Supporting Information

Biotransformation of ursolic acid by *Circinella muscae* and their anti-neuroinflammatory activities of metabolites

Chengjiao Chu [a], Kainan Song [a], Yongzheng Zhang [a], Min Yang [a], Boyi Fan [a,*], Huilian Huang [b], Guangtong Chen [a,*]

[a] *School of Pharmacy, Nantong University, Nantong, China*

[b] *Key Laboratory of Modern Preparation of TCM, Jiangxi University of Traditional Chinese Medicine, ministry of education, Nanchang, China*

Abstract

In this study, the biotransformation of ursolic acid by *Circinella muscae* CGMCC 3.2695 was investigated. Scaled-up biotransformation reactions yielded ten metabolites. Their structures were established based on extensive NMR and HR-ESI-MS data analyses, and four of them are new compounds. *C. muscae* could selectively catalyze hydroxylation, lactonization, carbonylation and carboxyl reduction reactions. Furthermore, all the identified metabolites were evaluated for their anti-neuroinflammatory activities in LPS-induced BV-2 cells. Most metabolites displayed pronounced inhibitory effect on nitric oxide (NO) production. The results suggested that biotransformed derivatives of ursolic acid might be served as potential neuroinflammatory inhibitors.

[*]Corresponding author at: School of Pharmacy, Nantong University, 19 Qixiu Road, Nantong, Jiangsu 226001, China. Tel/fax.: +86 513 85051749

Email address: fanboyi@ntu.edu.cn (B-Y. Fan), guangtong_chen@163.com (G-T. Chen).

3. Experimental Section

3.1. *General Experimental Procedures*

The IR spectrum was recorded using a Nicolet iS10 FT/IR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). 1D and 2D NMR spectra were recorded on Varian INOVA 500 spectrometers. HR-ESI-MS and ESI-MS data were obtained on a Finnigan LCQ^{DECA} instrument (Thermo Finnigan, San Jose, CA, USA). HPLC was performed on a Shimadzu Liquid Chromatography LC-20AD with an YMC packed J'sphere ODS-H80 column (250 × 10 mm, 4 μm, 80 Å) using a Shimadzu UV detector SPD-20A.

3.2. *Substrate and organisms*

The substrate ursolic acid (No. SU675301-0024, purity≥98% by HPLC) and L-NMMA (No. TOCO077110-0016, purity≥98% by HPLC) were purchased from Sinopharm Chemical Reagent Co., China. *Circinella muscae* CGMCC 3.2695 was purchased from China General Microbiological Culture Collection Center, and were maintained on potato slants solidified with agar at 4 ^{o}C. BV-2 cell line was obtained from the Chinese Academy of Sciences Cell Bank.

3.3. *Biotransformation*

The fungi were cultured by two-stage liquid fermentation as previously reported. The fungal inoculum was transferred to 1000 mL flasks each containing 400 mL of liquid potato dextrose medium, and incubated on a rotary shaker at 26 ^{o}C 160 rpm for 48 h. Then 25 mg of ursolic acid (UA, 25 mg/mL in DMSO) 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.

3.4. *Isolation and purification of metabolites*

The residue (0.93 g) was purified on a ODS C_{18} open columns (100 g, 60 × 3 cm)

and eluted with 20% CH_3OH-H_2O, 60% CH_3OH-H_2O, 80% CH_3OH-H_2O, and CH_3OH to give the Fractions A, B, C and D, respectively. Fraction B was then purified on a semi-preparative HPLC (CH_3CN:H_2O, 40:60, flow rate, 2.5 mL/min) to yield compounds **1** (13.4 mg), **2** (20.1 mg), **3** (11.6 mg), **4** (22.7 mg), **5** (9.8 mg), and **6** (15.2 mg). Fraction C was then purified on a semi-preparative HPLC (CH_3CN:H_2O, 55:45, flow rate, 2.5 mL/min) to yield compounds **7** (14.4 mg), **8** (8.7 mg), **9** (6.2 mg), and **10** (8.1 mg).

3.4.1. *3β,7β,15α,21β-tetrahydroxy-uvaol (**3**)*

White amorphous power; $[\alpha]_D^{22}$: +84.7° (*c* = 0.1, MeOH); IR (KBr): ν_{max} = 3581, 3037, 2916, 1384, 1221, 1059 cm^{-1}; HR-ESI-MS: *m*/*z* = 489.3584 $[M-H]^-$ (calcd. 489.3586 for $C_{30}H_{49}O_5$); ^1H NMR (pyridine-d_6, 500 MHz) and ^{13}C NMR (pyridine-d_6, 125 MHz): see Tables S1 and S2.

3.4.2. *3β,7β,30-trihydroxy-ursolic acid (**4**)*

White amorphous power; $[\alpha]_D^{22}$: +71.3° (*c* = 0.1, MeOH); IR (KBr): ν_{max} = 3502, 3027, 2925, 1711, 1436, 1207, 1071 cm^{-1}; HR-ESI-MS: *m*/*z* = 487.3427 $[M-H]^-$ (calcd. 487.3429 for $C_{30}H_{47}O_5$); ^1H NMR (pyridine-d_6, 500 MHz) and ^{13}C NMR (pyridine-d_6, 125 MHz): see Tables S1 and S2.

3.4.3. *3-oxo-7β,21β-dihydroxy-ursolic acid (**6**)*

White amorphous power; $[\alpha]_D^{22}$: +77.2° (*c* = 0.1, MeOH); IR (KBr): ν_{max} = 3476, 3011, 2930, 1714, 1427, 1380, 1183 cm^{-1}; HR-ESI-MS: *m*/*z* = 485.3273 $[M-H]^-$ (calcd. 485.3272 for $C_{30}H_{45}O_5$); ^1H NMR (pyridine-d_6, 500 MHz) and ^{13}C NMR (pyridine-d_6, 125 MHz): see Tables S1 and S2.

3.4.4. *3β,7β,15α,20β-tetrahydroxy-uvaol (**7**)*

White amorphous power; $[\alpha]_D^{22}$: +86.8° (*c* = 0.1, MeOH); IR (KBr): ν_{max} = 3485,

3011, 2923, 1372, 1215, 1031 cm^{-1}; HR-ESI-MS: *m*/*z* = 489.3585 $[M-H]^-$ (calcd. 489.3586 for $C_{30}H_{49}O_5$); ^1H NMR (pyridine-d_6, 500 MHz) and ^{13}C NMR (pyridine-d_6, 125 MHz): see Tables S1 and S2.

3.5. *LPS-induced nitric oxide production*

The production of NO was indirectly determined by Griess reaction to measure the concentration of nitrite in the culture medium. DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin was applied to cultured BV-2 cells. BV-2 cells were seeded in 96-well microplates at a density of 8×10^4 cells/well and were incubated for 24 h. Cells were exposed to 100 ng/mL of LPS with various compounds concentrations for 48 h. The culture supernatant (100 μL) was mixed with Griess reagent and incubated at room temperature for 10 min. The absorbance was measured at 570 nm. L-NMMA was selected as positive control. An MTT assay was used for the cell viability assay. All experiments were performed in triplicate. The concentration required for 50% inhibition of NO production (IC_{50}) was calculated with GraphPad Prism 7.00 (GraphPad Software, Inc.).

Table S1 ^{1}H NMR data for metabolites **3**, **4**, **6**, and **7** (pyridine-d_5, 500 MHz).

No.	**3**	**4**	**6**	**7**
1	0.96, 1.63 m	0.93, 1.59 m	1.33, 1.74 m	0.98, 1.64 m
2	1.87 m	1.84, 1.91 m	2.46, 2.53 m	1.88 m
3	3.50 m	3.47 dd (10.1, 5.7)	-	3.51 m
5	1.05 m	1.05 m	1.60 m	1.10 m
6	1.87, 2.11 m	1.81, 2.00 m	1.82 m	1.45, 2.16 m
7	4.34 dd (11.2, 4.8)	4.27 dd (12.5, 5.4)	4.26 dd (9.7, 5.9)	4.37 dd (10.4, 3.2)
9	1.61 m	1.63 m	1.66 m	1.64 m
11	2.03 m	2.00, 2.07 m	1.94, 2.07 m	2.04 m
12	5.49 t (3.8)	5.68 m	5.68 m	5.55 m
15	4.81 dd (10.9, 5.9)	2.25, 2.87 m	2.28, 2.79 m	4.92 m
16	2.35 m	2.15, 2.33 m	2.24, 2.28 m	2.32, 2.50 m
18	1.88 m	2.86 d (12.4)	2.86 d (11.5)	2.45 m
19	1.90 m	2.17 m	1.85 m	2.15 m
20	1.25 m	1.34 m	1.37 m	-
21	3.84 td (10.7, 4.0)	2.01 m	3.87 m	1.85, 2.47 m
22	2.03 m 2.65 dd (13.0, 4.3)	2.05, 2.17 m	2.24, 2.70 m	2.01, 2.49 m
23	1.28 s	1.23 s	1.15 s	1.28 s
24	1.07 s	1.05 s	1.04 s	1.07 s
25	1.00 s	0.97 s	0.96 s	1.00 s
26	1.31 s	1.37 s	1.34 s	1.33 s
27	1.55 s	1.50 s	1.47 s	1.62 s
28	3.67 d (10.5) 4.03 d (10.5)	-	-	3.70 d (10.6) 4.15 d (10.6)
29	1.12 d (6.0)	1.22 d (6.7)	1.15 d (6.9)	1.31 d (6.7)
30	1.40 s (6.3)	3.93, 4.01 m	1.42 d (6.0)	1.45 s

Table S2 ^{13}C NMR data for metabolites **3**, **4**, **6**, and **7** (pyridine-d_5, 125 MHz).

No.	**3**	**4**	**6**	**7**
1	39.1	39.2	39.3	39.3
2	28.0	28.2	34.4	28.3
3	77.7	78.0	216.1	77.9
4	38.9	39.2	47.1	39.2
5	52.5	53.1	52.7	52.7
6	28.6	30.5	31.3	28.9
7	71.9	73.3	72.9	72.2
8	47.1	45.9	45.8	47.4
9	48.7	48.5	47.7	49.0
10	37.4	37.6	37.1	37.6
11	23.7	23.8	24.0	24.0
12	127.1	126.3	126.3	126.9
13	140.3	139.1	139.4	140.7
14	49.7	44.4	44.6	50.2
15	65.7	32.6	32.7	66.2
16	34.8	25.9	26.7	33.1
17	40.1	48.4	49.5	38.6
18	55.5	54.8	54.7	51.2
19	38.7	34.4	39.0	41.2
20	47.8	47.6	47.9	70.4
21	70.8	25.7	70.7	36.5
22	45.5	37.4	46.8	31.2
23	28.5	28.7	26.8	28.8
24	16.4	16.7	21.6	16.7
25	15.7	15.9	15.6	16.0
26	10.4	10.5	10.4	10.7
27	17.1	23.8	23.5	17.2
28	69.4	180.2	179.2	70.1
29	17.7	17.3	17.8	13.5
30	16.3	65.2	16.4	30.7

Table S3 Inhibitory effects of transformed products on NO production in LPS-activated BV-2 cells (mean ± SD, n = 3).

