SUPPORTING INFORMATION

Tunable Reagents for Multi-functional Bioconjugation: Reversible or Permanent Chemical Modification of Proteins and Peptides by Control of Maleimide Hydrolysis

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1. General Procedures

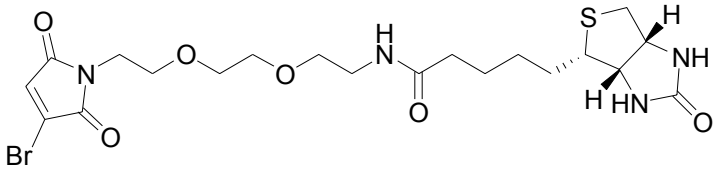
^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Bruker Avance 500 instrument operating at a frequency of 500 MHz for ^{1}H and 125 MHz for ^{13}C. ^{1}H NMR spectra were referenced to the $CDCl_3$ (7.26 ppm) signal. ^{13}C NMR spectra were referenced to the $CDCl_3$ (77.67 ppm) signal. Infra-red spectra were run on a PerkinElmer Spectrum 100 FT-IR spectrometer operating in ATR mode with frequencies given in reciprocal centimeters (cm^{-1}). Mass spectra and high resolution mass data for small molecules were recorded on a VG70-SE mass spectrometer (EI mode). Melting points were taken on a Gallenkamp heating block and are uncorrected. Lyophilised somatostatin, 3,4-dibromomaleimide, *N*-methylmaleamic acid and *N*-phenylmaleamic acid were purchased from Sigma-Aldrich and used without further purification. *N*-Phenylbromomaleimide[1], *N*-phenyldibromomaleimide[2], *N*-methylbromomaleimide[3] and *N*-fluoresceindibromomaleimide[3] were synthesized as described previously.

2. Protein / Peptide Mass Spectrometry

LC-MS was performed on protein samples using a Waters Acquity uPLC connected to Waters Acquity Single Quad Detector (SQD). Column: Acquity uPLC BEH C18 1.7µm 2.1 x 50 mm. Wavelength: 254 nm. Mobile Phase: 95:5 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid) Gradient over 4 min (to 5:95 Water (0.1% Formic Acid): MeCN (0.1% Formic Acid). Flow Rate: 0.6 mL/min. MS Mode: ES+. Scan Range: m/z = 85-2000. Scan time: 0.25 sec. Data obtained in continuum mode. The electrospray source of the MS was operated with a capillary voltage of 3.5 kV and a cone voltage of 50 V. Nitrogen was used as the nebulizer and desolvation gas at a total flow of 600 L/h.Total mass spectra for protein samples were reconstructed from the ion series using the MaxEnt 1 algorithm pre-installed on MassLynx software.

3. Synthesis of Cleavable Affinity Tag 11.

N-(2-(2-(2-(3-bromo-2,5-dioxo-pyrrol-1-yl)ethoxy)ethoxy)ethyl)-5-(2-oxo-1,3,3a,4,6,6a-hexahydrothieno(3,4-d)imidazol-6-yl)pentanamide 11

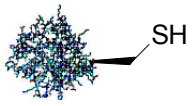


Monobromomaleic anhydride (45.0 mg, 0.25 mmol, 1 eq) was added in one portion to a solution of 2-(2-(2-(5-(2-oxo-1,3,3a,4,6,6a-hexahydrothieno(3,4-d)imidazol-6-yl)pentanoylamino)ethoxy)ethoxy)ethylammonium 2,2,2-trifluoroacetate[4] (124 mg, 0.25 mmol, 1 eq) in acetic acid (10 mL) and the reaction mixture heated to 170 °C for 3 h. Upon cooling to 21 °C toluene was added and the acetic acid azeotropically removed *in vacuo* (x2) to give crude product. Column chromatography (gradient 2-10% MeOH/CH_2Cl_2) yielded **11** as a white solid (70.0 mg, 0.13 mmol, 52% yield). m.p. 95-98 °C; $[\alpha]_D^{20.0}$ +65.1 (*c* 0.15, MeOH); ^{1}H NMR (600 MHz, CD_3OD) δ 7.17 (s, 1H, C*H*CBr), 4.51 (dd, J = 5.0, 8.0 Hz, 1H, NHC(O)NHC*H*), 4.33 (dd, J = 5.0, 8.0 Hz, 1H, NHC(O)NHC*H*), 3.77 (t, J = 5.5 Hz, 2H, OCH_2), 3.68 (t, J = 5.5 Hz, 2H, OCH_2), 3.63 (m, 2H, OCH_2), 3.58 (m, 2H, OCH_2), 3.53 (t, J = 5.5 Hz, 2H, NCH_2), 3.37 (t, J = 5.5 Hz, 2H, NCH_2), 3.24 (td, J = 5.0, 8.0 Hz, 1H, SC*H*), 2.95 (dd, J = 5.0, 12.5 Hz, 1H, SCH*H*), 2.73 (d, J = 12.5 Hz, 1H, SC*H*H), 2.26 (t, J = 7.0 Hz, 2H, NHC(O)CH_2CH_2CH_2), 1.69 (m, 4H, CH_2CH_2CH_2), 1.47 (quintet, J = 7.0 Hz, 2H, CH_2CH_2CH_2); ^{13}C NMR (150 MHz, CD_3OD) δ 176.12 (s), 170.13 (s), 166.97 (s), 166.08 (s), 133.63 (s), 132.05 (d), 71.22 (t), 71.11 (t), 70.61 (t), 68.69 (t), 63.35 (d), 61.61 (d), 57.03 (d), 41.09 (t), 40.31 (t), 39.09 (t), 36.75 (t), 29.78 (t), 29.50 (t), 26.87 (t); IR (MeOH) 3355, 2970, 1737 cm^{-1}; HRMS (ES) calcd for $C_{20}H_{29}N_4O_6$ NaSBr $[M+Na]^+$ 555.0889, observed 555.0905.

4. Protein and Peptide Modification

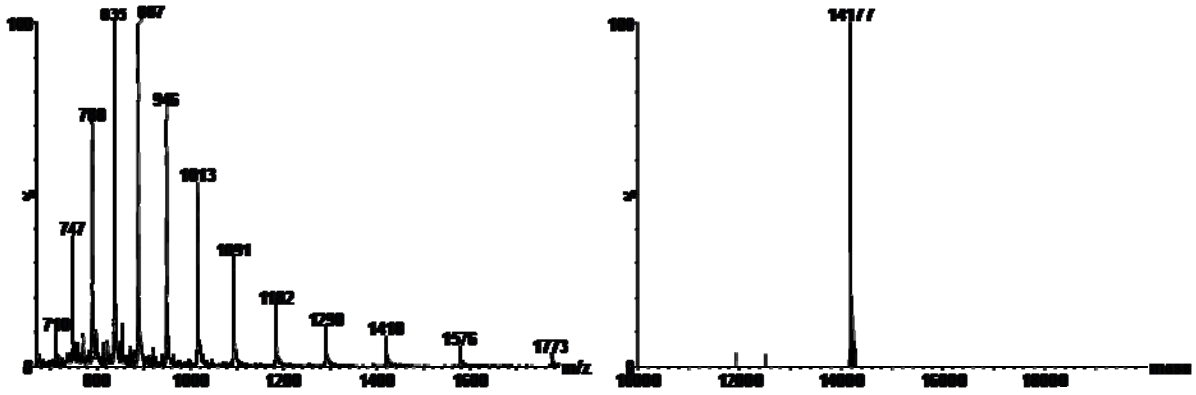
Sequence of isolated Grb2-SH2 L111C (residues 53-163) **1**: G I E M K P H P W F F G K I P R A K A E E M L S K Q R H D G A F L I R E S E S A P G D F S L S V K F G N D V Q H F K V C R D G A G K Y F L W V V K F N S L N E L V D Y H R S T S V S R N Q Q I F L R D I E Q V P Q Q P T Y V Q A G S R S H H H H H H Stop. Calculated mass = 14168

4.1 Cloning and Expression of Grb2-SH2 L111C Mutant 1

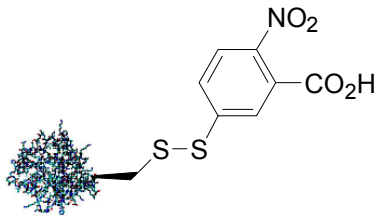


The DNA construct for the Grb2 SH2 domain contained the primary amino acid sequence 53-163 and was cloned on plasmid QE-60 (Qiagen).[5] The Grb2 SH2 L111C mutant was constructed by site-directed mutagenesis (Stratagene Kit) using oligonucleotides coding for the mutated residue. Both constructs were expressed in *Escherichia coli* (M15[pREP4], Qiagen) using a T5 promoter and a C-terminal 6-His

