

Supplementary Materials

Genome mining and metabolic profiling reveal cytotoxic cyclodipeptides in *Streptomyces hygrospinosus* var. *beijingensis*

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Table S1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Streptomyces strains		
<i>S. hygrospinosus</i> 26D9-414	<i>ani</i> and <i>tetr</i> deletion mutant	[18]
<i>S. hygrospinosus</i> Δcdp	<i>Ani</i> , <i>tetr</i> and <i>cdp</i> deletion mutant	This study
E. coli strains		
DH10B	General molecule cloning strain	Gibco BRL
ET12567/pUZ8002	Intergeneric conjugation strain	[38]
Plasmids		
pJTU1278	template plasmid for gene deletion	[39]
pIJ773	template for PCR amplification of <i>Apr</i> gene	[40]
pZDS-1	constructed plasmid for the <i>cdp</i> genes deletion	This study

Table S2. Primers used in this study

Primer	Sequence (5'–3')	Use(s)
cdp-L-P1	GGCCGCTCTAGAACTAGTGGATCCTGATGTTGGCGGCGAAGATGAC	Amplification of <i>cdp</i> fragment for insertion into pJTU1278 by one-step cloning.
cdp-L-P2	GAAGCAGCTCCAGCCTACACGATTGACCTGCGGCTGGATTC	
cdp-apr-P1	GAATCCAGCCGCGAGGTCAATCGTGTAGGCTGGAGCTGCTTC	Amplification of <i>Apr</i> cassette from pIJ773
cdp-apr-P2	TGTTGTGGCACCGCGACAAATTCGCGGGATCCGTCGACC	
cdp-R-P1	GGTCGACGGATCCCCGGAATTTGTCGCGGTGCCACAACA	Amplification of <i>cdp</i> fragment for insertion into pJTU1278 by one-step cloning.
cdp-R-P2	GTATCGATAAGCTTGATATCGAATTCAGCAGAAGATGAGCCCGAGTTC	
apr-P1	CAGCGTCGTGTTGGCATCGT	Verification of Δcdp mutant strain
apr-P2	ATCAGCGGTGGAGTGCAATGTC	
cdp1-P1	GGTCCGAGATCGTCAAGAGCAT	Verification of Δcdp mutant strain
cdp1-P2	GCAGCAGCATGGTGAGGTTCT	

Table S3. Cytotoxicity assay of compounds 1-5 (IC₅₀, μ M)

Compound	A549	HL60	SMMC-7721	SW480	MDA-MB-231
1	>40	>40	>40	>40	>40
2	>40	>40	>40	13.42 \pm 0.78	18.26 \pm 1.56
3	>40	>40	>40	>40	>40
4	>40	>40	>40	>40	>40
5	>40	>40	>40	>40	>40
cisplatin	-	-	-	14.99 \pm 0.09	14.03 \pm 0.75

^aHuman lung carcinoma (A549), human leukemia cell (HL60), human hepatocellular carcinoma (SMMC-7721), human colon cancer cell (SW480) and human breast carcinoma

(MDA-MB-231 were tested for cytotoxicity of CDPs and cisplatin was used as positive control.

Table S4. Antibacterial activity assay of compounds **1-9**.

Compound	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>B. subtilis</i>	<i>X.albilineans</i>	<i>C. albicans</i>	<i>C. sake</i>
1	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
2	>1.0	>1.0	>1.0	>1.0	0.25	1.0	1.0
3	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
4	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
5	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
6	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
7	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
8	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
9	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0	>1.0
apramycin	0.025	0.025	0.013	0.006	0.006	-	-
nystatin	-	-	-	-	-	0.004	0.002

Apramycin and nystatin as positive control.

Table S5. Ten selected mediums in this study.

Name	Composition
PYJ1	Glucose 40 g, glycerol 10 g, soluble starch 30 g, soytone 15 g, beef extract 10 g, peptone 6.5 g, yeast extract 0.5 g, MgSO ₄ 1 g, NaCl 2g, CaCO ₃ 2,4 g in 1.0 L of distilled H ₂ O. [41]
PYJ2	Sucrose 100 g, glucose 10 g, casamino acids 0.1 g, yeast extract 5 g, MOPS 21 g, K ₂ SO ₄ 0.25 g, MgCl ₂ ·6H ₂ O 10 g, FeSO ₄ ·7H ₂ O 0.001 g, MnCl ₂ ·7H ₂ O 0.001 g, ZnSO ₄ ·7H ₂ O 0.001 g in 1.0 L of distilled H ₂ O, pH 7.0. [42]
PYJ3	Soluble starch 15g, soy peptone 15 g, soybean meal 5 g, glycerol 15 g, CaCO ₃ 2 g, sea salt 30 g in 1.0 L of distilled H ₂ O. [43]
PYJ4	Glycerol 10 g, peptone 8.6 g, soybean meal 40 g, cottonseed meal 20 g, mannitol 97.3 g, ornithine 5.3 g, proline 5 g, CaCl ₂ 5 g, K ₂ HPO ₄ 8.4 g, MgSO ₄ ·7H ₂ O 5g, CuSO ₄ ·5H ₂ O 0.6 g, MnSO ₄ ·H ₂ O 0.1 g, FeSO ₄ ·7H ₂ O 0.05 g in 1.0 L of distilled H ₂ O. [44]
PYJ5	Corn starch 24 g, glucose 10 g, peptone 3 g, beef extract 3 g, yeast extract 5 g, CaCO ₃ 4 g in 1.0 L of distilled H ₂ O, pH 7.0. [45]
PYJ6	glucose 10 g, Dextrin 25 g, oat meal 20 g, cottonseed meal 10 g, fish meal 5 g, yeast extract 2 g, CaCO ₃ 3 g in 1.0 L of distilled H ₂ O, pH 7.2. [46]
PYJ7	Soluble starch 30g, glucose 15 g, glycerol 5 g, NaNO ₃ 5 g, NH ₄ Cl 5 g, K ₂ HPO ₄ 1 g, KH ₂ PO ₄ 2 g, MnSO ₄ ·7H ₂ O 2 g, CaCO ₃ 2 g in 1.0 L of distilled H ₂ O. (This research)
PYJ8	Dextrin 40 g, molasses 5 g, glucose 10 g, casamino acid 10 g, MgSO ₄ ·7H ₂ O 1 g, CaCO ₃ 2 g in 1.0 L of distilled H ₂ O, pH 7.5. (This research)
PYJ9	Soluble starch 10 g, peptone 10 g, glycerol 10 g, mannitol 5 g, (NH ₄) ₂ HPO ₄ 1 g in 1.0 L of distilled H ₂ O, pH 7.0. (This research)
PYJ10	glucose 25 g, soya flour 18.8 g, molasses 3.75 g, NZ Amine type A 1.25 g, CH ₃ COONa 8 g, CaCO ₃ 3 g in 1.0 L of distilled H ₂ O, pH 7.0. (This research)

