Supporting Information

Masked Cerulenin Enables a Dual-Site Selective Protein Crosslink

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A. General experimental methods.

Chemical solvents and reagents were obtained from Acros Organics, Alfa Aesar, Chem-Impex Int., Fischer Scientific, Fluka, Oakwood Chemical, Sigma-Aldrich, Spectrum Chemical Mfg. Corp., or TCI Chemicals. Deuterated NMR solvents were obtained from Cambridge Isotope Laboratories. All reactions were conducted with rigorously dried solvents that were purchased from Alfa Aesar. Reactions run under 1 L were stirred with Teflon coated stir bars using an IKAMAG RCT-basic stirrer (IKA GmbH). Unless otherwise noted, reactions under 200 mg, 50 to 5 mg and under 5 mg were conducted in flame dried 20 mL, 1 dram or ½ dram vials, respectively, equipped with a Teflon lined cap and a 3 mm stirbar. Mixtures were heated on flask or reaction block adapters for IKAMAG RCT-basic stirrers (Chemglass). Analytical Thin Layer Chromatography (TLC) was performed on Silica Gel 60 F254 precoated glass plates (EM Sciences). Visualization was achieved with UV light and/or an appropriate stain ($KMnO_4$, dinitrophenylhydrazine, ninhydrin, and ceric ammonium molybdate). Flash chromatography was carried out on Fischer Scientific Silica Gel, 230-400 mesh, grade 60 or SiliaFlash Irregular Silica Gel P60, 40-63 μm mesh, grade 60. Yields correspond to isolated, chromatographically, and spectroscopically homogeneous materials. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AVA300, a JEOL400, a Varian VX500 (equipped with an XSens Cold probe), or a Bruker Avance 800 (equipped with the triple resonance TXO cryoprobe) spectrometer. Chemical shift δ values for ^{1}H and ^{13}C spectra are reported in parts per million (ppm) and multiplicities are abbreviated as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. ^{13}C-NMR spectra were recorded with proton decoupling. NMR spectra from many of the compounds reported in this study were complicated due to isomers or tautomers preventing accurate tabulation. For these compounds, we provided expansions of the ^{1}H NMR spectra and tabulated the major peaks within the ^{13}C NMR spectra (peaks were selected with heights twice that of the baseline). Radio frequency interference was occasionally observed in ^{13}C NMR spectra collected on the XSens Cold probe with noise appearing at 150.4 ppm. FID files were processed using MestraNova 12.0.3 (MestreLab Research). Electrospray (ESI) mass spectrometric analyses were performed using a ThermoFinnigan LCQ Deca spectrometer. A Thermo Fisher Scientific LTQ Orbitrap XL mass spectrometer was used for high-resolution electrospray ionization mass spectrometry analysis (HR-ESI-MS). Spectral data and procedures are provided for all new compounds and copies of select spectra have been provided at the end of this document.

B. Protein expression and purification.

The following sections were used to prepare the proteins used within this study. All proteins were purified with a purity of >80% as determined by SDS-PAGE analyses using ImageJ software (NIH) to evaluate band intensity.

The *E. coli* carrier protein acyl carrier protein AcpP. The gene for native AcpP was synthesized with a C-terminal stop codon by Twist Bioscience and cloned into a pET-21 vector with NdeI and XhoI digestion sites. The addition of the stop codon was required to prevent expression of the C-terminal HiS6-tag on the vector. The AcpP gene was chemically transformed into *E. coli* BL21(DE3) cells and cultured in 5 mL of LB broth (LB broth Miller, RPI International) prepared at 25 g/L in deionized H_2O and autoclaved with 100 mg/L of ampicillin (added after autoclaving) for 12 h at 37 °C. This culture was added into LB broth (1 L) with 100 mg/L of ampicillin and incubated at 37 °C until the OD_{600} reached between 0.7±0.1. The culture was then induced by the addition of 0.5 mM IPTG (addition of 0.5 mL of 1 M IPTG)) at 18 °C. After shaking for 12 h at 18 °C, cell pellets were obtained through centrifugation and lysed by sonication in 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol. After centrifugation, the supernatant was collected and titrated slowly with an equivalent volume of isopropanol. Further centrifugation at 17,400 g for 40 min provided a supernatant containing AcpP in 50% aq. isopropanol. This supernatant was loaded onto a HiTrap Q HP anion exchange column and purified *via* FPLC (ÄKTA Pure) with a linear gradient of 0 to 1 M NaCl in 50 mM Tris • HCl buffer pH 7.4. Eluted fractions were evaluated by SDS-PAGE (12% acrylamide) analyses. The pure AcpP fractions were collected and subjected to media exchange by dialysis using 3.5K MWCO SnakeSkindialysis tubing (Thermo Fisher Scientific) with 50 mM Tris • HCl buffer pH 7.4 containing 150 mM NaCl. These samples were spin concentrated to 5 mg/mL using 3k spin concentrator (Millipore Amicon Ultra). The resulting sample was aliquoted, flash frozen, and stored at -80 °C.

Coenzyme A biosynthetic proteins CoaA, CoaD, and CoaE. CoaA, CoaD, and CoaE [Finzel K, Beld J, Burkart MD, Charkoudian LK. Utilizing Mechanistic Cross-Linking Technology to Study Protein-Protein Interactions: An Experiment Designed for an Undergraduate Biochemistry Lab. J Chem Educ. 2017 Mar 14;94(3):375-379] with *N*-terminal MBP-tags were recombinantly expressed in *E. coli* BL21 (DE3). Single colonies were grown in LB broth (5 mL) with 50 mg/L kanamycin for 12 h at 37 °C. Cultures were then added into LB broth (1 L) with 50 mg/L kanamycin and incubated at 37 °C until OD_{600} reached 0.7. The resulting cultures were cooled to 4 °C followed by induction with 0.5 μM IPTG (addition of 0.5 mL of 1 M IPTG). After shaking for 16 h at 18 °C, cell pellets were collected by centrifugation (2000 × g for 30 min) and lysed by sonication in lysis buffer (50 mM potassium phosphate, 150 mM NaCl, 10% glycerol, pH 7.5). The lysate was then centrifuged at 5,000 rpm for 1 h. The resulting supernatant was loaded onto an amylose column (New England BioLabs), washed with lysis buffer containing 10 mM maltose and eluted with lysis buffer containing 100 mM maltose. Sequential dialysis using 10K MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific) was performed with lysis buffer (1 L) for 12 h at 4 °C. The dialyzed protein was concentrated to 10 mg/mL and flash frozen in aliquots for storage at -80 °C.

The Acyl carrier protein phosphodiesterase (AcpH). AcpH [Kosa NM, Haushalter RW, Smith AR, Burkart MD. Reversible labeling of native and fusion-protein motifs. Nat Methods. 2012 Oct;9(10):981-4] with *N*-terminal HiS6-tag was expressed in *E. coli* BL21 (DE3) cells. The cells were cultured in LB broth (5 mL) with 50 mg/L kanamycin for 12 h at 37 °C. The culture was then added into LB broth (1 L) with 50 mg/L kanamycin and allowed to grow at 37 °C to reach an

OD_{600} of 0.6. Induction was performed with 1 mM IPTG (addition of 1 mL of 1 M IPTG) at 16 °C followed by shaking for 12 h at 16 °C. The cell pellets were collected by centrifugation (2,000 rpm for 30 min) and sonicated using lysis buffer (50 mM Tris • HCl, 500 mM NaCl, 10% glycerol, pH 7.5). The resulting solution was centrifuged to give clear supernatant, which was loaded onto column containing Ni-NTA resin (Thermo Fisher Scientific). The column was washed with 10 column volumes (CV) of the lysis buffer followed by lysis buffer containing 10 mM imidazole (2 × 10 mL). Lysis buffer containing 250 mM imidazole was used to elute AcpH. Sequential dialysis using 10K MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific) was performed with lysis buffer (1 L) for 12 h at 4 °C. The dialyzed protein was concentrated to 5 mg/mL using a 10k Amicon Ultra spin concentrator (Millipore) and flash frozen in aliquots for storage at -80 °C.

Apofication of *E. coli* AcpP. Native *E. coli* AcpP (described in the above paragraph on the preparation of AcpP) was obtained as a mixture of *apo*-AcpP and *holo*-AcpP. AcpH was used to convert the mixture of *apo*-AcpP and *holo*-AcpP to *apo*-AcpP. Briefly, a solution of 5 mg/mL AcpP mixture was incubated in 0.01 mg/mL AcpH in 50 mM Tris • HCl buffer pH 7.4, 10% glycerol, 5 mM $MnCl_2$, 10 mM $MgCl_2$, 0.25% DTT at 23 °C for 12 h. FPLC (ÄKTA PURE) and size exclusion purification on a HiLoad 16/600 A Superdex 75 pg column (GE Biosciences) were used to obtain pure *apo*-AcpP [Kosa NM, Haushalter RW, Smith AR, Burkart MD. Reversible labeling of native and fusion-protein motifs. Nat Methods. 2012 Oct;9(10):981-4]. The purified sample was spin concentrated to 5 mg/mL using 3k Amicon Ultra spin concentrator (Millipore). The resulting sample was aliquoted, flash frozen, and stored at -80 °C.

The *E. coli* partner protein FabA. HiS6-tagged recombinant FabA [Ishikawa F, Haushalter RW, Burkart MD. Dehydratase-specific probes for fatty acid and polyketide synthases. J Am Chem Soc. 2012 Jan 18;134(2):769-72] was expressed in *E. coli* K12, strain AG1. The cells were cultured in LB broth (5 mL) with 30 mg/L of chloramphenicol for 12 h at 37 °C. The culture was transferred into LB broth (1 L) with 30 mg/L of chloramphenicol and incubated at 37 °C until the OD_{600} reached 0.6. The culture was then induced by 1 mM IPTG (addition of 1 mL of 1 M IPTG) at 18 °C and left shaking for 12 h at 18 °C. Cell pellets were obtained through centrifugation (2,000 rpm for 30 min) and lysed by sonication in lysis buffer (100 mM potassium phosphate, 300 mM NaCl, pH 8.0). After centrifugation (5,000 rpm for 1 h) the resulting supernatant was loaded onto a Ni-NTA-column and washed with 10 CV of the lysis buffer followed by lysis buffer containing 10 mM imidazole (2 × 10 mL). Lysis buffer containing 250 mM imidazole was used to elute the protein. Sequential dialysis using 10K MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific) was performed with lysis buffer (1 L) for 12 h at 4 °C. The dialyzed protein was concentrated to 5 mg/mL using a 10k Amicon Ultra spin concentrator (Millipore) and flash frozen in aliquots for storage at -80 °C.

The *E. coli* partner protein FabD. FabD **[**Misson LE, Mindrebo JT, Davis TD, Patel A, McCammon JA, Noel JP, Burkart MD. Interfacial plasticity facilitates high reaction rate of *E. coli* FAS malonyl-CoA:ACP transacylase, FabD Proc Natl Acad Sci U S A. 2020 Sep 29;117(39):24224-24233] with one extra glycine at the *N*-terminal was expressed in BL21 (DE3). The cells were cultured in LB broth (5 mL) with 50 mg/L of kanamycin for 12 h at 37 °C then transferred into LB broth (1 L) with 50 mg/L of kanamycin and incubated at 37 °C until the OD_{600} reached 0.5. The culture was then induced by 0.5 mM IPTG (addition of 0.5 mL of 1 M IPTG) at 18 °C and left shaking for 18 h at 18 °C. Cell pellets were obtained through centrifugation (2,000 rpm for 30 min) and lysed by sonication in lysis buffer (25 mM MOPS, 1 mM EDTA, 1 mM BME, pH 7.1). After centrifugation (5,000 rpm for 1 h), the supernatant was loaded onto HiTrap Q HP

anion exchange column and purified *via* FPLC (ÄKTA PURE) with a linear gradient of 0 to 1 M potassium chloride in the lysis buffer. The eluted FabD was concentrated to 5 mg/mL using a 10k Amicon Ultra spin concentrator (Millipore) and flash frozen in aliquots for storage at -80 °C.

The *E. coli* partner protein FabG. FabG [Heath RJ, Rock CO. Enoyl-acyl carrier protein reductase (FabI) plays a determinant role in completing cycles of fatty acid elongation in *Escherichia coli*. J Biol Chem. 1995 Nov 3;270(44):26538-42] with HiS6-tag was expressed in BL21 (DE3). The cells were incubated in LB broth (5 mL) with 100 mg/L of ampicillin for 12 h at 37 °C. The culture was transferred into LB broth (1 L) supplemented with 1% casamino acids, 0.4% glucose, 1 mM $MgCl_2$, and 100 mg/L of ampicillin and incubated at 37 °C until the OD_{600} reached between 0.6 and 0.8. The culture was then induced by 1 mM IPTG (addition of 1 mL of 1 M IPTG) at 37 °C and left shaking for 3 h. After centrifugation (2,000 rpm for 30 min), cell pellets were sonicated in lysis buffer (20 mM Tris • HCl, 500 mM NaCl, 1 mM β-mercaptoethanol, pH 7.4). After centrifugation (5,000 rpm for 1 h), the resulting supernatant was loaded onto a Ni-NTA-column and washed with 10 CV of the lysis buffer followed by Lysis buffer containing 10 mM imidazole (2 × 10 mL). Lysis buffer containing 250 mM imidazole was used to elute the protein. Sequential dialysis using 10K MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific) was performed with lysis buffer (1 L) for 12 h at 4 °C. The dialyzed protein was concentrated to 5 mg/mL using a 10k Amicon Ultra spin concentrator (Millipore) and flash frozen in aliquots for storage at -80 °C.

The *E. coli* partner proteins FabB, FabF, FabF*[C163A], or FabH. HiS6-tagged recombinant protein FabB [Mindrebo JT, Patel A, Kim WE, Davis TD, Chen A, Bartholow TG, La Clair JJ, McCammon JA, Noel JP, Burkart MD. Gating mechanism of elongating β-ketoacyl-ACP synthases. Nat Commun. 2020 Apr 7;11(1):1727], FabF [Mindrebo JT, Patel A, Kim WE, Davis TD, Chen A, Bartholow TG, La Clair JJ, McCammon JA, Noel JP, Burkart MD. Gating mechanism of elongating β-ketoacyl-ACP synthases. Nat Commun. 2020 Apr 7;11(1):1727], FabF*[C163A], or FabH was expressed in BL21(DE3) cells and cultured in 5 mL LB broth with 50 mg/L of kanamycin for 12 h at 37 °C. The culture was added into 1 L LB broth with 50 mg/L of kanamycin and incubated at 37 °C until the OD_{600} reached between 0.6 and 0.8. The culture was then induced by 0.5 mM IPTG (addition of 1 mL of 500 mM IPTG) at 37 °C and left shaking for 3 h. Cell pellets were obtained through centrifugation (2,000 rpm for 30 min). Cell pellets were sonicated in lysis buffer (50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol). The lysate was then centrifuged at 5,000 rpm for 1 h. The resulting supernatant was loaded onto a Ni-NTA-column (Thermo Fisher Scientific) and washed with 10 CV (column volume) of the lysis buffer followed by lysis buffer containing 10 mM imidazole (2 × 10 mL). Lysis buffer containing 250 mM imidazole was used to elute the protein. Sequential dialysis with 10K MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific) was performed with lysis buffer (1L) for 12 h at 4 °C. The dialyzed protein was concentrated to 5 mg/mL using a 10k Amicon Ultra spin concentrator (Millipore) and flash frozen in aliquots for storage at -80 °C.

The *E. coli* partner protein FabI. FabI [Tallorin L, Finzel K, Nguyen QG, Beld J, La Clair JJ, Burkart MD. Trapping of the Enoyl-Acyl Carrier Protein Reductase-Acyl Carrier Protein Interaction. J Am Chem Soc. 2016 Mar 30;138(12):3962-5] with HiS6-tag was expressed in BL21(DE3). The cells were cultured in LB broth (5 mL) with 50 mg/L of kanamycin for 12 h at 37 °C. The culture was transferred into LB broth (1 L) with 50 mg/L of kanamycin and incubated at 37 °C until the OD_{600} reached 0.8. The culture was then induced by 0.5 mM IPTG (addition of 0.5 mL of 1 M IPTG) at 18 °C and left shaking for 12 h at 18 °C. Cell pellets were obtained through

centrifugation (2,000 rpm for 30 min) and lysed by sonication in lysis buffer (50 mM potassium phosphate buffer pH 7.4, 250 mM NaCl, 20% glycerol). The lysate was then centrifuged at 5,000 rpm for 1 h. The resulting supernatant was loaded onto a Ni-NTA-column and washed with 10 CV of the lysis buffer followed by lysis buffer containing 10 mM imidazole (2 × 10 mL). Lysis buffer containing 250 mM imidazole was used to elute the protein. Sequential dialysis using 10K MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific) was performed with lysis buffer (1 L) for 12 h at 4 °C. The dialyzed protein was concentrated to 5 mg/mL using a 10k Amicon Ultra spin concentrator (Millipore) and flash frozen in aliquots for storage at -80 °C.

The *M. smegmatis* mycocerosic acid synthases (MAS) and MAS ΔKS•AT. HiS6-tagged recombinant protein MAS [Konno S, La Clair JJ, Burkart MD. Trapping the Complex Molecular Machinery of Polyketide and Fatty Acid Synthases with Tunable Silylcyanohydrin Crosslinkers Angew Chem Int Ed Engl. 2018 Dec 21;57(52):17009-17013] and HiS6-tagged MAS Δ KS•AT were expressed in BL21(DE3) and cultured in LB broth with 50 mg/L of kanamycin (5 mL) for 16 h at 37 °C. This culture was transferred into LB broth (1 L) with 50 mg/L of kanamycin incubated at 37 °C until the OD_{600} reached between 0.6 and 0.8. The culture was then induced by 0.5 mM IPTG (addition of 0.5 mL of 1 M IPTG) at 18 °C and left shaking for 12 h at 18 °C. Cell pellets were obtained through centrifugation (2,000 rpm for 30 min) and lysed by sonication in lysis buffer (50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol). The lysate was then centrifuged at 5,000 rpm for 1 h. The resulting supernatant was loaded onto a Ni-NTA-column and washed with 10 CV of lysis buffer followed by lysis buffer containing 10 mM imidazole (2 × 10 mL). Lysis buffer containing 250 mM imidazole was used to elute the protein. Sequential dialysis using 10K MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific) was performed with lysis buffer (1 L) for 12 h at 4 °C. The dialyzed protein was then subjected to further purification by HiLoad 16/600 Superdex 75 PG (GE Biosciences) size exclusion column through FPLC (ÄKTA Pure) to yield pure MAS or MAS Δ KS•AT. Pure proteins were concentrated to 32 mg/mL using a 10k Amicon Ultra spin concentrator (Millipore) for MAS ΔKS•AT and 100k Amicon Ultra spin concentrator (Millipore) for MAS and flash frozen in aliquots for storage at -80 °C.

The 4'-phosphopantetheinyl transferase (Sfp). Sfp [Finzel K, Beld J, Burkart MD, Charkoudian LK. Utilizing Mechanistic Cross-Linking Technology to Study Protein-Protein Interactions: An Experiment Designed for an Undergraduate Biochemistry Lab. J Chem Educ. 2017 Mar 14;94(3):375-379] with an *N*-terminal HiS6-tag was prepared by recombinant expression in *E. coli* BL21 (DE3). Single colonies were grown in LB broth (5 mL) with 100 mg/L ampicillin for 12 h at 37 °C. Cultures were then added into LB broth (1 L) with 100 mg/L ampicillin and incubated at 37°C until OD_{600} reached 0.7. The resulting cultures were cooled to 4 °C followed by induction with 0.5 mM IPTG (addition of 0.5 mL of 1 M IPTG). The culture was then subjected to shaking at 18 °C for 16 h and centrifugation (2000 × g for 30 min) for pellets collection. Cell pellets were lysed by sonication in lysis buffer (50 mM potassium phosphate,150 mM NaCl, 10% glycerol, pH 7.5). The lysate was then centrifuged at 5,000 rpm for 1 h. The resulting supernatant was loaded onto a Ni-NTA-column and washed with 10 CV of the lysis buffer followed by lysis buffer containing 10 mM imidazole (2 × 10 mL). Lysis buffer containing 250 mM imidazole was used to elute the protein. Sequential dialysis using 10K MWCO SnakeSkin dialysis tubing (Thermo Fisher Scientific) was performed with lysis buffer (1 L) for 12 h at 4 °C. The dialyzed protein was concentrated to 5 mg/mL using a 10K MWCO Amicon Ultra spin concentrator (Millipore) and flash frozen in aliquots for storage at -80 °C.

C. Fluorescent labeling, crosslinking studies, and protein crystallography.

The following protocols were used to generate the data presented in Fig. 3.

Fluorescent labeling protocol. Fluorescent labeling reactions (typically 5-10 μL) shown in Fig. 3 were quenched by SDS-loading dye (3 μL, 420 mM Tris pH 6.8, 600 mM DTT, 10% SDS, 30% glycerol, 0.012% bromophenol blue) and boiled on a water bath for 5 min. Samples were evaluated by SDS-PAGE using 12% acrylamide gels except for MAS and MAS Δ KS•AT which due to size were run on 8% SDS-PAGE gels. Fluorescent gels were imaged with a UV transilluminator at 365 nm and photos were taken using an iPhone 12 (Apple) prior to Coomassie Brilliant Blue staining. Once imaging was complete, the gels were immersed in fixation solution (50% MeOH with 10% acetic acid) and left shaking at 25 °C for 2 h. After fixation, all gels were washed with H_2O and stained with Coomassie Brilliant Blue at 25 °C for 1 h. The resulting gels were then destained with 10% EtOH at 25 °C overnight. All gels were imaged on a Perfection V19 scanner (Epson).

Probes and crosslinkers. Probes and crosslinkers described in this study were prepared as stock solutions at 10 mM for **1a**, **1b**, **2a**, **2b**, **7b**, and **8b**, 25 mM for (2*R*,3*S*)-**1b** and (2*S*,3*R*)-**1b**, 20 mM for **1c**, (2*R*,3*S*)-**1c**, **2c**, **5c**, **6c**, **8c**, or **9c** in DMSO unless noted otherwise. These stock solutions were added to proteins in buffer so that the DMSO content of each reaction was 1%.

Time course labeling of *E. coli* type II ketosynthases. Probes **1a**, **1b**, **2a**, or **2b** were added from DMSO stock solutions to provide 100 μM probe in 20 μM FabB, 20 μM FabF, or 20 μM FabH in 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol, 1% DMSO at 25 °C. The reaction was sampled at seven time points from 0 to 21 h and evaluated by SDS-PAGE analyses. The results are shown in Fig. 3a-b, Supporting Fig. S4 and Supporting Fig. S5.

Labeling of type I ketosynthases. Probes **1a**, **1b**, **2a**, or **2b** were added from DMSO stock solutions to provide 100 μM probe in 50 μM MAS or 50 μM MAS Δ KS•AT in 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol at 25 °C for 21 h. The final concentration of DMSO was controlled to be 1%. The reactions were evaluated by SDS-PAGE analyses. The results are shown in Fig. 3c, Supporting Fig. S4 and Supporting Fig. S5.

Labeling of type I ketosynthases. Probes **1a** or **1b** was added from DMSO stock solutions to provide 100 μM probe in 20 μM FabB, FabF, or FabH in 100 mM Tris • HCl buffer pH 7.5 at 25 °C for 21 h. **1c**-*crypto*-AcpP was added in a similar fashion with a final concentration of 100 μM. For competitive binding, cerulenin or iodoacetamide was added into FabB, FabF, or FabH and incubated for 30 min at 25 °C prior to the addition of probes or *crypto*-AcpP. The fluorescent labeling or the crosslinking was allowed to proceed for 4 h. The reactions were evaluated by SDS-PAGE analyses. The results are shown in Supporting Fig. 13.

Probe labeling selectivity of *E. coli* FAS partner proteins. Probes **1a** or **1b** were added from DMSO stock solutions to provide 100 μM probe in 20 μM FabF (a positive control), 20 μM FabF (C163A) mutant, 20 μM FabG, 20 μM FabA, 20 μM FabI, or 20 μM FabD in 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol at 25 °C for 21 h. The final concentration of DMSO was controlled to be 1%. The reactions were evaluated by SDS-PAGE analyses. The results are shown in Fig. 3d and Supporting Fig. S4.

Enantiopure probes labeling efficiency. Probes (2*R*,3*S*)-**1b** or 2*S*,3*R*)-**1b** were added from DMSO stock solutions to provide 250 μM probe in 50 μM FabF* (C163A) mutant, 50 μM FabF, 50 μM FabB, or 50 μM FabH in 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol at 25 °C. The final concentration of DMSO was controlled to be 1%. Two time points at 3 h and 21

h were taken and evaluated by SDS-PAGE analyses. The results are shown in Fig. 3f and Supporting Fig. S6.

Tunable labeling of type II ketosynthase FabF. Probes **7b** or **8b** were added from DMSO stock solutions to provide 100 μM probe in 20 μM FabF in 100 mM Tris • HCl buffer pH 7.5 with 0 to 1M KF at 25 °C for 21 h. As a positive control, 100 μM **1b** was incubated with 20 μM FabF without KF at 25 °C for 21 h. The final concentration of DMSO was controlled to be 1%. As an additional control, FabF was incubated with 200 μM (+)-cerulenin (EMD Millipore Corp.) from a DMSO stock solution of 20 mM (+)-cerulenin at 25 °C for 30 min. Excess unbound (+)-cerulenin was removed by a buffer exchange with an Omega Nanosep 10K MWCO (Pall Corp.) eluting with 100 mM Tris • HCl buffer pH 7.5 prior to fluorescent labeling experiments. The reactions were evaluated by SDS-PAGE analyses. The results are shown in Fig. 3h and Supporting Fig. S6.

Crosslinking protocol. Crosslinking reactions (typically 5-10 μL) shown in Fig. 3 were quenched by SDS-loading dye (3 μL, 420 mM Tris pH 6.8, 600 mM DTT, 10% SDS, 30% glycerol, 0.012% bromophenol blue) and boiled on a water bath for 5 min. Samples were evaluated by SDS-PAGE using 12% acrylamide gels except for MAS which due to size was run on 8% SDS-PAGE gels. The gels were immersed in fixation solution (50% MeOH with 10% acetic acid) and left shaking at 25 °C for 2 h. After fixation, all gels were washed with H_2O and stained with Coomassie Brilliant Blue at 25 °C for 1 h. The resulting gels were then destained with 10% EtOH at 25 °C overnight. All gels were imaged on a Perfection V19 scanner (Epson).

***E. coli apo*-AcpP loading.** The synthesized crosslinkers were loaded onto *apo*-AcpP by a one-pot chemoenzymatic method [Worthington AS, Burkart MD. One-pot chemo-enzymatic synthesis of reporter-modified proteins. Org Biomol Chem. 2006 Jan 7;4(1):44-6]. The reaction included 1 mg/mL *apo*-AcpP, 0.01 mg/mL CoaA, 0.01 mg/mL CoaD, 0.01 mg/mL CoaE, 0.04 mg/mL Sfp, 12.5 mM $MgCl_2$, 1 mM DTT, 8 mM ATP, and 0.2 mM crosslinker **1c**, (2*R*,3*S*)-**1c**, **2c**, **5c**, **6c**, **8c**, or **9c** (Fig. 2 and Scheme 2) in a buffer containing 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol. The final concentration of DMSO was controlled to be 1%. After overnight incubation at rt, the mixture was subjected to purification on a HiLoad 16/600 Superdex 75 PG (GE Biosciences) size exclusion column through FPLC (ÄKTA Pure) using 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol to yield pure *crypto*-AcpP. The loading of crosslinkers onto AcpP was evaluated by urea gel (20% acrylamide) with the purified *crypto*-AcpPs. As reported previously, *apo*-, *holo*- and *crypto*-AcpPs travel different distances on the urea gel. The purified *crypto*-AcpP was spin concentrated to 5 mg/mL using 3k spin concentrator (Millipore Amicon Ultra). The resulting sample was aliquoted, flash frozen, and stored at -80 °C.

Crosslinking of *crypto*-AcpP with *E. coli* ketosynthases. *Crypto*-AcpP loaded with **1c** (50 μM) or **2c** (50 μM) was incubated with 20 μM FabF, 20 μM FabB, or 20 μM FabH in 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol at 25 °C for 21 h. The reactions were evaluated by SDS-PAGE analyses. The results are shown in Fig. 3e and Supporting Fig. S6.

Crosslinking of 1c *crypto*-AcpP with *E. coli* partner proteins. *Crypto*-AcpP loaded with **1c** (100 μM) was incubated with 20 μM FabF (C163A) mutant, 20 μM FabG, 20 μM FabA, 20 μM FabI, or 20 μM FabD in 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol at 25 °C for 21 h. The reactions were evaluated by SDS-PAGE analyses. The results are shown in Fig. 3e and Supporting Fig. S6.

Crosslinking efficiency of enantiopure crosslinkers. *Crypto*-AcpP loaded with **1c** (100 μM) or (2*R*,3*S*)-**1c** (100 μM) was incubated with 20 μM FabF, 20 μM FabB, or 20 μM FabH in 50 mM

Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol at 25 °C. Two time points at 3 h and 21 h were taken. The reactions were evaluated by SDS-PAGE analyses. The results are shown in Fig. 3g and Supporting Fig. S6.

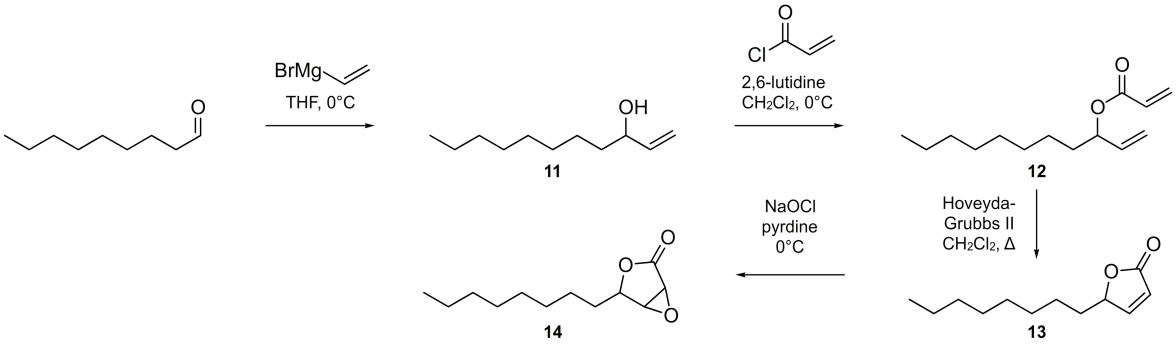
Switchable crosslinking with type II ketosynthase FabB. *Crypto*-AcpP bearing **5c** (50 μM), **6c** (50 μM), **8c** (50 μM), or **9c** (50 μM) was incubated with 10 μM FabB in 100 mM Tris • HCl buffer pH 7.5 with or without the addition of 50 mM KF at 25 °C for 21 h. The reactions were evaluated by SDS-PAGE analyses. The results are shown in Fig. 3i and Supporting Fig. S6.

***M. smegmatis* mycocerosic acid synthases (MAS) ACP loading and intramolecular crosslinking.** Crosslinkers were loaded onto the ACP of MAS *via* a one-pot chemoenzymatic reaction. The reaction contained 0.9 mg/mL MAS, 0.01 mg/mL CoaA, 0.01 mg/mL CoaD, 0.01 mg/mL CoaE, 0.04 mg/mL Sfp, 12.5 mM $MgCl_2$, 1 mM DTT, 8 mM ATP, and 8.8 μM masked crosslinkers from 10 × stock solution of **5c**, **6c**, **8c**, or **9c** in DMSO in 100 mM Tris • HCl pH 7.5. The final concentration of DMSO was controlled to be 1%. After 3 h incubation at rt, the mixture was subjected to buffer exchange to 100 mM Tris • HCl pH 7.5 using 3 column volumes of buffer on a 100k spin concentrator (Millipore Amicon Ultra). Protein was concentrated to afford a 10 mg/mL solution of MAS with the crosslinker loaded onto the ACP domain. The second unmasking step was conducted by treating a 4.4 μM solution of the resulting MAS protein with 50 mM KF in 100 mM Tris • HCl pH 7.5 for 21 h. The intramolecular crosslinking efficiency was evaluated by 8% SDS-PAGE gel analyses. Intramolecular crosslinking within MAS was visualized as a higher molecular weight band by SDS-PAGE [Konno S, La Clair JJ, Burkart MD. Trapping the Complex Molecular Machinery of Polyketide and Fatty Acid Synthases with Tunable Silylcyanohydrin Crosslinkers Angew Chem Int Ed Engl. 2018 Dec 21;57(52):17009-17013]. The results are shown in Fig. 3i and Supporting Fig. S6.

Protein crystallization, data processing, and structure refinement. *Crypto*-AcpP loaded with (2*R*,3*S*)-**1c** (100 μM) was incubated with 20 μM FabB in 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol at 25 °C for 21 h. FPLC (ÄKTA PURE) and size exclusion purification on a HiLoad 16/600 A Superdex 75 pg column (GE Biosciences) were used to obtain pure FabB crosslinked with *crypto*-AcpP loaded with (2*R*,3*S*)-**1c**. The crosslinked complex was buffer exchanged and concentrated to 9 mg/mL using an Amicon ultra centrifugal filter with 30 kDa MW cuttoff in 12.5 mM Tris • HCl buffer pH 7.4, 25 mM NaCl. For protein crystallization, the crosslinked complex was grown by vapor diffusion at 6 °C. The crosslinked complex (1 μL, 9 mg/mL) was mixed with 2 μL of the corresponding crystallographic solution. The mixture was placed inverted over 500 μL well solution (hanging-drop approach. The crosslinked complex of FabB and *crypto*-AcpP loaded with (2*R*,3*S*)-**1c** crystallized in 24% (*w*/*v*) PEG 8K, 0.2 M sodium acetate, 0.1 M sodium cacodylate pH 6.0. The protein crystallized in this condition as needle-like crystals. X-ray diffraction data (Table S1) was collected at the Advanced Light Source synchrotron at Berkeley. The collected reflection data was indexed by *iMosflm* [Battye TG, Kontogiannis L, Johnson O, Powell HR, Leslie AG. iMOSFLM: a new graphical interface for diffraction-image processing with MOSFLM. Acta Crystallogr D Biol Crystallogr. 2011 Apr;67(Pt 4):271-81] and scaled by *AIMLESS* [Winn MD, Ballard CC, Cowtan KD, Dodson EJ, Emsley P, Evans PR, Keegan RM, Krissinel EB, Leslie AG, McCoy A, McNicholas SJ, Murshudov GN, Pannu NS, Potterton EA, Powell HR, Read RJ, Vagin A, Wilson KS. Overview of the CCP4 suite and current developments. Acta Crystallogr D Biol Crystallogr. 2011 Apr;67(Pt 4):235-42]. Molecular replacement was accomplished by *PHASER* [McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. J Appl Crystallogr. 2007 Aug 1;40(Pt

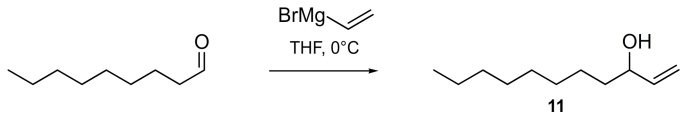
4):658-674] using a reported crosslinked structure of FabB and AcpP (PDB: 6OKC). Density map and model refinement were performed on *REFMAC5* [Murshudov GN, Skubák P, Lebedev AA, Pannu NS, Steiner RA, Nicholls RA, Winn MD, Long F, Vagin AA. REFMAC5 for the refinement of macromolecular crystal structures. Acta Crystallogr D Biol Crystallogr. 2011 Apr;67(Pt 4):355-67] and *Coot* [Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. Acta Crystallogr D Biol Crystallogr. 2010 Apr;66(Pt 4):486-501]. Crosslinker (2*R*,3*S*)-**1c** and covalent linkages between the protein and crosslinker were generated through *AceDRG* [Long F, Nicholls RA, Emsley P, Graæulis S, Merkys A, Vaitkus A, Murshudov GN. AceDRG: a stereochemical description generator for ligands. Acta Crystallogr D Struct Biol. 2017 Feb 1;73(Pt 2):112-122] for modeling. All the X-ray crystallography data processing modules were performed on *CCP4i2* suite [Potterton L, Agirre J, Ballard C, Cowtan K, Dodson E, Evans PR, Jenkins HT, Keegan R, Krissinel E, Stevenson K, Lebedev A, McNicholas SJ, Nicholls RA, Noble M, Pannu NS, Roth C, Sheldrick G, Skubak P, Turkenburg J, Uski V, von Delft F, Waterman D, Wilson K, Winn M, Wojdyr M. CCP4i2: the new graphical user interface to the CCP4 program suite. Acta Crystallogr D Struct Biol. 2018 Feb 1;74(Pt 2):68-84]. The crystal structure has been deposited on Protein Data Bank as 8SMS.

D. Synthesis of racemic epoxylactone **14** from nonanal.



Scheme S1. A four-step synthesis was developed to produce epoxylactone **14** from nonanal. Ester **12** was prepared by a two-step process. Ring closing metathesis was used to convert **12** to **13**, which in turn was epoxidized to yield **14** in 46% overall yield from nonanal.

Synthesis of undec-1-en-3-ol (11)



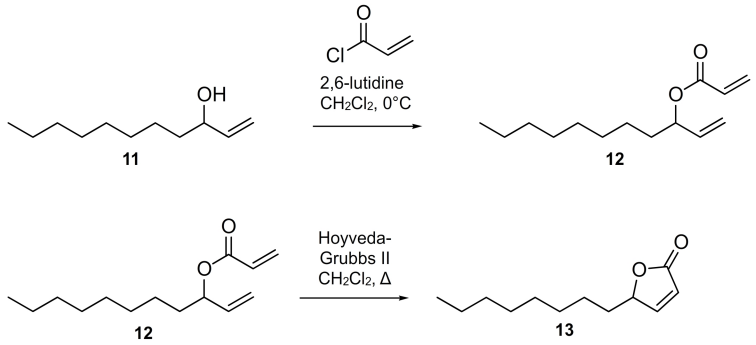
Reagents:

Nonanal, 97% (Alfa Aesar): used without further purification
Vinylmagnesium chloride, 2M in THF (Acros Organics): used without further purification

Undec-1-en-3-ol (11). Nonanal (1.20 mL, 7.03 mmol) dissolved in THF (12 mL) was cooled to 0 °C followed by the dropwise addition of vinylmagnesium chloride in THF (2M, 5.30 mL, 10.6 mmol). The mixture was stirred at rt overnight. Satd. NH_4Cl (10 mL) was added to the mixture at 0 °C and warmed to rt. The mixture was extracted with EtOAc (3 × 15 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure allyl alcohol **11** (1.42 g, 99%) was obtained as a clear oil.

Allyl alcohol **11**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.85 (ddd, J = 17.1, 10.4, 6.2 Hz, 1H), δ 5.20 (dt, J = 17.2, 1.5 Hz, 1H), δ 5.08 (dt, J = 10.4, 1.4 Hz, 1H), 4.07 (m, 1H), 1.85 (s, 1H), 1.50 (m, 2H), 1.26 (m, 12H), 0.85 (t, J = 6.7 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 141.5, 114.6, 73.4, 37.1, 32.0, 29.7, 29.4, 25.5, 22.8, 14.2.

Synthesis of 5-Octylfuran-2(5H)-one (13)



Reagents:

Acryloyl chloride (contains 400 ppm phenothiazine as stabilizer), ≥97% (Sigma Aldrich): used without further purification
2,6-Lutidine 99.5% (Chem-Impex International, Inc.): used without further purification
Propionyl chloride, 99% (Chem-Impex International, Inc.): used without further purification

2nd Generation Hoyveda-Grubb's catalyst, 97% (Sigma Aldrich): used without further purification

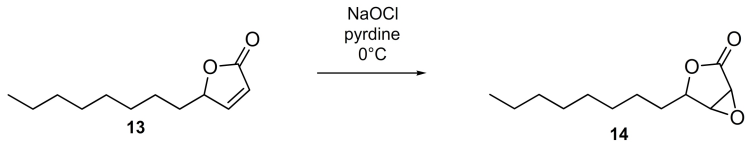
The conversion of **11** to **13** was run in two steps without detailed intermediate characterization.

Undec-1-en-3-yl acrylate (12). Allyl alcohol **11** (600.0 mg, 3.565 mmol) was dissolved in CH_2Cl_2 (7.2 mL). After cooling to 0 °C, 2,6-lutidine (0.83 mL, 7.13 mmol) was added. After 10 min, acryloyl chloride (0.36 mL, 4.5 mmol) was added in a dropwise manner. The mixture was vigorously stirred overnight at rt. Satd. $NaHCO_3$ (5 mL) and brine (5 mL) were added, and the mixture was separated followed by extraction of the aqueous phase with CH_2Cl_2 (3 × 15 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure ester **12** (0.55 g, 69%) was obtained as a yellow oil by flash chromatography, eluting with a gradient of hexanes to 5:95 EtOAc/hexanes.

5-Octylfuran-2(5H)-one (13). Ester **12** (238.0 mg, 1.061 mmol) in CH_2Cl_2 (110 mL) was added to a two-necked flask (500 mL) equipped with an additional funnel (125 mL). The mixture was allowed to reflux, and 2nd Generation Hoveyda-Grubb's catalyst (90.1 mg, 0.106 mmol) dissolved in CH_2Cl_2 (100 mL) was added dropwise over 30 min. The mixture was refluxed overnight, cooled, and concentrated by rotary evaporation. Pure butenolide **13** (187 mg, 90%) was obtained by flash chromatography, eluting with a gradient of hexanes to 1:5 acetone/hexane.

Butenolide **13**: ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.45 (dd, J = 5.7, 1.5 Hz, 1H), 6.10 (dd, J = 5.7, 2.0 Hz, 1H), 5.03 (ddt, J = 7.3, 3.4, 1.7 Hz, 1H), 1.75 (m, 1H), 1.65 (m, 1H), 1.43 (m, 2H), 1.25 (m, 10H), 0.86 (t, J = 6.9 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 173.4, 156.5, 121.6, 83.6, 33.3, 31.9, 29.4, 29.4, 29.3, 25.1, 22.8, 14.2; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{12}H_{21}O_2$ $[M+H]^+$:197.1542, found 197.1536.

Synthesis of epoxylactone 14



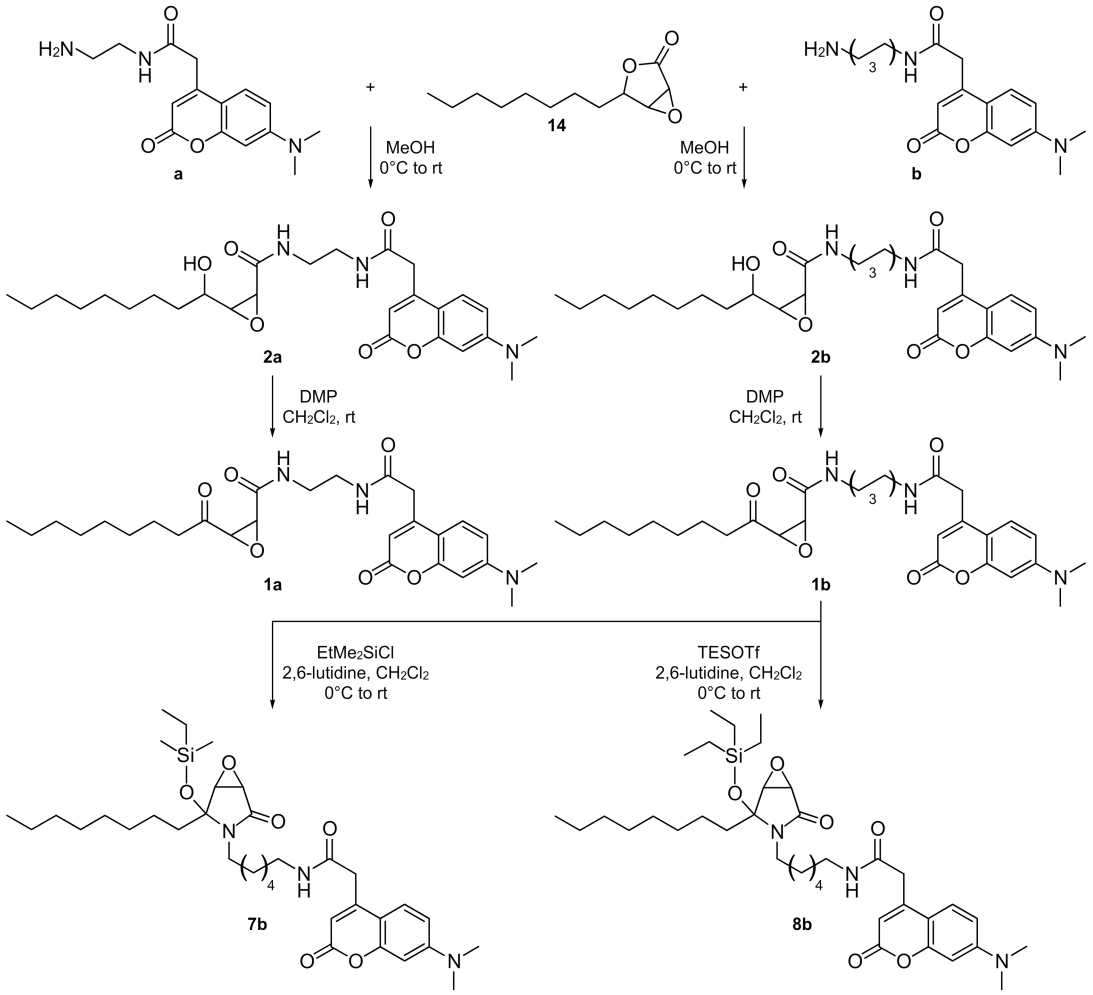
Reagents:

Pyridine, 99% (Fischer Scientific): used without further purification
NaOCl, 2M, 10-15% active chlorine (Spectrum Chemical Mfg. Corp.): used without further purification.

4-Octyl-3,6-dioxabicyclo[3.1.0]hexan-2-one (14). Butenolide **13** (180.3 mg, 0.9185 mmol) was dissolved in pyridine (3.7 mL). The mixture was cooled to 0 °C followed by the dropwise addition of NaOCl (2M, 10-15% active chlorine, 1.60 mL). The reaction was allowed to stir for 3 h at 0 °C. The completion of the reaction was monitored by TLC, and additional NaOCl might be required. The mixture was quenched with satd. $NaHCO_3$ (10 mL) and extracted with EtOAc (3 × 15 mL). The combined organic layers were then washed with brine (5 × 3 mL), dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure epoxylactone **14** (144 mg, 74%) was obtained by flash chromatography, eluting with a gradient of hexane to 1:3 EtOAc/hexanes as a pale-yellow oil.

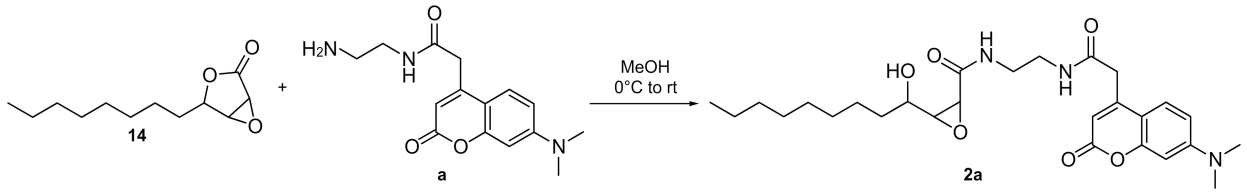
Epoxylactone **14**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.57 (t, J = 6.5 Hz, 1H), 3.96 (d, J = 2.5 Hz, 1H), 3.77 (dd, J = 2.4, 0.6 Hz, 1H), 1.68 (m, 2H), 1.45 (m, 2H), 1.28 (m, 10H), 0.87 (t, J = 6.3 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 170.5, 80.0, 58.2, 49.9, 32.2, 31.9, 29.4, 29.4, 29.3, 24.4, 22.8, 14.2; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{13}H_{24}O_4Na$ $[M+MeOH+Na]^+$:267.1572, found 267.1569.

E. Synthesis of probes **1a**, **1b**, **2a**, **2b**, **7b**, and **8b** from epoxylactone **14**



Scheme S2. Synthesis of probes **1a**, **1b**, **2a**, **2b**, **7b**, and **8b** from epoxylactone **14**. An amidation of epoxylactone **14** with fluorescent tags **a** or **b** (see Fig. 2b) gave **2a** or **2b**, respectively, which were sequentially oxidized into **1a** or **1b**. Utilizing the tautomerization of cerulenin (Fig. 1a), a single step silylation was conducted to furnish **7b** and **8b** from **1b**.

Synthesis of fluorescent probe 2a.



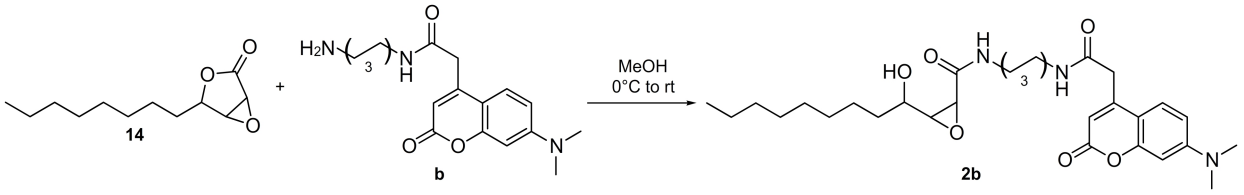
Reagents:

Fluoresent tag **a** was prepared according to: [Alexander MD, Burkart MD, Leonard MS, Portonovo P, Liang B, Ding X, Joullié MM, Gulledge BM, Aggen JB, Chamberlin AR, Sandler J, Fenical W, Cui J, Gharpure SJ, Polosukhin A, Zhang HR, Evans PA, Richardson AD, Harper MK, Ireland CM, Vong BG, Brady TP, Theodorakis EA, La Clair JJ. A central strategy for converting natural products into fluorescent probes. Chembiochem. 2006 Mar;7(3):409-16]

***N*-(2-(2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamido)ethyl)-3-(1-hydroxynonyl)oxirane-2-carboxamide (2a).** Fluorescent tag **a** (21.0 mg, 0.0726 mmol) was added to a mixture of epoxylactone **14** (10.3 mg, 0.0484 mmol) in MeOH (100 µL) at 0 °C. After warming and stirring overnight at rt, the solvent was removed by airflow. Pure probe **2a** (22.1 mg, 91%) was obtained as a yellow wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/CH_2Cl_2.

Probe **2a**: ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.49 (d, *J* = 8.9 Hz, 1H), 7.13 (t, *J* = 5.0 Hz, 1H), 6.86 (t, *J* = 5.8 Hz, 1H), 6.60 (dd, *J* = 9.0, 2.5 Hz, 1H), 6.42 (d, *J* = 2.6 Hz, 1H), 6.05 (s, 1H), 3.60 (s, 2H), 3.51 (td, *J* = 7.4, 4.9 Hz, 1H), 3.45 (d, *J* = 4.6 Hz, 1H), 3.38 (m, 2H), 3.32 (m, 2H), 3.10 (dd, *J* = 7.0, 4.6 Hz, 1H), 3.02 (s, 6H), 1.62 (m, 2H), 1.47 (m, 1H), 1.35 (m, 1H), 1.24 (m, 10H), 0.85 (t, *J* = 6.8 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 169.4, 168.7, 162.6, 156.0, 153.2, 150.3, 125.9, 110.2, 109.4, 108.5, 98.1, 68.8, 60.7, 54.5, 40.3, 40.2, 39.9, 39.2, 35.0, 32.0, 29.8, 29.7, 29.4, 25.3, 22.8, 14.3; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{27}H_{40}N_3O_6$ $[M+H]^+$:502.2918, found 502.2910.

Synthesis of probe 2b.



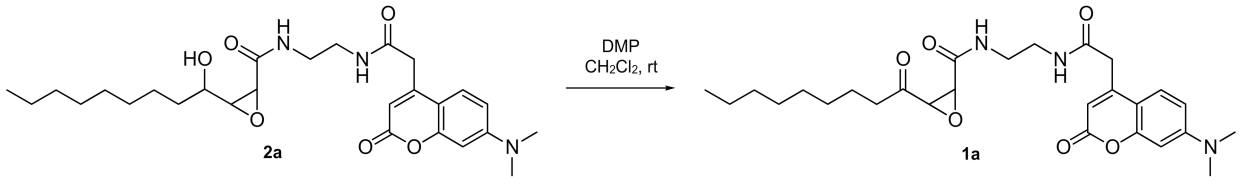
Reagents:

Fluoresent tag **b** was prepared according to: [Alexander MD, Burkart MD, Leonard MS, Portonovo P, Liang B, Ding X, Joullié MM, Gulledge BM, Aggen JB, Chamberlin AR, Sandler J, Fenical W, Cui J, Gharpure SJ, Polosukhin A, Zhang HR, Evans PA, Richardson AD, Harper MK, Ireland CM, Vong BG, Brady TP, Theodorakis EA, La Clair JJ. A central strategy for converting natural products into fluorescent probes. Chembiochem. 2006 Mar;7(3):409-16].

***N*-(6-(2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamido)hexyl)-3-(1-hydroxynonyl)oxirane-2-carboxamide (2b).** Fluorescent tag **b** (11.0 mg, 0.0319 mmol) was added to a mixture of epoxylactone **14** (4.51 mg, 0.0213 mmol) in MeOH (100 µL) at 0 °C. After warming and stirring overnight, the solvent was removed by airflow. Pure probe **2b** (9.95 mg, 84%) was obtained as a yellow wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/CH_2Cl_2.

Probe **2b**: ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.51 (d, *J* = 9.0 Hz, 1H), 6.62 (dd, *J* = 9.0, 2.5 Hz, 1H), 6.48 (d, *J* = 2.6 Hz, 1H), 6.27 (m, 2H), 6.08 (s, 1H), 3.64 (d, *J* = 2.3 Hz, 2H), 3.51 (d, *J* = 4.6 Hz, 1H), 3.35 (td, *J* = 7.7, 5.4 Hz, 1H), 3.28 (dq, *J* = 13.0, 6.4 Hz, 1H), 3.18 (p, *J* = 6.6 Hz, 2H), 3.12 (m, 2H), 3.05 (s, 6H), 1.65 (m, 2H), 1.44 (m, 6H), 1.26 (m, 14H), 0.87 (t, *J* = 6.9 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 168.3, 167.7, 162.5, 156.1, 153.3, 150.1, 126.0, 110.6, 109.4, 108.5, 98.2, 68.7, 60.6, 54.9, 41.0, 40.3, 39.0, 38.1, 35.1, 32.0, 29.8, 29.7, 29.6, 29.6, 29.4, 28.8, 25.3, 25.1, 25.1, 22.8, 14.3; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{48}N_3O_6$ $[M+H]^+$:558.3544, found 558.3534.

Synthesis of probe 1a



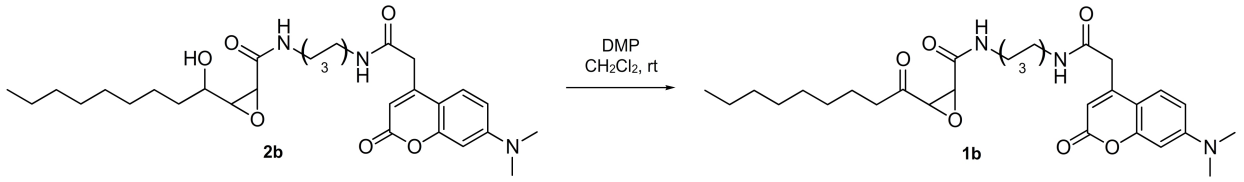
Reagents:

Dess-Martin periodinane, 95% (Astatech, Inc): used without further purification

***N*-(2-(2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamido)ethyl)-3-nonanoyloxirane-2-carboxamide (1a).** Probe **2a** (10.0 mg, 0.0199 mmol) was dissolved in CH_2Cl_2 (100 µL). Dess-Martin periodinane (11.8 mg, 0.0279 mmol) was added into the mixture at rt, and stirred overnight. The mixture was quenched with satd. Na_2S2O_3 (200 µL) and satd. $NaHCO_3$ (200 µL) and extracted with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure probe **1a** (5.0 mg, 50%) was obtained as a yellow wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/CH_2Cl_2

Probe **1a**: ^{1}H-NMR (500 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers and tautomers within **1a**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (125 MHz, $CDCl_3$) δ 204.6, 169.9, 169.9, 168.8, 165.1, 162.0, 162.0, 156.2, 156.2, 156.2, 153.2, 153.2, 149.3, 149.3, 129.6, 125.9, 125.5, 111.3, 109.3, 109.3, 108.6, 108.6, 98.5, 98.5, 89.1, 89.1, 57.7, 55.6, 52.8, 41.8, 41.1, 40.4, 40.3, 39.6, 39.0, 39.0, 37.2, 34.9, 31.9, 31.9, 29.8, 29.7, 29.6, 29.6, 29.4, 29.3, 29.3, 29.2, 29.1, 23.2, 23.1, 22.8, 14.2; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{27}H_{37}N_3O_6Na$ $[M+Na]^+$:522.2580, found 522.2580.

Synthesis of probe 1b



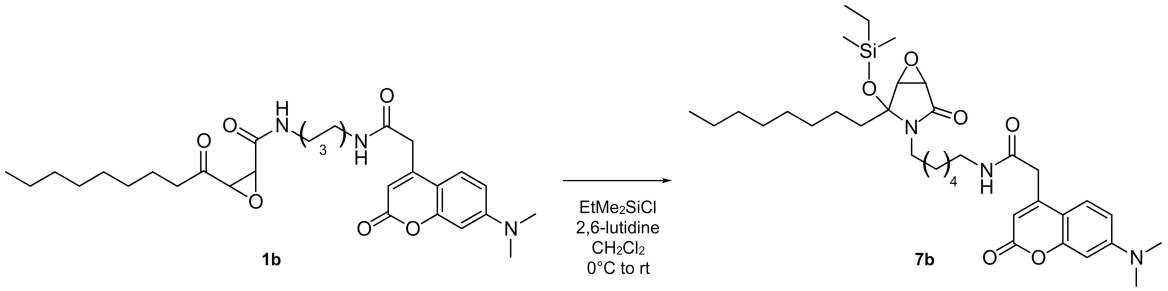
Reagents:

Dess-Martin periodinane, 95% (Astatech, Inc): used without further purification

***N*-(6-(2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamido)hexyl)-3-nonanoyloxirane-2-carboxamide (1b).** Probe **2b** (10.0 mg, 0.0179 mmol) was dissolved in CH_2Cl_2 (100 µL). Dess-Martin periodinane (10.6 mg, 0.0251 mmol) was added into the mixture at rt, and stirred overnight. The mixture was quenched with satd. Na_2S2O_3 (200 µL) and satd. $NaHCO_3$ (200 µL) and extracted with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure probe **1b** (4.5 mg, 45%) was obtained as a yellow wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/CH_2Cl_2.

Probe **1b**: ^{1}H-NMR (500 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers and tautomers within **1b**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (125 MHz, $CDCl_3$) δ 203.1, 168.0, 164.9, 162.0, 156.2, 153.2, 150.0, 125.9, 110.6, 109.3, 108.4, 98.3, 58.5, 55.7, 41.2, 41.1, 40.3, 39.5, 38.6, 31.9, 29.4, 29.3, 29.3, 29.2, 29.1, 25.9, 25.8, 23.3, 22.8, 14.2; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{45}N_3O_6Na$ $[M+Na]^+$:578.3206, found 578.3193.

Synthesis of caged probe 7b



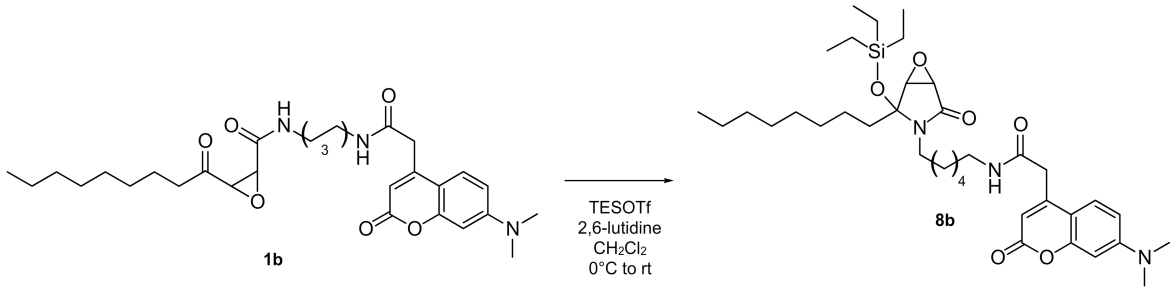
Reagents:

2,6-Lutidine 99.46% (Chem-Impex International, Inc.): used without further purification
Chloroethyldimethylsilane 97% (TCI): used without further purification.

2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)-*N*-(6-(2-((ethyldimethylsilyl)oxy)-2-octyl-4-oxo-6-oxa-3-azabicyclo[3.1.0]hexan-3-yl)hexyl)acetamide (7b). Probe **1b** (5.0 mg, 0.0090 mmol) was dissolved in CH_2Cl_2 (100 µL). The mixture was then cooled to 0°C prior to the addition of 2,6-lutidine (3.1 µL, 0.027 mmol) and chloroethyldimethylsilane (3.8 µL, 0.027 mmol). The reaction was stirred overnight at rt. The reaction was quenched with satd. $NaHCO_3$ (200 µL) and extracted with CH_2Cl_2 (3 × 1 mL). Pure caged probe **7b** (2.7 mg, 48%) was obtained by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/CH_2Cl_2

Caged probe **7b**: ^{1}H-NMR (400 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **7b**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 169.9, 168.4, 168.0, 161.9, 156.2, 153.1, 150.0, 126.0, 110.9, 109.6, 98.7, 91.5, 56.9, 55.0, 53.3, 53.2, 52.1, 41.0, 40.5, 39.6, 39.3, 38.8, 38.5, 36.8, 32.0, 31.9, 30.0, 29.7, 29.6, 29.4, 29.3, 29.2, 29.2, 28.9, 28.8, 26.5, 26.3, 26.0, 26.0, 24.5, 23.5, 22.8, 22.8, 14.3, 9.7, 7.0, 6.9, -0.1, -0.4, -0.5, -0.7; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{35}H_{55}N_3O_6SiNa$ $[M+Na]^+$: 664.3758, found 664.3747.

Synthesis of caged probe 8b



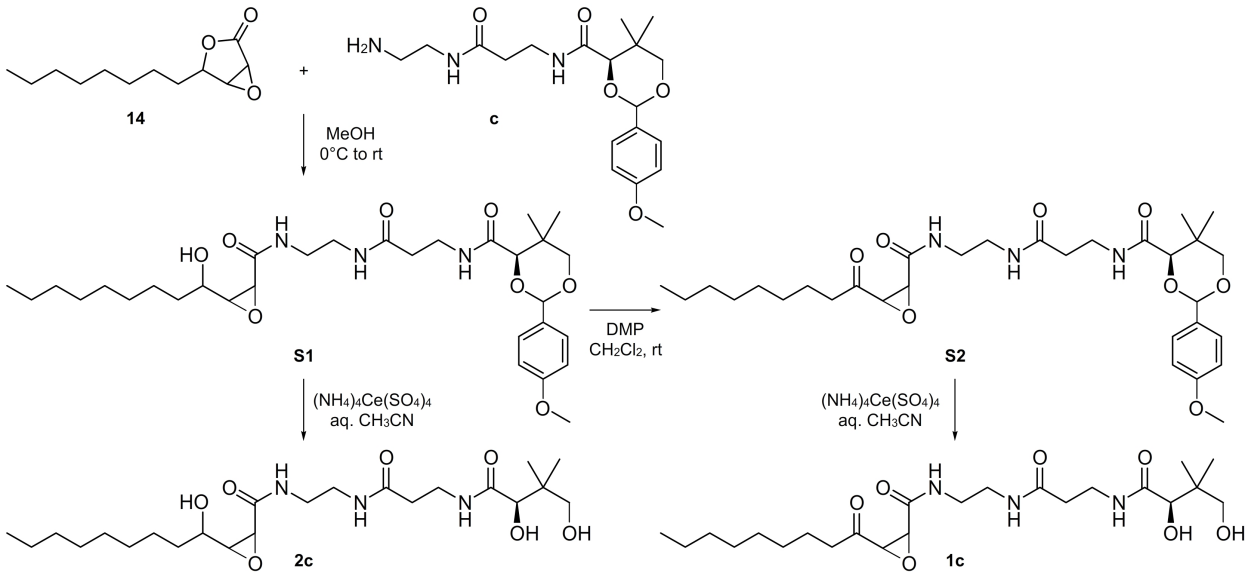
Reagents:

2,6-Lutidine 99.46% (Chem-Impex International, Inc.): used without further purification
Triethylsilyl trifluoromethanesulfonate 98% (Alfa Aesar): used without further purification

2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)-*N*-(6-(2-octyl-4-oxo-2-((triethylsilyl)oxy)-6-oxa-3-azabicyclo[3.1.0]hexan-3-yl)hexyl)acetamide (8b). Caged probe **1b** (4.0 mg, 0.0072 mmol) was added dissolved in CH_2Cl_2 (100 µL). The solution was then cooled to 0°C prior to the addition of 2,6-lutidine (2.5 µL, 0.022 mmol) and TESOTf (4.9 µL, 0.022 mmol). The reaction was stirred overnight at rt. The reaction was quenched with satd. $NaHCO_3$ (200 µL) and extracted with CH_2Cl_2 (3 × 1 mL). Pure probe **8b** (2.0 mg, 40%) was obtained by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/CH_2Cl_2

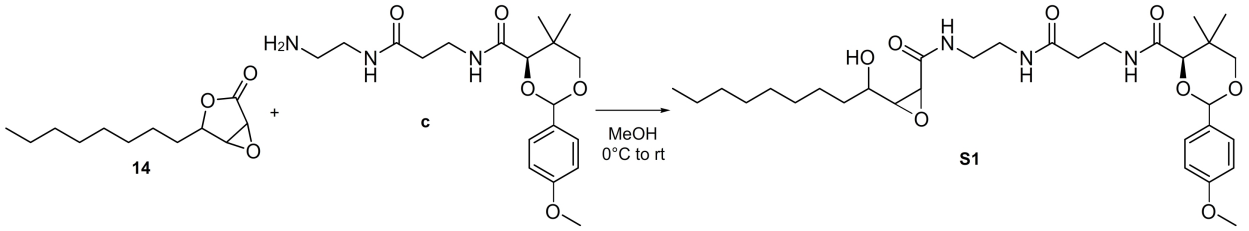
Caged probe **8b**: ^{1}H-NMR (300 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **8b**, the ^{1}H-NMR data was not tabulated;^{13}C-NMR (125 MHz, $CDCl_3$) δ 169.8, 168.0, 161.9, 156.2, 153.2, 149.9, 125.9, 110.6, 109.3, 108.5, 98.4, 91.3, 90.2, 56.8, 55.1, 53.6, 53.1, 53.1, 52.0, 51.1, 41.0, 40.3, 39.6, 38.8, 36.9, 32.0, 31.9, 29.9. 29.7, 29.7, 29.5, 29.3, 29.3, 29.2, 29.2, 28.8, 28.7, 14.3, 7.1, 7.0, 6.8, 6.2, 6.0, 5.9; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{37}H_{59}N_3O_6SiNa$ $[M+Na]^+$:692.4071, found 692.4060.

F. Synthesis of crosslinkers 1c and 2c and crosslinkers 8c and 9c.



Scheme S3. Synthesis of crosslinkers 1c and 2c from 14. Epoxylactone **14** was amidated with pantetheine amine **c (**Fig. 2b) to **S1**, which was oxidized to furnish central intermediate **S2**. A chemoselective deprotection using $(NH_4)_4Ce(SO_4)_4$ converted intermediate **S1** and **S2** to respective crosslinkers **2c** and **1c**, respectively.

Synthesis of intermediate S1



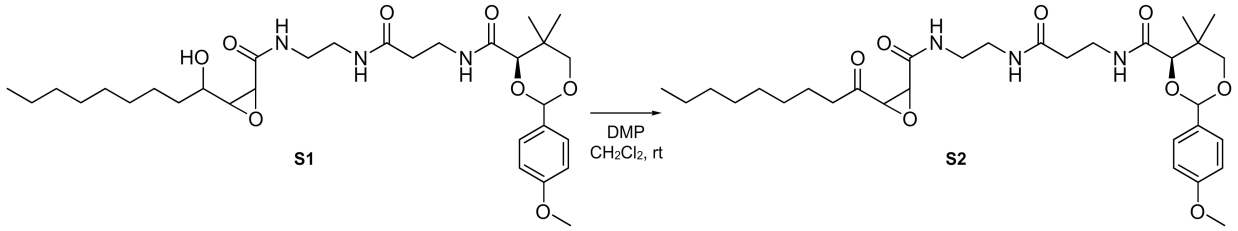
Reagents:

PMP-protected pantetheine amine was prepared according to: [Beld J, Cang H, Burkart MD. Visualizing the chain-flipping mechanism in fatty-acid biosynthesis. Angew Chem Int Ed Engl. 2014 Dec 22;53(52):14456-61].

(4*R*)-*N*-(3-((2-(3-(1-hydroxynonyl)oxirane-2-carboxamido)ethyl)amino)-3-oxopropyl)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamide (S1). Epoxylactone **14** (16.0 mg, 0.0752 mmol) was dissolved in MeOH (400 µL). Amine **c** (57.0 mg, 0.150 mmol) was added into the mixture at rt, and the reaction was stirred overnight. The solvent was then removed by airflow. Pure intermediate **S1** (40.9 mg, 92%) was obtained by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/CH_2Cl_2.

Intermediate **S1**: ^{1}H-NMR (400 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **S1**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 171.8, 171.8, 170.2, 170.1, 168.4, 168.3, 160.4, 130.2, 130.1, 127.7, 127.7, 113.9, 101.6, 101.5, 84.0, 83.9, 78.5, 68.8, 68.6, 60.7, 55.5, 54.5, 54.4, 54.4, 39.4, 39.3, 39.1, 36.5, 36.4, 35.6, 35.5, 35.1, 34.9, 33.2, 32.0, 29.8, 29.7, 29.4, 25.3, 25.2, 22.8, 22.0, 19.3, 14.3; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{49}N_3O_8Na$ $[M+Na]^+$:614.3418, found 614.3411.

Synthesis of intermediate S2



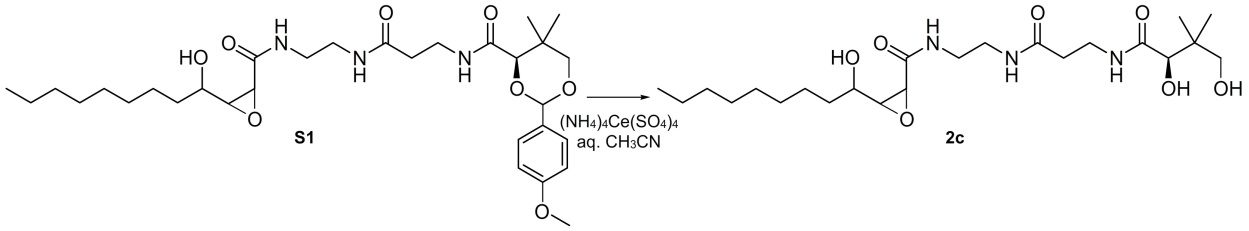
Reagents:

Dess-Martin periodinane, 95% (Astatech, Inc): used without further purification

(4*R*)-2-(4-methoxyphenyl)-5,5-dimethyl-*N*-(3-((2-(3-nonanoyloxirane-2-carboxamido)ethyl)amino)-3-oxopropyl)-1,3-dioxane-4-carboxamide (S2). Intermediate **S1** (8.0 mg, 0.014 mmol) was dissolved in CH_2Cl_2 (200 µL). Dess-Martin periodinane (8.0 mg, 0.019 mmol) was added into the mixture at rt, and the reaction was stirred overnight. The reaction was quenched with satd. Na_2S2O_3 (300 µL) and satd. $NaHCO_3$ (300 µL) and extracted with CH_2Cl_2 (3 × 3 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure central intermediate **S2** (7.0 mg, 88%) was obtained by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/ CH_2Cl_2.

Central intermediate **S2**: ^{1}H-NMR (400 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers and tautomers within **S2**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 204.4, 171.6, 171.6, 169.7, 169.5, 165.2, 165.0, 160.4, 160.4, 130.3, 130.2, 127.7, 127.7, 113.2, 113.2, 101.6, 101.5, 84.1, 84.0, 78.7, 58.0, 58.0, 55.8, 55.7, 55.5, 41.6, 41.6, 39.1, 39.1, 39.0, 38.8, 36.5, 36.3, 35.2, 35.2, 33.2, 33.2, 32.0, 31.9, 29.4, 29.3, 29.2, 29.1, 23.3, 23.3, 22.8, 22.7, 22.0, 19.3, 19.3, 14.3, 14.2; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{47}N_3O_8Na$ $[M+Na]^+$:612.3261, found 612.3251.

Synthesis of Crosslinker 2c.



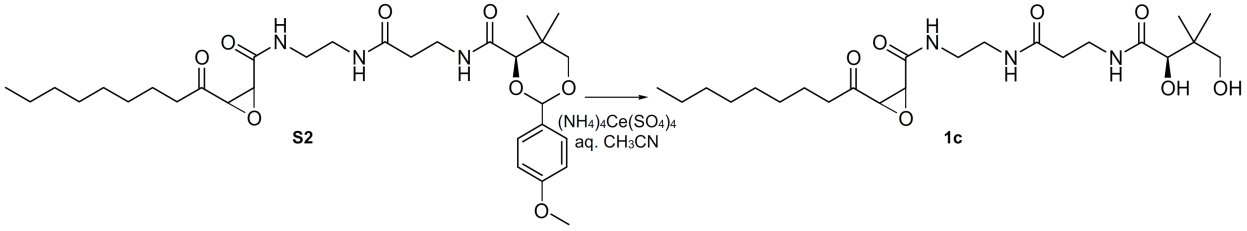
Reagents:

$(NH_4)_4Ce(SO_4)_4$ • 2 H_2O, 95% (Alfa Aesar): used without further purification

***N*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl)-3-(1-hydroxynonyl)oxirane-2-carboxamide (2c).** $(NH_4)_4Ce(SO_4)_4$ • 2 H_2O (1.1 mg, 0.0017 mmol) was added at rt to **S1** (10.0 mg, 0.0170 mmol) was dissolved into 9:1 CH_3CN/H_2O (100 μL). The reaction was allowed to proceed at rt for 1 h. The completion of the reaction was monitored by TLC and quenched with satd. $NaHCO_3$ (200 μL). The mixture was extracted with CH_2Cl_2 (3 × 2 mL). Pure crosslinker **2c** (3.6 mg, 45%) was obtained as a clear wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/CH_2Cl_2

Crosslinker **2c**: ^{1}H-NMR (300 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **2c**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (125 MHz, CD_3OD) δ 175.1, 175.0, 173.2, 173.1, 169.2, 169.1, 76.1, 69.1, 67.9, 60.1, 54.1, 52.4, 46.7, 39.2, 38.9, 38.8, 38.7, 35.5, 35.4, 35.2, 32.0, 29.7, 29.6, 29.3, 25.0, 22.6, 20.2, 19.7, 13.3, 8.1, 6.4; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{23}H_{43}N_3O_7Na$ $[M+Na]^+$:496.2999, found 496.2993.

Synthesis of crosslinker 1c

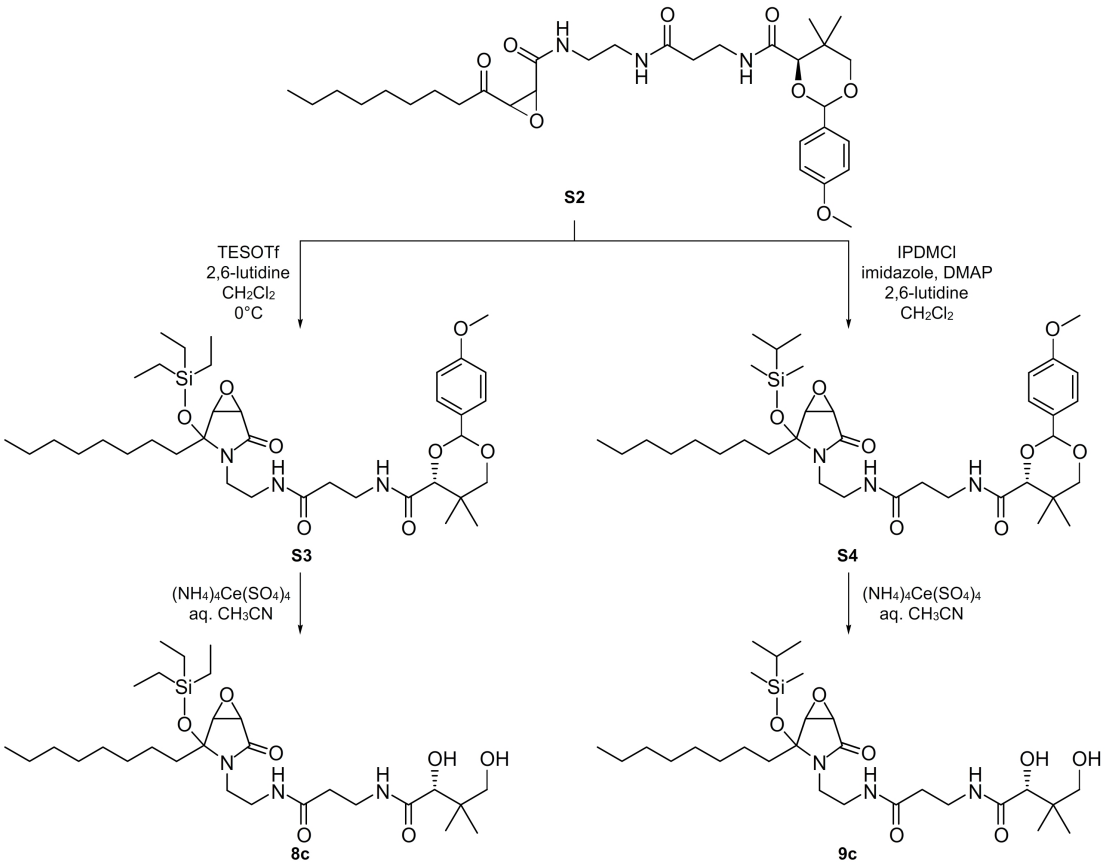


Reagents:

$(NH_4)_4Ce(SO_4)_4$ • 2 H_2O , 95% (Alfa Aesar): used without further purification

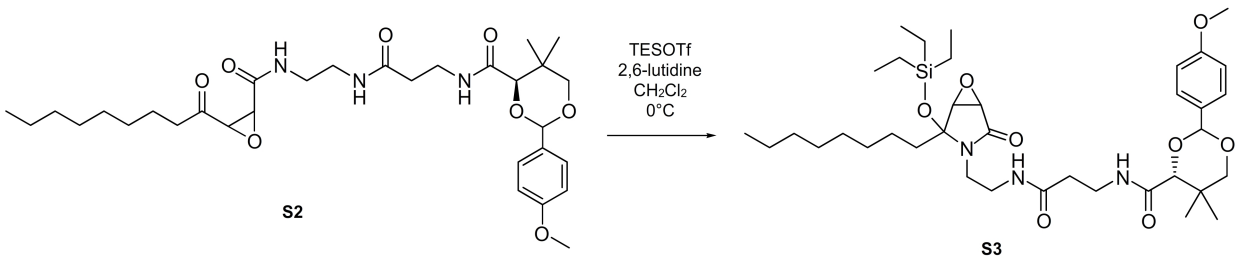
***N*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl)-3-nonanoyloxirane-2-carboxamide (1c)**. $(NH_4)_4Ce(SO_4)_2$ • 2 H_2O (2.1 mg, 0.0034 mmol) was added at rt to **S2** (8.0 mg, 0.017 mmol) dissolved into 9:1 CH_3CN/H_2O (100 µL). The reaction was allowed to proceed at rt for 1 h. The completion of the reaction was monitored by TLC and quenched with satd. $NaHCO_3$ (200 µL). The mixture was extracted with CH_2Cl_2 (3 × 2 mL). Pure crosslinker **1c** (4.1 mg, 51%) was obtained as a clear wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/CH_2Cl_2.

Crosslinker **1c**: ^{1}H-NMR (400 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers and tautomers within **1c**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (125 MHz, $CDCl_3$) δ 205.2, 204.9, 175.7, 174.0, 173.8, 173.6, 173.4, 173.2, 172.2, 172.0, 171.6, 171.5, 165.3, 165.2, 89.7, 89.6, 89.6, 89.5, 78.2, 78.0, 77.9, 77.8, 76.6, 71.2, 71.1, 71.0, 70.9, 58.3, 57.9, 57.8, 56.2, 56.1, 56.0, 55.9, 52.6, 52.5, 51.8, 51.7, 41.9, 41.7, 40.8, 40.2, 39.5, 39.4, 39.4, 39.4, 39.2, 39.2, 39.1, 39.1, 39.1, 37.1, 36.2, 36.1, 35.6, 35.3, 35.1, 35.0, 34.6, 34.5, 34.4, 32.0, 31.9, 31.9, 30.1, 30.0, 29.7, 29.6, 29.5, 29.4, 29.3, 29.3, 29.2, 29.1, 29.1, 24.0, 24.0, 23.4, 23.3, 23.1, 23.1, 22.8, 22.8, 22.2, 22.0, 21.8, 21.8, 21.7, 21.5, 21.4, 21.3, 20.7, 20.6, 20.4, 20.2, 14.3, 14.3; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{23}H_{41}N_3O_7Na$ $[M+Na]^+$:494.2843, found 494.2836.



Scheme S4. Synthesis of caged crosslinkers 8c and 9c from central intermediate S2. Utilizing the tautomerization of the cerulenin moiety, a single step silylation was able to trap intermediates **S3** and **S4** in their cyclic form. A chemoselective deprotection using $(NH_4)_4Ce(SO_4)_4$ converted intermediates **S3** and **S4** to caged crosslinkers **8c** and **9c**, respectively.

Synthesis of intermediate S3



Reagents:

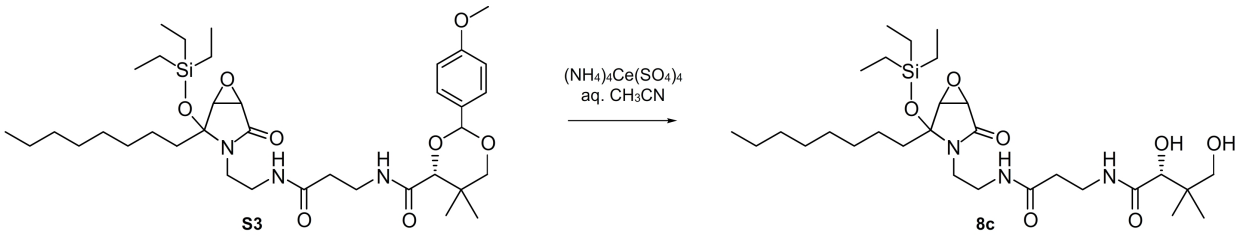
Triethylsilyl trifluoromethanesulfonate, >98.0% (TCI Chemicals): used without further purification

2,6-Lutidine 99.46% (Chem-Impex International, Inc.): used without further purification

(4*R*)-2-(4-methoxyphenyl)-5,5-dimethyl-*N*-(3-((2-(2-octyl-4-oxo-2-((triethylsilyl)oxy)-6-oxa-3-azabicyclo[3.1.0]hexan-3-yl)ethyl)amino)-3-oxopropyl)-1,3-dioxane-4-carboxamide (S3). Central intermediate **S2** (10.0 mg, 0.0170 mmol) was dissolved into CH_2Cl_2 (100 µL) and cooled to 0 °C. 2,6-Lutidine (5.9 µL, 0.051 mmol) and triethylsilyl trifluoromethanesulfonate (11.5 µL, 0.051 mmol) was added dropwise. The reaction was allowed to proceed at 0 °C for 1 h. The completion of the reaction was monitored by TLC. The reaction mixture was then diluted with CH_2Cl_2 (1 mL) and satd. $NaHCO_3$ (1 mL).. Extraction was performed with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure intermediate **S3** (8.6 mg, 70%) was obtained as a clear wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 1:99 MeOH/ CH_2Cl_2

Intermediate **S3**: ^{1}H-NMR (300 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **S3**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 171.4,169.5, 160.3, 130.4, 127.6, 113.8, 101.4, 91.4, 84.0, 78.7, 57.0, 55.4, 51.8, 38.3, 36.7, 35.9, 35.0, 33.2, 31.9, 29.8, 29.6, 29.3, 24.5, 22.8, 22.0, 19.3, 14.2, 7.1, 7.0, 6.7, 6.2, 6.0, 5.9; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{37}H_{61}N_3O_8SiNa$ $[M+Na]^+$:726.4126, found 726.4118.

Synthesis of caged crosslinker 8c



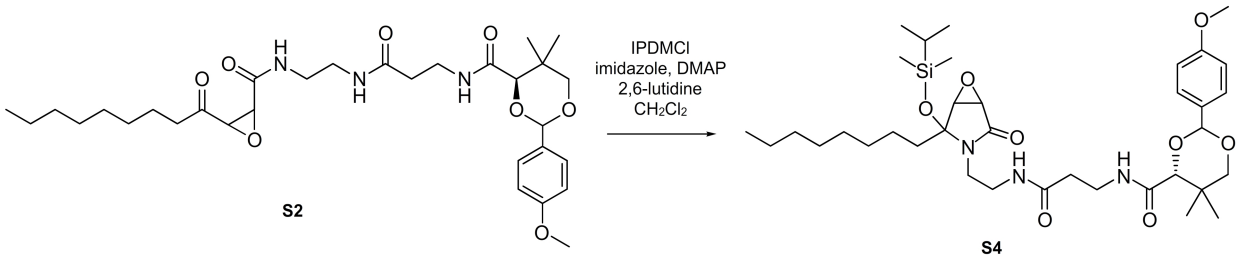
Reagents:

$(NH_4)_4Ce(SO_4)_4$ • 2 H_2O , 95% (Astatech, Inc): used without further purification

(2*R*)-2,4-dihydroxy-3,3-dimethyl-*N*-(3-((2-(2-octyl-4-oxo-2-((triethylsilyl)oxy)-6-oxa-3-azabicyclo[3.1.0]hexan-3-yl)ethyl)amino)-3-oxopropyl)butanamide (8c). $(NH_4)_4Ce(SO_4)_2$ • 2 H_2O (11.0 mg, 0.0180 mmol) was added to **S3** (8.6 mg, 0.012 mmol) in 9:1 CH_3CN/H_2O (200 µL). The reaction was allowed to proceed at rt for 1 h . The reaction was monitored by TLC and quenched with satd. $NaHCO_3$ (200 µL) and extracted with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure caged crosslinker **8c** (3.0 mg, 43%) was obtained as a clear wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/ CH_2Cl_2.

Caged crosslinker **8c**: ^{1}H-NMR (800 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **8c**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (200 MHz, $CDCl_3$) δ 173.7, 173.6, 173.5, 173.4, 171.9, 171.9, 171.8, 171.8, 171.5, 171.5, 170.3, 170.1, 91.6, 90.8, 90.5, 78.1, 78.0, 78.0, 71.2, 71.2, 71.1, 71.0, 57.1, 57.0, 55.2, 55.1, 52.8, 52.8, 51.8, 51.8, 40.8, 40.3, 40.3, 40.2, 39.6, 39.5, 39.4, 39.1, 38.3, 38.3, 38.2, 37.0, 36.8, 36.0, 35.9, 35.9, 35.4, 35.3, 35.3, 35.2, 32.0, 31.9, 29.9, 29.7, 29.6, 29.5, 29.5, 29.3, 29.3, 24.5, 24.5, 23.7, 22.8, 22.8, 22.2, 22.1, 22.1, 22.0, 20.7, 20.6, 20.5, 20.4, 14.2, 14.2, 7.1, 7.0, 6.9, 6.3, 6.3, 6.1, 6.1; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{29}H_{55}N_3O_7SiNa$ $[M+Na]^+$:608.3707, found 608.3700.

Synthesis of intermediate S4



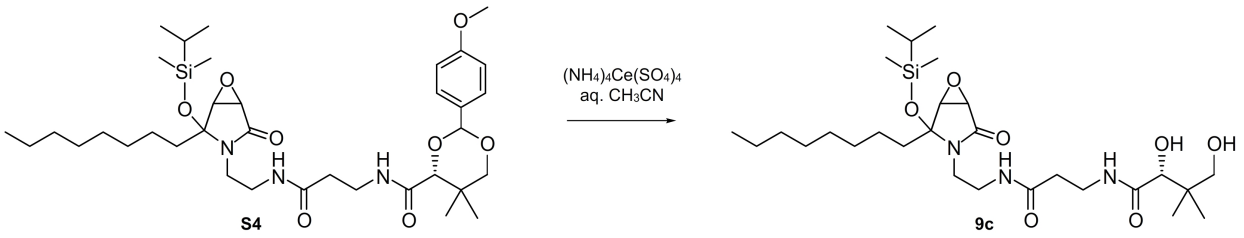
Reagents:

Dimethylisopropylchlorosilane, 90.0% (TCI Chemicals): used without further purification
Imidazole, 99% (Sigma Aldrich): used without further purification
DMAP (4-Dimethylaminopyridine), 99% (Alfa Aesar): used without further purification
2,6-Lutidine 99.46% (Chem-Impex International, Inc.): used without further purification
.

(4*R*)-*N*-(3-((2-(2-((isopropyldimethylsilyl)oxy)-2-octyl-4-oxo-6-oxa-3-azabicyclo[3.1.0]hexan-3-yl)ethyl)amino)-3-oxopropyl)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamide (S4). Intermediate **S2** (15.0 mg, 0.0254 mmol) was dissolved in CH_2Cl_2 (100 µL). Imidazole (0.3 mg, 0.004 mmol) and DMAP (0.3 mg, 0.003 mmol) were added into the reaction mixture followed by dropwise addition of 2,6-lutidine (10.1 µL, 0.086 mmol). Isopropyldimethylsilyl chloride (0.050 mL, 0.320 mmol) was added into the reaction, and the reaction was allowed to proceed at rt overnight. Satd. $NaHCO_3$ (500 µL) was added, and the mixture was separated followed by extraction of the aqueous phase with CH_2Cl_2 (3 × 2 mL). The combined organic phases were washed with brine (3 × 0.5 mL), dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure intermediate **S4** (6.5 mg, 37%) was obtained as a clear wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/ CH_2Cl_2.

Intermediate **S4**: ^{1}H-NMR (300 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **S4**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 172.3, 171.4, 171.4, 171.2, 171.2, 170.0, 169.5, 169.5, 169.3, 160.3, 160.3, 130.2, 127.8, 127.7, 127.7, 127.6, 113.9, 113.8, 101.4, 101.4, 91.5, 91.4, 84.0, 78.7, 57.0, 55.5, 51.8, 39.3, 38.4, 35.9, 35.0, 33.2, 32.0, 32.0, 31.9, 30.1, 29.8, 29.7, 29.7, 29.6, 29.5, 29.3, 29.3, 24.4, 22.8, 22.8, 22.0, 22.0, 19.3, 19.3, 17.0, 16.9, 16.9, 15.5, 15.5, 14.2, 14.2, -1.4, -2.1, -2.3, -2.4, -2.6; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{36}H_{59}N_3O_8SiNa$ $[M+Na]^+$:712.3969, found 712.3961.

Synthesis of caged crosslinker 9c



Reagents:

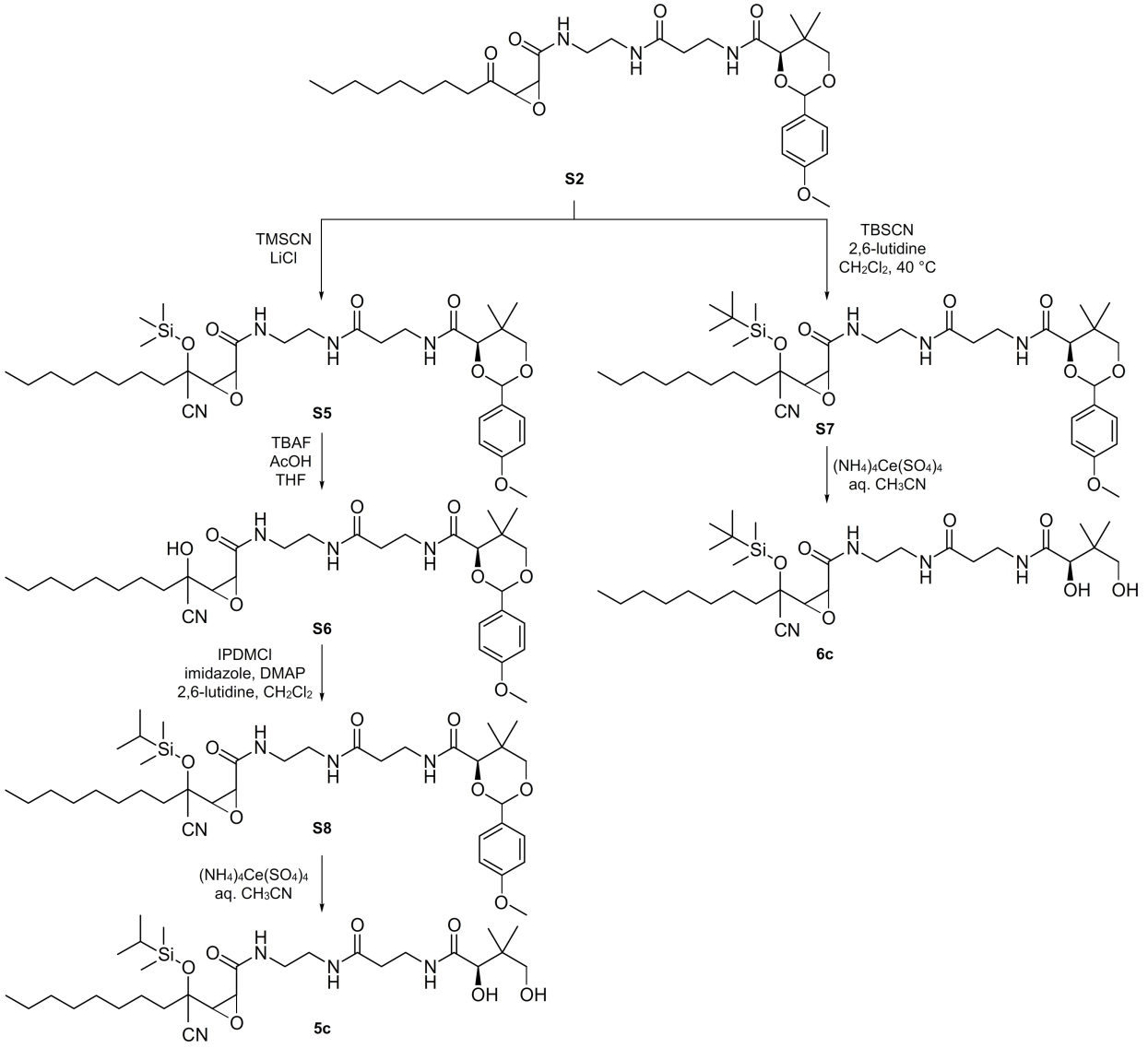
$(NH_4)_4Ce(SO_4)_4$ • 2 H_2O, 95% (Astatech, Inc): used without further purification

(2*R*)-2,4-dihydroxy-*N*-(3-((2-(2-((isopropyldimethylsilyl)oxy)-2-octyl-4-oxo-6-oxa-3-azabicyclo[3.1.0]hexan-3-yl)ethyl)amino)-3-oxopropyl)-3,3-dimethylbutanamide (9c).

$(NH_4)_4Ce(SO_4)_2$ • 2 H_2O (12.7 mg, 0.0200 mmol) was added to **S4** (6.0 mg, 0.010 mmol) in 9:1 CH_3CN/H_2O (100 µL). The reaction was allowed to proceed at rt for 1 h. The reaction was monitored by TLC. Satd. $NaHCO_3$ (200 µL) was added, and the mixture was separated followed by extraction of the aqueous phase with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure caged crosslinker **9c** (2.5 mg, 43%) was obtained by flash chromatography, eluting with a gradient of CH_2Cl_2 to 3:97 $MeOH/CH_2Cl_2$.

