Supplemental material

ANTIBACTERIAL AND CYTOTOXIC ACTIVITIES OF UNDESCRIBED CASSIARIC ACID AND OTHER CONSTITUENTS FROM *CASSIA ARAEREH* STEM BARKS

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ABSTRACT:

A new lupane-type triterpene, 2*α*,3*β*-dihydroxylupan-29-oic acid (**1**), and one new ceramide derivative: (2*S**,2'*R**,3*S**,4*R**,5*R**,7'*E*,11*E*,12'*E*,20*E*)-*N*-[2'-hydroxyoctadeca-6,11-dienoyl]-2-aminohexacosa-11,20-diene-1,3,4,5-tetrol (**2**) were isolated from the ethyl acetate fraction obtained from the methanol extract of the stem barks of *Cassia arereh* together with seven known compounds. Their structures were characterized using two-dimensional NMR, mass spectrometry, and compared with reported data. To date, this is the first report of the isolation of a multiple double bonds sphingolipid type **(2)** from this genus**.** The ethyl acetate extract and selected isolates were examined for antimicrobial and cytotoxic activities *in vitro*. Betulinaldehyde (**5**) has shown to be active against all bacterial strains whereas, cassiaric acid (**1)** and betulinic acid (**6)** have demonstrated to be moderately active. In addition, cassiaric acid (**1**) showed the best cytotoxic result against HeLa and MCF-7 cell lines tested with IC_{50} 75.00 µM, while lupeol (**3)** and betulinic acid (**6)** displayed weak cytotoxicity at 100.00 µM.

Keywords: *Cassia arereh*, Fabaceae, cassiaric acid, lupane triterpenoid derivative, cassiaramide, sphingolipid, antimicrobial and cytotoxicity.

Experimental

General experimental procedures

Melting points were uncorrected and measured on a Mettler Toledo instrument. IR spectra were taken on a Jasco FT-IR-4600 type A spectrometer. GC-MSD analyses were carried out on a GCMS-QP2010SE (Shimadzu), and HRESIMS spectra were determined on microTOF-focus and microTOF-Q III mass spectrometers (Bruker). 1D and 2D NMR spectra were recorded on Bruker Avance III HD 400 (400 MHz, ^{1}H) or 500 (500 MHz, ^{1}H) instruments using deuterated solvent; the chemical shifts (δ) are reported in parts per million (ppm) using TMS as the internal reference. For the phytochemical study, silica gel 60 (230–400 mesh E. Merck, Darmstadt, Germany), sephadex LH-20 (25-100 μm Sigma Aldrich, South Africa) were employed for column chromatography and thin-layer chromatography (TLC) plates (silica gel 60 F254, thickness 0.2 mm) were obtained from Merck. The solvent mixing systems for elution were mainly CH_2Cl_2/MeOH for silica gel and pure MeOH for Sephadex LH-20.

Plant material

Stem barks of *C. arereh* Delile (Fabaceae) were collected at Yagoua in the North Region of Cameroon in April 2015 and identified by a botanist. A voucher specimen (N° 24093 SFRCAM) was deposited at the National Herbarium in Yaoundé, Cameroon.

Extraction and isolation

Air-dried stem barks of *C. arereh* (685.0 g) were powdered and extracted with methanol at room temperature for 48 h. After filtering to remove solids, the filtrate was concentrated to yield an extract (52.1 g). Approximately 50 g of this extract was suspended in MeOH–H_2O (2:8). The methanolic aqueous layer was partitioned with EtOAc and extracted (250 mL x 8) to afford (19.5 g) of a powder after concentration. The powder was eluted with CH_2Cl_2–MeOH of increasing polarity (90:10 to 5:1, MeOH) on a normal-phase silica gel column, to afford seven compounds (**2-8**). Compound **6** (40 mg) was washed with pentane, compounds **3** (11.5 mg), **7** (8.5 mg) and **8** (12.1 mg) with acetone, in order to purify them. Compound **2** (6.2 mg) and a solid containing **4** and **5** (18.2 mg) were also isolated. Further, this solid was purified by repeatedly Sephadex LH-20 with MeOH, until 12.9 and 3.8 mg of **5** and **4** were obtained respectively. The other sub-fractions were combined into five separate fractions (F_1–F_5) on the basis of TLC composition profile. Fraction F_2 (2.2 g) was subjected to repeated sephadex LH-20 with MeOH to afford precipitates that were filtered with acetone to give

compound **9** (18.4 mg). Fraction F_3 (250 mg) was also subjected to sephadex LH-20 column, eluting with MeOH to yield compound **1** (11.5 mg).

Dimethyl disulfide (DMDS) derivatives

The derivatives were prepared based on a previously described method (Khedr et al. 2018). Briefly, ceramide, **2** (1.0 mg) was dissolved in dimethyl disulfide (DMDS 0.5 mL), then iodine (1.0 mg) added. The resulting mixture was kept at room temperature for 24 h. Subsequently, the reaction was quenched with aqueous $Na_2S_2O_3$ (5%), and extracted with *n*-hexane (3 x 5 mL); the organic layer was concentrated to provide the mixture of DMDS derivatives.

*Methanolysis of compound **2***

Compound **2** (2.0 mg) was subjected to methanolysis in 2.5 mL of 1N HCl in MeOH at 80 °C for 18 h. Upon cooling, H_2O (10 mL) was added to the reaction mixture (Khedr et al. 2018); the aqueous layer extracted with n-hexane (3 × 5 mL) and concentrated to provide a fatty acid methyl ester (FAME).

Spectroscopic data of compounds

Cassiaric acid (**1**): White solid; m.p. 230-232 °C ; $[\alpha]_D^{21}$ = + 26.9 (*c* 0.1 MeOH); UV (MeOH) λ_{max}(log ε) 206 (3.18), 219 (3.21), 295 (2.54), 333 (2.08) nm; IR (KBr) ν_{max} 3467, 2942, 1672, 1457, 1376, 1239, 1035, 885, 722 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1 ; HRESIMS *m/z* = 473.3649 $[M-H]^-$ (calcd. for $C_{30}H_{49}O_4$: 473.3650),

Cassiaramide (**2**): White amorphous powder; m.p. 123-125 °C ; $[\alpha]_D^{21}$ = + 16.9 (*c* 0.1 $CHCl_3$ /MeOH), IR (KBr) ν_{max} 3331; 1622; 1544; 1067; 1022; 964; 722 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2 ; HRESIMS *m/z* 718.6014 $[M-H]^-$ (calcd. for $C_{44}H_{80}NO_6$: 718.6017).

Antimicrobial assay

To determinate the antibacterial activity of extract and metabolites, Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were assayed against four clinical isolates of Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Haemophilus influenza*, *Klebsiella pneumonia*), using the microdilution method following CLSI guidelines (CLSI, 2013).

The strains have been given by Pr Gonshu Hortense, Head of Laboratory of Bacteriology (Centre Hospitalier Universitaire de Yaoundé) and maintained in the Microbiology Laboratories at the University of Yaoundé I, Yaoundé, Cameroon. Extract, pure compounds and antibiotic standard (levofloxacin) were accurately weighed and dissolved in a minimum

amount of DMSO. For each sample, a stock solution of crude extract was prepared at 100,000 µg/mL, pure compounds/Levofloxacin at 1,000 µg/mL. From 100 µL of each stock solution, a series of geometric dilutions of reason 1/2 were made in 100 µL of broth contained in the wells of the microplate. The series of dilutions allowed us to obtain a concentration range from 50,000 µg/mL to 48.8 µg/mL for the plant extract and 500 to 0.488 µg/mL for the compounds/Levofloxacin. 100 µL of loading inoculum 10^6 CFU/mL were subsequently seeded. The final ranges of test concentrations were 25,000 - 24.4 µg/mL (extract) and 250-0.244 µg/mL (pure compounds/Levofloxacin). Two controls were added to the series, a positive control (Mueller Hinton broth and microorganism) and a negative control (Mueller Hinton broth and plant extract or compound solution). The tests were carried out in triplicate and the microplates incubated for 24 h at 37°C. 10 µL of a 0.2% (w/v) resazurin sodium salt solution was added to each well and the plates were re-incubated for 2 h. Visual change of the solution from blue to pink indicated that the bacteria were still alive.

MIC was determined as the lowest concentration of sample that inhibited visible bacterial growth and where no color change could be observed and the MBC was the lowest concentration of antimicrobial agent that kills more than 99.9% of the initial bacterial population and when no visible growth of the bacteria was observed on the plates (CLSI, 2013).

Cytotoxicity assay

The cytotoxic activities of the isolated compounds were evaluated human cervical HeLa and human breast MCF-7 cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983), according to the published procedure. After treatment, MTT was added to all samples with at a concentration of 5 mg/ml.

HeLa cells were seeded in 96-well format at 10 000 cells per well and incubated for 24 hours to allow for attachment. Cells were treated with compounds in duplicate using various protocols: 24- and 48-hour treatments of **6** at 75 µM, Only a 48-hour treatment of **9** at 5 µM and 150 µM, 75 µM and 5 µM treatments of the remaining compounds for both 24- and 48-hours Vehicle control samples were treated with 0.1% (v/v) DMSO for 24- and 48-hours. This was the commonly recommended concentration, which did show to mostly maintain 100% cell viability which will be used in further experiments as a reference for a negative result of cytotoxicity (Komissarova et al. 2005; Evans et al. 2019). MCF-7 cells were also seeded at 10 000 cells per well in 96-well plates and incubated for 24 hours. Cells were treated with all 10 compounds at 100, 75, 50, 25 and 5 µM in technical duplicates for 24- and 48-hours respectively. 0.1 % DMSO was used to treat cells for 24- and 48-hours, acting as the vehicle

control. The positive control was set-up at 24- and 48-hour exposures of 1, 0.75 and 0.5 µM of camptothecin. Cells were incubated at 37 °C for 4 hours to allow time for cells to metabolize MTT into formazan product. Formazan was solubilized in DMSO and absorbances measured at 570 nm and analyzed using the SpectraMax Paradigm Microplate reader. The percentages of cell viabilities (percentage of cells remaining after treatment) were calculated using Microsoft Excel using the following formula:

$$\%\ Cell\ Viability = \frac{Absorbance\ (treated\ samples) - Absorbance\ (blank)}{Absorbance\ (untreated\ samples) - Absorbance\ (blank)} \times 100$$

The averages and standard deviations of individual cell viability results from all biological replicates were determined where possible in Excel (data represented as bar graphs in results and discussion). camptothecin was used as the positive control.

Table S1. ^{1}H and ^{13}C NMR spectroscopic data for compound **1** in DMSO.

Position	**1**	
	δ_C	δ_H (J in Hz)
1	42.0	1.08-1.11 (m)[1]
		1.46-1.48 (m)[1]
2	65.2	3.75-3.80 (m)[1]
3	78.2	3.14[2] bd (2.5)
4	38.3	-
5	47.8	1.09-1.12 (m)[1]
6	17.8	1.09-1.12 (m)[1]
		1.32-1.35 (m)[1]
7	34.1	1.31-1.35 (m)[1]
8	40.8	-
9	49.6	1.30-1.35 (m)[1]
10	38.2	-
11	23.5	1.69-1.71 (m)[1]
		-
12	26.6	1.33-1.35 (m)[1]
		1.52-1.55 (m)[1]
13	37.4	1.62-1.65 (m)[1]
14	42.9	-
15	20.6	1.31-1.35 (m)[1]
		1.45-1.47 (m)[1]
16	35.4	1.22-1.26 (m)[1]
		1.41-1.44 (m)[1]
17	42.9	-
18	48.2	1.37-1.39 (m)[1]
19	43.0	1.70-1.71 (m)[1]
20	41.6	2.57-2.59 (m)[1]
21	27.1	0.91-0.94 (m)[1]
		1.61-1.65 (m)[1]
22	39.6	1.00-1.08 (m)[1]

		1.25-1.29 (m)[1]
23	29.1	0.85 s
24	21.9	0.75 s
25	16.0	0.97 s
26	17.1	0.81 s
27	14.5	0.86 s
28	17.9	0.70 s
29	177.1	-
		-
30	17.5	1.00 d (6.9)

[1] Overlapped signal. [2] Broad signal

Table S2. ^{1}H and ^{13}C spectroscopic data for compound **2** in $CDCl_3$ and CD_3OD.

Position	**2**	
	δ_C	δ_H (J in Hz)
1	60.8	3.77 dd (4.6, 9.9)
2	51.4	4.12 dddd (3.6, 4.4, 8.6, 8.8)
3	74.9	3.56-3.58 (m)[2]
4	72.0	3.52-3.54 (m)[2]
5	72.1	3.54-3.57 (m)[2]
6	31.9	1.67-1.69 (m)[1]
		1.40-1.42 (m)[1]
7	24.9	1.42-1.44 (m)[1]
8	29.2	1.31-1.35 (m)[1]
9	29.3	1.27-1.29 (m)[1]
10	32.4	2.02-2.08 (m)[1]
11	130.4	5.40 (brs)
12	129.9	5.40 (brs)
13	32.3	1.97-2.00 (m)[1]
14	29.3	1.27-1.29 (m)[1]
15-18	29.3	1.27-1.29 (m)[1]
19	32.4	2.02-2.08 (m)[1]
20	129.9	5.40 (brs)
21	130.4	5.40 (brs)
22	32.3	1.97-2.00 (m)[1]
23	29.2	1.11 (brs)
24	31.4	1.28-1.31 (m)[1]
25	22.4	1.27-1.29 (m)[1]
26	13.4	0.89 t (6.7)
NH	-	7.38 d (8.0)

1′	175.6	-
2′	71.6	4.04 dd (3.6, 8.0)
3′	34.4	1.77-1.80 (m)[1]
		1.61-1.63 (m)[1]
4′	24.9	1.44-1.45 (m)[1]
5′	29.3	1.28-1.30 (m)[1]
6′	32.4	2.02-2.08 (m)[1]
7′	130.4	5.40 (brs)
8′	130.1	5.40 (brs)
9′	32.3	1.97-2.00 (m)[1]
10′	29.4	1.76-1.79 (m)[1]
11′	32.4	2.02-2.08 (m)[1]
12′	130. 4	5.40 (brs)
13′	130.1	5.40 (brs)
14′	32.3	1.97-2.00 (m)[1]
15′	29.3	1.27-1.29 (m)[1]
16′	31.5	1.27-1.29 (m)[1]
17′	22.5	1.29-1.31 (m)[1]
18′	13.4	0.89 t (6.7)

[1] Overlapped signal. [2] Broad signal

Table S3. Inhibition parameters (MIC and MBC) (μg/mL) of pure compounds and MeOH extract on bacterial strains.

