

**Enabling Olefin Metathesis on Proteins:
Chemical Methods for Installation of *S*-Allyl Cysteine**

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General Considerations

Melting points (m.p.) were recorded on a Leica Galen III hot stage microscope equipped with a Testo 720 thermocouple probe and are uncorrected. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a Bruker AV400 (400 MHz) or a Bruker DPX200 (200 MHz) spectrometer, as indicated. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker AV400 (100 MHz) spectrometer or on a Bruker DPX200 (50 MHz) spectrometer, as indicated. All chemical shifts are quoted on the δ scale in ppm using residual solvent as the internal standard (^1H NMR: $\text{CDCl}_3 = 7.26$; $\text{DMSO-d}_6 = 2.50$ and ^{13}C NMR: $\text{CDCl}_3 = 77.0$; $\text{DMSO-d}_6 = 39.5$). Coupling constants (J) are reported in Hz with the following splitting abbreviations: s = singlet, d = doublet, t = triplet, q = quartet.

Infrared (IR) spectra were recorded on a Bruker Tensor 27 Fourier Transform spectrophotometer using thin films on NaCl plates for liquids and oils and KBr discs for solids and crystals. Absorption maxima (ν_{max}) are reported in wavenumbers (cm^{-1}).

Low resolution mass spectra (LRMS) were recorded on a Waters Micromass LCT Premier TOF spectrometer Waters using electrospray ionization (ESI). Nominal m/z values are reported in Daltons.

Elemental microanalysis was obtained through the London Metropolitan University Elemental Analysis Service.

Thin layer chromatography (TLC) was carried out using Merck aluminium backed sheets coated with 60F₂₅₄ silica gel. Visualization of the silica plates was achieved using a UV lamp ($\lambda_{\text{max}} = 254$ nm), and/or ammonium molybdate (5% in 2 M H_2SO_4), and/or potassium permanganate (5% KMnO_4 in 1M NaOH with 5% potassium carbonate). Flash column chromatography was carried out using BDH PROLAB[®] 40-63 mm silica gel (VWR). Mobile phases are reported in % volume of more polar solvent in less polar solvent for binary systems (*e.g.* 20% EtOAc in petrol = 1:4 ethyl acetate:petrol).

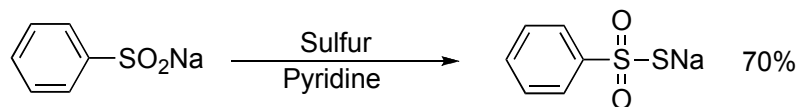
Supplementary Information

Dimethylformide (DMF), ethyl acetate (EtOAc), diethyl ether (Et₂O), and ethanol (EtOH) were HPLC grade and used as received. Distilled water was used for chemical reactions and Milli-Q[®] purified water for protein manipulations. Reagents were purchased from Alfa Aesar or Aldrich and used as supplied. Brine refers to a saturated solution of sodium chloride. Anhydrous magnesium sulfate (MgSO₄) was used as a drying agent after reaction workup where indicated.

Protein Mass Spectrometry

Liquid chromatography-mass spectrometry (LC-MS) was performed on a Micromass LCT (ESI-TOF-MS) coupled to a Waters Alliance 2790 HPLC using a Phenomenex Jupiter C4 column (250 × 4.6 mm × 5 μm). Water:acetonitrile, 95:5 (solvent A) and acetonitrile (solvent B), each containing 0.1% formic acid, were used as the mobile phase at a flow rate of 1.0 mL min⁻¹. The gradient was programmed as follows: 95% A (5 min isocratic) to 100% B after 15 min then isocratic for 5 min. The electrospray source was operated with a capillary voltage of 3.2 kV and a cone voltage of 25 V for subtilisin *Bacillus lentus* (SBL). Nitrogen was used as the nebulizer and desolvation gas at a total flow of 700 L/hr. Spectra were calibrated using a calibration curve constructed from a minimum of 17 matched peaks from the multiply charged ion series of equine myoglobin obtained at a cone voltage of 25V. Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.1 from Waters) according to manufacturer's instructions.

Sodium phenylthiosulfonate

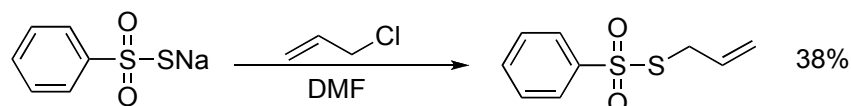


Sodium benzenesulfinate (10.00 g, 61.0 mmol) and sulfur (1.95 g, 61.0 mmol) were dissolved in anhydrous pyridine (60 mL) to give a yellow solution. The reaction was stirred for 1 h at room temperature under argon, after which time a white suspension had formed. The solid formed was isolated by filtration and washed with Et₂O. Recrystallization from EtOH afforded sodium phenylthiosulfonate (8.40 g, 70%) as a white crystalline solid. Spectroscopic data was consistent with that previously reported.¹ m.p. = 294–295 °C (Lit¹ = 287 °C). IR (ν_{max}, KBr): 1936, 1900, 1818, 1771, 1684, 1607, 1583, 1475, 1443, 1390, 1333, 1311. ¹H NMR (400 MHz, DMSO-d₆)

Supplementary Information

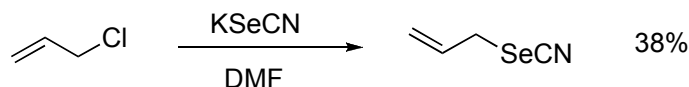
$\delta_{\text{H}} = 7.19\text{--}7.50$ (3H, m, *m,p*-ArH), 7.75 (2H, dd, $J = 7.7, 1.5$, *o*-ArH). ^{13}C NMR (100 MHz, DMSO- d_6): $\delta_{\text{C}} = 124.0, 127.8, 129.0$ (3° Ar), 154.9 (4° Ar). LRMS m/z (ESI $^+$): Found 173.0 [M-Na] $^-$; $\text{C}_6\text{H}_5\text{O}_2\text{S}_2$ requires 173.0.

Allyl phenylthiosulfonate (3)



Sodium phenylthiosulfonate (2.00 g, 10.19 mmol) was added to a 50 mL round bottom flask containing allyl chloride (2.50 mL, 30.58 mmol) in DMF (10 mL). The reaction was stirred at room temperature for 3 hours. After this time, the reaction was diluted with water (100 mL) and transferred to a separatory funnel. The aqueous layer was extracted with Et_2O (2×100 mL). The combined Et_2O layers were washed sequentially with H_2O (100 mL) and brine (100 mL) and then dried (MgSO_4) and filtered. The solvent was removed under reduced pressure and the resulting residue was purified by column chromatography (5% EtOAc in petrol). The titled compound was isolated as a clear oil (830 mg, 38%). IR (ν_{max} , film): 1447, 1325, 1144, 1078, 988, 929, 755, 716, 685, 598, 537. ^1H NMR (400 MHz, CDCl_3): $\delta_{\text{H}} = 3.68$ (2H, dt, $J = 1.1, 7.1$, SCH_2), 5.09 (1H, dd, $J = 10.0, 1.1$, $\text{HC}=\text{CHH}$ *cis*), 5.21 (1H, dd, $J = 17.0, 1.1$, $\text{HC}=\text{CHH}$ *trans*), 5.70 (1H, m, $J = 17.0, 10.0, 7.1$, $\text{HC}=\text{CH}_2$), 7.51–7.59 (2H, m, *m*-ArH), 7.60–7.68 (1H, m, *p*-ArH), 7.93 (2H, d, $J = 7.3$, *o*-ArH). ^{13}C NMR (100 MHz, CDCl_3): $\delta_{\text{C}} = 38.8$ (SCH_2), 120.0 ($\text{HC}=\text{CH}_2$), 126.9, 129.2, 130.4, 133.7 (includes 3° Ar and $\text{HC}=\text{CH}_2$), 144.8 (4° Ar). Anal. Calcd. for $\text{C}_9\text{H}_{10}\text{O}_2\text{S}_2$: C, 50.44; H, 4.70; S, 29.92. Found C, 50.36; H, 4.76; S, 30.00.

Allyl selenocyanate (6)



The synthesis of allyl selenocyanate was adapted from the literature.² KSeCN (3.27 g, 22.72 mmol) was added to a 100 mL round bottom flask and dissolved in DMF (25 mL). The solution was placed under an atmosphere of nitrogen and allyl chloride (3.72 mL, 45.43 mmol) was added

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slowly to the stirred solution. The reaction was stirred for 20 minutes at room temperature and then diluted with Et₂O (200 mL) and washed sequentially with H₂O (2 × 200 mL) and brine (200 mL). The organic layer was dried (MgSO₄), filtered, and concentrated under reduced pressure. The product was isolated as a pale yellow liquid and was sufficiently pure to use in subsequent manipulations (1.25 g, 38%). This material has a sharp, lingering odor and should be used only in a well-ventilated fume hood. IR (ν_{\max} , film): 2151, 1633, 1432, 1403, 1197, 987, 928, 851, 686. ¹H NMR (200 MHz, CDCl₃): δ_{H} = 3.65 (2H, dt, J = 7.2, 1.0, CH₂SeCN), 5.23 (1H, dd, J = 1.0, 9.9, CHH=CH), 5.32 (1H, dq, J = 1.0, 16.7, CHH=CH), 6.00 (1H, m, H₂C=CH). ¹³C NMR (50 MHz, CDCl₃): δ_{C} = 31.3 (CH₂SeCN), 101.4 (C≡N), 120.6 (CH=CH₂), 131.6 (CH=CH₂).

Sequence of subtilisin *Bacillus lentus* (SBL) mutant S156C

PDB code for wild type = 1GCI

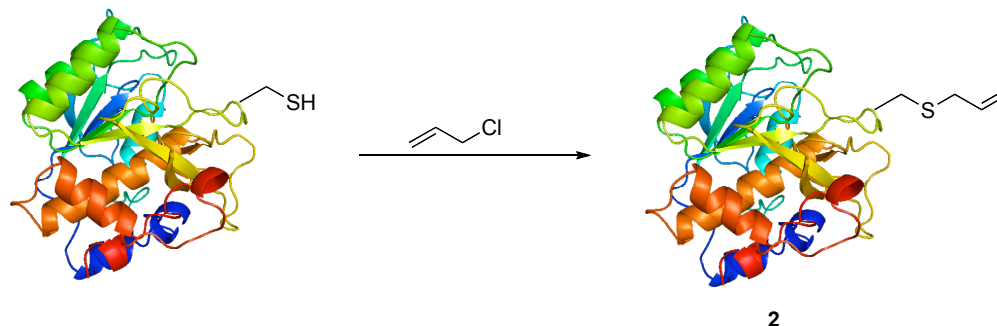
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Calculated average isotopic mass = 26714.5