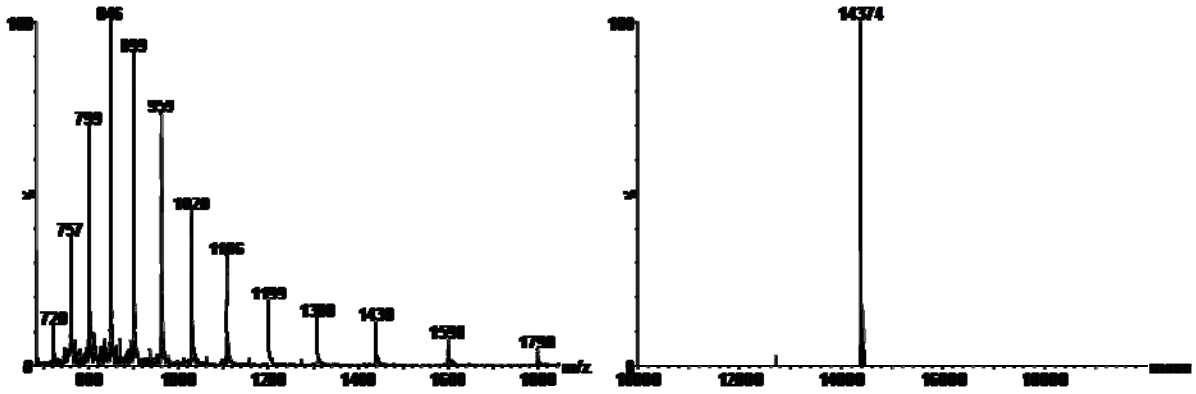
Tag was incorporated to aid purification. Cultures (1 L) were grown at 37 ^{0}C in Terrific Broth. from a single colony, and expression was induced with 1.0 mM isopropyl β-D-1-thiogalactopyranoside when an O.D.$_{\lambda 600}$ of 0.9 was attained. Cultures were allowed to express protein for 3 h before the cells were pelletised. Pellets were lysed in 0.1M sodium phosphate, 300 mM NaCl, 50 mM imidazole, pH 7.2 containing a protease inhibitor cocktail (Roche). The lysate was centrifuged, and the supernatant was applied to a Ni-NTA column (Qiagen). Grb2-SH2 L111C was eluted from the Ni-NTA column with 0.1M sodium phosphate, 300 mM NaCl, 200 mM imidazole at pH 7.2 . The collected Grb2 SH2 L111C **1** was ~95% pure as visualized by Coomassie-stained SDS–PAGE. Dimerization of Grb2 SH2 domain through domain-swapping has been previously observed.[6] Dimeric and monomeric Grb2-SH2 were separated on a Sephacryl S-100 column (320 mL) that had been pre-equilibrated with 0.1 M sodium phosphate and 150 mM NaCl at pH 8.0. Two peaks eluted, corresponding to the molecular weights of monomer (~14 kDa) and dimer (~28 kDa) Grb2-SH2. Almost, 60% of the Grb2-SH2 L111C domain **1** eluted from the column as monomer. Separated monomer and dimer were found to be surprisingly kinetically stable, as very little interconversion was seen over a course of months at 4 ^{0}C. The monomer was concentrated using Amicon® Ultra-4 centrifugal filter units (Millipore) and the final concentration of the protein was determined by absorbance at 280nm using the extinction coefficient obtained by McNemar and coworkers (15,600M^{-1}).[7] The protein was frozen at 2 mg/mL concentration in 100 μL aliquots which were thawed as required for experiments.The mass of the monomeric protein **1** (mass = 14177) was obtained using LC-MS



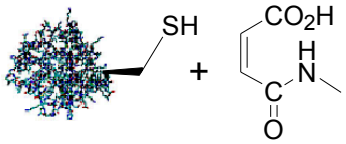
4.2 Grb2-SH2 L111C Mutant / Ellman's Reagent Adduct



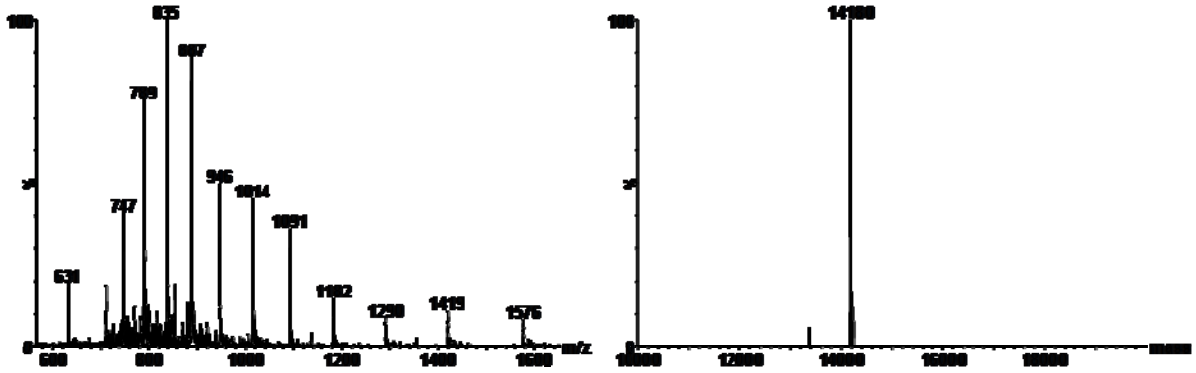
To a solution of Grb2-SH2 (L111C) **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added Ellman's reagent (5 µL, 282 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 10 mins after which the mixture was analysed by LC-MS. Analysis showed that a single reaction had occurred yielding a single product with a mass of 14374 showing that C111 was available for functionalisation



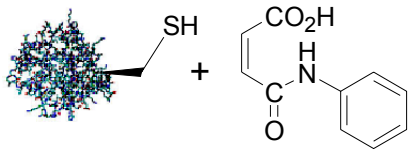
4.3 Reaction of Grb2-SH2 Domain L111C with *N*-Methylmaleamic Acid



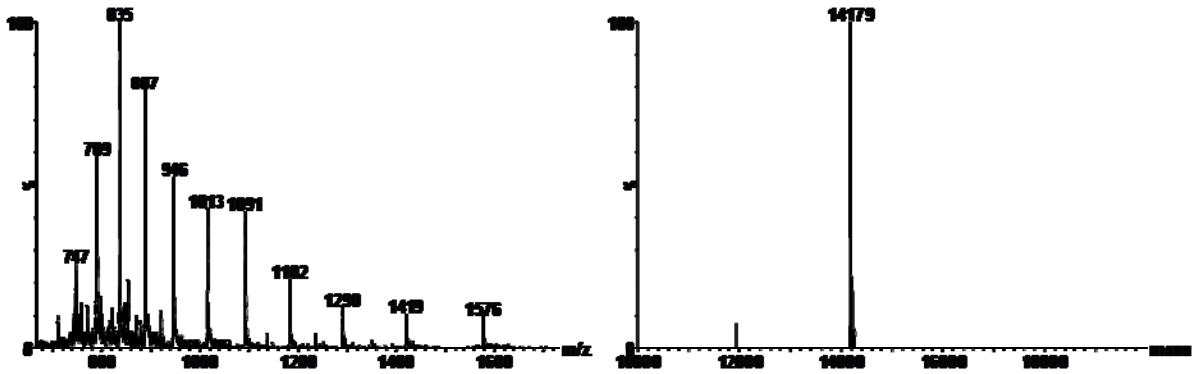
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added *N*-methylmaleamic acid (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that no reaction had occurred. Heating the reaction at 37°C for a further 2 h also yielded no conversion.



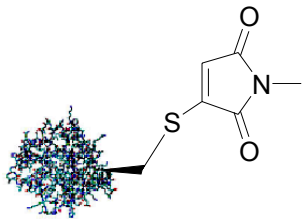
4.4 Reaction of Grb2-SH2 Domain L111C with *N*-Phenylmaleamic Acid



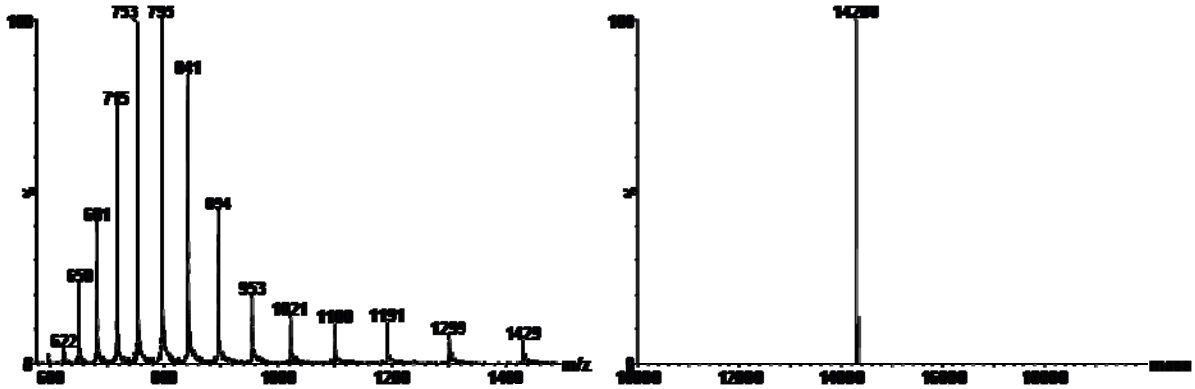
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added *N*-phenylmaleamic acid (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that no reaction had occurred. Heating the reaction at 37°C for a further 2 h also yielded no conversion.



