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Supplementary Information For

Semi-Synthesis of an Evasin from Tick Saliva Reveals a Critical Role of Tyrosine Sulfation for Chemokine Binding and Inhibition

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This PDF file includes:

Table of Contents

Materials

Peptide grade N,N-dimethylformamide (DMF) was obtained from Labscan. Amino acids, coupling reagents and resins for Fmoc-solid-phase peptide synthesis (SPPS) were obtained from either Novabiochem or GL Biochem. SPPS was performed in polypropylene syringes equipped with Teflon filters, purchased from Torviq. Analytical reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on either a Waters Acquity UPLC system equipped with PDA eλ detector (λ = 210-400 nm), Sample Manager FAN and Quaternary Solvent Manager (H-Class) modules or a Waters System e2695 separations module with an Alliance series column heater at 40 °C and 2489 UV/VIS Detector, operating at 215 and 280 nm, and 2475 FLR Detector operating at excitation wavelength of 280 nm and emission wavelength of 347 nm. Proteins were analysed using an Xbridge BEH 5 µm, 2.1 x 150 mm wide-pore column (C-18) at a flow rate of 0.2 mL min^{-1} on the HPLC system or Waters Acquity UPLC BEH 1.7 µm 2.1 x 50 mm wide-pore column (C-18) at a flow rate of 0.6 mL min^{-1} on the UPLC system using a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) in a linear gradient as indicated. Peptides (N-terminal (sulfated) constructs) were analysed using a Waters Acquity UPLC BEH 1.7 µm 2.1 x 50 mm small pore column (C-18) at a flow rate of 0.6 mL min^{-1} on the UPLC system using a mobile phase of 0.1% trifluoroacetic acid in water (Solvent A) and 0.1% trifluoroacetic acid in acetonitrile (Solvent B) in a linear gradient as indicated. Analysis of the chromatograms was conducted using Empower 3 Pro software (2010) and retention times (R_t min) of pure peptides and proteins are reported with the gradients specified.

Preparative and semi-preparative RP-HPLC was performed using a Waters 2535 Quaternary Gradient system interfaced with a Waters 2489 UV/VIS Detector module operating at 214, 215 or 280 and a Waters Fraction Collector III. Preparative reversed phase HPLC was performed using Waters Sunfire C18 Column (5 µm, 30 x 150 mm) at a flow rate of 38 mL min^{-1}. Semi-preparative reversed-phase HPLC was performed using a Waters Xbridge Peptide BEH prep, C18 300 Å column (5 µm, 10 x 250 mm) at a flow rate of 4 mL min^{-1}. Proteins bearing free sulfated tyrosine residues were purified using a mobile phase of 10 mM NH_3 in H_2O (Solvent A – using a 30% NH_4OH solution) and 10 mM NH_3 in acetonitrile (Solvent B - using a 30% NH_4OH solution) using the indicated gradient. A mobile phase of 0.1% trifluoroacetic acid in water (Solvent A) and 0.1% trifluoroacetic acid in acetonitrile (Solvent B) were used in all other cases, using the linear gradients specified. After lyophilization, peptides/proteins were isolated as TFA or ammonia salts depending on the chromatographic eluent.

Liquid chromatography-mass spectrometry (LC-MS) was conducted on a Shimadzu UPLC-MS consisting of a LC-M20A pump and an SPD-M30A diode array detector coupled to a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode unless otherwise stated. Separations on the UPLC-MS system were performed using a Waters Acquity UPLC BEH 1.7 µm 2.1 x 50 mm (C-18) wide-pore column for proteins and the small-pore variant for peptides at a flow rate of 0.6 mL min^{-1}. Separations were done using a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 8 minutes.

Low-resolution mass spectra were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. Low resolution MALDI-TOF mass spectra were recorded on a Bruker Autoflex TM Speed MALDI-TOF mass spectrometer operating in linear, negative mode using a matrix of 10 mg/mL sinapinic acid in water/acetonitrile (1:1 v/v) with no TFA.

Fmoc-Strategy SPPS General Procedures

2-Chlorotrityl Chloride Resin Loading

2-Chlorotrityl Chloride Resin (0.7 mmol/g loading) was swollen in dry CH_2Cl_2 for 30 min, followed by washing with CH_2Cl_2 (10 x 3 mL). A solution of Fmoc-Thr(*t*Bu)-OH (2 eq.) and *i*Pr_2NEt (4 eq.) in CH_2Cl_2 (final concentration 0.1 M of amino acid) was added to the resin, which was shaken at room temperature for 16 hours. The resin was then washed with CH_2Cl_2 (5 x 3 mL), DMF (5 x 3 mL) and CH_2Cl_2 (5 x 3 mL) prior to capping *via* treatment with 17:2:1 v/v CH_2Cl_2:MeOH:*i*Pr_2NEt for 20 minutes at room temperature. The resin was then washed again with CH_2Cl_2 (5 x 3 mL) prior to determination of the estimated loading of the first amino acid.

Loading Estimation of the First Amino Acid

The resin was treated with piperidine/DMF (1:4, 3 mL, 3 x 5 min) and then washed with DMF (5 x 3 mL), CH_2Cl_2 (5 x 3 mL) and DMF (5 x 3 mL). The combined deprotection solutions were then made up to 10 mL with fresh piperidine/DMF (1:4). The solution was diluted 50-100 fold with fresh piperidine/DMF (1:4) and the UV absorbance of the piperidine-fulvene adduct measured (λ = 301 nm, ε = 7800 $M^{-1}cm^{-1}$) to estimate the amount of amino acid loaded onto the resin.

Peptide Assembly via Iterative SPPS

Peptides were assembled by stepwise Fmoc-SPPS on a 0.07-0.1 mmol scale. Coupling of each amino acid (4 eq.) was achieved using PyBOP (4 eq.) and NMM (8 eq.) in DMF (0.1 M) over 1 hour at room temperature. Capping steps were introduced after all steps unless otherwise specified using acetic anhydride/pyridine (1:9, v/v, 3 mL) for 3 minutes at room temperature. Fmoc-deprotection steps were performed by treatment with 20% piperidine in DMF at room temperature (3 x 5 min). Following each coupling, capping or deprotection step, the resin was washed with DMF (5 x 3 mL), CH_2Cl_2 (5 x 3 mL) and DMF (5 x 3 mL).

Coupling of Fmoc-Tyr(SO_3nP)-OH

A solution of amino acid (2.0 eq.), DIC (2.0 eq.) and HOAt (2.0 eq.) in DMF (0.1 M) was added to the resin and shaken at room temperature for 16 hours. The resin was then washed with DMF (5 x 3 mL), CH_2Cl_2 (5 x 3 mL) and DMF (5 x 3 mL) and a capping step was performed as described above. Synthesis of the desired fragment was then completed using iterative Fmoc-SPPS.

Peptide Assembly via Iterative Fully Automated SPPS

Peptides were assembled by stepwise Fmoc-SPPS on a Protein Technologies Symphony peptide synthesiser, operating on a 0.07-0.1 mmol scale. Activation of entering Fmoc-protected amino acids (0.3 M solution in DMF) was performed using 0.3 M Oxyma in DMF/ 0.3 M DIC in DMF (1:1:1 molar ratio), with an 8-equivalent excess over the initial resin loading. Coupling steps were performed for 45 mins at 25 °C. Capping steps were introduced after each coupling step unless otherwise specified and performed by treatment with a 0.3 M acetic anhydride/ 0.3 M *i*Pr_2NEt solution in DMF (1 x 3 min). Fmoc-deprotection steps were performed by treatment of the resin with a 20 vol.% piperidine solution in DMF at room temperature (2 x 3 min). Following each coupling, capping or deprotection step, the resin was washed with DMF (4 x 30 sec). Upon complete assembly of the peptide chain, the resin was washed with CH_2Cl_2 (5 x 30 sec).

Cleavage from the Resin and thioesterification

The resin-bound peptide was washed with CH_2Cl_2 (3 x 5 mL) before treating with a HFIP/CH_2Cl_2 mixture (3:7) and allowed to shake at room temperature for 2 hours. At this point, the resin was filtered and washed with fresh cleavage cocktail. The combined cleavage solutions were concentrated under nitrogen flow and dried in vacuo to afford the crude sidechain protected peptide.

Solution-Phase Thioesterification

The crude, sidechain protected peptide was dissolved in DMF followed by the addition of 5 equivalents of *i*Pr_2NEt and 30 equivalents (both compared to estimated loading on resin) of ethyl-3-mercaptopropionate. Subsequently 5 equivalents of PyBOP in DMF (ice-cold) was added and the reaction was left for 3 hours at -30 °C under an argon atmosphere. The solution was concentrated under nitrogen flow.

Cleavage of the protecting groups and work-up

The thioesterified peptide was treated with an ice-cold TFA, TIS, water mixture (90:5:5, 5 mL) and stirred at room temperature for 2 hours. The cleavage solution was then concentrated in vacuo to ~1 mL volume and ice-cold diethyl ether (40 mL) was added to precipitate the crude peptide. The precipitate was then collected *via* centrifugation and washed with diethyl ether to remove any remaining scavengers. Residual diethyl ether was removed under gentle nitrogen flow and the crude peptide dissolved in 0.1% TFA aqueous buffer (with minimal addition of acetonitrile to aid dissolution, if necessary). The crude peptide was analysed by LC-MS (ESI) and purified by RP-HPLC.

Mammalian cell expression of ACA-01-Myc-His6

Suspension-adapted HEK Expi293F™ cells (Thermo Fisher Scientific, Waltham, MA, USA) were grown to a density of $1.8x10^6$ cells mL^{-1} in Expi293™ Expression Medium (Thermo Fisher Scientific). ACA-01 with a C-terminal Myc-His6 tag was cloned into a pSecTag2a expression vector and subsequently used for transfection of the cells. For this purpose, 96 µg of DNA was first diluted in 5.1 mL of PBS and vortexed briefly, followed by the addition of 386 ug of polyethylenimine (PEI) (Polysciences, Warrington, PA, USA). The mixture was vortexed again and incubated at room temperature for 20 min before adding to 48 mL of HEK Expi293F™ cell culture. The cells were incubated for 68 h at 37 °C with 5% CO_2 and horizontal orbital shaking at 130 rpm. Media, containing the secreted protein, was harvested and subject to immobilized nickel ion affinity chromatography using a 1 mL HisTrap column, equilibrated in 10 mM HEPES, 150 mM NaCl, pH 7.5. The bound protein was eluted using 250 mM imidazole. The protein containing elution fractions were pooled and concentrated to a small volume. The concentrated sample was loaded on a Superdex 75 HiLoad 16/600 column (size exclusion chromatography) and eluted using 10 mM HEPES, 150 mM NaCl, pH 7.5. Protein was pooled, aliquoted, snap frozen and stored at -80 °C. Protein purification was analysed by SDS-PAGE and monitoring UV absorbance at 280 nm.

Identification of *in vivo* sulfation via LCMS/MS

Protein preparation and digestion were performed as described previously with modifications(1). Briefly, 10 µg of purified ACA-01 protein expressed in HEK293 cells was added to 25 µl of buffer containing 0.1 M Tris-HCl pH 8.0 and 5 mM dithiothreitol (DTT) before being heated to 37 °C for 30 minutes to reduce the disulfide bonds in the protein. Chloroacetamide was then added to 20 mM final concentration and heated to 37 °C for 30 minutes to alkylate cysteine residues and quench remaining DTT. The protein was digested to peptides by the addition of either 200 ng trypsin, or chymotrypsin, followed by incubation at 37 °C for 16 h. For peptide clean-up C18 StageTips containing two layers of 3M Empore C18 disks (Cat No. 66883-U, Sigma) were used in the Spin96 device. Each tip was wetted with 100 µL of 100% acetonitrile and centrifuged at 1,000 x g for 1 minute. Following wetting, each StageTip was equilibrated with 100 µL of H_2O (neutral pH), with centrifugation for each at 1,000 x g for 3 minutes. Each StageTip was then loaded with the digested peptides (neutral pH). The peptides were washed twice with 100 µL of water (neutral pH). To elute the peptides, 100 µL of 50% acetonitrile in H_2O (neutral pH) was added to each tip and centrifuged as above for 5 minutes. Peptides were dried using a GeneVac EZ-2 using the HPLC setting at 35 °C for 1.25 h. Dried peptides were resuspended in 30 µL of H_2O (neutral pH) and stored at 4 °C until analysed by LC-MS/MS.

Using a Thermo Fisher Dionex RSLCnano uHPLC, peptides in water (injection volume 3 µL) were directly injected onto a 45 cm x 75 µm C18 (Dr. Maisch, Ammerbuch, Germany, 1.9 µm) fused silica analytical column with a ~10 µm pulled tip, coupled online to a nanospray ESI source. Peptides were resolved over gradient from 5% acetonitrile to 40% acetonitrile over 45 minutes with a flow rate of 300 nL min^{-1}. Formic acid at 0.1% final concentration was included in both buffers used to form the gradient. Peptides were ionized by electrospray ionization at 2.3 kV and the ion transfer capillary temperature was set to 150 °C to minimize artefactual loss of labile sulfate groups during peptide ion transfer. Tandem mass spectrometry analysis was carried out on a Fusion Lumos mass spectrometer (ThermoFisher) using either EThcD, or HCD fragmentation. RAW MS data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD016778, username: reviewer51373@ebi.ac.uk, password: 2rBVtbs9. RAW data were analysed using Byonic (Protein Metrics) and the search output has also been uploaded to the ProteomeXchange Consortium under the same identifier. Peptide and protein level identification were both set to a false discovery rate of 1% using a target- decoy based strategy. The database supplied to the search engine for peptide identifications contained a subset of secreted human

proteins from the UniProt database(2) and the full-length ACA-01 protein sequence. Mass tolerance was set to 7 ppm for precursor ions and MS/MS mass tolerance was 20 ppm. Enzyme specificity was set to semi-specific N-ragged digestion with either chymotrypsin (cleavage C-terminal to FYWLM) or trypsin (cleavage C-terminal to KR), having a maximum of 2 missed cleavages permitted. Oxidation of Met (common2), pyro-Glu (with peptide N-term Gln or Glu) (rare1), sulfation of tyrosine (common2) and protein N-terminal acetylation (rare1) were set as variable modifications. Two common modifications and one rare modification were allowed per peptide spectral match. Carbamidomethyl on Cys was searched as a fixed modification. Extracted ion chromatograms were generated using Qual Browser (Thermo) with a 5 ppm error tolerance.

Bacterial expression of ACA-01 (17-97) (10)

A synthetic gene encoding N-terminally truncated ACA-01 (17-97) from *Amblyoma cajenennse* fused to an N-terminal 6xHis-SUMO tag (**10**) was subcloned into the NcoI and XhoI restriction sites of the bacterial expression plasmid pET28a by Genscript (Piscataway, NJ, USA); codon usage was optimized for protein production in *Escherichia coli.* BL21(DE3) *E. coli* cells transformed with the expression plasmid were grown overnight, shaking at 37 °C, in 10 mL of LB medium supplemented with kanamycin sulfate (25 μg mL^{-1}). The overnight culture (10 mL) was used to inoculate 1 L of the same medium, which was grown, shaking at 37 °C, to mid-log phase (OD_{600} of ~0.6-0.8). Protein expression was then induced by addition of isopropyl β-d-1-thiogalactopyranoside (IPTG; 1 mM) and the culture continued growing overnight at 25 °C. Cells were harvested by centrifugation at 5000 rpm, 4 °C for 20 minutes using Thermo Scientific RC BIOS. Subsequently the pellet was lysed in 25 mL of 1 M NaCl, 10 mM Tris HCl pH 8.0, 1 x cOmplete EDTA-free protease inhibitor, 10 μg mL^{-1} DNase I, 10 μg mL^{-1} RNase, and 100 μg mL^{-1} lysozyme followed by homogenizing using Emulsiflex C3 homogenizer. Centrifugation using Thermo Scientific Sorvall RC 6+ separated soluble from insoluble fraction. The protein was initially purified on ÄKTA pure 25 with a HiTrap TALON Crude column at a flow rate of 5 mL min^{-1} (elution buffer: 500 mM NaCl, 10 mM Tris HCl, 150 mM imidazole, pH 8.0). The 6xHis-SUMO tag was cleaved from the ACA-01(17-97) by Ulp1 protease (0.5 mg – pFGET19_Ulp1 was a gift from Hideo Iwai; Addgene plasmid #64697; http://n2t.net/addgene:64697; RRID:Addgene_64697) during overnight dialysis to remove imidazole. The cleaved protein (**5**) was separated from the 6xHis-SUMO tag and Ulp1 protease by reverse HiTrap TALON Crude on same system. The protein was further desalted using RP-HPLC (isocratic gradient 50% B, 0.1% TFA, H_2O/MeCN), yielding the N-terminally truncated protein as a fluffy white solid after lyophilization (3-4 mg per litre culture).

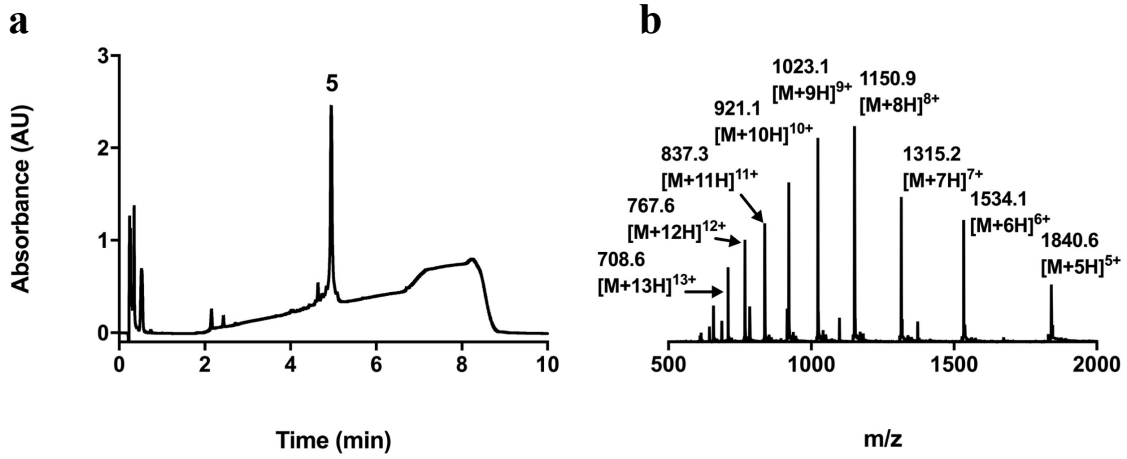
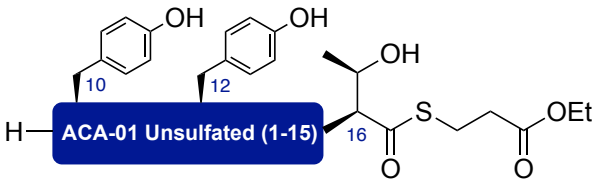


Figure S1. Analytical HPLC trace (**a**) and mass spectrum (**b***)* of purified protein **5**. Analytical HPLC Rt 5.00 min (0 to 50% over 5 min, 0.1% TFA, λ = 214 nm). Calculated Mass $[M+5H]^{5+}$: 1840.9, $[M+6H]^{6+}$:1534.2, $[M+7H]^{7+}$: 1315.2, $[M+8H]^{8+}$: 1150.9, $[M+9H]^{9+}$: 1023.2, $[M+10H]^{10+}$: 921.0, $[M+11H]^{11+}$: 837.3, $[M+12H]^{12+}$: 767.6, $[M+13H]^{13+}$: 708.7, Mass Found (ESI) 1840.3 $[M+5H]^{5+}$, 1533.8 $[M+6H]^{6+}$, 1315.0 $[M+7H]^{7+}$, 1150.8 $[M+8H]^{8+}$, 1023.1 $[M+9H]^{9+}$, 921.0 $[M+10H]^{10+}$, 837.3 $[M+11H]^{11+}$, 767.7 $[M+12H]^{12+}$, 708.7 $[M+13H]^{13+}$.

Synthesis of sulfated ACA-01 thioester fragments

ACA-01 Unsulfated Thioester Fragment synthesis



H-ENTQQEEQDYDYGTDT-S(CH$_2$)$_2$COOEt (6)

Peptide thioester **6** was prepared by loading of Fmoc-Thr(*t*Bu)-OH to 2-chlorotrityl chloride resin (100 µmol) and extended *via* Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment and cleavage of resin using HFIP and CH_2Cl_2, thioesterification in solution with 3-mercaptopropionate (380 µL, 30 eq.) was completed as outlined in the general procedures. Cleavage of all the acid labile protecting groups was achieved *via* treatment of the crude peptide thioester with a solution of TFA/TIS/H_2O (90:5:5 v/v/v, 5 mL) for 2 hours at room temperature. The cleavage solution was then concentrated under nitrogen flow and the crude thioester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 40% B over 50 min, 0.1% TFA) afforded **6** as a fluffy white solid after lyophilisation (51 mg, 25% yield).

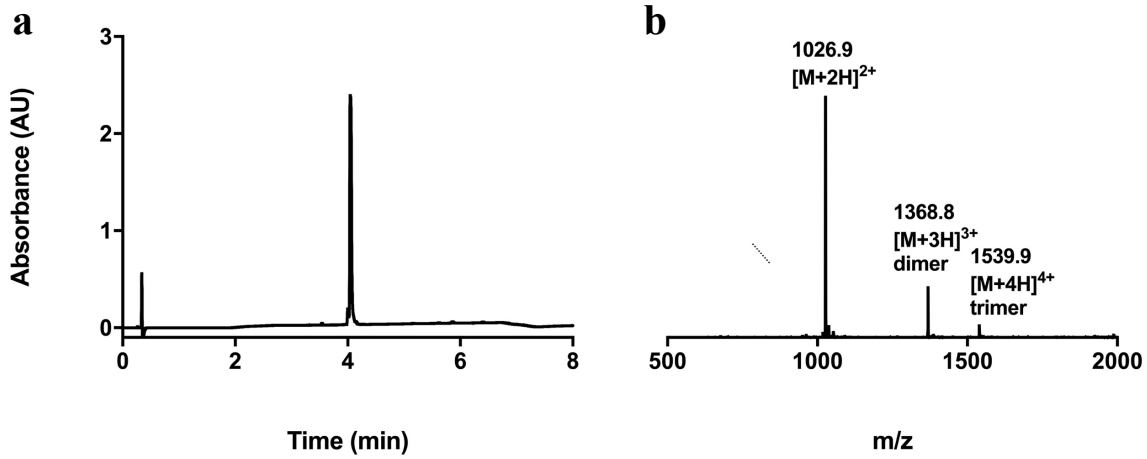
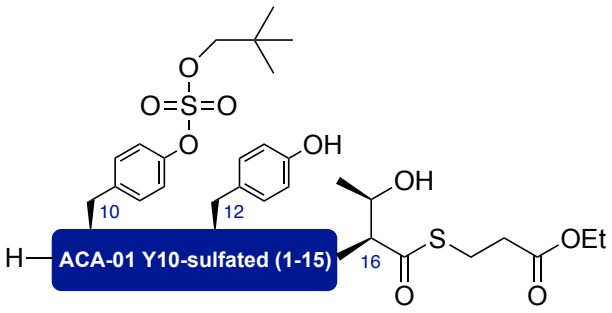


Figure S2. Analytical HPLC trace (**a**) and mass spectrum (**b***)* of purified peptide **6** thioester. Analytical HPLC Rt 4.03 min (0 to 50% over 5 min, 0.1% TFA, λ = 214 nm). Calculated Mass $[M+2H]^{2+}$: 1026.4, $[M+3H]^{3+}$ (dimer): 1368.2, $[M+4H]^{4+}$ (trimer): 1539.0, Mass Found (ESI) 1026.9 $[M+2H]^{2+}$, 1368.8 $[M+3H]^{3+}$ (dimer), 1539.9 $[M+4H]^{4+}$ (trimer).

ACA-01 Tyr10-sulfated Thioester Fragment Synthesis



H-ENTQQEEQDsYDYGTDT-S(CH_2)$_2$COOEt (7)

Peptide thioester **7** was prepared by loading of Fmoc-Thr(*t*Bu)-OH to 2-chlorotrityl chloride resin (60 µmol) and extended *via* Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment and cleavage of resin using HFIP and CH_2Cl_2, thioesterification in solution with 3-mercaptopropionate (228 µL, 30 eq.) was completed as outlined in the general procedures. Cleavage of all the acid labile protecting groups was achieved *via* treatment of the crude peptide thioester with a solution of TFA/TIS/H_2O (90:5:5 v/v/v, 5 mL) for 2 hours at room temperature. The cleavage solution was then concentrated under nitrogen flow and the crude thioester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 40% B over 50 min, 0.1% TFA) afforded **7** as a fluffy white solid after lyophilisation (13.9 mg, 11% yield).

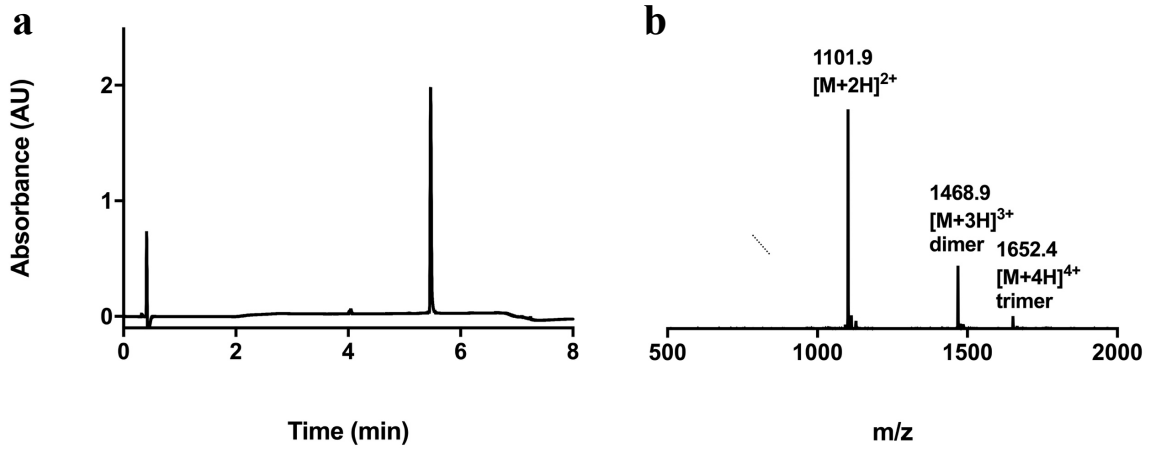
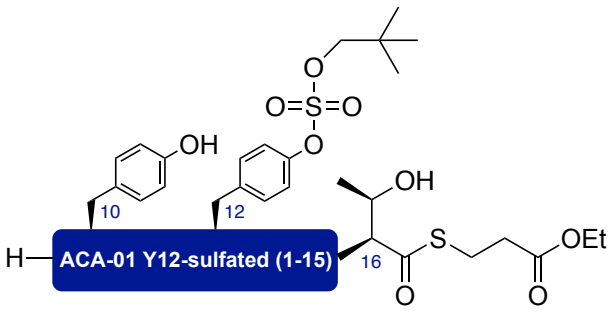


Figure S3. Analytical HPLC trace (**a**) and mass spectrum (**b***)* of purified peptide **7** thioester. Analytical HPLC Rt 5.45 min (0 to 50% over 5 min, 0.1% TFA, λ = 214 nm). Calculated Mass $[M+2H]^{2+}$: 1101.4, $[M+3H]^{3+}$ (dimer): 1468.1, $[M+4H]^{4+}$ (trimer): 1651.5, Mass Found (ESI) 1101.9 $[M+2H]^{2+}$, 1468.9 $[M+3H]^{3+}$ (dimer), 1652.4 $[M+4H]^{4+}$ (trimer).

ACA-01 Tyr12-sulfated Thioester Fragment Synthesis



H-ENTQQEEQDYDsYGTDT-S(CH$_2$)$_2$COOEt (8)

Peptide thioester **8** was prepared by loading of Fmoc-Thr(*t*Bu)-OH to 2-chlorotrityl chloride resin (60 µmol) and extended *via* Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment and cleavage of resin using HFIP and CH_2Cl_2, thioesterification in solution with 3-mercaptopropionate (228 µL, 30 eq.) was completed as outlined in the general procedures. Cleavage of all the acid labile protecting groups was achieved *via* treatment of the crude peptide thioester with a solution of TFA/TIS/H_2O (90:5:5 v/v/v, 5 mL) for 2 hours at room temperature. The cleavage solution was then concentrated under nitrogen flow and the crude thioester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 40% B over 50 min, 0.1% TFA) afforded **8** as a fluffy white solid after lyophilisation (15.7 mg, 12% yield).

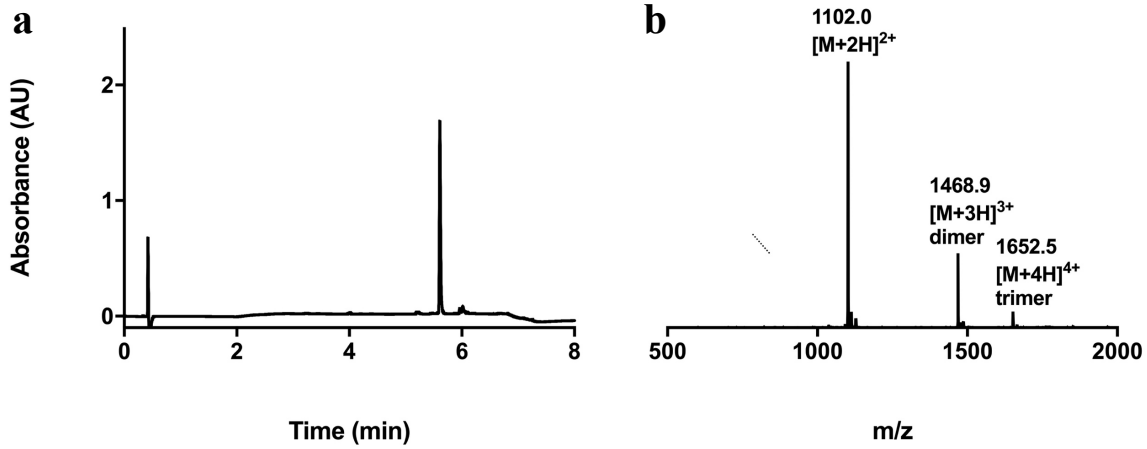
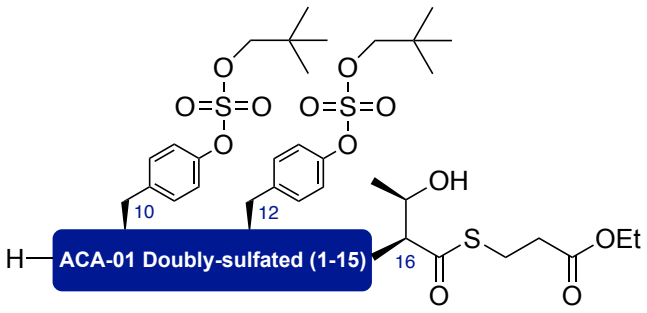


Figure S4. Analytical HPLC trace (**a**) and mass spectrum (**b***)* of purified peptide **8** thioester. Analytical HPLC Rt 5.59 min (0 to 50% over 5 min, 0.1% TFA, λ = 214 nm). Calculated Mass $[M+2H]^{2+}$: 1101.4, $[M+3H]^{3+}$ (dimer): 1468.1, $[M+4H]^{4+}$ (trimer): 1651.5, Mass Found (ESI) 1102.0 $[M+2H]^{2+}$, 1468.9 $[M+3H]^{3+}$ (dimer), 1652.5 $[M+4H]^{4+}$ (trimer).

ACA-01 Tyr10+Tyr12-sulfated Thioester Fragment Synthesis



Peptide thioester **9** was prepared by loading of Fmoc-Thr(*t*Bu)-OH to 2-chlorotrityl chloride resin (60 µmol) and extended *via* Fmoc-strategy SPPS, through a combination of automated and manual synthesis, with the N-terminal amino acid coupled as a Boc-protected amino acid. Upon completion of the resin-bound peptide fragment and cleavage of resin using HFIP and CH_2Cl_2, thioesterification in solution with 3-mercaptopropionate (228 µL, 30 eq.) was completed as outlined in the general procedures. Cleavage of all the acid labile protecting groups was achieved *via* treatment of the crude peptide thioester with a solution of TFA/TIS/H_2O (90:5:5 v/v/v, 5 mL) for 2 hours at room temperature. The cleavage solution was then concentrated under nitrogen flow and the crude thioester precipitated from ice-cold diethyl ether. Purification of the crude peptide by preparative RP-HPLC (0 to 60% B over 60 min, 0.1% TFA) afforded **9** as a fluffy white solid after lyophilisation (6.3 mg, 4% yield).

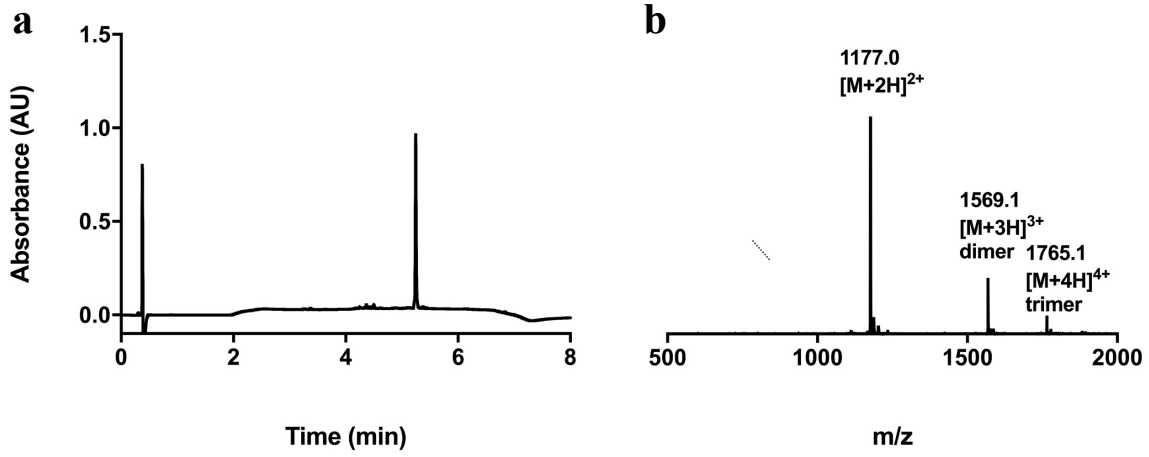
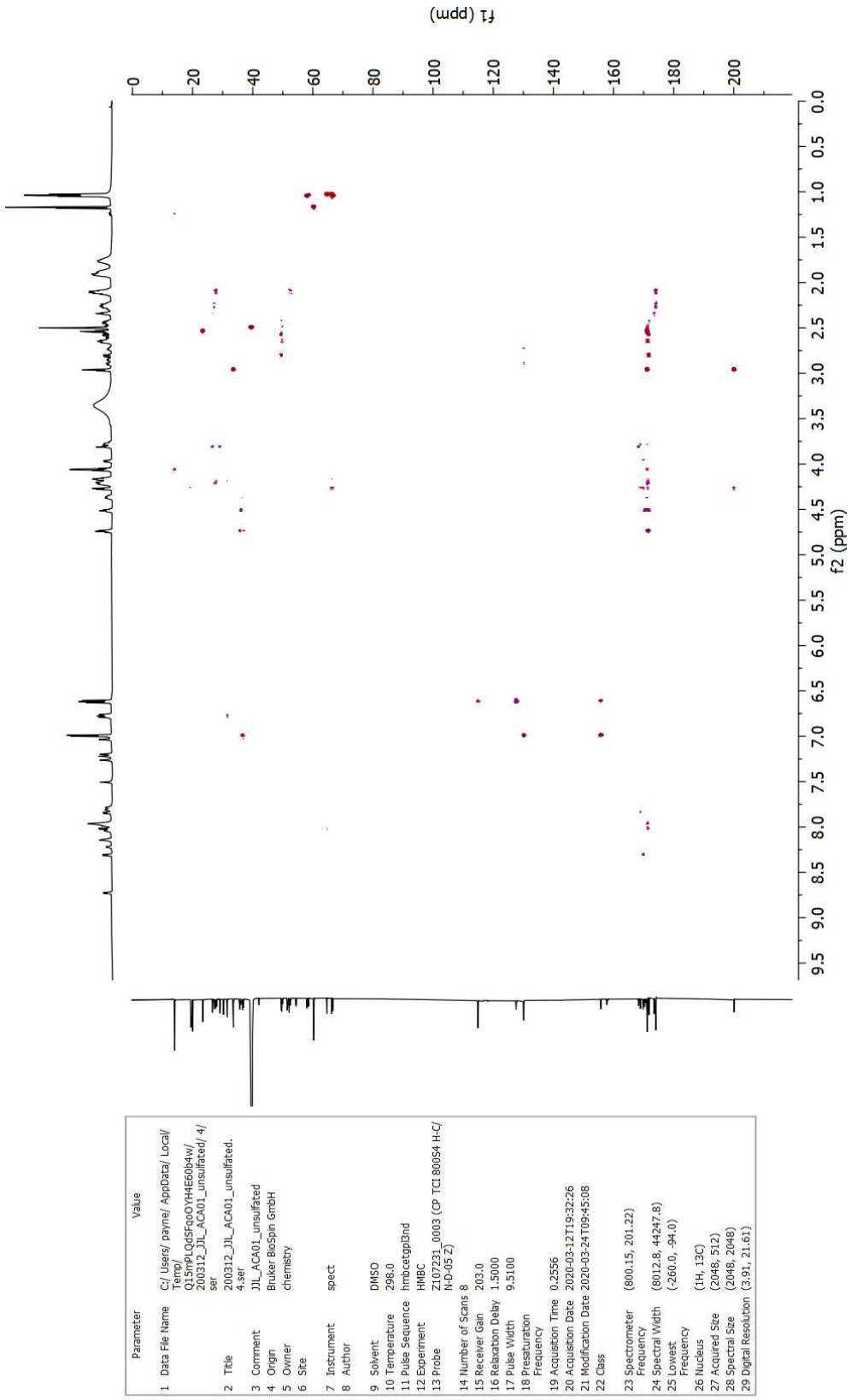


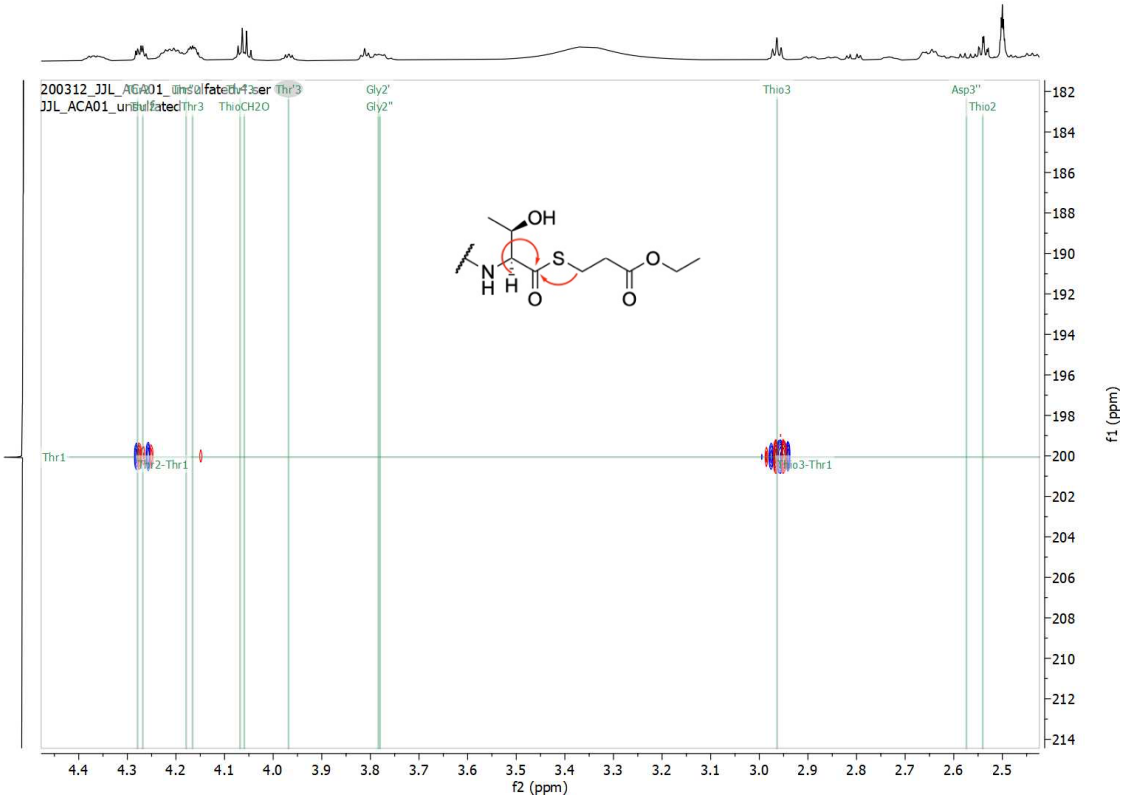
Figure S5. Analytical HPLC trace (**a**) and mass spectrum (**b***)* of purified peptide **9** thioester. Analytical HPLC Rt 5.22 min (0 to 70% over 5 min, 0.1% TFA, λ = 214 nm). Calculated Mass $[M+2H]^{2+}$: 1176.4, $[M+3H]^{3+}$ (dimer): 1568.1, $[M+4H]^{4+}$ (trimer): 1764.0, Mass Found (ESI) 1177.0 $[M+2H]^{2+}$, 1569.1 $[M+3H]^{3+}$ (dimer), 1765.1 $[M+4H]^{4+}$ (trimer).