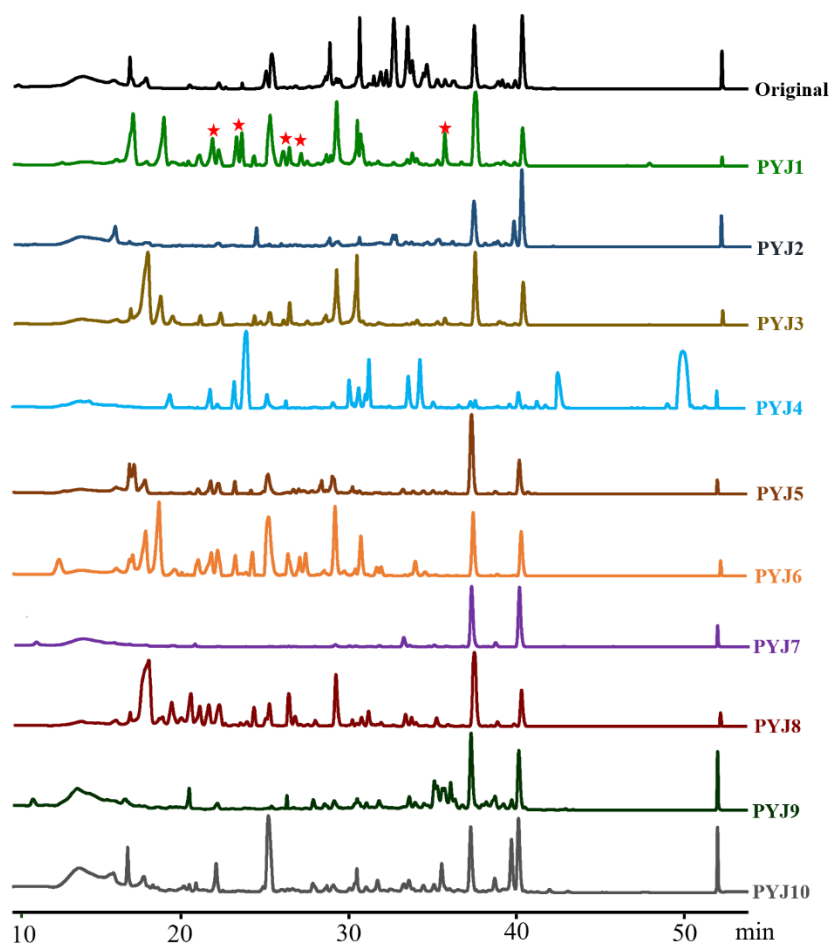


Figure S1. HPLC analysis of fermentation products of *S. hygrospinosus* 26D9-414 from eleven different media.

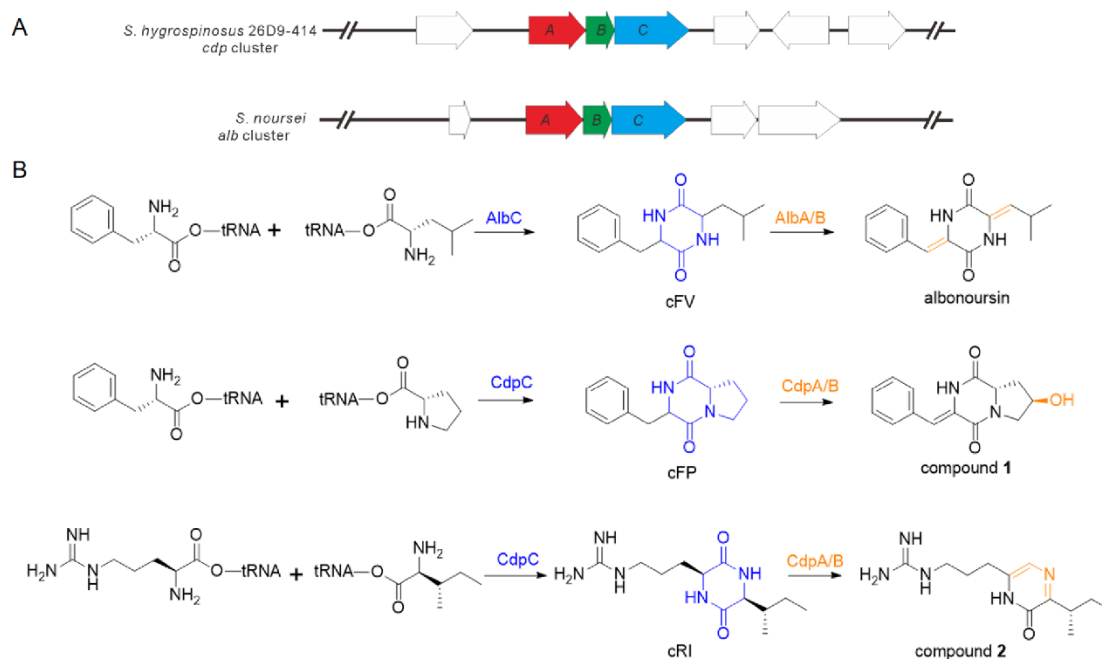


Figure S2. Biosynthetic analysis of CDPs. (A) Comparative analysis of *cdp* cluster with the reported *alb* cluster. Proteins encoded by *alb* cluster for albonoursin biosynthesis: AlbA, cyclic dipeptide synthase (CDPS), AlbB and AlbC, cyclic dipeptide oxidase (CDO) [21]. (B) biosynthetic machineries for CDPs (compounds **1** and **2**) in this study.

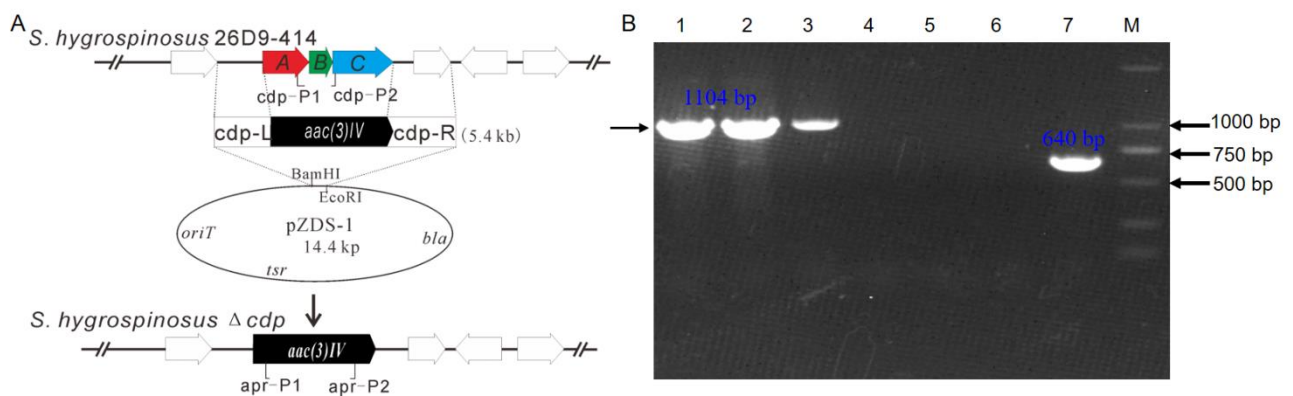


Figure S3. Schematic construction and verification of *S. hygrospinosus* Δcdp mutant. **(A)** Schematic representation of *cdpA-C* fragment replaced by *aac(3)IV* apramycin resistance gene, and then the mutated shuttle plasmid pZDS-1 was introduced into *S. hygrospinosus* 26D9-414 by intergeneric conjugation. **(B)** PCR verification of *S. hygrospinosus* Δcdp mutants using primers apr-L/R and cdp-L/R. Lane 1 and 2, genome of two clone of *S. hygrospinosus* Δcdp mutant were used as template using primers apr-L/R; lane 3, pIJ773 was used as template for positive control; lane 4, genome of *S. hygrospinosus* 26D9-414 used as template for negative control; lane 5 and 6, genome of two clone of *S. hygrospinosus* Δcdp mutant were used as template using primers cdp-L/R; lane 7, genome of *S. hygrospinosus* 26D9-414 used as template for negative control; M, LD DSTM 2000 kb DNA ladder.

