Supporting information

1. Experimental procedures

Peptides

The FITC labelled and acetylated 11-mer B-Raf pSer365 were purchased from GenScript. FITC labelled and acetylated 17-mer B-Raf mutant peptides were synthesized via Fmoc solid-phase peptide synthesis (SPPS) manually or using an automated Intavis MultiPepRsi peptide synthesizer and H-Rink amide MBHA resin (Novabiochem, 0.51 mmol/g loading). FITC (Sigma-Aldrich) was attached to the N-terminus of the peptides with an Fmoc-O1Pen-OH linker (Iris Biotech GmbH). The peptides were purified via reverse-phase preparative HPLC using a C18 XBridge Prep OBD column (19 mm × 250 mm); mobile phase A, water (0.1% FA); mobile phase B, MeCN (0.1% FA); flow rate, 15-20 mL/min.

Protein

14-3-3σΔC (C-terminally truncated after T231) was expressed in BL21 (DE3) competent cells *via* a pProEX HTb plasmid. Protein expression was induced with 0.4 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 18 °C. After spinning down and cell lysis of the expression culture, the protein was purified on a Ni^{2+}-NTA column. The His_6-tag was cleaved with Tobacco Etch Virus (TEV) protease in 1:0.05 mg ratio and a second nickel-affinity chromatography was performed followed by size exclusion chromatography (Superdex75) in 25 mM Hepes pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM β-mercaptoethanol. PPAR protein was provided by Auke Koops. BSA protein was purchased from Sigma-Aldrich and desalted prior to use. Tau protein was provided by Maxime Oetelaar.

Crystallography

The crystallization of 14-3-3σΔC/B-Raf pSer365 complex was performed according to a c-Jun (sequence: RNRVAA(pS)SKCRKRK) replacement method[1]. Crystals of the 14-3-3σΔC/c-Jun complexes were grown by first mixing 14-3-3σΔC and c-Jun peptide in a molar ratio of 1:1 with a final protein concentration of 12 $mg \cdot mL^{-1}$ in crystallization buffer (20 mM Hepes pH 7.5, 2 mM $MgCl_2$, and 2 mM β-mercaptoethanol). After 1 h incubation on ice, the solution of 14-3-3σΔC/c-Jun complex was mixed with precipitation buffer (0.1 M Hepes pH 7.5, 28% (v/v) PEG400, 0.2 $CaCl_2$, 5% (v/v) glycerol, 2 mM β-mercaptoethanol) in a 1:1 ratio for crystallization using the hanging drop method (2 µL droplet). The 14-3-3σΔC/c-Jun co-crystals grew within 2 weeks at 4 °C.

Next, 1 µL of 5 mM B-Raf pS365 (sequence: RDRSS(pS)APNVH) in crystallization buffer was pipetted to the above droplet to soak the 14-3-3σΔC/c-Jun crystals. After 23 days, the crystals were fished and directly flash-frozen in liquid nitrogen. Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF

Grenoble, France) on the automated beamline ID30B. Data were processed using Dials.[2] Molecular replacement was carried out using Phaser with PDB ID 4JC3.[3] The obtained model was subjected to reiterative rounds of model building and refinement using Coot[4] and Phenix.[5] Figures were prepared using Pymol software.

Crosslinking experiment

All the crosslinking experiments were performed in a BTP buffer (20 mM BTP, 100 µM TCEP, 2 mM $MgCl_2$, pH 7.0). A mixture of 8 µM 14-3-3σΔC and 80 µM compound with or without 16 µM acetylated peptide was incubated at 10 °C overnight. Two-step crosslinking experiments, used for the Tau peptide and Tau protein, were performed by pre-incubating 14-3-3σΔC and compound for 5 min to preform the 14-3-3-compound conjugate. Then the bivalent Tau peptide or Tau protein was added, and the resulting mixture incubated at 10 °C overnight.

Intact mass spectrometry experiments

Intact mass spectrometry experiments were performed using a High-Resolution LC-MS system consisting of a Waters ACQUITY UPLC I-Class system coupled to a Xevo G2 Quadrupole Time of Flight (Q-TOF) mass spectrometer operated in positive electrospray ionization (ESI) mode and scanning between 500-2000 m/z. A Polaris 3 C18-A reverse phase column (2.0 x 100 mm, Agilent) was used with a gradient of 15-60% MeCN in water (0.1% FA) and a flow rate of 0.3 mL/min using a column oven temperature set at 60 °C. Deconvolution of the m/z spectra was done using the MaxENT1 algorithm in the Masslynx v4.1 (SCN862, Waters) software to determine the intact protein mass.

Fluorescence anisotropy assay

Fluorescence anisotropy assays were performed in a HEPES buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween 20, pH 7.4, 1 mg/mL BSA). The anisotropy was measured in black round-bottom 384-well plate with a Tecan Infinite F500 plate reader. To determine the K_D values, 14-3-3 protein was titrated in a 1:1 dilution series to 50 nM fluorescein-labelled peptide. For compound titration, each compound was titrated in a 1:1 dilution series to a constant concentration of 14-3-3 protein (1.5 µM for B-Raf pS365) and fluorescein-labelled peptide (50 nM). A final sample volume of 10 µL per well in triplicate was prepared. Data were fitted with 'Hill' equation and graphs were made using OriginLab2020.

Digestion experiment

The digestion experiment was performed in a Tris buffer (50 mM Tris, 1 mM $CaCl_2$, pH 7.6). Prior to digestion, protein samples denatured at 95 °C for 5 min. Each sample (25 µL) was then diluted to a volume of 78 µL in Tris buffer, containing methoxyamine (80 µM). Note, that methoxyamin is added to inhibit the retro-Michael addition cleavage between the histidine and the cyclohexanone. Trypsin was then added (10 µL, 30 ng/µL, sequencing grade, Promega) and the mixture was incubated overnight at 37 °C. The digestion reaction was

then quenched by adding acetonitrile (10 µL) and formic acid (FA, 2 µL). The sample was centrifuged for 10 min and the supernatant was collected for LC-MS/MS measurement.

LC-MS/MS measurement

The LC-MS/MS measurements were performed using a High-Resolution LC-MS system consisting of a Waters ACQUITY UPLC I-Class system coupled to a Xevo G2 Quadrupole Time of Flight (Q-TOF) mass spectrometer operated in positive electrospray ionization (ESI) mode. A Polaris 3 C18-A reverse phase column (2.0 x 100 mm, Agilent) was used with a gradient of 5-90% MeCN in water (0.1% FA) and a flow rate of 0.3 mL/min using a column oven temperature set at 50 °C. Mass spectra were collected with ramped collision energy (CE) from 20-50 eV over the range of 200-2000 m/z.

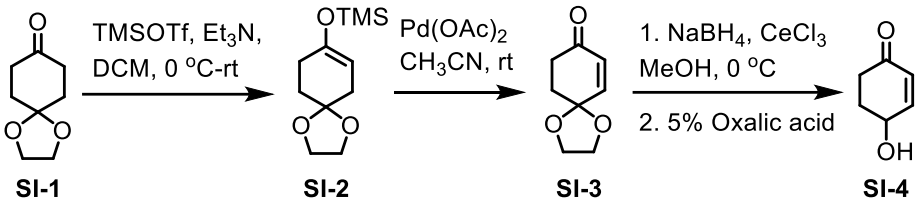
SDS-PAGE analysis

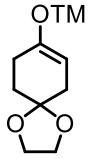
The SDS-page (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) experiments were performed in a SDS-page running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Before electrophoresis, the samples were mixed with 2x Laemmli loading buffer (100 mM Tris, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, DTT, pH 6.8) in a 1:1 ratio. The samples (10 µL) were loaded into the gel lanes and the electrophoresis was performed under a constant voltage of 140 V for 1 h. The gel was later stained with Coomassie.

Chemistry

General information. Starting materials were purchased from Sigma-Aldrich, TCI, ABCR or Fluorochem and used as received without purification. Dry solvents were obtained from a MBRAUN Solvent Purification System (MB-SPS-800). Deuterated solvents were purchased from Cambridge Isotope Laboratories and used as received without further purification. All reactions were carried out open to air unless otherwise stated, and monitored by analytical thin-layer chromatography (TLC) with aluminium-backed silica (Merck silica gel 60 F254). Visualization of TLC plates was performed by ultraviolet light (254 nm) or by staining. Flash column chromatography was carried out on an automated Grace Reveleris X2 chromatograph with pre-packed silica columns (Büchi). Solvents were evaporated *in vacuo* using a Büchi rotary evaporator. LC-MS analysis was carried out using a Thermo Fisher LCQ Fleet Ion Trap Mass Spectrometer and a C18 Jupiter SuC4300A 150 × 2.0 mm column with a gradient of 5-100% MeCN in water (+ 0.1% FA) over 15min. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were obtained on a Bruker Avance 400 MHz spectrometer at ambient temperature. Chemical shifts (δ) are reported in ppm and are referenced to residual solvent peak of $CDCl_3$ (^{1}H NMR: δ = 7.26 ppm; ^{13}C NMR: δ = 77.16 ppm). Peaks are reported as: multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets), integration, and coupling constant (J) in hertz (Hz).

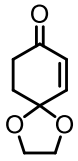
Synthesis of 2-Cyclohexen-1-one intermediate (SI-4). The synthetic route was adapted from literature reported by James *et al*[6] and Yu-Jen *et al*.[7] **SI-1** was purchased from Sigma-Aldrich.





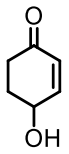
SI-2

To a 1000 mL round bottom flask was added **SI-1** (5 g, 32 mmol) and triethylamine (13 mL, 96 mmol) in anhydrous DCM (378 mL). To the solution was then added trimethylsilyl trifluoromethanesulfonate (6.3 mL, 35.2 mmol) dropwise at 0 °C (ice bath) over 10 min under a nitrogen atmosphere. The reaction mixture was brought to room temperature, stirred for 15 min, and quenched with sat. Na_2CO_3 (20 mL). The resulting mixture was washed with water (3 x 100 mL). The organic phase was separated, dried over Na_2SO_4, filtered and concentrated to furnish **SI-2** as pale-yellow liquid (6.7 g, 92%). The resulting material was used directly in the next step without further purification. Experimental NMR spectra are consistent with the reported ones.[6]



SI-3

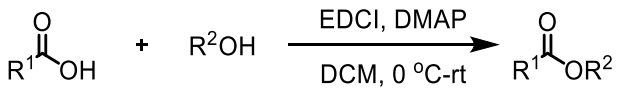
To a 100 mL round bottom flask was added **SI-2** (0.5 g, 2.2 mmol) in MeCN (35 mL). To the solution was then added $Pd(OAc)_2$ (0.5 g, 2.2 mmol). The reaction mixture was stirred overnight at room temperature. The resulting mixture was filtered through a short pad of celite. The filtrate was concentrated. The resulting material was purified using flash column chromatography (0-70% EtOAc in hexane) to furnish **SI-3** (0.32 g, 94%) as pale-yellow liquid. Experimental NMR spectra are consistent with the reported ones.[6]



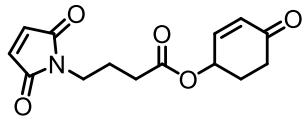
SI-4

To a 50 mL round bottom flask was added **SI-3** (654 mg, 4.2 mmol) and $CeCl_3.7H_2O$ (1.5 g, 4.2 mmol) in MeOH (10.5 mL). To the solution was then added $NaBH_4$ (80 mg, 2.1mmol) slowly in 3 portions at 0 °C (ice bath) while stirring. The reaction mixture was stirred for around 30min. Once **SI-3** was fully consumed as indicated by TLC, the reaction was quenched with ice water (2 mL). To the mixture was then added 5% oxalic acid (4 mL) and stirring was continued for another 30min. Sat. $NaHCO_3$ was added to adjust the pH to 6-7. The resulting mixture was extracted with DCM (3 x 15 mL). The organic layers were combined, dried over Na_2SO_4, filtered and concentrated. The resulting material was purified using flash column chromatography (50-100% EtOAc in hexane) to furnish **SI-4** (100 mg, 21%) as colorless liquid. Experimental NMR spectra are consistent with the reported ones.[7]

General procedure for synthesis of analogues 1-10. A Steglich esterification using EDCI as an activating reagent and DMAP as a catalyst. Compound **11** was purchased from abcr.

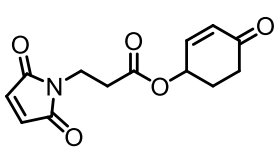


To a round bottom flask was added carboxylic acid in dry DCM (0.1 M). To the solution was added EDCI and a small grain of DMAP at 0 °C (ice bath) under nitrogen atmosphere. The mixture was stirred for around 15min and then alcohol was added. The reaction mixture was brought to room temperature and stirred overnight. Then extra DCM (2-4 mL) was added to dilute the mixture. The resulting mixture was washed with water (3 x 5 mL). The organic phase was separated, dried over Na_2SO_4, filtered and concentrated. The resulting material was purified as specified.



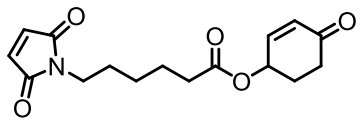
1

1 was prepared according to the general procedure using 4-Maleimidobutyric acid (47.6 mg, 0.26 mmol), **SI-4** (24.7 mg, 0.22 mmol), EDCI (63.2 mg, 0.33 mmol) and catalytic amount of DMAP. Flash column chromatography (30-80% EtOAc in hexane) was performed to furnish **1** (14 mg, 23%) as pale-yellow oil. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 6.82 (d, *J* = 10.3, 1H), 6.67 (s, 2H), 5.98 (d, *J* = 10.3, 1H), 5.60 – 5.46 (m, 1H), 3.54 (t, *J* = 6.8 Hz, 2H), 2.55 (dt, *J* = 16.9, 5.1 Hz, 1H), 2.47 – 2.23 (m, 4H), 2.13 – 1.99 (m, 1H), 1.89 (p, *J* = 7.1 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.9, 171.8, 170.7, 147.6, 134.2, 130.9, 67.9, 36.9, 35.0, 31.3, 28.7, 23.7. LRMS (ESI+) *m/z* 300 (M+Na).



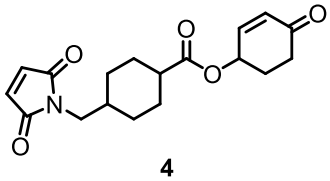
2

2 was prepared according to the general procedure using 3-Maleimidopropionic acid (44.0 mg, 0.26 mmol), **SI-4** (24.7 mg, 0.22 mmol), EDCI (63.2 mg, 0.33 mmol) and catalytic amount of DMAP. Flash column chromatography (30-80% EtOAc in hexane) was performed to furnish **2** (5.2 mg, 9%) as pale-yellow oil. ^{1}H NMR (400 MHz, CD_2Cl_2) δ 6.80 (d, *J* = 10.3, 1H), 6.68 (s, 2H), 5.98 (d, *J* = 10.3, 1H), 5.53 (m, 1H), 3.80 (t, *J* = 7.0 Hz, 2H), 2.65 (t, *J* = 6.9 Hz, 2H), 2.54 (dt, *J* = 16.9, 5.2 Hz, 1H), 2.45 – 2.23 (m, 2H), 2.10 – 1.99 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.8, 170.30, 170.08, 147.2, 134.3, 131.0, 68.2, 34.9, 33.61, 33.14, 28.6. LRMS (ESI+) m/z 286 (M+Na).



3

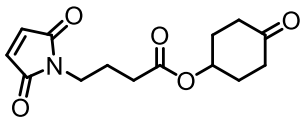
3 was prepared according to the general procedure using 6-Maleimidocaproic acid (54.9 mg, 0.26 mmol), **SI-4** (24.7 mg, 0.22 mmol), EDCI (63.2 mg, 0.33 mmol) and catalytic amount of DMAP. Flash column chromatography (30-80% EtOAc in hexane) was performed to furnish **3** (10 mg, 15%) as pale-yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.84 (d, *J* = 10.2, 1H), 6.70 (s, 2H), 6.06 (d, *J* = 10.3, 1H), 5.57 (m, 1H), 3.52 (t, *J* = 7.2 Hz, 2H), 2.62 (dt, *J* = 16.9, 5.1 Hz, 1H), 2.45 (m, 1H), 2.35 (m, 3H), 2.16 – 2.02 (m, 1H), 1.65 (dp, *J* = 23.0, 7.5 Hz, 4H), 1.34 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.9, 172.7, 170.8, 147.7, 134.1, 130.8, 67.6, 37.6, 35.0, 34.0, 28.71, 28.17, 26.2, 24.3. LRMS (ESI+) m/z 328 (M+Na).



4

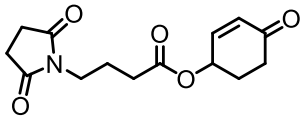
4 was prepared according to the general procedure using N-[4-(-Carboxycyclohexylmethyl)]maleimide (51.2 mg, 0.22 mmol), **SI-4** (20 mg, 0.18 mmol), EDCI (51.7 mg, 0.27 mmol) and catalytic amount of DMAP. Flash column

chromatography (30-80% EtOAc in hexane) was performed to furnish **4** (20 mg, 33.5%) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.81 (d, *J* = 10.2, 1H), 6.70 (s, 2H), 6.05 (d, *J* = 10.2, 1H), 5.63 – 5.45 (m, 1H), 3.38 (d, *J* = 6.9 Hz, 2H), 2.61 (dt, *J* = 16.9, 5.1 Hz, 1H), 2.44 (m, 1H), 2.38 – 2.21 (m, 2H), 2.13 – 1.95 (m, 3H), 1.81 – 1.62 (m, 3H), 1.41 (qd, *J* = 13.0, 3.3 Hz, 2H), 1.02 (qd, *J* = 13.0, 3.6 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.9, 174.9, 171.0, 147.7, 134.0, 130.9, 67.4, 43.6, 43.0, 36.3, 35.0, 29.6, 28.66, 28.17. LRMS (ESI+) m/z 354 (M+Na).



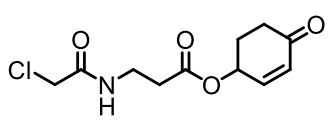
5

5 was prepared according to the general procedure using 4-Maleimidobutyric acid (80.5 mg, 0.44 mmol), 4-Hydroxycyclohexanone (60.4 mg, 0.53 mmol), EDCI (126.5 mg, 0.66 mmol) and catalytic amount of DMAP. Flash column chromatography (30-80% EtOAc in hexane) was performed to furnish **5** (102.2 mg, 83.2%) as pale-yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.68 (s, 2H), 5.14 (m, 1H), 3.56 (t, *J* = 6.9, 2H), 2.52 (m, 2H), 2.32 (m, 4H), 2.04 (m, 4H), 1.91 (p, *J* = 7.3 Hz, 2H). LRMS (ESI+) m/z 302 (M+Na).