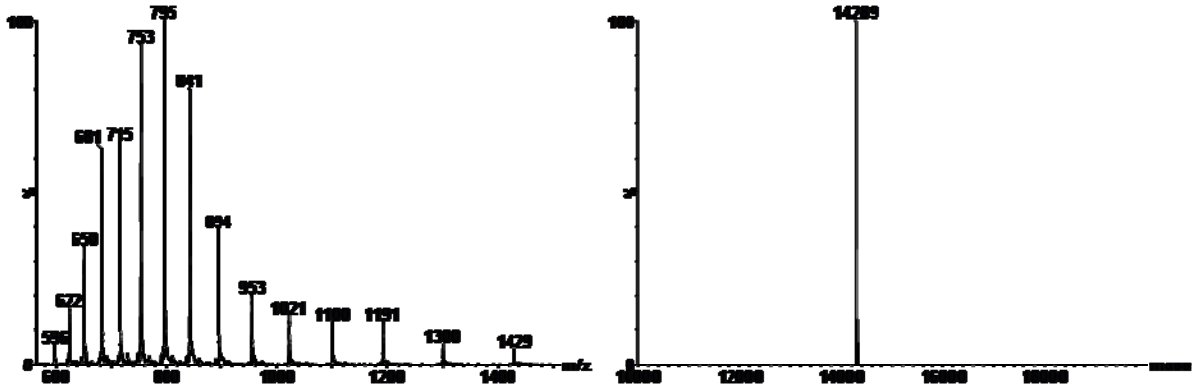
4.5 Grb2-SH2 Domain L111C / *N*-Methylbromomaleimide Adduct



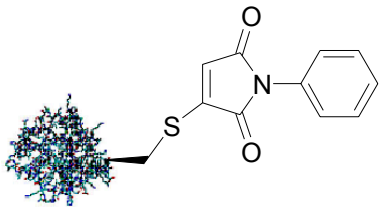
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added *N*-methylbromomaleimide (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 1 h. Analysis using LC-MS showed that the desired product had been formed in quantitative conversion (mass = 14288)[3].



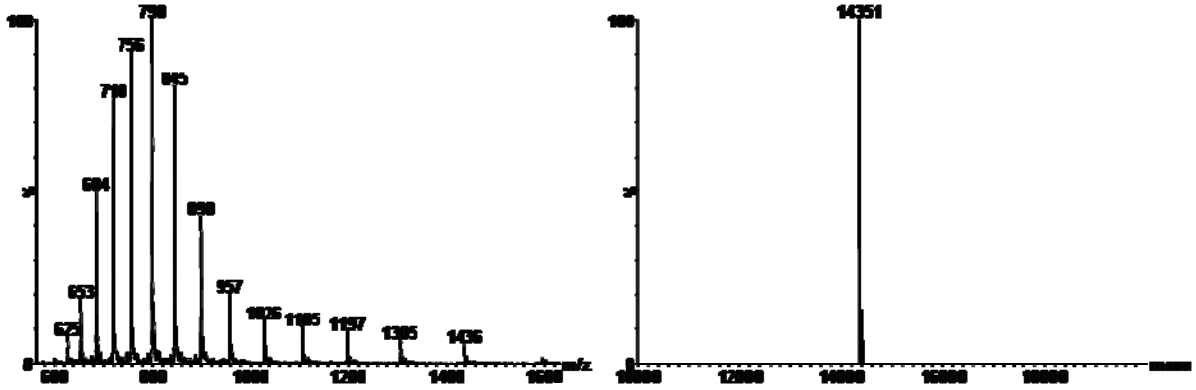
The solution of Grb2-SH2 domain L111C / *N*-methylbromomaleimide adduct was heated at 37°C for 4 h. The construct was stable to hydrolysis under these conditions (mass = 14289).



4.6 Grb2-SH2 Domain L111C / *N*-Phenylbromomaleimide Adduct 3

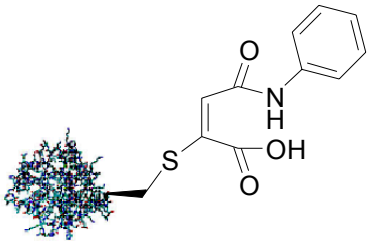


To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added *N*-phenylbromomaleimide **2** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 1 h. Analysis using LC-MS showed that the desired product **3** had been formed in quantitative conversion (mass = 14351).



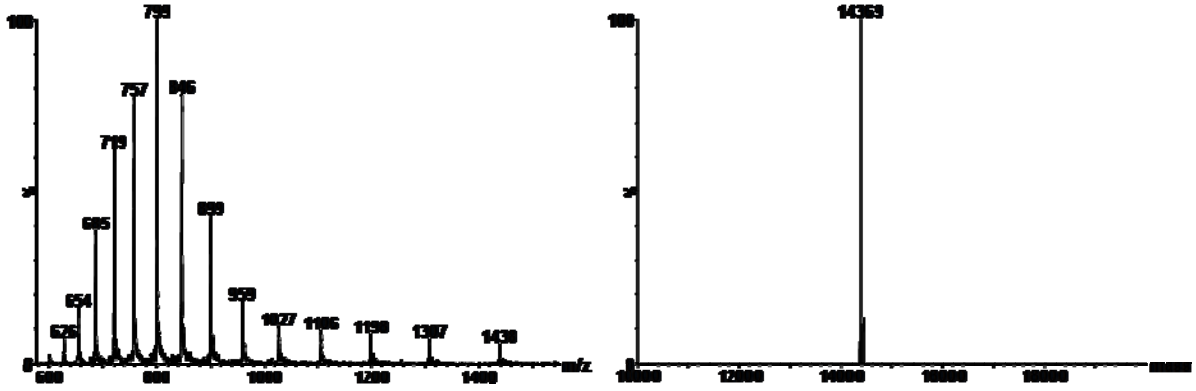
The mixture was treated with Ellman's reagent (5 µL, 282 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 10 mins after which the mixture was analysed by LC-MS. Analysis showed that no reaction with Ellman's reagent was evident highlighting that *N*-phenylbromomaleimide functionalisation had occurred at C111.

4.7 Hydrolysed Grb2-SH2 Domain L111C / *N*-Phenylbromomaleimide Adduct 4

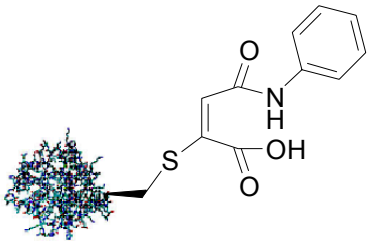


To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added *N*-phenylbromomaleimide **2** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 1 h. Analysis using LC-MS showed that protein / *N*-phenylbromomaleimide adduct **3** had been formed (mass = 14351).

Heating of the mixture at 37°C for 4 h gave the desired product **4** in quantitative conversion (mass = 14369).



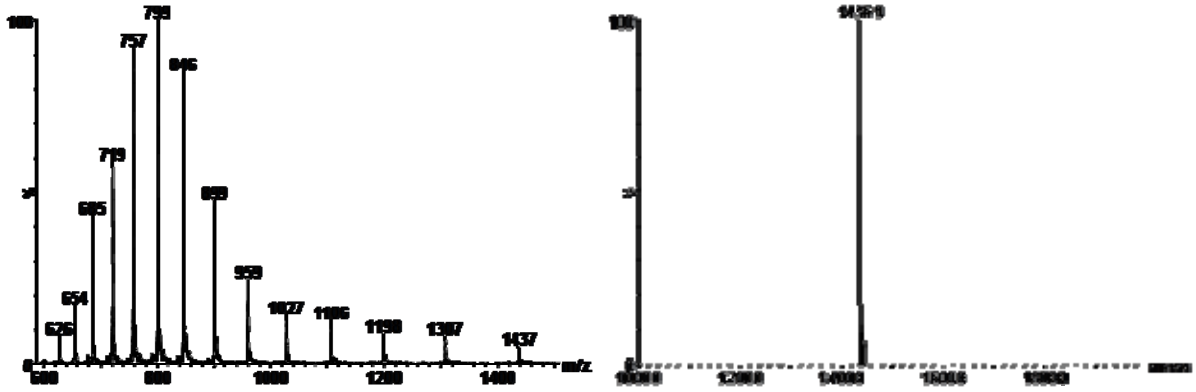
4.8 Treatment of Hydrolysed Grb2-SH2 Domain L111C / *N*-Phenylbromomaleimide Adduct 4 with excess 2-Mercaptoethanol



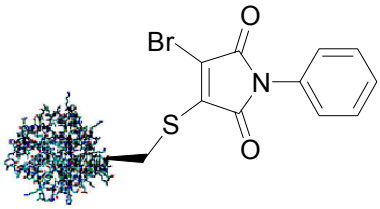
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added *N*-phenylbromomaleimide **2** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 1 h. Analysis using LC-MS showed that protein / *N*-phenylbromomaleimide adduct **3** had been formed (mass = 14351).

Heating of the mixture at 37°C for 4 h gave the desired product **4** (mass = 14369).