Samples	Bacteria strains											
	Escherichia coli			*Klebsiella pneumoniae*			*Pseudomonas aeruginosa*			*Haemophilus influenza*		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
1	**62.50**	**62.5**	1.00	**62.50**	125	2.00	**15.63**	**15.63**	1.00	**62.50**	125	2.00
5	**31.25**	125	4.00	**31.25**	**62.5**	2.00	**15.63**	**62.50**	4.00	125	125	1.00
6	**62.50**	125	2.00	**31.25**	**62.5**	2.00	125	125	1.00	**62.50**	125	2.00
Extract	3125	12500	4.00	3125	6250	2.00	1563	6250	4.00	6250	12500	2.00
Levofloxa-cine	3.91	7.81	2.00	7.81	62.5	8.00	7.81	15.63	2.00	7.81	15.63	2.00

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration. In bold are values of significant activity (Kuete, 2010).

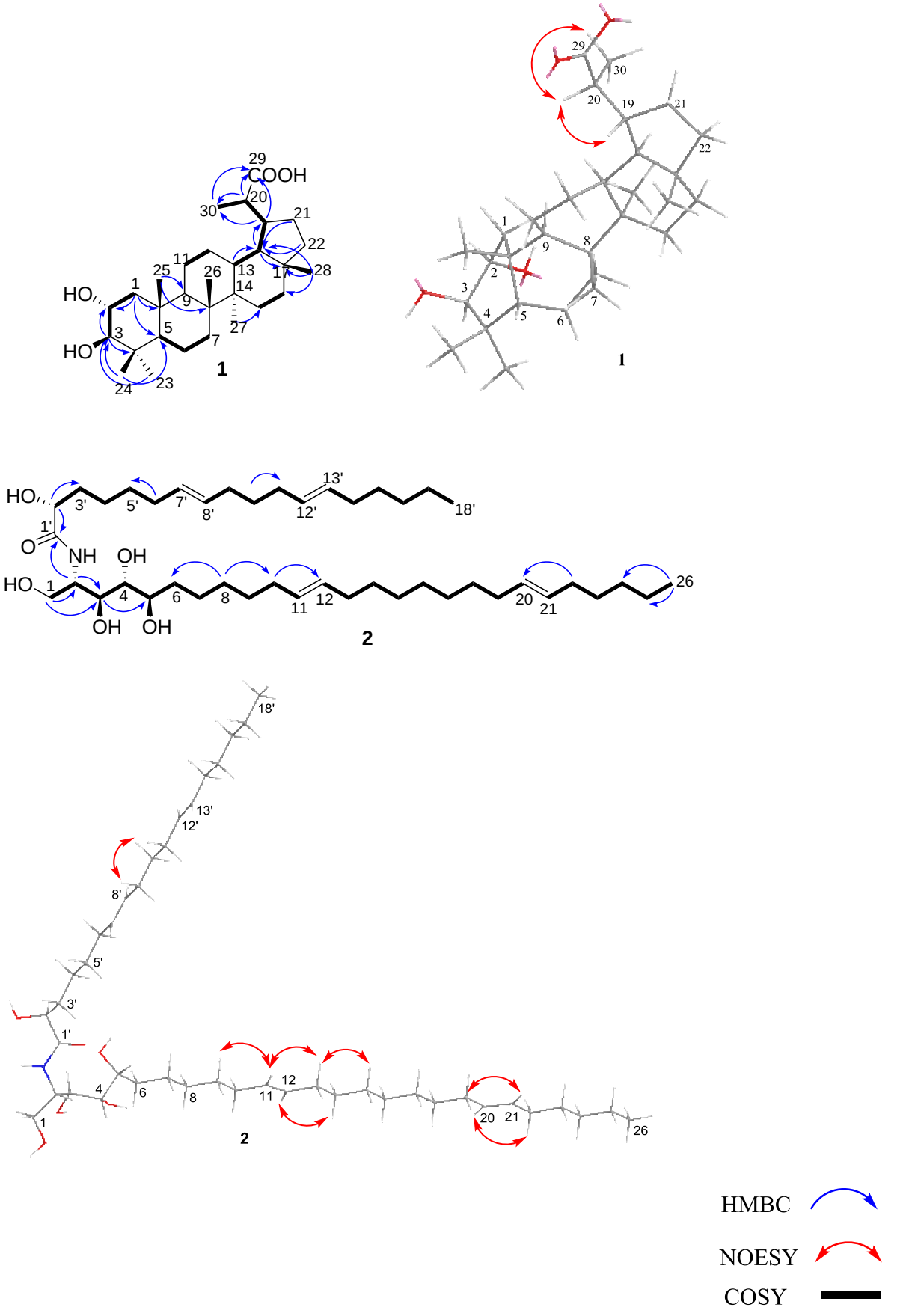


Figure. S1. Key 2D NMR correlations of cassiaric acid **1** and cassiaramide **2.**

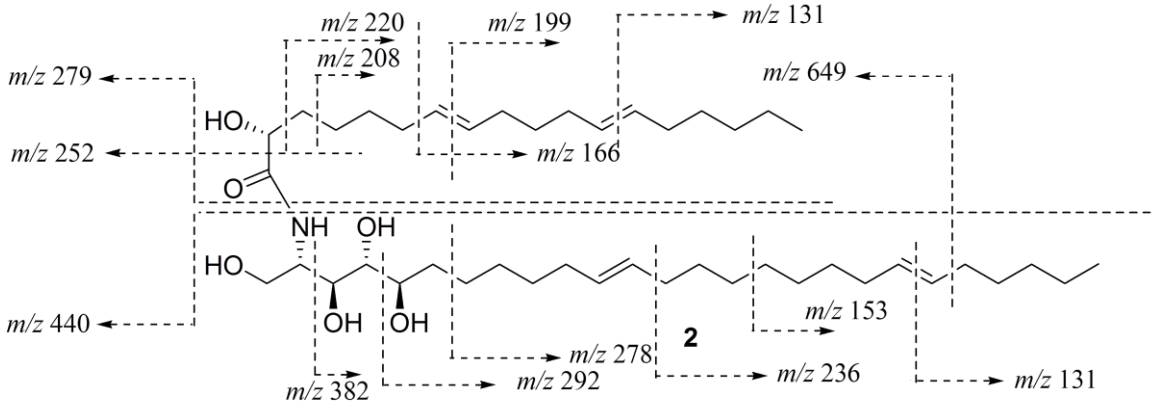


Figure S2. Possible fragmentation pattern of **2,** included methylthiolated ions m/z= 199 and 131**).**

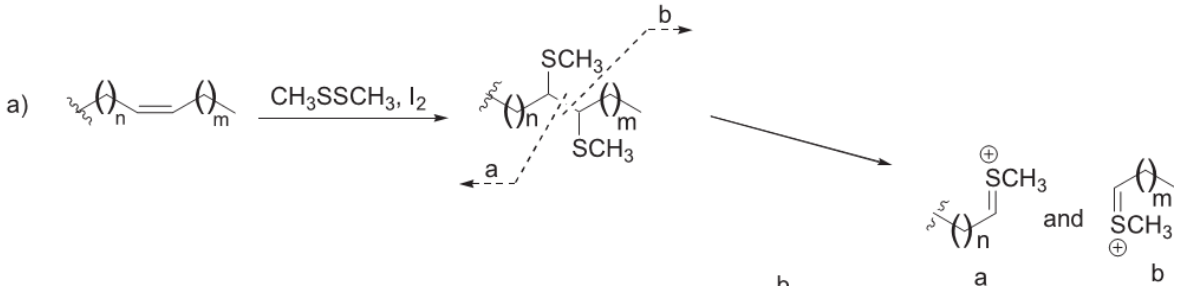
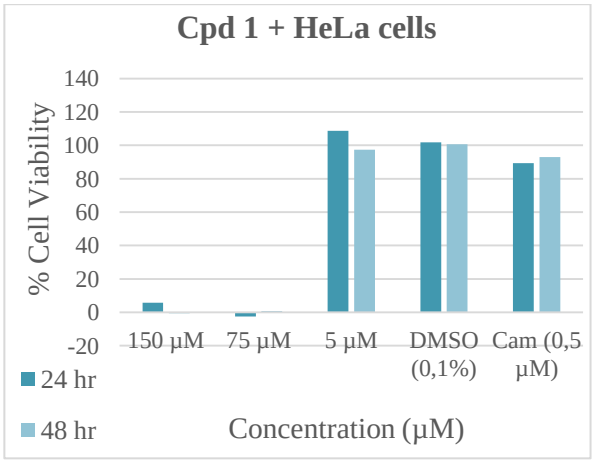
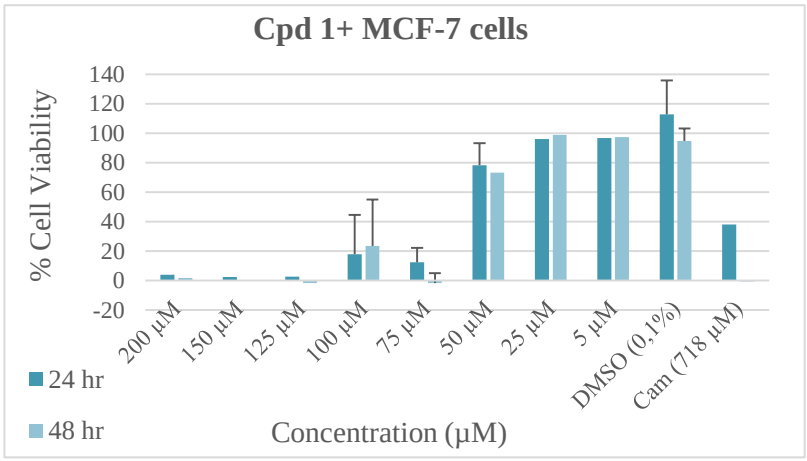
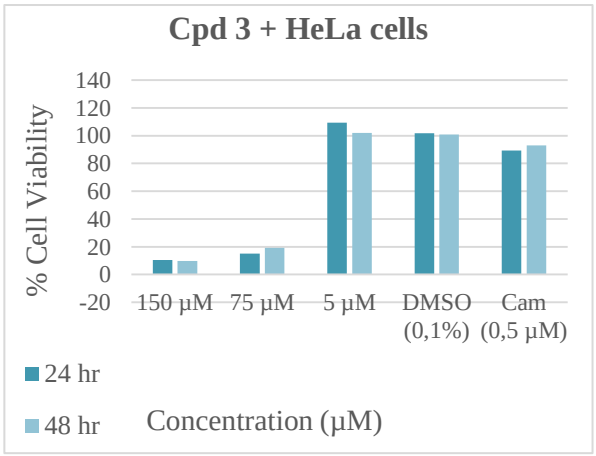
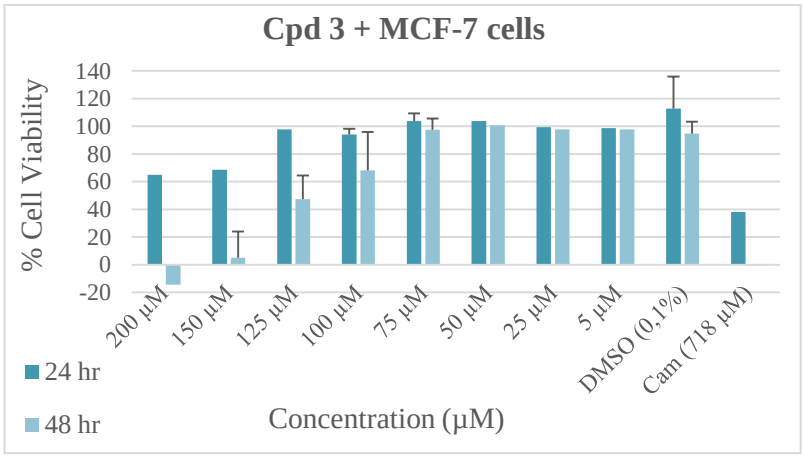


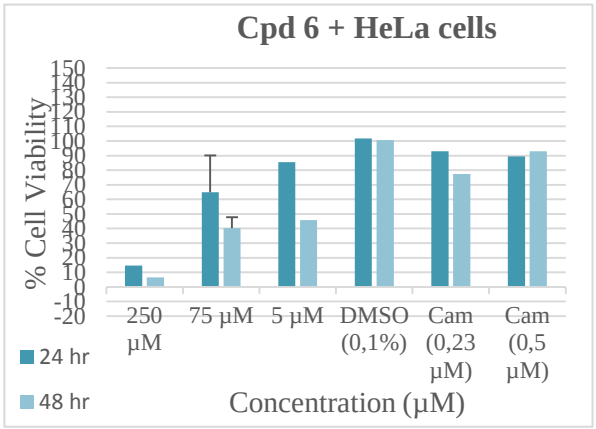
Figure S3. Mass Spectra fragmentation of methylthiolated alkenyl chains of ceramide **2**.

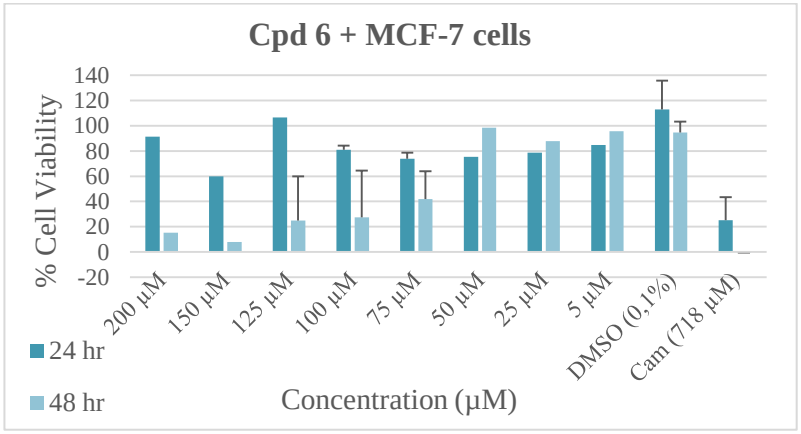


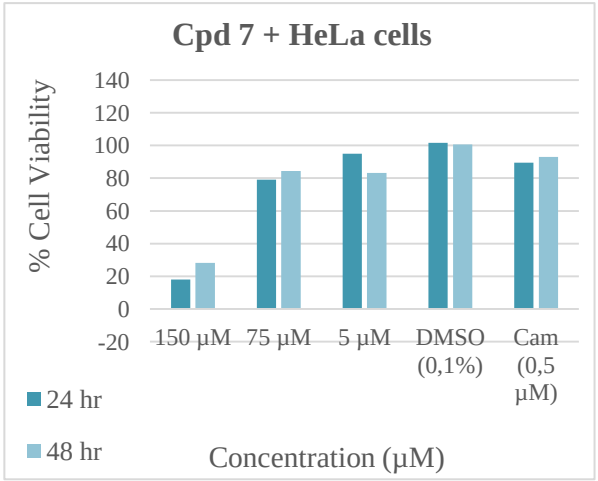


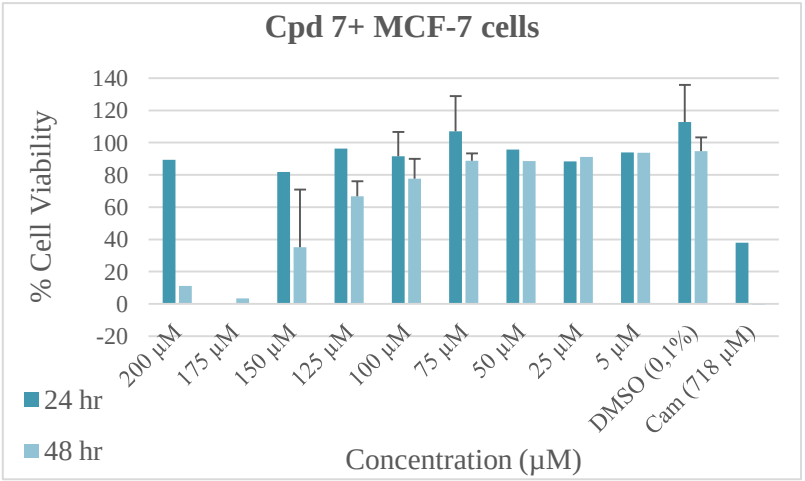

Cpd 3 + HeLa cells
% Cell Viability
140
120
100
80
60
40
20
0
-20
150 µM
75 µM
5 µM
DMSO (0,1%)
Cam (0,5 µM)
24 hr
48 hr
Concentration (µM)

