Treasures from the deep, characellides as anti-inflammatory lipoglycotripeptides from the sponge *Characella pachastrelloides*.

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Experimental

1. *General Experimental Procedures*

Optical rotations were measured on a UNIPOL 1000 Polarimeter. VCD experiments were measured using Biotools chiral/R-2X polarimeter. UV and ECD measurements were obtained on a Jasco J-815 spectrophotometer. NMR experiments were measured on a 600 MHz equipped with a cryoprobe (Varian). Chemical shifts (δ in ppm) are referenced to carbons (δ_C 39.5 or 49.0) and residual protons (δ_H 2.50 or 3.31) signals of DMSO-d_6 or MeOH-d_4. High Resolution Electrospray Ionsition Mass Spectrometry (HRESIMS) data was obtained from a qTOF Agilent 6540 in ESI(+). Preparative HPLC was preformed using a Jasco PU-2087 Plus equipped with a UV-Vis detector UV 2075 Plus and then by Agilent 1260 analytical HPLC series equipped with a DAD detector.

2. *Biological Material and Identification*

The specimen was collected using the remotely operated vehicle *Holland I* during the cruise CE16006 of RV *Celtic Explorer* at 809 m depth (48.6509° N, 10.4846° W). The sponge appeared as a large white barrel sponge. All epibionts were removed from the surface of the sponge and a small amount was retained in 96% ethanol as a voucher specimen for DNA analysis and morphological identification. The rest of the sample was lyophilized and stored at -20 °C.

Light microscope slides of the spicules and thick section mounts were made according to the methods detailed in Vacelet (2006). DNA was extracted from choanosomal tissue using E.Z.N.A. tissue DNA kits following the manufacturer's instructions. The CO1 barcoding region was amplified using the following primers; LCO1490 and HCO2198 (Folmer et al., 1994).The protocols for PCR followed those detailed in Morrow et al. (2012). The sequence was blast searched against those in GenBank, the search returned 100% identity with the GenBank sequence HM592672. A voucher specimen (BVD #1826) is deposited at the Ryan Institute, National University of Ireland, Galway, Ireland and a second one at the National Museum of Northern Ireland, Belfast (BELUM.Mc2018.5).

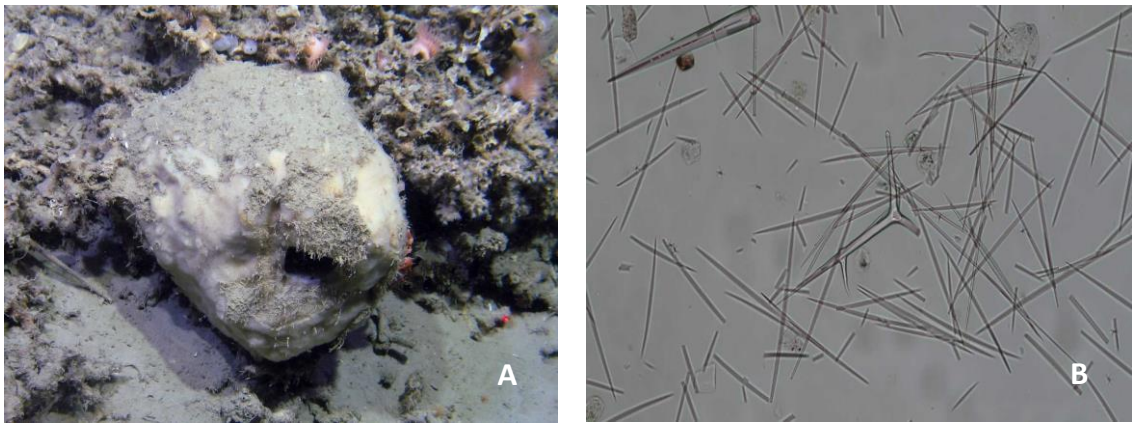


Figure S1. Illustrations of the specimen of *Characella pachastrelloides*: A-Photography *in situ* (Whittard canyon, Celtic continental margin), B- Image of spicules under a light microscope.

3. *Extraction and Isolation*

The sponge (330 g) was ground into a power and extracted with MeOH −CH_2Cl_2 (1:1) aided by ultrasonification. The crude extract (20.6 g) was fractionated by RP-C18 vacuum liquid chromatography (elution with a decreasing polarity gradient of H_2O, H_2O; MeOH (1:1), H_2O: MeOH (1:3), MeOH and MeOH:CH_2Cl_2 (1:1). The MeOH fraction (628 mg) fraction was separated into subfractions by RP-HPLC on a semipreparative T3 column, 250 mm × 19 mm, 5 μm (Xselect, Waters, Milford, CT, USA), using a mobile phase of water (A) and acetonitrile (B), both under acidic conditions (0.1% TFA) with a flow rate of 5 mL/min. The method was developed on 36 min acquisition time: isocratic 45% B for 4 min, then linear gradient to 68% B at 17 min, held at 68% B for 13 min, back to 45% B in 1 min, and held at that percentage of B for 5 min. Subfraction CK-1826-F4-P2 (t_R 6 min, 8.9 mg) contained the mixture of characellides A (**1**) and B (**2**). The mixture was then repurified using BEH Amide 4.6 x 250mm 5 μm (Xbridge, Waters, Milford, CT, USA) using a mobile phase of water (A) and acetonitrile (B) both buffered with 0.1% TFA, with a flow rate of 1 mL/min under isocratic conditions at 89% B, yielding characellide A (t_R 10 min, 3.42 mg, $11.2.10^{-4}$ % w/w) and B (t_R 14 min, 1.64 mg, $4.97.10^{-4}$ % w/w). The H_2O/MeOH (1:3) fraction (223 mg) was subjected to HPLC purification using the same column and system mentioned for the purification of the MeOH fraction. The method was developed on 30 min acquisition time: isocratic 40% B for 4 min, then linear gradient to 85% B for 13 min, held at 85% B for 7 min, back to 40% B in 1 min, and held at that percentage of B for 5 min, yielding a mixture of characellides C (**3**) and D (**4**) (t_R 5 min, 4.3 mg) Unfortunately, characellides C and D were unsuccessfully separated using the amide column.

Characellide A (**1**). Yellowish oily solid; [α]D -30 (c 0.1, MeOH); λ_{max} (log *s*) 225 (4.24) nm; ^{1}H NMR and ^{13}C NMR data see Table 1; HRESIMS *m/z* 867.4705 $[M+H]^+$ (867.4710 calcd. for $C_{41}H_{67}N_6O_{14}$, Δ –0.6 ppm).

Characellide B (**2**). Yellowish oily solid; [α]D -60 (c 0.2, MeOH); λ_{max} (log ε) 225 (4.24) nm; ^{1}H NMR and ^{13}C NMR data see Table 1; HRESIMS *m/z* 867.4700 $[M+H]^+$ (867.4710 calcd. for $C_{41}H_{67}N_6O_{14}$, Δ –1.1 ppm).

Marfey's Method

500 μg of **1** in 2 M HCl (1 mL) was heated to 100°C for 12 hours in a sealed vial. The sample was dried and treated with 1 M $NaHCO_3$ (200 μL) and D-FDAA (1% in acetone, 400 µL) at 40 °C for 1 h, and neutralized with 1 M HCl (200 μL). 50 mM of each amino acid (L and D-*O*Me-Tyr, L and D-Asp, L, D, L-*allo* and D-*allo*-Thr) in H_2O_{dd} was treated with 1M $NaHCO_3$ (20 μL) and D-FDAA (1% solution in acetone, 100 μL) at 40 °C for 1 h, after which the reaction was neutralized with 1M HCl (20 μL), diluted with acetonitrile (810 μL) and filtered (0.22 μm, PTFE). All solutions were filtered (0.22 nm PTFE) prior to UPLC-HRESIMS analysis. 10 μL of each solution was injected and analyzed on a UPLC-DAD-HRESIMS system, on a Kinetex C8, 1.7 μm, 100 x 2.1 mm (Phenomenex). The flowrate was fixed at 0.6 ml/min. The method for the analysis of the aspartic acid and threonine derivatives was developed on 25 min acquisition time: isocratic 15% B for 5 min, then linear gradient to 20% B for 13 min, followed by another gradient at 100% B for 1 min and held at 100% B for 3 min, back to 15% B in 1 min, and held at that percentage of B for 2 min. The t_R of each amino acid were as followed: L-Asp (5.69 min), D-Asp (8.09 min), L-Thr (5.58 min), L-*allo*-Thr (5.80 min), D-*allo*-Thr (8.29 min) and D-Thr (11.63 min). The method for the analysis of the *O*Me-Tyrosine derivatives was developed on 25 min acquisition time: isocratic 25% B for 5 min, then linear gradient to 40% B for 13 min, followed by another gradient at 100% B for 1 min and held at 100% B for 3 min, back to 25% B in 1 min, and held at that percentage of B for 2 min. The t_R of each amino acid were as followed: L-*O*Me-Tyr (10.07 min) and D-*O*Me-Tyr (13.64 min).

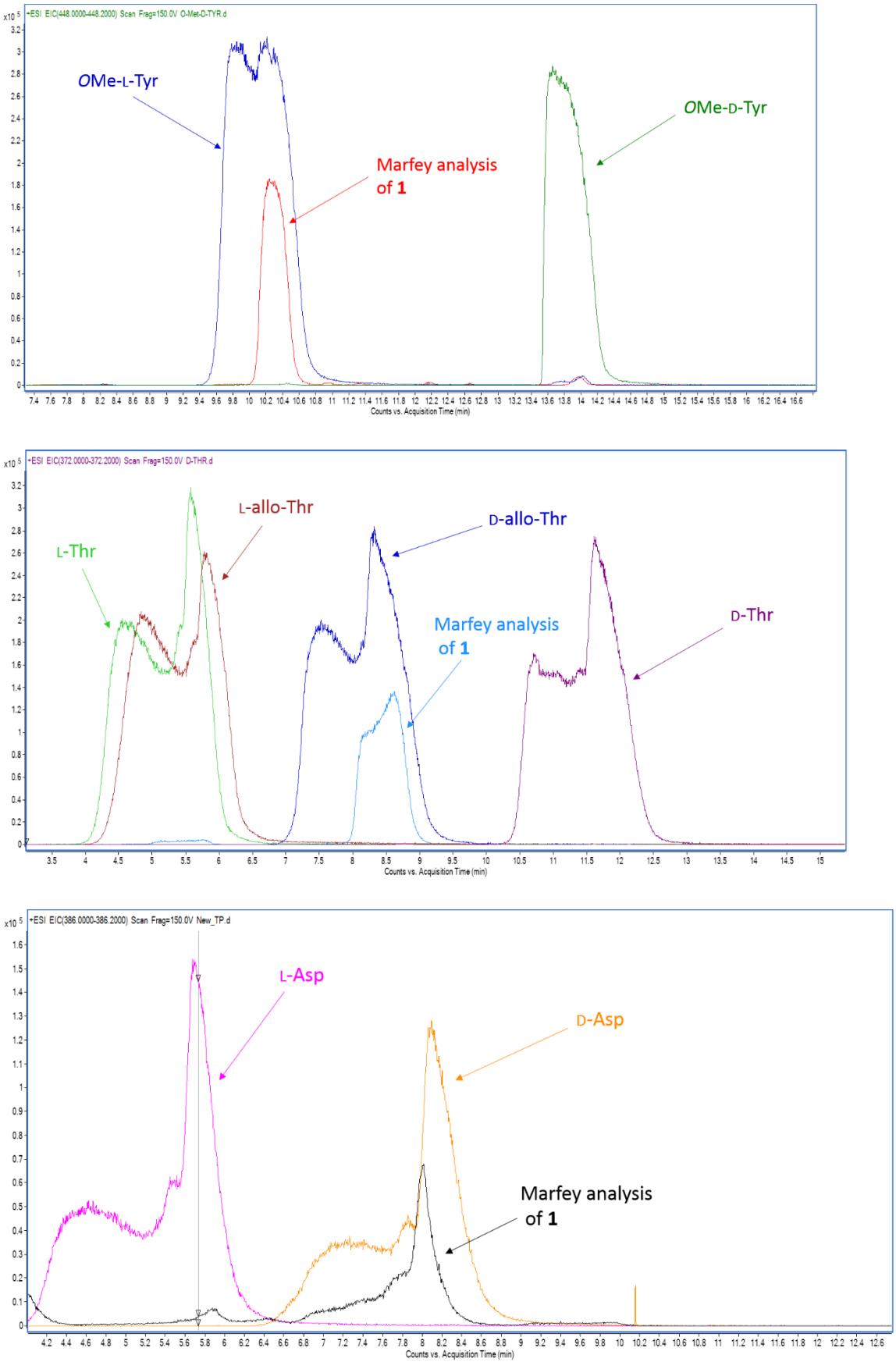


Figure S2. MS chromatogram of the three amino acids present in characellide A (**1**).

UPLC-HRMS/MS analyses

Fractions 2 – 5 were prepare for MS/MS analysis at a concentration of 10mg/mL in MeOH. Separation was performed on a BEH C18 column using a standard gradient of A H_2O and B CH_3CN gradient with 0.1% formic acid (FA): from 10%B during 2 min until 100%B at 12 min and 3 min of isocratic at this concentration. Mass spectra were recorded in positive mode on a qTOF Agilent 6540 in ESI(+).

Molecular Networking Analysis

A molecular network was created using the online workflow at GNPS. The data was filtered by removing all MS/MS peaks within +/- 17 Da of the precursor m/z. MS/MS spectra were window filtered by choosing only the top 6 peaks in the +/- 50Da window throughout the spectrum. The data was then clustered with MS-Cluster with a parent mass tolerance of 2.0 Da and a MS/MS fragment ion tolerance of 0.5 Da to create consensus spectra. Further, consensus spectra that contained less than 2 spectra were discarded. A network was then created where edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. Further edges between two nodes were kept in the network if and only if each of the nodes appeared in each other's respective top 10 most similar nodes.

After file conversion using Proteowizard (version 3.18212), a molecular network was generated using the GNPS platform. Data can be accessed at https://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=f0f277796394477e8d186099aa5ee1af. The molecular network was visualized via Cytoscape (version 3.6.1).

Biological Activities

Cell Culture

Microglia BV-2 cell line was obtained from InterLab Cell Line Collection (ICLC) (Genova, Italy), number ATL03001. Cells were maintained in Roswell Park Memorial Institute Medium (RPMI) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and 100 μg/mL streptomycin at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air. Cells were dissociated twice a week using 0.05% trypsin/EDTA.

Cell viability

The MTT assay was used to analyze cell viability as previously described.[1] Briefly, microglia BV-2 cell line was grown in 96 well plate at a density of 4 x 10^4 cells per well. Cells were

exposed to different compounds concentration (0.001, 0.01, 0.1, 1 and 10 μM) for 24 hours. Then, the cells were rinsed and incubated with MTT (500 μg/mL) diluted in a saline buffer for 1h at 37 ºC. The resulting formazan crystals were dissolved with 5% sodium dodecyl sulfate (SDS) and the absorbance values were obtaining using a spectrophotometer plate reader (595 nm). Saponin was used as a cellular death control and its absorbance was substrate from the other data.

Measurement of intracellular ROS production

The intracellular ROS levels in microglia activation were performed using 7′,2′-dichlorofluorescein diacetate (DCFH-DA), as previously described.[2] Cells were pre-treated with different compounds concentration (0.001, 0.01, 0.1, 1 and 10 μM) 1 hour prior to the stimulation with LPS (500 ng/mL) for 24 h. Afterwards, cells were rinsed twice with saline solution and incubated 1 h at 37 ºC with 20 μM DCFH-DA. Then, cells were washed and keep with saline solution for 30 min at 37 ºC. Intracellular production of ROS was measured by fluorescence detection of dichlorofluorescein (DCF) as the oxidized product of DCFH-DA on a spectrophotometer plate reader (495 nm excitation and 527 nm emission).

Mitochondrial membrane potential variations assay

The variations in mitochondrial membrane potential were determined with the tetramethylrhodamine methyl ester (TMRM) assay.[3] 24 hours prior to the experiments, the cells were pre-treated with compounds (0.001, 00.1, 0.1, 1 and 10 μM) for 1 hour and then stimulated with LPS (500 ng/mL) for 24h. Cells were washed twice with saline buffer and incubated with TMRM (1 μM) during 30 min at 37ºC. After, the cells were solubilized with 50% water/DMSO. Fluorescence was measured at 536 nm excitation and 590 nm emission in a spectrophotometer plate reader.

Nitrite determination

The NO concentration in the culture media was established by measuring nitrite formed by the oxidation of NO, using the Griess reagent kit, according to manufacturer's instructions. The detection limit of this method is 1 μM. Briefly, microglia cells were seeded in 12-well plate at a density of 1 x 10^6 cells per well and pre-incubated with compounds (0.1, 1 and 10 μM) 1 hour and then were stimulated with LPS (1 μg/mL) for 24 h. Thereafter, in a microplate were mixed: 150 μL of cells supernatant, 130 μL of deionized water and 20 μL

of Griess Reagent and was incubated for 30 min at room temperature. The absorbance was measured on a spectrophotometer plate reader at a wavelength of 548 nm.

Statistical analysis

Results were expressed as mean ± SEM of a minimum of three experiments and were performed by duplicate or triplicate. Comparisons were analysed using Student´s t-test or one-way ANOVA with Dunnett´s post hoc analysis. P values < 0.05 were considered statistically significant.

1. Sanchez, J. A.; Alfonso, A.; Leiros, M.; Alonso, E.; Rateb, M. E.; Jaspars, M.; Houssen, W. E.; Ebel, R.; Tabudravu, J.; Botana, L. M., Identification of Spongionella compounds as cyclosporine A mimics. *Pharmacological research* **2016**.

2. Leiros, M.; Alonso, E.; Rateb, M. E.; Houssen, W. E.; Ebel, R.; Jaspars, M.; Alfonso, A.; Botana, L. M., Gracilins: Spongionella-derived promising compounds for Alzheimer disease. *Neuropharmacology* **2015,** *93*, 285-93.

3. White, M. G.; Wang, Y.; Akay, C.; Lindl, K. A.; Kolson, D. L.; Jordan-Sciutto, K. L., Parallel high throughput neuronal toxicity assays demonstrate uncoupling between loss of mitochondrial membrane potential and neuronal damage in a model of HIV-induced neurodegeneration. *Neurosci Res* **2011,** *70* (2), 220-9.

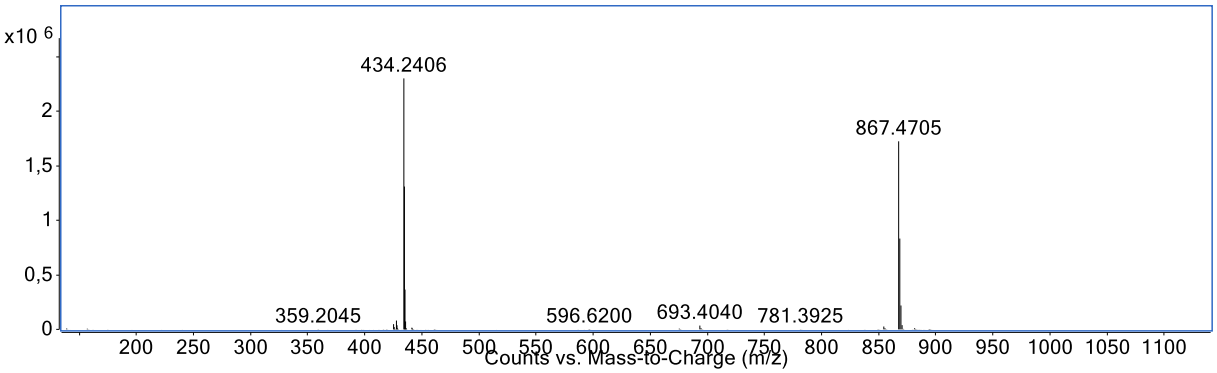


Figure S3. HRESIMS analysis of characellide A (**1**).