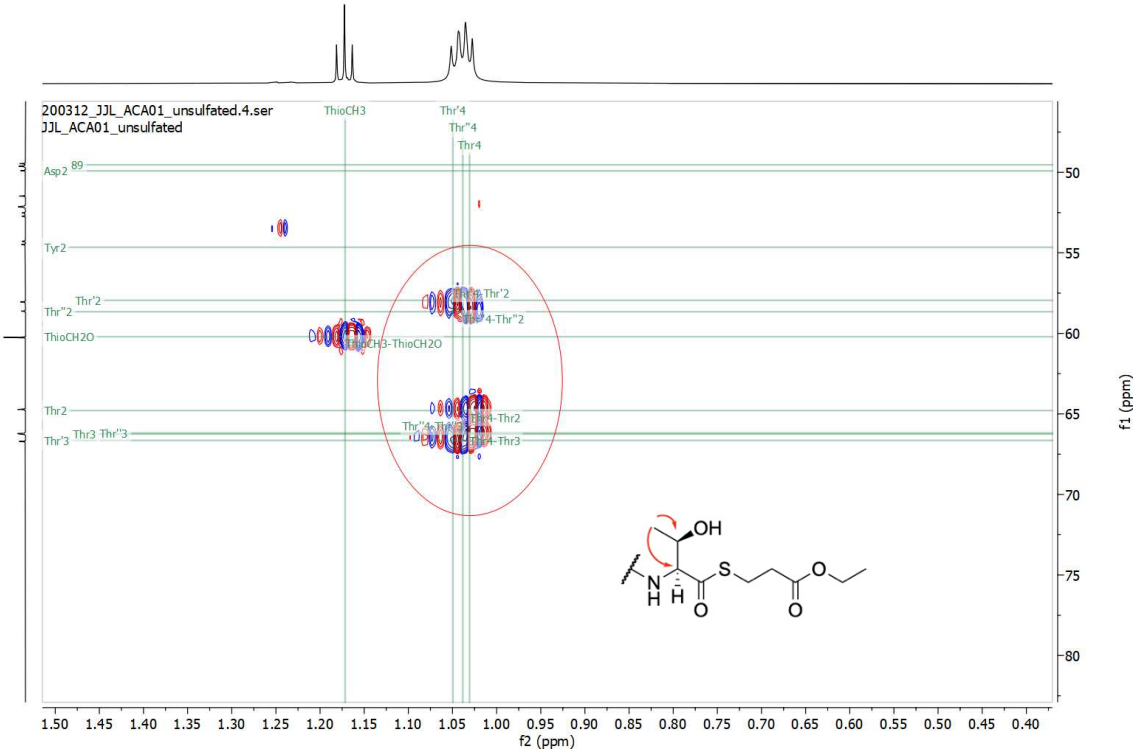
1D and 2D NMR data of (sulfo)peptide thioesters 6-9

HMBC of ACA-01 unsulfated thioester (6) (800/200 MHz, d_6-DMSO)

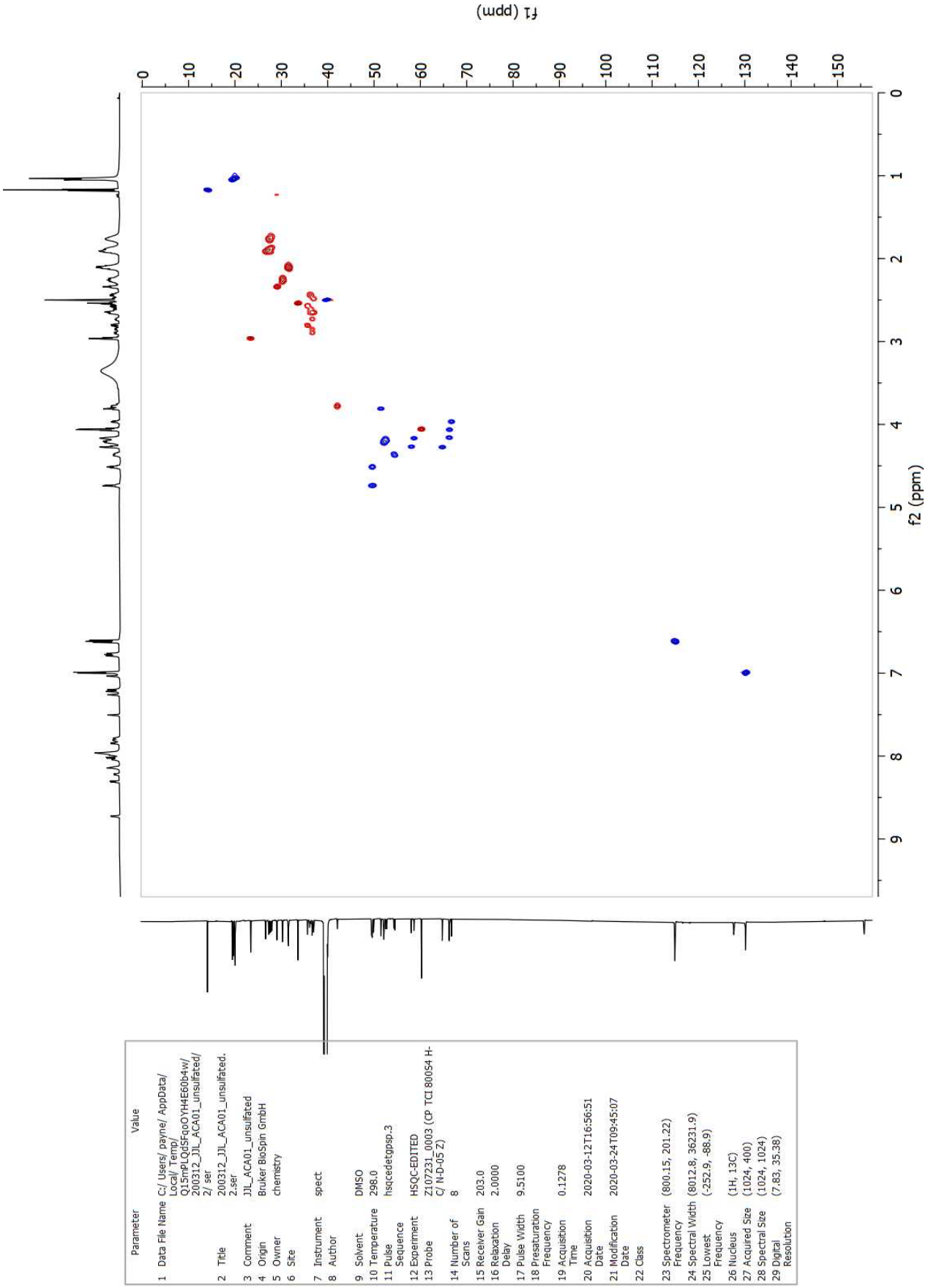


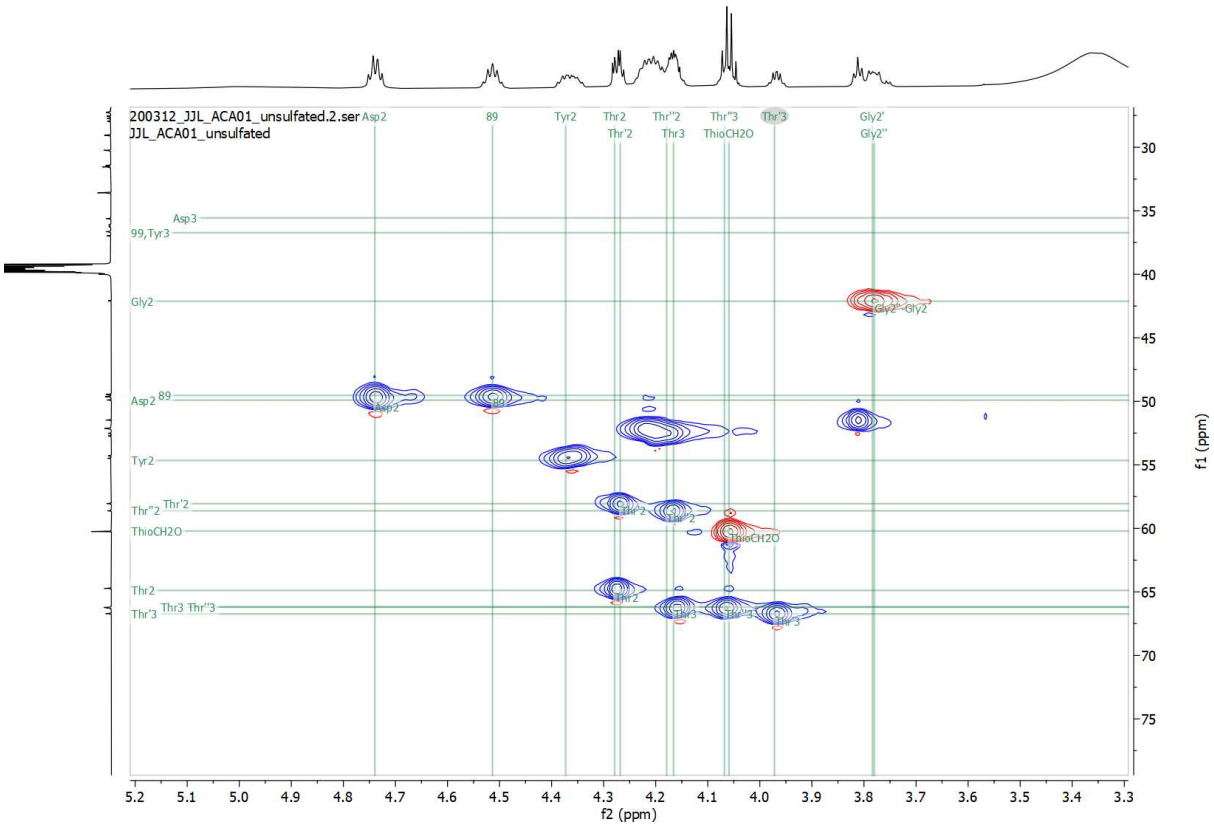
	Parameter	Value
1	Data File Name	C:/ Users/ payne/ AppData/ Local/ Temp/ Q15MPLQdSFqoOYH4E60b4w/ 200312_JJL_ACA01_unsulfated/ 4/ ser
2	Title	200312_JJL_ACA01_unsulfated. 4.ser
3	Comment	JJL_ACA01_unsulfated
4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	hmbcetgpl3nd
12	Experiment	HMBC
13	Probe	Z107231_0003 (CP TCI 800S4 H-C/ N-D-05 Z)
14	Number of Scans	8
15	Receiver Gain	203.0
16	Relaxation Delay	1.5000
17	Pulse Width	9.5100
18	Presaturation Frequency	
19	Acquisition Time	0.2556
20	Acquisition Date	2020-03-12T19:32:26
21	Modification Date	2020-03-24T09:45:08
22	Class	
23	Spectrometer Frequency	(800.15, 201.22)
24	Spectral Width	(8012.8, 44247.8)
25	Lowest Frequency	(-260.0, -94.0)
26	Nucleus	(1H, 13C)
27	Acquired Size	(2048, 512)
28	Spectral Size	(2048, 2048)
29	Digital Resolution	(3.91, 21.61)


200312_JJL_ACA01_unsulfated.4.ser
JJL_ACA01_unsulfated
Thr'3
Thr3
ThioCH2O
Gly2'
Gly2"
Thio3
Asp3''
Thio2
OH
N
H
H
S
O
O
O
Thr1
Thr2-Thr1
Thio3-Thr1
f2 (ppm)
f1 (ppm)

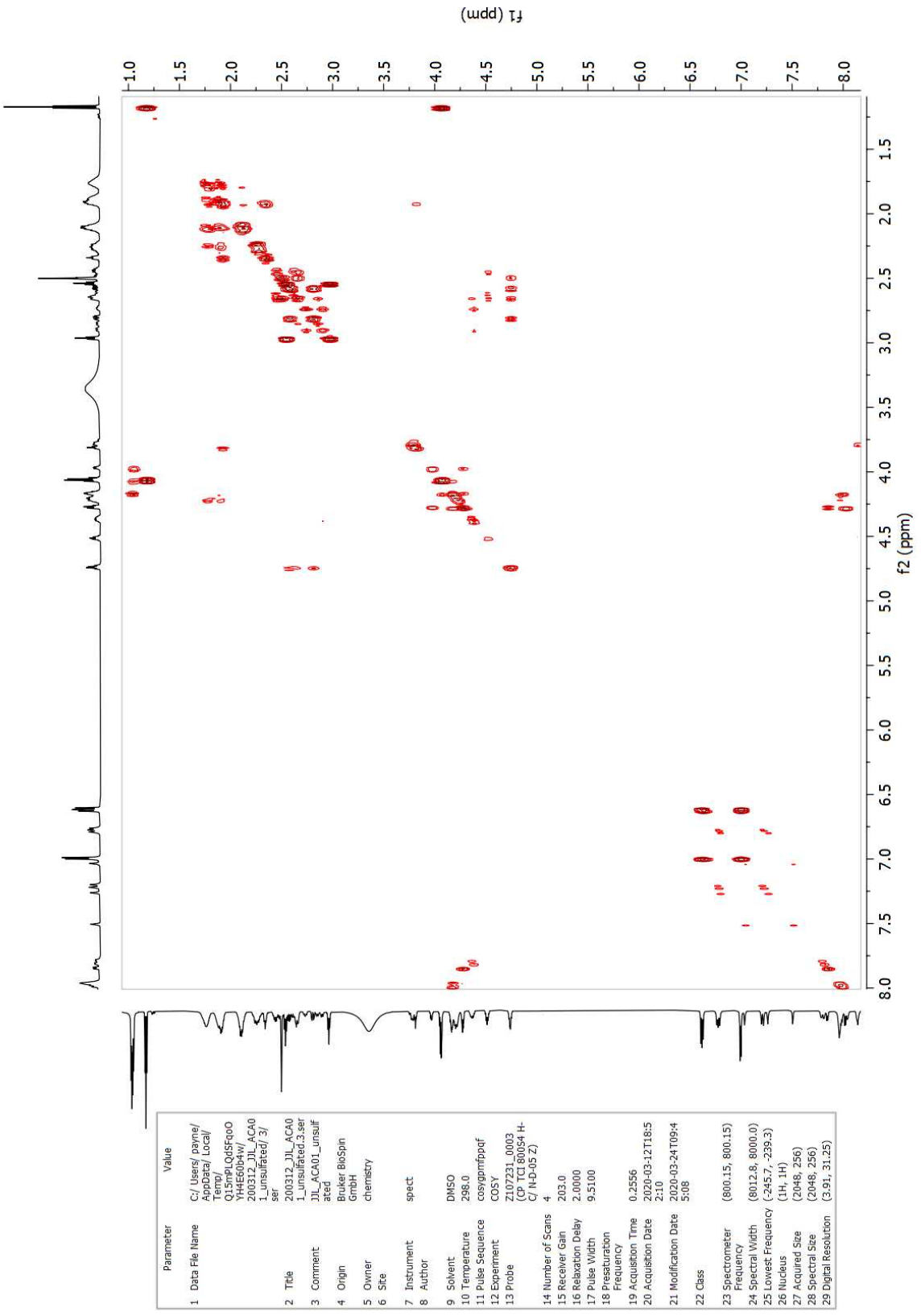

200312_JJL_ACA01_unsulfated.4.ser
JJL_ACA01_unsulfated
ThioCH3
Thr'4
Thr''4
Thr4
Asp2
Tyr2
Thr'2
Thr''2
ThioCH2O
Thr2
Thr'3
Thr3
Thr''3
ThioCH3-ThioCH2O
OH
N
H
H
S
O
O
O
f2 (ppm)
f1 (ppm)

HSQC of ACA-01 unsulfated thioester (6) (800/200 MHz, d_6-DMSO)




200312_JJL_ACA01_unsulfated.2.ser
JJL_ACA01_unsulfated
Asp2
89
Tyr2
Thr2
Thr'2
Thr''2
Thr3
Thr''3
ThioCH2O
Thr'3
Gly2'
Gly2''
Asp3
99,Tyr3
Gly2
Asp2
89
Tyr2
Thr''2
Thr'2
ThioCH2O
Thr2
Thr'3
Thr3
Thr''3
f2 (ppm)
f1 (ppm)

COSY of ACA-01 unsulfated thioester (6) (800 MHz, d_6-DMSO)



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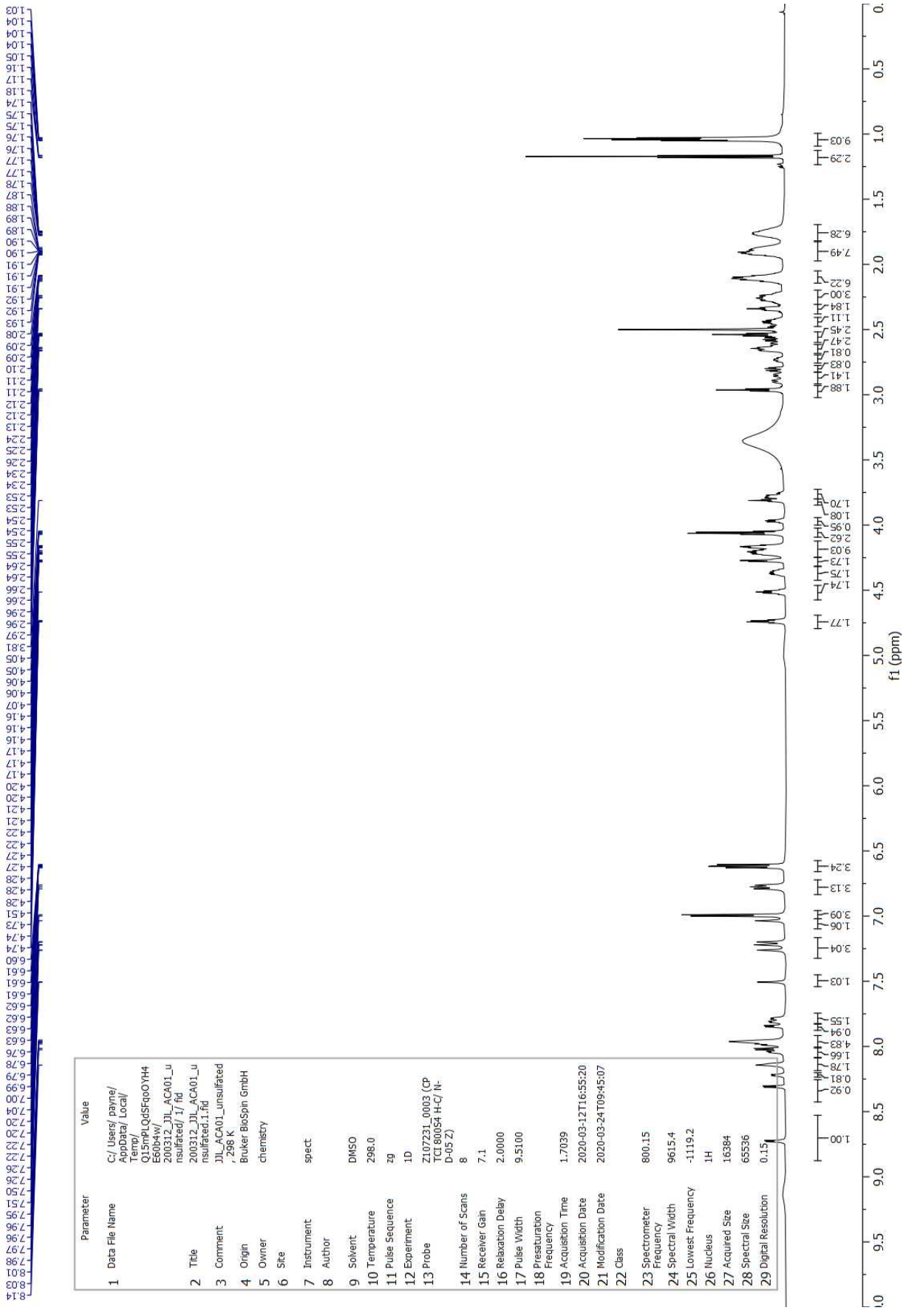
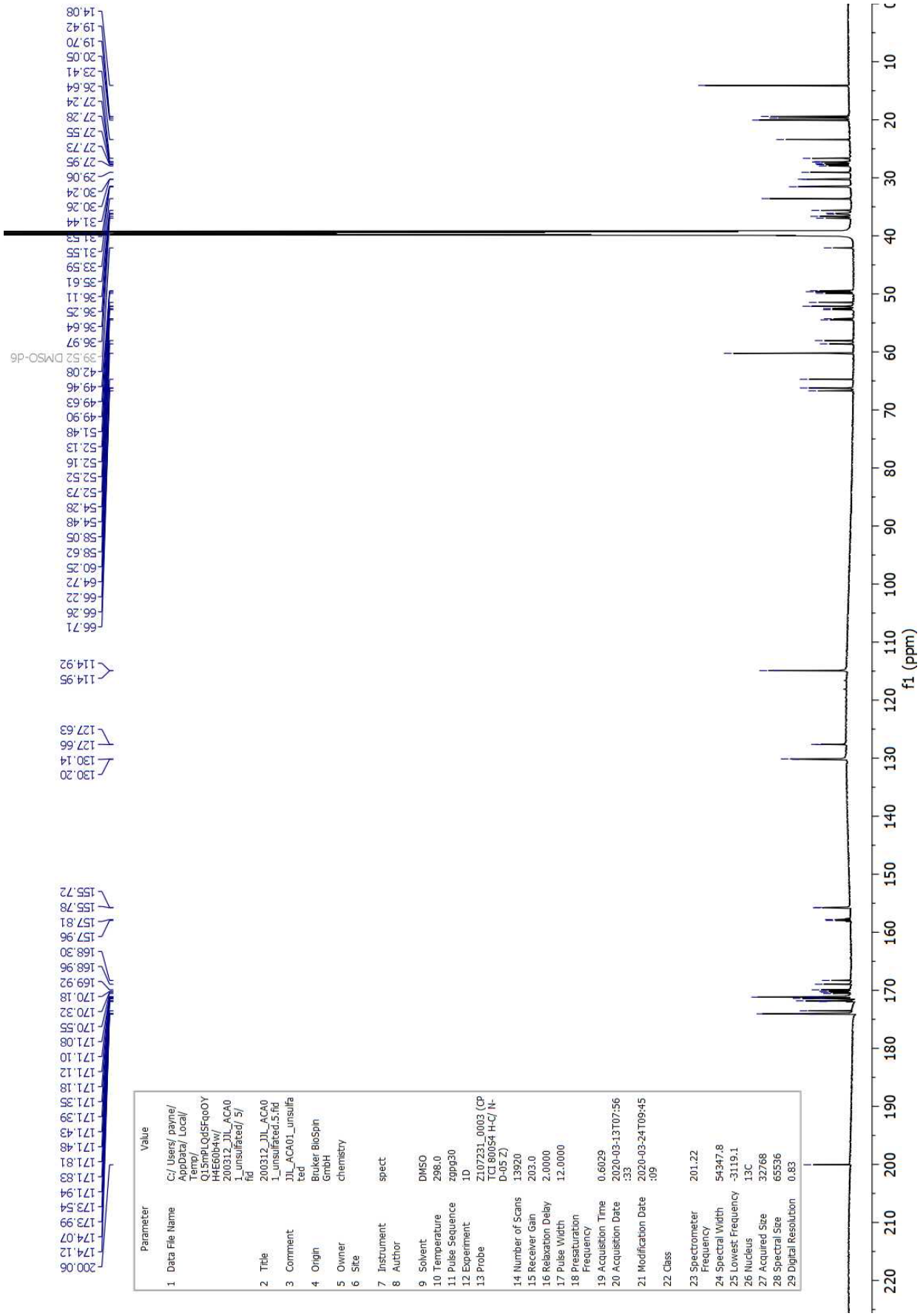
1	Data File Name	C:/ Users/ payne/ AppData/ Local/ Temp/ Q15mPLQdSFqoO YH4E60b4w/ 200312_JIL_ACA0 1_unsulfated/ 3/ ser
2	Title	200312_JIL_ACA0 1_unsulfated.3.ser
3	Comment	JIL_ACA01_unsulf ated
4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	cosygpmfppqf
12	Experiment	COSY
13	Probe	Z107231_0003 (CP TCI 80054 H-C/ N-D-05 Z)
14	Number of Scans	4
15	Receiver Gain	203.0
16	Relaxation Delay	2.0000
17	Pulse Width	9.5100
18	Presaturation Frequency	
19	Acquisition Time	0.2556
20	Acquisition Date	2020-03-12T18:5 2:10
21	Modification Date	2020-03-24T09:4 5:08
22	Class	
23	Spectrometer Frequency	(800.15, 800.15)
24	Spectral Width	(8012.8, 8000.0)
25	Lowest Frequency	(-245.7, -239.3)
26	Nucleus	(1H, 1H)
27	Acquired Size	(2048, 256)
28	Spectral Size	(2048, 256)
29	Digital Resolution	(3.91, 31.25)

^{1}H NMR of ACA-01 unsulfated thioester (6) (800 MHz, d_6-DMSO)

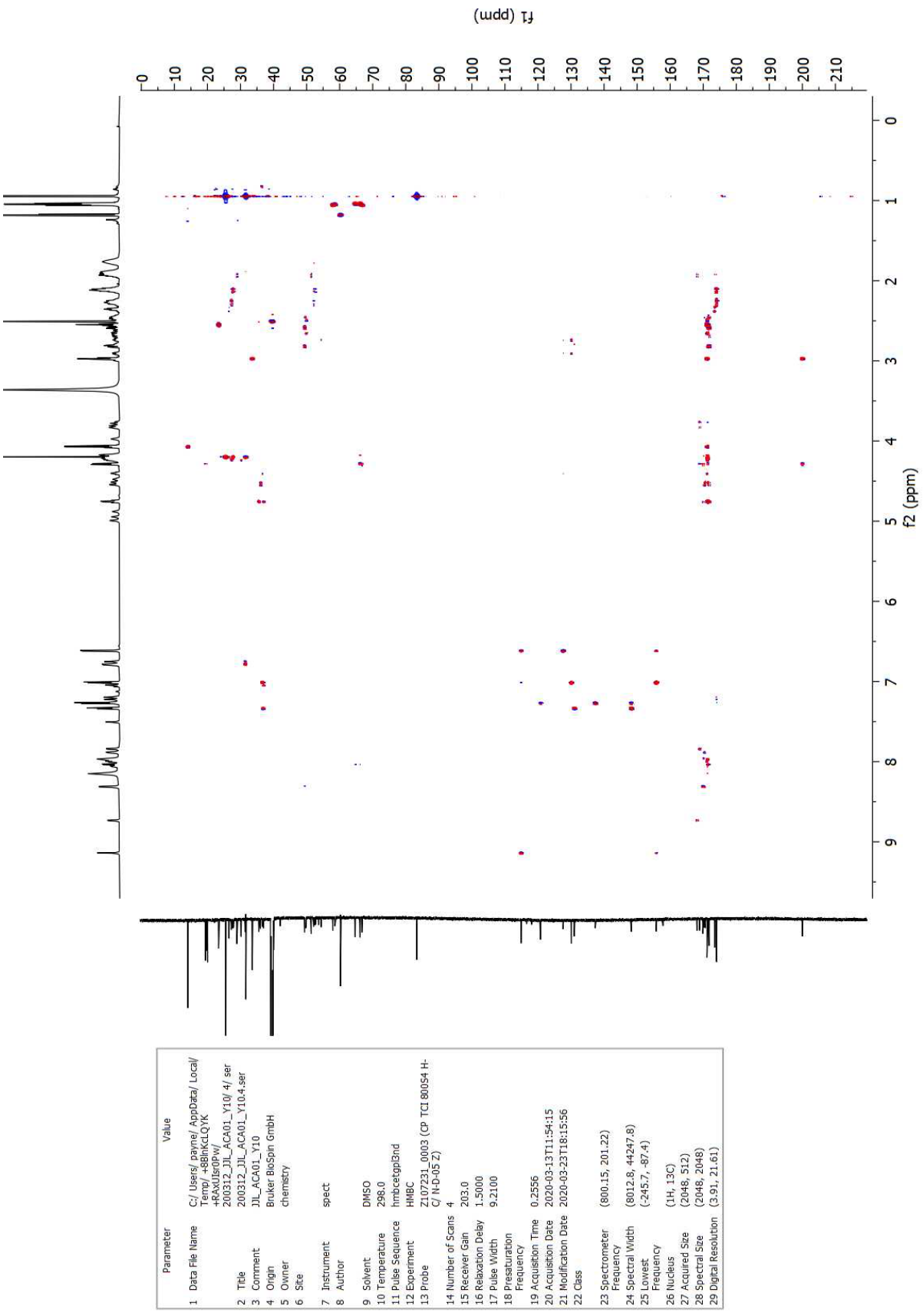


	Parameter	Value
1	Data File Name	C:/ Users/ payne/ AppData/ Local/ Temp/ Q15mPLQdSFqoOYH4 E60B4w/ 200312_JJL_ACA01_unsulfated/ 1/ fid
2	Title	200312_JJL_ACA01_unsulfated.1.fid
3	Comment	JJL_ACA01_unsulfated , 298 K
4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	zg
12	Experiment	1D
13	Probe	Z107231_0003 (CP TCI 800S4 H-C/ N-D-05 Z)
14	Number of Scans	8
15	Receiver Gain	7.1
16	Relaxation Delay	2.0000
17	Pulse Width	9.5100
18	Presaturation Frequency	
19	Acquisition Time	1.7039
20	Acquisition Date	2020-03-12T16:55:20
21	Modification Date	2020-03-24T09:45:07
22	Class	
23	Spectrometer Frequency	800.15
24	Spectral Width	9615.4
25	Lowest Frequency	-1119.2
26	Nucleus	1H
27	Acquired Size	16384
28	Spectral Size	65536
29	Digital Resolution	0.15

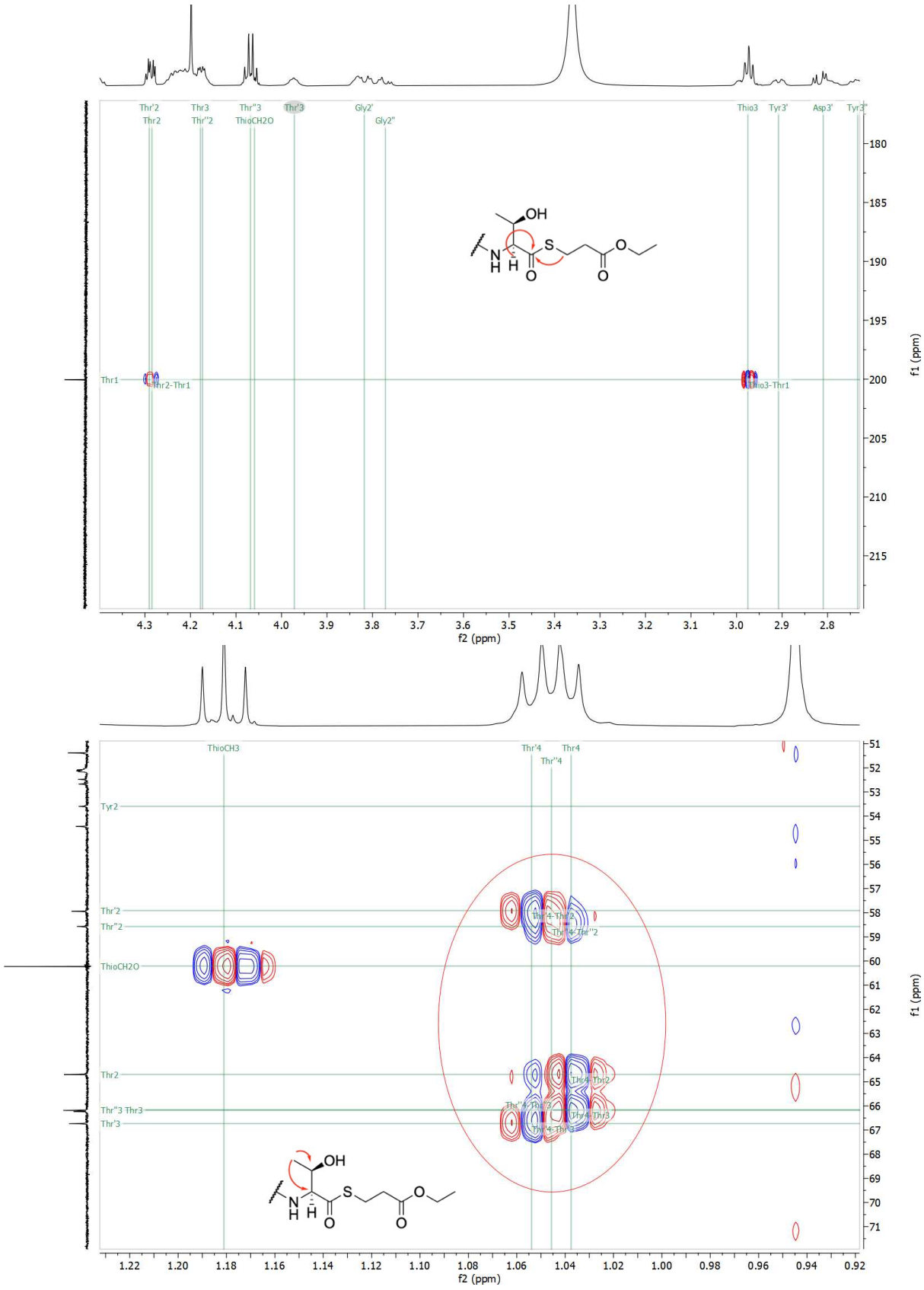
13C NMR of ACA-01 unsulfated thioester (6) (200 MHz, d_6-DMSO)



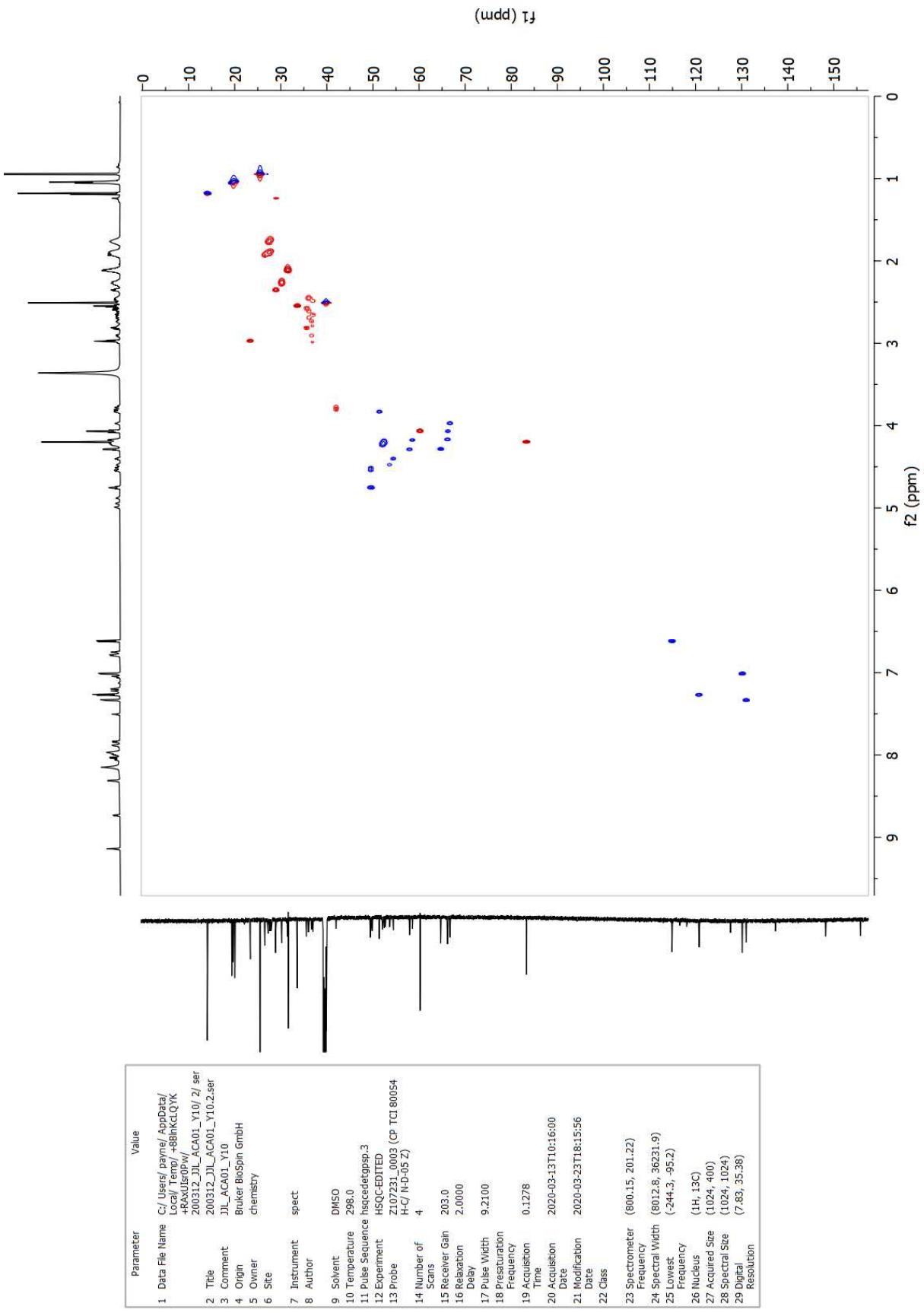
HMBC of ACA-01 Tyr10-sulfated thioester (7) (800/200 MHz, d_6-DMSO)