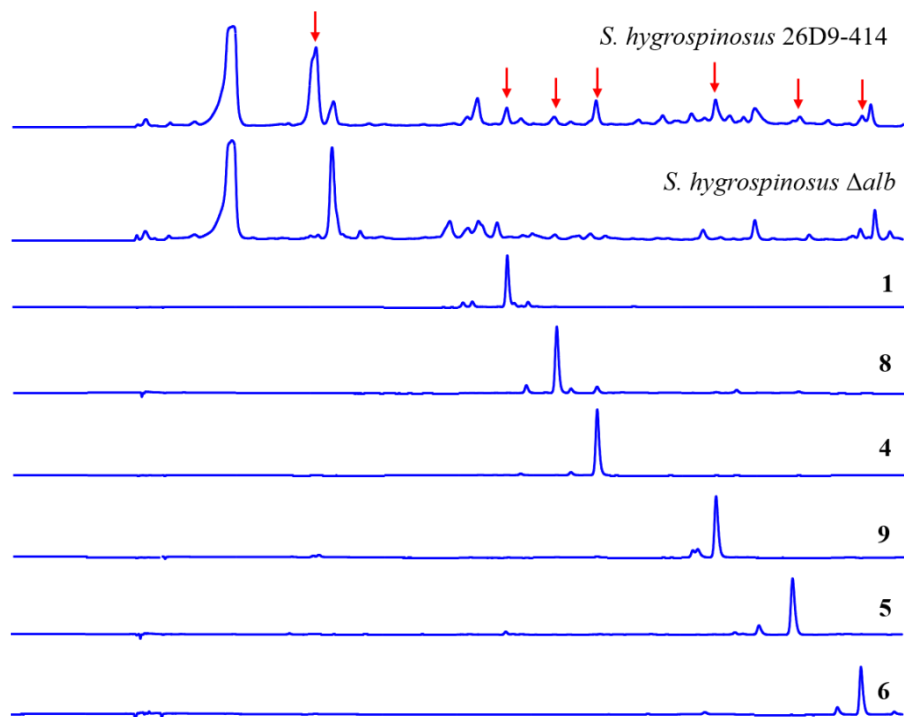
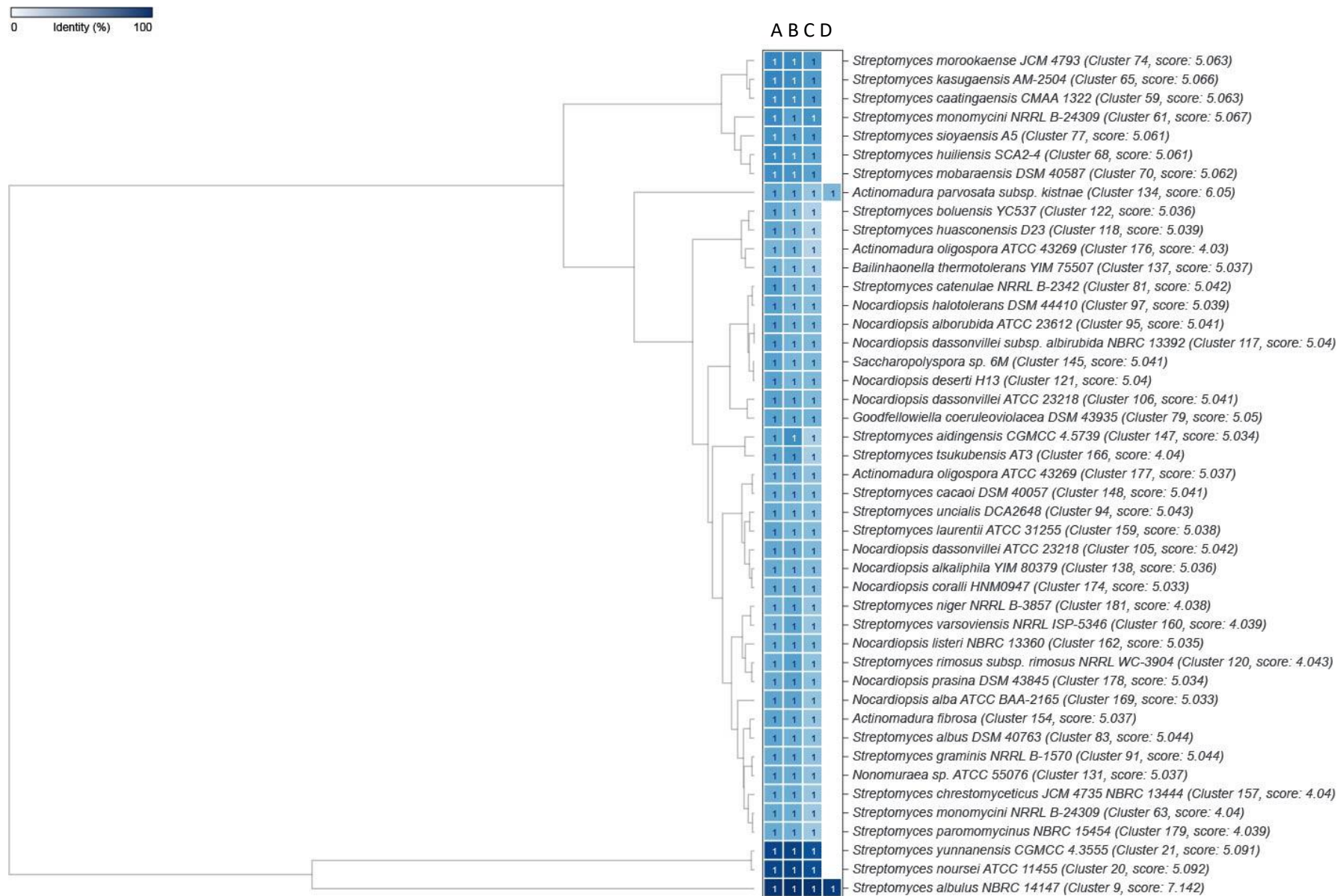


Figure S4. HPLC analysis of metabolites in *S. hygrospinosus* 26D9-414 and in *S. hygrospinosus* Δcdp mutant.



Figures S5. Distribution statistics of putative *cdp* gene analogues.

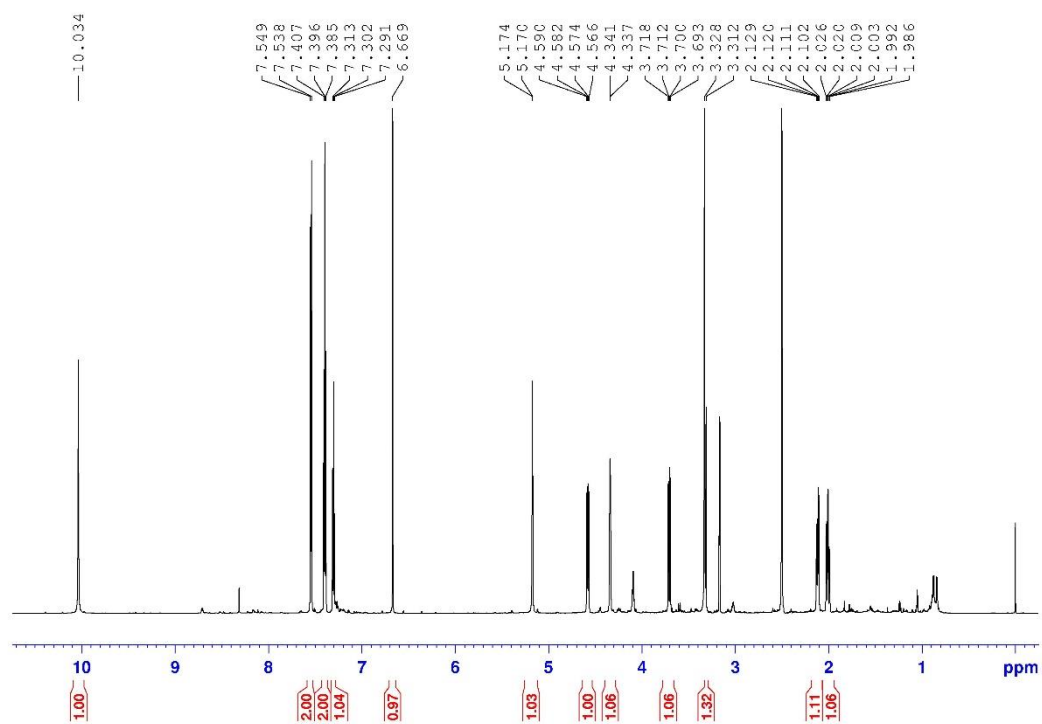


Figure S6. ¹H NMR spectrum of **1** recorded in DMSO-*d*₆ (600 MHz)