Compounds	IC_{50} (μM)	Cell viability (%)
UA	43.86 ± 2.46	104.52 ± 6.72
1	14.06 ± 1.27	107.09 ± 9.24
2	6.09 ± 1.22	98.64 ± 4.82
3	10.50 ± 1.01	98.96 ± 5.76
4	15.23 ± 1.24	118.46 ± 7.56
5	> 100	101.32 ± 5.23
6	39.70 ± 1.82	106.47 ± 6.70
7	58.61 ± 3.18	101.63 ± 4.61
8	30.85 ± 1.34	100.28 ± 8.89
9	> 100	99.82 ± 1.52
10	> 100	100.05 ± 2.75
L-NMMA[a]	22.46 ± 1.08	112.71 ± 5.04

[a] L-NMMA as a positive control

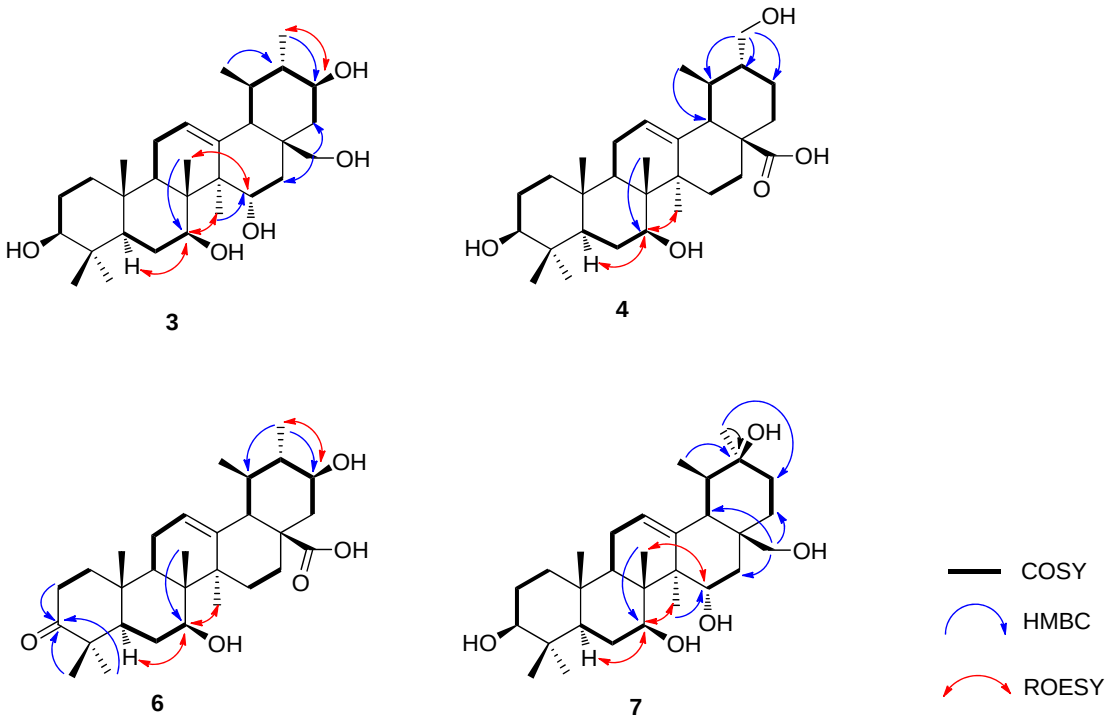

Fig. S1 Selected COSY, HMBC and ROESY correlations of metabolites **3**, **4**, **6** and **7**.

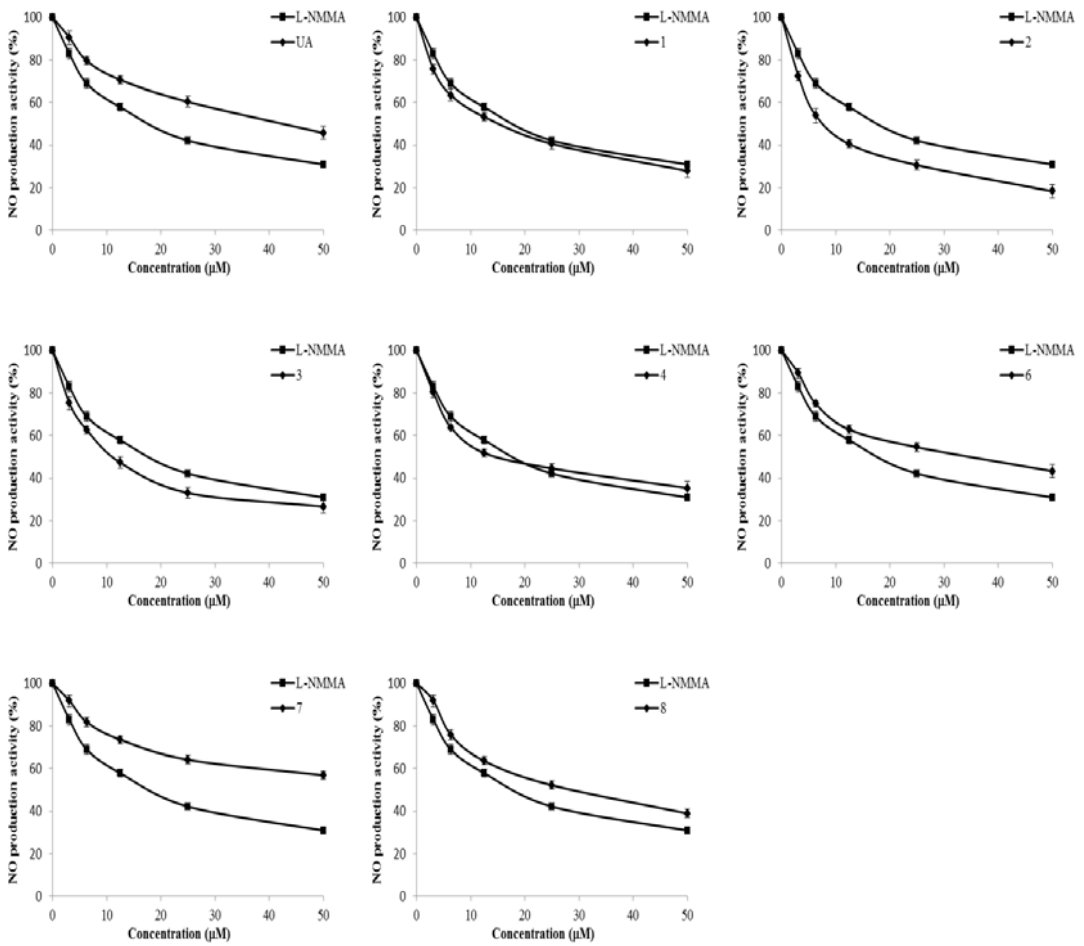

Fig. S2 Dose-effect result of inhibitory effect of UA, **1**-**4** and **6**-**8** on NO production (n = 3).

