SUPPORTING INFORMATION

Bromopyridazinedione-mediated protein and peptide bioconjugation

Vijay Chudisama,[a] Mark E. B. Smith,[a] Felix F. Schumacher,[a] Danai Papaioannou,[b] Gabriel Waksman,[b] James R. Baker[a] and Stephen Caddick*[a]

a. Department of Chemistry, UCL, 20 Gordon Street, London, WC1H 0AJ, U.K.; b. Institute of Structural Molecular Biology at UCL/Birkbeck, Malet Street, London, WC1E 7HX, U.K.

1. General Procedures

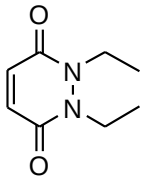
^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Bruker Avance 600 instrument operating at a frequency of 600 MHz for ^{1}H and 150 MHz for ^{13}C. ^{1}H NMR spectra were referenced to the $CDCl_3$ (7.26 ppm) signal. ^{13}C NMR spectra were referenced to the $CDCl_3$ (77.67 ppm) signal. 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 and CI mode). Maleic hydrazide and lyophilised somatostatin were purchased from Sigma-Aldrich and used without further purification.

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 1.7 µm 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 s. 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 Bromopyridazinediones and Intermediates.

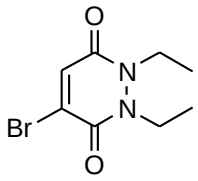
3.1 1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione 5



To a solution of maleic anhydride (98 mg, 1.0 mmol) in glacial AcOH (3 mL) was added *N*,*N*'-diethylhydrazine.2HCl (161 mg, 1.0 mmol) and the reaction mixture heated at 130 °C for 16 h. The solvent was removed *in vacuo* and the crude residue purified by column chromatography (50% EtOAc/Petrol to neat EtOAc) to give 1,2-

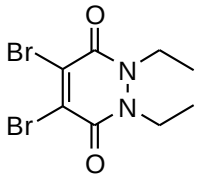
diethyl-1,2-dihydro-pyridazine-3,6-dione as a white solid (121 mg, 0.72 mmol, 72%): ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.85 (s, 2H), 4.11 (q, *J* = 7.0 Hz, 4H), 1.25 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 157.6 (s), 134.7 (d), 40.2 (t), 13.3 (q); IR (solid) 2984, 1636, 1590 cm^{-1}; LRMS (CI) 169 (100, $[M+H]^+$); HRMS (CI) calcd for $C_8H_{13}N_2O_2$ $[M+H]^+$ 169.0977, observed 169.0980.

3.2 4-Bromo-1,2-diethyl-1,2-dihydro-pyridazine-3,6-dione 7



To a solution of 1,2-diethyl-1,2-dihydro-pyridazine-3,6-dione (168 mg, 1.0 mmol) in CH_2Cl_2 (3 mL) was added bromine (1.59 g, 0.51 mL, 10.0 mmol) and the reaction mixture stirred at 20 °C for 16 h. The solvent and excess bromine were removed *in vacuo* and then was added NEt_3 (405 mg, 558 μL, 4.0 mmol) and CH_2Cl_2 (3 mL) and the reaction mixture stirred at 20 °C for 16 h. The solvent was removed *in vacuo* and the crude residue purified by column chromatography (50% EtOAc/Petrol to neat EtOAc) to give 4-bromo-1,2-diethyl-1,2-dihydro-pyridazine-3,6-dione as a yellow solid (227 mg, 0.92 mmol, 92%): ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.31 (s, 1H), 4.14 (q, *J* = 7.0 Hz, 2H), 4.07 (q, *J* = 7.0 Hz, 2H), 1.26 (t, *J* = 7.0 Hz, 3H), 1.22 (t, *J* = 7.0 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 156.2 (s), 154.3 (s), 136.0 (d), 133.7 (s), 41.9 (t), 40.7 (t), 13.3 (q), 13.3 (q); IR (solid) 3058, 2979, 2938, 1631, 1595 cm^{-1}; LRMS (CI) 249 (100, $[M^{81}Br+H]^+$), 247 (100, $[M^{79}Br+H]^+$); HRMS (CI) calcd for $C_8H_{12}BrN_2O_2$ $[M^{81}Br+H]^+$ 249.0082, observed 249.0086.

3.3 4,5-Dibromo-1,2-diethyl-1,2-dihydro-pyridazine-3,6-dione 8

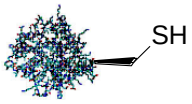


To a solution of 4-bromo-1,2-diethyl-1,2-dihydro-pyridazine-3,6-dione (248 mg, 1.0 mmol) in CH_2Cl_2 (3 mL) was added bromine (1.59 g, 0.51 mL, 10.0 mmol) and the reaction mixture stirred at 50 °C for 72 h. The solvent and excess bromine were removed *in vacuo* and then was added NEt_3 (405 mg, 558 μL, 4.0 mmol) and CH_2Cl_2 (3 mL) and the reaction mixture stirred at 20 °C for 16 h. The solvent was removed *in vacuo* and the crude residue purified by column chromatography (50% EtOAc/Petrol to neat EtOAc) to give 4,5-dibromo-1,2-diethyl-1,2-dihydro-pyridazine-3,6-dioneas a yellow solid (295 mg, 0.91 mmol, 91%): ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.17 (q, *J* = 7.0 Hz, 4H), 1.28 (t, *J* = 7.0 Hz, 6H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 153.3 (s), 136.1 (s), 42.4 (t), 13.2 (q); IR (solid) 2979, 2937, 1630, 1574 cm^{-1}; LRMS (EI) 328 (50, $[M^{81}Br^{81}Br]^{+\bullet}$), 326 (100, $[M^{81}Br^{79}Br]^{+\bullet}$), 324 (50, $[M^{79}Br^{79}Br]^{+\bullet}$); HRMS (EI) calcd for $C_8H_{10}Br_2N_2O_2$ $[M^{79}Br^{79}Br]^{+\bullet}$ 323.9104, observed 323.9097.

