Electronic Supplementary Material (ESI) for Chemical Communications.

Rational Design of Reversible and Irreversible Cysteine Sulfenic Acid-Targeted Linear C-Nucleophiles

Vinayak Gupta and Kate S. Carroll*

Department of Chemistry, The Scripps Research Institute, Jupiter, Florida, 33458.

*Correspondence should be addressed to K.S.C. (kcarroll@scripps.edu).

Contents:

Synthetic Methods and Materials.

All reactions were conducted in flame-dried glassware under nitrogen pressure with dry solvents, unless otherwise noted. Tetrahydrofuran (THF) was freshly distilled from sodium/benzophenone. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific and used as received. Silica gel P60 (Sorbent Technologies) was used for column chromatography. Reactions were monitored by thin layer chromatography (TLC) carried out using Analtech 60 F254 silica gel (precoated sheets, 0.25 mm thick). ^{1}H-NMR and ^{13}C-NMR spectra were collected in DMSO-d_6 or $CDCl_3$ (Cambridge Isotope Laboratories, Cambridge, MA) at 400 and 100 MHz respectively, using a Bruker AM-400 instrument with chemical shifts relative to residual $CHCl_3$ (7.27 and 77.37 ppm). Low resolution mass spectral analyses were carried out on an Agilent LC/MS system. All spectra are available upon request.

Instrumentation for kinetics assay (Rate Studies)

The LC-MS used was Agilent technologies 1220 Infinity LC and Agilent Technologies 6120 quadrupole MS. The column used was Agilent Poroshell 120 SB C-18, 2.7 μM particle size and dimensions were 3.0 x 50 mm. LC-MS grade solvents were used which were buffered with 0.1% Formic acid (LC-MS grade). Data was analyzed by LC/MSD ChemStation (Rev. B.04.03-SP1).

KaleidaGraph (version 4.1.1) was used for graphing and further data analysis to obtain pseudo unimolecular 1st order rate constants, 2nd order rate constants and pH plots.

Following equations were used for the purpose of graphing:

Pseudo unimolecular 1st order rate constant (k_{obs}) -

$$m1 * (1 - \exp(-m2 * m0)); m1 = xx; m2 = yy;$$

Sample Preparation and General LC-MS Assay

Sample preparation - Stock solutions of nucleophiles were prepared in DMSO (100 mM concentration). Stock solutions were then diluted to appropriate concentration in PBS (10 mM, pH = 7.4). The pH was checked for each stock solution and was found to be in range of 7.40 – 7.45. Similarly, a 100 mM stock solution of dipeptide sulfenamide was prepared in acetonitrile and diluted to appropriate concentration in acetonitrile.

Assay - For each rate study, to a 2 mL solution of the nucleophile, was added 1 mL solution of cyclic sulfenamide. Effective concentrations were: Cyclic sulfenamide – 10, 25 or 50 or 100 μM; Nucleophile – 50, 125 or 250 μM, 1.0 mM or higher. Resulting reaction mixture was quenched after regular intervals by taking out a measured aliquot (300 μL) and adding it to a LC-MS vial containing formic acid (100 μL) and analyzing it by LC-MS. Area under the curve for the product formation at each time point was obtained from LC and plotted against time to get k_{obs}.

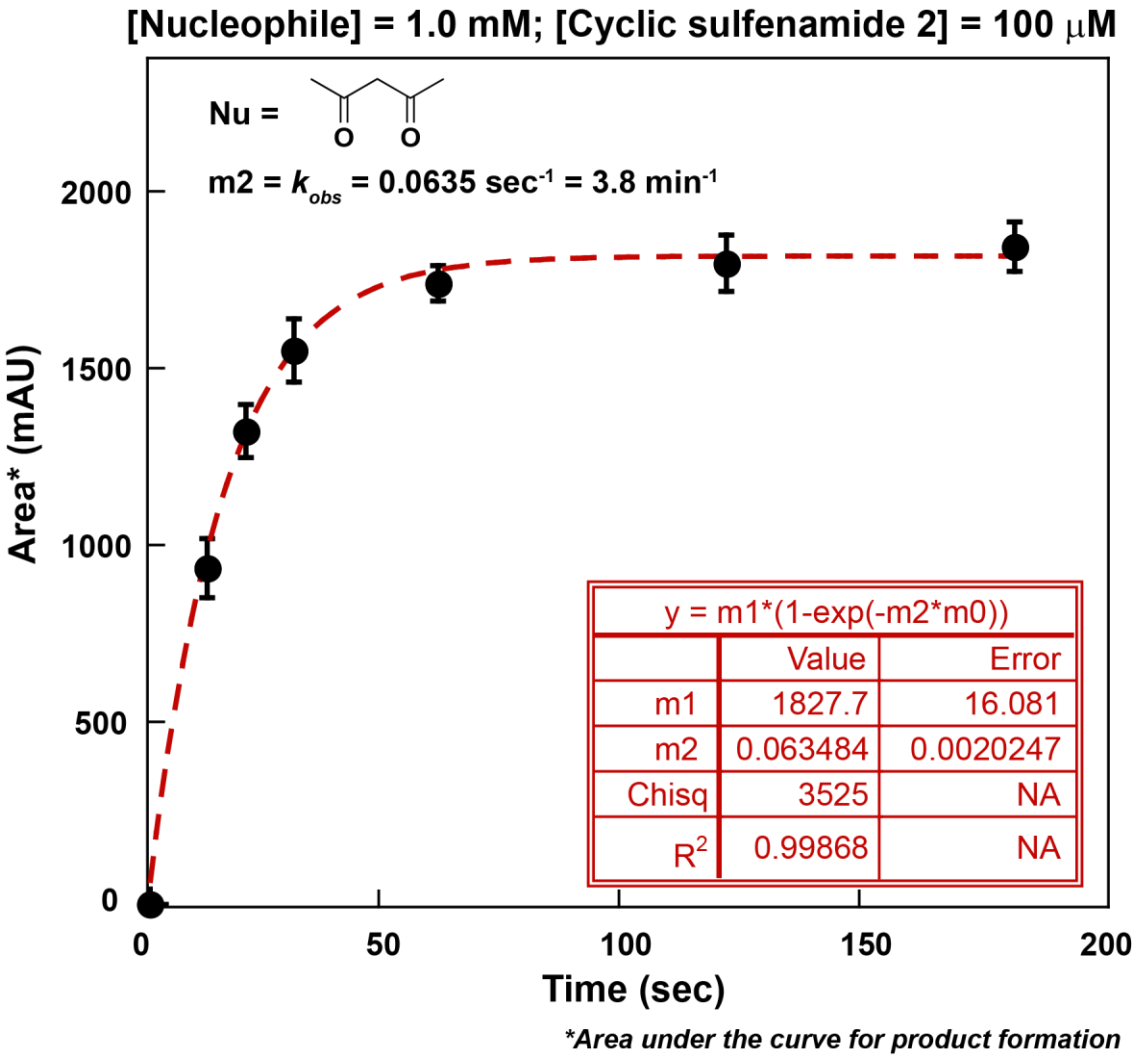
LC-MS method – The gradient was started at 95% H_2O – 5% acetonitrile (0 minutes) with a flow rate of 1 ml/min. The gradient was changed to 0% H_2O – 100% acetonitrile over 5 min. with same flow rate. This gradient was maintained for 2 min. Total run time was 7 minutes followed by a 1.4 min. of post-time. The LC trace was obtained by monitoring 190 nm wavelength.

Assay approximation - In the cases of kinetically faster nucleophiles (where < 1 mM nucleophile concentrations were used), the k_{obs} was adjusted to [Nu] = 1 mM by multiplying the observed rate values with appropriate factor for comparison purposes. The correction presupposes that the same rate law applies throughout the entire concentration range, which may or may not hold true.

Standard deviation for k_{obs} reported are $\leq$ 7.5% and representative plot for each nucleophile is shown here.

Electrospray ionization mass spectrometry (ESI-MS). The molecular mass of Gpx3-S-Nucleophile adduct was measured on a LTQ XL linear Ion trap mass spectrometer (Thermo Scientific) connected to a liquid chromatography (LC) system. Each Gpx3-S-nucleophile adduct (3 μM, 8 μL) was separated and desalted onto a C8 column and subsequently analyzed in the mass spectrometer. Spectra were acquired in the positive ion mode. The deconvolution program MagTran was used to obtain the mass spectra.

Sample rate plot –



Sample MS plot – For the reaction of dipeptide-SOH **(2)** with acetylacetone **(3a)** showing the thioether adduct formation (m/z 467.2)

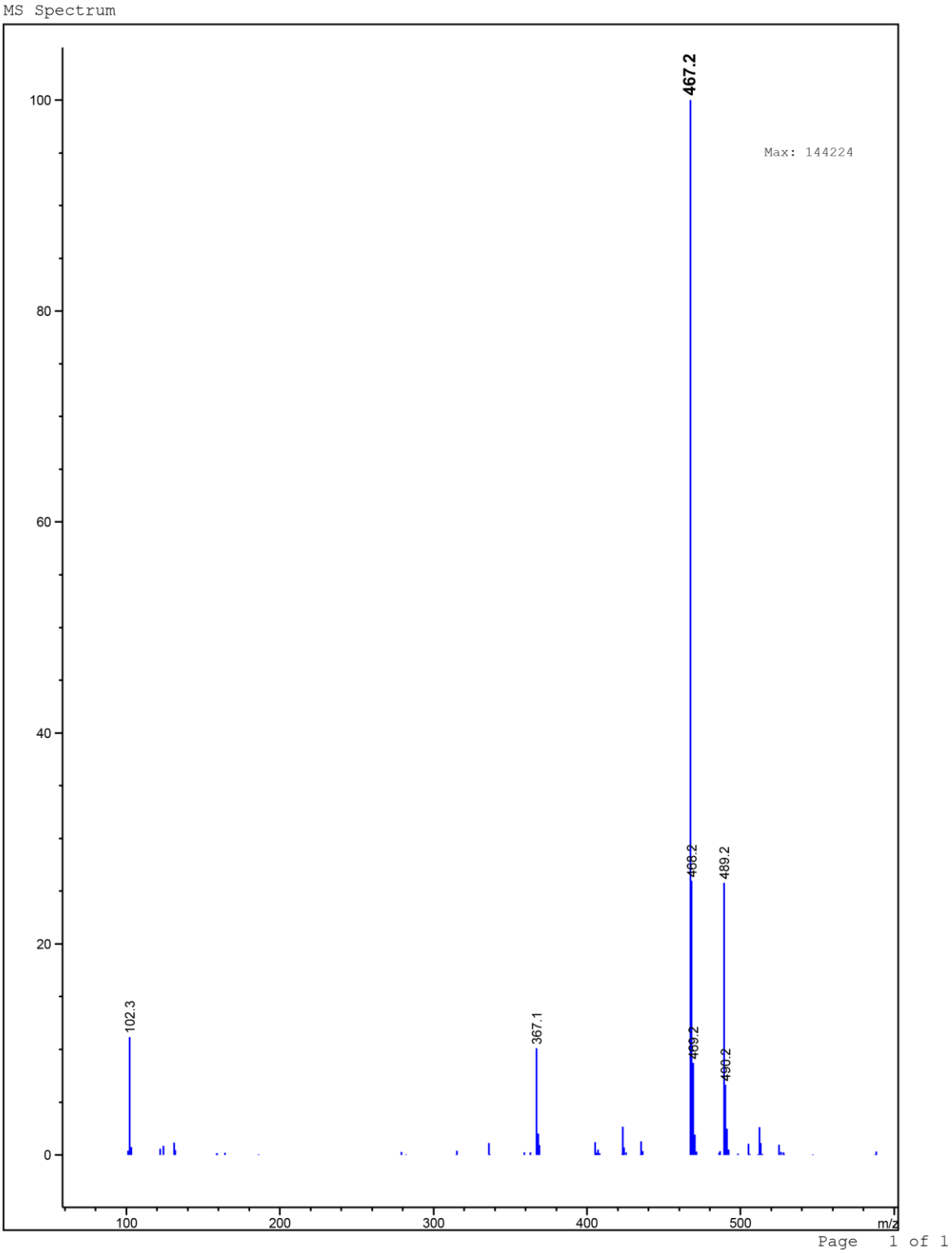


Fig. S1 (A) Bromomaleimides are used as reversible thiol alkylating reagents. (B) Tuned acrylonitrile-based Michael acceptors have been used as reversible covalent kinase inhibitors. (C) Biological cysteine oxoforms formed by the action of ROS. (D) Dipeptide-based small molecule sulfenic acid model was used to screen cyclic C-nucleophiles which formed stable thioether adducts.

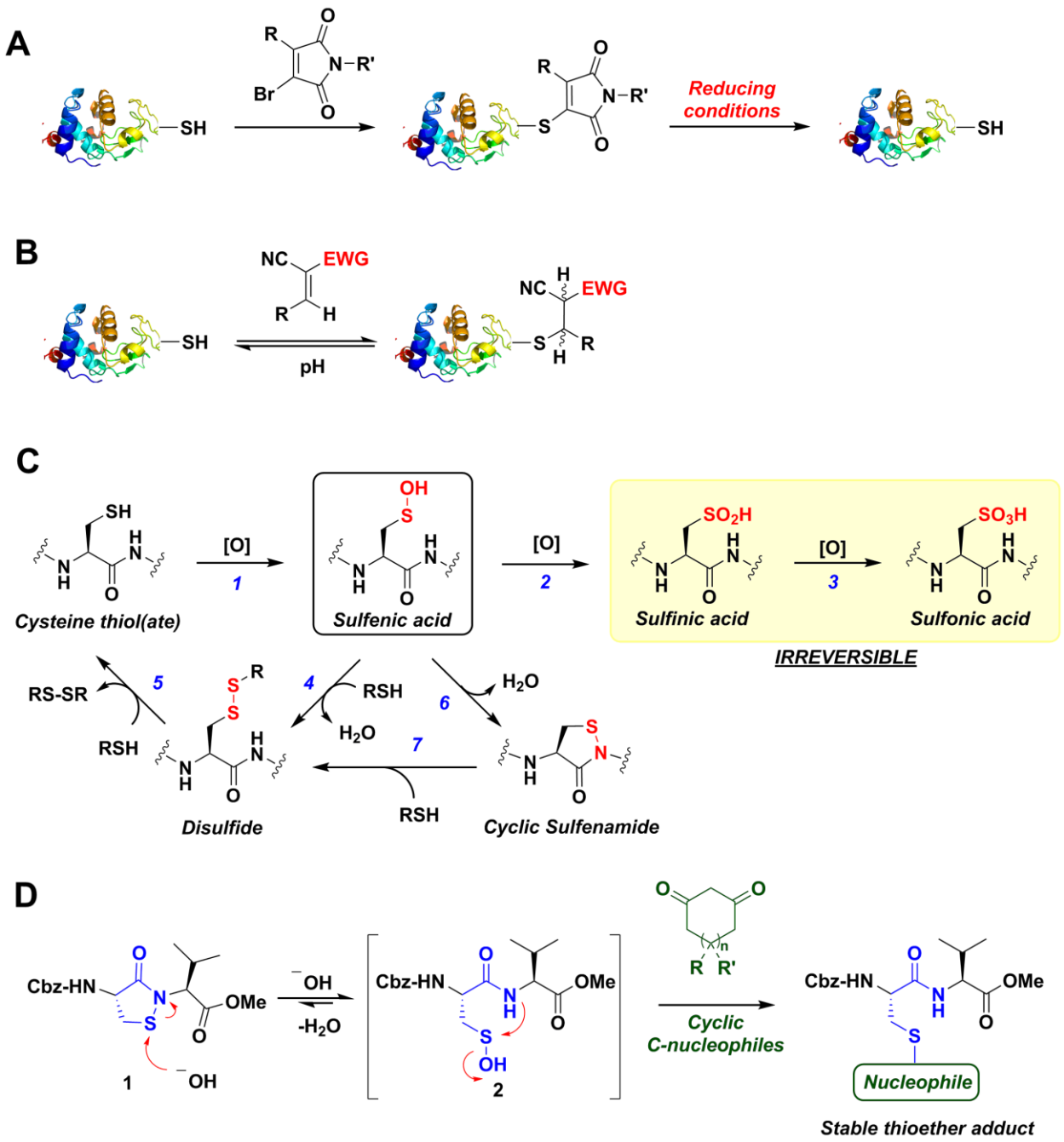
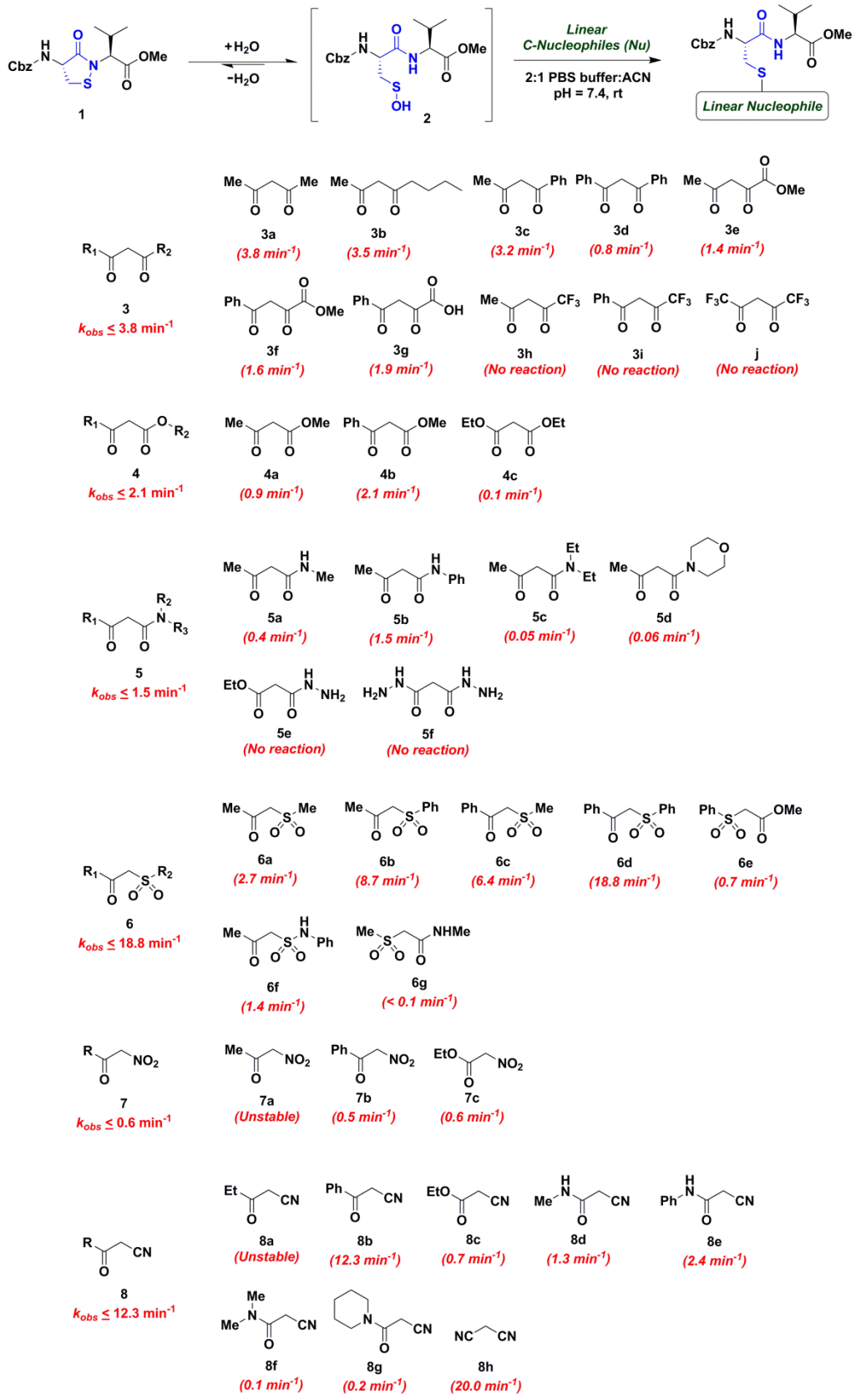


Fig. S2 Expanded list of linear C-nucleophiles screened against dipeptide-SOH 2.



In the work described here, we employed the same mass spectrometery-based assay to screen *linear C-nucleophiles*. As discussed previously, unlike cyclic 1,3-dicarbonyls, linear 1,3-dicarbonyl compounds form intermolecular hydrogen bond with water molecules thus making keto form dominant, we argued that this orthogonal behaviour may lead to difference in reactivity trends towards Cys-SOH and corresponding stability of thioether adducts. This study would further increase the diversity of the 'chemical tool-box' of sulfenic acid specific nucleophiles. Initial proof of our hypothesis was observed by the reactivity of 2,4-pentanedione (**3a**, pKa = 8.99) towards dipeptide sulfenic acid **2**. It exhibits a 4:1 ratio of keto:enol tautomers and showed a k_{obs} of 3.8 min^{-1} which is almost 200-fold higher compared to corresponding 5-membered cyclic analog (cyclopentane-1,3-dione, k_{obs} = 0.02 min^{-1}).[14] Next, we explored the effects of alkylation and arylation on the reactivity **3** towards dipeptide sulfenic acid **2**. Mono alkylated octane-2,4-dione **(3b)** and mono arylated 1-phenylbutane-1,3-dione **(3c)** showed k_{obs} of 3.5 min^{-1} and 3.2 min^{-1} respectively which are similar to the k_{obs} of **3a**. However, diarylated 1,3-diphenylpropane-1,3-dione **(3d)**, due to a shift in keto-enol equilibrium towards enol form, and enhanced stability of carbanion showed reduced k_{obs} of 0.8 min^{-1} which is similar to that of dimedone (k_{obs} = 0.8 min^{-1}). Methyl acetopyruvate **(3e)** and its phenyl analogs methyl 2,4-dioxo-4-phenylbutanoate **(3f)** and 2,4-dioxo-4-phenylbutanoic acid **(3g)** showed very similar k_{obs} of 1.4 min^{-1}, 1.6 min^{-1} and 1.9 min^{-1} respectively, for the reaction with dipeptide-SOH **2**. Replacing one or both methyls with trifluoromethyl results in complete loss of activity as shown by 1,1,1-trifluoropentane-2,4-dione **(3h)**, 4,4,4-trifluoro-1-phenylbutane-1,3-dione **(3i)** and 1,1,1,5,5,5-hexafluoropentane-2,4-dione **(3j)**. This lack of reactivity is attributed to the hydration of carbonyl group due to electron-withdrawing effect of $-CF_3$ group resulting in increased pK_a of reactive carbanion.

Next, we evaluated the effect of replacing one or both carbonyls with carboxylate ester, amide and hydrazide functional groups on the reactivity towards dipeptide sulfenic acid **2**. We argued that these substitutions would result in the increase in the pK_a of nucleophilic C-centre, resulting in the decreased viability of reactive carbanion formation at the physiological pH. This would correspond with the reduced rates of reactions of such nucleophiles towards **2**. As expected methyl acetoacetate **(4a)**, which has a pK_a (in H_2O) of 11, showed k_{obs} of 0.9 min^{-1} which is 4-fold less than **3a** and similar to that of dimedone. Our observation that β-ketoesters such as **4a** shows similar kinetics as dimedone, contradicts Furdui *et al.* report of enhanced kinetics of such nucleophiles. Methyl 3-oxo-3-phenylpropanoate **(4b)** reacted with **2** with k_{obs} of 2.1 min^{-1}. Furthermore,

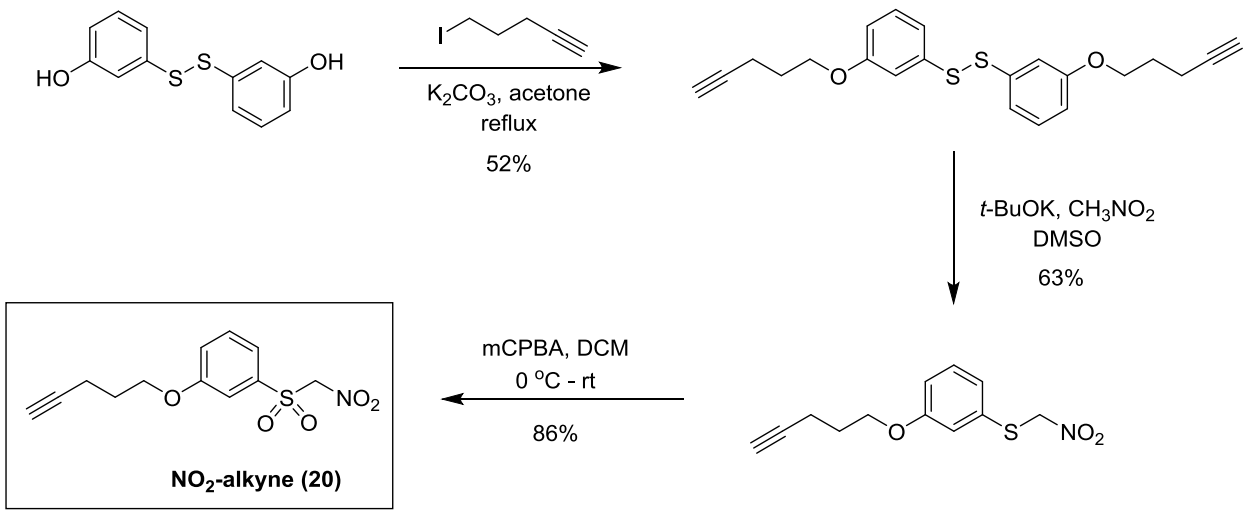
diethyl malonate **(4c)**, with a pK_a (in H_2O) of 13, showed even lower k_{obs} of 0.1 min^{-1}, a 40-fold rate reduction compared to **3a**. Similarly, *N*-methylacetoacetamide **(5a)** showed a k_{obs} of 0.4 min^{-1} and *N*-phenylacetoacetamide **(5b)** showed a k_{obs} of 1.5 min^{-1}. Reaction of *N,N*-diethylacetoacetamide **(5c)** and *N*-acetoacetylmorpholine **(5d)** with dipeptide-SOH **2** showed similar k_{obs} of 0.05 min^{-1} and 0.06 min^{-1} respectively. Lastly, owing to high pk_a values (> 15), ethyl malonyl hydrazide **(5e)** and malonic dihydrazide **(5f)** failed to react with dipeptide-SOH **2**.

Next, we evaluated the nucleophiles in which one of the carbonyls was replaced with a sulfone functional group. Simplest such derivative, methylsulfonylacetone **(6a)** successfully formed the thioether adduct with dipeptide-SOH **2** with a k_{obs} of 2.7 min^{-1}, similar to that of **3a**. Interestingly, phenylsulfonylacetone **(6b)** and 2-methylsulfonylacetophenone **(6c)** showed enhanced k_{obs} of 8.7 min^{-1} and 6.4 min^{-1} respectively. Diarylated derivative 2-phenylsulfonylacetophenone **(6d)** showed the best in class k_{obs} of 18.8 min^{-1} which is 5-fold higher than that of **3a**. As discussed in the manuscript, this structure-reactivity breakthrough led to the development of designer nucleophiles with enhanced kinetics. Continuing on, we evaluated the ester and amide derivatives of **6**. Methyl(phenylsulfonyl)acetate **(6e)** showed a reduced k_{obs} of 0.7 min^{-1}. Reaction of 2-oxo-*N*-phenylpropane-1-sulfonamide (6f) successfully reacted with dipeptide-SOH **2** with k_{obs} of 1.4 min^{-1}. Lastly, *N*-methyl-2-(phenylsulfonyl)acetamide **(6g)** was too slow to react with with dipeptide-SOH **2**. It is important to notice the difference in the trend here compared to the trends observed with derivatives of **3**. Mono- and di-arylation of **3** resulted in decrease in reactivity (due to enhanced enol character/stabilization of C-3 carbanion) whereas, in case of **6**, such substitution results in increase in the reactivity emphasizing the fine balance between the keto-enol tautomerism and stability/reactivity of carbanion (due to more/less resonance forms) on the reactivity of nucleophiles towards sulfenic acid.

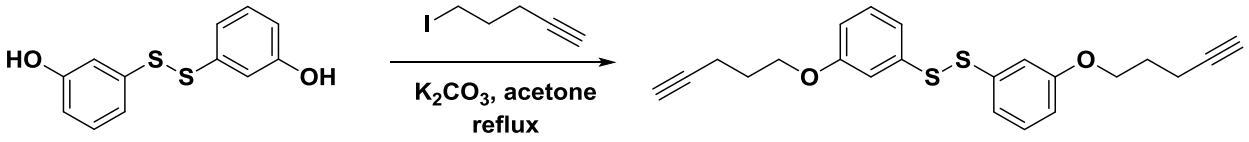
Next, we evaluated the nucleophilic reactivity of molecules in which one (or both) of the carbonyls is replaced with an electron-withdrawing group (EWG). We next evaluated the nucleophiles containing nitro ($-NO_2$) functionality. Although simple nitroacetone **(7a)** proved volatile and unstable, 2-nitroacetophenone **(7b)** owing to its predicted pk_a similar to that of 1,3-cyclohexanedione showed similar k_{obs} of 0.5 min^{-1} towards dipeptide-SOH **2**. Ethyl 2-nitroacetate **(7c)** was slightly more reactive with k_{obs} of 0.6 min^{-1}. 3-Oxopentanenitrile **(8a)** was unstable but with a predicted pK_a of 7.78, benzoylacetonitrile **(8b)** showed k_{obs} of 12.3 min^{-1} for the reaction with dipeptide-SOH **2**. Ethyl 2-cyanoacetate **(8c)** reacted with dipeptide-SOH **2** with

k_{obs} of 0.7 min^{-1}. Amide derivatives also exhibited slow kinetics. For example, 2-cyano-*N*-methylacetamide **(8d)** and 2-cyano-*N*-phenylacetamide **(8e)** showed k_{obs} of 1.3 min^{-1} and 2.4 min^{-1} respectively. 2-Cyano-*N,N*-dimethylacetamide **(8f)** and 1-cyanoacetylpiperidine **(8g)** reacted with dipeptide-SOH **2** with k_{obs} of 0.1 min^{-1} and 0.2 min^{-1} respectively. Commercially available malononitrile **(8h)** was simplest nucleophile in which both carbonyls were replaced with cyano groups, reacted successfully with dipeptide-SOH **2** with a k_{obs} of 20 min^{-1}, a 25-fold increase compared to dimedone.

Scheme S1. Synthesis of 20



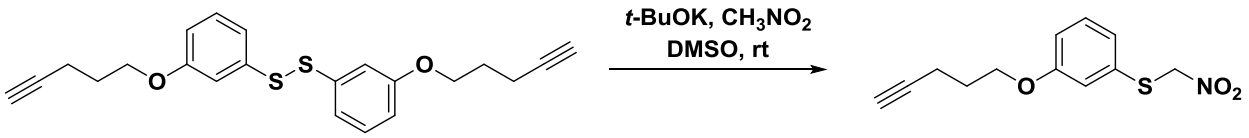
Synthesis of 1,2-bis(3-(pent-4-yn-1-yloxy)phenyl)disulfane



To a stirred solution of bisphenol (1.25 g, 5 mmol) in anhydrous acetone (100 ml) was added anhydrous potassium carbonate (3.46 g, 25 mmol) followed by 5-iodopentyne (2.15 g, 11 mmol). Resulting reaction mixture was refluxed with monitoring. The reaction was complete after 48 hr of reflux. The reaction was quenched by the addition of water and acetone was removed *in vacuo*. The residue was taken in EtOAc and was washed twice with HCl (0.5 N), water, sodium bicarbonate solution (5% aqueous), water and brine. Organic layer was then dried over anhydrous magnesium sulfate, filtered and evaporated to give the crude product. Purification by column chromatography using 5% to 10% EtOAc:Hexane resulted in the recovery of pure product.

^{1}H-NMR (in CDCl3, 400 MHz): δ 7.17 – 7.30 (m, 2H), 7.00 – 7.15 (m, 4H), 6.70 – 6.85 (m, 2H), 4.06 (t, 4H, J = 6.1 Hz); 2.42 (dt, 4H, J1 = 7.0 Hz, J2 = 2.5 Hz); 1.90 – 2.10 (m, 6H); ^{13}C-NMR (in CDCl3, 100 MHz): δ 159.5, 138.4, 130.1, 119.8, 113.8, 113.3, 83.5, 69.2, 66.3, 28.2, 15.3. LR-MS: $C_{22}H_{22}O_2S_2$; Calculated: 382.54; Observed: 383.1 (M^++1), 405.1 (M^++Na).

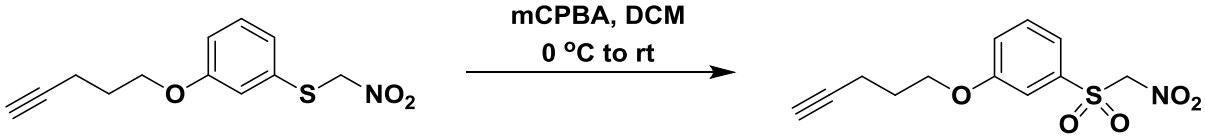
Synthesis of (nitromethyl)(3-(pent-4-yn-1-yloxy)phenyl)sulfane



To anhydrous DMSO (5 ml) was added potassium *tert*-butoxide (0.561 g, 5 mmol) at rt under nitrogen pressure and stirred till it became a homogeneous solution. To this solution, was added anhydrous nitromethane (0.244 g, 4 mmol) and resulting yellow slurry was stirred for 15 minutes. Finally, to this slurry was added dropwise the disulfide (0.764 g, 2 mmol) in a solution of DMSO (5 ml). Resulting yellow reaction mixture was stirred at rt with monitoring. The reaction was complete after 5 h of stirring. It was neutralized by the addition of 1 N HCl solution. The aqueous layer was then extracted with DCM. Organic phase were combined and washed with water and brine. Organic layer was then dried over anhydrous magnesium sulfate, filtered and evaporated to give the crude product. Purification by column chromatography using 10% EtOAc:Hexane resulted in the recovery of pure product.

^{1}H-NMR (in CDCl3, 400 MHz): δ 7.18 – 7.26 (m, 1H), 7.00 – 7.07 (m, 2H), 6.85 – 6.90 (m, 1H), 5.45 (s, 2H), 4.05 (t, 2H, J = 6.1 Hz); 2.39 (dt, 2H, J1 = 7.0 Hz, J2 = 2.7 Hz); 1.94 – 2.02 (m, 3H); ^{13}C-NMR (in CDCl3, 100 MHz): δ 159.6, 133.0, 130.7, 124.1, 117.9, 115.7, 83.4, 79.5, 69.3, 66.5, 28.2, 15.3. LR-MS: $C_{12}H_{13}NO_3S$; Calculated: 251.30; Observed: 252.1 (M^++1).

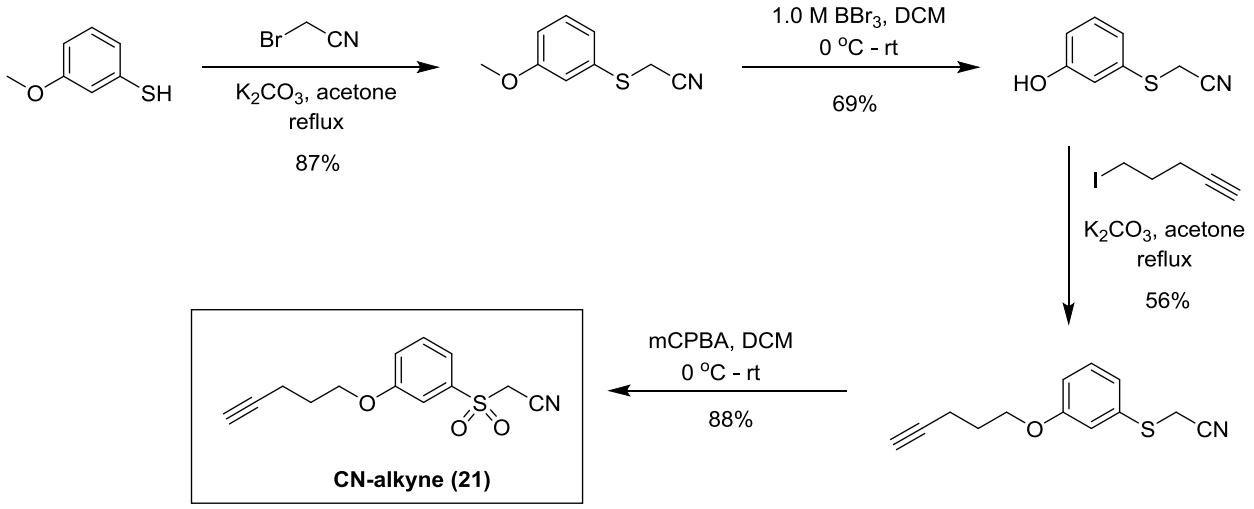
Synthesis of 1-((nitromethyl)sulfonyl)-3-(pent-4-yn-1-yloxy)benzene



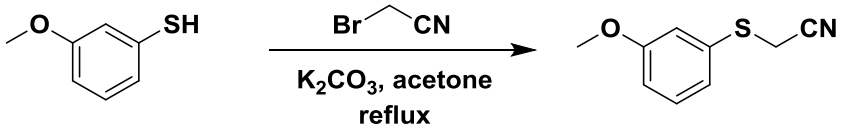
To a stirred solution of (nitromethyl)(3-(pent-4-yn-1-yloxy)phenyl)sulfane (0.25 g, 1.0 mmol) in DCM (50 ml) was added *m*-CPBA (0.62 g, 5 mMol) in batches at 0 ^{o}C. Resulting reaction mixture was allowed to stir at rt with monitoring. After the completion (18 h), the reaction mixture was quenched by the addition of small amount of DMSO. Organic phase was washed with water, saturated sodium bicarbonate and brine, dried over anhydrous magnesium sulfate, filtered and evaporated to give the crude product which was then purified by silica gel column chromatography to recover the pure product in 86% yield.

^{1}H-NMR (in CDCl3, 400 MHz): δ 7.52 – 7.55 (m, 2H), 7.42 – 7.45 (m, 1H), 7.27 – 7.32 (m, 1H), 5.62 (s, 2H), 4.15 (t, 2H, J = 6.1 Hz); 2.43 (dt, 2H, J1 = 7.0 Hz, J2 = 2.6 Hz); 2.04 (p, 2H, J = 6.3 Hz), 2.01 (t, 1H, J = 2.7 Hz); ^{13}C-NMR (in CDCl3, 100 MHz): δ 159.8, 136.8, 131.0, 122.8, 121.5, 114.2, 90.4, 83.1, 69.5, 67.0, 28.0, 15.2. LR-MS: $C_{12}H_{13}NO_5S$; Calculated: 283.30; Observed: 284.0 (M^++1).

Scheme S2. Synthesis of 21



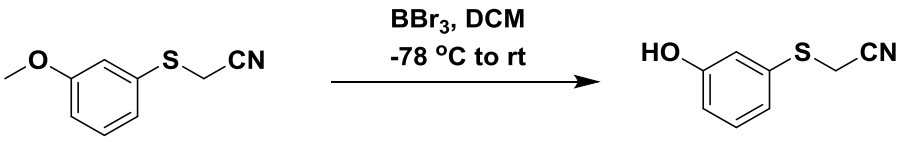
Synthesis of 2-((3-methoxyphenyl)thio)acetonitrile



To a stirred slurry of 3-methoxybenzenethiol (1.4 g, 10 mmol) and anhydrous potassium carbonate (2.8 g, 20 mmol, 2 eq) in acetone (40 ml), was added dropwise a solution of 2-bromoacetonitrile (1.44 g, 12 mmol) in anhydrous acetone (10 ml). Resulting reaction mixture was allowed to stir under reflux conditions with monitoring. After the completion, acetone was removed and reaction mixture was acidified by the addition of 0.1N HCl solution. Aqueous phase was extracted with ethyl acetate (3 x 50 ml). Combined organic layers were washed with brine, dried over anhydrous magnesium sulfate, filtered and evaporated to give the crude product which was then purified by silica gel column chromatography using a gradient of 15% - 20% EtOAc/Hexane to recover the pure product in 87% yield.

^{1}H-NMR (in CDCl3, 400 MHz): δ 7.27 – 7.35 (m, 1H), 7.12 – 7.15 (m, 1H), 7.08 – 7.11 (m, 1H), 6.90 – 6.95 (m, 1H), 3.85 (s, 3H), 3.60 (s, 2H); ^{13}C-NMR (in CDCl3, 100 MHz): δ 160.3, 133.4, 130.6, 124.2, 117.4, 116.7, 115.0, 55.6, 21.3. LR-MS: C_9H_9NOS; Calculated: 179.24; Observed: 180.1 (M^++1).

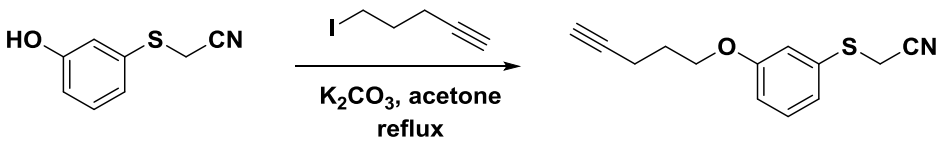
Synthesis of 2-((3-hydroxyphenyl)thio)acetonitrile



To a solution of 2-((3-methoxyphenyl)thio)acetonitrile (1.45 g, 8.1 mmol) in anhydrous DCM (40 ml) was added a solution of BBr_3 (1.0 M, 20 ml, 20 mmol) at -78 ^{o}C. Resulting reaction mixture was allowed to stir with steady increase in temperature till it reaches rt. At that stage, the reaction mixture was analyzed and found to be incomplete. So, it was allowed to stir at rt overnight. After the completion, reaction mixture was quenched with water. Organic later was separated, washed with brine, dried over anhydrous magnesium sulfate, filtered and evaporated to give the crude product. Crude was purified by column chromatography using a gradient of 20% - 30% EtOAc/Hexane to recover the pure product in 69% yield.

^{1}H-NMR (in CDCl3, 400 MHz): δ 7.24 – 7.29 (m, 1H), 7.08 – 7.12 (m, 1H), 7.04 – 7.07 (m, 1H), 6.83– 6.88 (m, 1H), 5.81 (bs, 1H), 3.60 (s, 2H); ^{13}C-NMR (in CDCl3, 100 MHz): δ 156.6, 133.4, 130.9, 124.3, 118.9, 116.9, 116.5, 21.3. LR-MS: C_8H_7NOS; Calculated: 165.21; Observed: 166.1 (M^++1).

Synthesis of 2-((3-(pent-4-yn-1-yloxy)phenyl)thio)acetonitrile



To a stirred solution of 2-((3-hydroxyphenyl)thio)acetonitrile (0.89 g, 5.4 mmol) in anhydrous acetone (50 ml) was added anhydrous potassium carbonate (1.5 g, 10.8 mmol) followed by 5-iodopentyne (1.26 g, 6.5 mmol). Resulting reaction mixture was refluxed with monitoring. The reaction was complete after 12 hr of reflux. The reaction was quenched by the addition of water and acetone was removed *in vacuo*. The residue was taken in EtOAc and was washed twice

with HCl (0.5 N), water, sodium bicarbonate solution (5% aqueous), water and brine. Organic layer was then dried over anhydrous magnesium sulfate, filtered and evaporated to give the crude product. Purification by column chromatography using 10% to 20% EtOAc:Hexane resulted in the recovery of pure product in 56% yield.