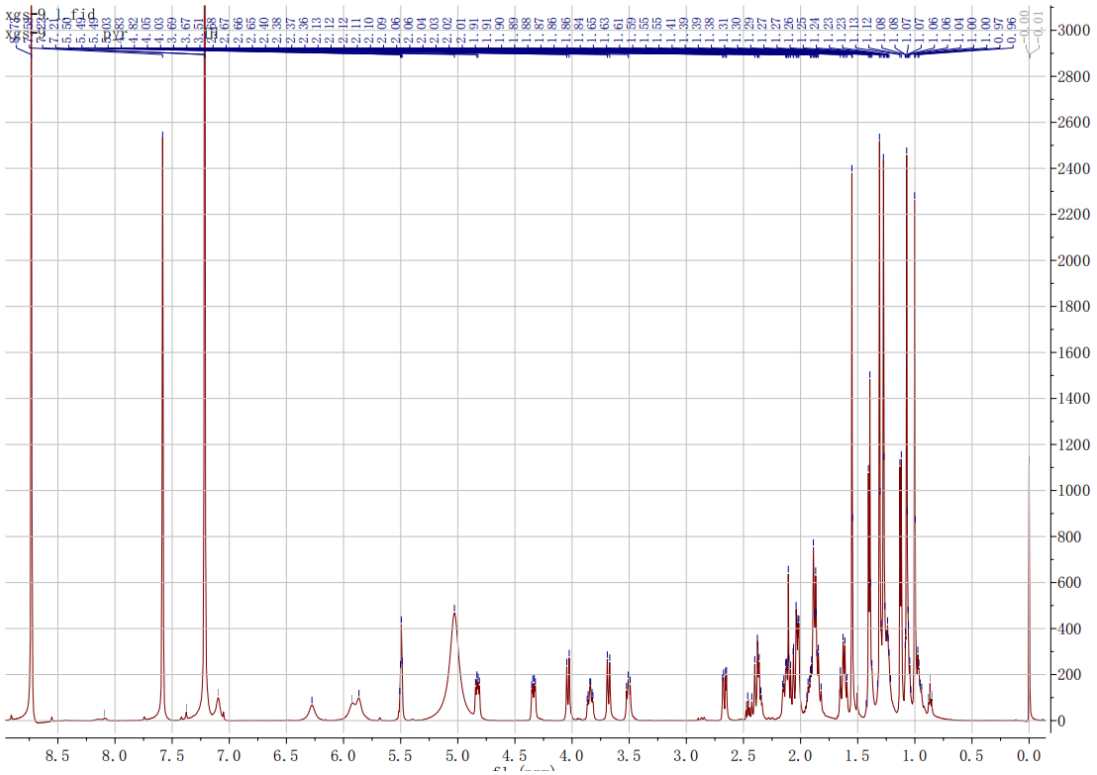

Fig. S3 ^{1}H NMR spectrum (500 MHz) of compound **3** in Pyridine-d_5

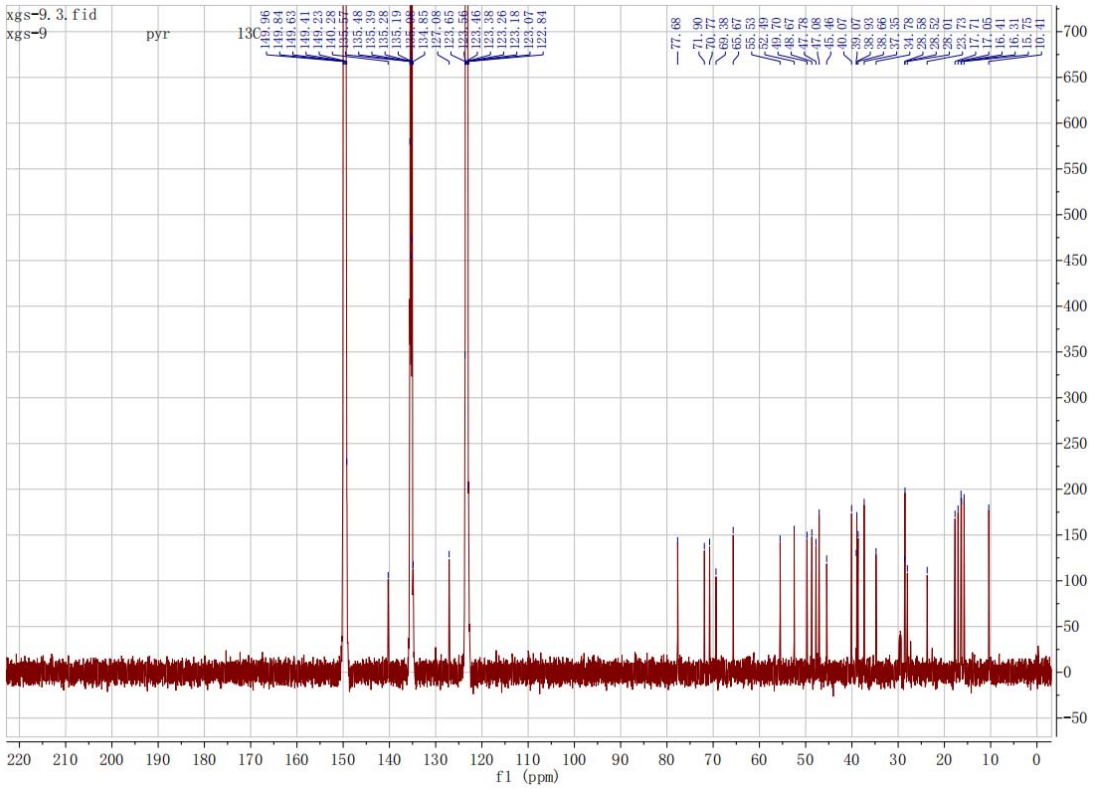

Fig. S4 ^{13}C NMR spectrum (125 MHz) of compound **3** in Pyridine-d_5

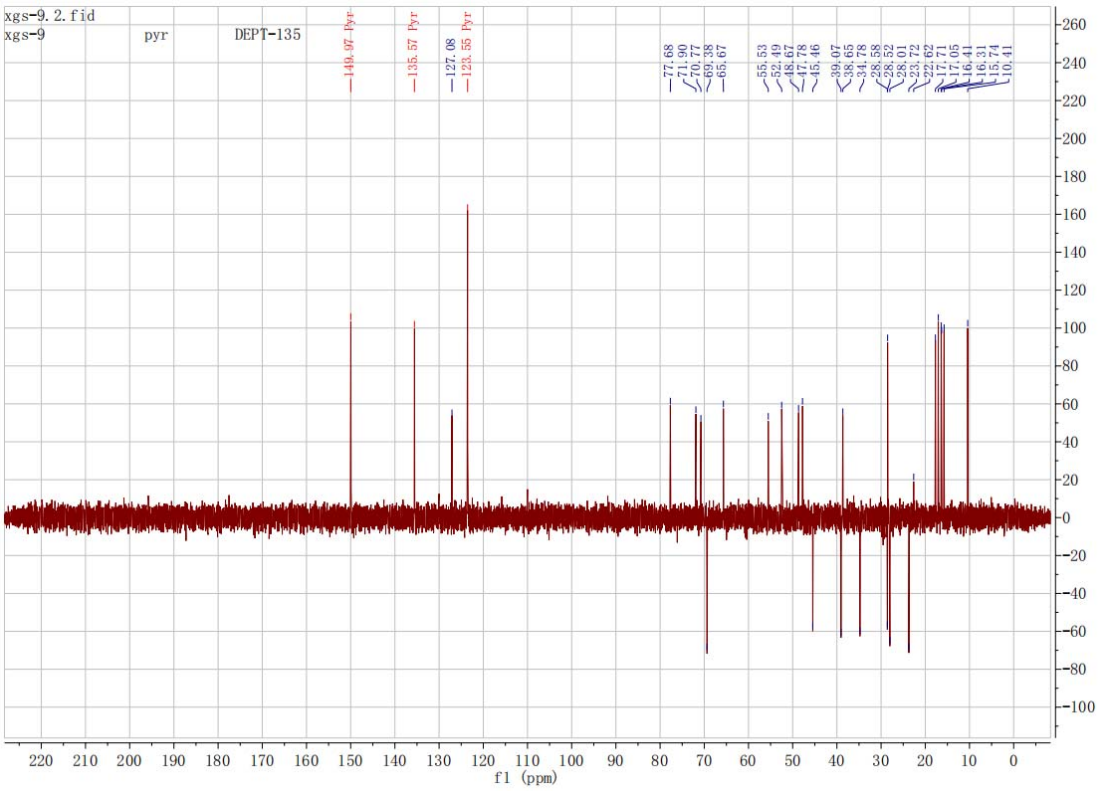

Fig. S5 DEPT-135 spectrum (125 MHz) of compound **3** in Pyridine-d_5

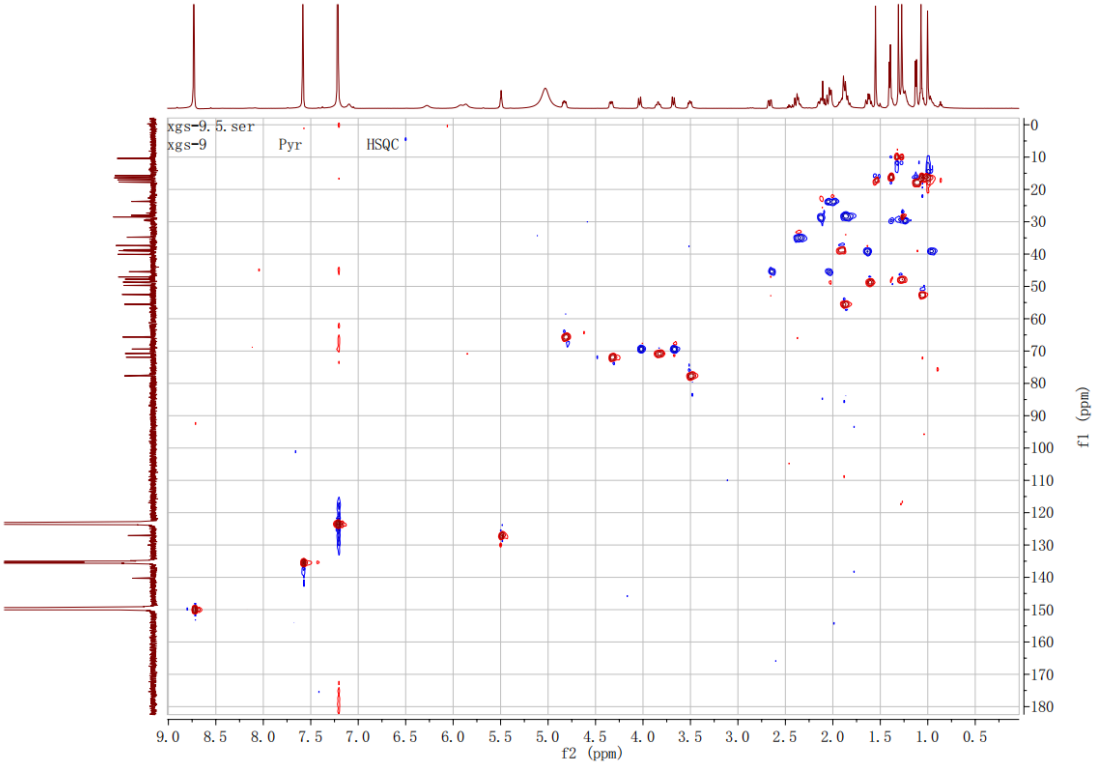

Fig. S6 HSQC spectrum (125 MHz) of compound **3** in Pyridine-d_5

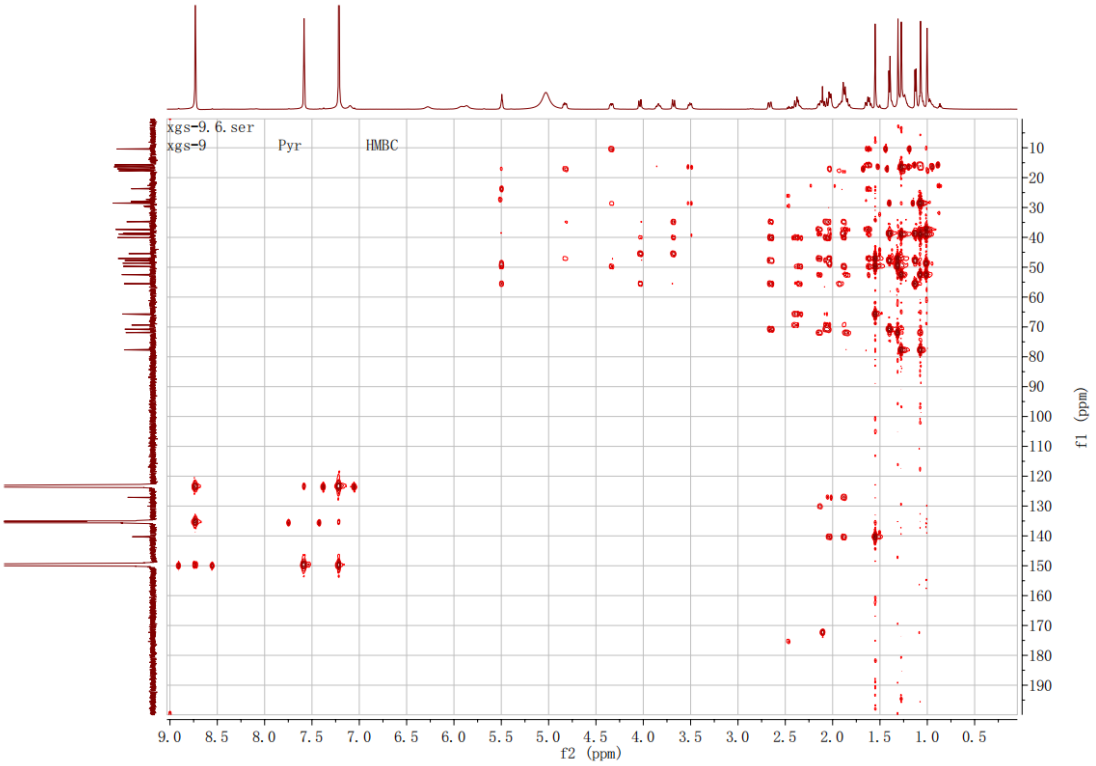