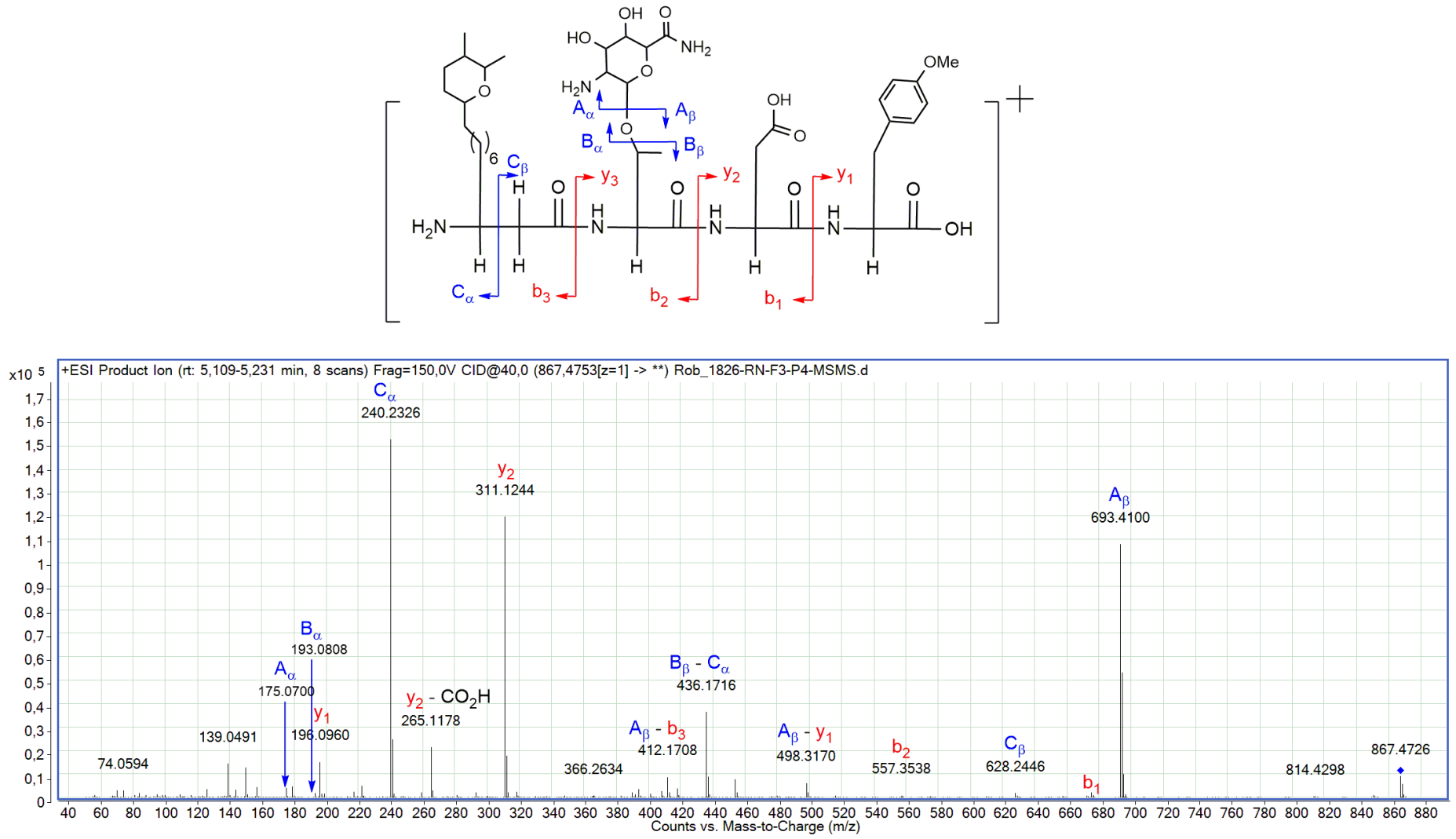


Figure S4. MS/MS analysis and fragmentation pattern of **1** and **2**.

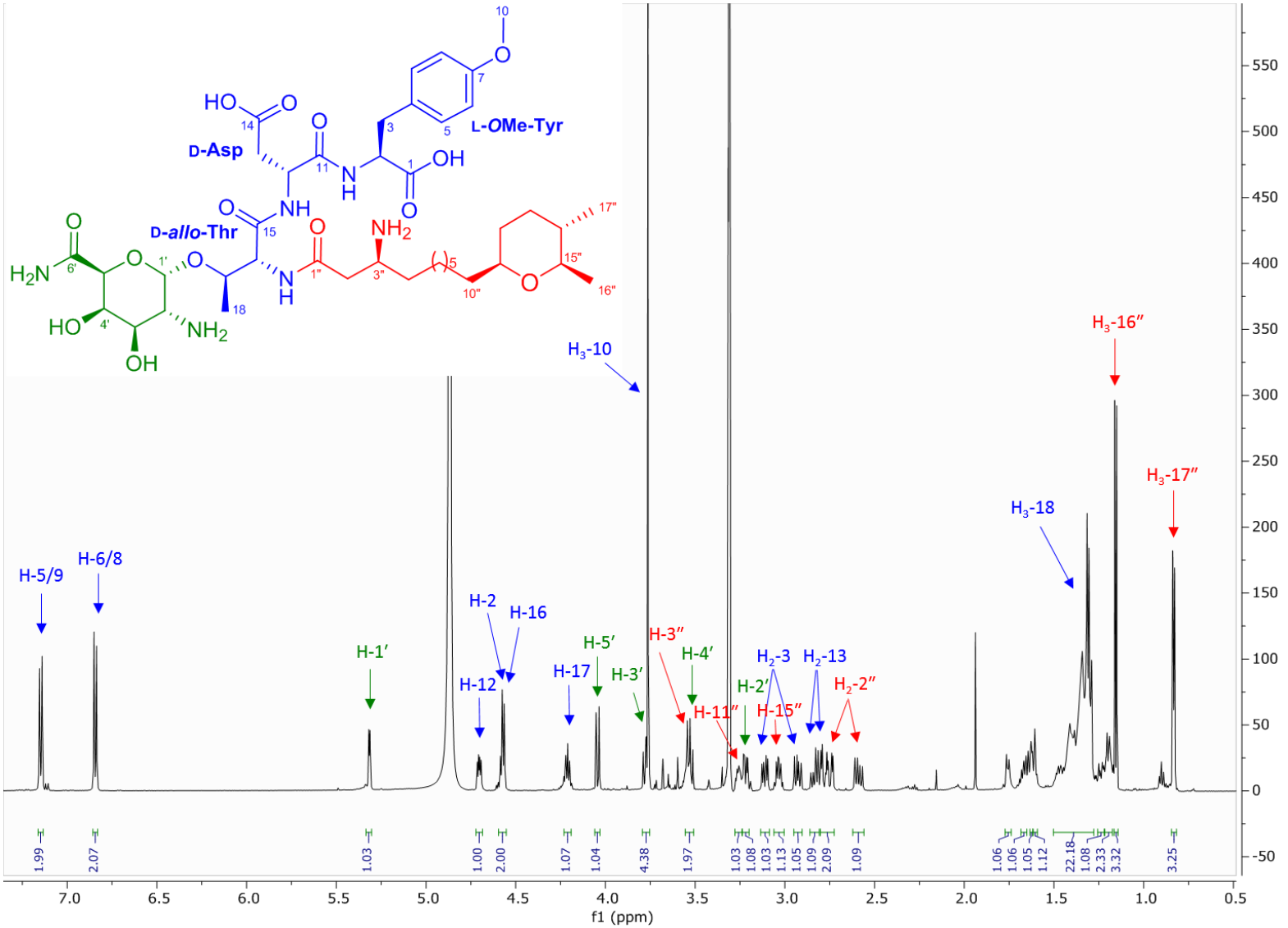


Figure S5. ^{1}H NMR spectrum of **1** in CD_3OD (600 MHz).

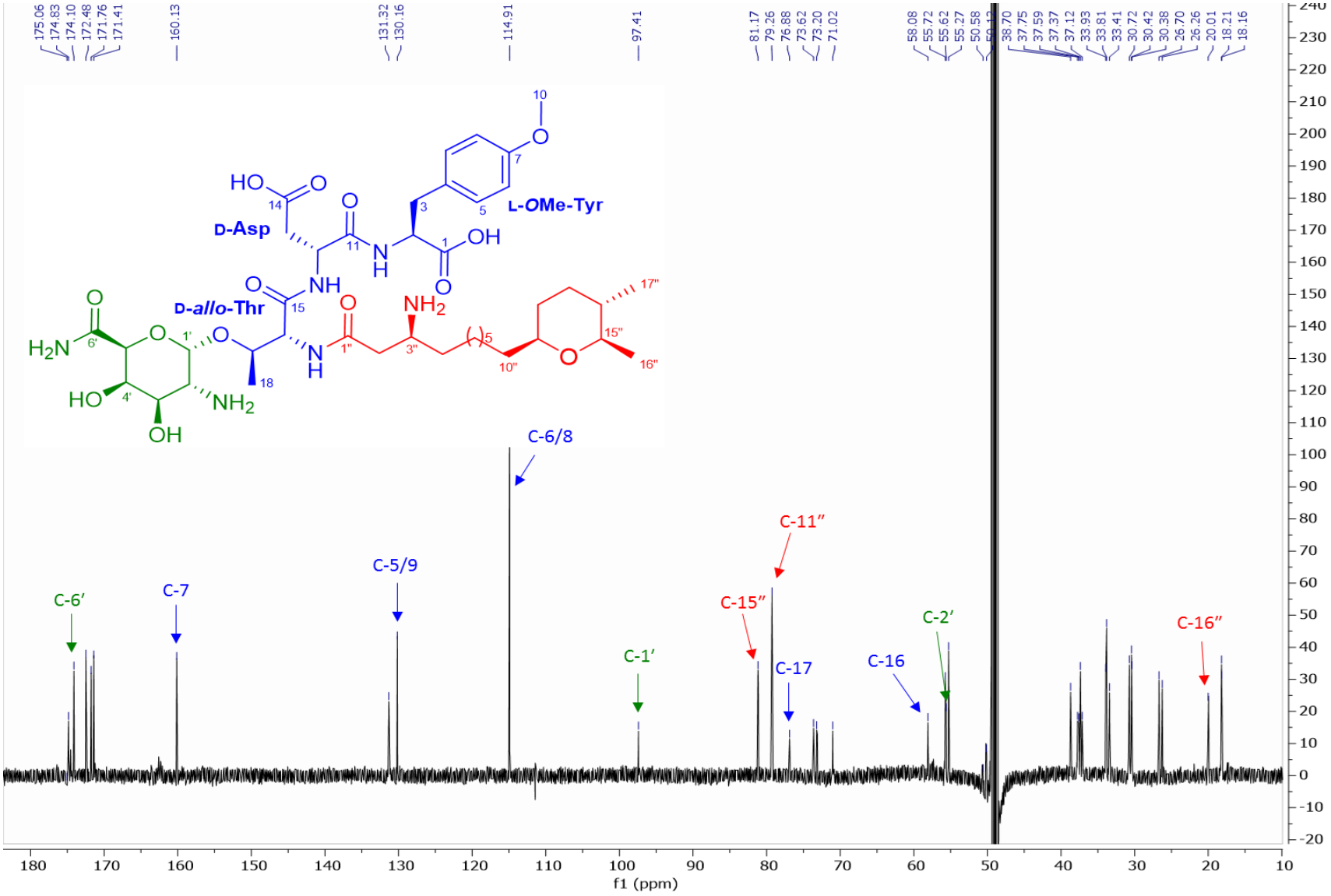


Figure S6. ^{13}C NMR spectrum of **1** in CD_3OD (150 MHz).

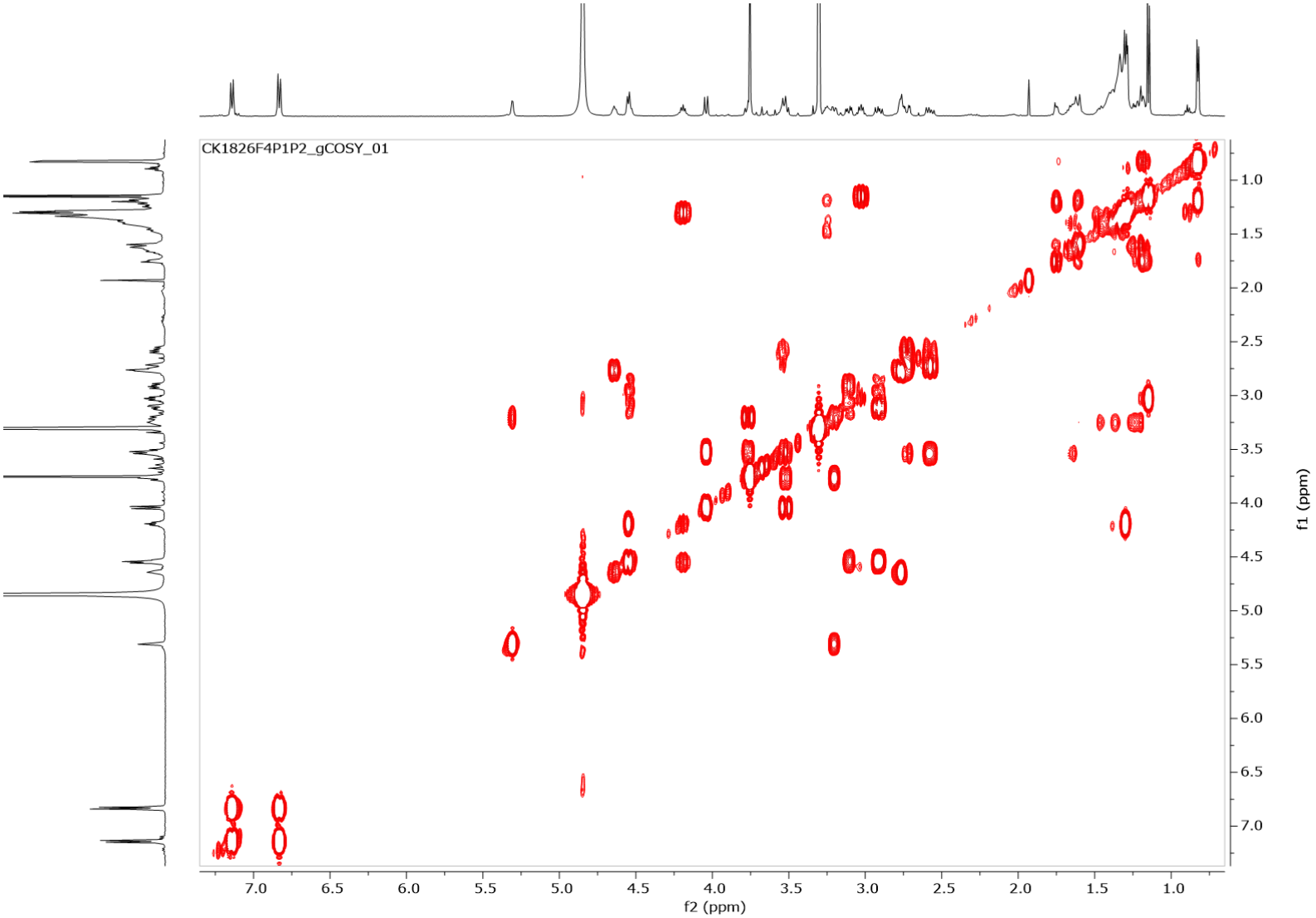


Figure S7. COSY NMR spectrum of **1** in CD_3OD (600 MHz).

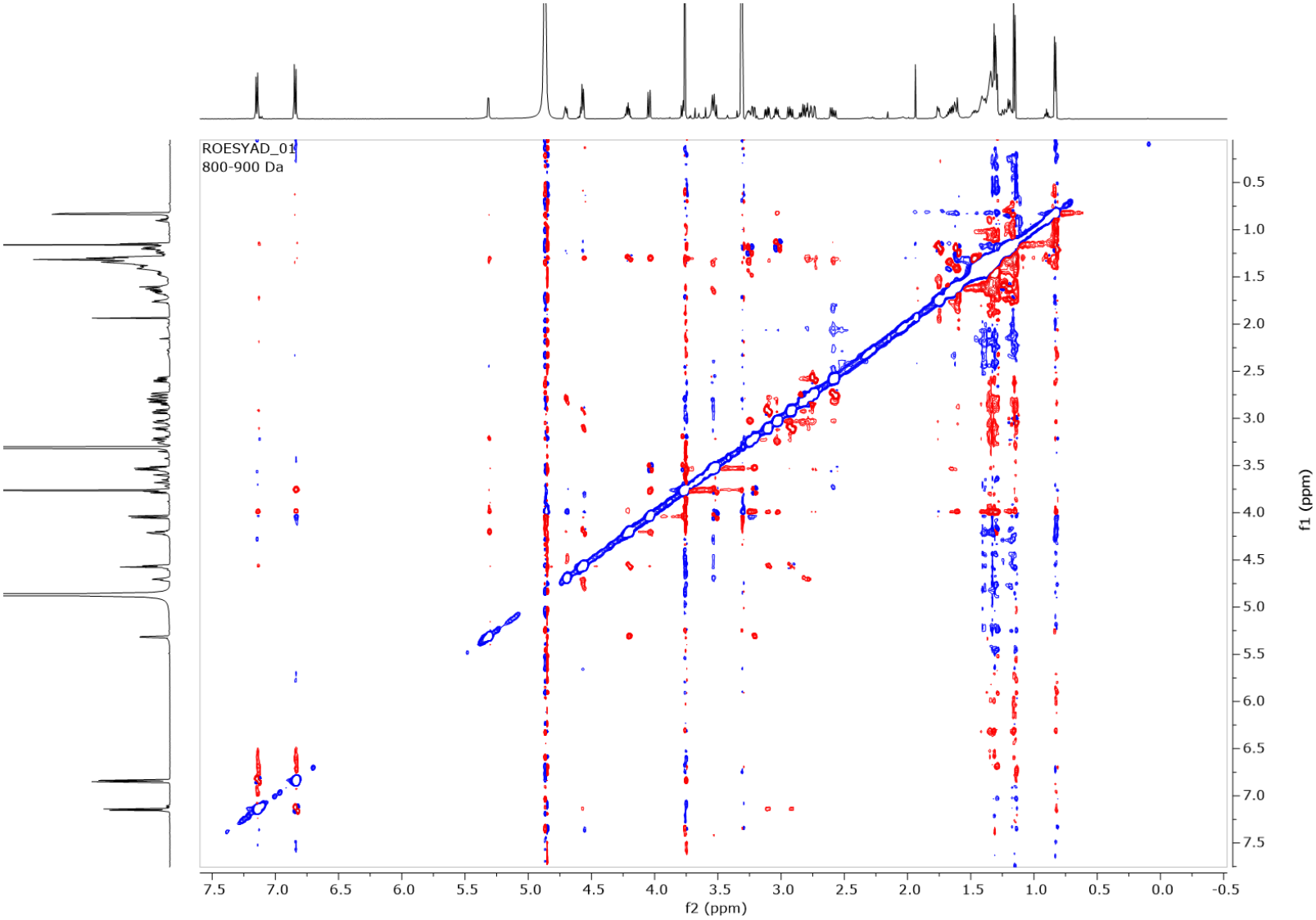


Figure S8. ROESY NMR spectrum of **1** in CD_3OD (600 MHz).