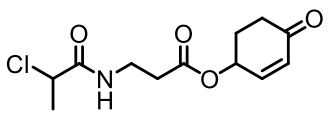
6

6 was prepared according to the general procedure using 4-Succinimidobutyric acid (10 mg, 0.05 mg), **SI-4** (6.7 mg, 0.06 mmol), EDCI (14.4mg, 0.08 mmol) and catalytic amount of DMAP. Flash column chromatography (30-80% EtOAc in hexane) was performed to furnish **6** (3.2 mg, 22.9%) as pale-yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.86 (d, *J* = 10.3, 1H), 6.06 (d, *J* = 10.3, 1H), 5.63 – 5.54 (m, 1H), 3.59 (t, *J* = 6.9 Hz, 2H), 2.72 (s, 4H), 2.67 – 2.58 (m, 1H), 2.50 – 2.31 (m, 4H), 2.11 (dddd, *J* = 13.3, 11.7, 8.8, 4.7 Hz, 1H), 1.94 (p, *J* = 7.1 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.9, 177.3, 171.9, 147.6, 130.9, 67.9, 37.9, 35.0, 31.5, 28.71, 28.20, 22.9. LRMS (ESI+) m/z 302 (M+Na).



7

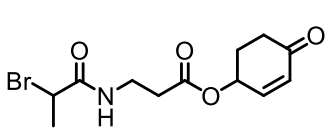
7 was prepared according to the general procedure using N-(2-Chloroacetyl)-beta-alanine (17,9 mg, 0.11 mmol), **SI-4** (10 mg, 0.09 mmol), EDCI (25.9 mg, 0.13 mmol) and catalytic amount of DMAP. Flash column chromatography (30-80% EtOAc in hexane) was performed to furnish **7** (4.5 mg, 19.2%) as pale-yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.14 (s, 1H), 6.85 (d, *J* = 10.2, 1H), 6.08 (d, *J* = 10.2, 1H), 5.62 (m, 1H), 3.62 (q, *J* = 6.1 Hz, 2H), 2.70 – 2.58 (m, 3H), 2.52 – 2.29 (m, 2H), 2.16 – 2.04 (m, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.6, 171.5, 166.0, 146.9, 131.2, 68.3, 42.6, 35.16, 34.88, 33.9, 28.7. LRMS (ESI+) m/z 282 (M+Na).



8

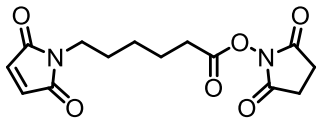
8 was prepared according to the general procedure using N-(2-Chloro-1-oxopropyl)-β-alanine (19.8 mg, 0.11mmol), **SI-4** (10 mg, 0.09 mmol), EDCI (25.9 mg, 0.13 mmol) and catalytic amount of DMAP. Flash column chromatography (30-80% EtOAc in hexane) was performed to furnish **8** (5.3 mg, 21.5%) as pale-yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.11 (s, 1H), 6.85 (d, *J* = 10.3, 1H), 6.07 (d, *J* = 10.3, 1H), 5.61 (m, 1H), 4.41 (q, *J* = 7.0 Hz, 1H), 3.59 (qd, *J* = 6.4, 2.7 Hz, 2H), 2.63 (m, 3H), 2.52 – 2.31 (m, 2H), 2.12 (m, 1H), 1.73 (d,

J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.7, 171.5, 169.7, 147.0, 131.1, 68.3, 55.86, 35.29, 34.89, 33.9, 28.7, 22.7. LRMS (ESI+) m/z 296 (M+Na).



9

9 was prepared according to the general procedure using N-(2-Bromo-1-oxopropyl)-β-alanine (24.6 mg, 0.11 mmol), **SI-4** (10 mg, 0.09 mmol), EDCI (25.9 mg, 0.13 mmol) and catalytic amount of DMAP. Flash column chromatography (30-80% EtOAc in hexane) was performed to furnish **9** (4.7 mg, 16.4%) as pale-yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.95 (s, 1H), 6.85 (d, *J* = 10.3, 1H), 6.07 (d, *J* = 10.3, 1H), 5.61 (m, 1H), 4.40 (q, *J* = 7.0 Hz, 1H), 3.58 (q, *J* = 6.1 Hz, 2H), 2.63 (m, 3H), 2.42 (m, 2H), 2.20 – 2.02 (m, 1H), 1.92 – 1.90 (s, 1H), 1.87 (d, *J* = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 197.7, 171.6, 169.5, 147.1, 131.1, 68.3, 45.0, 35.52, 34.89, 33.8, 28.7, 23.0. LRMS (ESI+) m/z 340 (M+Na).



10

10 was prepared according to the general procedure using 6-Maleimidocaproic acid (54.9 mg, 0.26 mmol), N-Hydroxysuccinimide (38.0 mg, 0.33 mmol), EDCI (63.2 mg, 0.33 mmol) and catalytic amount of DMAP. Flash column chromatography (30-80% EtOAc in hexane) was performed to furnish **10** (64.1 mg, 80%) as white solid. The obtained spectra was consistent with reported literature.[8]

2. Effective molarity and mass balance models

General remarks

In this section we describe the binding model between homodimeric 14-3-3 and pTau as illustrated in Figure 5 in the main text. Two Python codes were developed, specifically, the first script calculates effective molarities using a worm-like chain model and the second script calculates equilibrium concentrations by solving a mass balance equation. The Python codes were written in Scientific Python Development Environment (Spyder IDE 5.4.3) using Python version 3.9.12 and used the Numpy,[9] Scipy[10] and Matplotlib libraries.[11] Both codes are provided at the end of this section.

Worm-like chain model

The theoretical effective molarity of a random-coil peptide with a specific chain length was calculated using the worm-like chain (WLC) model as described by van Rosmalen *et al*.[12] A Python script was developed to compute the end-to-end distance probability distribution of a linker according to the WLC model. This script included the nonclosed form expression of $P_{\mathrm{WLC}}(R_e)$, the probability density for the end-to-end distance (R_{e}) of a random-coil peptide with a given linker length needed to bridge the two 14-3-3 binding sites, and the effective concentration (c_{eff}) based on Avogadro's number.

The following parameters were defined as follows:

End-to-end distance (R_{e}) = 35.4 Å

Length of one residue (b_0) = 3.8 Å

Persistence length (L_p) = 4.5 Å

Peptide linker lengths = [32-mer, 110-mer, 142-mer]

The resulting effective concentrations for the three peptide linker lengths, i.e., EM_32, EM_110 and EM_142, respectively, are given in Table S1.

Mass balance models

To interpret the results of the experimental SDS-PAGE experiments, we developed a general mass balance model. Equilibrium constants for monovalent binding each 14-3-3 binding motif to 14-3-3 were experimentally determined via a fluorescence anisotropy assay using a 16-mer peptide representing the pSer214, pSer324, and pSer356 binding motifs (Table S1). Bivalent binding was calculated using Equation S1, where K_D is defined as the experimentally determined binding constant of the second binding interaction and the effective molarity (EM) value was determined using the WLC model. Spyder was utilized to write Python code that expresses the equilibrium constant for each complex species and the mass balance equation.

$$K_D(bivalent) = \frac{K_D}{EM} \tag{S1}$$

Table S1. Experimentally determined dissociation constants for the three binding motifs (pSer214 (1), pSer324 (2), and pSer356 (3)) and the effective molarities as determined using the worm-like chain model. All values in μM.

```
K_D^1 = 12.5 # micromolar
K_D^2 = 15.9
K_D^3 = 586
EM_32 = 2845   # 2-3
EM_110 = 1438  # 1-2
EM_142 = 1096  # 1-3
```

At equilibrium, all individual reactions should be in equilibrium, such that we can express the equilibrium concentrations of all complex species (AB1, AB2, AB3, AB1B2, AB2B3, AB1B3, AB1B2B3, AB1_2, AB2_3, AB1_3, AB1_2B3, AB2_3B1, AB1_3B2) based on the K_D values and the concentrations of their constituents:

$$AB1 = A * B / K_D^1$$
$$AB2 = A * B / K_D^2$$
$$AB3 = A * B / K_D^3$$
$$AB1B2 = AB1 * B / K_D^2$$
$$AB2B3 = AB2 * B / K_D^3$$
$$AB1B3 = AB1 * B / K_D^3$$
$$AB1B2B3 = AB1B2 * B / K_D^3$$
$$AB1\_2 = AB1 / (K_D^2 / EM\_110)$$
$$AB2\_3 = AB2 / (K_D^3 / EM\_32)$$
$$AB1\_3 = AB1 / (K_D^3 / EM\_142)$$
$$AB1\_2B3 = AB1\_2 * B / K_D^3$$
$$AB2\_3B1 = AB2\_3 * B / K_D^1$$
$$AB1\_3B2 = AB1\_3 * B / K_D^2$$

Derivation of equilibrium concentrations

The mass-balance equation for dimeric 14-3-3 binding a trivalent Tau protein was expressed as the total concentration of individual complexes for a given input concentrations A_tot and B_tot as the sum of the concentrations of free molecules and the equivalent concentrations of the molecules in complexes:

(1) A = A_tot - (AB1 + AB2 + AB3 + AB1B2 + AB2B3 + AB1B3 + AB1B2B3 + AB1_2 + AB2_3 + AB1_3 + AB1_2B3 + AB2_3B1 + AB1_3B2)

(2) B = B_tot - (AB1 + AB2 + AB3 + 2*AB1B2 + 2*AB2B3 + 2*AB1B3 + 3*AB1B2B3 + AB1_2 + AB2_3 + AB1_3 + 2*AB1_2B3 + 2*AB2_3B1 + 2*AB1_3B2)

Python Code Worm-like chain model:

```
import numpy as np

# Constants
Re_angstrom = 35.4  # End-to-end distance in Ångströms
Re_nm = Re_angstrom * 0.1  # Convert end-to-end distance to nanometers
B0_angstrom = 3.8  # Bond length in Ångströms
B0_nm = B0_angstrom * 0.1  # Convert bond length to nanometers
Lp_angstrom = 4.5  # Persistence length in Ångströms
Lp_nm = Lp_angstrom * 0.1  # Convert persistence length to nanometers
Avogadro_number = 6.022e23  # Avogadro's number

# Peptide lengths in mers
peptide_lengths = [32, 110, 142]

# Function to calculate w for each peptide length
def calculate_w(L_nm, Re_nm, Lp_nm):
    w = ((5*Lp_nm)/(4*L_nm)) - ((2*Re_nm**2)/(L_nm**2)) +
((33*Re_nm**4)/(80*Lp_nm*L_nm**3)) + \
        ((79*Lp_nm**2)/(160*L_nm**2)) + ((329*Re_nm**2*Lp_nm)/(120*L_nm**3)) -
((6799*Re_nm**4)/(1600*L_nm**4)) + \
        ((3441*Re_nm**6)/(2800*Lp_nm*L_nm**5)) -
((1089*Re_nm**8)/(12800*Lp_nm**2*L_nm**6))
    return w

# Function to calculate Pwlc_r
def calculate_Pwlc_r(L_nm, Re_nm, Lp_nm, w):
    Pwlc_r = 4 * np.pi * Re_nm**2 * (1 - w) * (3 / (4 * np.pi * Lp_nm * L_nm))**1.5 *
np.exp(-3 * Re_nm**2 / (4 * Lp_nm * L_nm))
    return Pwlc_r

# Calculate, print w, Pwlc_r, and p_re for each peptide length
for length in peptide_lengths:
    L_nm = length * B0_nm  # Contour length in nanometers
    w = calculate_w(L_nm, Re_nm, Lp_nm)
    Pwlc_r = calculate_Pwlc_r(L_nm, Re_nm, Lp_nm, w)
    p_re_nm2 = Pwlc_r / (4 * np.pi * Re_nm**2)
    p_re_L = p_re_nm2 * 10**24

 # Convert p_re to per liter (L^-1), considering nm^2 to L conversion
    C_eff_M = p_re_L / Avogadro_number  # Calculate the effective concentration in mol/L
(Molarity)
    C_eff_uM = C_eff_M * 10**6  # Convert C_eff to micromolar
    print(f"Peptide length: {length} mers, w: {w}, P_wlc(r): {Pwlc_r}, p_re (L^-1):
{p_re_L}, C_eff (µM): {C_eff_uM}")
```

Python Code Mass balance model:

```
import numpy as np
import matplotlib.pyplot as plt
from scipy.optimize import fsolve

# Constants
KD1 = 12.5
KD2 = 15.9
KD3 = 586
EM_32 = 2845 # microM, values determined from the WLC model. The EM value is termed
C_eff
EM_110 = 1438
EM_142 = 1096
A_tot = 2

def equations(vars, B_tot):
    AB1, AB2, AB3, AB1B2, AB2B3, AB1B3, AB1B2B3, AB1_2, AB2_3, AB1_3, AB1_2B3, AB2_3B1,
AB1_3B2 = vars
    A = A_tot - (AB1 + AB2 + AB3 + AB1B2 + AB2B3 + AB1B3 + AB1B2B3 + AB1_2 + AB2_3 +
AB1_3 + AB1_2B3 + AB2_3B1 + AB1_3B2)
    B = B_tot - (AB1 + AB2 + AB3 + 2*AB1B2 + 2*AB2B3 + 2*AB1B3 + 3*AB1B2B3 + AB1_2 +
AB2_3 + AB1_3 + 2*AB1_2B3 + 2*AB2_3B1 + 2*AB1_3B2)
    return [
        A * B / KD1 - AB1,
        A * B / KD2 - AB2,
        A * B / KD3 - AB3,
        AB1 * B / KD2 - AB1B2,
        AB2 * B / KD3 - AB2B3,
        AB1 * B / KD3 - AB1B3,
        AB1B2 * B / KD3 - AB1B2B3,
        AB1 / (KD2 / EM_110) - AB1_2,
        AB2 / (KD3 / EM_32) - AB2_3,
        AB1 / (KD3 / EM_142) - AB1_3,
        AB1_2 * B / KD3 - AB1_2B3,
        AB2_3 * B / KD1 - AB2_3B1,
        AB1_3 * B / KD2 - AB1_3B2,
    ]

# Generate B concentrations from 1x10^-2 to 1x10^4 logarithmically spaced
B_concentrations = np.logspace(-2, 4, 1000)

# Dictionaries to store species concentrations and a list for free A
species_concentrations = {
    "AB1": [],
    "AB2": [],
    "AB3": [],
    "AB1B2": [],
    "AB2B3": [],
    "AB1B3": [],
    "AB1B2B3": [],
    "AB1_2": [],
    "AB2_3": [],
    "AB1_3": [],
    "AB1_2B3": [],
    "AB2_3B1": [],
    "AB1_3B2": [],
}
```

Python Code Mass balance model continued:

```
free_A_values = []  # To store the free A concentrations

# Initial guess for the first point
initial_guess = [0] * 13

# Solve the system for each B concentration
for B_tot in B_concentrations:
    solution, infodict, ier, mesg = fsolve(equations, initial_guess, args=(B_tot,),
full_output=True)
    if ier == 1:  # Check if fsolve converged successfully
        initial_guess = solution  # Use the current solution as the initial guess for
the next iteration
        for i, key in enumerate(species_concentrations.keys()):
            species_concentrations[key].append(solution[i])
        # Calculate and append free A
        free_A = A_tot - sum(solution)
        free_A_values.append(free_A)
    else:
        # If fsolve did not converge, append np.nan to indicate failed convergence
        for key in species_concentrations.keys():
            species_concentrations[key].append(np.nan)
        free_A_values.append(np.nan)
        print(f"Warning: fsolve did not converge for B_tot={B_tot}. Message: {mesg}")

# Plotting
plt.figure(figsize=(10, 6))
for key, values in species_concentrations.items():
    plt.plot(B_concentrations, values, label=key, linewidth=2)

# Add the plot for free A
plt.plot(B_concentrations, free_A_values, label='Free A', linewidth=2, linestyle='--',
color='black')

# Adding a dashed vertical red line at A_tot = 2
plt.axvline(x=2, color='red', linestyle='--', label='Tau Concentration')
plt.axvline(x=100, color='grey', linestyle='--', label='14-3-3 Concentration')
plt.tick_params(axis='both', labelsize=11)  # Adjusts both x and y axis tick label sizes

plt.xscale('log')
plt.xlabel('[14-3-3] / µM', fontsize=11)
plt.ylabel('Species Concentration / Free A (µM)', fontsize=11)
plt.title('hTau/14-3-3 Species and Free A as a Function of 14-3-3 Concentration')
plt.legend()
plt.show()

#continued on the next page
```

Python Code Mass balance model continued:

```
def print_species_at_B_concentration(B_target):
    """
    Prints the concentrations of all species and free A at a specified target
concentration of B.
    """
    # Find the closest B concentration in our calculated range to the target
    closest_B_index = np.abs(B_concentrations - B_target).argmin()
    closest_B = B_concentrations[closest_B_index]

    print(f"\nConcentrations of all species and free A at B concentration: {closest_B}
uM")
    for key in species_concentrations.keys():
        # Print the concentration of each species at the closest B concentration
        print(f"{key}: {species_concentrations[key][closest_B_index]} uM")
    # Additionally, print the concentration of free A
    print(f"Free A: {free_A_values[closest_B_index]} uM")

print_species_at_B_concentration(100)  # Adjust 100 uM to your specific target B
concentration

#end of code
```

3. Characterization and evaluation of the crosslinkers

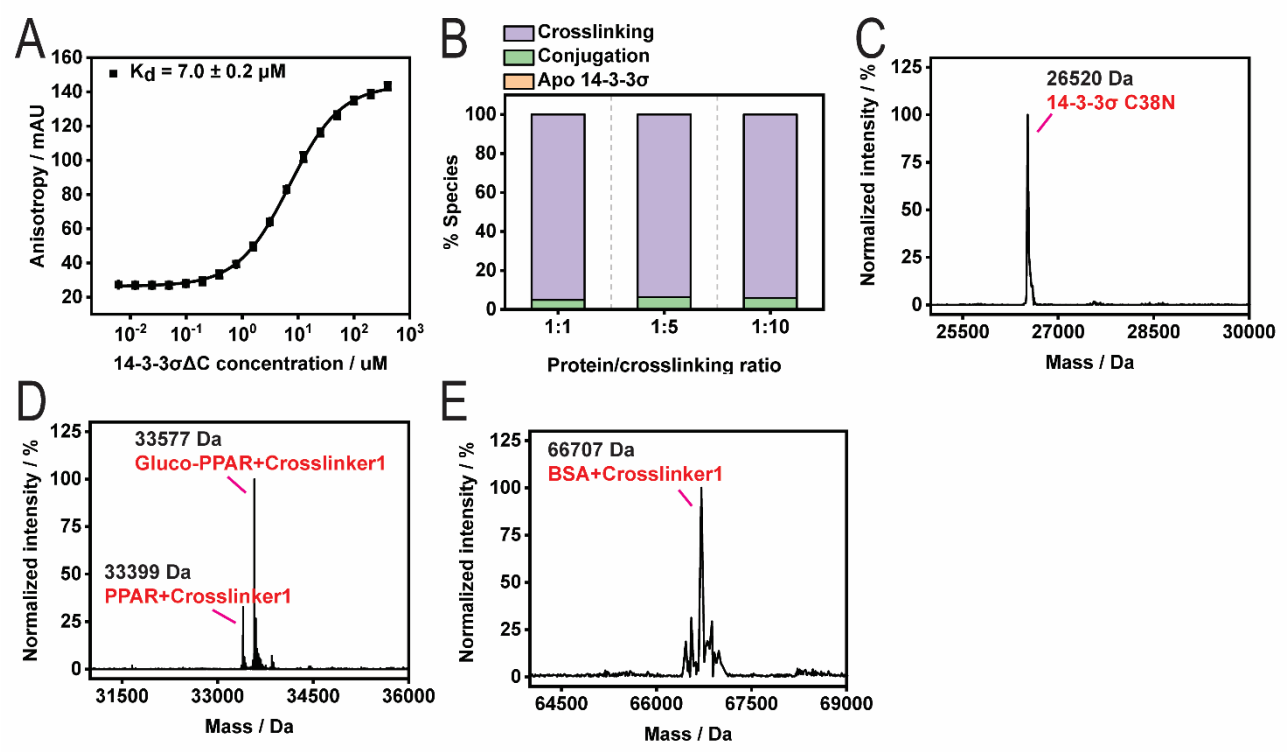


Figure S1. (A) Kd measurement of 14-3-3σ/B-Raf pS365 protein-peptide complex by FA assay. (B) Stoichiometric effect of crosslinker **1** on the crosslinking. (C) Deconvoluted mass spectrum of 14-3-3σ C38N protein incubated with crosslinker 1. (D) Deconvoluted mass spectrum of PPAR protein incubated with B-Raf pS365 and crosslinker 1. Notably, a significant portion of the PPARγ protein contains a glycosylation modification (+glucose), however, this does not interfere with the crosslinking experiment. (E) Deconvoluted mass spectrum of BSA protein incubated with B-Raf pS365 and crosslinker 1.