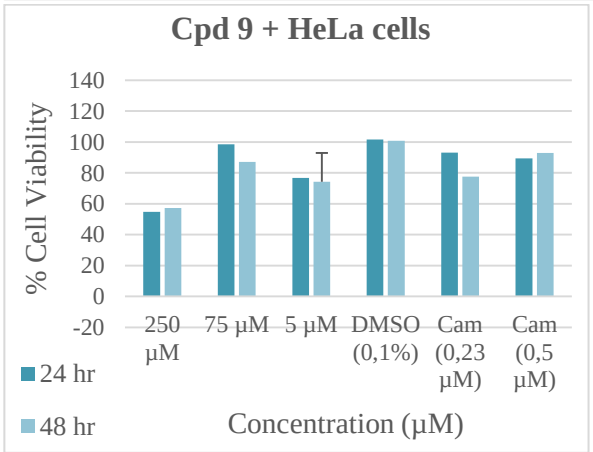

Cpd 3 + MCF-7 cells
% Cell Viability
140
120
100
80
60
40
20
0
-20
200 µM
150 µM
125 µM
100 µM
75 µM
50 µM
25 µM
5 µM
DMSO (0,1%)
Cam (718 µM)
24 hr
48 hr
Concentration (µM)


Cpd 6 + HeLa cells
% Cell Viability
250 µM
75 µM
5 µM
DMSO (0,1%)
Cam (0,23 µM)
Cam (0,5 µM)
24 hr
48 hr
Concentration (µM)


Cpd 6 + MCF-7 cells
% Cell Viability
200 µM
150 µM
125 µM
100 µM
75 µM
50 µM
25 µM
5 µM
DMSO (0,1%)
Cam (718 µM)
24 hr
48 hr
Concentration (µM)


Cpd 7 + HeLa cells
% Cell Viability
150 µM
75 µM
5 µM
DMSO (0,1%)
Cam (0,5 µM)
24 hr
48 hr
Concentration (µM)


Cpd 7+ MCF-7 cells
% Cell Viability
200 µM
175 µM
150 µM
125 µM
100 µM
75 µM
50 µM
25 µM
5 µM
DMSO (0,1%)
Cam (718 µM)
24 hr
48 hr
Concentration (µM)


Cpd 9 + HeLa cells
% Cell Viability
250 µM
75 µM
5 µM
DMSO (0,1%)
Cam (0,23 µM)
Cam (0,5 µM)
24 hr
48 hr
Concentration (µM)

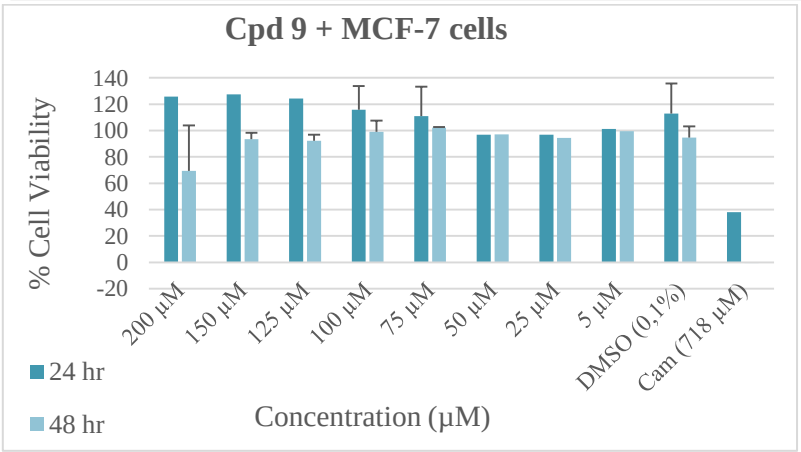

Cpd 9 + MCF-7 cells
% Cell Viability
200 µM
150 µM
125 µM
100 µM
75 µM
50 µM
25 µM
5 µM
DMSO (0,1%)
Cam (718 µM)
24 hr
48 hr
Concentration (µM)

Figure S4. Hela and MCF-7 cell viability (%) after treatment with different concentrations of test compounds **1**, **3**, **6**, **7** and **9** after 24 and 48 hours, in Hela and MCF7 cell lines**.**

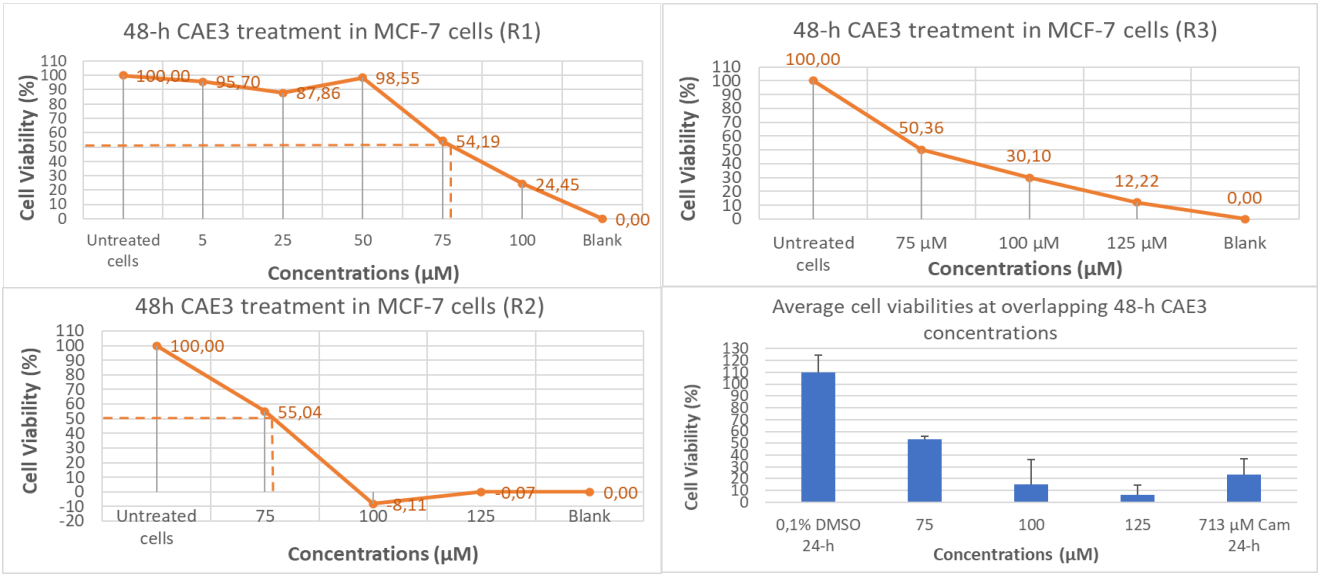


Figure S5. Three replicates MCF-7 cell viability (%) after treatment with different concentrations of test compound **6** after 48 hours, in MCF7 cell lines.

* **CAE3** = Compound **6**

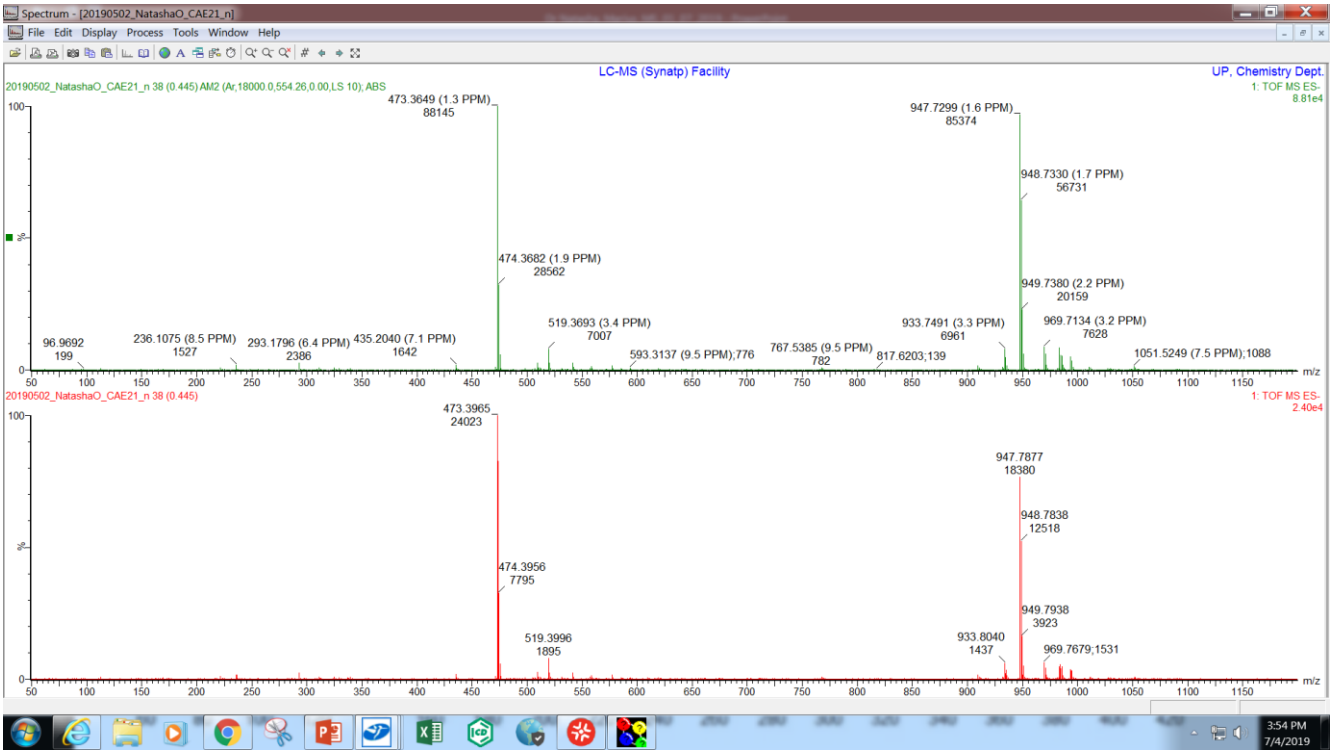


Figure S6. HR-ESI-MS spectrum of **1.**

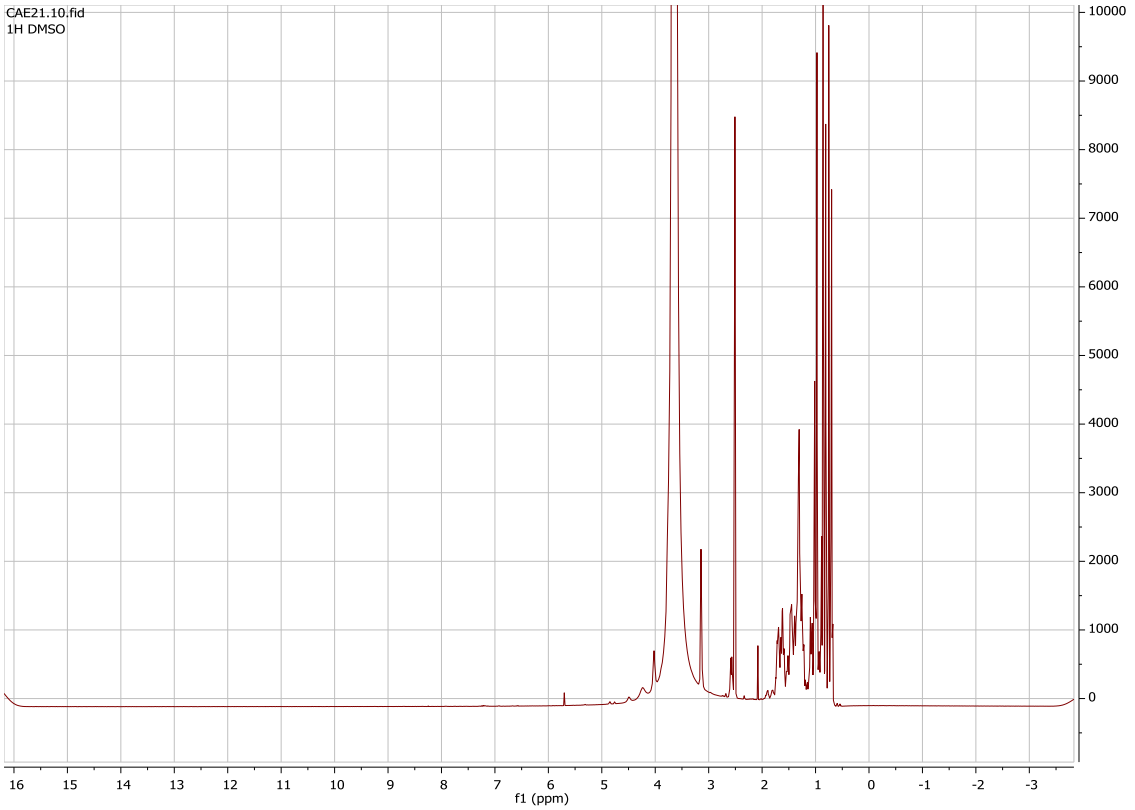


Figure S7. ^{1}H-NMR spectrum of **1**.