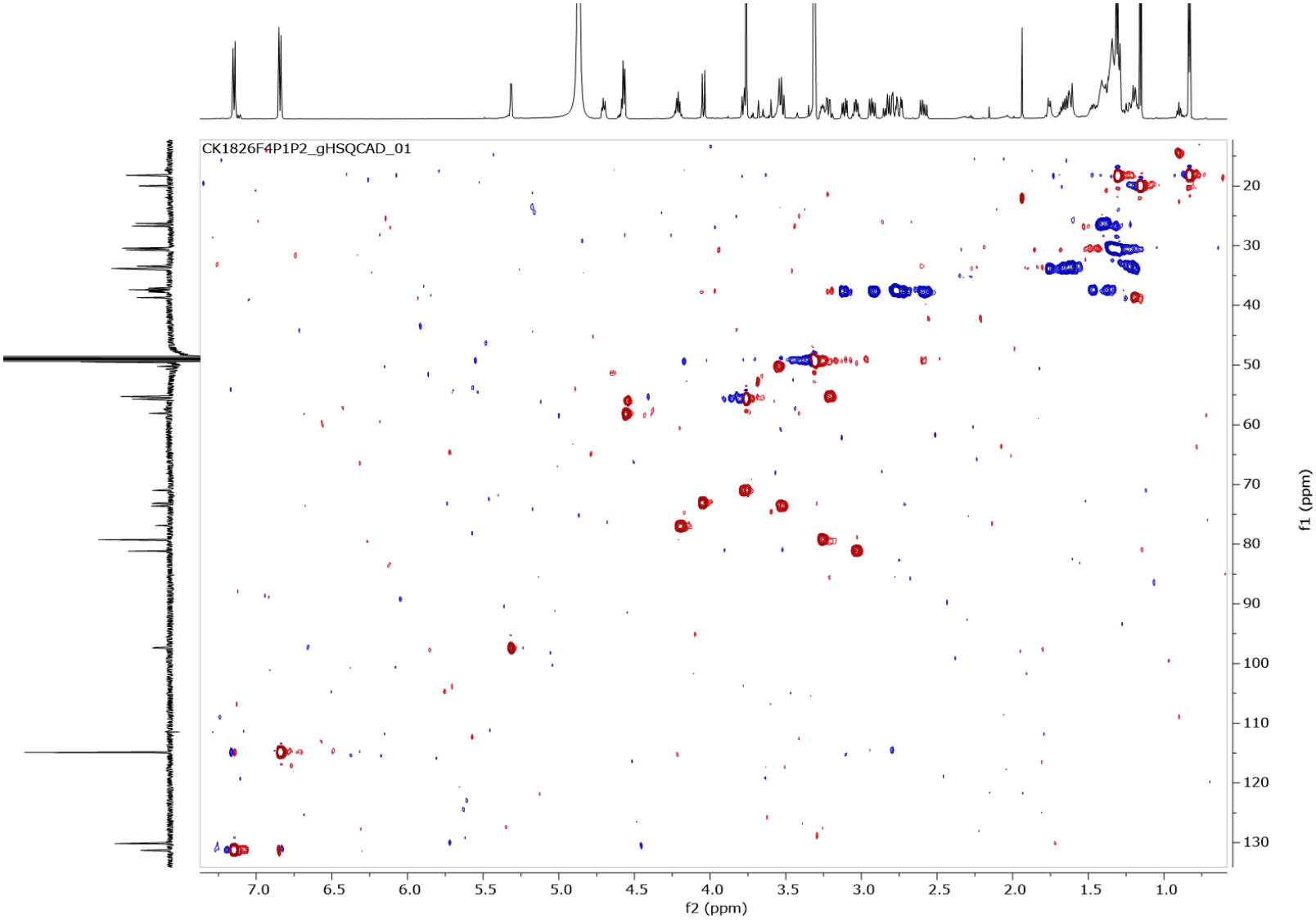


Figure S9. HSQC NMR spectrum of **1** in CD_3OD (600 MHz).

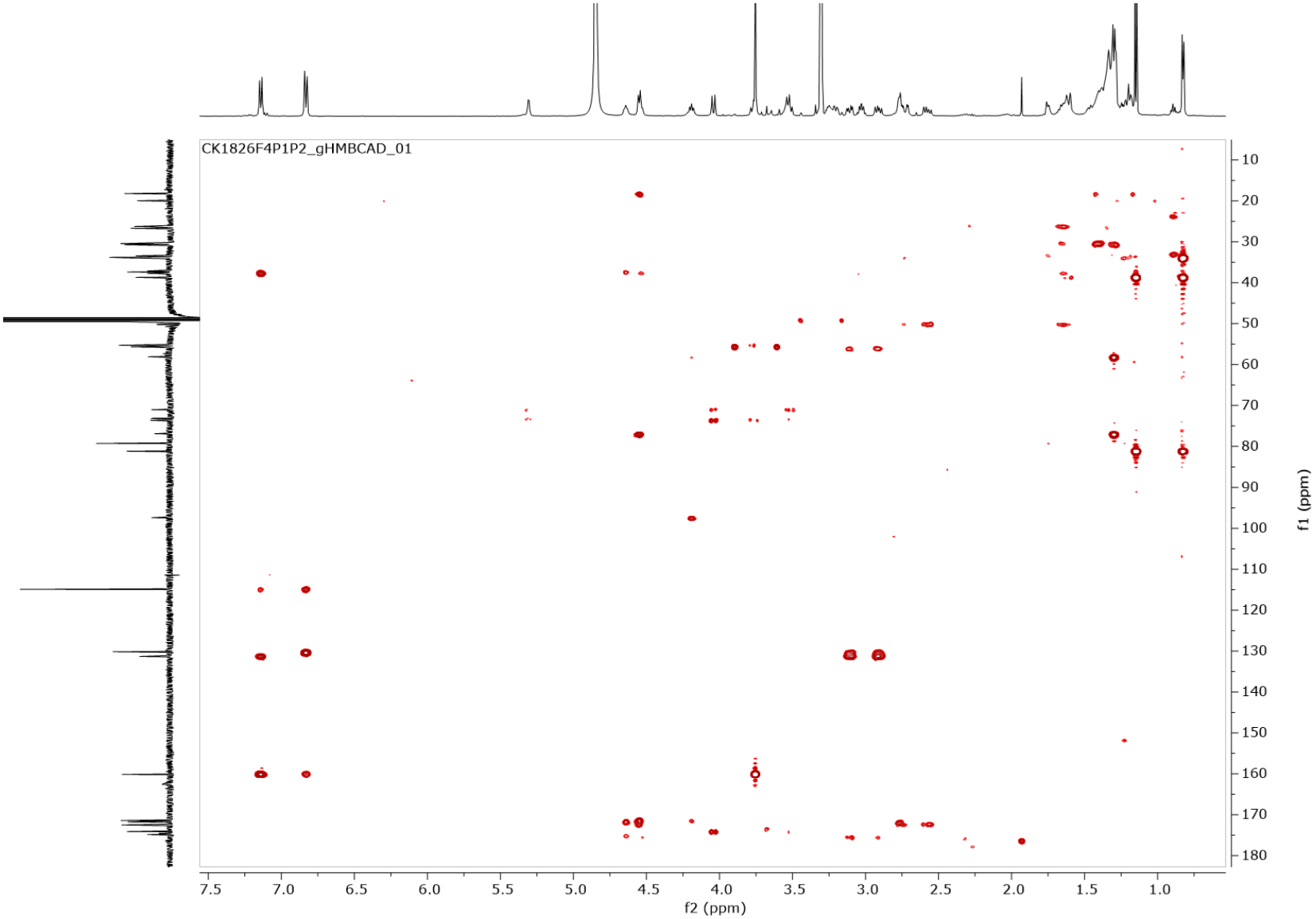


Figure S10. HMBC NMR spectrum of **1** in CD_3OD (600 MHz).

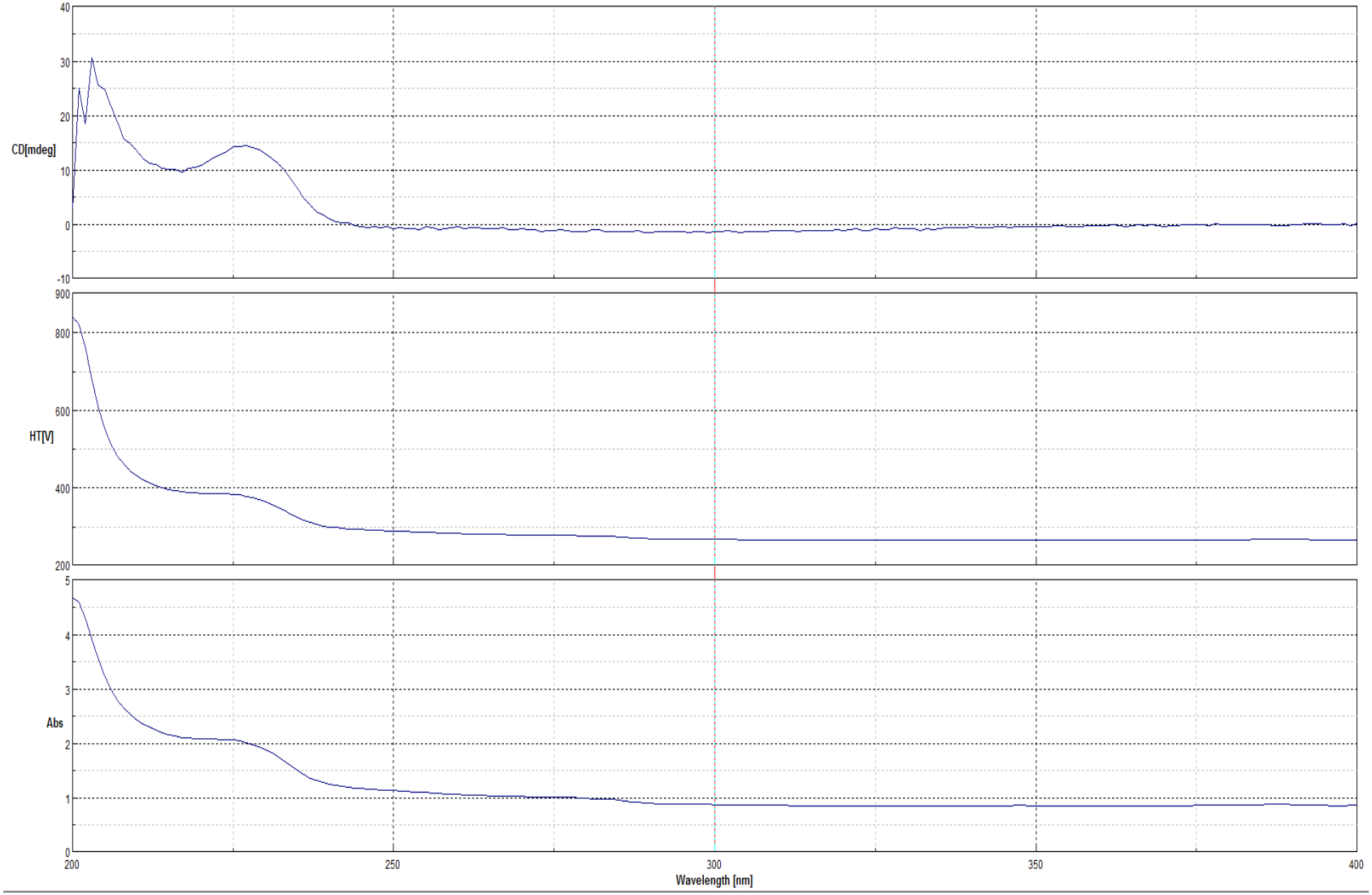


Figure S11. ECD and UV spectra of **1** in MeOH at 0.1 mg/ml.

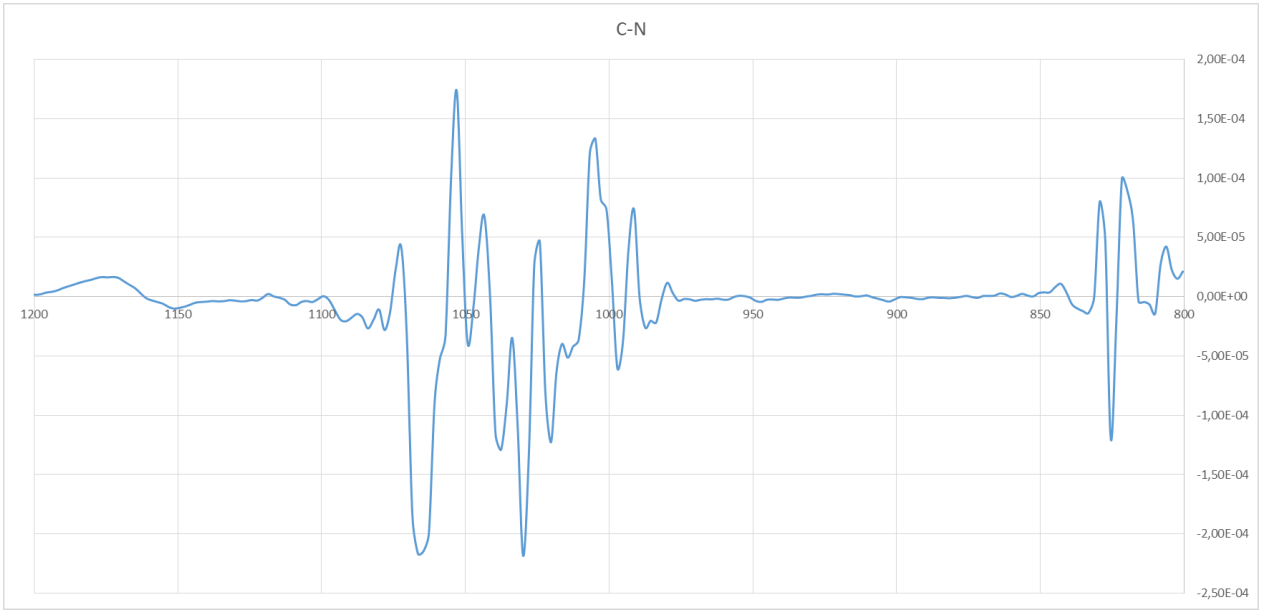


Figure S12. VCD spectrum of **1** at 5 mg/ml in DMSO-d_6 (800-1200 cm^{-1}).

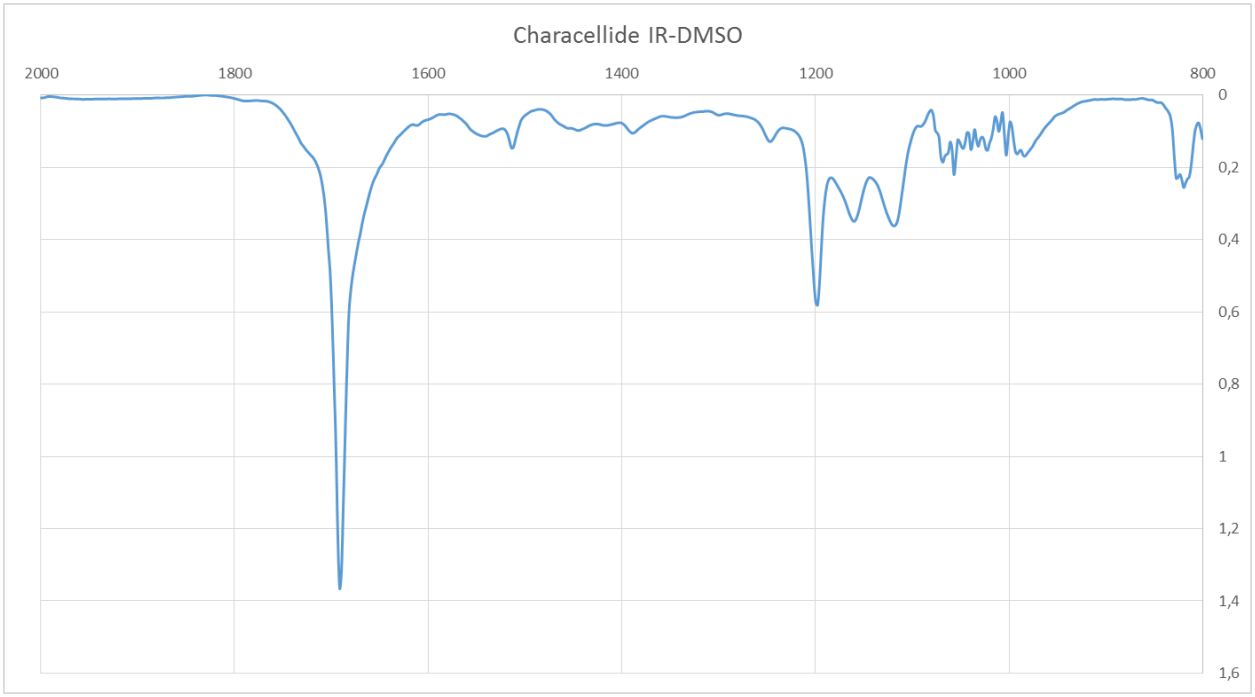


Figure S13. IR spectrum of **1** at 5 mg/ml in DMSO-d_6.

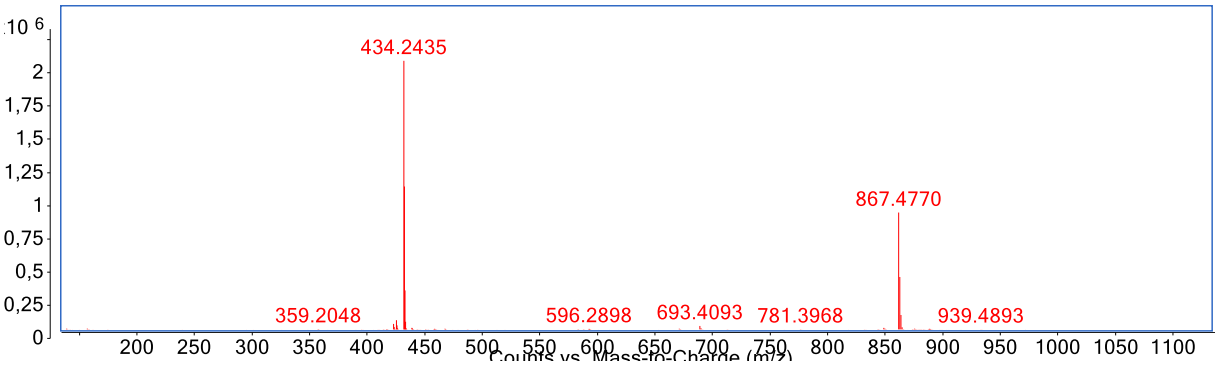


Figure S14. HRESIMS analysis of characellide B (**2**).