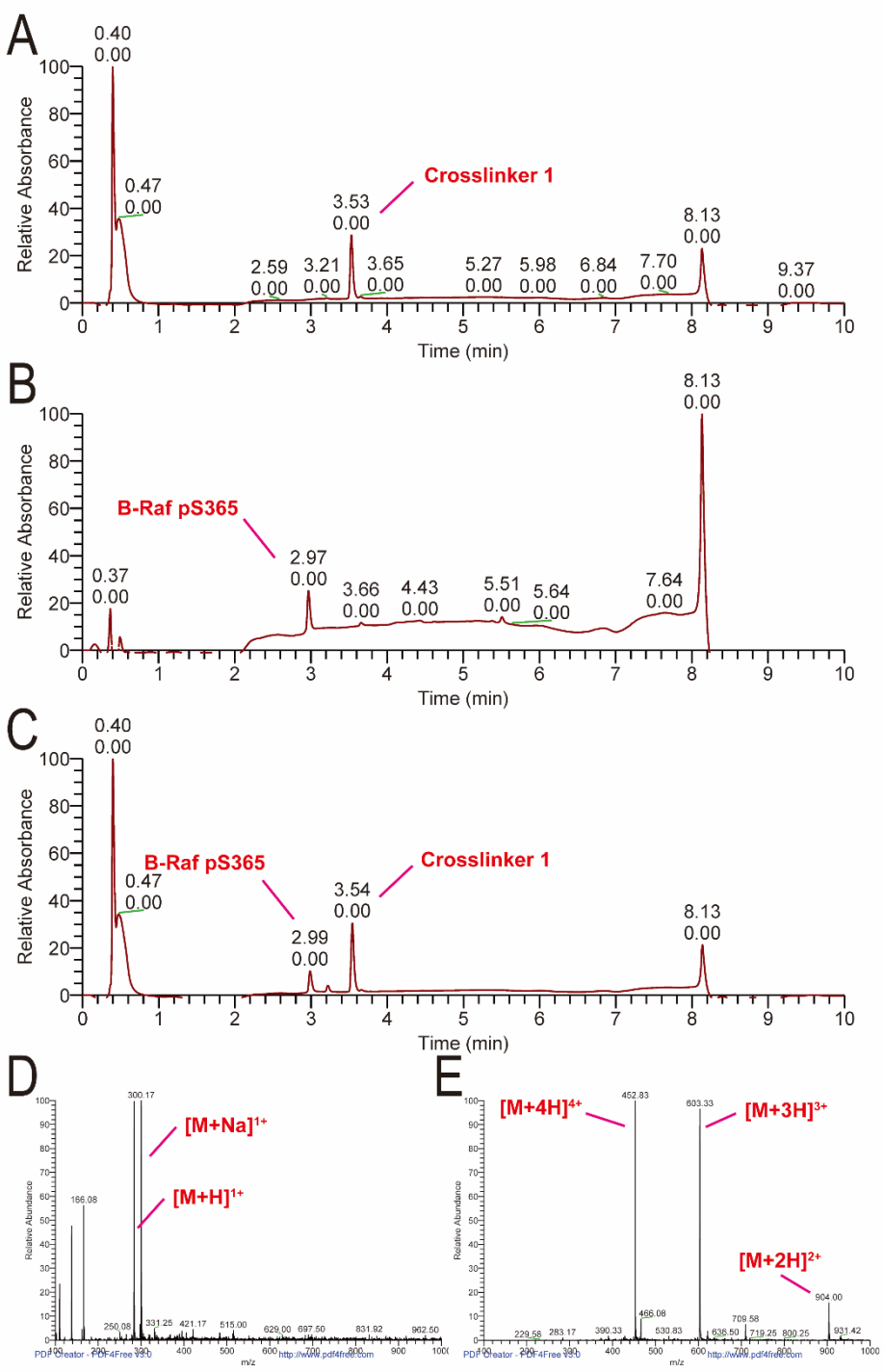


Figure S2. (A) HPLC chromatogram of crosslinker 1. (B) HPLC chromatogram of B-Raf pS365. (C) LC chromatogram of B-Raf pS365 incubated with crosslinker 1 in a 2:10 ratio overnight at 10 °C in BTP buffer at pH 7. (D) Mass spectrum of crosslinker 1. (E) Mass spectrum of B-Raf pS365.

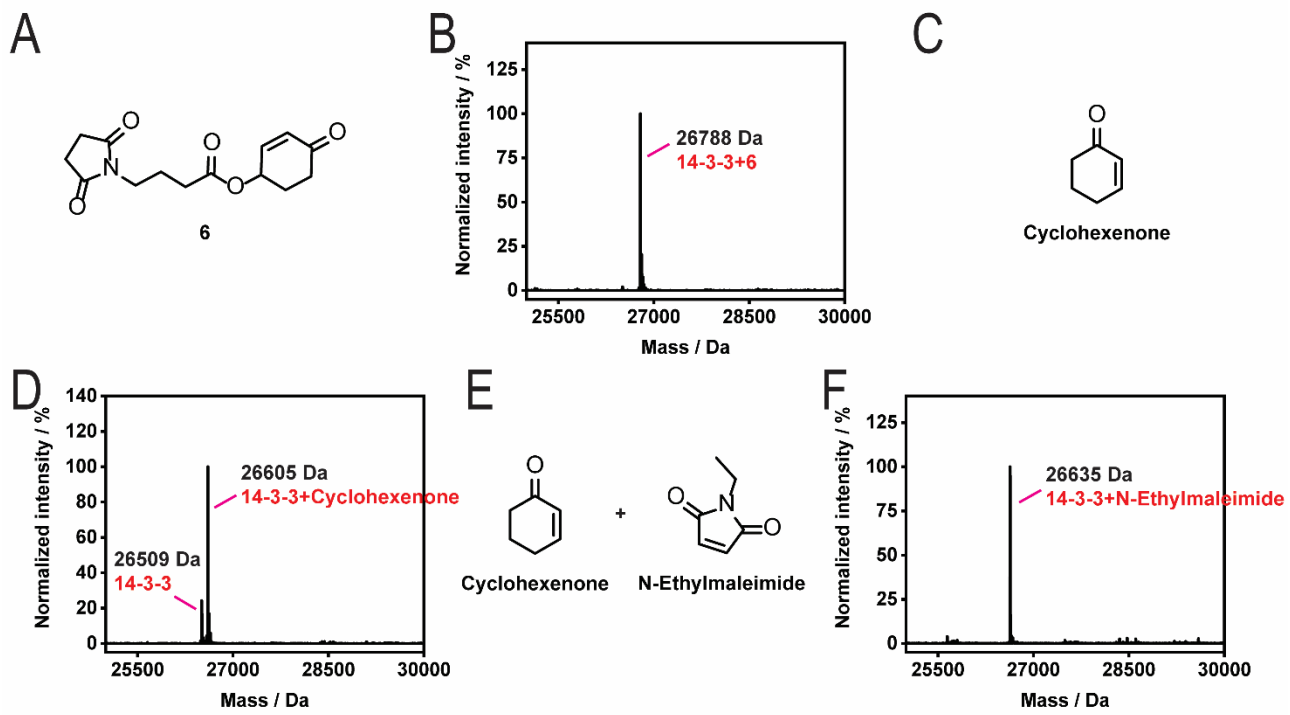


Figure S3. (A) Chemical structure of compound **6**. (B) Deconvoluted mass spectrum of 14-3-3σ incubated with B-Raf pS365 and compound **6**. (C) Chemical structure of cyclohexenone electrophile. (D) Deconvoluted mass spectrum of 14-3-3σ incubated with cyclohexenone electrophile in a 1:10 ratio overnight at 10 °C in BTP buffer at pH 7. (E) Chemical structure of cyclohexenone and maleimide electrophiles. (F) Deconvoluted mass spectrum of 14-3-3σ incubated with both cyclohexenone and N-ethylmaleimide (NEM) electrophiles in a 1:10:10 ratio overnight at 10 °C in BTP buffer at pH 7.

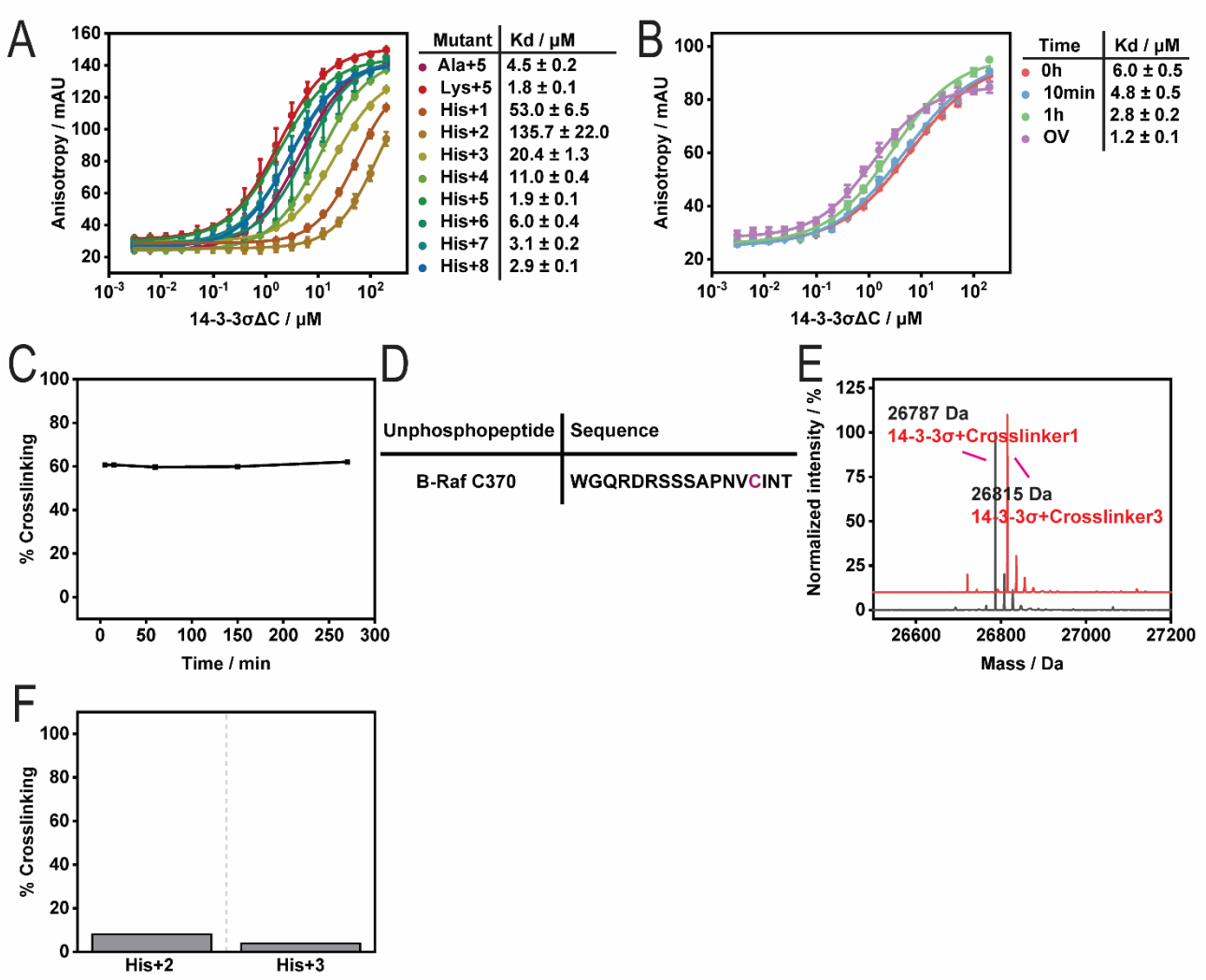


Figure S4. (A) K_d measurement of 14-3-3σ in complex with the mutated peptides by FA assay. (B) Time dependent K_d measurement of 14-3-3σ/Cys+5 complex. (C) Percentage of crosslinking of 8 μM 14-3-3σ and 16 μM Cys+5 peptide by 80 μM crosslinker 1 over 4.5 h. (D) Sequences of unphosphorylated peptides B-Raf C370. (E) Deconvoluted mass spectra of 14-3-3σ protein incubated with unphospho-peptides B-Raf C370 and crosslinker **1** or **3**. (F) Percentage of crosslinking of 14-3-3σ with B-Raf His+2 and B-Raf His+3 mutants respectively with crosslinker **1** under increased peptide concentration (16 eq.).

4. Probing 14-3-3/Tau interaction

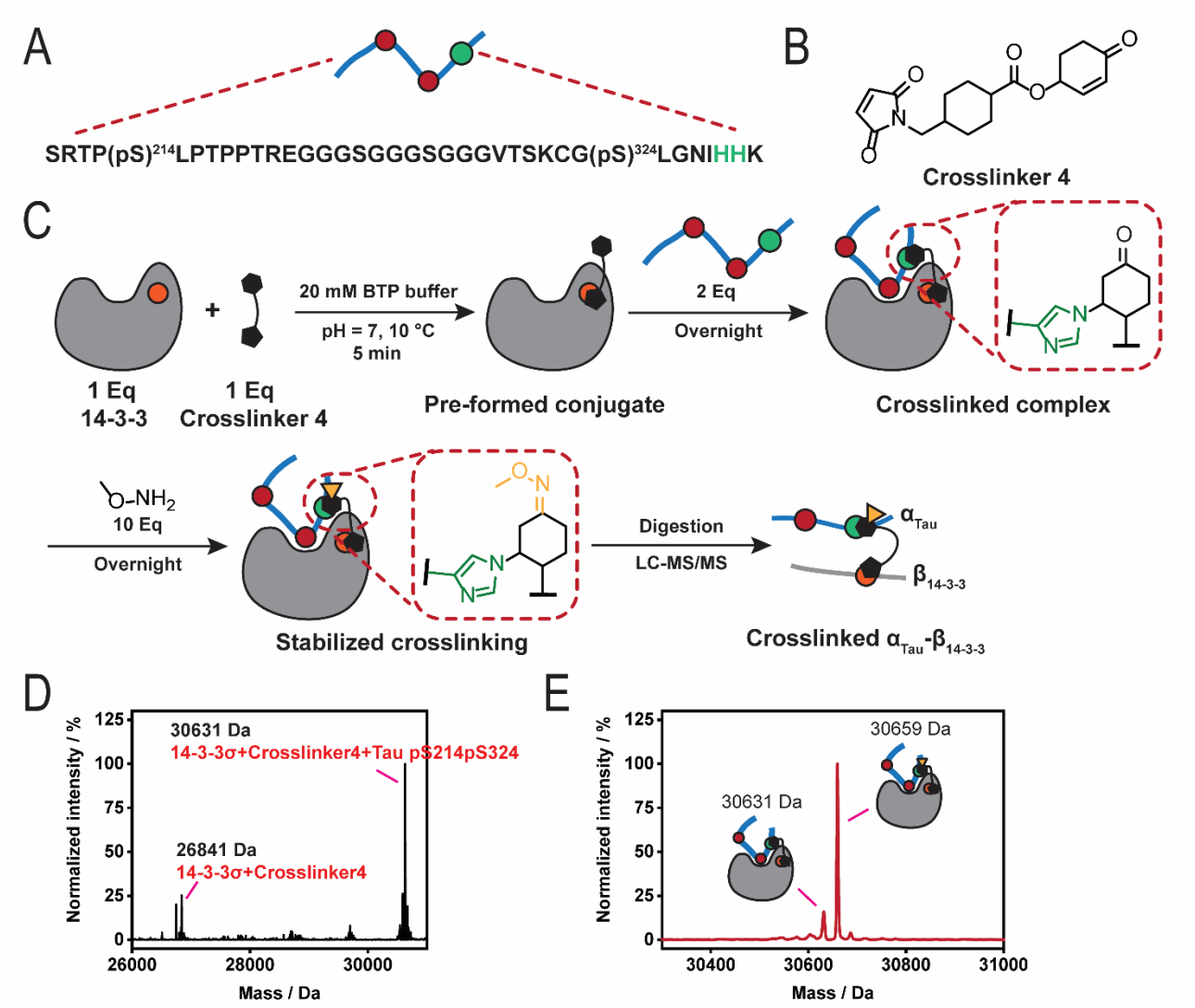


Figure S5. (A) Schematic representation and sequence of bivalent Tau peptide. (B) Chemical structure of crosslinker 4. (C) Schematic representation of the workflow of the 14-3-3 binding motif confirmation via crosslinking and tandem mass spectrometry. (D) Deconvoluted mass spectrum derived from crosslinking 14-3-3σ/Tau pS214pS324 complex with 1 eq. crosslinker 4 in two steps. Result showed satisfying crosslinking yield (74 %). (E) Deconvoluted mass spectrum of the methoxyamine treated crosslinked 14-3-3σ/Tau pS214pS324.

