, cytokines

Legends for Figures and Tables

Experimental section

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Table S1. ^{1}H-^{13}C HMBC correlations of compound **1**.

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Table S2. ^{1}H-^{13}C HMBC correlations of acetylated compound **2**.

Table S3. 600 MHz ^{13}C-NMR data of compounds **1-4** in CD_3OD.

Experimental section

General experimental procedures

ESI-MS measurements were obtained on a Waters UPLC-QToF SYNAPT G1 HDMS instrument in positive ionization mode. ^{1}H NMR and ^{13}C NMR experiments were performed on a 600 MHz Varian NMR instrument. Silica gel (Kieselgel GF254 15 mm, Merck) and solid phase extraction C-18 cartridges (Supelco 140 ml PP tubes, Sigma Aldrich) were used for column chromatography and silica gel 60 F254 on aluminum sheets (Merck) for thin-layer chromatography (TLC). Plates were sprayed with 5% ethanolic sulfuric acid followed by 1% ethanolic vanillin and baked for 5 min at 100 °C.

Plant Material

Aerial parts of S. *frutescens* were collected in Petrusburg, Free State, South Africa. A voucher specimen (twigs containing flowers) was sent to the South African National Biodiversity Institute (SANBI) for verification of identity and the plant species was identified as *Sutherlandia frutescens* (L.) R. B.r. (Voucher specimen number 39250002).

Extraction and isolation

A quantity of 200 g of dry, powdered material was extracted three times with absolute ethanol (2.0 L of 99.9% EtOH) overnight at room temperature, after which aliquots were combined and dried under vacuum to obtain 51 g of dry extract. Of this dry extract, 10 g was suspended in 100 ml of methanol/water (ratio 9:1 v/v) and sequentially partitioned with hexane, dichloromethane and ethyl acetate, respectively to give 1.52 g, 0.42 g and 1.13 g, respectively. While partitioning with ethyl acetate, three layers were observed and the middle layer was an emulsion layer. The water and emulsion layers had masses of 3.25g and 3.13 g, respectively, when dried. A mass of 416 mg of the dichloromethane extract was then chromatographed on a silica gel column using a stepwise gradient mixture of approximately

500 ml hexane:ethyl acetate (v/v) in a ratio of 6:4, 5:5, 4:6, 2:8, 0:9 as gradient mobile phase to give 5 fractions. The last fraction was chromatographed using preparative thin layer chromatography (prep-TLC) using chloroform: methanol (9.5:0.5) as a mobile phase and compound **(1)** (2.2 mg) was isolated as a pure white powder. A mass of 3.0 g of emulsion extract was subjected to silica gel vacuum liquid chromatography (VLC) using a stepwise gradient solvent system of 500 ml of $CHCl_3$/MeOH (v/v) in a ratio of 10:0, 9:1, 7:3, 5:5, 4:6, 1:9 and 0:10 as mobile phase. Similar fractions were combined to give 7 fractions (1-7). Fraction 1 was further partitioned on a silica gel flash column and was eluted with $CHCl_3$:MeOH (ratio 10:0, 9:1, 7:3, 5:5, 0:10) and similar fractions were combined to obtain 5 fractions (A-E). Fraction A was then purified using a reverse phase C-18 solid phase extraction (SPE) column as a stationary phase and was eluted with acetonitrile:MeOH (10:0, 9:1, 7:3) solvent systems. Further purification of obtained semi-pure compounds was accomplished by prep-TLC using an ethyl acetate:methanol:acetic acid (9:1:1 v/v) solvent system and compounds **(3)** (6.9 mg) and **(4)** (3.1 mg) were obtained as pure white powders.

Acetylation of the extract

A quantity of 74.5mg emulsion extract was acetylated by dissolving it in a mixture of pyridine-acetic anhydride (1:1) in a round-bottomed flask. The reaction mixture was stirred for 48h at room temperature and then 3ml ethanol was added to react with the excess acetic anhydride. The crude mixture was then partitioned using a gravitational column chromatography employing hexane:ethyl acetate (8:2 and 7:3) as eluent and increasing the polarity to chloroform:methanol (9.5:0.5 and 8:2) to obtain three semi-pure fractions (1-3). Fraction 1 (Fraction Ac) was further purified using prep-TLC with chloroform-methanol (9:1 v/v) as the solvent system and compound **(2)** (2.1 mg) was obtained as a pure colourless oil.

Immunomodulation assay

The immune-potentiating ability of extracts, fractions and compounds isolated from *S. frutescens* was assessed using a flow cytometry-based, multiplex cytokine bead assay (CBA) system. The BD CBA Human Th1/Th2/Th17 cytokine kit was employed. Samples were incubated with aliquots of whole blood and then tested for the expression of cytokines. Aliquots of whole blood (125 µl) were separately introduced into a 15 ml conical polypropylene (PP) tube and then 100 µg/ml of sample (plant extract, fraction or pure compounds) dissolved in 0.1 % DMSO were added to each tube followed by growth medium (RPMI 1640) to make a final volume of 1250 µl. A positive control, 0.3 µg/ml phytohaemagglutin A (PHA), was included in these experiments. Negative controls consisted of unstimulated controls containing the same amount of blood as other samples followed by the addition of an equal volume of solvent that had been used to dissolve the plant extract samples. RMPI 1640 was then added to the cells and the control tubes to make up a volume of 1250µl. The tubes were incubated at 37℃ in the presence of 5% carbon dioxide (CO_2) and at 100% relative humidity for 3 days. On day 4, 500µl of supernatant medium was removed from each tube and transferred into separate labelled Sarsted tubes and stored at -80℃. To harvest the cells, 2 mM EDTA was used and 15 ml sterile polypropylene Sterilin tubes were vortexed and incubated at room temperature (RT) for 10 minutes before being centrifuged at 450 *g* (1400 rpm) for 7 min at room temperature and the supernatant decanted. This was followed by the addition of 5 ml of phosphate buffer saline (PBS) to the pellet. The tubes were centrifuged again and the supernatant discarded. For white blood cell staining, a fixable viability dye was used, vortexed and incubated at room temperature for 20 minutes in the dark and the steps to follow were also performed in the dark. An aliquot of 5 ml PBS with 1% fetal calf serum was added into the tubes followed by centrifugation at 450 *g* (1400 rpm) for 7 minutes at RT and the supernatant liquid was discarded. Blood samples

were then lysed with 1X FACSlyse solution in a ratio of 1:10 to the sample volume and then the tubes were vortexed well and incubated for 10 min at room temperature (RT). The tubes were then centrifuged at 600*g* (1600 rpm) for 7 min at RT followed by decanting of the supernatant liquid. Cell pellets were re-suspended in 0.5-1 ml cryo-solution and transferred into appropriately labelled cryovials. These tubes were then transferred into Mr Frosty containers and stored in a -80℃ freezer.