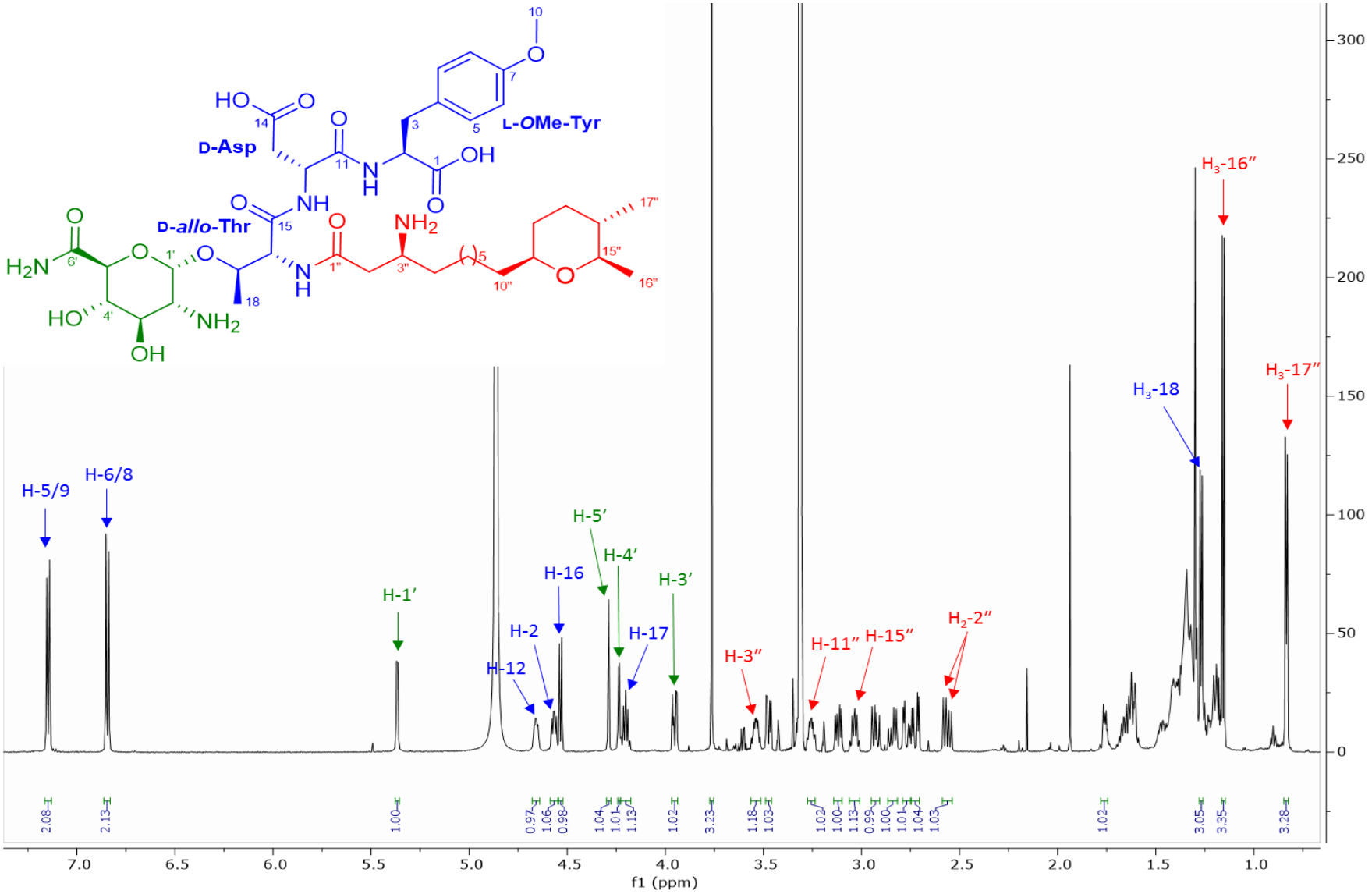


Figure S15. ^{1}H NMR spectrum of **2** in CD_3OD (600 MHz).

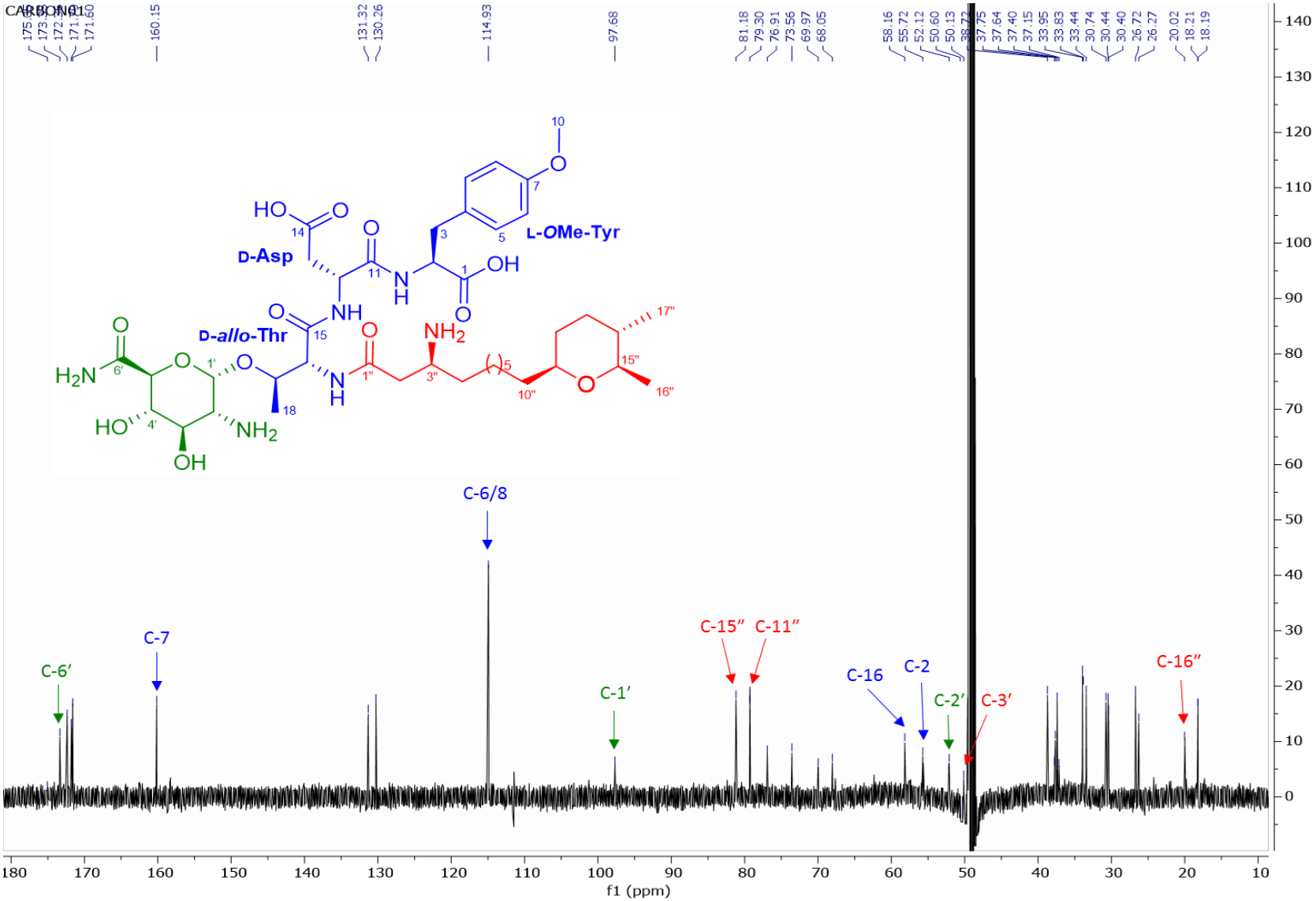


Figure S16. ^{13}C NMR spectrum of **2** in CD_3OD (150 MHz).

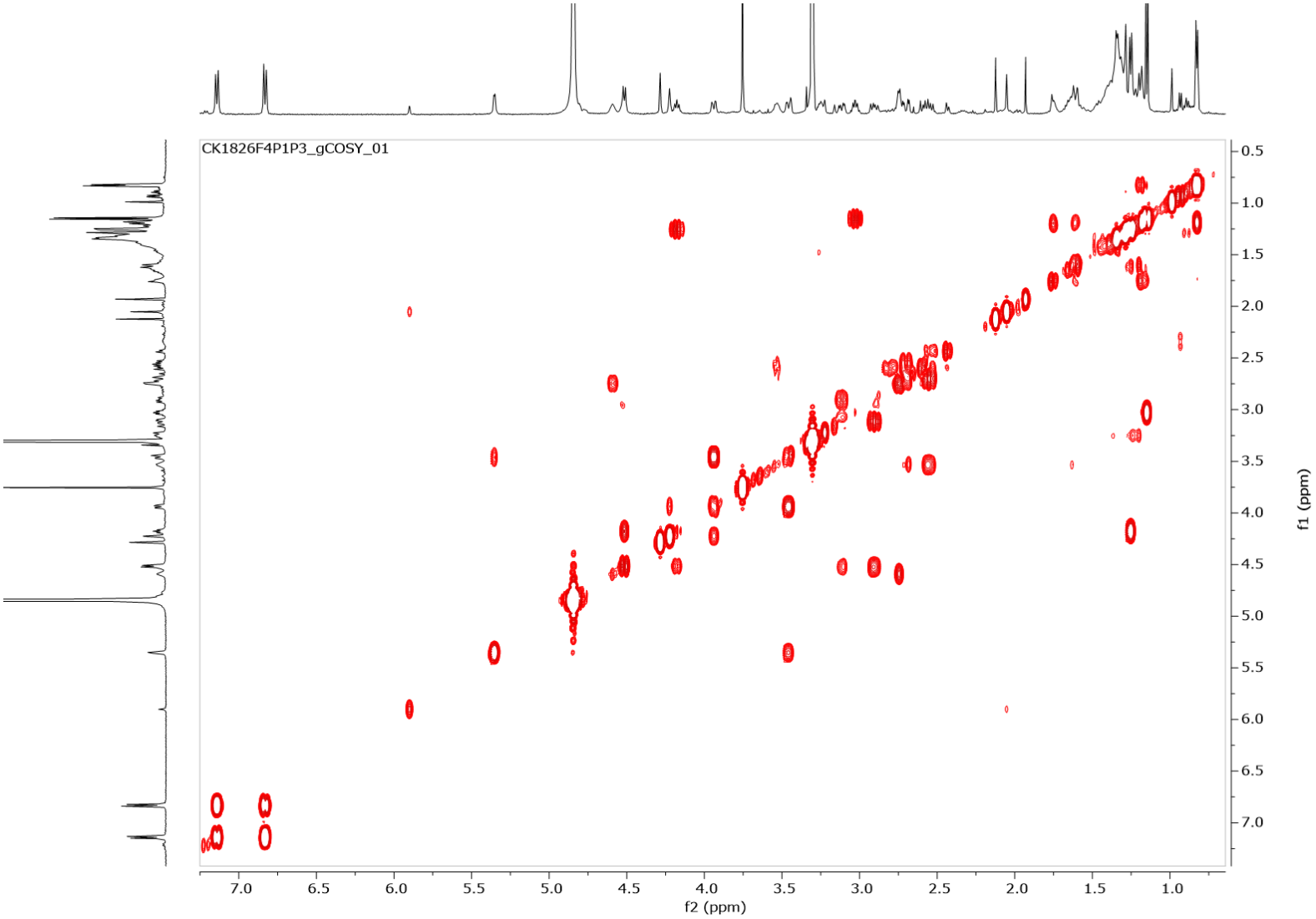


Figure S17. COSY NMR spectrum of **2** in CD_3OD (600 MHz).

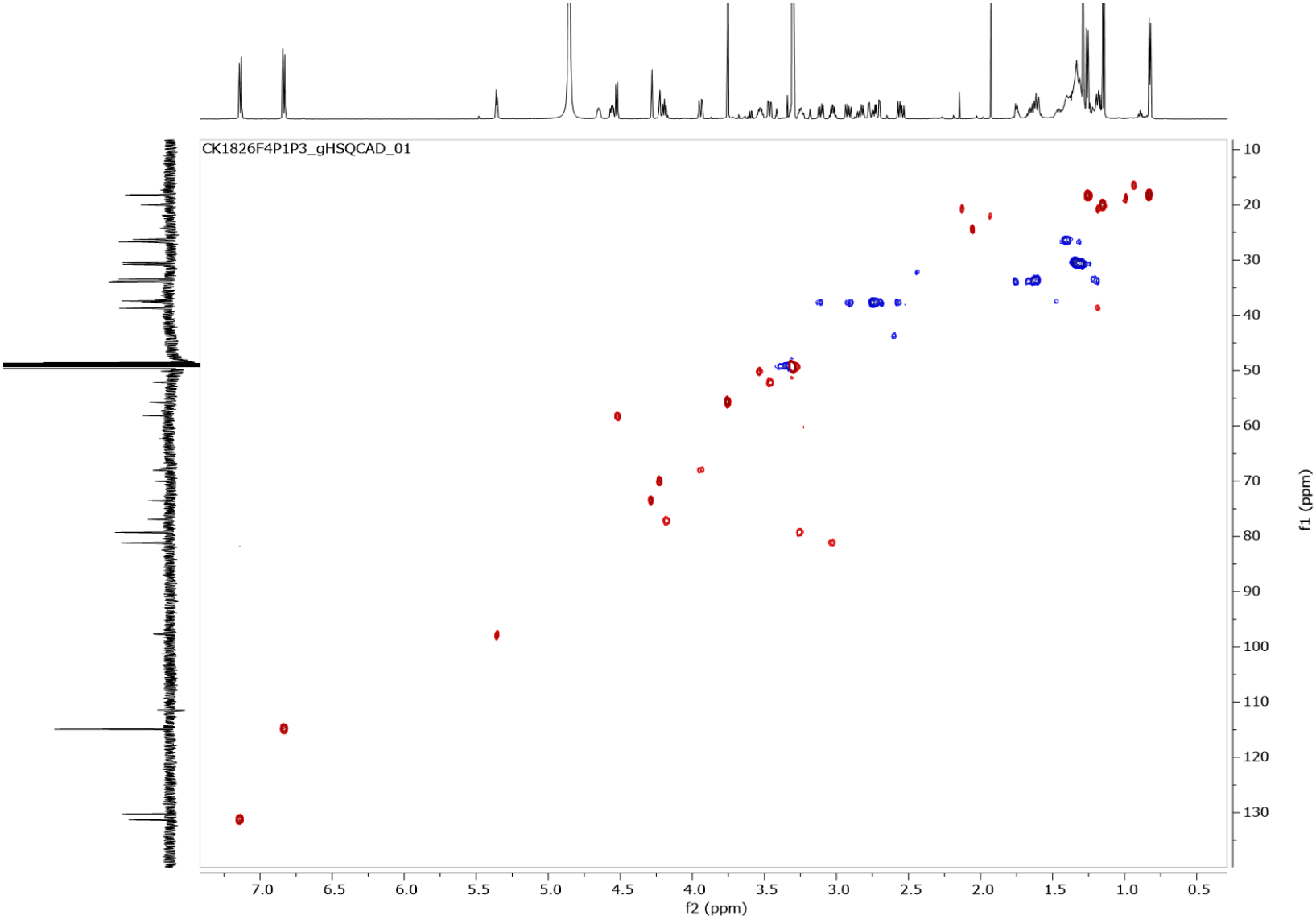


Figure S18. HSQC NMR spectrum of **2** in CD_3OD (600 MHz).

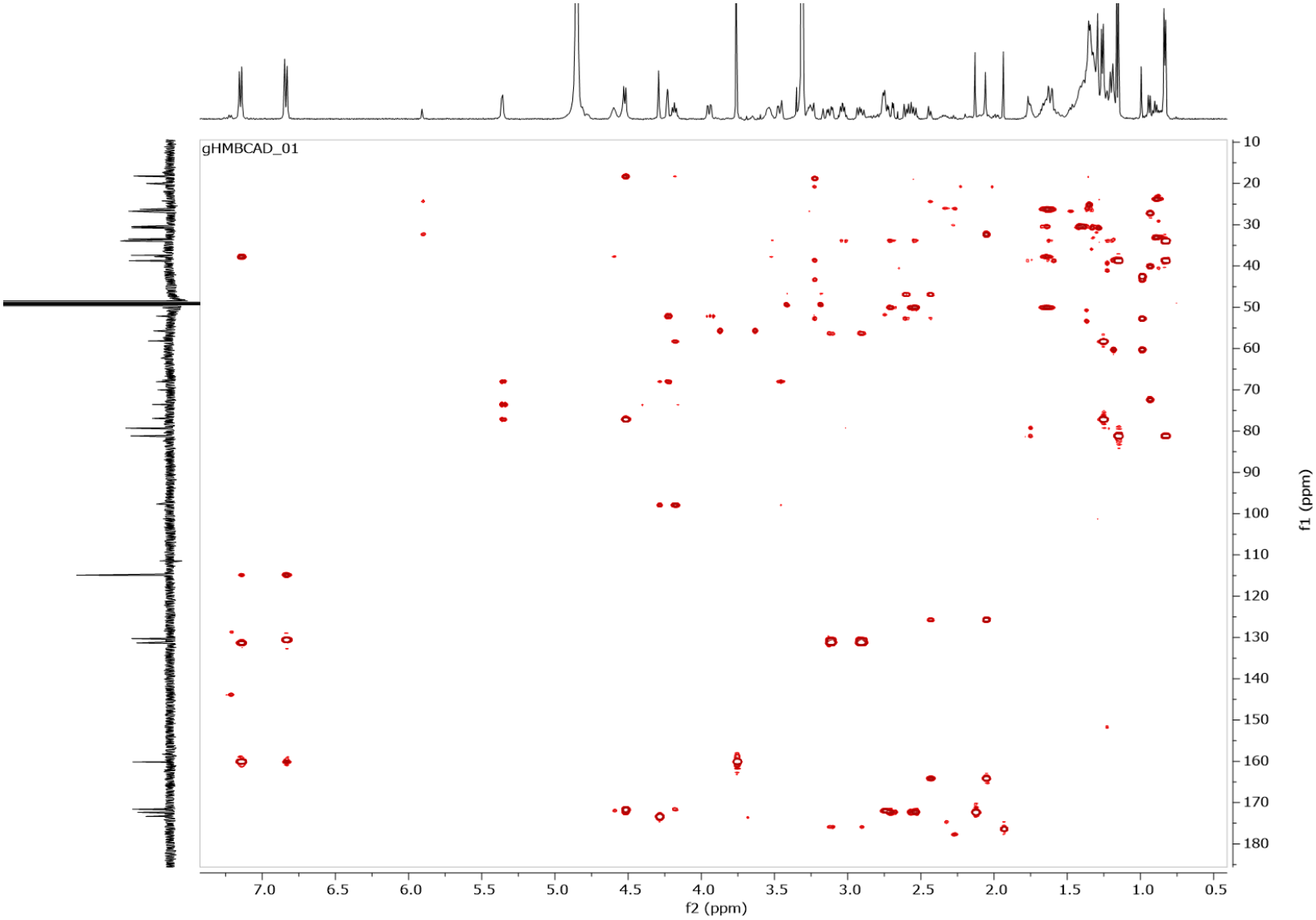


Figure S19. HMBC NMR spectrum of **2** in CD_3OD (600 MHz).