A

Peptide	Observed (monoisotopic)	Theoretical (monoisotopic)
GEELSCEER-CG(pS)LGNIHHK	$[M+3H]^{3+}$ = 852.7011 $[M+4H]^{4+}$ = 639.7750 $[M+5H]^{5+}$ = 512.0248	$[M+3H]^{3+}$ = 852.7013 $[M+4H]^{4+}$ = 639.7778 $[M+5H]^{5+}$ = 512.0237
GEELS(C)^{modified}EER	$[M+H]^{1+}$ = 1411.6086	$[M+H]^{1+}$ = 1411.6046
CG(pS)LGNIHHK	$[M+H]^{1+}$ = 1145.4935	$[M+H]^{1+}$ = 1145.4922

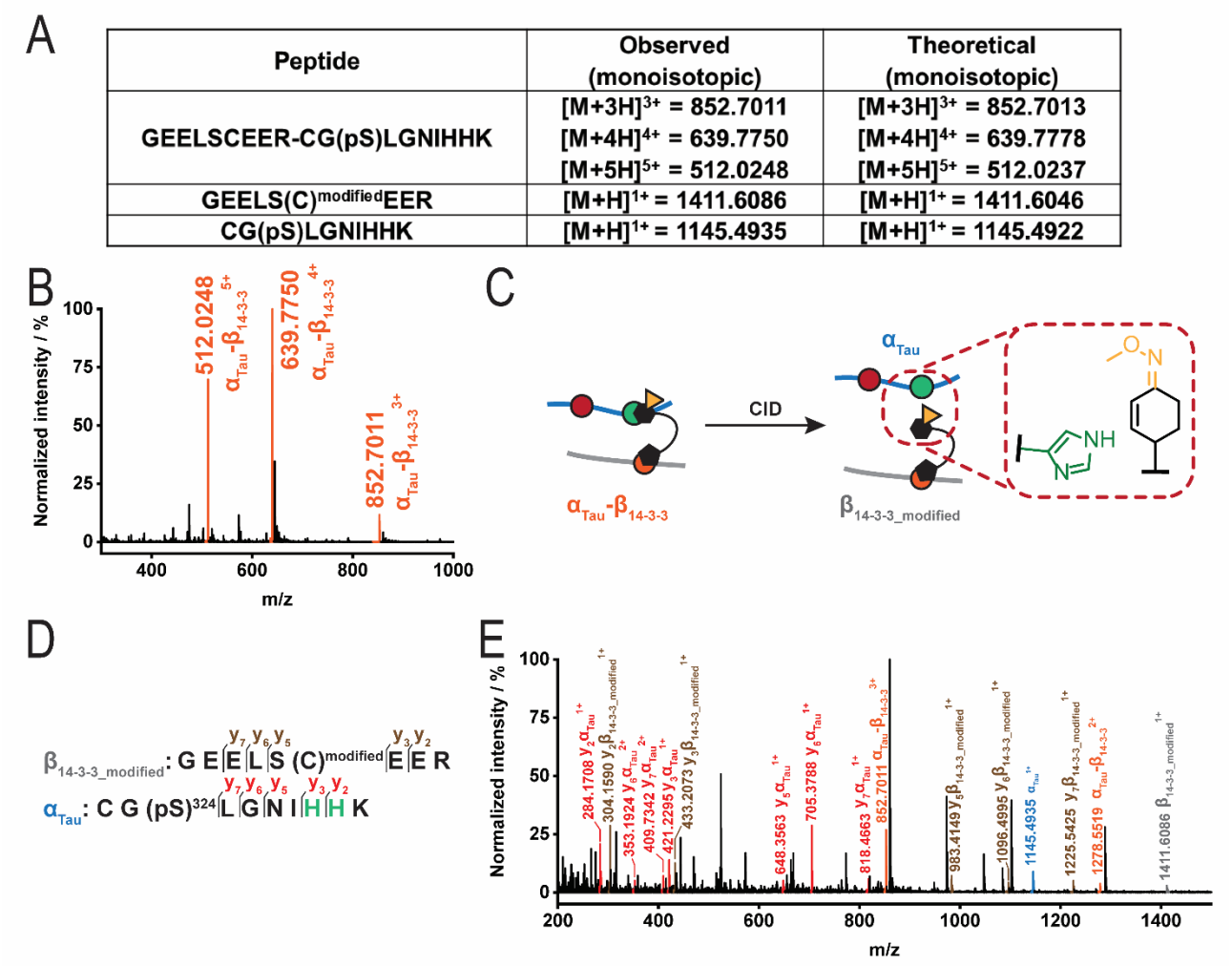
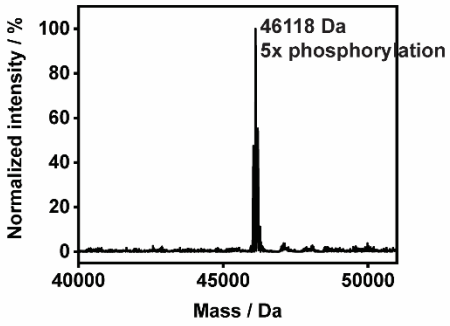


Figure S6. (A) Mass accuracies for ions observed in tandem mass spectrometry. (B) MS1 spectrum of α_{Tau}-$\beta_{14\text{-}3\text{-}3}$ crosslinked peptide. α_{Tau} and $\beta_{14\text{-}3\text{-}3}$ were termed according to Schilling et al. The m/z values are given as monoisotopic. (C) Schematic representation of the collision-induced dissociation of the α_{Tau}-$\beta_{14\text{-}3\text{-}3}$ crosslinked peptide in the MS2 stage. (D-E) MS2 spectrum of the α_{Tau}-$\beta_{14\text{-}3\text{-}3}$ crosslinked peptide. y-fragment ion peaks of α_{Tau} and $\beta_{14\text{-}3\text{-}3\_modified}$ are highlighted in red and brown respectively.

A

AEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDA
GLKESPLQTPTEDGSEEPGSETSDAKSTPTAEDVTAPLVDEGAP
GKQAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHVTQARM
VSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANA
TRIPAKTPPAPKTPPSSGEPPKSGDRSGYSSPGSPGTPGSRSRTP
SLPTPPTREPKKVAVVRTPPKSPSSAKSRLQTAPVPMPDLKNVK
SKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVP
GGGSVQIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLD
FKDRVQSKIGSLDNITHVPGGGNKKIETHKLTFRENAKAKTDHGA
EIVYKSPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATLADEVSASL
AKQGL

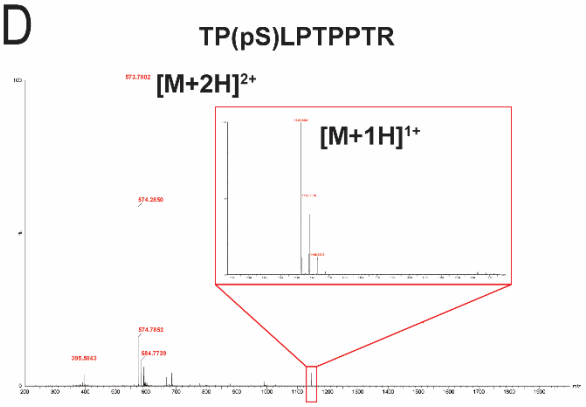
B



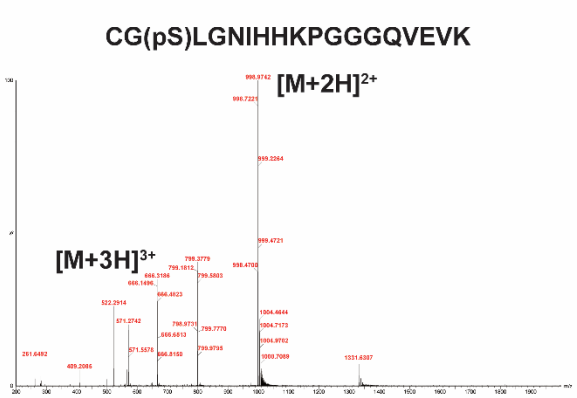
C

No.	Phosphorylation site	Observed (monoisotopic)	Theoretical (monoisotopic)
1	TP(pS)^{214}LPTPPTR	$[M+H]^{1+}$ = 1146.5531 $[M+2H]^{2+}$ = 573.7802	$[M+H]^{1+}$ = 1146.5555 $[M+2H]^{2+}$ = 573.7814
2	CG(pS)^{314}LGNIHHKPGGGQVEVK	$[M+2H]^{2+}$ = 998.9742 $[M+3H]^{3+}$ = 666.3186	$[M+2H]^{2+}$ = 998.9748 $[M+3H]^{3+}$ = 666.3189
3	IG(pS)^{356}LDNITHVPGGGNK	$[M+2H]^{2+}$ = 829.9001 $[M+3H]^{3+}$ = 553.6016	$[M+2H]^{2+}$ = 829.8985 $[M+3H]^{3+}$ = 553.6014
4	HL(pS)^{409}NVSSTG(pS)^{416}IDMVDSPQLAT LADEVSASLAK	$[M+3H]^{3+}$ = 1135.1873 $[M+4H]^{4+}$ = 851.6382	$[M+3H]^{3+}$ = 1135.1840 $[M+4H]^{4+}$ = 851.6399

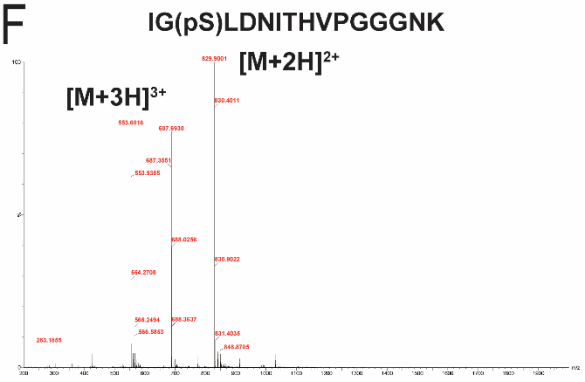
D



E



F



G

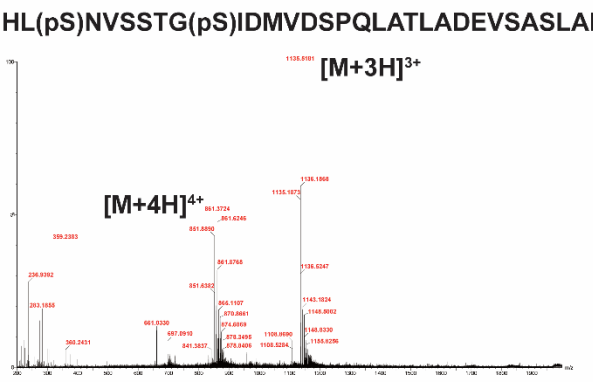
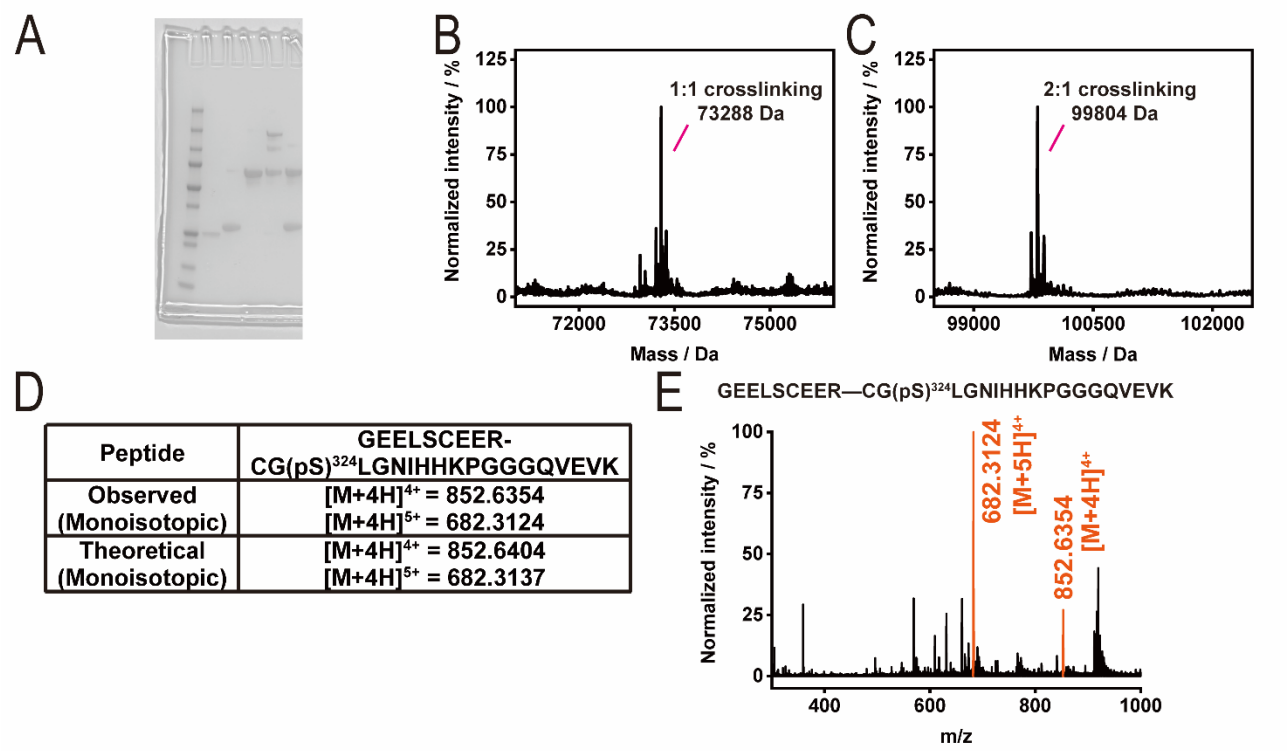


Figure S7. (A) Amino acid sequence of Tau protein used in the study. PKA phosphorylated serines are highlighted in red. (B) Deconvoluted mass spectrum of PKA phosphorylated Tau protein. (C) Summary of the identified phospho-peptides derived from tryptic digestion. All ion masses are shown as monoisotopic. (D-G) Mass spectra of the phospho-peptides.



Peptide	GEELSCEER-CG(pS)^{324}LGNIHHKPGGGQVEVK
Observed (Monoisotopic)	$[M+4H]^{4+}$ = 852.6354 $[M+4H]^{5+}$ = 682.3124
Theoretical (Monoisotopic)	$[M+4H]^{4+}$ = 852.6404 $[M+4H]^{5+}$ = 682.3137

Figure S8. (A) The original SDS-page gel of the crosslinking reaction between 14-3-3σ and hTau. (B) and (C) Deconvoluted mass spectra of 1:1 crosslinking and 2:1 crosslinking of 14-3-3σ and pTau. (D) Summary of the identified crosslinked peptide derived from tryptic digestion. All ion masses are shown as monoisotopic. (E) Mass spectrum correlated with the crosslinked peptide derived from crosslinking of Cys38 of 14-3-3σ and pSer324 site of pTau.

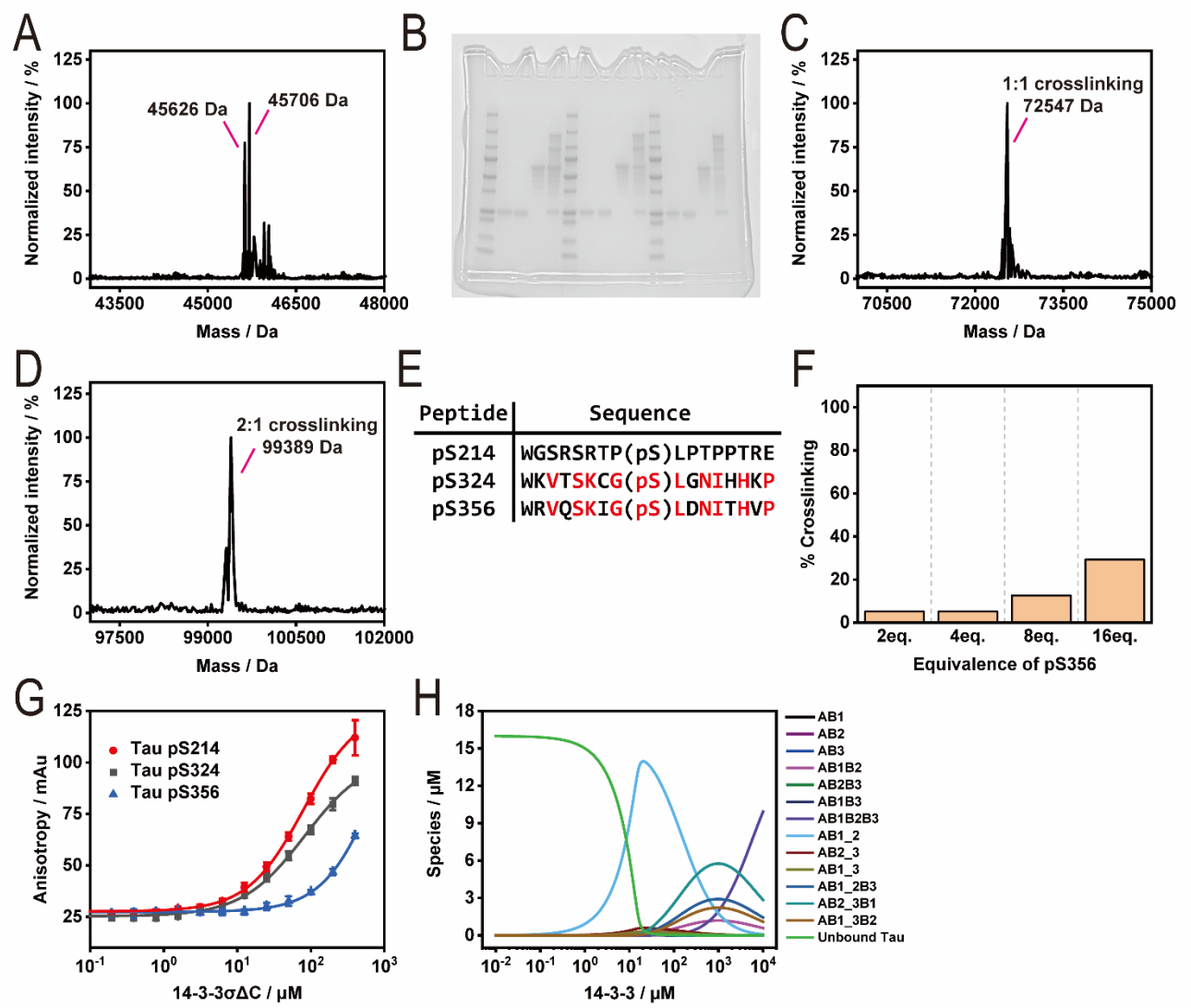


Figure S9. (A) Deconvoluted mass spectra of pTau$_{324,356}$ mutant. (B) The original SDS-page gel of the crosslinking reaction between 14-3-3σ and pTau$_{324,356}$. (C) and (D) Deconvoluted mass spectra of 1:1 crosslinking and 2:1 crosslinking of 14-3-3σ and pTau$_{324,356}$ mutant respectively. (E) Peptides representing Tau pS214, pS324 and pS356 sites. (F) Percentage of crosslinking of 14-3-3σ and Tau pS356 peptide with crosslinker **4** under different peptide concentrations (up to 16 eq.). (G) Fluorescence anisotropy (FA) measurement of titrating 14-3-3σ to 50 nM FITC-labelled Tau pS214, pS324 and pS356 peptides respectively. (H) Species distribution at different concentrations of 14-3-3γ at a constant concentration of 16 μM hTau was applied.

