Supporting Information

Discovery and Biosynthesis of Guanipiperazine from a NRPS-like Pathway

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Experimental Procedures

General experimental procedures.

The NMR spectra were collected on Bruker Avance 400 for 400 MHz for ^{1}H and 100 MHz for ^{13}C nuclei. UV spectrum was recorded on a Nanodrop 2000 spectrometer with a 10 mm cuvette. LC-MS analysis was performed on an Agilent 6530 TOF LC/MS mass spectrometer with a Poroshell 120 EC-C18 column (4.6×50 mm, 2.7 um). MPLC was conducted on Biotage Isolera One MPLC system using a Biotage SNAP Cartridge Ultra C18 60 g column and semipreparative RP-HPLC was performed on an Agilent 1200 HPLC with an Agilent Eclipse XDB-C18 column (5 μm, 250×9.4 mm). The HPLC analysis was run on an Agilent 1200 infinity HPLC equipped with a Poroshell 120 EC-C18 column (4.6×50 mm, 2.7 um). Sephadex LH-20 was purchased from GE Biotechnology. All chemicals used in the study were of analytical grade. The enzymes for PCR were purchased form Vazyme.

PCR procedures for targeted genes.

Fragments were respectively amplified by the condition：step 1: 95℃ for 3 min; step 2: 95℃ for 15 s; step 3: Tm for 15 s; step 4: 72℃ by 30s/kb; step 5: step 2 to step 4 for 35 cycles; step 6: 72℃ 10 min, then the productions were puried by gel.

Construction of plasmids for heterologous expression.

gupB, *gupA-B*, *gupA-C*, and *gupA-D* were respectively amplified with the primer pairs gupB-152-F/R, gupA-B-152-F/R, gupA-C-152-F/R, and gupA-D-152-F/R, and then were cloned into the *Eco*RI and *Bcu*I sites of pSET152 to generate pHG9101, pHG9102, pHG9103, and pHG9104, respectively.

Heterologous expression, fermentation, extraction and HPLC detection.

The plasmids pHG9101-pHG9106 were transferred into the *E. coli*/ET12567 (pUZ8002) strain individually, and further introduced into *Streptomyces lividans* TK24 by conjugation.[1] The recombinant strains were grown on ISP4 agar medium supplemented with 50 μg/mL of apramycin for sporulation. After growing at 30 ℃ for 10 days, the medium was soakd with methanol. The afforded methanol extract was directly used for HPLC analysis. Each sample (10 μL) was injected into the column and first eluted with a linear gradient of 10% methanol to 90% methanol in water for 13 min, and finally with 100% methanol for 3 min at a flow rate of 0.5 mL/min.

Construction of in-frame deletion in plasmid pHG9104.

The gene inactivation was performed by pKOV-Amp and pDF25 to generate the in-frame deletion mutants.[2] Briefly, the up- and downstream homology arms were amplified with primers upF/R and downF/R listed in Table S2 using genomic DNA as template, respectively. The purified PCR products were ligated to linearized pKOV-Amp to generate plasmids pHG9105-pHG9106. After confirmed by DNA sequencing, plasmids were used for in-frame deletion of each gene in *E. coli*, following the previouse reported procedure.[3] The genotype of mutated cosmid were confirmed by PCR and DNA sequencing. The afforded mutated cosmid were transformed into *S. lividans* TK24 through conjugation as mentionbed above. The resulting mutant strains were used for further fermentations.

Isolation of compounds 1 and 2.

The strain haboring plasimids were cultivated on ISP4 solid midium for 10 days, 4 liters midium were carried out for fermentation，then were extracted by methanol, extract was fractionated by MPLC over ODS column eluted with a linear gradient MeOH-H_2O system from 10% MeOH to 100% MeOH to give ten fractions. The fractions were subjected for TLC analysis with the system containing dichloromethane and MeOH(5:1). The same fractions were combined and concentrated, and further fractionated by Sephadex LH-20 column using MeOH to give several fractions. The target fraction was purified by semi-HPLC with a gradient solvent system from 30% to 70% MeOH in H_2O system over 25 min with 2.0 mL/min flow rate to give **1** and **2** (1 mg, t_R: 20.5 min).

Isolation of compounds 3, **4 and 6.**

The strains haboring plasimids were cultivated on ISP4 solid midium for 10 days, 3 liters midium were carried out for fermentation， then were extracted by methanol, extract was fractionated by MPLC over ODS column eluted with a linear gradient MeOH-H_2O system from 10% MeOH to 100% MeOH to give ten fractions. The fractions were subjected for TLC analysis with the system containing dichloromethane and MeOH(5:1). The same fractions were combined and concentrated, and further fractionated by Sephadex LH-20 column using MeOH to give several fractions. The target fraction was purified by semi-HPLC, **3** was purified with a gradient solvent system from 50% to 90% MeOH in H_2O system over 20 min with 2.0 mL/min flow rate, **4** was purified with a isocratic solvent system 12% ACN in H_2O system over 13 min with 2.0 mL/min flow rate, **6** was purified with a isocratic solvent system 13% ACN in H_2O system over 15 min with 2.0 mL/min flow rate.

Procedure for MICs of 1 and 2.[3]

*Saccharomyces cerevisiae w*ere cultured in a 5 mL scale at 30 ℃ for overnight, and then 1 mL culture medium was mixed with 100 mL YPD (1% Yeast Extract, 2% Peptone, 2% Glucose), 100 μL mixture was added into the hole of 96-well plate. The guanipiperazine was prepared with a 10 mM concentration by DMSO. Gradient Guanipiperazine was added into 96-well

plate controled by the equal volume DMSO, after cultured at 30 ℃ for 12h, OD_{600} was collected on a Nanodrop 2000 spectrometer. For each sample, three parallel groups were set, and the average value were adopted for analysis. Firstly, a gradient from 0.1 mM to 2 mM with 0.1 mM ladder concentration was used, as for compound **1**, the inhibitory concentration was range to 0.9~1.1 mM, as for compound **2**, the inhibitory concentration was range to 1.1~1.3 mM. For a meticulous measure, a ladder with 0.01 mM concentration was adopted, which finally prove the inhibitory concentrations for **1** and **2** were 1.01 and 1.26 mM, equivalent to 4.5 and 5.6 μg/mL, respectively.

Chemical complementation of compound 3 and 4 into Δ*gupB* mutant.

The Δ*gupB* mutant were cultured in a 200 ml scale at 30°C in fermentation medium. After 24 hours cultivation, compounds **3** or **4** (1.5 mg, 2 μmol) dissolved in DMSO were supplemented into fermentation broth individually and cultured for another 12 hours. The metabolic extract was analyzed by LC-MS using the method mentioned above.

Protein Expression and Purification.

DNA fragments containing target genes including *GupA, GupB, GupC, GupD, Ctg_5604,* and *Ctg_7941* were amplified from genomic DNA of *S. chrestomyceticus* NA4264 with primers listed in Table S2. The purified PCR products were ligated with linearized pET28a (linearized by *Nde*I and *Hin*dIII) to afford pHG9107-pHG9112. The pHG9107-pHG9112 were further introduced into *E. coli* BL21(DE3), respectively. The transformants were cultivated in 400 mL LB medium supplemented with 50 μg/mL kanamycin at 37 ℃ (220 rpm) until OD_{600} value reached around 0.6. The culture was cooled to 4 ℃ and induced with 0.1 mM IPTG, continued to cultivate at 16 ℃ (220 rpm) for 20 h. After centrifugation at 8000 rpm for 10 min, cells were resuspended in lysis buffer (100 mM Tris, pH 8.0, 15 mM imidazole, 300 mM NaCl, 10 % glycerol) and lysed on ice by sonication. After centrifugation at 15000 rpm for 30 min, the supernatant was filtered and purified by ÄKTA FPLC system equipped with a 5 mL Histrap HP column (GE lifesciences).The proteins were pooled and desalted by a PD10 column (GE Healthcare) with 100 mM phosphate buffer (pH 8.0) and 10% glycerol and stored at -80 ℃.

Adenylation activities of A domain

To evaluate the adenylation activity of A domain in GupB, we conducted a coupled continuous assay for inorganic pyrophosphate using a unique fluorogenic pyrophosphate sensor (Sigma-Aldrich Pyrophosphate Assay Kit MAK168). Briefly, the presence of pyrophosphate results in the production of a fluorescent product (λ_{ex}=316 /λ_{em}=456 nm) proportional to the pyrophosphate present. The A domain specificity assay against different amino acid substrates was conducted in a 50 μL reaction volume containing 50 mM Tris-HCl, pH 7.5, 60 μM NRPS protein,12.5 mM MgCl2, 2.0 mM TCEP, 2 mM amino acid, 4 mM ATP. The control group was treated using the boiled protein. After reacting for 30 min at 25 ℃, an equal volume of the master reaction mix were added to each of the sample, and the reaction was incubated at room temperature. After 20 mins, the fluorescence intensity (λ_{ex} = 316/λ_{em} = 456 nm) was determined by a microplate reader. After deducting the data of the control group, the relative fluorescence intensity was calculated.

***In vitro* biochemical assay of GupA, GupB.**

The *holo*-GupB was achieved by a mixture (50 μL) containing 50 μM *apo*-GupB, 0.8 mM CoA, 2 mM TCEP, 12.5 mM $MgCl_2$, 20 μM Sfp for 45 min. Co-reaction of GupA and *holo*-GupB was carried out in a 100 μL reaction system containing 50 mM Tris-HCl buffer (pH 7.5), 25 μM *apo*-GupB, 2 mM L-Tyr, 4 mM ATP, 1 mM NADH, 2 mM TCEP, 12.5 mM $MgCl_2$, 8 μM GupA, 1 mM F420, 1.6 μM FGD, 2.5 mM glucose-6-phosphate. After incubation at 30 ℃ for 2 h, the reaction was quenched by adding 100 μL methanol.Then the mixture was centrifuged at 15,000 g for 10 min and the supernatant was analyzed by LC-MS. The LC-MS analysis was performed using a 18 min solvent gradient from 10% to 70% (0–18 min) methanol in water supplied with 0.1 TFA at a flow rate of 0.5 mL/min.

***In vitro* biochemical assay of GupC.**

The GupC catalyzed reaction was carried out in a 100 μL reaction system containing 50 mM MES buffer (pH 5.5), 134 μM substrate, 1.5 mM NADPH, 8 μM FDR, 10 μM FDX and 27 μM GupC. After incubation at 30 ℃ for 2 h, the reaction was quenched by adding 100 μL methanol. The LC-MS analysis was performed using a 18 min solvent gradient from 10% to 50% (0–18 min) methanol in water supplied with 0.1 TFA at a flow rate of 0.5 mL/min.

***In vitro* biochemical assay of CupD.**

The reaction mixture (100 μL) contained 17 μM GupD, 1.5 mM NADPH, 8 μM FDR, 10 μM FDX, 1 mM GTP, 70 μM compound **6**, and 50 mM PIPES buffer (pH 6.5). After incubation at 30 ℃ for 4 h, the reaction was quenched by adding 100 μL methanol. Then the mixture was centrifuged at 15,000 g for 10 min and the supernatant was analyzed by LC-MS. The LC-MS analysis was performed using a 18 min solvent gradient from 10% to 50% (0–18 min) methanol in water supplied with 0.1 TFA at a flow rate of 0.5 mL/min.

Sequence similarity network (SSN) analysis.[4]

R domain was used as the search query in antiSMASH database and our own, 1,230 NRPS BGCs containing a terminal R domain were retrieved. After manually removing the multi-modular NRPS BGCs, we were left with 608 single-module NRPS-like BGCs. For SSN analysis, an initial sequence identity 10% was used from the local blast analysis (all vs all). Cytoscape

3.7.0 were used for network visualization. Both self loops and duplicate loops were deleted. Finally, the sequence identity 40% was chosen for figure generation.

Physical data for isolated compounds

Compound **1**: light brown powder; NMR data see Table S4; HRESIMS *m/z* 446.1937 [M+H]+ (calcd for $C_{23}H_{23}N_7O_3$, 446.1935); UV (MeOH): λ_{max} (log ε) =229 nm (2.93), 314 nm (1.78).

Compound **2**: white powder; NMR data see Table S5, HRESIMS *m/z* 446.1939 [M+H]+ (calcd for $C_{23}H_{23}N_7O_3$, 446.1935); UV (MeOH): λ_{max} (log ε) =229 nm (2.92), 280 nm (2.63).

Compound **3**: white powder; NMR data see Table S6, HRESIMS *m/z* 293.1283 $[M+H]^+$ (calcd for $C_{18}H_{16}N_2O_2$, 293.1285) ; UV (MeOH): λ_{max} (log ε) =229 nm (3.21), 277 nm (3.13).

Compound **4**: Light yellow oil; NMR data see Table S7, HRESIMS *m/z* 299.1754 $[M+H]^+$ (calcd for $C_{18}H_{22}N_2O_2$, 299.1754) ; UV (MeOH): λ_{max} (log ε) =218 nm (3.22), 277 nm (2.76).

Compound **6**: Light yellow oil; NMR data see Table S8, HRESIMS *m/z* 297.1596 $[M+H]^+$ (calcd for $C_{18}H_{20}N_2O_2$, 297.1598) ; UV (MeOH): λ_{max} (log ε) =228 nm (3.29), 279nm (2.75).

Table S1. Deduced gene functions of in the *gup* BGC.[a]

ORF	Amino acids[b]	Blastp homologue	Identity/coverage [%]	Protein ID[c]
GupA	162	$F_{420}H_2$-dependent reductase	24/52	O06553.1
GupB	1074	non-ribosomal peptide synthetase	97/99	GCD40661.1
GupC	417	cytochrome P450	94/90	KOG65251.1
GupD	409	cytochrome P450	79/99	GGT86394.1
Ctg_7941	428	ferredoxin reductase	93/99	KOT90529.1
Ctg_5604	106	ferredoxin	100/100	KOT40977.1

[a] The sequence has been deposited in GenBank with the accession number MW051641.[b] Numbers are in amino acids. [c] Given in numbers are NCBI accession numbers.