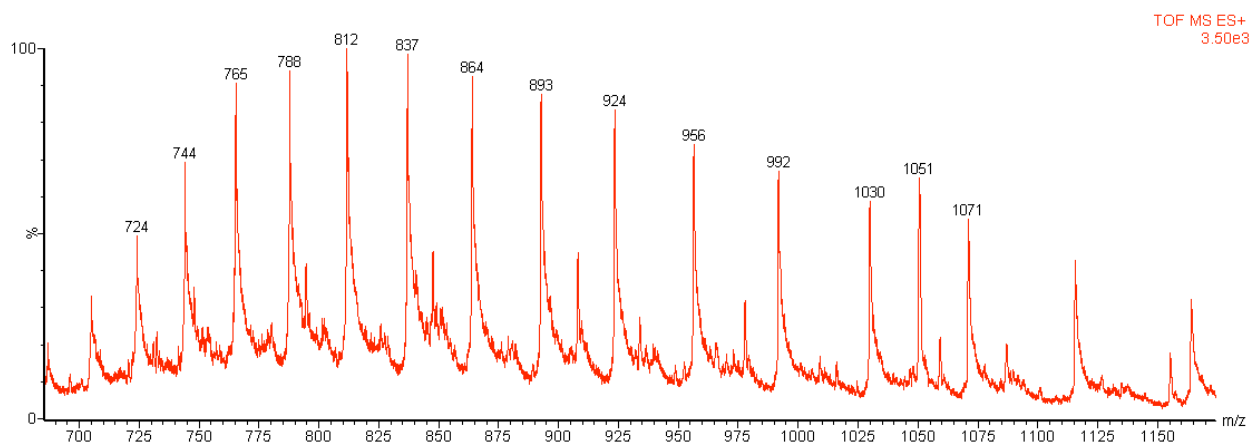
Supplementary Information

Allylation of SBL-S156C with Allyl Chloride

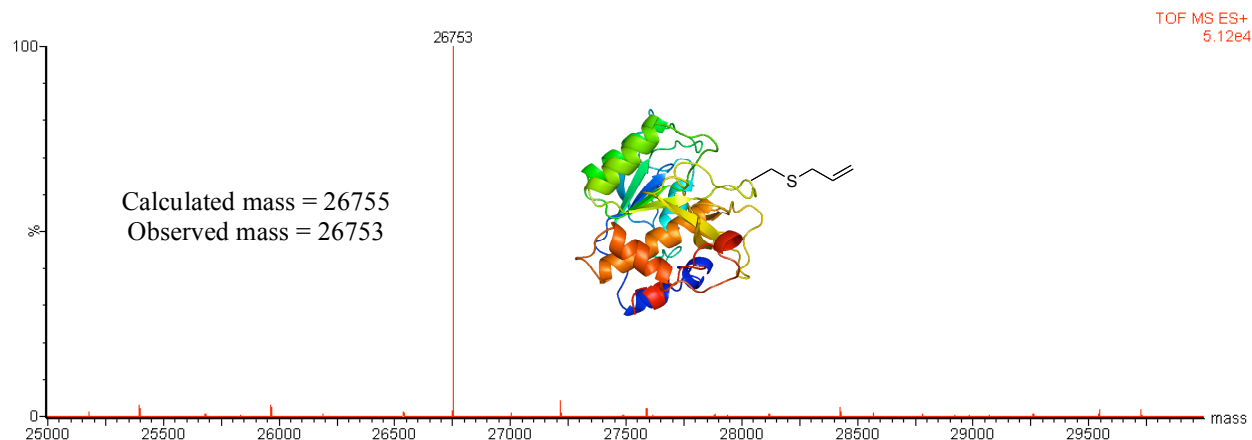
Method A: Direct Allylation



SBL-S156C (2.5 mL, 1 mg/mL, pH 8.0 sodium phosphate, 94 nmol) was added to a 15 mL Falcon tube and stored on ice until needed. Allyl chloride was prepared as a 0.65 M solution in DMF. A 147 μ L aliquot of the allyl chloride solution (96 μ mol) was added to the protein solution and the reaction was vortexed immediately upon addition. The reaction was incubated at 37 $^{\circ}$ C for 30 minutes and then analyzed directly by LC-MS, confirming full conversion to the allylated product SBL-156-Sac (26755 calculated mass, found 26753). Small molecules were removed with a PD10 column (GE Healthcare), eluting with 3.5 mL 50 mM sodium phosphate buffer (pH 8.0). The sample was then split into 200 μ L aliquots, flash frozen, and stored at -20 $^{\circ}$ C. ESI-MS are shown below.

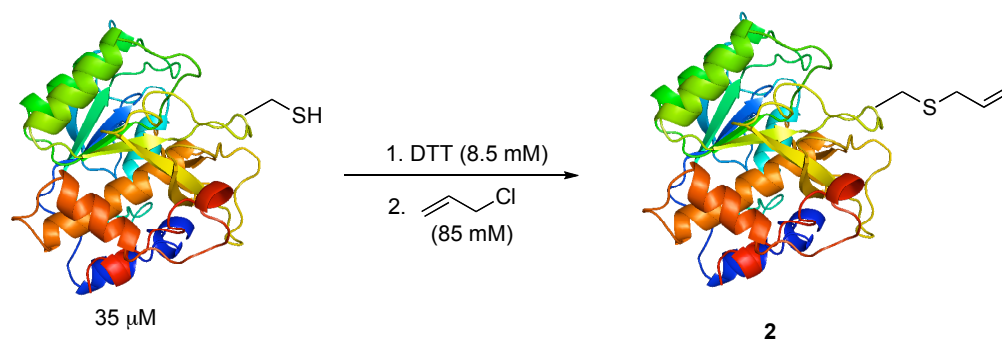


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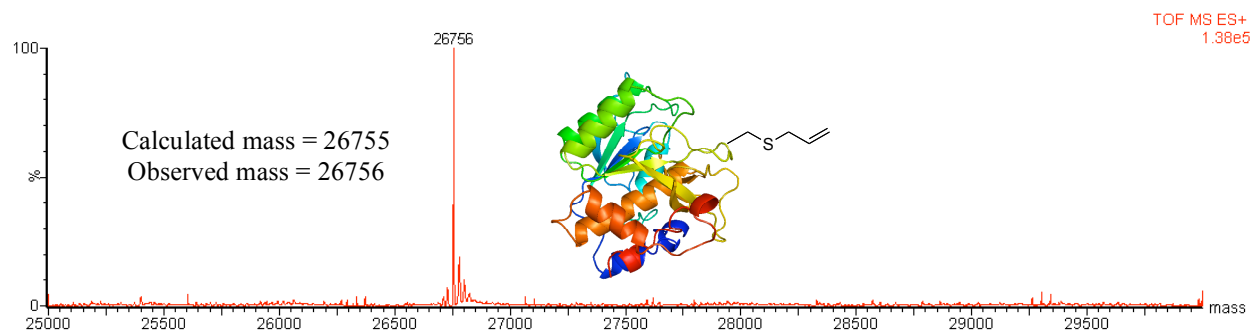
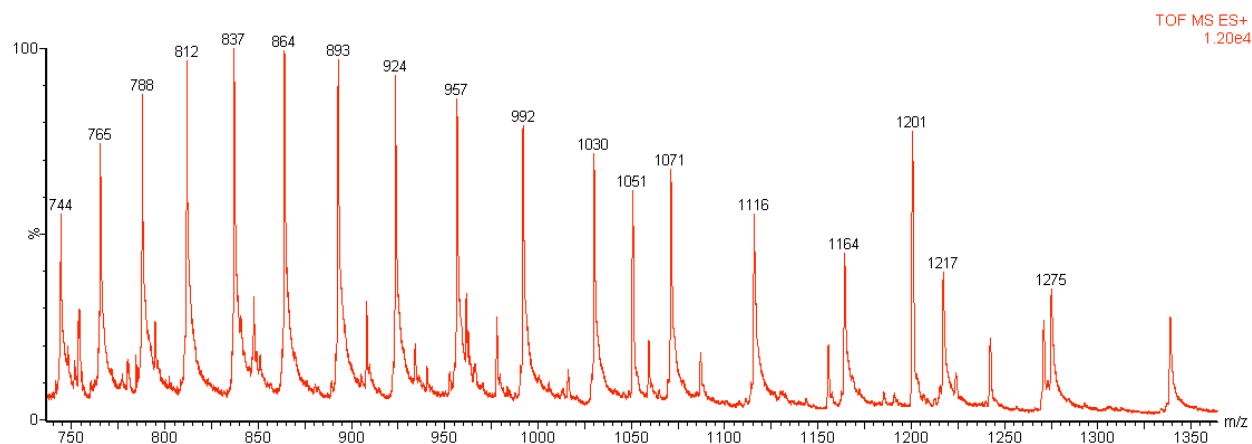
Allylation of SBL-S156C with Allyl Chloride

Method B: Pre-reduction with DTT

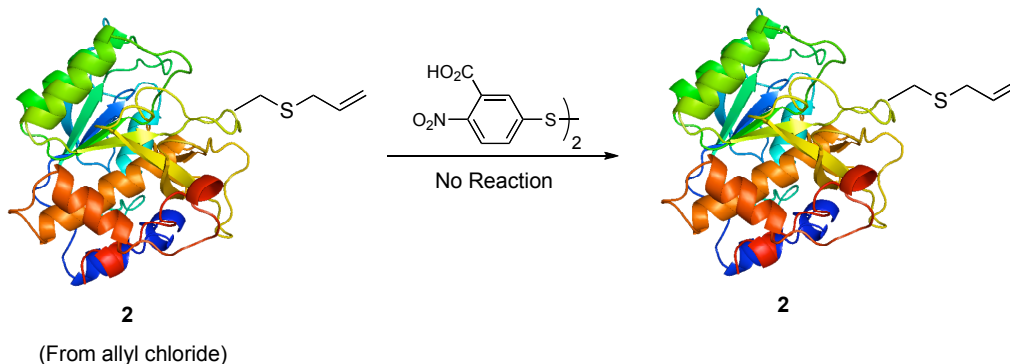


SBL-S156C (2.5 mL, 1.0 mg/mL, pH 8.0 sodium phosphate, 94 nmol) was added to a 15 mL Falcon tube and stored on ice. Dithiothreitol (DTT) (3.6 mg, 23 μ mol) was added as a solid to reduce any contaminant disulfide. The solution was vortexed and then shaken for 10 minutes at room temperature. Allyl chloride (19 μ L, 230 μ mol) was then added as a solution in DMF (200 μ L). The mixture was vortexed and then shaken at 37 $^{\circ}$ C for 30 min. LC-MS analysis revealed full conversion to the allylated product. (26755 = calculated mass, found 26756). The reaction mixture was passed through a PD10 column previously equilibrated with pH 8.0 sodium phosphate (50 mM). The product was split into 200 μ L aliquots and flash frozen. ESI-MS are shown below.