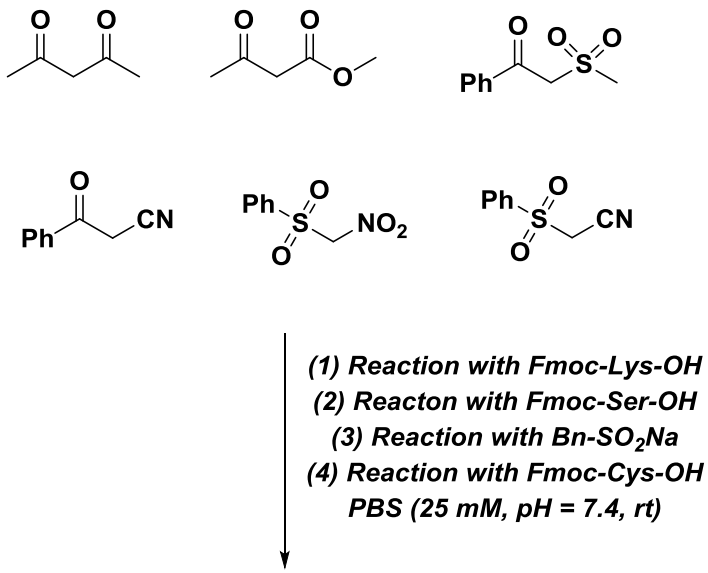
^{1}H-NMR (in CDCl3, 400 MHz): δ 7.30 (t, 1H, J = 8.0 Hz), 7.10 – 7.14 (m, 1H), 7.07 – 7.10 (m, 1H), 6.88– 6.93 (m, 1H), 4.09 (t, 2H, J = 6.1 Hz); 3.58 (s, 2H), 2.42 (dt, 2H, J^1 = 7.0 Hz, J^2 = 2.7 Hz), 1.97 – 2.06 (m, 3H); ^{13}C-NMR (in CDCl3, 100 MHz): δ 159.6, 133.4, 130.6, 124.2, 118.0, 116.7, 115.4, 83.5, 69.2, 66.4, 28.2, 21.3, 15.3. LR-MS: $C_{13}H_{13}NOS$; Calculated: 231.31; Observed: 232.1 (M^++1).

Synthesis of 2-((3-(pent-4-yn-1-yloxy)phenyl)sulfonyl)acetonitrile

To a stirred solution of 2-((3-(pent-4-yn-1-yloxy)phenyl)thio)acetonitrile (0.65 g, 2.8 mmol) in DCM (50 ml) was added *m*-CPBA (1.73 g, 7 mmol) in batches at 0 ^{o}C. Resulting reaction mixture was allowed to stir at rt with monitoring. After the completion (18 h), the reaction mixture was quenched by the addition of small amount of DMSO. Organic phase was washed with water, saturated sodium bicarbonate and brine, dried over anhydrous magnesium sulfate, filtered and evaporated to give the crude product which was then purified by silica gel column chromatography using a gradient of 10% - 20% EtOAc/Hexane to recover the pure product in 88% yield.

^{1}H-NMR (in CDCl3, 400 MHz): δ 7.59 – 7.63 (m, 1H), 7.53 – 7.58 (t, 1H, *J* = 8.0 Hz), 7.50 – 7.52 (m, 1H), 7.27 – 7.31 (m, 1H), 4.18 (t, 2H, *J* = 6.1 Hz); 4.07 (s, 2H), 2.44 (dt, 2H, J^1 = 7.0 Hz, J^2 = 2.6 Hz), 2.05 (p, 2H, *J* = 6.2 Hz), 2.01 (t, 1H, *J* = 2.7 Hz); ^{13}C-NMR (in CDCl3, 100 MHz): δ 159.9, 137.9, 131.1, 122.6, 121.1, 113.8, 110.6, 83.1, 69.5, 67.1, 45.9, 28.0, 15.3. LR-MS: $C_{13}H_{13}NO_3S$; Calculated: 263.31; Observed: 264.1 (M^++1).

Scheme S3. Cross-reactivity profiles of various linear nucleophiles with biological electrophiles/nucleophiles.



Procedure: A 100 mM solution of each nucleophile was prepared in DMSO. Solutions of Fmoc-Lys-OH (100 mM, in PBS buffer), Fmoc-Ser-OH (100 mM in DMSO), Bn-SO_2Na (100 mM in PBS buffer) and Fmoc-Cys-OH (100 mM in DMSO) were prepared. 10 μL of nucleophile solution and 10 μL of each biological nucleophile/electrophile solution was added to 980 μL of PBS buffer (25 mM, pH = 7.4, rt). Effective concentrations were as follow:

(1) Linear Nucelophile = 1 mM

(2) Biological nucleophile/electrophile = 1mM

(3) Buffer = 25 mM

Each reaction mixture was analyzed by LC-MS at various intervals.

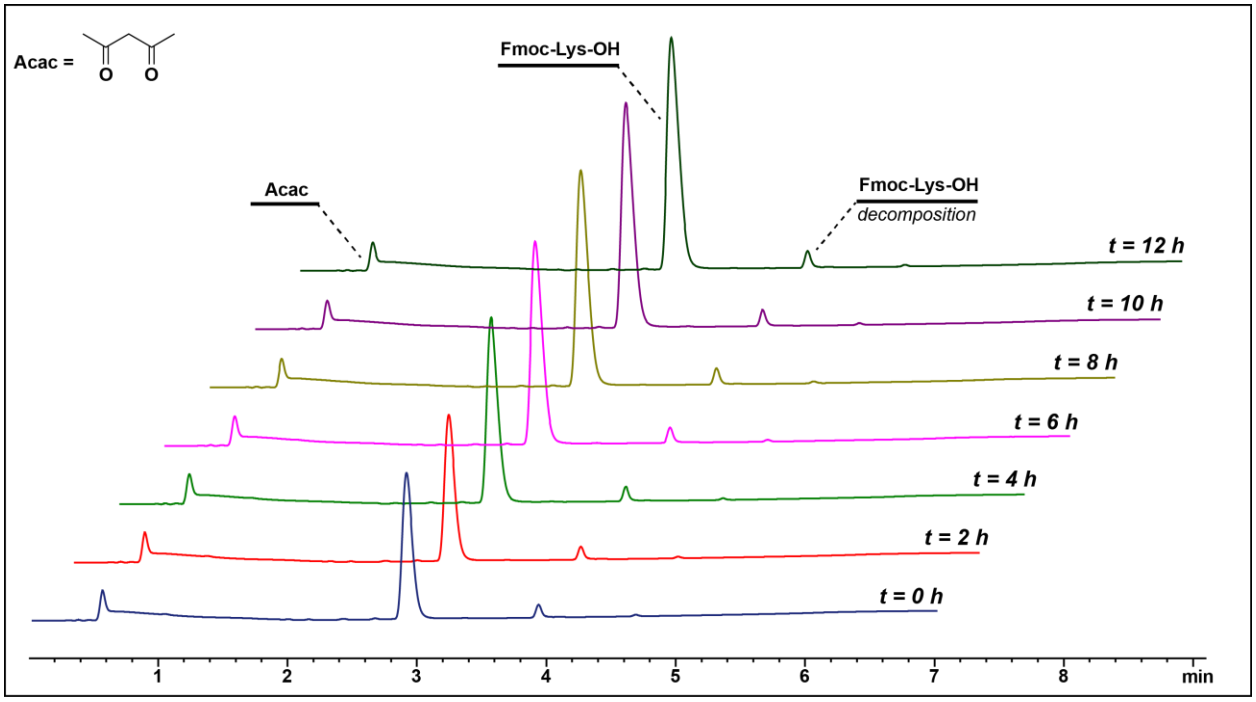


Fig. S3. Reaction of acetyl acetone **3a** (Acac, 1 mM) with Fmoc-Lys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

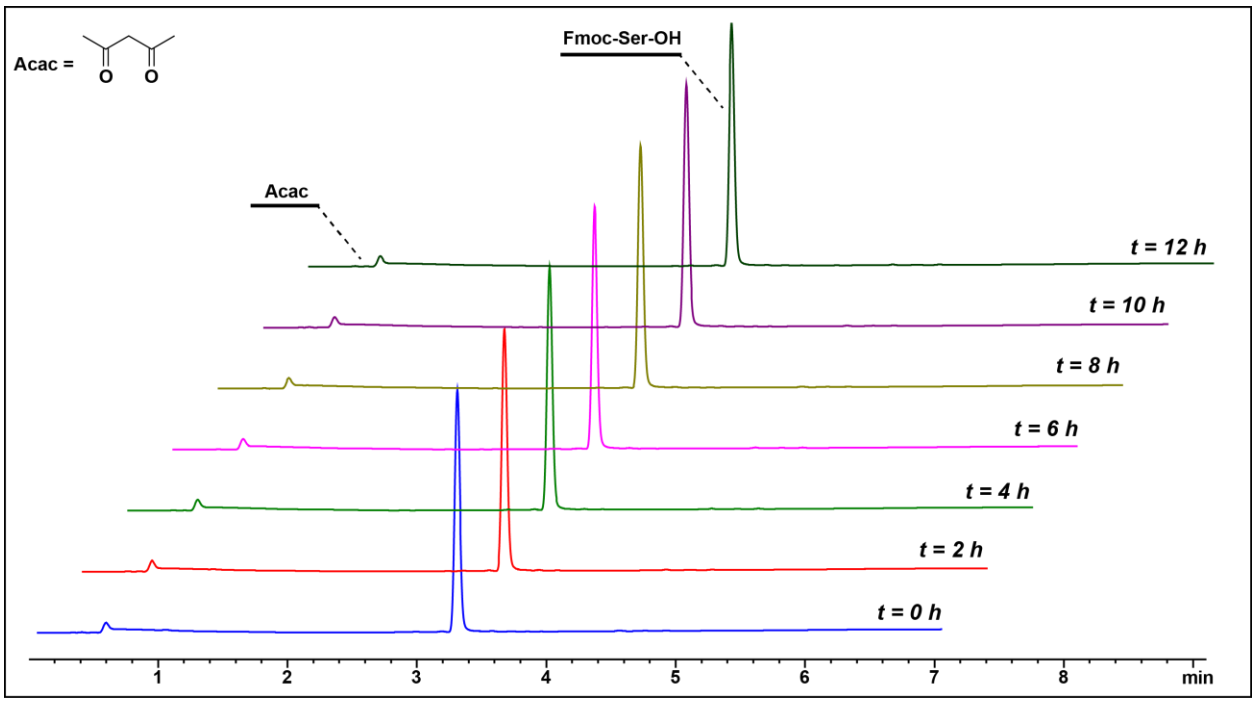


Fig. S4. Reaction of acetylacetone **3a** (Acac, 1 mM) with Fmoc-Ser-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

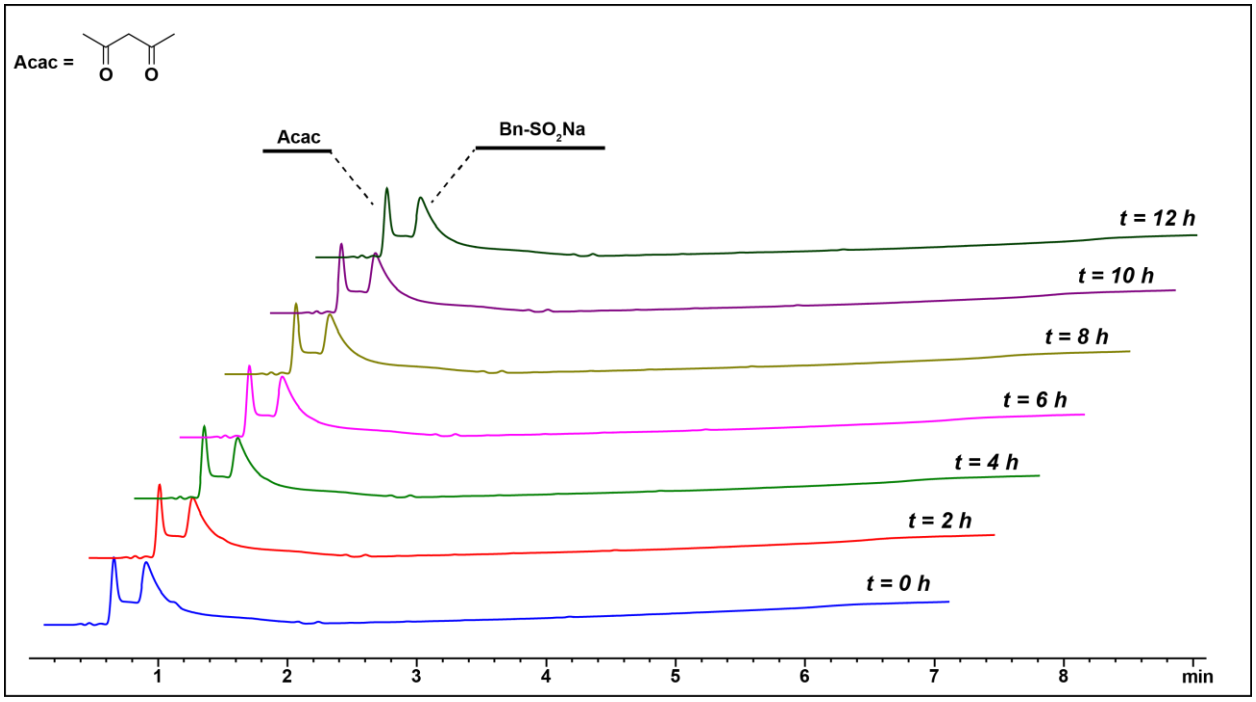


Fig. S5 Reaction of acetylacetone **3a** (Acac, 1 mM) with benzyl sulfinate sodium salt (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

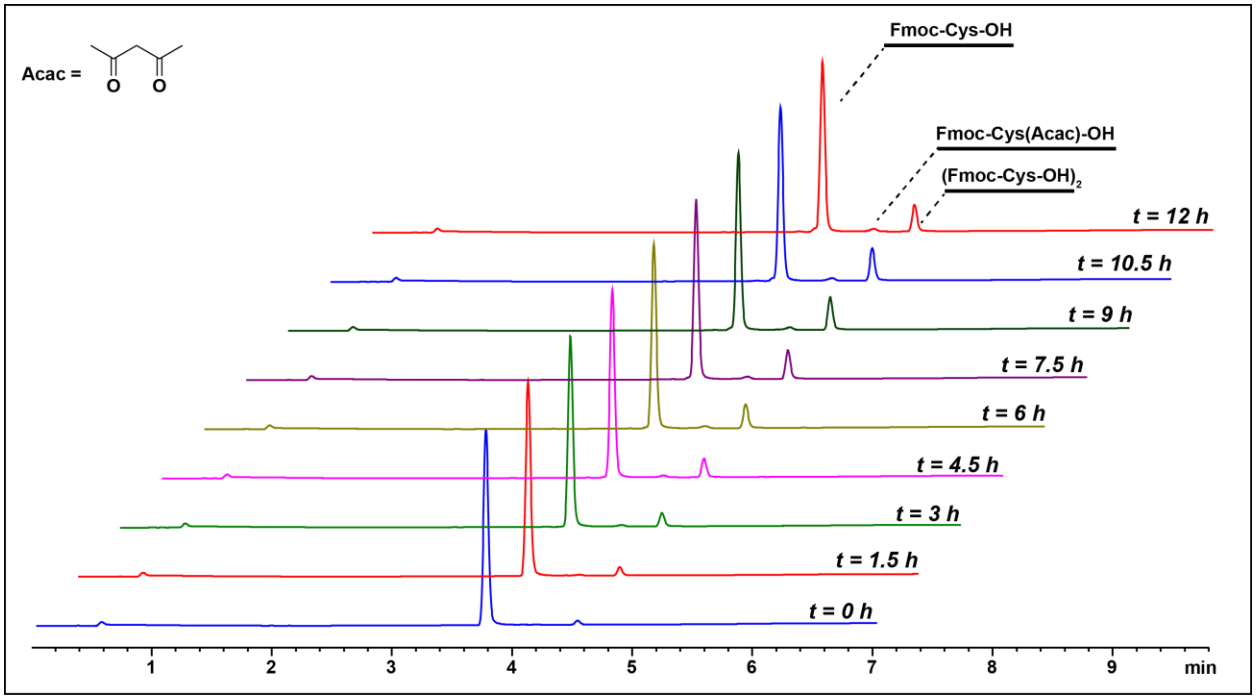


Fig. S6 Reaction of acetylacetone **3a** (Acac, 1 mM) with Fmoc-Cys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over a course of 12 h.

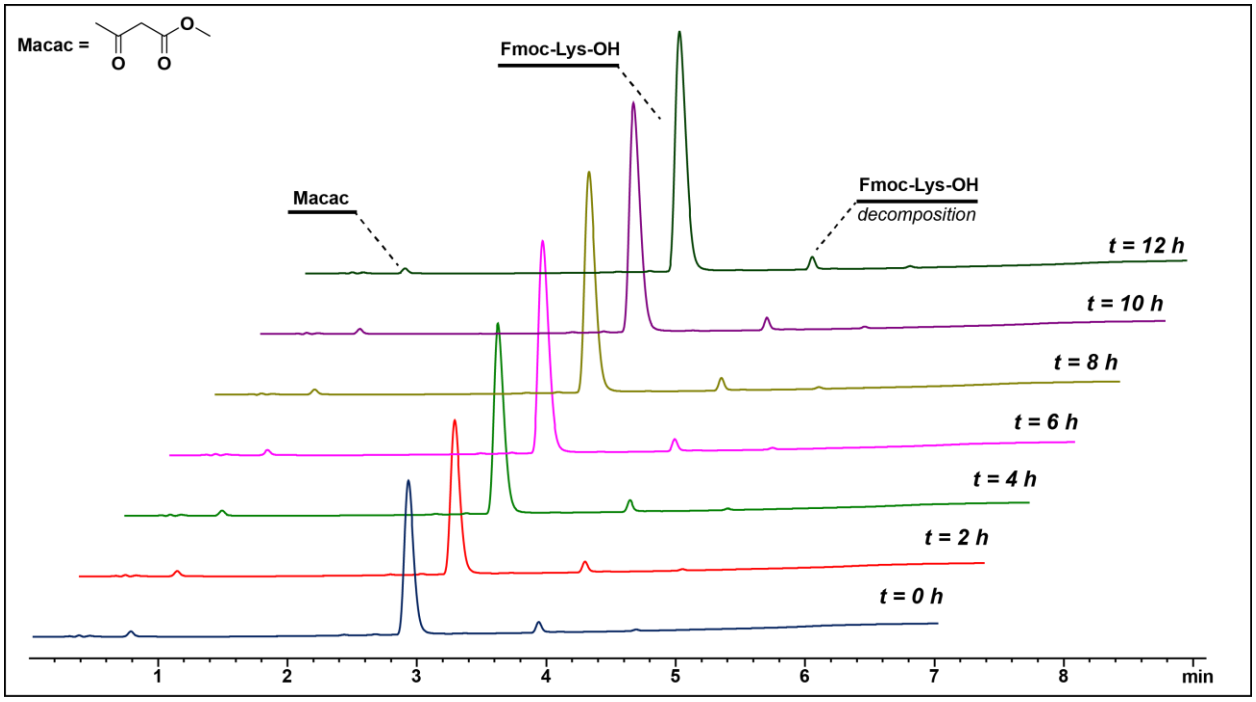


Fig. S7 Reaction of methyl acetoacetate (Macac, 1 mM) with Fmoc-Lys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

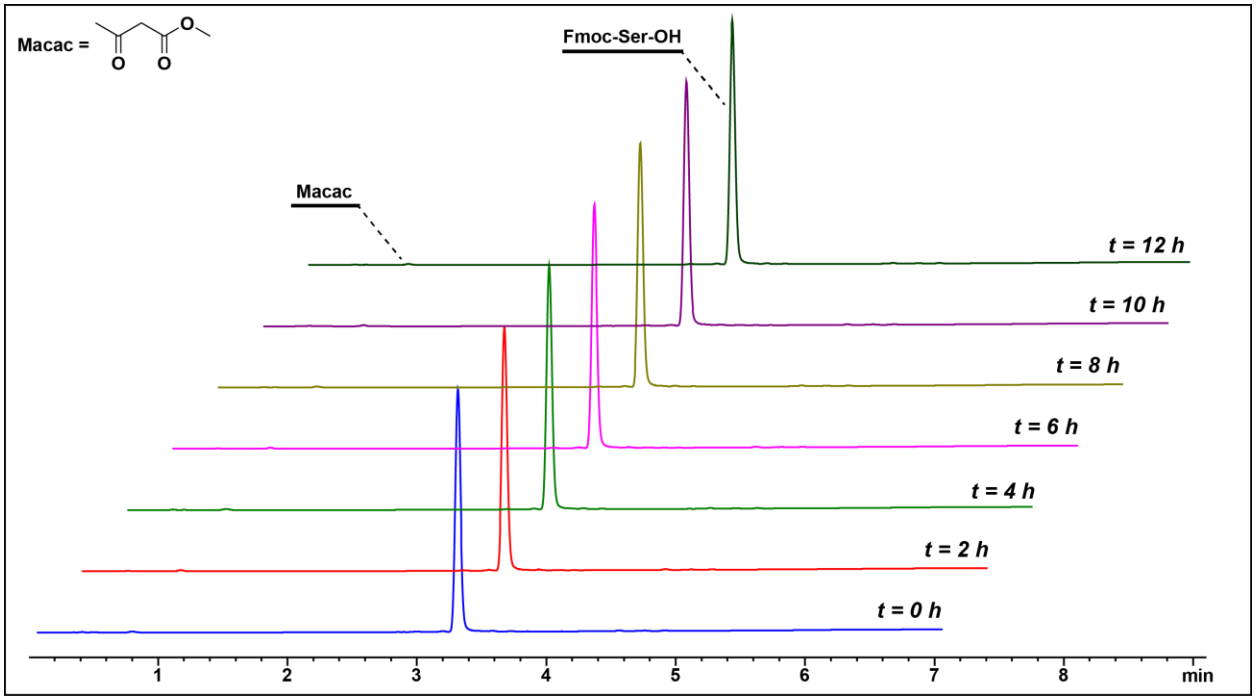


Fig. S8 Reaction of methyl acetoacetate (Macac, 1 mM) with Fmoc-Ser-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

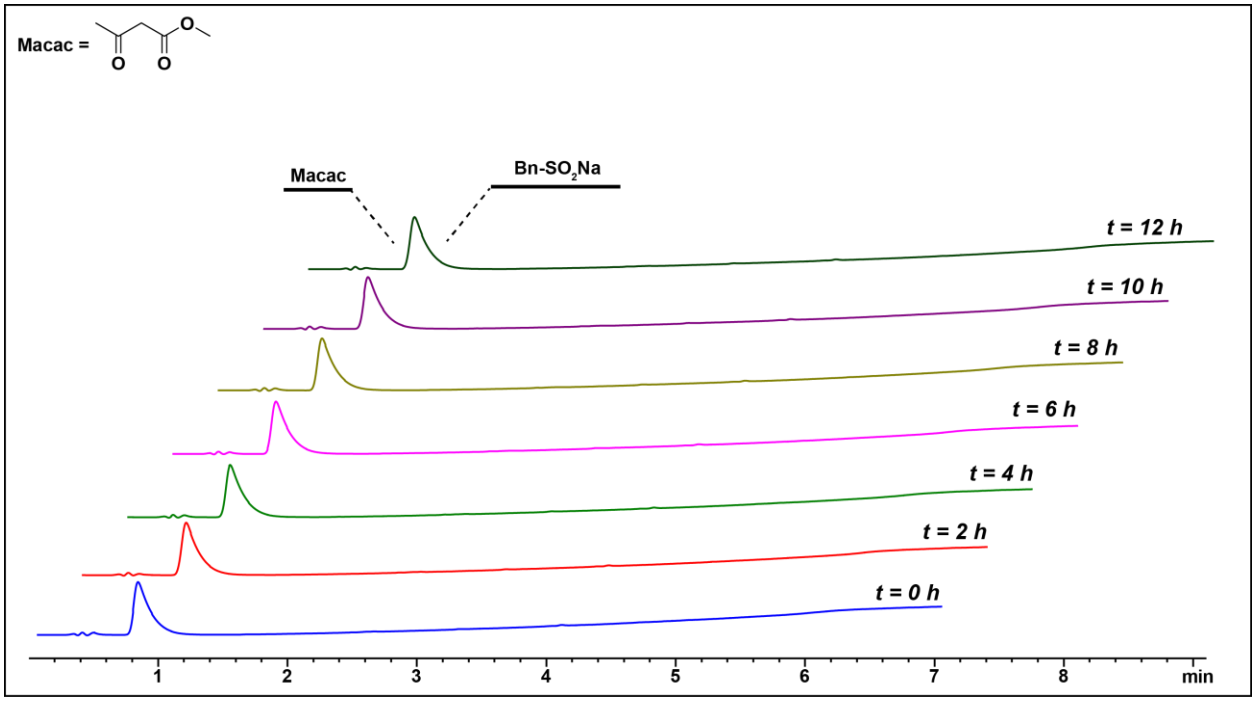


Fig. S9 Reaction of methyl acetoacetate (Macac, 1 mM) with benzyl sulfinate sodium salt (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

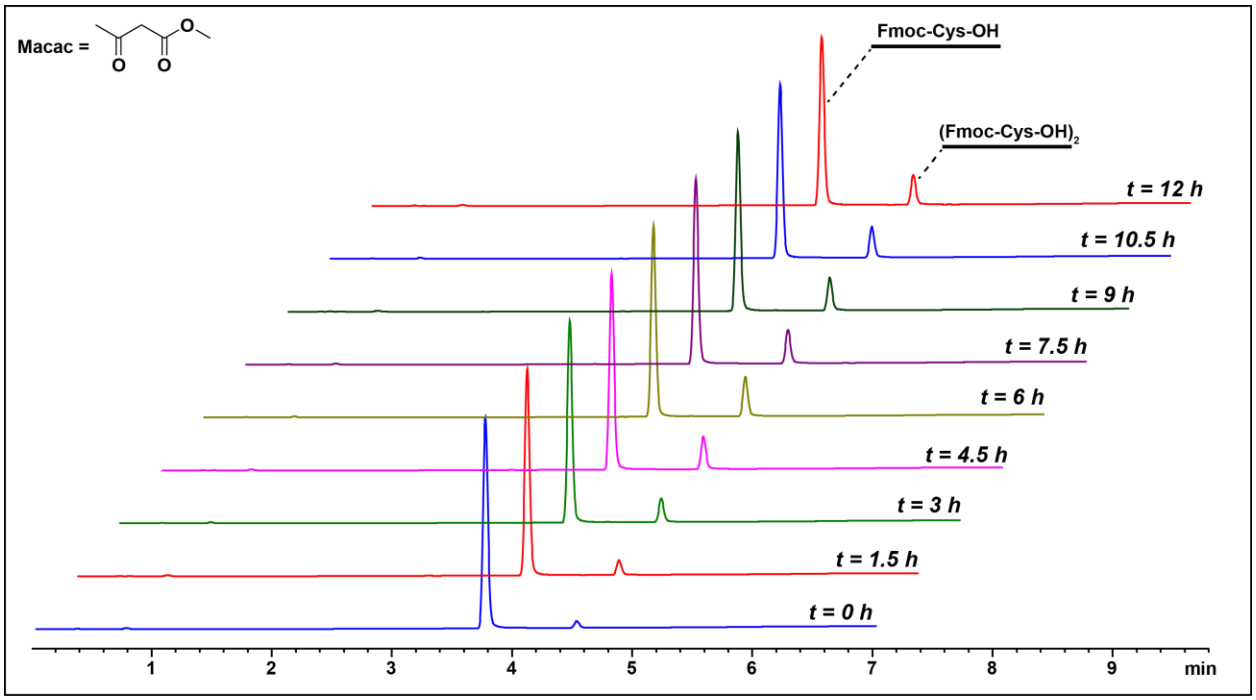


Fig. S10 Reaction of methyl acetoacetate (MAcac, 1 mM) with Fmoc-Cys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over a course of 12 h.

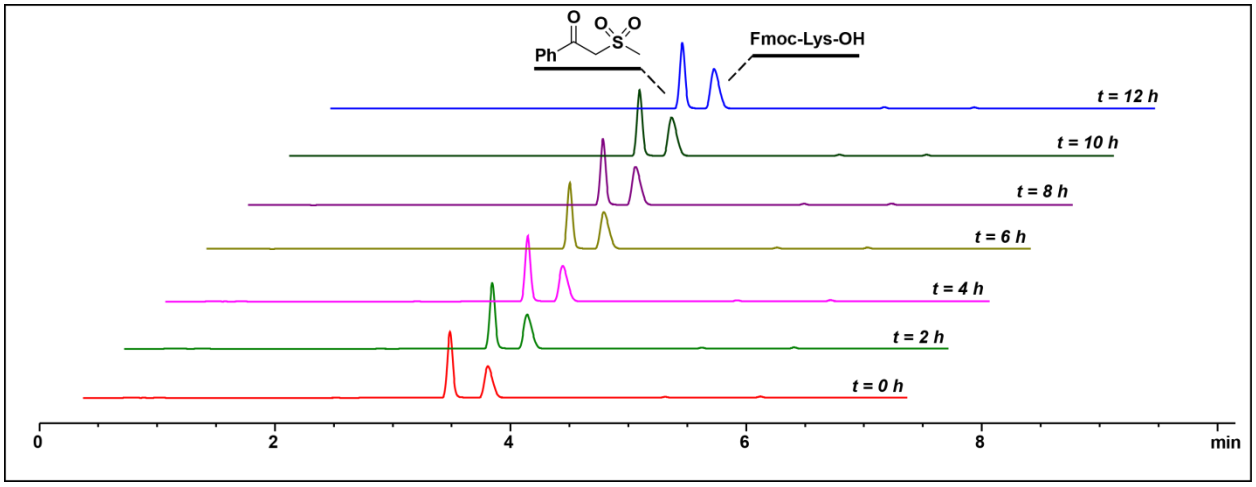


Fig. S11 Reaction of 2-(methylsulfonyl)-1-phenylethan-1-one (MSP, 1 mM) with Fmoc-Lys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

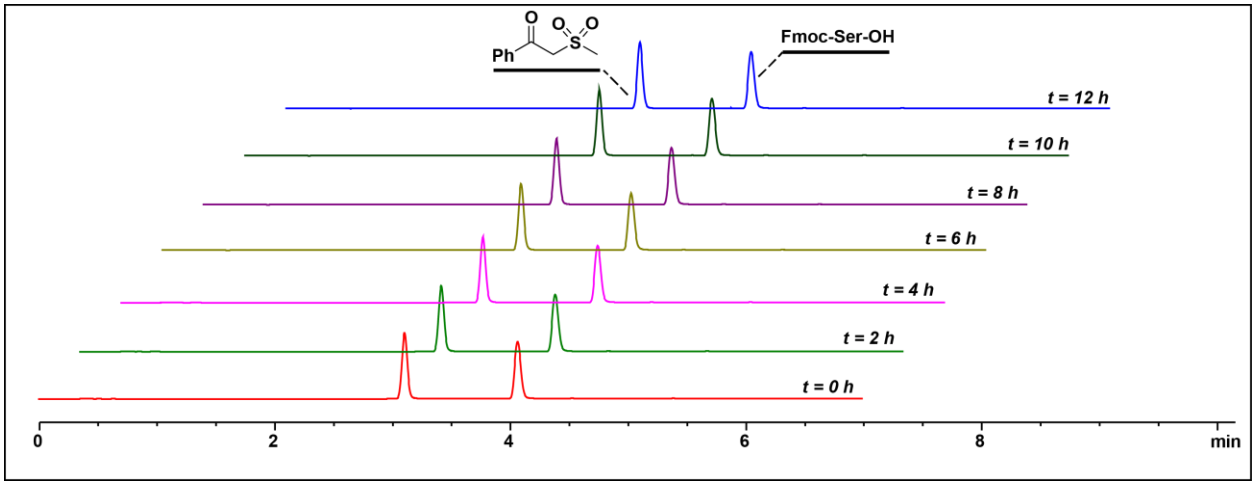


Fig. S12 Reaction of 2-(methylsulfonyl)-1-phenylethan-1-one (MSP, 1 mM) with Fmoc-Ser-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

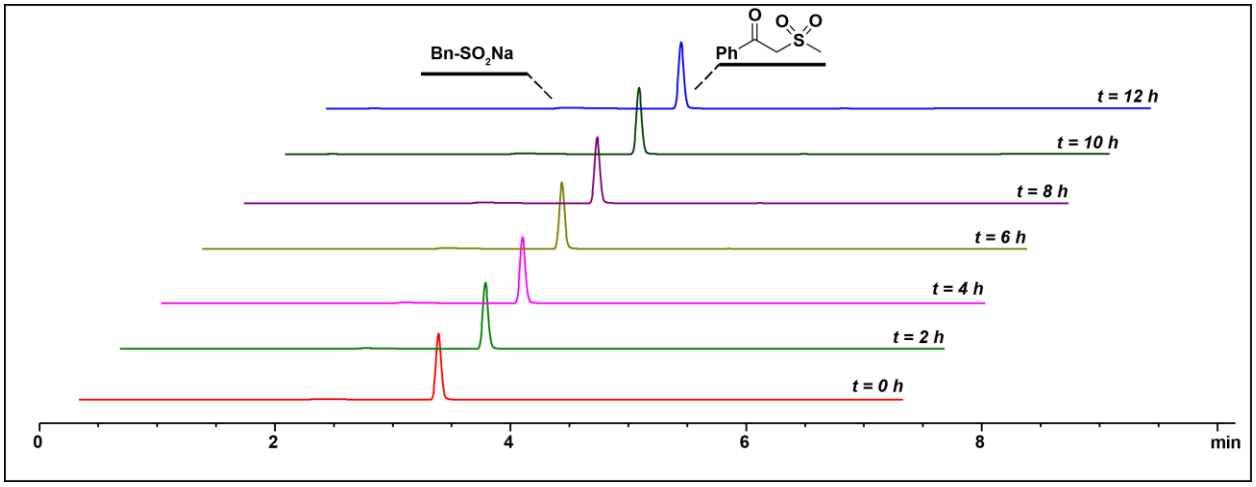


Fig. S13 Reaction of 2-(methylsulfonyl)-1-phenylethan-1-one (MSP, 1 mM) with benzyl sulfinate sodium salt (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

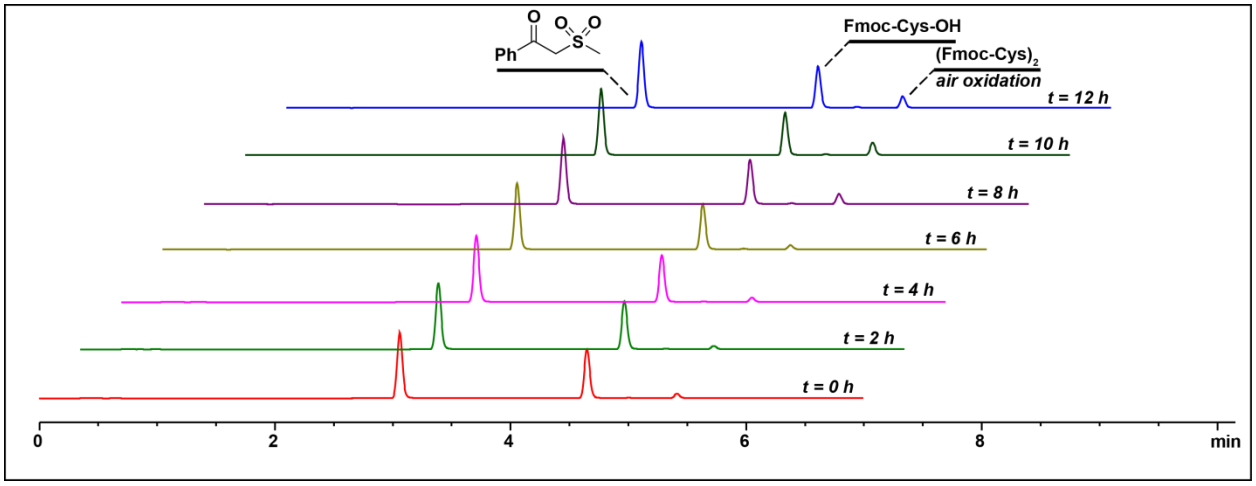


Fig. S14 Reaction of 2-(methylsulfonyl)-1-phenylethan-1-one (MSP, 1 mM) with Fmoc-Cys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over a course of 12 h.

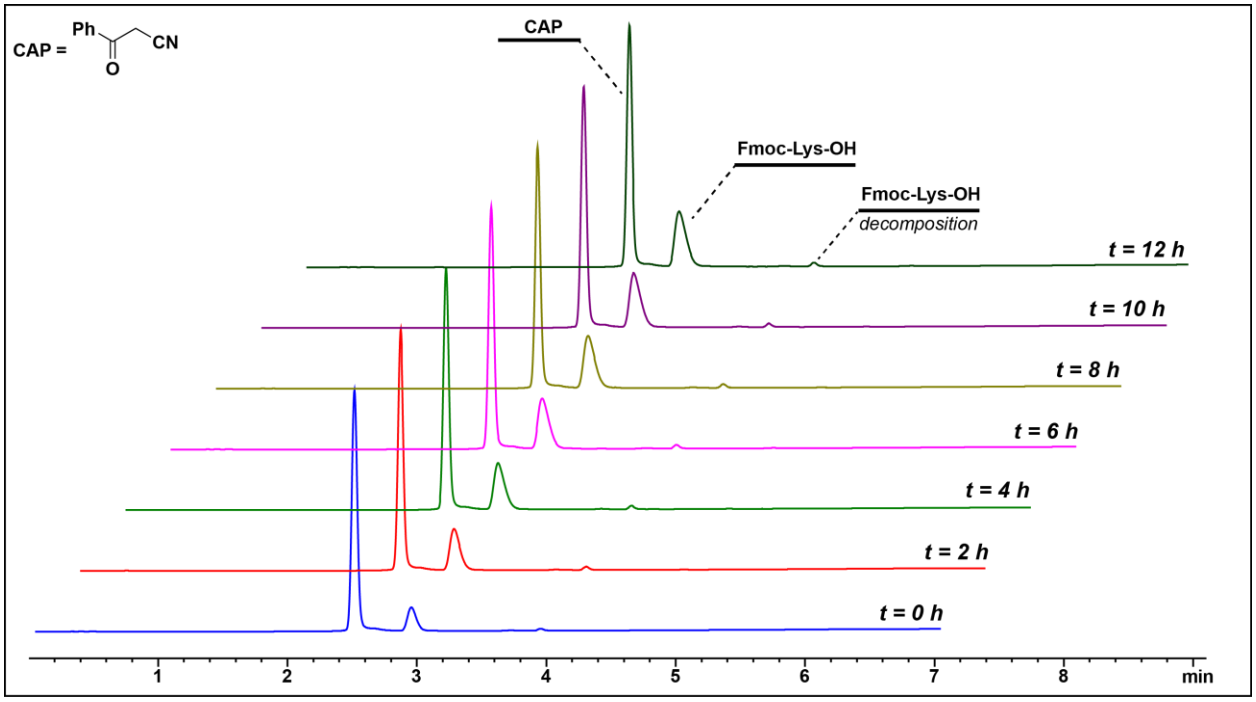


Fig. S15 Reaction of 2-cyanoacetophenone (CAP, 1 mM) with Fmoc-Lys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

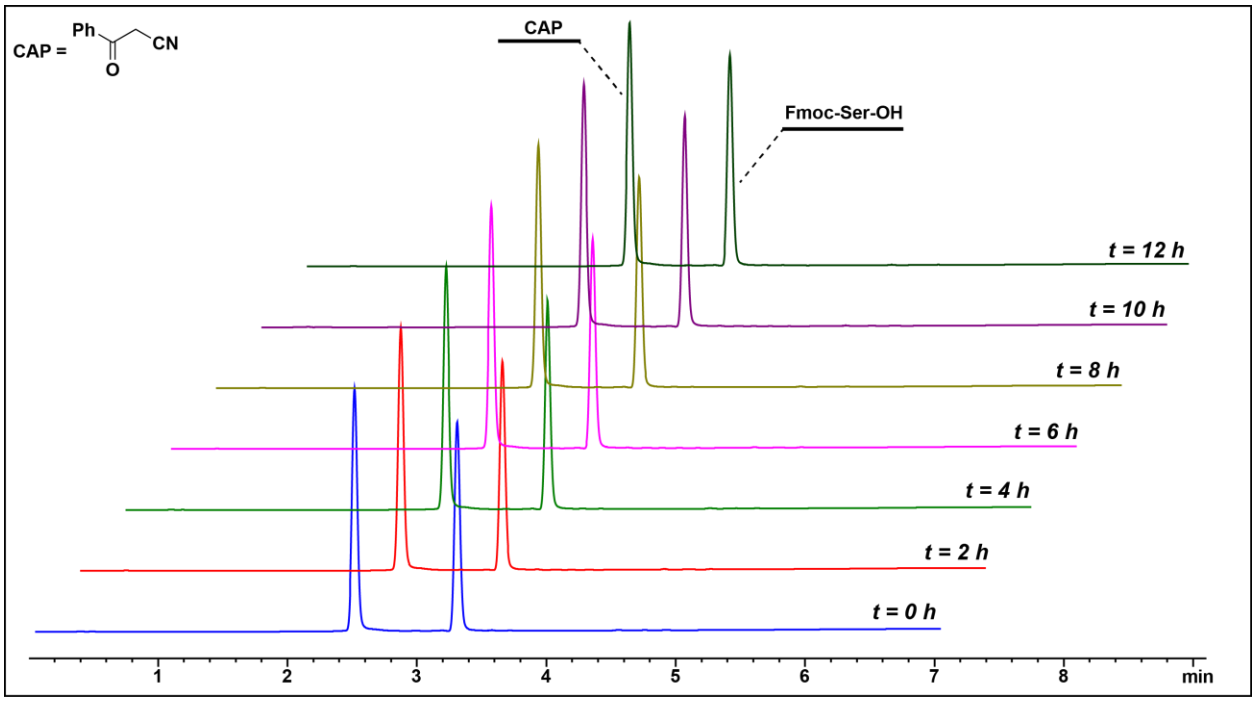


Fig. S16 Reaction of 2-cyanoacetophenone (CAP, 1 mM) with Fmoc-Ser-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

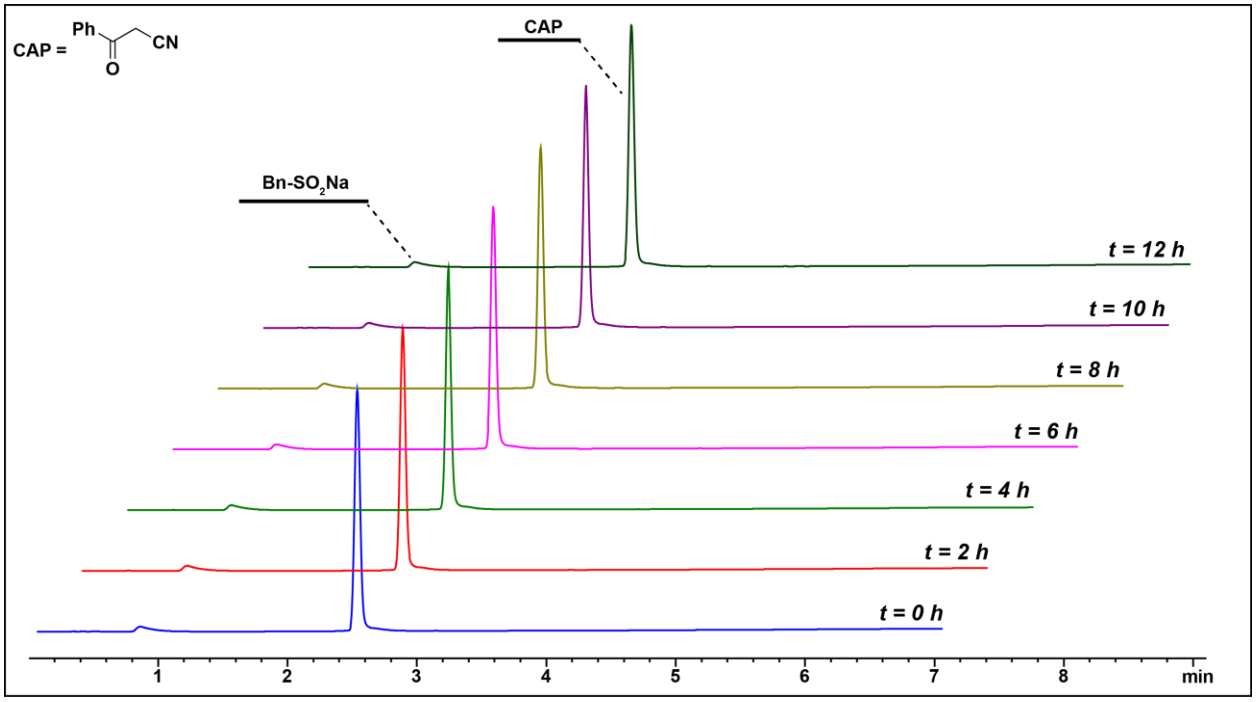


Fig. S17 Reaction of 2-cyanoacetophenone (CAP, 1 mM) with benzyl sulfinate sodium salt (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

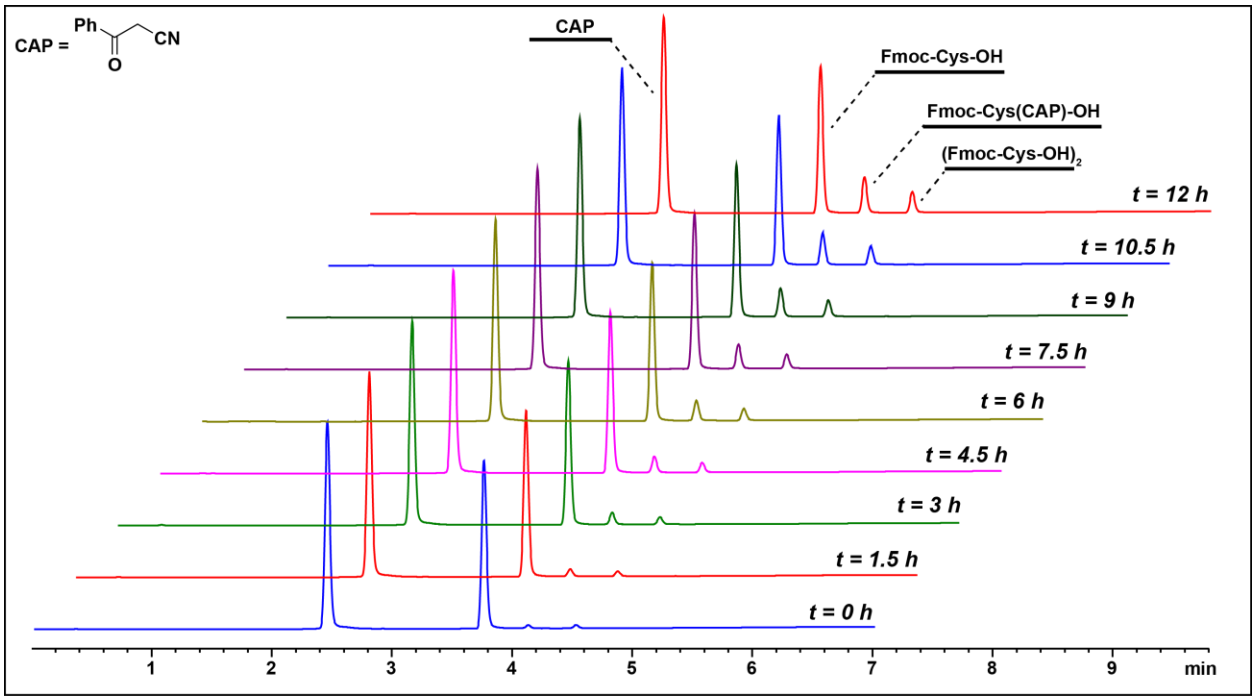


Fig. S18 Reaction of cyanoacetophenone (CAP, 1 mM) with Fmoc-Cys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over a course of 12 h.