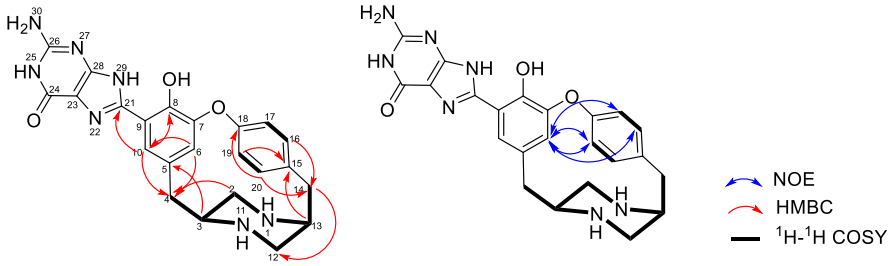
Table S2. Bacterial plasmids and strains.

Plasmid/Strain	Relevant characteristics	Source
Plasmid		
pSET152	Apr^r, integrated vector for heterlogous expression	5
pUZ8002	Kan^r, including tra for conjugation	5
pKOV-Amp	plasmid used for gene disruption, temperature sensitive	6
pDF25	plasmid used for gene disruption, temperature sensitive	7
pET28a(+)	Kan^r, protein expression vector	Novagen
pHG9101	The plasmid for heterologous expression of *gupB*	This study
pHG9102	The plasmid for heterologous expression of *gupA-B*	This study
pHG9103	The plasmid for heterologous expression of *gupA-C*	This study
pHG9104	The plasmid for heterologous expression of *gupA-D*	This study
pHG9105	pKOV derived plasmid for disruption of *gupB*	This study
pHG9106	pKOV derived plasmid for disruption of *gupC*	This study
pHG9107	pET28a(+) derived plasmid for expressing N-terminal His-tag GupA	This study
pHG9108	pET28a(+) derived plasmid for expressing N-terminal His-tag GupB	This study
pHG9109	pET28a(+) derived plasmid for expressing N-terminal His-tag GupC	This study
pHG9110	pET28a(+) derived plasmid for expressing N-terminal His-tag GupD	This study
pHG9111	pET28a(+) derived plasmid for expressing N-terminal His-tag Ctg_7941	This study
pHG9112	pET28a(+) derived plasmid for expressing N-terminal His-tag Ctg_5604	This study
***E. coli* strains**		
DH5α	General cloning host	8
DH10B	General cloning host	8
BL21 (DE3)	Heterologous host for protein expression	NEB
ET12567 (pUZ8002)	Methylation-deficient host used for *E. coli-Streptomyces* intergeneric conjugation	9
Strains		
S. lividans TK24	Model actinomycete used for gene heterologous expression.	10
HG9101	*S. lividans* TK24 carrying pHG9101	This study
HG9102	*S. lividans* TK24 carrying pHG9102	This study
HG9103	*S. lividans* TK24 carrying pHG9103	This study
HG9104	*S. lividans* TK24 carrying pHG9104	This study
HG9105	*S. lividans* TK24 carrying*ΔgupB* /pHG9104	This study
HG9106	*S. lividans* TK24 carrying*ΔgupC* /pHG9104	This study

Table S3. Primers used in this study.

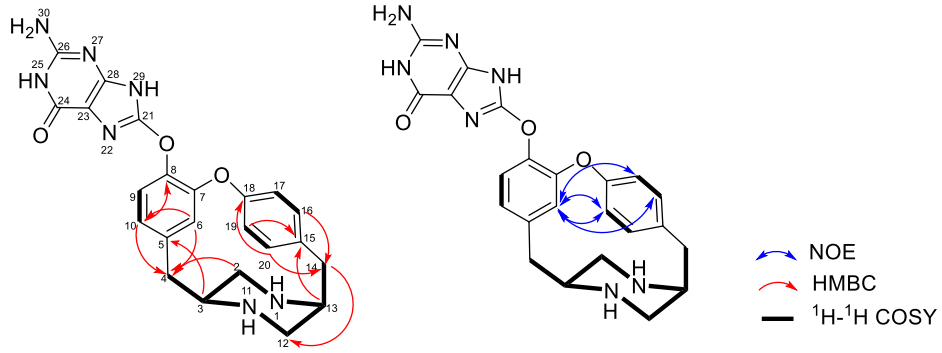
Name	Sequence
gupB-152-F	GCTGCATGCATACGTACTAGTcccaccgctacctggacg
gupB-152-R	CTATGACATGATTACGAATTCtcatcggctgtgctccgt
gupA-B-152-F	GCTGCATGCATACGTACTAGTgccggtaagcccagatgt
gupA-B-152-R	CTATGACATGATTACGAATTCtcatcggctgtgctccgt
gupA-C-152-F	GCTGCATGCATACGTACTAGTgccggtaagcccagatgt
gupA-C-152-R	CTATGACATGATTACGAATTCtcaccagcgcacgggaag
gupA-D-152-F	GCTGCATGCATACGTACTAGTgccggtaagcccagatgt
gupA-D-152-R	CTATGACATGATTACGAATTCtcaccaggtgaccgggac
ΔgupB-UF	GGTAACTGTCAGACCGGATCC cacctcatacgtaccggg
ΔgupB-UR	GGCGGTGCGGACCCGCTGggcgtcggaggacaggct
ΔgupB-DF	AGCCTGTCCTCCGACGCCcagcgggtccgcaccgcc
*ΔgupB-D*R	CCGGTCGACTCTAGAGGATCC gatctcccggtcctcgta
ΔgupC-UF	GGTAACTGTCAGACCGGATCC gggcactccctgaccgcc
ΔgupC-UR	GGTCCGGGCCAGGTCGAGgtccgggacgggggcgtc
ΔgupC-DF	GACGCCCCCGTCCCGGACctcgacctggcccggacc
*ΔgupC-D*R	CCGGTCGACTCTAGAGGATCC gttggggacaagcacgta
ΔgupB-PO-F	atgaccgacacgtacgtt
ΔgupB- PO-R	tcatcggctgtgctccgt
ΔgupB-NE-F	gtcacccgcaccctcgcc
ΔgupB- NE-R	cgcgtcctcgtagcggct
ΔgupC-PO-F	tacgacgcgtgggtcgcc
ΔgupC- PO-R	catgcactgggcggcgac
ΔgupC-NE-F	gtgctgtgtgagaccgtc
ΔgupC- NE-R	gaggtcgccggggctgcc
GupA-pET28a-F	GTGCCGCGCGGCAGCCATATGctgcccgccatgtcg
GupA-pET28a-R	GCTCGAGTGCGGCCGCAAGCTTtcagcgctgggcggcttc
GupB-pET28a-F	GTGCCGCGCGGCAGCCATATGaccgacacgtacgtttcttcac
GupB-pET28a-R	GCTCGAGTGCGGCCGCAAGCTTtcatcggctgtgctccgt
GupC-pET28a-F	GTGCCGCGCGGCAGCCATATGagccgaaccgaagccgcc
GupC-pET28a-R	GCTCGAGTGCGGCCGCAAGCTTtcaccagcgcacgggaag
GupD-pET28a-F	GTGCCGCGCGGCAGCCATATGtccacaccaccgcaagcg
GupD-pET28a-R	GCTCGAGTGCGGCCGCAAGCTTtcaccaggtgaccgggac
Ctg_7941-pET28a-F	AAGAAGGAGATATACATATGggtgcgccgcaggct
Ctg_7941-pET28a-R	CTCGAGTGCGGCCGCAAGCTTcgtcacgtccctttctcc
Ctg_5604-pET28a-F	GTGCCGCGCGGCAGCCATATGacccccccgccgcatc
Ctg_5604-pET28a-R	GCTCGAGTGCGGCCGCAAGCTTtcacgcgagggcgttccg

Table S4. ^{1}H and ^{13}C NMR data of **1** in DMSO-d_6.[a]



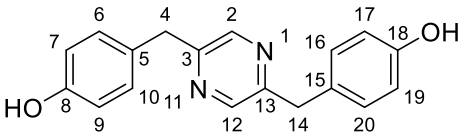
No.	δ_C	δ_H (mult, *J*, Hz)
2	42.9, CH_2	2.62 (t, 1.08), 2.40 (t, 7.72)
3	52.1, CH	2.92 (dd, 7.72, 1.44)
4	34.3, CH_2	2.52 (t, 1.24), 2.83 (dd, 11.76, 1.88)
5	126.7, C	
6	121.6, CH	6.83 (s)
7	146.8, C	
8	144.4, C	
9	112.9, C	
10	118.8, CH	7.20 (s)
12	47.9, CH_2	3.05 (dd, 8.72, 3.84), 3.45 (d, 8.76)
13	48.7, CH	3.59 (m)
14	37.7, CH_2	3.20 (ddd, 15.32, 5.44, 2.16)
15	135.3, C	
16	132.6, CH	7.57 (dd, 8.0, 2.0)
17	125.5, CH	7.27 (dd, 8.0, 2.0)
18	160.2, C	
19	124.1, CH	6.92 (dd, 8.4. 1.8)
20	132.0, CH	6.79 (dd, 8.4, 1.8)
21	151.2, C	
23	117.5, C	
24	155.2, C	
26	153.9, C	
28	152.6, C	
30-NH		6.68(s)

Table S5. ^{1}H and ^{13}C NMR data of **2** in DMSO-d_6.[a]



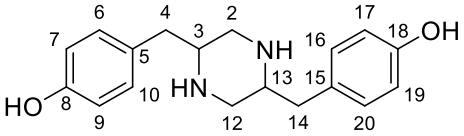
No.	δ_C	δ_H (mult, *J*, Hz)
2	43.0, CH_2	2.64 (d, 13.4), 2.38 (m)
3	52.1, CH	2.92 (dd, 3.84, 1.56)
4	34.6, CH_2	2.57 (dd, 13.6, 6.2), 2.83 (dd, 13.6, 5.8)
5	135.3, C	
6	121.8, CH	6.27 (s)
7	153.7, C	
8	138.5, C	
9	111.7, CH	7.09 (d, 8.0)
10	122.6, CH	6.62 (dd, 8.0, 1.8)
12	47.2, CH_2	3.01 (dd,14.4, 4.16), 3.42 (dd, 14.4, 8.64)
13	48.8, CH	3.57 (brs)
14	37.8, CH_2	3.17 (dd, 14.4, 4.88), 3.23 (dd, 14.4, 5.64,)
15	135.9, C	
16	132.7, CH	7.55 (dd, 8.0, 1.8)
17	123.8, CH	6.93 (dd, 8.0, 1.8)
18	159.7, C	
19	125.3, CH	7.24 (dd, 8.4, 2.2)
20	131.9, CH	7.64 (dd ,8.4, 2.2)
21	158.0, C	
23	121.4, C	
24	155.2, C	
26	158.5, C	
28	158.7, C	

Table S6. ^{1}H NMR data of **3** in DMSO-d_6.[a]



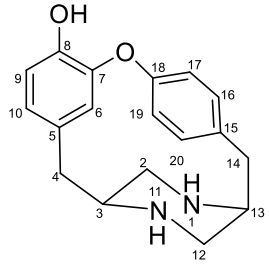
No.	δ_H (mult, *J*, Hz)
2	8.43 (s)
4	3.94 (s)
6	6.60 (d, 8.4)
7	7.04 (d, 8.4)
9	7.04 (d, 8.4)
10	6.60 (d, 8.4)
12	8.43 (s)
14	3.94 (s)
16	6.60 (d, 8.4)
17	7.04 (d, 8.4)
19	6.60 (d, 8.4)
20	7.04 (d, 8.4)

Table S7. ^{1}H NMR data of **4** in DMSO-d_6.



No.	δ_H (mult, *J*, Hz)
2	2.98 (m)
3	3.70 (m)
4	3.20 (m)
6	6.76 (d, 8.4)
7	7.11 (d, 8.4)
9	7.11(d, 8.4)
10	6.76 (d, 8.4)
12	2.98 (m)
13	3.70 (m)
14	3.20 (m)
16	6.76 (d, 8.4)
17	7.11 (d, 8.4)
19	7.11(d, 8.4)
20	6.76 (d, 8.4)

Table S8. ^{1}H NMR data of **6** in DMSO-d_6.[a]



No.	δ_H (mult, *J*, Hz)
2	2.63 (d, 7.4), 2.36 (m)
3	2.93 (brd)
4	2.50 (dd, 6.20, 2.28)
	2.76 (dd, 5.68, 1.88)
6	5.89 (s)
9	7.21 (dd, 8.0, 2.4)
10	6.62 (d, 8.0)
12	3.04 (dd, 13.3, 5.6)
	3.45 (d, 13.3)
13	3.53 (brs)
14	3.17 (dd, 14.7, 3.6)
	3.23 (dd, 14.7, 5.4)
16	7.55 (dd, 8.4, 2.2)
17	6.89 (dd, 8.4, 2.2)
19	7.62 (dd, 8.4, 2.2)
20	6.38 (dd, 8.4, 2.2)