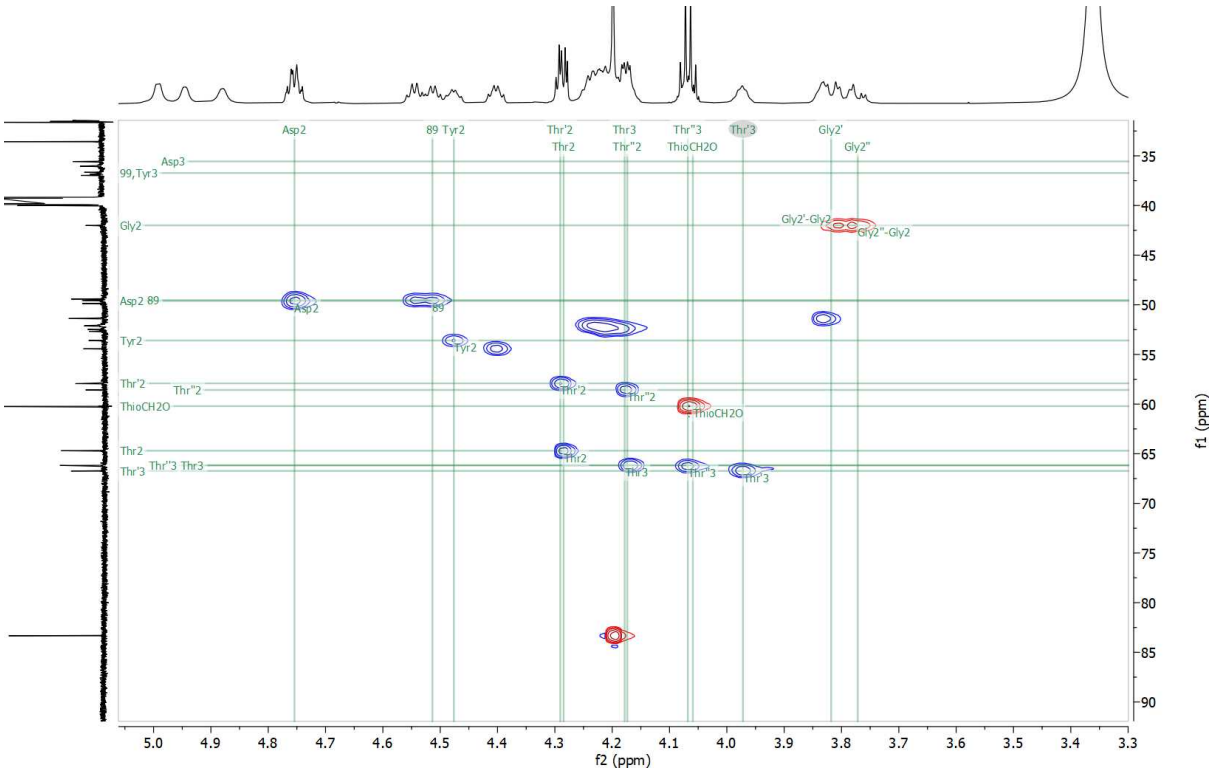


	Parameter	Value
1	Data File Name	C:/ Users/ payne/ AppData/ Local/ Temp/ +8BlnlKcLQYK +RAxUSr0Pw/ 200312_JIL_ACA01_Y10/ 4/ ser
2	Title	200312_JIL_ACA01_Y10.4.ser
3	Comment	JIL_ACA01_Y10
4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	hmbcetgpl3nd
12	Experiment	HMBC
13	Probe	Z107231_0003 (CP TCI 80054 H-C/ N-D-05 Z)
14	Number of Scans	4
15	Receiver Gain	203.0
16	Relaxation Delay	1.5000
17	Pulse Width	9.2100
18	Presaturation Frequency	
19	Acquisition Time	0.2556
20	Acquisition Date	2020-03-13T11:54:15
21	Modification Date	2020-03-23T18:15:56
22	Class	
23	Spectrometer Frequency	(800.15, 201.22)
24	Spectral Width	(8012.8, 44247.8)
25	Lowest Frequency	(-245.7, -87.4)
26	Nucleus	(1H, 13C)
27	Acquired Size	(2048, 512)
28	Spectral Size	(2048, 2048)
29	Digital Resolution	(3.91, 21.61)

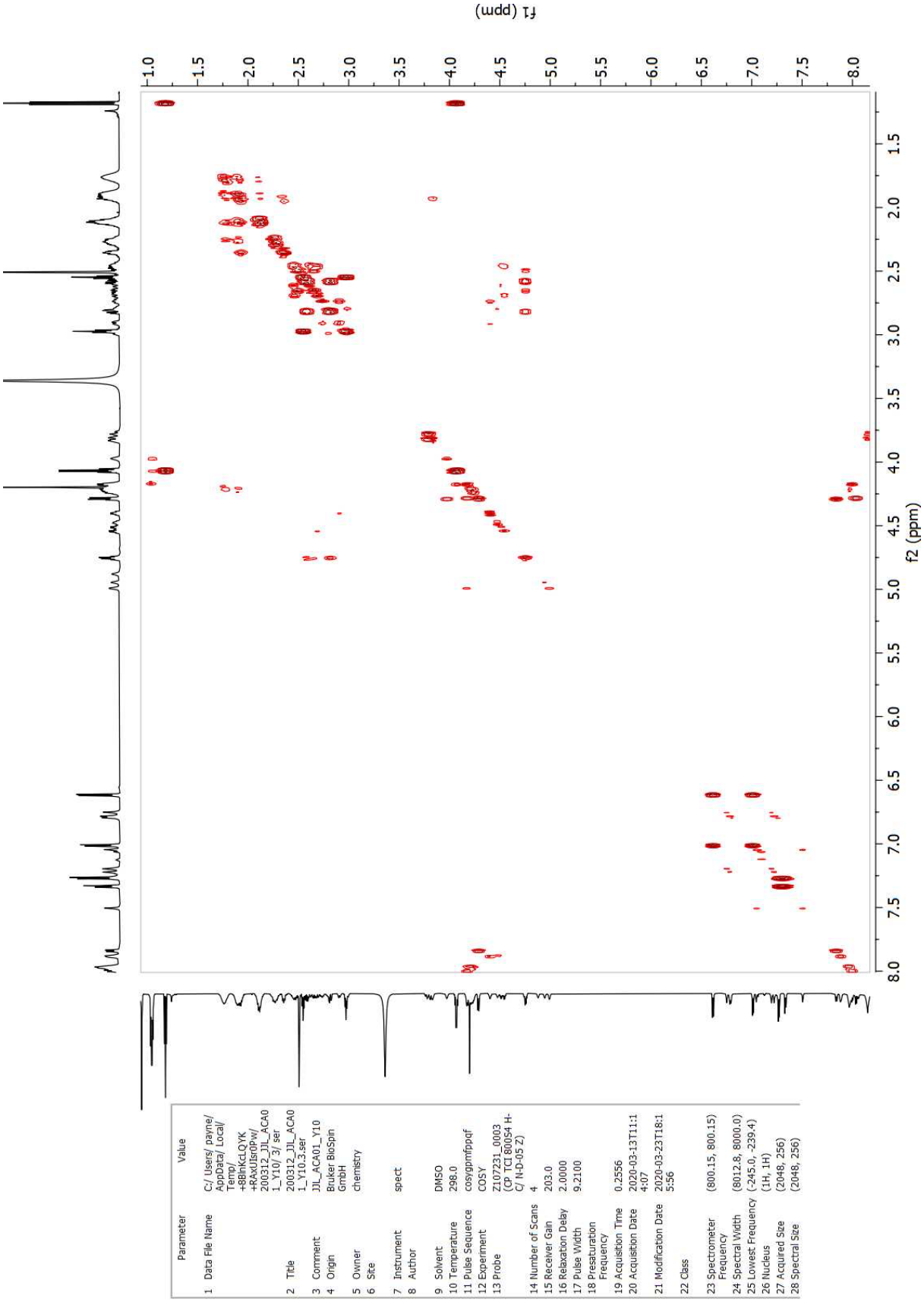

Thr'2
Thr2
Thr3
Thr''2
Thr''3
ThioCH2O
Thr'3
Gly2'
Gly2''
Thio3
Tyr3'
Asp3'
Tyr3''
Thr1
Thr2-Thr1
Thio3-Thr1
f2 (ppm)
f1 (ppm)
ThioCH3
Thr'4
Thr4
Thr''4
Tyr2
Thr'2
Thr''2
ThioCH2O
Thr2
Thr''3 Thr3
Thr'3
f2 (ppm)
f1 (ppm)

HSQC of ACA-01 Tyr10-sulfated thioester (7) (800/200 MHz, d_6-DMSO)



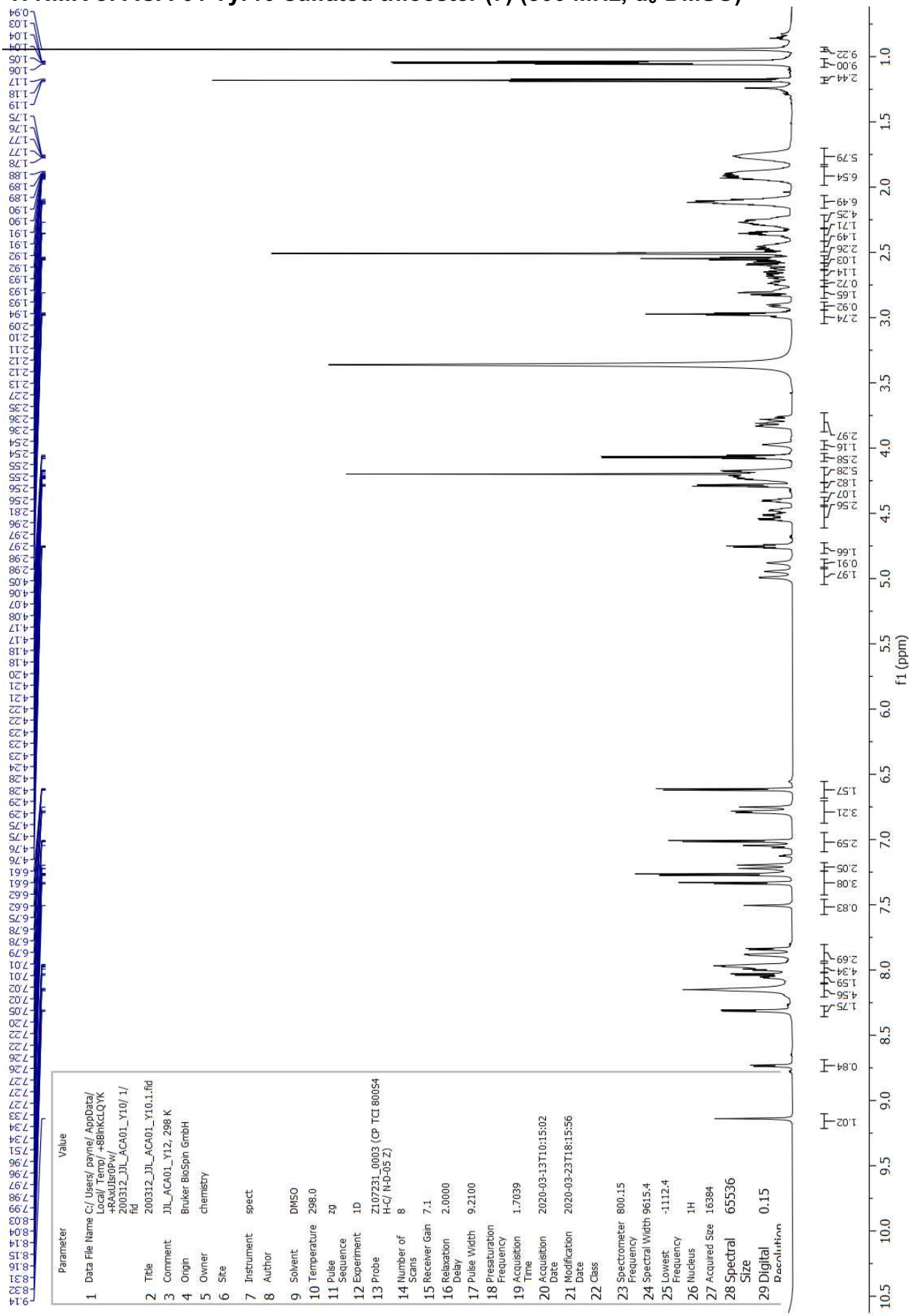

Asp2
89 Tyr2
Thr'2
Thr2
Thr3
Thr"2
Thr"3
ThioCH2O
Thr'3
Gly2'
Gly2"
Asp3
99,Tyr3
Gly2
Gly2'-Gly2
Gly2"-Gly2
Asp2 89
Tyr2
Thr'2
Thr"2
ThioCH2O
Thr2
Thr'3 Thr"3 Thr3
f2 (ppm)
f1 (ppm)

COSY of ACA-01 Tyr10-sulfated thioester (7) (800 MHz, d_6-DMSO)



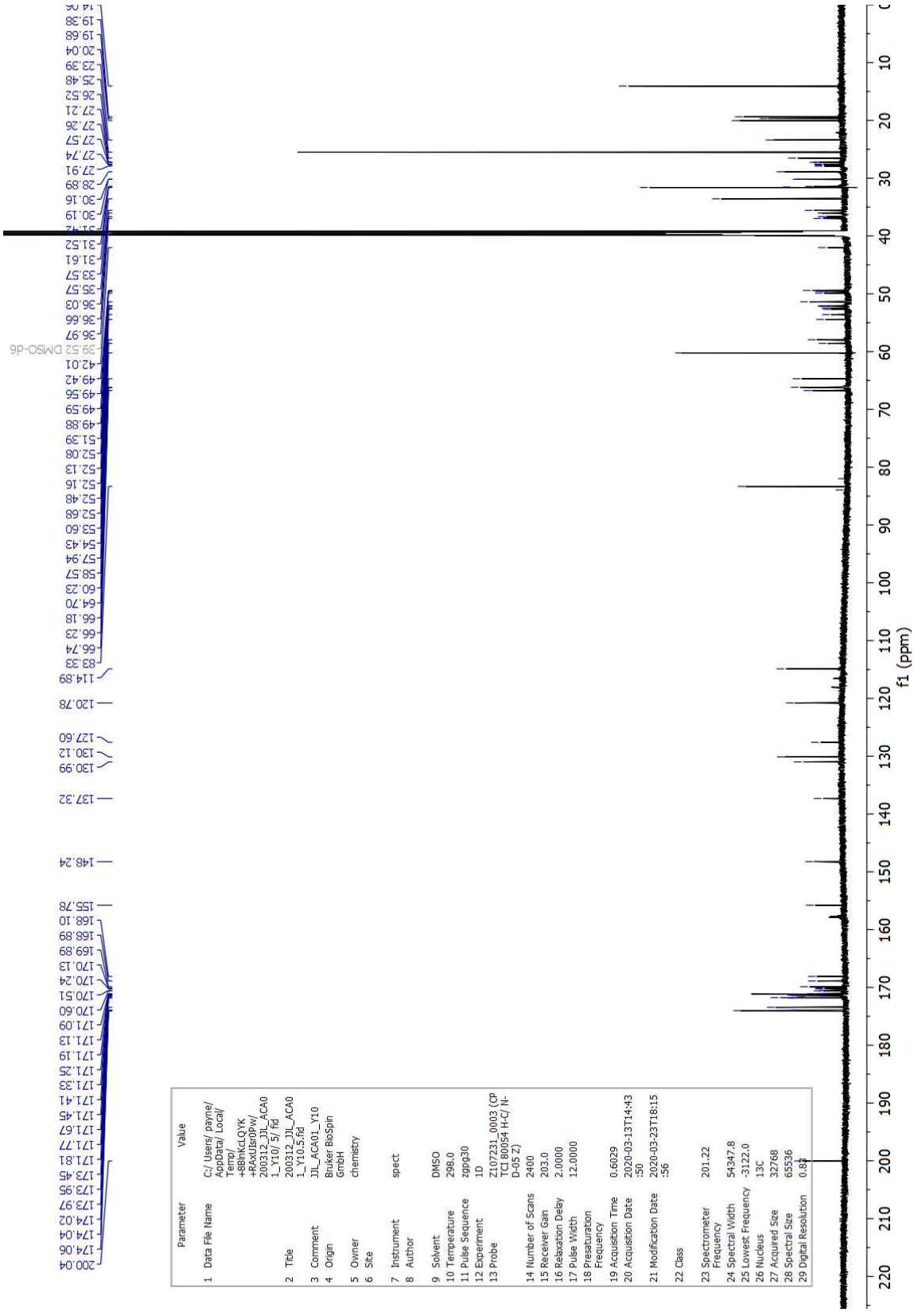
	Parameter	Value
1	Data File Name	C:/ Users/ payne/ AppData/ Local/ Temp/ +8BfnKcLQYK +RAxUIsr0Pw/ 200312_JIL_ACA0 1_Y10/ 3/ ser
2	Title	200312_JIL_ACA0 1_Y10 3.ser
3	Comment	JIL_ACA01_Y10
4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	cosygpmfppqf
12	Experiment	COSY
13	Probe	Z107231_0003 (CP TCI 80054 H-C/ N-D-05 Z)
14	Number of Scans	4
15	Receiver Gain	203.0
16	Relaxation Delay	2.0000
17	Pulse Width	9.2100
18	Presaturation Frequency	
19	Acquisition Time	0.2556
20	Acquisition Date	2020-03-13T11:1 4:07
21	Modification Date	2020-03-23T18:1 5:56
22	Class	
23	Spectrometer Frequency	(800.15, 800.15)
24	Spectral Width	(8012.8, 8000.0)
25	Lowest Frequency	(-245.0, -239.4)
26	Nucleus	(1H, 1H)
27	Acquired Size	(2048, 256)
28	Spectral Size	(2048, 256)

^{1}H NMR of ACA-01 Tyr10-sulfated thioester (7) (800 MHz, d_6-DMSO)

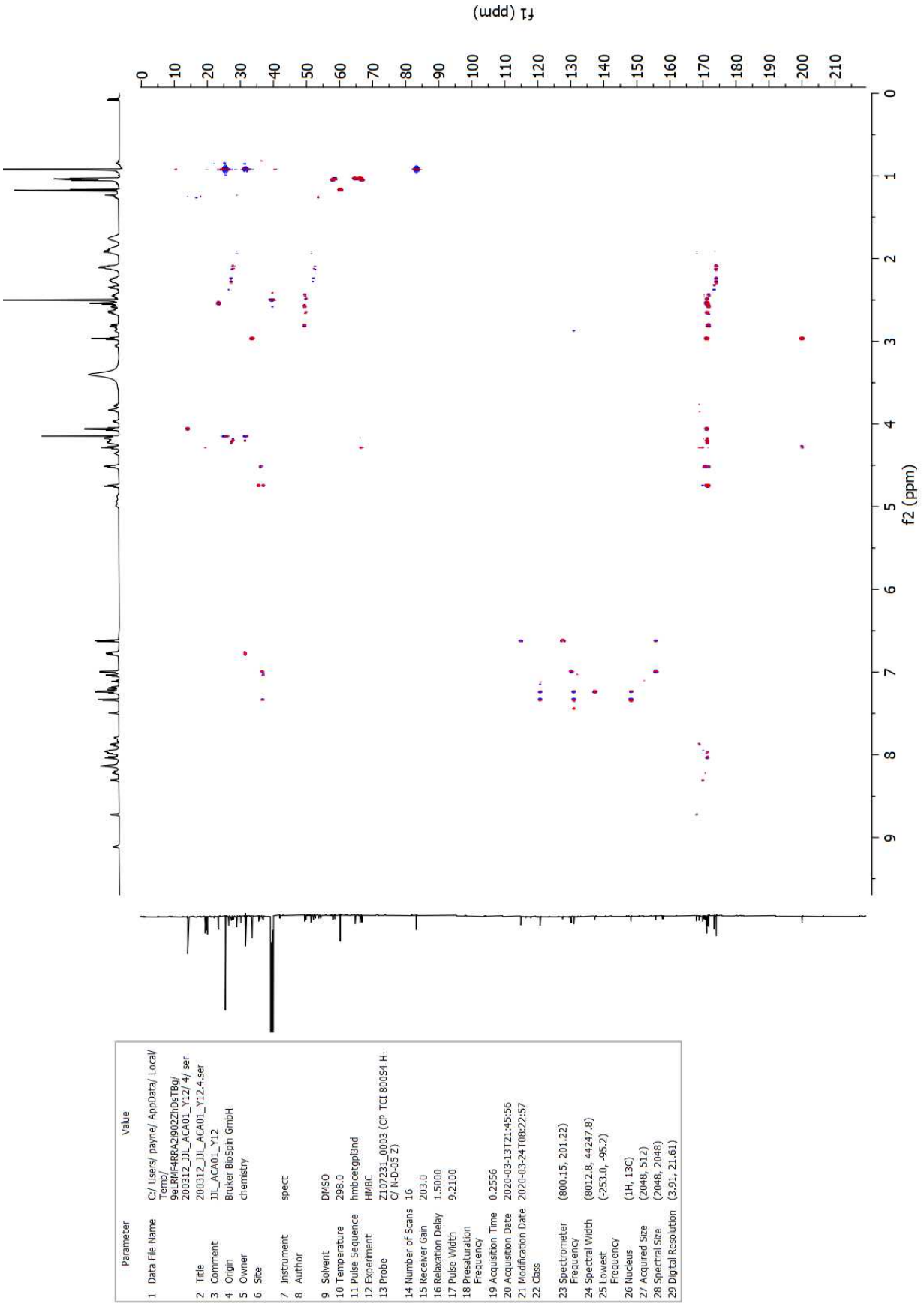


	Parameter	Value
1	Data File Name	C:/ Users/ payne/ AppData/ Local/ Temp/ +8BnKcLQYK +RAxUIsr0Pw/ 200312_JJL_ACA01_Y10/ 1/ fid
2	Title	200312_JJL_ACA01_Y10.1.fid
3	Comment	JJL_ACA01_Y12, 298 K
4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	zg
12	Experiment	1D
13	Probe	Z107231_0003 (CP TCI 80054 H-C/ N-D-05 Z)
14	Number of Scans	8
15	Receiver Gain	7.1
16	Relaxation Delay	2.0000
17	Pulse Width	9.2100
18	Presaturation Frequency	
19	Acquisition Time	1.7039
20	Acquisition Date	2020-03-13T10:15:02
21	Modification Date	2020-03-23T18:15:56
22	Class	
23	Spectrometer Frequency	800.15
24	Spectral Width	9615.4
25	Lowest Frequency	-1112.4
26	Nucleus	1H
27	Acquired Size	16384
28	Spectral Size	65536
29	Digital Resolution	0.15

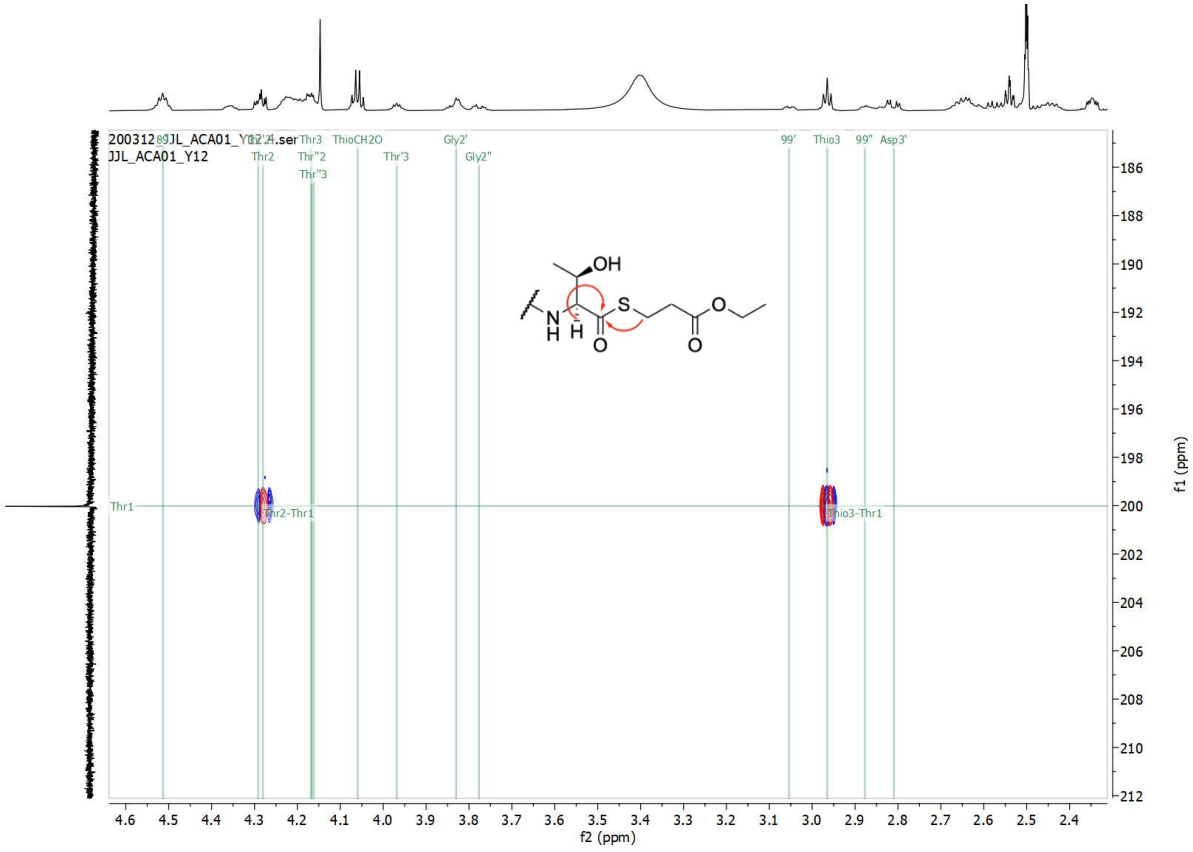
^{13}C NMR of ACA-01 Tyr10-sulfated thioester (7) (200 MHz, d_6-DMSO)

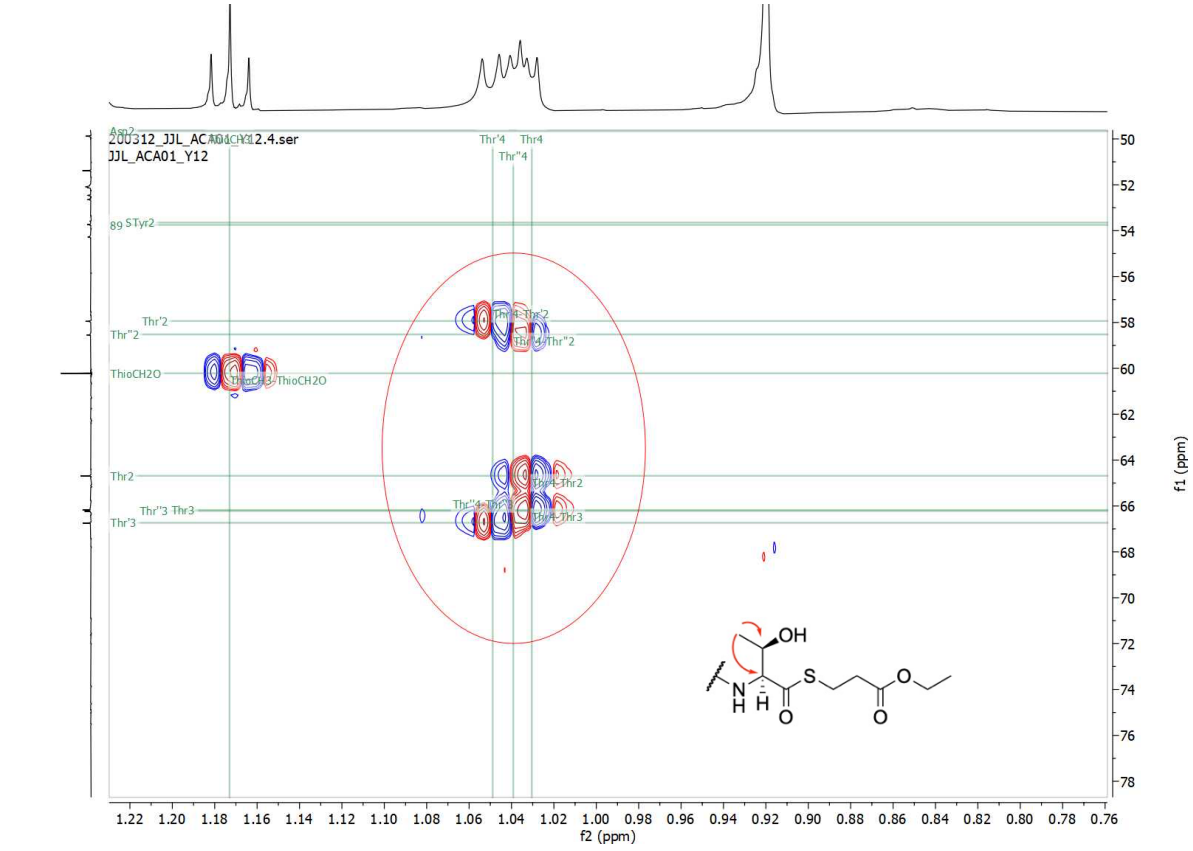


HMBC of ACA-01 Tyr12-sulfated thioester (8) (800/200 MHz, d_6-DMSO)

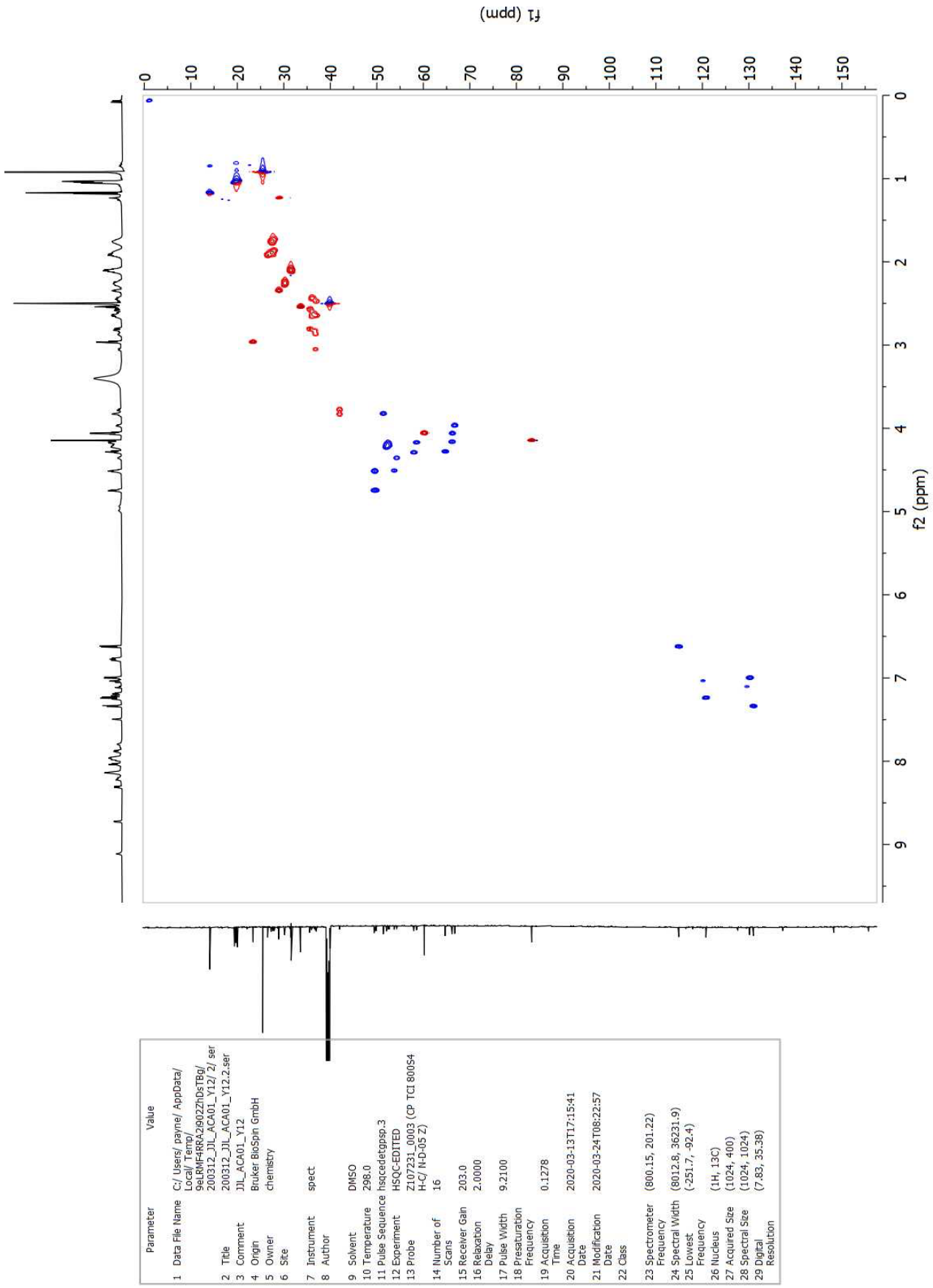


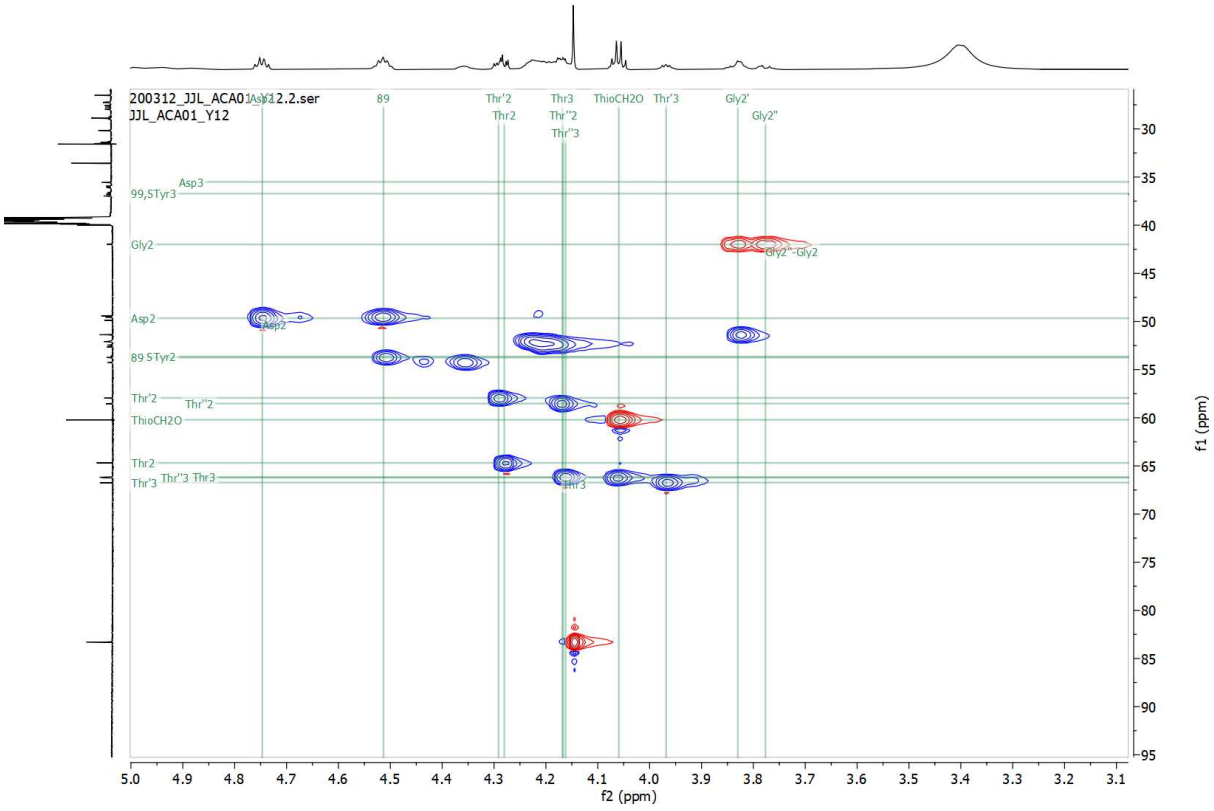
	Parameter	Value
1	Data File Name	C:/ Users/ payne/ AppData/ Local/ Temp/ 9eLRMF4RRA2902ZhDsTBg/ 200312_JIL_ACA01_Y12/ 4/ ser
2	Title	200312_JIL_ACA01_Y12.4.ser
3	Comment	JIL_ACA01_Y12
4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	hmbcetgpl3nd
12	Experiment	HMBC
13	Probe	Z107231_0003 (CP TCI 80054 H-C/ N-D-05 Z)
14	Number of Scans	16
15	Receiver Gain	203.0
16	Relaxation Delay	1.5000
17	Pulse Width	9.2100
18	Presaturation Frequency	
19	Acquisition Time	0.2556
20	Acquisition Date	2020-03-13T21:45:56
21	Modification Date	2020-03-24T08:22:57
22	Class	
23	Spectrometer Frequency	(800.15, 201.22)
24	Spectral Width	(8012.8, 44247.8)
25	Lowest Frequency	(-253.0, -95.2)
26	Nucleus	(1H, 13C)
27	Acquired Size	(2048, 512)
28	Spectral Size	(2048, 2048)
29	Digital Resolution	(3.91, 21.61)


200312_JJL_ACA01_Y12.4.ser
JJL_ACA01_Y12
Thr2
Thr"2
Thr"3
Thr3
ThioCH2O
Thr'3
Gly2'
Gly2"
99'
Thio3
99"
Asp3'
Thr1
Thr2-Thr1
Thio3-Thr1
OH
N
H
H
O
S
O
O
f2 (ppm)
f1 (ppm)

