New phenoxazinone-related alkaloids from strain *Streptomyces* sp. KIB-H1318

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Contents

General experimental procedures

The UV data was detected by Shimadzu UV2401PC (Shimadzu Corp., Japan). The IR spectrum was recorded on a Bruker Tensor-27 spectrometer (Bruker Corp., Germany). NMR spectra were observed by Bruker Avance III 600 (Bruker Corp., Switzerland) with ^{1}H NMR at 600 MHz and ^{13}C NMR at 150 MHz in DMSO-d_6. The chemical shifts are represented in ppm and are referenced to solvent residual in both ^{1}H NMR (2.50 ppm) and ^{13}C NMR (39.2 ppm) spectra, coupling constants (*J*) were expressed in Hz. HRESIMS analysis was carried out on an Agilent Auto SpecPremier G6230 mass spectrometer and Shimadzu UPLC-IT-TOF mass spectrometer, general ESIMS on an API QSTAR time-of-flight spectrometer. Column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (25–100 μm, Pharmacia Biotech Ltd., Sweden). Thin-layer chromatography (TLC) was performed using precoated silica gel GF254 plates (0.25 mm in thickness for analysis, Qingdao Marine Chemical Inc., China) with various solvent systems, and spots were visualized by UV light (254 nm) and colorated by iodine, or by spraying heated silica gel plates with 10% H_2SO_4 in MeOH. Preparative HPLC was conducted on a Hitachi Chromaster system (Hitachi Ltd., Japan) equipped with an YMC−Triart C18 column (250 × 10 mm i.d., 5 μm), using a flow rate of 3.0 mL/min at a column temperature of 28 ℃, and detection was performed with a DAD detector.

Biological material

Strain KIB-H1318 was isolated from a soil sample collected in Yuxi, Yunnan province, China, in 2015. Its 16S rRNA gene sequence (GenBank accession No. MG686580) of this strain showed 99% identity with *Streptomyces omiyaensis* strain 13647K (GenBank accession No. EU741141).

Fermentation and isolation

The seed medium was prepared in 250 mL baffle Erlenmeyer flasks. Each flask was filled with 50 mL of Tryptone Soy Broth (30 g/L, pH was not adjusted) and cultivated for 48 hours at 28 ℃ on a rotary shaker (200 rpm). Every fermentation flask was inoculated with 10 mL seed medium and cultivated for 7 days at 28 ℃ on a rotary shaker (200 rpm). Each flask was filled with 250 mL of medium consisted of D-glucose 30.0 g, beef extract 5.0 g, peptone 1.0 g, NaCl 5.0 g, $CaCO_3$ 2.5 g, trace elements 0.1% (v/v) in 1.0 liter deionized water (pH was adjusted to 7.0). Trace elements contained the following supplements: $FeSO_4 \cdot 7H_2O$ 1.0 g, $CuSO_4 \cdot 5H_2O$ 0.45 g, $ZnSO_4 \cdot 7H_2O$ 1.0 g, $MnSO_4 \cdot 4H_2O$ 0.1 g, K_2MnO_4 0.1 g in 1.0 liter deionized water. The culture broth (20 L) of strain was centrifuged at 4000 rpm for 20 min. Then the supernatant was extracted with ethyl acetate (6 × 5 L) and concentrated in vacuo. The mycelium was extracted by acetone (3 × 0.5 L) and concentrated in vacuo, and the extract was partitioned with ethyl acetate twice and concentrated. Both organic parts were combined and evaporated to remove the solvent. The residue (5.0 g) was subjected on silica gel chromatography. Eluting with petroleum ether-ethyl acetate gradient solvent (100:0, 80:20, 50:50, 0:100 v/v) and MeOH, four fractions A to D were yielded. The elute fraction of B (300 mg) was further separated by Sephadex LH-20 chromatography eluting with MeOH into fractions B-1~5, which were combined based on TLC analyses. Fraction B-2 (100 mg) was subjected to semi-preparative HPLC (Hitachi HPLC system, YMC−Triart C18 column, 250 × 10 mm, DAD detector) using a flow rate of 3 mL/min and isocratic elution with 60% MeOH in H_2O, and obtained compounds **4** (3.0 mg) and **5** (4.0 mg). Similarly the elute fraction of D (400 mg) was further separated by Sephadex LH-20 chromatography into fractions D-1~7. Fraction D-3 (80 mg) was subjected to semi-preparative HPLC using a flow rate of 3 mL/min and isocratic elution with 55% MeOH in H_2O, and obtained compounds **1** (7.0 mg), **2** (4.0 mg) and **3** (5.0 mg).

Antimicrobial assays

For plate diffusion assays,[1] 30 μg of the compounds was dissolved in DMSO and dropped on paper disks (Ø 6 mm, thickness 0.5 mm). These were dried under sterile conditions and put on agar plates inoculated with the testing organism (four bacteria, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8099 and *Xanthomonas oryzae* pv. *oryzae* RS105, and eight fungi, *Saccharomyces cerevisiae*, *Penicillium decumbens* ATCC 10436, *Candida albicans*, *Gibberella zeae*, *Fusarium oxysporum* Schl.f.sp. *vasinfectum*, *Fusarium oxysporum*, *Curvularia lunata* (Walk) Boed and *Rhizoctonia solani* Kühn.). The plates were cultivated at 37 ℃ (bacteria) for 24 h or 28 ℃ (fungi) for 48 h, and the zones of inhibition were measured. Kanamycin (10 μg/disc) and nystatin (10 μg/disc) were used as positive control for bacteria and fungi respectively. DMSO was used as blank control.

Cytotoxicity

The cytotoxicity of compounds **1**–**3** against cervical cancer Hela, hepatocellular carcinoma SMMC-7721, lung cancer A-549, breast adenocarcinoma MCF-7 and colon carcinoma SW480 was assessed using the MTS method.[2] DDP and taxol (Sigma, USA) were used as positive controls. Briefly, all cells were cultured in RPMI-1640 or DMEM medium (Hyclone, Logan, UT, USA), which were supplemented with 10% fetal bovine serum (Hyclone, USA) at 37 ℃ in a humidified atmosphere containing 5% CO_2. Cells were seeded at 1 $\times 10^4$ cells per well into 96-well cell culture plates and then incubated at 37 ℃ for 12~24 h. The tested compounds of different concentrations (0.064, 0.32, 1.6, 8.0, 40 μM) were added into the 96-well plates and cultivated for 48 h. Then 20 μL of MTS was added to each well and the incubation continued for 4 h at 37 ℃. Finally, the optical density was measured at 492 nm using a Multiskan FC plate reader (Thermo Scientific, USA). The IC_{50} value of each compound was calculated by the Reed and Muench method.[3]

Table S1. Antimicrobial activity of **4** and **5**. [a, b]

Strains	exfoliazone (**4**) (30 μg/disc)	viridobrunnine A (**5**) (30 μg/disc)	Kanamycin (10 μg/disc)	Nystatin (10 μg/disc)
Escherichia coli ATCC 8099	–	**7**	15	–
Staphylococcus aureus ATCC 6538	12	10	15	–
Bacillus subtilis ATCC 6633	8	8	22	–
Xanthomonas oryzae pv. *oryzae* RS105	–	–	31	–
Saccharomyces cerevisiae	–	–	–	14
Penicillium decumbens ATCC 10436	–	–	–	10
Candida albicans	–	–	–	–
Gibberella zeae	–	–	–	8
Fusarium oxysporum Schl.f.sp. *vasinfectum*	–	–	–	10
Fusarium oxysporum	–	–	–	10
Curvularia lunata (Walk) Boed	–	–	–	28
Rhizoctonia solani Kühn	–	–	–	10

[a] Diameter of inhibition zones in the plate diffusion assay in mm. "–" No activity

[b] Data given were from three duplicates.

