

A class of benzofuranoindoline-bearing heptacyclic fungal RiPPs with anticancer activities

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Supplementary Methods

Computational protocol to identify low-energy conformers of the stereoisomers of 1. To identify the low-energy conformers for each possible stereoisomer of compound **1** (**Supplementary Fig. 9**), careful conformer search was performed using the Conformer-Rotamer Ensemble Sampling Tool (CREST)¹ program (v2.1.2) utilizing the metadynamics sampling with genetic z-matrix crossing (iMTD-GC) method. We surmised that sampling conformers of the medium-sized 11-membered ring (highlighted in yellow in **Supplementary Fig. 9**) could be challenging for the automated conformer search algorithm. Therefore, to ensure the located conformers are global minima, we performed CREST conformer searches using different input geometries of each stereoisomer. These CREST input geometries were constructed using different strategies, including using the conformer generation tool in RDKit² and manually modifying the crystal structure of a previously reported 11-membered cyclic amide (CCDC Number:1120267). In the latter approach, both ring-flip conformers of the 11-membered ring were considered. All amide bonds have the trans conformation in the CREST input geometries. The amino group was protonated, and the carboxylic acid was deprotonated, based on their expected protonation state at the experimental pH. Each input structure was pre-optimized using the xtb³ with the ALPB⁴ implicit solvation model and water as the solvent. Then the optimized geometries were used as input for CREST conformer search. The CREST conformer search was performed with the GFN2-xTB//GFN-FF⁵ method in aqueous solution using the ALPB⁴ solvation model. Conformers were filtered by using a 0.5 Å root-mean-square-deviation (RMSD) threshold and an energy threshold of 12 kcal/mol. After the CREST conformer search, cluster analysis via k-means clustering was used to generate ten distinct clusters of conformers. Ten representative conformers from the clustering analysis, along with the ten lowest-energy conformers directly obtained from the CREST ensemble, were subjected to density functional theory (DFT) geometry optimization. DFT calculations were carried out using Gaussian 16. Geometry optimizations and vibrational frequency calculations were performed with the M06-2X^{6,7} functional with the 6-31G(d) basis set, incorporating the SMD implicit solvation model with water as solvent.⁸ Single-point energies were calculated using the M06-2X functional with the 6-311+G(d,p) basis set and the same solvation model. The resulting conformer ensemble was analysed using the Boltzmann average based on the single-point energy-corrected Gibbs free energies (**Supplementary Fig. 10**). Boltzmann-averaged NMR shielding tensors were calculated using the Gauge-Invariant Atomic Orbital (GIAO) method at the B3LYP/6-311+G(2d,p)/SMD(H₂O) level of theory.⁹⁻¹¹ Boltzmann-averaged electronic circular dichroism (ECD) spectra were computed using time-dependent DFT (TD-DFT) at the CAM-B3LYP/6-311+G(d,p) level of theory with 50 electronic states included in each calculation. The Boltzmann-averaged ECD spectra were plotted using SpecDis software.¹² Finally, the DP4+ analysis was performed following previous report¹³ and provided probabilities (%) for four isomers of compound **1**.

Immunofluorescence analysis. Jurkat cells were cultured with 500 nM **2-L6** and equivalent DMSO, respectively for 12 h. The collected cells were washed with PBS three times and fixed cells by adding 4% PFA, then incubated for 25-30 min at RT. Wash with PBS twice and permeabilized cells by adding 0.1% Triton X-100 in PBS and incubate for 10 min at RT. Wash with PBS three times with 5 min incubation each. Block cells with 5%-Bovine Serum Albumin (BSA) in PBS for 30 mins. Dilute alpha Tubulin monoclonal antibody (B-5-1-2), Alexa Fluor™ 488 (Invitrogen, 322588) in 5%-BSA in PBS and incubate overnight (4°C). Wash with PBS three times with 5 min incubation each. Take the cover slip upside down onto a glass slide with mounting

media with antifade reagent (Thermofisher, ProLong Gold, P36941) on top of it. Seal using nail polish and let it dry for 30 min. The samples were analyzed by Zeiss Elyra 7 Lattice/SIM with TIRF.

Western Blot analysis. Jurkat cells were cultured with 500, 250, 100, 50 nM **2-L₆** and equivalent DMSO, respectively for 12 h. Then cells were collected and washed with cold PBS three times. Cells lysates were obtained using RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, and protease inhibitors, pH 8.0). Centrifuge for 20 min at 15000 g at 4°C and then discard the pellet. Protein quantification assays were performed with Pierce™ BCA Protein Assay Kits (Thermofisher, 23225). After determining the concentration of proteins, equivalent 2X Laemmli sample buffer were added and heated at 95°C for 5 mins. After running the SDS-PAGE gel, the proteins were transferred from the gel to the PVDF membrane (Millipore, IPFL00010) pre-activated by MeOH using transfer buffer (25 mM Tris-HCl, 190 mM glycine, 0.1% (w/v) SDS, and 10% (v/v) methanol, pH 8.3). Then the membrane was blocked for 2 h at room temperature using blocking buffer (5% skim milk with TBST buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% (w/v) Tween-20, pH 7.4)). Incubate the membrane with appropriate dilutions of E2F1 Monoclonal Antibody (KH95) (Invitrogen, 32-1400) and RRM2 Monoclonal Antibody (OTI1F2) (Invitrogen, MA5-27318) in blocking buffer at 4°C overnight. Wash the membrane in three washes of TBST, 5 min each. Incubate the membrane with the dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 h. Wash the membrane in three washes of TBST, 5 min each. The image acquisition used ImageQuant™ LAS 4000 imager.

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Supplementary Tables

Supplementary Table 1. Fungal strains used in this study.

Strain	Characteristics	source
<i>A. flavus</i> CA14 (Δ pyrG)	Wild type <i>A. flavus</i> used in this study	
<i>A. clavatus</i> NRRL1	Wild type <i>A. clavatus</i> used in this study	NRRL
<i>A. versicolor</i> NRRL238	Wild type <i>A. versicolor</i> used in this study	NRRL
<i>A. sydowii</i> NRRL254	Wild type <i>A. sydowii</i> used in this study	NRRL
<i>A. brasiliensis</i> NRRL26652	Wild type <i>A. brasiliensis</i> used in this study	NRRL
<i>A. niger</i> NRRL326	Wild type <i>A. niger</i> used in this study	NRRL
<i>A. tubingensis</i> NRRL365	Wild type <i>A. tubingensis</i> used in this study	NRRL
<i>A. wentii</i> NRRL375	Wild type <i>A. wentii</i> used in this study	NRRL
<i>A. acidus</i> NRRL4750	Wild type <i>A. acidus</i> used in this study	NRRL
<i>A. zonatus</i> NRRL5079	Wild type <i>A. zonatus</i> used in this study	NRRL
<i>A. aculeatus</i> NRRL5094	Wild type <i>A. aculeatus</i> used in this study	NRRL
<i>A. oryzae</i> NSAR1 (<i>niaD</i> -, <i>sC</i> -, Δ argB, <i>adeA</i> -)	Wild type <i>A. oryzae</i> used in this study	
Δ apgB	<i>apgB</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgC	<i>apgC</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgD	<i>apgD</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgQ	<i>apgQ</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgE	<i>apgE</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgF	<i>apgF</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgG	<i>apgG</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgA	<i>apgA</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgYa	<i>apgYa</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgYb	<i>apgYb</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgYc	<i>apgYc</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgYd	<i>apgYd</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgYe	<i>apgYe</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgYf	<i>apgYf</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgH	<i>apgH</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgI	<i>apgI</i> konckout mutant of <i>A. flavus</i> CA14	This study
Δ apgA::apgAr	Replacement of <i>apgA</i> by <i>apgAr</i> in <i>A. flavus</i> CA14	This study
Δ apgA::apgAr-Q1H	Replacement of <i>apgA</i> by <i>apgAr-Q1H</i> in <i>A. flavus</i> CA14	This study
Δ apgA::apgAr-Q1S	Replacement of <i>apgA</i> by <i>apgAr-Q1S</i> in <i>A. flavus</i> CA14	This study
Δ apgA::apgAr-Q1N	Replacement of <i>apgA</i> by <i>apgAr-Q1N</i> in <i>A. flavus</i> CA14	This study
Δ apgA::apgAr-Q1L	Replacement of <i>apgA</i> by <i>apgAr-Q1L</i> in <i>A. flavus</i> CA14	This study

$\Delta apgA::apgA_T-G4S$	Replacement of <i>apgA</i> by <i>apgA_T-G4S</i> in <i>A. flavus</i> CA14	This study
$\Delta apgA::apgA_T-A5S$	Replacement of <i>apgA</i> by <i>apgA_T-A5S</i> in <i>A. flavus</i> CA14	This study
$\Delta apgA::apgA_T-A5T$	Replacement of <i>apgA</i> by <i>apgA_T-A5T</i> in <i>A. flavus</i> CA14	This study
$\Delta apgA::apgA_T-G7S$	Replacement of <i>apgA</i> by <i>apgA_T-G7S</i> in <i>A. flavus</i> CA14	This study
$\Delta apgA::apgA_T-G8S$	Replacement of <i>apgA</i> by <i>apgA_T-G8S</i> in <i>A. flavus</i> CA14	This study
$\Delta apgA::apgA_T-I9V$	Replacement of <i>apgA</i> by <i>apgA_T-I9V</i> in <i>A. flavus</i> CA14	This study
$\Delta apgA::apgA_T-I9L$	Replacement of <i>apgA</i> by <i>apgA_T-I9L</i> in <i>A. flavus</i> CA14	This study

Supplementary Table 2. Plasmids used in this study.

Plasmids	Description of Plasmid
pET-ApgG _T	ApgG _T expression in <i>E. coli</i> BL21(DE3)
pET-ApgG _T -D121A	ApgG _T -D121A expression in <i>E. coli</i> BL21(DE3)
pET-ApgG _T -E168A	ApgG _T -E168A expression in <i>E. coli</i> BL21(DE3)
pET-ApgG _T -E169A	ApgG _T -E169A expression in <i>E. coli</i> BL21(DE3)
pET-ApgG _T -D216A	ApgG _T -D216A expression in <i>E. coli</i> BL21(DE3)
pET-ApgG _T -D281A	ApgG _T -D281A expression in <i>E. coli</i> BL21(DE3)
pET-ApgG _T -F305A	ApgG _T -F305A expression in <i>E. coli</i> BL21(DE3)
pET-ApgG _T -W308A	ApgG _T -W308A expression in <i>E. coli</i> BL21(DE3)
pET-ApgG _T -H309A	ApgG _T -H309A expression in <i>E. coli</i> BL21(DE3)
CCNF KO	CCNF knockout in Jurkat cells
CLASP2 KO	CLASP2 knockout in Jurkat cells
KEAP1 KO	KEAP1 knockout in Jurkat cells
SLC46A3 KO	SLC46A3 knockout in Jurkat cells
STMN1 KO	STMN1 knockout in Jurkat cells
pYTR-ApgG-GFP	Expression of ApgG-GFP in <i>A. nidulans</i>
pYTR-ApgG	Expression of ApgG in <i>A. nidulans</i>
pYTR-ApgYa-GFP	Expression of ApgYa-GFP in <i>A. nidulans</i>
pYTR-ApgYe-GFP	Expression of ApgYe-GFP in <i>A. nidulans</i>

Supplementary Table 3. Primers used in this study.

Primer	Sequence (5'→3')
ApgB-up-F	cagaacctgcaccattgcatg
ApgB-up-R	CTGGTATATTGTTCTGAGATCCATAGGATCCgaaaccggaggccttctaac
ApgB-dn-F	GAATAATATGCCTGCCTCGAGATATGGACATgggctgatcactctaggatg
ApgB-dn-R	cgagaatcgagagaacactaaaattg
ApgC-up-F	ccggtgtgattaccgaagcc

ApgC-up-R	CTGGTATATTGTTCTGAGATCCATAGGATCCgaatcacaggggtaaatcagagc
ApgC-dn-F	GAATAATATGCCTGCCTCGAGATATGGACATtattctagcactccgaaccacag
ApgC-dn-R	ccgatgctctgcatcacgcac
ApgD-up-F	ttctctaccggcgtgtatgcg
ApgD-up-R	CTGGTATATTGTTCTGAGATCCATAGGATCCttgggtgggatggatgaagggt
ApgD-dn-F	GAATAATATGCCTGCCTCGAGATATGGACATcgagcgacgtgtcggttca
ApgD-dn-R	cgagtacagcatgggatcgaac
ApgQ-up-F	GAAATTAACCCTCACTAAAGGGAgtttatcacgccgcggag
ApgQ-up-R	GTTCTGAGATCCATAGGATCCacatgtgagtgttgagaaag
ApgQ-dn-F	CTGCCTCGAGATATGGACATtttgccgtgactttcctcaaatg
ApgQ-dn-R	GAACAAAAACATGCAGGAAACggcatcggaggtgcattcac
ApgE-up-F	ggaccctcataaacacatcctc
ApgE-up-R	CTGGTATATTGTTCTGAGATCCATAGGATCCctcctgggttaatatggaccg
ApgE-dn-F	GAATAATATGCCTGCCTCGAGATATGGACATggttgcaagacgaaggcca
ApgE-dn-R	tgtcgcgcattggtgcaact
ApgF-up-F	CGAAATTAACCCTCACTAAAGGGAagaaacctactgaaatgcaaagc
ApgF-up-R	GGTATATTGTTCTGAGATCCATAGGATCCaggtctagaatgtgtttgactc
ApgF-dn-F	ATGCCTGCCTCGAGATATGGACATgagatggtcactactacgggc
ApgF-dn-R	CAACTGCACAGAACAAAAACATGCAGGAAACgctgcgcattgggcggtgga
ApgG-up-F	GAAATTAACCCTCACTAAAGGGAgtccaagctctcgagatgg
ApgG-up-R	GTTCTGAGATCCATAGGATCCgcctattctacgaactacggc
ApgG-dn-F	CCTGCCTCGAGATATGGACATgaggggtgtatgacaatgtgt
ApgG-dn-R	CAGAACAAAAACATGCAGGAAACgtttcttgcaccgctgtg
ApgA-up-F	GAAATTAACCCTCACTAAAGGGAaattgtccaactctctcagac
ApgA-up-R	GTTCTGAGATCCATAGGATCCtactgtcagtaactttgg
ApgA-dn-F	CCTGCCTCGAGATATGGACATggtctagcggatgccaaga
ApgA-dn-R	AGAACAAAAACATGCAGGAAACcttatctccgggaaagagct
ApgYa-up-F	GAAATTAACCCTCACTAAAGGGAgagcagtaattagatgcacc
ApgYa-up-R	TTCTGAGATCCATAGGATCCgacactggaaggtatctctaacc
ApgYa-dn-F	CTGCCTCGAGATATGGACATacttttgtattacggcatg
ApgYa-dn-R	CAGAACAAAAACATGCAGGAAACgacctataaggtgtgtacatc
ApgYb-up-F	GAAATTAACCCTCACTAAAGGGAcgaccataagcagatgcacg
ApgYb-up-R	GTTCTGAGATCCATAGGATCCcgtgtgttagatctcctatcc
ApgYb-dn-F	CCTGCCTCGAGATATGGACATcgtcgaagtggcgtatctggag
ApgYb-dn-R	CAGAACAAAAACATGCAGGAAACcgaaacccatatacttcacg
ApgYc-up-F	GAAATTAACCCTCACTAAAGGGAacttttctcattgagaggtgg
ApgYc-up-R	GTTCTGAGATCCATAGGATCCcagtgtgtcatgttgccgac
ApgYc-dn-F	CCTGCCTCGAGATATGGACATtgtgggtgacctaaacaatacg

ApgYc-dn-R	AACAAAAACATGCAGGAAACatctcccggtgcactcta
ApgYd-up-F	GAAATTAACCCTCACTAAAGGGAgttagcgtataacttatccaac
ApgYd-up-R	GTTCTGAGATCCATAGGATCCagttcttttggcgaagg
ApgYd-dn-F	CTGCCTCGAGATATGGACATgttatctaaccctaggttttg
ApgYd-dn-R	GAACAAAAACATGCAGGAAACctctgccactccagaatagc
ApgYe-up-F	GAAATTAACCCTCACTAAAGGGAaccagcaccgtattctteg
ApgYe-up-R	GTTCTGAGATCCATAGGATCCtgggcttccgtgataccgac
ApgYe-dn-F	CTGCCTCGAGATATGGACATgggaagtcgagtagcaaagag
ApgYe-dn-R	GAACAAAAACATGCAGGAAACggttgttcagacttgaagaag
ApgYf-up-F	GAAATTAACCCTCACTAAAGGGAcacggaagccaaaagtgc
ApgYf-up-R	GTTCTGAGATCCATAGGATCCgaacaagtcctacctcgcctg
ApgYf-dn-F	CTGCCTCGAGATATGGACATaccgggctcttgccttctg
ApgYf-dn-R	GAACAAAAACATGCAGGAAACacattcacatccatgcctc
ApgH-up-F	CGAAATTAACCCTCACTAAAGGGAacctgtcagcaattgtctatg
ApgH-up-R	CTGGTATATTGTTCTGAGATCCATAGGATCCcaaagttcgtgatgggtgtag
ApgH-dn-F	GAATAATATGCCTGCCTCGAGATATGGACATgtgagtgggaggtgacaggt
ApgH-dn-R	ACCCAACCTGCACAGAACAAAAACATGCAGGAAACtgatcttctgtaccctctg
ApgI-up-F	caggtctgactggttccaggattg
ApgI-up-R	GTATATTGTTCTGAGATCCATAGGATCCgtctactcacggaagtttctctg
ApgI-down-F	CTGCCTCGAGATATGGACATatatgagcggtagtgagactgttg
ApgI-down-R	caatgcacccagaatgaacagtac
pyrG-F	GGATCCTATGGATCTCAGAAC
pyrG-R	ATGTCCATATCTCGAGGCAG
pyrG-upR	TCCTCCTCCTCCGAAGGAGAG
pyrG-dnF	GTTTGGACTTATCGCTATGTCCTTG
ApgB-V-F	catgatgcctttttcacgtgtcc
ApgB-V-R	gcgataatggtgcagctgaatg
ApgC-F	cctgcatggttgctatgacg
ApgC-R	gtagcagggtggccagctcg
ApgD-F	ctcatacctagcagttggaagc
ApgD-R	cctctctgttctgttcaacgga
ApgQ-V-F	gtcaacgcctcattcagcttc
ApgQ-V-R	gtctgcttcggcattatggc
ApgE-V-F	cgccatcaatgggacccatg
ApgE-V-R	cgtacgcttctgatatcagccc
ApgF-V-F	acctccaattcgagcgctacttc
ApgF-V-R	tcggcaatacgttgaccgaaac
ApgG-V-F	tgctggaggaggagatcagg

ApgG-V-R	agctcacatgaagctcagc
ApgYa-V-F	gatgagagctgtgaacccaaag
ApgYa-V-R	gtgtgtcatgttgccgactac
ApgYb-V-F	caccgtgctgacattgattacg
ApgYb-V-R	ccacaatgaatagcgatcgc
ApgYc-V-F	cagctatggcgaagctattgac
ApgYc-V-R	caactgacagtgtatactagcgcc
ApgYd-V-F	gttgaactccagtgttgtgtc
ApgYd-V-R	tgctcgttgactgcattgcc
ApgYe-V-F	cggcaaagagcttgccagtag
ApgYe-V-R	agcaagaagagaaggaagcgc
ApgYf-V-F	gactttccgcacgataatggac
ApgYf-V-R	cacgtgcaggcacctgtcaag
ApgH-V-F	cacacattcatcatgcaagatgcc
ApgH-V-R	gacgtactatatttgcctcctgg
ApgI-V-F	ctgatgacagggtaagagattc
ApgI-V-R	gtccgcagcttgctgaagagtac
ApgA _T -1F	cggaccatagcttgctcagctc
ApgA _T -1R	CGCGTTTATTCTTGTTGACATGGAGCTATTAAATCAgatgcctccccatgcaccgta
trpCt-F	cgggtgcatggggaggcattcTGATTAAATAGCTCCATGTCAACAAG
trpCt-R	CTGGTATATTGTTCTGAGATCCATAGGATCCAAAGAAGGATTACCTCTAAACAAGTG
pyrG-F	GTACACTTGTTTAGAGGTAATCCTTCTTTGGATCCTATGGATCTCAGAACAATATAC
pyrG-R	ctgtgaacccaaagtactgagcagtaaATGTCCATATCTCGAGGCAGG
ApgA _T -2F	TGCCTGCCTCGAGATATGGACATtactgctcagtaactttgggttc
ApgA _T -2R	catacttcgcagtgaggggatac
ApgA _T (Q1H)-1R	TCTTGTTGACATGGAGCTATTAAATCAgatgcctccccatgcaccgtaaagGtgttccg
ApgA _T (Q1S)-1R	TCTTGTTGACATGGAGCTATTAAATCAgatgcctccccatgcaccgtaaagagattccg
ApgA _T (Q1N)-1R	TCTTGTTGACATGGAGCTATTAAATCAgatgcctccccatgcaccgtaaagagattccg
ApgA _T (Q1L)-1R	GCTATTAAATCAgatgcctccccatgcaccgtaaagaagtccgaatatcgtccacag
ApgA _T (G4S)-1R	TCTTGTTGACATGGAGCTATTAAATCAgatgcctccccatgcGCTgtacagctgttccg
ApgA _T (A5S)-1R	TCTTGTTGACATGGAGCTATTAAATCAgatgcctccccatGCtaccgtacagctgttccg
ApgA _T (A5T)-1R	TCTTGTTGACATGGAGCTATTAAATCAgatgcctccccatGGTaccgtacagctgttccg
ApgA _T (G7S)-1R	CTTGTTGACATGGAGCTATTAAATCAgatgccGCTccatgcaccgtacagctgttccg
ApgAT(G8S)-1R	CTTGTTGACATGGAGCTATTAAATCAgatGCTaccatgcaccgtacagctgttccg
ApgA _T (I9L)-1R	TCTTGTTGACATGGAGCTATTAAATCACAGcctccccatgcaccgtacagctgttccg
ApgA _T (I9L)-F	cagctgtacgggtgcatggggaggcCTGTGATTAAATAGCTCCATGTCAACAAGA
ApgAT(I9V)-1R	TCTTGTTGACATGGAGCTATTAAATCACACgcctccccatgcaccgtacagctgttccg
ApgG _T -F	gtttaactttaagaaggagatataccatggacgtcatggaaacttatcc

ApgG-R	caaattatcattagtgatgggtgggtgatgggtatccataaacccctgcage
PET-F	catcaccaccaccatcactaatga
PET-R	ggtatatctcctcttaaagttaacaaaattatttctagag
ApgG _T -D137A-f	gaaggattcatcggcgcaatcGCGagcgcagctccgtgcagtac
ApgG _T -D137A-r	gagctgcgctCGcgattgcgccgatgaatccttc
ApgG _T -D184A-f	catacaagtcattttcacggacgggGCGgagagcttcggcaatacgttg
ApgG _T -D184A-r	ctctcCGCaccgtccgtgaaaatgacttgatg
ApgG _T -D185A-f	gtcattttcacggacggtagGCGagcttcggcaatacgttgac
ApgG _T -D185A-r	gctCGCctcaccgtccgtgaaaatgacttg
ApgG _T -D232A-f	ctactgtgctattgGCGcttctgggagccaagaatccgcagatc
ApgG _T -D232A-r	cttggctcccagaagCGCcaatagcacaagtagagagatcgaggaaag
ApgG _T -E297A-f	caacctgtcgaggatGCGcaggttcattctctggcctag
ApgG _T -E297A-r	gaacctgCGCctctcgacaggttgccgcttaag
ApgG _T -F321A-f	ctactacgggggagGCGcctagtgtttggcacacaccagac
ApgG _T -F321A-r	caaacactaggCGCctccccgtagtagggtcag
ApgG _T -W325A-f	gagtttctagtgttGCGcacacaccagacgatgatgaaaac
ApgG _T -W325A-r	gtctggtgtgtgCGCaactaggaaactccccgtag
ApgG _T -H326A-f	gagtttctagtgtttggGCGacaccagacgatgatgaaaaccatttg
ApgG _T -H326A-r	cgtctggtgtCGCcaaactaggaaactccccgtag
ZRBS2948	CCTCAGGCCAGGCTTTTGAGCT
ZRBS2949	GGAGCGGGCATGTAACAGGAGT
ZRBS2950	CCAGCCTGGGCAACAAGGTGAG
ZRBS2951	AGGCAAAGAAAGTGATTGCATGT
ZRBS2952	ACTTCCTCCTTGTCCCCCTCGTCT
ZRBS2953	AGTGTTTCATGCTTGCTGTGGCT
ZRBS2954	TCCACCACCTCCGCAGCAAAAC
ZRBS2955	GCGCGTCGACTGTCTTGCCCTAG
ZRBS2956	CATCCTTGACAGGGAGCATGC
ZRBS2957	TCACCCAGCAGTGCCCTCATGT
ZRBS2958	AGGCAAAGCAAGCTCCCCAAAA
ZRBS2959	GTCCCGCCCATCCACCAGAGAT
ZRBS2960	CCCAGACTCACCTCTGCAGCT
ZRBS2961	TCTGTCTGTCTGTCCCCGCAG
ZRBS2962	TGGAGGGAGAAAGGCCAGCTCC
ZRBS2963	AAGGGAGGTGGCTCTTGCTGCT
ZRBS2964	TCGTTGACGCCGAACCTTCCTGC
ZRBS2965	GGGTTGTAACAGTCCAGGGCGC
ZRBS2966	TCCCGCTCACCTCCCCAAAAT

ZRBS2967	TTCATGGCCCACAAGGTGGTGC
ZRBS2948	CCTCAGGCCAGGCTTTTGAGCT
ZRBS2949	GGAGCGGGCATGTAACAGGAGT
ZRBS2950	CCAGCCTGGGCAACAAGGTGAG
ZRBS2951	AGGCAAAGAAAGTGATTGCATGT
ZRBS2757	CATGCGTCTCGCTGGTGGTCGGGCGAGACTCCG
ZRBS2758	CATGCGTCTCGCCAGGGCAAGGGTCTGGG
ZRBS2759	CATGCGTCTCGATCCCGTATTGACGCCGGGC
ZRBS2760	AGTGCGTCTCCGGATAATACCGCGCCACATAGCA
ZRBS2761	AGTGCGTCTCCAGGTGGTACCTTAATTAACCAAAGTGGATCTC
ZRBS2762	AGTGCGTCTCCACCTCGAGCGGCCCAAGC
ZRBS2763	AGTGCGTCTCCTTCTGCGCCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
ZRBS2764	AGTGCGTCTCGAGAAGTACTCCATGAGGGGGCACCCGGATTG
ZRBS2765	AGTGCGTCTCGGCACCACCTAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG
ZRBS2766	AGTGCGTCTCGGTGCTACTTGATCCAGGGGGCACCCGGATTG
ZRBS2767	GCTTCGTCTCCCTTCTCGCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
ZRBS2768	GCTTCGTCTCGGAAGTGTGTCCTCAGGGGGCACCCGGATTG
ZRBS2769	GCTTCGTCTCGTGATCTACACCGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG
ZRBS2770	GCTTCGTCTCGATCAGGCGGCCAGGGGGCACCCGGATTG
ZRBS2771	GCTTCGTCTCGTCCCGTATTGACGCCGGGC
ZRBS2772	ACGACGTCTCGACCTCGAGCGGCCCAAGC
ZRBS2773	ACGACGTCTCGAGAAGAAGCCAGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGC
ZRBS2774	ACGACGTCTCCTTCTGGTAGGTAAAGGGGGCACCCGGATTG
ZRBS2775	ACGACGTCTCCCTTCCCTGGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGG
ZRBS2776	ACGACGTCTCAAAGATCCTTCTCAGGGGGCACCCGGATTG
ZRBS2777	ACGACGTCTCCTCGGAATTCGCTAGCTAGGTCTTGAAAG
TKOv3-i5-U6-F1-D501	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTCCCTACACGAC GCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F1-D502	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTCCCTACACGAC GCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F1-D503	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTCCCTACACGAC GCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F1-D504	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTCCCTACACGAC GCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F1-D505	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTCCCTACACGAC GCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F1-D506	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTCCCTACACGAC GCTCTTCCGATCTTTGTGGAAAGGACGAAACACCG

TKOv3-i5-U6-F2-D501	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGAC GCTCTTCCGATCTGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F2-D502	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGAC GCTCTTCCGATCTGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F2-D503	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACGAC GCTCTTCCGATCTGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F2-D504	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACGAC GCTCTTCCGATCTGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F2-D505	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGAC GCTCTTCCGATCTGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F2-D506	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGAC GCTCTTCCGATCTGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F3-D501	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGAC GCTCTTCCGATCTCAGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F3-D502	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGAC GCTCTTCCGATCTCAGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F3-D503	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACGAC GCTCTTCCGATCTCAGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F3-D504	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACGAC GCTCTTCCGATCTCAGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F3-D505	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGAC GCTCTTCCGATCTCAGTTGTGGAAAGGACGAAACACCG
TKOv3-i5-U6-F3-D506	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGAC GCTCTTCCGATCTCAGTTGTGGAAAGGACGAAACACCG
TKOv3-i7-U6-R-D701	CAAGCAGAAGACGGCATAACGAGATATTACTCGGTGACTGGAGTTCAGACGTGTGCT CTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
TKOv3-i7-U6-R-D702	CAAGCAGAAGACGGCATAACGAGATTCCGGAGAGTGACTGGAGTTCAGACGTGTGCT CTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
TKOv3-i7-U6-R-D703	CAAGCAGAAGACGGCATAACGAGATCGCTCATTGTGACTGGAGTTCAGACGTGTGCTC TTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
TKOv3-i7-U6-R-D704	CAAGCAGAAGACGGCATAACGAGATGAGATTCCGTGACTGGAGTTCAGACGTGTGCT CTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
TKOv3-i7-U6-R-D705	CAAGCAGAAGACGGCATAACGAGATATTCAGAAGTGACTGGAGTTCAGACGTGTGCT CTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC
TKOv3-i7-U6-R-D706	CAAGCAGAAGACGGCATAACGAGATGAATTCGTGTGACTGGAGTTCAGACGTGTGCT CTTCCGATCTACTTGCTATTTCTAGCTCTAAAAC

Supplementary Table 4. Proteins encoded in *apg* gene cluster.

Gene	Amino Acid length	NCBI Accession Number	Putative function	Protein homologue	Coverage /Identify (%)	Organism
<i>apgB</i>	282	KAF7617334.1	Hypothetical protein	XP_022391110.1	99/75	<i>Aspergillus bombycis</i>
<i>apgC</i>	525	KAF7617335.1	Amino acid transporter	EIT81401.1	100/97.52	<i>Aspergillus oryzae</i>
<i>apgD</i>	542	KAF7617336.1	Amino acid permease	OOO06478.1	100/99.63	<i>Aspergillus oryzae</i>
<i>apgQ</i>	390	KAF7617337.1	Tyrosinase	OOO06479.1	100/99.74	<i>Aspergillus oryzae</i>
<i>apgE</i>	426	KAF7617338.1	Transporter	OOO06480.1	100/85.21	<i>Aspergillus oryzae</i>
<i>apgF</i>	88	KAF7617339.1	Hypothetical protein	-	-	-
<i>apgG</i>	363	KAF7617340.1	Glutaminyl-peptide cyclotransferase	OOO06481.1	100/99.72	<i>Aspergillus oryzae</i>
<i>apgA</i>	252	XP_041147057.1	Precursor peptide	OOO06482.1	100/86.91	<i>Aspergillus oryzae</i>
<i>apgYa</i>	244	KAF7617342.1	DUF3328 Oxidase ustYa	QRD83460.1	100/73.38	<i>Aspergillus oryzae</i>
<i>apgYb</i>	217	KAF7617343.1	DUF3328 Oxidase ustYa	KAB8201375.1	100/90.32	<i>Aspergillus parasiticus</i>
<i>apgYc</i>	239	KAF7617344.1	DUF3328 Oxidase ustYa	KAE8317681.1	100/92.47	<i>Aspergillus transmontanensis</i>
<i>apgYd</i>	320	KAF7617345.1	DUF3328 Oxidase ustYa	XP_031920864.1	87/91.04	<i>Aspergillus parasiticus SU-1</i>
<i>apgYe</i>	328	KAF7617346.1	DUF3328 Oxidase ustYa	KAB8274923.1	97/97.91	<i>Aspergillus minisclerotigenes</i>
<i>apgYf</i>	283	KAF7617347.1	DUF3328 Oxidase ustYa	KAB8274924.1	100/97.71	<i>Aspergillus minisclerotigenes</i>
<i>apgH</i>	222	KAF7617348.1	Hypothetical protein	OOO06487.1	100/99.55	<i>Aspergillus oryzae</i>
<i>apgI</i>	344	KAF7617349.1	Transcription factor	OOO06488.1	100/99.71	<i>Aspergillus oryzae</i>

Supplementary Table 5. sDP4+, uDP4+ and DP4+ probabilities (%) for four isomers of compound 1.

	Isomer 1	Isomer 2	Isomer 3	Isomer 4
sDP4+ (H data)	100.00%	0.00%	0.00%	0.00%
sDP4+ (C data)	36.25%	0.00%	63.75%	0.00%
sDP4+ (all data)	100.00%	0.00%	0.00%	0.00%
uDP4+ (H data)	60.04%	18.18%	21.78%	0.00%
uDP4+ (C data)	100.00%	0.00%	0.00%	0.00%
uDP4+ (all data)	100.00%	0.00%	0.00%	0.00%
DP4+ (H data)	100.00%	0.00%	0.00%	0.00%
DP4+ (C data)	100.00%	0.00%	0.00%	0.00%
DP4+ (All data)	100.00%	0.00%	0.00%	0.00%

The DP4+ results strongly supported isomer 1 as the most likely configuration in high confidence, with 100.00% DP4+ probability (highlighted in the red box).

Supplementary Table 6. Antimicrobial activity of 1-4.

	Gram-positive <i>Bacillus subtilis</i>	Gram-positive <i>Staphylococcus aureus</i>	Gram-negative <i>Salmonella Typhimurium</i>	Gram-negative <i>Escherichia coli</i>	<i>Candida albicans</i>
1	No halo	No halo	No halo	No halo	No halo
2	No halo	No halo	No halo	No halo	No halo
3	No halo	No halo	No halo	No halo	No halo
4	No halo	No halo	No halo	No halo	No halo

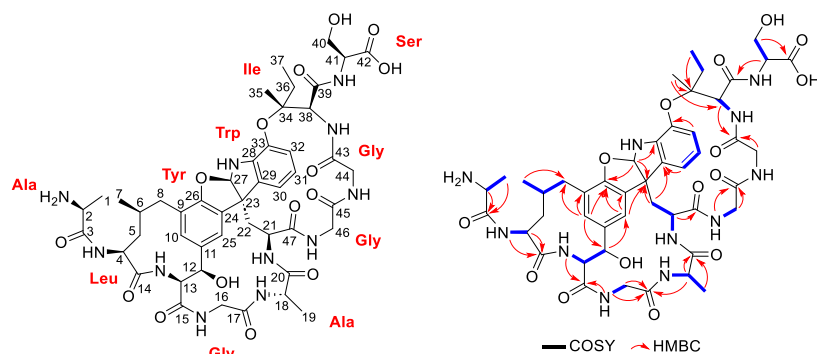
Supplementary Table 7. Crystal Diffraction and refinement data.

	ApgG-diamond	ApgG-rod
PDB Code	8VPL	8VPM
Data collection		
Wavelength (Å)	0.9787	0.9787
Space group	<i>P3₁21</i>	<i>P6₄</i>
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	70.27	123.95
	70.27	123.95
	145.17	46.08
<i>α</i> , <i>β</i> , <i>γ</i> (°)	90, 90, 120	90, 90, 120
Resolution (Å) ¹	35.13 - 1.84	40.57 - 2.15
	1.91 - 1.84	2.23 - 2.15
R _{sym} or R _{merge} ¹	0.04509 (0.9069)	0.08973 (0.8701)
<i>I</i> / <i>σI</i> ¹	26.44 (2.48)	17.19 (3.03)
CC ^{1/2} ¹	0.999 (0.82)	0.998 (0.851)
Completeness (%)	99.87 (99.14)	99.95 (99.86)
No. unique reflections ¹	36676 (3568)	22281 (2214)
Refinement		
B-factors		
Protein	47.15	45.45
Water	41.17	29.66
Resolution (Å)	1.84	2.15
No. reflections	36675 (3568)	22279 (2214)
R _{work} /R _{free}	0.24/0.26	0.21/0.25
Wilson B	41.03	41.4
Ramachandran		
Favored (%)	97.94	98.48
Outlier (%)	0	0
R.m.s. deviations		
Bond lengths (Å)	0.009	0.01
Bond angles (°)	1.15	1.16

¹Data in the highest resolution shell is shown in the parenthesis.

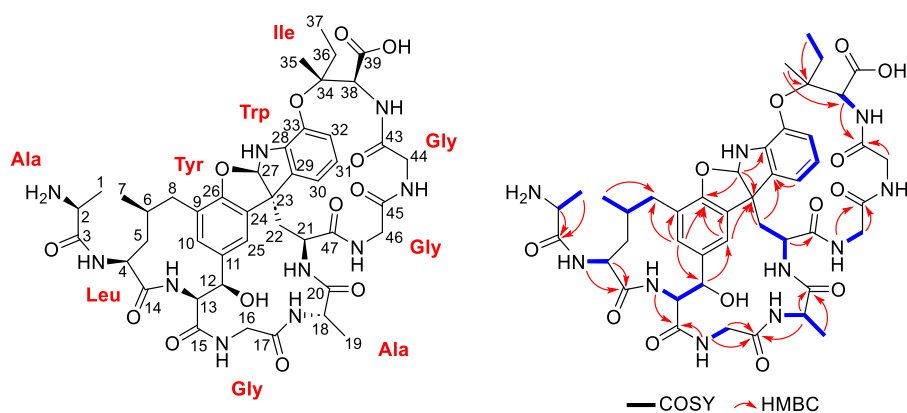
²B-factor of metal ions and their protein nucleotide ligands.

Supplementary Table 8. NMR data of 1.



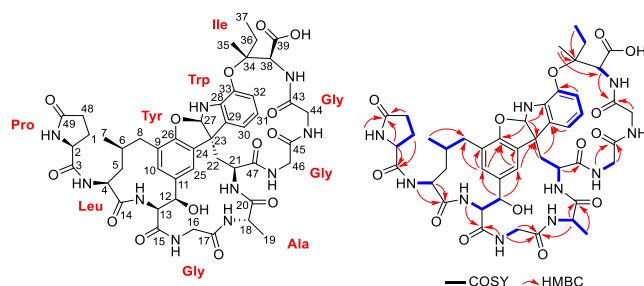
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Ala	1	1.34, d (6.9)	16.6	Ile	34		85.5
	2	4.01, q (6.9)	48.8		35	1.38, ov.	17.7
	3		171.0		36	1.78, m	31.2
Leu	4	4.11	54.1		37	0.91, ov.	7.7
	5	1.73, ov.	35.2		38	4.97, s	61.4
		1.56, m			39		170.3
	6	2.08, ov.	29.8	Ser	NH	8.21, d (9.5)	
	7	0.93, ov.	7.7		40	3.81, ov.	61.3
						3.74, ov.	
	8	2.32, m	35.1		41	4.37, t (4.6)	55.2
		1.74, ov.					
Tyr	14		169.7		42		172.6
	9		120.2	Gly	43		171.3
	10	6.67, s	128.5		44	3.92, d (16.4)	43.1
						3.66, d (16.4)	
	11		131.8	Gly	45		171.6
	12	4.79	72.1		46	3.84, ov.	42.7
	13	3.48, ov.	48.0		NH	8.56, m	
	15		174.2	Ala	18	4.55, t (6.8)	48.1
	24		128.7		19	1.11, d (6.8)	15.9
	25	7.39, s	117.1		20		173.9
					NH	8.57, m	
	26		157.4	Gly	16	3.76, ov.	43.4
						3.47, ov.	
Trp	21	3.80, ov.	52.6		17		170.1
	22	2.77, d (16.0)	37.1		NH	8.16, m	
		2.12, dd (16, 5.4)					
	23		61.4				
	27	6.38, s	101.2				
	NH	5.98, s					
	28		138.8				
	29		133.7				
	30	6.72, ov.	116.7				
	31	6.59, t (7.8)	120.7				
	32	6.73, ov.	119.8				
	33		140.7				
	47		176.0				
	NH	8.69, s					

Supplementary Table 9. NMR data of 2.



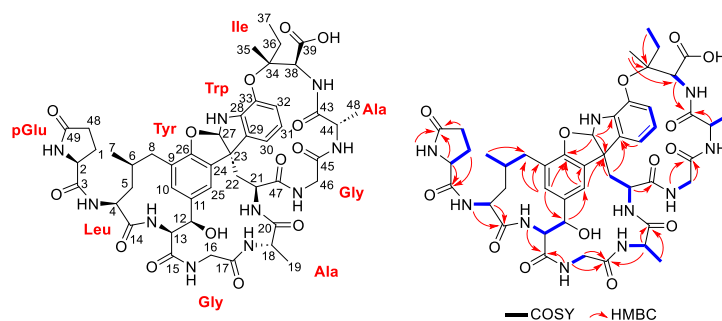
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Ala	1	1.34, d (7.1)	16.6	Ile	34		85.3
	2	4.00, m	48.9		35	1.31, s	18.3
	3		171.1		36	1.80, m	31.3
Leu	4	4.11, br s	54.1		37	0.90, ov.	7.7
	5	1.65, m	35.1		38	4.66	63.5
		1.56, m			39		174.8
	6	2.08, ov.	29.9		NH	7.81, d (9.6)	
	7	0.92, ov.	7.7	Gly	43		170.8
	8	2.31, m	35.1		44	3.94, ov.	43.4
		1.73, ov.				3.69, ov.	
	14		174.8		NH	7.62, br s	
Tyr	NH			Gly	45		174.1
	9		119.2		46	3.79, ov.	42.7
	10	6.66, ov.	128.7			3.90, ov.	
	11		131.8		NH	8.52, s	
	24		128.6	Ala	18	4.55	48.1
	25	7.39, s	117.2		19	1.11, d (6.9)	15.9
	26		157.4		20		173.9
	12	4.78	72.1		NH	8.16, br s	
	13	3.58	52.8	Gly	16	3.76, ov.	43.4
	15		174.2			3.48, ov.	
Trp	NH	8.30, brs			17		170.1
	27	6.36, s	101.4		NH	7.58, m	
	23		61.3				
	28		138.7				
	NH	8.69, brs					
	29		133.4				
	30	6.70, d (7.7)	119.2				
	31	6.54, m	120.6				
	32	6.66, ov.	116.5				
	33		140.8				
	21	3.81, ov.	52.5				
	22	2.06, ov.	36.9				
		2.74, d (17.5)					
	47		175.8				
	NH	8.69, s					

Supplementary Table 10. NMR data of 3.



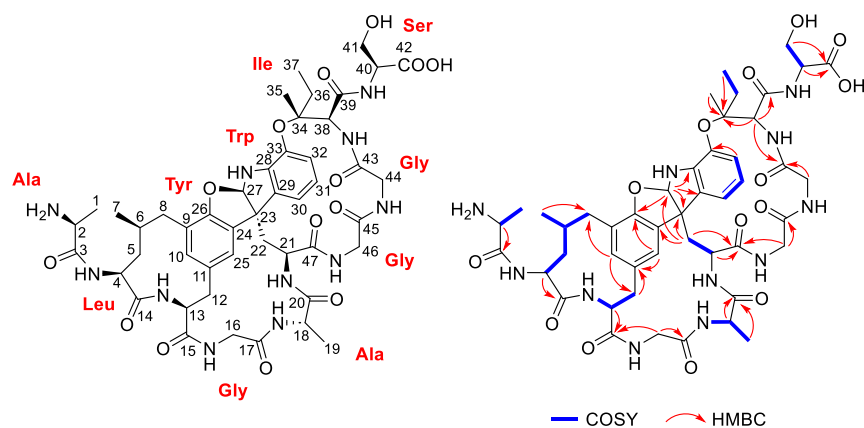
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Pro	1	2.38, m	25.3	Ile	34		85.3
		1.81, m					
	2	4.26, m	56.7		35	1.35, s	18.6
	3		175.0		36	1.79, ov.	31.4
	48	2.25, m	29.1		37	0.89, ov.	7.7
	49		182.3		38	4.70	62.2
	NH	7.80, s			39		172.6
Leu	4	4.08, m	54.1		NH	7.96, m	
	5	1.67, m	35.3	Gly	43		170.9
		1.51, m			44	3.96, ov.	43.5
	6	2.01, ov.	29.8			3.72, ov.	
	7	0.89, ov.	7.7		NH	7.66, br s	
	8	2.29, ov.	35.0				
		1.70, ov.					
	14		168.1				
Tyr	NH	7.85, br s		Gly	45		171.6
	9		120.5		46	3.91, ov.	42.8
	10	6.64, s	128.7			3.80, m	
	11		131.8		NH	8.53	
	24		128.6	Ala	18	4.56	48.0
	25	7.39, s	117.2		19	1.10, d (6.9)	15.9
	26		157.4		20		173.8
	12	4.77	72.1		NH	8.12, br s	
	13	3.71, ov.	55.5	Gly	16	3.72, ov.	43.5
	15		174.2			3.47, m	
	NH				17		170.1
Trp	27	6.36, s	101.4		NH	7.60, t (6.2)	
	23		61.4				
	28		138.8				
	NH	8.53, t (5.8)					
	29		133.5				
	30	6.68, ov.	116.6				
	31	6.54, m	120.6				
	32	6.71, ov.	119.5				
	33		140.7				
	21	3.81, ov.	52.5				
	22	2.74, d (17.4)	36.6				
		2.06, m					
	47		175.8				
	NH	8.66, s					

Supplementary Table 11. NMR data of 4.



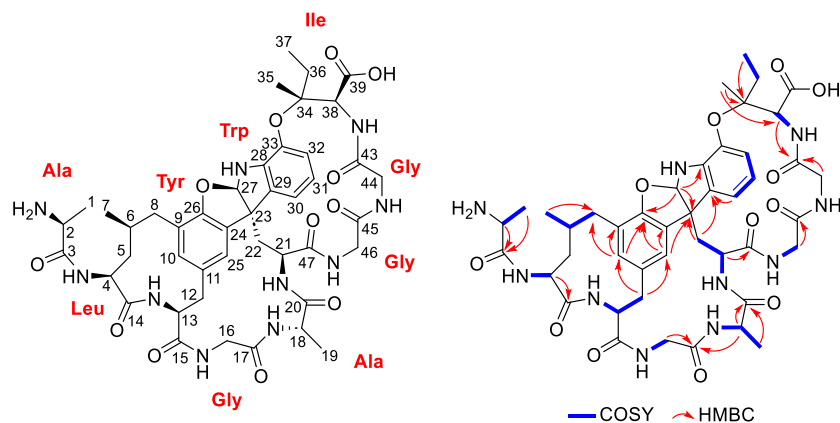
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Pro	1	2.26, m	29.1	Ile	34		85.1
	2	4.26, m	56.6		35	1.33, s	18.5
	3		175.1		36	1.91, ov. 1.76, ov.	30.9
	48	2.40, m 1.82, ov.	25.3		37	0.91, ov.	7.8
	49		182.3		38	3.71, ov.	55.6
Leu	NH	7.81, s			39		172.7
	4	4.09, m	54.1	Ala	NH	7.83, ov.	
	5	1.70, ov. 1.50, m	35.2		43		175.0
	6	2.04, m	29.9		44	4.13, m	50.8
	7	0.90, ov.	7.8		48	1.25, d (7.1)	16.9
	8	2.35, ov. 1.70, ov.	35.0	Gly	NH	8.09, d (4.4)	
					45		170.3
					46	3.73, ov. 3.47, m	43.4
	14		169.1		NH	7.59, t (5.9)	
Tyr	NH			Ala	18	4.55	48.0
	9		120.3		19	1.10, d (6.9)	15.9
	10	6.66, s	128.7		20		173.8
	11		131.7	Gly	NH	8.13, ov.	
	24		128.6		16	3.90, ov. 3.76, ov.	42.4
	25	7.40, s	117.3		17		170.2
	26		157.1				
	12	4.78, ov.	72.0				
	13		56.5				
	15		174.1				
Trp	NH						
	27	6.33, s	101.4				
	23		61.7				
	28		138.6				
	NH	5.93, s					
	29		133.7				
	30	6.69, d (7.4)	115.8				
	31	6.59, t (7.7)	120.5				
	32	6.75, d (8.1)	118.2				
	33		141.0				
	21	3.75, ov.	52.5				
	22	2.73, d (17.4) 2.17, m	37.6				
	47		175.6				
	NH	8.60, s					

Supplementary Table 12. NMR data of 5.



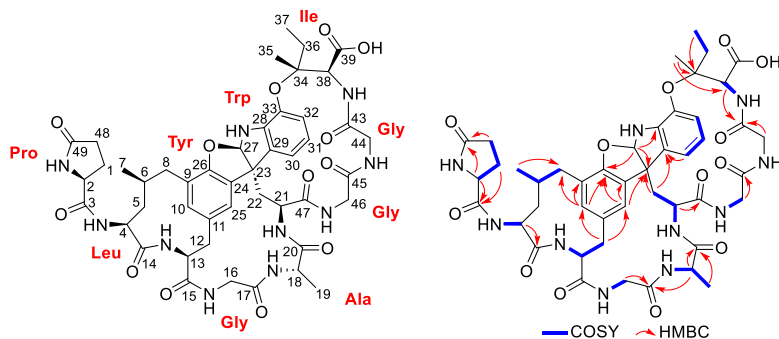
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Ala	1	1.30, d (6.7)	17.1	Ile	34		85.4
	2	3.91, ov.	49.2		35	1.27, s	17.7
	3		172.4		36	1.68, m	31.3
Leu	4	4.15, ov.	54.1		37	0.82, ov.	7.8
	5	1.72, ov.	35.5		38	4.93	61.3
		1.56, ov.					
	6	2.08, ov.	29.5		39		169.5
	7	0.82, ov.	7.8	Ser	40	3.69, ov.	62.1
	8	2.67, m	36.2		41	4.12, ov.	57.5
	14		169.5		42		175.5
	9		121.0	Gly	43		170.3
Tyr	10	6.65, s	131.9		44	3.95, ov.; 3.66, ov.	43.2
	11		129.6	Gly	45		170.3
	12	1.55, m; 1.72, m	35.5		46	3.74, ov.; 3.56, ov.	43.5
	13	3.88, ov.	51.8		NH	8.17, s	
	15		175.6	Ala	18	4.54	48.1
	24		128.5		19	1.12, d (6.8)	15.8
	25	7.03, s	121.1		20		174.0
	26		156.7	Gly	16	3.87, ov.	42.8
	21	3.70, ov.	52.7		17		171.7
Trp	22	2.08	37.1		NH	8.21, s	
		2.70					
	23		61.3				
	27	6.30, s	100.9				
	28		138.9				
	29		133.5				
	30	6.57	116.6				
	31	6.35	120.5				
	32	6.49	119.6				
	33		140.5				
	47		175.8				

Supplementary Table 13. NMR data of 6.



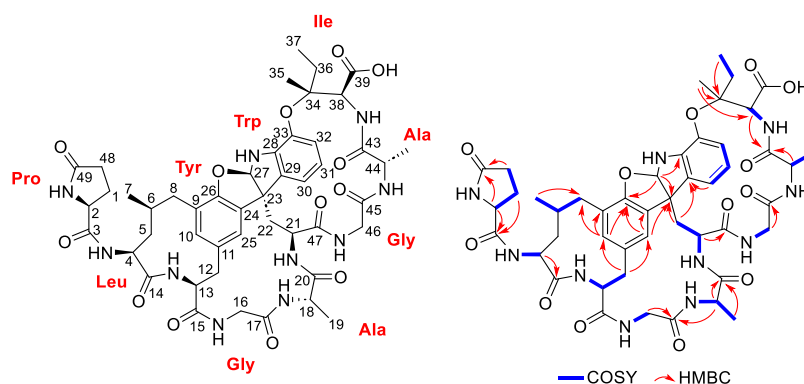
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Pro	1	2.17, ov.	29.4	Ile	34		84.2
	2	4.22, m	54.1		35	1.58, s	19.0
	3		173.8		36	1.98, m	31.6
Leu	4	4.43, m	52.4		37	1.13, ov.	7.6
	5		23.9		38	4.99, s	60.4
Tyr	6	2.24, ov.	29.6	Gly	39		171.2
	7	1.14, ov.			43		170.8
	8	2.48, ov.	34.9	Gly	44	4.00, m	43.4
	14				45	3.90, ov.	
	9		129.8	Ala	46	4.14, ov.	174.6
	10	6.84, s	133.6		18	4.60, q (7.1)	42.7
	11			Ala	19	1.31, d (7.2)	48.0
	24		129.5		20		15.8
	25	7.12, s	139.0	Gly	16		173.3
	26		156.7		17	3.77, m	43.4
Trp	12	1.72, m	36.1				170.0
	13		51.7				
	15		174.3				
	27	6.36, s	101.1				
	23		61.3				
	28		139.7				
	29		133.6				
	30	6.75, d (7.4)	117.1				
	31	6.65, t (7.8)	120.8				
	32	6.80, d (8.1)	119.9				
	33		140.7				
	21	3.91, ov.	52.5				
	22	2.82, d (15.3)	37.4				
		2.30, ov.					
	47		170.4				

Supplementary Table 14. NMR data of 7.