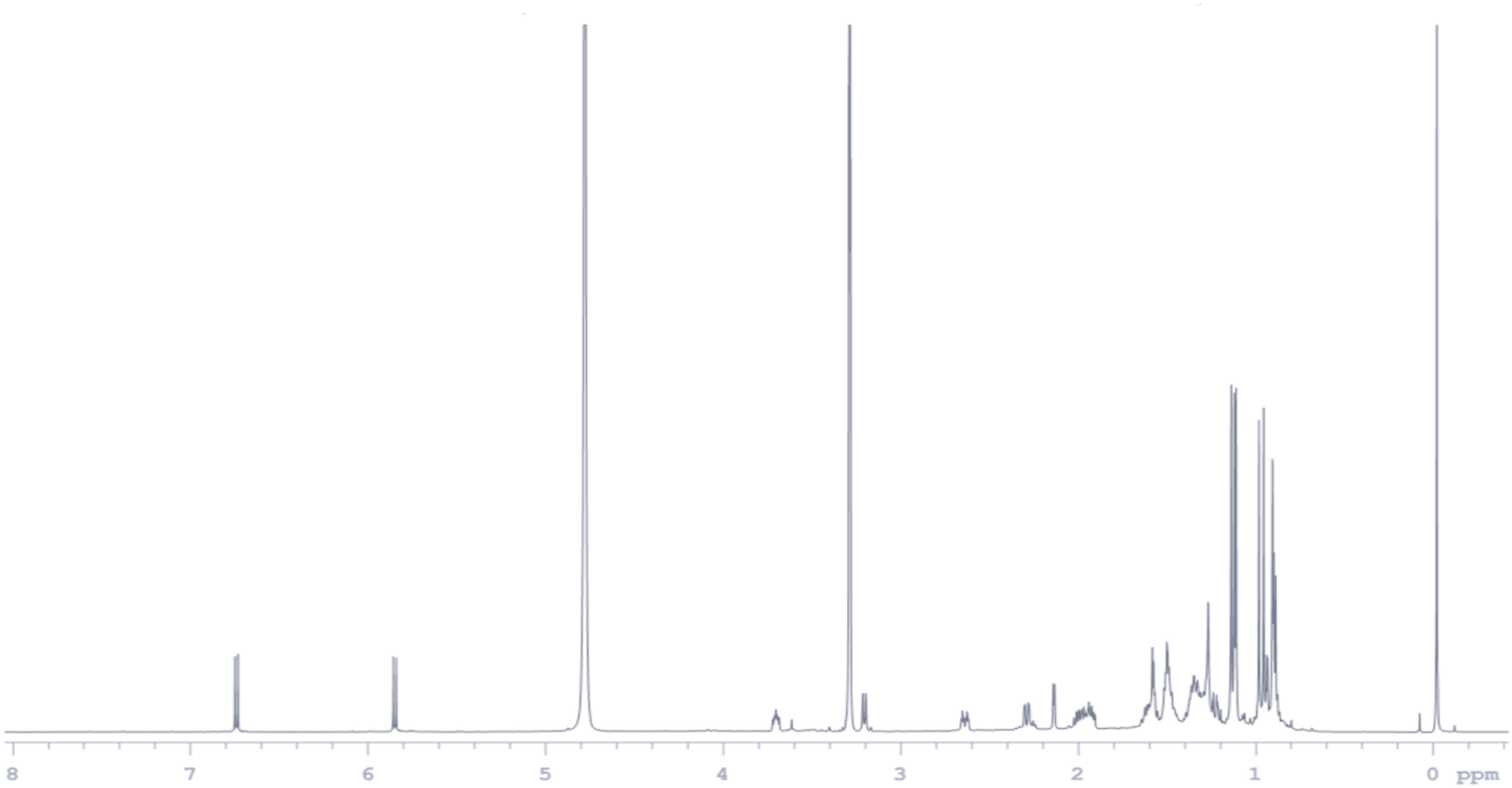


Figure S1. ^{1}H NMR of Compound **1.**

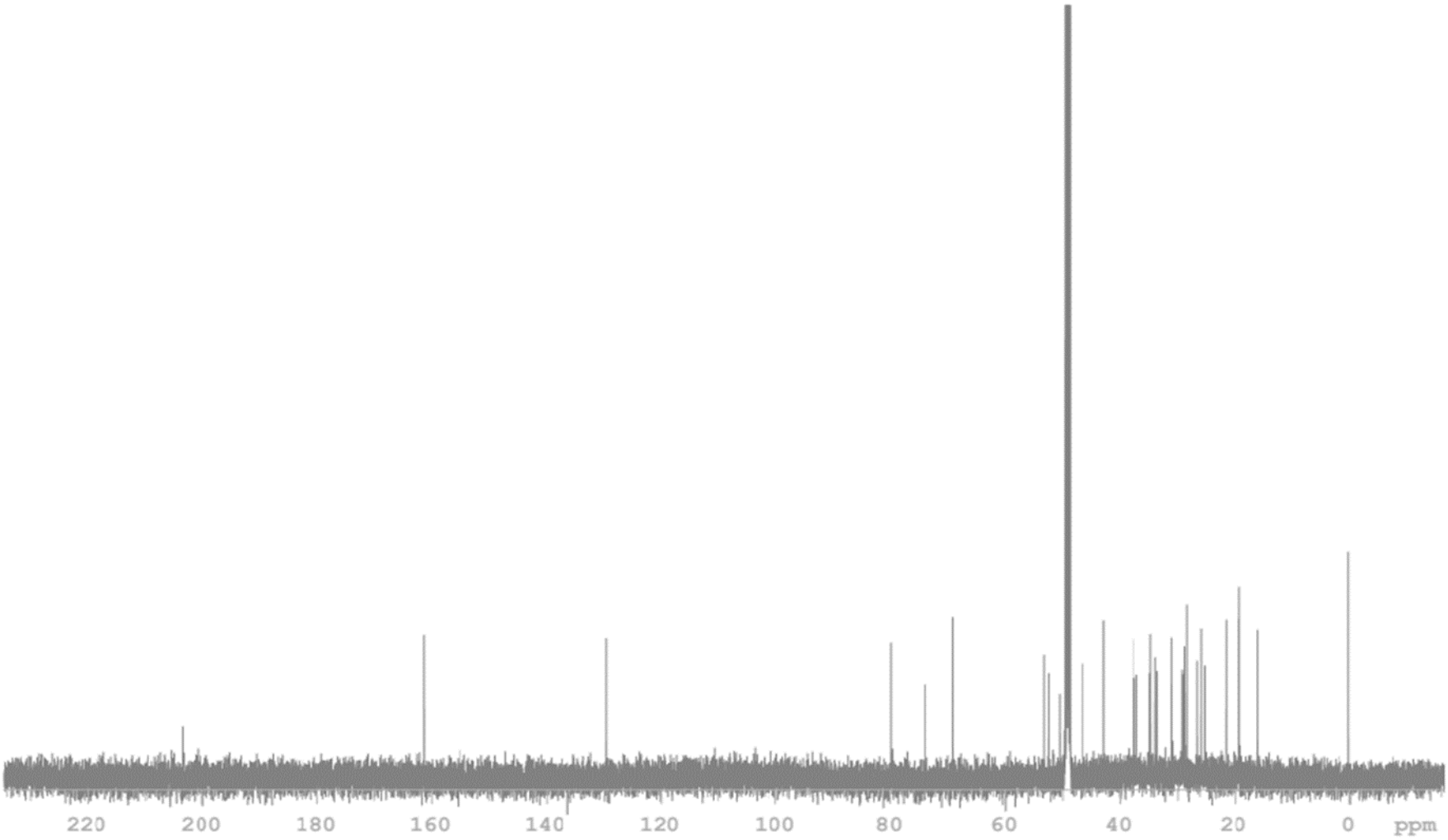


Figure S2. ^{13}C NMR of Compound **1.**

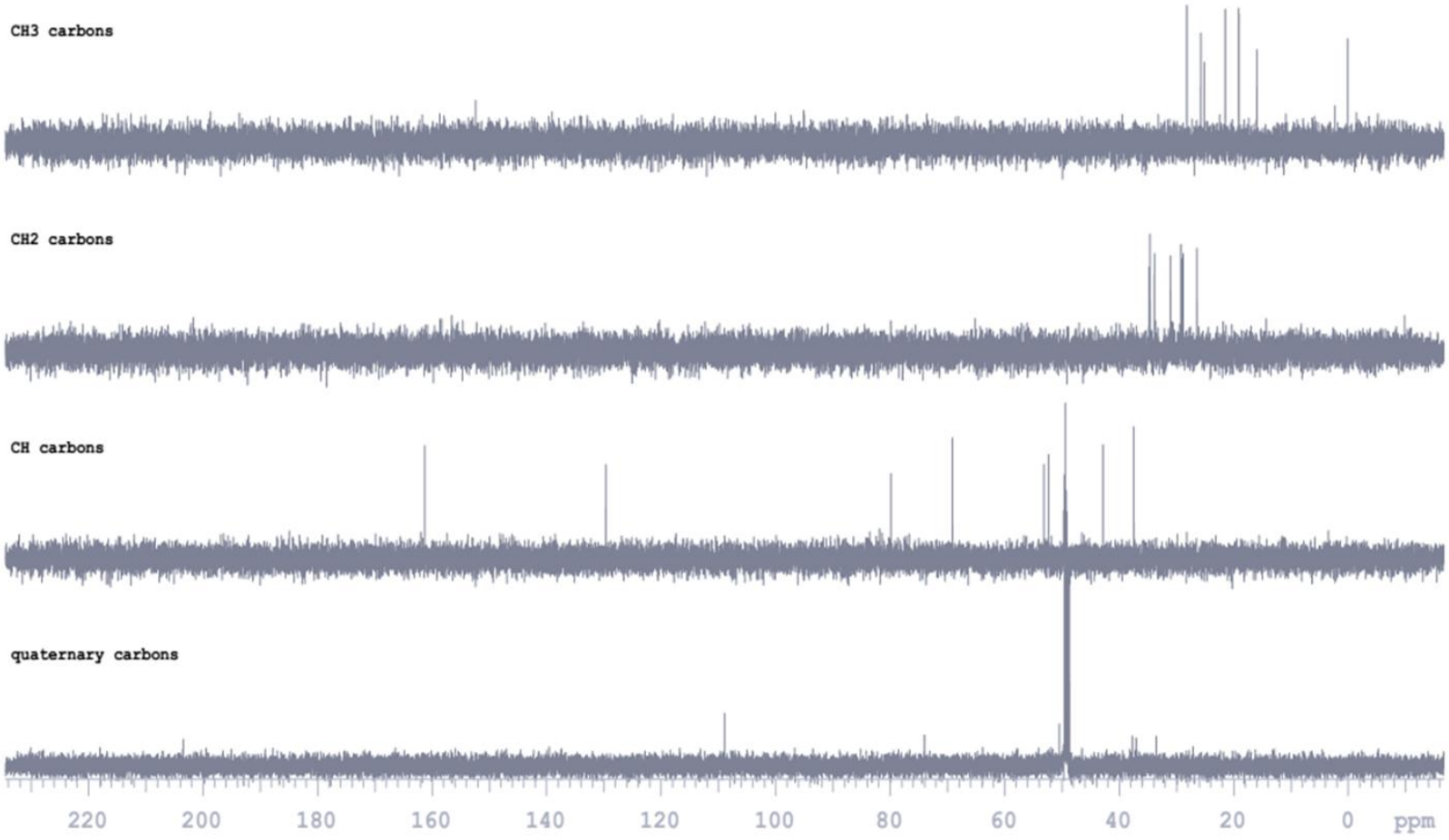
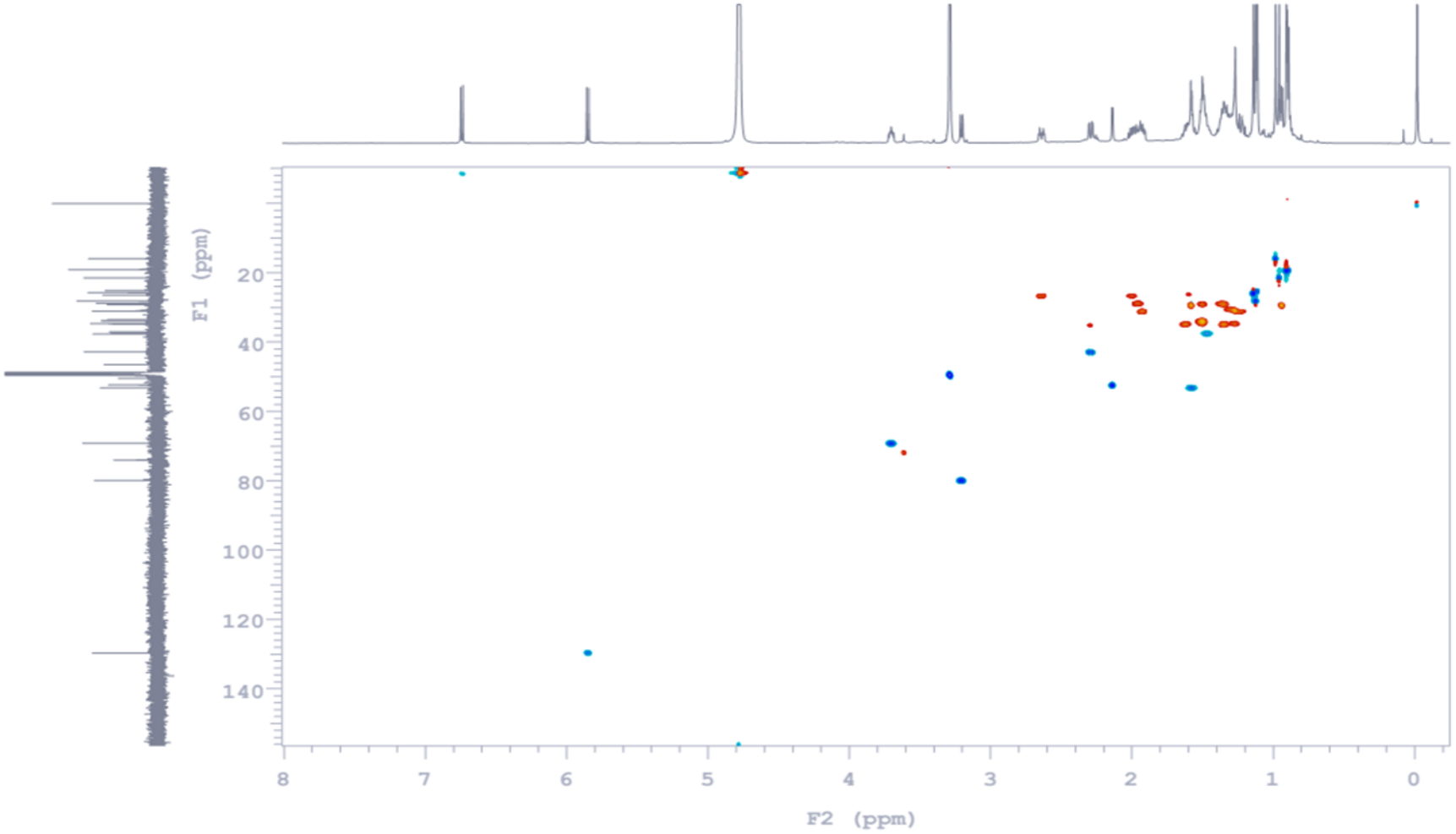


Figure S3. DEPT of Compound of **1**.