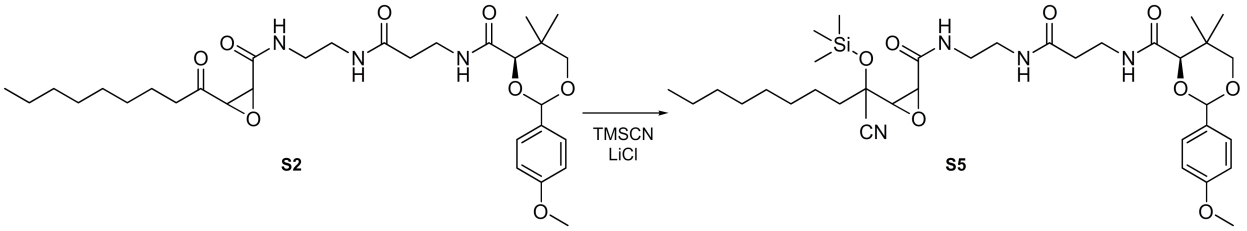
Caged crosslinker **9c**: ^{1}H-NMR (800 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **9c**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (200 MHz, $CDCl_3$) δ 174.0, 173.9, 173.9, 173.8, 172.2, 172.2, 172.1, 172.0, 171.8, 171.7, 170.5, 170.3, 91.9, 91.1, 90.9, 78.3, 78.2, 78.2, 71.4, 71.4, 71.3, 57.4, 57.3, 55.4, 55.3, 53.3, 52.1, 52.1, 41.0, 40.6, 40.4, 40.4, 39.8, 39.8, 39.6, 39.4, 38.7, 38.6, 38.5, 38.5, 37.1, 37.0, 36.2, 36.2, 36.2, 35.7, 35.6, 35.5, 35.5, 32.2, 32.2, 30.1, 29.9, 29.9, 29.8, 29.8, 29.6, 29.5, 24.7, 24.7, 23.8, 23.1, 23.0, 22.4, 22.3, 22.3, 22.2, 20.9, 20.9, 20.8, 20.7, 17.3, 17.1, 17.1, 15.8, 15.8, 15.8, 14.5, 14.5, -1.1, -1.7, -1.8, -1.9, -2.0, -2.2, -2.3; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{28}H_{53}N_3O_7SiNa$ $[M+Na]^+$:594.3545, found 594.3537.

G. Synthesis of caged crosslinker 5c and 6c.



Scheme S5. Synthesis of crosslinkers **5c** and **6c** from central intermediate **S2**. For caged crosslinker **5c**, cyanohydrin intermediate **S6** was obtained through a cyanosilylation and a desilylation of **S2**. A dimethylisopropylsilyl group can be introduced to **S6** providing **S8**. Finally, chemoselective deprotection of the acetal gives caged crosslinker **5c**. A *tert*-butyldimethylsilyl cyanohydrin can be directly installed onto the ketone moiety of central intermediate **S2**, and the sequential deprotection gives caged crosslinker **6c**.

Synthesis of intermediate S5



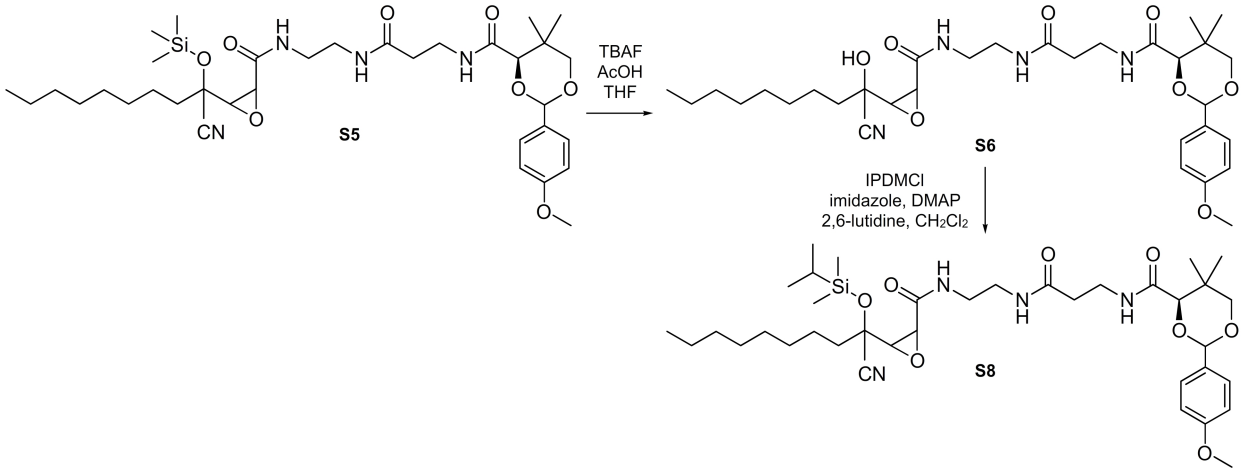
Reagents:

TMSCN, 98% (Sigma Aldrich): used without further purification
LiCl, ultra dry, 99.9% (metal basis) (Thermo Fisher Scientific): used without further purification

(4*R*)-*N*-(3-((2-(3-(1-cyano-1-((trimethylsilyl)oxy)nonyl)oxirane-2-carboxamido)ethyl)amino)-3-oxopropyl)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamide (S5). TMSCN (88.3 mg, 0.890 mmol) was added to central intermediate **S2** (35.0 mg, 0.0593 mmol) and LiCl (1.0 mg, 0.02 mmol) at rt. The reaction was allowed to proceed at rt for 1 h at which point the solvent was removed by airflow. Pure intermediate **S5** (33.9 mg, 83%) was obtained as a clear wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 3:97 MeOH/CH_2Cl_2.

Intermediate **S5**: ^{1}H-NMR (400 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **S5**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 171.8, 171.4, 171.4, 171.2, 171.1, 171.0, 169.4, 160.3, 160.3, 130.3, 127.6, 113.9, 113.8, 101.4, 101.4, 91.5, 84.0, 84.0, 78.6, 57.0, 55.5, 55.4, 51.8, 39.9, 39.8, 38.3, 36.4, 35.9, 35.0, 33.2, 31.9, 31.9, 29.8, 29.7, 29.4, 29.3, 29.3, 24.4, 22.8, 22.7, 22.0, 19.2, 14.2, 1.8, 1.3, 1.1; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{35}H_{57}N_4O_8Si$ $[M+H]^+$:689.3946, found 689.3936.

Synthesis of intermediate S8



Reagents:

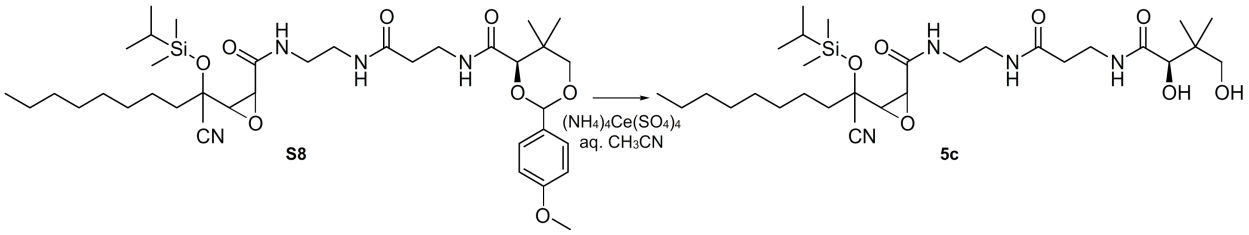
TBAF • H_2O, 98.0% (TCI): used without further purification
AcOH, glacial (certificed ACS), (Fisher Scientific): used without further purification
Molecular sieves, activated, type 4A, 8-12 mesh, (Spectrum): used without further processing
Dimethylisopropylchlorosilane, 90.0% (TCI Chemicals): used without further purification
Imidazole, 99% (Sigma Aldrich): used without further purification
DMAP (4-Dimethylaminopyridine), 99% (Alfa Aesar): used without further purification
2,6-Lutidine 99.46% (Chem-Impex International, Inc.): used without further purification

(4*R*)-*N*-(3-((2-(3-(1-cyano-1-hydroxynonyl)oxirane-2-carboxamido)ethyl)amino)-3-oxopropyl)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamide (S6). 4Å Molecular sieves (5.0 mg) were added to **S5** (21.0 mg, 0.0305 mmol) in THF (100 µL). Glacial acetic acid (5.2 µL, 0.091 mmol) was added followed by the addition of TBAF • H_2O (12.8 mg, 0.0457 mmol). The reaction was allowed to stir for 30 min. After completion, which was monitored by TLC analyses, the mixture was diluted with EtOAc (1 mL) followed by the addition of 5% aq. citric acid (500 µL) and then followed by $NaHCO_3$ (500 µL). The mixture was extracted with aqueous phase with EtOAc (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator to afford pale yellow crude **S6**, which was used directly for the next step.

(4*R*)-N-(3-((2-(3-(1-cyano-1-((isopropyldimethylsilyl)oxy)nonyl)oxirane-2-carboxamido)ethyl)amino)-3-oxopropyl)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamide (S8). Imidazole (4.1 mg, 0.060 mmol), DMAP (0.7 mg, 0.006 mmol), along with 2,6-lutidine (4.1 µL, 0.036 mmol) were added to **S6** (25.0 mg, 0.040 mmol) in CH_2Cl_2 (200 µL) at rt. IPDMCl (9.3 µL, 0.060 mmol) was added into the reaction and allowed to proceed at rt overnight. Satd. $NaHCO_3$ (200 µL) was added, and the mixture was separated followed by extraction of the aqueous phase with CH_2Cl_2 (3 × 2 mL). The combined organic phases dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure intermediate **S8** (6.0 mg, 26% was obtained as a clear wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/ CH_2Cl_2

Intermediate **S8**: ^{1}H-NMR (500 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **S8**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (125 MHz, $CDCl_3$) δ 174.0, 173.9, 169.9, 169.6, 167.6, 167.5, 160.4, 160.3, 130.3, 130.2, 127.7, 127.7, 127.7, 127.6, 127.6, 127.6, 113.9, 113.9, 113.8, 101.5, 101.4, 101.4, 101.4, 92.9, 92.8, 86.1, 84.0, 84.0, 83.9, 78.6, 78.6, 78.6, 76.6, 61.1, 56.1, 55.5, 55.5, 49.9, 33.3, 33.2, 31.9, 29.9, 29.8, 29.5, 29.5, 29.3, 29.3, 29.3, 29.3, 25.8, 25.8, 25.7, 25.5, 23.3, 22.8, 22.0, 22.0, 22.0, 19.3, 19.3, 18.1, 18.1, 17.6, 14.3, 14.3, -2.5, -4.5, -4.6, -4.6, -4.7; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{37}H_{61}N_4O_8Si$ $[M+H]^+$:717.4259, found 717.4256.

Synthesis of caged crosslinker 5c



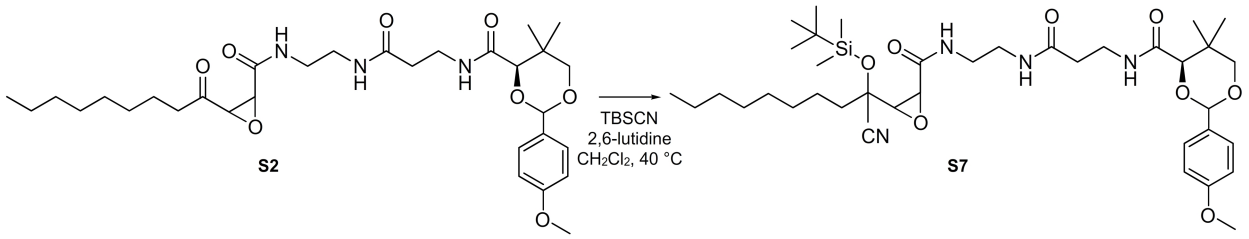
Reagents:

$(NH_4)_4Ce(SO_4)_4$ • 2 H_2O , 95% (Astatech, Inc): used without further purification

3-(1-cyano-1-((isopropyldimethylsilyl)oxy)nonyl)-*N*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl)oxirane-2-carboxamide (5c). $(NH_4)_4Ce(SO_4)_2$ • 2 H_2O (9.7 mg, 0.015 mmol) was added at rt to **S8** (5.5 mg, 0.0077 mmol) in 9:1 CH_3CN/H_2O (200 µL). The reaction was allowed to proceed at rt for 1 h. Once complete by TLC monitor, the reaction was quenched with satd. $NaHCO_3$ (400 µL) and extracted with CH_2Cl_2 (3 × 3 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure caged crosslinker **5c** (3.0 mg, 47%) was obtained as a clear wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/ CH_2Cl_2

Caged crosslinker **5c**: ^{1}H-NMR (400 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **5c**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.7, 173.6, 172.0, 171.9, 171.5, 171.5, 91.6, 71.0, 51.8, 39.5, 39.5, 35.2, 31.9, 29.8, 29.6, 29.3, 24.4, 22.8, 22.0, 20.6, 20.5, 17.0, 16.9, 16.8, 15.5, 15.5, 14.2, -1.3, -2.2; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{29}H_{55}N_4O_7Si$ $[M+H]^+$:599.3841, found 599.3827.

Synthesis of intermediate S7



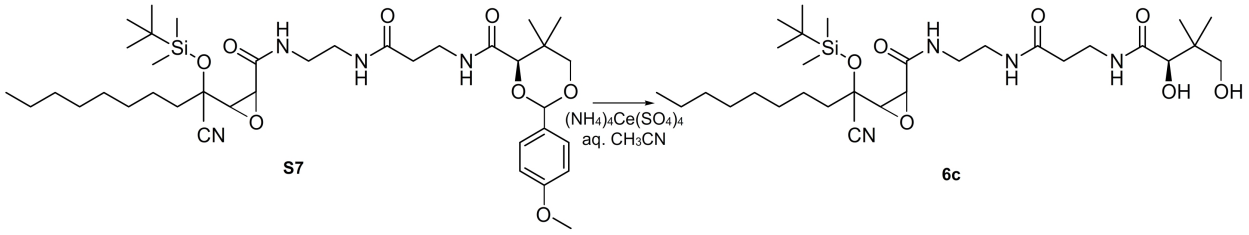
Reagents:

TBSCN, 97% (Sigma Aldrich): used without further purification
LiCl, ultra dry, 99.9% (metal basis) (Thermo Fisher Scientific): used without further purification

(4*R*)-*N*-(3-((2-(3-(1-((tert-butyldimethylsilyl)oxy)-1-cyanononyl)oxirane-2-carboxamido)ethyl)amino)-3-oxopropyl)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamide (S7). LiCl (0.1 mg, 0.002 mmol) was added to **S2** (2.0 mg, 0.0034 mmol) in THF (50 μL). TBSCN (1.9 mg, 0.014 mmol) was added into the reaction mixture at rt. The reaction was stirred overnight. After airflow, the mixture was extracted with brine (1 mL) and CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator to afford intermediate **S7** (1.0 mg, 40%).

Intermediate **S7**: ^{1}H-NMR (500 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **S7**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (125 MHz, $CDCl_3$) δ 174.0, 173.9, 169.9, 169.6, 167.6, 167.5, 160.4, 160.3, 130.3, 130.2, 127.7, 127.7, 127.7, 127.6, 127.6, 127.6, 113.9, 113.9, 113.8, 101.5, 101.4, 101.4, 101.4, 92.9, 92.8, 86.1, 84.0, 84.0, 83.9, 78.6, 78.6, 78.6, 76.6, 61.1, 56.1, 55.5, 55.5, 33.2, 33.2, 33.2, 31.9, 29.8, 29.5, 29.5, 29.3, 29.3, 25.8, 25.8, 25.7, 25.5, 23.3, 22.8, 22.0, 22.0, 19.3, 19.3, 18.1, 18.1, 17.6, 14.3, 14.3, -2.5, -4.5, -4.6, -4.6, -4.7; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{38}H_{63}N_4O_8Si$ $[M+H]^+$:731.4416, found 731.4407.

Synthesis of caged crosslinker 6c



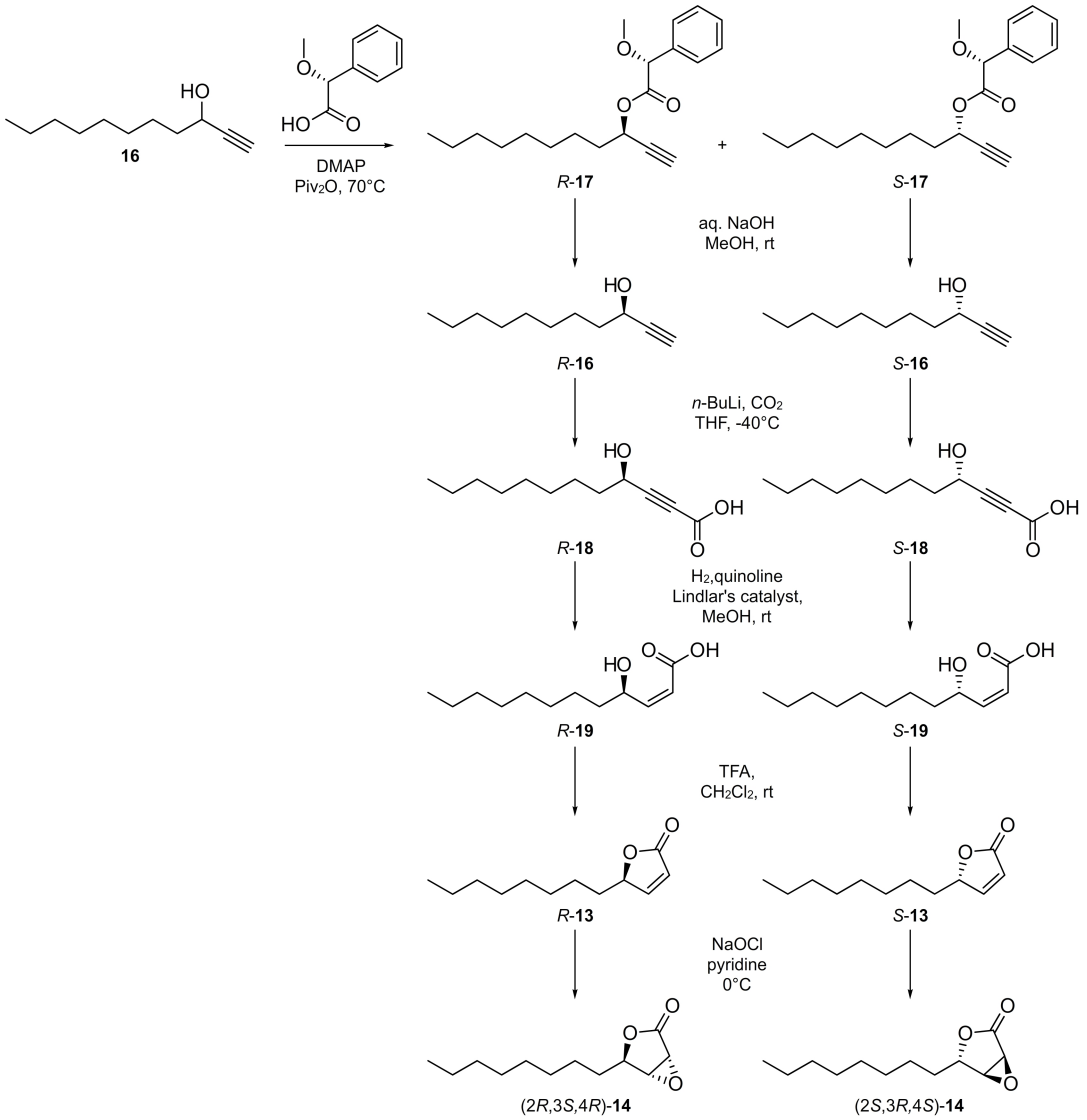
Reagents:

$(NH_4)_4Ce(SO_4)_4$ • 2 H_2O , 95% (Astatech, Inc): used without further purification

3-(1-((tert-butyldimethylsilyl)oxy)-1-cyanononyl)-N-(2-(3-((R)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl)oxirane-2-carboxamide (6c). $(NH_4)_4Ce(SO_4)_2$ • 2 H_2O (3.5 mg, 0.0055 mmol) was added at rt to **S7** (2.0 mg, 0.0027 mmol) dissolved in 9:1 CH_3CN/H_2O (100 µL). The reaction was stirred at rt for 1 h. Once complete by TLC monitor, the reaction was quenched with satd. $NaHCO_3$ (200 µL) and extracted with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure caged crosslinker **6c** (0.7 mg, 43%) was obtained as a clear wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5:95 MeOH/ CH_2Cl_2

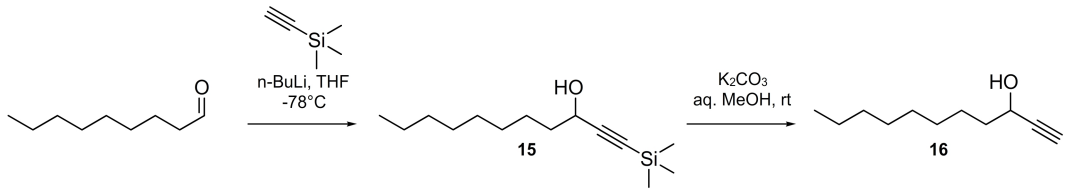
Caged crosslinker **6c**: ^{1}H-NMR (800 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to isomers within **6c**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (200 MHz, $CDCl_3$) 177.0, 173.1, 167.7, 131.1, 129.0, 93.5, 86.2, 86.2, 79.2, 77.4, 76.8, 71.7, 66.1, 61.5, 40.1, 40.0, 39.6, 39.1, 37.5, 33.8, 33.6, 33.6, 33.4, 32.0, 31.7, 29.9, 29.9, 29.6, 29.5, 29.3, 29.3, 28.7, 25.9, 25.9, 25.8, 25.8, 25.6, 25.6, 25.6, 23.9, 23.3, 23.3, 22.8, 22.7, 21.7, 20.5, 14.3, -4.5, -4.5, -4.5, -4.5; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{30}H_{56}N_4O_7SiNa$ $[M+Na]^+$:635.3816, found 635.3807.

H. The synthesis of crosslinkers (2*R*,3*S*,4*R*)-14 and (2*S*,3*R*,4*S*)-14.



Scheme S6. Synthesis of (2*R*,3*S*,4*R*)-**14** and (2*S*,3*R*,4*S*)-**14** from **16**. Esterification of **16** with a chiral acid allowed the separation of enantiomeric esters *R*-**17** and *S*-**17**. The enantiomeric esters were subjected to 4-step process including ester hydrolysis, carboxylation, controlled reduction, and lactonization to yield butenolide *R*-**13** and S-**13**. An asymmetric epoxidation to afford epoxylactones (2*R*,3*S*,4*R*)-**14** and (2*S*,3*R*,4*S*)-**14**.

Synthesis of alcohol 16



Reagents:

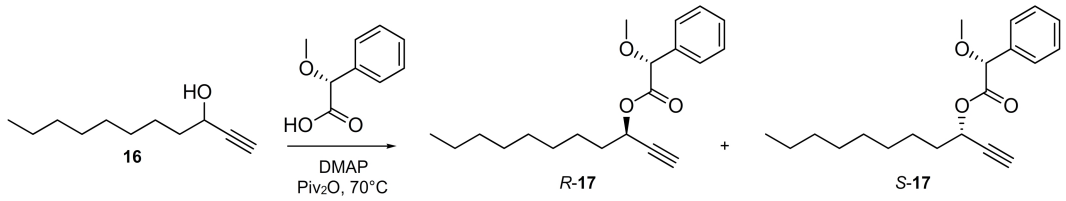
Trimethylsilylacetylene, 98% (Fisher scientific): used without further purification
n-BuLi, 2.5M in hexanes (ACROS Organics): used without further purification
Nonanal, 97%, (Alfa Aesar): used without further purification
K_2CO_3, 99% (Alfa Aesar): used without further purification

1-(trimethylsilyl)undec-1-yn-3-ol (15). *n*-BuLi (8.04 mL, 2.5M, 20.1 mmol) was added dropwise to trimethylsilylacetylene (2.90 mL, 20.4 mmol) in THF (132 mL) at -78 °C. After 40 min, nonanal (3.14 mL, 18.3 mmol) was added dropwise. After 1 h was warmed up to 0 °C, satd. NH_4Cl (40 mL) was added, and the mixture was separated followed by extraction of the aqueous phase with Et_2O (3 × 50 mL). The combined organic phases were washed with brine (3 × 10 mL), dried over Na_2SO_4, filtered and concentrated on a rotary evaporator to afford crude alcohol **15** as a colorless oil.

Undec-1-yn-3-ol (16). Crude alcohol **15** (4.40 g, 18.3 mmol) was dissolved in satd. K_2CO_3 in MeOH (220 mL) and H_2O (16 mL). The reaction was allowed to stir for 2 h at rt. After completion by TLC analysis, the mixture was concentrated on a rotary evaporator. Satd. NH_4Cl (60 mL) was added, and the mixture was separated followed by extraction of the aqueous phase with EtOAc (3 × 60 mL). The combined organic phases were washed with brine (3 × 10 mL), dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure alcohol **16** (2.91 g, 95%) was obtained as a colorless oil by flash chromatography, eluting with a gradient of hexane to 15:85 EtOAc/hexane.

Alcohol **16**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.36 (td, J = 6.6, 2.2 Hz, 1H), 2.45 (m, 1H), 2.02 (s, 1H), 1.70 (m, 2H), 1.44 (m, 2H), 1.27 (m, 10H), 0.87 (t, J = 6.6 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 85.1, 72.9, 62.4, 37.7, 32.0, 29.6, 29.3, 25.1, 22.8, 14.2; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{11}H_{21}O$ $[M+H]^+$:169.1593, found 169.1538.

Synthesis of esters *R*-17 and ***S*-17**



Reagents:

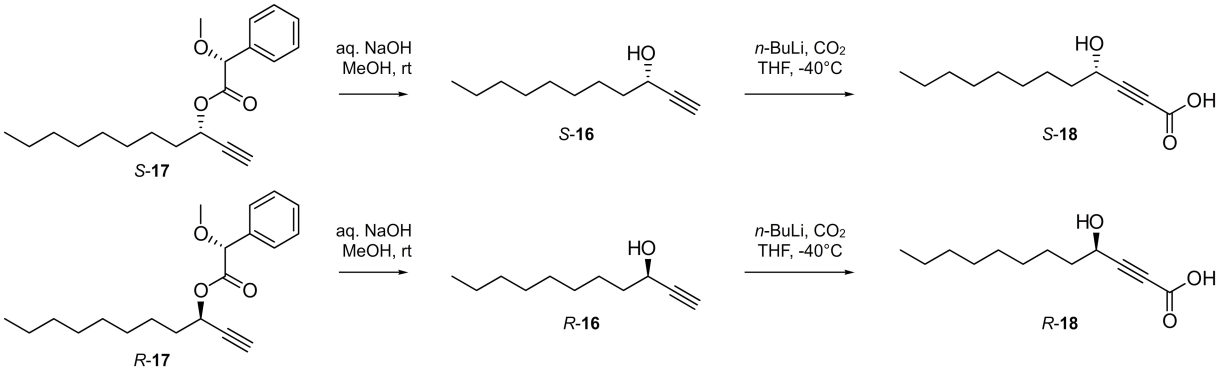
(*R*)-(-)-α-Methoxyphenylacetic acid, >98.0% (TCI): used without further purification
Trimethylacetic anhydride, 99% (Acros Organics): used without further purification

(*R*)-undec-1-yn-3-yl (*R*)-2-methoxy-2-phenylacetate (*R*-17) and (*S*)-undec-1-yn-3-yl (*R*)-2-methoxy-2-phenylacetate (*S*-17). Alcohol **16** (1.25 g, 7.43 mmol), (*R*)-(−)-α-methoxyphenylacetic acid (1.36 g, 8.2 mmol), DMAP (136.1 mg, 1.114 mmol) were dissolved in pivalic anhydride (3.11 g, 16.7 mmol). The mixture heated for 2 h at 70 °C. After completion by was confirmed by TLC analysis, satd. $NaHCO_3$ (10 mL) was added, and the mixture was separated followed by extraction of the aqueous phase with CH_2Cl_2 (3 × 20 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure ester *R*-**17** (0.97 g, 41%) and ester *S*-**17** (0.93 mg, 40%) were obtained by flash chromatography, eluting with a gradient of hexanes to 1:10 Et_2O/hexanes.

Ester *R*-**17**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.36 (m, 1H), 7.27 (m, 2H), 7.23 (m, 2H), 5.29 (td, *J* = 6.7, 2.2 Hz, 1H), 4.70 (s, 1H), 3.33 (s, 3H), 2.27 (d, *J* = 2.1 Hz, 1H), 1.68 (m, 2H), 1.29 (m, 2H), 1.16 (s, 10H), 0.78 (t, *J* = 6.8 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 169.8, 135.8, 128.8, 128.6, 127.2, 82.6, 80.4, 74.0, 64.6, 57.4, 34.5, 31.8, 29.4, 29.2, 29.0, 24.8, 22.7, 14.1; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{20}H_{28}O_3Na$ $[M+Na]^+$:339.1936, found 339.1929.

Ester *S*-**17**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.35 (m, 2H), 7.25 (m, 3H), 5.31 (td, *J* = 6.6, 2.2 Hz, 1H), 4.70 (s, 1H), 3.32 (s, 3H), 2.38 (d, *J* = 2.2 Hz, 1H), 1.54 (m, 2H), 1.15 (m, 10H), 0.79 (t, *J* = 6.9 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 169.5, 136.1, 128.7, 128.5, 127.2, 82.2, 80.7, 73.9, 64.2, 57.3, 34.4, 31.8, 29.2, 29.0, 28.8, 24.4, 22.6, 14.1; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{20}H_{28}O_3Na$ $[M+Na]^+$:339.1936, found 339.1928.

Two-step synthesis of *S*-18 and *R*-18



Reagents:

n-BuLi, 2.5M in toluene (Thermo Fisher): used without further purification

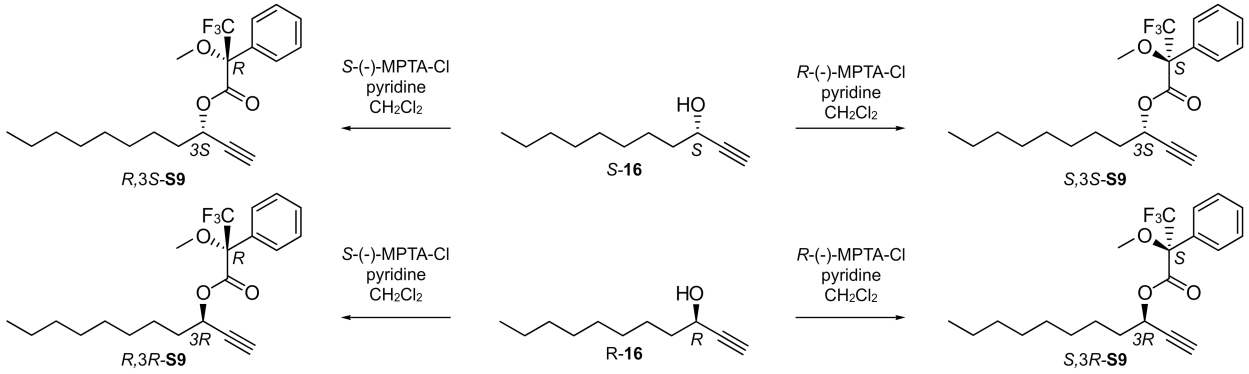
(3*S*)-Undec-1-yn-3-ol (*S*-16): Ester *S*-**17** (0.230 g, 0.727 mmol) was dissolved in MeOH (8.2 mL) and H_2O (2 mL). A 1M solution of NaOH (2 mL) was added dropwise. After completion of the reaction by TLC analyses, the mixture was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic phases were washed with brine (3 × 5 mL), dried over Na_2SO_4, filtered and concentrated on a rotary evaporator to afford crude *S*-**16**. The enatiopurity of this material was checked by Mosher's analyses (shown in next page), and crude *S*-**16** (120.7 mg, which was used directly for the next step.

(4*S*)-4-hydroxydodec-2-ynoic acid (*S*-18): Crude *S*-**16** (120.0 mg, 0.7131 mmol) was dissolved THF (3.7 mL). After cooling to -40 °C, *n*-BuLi (0.689 mL, 2.5M 1.72 mmol) was added dropwise. After stirring for 30 min at -40 °C, CO_2 was bubbled through the reaction for 1 h. Brine (12 mL) was added, and the aqueous phase was washed with hexanes (4 × 2 mL). A 1 M solution of HCl was added to the aqueous layer until the pH reached 3, and aqueous phase was extracted of the aqueous phase with EtOAc (3 × 20 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator to afford crude *S*-**18** (147.2 mg), which was used directly for the next step.

(3*R*)-Undec-1-yn-3-ol (*R*-16): Ester *R*-**17** (0.230 g, 0.727 mmol) was dissolved in MeOH (8.2 mL) and H_2O (2 mL). A 1M solution of NaOH (2 mL) was added dropwise. After completion of the reaction by TLC analyses, the mixture was extracted with CH_2Cl_2 (3 × 20 mL). The combined organic phases were washed with brine (3 × 5 mL), dried over Na_2SO_4, filtered and concentrated on a rotary evaporator to afford crude *R*-**16**. The enatiopurity of this material was checked by Mosher's analyses, and crude *R*-**16** (118.5 mg). was carried on to the next step.

(4*R*)-4-hydroxydodec-2-ynoic acid (*R*-18): Crude *R*-**16** 115.0 mg, 0.7131 mmol) was dissolved THF (3.7 mL). After cooling to -40 °C, *n*-BuLi (0.656 mL, 2.5M, 1.640 mmol) was added dropwise. After stirring for 30 min at -40 °C, CO_2 was bubbled through the reaction for 1 h. Brine (12 mL) was added, and the aqueous phase was washed with hexanes (4 × 2 mL). A 1 M solution of HCl was added to the aqueous layer until the pH reached 3, and aqueous phase was extracted of the aqueous phase with EtOAc (3 × 20 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator to afford crude *R*-**18** (145.0 mg), which was used directly for the next step.

Mosher analysis of alkynols *S*-16 and *R*-16.



Reagents:

(*S*)-(+)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride, 99% (Sigma Aldrich): used without further purification
(*R*)-(-)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride, 99% (Sigma Aldrich): used without further purification
Pyridine, 99% (Fischer Scientific): used without further purification

(*3R*)-undec-1-yn-3-yl-(*R*)-3,3,3-trifluoro-2-methoxy-2-phenylpropanoate (*R,3R*-S9): Pyridine (14.9 µL, 0.184 mmol) and (*S*)-MTPA-Cl (21.1 uL, 0.113 mmol) were added sequentially to a solution of *R*-**16** (10.0 mg, 0.0594 mmol) in CH_2Cl_2 (100 µL). The reaction mixture was stirred at rt for 2 h before being extracted with H_2O (1 mL) and Et_2O (3 × 4 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure *R,*3*R*-**S9** (13.8 mg, 60%) was obtained by flash chromatography on a silica plug (10% Et_2O in hexane).

Ester *R,*3*R*-**S9**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.55 (m, 2H), 7.40 (m, 3H), 5.54 (td, *J* = 6.7, 2.2 Hz, 1H), 3.60 (bs, 3H), 2.54 (d, *J* = 2.2 Hz, 1H), 1.79 (m, 2H), 1.24 (m, 12H), 0.87 (t, *J* = 6.9 Hz, 3H).

(*3S*)-undec-1-yn-3-yl-(*R*)-3,3,3-trifluoro-2-methoxy-2-phenylpropanoate (*R,3S*-S9): Pyridine (14.9 µL, 0.184 mmol) and (*S*)-MTPA-Cl (21.1 uL, 0.113 mmol) were added sequentially to a solution of *S*-**16** (10.0 mg, 0.0594 mmol) in CH_2Cl_2 (100 µL). The reaction mixture was stirred at rt for 2 h before being extracted with H_2O (1 mL) and Et_2O (3 × 4 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure *R,*3*S*-**S9** (15.3 mg, 66%) was obtained by flash chromatography on a silica plug (10% Et_2O in hexane).

Ester *R,*3*S*-**S9**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.53 (m, 2H), 7.40 (m, 3H), 5.51 (td, *J* = 6.7, 2.1 Hz, 1H), 3.55 (bs, 3H), 2.49 (d, *J* = 2.1 Hz, 1H), 1.85 (m, 2H), 1.25 (m, 12H), 0.87 (t, *J* = 7.0 Hz, 3H).

(*3R*)-undec-1-yn-3-yl-(*S*)-3,3,3-trifluoro-2-methoxy-2-phenylpropanoate (*S,3R*-S9): Pyridine (14.9 µL, 0.184 mmol) and (*R*)-MTPA-Cl (21.1 uL, 0.113 mmol) were added sequentially to a solution of *R*-**16** (10.0 mg, 0.0594 mmol) in CH_2Cl_2 (100 µL). The reaction mixture was stirred at rt for 2 h before being extracted with H_2O (1 mL) and Et_2O (3 × 4 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure *S,*3*R*-**S9** (16.1 mg, 70.5%) was obtained by flash chromatography on a silica plug (10% Et_2O in hexane).

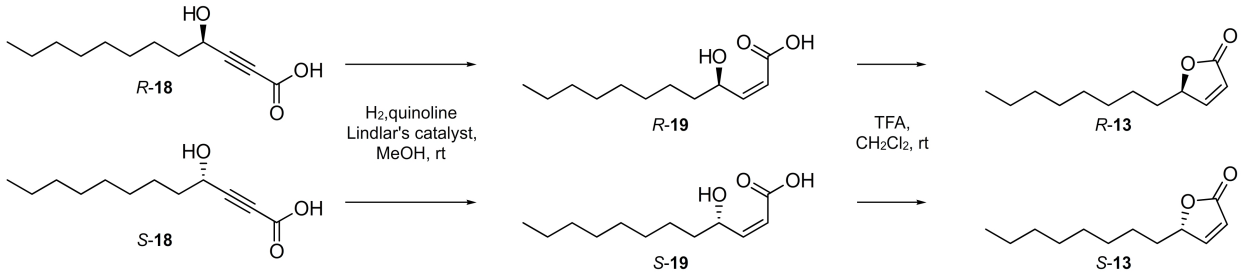
Ester *S,*3*R*-**S9**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.54 (m, 2H), 7.40 (m, 3H), 5.51 (td, *J* = 6.7, 2.1 Hz, 1H), 3.55 (bs, 3H), 2.49 (d, *J* = 2.1 Hz, 1H), 1.86 (m, 2H), 1.27 (m, 12H), 0.88 (t, *J* = 7.0 Hz, 3H).

(*3S*)-undec-1-yn-3-yl-(*S*)-3,3,3-trifluoro-2-methoxy-2-phenylpropanoate (*S,3S*-S9): Pyridine (14.9 μL, 0.184 mmol) and (*R*)-MTPA-Cl (21.1 uL, 0.113 mmol) were added sequentially to a solution of *S*-**16** (10.0 mg, 0.0594 mmol) in CH_2Cl_2 (100 μL). The reaction mixture was stirred at rt for 2 h before being extracted with H_2O (1 mL) and Et_2O (3 × 4 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure *S,*3*S*-**S9** (14.8 mg, 65%) was obtained by flash chromatography on a silica plug (10% Et_2O in hexane).

Ester *S,*3*S*-**S9**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.55 (m, 2H), 7.40 (m, 3H), 5.54 (td, *J* = 6.7, 2.2 Hz, 1H), 3.60 (bs, 3H), 2.53 (d, *J* = 2.1 Hz, 1H), 1.79 (m, 2H), 1.25 (m, 12H), 0.88 (t, *J* = 7.0 Hz, 3H).

Since the phenyl group of MTPA is in close proximity to the propargyl proton on *S*,3*R*-**S9** compared with the one on *R*,3*R*-**S9**, the chemical shift of the propargyl proton of *S*,3*R*-**S9** is more shielded (2.49 ppm) while the one on *R*,3*R*-**S9** is more deshielded (2.54 ppm). Similar result was observed from *R*,3*S*-**S9** (2.49 ppm) and *S*,3*S*-**S9** (2.53 ppm).

Two step synthesis of butenolides *S*-13 or *R*-13



Reagents:

Lindlar's catalyst, (Strem Chemicals Inc.): used without further purification
Quinoline, >97.0% (TCI): used without further purification
Trifluoroacetic acid (TFA), >99.9% (EMD chemicals): used without further purification

(*R*,*Z*)-4-hydroxydodec-2-ynoic acid (*R*-19). Propiolic acid *R*-**18** (145.0 mg, 0.6407 mmol), quinoline (7.6 µL, 0.064 mmol), and Lindlar's catalyst (38.7 mg, 0.363 mmol) was dissolved in MeOH (23 mL). With stirring, H_2 gas was bubbled through the reaction mixture for 4.5 min. The resulting mixture was filtered and concentrated under reduced pressure to give *R*-**19** (137.3 mg), which was used directly for the next step.

(*R*)-5-octylfuran-2(5H)-one (*R*-13): Carboxylic acid *R*-**19** (135.0 mg, 0.6299 mmol) was dissolved in CH_2Cl_2 (2 mL). With stirring, trifluoroacetic acid (7.3 µL, 0.094 mmol) was added. The reaction reached completion after 10 min. The reaction mixture was then diluted with CH_2Cl_2 (3 mL) and subjected to an extraction with satd. $NaHCO_3$ (3 mL) and CH_2Cl_2 (3 × 5 mL). The combined organic phases were dried over Na_2SO_4, filtered, and concentrated on a rotary evaporator. Pure butenolide *R*-**13** (49.8 mg, 32% from *S-17*) was obtained by flash chromatography, eluting with a gradient of hexanes to 1:3 EtOAc/hexane.

Butenolide *R*-**13**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.44 (dd, J = 5.7, 1.5 Hz, 1H), 6.10 (dd, J = 5.7, 2.0 Hz, 1H), 5.03 (td, J = 5.5, 1.8 Hz, 1H), 1.73 (m, 2H), 1.44 (m, 2H), 1.27 (m, 10H), 0.88 (t, J = 6.5 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.4, 156.5, 121.7, 83.6, 33.3, 31.9, 29.5, 29.4, 29.3, 25.1, 22.8, 14.2; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{12}H_{21}O_2$ $[M+H]^+$:197.1542, found 197.1536.

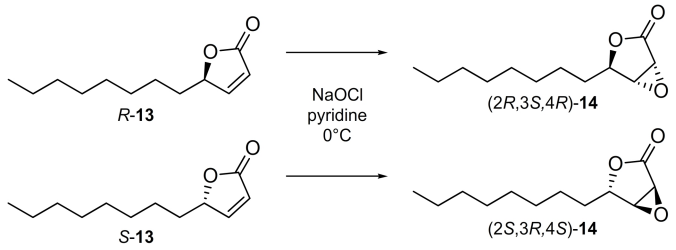
(*S*,*Z*)-4-hydroxydodec-2-ynoic acid (*S*-19). Propiolic acid *S*-**18** (147.2 mg, 0.6504 mmol), quinoline (7.8 µL, 0.066 mmol), and Lindlar's catalyst (39.3 mg, 0.369 mmol) was dissolved in MeOH (23 mL). With stirring, H_2 gas was bubbled through the reaction mixture for 4.5 min. The resulting mixture was filtered and concentrated under reduced pressure to give crude *S*-**19** (129.6 mg), which was used directly for the next step.

(*S*)-5-octylfuran-2(5H)-one (*S*-13): Carboxylic acid *S*-**19** (129.6 mg, 0.6047 mmol) was dissolved in CH_2Cl_2 (2 mL). With stirring, trifluoroacetic acid (7.0 µL, 0.091 mmol) was added. The reaction reached completion after 10 min. The reaction mixture was then diluted with CH_2Cl_2 (3 mL) and subjected to an extraction with satd. $NaHCO_3$ (3 mL) and CH_2Cl_2 (3 × 5 mL). The combined organic phases were dried over Na_2SO_4, filtered, and concentrated on a rotary

evaporator. Pure butenolide *S*-**13** (49.8 mg, 32% from *S*-***17***) was obtained by flash chromatography, eluting with a gradient of hexanes to 1:3 EtOAc/hexane.

Butenolide ***S*-13**: ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.44 (dd, *J* = 5.7, 1.5 Hz, 1H), 6.09 (dd, *J* = 5.7, 2.0 Hz, 1H), 5.03 (td, *J* = 5.5, 2.7 Hz, 1H), 1.70 (m, 2H), 1.44 (m, 2H), 1.26 (m, 10H), 0.87 (t, *J* = 7.0, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 173.3, 156.6, 121.5, 83.5, 33.2, 31.8, 29.4, 29.3, 29.2, 25.0, 22.7, 14.1; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{12}H_{21}O_2$ $[M+H]^+$:197.1542, found 197.1538.

Synthesis of epoxylactones (2*S*,3*R*,4*S*)-14 and (2*R*,3*S*,4*R*)-14.



Reagents:

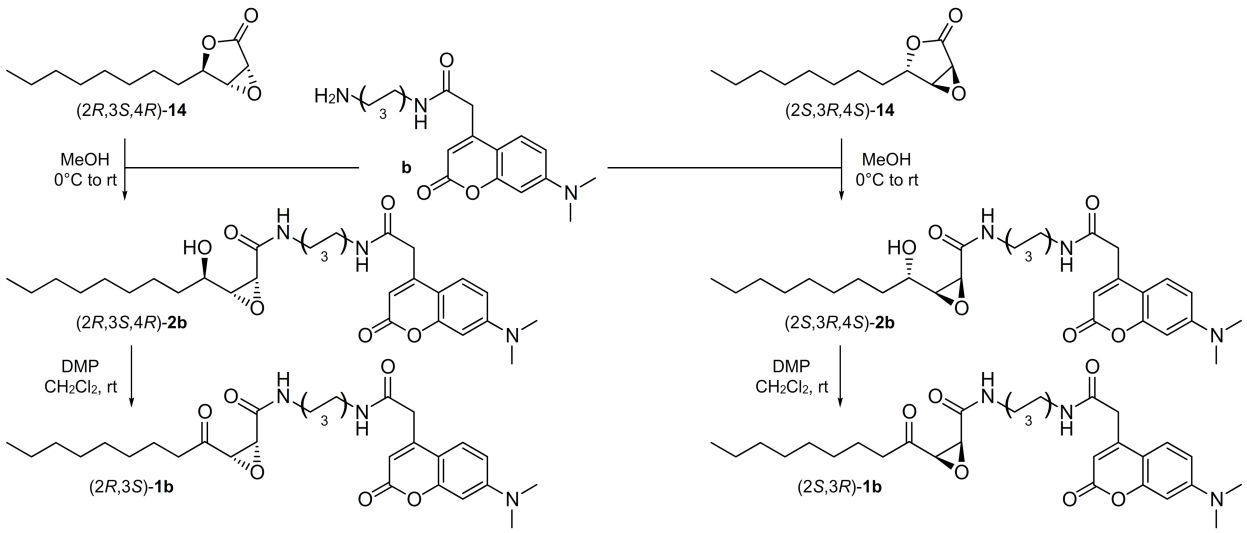
Pyridine, 99% (Fischer Scientific): used without further purification
NaOCl, 2M, 10-15% active chlorine (Spectrum Chemical Mfg. Corp.): used without further purification

(2*S*,3*R*,4*S*)-4-octyl-3,6-dioxabicyclo[3.1.0]hexan-2-one ((2*R*,3*S*,4*R*)-14). Butenolide *S*-**13** (50.0 mg, 0.255 mmol) was dissolved in pyridine (4 mL). The mixture was cooled to 0 °C followed by dropwise addition of NaOCl (2M, 0.44 mL, 0.88 mmol). The reaction was allowed to stir for 3 h at 0 °C. The completion of the reaction was monitored through TLC, and additional amount of NaOCl might be added. The mixture was quenched with satd. $NaHCO_3$ (10 mL), extracted with EtOAc (3 × 15 mL) and washed with brine (5 × 3 mL). The organic phases were dried over Na_2SO_4, filtered, and concentrated on a rotary evaporator. Pure epoxylactone (2*S*,3*R*,4*S*)-**14** (35.1 mg, 65%) was obtained as a pale yellow oil, by flash chromatography, eluting with a gradient of hexanes to 1:3 EtOAc/hexane.

Epoxylactone (2*S*,3*R*,4*S*)-**14**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.58 (t, *J* = 6.6 Hz, 1H), 3.98 (d, *J* = 2.5 Hz, 1H), 3.79 (d, *J* = 2.5 Hz, 1H), 1.70 (m, 2H), 1.47 (m, 2H), 1.29 (m, 10H), 0.87 (t, *J* = 6.3 Hz, 3H); ^{13}C-NMR NMR (100 MHz, $CDCl_3$) δ 170.5, 80.0, 79.9, 58.2, 49.9, 32.2, 31.9, 29.4, 29.3, 29.2, 24.3, 22.7, 14.2; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{12}H_{20}O_3Na$ $[M+Na]^+$:235.1310, found 235.1303.

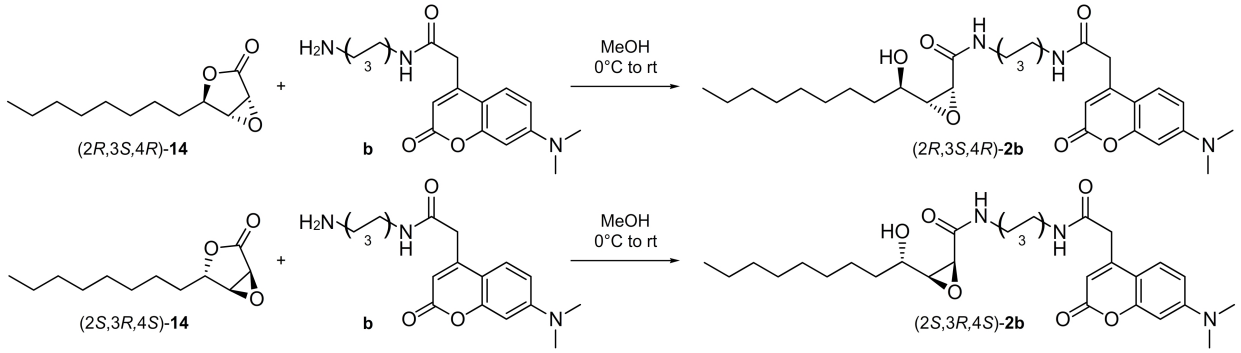
(2*R*,3*S*,4*R*)-4-octyl-3,6-dioxabicyclo[3.1.0]hexan-2-one ((2*R*,3*S*,4*R*)-14). Butenolide *R*-**13** (80.0 mg, 0.408 mmol) was dissolved in pyridine (2 mL). The mixture was cooled to 0 °C followed by dropwise addition of NaOCl (2M, 0.7 mL, 1.6 mmol). The reaction was allowed to stir for 3 h at 0 °C. The completion of the reaction was monitored through TLC, and additional amount of NaOCl might be added. The mixture was quenched with satd. $NaHCO_3$ (10 mL), extracted with EtOAc (3 × 15 mL) and washed with brine (5 × 3 mL). The organic phases were dried over Na_2SO_4, filtered, and concentrated on a rotary evaporator. Pure epoxylactone (2*R*,3*S*,4*R*)-**14** (60.7 mg, 70%) was obtained as a pale yellow oil, by flash chromatography, eluting with a gradient of hexanes to 1:3 EtOAc/hexane.

Epoxylactone (2*R*,3*S*,4*R*)-**14**: NMR data are identical to (2*S*,3*R*,4*S*)-**14** and a reported above; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{12}H_{20}O_3NaCH_3OH$ $[M+MeOH+Na]^+$:267.1572, found 267.1564.



Scheme S7. Synthesis of enantiomeric fluorescent probes (2*R*,3*S,*4*R*)-**2b**, (2*S*,3*R,*4*S*)-**2b**, (2*R*,3*S*)-**1b**, (2*S*,3*R*)-**1b**. A two-step procedure was developed to append tag **b** on to (2*R*,3*S,*4*R*)-**14** or (2*S*,3*R,*4*S*)-**14** and then convert the resulting alcohols (2*R*,3*S,*4*R*)-**2b** and (2*S*,3*R,*4*S*)-**2b** to (2*R*,3*S*)-**1b** and (2*S*,3*R*)-**1b**, respectively.

Synthesis of probe (2*S*,3*R*,4*S*)-2b and (2*R*,3*S*,4*R*)-2b



Reagents:

Fluoresent tag **b** was prepared according to: [Alexander MD, Burkart MD, Leonard MS, Portonovo P, Liang B, Ding X, Joullié MM, Gulledge BM, Aggen JB, Chamberlin AR, Sandler J, Fenical W, Cui J, Gharpure SJ, Polosukhin A, Zhang HR, Evans PA, Richardson AD, Harper MK, Ireland CM, Vong BG, Brady TP, Theodorakis EA, La Clair JJ. A central strategy for converting natural products into fluorescent probes. Chembiochem. 2006 Mar;7(3):409-16].

(2*R*,3*S*)-N-(6-(2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamido)hexyl)-3-((*R*)-1-hydroxynonyl)oxirane-2-carboxamide ((2*R*,3*S*,4*R*)-2b). Fluorescent tag **b** (48.8 mg. 0.141 mmol) was added to a mixture of epoxylactone (2*R*,3*S*,4*R*)-**14** (15.0 mg, 0.0707 mmol) in MeOH (100 μL). The reaction was stirred overnight was then evaporated by airflow. Pure probe (2*R*,3*S*,4*R*)-**2b** (34.3 mg, 87%) was obtained as a pale yellow oil, by flash chromatography, eluting with a gradient of hexanes to 1:3 EtOAc/hexane.

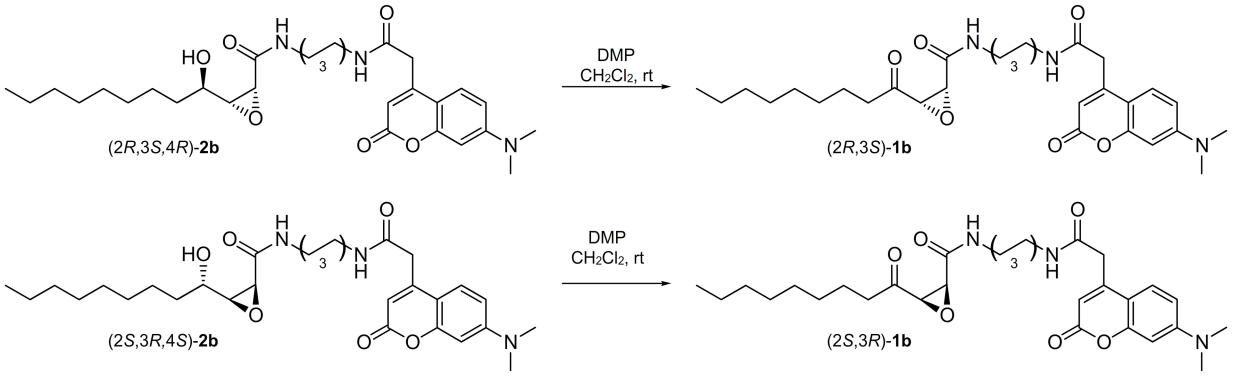
Probe (2*R*,3*S*,4*R*)-**2b:** ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.51 (d, *J* = 9.0 Hz, 1H), 6.63 (dd, *J* = 8.9, 2.6 Hz, 1H), 6.48 (d, *J* = 2.5 Hz, 1H), 6.33 (m, 1H), 6.28 (t, *J* = 6.2 Hz, 1H), 6.08 (s, 1H), 3.63 (bs, 2H), 3.51 (d, *J* = 4.6 Hz, 1H), 3.35 (td, *J* = 7.9, 5.3 Hz, 1H), 3.27 (dq, *J* = 13.1, 6.5 Hz, 1H), 3.18 (m, 2H), 3.12 (m, 2H), 3.05 (s, 6H), 1.65 (m, 2H), 1.51 (m, 2H), 1.42 (m, 2H), 1.25 (m, 16H), 0.86 (t, *J* = 7.0 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 168.3, 167.6, 162.4, 156.1, 153.1, 150.2, 126.0, 110.7, 109.5, 108.7, 98.3, 68.7, 65.8, 60.6, 54.9, 40.9, 40.3, 39.0, 38.1, 35.1, 32.0, 29.8, 29.7, 29.5, 29.4, 28.8, 25.3, 25.2, 25.0, 22.8, 14.3; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{48}N_3O_6$ $[M+H]^+$:558.3544, found 558.3543.

(2*S*,3*R*)-N-(6-(2-(7-(dimethylamino)-2-oxo-2*H*-chromen-4-yl)acetamido)hexyl)-3-((*S*)-1-hydroxynonyl)oxirane-2-carboxamide ((2*S*,3*R*,4*S*)-2b). Fluorescent tag **b** (45.6 mg. 0.132 mmol) was added to a mixture of epoxylactone (2*S*,3*R*,4*S*)-**14** (14.0 mg , 0.0659 mmol) in MeOH (100 μL). The reaction was stirred overnight was then evaporated by airflow. Pure probe (2*S*,3*R*,4*S*)-**2b** (31.3 mg, 85%) was obtained as a pale yellow oil, by flash chromatography, eluting with a gradient of hexanes to 1:3 EtOAc/hexane

Probe (2*S*,3*R*,4*S*)-**2b**: ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.52 (d, *J* = 8.9 Hz, 1H), 6.65 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.50 (bs, 1H), 6.38 (bs, 1H), 6.32 (m, 2H), 6.09 (s, 1H), 3.64 (bs, 2H), 3.51 (d, *J* = 4.6 Hz, 1H), 3.35 (td, *J* = 7.8, 5.2 Hz, 1H), 3.27 (dt, *J* = 13.1, 6.5 Hz, 1H), 3.18 (q, *J* = 6.8 Hz, 2H), 3.11 (m, 2H), 3.05 (s, 6H), 1.66 (m, 2H), 1.50 (m, 2H), 1.42 (m, 2H), 1.25 (m, 16H), 0.86 (t,

J = 6.8 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 168.3, 167.7, 162.4, 156.0, 152.9, 150.2, 126.1, 110.9, 109.7, 109.0, 98.6, 68.8, 60.6, 54.9, 43.6, 40.9, 40.5, 39.1, 38.2, 35.1, 32.0, 29.8, 29.7, 29.6, 29.5, 28.9, 25.4, 25.3, 25.1, 22.8, 14.3; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{48}N_3O_6$ $[M+H]^+$:558.3544, found 558.3535.

Synthesis of probes (2*S*,3*R*)-1b and (2*R*,3*S*)-1b



Reagents:

Dess-Martin periodinane, 95% (Astatech, Inc): used without further purification

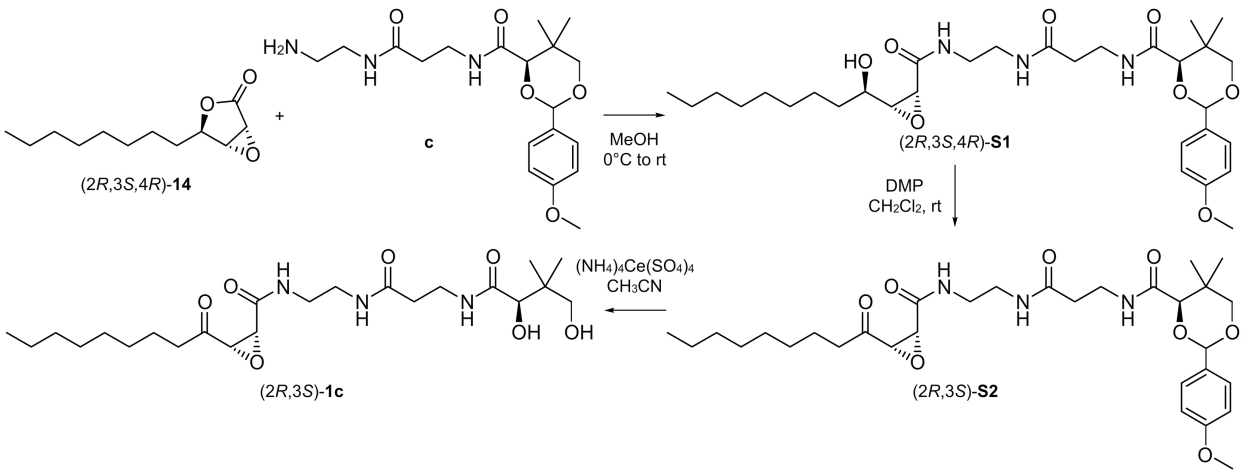
(2*R*,3*S*)-N-(6-(2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamido)hexyl)-3-nonanoyloxirane-2-carboxamide ((2*R*,3*S*)-1b). (2*R*,3*S*,4*R*)-**2b** (7.0 mg, 0.013 mmol) was dissolved in CH_2Cl_2 (100 µL). Dess-Martin periodinane (6.4 mg, 0.015 mmol) was added into the mixture at rt, and the reaction was stirred overnight. The reaction was quenched with satd. Na_2S2O_3 (0.2 mL) and satd. $NaHCO_3$ (0.2 mL) and extracted with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure probe (2*R*,3*S*)-**1b** (4.6 mg, 66%) was obtained as a yellow wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5% MeOH in CH_2Cl_2

Probe (2*R*,3*S*)-**1b**: ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.51 (d, *J* = 9.0 Hz, 1H), 6.63 (dd, *J* = 9.0, 2.6 Hz, 1H), 6.50 (d, *J* = 2.5 Hz, 1H), 6.42 (t, *J* = 6.1 Hz, 1H), 6.08 (s, 1H), 6.05 (t, *J* = 6.0 Hz, 1H), 3.85 (d, *J* = 5.2 Hz, 1H), 3.72 (d, *J* = 5.3 Hz, 1H), 3.62 (s, 2H), 3.19 (q, *J* = 6.6 Hz, 2H), 3.15 (m, 1H), 3.11 (m, 1H), 3.05 (s, 6H), 2.54 (m, 2H), 2.50 (m, 1H), 1.54 (m, 2H), 1.43 (m, 2H), 1.38 (m, 2H) 1.23 (m, 14H), 0.85 (t, *J* = 6.9 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 203.1, 168.0, 164.9, 162.0, 156.1, 153.1, 150.1, 126.0, 110.6, 109.4, 108.6, 98.4, 58.6, 55.7, 41.2, 41.0, 40.4, 39.5, 38.6, 31.9, 29.3, 29.3, 29.2, 29.1, 29.0, 25.9, 25.8, 23.2, 22.7, 14.2; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{45}N_3O_6Na$ $[M+Na]^+$:578.3206, found 578.3197.

(2*S*,3*R*)-N-(6-(2-(7-(dimethylamino)-2-oxo-2H-chromen-4-yl)acetamido)hexyl)-3-nonanoyloxirane-2-carboxamide ((2*S*,3*R*)-1b). (2*S*,3*R*,4*S*)-**2b** (10.0 mg, 0.0179 mmol) was dissolved in CH_2Cl_2 (100 µL). Dess-Martin periodinane (9.13 mg, 0.0215 mmol) was added into the mixture at rt, and the reaction was stirred overnight. The reaction was quenched with satd. Na_2S2O_3 (0.2 mL) and satd. $NaHCO_3$ (0.2 mL) and extracted with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure probe (2*S*,3*R*)-**1b** (7.0 mg, 70%) was obtained as a yellow wax by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5% MeOH in CH_2Cl_2.

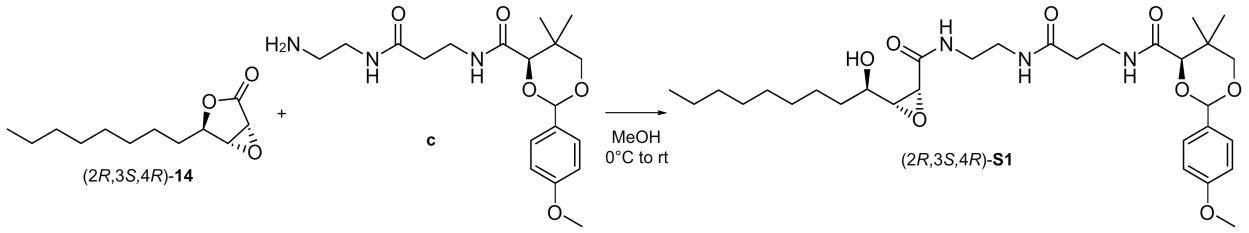
Probe (2*S*,3*R*)-**1b**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.52 (d, *J* = 9.1 Hz, 1H), 6.68 (dd, *J* = 9.0, 2.6 Hz, 1H), 6.56 (d, *J* = 2.5 Hz, 1H), 6.39 (m, 2H), 6.09 (s, 1H), 5.95 (m, 1H), 3.85 (d, *J* = 5.3 Hz, 3.72 (d, *J* = 5.3 Hz, 1H), 3.62 (s, 2H), 3.20 (q, *J* = 6.4 Hz, 2H), 3.12 (dd, *J* = 13.7, 6.4 Hz, 2H), 3.06 (s, 6H), 3.01 (s, 2H), 2.93 (s, 1H), 2.53 (m, 2H), 1.55 (m, 4H), 1.41 (m, 2H), 1.23 (m, 14H),

0.86 (t, J = 6.7 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 203.1, 168.0, 164.9, 156.2, 153.2, 151.3, 150.0, 148.4, 142.6, 126.0, 110.7, 109.4, 98.4, 58.7, 55.7, 41.2, 41.1, 40.4, 39.5, 38.6, 31.9, 29.4, 29.3, 29.2, 29.2, 29.0, 25.9, 25.8, 23.3, 22.8, 14.3; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{45}N_3O_6Na$ $[M+Na]^+$:578.3206, found 578.3205.



Scheme S8. Synthesis of crosslinker (2*R*,3*S*)-**1c**. A three-step process was used to convert (2*R*,3*S*,4*R*)-**14** to crosslinker (2*R*,3*S*)-**1c**. This began by addition of amine **c** to (2*R*,3*S*,4*R*)-**14** which was followed by oxidation of alcohol (2*R*,3*S*,4*R*)-**S1** to ketone (2*R*,3*S*)-**S2** and deprotection to (2*R*,3*S*)-**1c**.

Synthesis of intermediate (2*R*,3*S*,4*R*)-S1



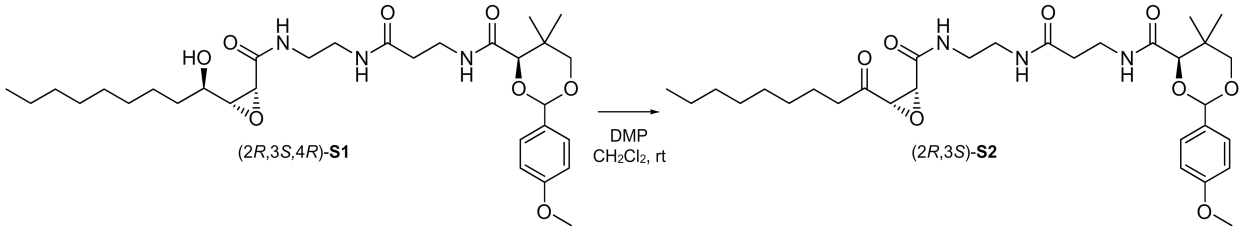
Reagents:

PMP-protected pantetheine amine was prepared according to: [Beld J, Cang H, Burkart MD. Visualizing the chain-flipping mechanism in fatty-acid biosynthesis. Angew Chem Int Ed Engl. 2014 Dec 22;53(52):14456-61].

(4*R*)-N-(3-((2-((2*R*,3*S*)-3-((*R*)-1-hydroxynonyl)oxirane-2-carboxamido)ethyl)amino)-3-oxopropyl)-2-(4-methoxyphenyl)-5,5-dimethyl-1,3-dioxane-4-carboxamide ((2*R*,3*S*,4*R*)-S1). Epoxy lactone (2*R*,3*S*,4*R*)-**14** (15.0 mg, 0.0707 mmol) was dissolved in MeOH (400 µL). Amine **c** (53.6 mg, 0.141 mmol) was added into the mixture at rt, and the reaction was stirred overnight. The reaction mixture was then air dried. Pure intermediate (2*R*,3*S*,4*R*)-**S1** (36.1 mg, 86%) was obtained by flash chromatography, eluting with a gradient of CH_2Cl_2 to 3% MeOH in CH_2Cl_2.

Intermediate (2*R*,3*S*,4*R*)-**S1**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.41 (d, *J* = 8.7 Hz, 2H), 6.99 (t, *J* = 6.4 Hz, 1H), 6.91 (d, *J* = 8.8 Hz, 2H), 6.91 (m, 1H), 6.72 (t, *J* = 5.9 Hz, 1H), 5.47 (s, 1H), 4.14 (s, 1H), 3.82 (s, 3H), 3.69 (m, 5H), 3.54 (m, 1H), 3.46 (m, 4H), 3.41 (m, 1H), 3.29 (m, 2H), 3.10 (m, 2H), 2.39 (m, 2H), 1.62 (m, 2H), 1.47 (m, 1H), 1.38 (m, 1H), 1.27 (m, 10H), 1.10 (s, 3H), 1.08 (s, 3H), 0.87 (t, *J* = 6.7 Hz, 3H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 171.6, 170.4, 168.3, 160.5, 130.1, 127.8, 113.9, 101.7, 84.0, 78.6, 68.7, 60.7, 55.5, 54.4, 39.5, 39.2, 36.6, 35.7, 34.7, 33.2, 32.0, 29.8, 29.7, 29.4, 25.4, 22.8, 22.0, 19.3, 14.3.; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{50}N_3O_8$ $[M+H]^+$:592.3599, found 592.3596.

Synthesis of intermediate (2*R*,3*S*)-S2



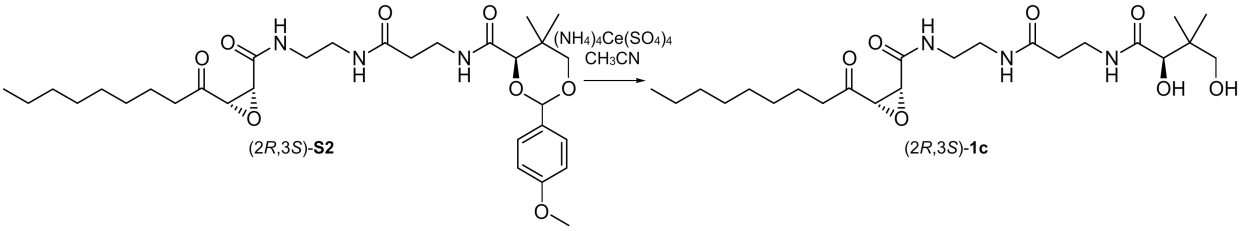
Reagents:

Dess-Martin periodinane, 95% (Astatech, Inc): used without further purification

(4*R*)-2-(4-methoxyphenyl)-5,5-dimethyl-*N*-(3-((2-((2*R*,3*S*)-3-nonanoyloxirane-2-carboxamido)ethyl)amino)-3-oxopropyl)-1,3-dioxane-4-carboxamide ((2*R*,3*S*)-S2). Intermediate (2*R*,3*S*,4*R*)-**S1** (20.0 mg, 0.0338 mmol) was dissolved in CH_2Cl_2 (200 µL). Dess-Martin periodinane (17.2 mg, 0.0406 mmol) was added into the mixture at rt, and the reaction was stirred overnight. The reaction was quenched with satd. Na_2S2O_3 (0.3 mL) and satd. $NaHCO_3$ (0.3 mL) and extracted with CH_2Cl_2 (3 × 3 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure intermediate (2*R*,3*S*)-**S2** (16.4 mg, 82%) was obtained by flash chromatography, eluting with a gradient of CH_2Cl_2 to 3% MeOH in CH_2Cl_2. Trace amounts of Dess-Martin periodinane by-products were observed in (2*R*,3*S*)-**S2**.

Intermediate (2*R*,3*S*)-**S2**: ^{1}H-NMR (500 MHz, $CDCl_3$) δ ^{1}H-NMR (500 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to tautomers within (2*R*,3*S*)-**S2**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (125 MHz, $CDCl_3$) δ 204.4, 171.7, 169.5, 164.8, 160.4, 132.2, 130.2, 127.7, 114.4, 113.9, 101.6, 84.0, 78.7, 58.0, 55.8, 55.5, 41.7, 39.0, 38.8, 36.4, 35.2, 33.2, 31.9, 29.3, 29.2, 29.1, 23.2, 22.8, 22.0, 19.3, 14.2; HRMS (ES-ESI-TOFMS) *m/z* calcd. for $C_{31}H_{47}N_3O_8Na$ $[M+Na]^+$:612.3261, found 612.3258.

Synthesis of crosslinker (2*R*,3*S*)-1c

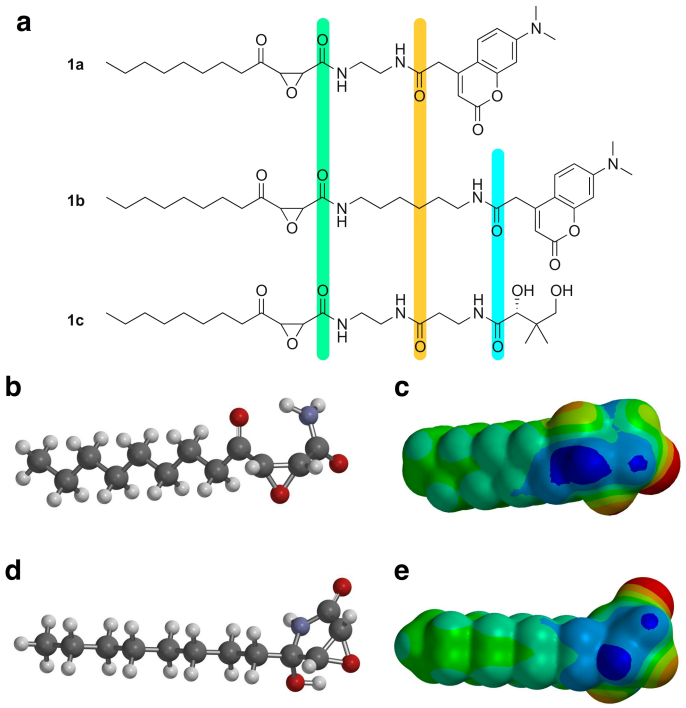


Reagents:

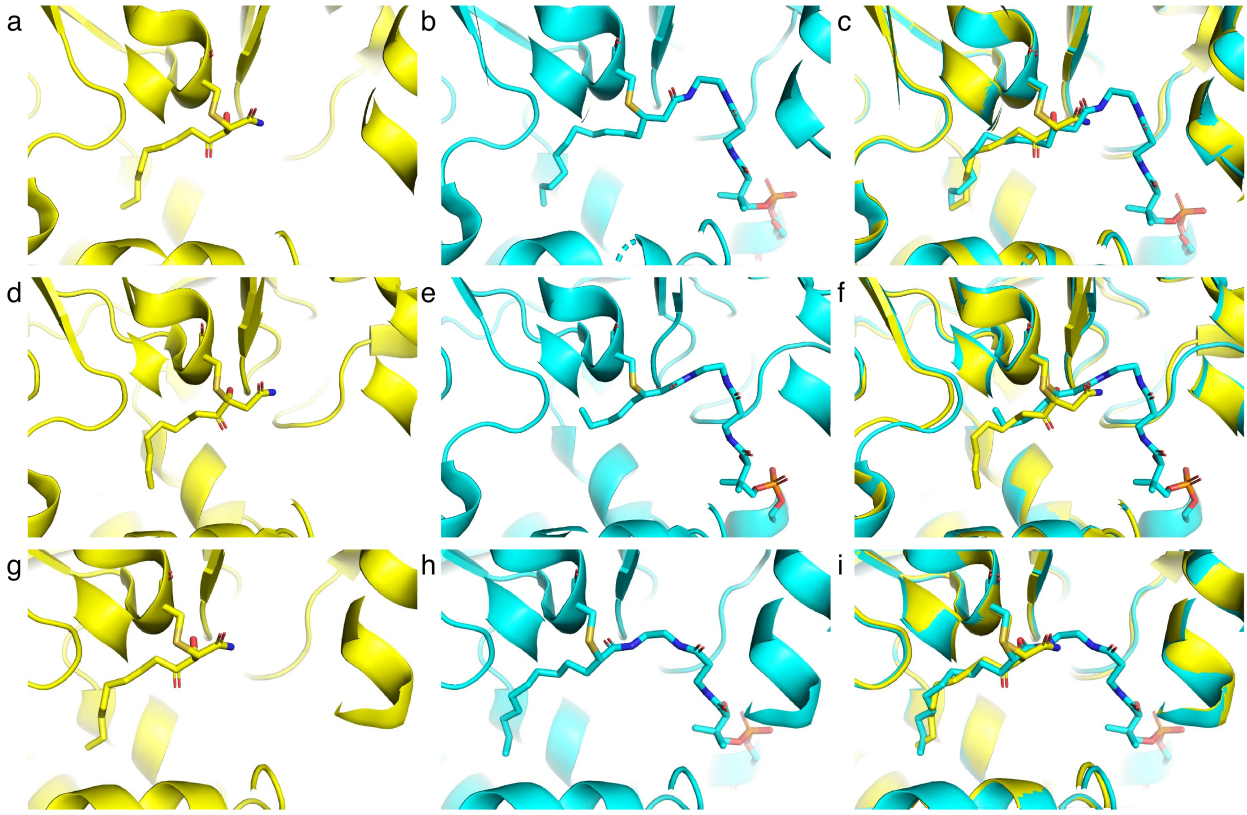
$(NH_4)_4Ce(SO_4)_4$ • 2 H_2O, 95% (Alfa Aesar): used without further purification

(2*R*,3*S*)-*N*-(2-(3-((*R*)-2,4-dihydroxy-3,3-dimethylbutanamido)propanamido)ethyl)-3-nonanoyloxirane-2-carboxamide ((2*R*,3*S*)-1c). (2*R*,3*S*)-**S2** (5.0 mg, 0.0085 mmol) was dissolved into 9:1 CH_3CN/H_2O (100 µL). $(NH_4)_4Ce(SO_4)_4$ • 2 H_2O (11.0 mg, 0.0174 mmol) was added at rt. The reaction was allowed to proceed at rt for 1 h. The reaction was monitored by TLC and quenched with satd. $NaHCO_3$ (0.2 mL) and extracted with CH_2Cl_2 (3 × 2 mL). The combined organic phases were dried over Na_2SO_4, filtered and concentrated on a rotary evaporator. Pure crosslinker (2*R*,3*S*)-**1c** (4.1 mg, 59%) was obtained by flash chromatography, eluting with a gradient of CH_2Cl_2 to 5% MeOH in CH_2Cl_2.

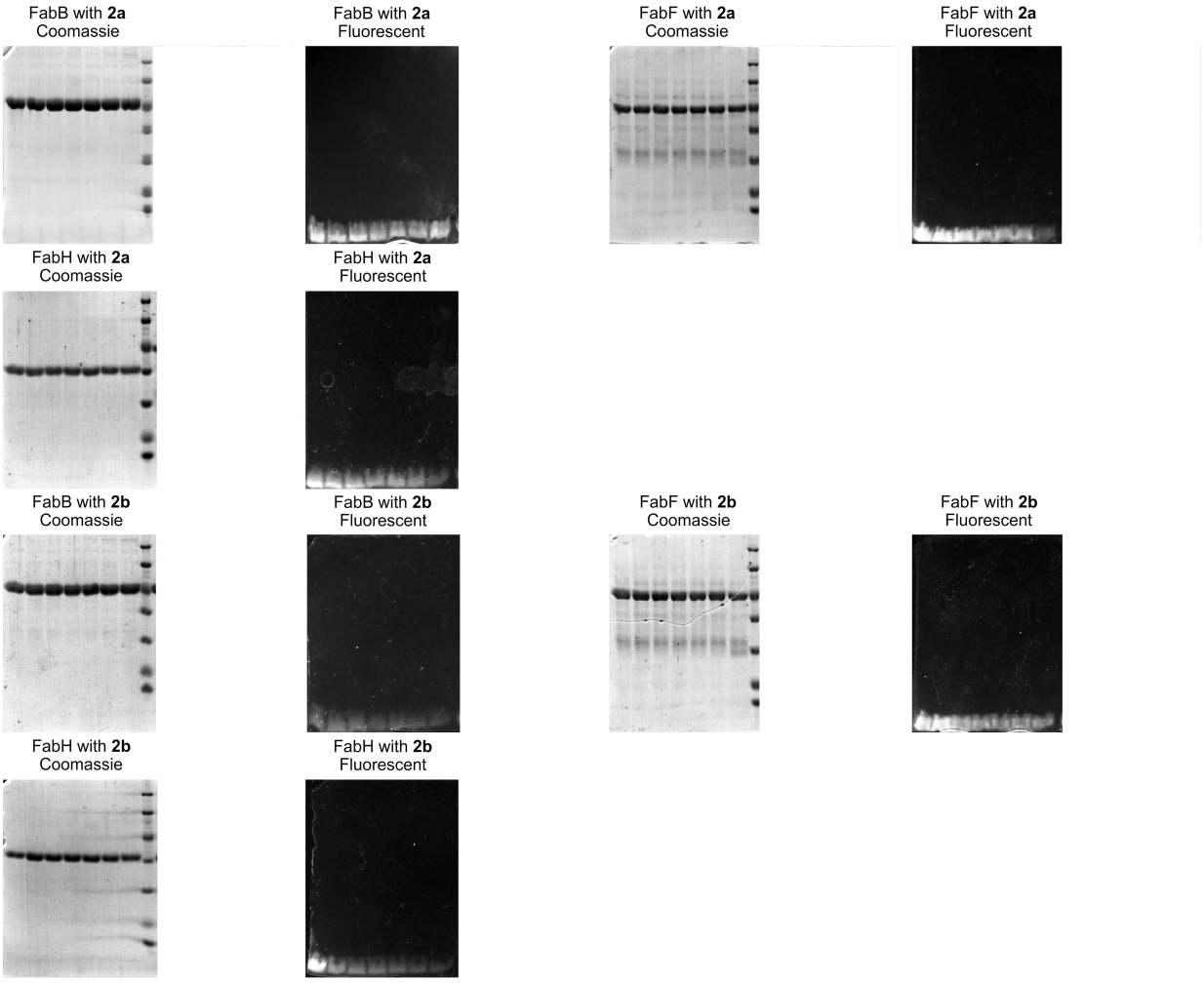
Crosslinker (2*R*,3*S*)-**1c**: ^{1}H-NMR (500 MHz, $CDCl_3$) has been placed in the SI with expansions. Due to tautomers within (2*R*,3*S*)-**1c**, the ^{1}H-NMR data was not tabulated; ^{13}C-NMR (200 MHz MHz, $CDCl_3$) δ 204.9, 173.7, 173.2, 172.1, 171.4, 170.1, 165.3, 131.2 (impurity), 129.0 (impurity), 89.7, 89.6, 78.5, 78.0, 71.3, 71.0, 70.7, 58.4, 58.0, 56.2, 56.0, 53.6, 52.7, 51.8, 41.8, 40.9, 39.6, 39.5, 39.4, 39.3, 39.2, 39.2, 37.8, 37.2, 36.3, 36.2, 36.2, 35.6, 35.5, 35.0, 34.6, 32.0, 32.0, 31.9, 30.1, 29.9, 29.7, 29.6, 29.5, 29.4, 29.3, 29.3, 29.2, 24.0, 23.4, 23.1, 22.8, 22.8, 21.9, 21.9, 21.7, 21.4, 20.8, 20.6, 14.3; HRMS (HR-ESI-TOFMS) *m/z* calcd. for $C_{23}H_{41}N_3O_7Na$ $[M+Na]^+$:494.2843, found 494.2840.



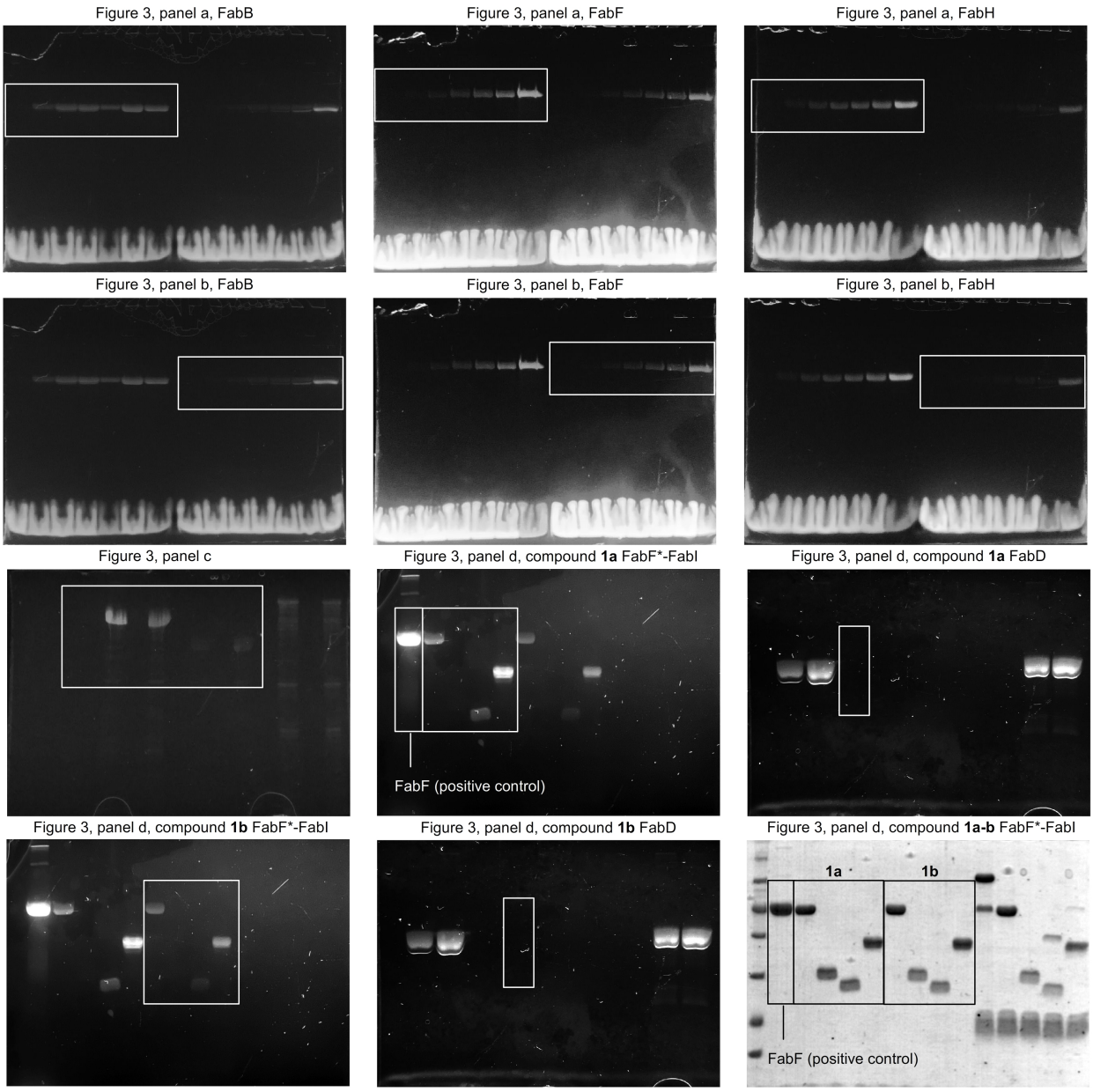
Supporting Figure S1. Structural analyses. **a)** Structural comparison of fluorescent probes **1a** and **1b** to crosslinker **1c**. Structural modifications within this probe set are deflected by common functionality (blue and green) and different functionality (orange). Structure and electrostatic potential map generated by Spartan'20 through B3LYP/6-311+G**, including **b)** structure of linear tautomer of cerulenin; **c)** electron static map of linear tautomer of cerulenin; **d)** structure of cyclic tautomer of cerulenin; and **e)** electron static map of cyclic tautomer of cerulenin.



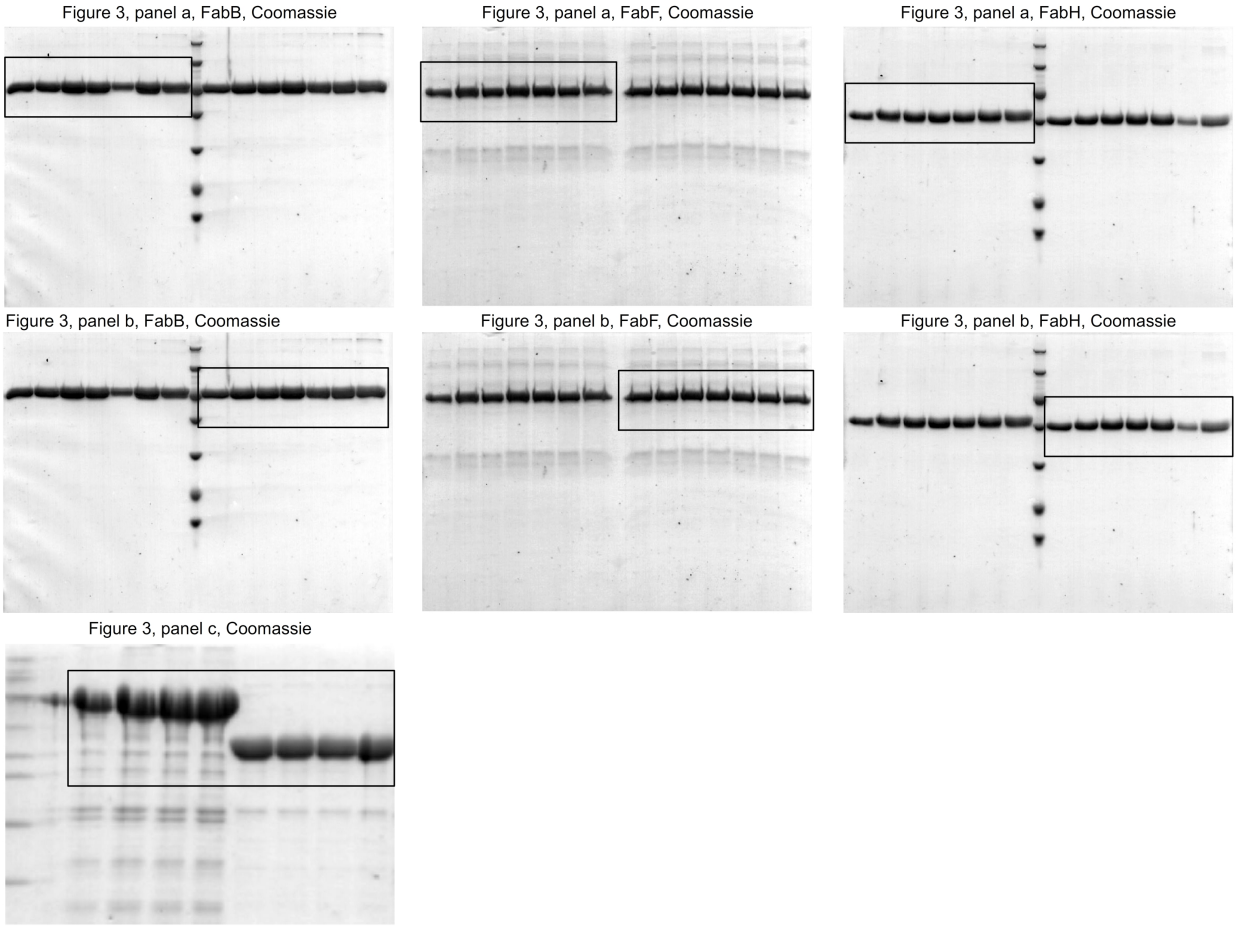
Supporting Figure S2. Cerulenin bound KSs, KS=AcpP crosslinked complexes. a) Covalent inhibition of *E. coli* FabB active Cys163 residue by cerulenin (PDB:1FJ8) [Price AC, Choi KH, Heath RJ, Li Z, White SW, Rock CO. Inhibition of beta-ketoacyl-acyl carrier protein synthases by thiolactomycin and cerulenin. J Biol Chem. 2001 Mar 2;276(9):6551-9]. **b**) Reactive site of *E. coli* FabB crosslinked with C12-α-bromopantetheineamide loaded AcpP (PDB:6OKC) [Mindrebo JT, Patel A, Kim WE, Davis TD, Chen A, Bartholow TG, La Clair JJ, McCammon JA, Noel JP, Burkart MD. Gating mechanism of elongating β-ketoacyl-ACP synthases. Nat Commun. 2020 Apr 7;11(1):1727]. **c**) Overlay of cerulenin bound *E. coli* FabB (yellow) and *E. coli* FabB crosslinked with C12-α-bromopantetheineamide loaded AcpP (blue). **d**) Covalent inhibition of *E. coli* FabB active residue Cys163 by cerulenin (PDB:1FJ8). **e)** Reactive site of *E. coli* FabB crosslinked with *trans*-C14-chloroacrylate pantetheineamide loaded AcpP (blue, PDB: 7SQI) [Chen A, Mindrebo JT, Davis TD, Kim WE, Katsuyama Y, Jiang Z, Ohnishi Y, Noel JP, Burkart MD. Mechanism-based cross-linking probes capture the *Escherichia coli* ketosynthase FabB in conformationally distinct catalytic states. Acta Crystallogr D Struct Biol. 2022 Sep 1;78(Pt 9):1171-1179]. **f**) Overlay of cerulenin bound *E. coli* FabB (yellow) and *E. coli* FabB crosslinked with *trans*-C14-chloroacrylate pantetheineamide loaded AcpP (blue). **g)** Covalent inhibition of *B. subtillis* FabF active residue Cys163 by cerulenin (PDB:4LS8 [Trajtenberg F, Altabe S, Larrieux N, Ficarra F, de Mendoza D, Buschiazzo A, Schujman GE. Structural insights into bacterial resistance to cerulenin. FEBS J. 2014 May;281(10):2324-38]. **h**) Reactive site of *E. coli* FabF crosslinked with *trans*-C8 chloroacrylate pantetheineamide loaded AcpP (PDB: 7L4L) [Mindrebo JT, Chen A, Kim WE, Re RN, Davis TD, Noel JP, Burkart MD Structure and mechanistic analyses of the gating mechanism of elongating ketosynthases. ACS Catal. 2021 Jun 18;11(12):6787-6799]. **i**) Overlay of cerulenin bound *B. subtillis* FabF (yellow) and *E. coli* FabF crosslinked with *trans*-C8 chloroacrylate pantetheineamide loaded AcpP (blue).



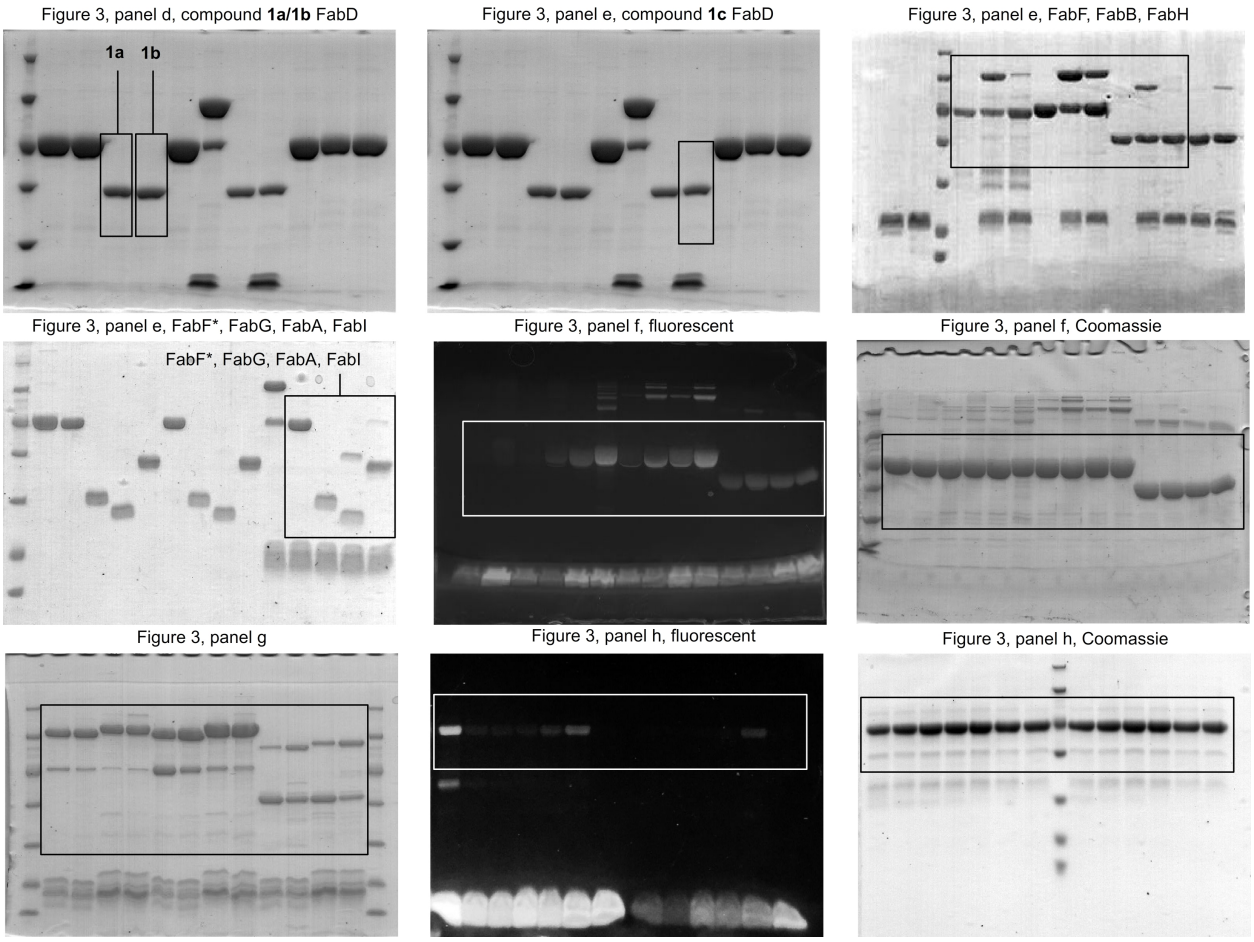
Supporting Figure S3. Additional gel images. Fluorescent images are from transillumination at 365 nm. Coomassie Brilliant Blue stained gels were imaged directly. Boxes show the cropped region used in each figure. Lanes 1-7 of the gels demonstrated the time course labeling of probes **2a** or **2b** on FabB, FabF, and FabH. The experimental procedure was described above.