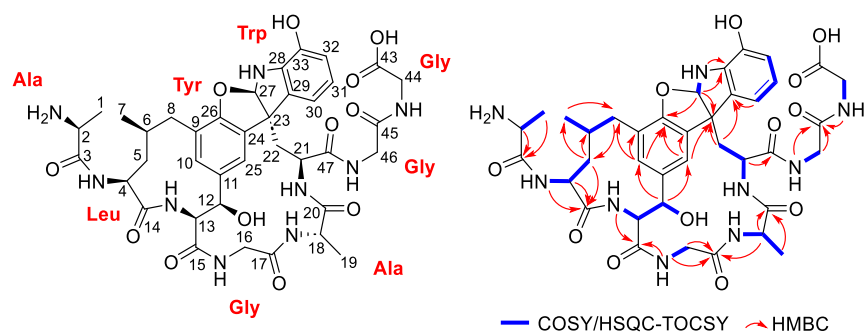
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Pro	1	2.53, ov. 2.07, m	25.4	Ile	34		84.2
	2	4.35, dd (8.7, 4.1)	56.6		35	1.58, s	18.7
	3		173.8		36	1.98, m	32.1
	48	2.40, m 2.35, m	29.0		37	1.13, ov.	7.1
	49		180.5		38	4.99, s	59.7
Leu	4	4.43, m	53.5		39		171.2
	5		23.9	Gly	43		170.8
					44	4.00, m 3.90, ov.	43.2
	6	2.24, ov.	29.8	Gly	45		174.6
	7	1.14, ov.			46	4.14, ov.	43.2
	8	2.48, ov.	34.9	Ala	18	4.60, q (7.1)	48.2
	14				19	1.31, d (7.2)	16.0
Tyr	9		129.7		20		173.3
	10	6.84, s	131.3	Gly	16	3.77, m	43.4
	11				17		170.0
	24		129.5				
	25	7.12, s	139.7				
	26		157.6				
	12	1.72, m	35.8				
	13		50.6				
	15		174.3				
Trp	27	6.44, s	101.0				
	23		61.4				
	28		139.7				
	29		133.7				
	30	6.75, d (7.4)	116.4				
	31	6.65, t (7.8)	119.8				
	32	6.80, d (8.1)	118.9				
	33		140.7				
	21	3.91, ov.	52.5				
	22	2.82, d (15.3) 2.30, ov.	37.4				
	47		170.4				

Supplementary Table 15. NMR data of 8.



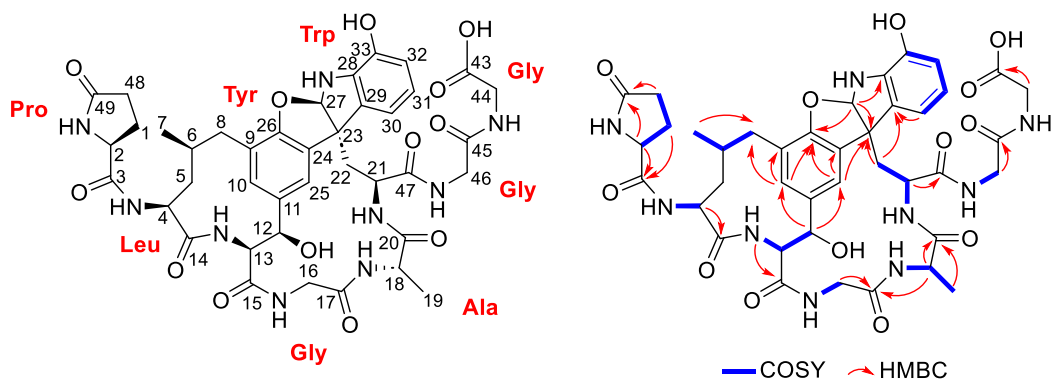
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Pro	1	2.53, ov. 2.07, m	25.4	Ile	34		84.2
	2	4.35, dd (8.7, 4.1)	56.6		35	1.58, s	18.7
	3		173.8		36	1.98, m	32.1
	48	2.40, m 2.35, m	29.0		37	1.13, ov.	7.1
	49		180.5		38	4.99, s	59.7
Leu	4	4.43, m	53.5	Gly	39		171.2
	5		23.9		43		170.8
					44	4.00, m 3.90, ov.	43.2
	6	2.24, ov.	29.8		45		174.6
	7	1.14, ov.			46	4.14, ov.	43.2
Tyr	8	2.48, ov.	34.9	Ala	18	4.60, q (7.1)	48.2
	14				19	1.31, d (7.2)	16.0
	9		129.7		20		173.3
	10	6.84, s	131.3		16	3.77, m	43.4
	11				17		170.0
Trp	24		129.5				
	25	7.12, s	139.7				
	26		157.6				
	12	1.72, m	35.8				
	13		50.6				
	15		174.3				
	27	6.44, s	101.0				
	23		61.4				
	28		139.7				
	29		133.7				
	30	6.75, d (7.4)	116.4				
	31	6.65, t (7.8)	119.8				
	32	6.80, d (8.1)	118.9				
	33		140.7				
	21	3.91, ov.	52.5				
	22	2.82, d (15.3) 2.30, ov.	37.4				
	47		170.4				

Supplementary Table 16. NMR data of 9.



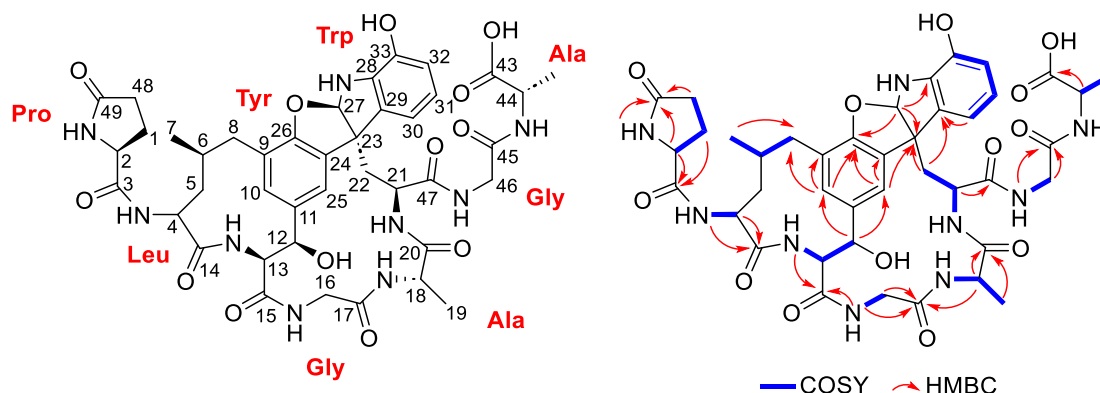
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Ala	1	1.21, ov.	16.6	Gly	43		171.2
	2	3.69, ov.	49.3		44	3.70, ov.	42.9
	3		175.8			3.86, ov.	
Leu	4	4.07, m	54.4		NH	8.24, s	
	5	1.76, m	34.6	Gly	45		170.9
		1.60, m			46	3.55, ov.	42.9
	6	2.13, m	30.3		NH	7.61, s	
	7	0.90, br s	7.7	Ala	18	4.58	48.7
	8	2.35, m	35.3		19	1.26, ov.	16.6
	14		173.6		20		174.9
Tyr	9		117.2	Gly	16	3.80, ov.	43.7
	10	6.56, s	128.9			3.52, ov.	
	11		132.5		17		171.3
	24		129.9				
	25	7.33, s	117.6				
	26		156.7				
	12	4.67	71.6				
	13	3.65, ov.	55.8				
	15		173.6				
Trp	27	6.23, s	102.1				
	23		61.0				
	28		135.0				
	NH	6.19, s					
	29		134.7				
	30	6.81, ov.	114.5				
	31	6.67, t (7.4)	122.0				
	32	6.58, ov.	115.5				
	33		141.6				
	21	3.26, m	51.4				
	22	2.48, d (17.7)	38.2				
		2.97, d (19.2)					
	47		176.2				
	NH	8.75, d (6.5)					

Supplementary Table 17. NMR data of 10.



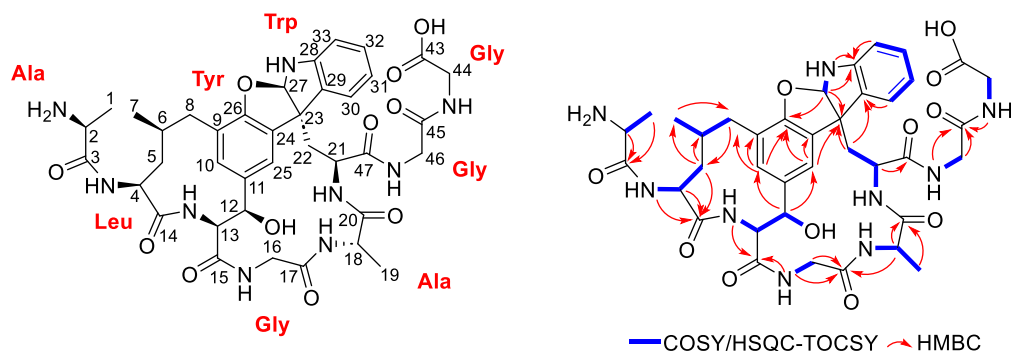
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Pro	1	2.03, m	31.2	Gly	43		173.7
	2	1.97, m			44	4.13, d (16.0)	42.4
		4.48, m	56.1			3.75, d (16.0)	
	3		174.0	Gly	45		170.3
	48	2.04, m	31.5		46	4.02, d (16.3)	43.0
		1.98, m				3.91, d (16.3)	
	49		174.5	Ala	18	4.71, m	48.2
Leu	4	4.10, ov.	53.3		19	1.31, d (7.3)	17.4
	5	1.61, m	34.4	Gly	20		173.1
					16	3.87, ov.	45.0
					17		169.4
	6	2.26, m	28.3				
	7	1.18, d (6.5)	23.2				
	8	2.67, m	34.4				
		2.54, m					
	14						
Tyr	9		120.3				
	10	6.74, ov.	127.6				
	11		130.7				
	24						
	25	7.31, s	116.4				
	26		158.3				
	12	5.43, m	70.9				
	13	3.16, m	61.2				
	15		171.4				
Trp	27	6.58, s	100.9				
	23		61.1				
	28		139.3				
	29		133.7				
	30	6.79, d (7.6)	115.5				
	31	6.61, t (7.8)	119.3				
	32	6.74, ov.	117.4				
	33		158.3				
	21	4.60, m	50.2				
	22	2.43, m	41.6				
	47		174.5				

Supplementary Table 18. NMR data of 11.



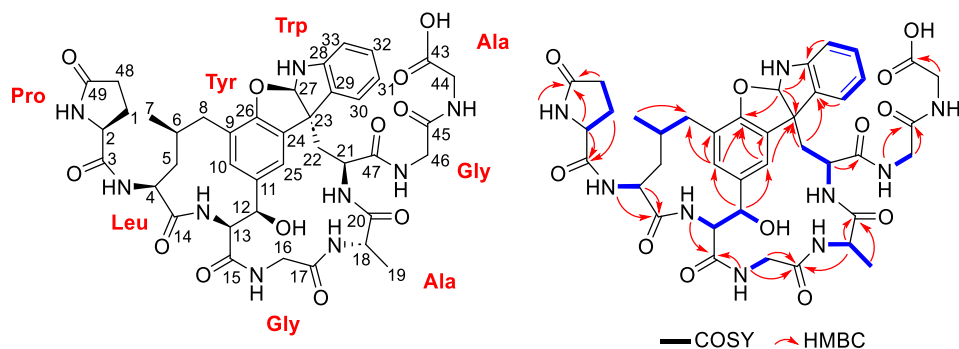
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Pro	1	2.04, m	25.4	Trp	27	6.41, s	102.2
	2	2.49, m			23		61.4
	3	4.33, ov.	56.2		28		135.4
	48	2.39, m	29.0		29		134.1
		2.33, ov.					
	49		180.3		30	6.84, d (7.2)	113.7
Leu	4	4.26, m	54.2		31	6.62, t (7.7)	120.2
	5	1.78, m	35.4		32	6.56, d (7.1)	114.3
	6	2.28, m	30.5		33		141.8
	7	1.14, m	23.9		21	3.92, m	56.3
					22	3.15, m	39.5
	8	2.60, m	35.4			2.55, ov.	
		2.53, ov.			47		172.6
	14			Gly	43		175.6
Tyr	9		130.7		44	4.34, ov.	48.7
	10		128.2	Gly	45		169.9
	11		130.9		46	4.12, m	42.6
						3.63, d (17.3)	
	24			Ala	18	4.77, m	48.5
	25	7.43, s	118.1		19	1.40, d (7.4)	16.4
	26		158.0		20		173.7
	12	4.83, ov.	72.4	Gly	16	3.85, d (17.8)	43.6
						3.69, d (17.8)	
	13				17		172.6
	15		173.4				

Supplementary Table 19. NMR data of 12.



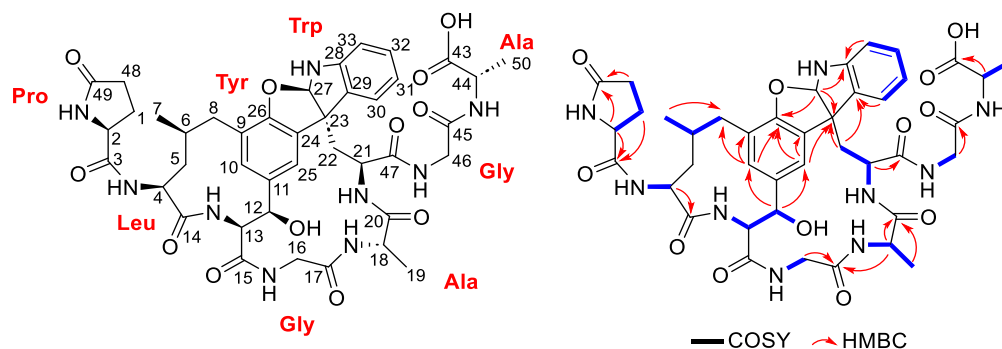
Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Ala	1	1.19, ov.	20.0	Gly	43		174.6
	2	3.48, ov.	49.4		44	3.49, ov.	41.4
	3		173.9			3.58, ov.	
Leu	4	4.29, br s	51.7		NH	7.88, s	
	5	1.37, m	35.9	Gly	45		170.8
		1.50, m			46	3.33, ov.	43.1
	6	2.21, br s	29.1		NH	7.71, s	
	7	1.05, br s	24.2	Ala	18	4.55, m	47.1
	8	2.30, m	34.5		19	1.22, d (6.8)	16.8
		2.41, m			20		171.8
	14		167.9		NH	8.18, s	
Tyr	NH	8.04, s		Gly	16	3.77, ov.	41.9
	9		118.8			3.63, ov.	
	10	6.80, s	127.0		17		168.3
	11		131.9		NH	7.90, s	
	24		129.2				
	25	7.22, s	117.5				
	26		156.9				
	12	4.71, br s	70.8				
	13	3.72, ov.	55.3				
	15		171.2				
Trp	NH	8.32, s					
	27	6.22, s	100.5				
	23		59.7				
	28		147.5				
	NH	7.36, s					
	29		132.7				
	30	6.53, d (7.5)	107.4				
	31	6.59, t (7.5)	117.3				
	32	6.96, t (7.5)	127.3				
	33	7.15, d (6.5)	121.9				
	21	3.19, ov.	50.5				
	22	2.44, m	38.1				
		2.81, ov.					
	47		171.6				
	NH	8.67, s					

Supplementary Table 20. NMR data of 13.



Amino Acids	No.	δ_H (<i>J</i> in Hz)	δ_C	Amino Acids	No.	δ_H (<i>J</i> in Hz)	δ_C
Pro	1	2.49, ov. 2.04, ov.	25.4	Gly	43		172.3
	2	4.33, m	56.3		44	3.78, d (17.5) 4.02, d (17.5)	40.8
	3		174.0	Gly	45		170.6
	48	2.38, m 2.32, m	29.0		46	4.13, d (17.0) 3.70, ov.	42.5
	49		180.3				
Leu	4	4.26, m	54.2	Ala	18	4.75, m	48.5
	5	1.78, m	35.5		19	1.38, d (7.3)	16.3
	6	2.28, ov.	30.5		20		174.0
	7	1.13,		Gly	16	3.86, d (18.0) 3.69, ov.	43.6
	8	2.52, m 2.58, m	35.4		17		169.9
Tyr	14		172.3				
	9		118.1				
	10	6.77, s	128.2				
	11		130.3				
	24		130.8				
Trp	25	7.45, s	118.1				
	26		158.0				
	12	4.83, ov.	72.4				
	13	3.93, m	56.3				
	15		173.4				
	27	6.38, s	101.8				
	23		60.6				
	28		148.1				
	29		132.5				
	30	7.26, d (7.4)	122.3				
	31	6.70, t (7.4)	118.7				
	32	7.00, t (7.7)	127.8				
	33	6.62, d (7.7)	108.7				
	21	3.45, m	51.4				
	22	2.59, m	39.2				
	47		172.5				

Supplementary Table 21. NMR data of 14.



Amino Acids	No.	δ_H (J in Hz)	δ_C	Amino Acids	No.	δ_H (J in Hz)	δ_C
Pro	1	2.04, m	25.4	Gly	43		175.0
	2	4.33, ov.	56.4		44	4.37, q (7.3)	48.3
	3		174.4		50	1.47, d (7.3)	16.0
	48	2.33, ov. 2.39, m	29.1				
	49		180.3	Gly	45		170.0
Leu	4	3.93, ov.	56.3		46	4.12, m 3.63, m	42.5
	5	1.78, m 1.57, m	35.3	Ala	18	4.78, q (7.2)	48.5
	6	2.04, m	25.4		19	1.40, d (7.2)	16.4
	7	1.13, ov.			20		173.8
	8	2.52, ov.	35.5	Gly	16	3.85, ov. 3.69, ov.	43.7
	14		171.8		17		169.9
Tyr	9		119.9				
	10	7.46, s	118.1				
	11		134.0				
	24		130.8				
	25	6.77, s	128.2				
	26		158.0				
	12	4.84, ov.	72.6				
	13	3.93, ov.	56.3				
	15		173.5				
Trp	27	6.38, s	101.9				
	23		60.6				
	28		148.1				
	29		132.5				
	30	7.26, d (7.5)	122.3				
	31	6.70, t (7.4)	118.7				
	32	7.00, ov.	127.8				
	33	6.62, d (7.8)	108.7				
	21	3.44, m	51.6				
	22	2.57, ov.	39.5				
	47		172.9				

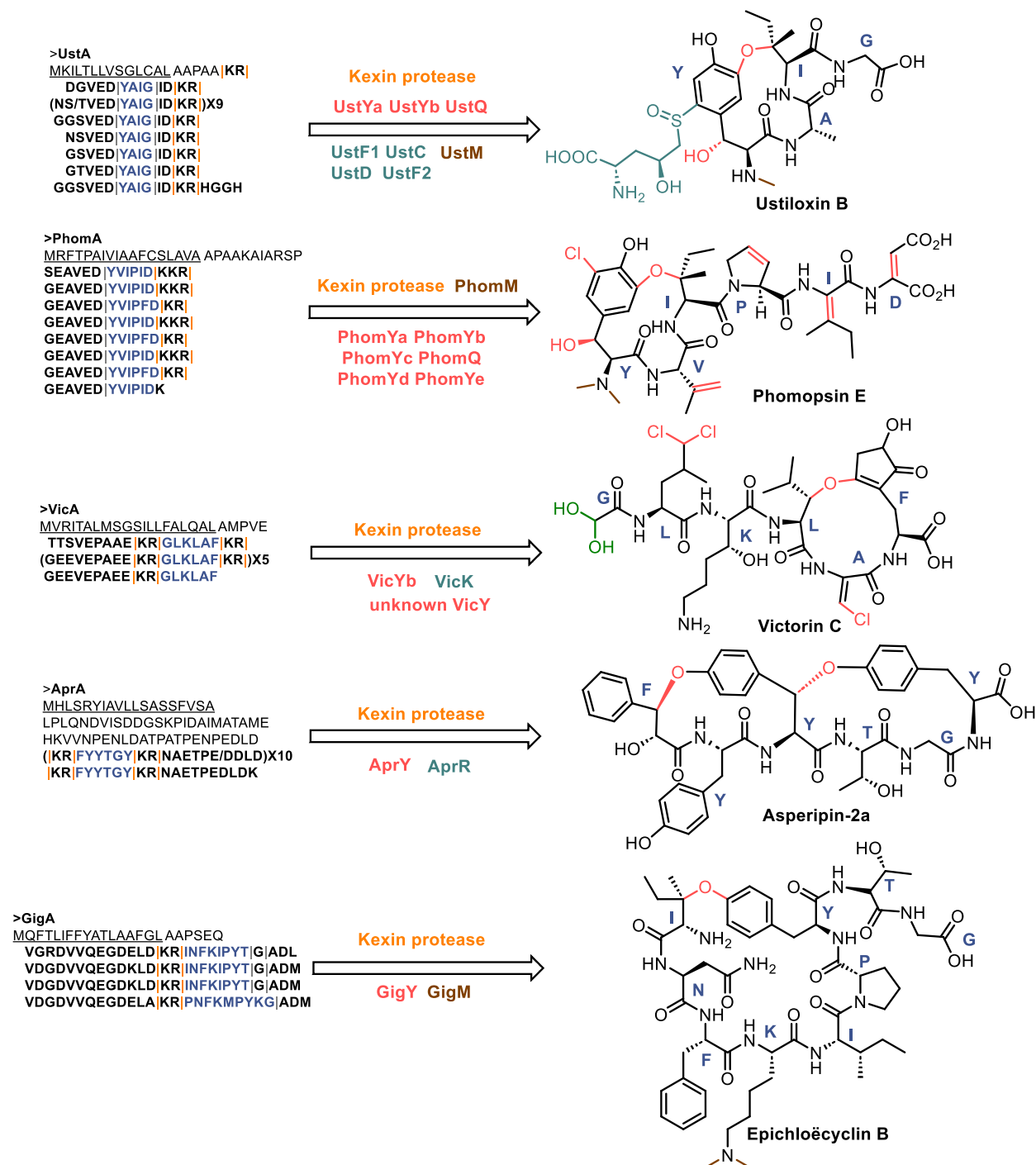
Supplementary Table 22. HRMS data of asperigimycin derivatives.

Compound	Molecular Formula	Experimental Mass [M + H] ⁺	Calculated Mass [M + H] ⁺
Asperigimycin A	[C ₄₇ H ₆₂ N ₁₁ O ₁₅] ⁺	1020.4413	1020.4421
Asperigimycin B	[C ₄₄ H ₅₇ N ₁₀ O ₁₃] ⁺	933.4097	933.4101
Asperigimycin C	[C ₄₆ H ₅₇ N ₁₀ O ₁₄] ⁺	973.4047	973.4050
Asperigimycin D	[C ₄₇ H ₅₉ N ₁₀ O ₁₄] ⁺	987.4206	987.4207
3-Q1H	[C ₄₇ H ₅₉ N ₁₂ O ₁₃] ⁺	999.4321	999.4325
3-Q1S	[C ₄₄ H ₅₇ N ₁₀ O ₁₄] ⁺	949.4056	949.4056
3-Q1L	[C ₄₇ H ₆₃ N ₁₀ O ₁₃] ⁺	975.4576	975.4575
3-Q1N	[C ₄₅ H ₅₈ N ₁₁ O ₁₄] ⁺	976.4158	976.4165
3-G4S	[C ₄₇ H ₅₉ N ₁₀ O ₁₅] ⁺	1003.4159	1003.4161
3-A5S	[C ₄₆ H ₅₇ N ₁₀ O ₁₅] ⁺	989.4005	989.4005
3-A5T	[C ₄₇ H ₅₉ N ₁₀ O ₁₅] ⁺	1003.4151	1003.4161
3-G7S	[C ₄₇ H ₅₉ N ₁₀ O ₁₅] ⁺	1003.4152	1003.4161
3-G8S	[C ₄₇ H ₅₉ N ₁₀ O ₁₅] ⁺	1003.4153	1003.4161
3-I9L	[C ₄₆ H ₅₇ N ₁₀ O ₁₄] ⁺	973.4054	973.4056
3-I9V	[C ₄₅ H ₅₅ N ₁₀ O ₁₄] ⁺	959.3895	959.3899
5	[C ₄₇ H ₆₂ N ₁₁ O ₁₄] ⁺	1004.4482	1004.4472
6	[C ₄₄ H ₅₇ N ₁₀ O ₁₂] ⁺	917.4157	917.4152
7	[C ₄₆ H ₅₇ N ₁₀ O ₁₃] ⁺	957.4089	957.4101
8	[C ₄₇ H ₅₉ N ₁₀ O ₁₃] ⁺	971.4264	971.4258
9	[C ₃₈ H ₄₈ N ₉ O ₁₂] ⁺	822.3417	822.3417
10	[C ₄₀ H ₄₈ N ₉ O ₁₃] ⁺	862.3342	862.3366
11	[C ₄₁ H ₅₀ N ₉ O ₁₃] ⁺	876.3504	876.3523
12	[C ₃₈ H ₄₈ N ₉ O ₁₁] ⁺	806.3454	806.3468
13	[C ₄₀ H ₄₈ N ₉ O ₁₂] ⁺	846.3412	846.3417
14	[C ₄₁ H ₅₀ N ₉ O ₁₂] ⁺	860.3585	860.3573
15	[C ₄₆ H ₆₀ N ₁₁ O ₁₄] ⁺	990.4321	990.4316
16	[C ₄₇ H ₆₂ N ₁₁ O ₁₄] ⁺	1004.4482	1004.4472
2-L ₁	[C ₄₈ H ₆₃ N ₁₀ O ₁₄] ⁺	1003.4533	1003.4525
2-L ₂	[C ₅₇ H ₆₅ N ₁₀ O ₁₄] ⁺	1113.4681	1113.4682
2-L ₃	[C ₅₅ H ₆₉ N ₁₀ O ₁₄] ⁺	1093.4991	1093.4995
2-L ₄	[C ₅₆ H ₇₉ N ₁₀ O ₁₄] ⁺	1115.5783	1115.5777
2-L ₅	[C ₅₅ H ₇₅ N ₁₀ O ₁₄] ⁺	1099.5465	1099.5464
2-L ₆	[C ₅₅ H ₇₇ N ₁₀ O ₁₄] ⁺	1101.5620	1101.5621
2-L ₇	[C ₆₂ H ₉₁ N ₁₀ O ₁₄] ⁺	1199.6731	1199.6716

Supplementary Table 23. Expression level revealed by Depmap.

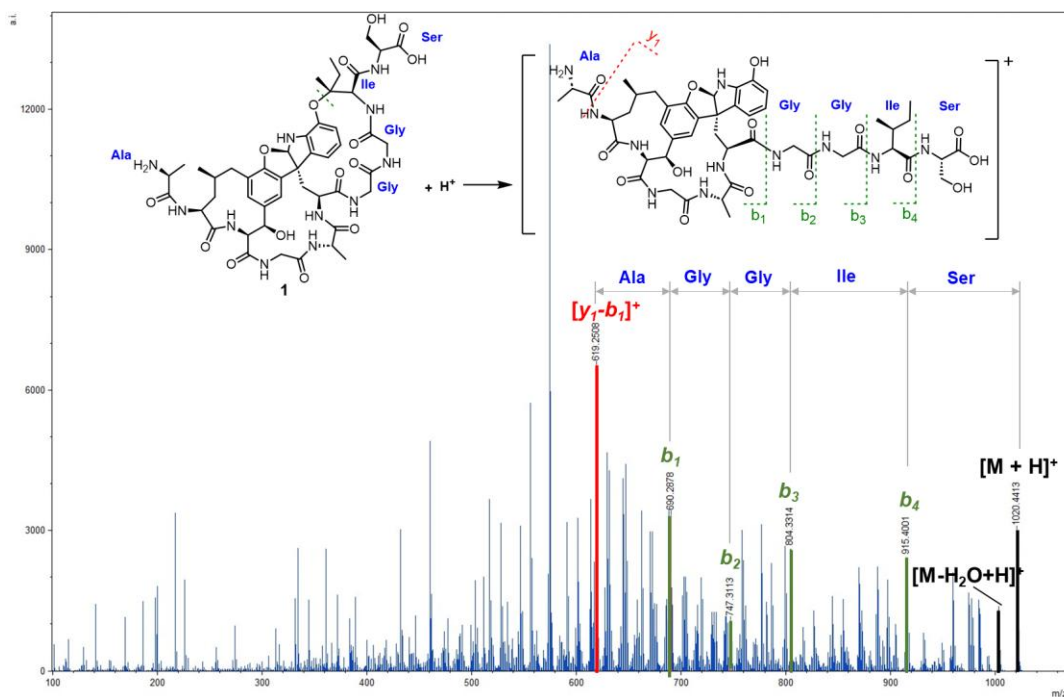
Depmap ID	Expression Public 23Q4	Cell Line Name
ACH-000995	4.148120631	JURKAT
ACH-000220	2.767654798	MINO
ACH-001574	2.49057013	MOLM14
ACH-000551	0.918386234	K562
ACH-000406	0	U937
ACH-000019	2.153805336	MCF7
ACH-001086	0.604071324	HELA
ACH-000739	0	HEPG2

Supplementary Figures

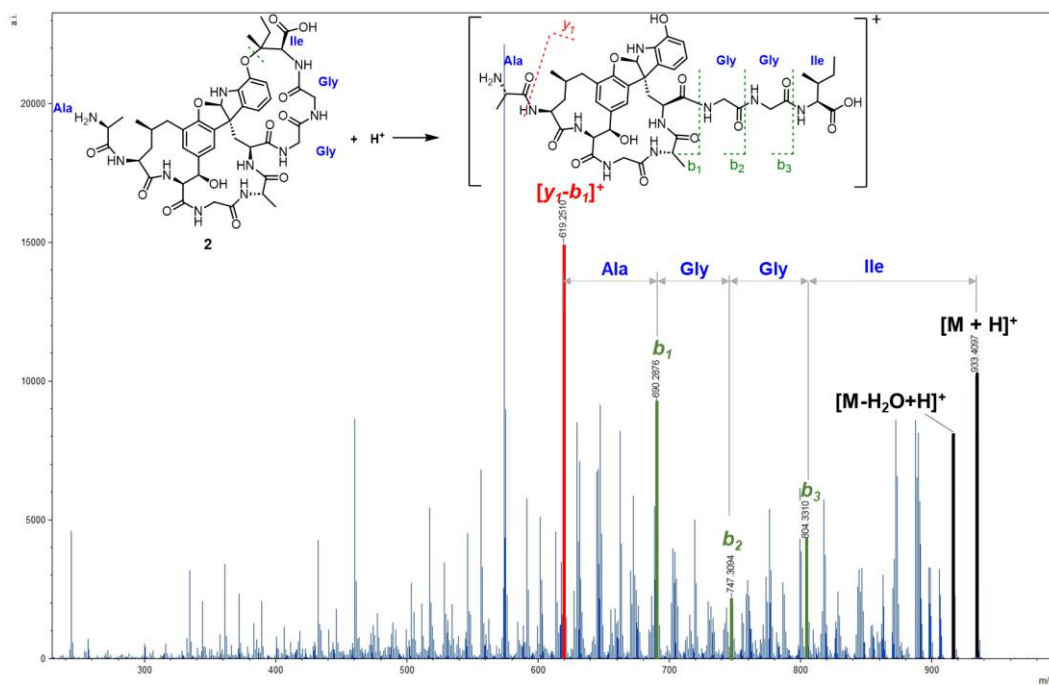


Supplementary Fig. 1. Proposed biosynthetic pathways of known ascomycetes-derived fungal RiPPs.

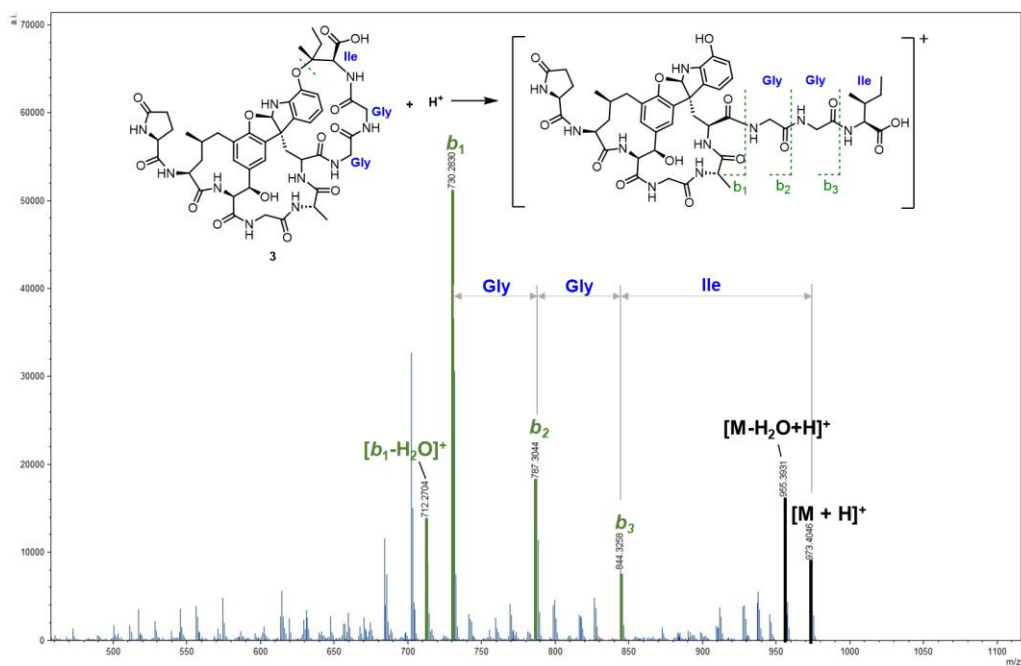
The underlined sequences indicate the proposed signal peptide in precursor peptides. Orange vertical lines mark predicted recognition sites for kexin endopeptidases. The core peptide is highlighted in blue.



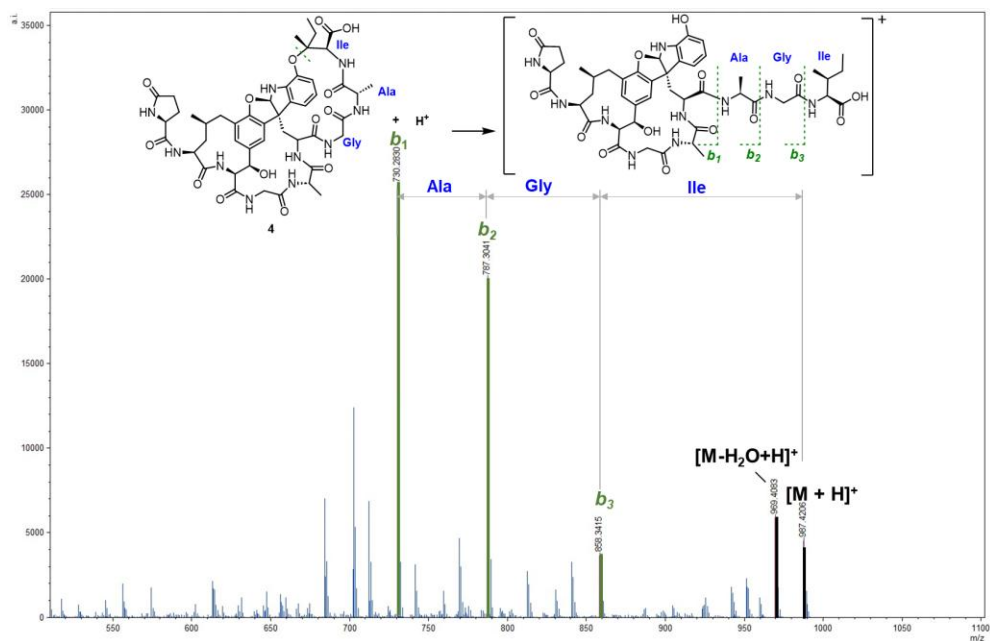
Supplementary Fig. 2. MS/MS spectrum of parent ion m/z 1020 $[M + H]^+$ in Fig. 1a and analysis with determined structure.



Supplementary Fig. 3. MS/MS spectrum of parent ion m/z 933 $[M + H]^+$ in Fig. 1a and analysis with determined structure.



Supplementary Fig. 4. MS/MS spectrum of parent ion m/z 973 $[M + H]^+$ in Fig. 1a and analysis with determined structure.