Supplementary Information



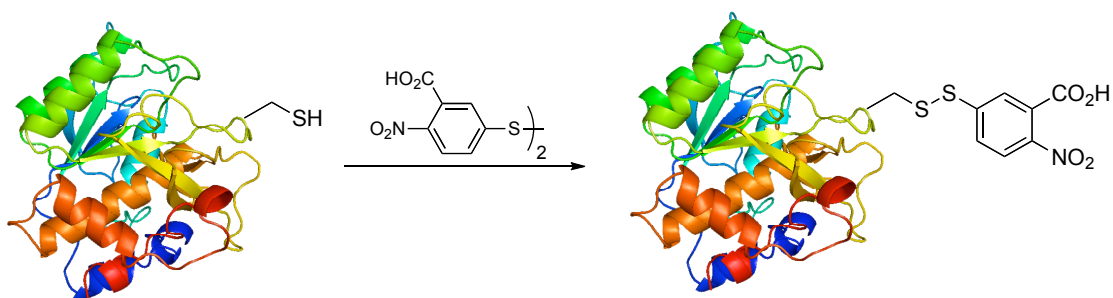
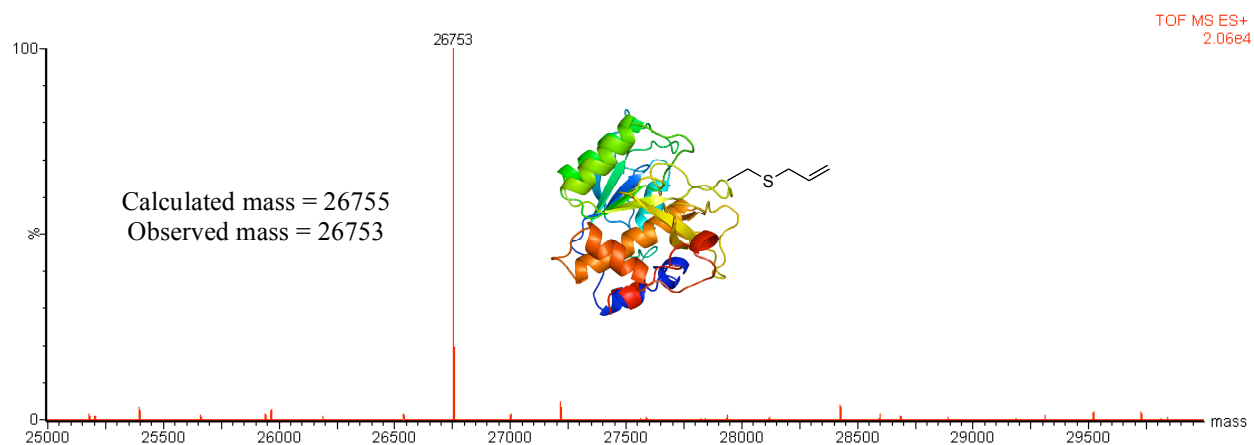
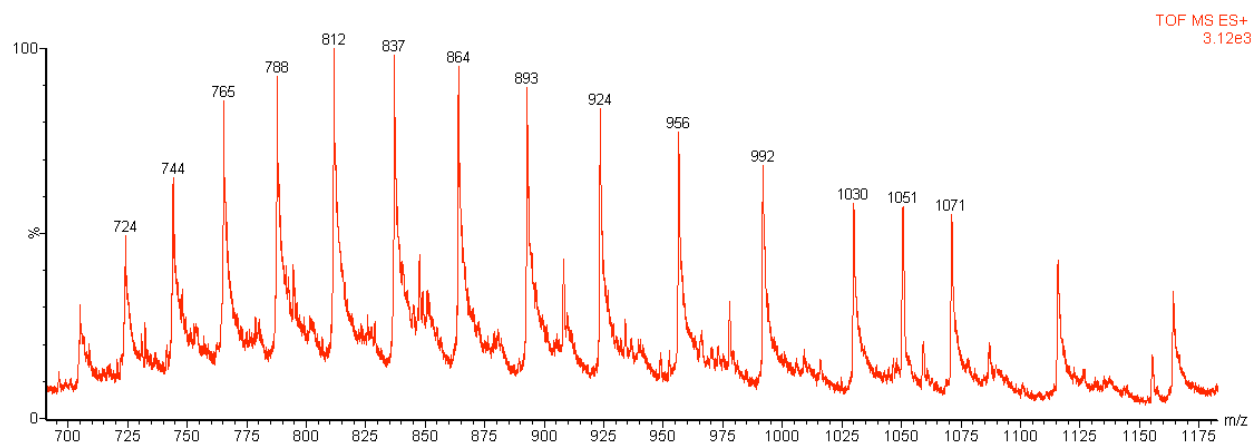
Ellman's Test to Confirm Reaction at Cysteine



A buffered solution of Ellman's reagent was prepared by dissolving 3.0 mg of Ellman's reagent in 300 μL of 50 mM sodium phosphate buffer (pH 8). A 20 μL of the Ellman's solution (0.5 μmol) was added to SBL-156Sac (**2**) (100 μL , 0.7 mg/mL, 3 nmol, prepared from Method A above) in an eppendorf tube. The reaction was vortexed and shaken at room temperature for 10 minutes. The reaction was then analyzed by LC-MS, revealing only the starting material (26755

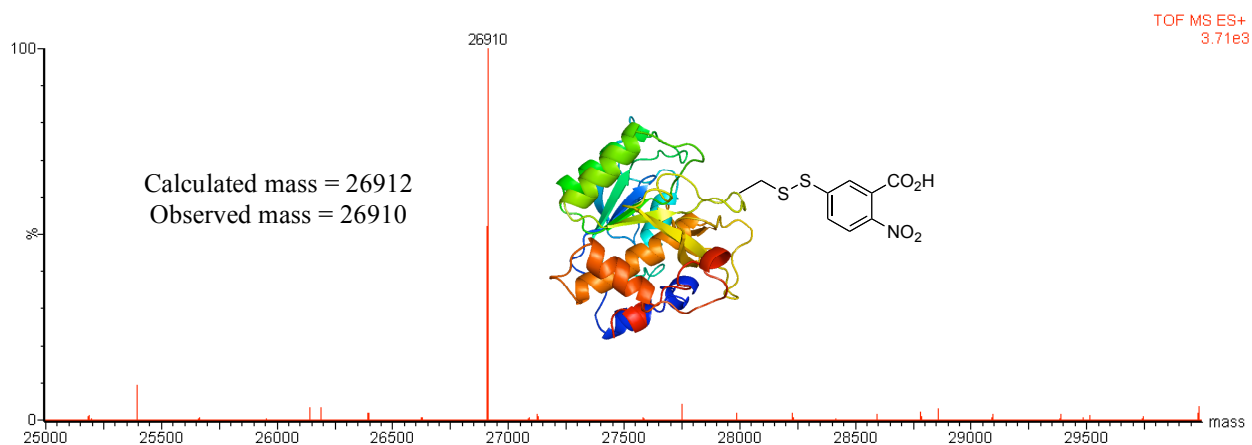
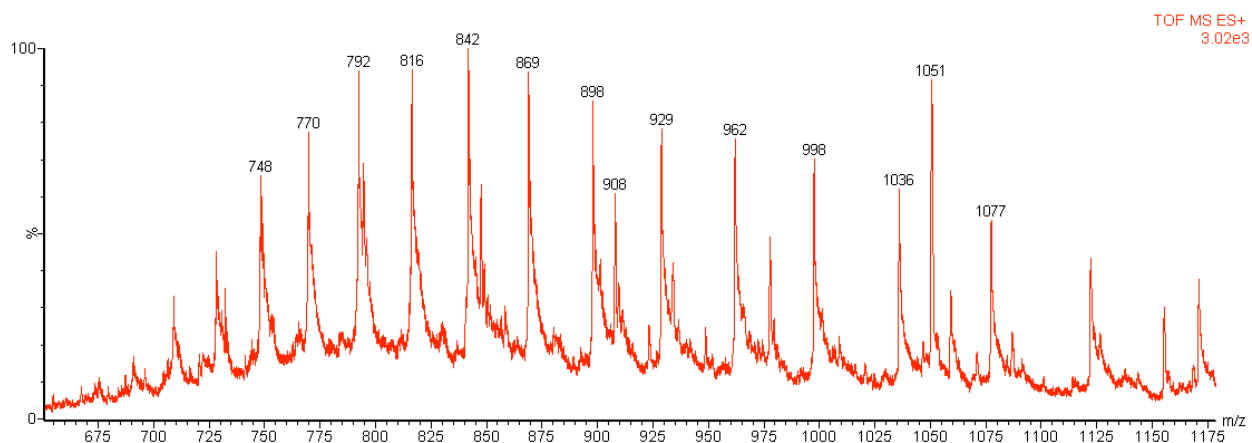
Supplementary Information

calculated mass, found 26753). This assay indicates that all free thiol is consumed, thus the allylation occurred at cysteine. Under identical conditions, the unmodified SBL-S156C forms the Ellman disulfide (see next).

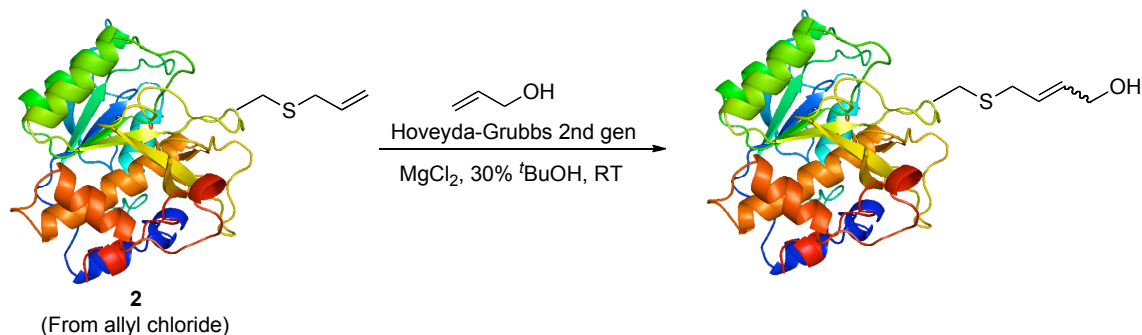


Supplementary Information

An SBL-S156C solution (100 μ L, 0.71 mg/mL) was prepared by diluting 71 μ L of SBL-S156C (1 mg/mL) to 100 μ L with 50 mM sodium phosphate buffer (pH 8.0). A 20 μ L aliquot of the Ellman's solution prepared above was added to protein solution. The solution turned bright yellow immediately upon addition of the Ellman's reagent. The reaction was shaken for 10 minutes at room temperature and was analyzed directly with LC-MS. Full conversion to the SBL-S156C Ellman's adduct was observed (26912 calculated mass, found 26910).