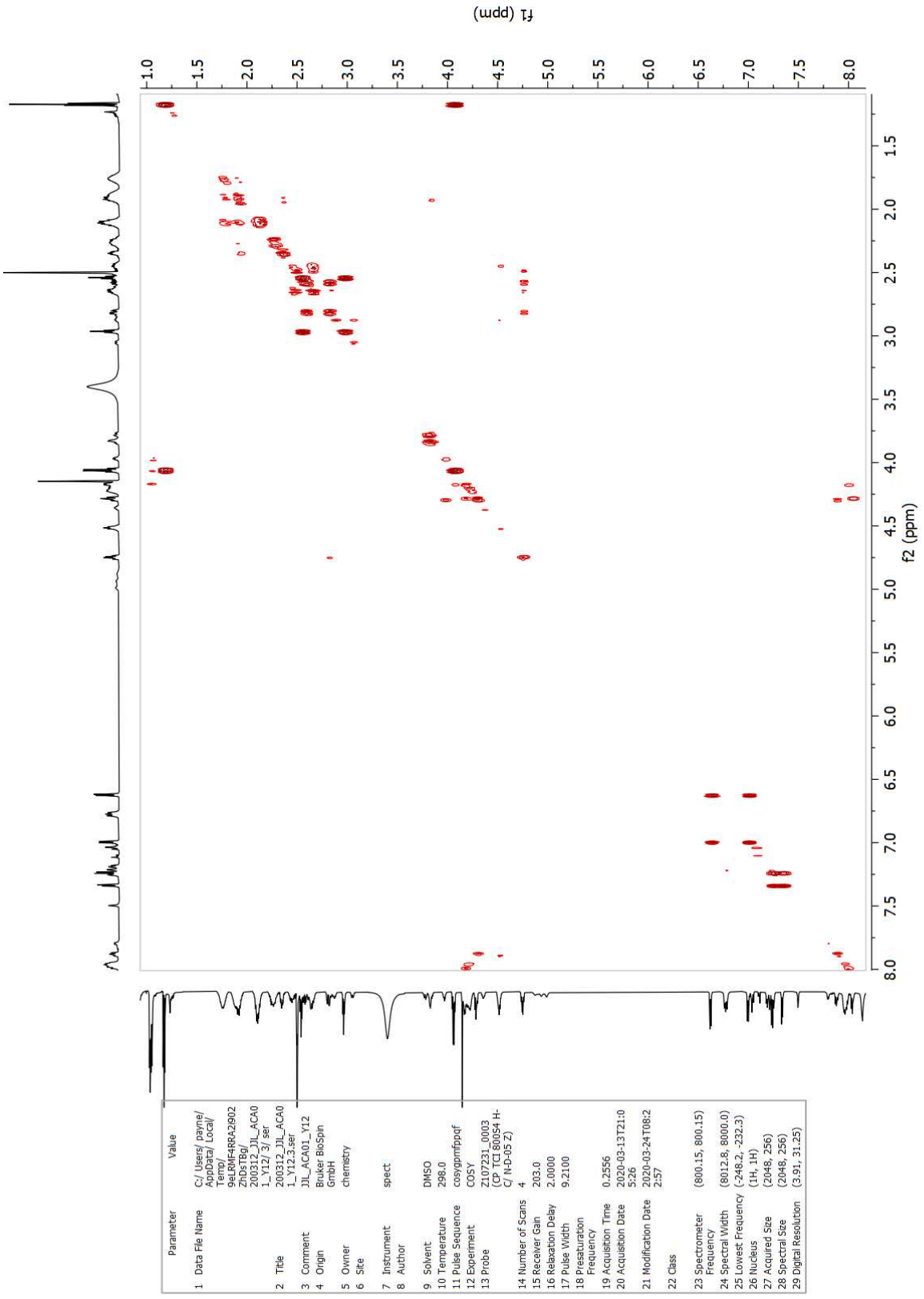

JJL_ACA01_Y12
Thr'4
Thr4
Thr"4
89 STyr2
Thr'2
Thr"2
ThioCH2O
Thr2
Thr"3 Thr3
Thr'3
OH
N
H
H
O
S
O
O
f2 (ppm)
f1 (ppm)

HSQC of ACA-01 Tyr12-sulfated thioester (8) (800/200 MHz, d_6-DMSO)

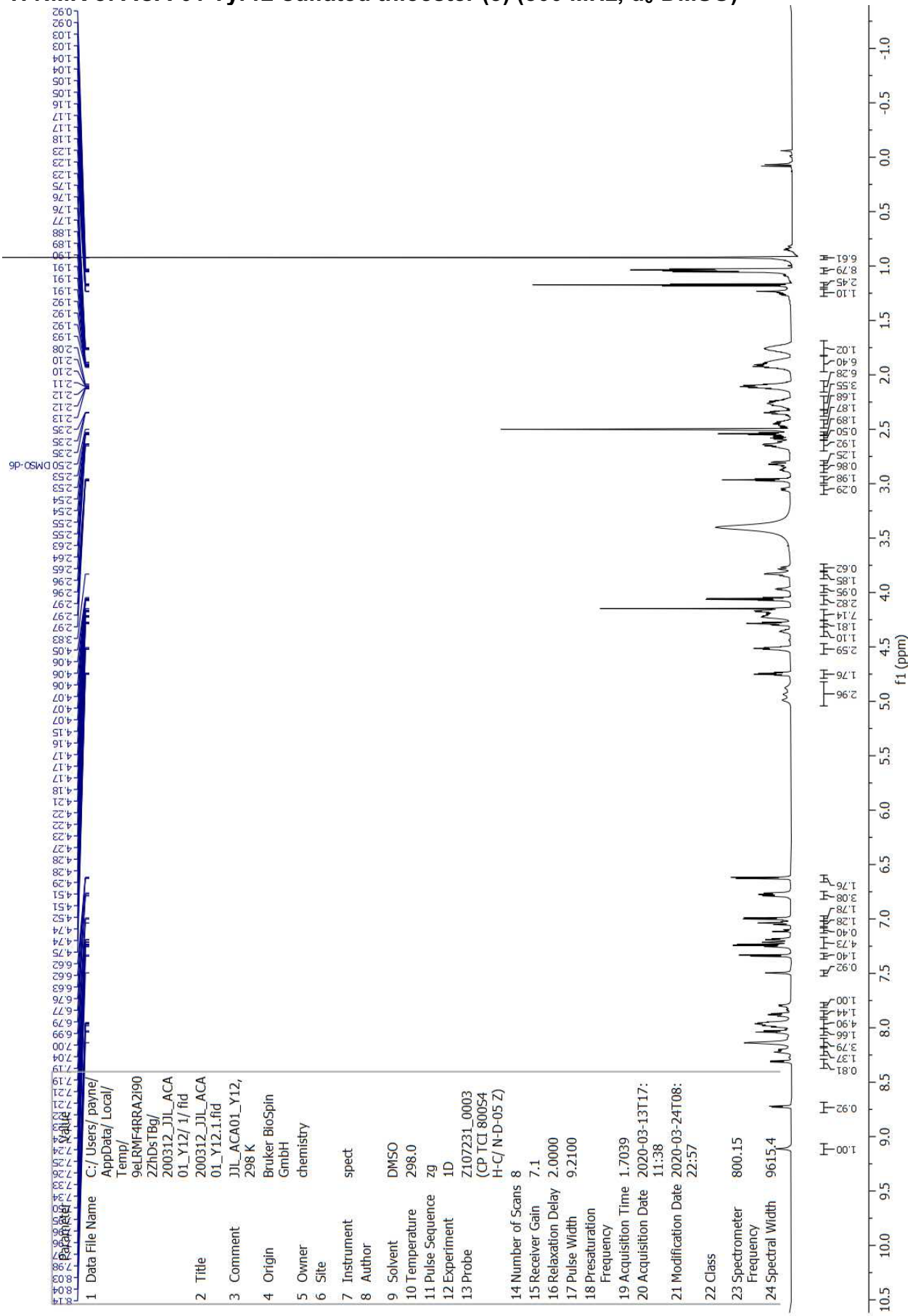



200312_JJL_ACA01_Y12.2.ser
JJL_ACA01_Y12
Asp2
89
Thr'2
Thr2
Thr3
Thr''2
Thr''3
ThioCH2O
Thr'3
Gly2'
Gly2''
Asp3
99,STyr3
Gly2
Gly2''-Gly2
Asp2
Asp2
89 STyr2
Thr'2
Thr''2
ThioCH2O
Thr2
Thr'3
Thr''3
Thr3
Thr3
f2 (ppm)
f1 (ppm)

COSY of ACA-01 Tyr12-sulfated thioester (8) (800 MHz, d_6-DMSO)

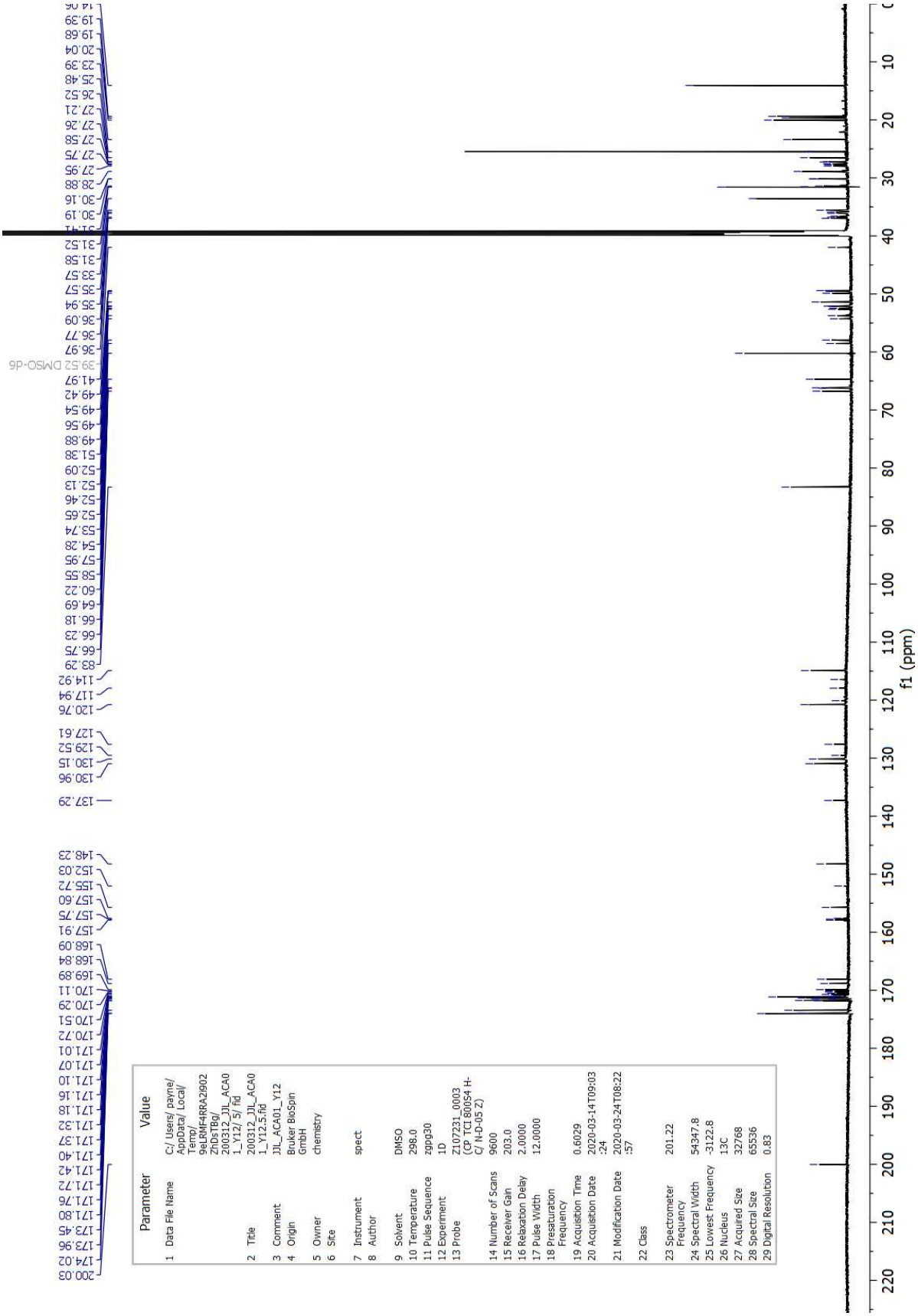


^{1}H NMR of ACA-01 Tyr12-sulfated thioester (8) (800 MHz, d_6-DMSO)

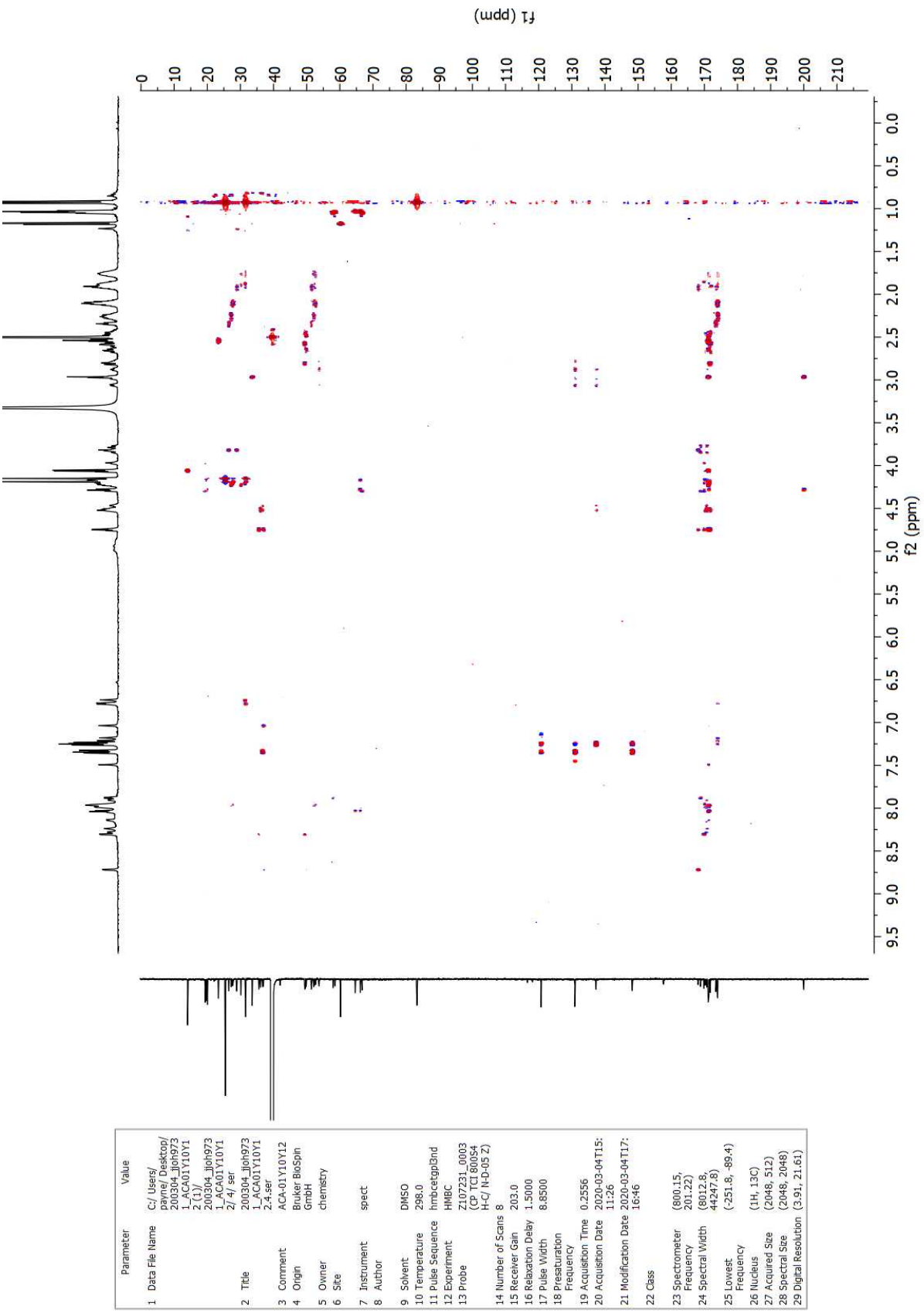


Parameter	Value
1 Data File Name	C:/ Users/ payne/ AppData/ Local/ Temp/ 9eLRMF4RRA2i90 2ZhDsTBg/ 200312_JJL_ACA 01_Y12/ 1/ fid
2 Title	200312_JJL_ACA 01_Y12.1.fid
3 Comment	JJL_ACA01_Y12, 298 K
4 Origin	Bruker BioSpin GmbH
5 Owner	chemistry
6 Site	
7 Instrument	spect
8 Author	
9 Solvent	DMSO
10 Temperature	298.0
11 Pulse Sequence	zg
12 Experiment	1D
13 Probe	Z107231_0003 (CP TCI 800S4 H-C/ N-D-05 Z)
14 Number of Scans	8
15 Receiver Gain	7.1
16 Relaxation Delay	2.0000
17 Pulse Width	9.2100
18 Presaturation Frequency	
19 Acquisition Time	1.7039
20 Acquisition Date	2020-03-13T17: 11:38
21 Modification Date	2020-03-24T08: 22:57
22 Class	
23 Spectrometer Frequency	800.15
24 Spectral Width	9615.4

^{13}C NMR of ACA-01 Tyr12-sulfated thioester (8) (200 MHz, d_6-DMSO)



HMBC of ACA-01 Tyr10+Tyr12-sulfated thioester (9) (800/200 MHz, d_6-DMSO)



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3	Comment	ACA-01Y10Y12
4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	hmbcetgpl3nd
12	Experiment	HMBC
13	Probe	Z107231_0003 (CP TCI 800S4 H-C/ N-D-05 Z)
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18	Presaturation Frequency	
19	Acquisition Time	0.2556
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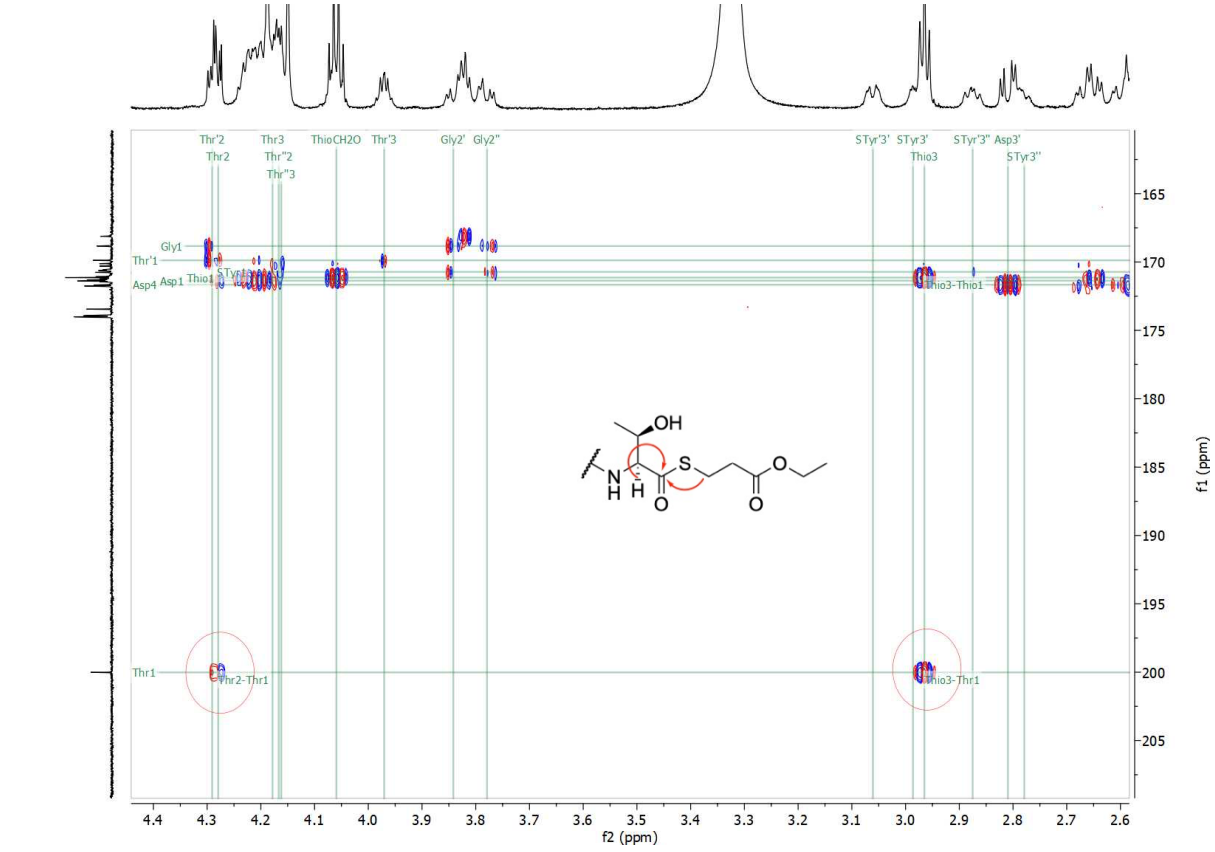
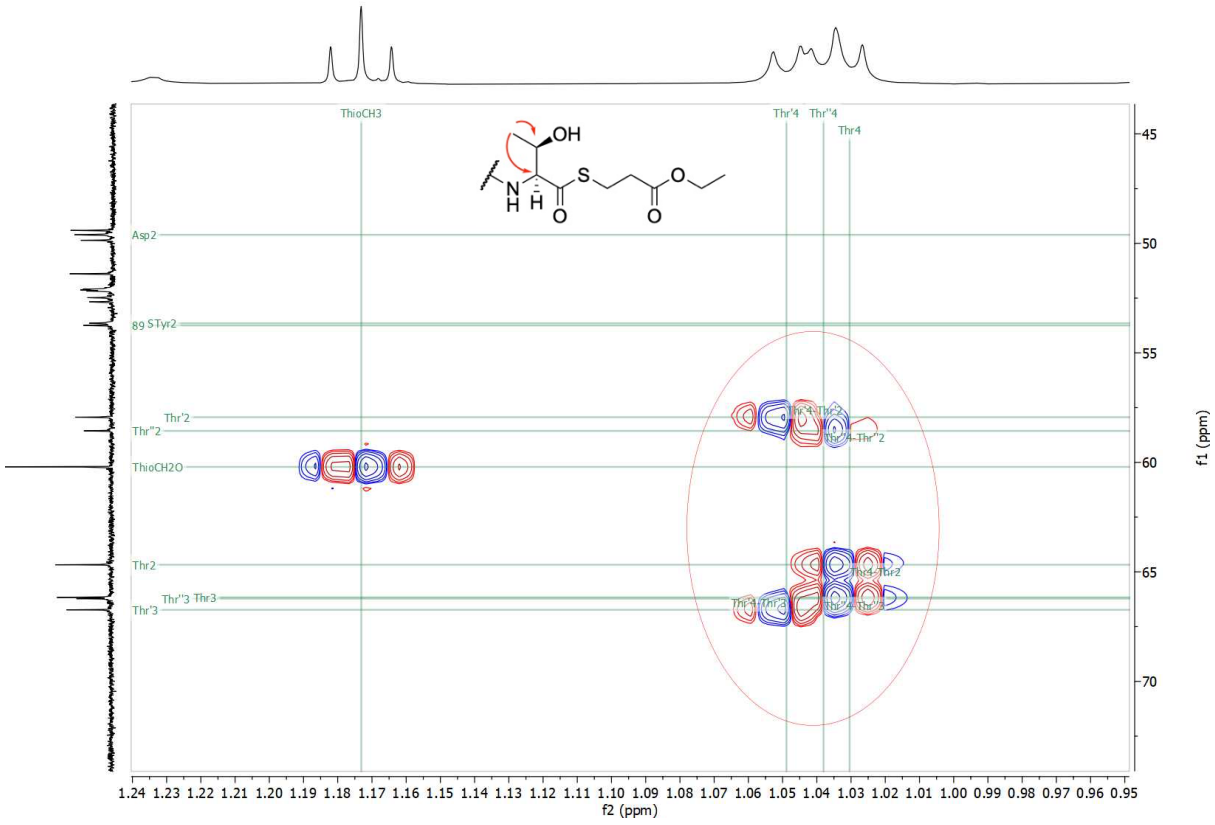
27	Acquired Size	(2048, 512)
28	Spectral Size	(2048, 2048)
29	Digital Resolution	(3.91, 21.61)


Thr'2
Thr2
Thr3
Thr''2
Thr''3
ThioCH2O
Thr'3
Gly2'
Gly2''
STyr'3'
STyr3'
Thio3
STyr'3''
Asp3'
STyr3''
Gly1
Thr'1
Asp4
Asp1
Thio1
Thio3-Thio1
Thr1
Thr2-Thr1
Thio3-Thr1
OH
N
H
H
S
O
O
O
165
170
175
180
185
190
195
200
205
f1 (ppm)
4.4
4.3
4.2
4.1
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
3.0
2.9
2.8
2.7
2.6
f2 (ppm)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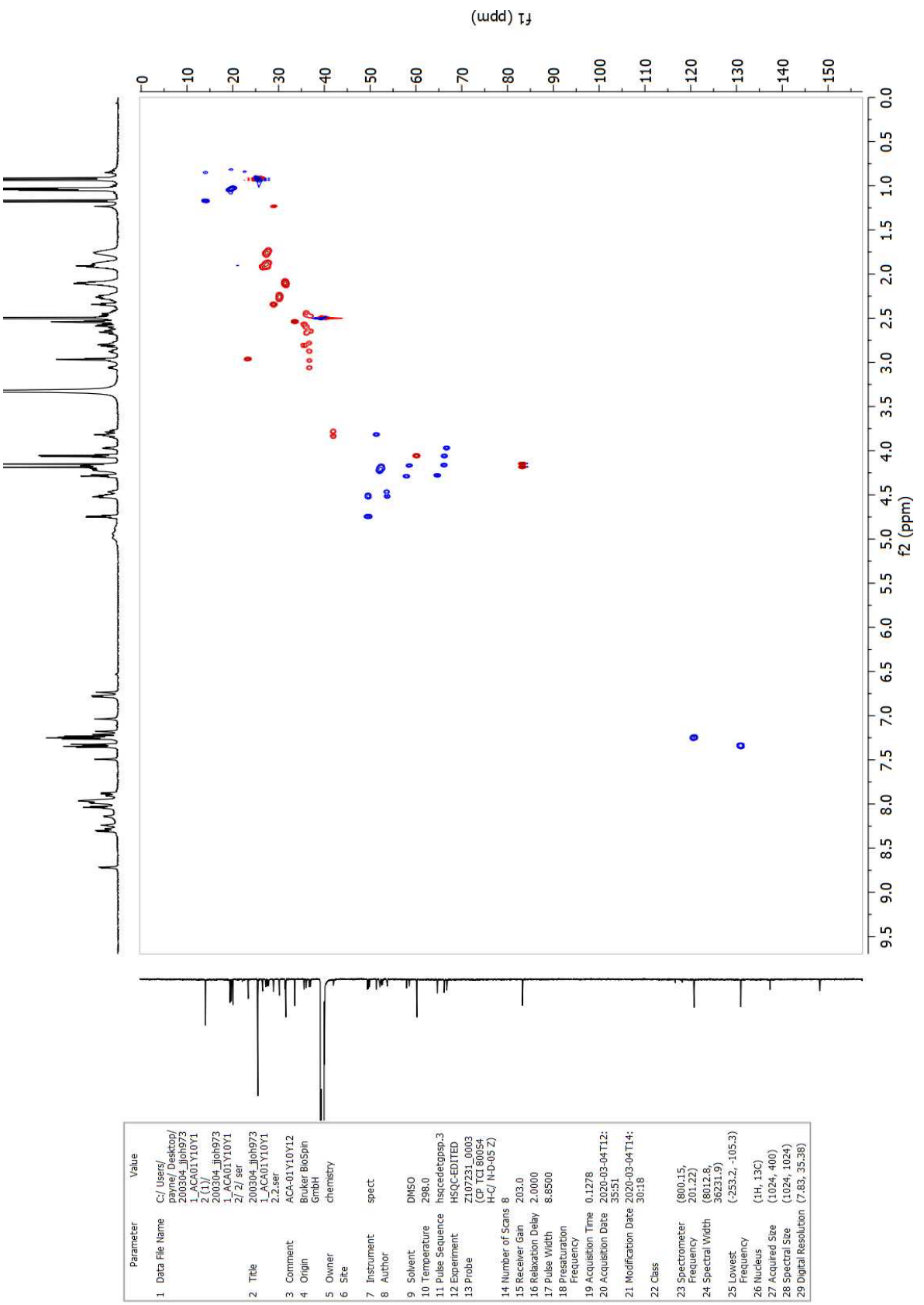
Thr'4
Thr''4
Thr4
OH
N
H
H
S
O
O
O
Asp2
89 STyr2
Thr'2
Thr''2
ThioCH2O
Thr2
Thr''3 Thr3
Thr'3
Thr4-Thr2
Thr''4-Thr''2
Thr4-Thr3
Thr''4-Thr''3
45
50
55
60
65
70
f1 (ppm)
1.24
1.23
1.22
1.21
1.20
1.19
1.18
1.17
1.16
1.15
1.14
1.13
1.12
1.11
1.10
1.09
1.08
1.07
1.06
1.05
1.04
1.03
1.02
1.01
1.00
0.99
0.98
0.97
0.96
0.95
f2 (ppm)

HSQC of ACA-01 Tyr10+Tyr12-sulfated thioester (9) (800/200 MHz, d_6-DMSO)



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3	Comment	ACA-01Y10Y12
4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
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11	Pulse Sequence	hsqcedetgpsp.3
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13	Probe	Z107231_0003 (CP TCI 80054 H-C/ N-D-05 Z)
14	Number of Scans	8
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24	Spectral Width	(8012.8, 36231.9)
25	Lowest Frequency	(-253.2, -105.3)
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27	Acquired Size	(1024, 400)
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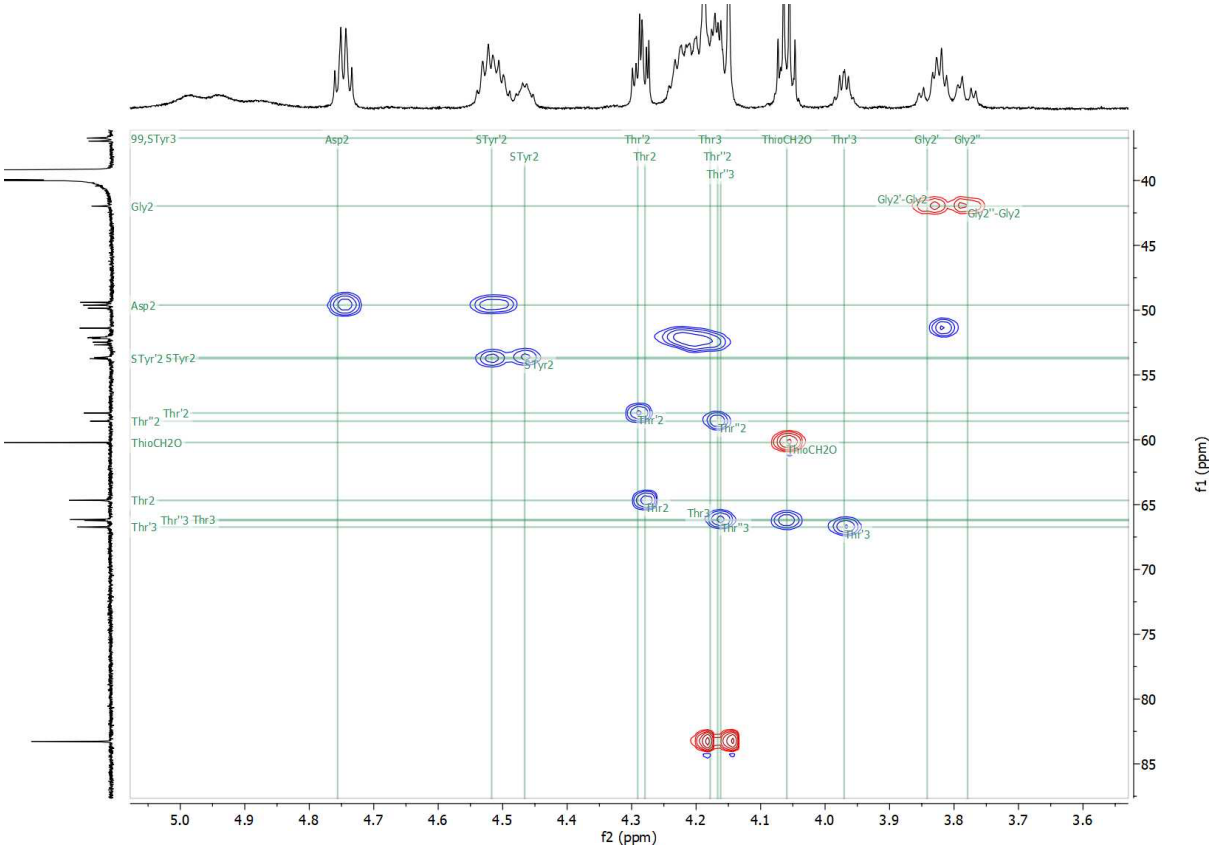
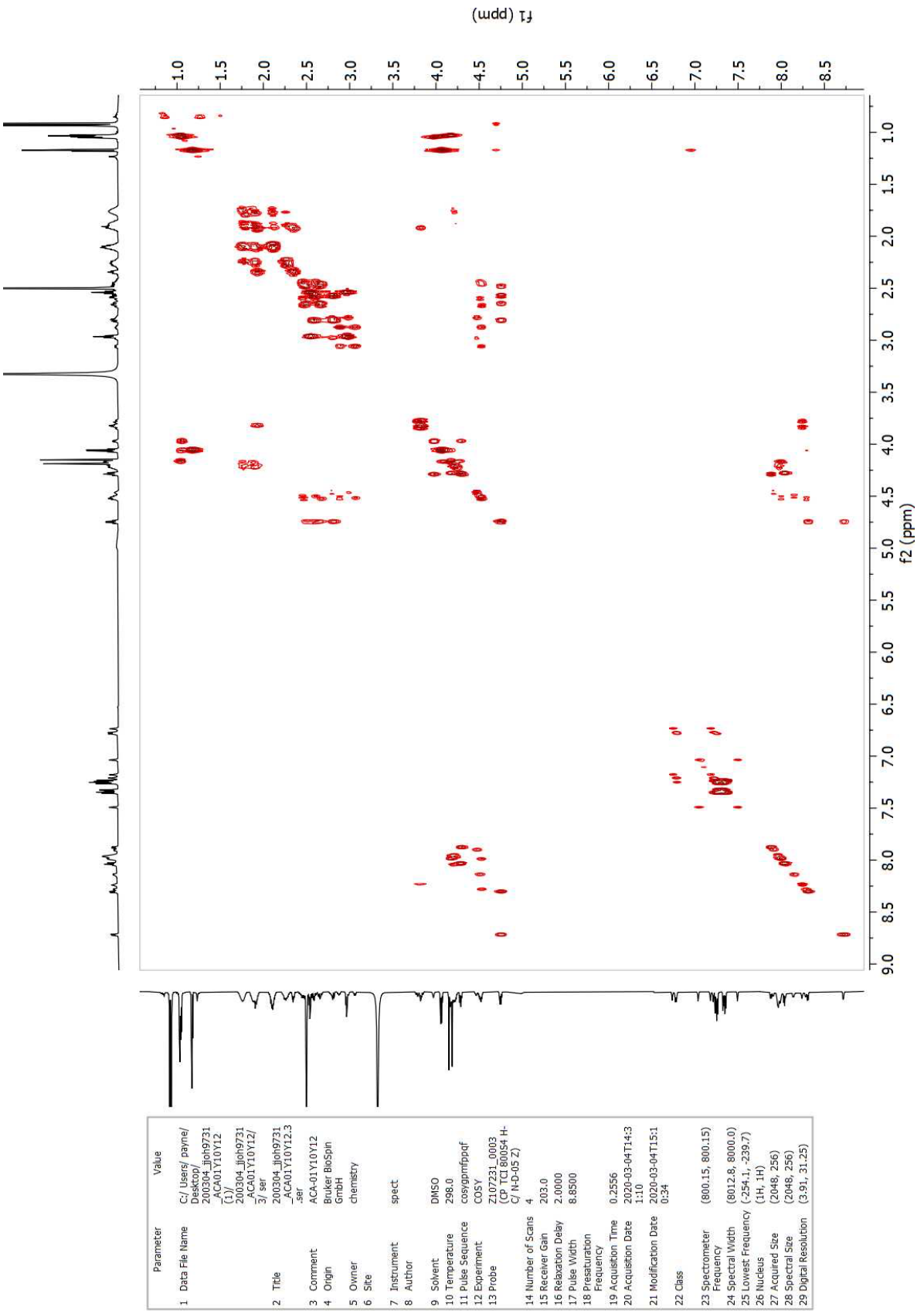
29	Digital Resolution	(7.83, 35.38)


99,STyr3
Asp2
STyr'2
STyr2
Thr'2
Thr2
Thr3
Thr''2
Thr''3
ThioCH2O
Thr'3
Gly2''
Gly2
Gly2'-Gly2
Gly2''-Gly2
Asp2
STyr'2 STyr2
Thr''2
Thr'2
ThioCH2O
Thr2
Thr'3
Thr''3 Thr3
f2 (ppm)
f1 (ppm)

COSY of ACA-01 Tyr10+Tyr12-sulfated thioester (9) (800 MHz, d_6-DMSO)



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7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	cosygpmfppqf
12	Experiment	COSY
13	Probe	Z107231_0003 (CP TCI 800S4 H-C/ N-D-05 Z)
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15	Receiver Gain	203.0
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18	Presaturation Frequency	
19	Acquisition Time	0.2556
20	Acquisition Date	2020-03-04T14:3 1:10
21	Modification Date	2020-03-04T15:1 0:34
22	Class	
23	Spectrometer Frequency	(800.15, 800.15)
24	Spectral Width	(8012.8, 8000.0)
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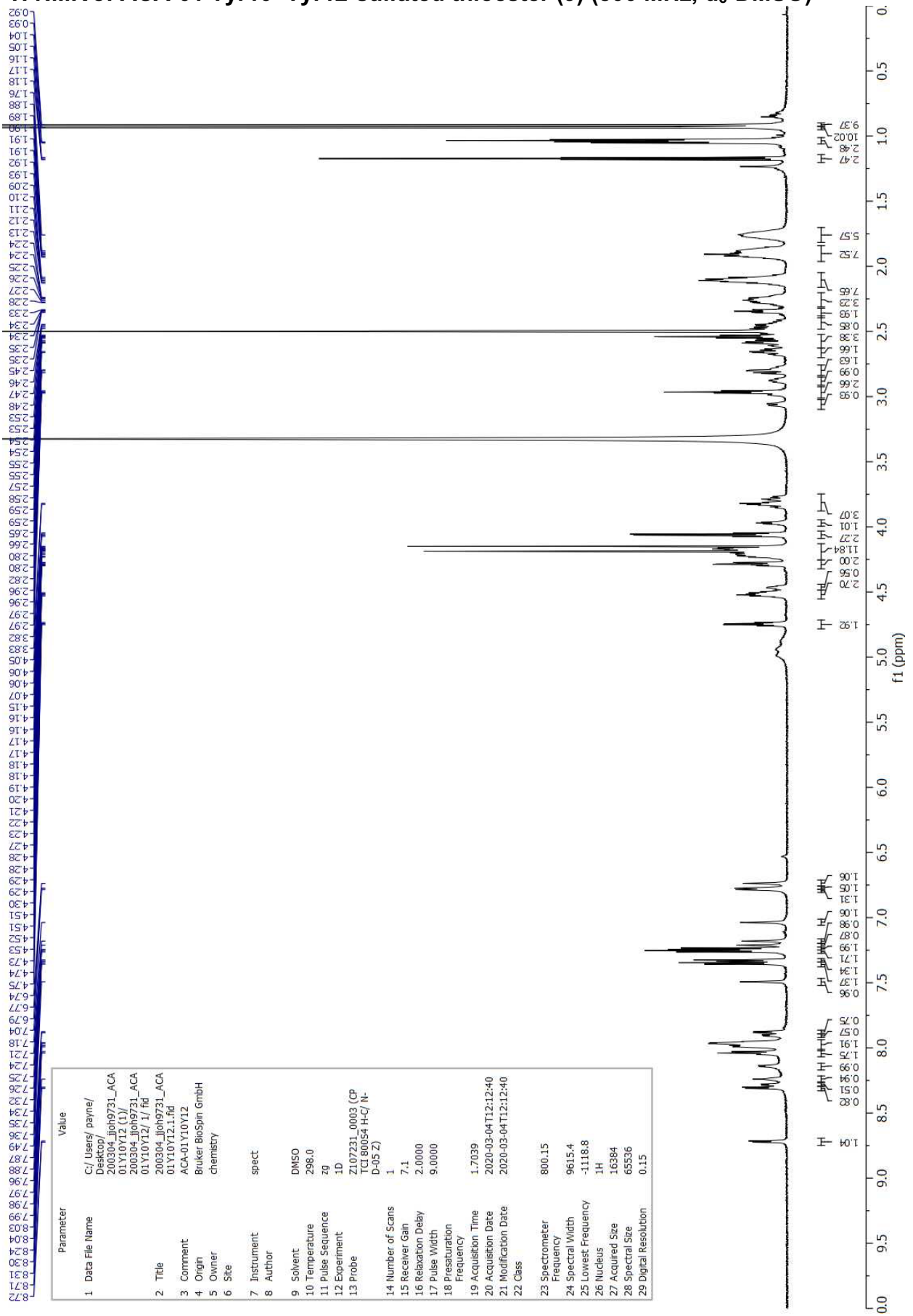
26	Nucleus	(1H, 1H)
27	Acquired Size	(2048, 256)
28	Spectral Size	(2048, 256)
29	Digital Resolution	(3.91, 31.25)