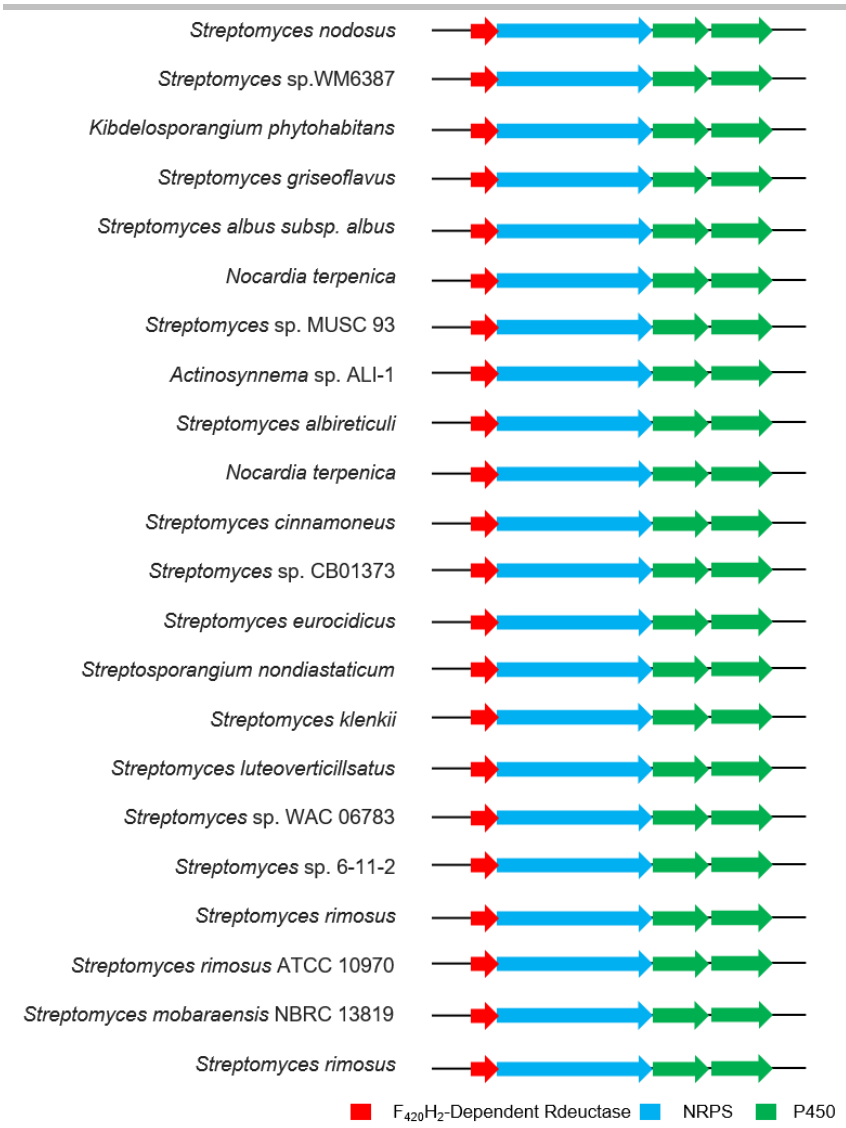


Figure S1. The *gup* homologous gene clusters obtained from NCBI database.

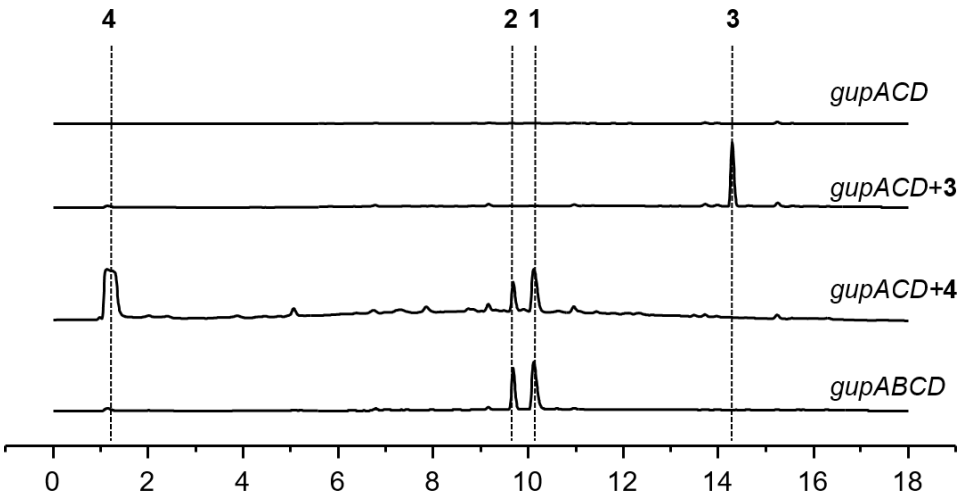


Figure S2. Chemical complementation of compounds into TK24/*gupACD* strain.

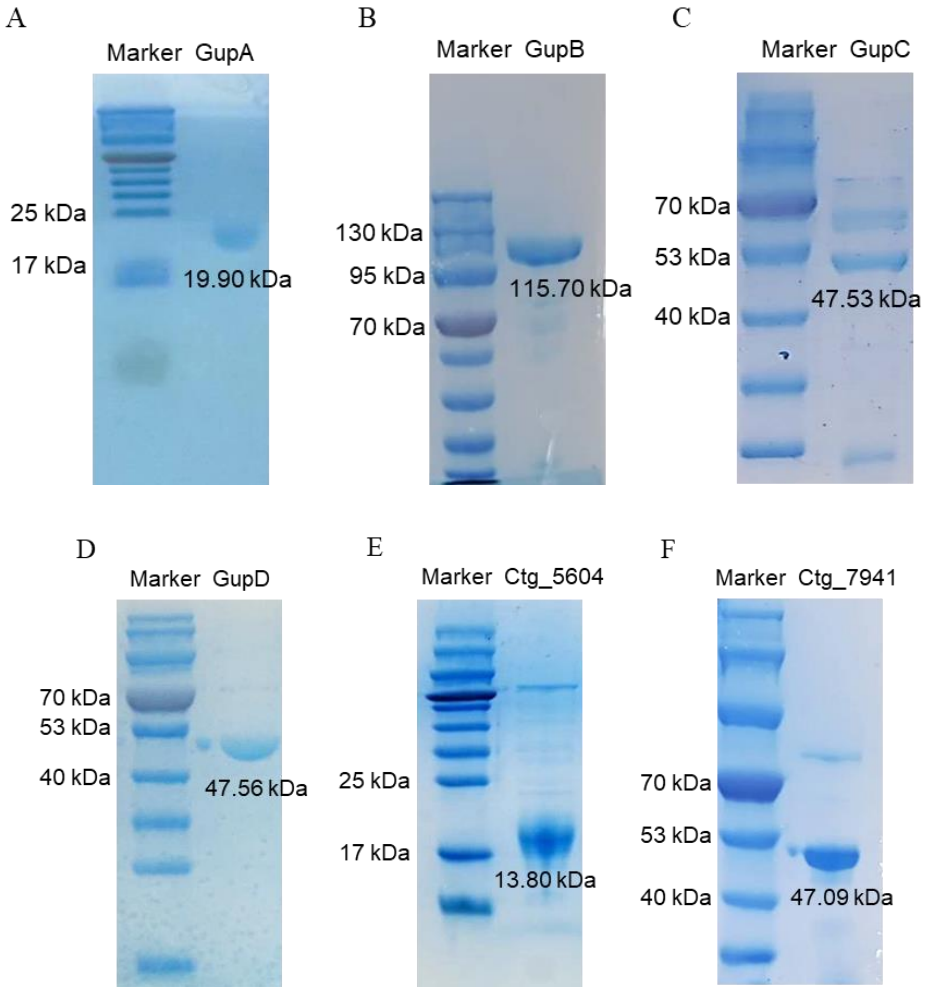


Figure S3. SDS-PAGE analysis of reconstituted proteins. A) GupA (calculated molecule weight 19.90kDa); B) GupB (calculated molecule weight 115.70kDa); C) GupC (calculated molecule weight 47.53kDa); D) GupD (calculated molecule weight 47.56kDa); E) Ctg_5604 (Fdx, calculated molecule weight 13.80kDa); F) Ctg_7941 (Fdr, calculated molecule weight 47.09kDa).

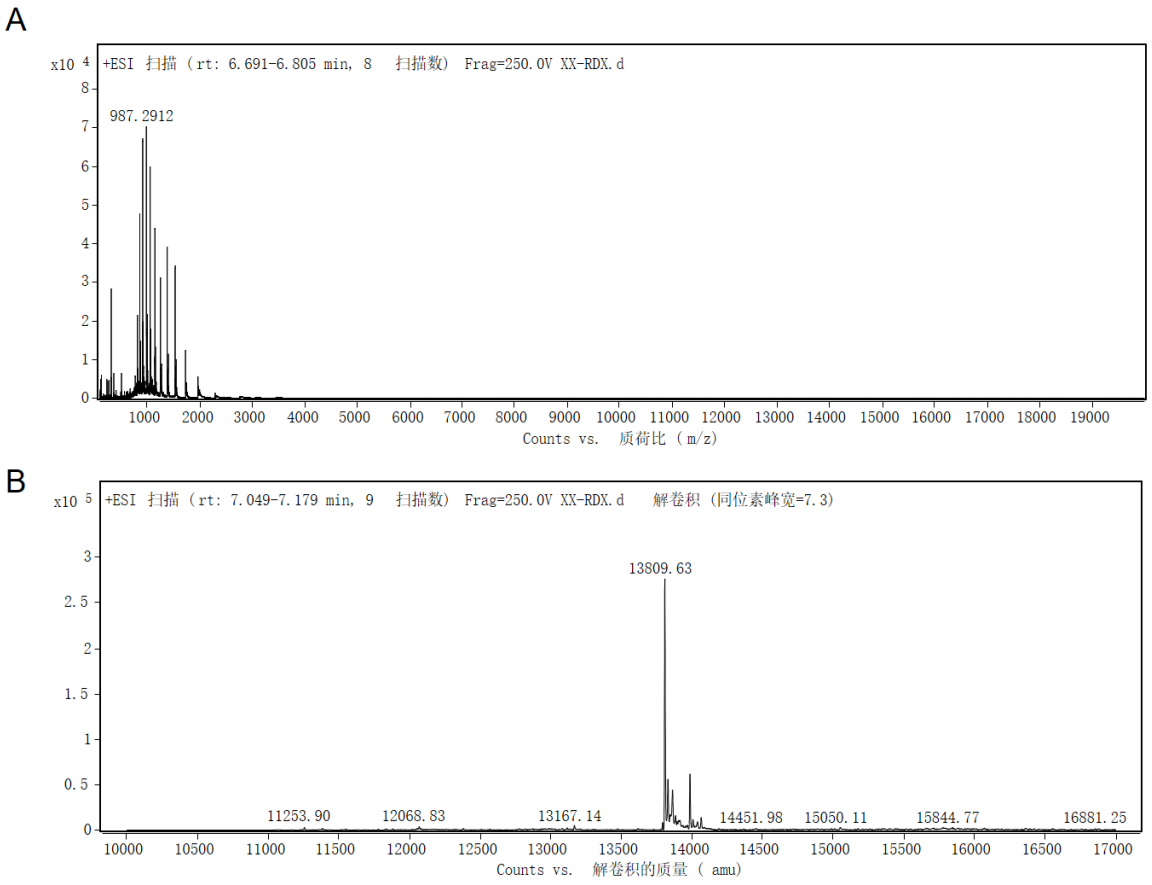


Figure S4. LC-MS analyses of the purified ferredoxin (ctg_5604). A) The *m/z* values of average protein masses of the 14^{+} charge states (MS1) are shown. B) The molecular weight of ctg_5604.

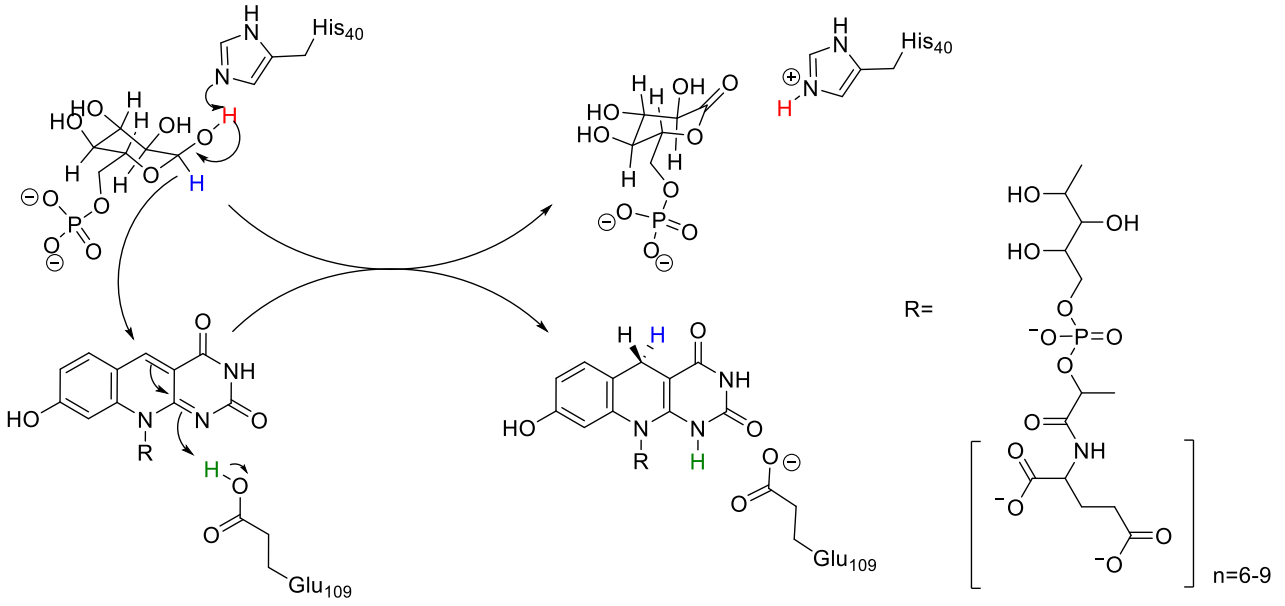


Figure S5. FGD-catalyzed reaction. FGD utilizes the oxidized form of cofactor F_{420} to convert glucose-6-phosphate (G6P) 6-phosphogluconolactone, thus yielding the reduced cofactor $F_{420}H_2$[11].