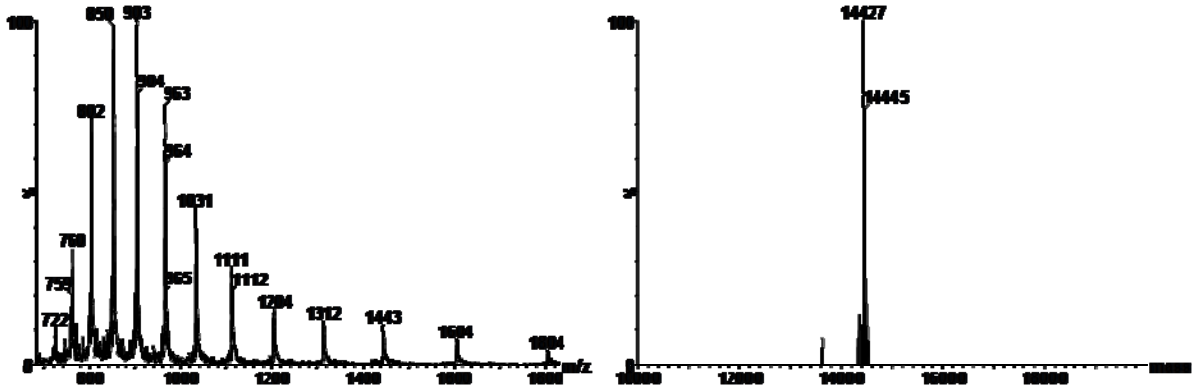
The mixture was treated with 2-mercaptoethanol (5 µL, 282 mM solution in H_2O), vortexed for 1 s and maintained at 37°C for 4 h after which the mixture was analysed by LC-MS. Analysis showed that hydrolysed protein / *N*-phenylbromomaleimide adduct **4** was stable to these conditions.



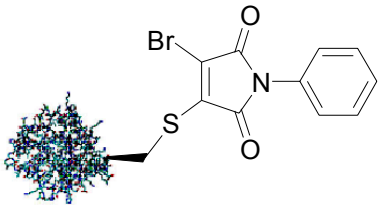
4.9 Grb2-SH2 Domain L111C / *N*-Phenyldibromomaleimide Adduct 6 at pH 8



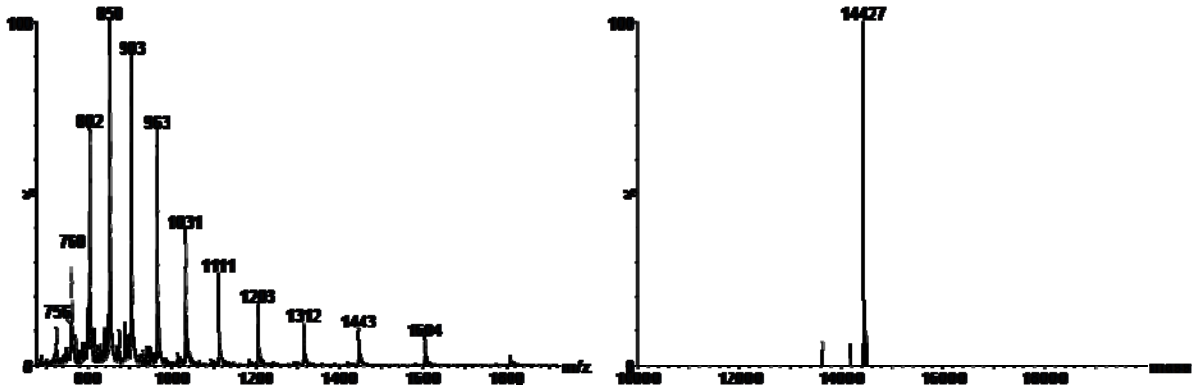
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added *N*-phenyldibromomaleimide **5** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that a mixture of the expected product **6** (mass = 14427) and its hydrolysed derivative **7** (mass = 14445) had been formed in a 7:3 ratio.



4.10 Grb2-SH2 Domain L111C / *N*-Phenyldibromomaleimide Adduct 6 at pH 6

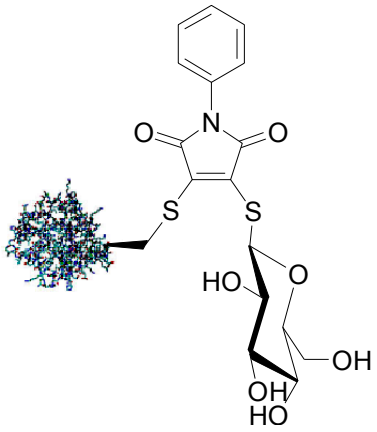


To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-phenyldibromomaleimide **5** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that the desired product **6** had been formed in >95% conversion (mass = 14427).



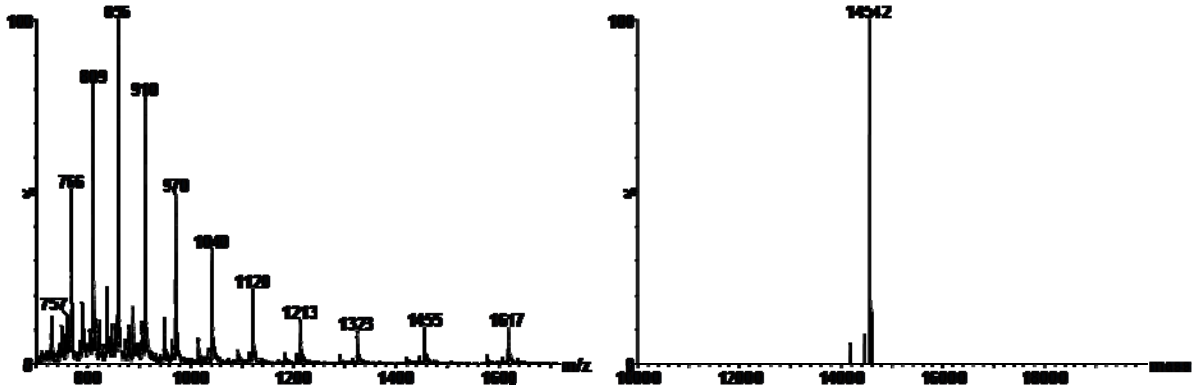
The mixture was treated with Ellman's reagent (5 µL, 282 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 10 mins after which the mixture was analysed by LC-MS. Analysis showed that no reaction with Ellman's reagent was evident highlighting that *N*-phenyldibromomaleimide functionalisation had occurred at C111.

4.11 Grb2-SH2 Domain L111C / *N*-Phenyldibromomaleimide / β-1-Thioglucose Adduct 9

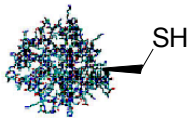


To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-phenyldibromomaleimide **5** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that protein / *N*-phenyldibromomaleimide adduct **6** had been formed (mass = 14427).

The mixture was treated with β-1-thioglucose, sodium salt **8** (5 µL, 2.82 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that the desired product **9** (mass = 14542) was formed in >90% conversion.



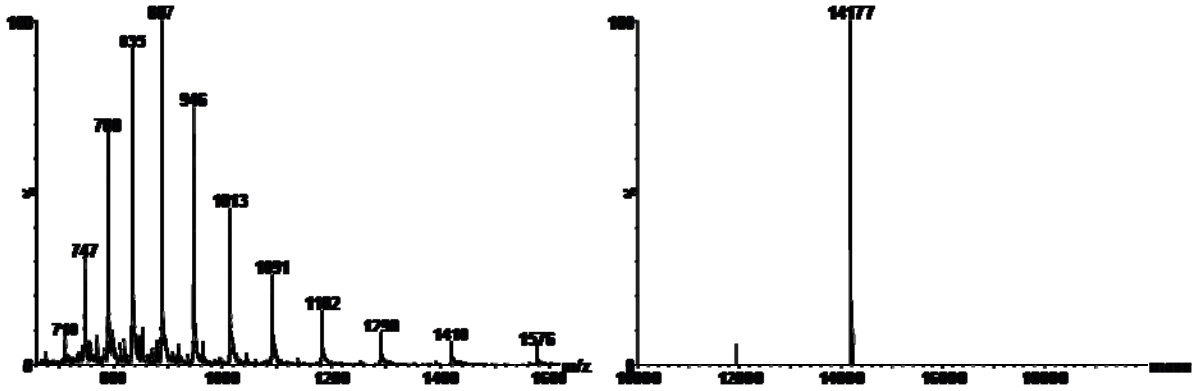
4.12 2-Mercaptoethanol-mediated Cleavage of Grb2-SH2 Domain L111C / *N*-Phenyldibromomaleimide / β-1-Thioglucose Adduct 9 to yield Protein 1



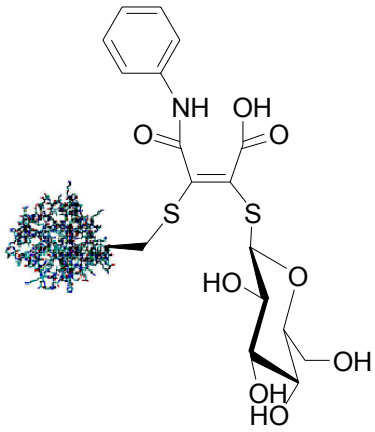
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-phenyldibromomaleimide **5** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that protein / *N*-phenyldibromomaleimide adduct **6** had been formed (mass = 14427).

The mixture was treated with β-1-thioglucose, sodium salt **8** (5 µL, 2.82 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that the desired product **9** (mass = 14542) was formed.

The mixture was treated with 2-mercaptoethanol (5 µL, 282 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 2 h after which the mixture was analysed by LC-MS. Analysis showed that the protein / *N*-phenyldibromomaleimide / β-1-thioglucose **9** had been cleanly cleaved yielding the Grb2-SH2 (L111C) **1** (mass = 14177) in quantitative conversion.