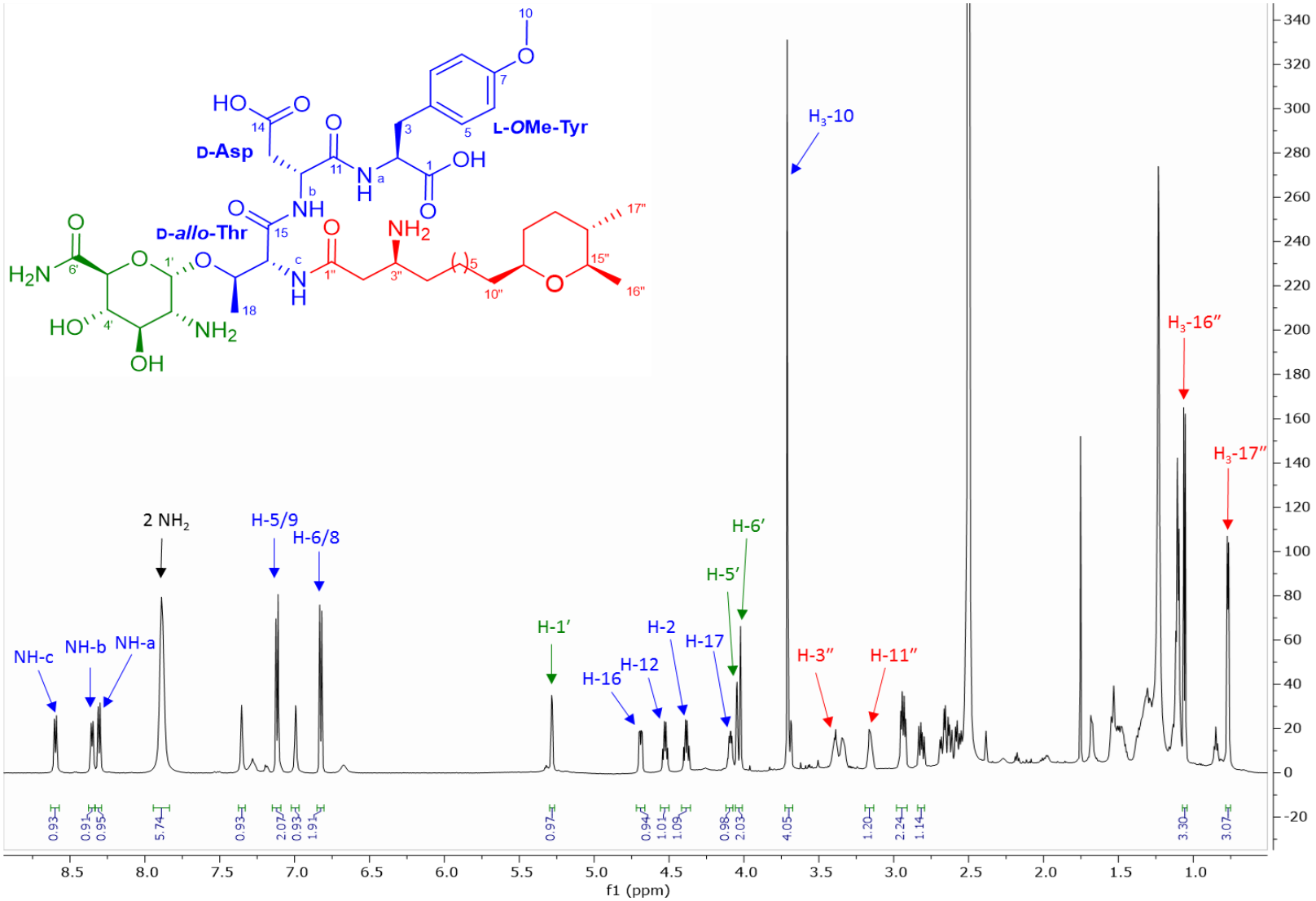


Figure S20. ^{1}H NMR spectrum of **2** in DMSO-d_6 (600 MHz).

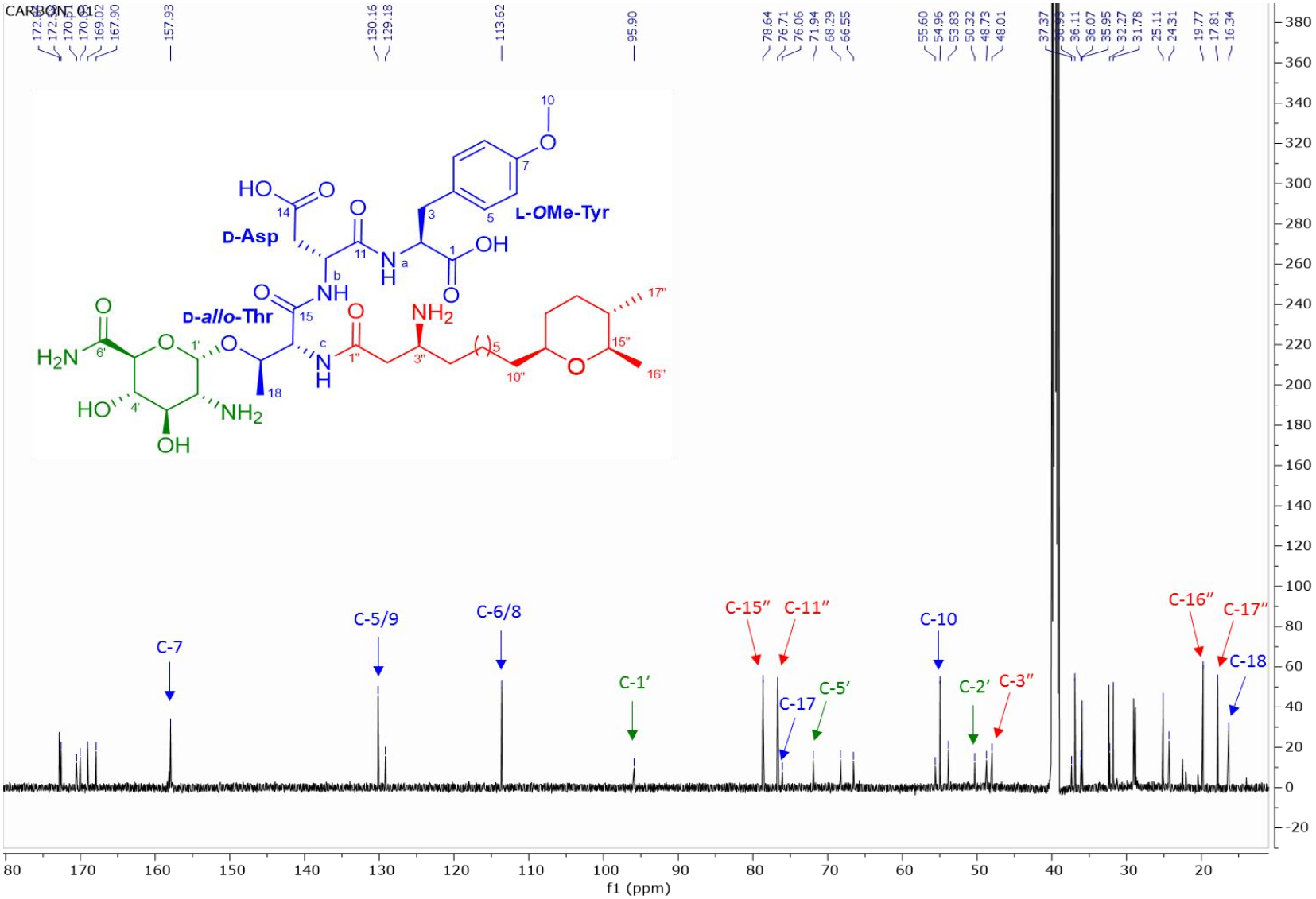


Figure S21. ^{13}C NMR spectrum of **2** in DMSO-*d*$_6$ (150 MHz).

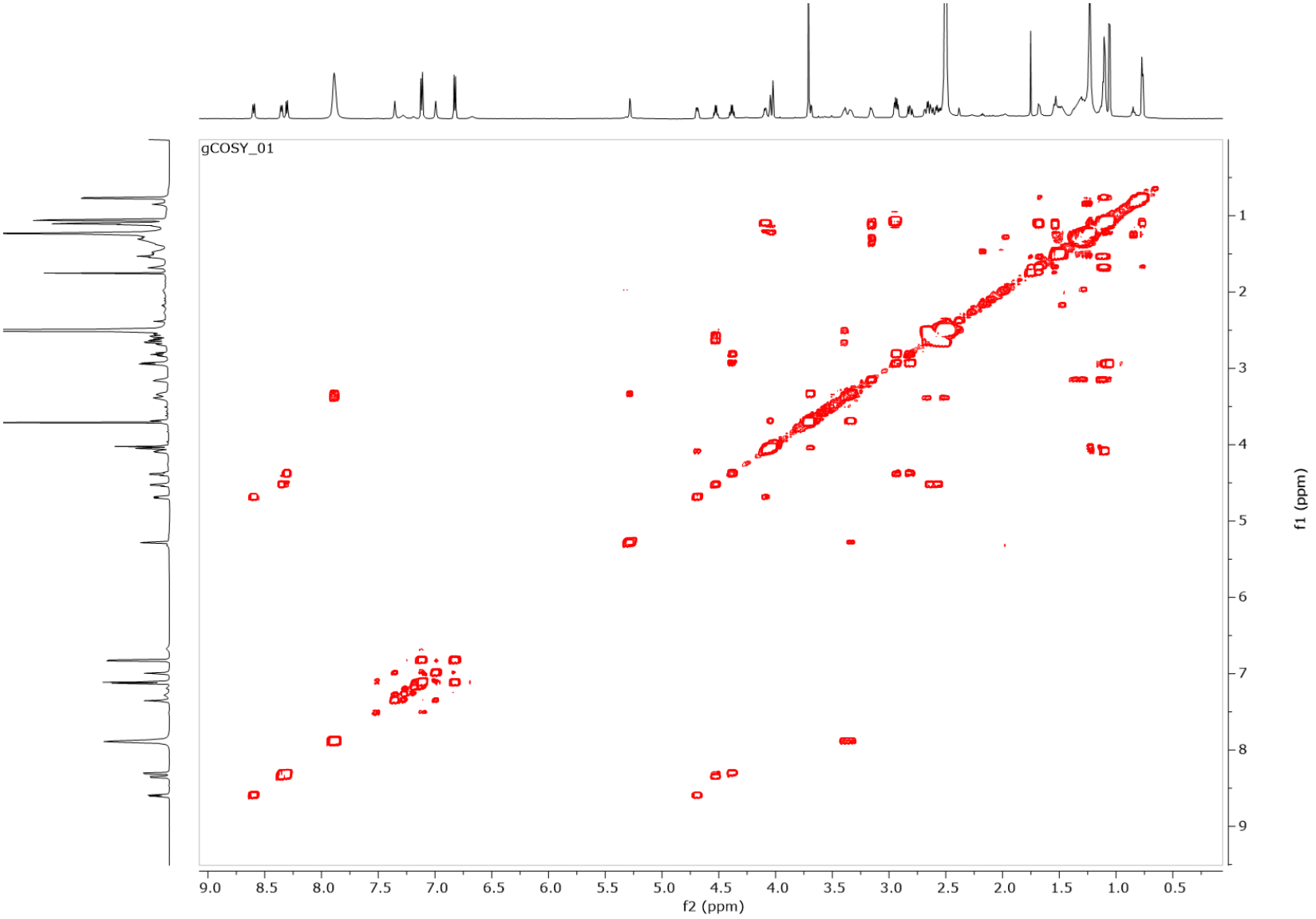


Figure S22. COSY NMR spectrum of **2** in DMSO-d_6 (600 MHz).

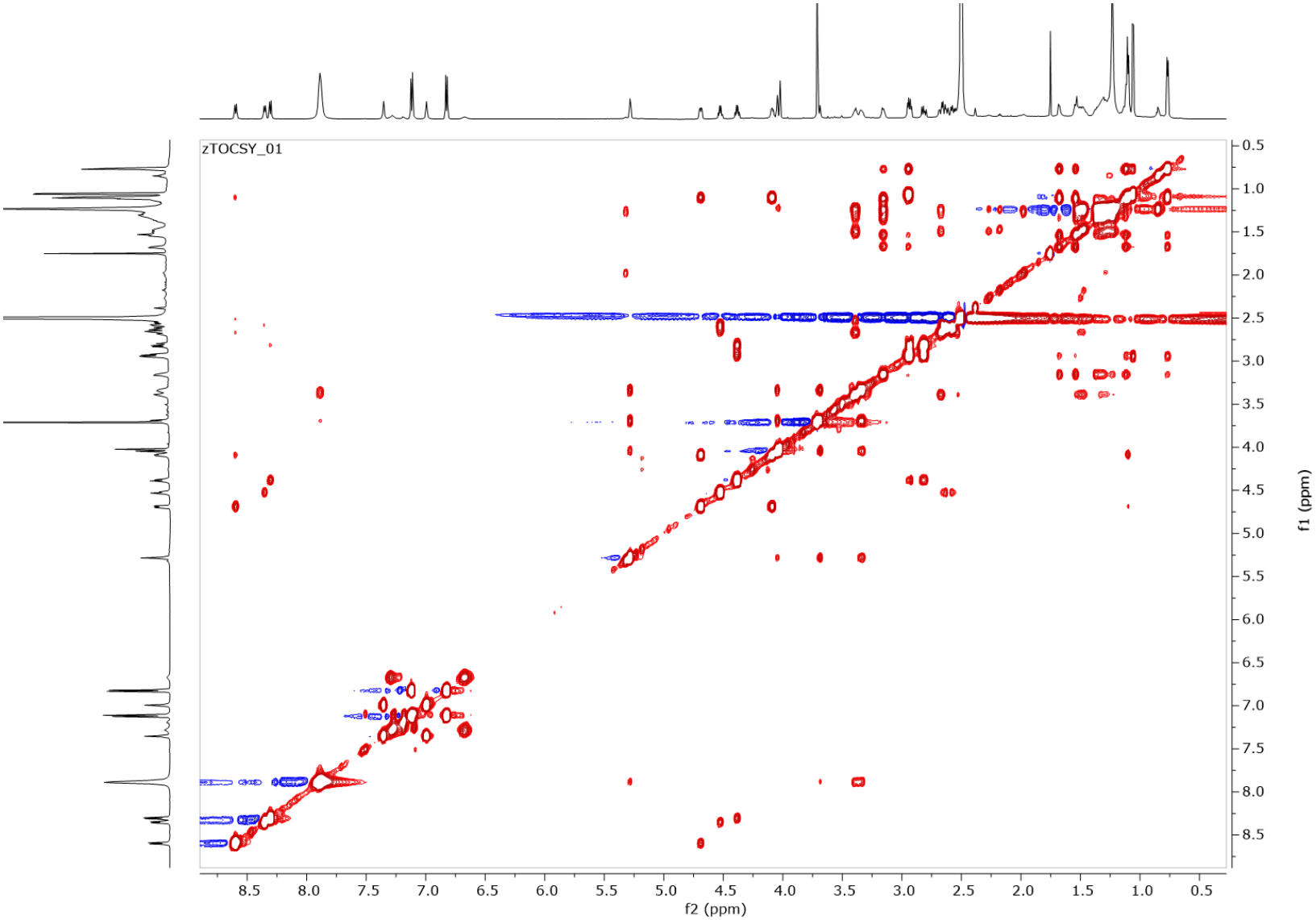


Figure S23. TOCSY NMR spectrum of **2** in DMSO-d_6 (600 MHz).

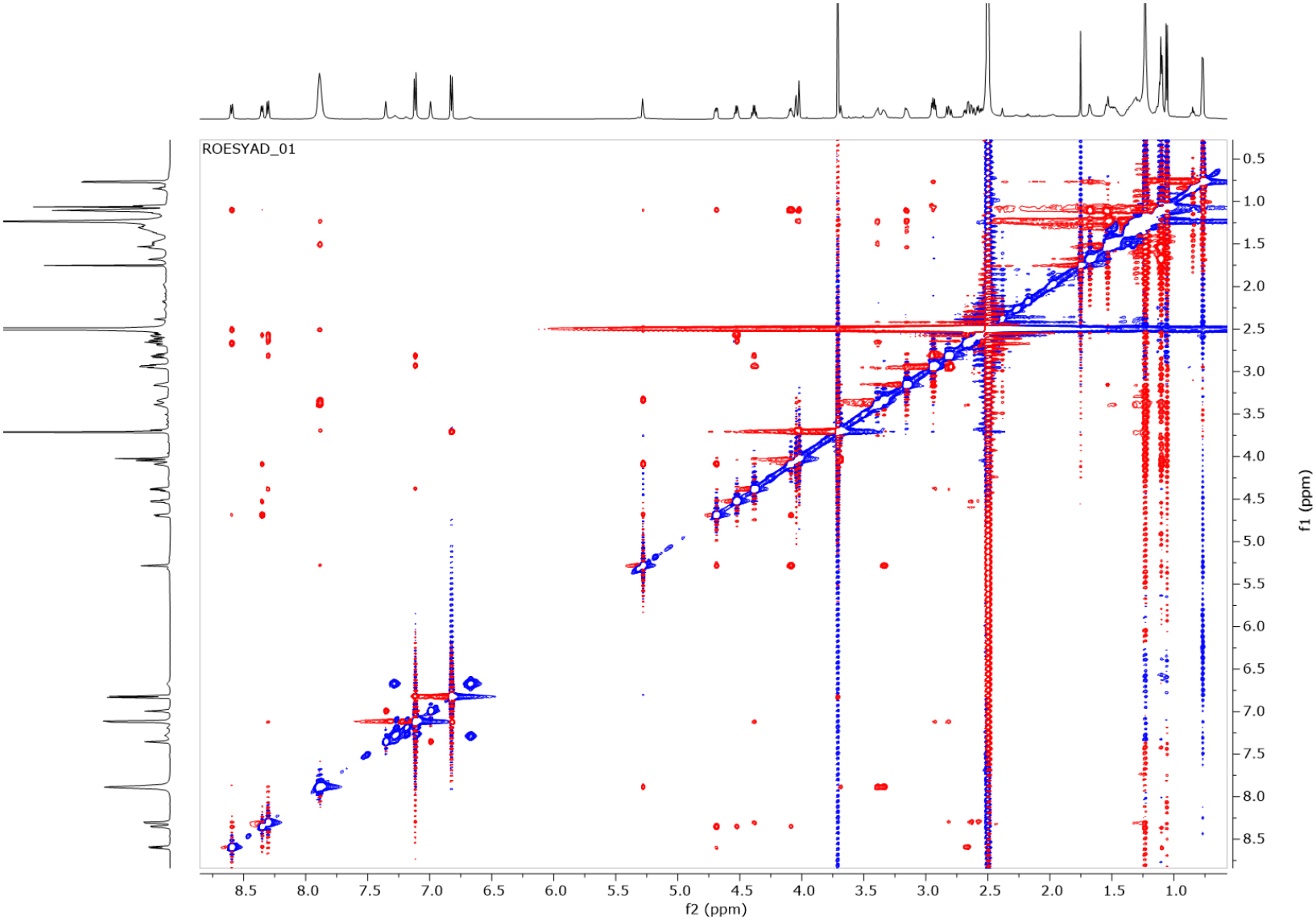


Figure S24. ROESY NMR spectrum of **2** in DMSO-d_6 (600 MHz).