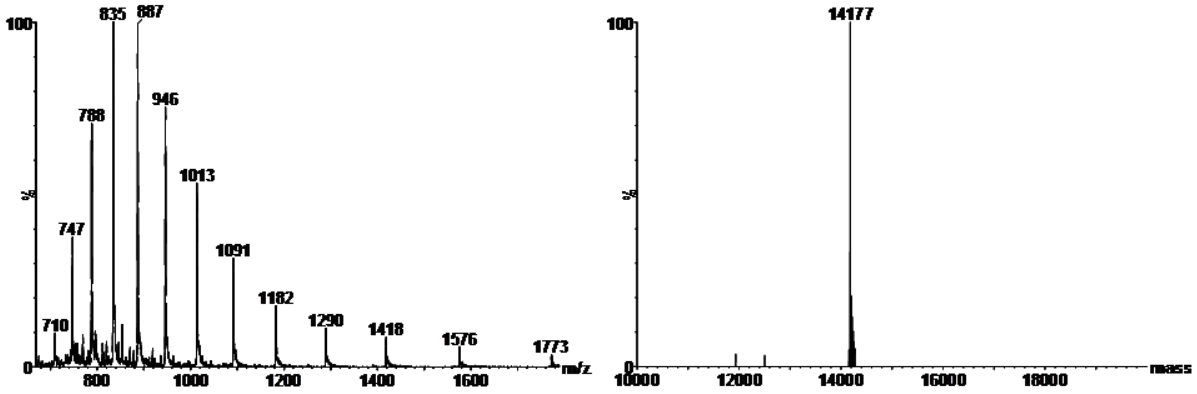
4. Protein and Peptide Modification

Sequence of isolated Grb2-SH2 L111C (residues 53-163) **3**: M 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.

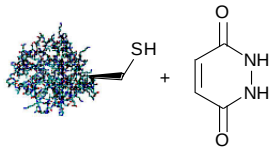
4.1 Cloning and Expression of Grb2-SH2 L111C Mutant 3[1]



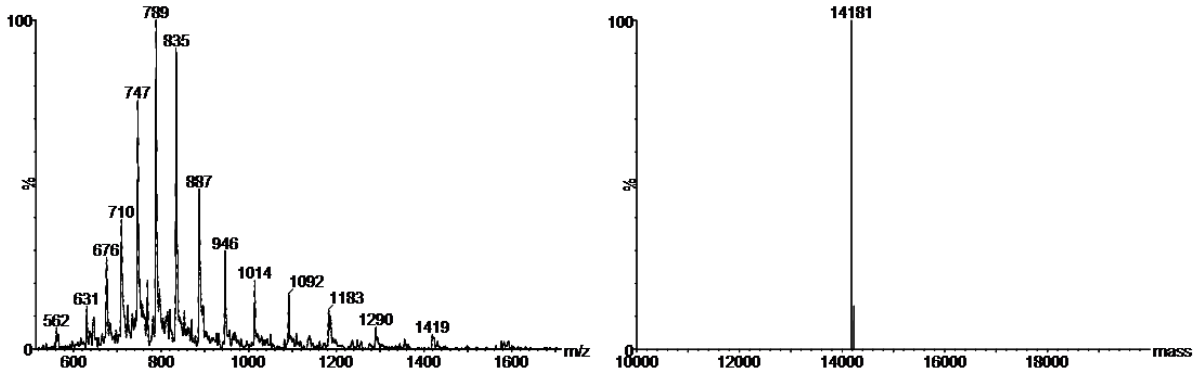
The DNA construct for the Grb2 SH2 domain contained the primary amino acid sequence 53-163 and was cloned on plasmid QE-60 (Qiagen).[2] The Grb2 SH2 L111C mutant was constructed by site-directed mutagenesis (Stratagene Kit) using oligonucleotides coding for the mutated residue. Both constructs were expressed 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 ^{o}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 0.1 M 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 0.1 M sodium phosphate, 300 mM NaCl, 200 mM imidazole at pH 7.2. The collected Grb2 SH2 L111C **3** was ~95% pure as visualized by Coomassie-stained SDS–PAGE. Dimerization of Grb2 SH2 domain through domain-swapping has been previously observed.[3] 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 **3** 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 ^{o}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 280 nm using the extinction coefficient obtained by McNemar and coworkers (15,600 M^{-1}).[4] The protein was frozen at 1 mg/mL concentration in 100 μL aliquots which were thawed as required for experiments.The mass of the monomeric protein **3** (mass = 14177) was obtained using LC-MS.



