Supporting Information

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Uvaol-28-D-(*R*)-pyroglutamic acid ester: a new triterpenoid compound from *Circinella muscae CGMCC 3.2695* and its activity assessment in transgenic zebrafish

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S.1: General Experimental Procedures

The NMR was measured in $CDCl_3$ using a Bruker AVANCE NEO-600 spectrometer (Bruker BioSpin AG, CH), and HR-ESI-MS analysis was conducted on an Agilent 6540 UHD Accurate Mass Q-TOF (Agilent Technologies, USA). Reversed-phase preparative high-performance liquid chromatography (HPLC) was carried out on a Shimadzu LC-20AD instrument with a SPD-20A UV detector and using YMC-Pack ODS-A (5 μm, 12.0 × 250 mm) columns (Shimadzu Corp., JP). Chromatographic grade acetonitrile and methanol was purchased from Merck Company, Shanghai, China. All organic solvents were of analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd, Beijing, China.

S.2: Biotransformation, isolation and purification

Circinella muscae CGMCC 3.2695 were cultured by two-stage liquid fermentation according previously described [1]. The fungal inoculum was transferred to 1000 mL flasks each containing 400 mL of liquid potato dextrose medium, and incubated on a rotary shaker with 160 rpm at 26 °C for 24 h. Then 1000 mg of uvaol (30 mg/mL in ethanol) were added to each flask. After 7 days of incubation, the cultures were pooled and filtered. The filtrates were extracted with ethyl acetate for three times. The organic layer was collected, and the solvent was removed by a rotary evaporator. The crude extracts of biotransformation of uvaol with C. muscae (500 mg) was purified on an ODS C18 open columns (100 g, 60 X 3 cm) and eluted with 10%, 40%, 80% CH_3OH aqueous solution, and 100% CH_3OH to give the Fractions A, B, C and D, respectively. Fraction C was refined by semi-preparative HPLC (CH_3CN:H_2O, 65:35, flow rate, 3.0 mL/min) to yield compound UPE (compound **1**, 37.6 mg, 3.76%, t_R = 15.5 min, purity >96%), compound **2** (18.6 mg, 1.86%, t_R = 25.4 min), compound **3** (19.5 mg, 1.95%, t_R = 31.1 min), and compound **4** (23.6 mg, 2.36%, t_R = 38.4 min).

S.3: Zebrafish

The transgenic zebrafish lines of *Tg (kdrl:EGFP)* with vascular endothelial growth factor receptor gene *kdrl* is marked with EGFP, and *Tg(hb9:mcherry)* with motor neuron gene *hb9* is marked with mcherry were offered by the Zebrafish Center Key Laboratory of Neuroregeneration of Nantong University. The transgenic zebrafish were maintained and raised on standard conditions according to previous reports [2].

S.4: Zebrafish drug treatments

The drug administration experiments were carried out as described in the literature [3]. In brief, the *Tg (kdrl:EGFP)* and *Tg(hb9:mcherry)* embryos were obtained by natural mating and maintained in E3 solution at 28 °C. We removed the abnormal embryos using anatomical microscope after 8 hours post fertilization (hpf). Each well of 96-well plate were loaded with 10 healthy embryos, and then replaced E3 solutions with UPE treatment solutions (0.1, 1, 10, 100, and 1000 μg/mL). At 40 hpf, the zebrafish embryos were collected, anesthetized, and embedded for a Leica TCS-SP8 confocal imaging. Image J software (version 1.47) was used to analysis.

S.5: Locomotion Analysis of Zebrafish

The swimming distance assays of zebrafish were performed as previously reported [2]. In brief, E3 solutions were replaced with CA-4 (8 ng/mL), CA-4 (8 ng/mL) + UPE (1000 μg/mL) treatment solutions at 120 hpf, respectively. One larval zebrafish is maintained in each well of a 24-well plate filled with 1 mL of E3 medium. The Zebralab Video-Track system was used to detect swimming distance, and ESOvision behavioral analysis software was used for analysis.

S.6: Statistical analysis

GraphPad Prism (version 7.0, Graphpad Software, La Jolla, California) was used to analyze the data statistically. All data were expressed as mean ± S. D. A one-way analysis of variance (ANOVA) was used to perform statistical analysis, $p < 0.05$ was considered statistically significant.

S.7: DP4+ Quantum-chemical calculations

Spartan'24 software (version 1.3.1, Wavefunction Inc., Irvine, California) was used to search the lowest-energy conformers of UPE isomers and perform DP4+ analysis [4]. Following parameters were used during the step-wise lowest-energy conformer search : Hartree-Fock calculations at 3-21G level, then DFT ωB97X-D theory at 6-31G* level, and then DFT ωB97X-V/6-311+G(2df,2p)[6-311G*] parameters used for the calculation of ^{13}C-NMR chemical shifts of UPE isomers.

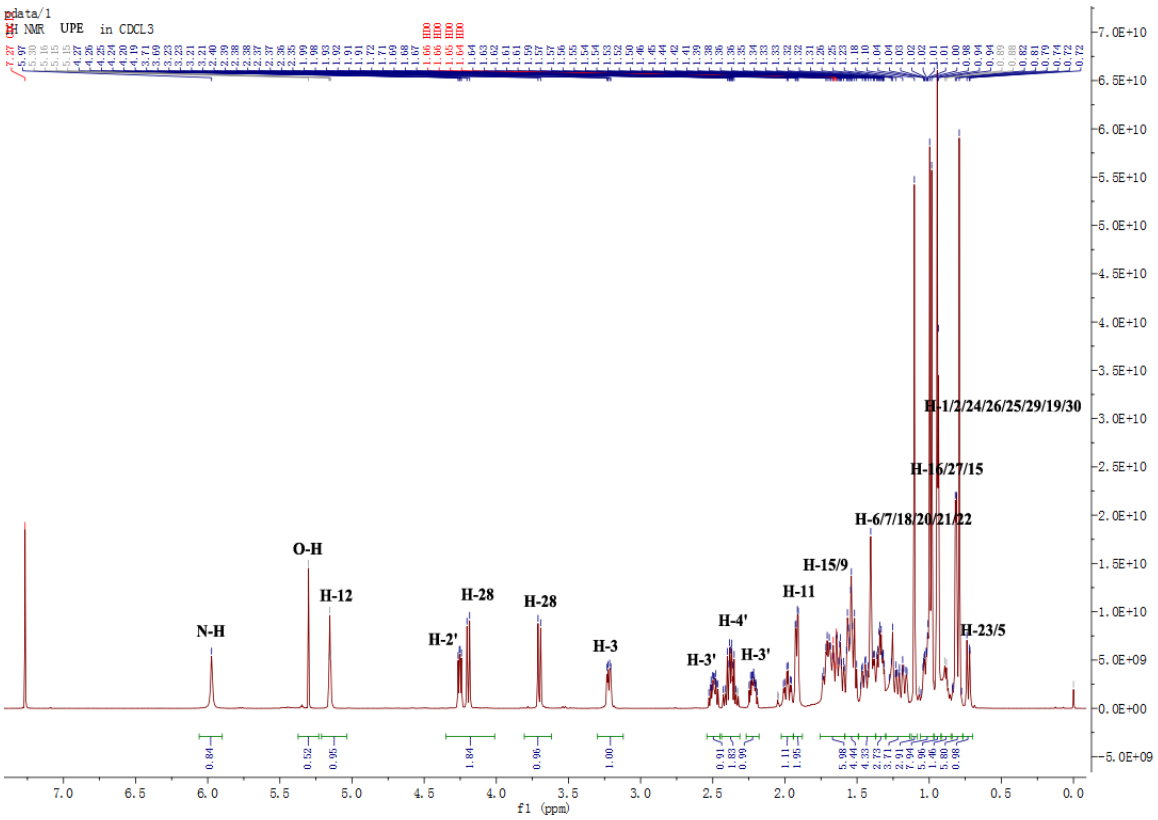


Figure S1. ^{1}H-NMR spectrum (600 MHz) of UPE in $CDCl_3$.