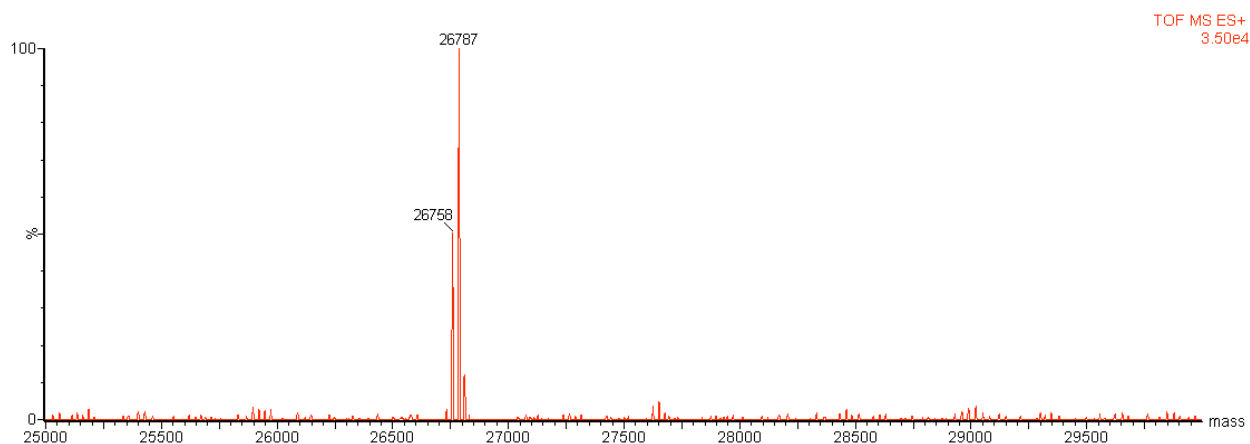
Olefin Metathesis on SBL-156Sac (2) from Allyl Chloride Allylation



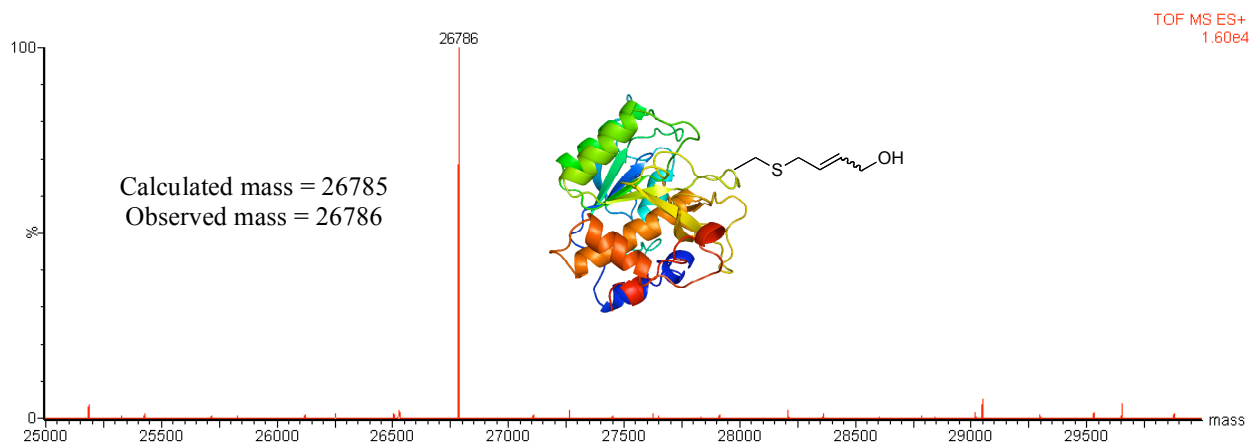
Supplementary Information

All manipulations were carried out at room temperature. The protein was prepared as described above by allylation with allyl chloride. A solution of Hoveyda-Grubbs 2nd generation catalyst (HG-II) in ^tBuOH was prepared by vortexing and gently warming 2.2 mg of HG-II in 355 μ L ^tBuOH. To an 1.5 mL eppendorf tube containing SBL-156Sac (250 μ L, 0.57 mg/mL, 5.3 nmol) was added MgCl₂·6H₂O (10.8 mg, 53 μ mol) followed by an aliquot of the HG-II/^tBuOH solution (107 μ L, 1.1 μ mol). The reaction was vortexed after each addition to homogenize. Allyl alcohol (3.6 μ L, 53 μ mol) was then added to the mixture. The reaction was vortexed and then shaken at room temperature. LC-MS after 1 hour of reaction time revealed formation of product. Full conversion to the CM product was observed after 2 hours (26785 calculated mass, found 26786).

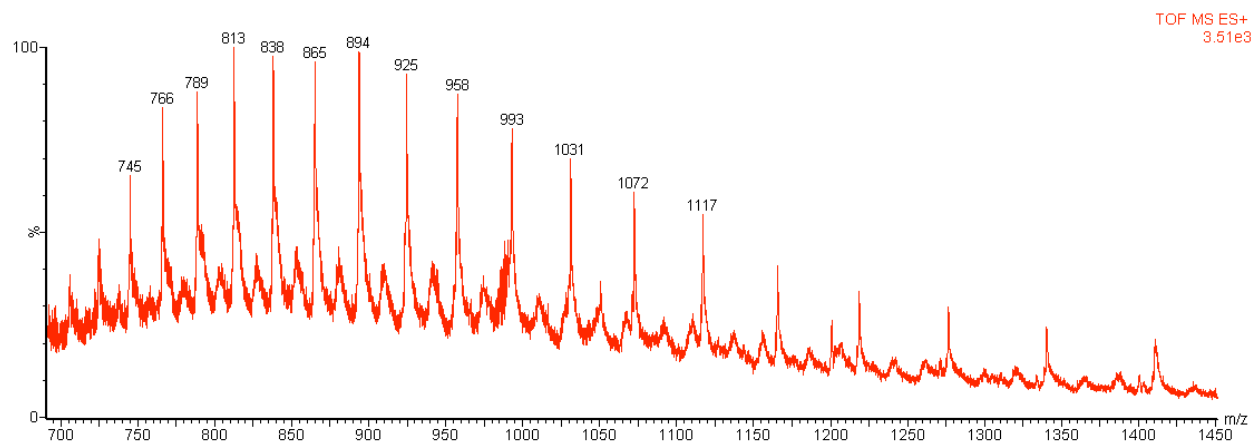
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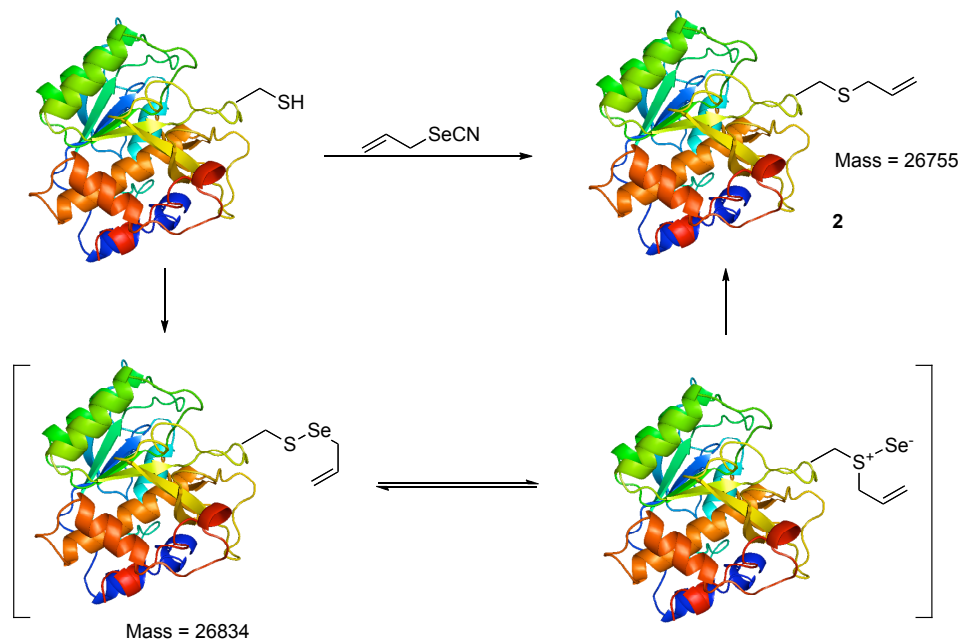
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Supplementary Information



Allylation of SBL-S156C with Allyl Selenocyanate



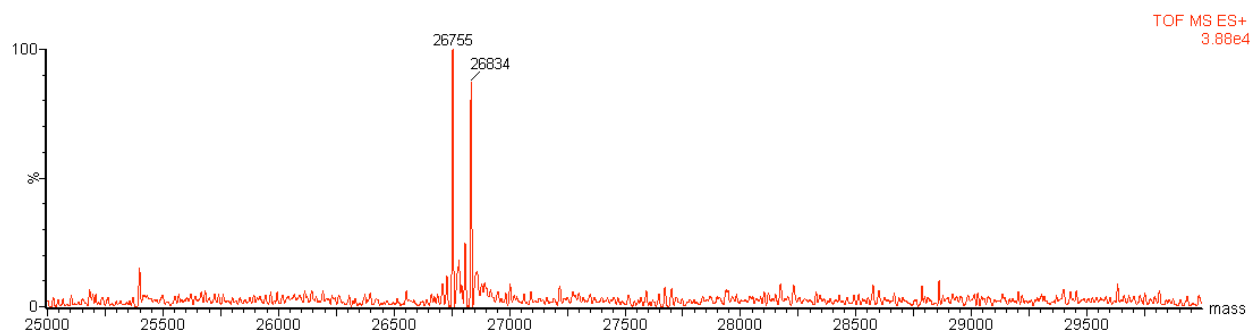
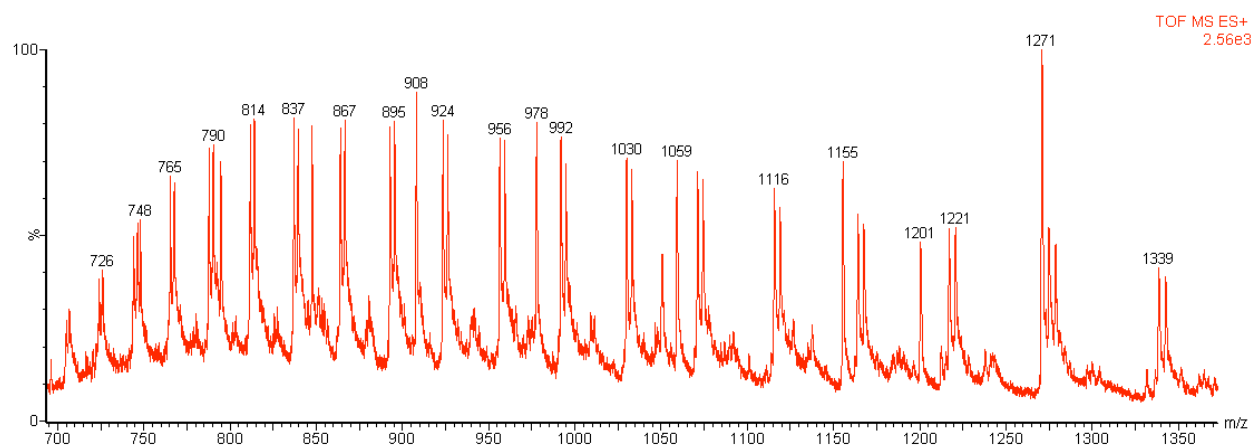
All manipulations were carried out in a well-ventilated hood. SBL-S156C (200 μ L, 1.0 mg/mL, pH 8.0 sodium phosphate, 7.5 nmol) was added to a 1.0 mL plastic tube and stored on ice. A stock solution of allyl selenocyanate (11.9 mg) was prepared in DMF (541 μ L). A 5 μ L aliquot of the selenide solution (0.75 μ mol) was added to the protein sample. The reaction was shaken for 10 minutes at room temperature and a 30 μ L aliquot was analyzed by LC-MS. A mixture of selenenyl sulfide and allylcysteine was observed (\sim 5:4). (26834 calculated for selenenyl sulfide; 26755 calculated for allylsulfide). After 1 hour of total reaction time, the reaction was analyzed

Supplementary Information

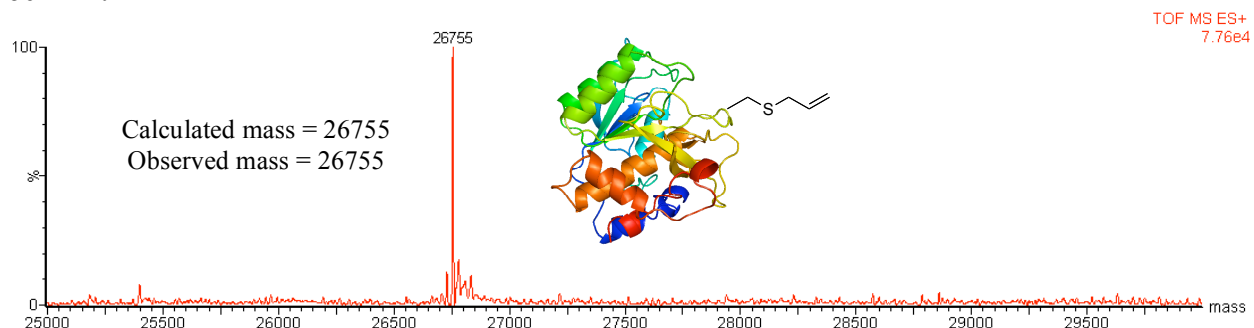
by LC-MS. Full conversion to the allyl sulfide was observed. (26755 = calculated mass; 26755 found). ESI-MS shown below.