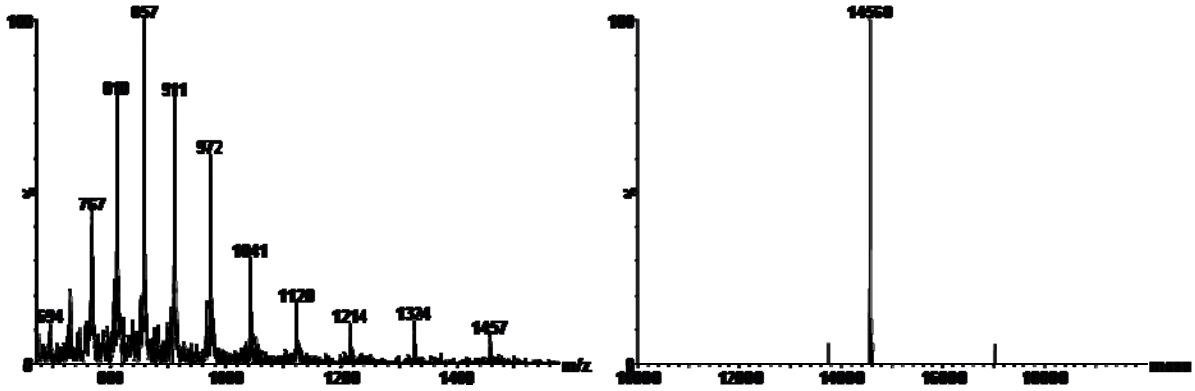
4.13 Hydrolysed Grb2-SH2 Domain L111C / *N*-Phenyldibromomaleimide / β-1-Thioglucose Adduct 10



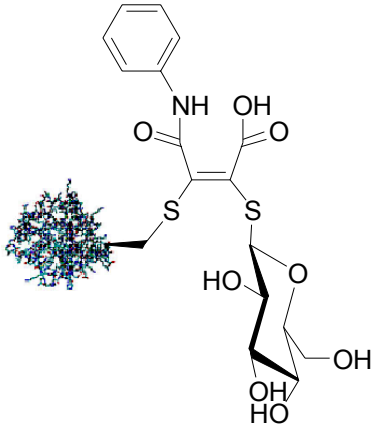
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-phenyldibromomaleimide **5** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that protein / *N*-phenyldibromomaleimide adduct **6** had been formed (mass = 14427).

The mixture was treated with β-1-thioglucose, sodium salt **8** (5 µL, 2.82 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 2 h after which the mixture was analysed by LC-MS. Analysis showed that the protein / *N*-phenyldibromomaleimide / β-1-thioglucose **9** (mass = 14542) was formed.

Dialysis (100 mM sodium phosphate, 150 mM NaCl, pH 8.0, 1L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 12 h at 4°C gave the desired product **10** in >95% conversion (mass = 14560).



4.14 Treatment of Hydrolysed Grb2-SH2 Domain L111C / *N*-Phenyldibromomaleimide / β-1-Thioglucose Adduct 10 with an Excess of 2-Mercaptoethanol

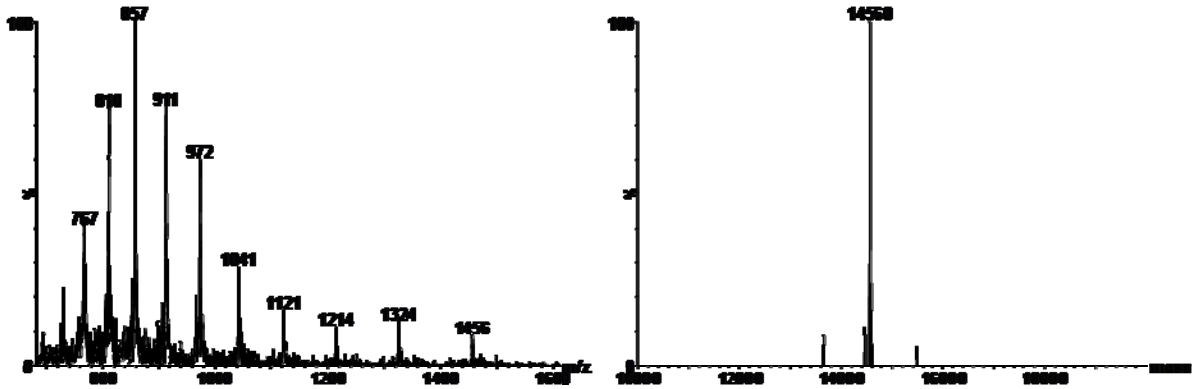


To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-phenyldibromomaleimide **5** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that protein / *N*-phenyldibromomaleimide adduct **6** had been formed (mass = 14427).

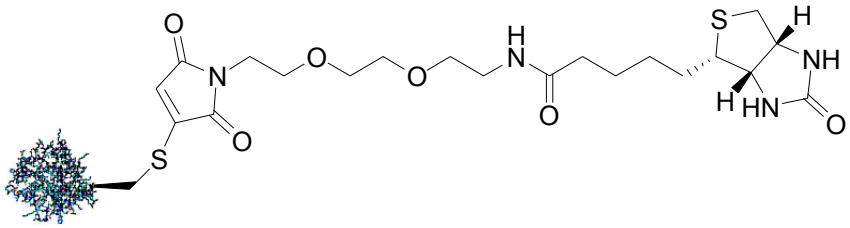
The mixture was treated with β-1-thioglucose, sodium salt **8** (5 µL, 2.82 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 2 h after which the mixture was analysed by LC-MS. Analysis showed that the protein / *N*-phenyldibromomaleimide / β-1-thioglucose **9** (mass = 14542) was formed.

Dialysis (100 mM sodium phosphate, 150 mM NaCl, pH 8.0, 1L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 12 h at 4°C gave product **10** (mass = 14560).

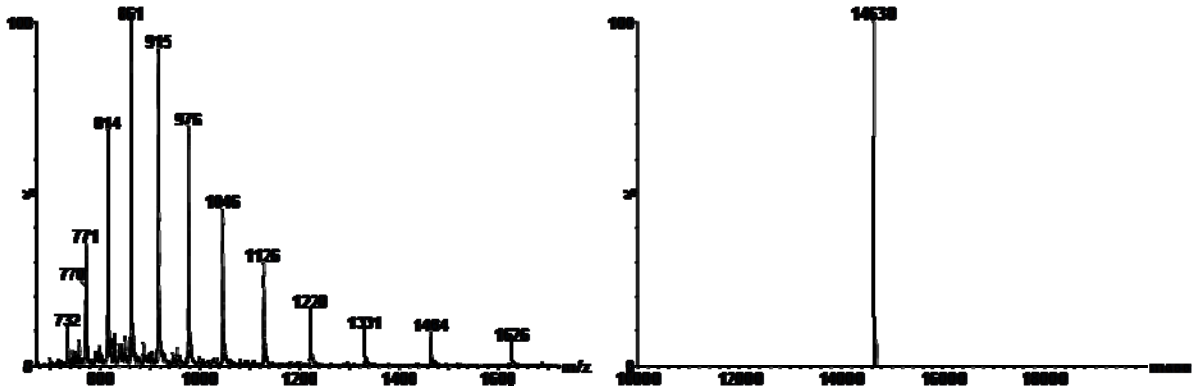
The mixture was treated with 2-mercaptoethanol (5 µL, 282 mM solution in H_2O), vortexed for 1 s and maintained at 37°C for 4 h after which the mixture was analysed by LC-MS. Analysis showed that hydrolysed protein / *N*-phenyldibromomaleimide / β-1-thioglucose **10** adduct was stable to these conditions.



4.15 Grb2-SH2 Domain L111C / Biotin-PEG-bromomaleimide Adduct 12

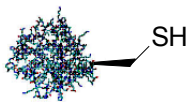


To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added biotin-PEG-bromomaleimide **11** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 1 h. Analysis using LC-MS showed that the desired product **12** had been formed in quantitative conversion (mass = 14630).



The mixture was treated with Ellman's reagent (5 µL, 282 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 10 mins after which the mixture was analysed by LC-MS. Analysis showed that no reaction with Ellman's reagent was evident highlighting that biotinylation had occurred at C111.

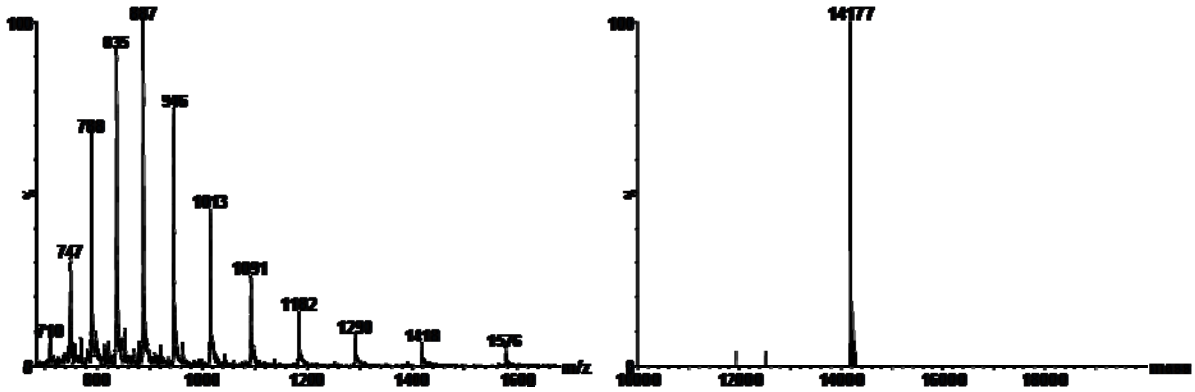
4.16 2-Mercaptoethanol-mediated Cleavage of Grb2-SH2 Domain L111C / Biotin-PEG-bromomaleimide Adduct 12 to Yield Protein 1



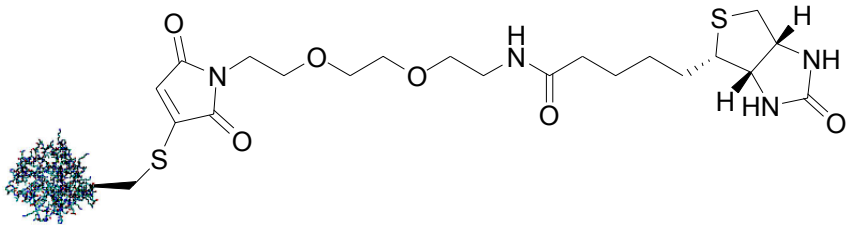
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added biotin-PEG-bromomaleimide **11** (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 1 h. Analysis using LC-MS showed that the protein / biotin-PEG-bromomaleimide adduct **12** had been formed (mass = 14630).