Fig. S7 HMBC spectrum (125 MHz) of compound **3** in Pyridine-d_5

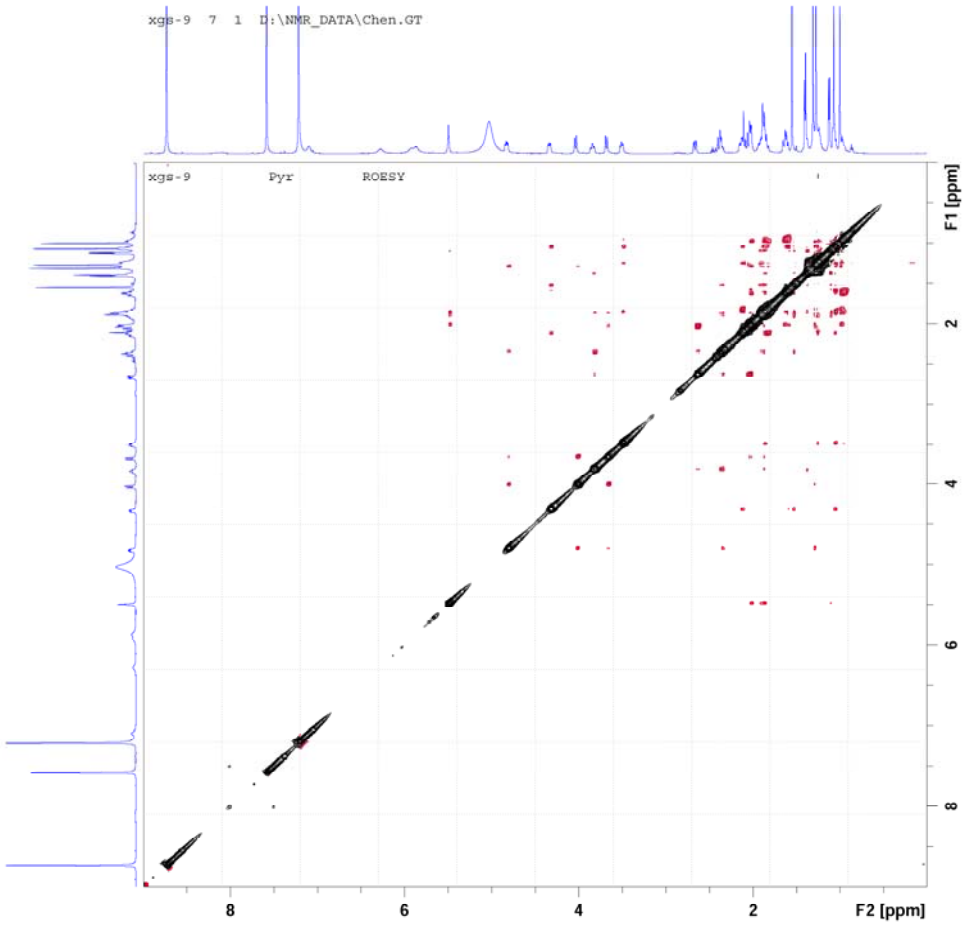

Fig. S8 ROESY spectrum (500 MHz) of compound **3** in Pyridine-d_5

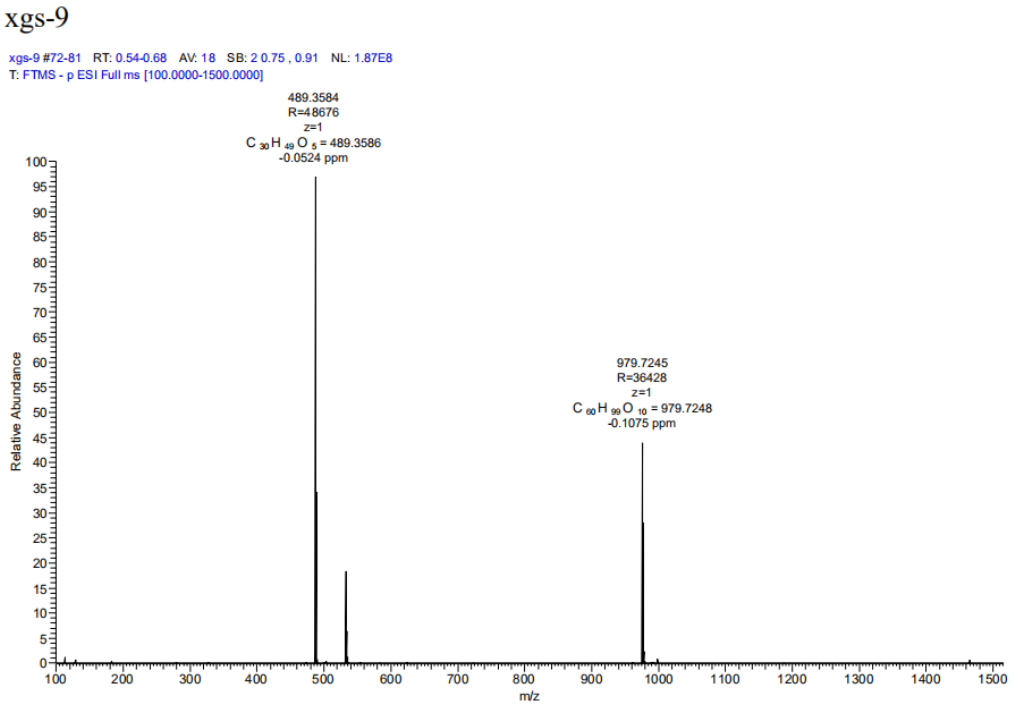

Fig. S9 HR-ESI-MS spectrum of compound **3**

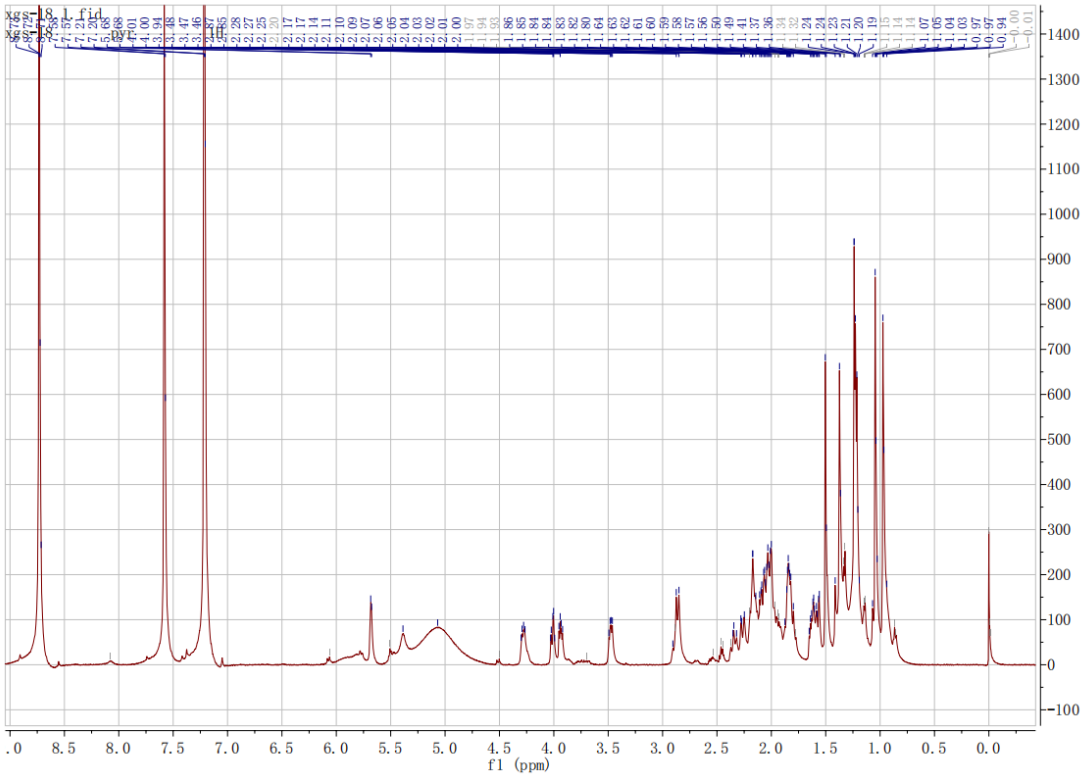

Fig. S10 ^{1}H NMR spectrum (500 MHz) of compound **4** in Pyridine-d_5

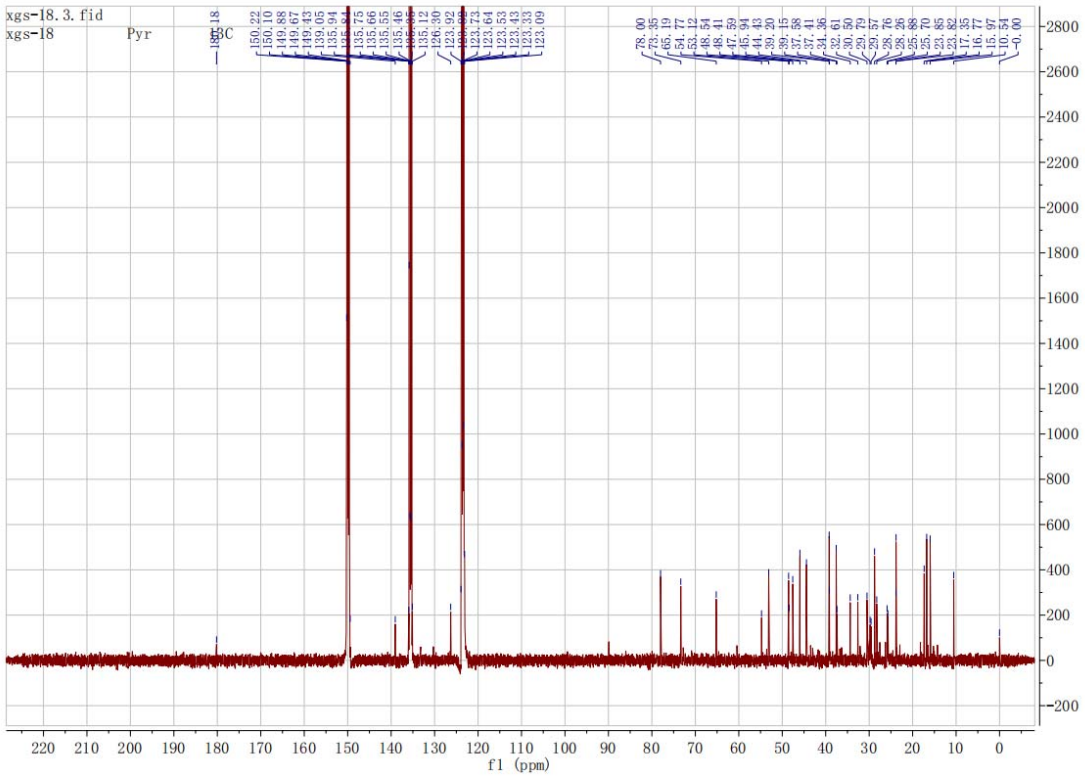

Fig. S11 ^{13}C NMR spectrum (125 MHz) of compound **4** in Pyridine-d_5

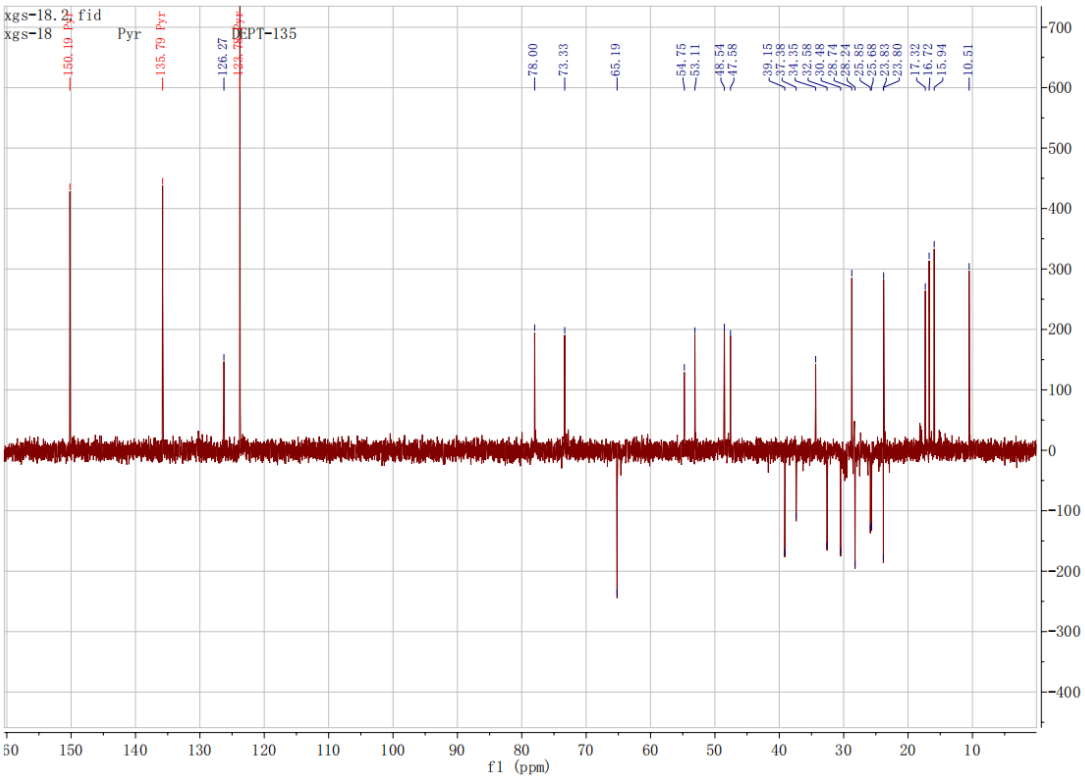