Supplementary Fig. 5. MS/MS spectrum of parent ion m/z 987 $[M + H]^+$ in Fig. 1a and analysis with determined structure.

```

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GQDNEAIPFYK | KR |
ADNSEAIPFYE | KR |
AGDNEAIPFYQ | RR |
ADSSEAIPFYE | KR |
EESEVIPFYQ | RR |
ADNSEAIPFYE | KR |
AEESEAIPFYQ

>UstA
MKLILTL LVSGLCALAAPAAKR
  DGVEDYAIGID | KR |
(NSVEDYAIGID | KR | ) x4
  NTVEDYAIGID | KR |
  NSVEDYAIGID | KR |
  NTVEDYAIGID | KR |
  NSVEDYAIGID | KR |
  NSVEDYAIGID | KR |
  GGSVEDYAIGID | KR |
  NSVEDYAIGID | KR |
  NSVEDYAIGID | KR |
  GSVEDYAIGID | KKR |
  GTVEDYAIGID | KR |
  GGSVEDYAIGID | KR | HGGH

>XP_041150036.1
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SGDAEAIPYYP | KR |
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SEEAEAIPYYP | KR |
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SEEAEAIPYYP | KR |
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  DQKGTTVDDISEQLYGAWGGI | KR |
  DQGGTTVDDISEALYGAWGGIS | KR |
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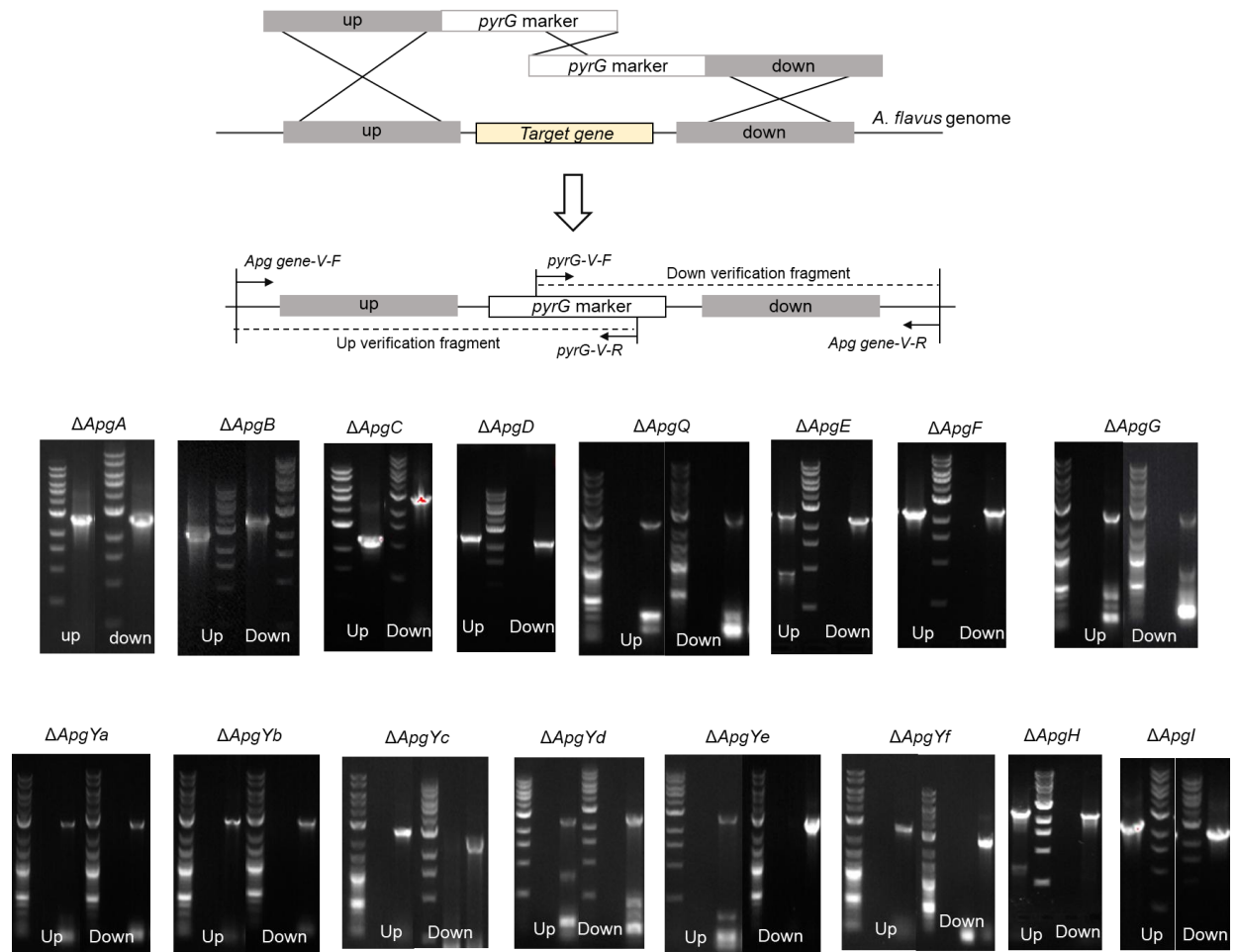
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FYYTGY | KR | NAETPDDL | KR |
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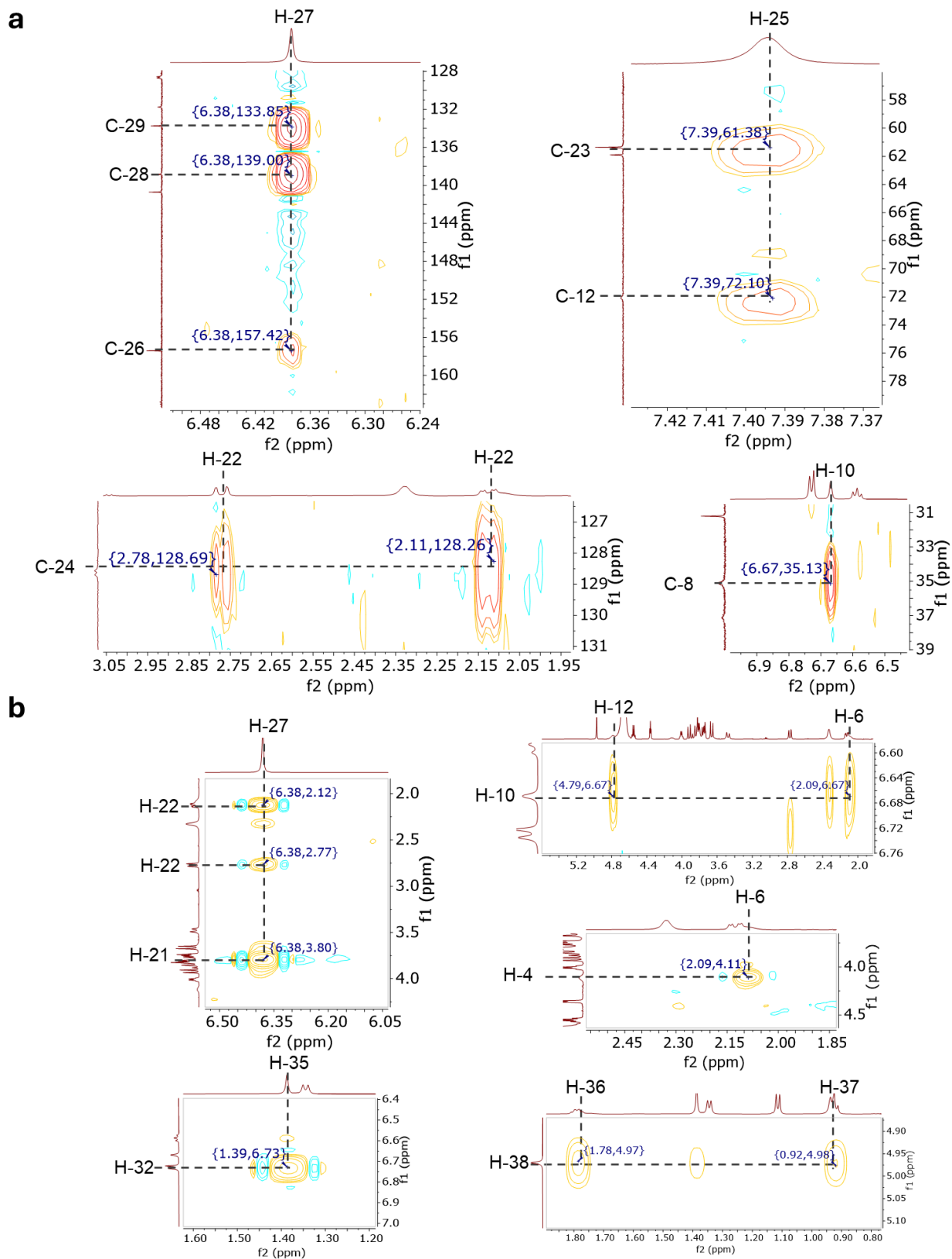
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Supplementary Fig. 6. Six candidate precursor peptides of fungal RiPPs in *Aspergillus flavus* revealed by bioinformatic analysis.

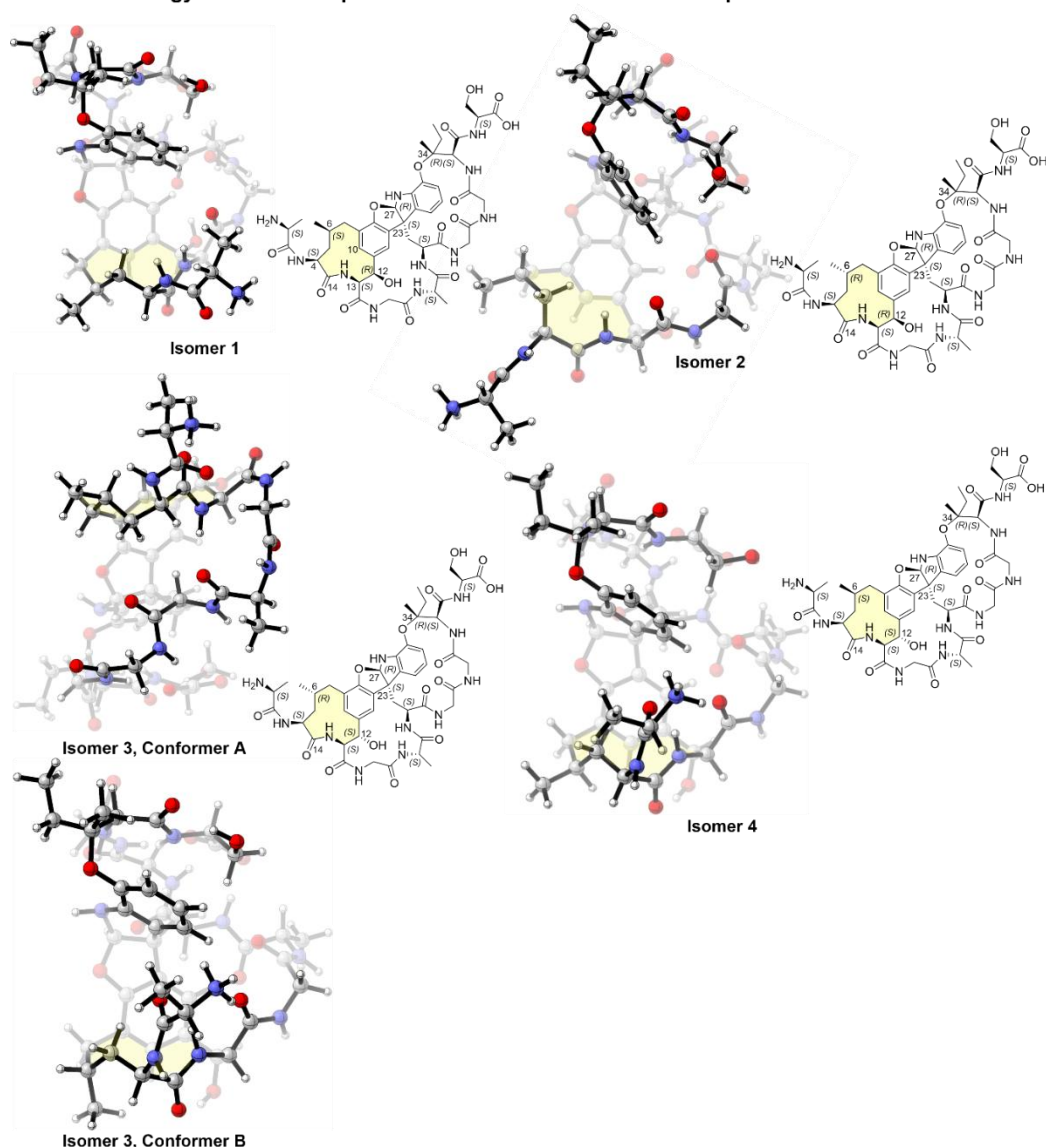
XP_041147057.1 (ApgA), highlighted in the yellow box, is the proposed precursor peptide involved in the asperigimycins biosynthesis. The orange vertical lines indicate predicted recognition sites for kexin endopeptidases.



Supplementary Fig. 7. PCR verification of *apg* mutants. Scheme of the fusion PCR-based gene deletion methods using a split marker strategy with *pyrG* as selection marker. Mutants were verified by PCR with primers listed in Table 3.

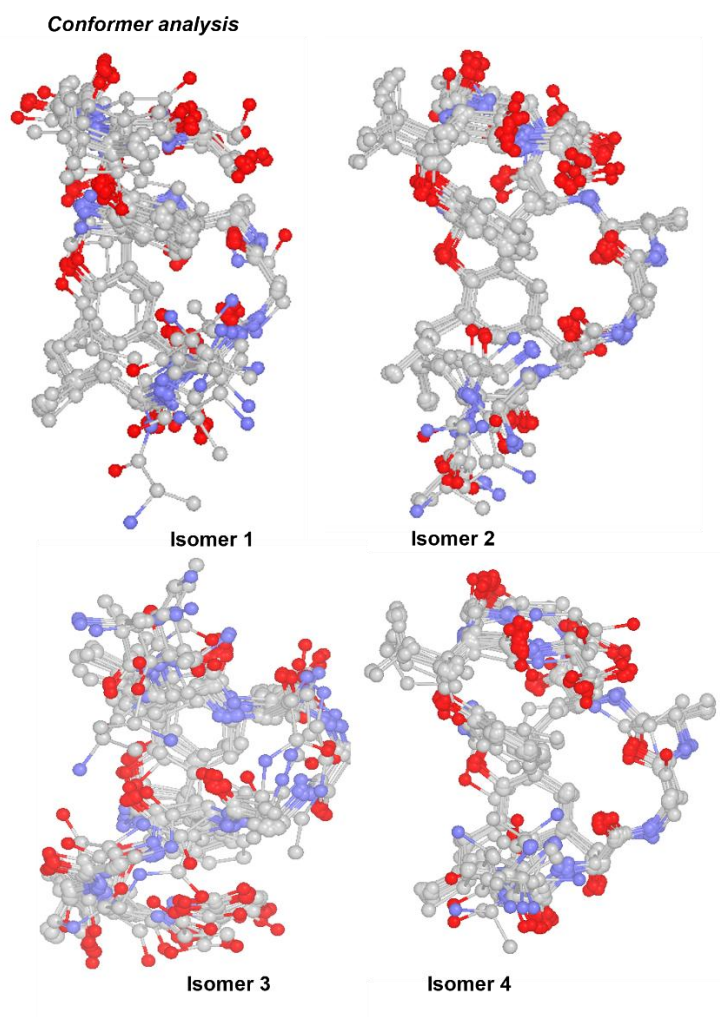


Lowest energy conformer of potential stereoisomers 1 to 4 of compound 1

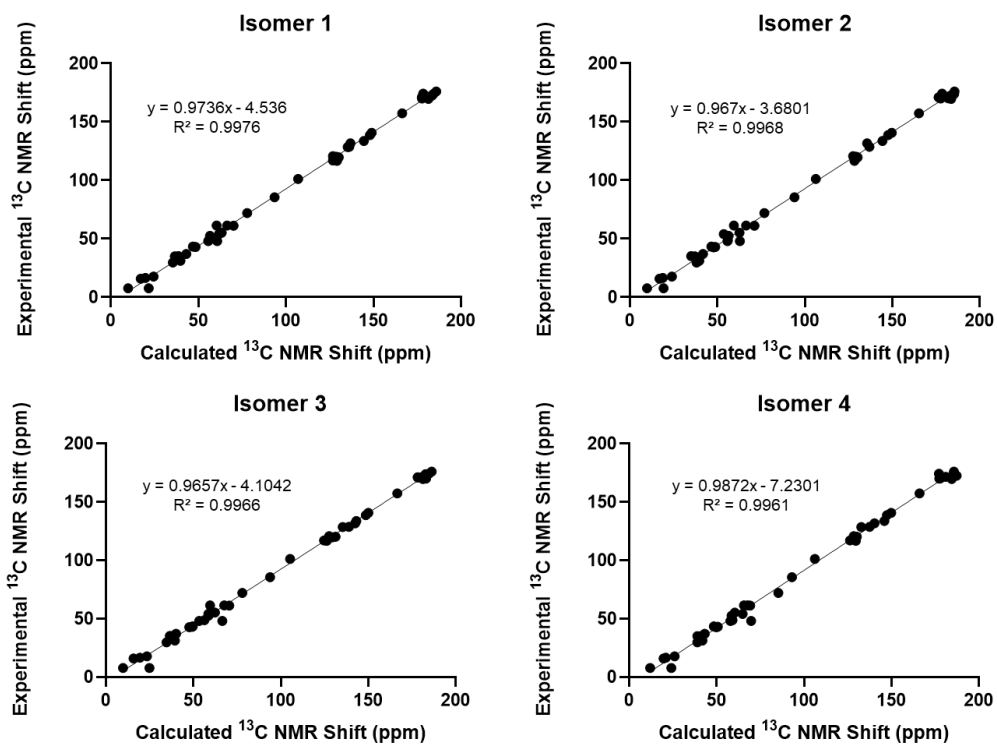


Supplementary Fig. 9. Lowest-energy conformer of four plausible stereoisomers of compound 1.

In the lowest-energy conformer of Isomer 1, the substituents on C6 (Me), C4 (acylamino), and C12 (OH) are all in the pseudoequatorial positions, whereas in Isomer 2 and 4 that each involves an inverted stereocenter, the C6-Me and C12-OH groups, respectively, are located at the pseudoaxial positions, leading to increased transannular strain within the 11-membered ring. In Isomer 3, the stereocenters at both C6 and C12 are inverted, which would place both C6-Me and C12-OH groups at the pseudoaxial positions, leading to significant transannular strain. Indeed, this conformer (Conformer B) was less stable than its ring-flip conformer (Conformer A) by 9.8 kcal/mol based on DFT calculations. However, in this ring-flipped conformer of Isomer 3, one of the amide bonds adopts the *cis* conformation, which is expected to be disfavoured compared with the other ring-flip conformer as in the lowest-energy conformers of Isomers 1, 2, and 4 that involve *trans* conformation for all amide bonds.

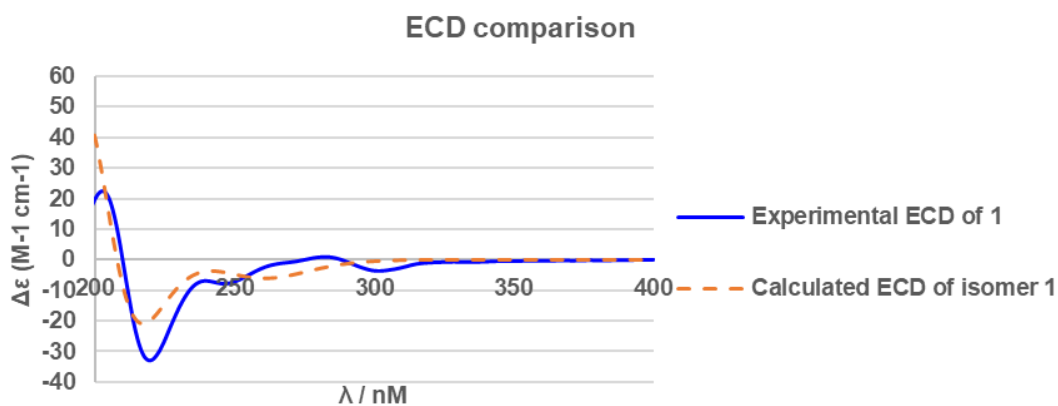


Supplementary Fig. 10. Overlay of generated conformer ensembles of four probable stereoisomers of compound 1.

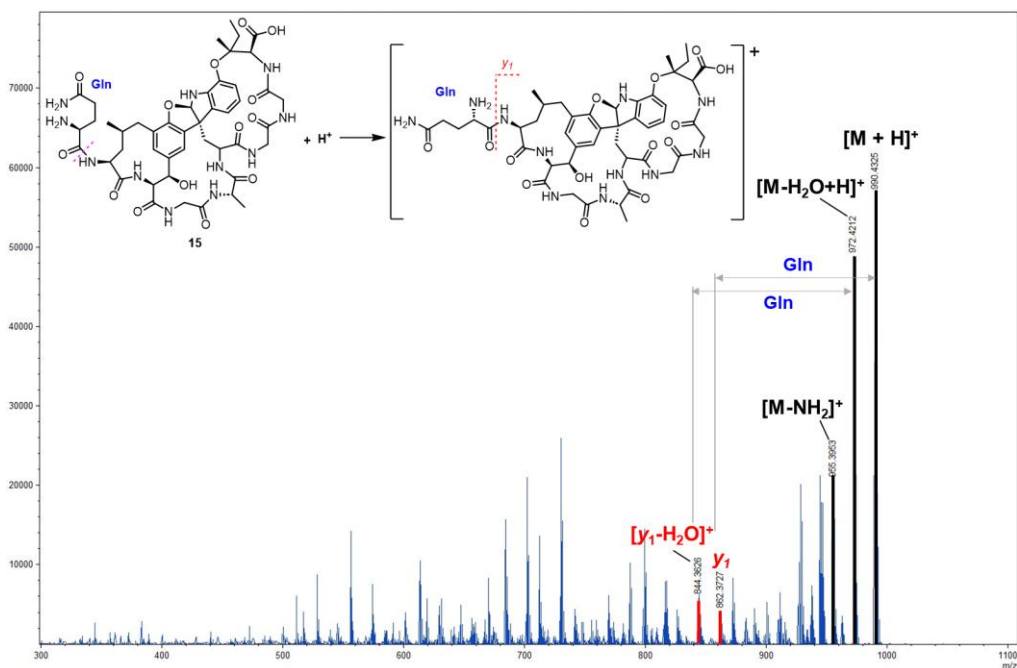


Supplementary Fig. 11. Correlation plots of experimental ^{13}C NMR chemical shifts versus the corresponding calculated data for isomers 1-4.

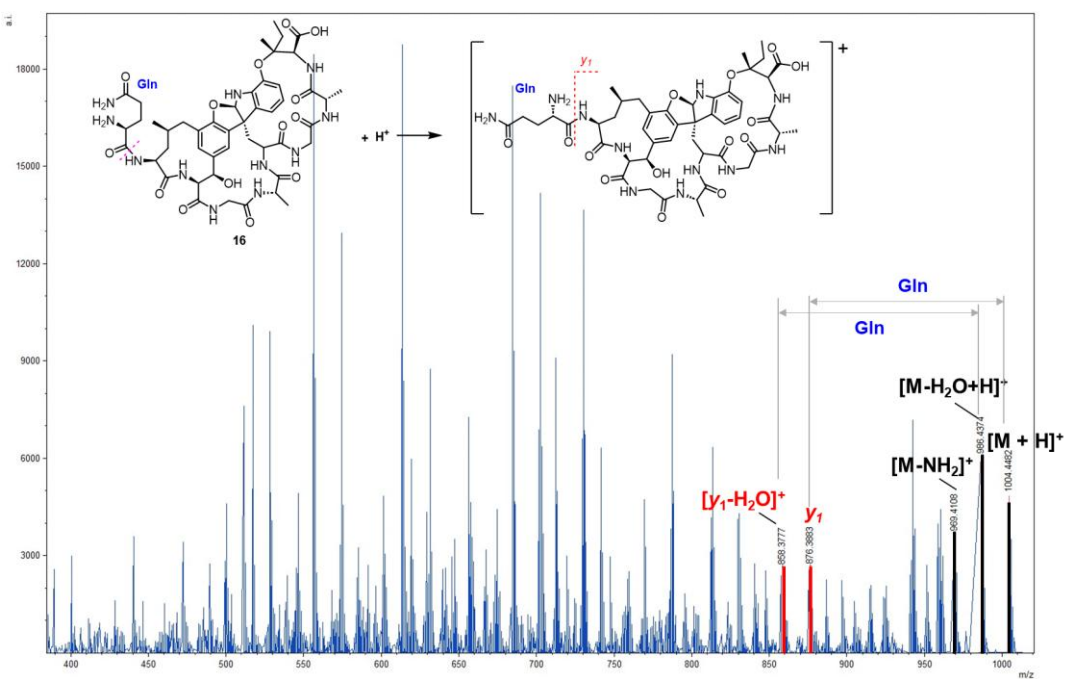