Table S2. Cytotoxicity of **2** against two human cancer cell lines (IC_{50} μM)

Compound	Hela	SW480
2	36.8	37.8
DDP[a]	6.65	13.1
Taxol[a]	<0.008	<0.008

[a] Positive control.

Table S3. NMR spectroscopic data for compound **1** (DMSO-d_6, δ in ppm)

Position	δ_C, type	δ_H, (J in Hz)	COSY	HMBC
1	97.4, CH	6.54, s		143.6, 148.2, 149.3, 179.9
2	143.6, C			
3	179.9, C			
4	103.5, CH	6.46, s		143.6, 148.2, 149.3, 179.9,
4a	149.3, C			
5a	140.9, C			
6	115.7, CH	7.49, overlapped	7.43	133.6, 140.1, 140.9
7	127.6, CH	7.43, overlapped	7.49, 7.62, 4.56	62.1, 125.5, 133.6, 140.9
8	140.1, C			
9	125.5, CH	7.62, s	7.43, 4.56	62.1, 127.6, 133.6, 140.9
9a	133.6, C			
10a	148.2, C			
11	62.1, CH_2	4.56, s	5.38, 7.43, 7.62	125.5, 127.6, 140.1
11-OH		5.38, br s	4.56	
12-NH		8.67, s		97.4, 115.7,119.6,129.6,148.2,179.9
1'	129.6, C			
2'	115.7, CH	8.20, s	6.95	119.6, 126.5, 129.6, 144.5
3'	126.5, C			
4'	144.5, C			
4'-OH		9.73, br s		
5'	115.3, CH	6.91, d (8.4)	6.95	126.5, 129.6, 144.5
6'	119.6, CH	6.95, d (8.4)	6.91, 8.20	115.7, 144.5
7'-NH				
8'	160.2, CH	8.31, s		126.5

Table S4. NMR spectroscopic data for compound **2** (DMSO-d_6, δ in ppm)

Position	δ_C, type	δ_H, (J in Hz)	COSY	HMBC	ROESY
1	97.5, CH	6.57, s		143.4, 148.2, 149.4, 179.9	
2	143.4, C				
3	179.9, C				
4	103.5, CH	6.46, s		143.4, 148.2, 149.4, 179.9	
4a	149.4, C				
5a	140.9, C				
6	115.7, CH	7.49, d (8.4)	7.43	133.6, 140.1, 140.9	
7	127.7, CH	7.43, d (8.4)	7.49, 7.62, 4.57	62.2, 125.5, 140.9, 133.6	
8	140.1, C				
9	125.5, CH	7.62, s	7.43, 4.57	62.2, 127.7, 133.6, 140.9	
9a	133.6, C				
10a	148.2, C				
11	62.2, CH_2	4.57, s	7.43, 7.62, 5.39	125.5, 127.7, 133.6, 140.1,	
11-OH		5.39, br s	4.57		
12-NH		8.67, s		97.5, 116.8, 119.8, 129.9, 148.2, 179.9	
1'	129.9, C				
2'	116.8, CH	7.90, d (2.3)	6.97	119.8, 126.9, 129.9, 145.2	
3'	126.9, C				
4'	145.2, C				
4'-OH					
5'	116.0, CH	6.90, d (8.5)	6.97	126.9, 129.9, 145.2	
6'	119.8, CH	6.97, dd (8.5, 2.3)	6.90, 7.90	116.8, 129.9, 145.2	
7'-NH		9.40, s			7.90, 2.12
8'	169.1, C				
9'	23.8, CH_3	2.12, s		169.1	

Table S5. NMR spectroscopic data for compound **3** (DMSO-d_6, δ in ppm)

Position	δ_C, type	δ_H, (J in Hz)	COSY	HMBC
1	94.1, CH	5.66, s		146.0, 148.3, 179.5
2	148.3, C			
3	179.5, C			
4	95.6, CH	5.76, s		146.0, 148.3, 178.9
4a	146.0, C			
5a	148.9, C			
6	115.8, CH	6.89, d (8.0)	6.99	124.9, 133.7, 146.0 (weak), 148.9
7	124.7, CH	6.99, d (8.0)	6.89, 7.26	62.6, 120.7, 148.9
8	133.7, C			
9	120.7, CH	7.26, s	6.99	62.6, 115.8, 124.7, 133.7, 148.9
9a	124.9, C			
10-NH_2		8.81, s		
10a	178.9, C			
11	62.6, CH_2	4.41, s	5.16	120.7, 124.7, 133.7
11-OH		5.16, br s	4.41	
12-NH		9.29, s		94.1, 117.6, 120.5, 179.5,
1'	128.5, C			
2'	117.6, CH	7.84, d (1.8)	6.93	120.5, 126.8, 128.5, 145.9
3'	126.8, C			
4'	145.9, C			
4'-OH		10.16, br s		
5'	115.5, CH	6.89, d (8.0)	6.93	126.8, 128.5
6'	120.5, CH	6.93, dd (8.5, 1.8)	6.89, 7.84	117.6, 145.9
7'-NH		9.36, s		117.6, 126.8, 145.9, 169.1
8'	169.1, C			
9'	23.8, CH_3	2.11(3H, s)		169.1

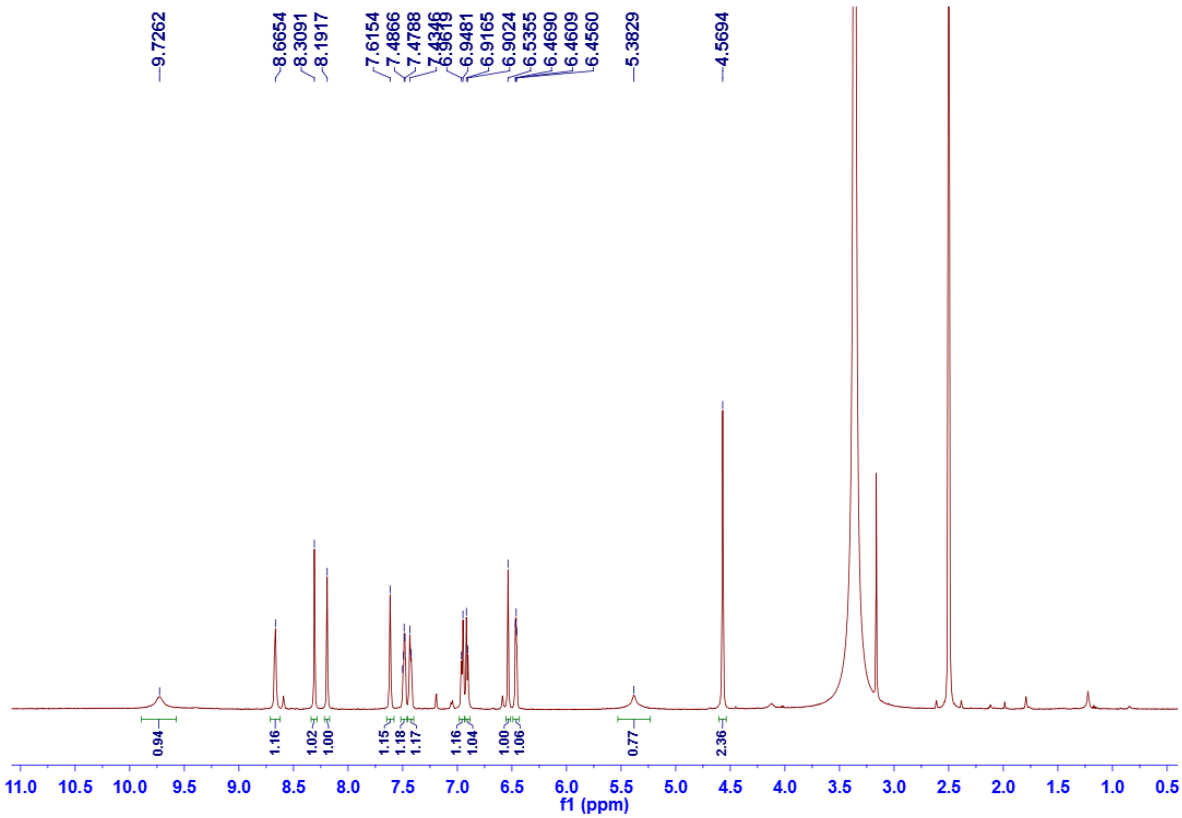

Figure S1. ^{1}H NMR spectrum of **1** (in DMSO-d_6, 600 MHz)