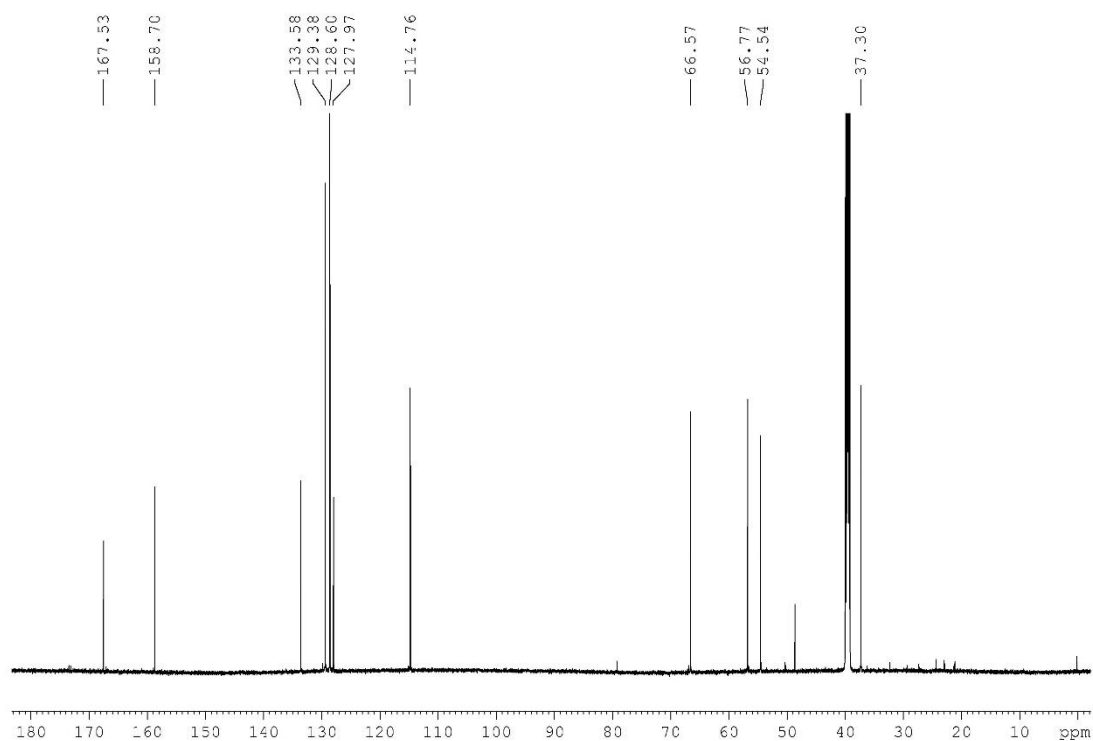


Figure S7. ¹³C NMR spectrum of **1** recorded in DMSO-*d*₆ (150 MHz)

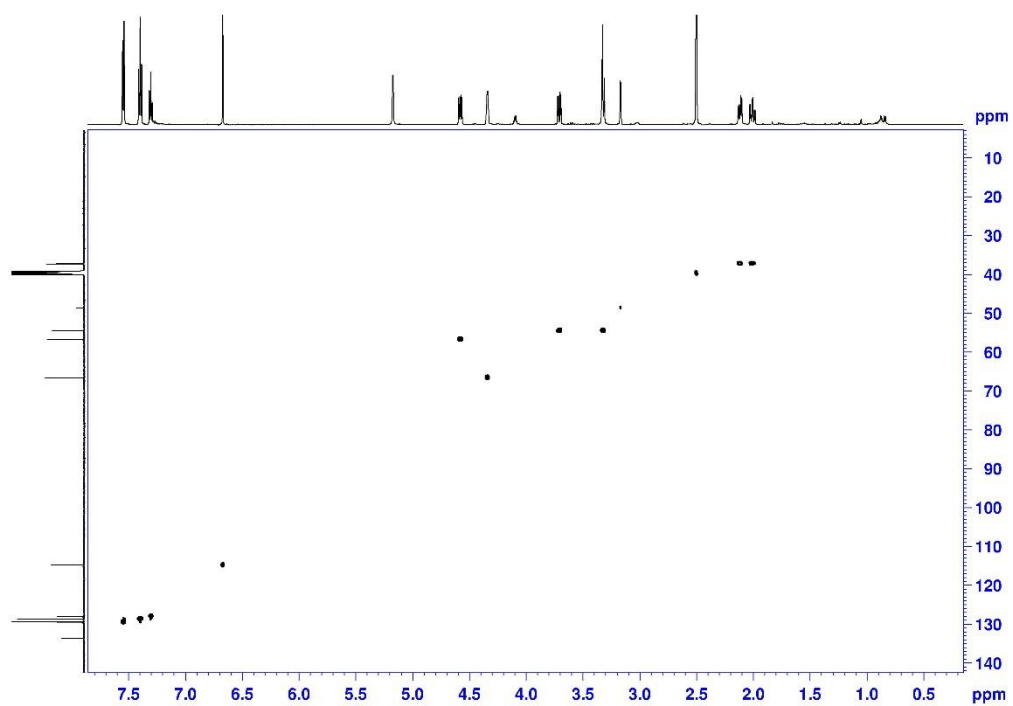


Figure S8. HSQC spectrum of **1** recorded in DMSO- d_6 (600 MHz)

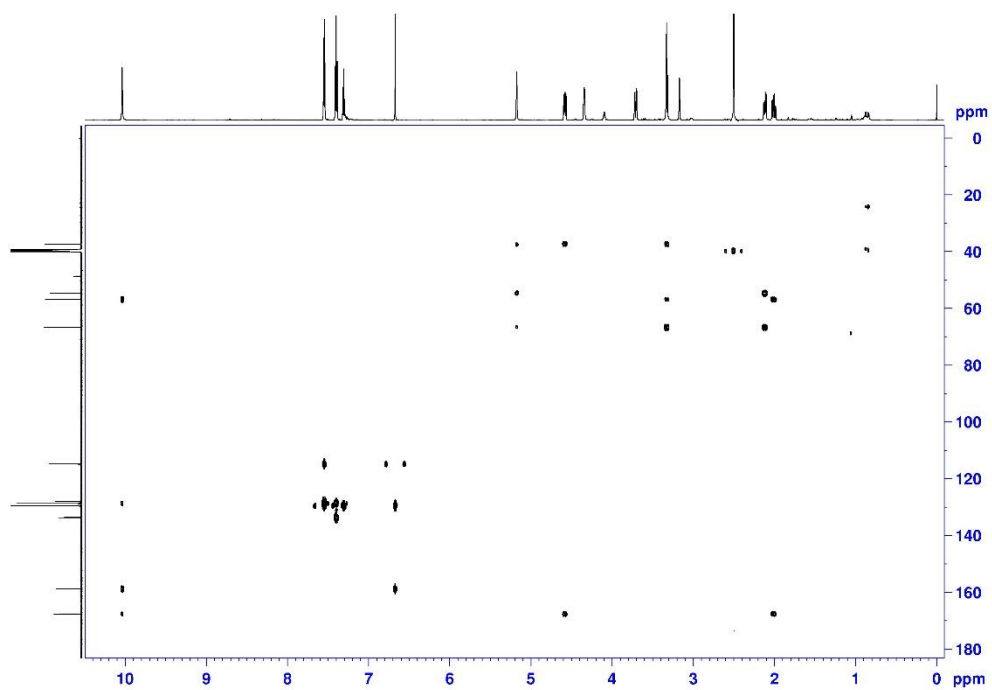


Figure S9. HMBC spectrum of **1** recorded in DMSO- d_6 (600 MHz)