This transformation was scaled up using 1.0 mL protein (1.0 mg/mL, pH 8.0 sodium phosphate, 37 nmol) and 0.55 mg allyl selenocyanate (3.7 μ mol in 25 μ L DMF). Complete conversion was observed after 1 hour at room temperature. Small molecules were removed by passing the sample through a PD10 column previously equilibrated with pH 8.0 sodium phosphate (50 mM). This sample was used for subsequent metathesis reactions.

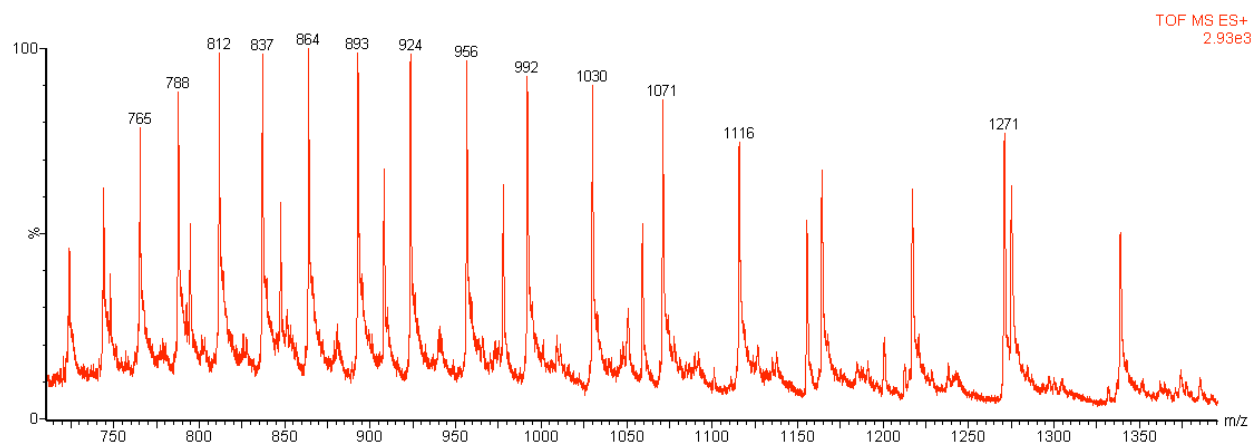
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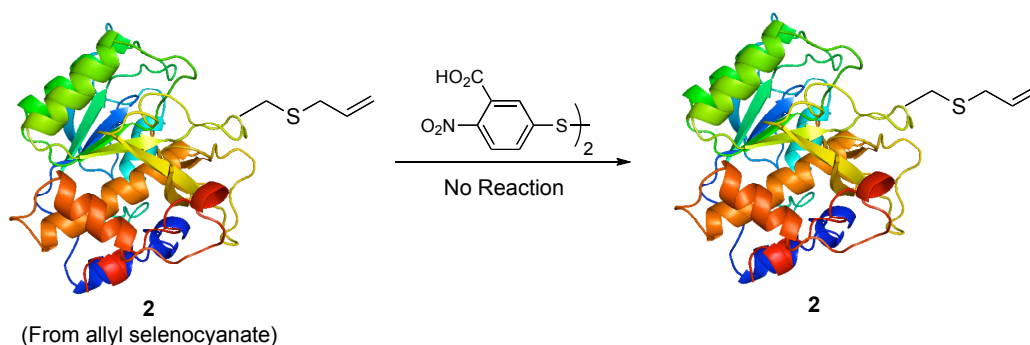
60 min:



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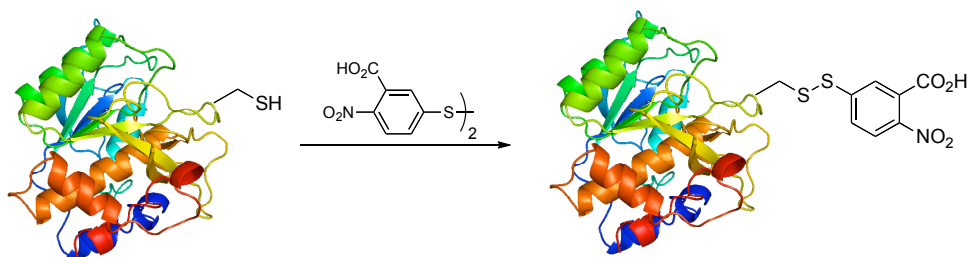
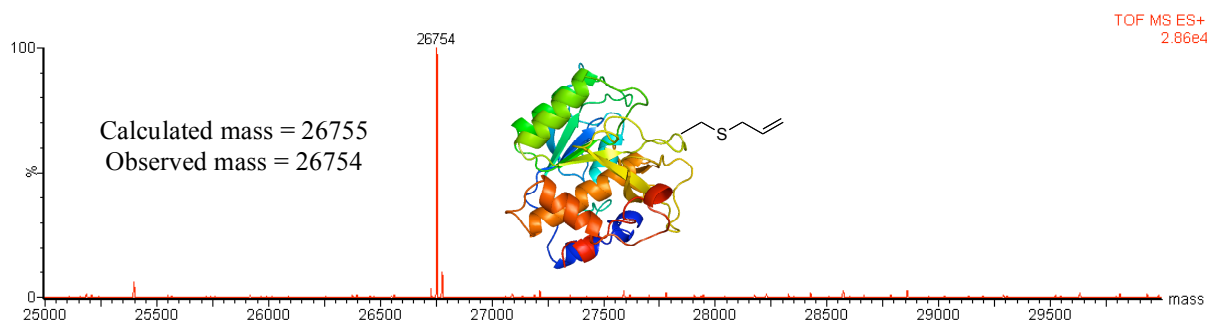
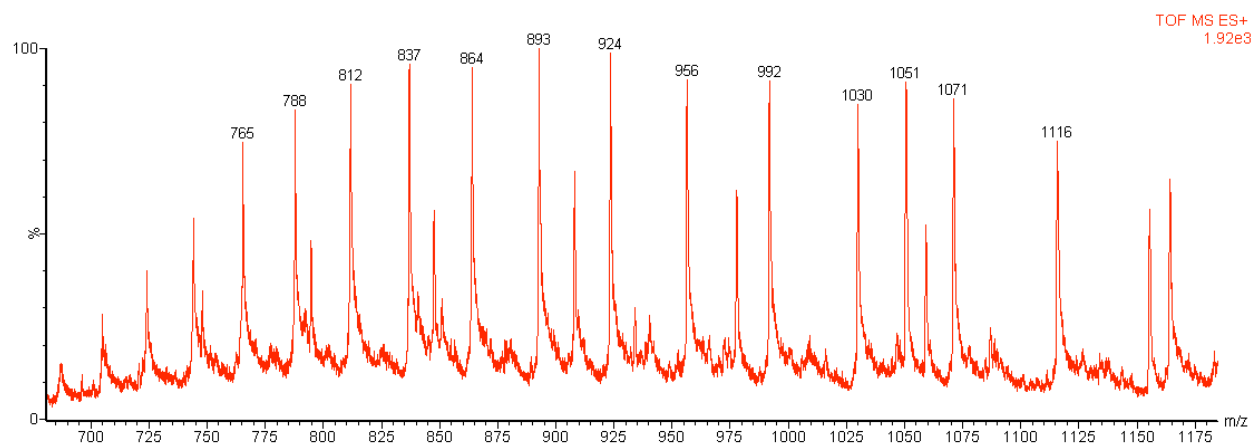


Ellman's Test to Confirm Reaction at Cysteine



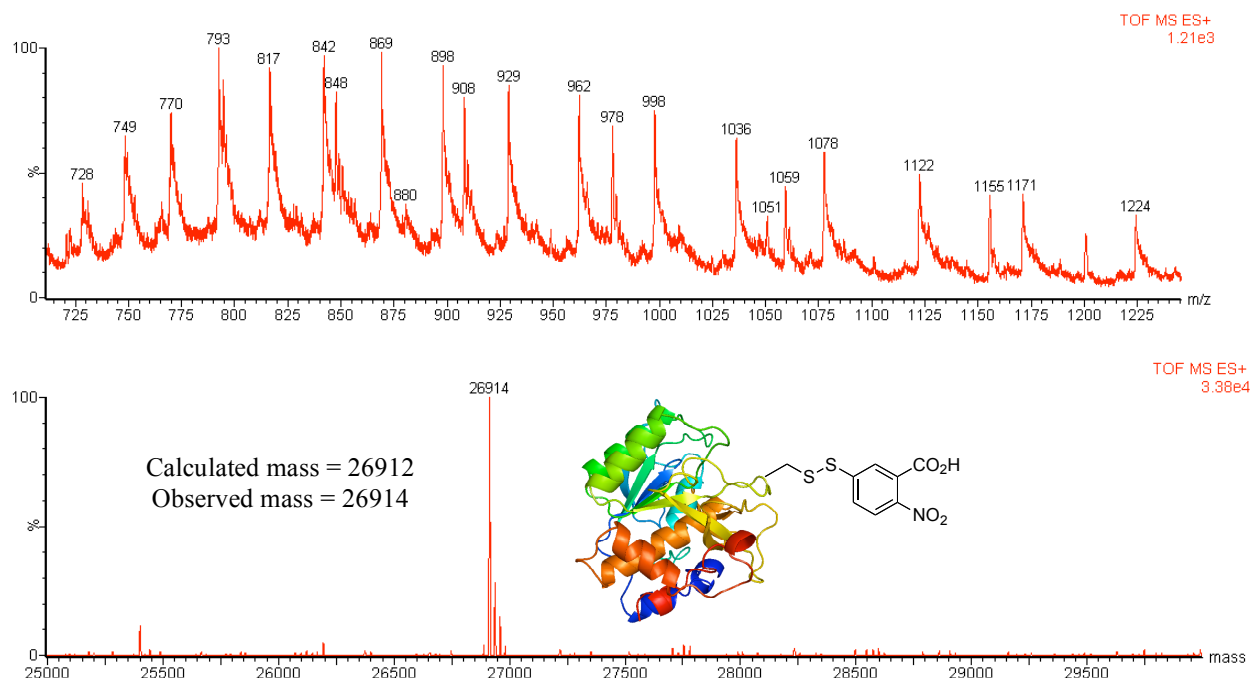
SBL-156Sac (**2**) (prepared above from allyl selenocyanate and purified by PD10) (100 μ L, 0.3 mg/mL, 1.1 nmol) was added to 250 μ L plastic tube. A stock solution of Ellman's reagent was prepared by dissolving 1.1 mg in 244 μ L of pH 8.0 sodium phosphate (50 mM). 20 μ L of the Ellman's solution (217 nmol) was added to the protein sample and shaken 10 minutes at room temperature. The reaction was analyzed by LC-MS and only allyl cysteine was observed, indicating no free cysteine was present in the protein and the initial allylation occurred at cysteine. (26755 = calculated mass for allyl cysteine; 26754 observed). Under identical conditions SBL-S156C gave full conversion to the Ellman's disulfide (see below). ESI-MS are shown below.