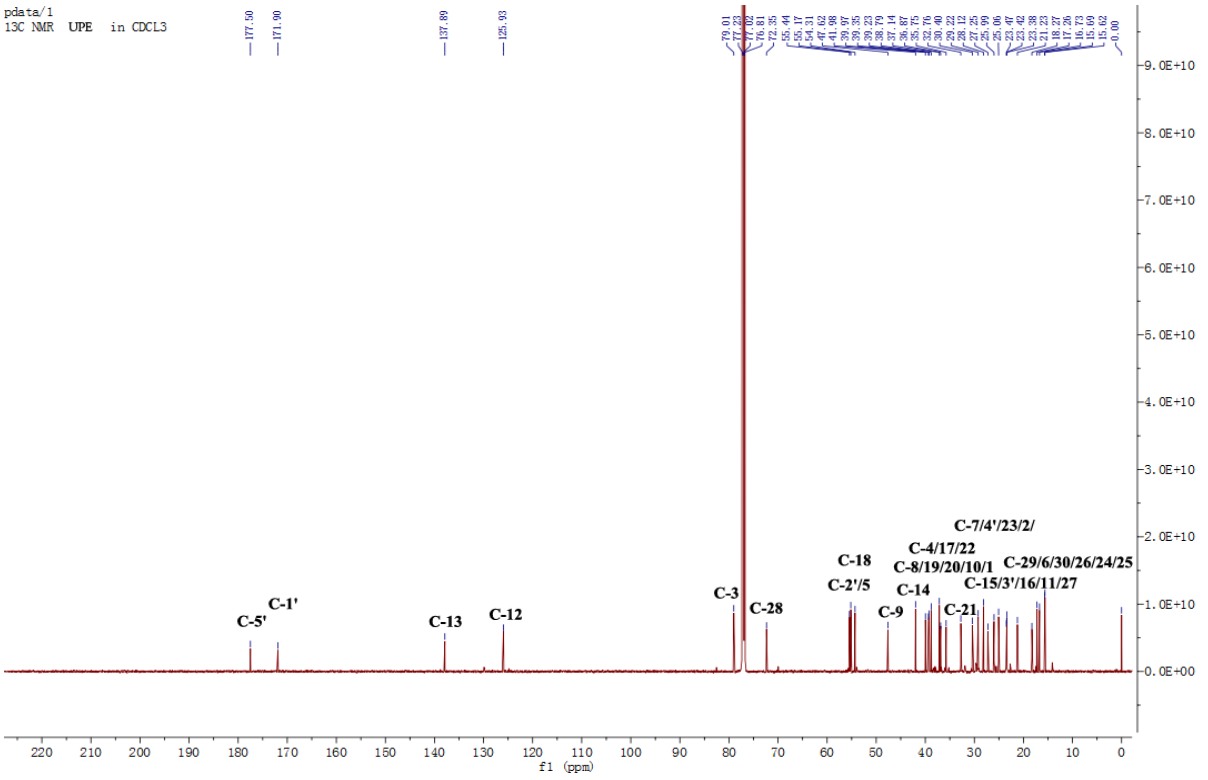


Figure S2. ^{13}C-NMR spectrum (150 MHz) of UPE in $CDCl_3$.

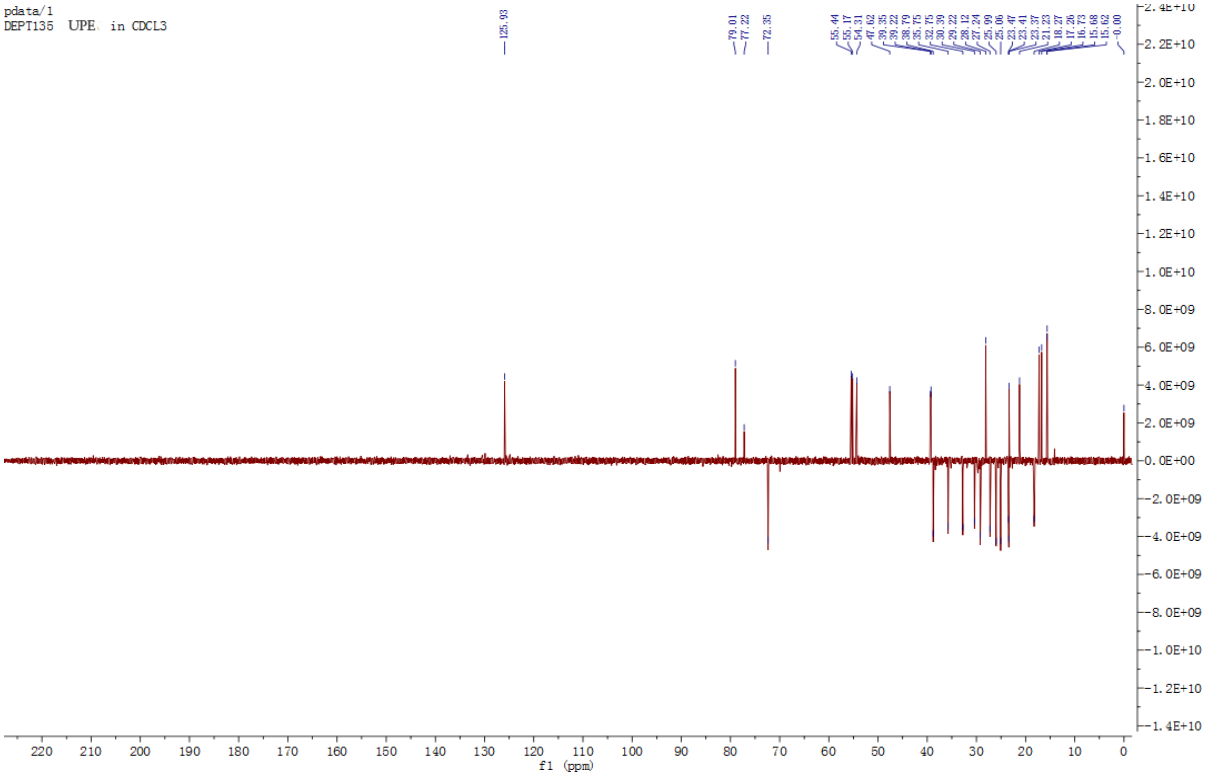


Figure S3. DEPT-135 spectrum (150 MHz) of UPE in $CDCl_3$.

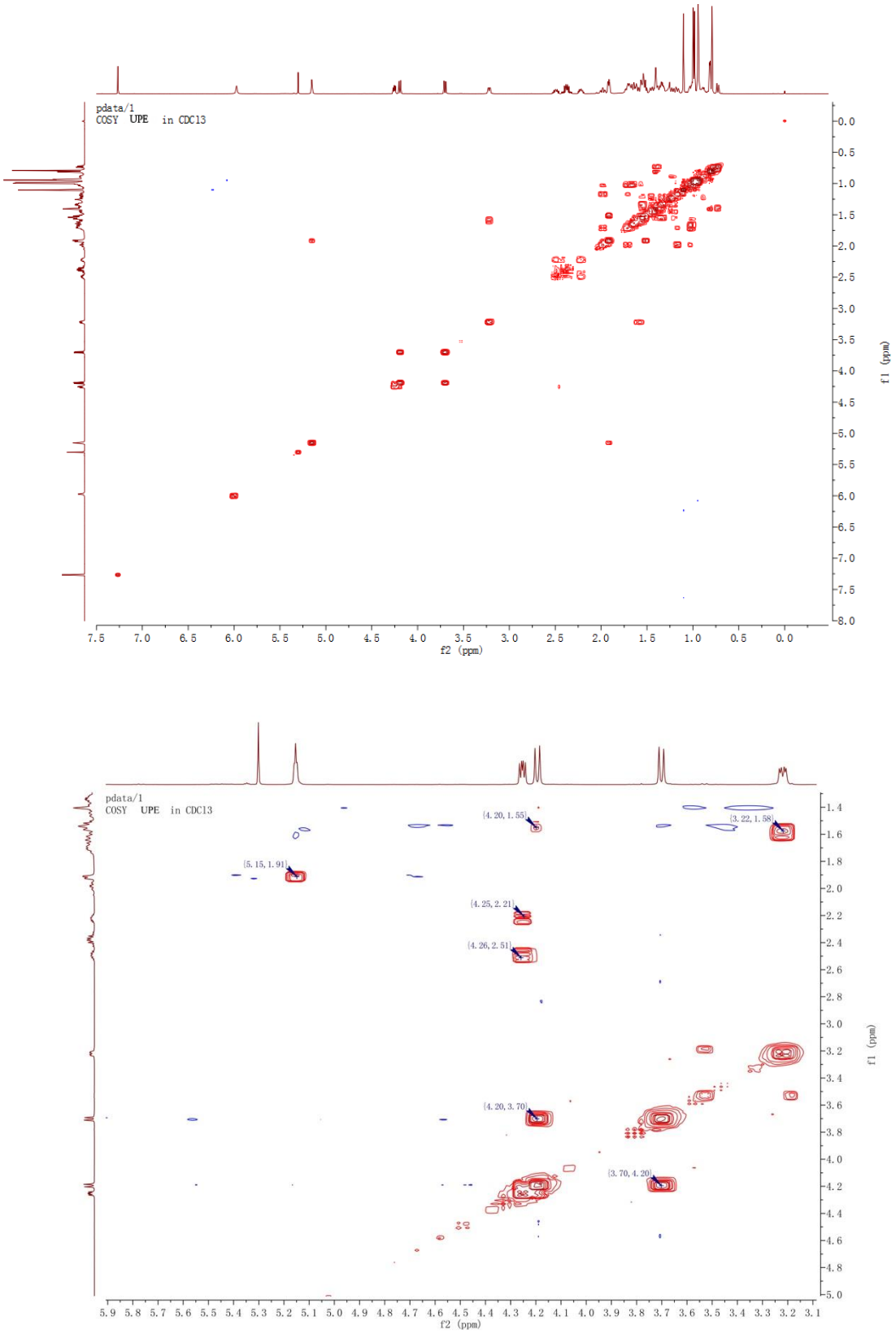


Figure S4. COSY spectrum (600 MHz) of UPE in $CDCl_3$ and its expanded downfield region.