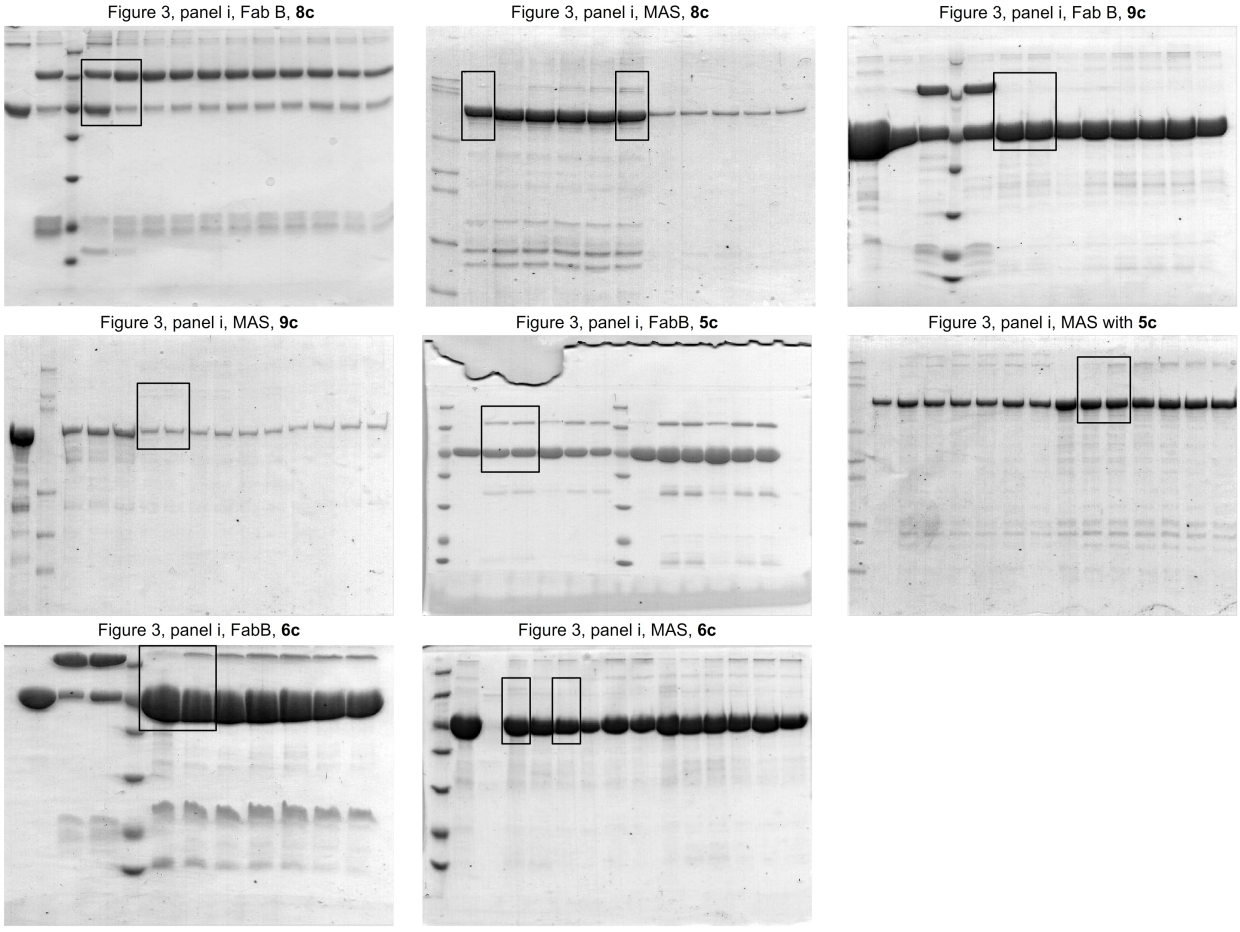
Supporting Figure S4. Full images of gels from Figure 3. Fluorescent images are from transillumination at 365 nm. Coomassie Brilliant Blue stained gels were imaged directly. Boxes show the cropped region used in each figure. The usage in Figure 3 is labeled above each image.



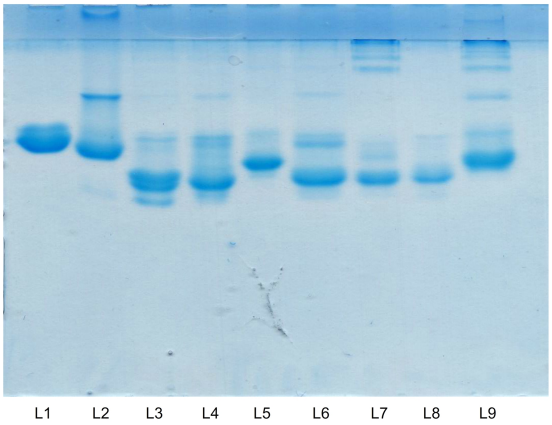
Supporting Figure S5. Full images of gels from Figure 3. Coomassie Brilliant Blue stained gels were imaged directly. Boxes show the cropped region used in each figure. The usage in Figure 3 is labeled above each image.



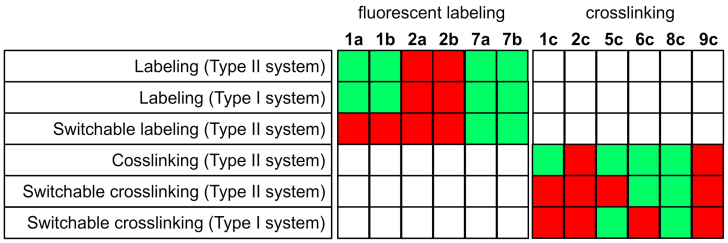
Supporting Figure S6. Full images of gels from Figure 3. Fluorescent images are from transillumination at 365 nm. Coomassie Brilliant Blue stained gels were imaged directly. Boxes show the cropped region used in each figure. The usage in Figure 3 is labeled above each image.



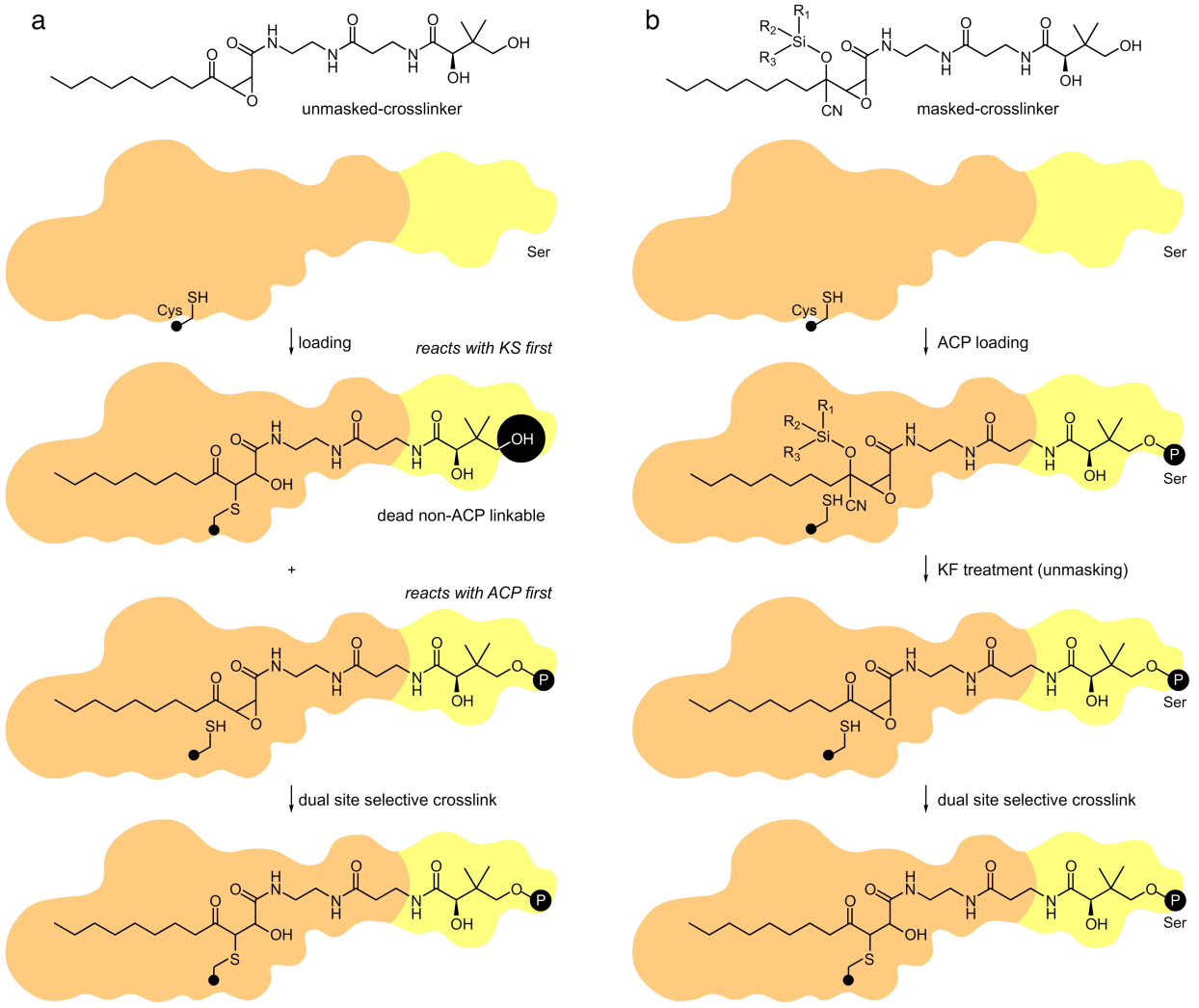
Supporting Figure S7. Full images of gels from Figure 3. Coomassie Brilliant Blue stained gels were imaged directly. Boxes show the cropped region used in each figure. The usage in Figure 3 is labeled above each image.



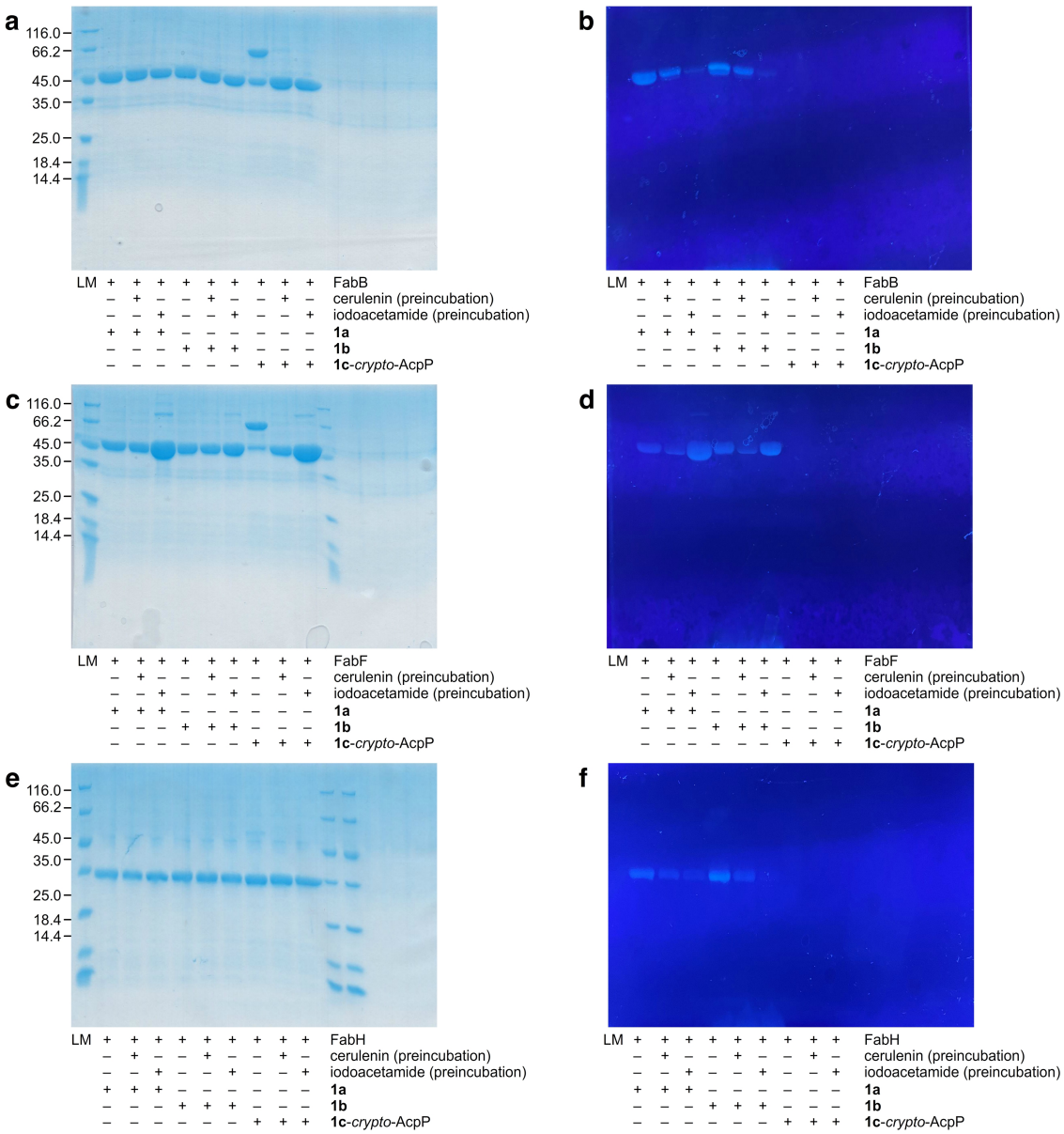
Supporting Figure S8. UREA-PAGE gel (20% acrylamide) demonstrating band shifts for select AcpP states including: (L1) *apo*-AcpP; (L2) *holo*-AcpP; (L3) **2c**-*crypto*-AcpP; (L4) **1c**-*crypto*-AcpP; (L5) (2*R*,3*S*)-**1c**-*crypto*-AcpP; (L6) **8c**-*crypto*-AcpP; (L7) **9c**-*crypto*-AcpP; (L8) **5c**-*crypto*-AcpP; and, (L9) **6c**-*crypto*-AcpP.



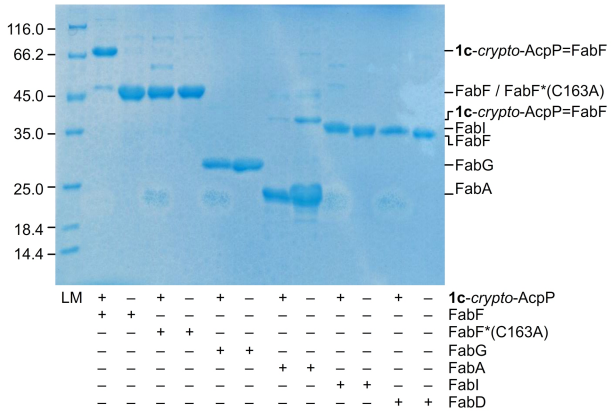
Supporting Figure S9. Summary of probes that are suitable in labeling, switchable labeling, crosslinking, and switchable crosslinking in type II and type I systems.



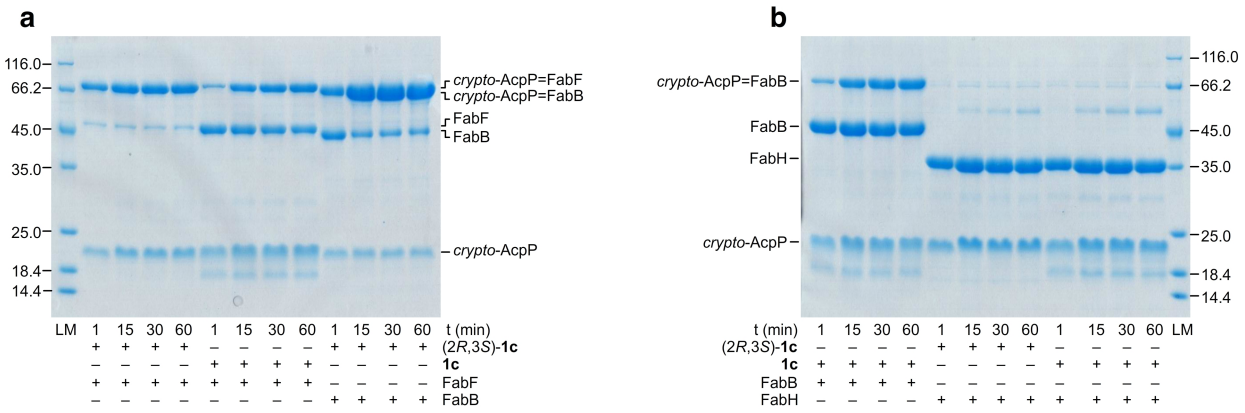
Supporting Figure S10. Crosslinking mechanism. **a)** Reaction of an unmasked crosslinker onto an ACP and KS pair. As desired, a crosslinker is loaded onto the active serine of an ACP through "one-pot" chemoenzymatic reaction by CoaA, CoaD, CoaE, and Sfp to form a *crypto*-ACP. Unless the KS reactive unit is masked, the reaction is complicated by a competitive, direct reaction of the crosslinker with the KS domain. This results in a labeled KS domain that cannot be appended onto the ACP, as noted by the dead non-ACP linkable state. This issue (dead non-ACP linkable state) is a particular problem when the ACP and KS are within the same protein, as it can be very challenging to selectively purify the unwanted dead-non-ACP linkable state from the desired ACP=KS crosslinked protein. **b)** A masked crosslinker is loaded onto the ACP to form a *crypto*-ACP. After the removal of the excess crosslinker, sequential unmasking can allow the crosslinker to bind to and react with KS domain covalently.



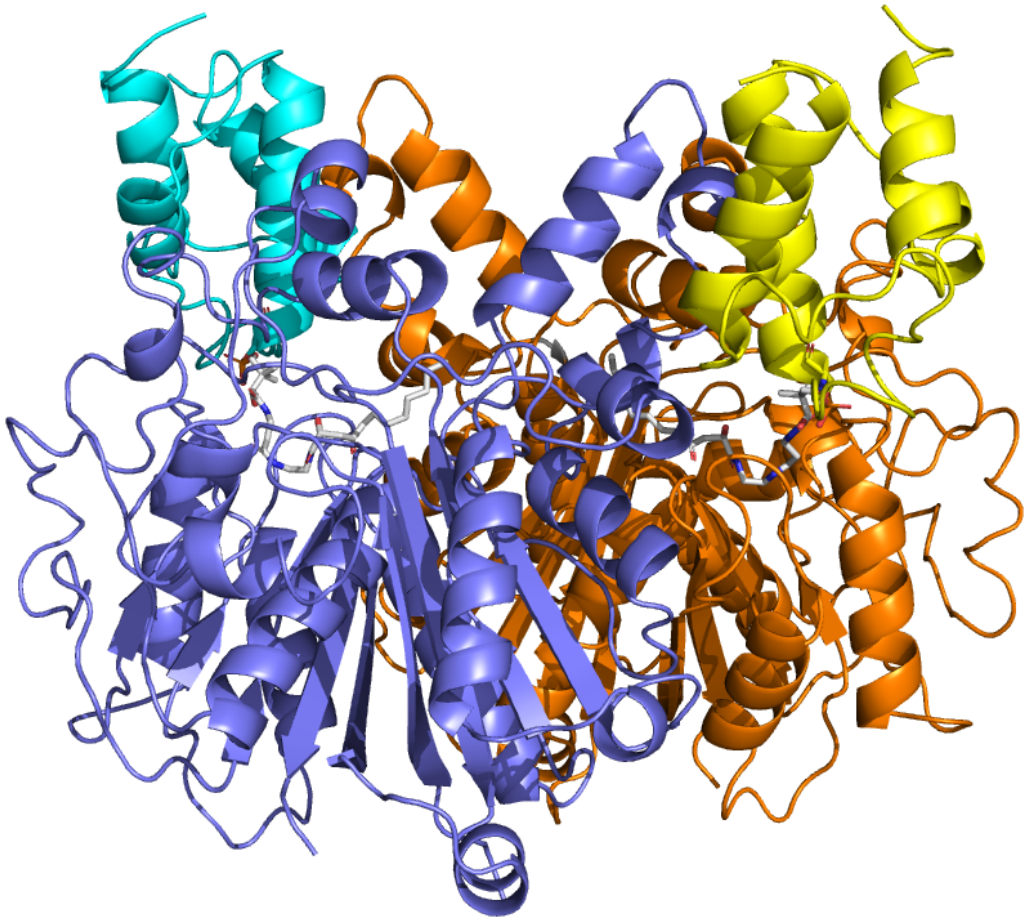
Supporting Figure S11. SDS-PAGE gels (12%) depicting active site inhibition studies. **a)** A gel stained with Coomassie Brilliant Blue or **b)** fluorescent imaged (UV transilluminator at 365 nm) depicting the blockage of labeling with **1a**, **1b**, or **1c**-*crypto*-AcpP by the preincubation of FabB with either cerulenin or iodoacetamide. For each band, the FabB was first incubated with the inhibitor (cerulenin or iodoacetamide) and then fluorescently stained with **1a** or **1b** or crosslinked with **1c**-*crypto*-AcpP. **c)** A gel stained with Coomassie Brilliant Blue or **d)** fluorescent imaged (UV transilluminator at 365 nm) depicting the blockage of labeling with **1a**, **1b**, or **1c**-*crypto*-AcpP by the preincubation of FabF with either cerulenin or iodoacetamide. For each band, the FabF was first incubated with the inhibitor (cerulenin or iodoacetamide) and then fluorescently stained with **1a** or **1b** or crosslinked with **1c**-*crypto*-AcpP. **e)** A gel stained with Coomassie Brilliant Blue or **f)** fluorescent imaged (UV transilluminator at 365 nm) depicting the blockage of labeling with **1a**, **1b**, or **1c**-*crypto*-AcpP by the preincubation of FabH with either cerulenin or iodoacetamide. For each band, the FabH was first incubated with the inhibitor (cerulenin or iodoacetamide) and then fluorescently stained with **1a** or **1b** or crosslinked with **1c**-*crypto*-AcpP.



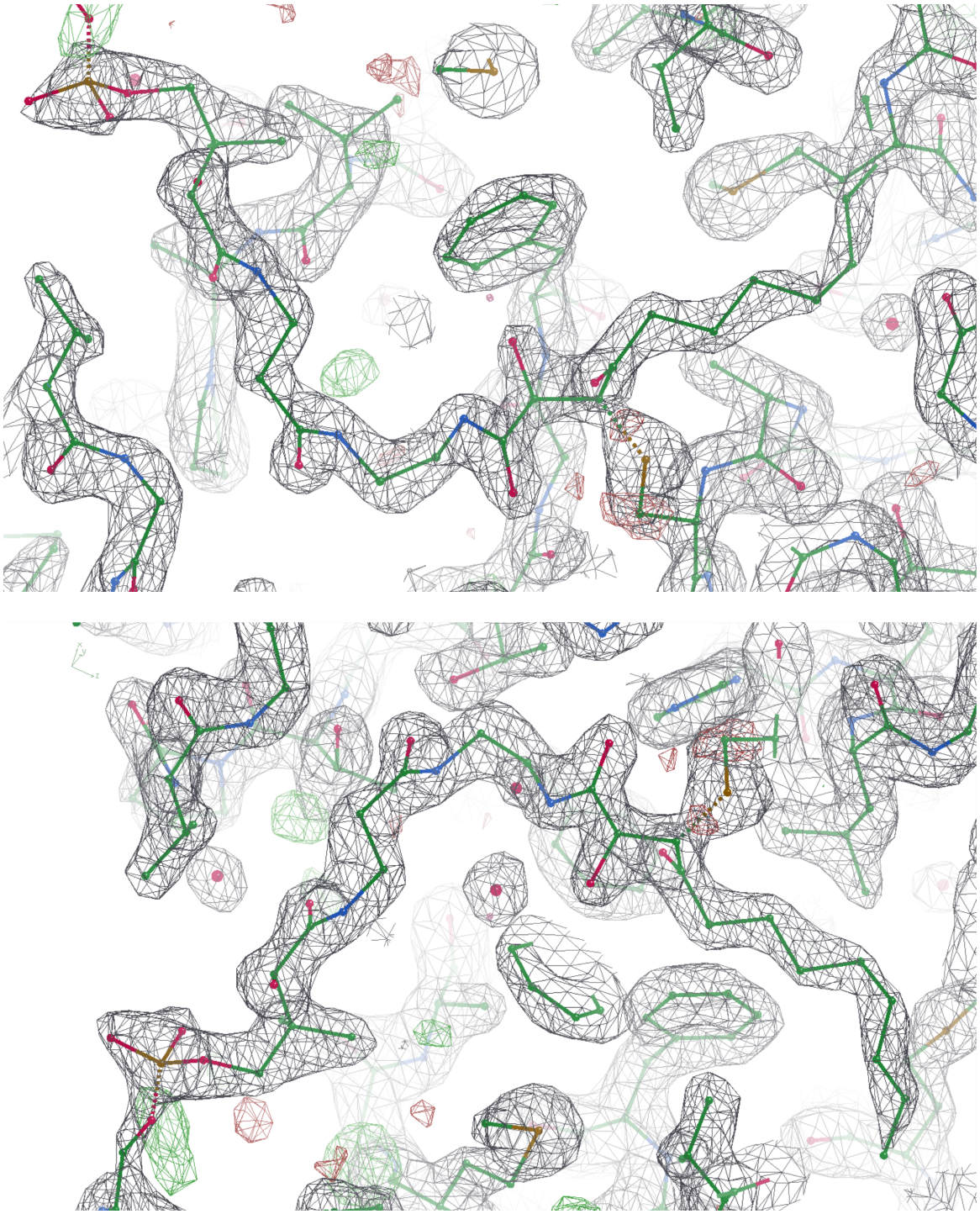
Supporting Figure S12. Crosslinking of **1c-*crypto*-AcpP** with *E. coli* partner proteins. *Crypto*-AcpP loaded with **1c** (200 µM) was incubated with 20 µM FabF, 20 µM FabF (C163A) mutant, 20 µM FabG, 20 µM FabA, 20 µM FabI, or 20 µM FabD in 50 mM Tris • HCl buffer pH 7.4, 150 mM NaCl, 10% glycerol at 25 °C for 21 h. The reactions were evaluated by SDS-PAGE analyses.



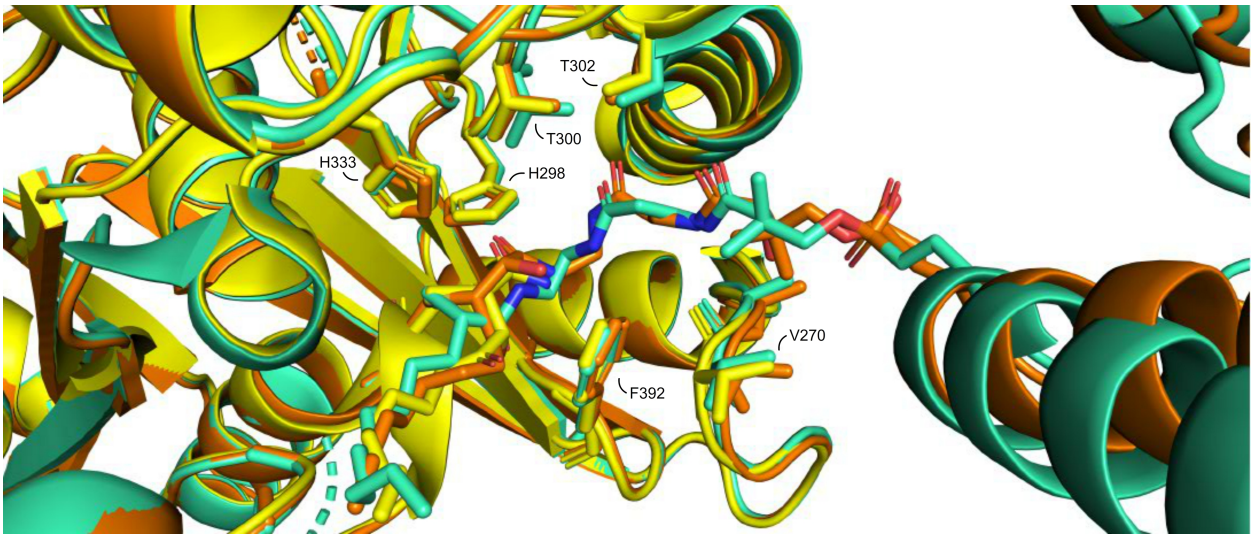
Supporting Figure S13. SDS-PAGE gels (12%) depicting time course crosslinking studies. **a)** Time course crosslinking of (2*R*,3*S*)-**1c**-*crypto*-AcpP crosslinking with FabF, **1c**-*crypto*-AcpP crosslinking with FabF, and (2*R*,3*S*)-**1c**-*crypto*-AcpP crosslinking with FabB. **b)** Time course crosslinking of **1c**-*crypto*-AcpP crosslinking with FabB, (2*R*,3*S*)-**1c**-*crypto*-AcpP crosslinking with FabH, and **1c**-*crypto*-AcpP crosslinking with FabH.



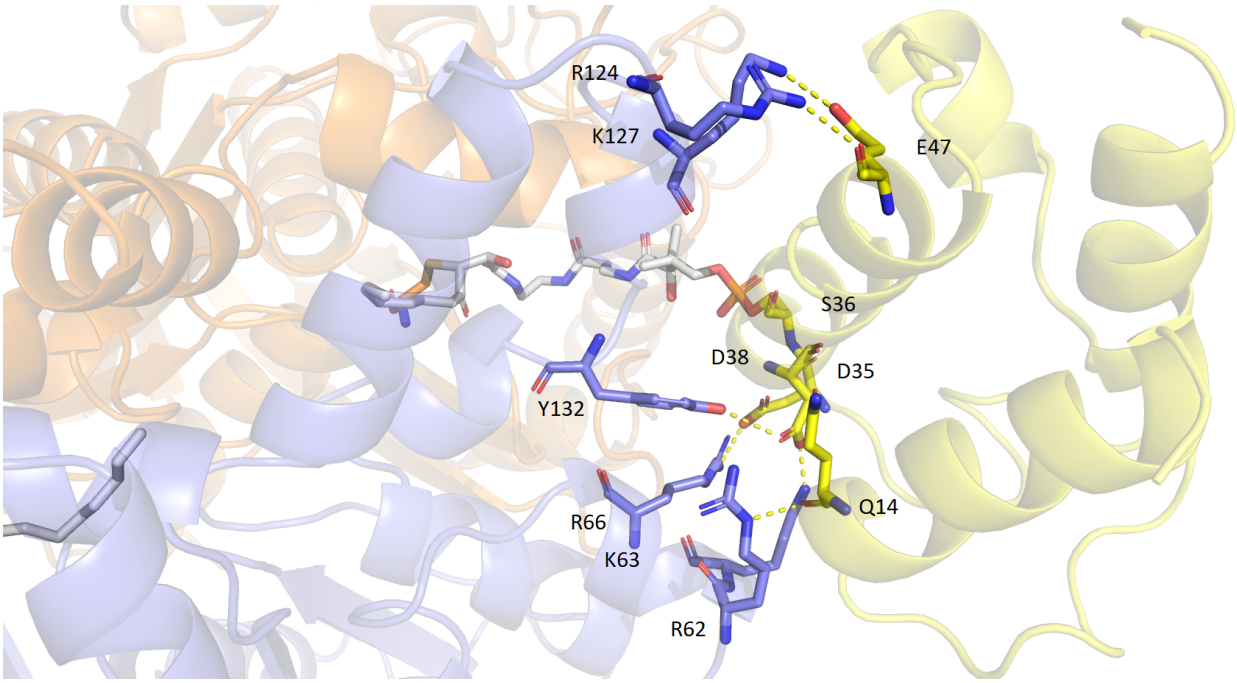
Supporting Figure S14. Crystal structure of *E. coli* FabB crosslinked with (2*R*,3*S*)-**1c**-*crypto*-AcpP with KS dimer chain A and chain B colored in orange and purple respectively. AcpP monomer chain C and chain D are colored in light blue and yellow respectively. Crosslinkers (2*R*,3*S*)-**1c** are in white color.



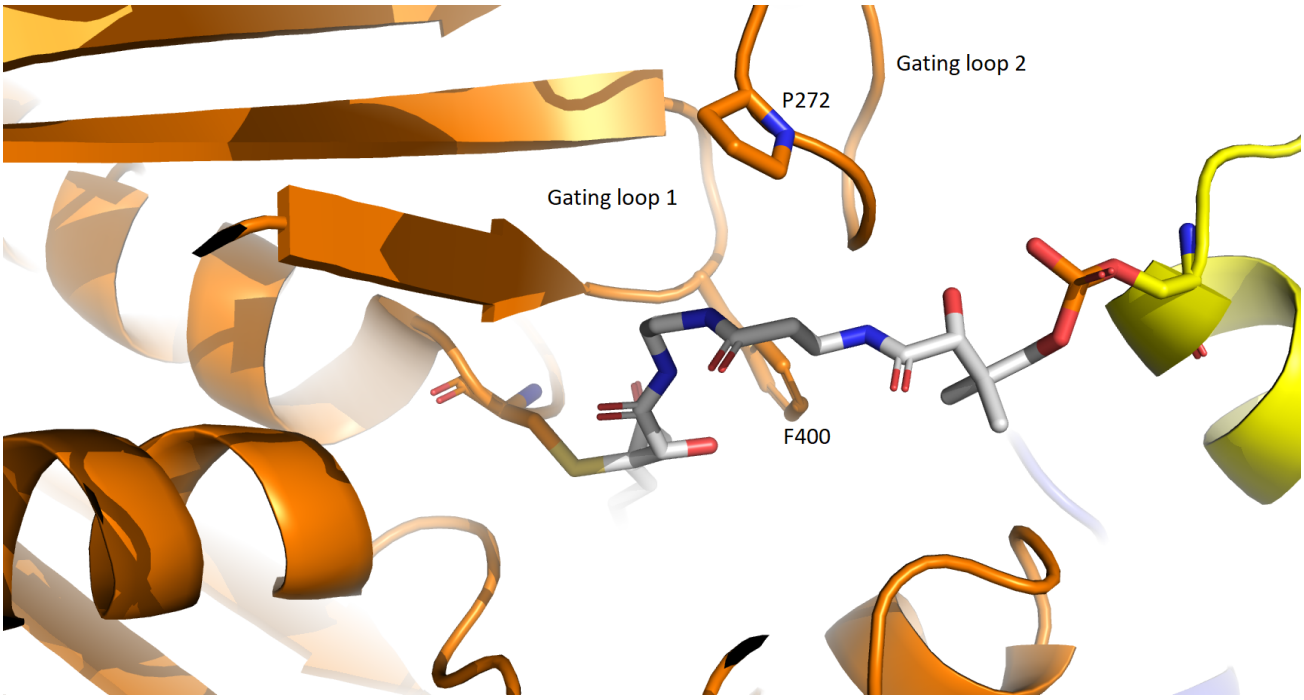
Supporting Figure S15. The front (top) and back (bottom) views of the modeling of AcpP tethered (chain D) crosslinker (2*R*,3*S*)-**1c**-*crypto*-AcpP into the density map. The density map was contoured at a sigma value of 1.5.



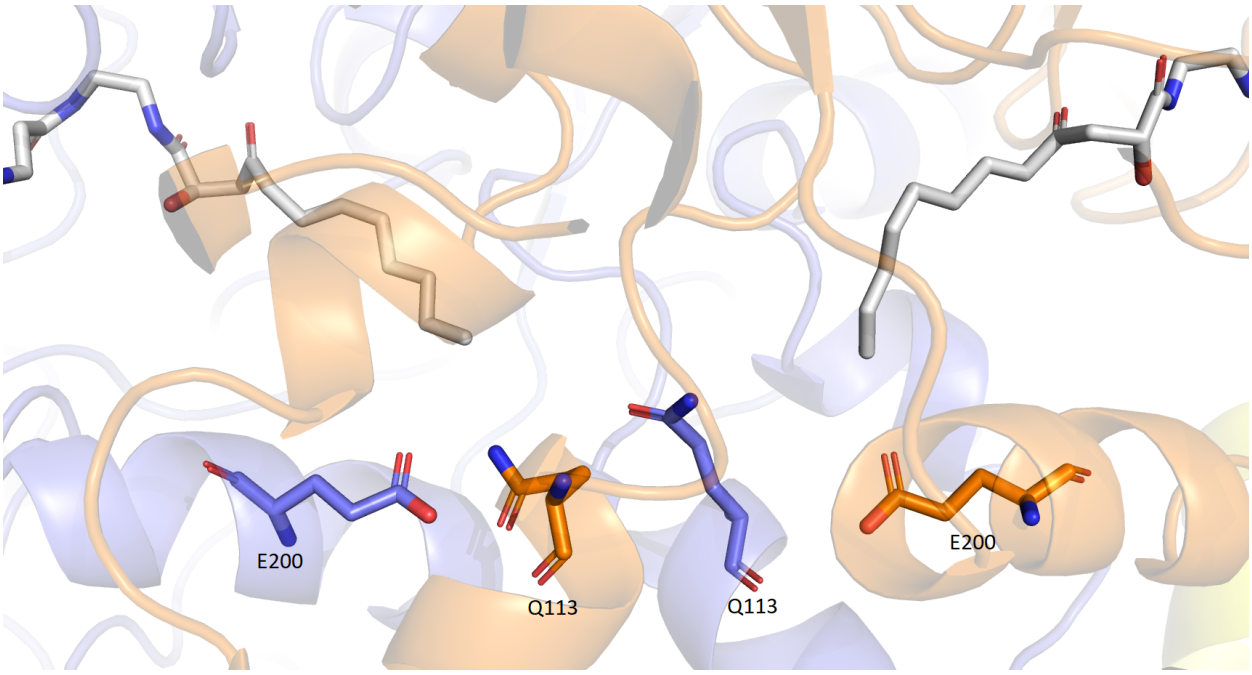
Supporting Figure S16. Structural overlay of cerulenin bound FabB (yellow), FabB crosslinked with C12αBr-*crypto*AcpP (cyan), and FabB crosslinked with (2*R*,3*S*)-**1c**-*crypto*-AcpP.



Supporting Figure S17. Protein-protein interface formed within R62, K63, R66, R124, K127 as well as Y132 from FabB (chain B in purple) and Q14, D35, D38, as well as E47 from AcpP (chain D in yellow).



Supporting Figure S18. The entrance of the substrate binding tunnel demonstrating the gating loop 1 and loop 2 at the gate closed conformation.

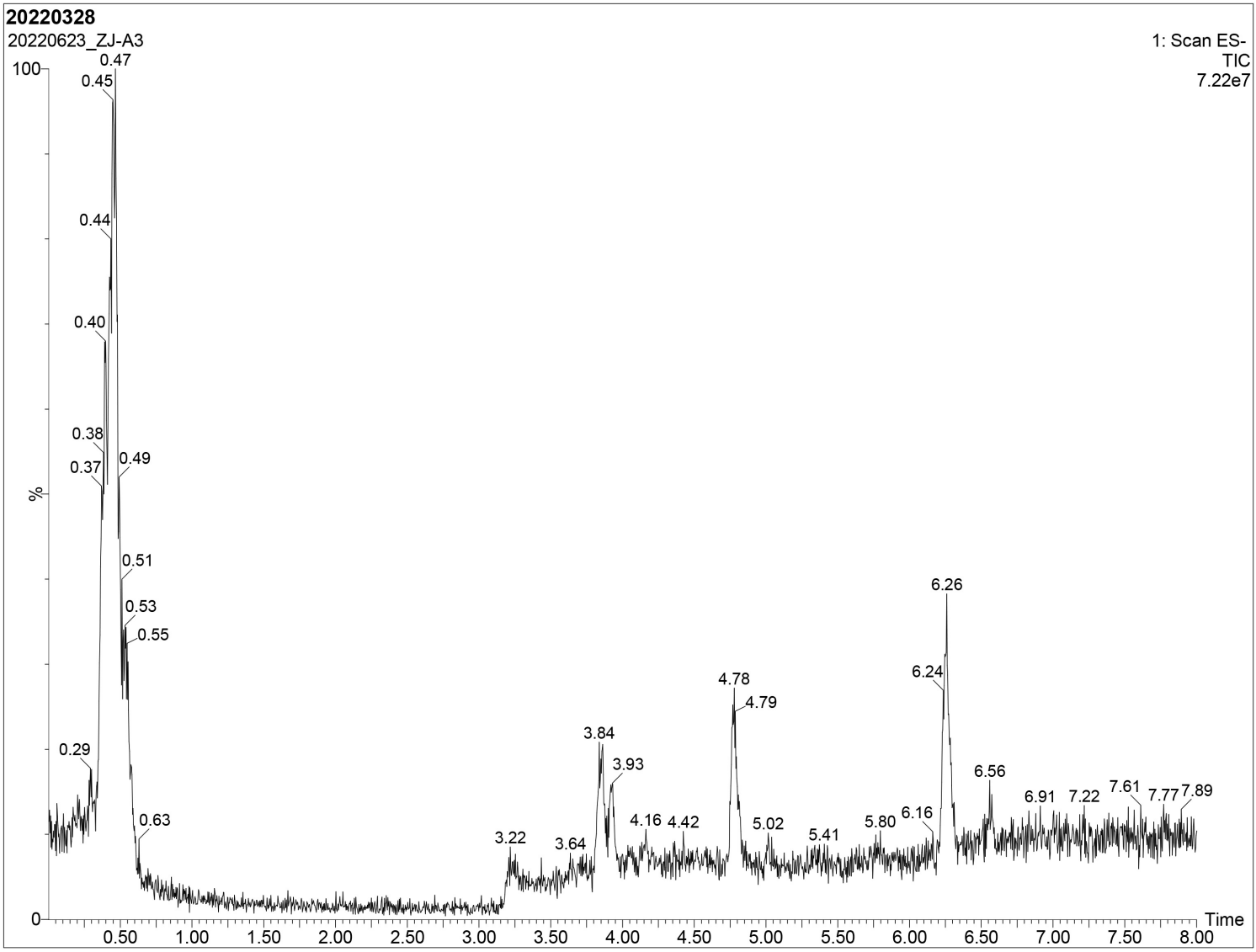


Supporting Figure S19. FabB asymmetric substrate pockets showing the back gate consisting of two sets of E200 and Q113. Q113 (FabB chain B in purple) is more proximal to the dimer interface expanding one of the substrate pockets while the other Q113 (FabB chain A in orange) is pushed to be distal to the dimer interface restricting the other pocket.

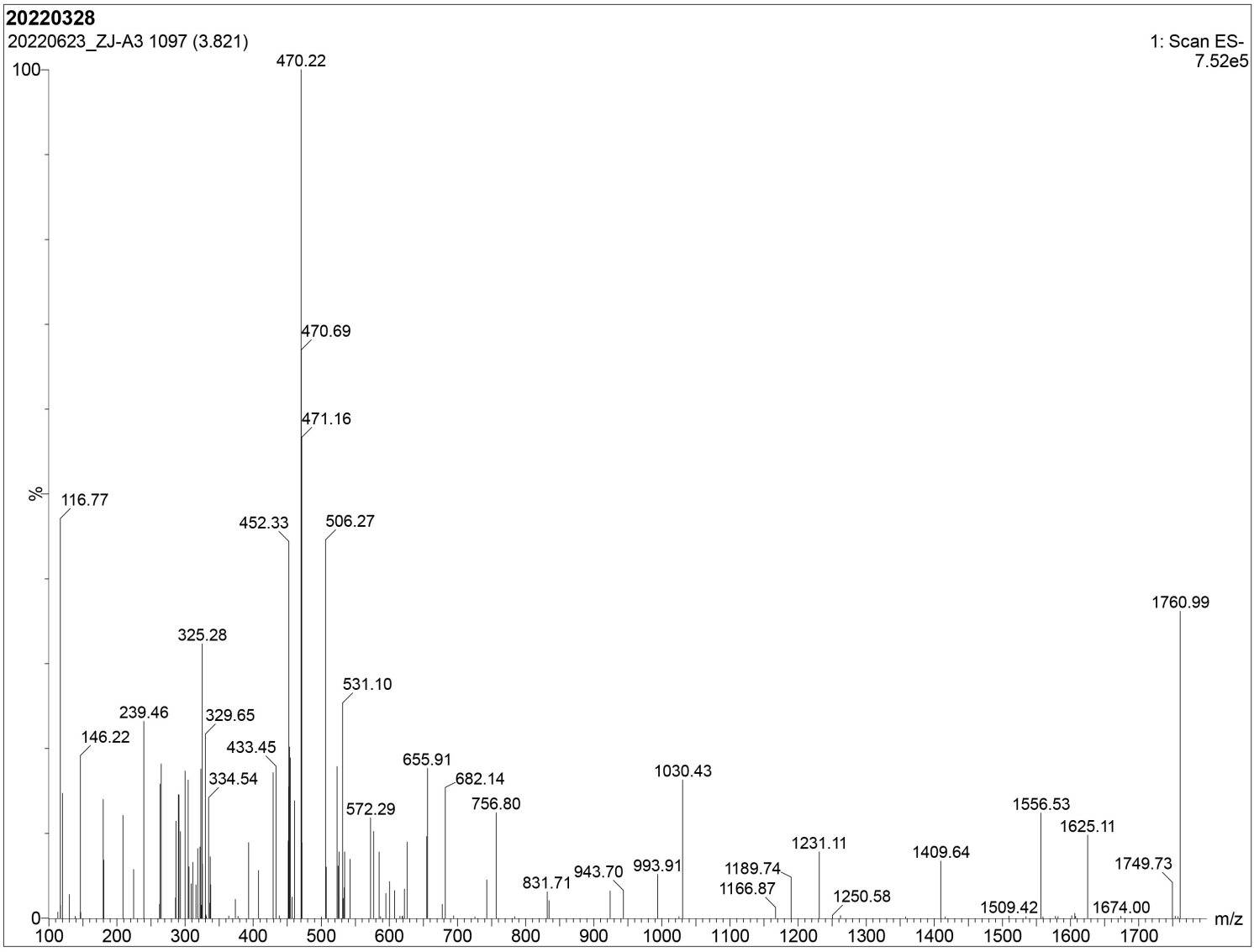
Structure (PDB code)	FabB=(2*R*,3*S*)-**1c**-AcpP (8SMS)
Wavelength (Å)	1
Resolution range (Å)	37.25-1.93
Space group	$P12_11$
a, *b*, *c* (Å)	59.03, 100.23, 78.31
α, β, γ (°)	90.00, 109.34, 90.00
Unique reflections	63659
Reflections used in refinement	63637
Reflections used for R_{free}	3057
R_{work}	0.201
R_{free}	0.255
Completeness	98.6% (97.1%)
Multiplicity	6.0 (5.4)
R_{merge}	0.17 (1.38)
I/σ	1.5
CC(1/2)	0.994 (0.650)
Protein residues	953
Solvent atoms	254
R.m.s.d., bond lengths (Å)	0.0083
R.m.s.d., angles (°)	1.565
Ramachandran favoured (%)	93.91%
Ramachandran allowed (%)	5.14%
Ramachandran outliers (%)	0.52%
Rotamer outliers (%)	2.88%
Clashscore	7.13
Average *B* factor (Å^2)	
Protein residues	31.02
Crosslinker	37.84
Water	28.53

Table S1. Data-collection and refinement statistics for the crystal structure of the crosslinked complex of FabB and (2*R*,3*S*)-**1c**-AcpP.

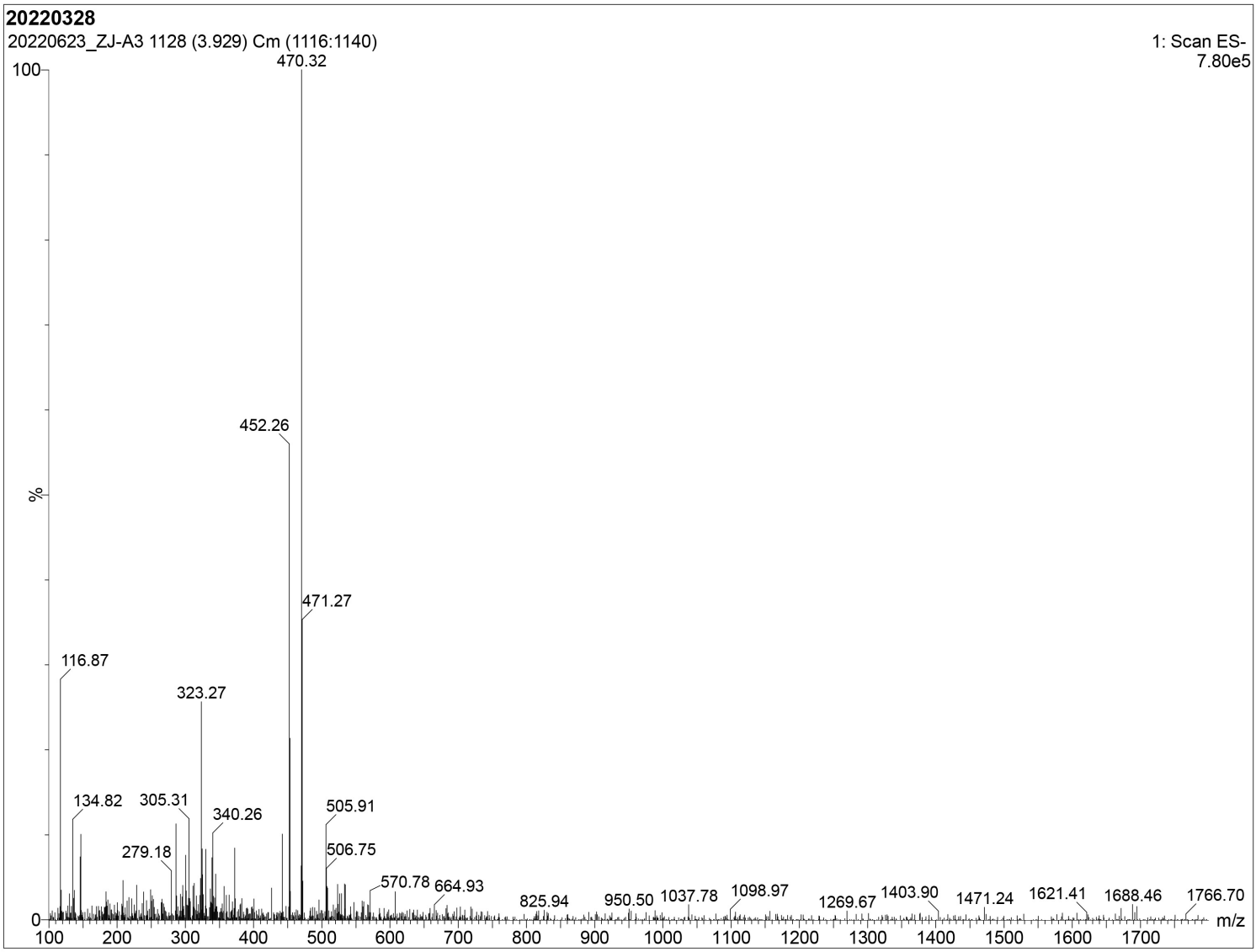
LC-MS spectral data from crosslinker **8c** incubated in CH_3CN for 12 h



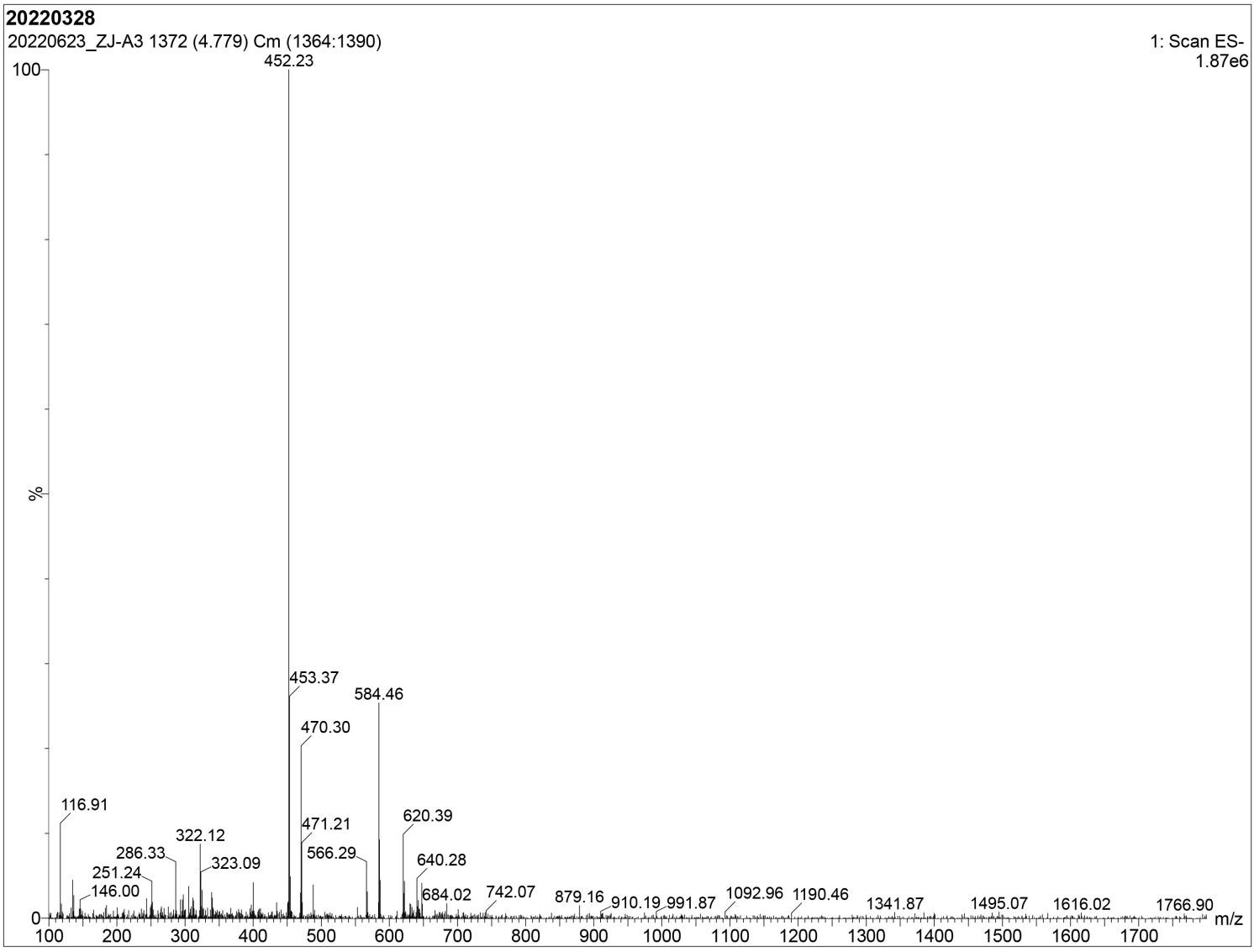
MS data from peak at 3.82 min from crosslinker **8c** incubated in CH_3CN for 12 h



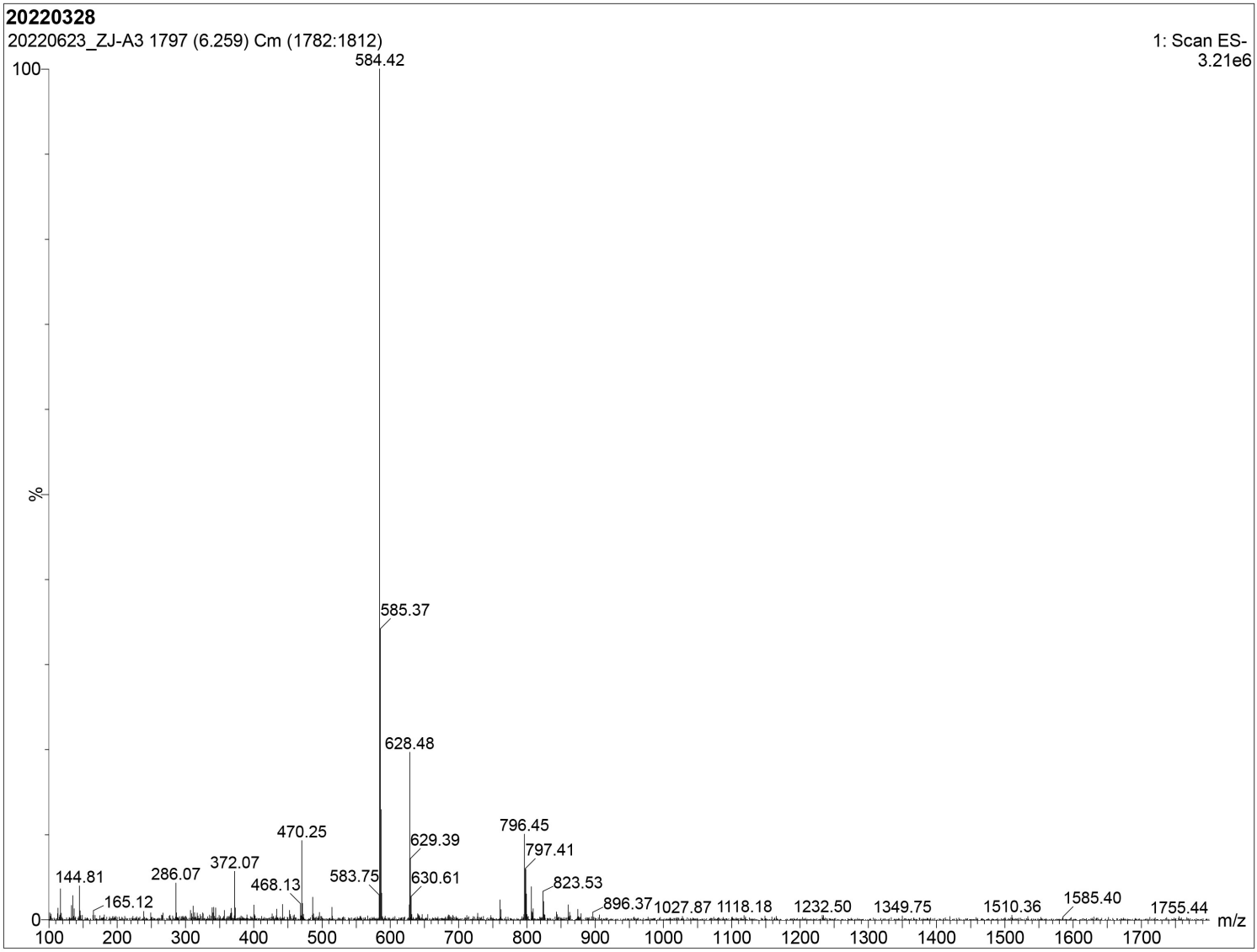
MS data from peak at 3.93 min from crosslinker **8c** incubated in CH_3CN for 12 h



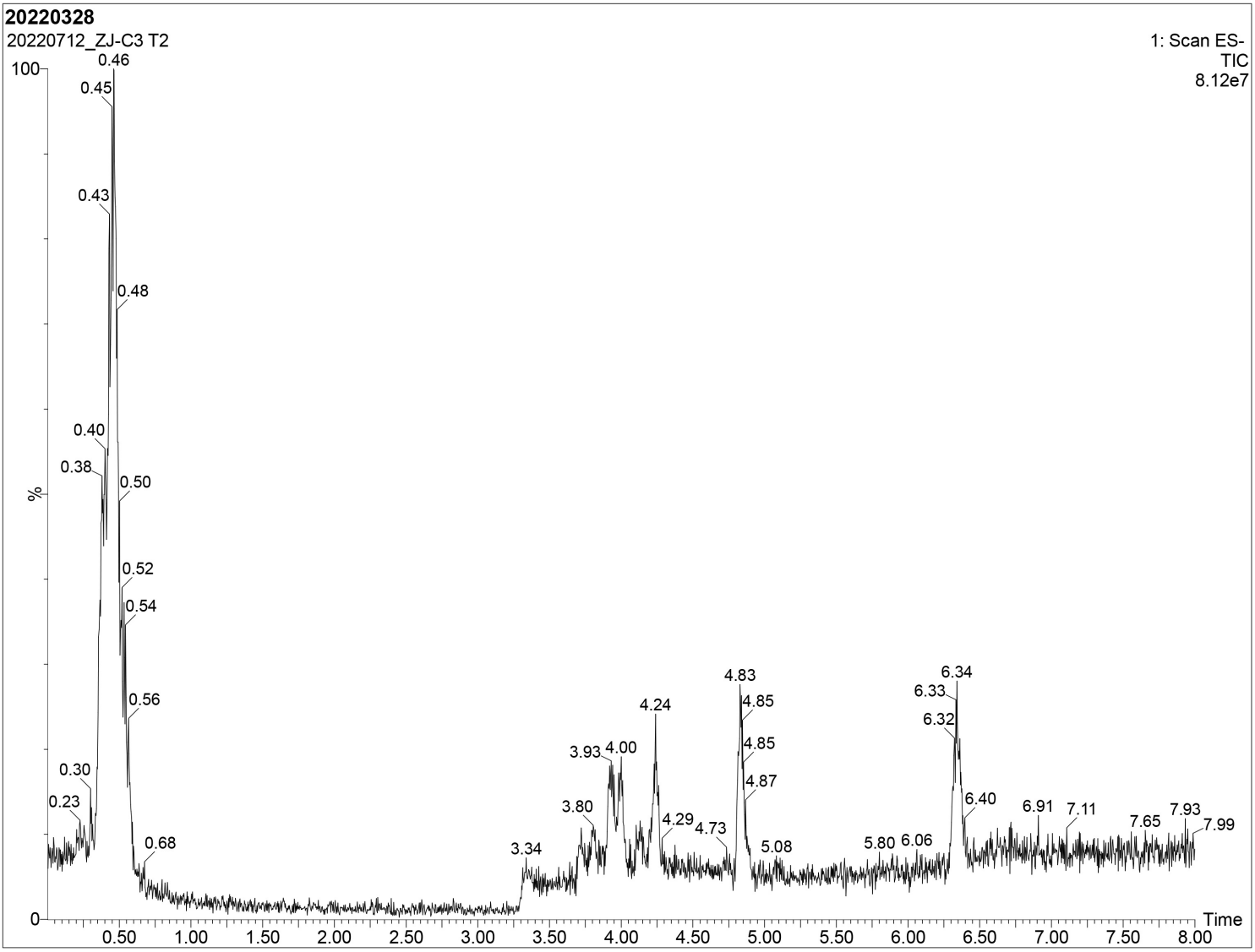
MS data from peak at 4.78 min from crosslinker **8c** incubated in CH_3CN for 12 h



MS data from peak at 6.26 min from crosslinker **8c** incubated in CH_3CN for 12 h

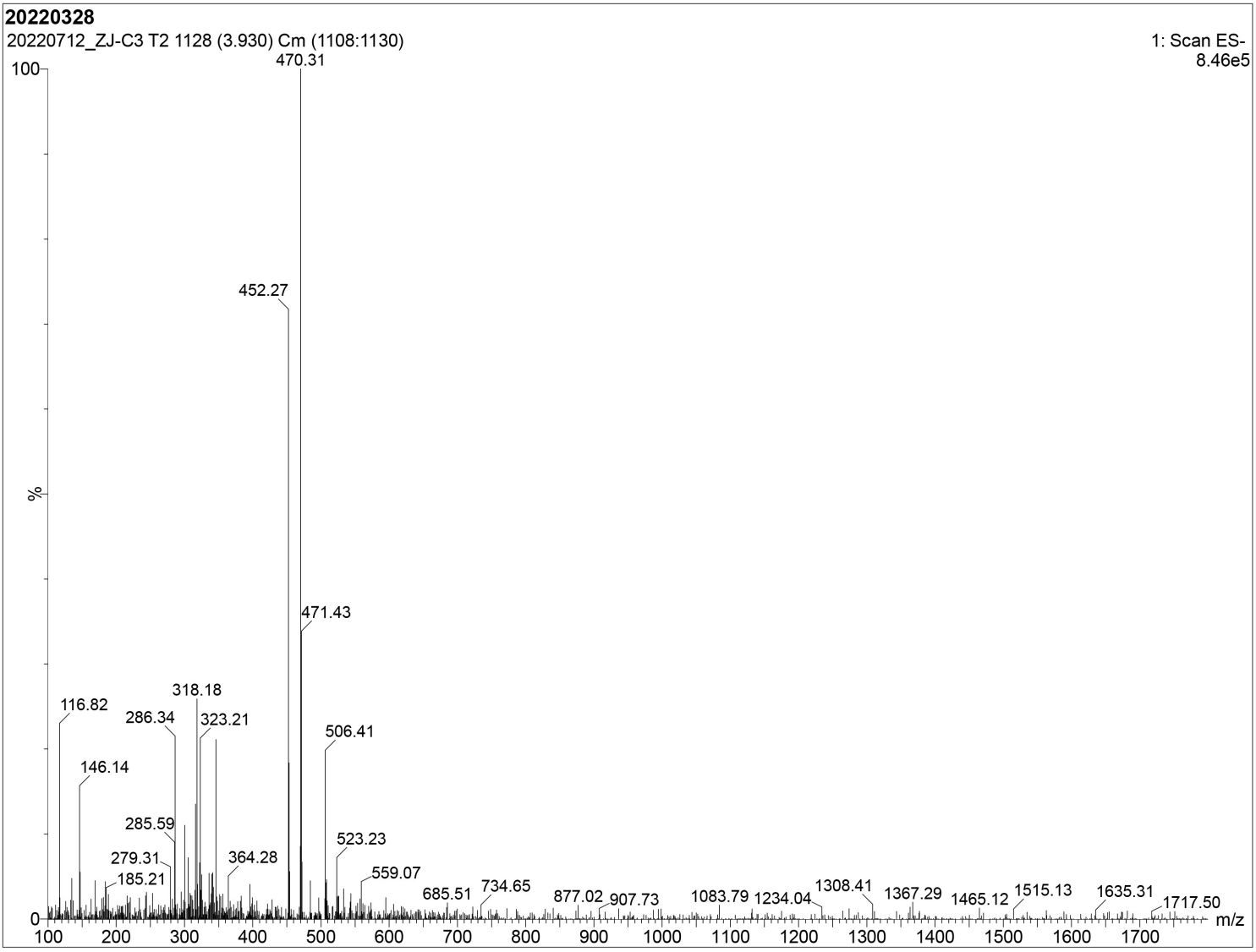


LC-MS spectral data from crosslinker **8c** incubated in buffer for 12 h

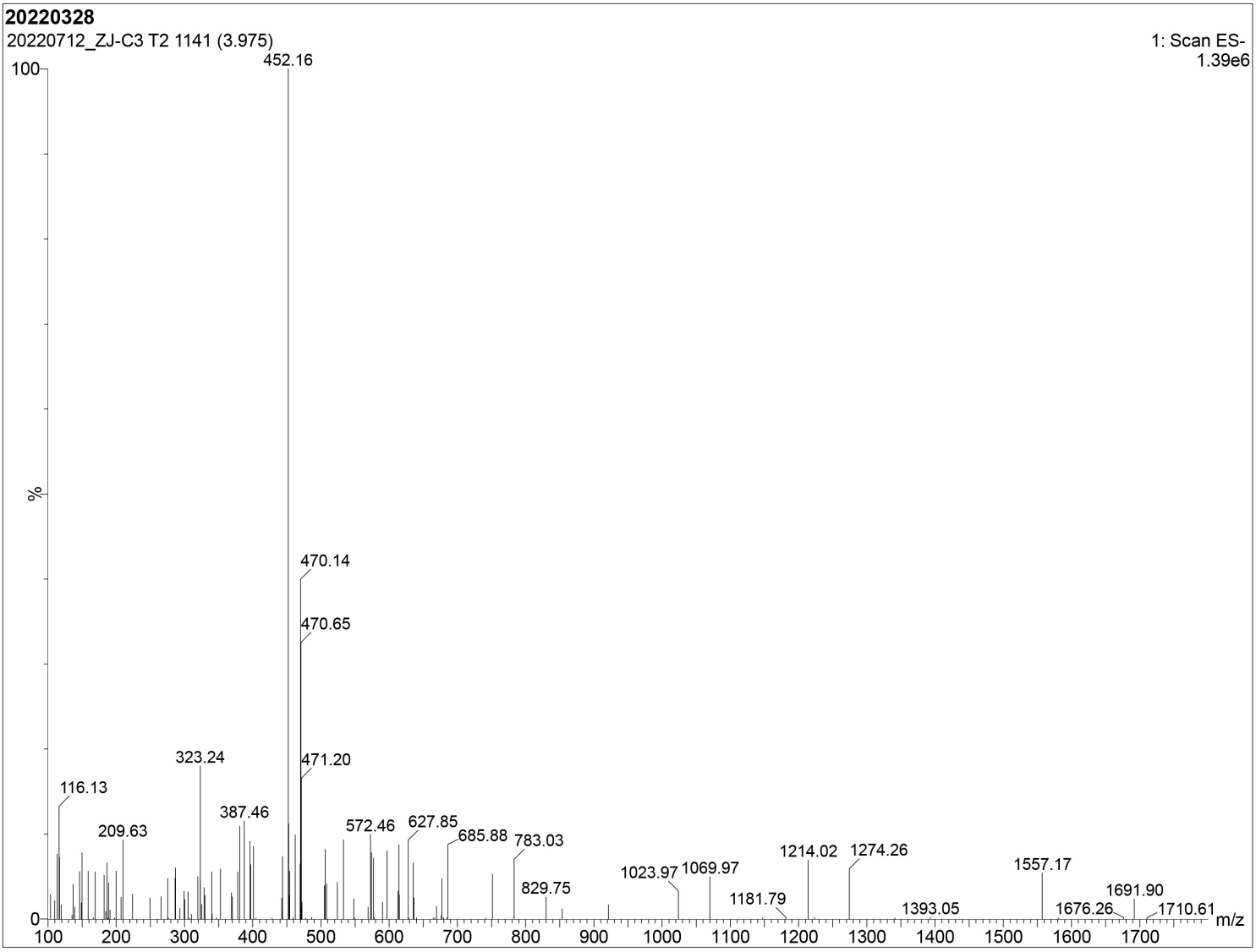


Buffer: 50 mM TRIS,150 mM NaCl,5% glycerol,pH 7.4

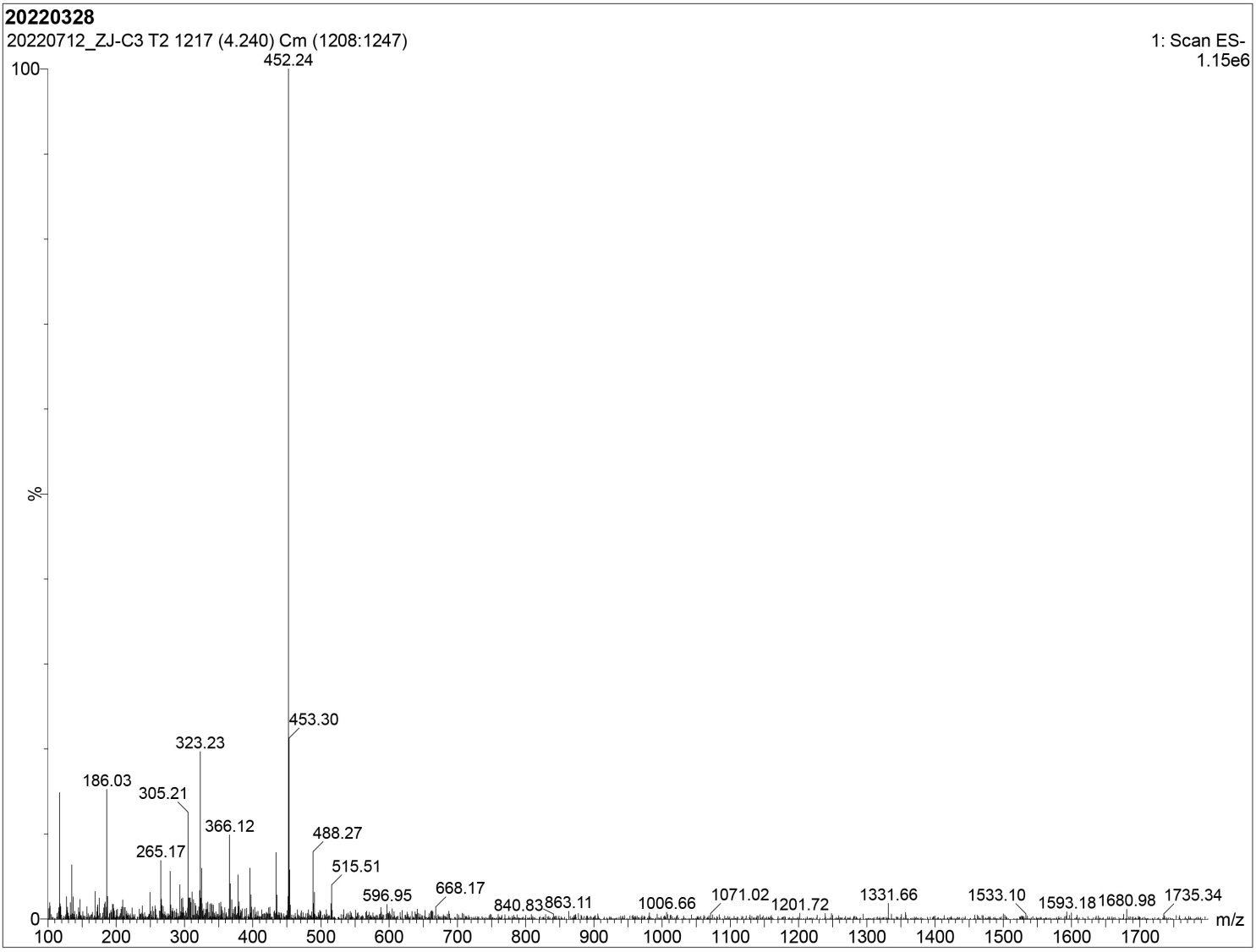
MS data from peak at 3.93 min from crosslinker **8c** incubated in buffer for 12 h



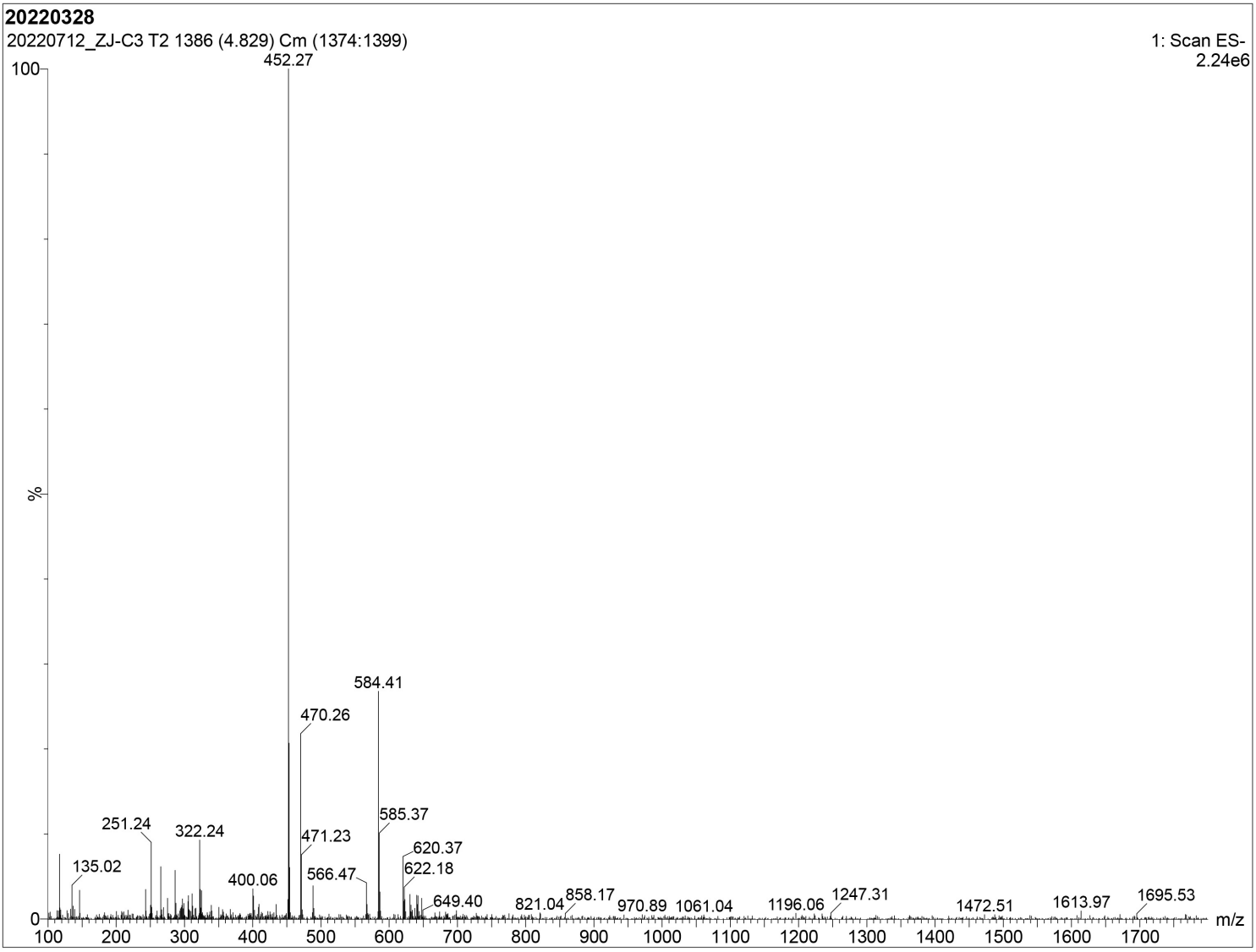
MS data from peak at 3.98 min from crosslinker **8c** incubated in buffer for 12 h



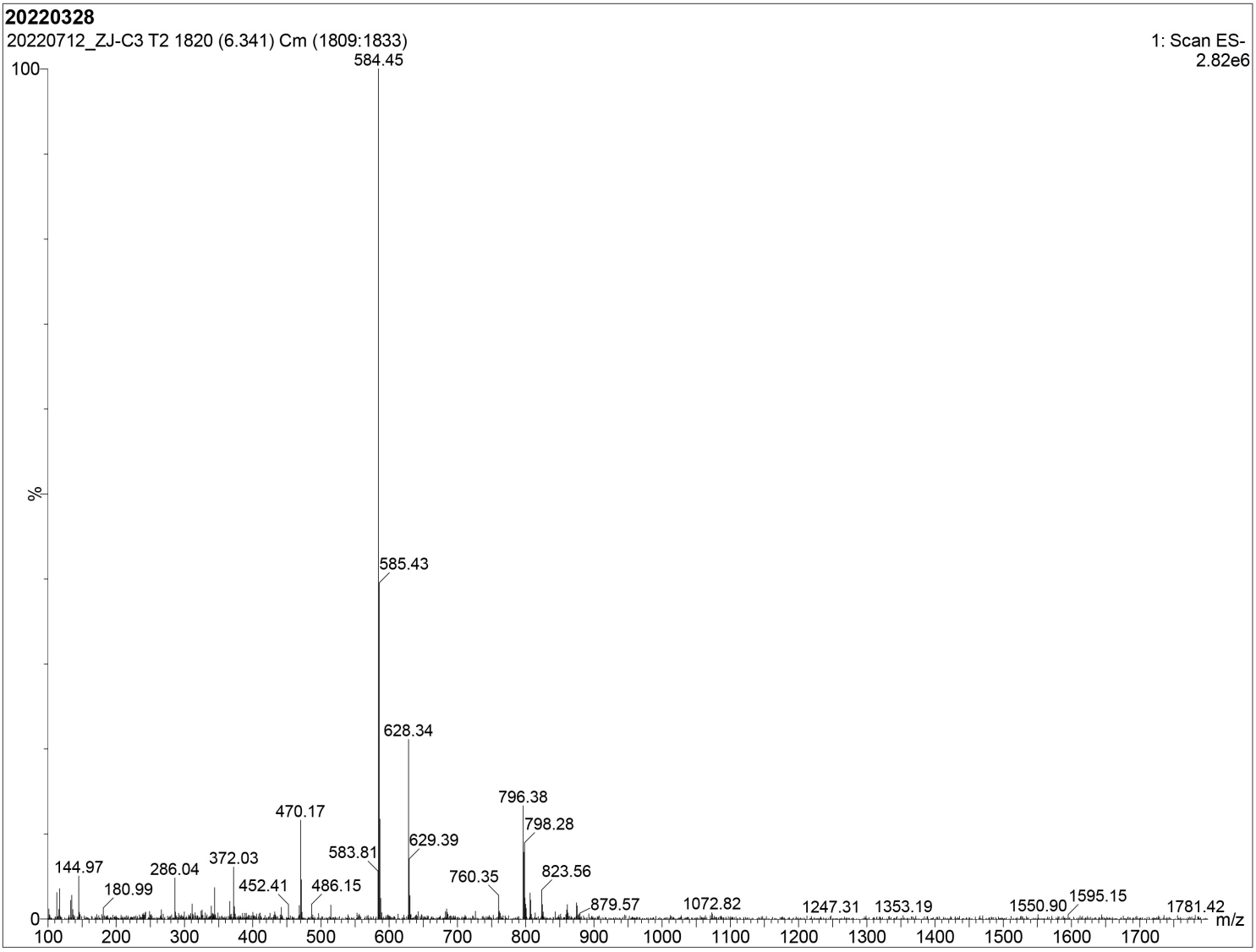
MS data from peak at 4.24 min from crosslinker **8c** incubated in buffer for 12 h



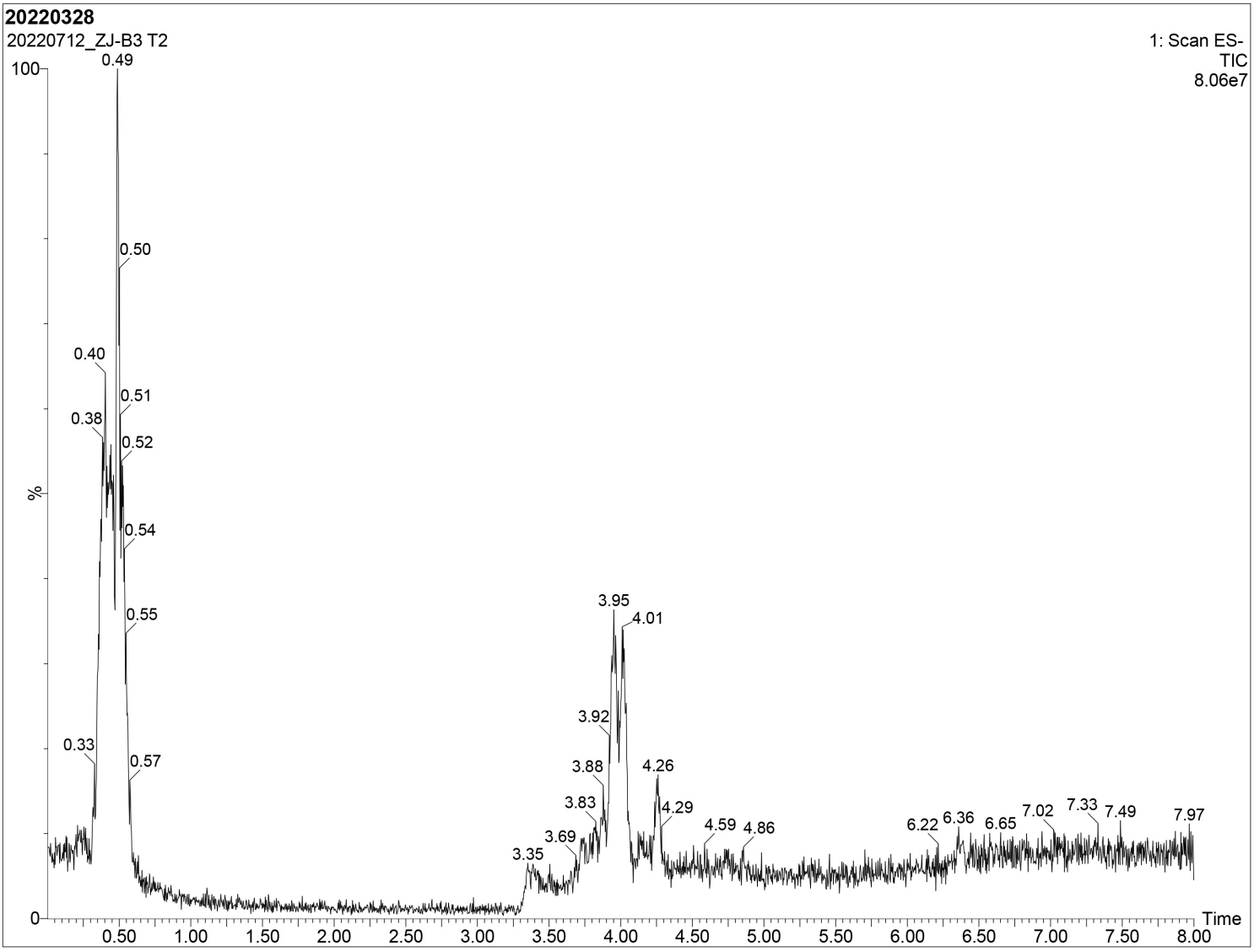
MS data from peak at 4.83 min from crosslinker **8c** incubated in buffer for 12 h



MS data from peak at 6.34 min from crosslinker **8c** incubated in buffer for 12 h

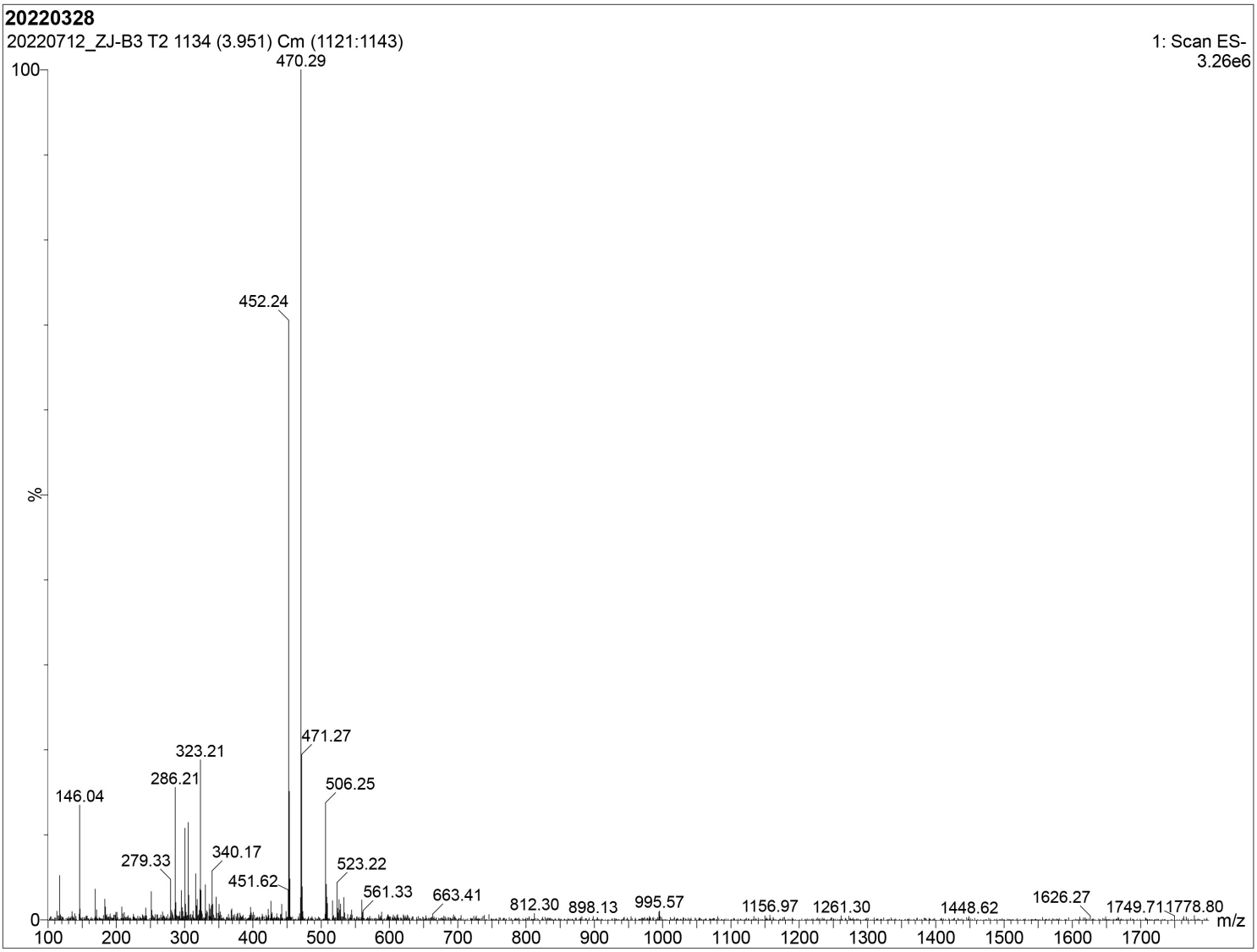


LC-MS spectral data from crosslinker **8c** incubated in fluoride buffer for 12 h

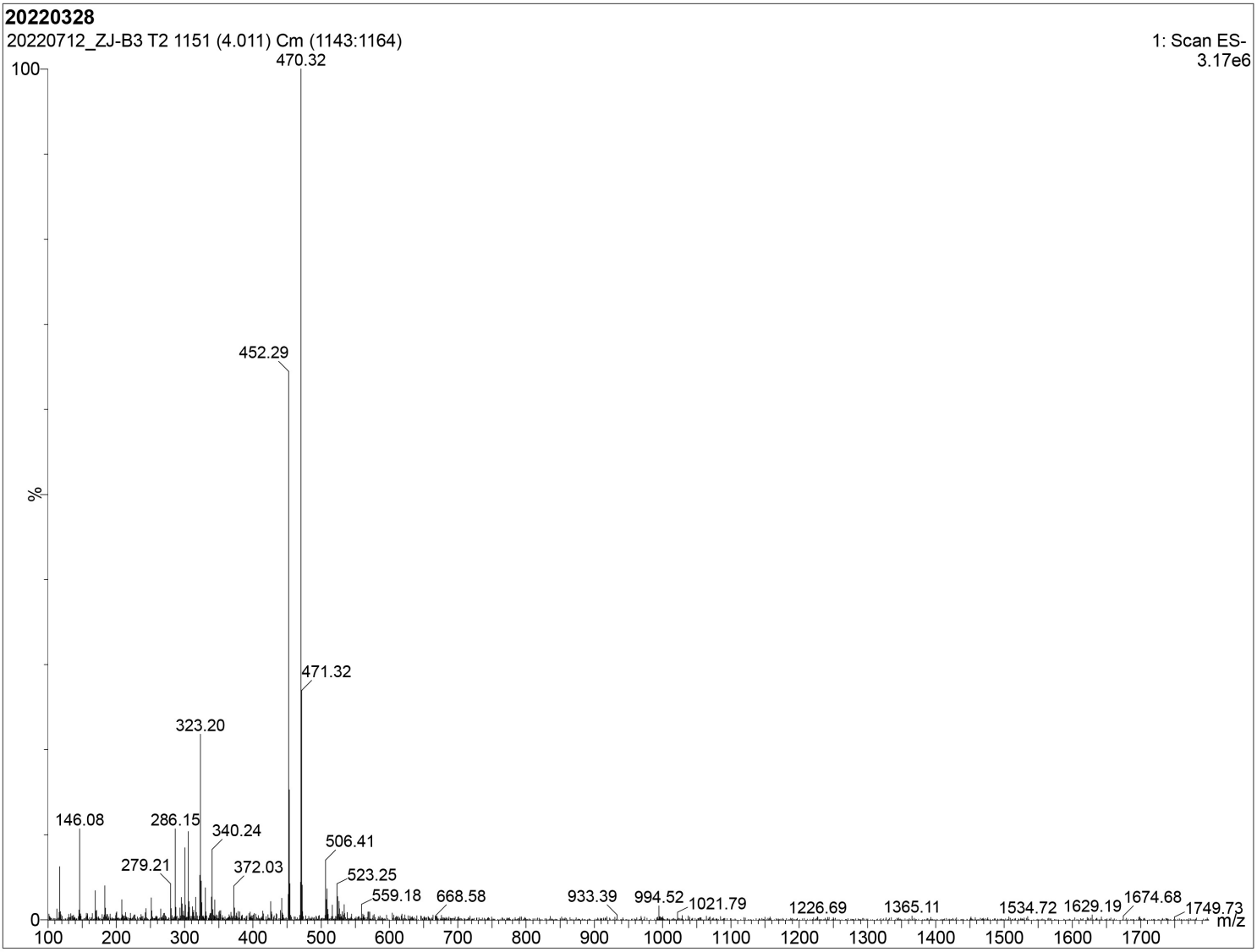


Fluoride buffer: 50 mM TRIS,150 mM NaCl,5% glycerol,pH 7.4 with 50 mM KF

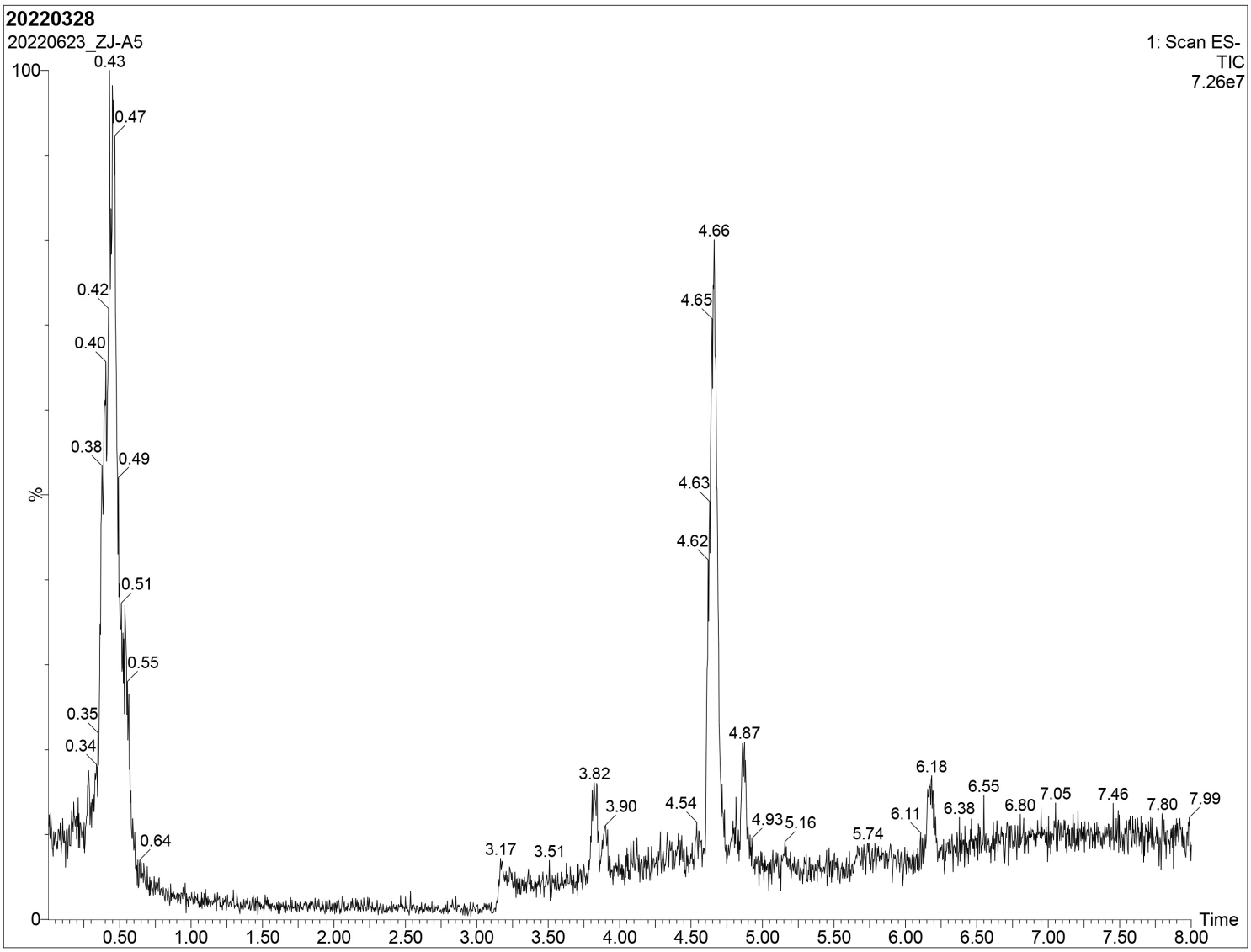
MS data from peak at 3.95 min from crosslinker **8c** incubated in fluoride buffer for 12 h