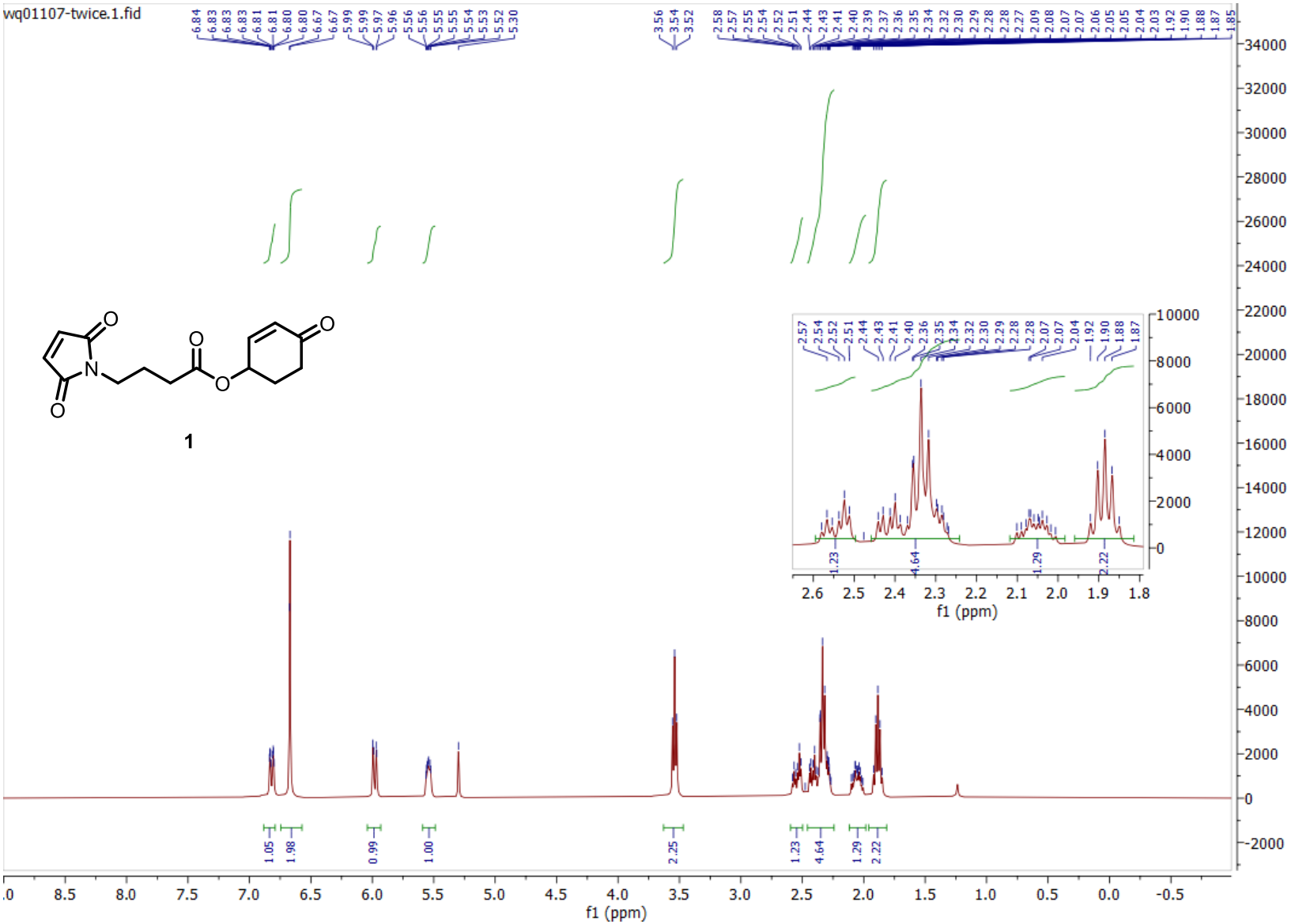
5. Crystallographic statistics

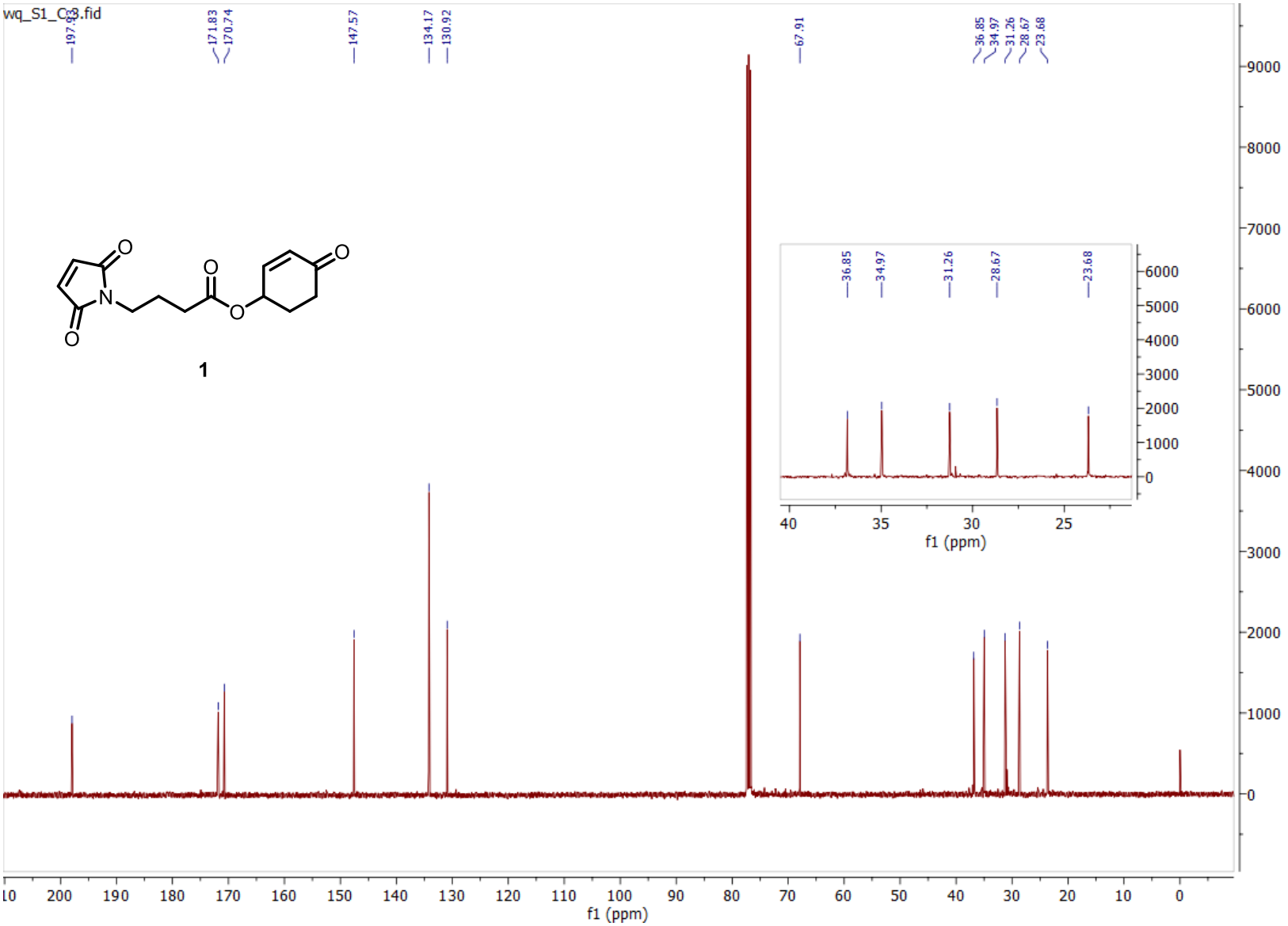
Tables S2. X-ray crystallography data collection and refinement statistics

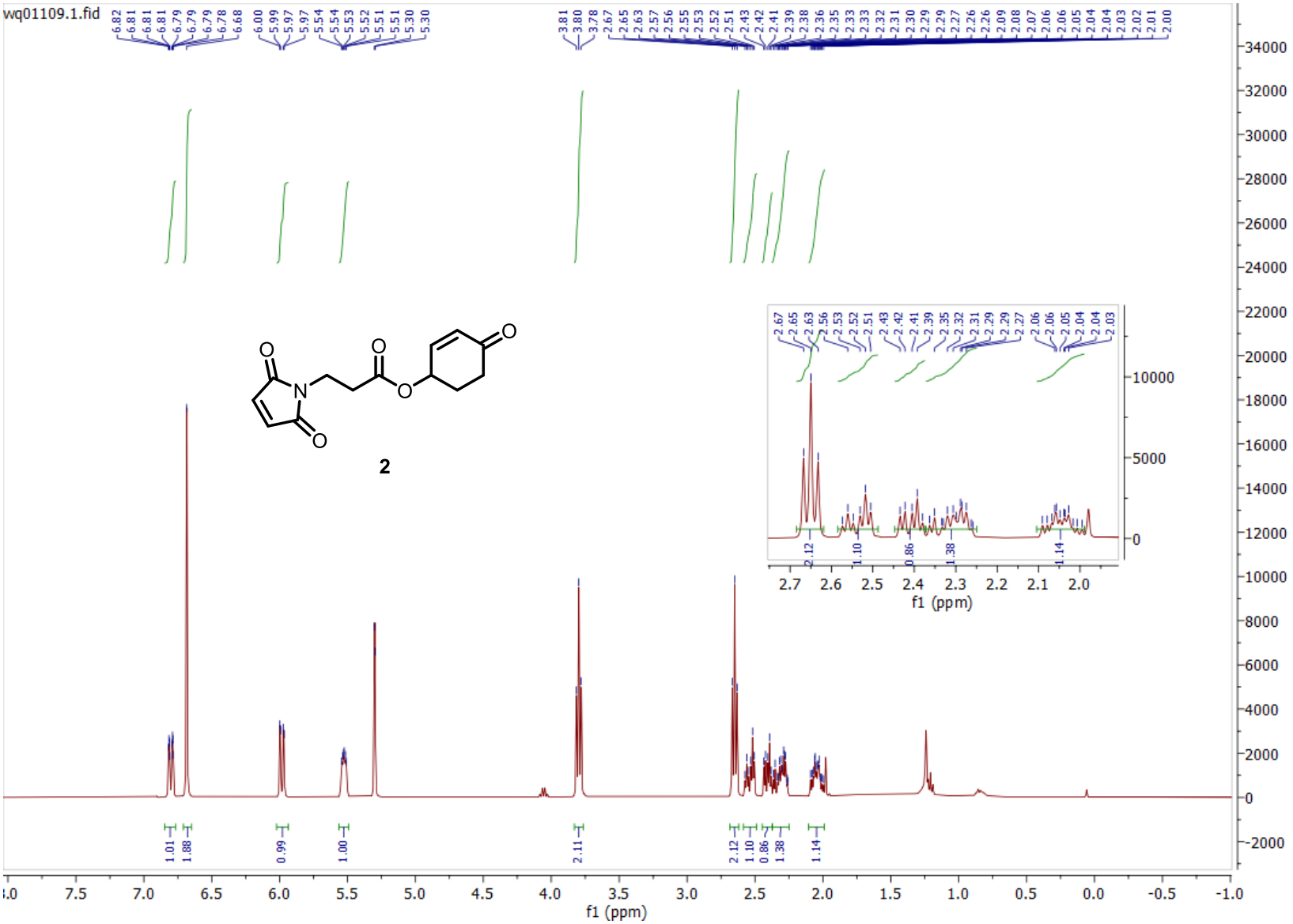
	14-3-3σ/B-Raf-pS365
Measuring date	05-02-2021
Measuring site	ESRF
Crystallization condition	c-Jun-pS277 replacement soaking
Data collection	
Wavelength (Å)	0.96863
Resolution range (Å)	57.626 - 2.299 (2.381 - 2.299)
Space group	C 2 2 21
Unit cell	
a, b, c (Å)	84.7824 115.2517 64.6707
No. of Total reflections	176653 (7277)
No. of Unique reflections	14439 (1417)
Multiplicity	12.2 (10.7)
Completeness (%)	99.92% (99.72%)
Mean I/sigma(I))	3.7 (3.0)
Wilson B-factor (Å2)	15.796
Rmerge (%)	0.388 (0.649)
Rmeas (%)	0.406 (0.693)
CC1/2 (%)	0.974 (0.946)
Refinement	
Reflections used in refinement	14439 (1417)
Reflections used for R-free	
Rwork	0.2336 (0.3640)
Rfree	0.2823 (0.4233)
RMS (bonds)	0.0013
RMS (angles)	0.50
Ramachandran favored (%)	97.38
Ramachandran allowed (%)	2.62
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	0.51
Clashscore	1.85
Average B-factor (Å)	25.4

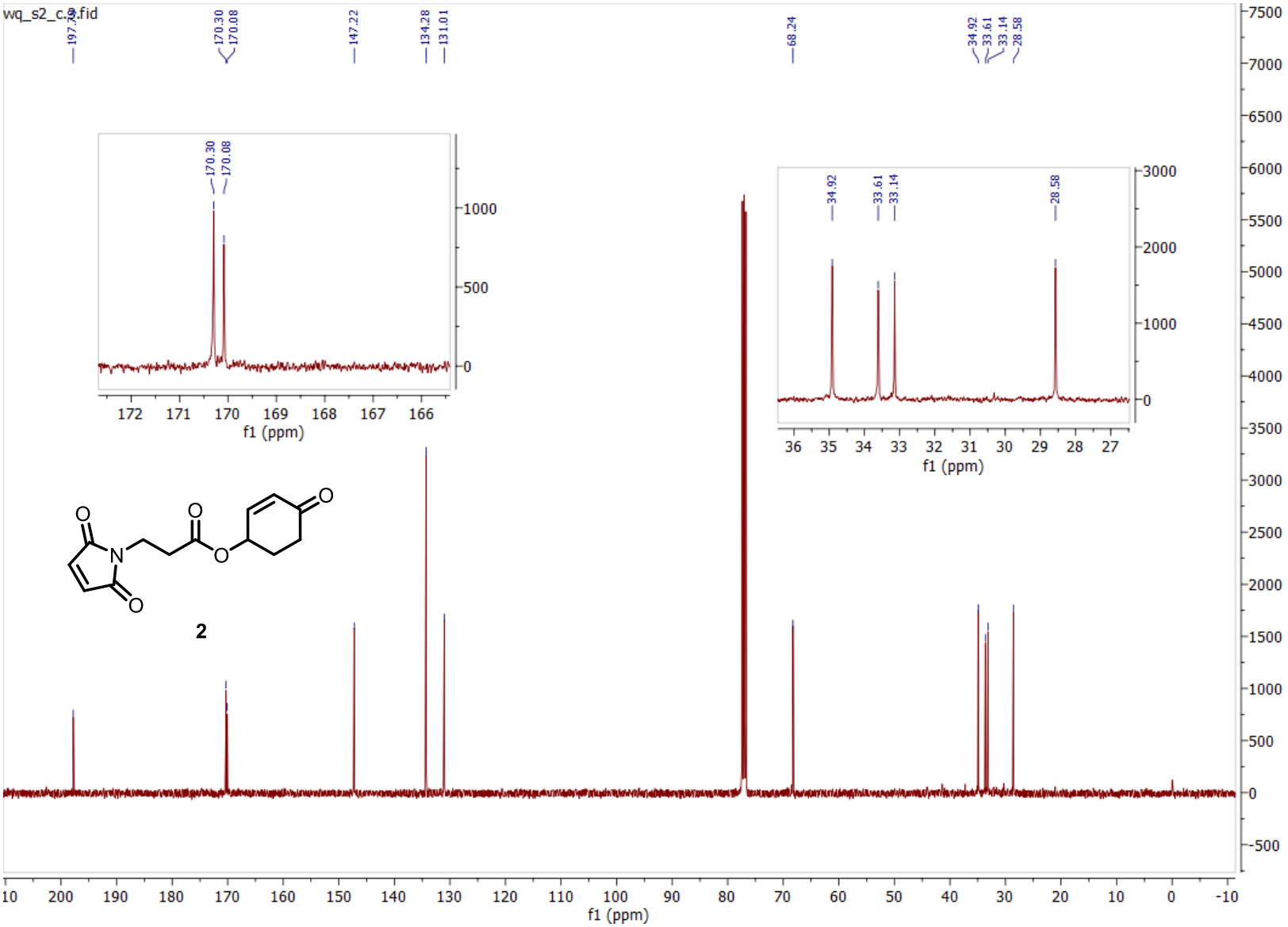
Statistics for the highest-resolution shell are shown in parentheses