The linear correlation coefficient (R^2 , 0.9976, 0.9968, 0.9966, and 0.9961 for the proposed isomers 1-4, respectively) were slightly in favor of **isomer 1**.



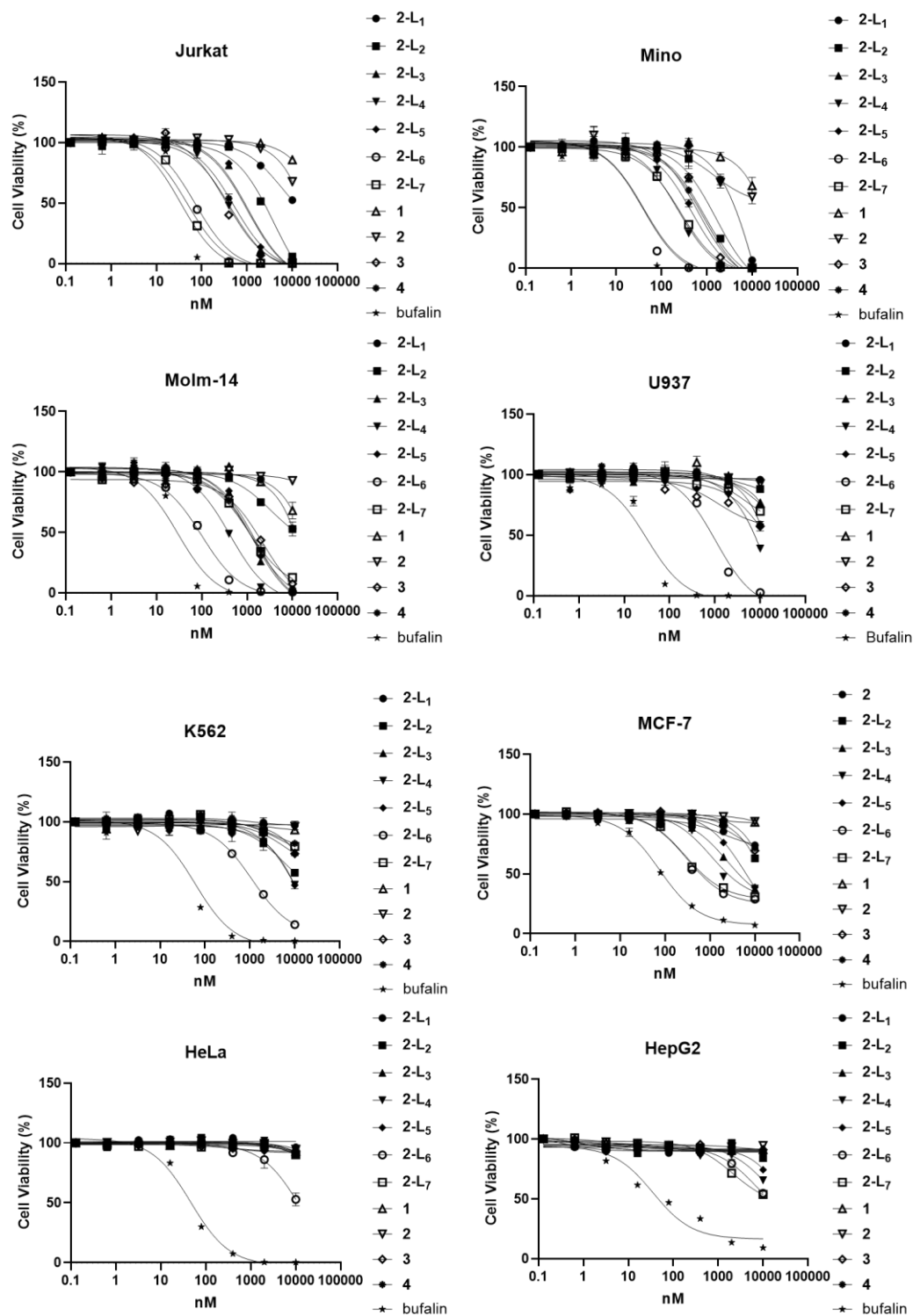
Supplementary Fig. 12. Experimental ECD of 1 and calculated ECD of isomer 1.



Supplementary Fig. 14. Analysis of MS/MS spectrum of 15.

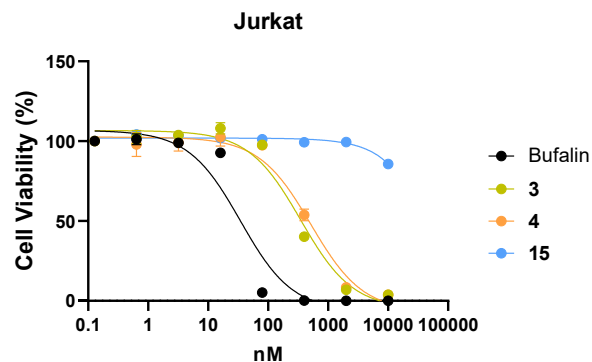


Supplementary Fig. 15. Analysis of MS/MS spectrum of 16.



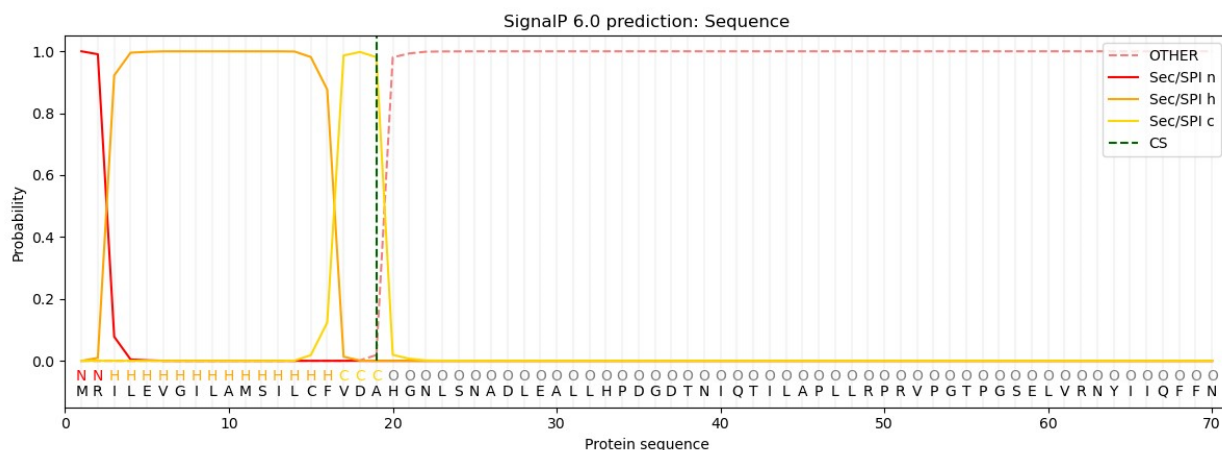
Supplementary Fig. 16. Dose-response curves of cell viability assays.

Data are mean $\pm$ s.d.; n = 3 biologically independent samples.

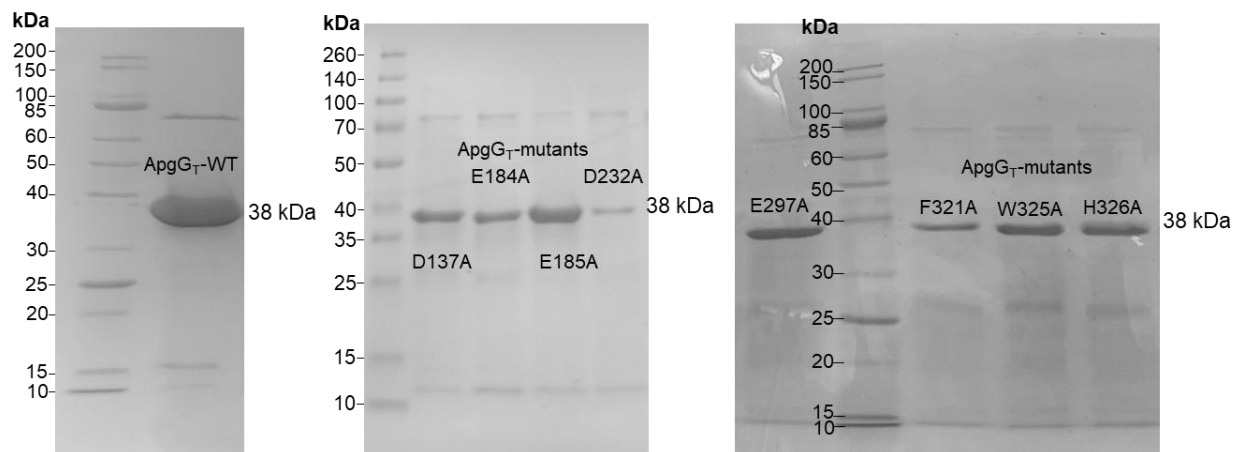


Supplementary Fig. 17. Cytotoxicity of 15 toward Jurkat cells.

15 featuring N-terminal glutamate exhibited no significant cytotoxicity toward Jurkat cells. In contrast, **3** and **4**, which contain an N-terminal pyroglutamate moiety, displayed obvious cytotoxic effects. Bufalin was used as the positive control. Data are mean $\pm$ s.d.; $n=3$ biologically independent samples.

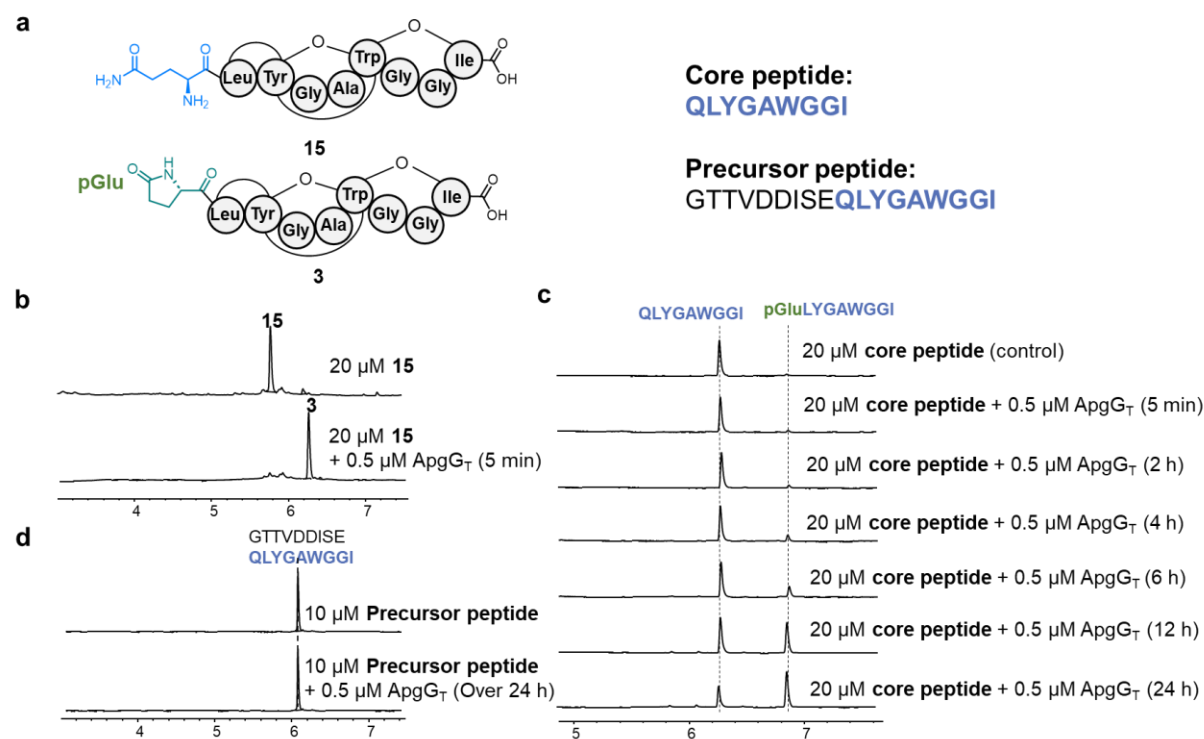


Supplementary Fig. 18. Analysis of signal peptide of ApgG by SignalP 6.0.

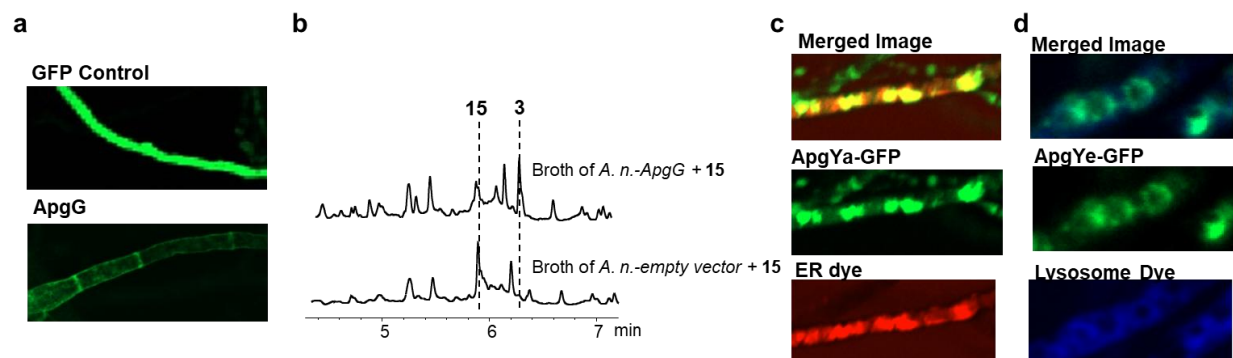


Supplementary Fig. 19. SDS-PAGE analysis of ApgG_T and its mutants.

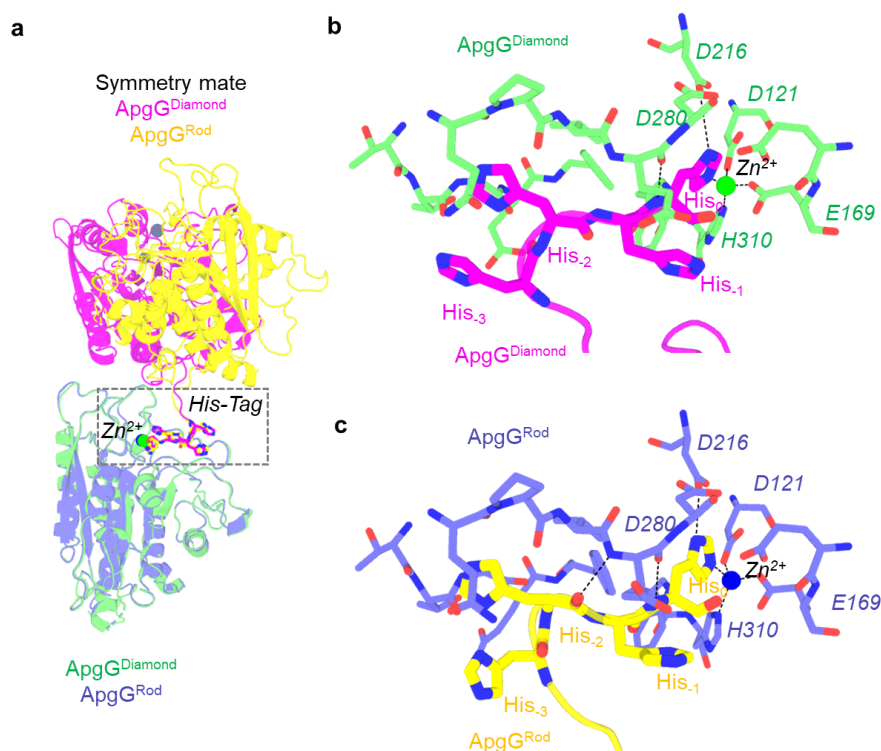
Protein Ladders displayed in the left panel and the right panel are from Millipore (Cat # MPSTD3), the ladder displayed in the middle panel is from Thermo Scientific (Cat # 26634).



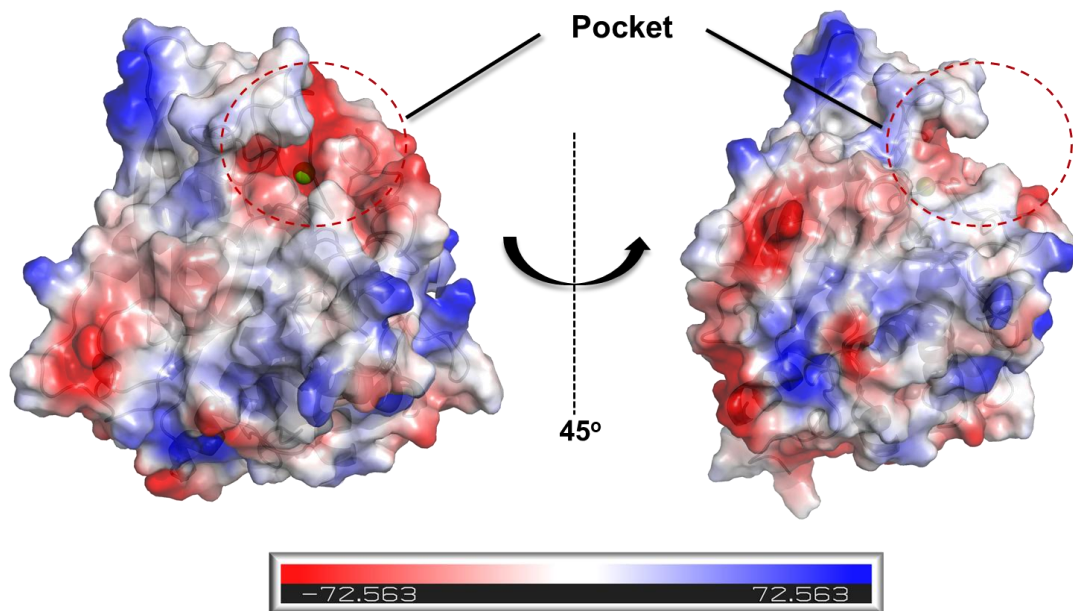
Supplementary Fig. 20. In vitro assays of ApgG with 15 and linear peptides. a. Structures of 15 and 3 and sequences of core peptides and precursor peptides used in vitro assays. **b.** In vitro assays of 15 and ApgG_T. **c.** In vitro assays of the core peptide of compound 3 with ApgG_T. **d.** In vitro assays of the precursor peptide of compound 3 with ApgG_T. All these reactions were incubated at 28°C.



Supplementary Fig. 21. Extracellular cyclization catalyzed by ApgG. **a.** Confocal images showing the subcellular localization of ApgG-GFP fusion proteins in *A. nidulans*. Two times of the experiment were repeated with the same results. **b.** HPLC analysis (280 nM) of the fermentation broth of *A. nidulans*-ApgG fed with **15**. The broth was incubated with **15** for 15 min at 30°C. **c.** Confocal images depicting the subcellular localization of ApgYa: merged images (top), ApgYa localization (middle), and ER localization using ER-Tracker Red (bottom). **d.** Confocal images showing the subcellular localization of ApgYe: merged images (top), ApgYe localization (middle), and lysosome localization using LysoTracker Deep Red (bottom).

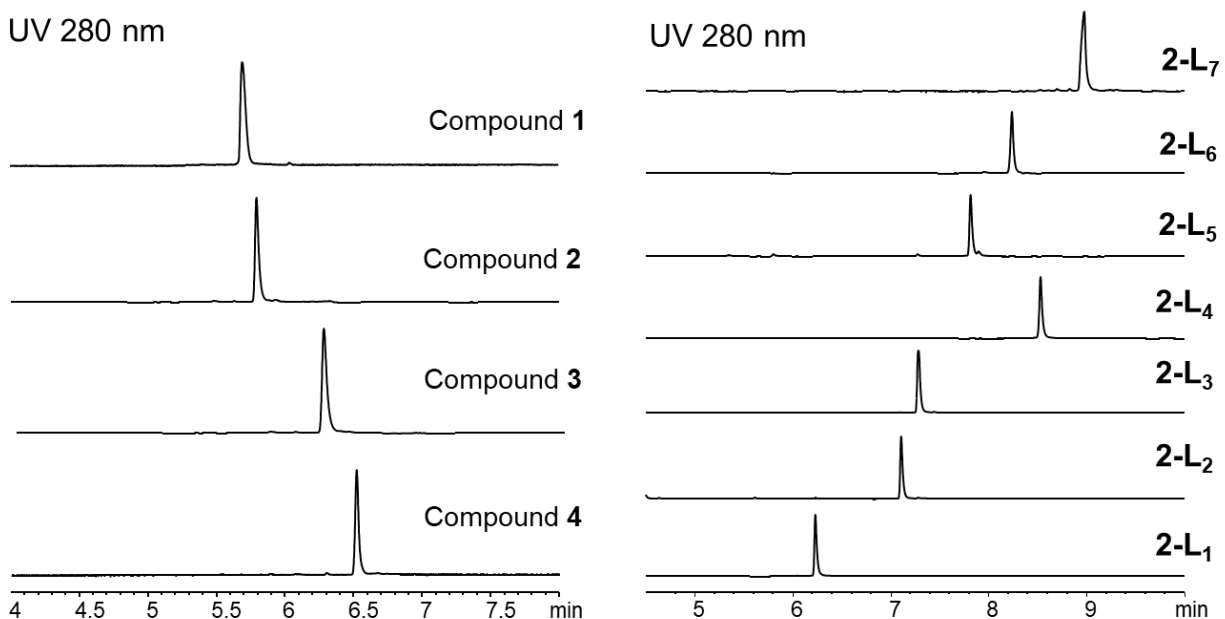


Supplementary Fig. 22. Crystal structures of ApgG^{Diamond} and ApgG^{Rod} as dimers. **a.** Overall structure of a single unit cell of ApgG^{Diamond} and ApgG^{Rod}, each single unit contains two monomers. **b. c.** Active site architectures of ApgG^{Diamond} (Carbon colored in Green) and ApgG^{Rod} (Carbon colored in Purple). The terminal histidine residue (His₀) of the other monomer (Carbon colored in magenta and yellow, respectively in ApgG^{Diamond} and ApgG^{Rod}) is positioned at the catalytic center, where it interacts with the catalytic Zn²⁺ cation and the D216 side chain.



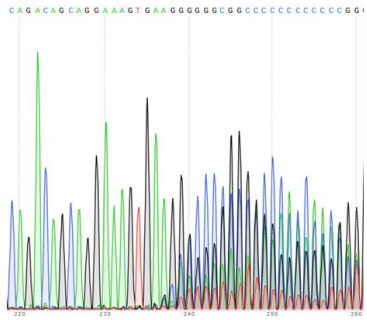
Supplementary Fig. 23. Electrostatic potential surface maps of ApgGr.

The active pocket is outlined in red and appears open and spacious across different surface views (with the left image rotated 45°). Positively charged residues are depicted in deeper shades of blue, while negatively charged residues are shown in deeper shades of red.