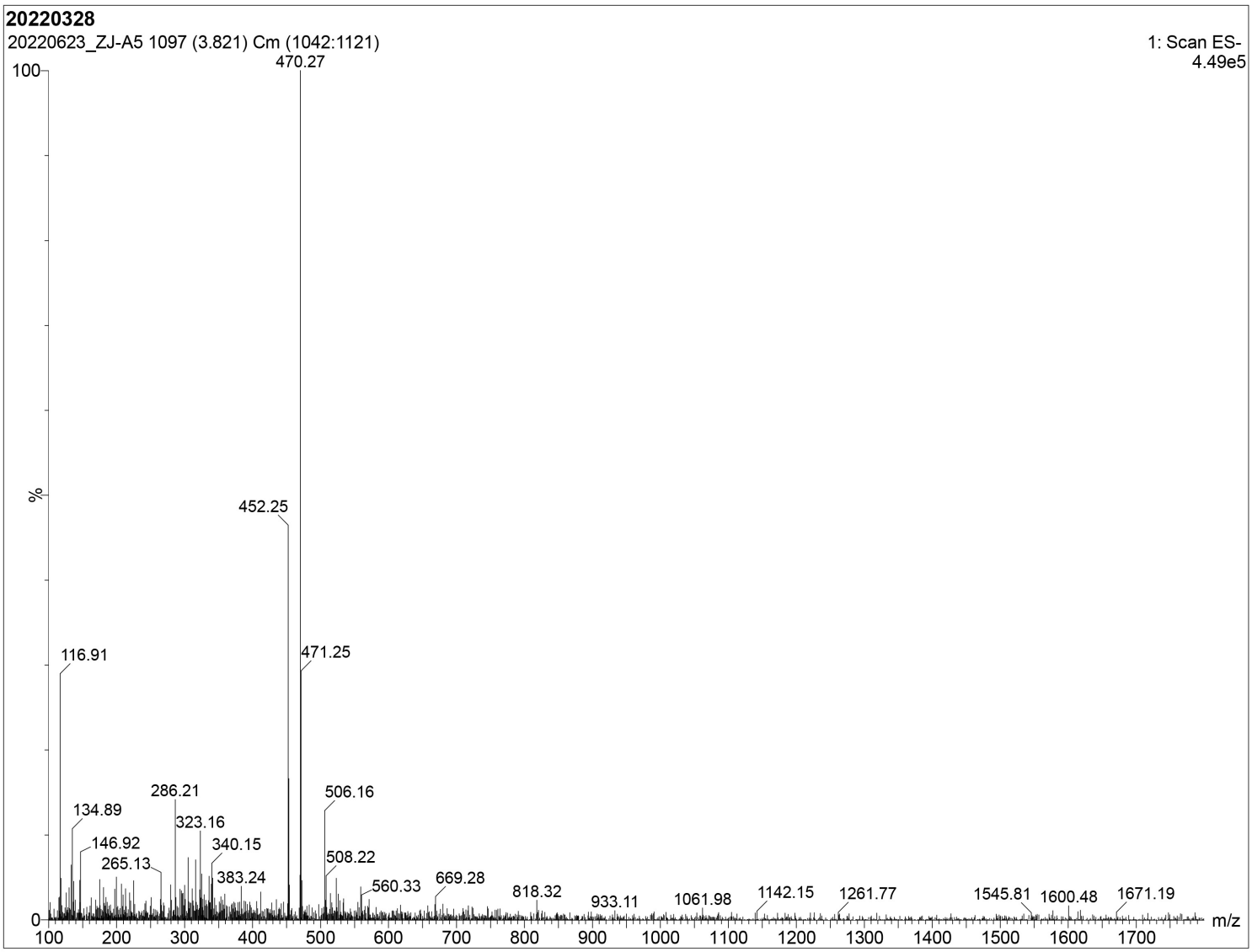
MS data from peak at 4.01 min from crosslinker **8c** incubated in fluoride buffer for 12 h



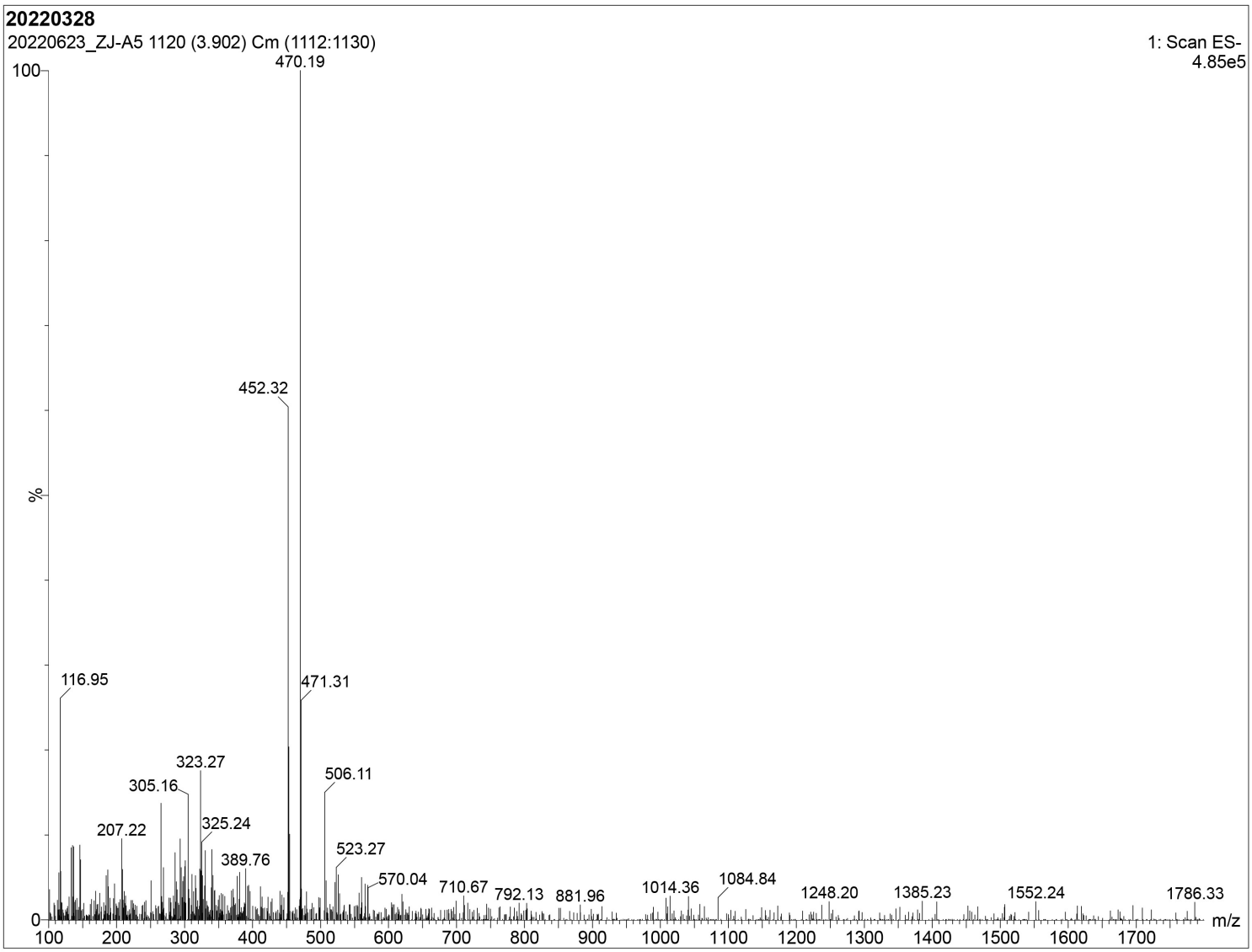
LC-MS spectral data from crosslinker **9c** incubated in CH_3CN for 12 h



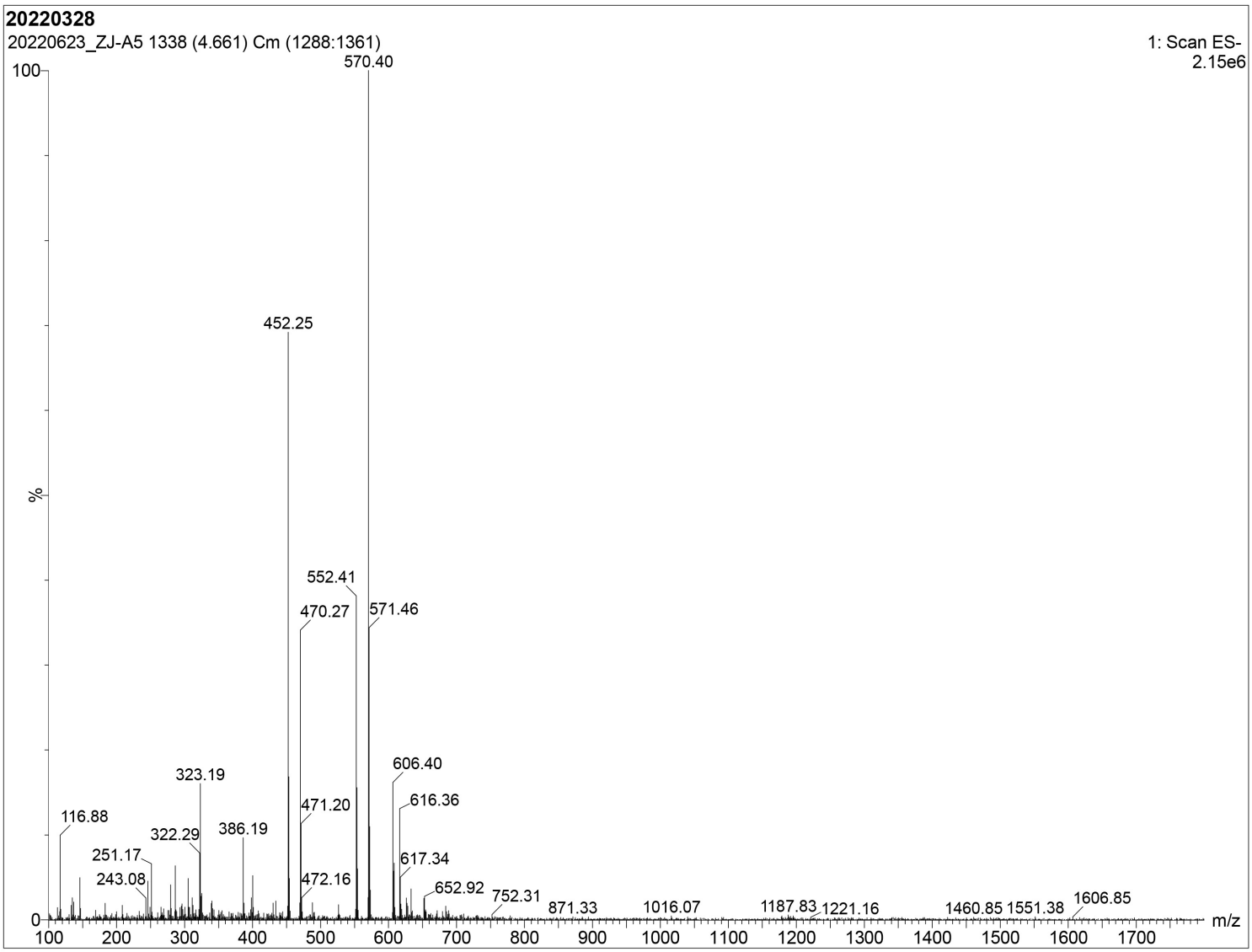
MS data from peak at 3.82 min from crosslinker **9c** incubated in CH_3CN for 12 h



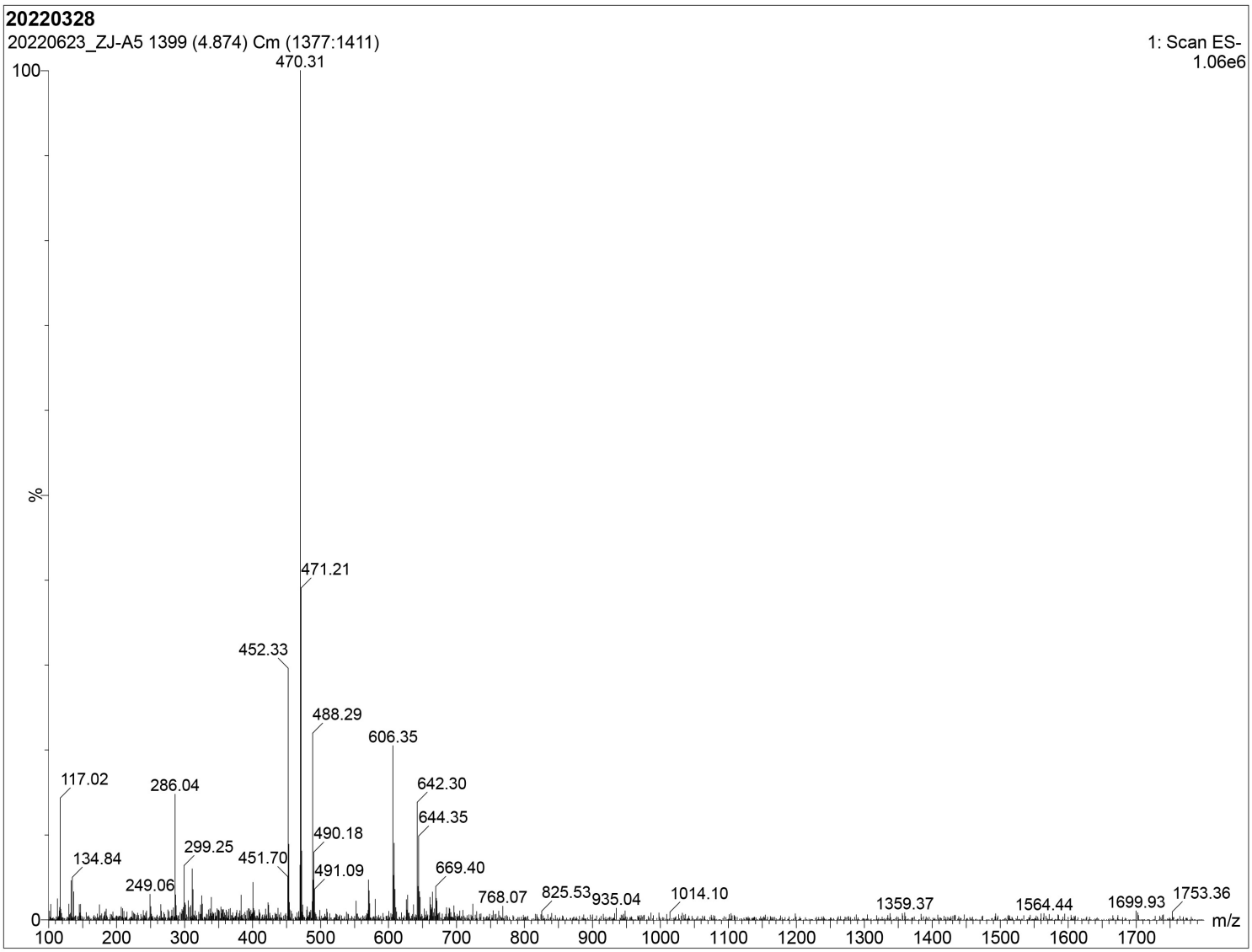
MS data from peak at 3.90 min from crosslinker **9c** incubated in CH_3CN for 12 h



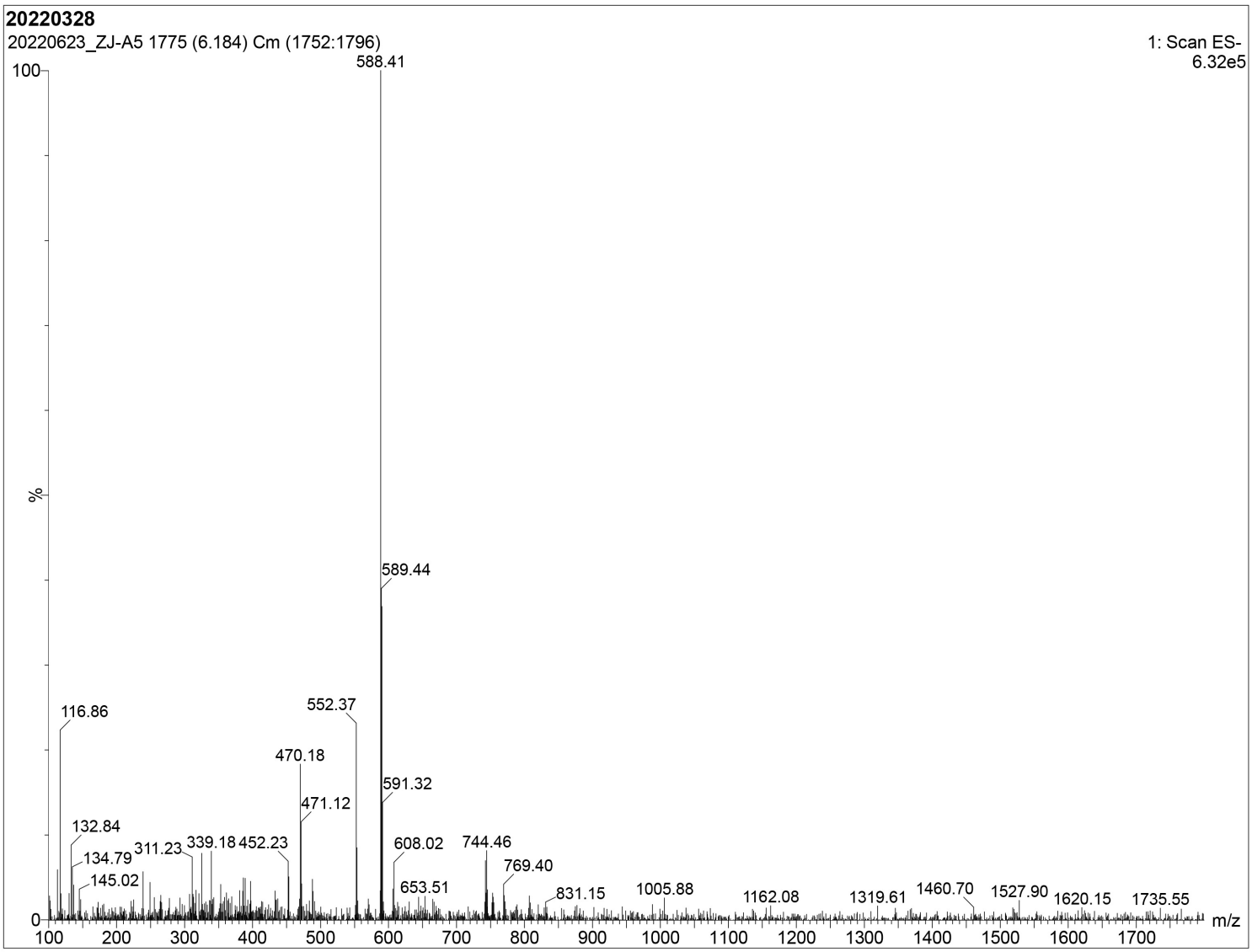
MS data from peak at 4.66 min from crosslinker **9c** incubated in CH_3CN for 12 h



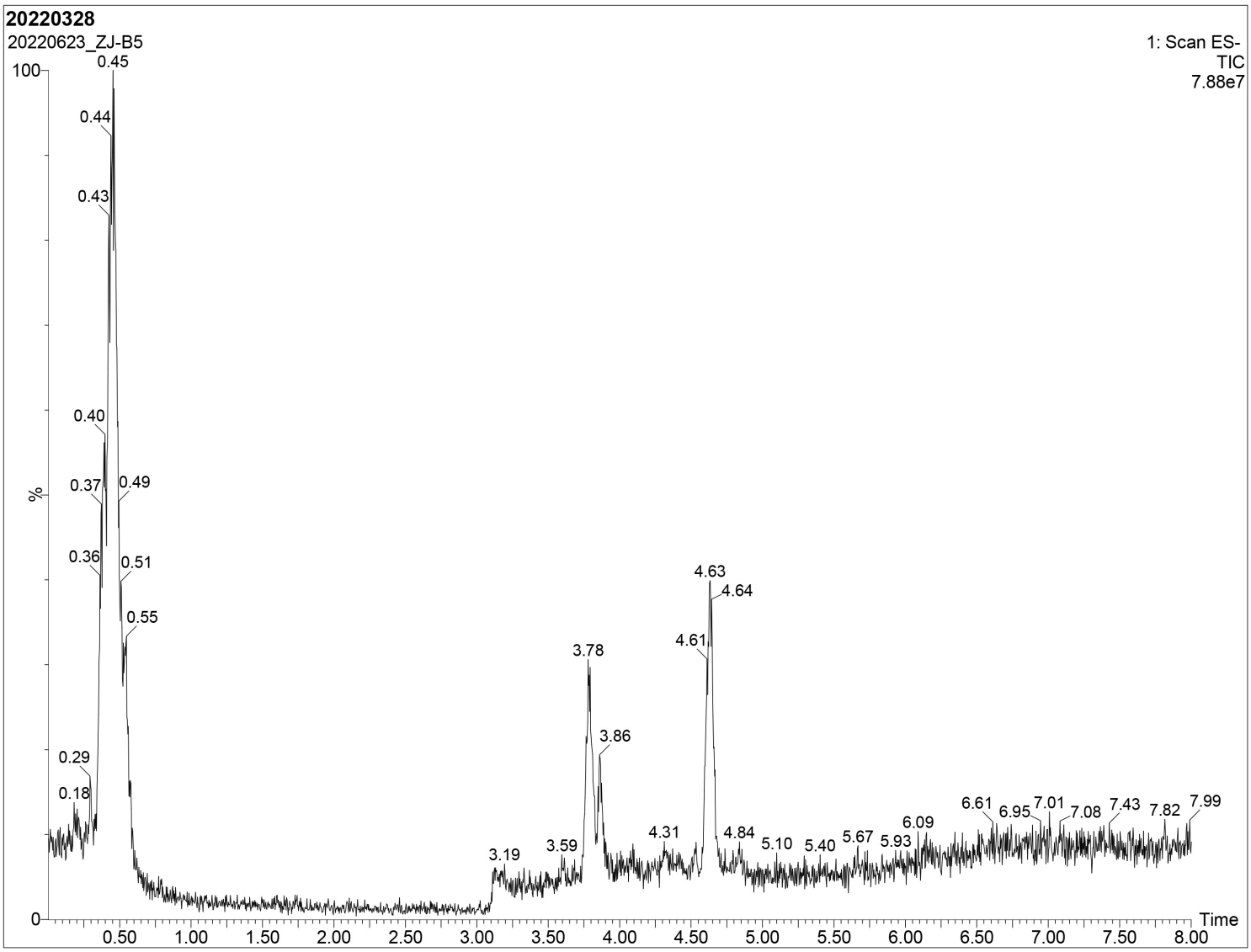
MS data from peak at 4.87 min from crosslinker **9c** incubated in CH_3CN for 12 h



MS data from peak at 6.18 min from crosslinker **9c** incubated in CH_3CN for 12 h

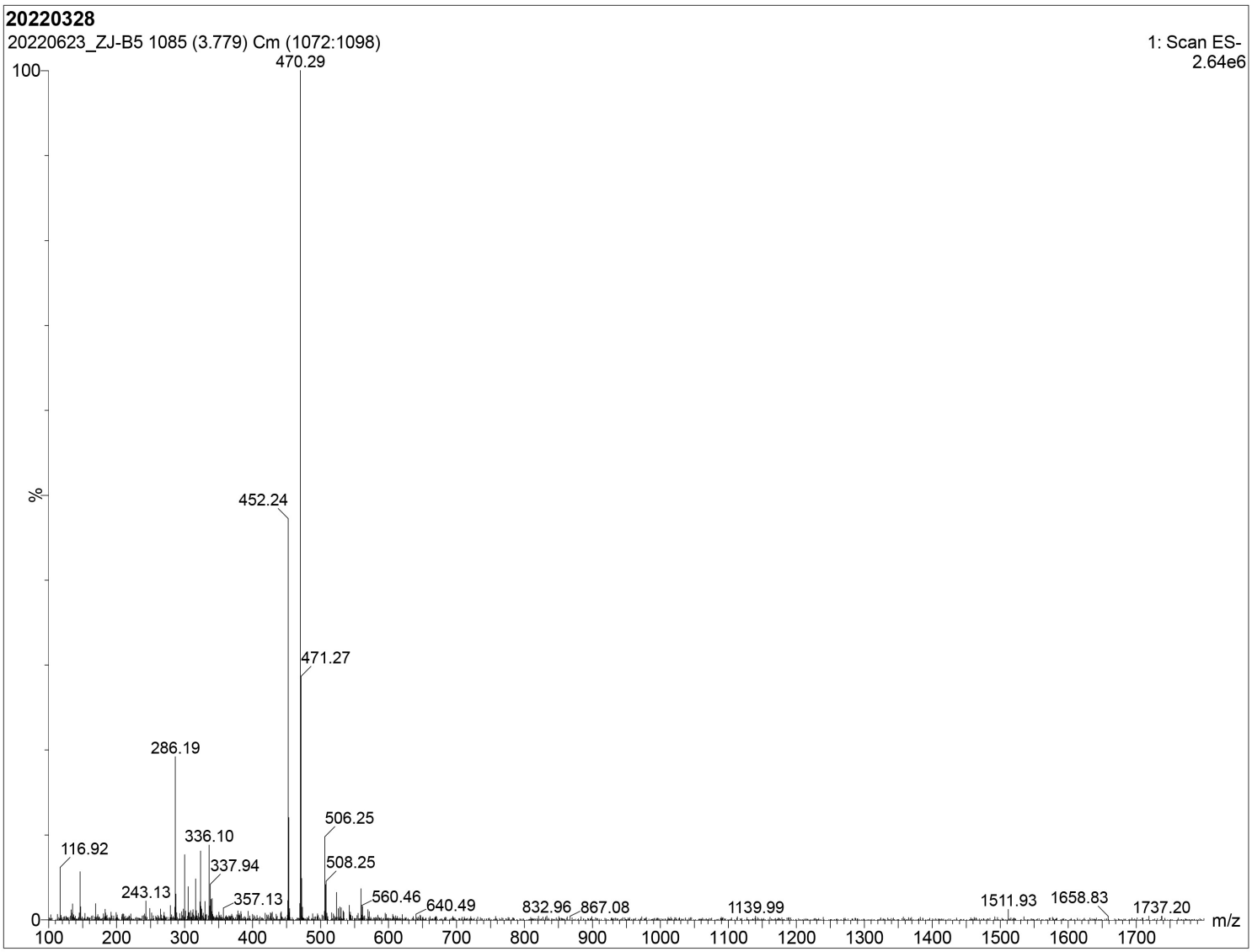


LC-MS spectral data from crosslinker **9c** incubated in buffer for 12 h

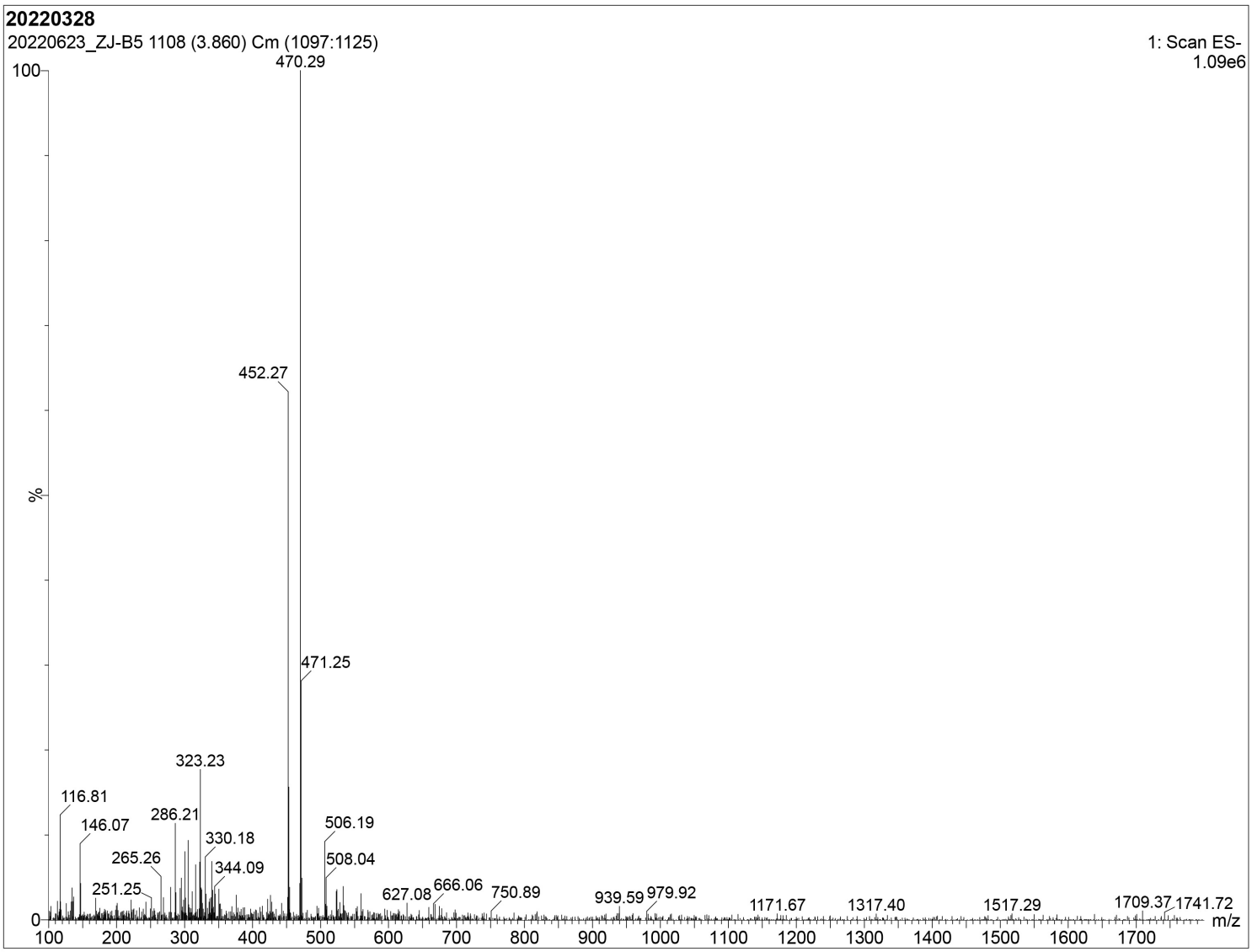


Buffer: 50 mM TRIS,150 mM NaCl,5% glycerol,pH 7.4

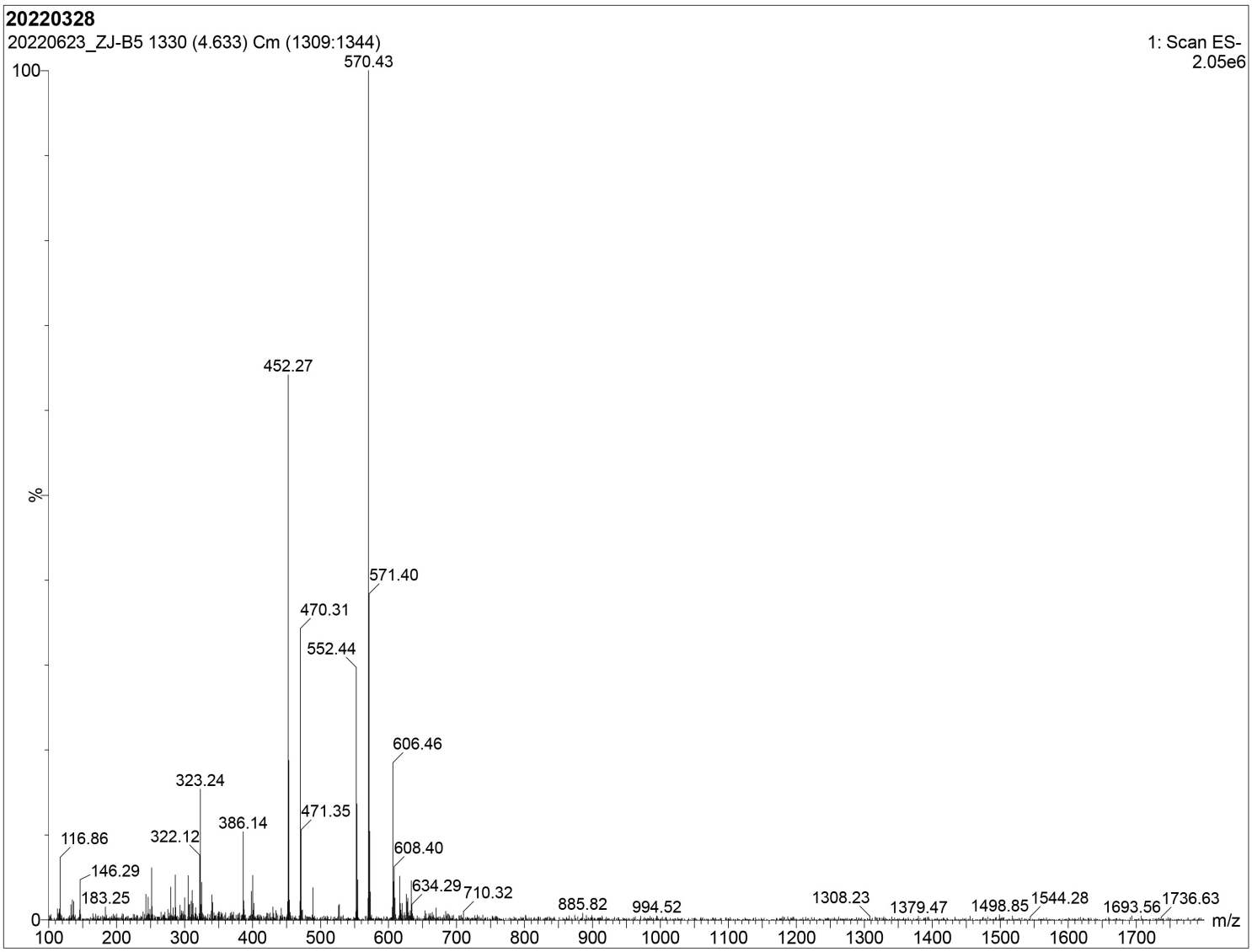
MS data from peak at 3.78 min from crosslinker **9c** incubated in buffer for 12 h



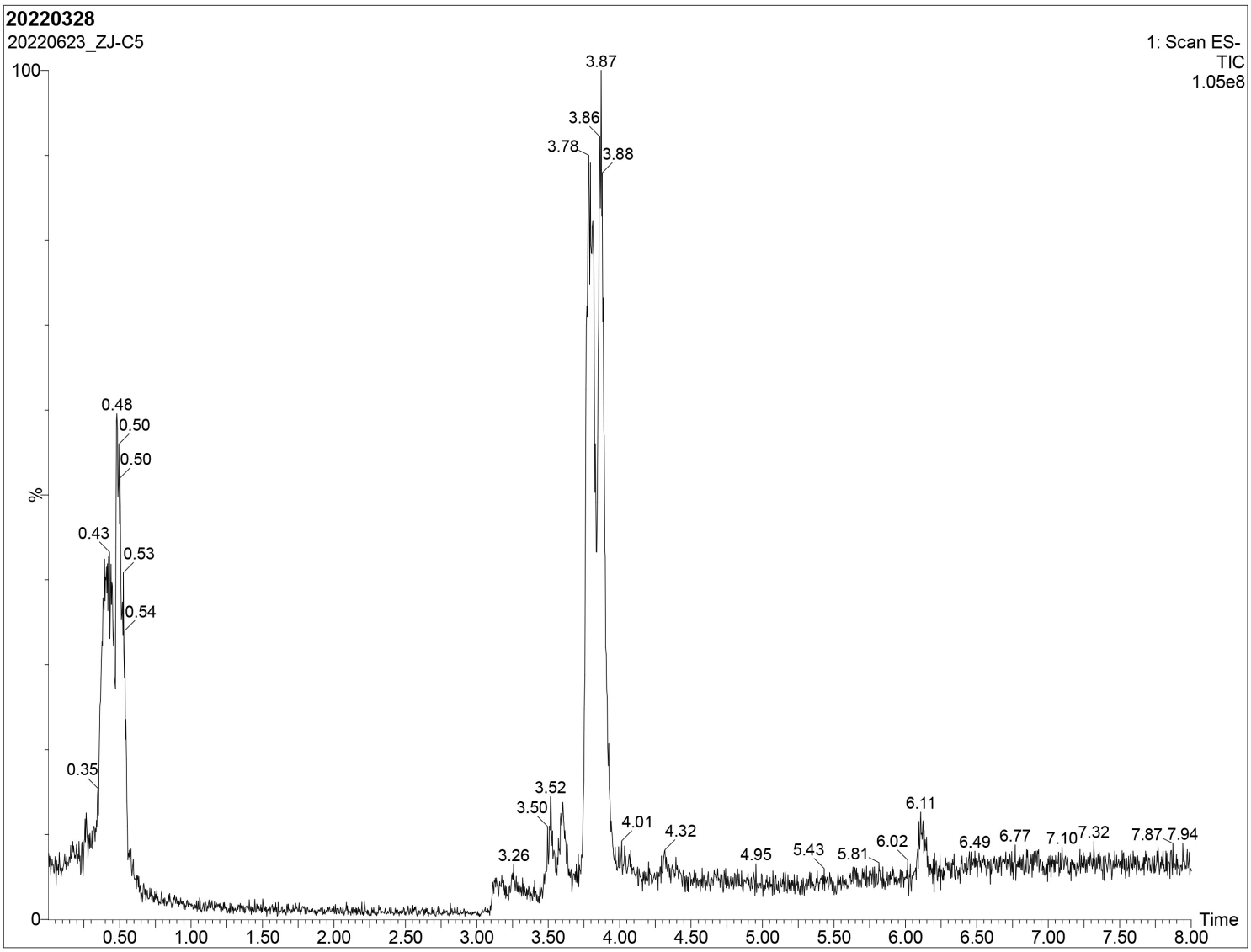
MS data from peak at 3.86 min from crosslinker **9c** incubated in buffer for 12 h



MS data from peak at 4.63 min from crosslinker **9c** incubated in buffer for 12 h

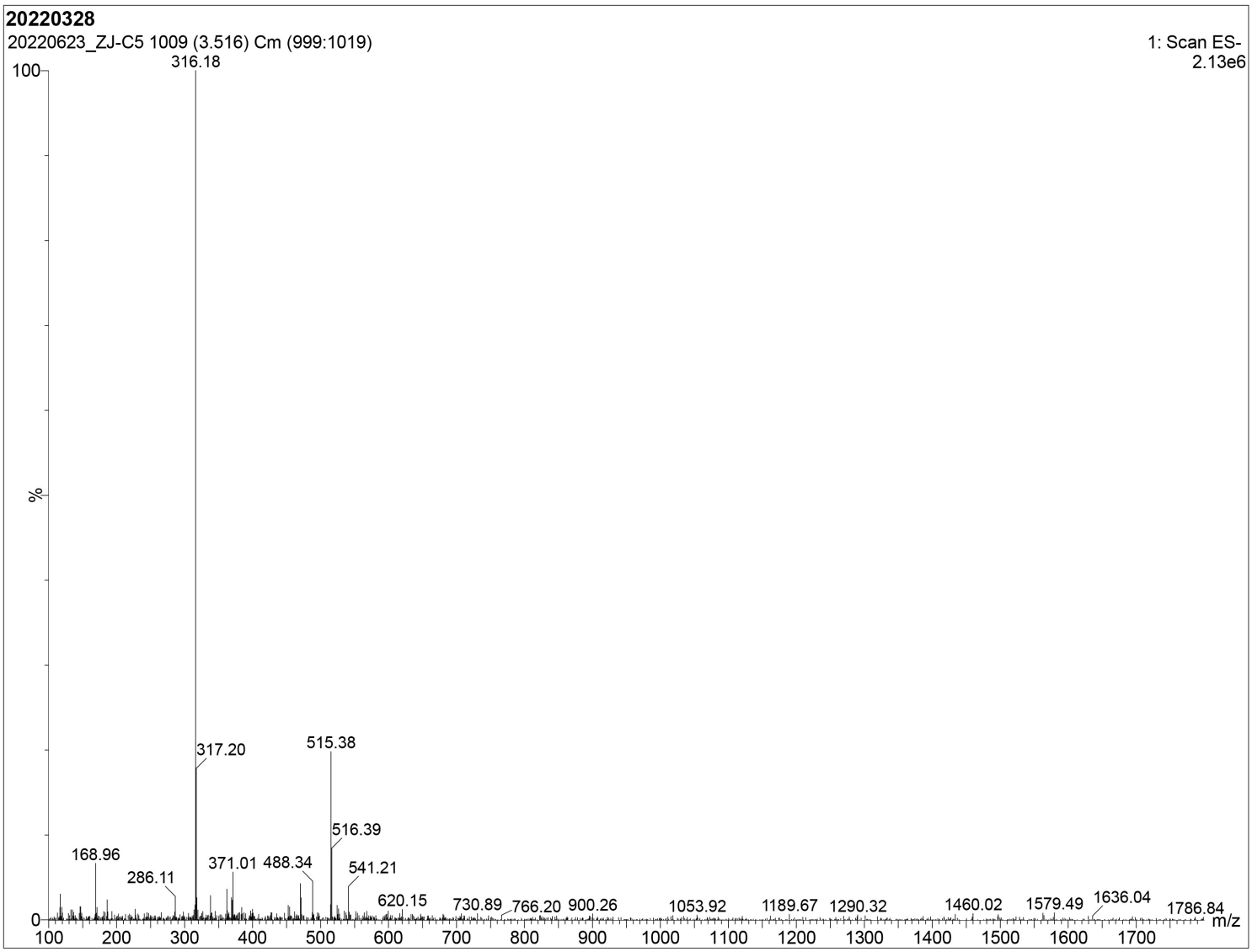


LC-MS spectral data from crosslinker **9c** incubated in fluoride buffer for 12 h

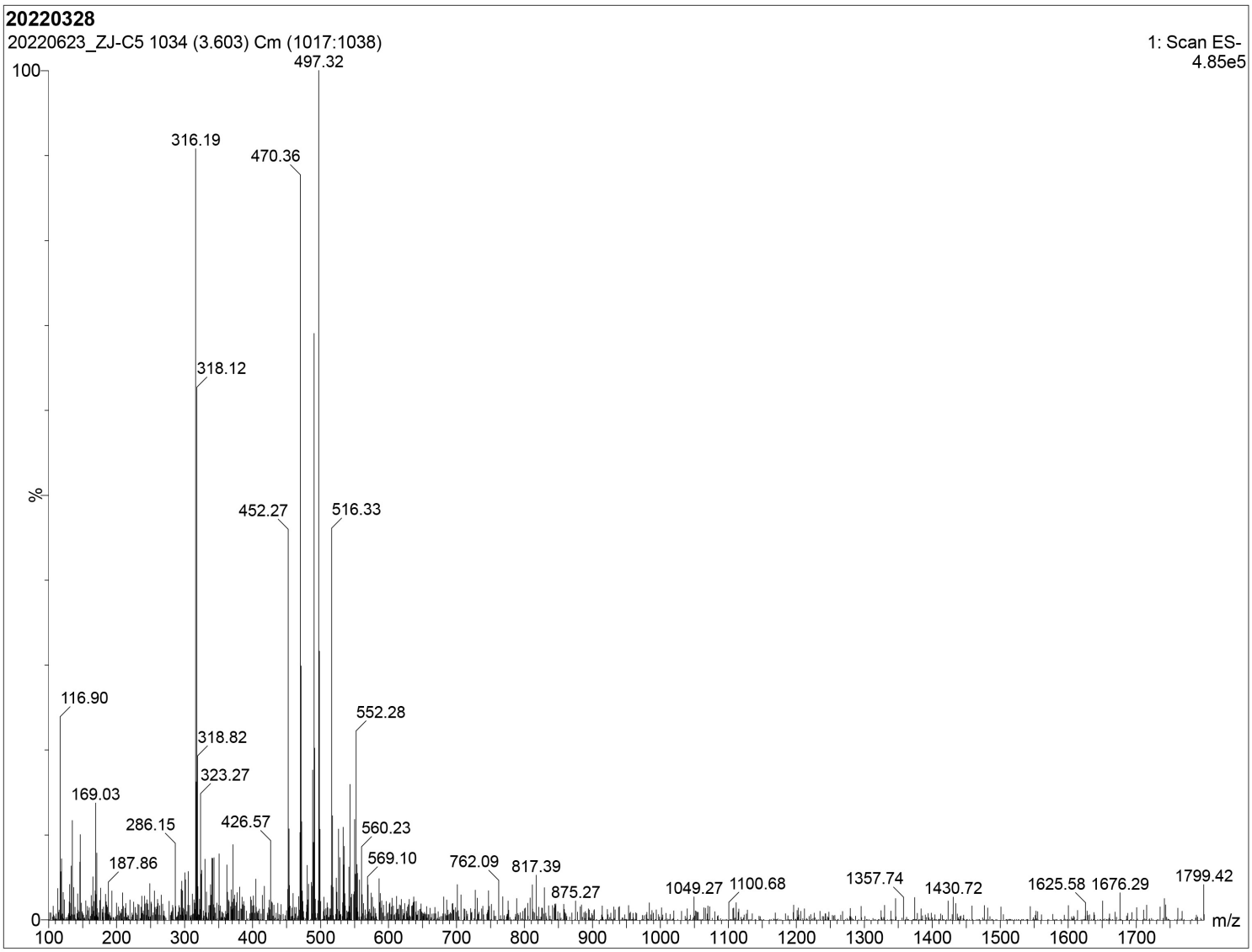


Fluoride buffer: 50 mM TRIS,150 mM NaCl,5% glycerol,pH 7.4 with 50 mM KF

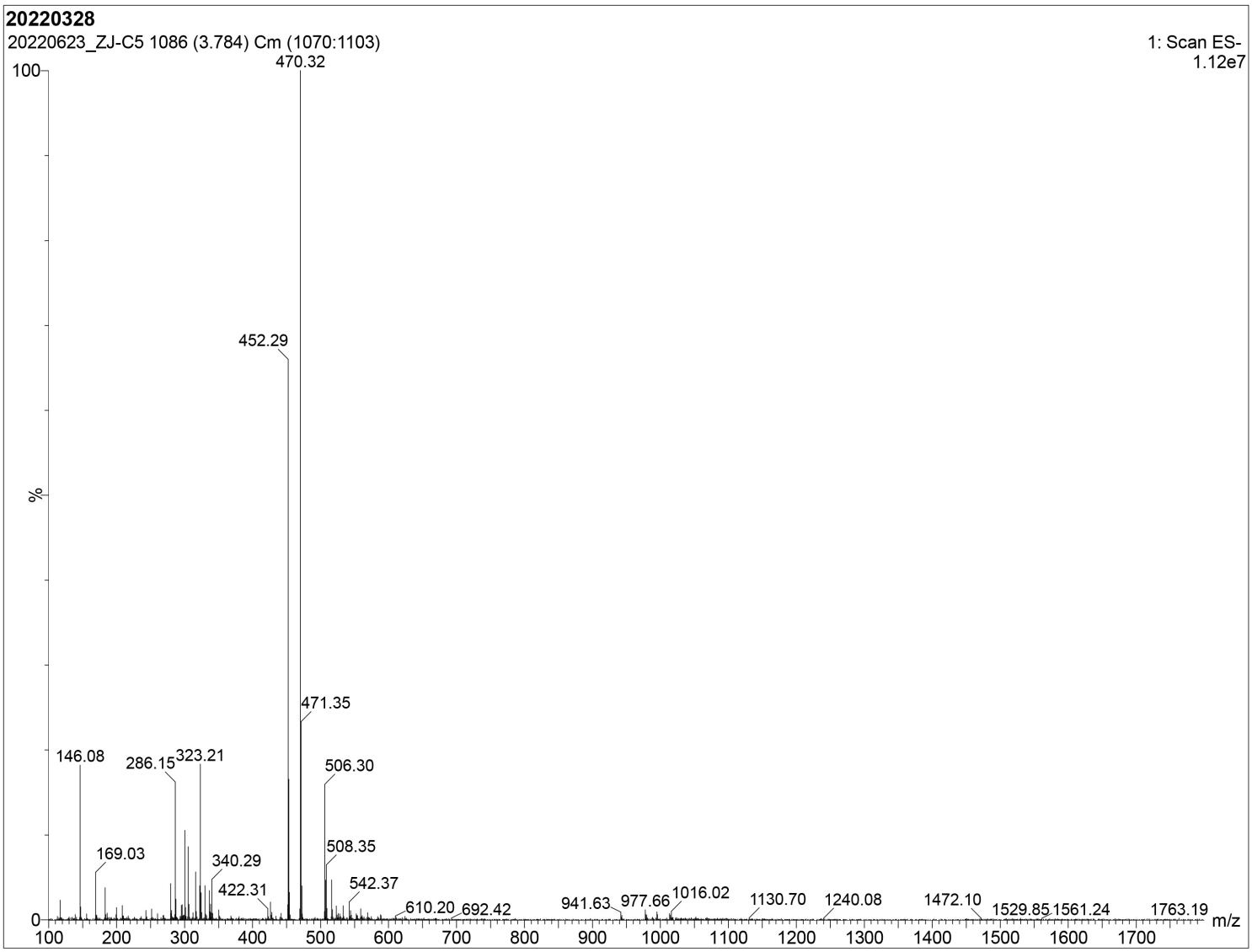
MS data from peak at 3.52 min from crosslinker **9c** incubated in fluoride buffer for 12 h



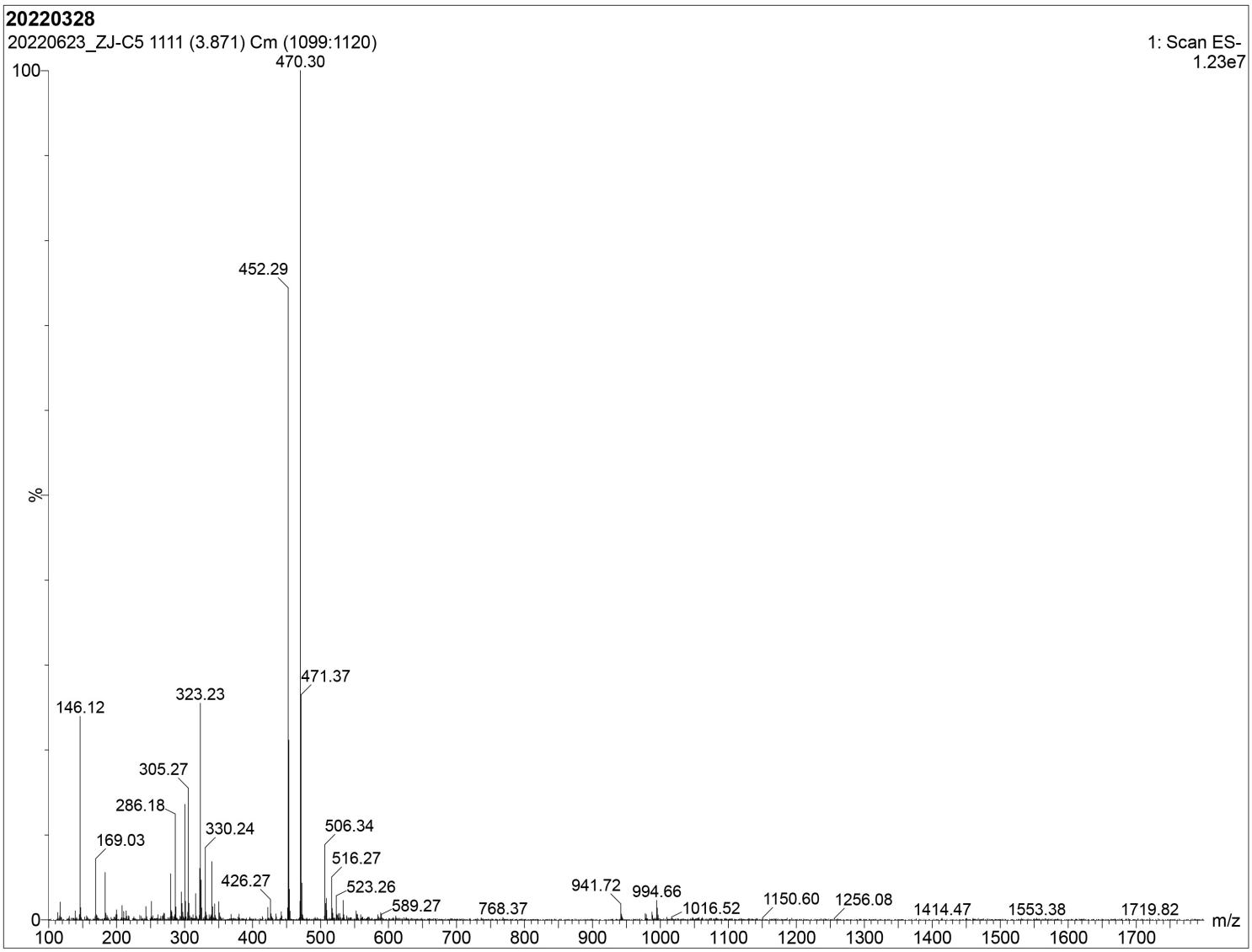
MS data from peak at 3.60 min from crosslinker **9c** incubated in fluoride buffer for 12 h



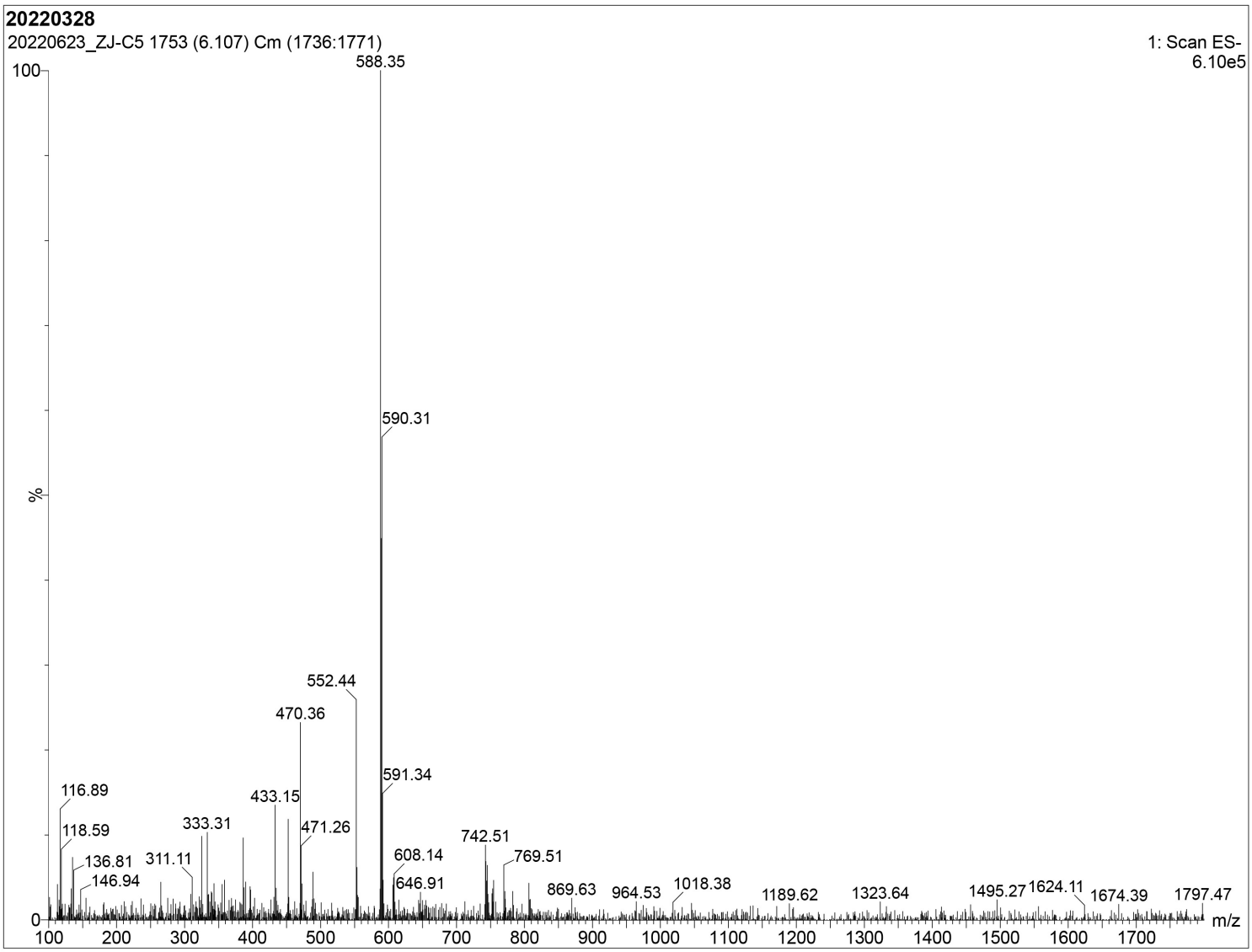
MS data from peak at 3.78 min from crosslinker **9c** incubated in fluoride buffer for 12 h



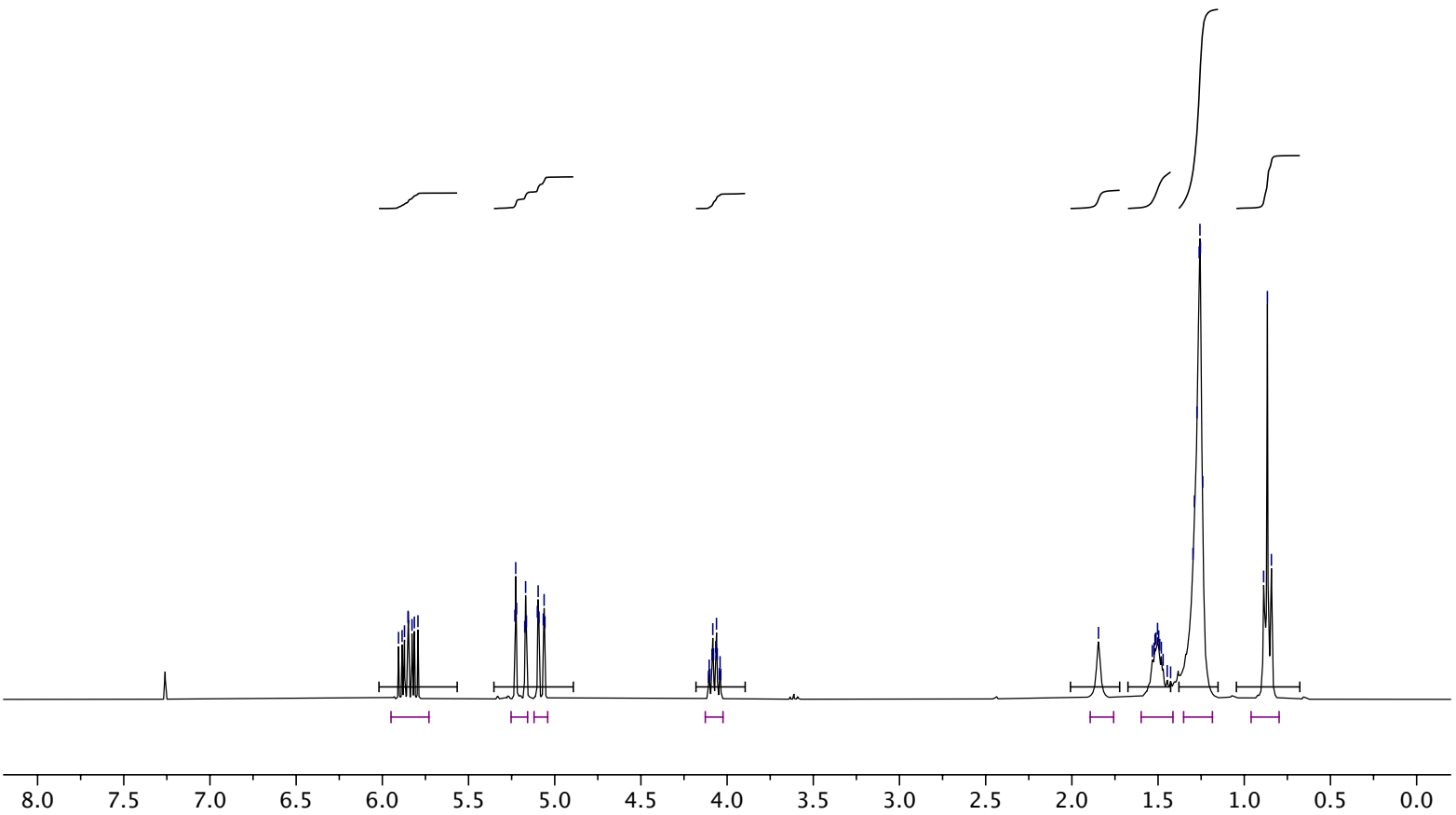
MS data from peak at 3.87 min from crosslinker **9c** incubated in fluoride buffer for 12 h

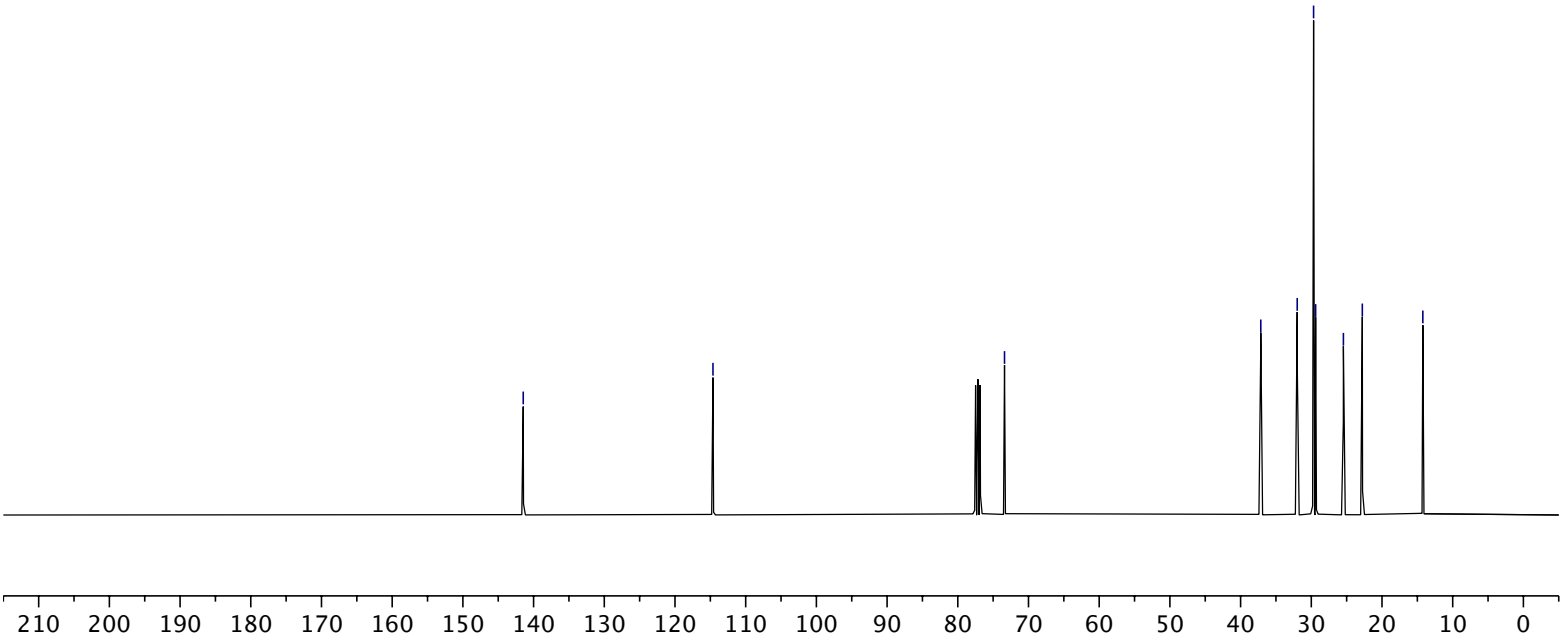


MS data from peak at 6.11 min from crosslinker **9c** incubated in fluoride buffer for 12 h

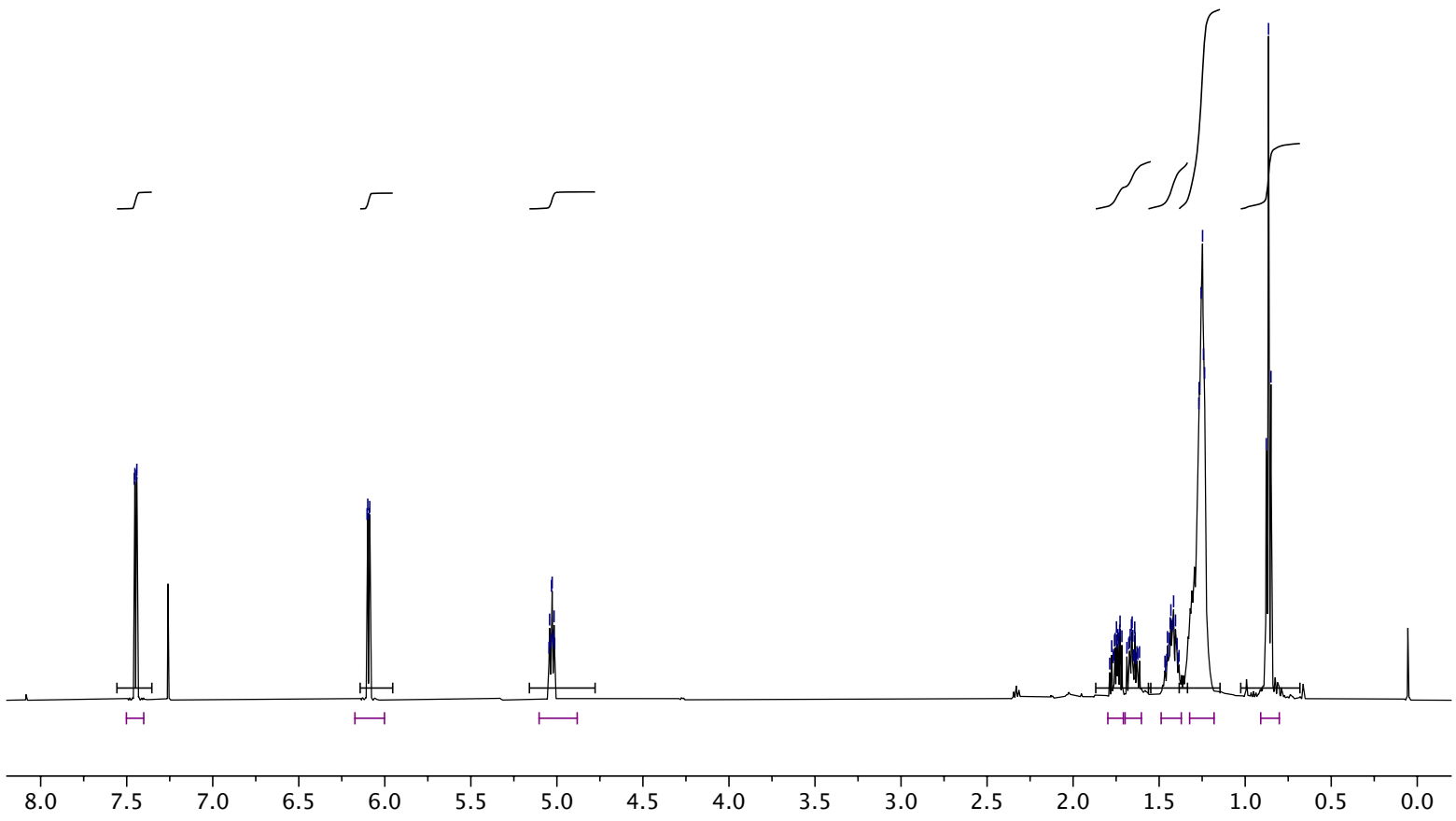


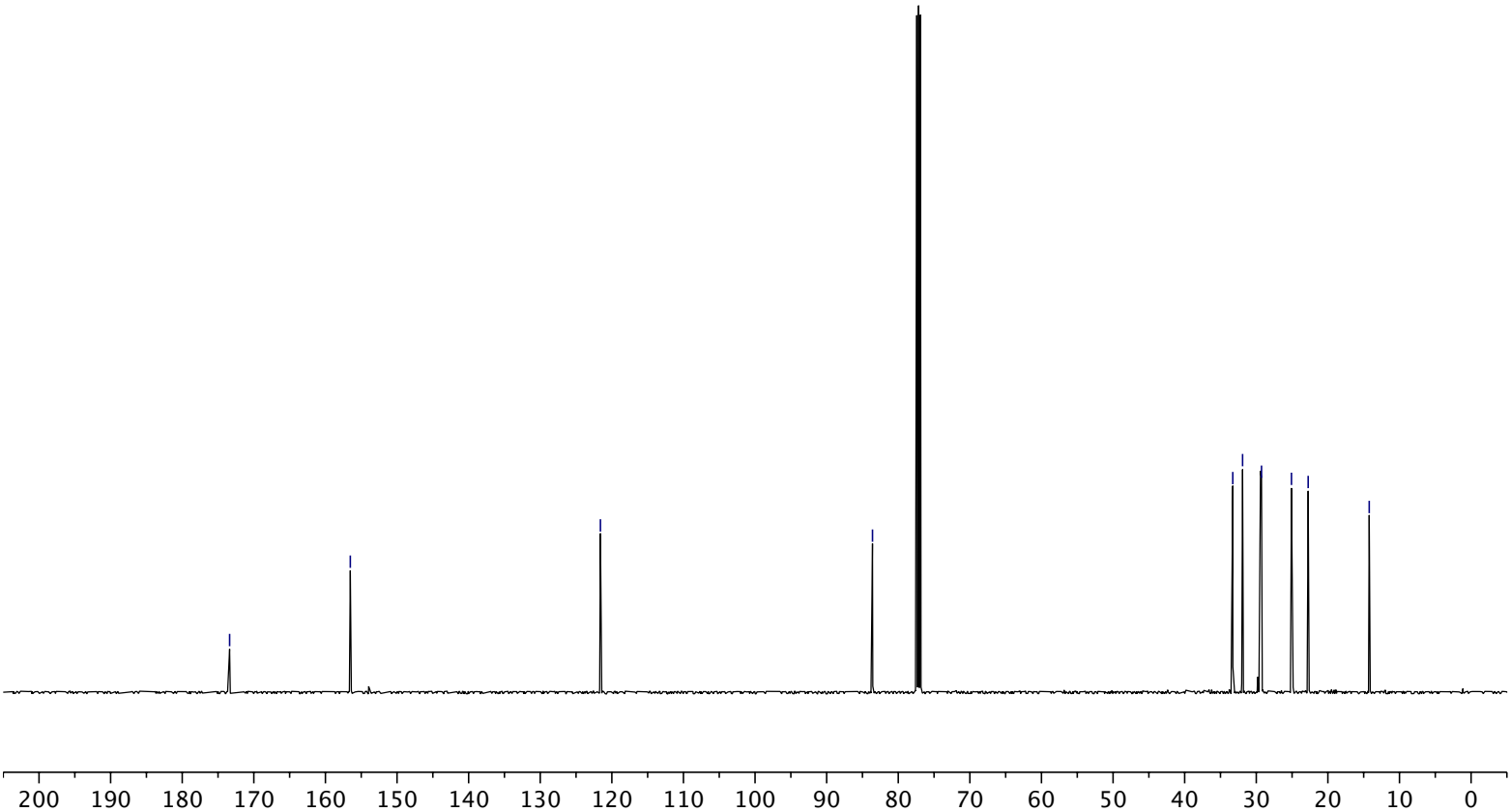
^{1}H–NMR (300 MHz) and ^{13}C–NMR (100 MHz) spectra of **11** in $CDCl_3$



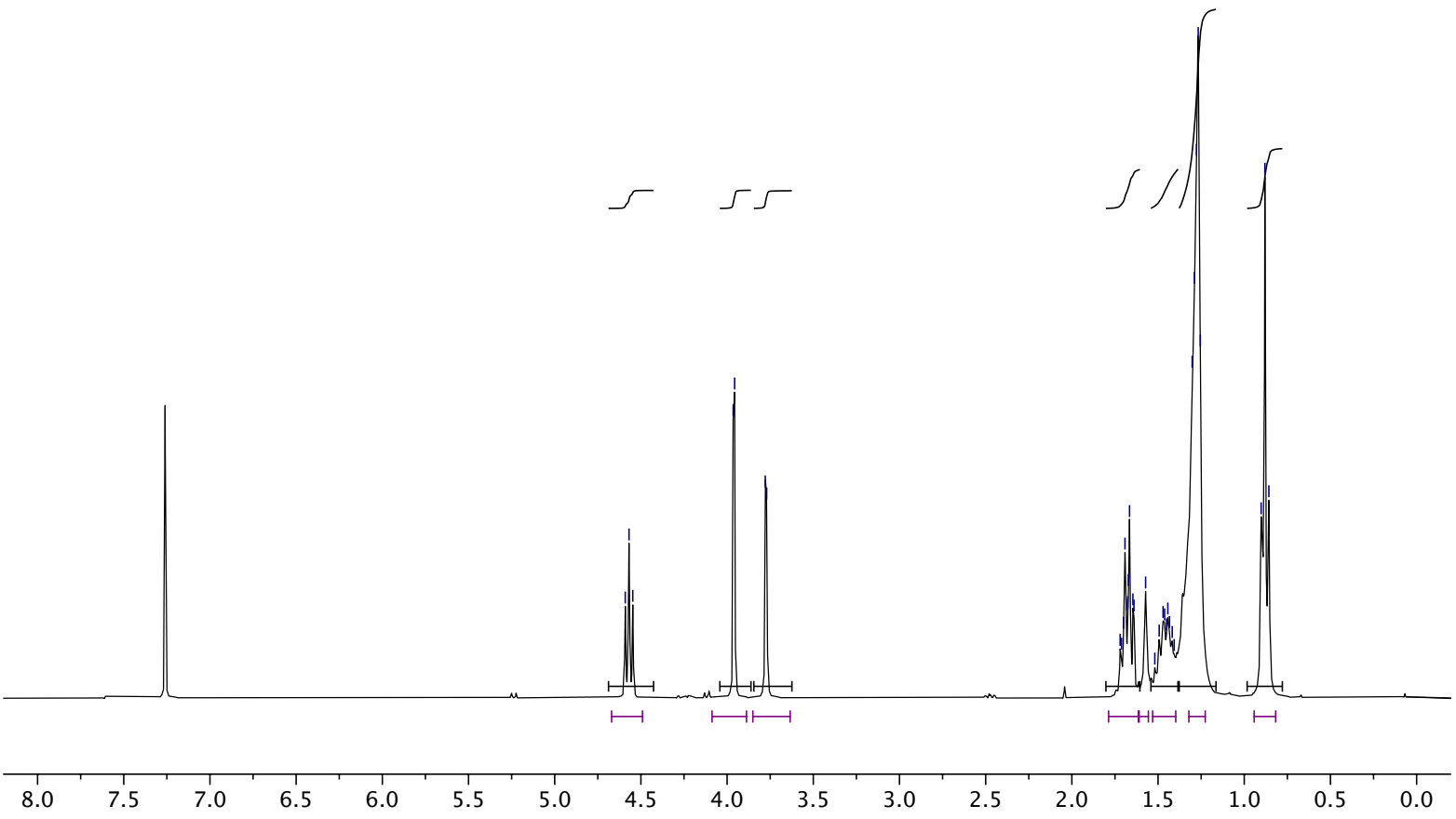


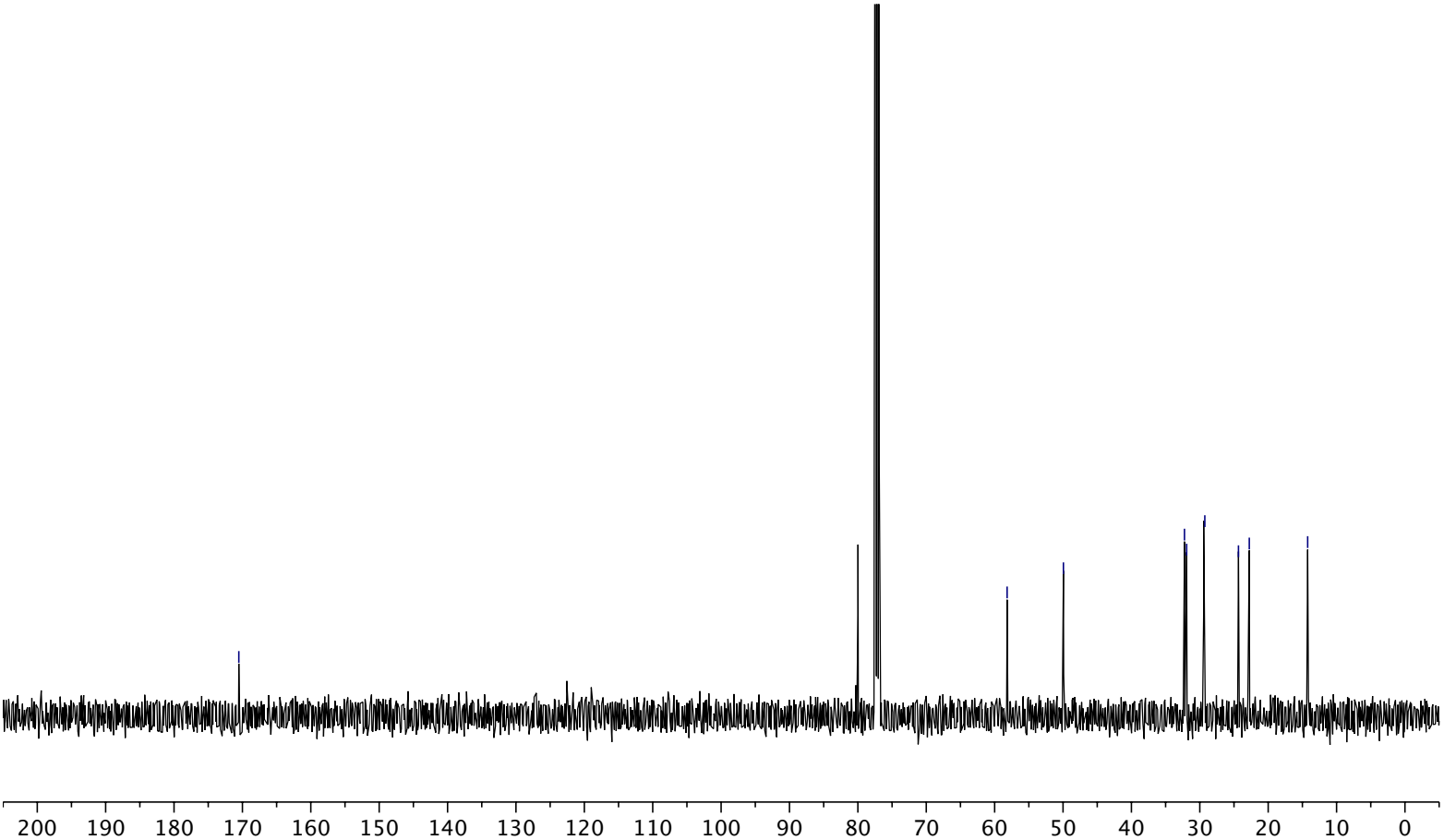
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **13** in $CDCl_3$



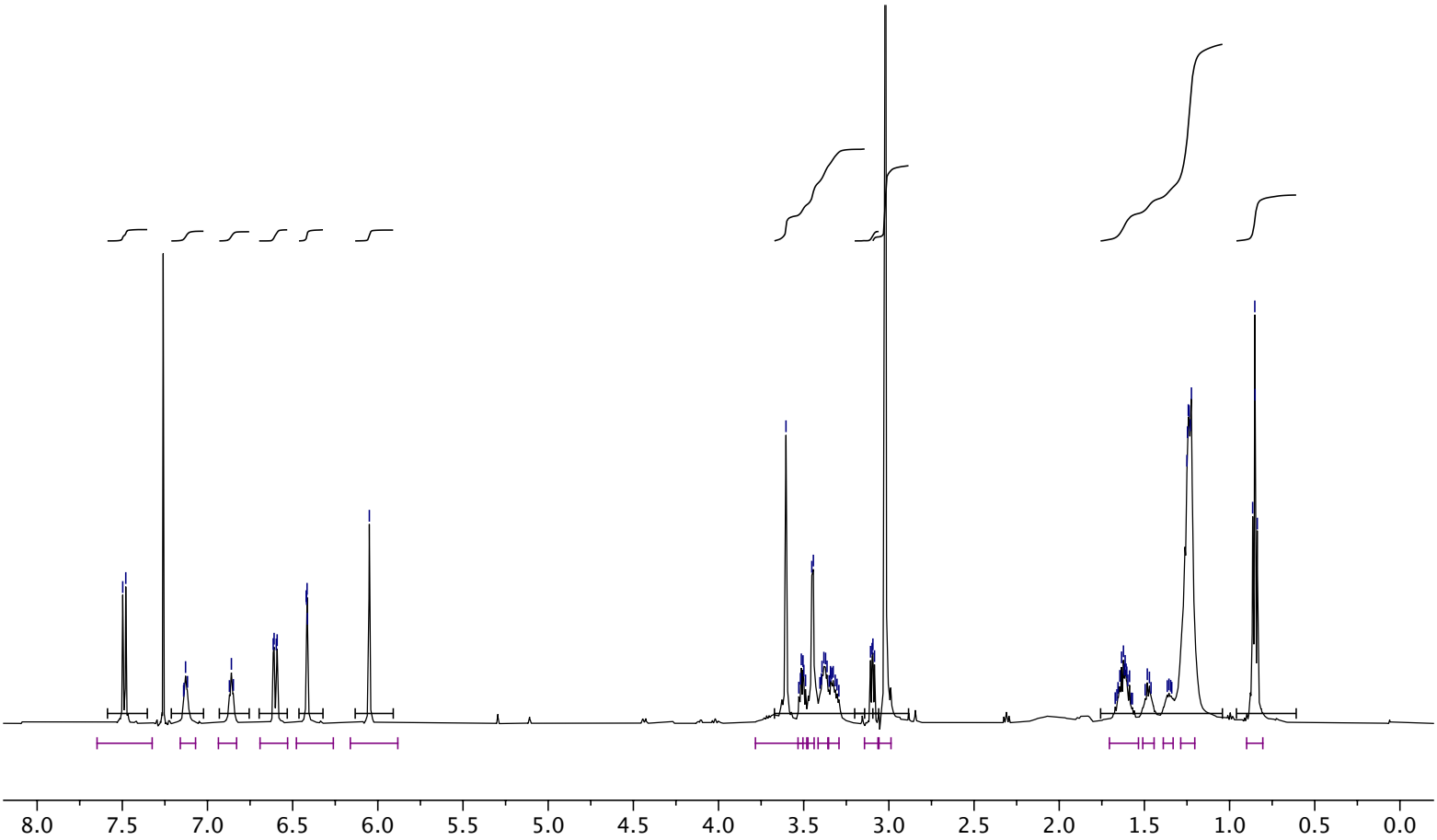


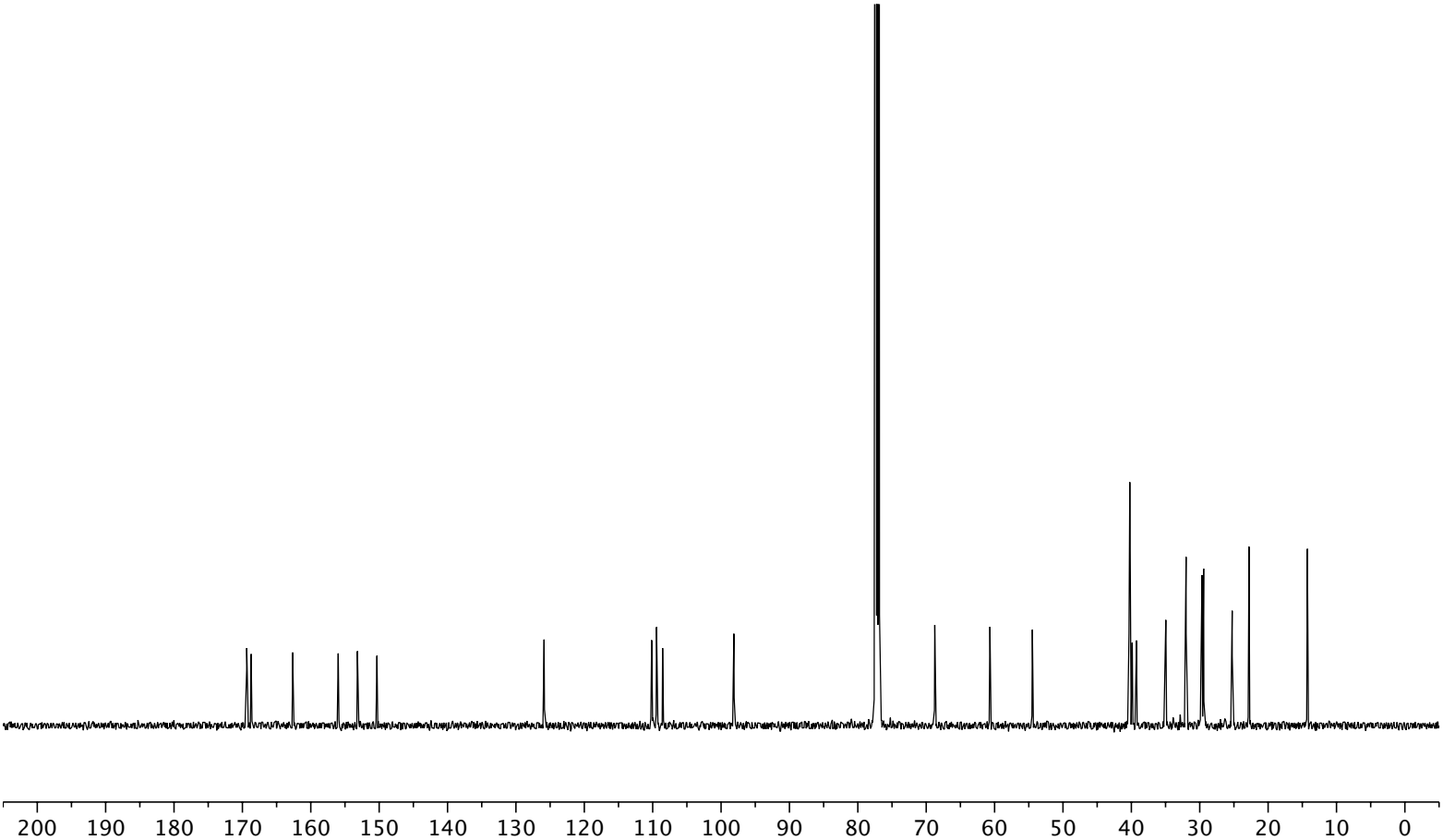
^{1}H–NMR (300 MHz) and ^{13}C–NMR (125 MHz) spectra of **14** in $CDCl_3$