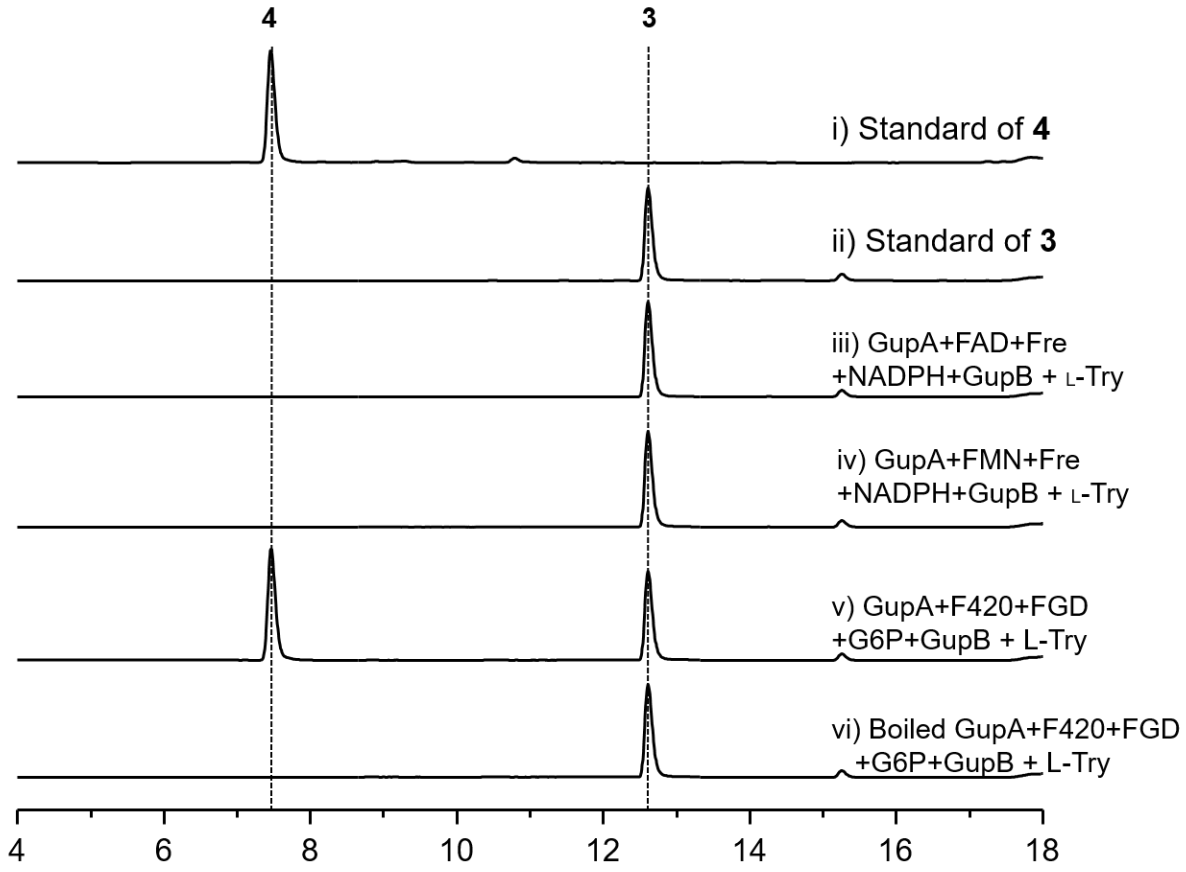


Figure S6. HPLC analysis (monitored at 280 nm) of *in vitro* GupB and GupA enzymatic reactions utilize iii) FAD iv) FMN v) F420 as reducing agents.

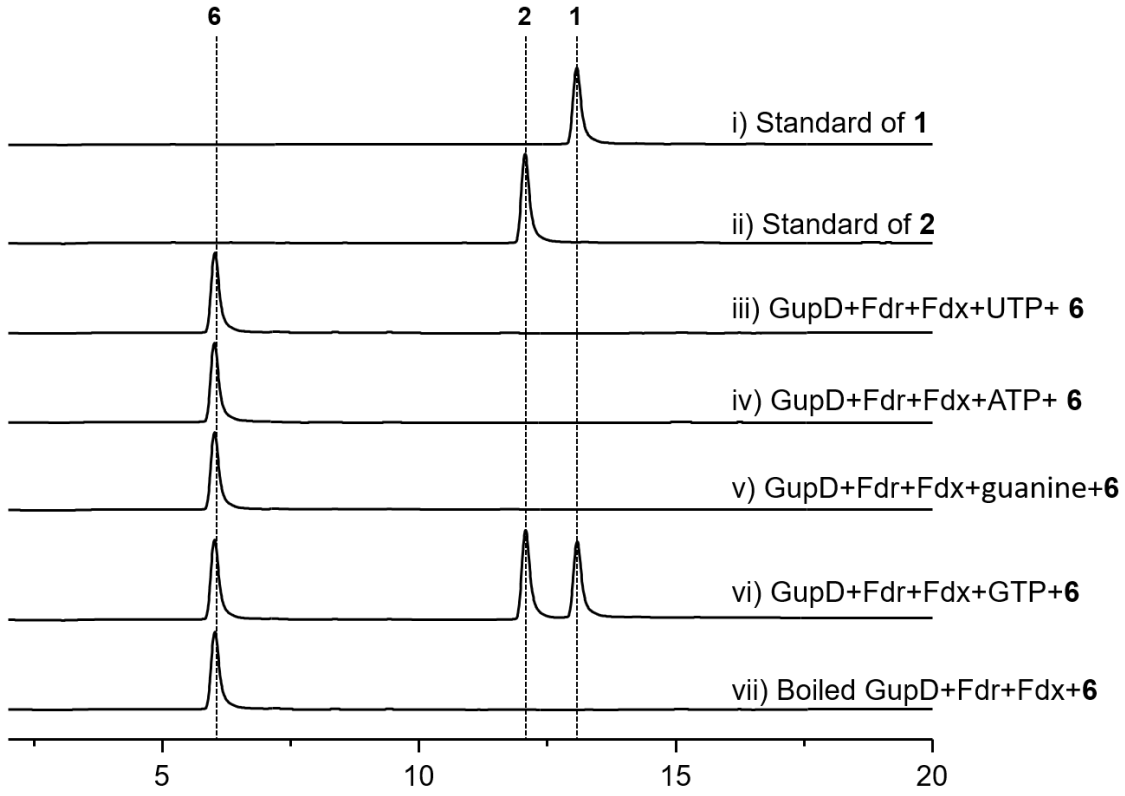


Figure S7. HPLC analysis (monitored at 280 nm) of *in vitro* GupD enzymatic reactions.

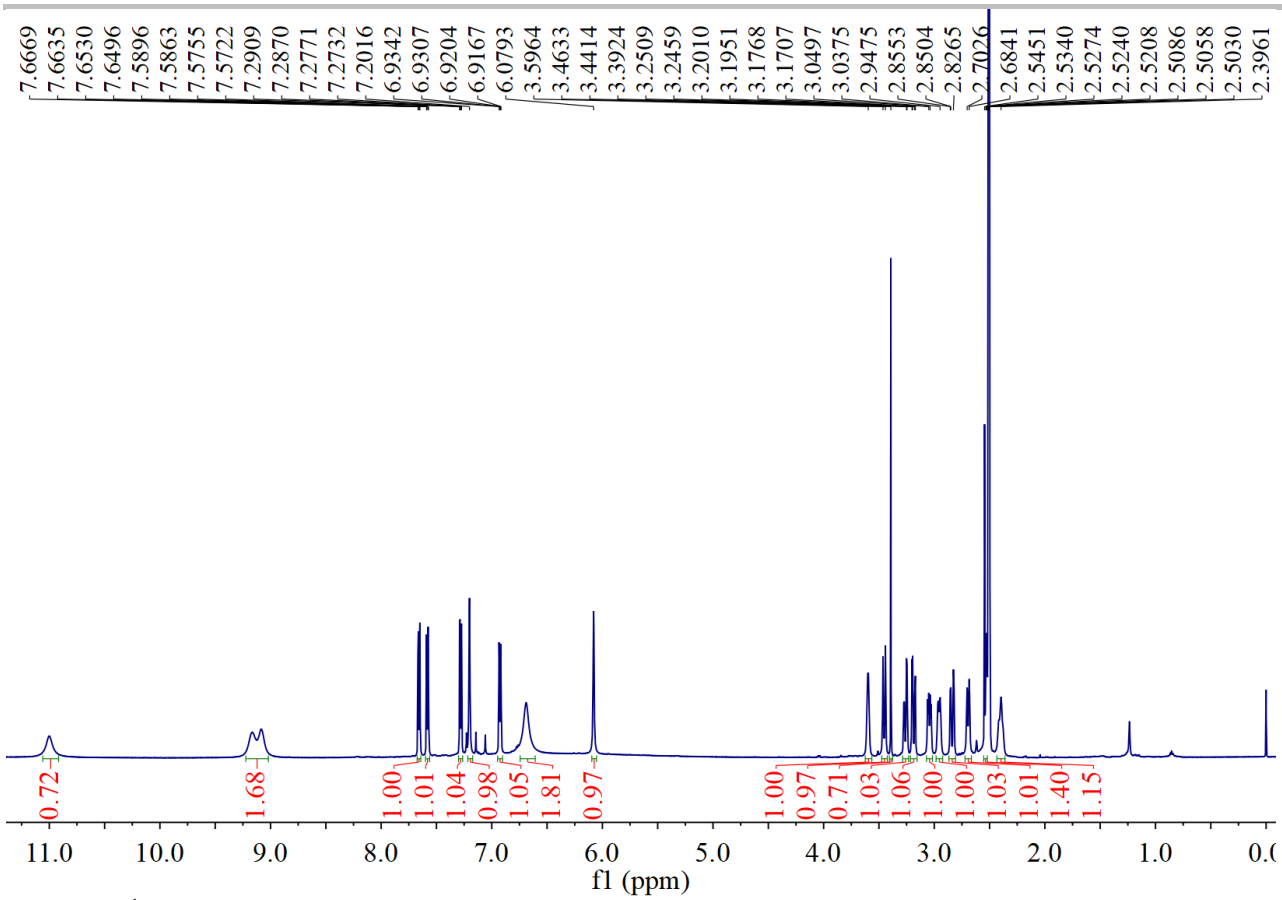


Figure S8. ^{1}H NMR spectrum of **1** in DMSO-d_6 at 400 MHz.

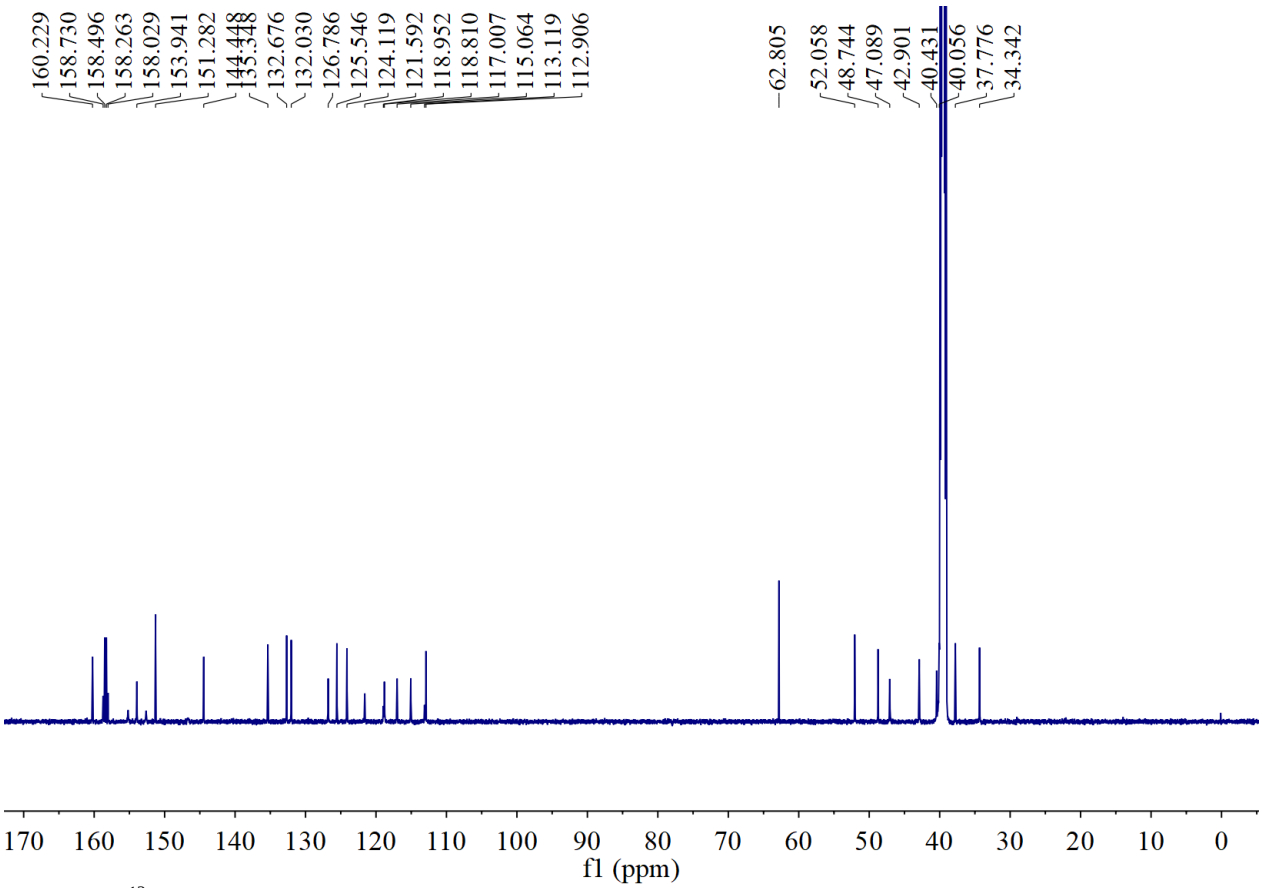


Figure S9. ^{13}C NMR spectrum of **1** in DMSO-d_6 at 100MHz.