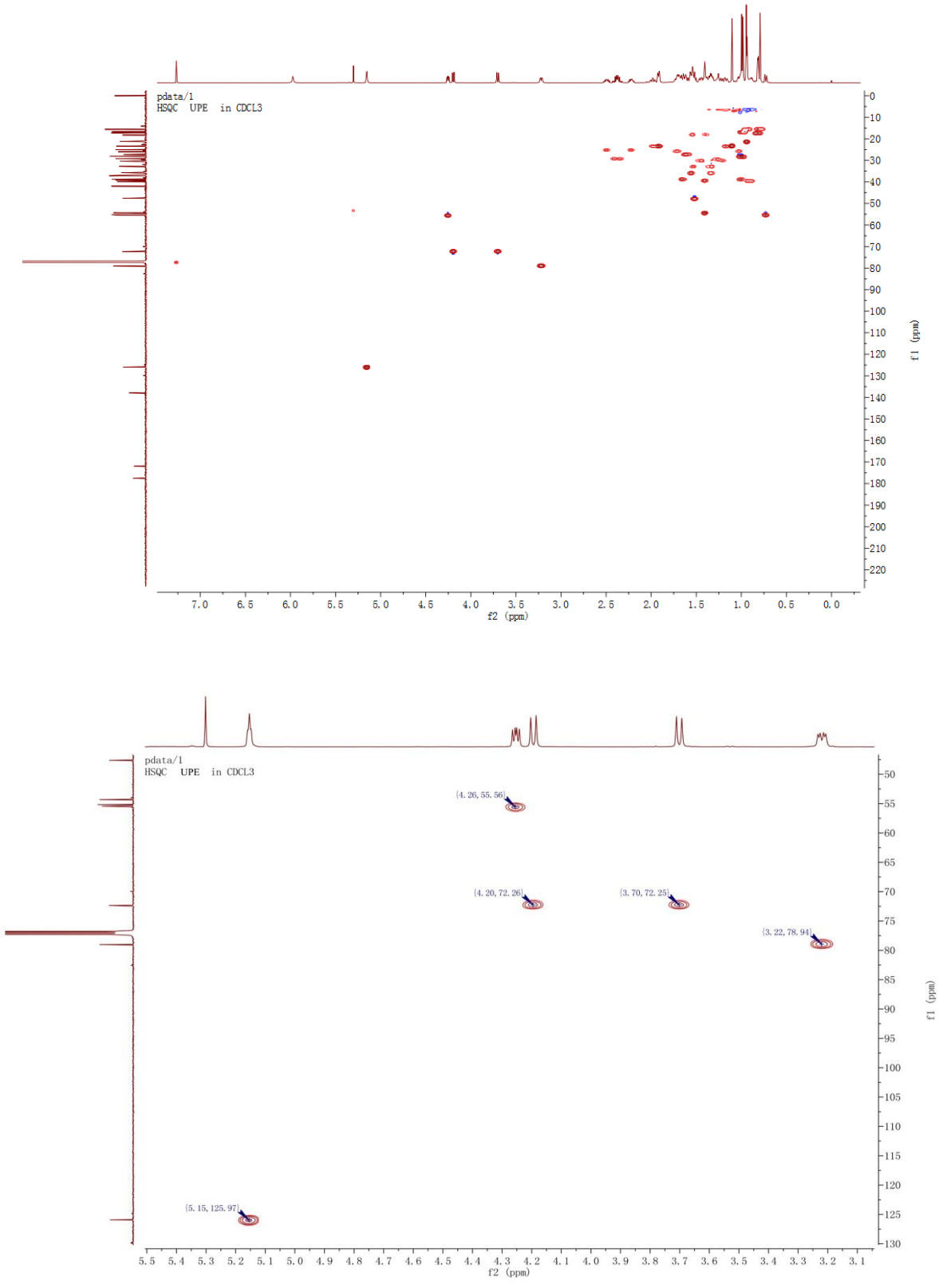


Figure S5. HSQC spectrum (150 MHz) of UPE in $CDCl_3$ and its expanded downfield region.

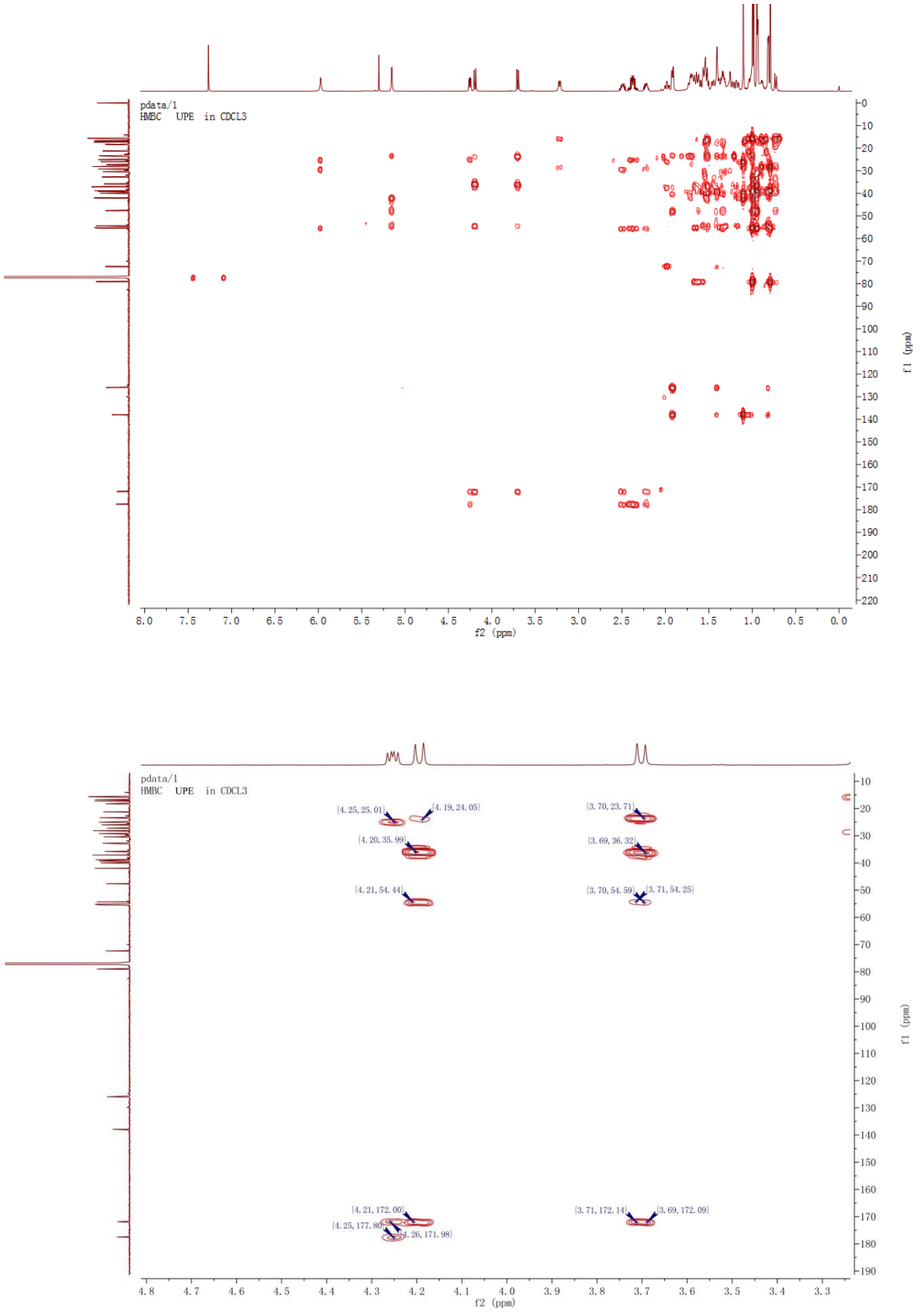


Figure S6. HMBC spectrum (150 MHz) of UPE in $CDCl_3$ and its expanded downfield region.