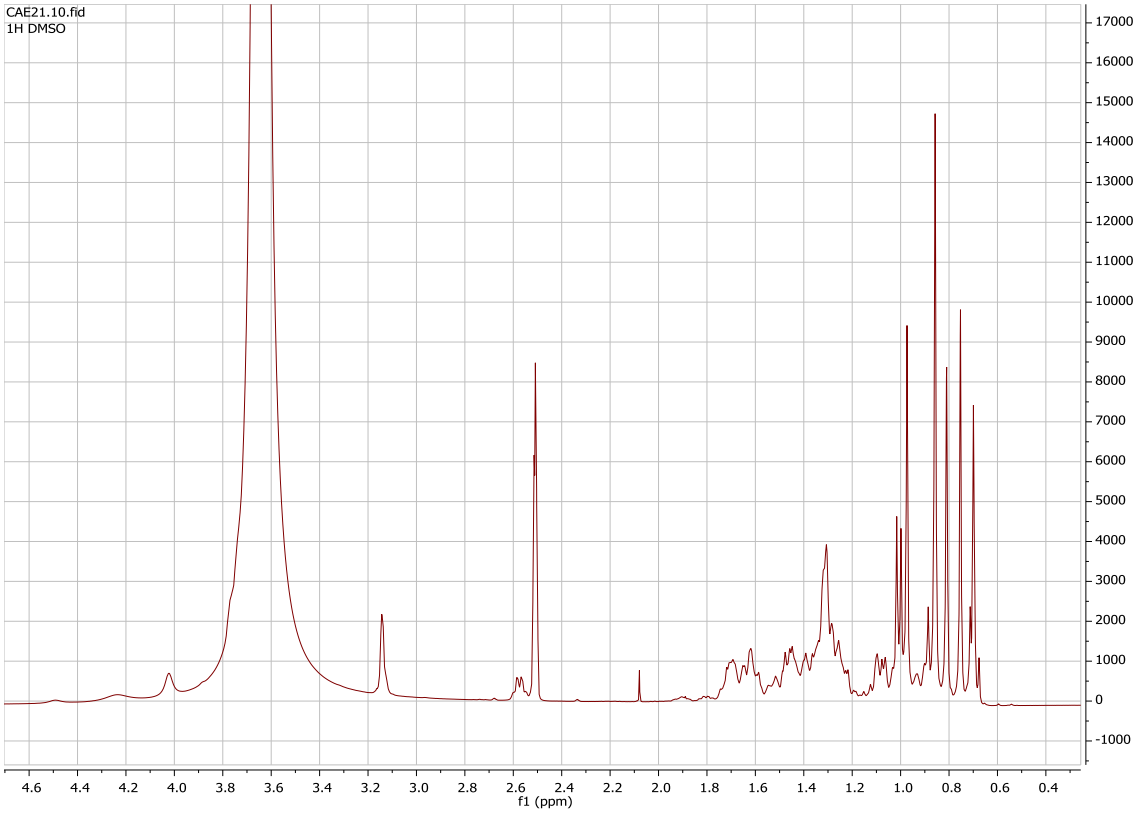


Figure S8. Expanded ^{1}H-NMR spectrum of **1**.

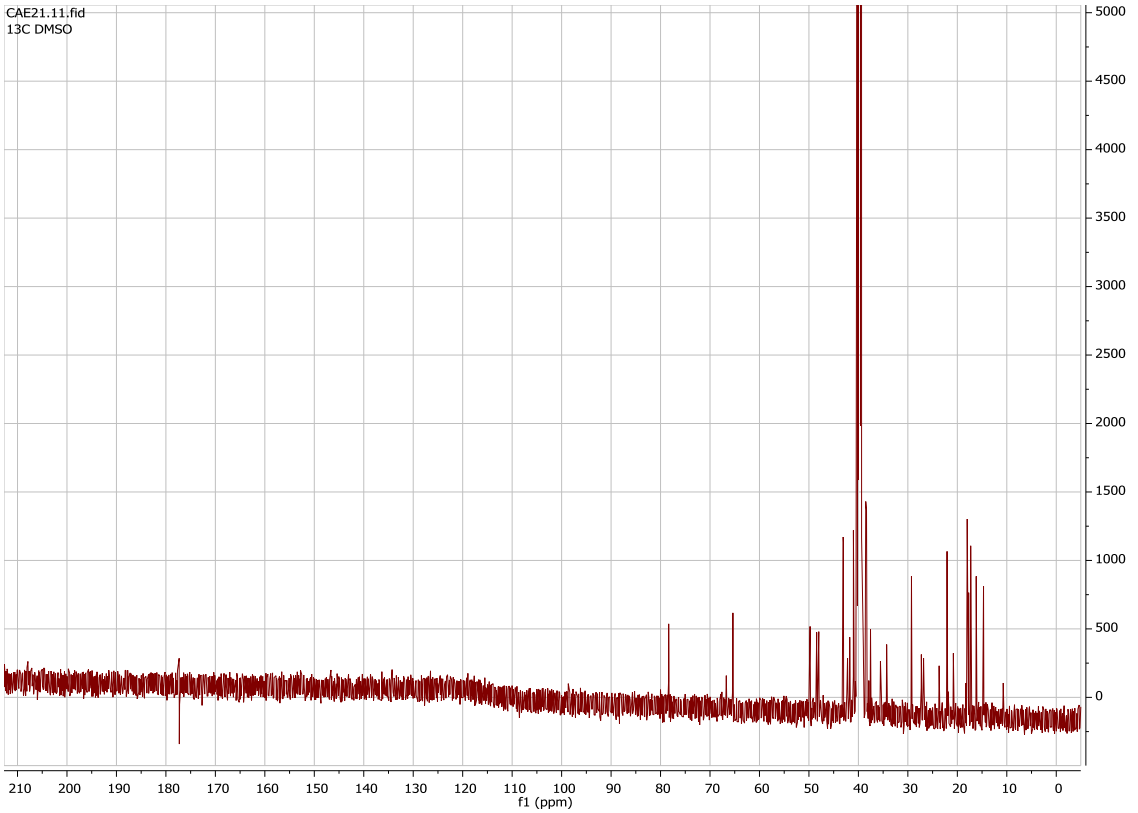


Figure S9. ^{13}C-NMR spectrum of **1**.

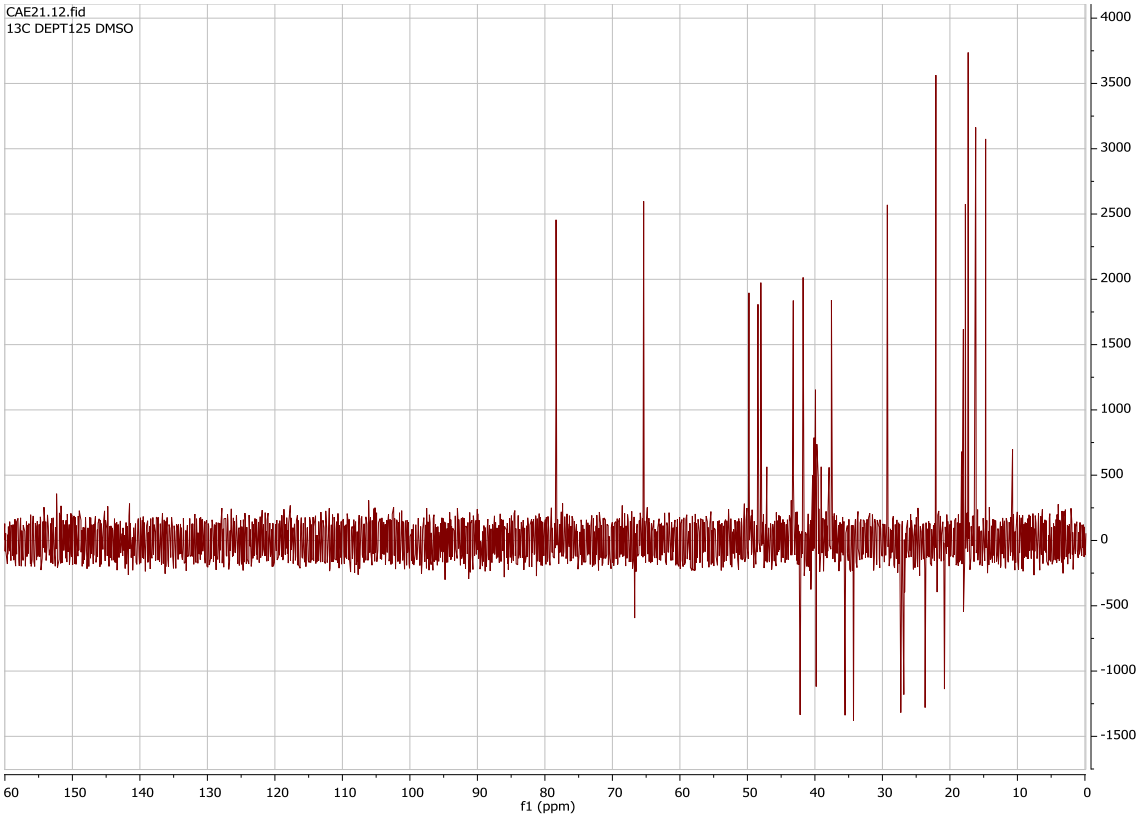


Figure S10. DEPT 135 spectrum of **1**.

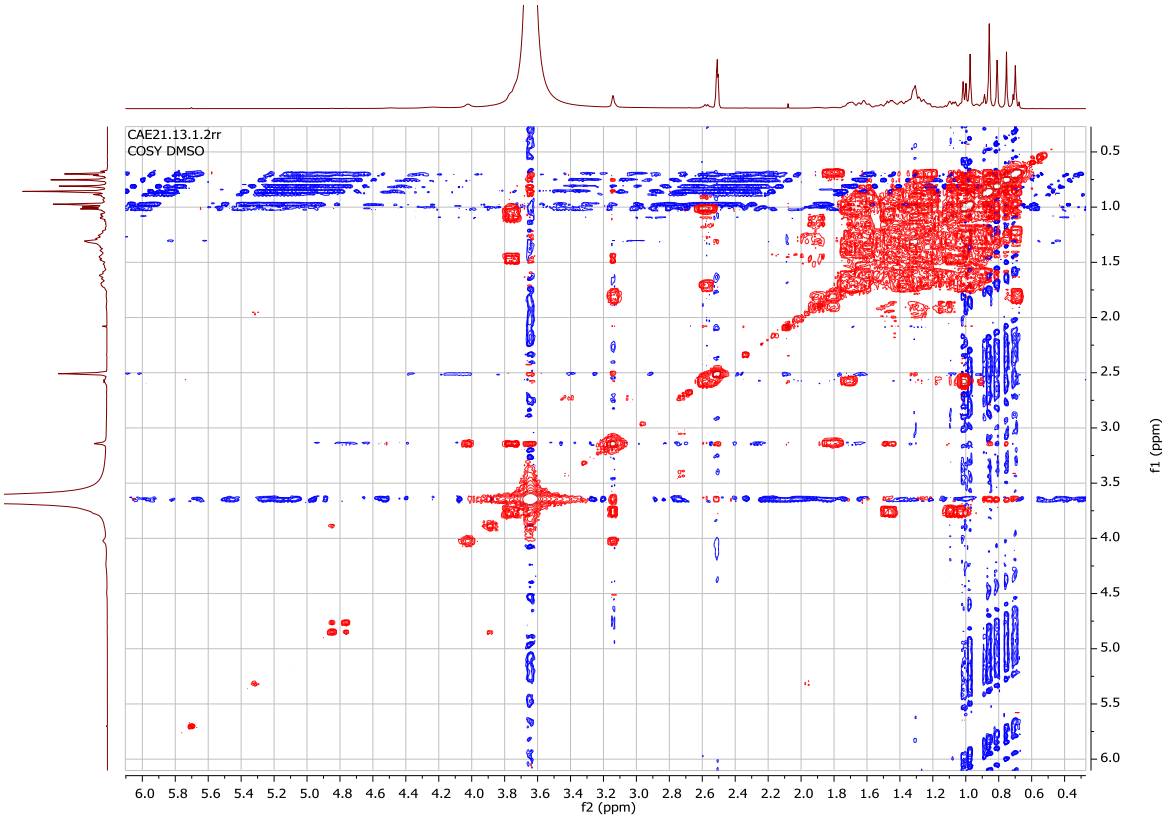


Figure S11. COSY spectrum of **1**.

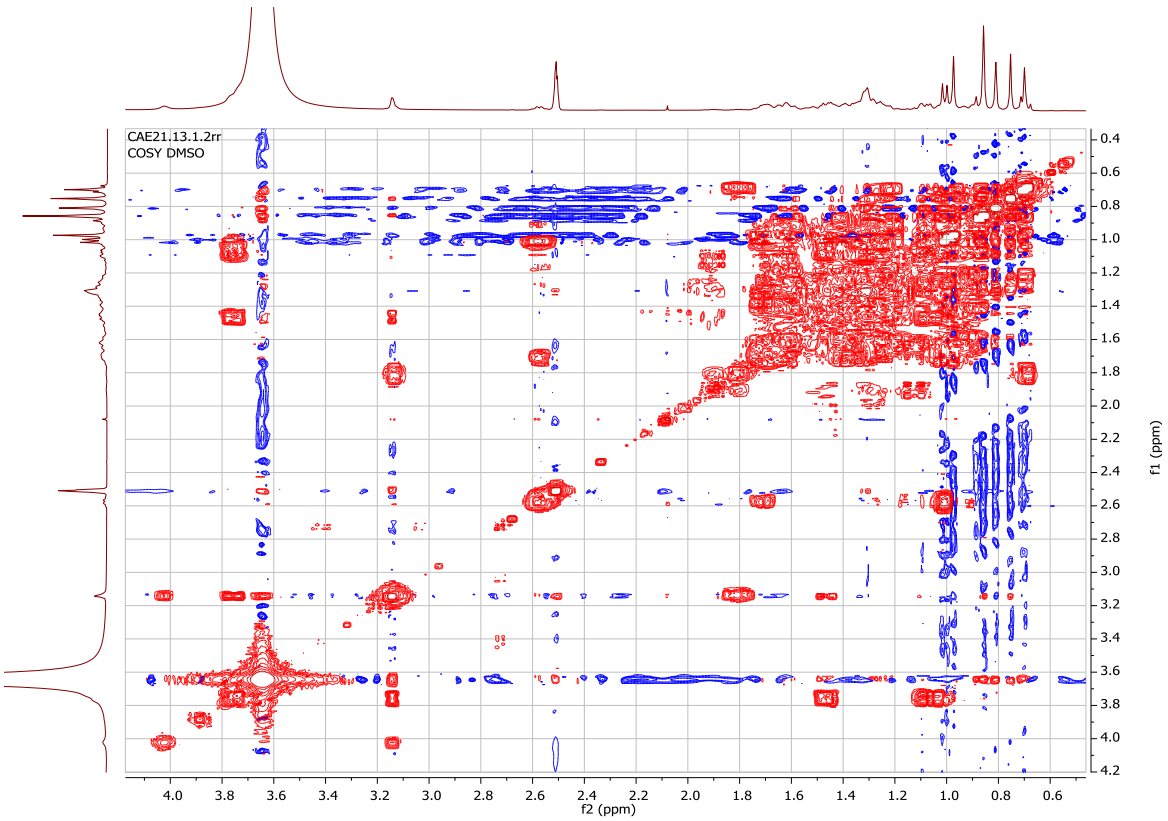


Figure S12. Expanded COSY spectrum of **1**.