F1 (ppm)
20
40
60
80
100
120
140
8
7
6
5
4
3
2
1
0
F2 (ppm)

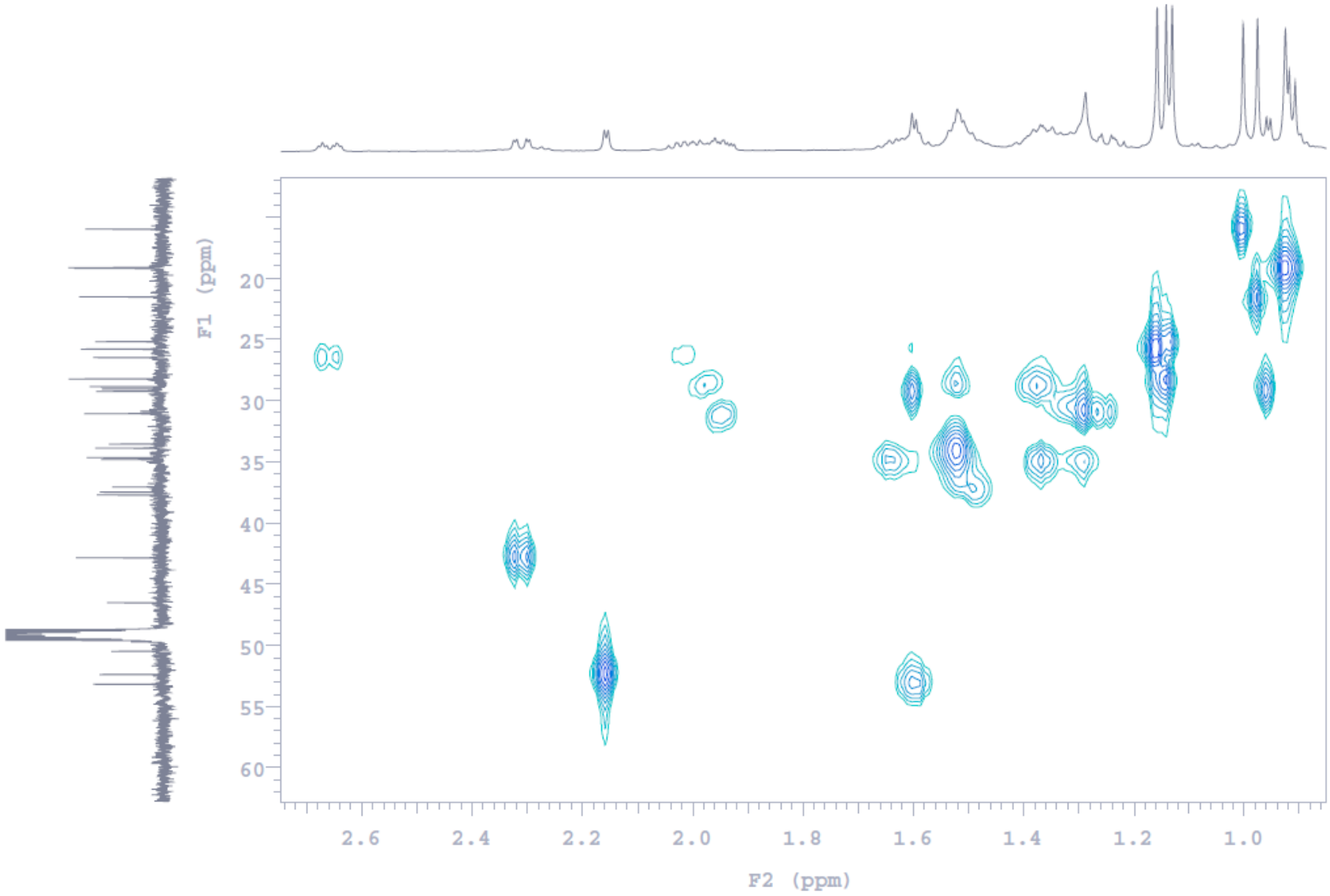

F1 (ppm)
20
25
30
35
40
45
50
55
60
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
F2 (ppm)

Figure S4. HSQC of Compound of **1**, with expanded spectrum.