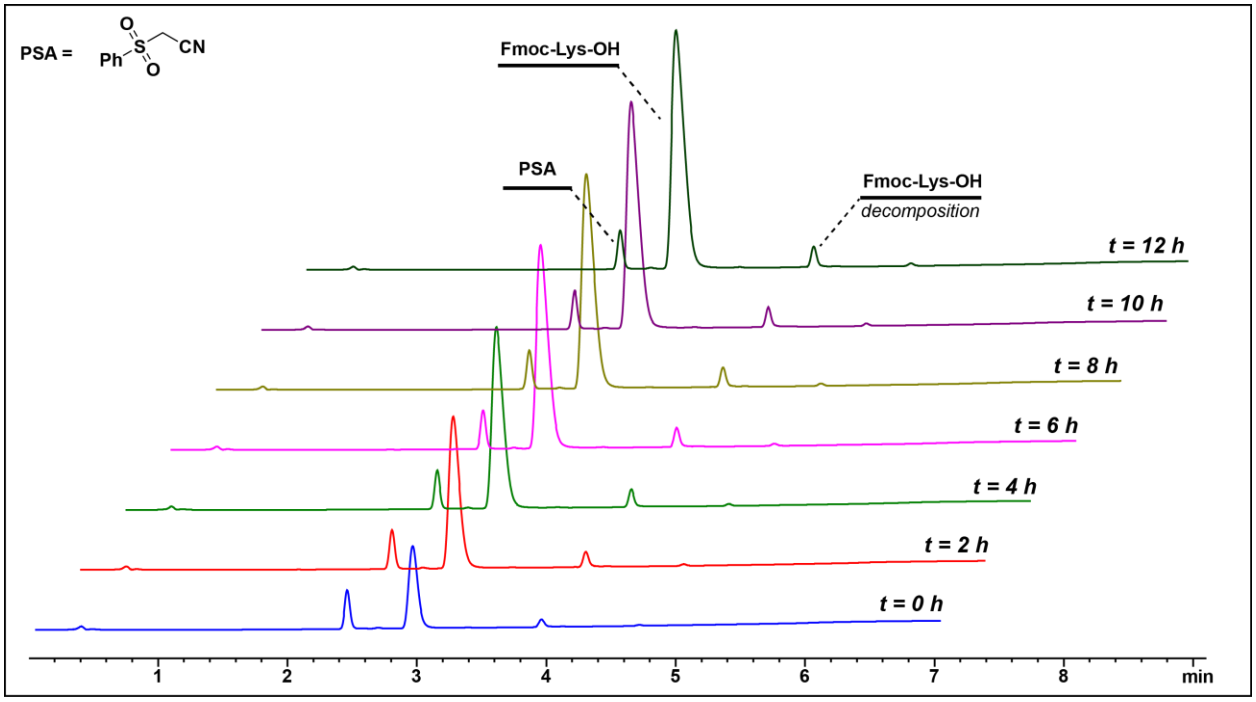


Fig. S19 Reaction of phenyl sulfonyl acetonitrile (PSA, 1 mM) with Fmoc-Lys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

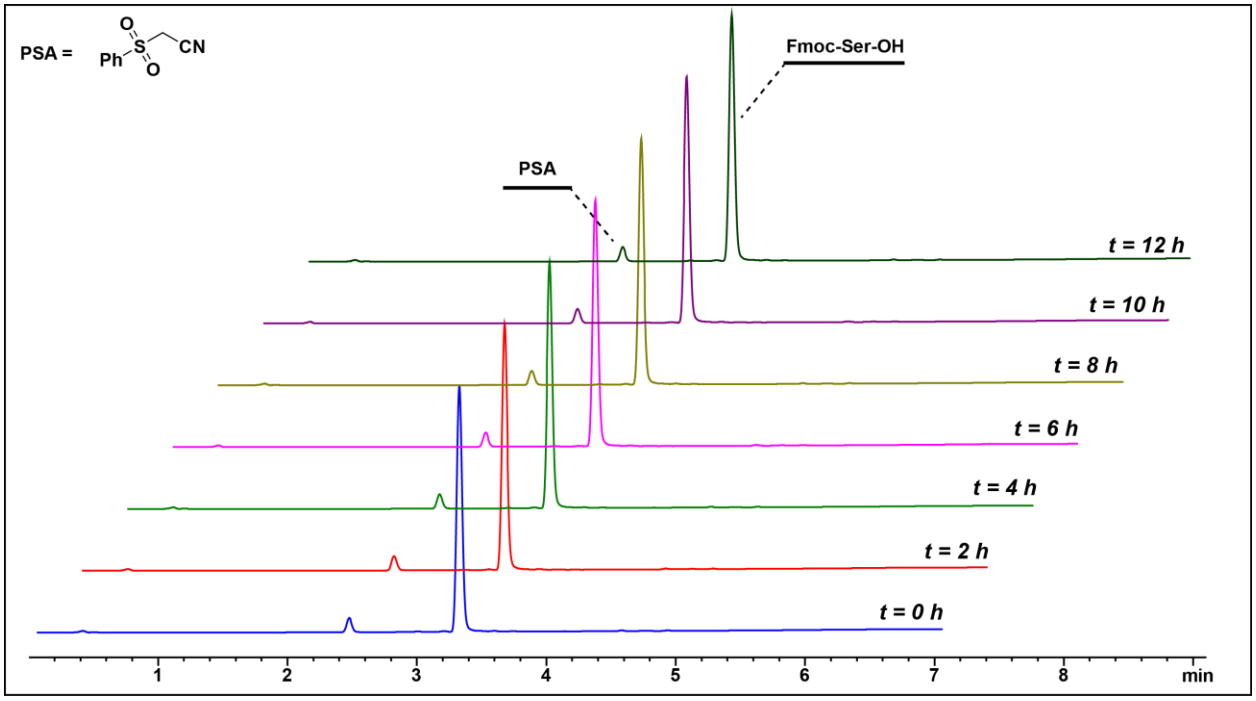


Fig. S20 Reaction of phenyl sulfonyl acetonitrile (PSA, 1 mM) with Fmoc-Ser-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

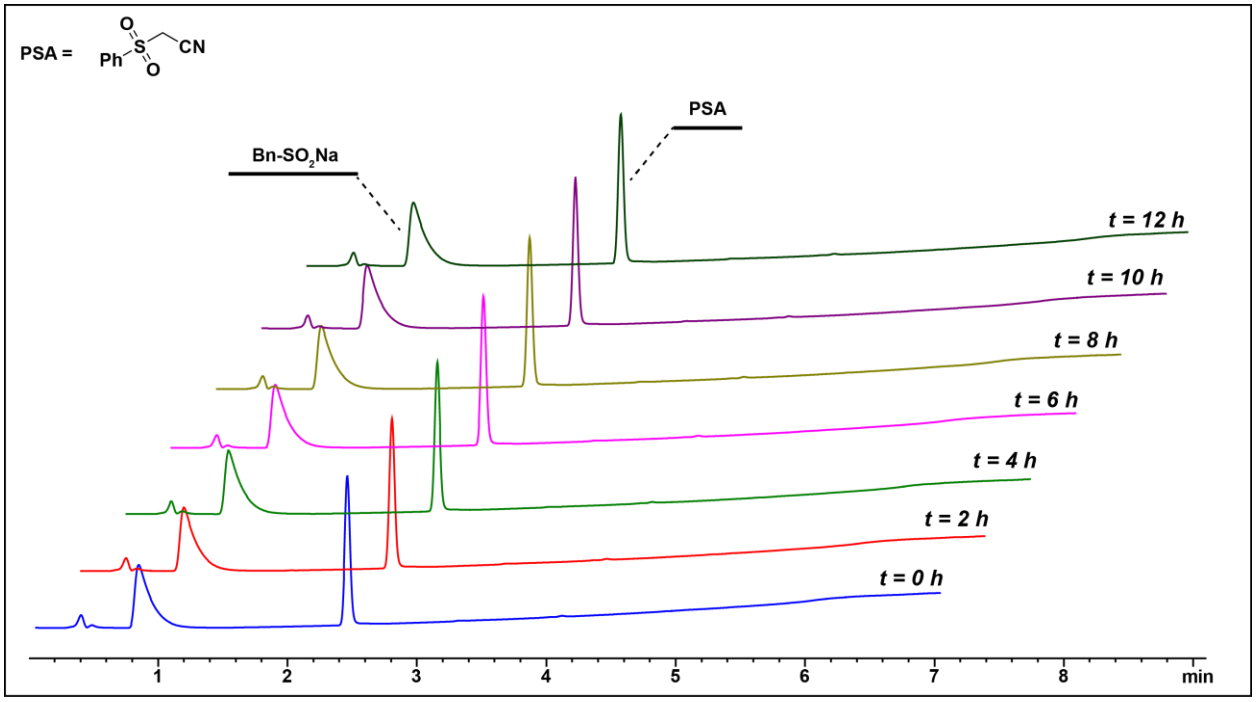


Fig. S21 Reaction of phenyl sulfonyl acetonitrile (PSA, 1 mM) with benzyl sulfinate sodium salt (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

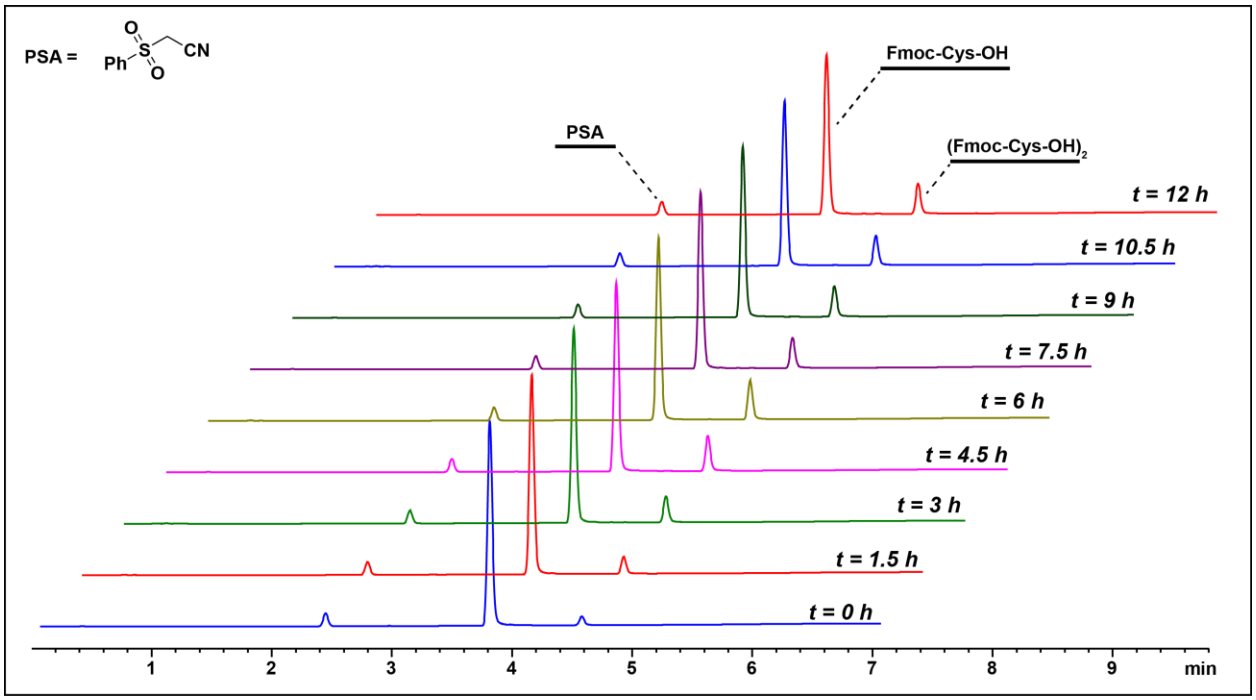


Fig. S22 Reaction of phenylsulfonylacetonitrile (PSA, 1 mM) with Fmoc-Cys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over a course of 12 h.

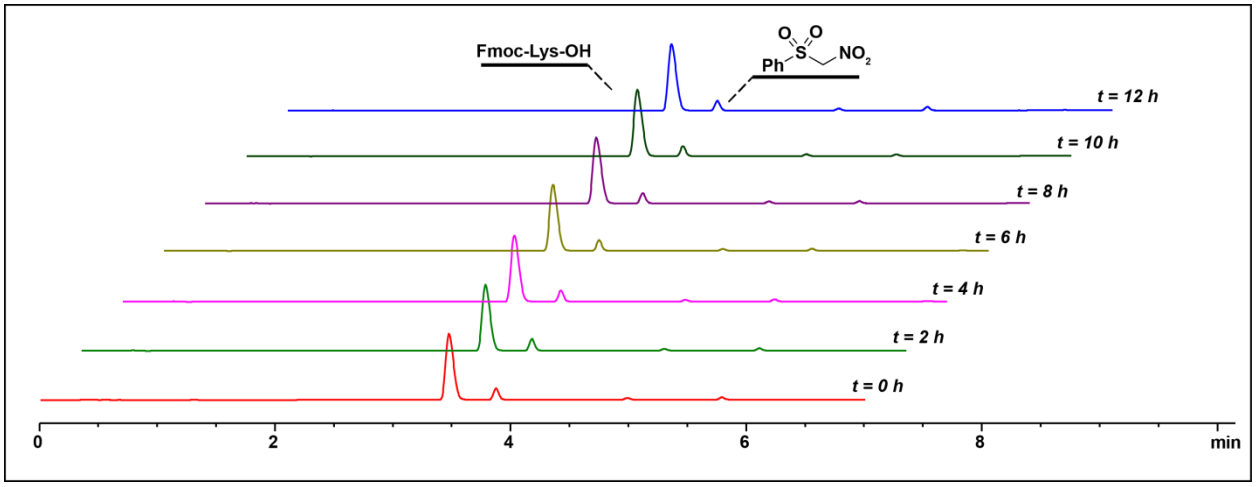


Fig. S23 Reaction of ((nitromethyl)sulfonyl)benzene (NSB, 1 mM) with Fmoc-Lys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

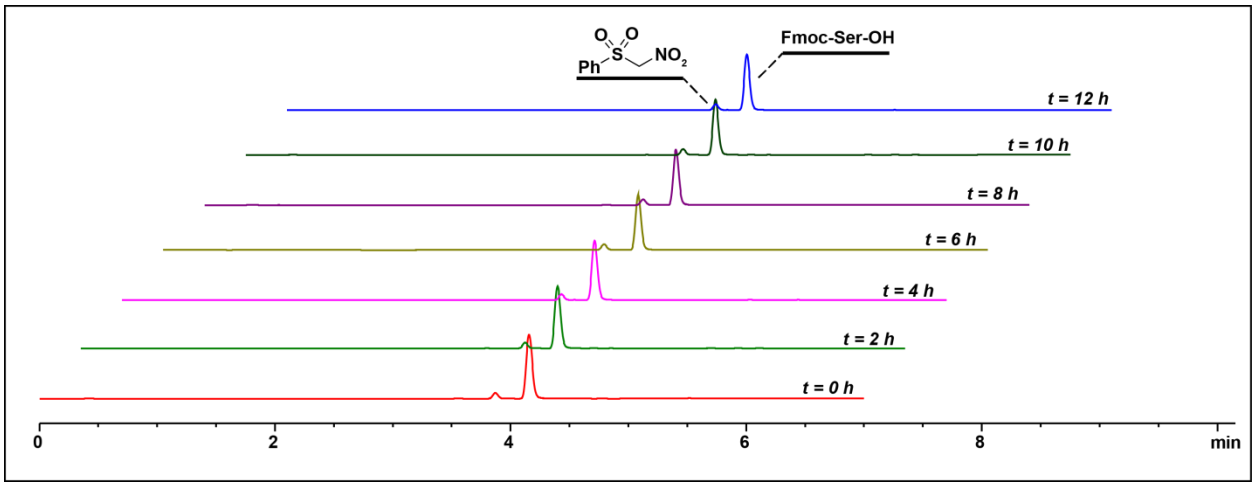


Fig. S24 Reaction of ((nitromethyl)sulfonyl)benzene (NSB, 1 mM) with Fmoc-Ser-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

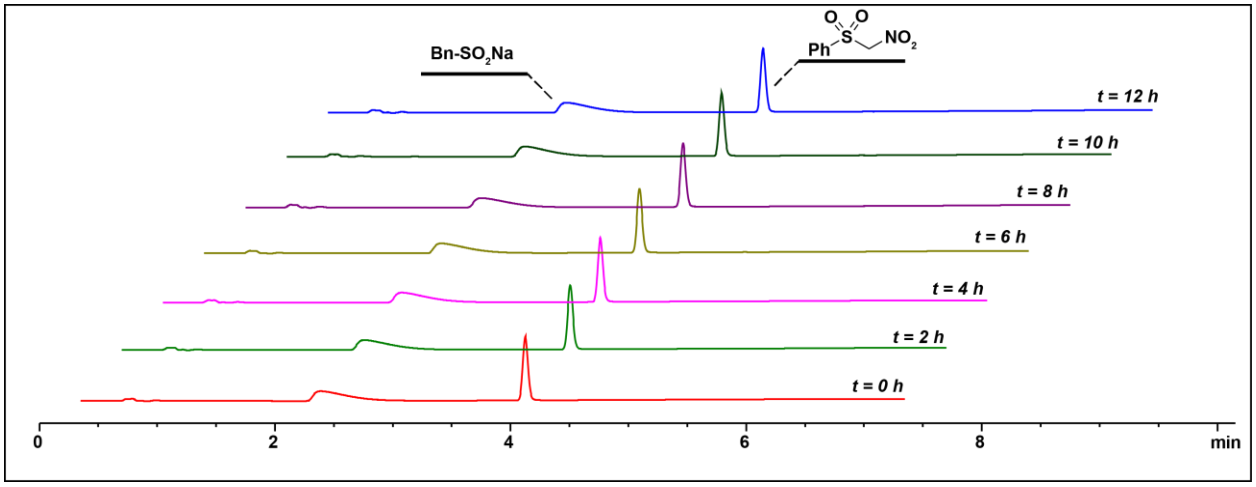


Fig. S25 Reaction of ((nitromethyl)sulfonyl)benzene (NSB, 1 mM) with benzyl sulfinate sodium salt (1 mM) in PBS (25 mM, pH = 7.4, rt) over 12 h.

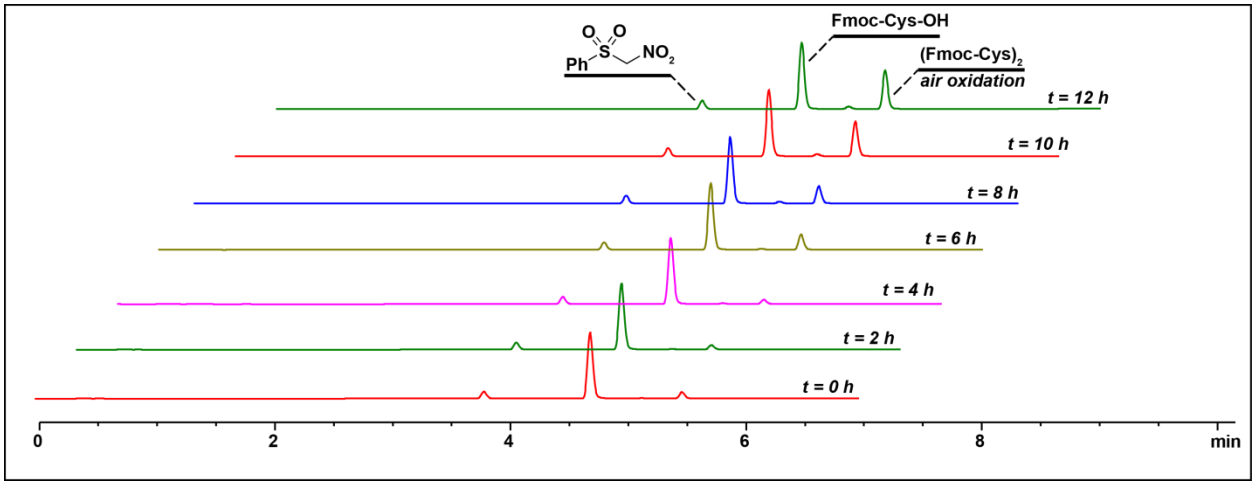
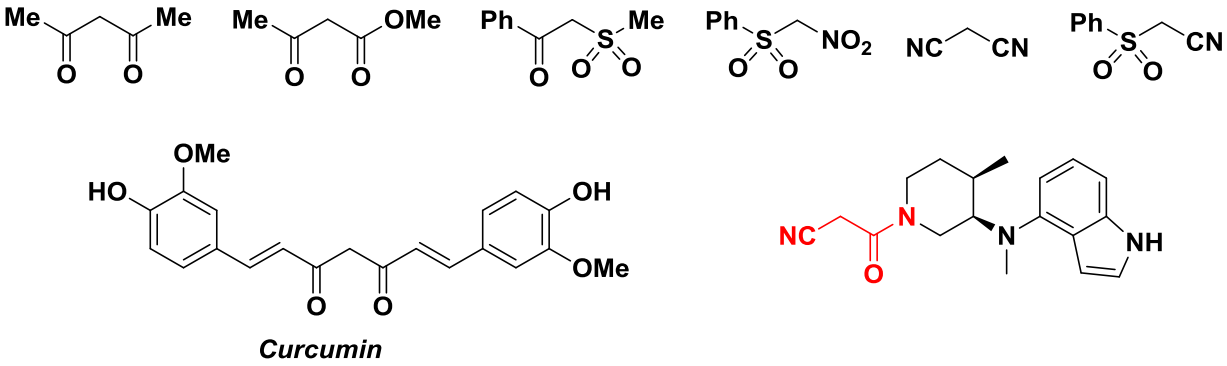


Fig. S26 Reaction of ((nitromethyl)sulfonyl)benzene (NSB, 1 mM) with Fmoc-Cys-OH (1 mM) in PBS (25 mM, pH = 7.4, rt) over a course of 12 h.

Scheme S4. Labeling of recombinant C64,82S Gpx3-SOH with various linear C-nucleophiles

Linear Nucleophiles tested:



Curcumin

[Gpx3] = 10 μM

[Nucleophile] = 1 mM

Reaction Conditions: Reducing (TCEP, 5 mM) and Oxidizing (1.5 eq H_2O_2)

Procedure:

(1) 100 mM stock solutions of nucleophiles and the electrophile were prepared in DMSO – If not soluble at room temperature, the eppendorf tubes were heated in 100 ^{o}C water bath for 2 minutes. 100 mM stock solutions were then diluted to 20 mM solution in Gpx3 labeling buffer (50 mM HEPES, 100 mM NaCl, pH = 7.4).

(2) C64, 82S Gpx3 was thawed at 0 ^{o}C and reduced with 50 mM (2M, 5 µL) DTT for 20 minutes on ice.

A 2 M solution of DTT in water was prepared (31 mg of DTT in 100 µl of water). 5 µl of this DTT solution was added to the protein solution (200 µl) giving an effective DTT concentration of 50 mM.

(3) Buffer exchange C64, 82S Gpx3 to labeling buffer (50 mM HEPES, 100 mM NaCl, pH = 7.4) using pre-equilibrated Nap-5 column. Nap-5 column was equilibrated by passing 10 ml of Gpx3 labeling buffer.

(4) Determine the conc. of C64, 82S Gpx3 by using A_{280} (E = 24410 $M^{-1}cm^{-1}$).

(5) Label 10 µM of C64, 82S Gpx3 with 1.5 eq of H_2O_2 and 1 mM nucleophile keeping DMSO $\leq$ 5%. Incubate for 1 h [REACTION VOLUME = 100 µl].

For each nucleophile, two reactions were performed:

(i) Protein incubated with TCEP + Nucleophile/electrophile (1 mM)

(ii) Protein + Nucleophile/electrophile (1 mM) + H_2O_2 (1.5 eq)

Order of addition was as follow:

(a) Appropriate amounts of Gpx3 labeling buffer were added to the eppy tubes.

(b) C64, 82S Gpx3 was added to the tube and the contents were mixed gently.

(c) For each nucleophile two tubes were prepared. In one, 5 mM TCEP was added to keep the Gpx3 in reduced form.

(c) Subsequently, nucleophile solution was added to each of the tubes. For each nucleophile, 5 µL of a 20 mM solution was used. This gave 1 mM effective probe concentration for each reaction and kept the DMSO concentration at < 5%.

(d) Hydrogen peroxide solution was added last. A 1 mM solution of hydrogen peroxide was prepared in water by serial dilution starting with commercially available 8.8 M solution. 1.5 µl of this 1 mM peroxide solution was used in each case to give an effective concentration of 15 µM.

(e) Reaction mixtures were incubated for 1 h at rt.

(6) The reaction mixtures were quenched by the filtration through pre-equilibrated P30 columns.

(7) During the filtration through P30 columns, each sample was buffer exchanged to 25 mM ammonium bicarbonate (pH 8.0).

(8) Each sample was then analyzed by LTQ-MS by injecting 3 µM concentration.

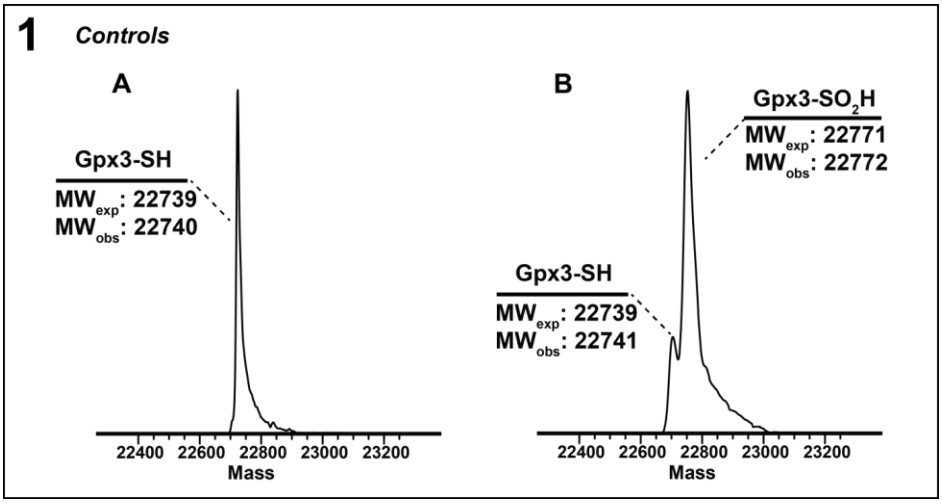


Fig. S27 C64, 82S Gpx3 controls. (A) C64, 82S Gpx3 (10 μM) under reducing conditions (TCEP, 5 mM); (B) C64, 82S Gpx3 (10 μM) under oxidizing conditions (H_2O_2, 15 μM).

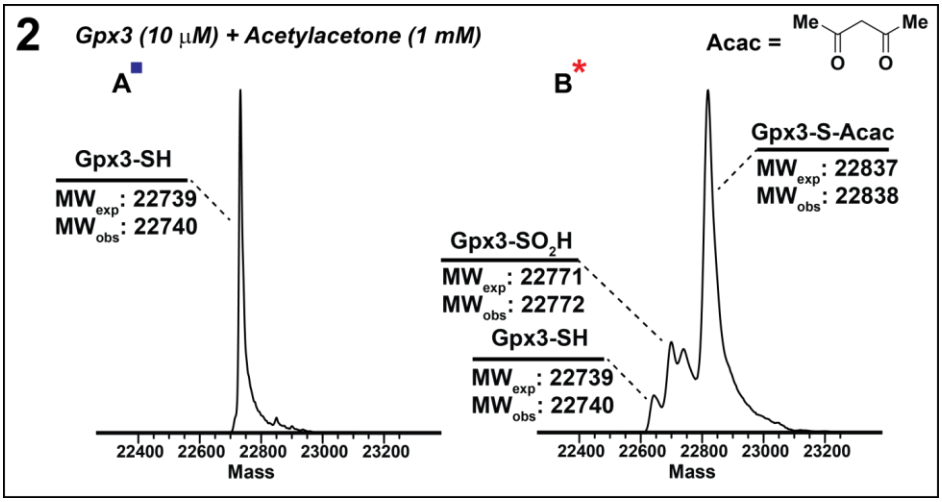


Fig. S28 C64, 82S Gpx3 labeling with acetylacetone (1 mM). (A) C64, 82S Gpx3 (10 μM) under reducing conditions (TCEP, 5 mM); (B) C64, 82S Gpx3 (10 μM) under oxidizing conditions (H_2O_2, 15 μM).

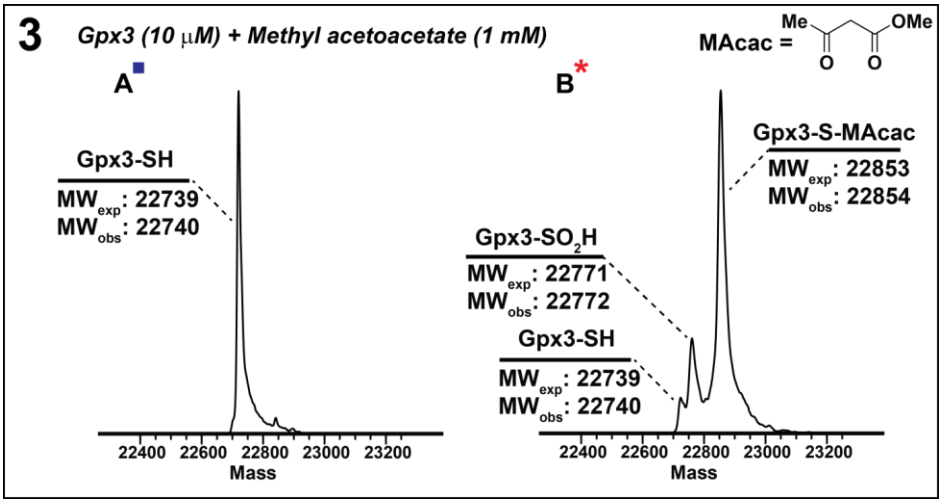


Fig. S29 C64, 82S Gpx3 labeling with methyl acetoacetate (1 mM). (A) C64, 82S Gpx3 (10 μM) under reducing conditions (TCEP, 5 mM); (B) C64, 82S Gpx3 (10 μM) under oxidizing conditions (H_2O_2, 15 μM).

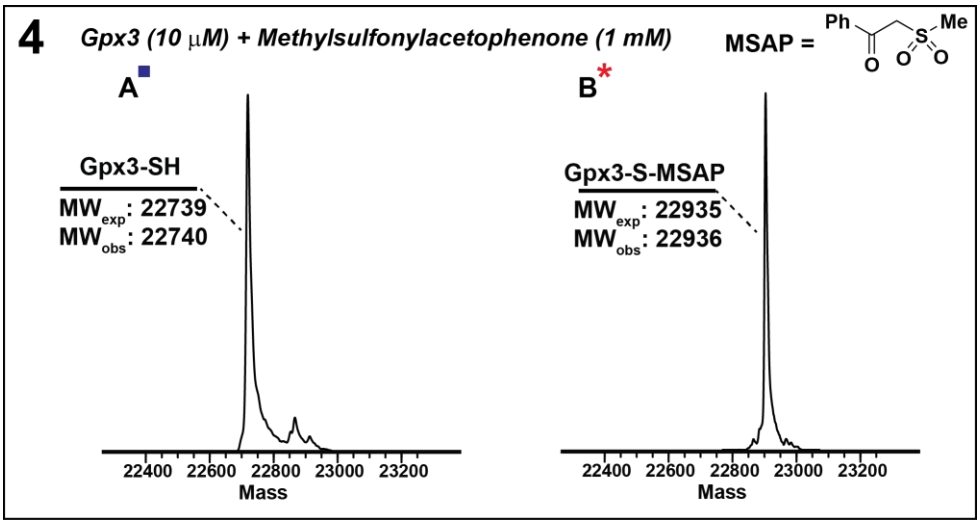


Fig. S30 C64, 82S Gpx3 labeling with methylsulfonylacetophenone (1 mM). (A) C64, 82S Gpx3 (10 μM) under reducing conditions (TCEP, 5 mM); (B) C64, 82S Gpx3 (10 μM) under oxidizing conditions (H_2O_2, 15 μM).

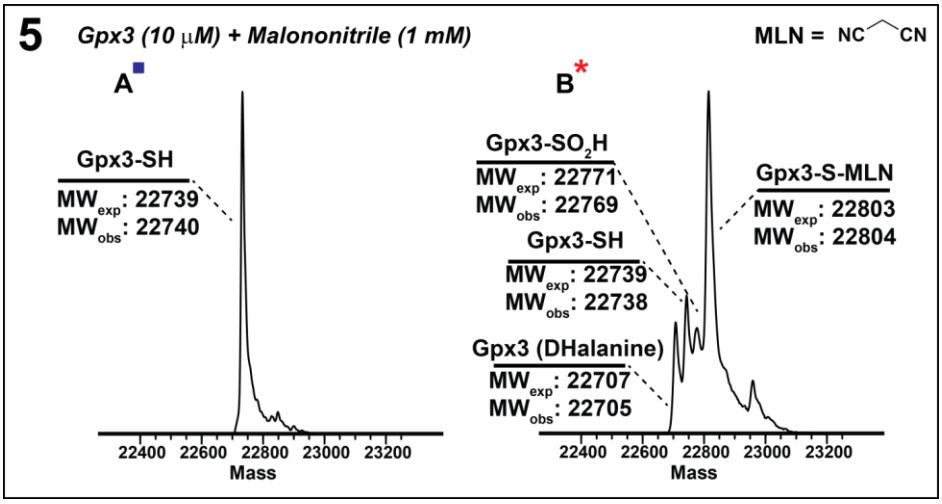


Fig. S31 C64, 82S Gpx3 labeling with malonitrile (1 mM). (A) C64, 82S Gpx3 (10 μM) under reducing conditions (TCEP, 5 mM); (B) C64, 82S Gpx3 (10 μM) under oxidizing conditions (H_2O_2, 15 μM).

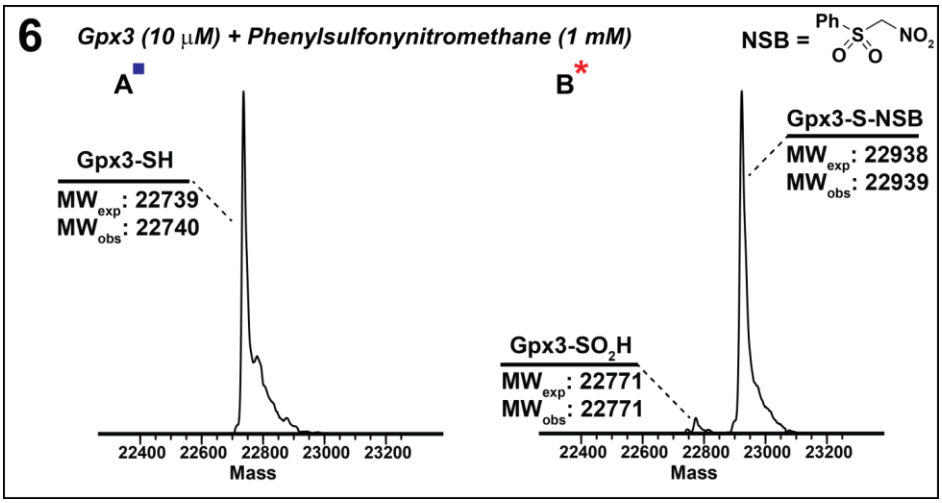


Fig. S32 C64, 82S Gpx3 labeling with phenylsulfonylnitromethane (1 mM). (A) C64, 82S Gpx3 (10 μM) under reducing conditions (TCEP, 5 mM); (B) C64, 82S Gpx3 (10 μM) under oxidizing conditions (H_2O_2, 15 μM).

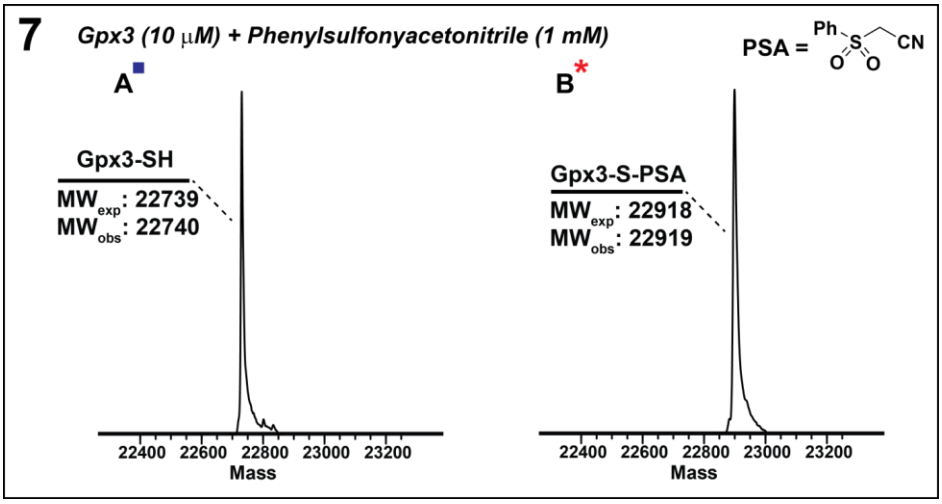


Fig. S33 C64, 82S Gpx3 labeling with phenylsulfonylacetonitrile (1 mM). (A) C64, 82S Gpx3 (10 μM) under reducing conditions (TCEP, 5 mM); (B) C64, 82S Gpx3 (10 μM) under oxidizing conditions (H_2O_2, 15 μM).

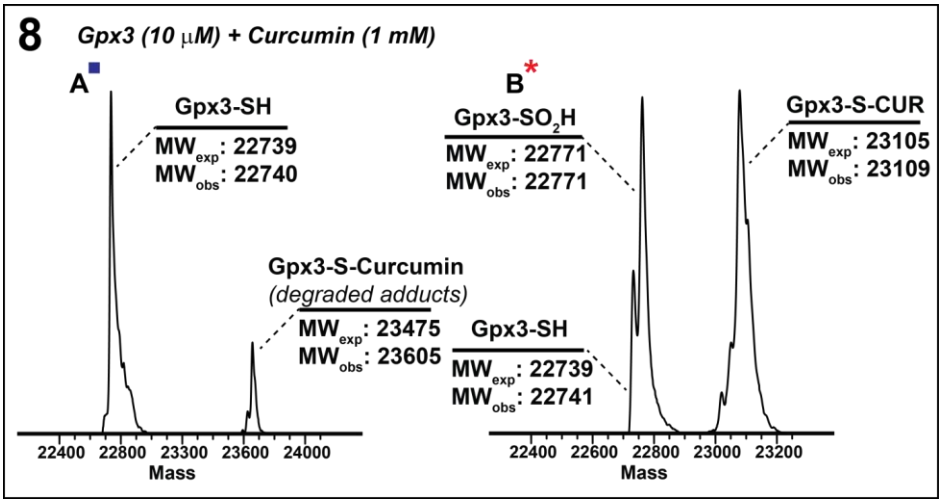


Fig. S34 C64, 82S Gpx3 labeling with curcumin (1 mM). (A) C64, 82S Gpx3 (10 μM) under reducing conditions (TCEP, 5 mM); (B) C64, 82S Gpx3 (10 μM) under oxidizing conditions (H_2O_2, 15 μM).