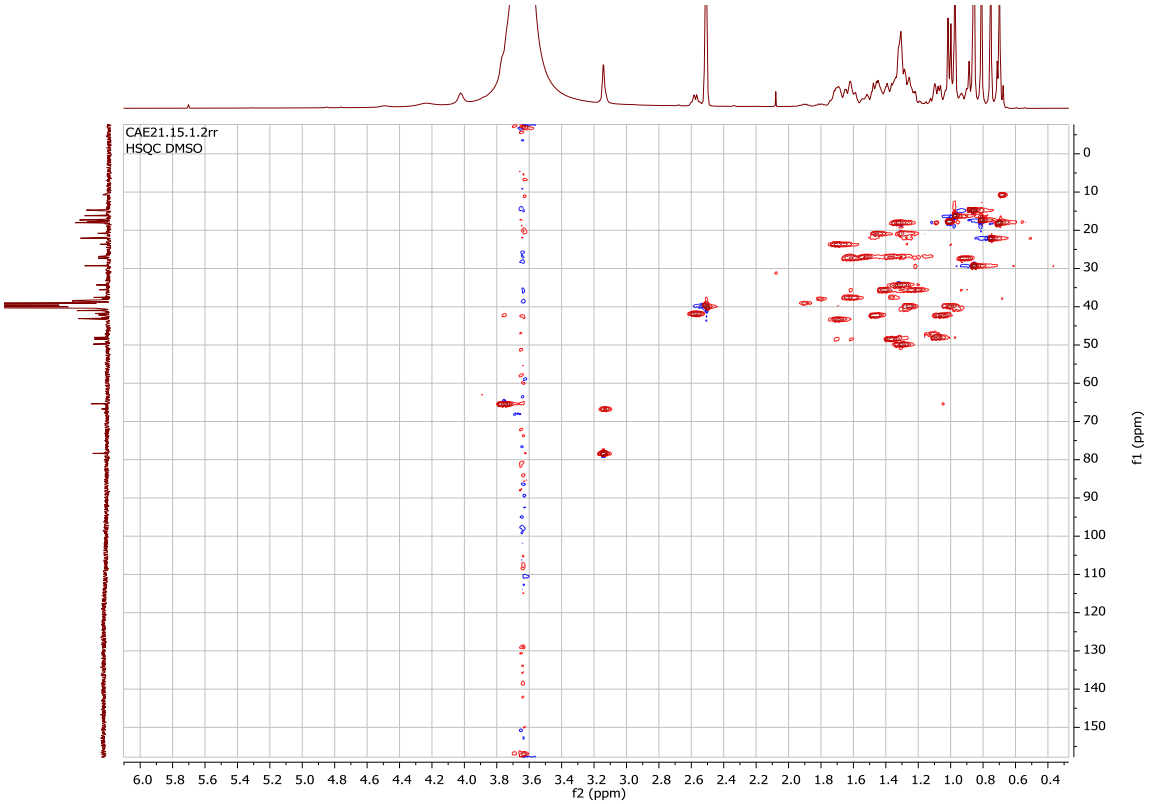


Figure S13. HSQC spectrum of **1**.

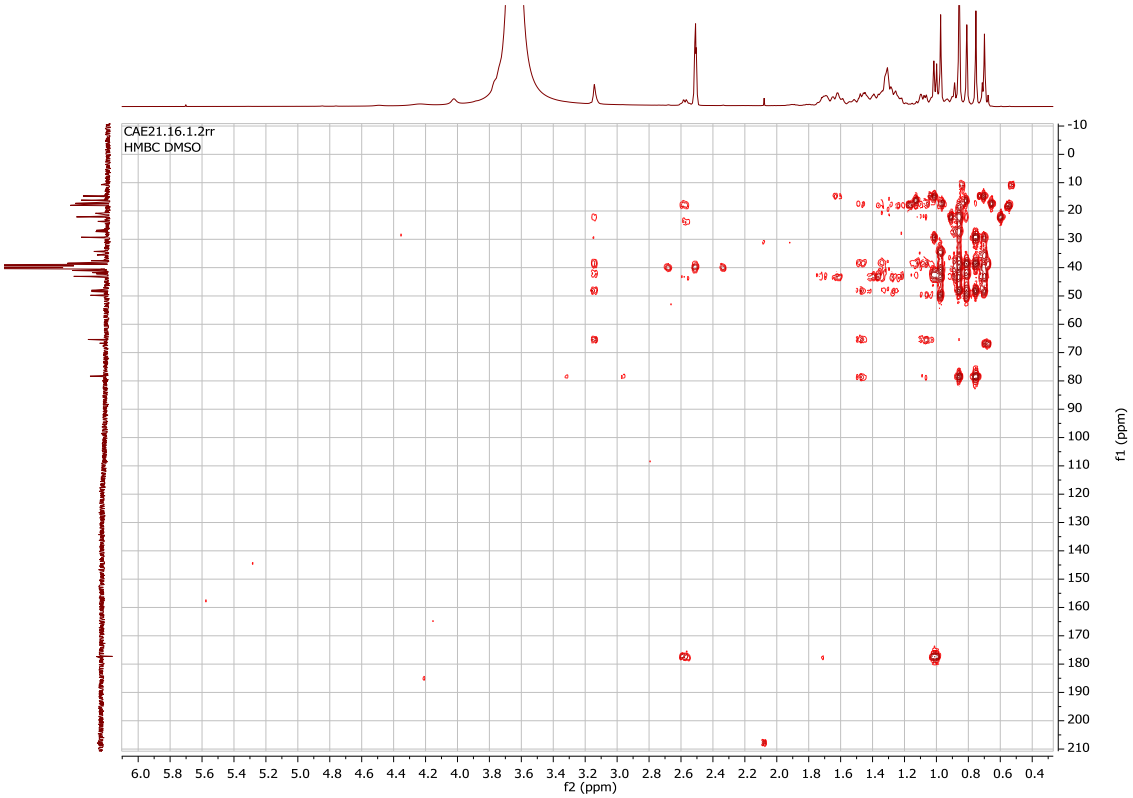


Figure S14. HMBC spectrum of **1**.

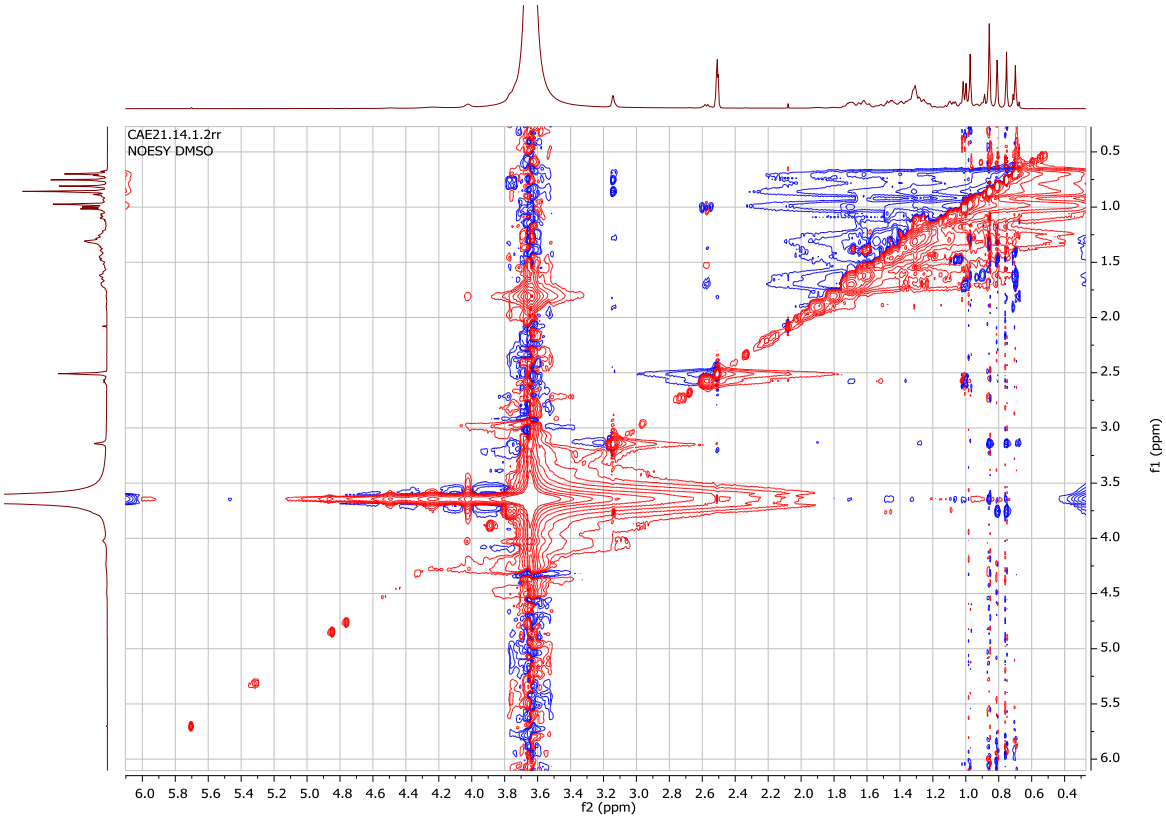


Figure S15 NOESY spectrum of **1**.

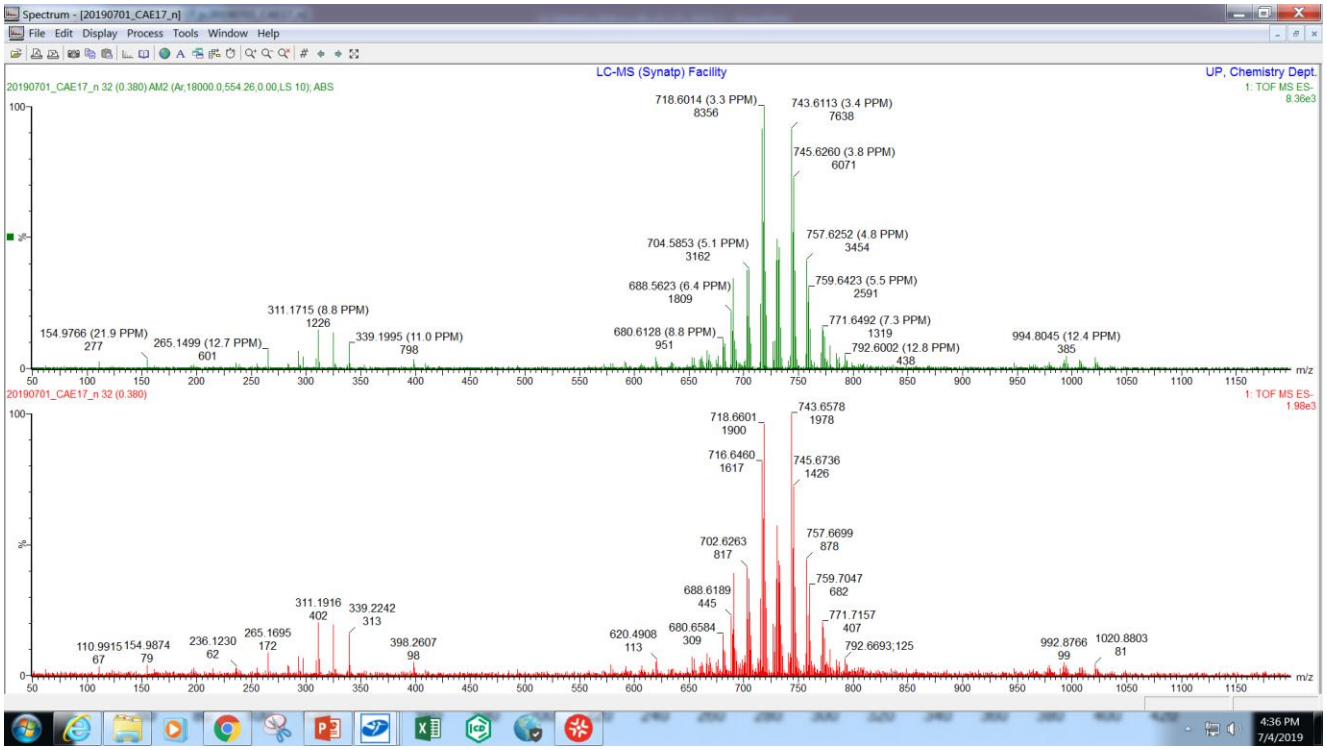


Figure S16. HR-ESI-MS spectrum of **2**.

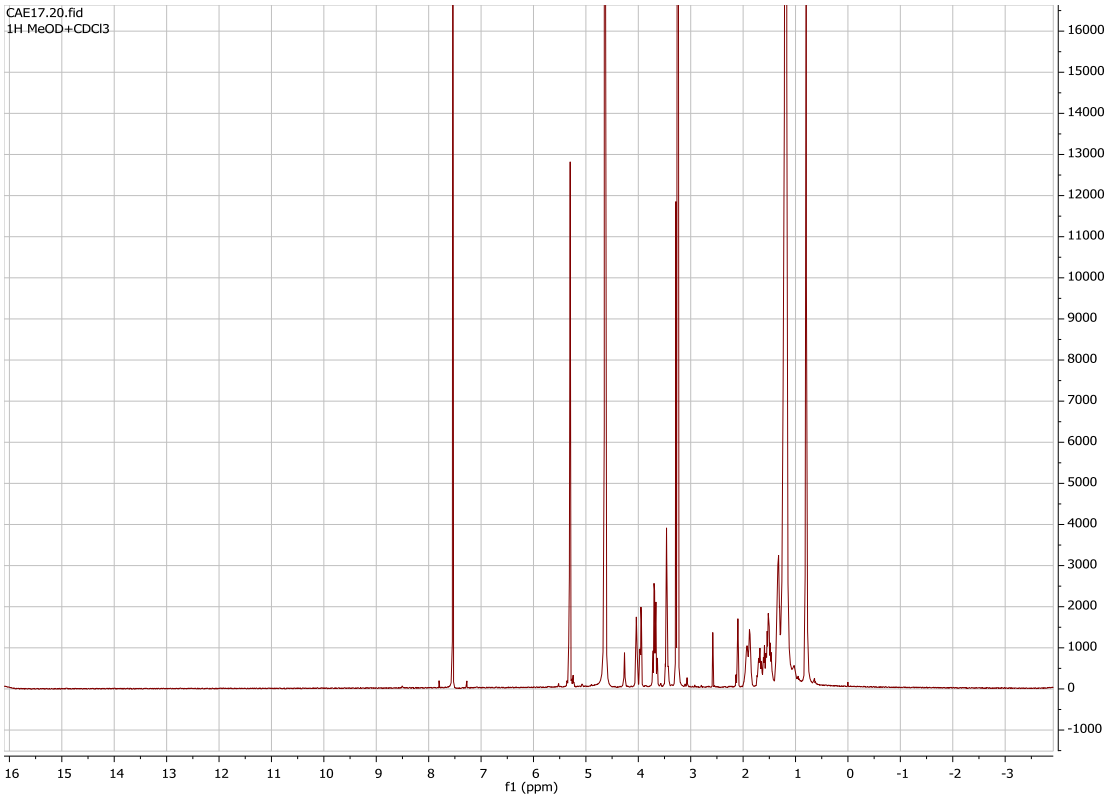


Figure S17. ^{1}H-NMR spectrum of **2**.