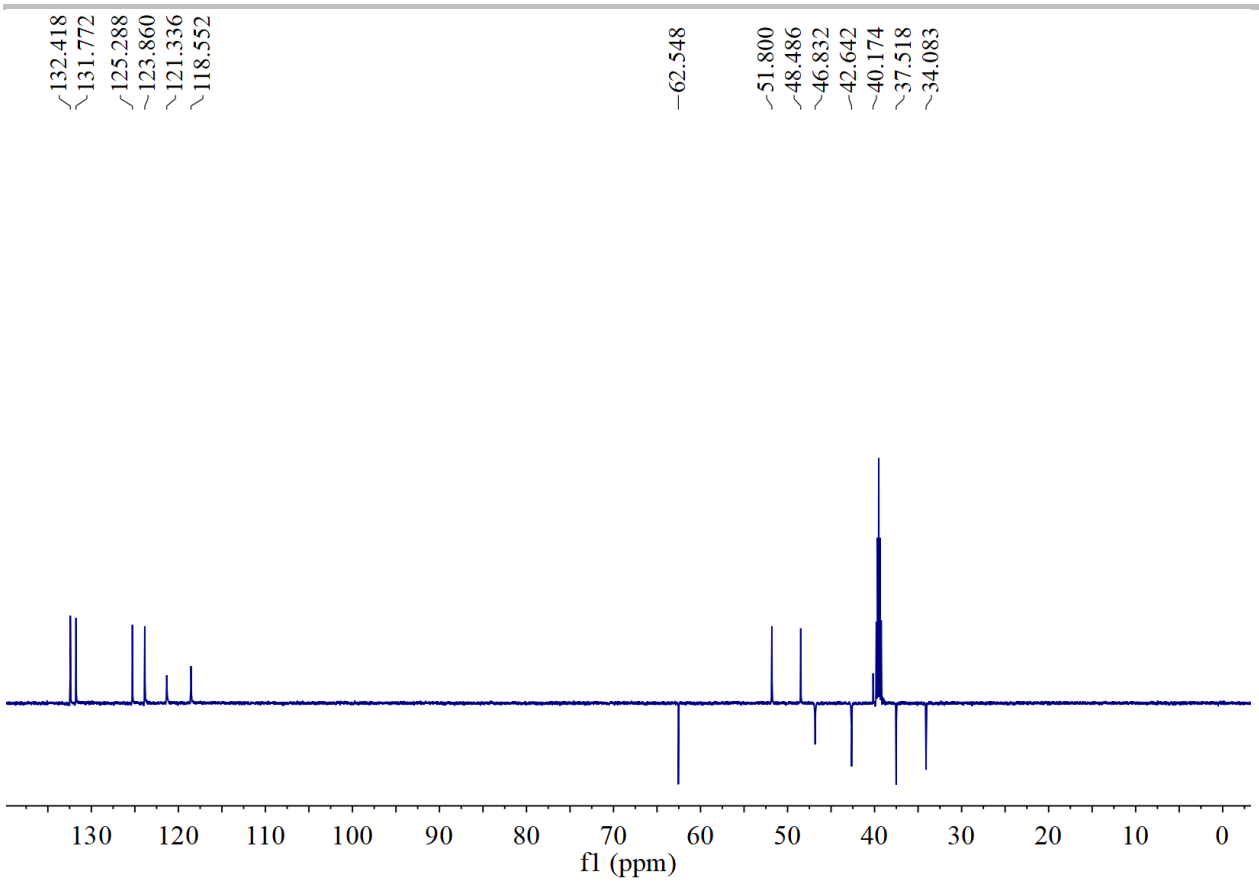


Figure S10. DEPT 135 spectrum of **1** in DMSO-d_6.

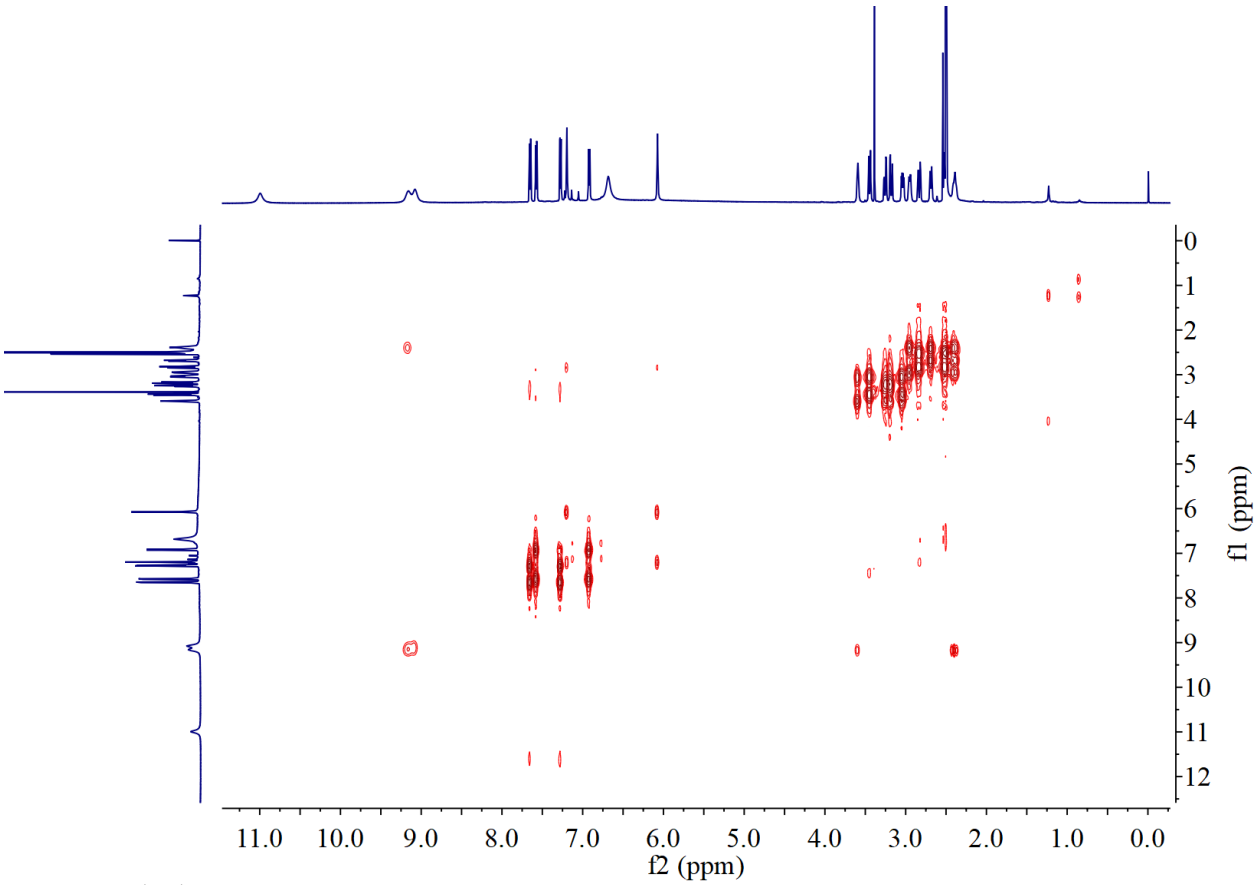


Figure S11. ^{1}H-^{1}H COSY NMR spectrum of **1** in DMSO-d_6.

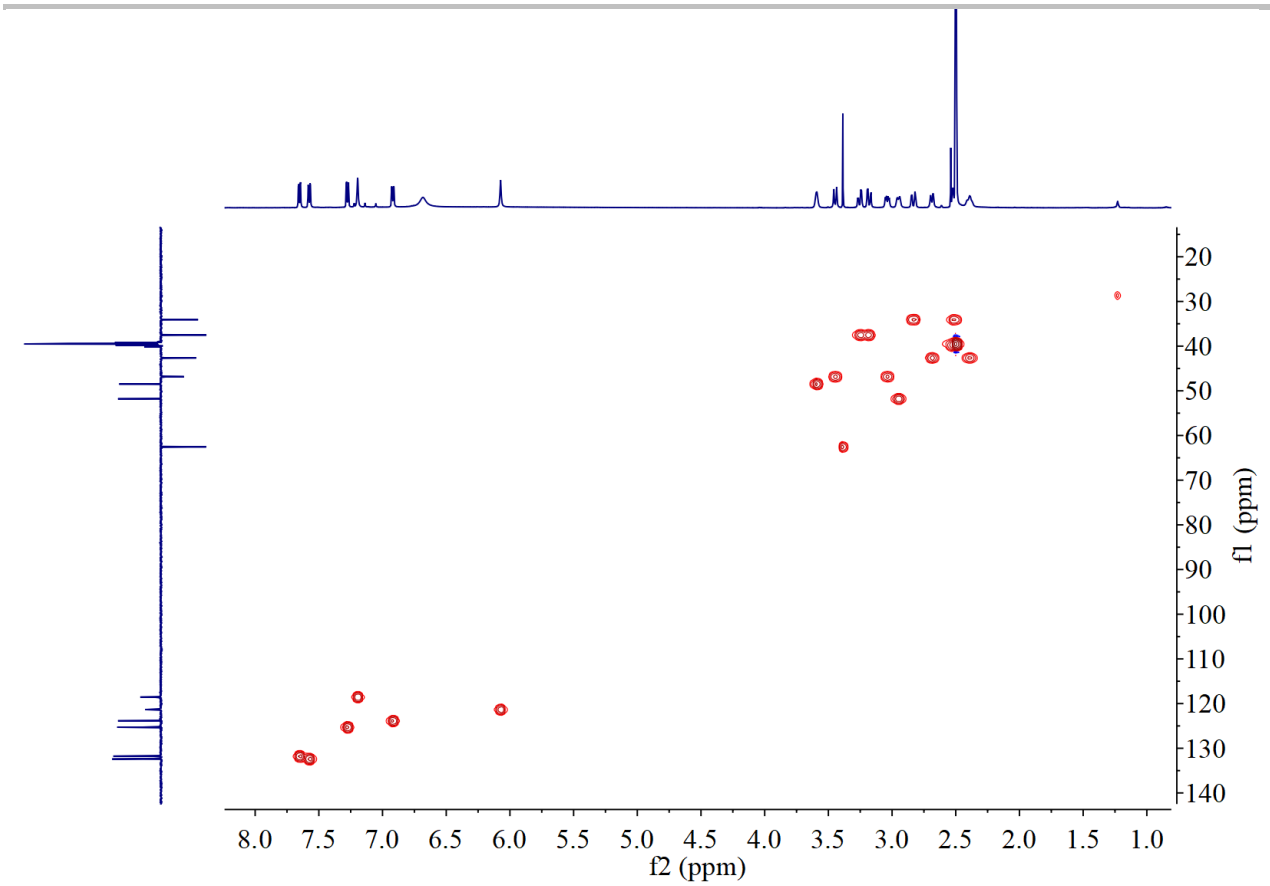


Figure S12. HSQC NMR spectrum of **1** in DMSO-d_6.

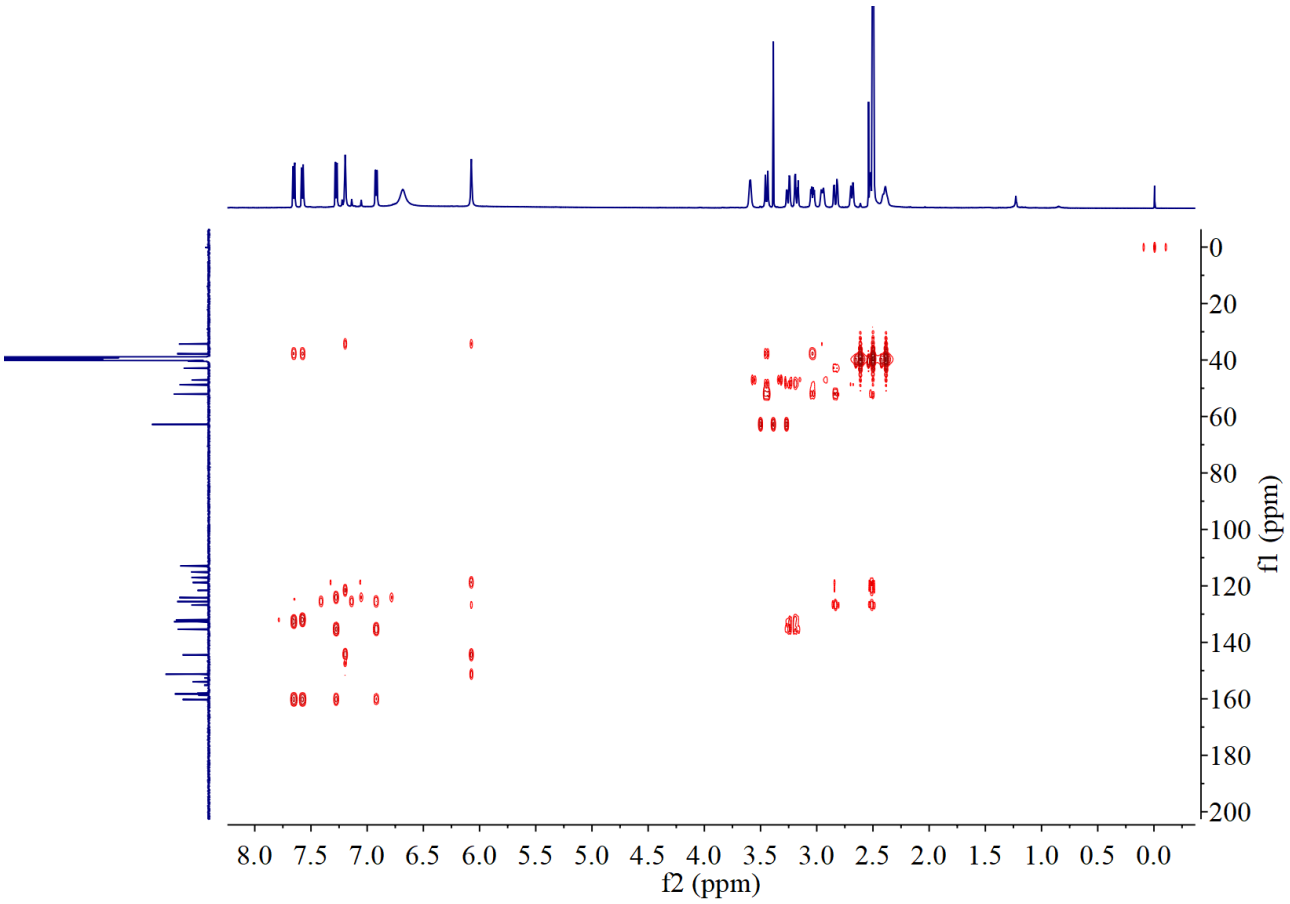


Figure S13. HMBC NMR spectrum of **1** in DMSO-d_6.