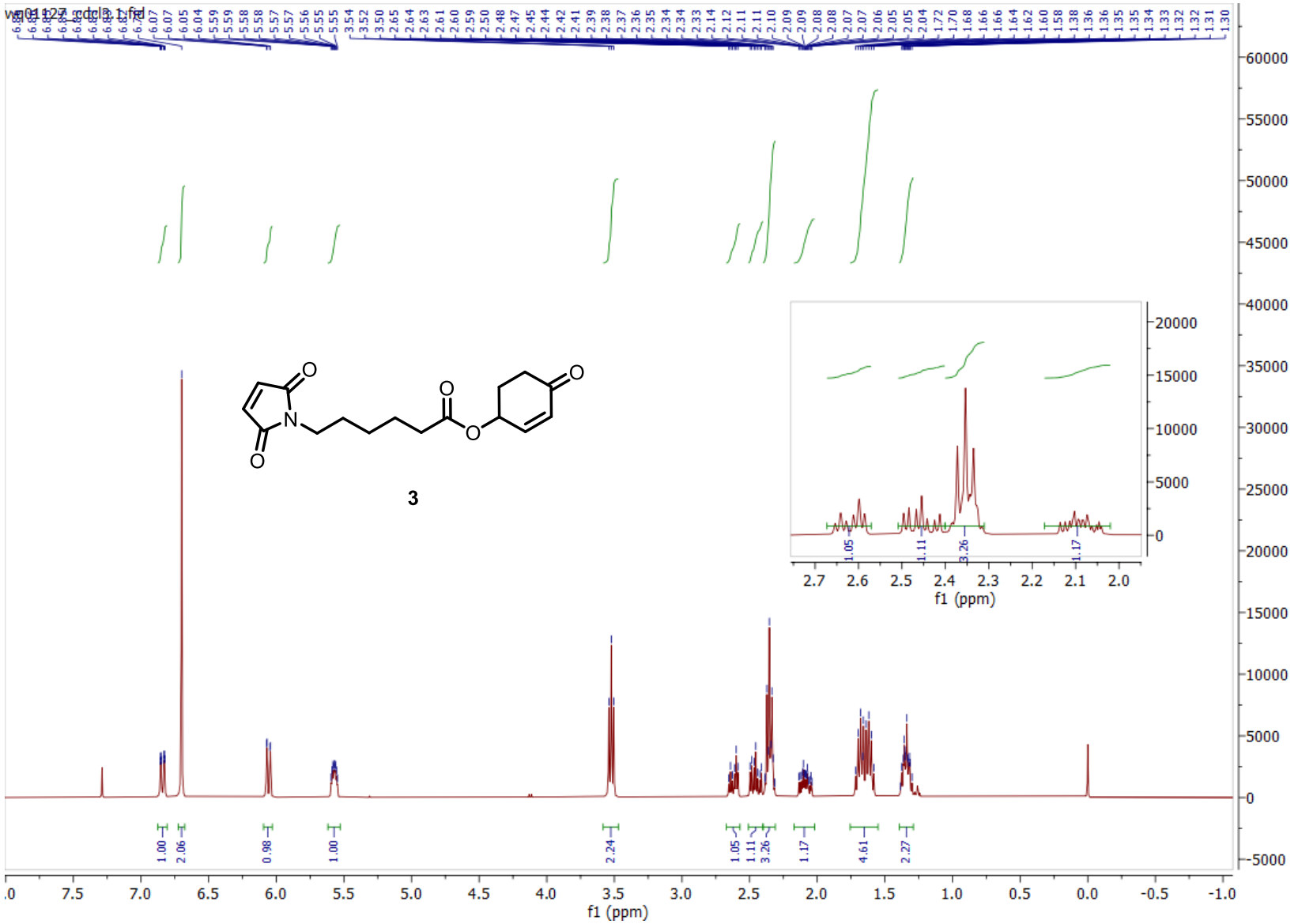
6. NMR spectra

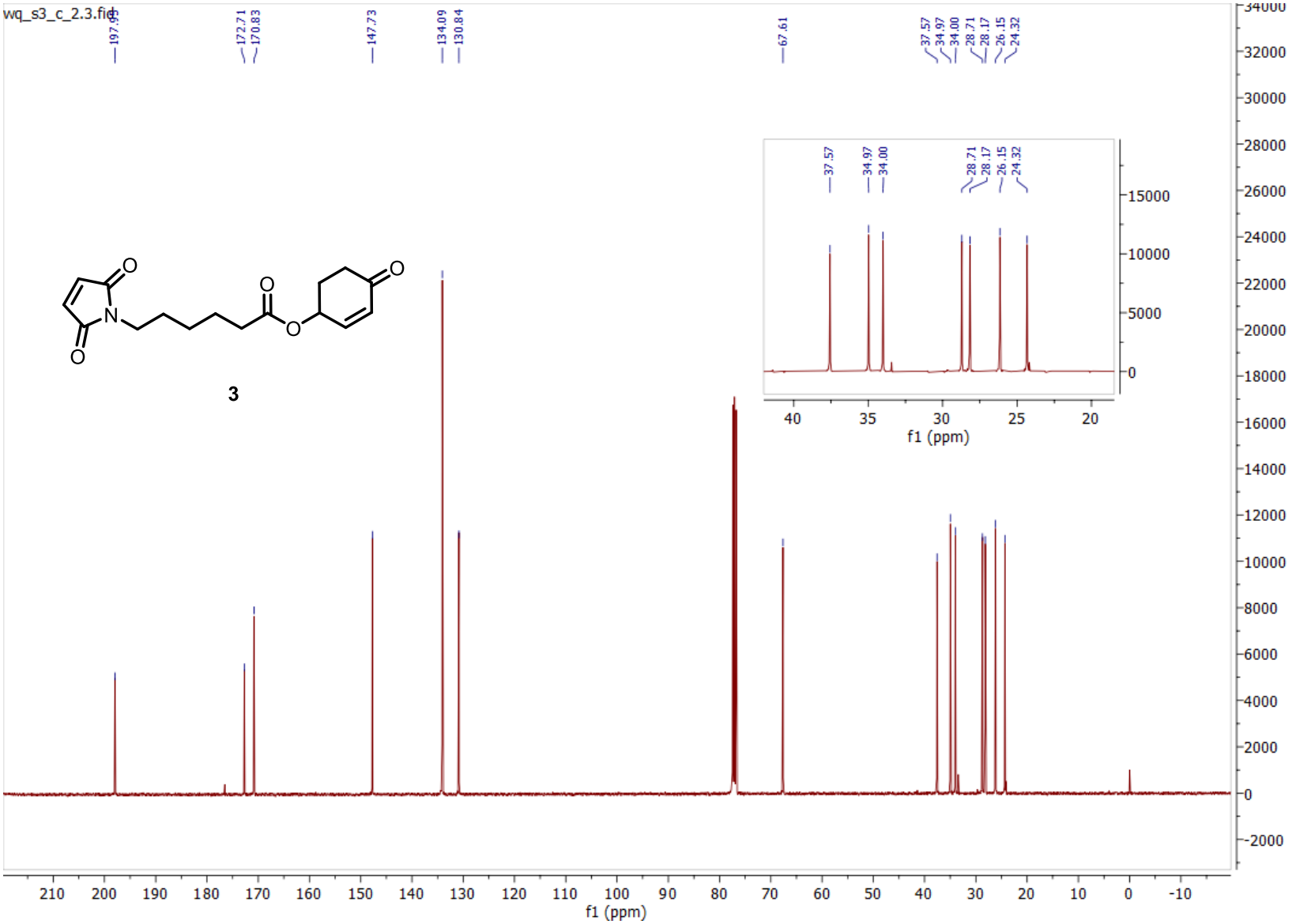


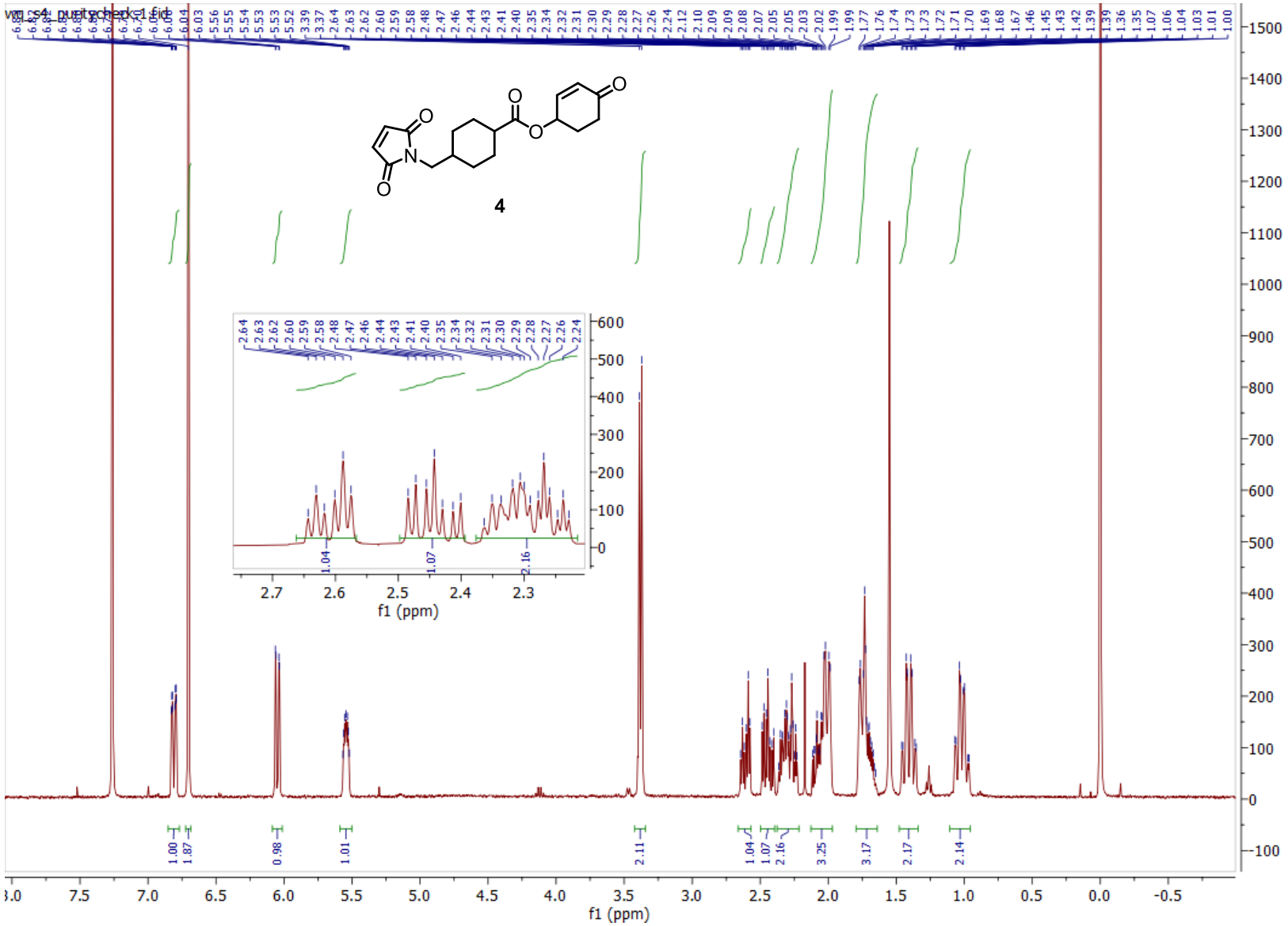

wq_S1_C13.fid
197.93
171.83
170.74
147.57
134.17
130.92
67.91
36.85
34.97
31.26
28.67
23.68
1
f1 (ppm)

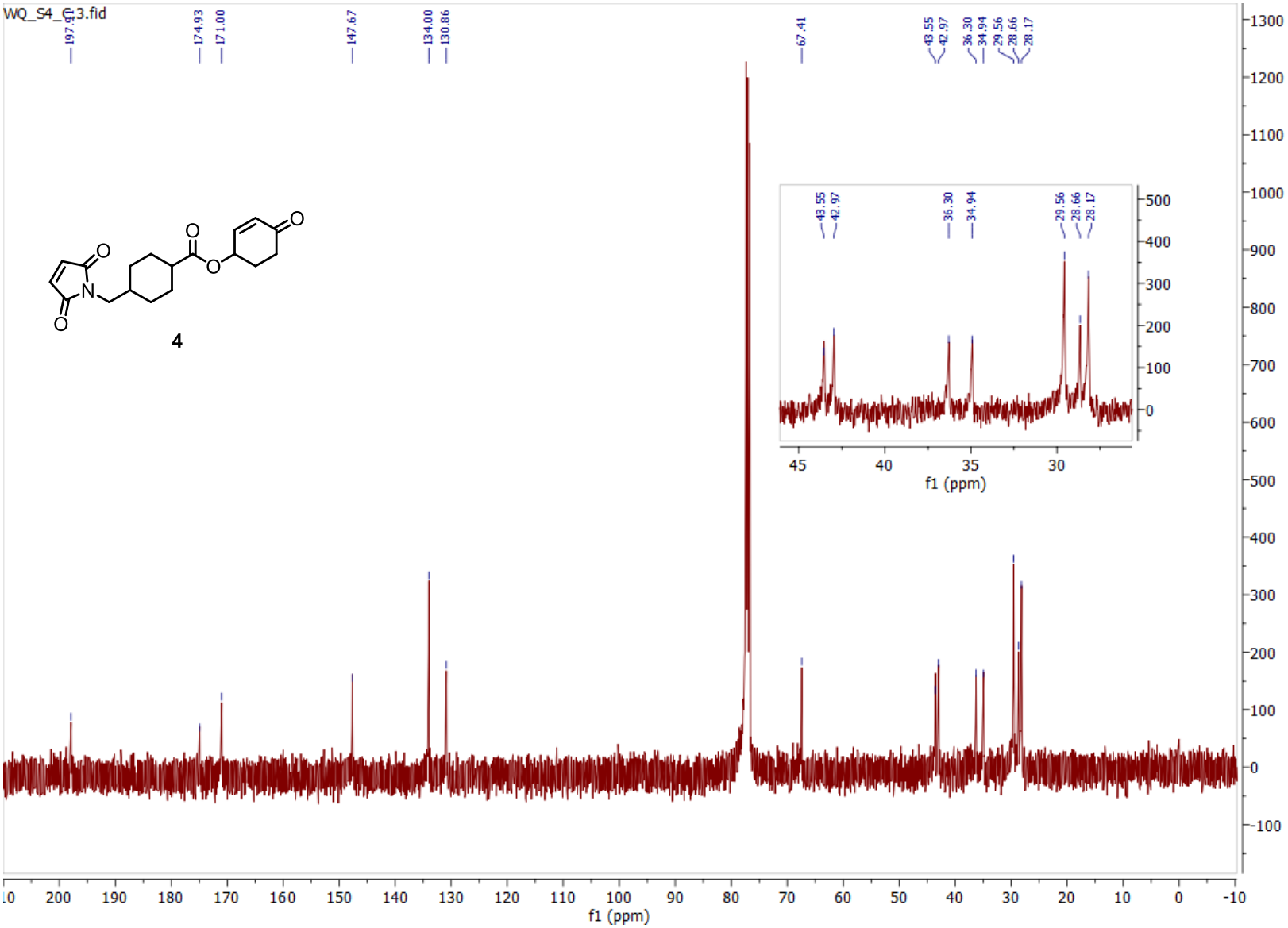

wq01109.1.fid
2
f1 (ppm)

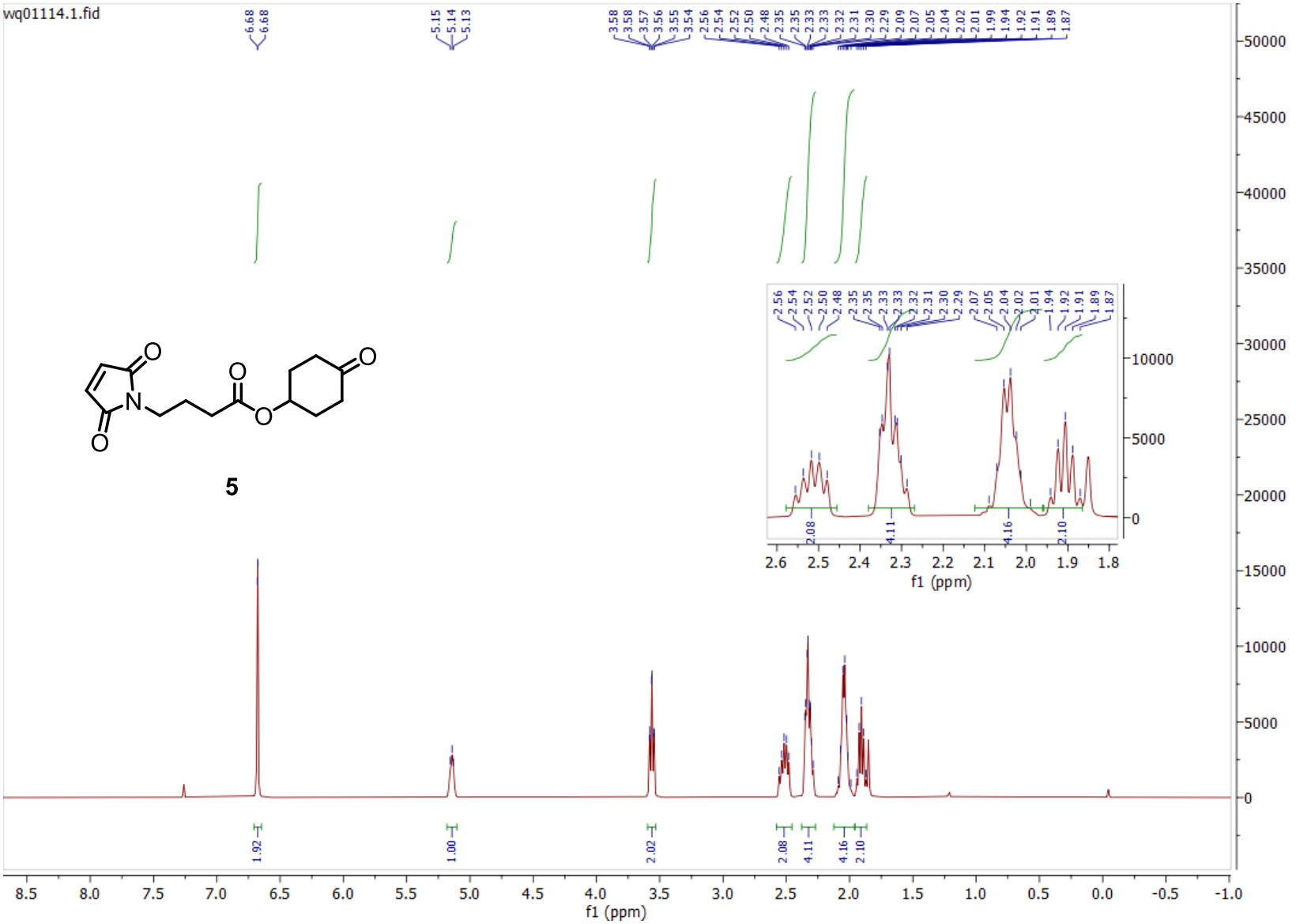

wq_s2_c.9.fid
197.73
170.30
170.08
147.22
134.28
131.01
68.24
34.92
33.61
33.14
28.58
f1 (ppm)
2