Fig. S12 DEPT-135 spectrum (125 MHz) of compound **4** in Pyridine-d_5

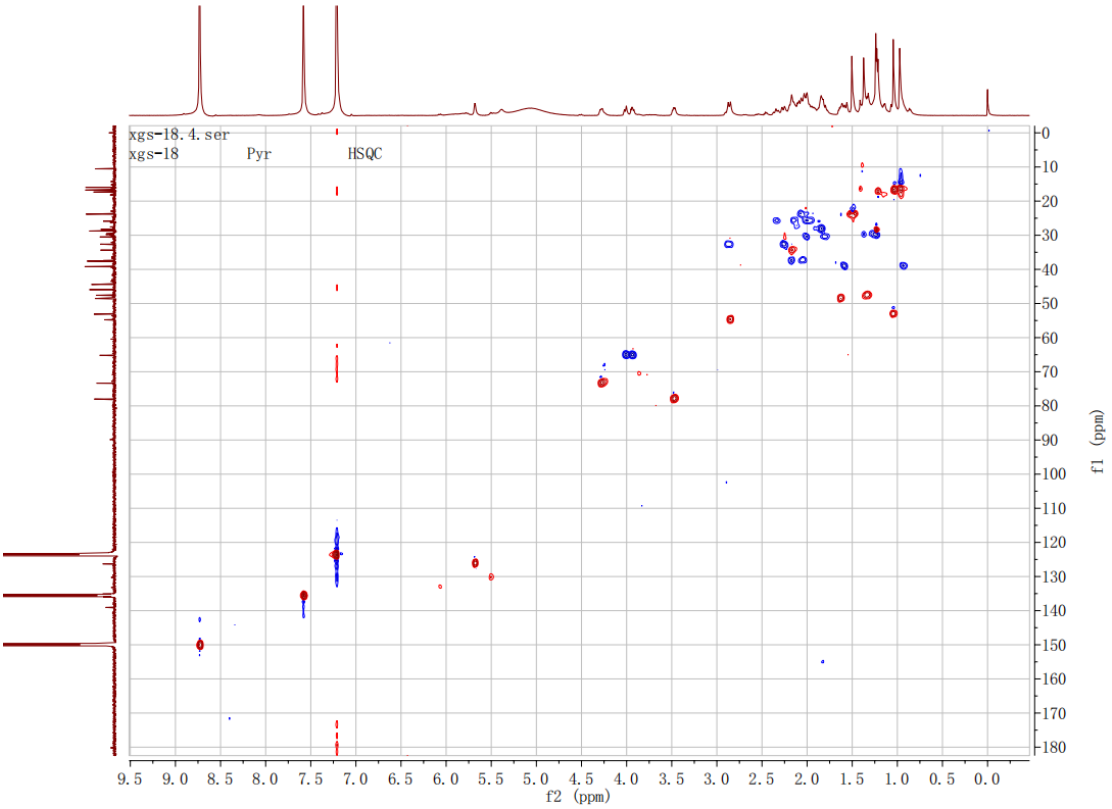

Fig. S13 HSQC spectrum (125 MHz) of compound **4** in Pyridine-d_5

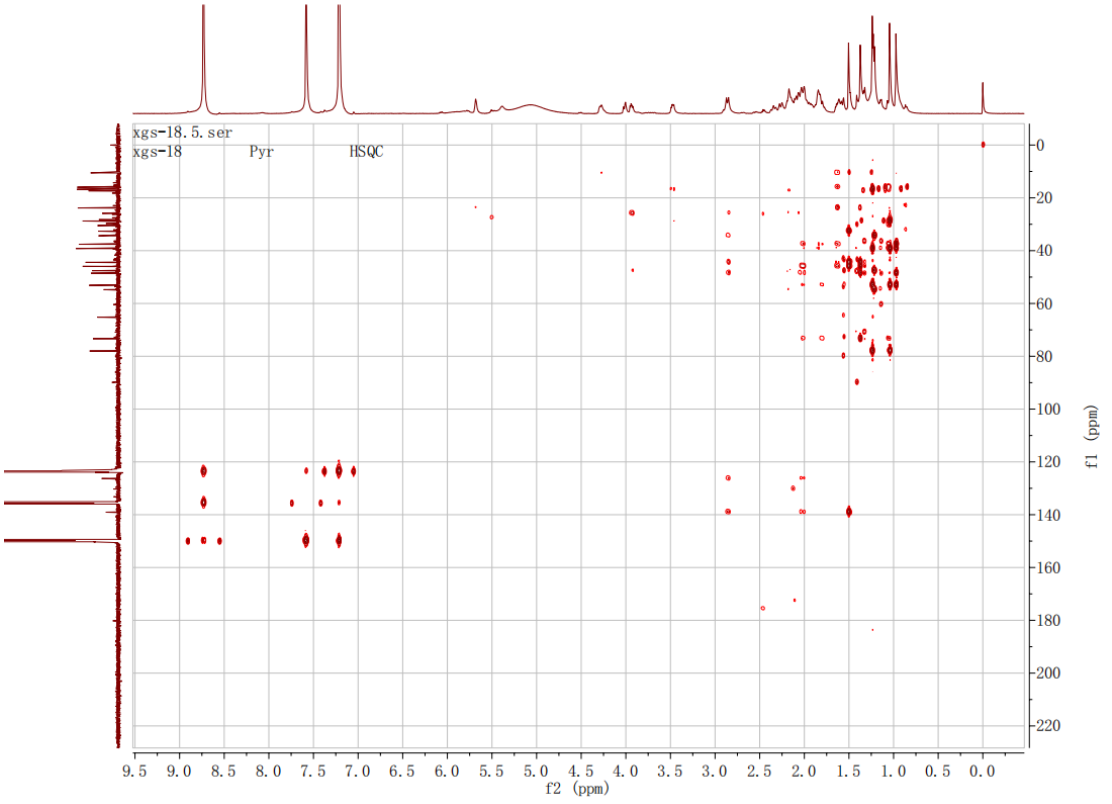

Fig. S14 HMBC spectrum (125 MHz) of compound **4** in Pyridine-d_5

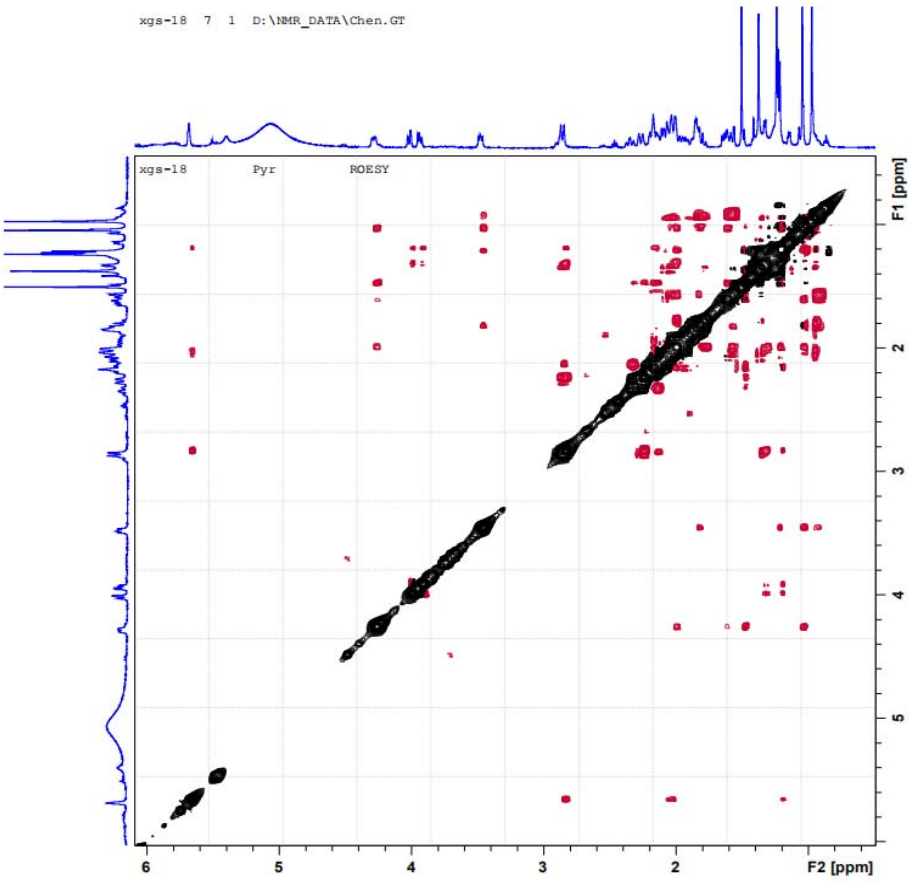

Fig. S15 ROESY spectrum (500 MHz) of compound **4** in Pyridine-d_5

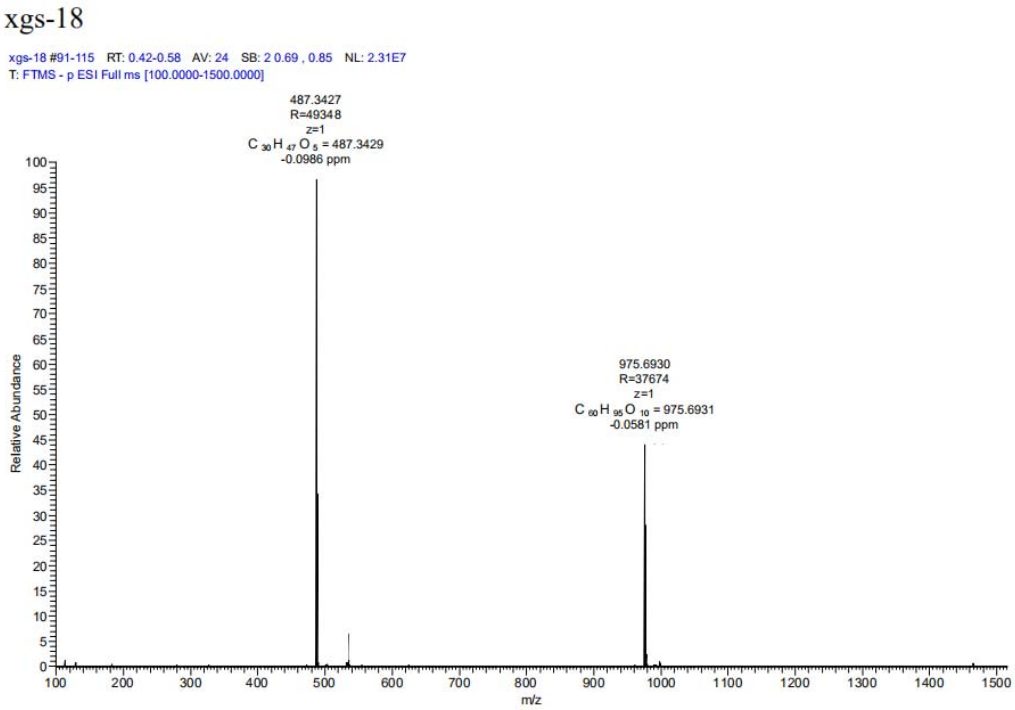

Fig. S16 HR-ESI-MS spectrum of compound **4**