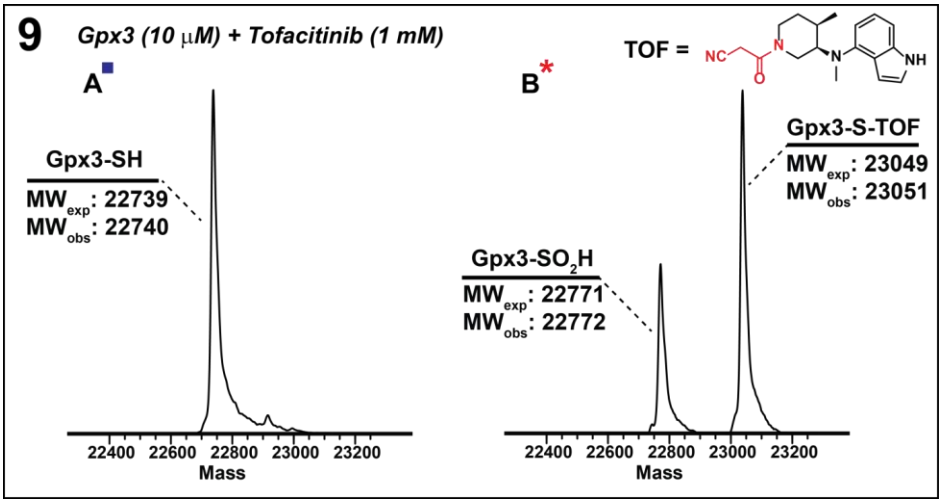
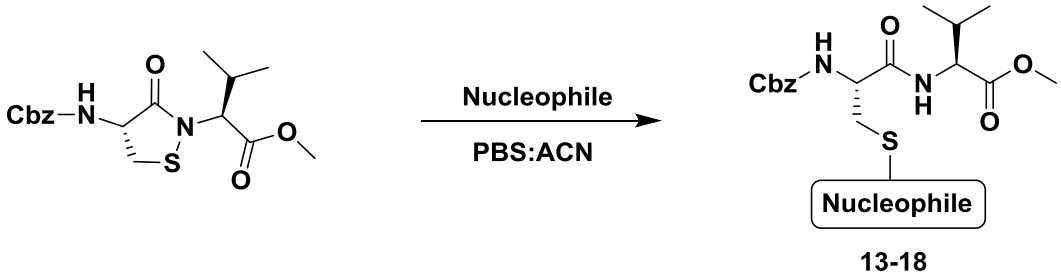


Fig. S35 C64, 82S Gpx3 labeling with tofacitinib (1 mM). (A) C64, 82S Gpx3 (10 μM) under reducing conditions (TCEP, 5 mM); (B) C64, 82S Gpx3 (10 μM) under oxidizing conditions (H_2O_2, 15 μM).

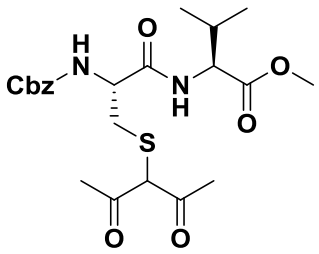
Scheme S5. Synthesis of dipeptide sulfenic acid adduct with various nucleophiles



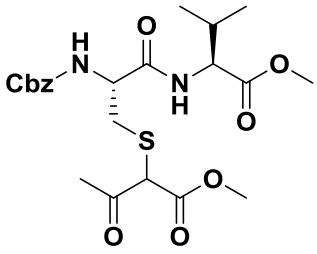
13-18

General Procedure: To a solution of dipeptide cyclic sulfenamide (36.6 mg, 0.1 mmol) in acetonitrile (2 ml) was added a solution of acetyl acetone (100 mg, 1 mmol) in PBS (4 ml, 10 mM, pH 7.4). Resulting reaction mixture was stirred at rt till completion. After completion, the reaction mixture was directly purified by prep-HPLC.

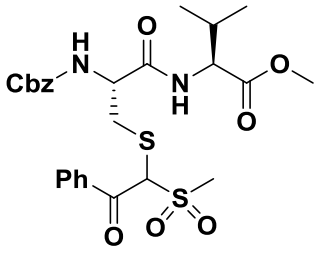
Adducts prepared:



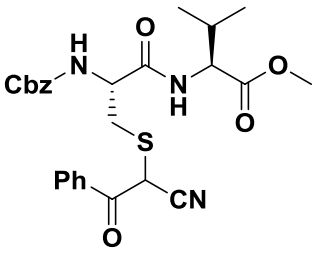
13



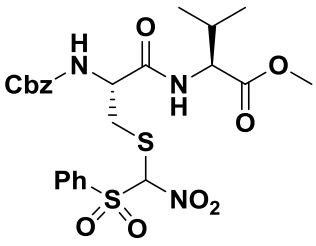
14



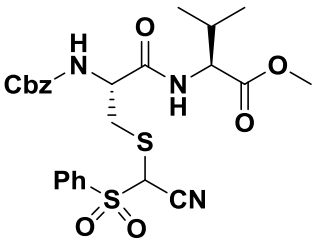
15



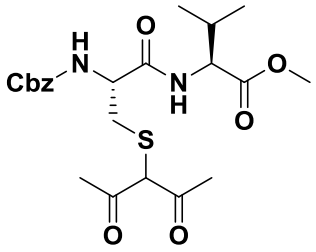
16



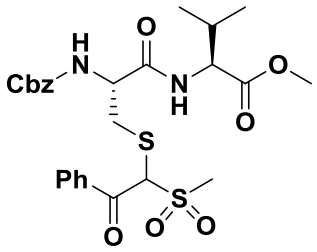
17



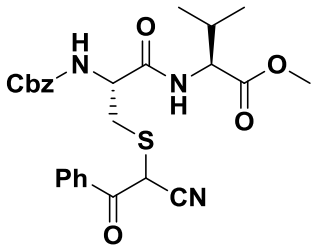
18



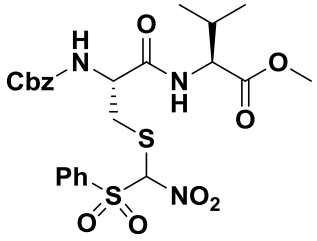
Yield: 78%. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.32 – 7.38 (m, 5H), 6.79 (d, 1H, J = 7.3 Hz), 5.64 (d, 1H, J = 7.6), 5.13 (s, 2H), 4.50 (dd, 1H, J_1 = 8.8 Hz, J_2 = 4.9 Hz), 4.33 (m, 1H), 3.74 (s, 3H), 2.94 – 3.02 (m, 1H), 2.82 – 2.92 (m, 1H), 2.38 (s, 6H), 2.15 (sep, 1H, J = 6.8), 0.89 (d, 3H, J = 6.9 Hz), 0.86 (d, 3H, J = 6.9 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 197.8, 172.2, 170.2, 156.4, 136.2, 128.9, 128.7, 128.5, 103.7, 67.7, 57.6, 54.4, 52.6, 38.6, 31.6, 24.7, 19.2, 18.0; LRMS: MF - $C_{22}H_{30}N_2O_7S$, MW_{exp} = 466.55, MW_{obs} = 467.2 (M^++1).



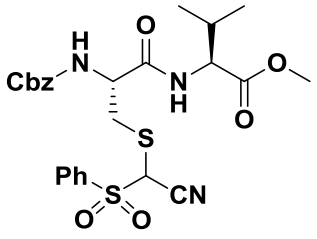
Yield: 82%.^{1}H-NMR (400 MHz, $CDCl_3$) δ 8.13 (d, 1H, J = 7.7 Hz), 8.05 (d, J = 7.6 Hz), 7.61 – 7.69 (m, 1H), 7.47 – 7.55 (m, 2H), 7.28 – 7.39 (m, 5H), 5.87 – 6.05 (m, 2H), 5.08 – 5.20 (m, 2H), 4.61 – 4.78 (m, 1H), 4.53 (dd, 0.5H, J1 = 8.8 Hz, J2 = 4.7 Hz), 4.44 (dd, 0.5H, J1 = 8.7 Hz, J2 = 4.9 Hz), 3.72 (s, 1H), 3.71 (s, 2H), 3.55 (dd, 0.5H, J1 = 14.7 Hz, J2 = 6.0 Hz), 3.07 – 3.27 (m, 3H), 2.08 – 2.28 (m, 1H), 0.96 (d, 1.5H, J = 6.9 Hz), 0.93 (d, 1.5H, J = 6.9Hz), 0.83 (t, 3H, J = 7.3 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 190.8, 190.7, 172.1, 172.1, 170.2, 169.7, 156.5, 156.3, 136.4, 136.2, 135.1, 135.1, 134.9, 129.8, 129.8, 129.4, 129.3, 128.9, 128.6, 128.6, 128.4, 128.3, 69.9, 68.3, 67.7, 67.5, 57.8, 57.8, 55.4, 53.8, 52.6, 52.5, 38.0, 37.0, 36.8, 36.6, 31.2, 31.0, 19.3, 19.2, 17.9, 17.8; LRMS: MF - $C_{26}H_{32}N_2O_8S2$, MW_{exp} = 564.67, MW_{obs} = 565.2 (M^++1).



Yield: 75%.^{1}H-NMR (400 MHz, DMSO-d6) *(mix of keto/enol)* δ 11.93 (bs, 0.7H), 8.20 – 8.40 (m, 1H), 7.69 (d, 1H, J = 8.0 Hz), 6.64 (d, 1H, J = 8.0 Hz), 7.40 – 7.57 (m, 3 H), 7.27 – 7.40 (m, 5H), 4.95 – 5.15 (m, 2H), 4.32 – 4.45 (m, 1H), 4.12 – 4.25 (m, 1H), 3.62 (s, 3H), 3.37 (bs, 1H), 3.09 (dd, 1H, J1 = 13.1 Hz, J2 = 4.3 Hz), 2.87 – 3.01 (m, 1H), 1.98 – 2.15 (m, 1.5H), 0.88 (d, 3H, J = 6.3 Hz), 0.85 (d, 3H, J = 9.6 Hz); ^{13}C-NMR (100 MHz, DMSO-d6) δ 171.7, 170.6, 170.4, 155.8, 136.8, 130.9, 128.3, 128.3, 127.8, 127.7, 65.6, 57.5, 54.1, 51.7, 36.3, 29.9, 18.9, 18.1; LRMS: MF - $C_{26}H_{29}N_3O_6S$, MW_{exp} = 511.59, MW_{obs} = 512.2 (M^++1).

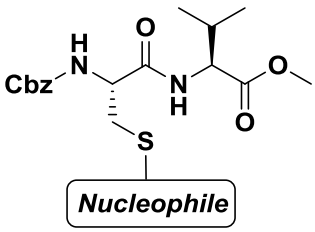


Yield: 65%.^{1}H-NMR (400 MHz, $CDCl_3$) *(mixture of stereoisomers)* δ 8.01 (d, 1H, J = 7.7 Hz), 7.95 (dd, 1H, J1 = 0.9 Hz, J2 = 8.1 Hz), 7.73 – 7.83 (m, 1H), 7.55 – 7.65 (m, 2H), 7.20 – 7.43 (m, 5H), 5.96 (d, 1H, J = 7.5 Hz), 5.89 (d, 1H, J = 8.3 Hz), 5.10 – 5.25 (m, 4H), 4.55 – 4.66 (m, 2H), 4.46 – 4.54 (m, 2H), 3.77 (s, 3H), 3.74 (s, 3H), 3.33 – 3.48 (m, 2H), 3.15 – 3.30 (m, 2H), 2.12 – 2.30 (m, 2H), 0.82 – 1.00 (m, 12H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 172.2, 171.9, 169.5, 156.3, 136.1, 136.0, 134.0, 131.0, 130.7, 129.8, 129.7, 129.0, 129.0, 128.8, 128.7, 128.5, 128.5, 103.9, 103.8, 68.0, 67.8, 58.2, 57.9, 54.7, 52.7, 52.7, 36.3, 36.0, 31.2, 31.0, 19.3, 19.2, 17.9, 17.9; LRMS: MF – $C_{24}H_{29}N_3O_9S_2$, MW_{exp} = 567.63, MW_{obs} = 568.2 (M^++1).



Yield: 88%.^{1}H-NMR (400 MHz, DMSO-*d6*) δ 8.15 – 8.35 (dd, 1H, J_1 = 32 Hz, J_2 = 8 Hz), 8.02 (s, 1H), 8.00 (s, 1H), 7.85 – 7.95 (m, 1H), 7.76 (t, 2H, J = 8.0 Hz), 7.25 – 7.40 (m, 5H), 6.58 (d, 1H, J = 28 Hz), 5.05 (s, 2H), 4.44 (dq, 1H, J_1 = 9.1 Hz, J_2 = 4.3 Hz), 4.14 – 4.22 9 (m, 1H), 3.64 (d, 3H, J = 4.4 Hz), 3.23 (dt, 1H, J1 = 13.9 Hz, J2 = 4.1 Hz), 3.03 (m, 1H), 1.97 – 2.12 (m, 1H), 0.88 (d, 3H, J = 5.7 Hz), 0.86 (d, 3H, J = 5.2 Hz); ^{13}C-NMR (100 MHz, DMSO-d6) δ 171.7, 169.9, 155.9, 136.8, 135.9, 134.3, 129.8, 128.4, 127.9, 127.7, 112.6, 65.7, 57.5, 56.0, 55.4, 51.9, 34.9, 29.9, 18.9, 18.1; LRMS: MF - $C_{25}H_{29}N_3O_7S_2$, MW_{exp} = 547.64, MW_{obs} = 548.2 (M^++1).

Scheme. S6. Stability of Dipeptide sulfenic acid – Linear nucleophile adducts under reductive conditions.



(1) Synthesize and prep-HPLC purify all analogs
(2) Confirm the formation by LC-MS, ^{1}H-NMR and ^{13}C-NMR

(a) Stability over 12 h at pH 7.4
(b) DTT (5 mM)
(c) GSH (5 mM)
(d) TCEP (5 mM)

Procedure: A 25 mM solution of dipeptide-S-Nu adduct was prepared in DMSO. Solutions of DTT (500 mM), GSH (500 mM) and TCEP (500 mM) were prepared in 50 mM HEPES, 100 mM NaCl (pH 7.4). 10 μL of dipeptide-S-Nu solution and 10 μL of each reducing agent solution was added to 980 μL of buffer (50 mM HEPES, 100 mM NaCl, pH 7.4, RT). Effective concentrations were as follow:

(1) Dipeptide-S-Nu = 250 μM

(2) Reducting agent = 5 mM

(3) Buffer = 50 mM HEPES, 100 mM NaCl, pH 7.4

Each reaction mixture was analyzed by LC-MS time course.

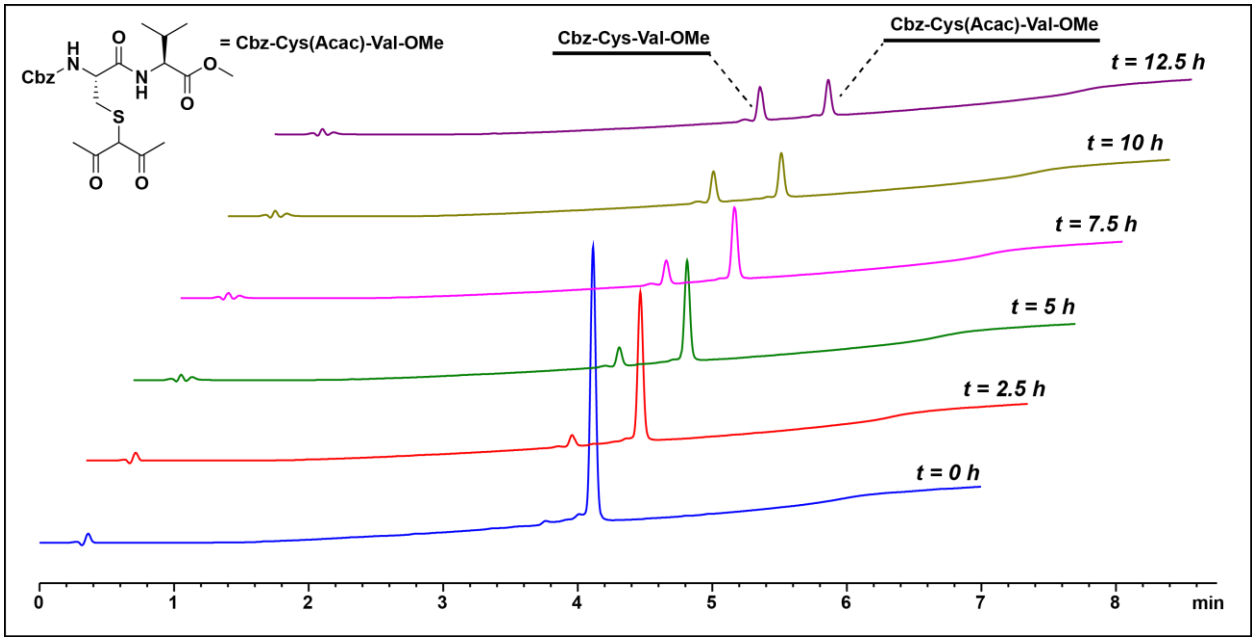


Fig. S36 Stability of Cbz-Cys(Acac)-Val-OMe **13** (250 μM) in buffer over a course of 12 h.

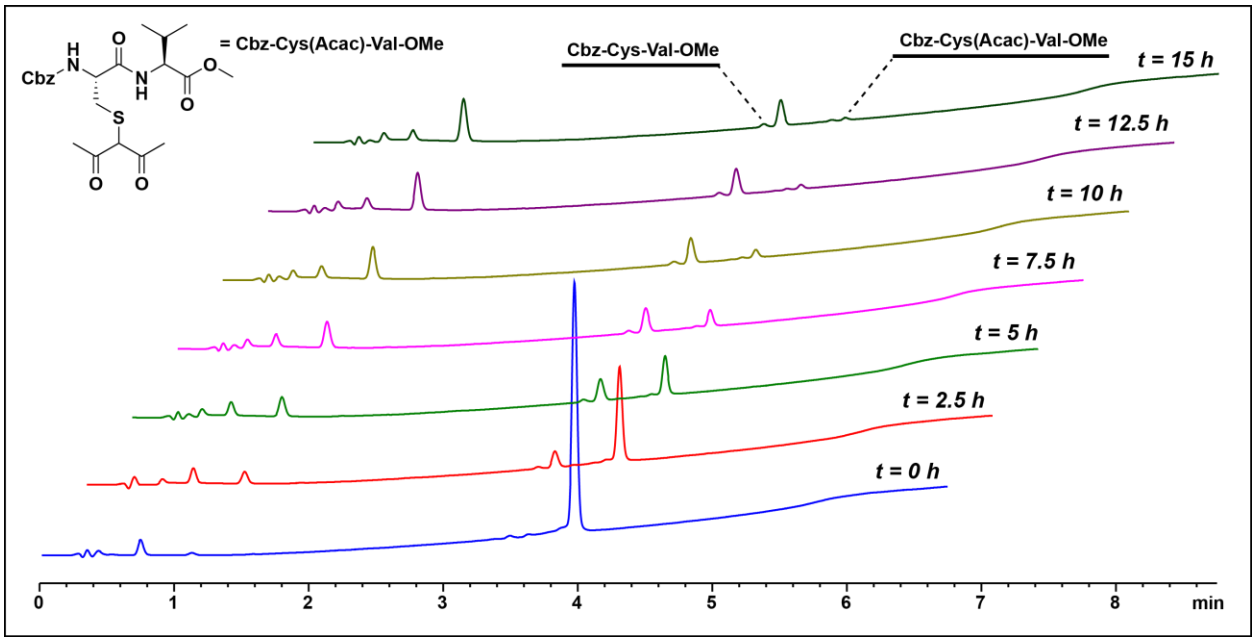


Fig. S37 Stability of Cbz-Cys(Acac)-Val-OMe **13** (250 μM) in presence of DTT (5 mM) in buffer over a course of 12 h.

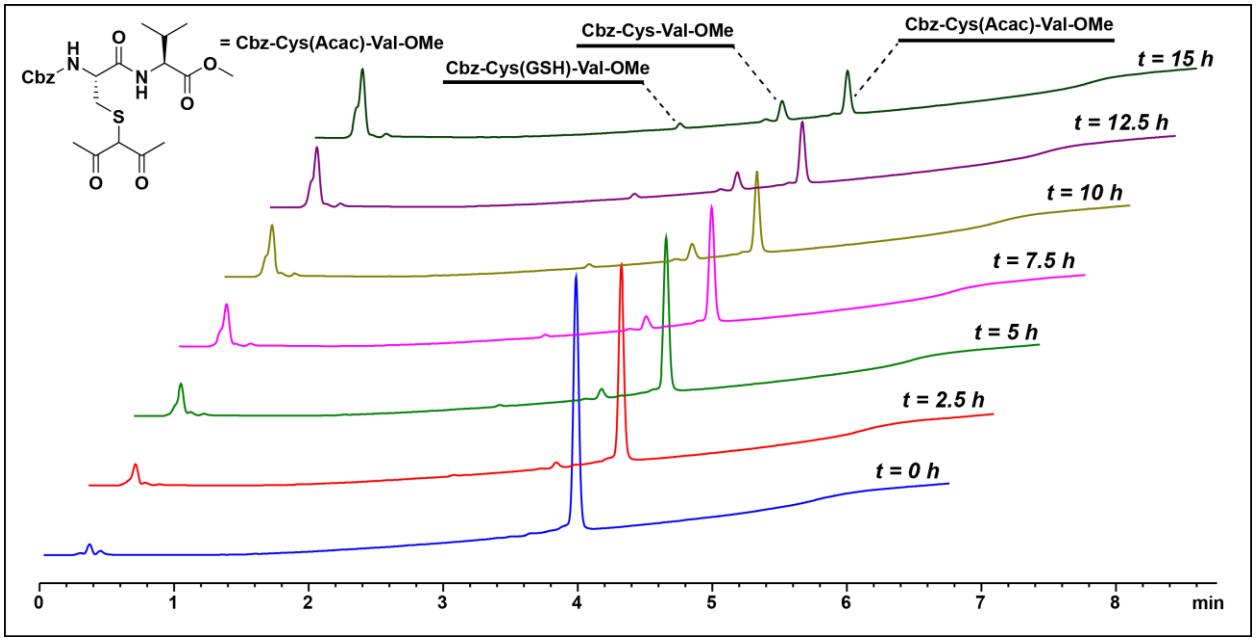


Fig. S38 Stability of Cbz-Cys(Acac)-Val-OMe **13** (250 μM) in presence of GSH (5 mM) in buffer over a course of 12 h.

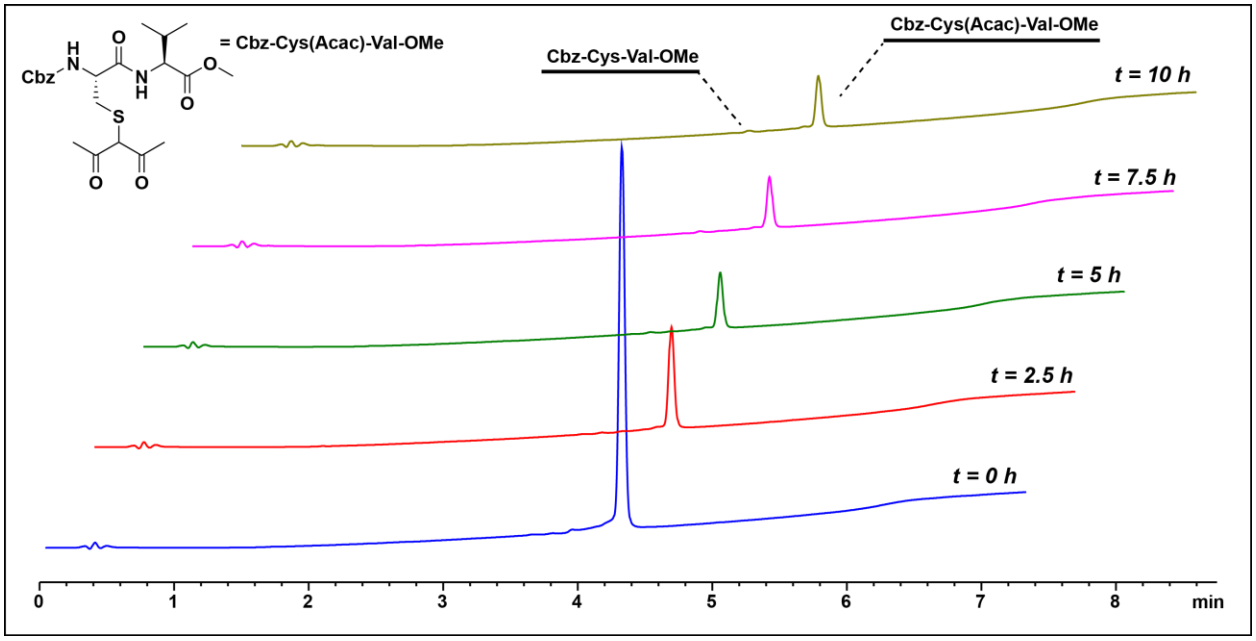


Fig. S39 Stability of Cbz-Cys(Acac)-Val-OMe **13** (250 μM) in presence of TCEP (5 mM) in buffer over a course of 12 h.

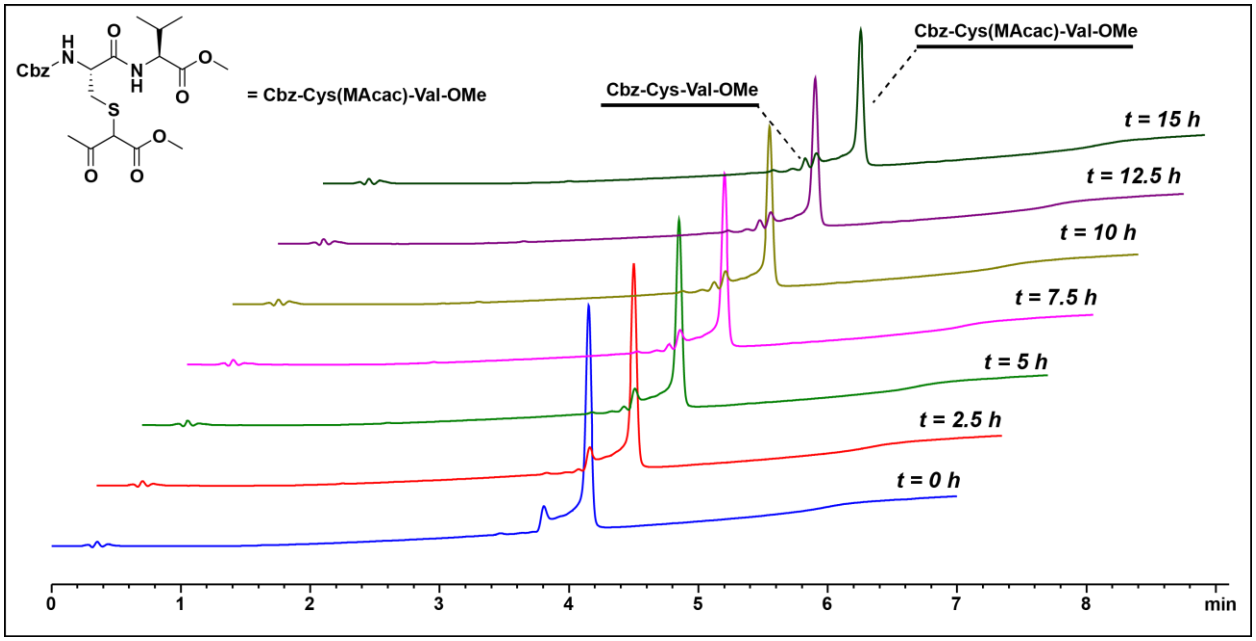


Fig. S40 Stability of Cbz-Cys(Macac)-Val-OMe **14** (250 μM) in buffer over a course of 12 h.

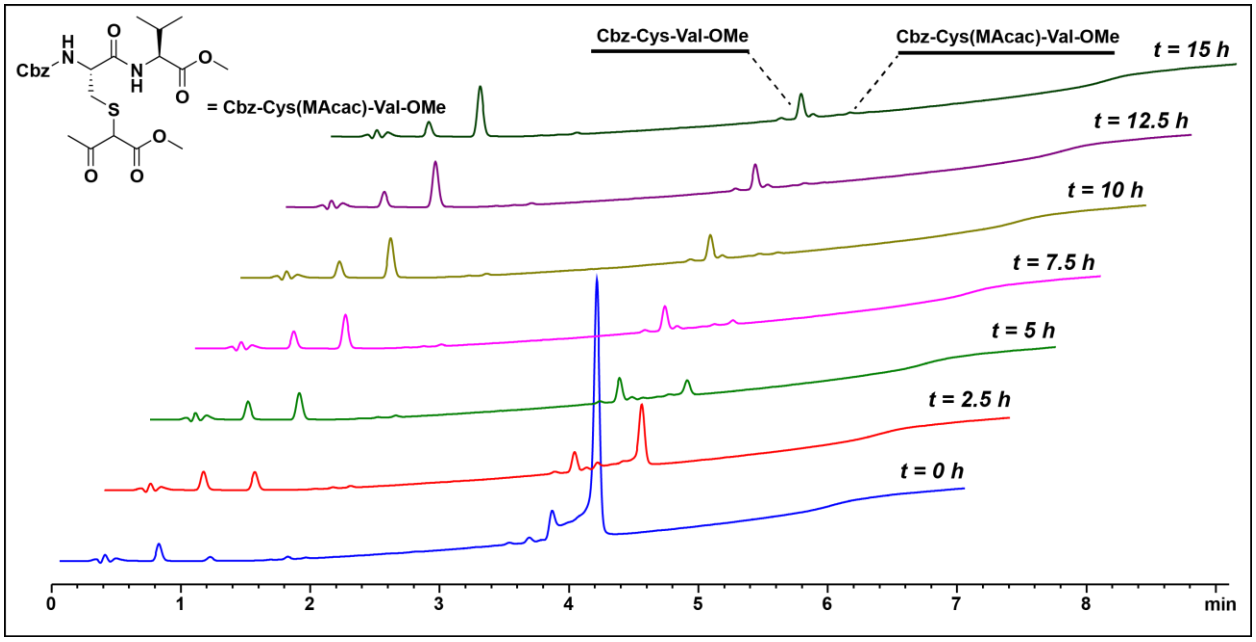


Fig. S41 Stability of Cbz-Cys(Macac)-Val-OMe **14** (250 μM) in presence of DTT (5 mM) in buffer over a course of 12 h.

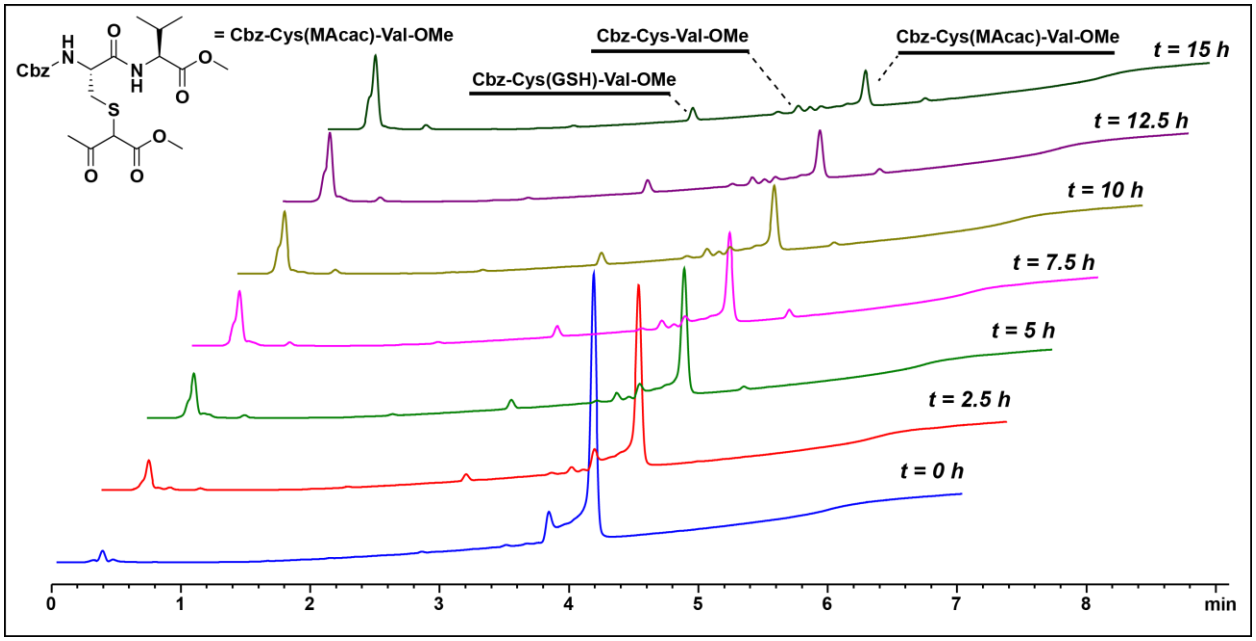


Fig. S42 Stability of Cbz-Cys(Macac)-Val-OMe **14** (250 μM) in presence of GSH (5 mM) in buffer over a course of 12 h.

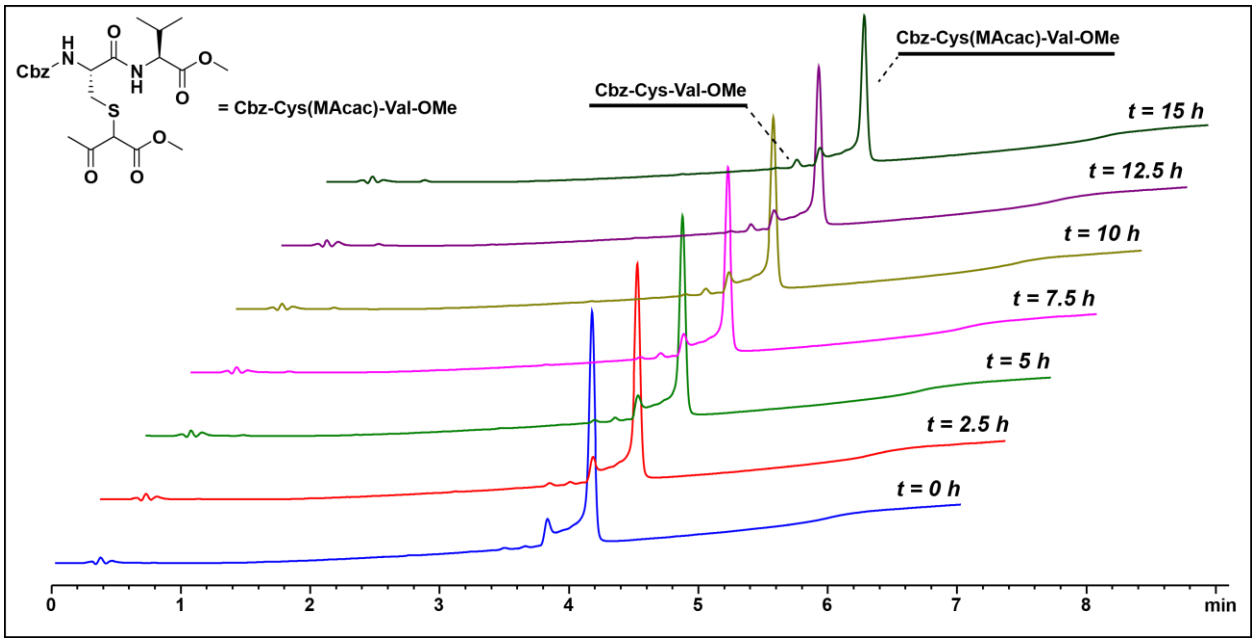


Fig. S43 Stability of Cbz-Cys(Macac)-Val-OMe **14** (250 μM) in presence of TCEP (5 mM) in buffer over a course of 12 h.

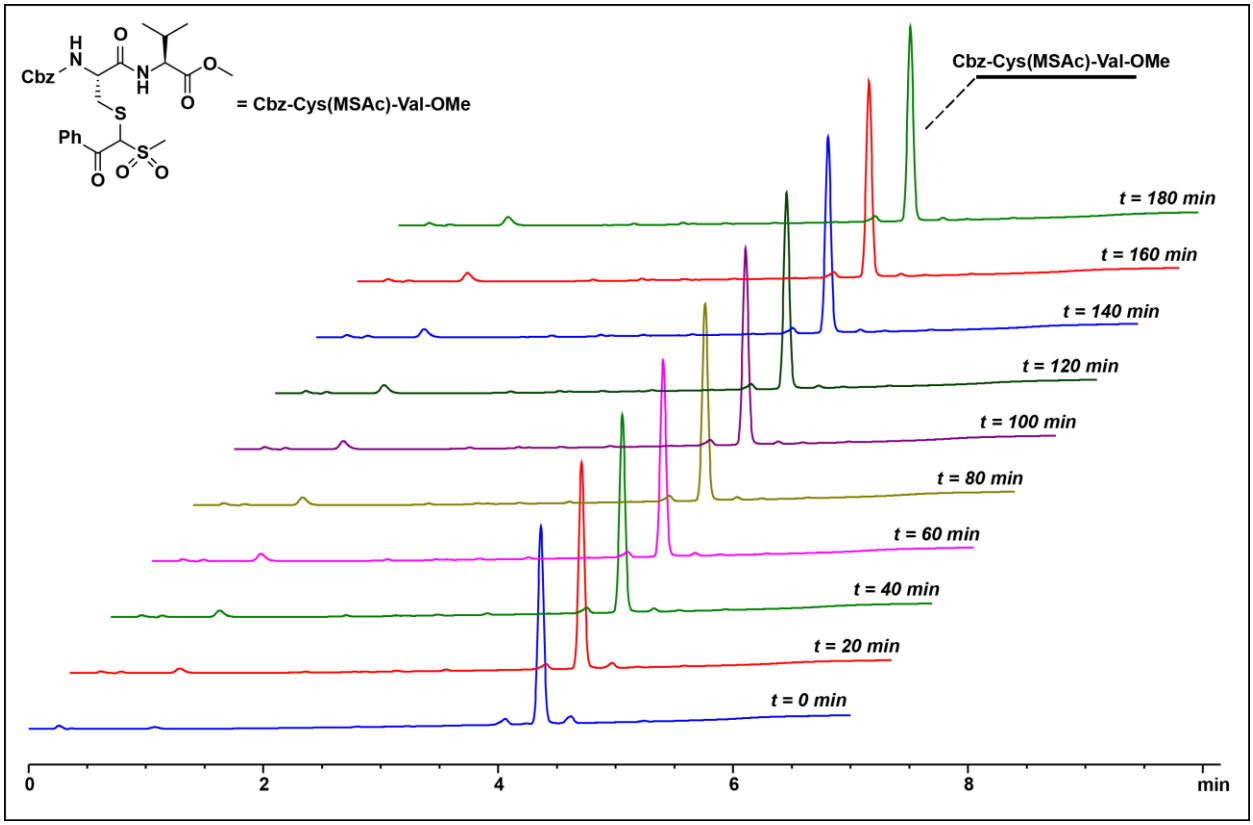


Fig. S44 LC trace of Cbz-Cys(MSAc)-Val-OMe **15** (250 μM) in buffer (pH 7.4) over 3 h.

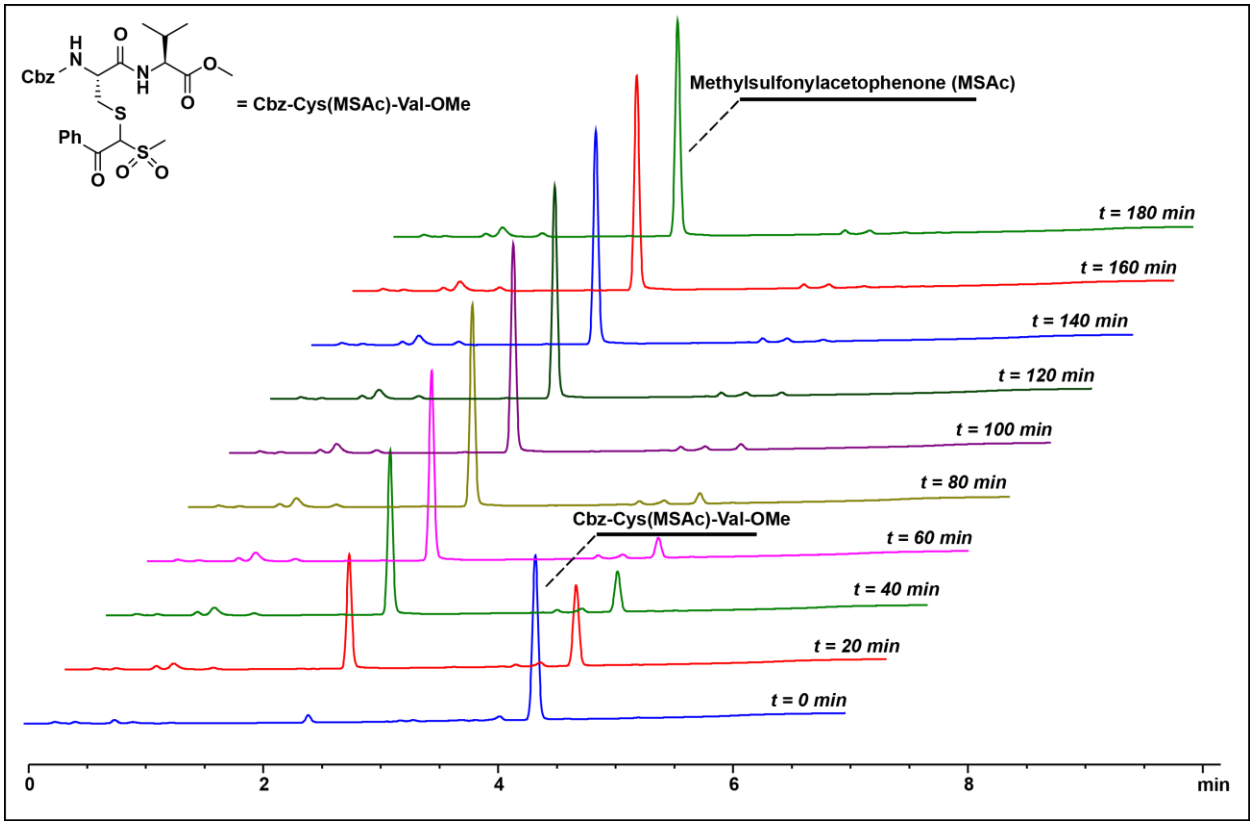


Fig. S45 LC trace of the reaction of Cbz-Cys(MSAc)-Val-OMe **15** (250 μM) with DTT (5 mM) over 3 h.

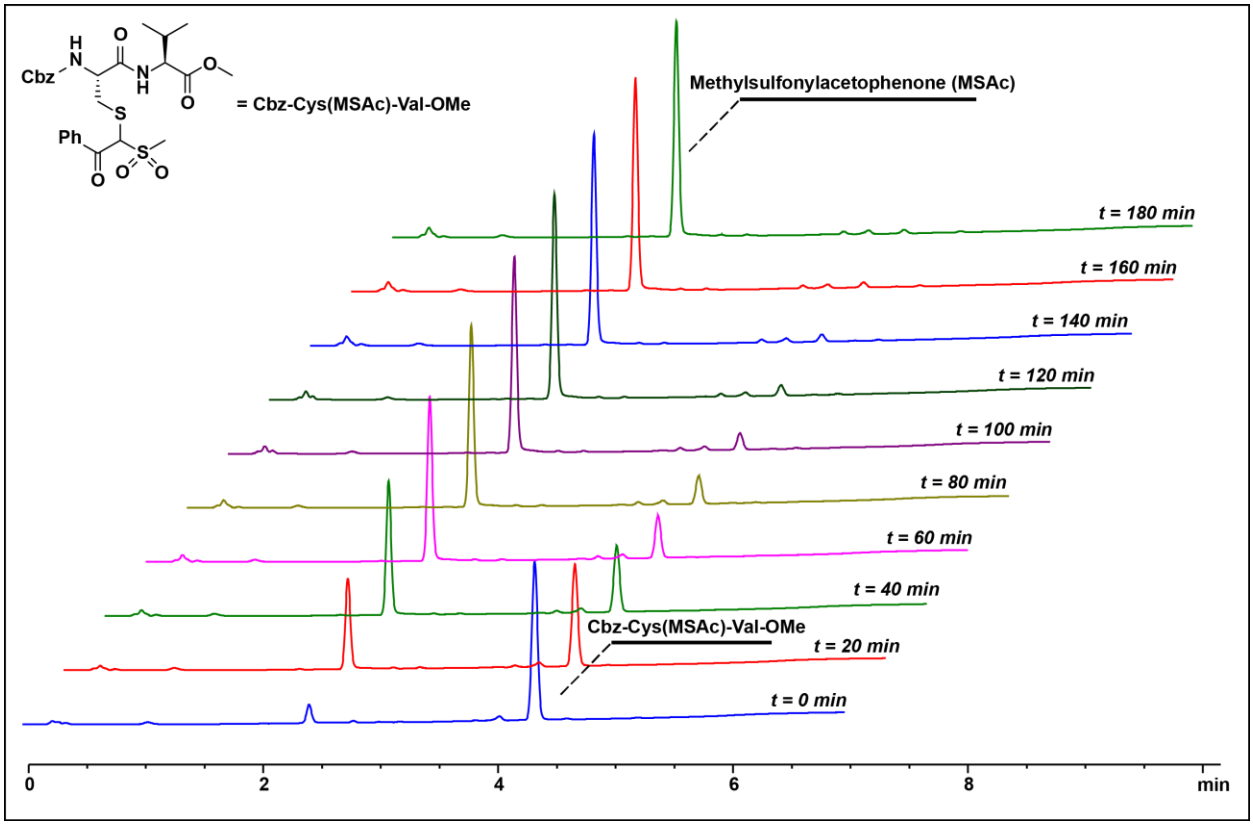


Fig. S46 LC trace of the reaction of Cbz-Cys(MSAc)-Val-OMe **15** (250 μM) with GSH (5 mM) over 3 h.