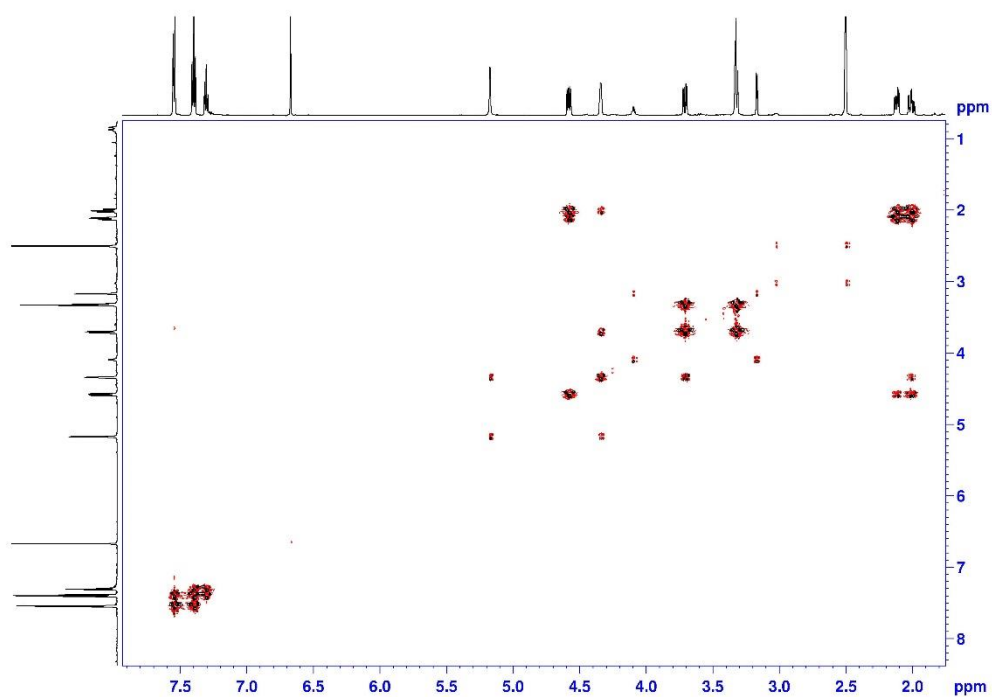


Figure S10. ^1H - ^1H COSY spectrum of **1** recorded in $\text{DMSO-}d_6$ (600 MHz)

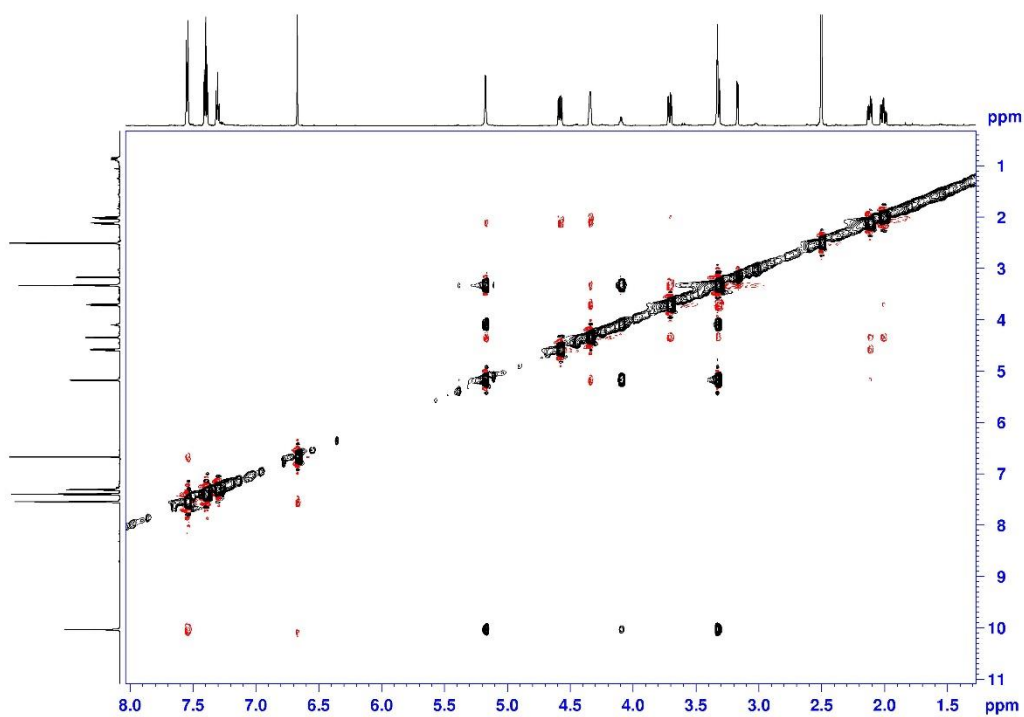


Figure S11. ^1H - ^1H NOESY spectrum of **1** recorded in $\text{DMSO-}d_6$ (600 MHz)