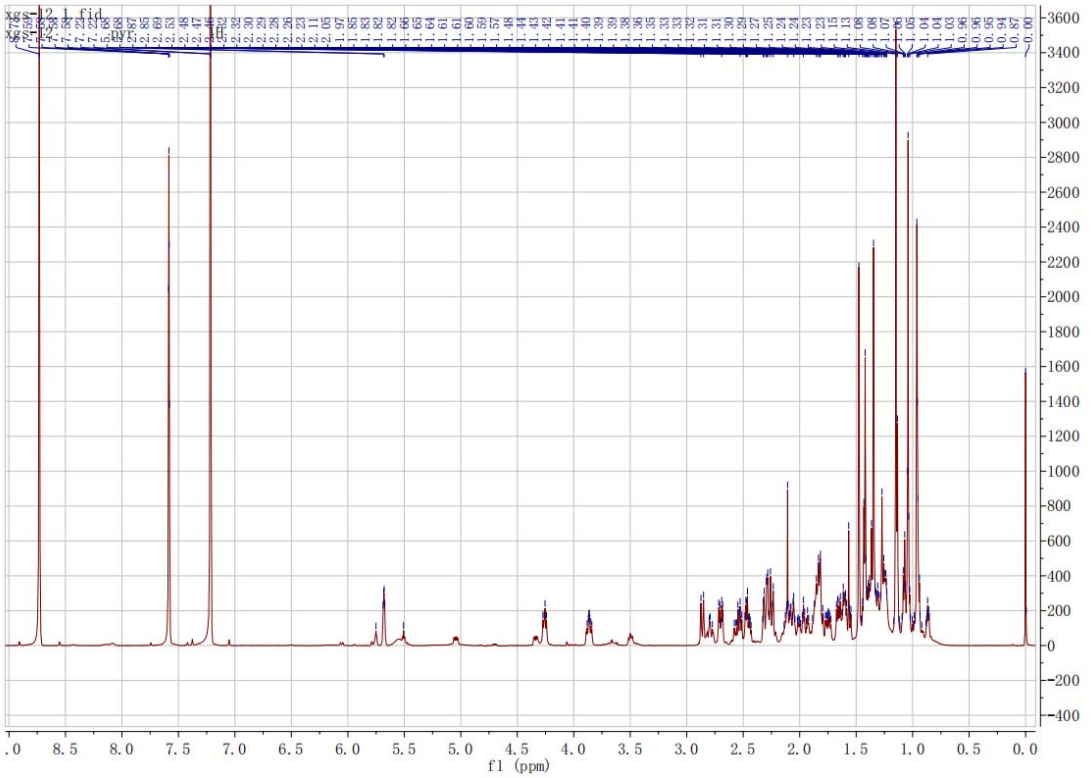

Fig. S17 ^{1}H NMR spectrum (500 MHz) of compound **6** in Pyridine-d_5

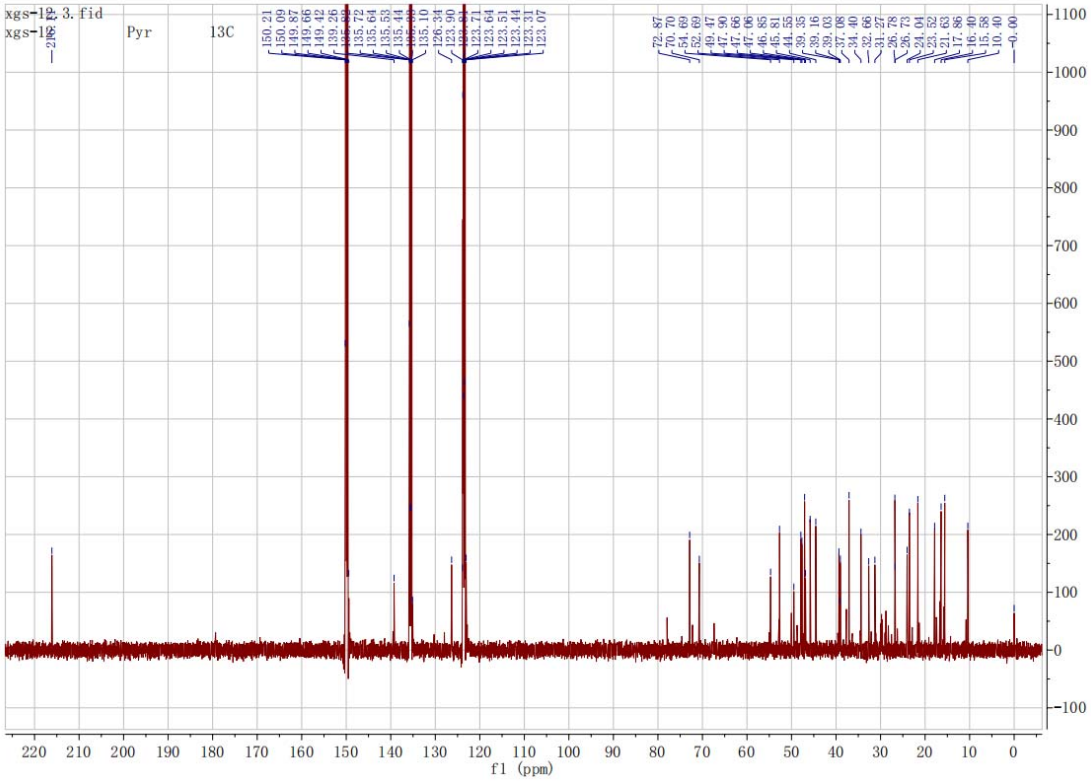

Fig. S18 ^{13}C NMR spectrum (125 MHz) of compound **6** in Pyridine-d_5

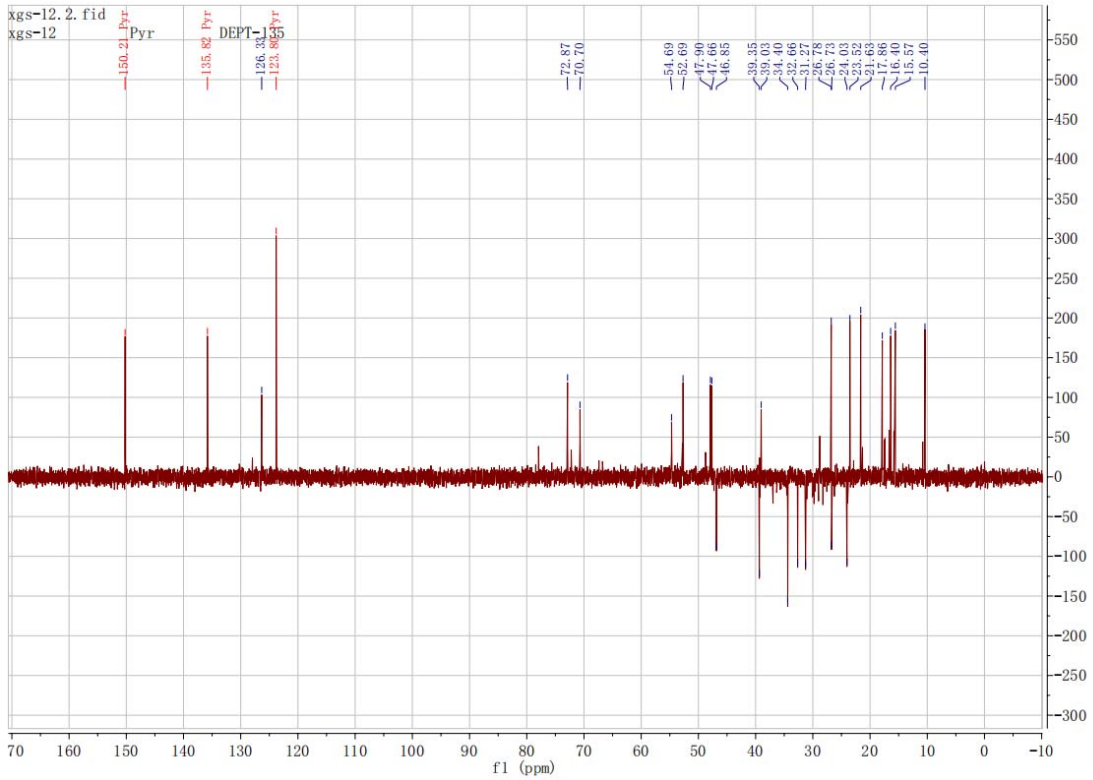

Fig. S19 DEPT-135 spectrum (125 MHz) of compound **6** in Pyridine-d_5

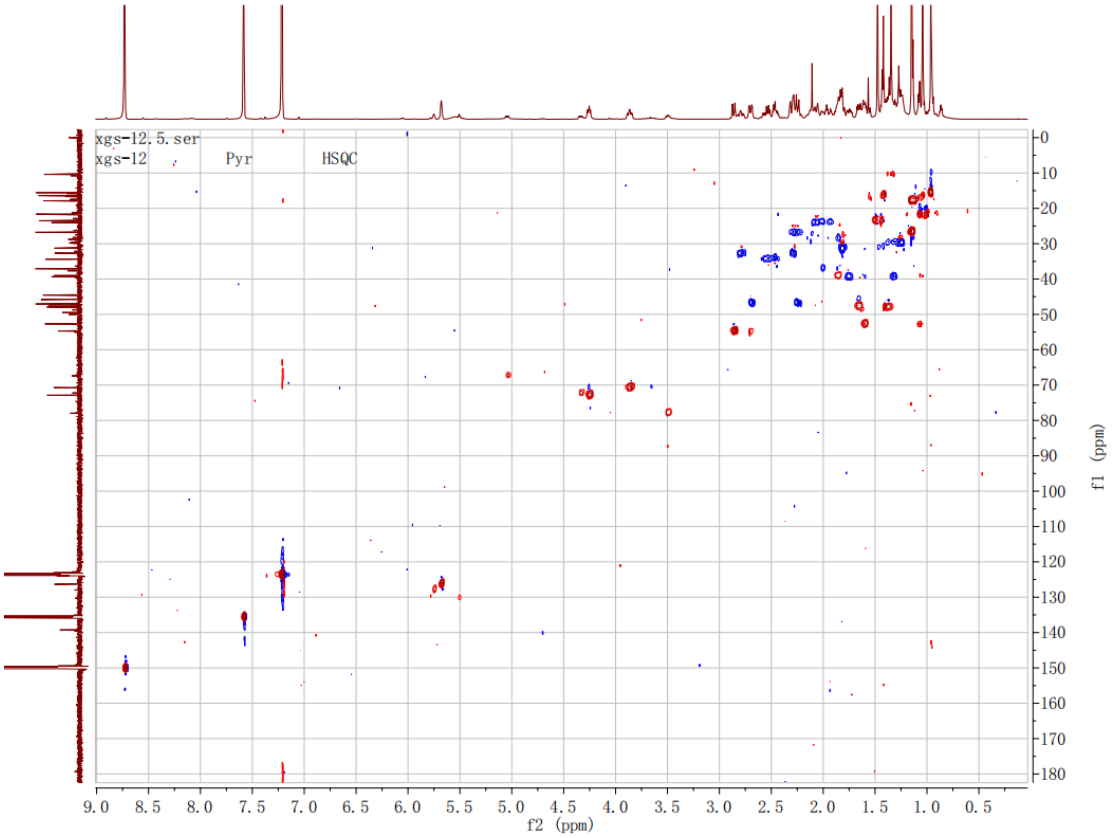

Fig. S20 HSQC spectrum (125 MHz) of compound **6** in Pyridine-d_5

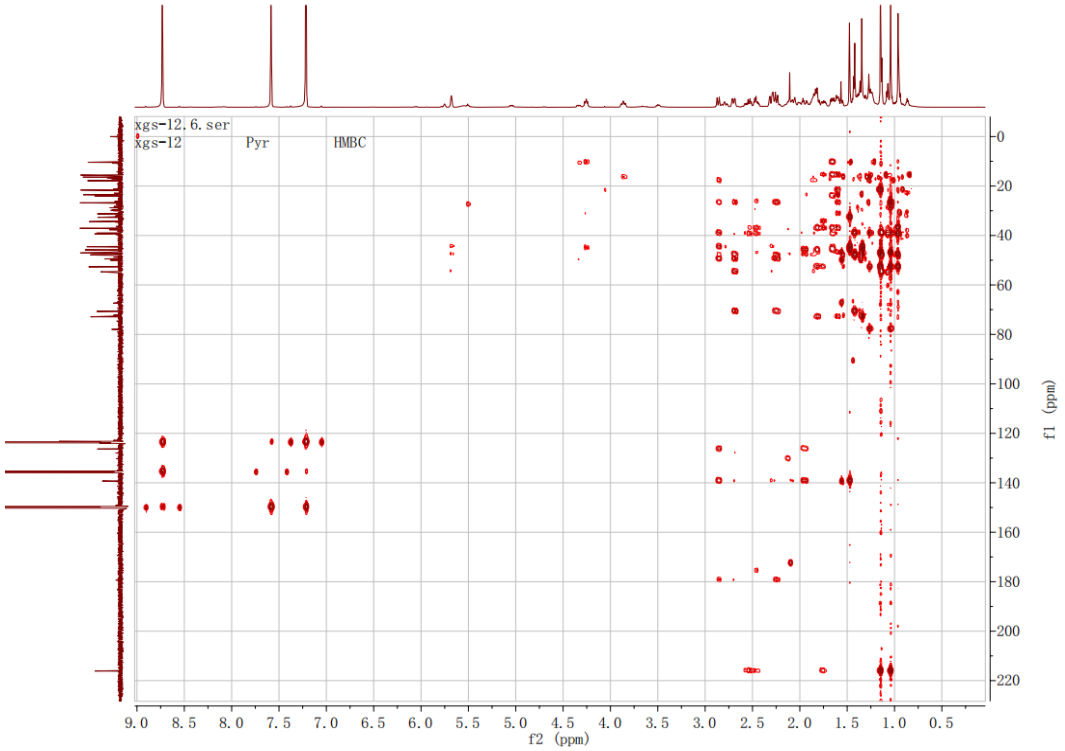