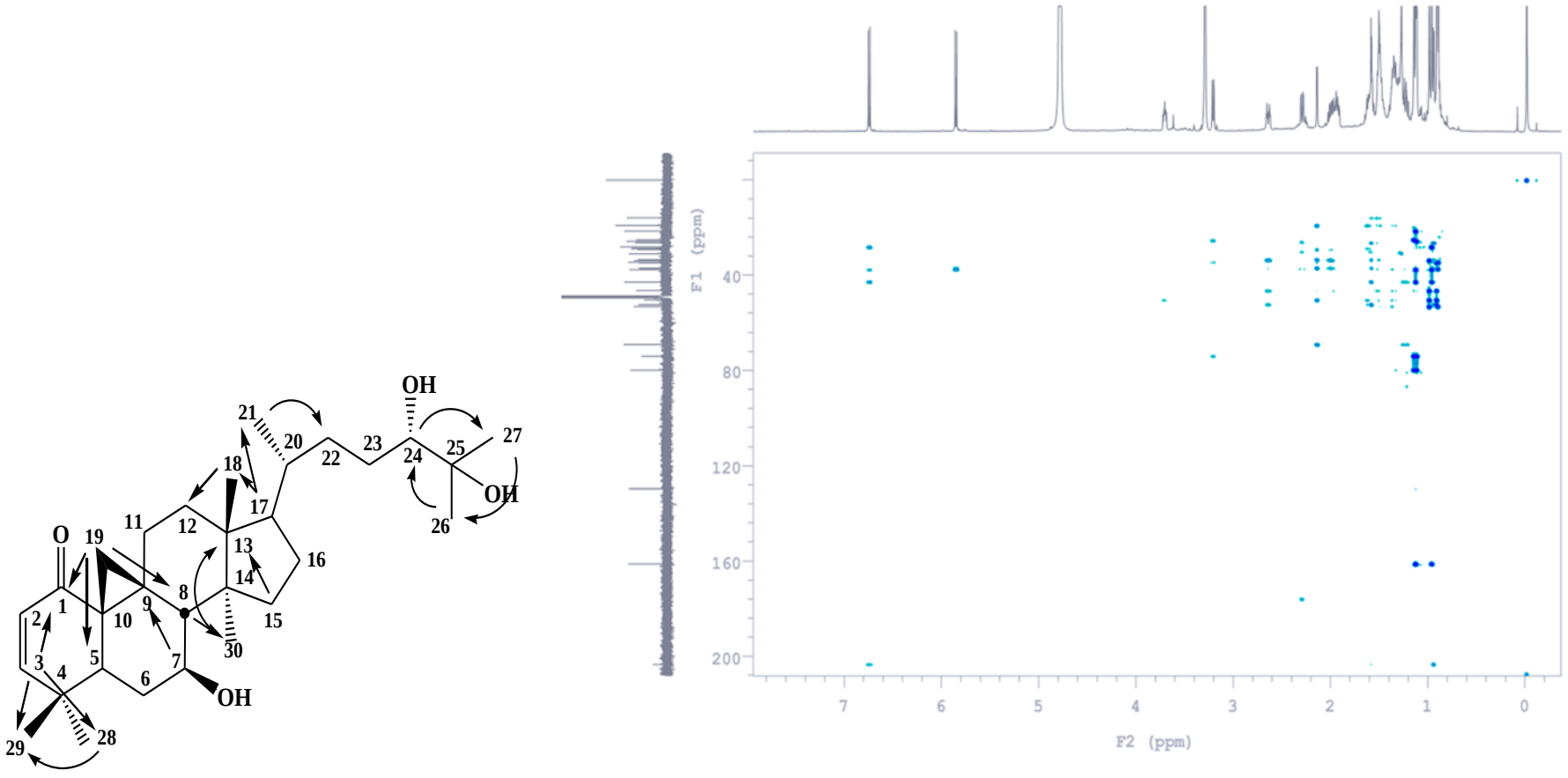


Figure S5. HMBC of Compound of **1**.

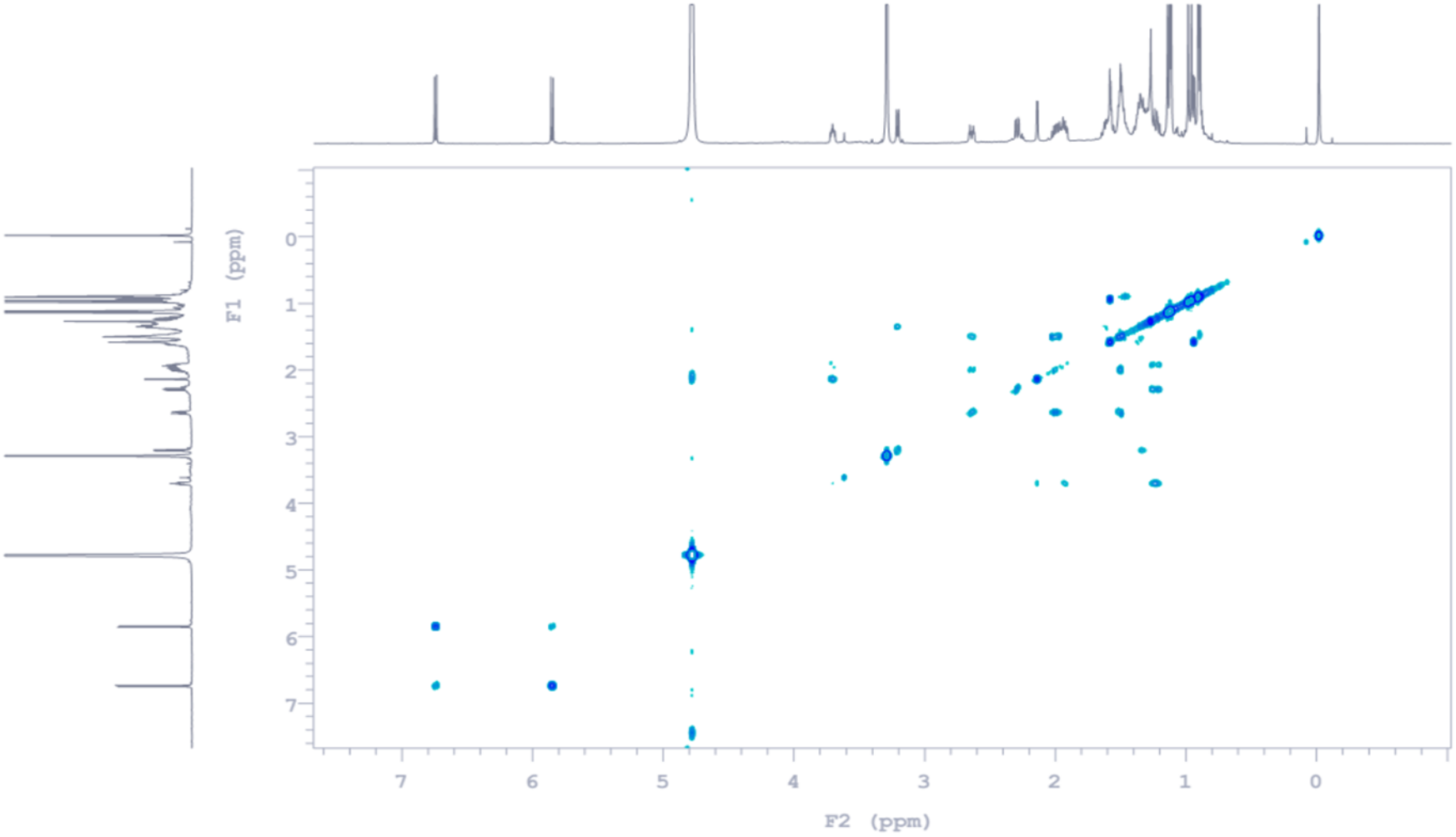
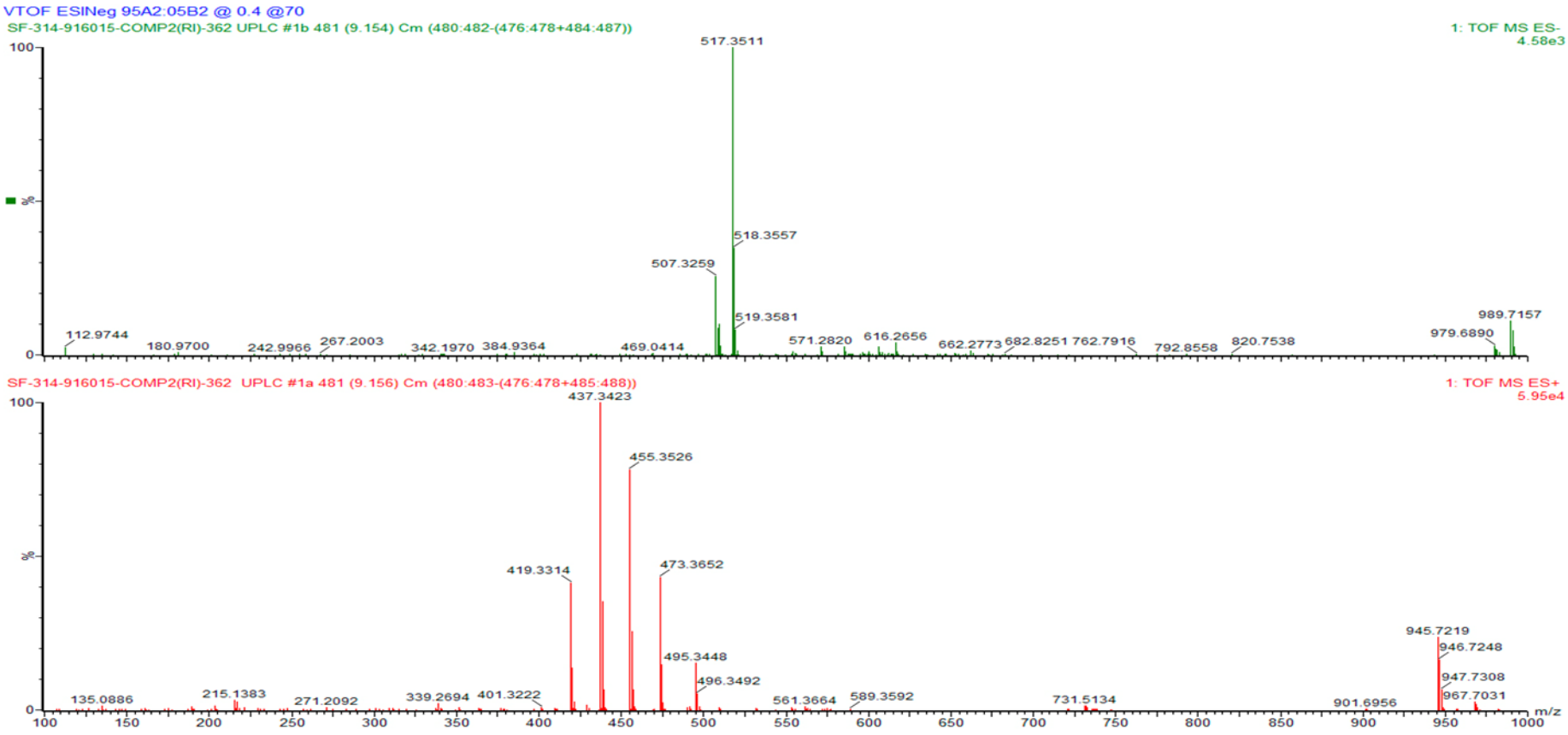