^{1}H NMR of ACA-01 Tyr10+Tyr12-sulfated thioester (9) (800 MHz, d_6-DMSO)



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4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
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12	Experiment	1D
13	Probe	Z107231_0003 (CP TCI 80054 H-C/ N-D-05 Z)
14	Number of Scans	1
15	Receiver Gain	7.1
16	Relaxation Delay	2.0000
17	Pulse Width	9.0000
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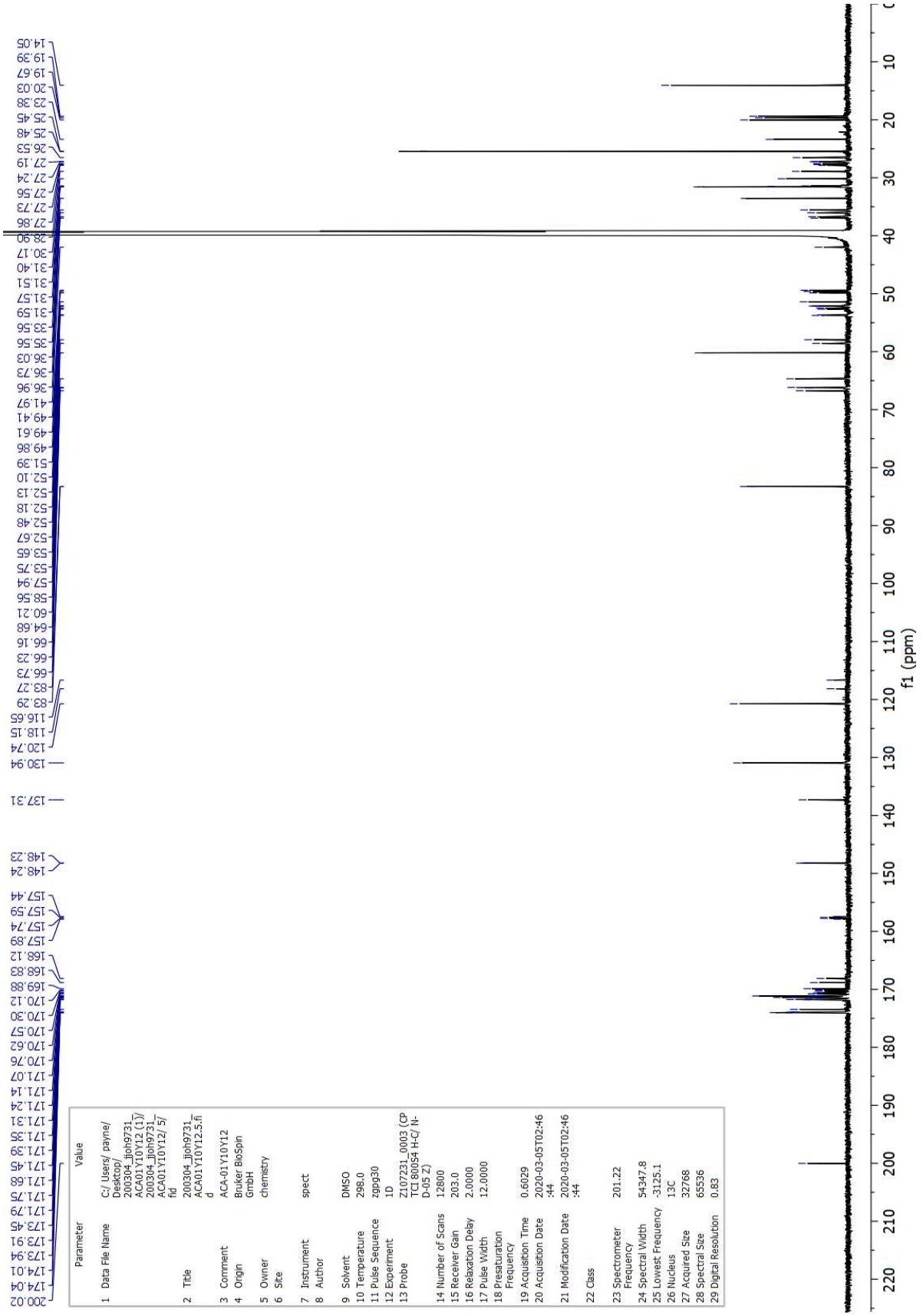
22	Class	
23	Spectrometer Frequency	800.15
24	Spectral Width	9615.4
25	Lowest Frequency	-1118.8
26	Nucleus	1H
27	Acquired Size	16384
28	Spectral Size	65536
29	Digital Resolution	0.15

^{13}C NMR of ACA-01 Tyr10+Tyr12-sulfated thioester (9) (200 MHz, d_6-DMSO)



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4	Origin	Bruker BioSpin GmbH
5	Owner	chemistry
6	Site	
7	Instrument	spect
8	Author	
9	Solvent	DMSO
10	Temperature	298.0
11	Pulse Sequence	zgpg30
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13	Probe	Z107231_0003 (CP TCI 80054 H-C/ N-D-05 Z)
14	Number of Scans	12800
15	Receiver Gain	203.0
16	Relaxation Delay	2.0000
17	Pulse Width	12.0000
18	Presaturation Frequency	
19	Acquisition Time	0.6029
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21	Modification Date	2020-03-05T02:46 :44
22	Class	
23	Spectrometer Frequency	201.22
24	Spectral Width	54347.8
25	Lowest Frequency	-3125.1
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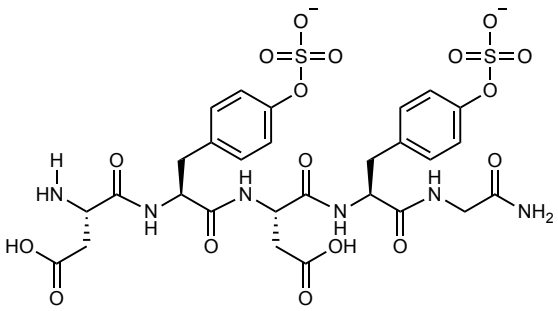
29	Digital Resolution	0.83

Synthesis of sulfated H-DYDYG-NH_2 fragment of ACA-01



H-DsYDsYG-NH_2 (15)

Peptide **15** was prepared by *via* Fmoc-strategy SPPS whereby Rink Amide resin (100 µmol, 135 mg, 0.74 mmol g^{-1}) was treated with 40 vol.% piperidine, 0.1 M HOBt in DMF (1.6 mL) for 3 min, drained, and then treated with 20 vol.% piperidine, 0.1 M HOBt in DMF (1.6 mL) for 10 min , drained, and washed with DMF (4 x 1.6 mL). The resin was then treated with a solution of Fmoc-Xaa-OH (400 µmol, 4 eq.) and Oxyma (57 mg, 400 µmol, 4 eq.) in DMF (800 µL), followed by a solution of DIC (63 µL, 400 µmol, 4 eq.) in DMF (800 µL) and shaken at 50 °C for 30 min. The resin was then drained and washed with DMF (4 x 1.6 mL) before being treated with a solution of 5 vol.% Ac_2O and 10 vol.% *i*Pr_2NEt in DMF (1.6 mL) for 5 min at rt, drained, washed with DMF (4 x 1.6 mL) and drained. The final residue was then Fmoc-deprotected by treatment with 20 vol.% piperidine, 0.1 M HOBt (3 mL) for 2 x 5 min at rt. The resin was then washed with DMF (5 x 3 mL) and CH_2Cl_2 (10 x 3 mL) before a portion of the resin (20 µmol) was cleaved with 90:5:5 v/v/v TFA:*i*Pr_3SiH:H_2O for 2 hours at room temperature. The resin was filtered and the deprotection solution evaporated under nitrogen flow before precipitating the crude peptide with diethyl ether (30 mL). The crude peptide was collected *via* centrifugation and then dissolved in aqueous 2 M NH_4OAc (5 mL) and heated at 50 °C for 16 h to remove the neopentyl protecting groups. Purification of the crude peptide by preparative RP-HPLC (0 to 40% B over 40 min, 0.1% formic acid) afforded **15** as a white solid after lyophilisation (1.13 mg, 8.2% yield).

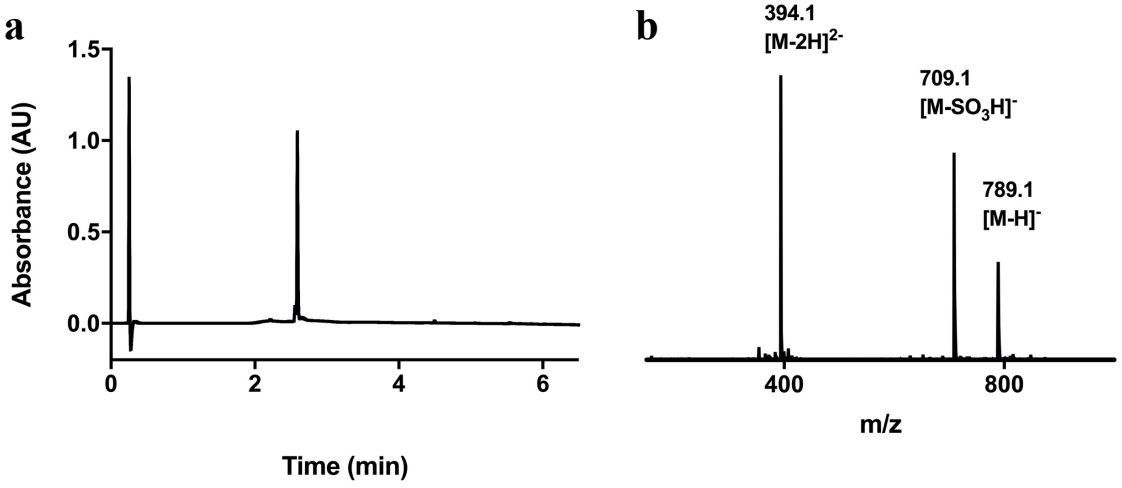


Figure S6. Analytical HPLC trace (**a**) and mass spectrum (**b***)* of purified peptide **15**. Analytical HPLC Rt 2.59 min (0 to 60% over 5 min, 0.1% TFA, λ = 214 nm). Calculated Mass $[M-2H]^{2-}$: 393.36, $[M-H]^-$: 787.7, Mass Found (ESI) 394.1 $[M-2H]^{2-}$, 789.1 $[M-H]^-$, 709.1 $[M-SO_3H]^-$

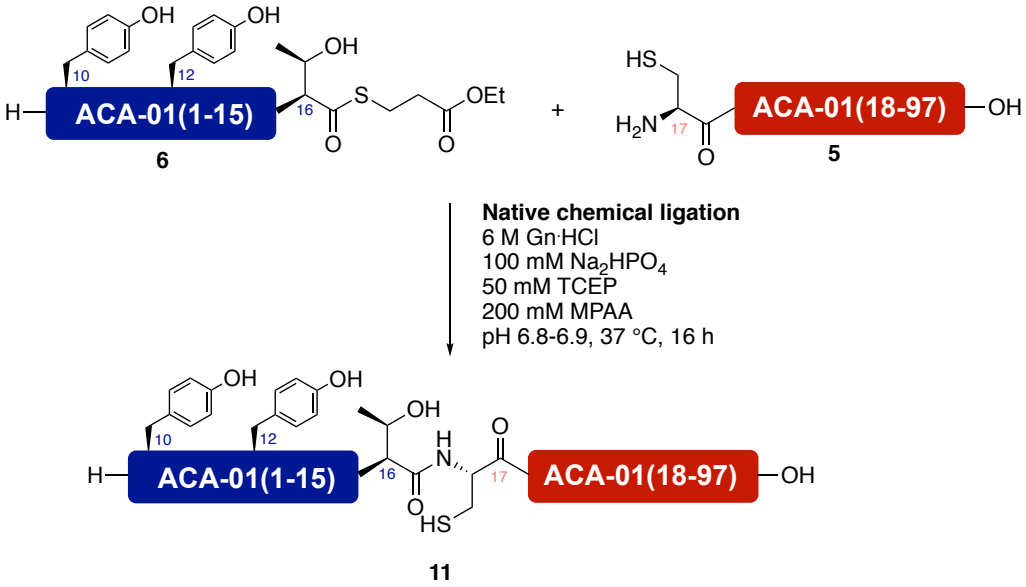
General procedures for native chemical ligation

A 2.5 mM solution of ACA-01 C-terminus (17-97) in ligation buffer (6 M Gdn·HCl, 100 mM Na_2HPO_4, 50 mM TCEP, 200 mM MPAA, adjusted to pH 7.2) was added to the appropriate thioester (2 equiv). The resulting solution was carefully readjusted to pH 6.7-6.9 with NaOH and incubated at 37 °C overnight. After complete conversion to the desired ligated product (judged by UPLC-MS analysis) the solution was diluted and purified by reversed-phase HPLC.

Analytical chromatography: Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector, operating at 215 or 280 nm, and 2475 FLR Detector operating at excitation of 280 nm and emission of 347 nm**,** fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} or Waters Acquity UPLC system fitted with Waters Acquity UPLC BEH 1.7 µm 2.1 x 50 mm small pore column (C-18) at a flow rate of 0.6 mL min^{-1}. For both a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) was used.

Semi-preparative chromatography: Waters HPLC fitted with a Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 250 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetonitrile (Solvent B).

Synthesis of ACA-01 Unsulfated via ligation



The native chemical ligation of **6** (0.9 mg, 0.44 µmol) and **5** (2 mg, 0.22 µmol) was performed according to the general procedure. Purification *via* semi-preparative HPLC (0-50% B over 50 min, 10 mM NH_3) followed by lyophilization afforded the target protein ACA-01 Unsulfated (**11**) as a white solid (1.0mg, 41%). Crude reaction and purified UV absorbance trace were obtained using Waters Acquity UPLC system fitted with a Waters Acquity UPLC BEH 300 Å, 1.7 µm, 2.1 x 50 mm C18 column (0-50% B over 5 min, 0.1% formic acid, λ=215 nm) at a flow rate of 0.6 mL min^{-1}. FLR trace was obtained using a Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector and 2475 FLR Detector, fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} (0-50% B over 30 min, 0.1% formic acid, exλ=280 nm emλ=347 nm). Low-resolution mass spectra of crude reaction and purified **11** were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. **11** was purified *via* semi-preparative reversed-phase HPLC using Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 250 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 50 min.

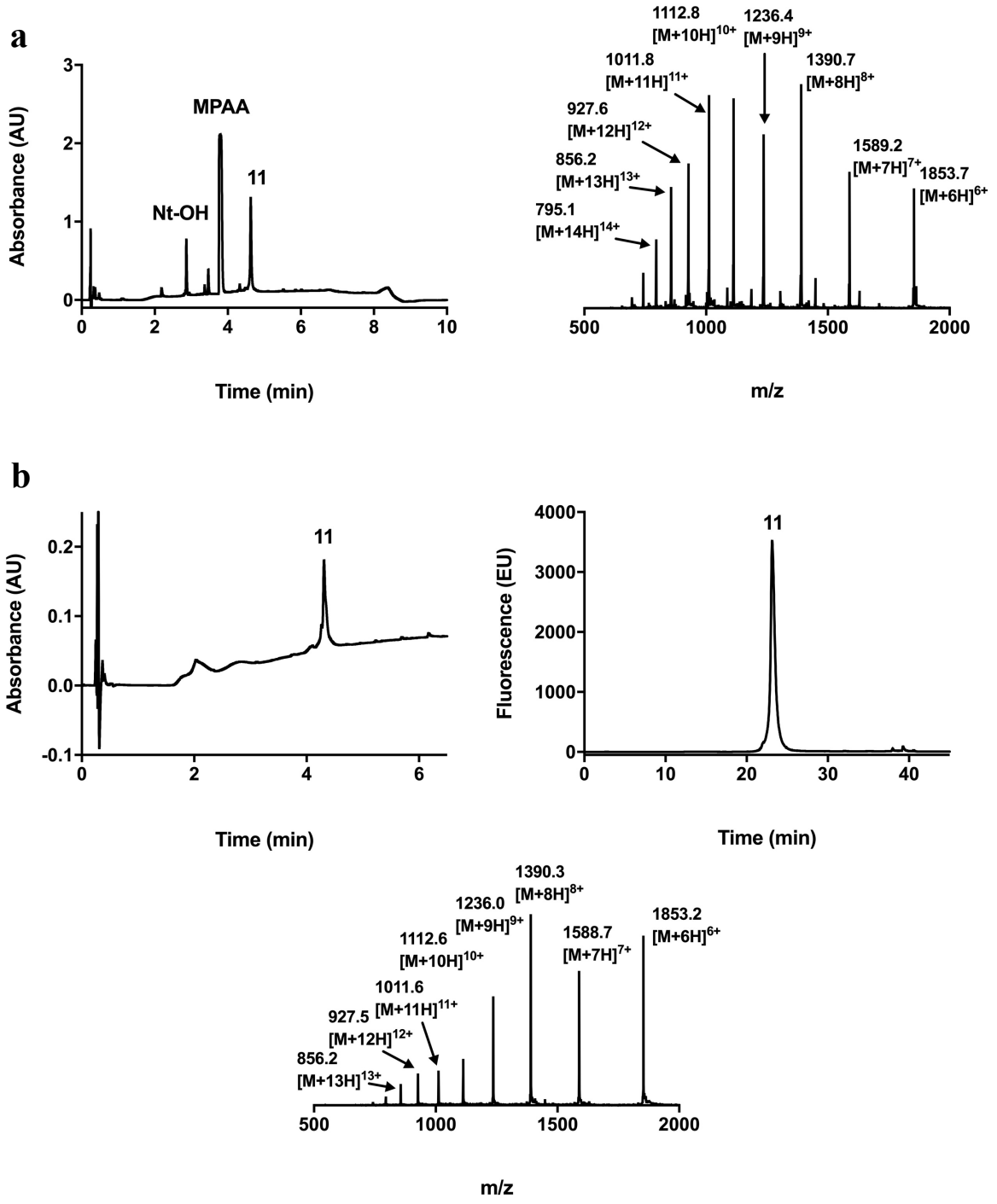
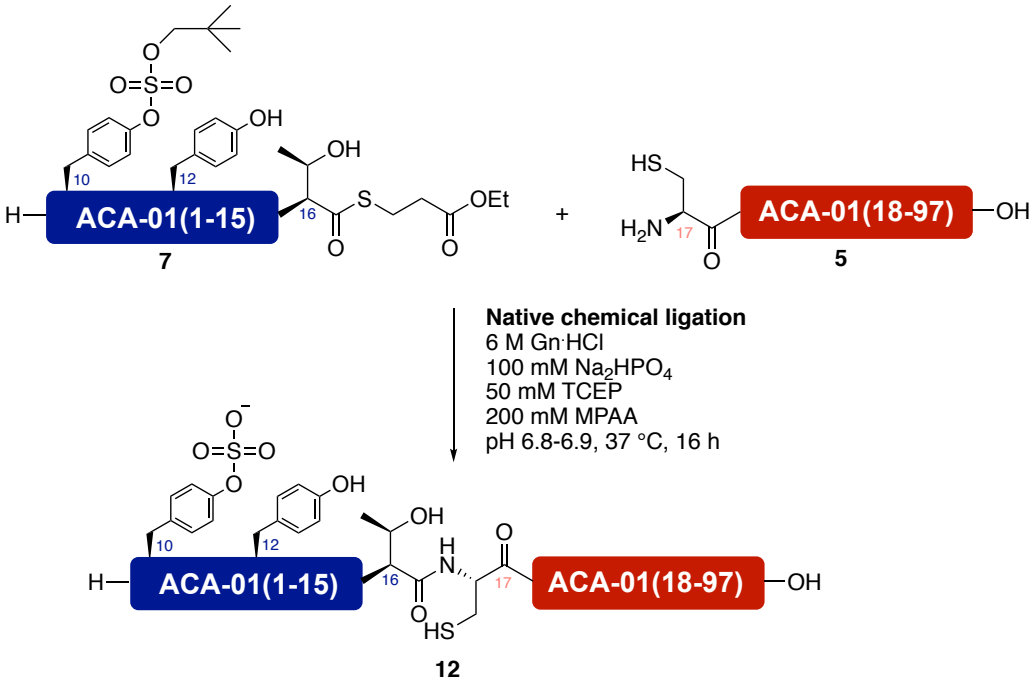


Figure S7. Analytical HPLC trace and mass spectrum of (**a**) crude ligation and (**b**) purified ACA-01 Unsulfated (**11**). UPLC (Purified final product) R_t 4.27 min (0-50% B over 30 minutes, 0.1% formic acid, λ=215 nm). Analytical HPLC (Purified final product) R_t 23.16 min (0-50% B over 30 minutes, 0.1% formic acid, exλ=280 nm emλ=347 nm). Calculated Mass $[M+6H]^{6+}$:1853.9, $[M+7H]^{7+}$: 1589.2, $[M+8H]^{8+}$: 1390.7, $[M+9H]^{9+}$: 1236.3, $[M+10H]^{10+}$: 1112.7, $[M+11H]^{11+}$: 1011.7, $[M+12H]^{12+}$: 927.5, $[M+13H]^{13+}$: 856.2, $[M+14H]^{14+}$: 795.1, Mass Found (ESI) 1853.4 $[M+6H]^{6+}$, 1588.9 $[M+7H]^{7+}$, 1390.4 $[M+8H]^{8+}$, 1236.1 $[M+9H]^{9+}$, 1112.6 $[M+10H]^{10+}$, 1011.6 $[M+11H]^{11+}$, 927.4 $[M+12H]^{12+}$, 856.1 $[M+13H]^{13+}$.

Synthesis of ACA-01 Tyr10-sulfated via ligation



The native chemical ligation of **7** (0.97 mg, 0.44 µmol) and **5** (2 mg, 0.22 µmol) was performed according to the general procedure. Purification *via* semi-preparative HPLC (0-40% B over 50 min, 10 mM NH_3) followed by lyophilization afforded the target sulfoprotein ACA-01 Y10-sulfated (**12**) as a white solid (1.5 mg, 60%). Crude reaction and purified UV absorbance trace were obtained using Waters Acquity UPLC system fitted with a Waters Acquity UPLC BEH 300 Å, 1.7 µm, 2.1 x 50 mm C18 column (0-50% B over 5 min, 0.1% formic acid, λ=215 nm) at a flow rate of 0.6 mL min^{-1}. FLR trace was obtained using a Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector and 2475 FLR Detector, fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} (0-50% B over 30 min, 0.1% formic acid, exλ=280 nm emλ=347 nm). Low-resolution mass spectra of crude reaction and purified **12** were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. **12** was purified *via* semi-preparative reversed-phase HPLC using Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 250 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetonitrile (Solvent B) and a linear gradient of 0-40% B over 50 min.

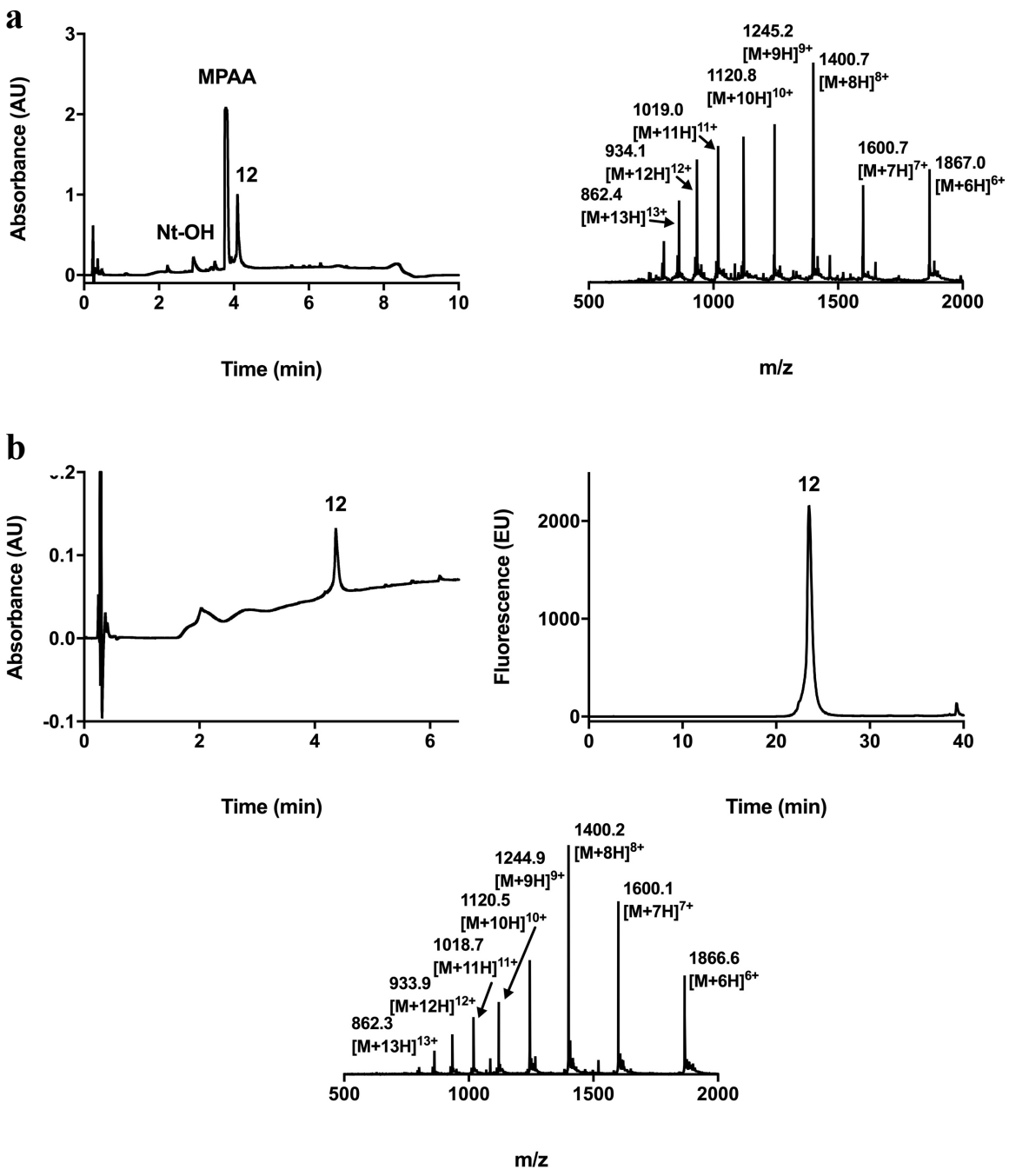
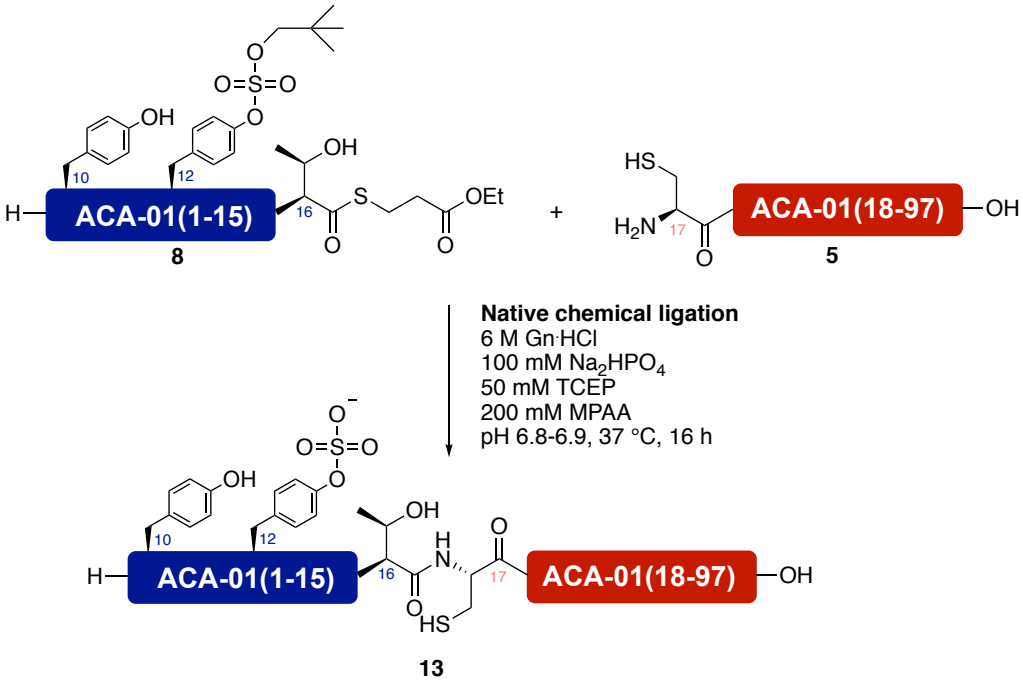


Figure S8. Analytical HPLC trace and mass spectrum of (**a**) crude ligation and (**b**) purified ACA-01 Tyr10-sulfated (**12**). UPLC (Purified final product) R_t 4.38 min (0-50% B over 5 minutes, 0.1% formic acid, λ=215 nm). Analytical HPLC (Purified final product) R_t 23.55 min (0-50% B over 30 minutes, 0.1% formic acid, exλ=280 nm emλ=347 nm). Calculated Mass $[M+6H]^{6+}$:1867.2, $[M+7H]^{7+}$: 1600.6, $[M+8H]^{8+}$: 1400.7, $[M+9H]^{9+}$: 1245.2, $[M+10H]^{10+}$: 1120.7, $[M+11H]^{11+}$: 1018.9, $[M+12H]^{12+}$: 934.1, $[M+13H]^{13+}$: 862.3, Mass Found (ESI) 1866.6 $[M+6H]^{6+}$, 1600.1 $[M+7H]^{7+}$, 1400.2 $[M+8H]^{8+}$, 1244.9 $[M+9H]^{9+}$, 1120.5 $[M+10H]^{10+}$, 1018.7 $[M+11H]^{11+}$, 933.9 $[M+12H]^{12+}$, 862.3 $[M+13H]^{13+}$.

Synthesis of ACA-01 Tyr12-sulfated via ligation



The native chemical ligation of **8** (2.34 mg, 0.92 µmol) and **5** (5 mg, 0.54 µmol) was performed according to the general procedure. Purification *via* semi-preparative HPLC (0-40% B over 50 min, 10 mM NH_3) followed by lyophilization afforded the target sulfoprotein ACA-01 Y12-sulfated (**13**) as a white solid (3.1 mg, 51%). Crude reaction and purified UV absorbance trace were obtained using Waters Acquity UPLC system fitted with a Waters Acquity UPLC BEH 300 Å, 1.7 µm, 2.1 x 50 mm C18 column (0-50% B over 5 min, 0.1% formic acid, λ=215 nm) at a flow rate of 0.6 mL min^{-1}. FLR trace was obtained using a Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector and 2475 FLR Detector, fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} (0-50% B over 30 min, 0.1% formic acid, exλ=280 nm emλ=347 nm). Low-resolution mass spectra of crude reaction and purified **13** were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. **13** was purified *via* semi-preparative reversed-phase HPLC using Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 250 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetonitrile (Solvent B) and a linear gradient of 0-40% B over 50 min.

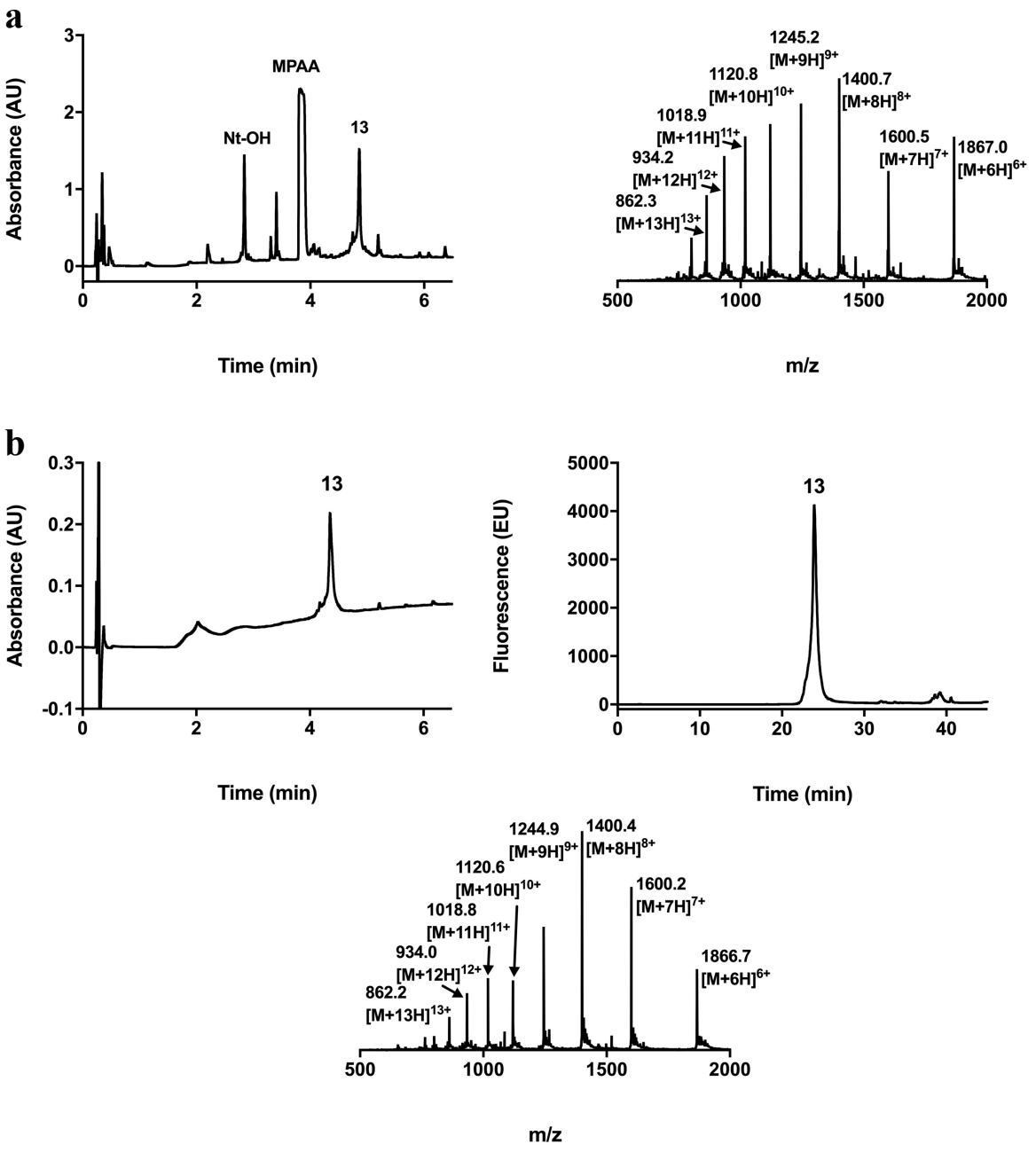
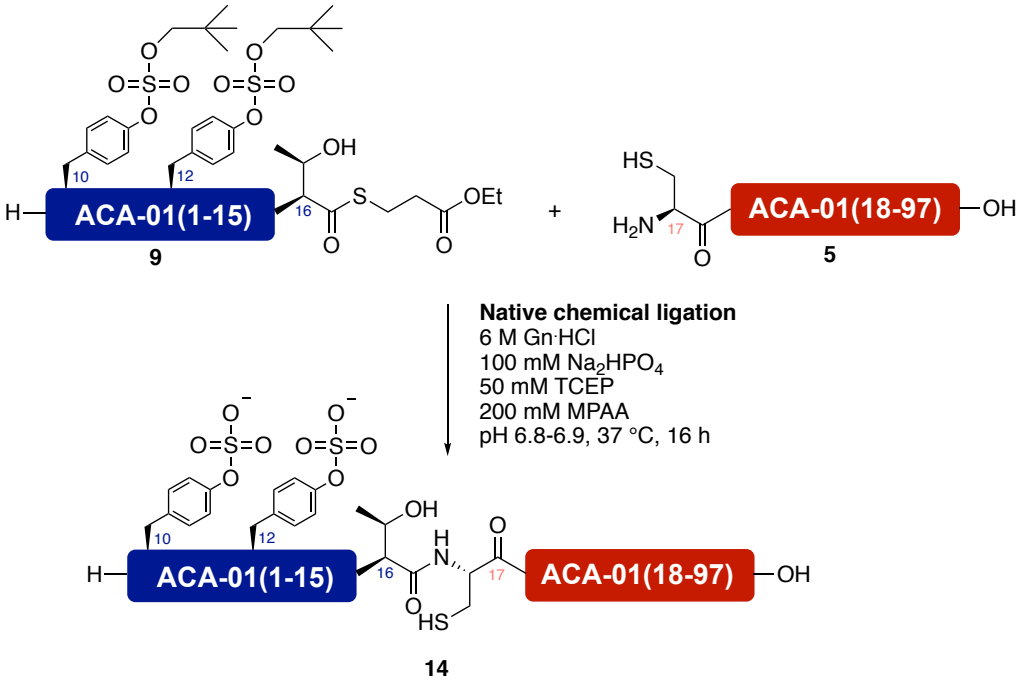


Figure S9. Analytical HPLC trace and mass spectrum of (**a**) crude ligation and (**b**) purified ACA-01 Tyr12-sulfated (**13**). UPLC (Purified final product) R_t 4.44 min (0-50% B over 5 minutes, 0.1% formic acid, λ=215 nm). Analytical HPLC (Purified final product) R_t 23.55 min (0-50% B over 30 minutes, 0.1% formic acid, exλ=280 nm emλ=347 nm). Calculated Mass $[M+6H]^{6+}$:1867.2, $[M+7H]^{7+}$: 1600.6, $[M+8H]^{8+}$: 1400.7, $[M+9H]^{9+}$: 1245.2, $[M+10H]^{10+}$: 1120.7, $[M+11H]^{11+}$: 1018.9, $[M+12H]^{12+}$: 934.1, $[M+13H]^{13+}$: 862.3, Mass Found (ESI) 1866.7 $[M+6H]^{6+}$, 1600.2 $[M+7H]^{7+}$, 1400.4 $[M+8H]^{8+}$, 1244.9 $[M+9H]^{9+}$, 1120.6 $[M+10H]^{10+}$, 1018.8 $[M+11H]^{11+}$, 934.0 $[M+12H]^{12+}$, 862.2 $[M+13H]^{13+}$.

Synthesis of ACA-01 Tyr10+Tyr12-sulfated via ligation



The native chemical ligation of **9** (1.69 mg, 0.72 µmol) and **5** (3.3 mg, 0.35 µmol) was performed according to the general procedure. Purification *via* semi-preparative HPLC (0-50% B over 70 min, 10 mM NH_3) followed by lyophilization afforded the target sulfoprotein ACA-01 doubly-sulfated (**14**) as a white solid (1.55 mg, 38%). Crude reaction trace and purified UV absorbance trace were obtained using Waters Acquity UPLC system fitted with a Waters Acquity UPLC BEH 300 Å, 1.7 µm, 2.1 x 50 mm C18 column (0-50% B over 5 min, 0.1% formic acid, λ=215 nm) at a flow rate of 0.6 mL min^{-1}. FLR trace was obtained using a Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector and 2475 FLR Detector, fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} (0-50% B over 30 min, 0.1% formic acid, exλ=280 nm emλ=347 nm). Low-resolution mass spectra of crude reaction and purified **14** were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. **14** was purified *via* semi-preparative reversed-phase HPLC using Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 250 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 70 min.