Fig. S21 HMBC spectrum (125 MHz) of compound **6** in Pyridine-d_5

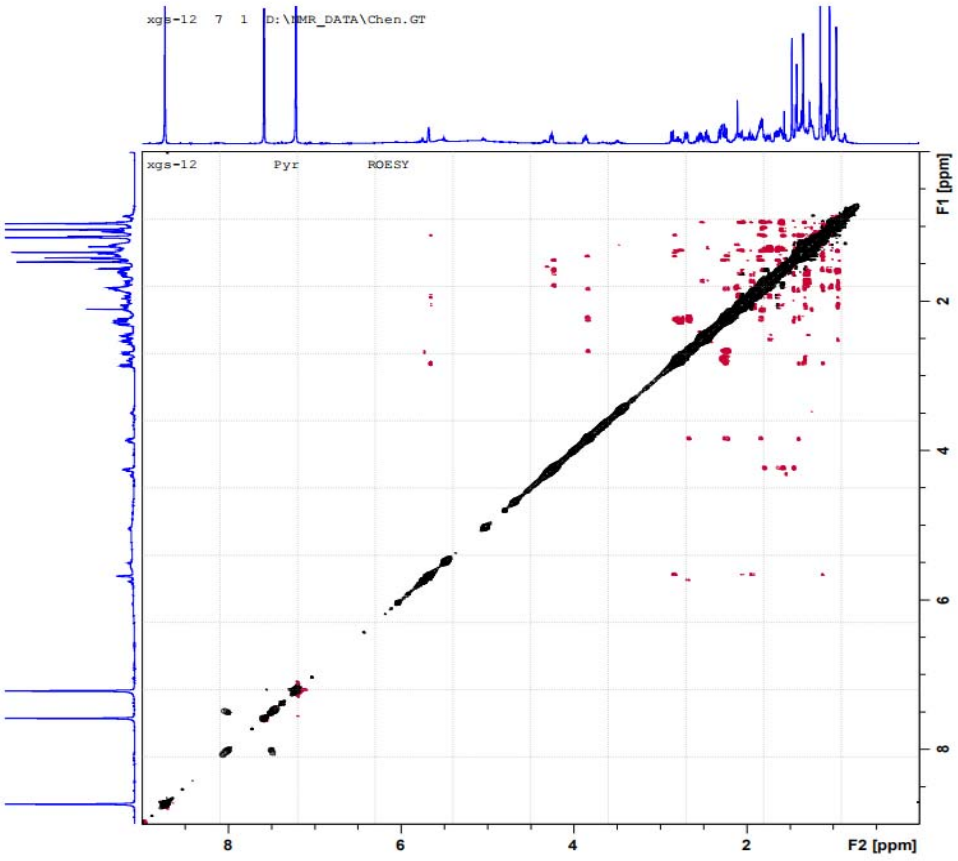

Fig. S22 ROESY spectrum (500 MHz) of compound **6** in Pyridine-d_5

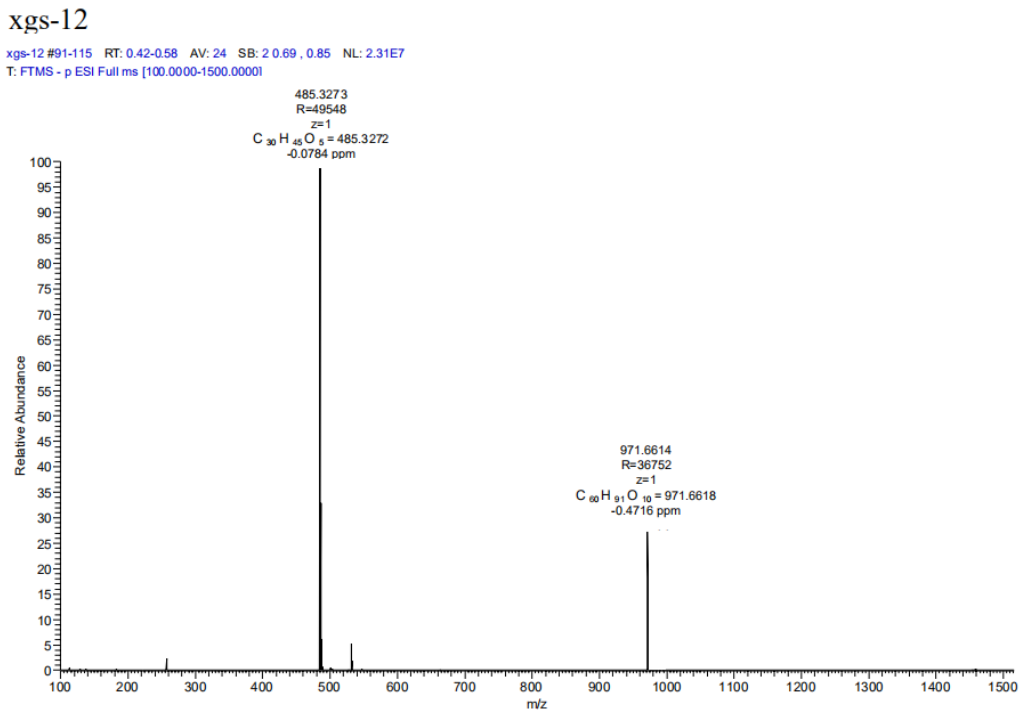

Fig. S23 HR-ESI-MS spectrum of compound **6**

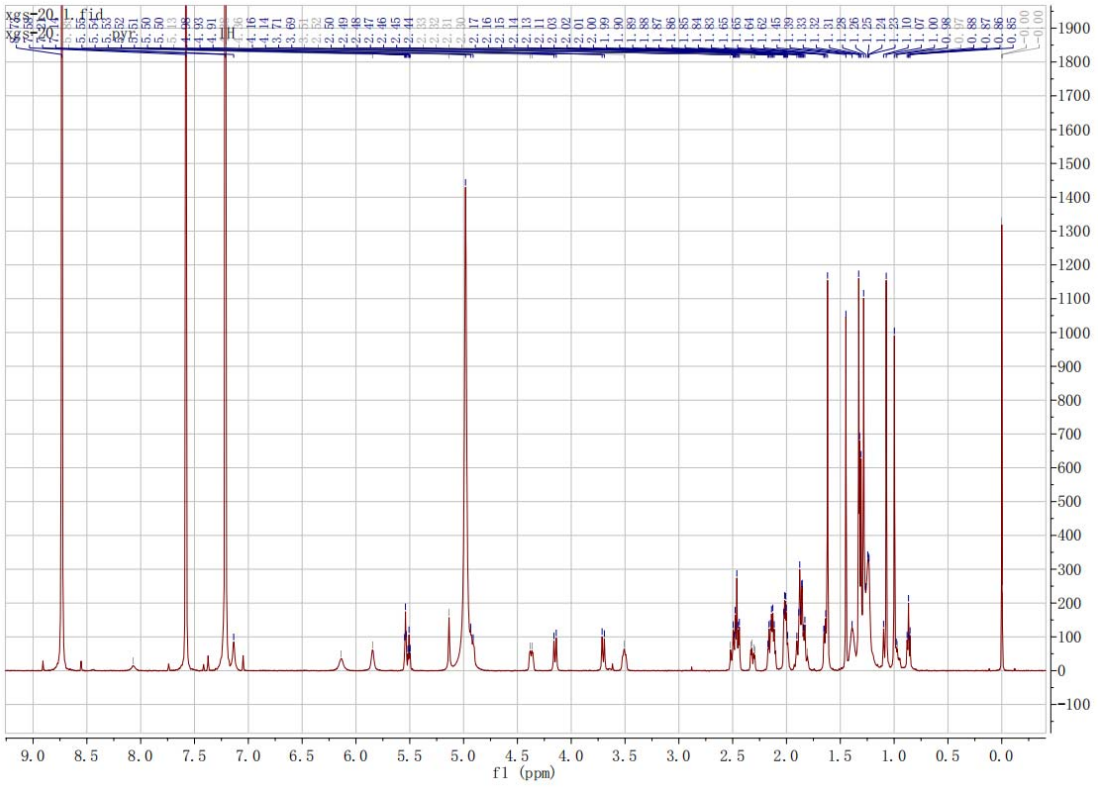

Fig. S24 ^{1}H NMR spectrum (500 MHz) of compound **7** in Pyridine-d_5

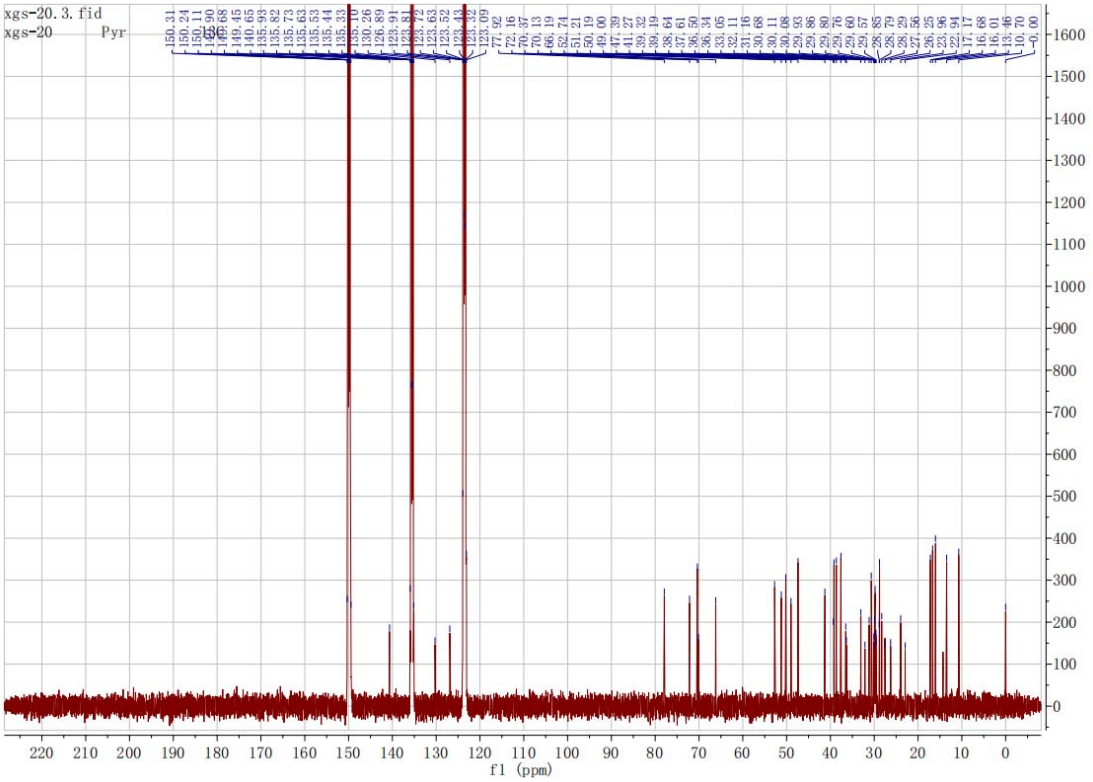

Fig. S25 ^{13}C NMR spectrum (125 MHz) of compound **7** in Pyridine-d_5

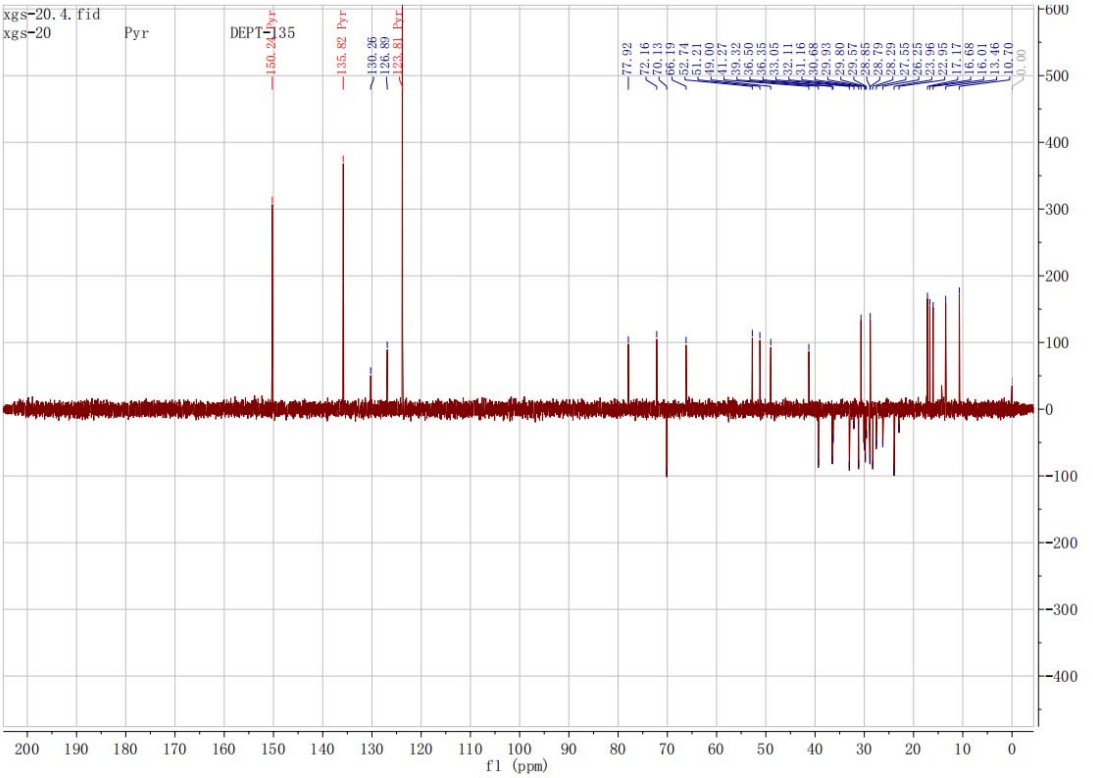