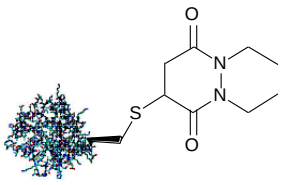
4.2 Attempted Reaction of Grb2-SH2 Domain L111C with 1,2-Dihydro-pyridazine-3,6-dione 4



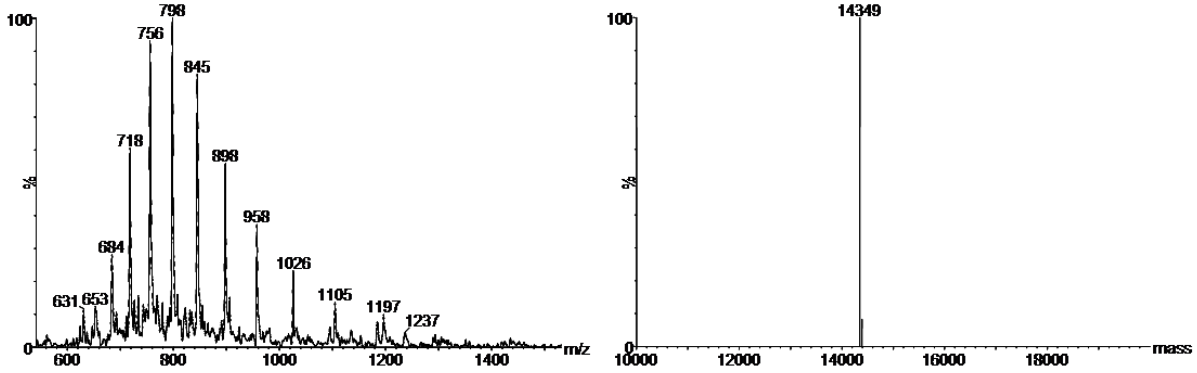
To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 ^{o}C was added pyridazinedione **4** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 ^{o}C for 16 h. Analysis using LC-MS showed that no reaction had occurred.



4.3 Grb2-SH2 Domain L111C / 1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione Adduct 6

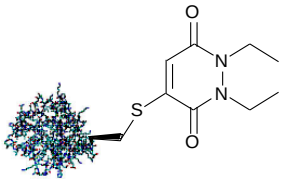


To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 ^{o}C was added pyridazinedione **5** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 ^{o}C for 16 h. Analysis using LC-MS showed that the desired product had been formed in quantitative conversion (mass = 14349).

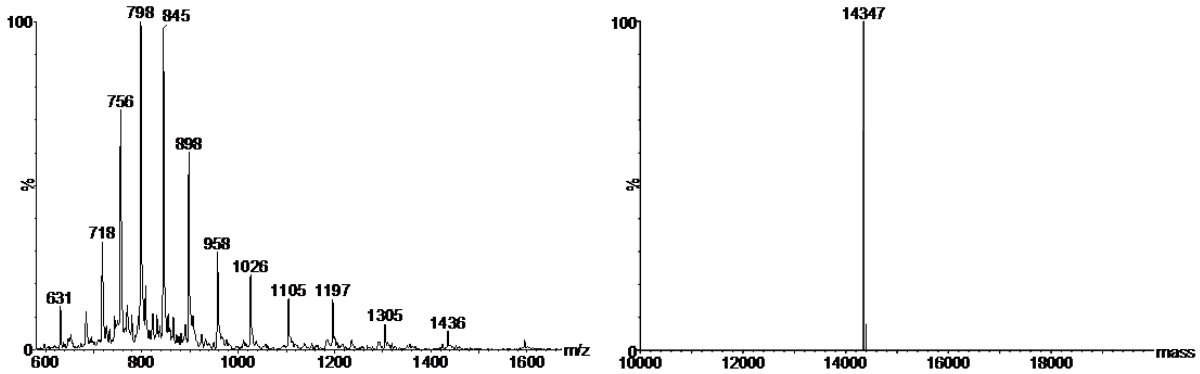


The mixture was treated with Ellman's reagent (5 μL, 141 mM solution in H_2O) at 0 ^oC. The mixture was vortexed for 1 s and maintained at 0 ^oC for 10 min after which the mixture was analysed by LC-MS. Analysis showed that no reaction with Ellman's reagent was evident thus highlighting that pyridazinedione functionalisation had occurred at C111.

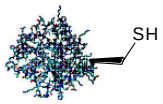
4.4 Grb2-SH2 Domain L111C / 4-Bromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione Adduct 9



To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 ^oC was added pyridazinedione **7** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 ^oC for 1 h. Analysis using LC-MS showed that the desired product had been formed in quantitative conversion (mass = 14347).

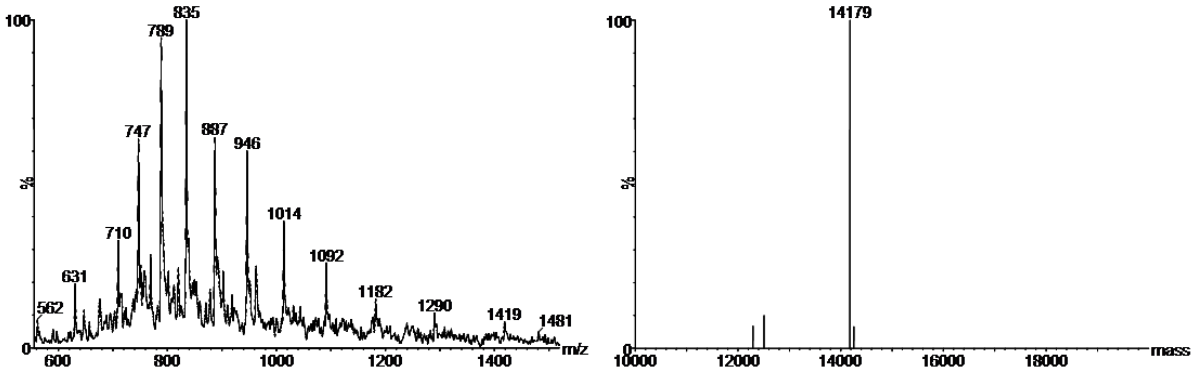


4.5 2-Mercaptoethanol-mediated Cleavage of Grb2-SH2 Domain L111C / 4-Bromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione Adduct 9 to yield Protein 3