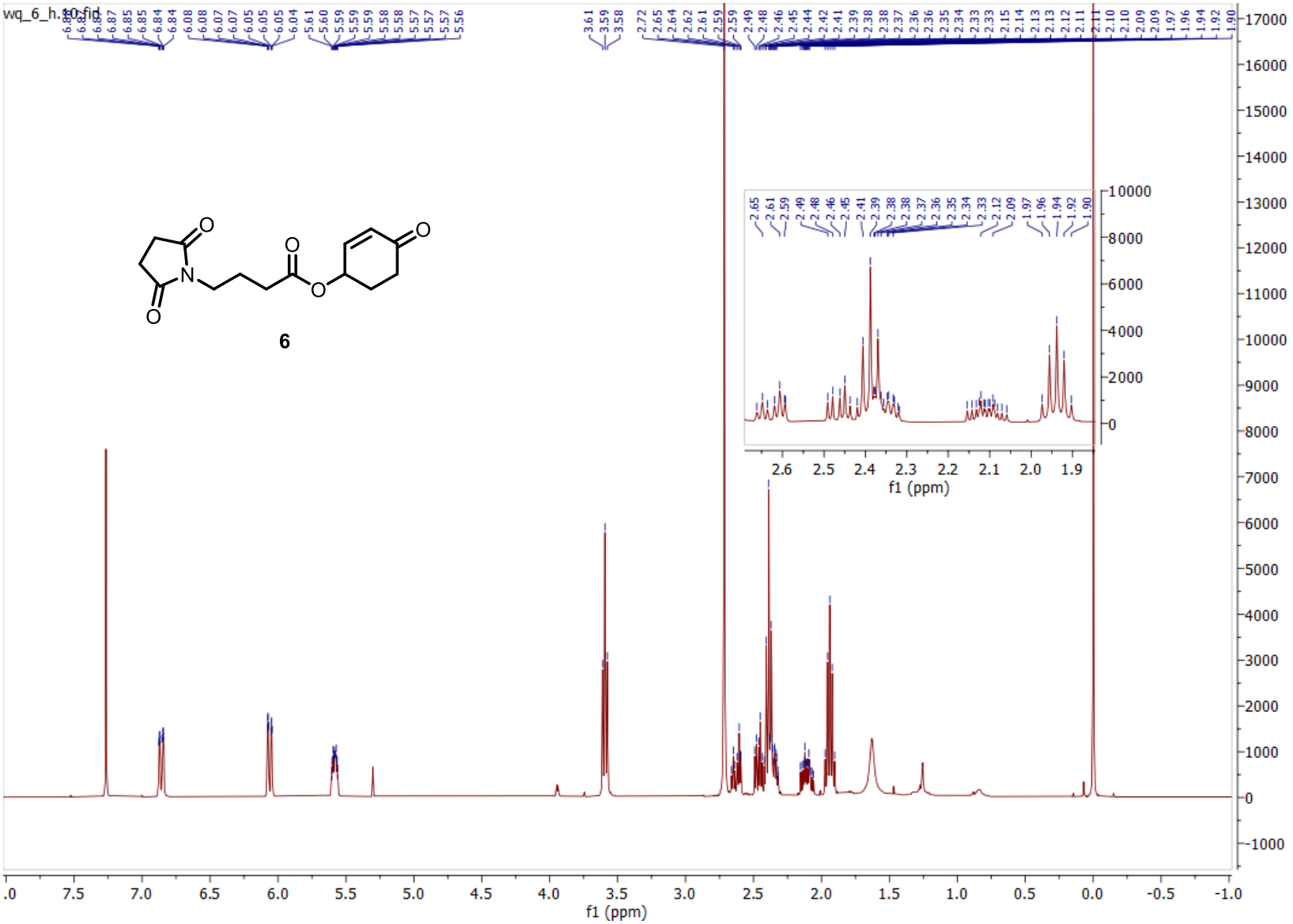

3
f1 (ppm)
1.00
2.06
0.98
1.00
2.24
1.05
1.11
3.26
1.17
4.61
2.27

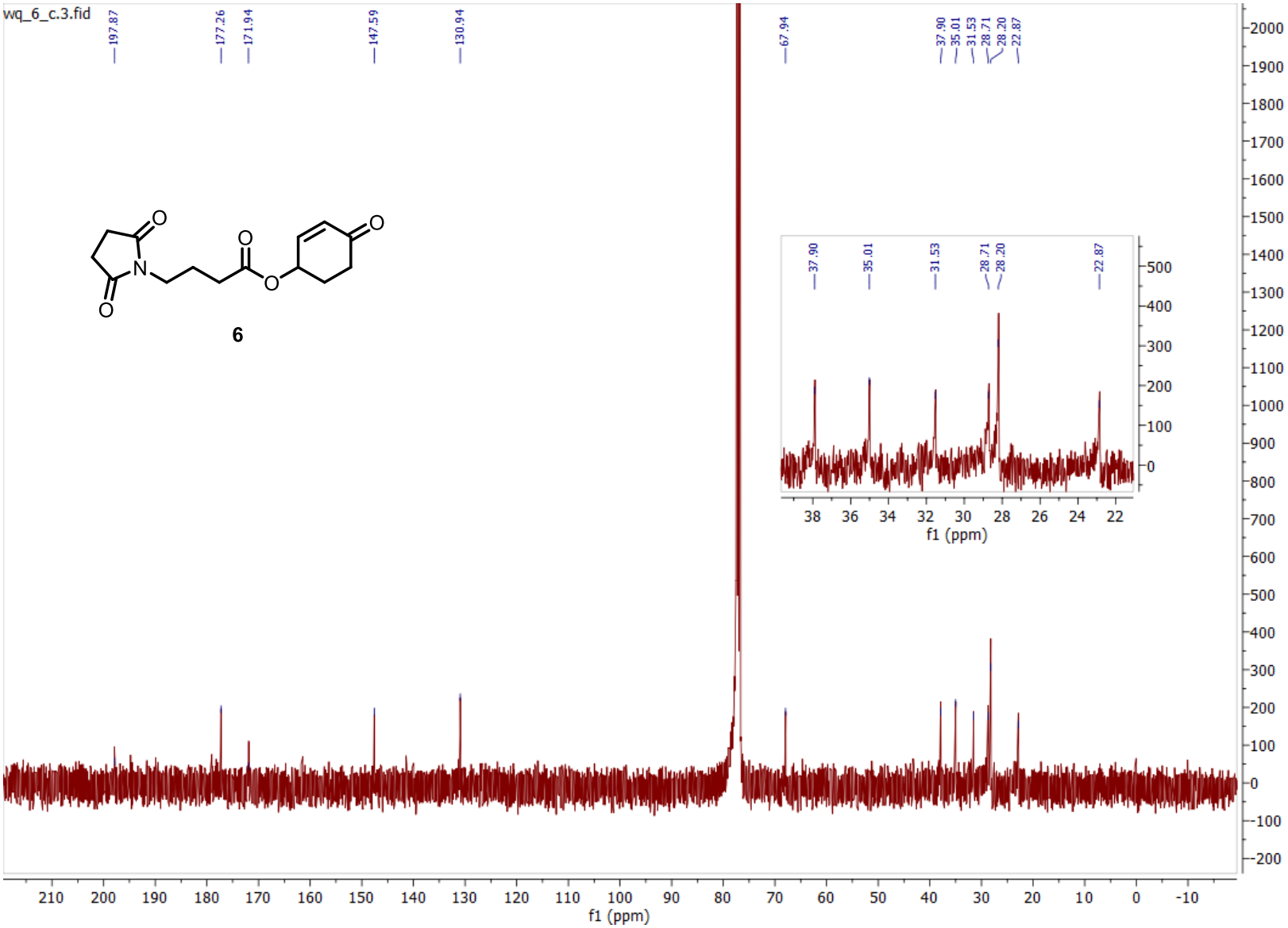

wq_s3_c_2.3.fid
197.99
172.71
170.83
147.73
134.09
130.84
67.61
37.57
34.97
34.00
28.71
28.17
26.15
24.32
3
f1 (ppm)
f1 (ppm)

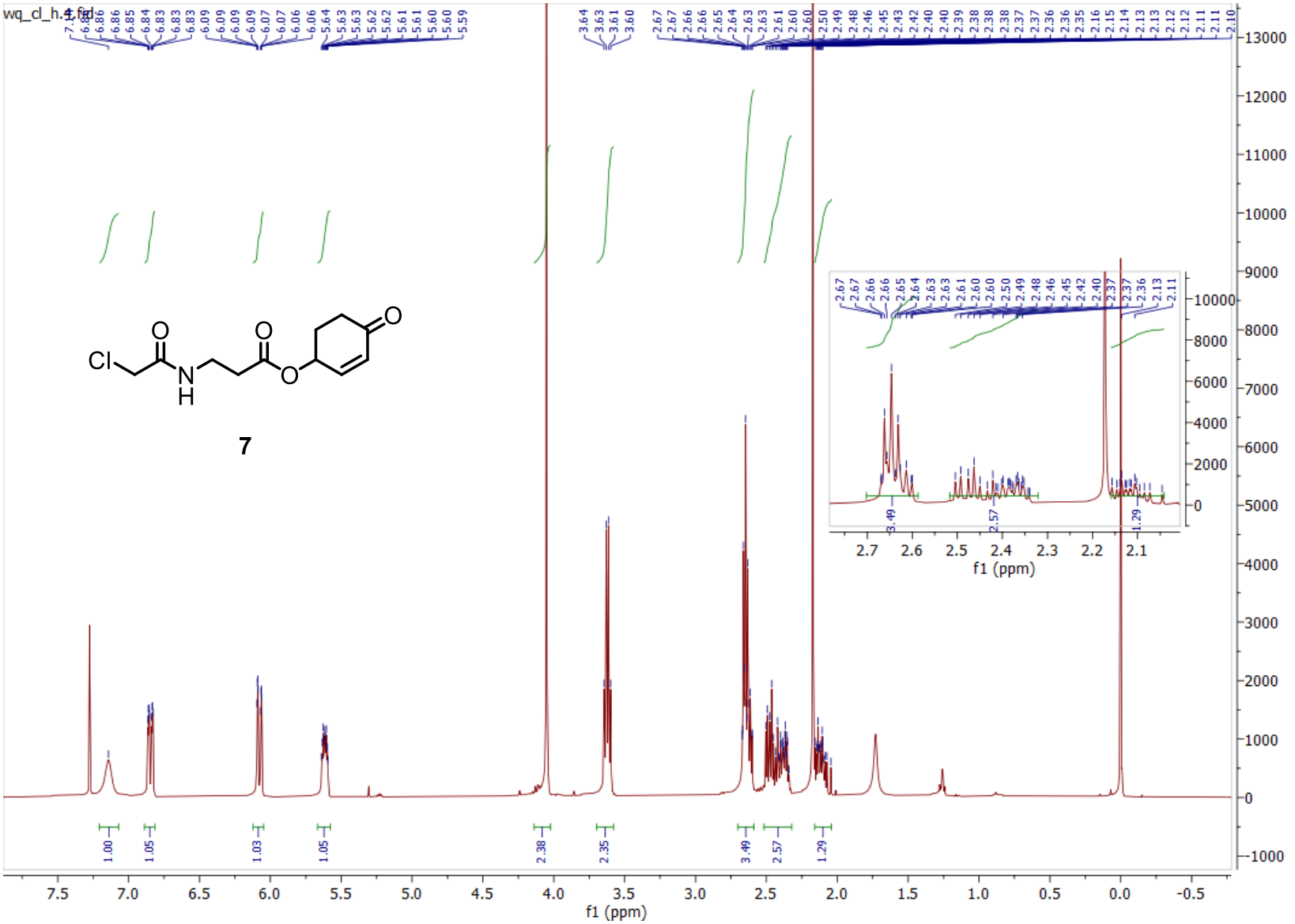

4
f1 (ppm)
f1 (ppm)

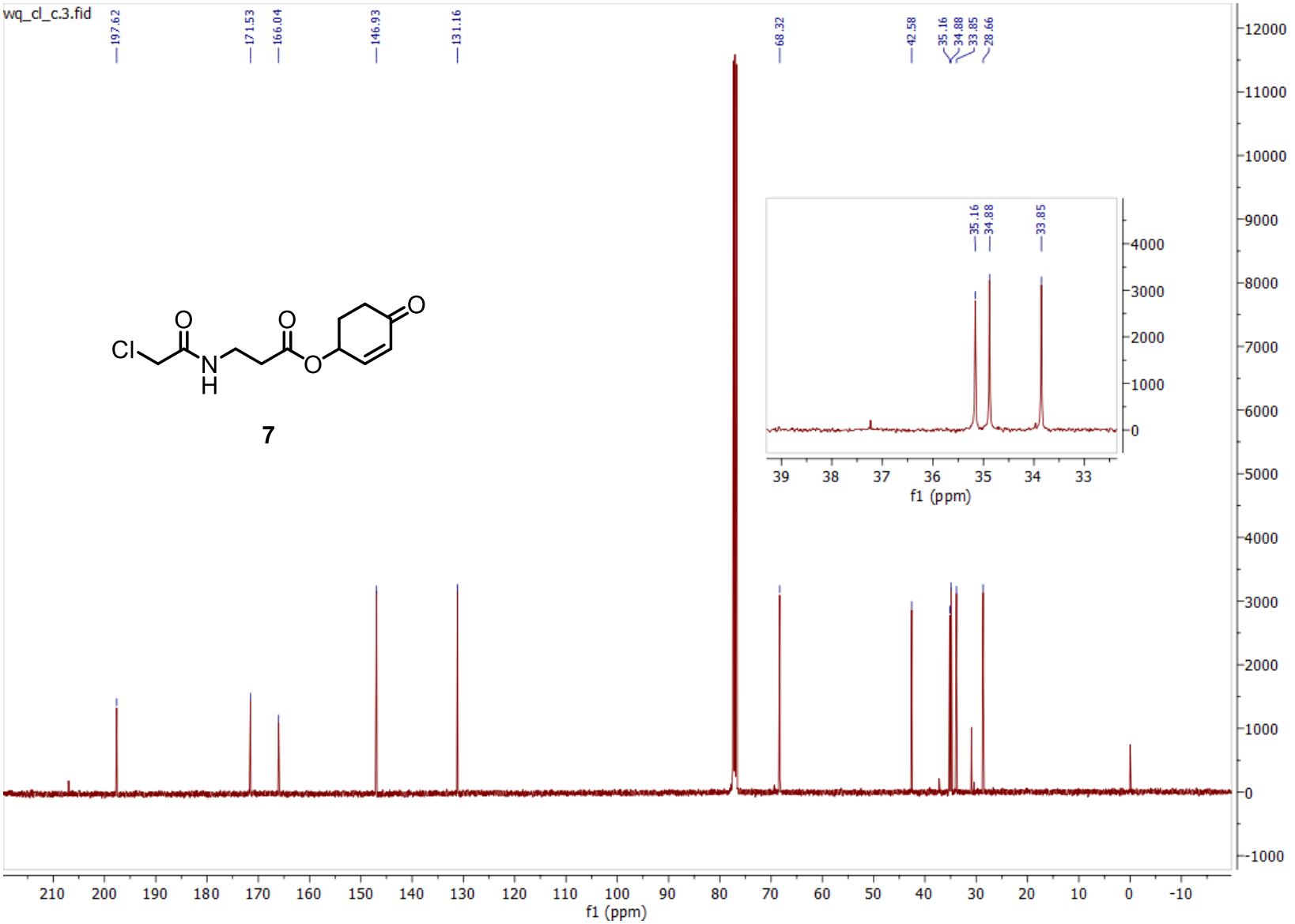

WQ_S4_C13.fid
197.41
174.93
171.00
147.67
134.00
130.86
67.41
43.55
42.97
36.30
34.94
29.56
28.66
28.17
4
f1 (ppm)