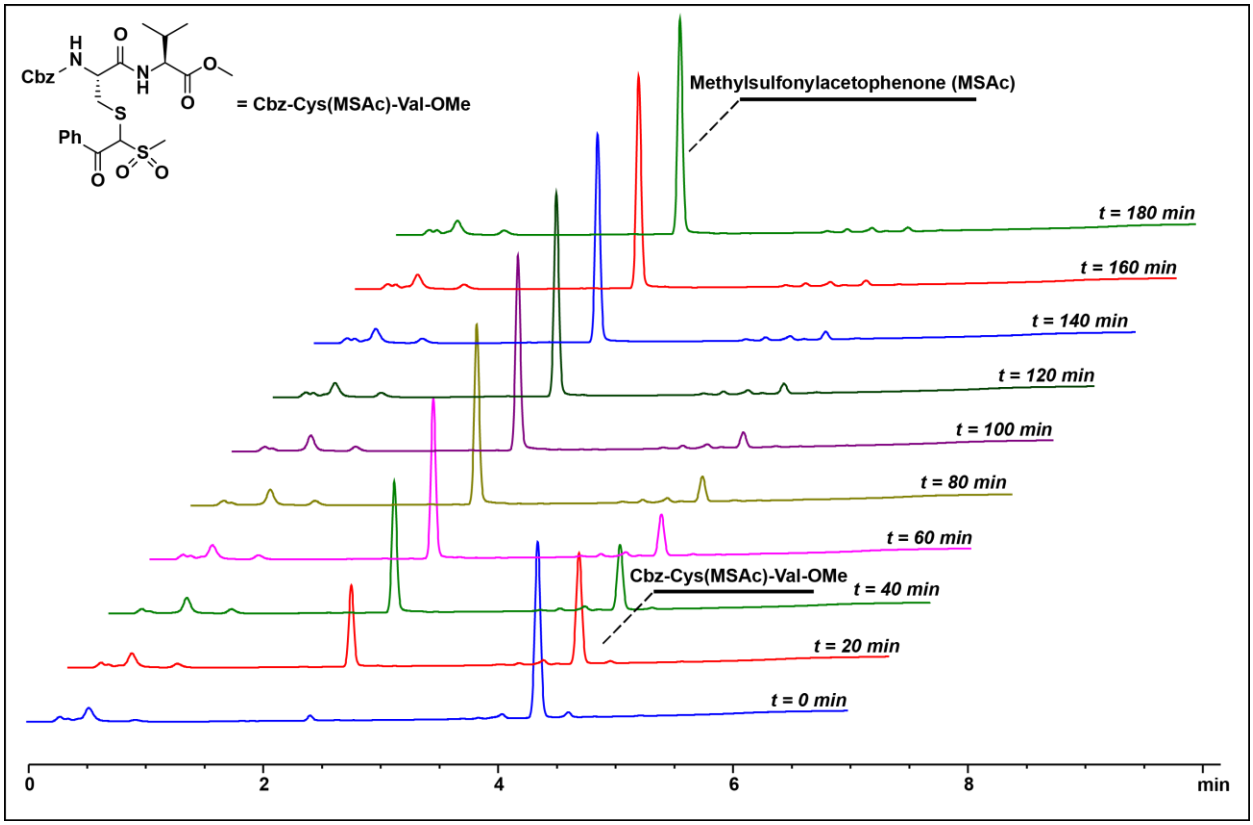


Fig. S47 LC trace of the reaction of Cbz-Cys(MSAc)-Val-OMe **15** (250 μM) with TCEP (5 mM) over 3 h.

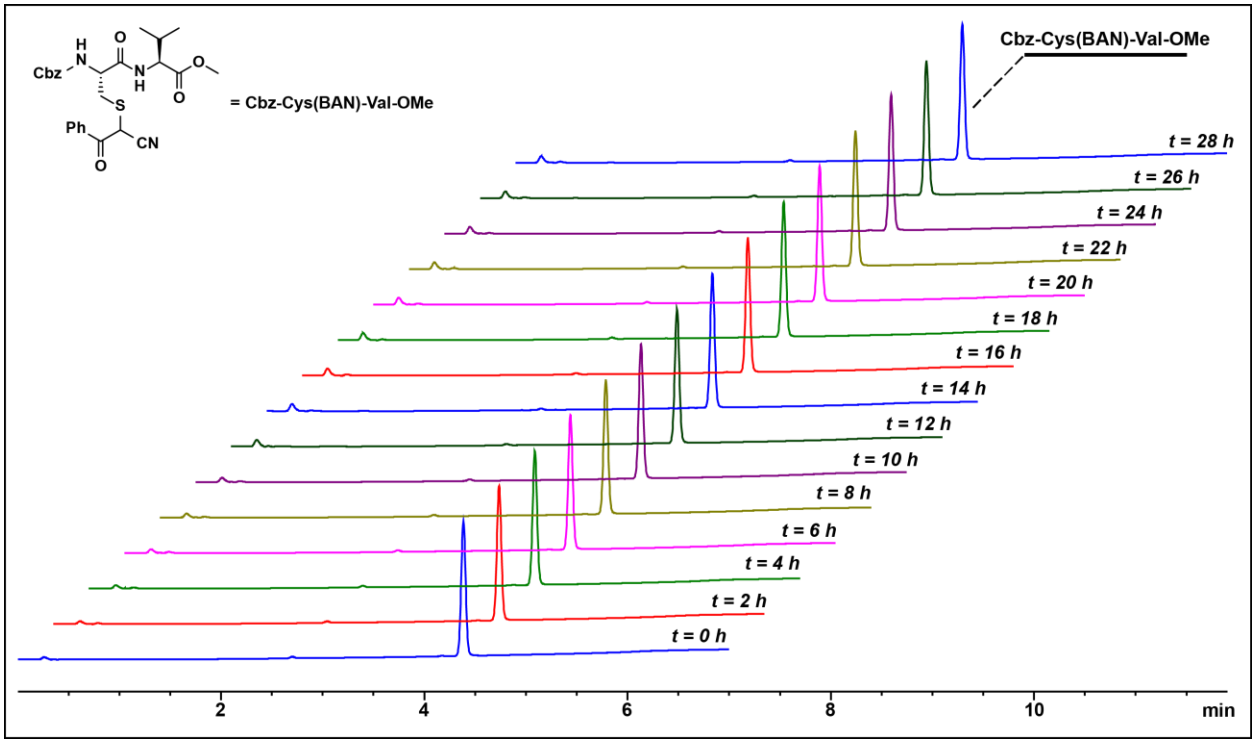


Fig. S48 Stability of Cbz-Cys(CAP)-Val-OMe **16** (250 μM) in buffer over a course of 28 h.

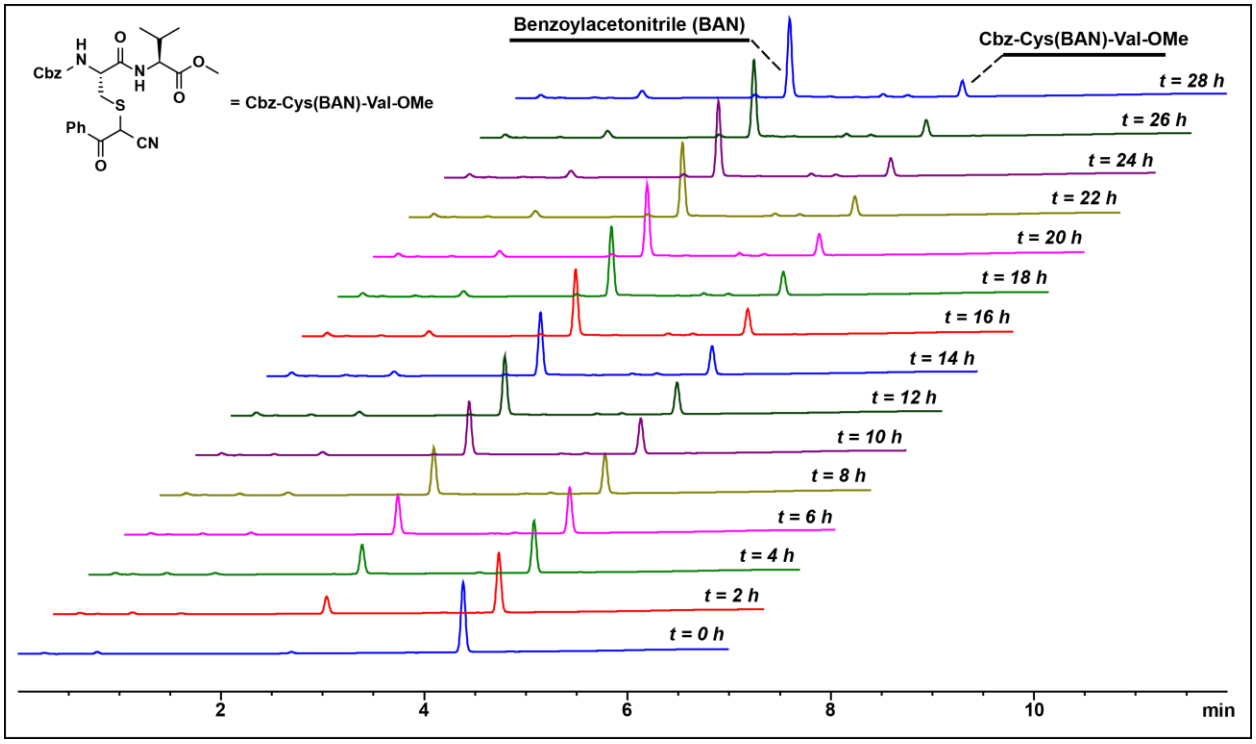


Fig. S49 Stability of Cbz-Cys(CAP)-Val-OMe **16** (250 μM) in presence of DTT (5 mM) in buffer over a course of 28 h.

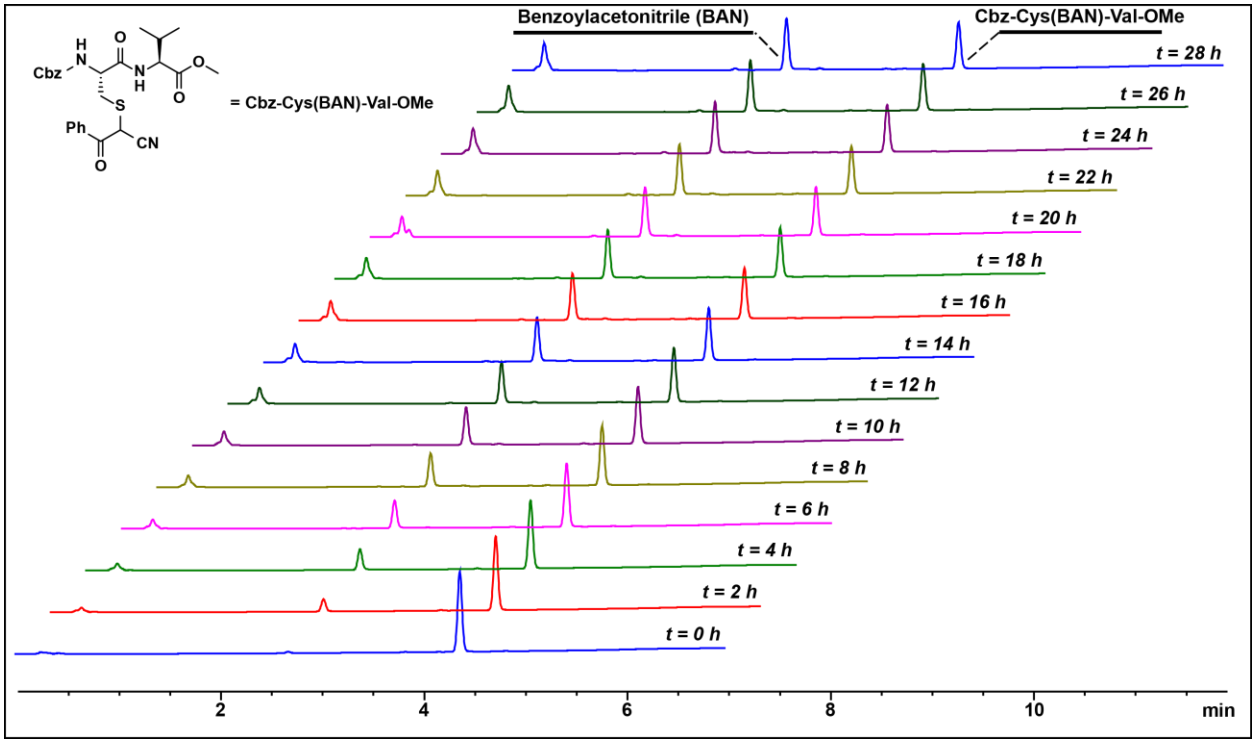


Fig. S50 Stability of Cbz-Cys(CAP)-Val-OMe **16** (250 μM) in presence of GSH (5 mM) in buffer over a course of 28 h.

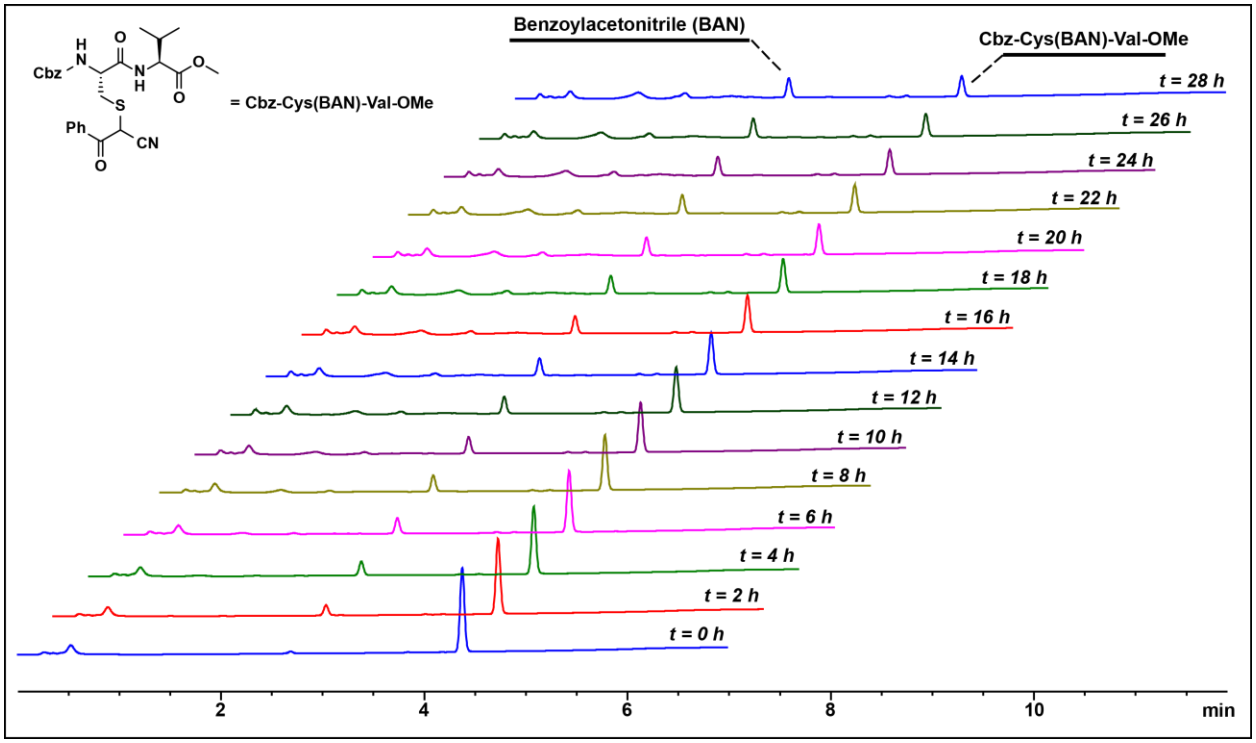


Fig. S51 Stability of Cbz-Cys(CAP)-Val-OMe **16** (250 μM) in presence of TCEP (5 mM) in buffer over a course of 28 h.

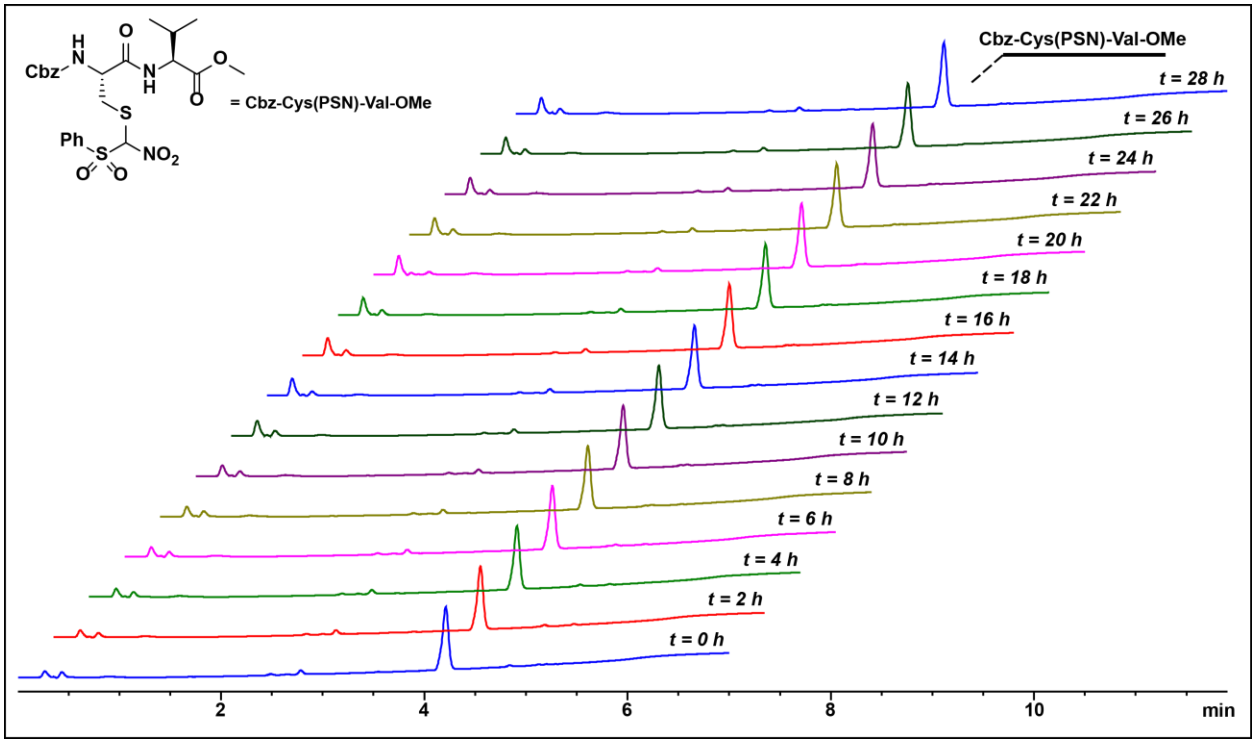


Fig. S52 LC trace of Cbz-Cys(PSN)-Val-OMe **17** (250 μM) in buffer (pH 7.4) over 28 h.

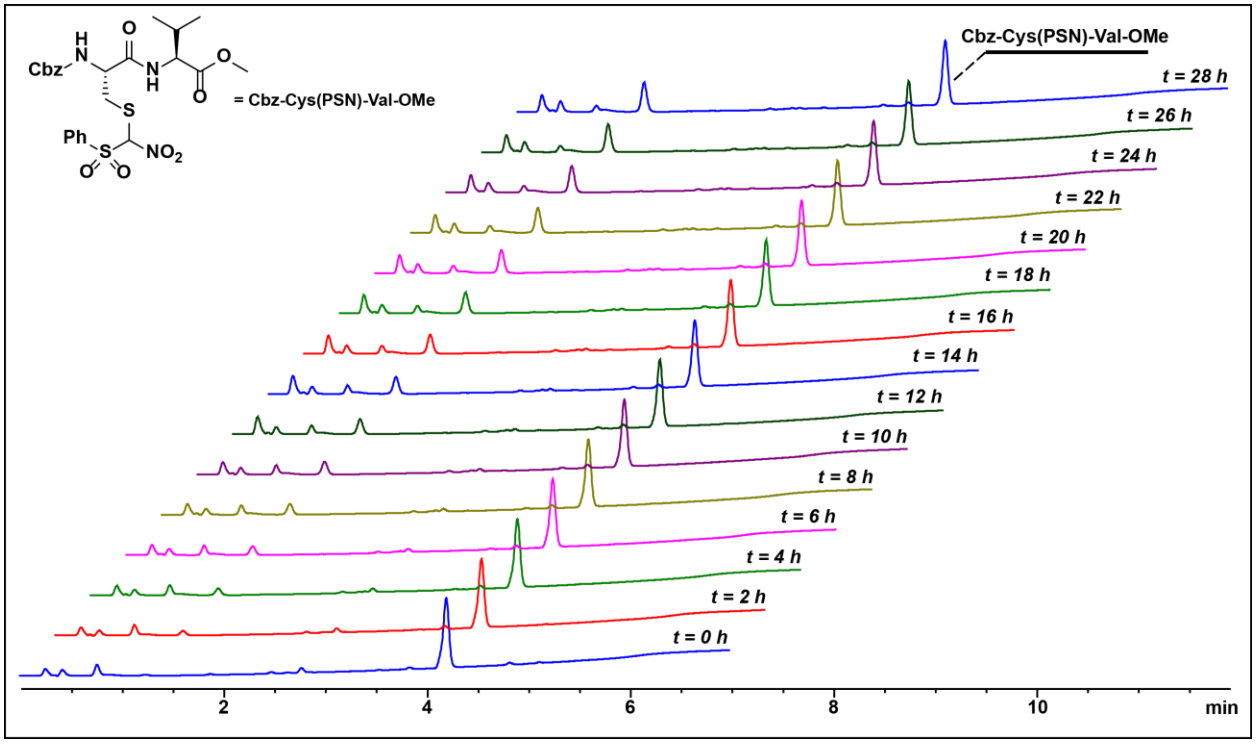


Fig. S53 LC trace of the reaction of Cbz-Cys(PSN)-Val-OMe **17** (250 μM) with DTT (5 mM) over 28 h.

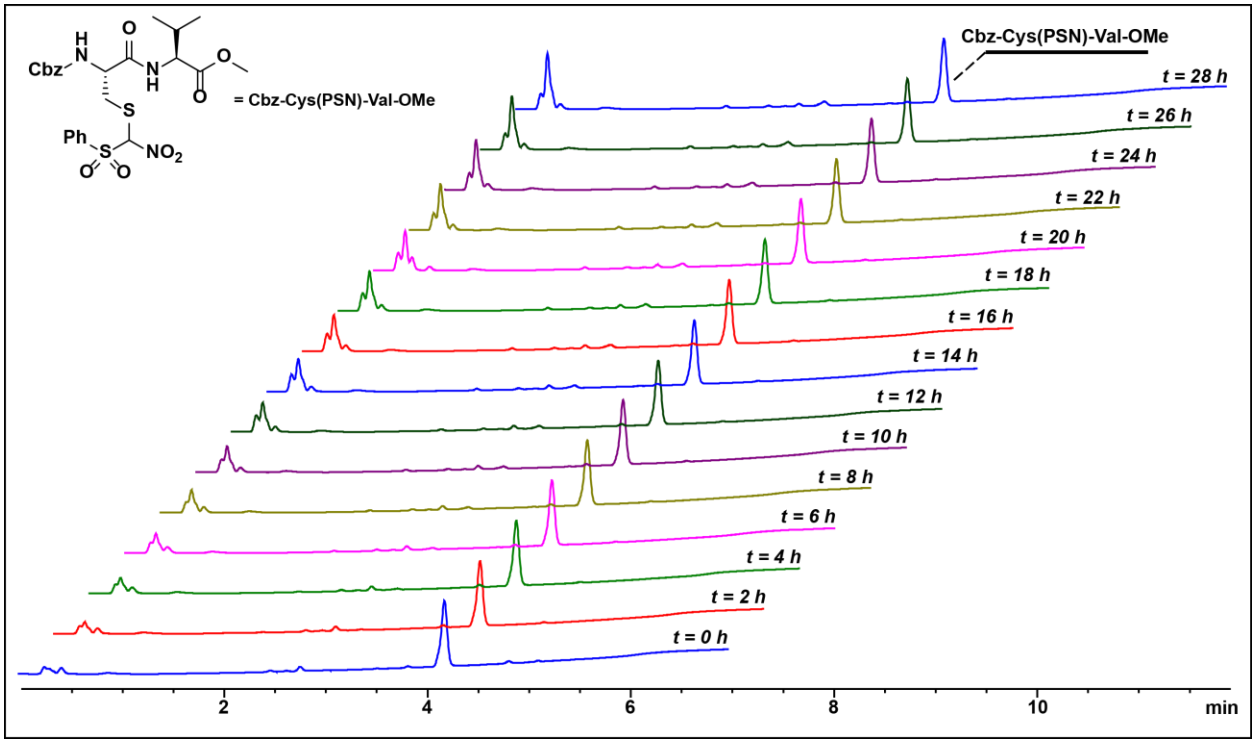


Fig. S54 LC trace of the reaction of Cbz-Cys(PSN)-Val-OMe **17** (250 μM) with GSH (5 mM) over 28 h.

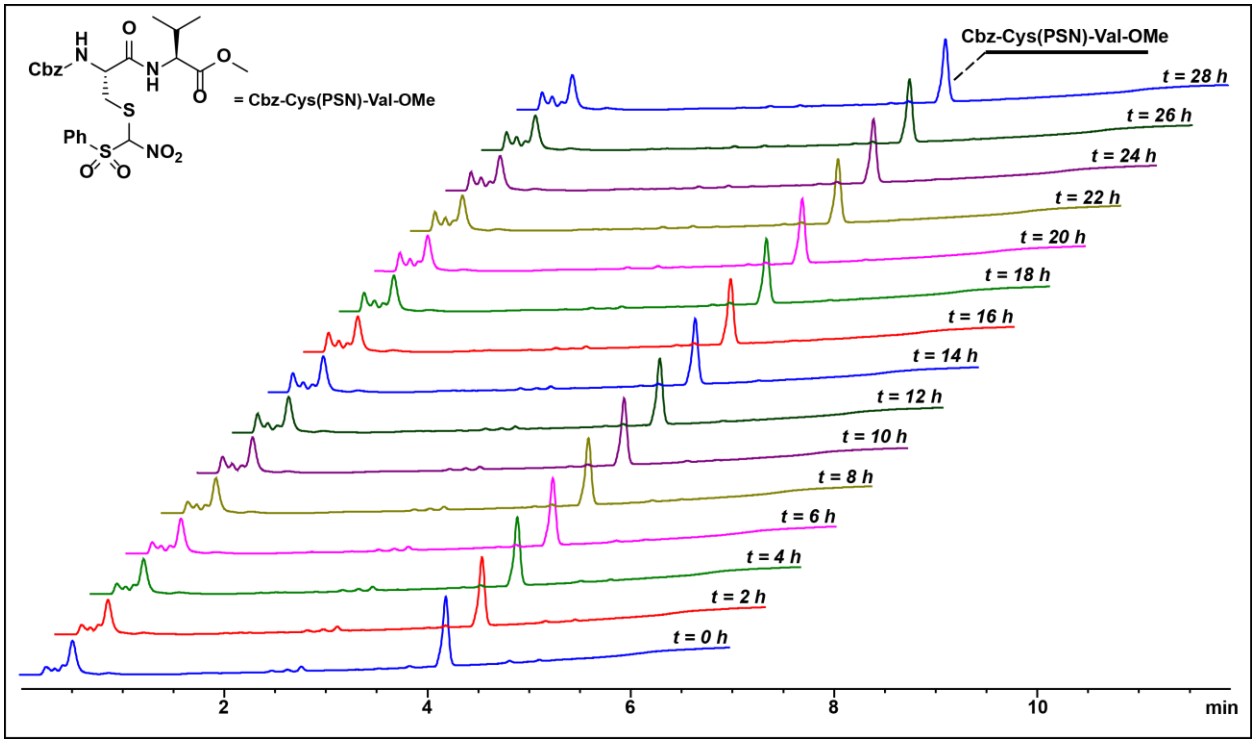


Fig. S55 LC trace of the reaction of Cbz-Cys(PSN)-Val-OMe **17** (250 μM) with TCEP (5 mM) over 28 h.

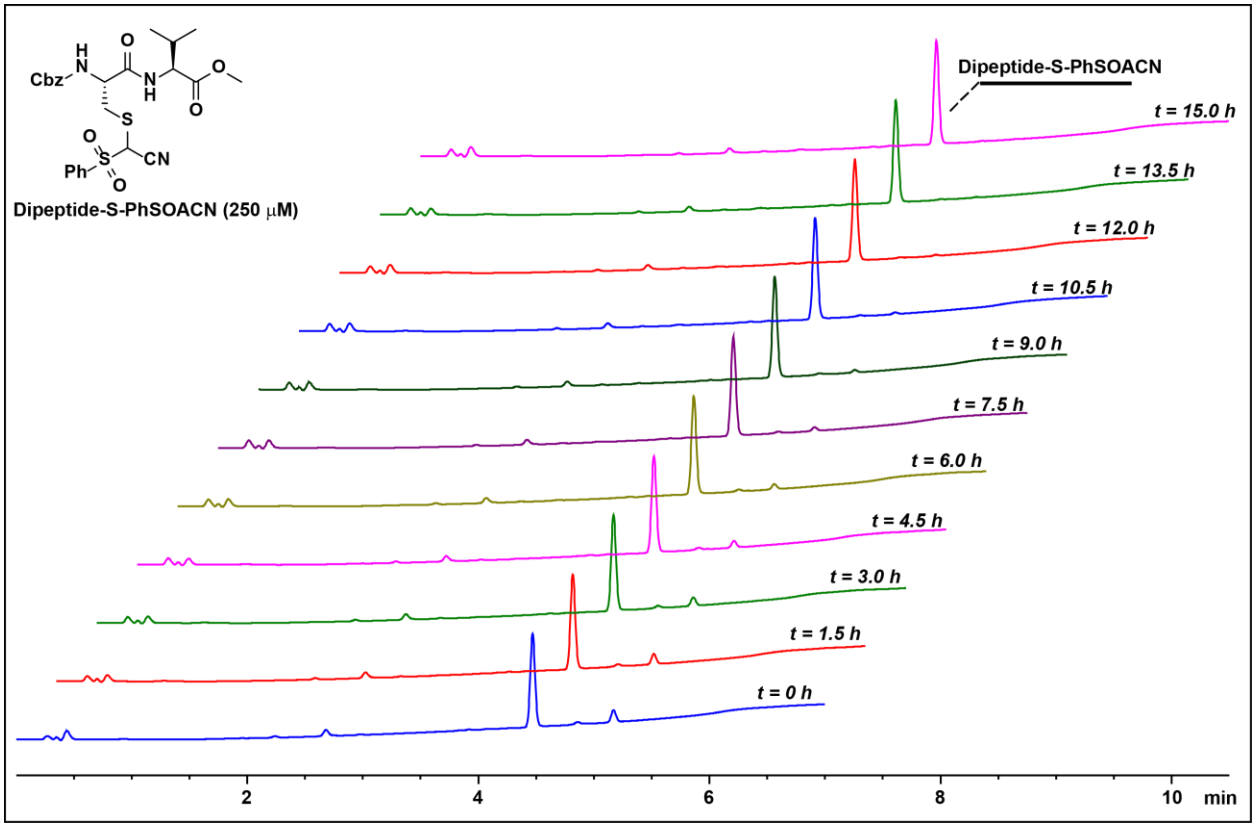


Fig. S56 Stability of Cbz-Cys(PSA)-Val-OMe **18** (250 μM) in buffer over a course of 12 h.

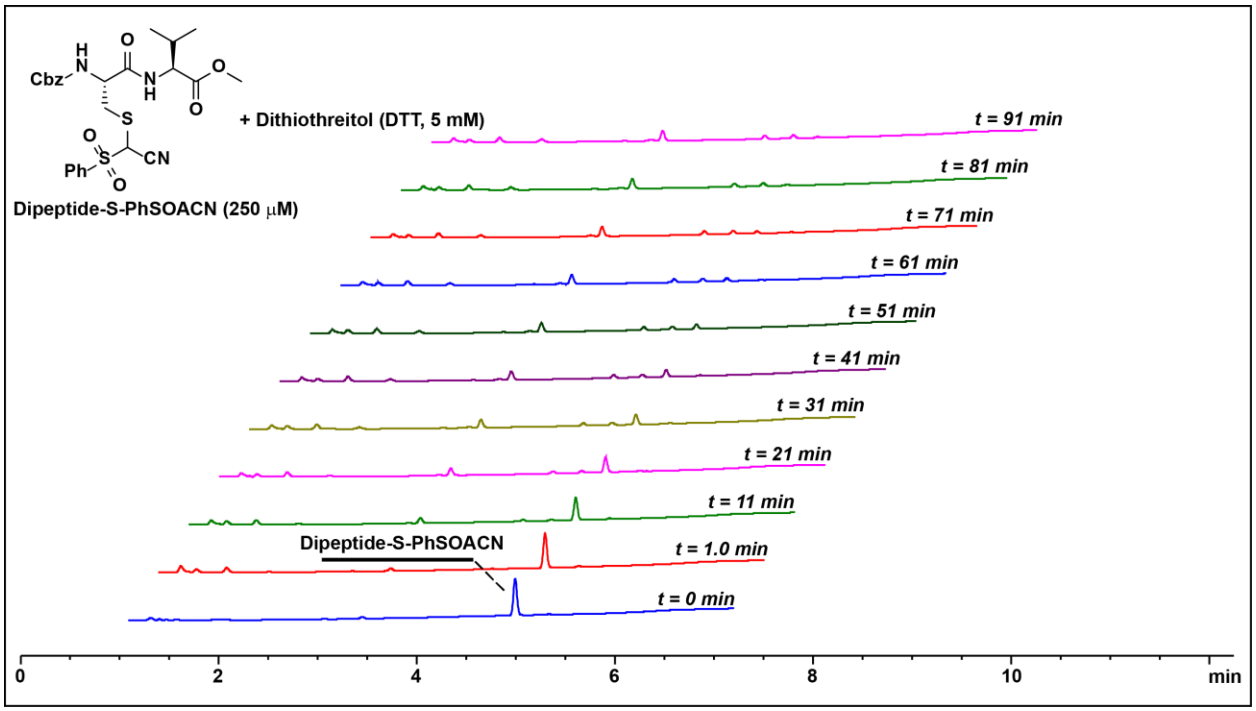


Fig. S57 Stability of Cbz-Cys(PSA)-Val-OMe **18** (250 μM) in presence of DTT (5 mM) in buffer over a course of 12 h.

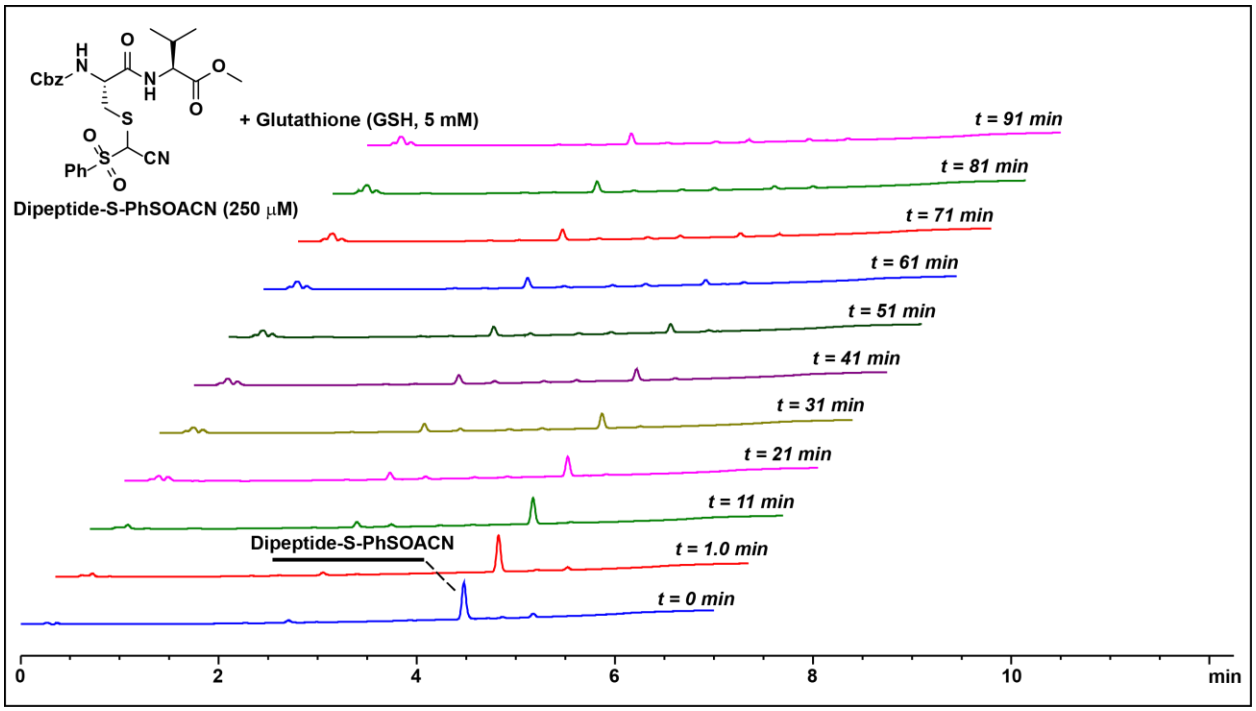


Fig. S58 Stability of Cbz-Cys(PSA)-Val-OMe **18** (250 μM) in presence of GSH (5 mM) in buffer over a course of 12 h.

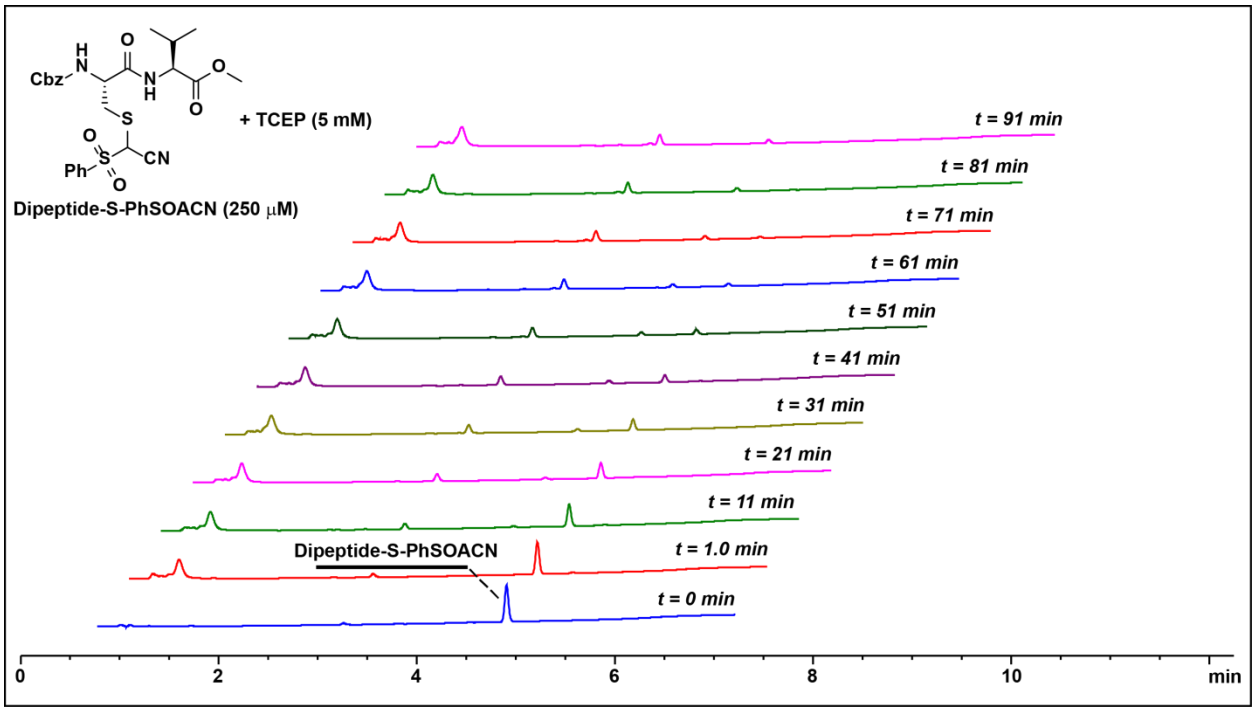


Fig. S59 Stability of Cbz-Cys(PSA)-Val-OMe **18** (250 μM) in presence of TCEP (5 mM) in buffer over a course of 12 h.

Scheme S7 Proposed mechanism for the loss of acetyl group.

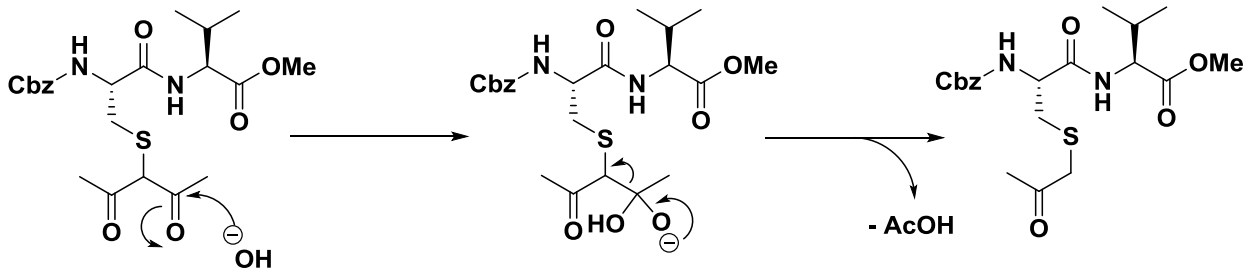
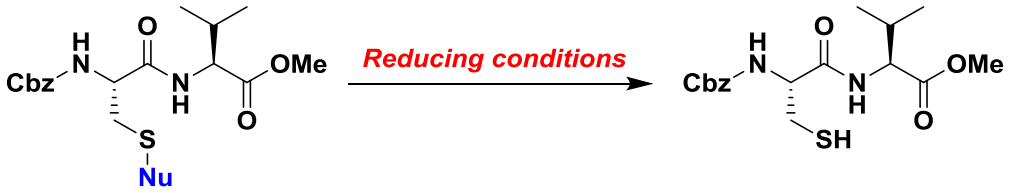
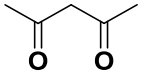
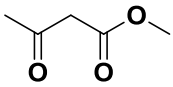
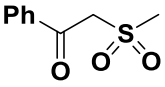
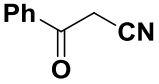
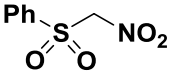
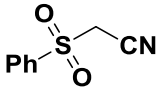
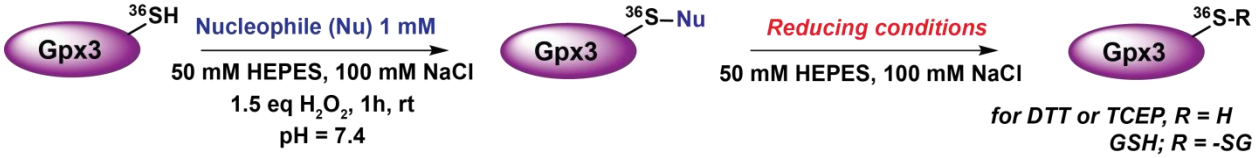


Table S1 Differential rates of reduction were observed for the thioether bonds of various *S*-nucleophile adducts.