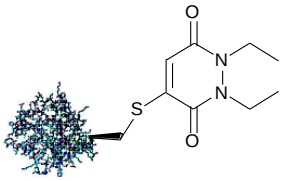


To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 °C was added pyridazinedione **7** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 °C for 1 h. Analysis using LC-MS showed that the protein / pyridazinedione conjugate **9** had been formed (mass = 14347).

The mixture was dialysed (100 mM sodium phosphate, 150 mM NaCl, pH 8.0, 1 L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 40 h at 4 °C. The mixture was then treated with 2-mercaptoethanol (5 μL, 141 mM solution in H_2O) at 20 °C. The mixture was vortexed for 1 s and maintained at 20 °C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that the protein / pyridazinedione conjugate **9** had been cleaved yielding the Grb2-SH2 (L111C) **3** (mass = 14179) in quantitative conversion.



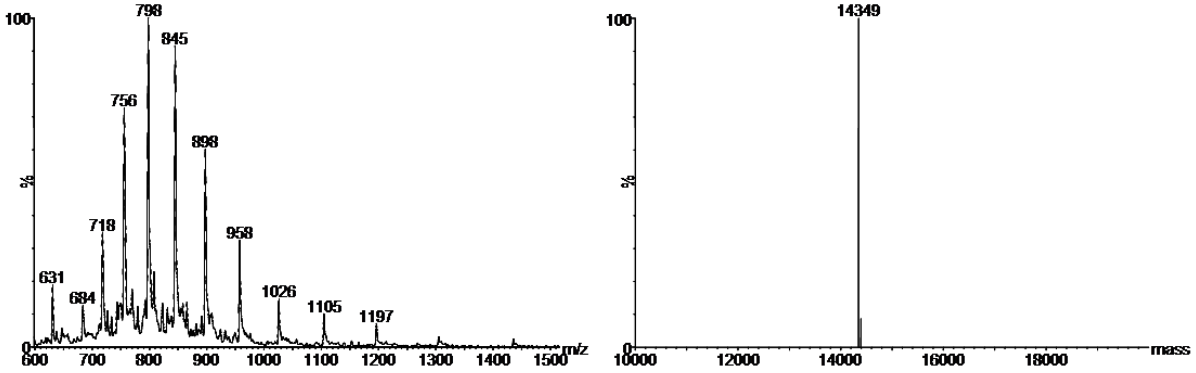
4.6 Hydrolytic Stability of Grb2-SH2 Domain L111C / 4-Bromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione Adduct 9 at 37 °C



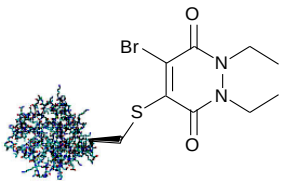
To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 °C was added pyridazinedione **7** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 °C for 1 h. Analysis using LC-MS showed that the protein / pyridazinedione conjugate **9** had been formed (mass = 14349).

The mixture was dialysed (100 mM sodium phosphate, 150 mM NaCl, pH 8.0, 1L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 40 h at 4 °C and then heated

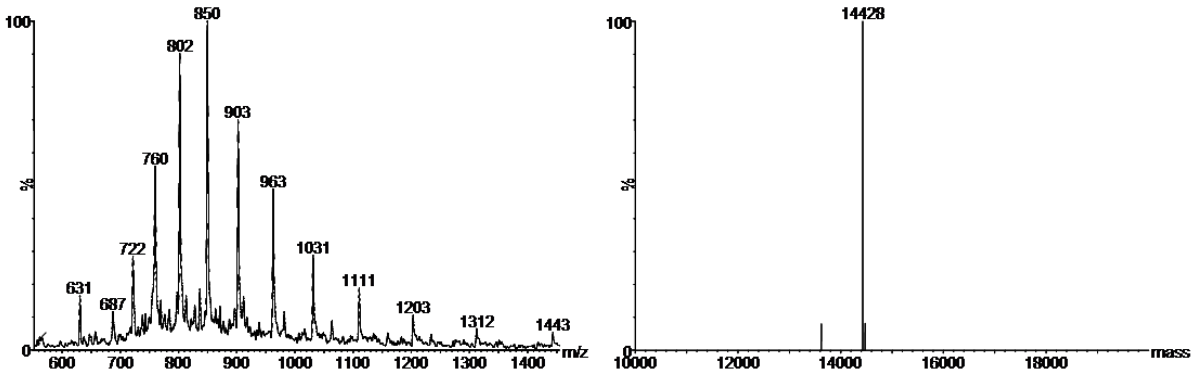
at 37 ^{o}C for 5 h. Analysis using LC-MS showed that conjugate **9** was hydrolytically stable under these conditions.