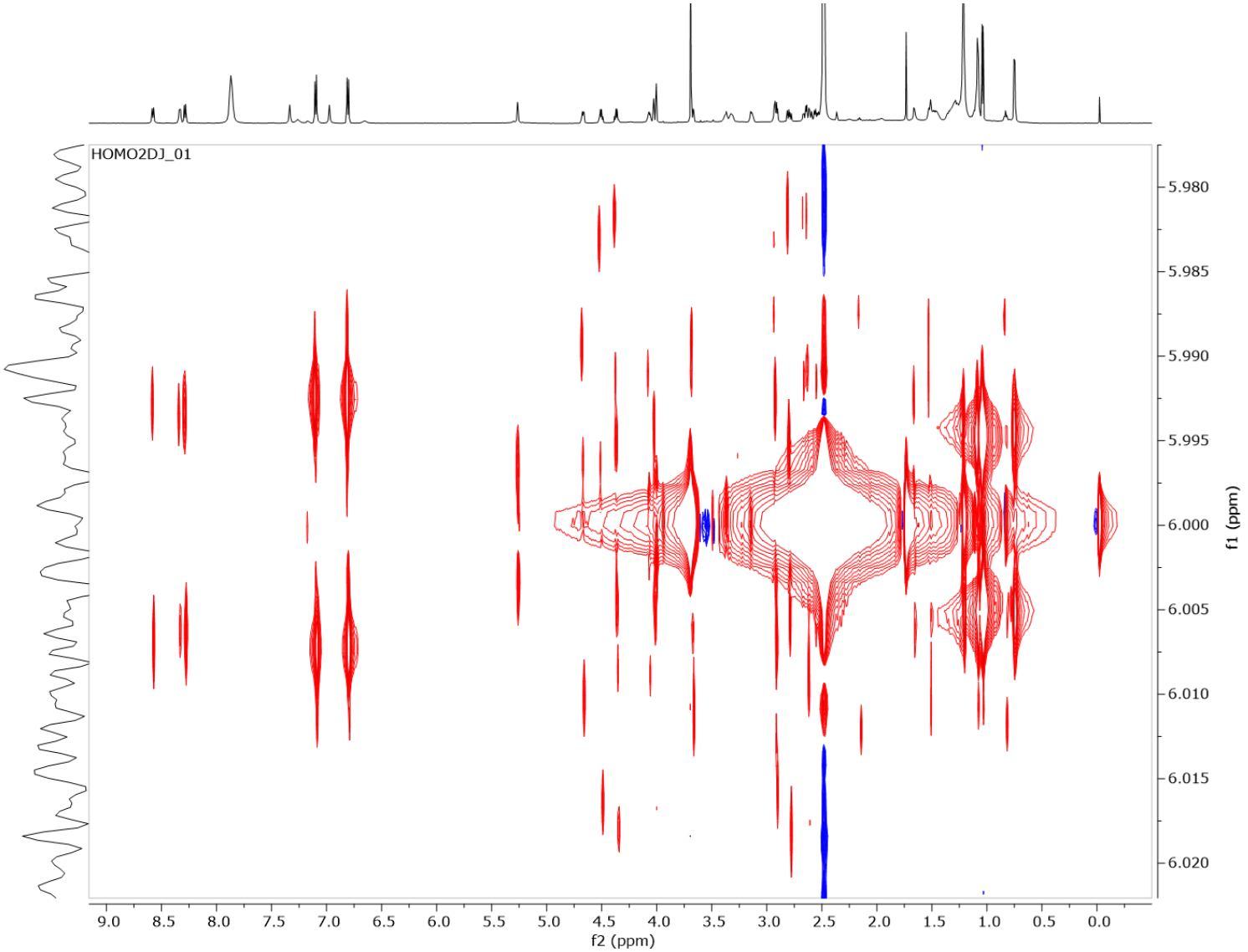


Figure S25. HOMO2DJ NMR spectrum of **2** in DMSO-d_6 (600 MHz).

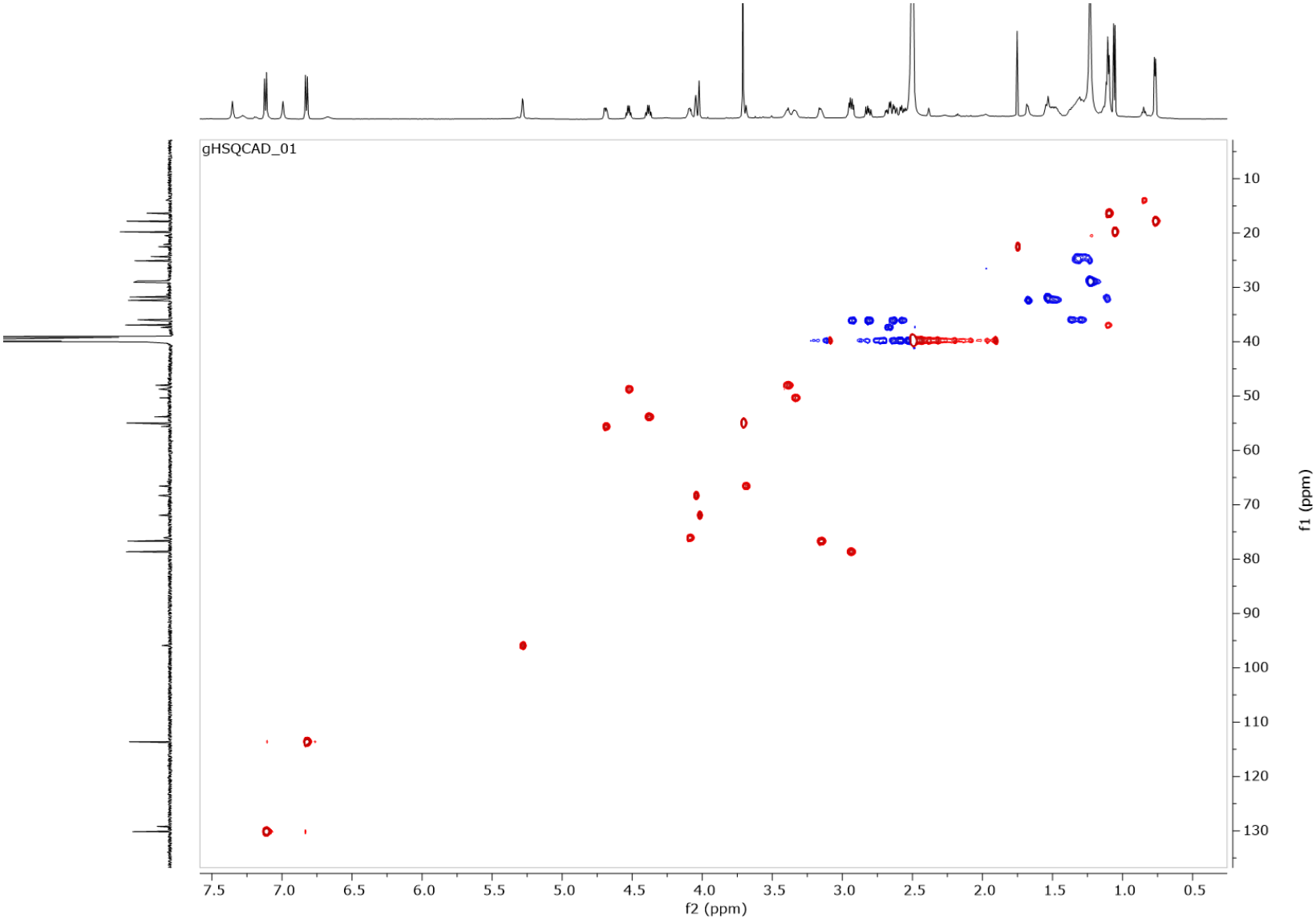


Figure S26. HSQC NMR spectrum of **2** in DMSO-*d*6 (600 MHz).

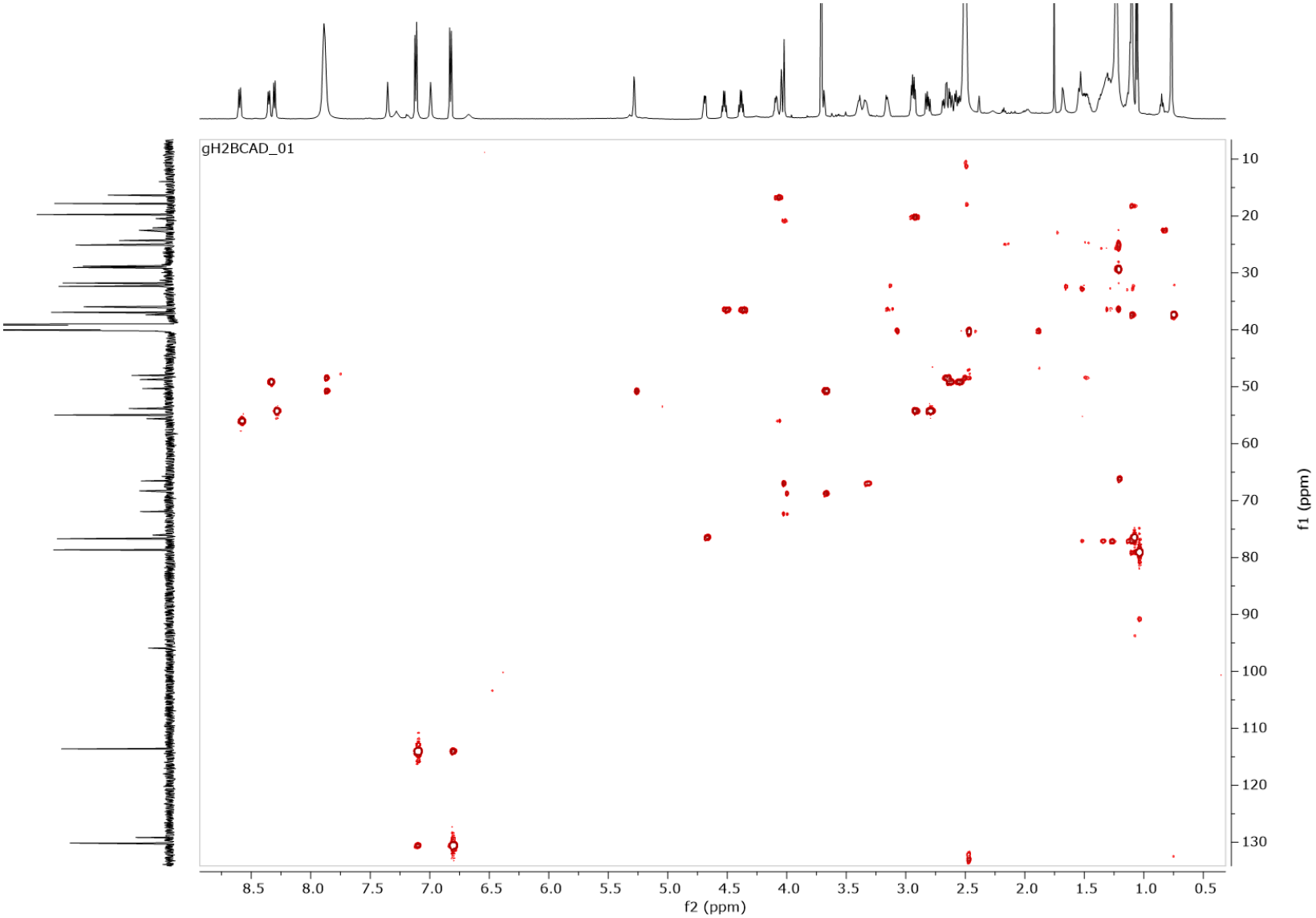


Figure S27. H2BC NMR spectrum of **2** in DMSO-d_6 (600 MHz).

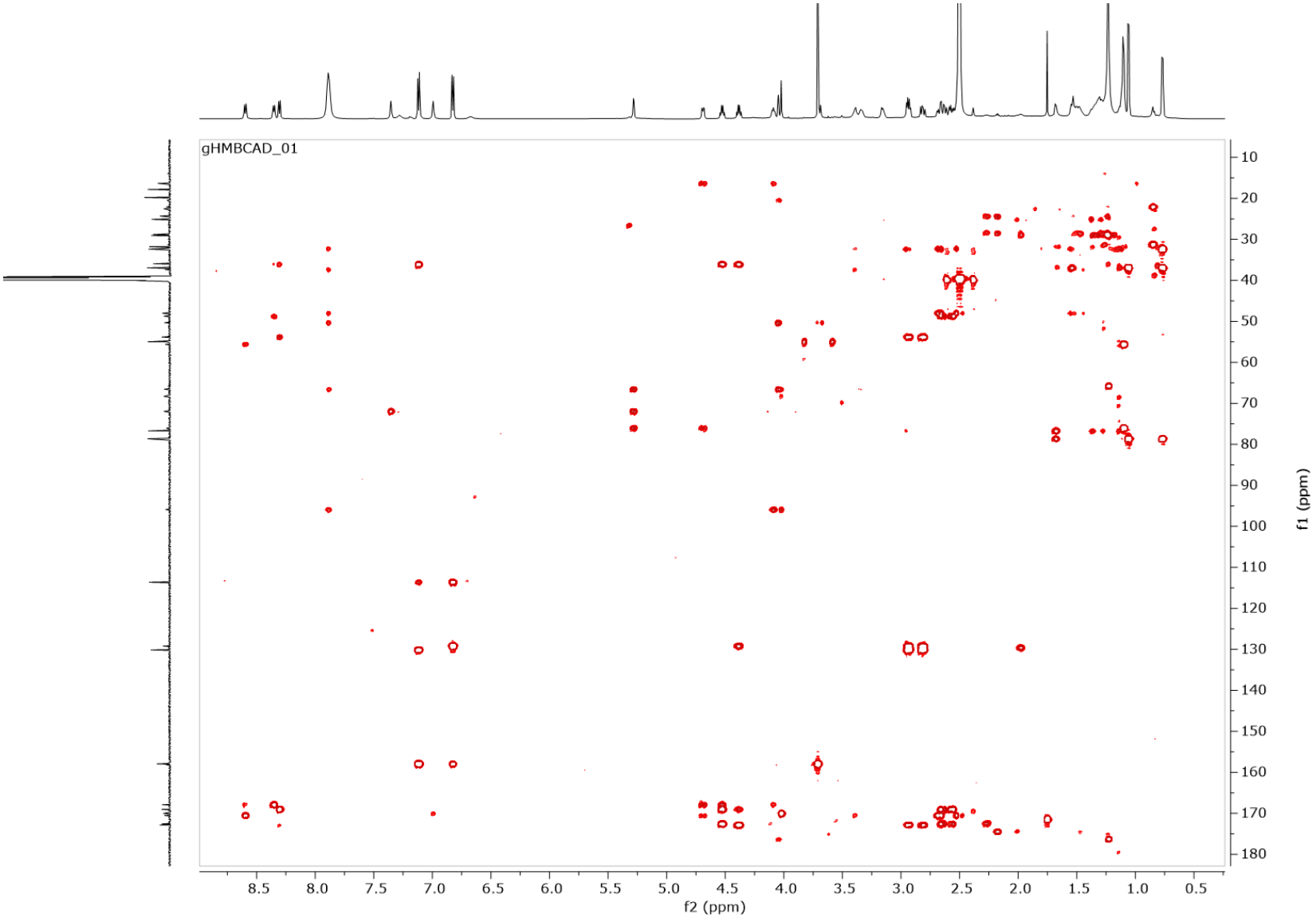


Figure S28. HMBC NMR spectrum of **2** in DMSO-d_6 (600 Mhz).

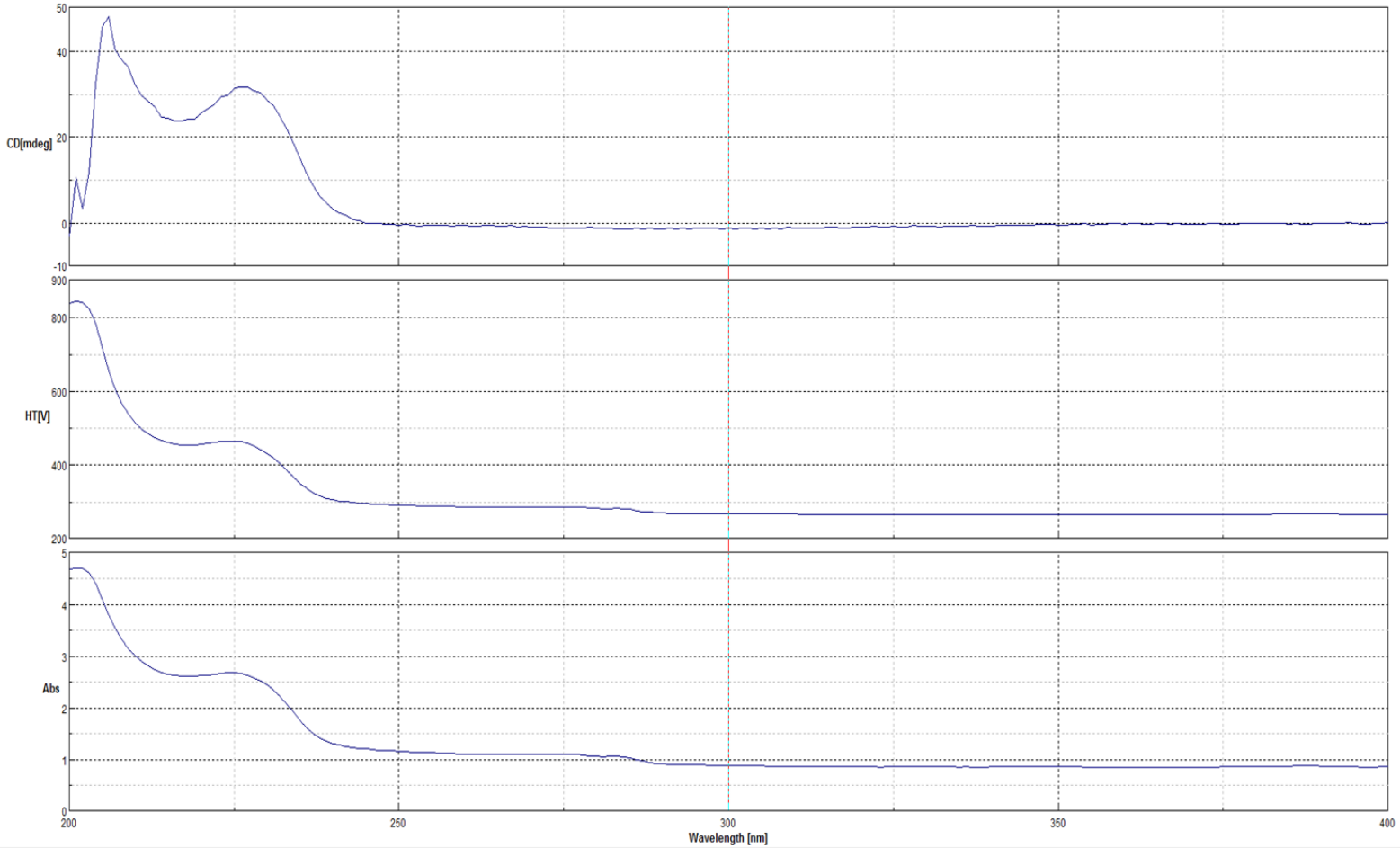
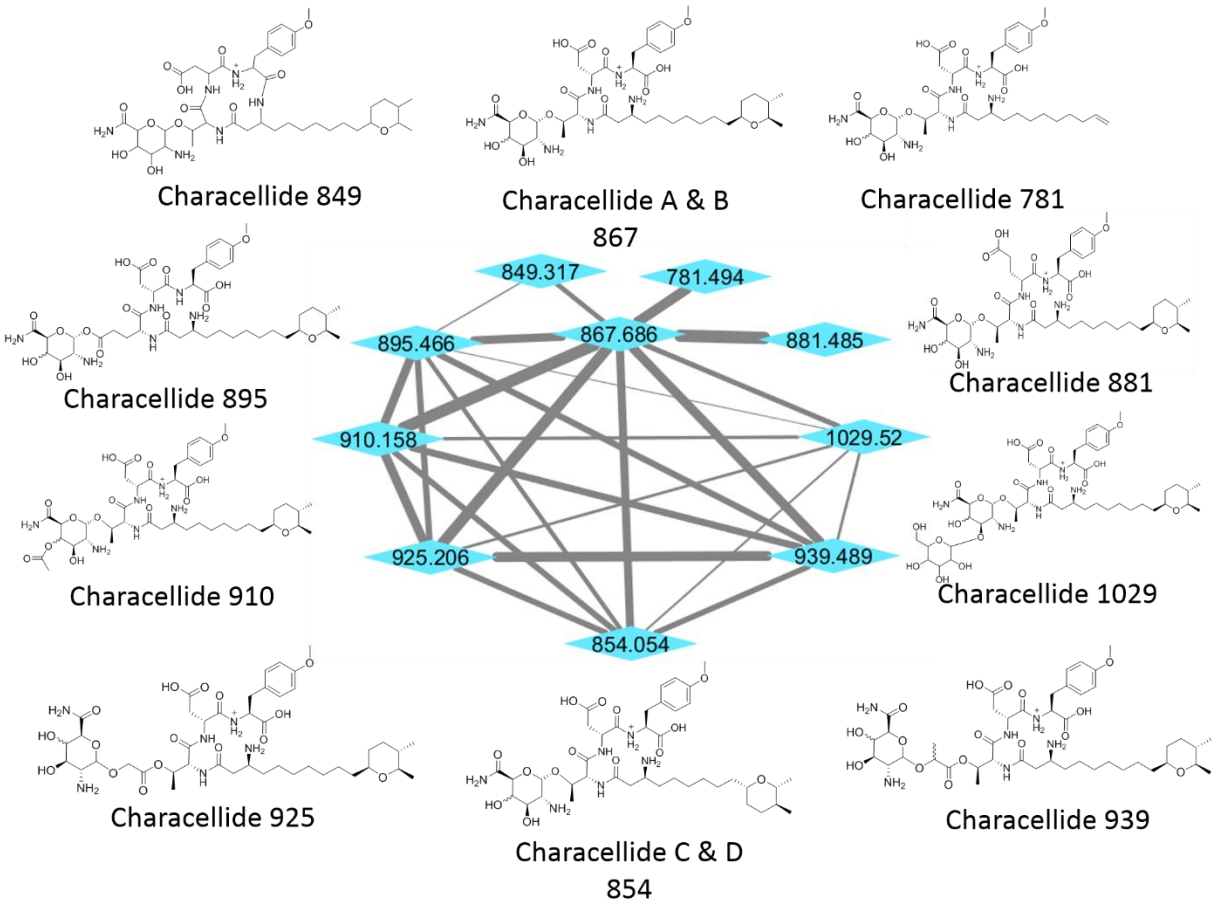


Figure S29. ECD and UV spectra of **2** at 0.2 mg/ml in MeOH.