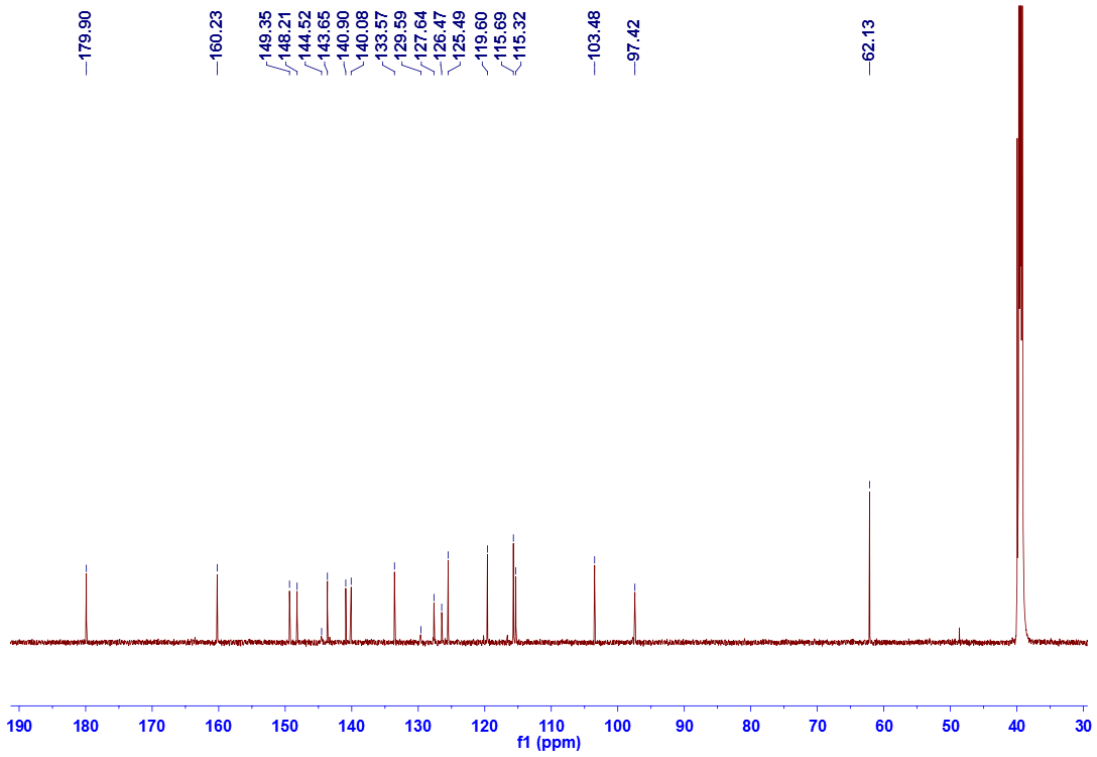

Figure S2. ^{13}C NMR spectrum of **1** (in DMSO-d_6, 150 MHz)

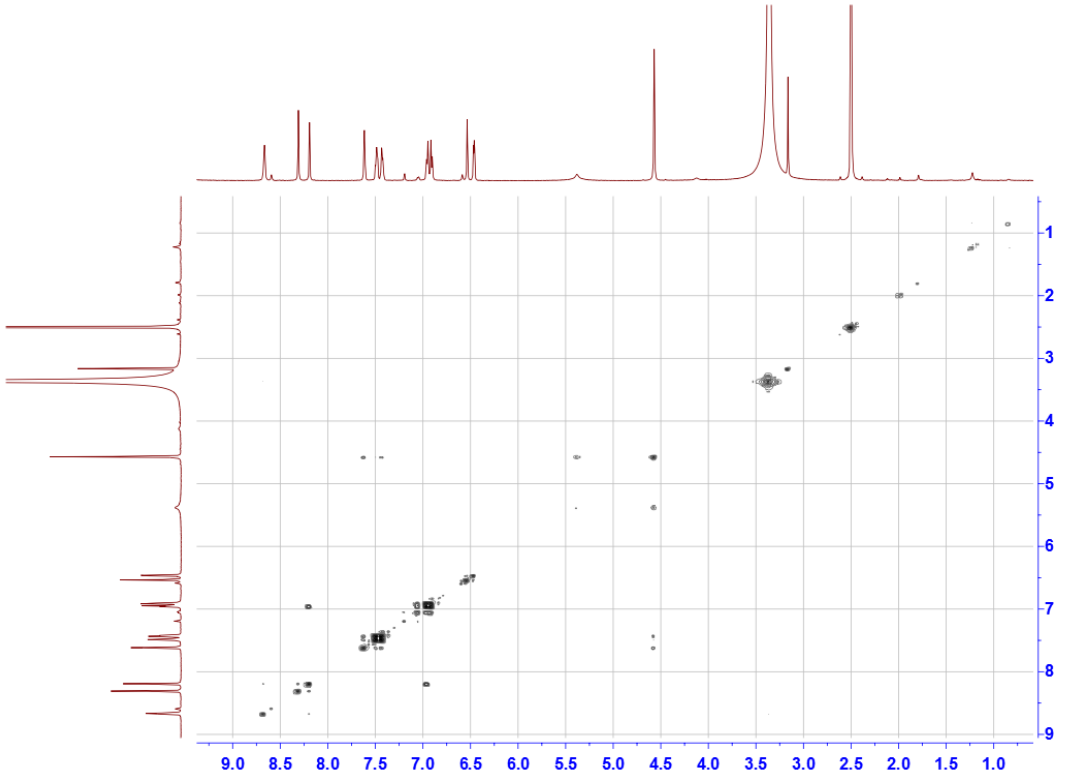

Figure S3. COSY NMR spectrum of **1** (in DMSO-d_6)