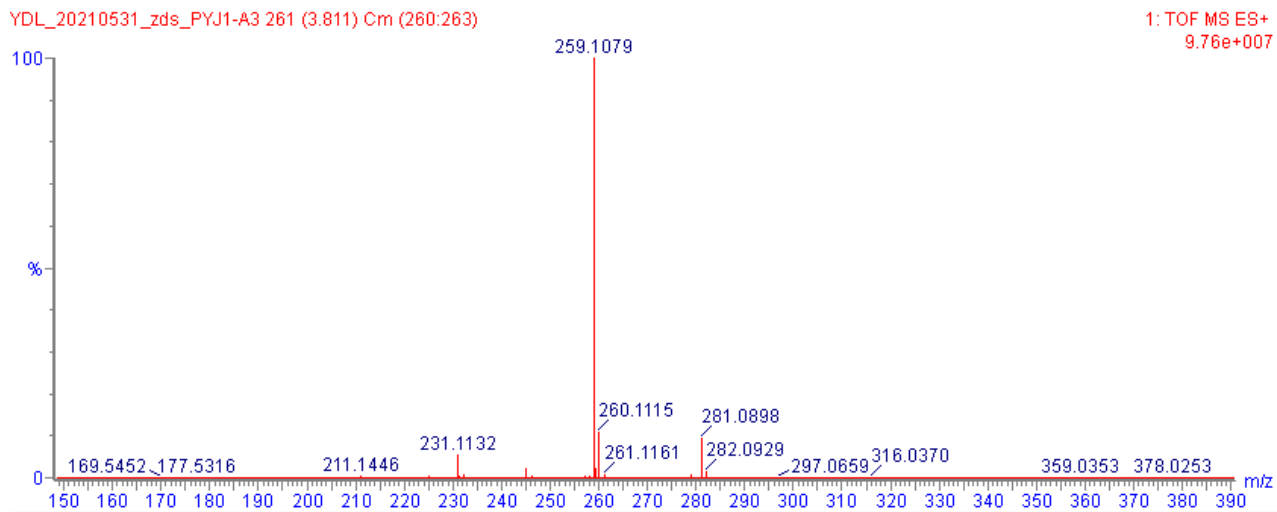


Figure S12. HR-ESI-MS spectra of **1**

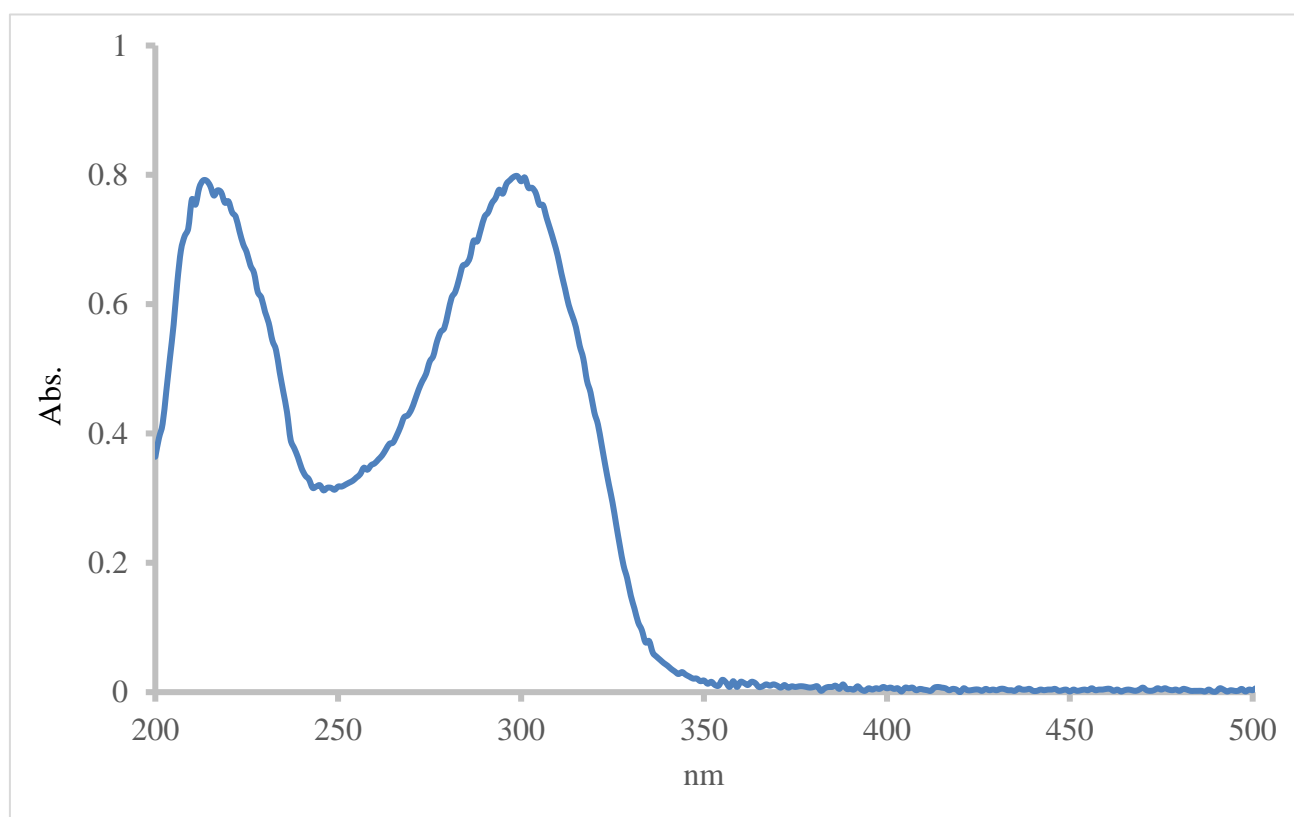


Figure S13. UV spectrum of **1**

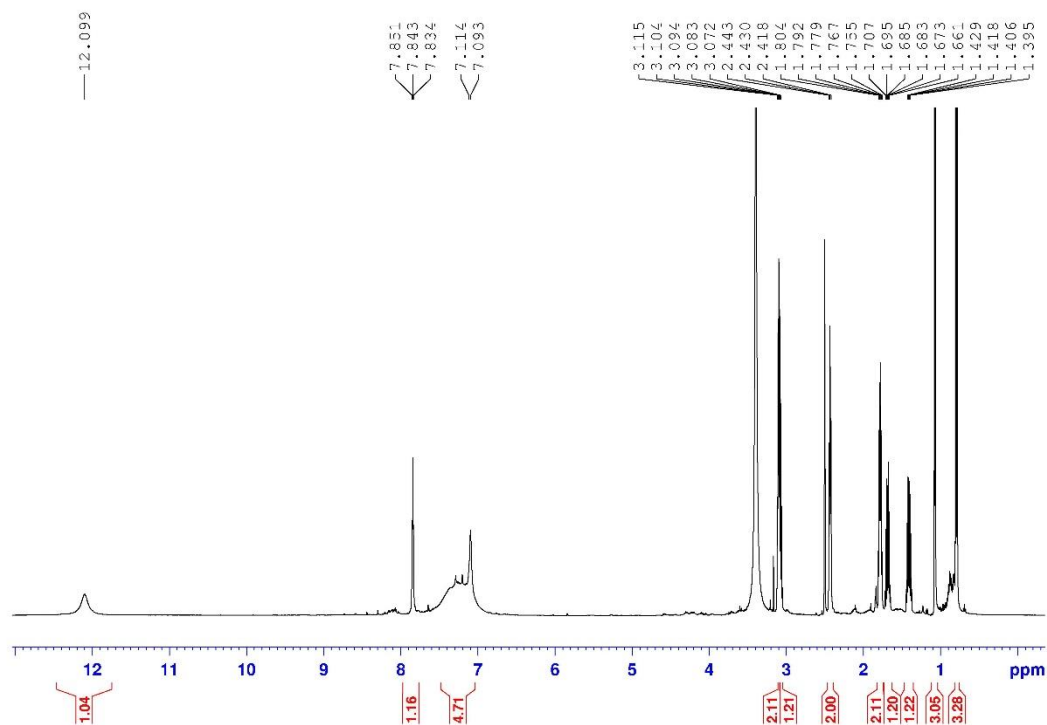


Figure S14. ¹H NMR spectrum of **2** recorded in DMSO-*d*₆ (600 MHz)

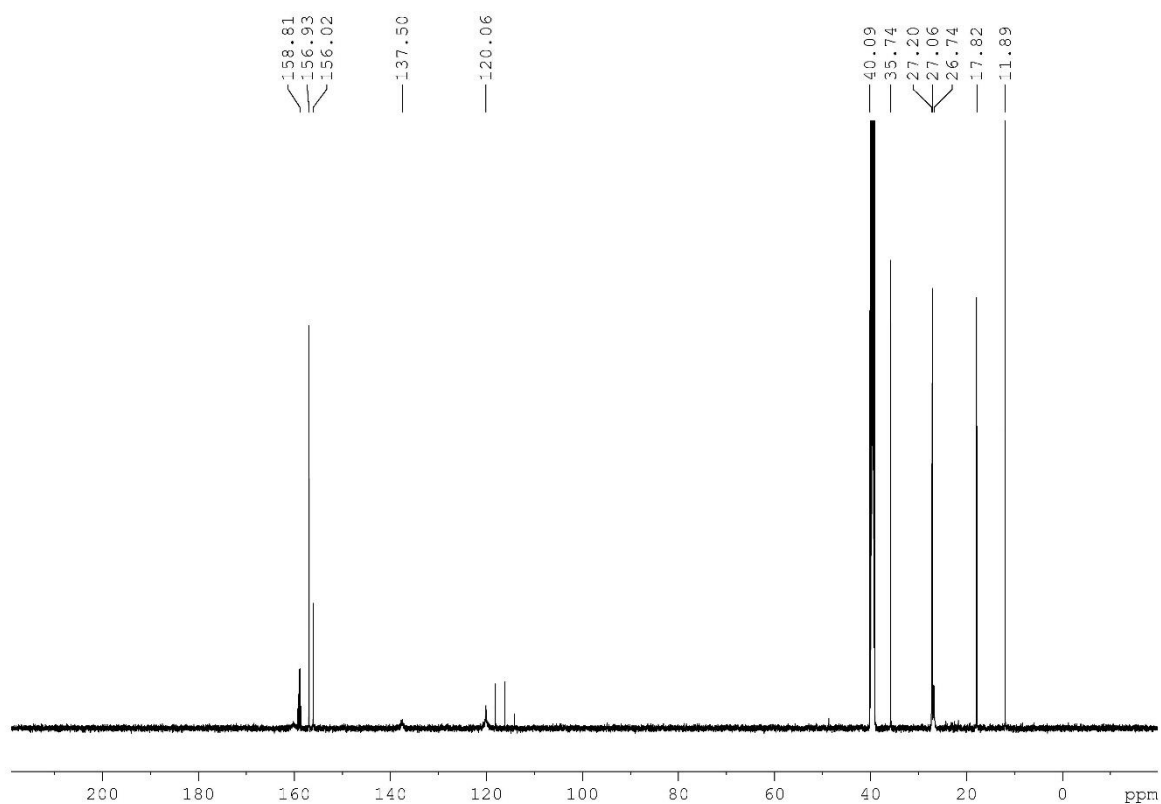


Figure S15. ¹³C NMR spectrum of **2** recorded in DMSO-*d*₆ (150 MHz)