Figure S6. COSY of Compound of **1.**


VTOF ESINeg 95A2:05B2 @ 0.4 @70
SF-314-916015-COMP2(RI)-362 UPLC #1b 481 (9.154) Cm (480:482-(476:478+484:487))
1: TOF MS ES-
4.58e3
517.3511
518.3557
507.3259
519.3581
989.7157
112.9744
180.9700
242.9966
267.2003
342.1970
384.9364
469.0414
571.2820
616.2656
662.2773
682.8251
762.7916
792.8558
820.7538
979.6890
SF-314-916015-COMP2(RI)-362 UPLC #1a 481 (9.156) Cm (480:483-(476:478+485:488))
1: TOF MS ES+
5.95e4
437.3423
455.3526
419.3314
473.3652
945.7219
946.7248
495.3448
496.3492
947.7308
967.7031
135.0886
215.1383
271.2092
339.2694
401.3222
561.3664
589.3592
731.5134
901.6956
m/z

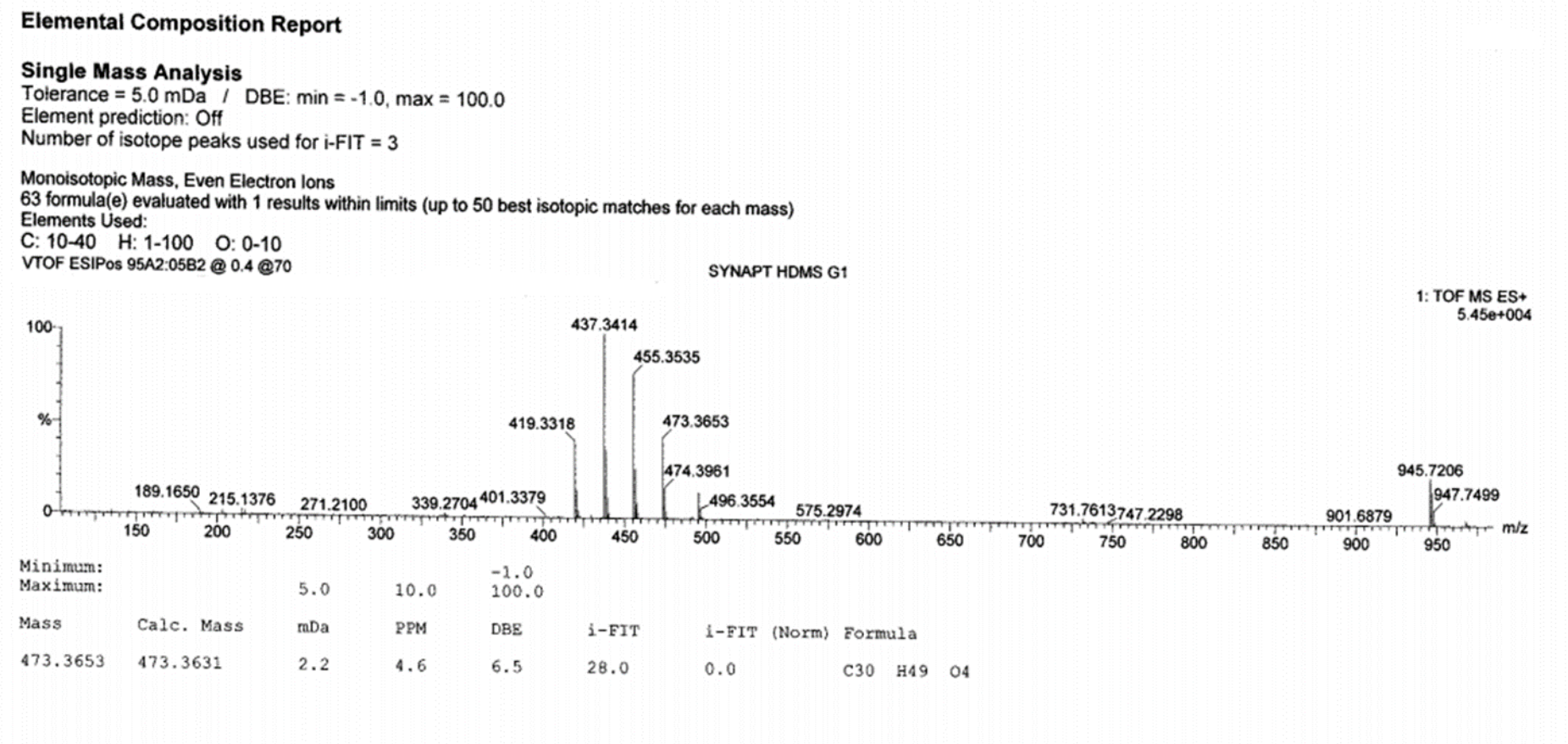


Figure S7. HRTOFMS spectrum (ESI^- and ESI^+) Compound of **1.**

Table S1. ^{1}H-^{13}C HMBC correlations of compound **1**.

Position	δ_H / ppm; *J* / Hz	δ_C / ppm	HMBC
1		203.49	
2	5.86 d (9.9)	129.66	C2
3	6.73 d (9.9)	161.31	C1, C5, C28, C29
4		37.06	
5		42.85	
6		28.85	
7	3.70	69.15	C9
8	2.14	52.39	C9, C13
9		50.49	
10		37.71	
11		34.81	
12		33.87	
13		46.52	
14		33.54	
15	1.58 2.64	26.47	C8, C13
16		31.06	
17		53.19	
18	0.98 s	15.97	C12, C17
19a	0.94 d (4.5)	29.23	C5, C8
19b	1.58 d (4.5)		
20		37.47	
21	0.89 d (6.6)	19.18	C17, C22

22		29.04	
23		34.66	
24	3.21	79.89	C22, C23, C27
25		74.04	
26	1.11 s	25.16	C24, C27
27	1.14 s	25.78	C24, C26
28	0.96 s	28.21	C29
29	1.12 s	21.51	C28
30	0.91 s	19.12	C8, C13