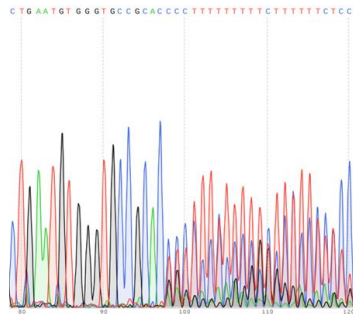


Supplementary Fig. 24. HPLC analysis (280 nm) of compounds for cytotoxicity test.

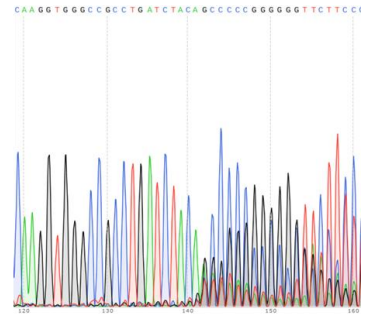
SLC46A3Δ



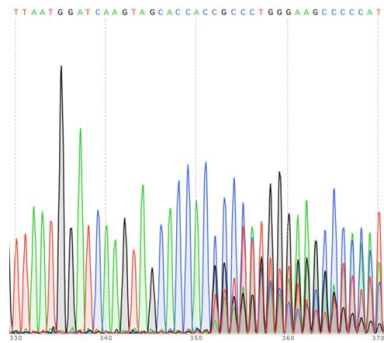
CCNFA



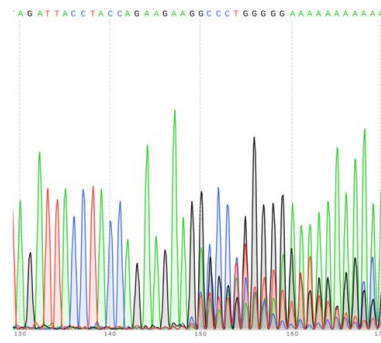
KEAP1Δ



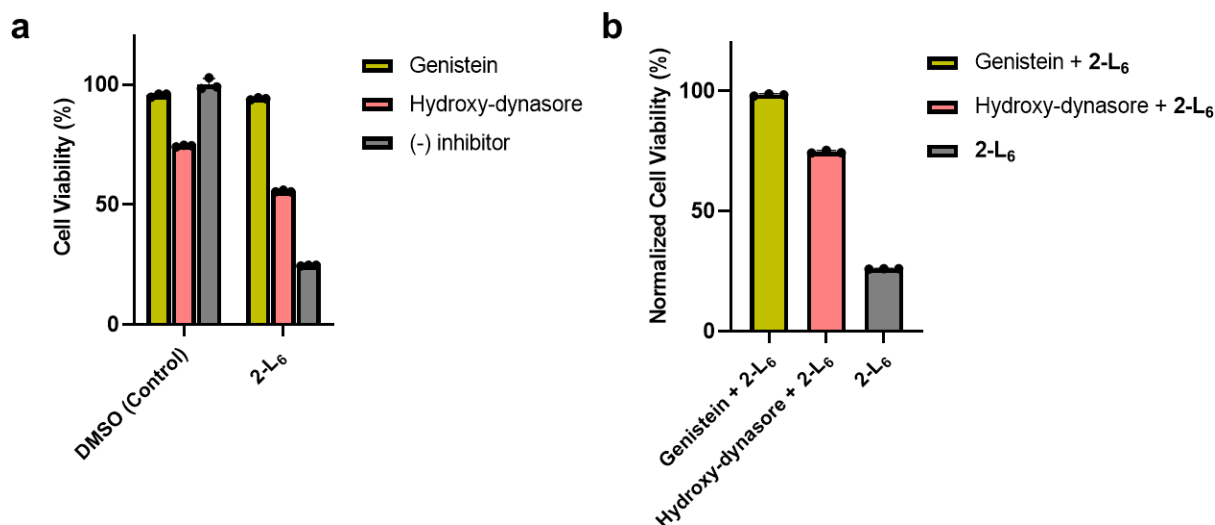
CLAPS2Δ



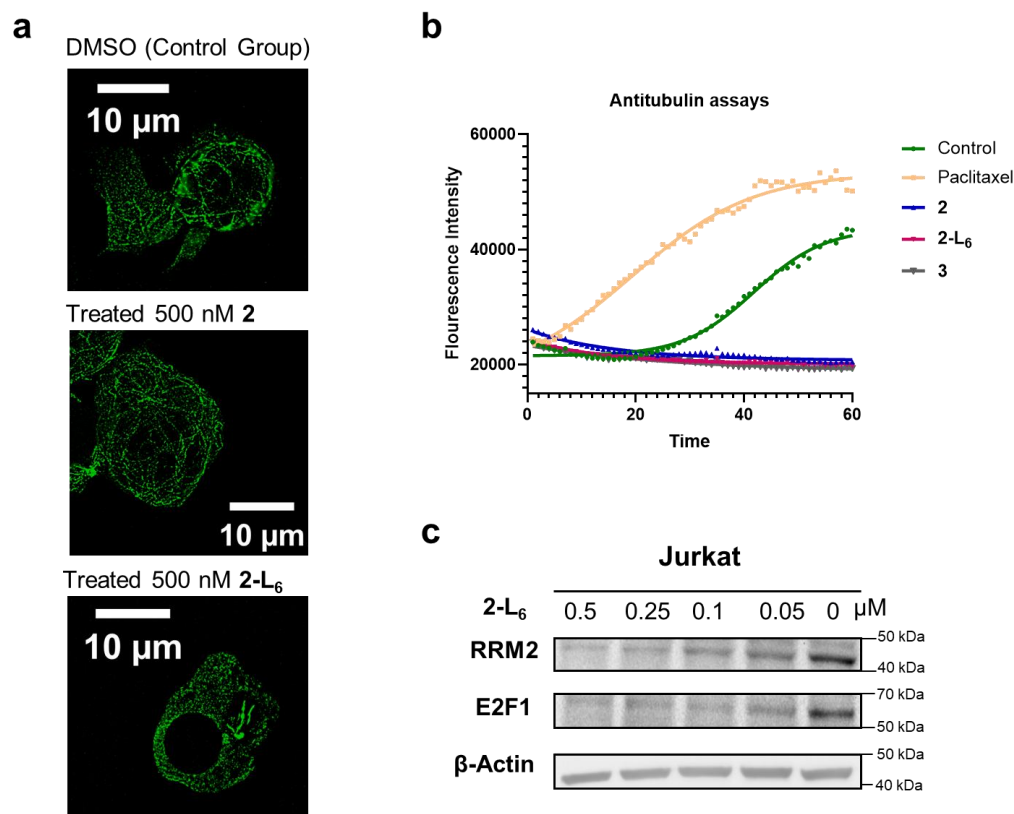
STMN1Δ



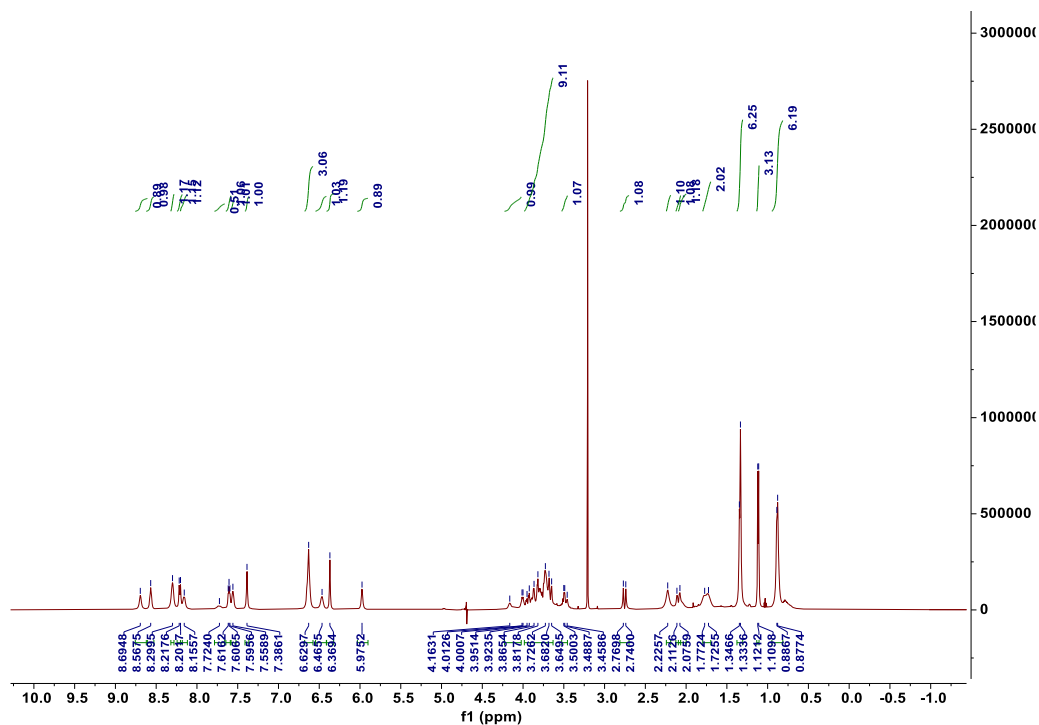
Supplementary Fig. 25. Sequencing results of *SLC46A3Δ*, *CCNFA*, *KEAP1Δ*, *CLAPS2Δ*, and *STMN1Δ*. It indicated that indels were successfully introduced into the correlated genes.



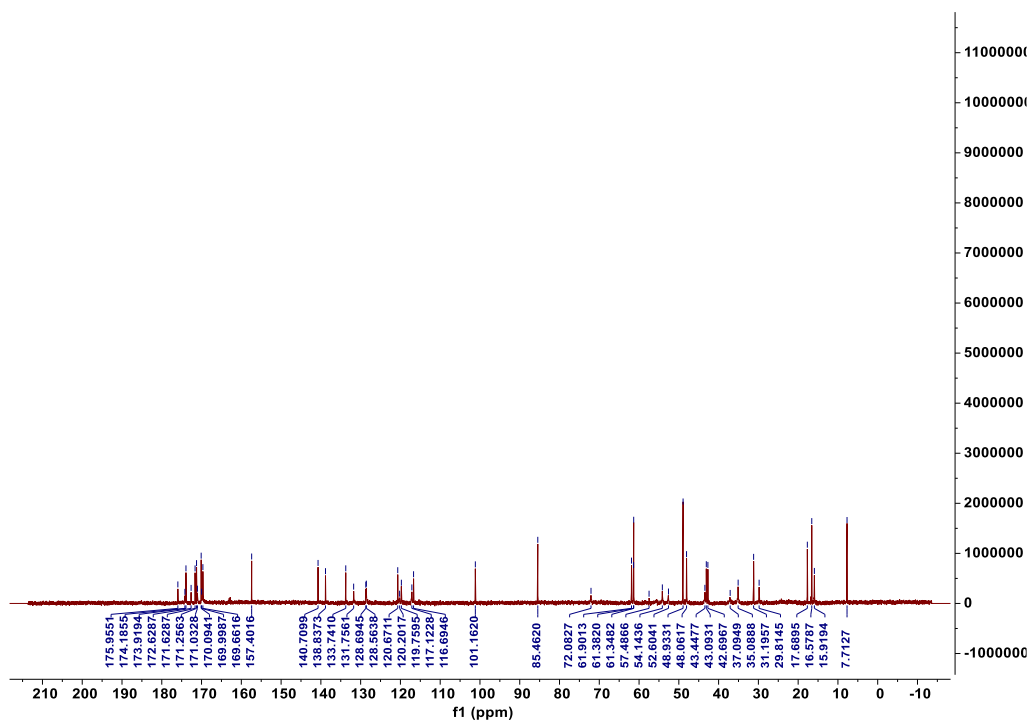
Supplementary Fig. 26. Cytotoxicity assays of 2-L₆ toward Jurkat with the treatment of endocytosis inhibitors. Jurkat cells were pre-incubated with 50 μ M genistein or 30 μ M hydroxy-dynasore for 1 h, followed by treatment with 5 μ M 2-L₆ at 37°C with 5% CO₂ overnight. **a.** Cell viability of Jurkat cells with inhibitors and 2-L₆ normalized by the group only treated with equivalent DMSO. In control group, 95% and 74% Jurkat cells survived treated with genistein and hydroxy-dynasore. The addition of 2-L₆ decreased cell viability to 94%, 56%, and 25% in Jurkat cells pretreated with genistein, hydroxy-dynasore, or DMSO, respectively. Despite the inherent cytotoxicity of endocytosis inhibitors toward Jurkat, they significantly mitigated the cytotoxicity of 2-L₆. **b.** Cell viability was normalized to cells treated with the respective inhibitors. Without inhibitors, 25% of cells survived with 2-L₆ treatment. In the present of genistein and hydroxy-dynasore, 98% and 74% of cells survived treated with 2-L₆, respectively. Cytotoxicity assays were tested with three biologically independent samples. Data are mean $\pm$ s.d.; n = 3 biologically independent samples.



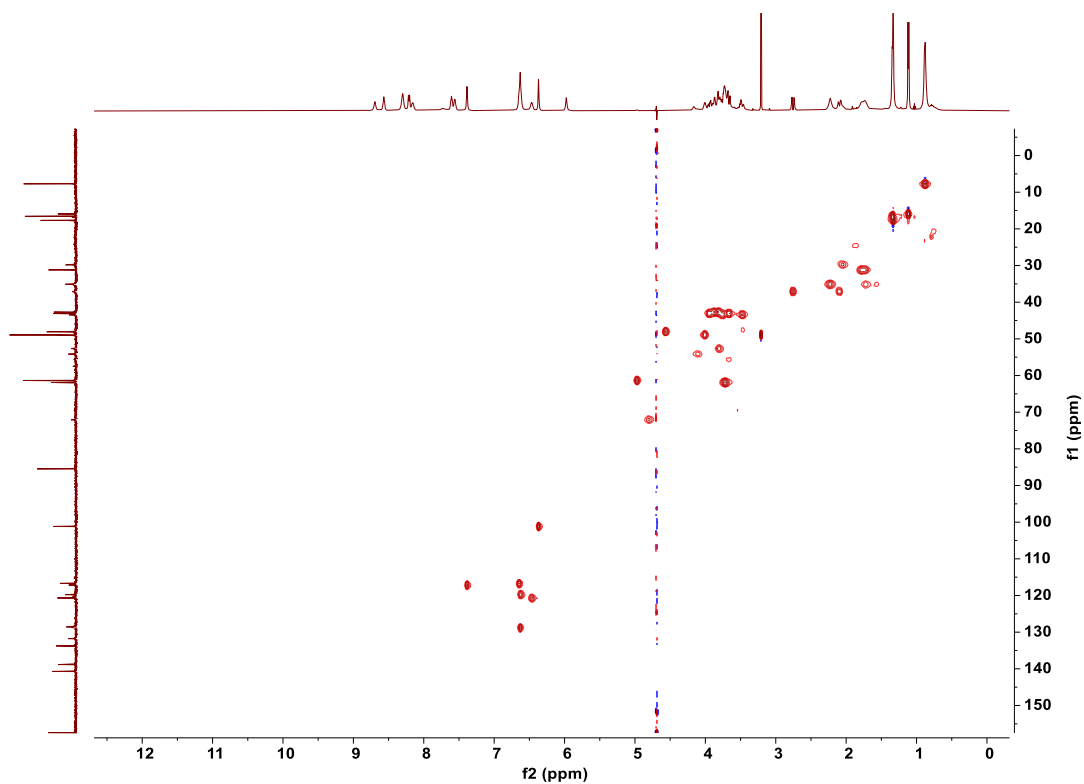
Supplementary Fig. 27. Potential effects of 2-L₆ on the CCNF-mediated pathway and tubulin polymerization. **a.** Immunofluorescence microscopy analysis of tubulin polymerization. Jurkat cells were incubated for 12 hours with either DMSO (top panel), 500 nM **2** (middle panel) or 500 nM **2-L₆** (bottom panel), two times of the experiment were repeated with the same results. **b.** In vitro tubulin polymerization assay. The fluorescence-based tubulin polymerization assay was performed using the Cytoskeleton kit (BK011P), following the manufacturer's protocol. Time-response curves for 10 μM **2-L₆** (pink), 10 μM **2** (blue), 10 μM **3** (gray), 5 μM paclitaxel (yellow, a tubulin polymerization enhancer), and DMSO control are shown. Compounds **2-L₆**, **2**, and **3** inhibited tubulin polymerization in vitro. **c.** Western blot analysis of E2F1 and RRM2 expression in Jurkat cells treated with increasing concentrations of **2-L₆** for 20 h.



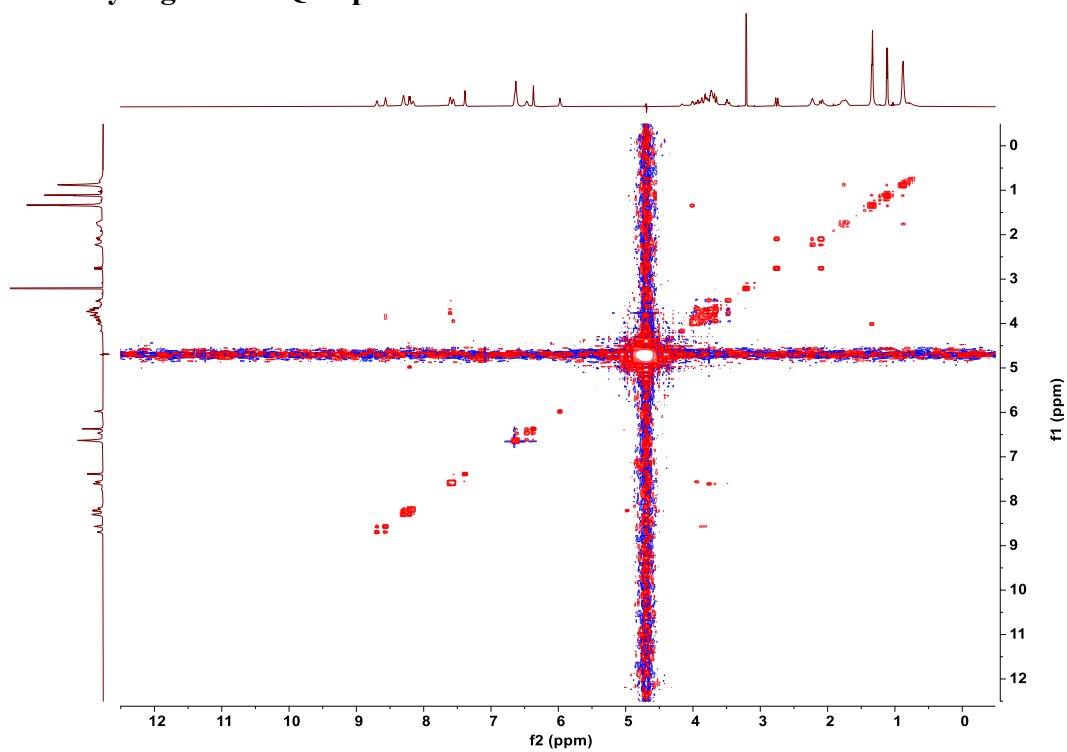
Supplementary Fig. 28. ¹H NMR (600 Hz, H₂O) spectrum of 1.



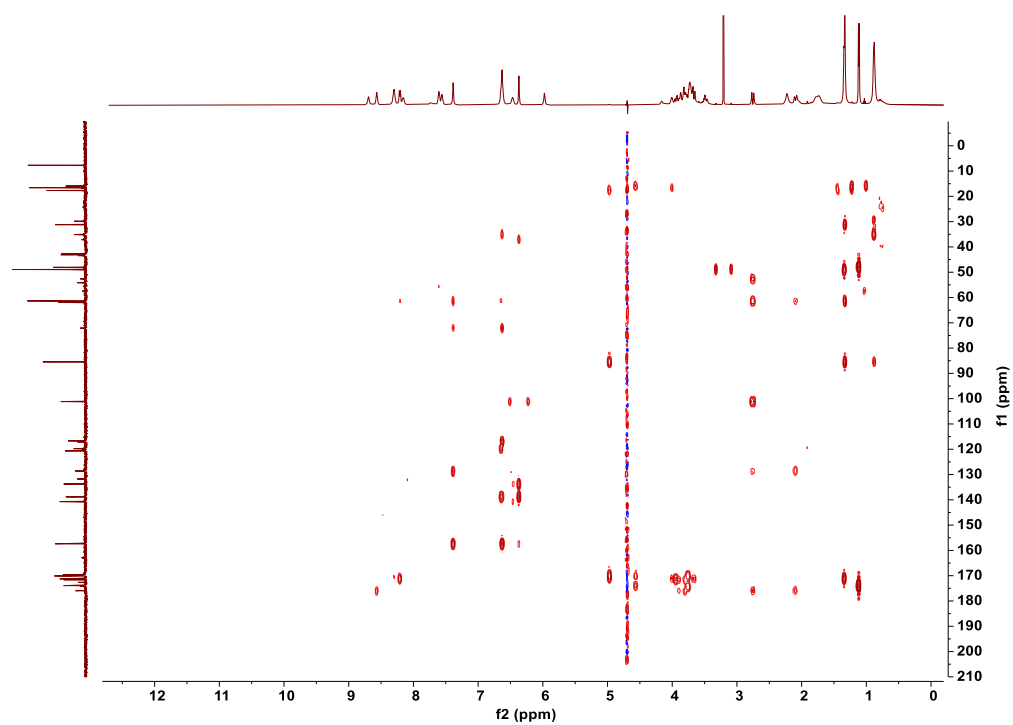
Supplementary Fig. 29. ¹³C NMR (150 Hz, H₂O) spectrum of 1.



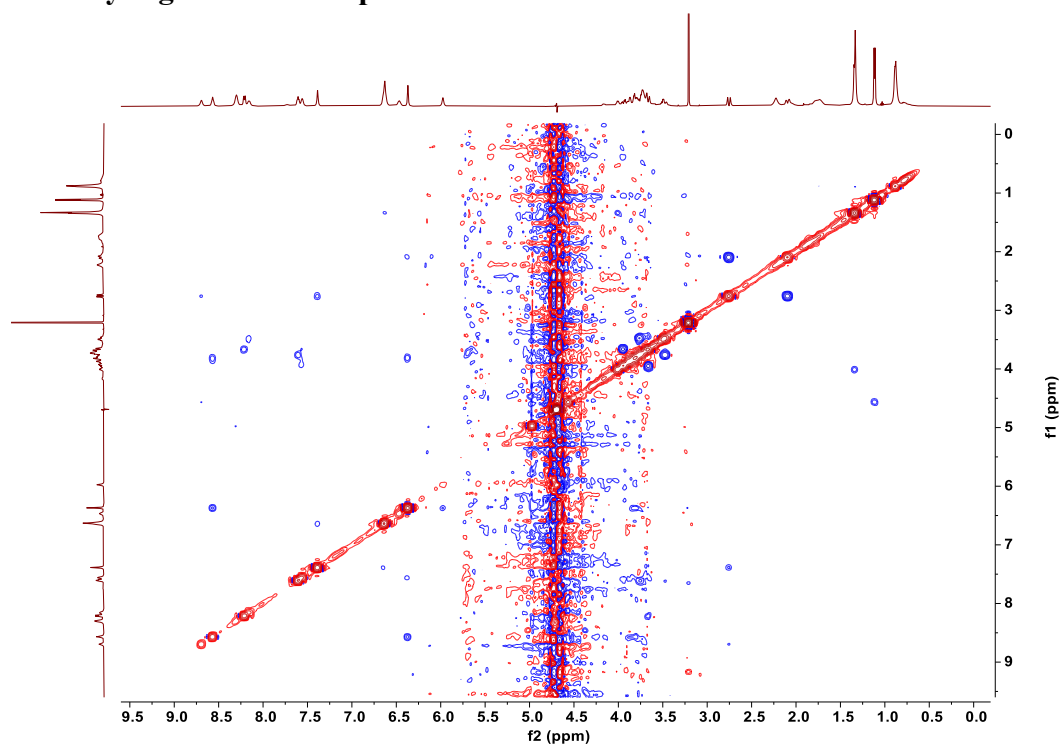
Supplementary Fig. 30. HSQC spectrum of 1.



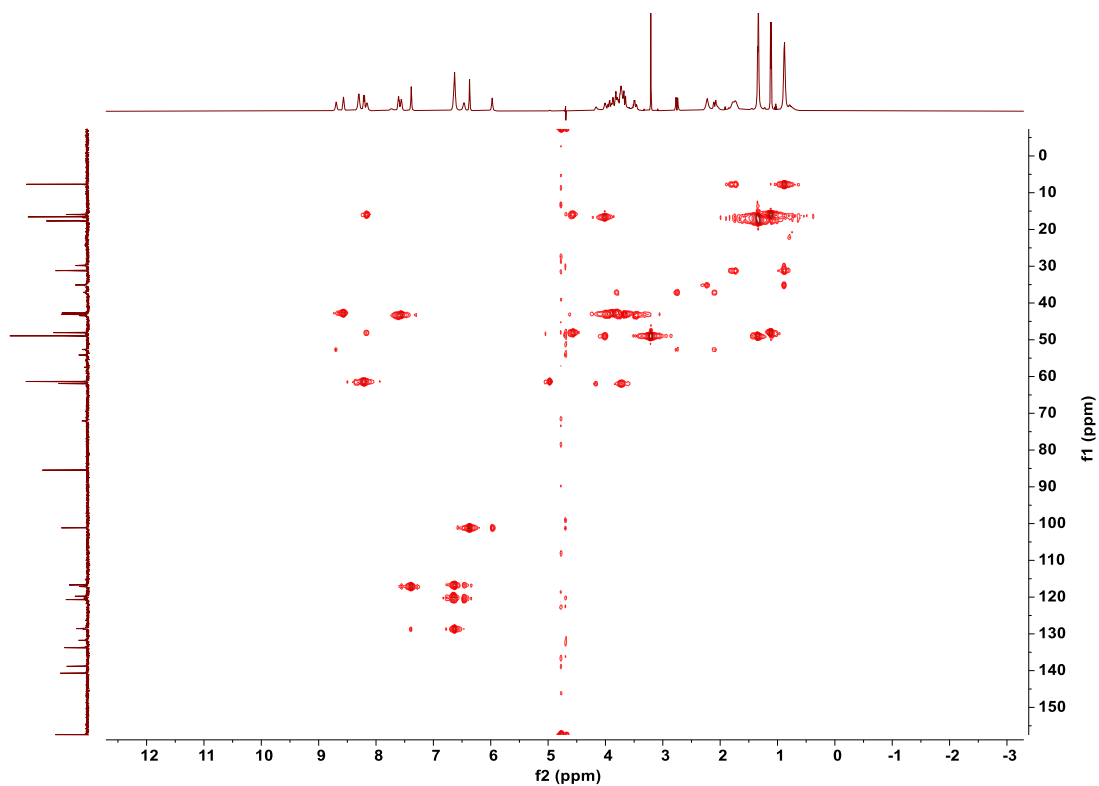
Supplementary Fig. 31. ^1H - ^1H COSY spectrum of 1.



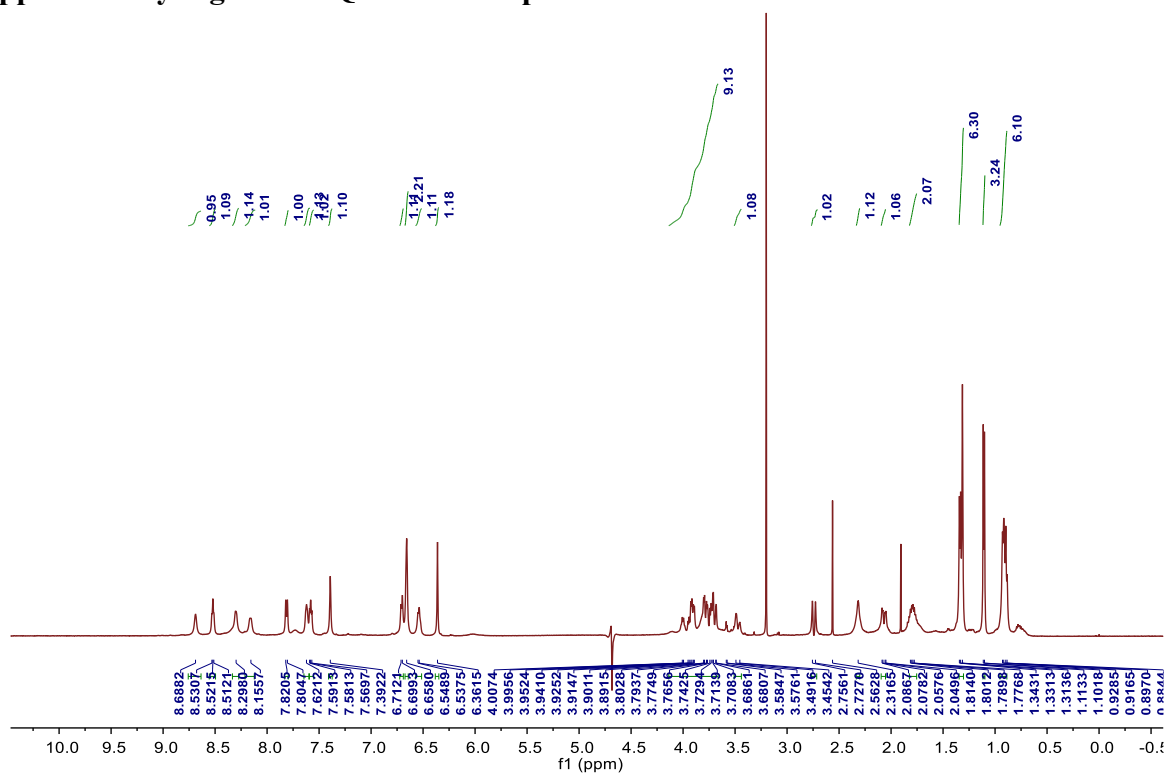
Supplementary Fig. 32. HMBC spectrum of 1.



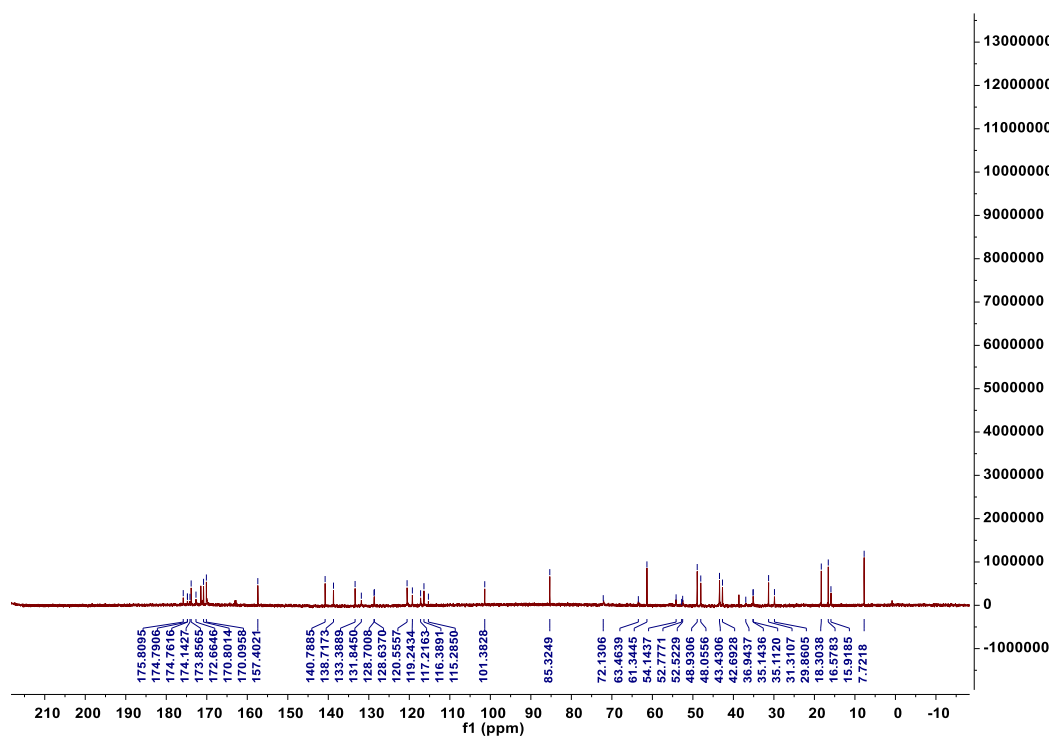
Supplementary Fig. 33. ROESY spectrum of 1.



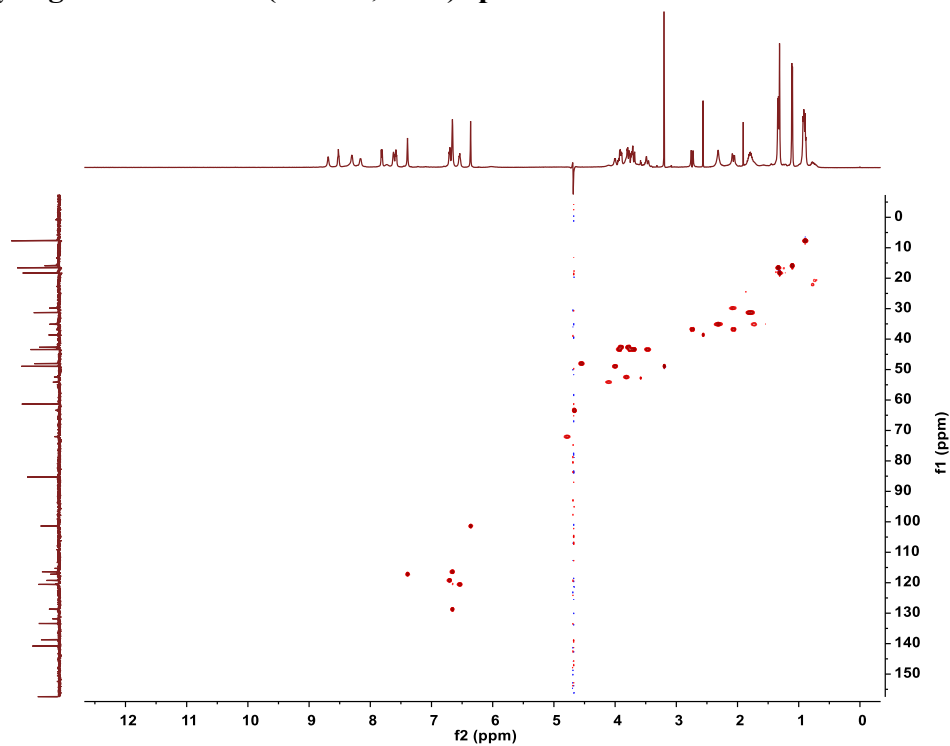