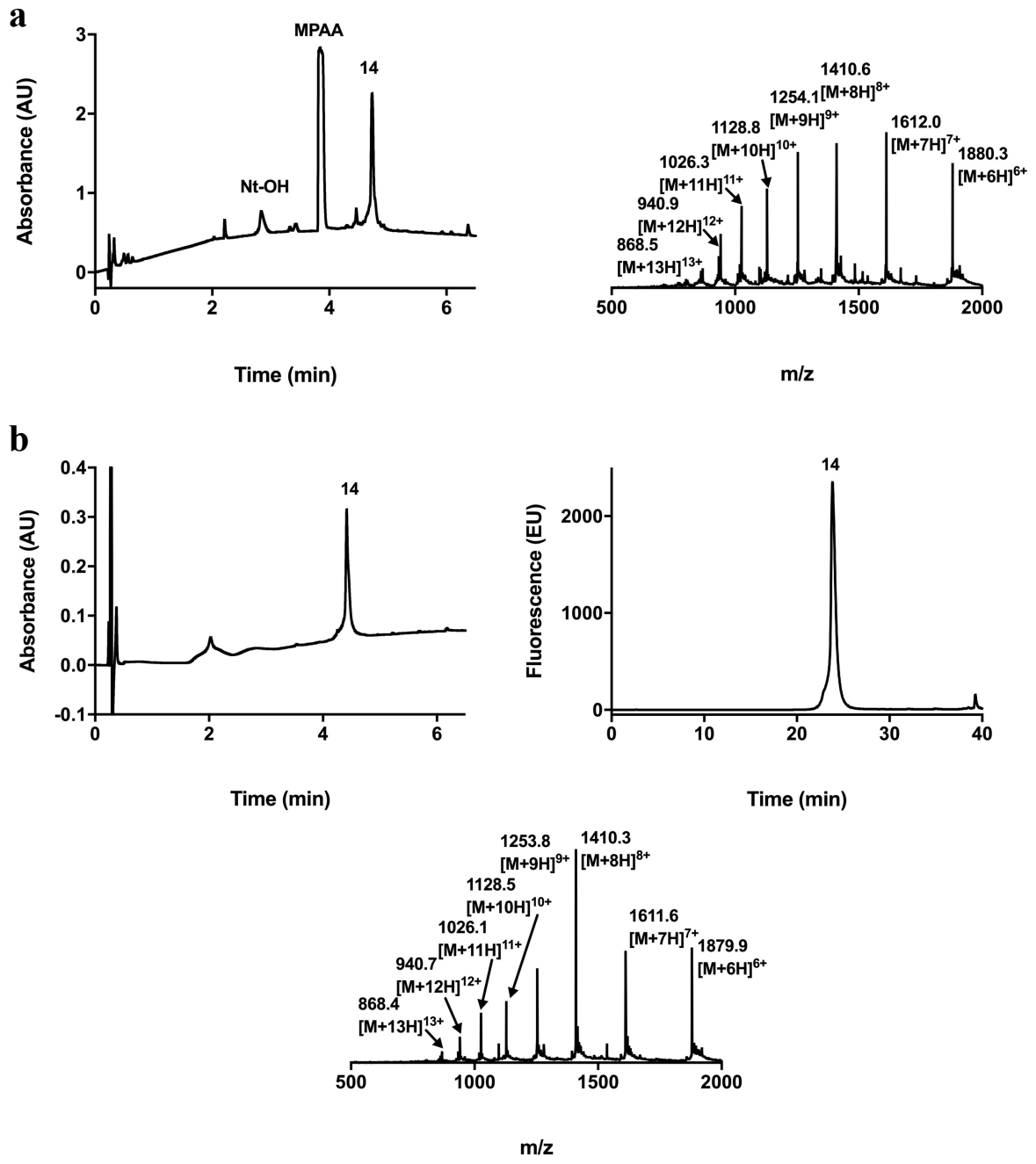


Figure S10. Analytical HPLC trace and mass spectrum of (**a**) crude ligation and (**b**) purified ACA-01 Tyr10 and Tyr12 sulfated (**14**). UPLC (Purified final product) R_t 4.40 min (0-50% B over 5 minutes, 0.1% formic acid, λ=215 nm). Analytical HPLC (Purified final product) R_t 23.88 min (0-50% B over 30 minutes, 0.1% formic acid, exλ=280 nm emλ=347 nm). Calculated Mass $[M+6H]^{6+}$:1880.6, $[M+7H]^{7+}$: 1612.1, $[M+8H]^{8+}$: 1410.7, $[M+9H]^{9+}$: 1254.0, $[M+10H]^{10+}$: 1128.7, $[M+11H]^{11+}$: 1026.2, $[M+12H]^{12+}$: 940.8, $[M+13H]^{13+}$: 868.5, Mass Found (ESI) 1879.9 $[M+6H]^{6+}$, 1611.6 $[M+7H]^{7+}$, 1410.3 $[M+8H]^{8+}$, 1253.8$[M+9H]^{9+}$, 1128.5$[M+10H]^{10+}$, 1026.1 $[M+11H]^{11+}$, 940.7 $[M+12H]^{12+}$, 868.4 $[M+13H]^{13+}$.

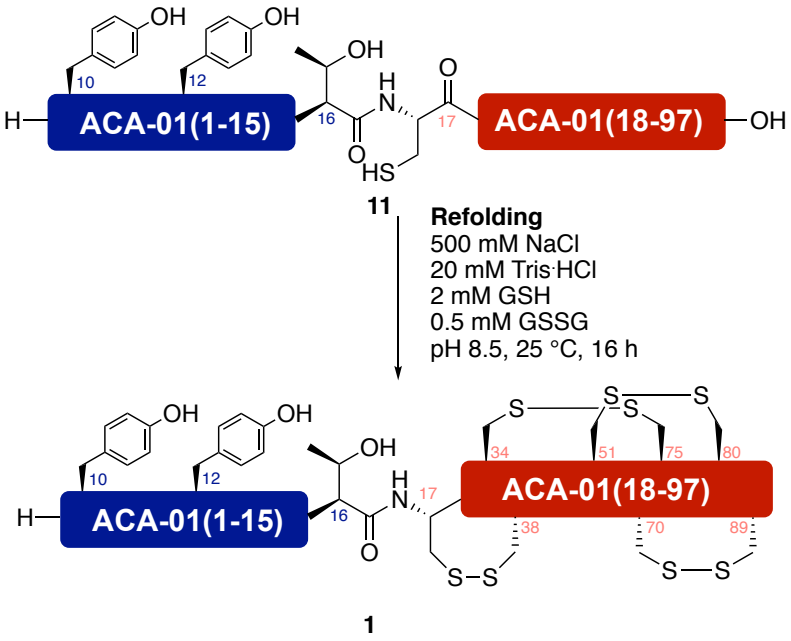
General procedures for refolding

The lyophilized proteins **11-14** were reconstituted in refolding conditions containing 500 mM NaCl, 20 mM Tris·HCl, 2 mM L-glutathione, 0.5 mM glutathione disulphide, pH 8.5 at a final concentration of 0.1 mg mL^{-1} and incubated overnight at room temperature. After complete conversion to the refolded proteins **1-4** (judged by UPLC-MS analysis), the solution was purified by reversed-phase HPLC.

Analytical chromatography: Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector, operating at 215 or 280 nm, and 2475 FLR Detector operating at excitation of 280 nm and emission of 347 nm*,* fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1}, or Waters Acquity UPLC system fitted with Waters Acquity UPLC BEH 1.7 µm 2.1 x 50 mm small pore column (C-18) at a flow rate of 0.6 mL min^{-1}. For both a mobile phase of 0.1% formic acid in water (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) was used.

Semi-preparative chromatography: Waters HPLC fitted with a Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 250 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetonitrile (Solvent B).

Refolding of Unsulfated ACA-01



The refolding of **11** (1.7 mg) was performed according to the general procedure. Purification *via* semi-preparative HPLC (0-50% B over 70 min, 10 mM NH_3) followed by lyophilization afforded the target folded unsulfated ACA-01 (**1**) as a white solid (0.55 mg, 32%; 14% over the ligation and refolding steps). Crude and purified traces were obtained using a Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector and 2475 FLR Detector, fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} (0-50% B over 30 min, 0.1% formic acid, exλ=280 nm emλ=347 nm or λ=215 nm). Low-resolution mass spectra of crude folding and purified **1** were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. **1** was purified *via* semi-preparative reversed-phase HPLC using Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 250 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 70 min.

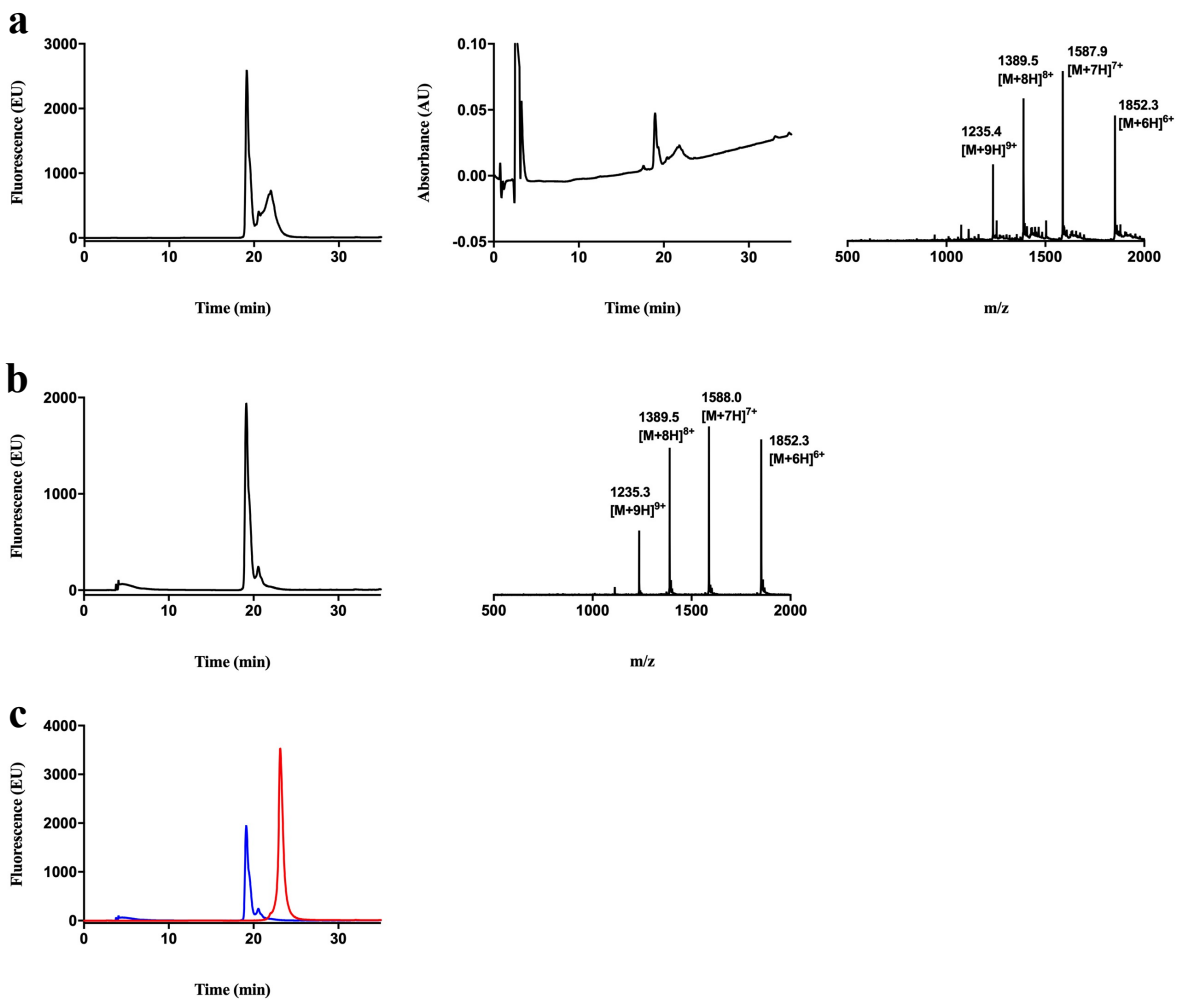
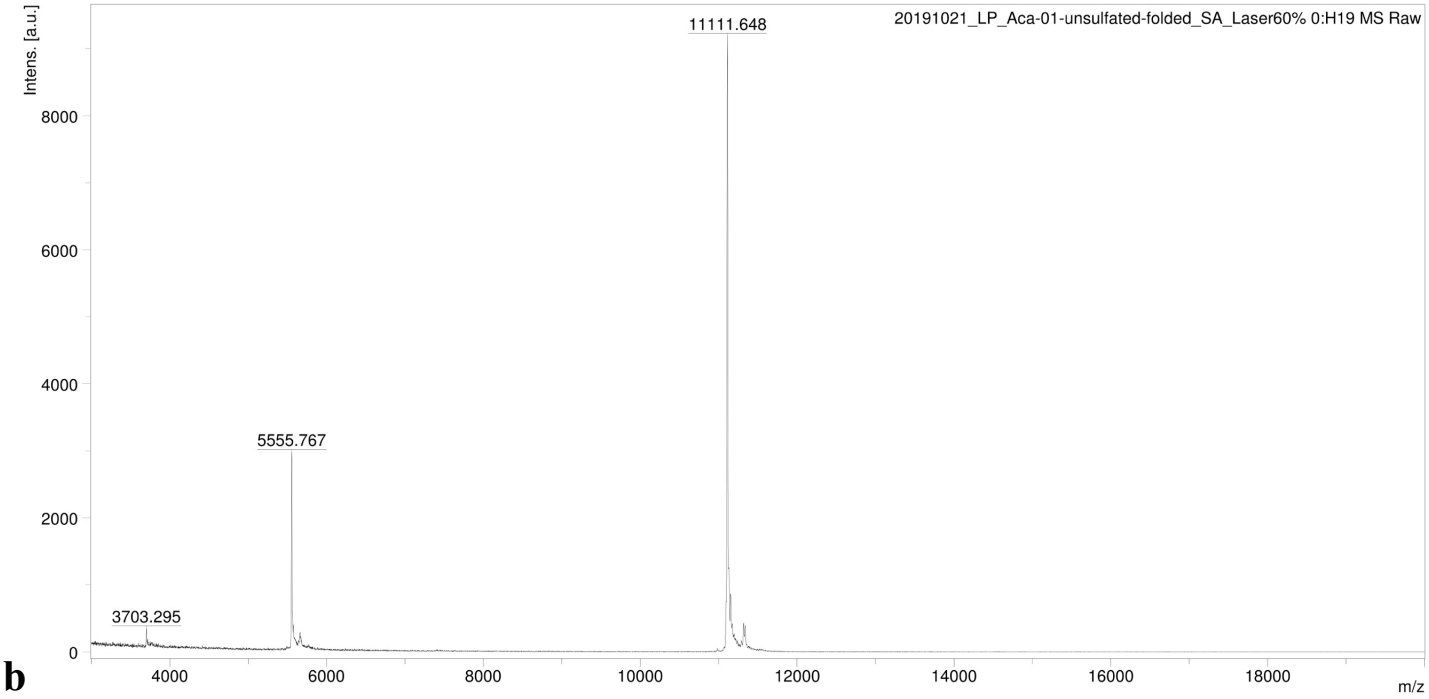


Figure S11. Analytical HPLC trace and mass spectrum of (**a**) crude refolding and (**b**) purified, folded ACA-01 Unsulfated (**1**). Comparison of both unfolded and folded conditions is shown in (**c**). Analytical HPLC (Purified final product) R_t 19.18 min (0-50% B over 30 minutes**,** 0.1% formic acid, exλ=280 nm emλ=347 nm). Calculated Mass $[M+6H]^{6+}$:1852.6, $[M+7H]^{7+}$: 1588.1, $[M+8H]^{8+}$: 1389.7, $[M+9H]^{9+}$: 1235.4, Mass Found (ESI) 1852.3 $[M+6H]^{6+}$, 1588.0 $[M+7H]^{7+}$, 1389.5 $[M+8H]^{8+}$, 1235.3 $[M+9H]^{9+}$. NB: the purity of the final folded protein was 95.2% as judged by integration of the chromatogram. The minor peak (R_t = 20.57 min) corresponds to differentially sulfated variants (see below).

a



b

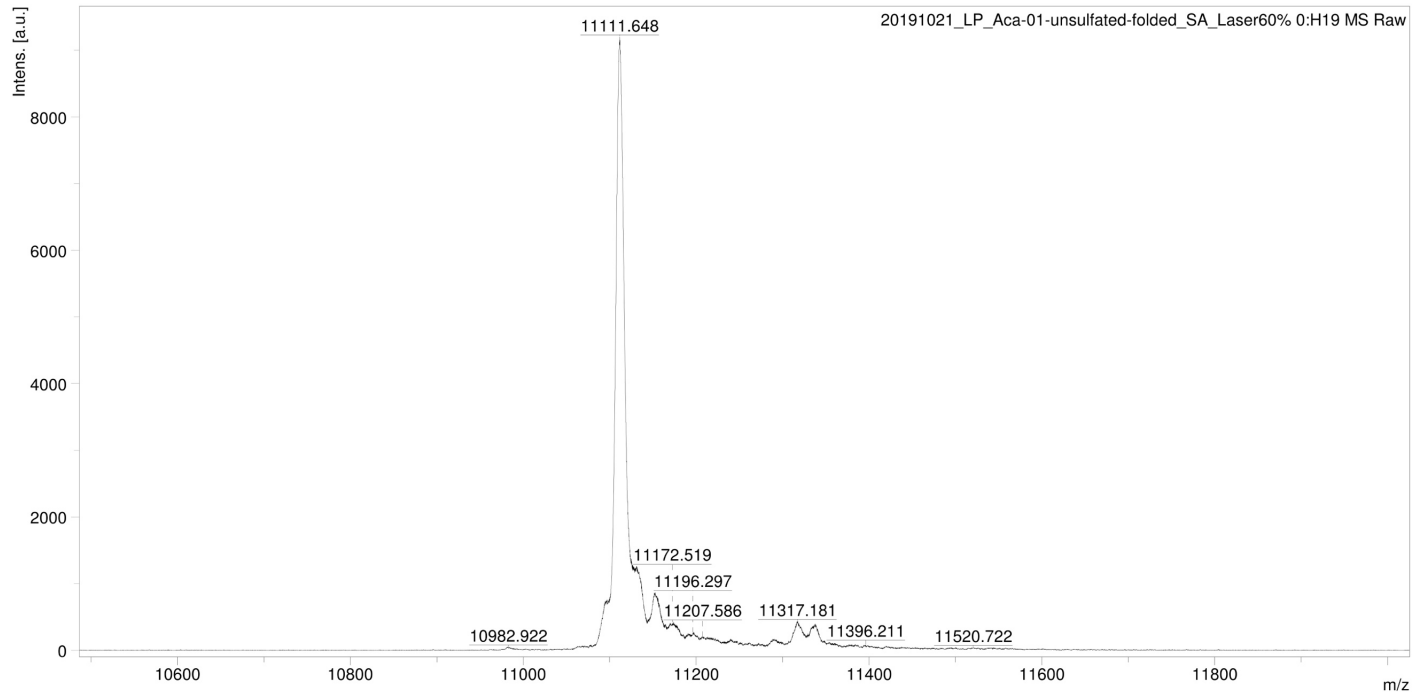
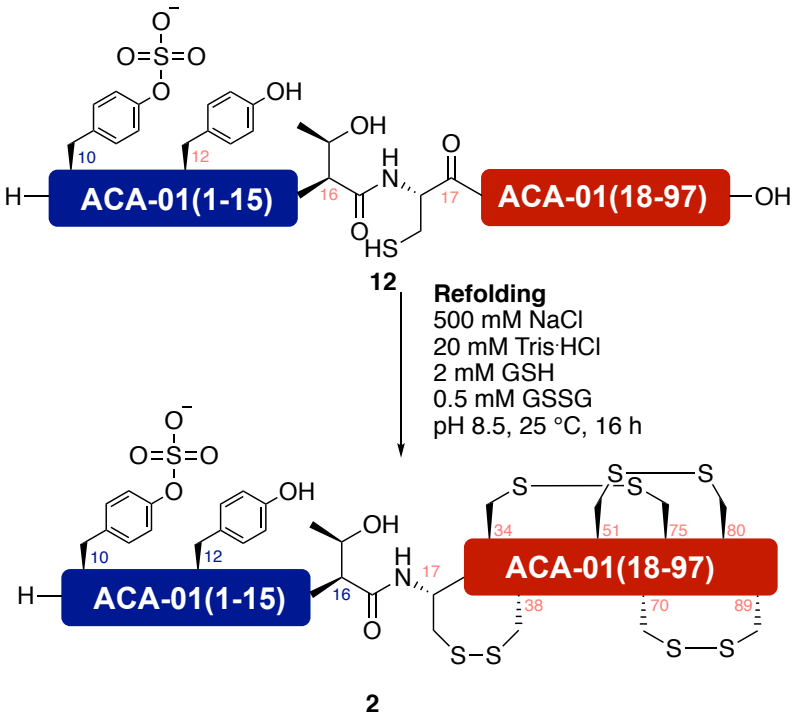


Figure S12. MALDI-TOF mass spectrum of ACA-01 Unsulfated (**1**) (**a**) 3000-20000 m/z (**b**) 10700-12000 m/z

Refolding of Tyr10-sulfated ACA-01



The refolding of **12** (1.6 mg) was performed according to the general procedure. Purification *via* semi-preparative HPLC (0-50% B over 70 min, 10 mM NH_3) followed by lyophilization afforded the target folded Y10-sulfated ACA-01 (**2**) as a white solid (0.78 mg, 48%, 26% over the ligation and refolding steps). Crude folding and purified UV absorbance trace were obtained using Waters Acquity UPLC system fitted with a Waters Acquity UPLC BEH 300 Å, 1.7 µm, 2.1 x 50 mm C18 column (0-50% B over 5 min, 0.1% formic acid, λ=215 nm) at a flow rate of 0.6 mL min^{-1}. Crude and purified FLR traces were obtained using a Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector and 2475 FLR Detector**,** fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} (0-50% B over 30 min, 0.1% formic acid, exλ=280 nm emλ=347 nm). Low-resolution mass spectra of crude folding and purified **2** were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. **2** was purified *via* semi-preparative reversed-phase HPLC using Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 25 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 70 min.

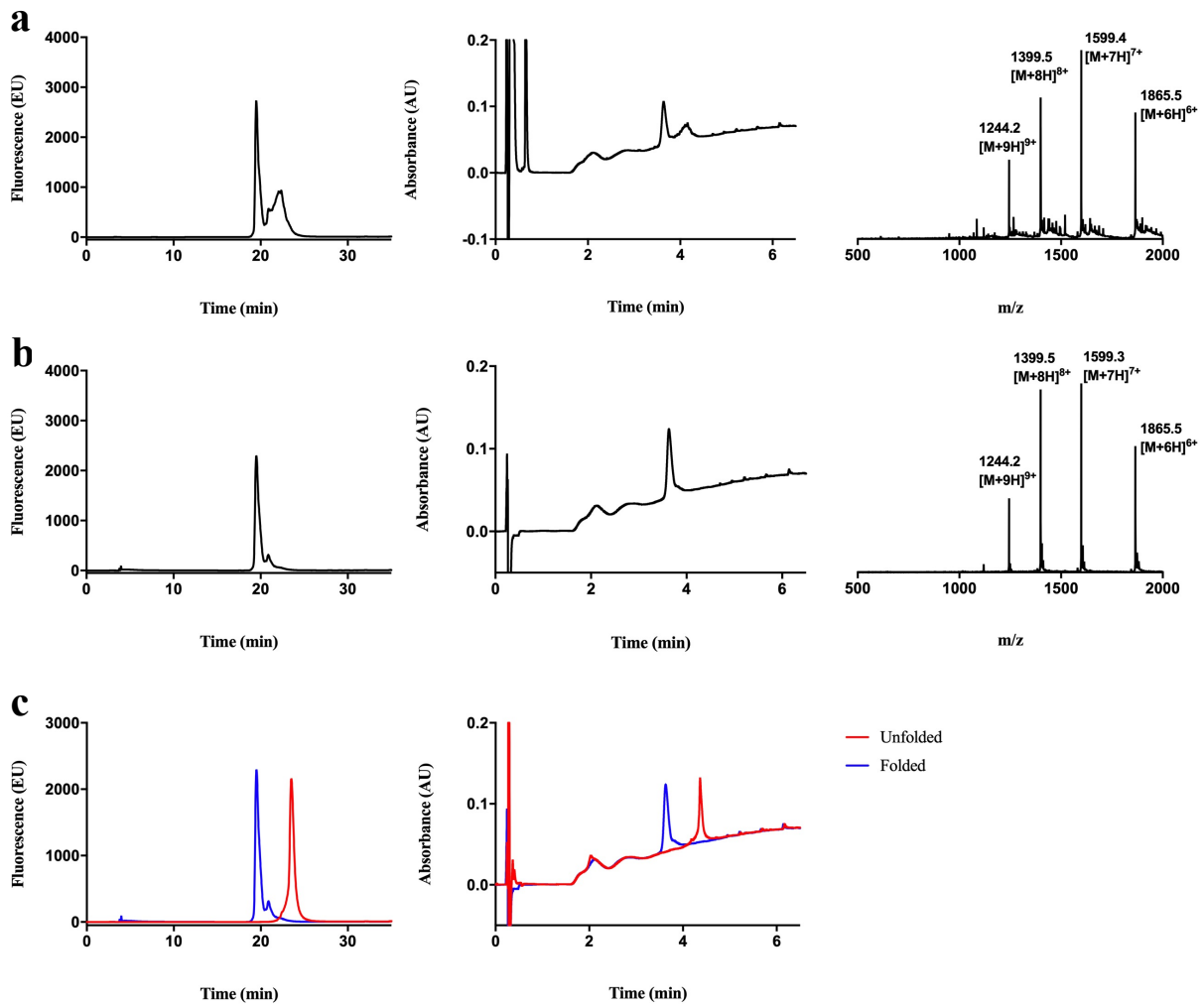
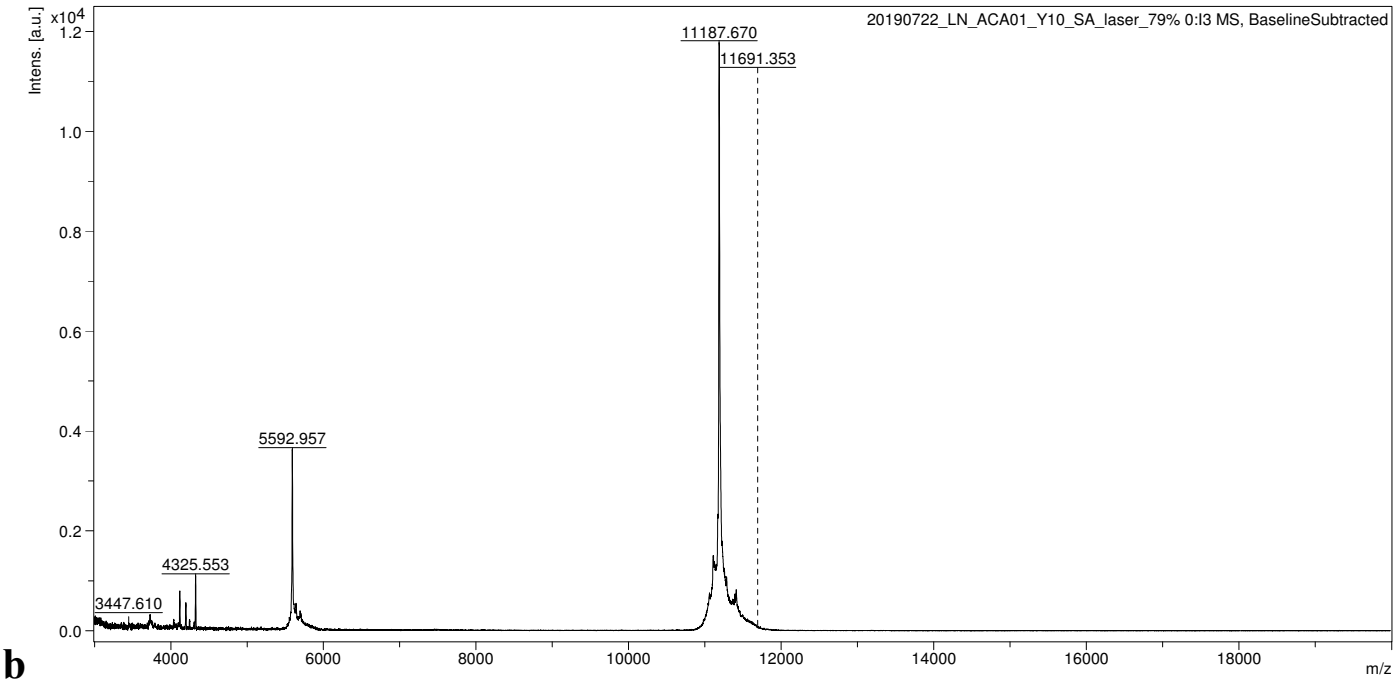


Figure S13. Analytical HPLC trace and mass spectrum of (**a**) crude refolding and (**b**) purified, folded Tyr10-sulfated ACA-01 (**2**). Comparison of both unfolded and folded conditions is shown in (**c**). Analytical HPLC (Purified final product) R_t 19.56 min (0-50% B over 30 minutes**,** 0.1% formic acid, exλ=280 nm emλ=347 nm). Calculated Mass $[M+6H]^{6+}$:1865.9, $[M+7H]^{7+}$: 1599.5, $[M+8H]^{8+}$: 1399.7, $[M+9H]^{9+}$: 1244.3, Mass Found (ESI) 1865.5 $[M+6H]^{6+}$, 1599.3 $[M+7H]^{7+}$, 1399.5 $[M+8H]^{8+}$, 1244.2 $[M+9H]^{9+}$. NB: the purity of the final folded protein was 95.3% as judged by integration of the chromatogram. The minor peak (R_t = 20.88 min) corresponds to differentially sulfated variants (see below).

a



b

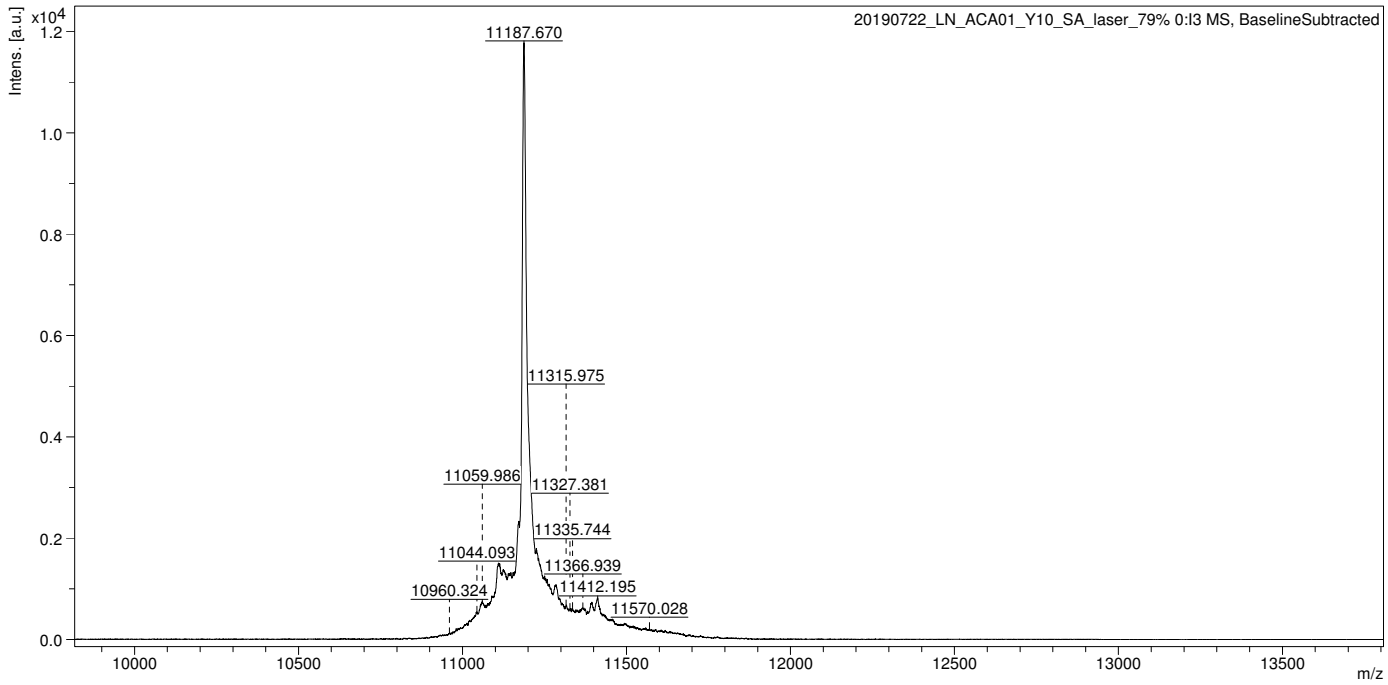
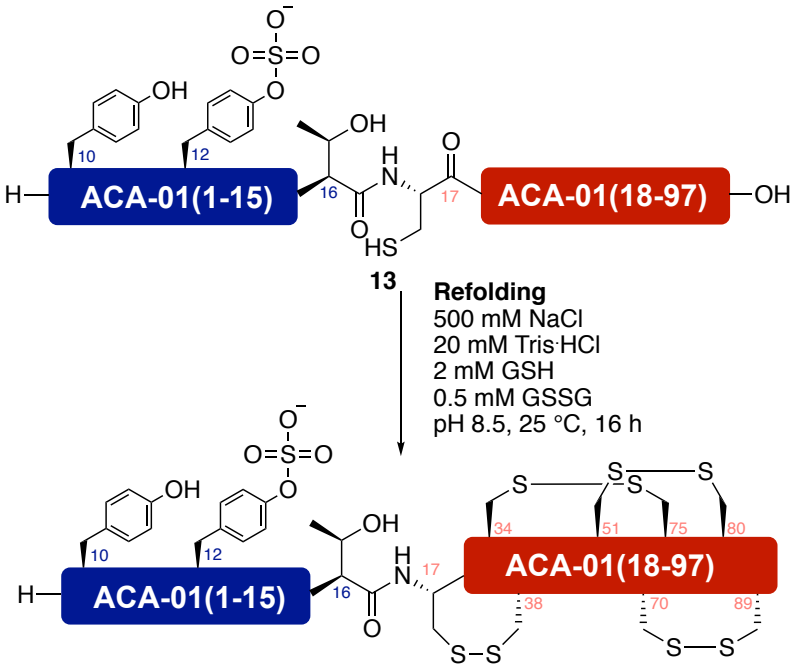


Figure S14. MALDI-TOF mass spectrum of Tyr10-sulfated ACA-01 (**2**) (**a**) 3000-20000 m/z (**b**) 9800-13800 m/z

Refolding of Tyr12-sulfated ACA-01



The refolding of **13** (1.17 mg) was performed according to the general procedure. Purification *via* semi-preparative HPLC (0-50% B over 70 min, 10 mM NH_3) followed by lyophilization afforded the target folded Y12-sulfated ACA-01 (**3**) as a white solid (0.57 mg, 48%, 24% over the ligation and refolding steps). Crude folding and purified UV absorbance trace were obtained using Waters Acquity UPLC system fitted with a Waters Acquity UPLC BEH 300 Å, 1.7 µm, 2.1 x 50 mm C18 column (0-50% B over 5 min, 0.1% formic acid, λ=215 nm) at a flow rate of 0.6 mL min^{-1}. Crude and purified FLR traces were obtained using a Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector and 2475 FLR Detector**,** fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} (0-50% B over 30 min, 0.1% formic acid, exλ=280 nm emλ=347 nm). Low-resolution mass spectra of crude folding and purified **3** were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. **3** was purified *via* semi-preparative reversed-phase HPLC using Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 250 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetontrile (Solvent B) and a linear gradient of 0-50% B over 70 min.