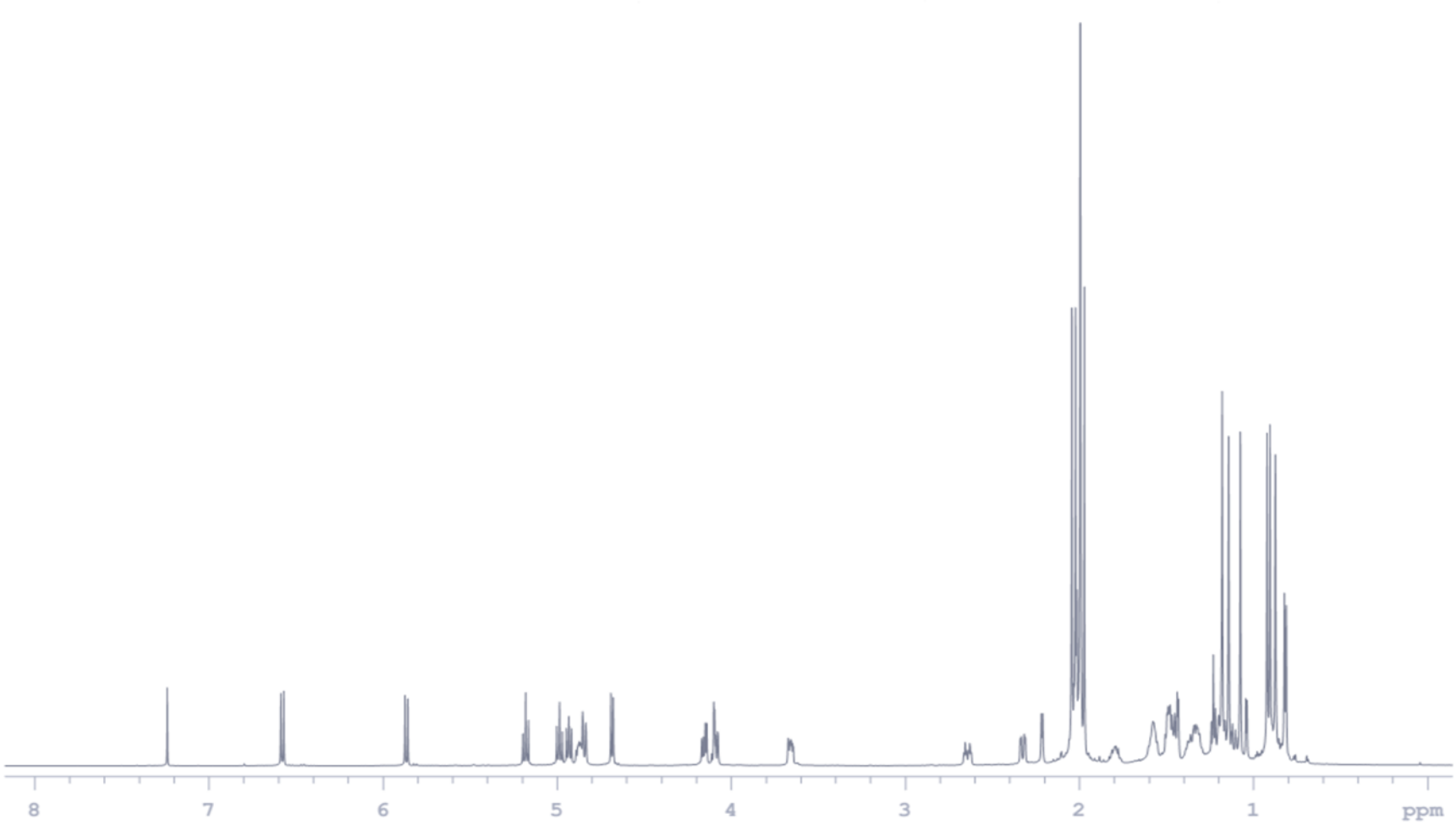


Figure S8. ^{1}H NMR Compound of **2.**

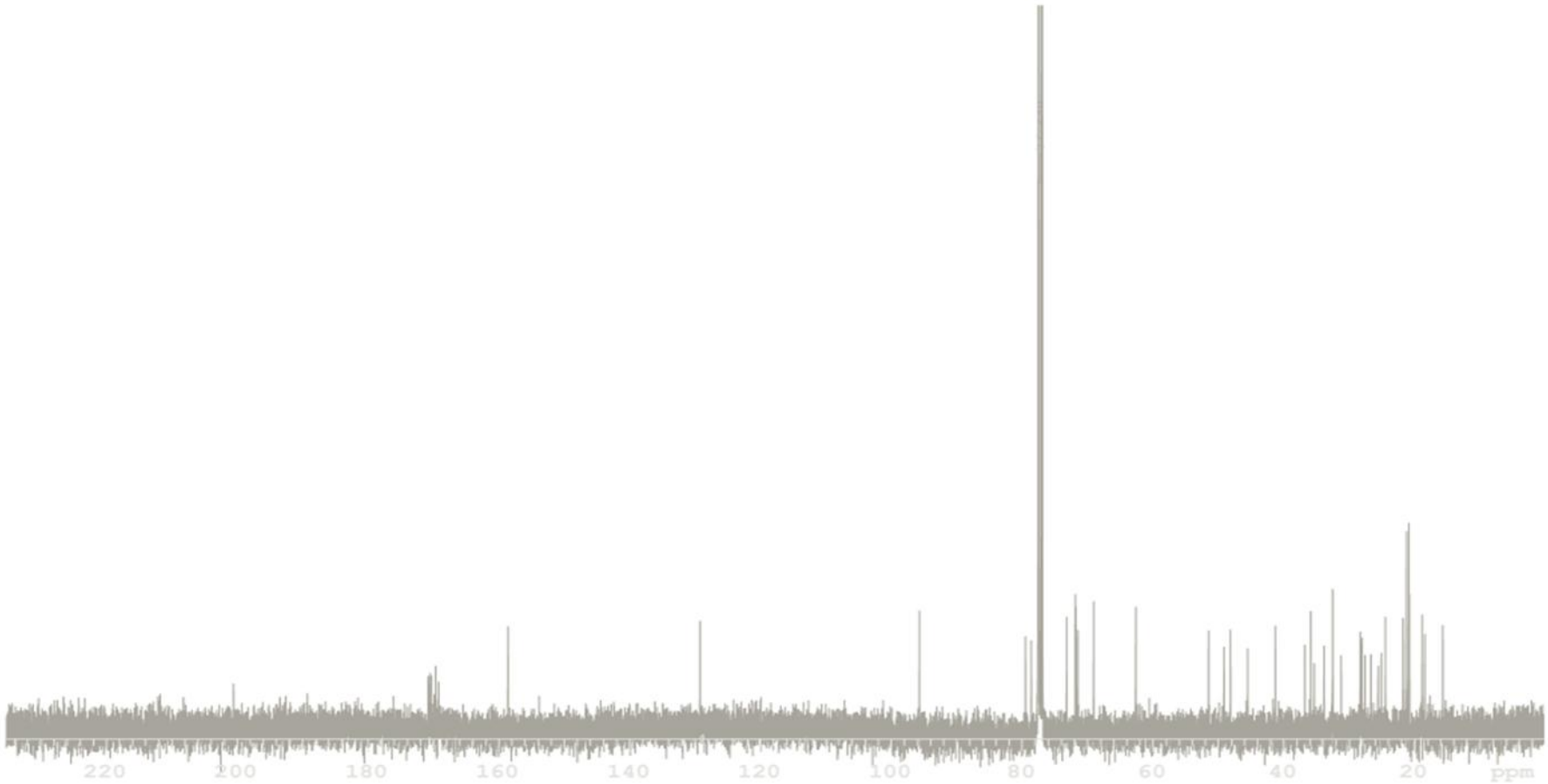


Figure S9. ^{13}C NMR Compound of **2.**

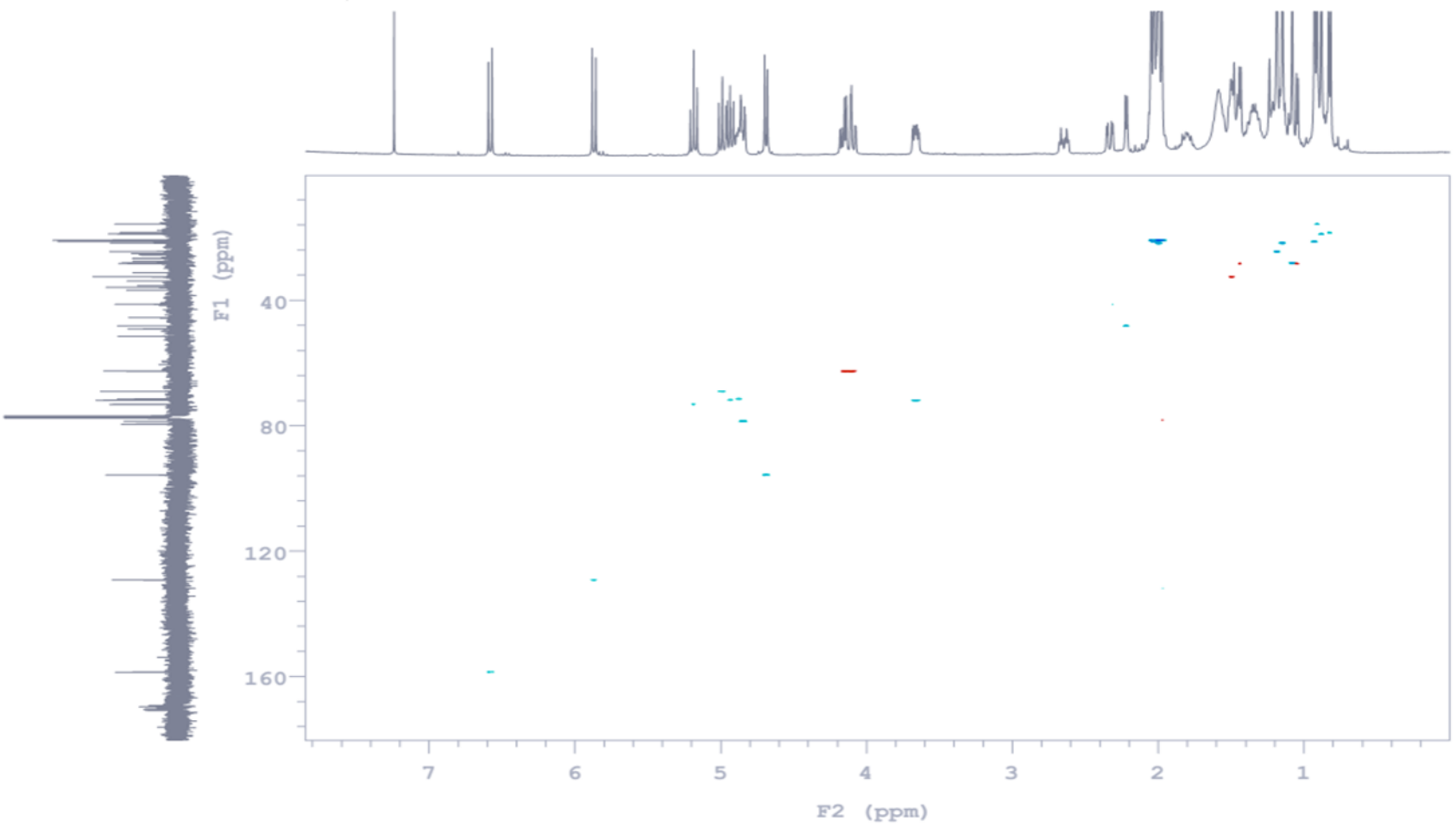