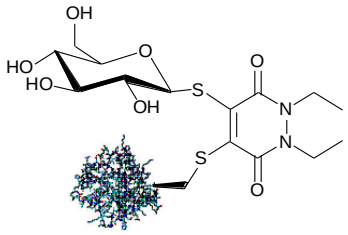
4.7 Grb2-SH2 Domain L111C / 4,5-Dibromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione Adduct 10



To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 ^{o}C was added pyridazinedione **8** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 ^{o}C for 1 h. Analysis using LC-MS showed that the desired product **10** had been formed (mass = 14428) in quantitative conversion.

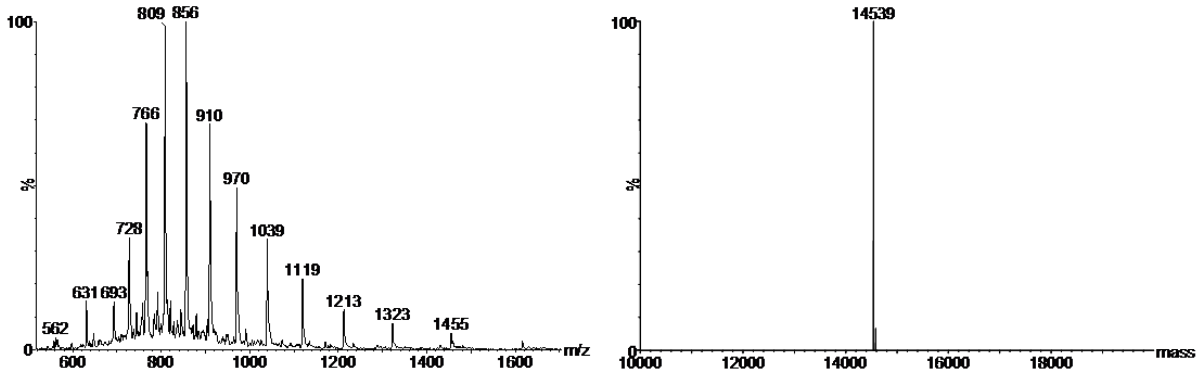


4.8 Grb2-SH2 Domain L111C / 4,5-Dibromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione / β-1-thioglucose Adduct 11

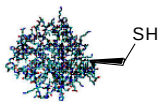


To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 °C was added pyridazinedione **8** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 °C for 1 h. Analysis using LC-MS showed that conjugate **10** had been formed (mass = 14428).

The mixture was dialysed (100 mM sodium phosphate, 150 mM NaCl, pH 8.0, 1 L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 40 h at 4 °C. The mixture was then treated with β-1-thioglucose, sodium salt (5 μL, 14.1 mM solution in H_2O) at 24 °C. The mixture was vortexed for 1 s and maintained at 24 °C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that the desired product **11** (mass = 14539) was formed in quantitative conversion.



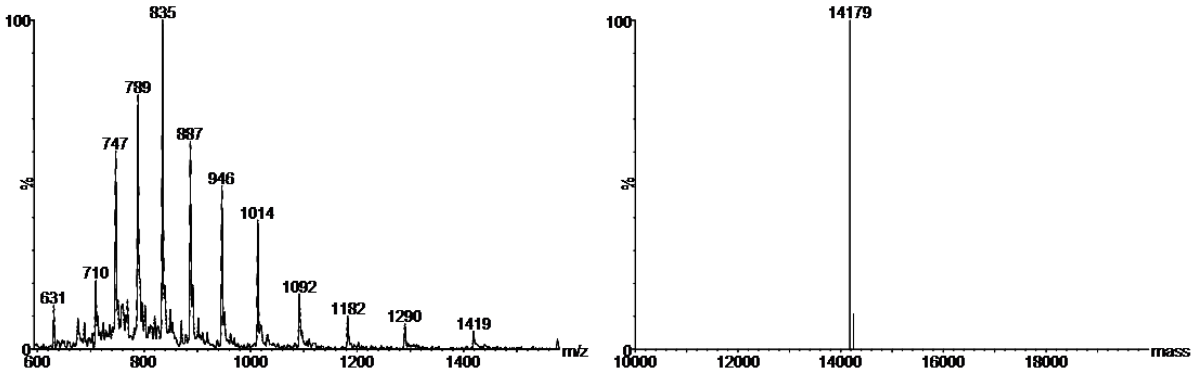
4.9 2-Mercaptoethanol-mediated Cleavage of Grb2-SH2 Domain L111C / 4,5-Dibromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione / β-1-thioglucose Adduct 11 to yield Protein 3



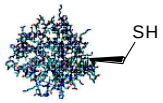
To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 °C was added pyridazinedione **8** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 °C for 1 h. Analysis using LC-MS showed that conjugate **10** had been formed (mass = 14428).

The mixture was dialysed (100 mM sodium phosphate, 150 mM NaCl, pH 8.0, 1 L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 40 h at 4 °C. The mixture was then treated with β-1-thioglucose, sodium salt (5 μL, 14.1 mM solution in H_2O) at 24 °C. The mixture was vortexed for 1 s and maintained at 24 °C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that conjugate **11** (mass = 14538) was formed.

The mixture was then treated with 2-mercaptoethanol (5 μL, 141 mM solution in H_2O) at 24 °C. The mixture was vortexed for 1 s and maintained at 24 °C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that conjugate **11** had been cleaved yielding Grb2-SH2 (L111C) **3** in quantitative conversion (mass = 14179).