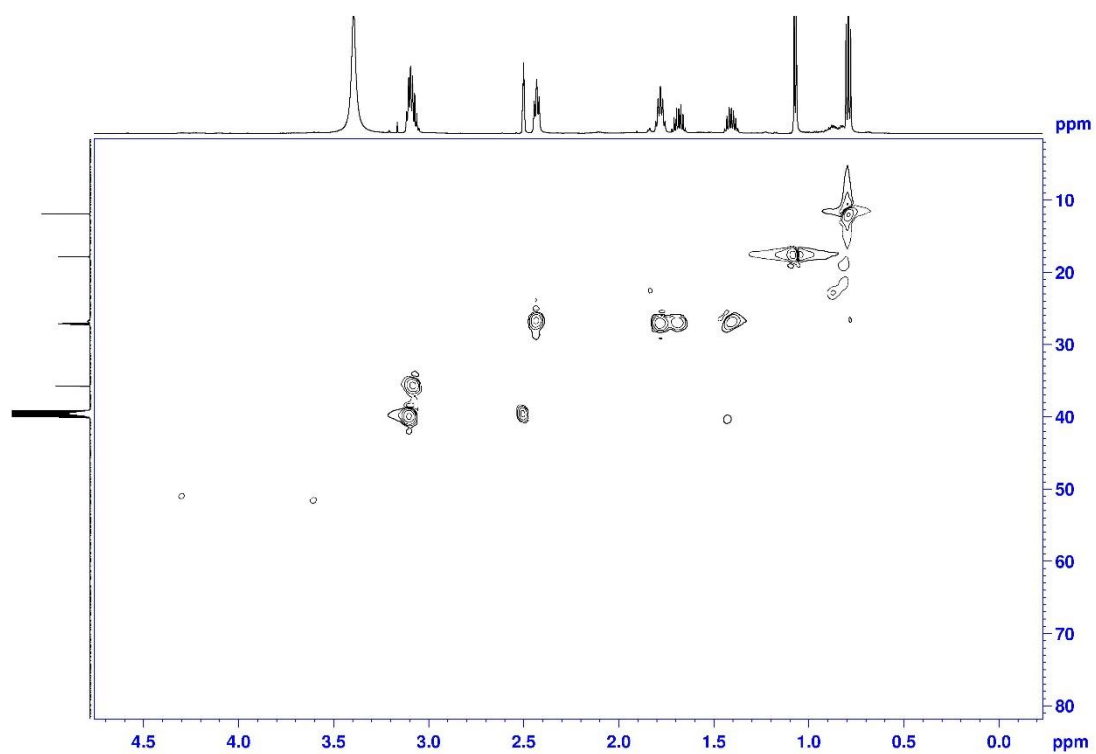


Figure S16. HSQC spectrum of **2** recorded in DMSO- d_6 (600 MHz)

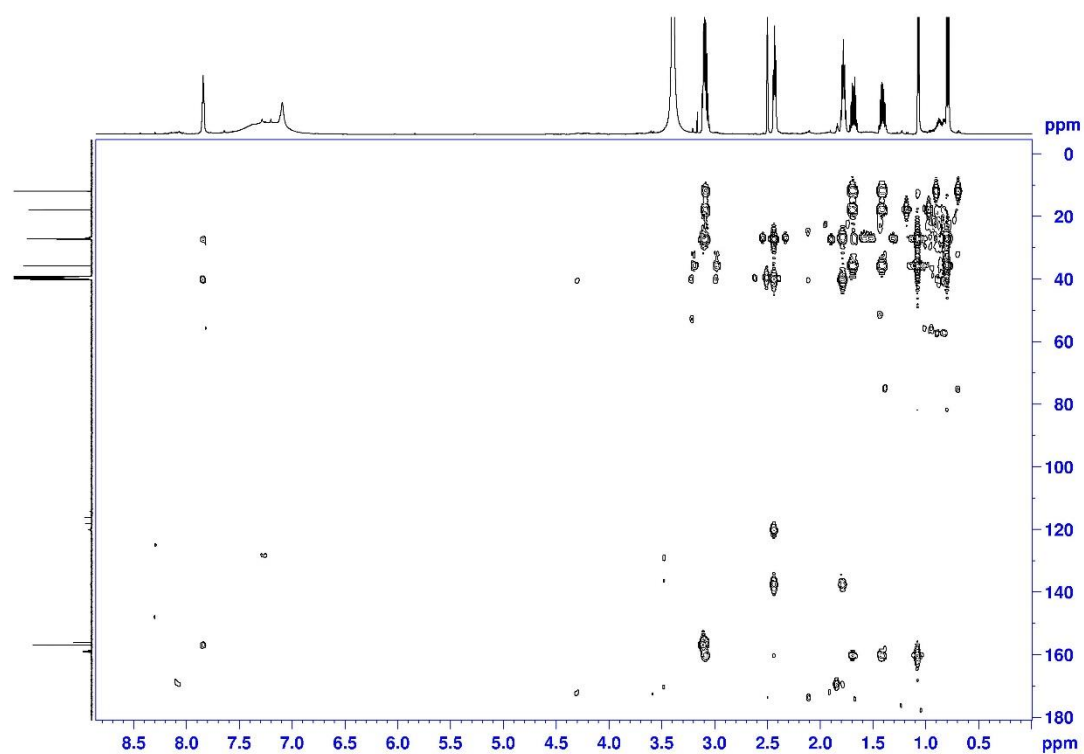


Figure S17. HMBC spectrum of **2** recorded in DMSO- d_6 (600 MHz)