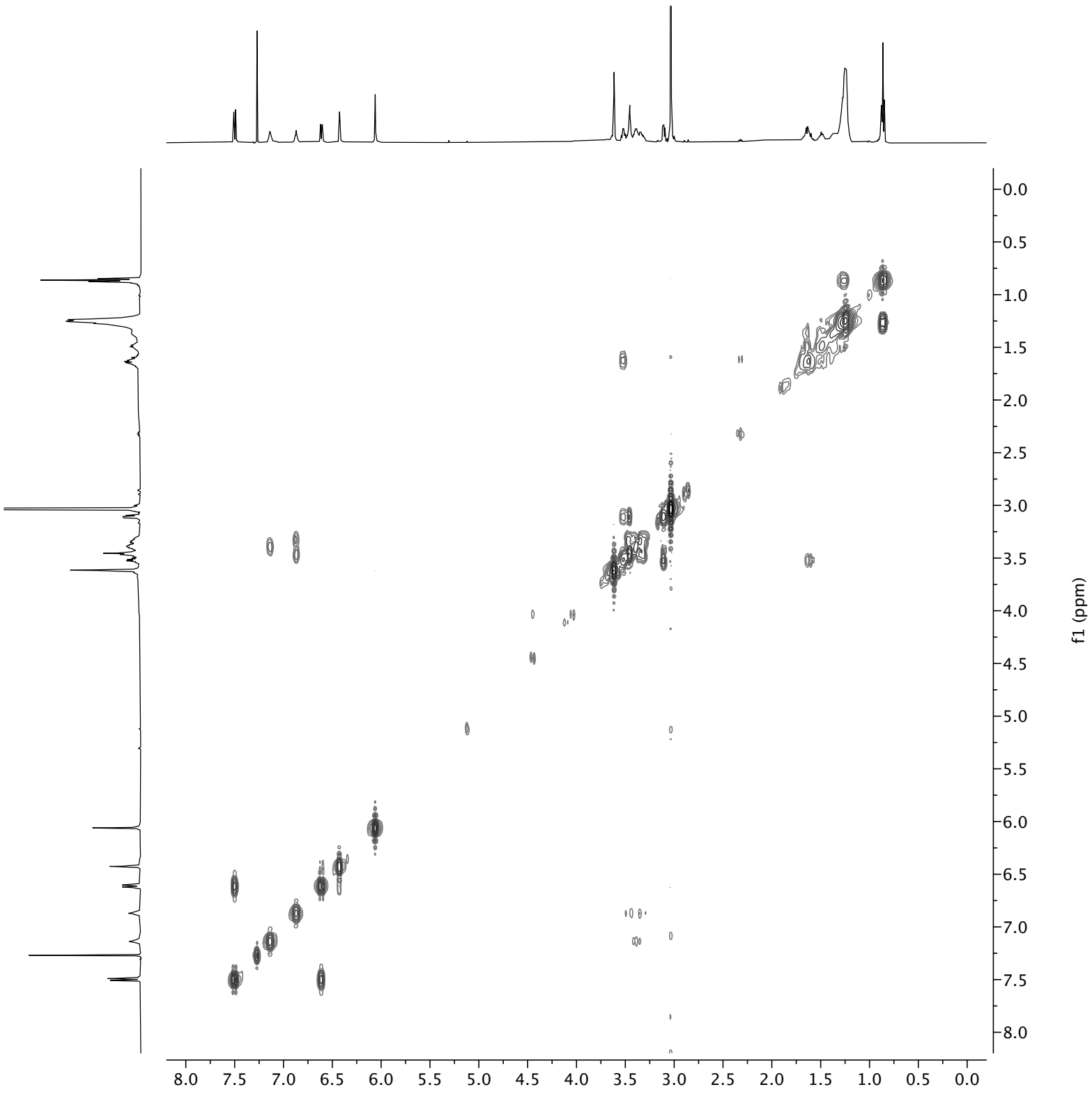


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **2a** in $CDCl_3$

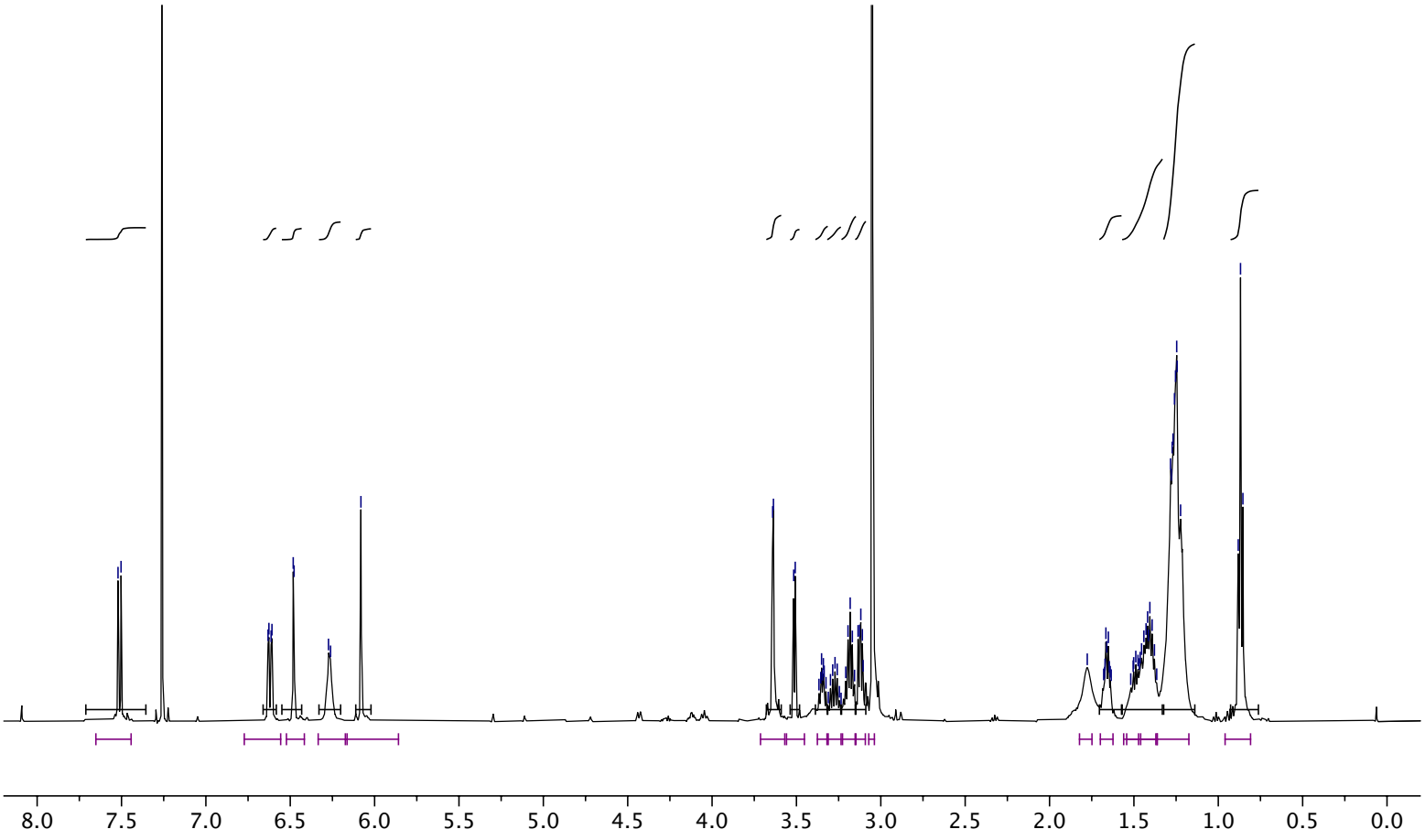


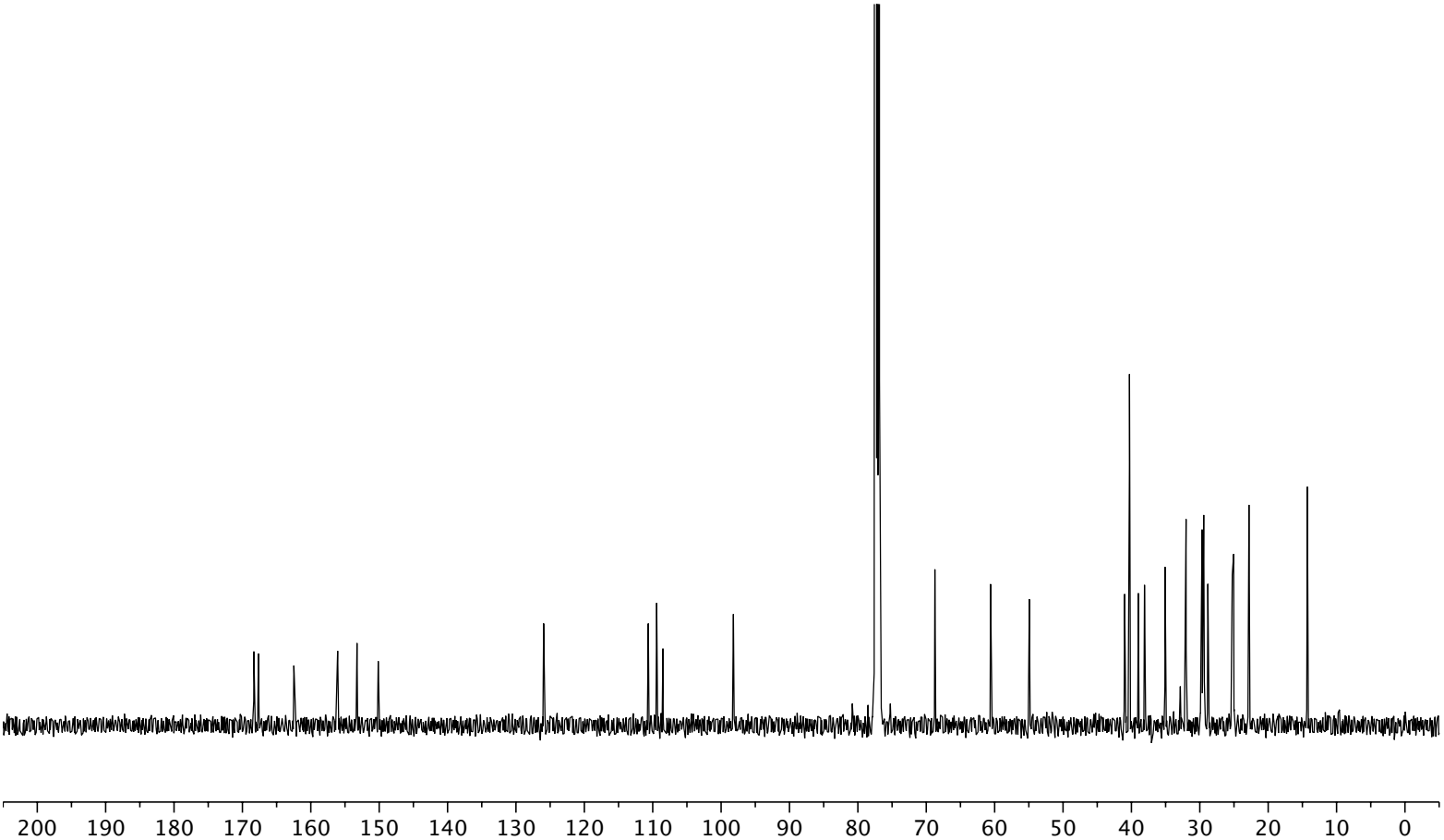


$^{1}H,^{1}H$-gCOSY (500 MHz) spectrum of **2a** in $CDCl_3$

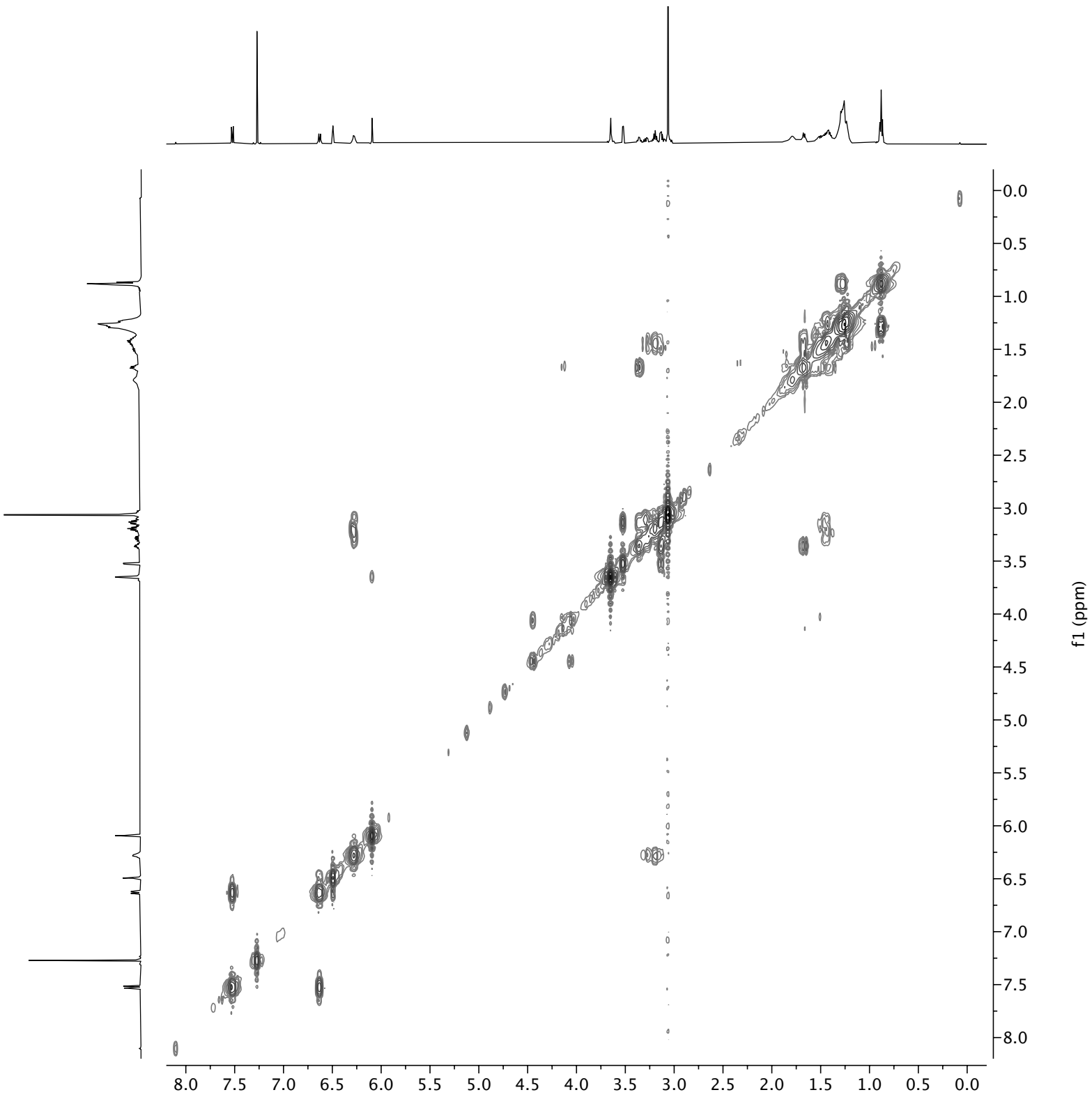


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **2b** in $CDCl_3$

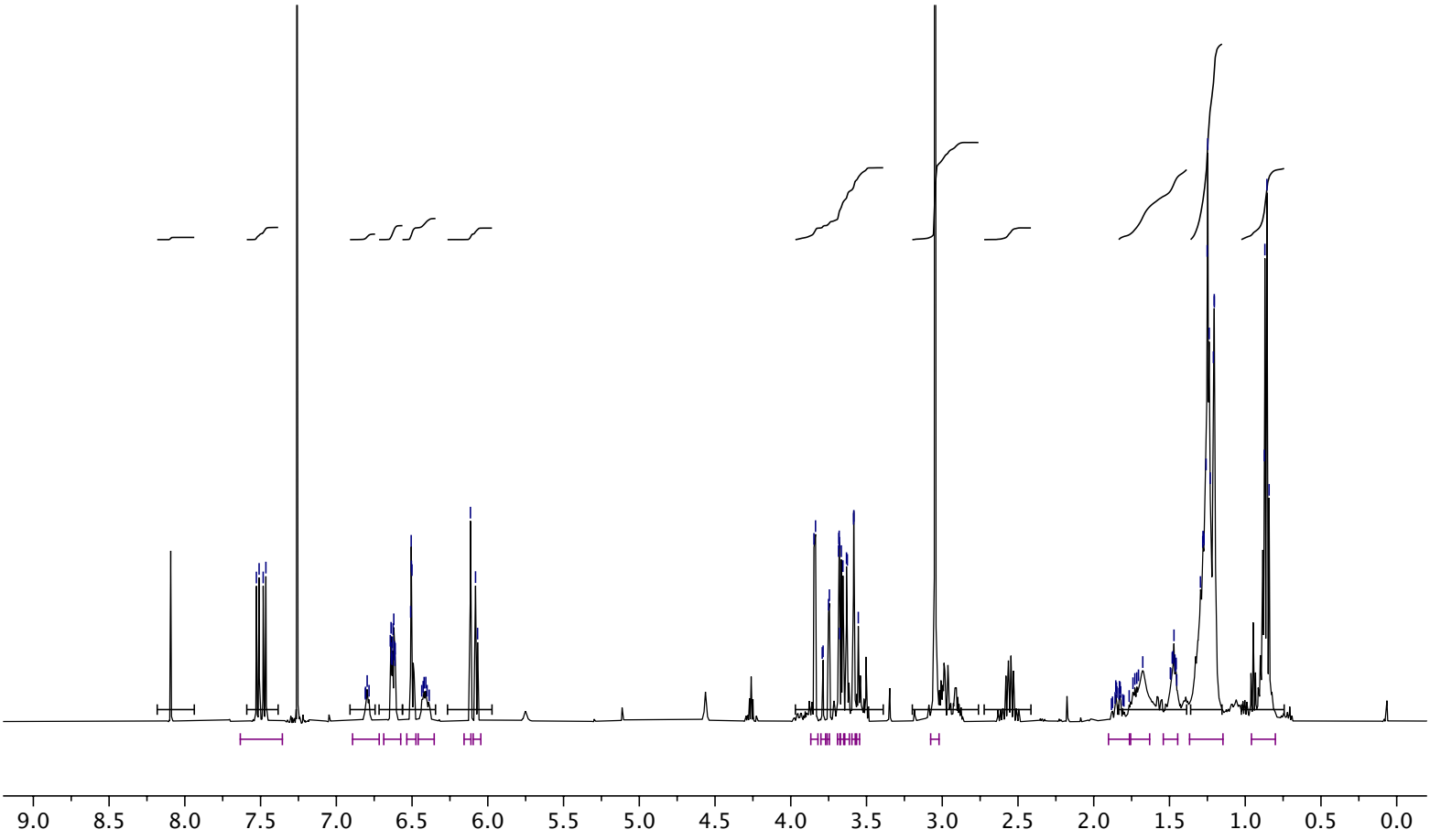


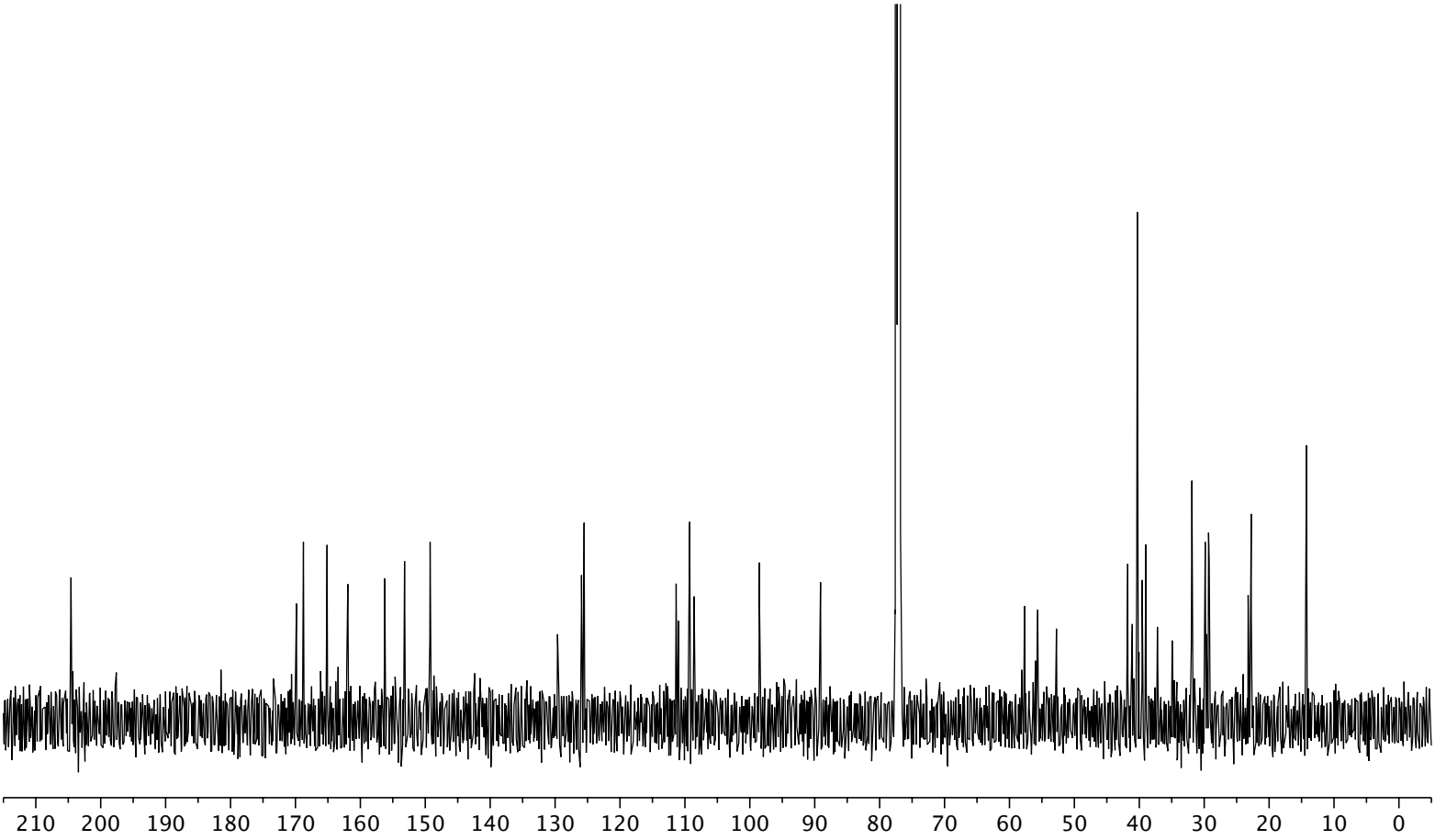


$^{1}H,^{1}H$–gCOSY (500 MHz) spectrum of **2b** in $CDCl_3$

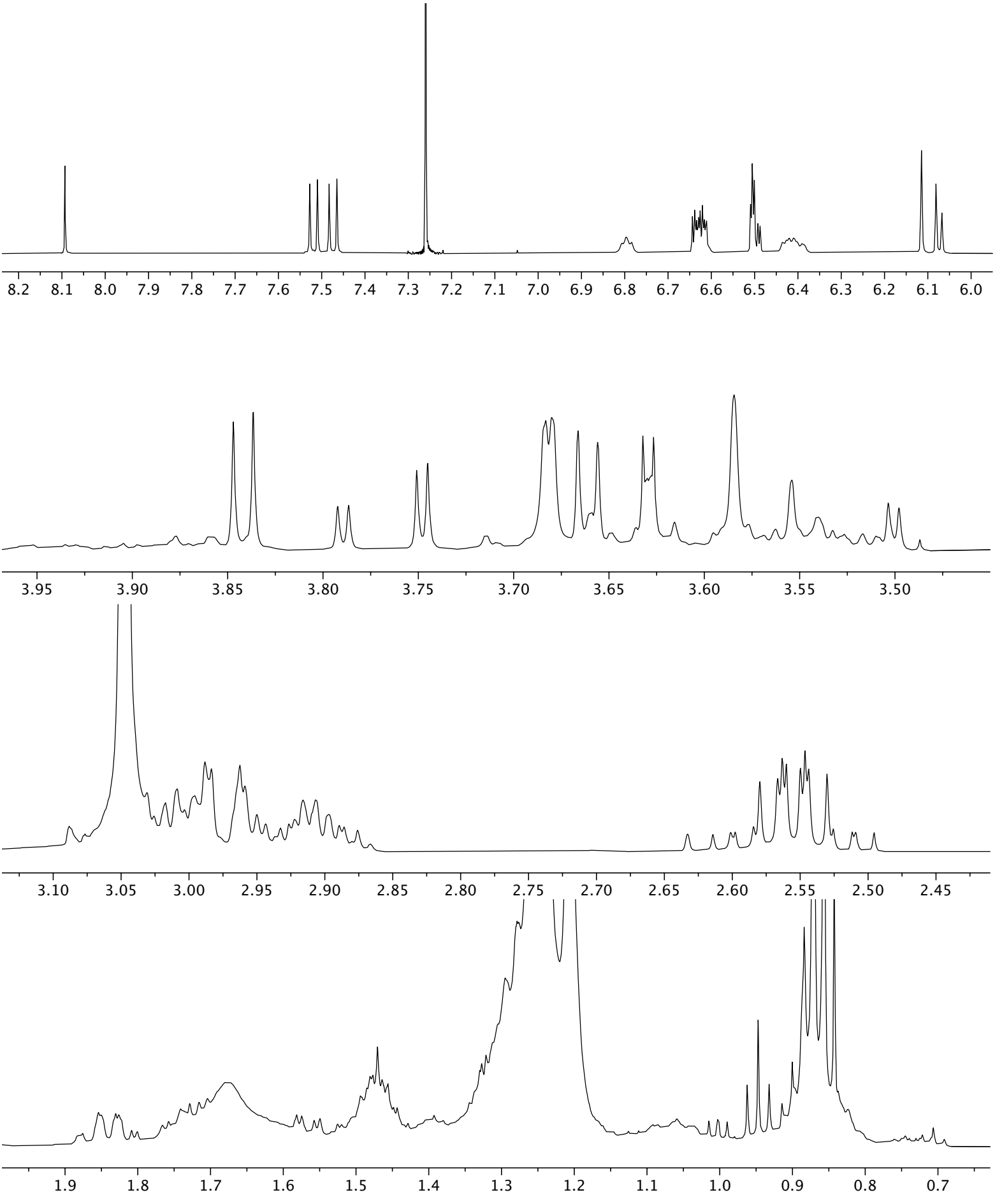


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **1a** in $CDCl_3$

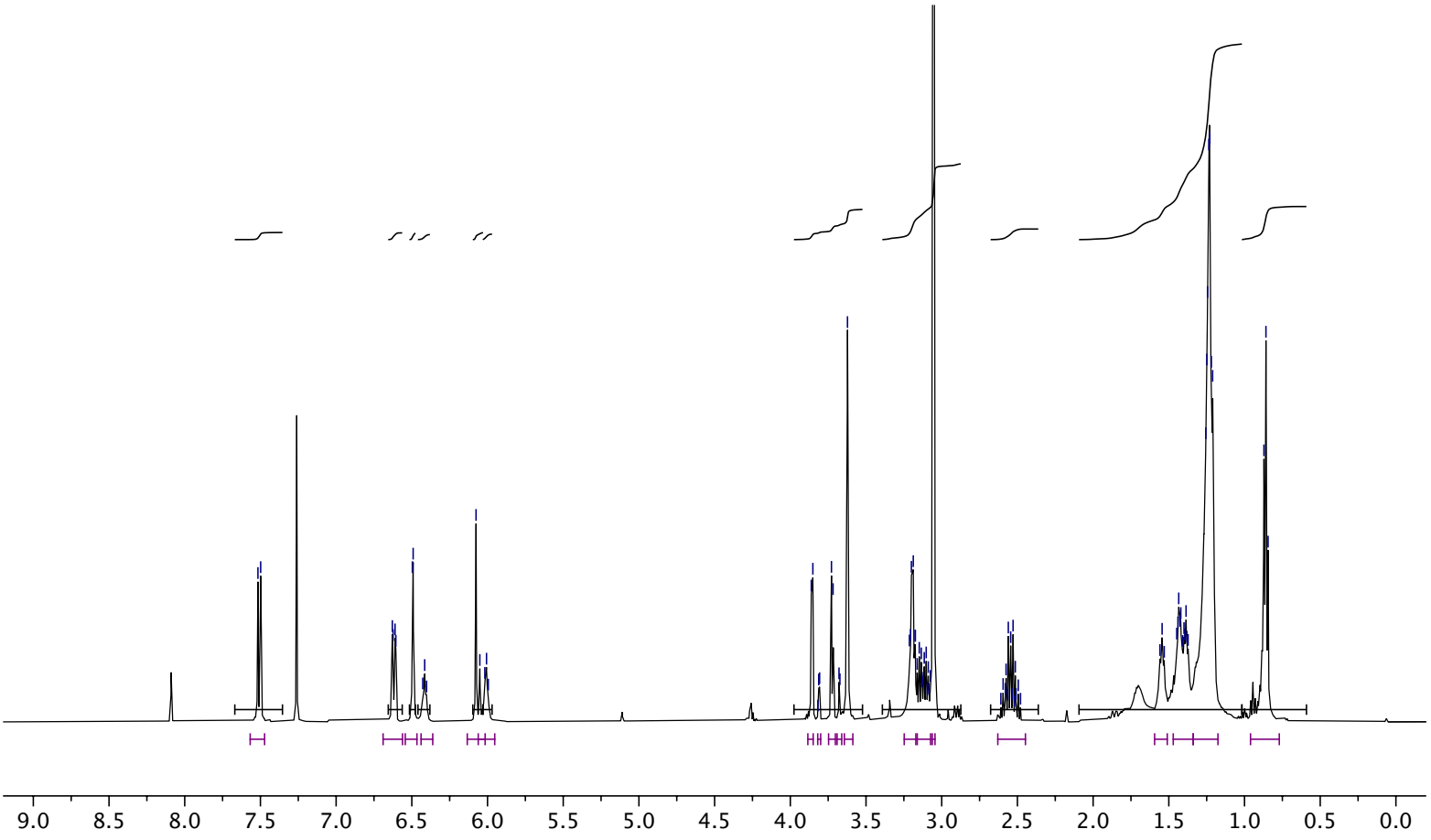


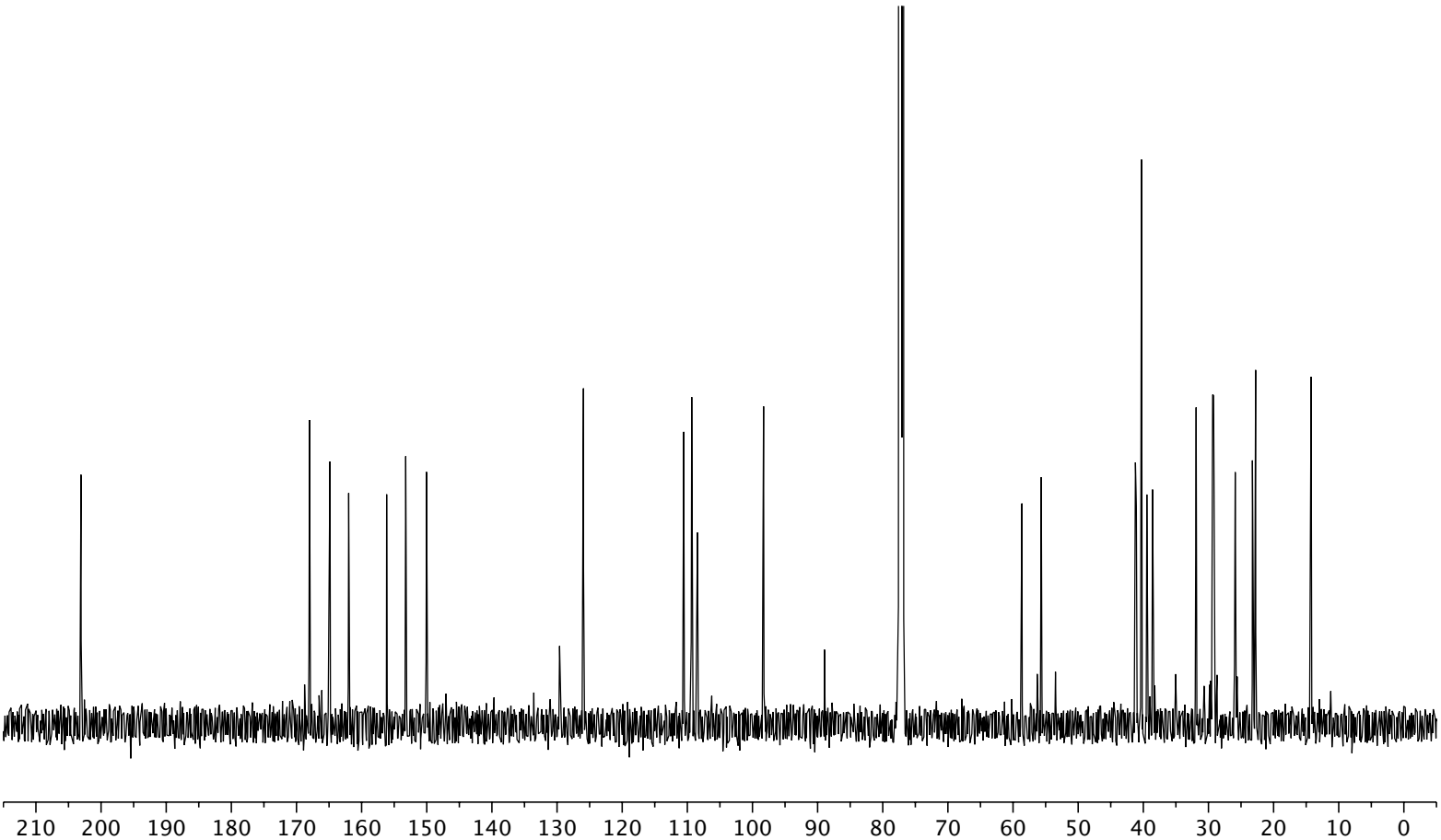


^{1}H–NMR (500 MHz) expansions for **1a** in $CDCl_3$

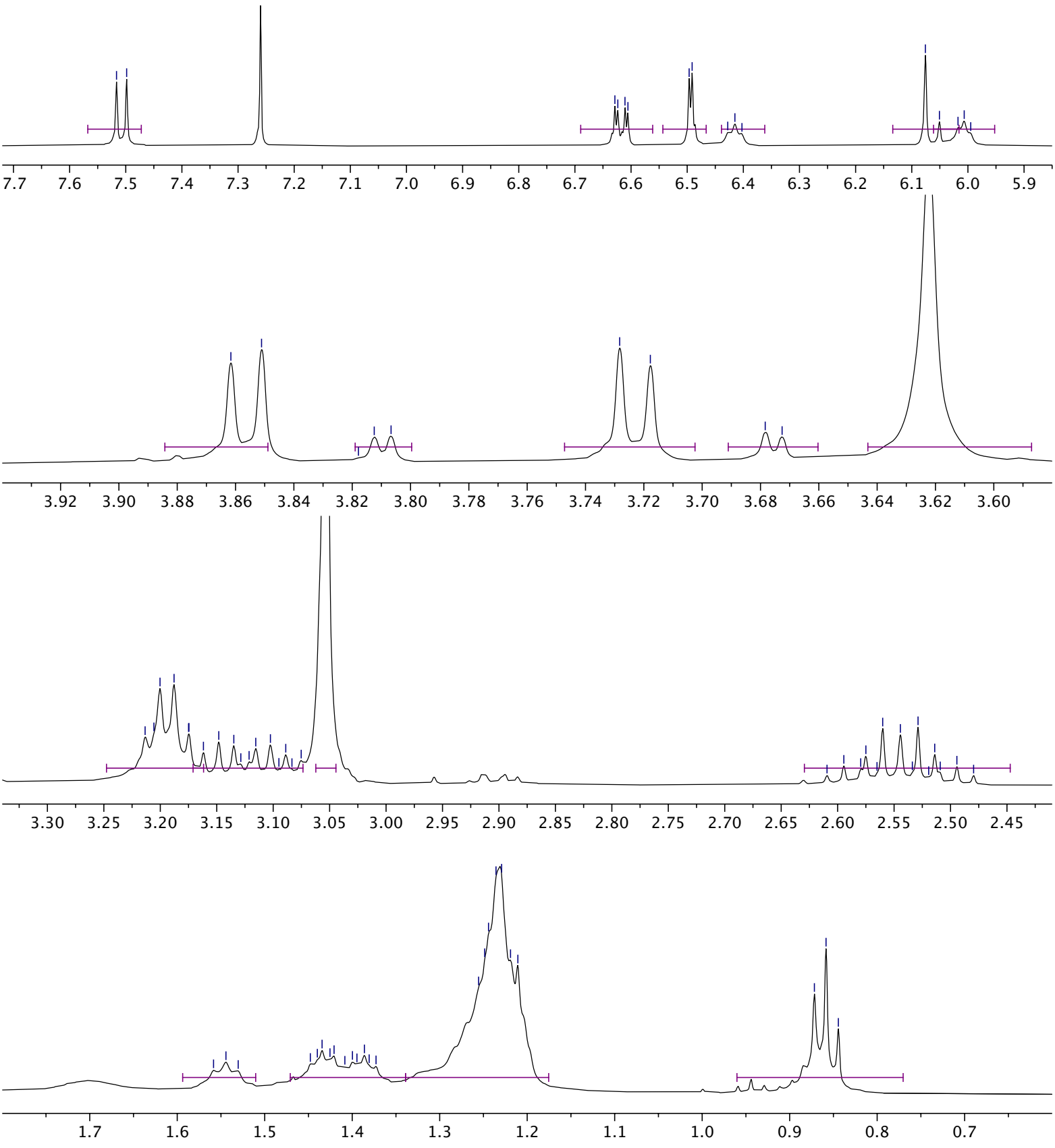


^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **1b** in $CDCl_3$

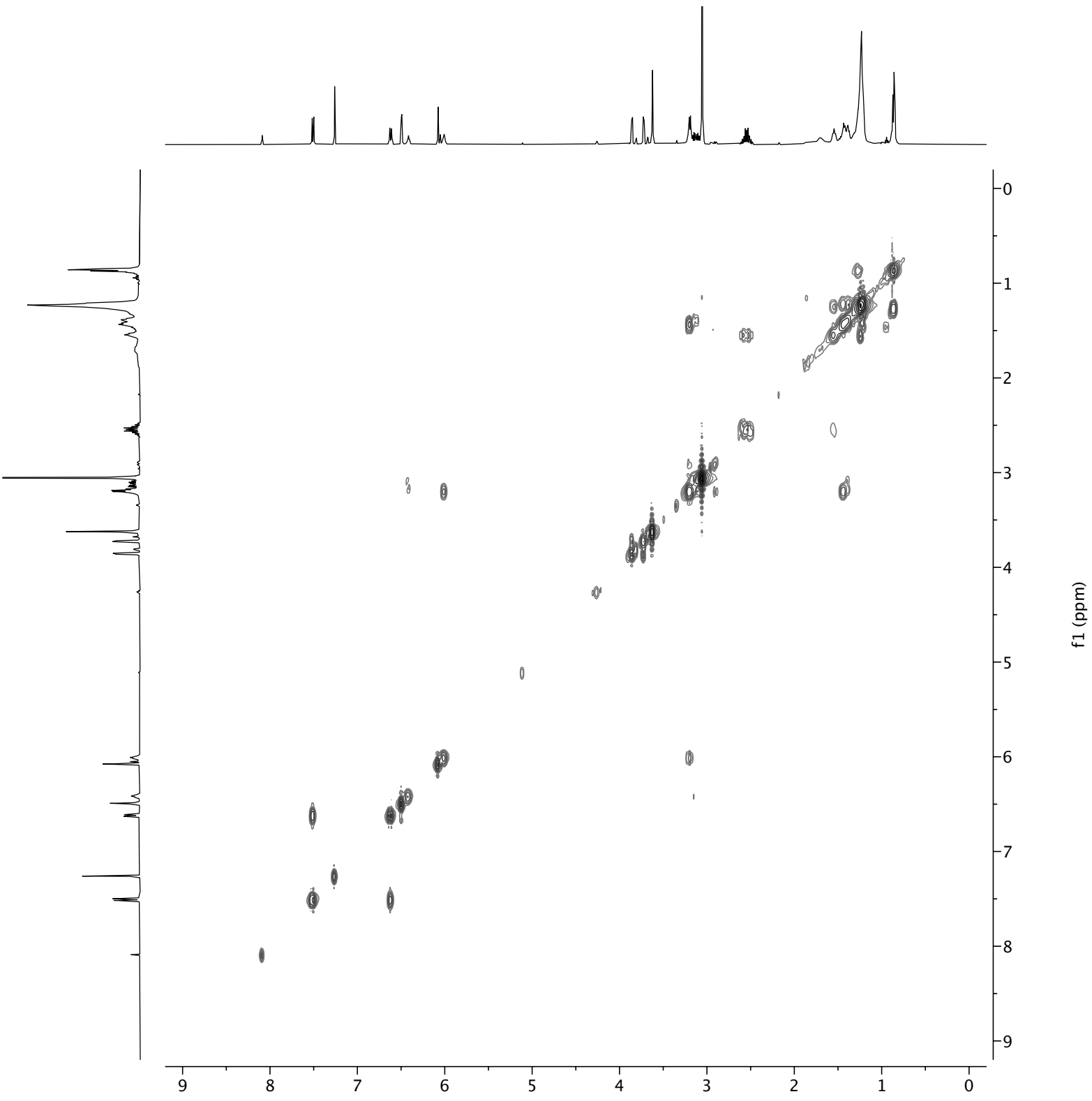




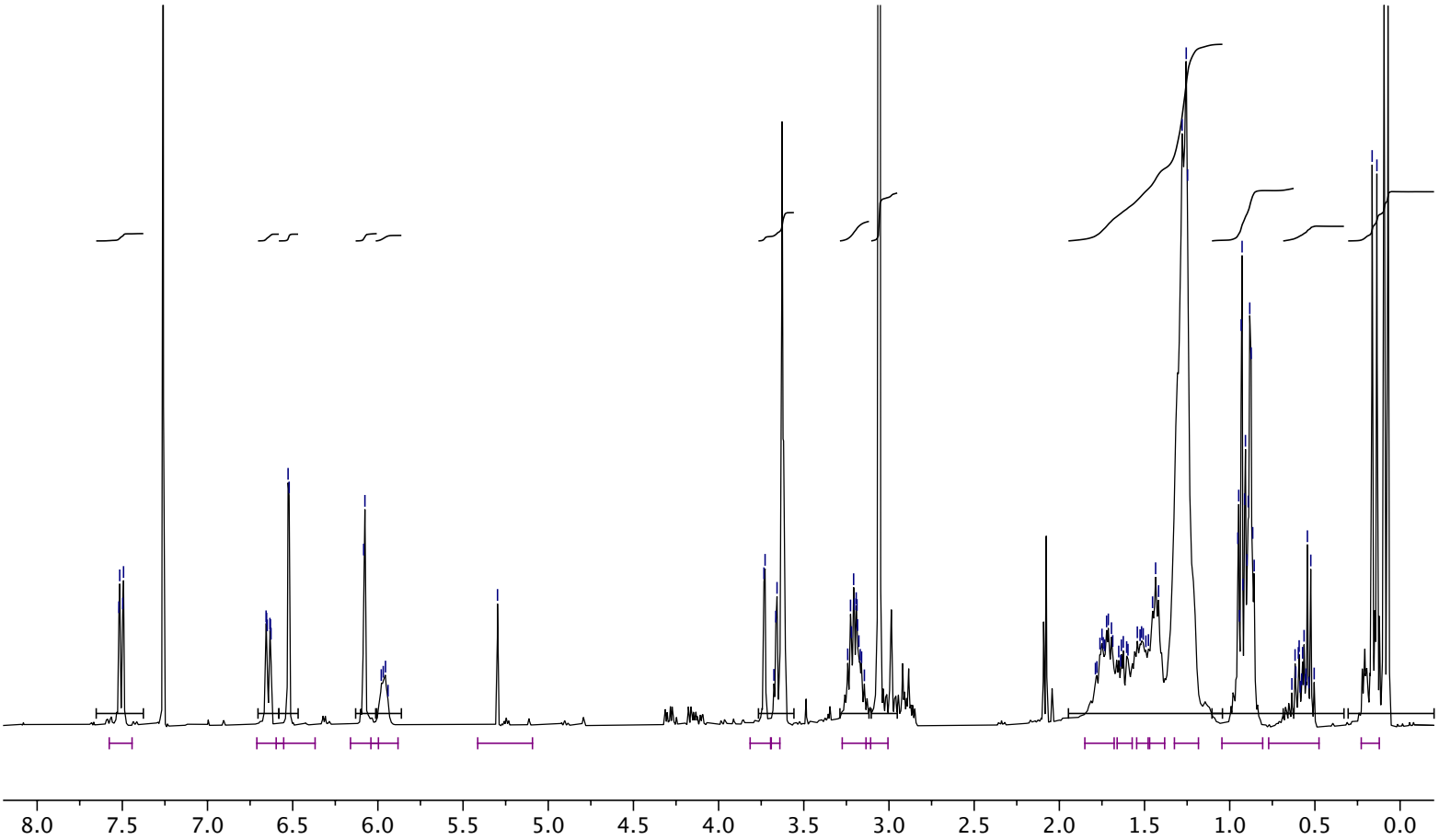
^{1}H–NMR (500 MHz) expansions for **1b** in $CDCl_3$

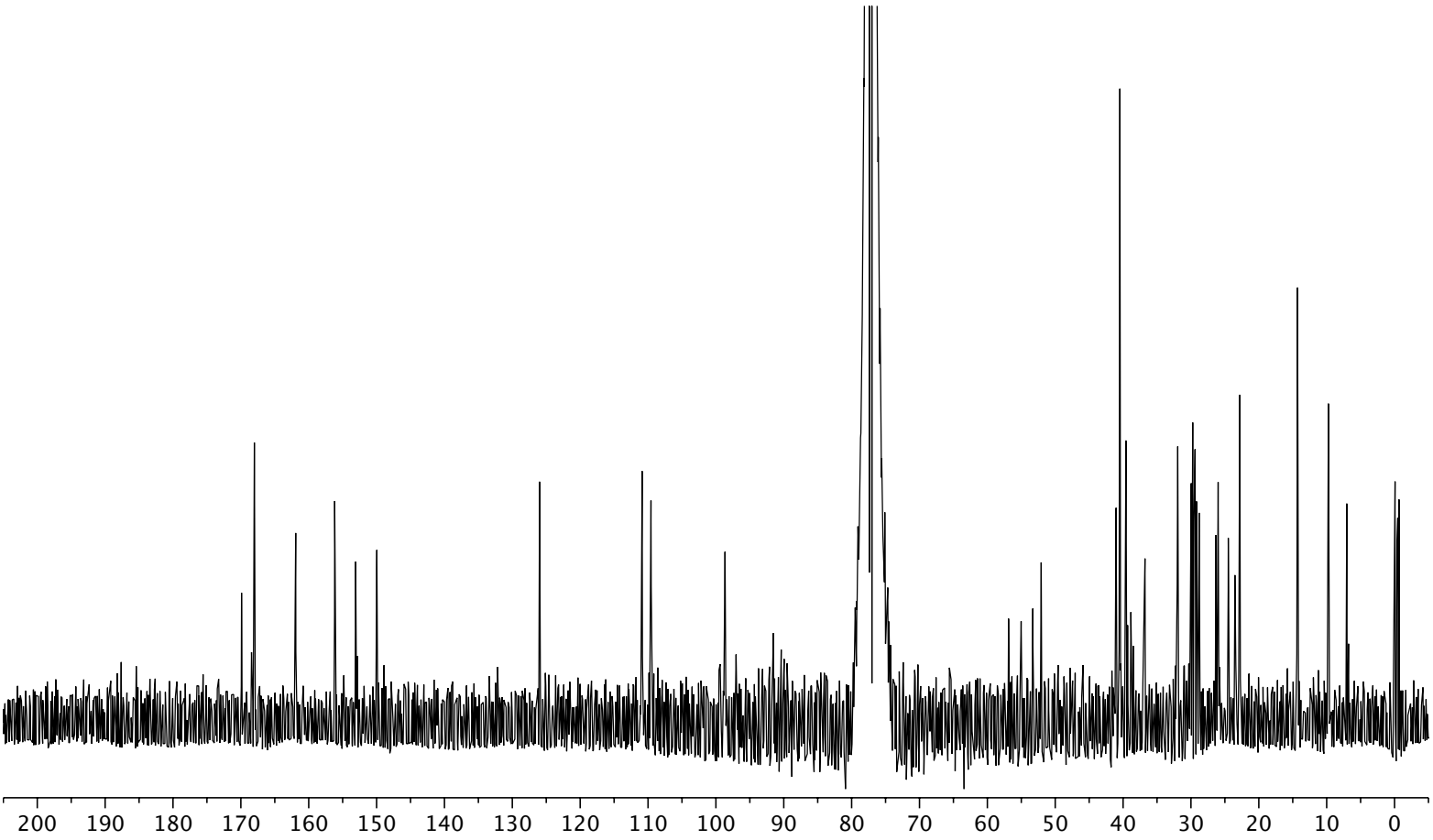


$^{1}H,^{1}H$–gCOSY (500 MHz) spectrum of **1b** in $CDCl_3$

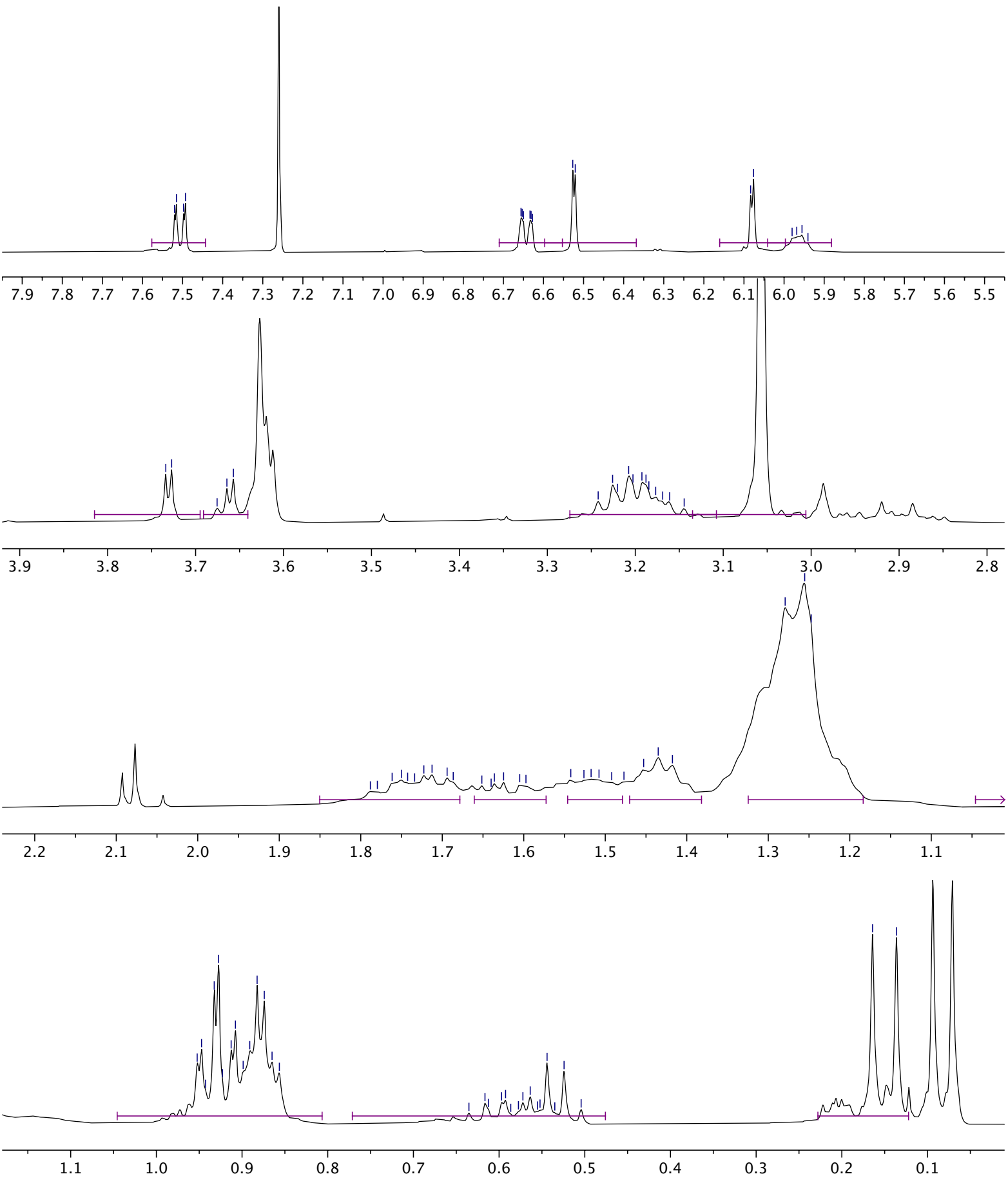


^{1}H–NMR (400 MHz) and ^{13}C–NMR (100 MHz) spectra of **7b** in $CDCl_3$

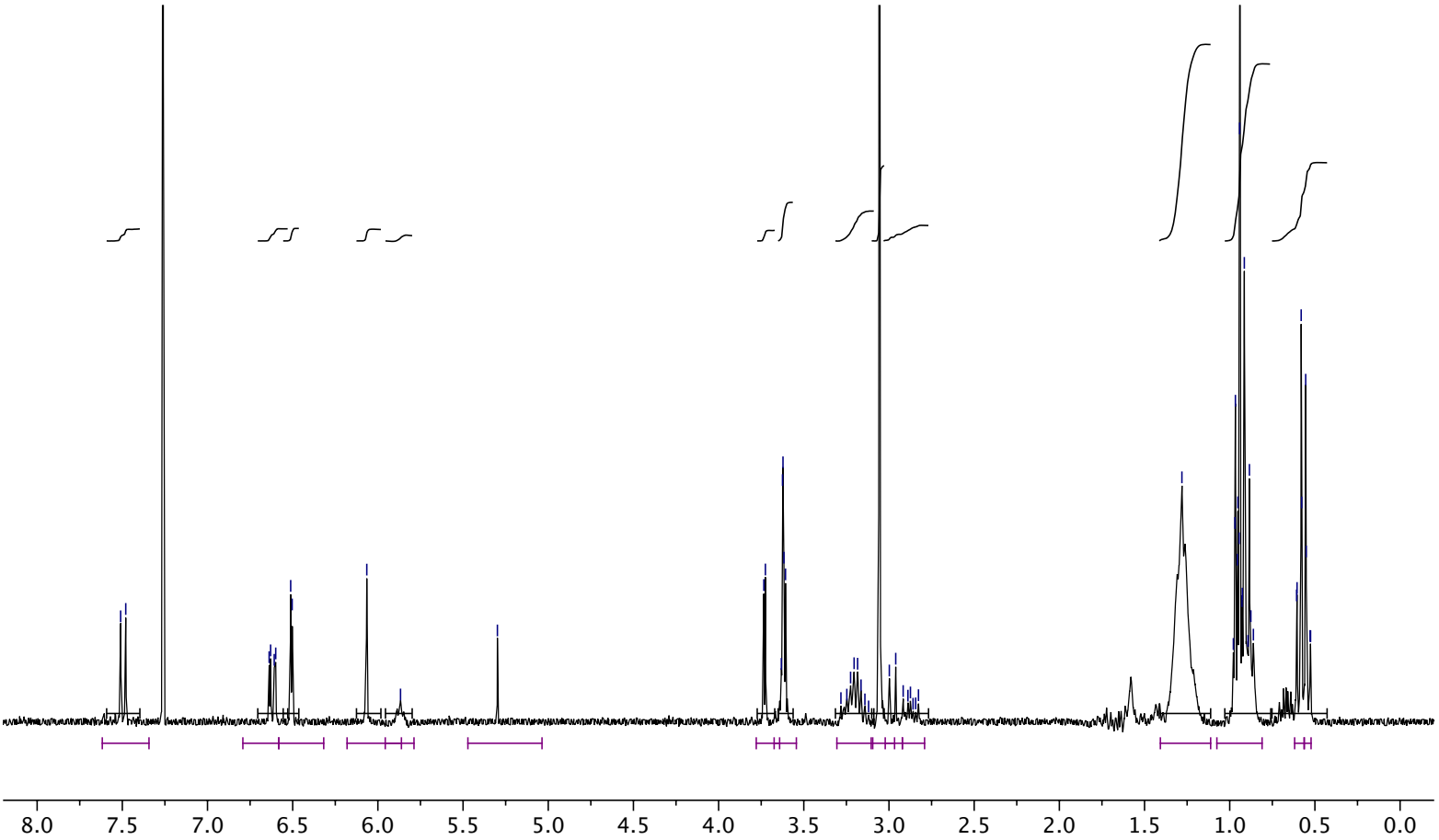


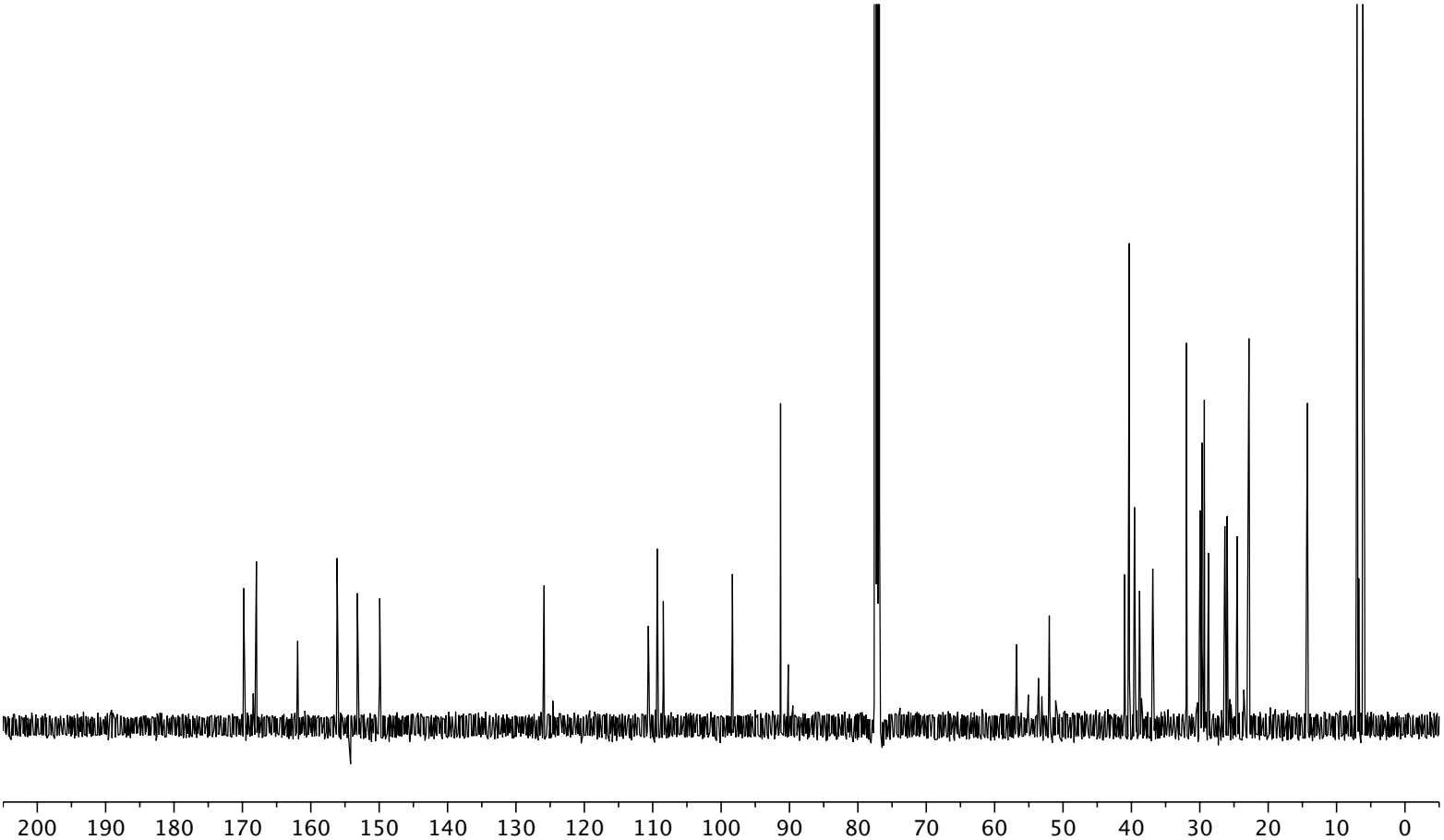


^{1}H–NMR (400 MHz) expansions for 7**b** in $CDCl_3$

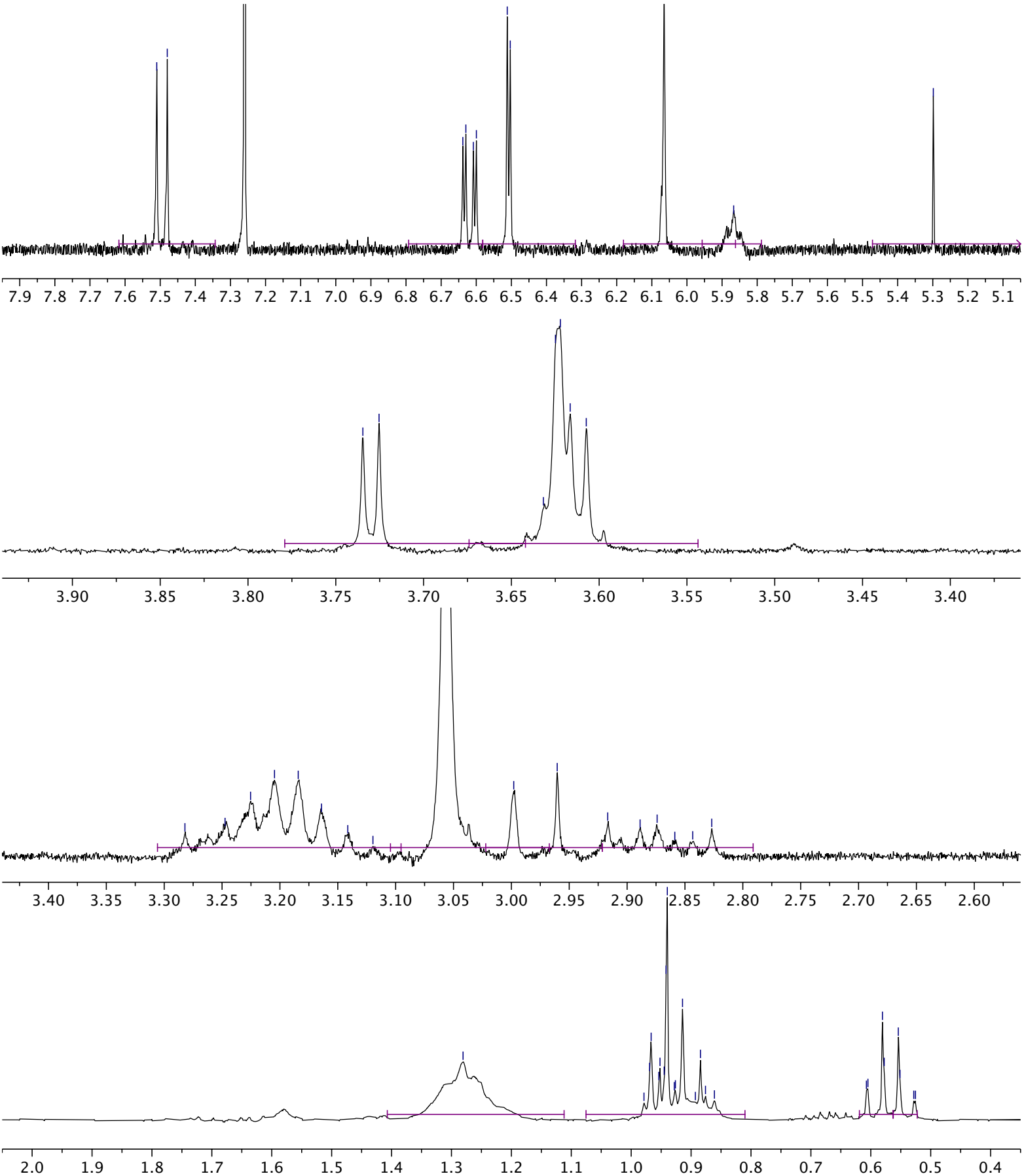


^{1}H-NMR (300 MHz) and ^{13}C-NMR (125 MHz) spectra of **8b** in $CDCl_3$

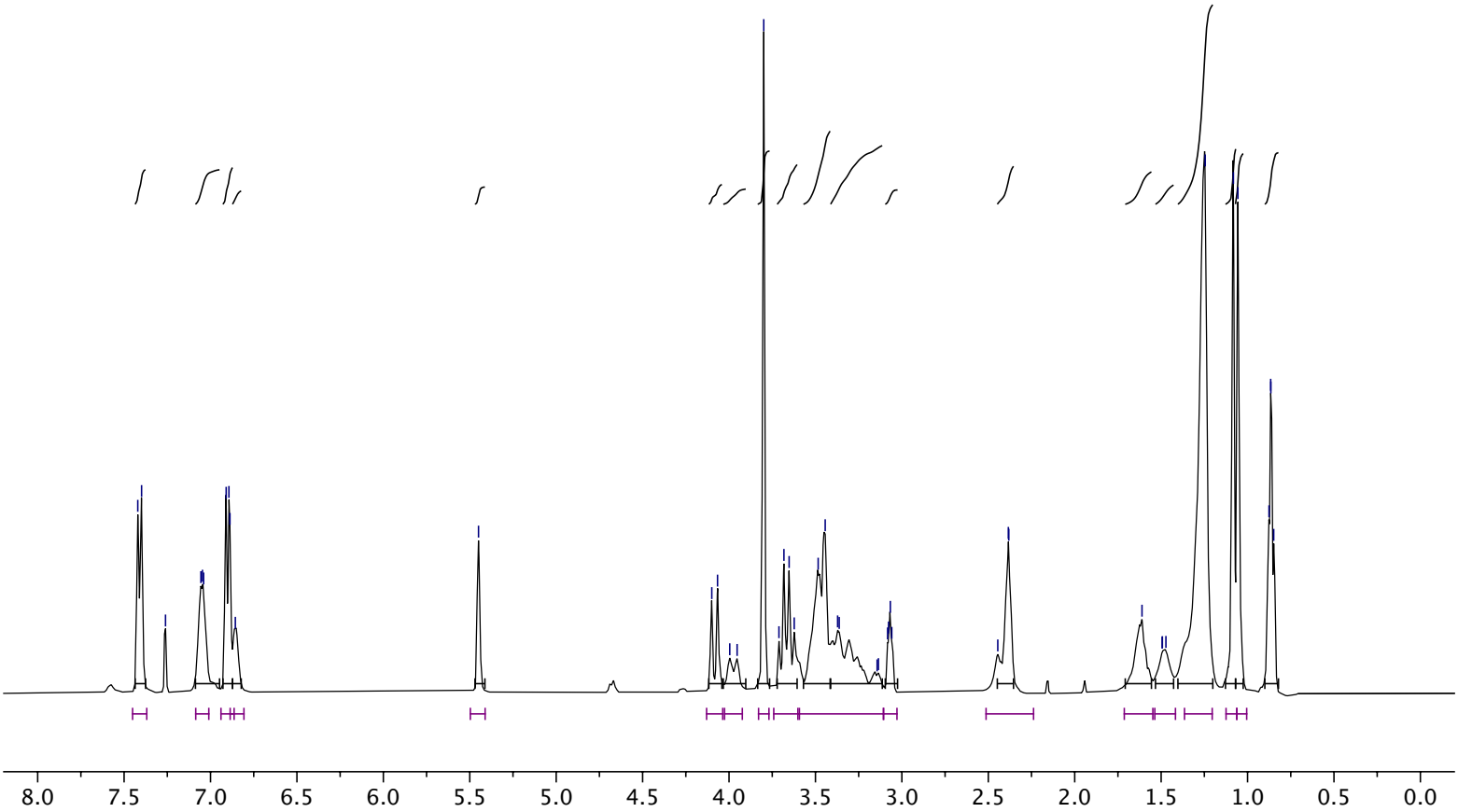


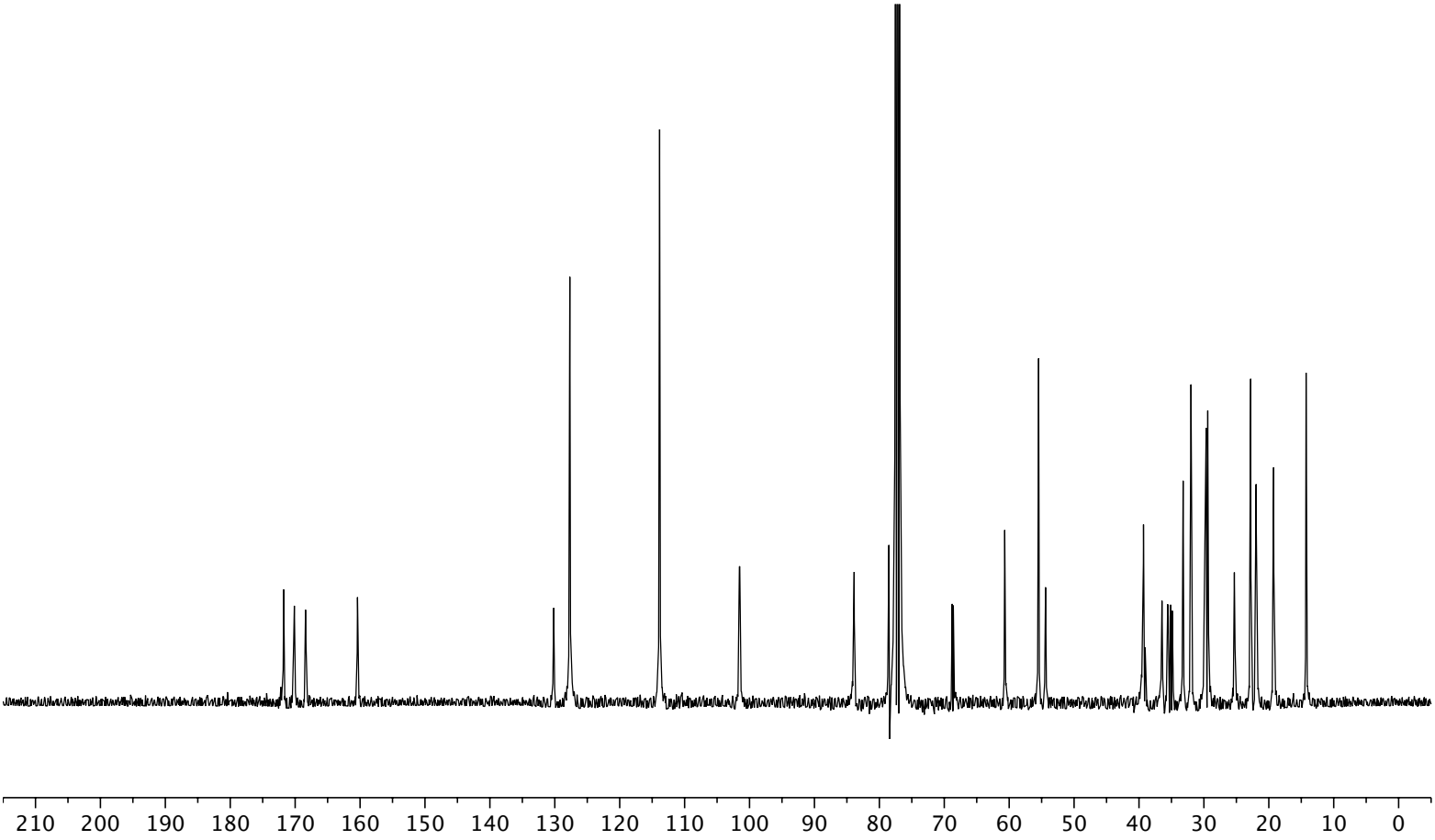


^{1}H-NMR (300 MHz) expansions for **8b** in $CDCl_3$

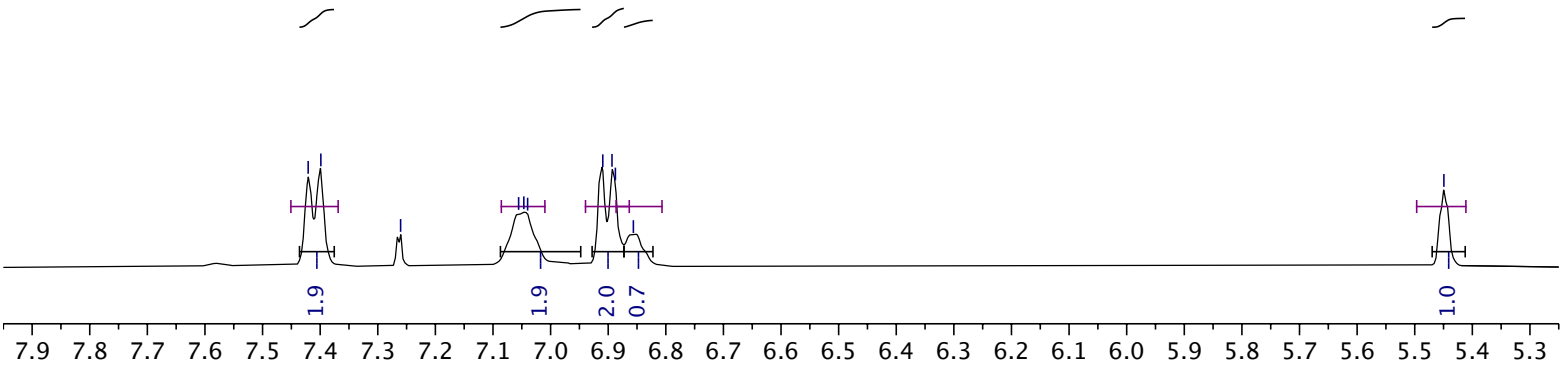


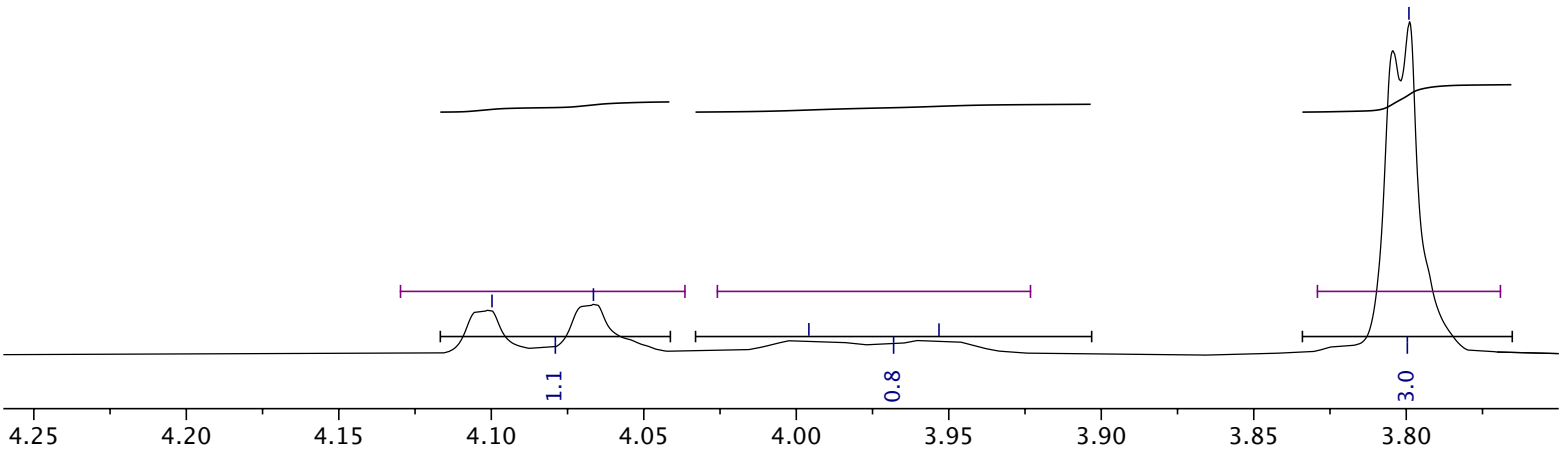
^{1}H–NMR (400 MHz) and ^{13}C–NMR (100 MHz) spectra of **S1** in $CDCl_3$

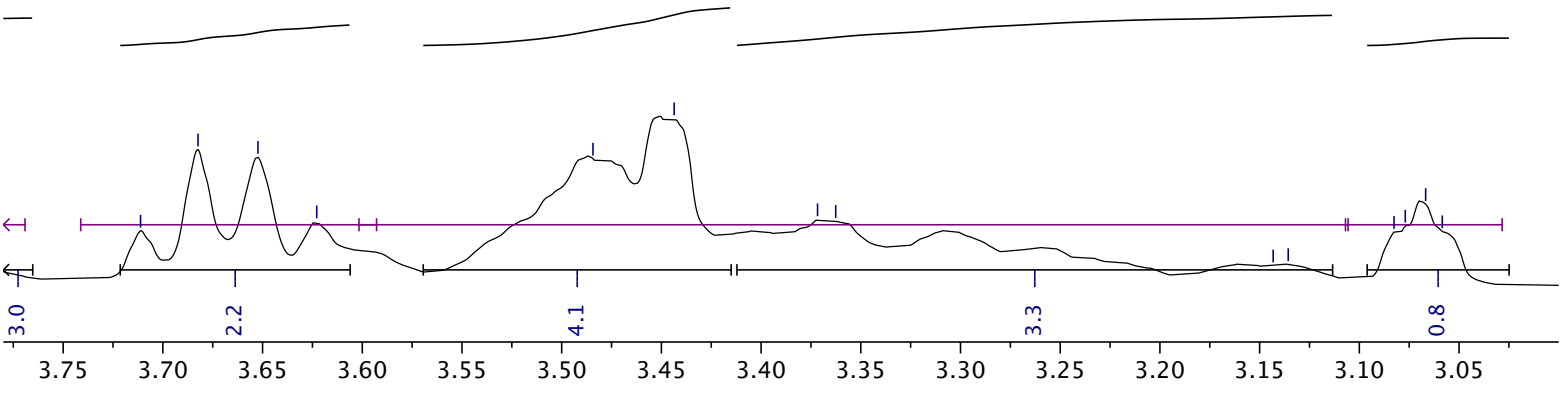


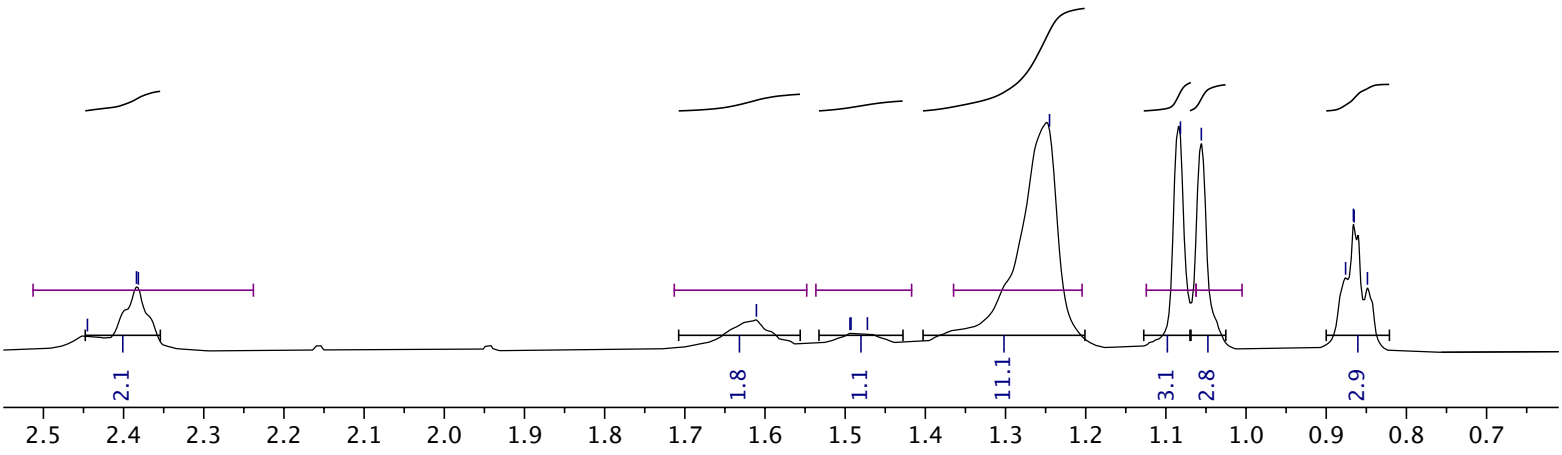


^{1}H–NMR (400 MHz) expansions for **S1** in $CDCl_3$

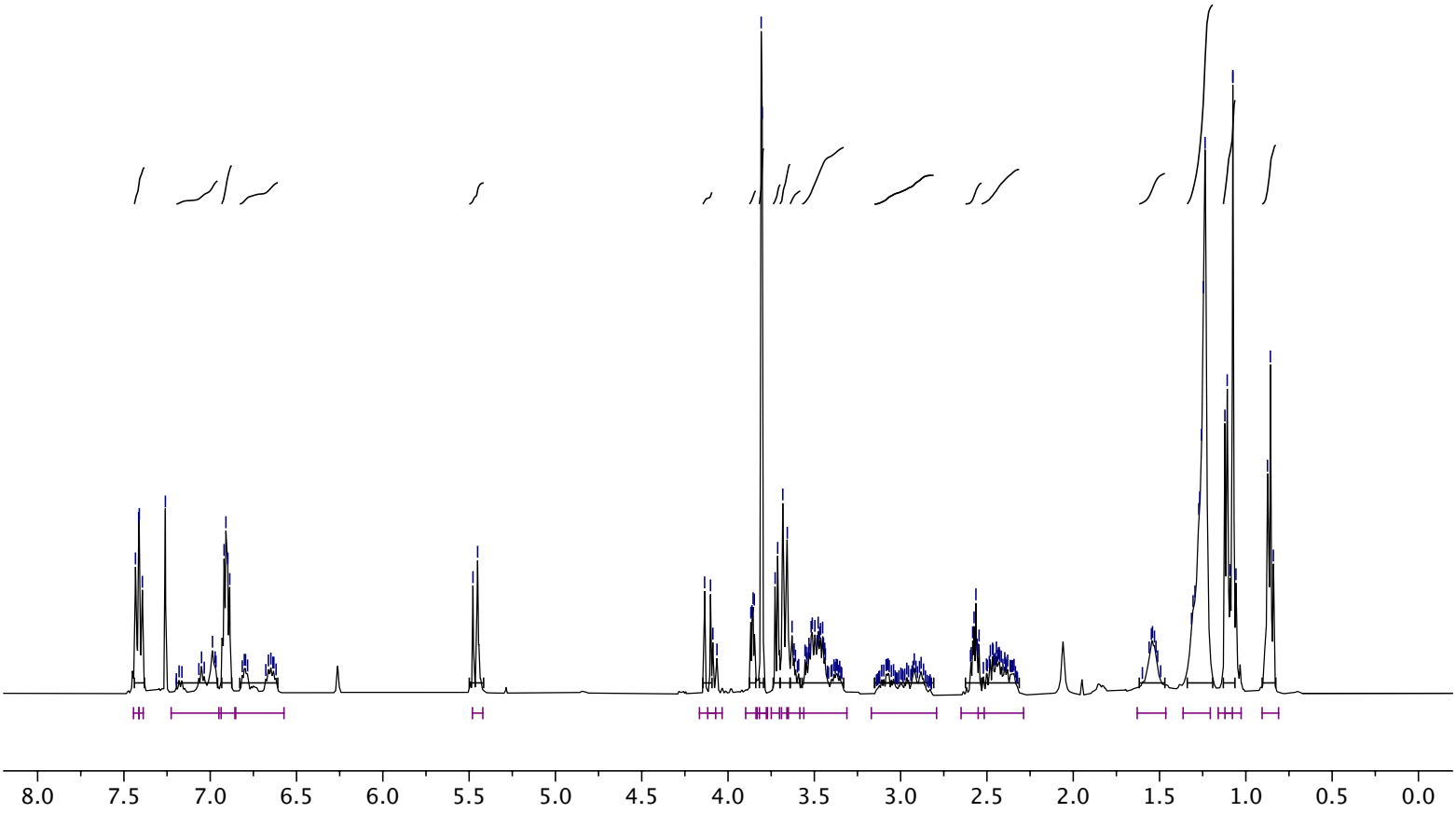


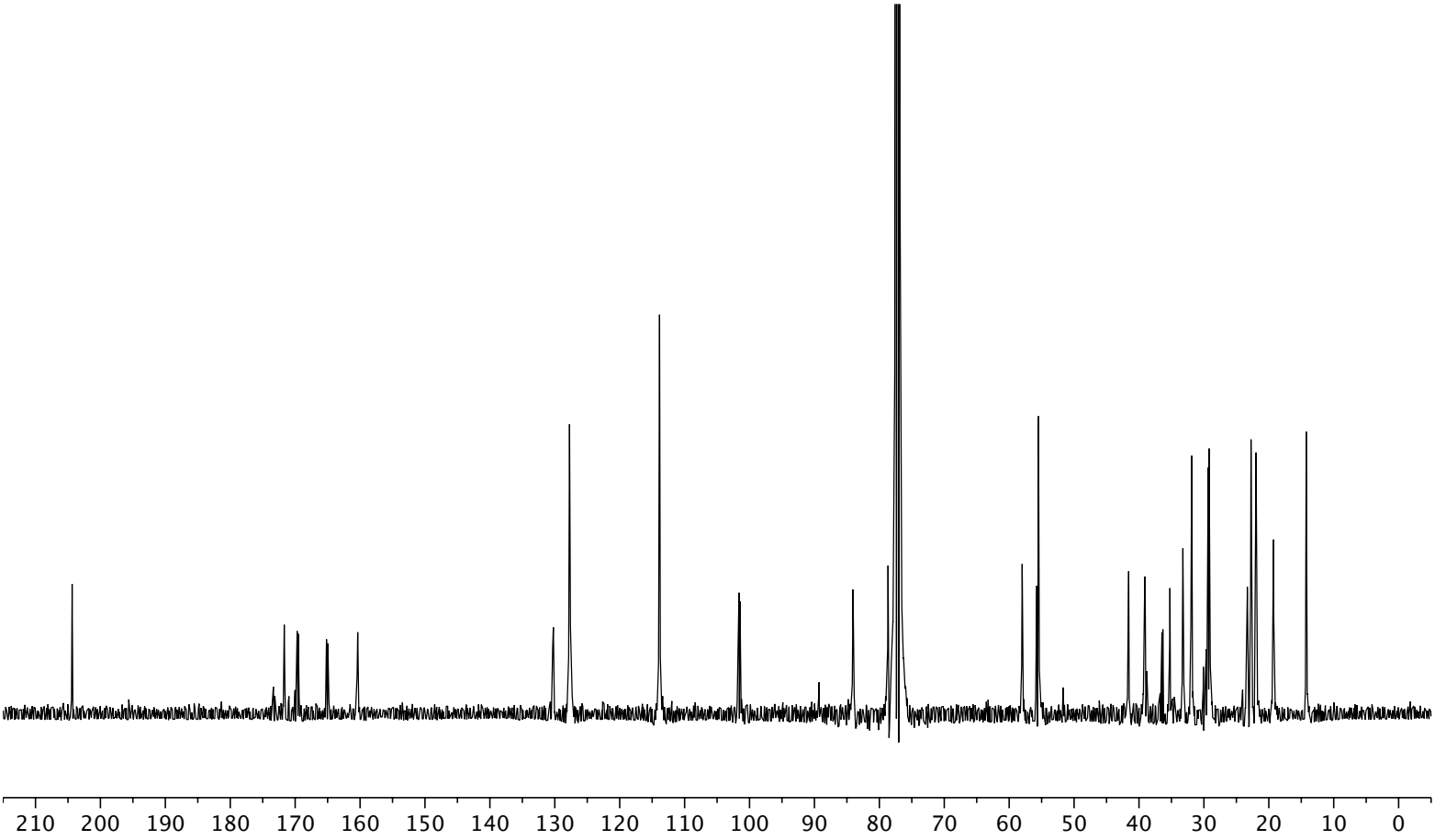




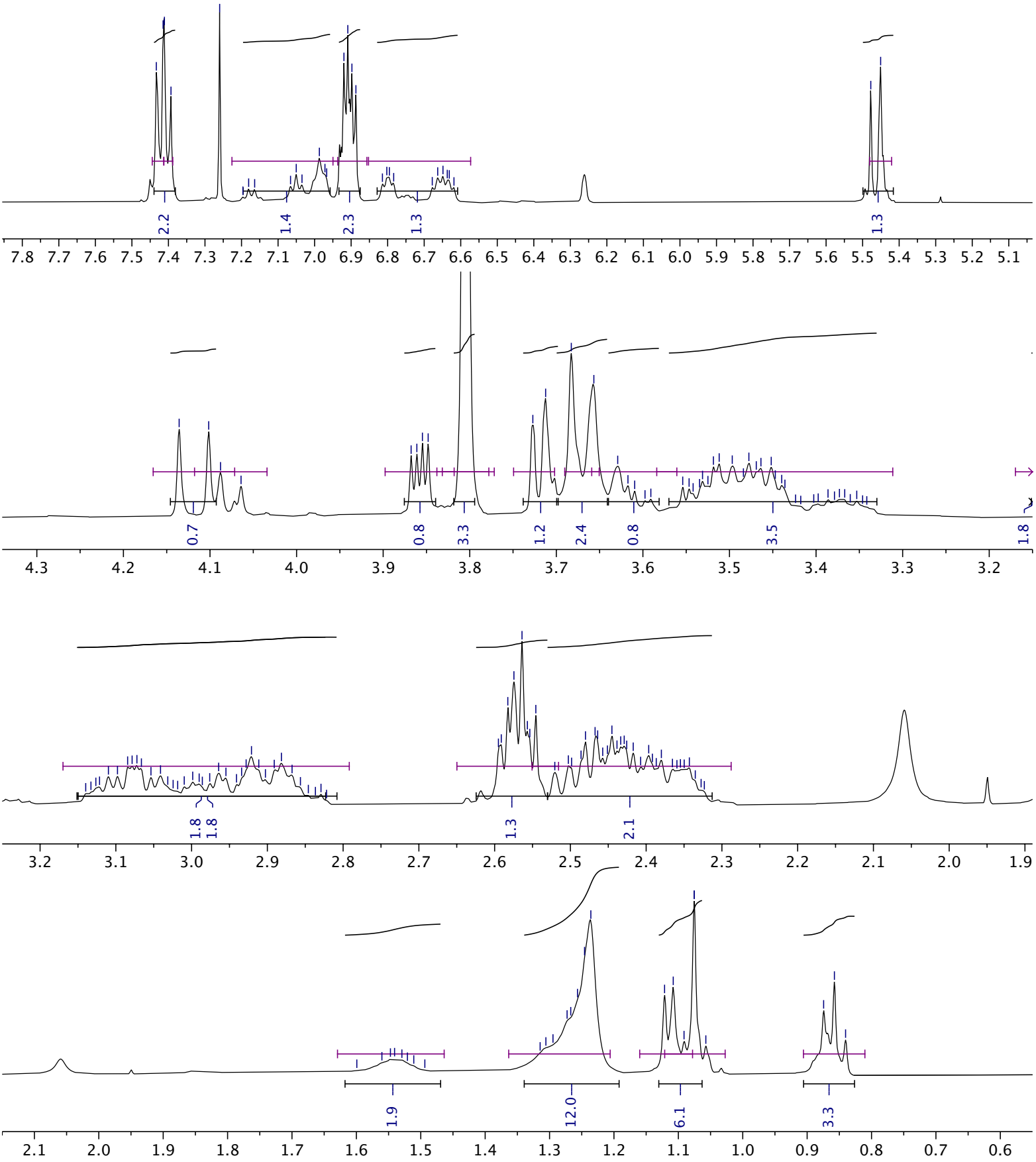


^{1}H–NMR (400 MHz) and ^{13}C–NMR (100 MHz) spectra of **S2** in $CDCl_3$

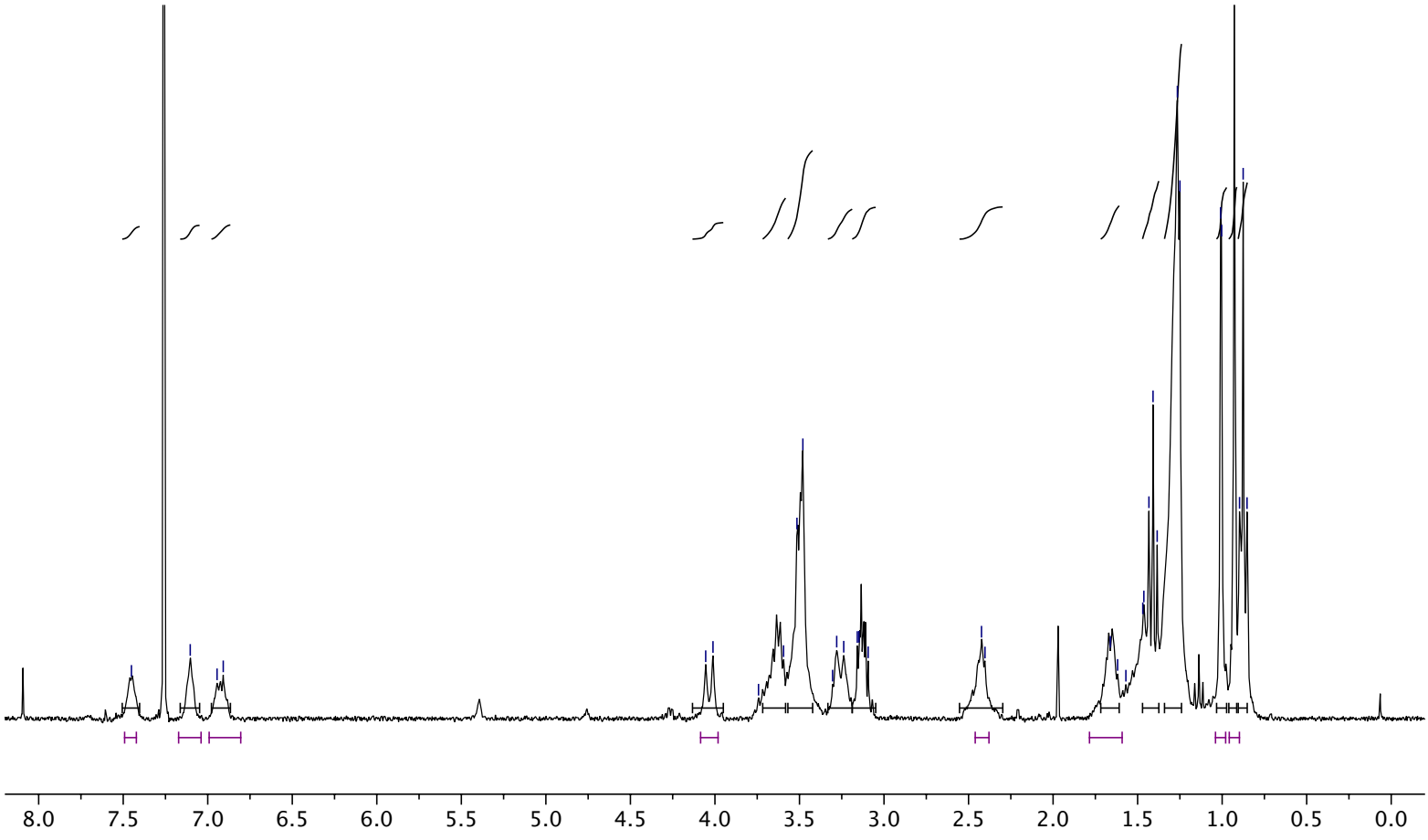


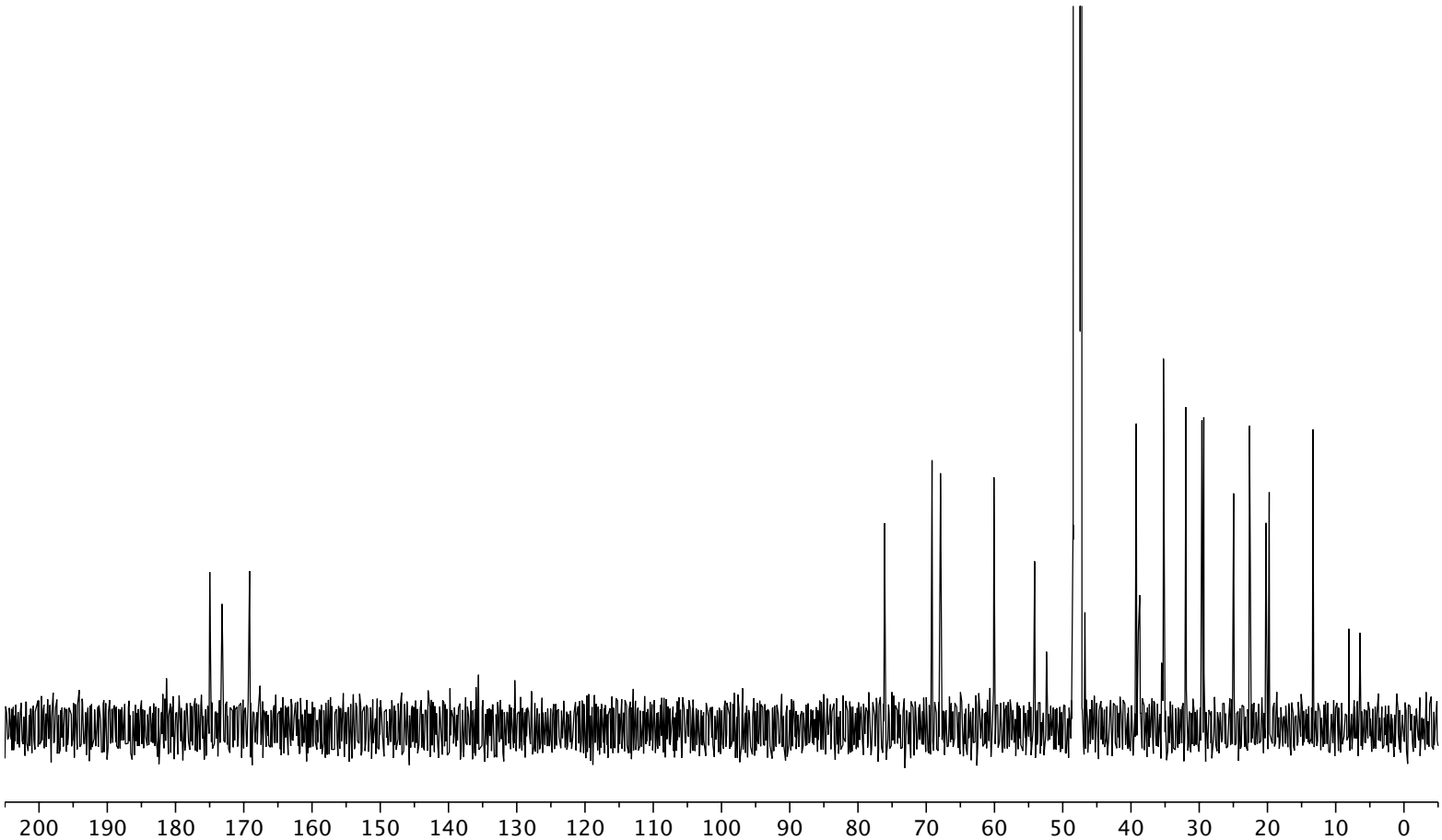


^{1}H–NMR (400 MHz) expansions for **S2** in $CDCl_3$

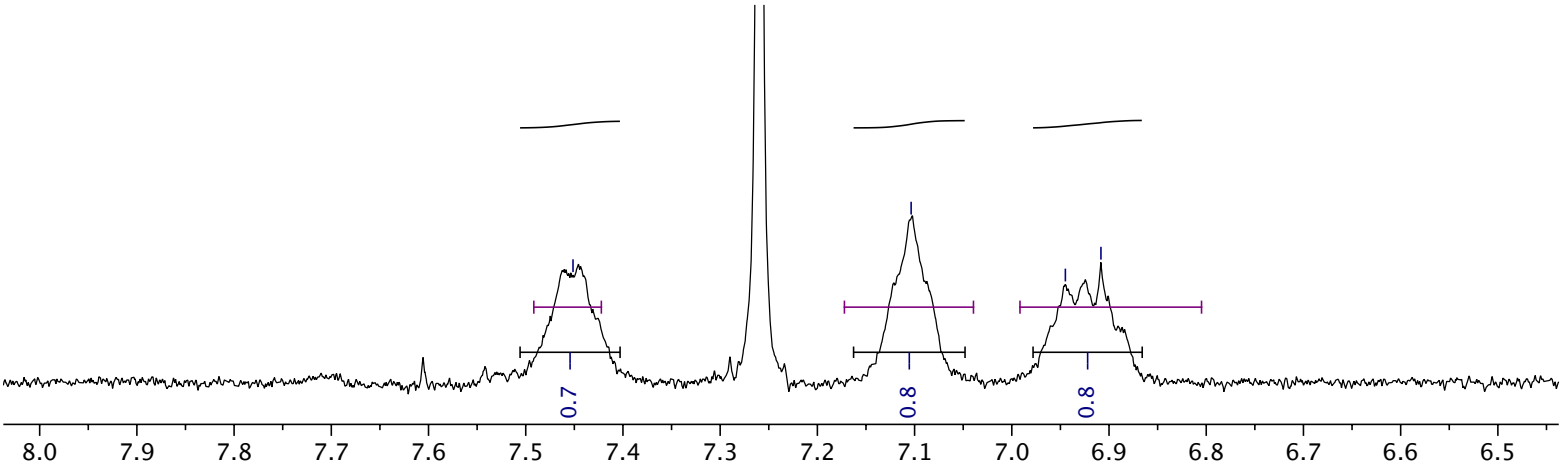


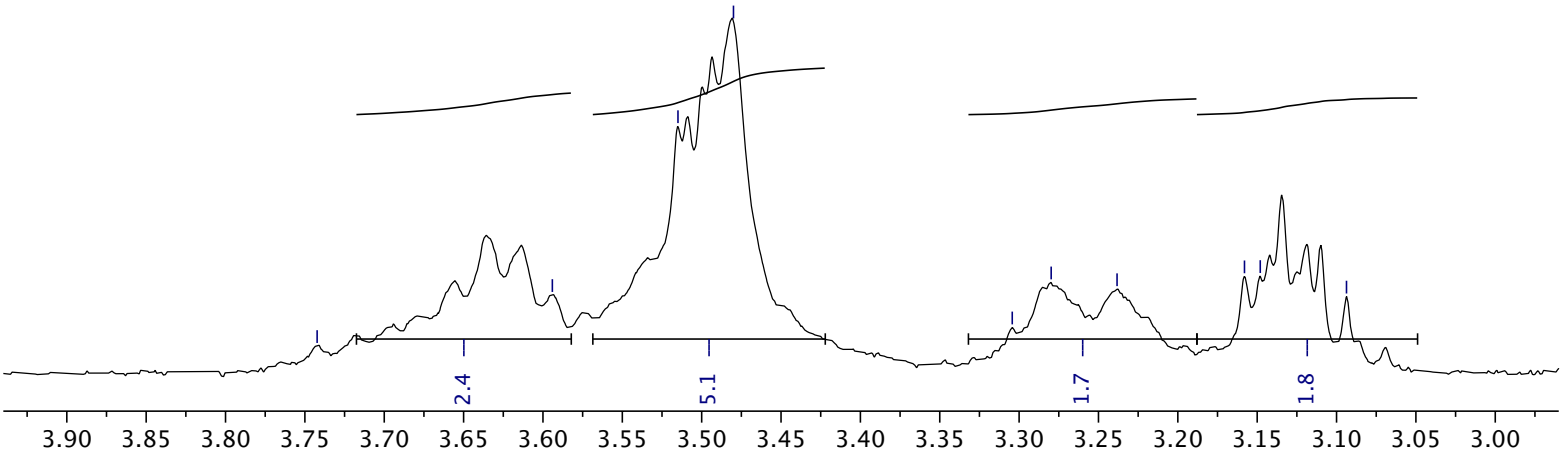
^{1}H-NMR (300 MHz) in $CDCl_3$ and ^{13}C-NMR (125 MHz) spectra of **2c** in CD_3OD