Characellide 849
Characellide A & B
867
Characellide 781
849.317
781.494
895.466
867.686
881.485
Characellide 895
Characellide 881
910.158
1029.52
Characellide 910
Characellide 1029
925.206
939.489
854.054
Characellide 925
Characellide C & D
854
Characellide 939

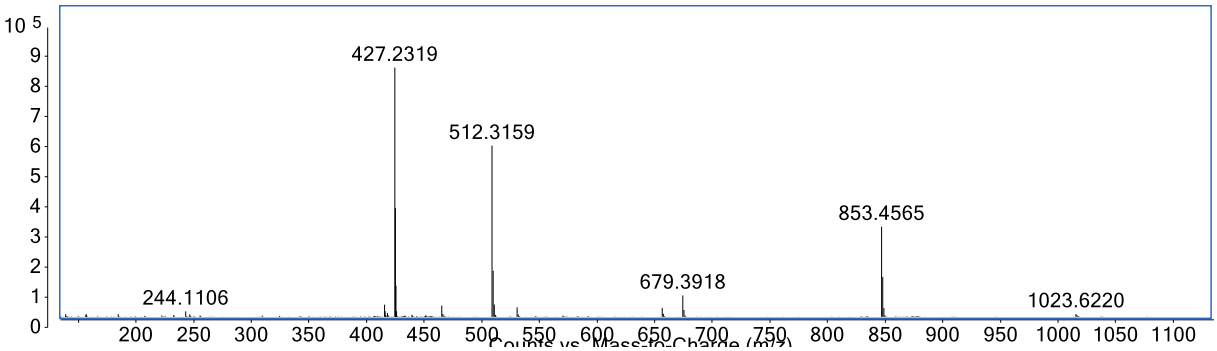


Figure S30. HRESIMS analysis of **3** and **4**.

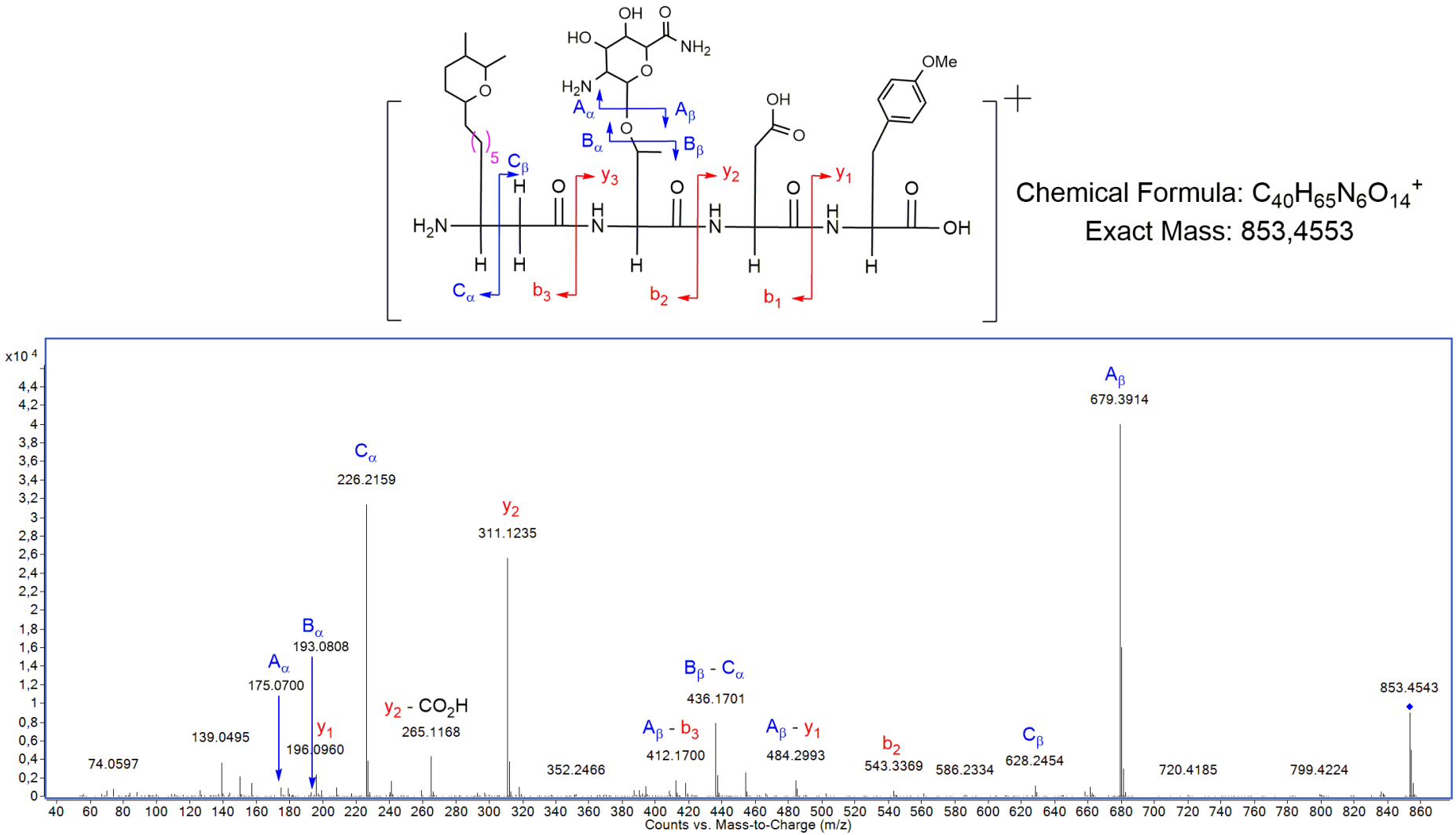


Figure S31. MS/MS analysis and fragmentation pattern of **3** and **4**.

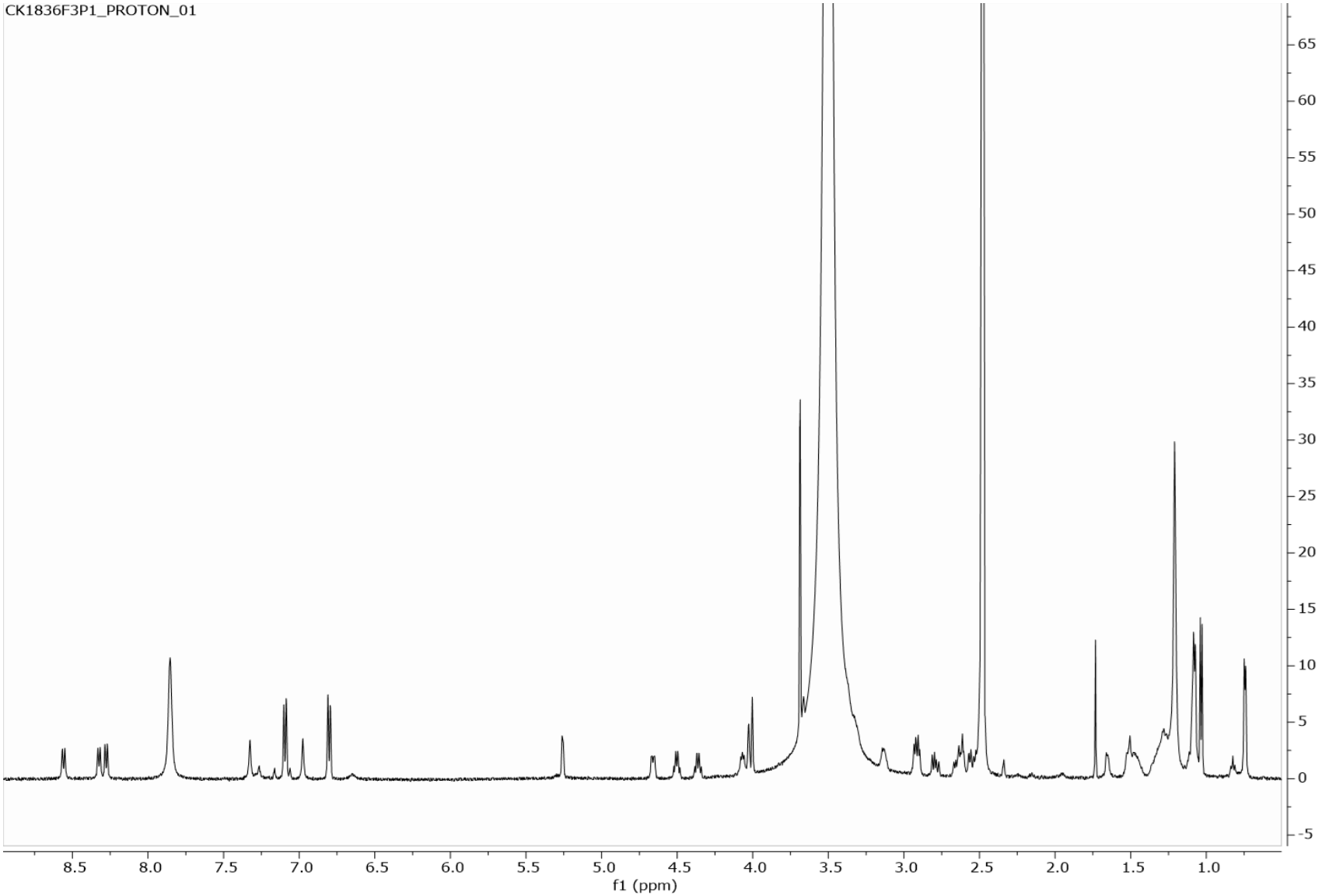


Figure S32. ^{1}H NMR spectrum of **3** and **4** in DMSO-d_6 (600 MHz).

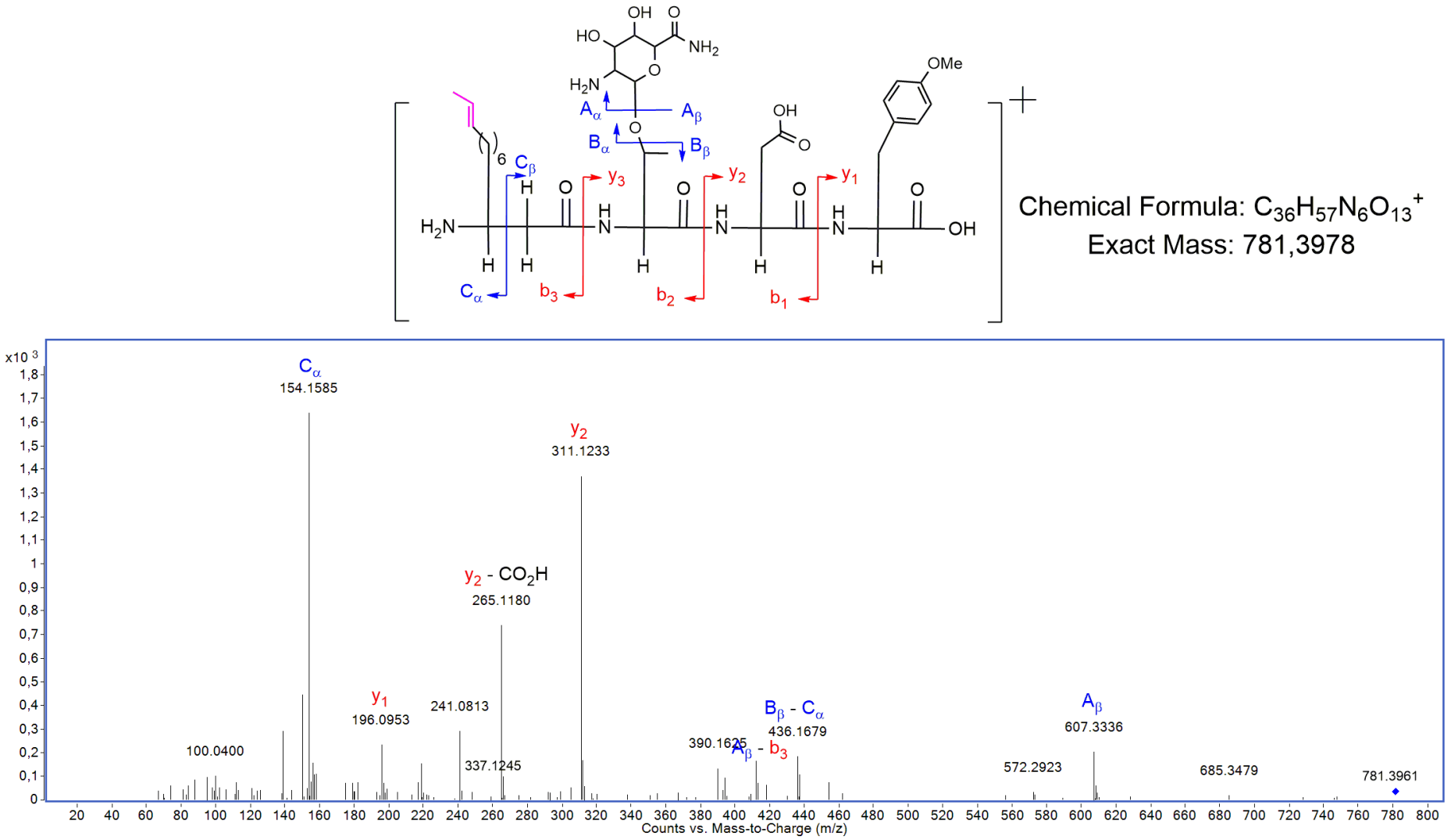


Figure S33. MS/MS analysis and fragmentation pattern of characellide 781.

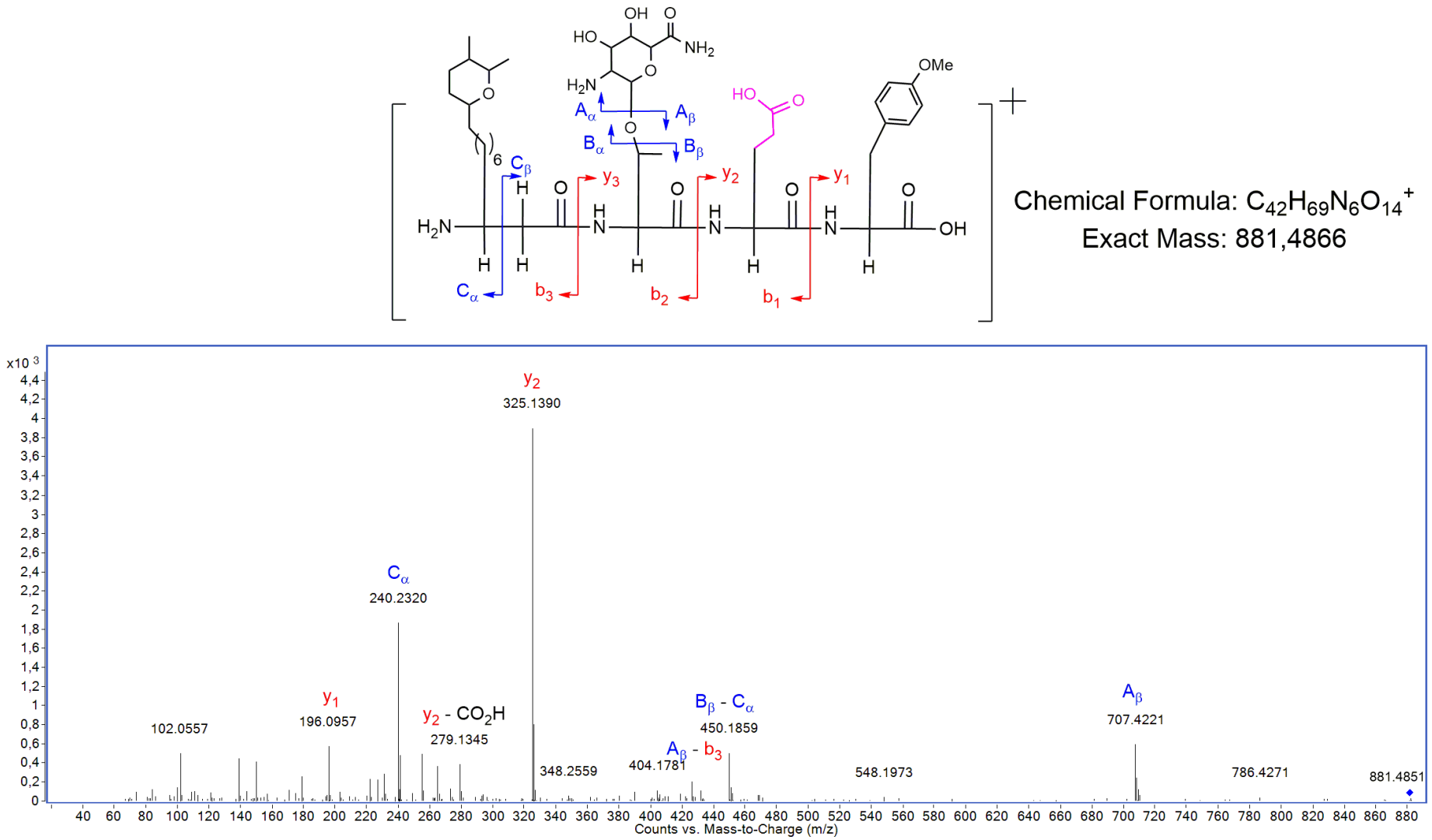


Figure S34. MS/MS analysis and fragmentation pattern of characellide 881.