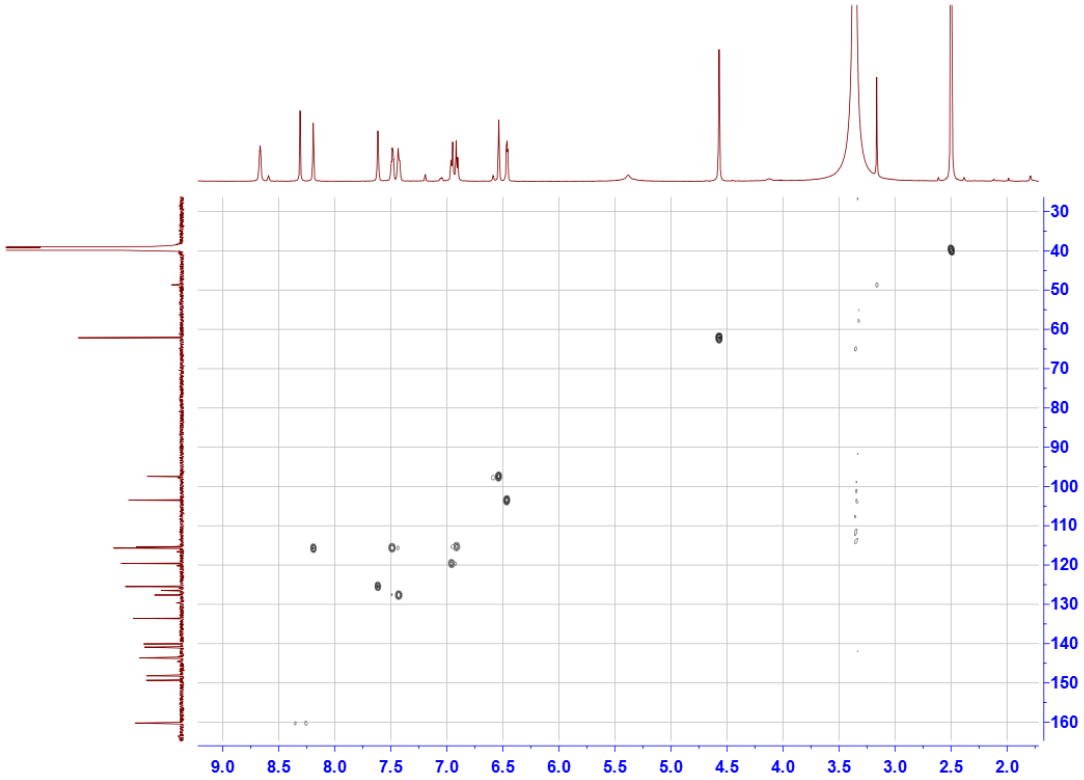

Figure S4. HSQC NMR spectrum of **1** (in DMSO-d_6)

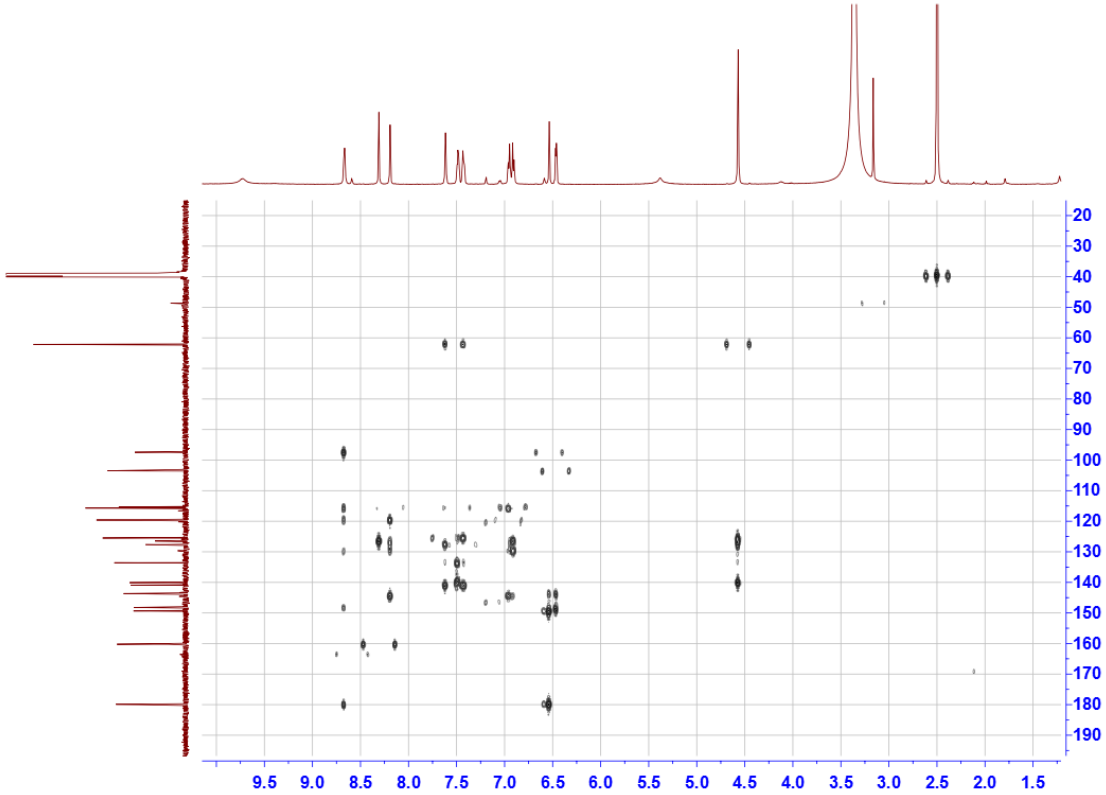

Figure S5. HMBC NMR spectrum of **1** (in DMSO-d_6)

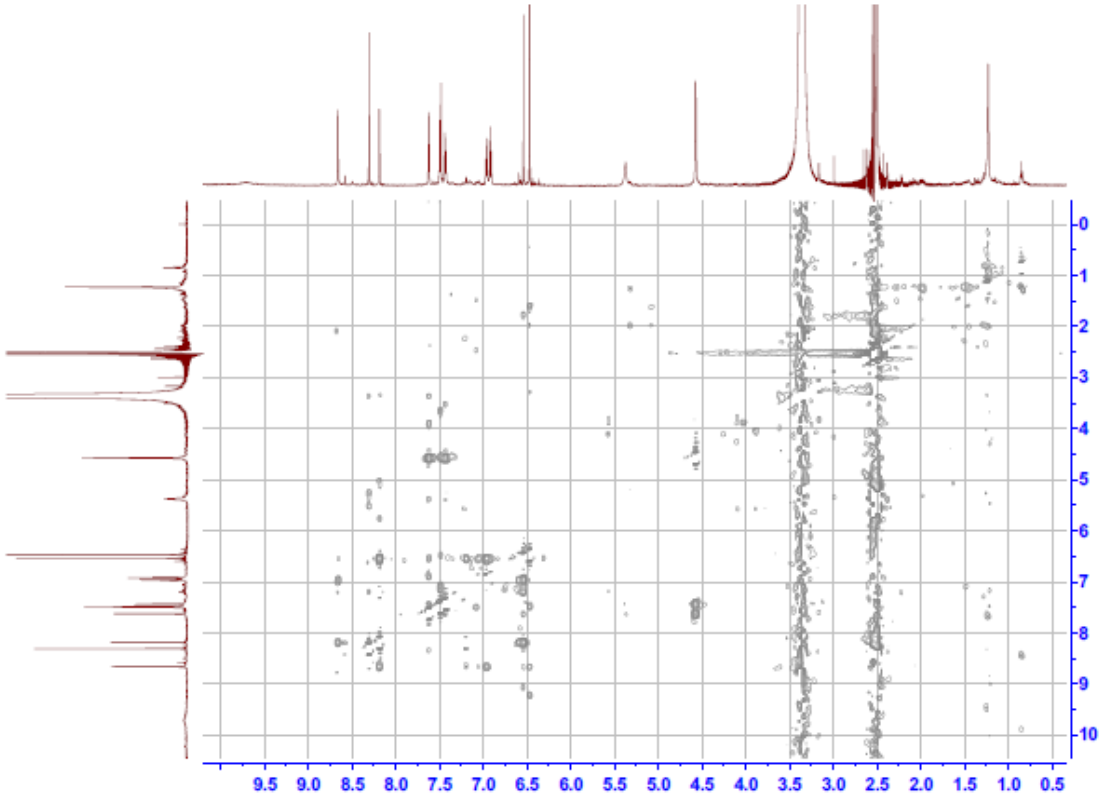

Figure S6. ROESY NMR spectrum of **1** (in DMSO-d_6)