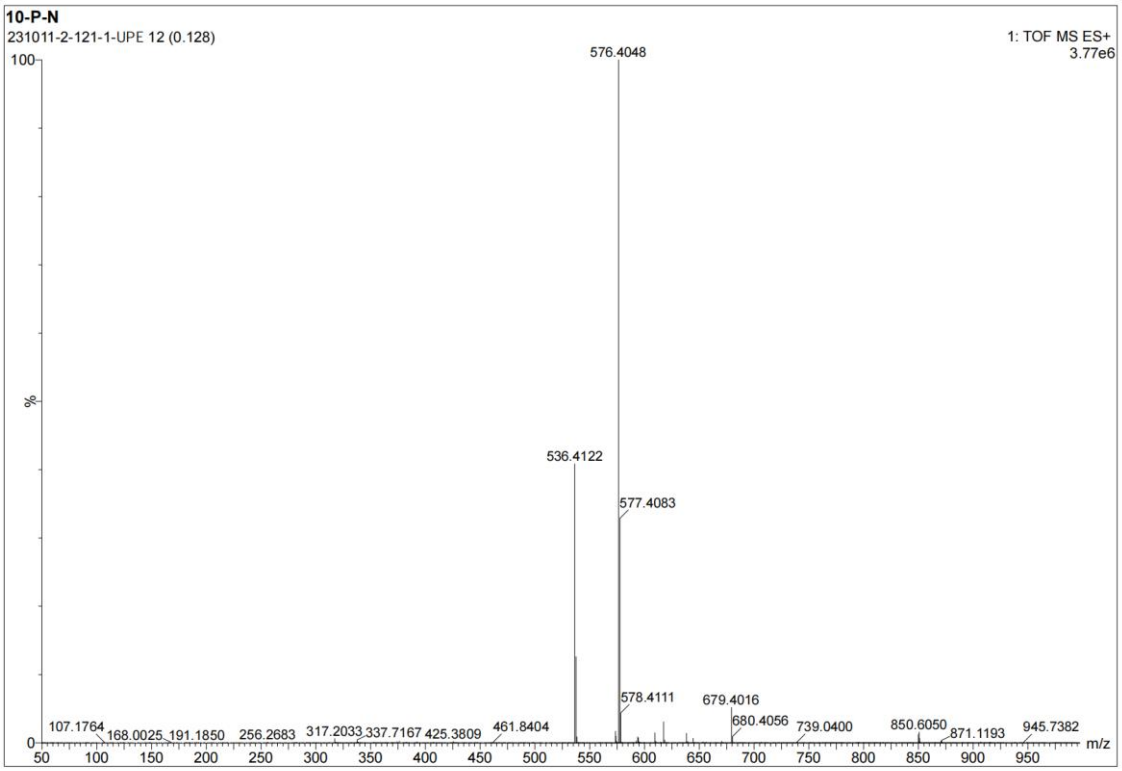


Figure S7. HR-ESI-MS spectrum of **UPE**.

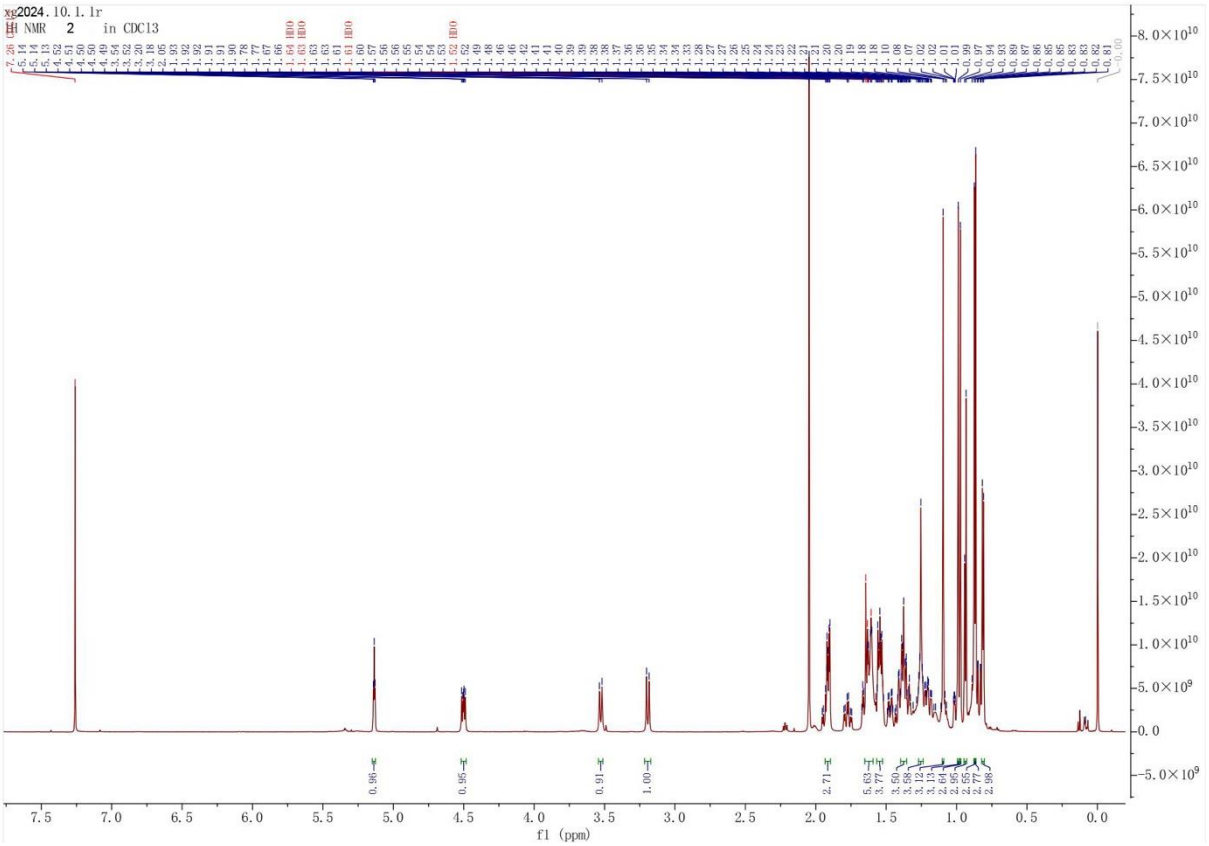


Figure S8: ^{1}H-NMR spectrum (600 MHz) of compounds **2**.

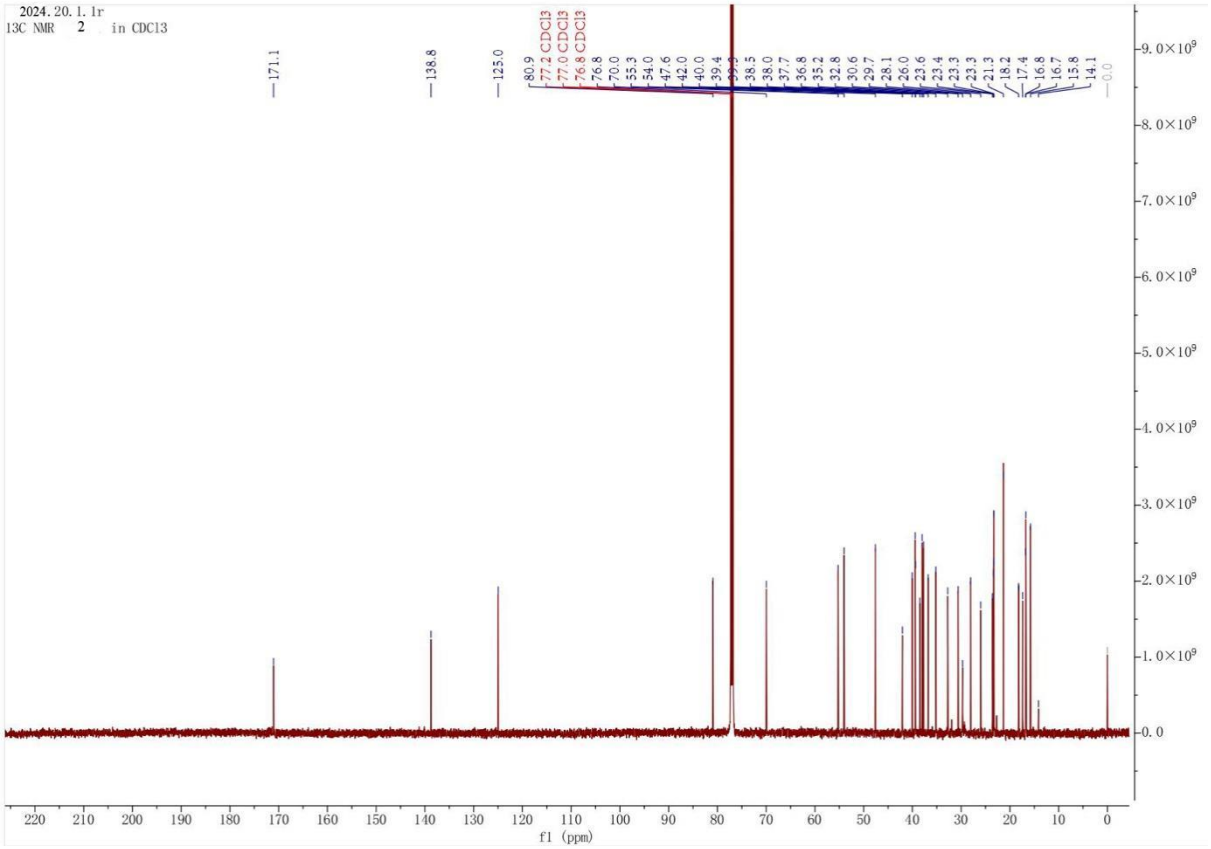


Figure S9: ^{13}C-NMR spectrum (150 MHz) of compounds **2**.