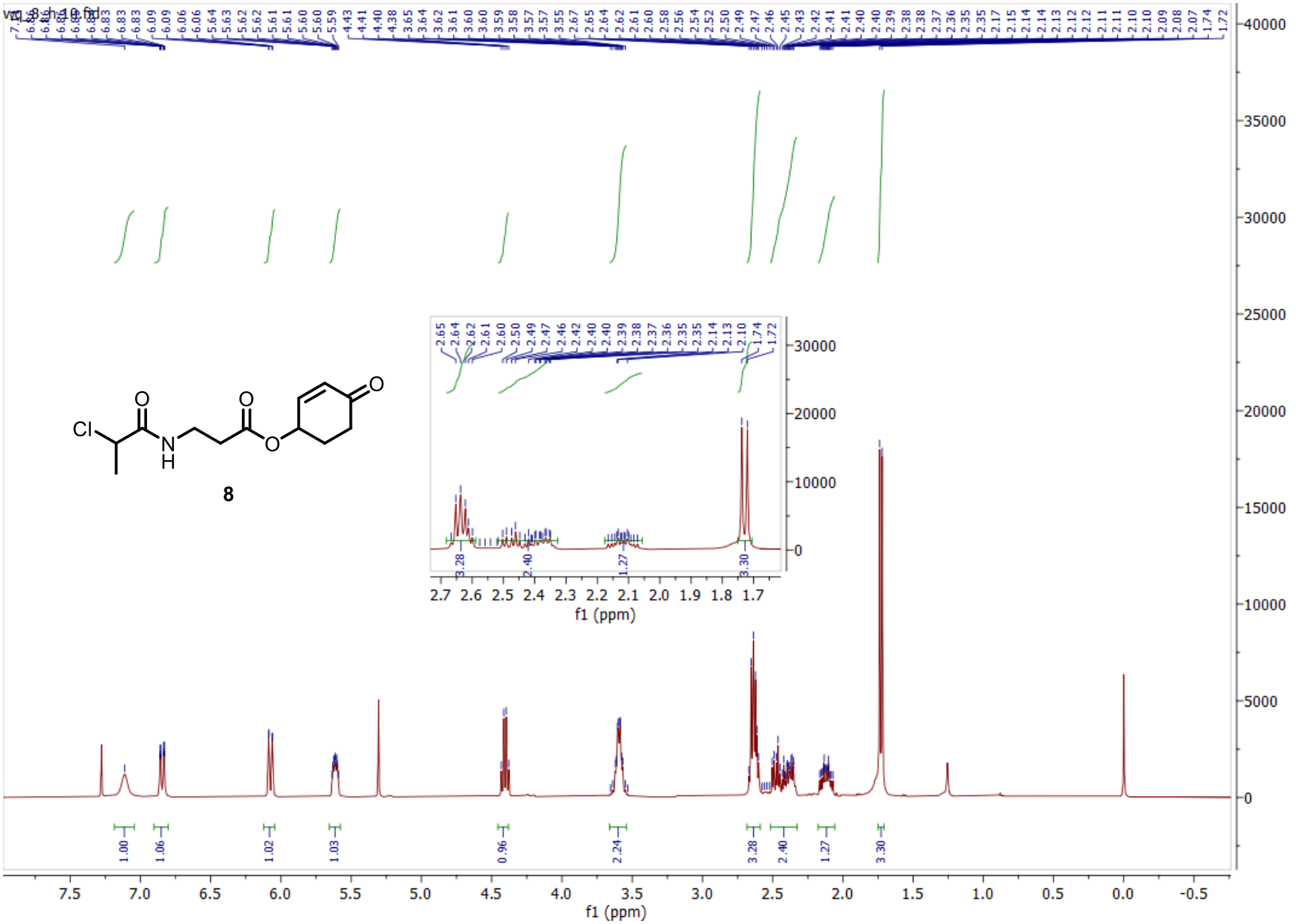

wq01114.1.fid
5
f1 (ppm)

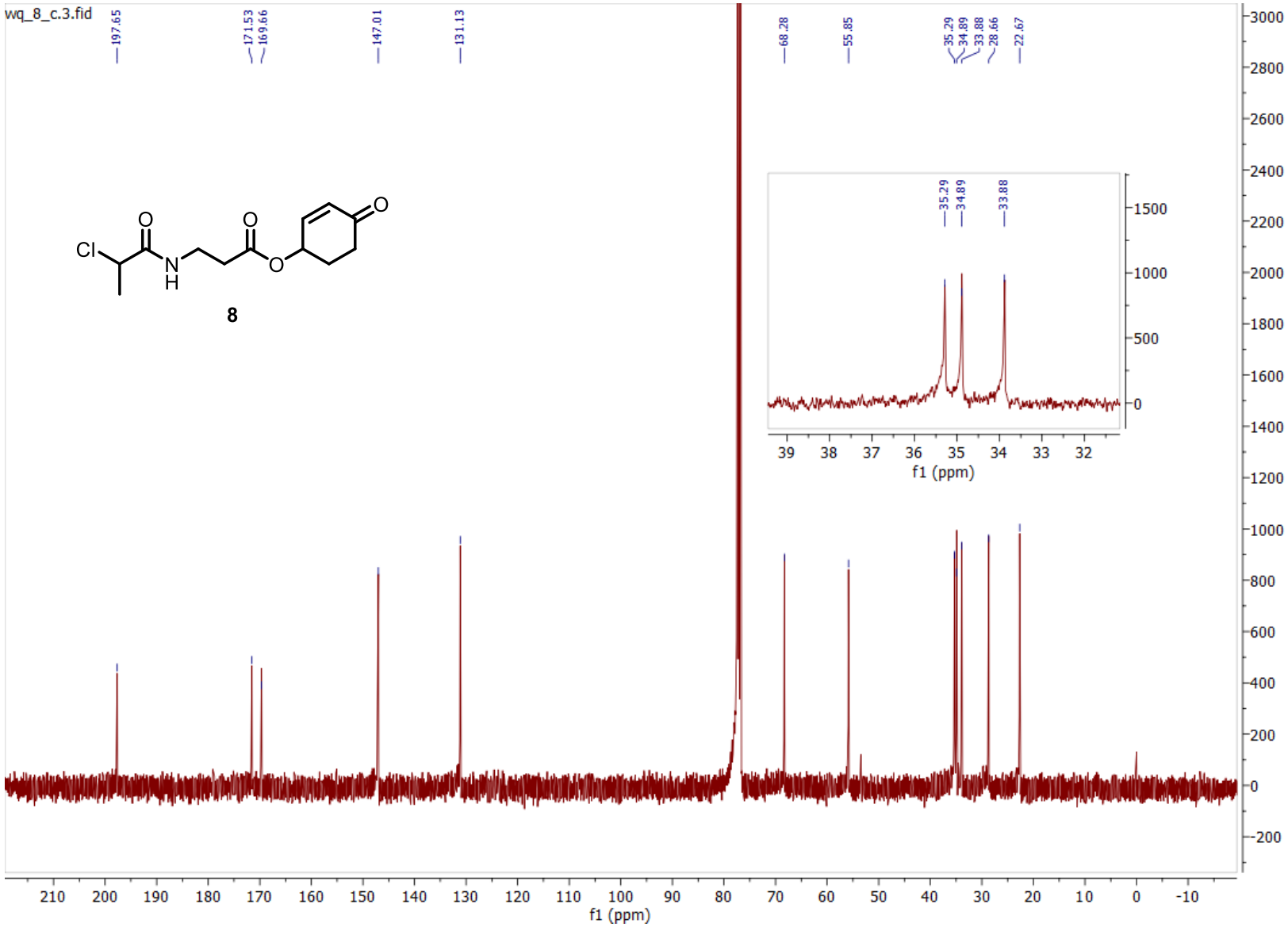

wq_6_h_10.fid
6
f1 (ppm)
f1 (ppm)

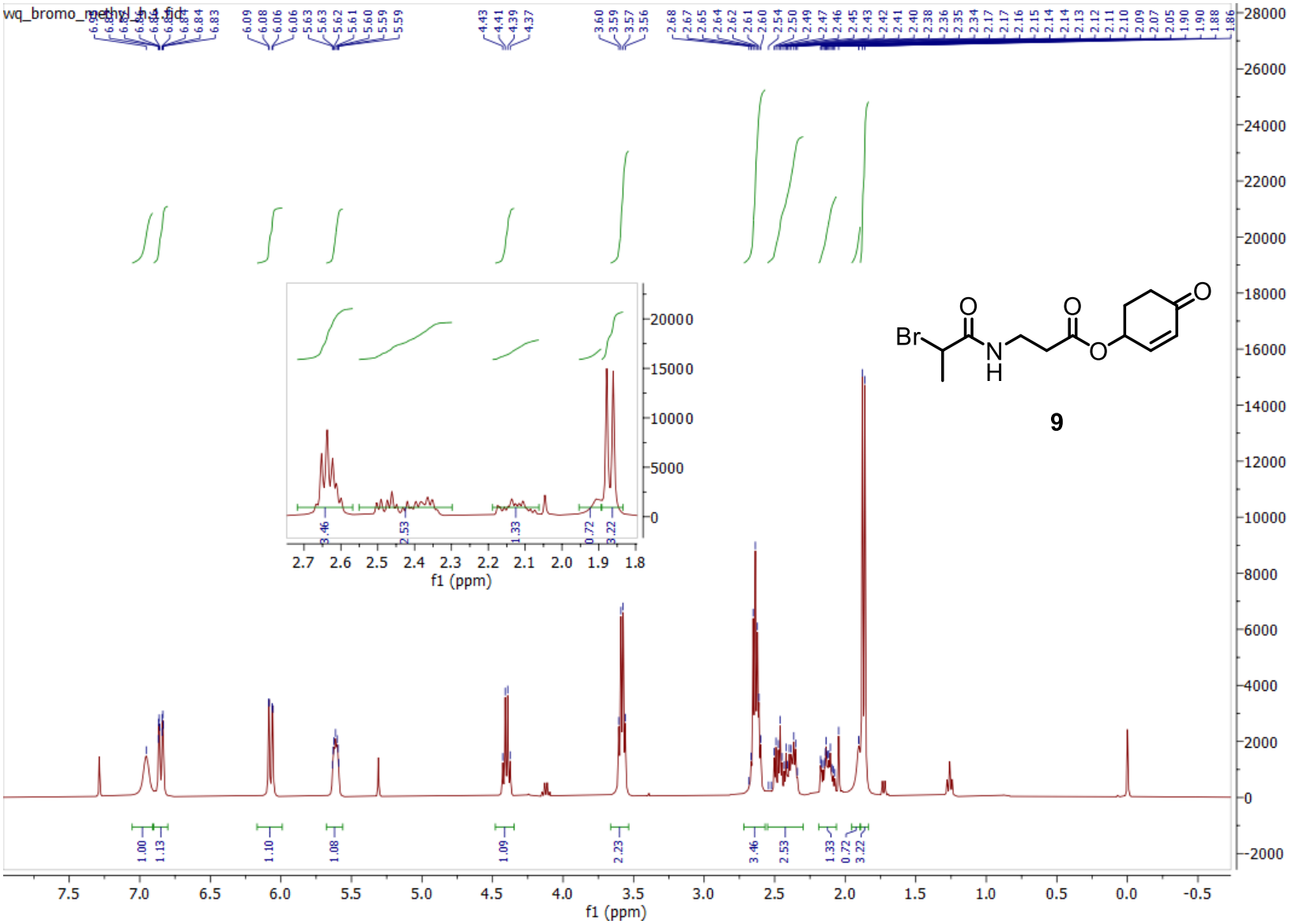

wq_6_c.3.fid
197.87
177.26
171.94
147.59
130.94
67.94
37.90
35.01
31.53
28.71
28.20
22.87
O
O
N
O
O
O
6
f1 (ppm)
f1 (ppm)

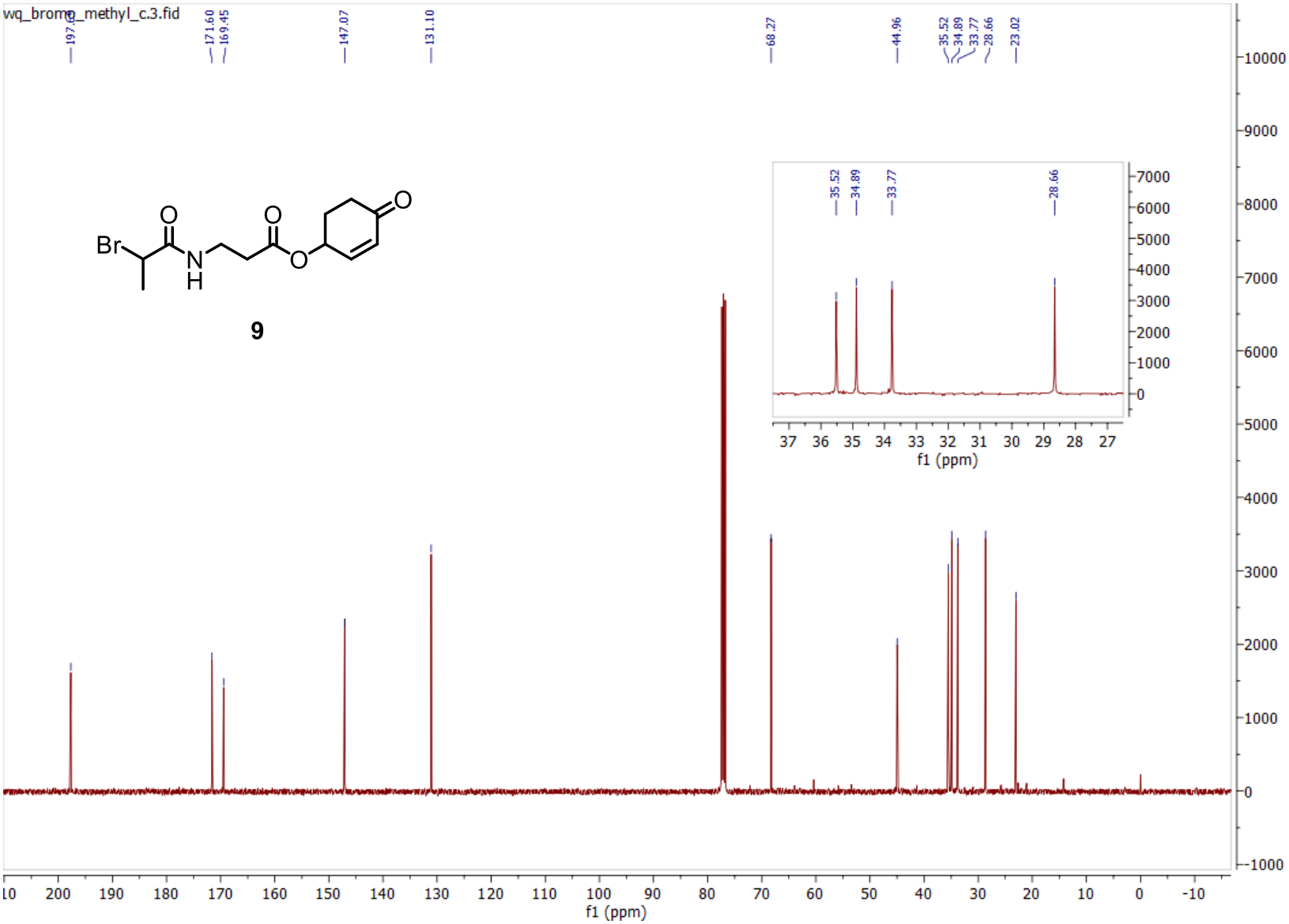

wq_cl_h.4.fid
Cl
O
N
H
O
O
O
7
1.00
1.05
1.03
1.05
2.38
2.35
3.49
2.57
1.29
f1 (ppm)


wq_cl_c.3.fid
197.62
171.53
166.04
146.93
131.16
68.32
42.58
35.16
34.88
33.85
28.66
Cl
O
N
H
O
O
O
7
f1 (ppm)
f1 (ppm)


Cl
O
N
H
O
O
O
8
f1 (ppm)
f1 (ppm)


wq_8_c.3.fid
197.65
171.53
169.66
147.01
131.13
68.28
55.85
35.29
34.89
33.88
28.66
22.67
Cl
O
N
H
O
O
O
8
f1 (ppm)
f1 (ppm)


wq_bromo_methyl_h_4.fid
f1 (ppm)
9


wq_bromo_methyl_c.3.fid
197.68
171.60
169.45
147.07
131.10
68.27
44.96
35.52
34.89
33.77
28.66
23.02
Br
O
N
H
O
O
9
f1 (ppm)
f1 (ppm)

7. Supporting references

(1) Ballone, A.; Lau, R. A.; Zweipfenning, F. P. A.; Ottmann, C. A new soaking procedure for X-ray crystallographic structural determination of protein-peptide complexes. *Acta Crystallogr. F Struct. Biol. Commun.* **2020**, *76* (Pt 10), 501-507. DOI: 10.1107/S2053230X2001122X

(2) Winter, G.; Waterman, D. G.; Parkhurst, J. M.; Brewster, A. S.; Gildea, R. J.; Gerstel, M.; Fuentes-Montero, L.; Vollmar, M.; Michels-Clark, T.; Young, I. D.; et al. DIALS: implementation and evaluation of a new integration package. *Acta Crystallogr. D Struct. Biol.* **2018**, *74* (Pt 2), 85-97. DOI: 10.1107/S2059798317017235

(3) McCoy, A. J.; Grosse-Kunstleve, R. W.; Adams, P. D.; Winn, M. D.; Storoni, L. C.; Read, R. J. Phaser crystallographic software. *J. Appl. Crystallogr.* **2007**, *40* (Pt 4), 658-674. DOI: 10.1107/S0021889807021206

(4) Emsley, P.; Cowtan, K. Coot: model-building tools for molecular graphics. *Acta Crystallogr. D Biol. Crystallogr.* **2004**, *60* (Pt 12 Pt 1), 2126-2132. DOI: 10.1107/S0907444904019158

(5) Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr. D Biol. Crystallogr.* **2010**, *66* (Pt 2), 213-221. DOI: 10.1107/S0907444909052925

(6) Crawford, J. J.; Kerr, W. J.; McLaughlin, M.; Morrison, A. J.; Pauson, P. L.; Thurston, G. J. Use of a highly effective intramolecular Pauson–Khand cyclisation for the formal total synthesis of (±)-α- and β-cedrene by preparation of cedrone. *Tetrahedron* **2006**, *62* (49), 11360-11370. DOI: 10.1016/j.tet.2006.05.044

(7) Chen, Y.-J.; Wang, H.-L.; Villarante, N. R.; Chuang, G. J.; Liao, C.-C. Substituent effect on the photochemistry of 4,4-dialkoxylated- and 4-hydroxylated cyclohexenones. *Tetrahedron* **2013**, *69* (46), 9591-9599. DOI: 10.1016/j.tet.2013.09.037

(8) Song, H. Y.; Ngai, M. H.; Song, Z. Y.; MacAry, P. A.; Hobley, J.; Lear, M. J. Practical synthesis of maleimides and coumarin-linked probes for protein and antibody labelling via reduction of native disulfides. *Org. Biomol. Chem.* **2009**, *7* (17), 3400-3406. DOI: 10.1039/b904060a

(9) Harris, C. R.; Millman, K. J.; van der Walt, S. J.; Gommers, R.; Virtanen, P.; Cournapeau, D.; Wieser, E.; Taylor, J.; Berg, S.; Smith, N. J.; et al. Array programming with NumPy. *Nature* **2020**, *585* (7825), 357-362. DOI: 10.1038/s41586-020-2649-2

(10) Virtanen, P.; Gommers, R.; Oliphant, T. E.; Haberland, M.; Reddy, T.; Cournapeau, D.; Burovski, E.; Peterson, P.; Weckesser, W.; Bright, J.; et al. SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat. Methods* **2020**, *17* (3), 261-272. DOI: 10.1038/s41592-019-0686-2

(11) Hunter, J. D. Matplotlib: A 2D Graphics Environment. *Comput. Sci. Eng.* **2007**, *9* (3), 90-95. DOI: 10.1109/MCSE.2007.55

(12) van Rosmalen, M.; Krom, M.; Merkx, M. Tuning the Flexibility of Glycine-Serine Linkers To Allow Rational Design of Multidomain Proteins. *Biochemistry* **2017**, *56* (50), 6565-6574. DOI: 10.1021/acs.biochem.7b00902