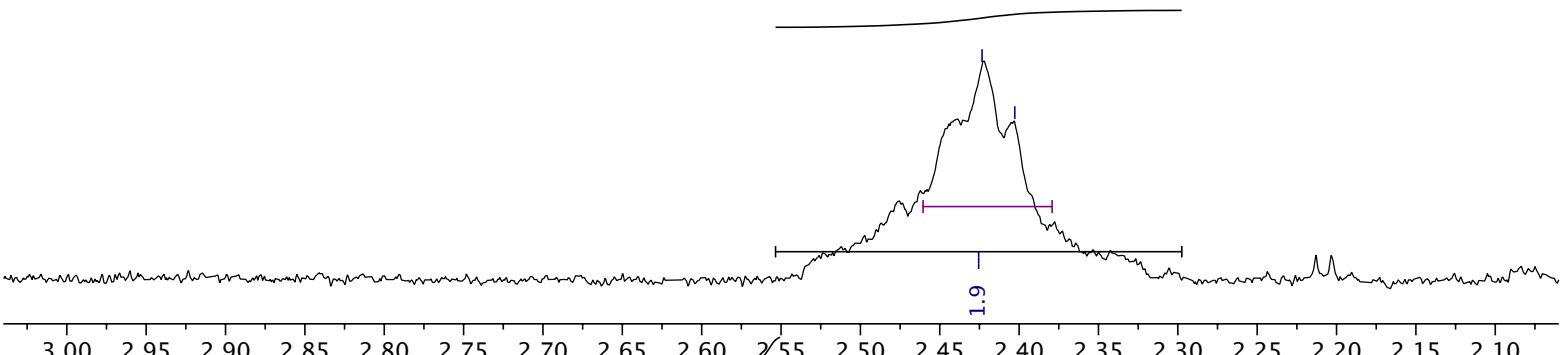


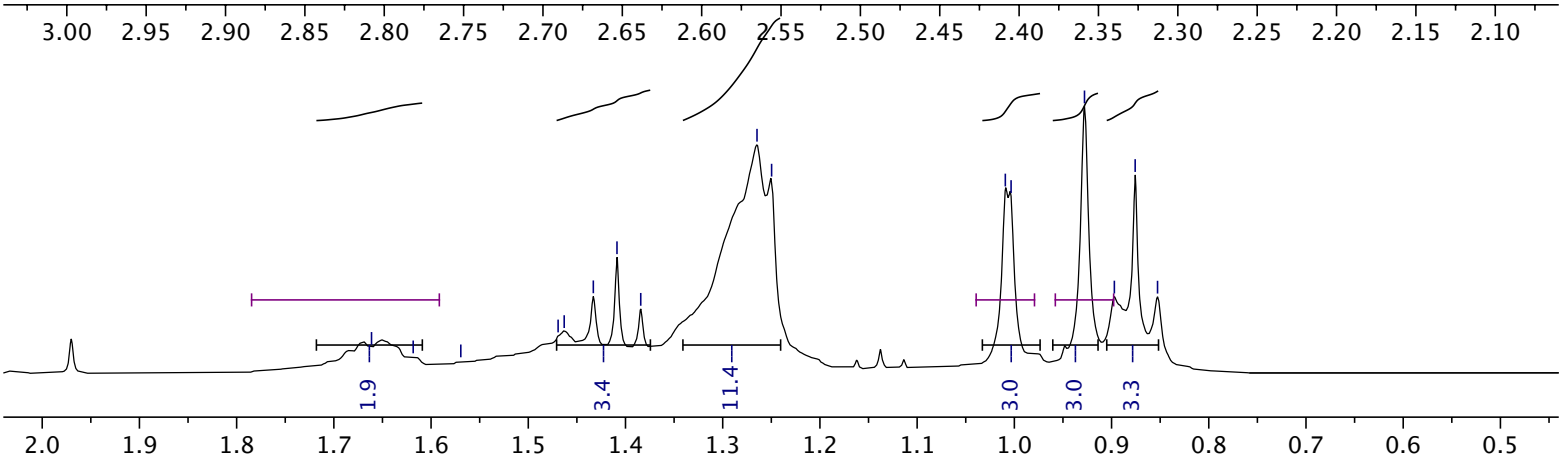


^{1}H–NMR (300 MHz) expansions for **S1** in $CDCl_3$

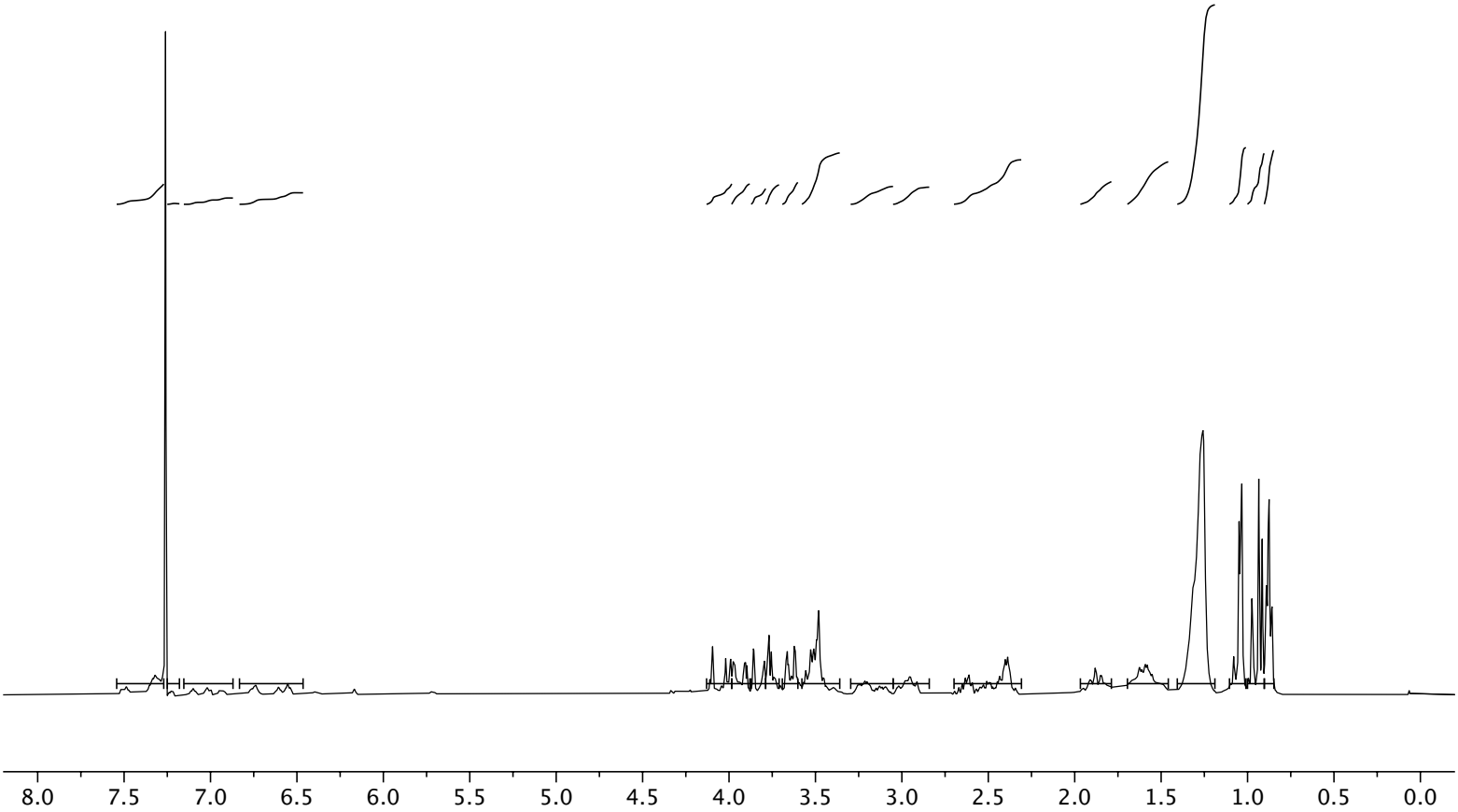


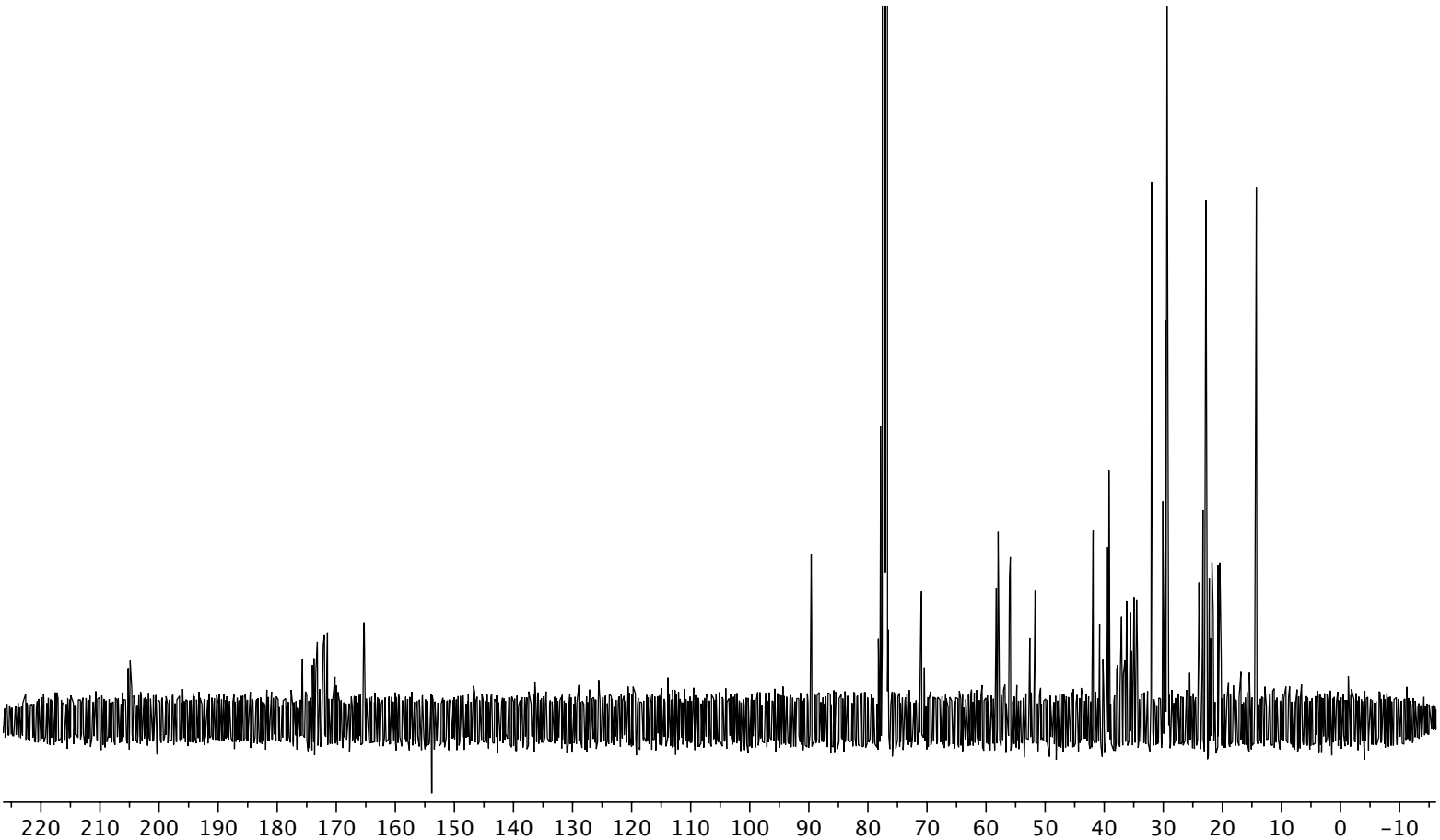




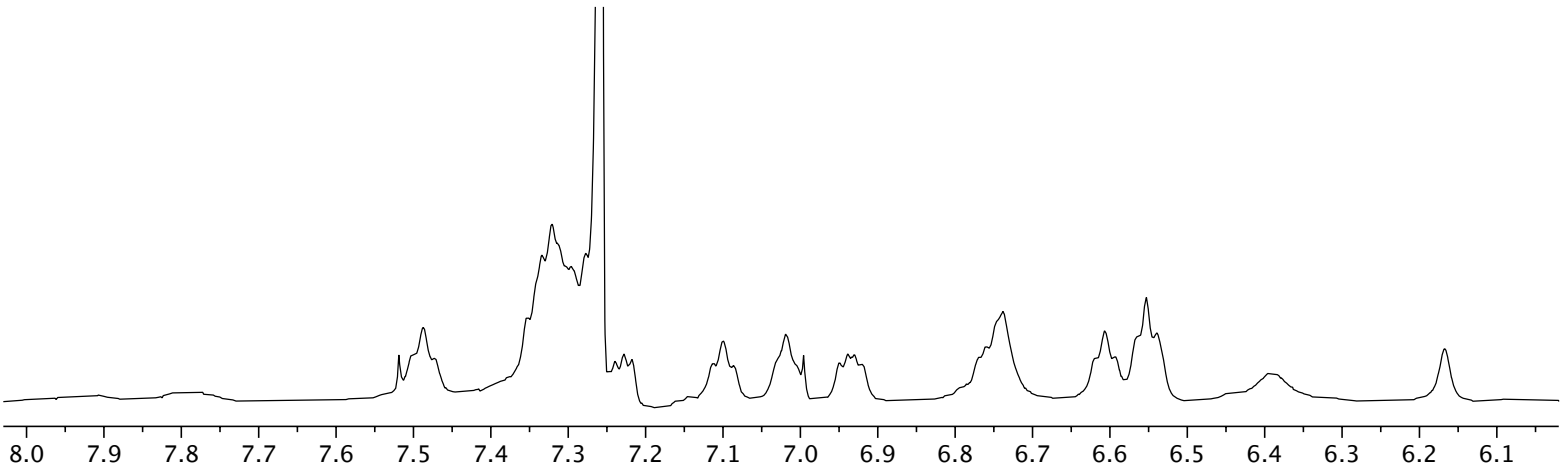


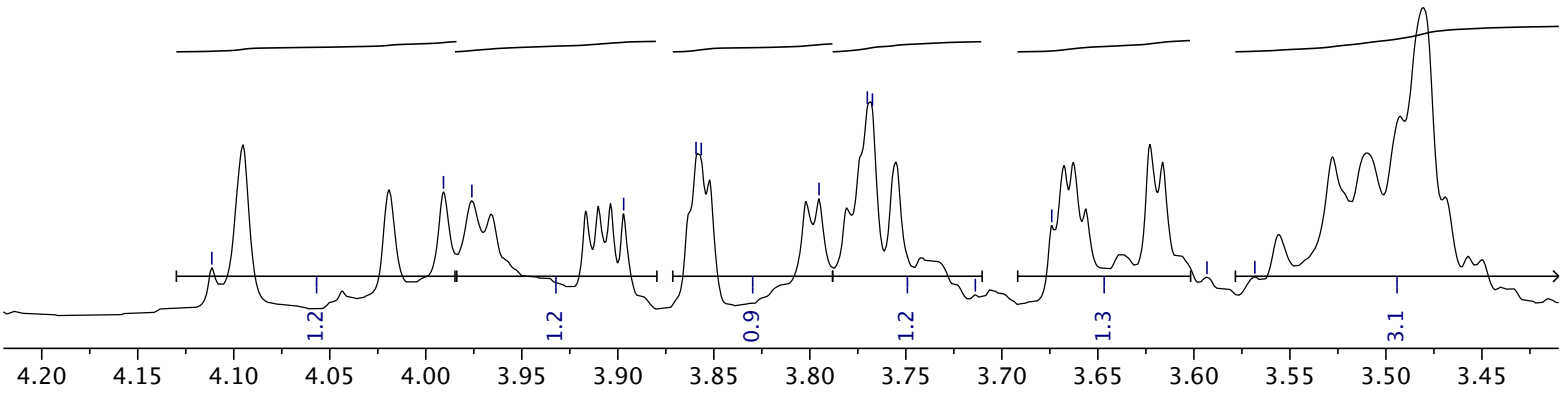
^{1}H–NMR (400 MHz) and ^{13}C–NMR (125 MHz) spectra of **1c** in $CDCl_3$

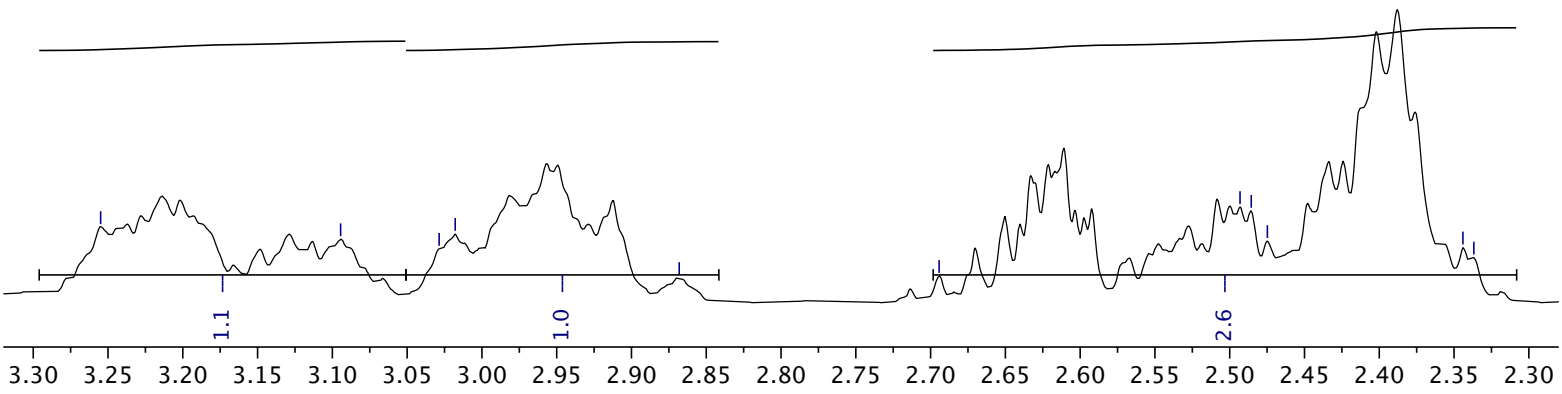


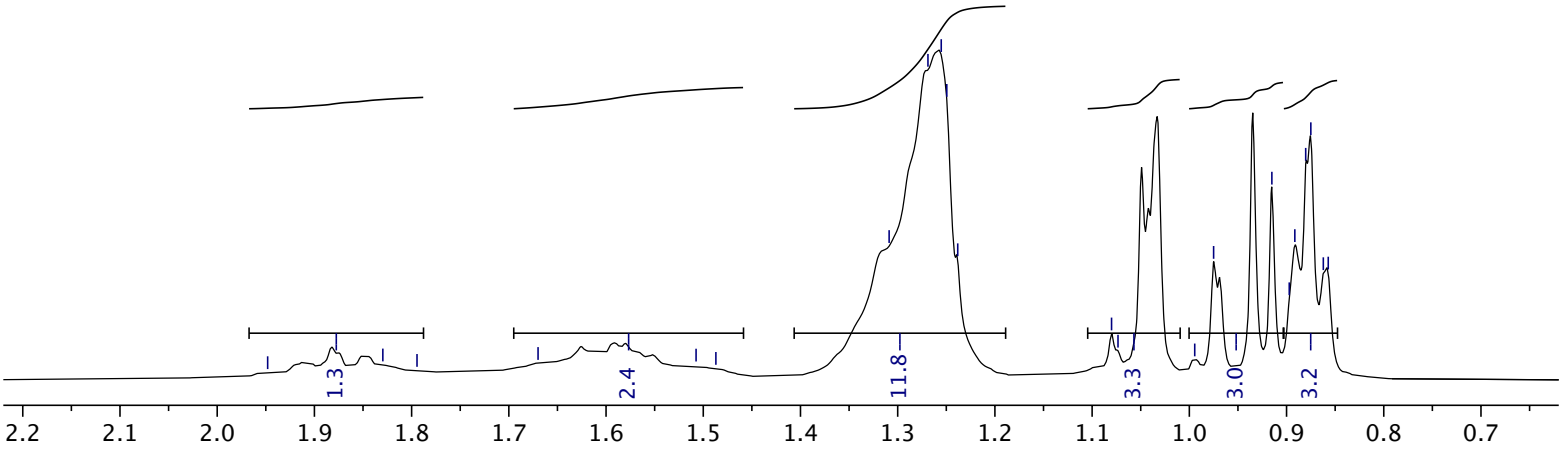


^{1}H-NMR (400 MHz) expansions for **1c** in $CDCl_3$

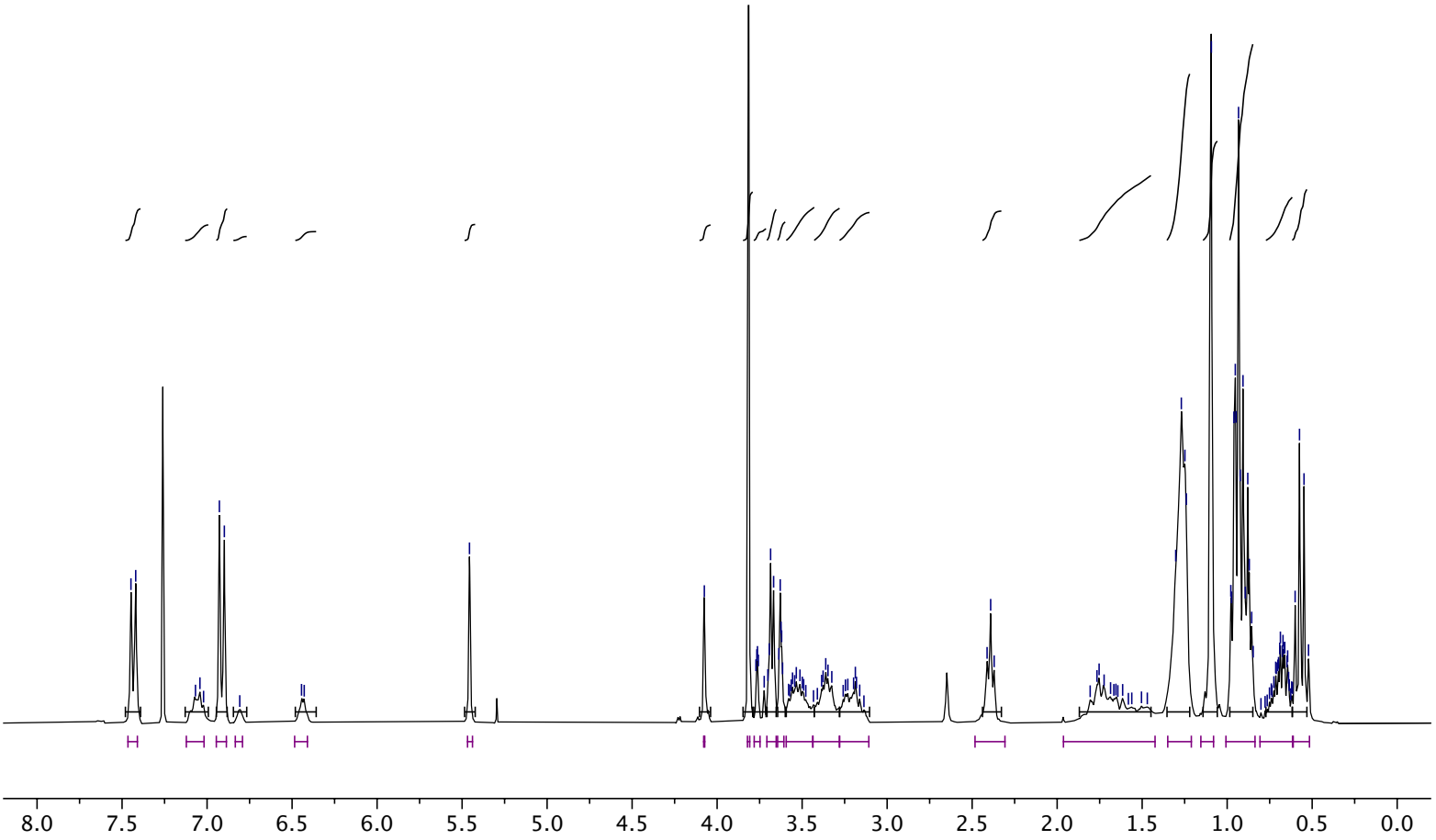


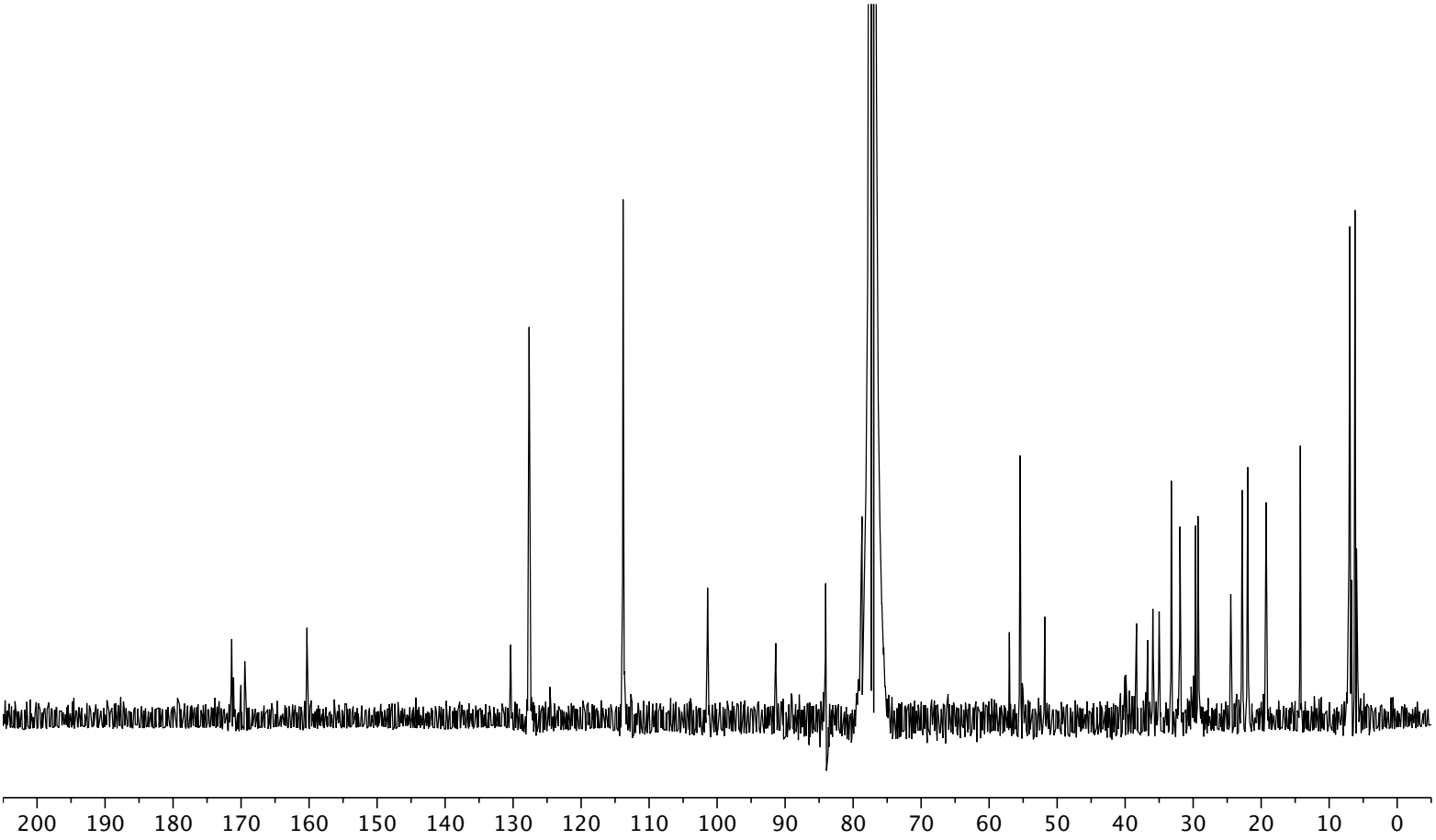




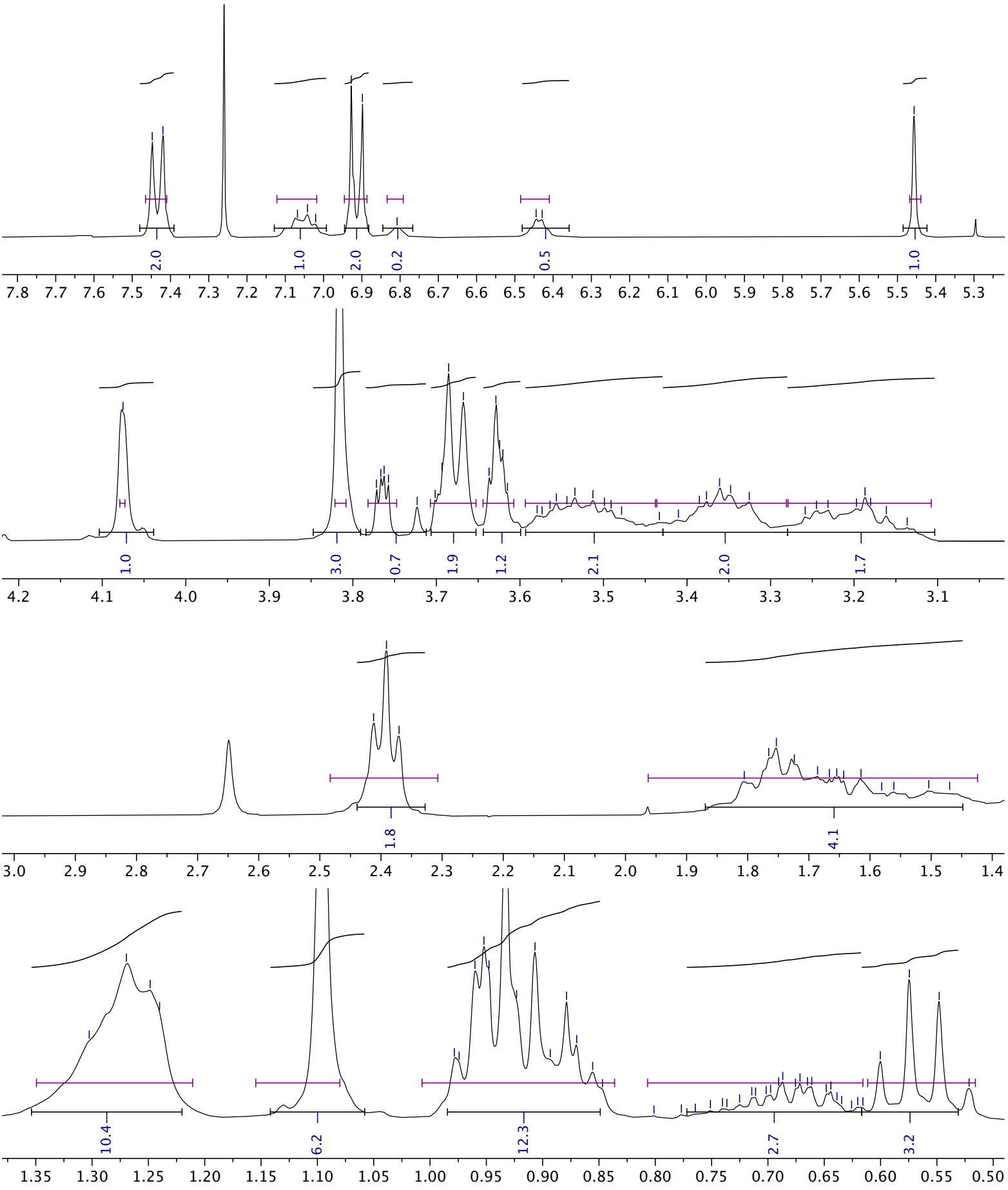


^{1}H–NMR (300 MHz) and ^{13}C–NMR (100 MHz) spectra of **S3** in $CDCl_3$

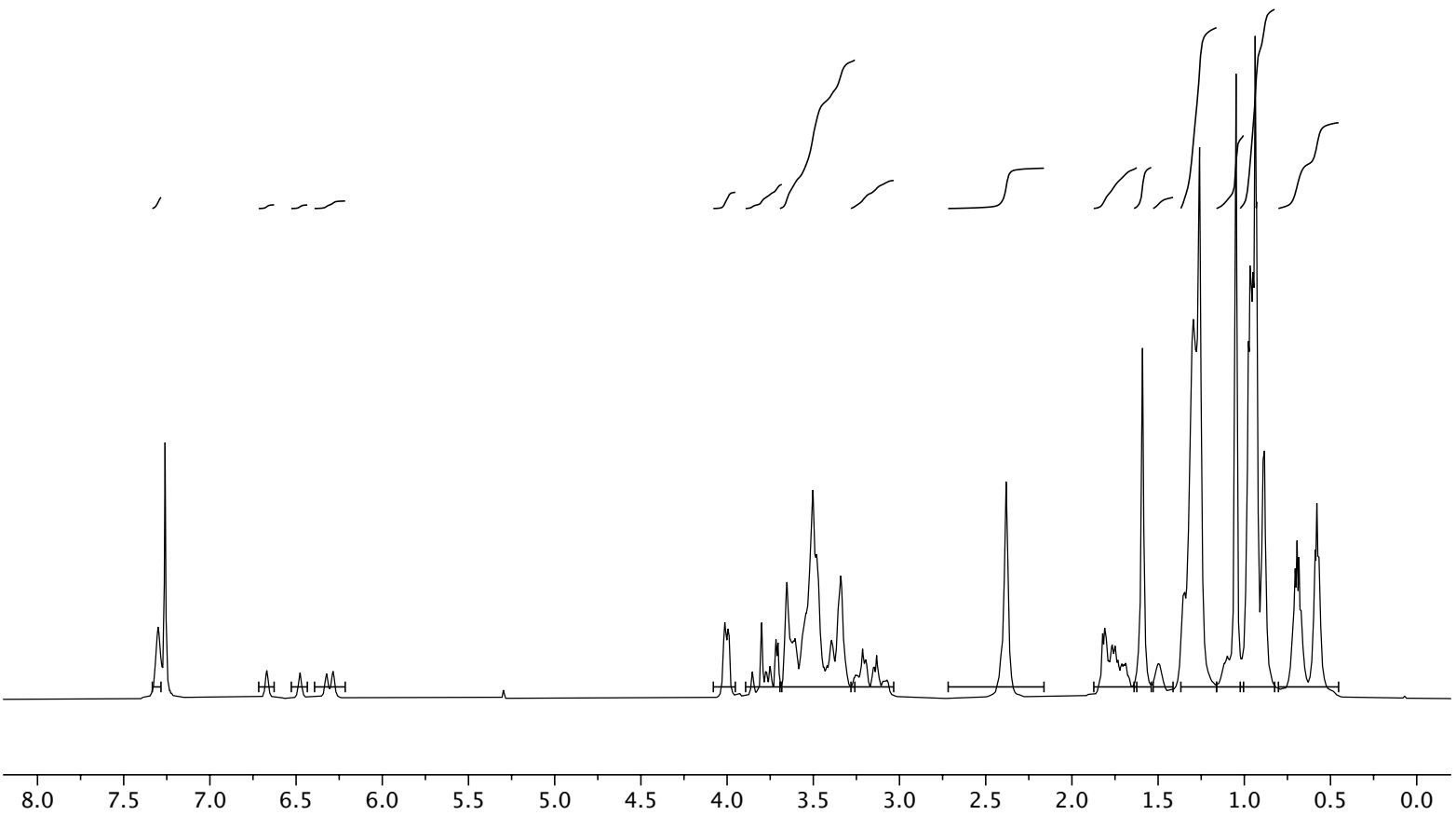


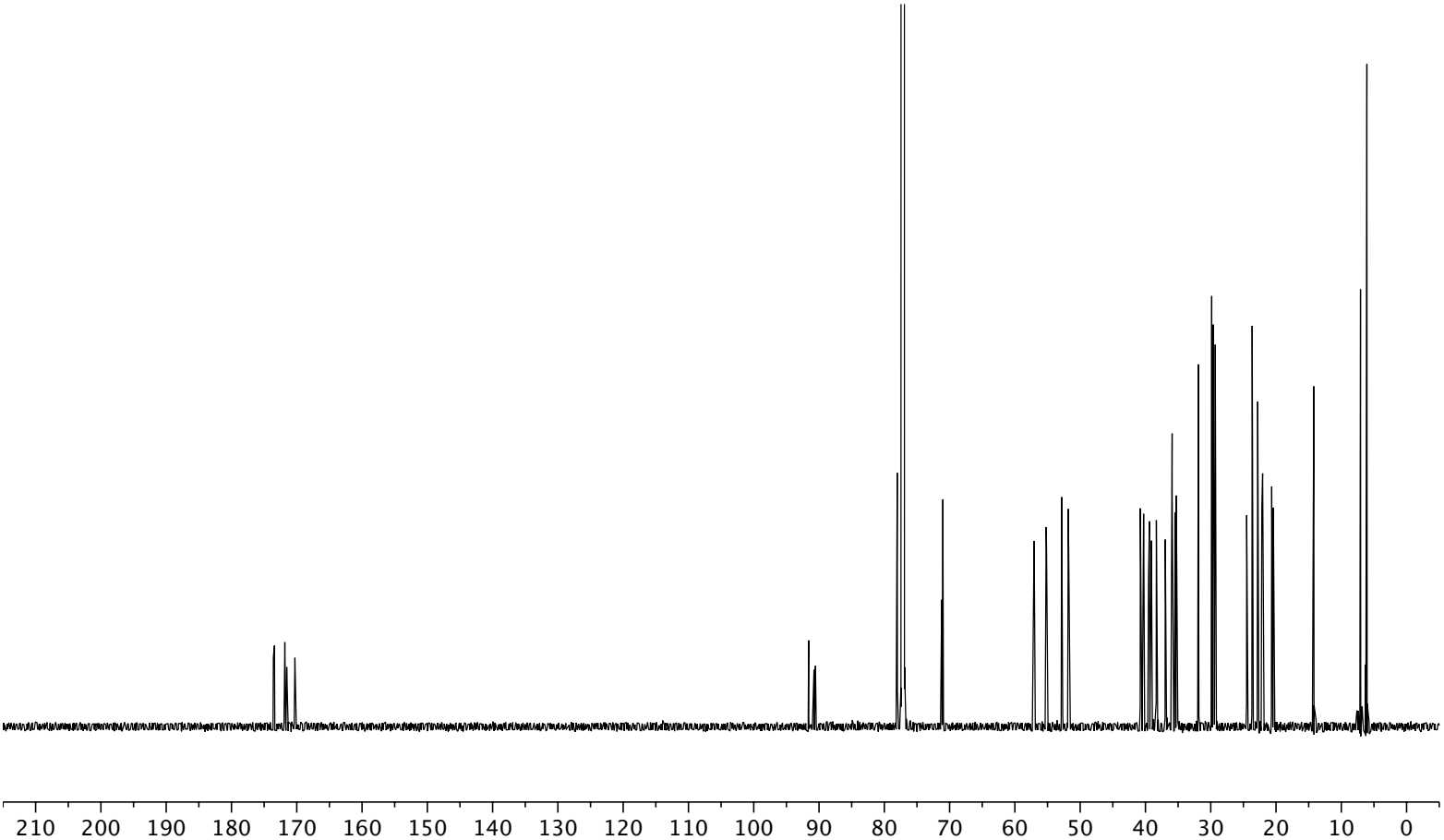


^{1}H-NMR (300 MHz) expansions for **S3** in $CDCl_3$

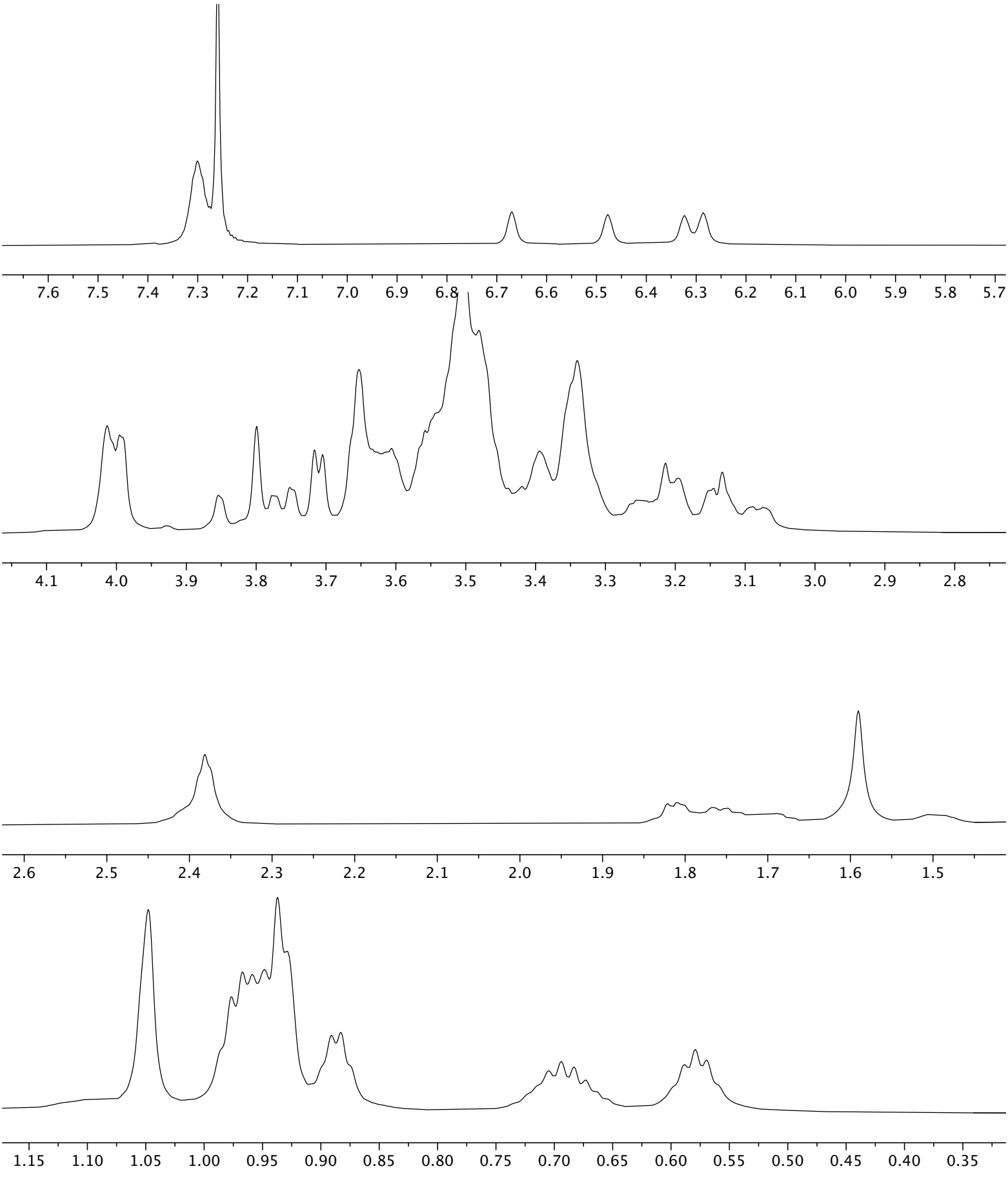


^{1}H–NMR (800 MHz) and ^{13}C–NMR (200 MHz) spectra of **8c** in $CDCl_3$

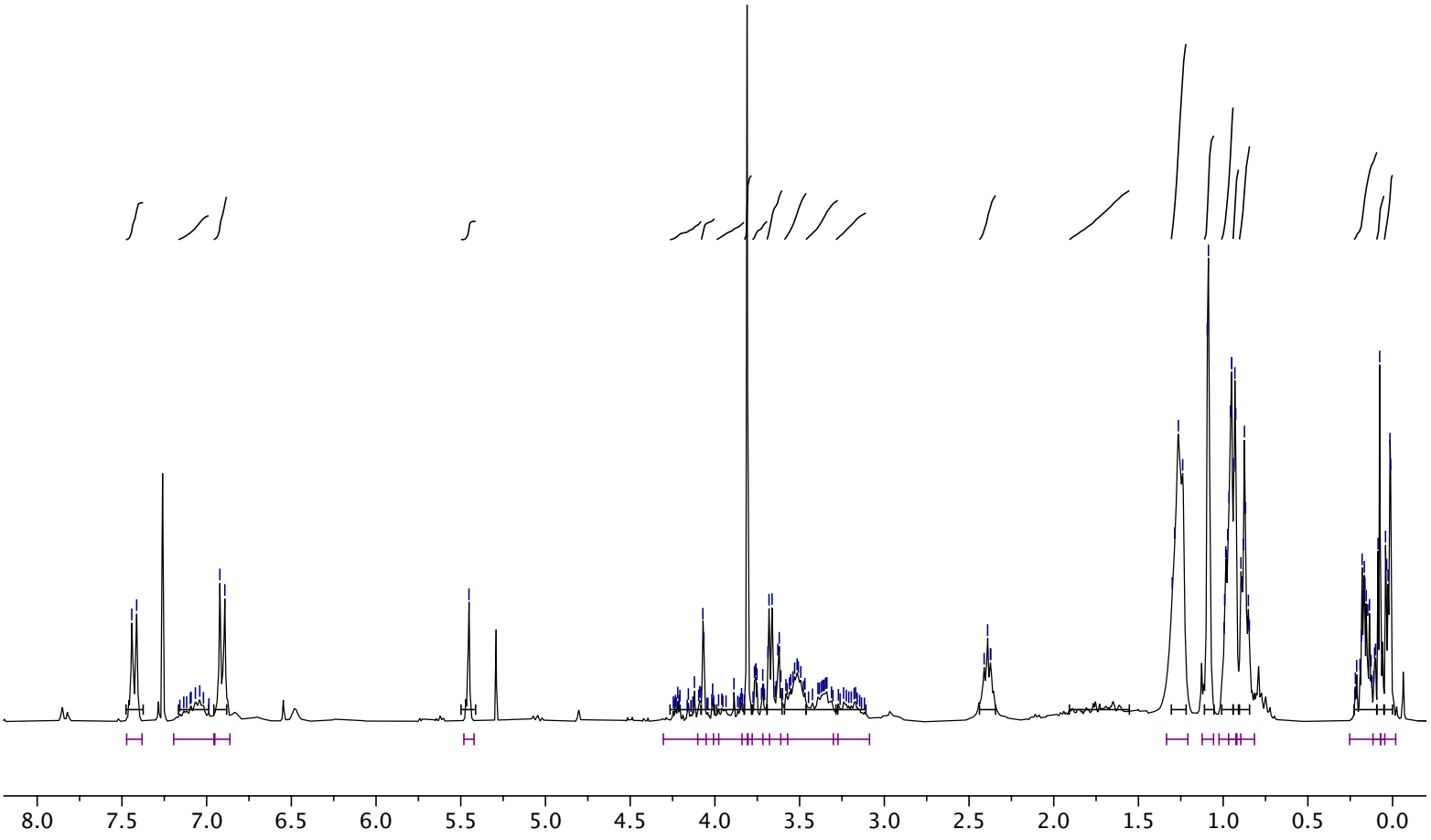


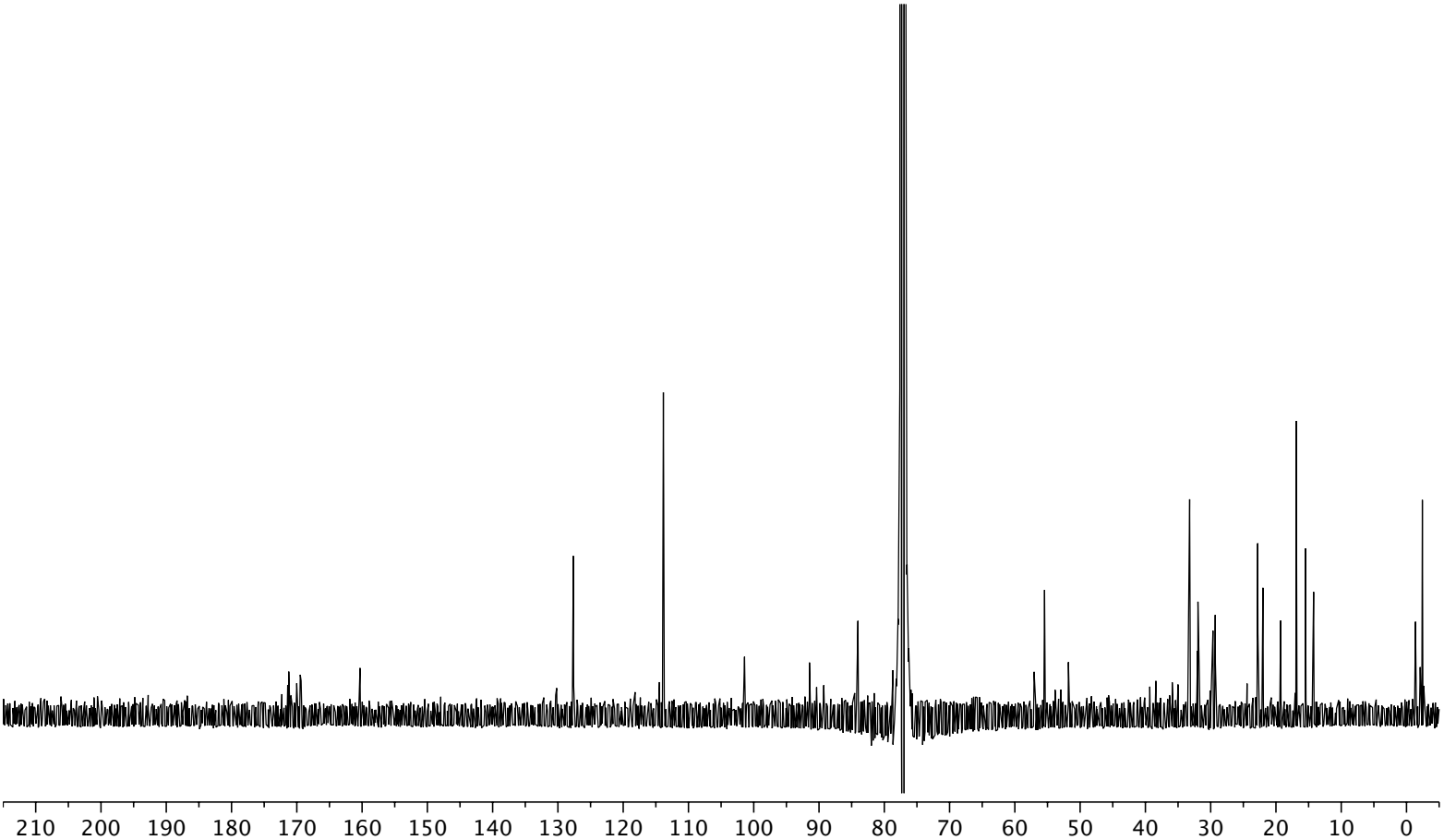


^{1}H–NMR (500 MHz) expansions for **8c** in $CDCl_3$

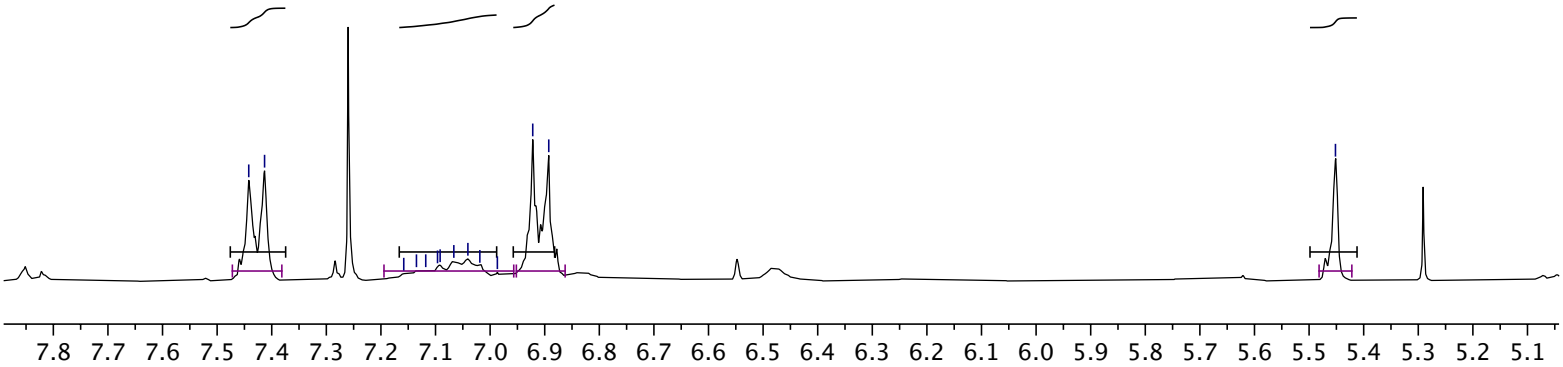


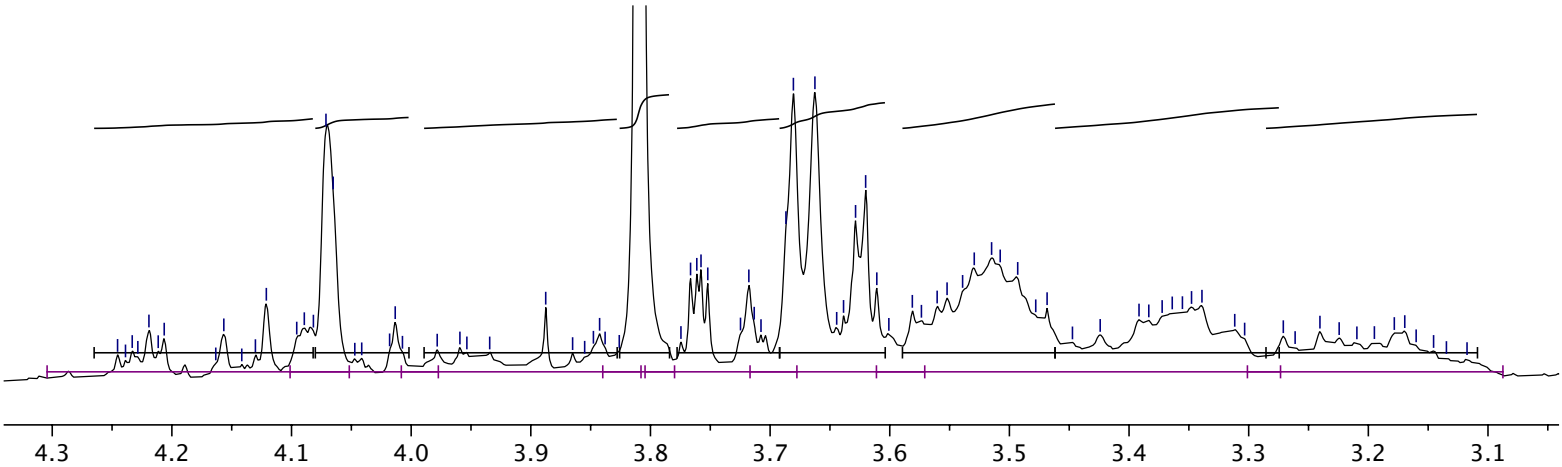
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **S4** in $CDCl_3$

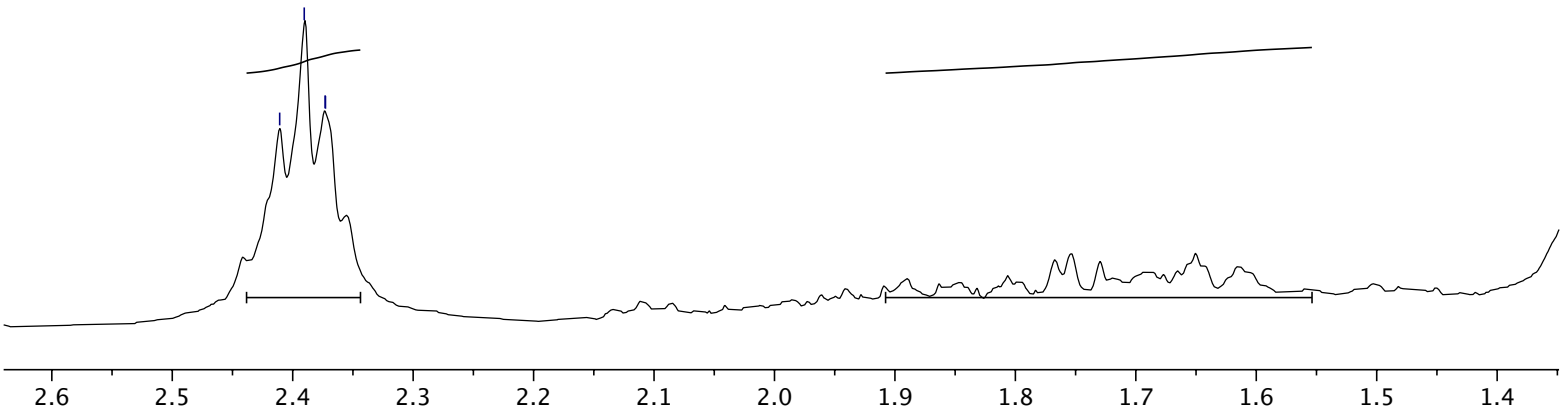


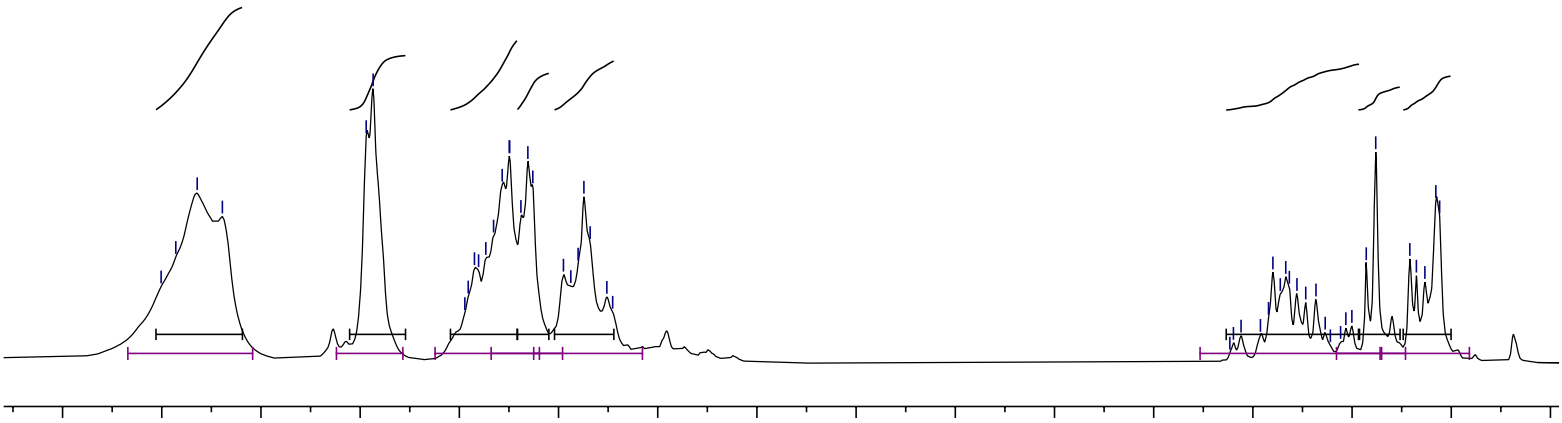


^{1}H-NMR (500 MHz) expansions for **S4** in $CDCl_3$

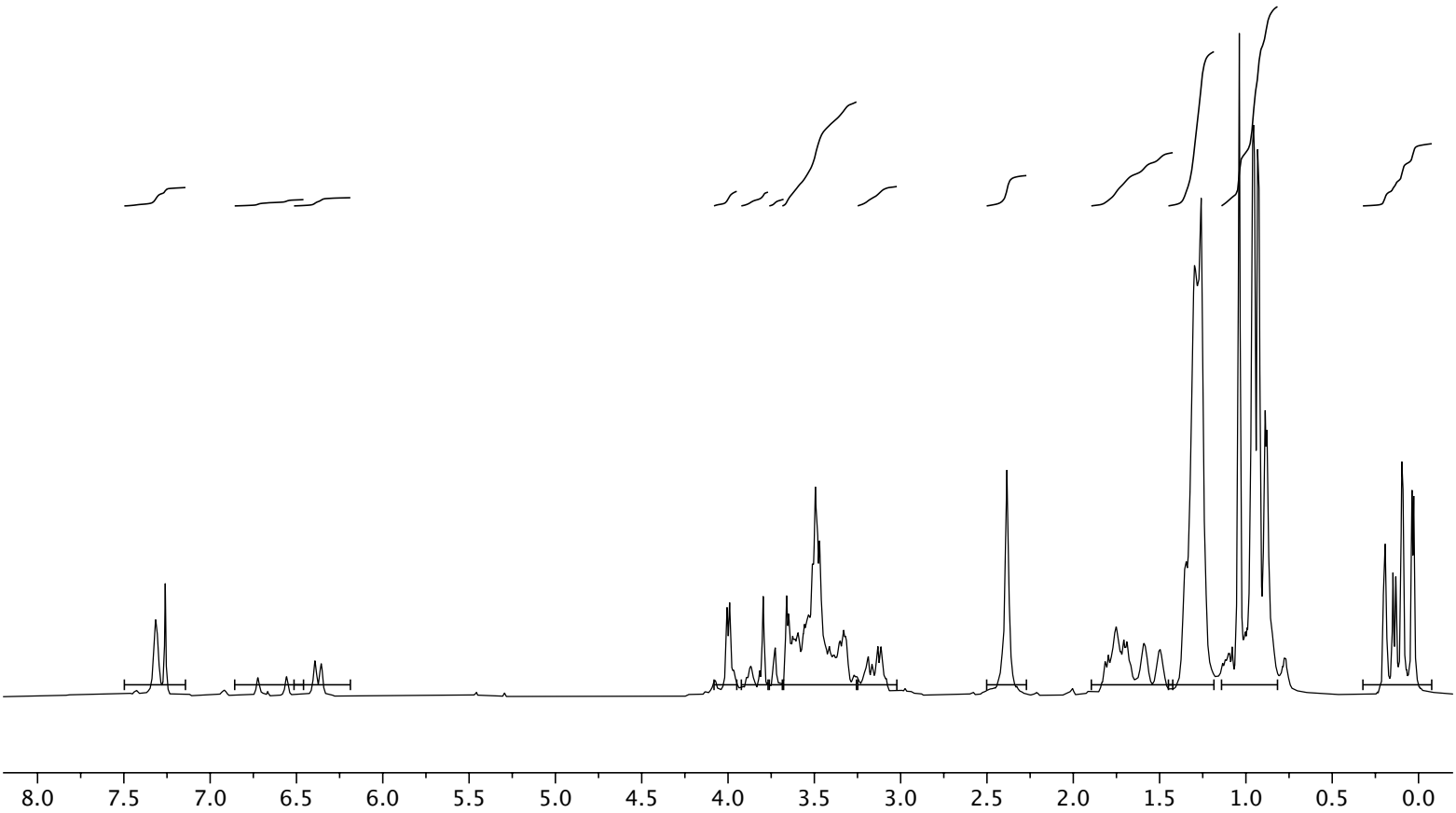


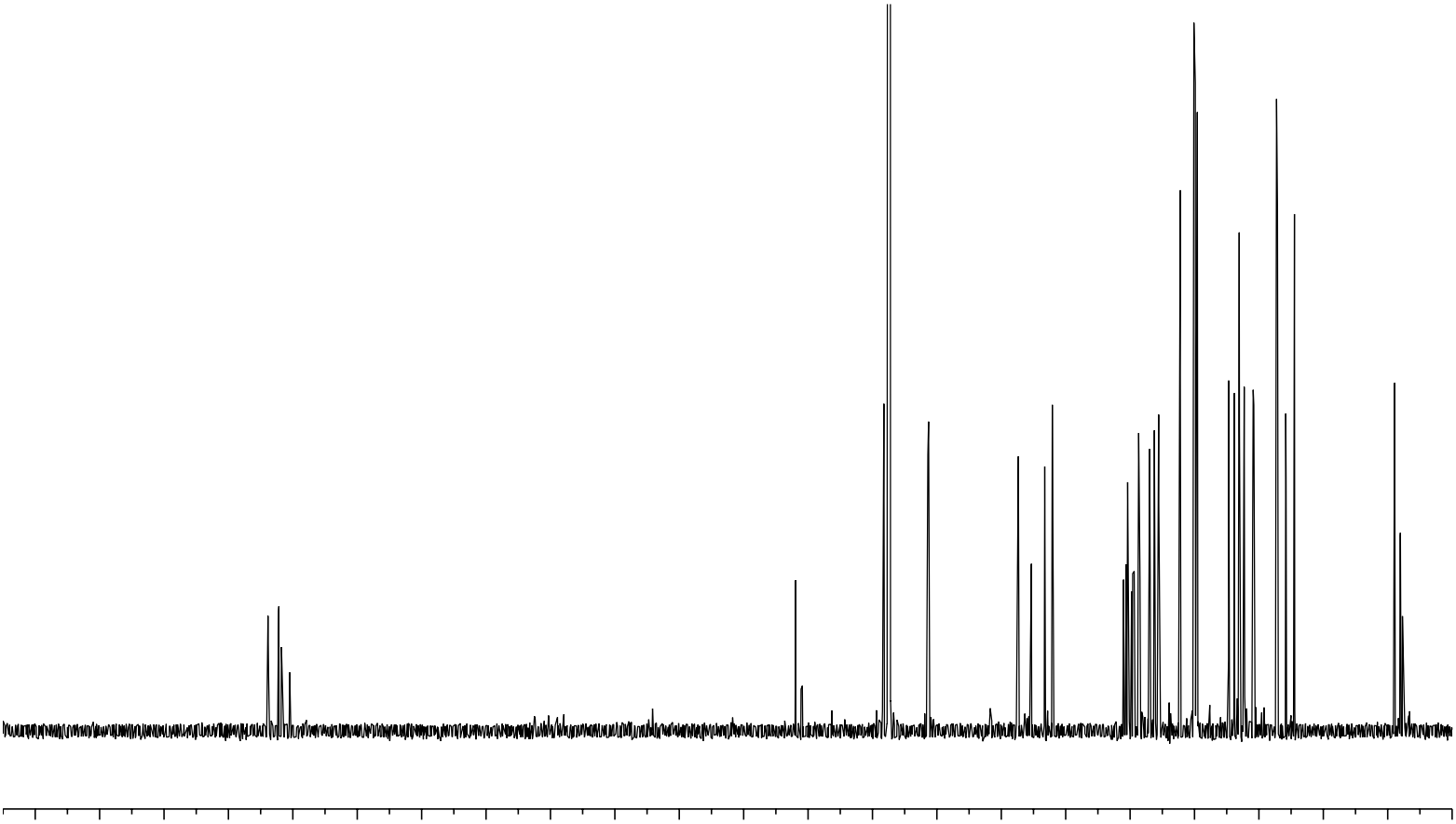




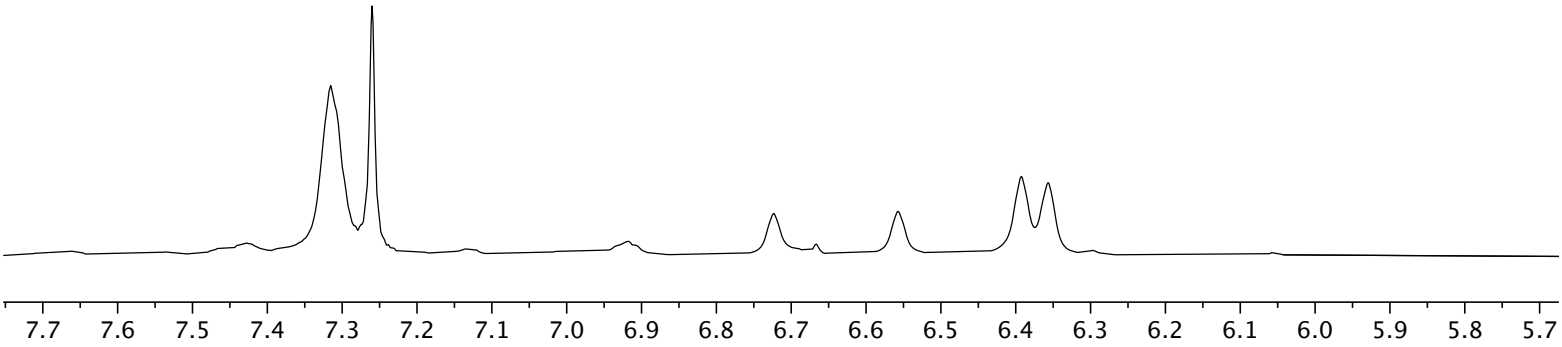


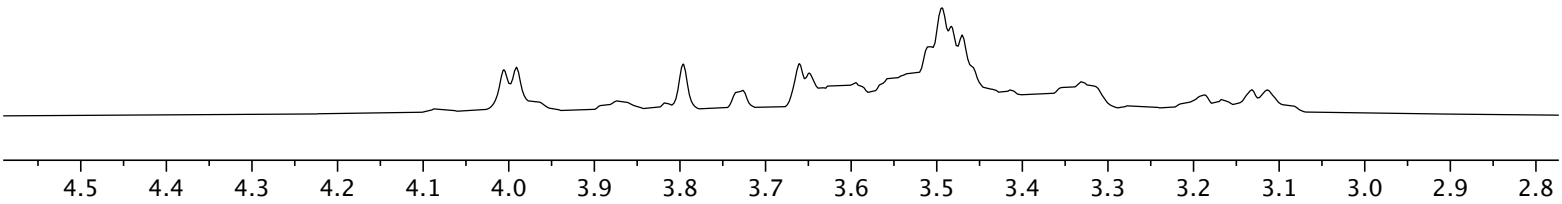
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **9c** in $CDCl_3$