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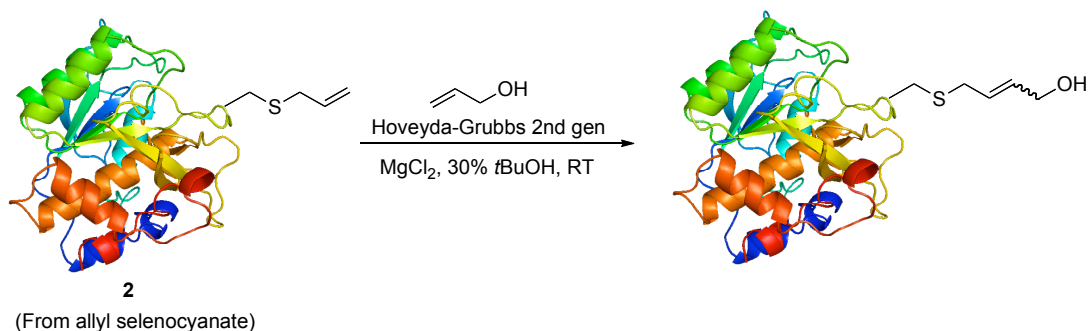


SBL-S156C (100 μ L, 0.3 mg/mL, 1.1 nmol) was added to 250 μ L plastic tube. A 20 μ L aliquot of the Ellman's solution prepared above (217 nmol) was added to the protein sample. The reaction was shaken 10 minutes at room temperature. The reaction was analyzed by LC-MS revealing full conversion to the Ellman disulfide adduct (26912 calculated mass, found 26914). ESI-MS are shown below.

Supplementary Information



Olefin Metathesis on SBL-156Sac (2) from Allyl Selenocyanate Allylation

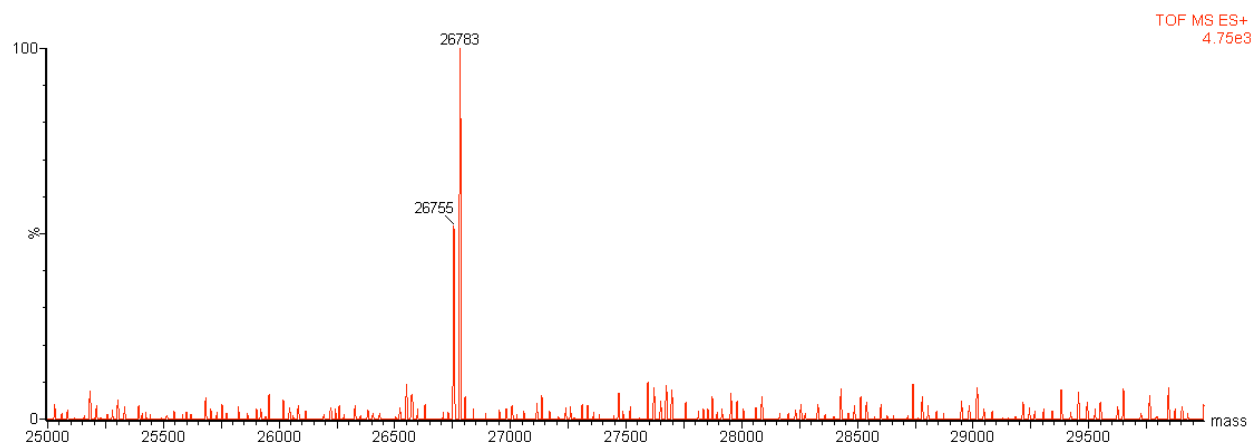


All manipulations were carried out at room temperature. The protein was prepared as described above by allylation with allyl selenocyanate. A solution of Hoveyda-Grubbs 2nd generation catalyst (HG-II) in *t*BuOH was prepared by vortexing and gently warming 1.4 mg of HG-II in 444 μ L *t*BuOH. To a 1.5 mL eppendorf tube containing SBL-156Sac (250 μ L, 0.29 mg/mL, 2.7 nmol) was added MgCl₂·6H₂O (5.5 mg, 27 μ mol) followed by an aliquot of the HG-II/*t*BuOH solution (107 μ L, 0.54 μ mol). The reaction was vortexed after each addition to homogenize. Allyl alcohol (1.8 μ L, 27 μ mol) was then added to the mixture. The reaction was vortexed and then shaken at room temperature. LC-MS after 1 hour of reaction time revealed formation of

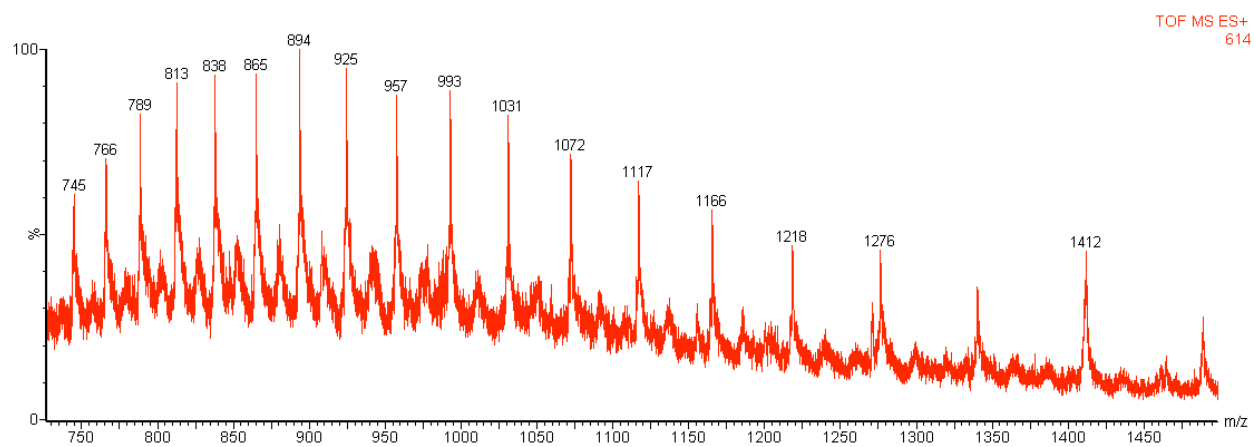
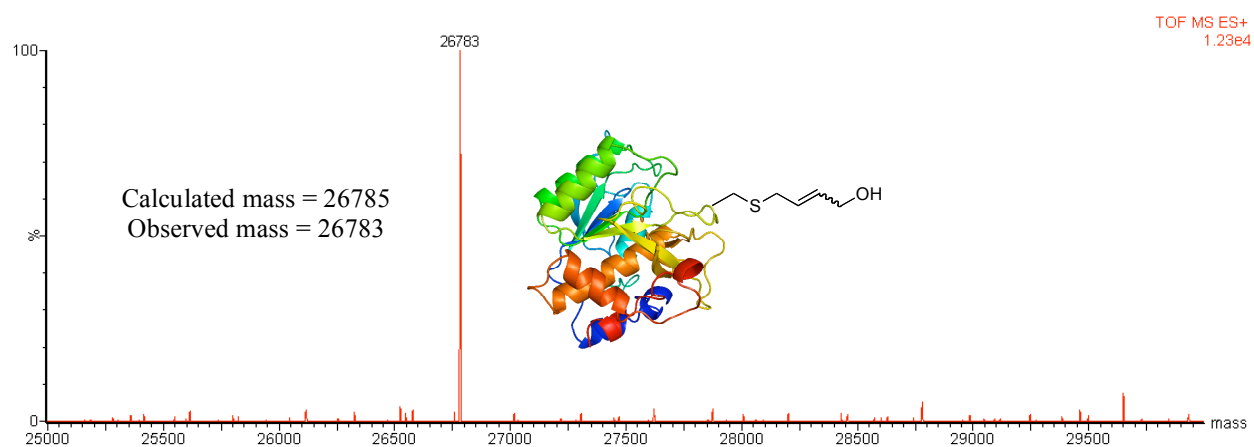
Supplementary Information

product. Full conversion to the CM product was observed after 2 hours (26785 calculated mass, found 26783). ESI-MS are shown below.

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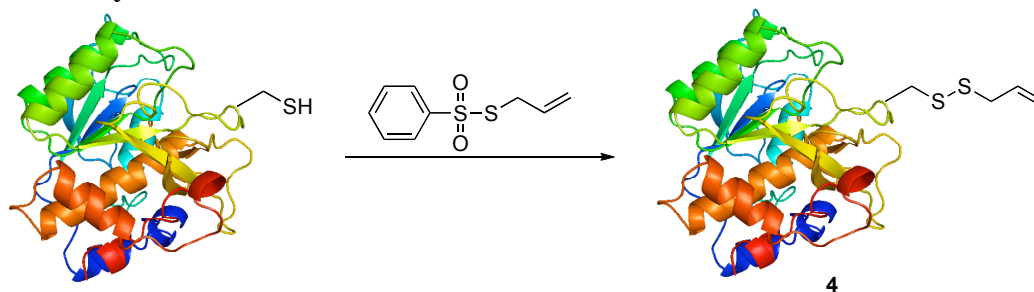


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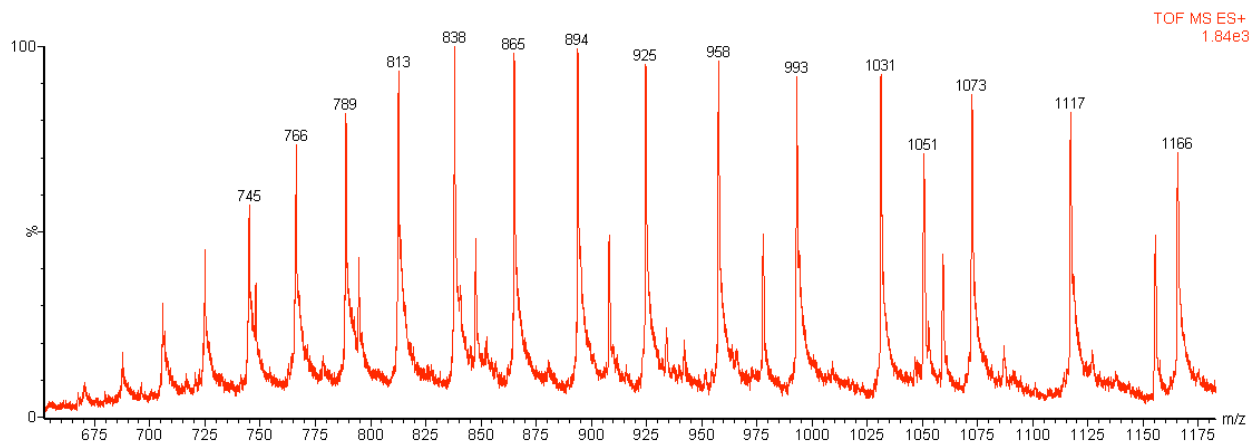