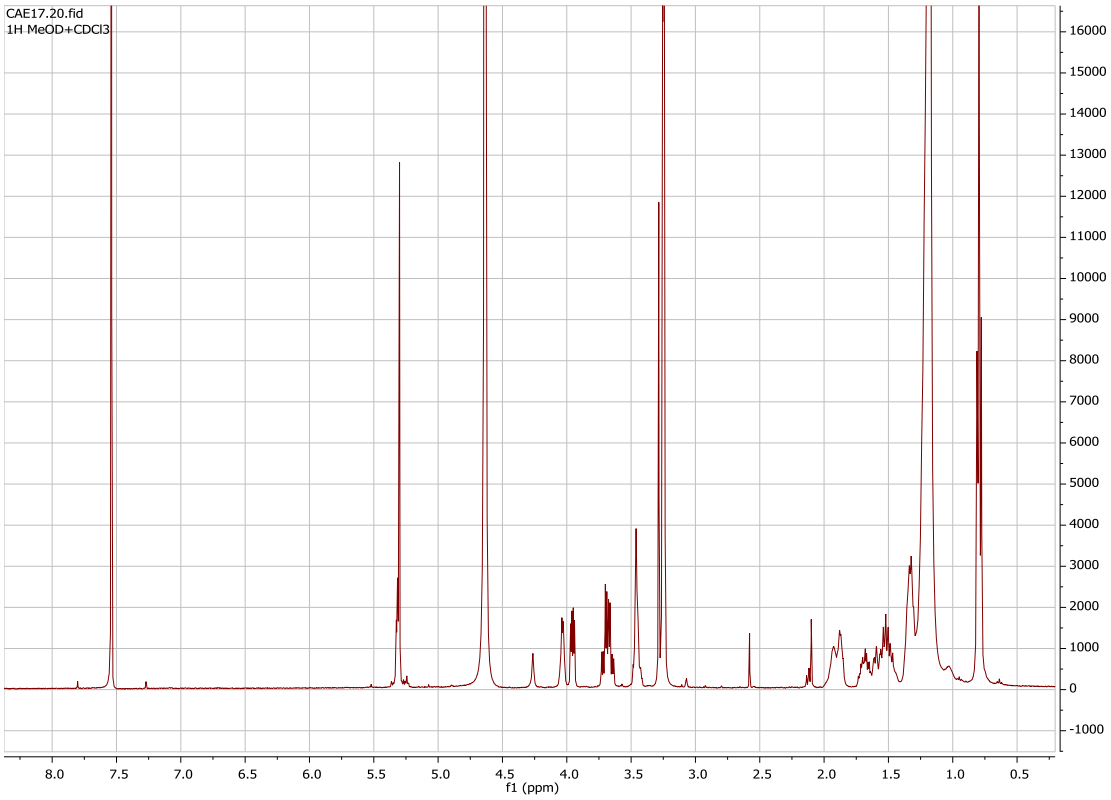


Figure S18. Expanded ^{1}H-NMR spectrum of **2**.

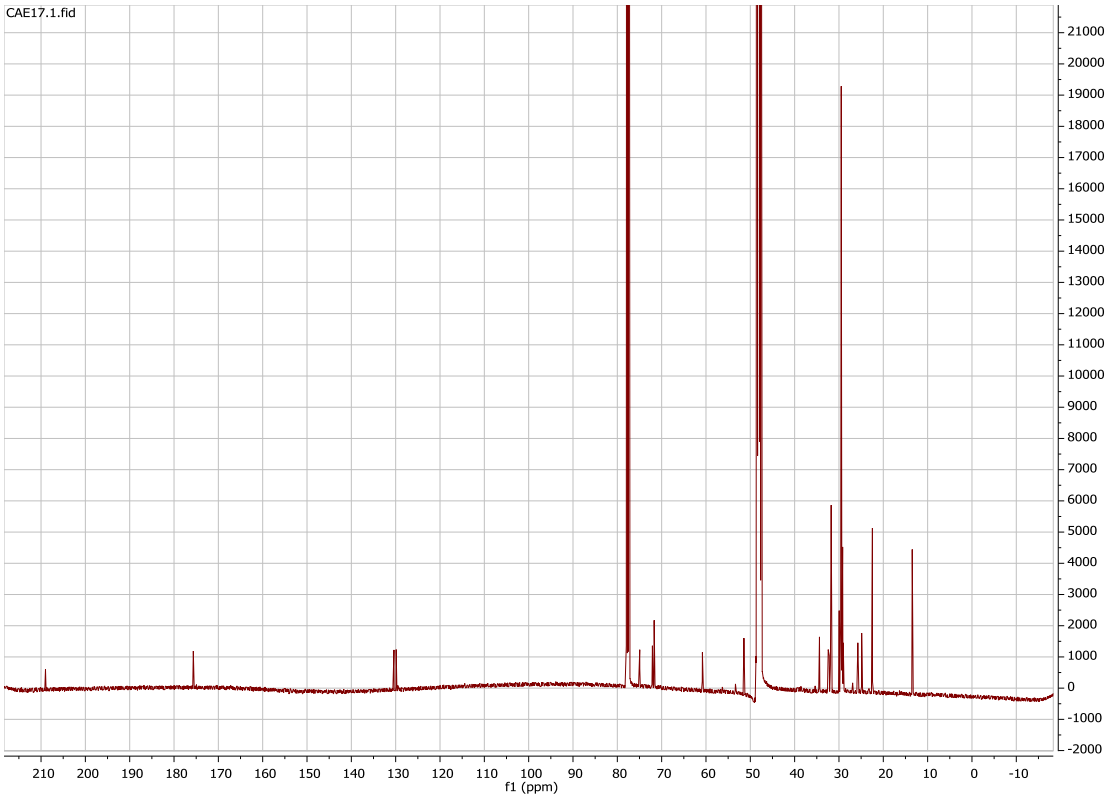


Figure S19. ^{13}C-NMR spectrum of **2**.

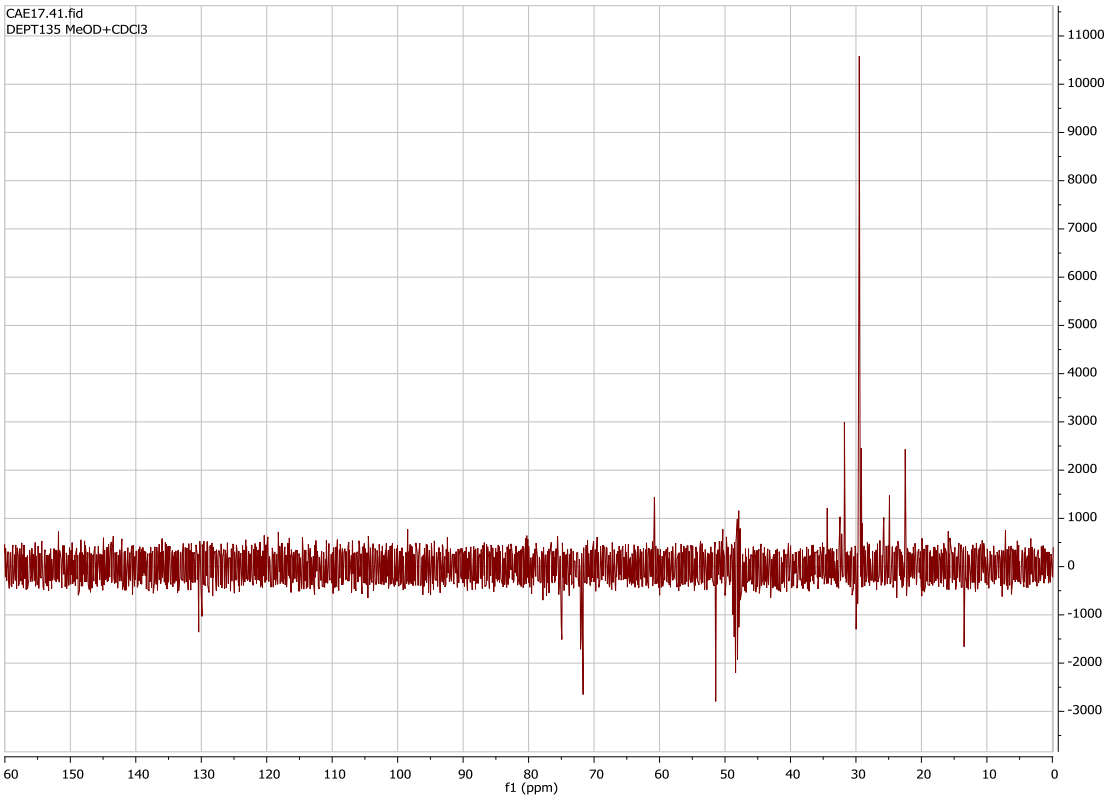


Figure S20. DEPT 135 spectrum of **2**.

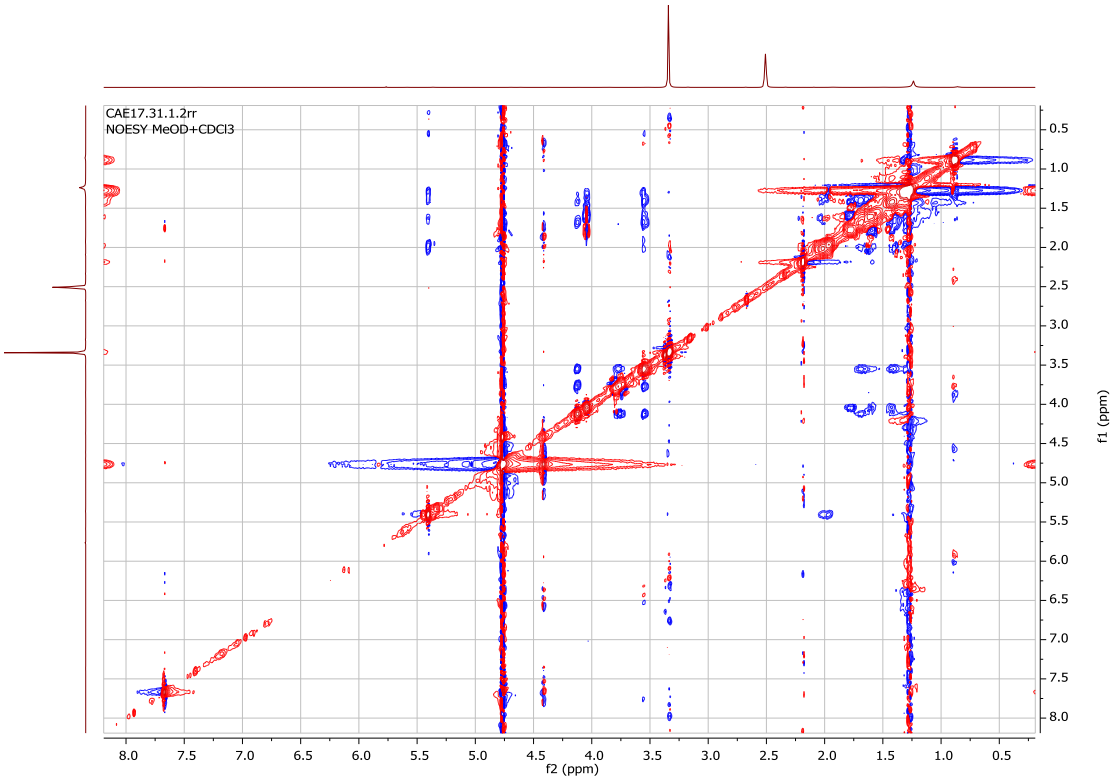


Figure S21. COSY spectrum of **2**.

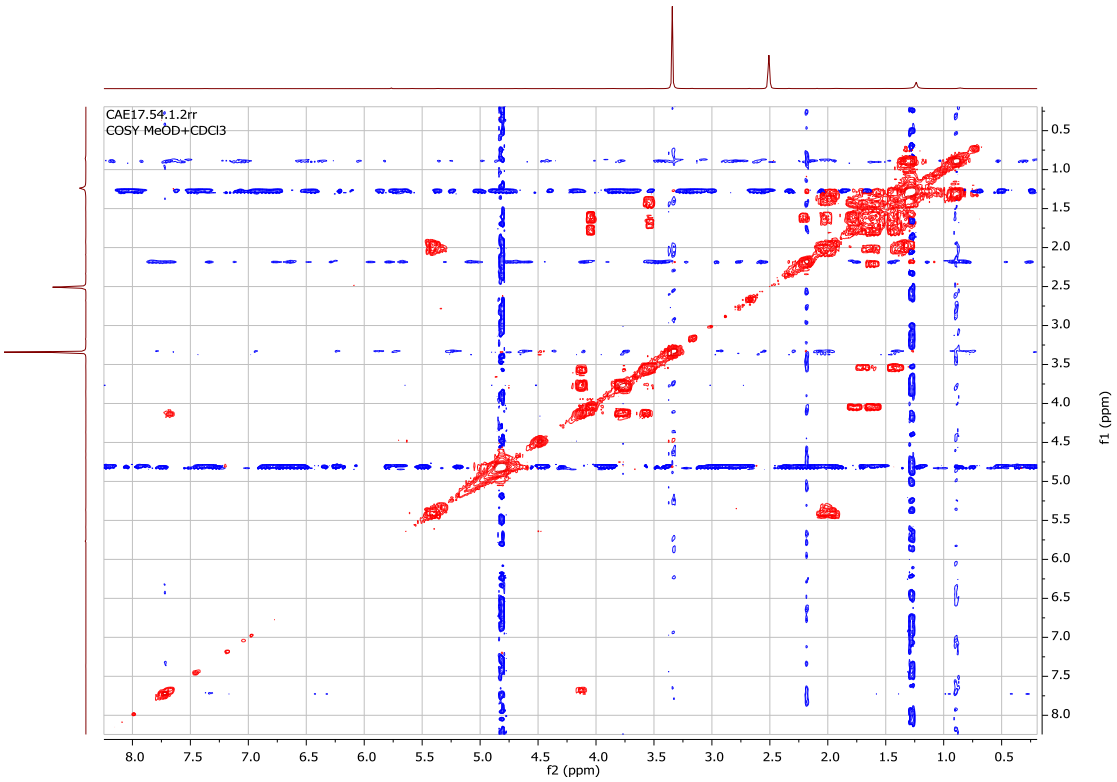


Figure S22. Expanded COSY spectrum of **2**.