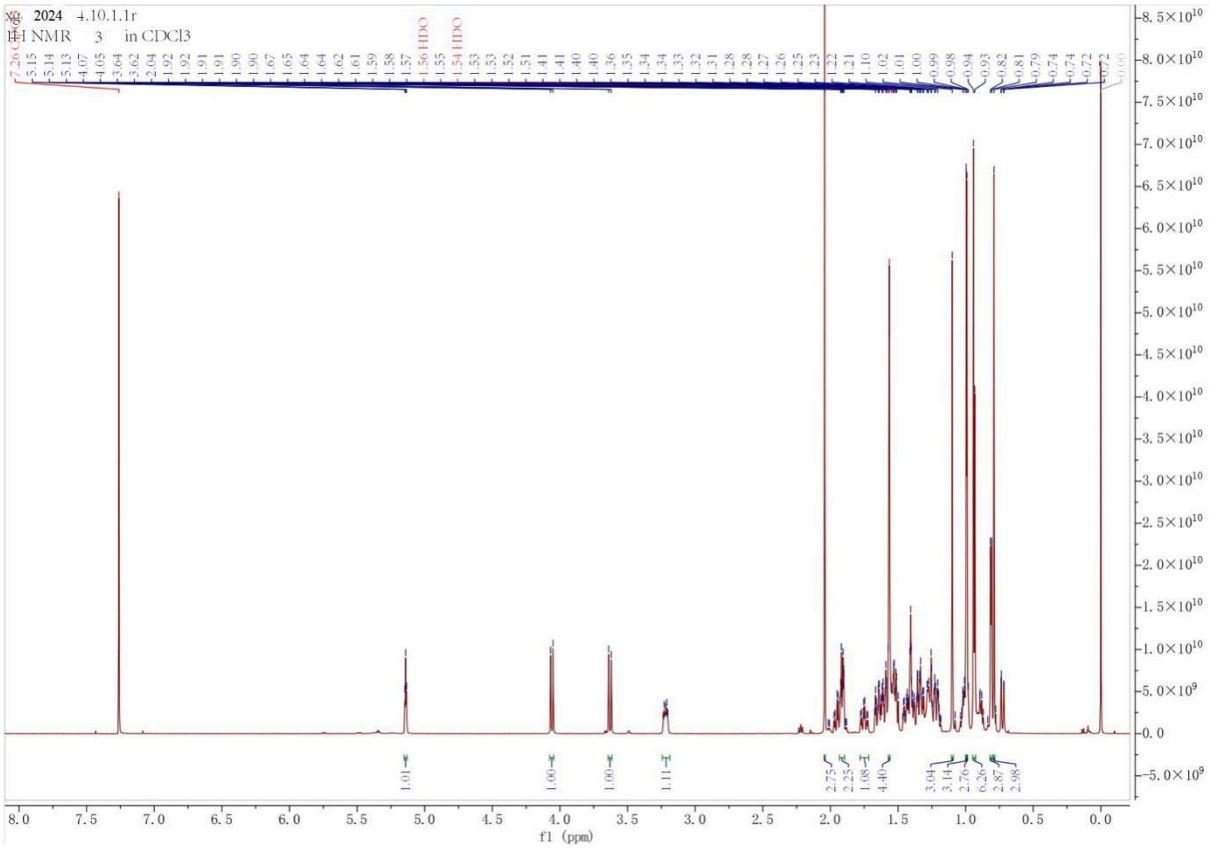


Figure S10: ^{1}H-NMR spectrum (600 MHz) of compounds **3**.

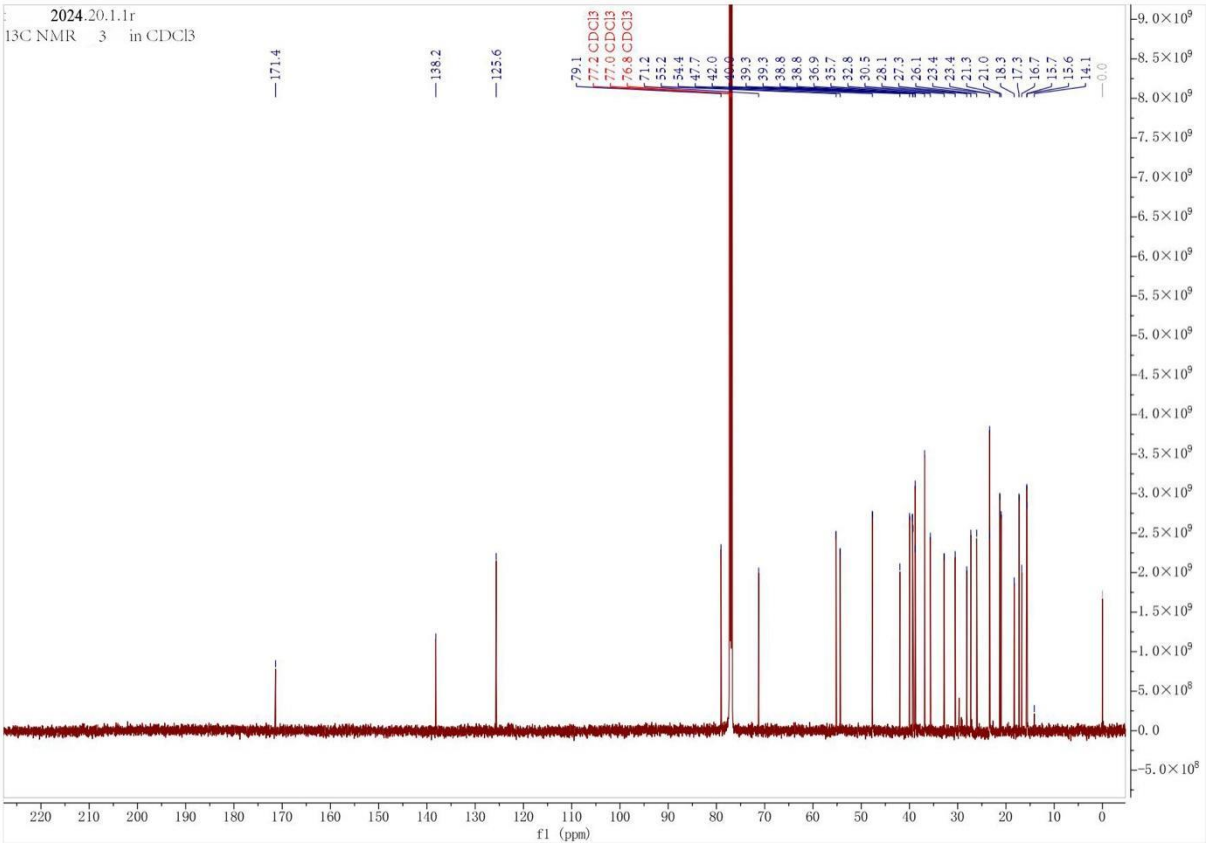


Figure S11. ^{13}C-NMR spectrum (150 MHz) of compounds **3**.