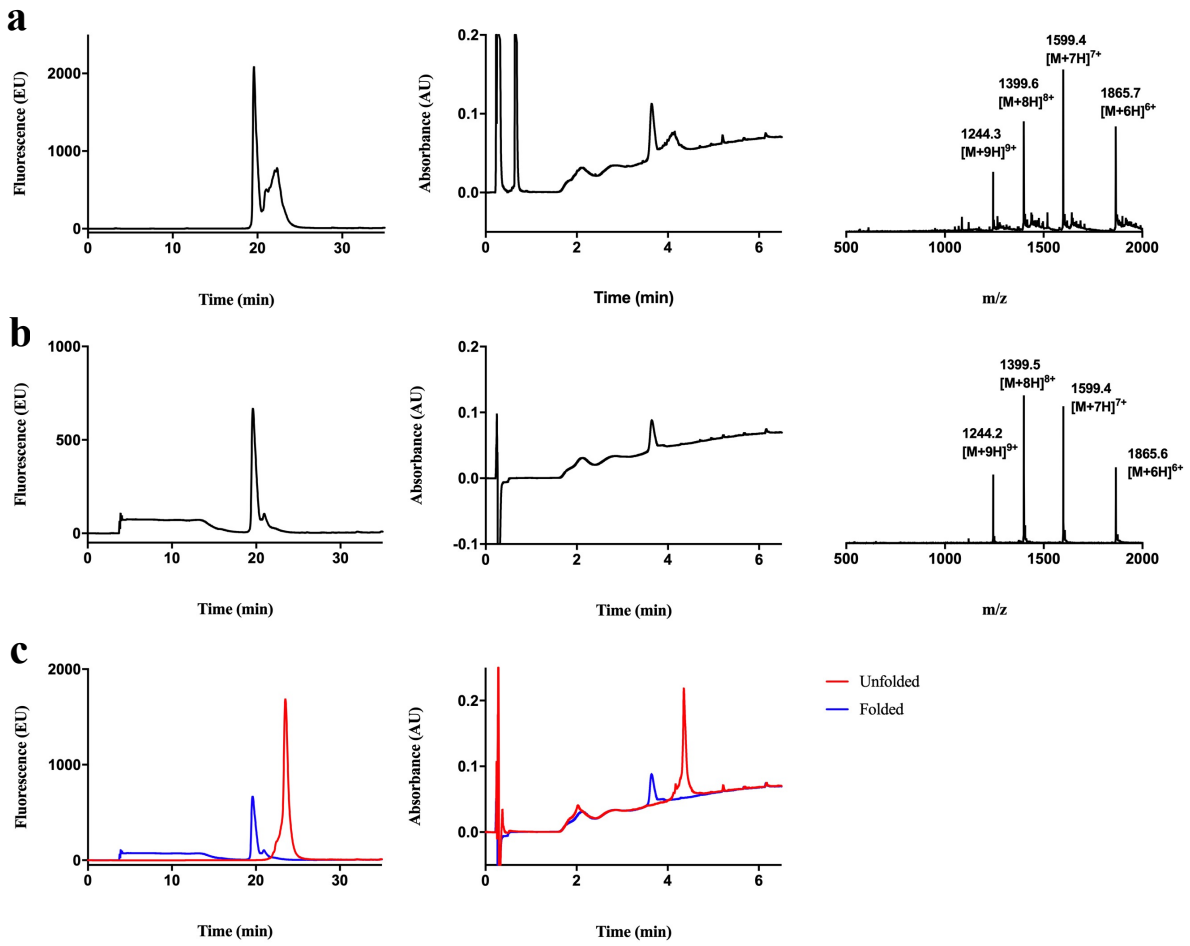
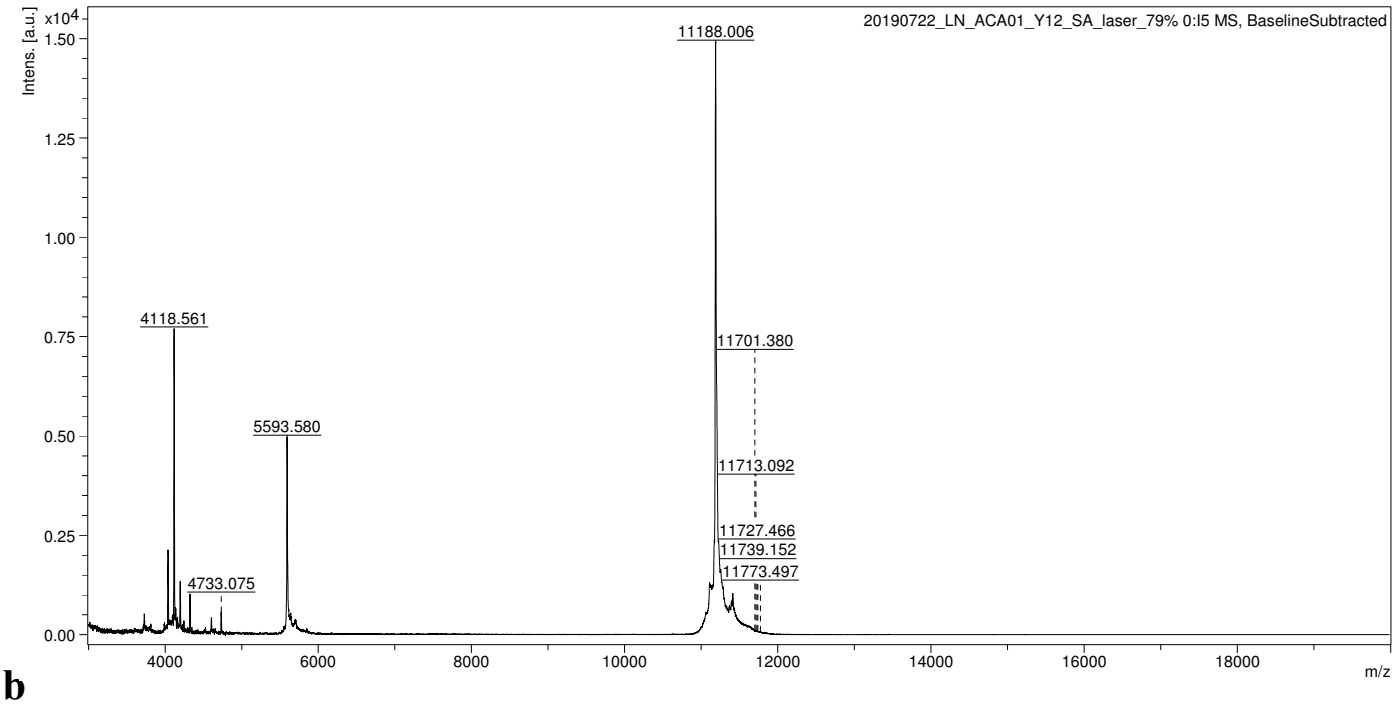


Figure S15. Analytical HPLC trace and mass spectrum of (**a**) crude refolding and (**b**) purified, folded Tyr12-sulfated ACA-01 (**3**). Comparison of both unfolded and folded conditions is shown in (**c**). Analytical HPLC (Purified final product) R_t 19.65 min (0-50% B over 30 minutes**,** 0.1% formic acid, exλ=280 nm emλ=347 nm). Calculated Mass $[M+6H]^{6+}$:1865.9, $[M+7H]^{7+}$: 1599.5, $[M+8H]^{8+}$: 1399.7, $[M+9H]^{9+}$: 1244.3, Mass Found (ESI) 1865.6 $[M+6H]^{6+}$, 1599.4 $[M+7H]^{7+}$, 1399.5 $[M+8H]^{8+}$, 1244.2 $[M+9H]^{9+}$. NB: the purity of the final folded protein was 95.5% as judged by integration of the chromatogram. The minor peak (R_t = 20.93 min) corresponds to differentially sulfated variants (see below).

a



b

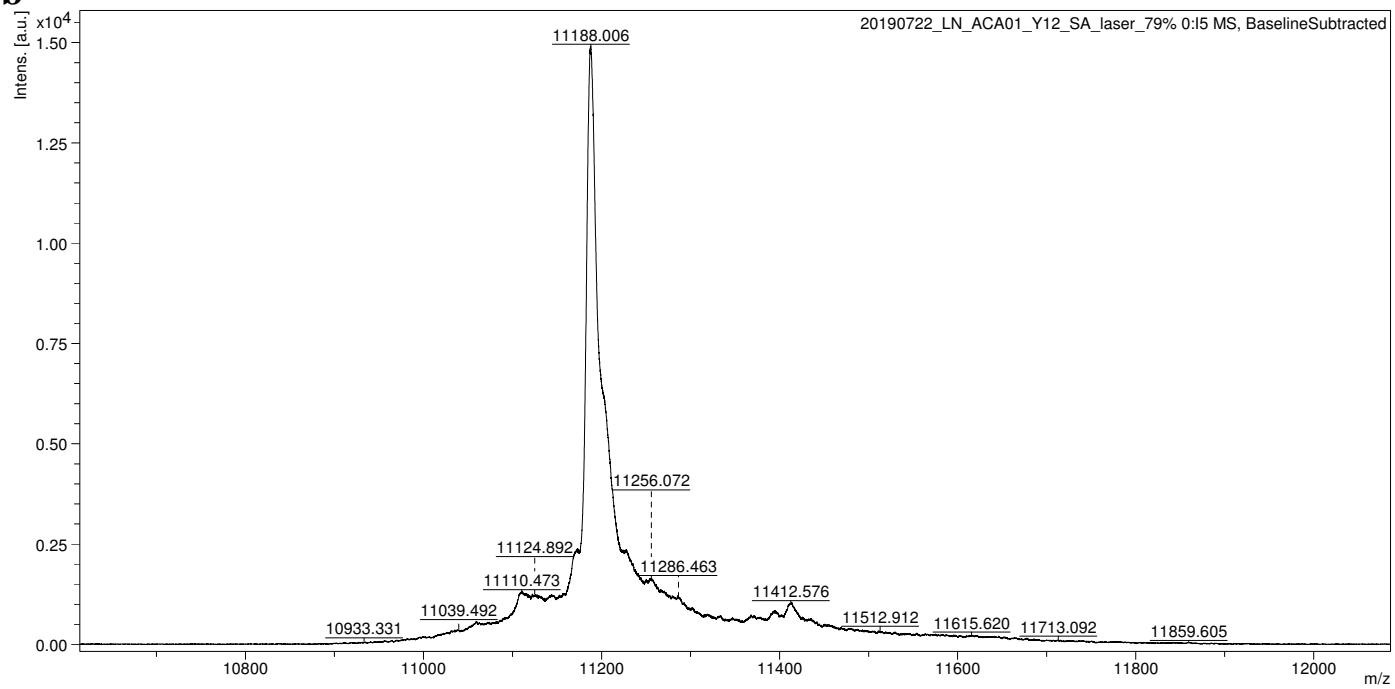
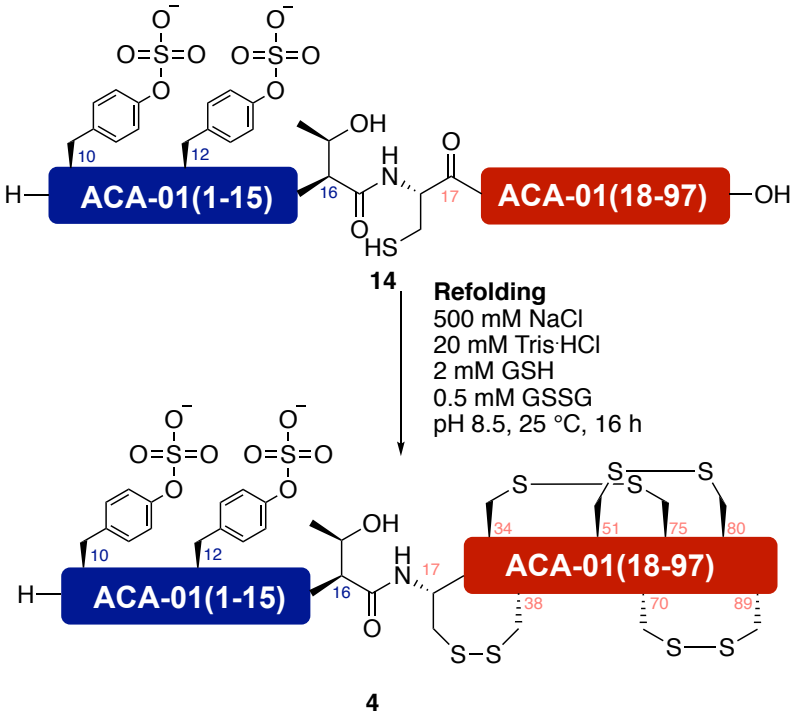


Figure S16. MALDI-TOF mass spectrum of Tyr12-sulfated ACA-01 (**3**) (**a**) 3000-20000 m/z (**b**) 10600-12100 m/z

Refolding of Tyr10+Tyr12-sulfated ACA-01



The refolding of **14** (1.85 mg) was performed according to the general procedure. Purification *via* semi-preparative HPLC (0-50% B over 70 min, 10 mM NH_3) followed by lyophilization afforded the target folded Tyr10+Tyr12-sulfated ACA-01 (**4**) as a white solid (0.53 mg, 28%, 9% over the ligation and refolding steps). Crude folding and purified UV absorbance trace were obtained using Waters Acquity UPLC system fitted with a Waters Acquity UPLC BEH 300 Å, 1.7 µm, 2.1 x 50 mm C18 column (0-50% B over 5 min, 0.1% formic acid, λ=215 nm) at a flow rate of 0.6 mL min^{-1}. Crude and purified FLR traces were obtained using a Waters System e2695 separations module with an Alliance series column heater at 30 °C and 2489 UV/VIS Detector and 2475 FLR Detector, fitted with an Xbridge BEH300 C18 5µm 2.1 x 150 mm column at a flow rate of 0.2 mL min^{-1} (0-50% B over 30 min, 0.1% formic acid, exλ=280 nm emλ=347 nm). Low-resolution mass spectra of crude folding and purified **4** were recorded on a Shimadzu 2020 mass spectrometer (ESI) operating in positive mode. **4** was purified *via* semi-preparative reversed-phase HPLC using Waters Xbridge Peptide BEH prep, C18 300 Å, 5 µm, 10 x 250 mm column at a flow rate of 4 mL min^{-1} using a mobile phase of 10 mM NH_3 in water (Solvent A) and 10 mM NH_3 in acetonitrile (Solvent B) and a linear gradient of 0-50% B over 70 min.

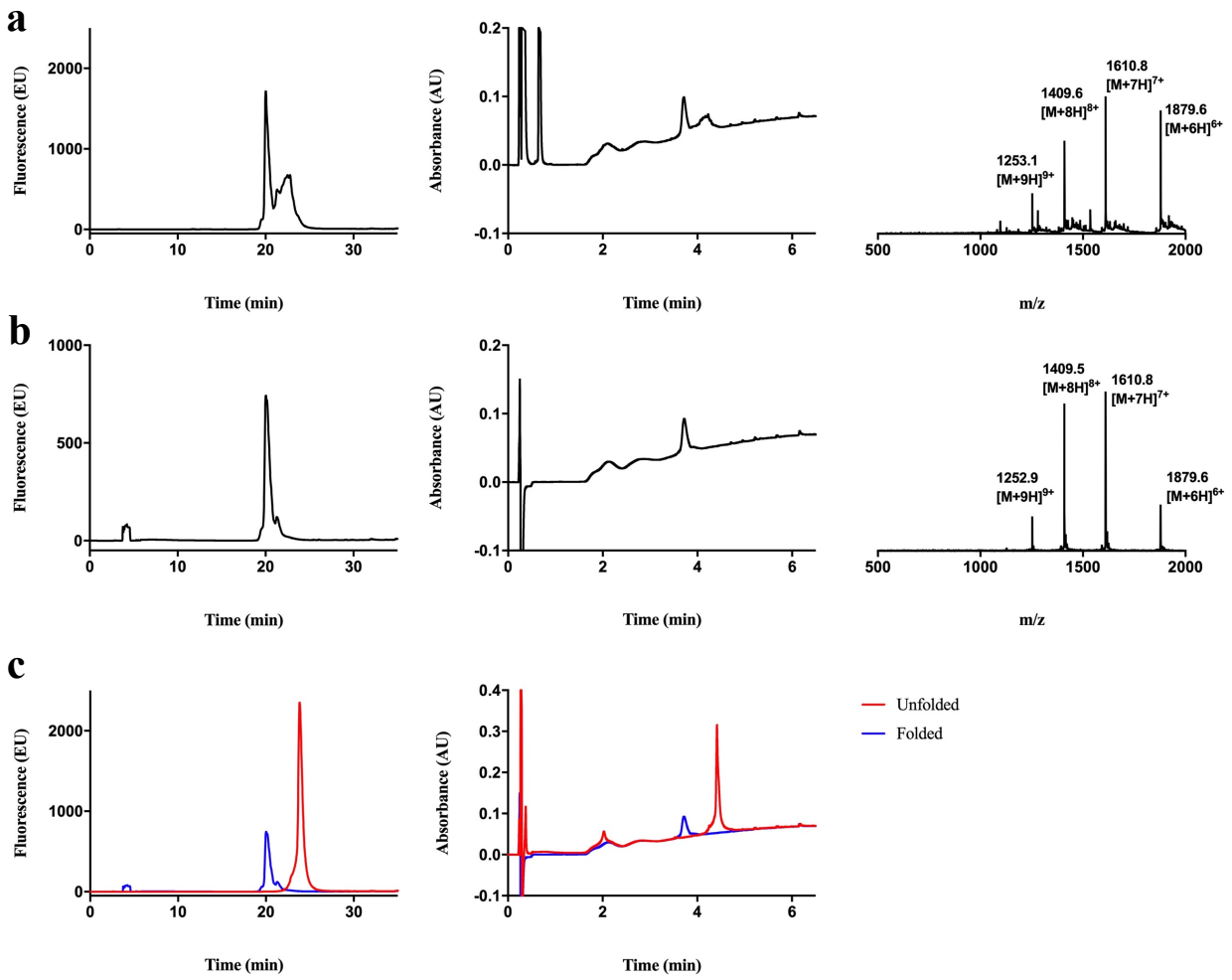
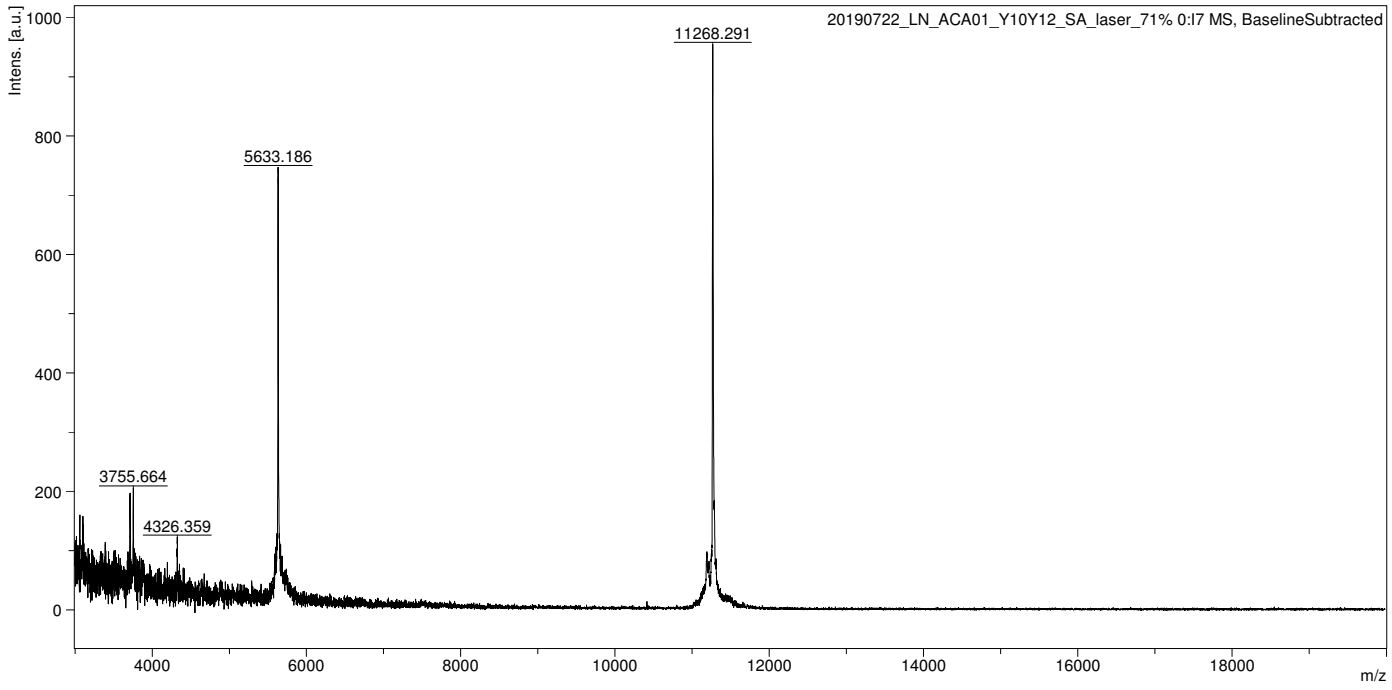


Figure S17. Analytical HPLC trace and mass spectrum of (**a**) crude refolding and (**b**) purified, folded Tyr10+Tyr12-sulfated ACA-01 (**4**). Comparison of both unfolded and folded conditions is shown in (**c**). Analytical HPLC (Purified final product) R_t 20.09 min (0-50% B over 30 minutes**,** 0.1% formic acid, exλ=280 nm emλ=347 nm). Calculated Mass $[M+6H]^{6+}$:1879.2, $[M+7H]^{7+}$: 1610.9, $[M+8H]^{8+}$: 1409.7, $[M+9H]^{9+}$: 1253.2, Mass Found (ESI) 1879.6 $[M+6H]^{6+}$, 1610.8 $[M+7H]^{7+}$, 1409.5 $[M+8H]^{8+}$, 1252.9 $[M+9H]^{9+}$. NB: the purity of the final folded protein was 96.4% as judged by integration of the chromatogram. The minor peak (R_t = 21.28 min) corresponds to differentially sulfated variants (see below).

a



b

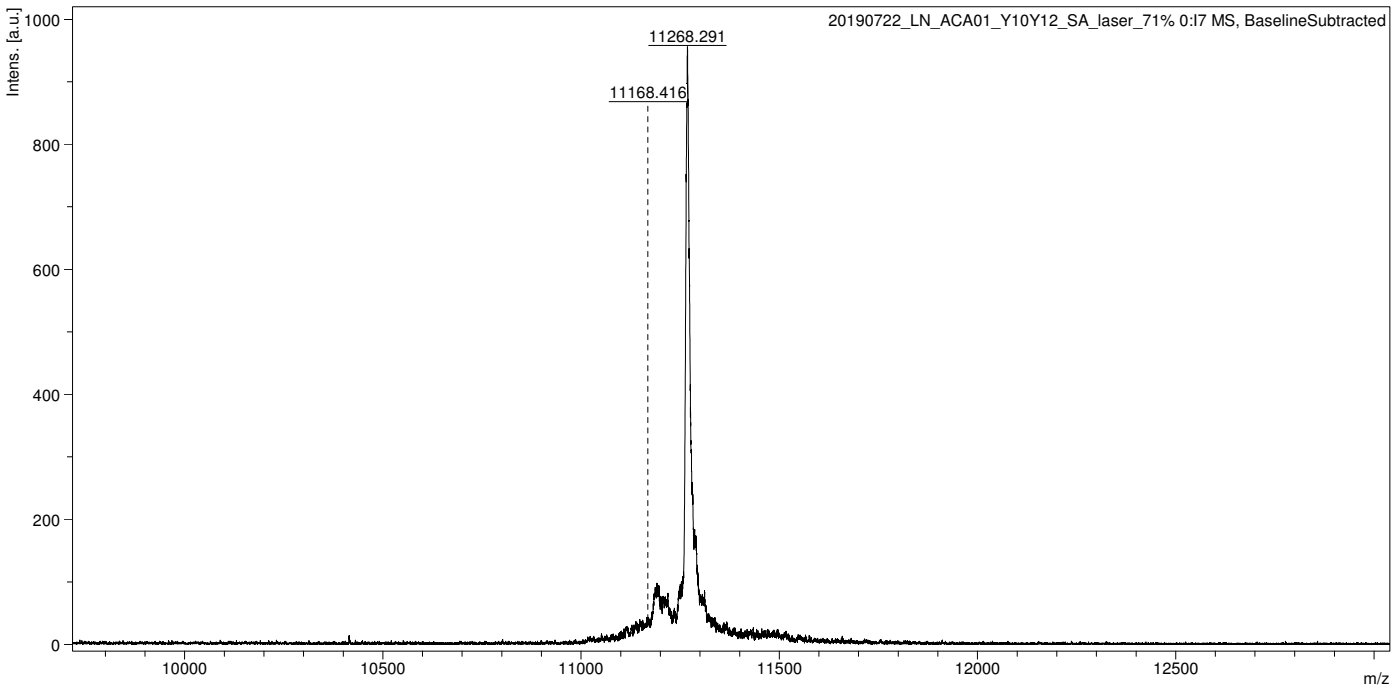


Figure S18. MALDI-TOF mass spectrum of doubly-sulfated ACA-01 (**4**) (**a**) 3000-20000 m/z (**b**) 9700-13000 m/z

Characterization of the minor differentially folded forms: To characterize the correctly and differentially folded forms we reanalyzed each chromatogram by integration of the peak areas for either the major folded form, or the minor unfolded forms. We also compared the observed disulfide bonding patterns from our LC-MS analysis for refolded synthetic (sulfo)proteins **1-4**, and our HEK293-expressed native folded protein. The disulfide bonds known to be present in the native form of the ACA-01 evasin (17-38, 51-80, 34-75, 70-89) was verified for the major peak for the semi-synthetic (>95%) and HEK293 expressed protein (see section on "determination of disulfide connectivity below"). In addition, we observed disulfide bonds corresponding to a differentially folded variant of the protein (17-34, 51-34, 51-89) in both the synthetic and HEK293 expressed protein. This analysis indicated that both the *in vitro* re-folding reaction and the native disulfide bond formation pathway within the HEK293 cell yields some misfolded product (~4-5% in the case of our semi-synthetic material). Therefore, the composition of our *in vitro* re-folded synthetic protein is similar to the native protein from mammalian cells.

Bacterial expression of ACA-01 (1-97) (rACA-01)

A synthetic gene encoding ACA-01 (1-97) fused to an N-terminal 6xHis-SUMO tag was subcloned using NcoI and XhoI restriction sites into the bacterial expression plasmid pET28a; codon usage was optimized for protein production in E. coli. BL21(DE3) E. coli cells transformed with the expression plasmid were incubated in starter cultures (10 mL LB supplemented with 30 µg mL^{-1} kanamycin) overnight at 37 °C, 180 rpm. Expression cultures (2 x 500 mL 2YT media supplemented with 30 µg mL^{-1} kanamycin) were inoculated with 1 mL saturated starter cultures (1:500) and incubated at 37 °C, while shaking at 180 rpm, until mid-log phase (OD_{600} of ~0.6). Cultures were supplemented with 0.5 mM IPTG and transferred to 28 °C for a further 16-18 h incubation, with shaking at 180 rpm. Cells were harvested by centrifugation at 3500 rpm, 4 °C for 20 minutes. Subsequently the pellet was lysed in 30 mL of 500 mM NaCl, 10 mM Tris HCl pH 8.0, 10% glycerol, 100 µg mL^{-1} lysozyme, and 10 µg mL^{-1} DNase. Sonication followed by centrifugation (15000 rpm, 40 min) separated soluble from insoluble fraction. The protein in the soluble fraction was initially purified on an ÄKTApurifier with a 5 mL HisTrap excel column at a flow rate of 5 mL min^{-1} (elution buffer: 500 mM NaCl, 20 mM Tris HCl, 200 mM imidazole, 10% glycerol, pH 8.0). Fractions containing protein (analysed using SDS-page and UV) were pooled and dialysed against phosphate-buffered saline before incubation with Ulp1 protease (1:100, Ulp1:ACA-01 (1-97)) 1 h at 30 °C. The Ulp1 protease had been previously expressed using plasmid pFGET19_Ulp1 (Addgene), which was a gift from Ivanhoe K.H. Leung (University of Auckland, New Zealand). The mixture of cleaved protein and tag was passed over a 5 mL HisTrap excel Column. ACA-01 (1-97) was collected in the flow through, which was concentrated and subjected to size exclusion chromatography, using a Superdex 75 100/300 GL column equilibrated in 10 mM HEPES, 150 mM NaCl, 10% glycerol, pH 7.5. Purified ACA-01 aliquots were concentrated, flash-frozen and kept at -80 °C until use.

Determination of disulfide connectivity

Protein preparation and digestion were performed as described previously with modifications(1). Briefly, 10 µg of purified ACA-01 protein expressed in HEK293 cells was added to 25 µl of buffer containing 4% (w/v) sodium deoxycholate, 0.1 M Tris-HCl pH 8.0 and heated to 95 °C for 10 minutes to denature the protein. The protein was digested to peptides by the addition of 75 µL water and either 200 ng trypsin, or chymotrypsin, followed by incubation at 37 °C for 16 h. An equal volume (100 µL) of 99% ethylacetate 1% TFA was added to the digested peptides and vortexed. For peptide clean-up mixed mode StageTips containing two layers of 3M Empore SDB-RPS discs (Sigma, Cat#66886-U) were used in the Spin96 device. Each tip was wetted with 100 µL of 100% acetonitrile and centrifuged at 1,000 x g for 1 minute. Following wetting, each StageTip was equilibrated with 100 µL of 0.1% TFA in H2O and 30% methanol/1% TFA with centrifugation for each at 1,000 x g for 3 minutes. Each StageTip was then

loaded with the equivalent of ~10 µg peptide (entire lower phase added). The peptides were washed twice with 100 ul of 99% ethylacetate 1% TFA, which was followed by one wash with 100 µl of 0.2% TFA in water. To elute, 100 µL of 5% ammonium hydroxide/80% acetonitrile was added to each tip and centrifuged as above for 5 minutes into a unskirted PCR plate. Samples in the PCR plate were dried using a GeneVac EZ-2 using the ammonia setting at 35 °C for 1 h. Dried peptides were resuspended in 30 µL of 5% formic acid and stored at 4 °C until analysed by LC-MS.

Using a Thermo Fisher Dionex RSLCnano uHPLC, peptides (injection volume 3 µL) were directly injected onto a 45 cm x 75 µm C18 (Dr. Maisch, Ammerbuch, Germany, 1.9 µm) fused silica analytical column with a ~10 µm pulled tip, coupled online to a nanospray ESI source. Peptides were resolved over gradient from 5% acetonitrile to 40% acetonitrile over 45 minutes with a flow rate of 300 nL min^{-1}. Formic acid at 0.1% final concentration was included in both buffers used to form the gradient. Peptides were ionized by electrospray ionization at 2.3 kV and the ion transfer capillary temperature was set to 300 °C. Tandem mass spectrometry analysis was carried out on a Fusion Lumos mass spectrometer (ThermoFisher) using either EThcD, or HCD fragmentation. RAW MS data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD016778, username: reviewer51373@ebi.ac.uk, password: 2rBVtbs9. RAW data were analysed using Byonic (Protein Metrics) and the search output has also been uploaded to the ProteomeXchange Consortium under the same identifier. Peptide and protein level identification were both set to a false discovery rate of 1% using a target- decoy based strategy. The database supplied to the search engine for peptide identifications contained either a subset of secreted human proteins from the UniProt database(2), or the Uniprot *E. coli* database and the full-length ACA-01 protein sequence. The disulfide bond search option for Byonic was applied only for the ACA-01 protein sequence. Mass tolerance was set to 7 ppm for precursor ions and MS/MS mass tolerance was 20 ppm. Enzyme specificity was set to semi-specific N-ragged digestion with either chymotrypsin (cleavage C-terminal to FYWLM) or trypsin (cleavage C-terminal to KR), having a maximum of 2 missed cleavages permitted. Oxidation of Met (common2), pyro-Glu (with peptide N-term Gln or Glu) (rare1), sulfation of tyrosine (common2) and protein N-terminal acetylation (rare1) were set as variable modifications. Two common modifications and one rare modification were allowed per peptide spectral match.

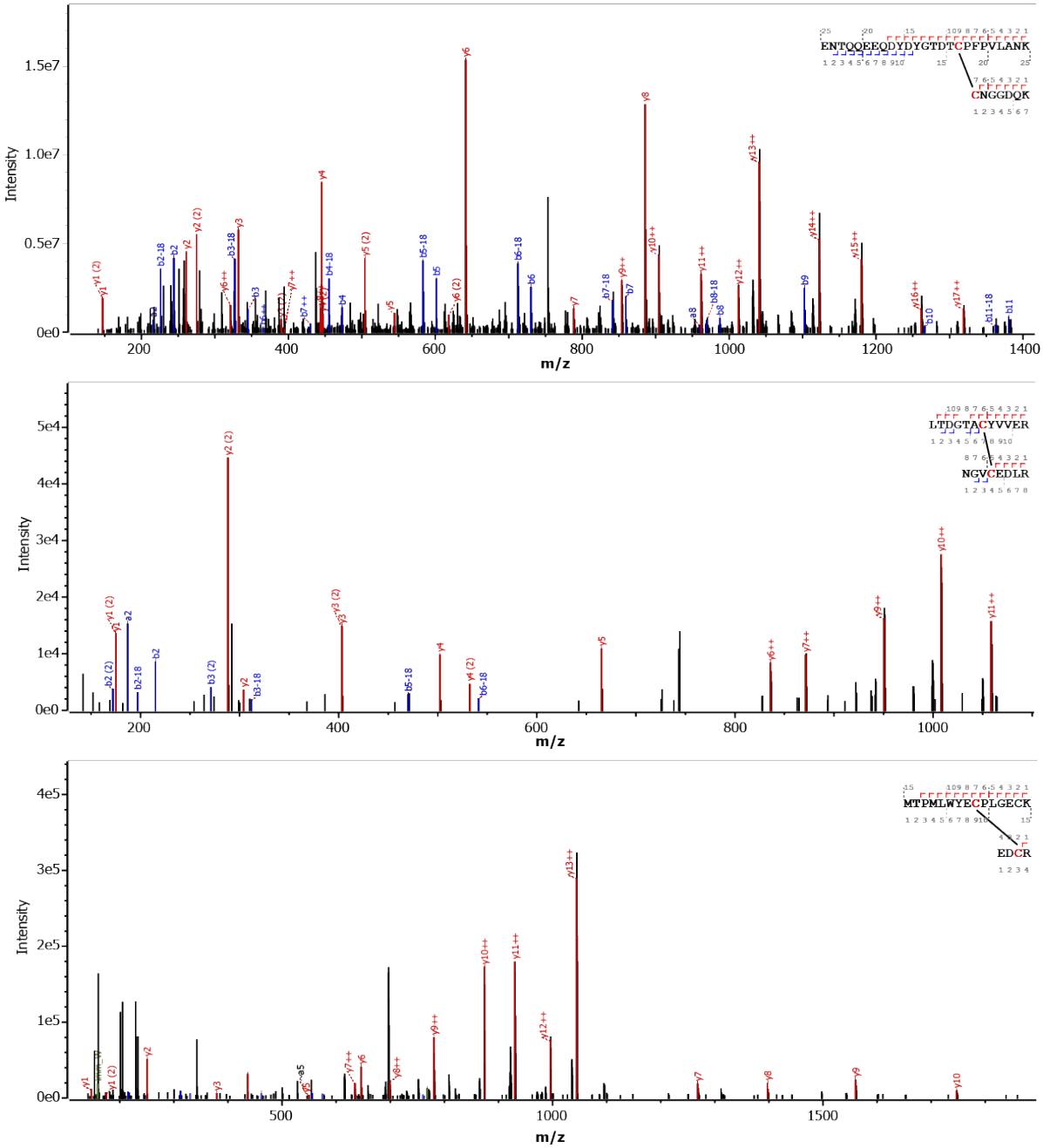


Figure S19. Tandem mass spectra of disulfide-linked peptides detected after trypsin digestion of purified and folded ACA-01 unsulfated (***1***) without reduction/alkylation.

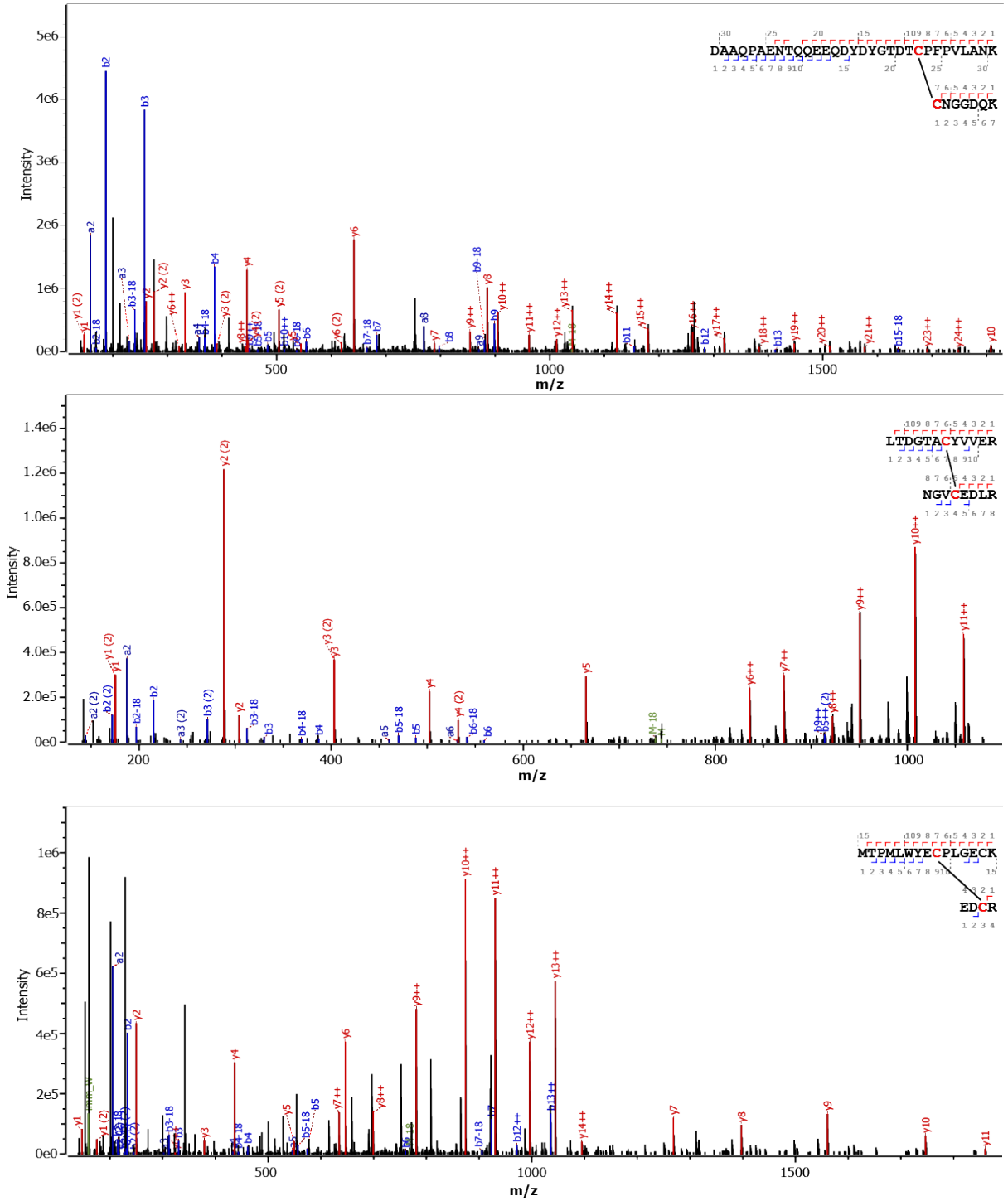


Figure S20. Tandem mass spectra of disulfide-linked peptides detected after trypsin digestion of purified ACA-01 expressed in HEK293 cells, without reduction/alkylation.

Chemokine Binding Fluorescence Anisotropy Assays

Chemokine binding assays, measuring the displacement of a fluorescent peptide Fl-R2D or Fl-R3D from six human CC chemokines, were conducted for each ACA-01 isoform as described previously(3). Briefly, a solution of the ACA-01 variant in 50 mM MOPS, pH 7.0 was serially 2-fold diluted (final concentration ranges from 1 μM to 15.6 nM) on a Greiner non-binding, black, flat-bottomed 96-well plate coated with a 0.001% w/v solution of poly-L-Lysine. A mixed solution of the fluorescent peptide (Fl-R2D for CCL2, CCL7 and CCL8; Fl-R3D for CCL11, CCL24 and CCL26; final concentration 10 nM) and chemokine (final concentration 100 nM) was added, such that the final volume in each well was 200 μL. Fluorescence anisotropy was measured at room temperature, using a BMG Labtech PHERAstar FS plate reader equipped with a fluorescence polarisation module with dedicated excitation and emission wavelengths of 485 and 520 nm respectively. Immediately before measurement, the focal height and intensity were adjusted by using a reference solution (200 μL 5 nM free fluorescein) in a separate well and specifying a polarisation value of 15 mP. Experiments were conducted in duplicate three times independently and the mean anisotropy was fitted by non-linear regression analysis using GraphPad Prism v.8.2.0 software to the equation for a 1:1 competitive displacement curve, described previously (4). Data were transformed to determine -log K_d (pK_d) values ± standard error and statistical analysis was conducted using one-way ANOVA to assess differences in pK_d.