Figure S10. HSQC Compound of **2.**

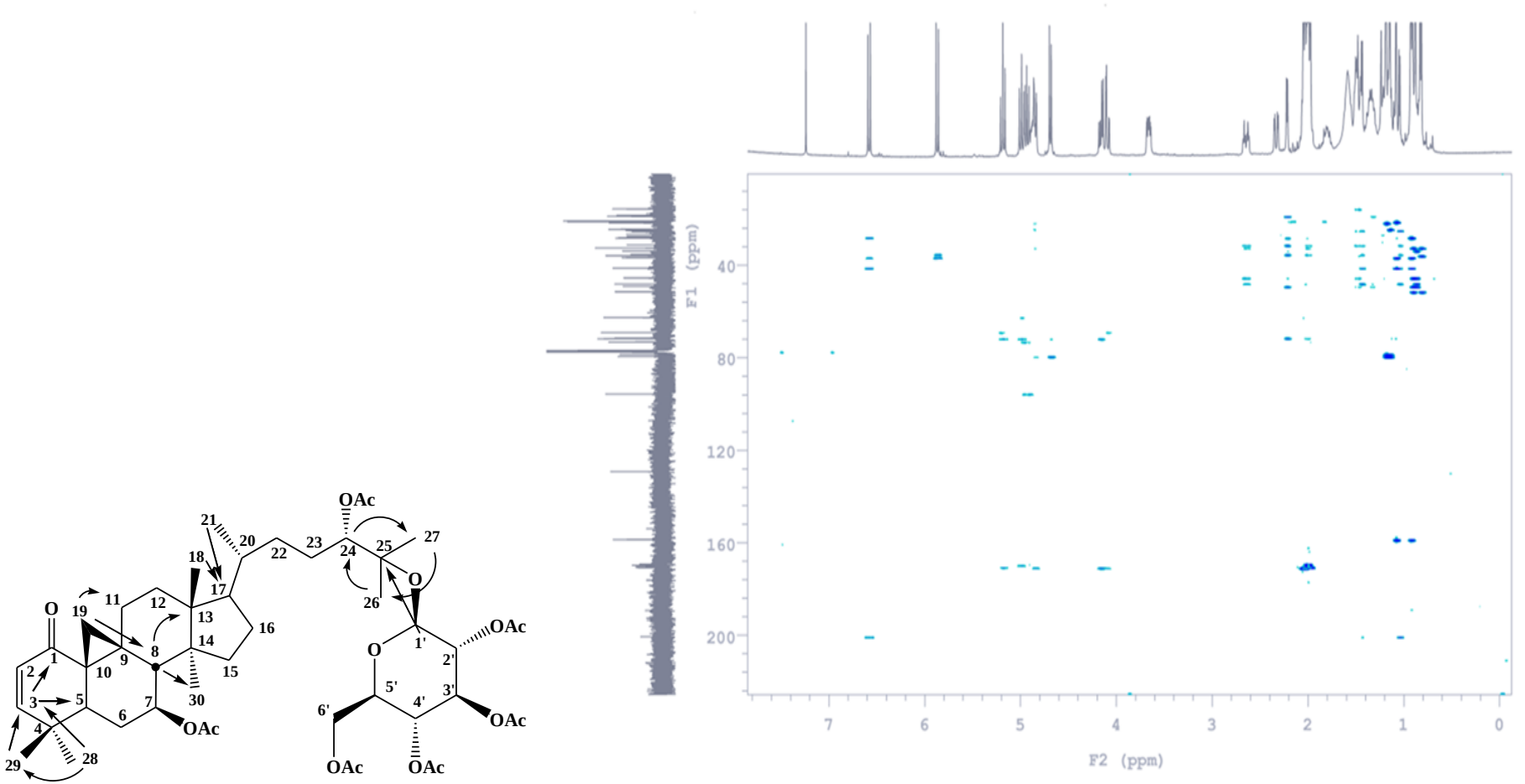


Figure S11. HMBC Compound of **2.**

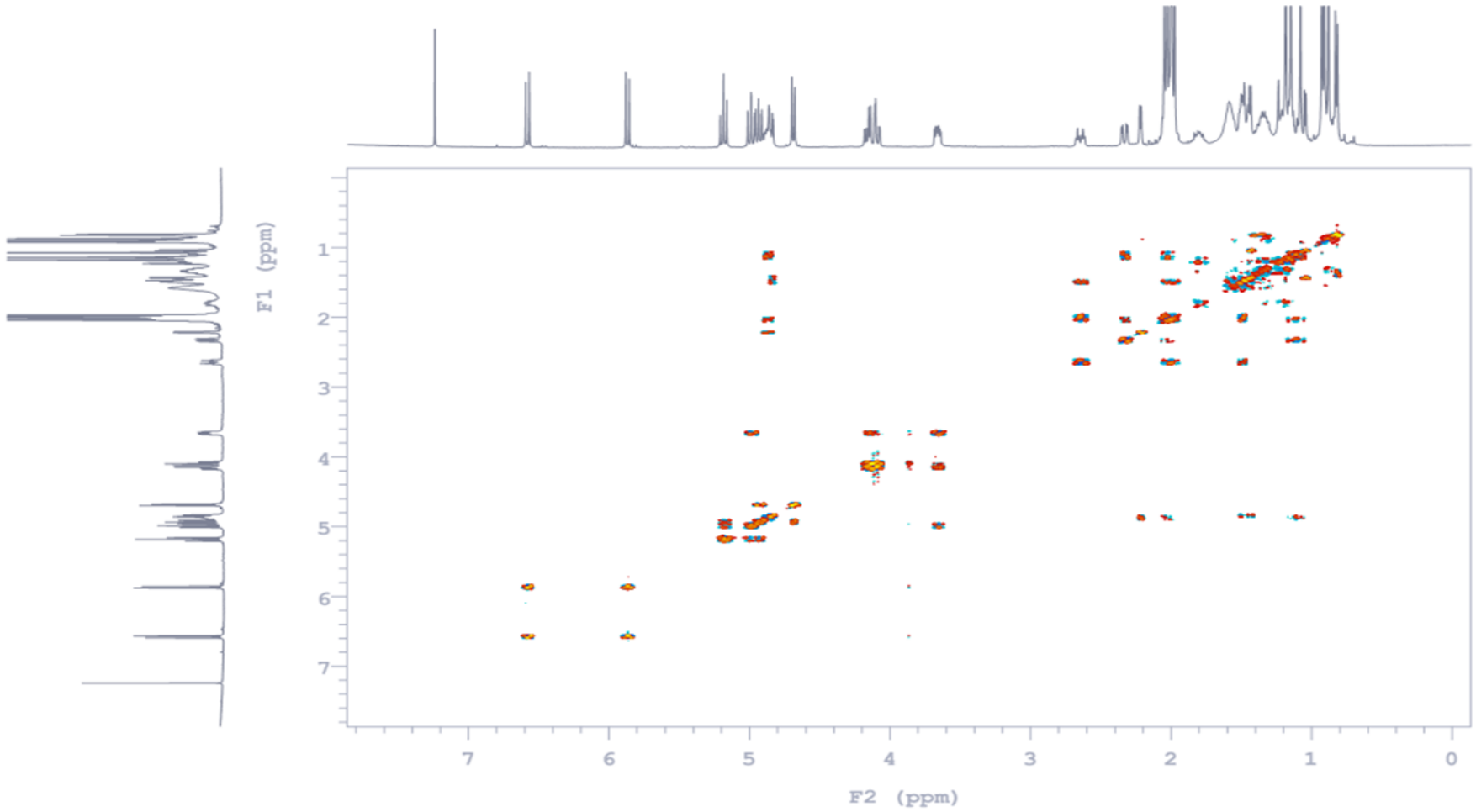
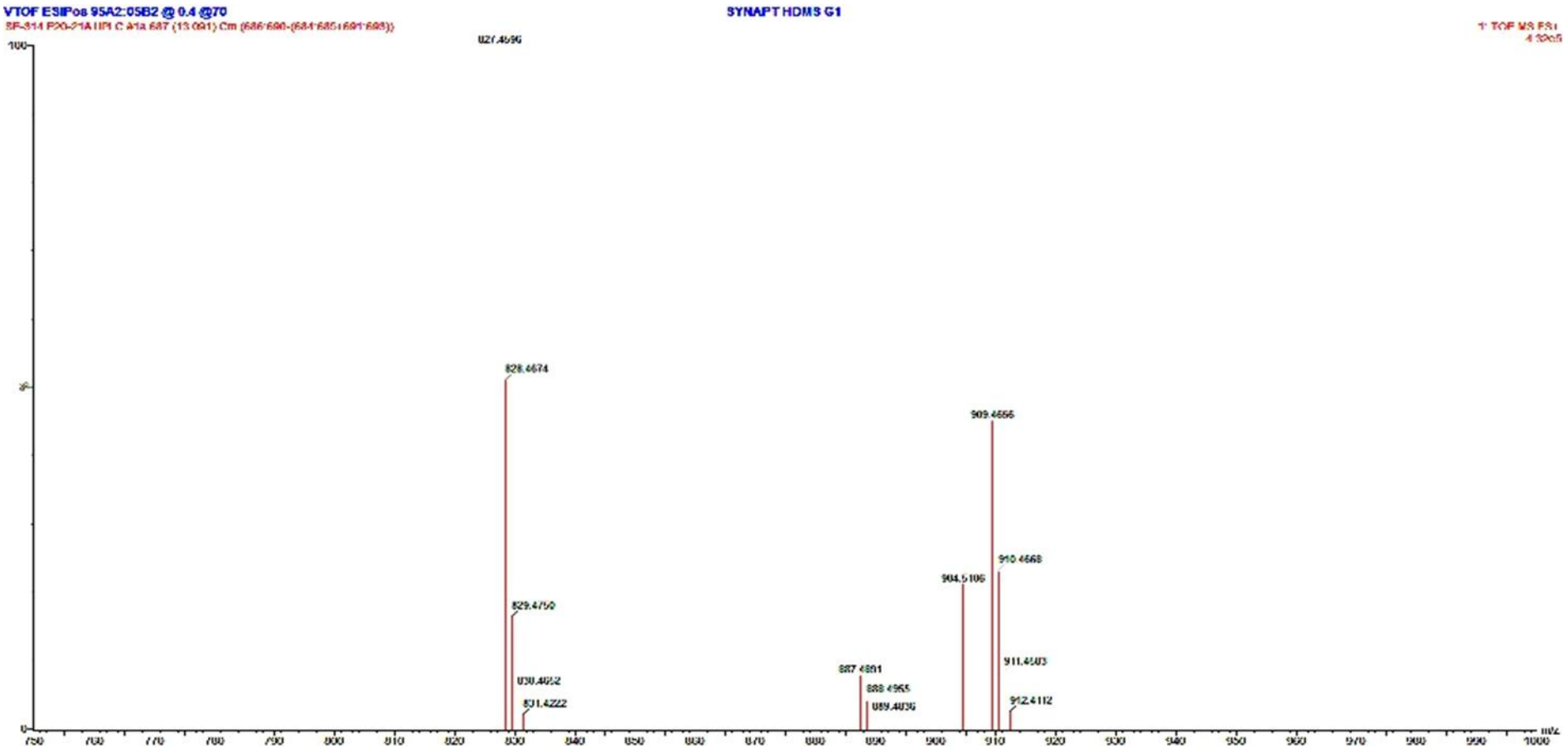