Supplementary Fig. 34. HSQC-TOCSY spectrum of 1.



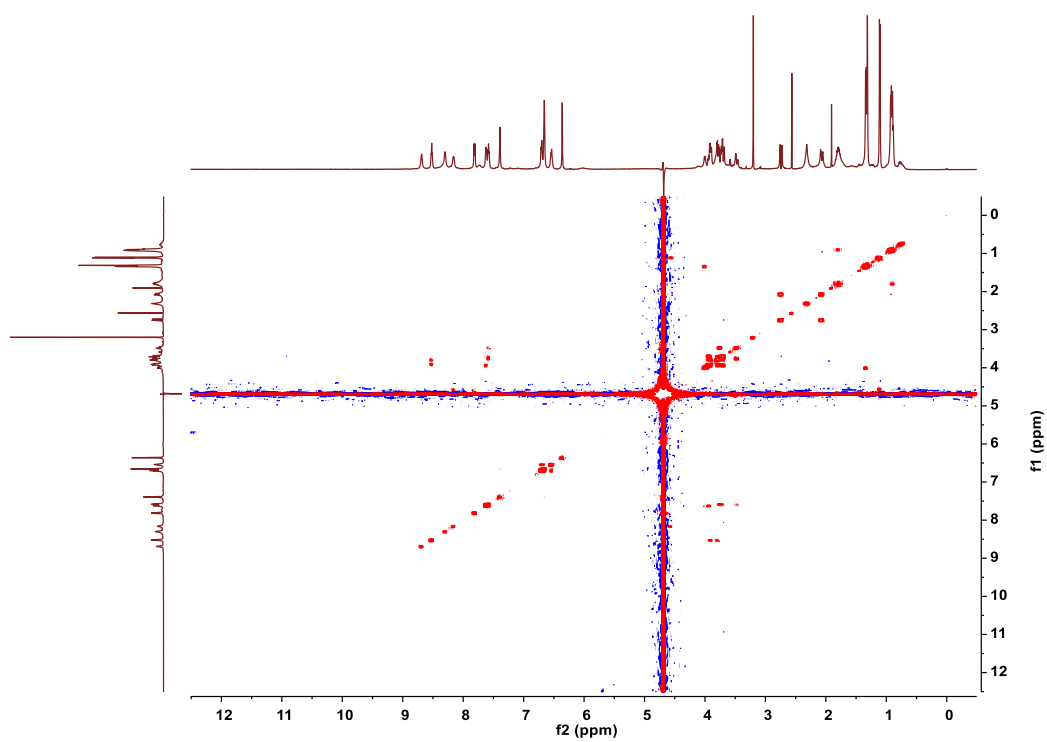
Supplementary Fig. 35. ^1H NMR (600 Hz, H_2O) spectrum of 2.



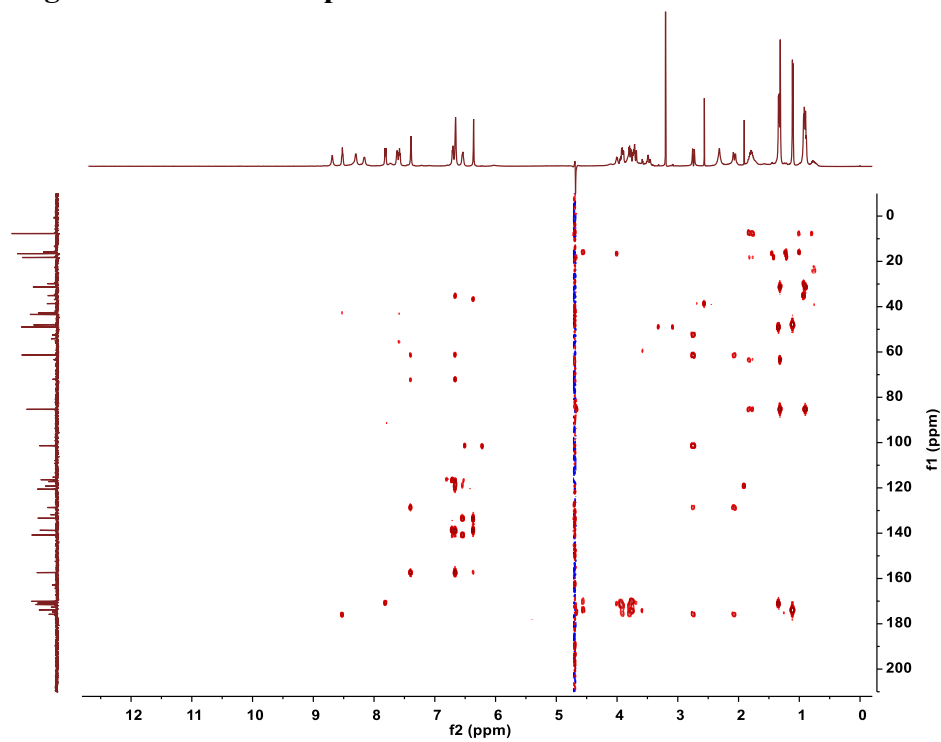
Supplementary Fig. 36. ¹³C NMR (150 Hz, H₂O) spectrum of 2.



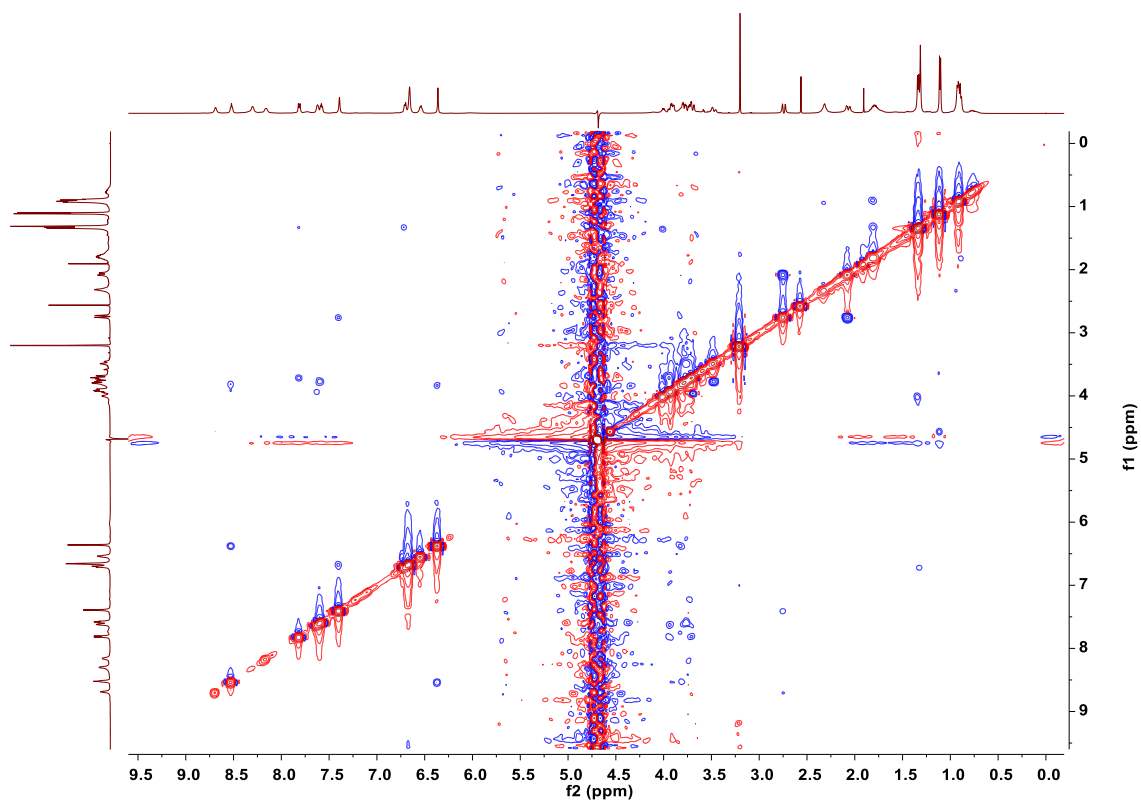
Supplementary Fig. 37. HSQC spectrum of 2.



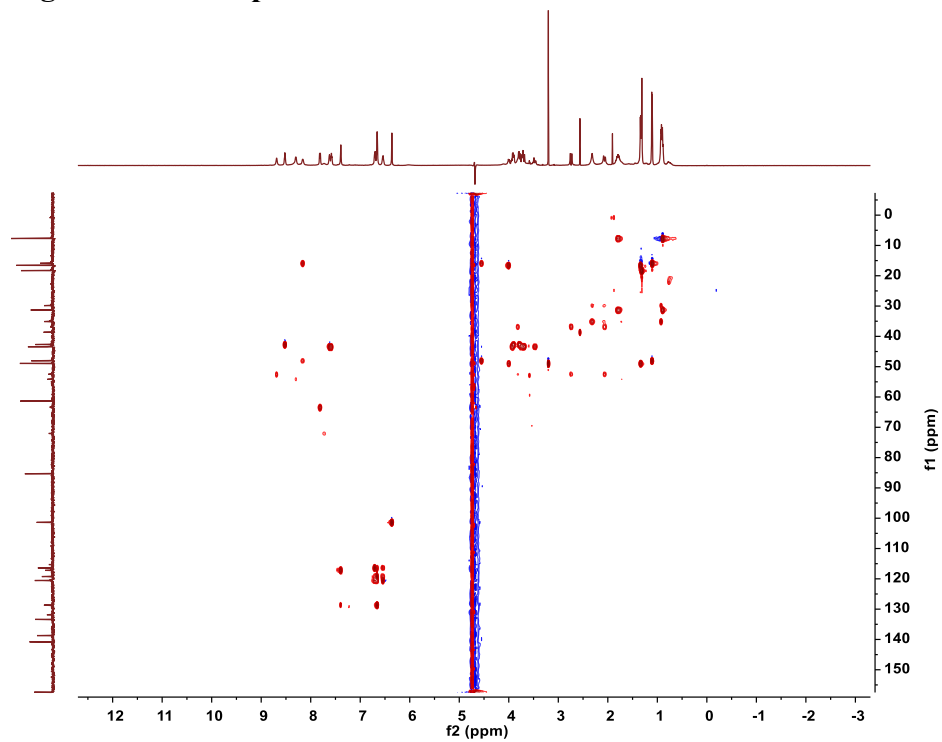
Supplementary Fig. 38. ^1H - ^1H COSY spectrum of 2.



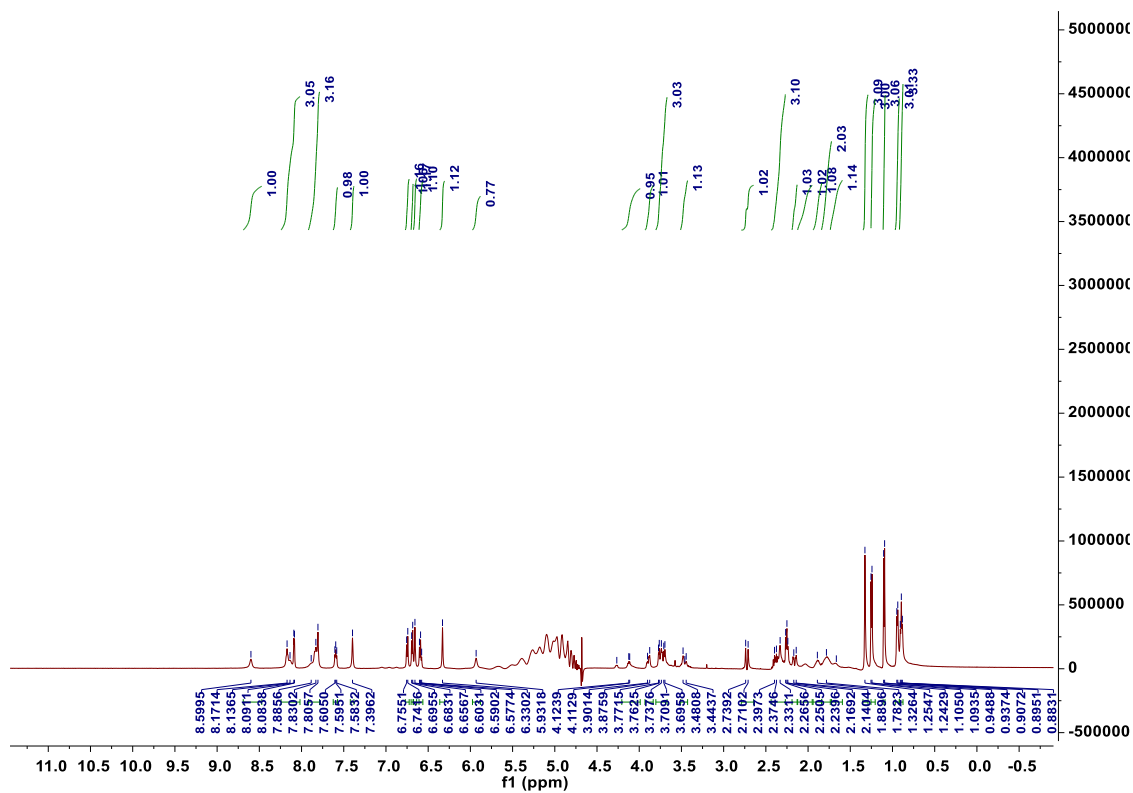
Supplementary Fig. 39. HMBC spectrum of 2.



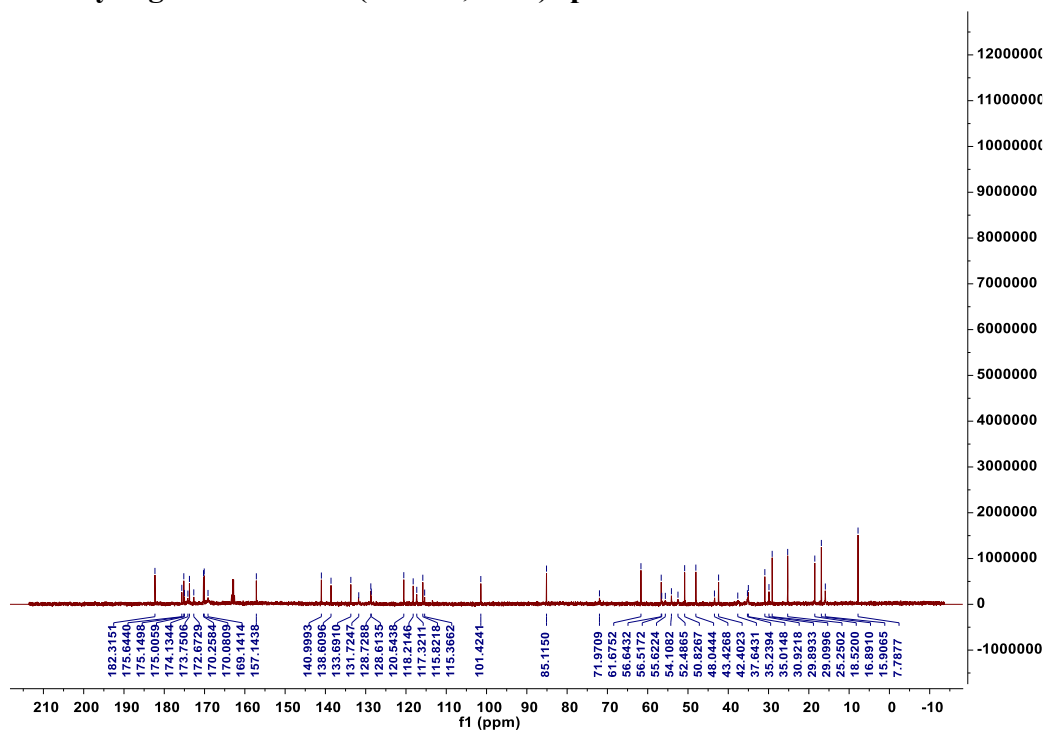
Supplementary Fig. 40. ROESY spectrum of 2.



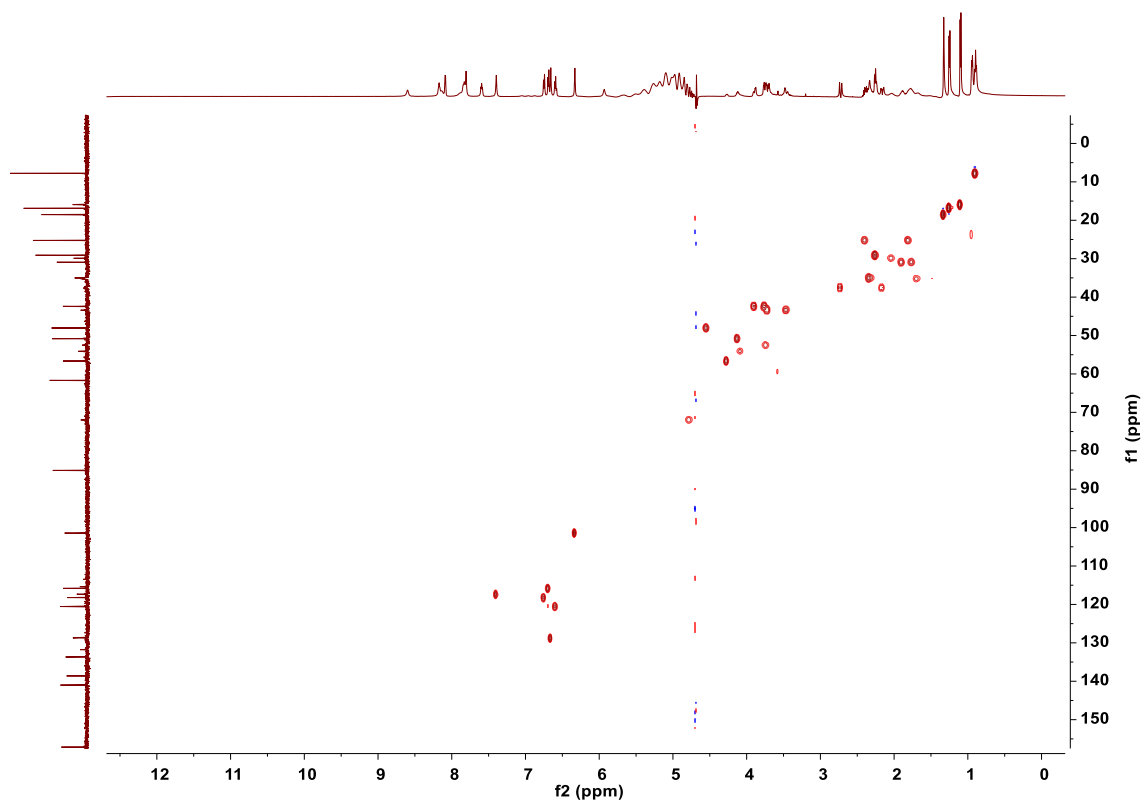
Supplementary Fig. 41. HSQC-TOCSY spectrum of 2.



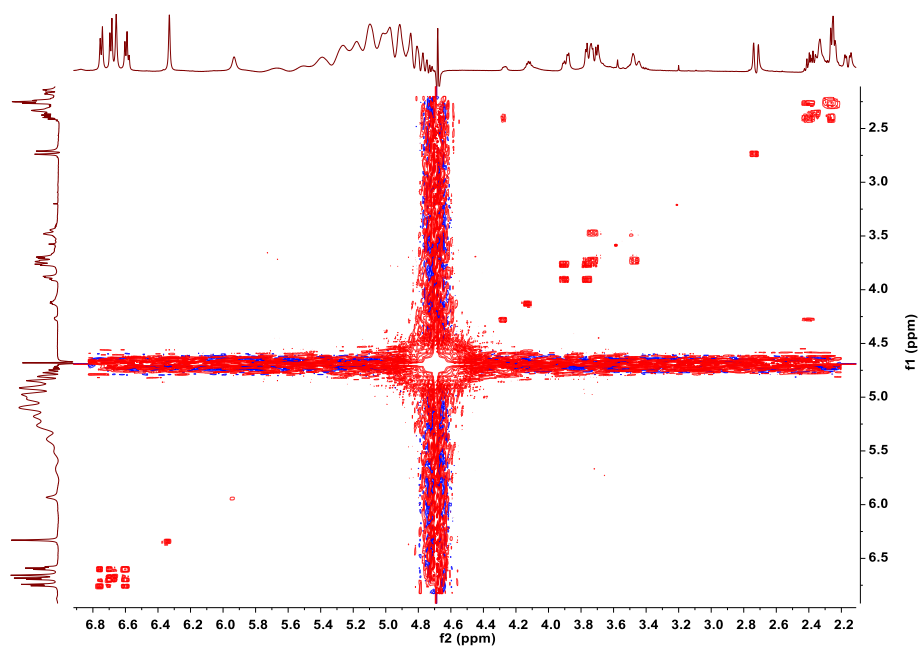
Supplementary Fig. 42. ¹H NMR (600 Hz, H₂O) spectrum of 3.



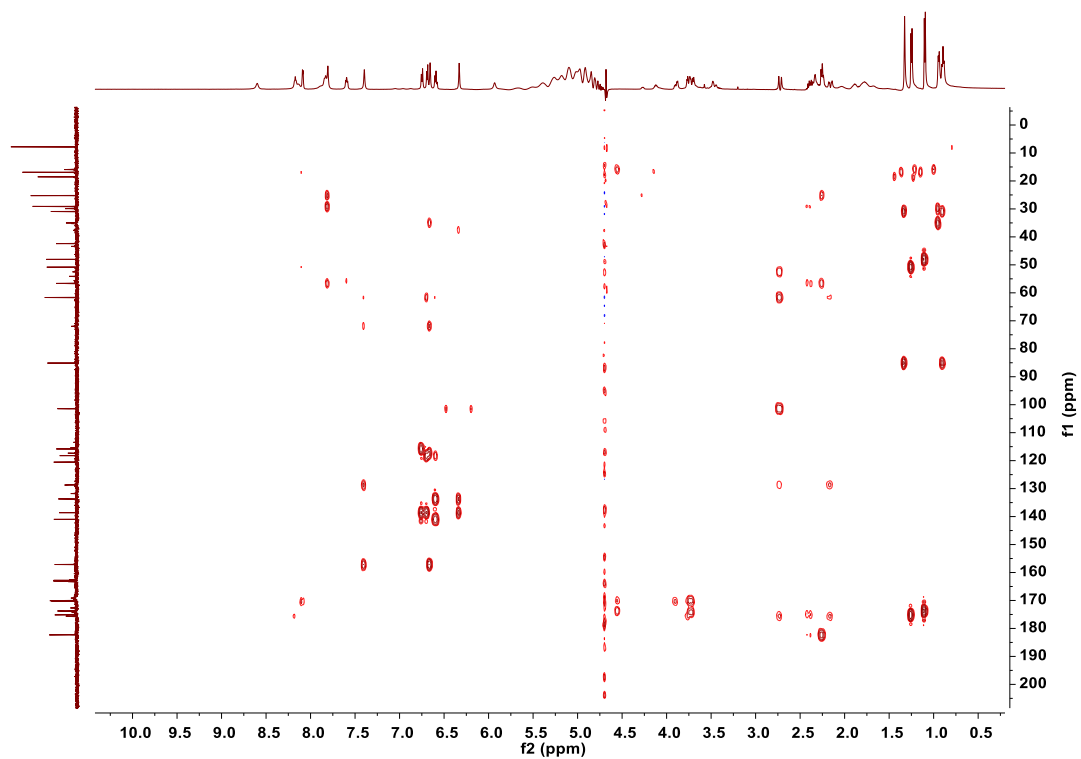
Supplementary Fig. 43. ¹³C NMR (150 Hz, H₂O) spectrum of 3.



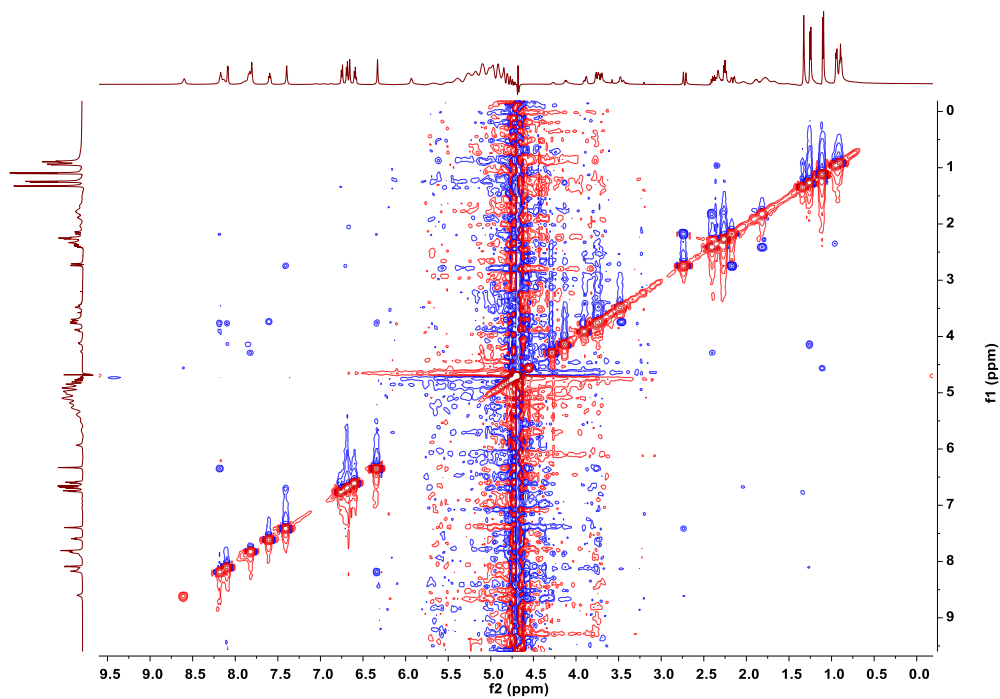
Supplementary Fig. 44. HSQC spectrum of 3.



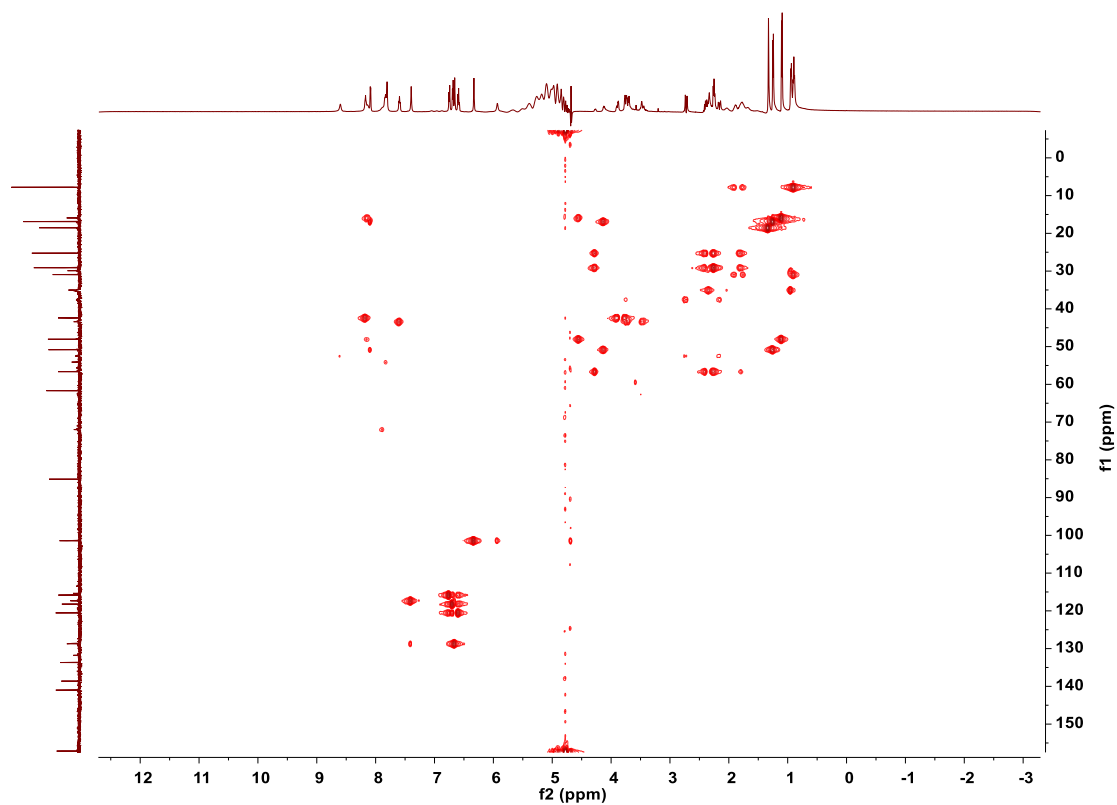
Supplementary Fig. 45. ^1H - ^1H COSY spectrum of 3.



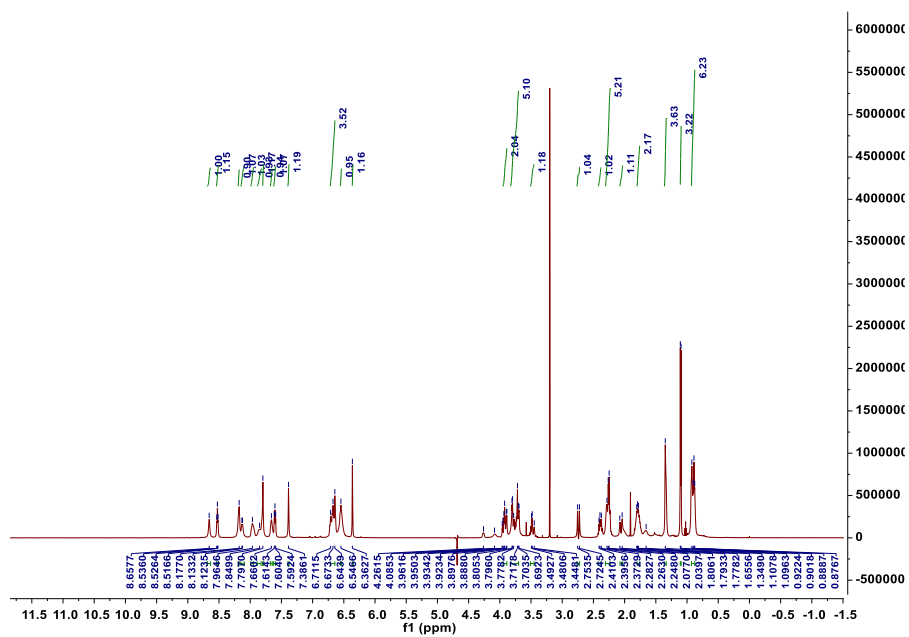
Supplementary Fig. 46. HMBC spectrum of 3.



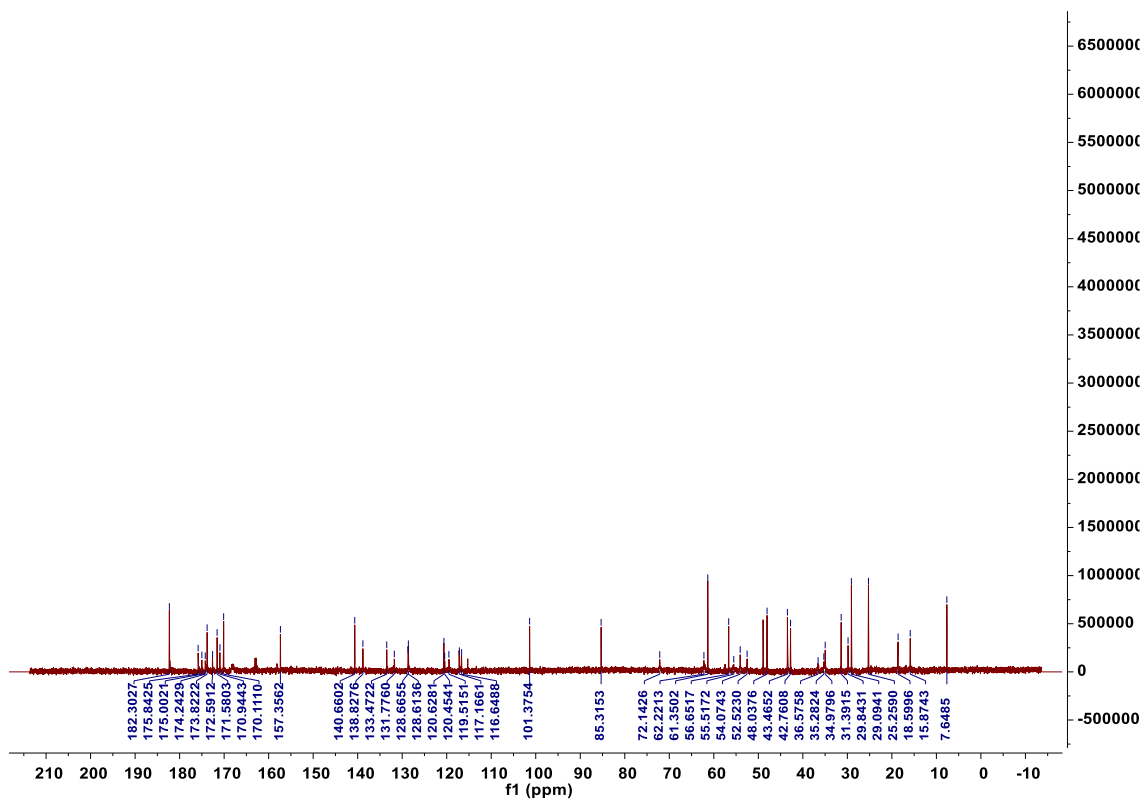
Supplementary Fig. 47. ROESY spectrum of 3.



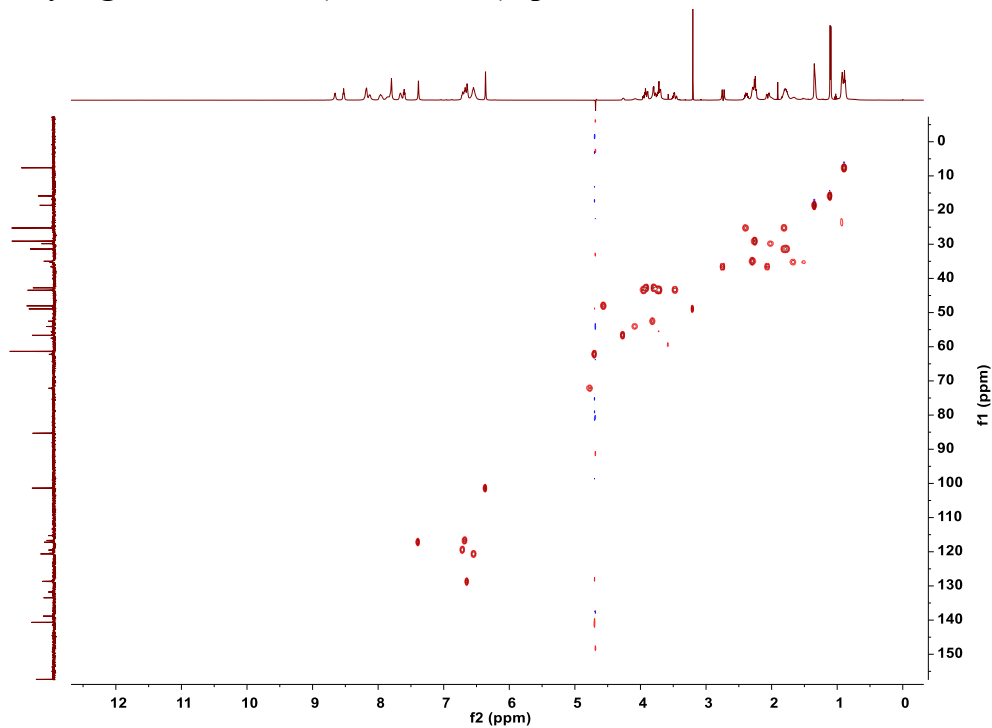
Supplementary Fig. 48. HSQC-TOCSY spectrum of 3.



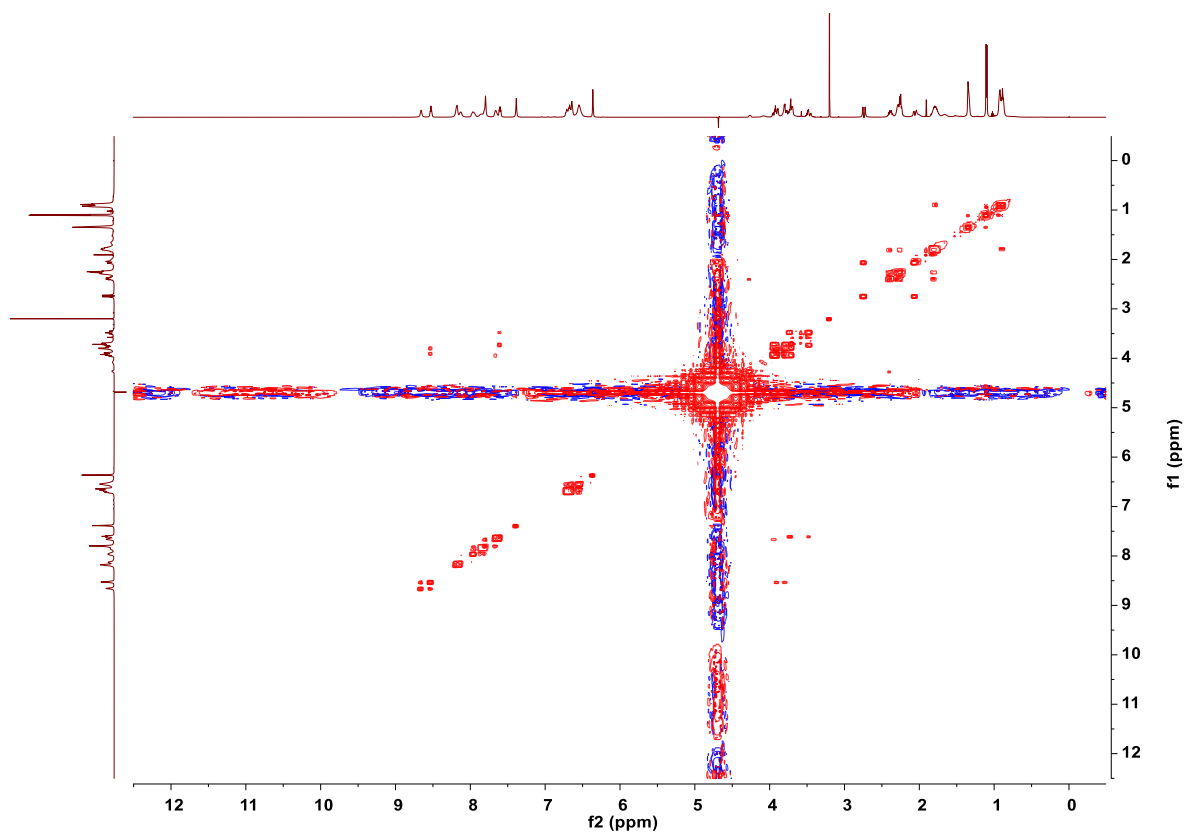
Supplementary Fig. 49. ^1H NMR (600 Hz, H_2O) spectrum of 4.



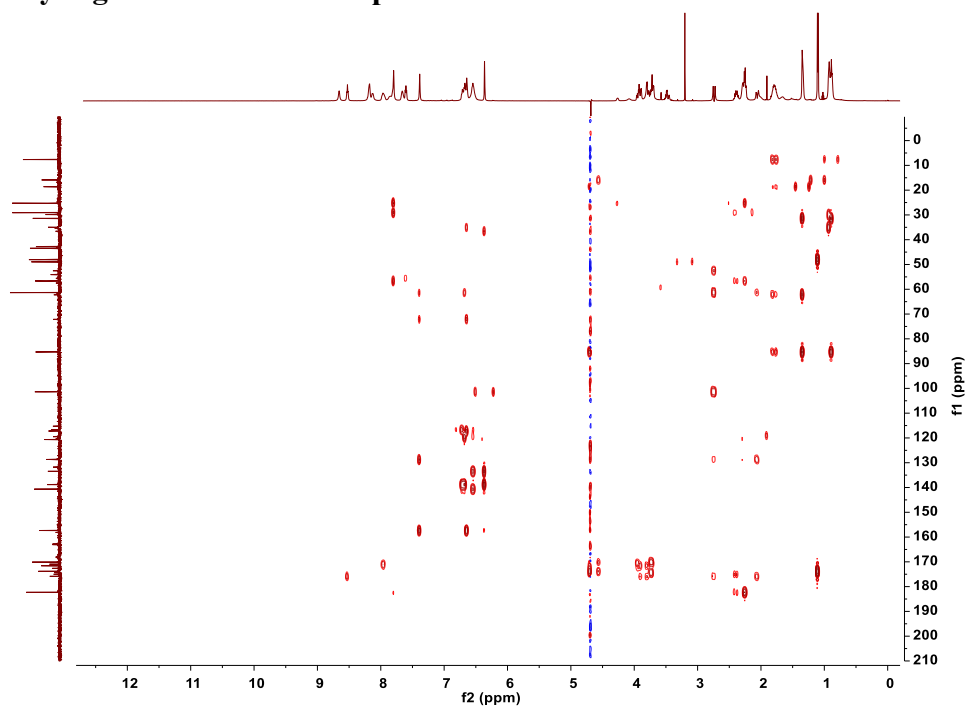
Supplementary Fig. 50. ¹³C NMR (150 Hz, H₂O) spectrum of 4.



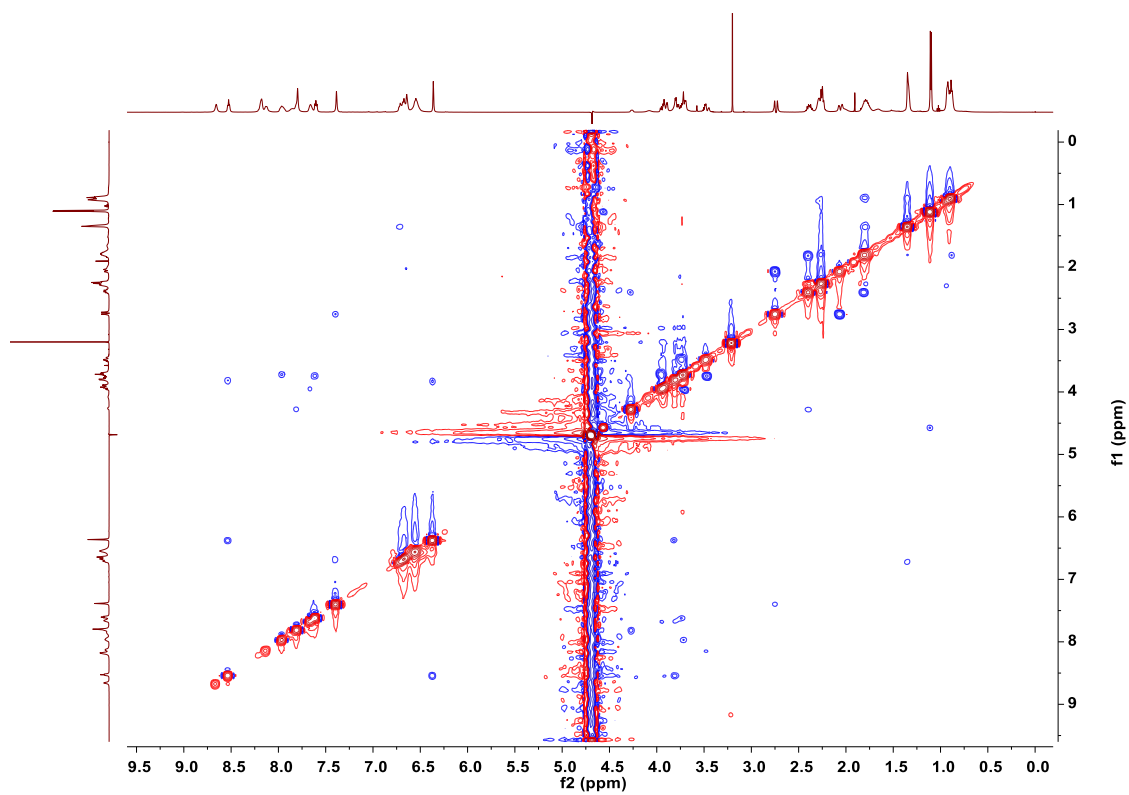
Supplementary Fig. 51. HSQC spectrum of 4.



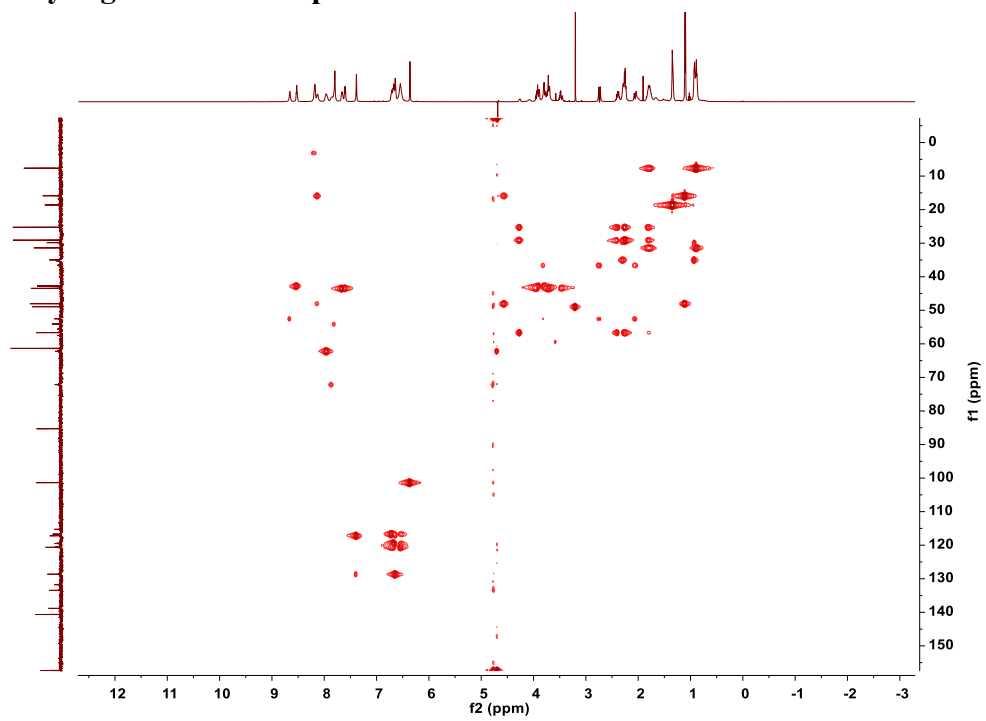
Supplementary Fig. 52. ^1H - ^1H COSY spectrum of **4**.



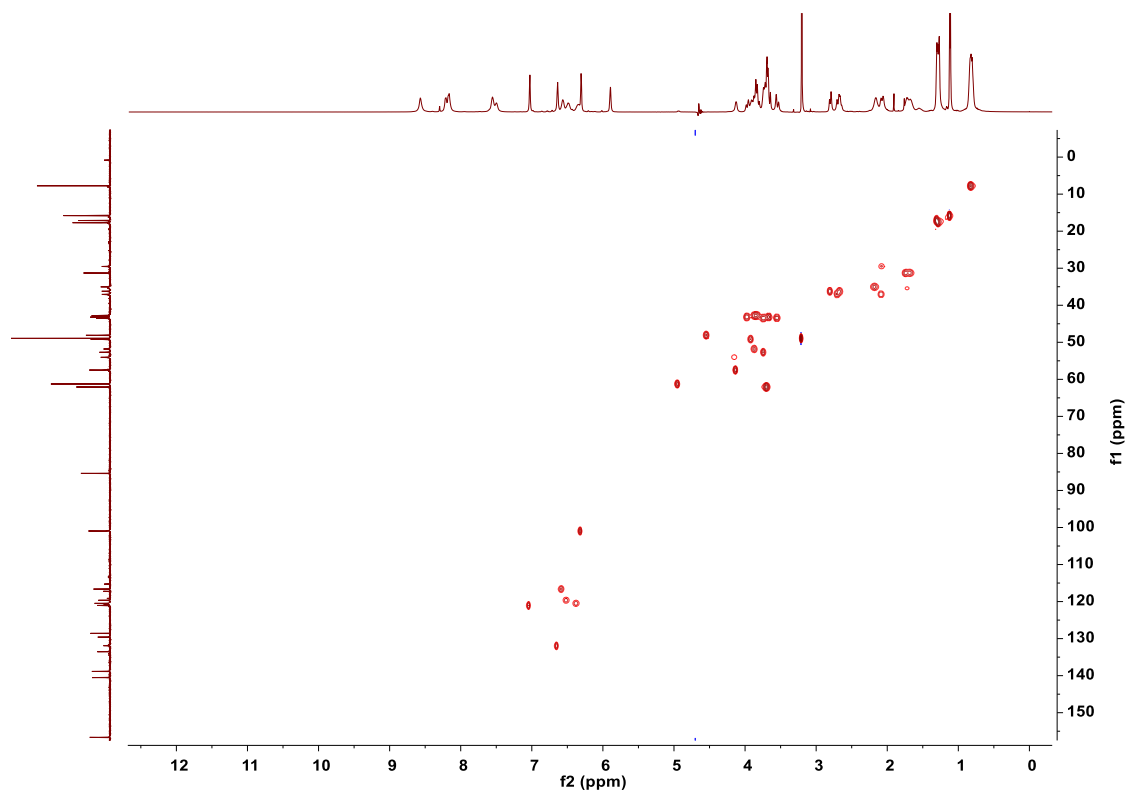
Supplementary Fig. 53. HMBC spectrum of **4**.



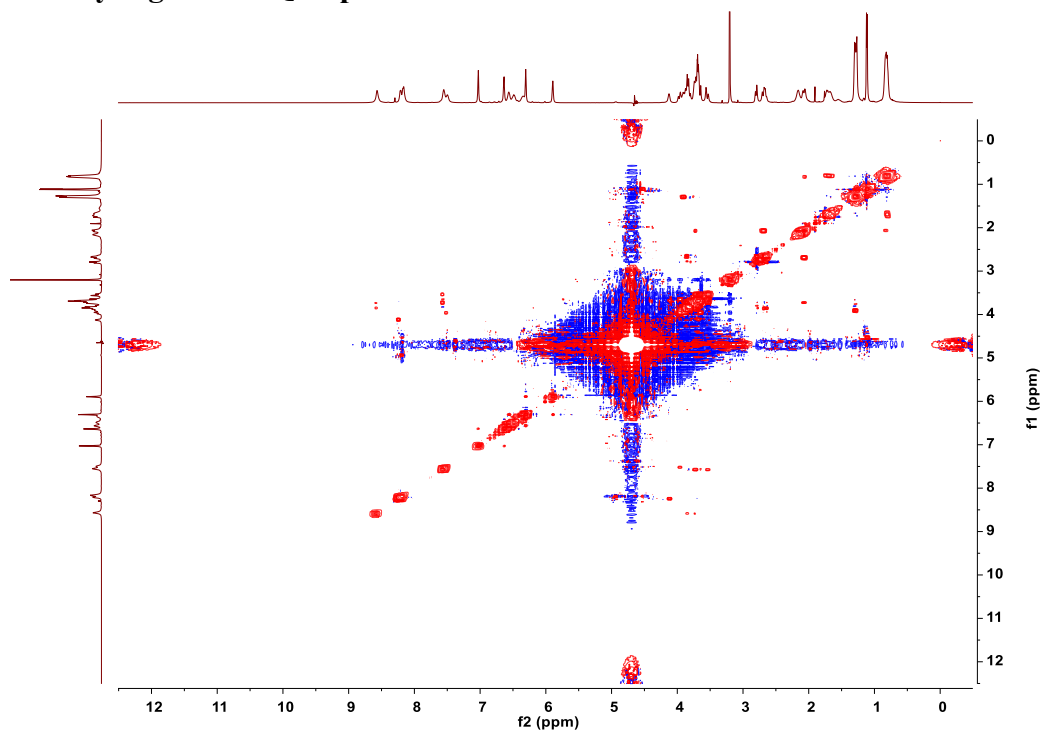
Supplementary Fig. 54. ROESY spectrum of 4.



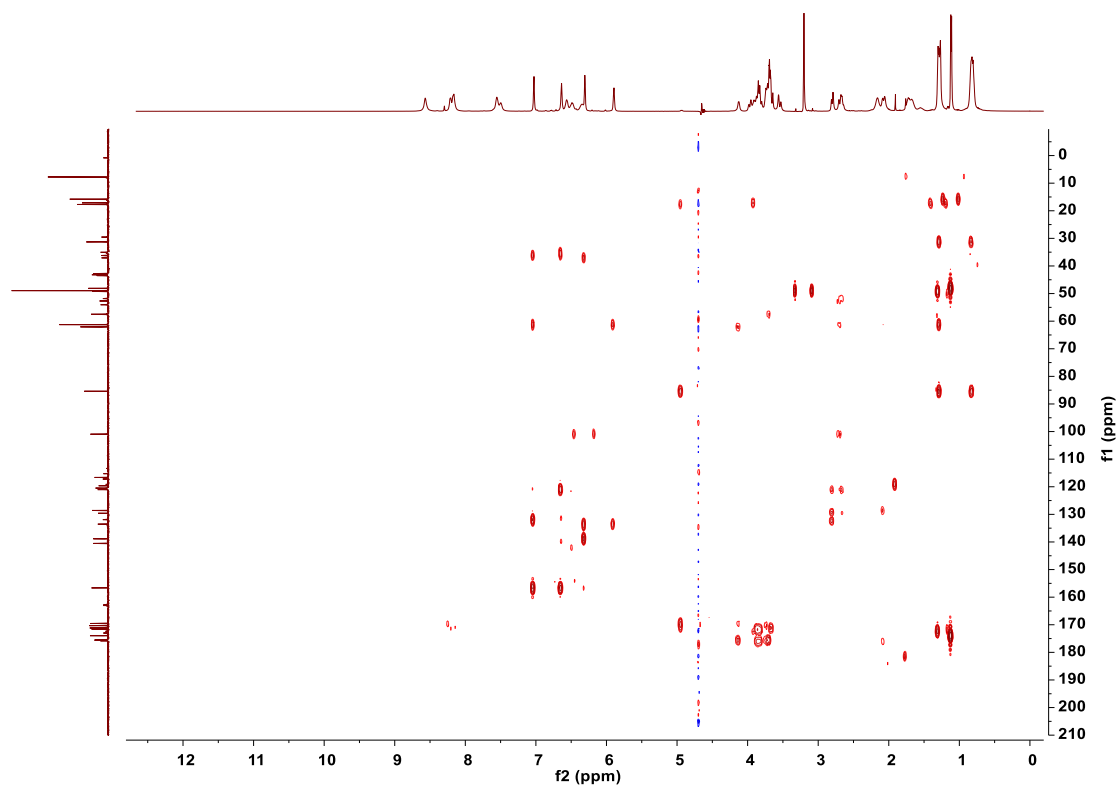
Supplementary Fig. 55. HSQC-TOCSY spectrum of 4.



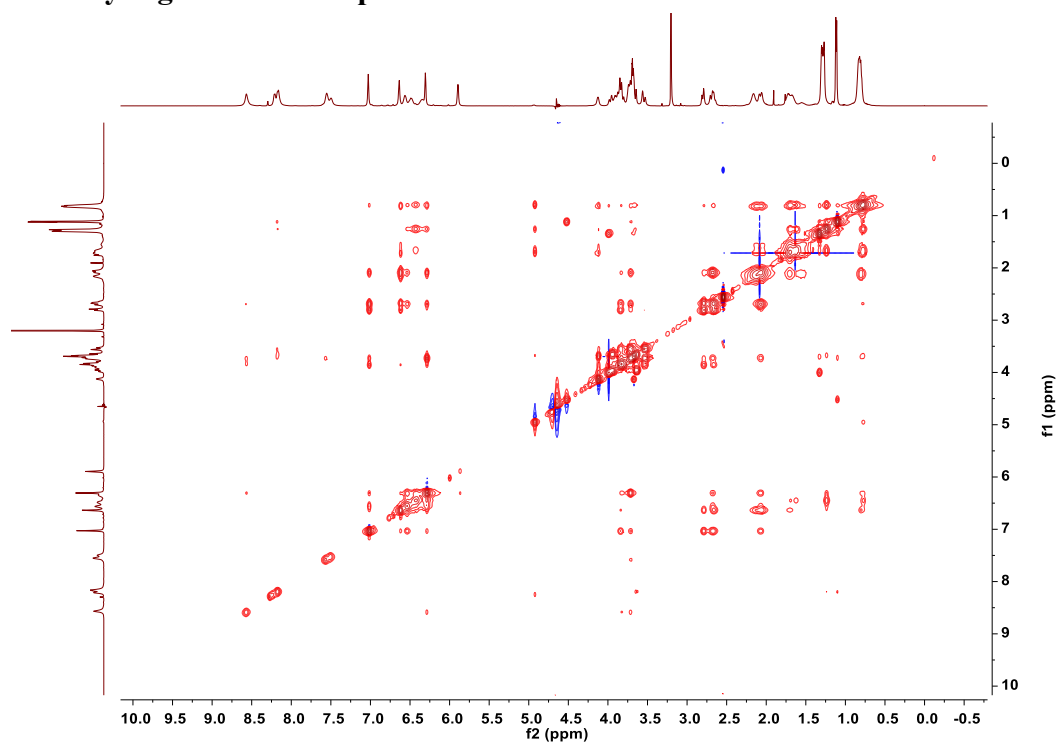
Supplementary Fig. 58. HSQC spectrum of **5**.



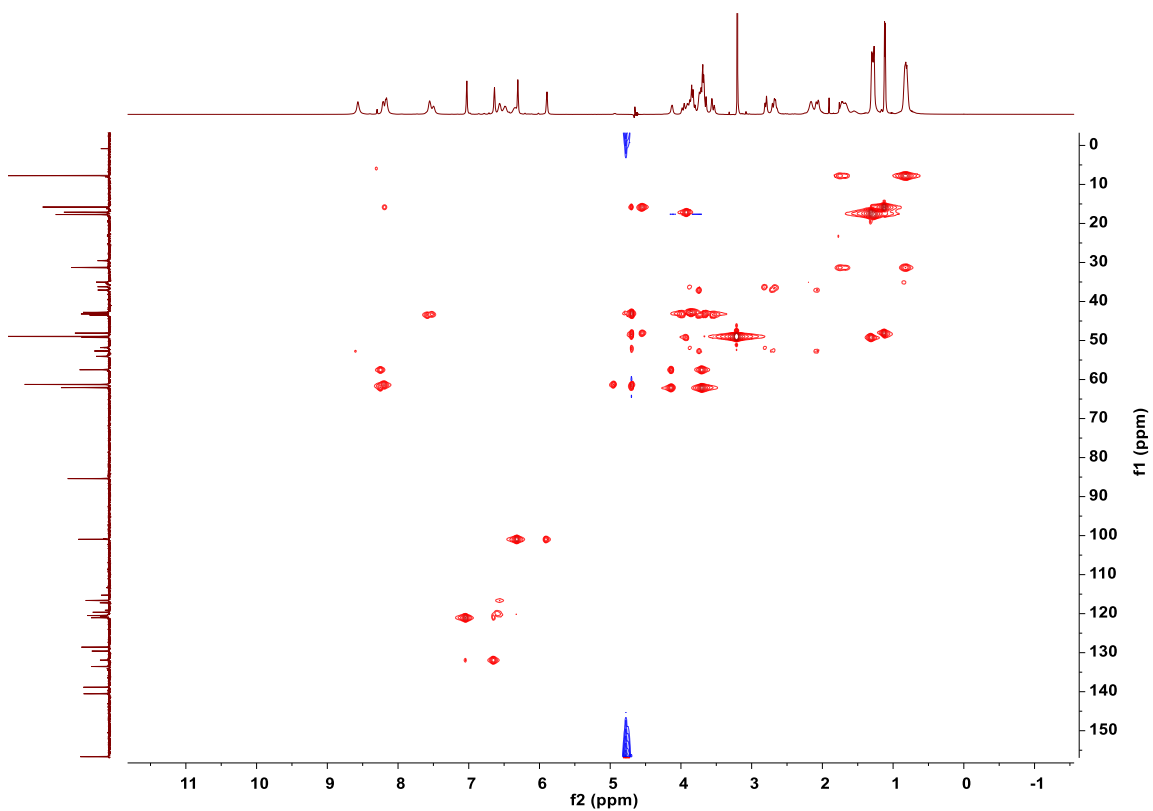
Supplementary Fig. 59. ^1H - ^1H COSY spectrum of **5**.



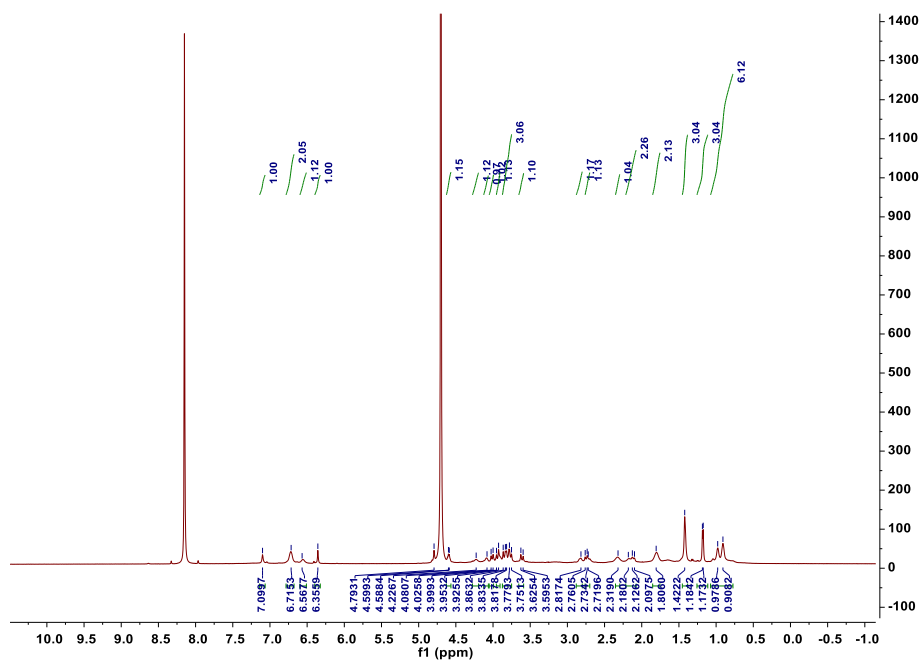
Supplementary Fig. 60. HMBC spectrum of 5.



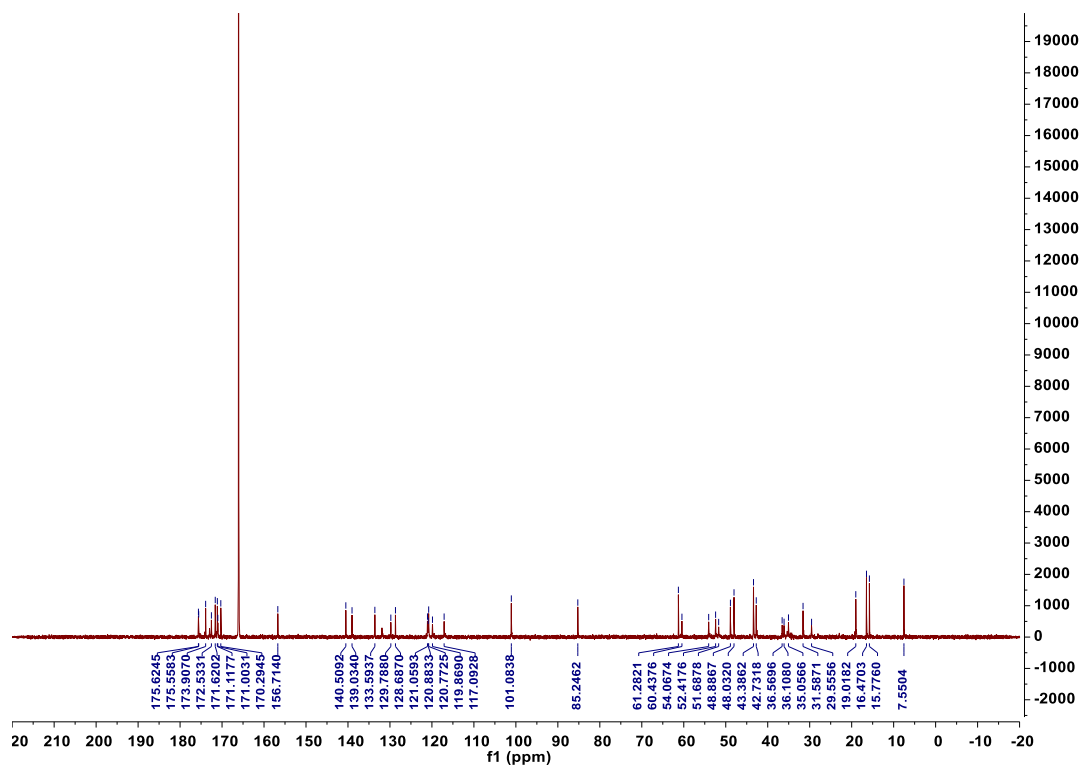
Supplementary Fig. 61. NOESY spectrum of 5.



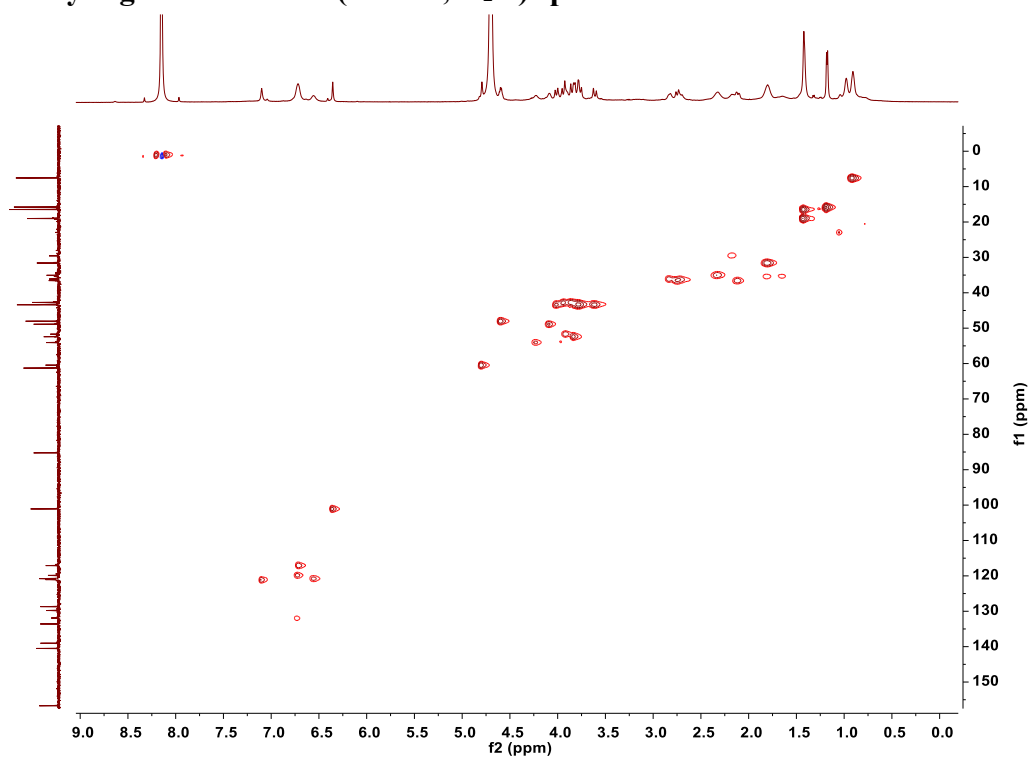
Supplementary Fig. 62. HSQC-TOCSY spectrum of 5.



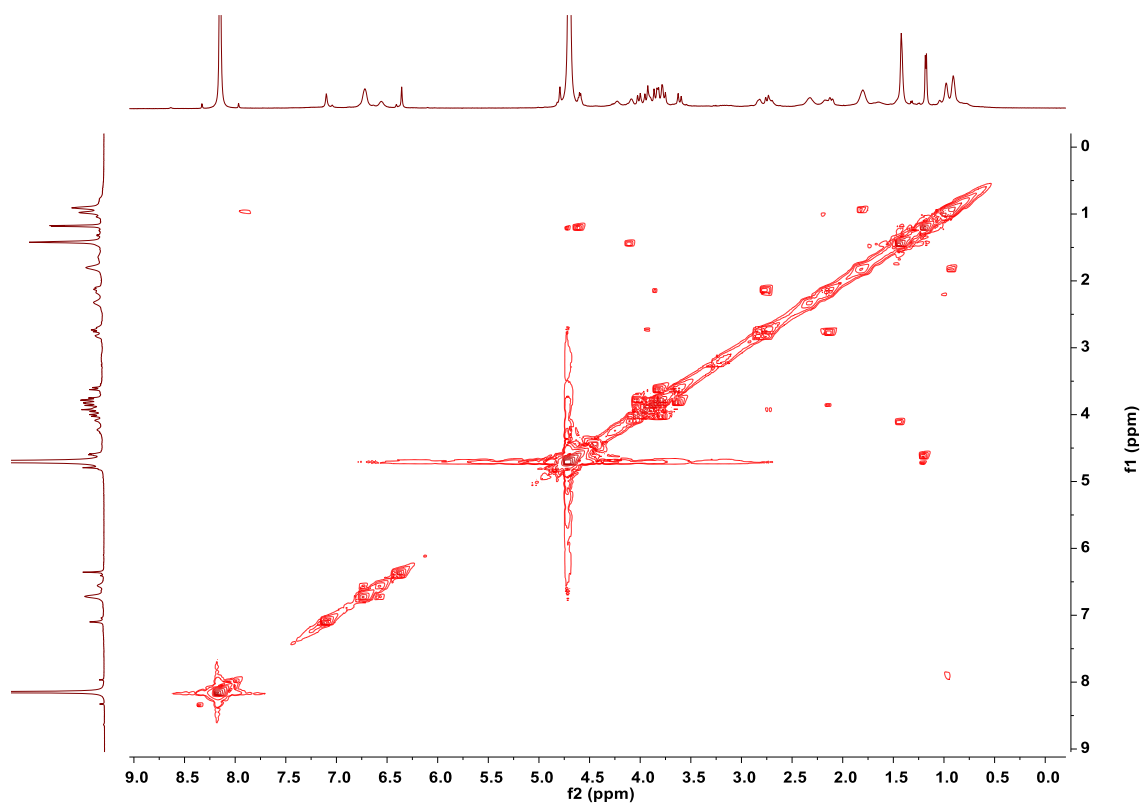
Supplementary Fig. 63. ^1H NMR (600 Hz, H_2O) spectrum of 6.



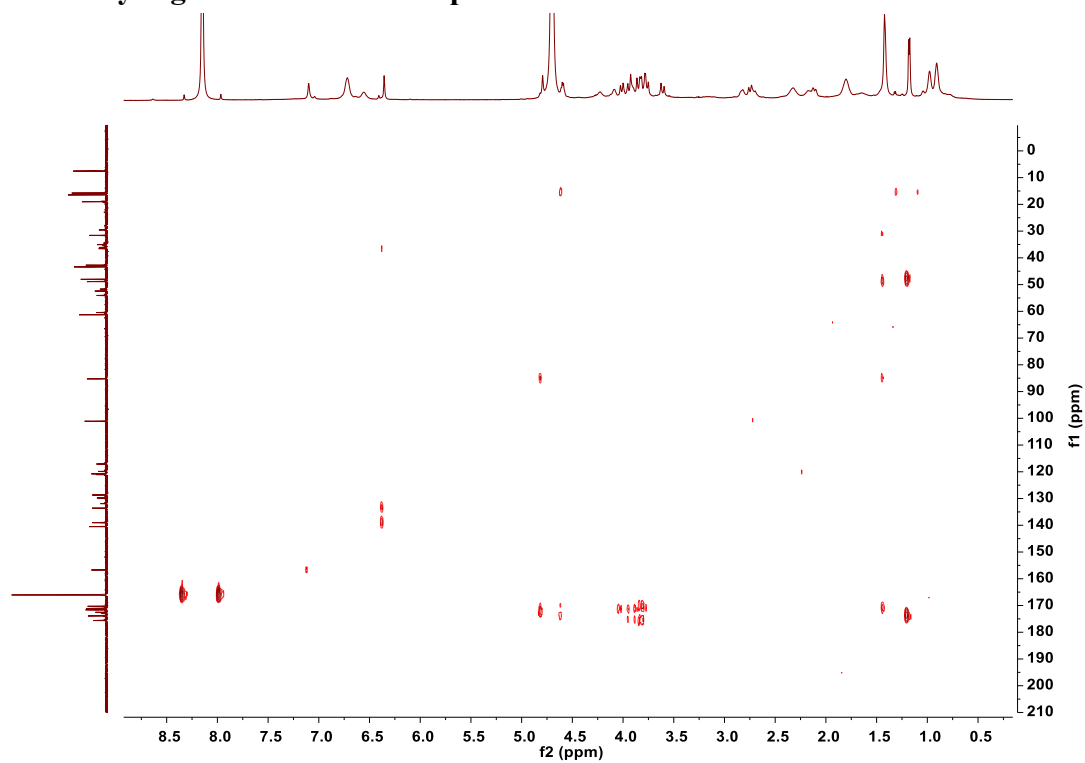
Supplementary Fig. 64. ^{13}C NMR (150 Hz, H_2O) spectrum of 6.



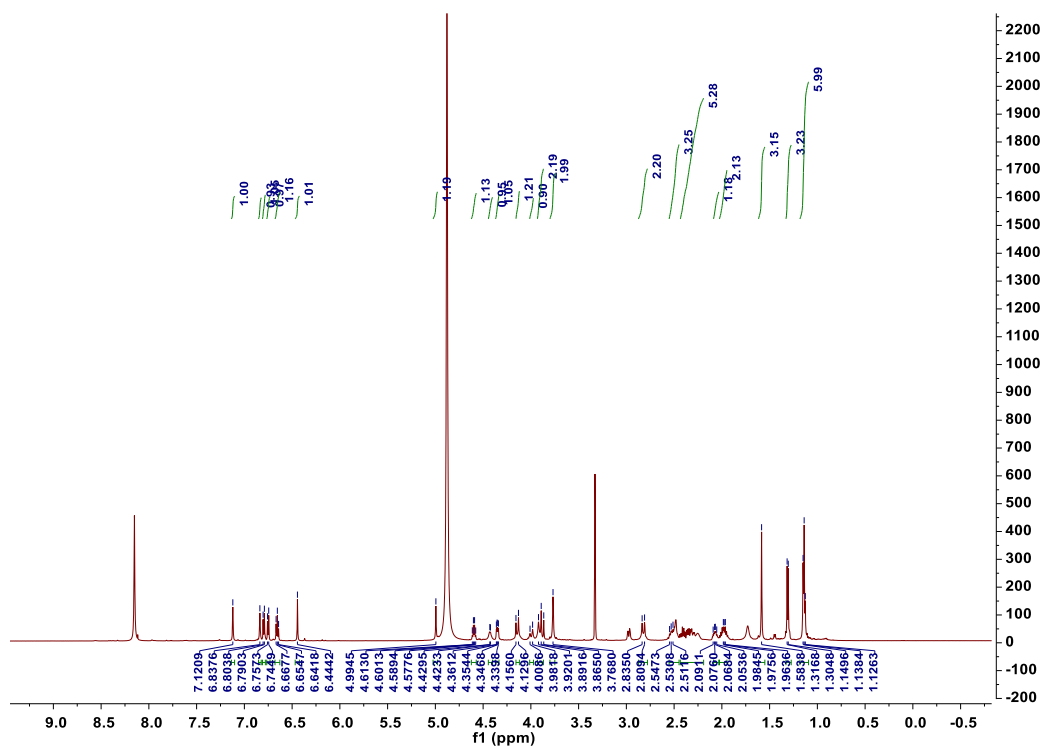
Supplementary Fig. 65. HSQC spectrum of 6.



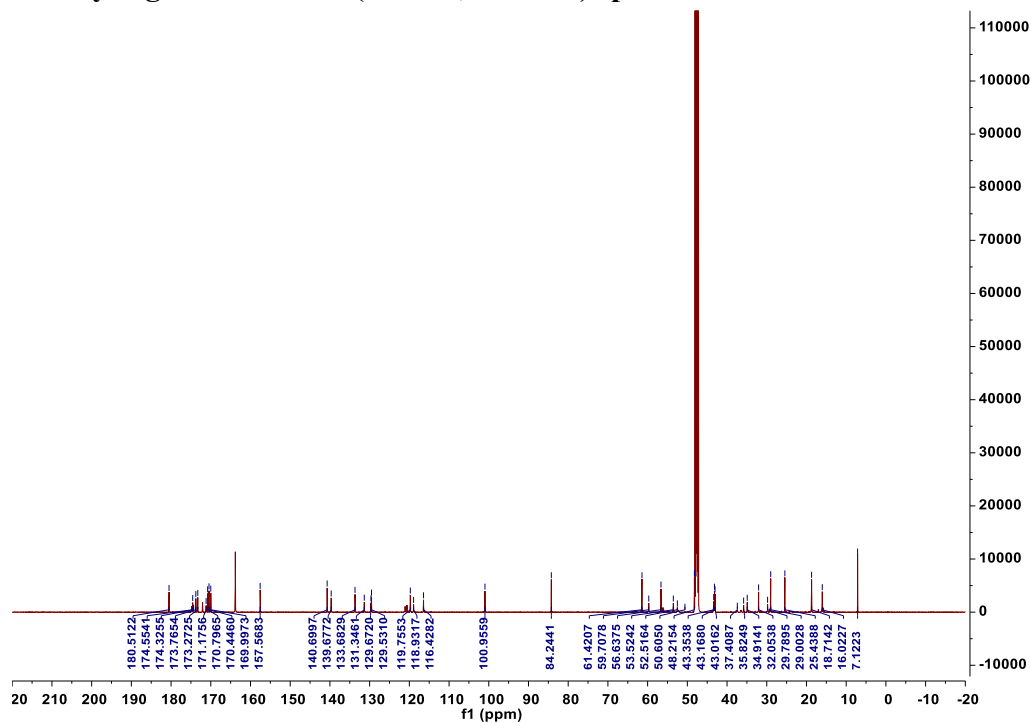
Supplementary Fig. 66. ^1H - ^1H COSY spectrum of 6.



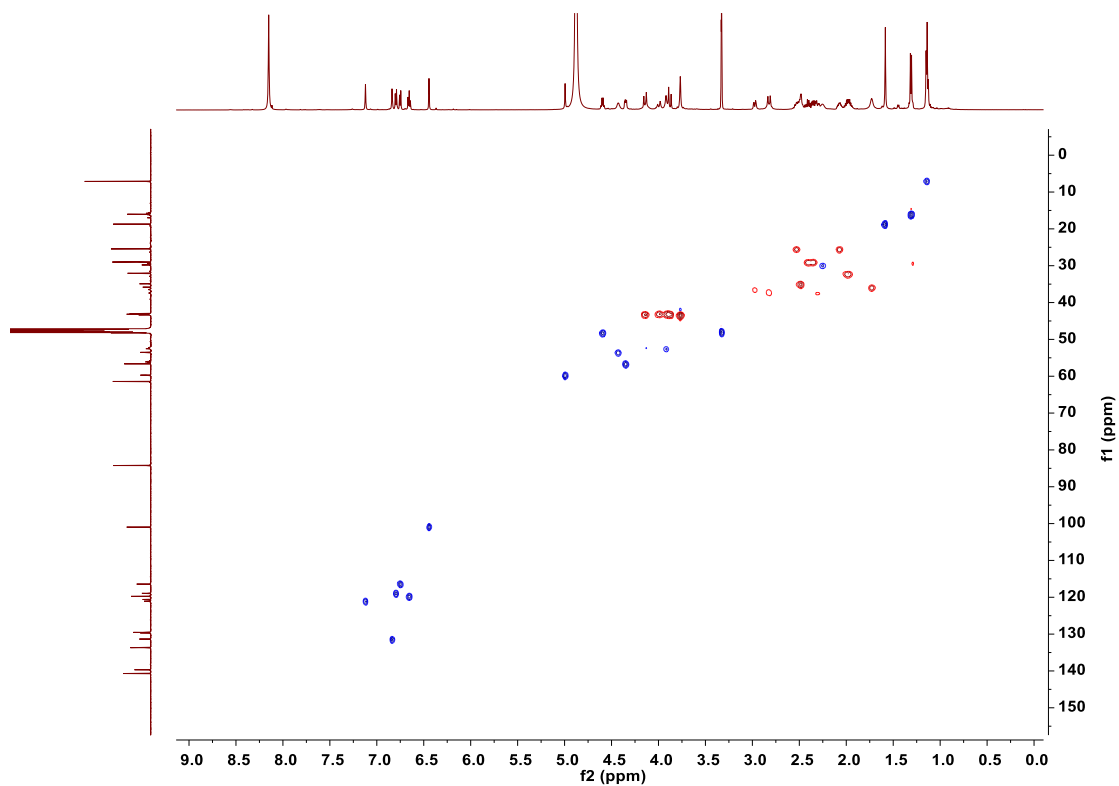
Supplementary Fig. 67. HMBC spectrum of 6.



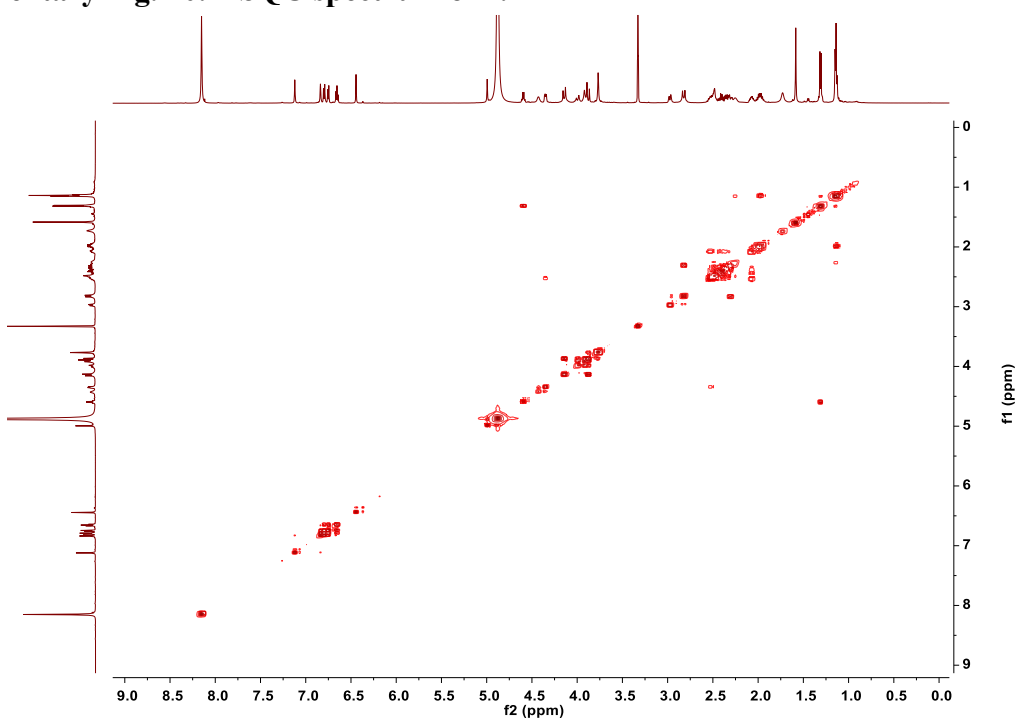
Supplementary Fig. 68. ^1H NMR (600 Hz, CD_3OD) spectrum of 7.



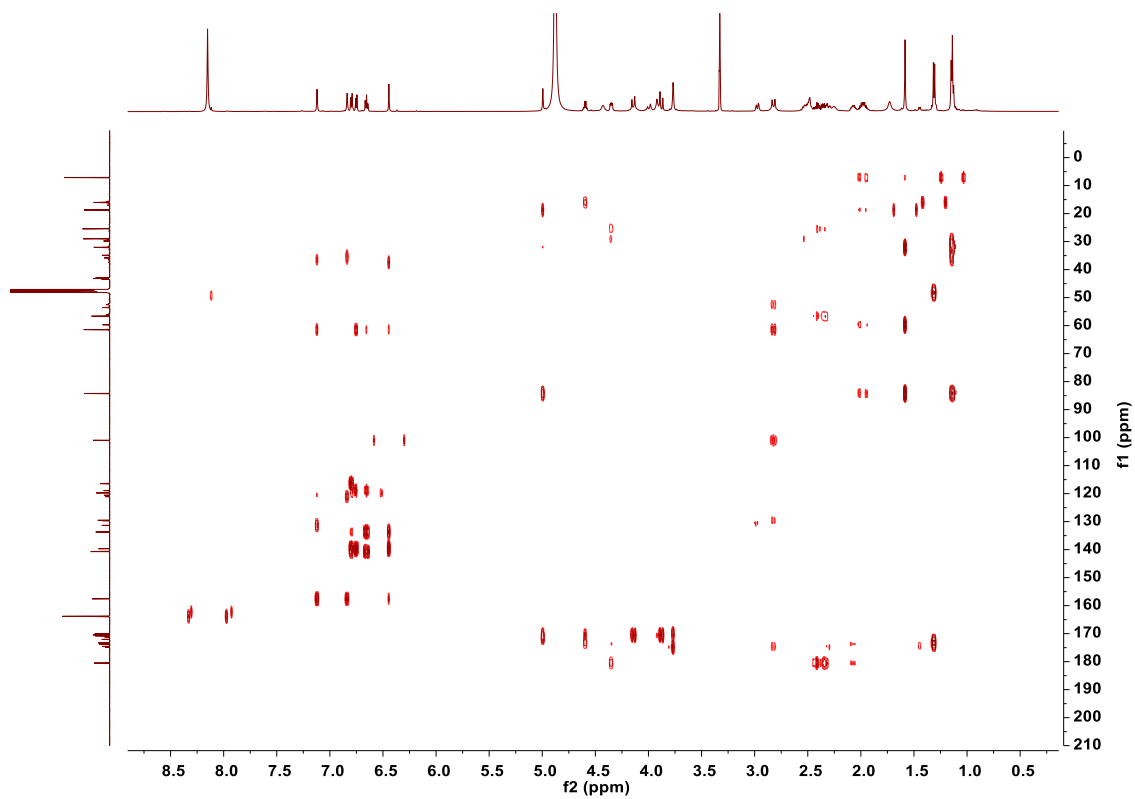
Supplementary Fig. 69. ^{13}C NMR (150 Hz, CD_3OD) spectrum of 7.



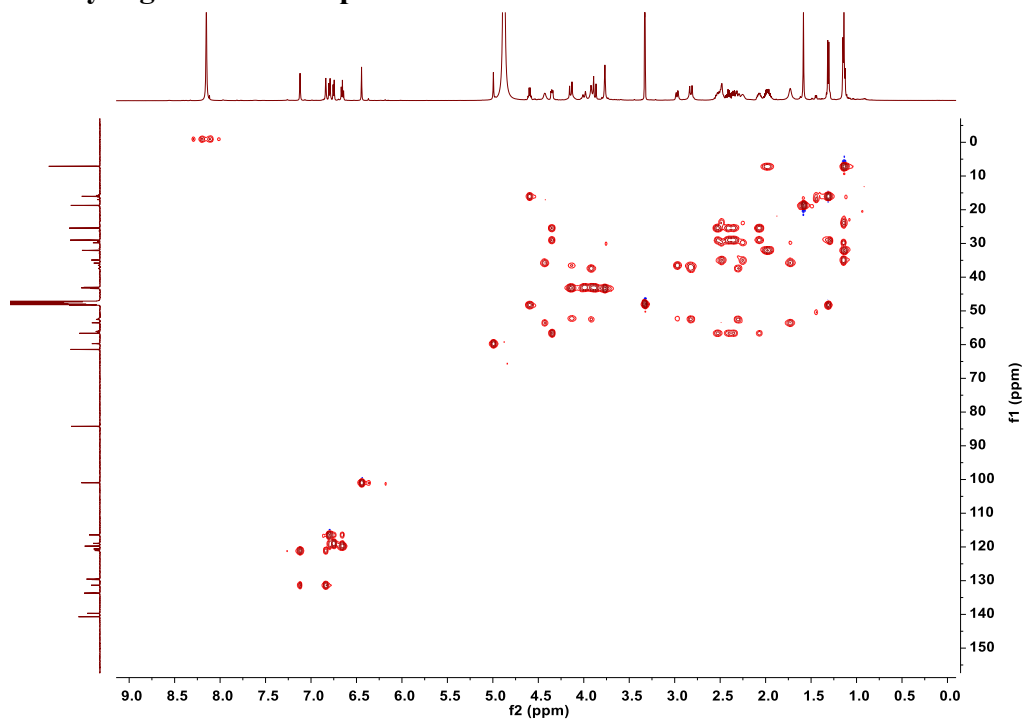
Supplementary Fig. 70. HSQC spectrum of 7.



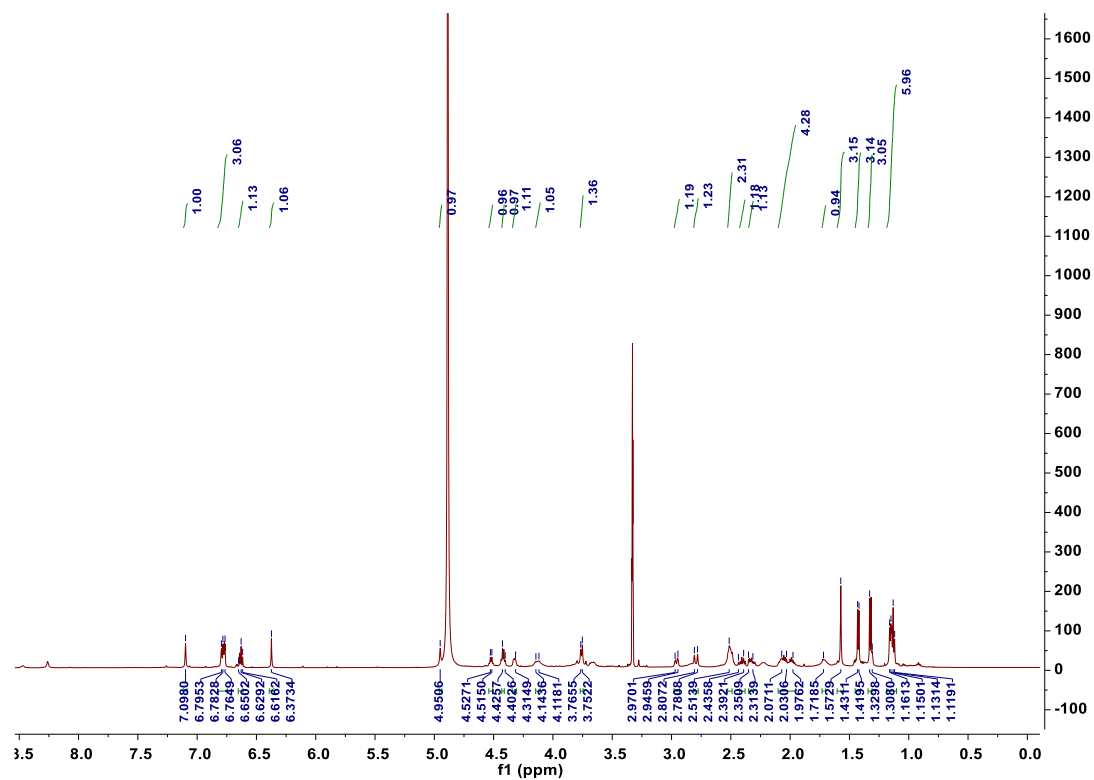
Supplementary Fig. 71. ^1H - ^1H COSY spectrum of 7.



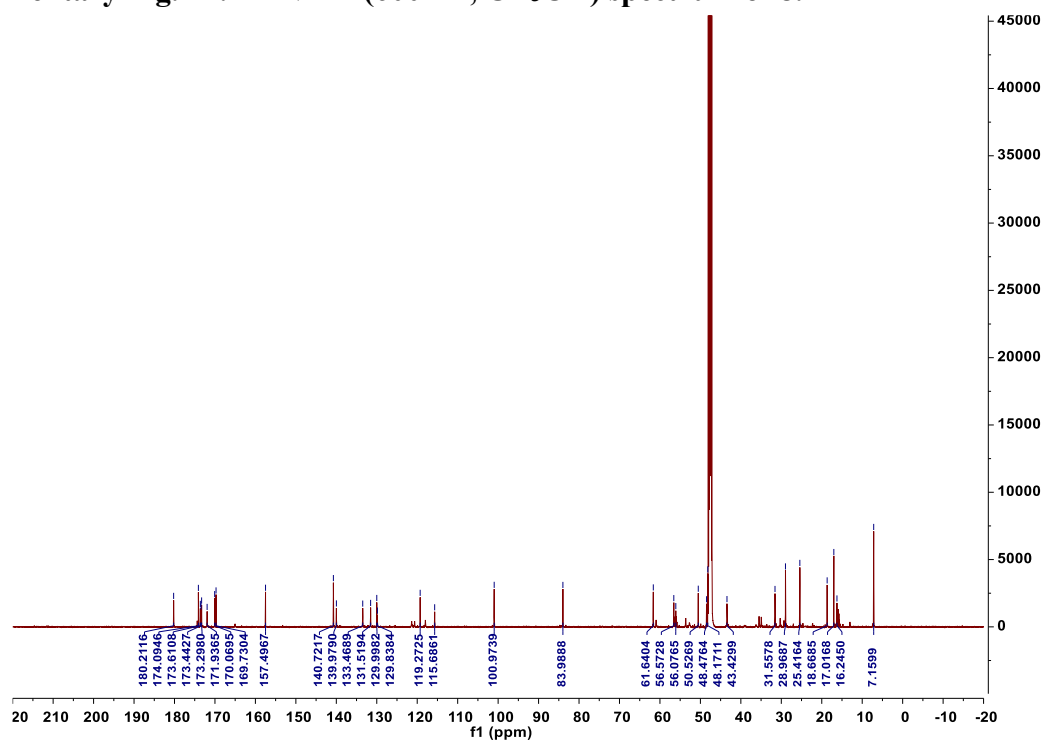
Supplementary Fig. 72. HMBC spectrum of 7.



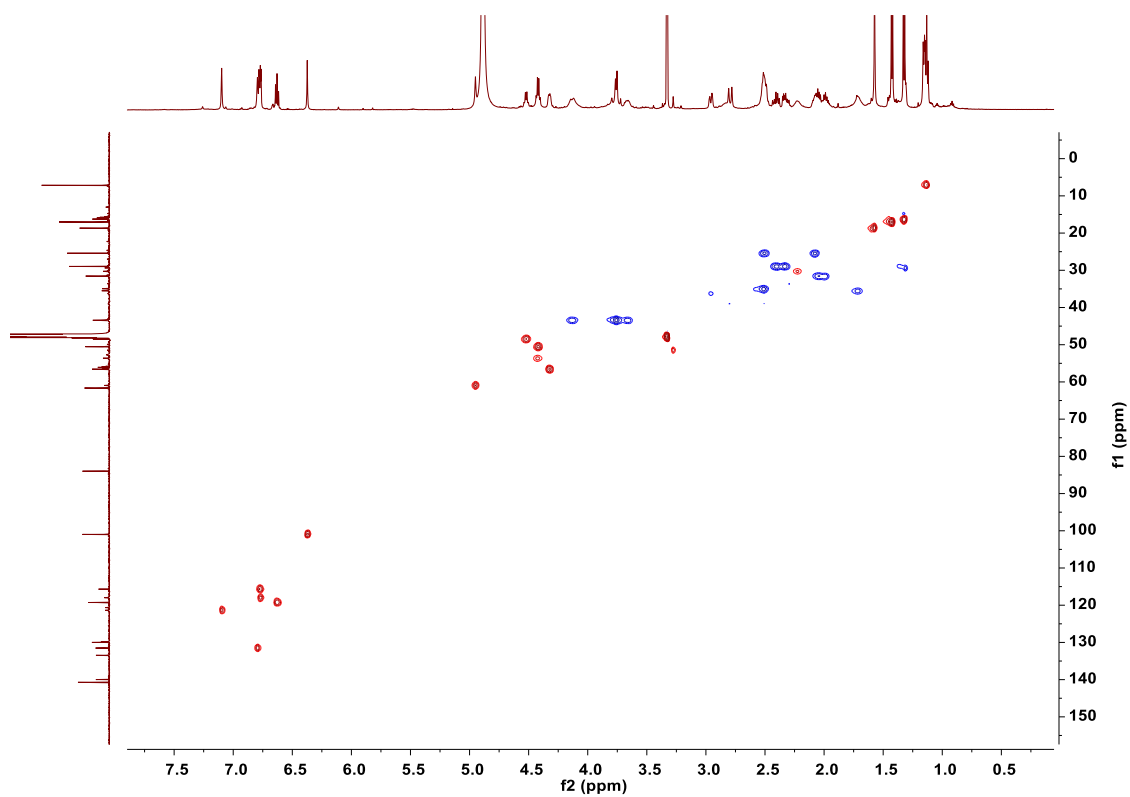
Supplementary Fig. 73. HSQC-TOCSY spectrum of 7.



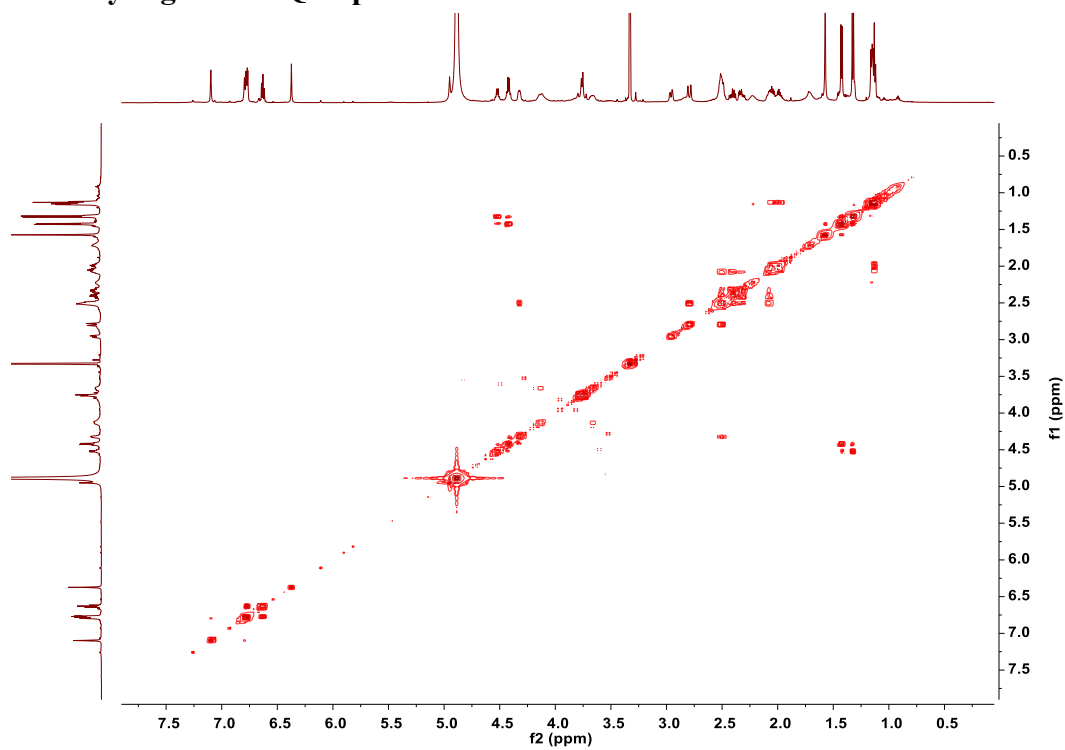