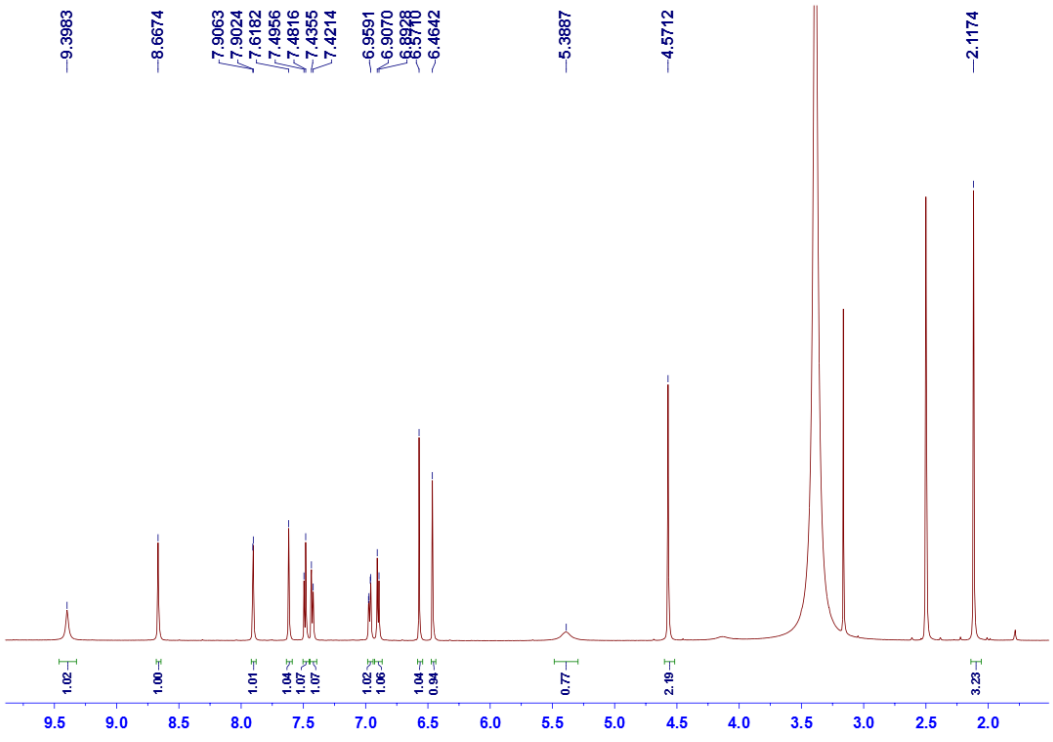

Figure S7. ^{1}H NMR spectrum of **2** (in DMSO-d_6, 600 MHz)

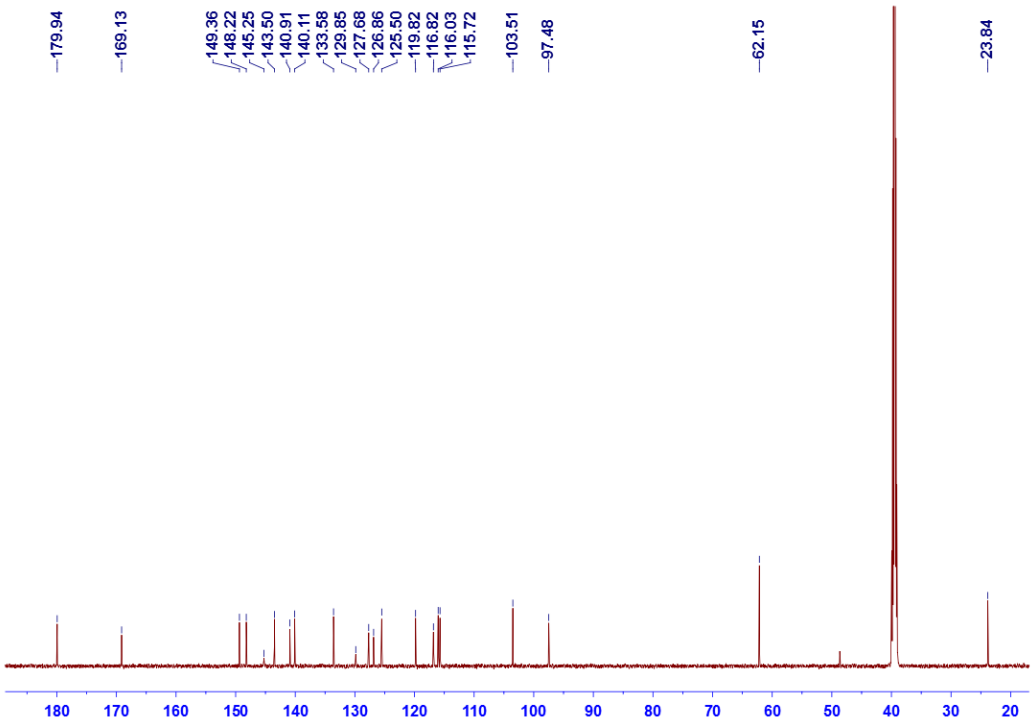

Figure S8. ^{13}C NMR spectrum of **2** (in DMSO-d_6, 150 MHz)

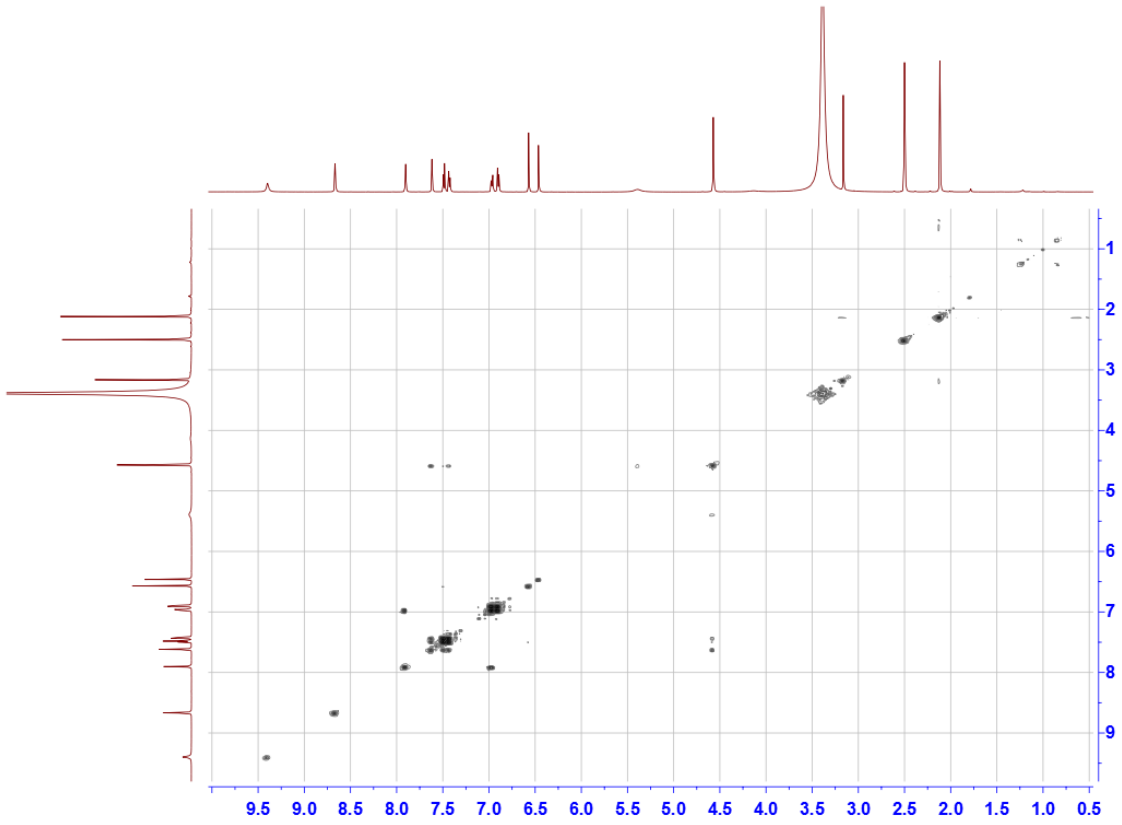

Figure S9. COSY NMR spectrum of **2** (in DMSO-d_6)