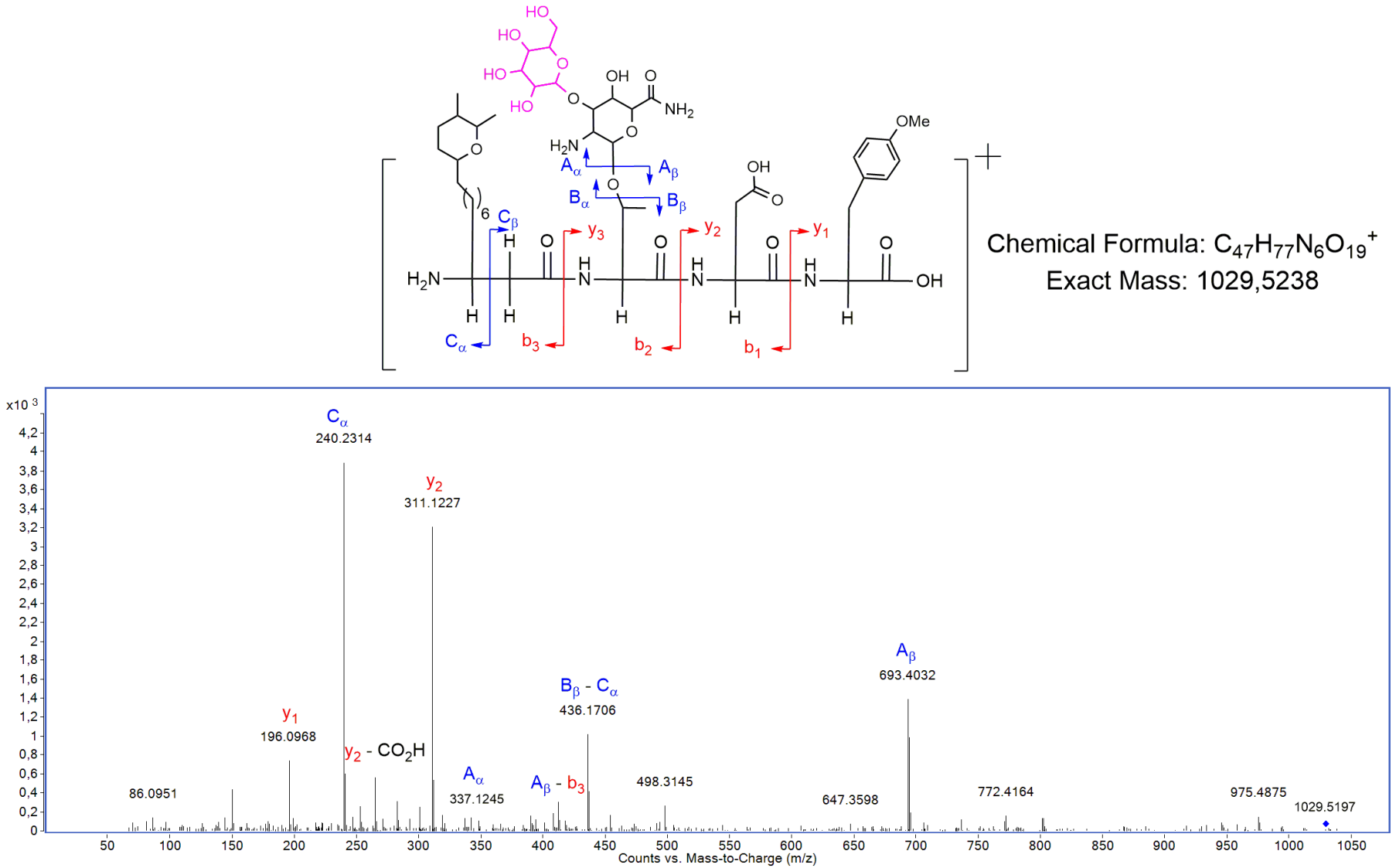


Figure S35. MS/MS analysis and fragmentation pattern of characellide 1029.

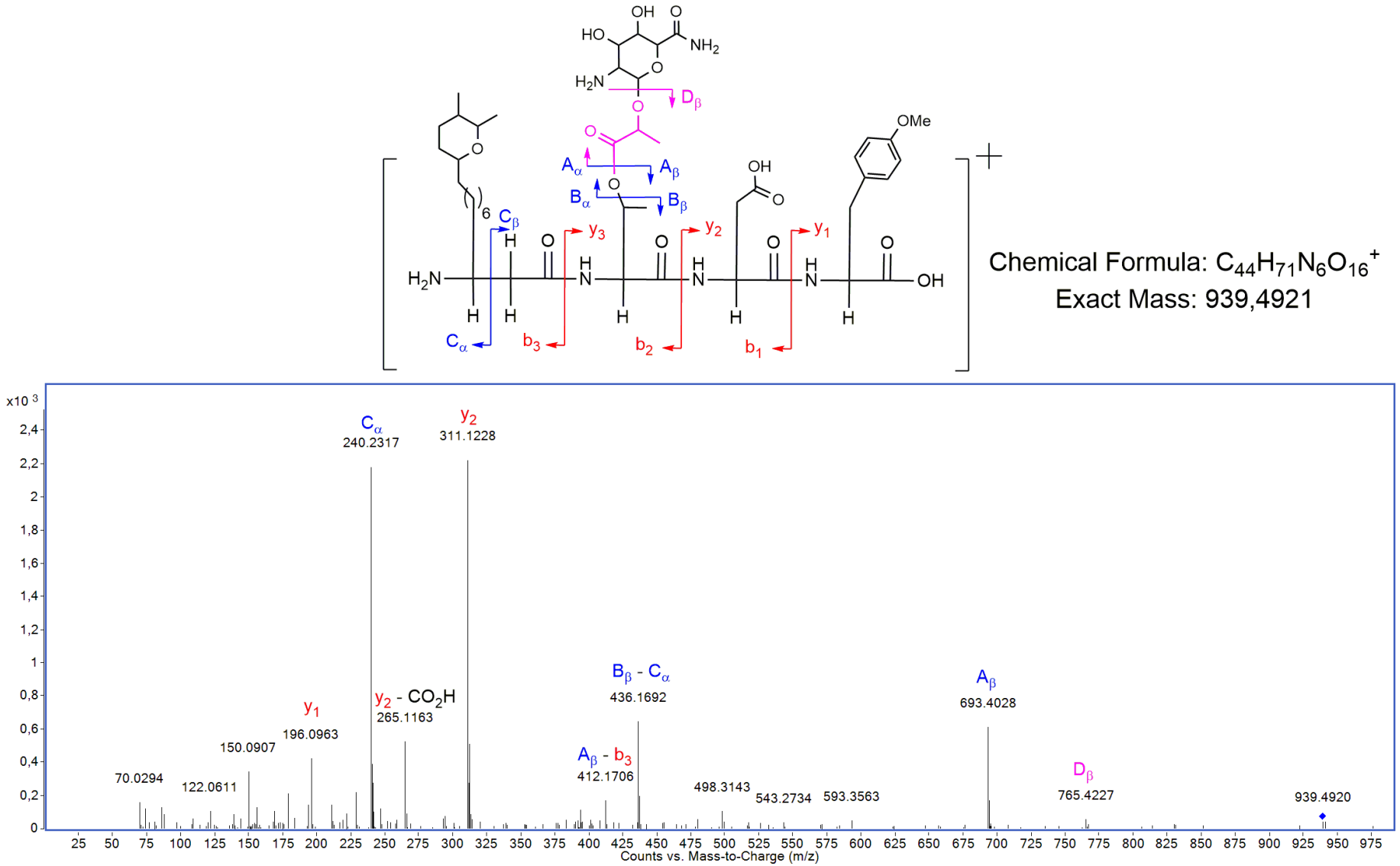


Figure S36. MS/MS analysis and fragmentation pattern of characellide 939.

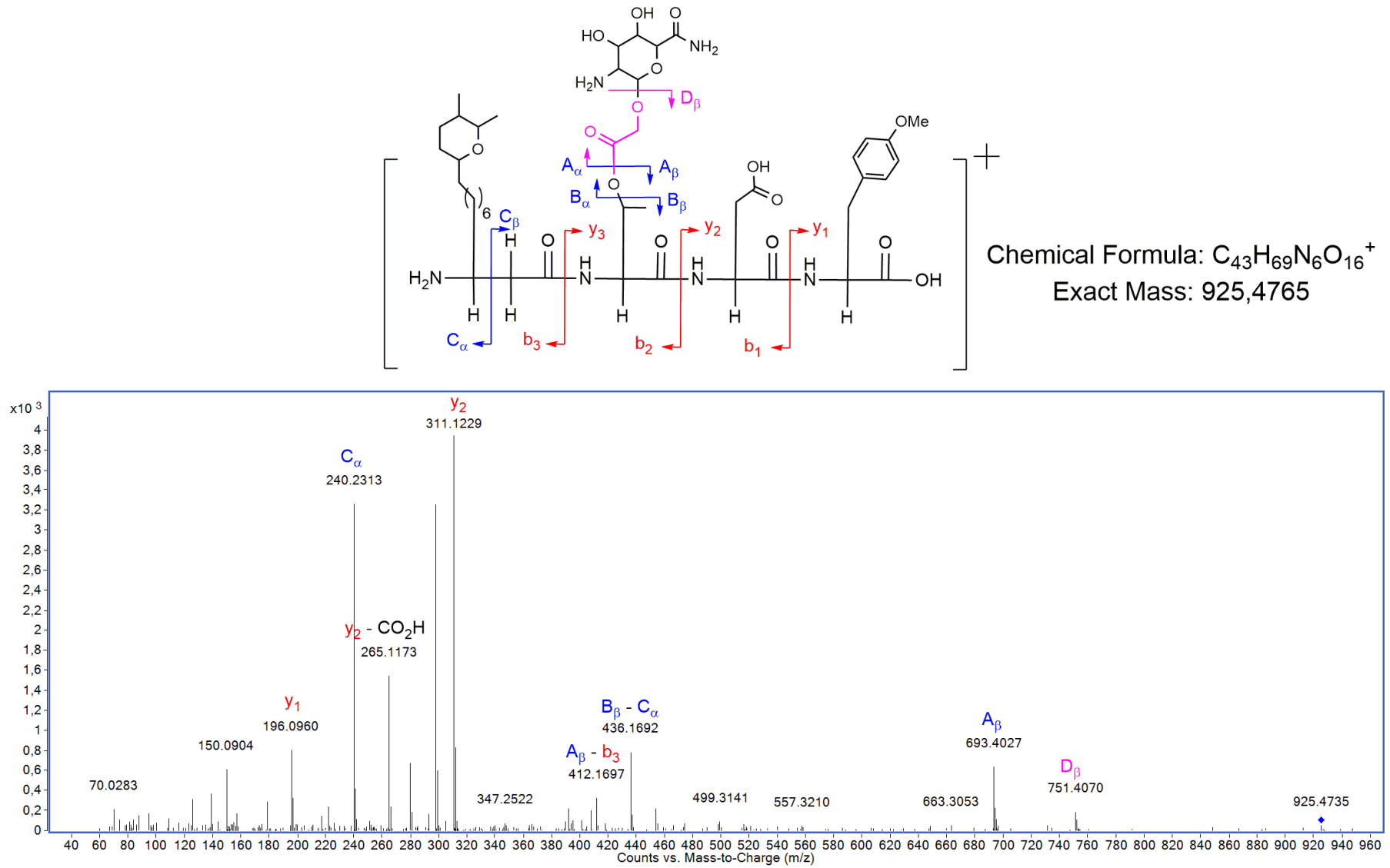
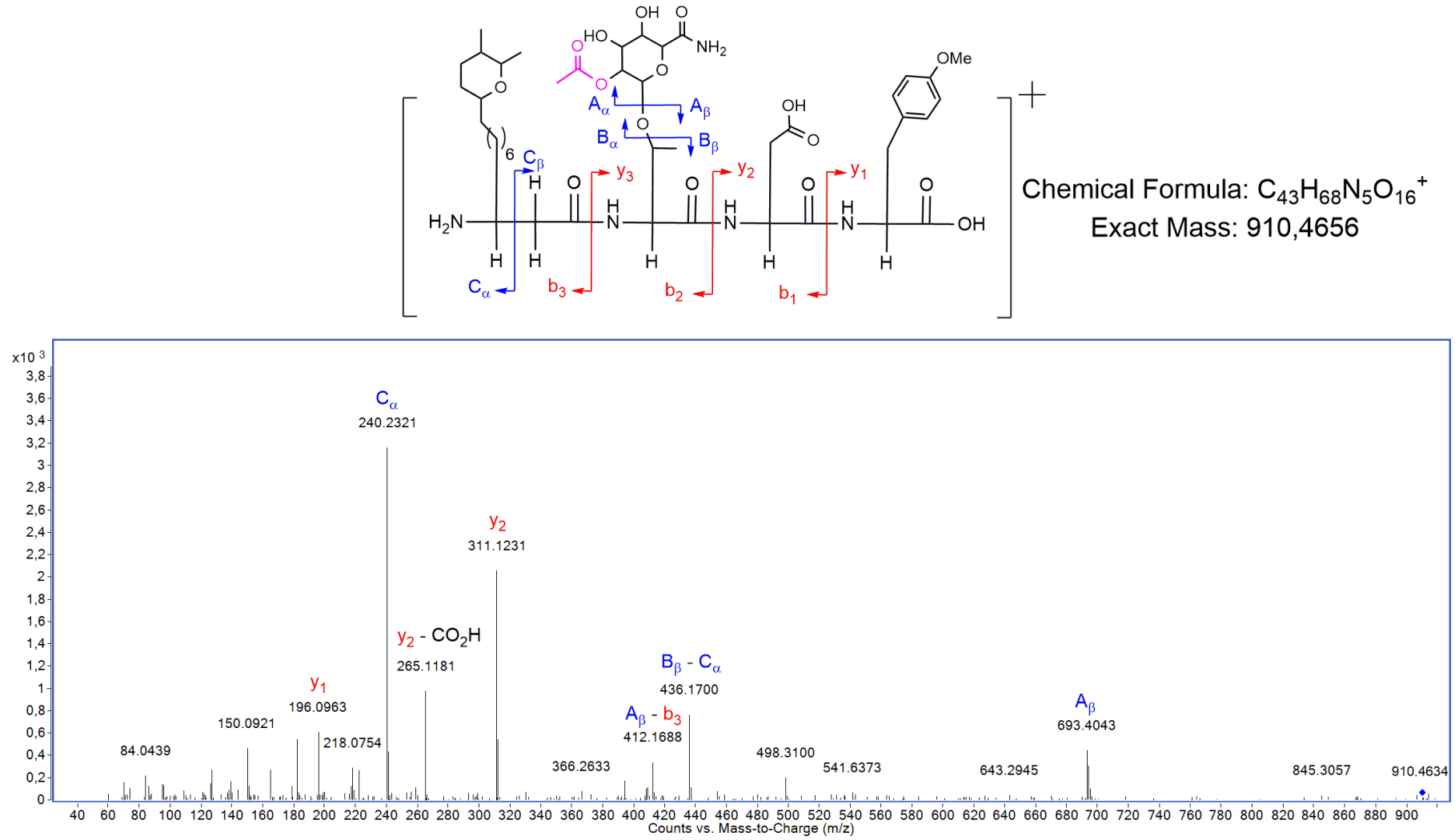


Figure S37. MS/MS analysis and fragmentation pattern of characellide 925.



FigureS38. MS/MS analysis and fragmentation pattern of characellide 910.

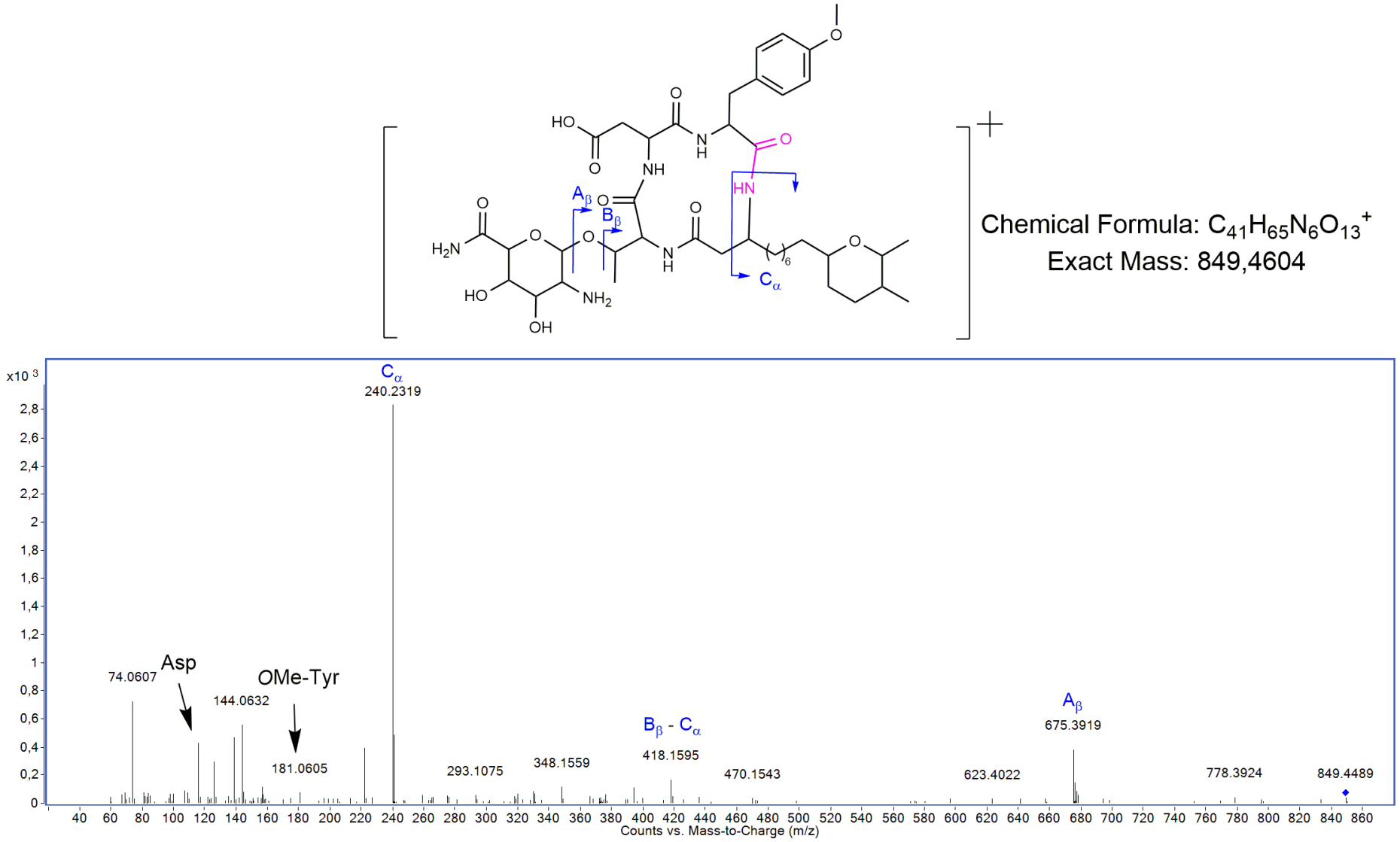


Figure S39. MS/MS analysis and fragmentation pattern of characellide 849.