Fig. S26 DEPT-135 spectrum (125 MHz) of compound **7** in Pyridine-d_5

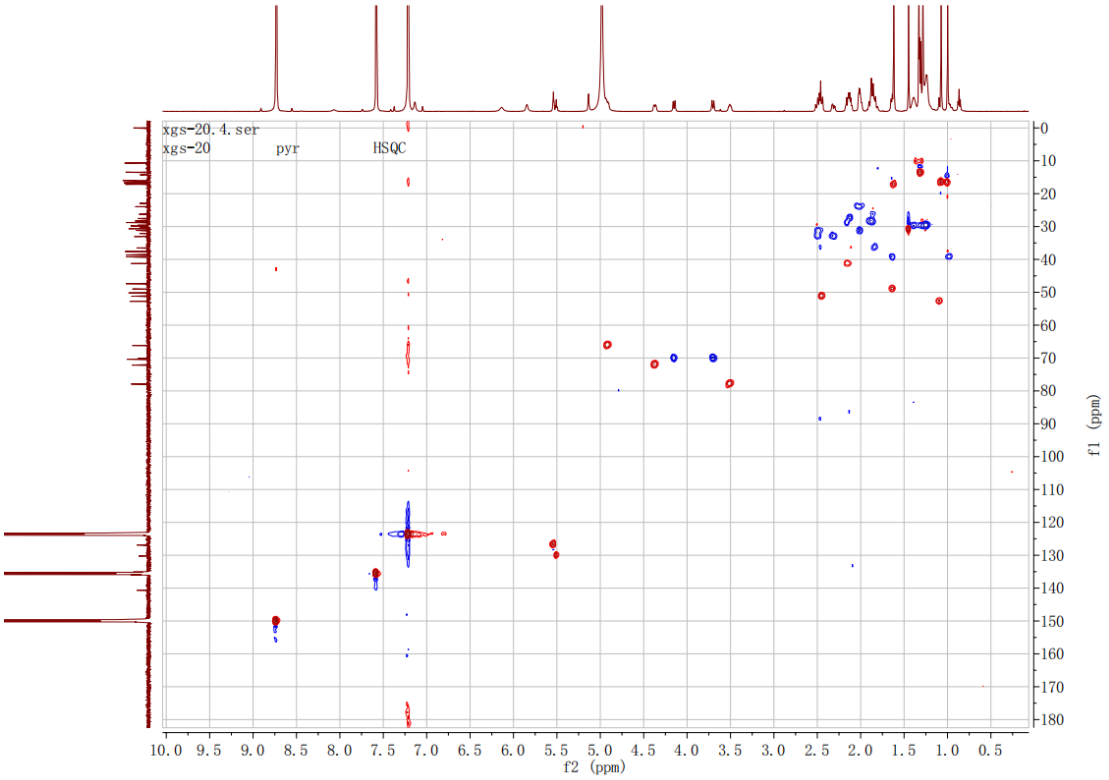

Fig. S27 HSQC spectrum (125 MHz) of compound **7** in Pyridine-d_5

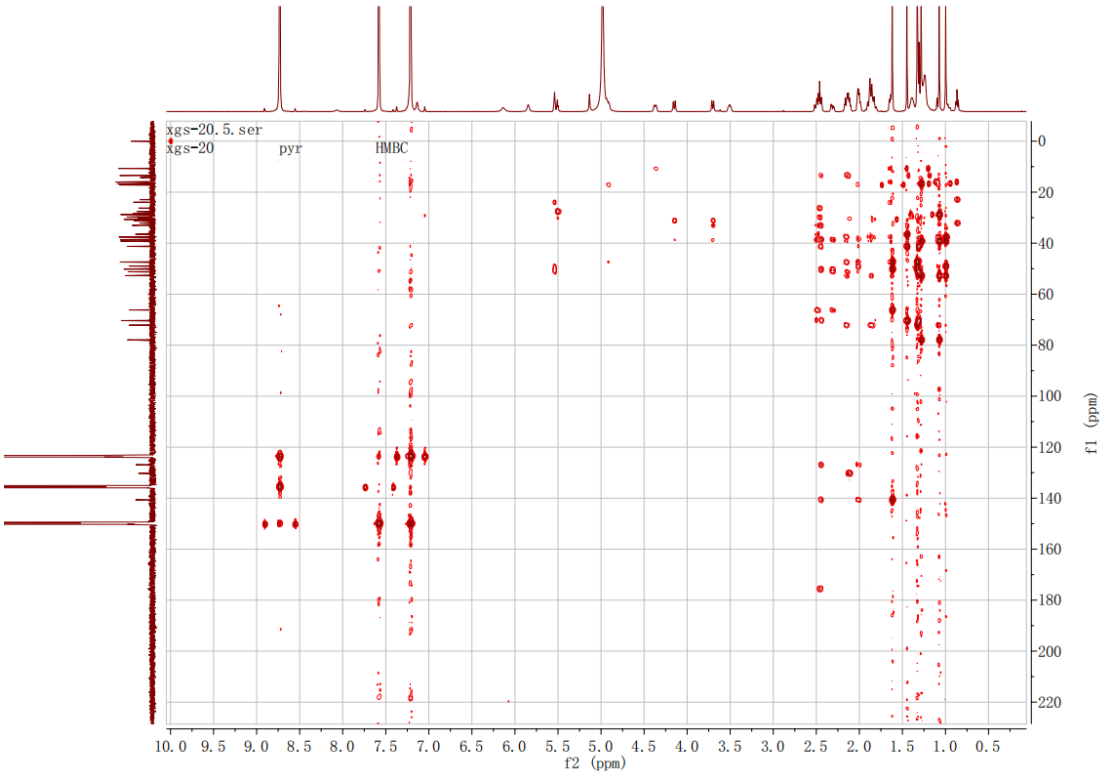

Fig. S28 HMBC spectrum (125 MHz) of compound **7** in Pyridine-d_5

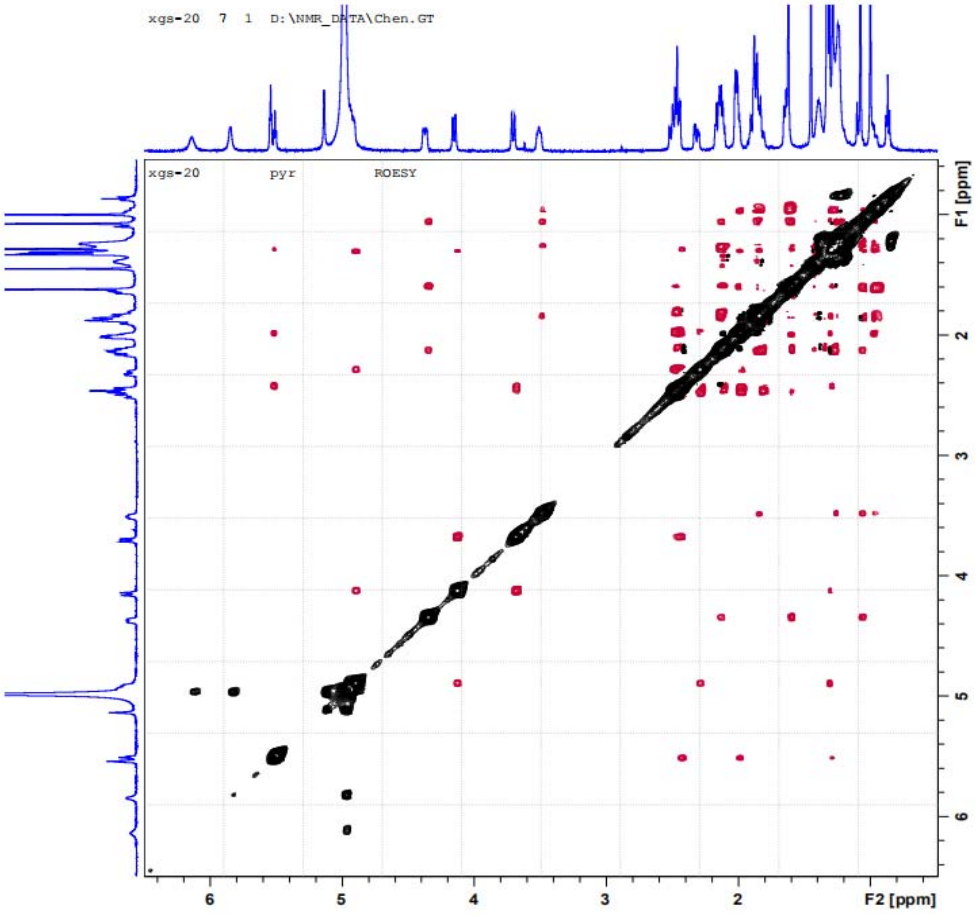

Fig. S29 ROESY spectrum (500 MHz) of compound **7** in Pyridine-d_5

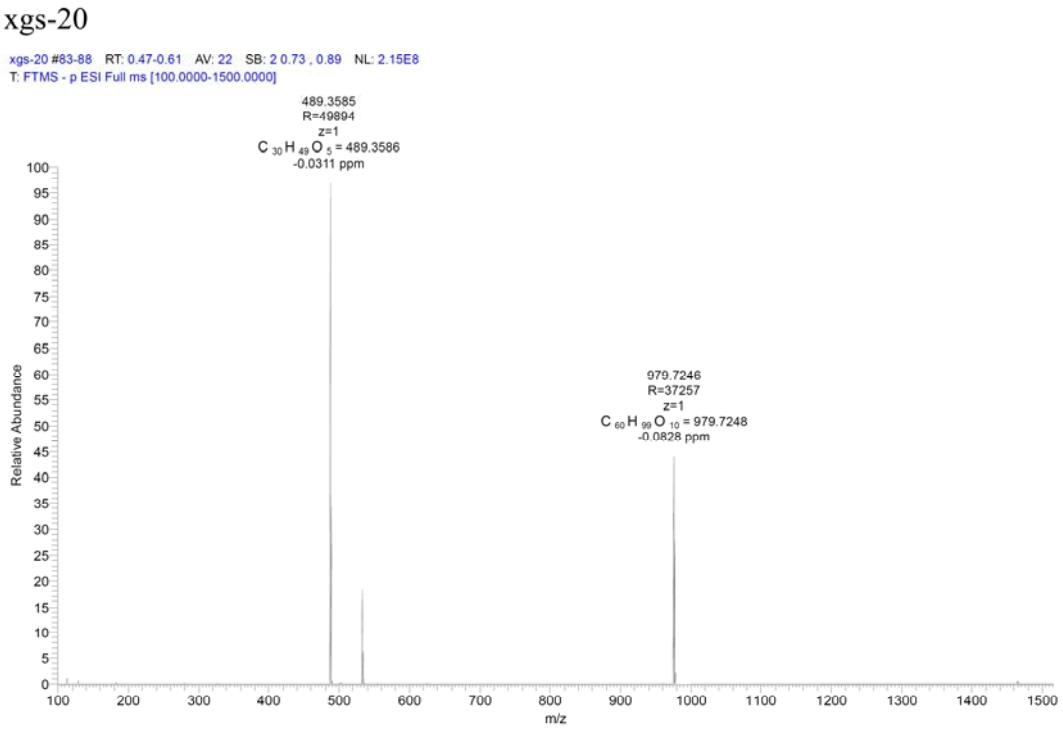

Fig. S30 HR-ESI-MS spectrum of compound **7**