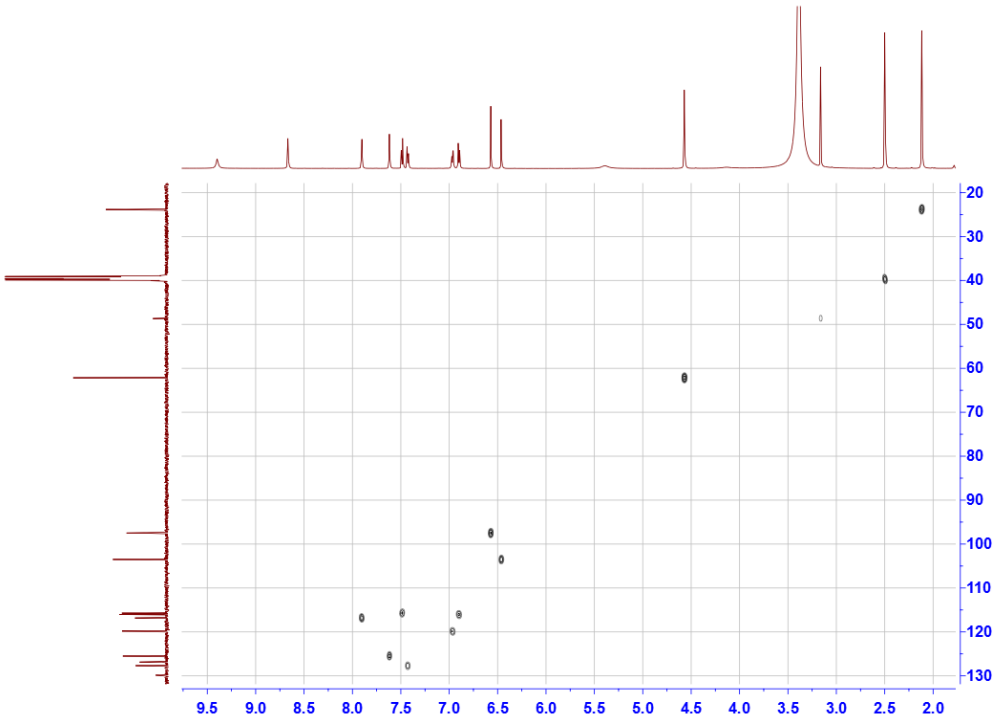

Figure S10. HSQC NMR spectrum of **2** (in DMSO-d_6)

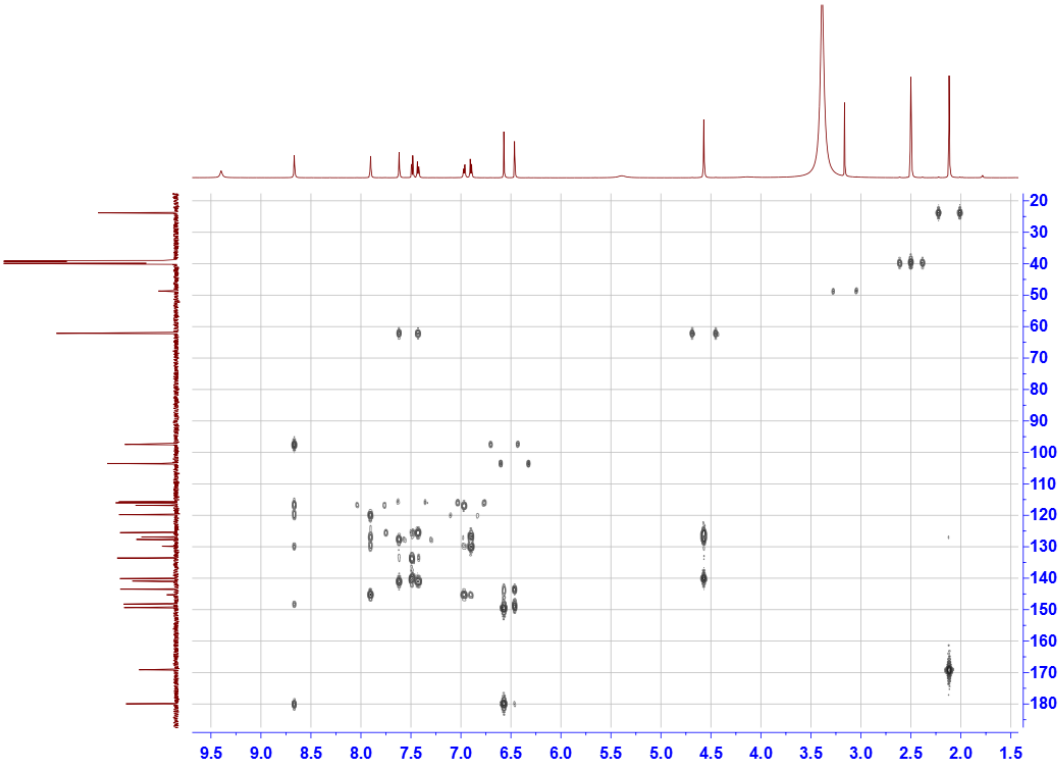

Figure S11. HMBC NMR spectrum of **2** (in DMSO-d_6)

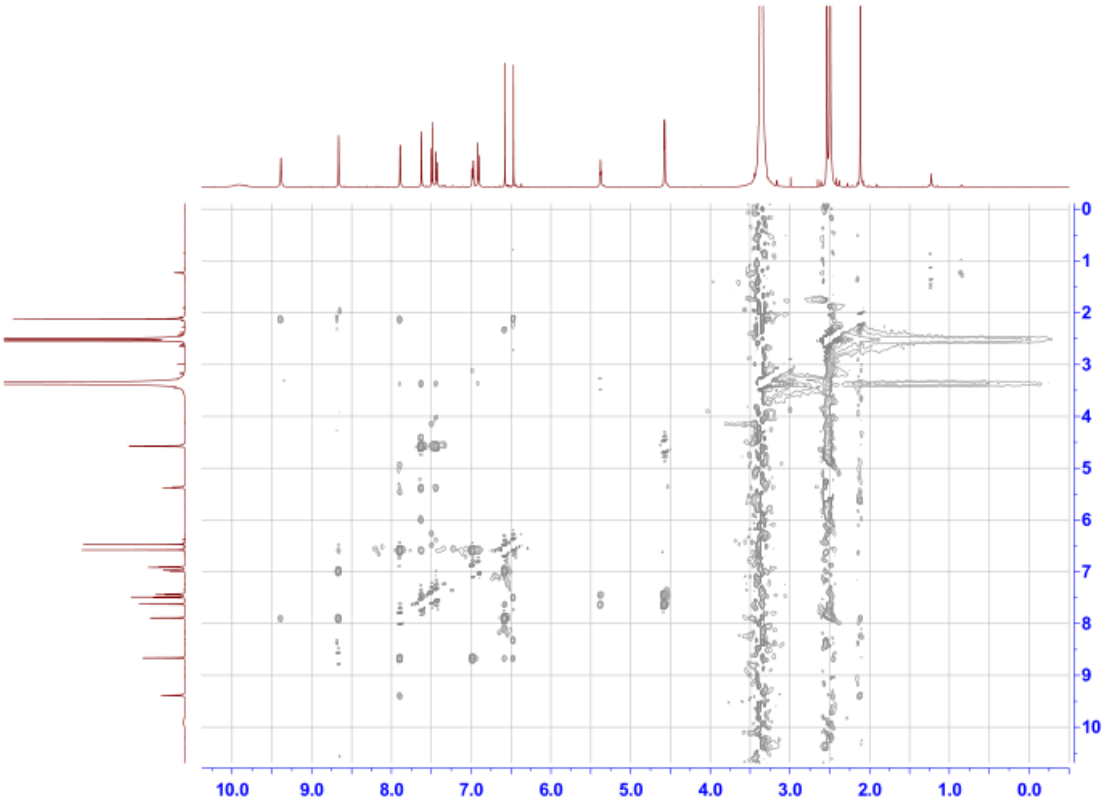

Figure S12. ROESY NMR spectrum of **2** (in DMSO-d_6)