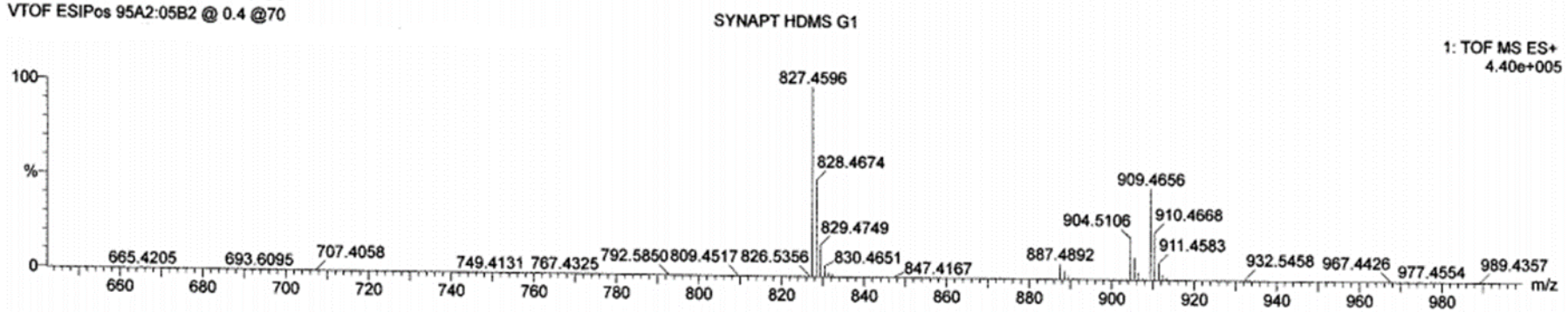
Figure S12. COSY Compound of **2.**


VTOF ESIPos 95A2:05B2 @ 0.4 @70
SYNAPT HDMS G1
827.4696
828.4674
829.4750
830.4852
831.4222
887.4891
888.4955
889.4036
904.5106
909.4656
910.4668
911.4503
912.4112
100
%
0
750
760
770
780
790
800
810
820
830
840
850
860
870
880
890
900
910
920
930
940
950
960
970
980
990
1000
m/z

Elemental Composition Report

Single Mass Analysis
Tolerance = 10.0 mDa / DBE: min = -1.5, max = 50.0
Element prediction: Off
Number of isotope peaks used for i-FIT = 3

Monoisotopic Mass, Even Electron Ions
118 formula(e) evaluated with 3 results within limits (up to 50 best isotopic matches for each mass)
Elements Used:
C: 40-60 H: 0-100 O: 10-30
VTOF ESIPos 95A2:05B2 @ 0.4 @70



Minimum:				-1.5			
Maximum:		10.0	10.0	50.0			
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	i-FIT (Norm)	Formula
887.4892	887.4793	9.9	11.2	13.5	24.3	0.2	C48 H71 O15
	887.4946	-5.4	-6.1	17.5	26.2	2.1	C52 H71 O12
	887.4852	4.0	4.5	4.5	27.0	2.9	C41 H75 O20

Figure S13. HRTOFMS spectrum (ESI+) of Compound of **2.**

Table S2. ^{1}H-^{13}C HMBC correlations of acetylated compound **2**.

Position	δ_H / ppm; J / Hz	δ_C / ppm	HMBC
1		200.58	
2	5.87 d (9.8)	129.25	C4, C10
3	6.58 d (9.8)	158.62	C1, C5
4		36.80	
5		41.31	
6		26.70	
7		71.49	
8	2.22	48.16	C10, C30, C13
9		31.24	
10		35.37	
11		25.10	C8
12		25.57	
13		49.15	
14		45.54	
15		33.86	
16		32.54	
17		51.51	
18	0.91	15.71	C17
19a	1.05		C5, C8, C11
19b	1.44	28.32	
20		35.89	
21	0.82 d (6.4)	18.44	C16, C22

22		25.57	
23		27.59	
24	4.85	78.62	C26, C27
25		79.51	
26	1.15 s	21.78	C27
27	1.19 s	24.48	C24
28	1.08 s	28.05	C3, C29
29	0.93 s	21.25	C3, C28
30	0.88 s	18.87	C7, C8,C9, C13
1′	4.69 d (8.0)	95.72	C2′, C25
2′		71.79	C1′
3′	5.185 t (9.4)	73.21	C2′
4′		69.08	C3′, C6′
5′		71.89	
6′a	4.09 dd (12.0, 2.4)	62.64	
6′b	4.16 dd (12.0, 5.6)		
Acetates			
1 (CH_3)	20.82		
2 (CH_3)	20.90		
3	169.23		
4	169.67		
5	170.32		
6	170.51		
7	170.79		

Table S3. 600 MHz ^{13}C-NMR data of compounds **1-4** in CD_3OD.

Position	1	2	3	4
1	203.49	213.5	200.58	72.75
2	129.66	48.9	129.25	31.57
3	161.31	78.4	158.62	77.85
4	37.06	40.2	36.80	37.97
5	42.85	38.8	41.31	46.76
6	28.85	31.0	26.70	31.27
7	69.15	69.5	71.49	77.09
8	52.39	51.4	48.16	53.87
9	50.49	30.8	31.24	135.82
10	37.71	40.6	35.37	85.36
11	34.81	29.1	25.10	122.36
12	33.87	34.0	25.57	38.33
13	46.52	46.6	49.15	46.19
14	33.54	50.6	45.54	46.57
15	26.47	34.5	33.86	35.05
16	31.06	28.9	32.54	29.31
17	53.19	53.2	51.51	51.70
18	15.97	15.8	15.71	15.61
19	29.23	24.9	28.32	42.77
20	37.47	37.5	35.89	37.17
21	19.18	19.2	18.44	18.94
22	29.04	34.8	25.57	29.06
23	34.66	29.1	27.59	34.82
24	79.89	78.9	78.62	78.07
25	74.04	81.7	79.51	81.67
26	25.16	23.2	21.78	22.73

27	25.78	21.9	24.48	23.17
28	28.21	24.9	28.05	27.00
29	21.51	22.7	21.25	23.93
30	19.12	19.1	18.87	20.32
1′		98.2	95.72	98.17
2′		75.5	71.79	75.51
3′		78.1	73.21	78.42
4′		71.8	69.08	71.85
5′		77.9	71.89	77.91
6′		62.9	62.64	62.94
Acetates				
1 (CH_3)			20.82	
2 (CH_3)			20.90	
3			169.23	
4			169.67	
5			170.32	
6			170.51	
7			170.79	