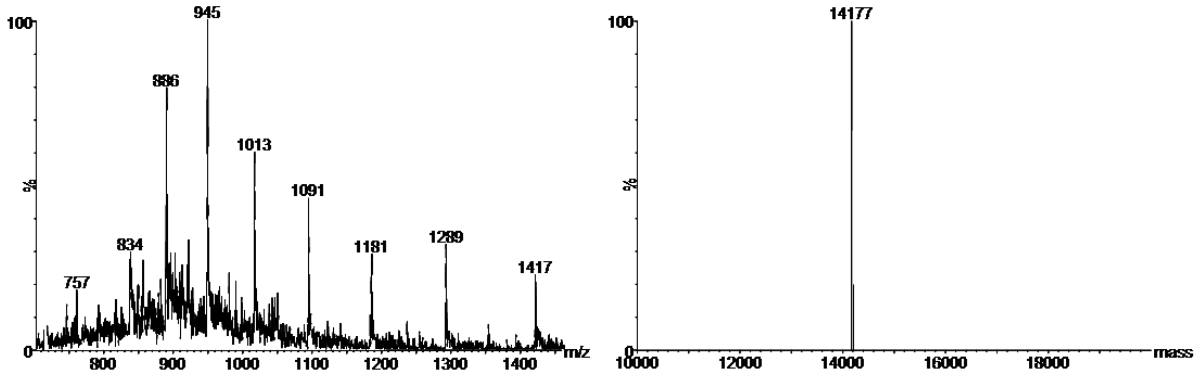
4.10 Glutathione-mediated Cleavage of Grb2-SH2 Domain L111C / 4,5-Dibromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione / β-1-thioglucose Adduct 11 to yield Protein 3 at Physiological pH and Temperature



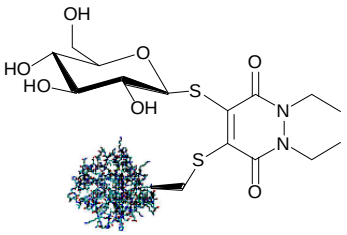
To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 °C was added pyridazinedione **8** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 °C for 1 h. Analysis using LC-MS showed that conjugate **10** had been formed (mass = 14428).

The mixture was dialysed (100 mM sodium phosphate, 150 mM NaCl, pH 7.2, 1 L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 40 h at 4 °C. The mixture was then treated with β-1-thioglucose, sodium salt (5 μL, 14.1 mM solution in H_2O) at 24 °C. The mixture was vortexed for 1 s and maintained at 24 °C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that conjugate **11** (mass = 14538) was formed.

The mixture was then treated with glutathione (5 μL, 20 mM solution in H_2O) at 37 °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 conjugate **11** had been cleaved yielding Grb2-SH2 (L111C) **3** (mass = 14177) in quantitative conversion.



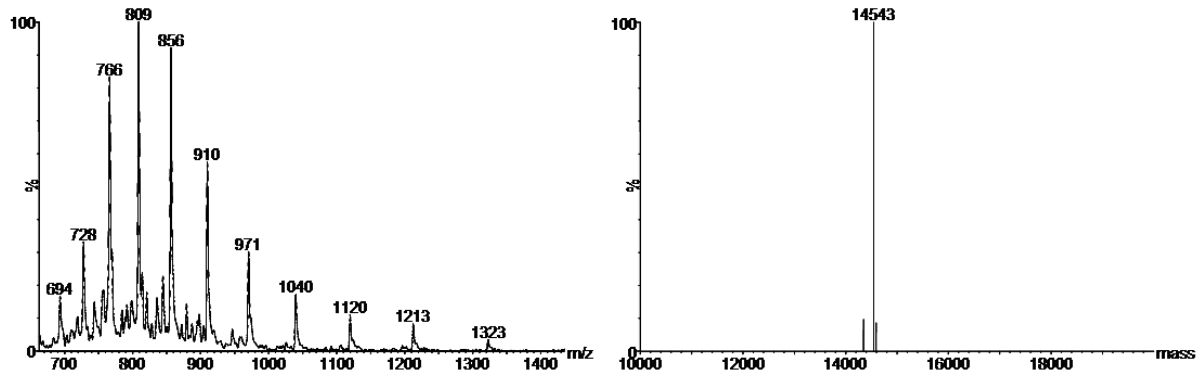
4.11 Hydrolytic Stability of Grb2-SH2 Domain L111C / 4,5-Dibromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione / β-1-thioglucose Adduct 11 at 37 °C



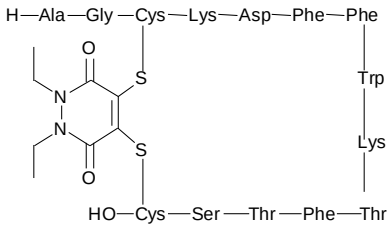
To a solution of model protein **3** (100 μL, [Protein] 1.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 37 °C was added pyridazinedione **8** (5 μL, 141 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 37 °C for 1 h. Analysis using LC-MS showed that conjugate **10** had been formed (mass = 14428).

The mixture was dialysed (100 mM sodium phosphate, 150 mM NaCl, pH 8.0, 1 L) (Slide-A-Lyzer®, 10,000 MWCO, Thermo Scientific) for 40 h at 4 °C. The mixture was then treated with β-1-thioglucose, sodium salt (5 μL, 14.1 mM solution in H_2O) at 24 °C. The mixture was vortexed for 1 s and maintained at 24 °C for 1 h after which the mixture was analysed by LC-MS. Analysis showed that conjugate **11** (mass = 14543) was formed.