The mixture was treated with 2-mercaptoethanol (5 µL, 282 mM solution in H_2O), vortexed for 1 s and maintained at 37°C for 4 h after which the mixture was analysed

by LC-MS. Analysis showed that desired product **1** was formed in quantitative conversion (mass = 14177).

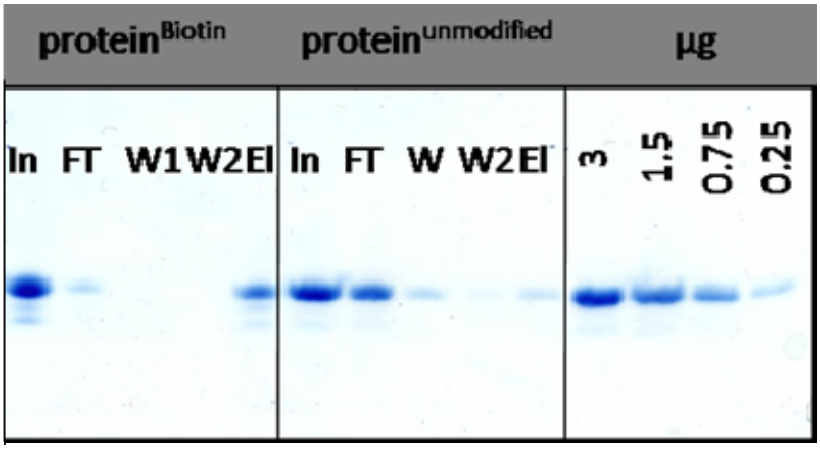


4.17 Pull-Down and Release of Grb2-SH2 Domain L111C / Biotin-PEG-bromomaleimide Adduct onto Neutravidin Coated Agarose Beads



To a solution of model protein **1** (200 µL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0°C was added biotin-PEG-bromomaleimide **11** (10 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 1 h. Analysis using LC-MS showed that the desired product **12** had been formed (mass = 14630).

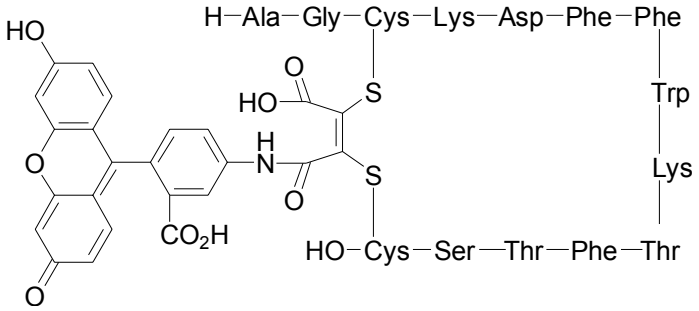
Protein / biotin-PEG-bromomaleimide adduct **12** (200 µL) and unmodified model protein **1** (200 µL) were washed independently with PBS buffer (3 x 500 µL), to remove any excess reagent **11**, in a concentrator (Vivaspin, cut off 10k) yielding protein solutions (300 µL) (Input fraction, **In**). For each of the protein solutions obtained, neutravidin-coated agarose beads (Pierce) (750 µL of 50% aqueous slurry) were washed with PBS (2 x 500 µL). Protein solution (300 µL) was then added to the beads and the mixture incubated at 4°C for 30 mins. The mixture was centrifuged and the flow through (**FT**) collected. The beads were washed with PBS (2 x 500 µL) and both wash fractions collected (**W1** and **W2**). Protein was released from the beads by incubation in PBS (300 µL) containing 2-mercaptoethanol (25 mM) for 2 h at 37°C. The sample was centrifuged and the eluant (**El**) containing cleaved Grb2-SH2 domain L111C collected.



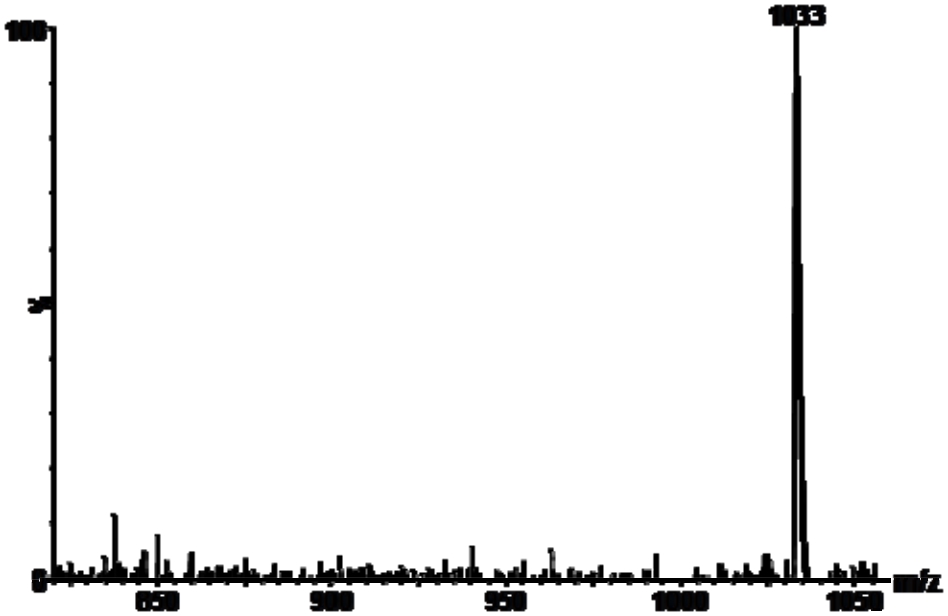
	Biotinyiated	*w/o Biotin*
	total protein per band (µg)	
IN	1.68	1.83
FT	0.06	1.07
W1		0.08
W2		
EL	0.75	0.06

The amount of protein recovered was determined as 46% by comparison with a protein series dilution on a SDS-PAGE gel *via* densitometry. However, correcting for non-specific, irreversibly bound protein (determined using the unmodified protein control) the corrected recovery was 77%.

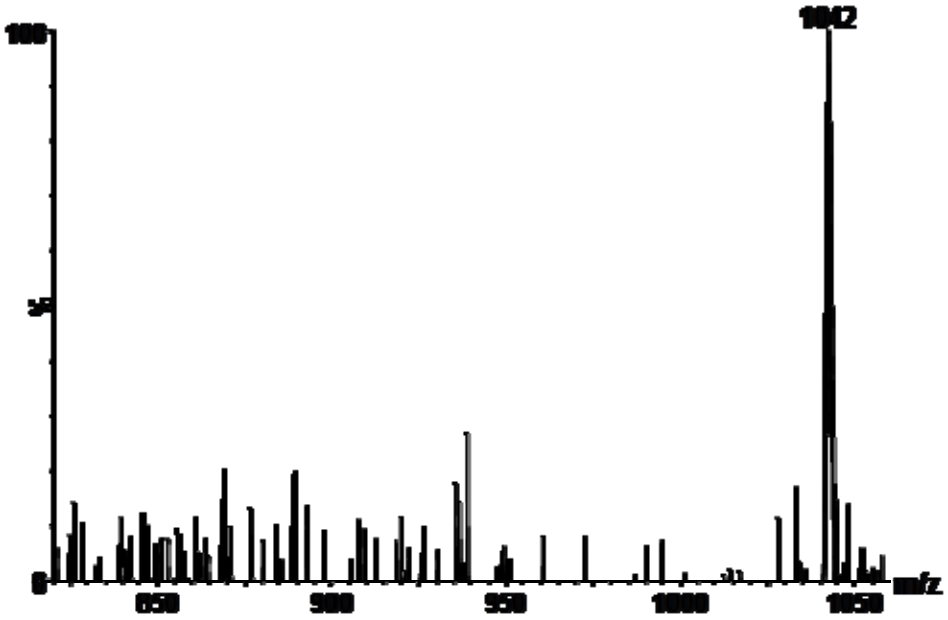
4.18 Hydrolysed Somatostatin / *N*-Fluoresceindibromomaleimide Adduct 14