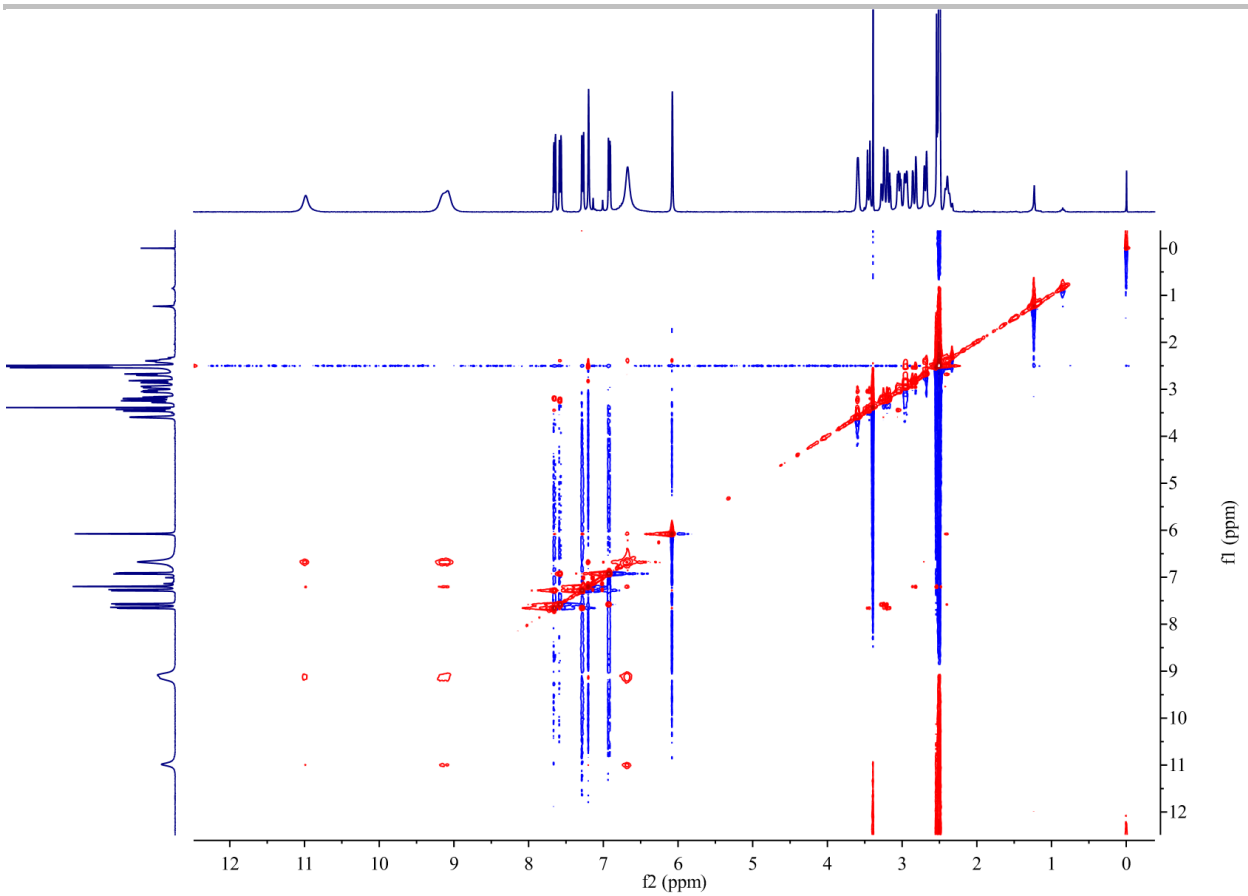


Figure S14. NOESY spectrum of **1** in DMSO-d_6.

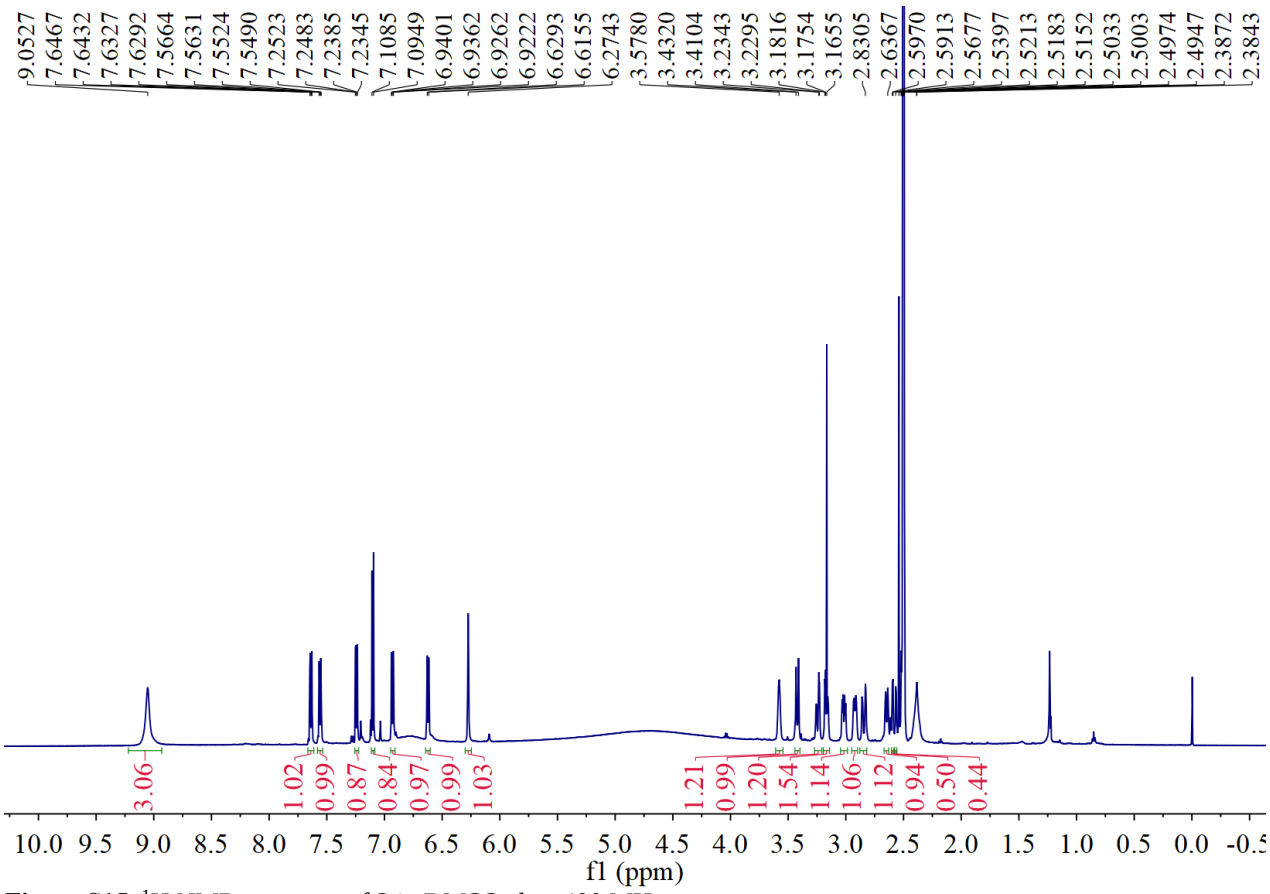


Figure S15. ^{1}H NMR spectrum of **2** in DMSO-d_6 at 400 MHz.

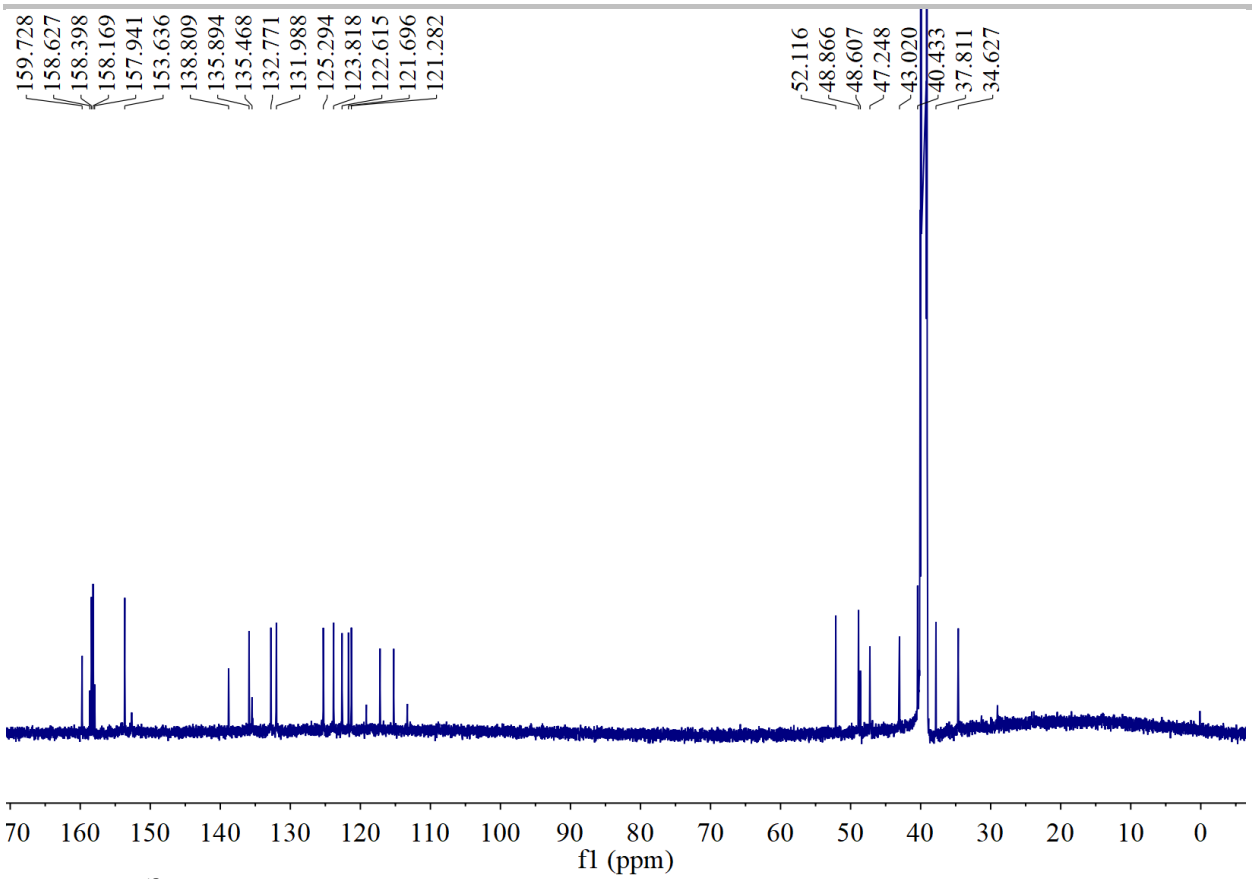


Figure S16. ^{13}C NMR spectrum of **2** in DMSO-d_6 at 100MHz.

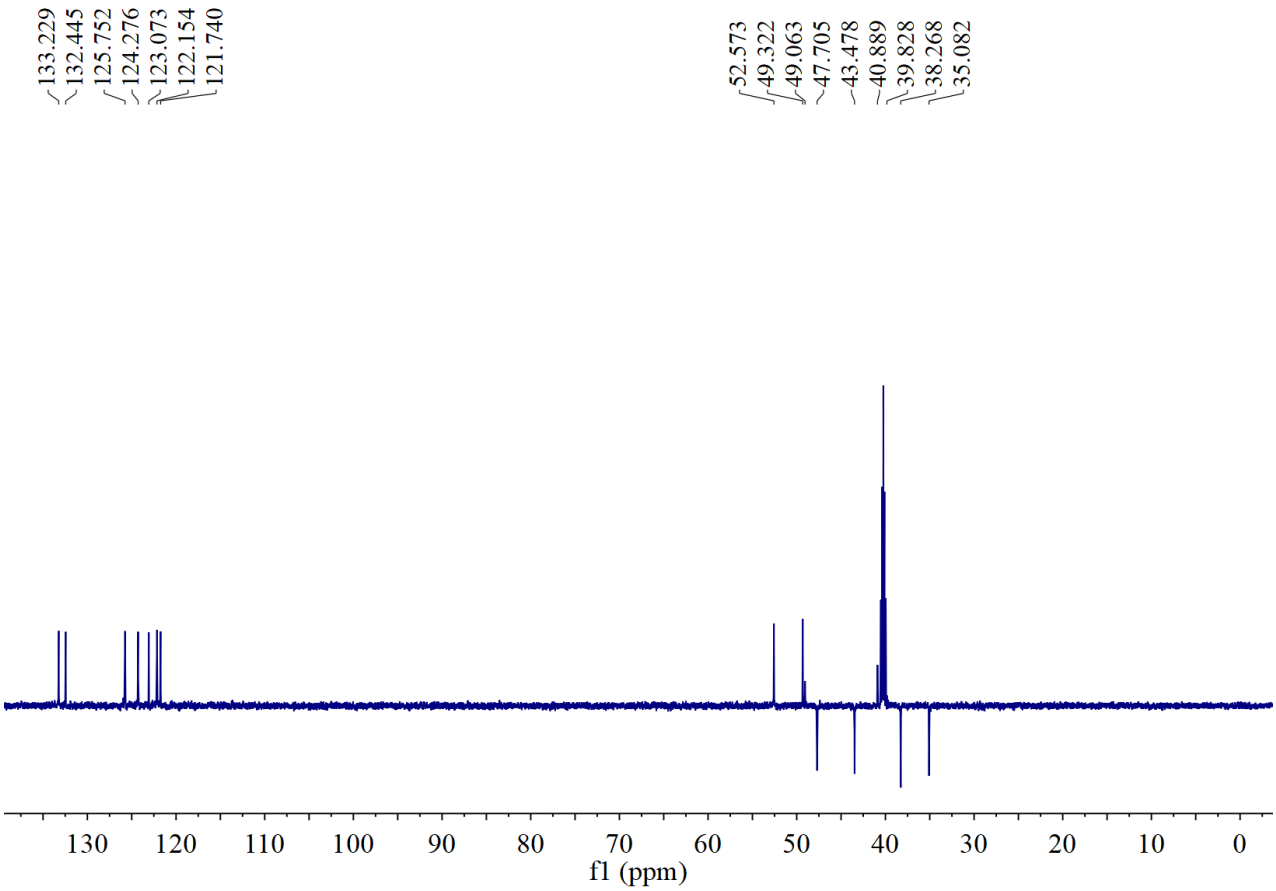


Figure S17. DEPT 135 spectrum of **2** in DMSO-d_6.