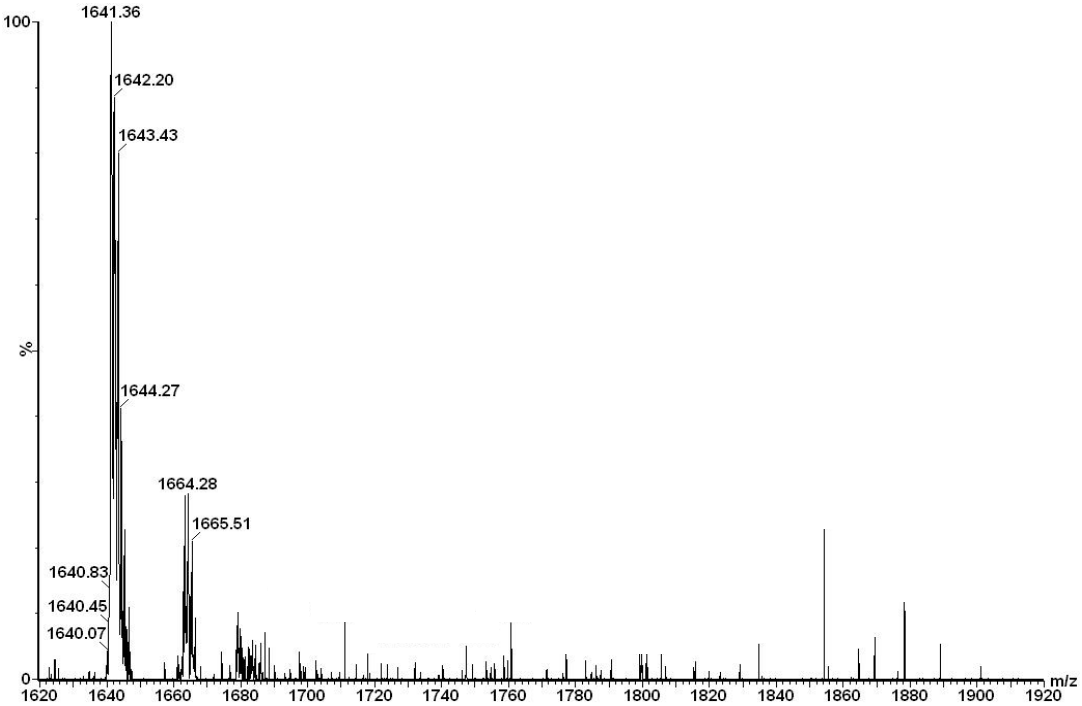
The mixture was then heated at 37 °C for 5 h. Analysis using LC-MS showed that conjugate **11** was hydrolytically stable under these conditions.



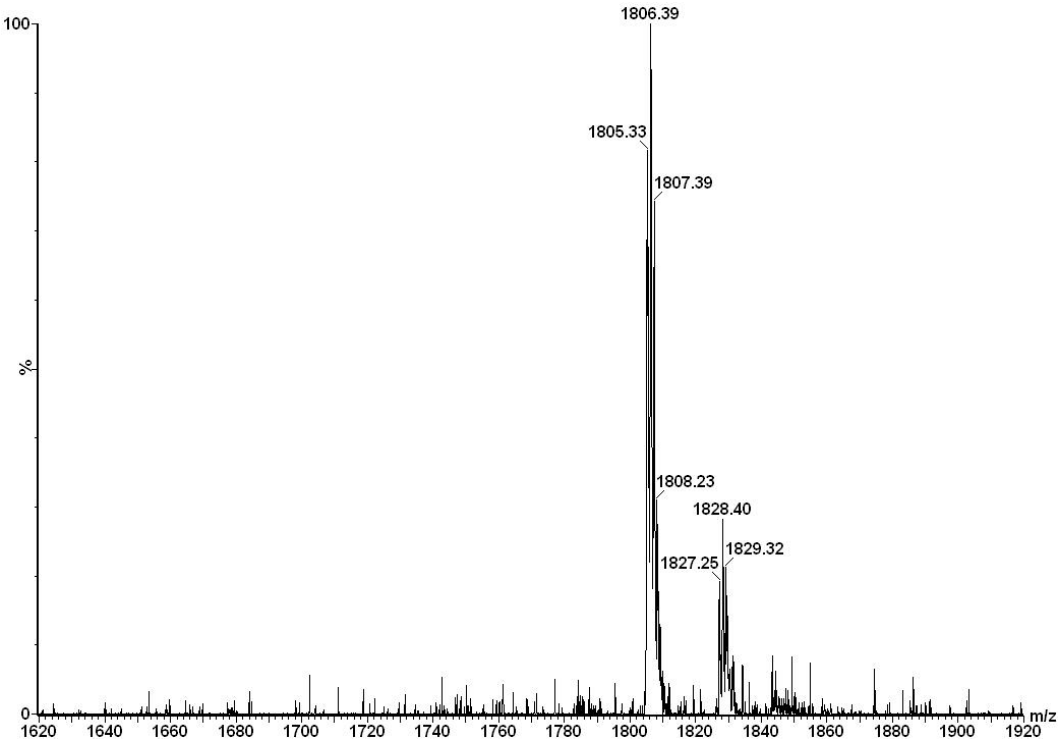
4.12 4,5-Dibromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione-bridged Somatostatin Adduct 13



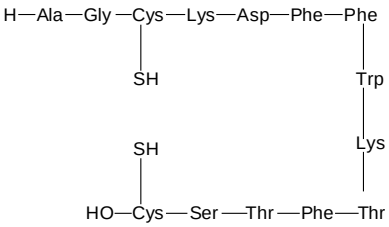
Lyophilised somatostatin (Sigma-Aldrich) **12** was solubilised in buffer (50 mM sodium phosphate, pH 6.2, 40% ACN, 2.5% DMF) to yield a concentration of 152.6 µM (0.25 mg/mL) and reduced with TCEP (1.1 eq.) for 1 h at 20 ^{o}C. Completeness of the reduction was confirmed by LC-MS (mass = 1641).