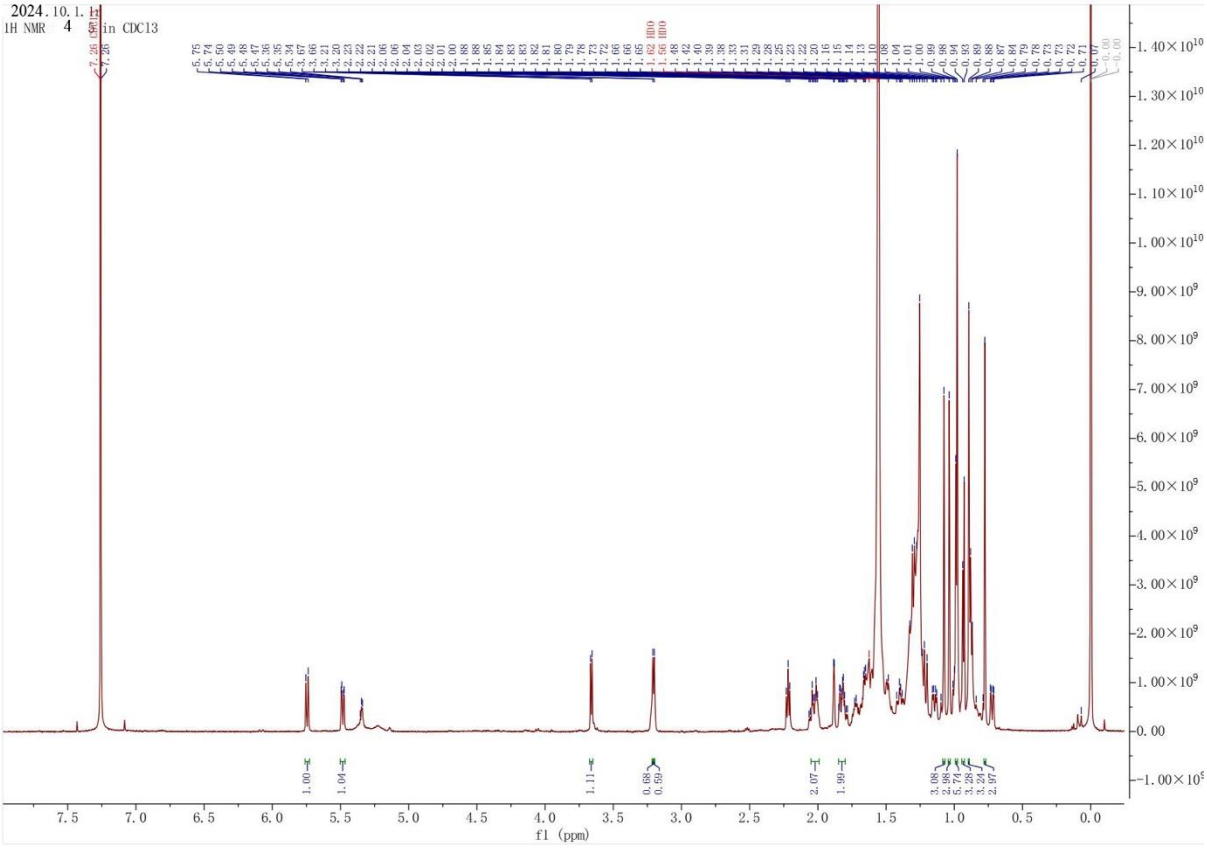


Figure S12. ^{1}H-NMR spectrum (600 MHz) of compounds **4**.

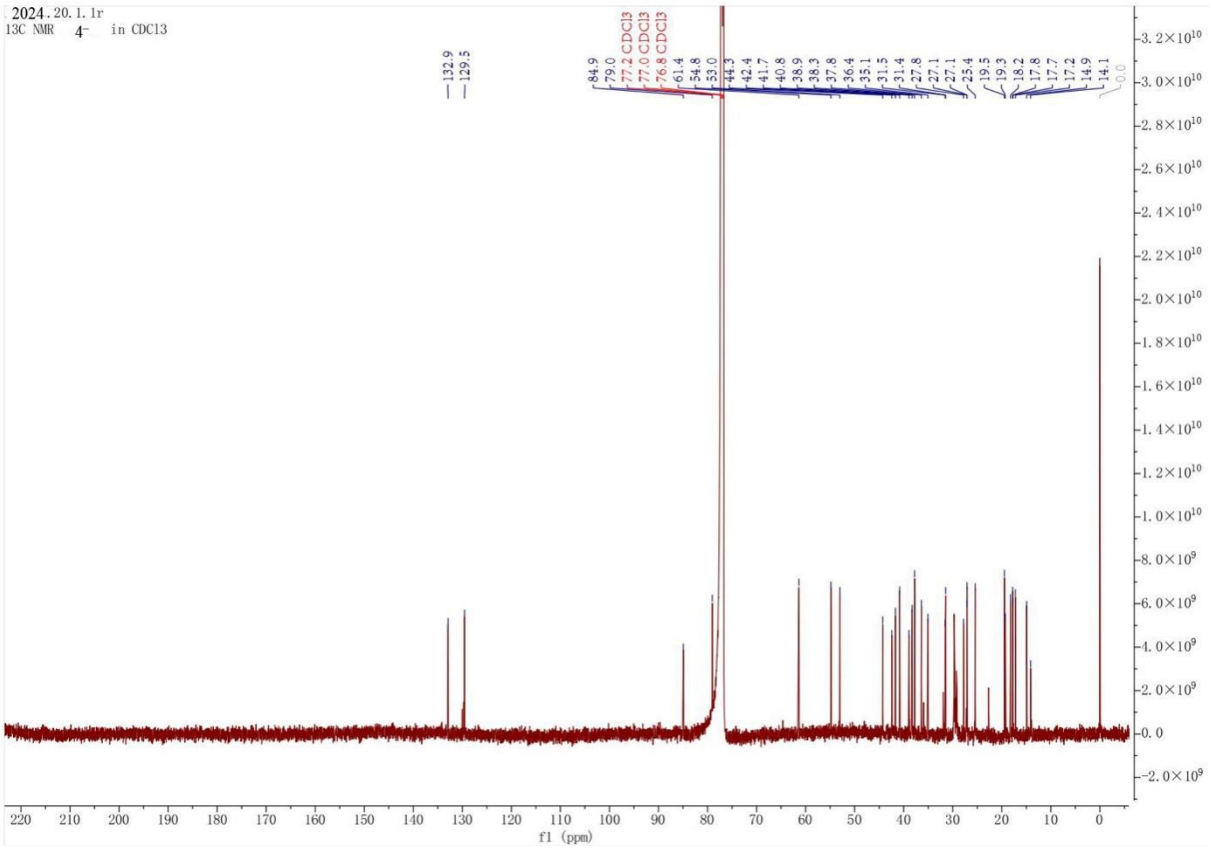


Figure S13. ^{13}C-NMR spectrum (150 MHz) of compounds **4**.

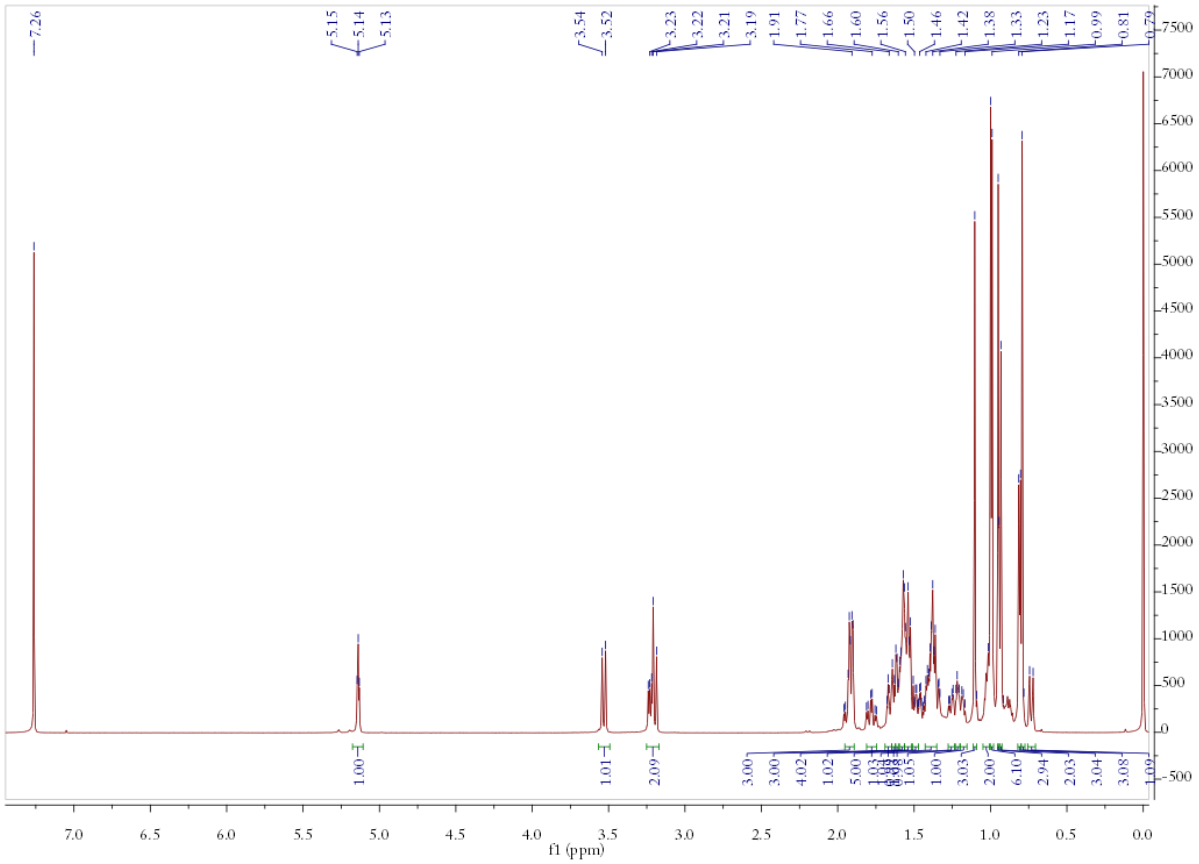


Figure S14. ^{1}H-NMR spectrum (600 MHz) of uvaol.