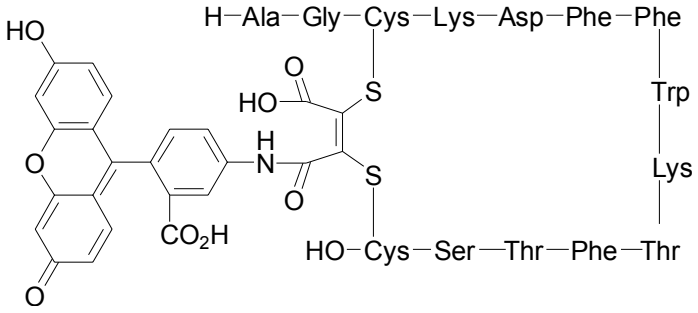
Lyophilised somatostatin (Sigma-Aldrich) was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40 % ACN, 2.5 % DMF) to yield a concentration of 152.6 µM (0.25 mg/ml) and reduced with TCEP (1.1 eq) for 1 h at ambient temperature. After the completeness of the reduction had been confirmed by LC-MS (M^{+2} = 820), *N*-fluorescein- dibromomaleimide (1.1 eq) was added for 10 min at room temperature and quantitative insertion of the maleimide into the opened disulfide bond to give **13** was confirmed by LC-MS (M^{+2} = 1033).[3]



The solution of conjugate **13** was dialysed (sodium phosphate, 50 mM, pH 8) (Slide-A-Lyzer®, 2,000 MWCO, Thermo Scientific) for 12 h at 4°C and subsequently heated at 37°C for 24 h. Analysis by LC-MS showed that the desired conjugate **14** had been formed in quantitative conversion (M^{+2} = 1042).



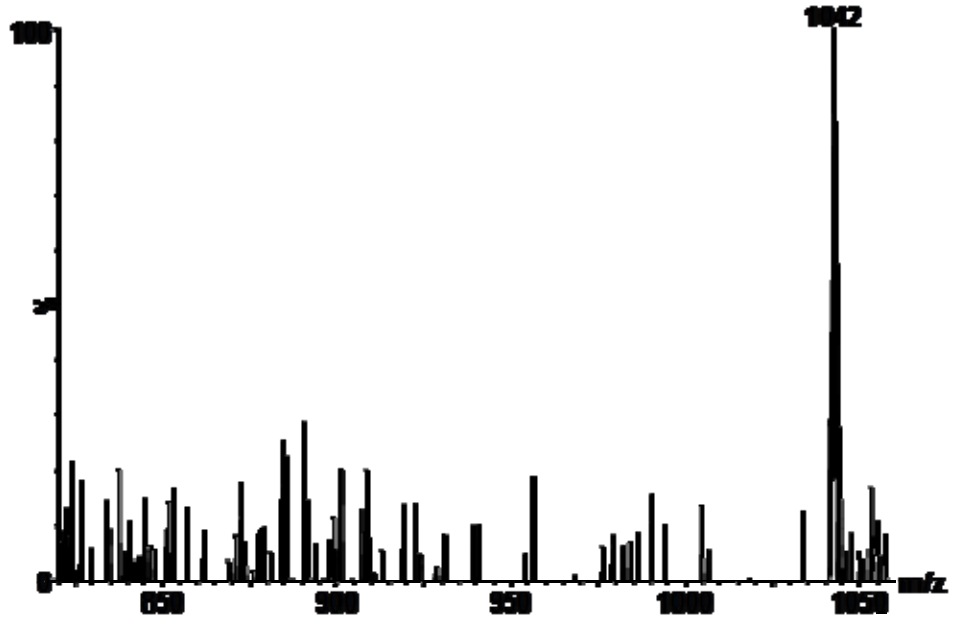
4.19 Treatment of Hydrolysed Somatostatin / *N*-Fluoresceindibromomaleimide Adduct 14 with an Excess of 2-Mercaptoethanol



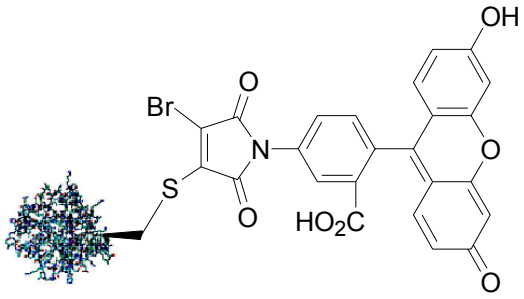
Lyophilised somatostatin (Sigma-Aldrich) was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40 % ACN, 2.5 % DMF) to yield a concentration of 152.6 µM (0.25 mg/ml) and reduced with TCEP (1.1 eq) for 1 h at ambient temperature. After the completeness of the reduction had been confirmed by LC-MS (M^{+2} = 820), *N*-fluorescein- dibromomaleimide (1.1 eq) was added for 10 min at room temperature and quantitative insertion of the maleimide into the opened disulfide bond to give **13** was confirmed by LC-MS (M^{+2} = 1033).[3]

The solution of conjugate **13** was dialysed (sodium phosphate, 50 mM, pH 8) (Slide-A-Lyzer®, 2,000 MWCO, Thermo Scientific) for 12 h at 4°C and subsequently heated at 37°C for 24 h. Analysis by LC-MS showed that conjugate **14** had been formed (M^{+2} = 1042).

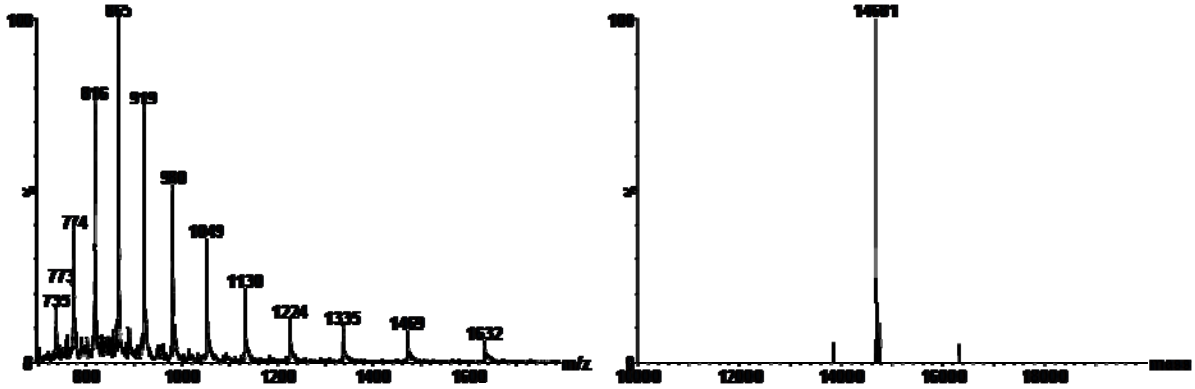
2-Mercaptoethanol (100 mol eq.) was added and the reaction was maintained at 37°C for 24 h. Analysis by LC-MS showed that the hydrolysed somatostatin / *N*-fluorescein dibromomaleimide adduct **14** was completely stable to these reaction conditions.



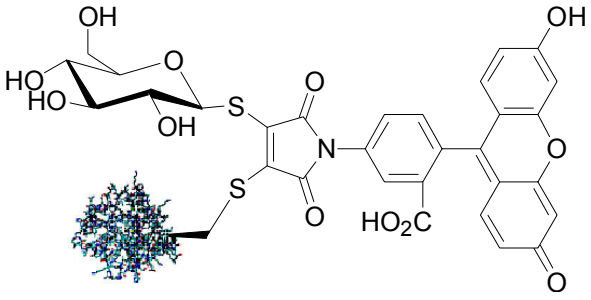
4.20 Grb2-SH2 Domain L111C / *N*-Fluoresceindibromomaleimide Adduct



To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-fluoresceindibromomaleimide (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that the desired product had been formed in >95% conversion (mass = 14681).



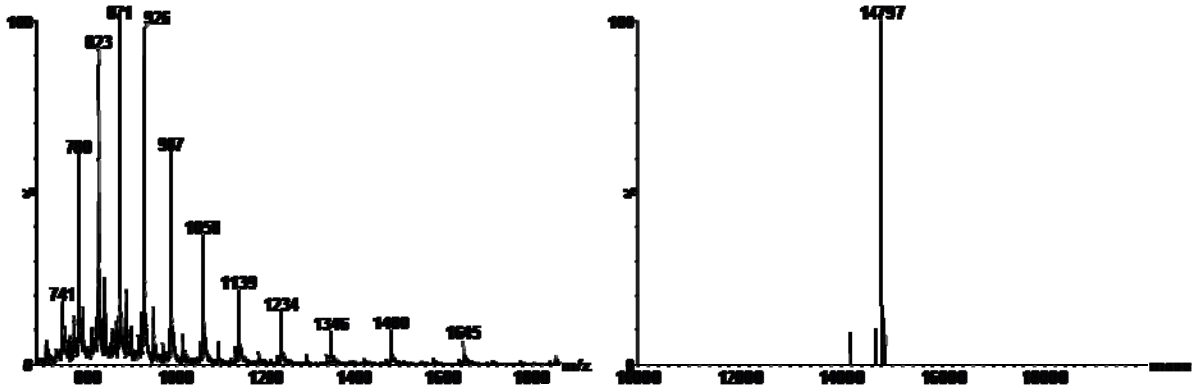
4.21 Grb2-SH2 Domain L111C / *N*-Fluoresceindibromomaleimide / β-1-thioglucose Adduct



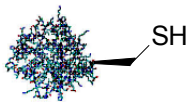
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-fluoresceindibromomaleimide (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that the protein / *N*-fluoresceindibromomaleimide adduct had been formed (mass = 14681).