#	Nucleophile (250 μM)	k_{obs} Buffer	k_{obs} DTT (5 mM)	k_{obs} TCEP (5 mM)	k_{obs} GSH (5 mM)
13		0.18 h^{-1}	0.42 h^{-1}	0.42 h^{-1}	0.36 h^{-1}
14		0.024 h^{-1}	0.9 h^{-1}	0.72 h^{-1}	0.54 h^{-1}
15		Stable	2.1 h^{-1}	1.5 h^{-1}	1.5 h^{-1}
16		Stable	0.06 h^{-1}	0.05 h^{-1}	0.02 h^{-1}
17		Stable	0.0054 h^{-1}	0.0035 h^{-1}	0.0032 h^{-1}
18		Stable	2.5 h^{-1}	2.4 h^{-1}	1.9 h^{-1}

Scheme S8. Stability of Gpx3-Nu adduct under reducing conditions



Procedure: The labeling of Gpx3-SOH was performed exactly as described in Scheme S3. For each nucleophile, in addition to the reduced & oxidized Gpx3 and nucleophile labeling controls the same labeling reaction was conducted in 12 different eppendorf tubes. The tubes were divided in 4 batches of three tubes each.

1st batch was incubated as is to test the stability of thioether adduct at 15 min, 30 min and 1 h.

2nd batch was incubated with DTT (5 mM) and stability was evaluated at 15 min, 30 min and 1h.

3rd batch was incubated with TCEP (5 mM) and stability was evaluated at 15 min, 30 min and 1h.

4th batch was incubated with GSH (5 mM) and stability was evaluated at 15 min, 30 min and 1h.

At each time point, the reaction was quenched by removing the reducing agents by filteration through P30 column. Subsequently, the samples were analyzed by LTQ-MS and results are summarized below.

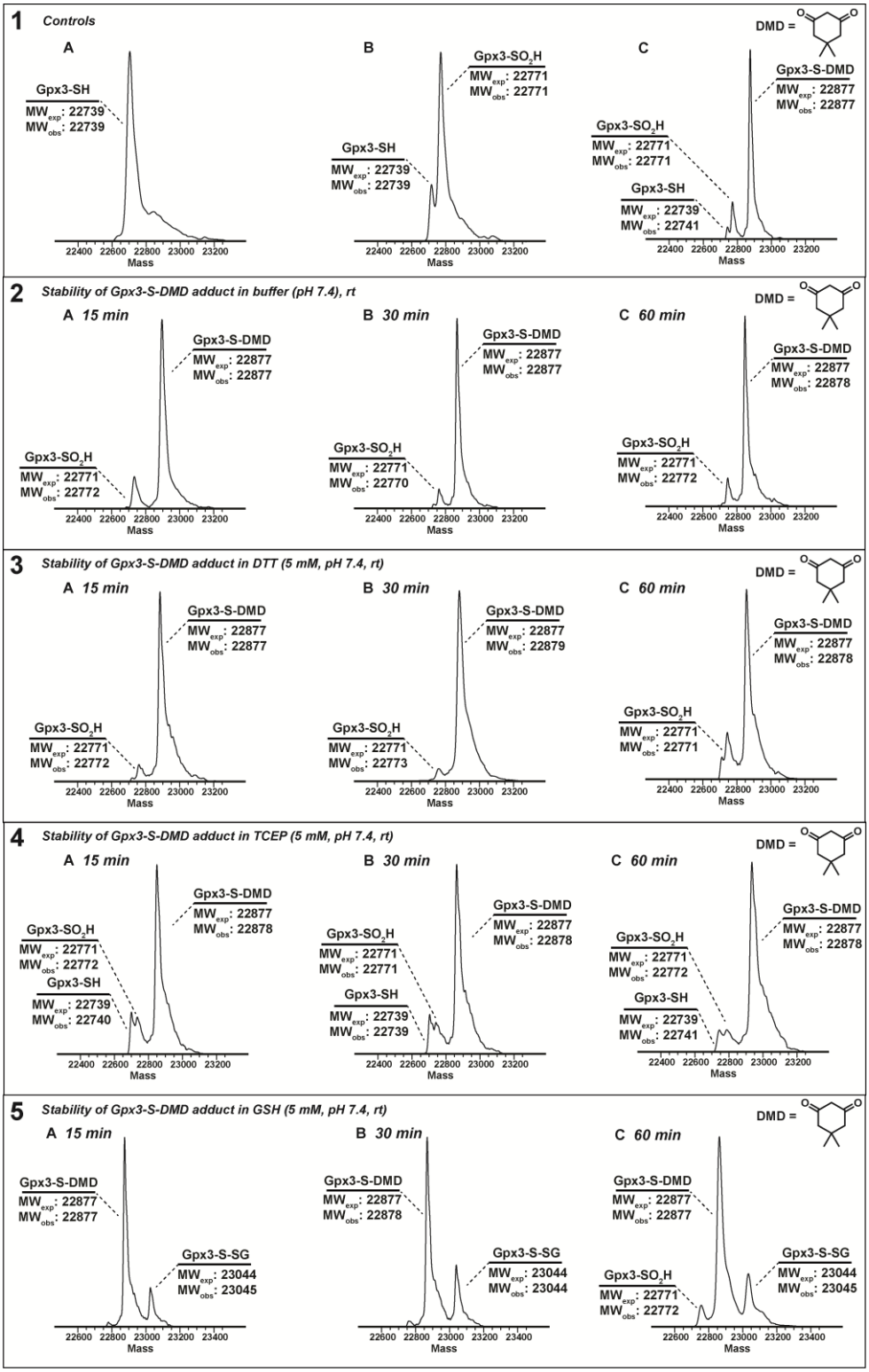


Fig. S60 Thioether adduct of Gpx3-SOH with dimedone showed stability under reducing conditions

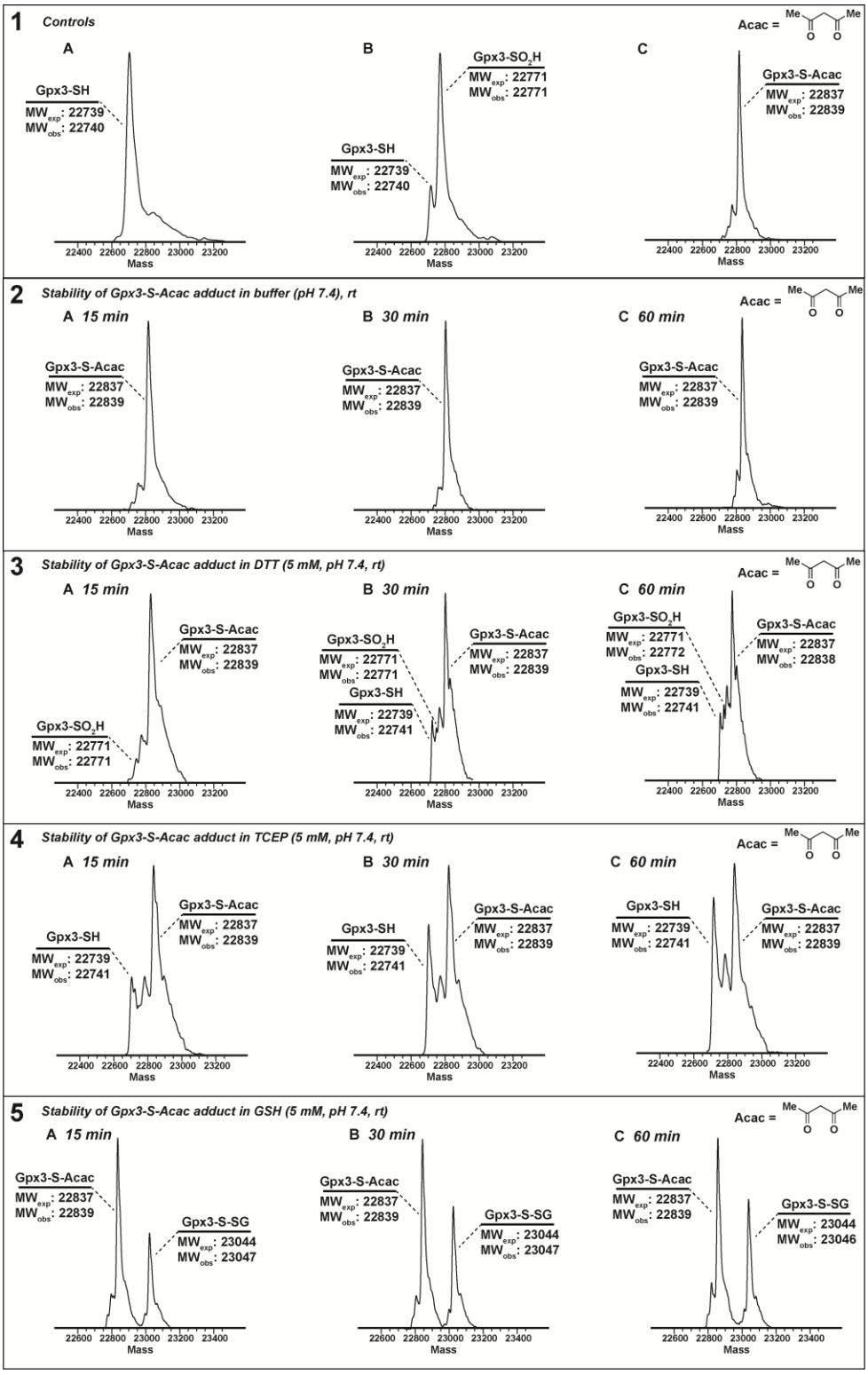


Fig. S61 Thioether adduct of Gpx3-SOH with acetylacetone 3a showed instability under reducing conditions

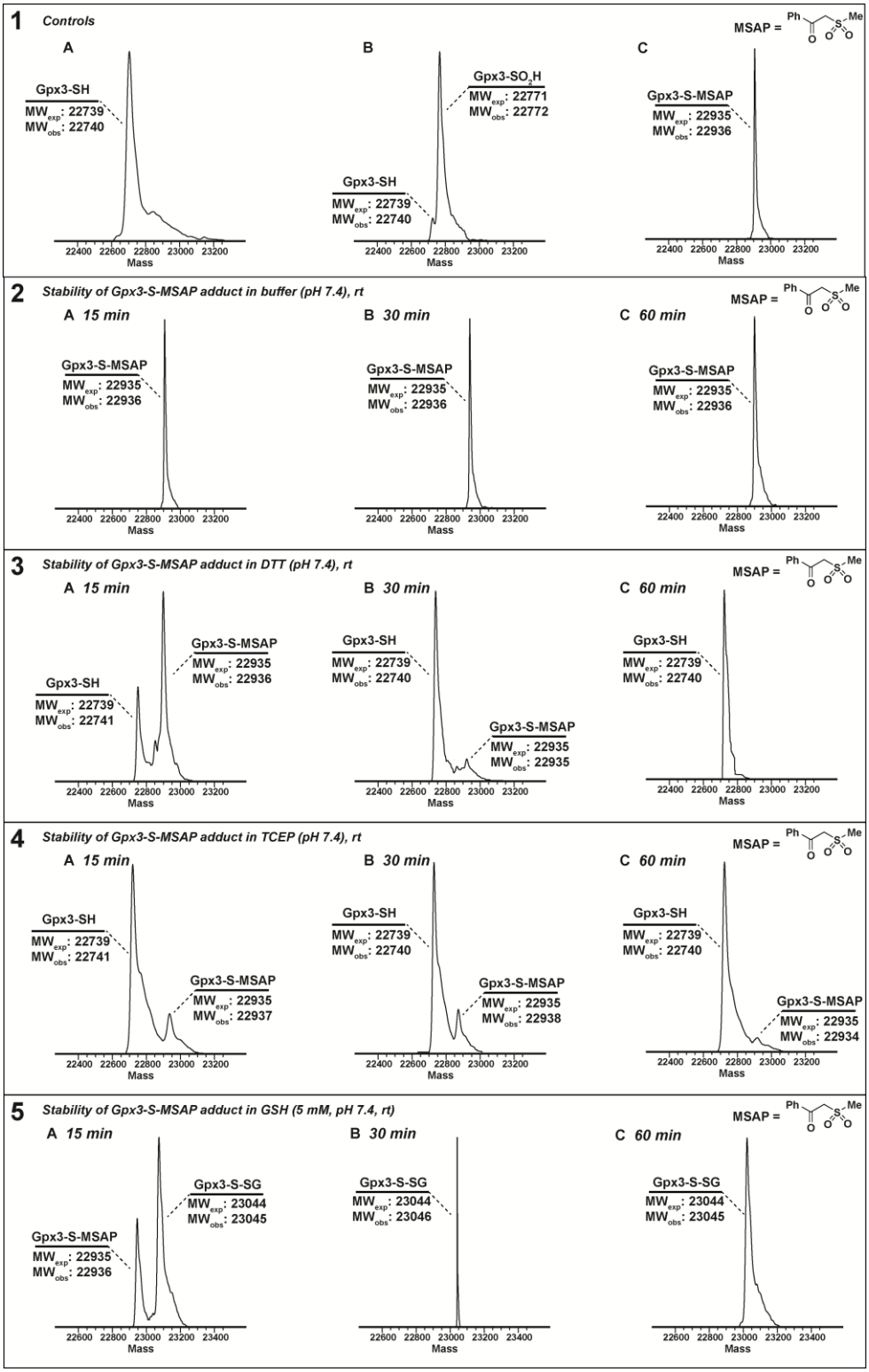


Fig. S62 Thioether adduct of Gpx3-SOH with 2-methylsulfonylacetophenone **(6c)** showed instability under reducing conditions

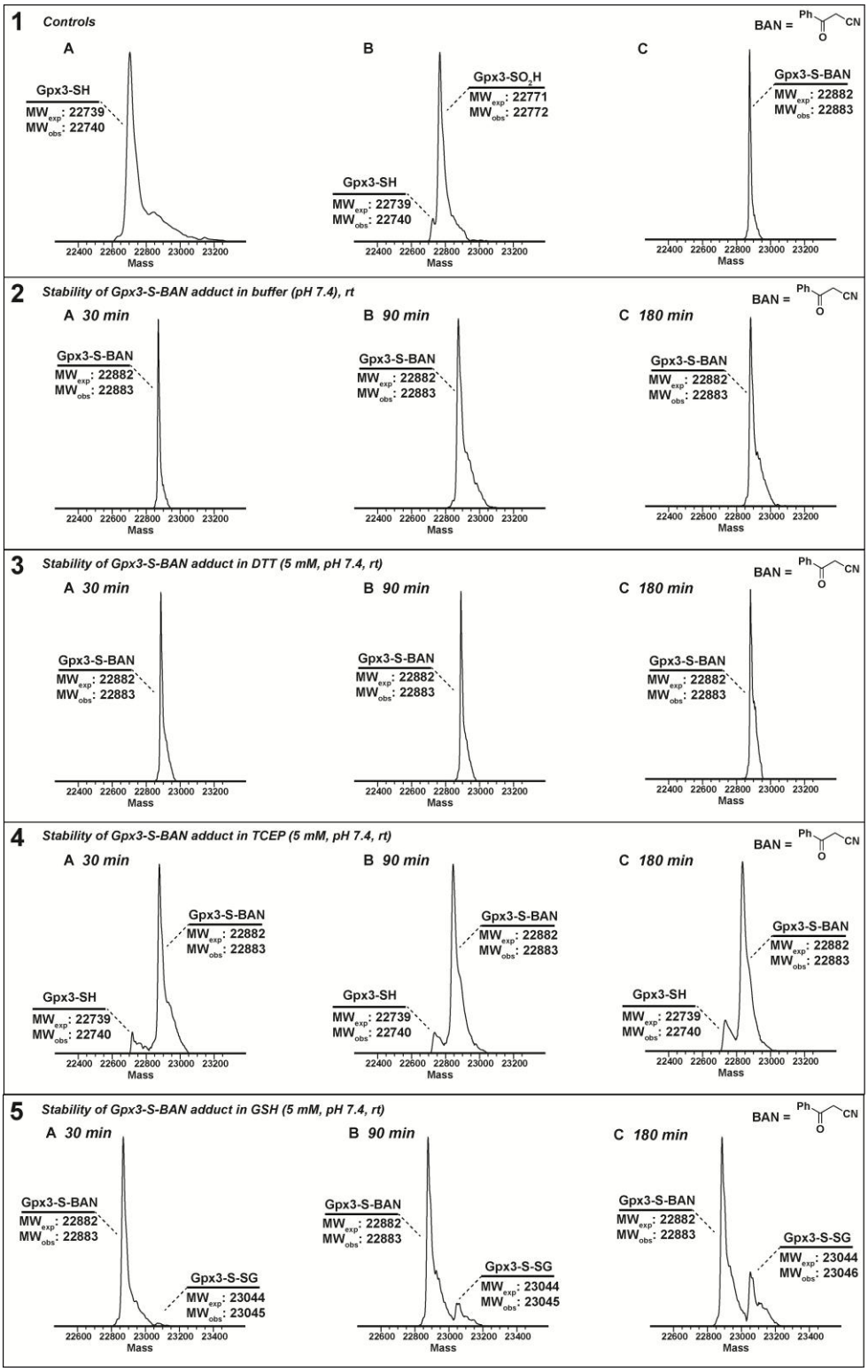


Fig. S63 Thioether adduct of Gpx3-SOH with benzoylacetonitrile **(8b)** showed stability under reducing conditions

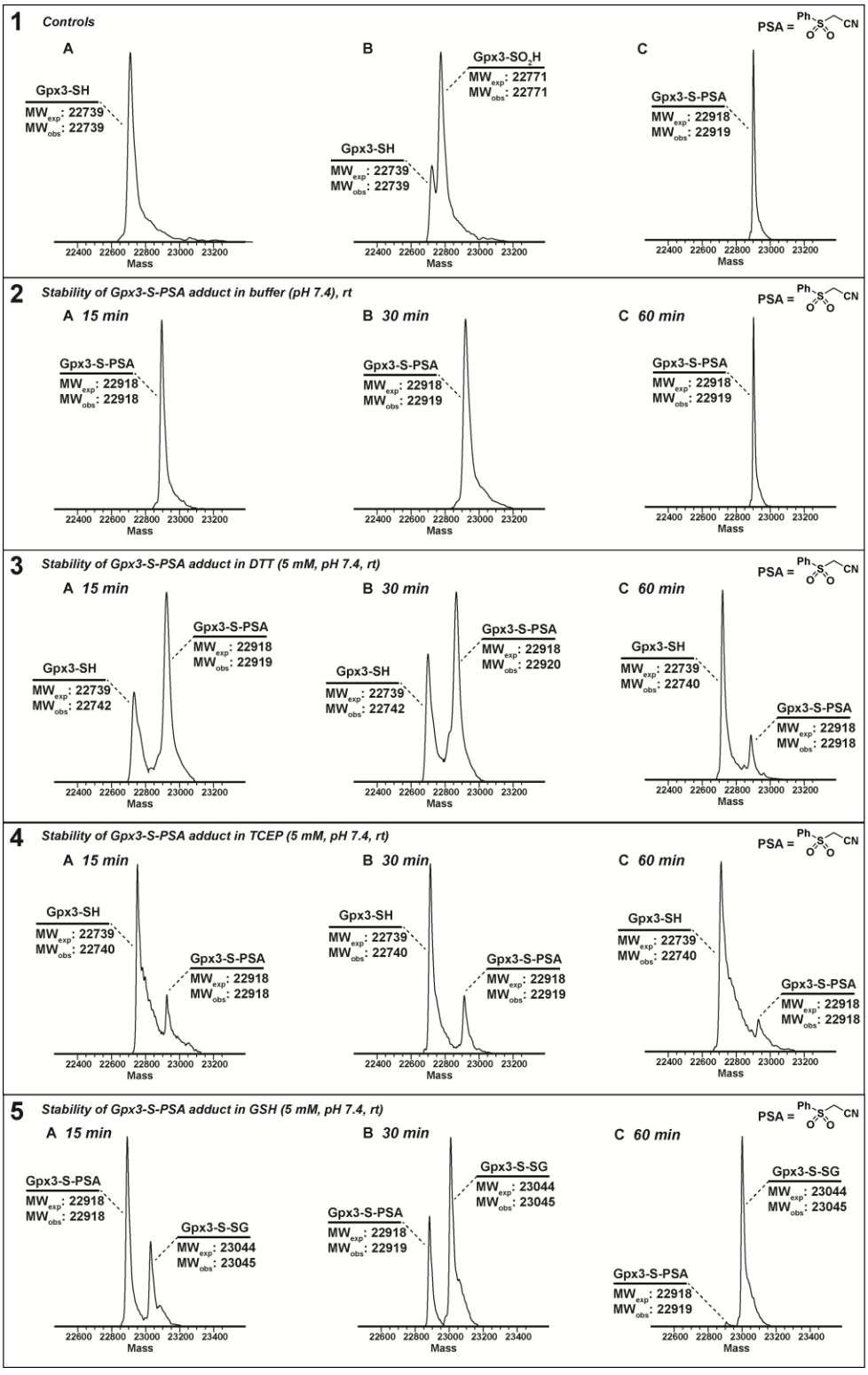


Fig. S64 Thioether adduct of Gpx3-SOH with phenylsulfonylacetonitrile **(10b)** showed instability under reducing conditions

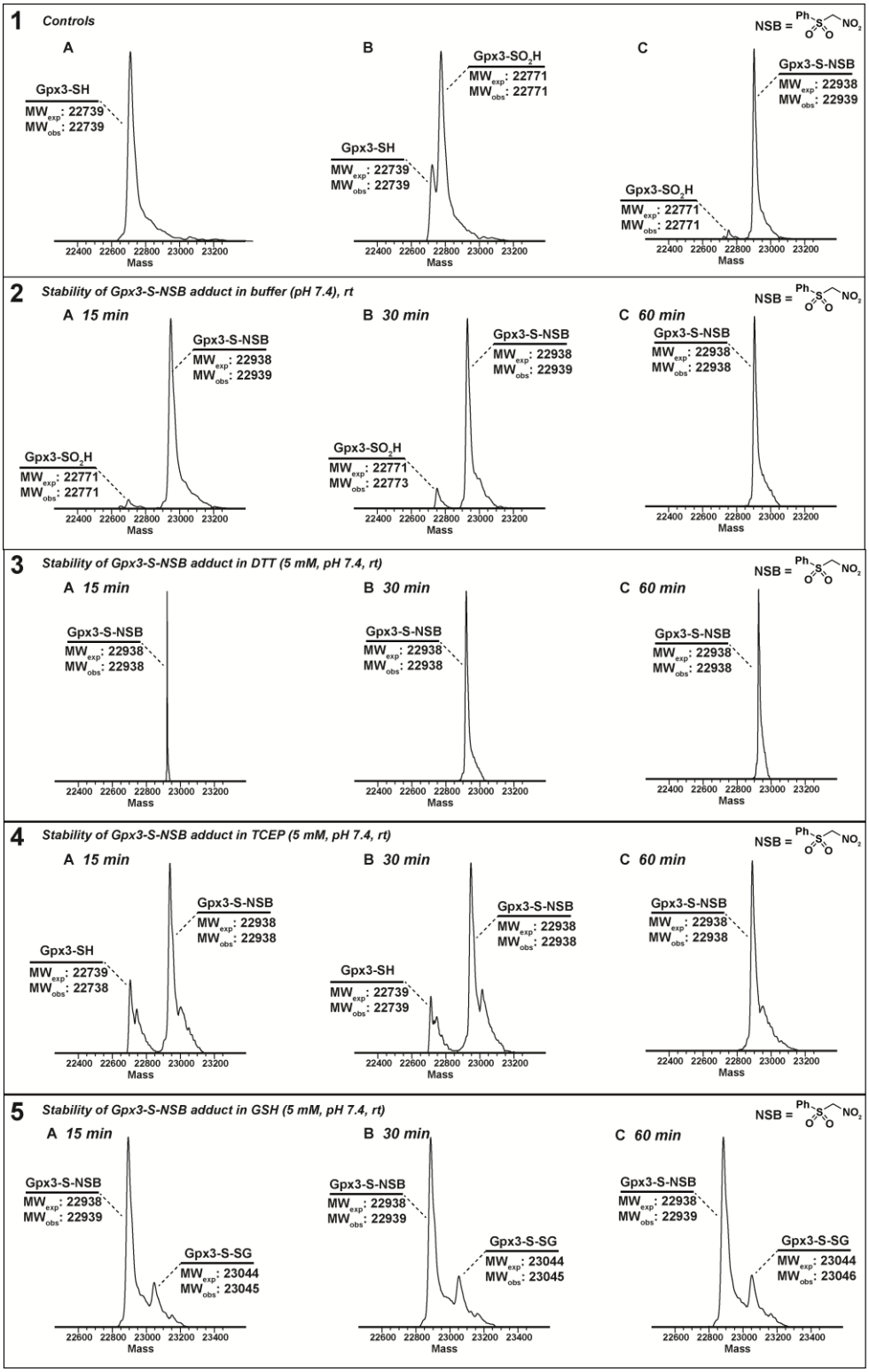
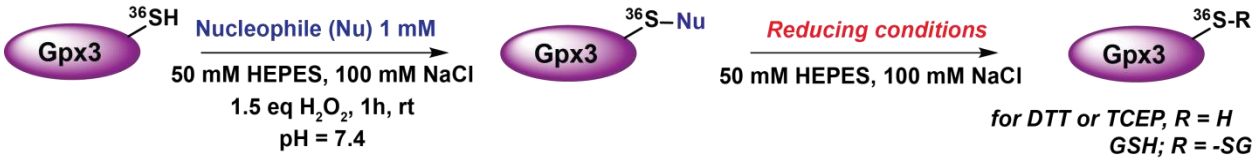


Fig. S65 Thioether adduct of Gpx3-SOH with phenylsulfonylnitromethane **(9)** showed instability under reducing conditions

Scheme S9. Stability/reversibility in Gpx3-SOH by western blot analysis



Expression, purification, and labeling of sulfenylated Gpx3. Recombinant Cys64Ser Cys82Ser Gpx3 was expressed and purified as previously described. Cys64Ser Cys82Ser Gpx3 was previously stored in 50 mM Tris HCl pH 7.4, 300 mM NaCl, 10% glycerol, and 5 mM DTT. DTT was removed from Gpx3 *via* spin filtration using P-30 micro BioSpin columns (BioRad) pre-equilibrated with Gpx3 buffer (50 mM HEPES pH 7.4, 100 mM NaCl). 10 μM Cys64Ser Cys82Ser Gpx3 was treated with 100 μM DYn-2 or 100 μM NO_2 – alkyne or 100 μM CN – alkyne or DMSO in the presence 15 μM H_2O_2 for 1 h at room temperature. Excess probe was removed by spin filtration, and alkyne-modified Cys64Ser Cys82Ser Gpx3 was biotinylated and analyzed as described below. After click chemistry, the reactions were quenched by the addition of EDTA solution (1 μl of 100 mM stock solution). The different biotinylated Cys64Ser Cys82Ser Gpx3 from above step were divided into two equal batches (50 μl each). To one, was added 5 mM GSH (5 μl, 55 mM stock solution) and other was treated with 5 μl of buffer. Resulting reaction mixtures were allowed to shake for 1 h at room temperature. After the completion, the reaction was quenched by filtering through preequilibrated Micro Bio-Spin™ P-30 gel columns (Bio-Rad, Catalog # 7326224).

Western blot. Protein samples were separated by SDS-PAGE (500 ng in each lane) using NOVEX Bolt 4 – 12% Bis-Tris Plus gels (Life Technologies) and transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad). After transfer, the membrane was washed with TBST (3 x 5 min.) and blocked with 3% BSA in TBST overnight at 4 °C. The membrane was washed with TBST (3 x 5 min.) and immunoblotting was performed with the following primary and secondary antibodies at the indicated dilutions in TBST, unless otherwise noted: streptavidin-HRP (GE Healthcare, 1:80000), His-HRP (Pierce, 1:50000). PVDF membrane was developed with chemiluminescence (GE Healthcare ECL Plus Western Blot Detection System) and imaged by film. Data was quantified by densitometry with ImageJ (Wayne Rasband, US National Institutes of Health, http://rsbweb.nih.gov/ij/). PVDF membranes were stripped using mild stripping buffer (200 mM glycine pH 2.2, 0.1% SDS, 1% Tween-20) according to established protocol (www.abcam.com) before reprobing.

HeLa cell lysate labeling with sulfenic acid specific probes under basal conditions

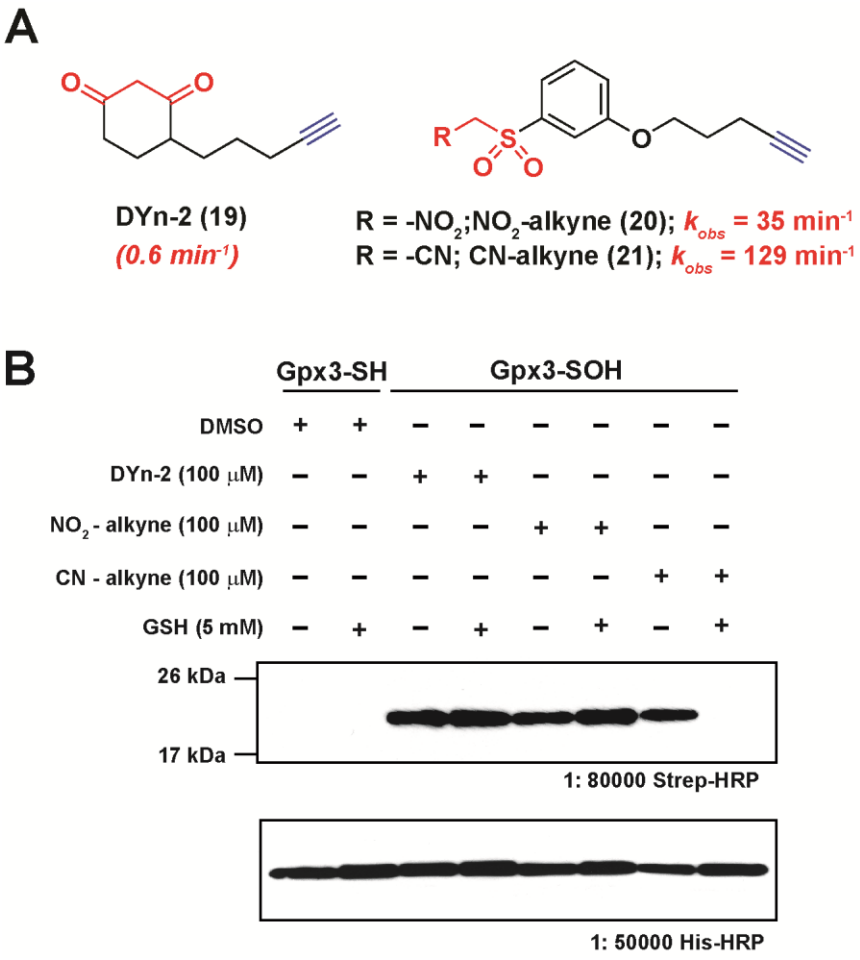
(a) Lysate Labeling - HeLa cell lysate was prepared as reported previously. The concentration was determined by BCA assay to be 3.31 mg/ml and 200 μl aliquots were frozen for future use. For the experiment, the HeLa cell lysate was thawed on ice and buffer exchanged to HEPES 50 mM, NaCl 100 mM (pH 7.4) using preequilibrated Micro Bio-Spin™ P-30 gel columns (Bio-Rad, Catalog # 7326224) or Micro Bio-Spin™ P-6 gel columns (Bio-Rad, Catalog # 7326222). The lysate recovered after filtration was aliquot in different eppendorf tubes (1.5 ml) to give effective concentration of 1.5 mg/ml. Appropriate amount of labeling buffer (HEPES 50 mM, NaCl 100 mM, pH 7.4) was added followed by the addition of probe (5 μl of 2 mM solution in 70:30 buffer:DMSO to give 100 μM effective concentration) to give a total reaction volume of 100 μl. Resulting reaction mixtures were allowed to shake at room temperature for 1 h. After the completion, the reaction was quenched by filtering using preequilibrated Micro Bio-Spin™ P-30 gel columns (Bio-Rad, Catalog # 7326224) or Micro Bio-Spin™ P-6 gel columns (Bio-Rad, Catalog # 7326222).

(b) BTTP click chemistry in lysate – To the filtered lysate recovered from following the above protocol was added biotin azide (2 μl of 5 mM stock solution to give effective concentration of 100 μM), followed by a solution of $CuSO_4$ (2 μl of 15 mM solution to give effective concentration of 250 μM) and BTTP (5 μl of 10 mM solution to give effective concentration of 500 μM). Finally, a solution of sodium ascorbate (2 μl of 125 mM to give effective concentration of 1.5 mM) was added. The total reaction volume was adjusted to 100 μl by the addition of 14 μl of buffer. Resulting reaction mixture was allowed to shake at room temperature for 1 h. The click reaction was quenched by the addition of EDTA solution (1 μl of 100 mM).

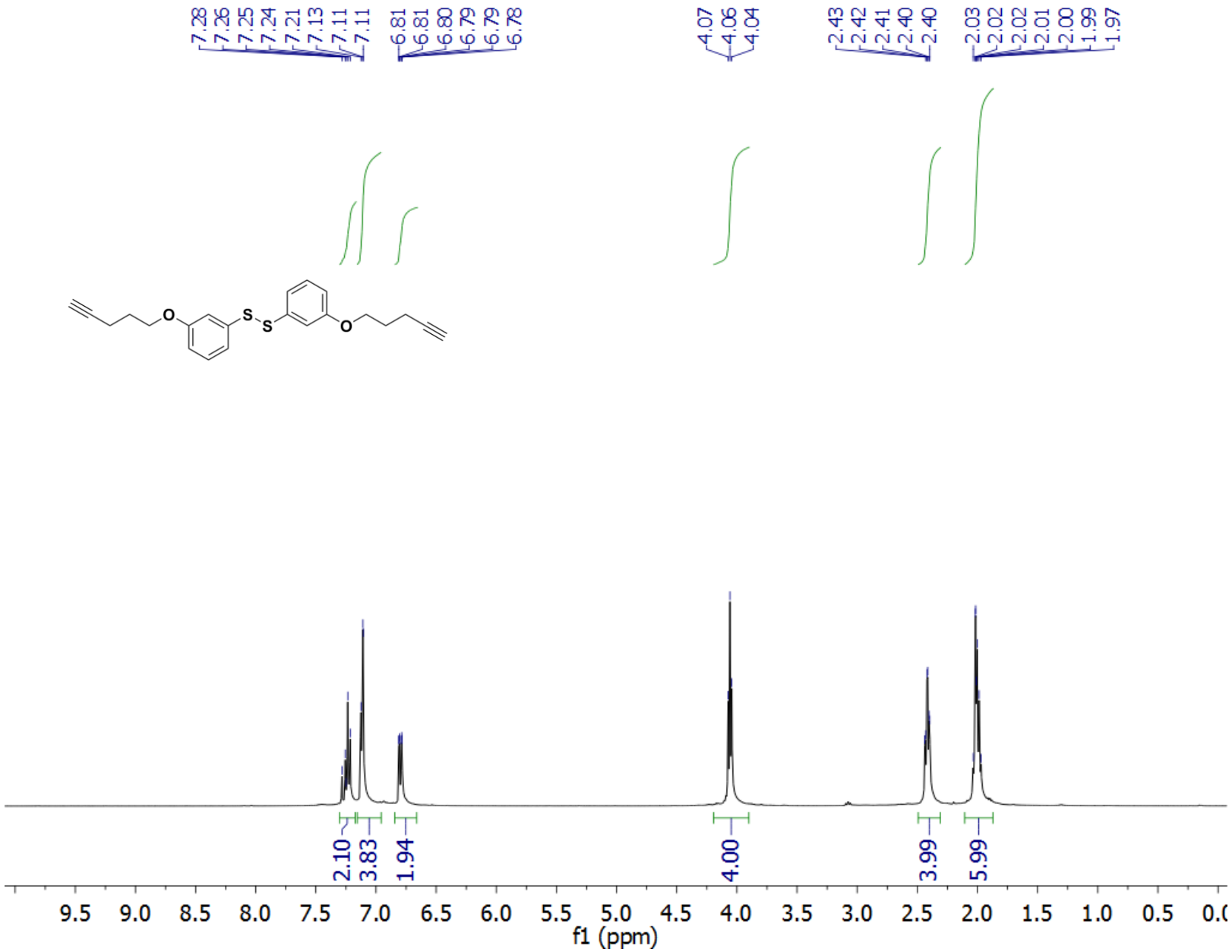
(c) Treatment of lysate with glutathione (GSH) – The reaction mixture from above step was divided into two equal batches (50 μl each). To one, was added 5 mM GSH (5 μl, 55 mM stock solution) and other was treated with 5 μl of buffer. Resulting reaction mixtures were allowed to shake for 1 h at room temperature. After the completion, the reaction was quenched by filtering through preequilibrated Micro Bio-Spin™ P-30 gel columns (Bio-Rad, Catalog # 7326224) or Micro Bio-Spin™ P-6 gel columns (Bio-Rad, Catalog # 7326222).

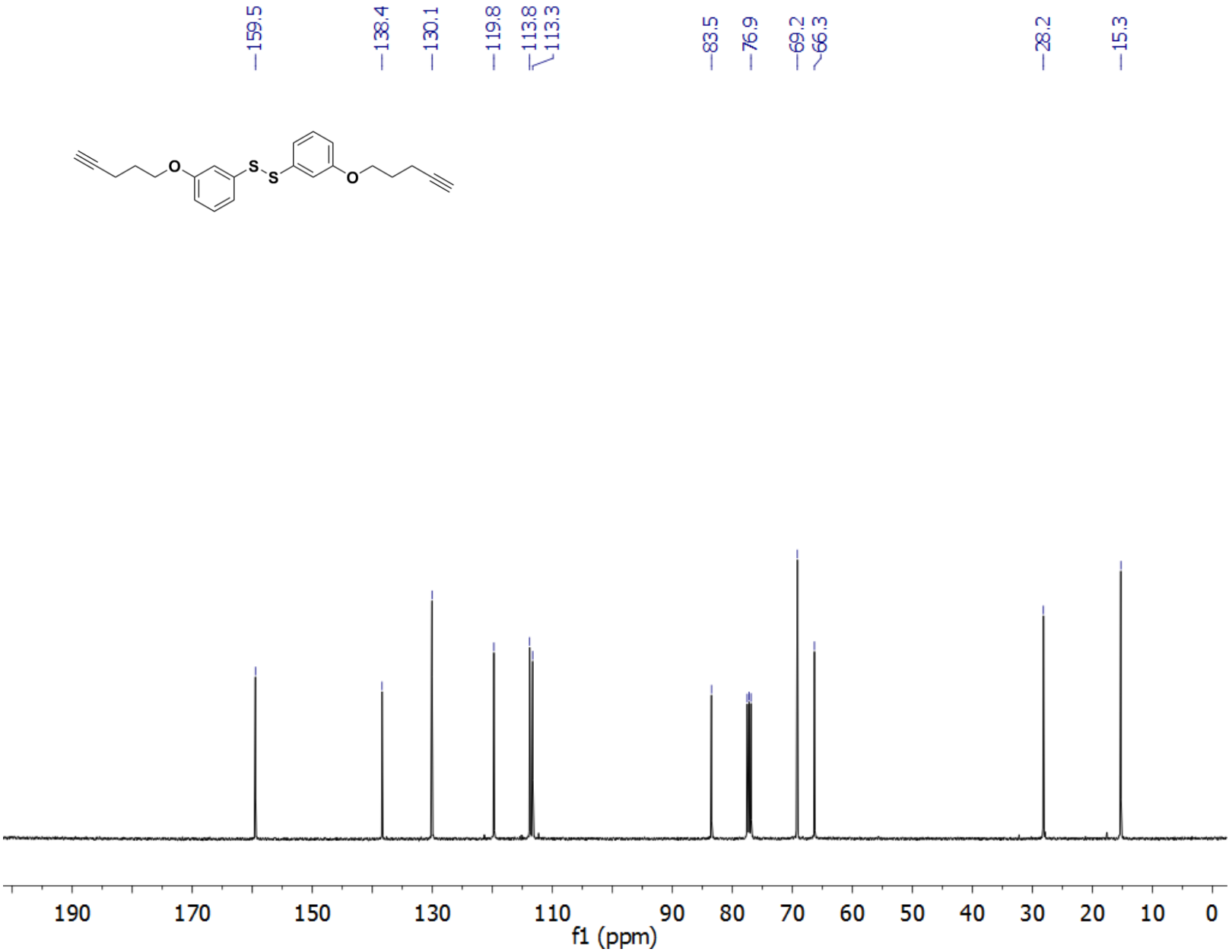
(d) Western blot. Labeled lysate samples were separated by SDS-PAGE (25 μg in each lane) using non-reducing NOVEX Bolt 4 – 12% Bis-Tris Plus gels (Life Technologies) and transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad). After transfer, the membrane was

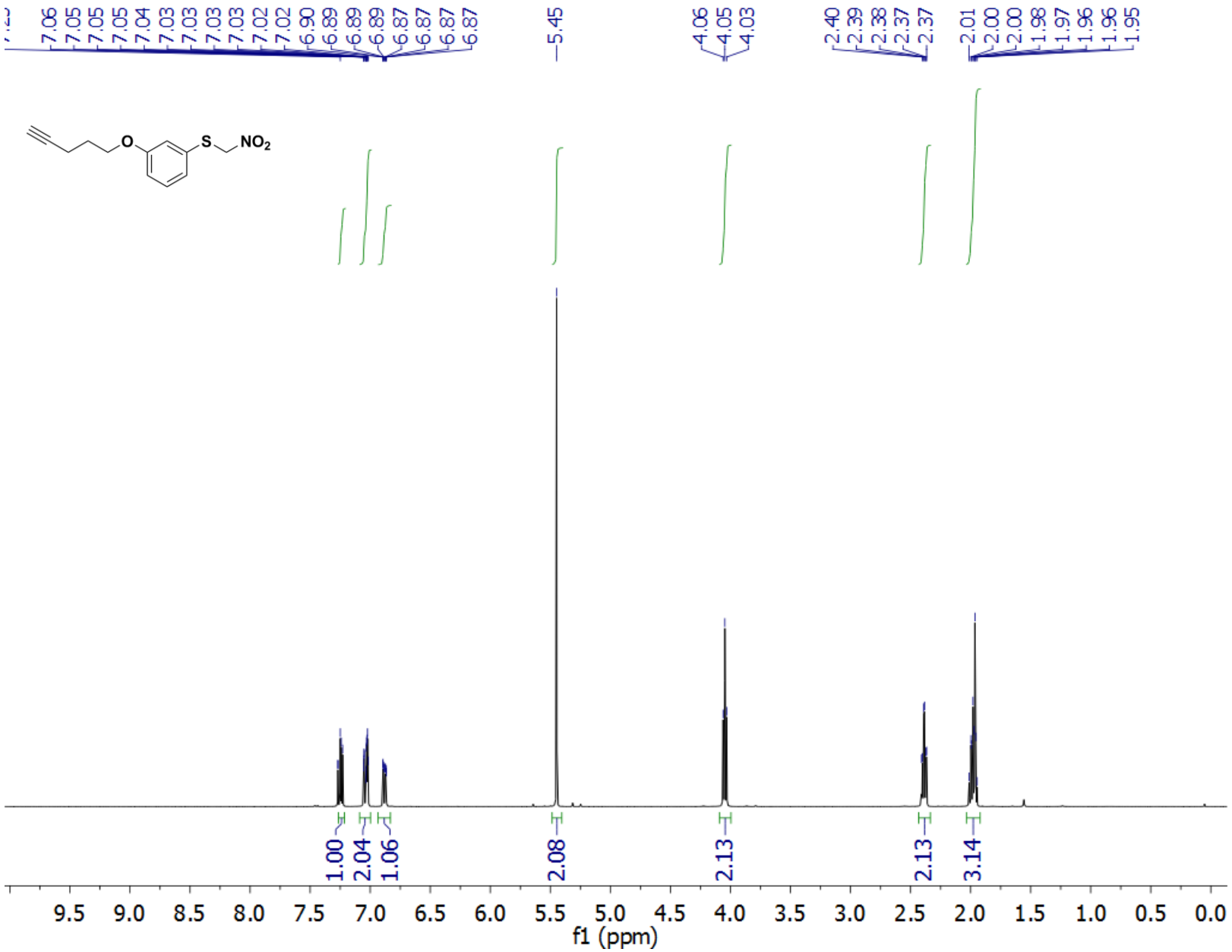
washed with TBST (3 x 5 min.) and blocked with 3% BSA in TBST overnight at 4 °C. The membrane was washed with TBST (3 x 5 min.) and immunoblotting was performed with the following primary and secondary antibodies at the indicated dilutions in TBST, unless otherwise noted: streptavidin-HRP (GE Healthcare, 1:80000), His-HRP (Pierce, 1:50000), β-actin (1:1000) rabbit anti-mouse IgG-HRP (Invitrogen, 1:40000). PVDF membrane was developed with chemiluminescence (GE Healthcare ECL Plus Western Blot Detection System) and imaged by film.