Supplementary Fig. 74. ^1H NMR (600 Hz, CD_3OD) spectrum of 8.



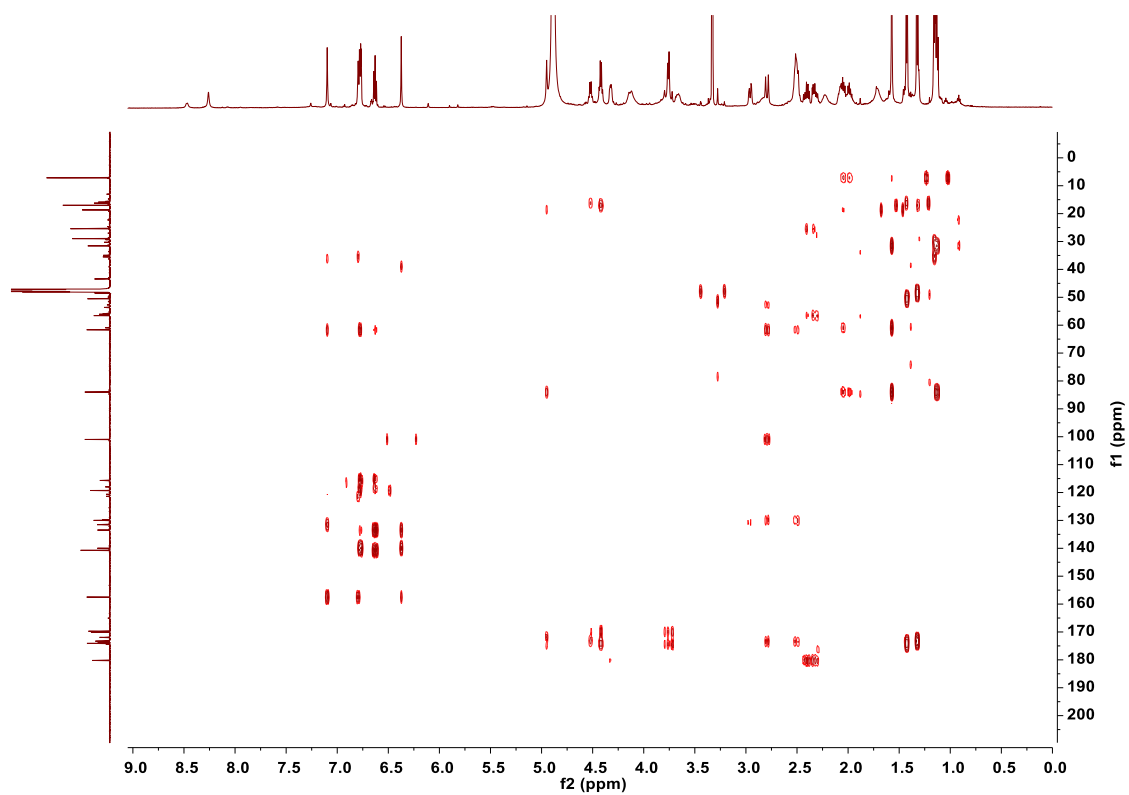
Supplementary Fig. 75. ^{13}C NMR (150 Hz, CD_3OD) spectrum of 8.



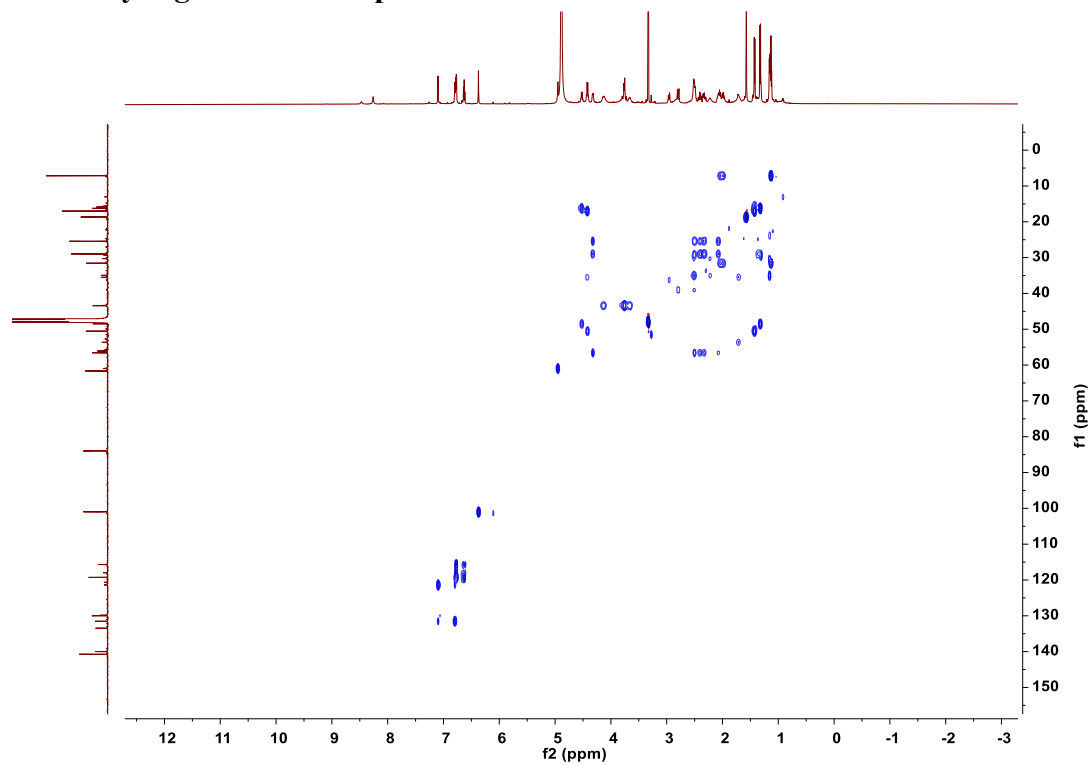
Supplementary Fig. 76. HSQC spectrum of 8.



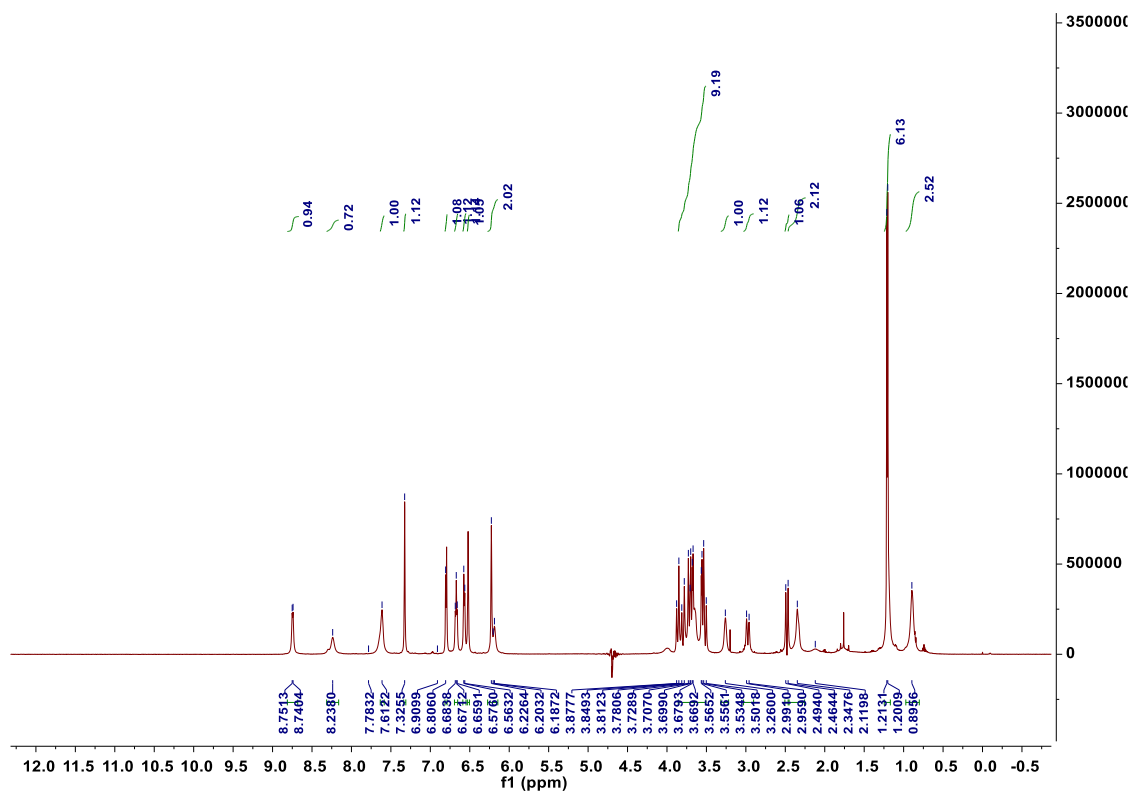
Supplementary Fig. 77. ^1H - ^1H COSY spectrum of 8.



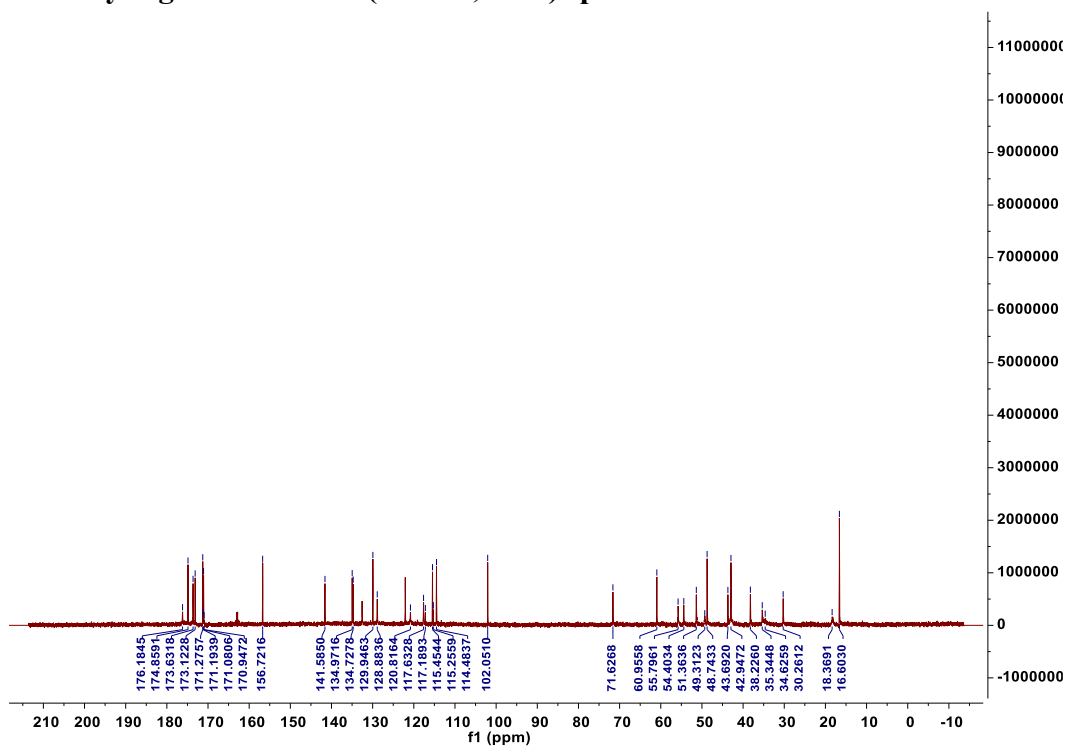
Supplementary Fig. 78. HMBC spectrum of 8.



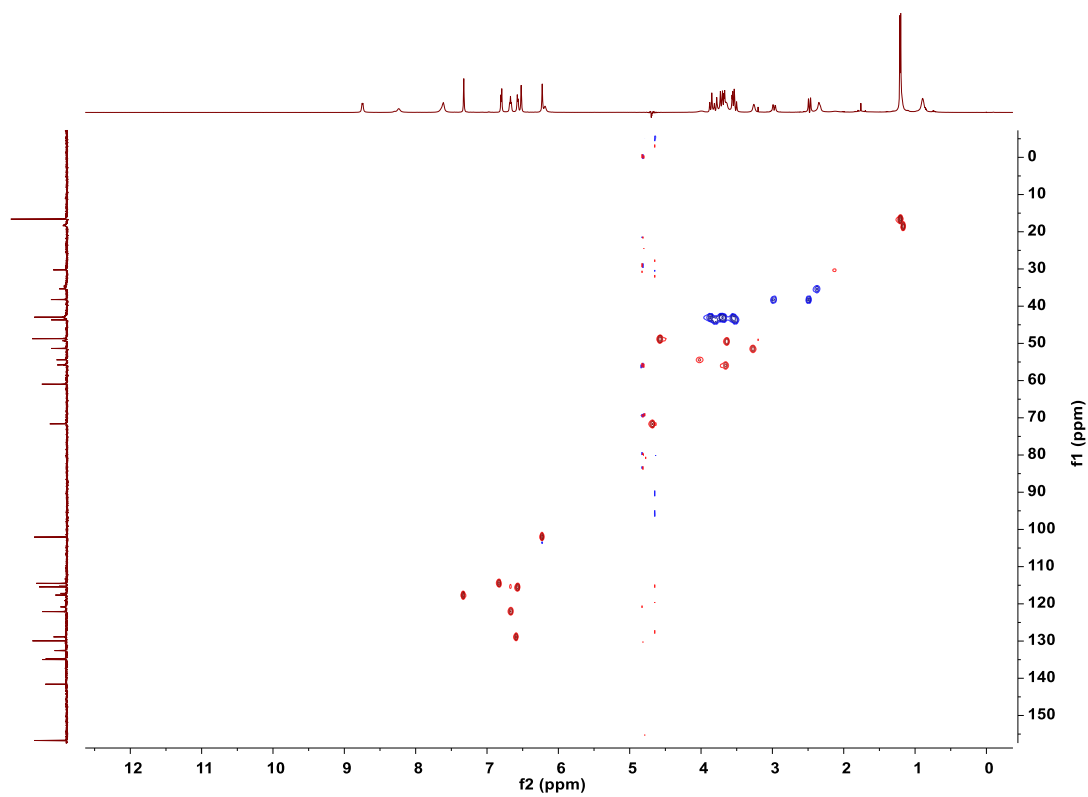
Supplementary Fig. 79. HSQC-TOCSY spectrum of 8.



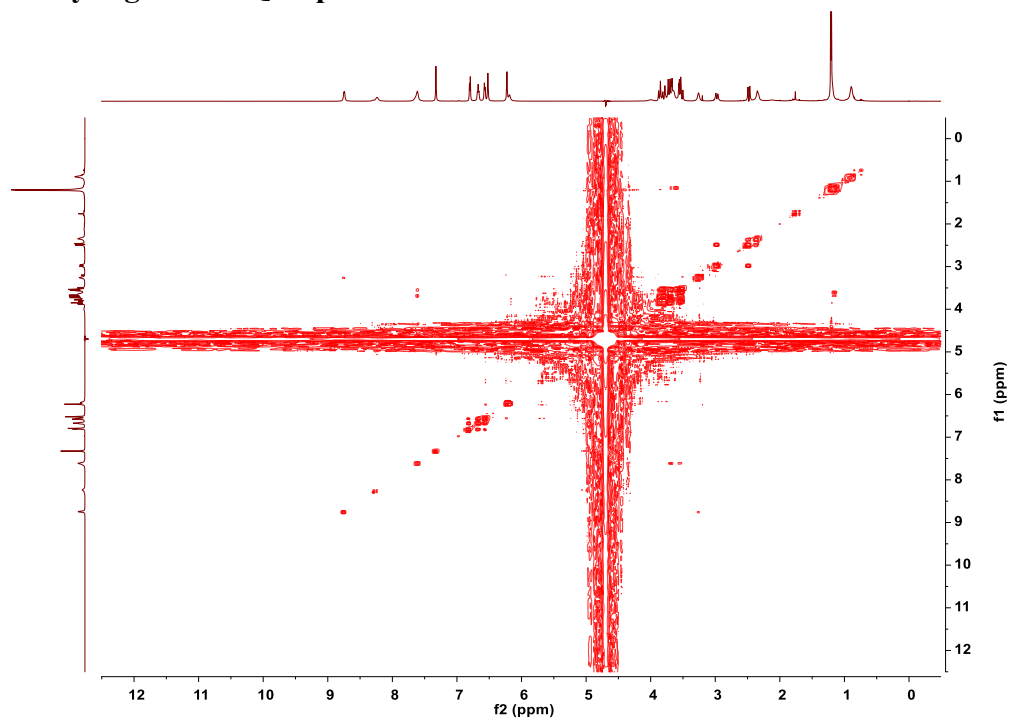
Supplementary Fig. 80. ¹H NMR (600 Hz, H₂O) spectrum of 9.



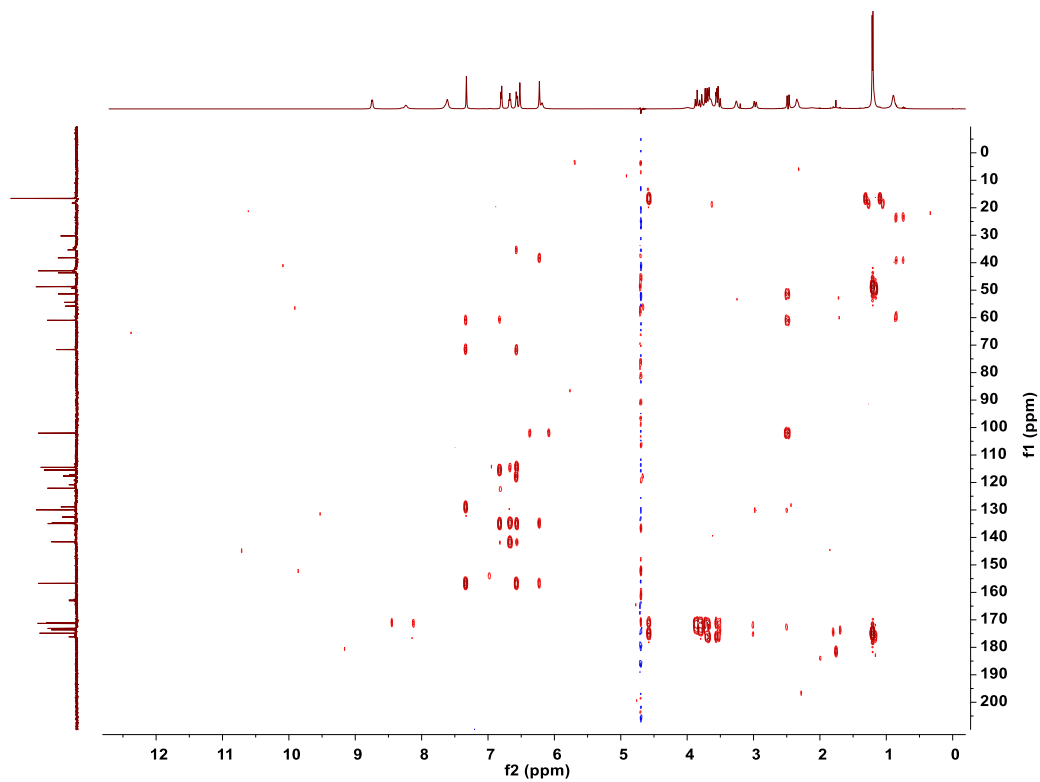
Supplementary Fig. 81. ¹³C NMR (150 Hz, H₂O) spectrum of 9.



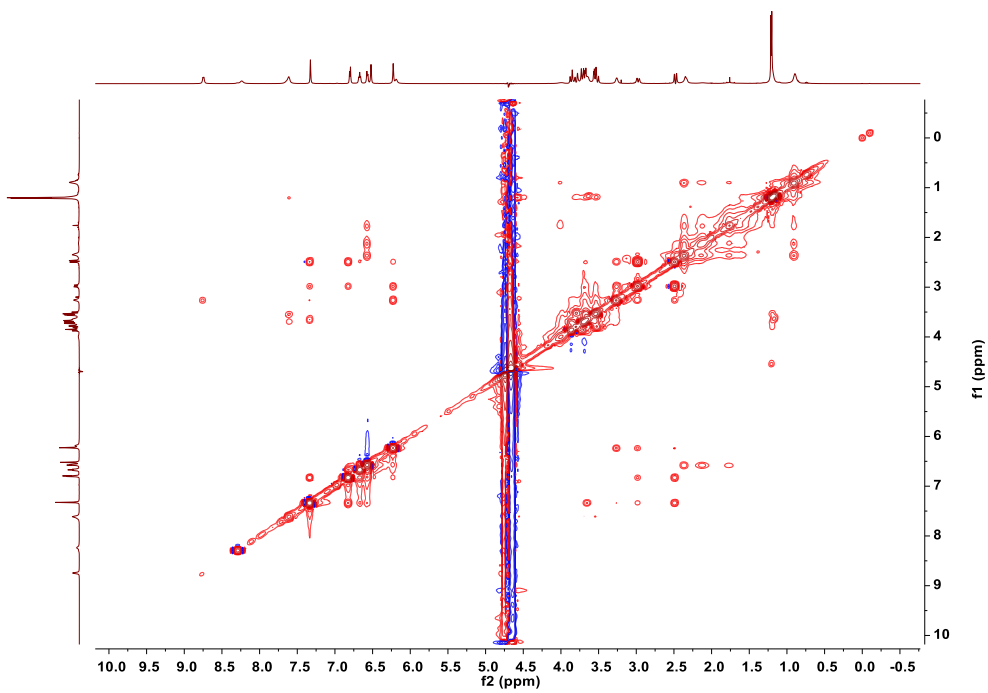
Supplementary Fig. 82. HSQC spectrum of 9.



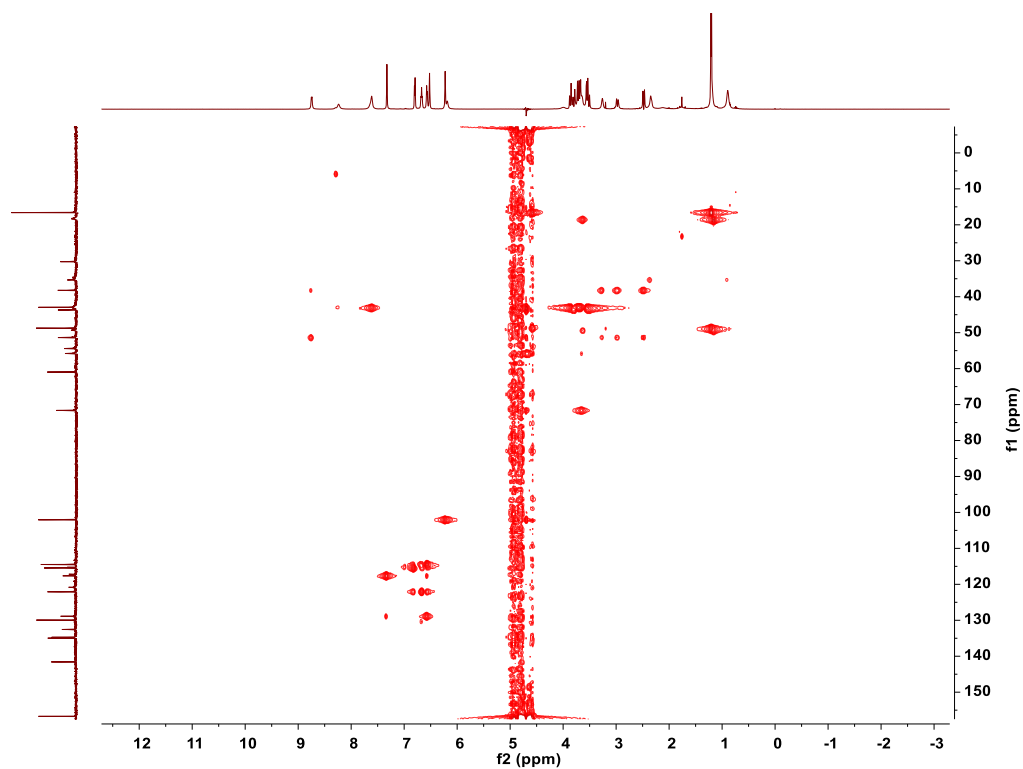
Supplementary Fig. 83. ^1H - ^1H COSY spectrum of 9.



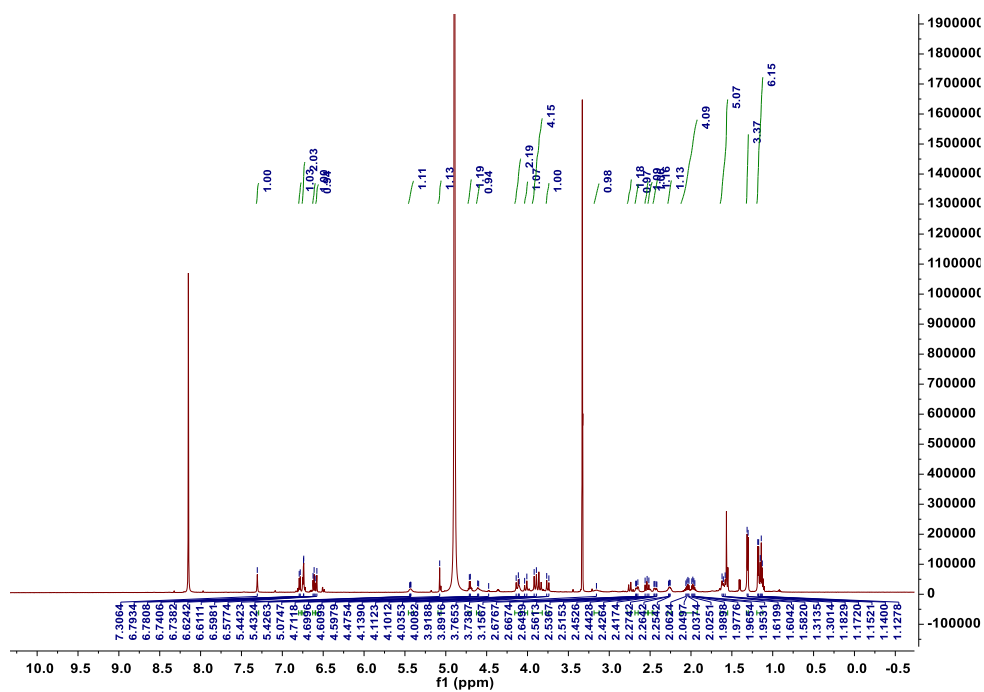
Supplementary Fig. 84. HMBC spectrum of 9.



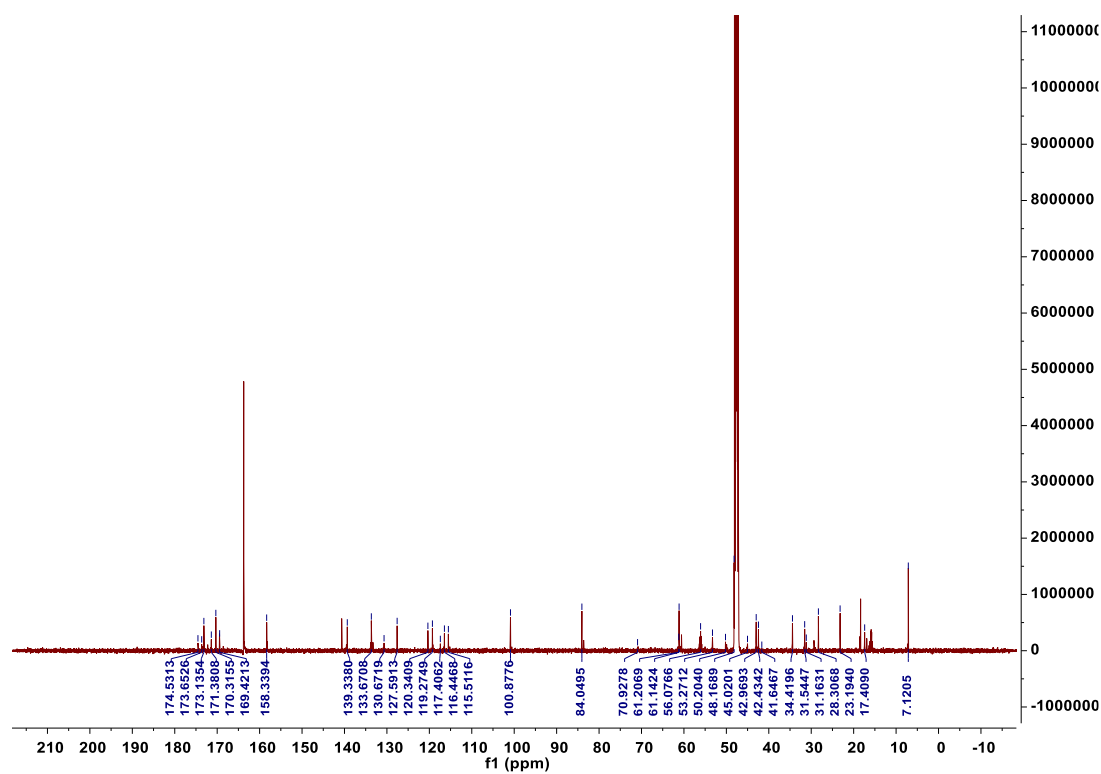
Supplementary Fig. 85. ROESY spectrum of 9.



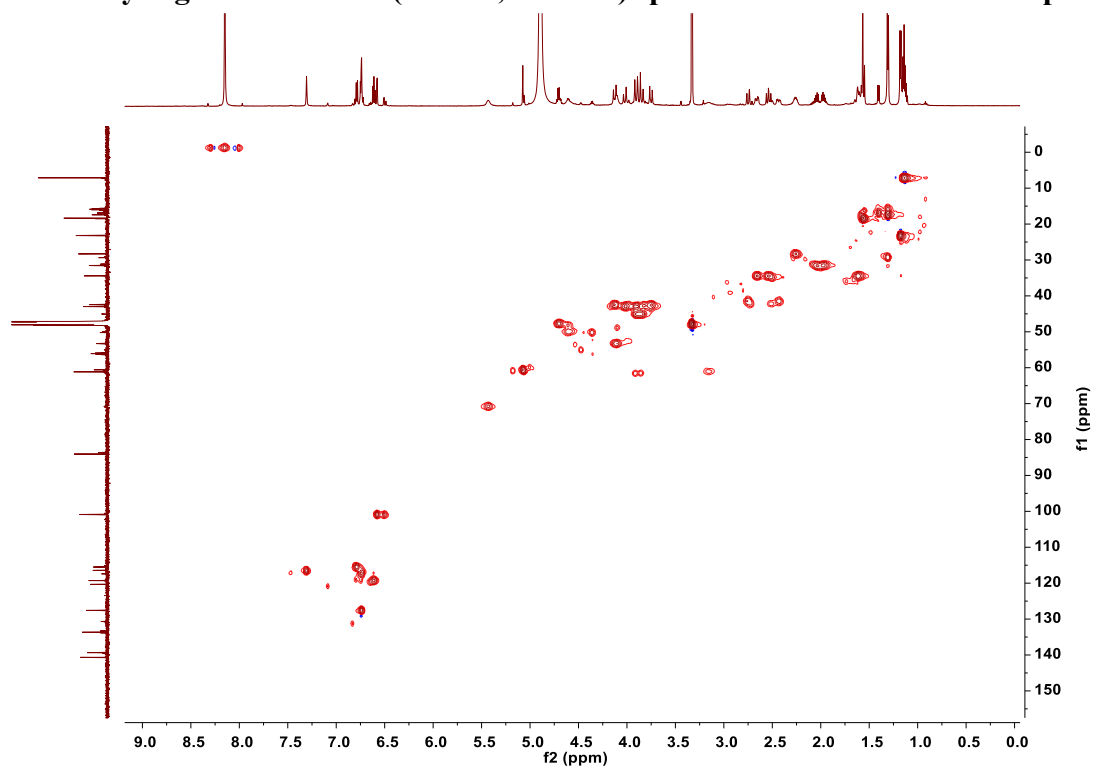
Supplementary Fig. 86. HSQC-TOCSY spectrum of 9.



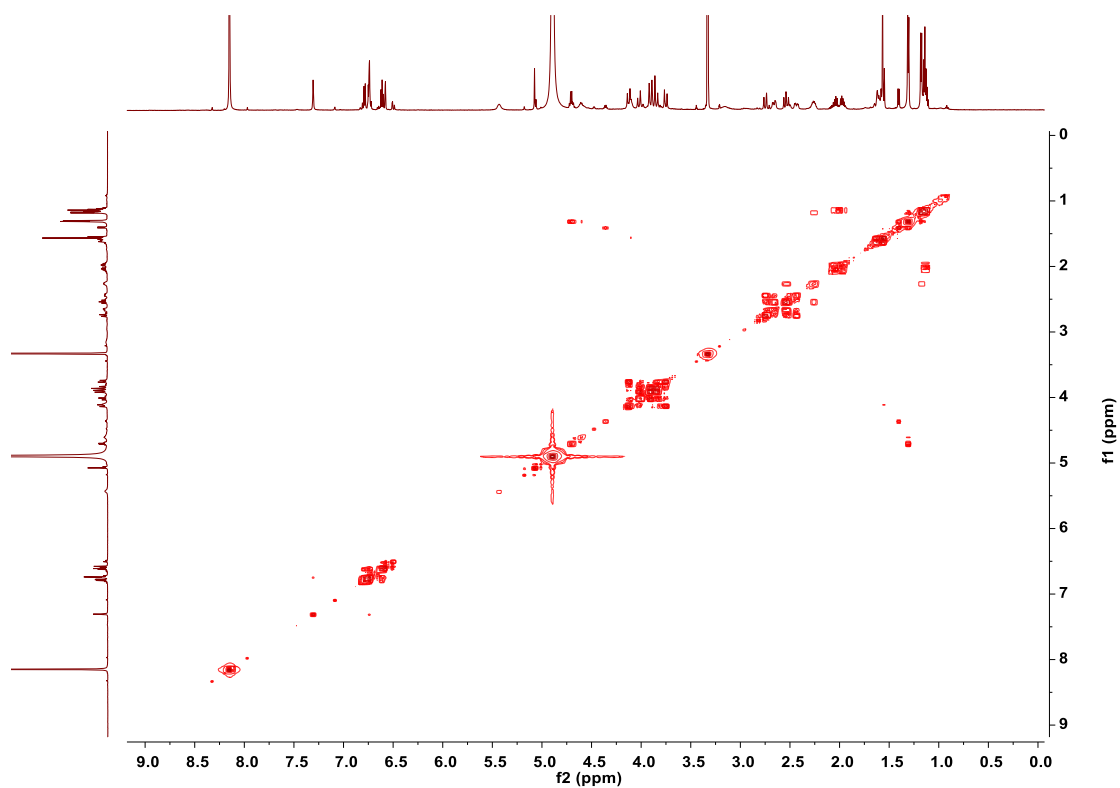
Supplementary Fig. 87. ^1H NMR (600 Hz, CD_3OD) spectrum of 10 with minor impurity.



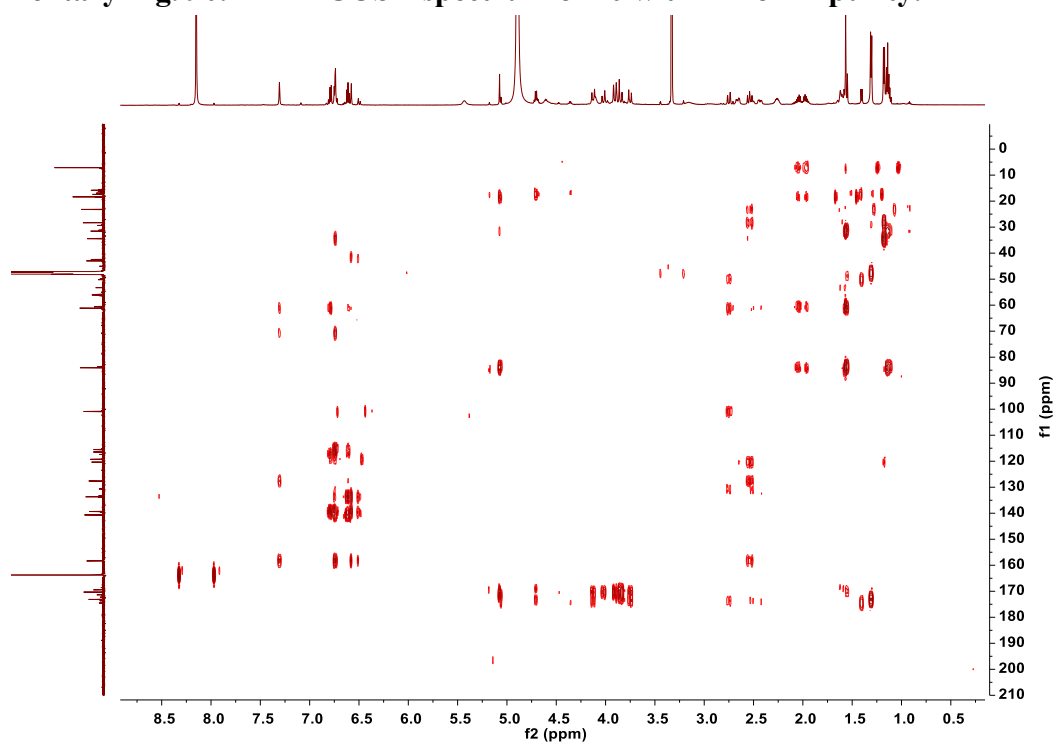
Supplementary Fig. 88. ^{13}C NMR (150 Hz, CD_3OD) spectrum of 10 with minor impurity.



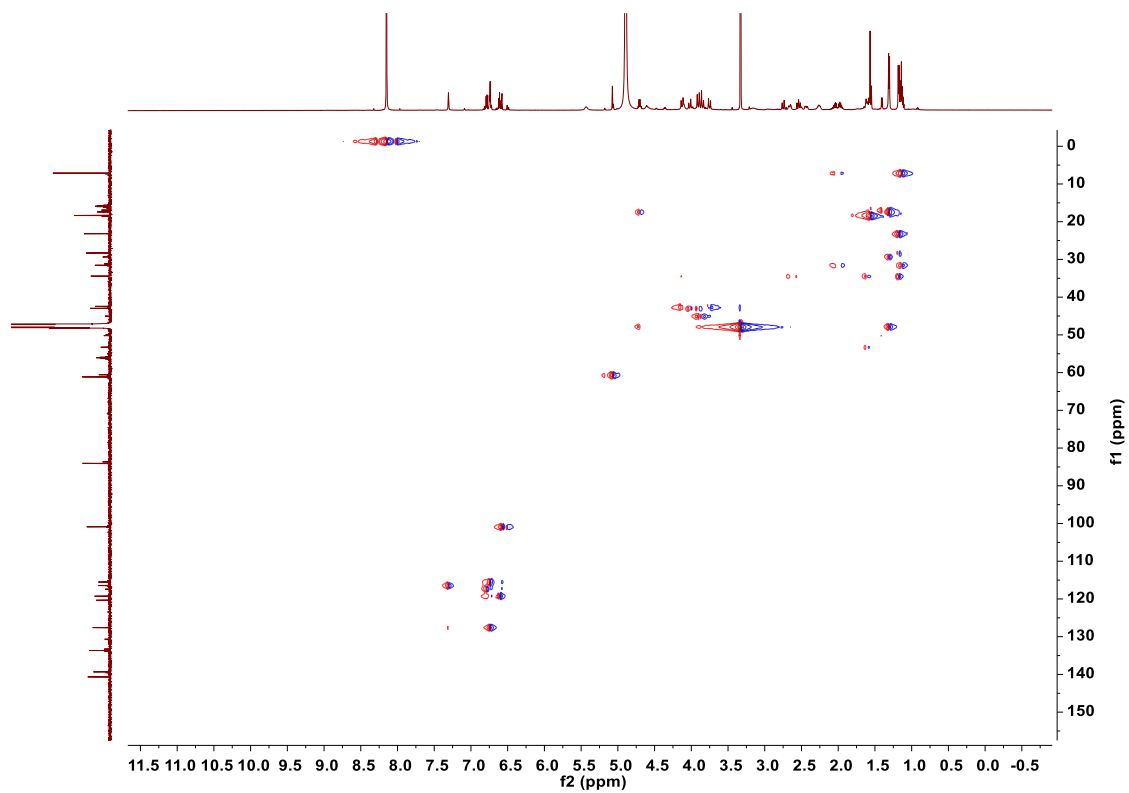
Supplementary Fig. 89. HSQC spectrum of 10 with minor impurity.



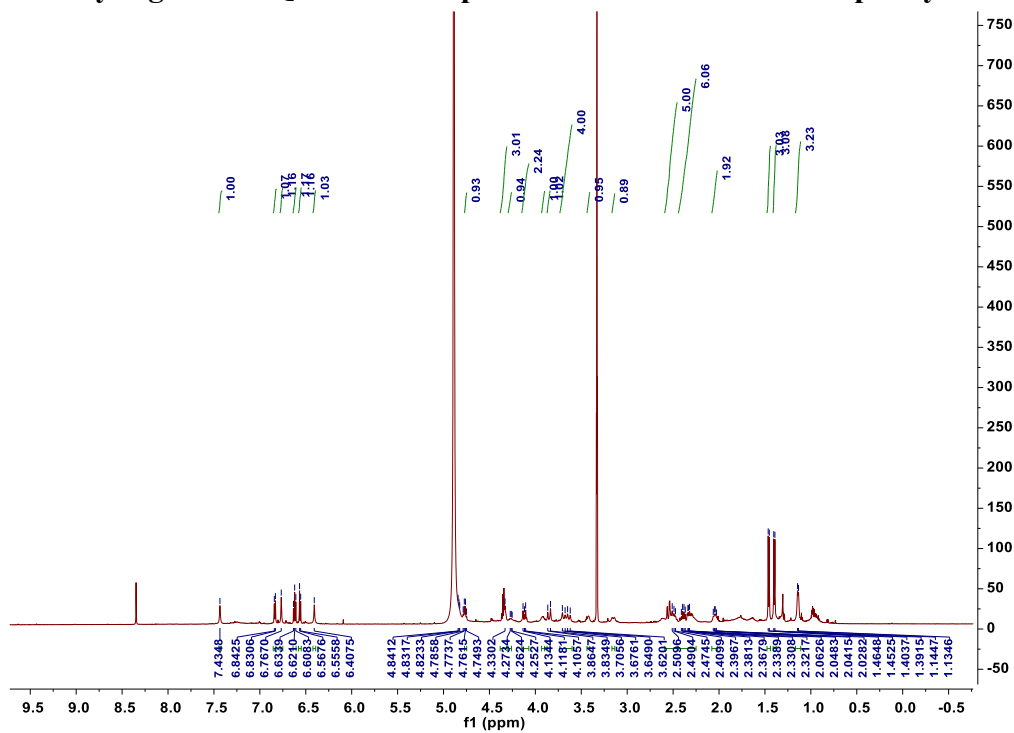
Supplementary Fig. 90. ^1H - ^1H COSY spectrum of 10 with minor impurity.



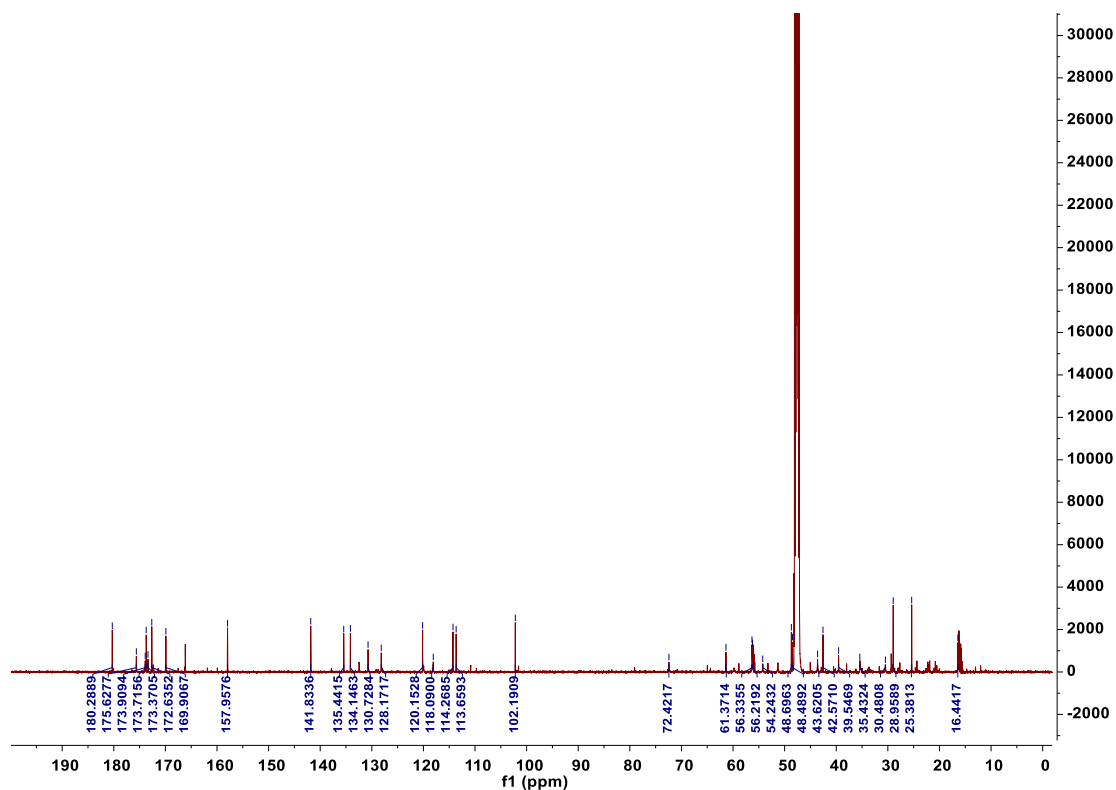
Supplementary Fig. 91. HMBC spectrum of 10 with minor impurity.



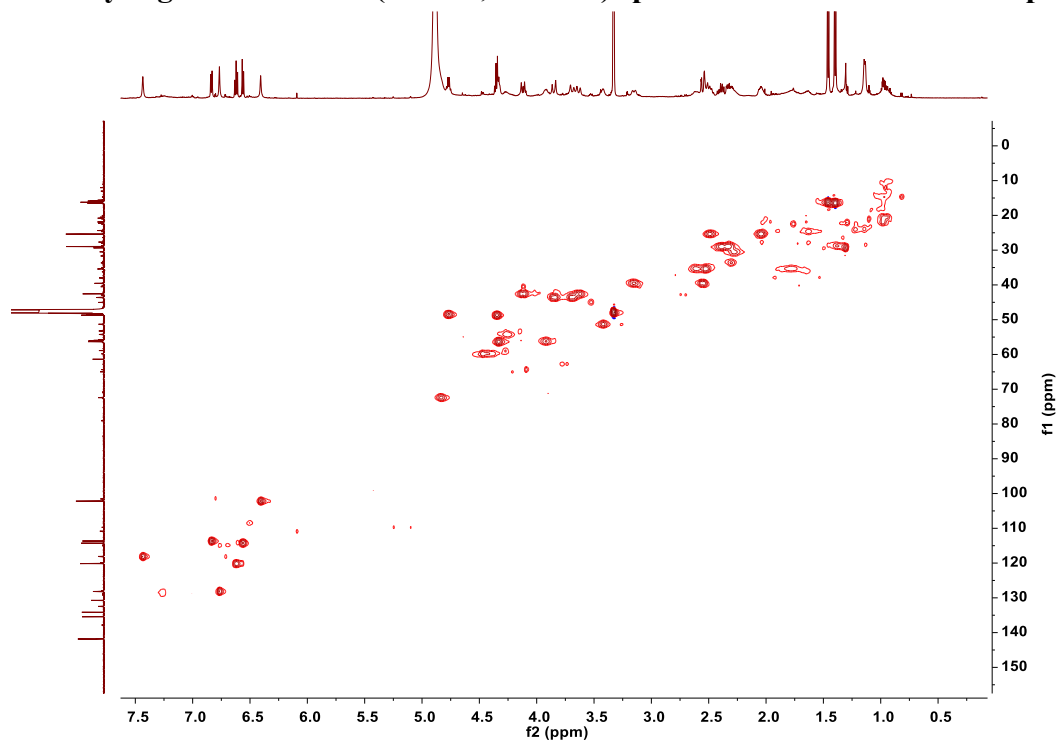
Supplementary Fig. 92. HSQC-TOCSY spectrum of 10 with minor impurity.



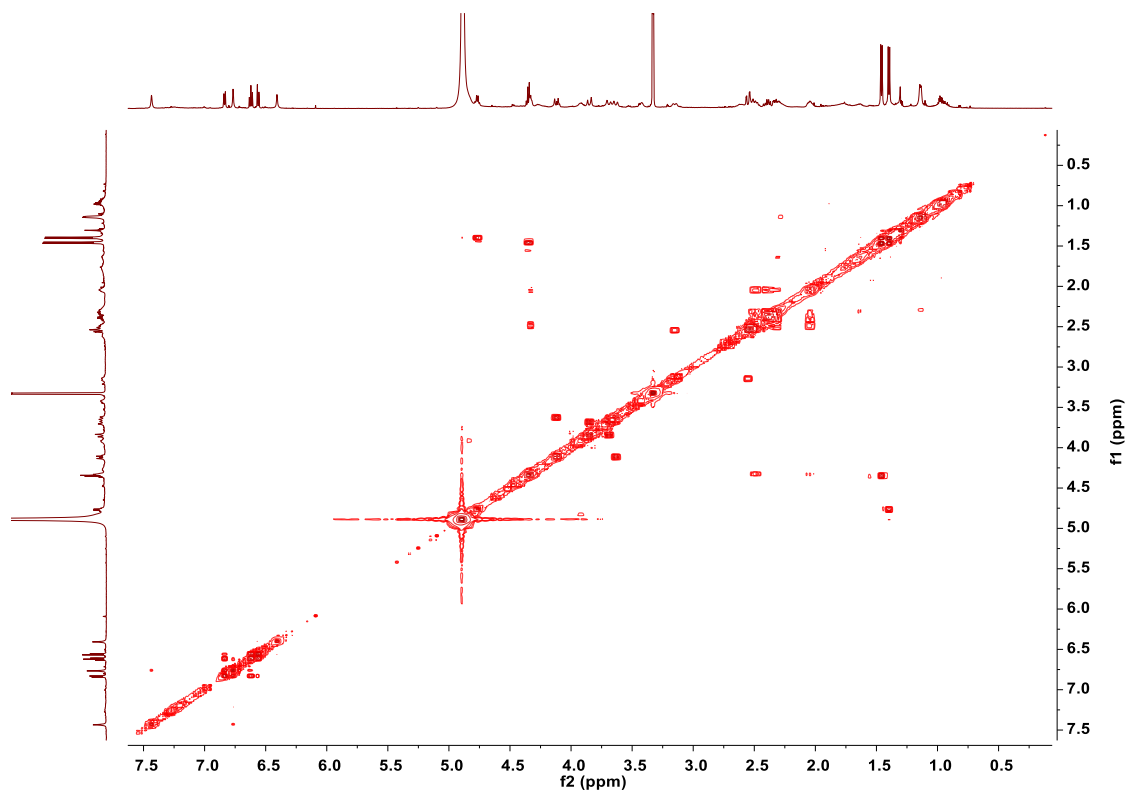
Supplementary Fig. 93. ^1H NMR (600 Hz, CD_3OD) spectrum of 11 with minor impurity.



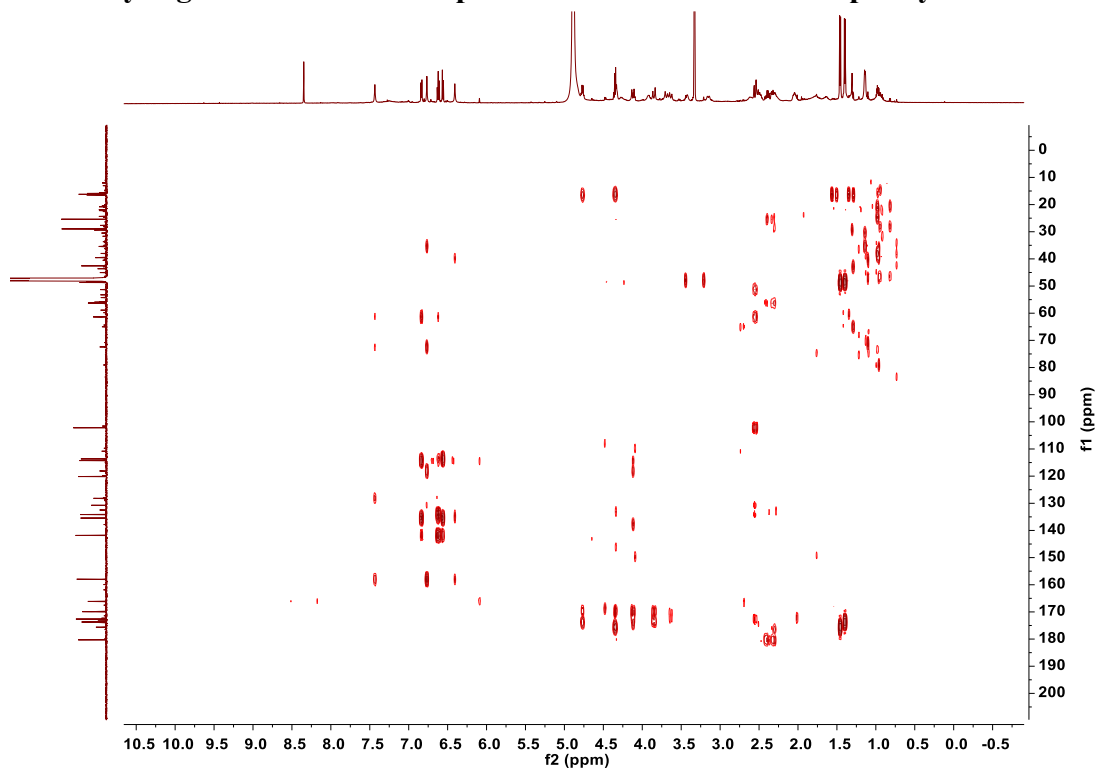
Supplementary Fig. 94. ^{13}C NMR (150 Hz, CD_3OD) spectrum of 11 with minor impurity.



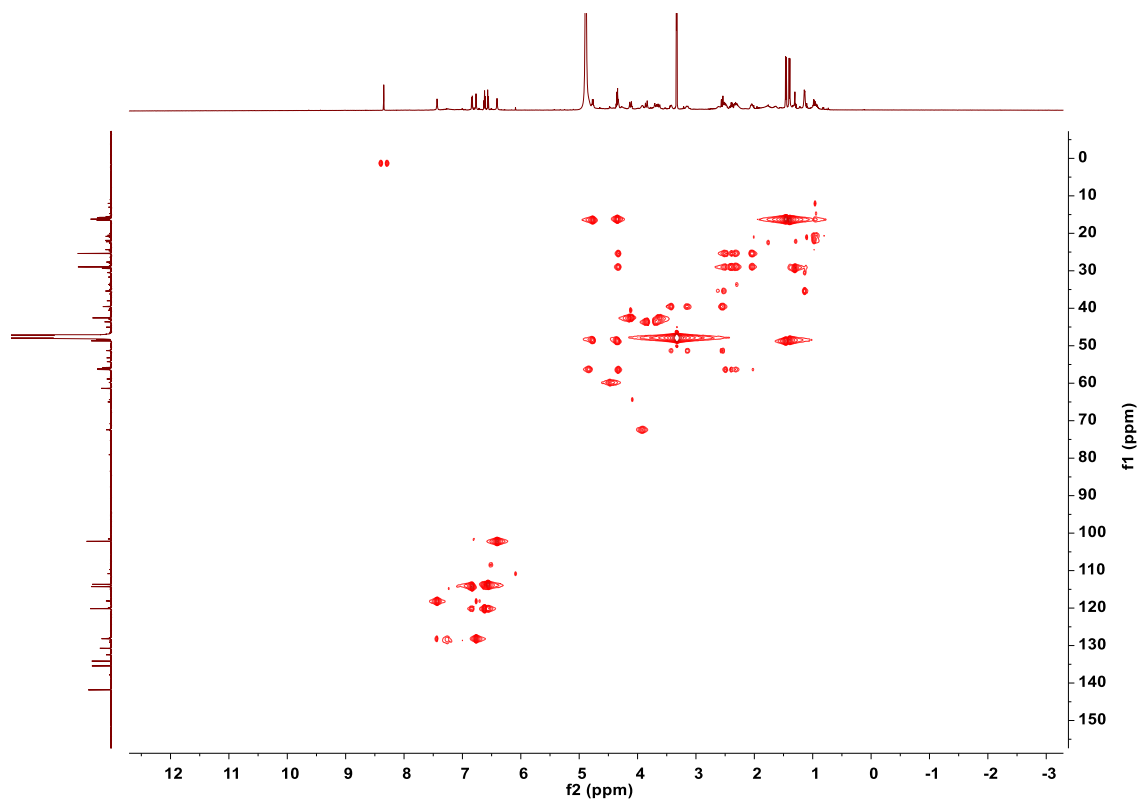
Supplementary Fig. 95. HSQC spectrum of 11 with minor impurity.



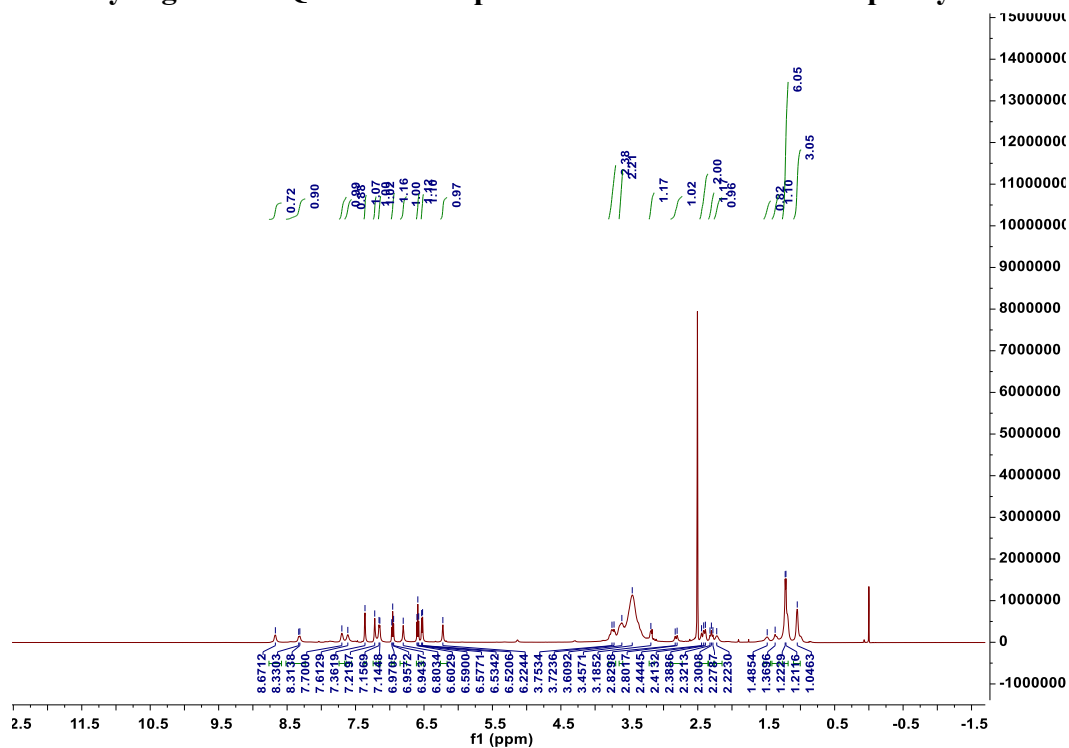
Supplementary Fig. 96. ^1H - ^1H COSY spectrum of 11 with minor impurity.



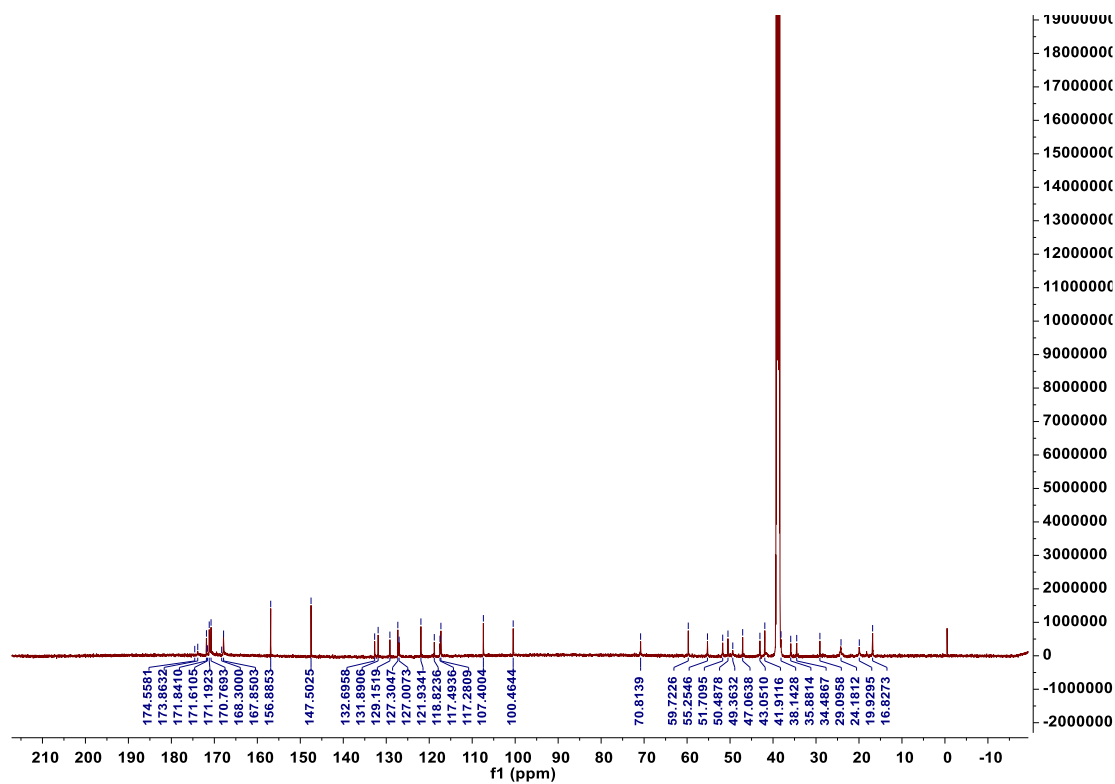
Supplementary Fig. 97. HMBC spectrum of 11 with minor impurity.



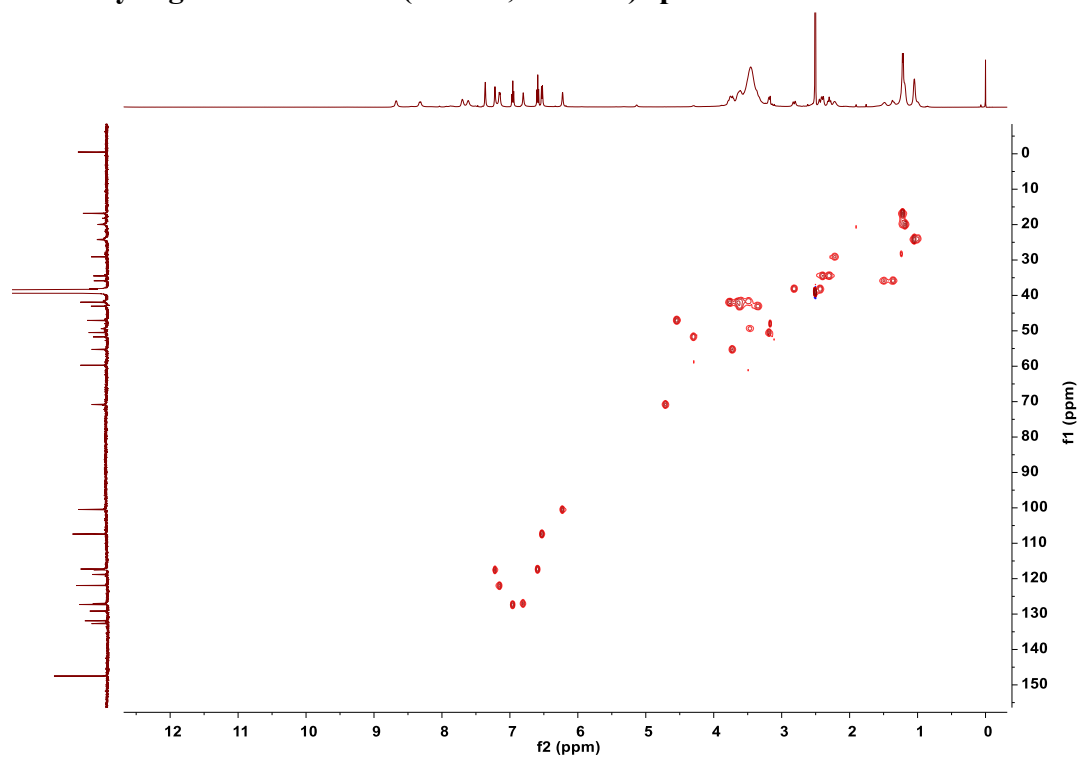
Supplementary Fig. 98. HSQC-TOCSY spectrum of 11 with minor impurity.



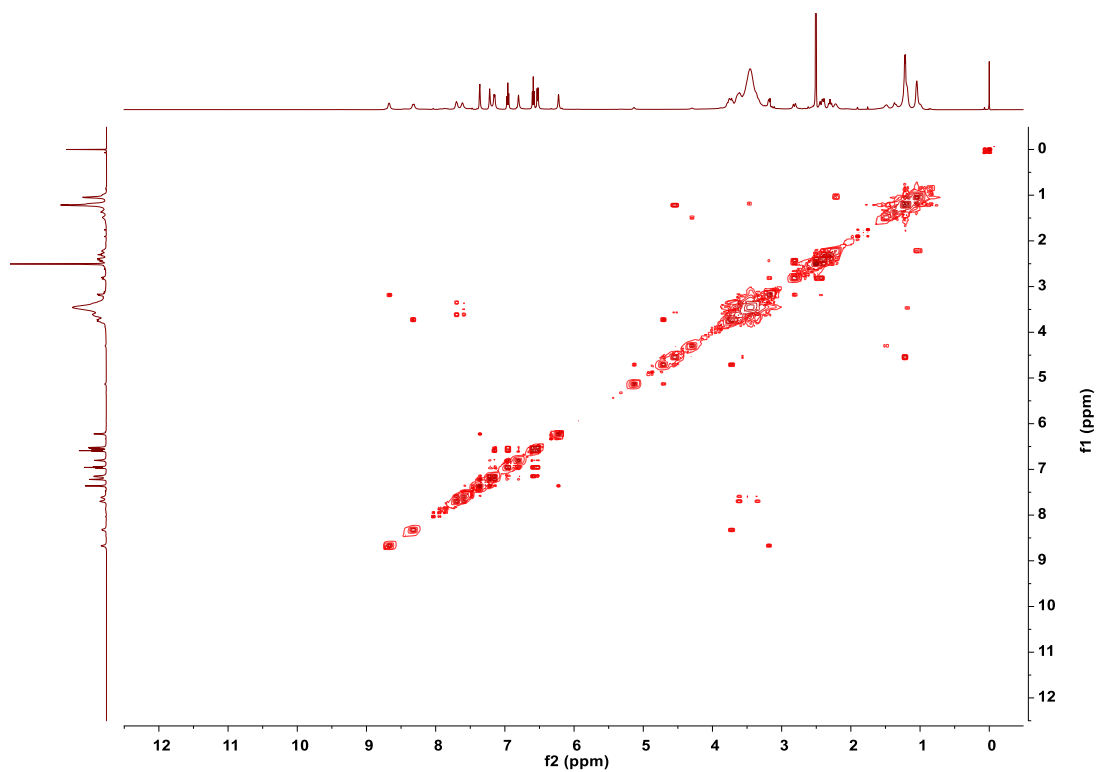
Supplementary Fig. 99. ^1H NMR (600 Hz, CD_3OD) spectrum of 12.



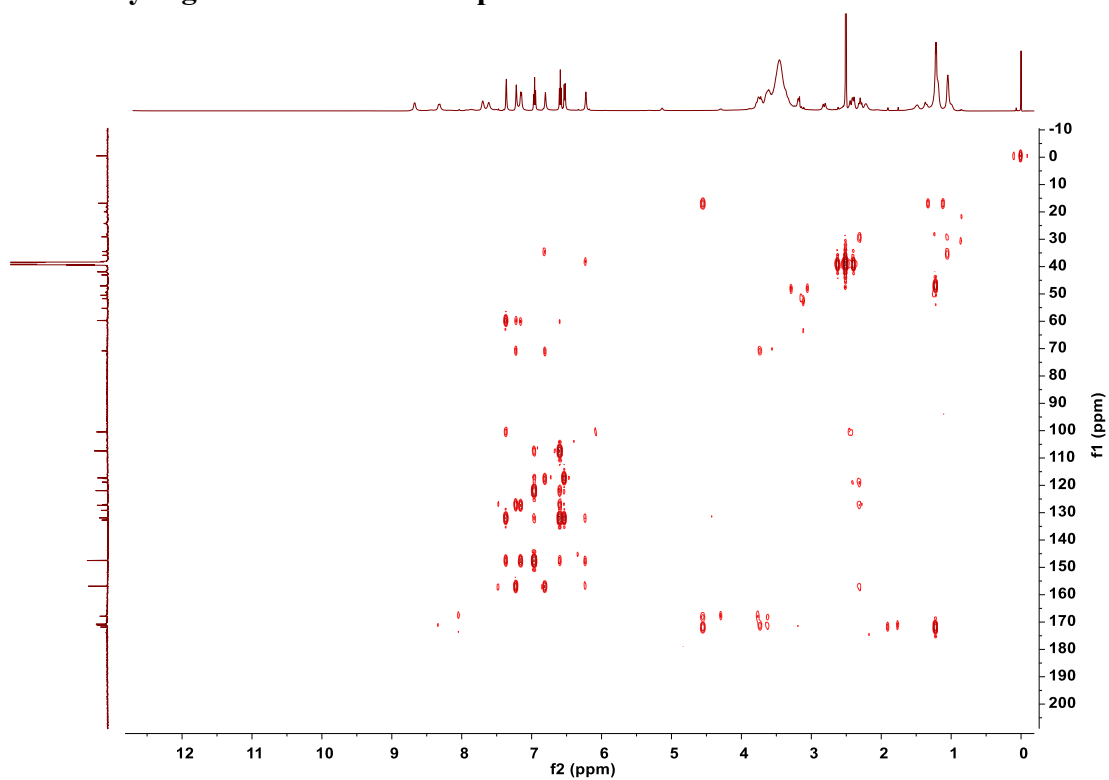
Supplementary Fig. 100. ^{13}C NMR (150 Hz, CD_3OD) spectrum of 12.



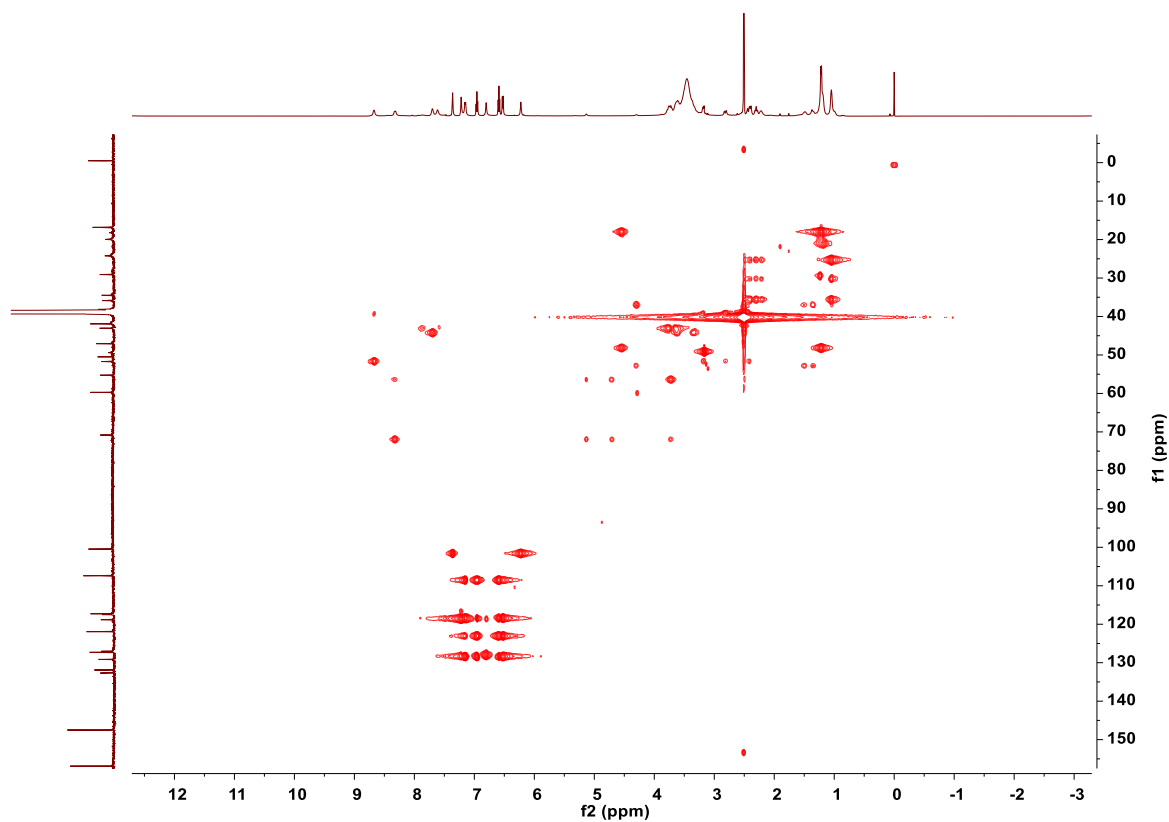
Supplementary Fig. 101. HSQC spectrum of 12.



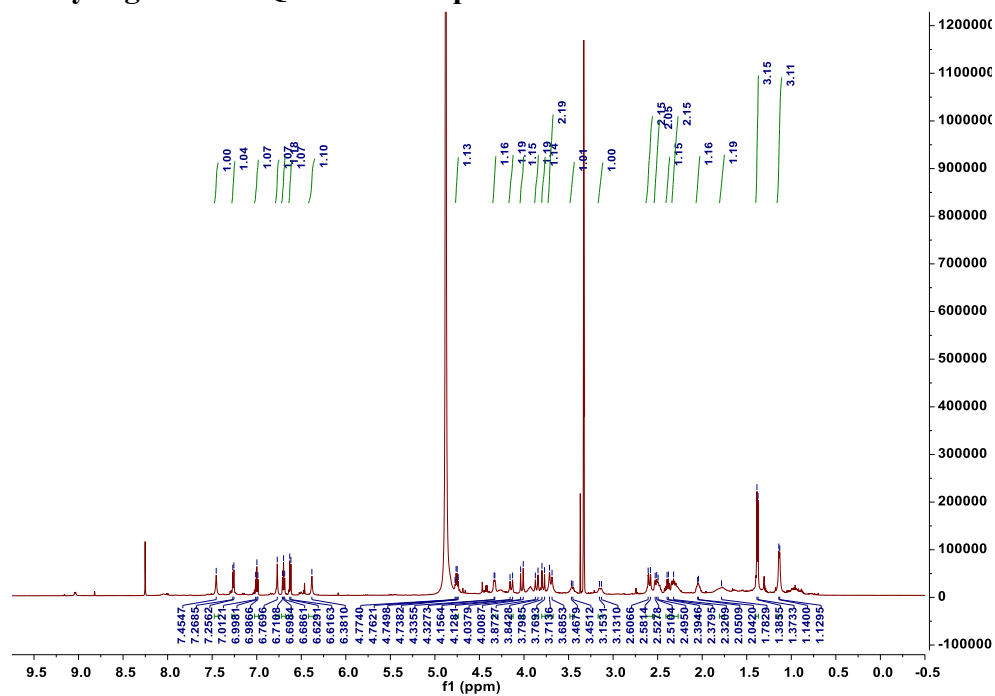
Supplementary Fig. 102. ^1H - ^1H COSY spectrum of 12.



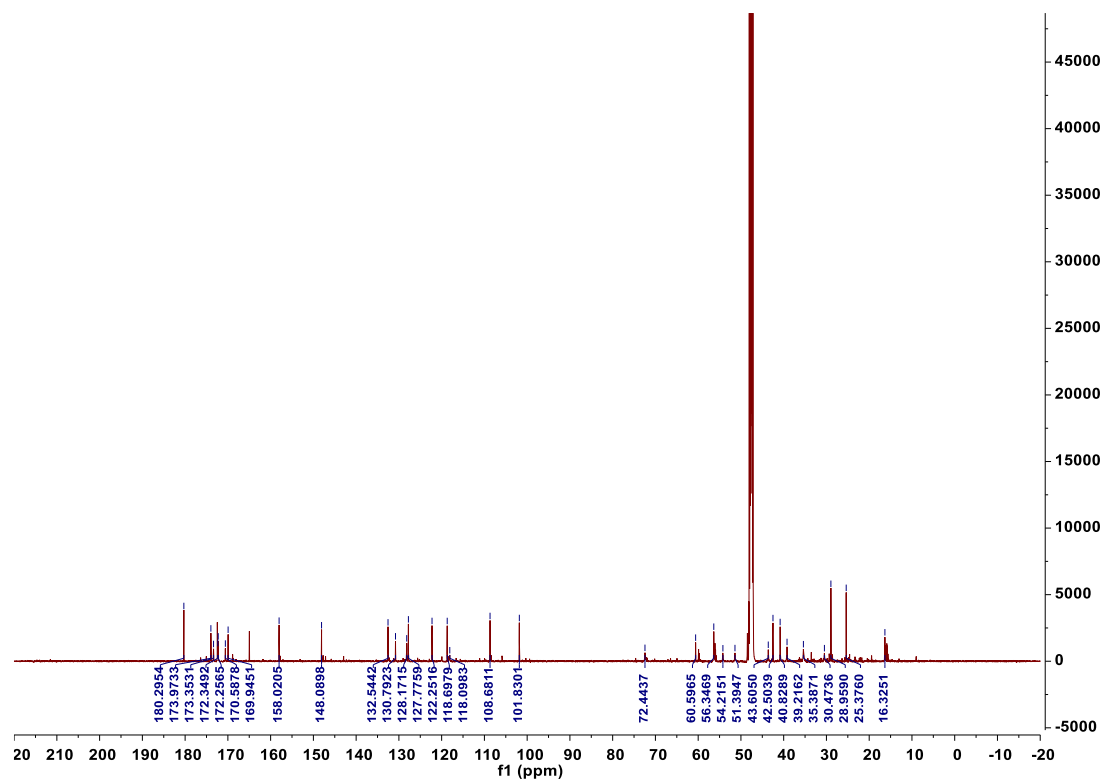
Supplementary Fig. 103. HMBC spectrum of 12.



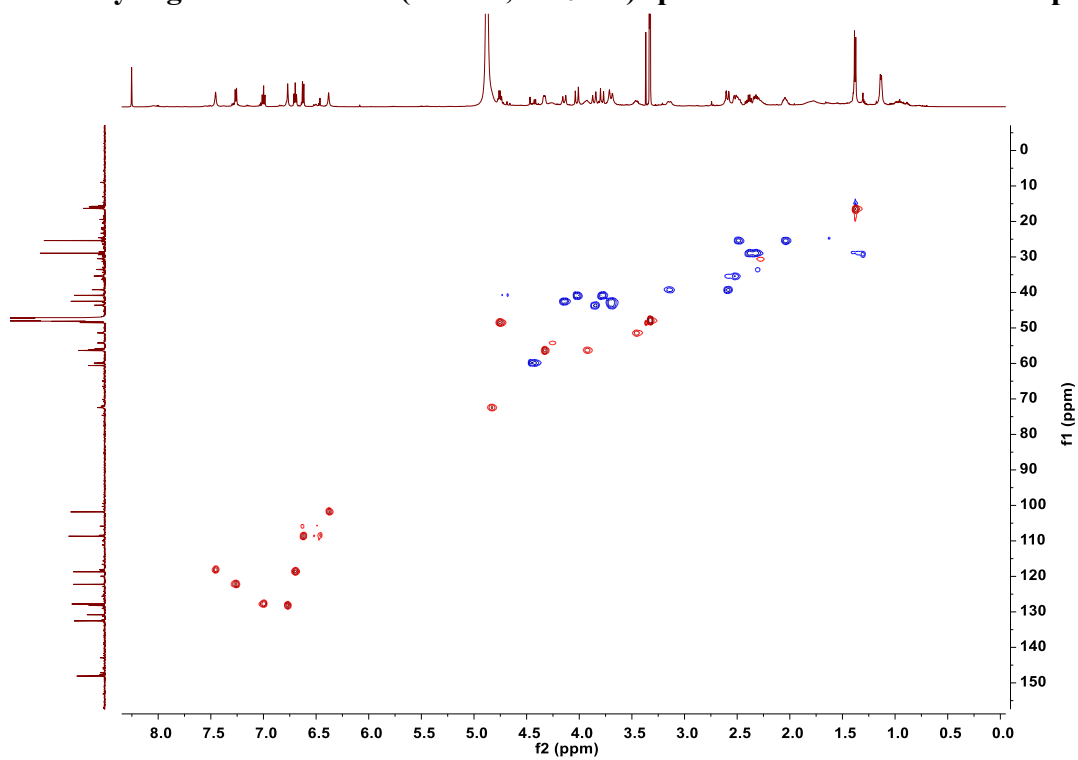
Supplementary Fig. 104. HSQC-TOCSY spectrum of 12.



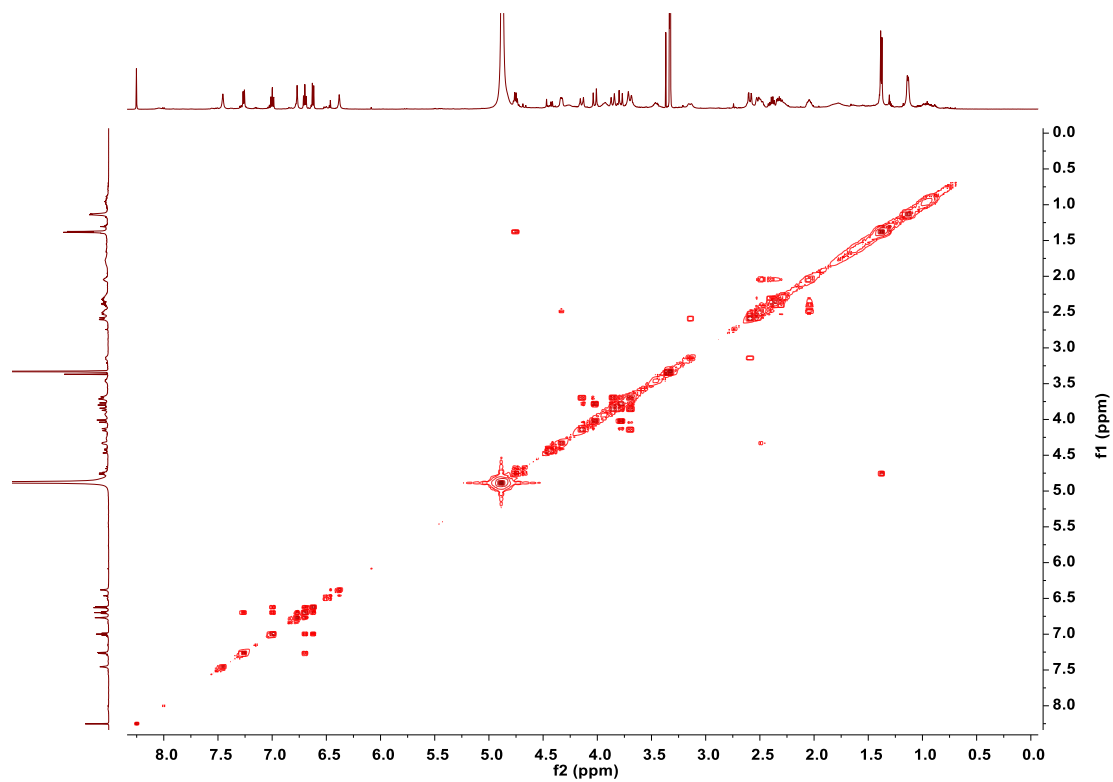
Supplementary Fig. 105. ^1H NMR (600 Hz, CD_3OD) spectrum of 13 with minor impurity.



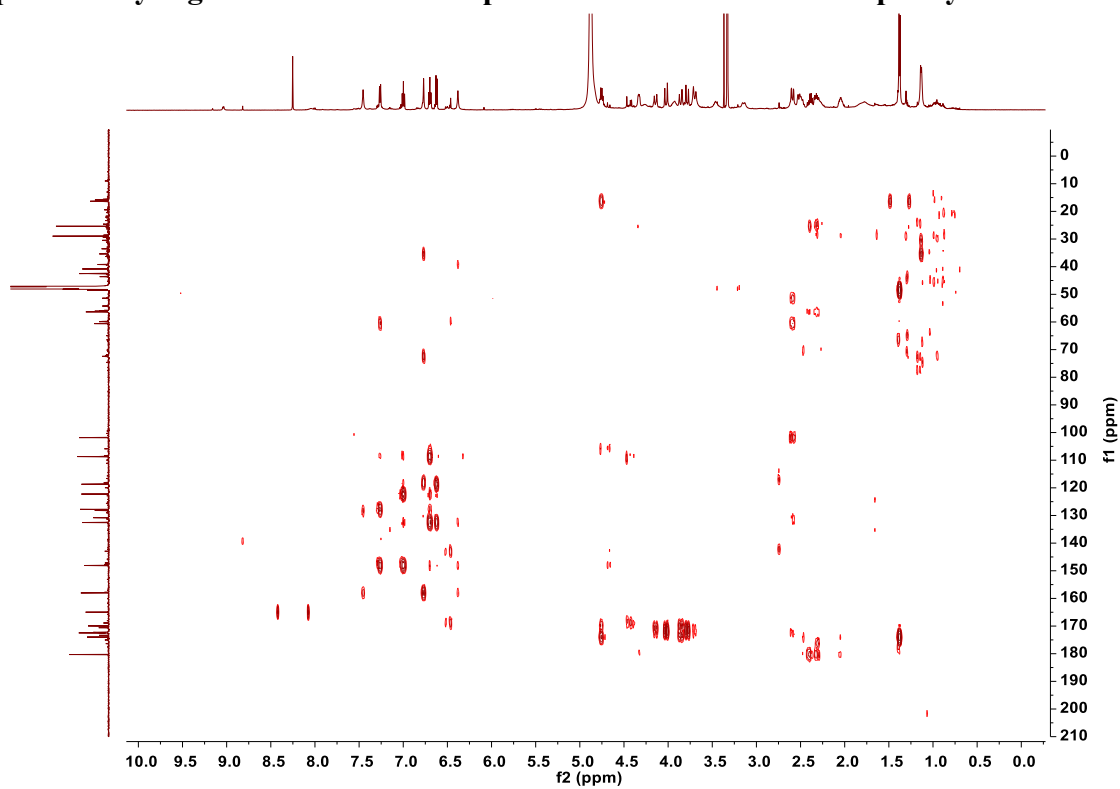
Supplementary Fig. 106. ¹³C NMR (150 Hz, CD₃OD) spectrum of 13 with minor impurity.



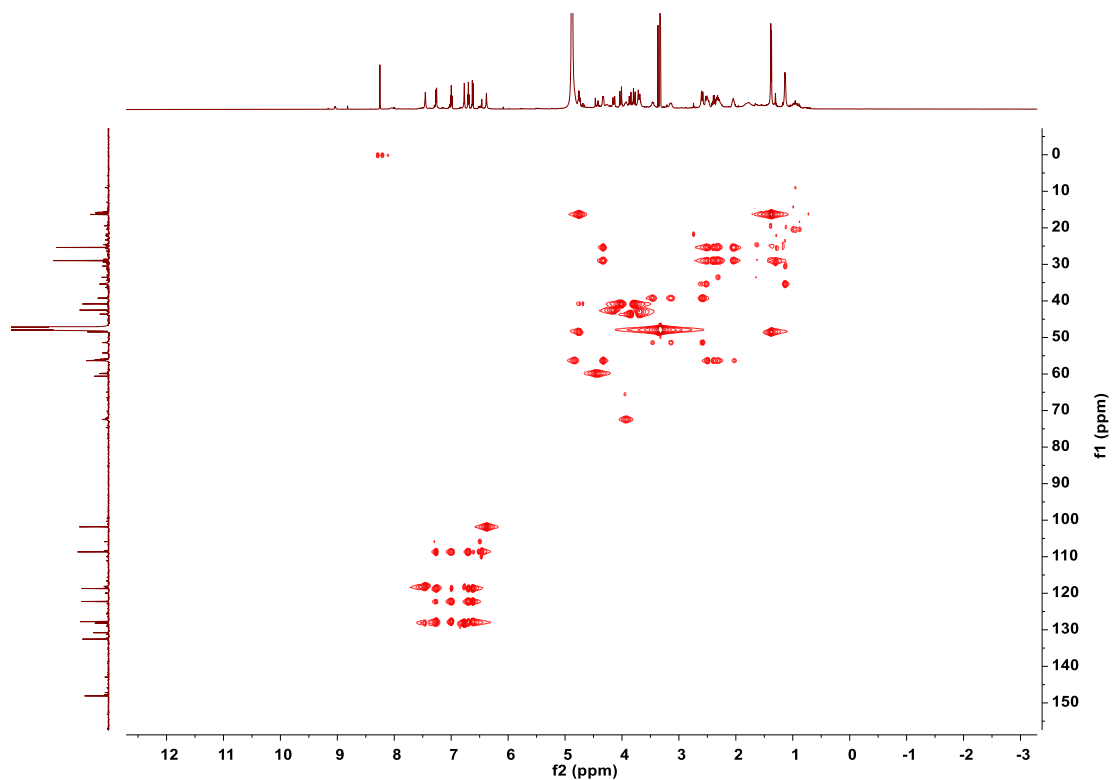
Supplementary Fig. 107. HSQC spectrum of 13 with minor impurity.