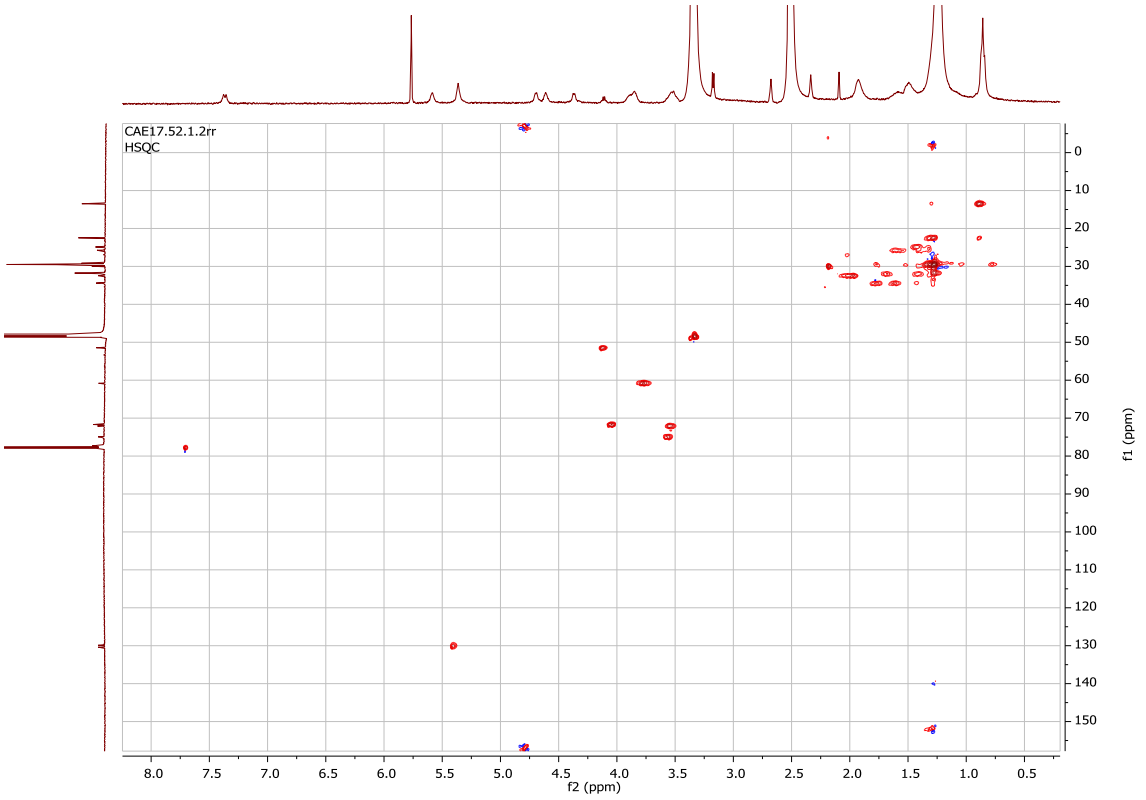


Figure S23. HSQC spectrum of **2**.

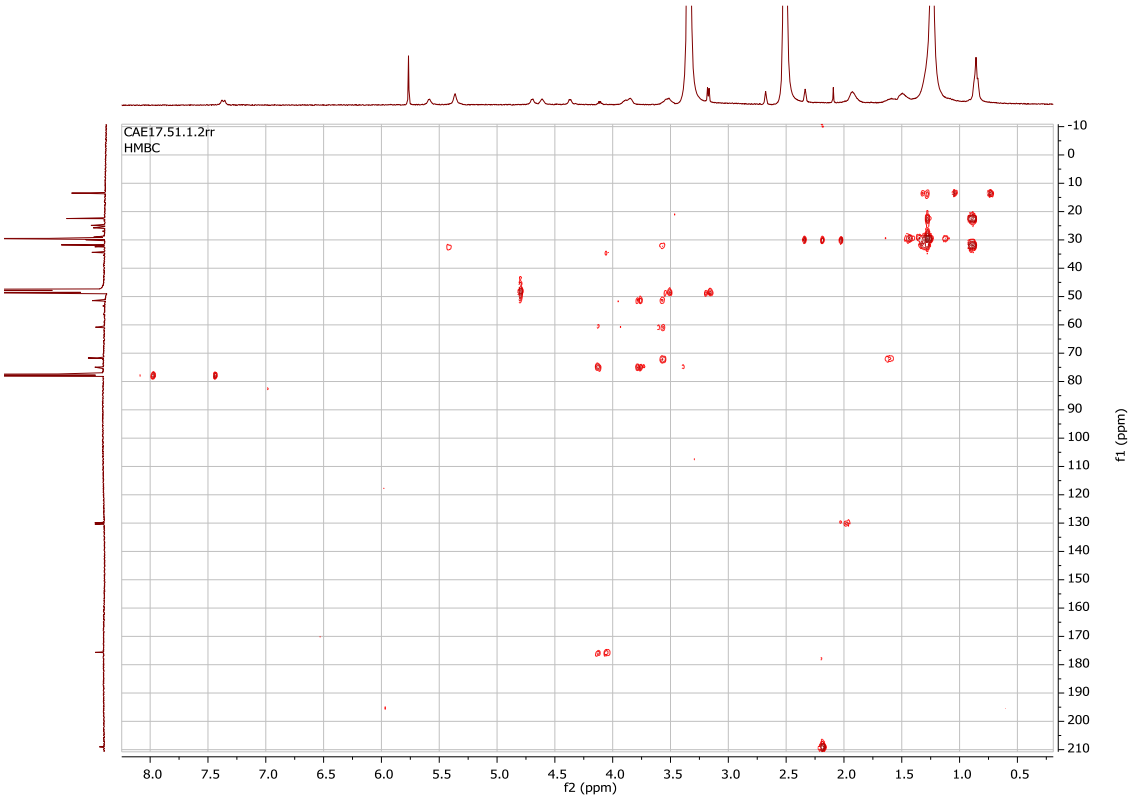


Figure S24. HMBC spectrum of **2**.

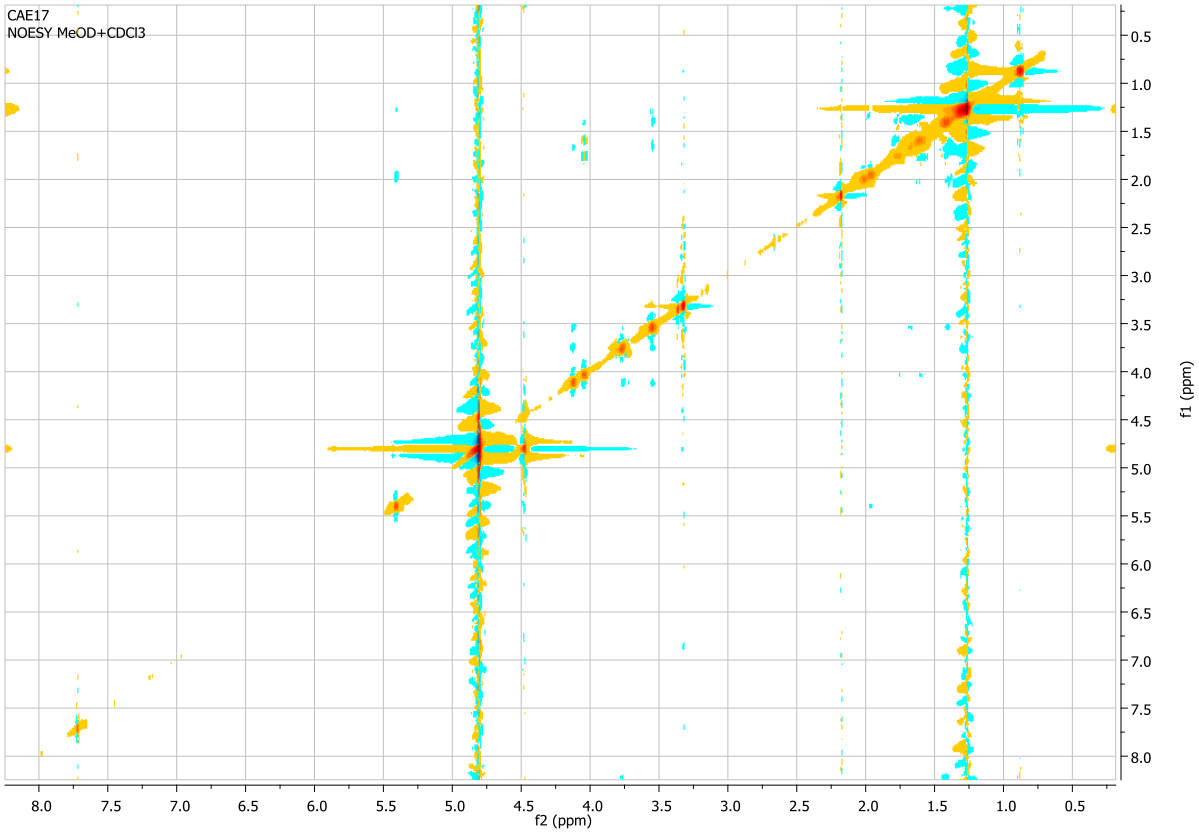
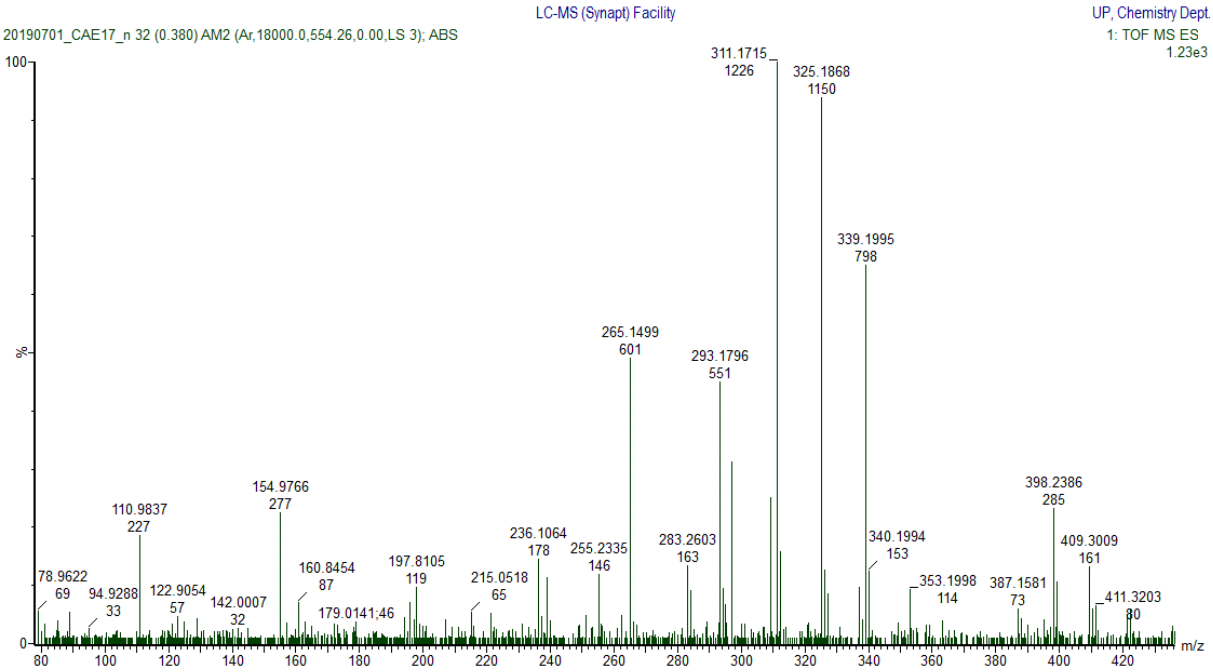


Figure S25. NOESY spectrum of **2**.



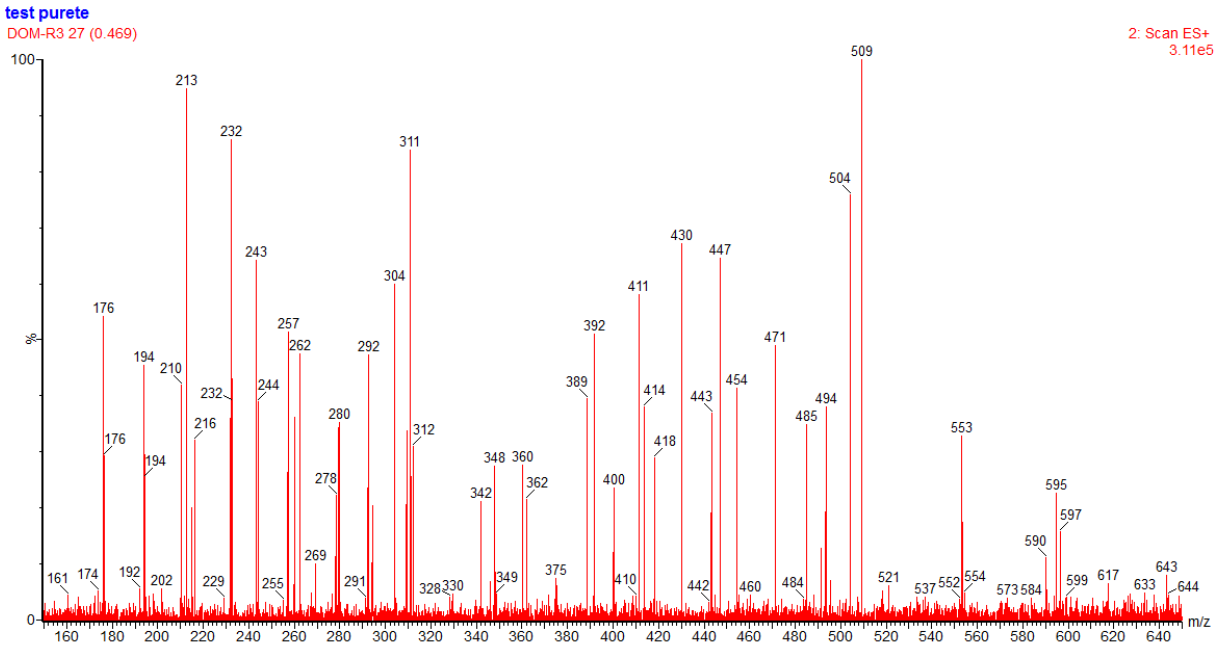
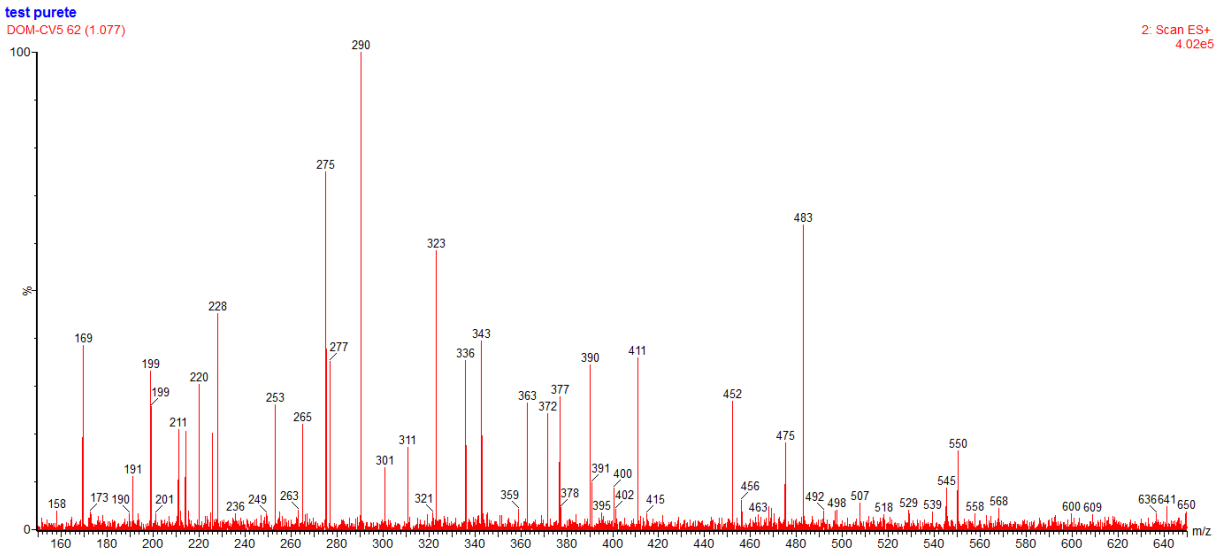