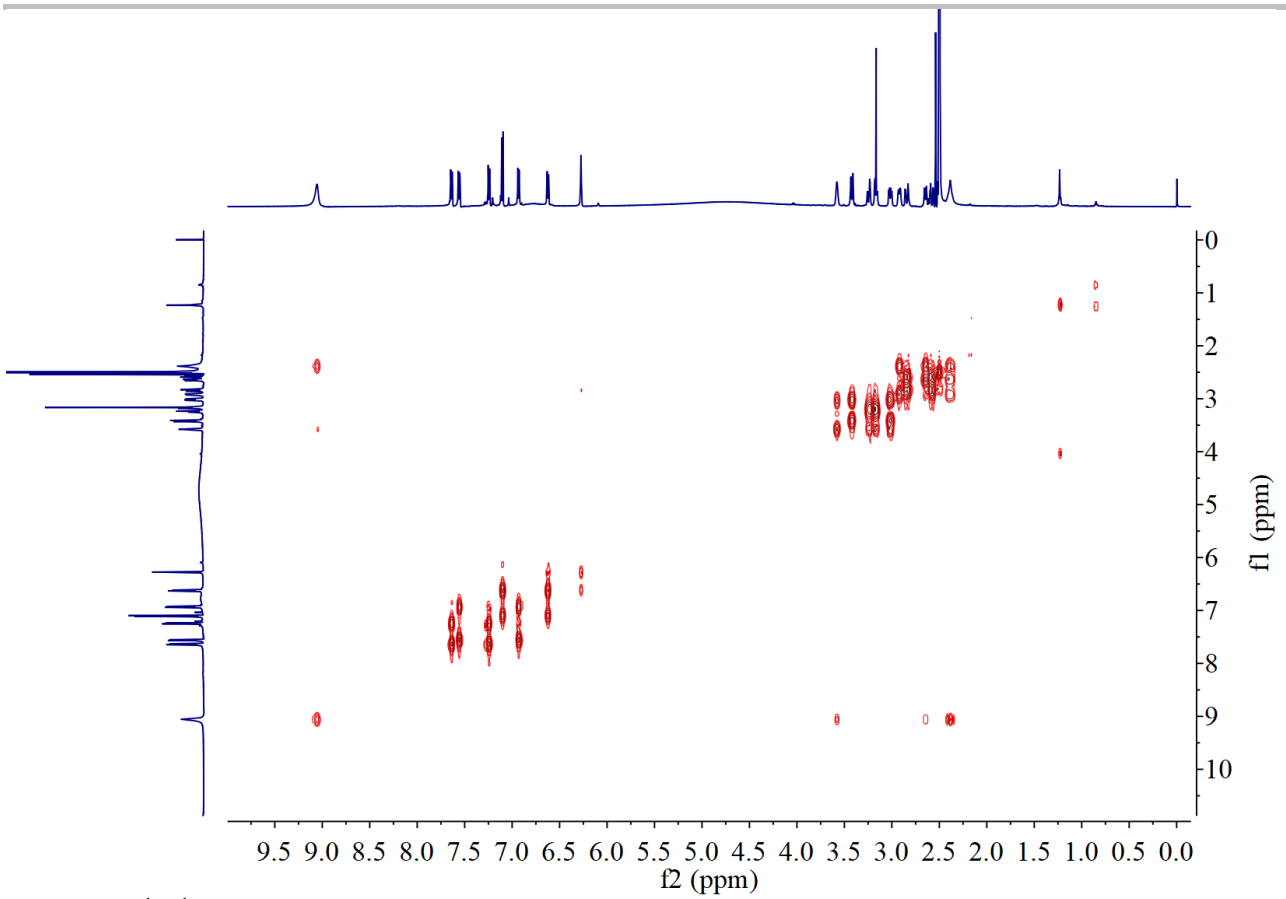


Figure S18. ^{1}H-^{1}H COSY NMR spectrum of **2** in DMSO-d_6.

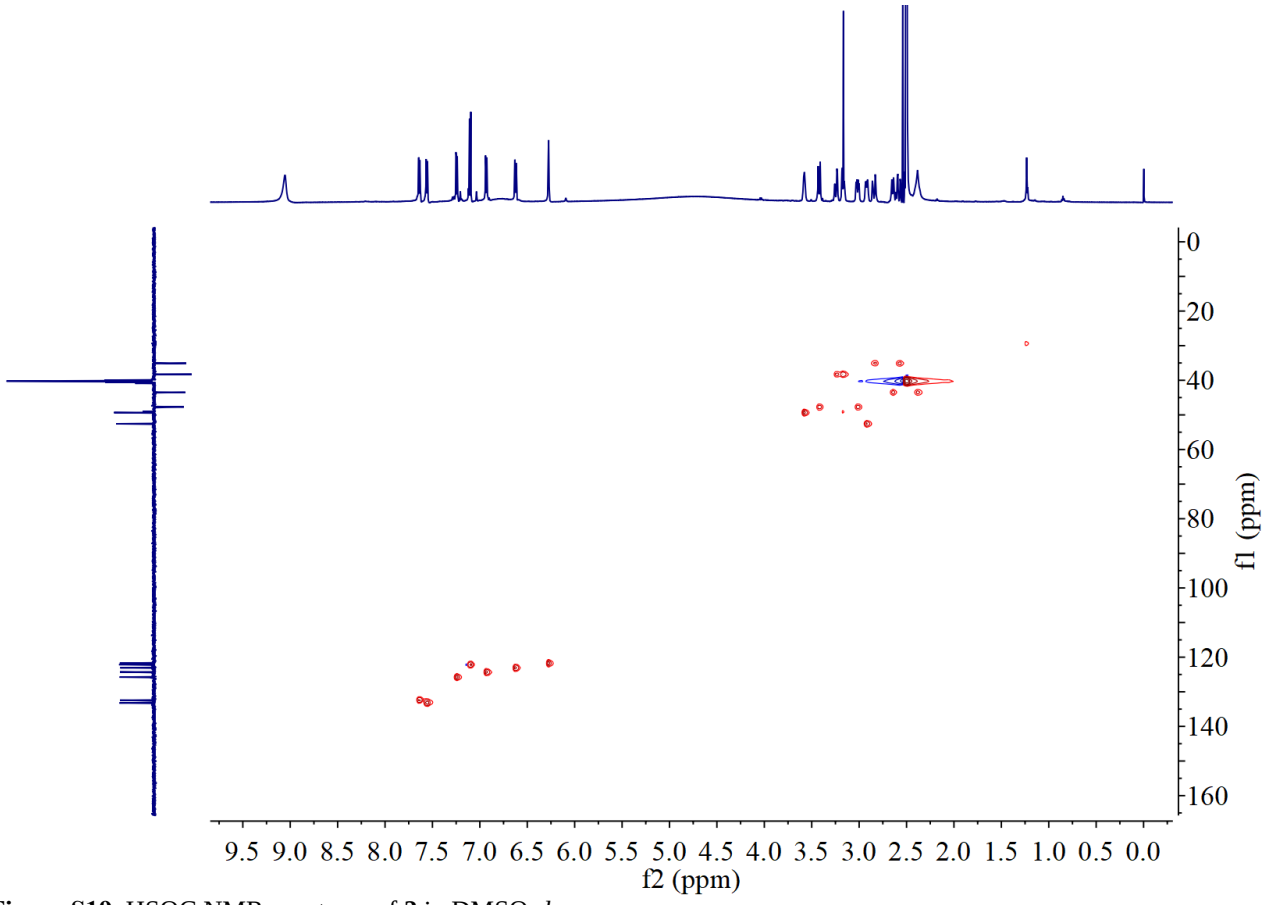


Figure S19. HSQC NMR spectrum of **2** in DMSO-d_6.

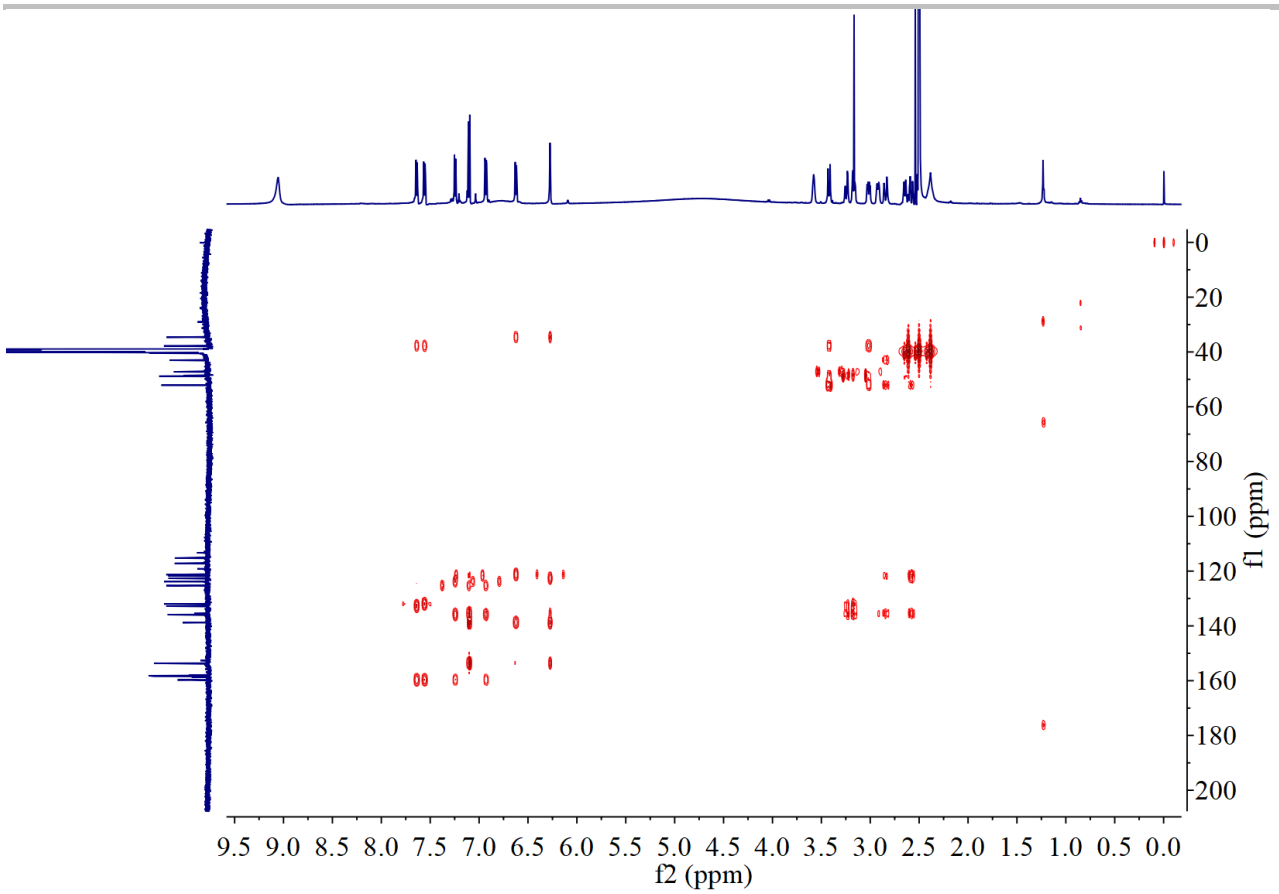


Figure S20. HMBC NMR spectrum of **2** in DMSO-d_6.