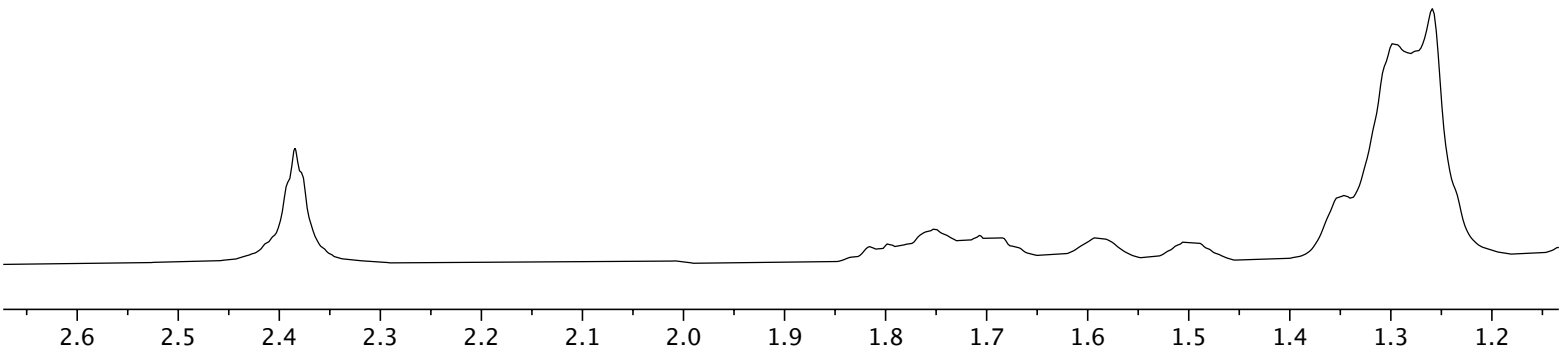


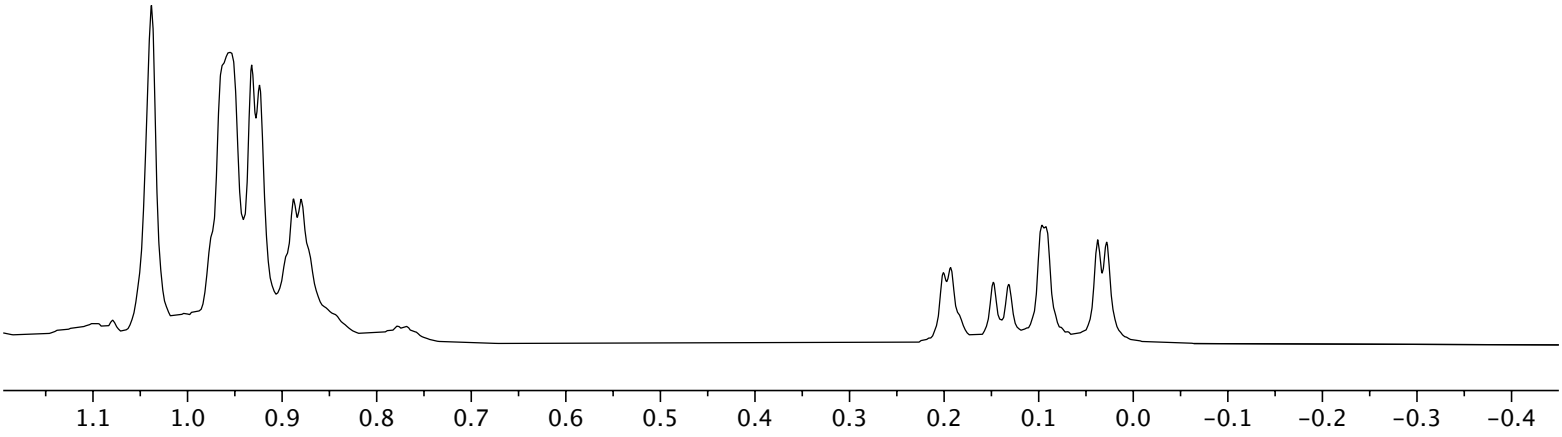


^{1}H–NMR (500 MHz) expansions for 9**c** in $CDCl_3$

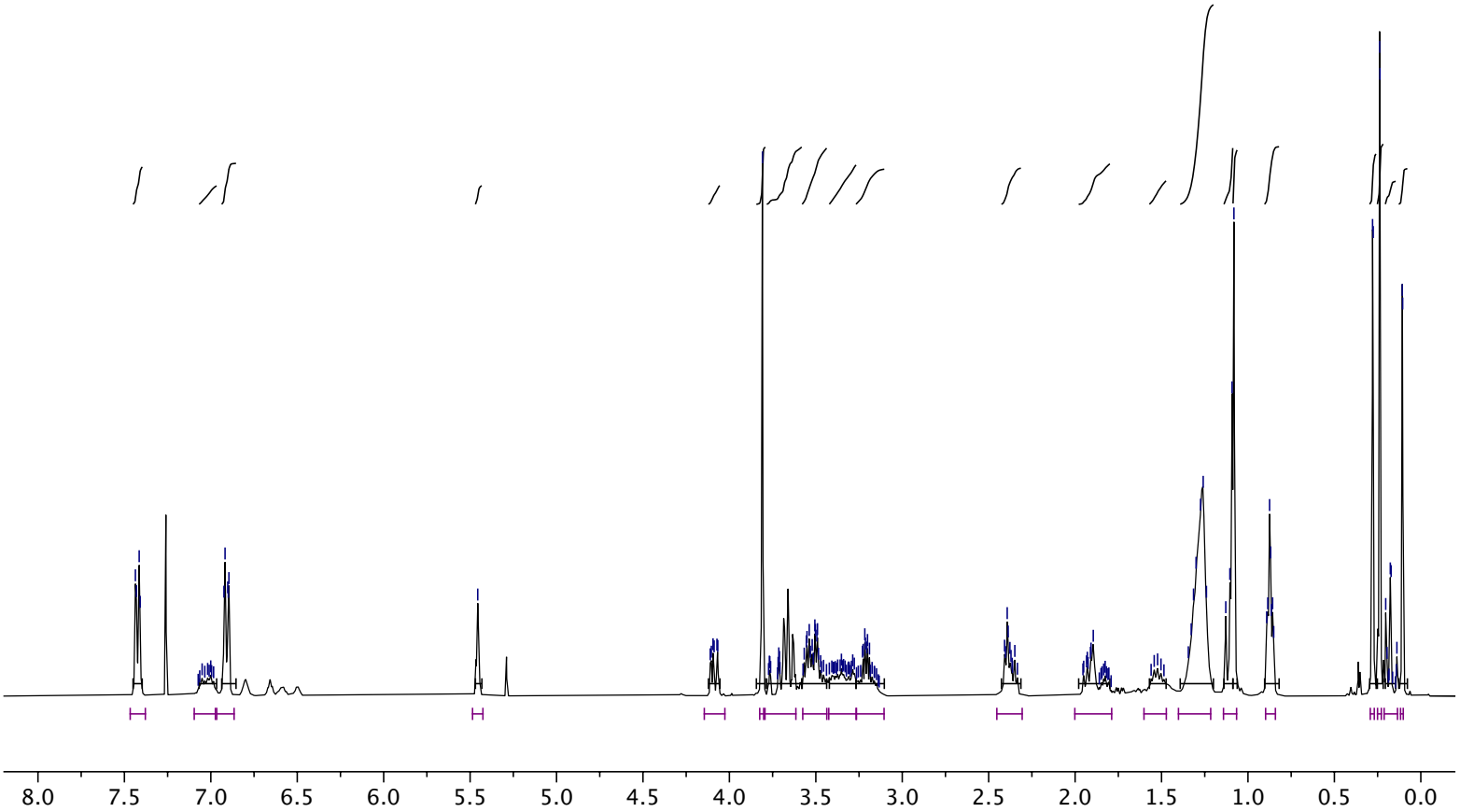


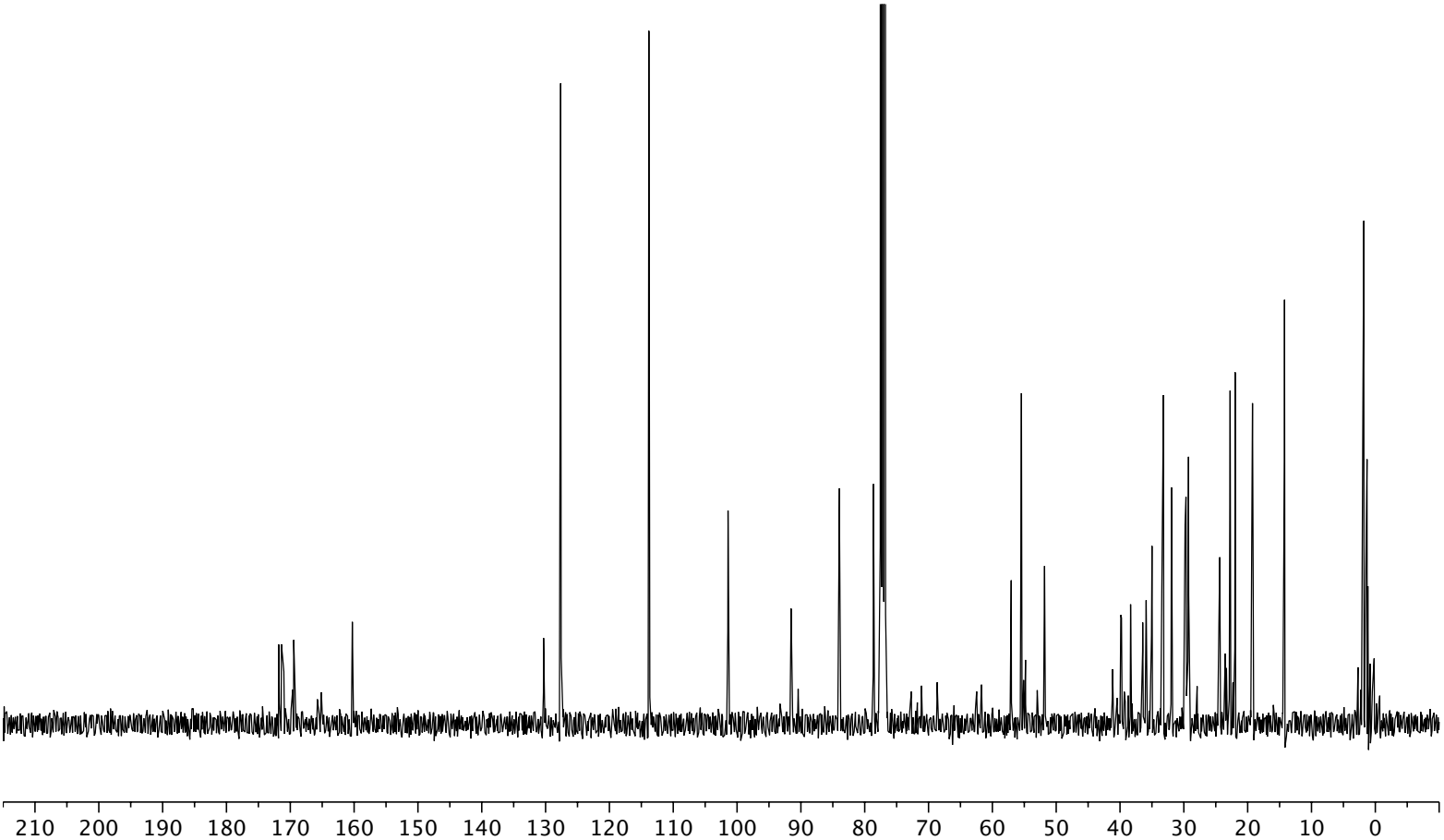




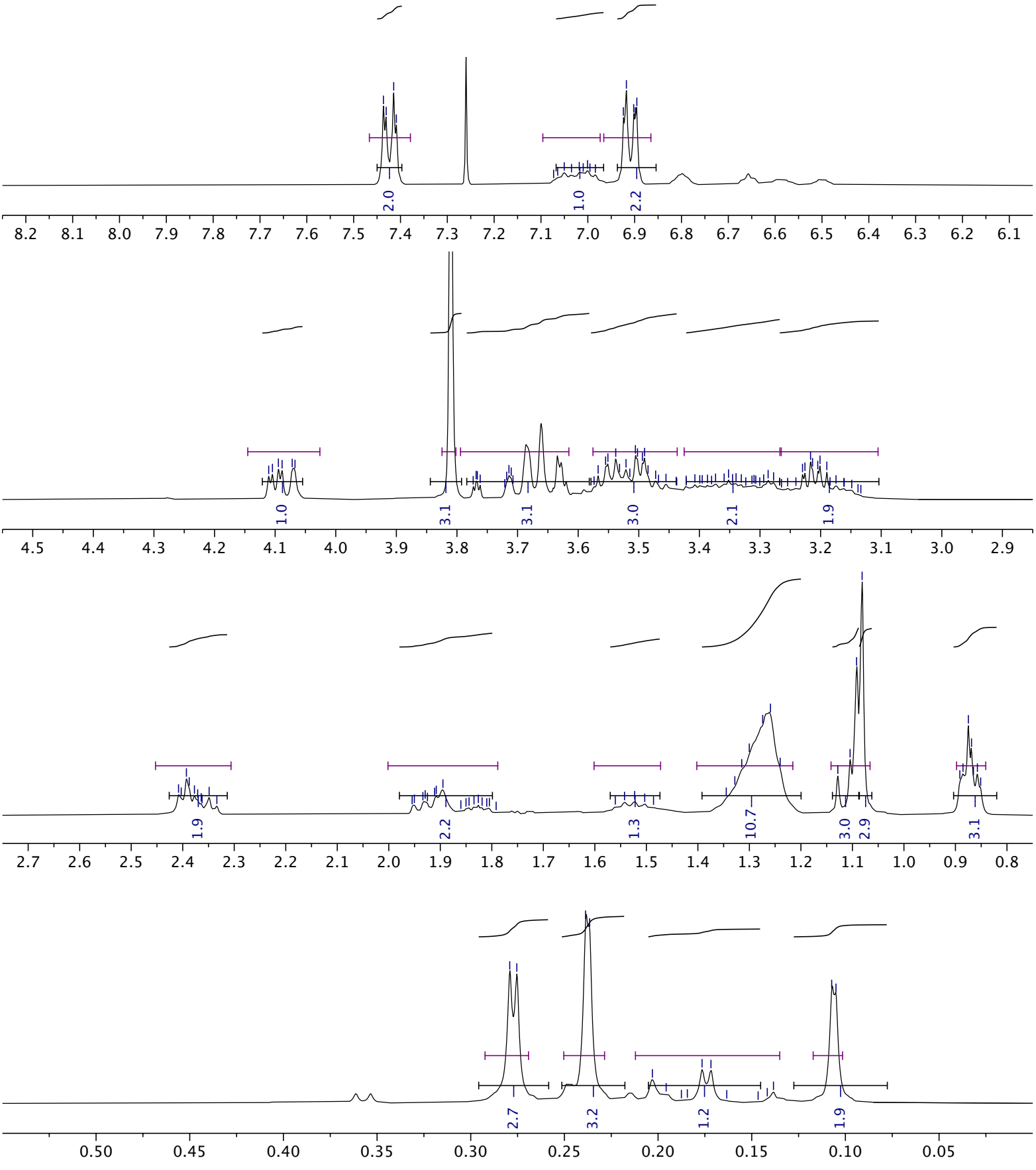


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **S5** in $CDCl_3$

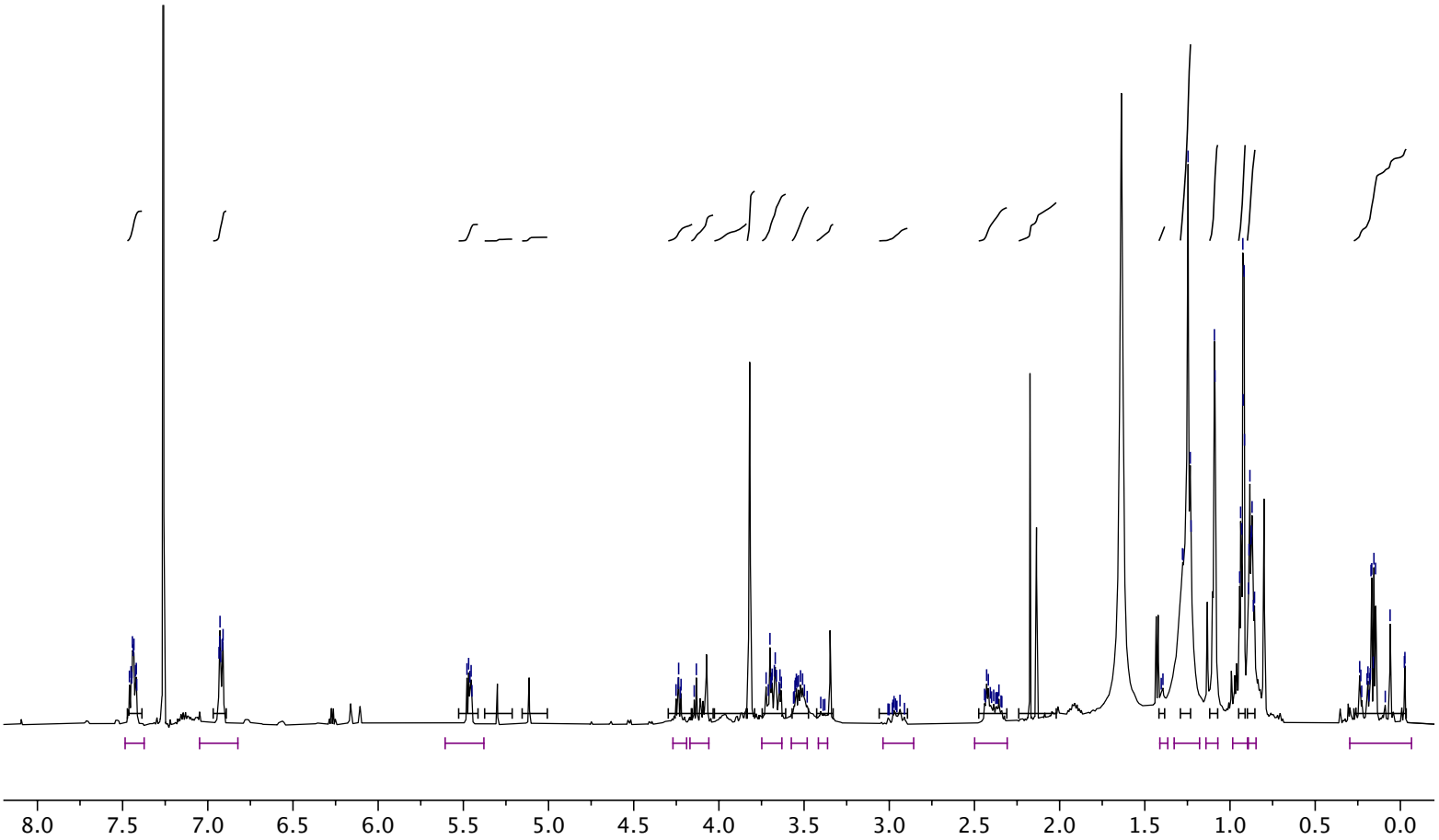


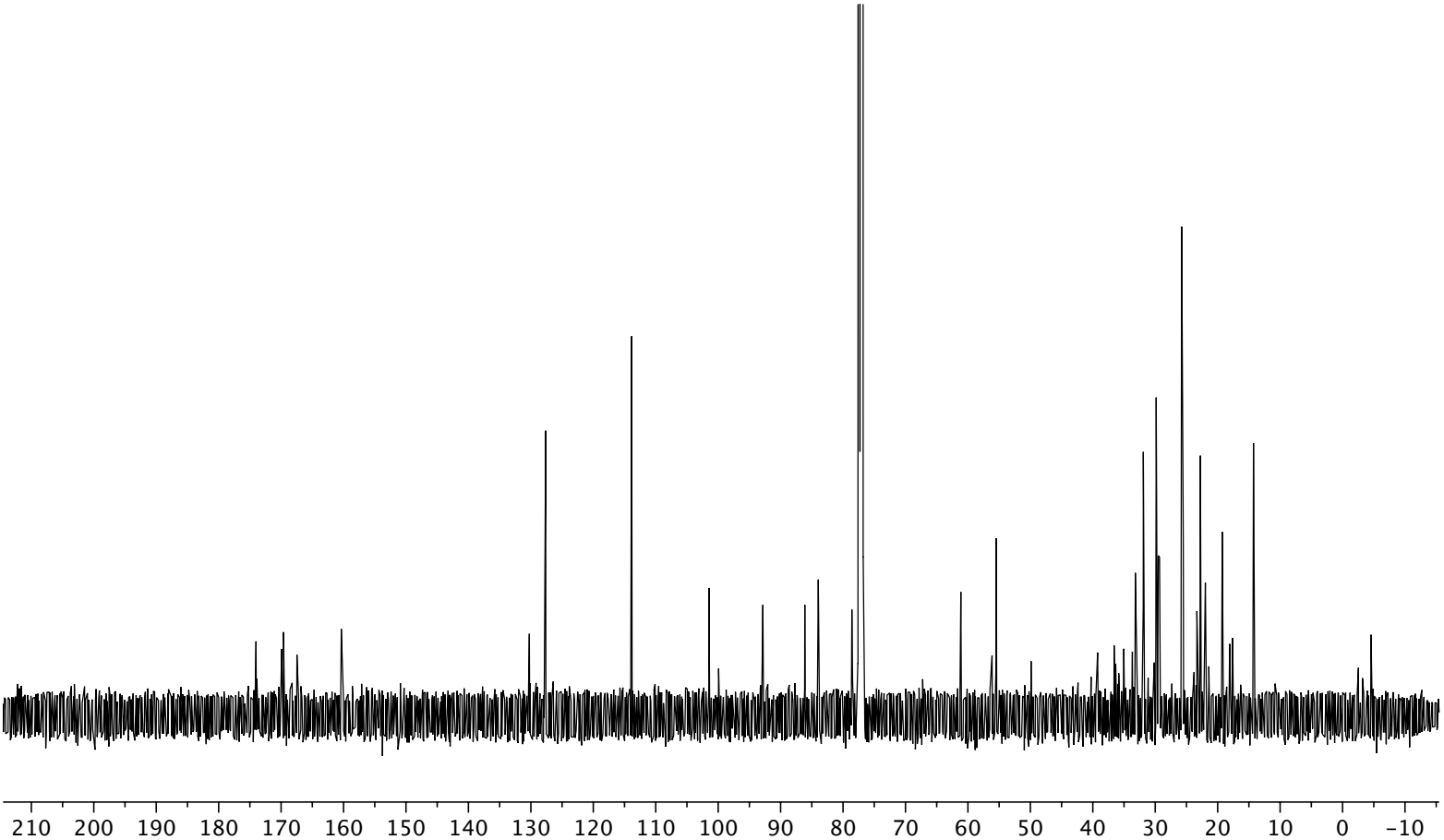


^{1}H–NMR (500 MHz) expansions for **S5** in $CDCl_3$

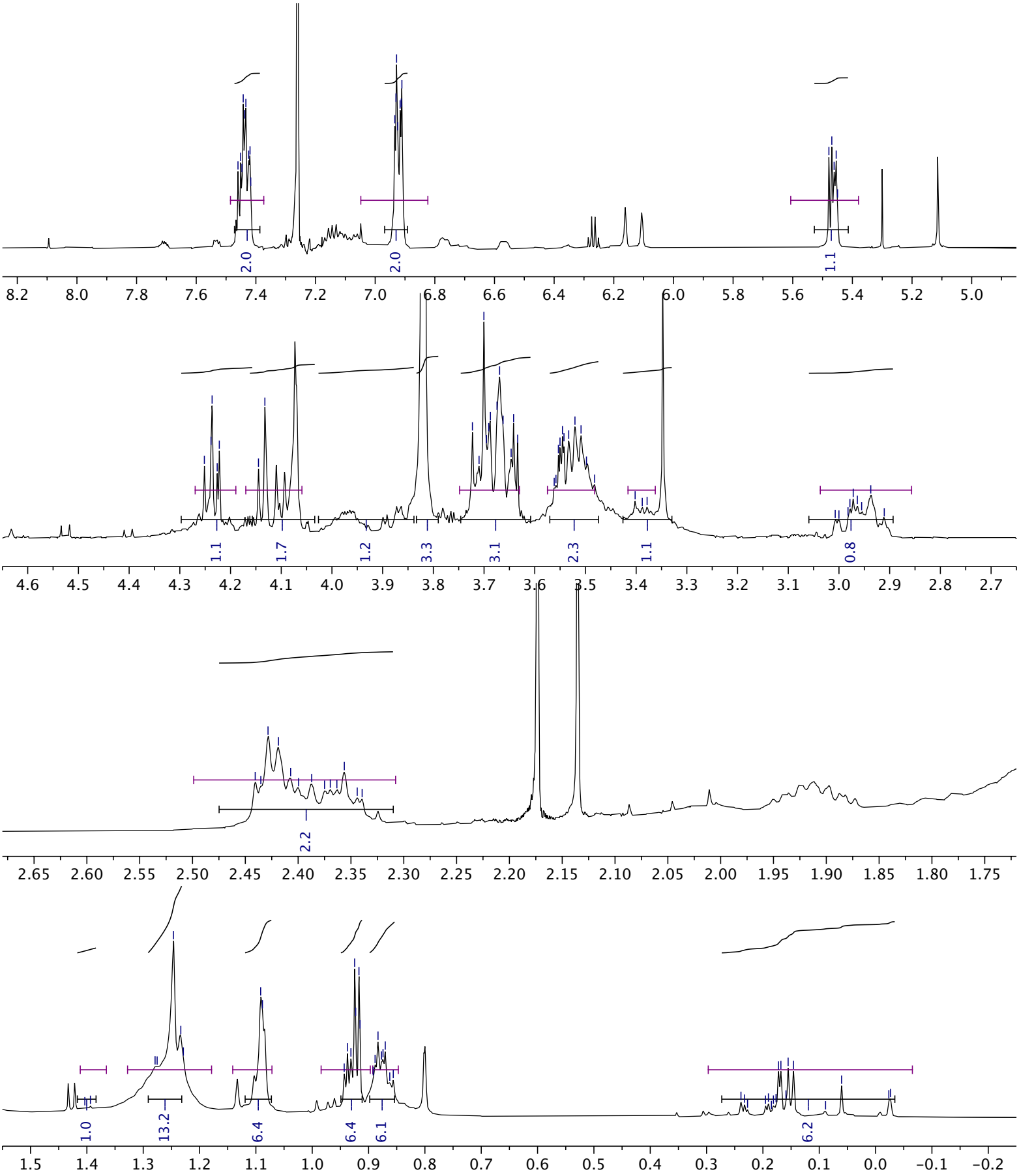


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **S8** in $CDCl_3$

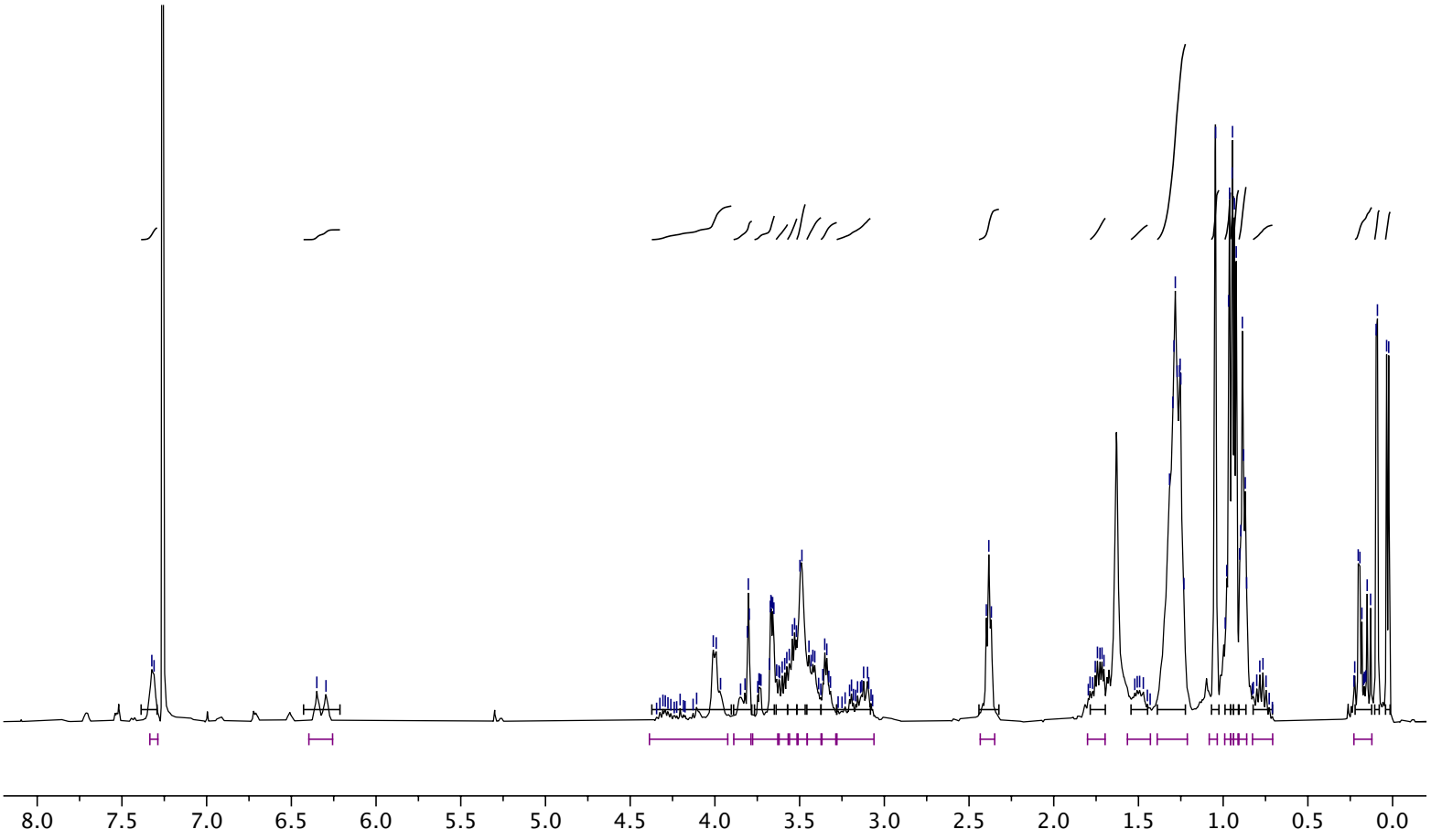


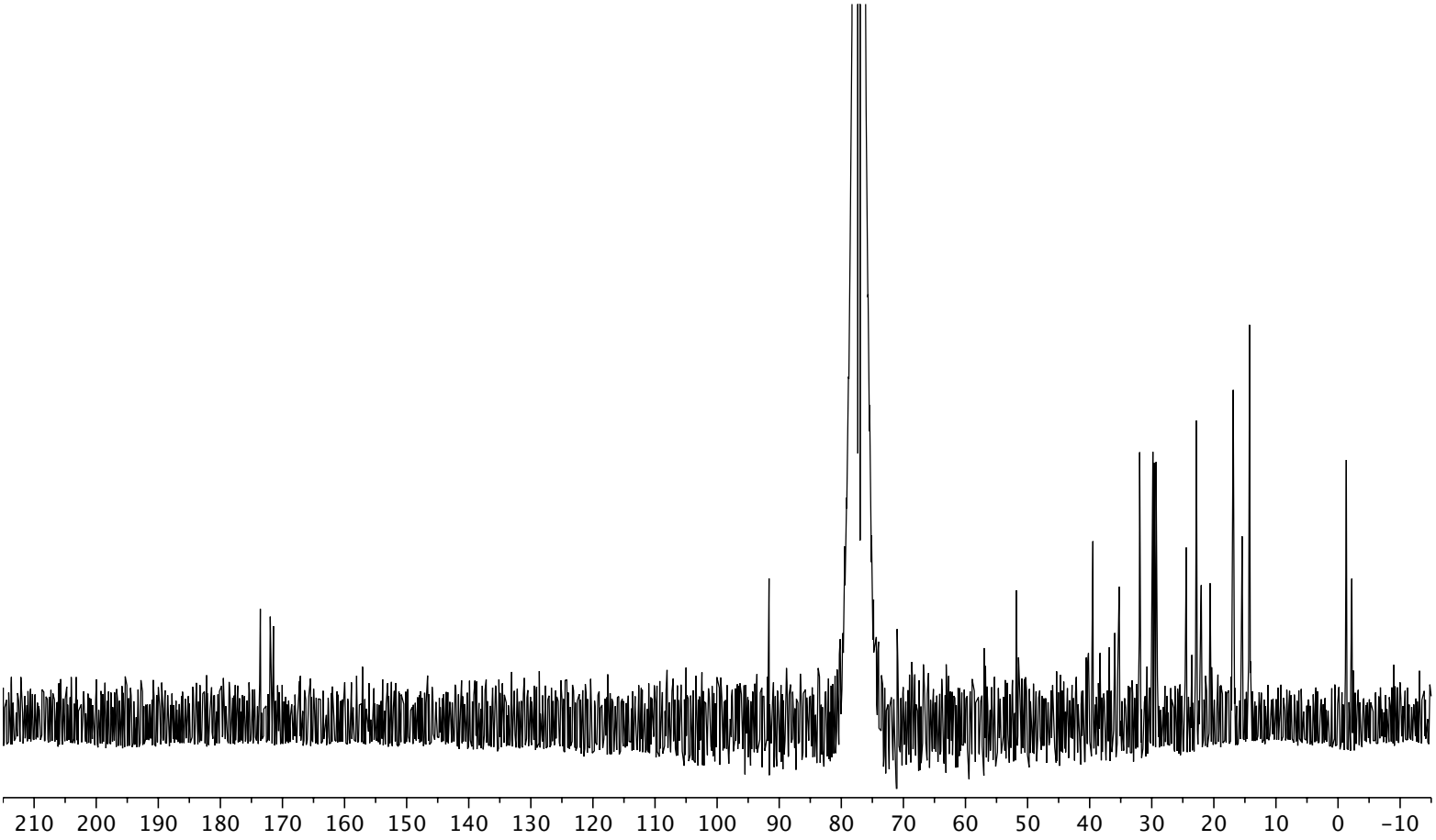


^{1}H-NMR (500 MHz) expansions for **S8** in $CDCl_3$

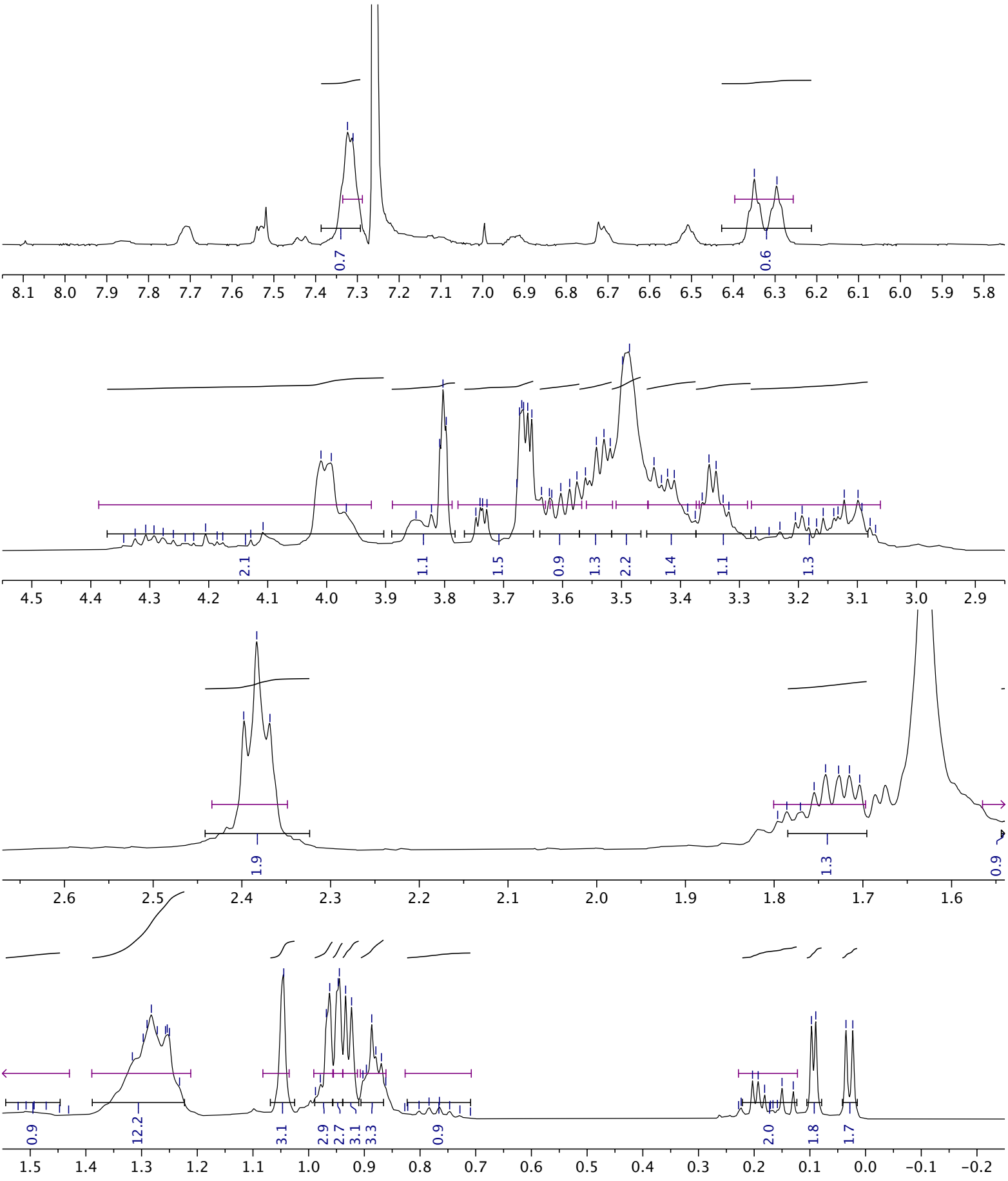


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **5c** in $CDCl_3$

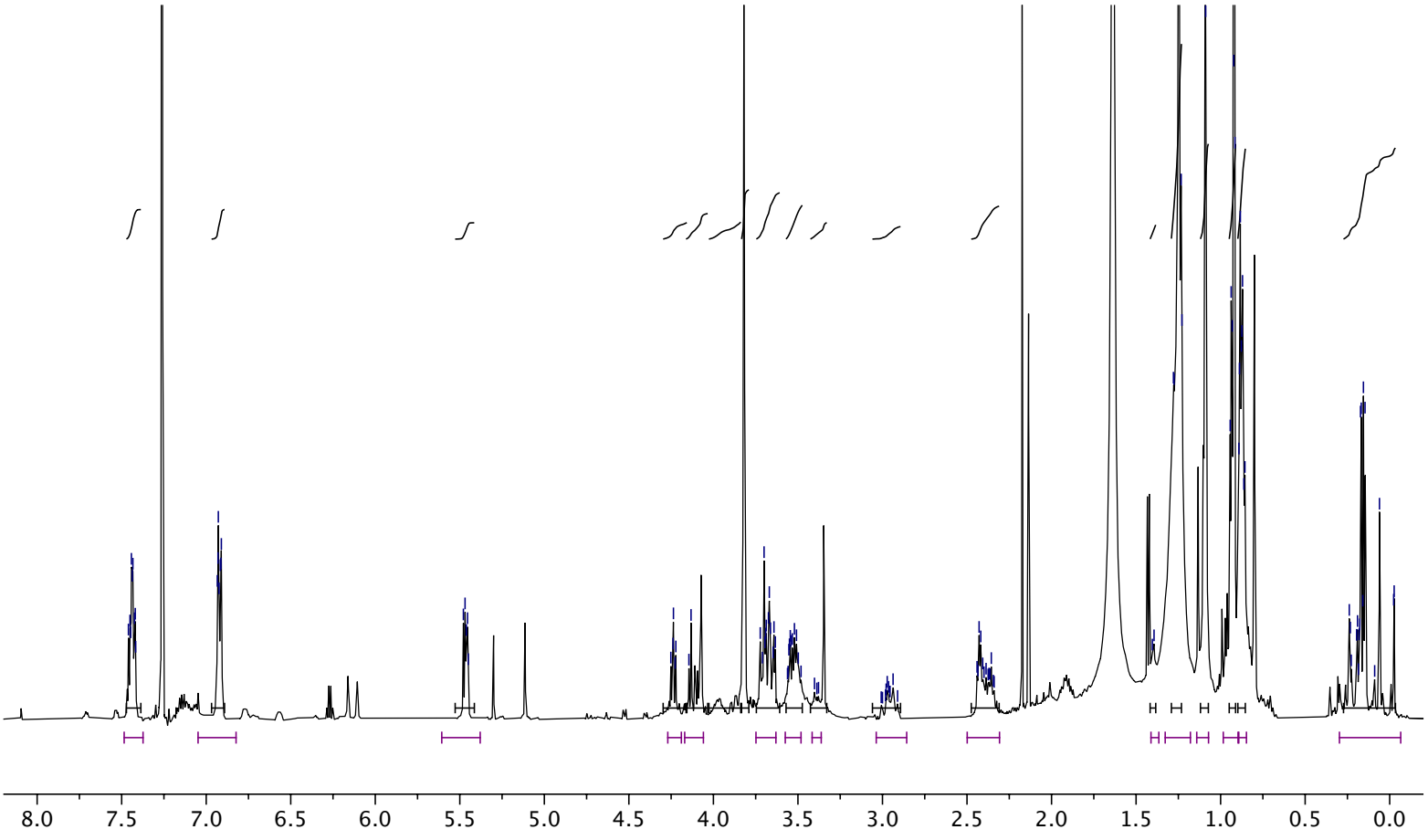


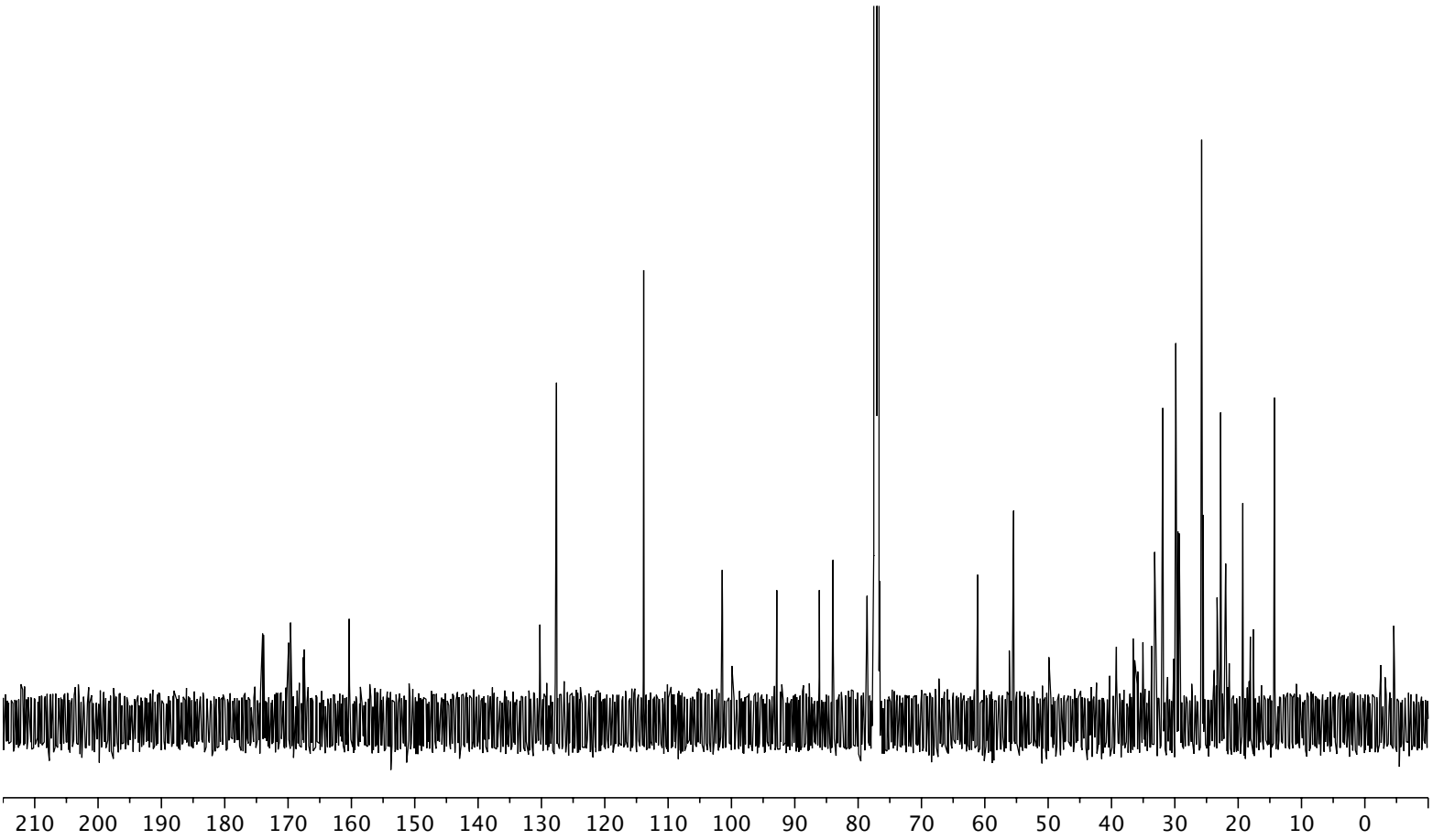


^{11}H-NMR (500 MHz) expansions for **5c** in $CDCl_3$

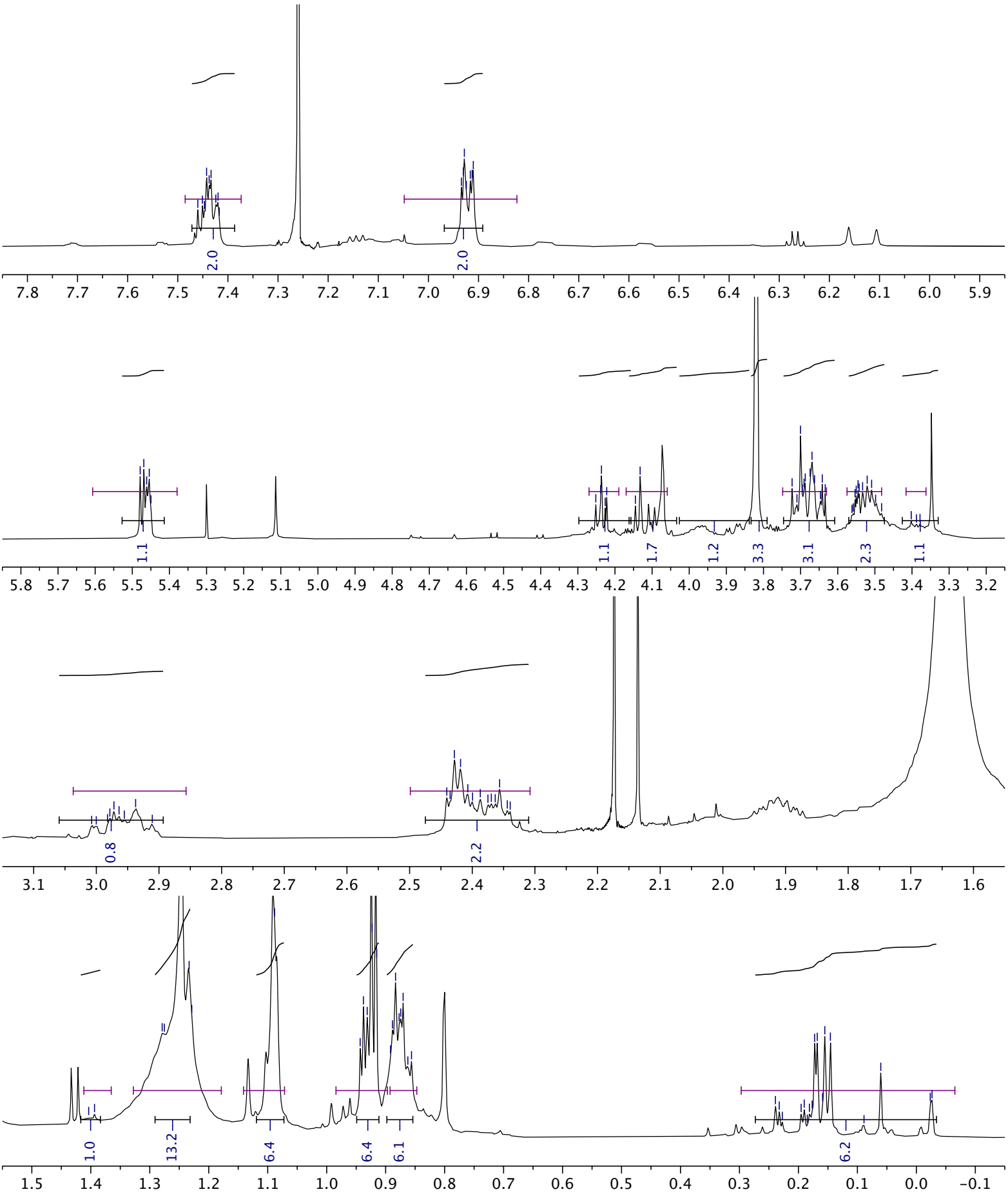


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **S7** in $CDCl_3$

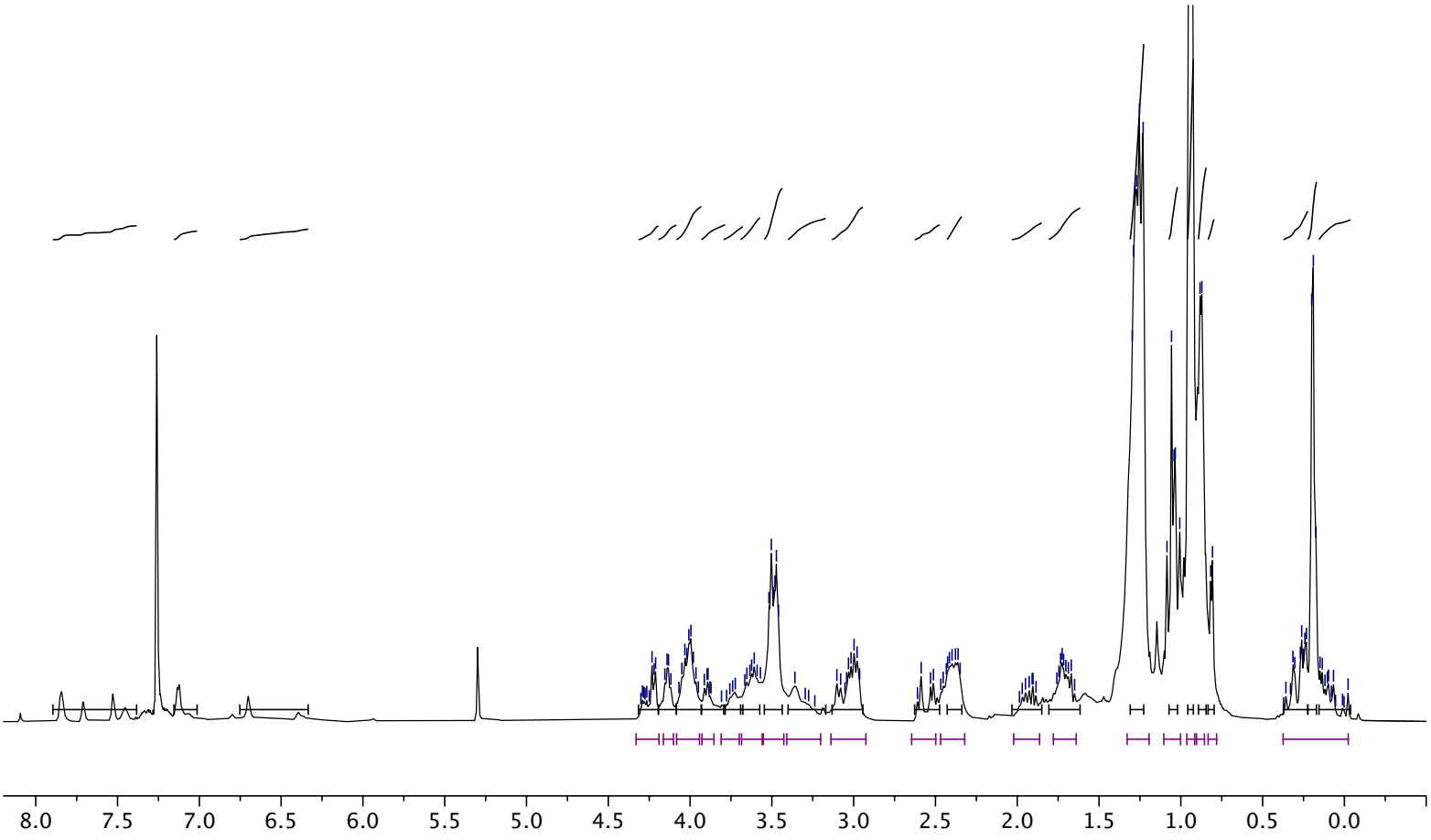


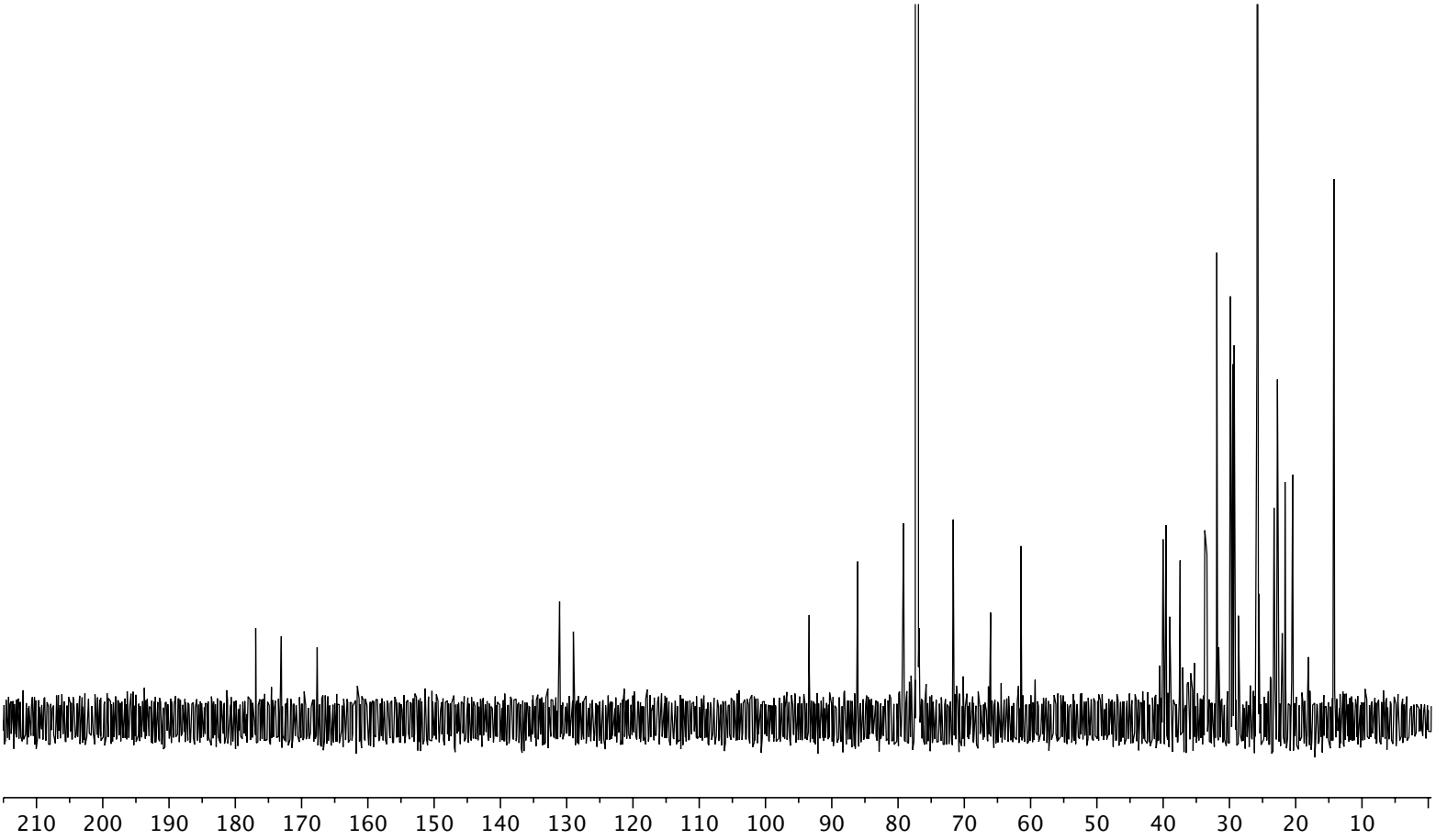


^{1}H-NMR (500 MHz) expansions for **S7** in $CDCl_3$

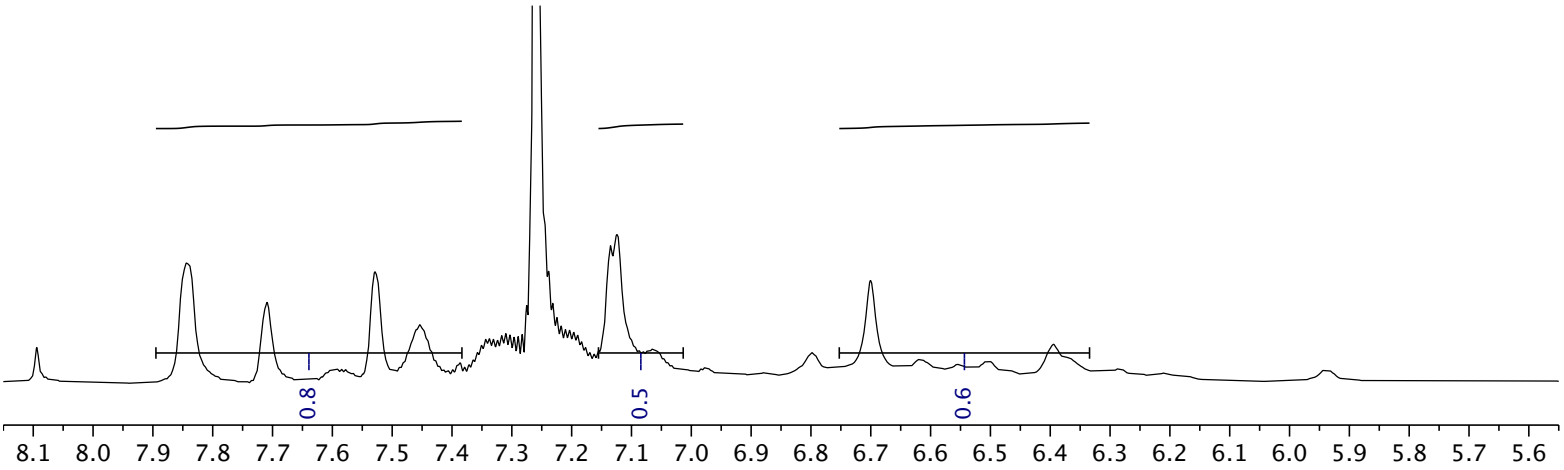


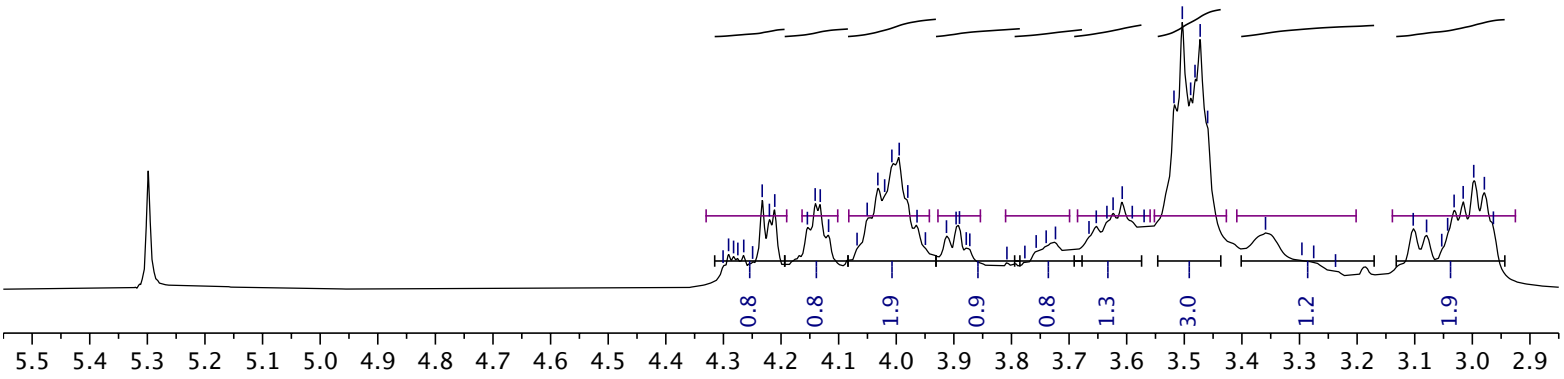
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **6c** in $CDCl_3$

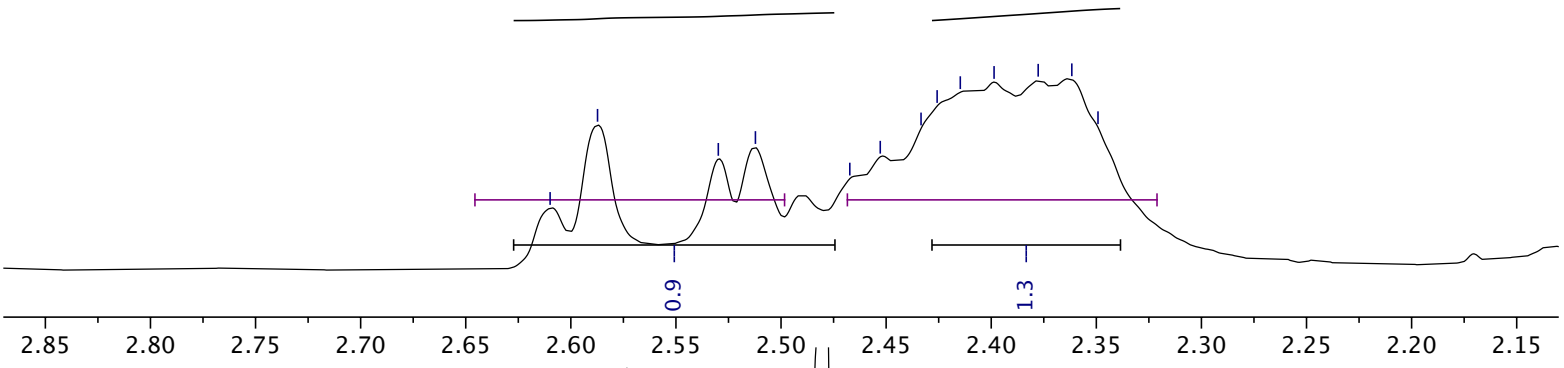


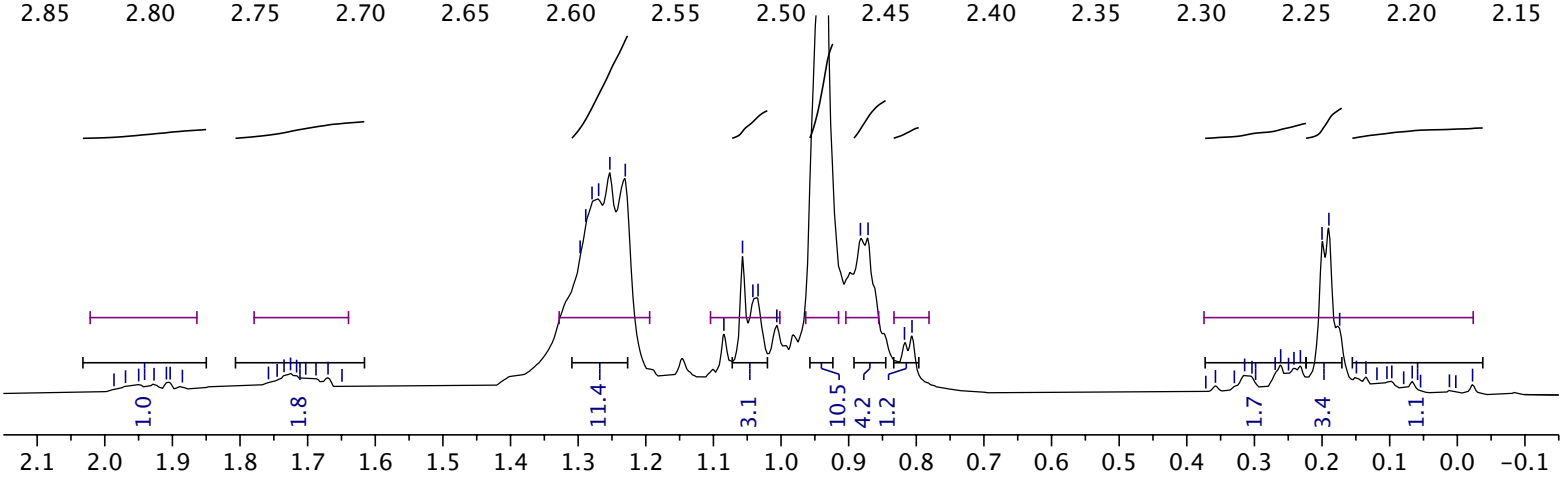


^{1}H–NMR (500 MHz) expansions for **6c** in $CDCl_3$

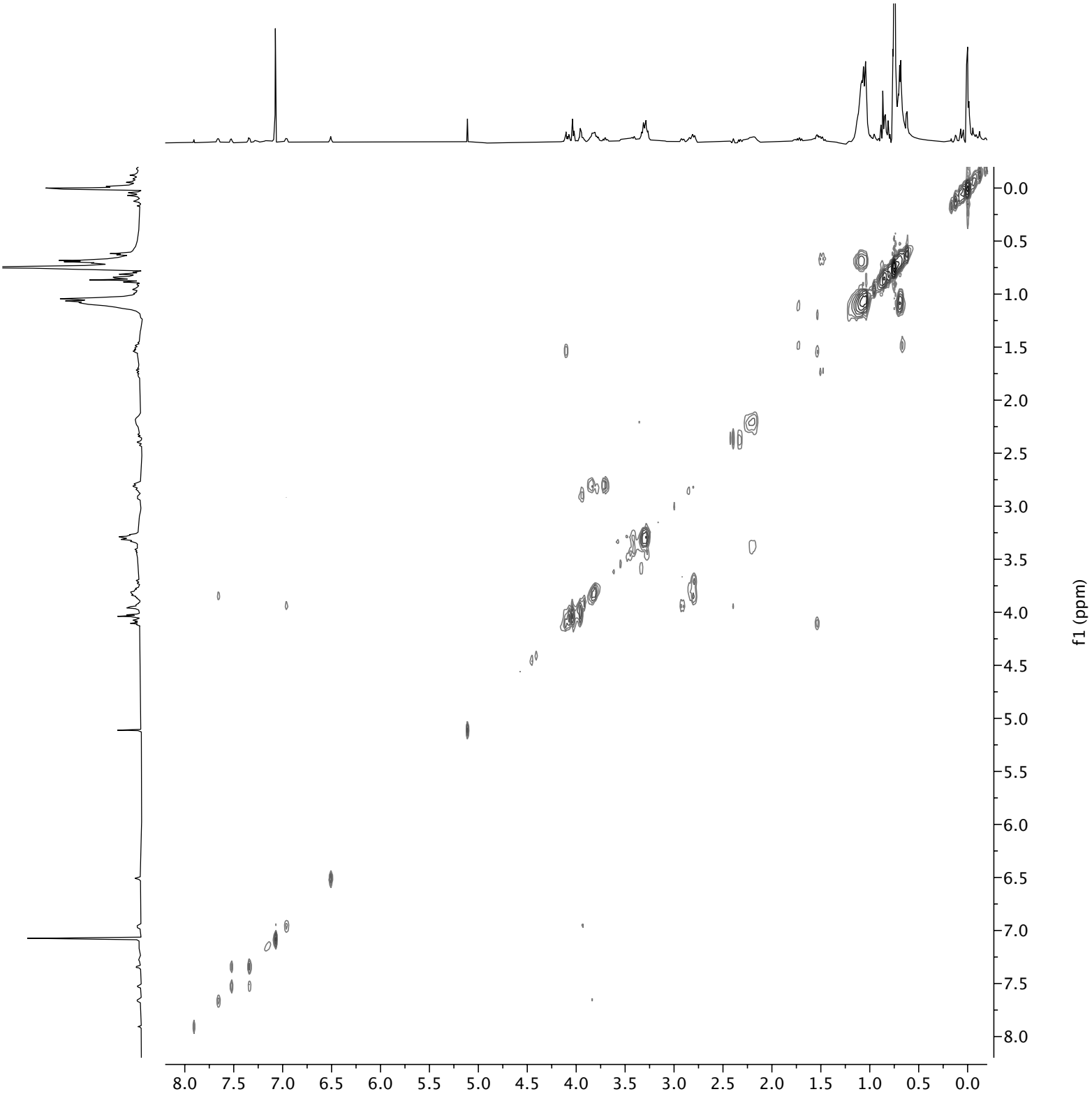




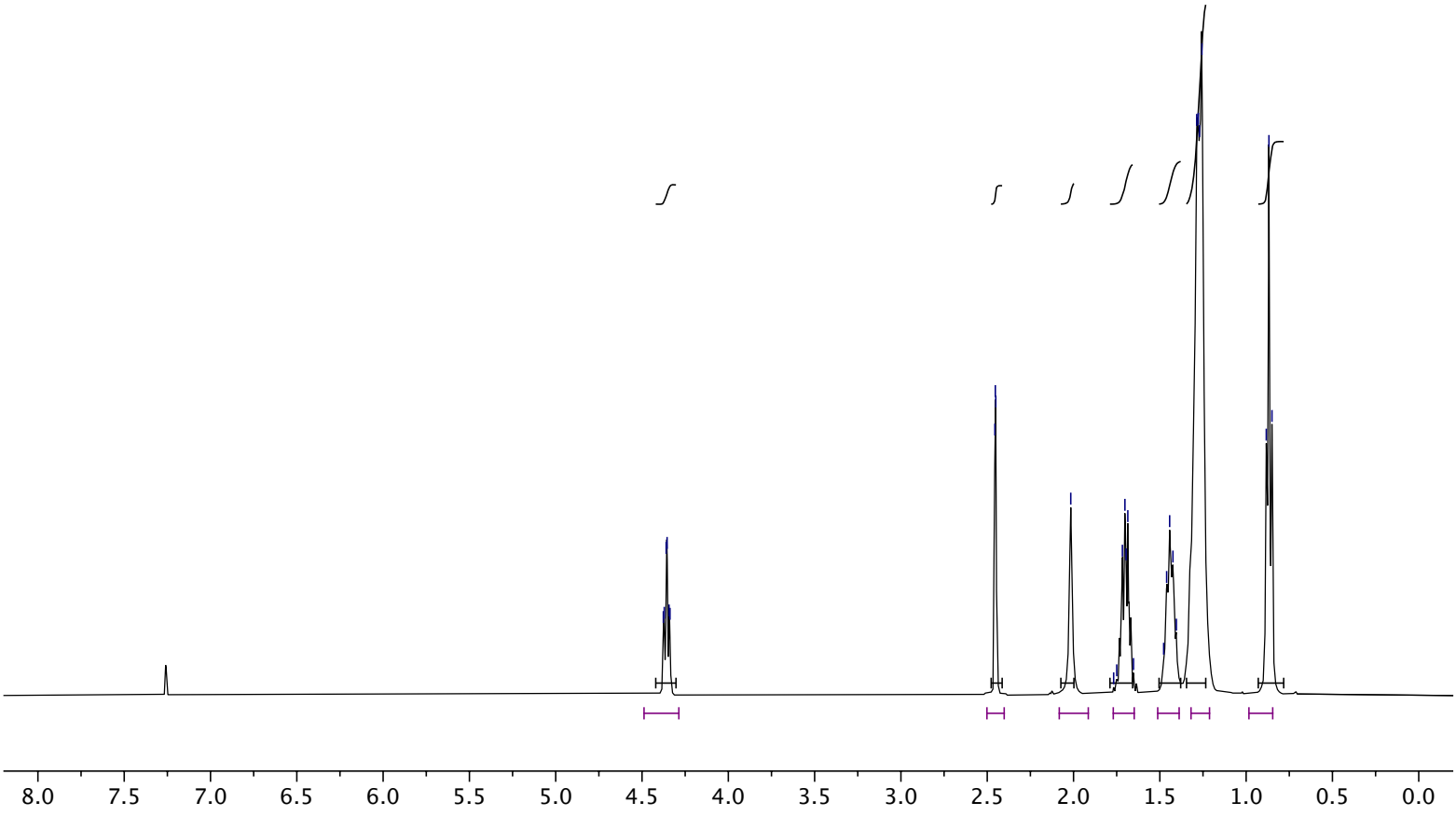


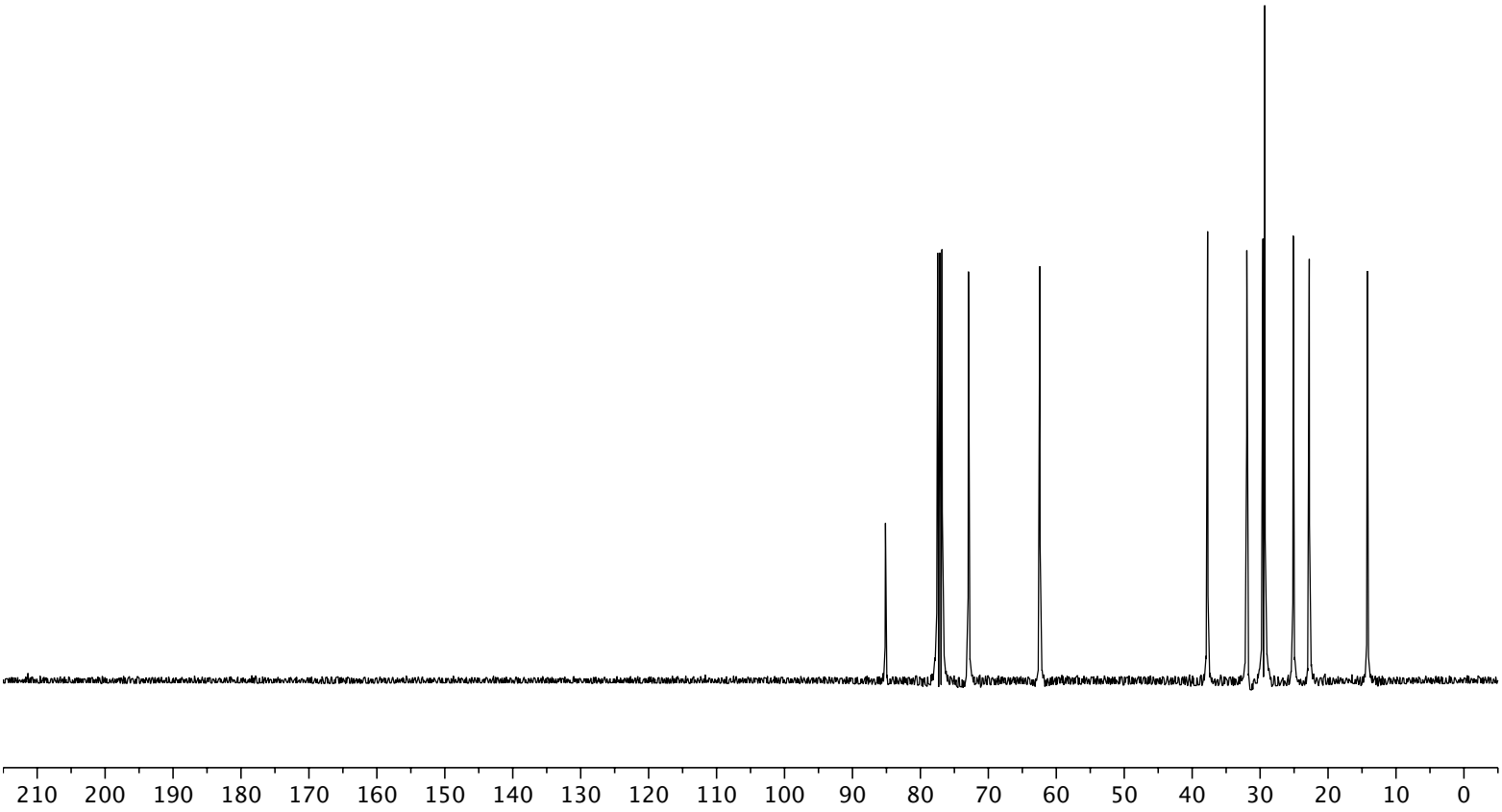


$^{1}H,^{1}H$–gCOSY (500 MHz) spectrum of **6c** in $CDCl_3$

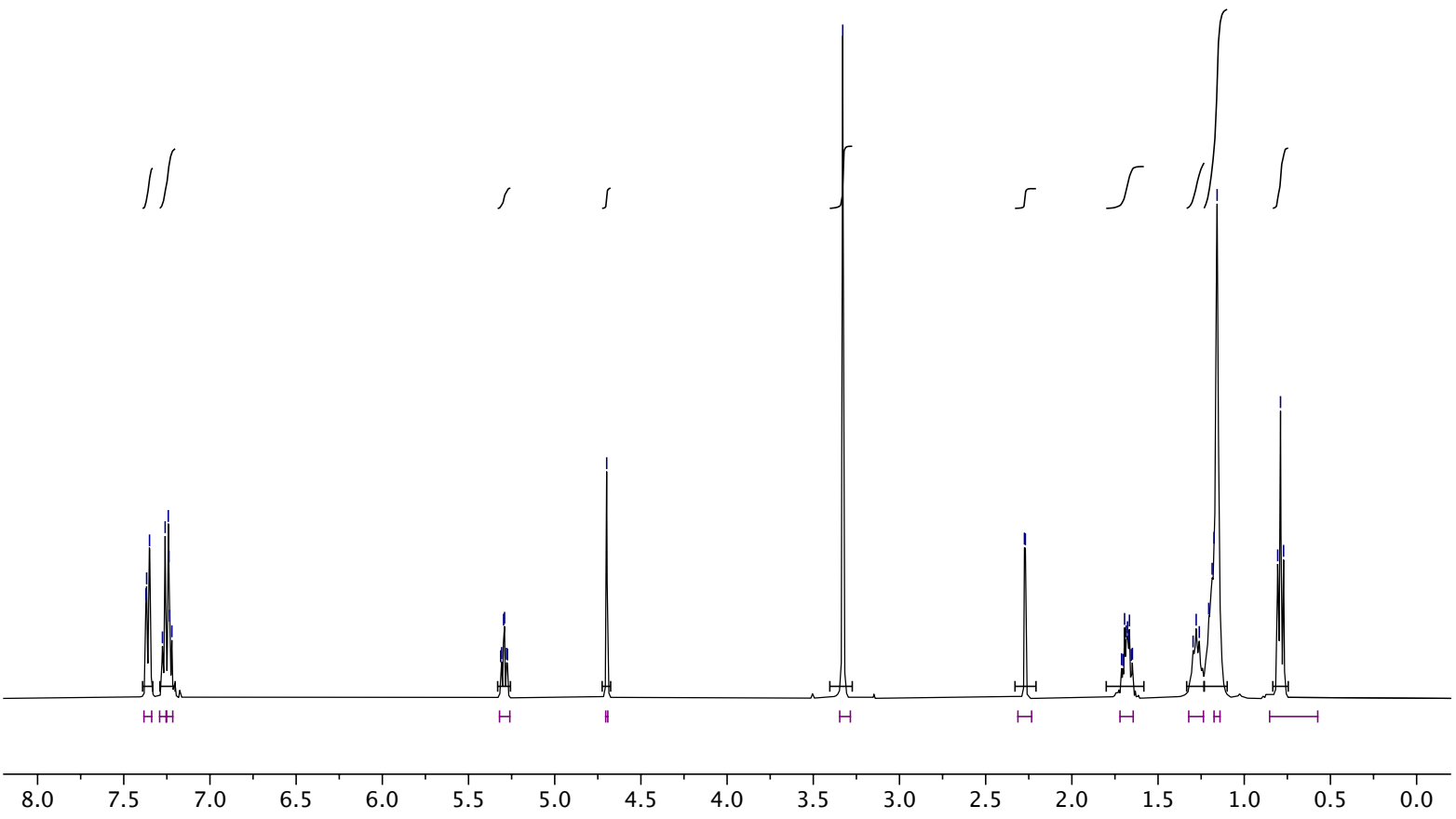


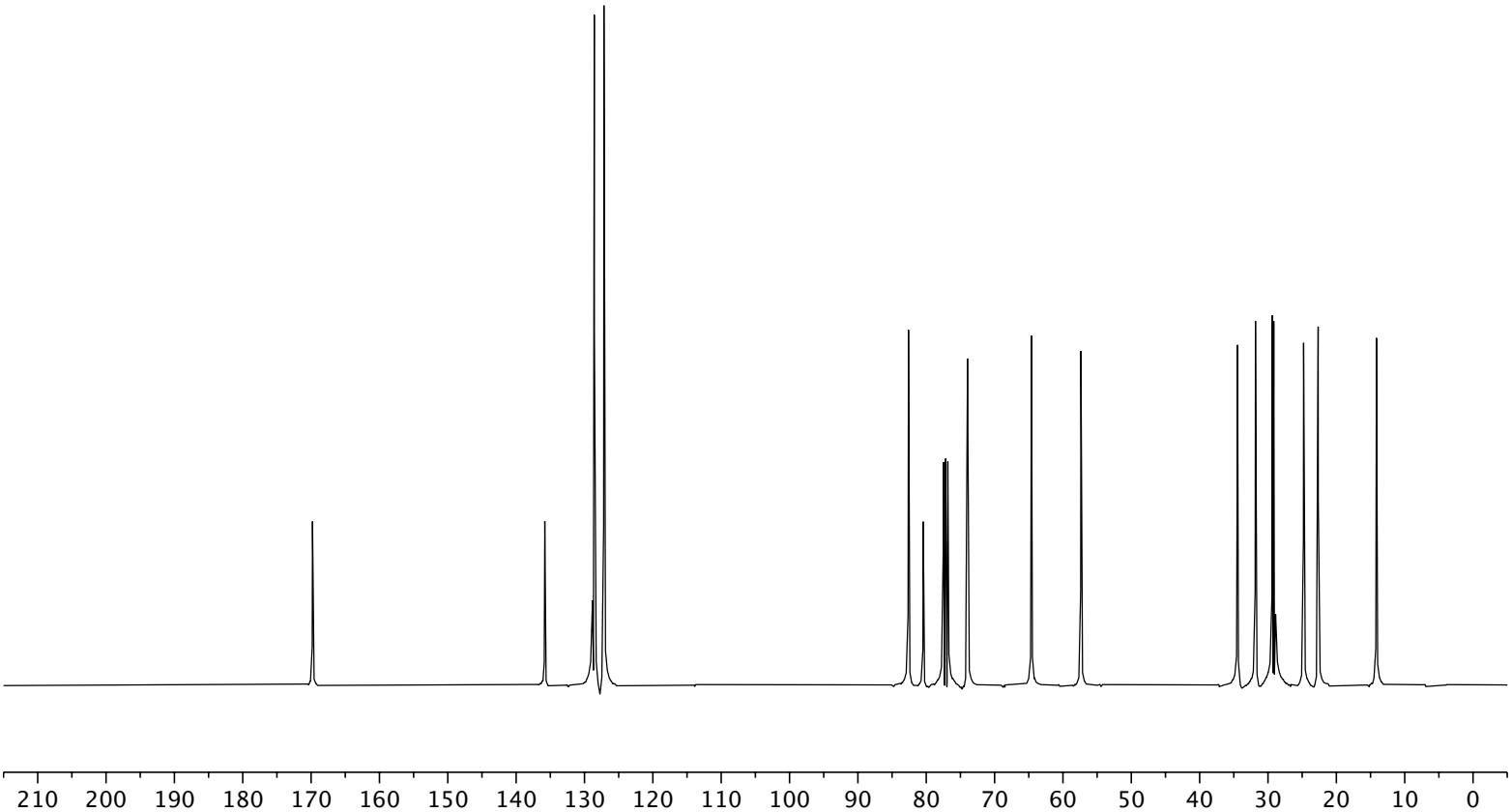
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of **16** in $CDCl_3$



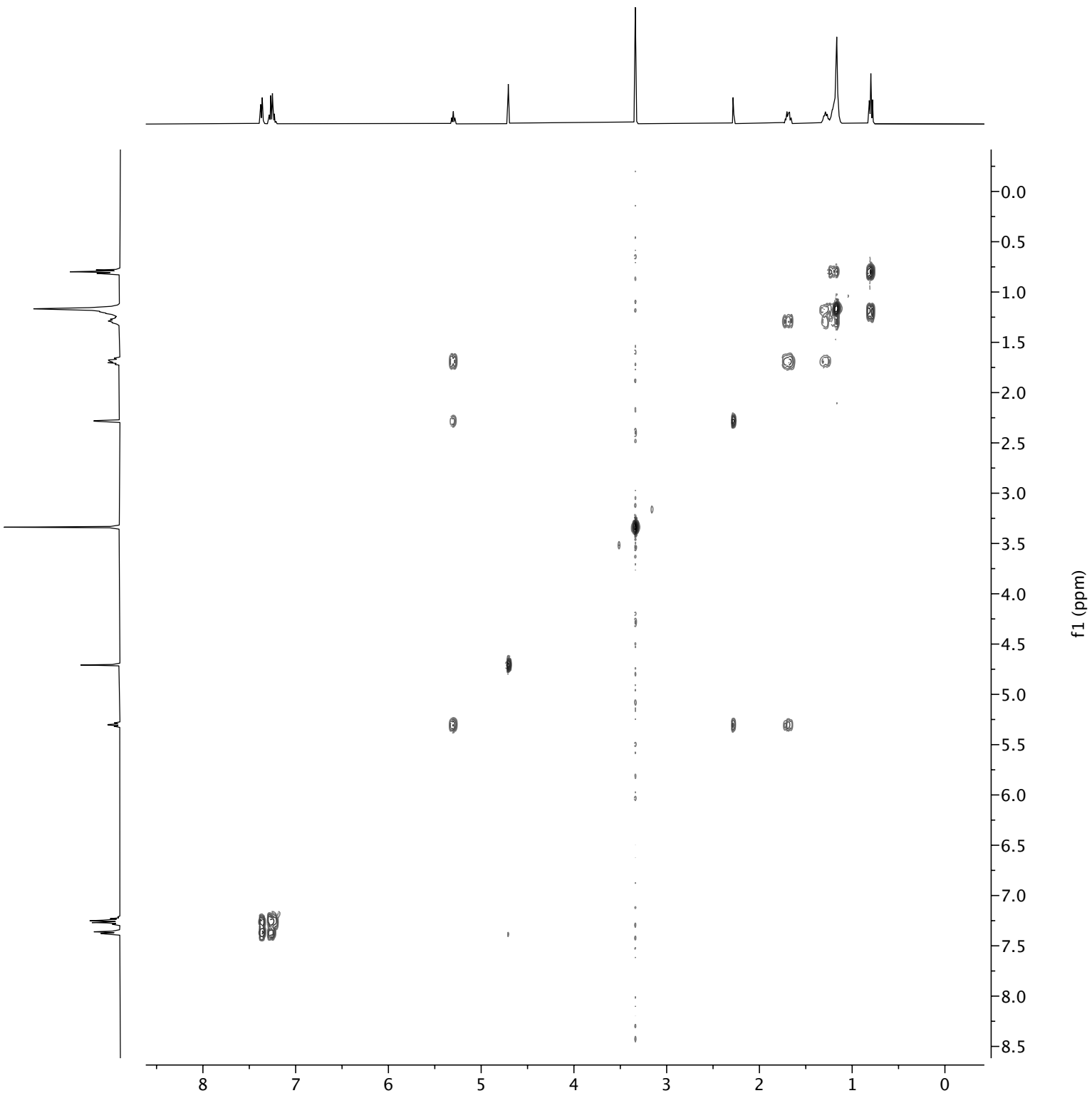


^{1}H–NMR (400 MHz) and ^{13}C–NMR (100 MHz) spectra of ***R*–17** in $CDCl_3$

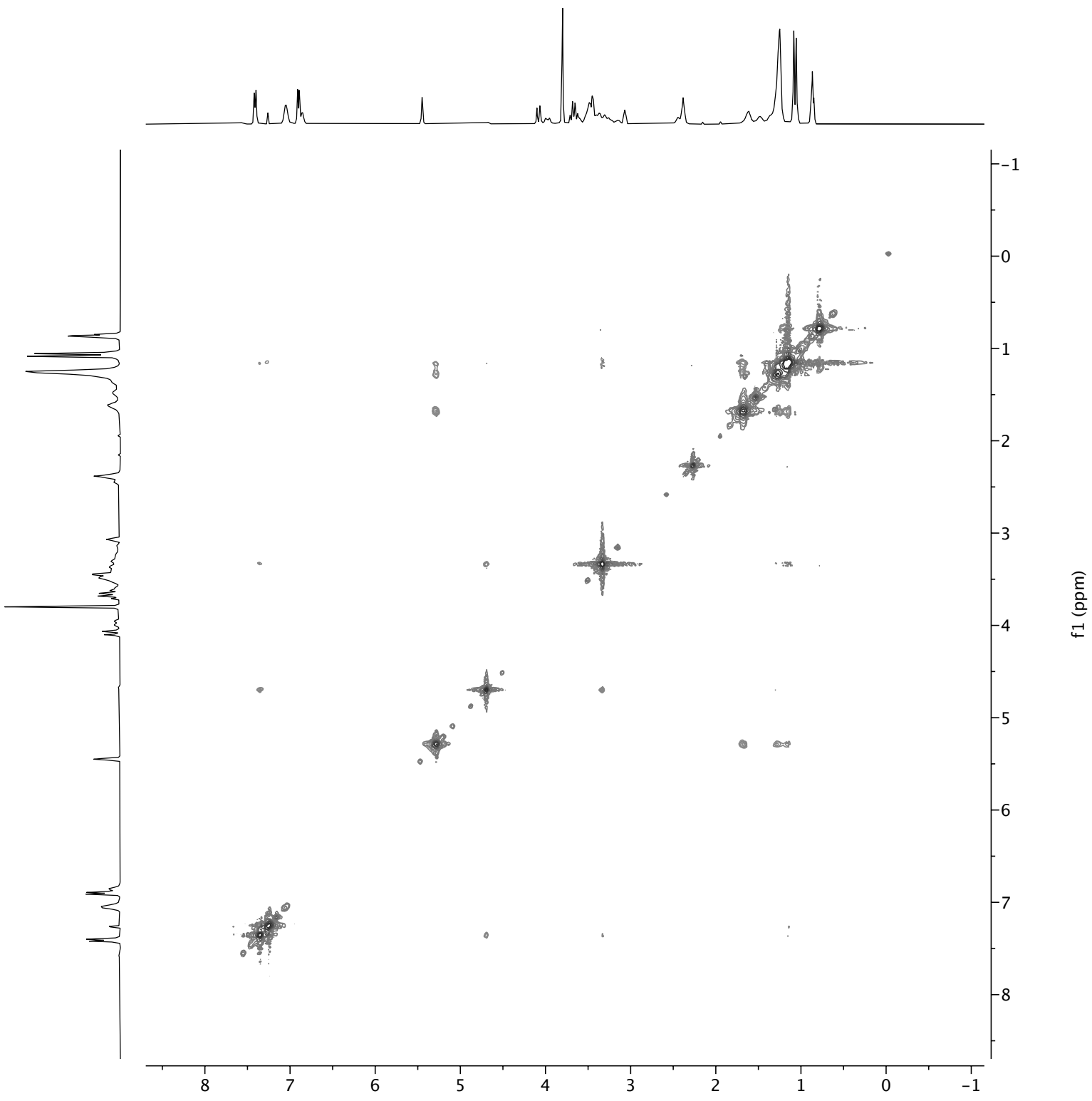




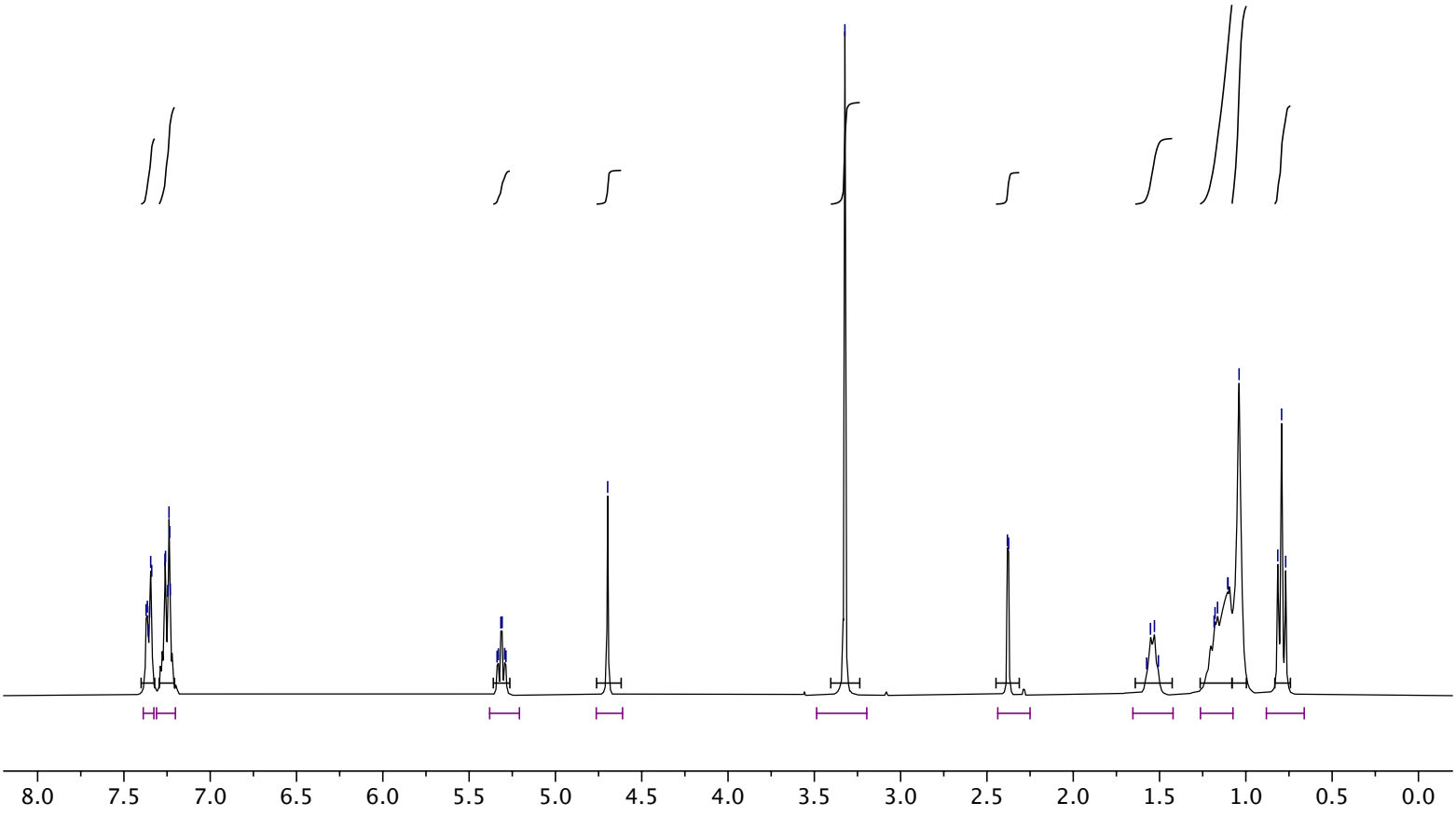
$^{1}H,^{1}H$-gCOSY (500 MHz) spectrum of *R*-**17** in $CDCl_3$

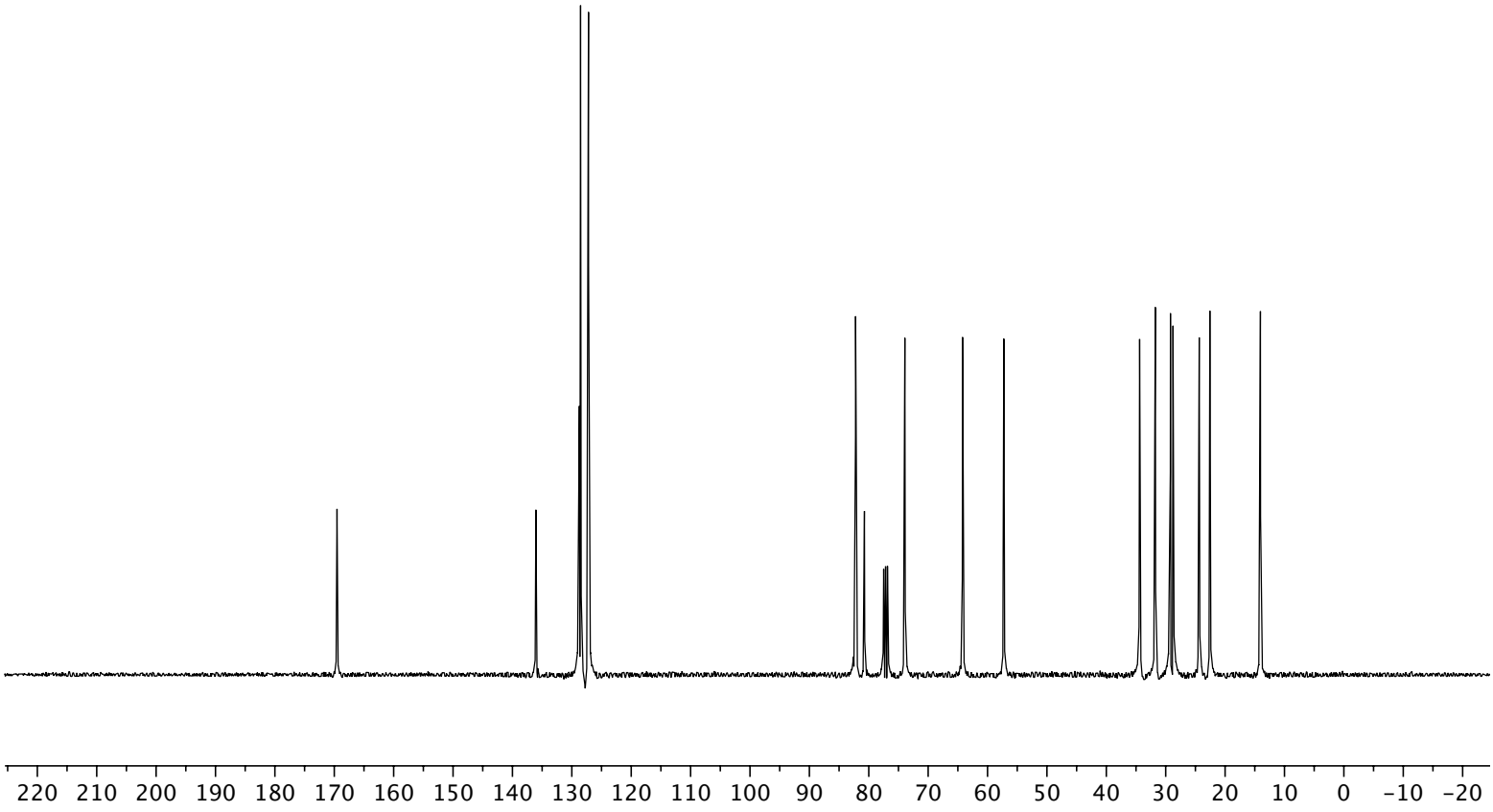


$^{1}H,^{1}H$–NOESY (500 MHz) spectrum of *R*–**17** in $CDCl_3$

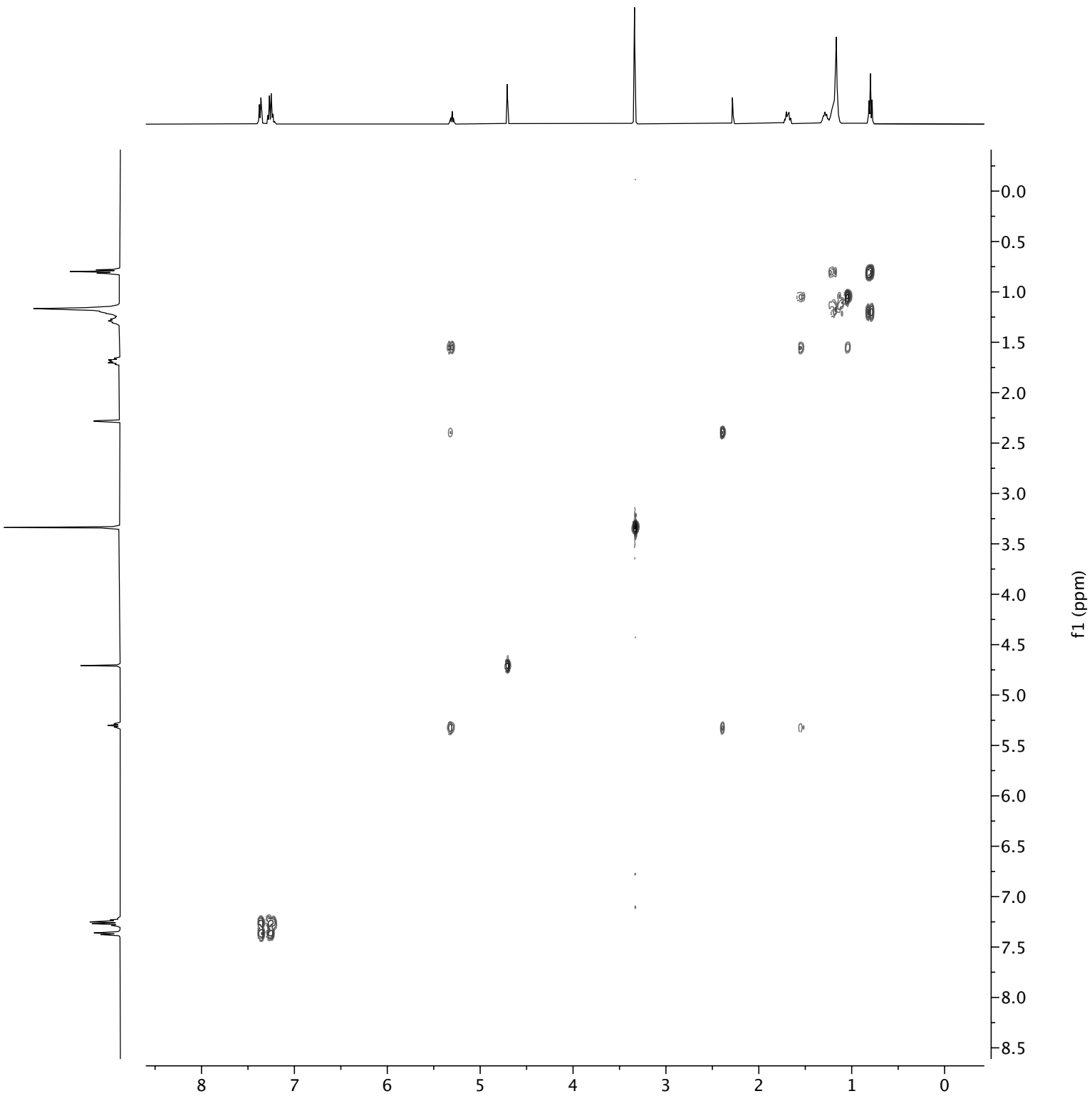


^{1}H–NMR (300 MHz) and ^{13}C–NMR (100 MHz) spectra of ***S*–17** in $CDCl_3$

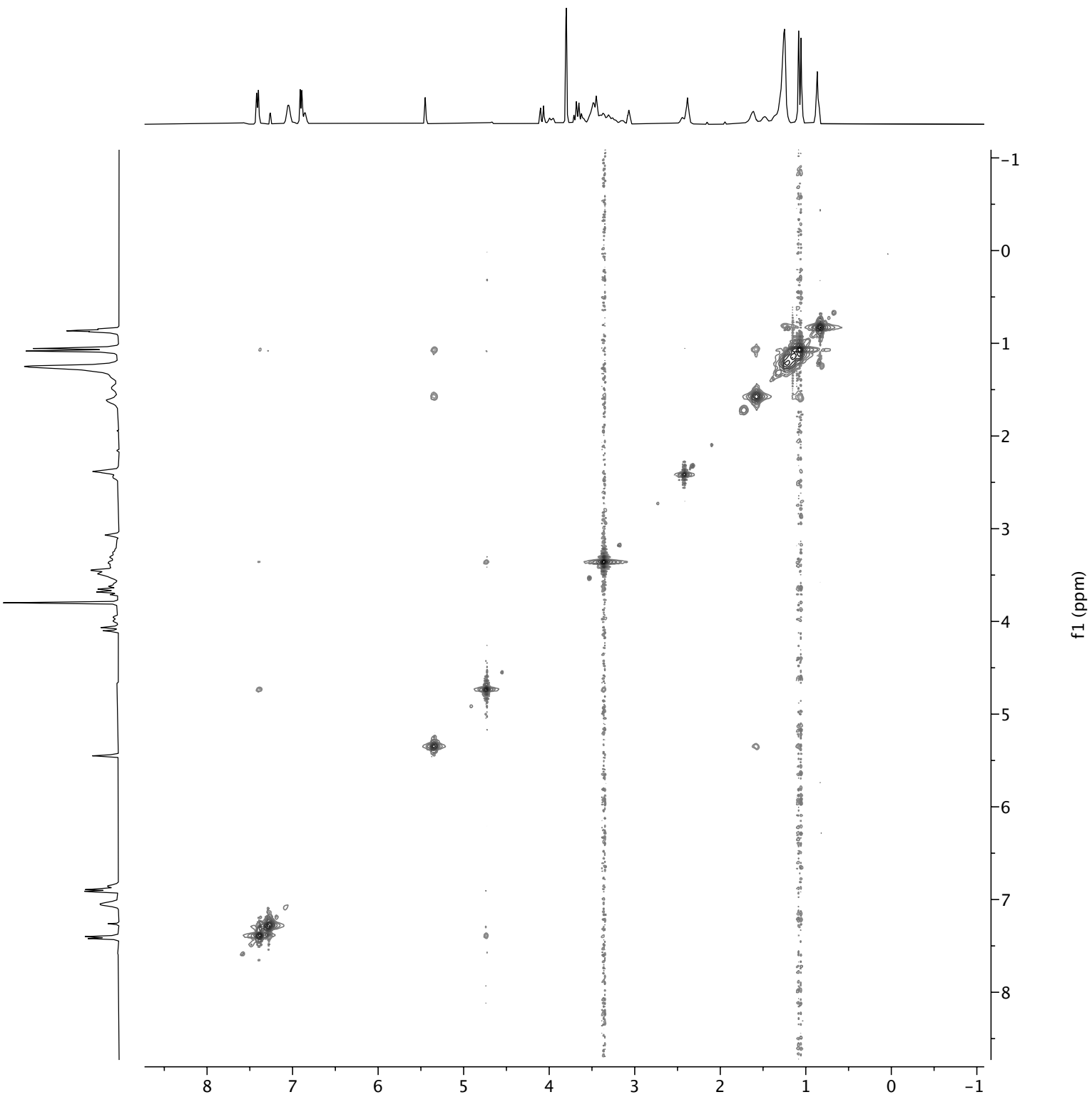




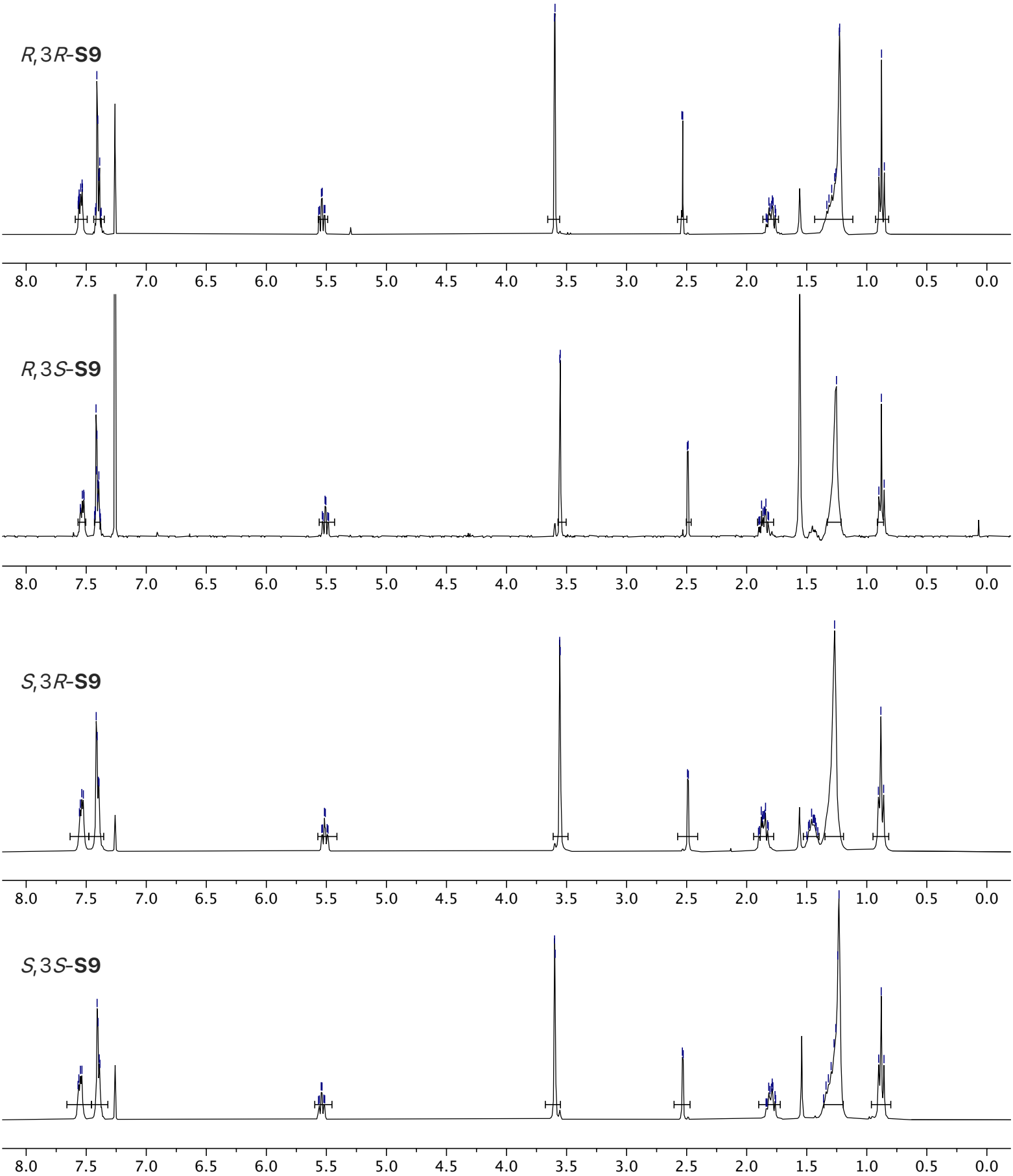
$^{1}H,^{1}H$-gCOSY (500 MHz) spectrum of *S*-**17** in $CDCl_3$



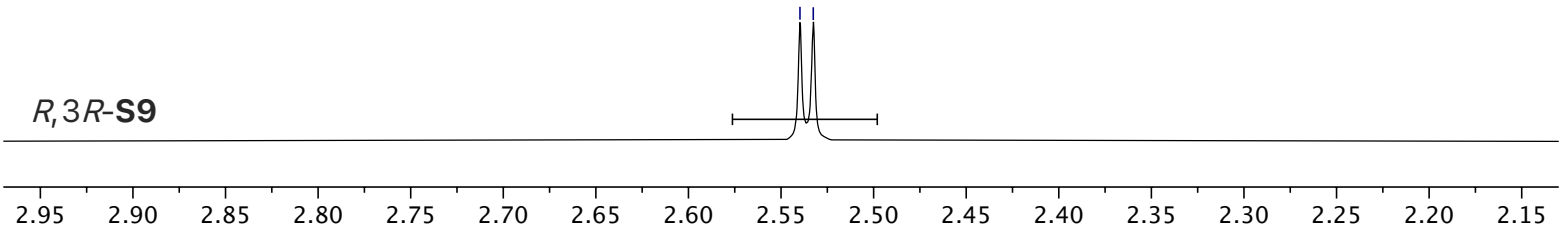
$^{1}H,^{1}H$–NOESY (500 MHz) spectrum of ***S*–17** in $CDCl_3$



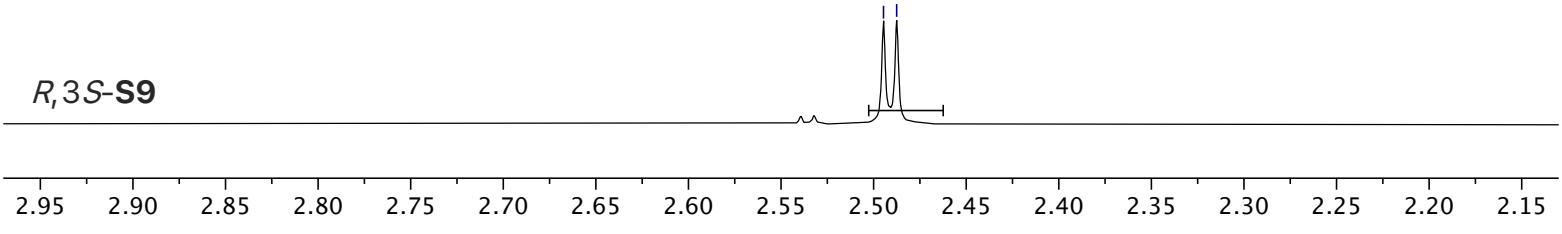
^{1}H–NMR (300 MHz) spectra of *R*,3*R*–**S9**, *R*,3S–**S9**, *S*,3*R*–**S9** and *S*,3*S*–**S9** in $CDCl_3$



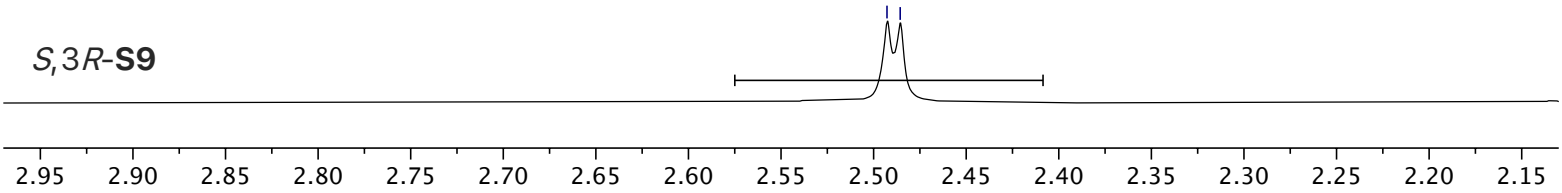
^{1}H–NMR (300 MHz) spectra of *R*,3*R*–**S9**, *R*,3S–**S9**, *S*,3*R*–**S9** and *S*,3*S*–**S9** in $CDCl_3$



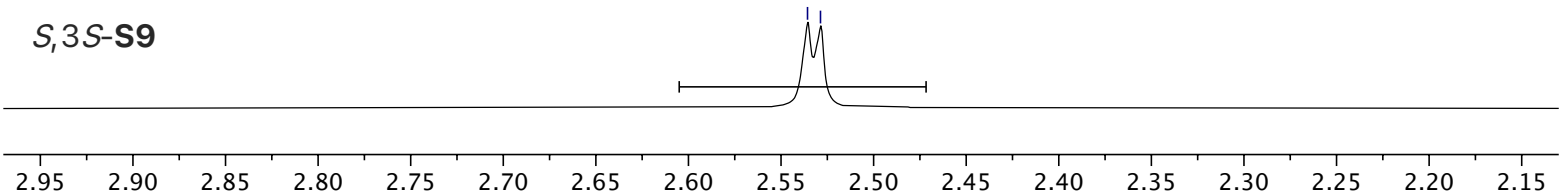
R,3*R*-**S9**



R,3*S*-**S9**

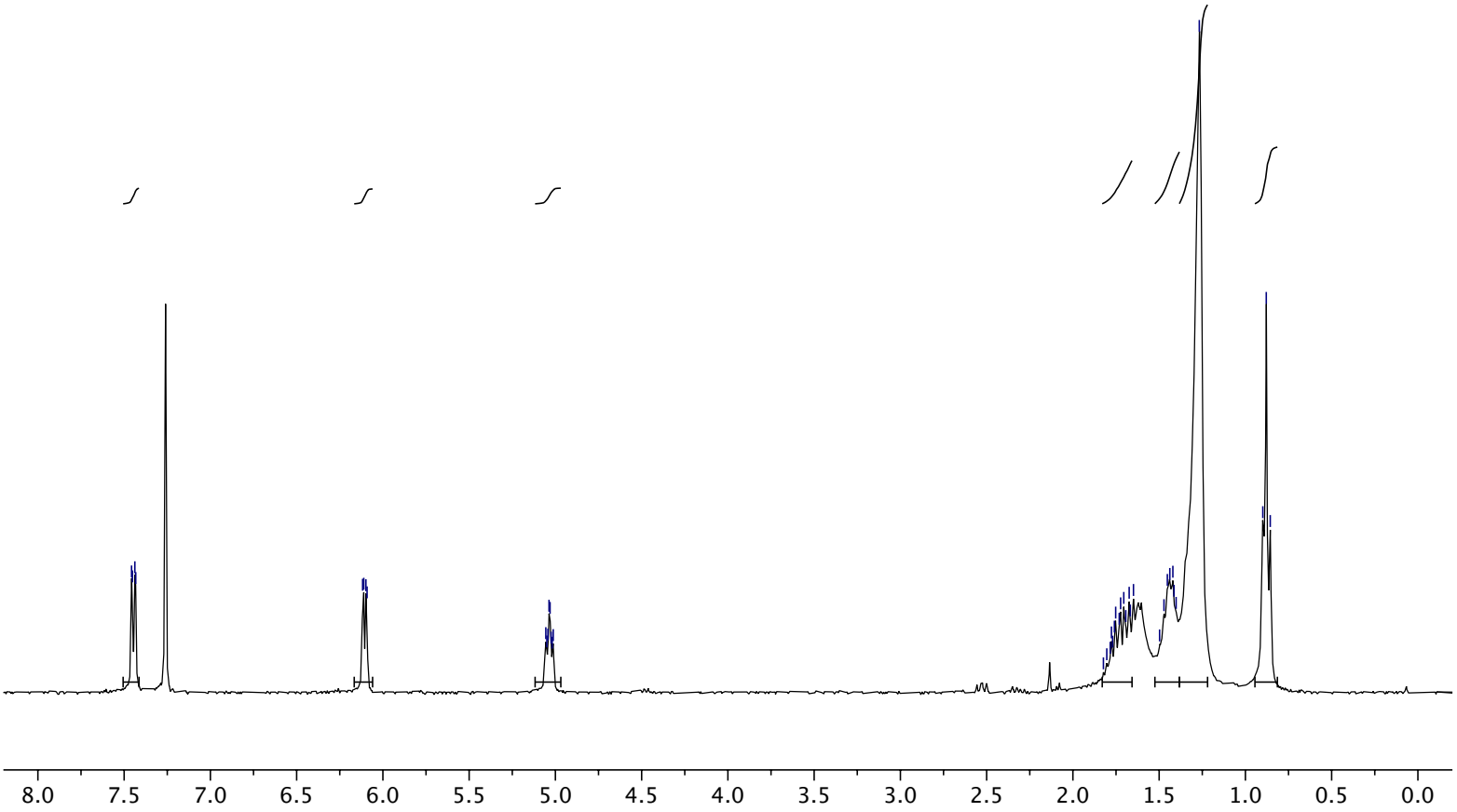


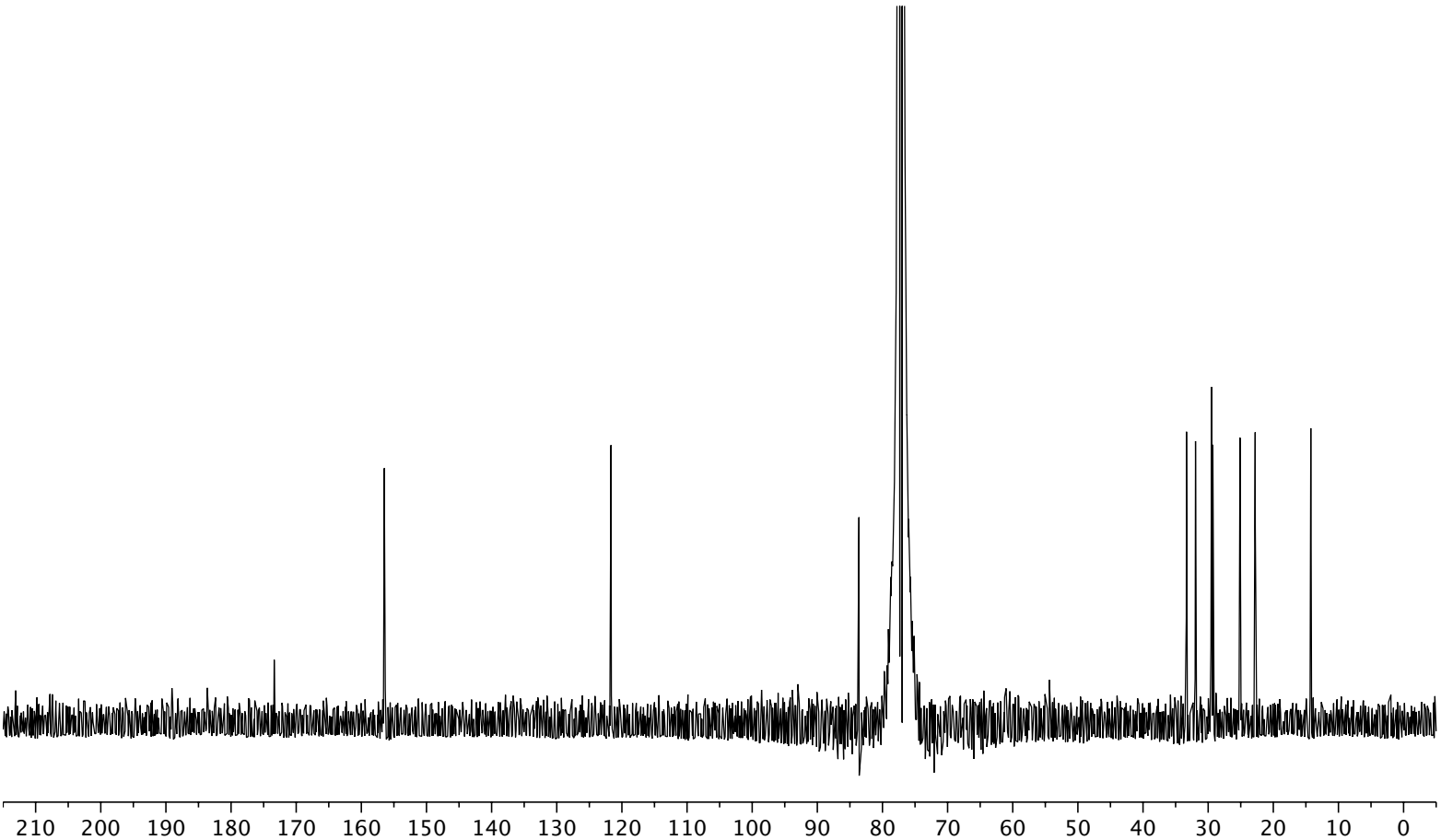
S,3*R*-**S9**



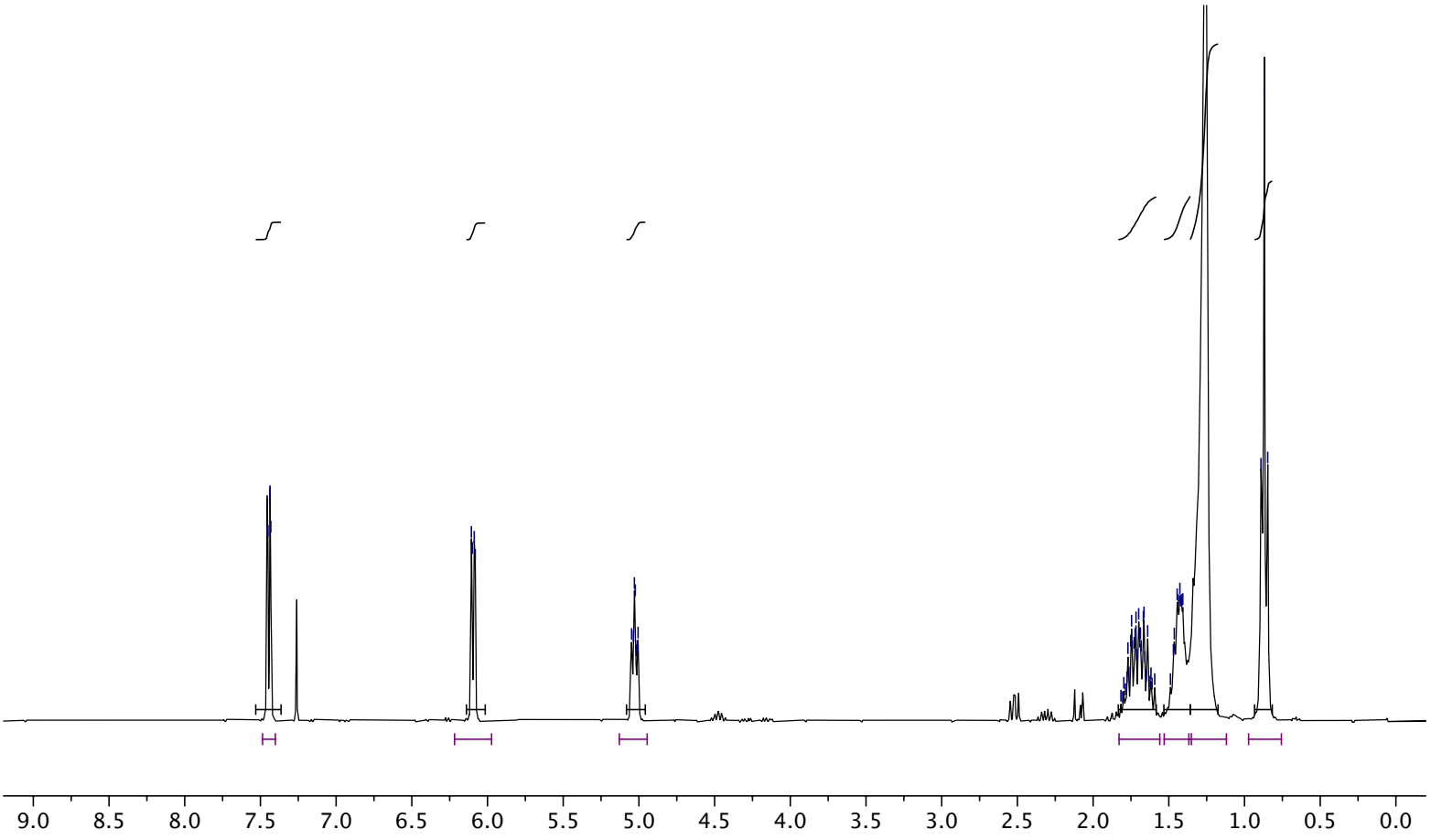
S,3*S*-**S9**

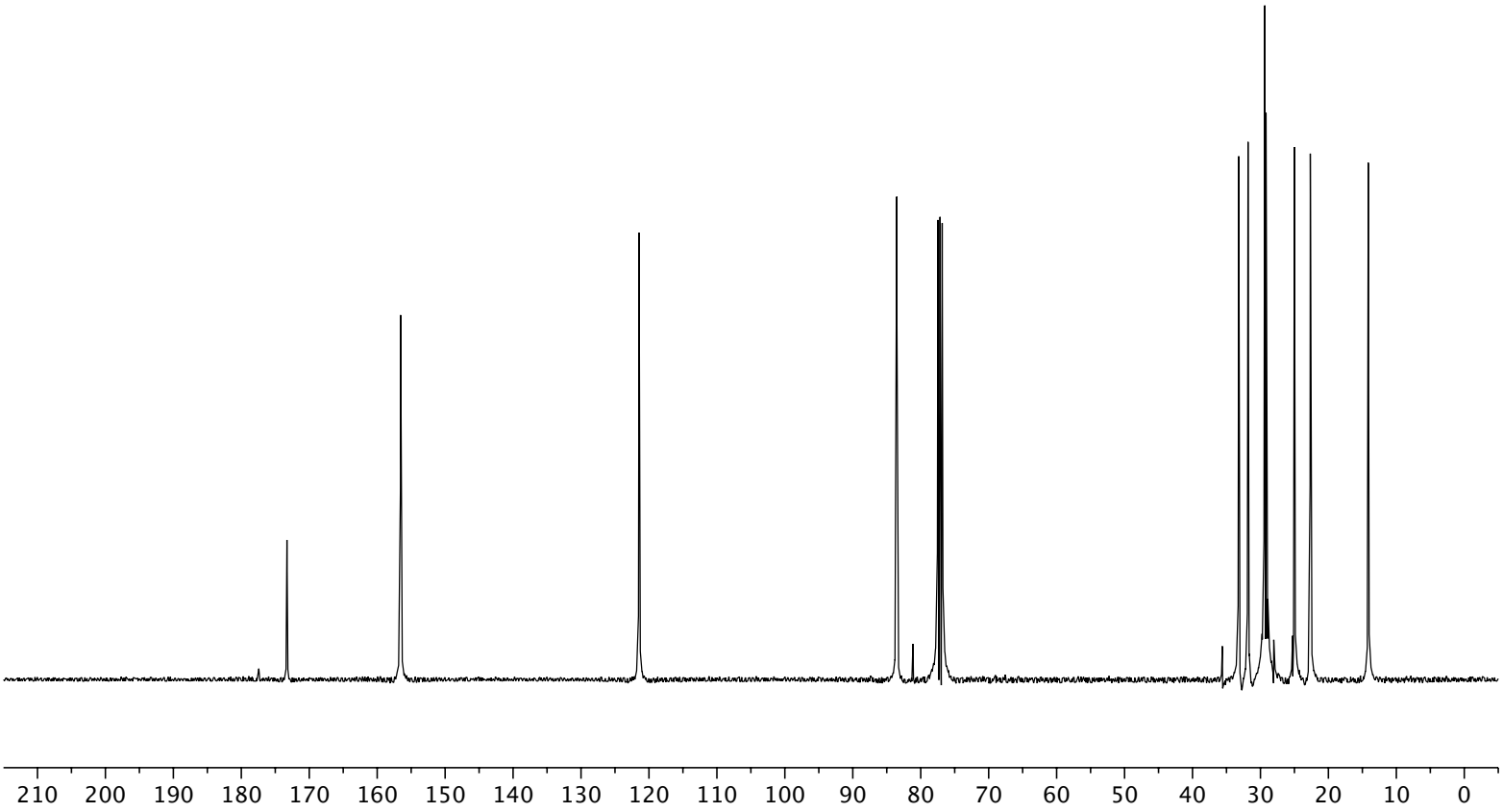
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of *R*–**13** in $CDCl_3$