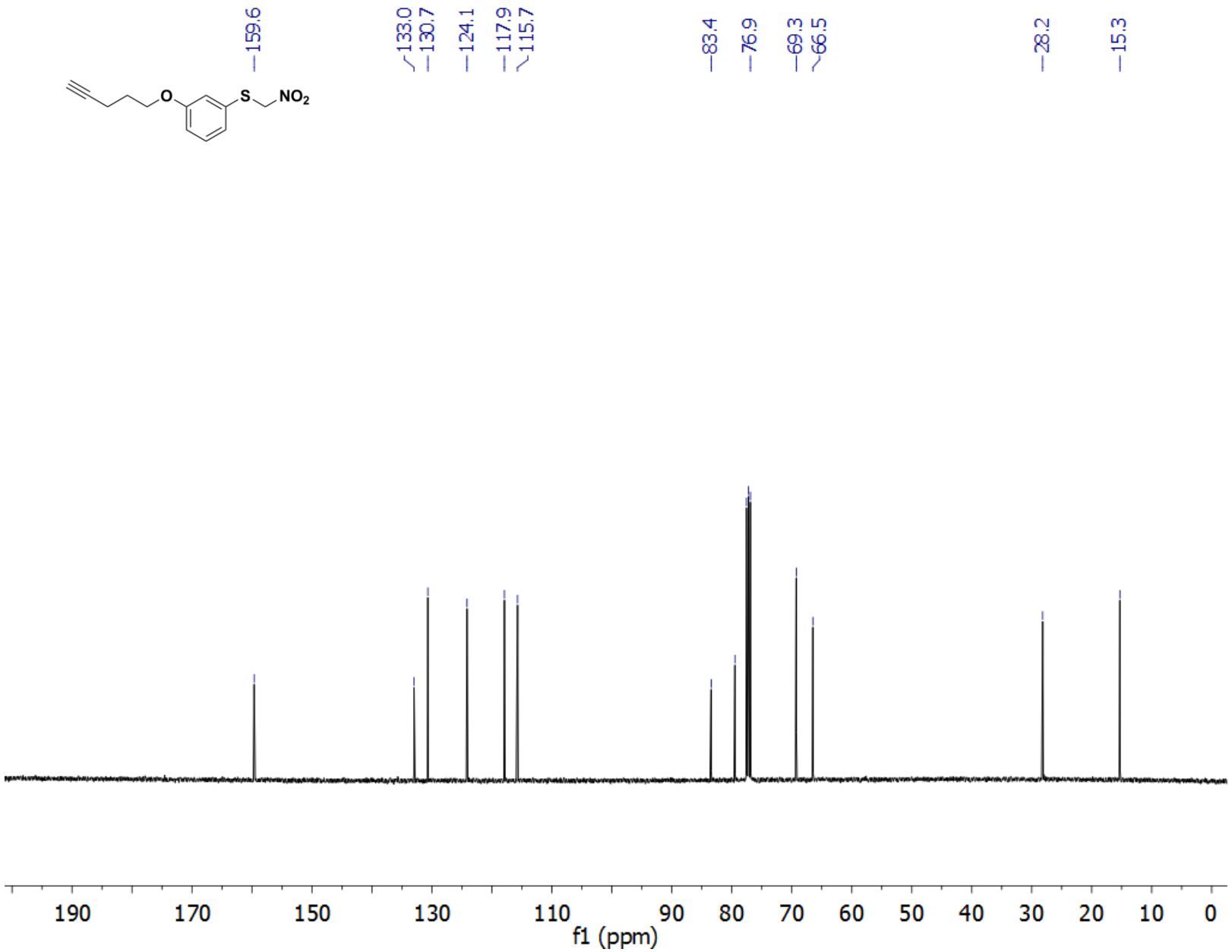


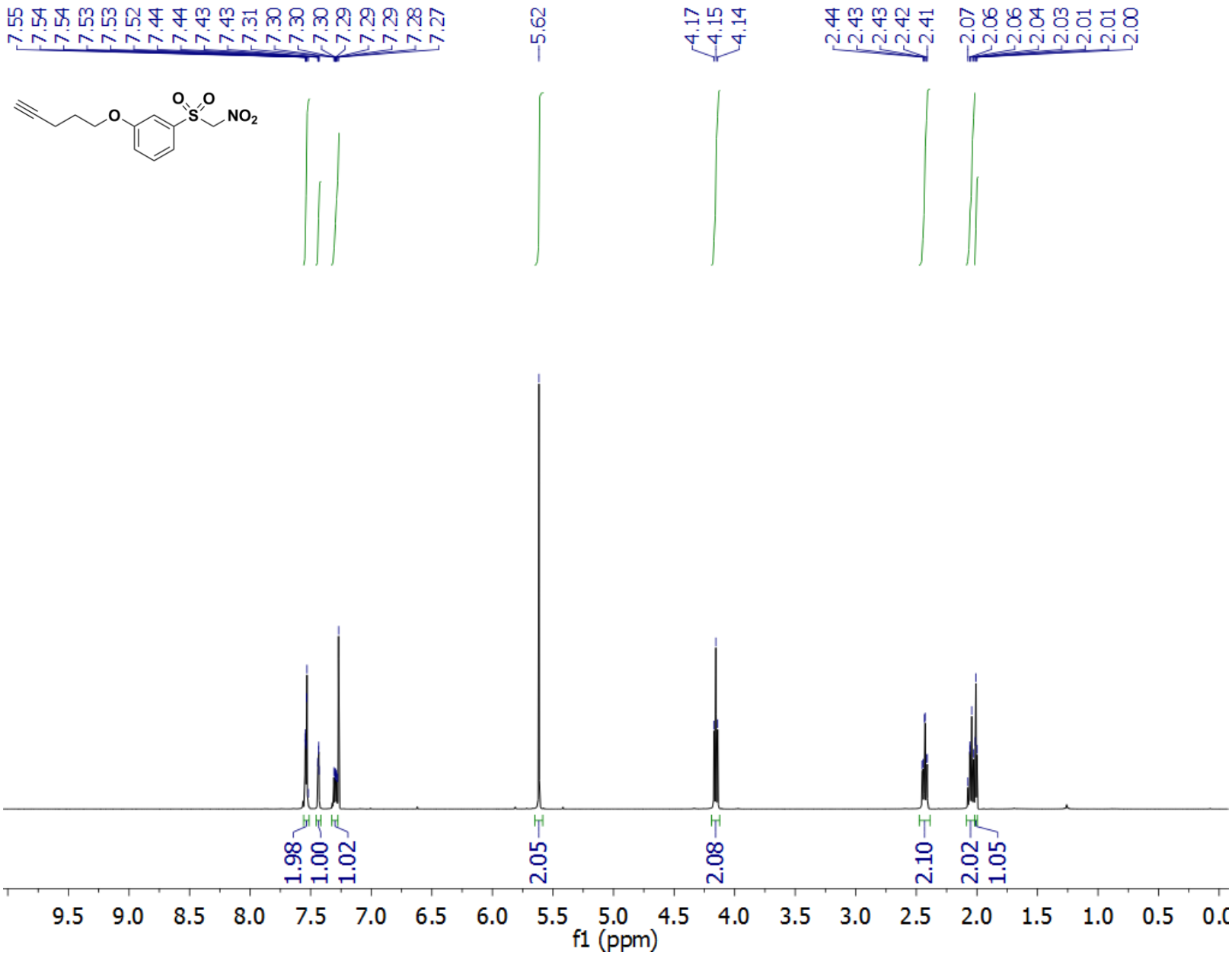
NMR Spectra

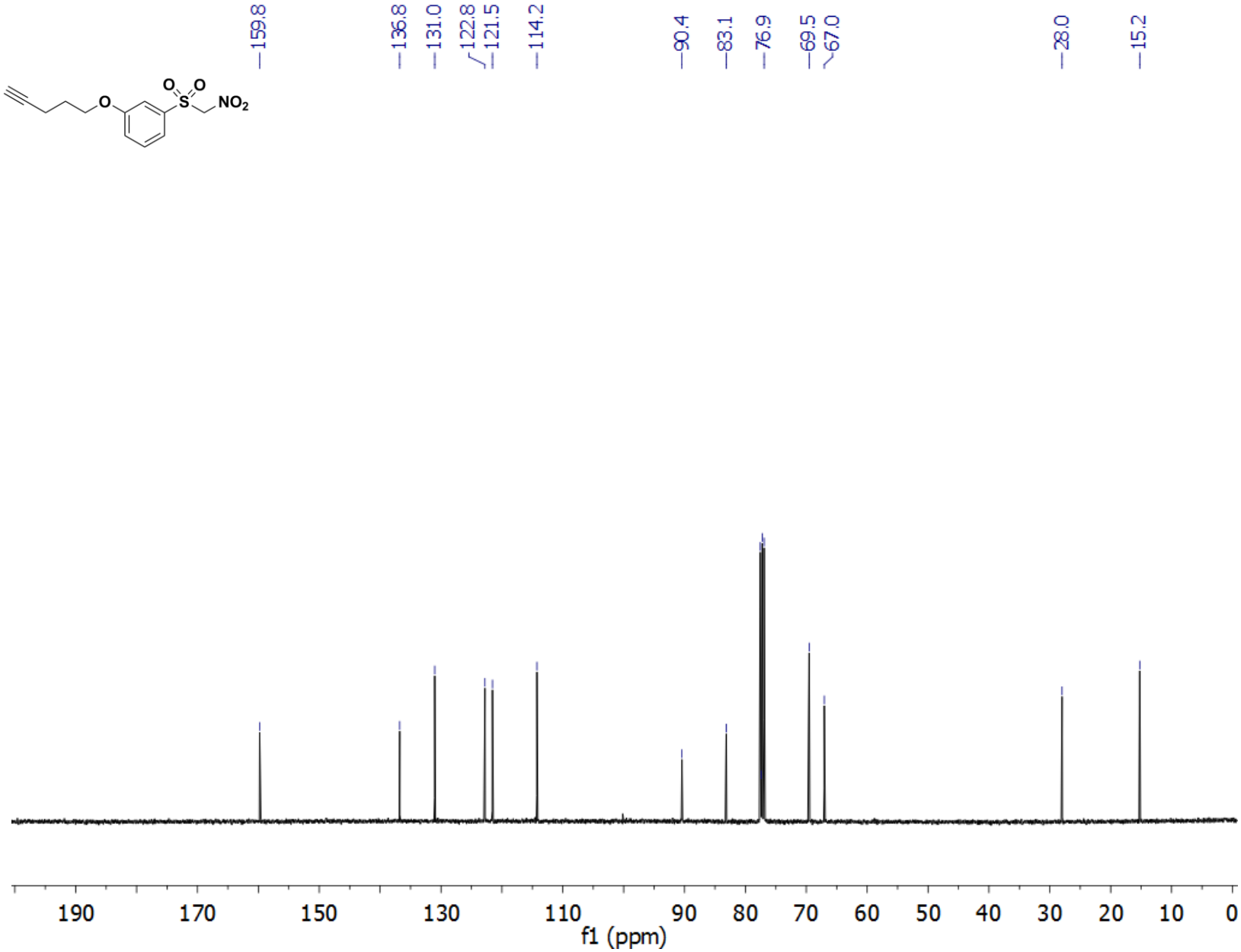

7.28
7.26
7.25
7.24
7.21
7.13
7.11
7.11
6.81
6.81
6.80
6.79
6.79
6.78
4.07
4.06
4.04
2.43
2.42
2.41
2.40
2.40
2.03
2.02
2.02
2.01
2.00
1.99
1.97
O
S
S
O
2.10
3.83
1.94
4.00
3.99
5.99
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

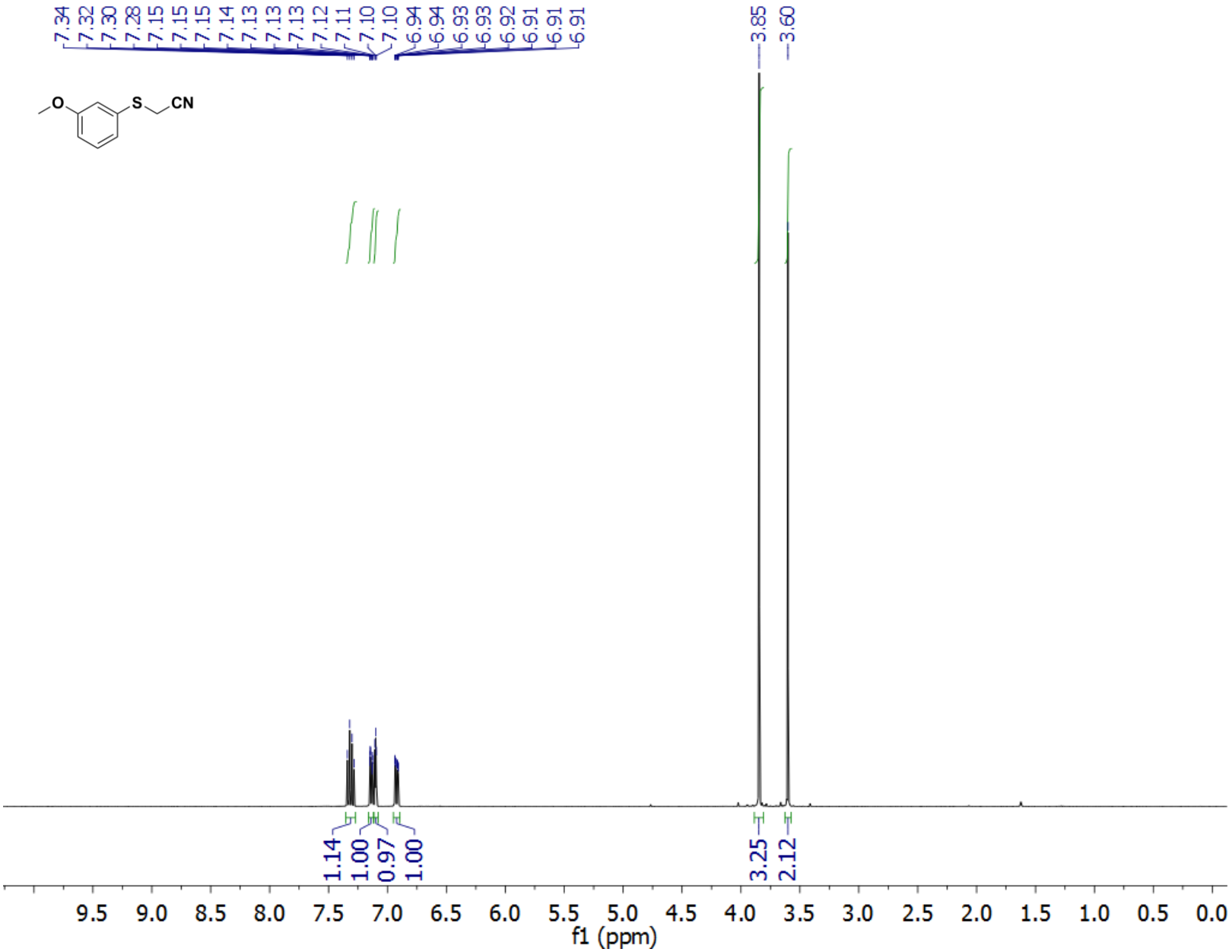

159.5
138.4
130.1
119.8
113.8
113.3
83.5
76.9
69.2
66.3
28.2
15.3
O
S
S
O
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

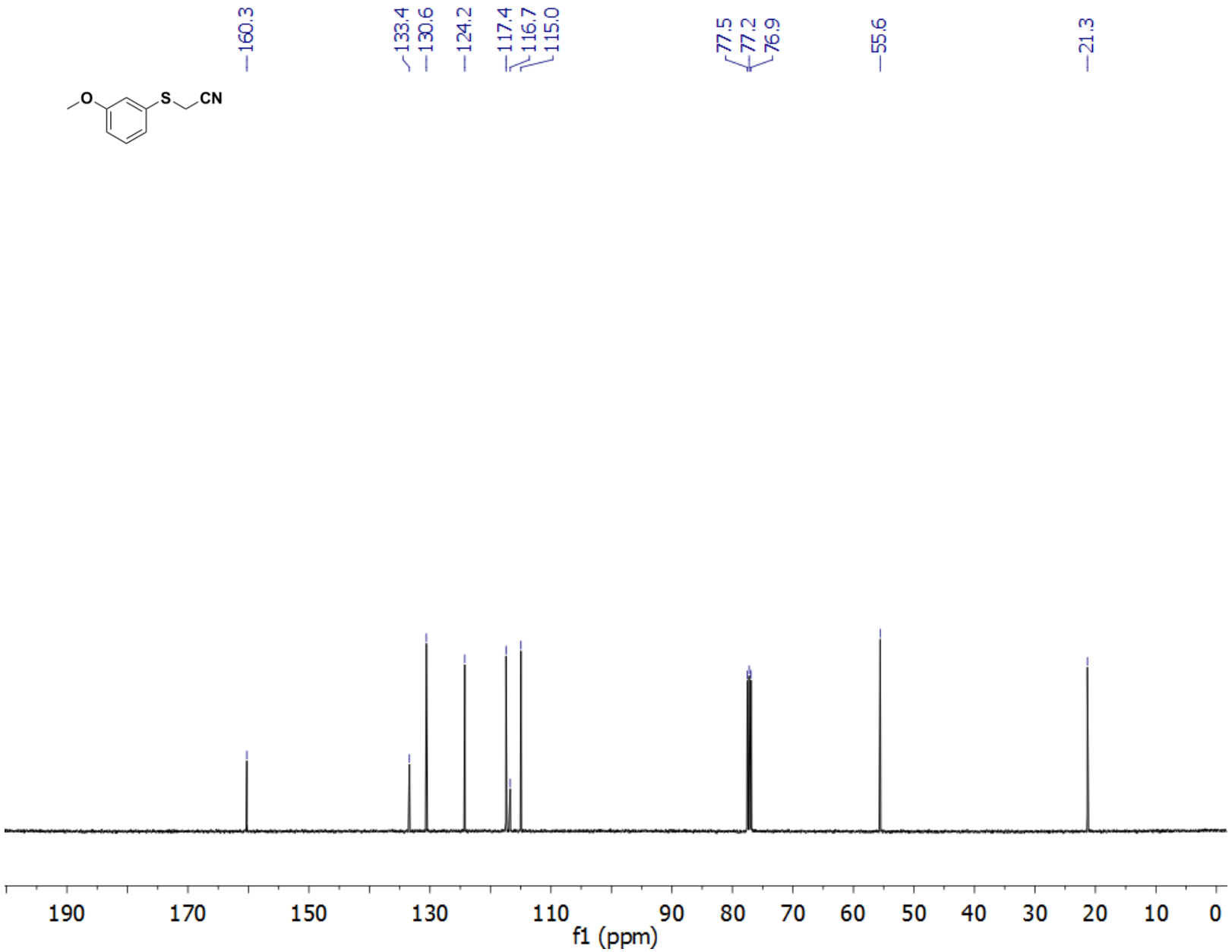

7.06
7.05
7.05
7.05
7.04
7.03
7.03
7.03
7.03
7.02
7.02
6.90
6.89
6.89
6.89
6.87
6.87
6.87
6.87
5.45
4.06
4.05
4.03
2.40
2.39
2.38
2.37
2.37
2.01
2.00
2.00
1.98
1.97
1.96
1.96
1.95
O
S
NO2
1.00
2.04
1.06
2.08
2.13
2.13
3.14
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

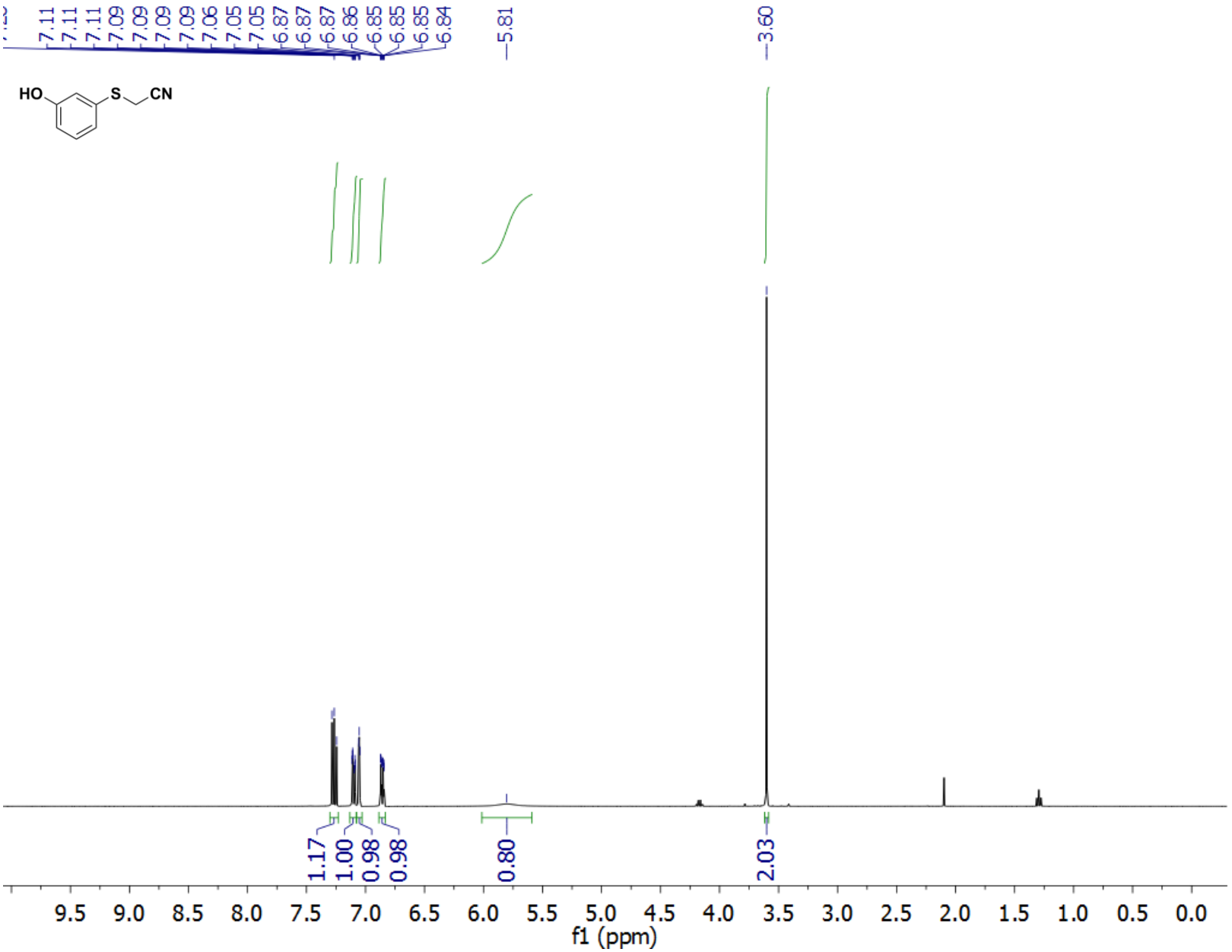

159.6
133.0
130.7
124.1
117.9
115.7
83.4
76.9
69.3
66.5
28.2
15.3
O
S
NO2
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

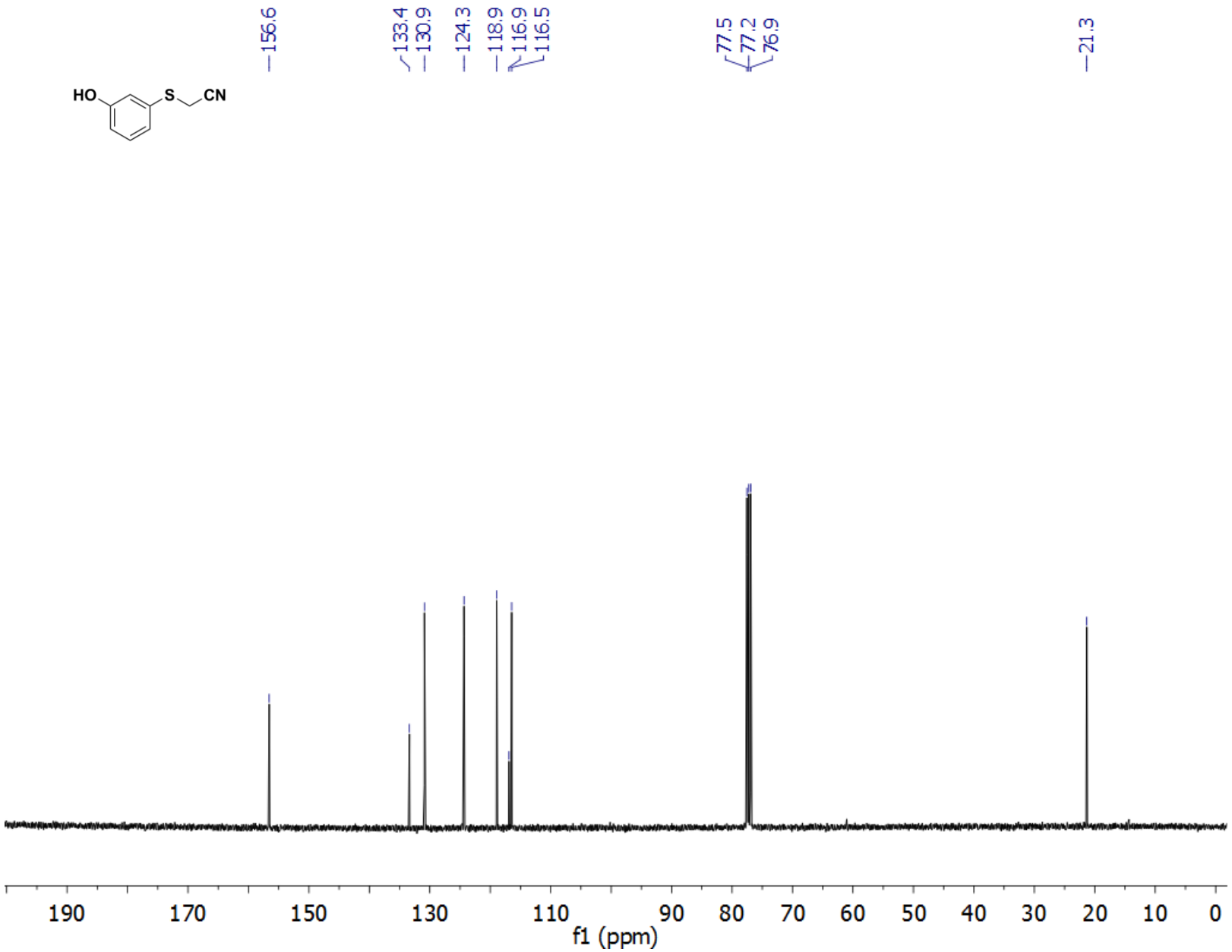

7.55
7.54
7.54
7.53
7.53
7.52
7.44
7.44
7.43
7.43
7.31
7.30
7.30
7.30
7.29
7.29
7.29
7.28
7.27
5.62
4.17
4.15
4.14
2.44
2.43
2.43
2.42
2.41
2.07
2.06
2.06
2.04
2.03
2.01
2.01
2.00
O
O
O
S
NO2
1.98
1.00
1.02
2.05
2.08
2.10
2.02
1.05
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

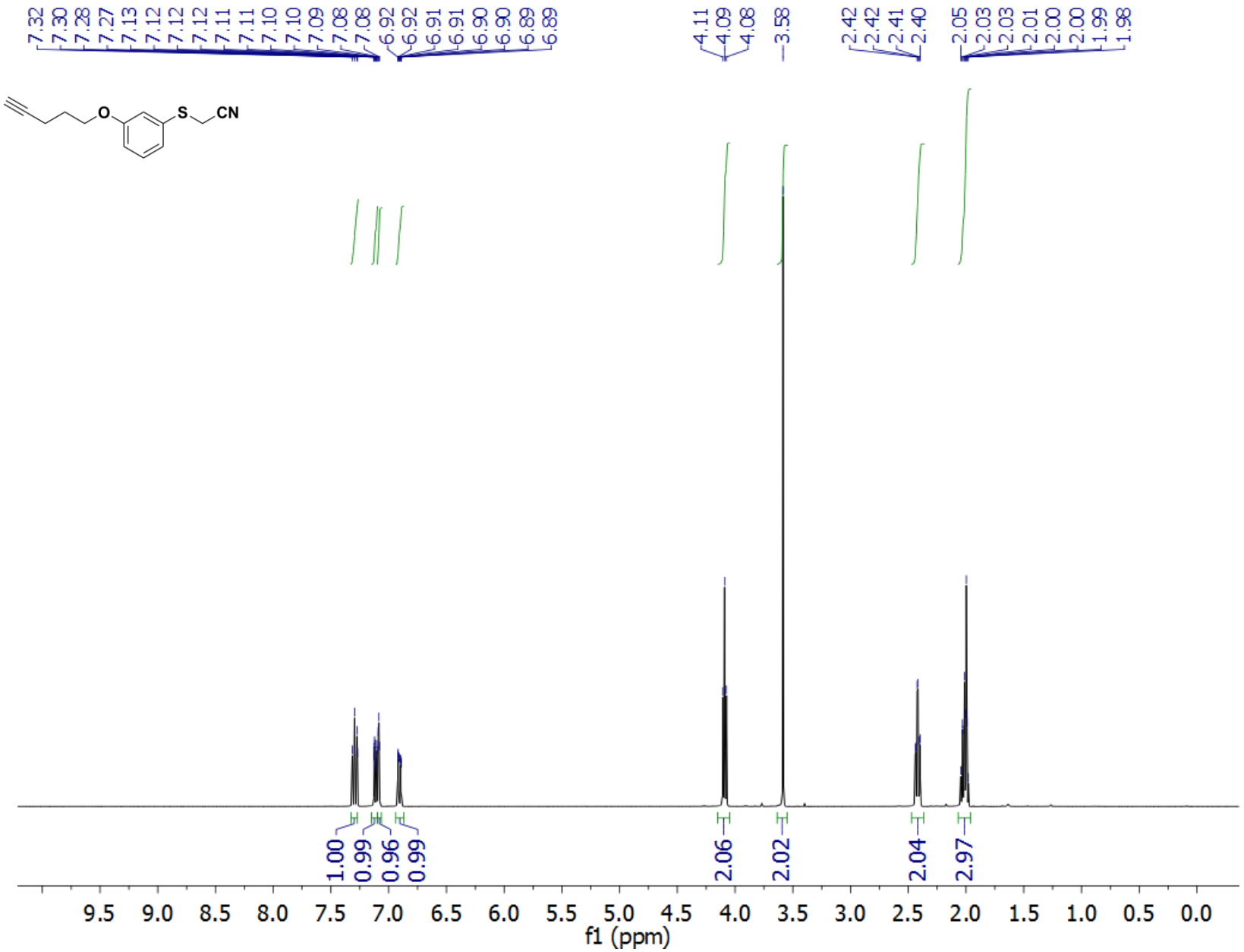

159.8
136.8
131.0
122.8
121.5
114.2
90.4
83.1
76.9
69.5
67.0
28.0
15.2
O
O
O
S
NO2
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

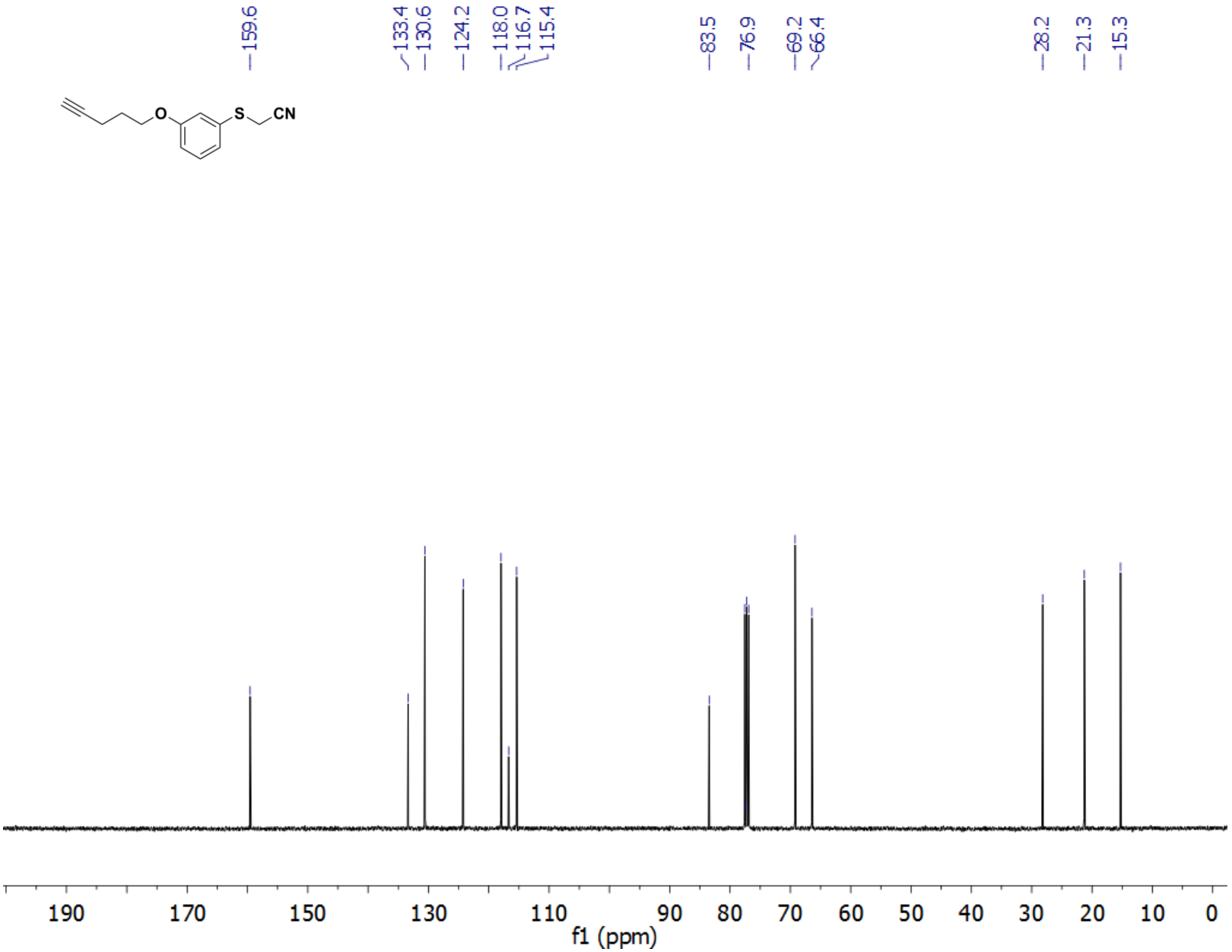

7.34
7.32
7.30
7.28
7.15
7.15
7.15
7.14
7.13
7.13
7.13
7.12
7.11
7.10
7.10
6.94
6.94
6.93
6.93
6.92
6.91
6.91
6.91
3.85
3.60
O
S
CN
1.14
1.00
0.97
1.00
3.25
2.12
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

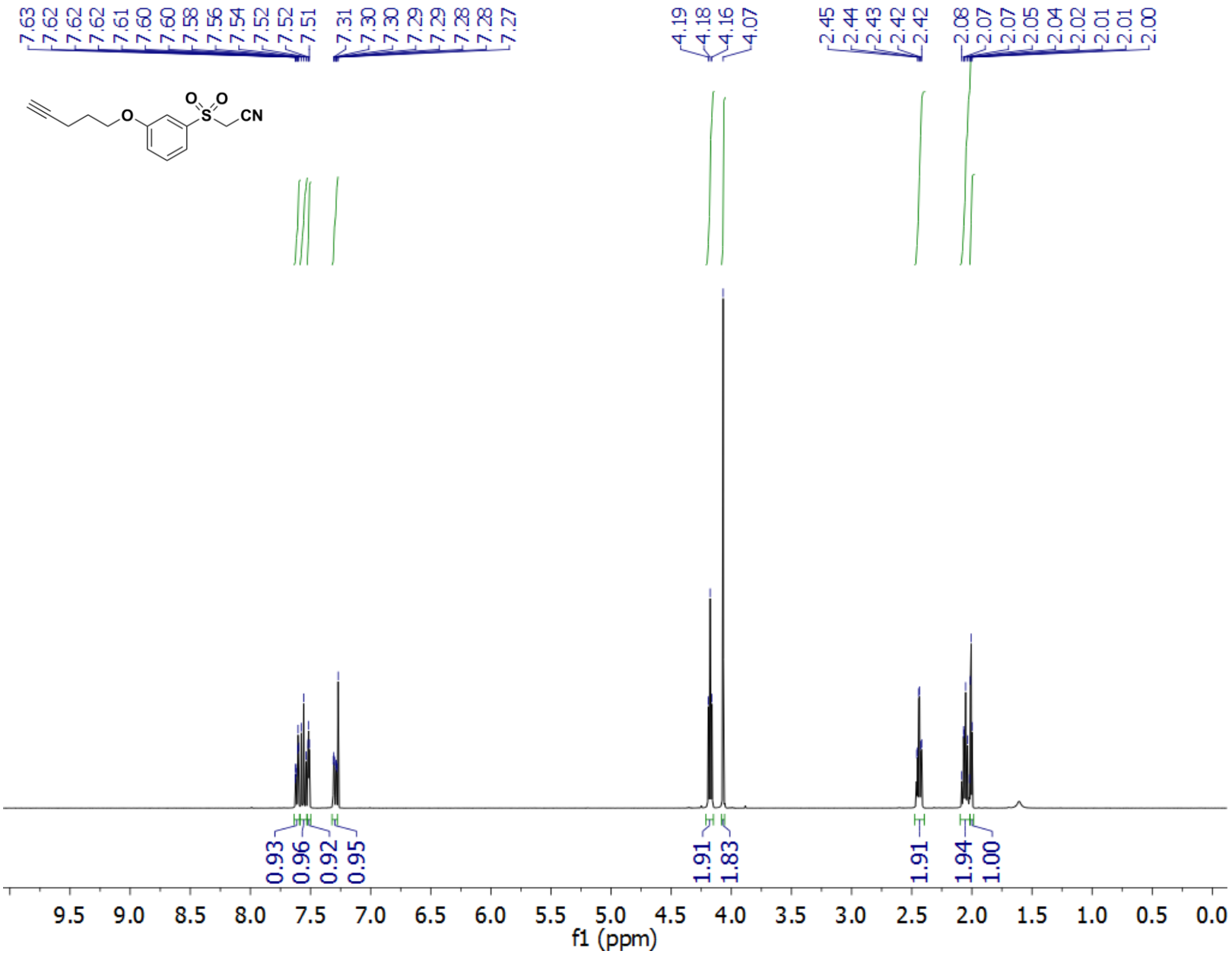

160.3
133.4
130.6
124.2
117.4
116.7
115.0
77.5
77.2
76.9
55.6
21.3
O
S
CN
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

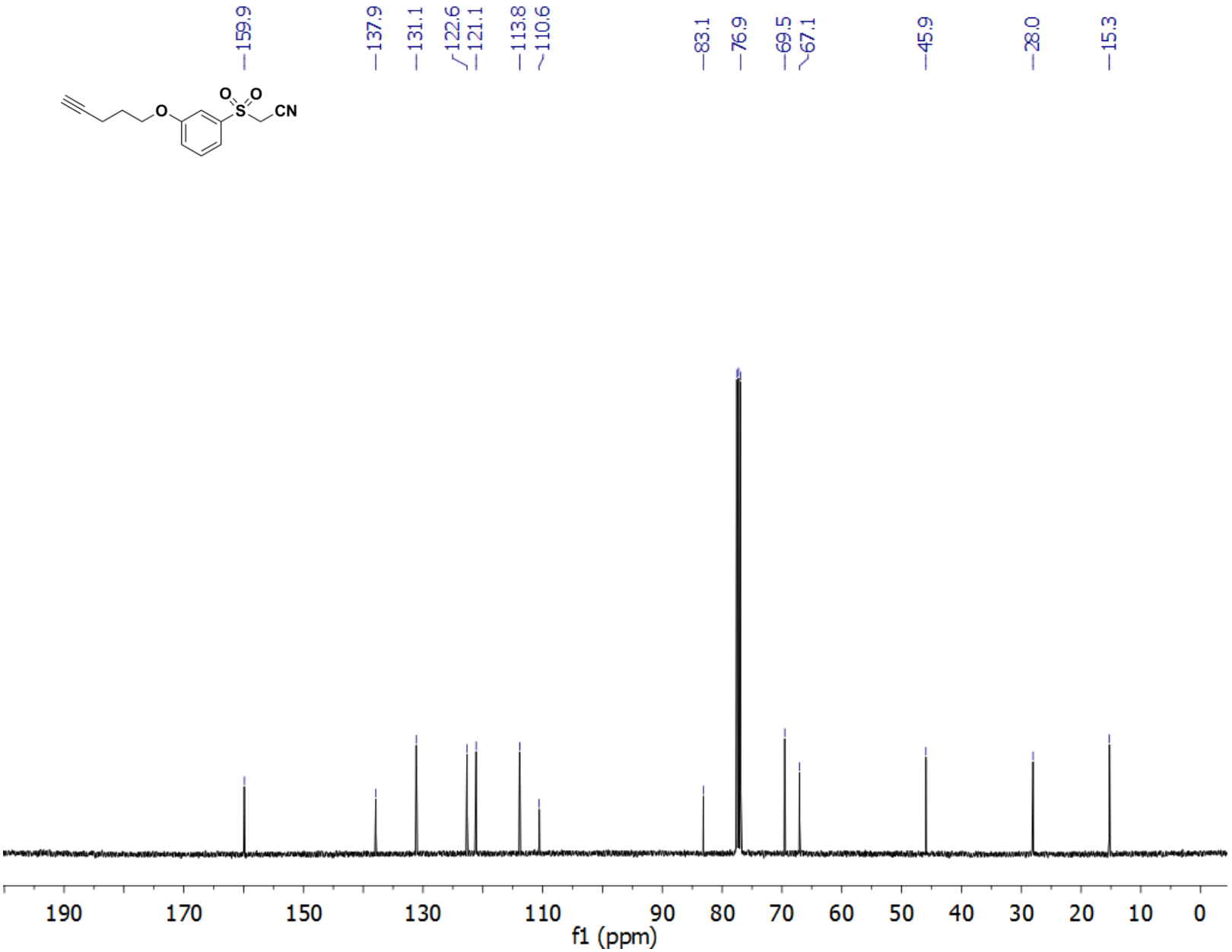

7.11
7.11
7.11
7.09
7.09
7.09
7.09
7.06
7.05
7.05
6.87
6.87
6.87
6.86
6.85
6.85
6.85
6.84
5.81
3.60
HO
S
CN
1.17
1.00
0.98
0.98
0.80
2.03
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