Figure S26. LC-MS and UPLC of FAME of **2**



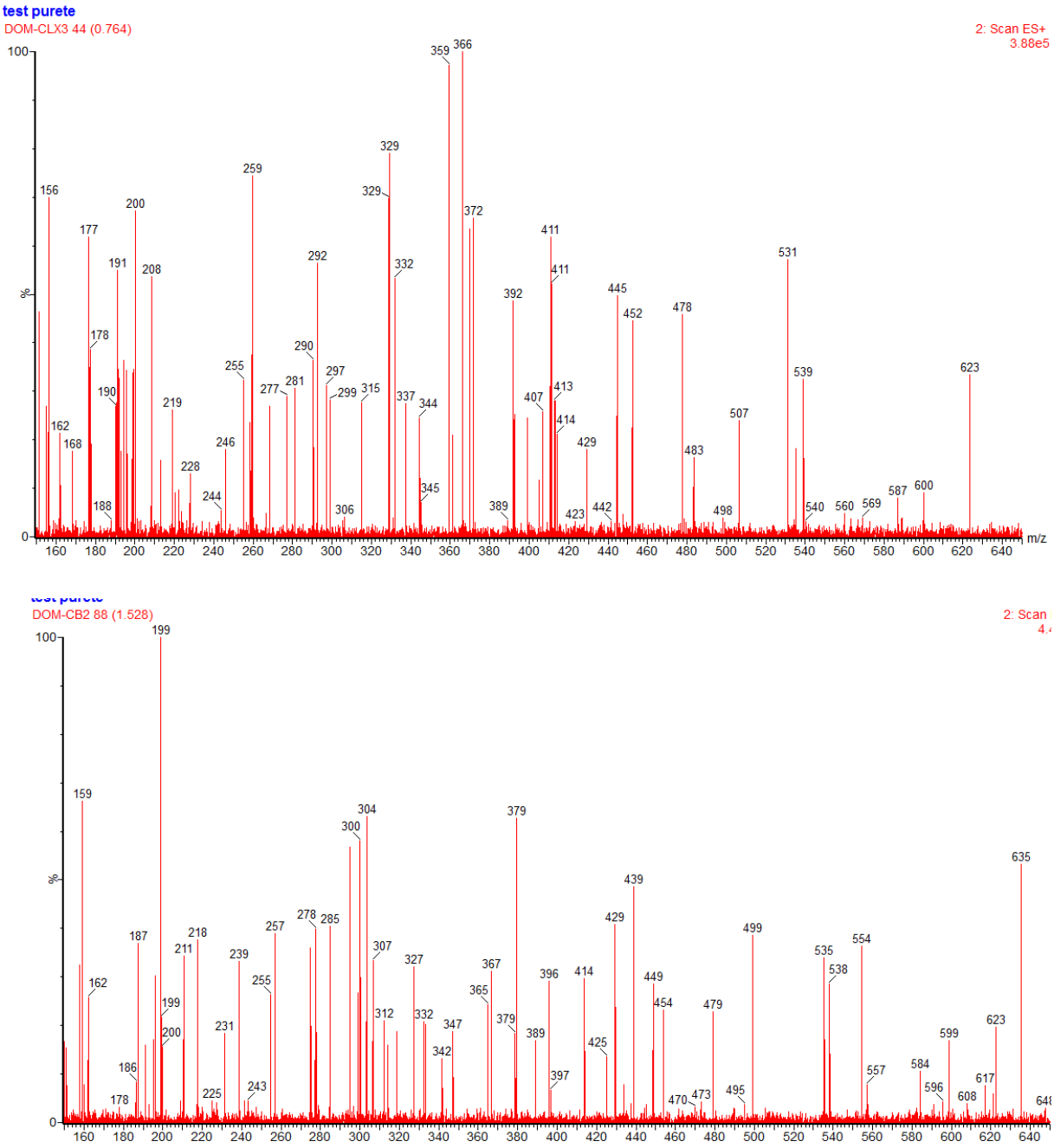
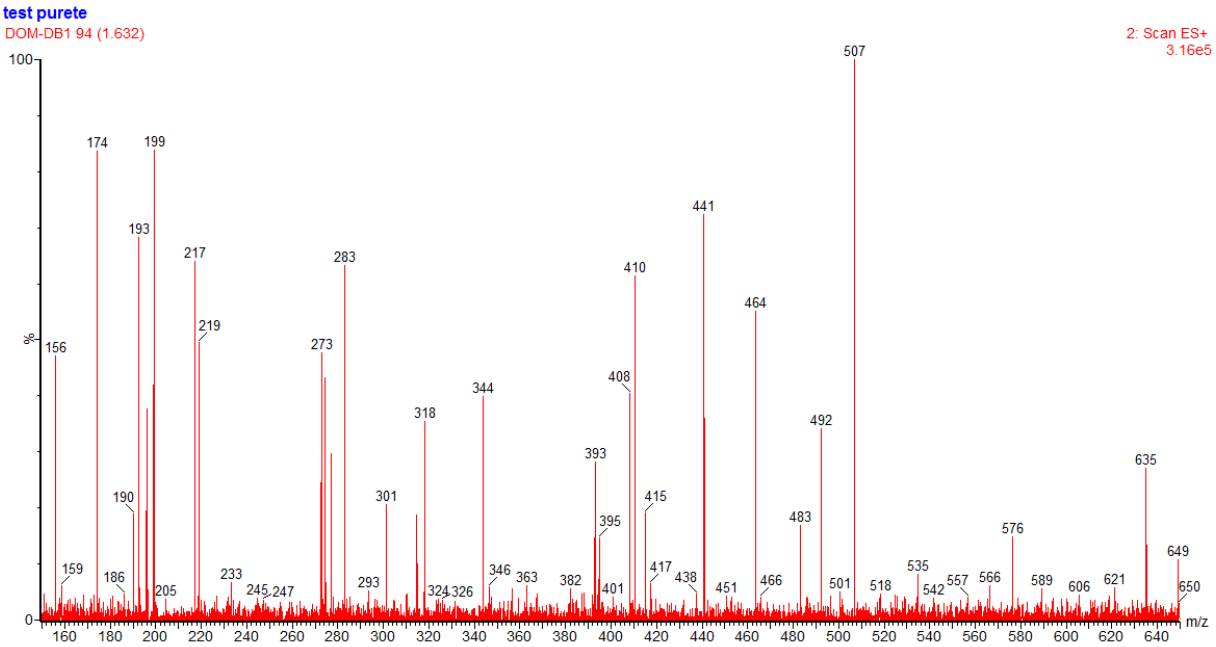


Figure S27. Fragment pattern of methylthiolated ions of **2** (UPLC).


test purete
DOM-DB1 94 (1.632)
2: Scan ES+
3.16e5
100
%
0
m/z
507
441
464
410
408
492
635
649
650
576
566
589
606
621
557
535
542
518
501
483
466
451
438
417
415
401
395
393
382
363
346
344
326
324
318
301
293
283
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247
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186
174
159
156
160
180
200
220
240
260
280
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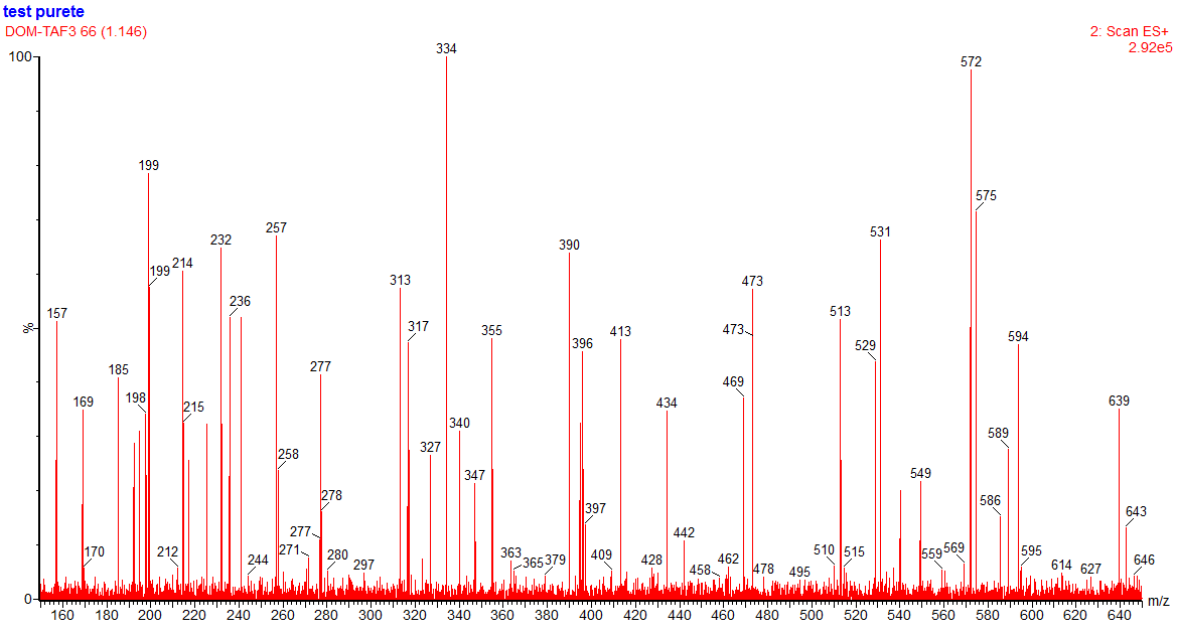
320
340
360
380
400
420
440
460
480
500
520
540
560
580
600
620
640


test purete
DOM-TAF3 66 (1.146)
2: Scan ES+
2.92e5
100
%
0
m/z
572
575
531
594
639
643
646
614
627
595
589
586
549
559
569
513
529
510
515
495
478
473
473
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214
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185
170
169
157
160
180
200
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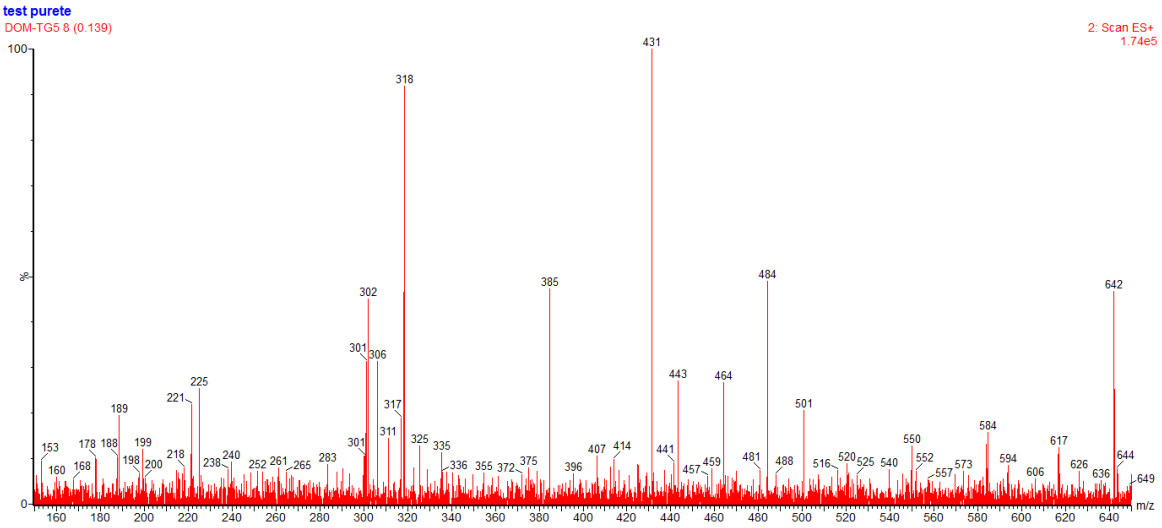

Figure S28. Cleavage ion fragment peaks issued from **2** (UPLC).

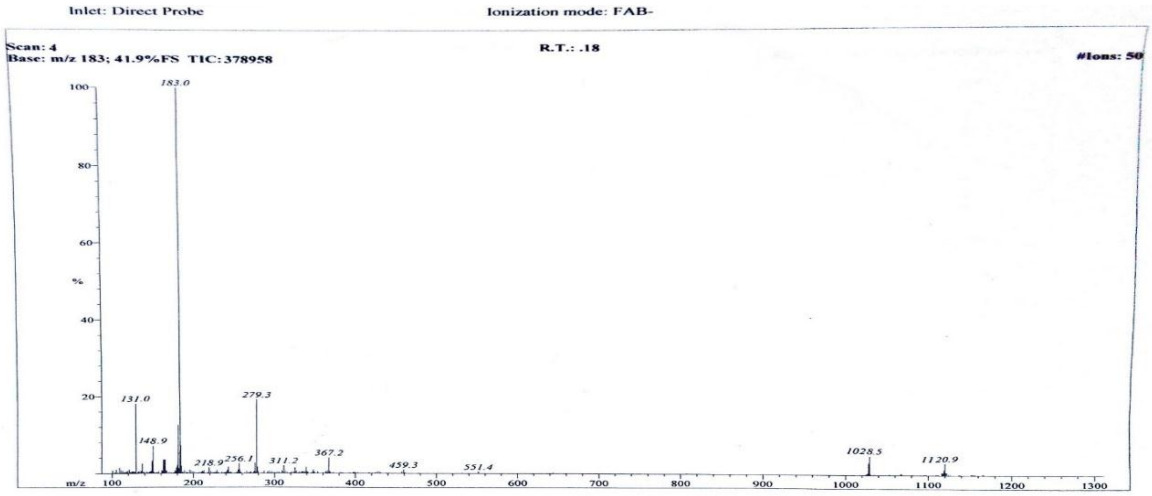

Figure S29. FABMS of cleavage ion fragment peaks issued from **2.**

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