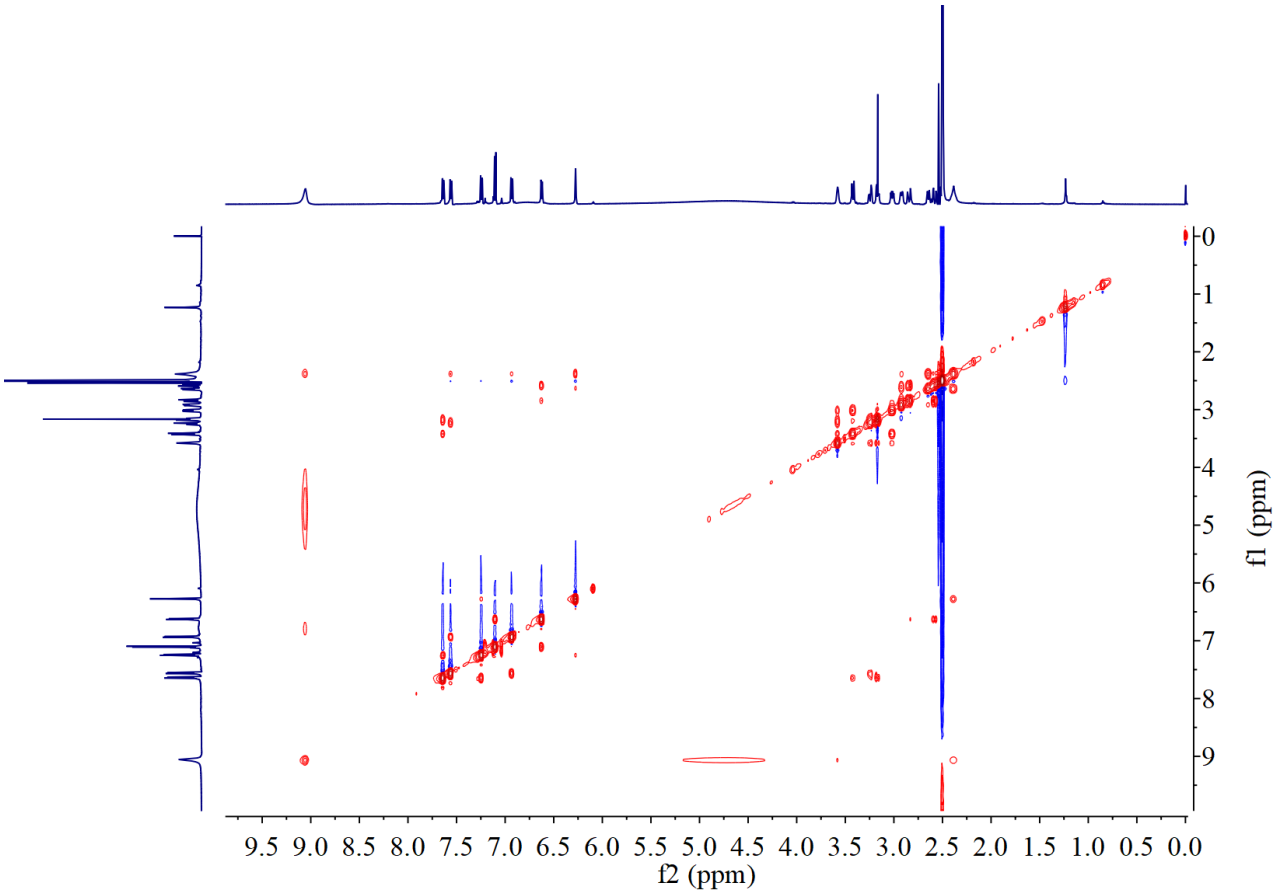


Figure S21. NOESY spectrum of **2** in DMSO-d_6.

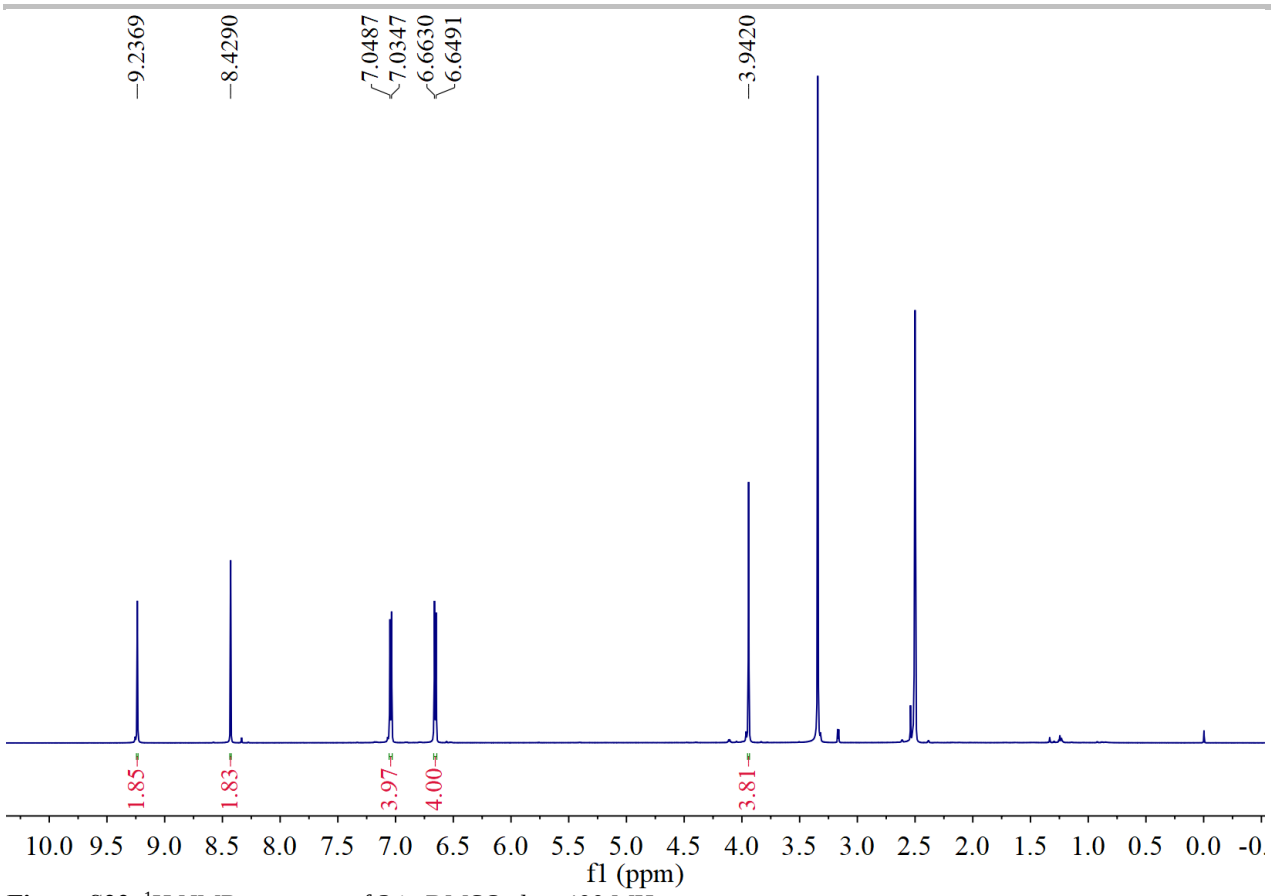


Figure S22. ^{1}H NMR spectrum of **3** in DMSO-d_6 at 400 MHz.

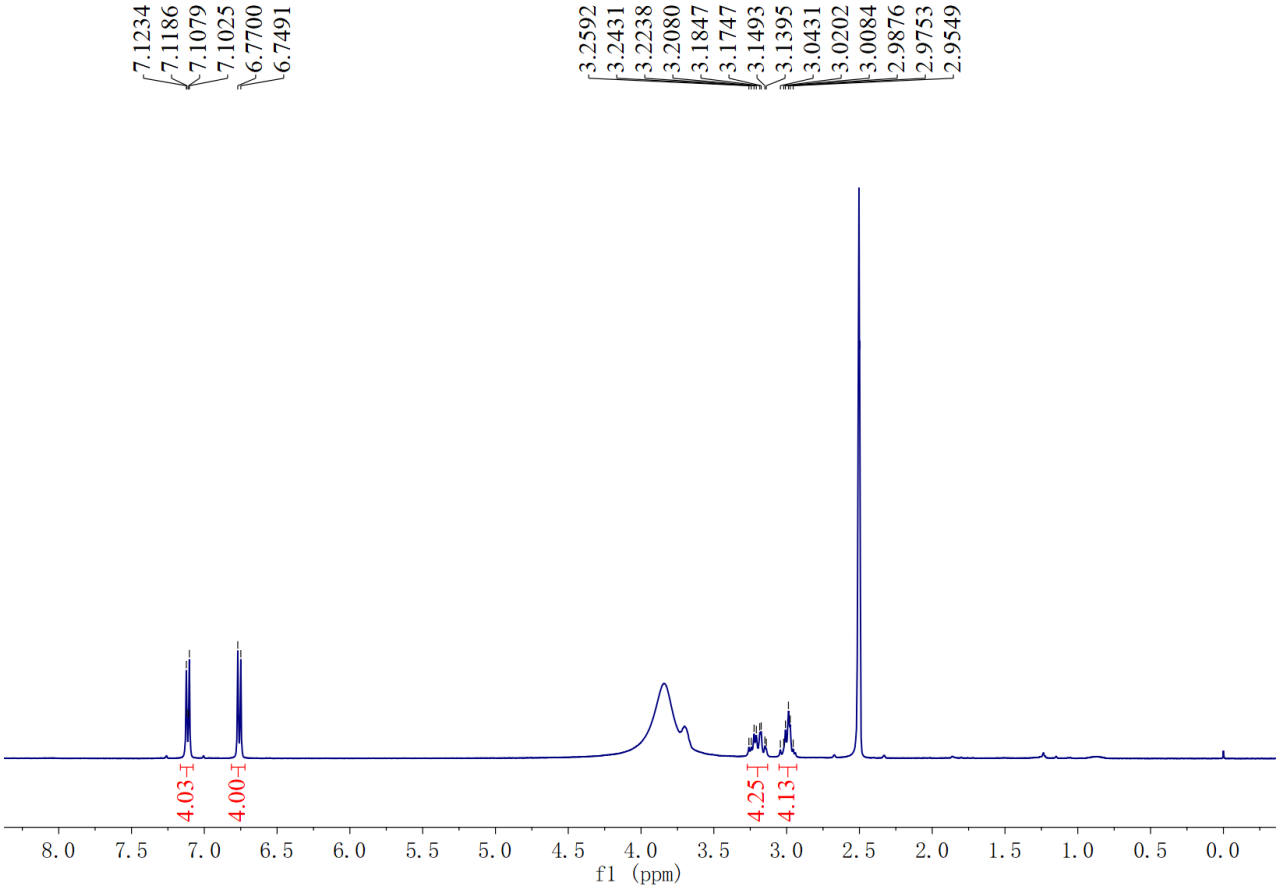


Figure S23. ^{1}H NMR spectrum of **4** in DMSO-d_6 at 400 MHz.

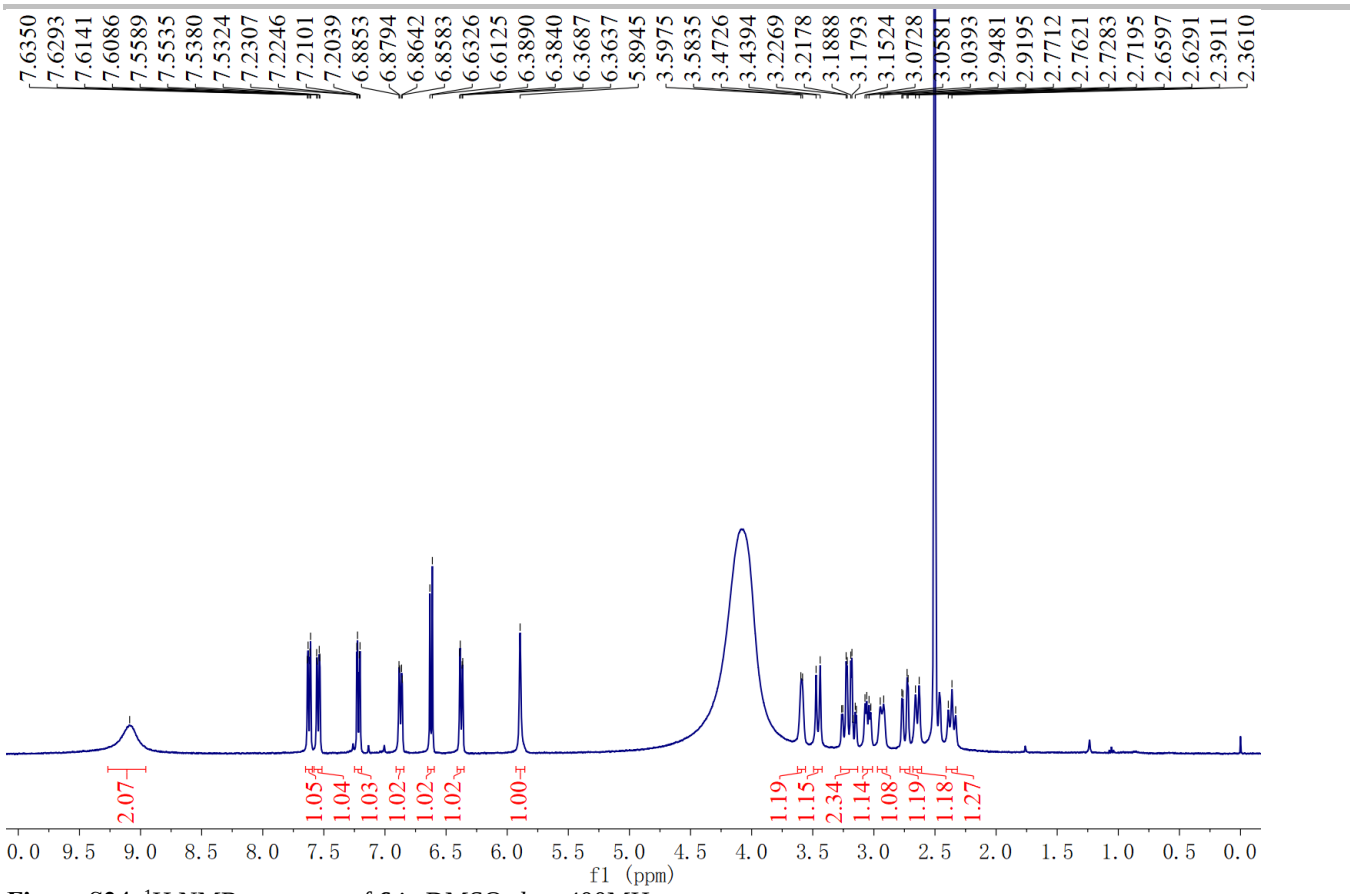


Figure S24. ^{1}H NMR spectrum of **6** in DMSO-d_6 at 400MHz.

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