The mixture was treated with β-1-thioglucose, sodium salt **8** (5 µL, 2.82 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that the protein / *N*-

fluoresceindibromomaleimide / β-1-thioglucose adduct was formed in >85% conversion (mass = 14797).



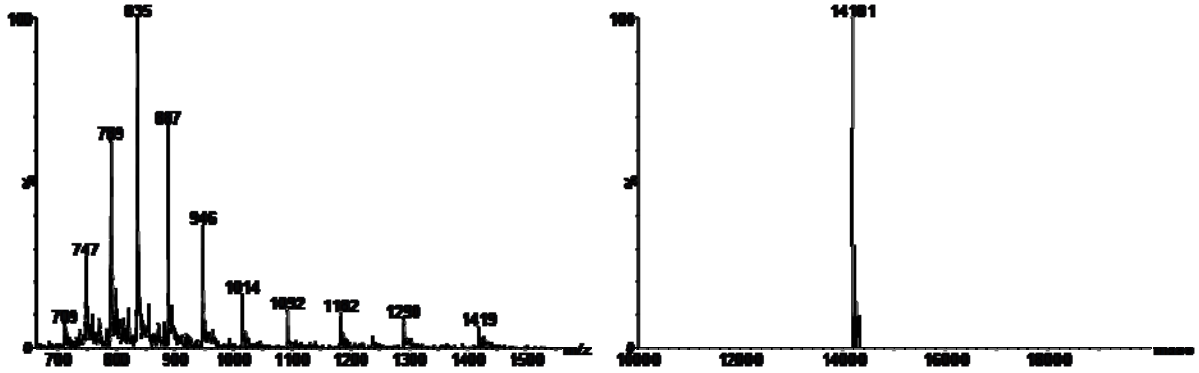
4.22 2-Mercaptoethanol-mediated Cleavage of Grb2-SH2 Domain L111C / *N*-fluoresceindibromomaleimide / β-1-thioglucose Adduct to Yield Protein 1



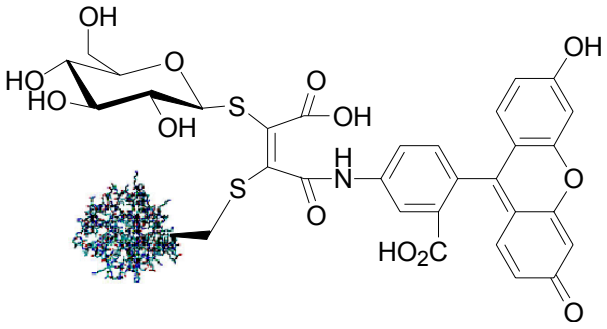
To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-fluoresceindibromomaleimide (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that the protein / *N*-fluoresceindibromomaleimide adduct had been formed (mass = 14681).

The mixture was treated with β-1-thioglucose, sodium salt **8** (5 µL, 2.82 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that the protein / *N*-fluoresceindibromomaleimide / β-1-thioglucose adduct was formed (mass = 14797).

The mixture was treated with 2-mercaptoethanol (5 µL, 282 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 2 h after which the mixture was analysed by LC-MS. Analysis showed that the protein / *N*-fluoresceindibromomaleimide / β-1-thioglucose adduct had been cleanly cleaved yielding the Grb2-SH2 (L111C) **1** in quantitative conversion (mass = 14181).

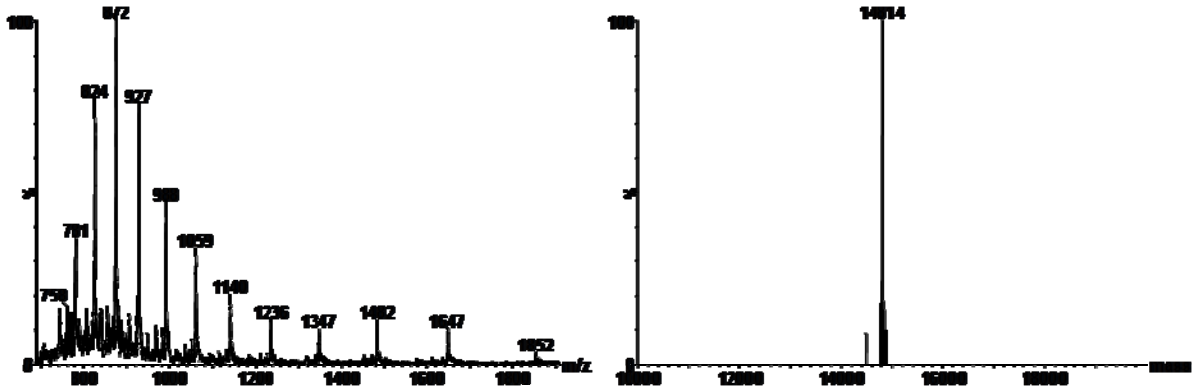


4.23 Hydrolysed Grb2-SH2 Domain L111C / *N*-Fluorescein Dibromomaleimide / β-1-Thioglucose Adduct 15

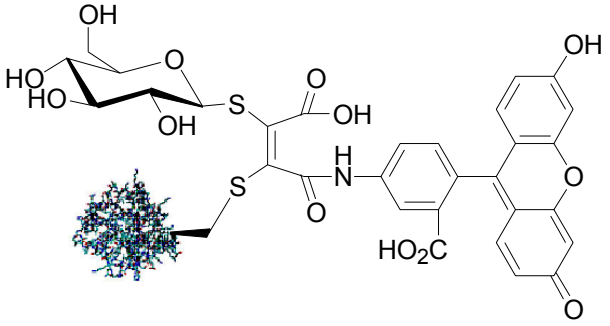


To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-fluoresceindibromomaleimide (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that the protein / *N*-fluoresceindibromomaleimide adduct had been formed (mass = 14681).

The mixture was treated with β-1-thioglucose, sodium salt **8** (5 µL, 2.82 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that the protein / *N*-fluoresceindibromomaleimide / β-1-thioglucose adduct was formed (mass = 14797). Dialysis (100 mM sodium phosphate, 150 mM NaCl, pH 8.0, 1L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 12 h at 4°C gave product **15** in >95% conversion (mass = 14814).



4.24 Treatment of Hydrolysed Grb2-SH2 Domain L111C / N-Fluorescein Dibromomaleimide / β-1-Thioglucose Adduct 15 with an excess of 2-Mercaptoethanol

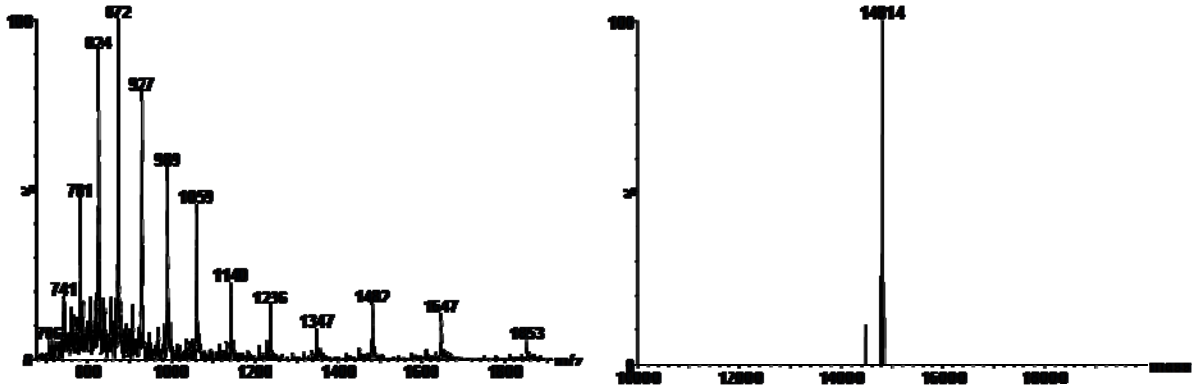


To a solution of model protein **1** (100 µL, [Protein] 2.0 mg/mL, 20 mM MES**,** 150 mM NaCl, pH 6) at 0°C was added *N*-fluoresceindibromomaleimide (5 µL, 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0°C for 2 h. Analysis using LC-MS showed that the protein / *N*-fluoresceindibromomaleimide adduct had been formed (mass = 14681).

The mixture was treated with β-1-thioglucose, sodium salt **8** (5 µL, 2.82 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 0°C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that the protein / *N*-fluoresceindibromomaleimide / β-1-thioglucose adduct was formed (mass = 14797).

Dialysis (100 mM sodium phosphate, 150 mM NaCl, pH 8.0, 1L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 12 h at 4°C gave product **15** (mass = 14814).

The mixture was treated with 2-mercaptoethanol (5 µL, 282 mM solution in H_2O) at 0°C. The mixture was vortexed for 1 s and maintained at 37°C for 4 h after which the mixture was analysed by LC-MS. Analysis showed that hydrolysed protein / *N*-fluoresceindibromomaleimide / β-1-thioglucose **15** adduct was stable to these conditions.



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5. The plasmid encoding Grb2 SH2 domain (residues 53-163) was kindly provided by Dr. Stephen F Martin (University of Texas, A., TX).
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