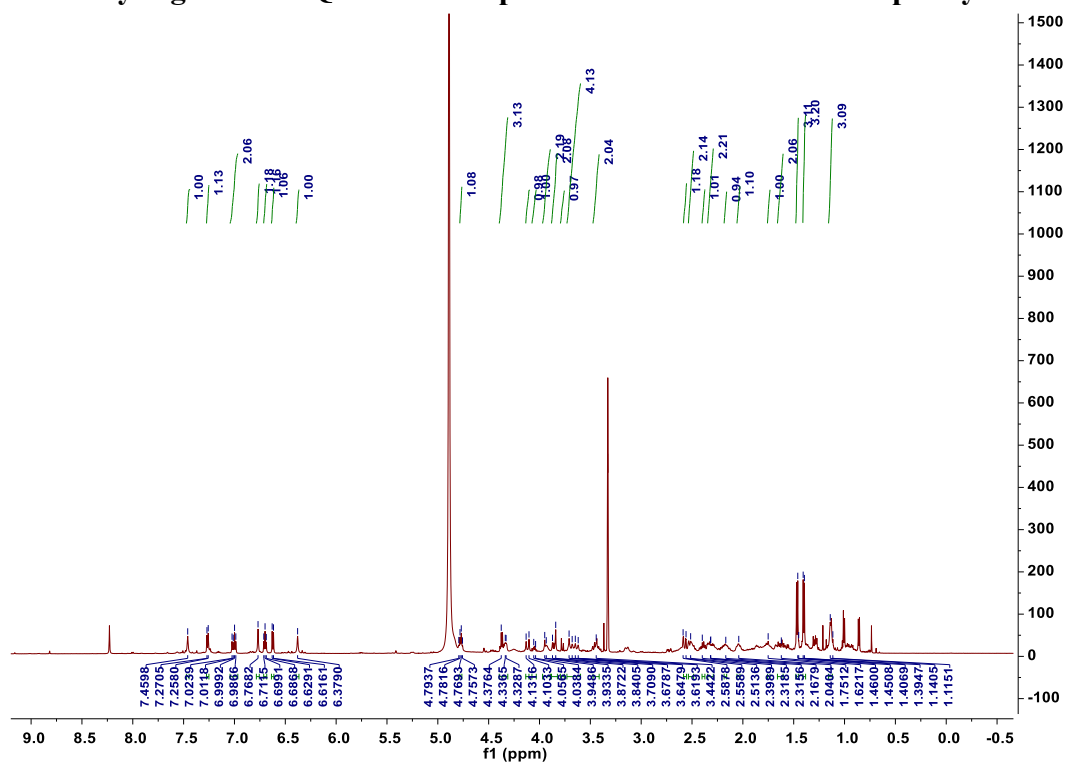
Supplementary Fig. 108. ^1H - ^1H COSY spectrum of 13 with minor impurity.



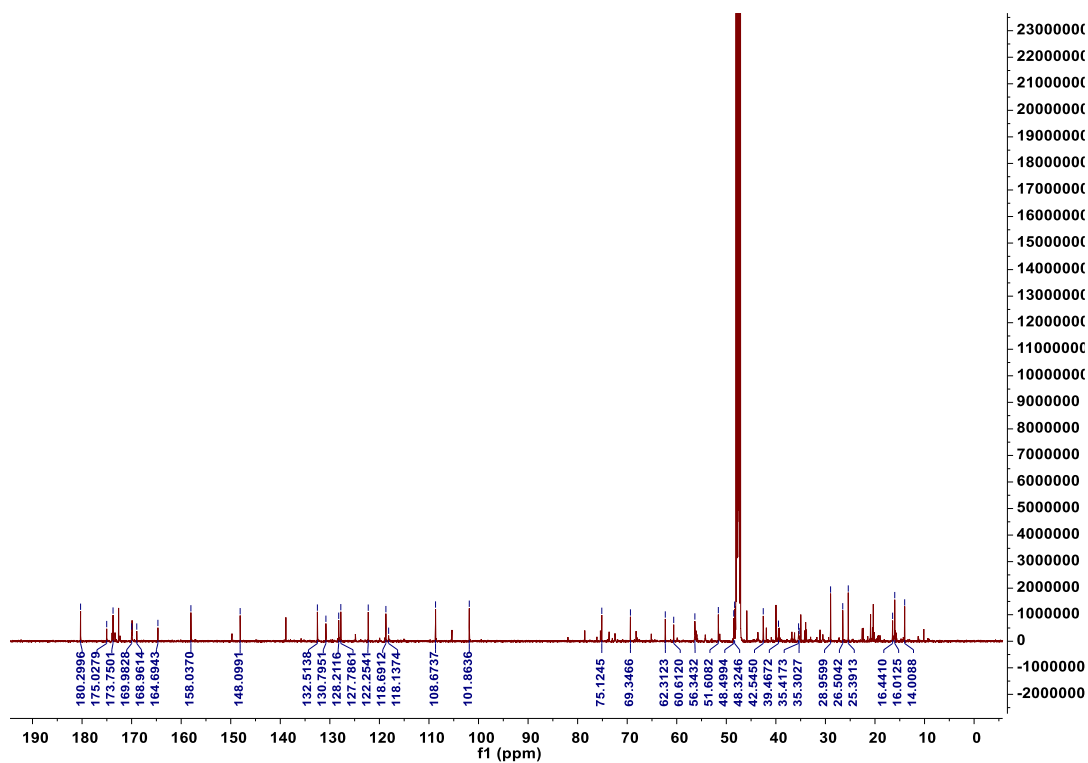
Supplementary Fig. 109. HMBC spectrum of 13 with minor impurity.



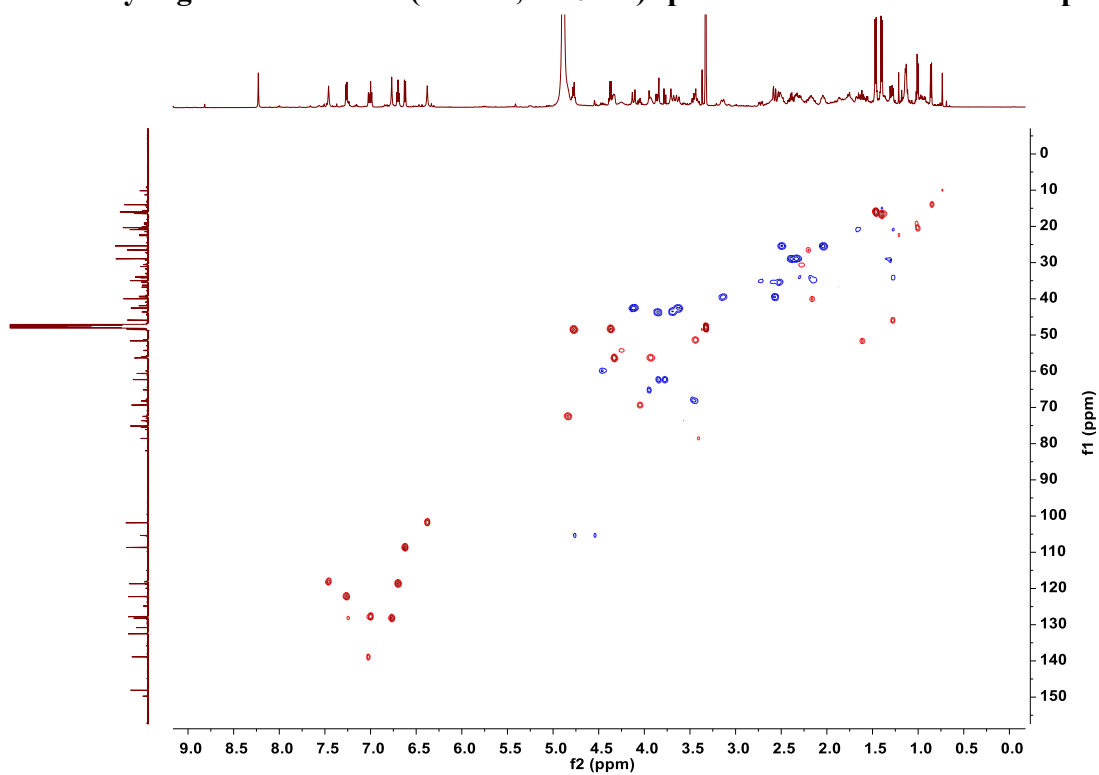
Supplementary Fig. 110. HSQC-TOCSY spectrum of 13 with minor impurity.



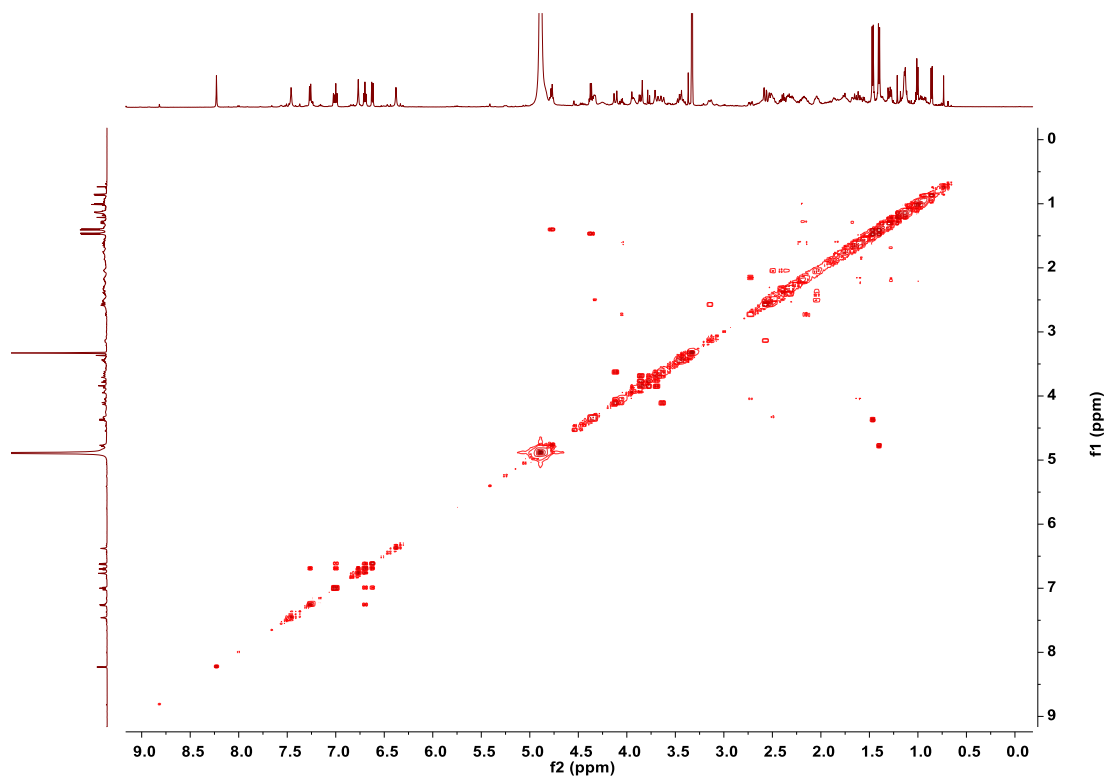
Supplementary Fig. 111. ^1H NMR (600 Hz, CD_3OD) spectrum of 14 with minor impurity.



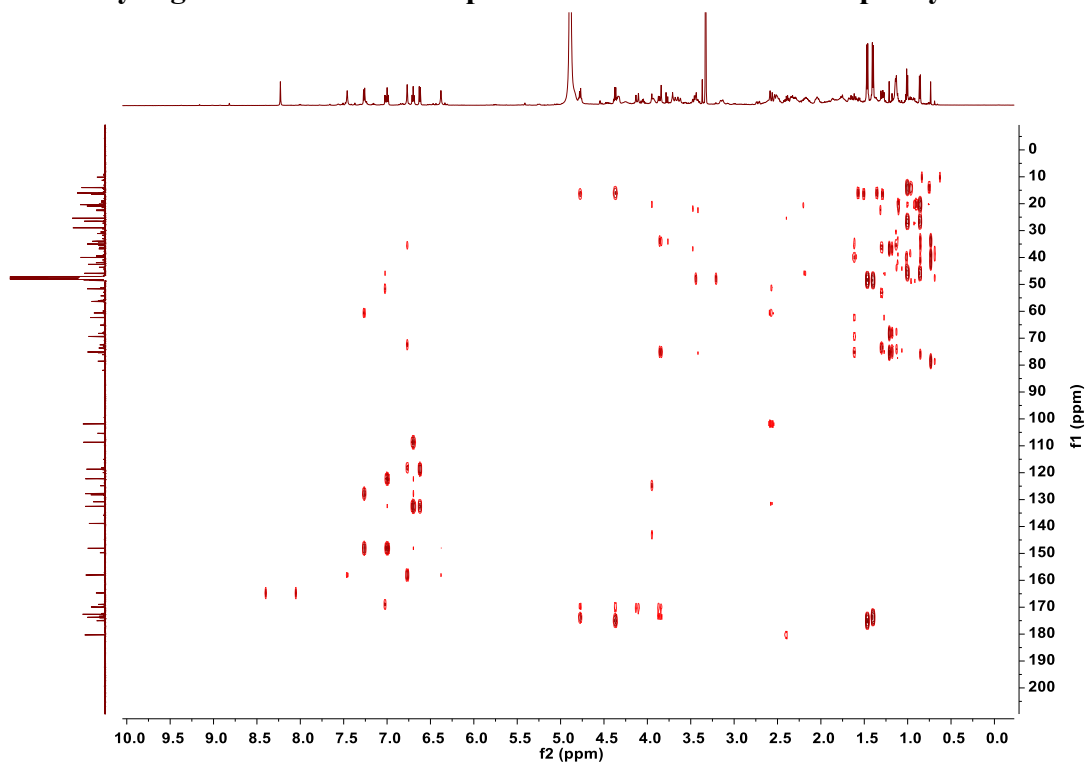
Supplementary Fig. 112. ¹³C NMR (150 Hz, CD₃OD) spectrum of 14 with minor impurity.



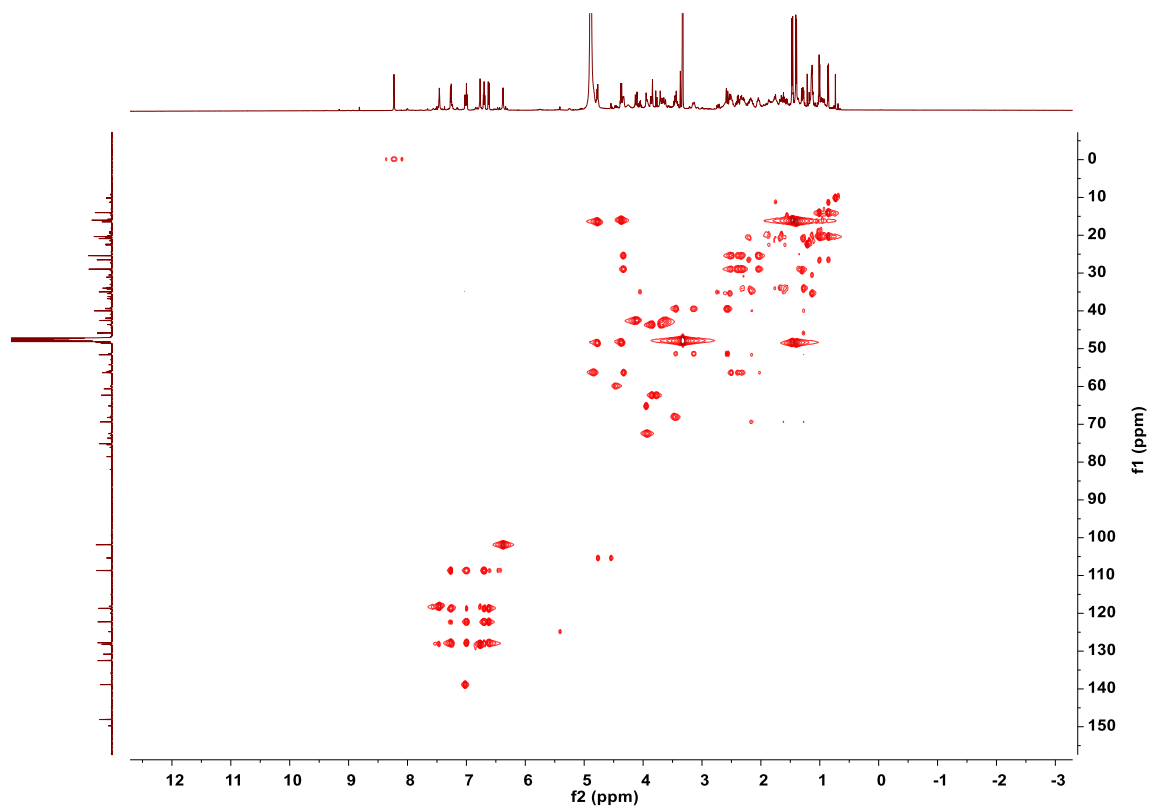
Supplementary Fig. 113. HSQC spectrum of 14 with minor impurity.



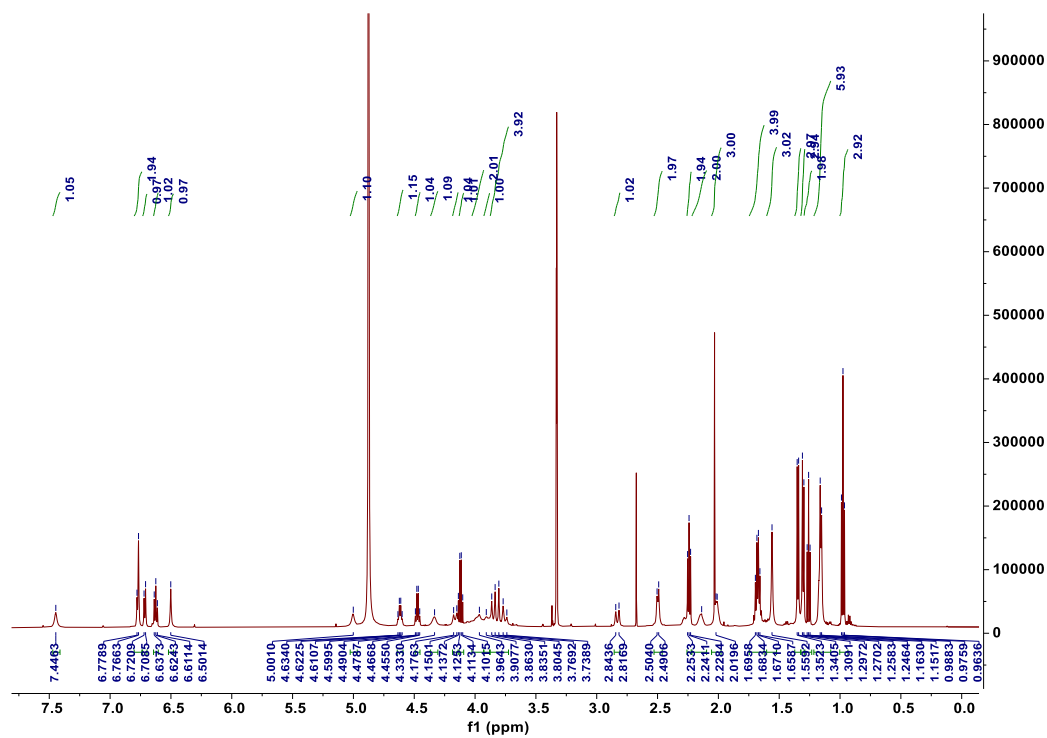
Supplementary Fig. 114. ^1H - ^1H COSY spectrum of 14 with minor impurity.



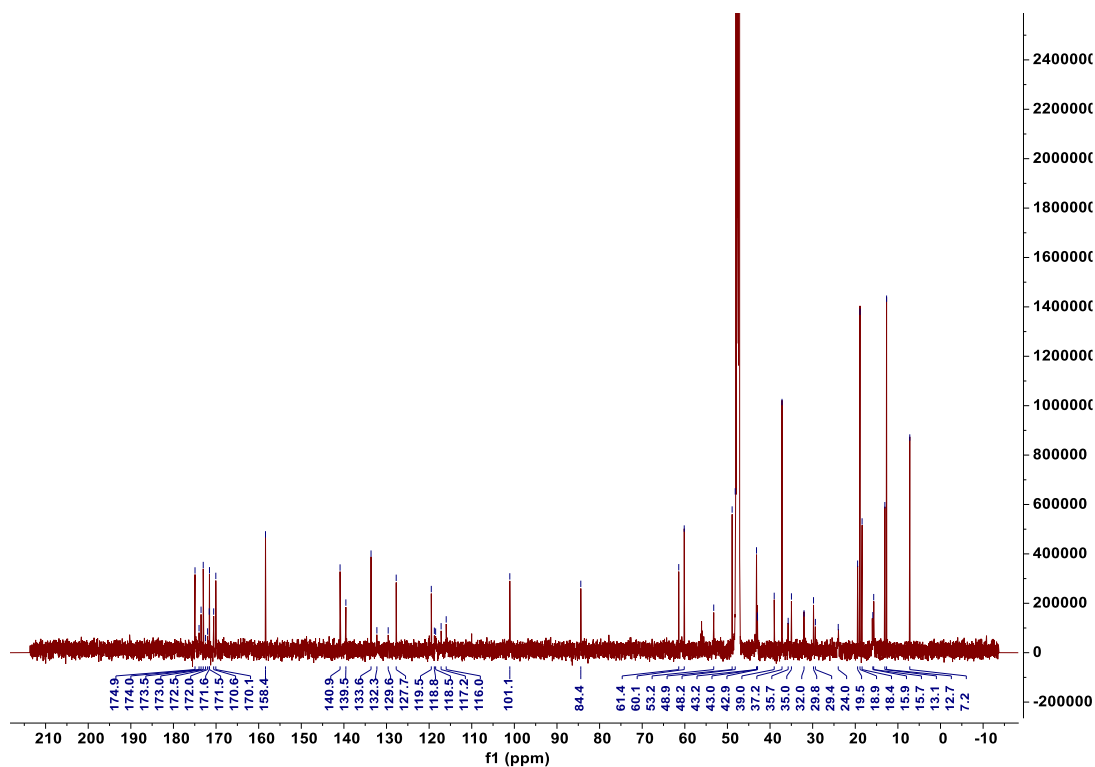
Supplementary Fig. 115. HMBC spectrum of 14 with minor impurity.



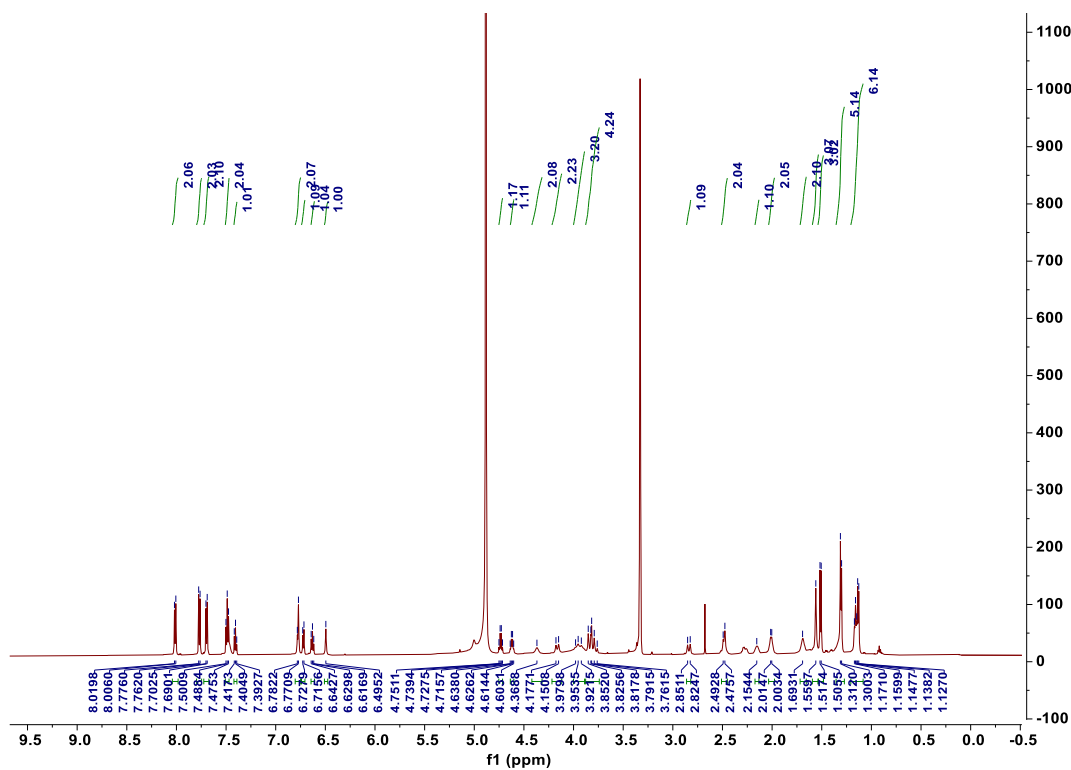
Supplementary Fig. 116. HSQC-TOCSY spectrum of 14 with minor impurity.



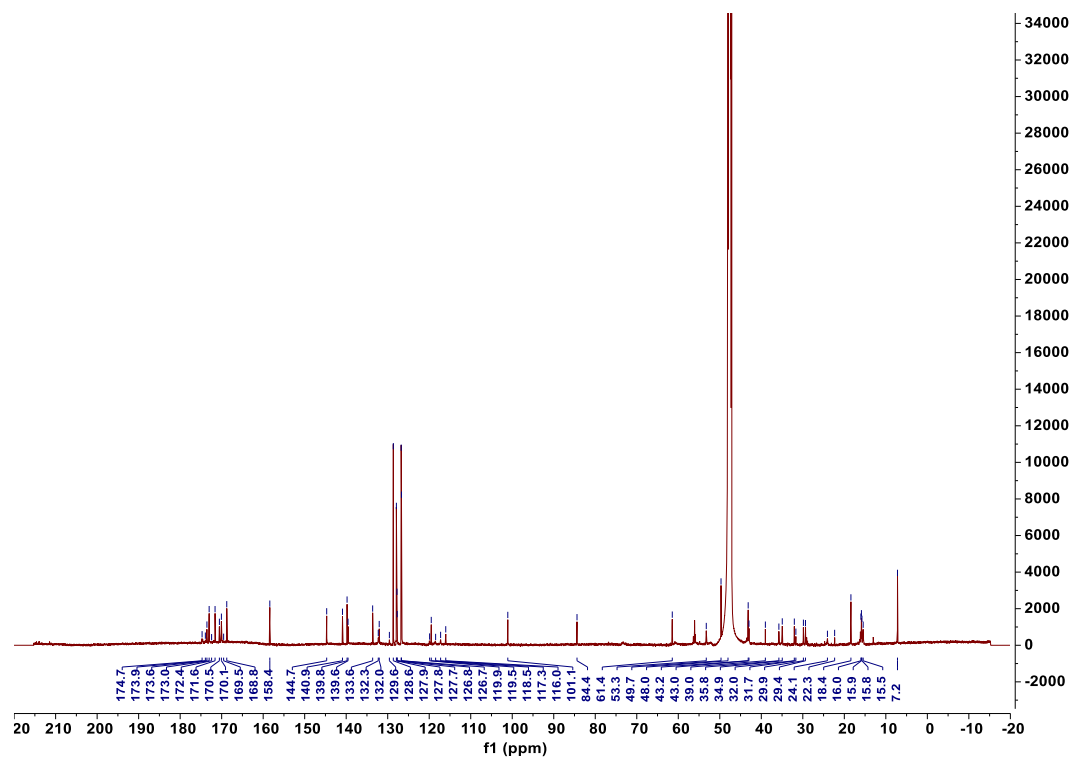
Supplementary Fig. 117. ^1H NMR (600 Hz, CD_3OD) spectrum of 2-L₁.



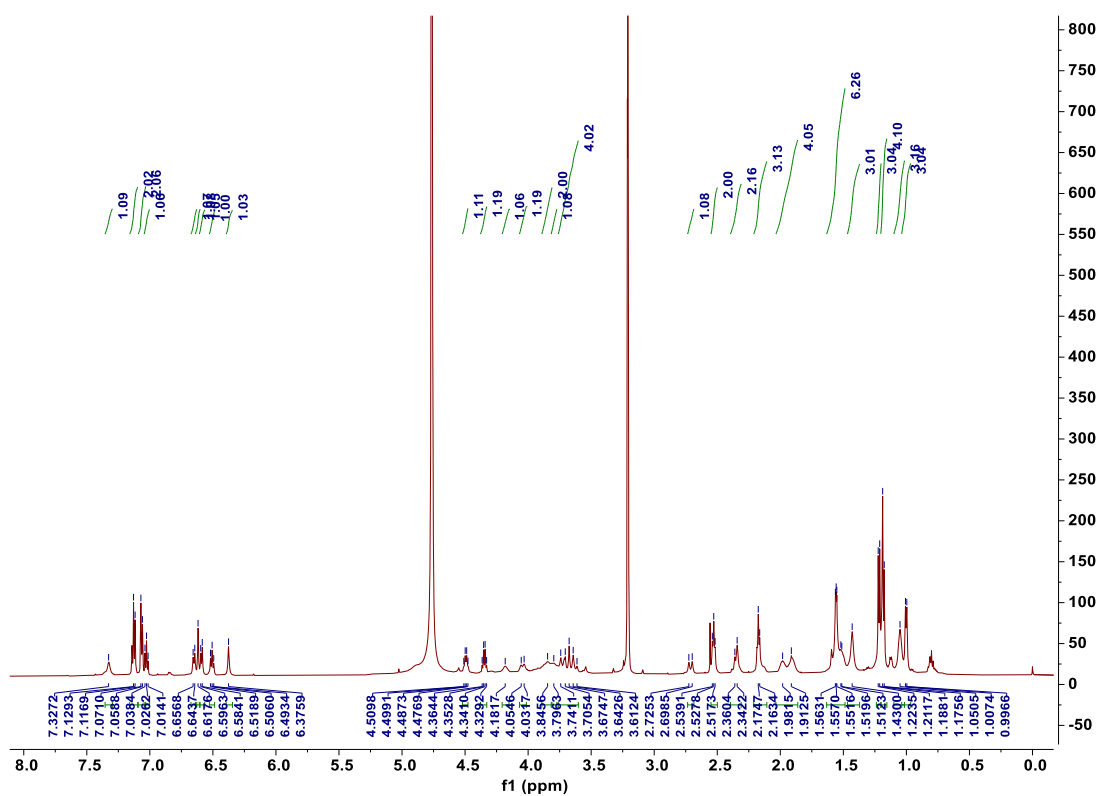
Supplementary Fig. 118. ^{13}C NMR (150 Hz, CD_3OD) spectrum of 2-L₁.



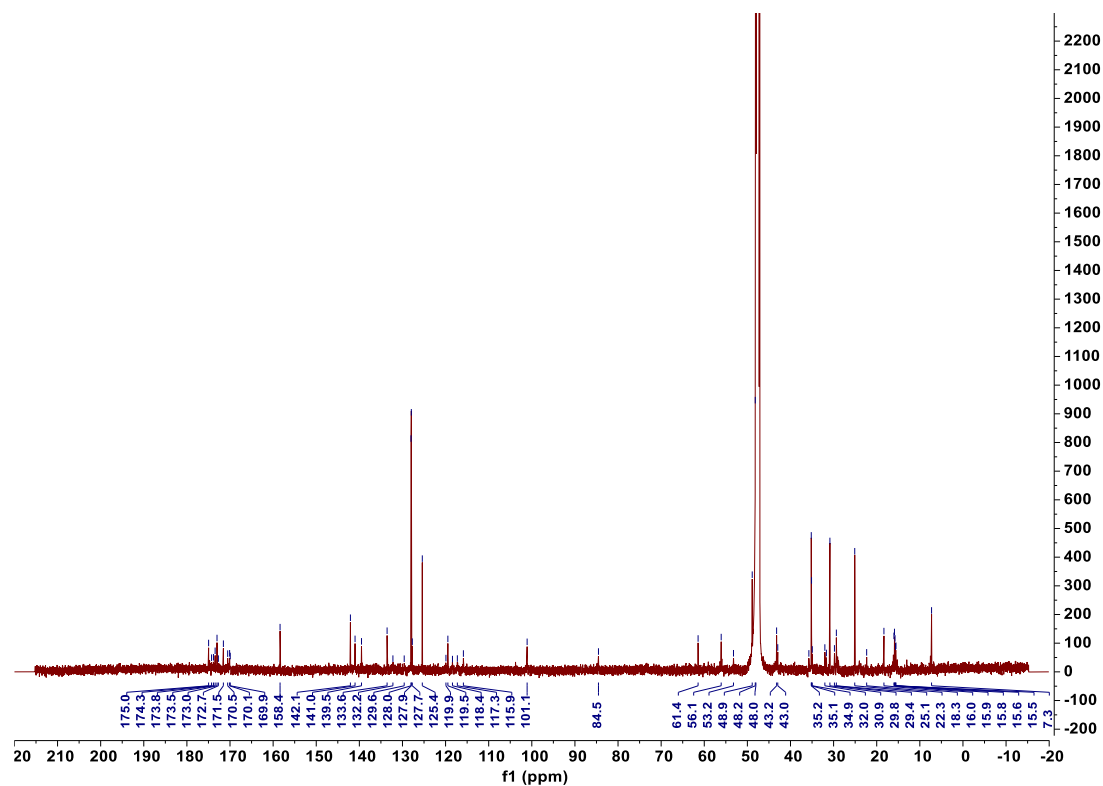
Supplementary Fig. 119. ^1H NMR (600 Hz, CD_3OD) spectrum of 2-L₂.



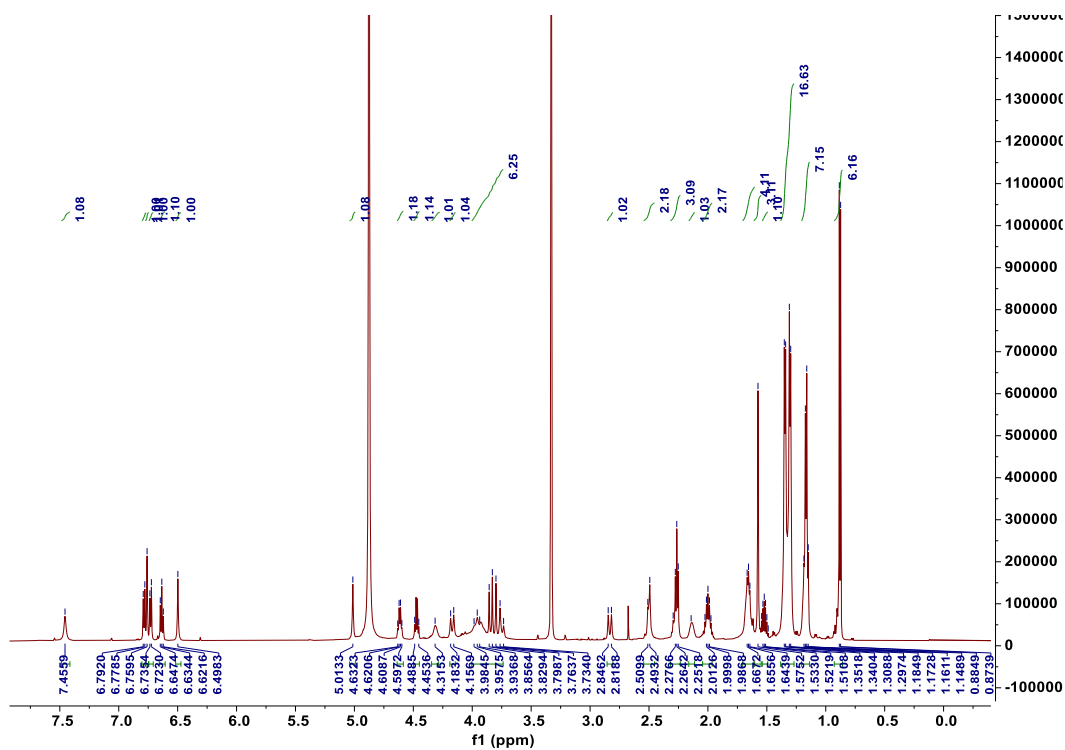
Supplementary Fig. 120. ^{13}C NMR (150 Hz, CD_3OD) spectrum of 2-L₂.



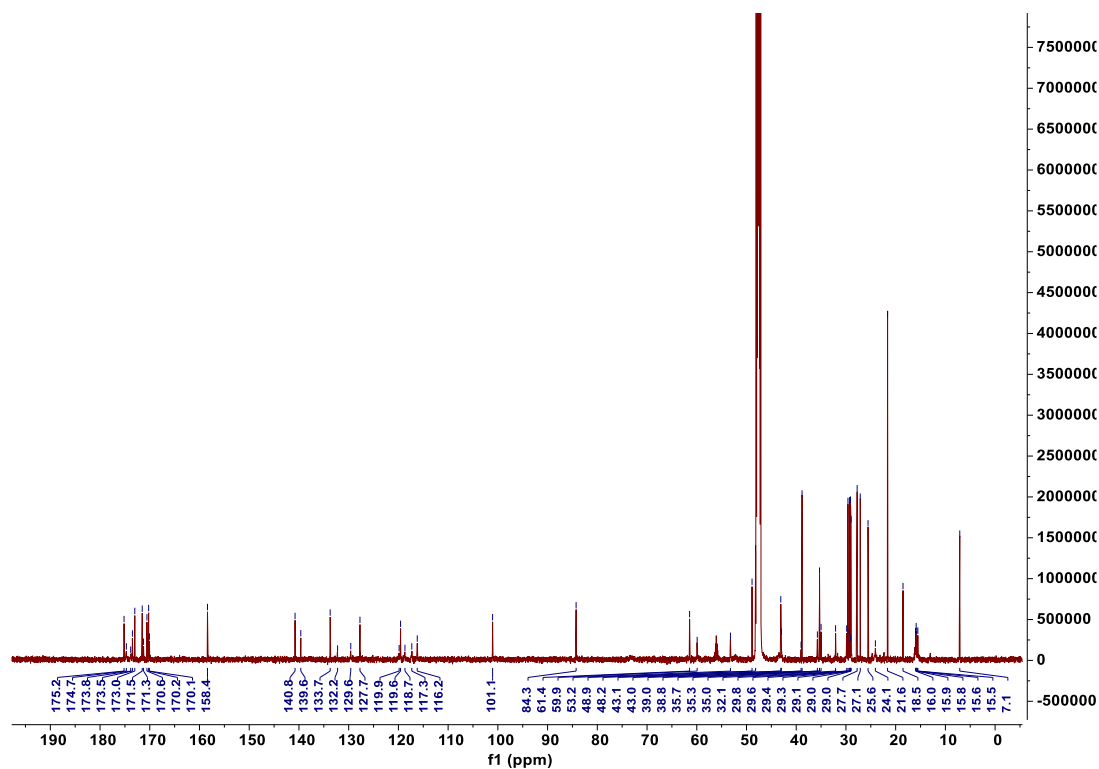
Supplementary Fig. 121. ^1H NMR (600 Hz, CD_3OD) spectrum of 2-L₃.



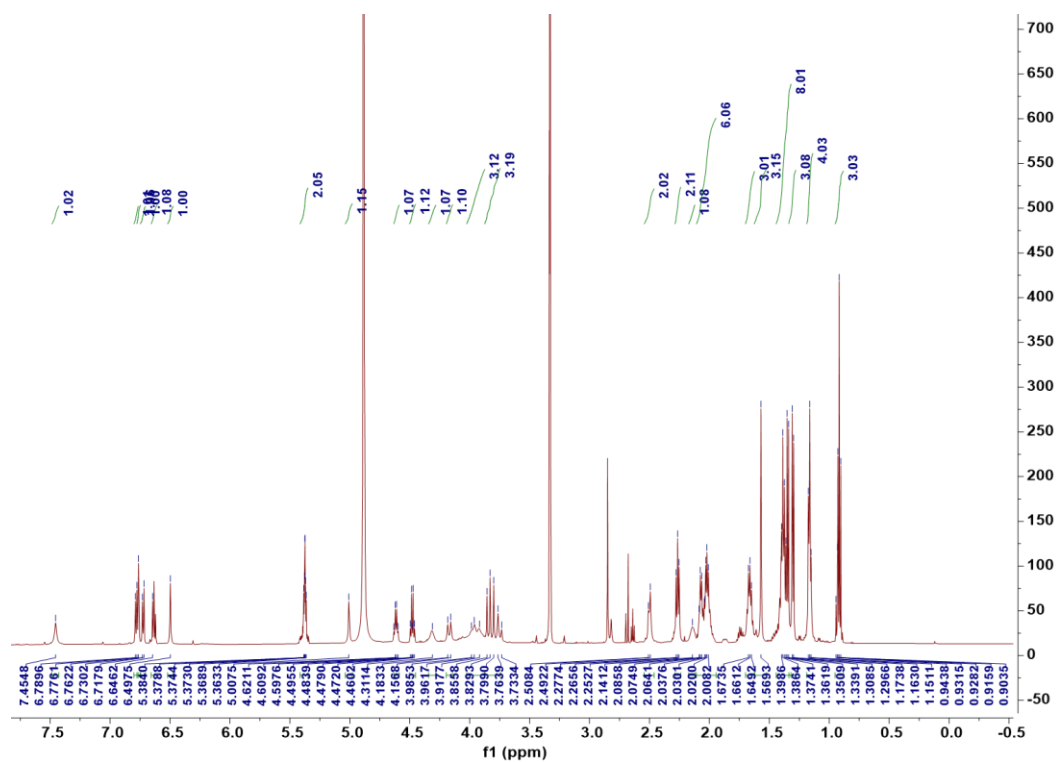
Supplementary Fig. 122. ¹³C NMR (150 Hz, CD₃OD) spectrum of 2-L₃.



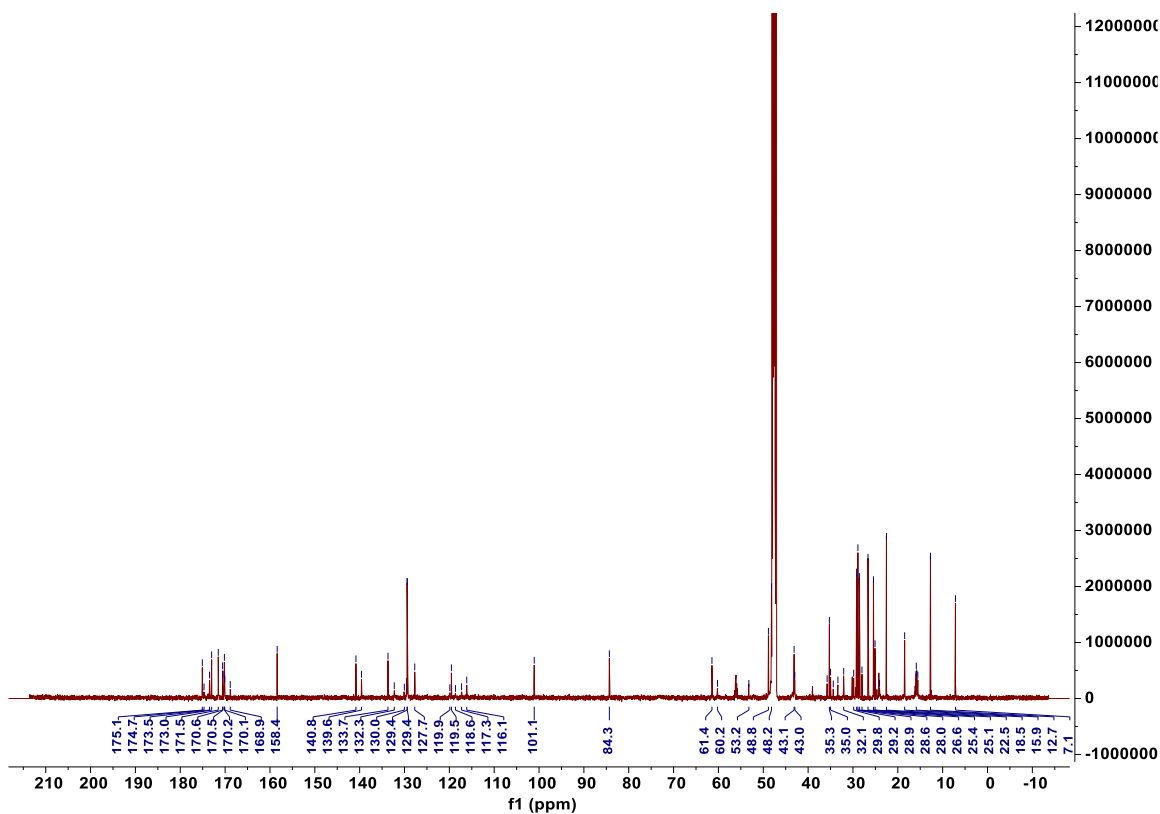
Supplementary Fig. 123. ¹H NMR (600 Hz, CD₃OD) spectrum of 2-L₄.



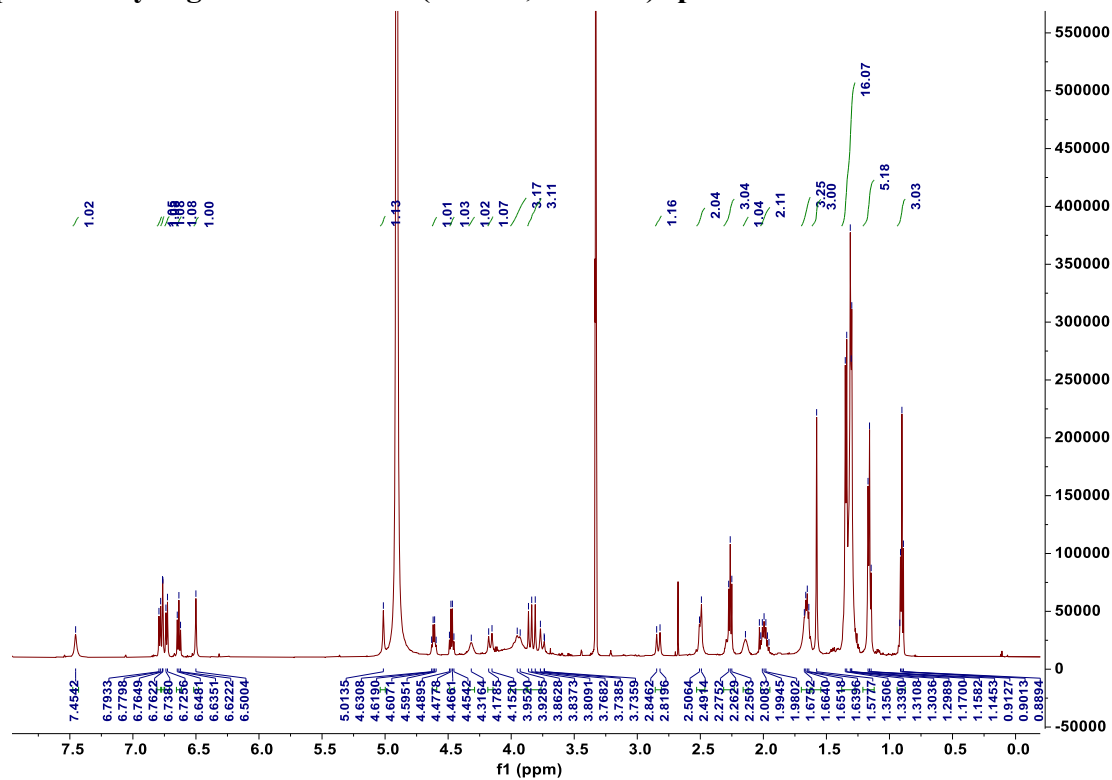
Supplementary Fig. 124. ^{13}C NMR (150 Hz, CD_3OD) spectrum of 2-L4.



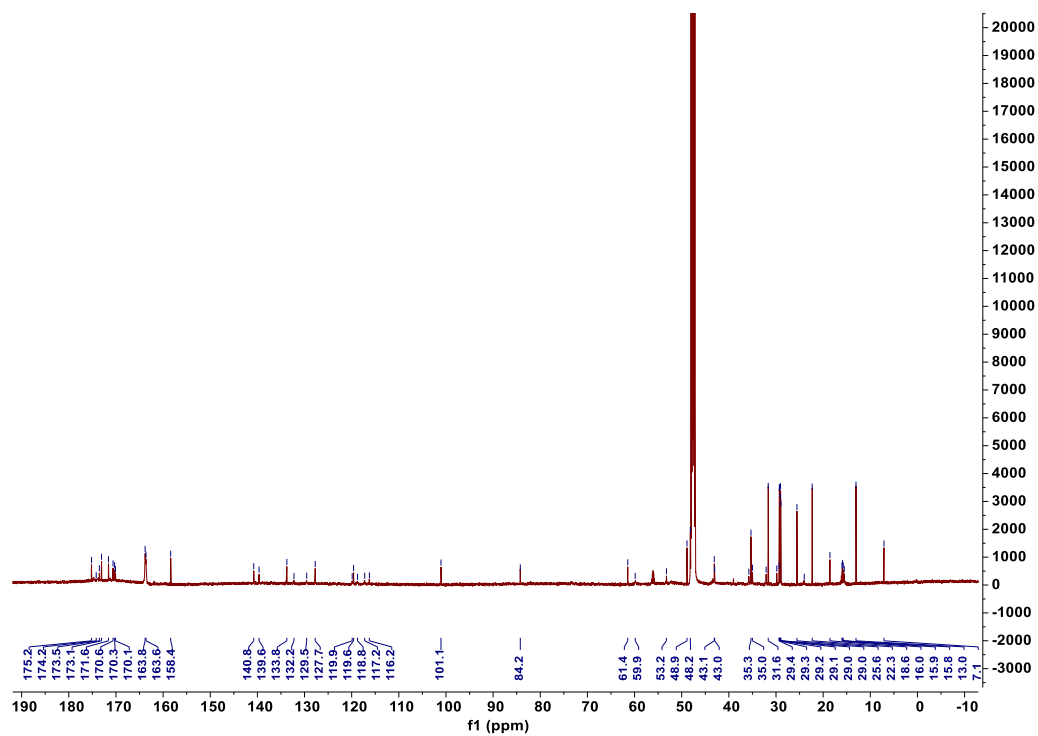
Supplementary Fig. 125. ^1H NMR (600 Hz, CD_3OD) spectrum of 2-L5.



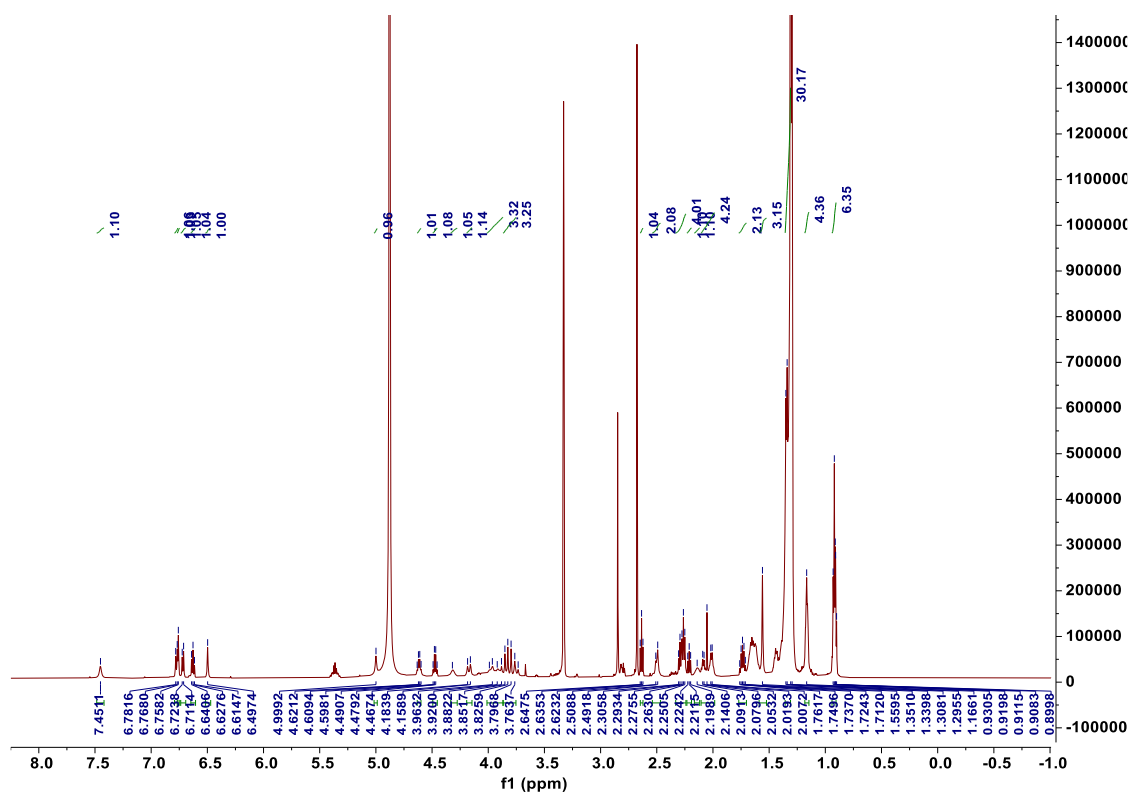
Supplementary Fig. 126. ¹³C NMR (150 Hz, CD₃OD) spectrum of 2-L₅.



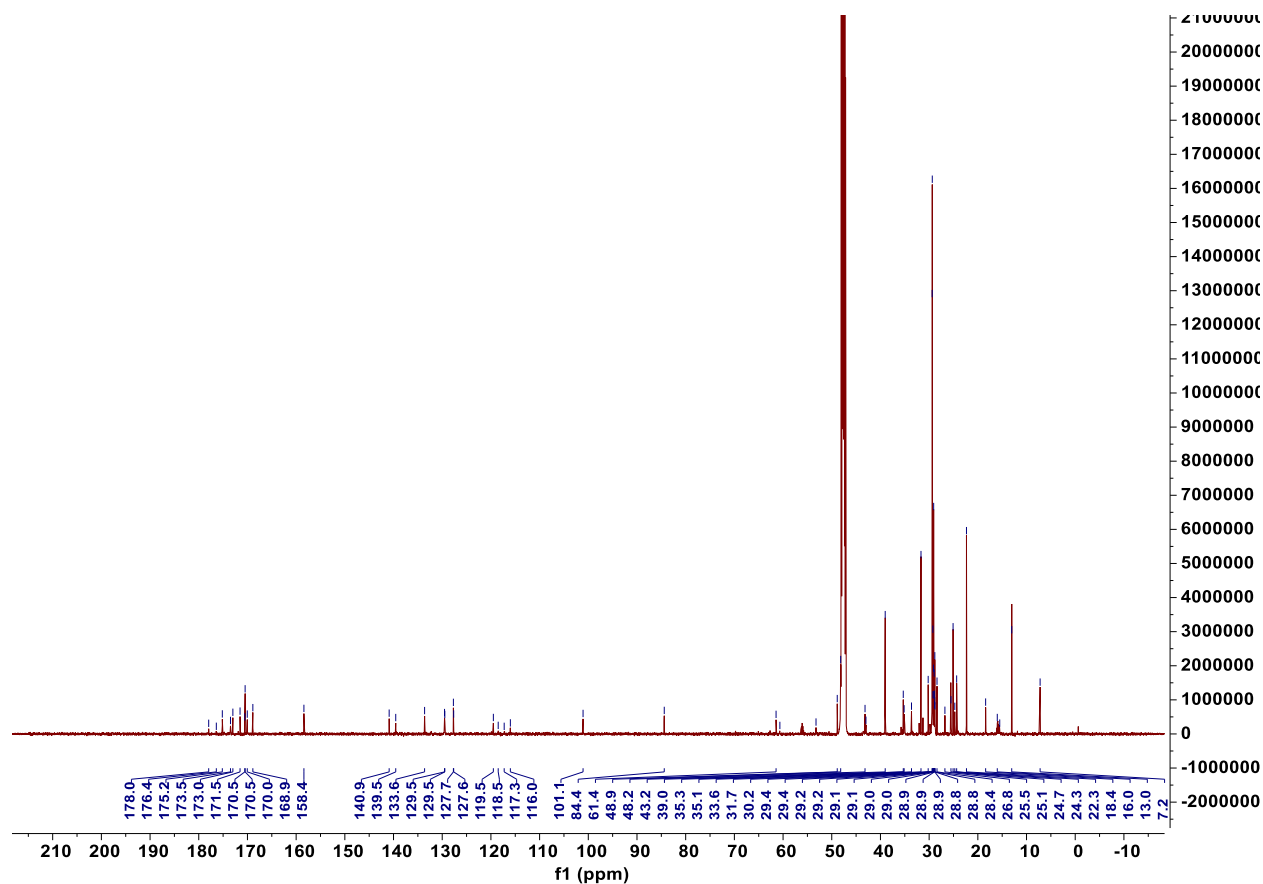
Supplementary Fig. 127. ¹H NMR (600 Hz, CD₃OD) spectrum of 2-L₆.



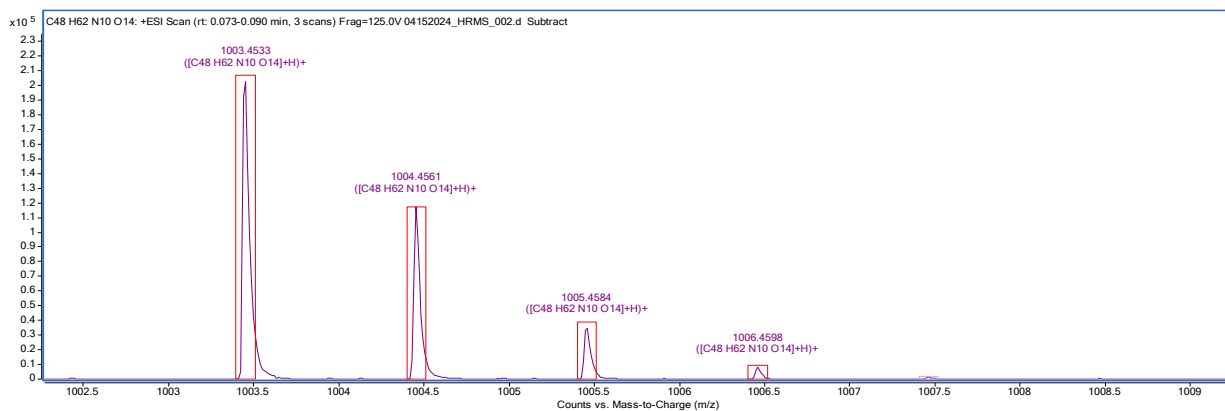
Supplementary Fig. 128. ¹³C NMR (150 Hz, CD₃OD) spectrum of 2-L6.



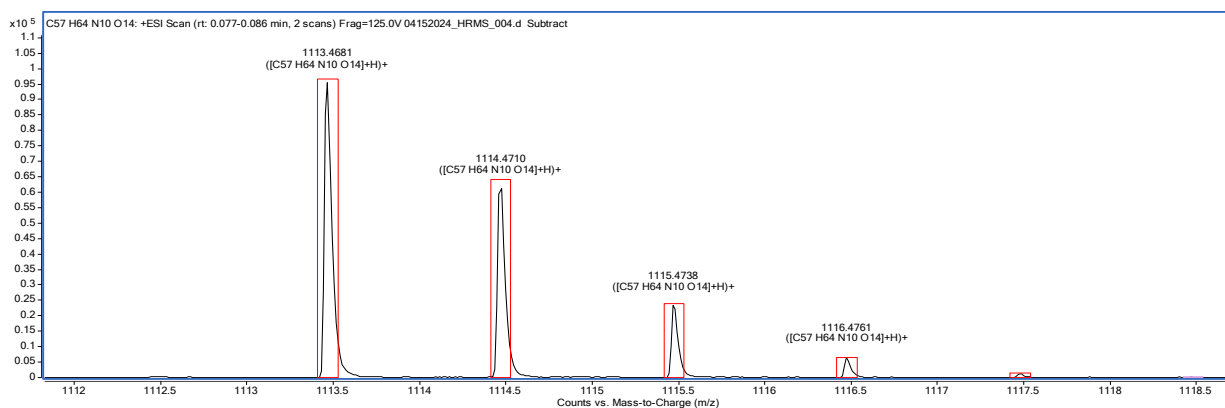
Supplementary Fig. 129. ¹H NMR (600 Hz, CD₃OD) spectrum of 2-L7.



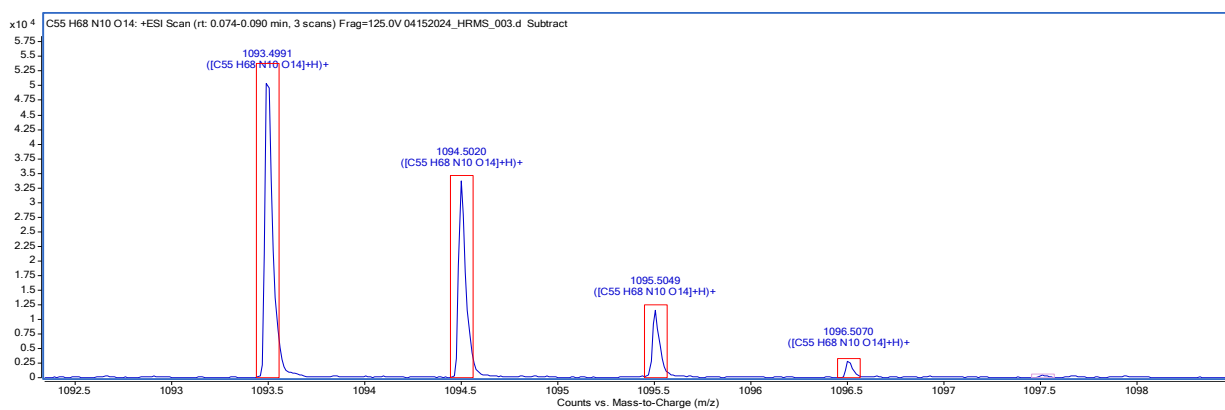
Supplementary Fig. 130. ^{13}C NMR (150 Hz, CD_3OD) spectrum of 2-L₇.



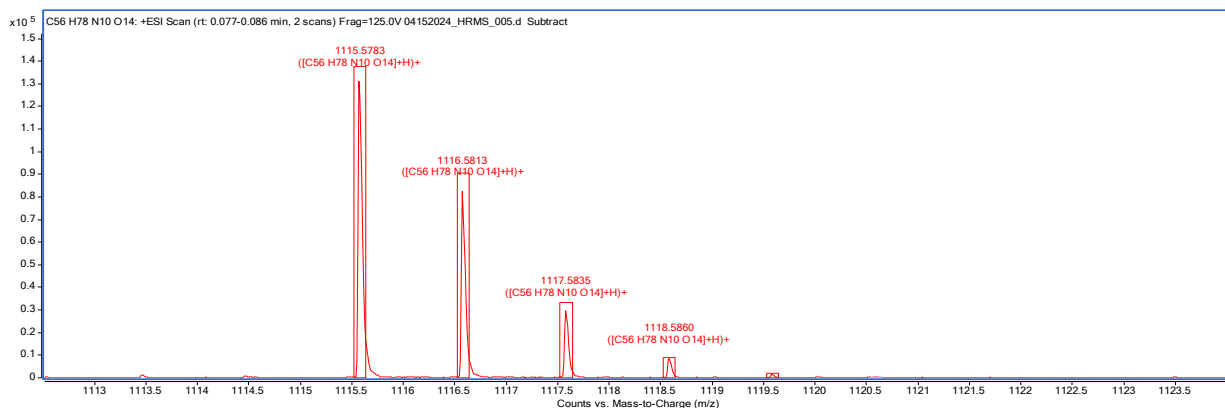
Supplementary Fig. 131. HRMS data for 2-L₁.



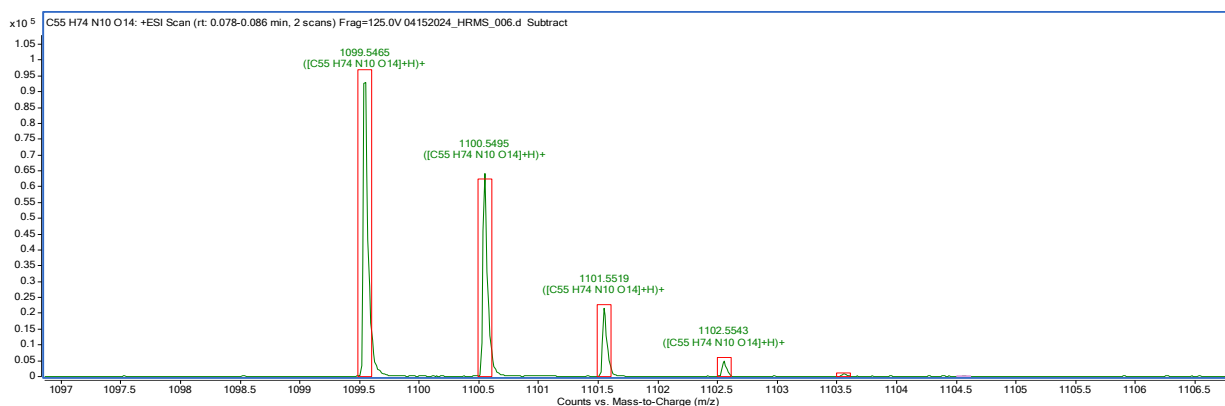
Supplementary Fig. 132. HRMS data for 2-L₂.



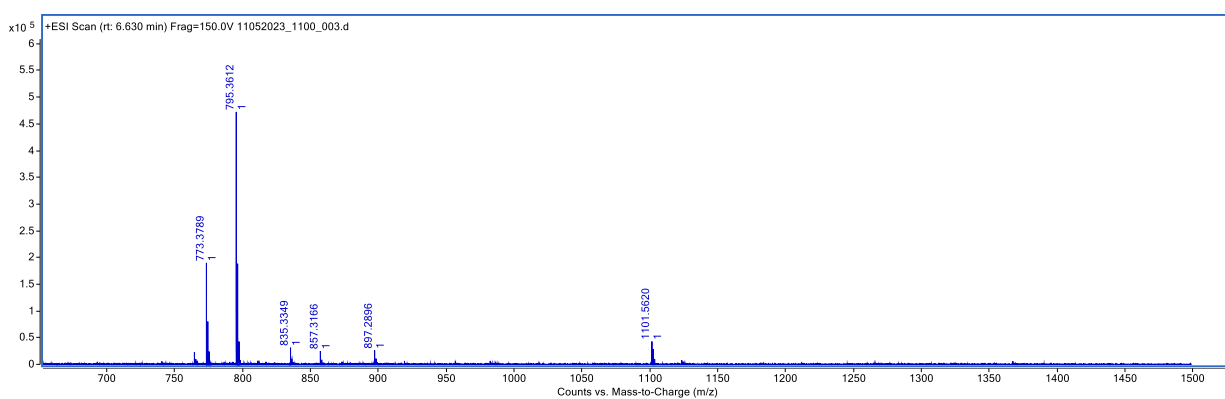
Supplementary Fig. 133. HRMS data for 2-L₃.



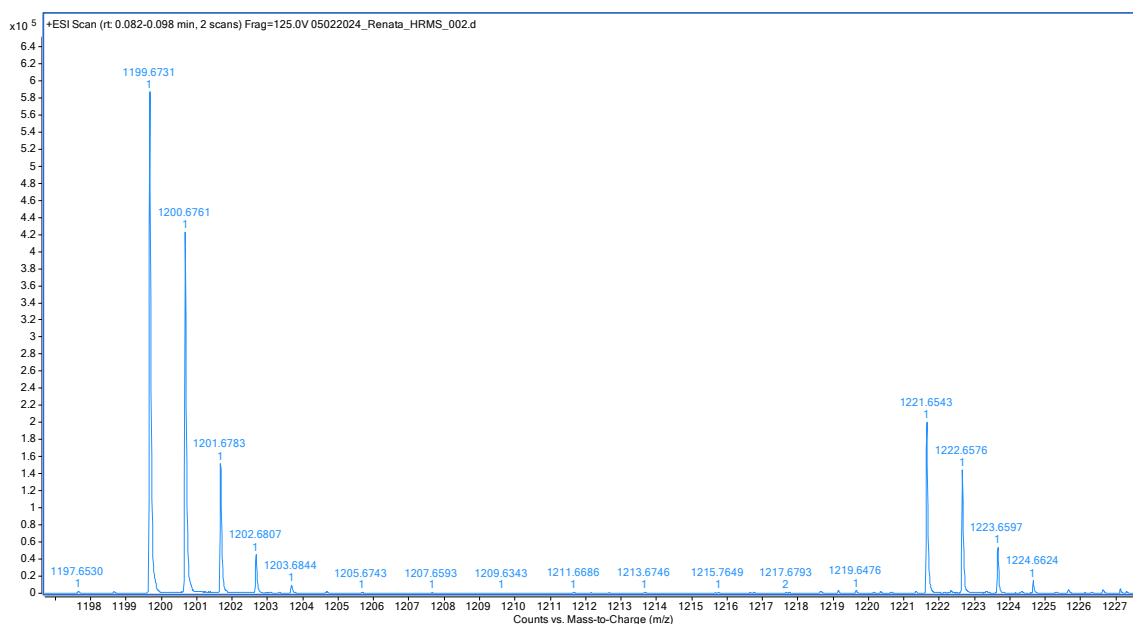
Supplementary Fig. 134. HRMS data for 2-L₄.



Supplementary Fig. 135. HRMS data for 2-L₅.



Supplementary Fig. 136. HRMS data for 2-L₆.

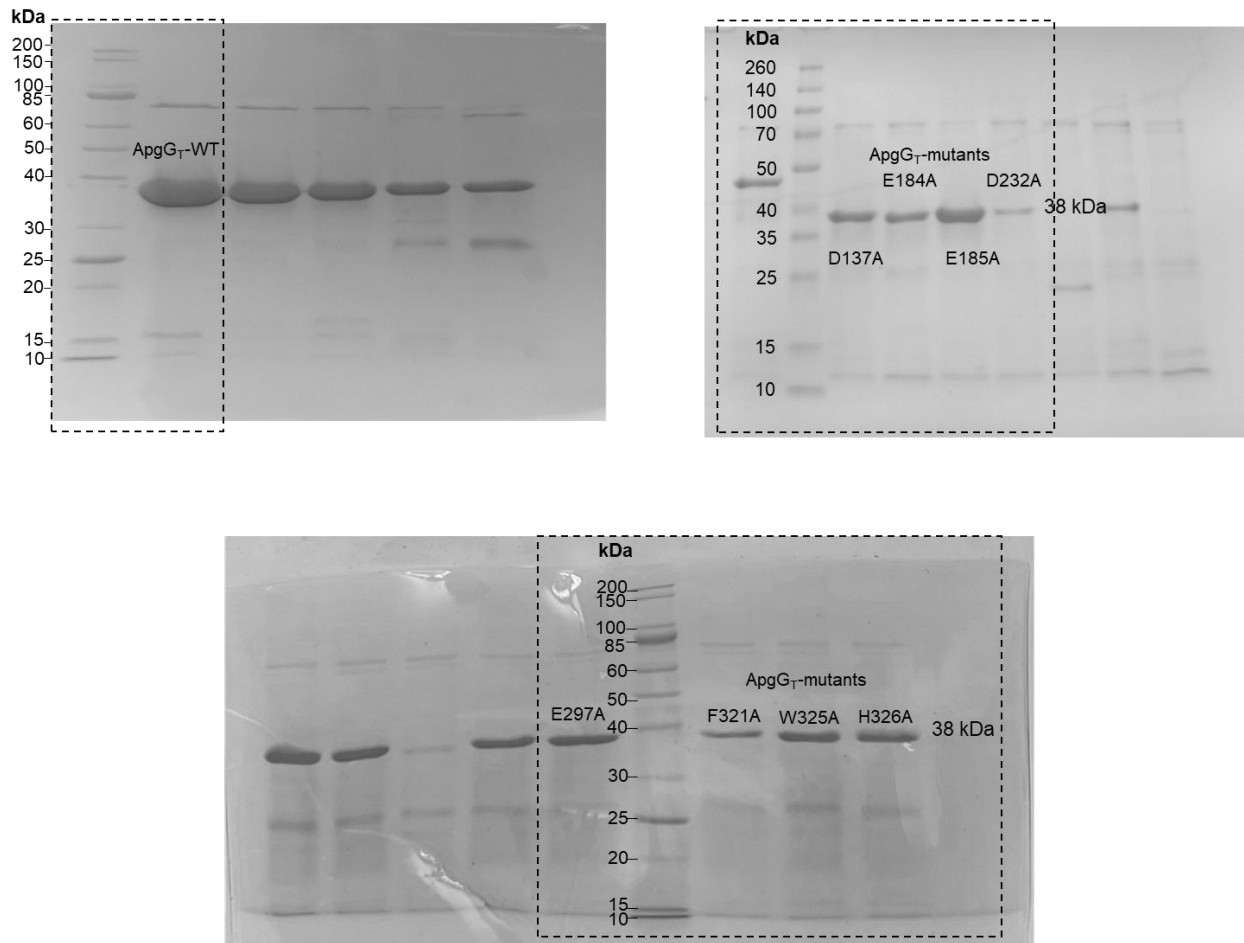


Supplementary Fig. 137. HRMS data for 2-L₇.

Uncropped DNA gels used in Supplementary Fig. 7.

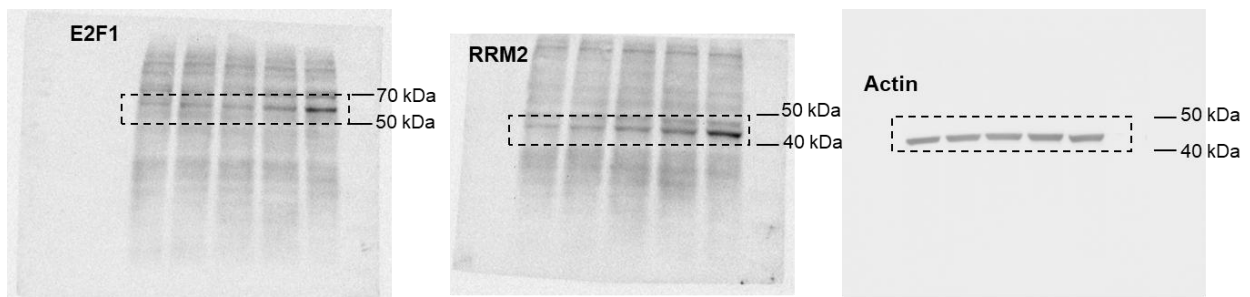


Uncropped SDS-PAGE-gels



Uncropped SDS-PAGE gels corresponding to Supplementary Fig. 19. Lanes highlighted by the dotted box indicate samples relevant to this study.

Uncropped Western Blot scans



The samples derive from the same experiment and Western blots were processed in parallel. Uncropped Western Blot scans used in Supplementary Fig. 27c.