Pyridazinedione **8** (5 mol eq) was added and the mixture maintained at 20 ^{o}C for 2 h. Analysis using LC-MS showed that conjugate **13** (mass = 1806) was formed.

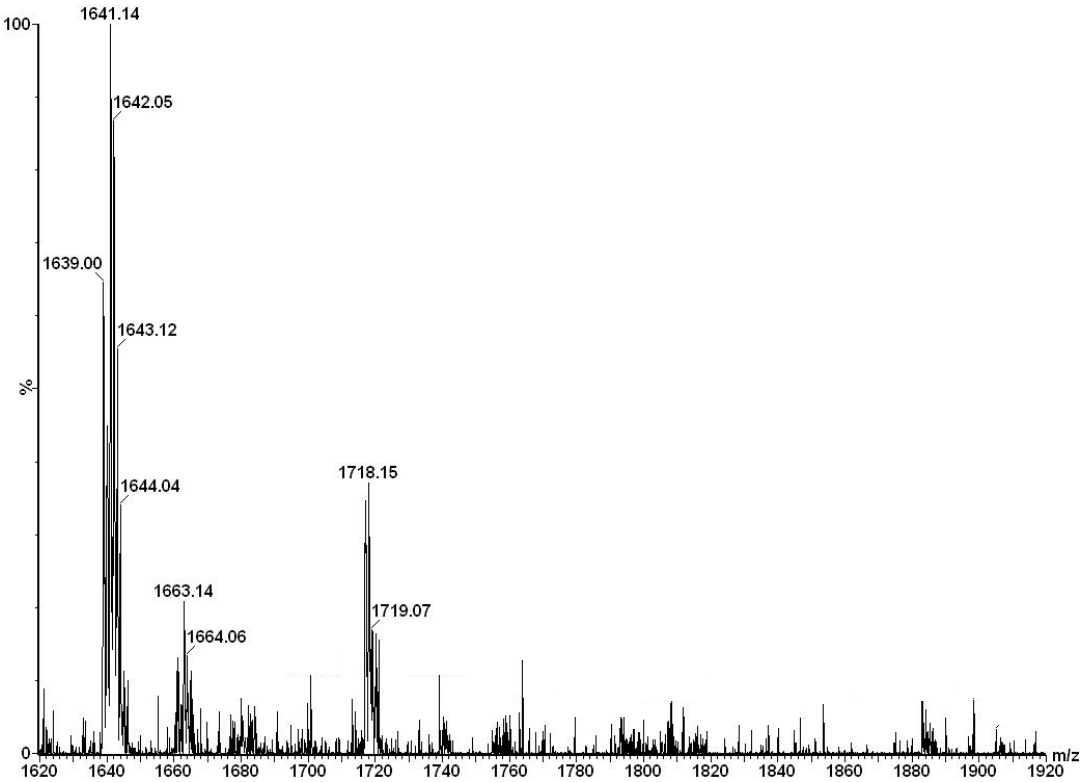


4.13 2-Mercaptoethanol-mediated Cleavage of 4,5-Dibromo-1,2-Diethyl-1,2-dihydro-pyridazine-3,6-dione-bridged Somatostatin Adduct 13 to yield Reduced Somatostatin 14



Lyophilised somatostatin (Sigma-Aldrich) **12** 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 20 ^{o}C. After completeness of the reduction had been confirmed by LC-MS (mass = 1641), pyridazinedione **8** (5 eq.) was added and the mixture maintained at 20 ^{o}C for 2 h. Analysis using LC-MS showed that conjugate **13** (mass = 1806) was formed.

The mixture was then treated with 2-mercaptoethanol (100 mol eq.) at 24 ^{o}C. The mixture was maintained at 24 ^{o}C for 72 h after which the mixture was analysed by LC-MS. Analysis showed that conjugate **13** had been cleaved yielding free reduced somatostatin **14** (mass = 1641) and a reduced somatostatin / 2-mercaptoethanol adduct (10-15 % total peptide) (mass = 1718).



References

1. Smith, M. E. B.; Schumacher, F. F.; Ryan, C. P.; Tedaldi, L. M.; Papaioannou, D.; Waksman, G.; Caddick, S.; Baker, J. R. *J. Am. Chem. Soc.*, **2010**, *132*, 1960-1965.
2. The plasmid encoding Grb2 SH2 domain (residues 53-163) was kindly provided by Dr. Stephen F Martin (University of Texas, A., TX).
3. Benfield, A. P.; Whiddon, B. B.; Clements, J. H.; Martin, S. F., *Arch. Biochem. Biophys.*, **2007**, 462, 47-53.
4. McNemar, C.; Snow, M. E.; Windsor, W. T.; Prongay, A.; Mui, P.; Zhang, R. M.; Durkin, J.; Le, H. V.; Weber, P. C., *Biochemistry*, **1997**, 36, 10006-10014.