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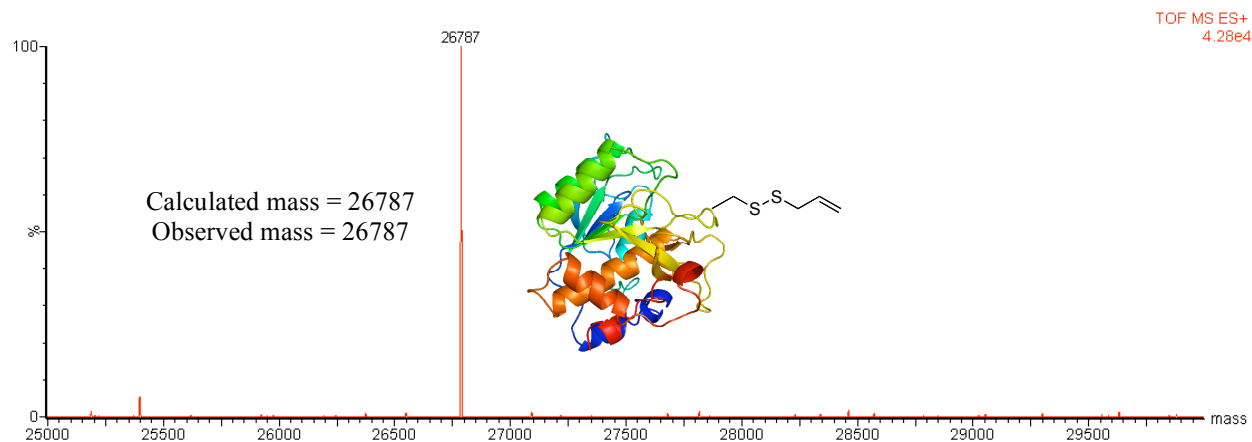
Synthesis of Allyl Disulfide on SBL-S156C



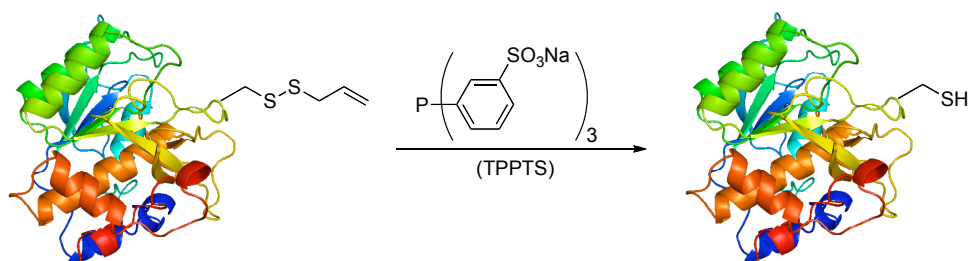
All manipulations were carried out at room temperature. A solution of allyl phenylthiosulfonate (**3**) in DMF was prepared by dissolving 3.3 mg of **3** in 406 μL DMF. 100 μL of allyl-PTS solution (0.8 mg, 3.7 μmol) was added to a 1.5 mL eppendorf tube containing SBL-S156C (1 mL, 1 mg/mL, 0.037 μmol , pH 8.0 sodium phosphate). The reaction was vortexed immediately and placed on shaker for 10 minutes. LC-MS analysis revealed full conversion to the allyl disulfide **4** (26787 calculated mass, found 26787). Small molecules were removed with a PD10 column (GE Healthcare), eluting with 3.5 mL 50 mM sodium phosphate buffer (pH 8.0). The sample was then split into 250 μL aliquots, flash frozen, and stored at $-20\text{ }^\circ\text{C}$. The final concentration of protein was 0.3 mg/mL.



Supplementary Information

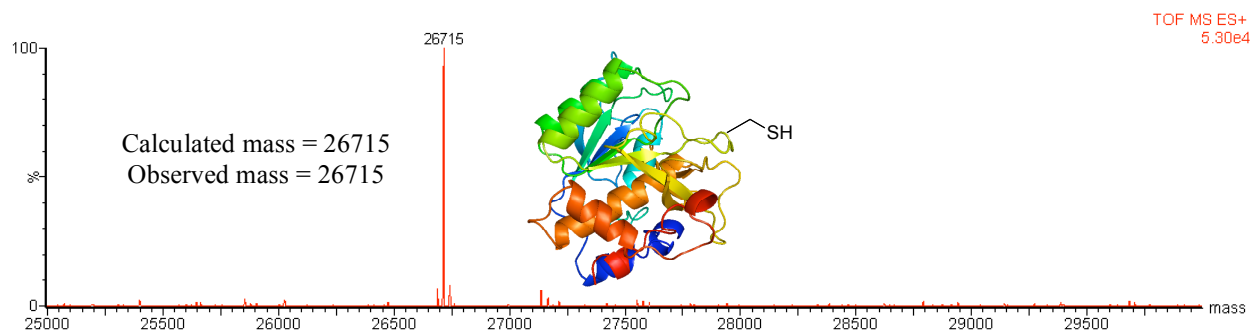
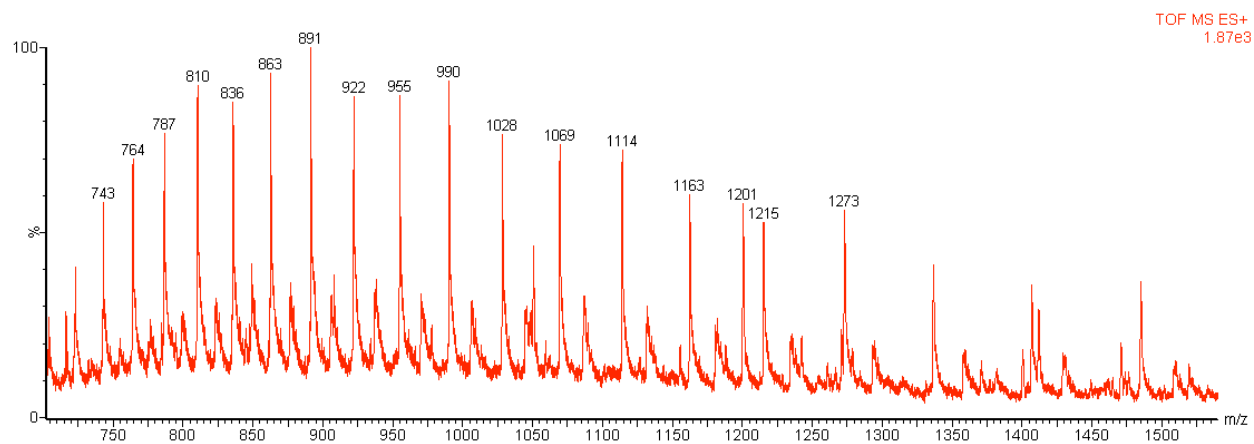


Attempted Desulfurization with TPPTS



SBL-156-Allyldisulfide **4** (250 μL , 0.29 mg/mL, pH 8.0 sodium phosphate, 2.7 nmol) was added to a 1.0 mL plastic tube and stored on ice. A stock solution of tris(3-sulfophenyl)phosphine trisodium salt (TPPTS, Aldrich) was prepared by dissolving 0.9 mg in 112.5 μL of H_2O . A 10 μL aliquot of the TPPTS solution (135 nmol) was added to the protein solution at room temperature. The reaction was shaken at room temperature for 15 minutes and then analyzed by LC-MS. Only free cysteine (product of reduction) was observed. (26715 = calculated mass; 26715 found). ESI-MS are shown below.

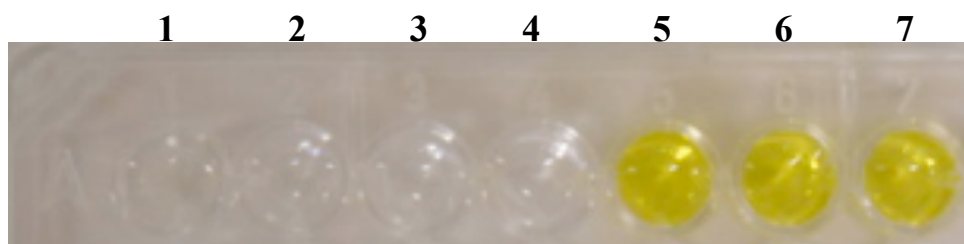
Supplementary Information



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Peptidase Activity Assay of Allylated SBL-S156C

SBL-S156C (unmodified), SBL-156Sac (from allyl chloride), and SBL-156Sac (from allyl selenocyanate) were prepared at a concentration of 0.1 mg/mL in pH 8.0 sodium phosphate (50 mM). 225 μ L aliquots of each sample were added to a 96-well plate. A 25 μ L aliquot of SucAAPFpNA (0.20 M in DMSO, Bachem) was added to each of the protein samples. All reactions turned yellow immediately upon addition of the peptide substrate. The yellow solution indicates liberation of *p*-nitroaniline (pNA), confirming peptidase activity of all samples. All protein solutions and the peptide solution alone at the same concentration are colorless (See below).

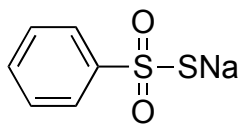


- Well 1: SucAAPFpNA
Well 2: SBL-S156C
Well 3: SBL-156Sac (from allyl chloride)
Well 4: SBL-156Sac (from allyl selenocyanate)
Well 5: SBL-S156C + SucAAPFpNA
Well 6: SBL-156Sac (from allyl chloride) + SucAAPFpNA
Well 7: SBL-156Sac (from allyl selenocyanate) + SucAAPFpNA