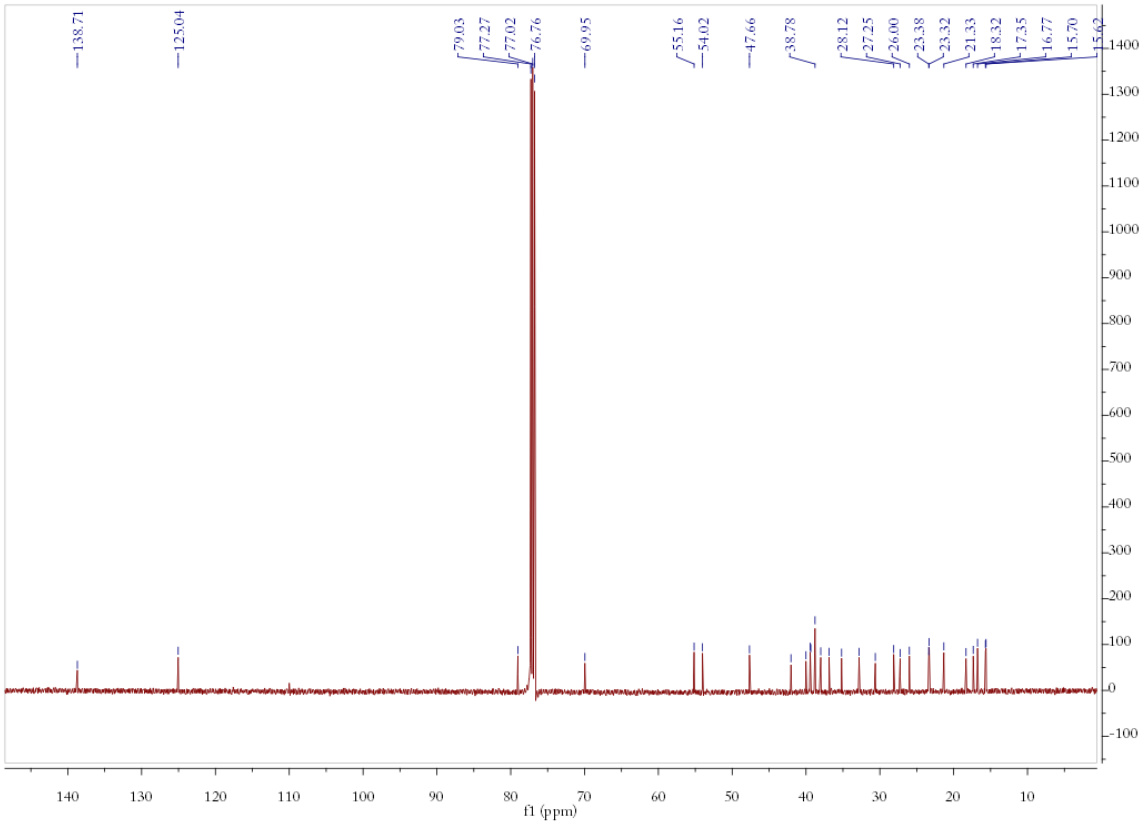


Figure S15. ^{13}C-NMR spectrum (150 MHz) of uvaol.

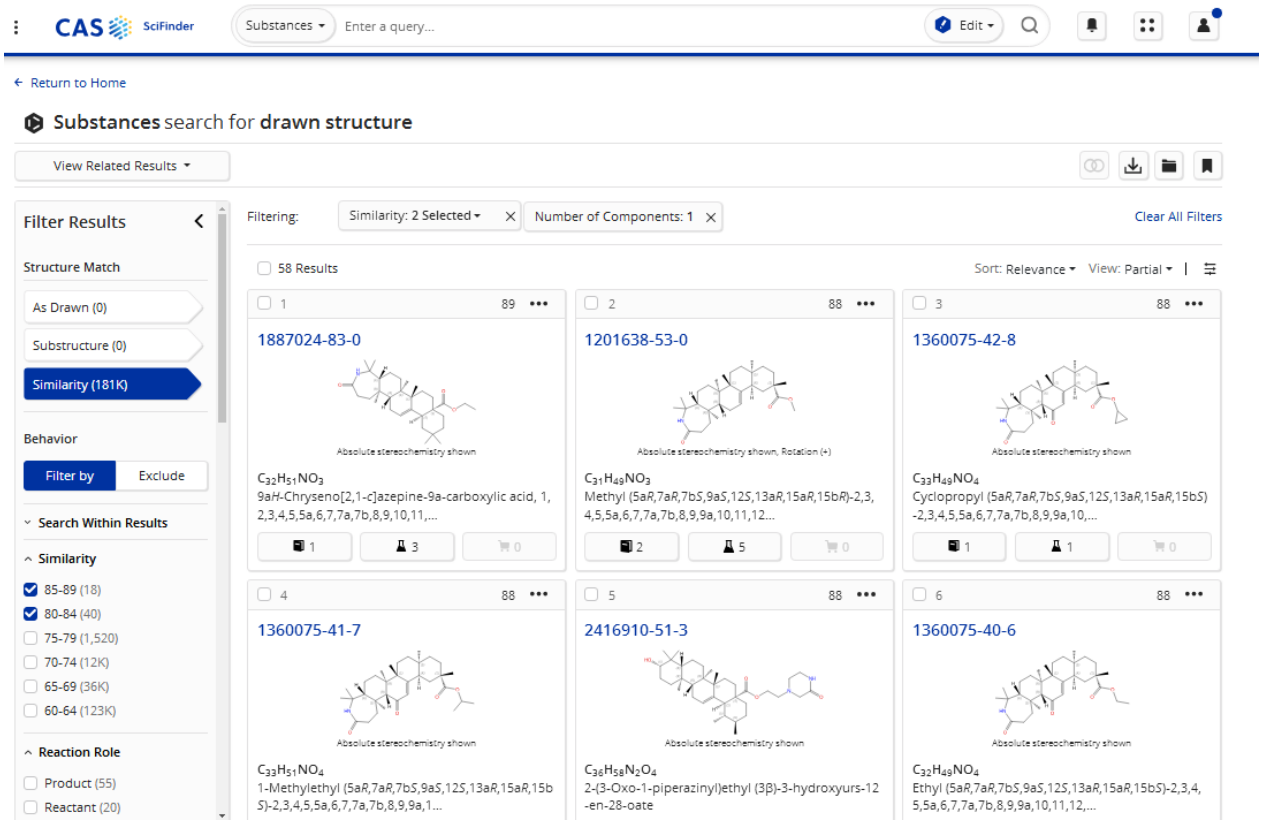
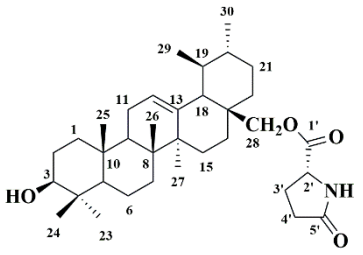


Figure S16. Scifinder search results of UPE.



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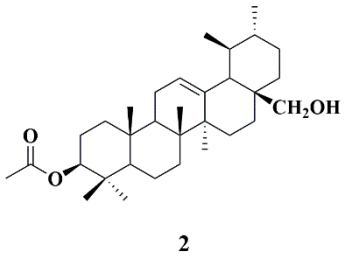

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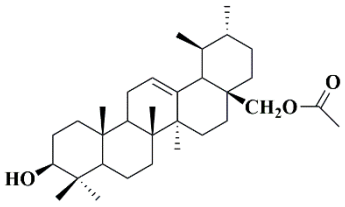

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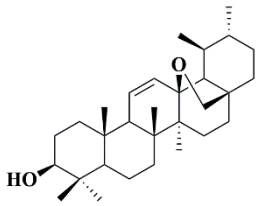
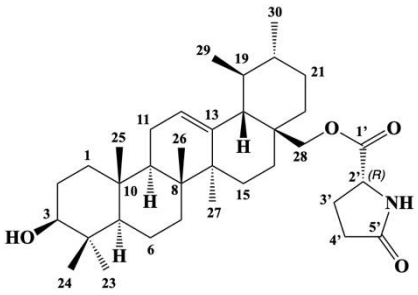


4

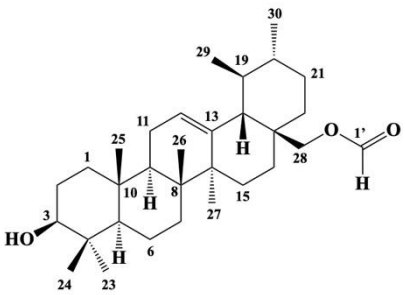
Table S1. NMR data for compounds UPE and **2-4** in $CDCl_3$ (δ in ppm and J in Hz).