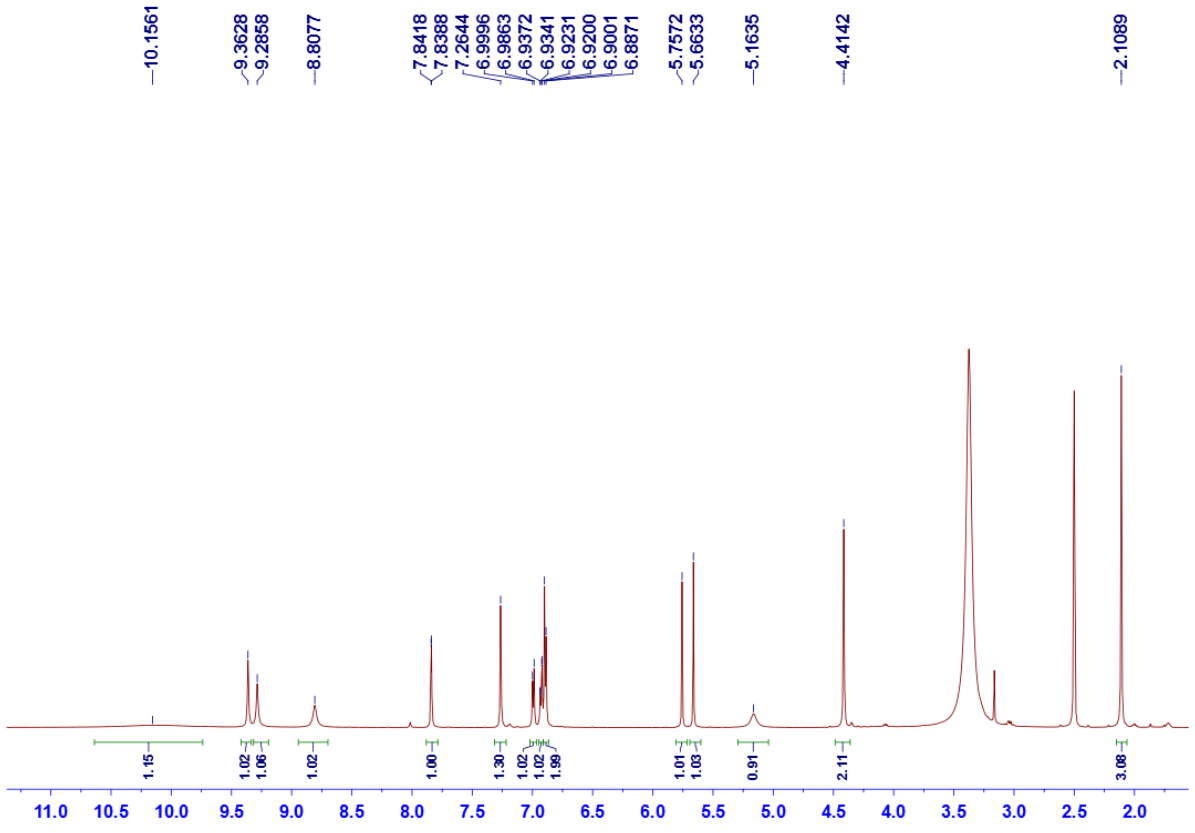

Figure S13. ^{1}H NMR spectrum of **3** (in DMSO-d_6, 600 MHz)

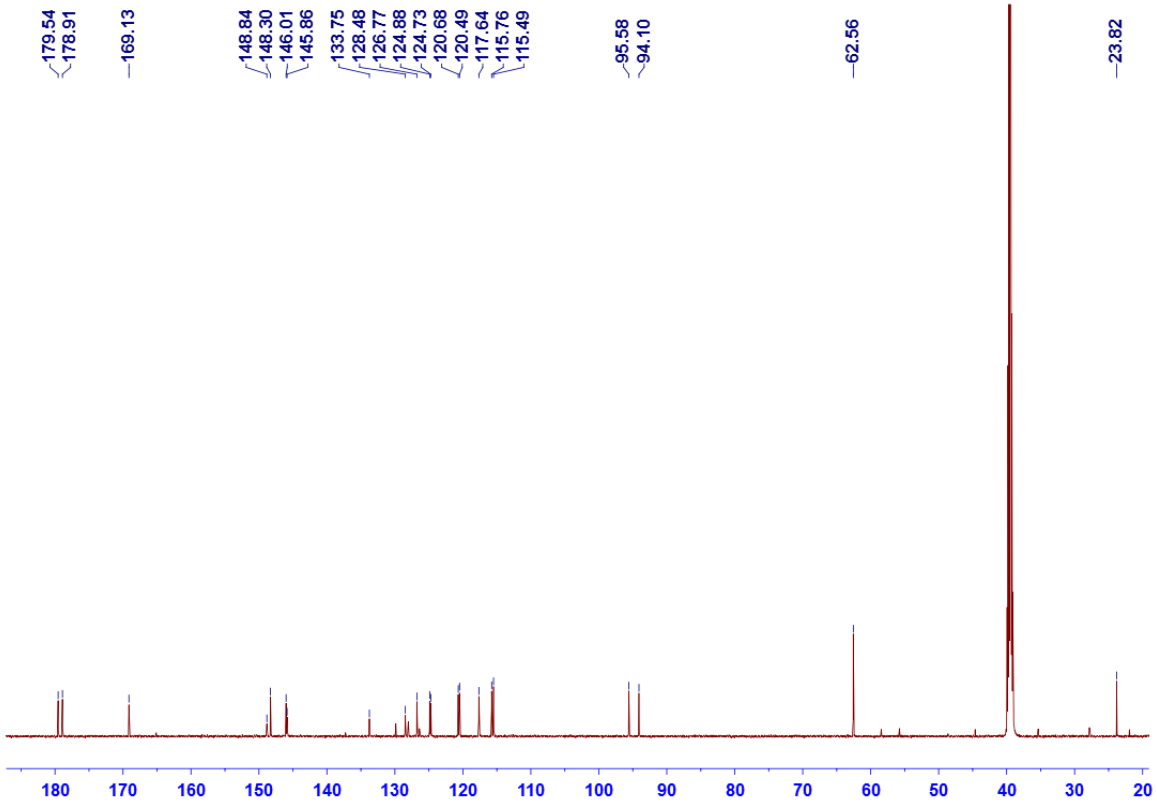

Figure S14. ^{13}C NMR spectrum of **3** (in DMSO-d_6, 150 MHz)