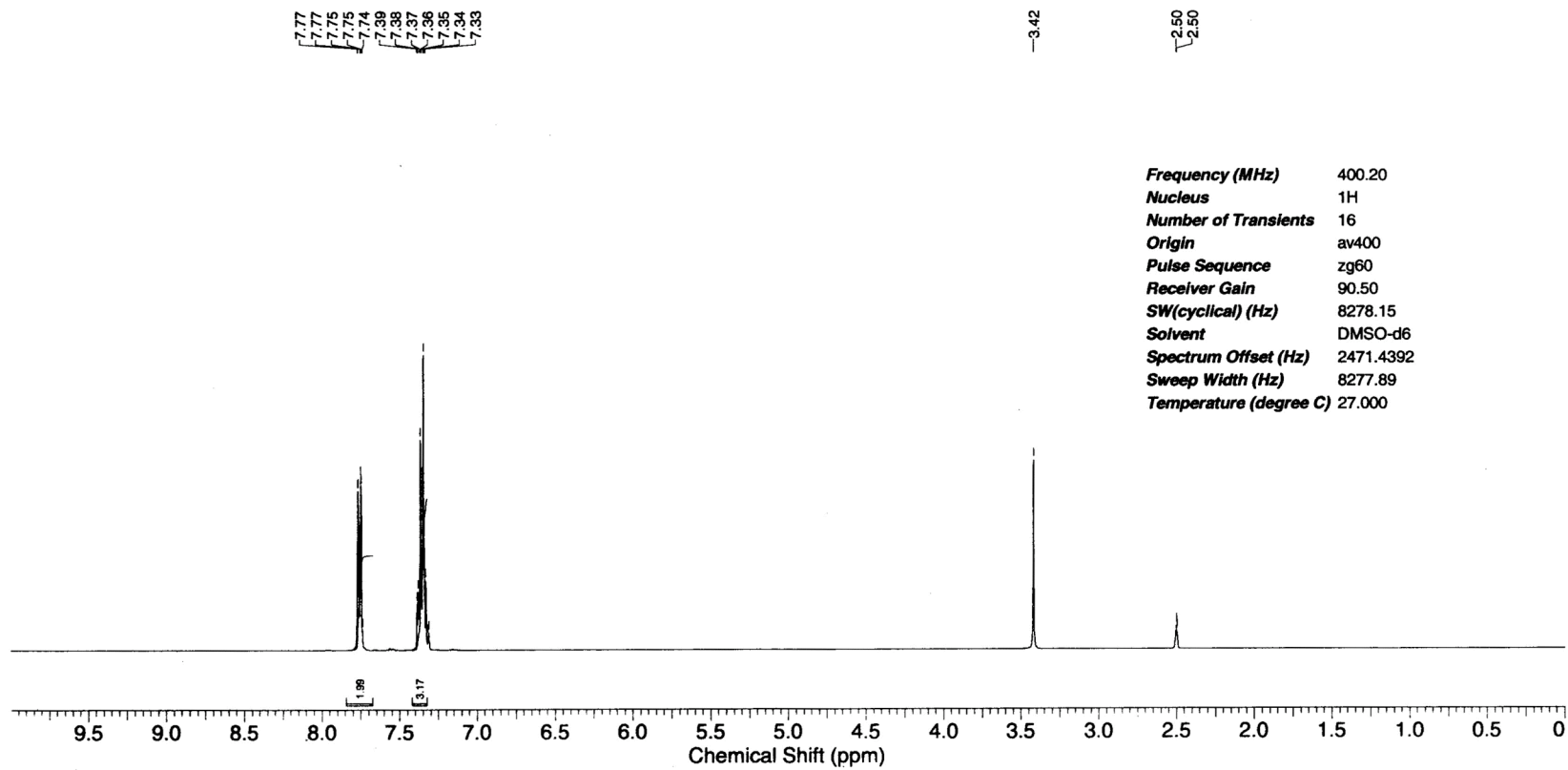
References:

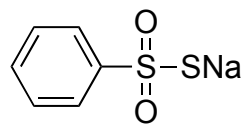
¹ R. Sato, T. Goto, Y. Takikawa, S. Takizawa, *Synthesis* 1980, 615.

² E. H. Riague, J.-C. Guillemin, *Organometallics* 2002, **21**, 68-73.

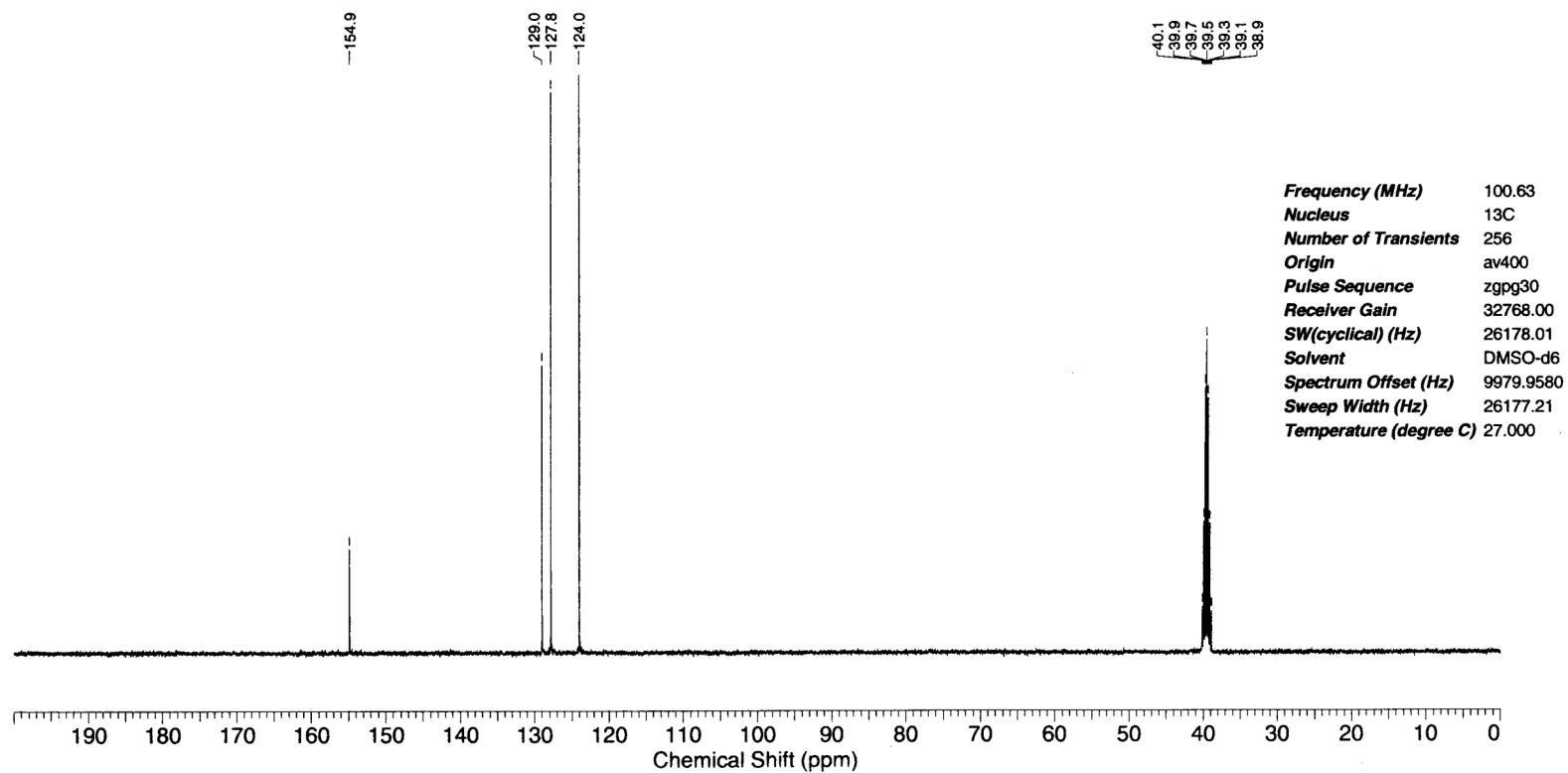


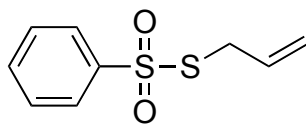
^1H NMR (400 MHz), DMSO- d_6



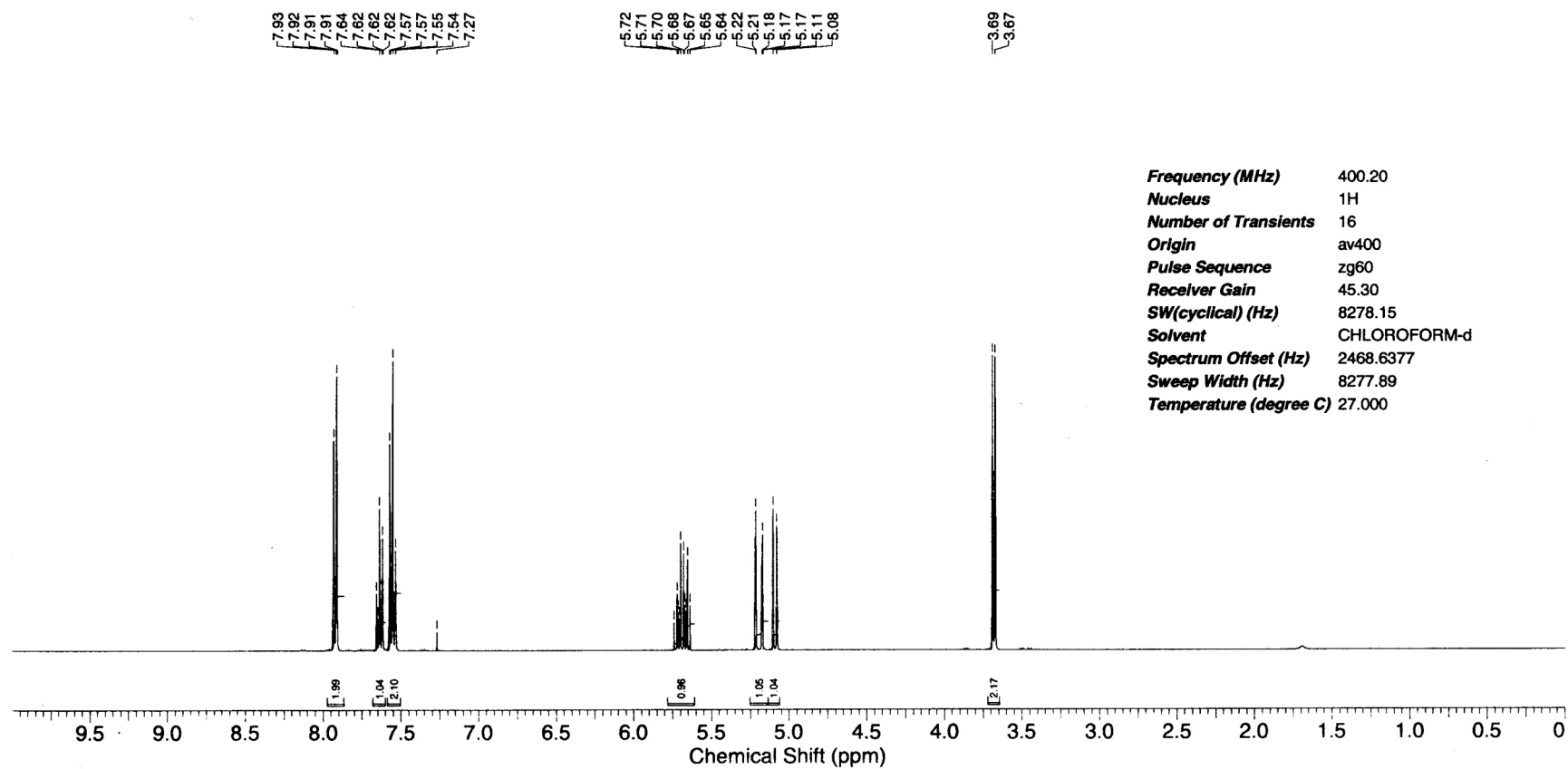


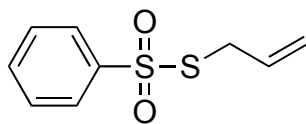
^{13}C NMR (100 MHz), DMSO- d_6





(3) ^1H NMR (400 MHz), CDCl_3





(3) ^{13}C NMR (100 MHz), CDCl_3