NO.	UPE		2 [6]		3 [7]		4 [8]	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	1.01 m, 1.64 m	38.9	1.01, 1.56 m	38.6	1.01, 1.65 m	38.8	1.83, 0.98 m	38.3
2	1.60 m	27.2	1.61 m	23.7	1.62 m	27.3	1.63 m	25.4
3	3.21 dd (11.3, 4.8)	79.2	4.51 m	81.1	3.21 m	79.1	3.21 s	79.0
4	-	38.9	-	37.9	-	36.9	-	38.9
5	0.72 bd (11.6)	55.6	0.82 m	54.2	0.72 m	54.4	0.72 m	54.8
6	1.38 m, 1.52 m	18.3	1.36, 1.55 m	18.4	1.36, 1.55 m	18.3	1.01, 1.00 m	17.7
7	1.32 m, 1.52 m	32.7	1.23, 1.41 m	32.9	1.23, 1.41 m	30.5	1.40, 1.26 m	31.4
8	-	40.1	-	40.2	-	40.0	-	42.4
9	1.50 m	47.6	1.53 m	47.7	1.53 m	47.7	1.88 m	53.0
10	-	36.9	-	36.9	-	38.8	-	36.4
11	1.91 m	23.6	1.91 m	23.4	1.91 m	23.4	5.75 d (10.4)	129.5
12	5.15 t (3.4)	126.1	5.14 t (3.2)	125.1	5.14 t (3.3)	125.6	5.48 dd (10.3, 3.0)	132.9
13	-	138.0	-	138.9	-	138.2	-	84.9
14	-	42.1	-	42.2	-	42.0	-	41.7
15	1.01 m, 1.70 m	26.1	1.02, 1.71 m	26.1	1.02, 1.71 m	26.1	1.25,1.24 m	29.7
16	1.17 bd (13.6), 1.98 td (4.8, 13.6)	23.6	1.98, 1.69 m	23.5	1.95, 1.68 m	23.4	2.01, 1.13 m	27.1
17	-	37.0	-	38.1	-	36.9	-	44.3
18	1.41 m	54.3	1.41 m	55.4	1.41 m	55.2	1.20 m	61.4
19	1.39 m	39.4	0.90 m	39.5	0.90 m	39.3	1.72 m	37.8
20	0.88 m	39.5	1.41 m	39.6	1.41 m	39.3	0.87 m	40.8
21	1.32 m, 1.52 m	32.9	1.34, 1.52 m	30.8	1.34, 1.52 m	32.8	1.49, 1.08 m	31.5
22	1.32 m, 1.54 m	35.9	1.34, 1.56 m	35.3	1.34, 1.56 m	35.7	1.57, 1.29 m	35.1
23	1.00 s	28.1	1.08 s	28.2	1.00 s	28.1	0.98 s	27.8

24	0.79 s	15.7	0.86 s	15.9	0.79 s	15.7	0.78 s	14.9
25	0.95 s	15.6	0.94 s	16.8	0.94 s	15.6	0.89 s	17.8
26	0.98 s	16.7	0.97 s	16.9	0.98 s	16.7	1.08 s	19.3
27	1.10 s	23.5	1.10 s	23.2	1.10 s	23.4	1.04 s	17.2
28	3.70 d (10.9), 4.19 d (10.9)	72.5	3.19 d (10.7), 3.53 d (10.7)	70.0	3.63 d (10.5), 4.06 d (10.5)	71.2	3.66 d (6.7)	76.8
29	0.81 d (4.6)	17.4	0.82 d (4.6)	21.4	0.82 d (4.6)	21.3	0.93 d (6.4)	19.5
30	0.93 d (4.9)	21.4	0.93 d (4.9)	17.5	0.93 d (4.9)	17.3	0.99 d (6.3)	18.2
1'	-	172.0	-	171.2	-	171.4		
2'	4.25 dd (8.7, 5.3)	55.6	2.05 s	21.5	2.04 s	21.0		
3'	2.21 m, 2.50 m	25.2						
4'	2.33 m, 2.37 m	29.4						
5'	-	177.7						



UPE



UFE

Table S2. NMR data for compounds UPE and Uvaol-28-formyl ester (UFE) in $CDCl_3$ (δ in ppm and J in Hz).

NO.	**UPE**		**UFE** [6]	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	1.01 m, 1.64 m	38.9	1.01 m, 1.65 m	39.2
2	1.60 m	27.2	1.62 m	28.1
3	3.21 dd (11.3, 4.8)	79.2	3.21 dd (11.5, 5.5)	79.0
4	-	38.9	-	38.9
5	0.72 bd (11.6)	55.6	0.72 m	55.2
6	1.38 m, 1.52 m	18.3	1.42, 1.57 m	18.3
7	1.32 m, 1.52 m	32.7	1.40, 1.57 m	32.7
8	-	40.1	-	40.2
9	1.50 m	47.6	1.53 m	47.6
10	-	36.9	-	36.9
11	1.91 m	23.6	1.93 m	23.3
12	5.15 t (3.4)	126.1	5.16 t (3.7)	125.8
13	-	138.0	-	138.0

14	-	42.1	-	42.1
15	1.01 m, 1.70 m	26.1	1.04 m, 1.18 m	26.1
16	1.17 bd (13.6), 1.98 td (4.8, 13.6)	23.6	1.96 m, 1.71 m	23.4
17	-	37.0	-	36.9
18	1.41 m	54.3	1.42 m	54.0
19	1.39 m	39.4	1.42 m	39.4
20	0.88 m	39.5	1.00 m	39.3
21	1.32 m, 1.52 m	32.9	1.27 m, 1.48 m	32.8
22	1.32 m, 1.54 m	35.9	1.40 m, 1.60 m	35.2
23	1.00 s	28.1	1.00 s	28.1
24	0.79 s	15.7	0.79 s	15.7
25	0.95 s	15.6	0.95 s	15.6
26	0.98 s	16.7	1.03 s	16.8
27	1.10 s	23.5	1.12 s	23.3
28	3.70 d (10.9), 4.19 d (10.9)	72.5	3.74 d (11.0), 4.15 d (11.0)	69.9
29	0.81 d (4.6)	17.4	0.84 d (5.6)	21.4
30	0.93 d (4.9)	21.4	0.93 d (5.8)	17.4
1'	-	172.0	8.08 s	161.3
2'	4.25 dd (8.7, 5.3)	55.6		
3'	2.21 m, 2.50 m	25.2		
4'	2.33 m, 2.37 m	29.4		
5'	-	177.7		

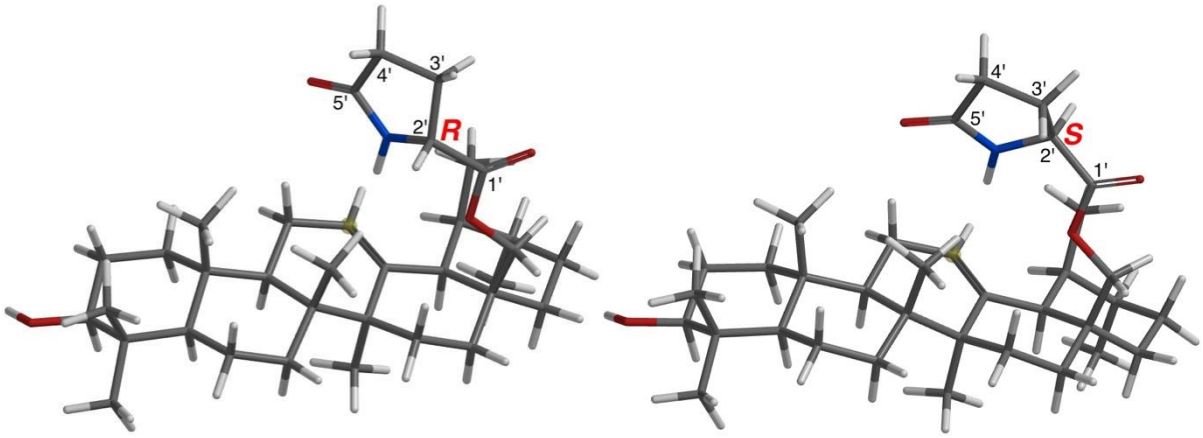


Figure S17. Quantum-chemistry calculated lowest-conformation of UPE-2'-(*R*) (at left) and UPE-2'-(*S*) (at right) isomers.

Table S3. DP4+ analysis of the lowest-energy conformers of UPE-2' isomers[4]

Carbon Number	Calculated ^{13}C-NMR Chemical shifts		Experimental ^{13}C-NMR Chemical shifts
	UPE-2'-(*R*)-isomer	**UPE-2'-(*S*)-isomer**	**UPE**
1	37.9	38.1	38.9
2	29.2	29.3	27.2
3	77.2	77.2	79.2
4	38.4	38.4	38.9
5	55.1	55.1	55.6
6	19.5	19.5	18.3
7	34.6	34.6	32.7
8	39.7	39.9	40.1
9	47.4	47.9	47.6
10	36.9	37.0	36.9
11	23.9	23.8	23.6
12	120.9	120.0	126.1
13	144.0	144.4	138.0
14	44.3	44.8	42.1
15	27.9	27.7	26.1
16	37.7	38.2	23.6
17	38.1	38.5	37.0
18	40.8	40.5	54.3
19	34.2	34.3	39.4
20	34.3	34.6	39.5
21	22.5	22.4	32.9
22	32.1	32.3	35.9
23	28.2	28.3	28.1
24	17.2	17.2	15.7
25	18.6	18.1	15.6
26	18.9	18.7	16.7
27	24.9	25.5	23.5
28	70.3	70.6	72.5
29	17.4	18.6	17.4
30	19.3	19.3	21.4
1'	172.9	174.4	172.0
2'	56.2	56.8	55.6
3'	25.1	27.4	25.2
4'	29.2	28.9	29.4
5'	173.9	174.8	177.7
DP4+%	**97.3**	2.7	
RMS	4.44	4.57	
Mean Abs.	2.78	2.95	

References

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