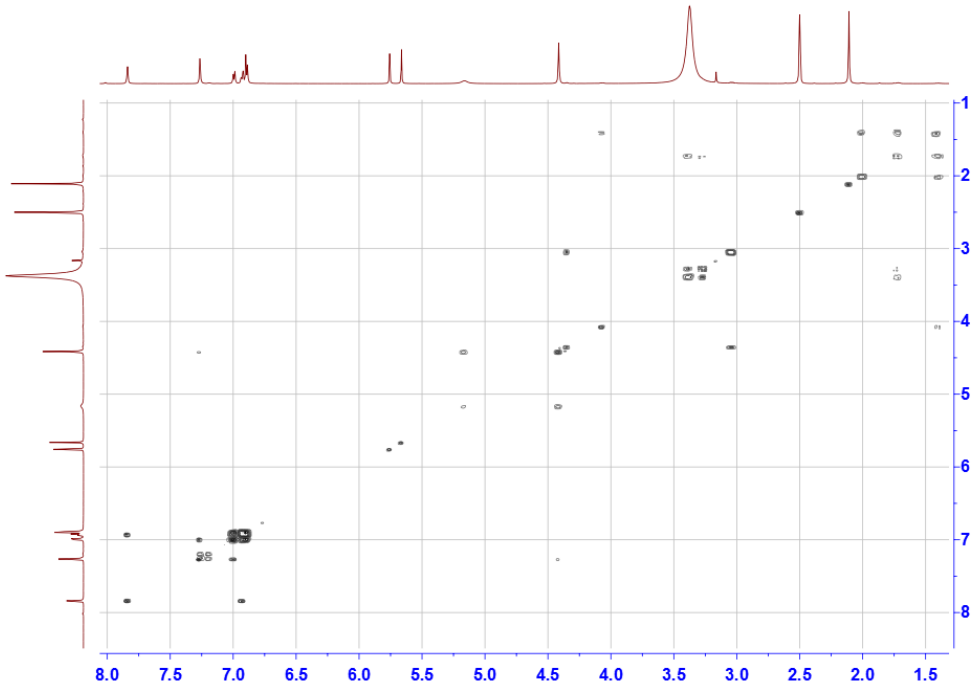

Figure S15. COSY NMR spectrum of **3** (in DMSO-d_6)

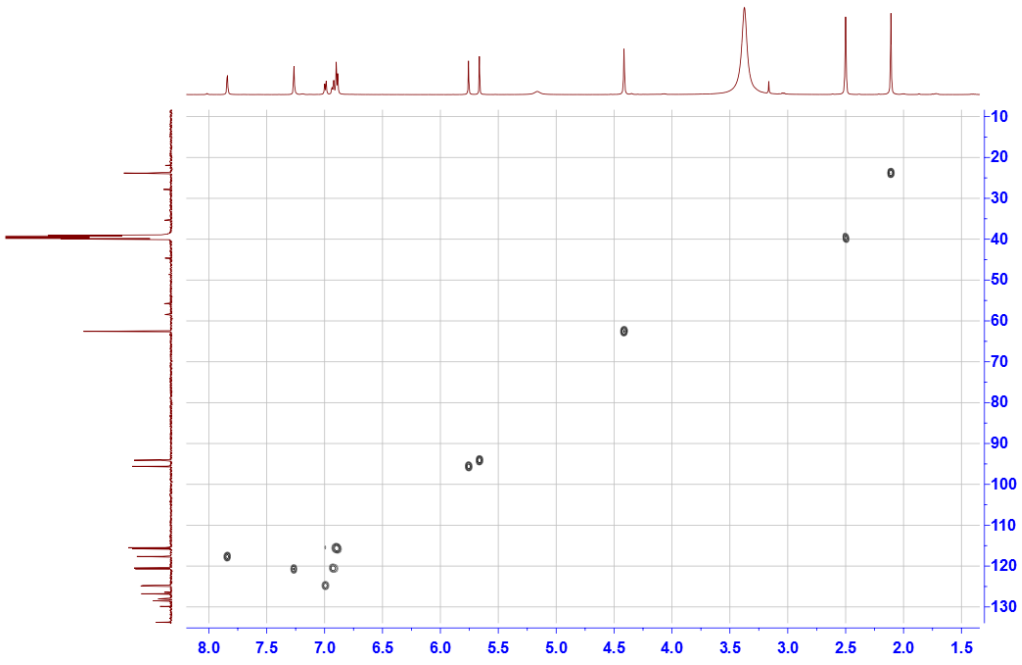

Figure S16. HSQC NMR spectrum of **3** (in DMSO-d_6)

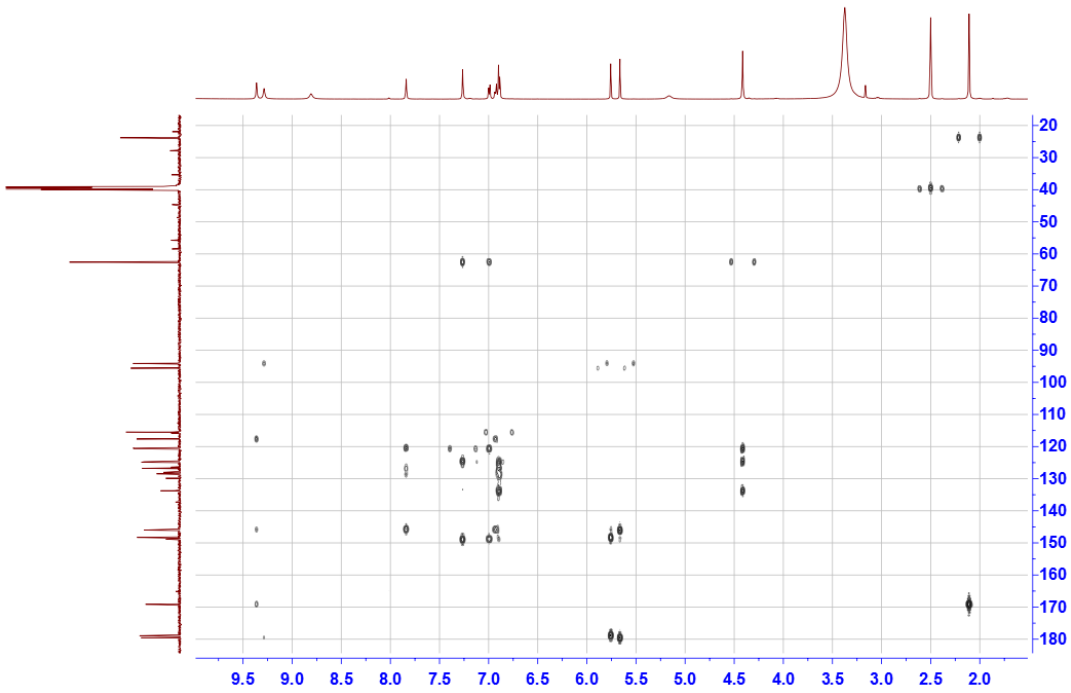

Figure S17. HMBC NMR spectrum of **3** (in DMSO-d_6)

Exfoliazone (**4**): orange powder; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.74 (1H, s, NH-12), 8.28 (1H, s, H-1), 7.77 (1H, s, H-9), 7.58 (1H, dd, J = 8.4, 2.0 Hz, H-7), 7.53 (1H, d, J = 8.4 Hz, H-6), 6.49 (1H, s, H-4), 5.46 (1H, s, 11-OH), 4.61 (2H, s, H-11), 2.23 (3H, s, H-2'); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 179.4 (C-3), 170.8 (C-1'), 149.1 (C-4a), 148.8 (C-10a), 141.7 (C-5a), 140.4 (C-8), 137.8 (C-2), 133.3 (C-9a), 130.2 (C-7), 126.7 (C-9), 115.9 (C-6), 113.4 (C-1), 103.8 (C-4), 62.0 (C-11), 24.4 (C-2'); ESIMS *m/z* 297 $[M+Na]^+$.

Viridobrunnine A: (**5**) light-brown powder; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.76 (1H, s, NH-12), 8.27 (1H, s, H-1), 7.85 (1H, s, H-9), 7.62 (1H, overlapped, H-7), 7.57 (1H, overlapped, H-6), 6.50 (1H, s, H-4), 5.18 (2H, s, H-11), 2.23 (3H, s, H-2'), 2.09 (3H, s, H-2"); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 179.6 (C-3), 170.9 (C-1"), 170.4 (C-1'), 149.2 (C-4a), 148.9 (C-10a), 142.6 (C-5a), 137.9 (C-2), 133.9 (C-8), 133.3 (C-9a), 131.6 (C-7), 128.8 (C-9), 116.3 (C-6), 113.3 (C-1), 104.0 (C-4), 64.6 (C-11), 24.5 (C-2'), 20.8 (C-2"); ESIMS *m/z* 327 $[M+H]^+$.

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