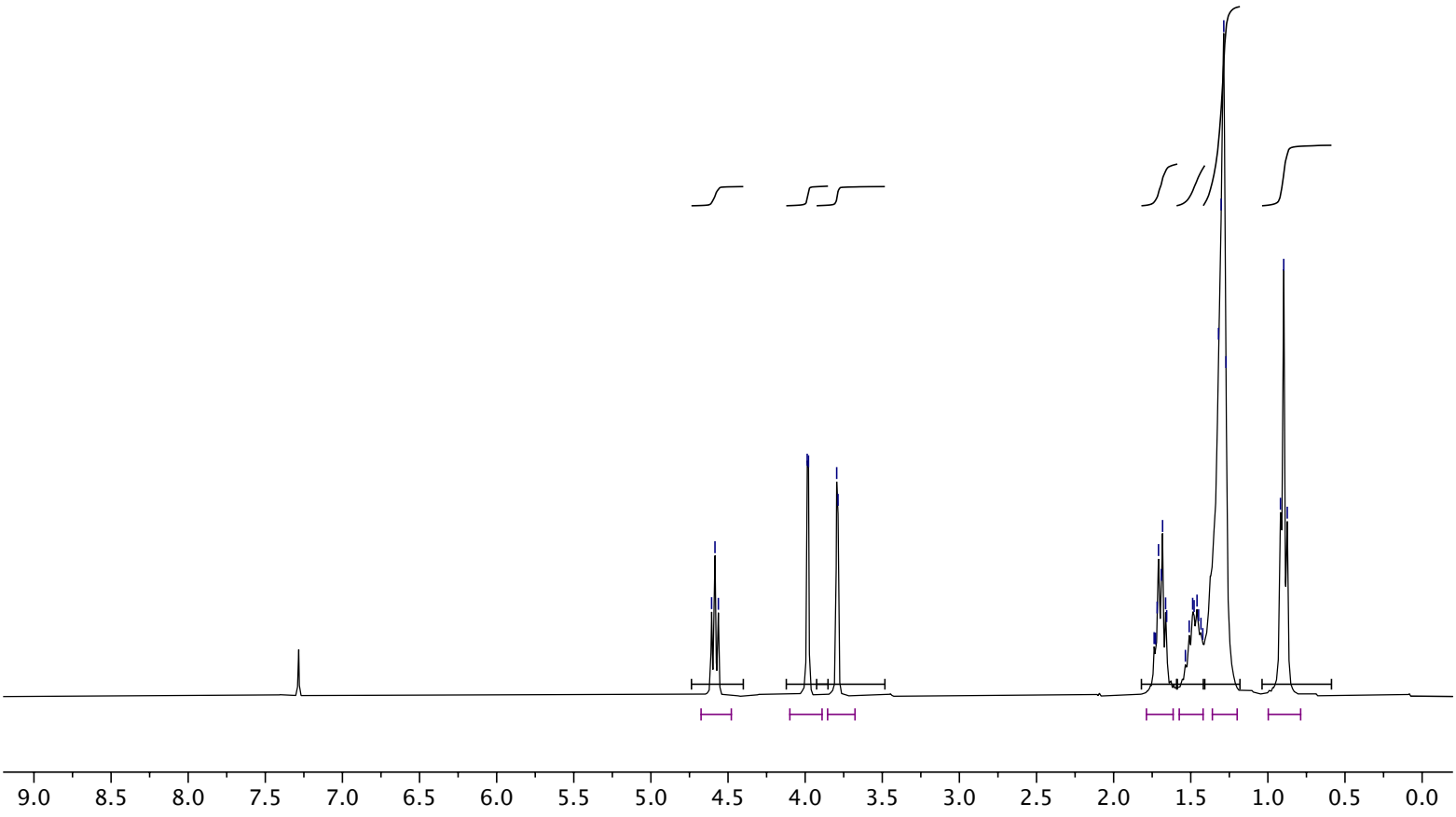


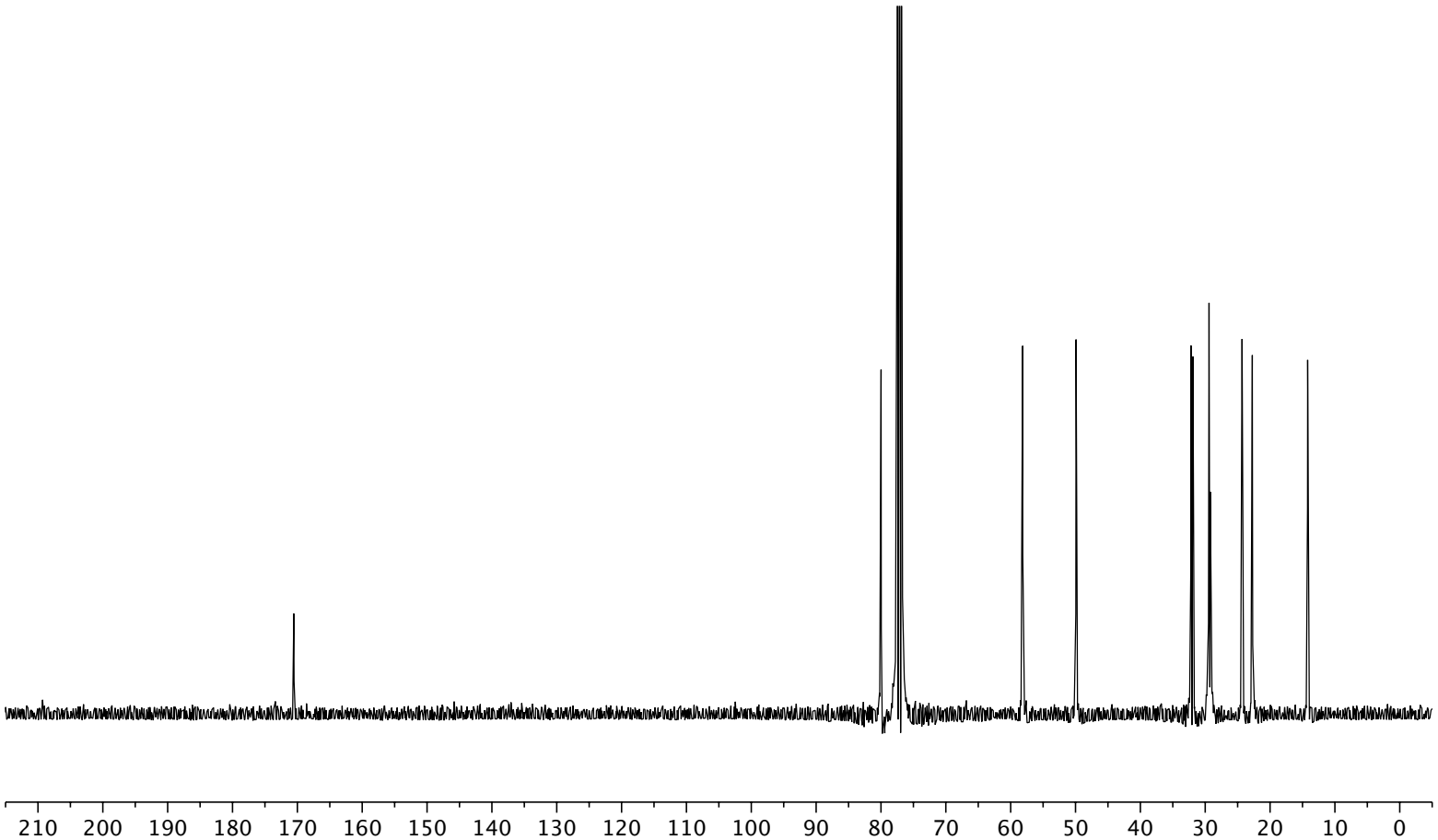
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of *S*–**13** in $CDCl_3$



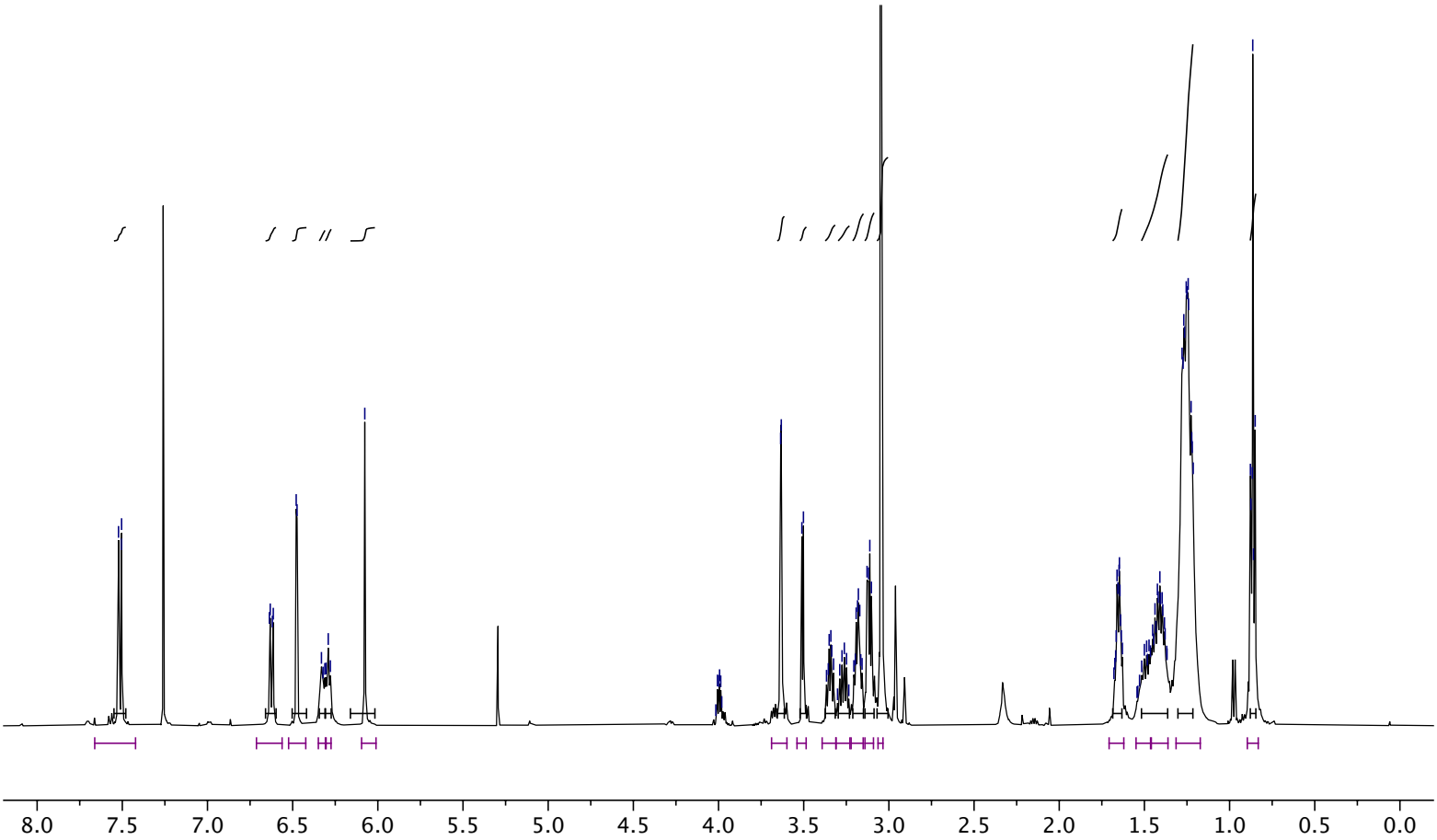


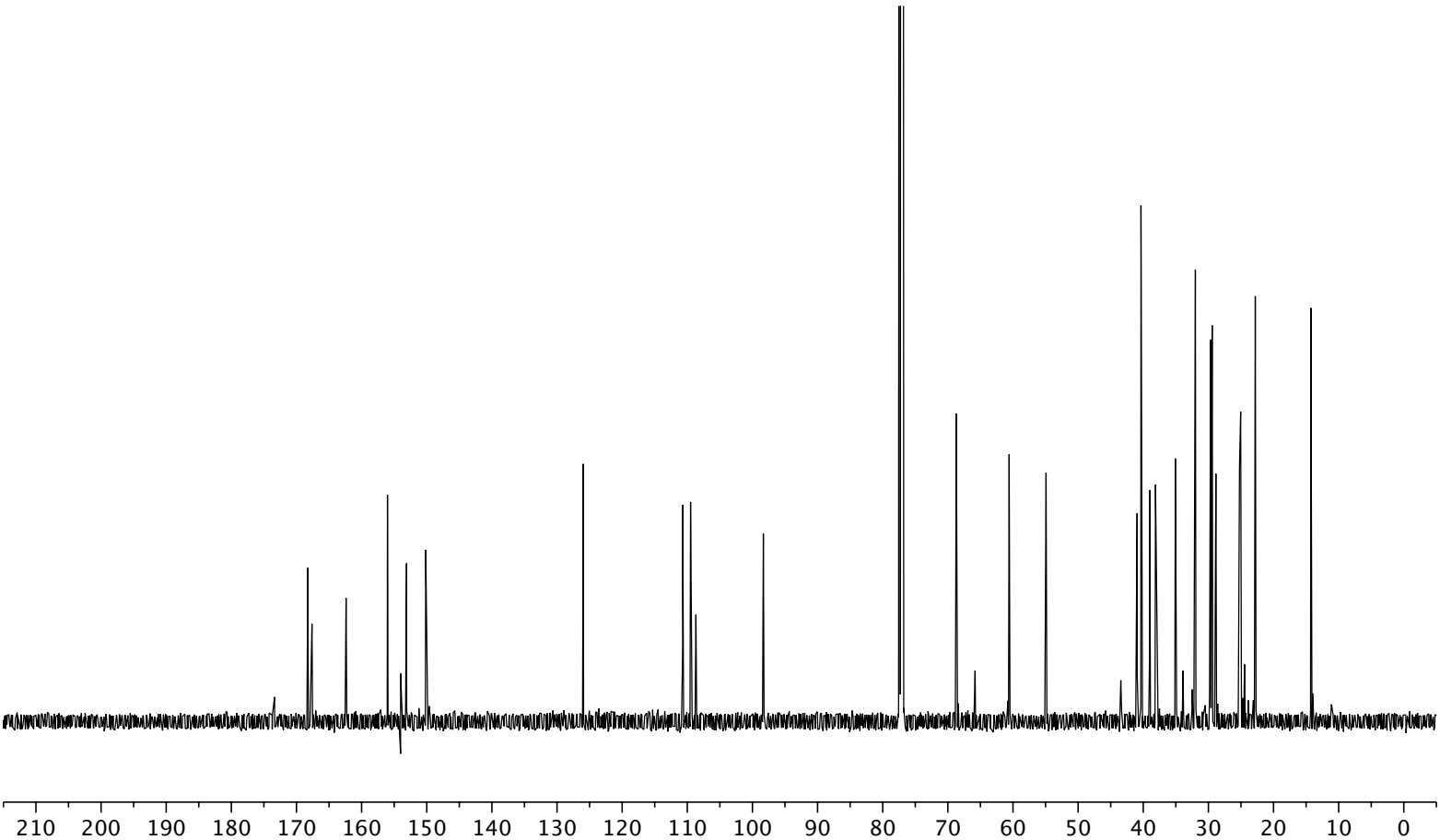
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of (2*S*,3*R*,4*S*)–**14** in $CDCl_3$



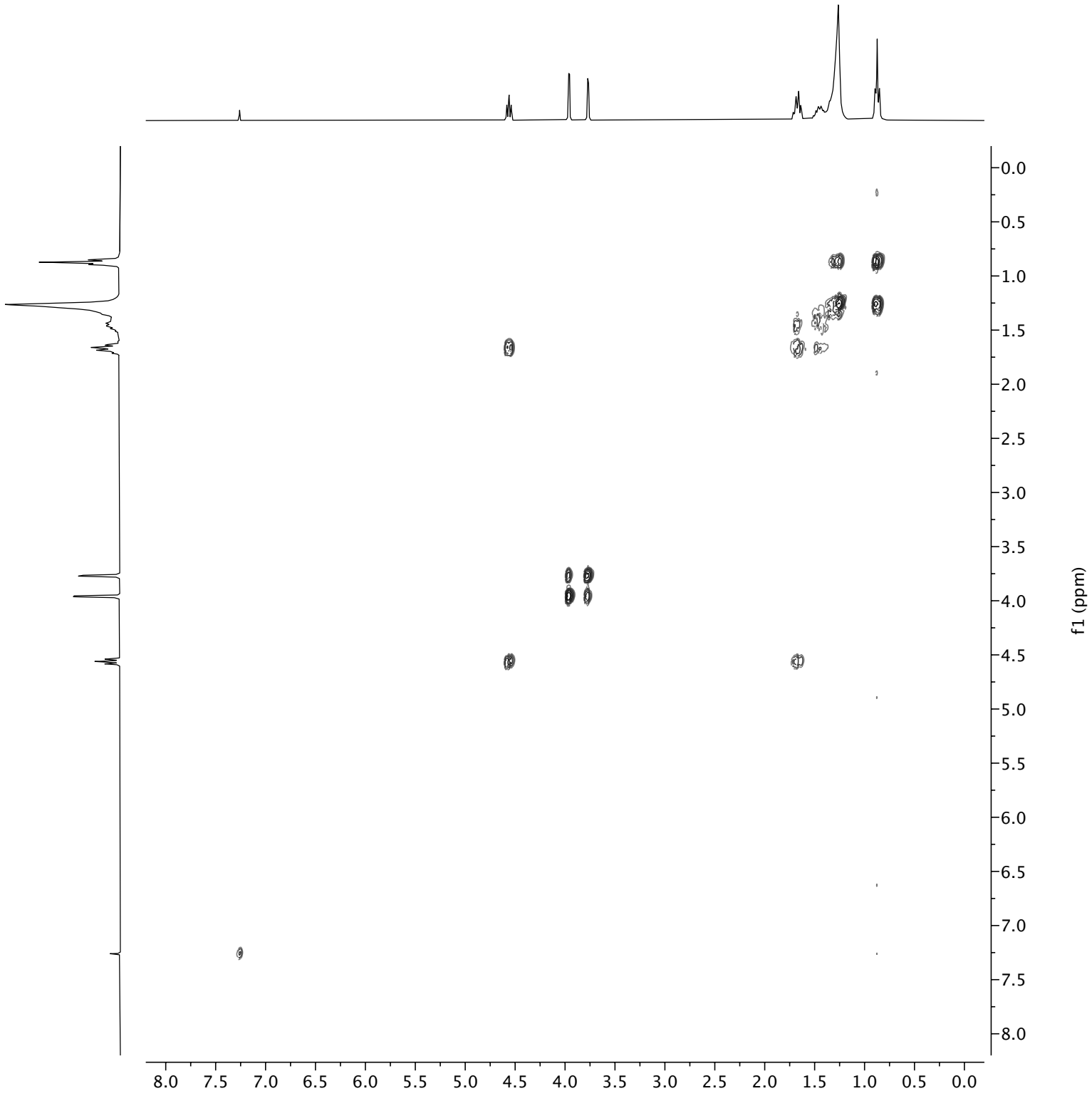


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of (2*R*,3*S*,4*R*)–**2b** in $CDCl_3$

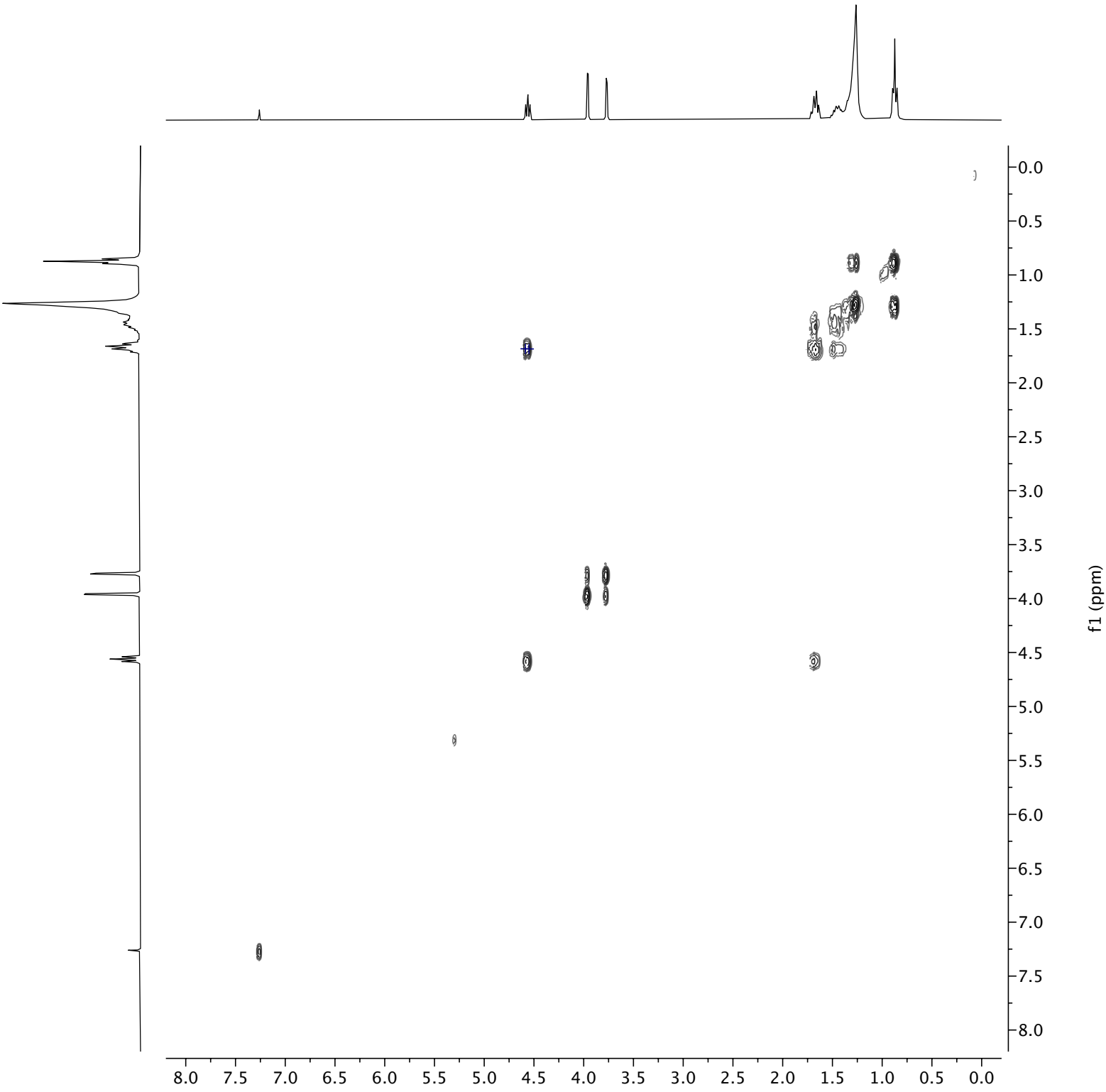




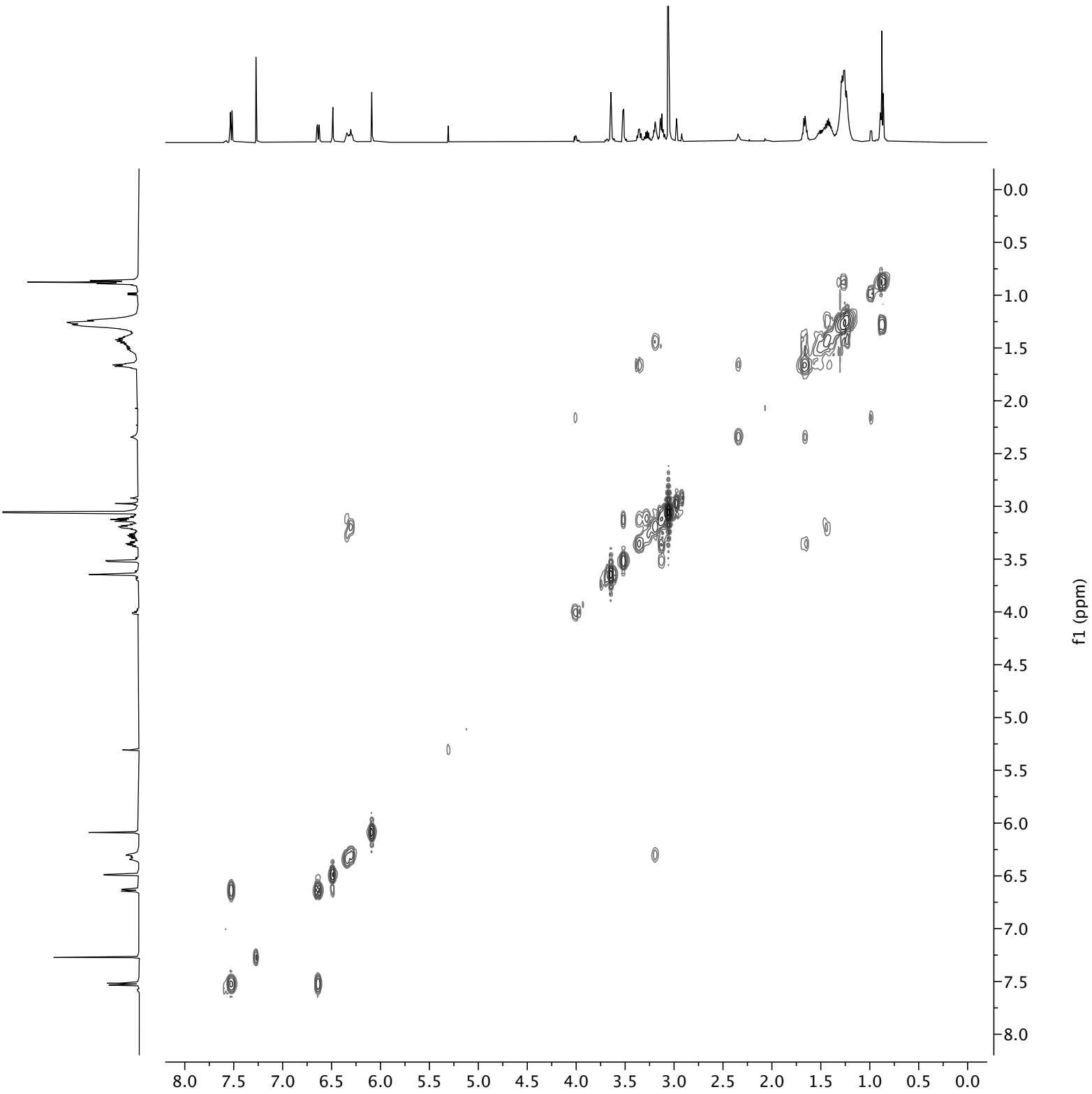
$^{1}H,^{1}H$–gCOSY (500 MHz) spectrum of (2*S*,3*R*,4*S*)–**14** in $CDCl_3$



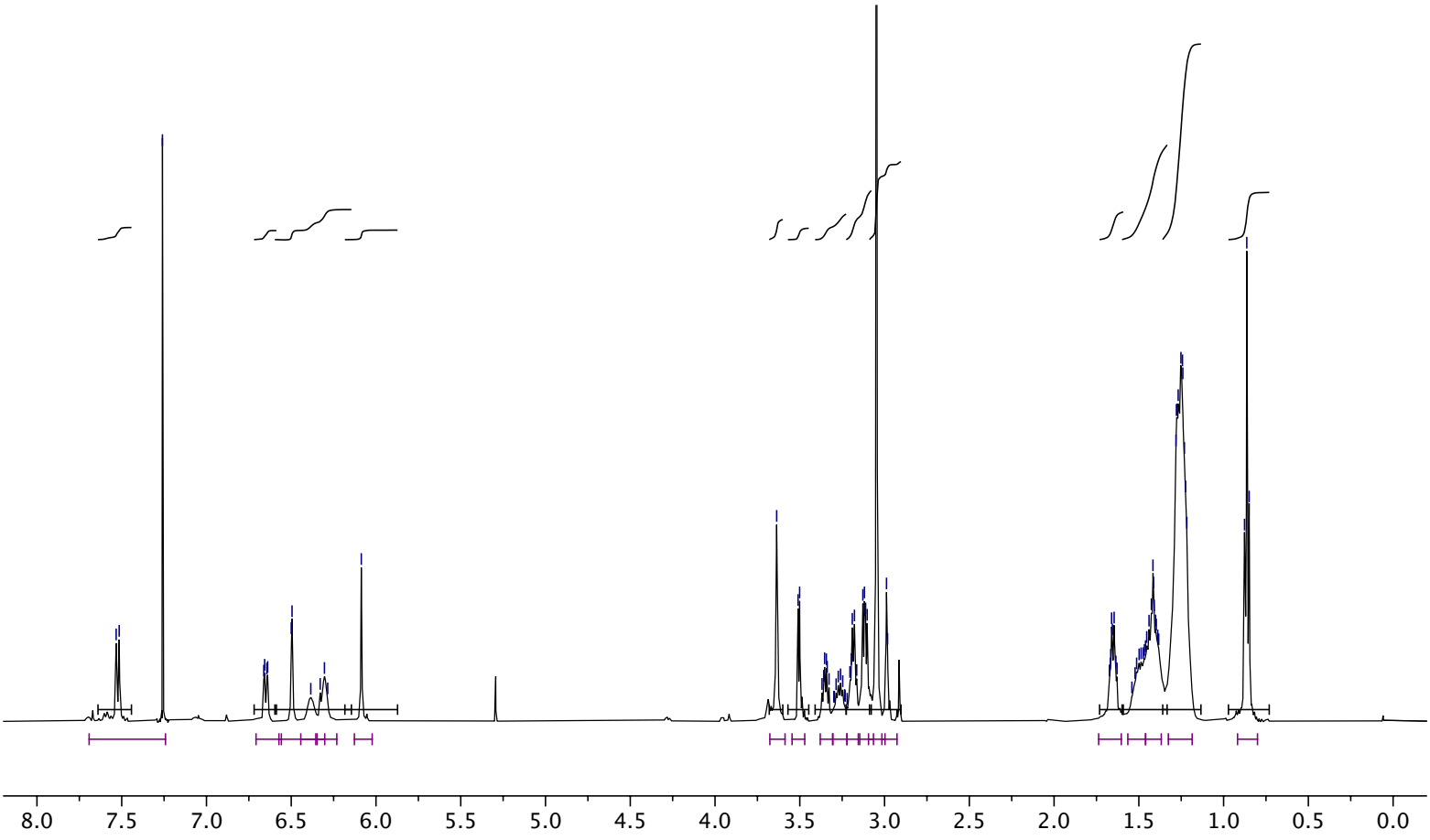
$^{1}H,^{1}H$–gCOSY (500 MHz) spectrum of (2*R*,3*S*,4*R*)–**14** in $CDCl_3$

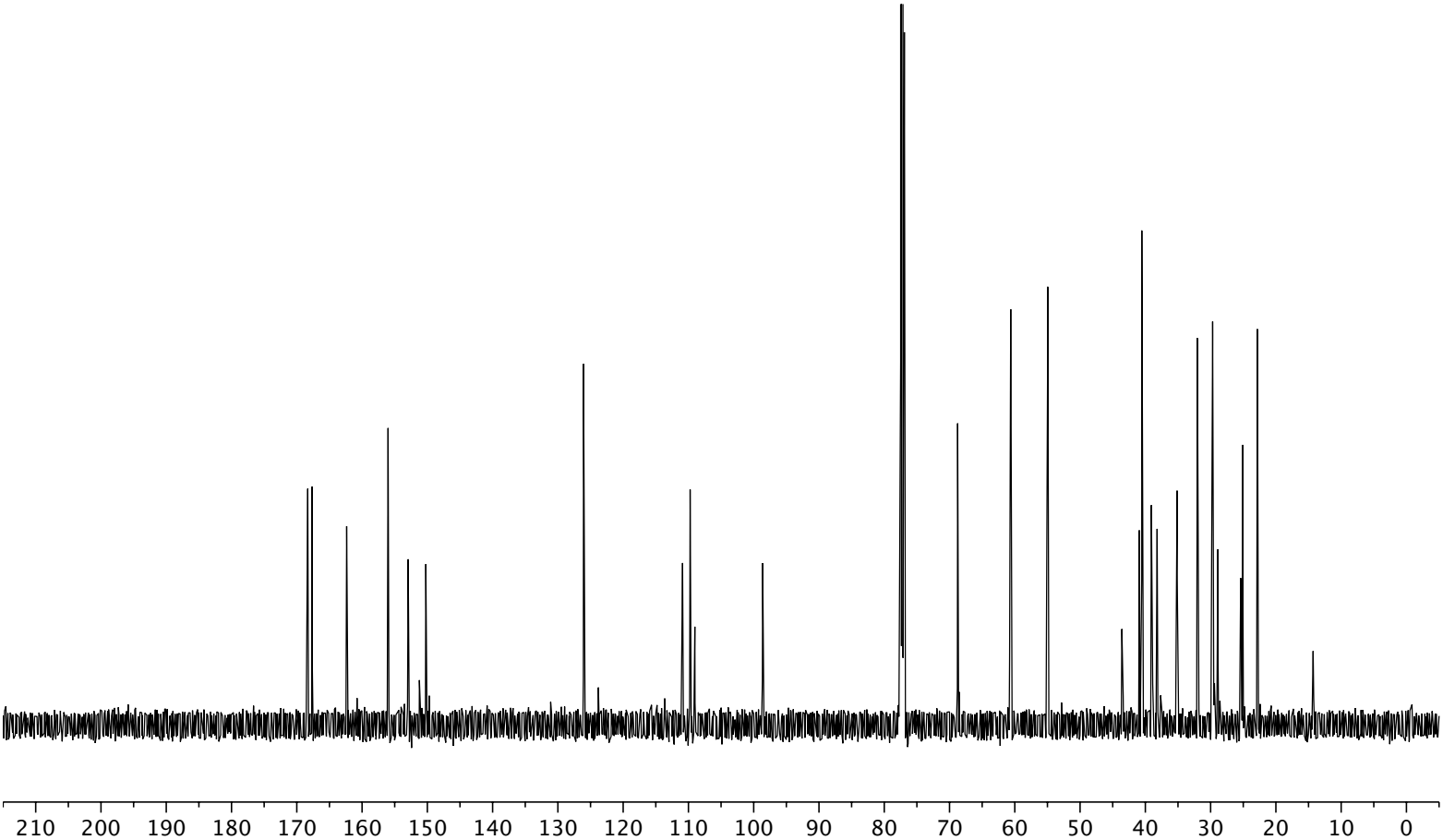


$^{1}H,^{1}H$–gCOSY (500 MHz) spectrum of (2*R*,3*S*,4*R*)–**2b** in $CDCl_3$

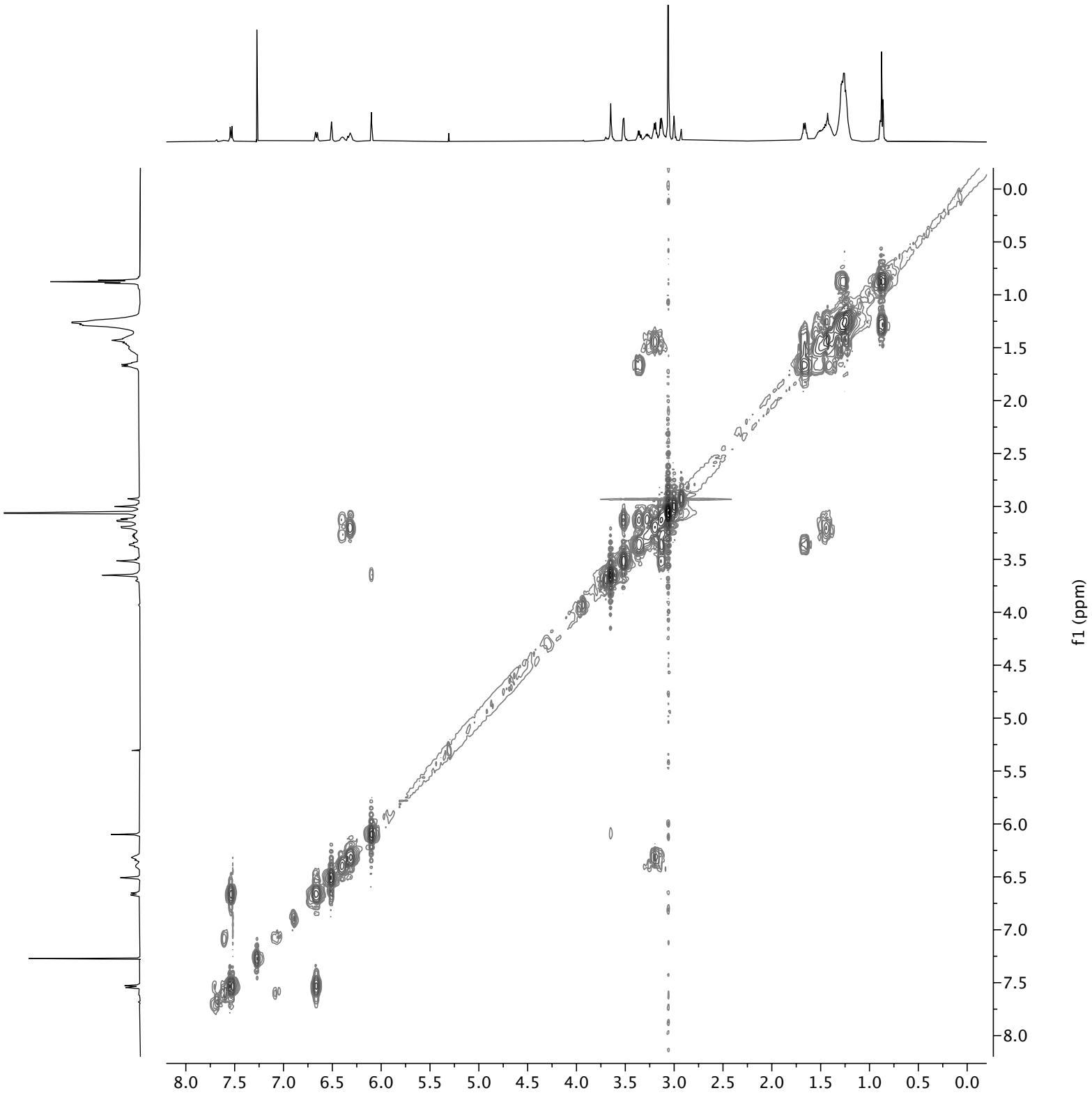


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of (2*S*,3*R*,4*S*)–**2b** in $CDCl_3$

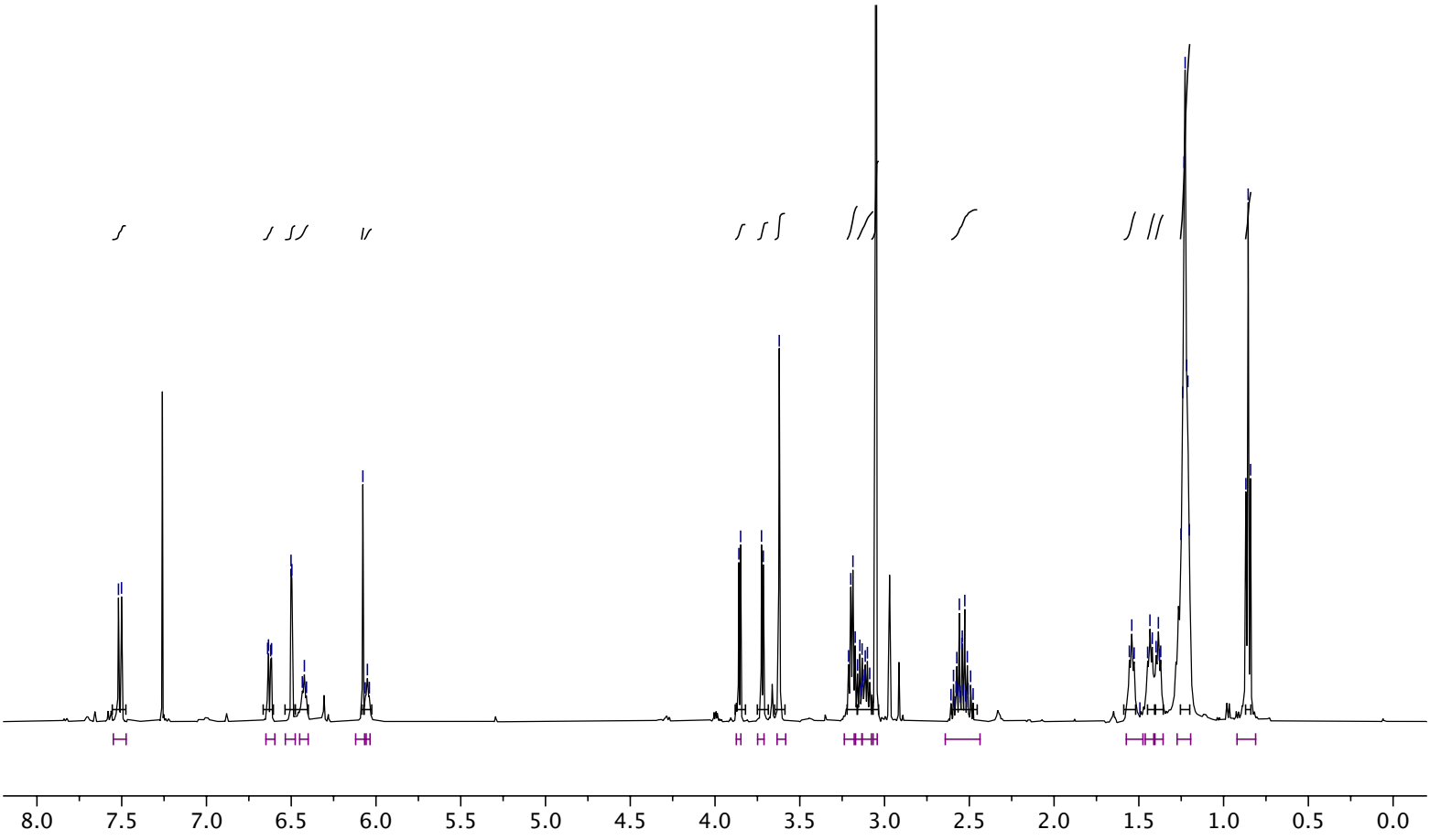


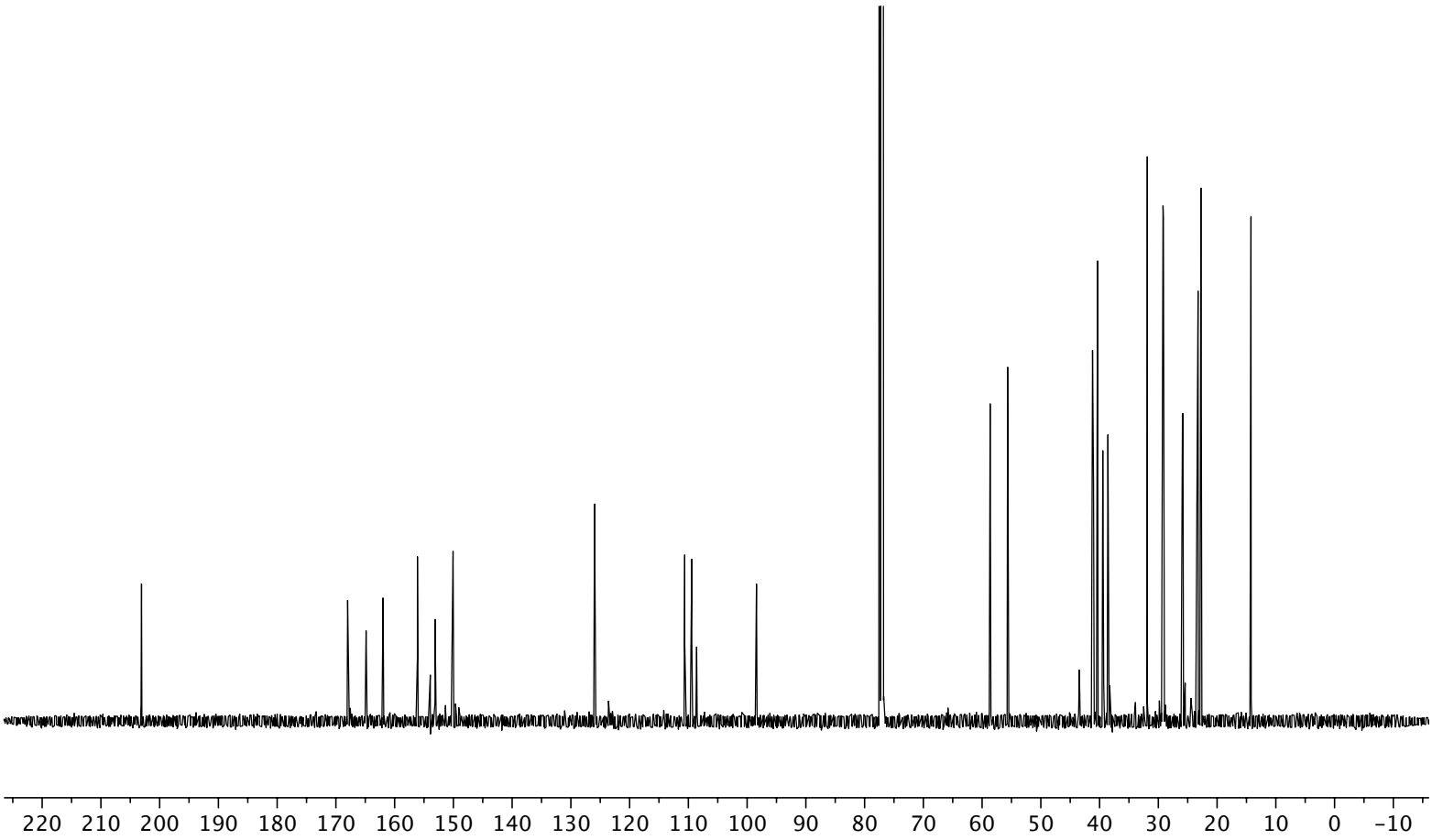


^{1}H,^{1}H–gCOSY (500 MHz) spectrum of (2*S*,3*R*,4*S*)–**2b** in $CDCl_3$

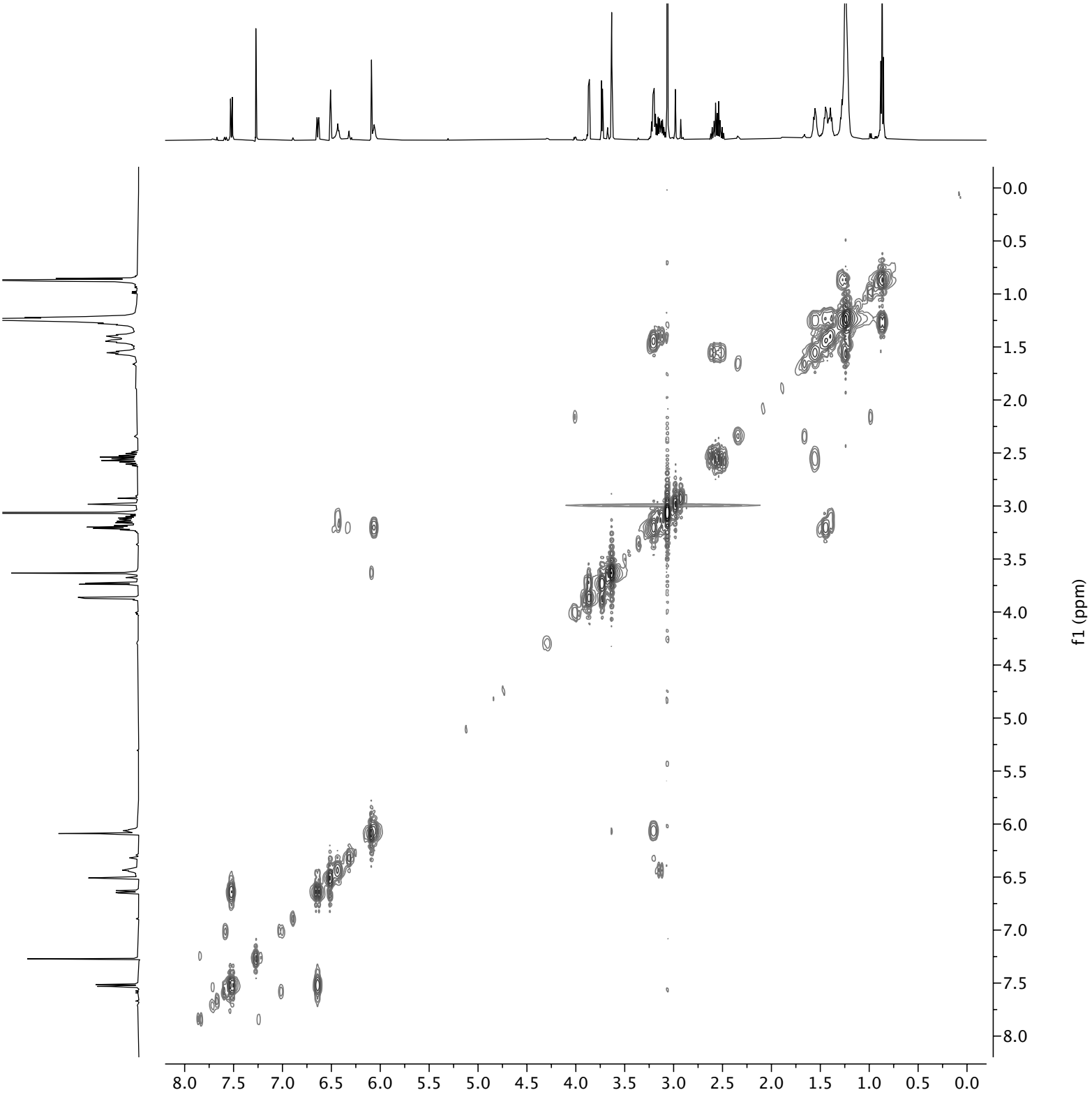


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of (2*R*,3*S*)–**1b** in $CDCl_3$

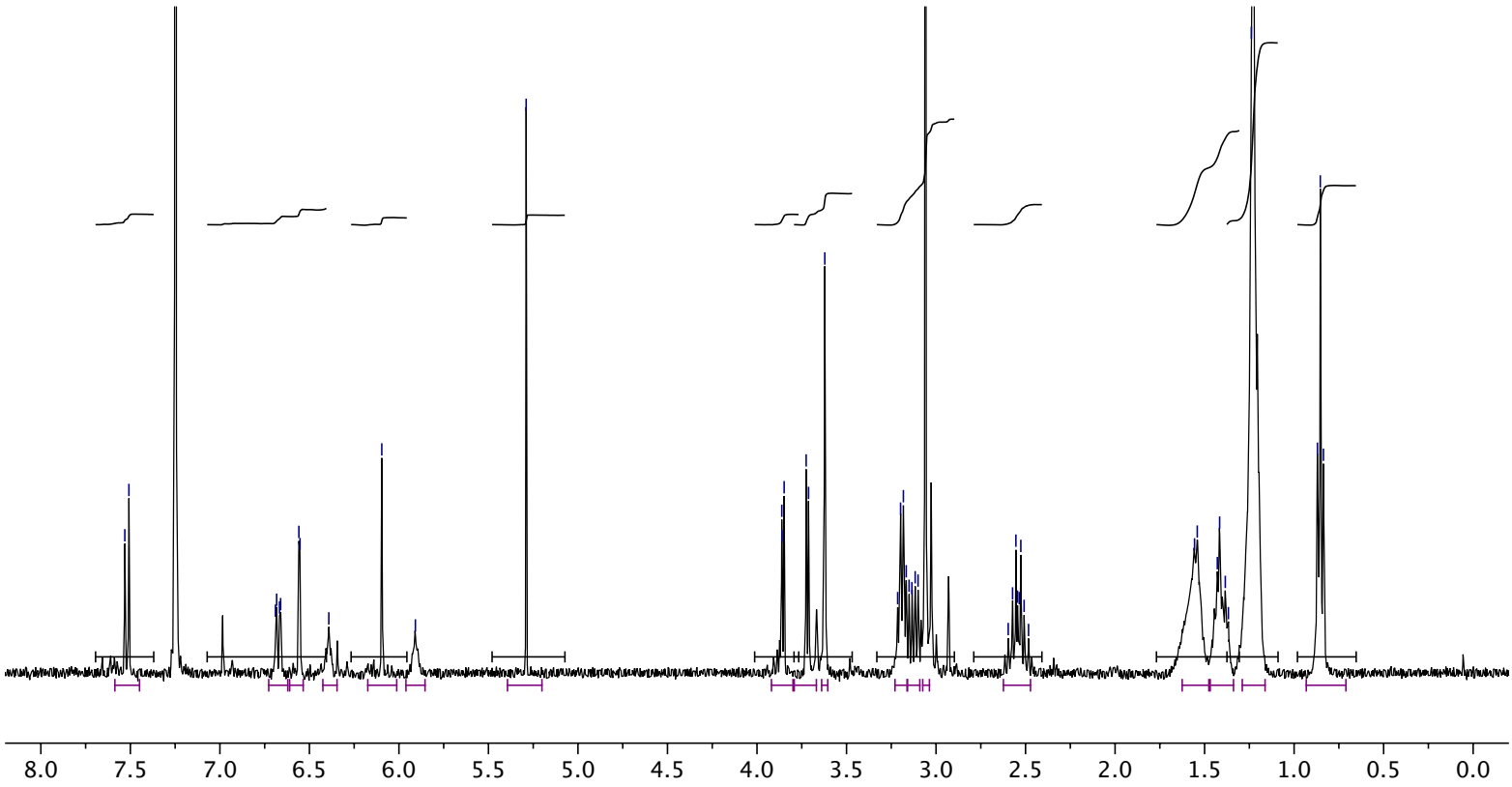


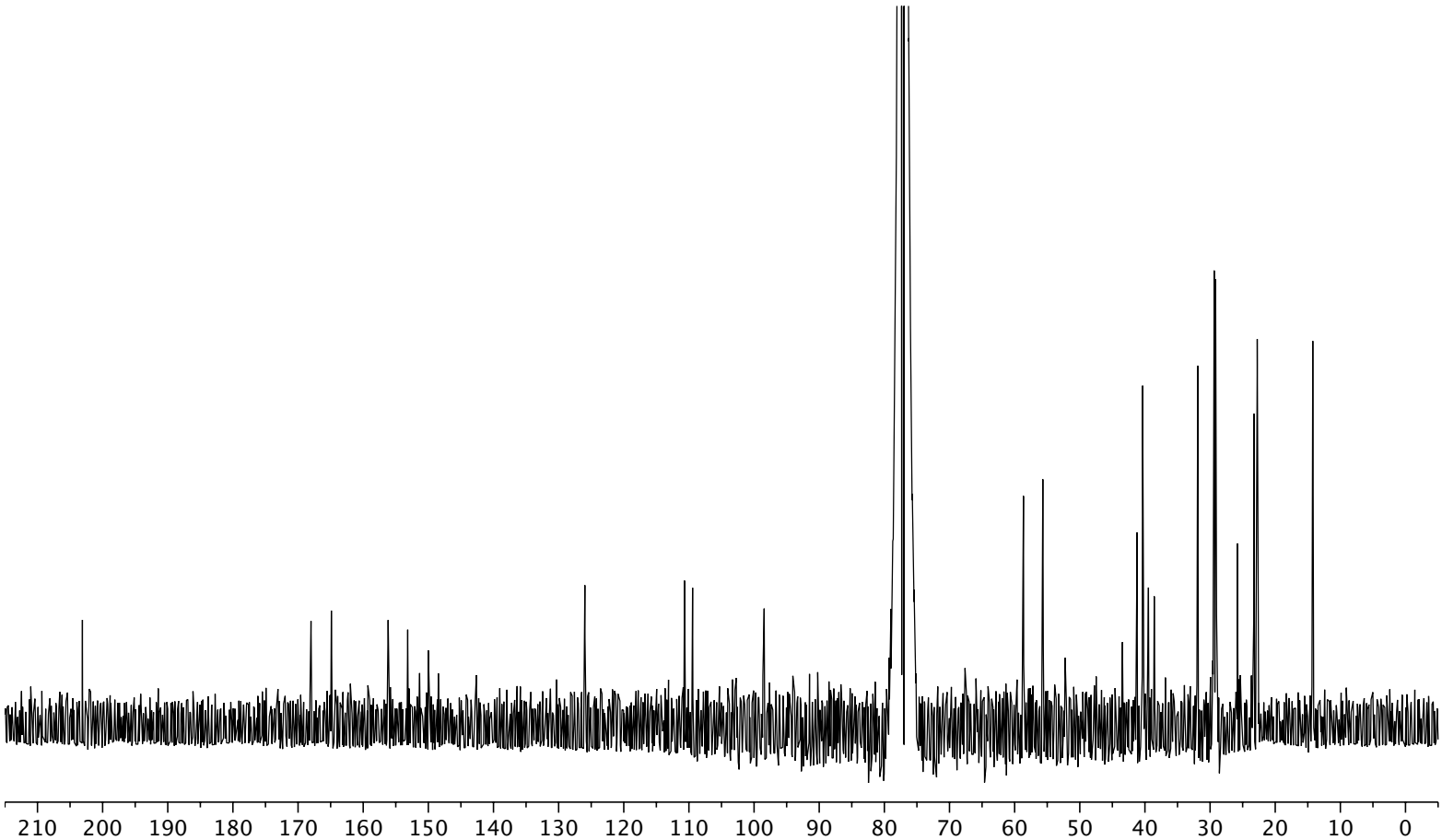


^{1}H,^{1}H–gCOSY (500 MHz) spectrum of (2*R*,3*S*)–**1b** in $CDCl_3$

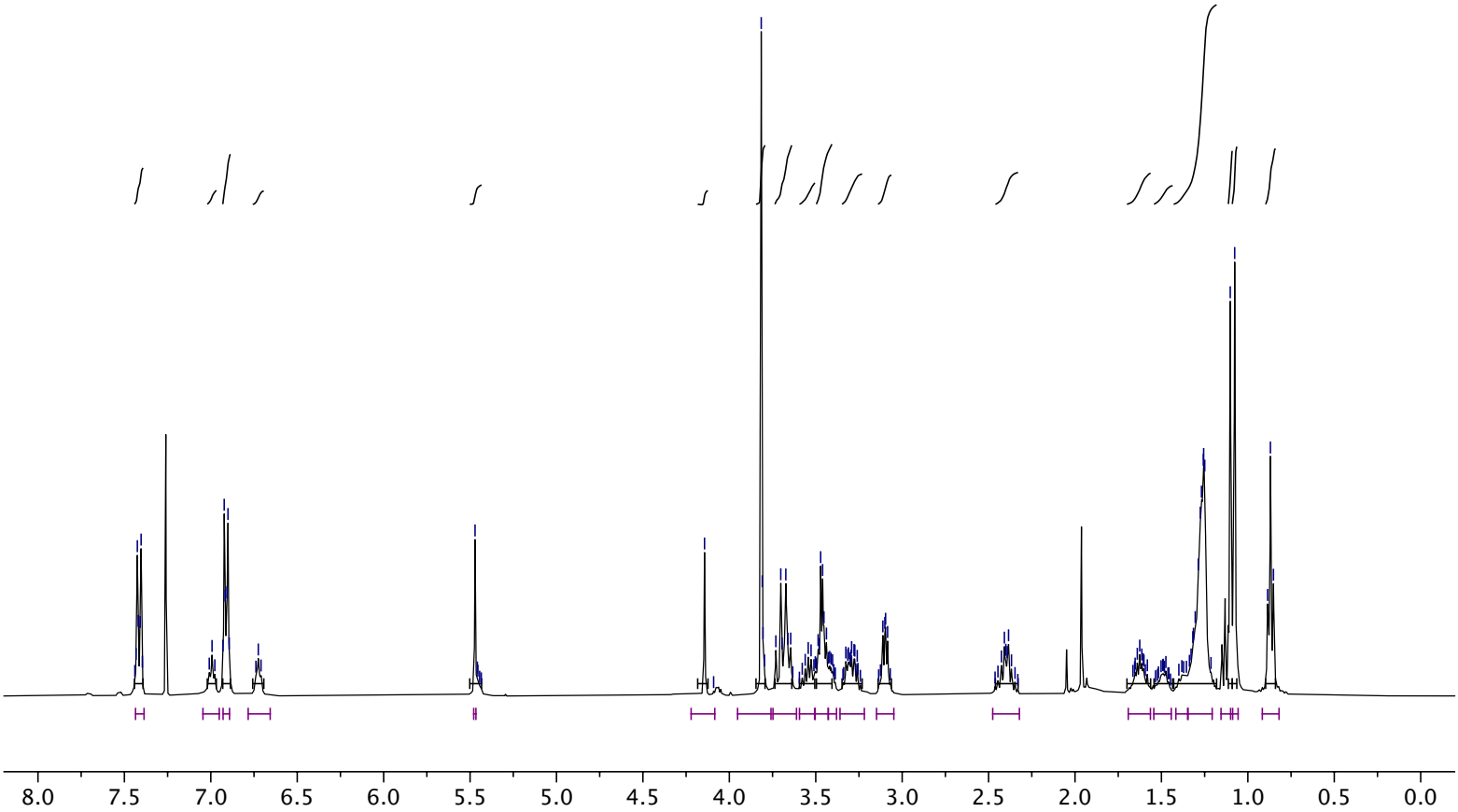


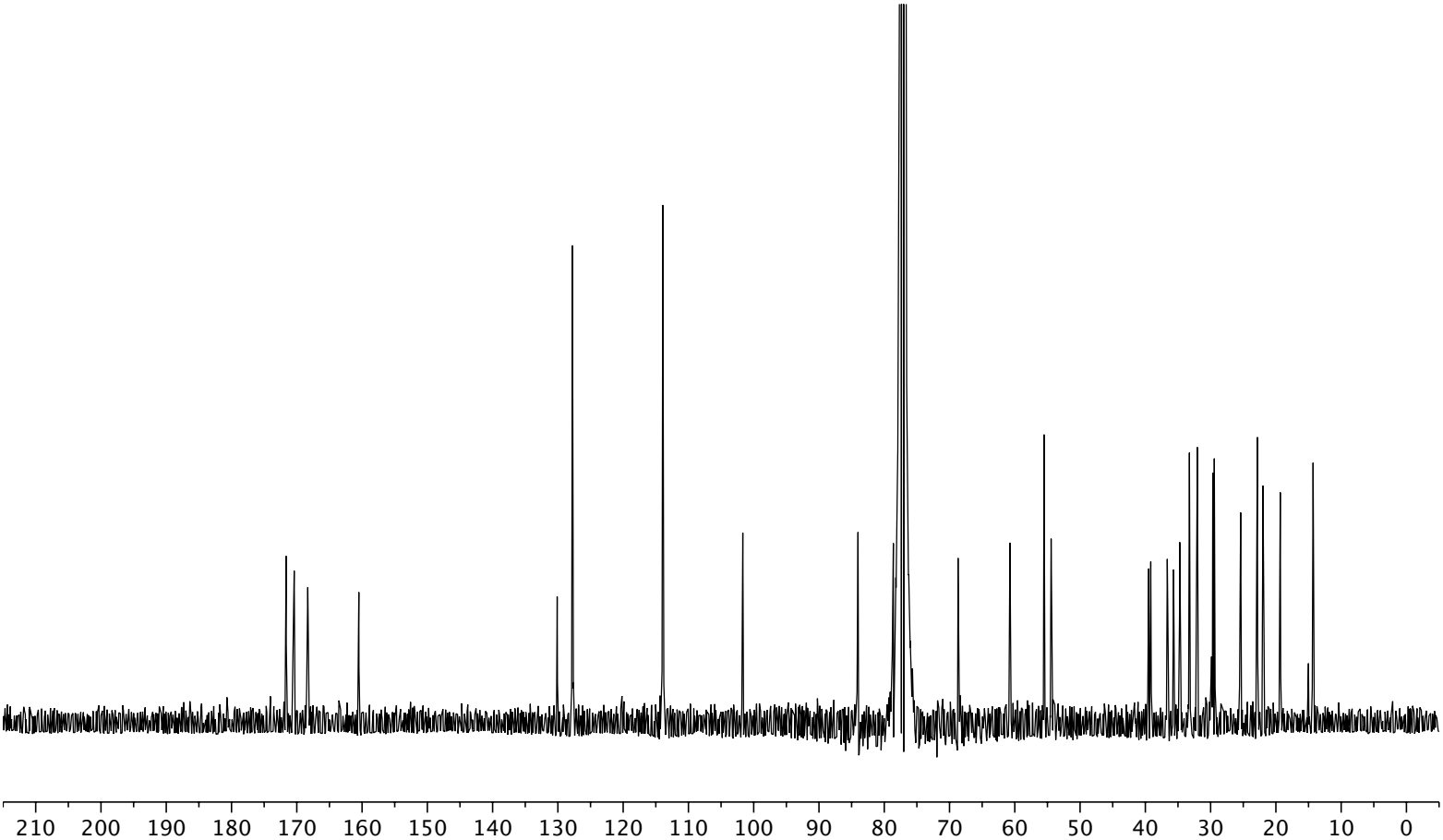
^{1}H–NMR (400 MHz) and ^{13}C–NMR (125 MHz) spectra of (2*S*,3*R*)–**1b** in $CDCl_3$



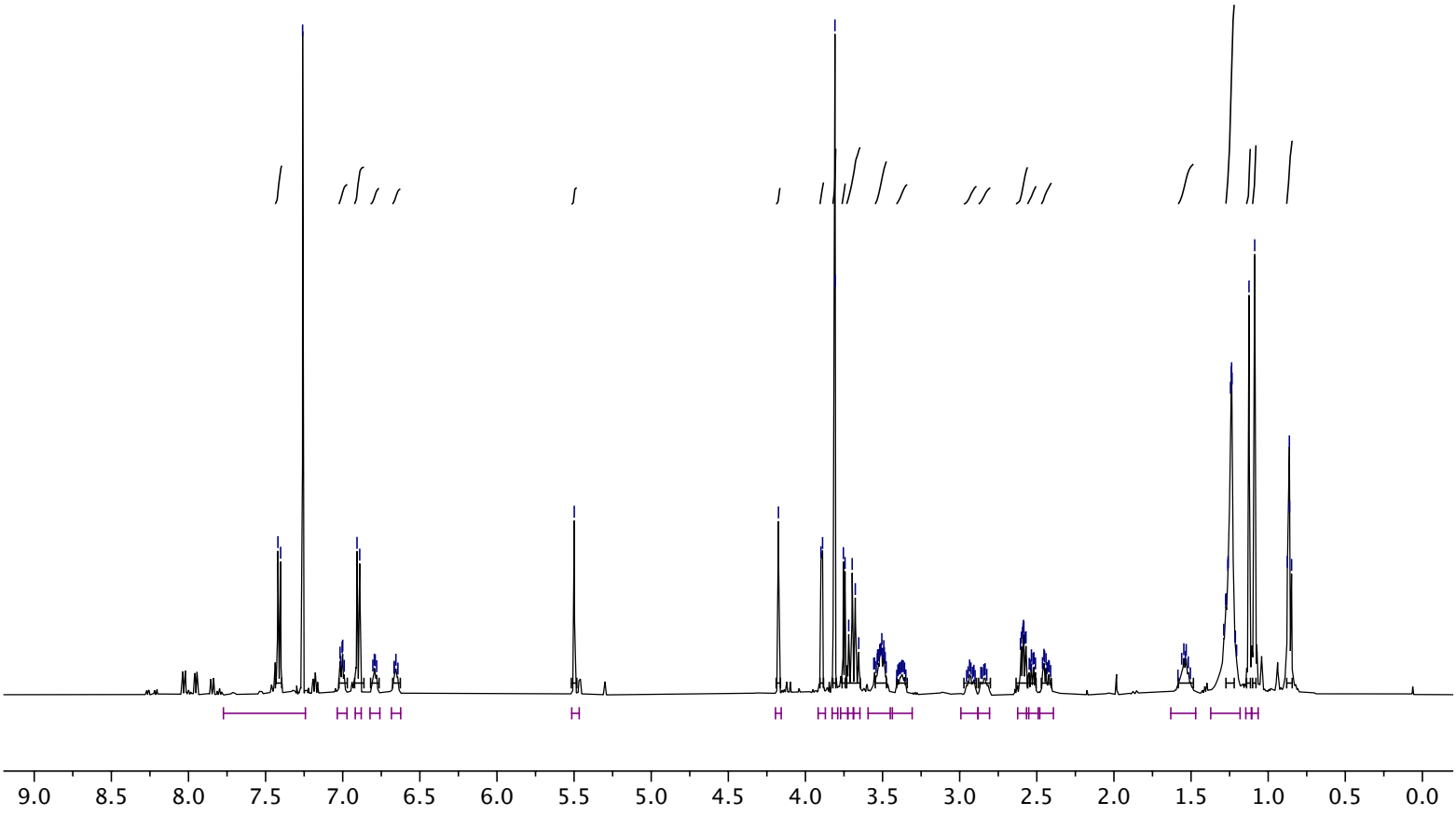


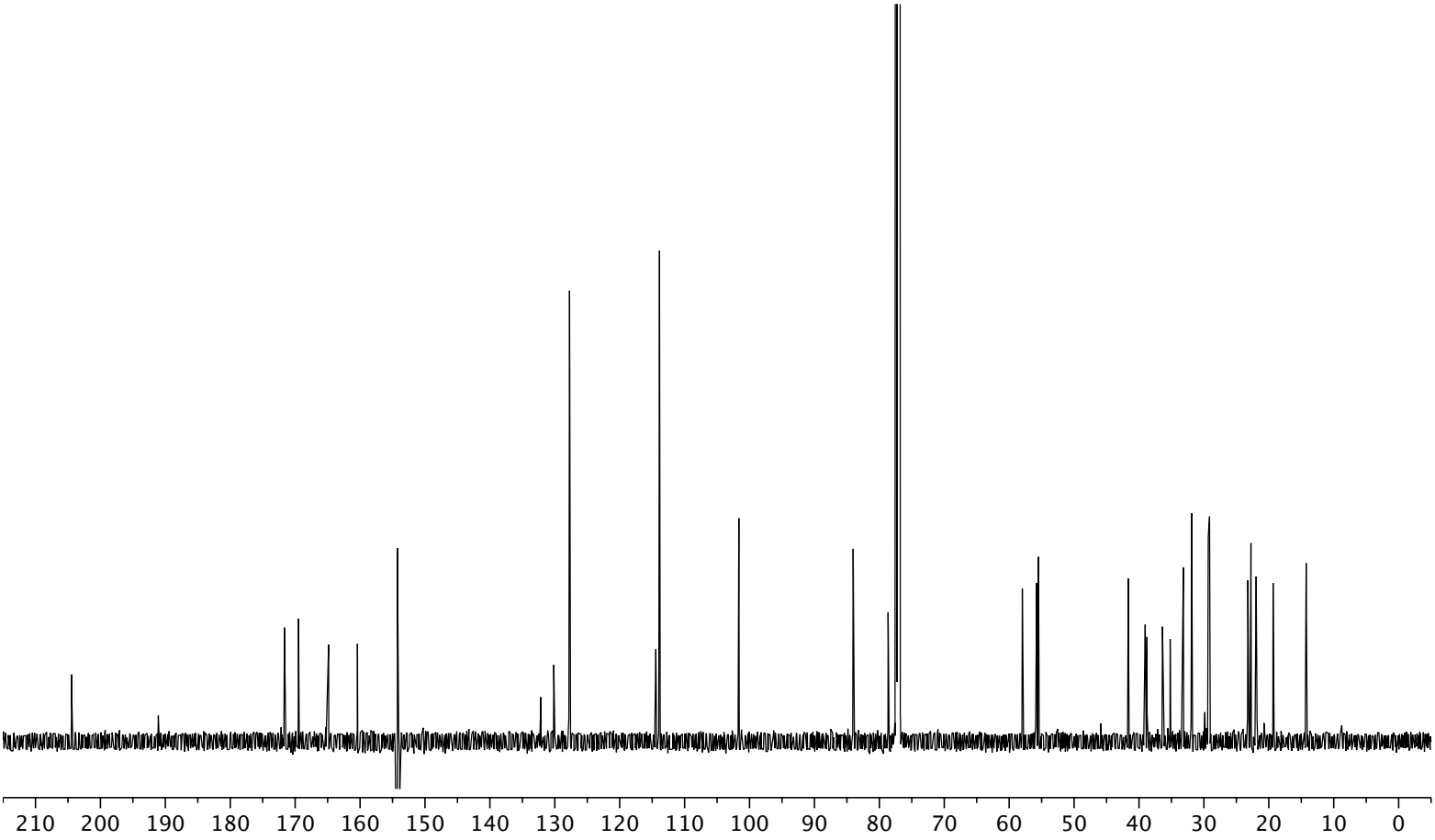
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of (2*R*,3*S*,4*R*)–**S1** in $CDCl_3$



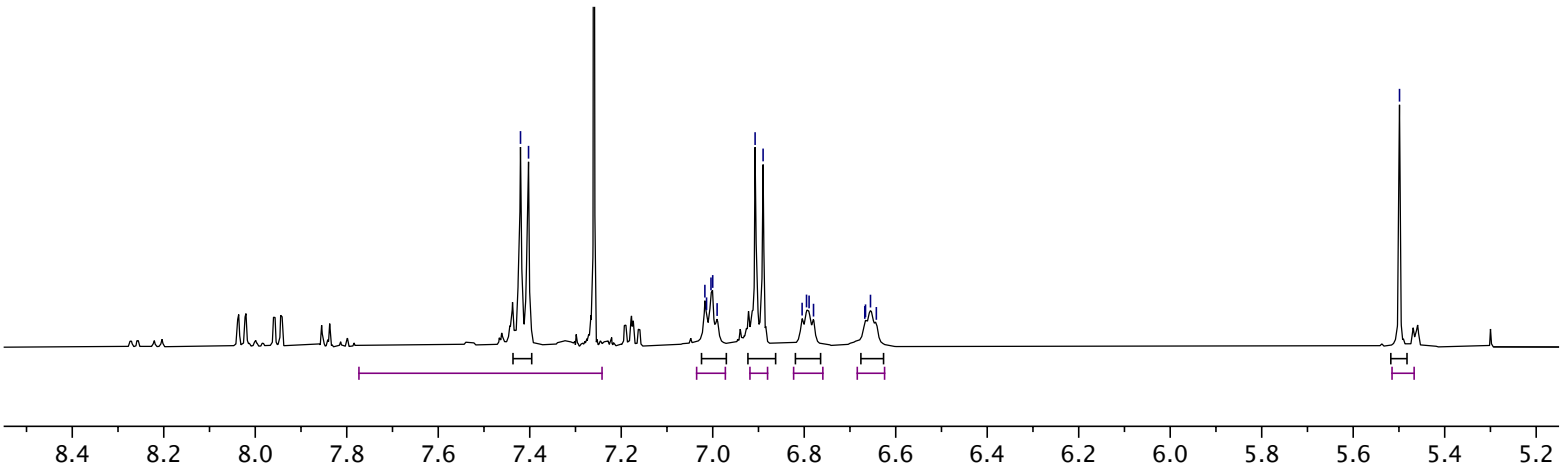


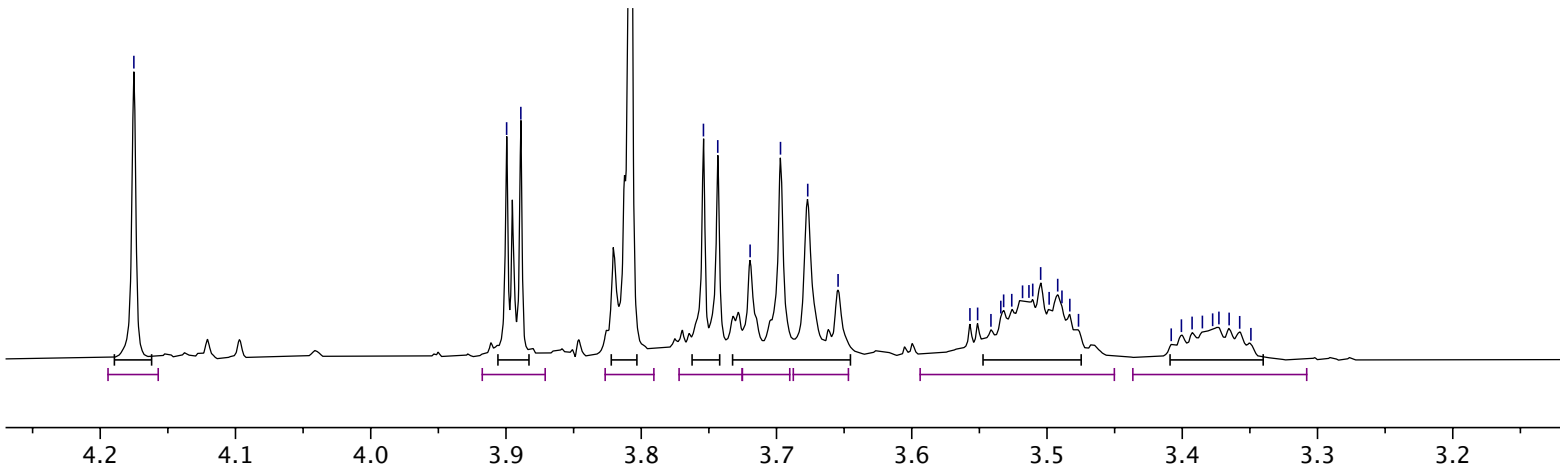
^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of (2*R*,3*S*)–**S2** in $CDCl_3$

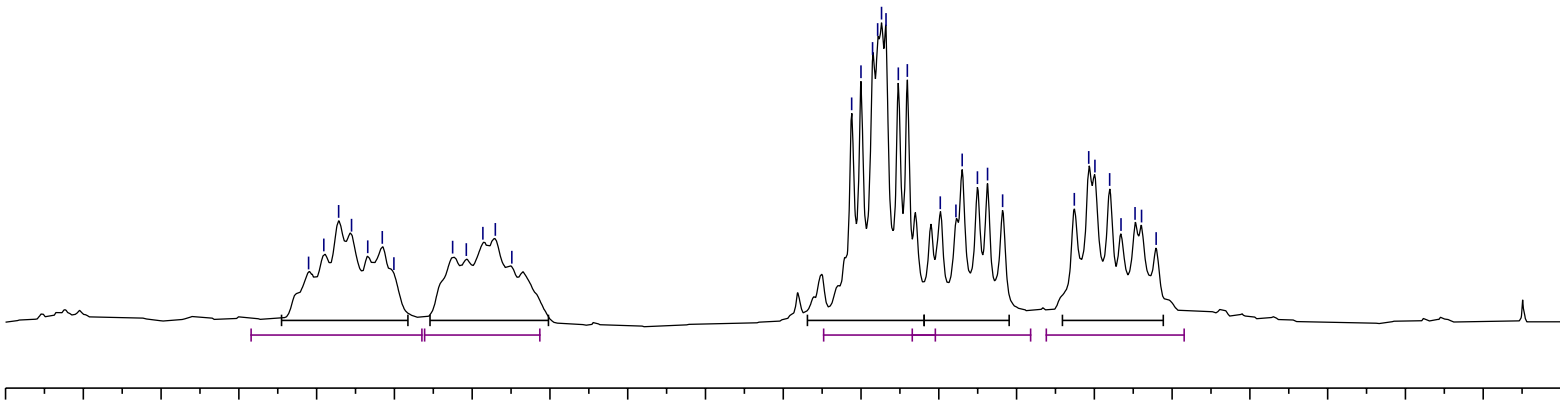


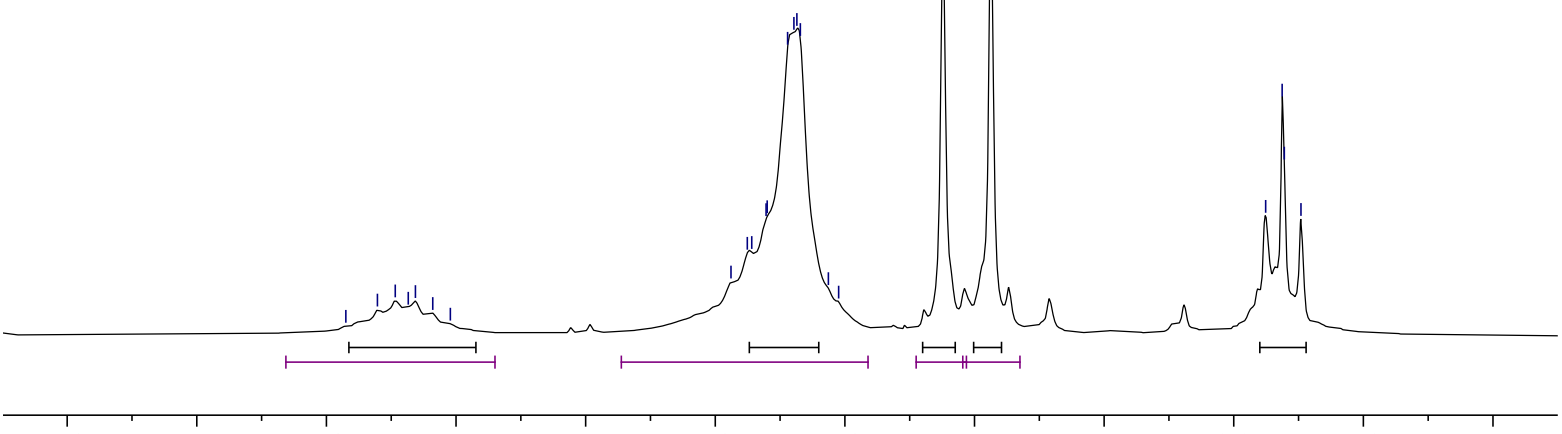


^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of (2*R*,3*S*)-**S2** in $CDCl_3$

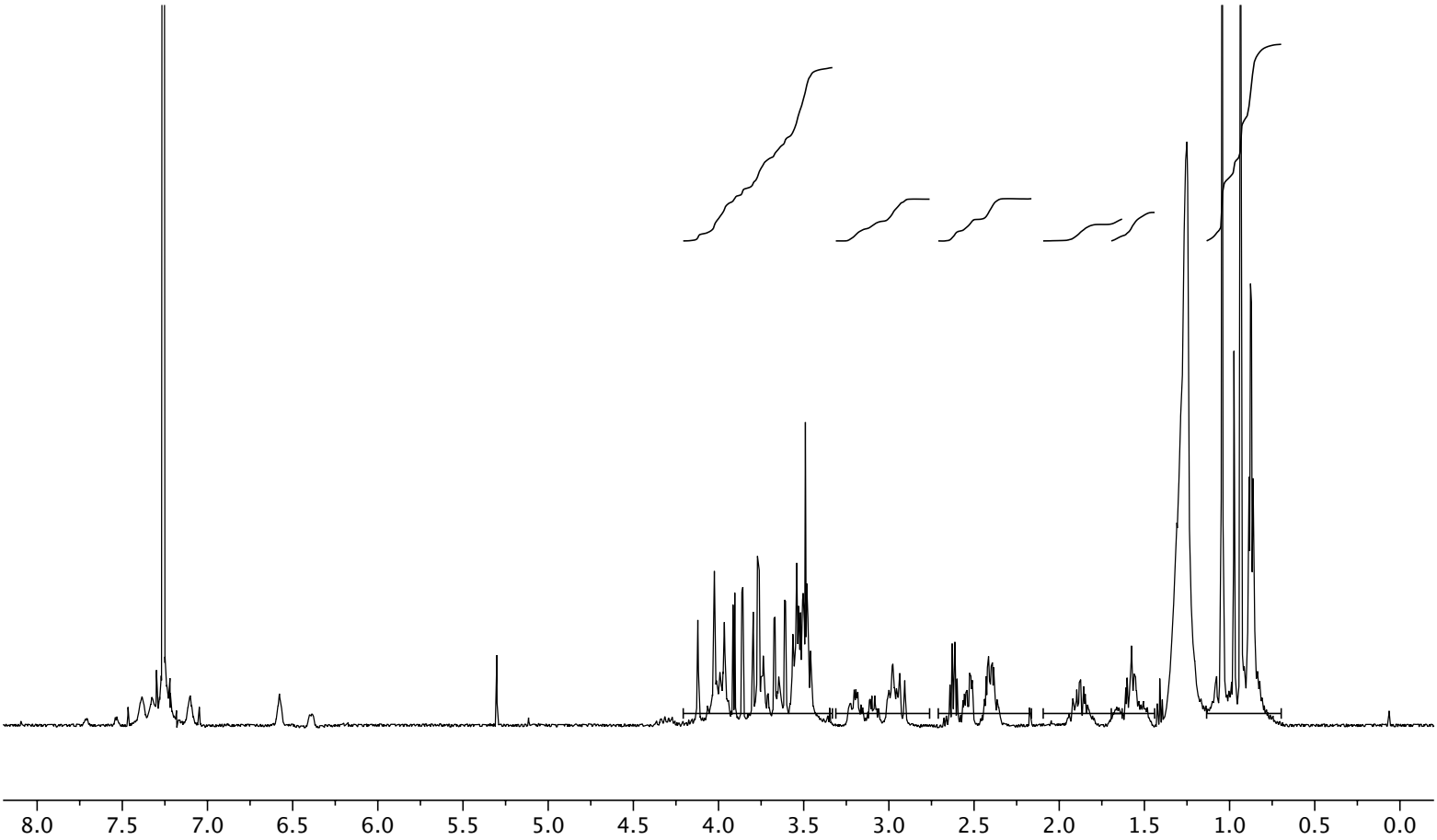


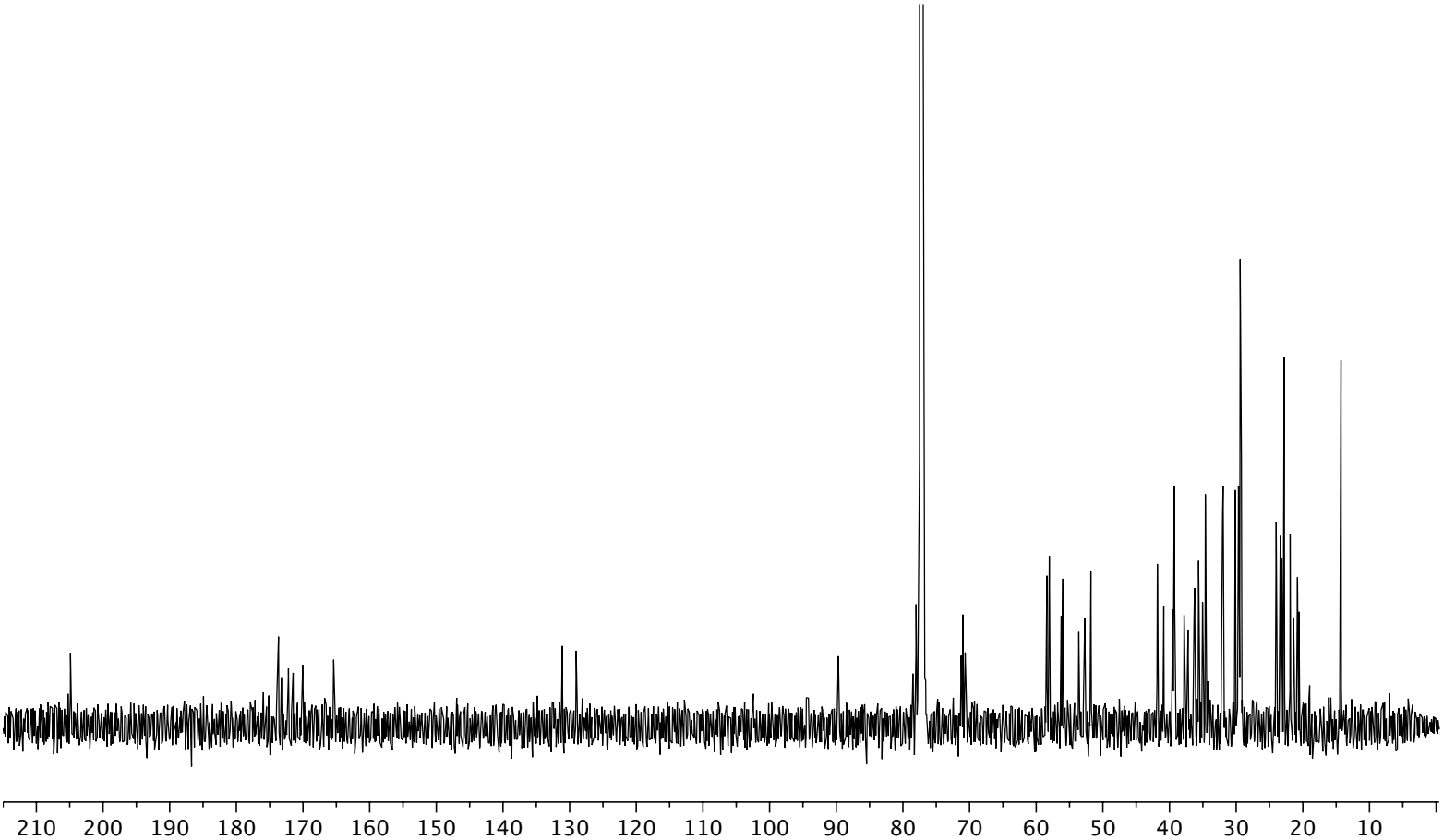




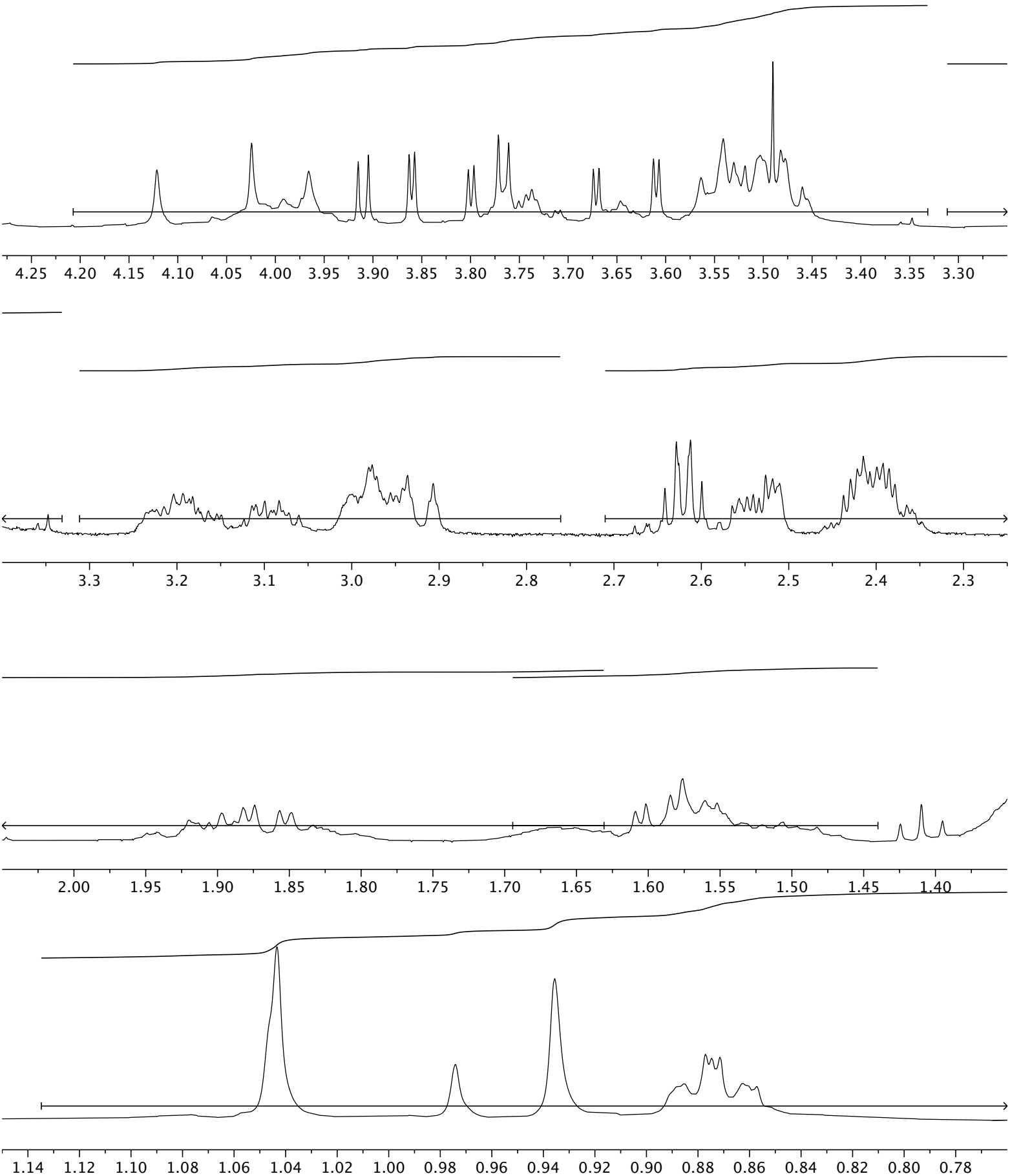


^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of (2*R*,3*S*)–**1c** in $CDCl_3$





^{1}H–NMR (500 MHz) and ^{13}C–NMR (125 MHz) spectra of (2*R*,3*S*)–**1c** in $CDCl_3$



$^{1}H,^{1}H$–gCOSY (500 MHz) spectrum of (2*R*,3*S*)–**1c** in $CDCl_3$