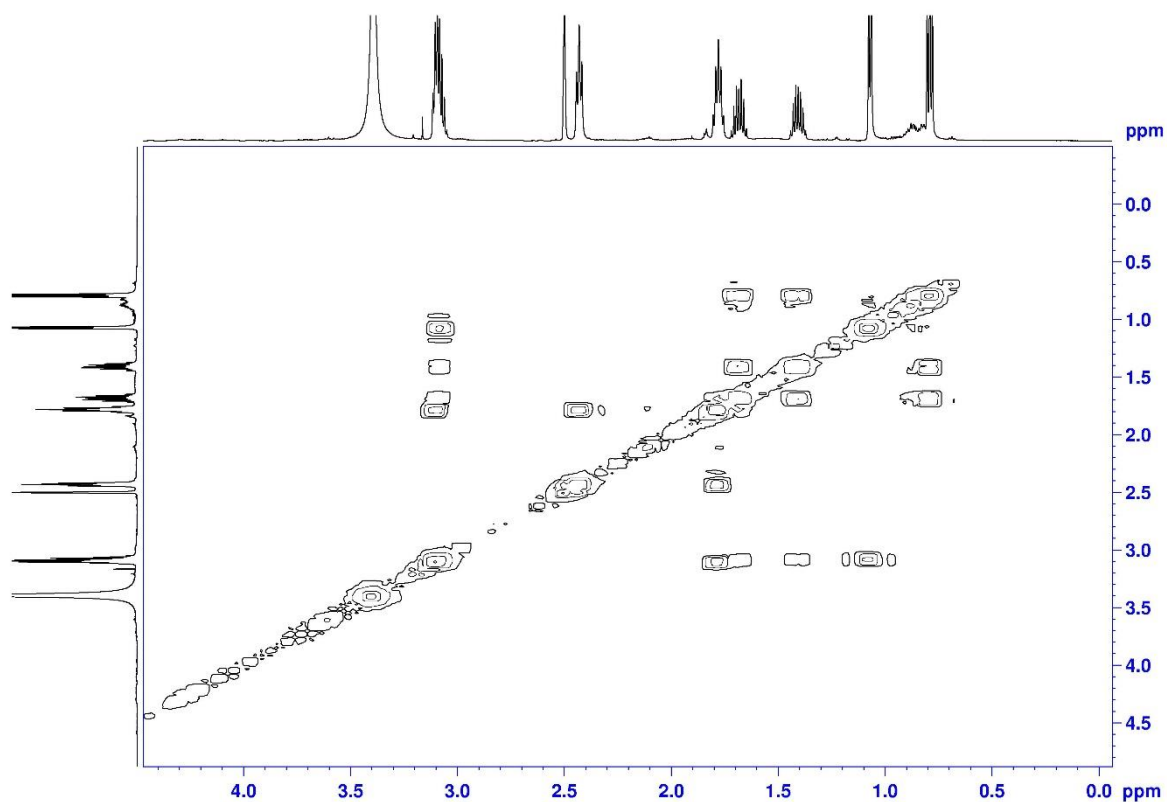


Figure S18. ^1H - ^1H COSY spectrum of **2** recorded in $\text{DMSO}-d_6$ (600 MHz)

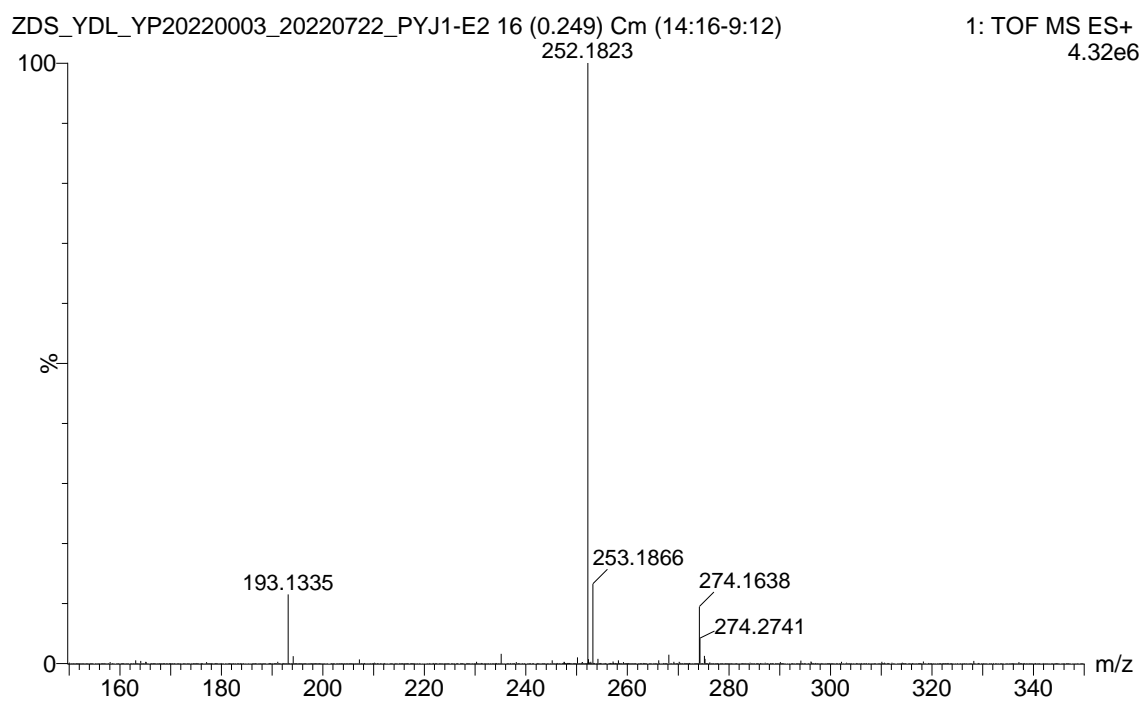


Figure S19. HR-ESI-MS spectra of **2**

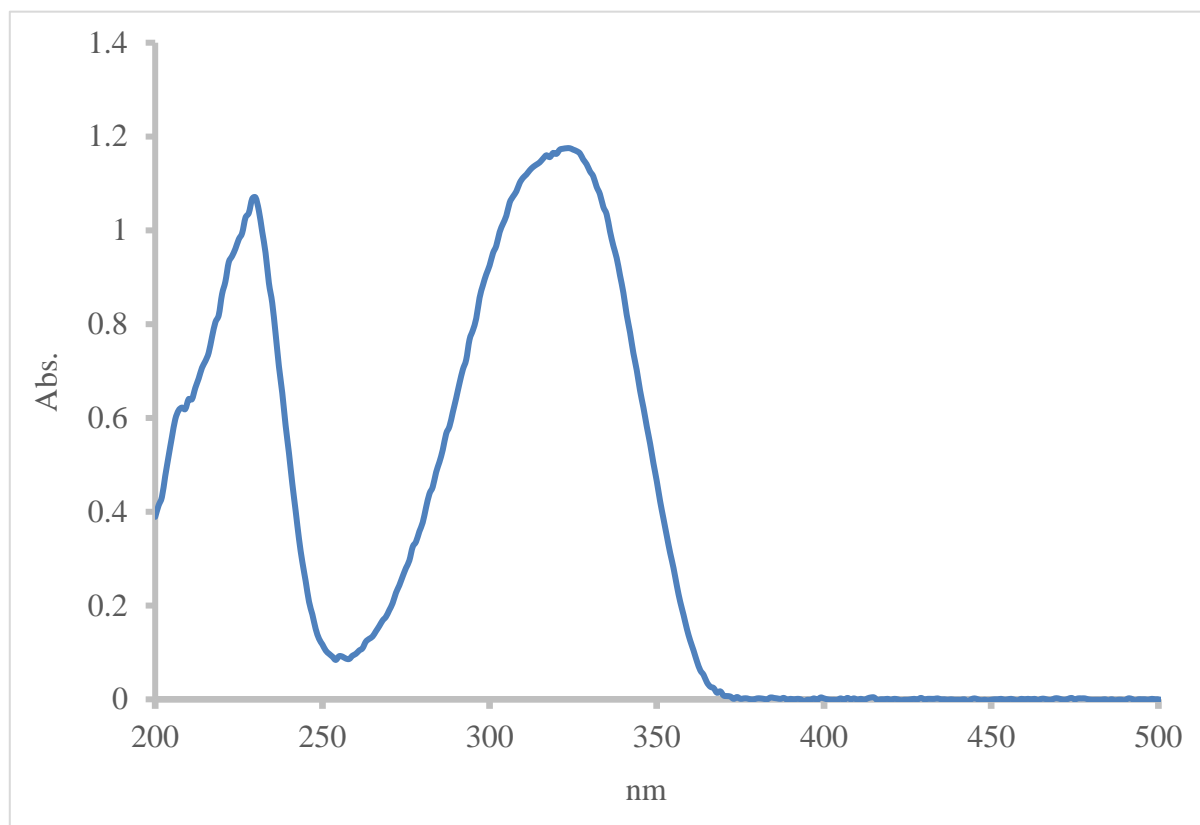


Figure S20. UV spectrum of **2**

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