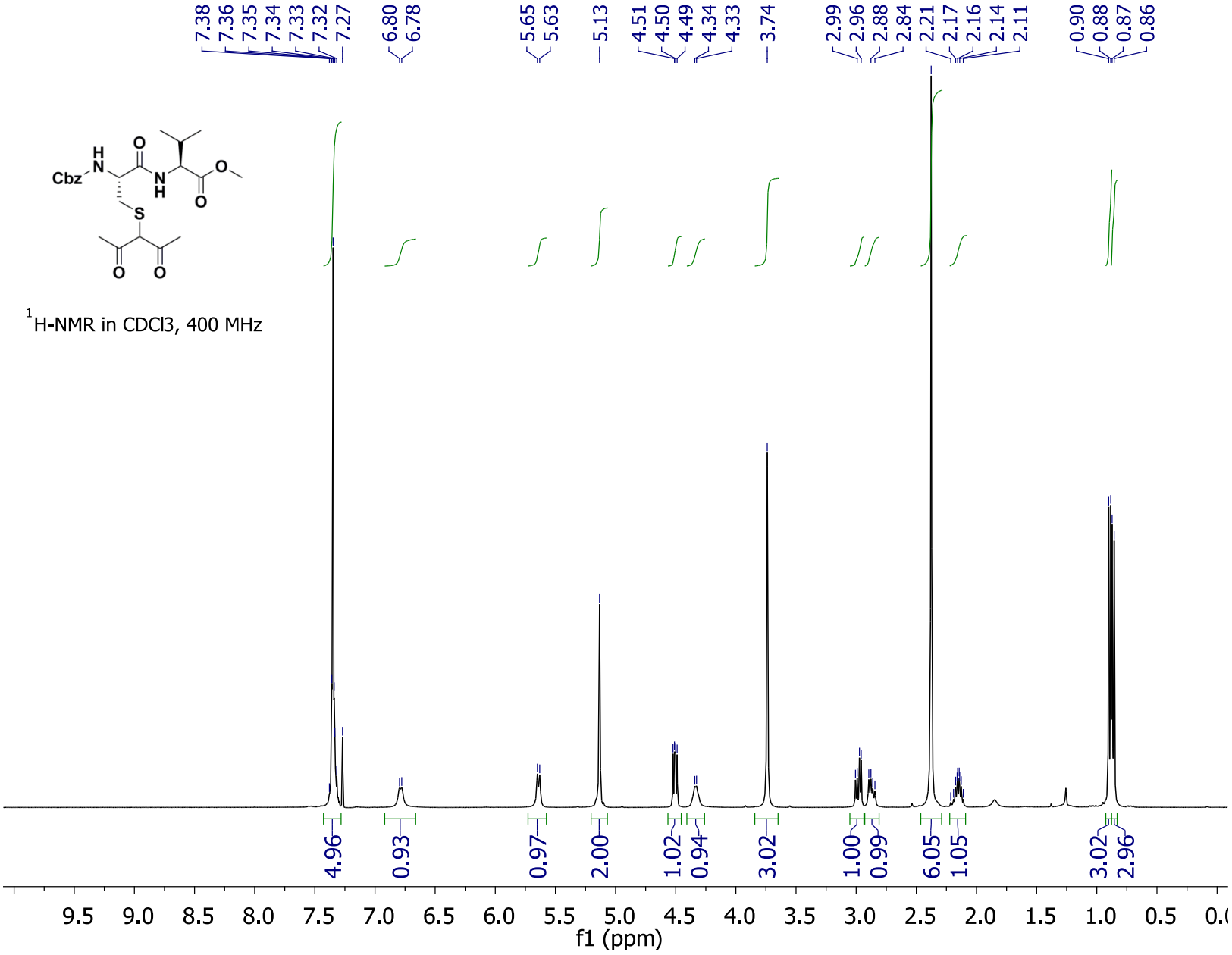

HO
S
CN
156.6
133.4
130.9
124.3
118.9
116.9
116.5
77.5
77.2
76.9
21.3
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

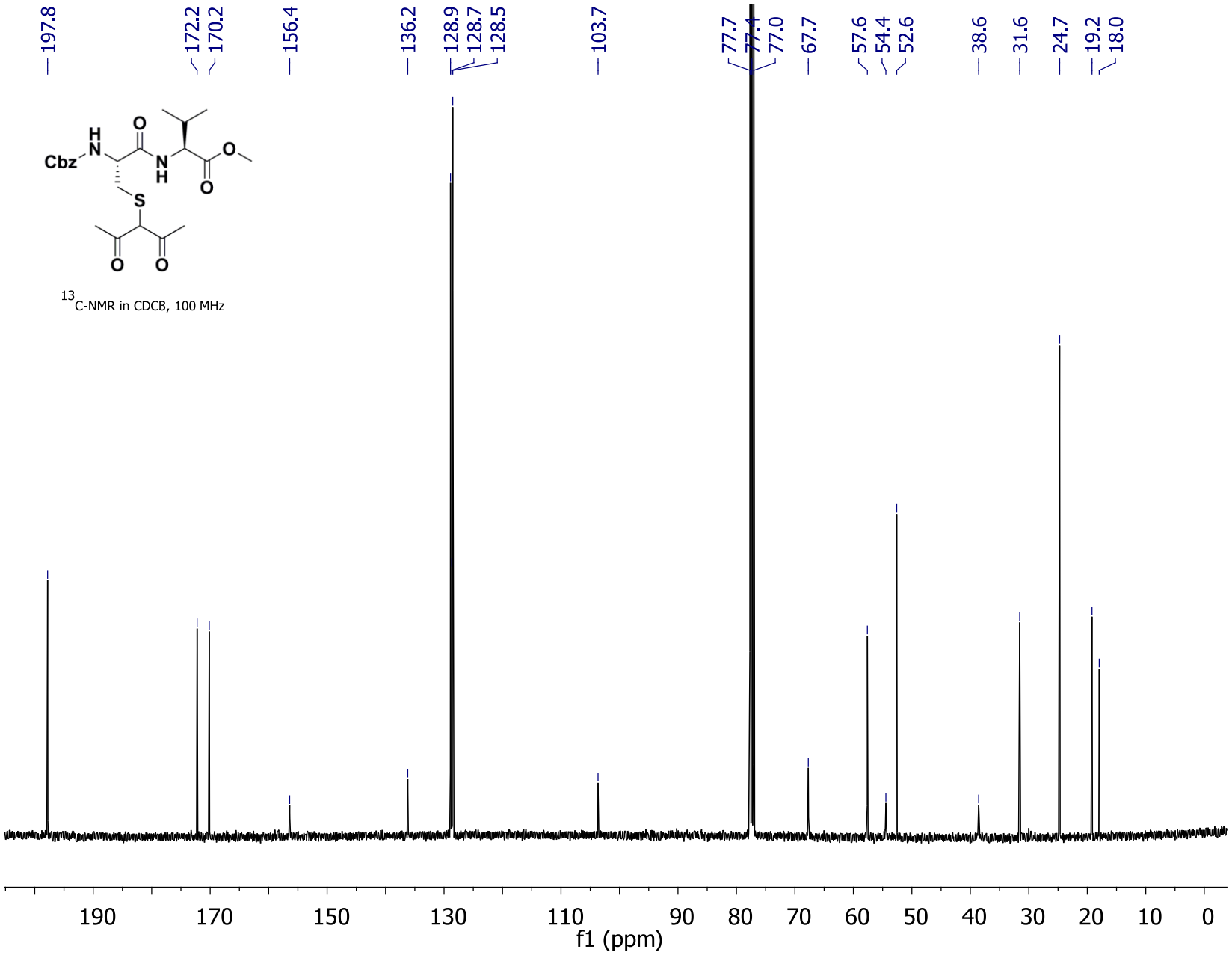

7.32
7.30
7.28
7.27
7.13
7.12
7.12
7.12
7.11
7.11
7.10
7.10
7.09
7.08
7.08
6.92
6.92
6.91
6.91
6.90
6.90
6.89
6.89
4.11
4.09
4.08
3.58
2.42
2.42
2.41
2.40
2.05
2.03
2.03
2.01
2.00
2.00
1.99
1.98
O
S
CN
1.00
0.99
0.96
0.99
2.06
2.02
2.04
2.97
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

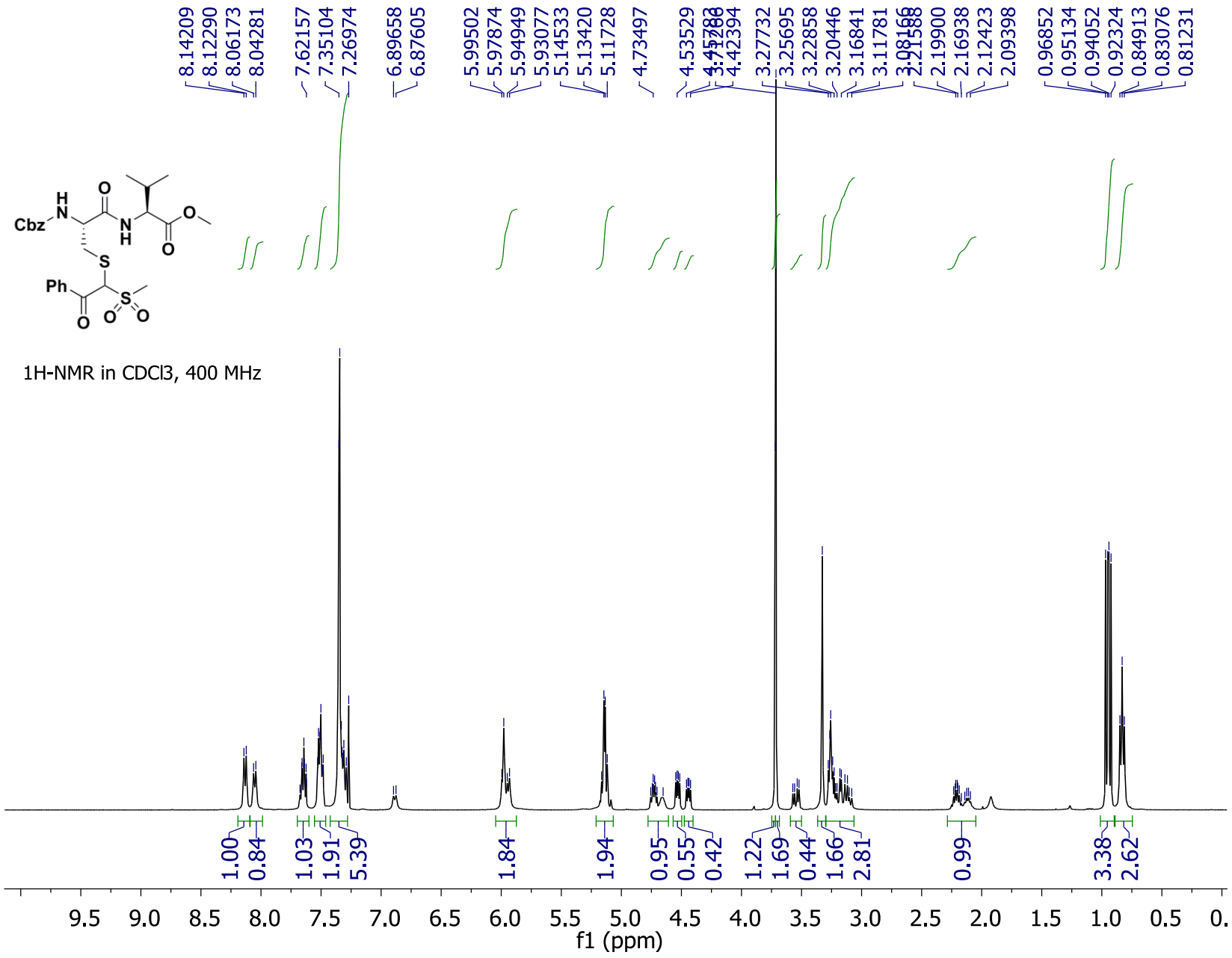

159.6
133.4
130.6
124.2
118.0
116.7
115.4
83.5
76.9
69.2
66.4
28.2
21.3
15.3
O
S
CN
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

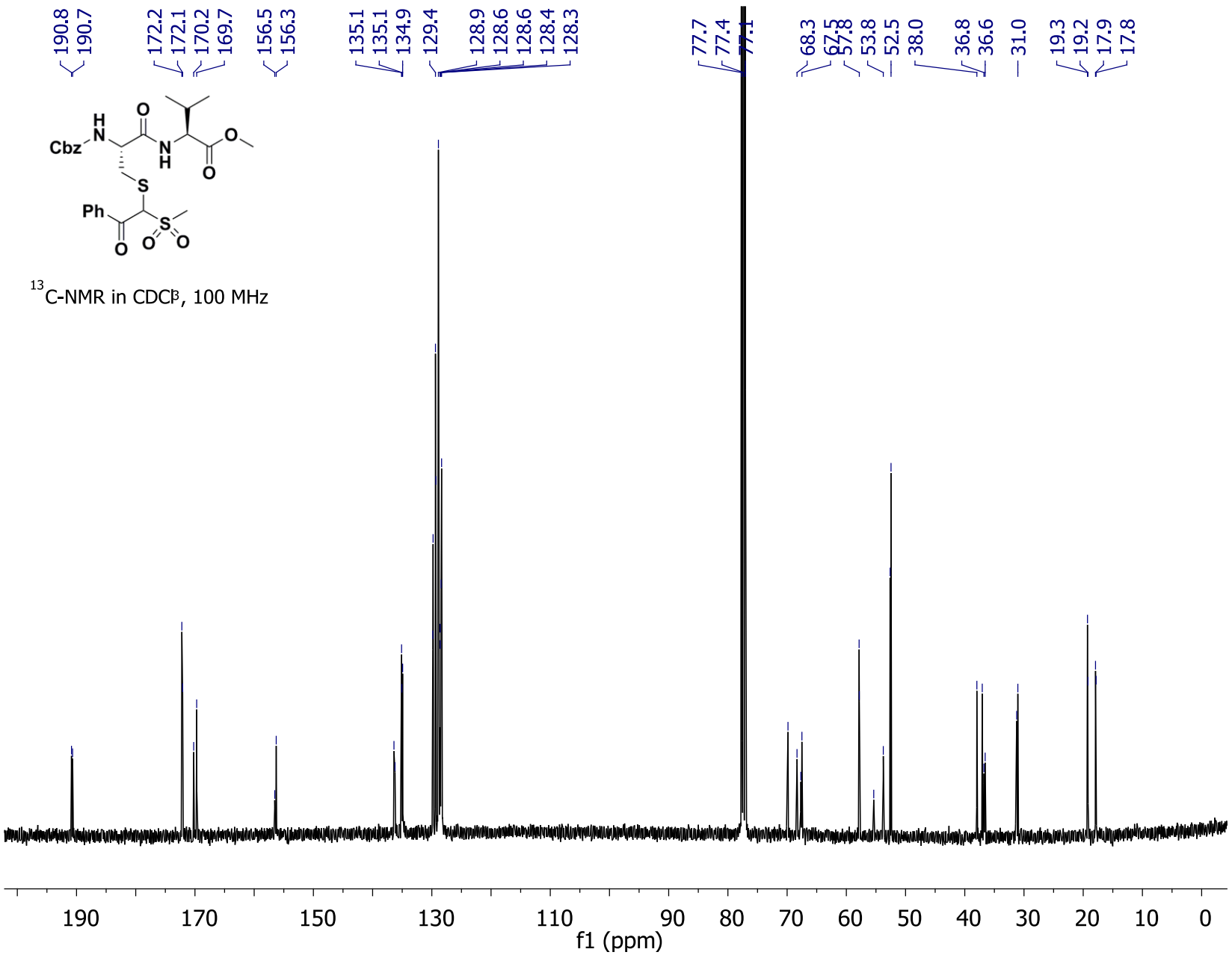

7.63
7.62
7.62
7.62
7.61
7.60
7.60
7.58
7.56
7.54
7.52
7.52
7.51
7.31
7.30
7.30
7.29
7.29
7.28
7.28
7.27
4.19
4.18
4.16
4.07
2.45
2.44
2.43
2.42
2.42
2.08
2.07
2.07
2.05
2.04
2.02
2.01
2.01
2.00
O
O
S
O
CN
0.93
0.96
0.92
0.95
1.91
1.83
1.91
1.94
1.00
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)

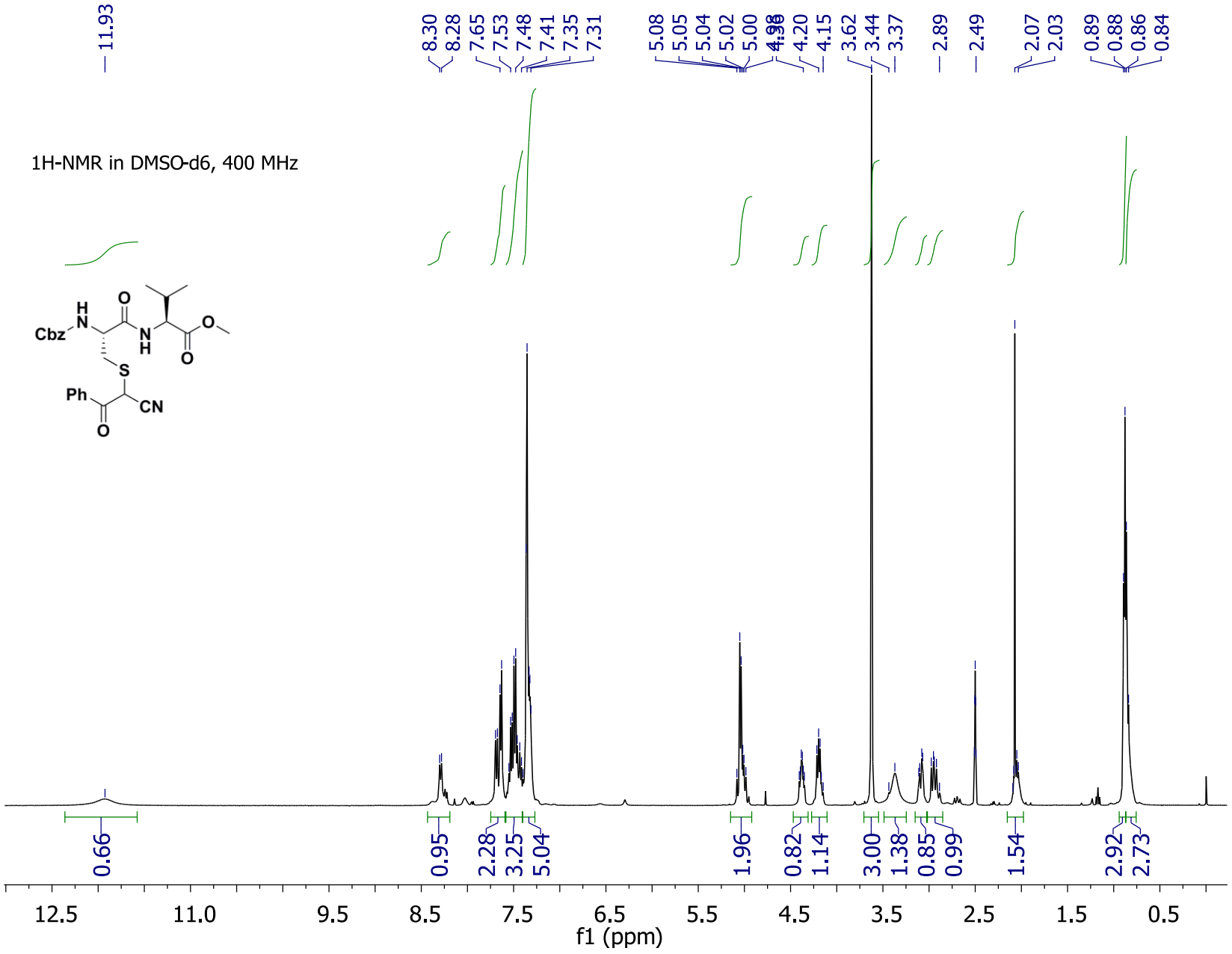

159.9
137.9
131.1
122.6
121.1
113.8
110.6
83.1
76.9
69.5
67.1
45.9
28.0
15.3
O
O
S
O
CN
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

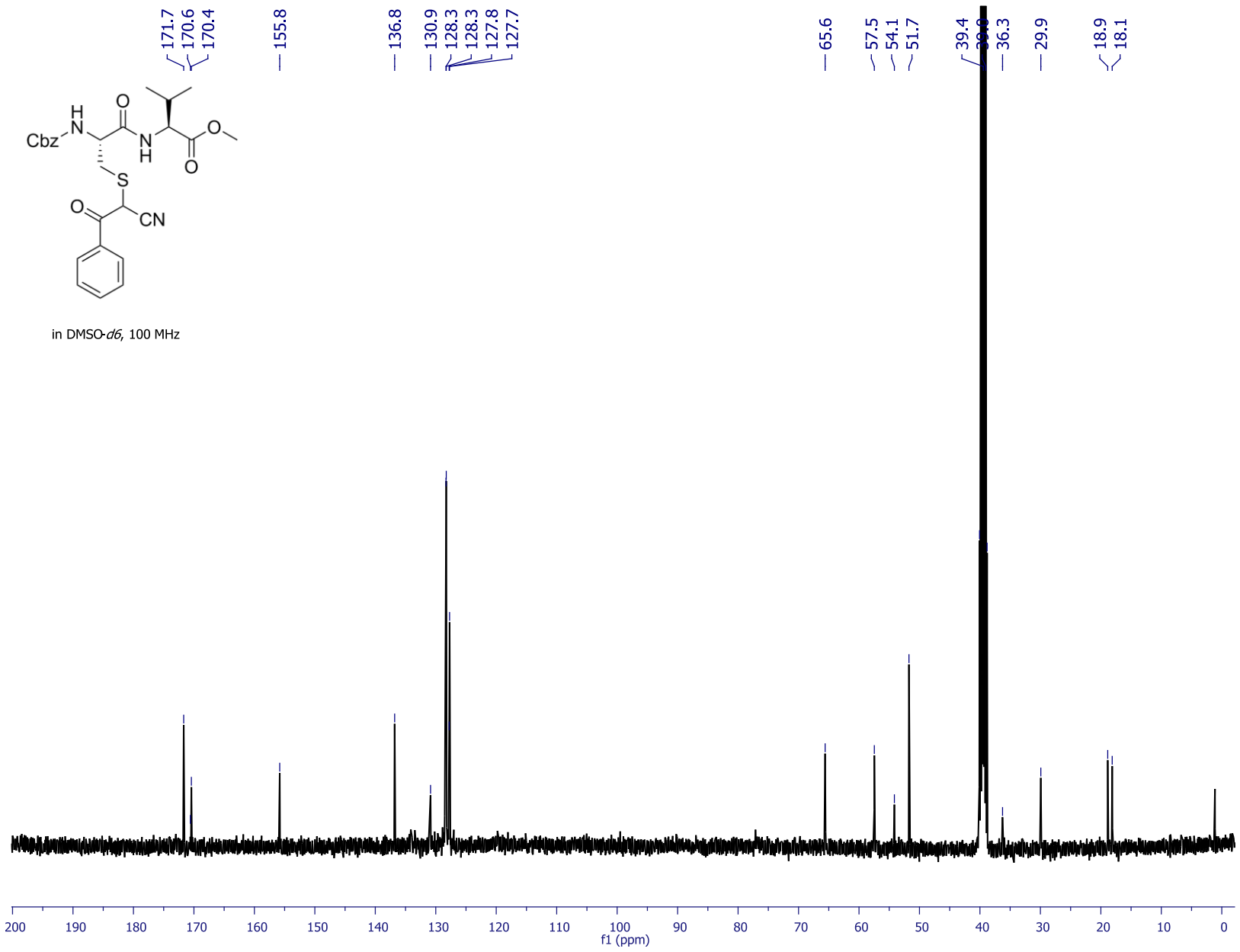

7.38
7.36
7.35
7.34
7.33
7.32
7.27
6.80
6.78
5.65
5.63
5.13
4.51
4.50
4.49
4.34
4.33
3.74
2.99
2.96
2.88
2.84
2.21
2.17
2.16
2.14
2.11
0.90
0.88
0.87
0.86
Cbz
H
N
O
N
H
O
O
S
O
O
1H-NMR in CDCl3, 400 MHz
4.96
0.93
0.97
2.00
1.02
0.94
3.02
1.00
0.99
6.05
1.05
3.02
2.96
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
f1 (ppm)

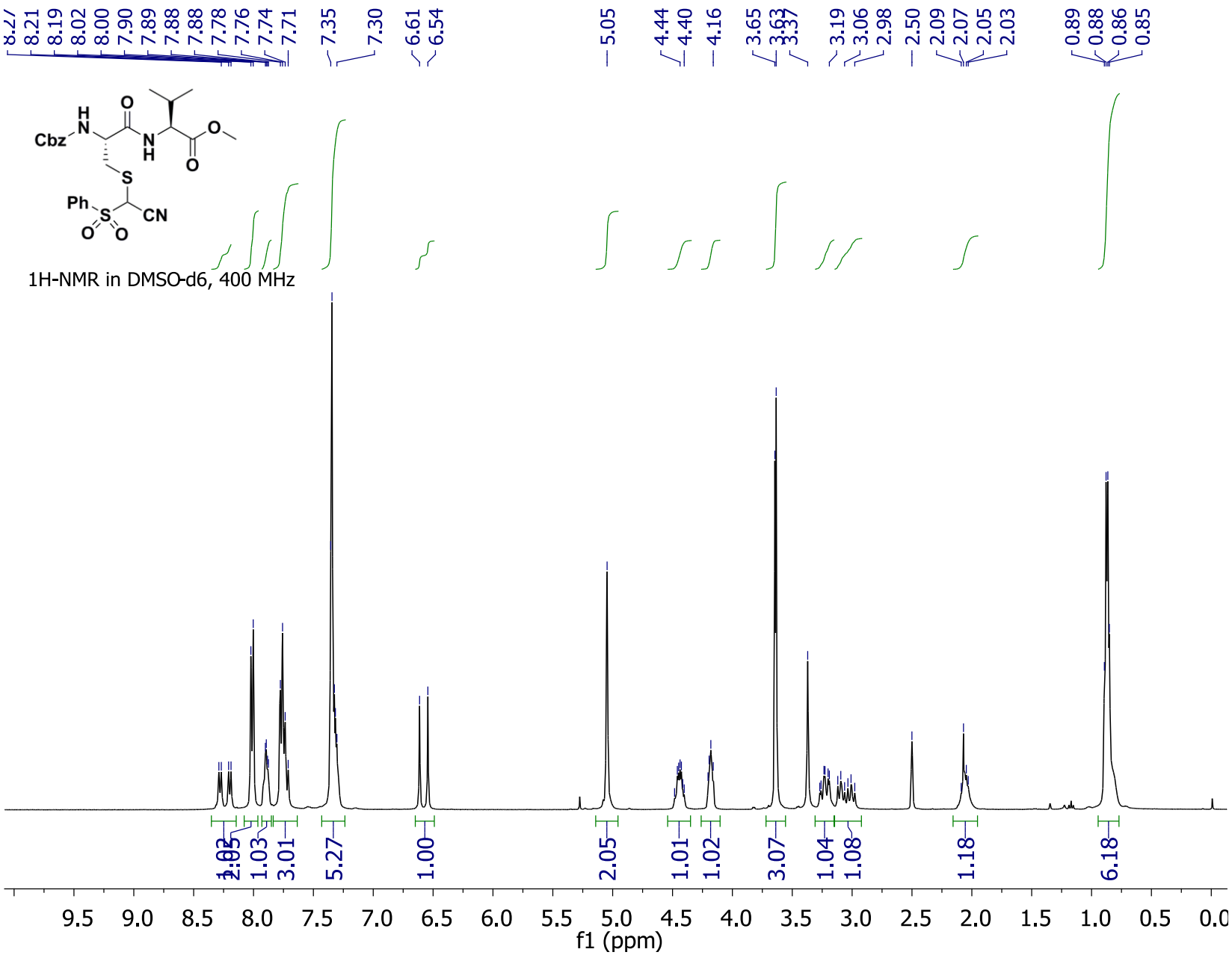

197.8
172.2
170.2
156.4
136.2
128.9
128.7
128.5
103.7
77.7
77.4
77.0
67.7
57.6
54.4
52.6
38.6
31.6
24.7
19.2
18.0
Cbz
H
N
O
N
H
O
O
S
O
O
13C-NMR in CDCB, 100 MHz
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

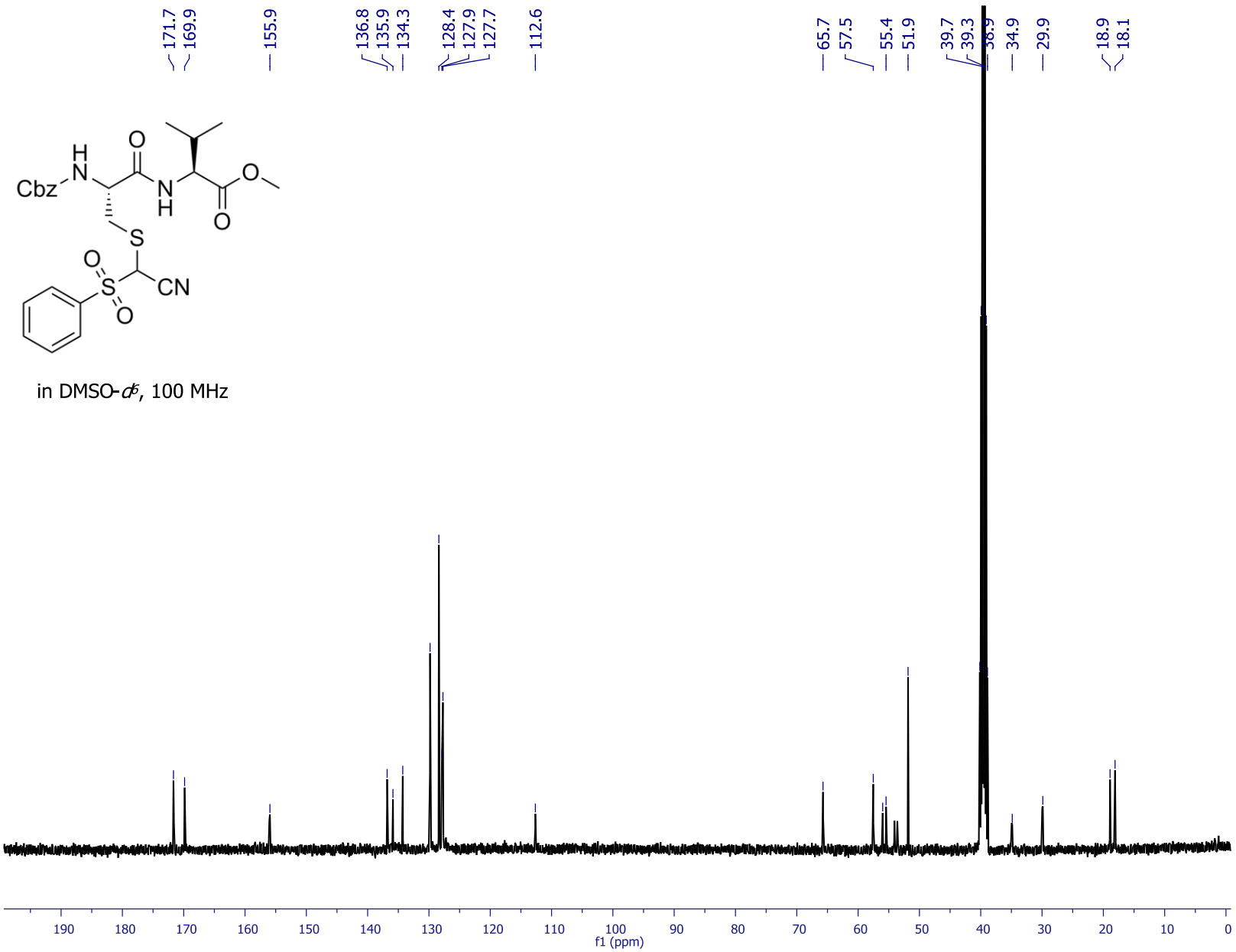

8.14209
8.12290
8.06173
8.04281
7.62157
7.35104
7.26974
6.89658
6.87605
5.99502
5.97874
5.94949
5.93077
5.14533
5.13420
5.11728
4.73497
4.53529
4.42394
3.27732
3.25695
3.22858
3.20446
3.16841
3.11781
2.19900
2.16938
2.12423
2.09398
0.96852
0.95134
0.94052
0.92324
0.84913
0.83076
0.81231
Cbz
Ph
1H-NMR in CDCl3, 400 MHz
1.00
0.84
1.03
1.91
5.39
1.84
1.94
0.95
0.55
0.42
1.22
1.69
0.44
1.66
2.81
0.99
3.38
2.62
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.
f1 (ppm)


190.8
190.7
172.2
172.1
170.2
169.7
156.5
156.3
135.1
135.1
134.9
129.4
128.9
128.6
128.6
128.4
128.3
77.7
77.4
77.1
68.3
67.5
57.8
53.8
52.5
38.0
36.8
36.6
31.0
19.3
19.2
17.9
17.8
Cbz
Ph
^{13}C-NMR in CDCl$_3$, 100 MHz
190
170
150
130
110
90
80
70
60
50
40
30
20
10
0
f1 (ppm)

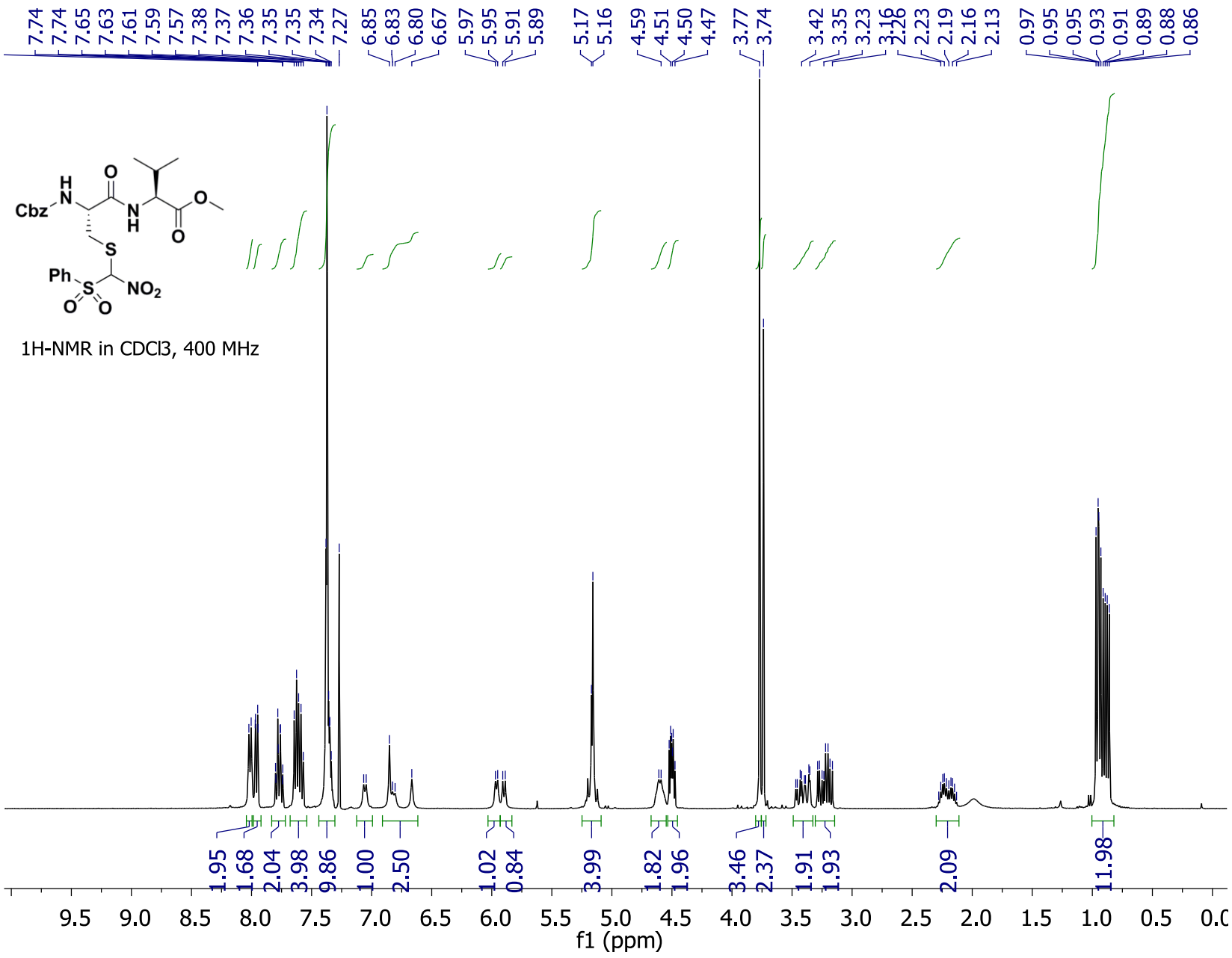

1H-NMR in DMSO-d6, 400 MHz
11.93
8.30
8.28
7.65
7.53
7.48
7.41
7.35
7.31
5.08
5.05
5.04
5.02
5.00
4.20
4.15
3.62
3.44
3.37
2.89
2.49
2.07
2.03
0.89
0.88
0.86
0.84
Cbz
Ph
CN
0.66
0.95
2.28
3.25
5.04
1.96
0.82
1.14
3.00
1.38
0.85
0.99
1.54
2.92
2.73
12.5
11.0
9.5
8.5
7.5
6.5
5.5
4.5
3.5
2.5
1.5
0.5
f1 (ppm)

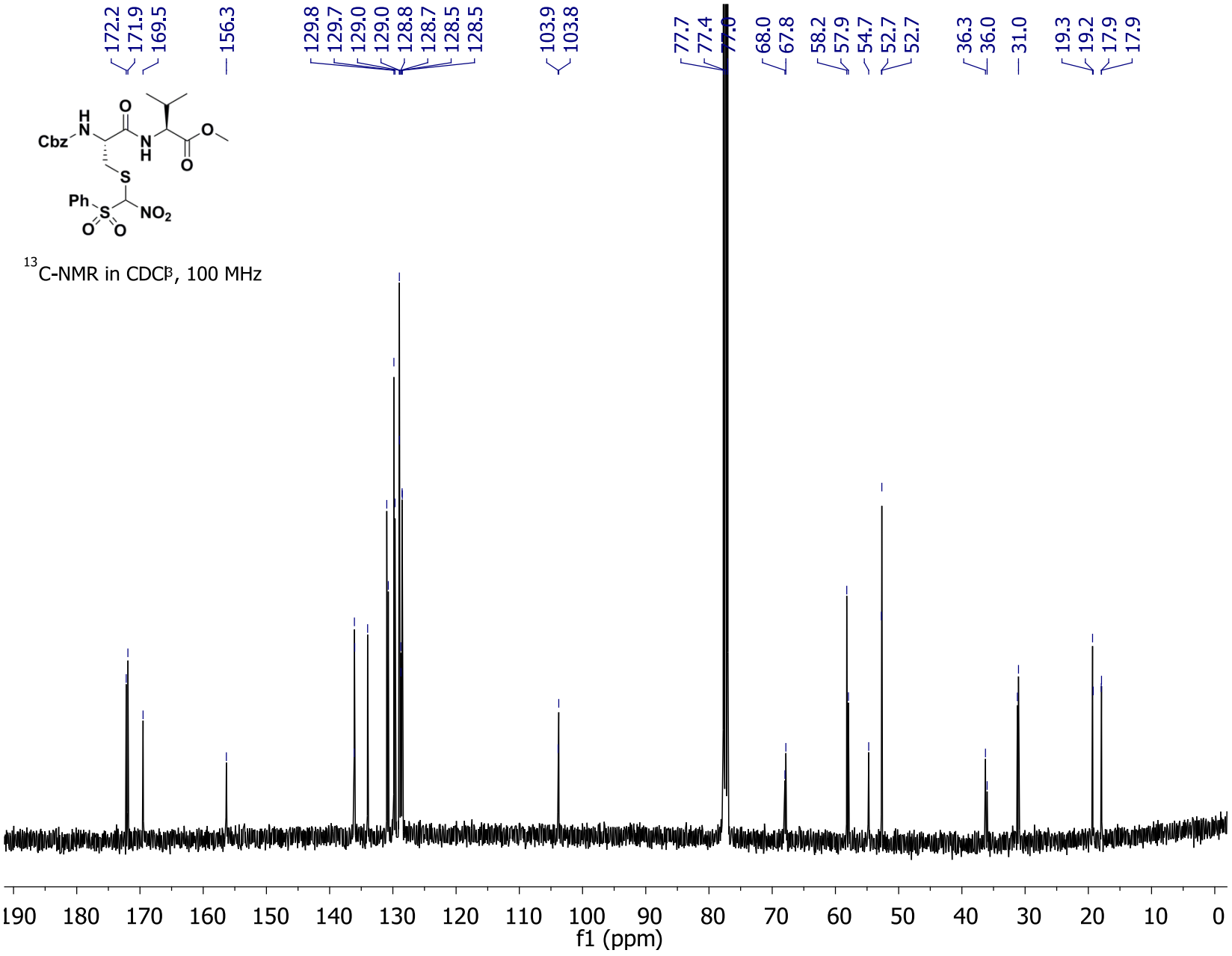

171.7
170.6
170.4
155.8
136.8
130.9
128.3
128.3
127.8
127.7
65.6
57.5
54.1
51.7
39.4
36.3
29.9
18.9
18.1
Cbz
H
N
O
N
H
O
O
S
O
CN
in DMSO-d6, 100 MHz
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


8.27
8.21
8.19
8.02
8.00
7.90
7.89
7.88
7.88
7.78
7.76
7.74
7.71
7.35
7.30
6.61
6.54
5.05
4.44
4.40
4.16
3.65
3.63
3.37
3.19
3.06
2.98
2.50
2.09
2.07
2.05
2.03
0.89
0.88
0.86
0.85
Cbz
Ph
CN
1H-NMR in DMSO-d6, 400 MHz
1.03
2.05
1.03
3.01
5.27
1.00
2.05
1.01
1.02
3.07
1.04
1.08
1.18
6.18
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)



in DMSO-d^6, 100 MHz


7.74 7.74 7.65 7.63 7.61 7.59 7.57 7.38 7.37 7.36 7.35 7.35 7.34 7.27 6.85 6.83 6.80 6.67 5.97 5.95 5.91 5.89 5.17 5.16 4.59 4.51 4.50 4.47 3.77 3.74 3.42 3.35 3.23 3.16 2.26 2.23 2.19 2.16 2.13 0.97 0.95 0.95 0.93 0.91 0.89 0.88 0.86
Cbz
Ph
NO2
1H-NMR in CDCl3, 400 MHz
1.95 1.68 2.04 3.98 9.86 1.00 2.50 1.02 0.84 3.99 1.82 1.96 3.46 2.37 1.91 1.93 2.09 11.98
9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


172.2
171.9
169.5
156.3
129.8
129.7
129.0
129.0
128.8
128.7
128.5
128.5
103.9
103.8
77.7
77.4
77.0
68.0
67.8
58.2
57.9
54.7
52.7
52.7
36.3
36.0
31.0
19.3
19.2
17.9
17.9
Cbz
Ph
NO2
13C-NMR in CDCl3, 100 MHz
190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)