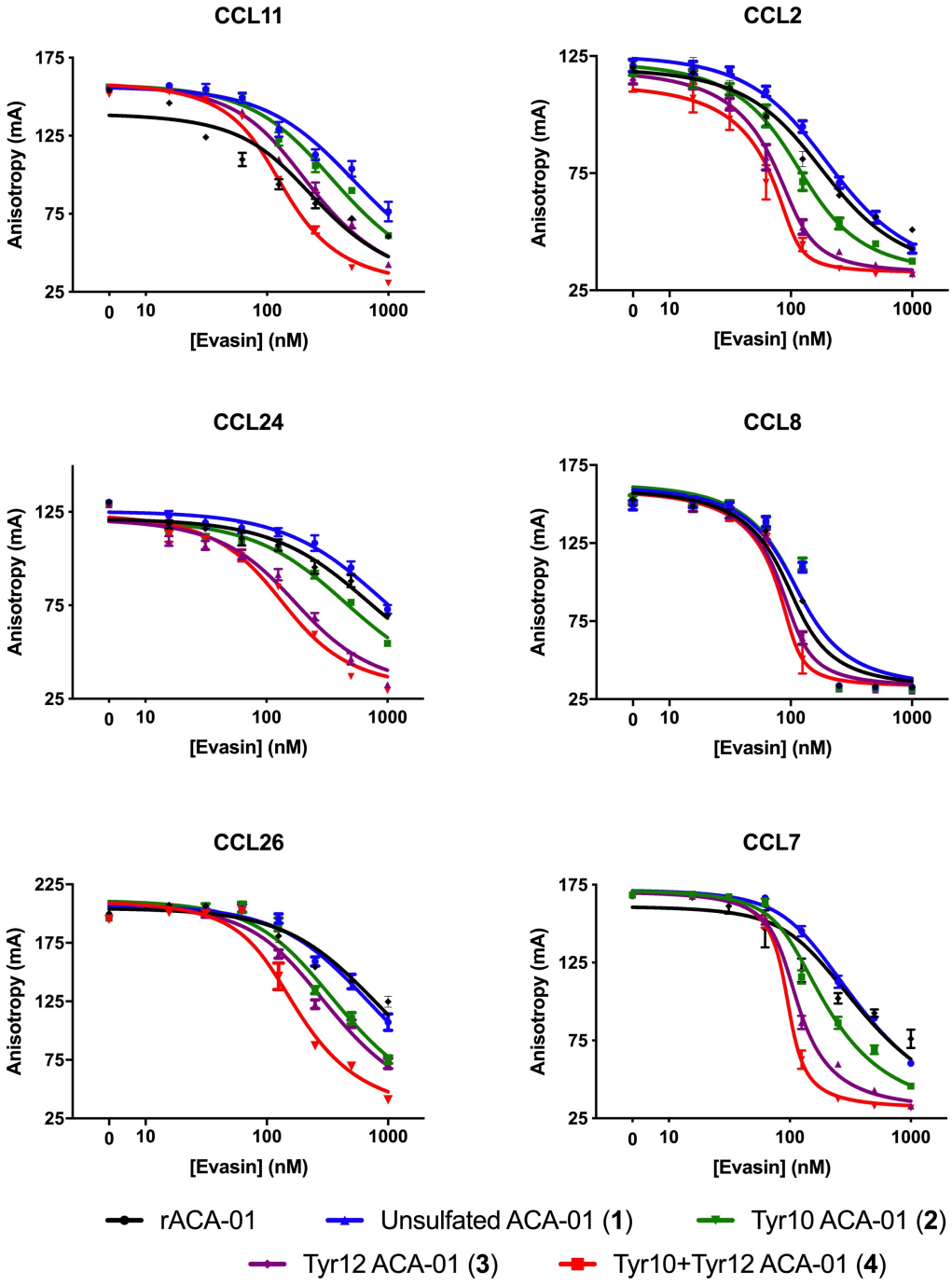


Figure S21. Binding of semi-synthetic ACA-01 (sulfo)proteins 10-13 and recombinantly expressed ACA-01 (E. coli) to human CCL2, CCL7, CCL8, CCL11, CCL24, and CCL26. Fluorescence anisotropy binding curves show that sulfation of ACA-01 at tyrosine residue 10 and/or 12 strongly improves binding affinity for each of the six human chemokines. Data points represent the mean anisotropy ± SEM from duplicate assays performed three times independently. Dose-response curves for binding against CCL11, CCL24, and CCL26 (each at 100 nM) were performed using increasing concentration of different isoforms of ACA-01 with FL-R3D as a probe, whereas for CCL2, CCL7, and CCL8 (each at 100 nM) FL-R2D was used as a probe (both at 10 nM). Under these conditions, the K_d is indicated by the shape of the curve rather than the midpoint. Solid lines are the best fits to a competitive displacement model.

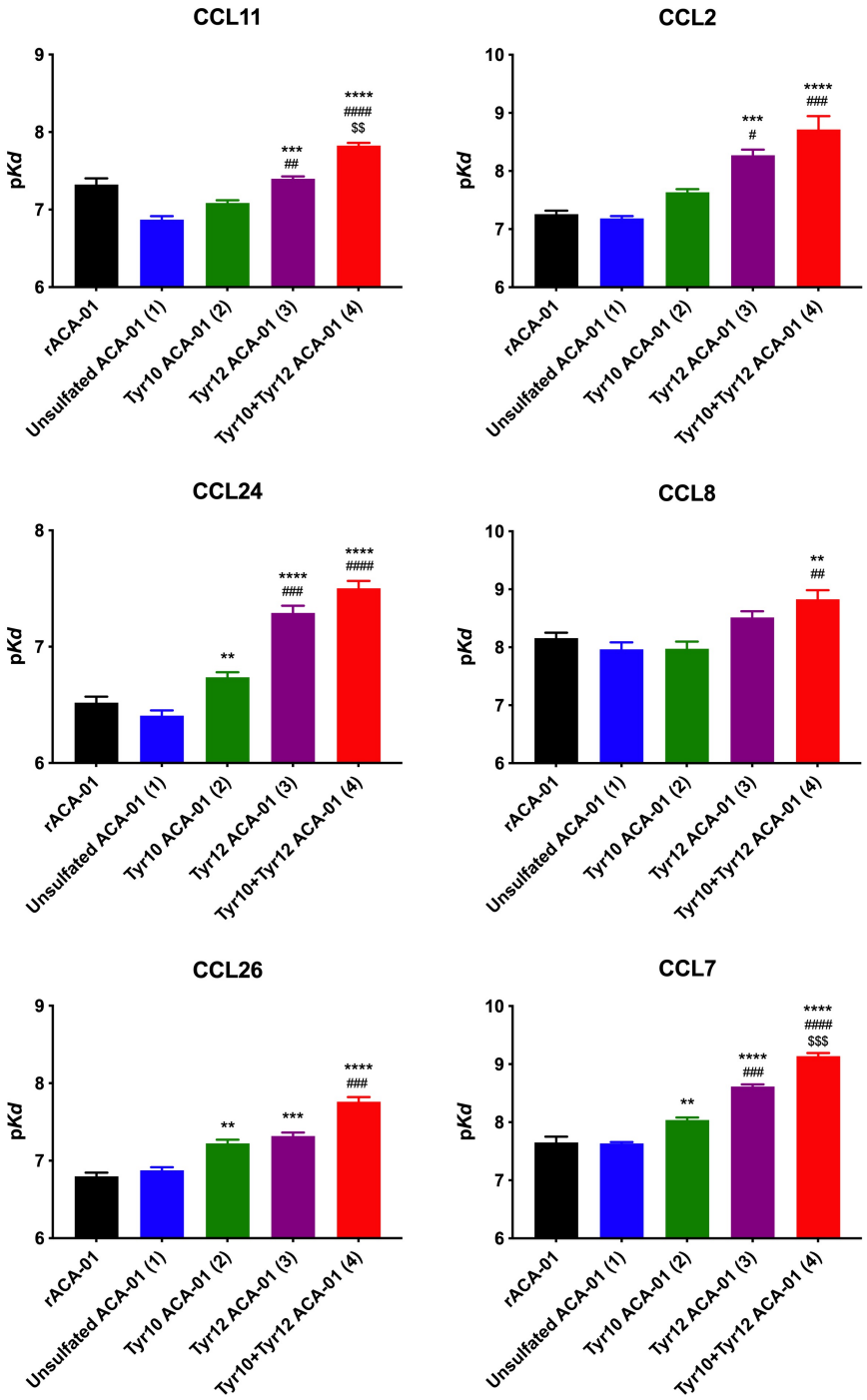


Figure S22. Chemokine binding affinity (pK_d) values, derived from the competitive fluorescence anisotropy data for all ACA-01 isoforms (1-4) and recombinantly expressed ACA-01 (E. coli). Each panel shows the binding affinity for all isoforms for one chemokine: CCL2, CCL7, CCL8, CCL11, CCL24, CCL26. Data represent the average ± SEM of values from three independent experiments, each recorded in duplicate. *, # and $ indicate significant differences from unsulfated ACA-01, Tyr10 ACA-01 and Tyr12-ACA01, respectively. Significance (one-way ANOVA) is shown as **, ##, $$ $p < 0.01$; ***, ###, $$$ $p < 0.001$; ****, ####, $$$$ $p < 0.0001$.

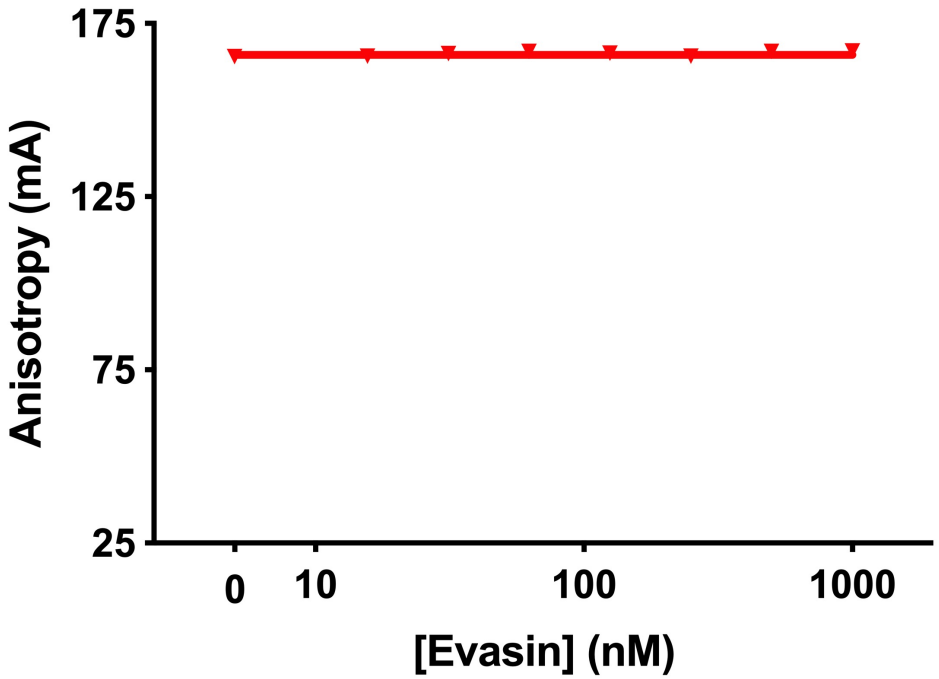


Figure S23. Binding of synthetic doubly sulfated ACA-01 fragment DYDYG (**15**) to CCL11. Fluorescence anisotropy binding curve shows that doubly sulfated ACA-01 peptide **15** does not displace the sulfopeptide probe FL-R3D from CCL11 at concentrations up to 1 μM. Data points represent the mean anisotropy ± SEM from duplicate assays performed three times independently. Dose-response curves for binding against CCL11 (at 100 nM) were performed using increasing concentration of ACA-01 sulfopeptide **15** with FL-R3D as a probe (at 10 nM).

Table S1. ΔpK_d values with SEM for ACA-01 Unsulfated (1) and Tyr10+Tyr12-sulfated ACA-01 (4).

	Unsulfated (1)		Doubly sulfated (4)	
Chemokine Pair	**ΔpK_d**	**SEM[ΔpK_d]**	**ΔpK_d**	**SEM[ΔpK_d]**
CCL2, CCL8	0.77	0.13	0.11	0.28
CCL2, CCL7	0.44	0.04	0.41	0.25
CCL2, CCL11	0.31	0.06	0.90	0.24
CCL2, CCL24	0.79	0.06	1.23	0.25
CCL2, CCL26	0.33	0.06	0.97	0.25
CCL8, CCL7	0.33	0.12	0.30	0.16
CCL8, CCL11	1.08	0.13	1.01	0.15
CCL8, CCL24	1.56	0.13	1.34	0.16
CCL8, CCL26	1.10	0.13	1.08	0.16
CCL7, CCL11	0.75	0.05	1.31	0.06
CCL7, CCL24	1.23	0.05	1.64	0.08
CCL7, CCL26	0.77	0.05	1.38	0.08
CCL11, CCL24	0.48	0.07	0.33	0.07
CCL11, CCL26	0.01	0.06	0.07	0.07
CCL24, CCL26	0.47	0.06	0.26	0.09

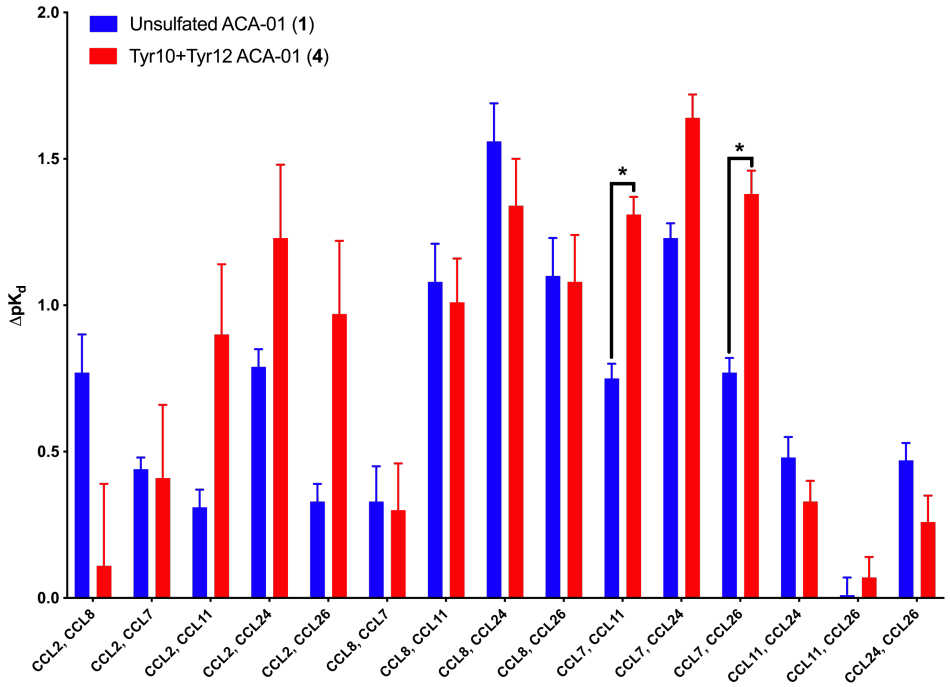


Figure S24. Effect of ACA-01 sulfation on chemokine-binding selectivity. The relative affinities, represented as ΔpK_d, for each pair of chemokines (amongst the six human CC chemokines: CCL2, CCL7, CCL8, CCL11, CCL24, and CCL26) are shown for unsulfated ACA-01 (**1**, blue bars) and Tyr10+Tyr12-sulfated ACA-01 (**4**, red bars). Statistical analysis was performed using an unpaired t-test with Holm-Sidak correction to account for multiple comparisons and no assumptions of identical SD values. Error bars represent SEM. Significant differences are annotated by * for $p<0.05$.

Inhibition of chemokine-mediated cyclic AMP signaling

Chemokine signaling via the receptor CCR2 or CCR3 was measured using a cyclic AMP (cAMP) biosensor assay as described previously(5, 6). We used this assay to investigate the ability of ACA-01 sulfoproteins **1-4** and recombinantly expressed ACA-01 to inhibit chemokine-mediated decrease in cAMP following pre-stimulation with forskolin. For this purpose, cMyc-FLAG-CCR2 or cMyc-FLAG-CCR3 FlpIn TREx HEK293 cells were transiently transfected with the CAMYEL cAMP bioluminescence resonance energy transfer biosensor(5) (ATCC). Cells were grown overnight in 10 cm dishes using Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX (Gibco, ThermoFisher Scientific) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S), before transfecting using 9:1 (w/w) PEI:DNA. Tetracycline (10 µg mL^{-1}) was added 24 hours after transfection to induce expression of cMyc-FLAG-CCR2 or cMyc-FLAG-CCR3 and cells were seeded (40 000 cells/well or 50 000 cells/well) in a white, poly-L-lysine coated 96-well plate (CulturePlates; PerkinElmer) and incubated overnight at 37 °C, 5% CO_2. The following day, cells were washed and equilibrated in Hank's Balanced Salt Solution (HBSS) for 30 minutes at 37 °C. For initial experiments to determine appropriate chemokine concentrations (EC_{80}) for detailed testing, concentration-response studies were performed for six different chemokines at two chemokine receptors (CCL2, CCL7 and CCL8 at CCR2; CCL11, CCL24 and CCL26 at CCR3) (Fig. S25). Cells were incubated with the Rluc substrate coelenterazine-h (Nanolight Technology) at a final concentration of 5 µM for 5 minutes, followed by a further 5-minute incubation with different concentrations of relevant chemokines. A final 5-minute incubation step was conducted after addition of forskolin (final concentration 10 µM), which directly stimulates the production of cAMP via adenylyl cyclase. The Rluc and yellow fluorescent protein emissions were then measured at 475 and 535 nm, respectively, using a BMG Labtech PHERAstar FS plate reader. Subsequent experiments to test evasin-mediated inhibition of the signaling response were performed as described above, except in the presence of a fixed concentration of chemokine (10 nM CCL2, 30 nM CCL8/CCL7, and 80 nM CCL11/CCL26, corresponding to approximately the EC_{80}) along with various concentrations of an evasin candidate that had been pre-incubated for ~15 minutes to allow binding of the evasin to the chemokine. Data were presented as a BRET ratio, calculated as the ratio of YFP to Rluc signals and expressed as the percentage inhibition of the forskolin-induced cAMP production.

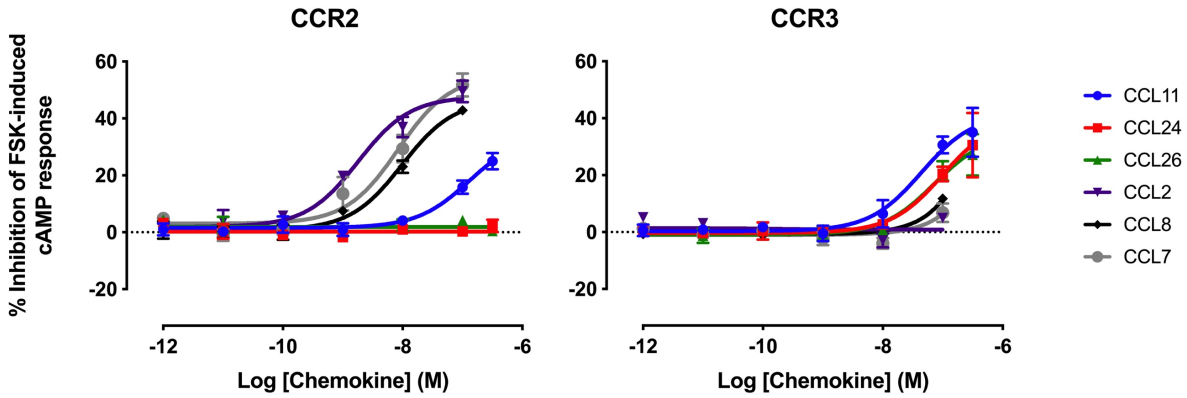


Figure S25. Chemokine-mediated inhibition of forskolin-induced cAMP production. Concentration-response curves are shown for inhibition of forskolin (10 µM)-induced production of cAMP in either cMyc-FLAG-CCR2 FlpIn TREx HEK293 cells or cMyc-FLAG-CCR3 FlpIn TREx HEK293 cells by the chemokines CCL2, CCL7, CCL8, CCL11, CCL24, and CCL26. Chemokine-mediated cAMP signaling was detected using a BRET biosensor (CAMYEL). Data points represent the average ± SEM of values from three independent experiments, each recorded in duplicate.

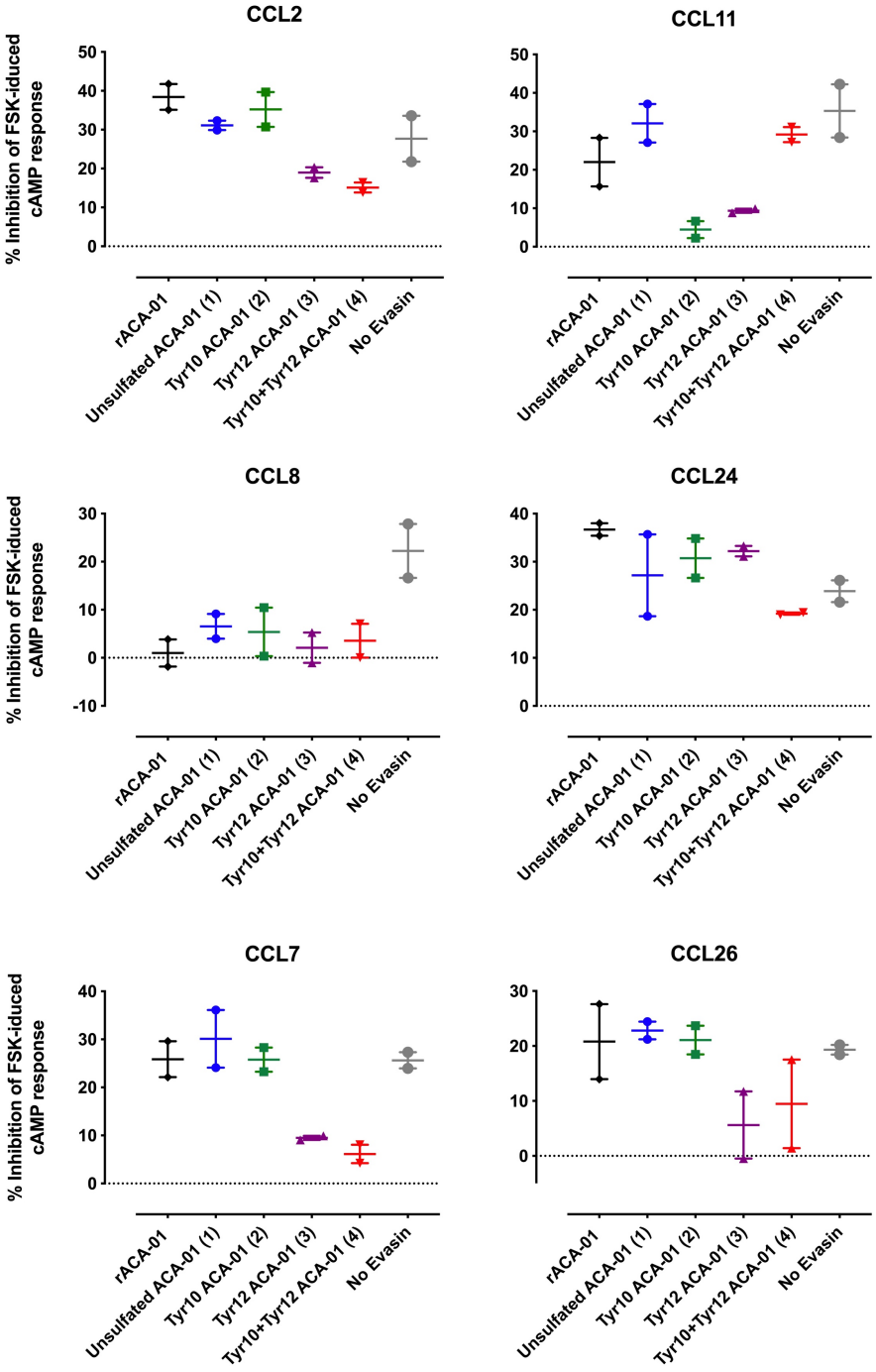


Figure S26. Single ACA-01 (sulfo)protein **1-4** concentration screen. Shown are the inhibition profiles of chemokine activity by ACA-01 (sulfo)proteins **1-4** at a concentration of 100 nM. Chemokines screened were CCL2 (10 nM), CCL8 (30 nM), and CCL7 (30 nM), acting at receptor CCR2 and CCL11 (80 nM), CCL24 (80 nM), and CCL26 (80 nM), acting at receptor CCR3. Chemokine activity was detected as the capacity of the chemokine to inhibit forskolin-induced production of cAMP, as detected via the BRET sensor, CAMYEL; thus ACA-01 (sulfo)proteins **1-4** potentially inhibit the cAMP-inhibitory activity of the chemokines. Data represent the duplicate values (symbols), recorded in a single experiment. The average ± SEM is represented with solid lines.

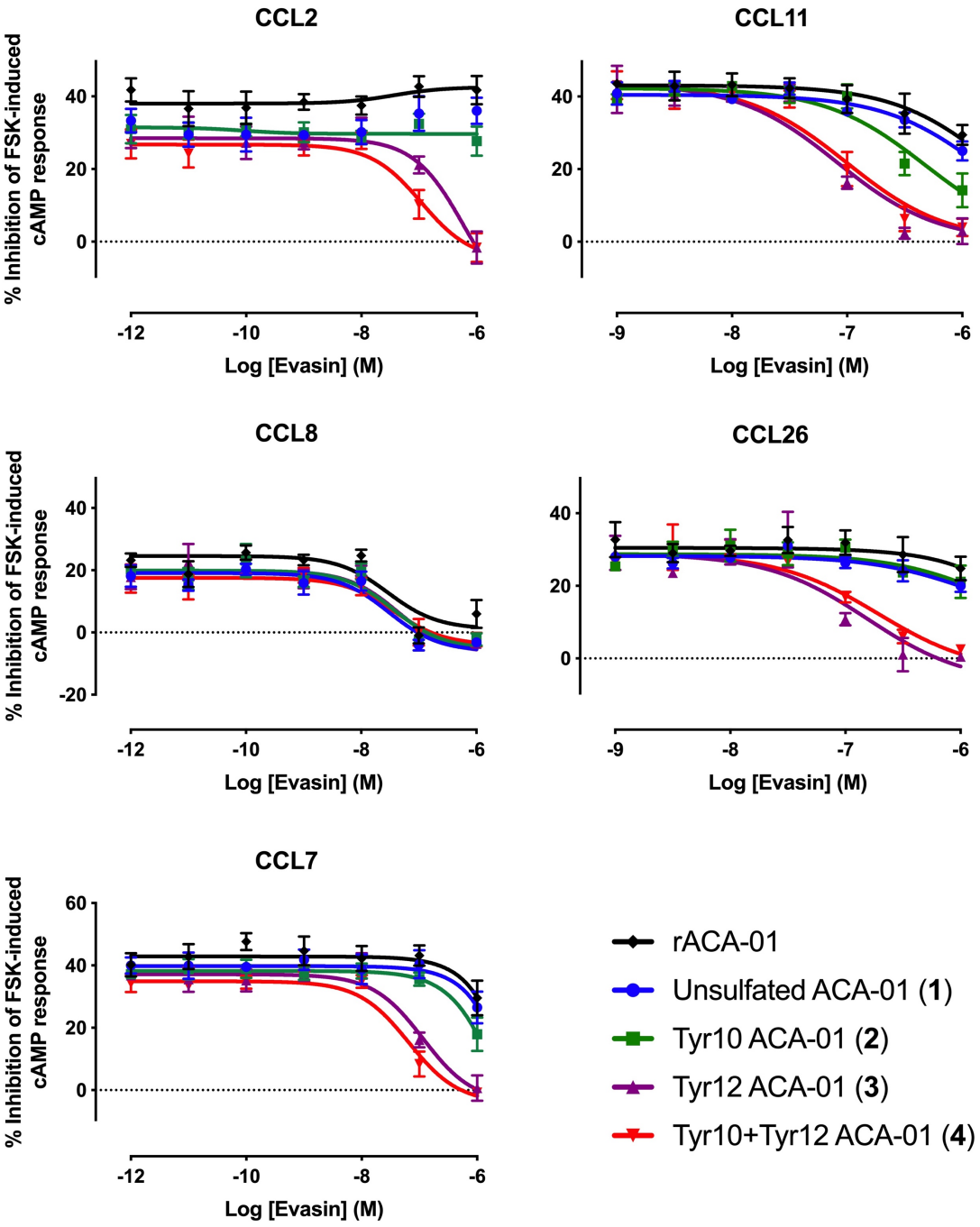


Figure S27. Inhibition of chemokine activity by ACA-01 (sulfo)proteins **1-4**. Shown are the inhibition profiles of chemokine activity by ACA-01 proteins **1-4** at a concentration ranging from 1 pM to 1 µM. Chemokines screened were CCL2 (10 nM), CCL8 (30 nM), CCL7 (30 nM), CCL11 (80 nM), and CCL26 (80 nM) acting at receptor CCR2 (CCL2, CCL7, CCL8) or CCR3 (CCL11 and CCL26) in FlpIn TREx HEK293 cells. Chemokine activity was detected as the capacity of the chemokine to inhibit forskolin-induced production of cAMP, as detected via the BRET sensor, CAMYEL; therefore, differentially sulfated ACA-01 proteins **1-4** potentially inhibit the cAMP-inhibitory activity of chemokines. Average ± SEM of three independent experiments, each conducted in duplicate are shown.

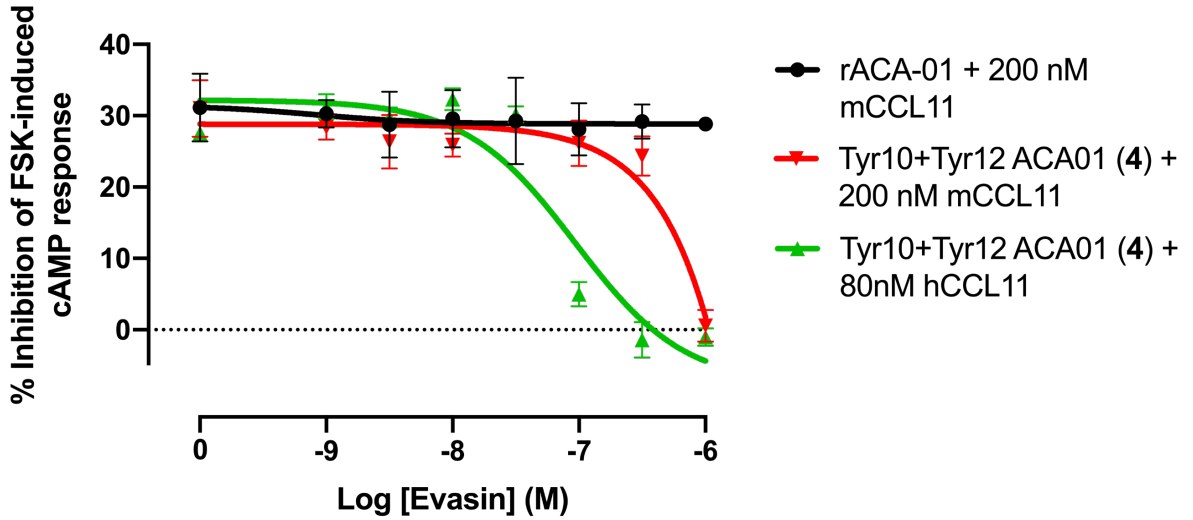


Figure S28. Inhibition of mouse CCL11 by ACA-01 sulfoprotein **4** and recombinantly expressed ACA-01 (rACA-01). Shown are the inhibition profiles of chemokine activity at a concentration ranging from 1 nM to 1 µM of evasin. Chemokines screened were human CCL11 (80 nM) and mouse CCL11 (200 nM) acting at human receptor CCR3 in FlpIn TREx HEK293 cells. Chemokine activity was detected as the ability of the chemokine to inhibit forskolin-induced production of cAMP, as detected *via* the BRET sensor, CAMYEL; therefore, sulfated ACA-01 protein **4** and recombinantly expressed ACA-01 potentially inhibit the cAMP-inhibitory activity of chemokines. Average ± SEM of three independent experiments, each conducted in duplicate are shown.

Statistical Analysis

One-way analysis of variance followed by Tukey's multiple comparison test was performed using GraphPad Prism Software version 8.2.0, unless otherwise mentioned. All data are shown as mean ± SEM or otherwise as described in figure legends. The significance is indicated for each experiment (*, #, $ $p < 0.05$; **, ##, $$ $p < 0.01$; ***, ###, $$$ $p < 0.001$; ****, ####, $$$$ $p < 0.0001$).

Western Blot

Western Blotting was performed using primary antibody against sulfotyrosine (Abcam, ab136481, 1:1000 dilution) and secondary antibody goat anti-mouse IgG H&L (HRP) (Biorad, 1706516, 1:2000 dilution).

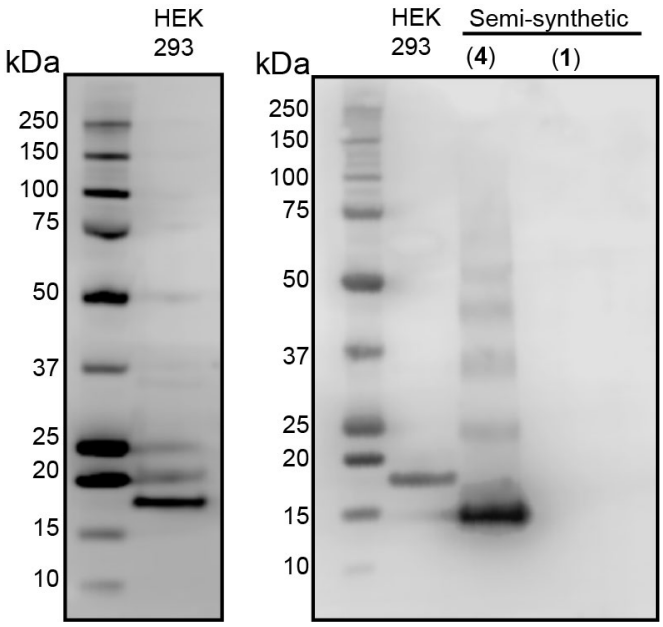


Figure S29. Uncropped versions of western blots. (***left***) Identification of sulfated tyrosine residues in mammalian expressed ACA-01-cMyc-His6 using a sulfotyrosine-specific antibody. (***right***) Identification of sulfated tyrosine residues in mammalian expressed ACA-01-cMyc-His6 as well as semi-synthetic ACA-01 proteins **4** (Tyr10+Tyr12 sulfated) and **1** (Unsulfated) using a sulfotyrosine-specific antibody.

Sulfinator prediction for other evasins

Sulfinator software was used to predict for tyrosine sulfation sites within 21 evasins.(7) Out of 21 validated (chemokine-binding) C_8 Class A Evasins, 12 (57%) are predicted to have sulfated Tyr sites - all except one in the first ~20 residues.

Class A Evasin Sequences

>EVA_1
EDDEDYGDLGGCPFLVAENKTGYPTIVACKQDCNGTTETAPNGTRCFSIGDEGLRRMTANLPYDCPL
GQCSNGDCIPKETYEVCYRRNWRDKKN

>EVA_4
EVPQMTSSSAPDLEEEDDYTAYAPLTCYFTNSTLGLLAPPNCSVLCNSTTTWFNETSPNNASCLLTV
DFLTQDAILQENQPYNCSVGHCDNGTCAGPPRHAQCW

>EVA_P467
AEKSLDSDSSGEDYELWTQGCPFLVAENRTGFGTTVSCQHNCNGAIEKVPEGEPCYTIGEDGLGRM
KLNLPYNCSLGECSGGVCVPNGRSDVCFKRTWEENNKAMA

>EVA_P546
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>EVA_P974
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>EVA_P983
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>EVA_P1180
EEPKDGYDYTEGCPFVVLGNGTHAKPAGCSHLCNGAPETLDDNMECYNVTEEVAKRMTPDIPYTC
WLGWCSKGECKRDNRTEVCYRGSERE

>EVA_P1181
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>EVA_P1182
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>EVA_P1183
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GWCSKGECKRDNRTEVCYRGSEEE

>EVA_P985
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>EVA_P991
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>EVA_P672
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>EVA_AAM_01
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>EVA_AAM_02
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VRTLTTYPNTSCAVGVCMKGTCVKNGTMEQCFKTP

>EVA_ACA_02
GIEGSGNLATSHEMDDCLDDNSTCVIQTLNTTGEPRPVGCVLKCKNSTQHLANGTECLGIPELAGVR
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>EVA_AMA_01
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SDVMQSDVPYYCPIGLCANGICKRSGLELNCWHDMPPPVSTDTAIENPTTSISSSAKL

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>EVA_ATR_01
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NRMQGNVTYTCPVGLCYRGVCERNGLGIDCWHNTPPPNSTNVTTNASTTTLPTSSRDL

>EVA_RPU_02
EVQNTTLAEEDYDTGCGYNIVITKNKTLVVNCTMDCQPKMLMNESEPCLFNSSVPYDHMQPHHNYT
CMEGICKNGTCVSPSNNITCWLPPPPVRYYPNETMVTSTIEPEA

Sulfinator Run 1: 8 Sequences – All C_8 Evasins

Protein / sequence name	Position	E-value	Sequence
EVA_1	6	[17]	DED**Y**GD d+dY+d
EVA_4	19	[12]	EEEDD**Y**TAYAPL +++ddY a
	22	[54]	DDYTA**Y**A---P ++ Y
	28	[54]	PLT-C**Y**FTN--S +l+ Yf++
EVA_P467	14	[3.4]	SSGED**Y**ELWTQGCPFLV +++dY+ + + +++
EVA_P546	10	[1.9]	QEEED**Y**DYGTDT ++++dY+ +++
	12	[17]	EEDYD**Y**-GTDT ++d dY +++
EVA_P974	10	[5.4]	QEEQD**Y**DYGTDT +++ dY+ +++
	12	[30]	EQDYD**Y**-GTDT + d dY +++

Sequence(s) processed:**8**

Sulfated tyrosines detected: **9** (of 35)

Number of proteins with at least one hit: **5**

No hits found in:
EVA_P1180
EVA_P1181
EVA_P983

Figure S30. Output for sulfinator of first series of 8 evasin sequences.

Sulfinator Run 2: 8 Sequences – All C_8 Evasins

Protein / sequence name	Position	E-value	Sequence
EVA_AAM_02	14	[4.2]	DNSTE**Y**YDYEEA +++ +Y + +e
	15	[32]	NSTEY**Y**DYEEAR ++++ Y+ e+ +
	17	[12]	TEYYD**Y**EEARC + dY+ea++
EVA_P1182	8	[12]	DND**Y**GGGCPFV d+dY++ +++
EVA_P672	21	[11]	TEDED**Y**E---DFFKP-V ed+dY+ d+f++
	31	[31]	EDE-D**Y**E---DFFKP-V e++ dY+ d+f++
EVA_P985	15	[35]	STDVD**Y**EE-LDA----N +d dY+e d+ +
EVA_P991	11	[46]	TQP-D**Y**DNSTDY + dY+++++
	17	[2.9]	DNSTD**Y**YNYEDF +++ dY + +++
	18	[53]	NSTDY**Y**-NYED +++d Y + e
	20	[54]	DYYN-**Y**EDFKC + + Y++ +

Sequence(s) processed:**8**

Sulfated tyrosines detected: **11** (of 38)

Number of proteins with at least one hit: **5**

No hits found in:
EVA_AAM_01
EVA_ACA_02
EVA_P1183

Figure S31. Output for sulfinator of second series of 8 evasin sequences.

Sulfinator Run 3: 5 Sequences – All C_8 Evasins

Protein / sequence name	Position	E-value	Sequence
EVA_APA_01	42	[19]	ENSTE**Y**LPNGTECLGLS e++++Y + e+++
EVA_RPU_02	12	[27]	AE-ED**Y**DTG--C + +dY+++ +
	56	[50]	NSSVP**Y**-DHMQ +++ Y ++++

Sequence(s) processed:**5**

Sulfated tyrosines detected: **3** (of 21)

Number of proteins with at least one hit: **2**

No hits found in:
EVA_AMA_01
EVA_ATR_01
EVA_ATR_02

Figure S32. Output for sulfinator of third series of 5 evasin sequences.

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