

Protein Modification, Bioconjugation, and Disulfide Bridging Using Bromomaleimides

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Abstract: The maleimide motif is widely used for the selective chemical modification of cysteine residues in proteins. Despite widespread utilization, there are some potential limitations, including the irreversible nature of the reaction and, hence, the modification and the number of attachment positions. We conceived of a new class of maleimide which would address some of these limitations and provide new opportunities for protein modification. We report herein the use of mono- and dibromomaleimides for reversible cysteine modification and illustrate this on the SH2 domain of the Grb2 adaptor protein (L111C). After initial modification of a protein with a bromo- or dibromomaleimide, it is possible to add an equivalent of a second thiol to give further bioconjugation, demonstrating that bromomaleimides offer opportunities for up to three points of attachment. The resultant protein–maleimide products can be cleaved to regenerate the unmodified protein by addition of a phosphine or a large excess of a thiol. Furthermore, dibromomaleimide can insert into a disulfide bond, forming a maleimide bridge, and this is illustrated on the peptide hormone somatostatin. Fluorescein-labeled dibromomaleimide is synthesized and inserted into the disulfide to construct a fluorescent somatostatin analogue. These results highlight the significant potential for this new class of reagents in protein modification.

Introduction

The selective chemical modification of cysteine residues in proteins is widely employed to enable a range of fundamental biological and biophysical studies.^{1,2} Cysteine is often the most nucleophilic residue in a protein and as such is generally the easiest to manipulate with chemical reagents in a selective manner. Furthermore, the relatively low natural abundance³ of cysteine makes the introduction of single cysteines via site-directed mutagenesis and subsequent chemical modification a very effective method to access modified proteins. A variety of electrophilic reagents have been developed in order to take advantage of the nucleophilic characteristics of cysteine, but of these the maleimide motif remains one of the most widely employed and there are numerous *N*-functionalized maleimide reagents available.⁴ The reaction of a cysteine residue with a maleimide is a highly selective process^{5,6} and is considered to be irreversible.^{7,8}

Despite the successful utilization of maleimide as a reagent for the chemical modification of proteins, there are limitations. The irreversible nature of the addition prevents any possibility for controlled disassembly of the conjugate regenerating the unmodified protein, which may be desirable for *in vitro* or *in vivo* applications.⁹

Furthermore, there are only two points of attachment, thus limiting the number of chemical or biological entities that can be attached to a system of interest. We report herein on a new class of maleimides, the bromomaleimides, which offer reversible protein modification, three points of attachment for efficient construction of bioconjugates, and the ability to insert into disulfide bonds, retaining their bridging character.

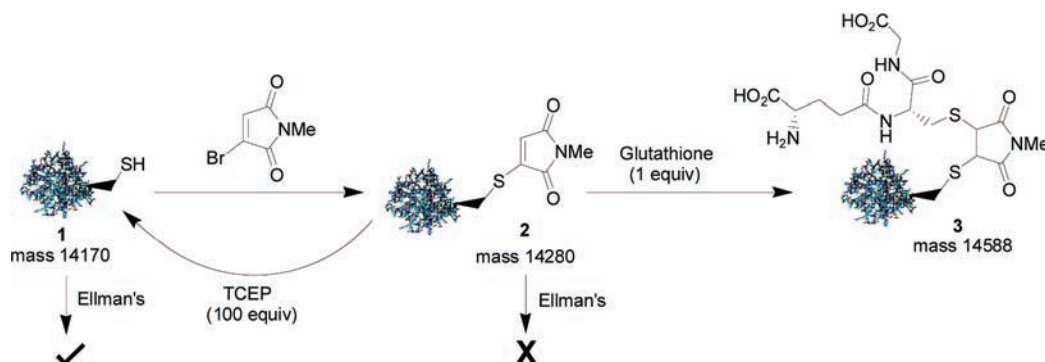
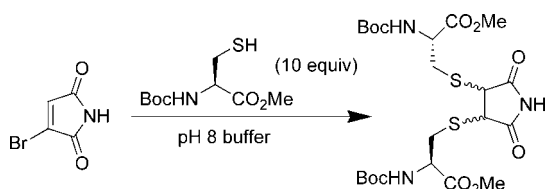
Results and Discussion

In prior work we had found that bromomaleimides could be used for the reversible chemical modification of a model system of the amino acid cysteine.¹⁰ In order to illustrate the applicability of this approach on proteins, we chose to examine a single point mutant (L111C) of the SH2 domain of the Grb2 adaptor protein **1**, a protein domain which does not otherwise contain any cysteine residues. We initially treated **1** with Ellman's reagent and observed clean formation of the expected mixed disulfide by LCMS, confirming the presence of an available cysteine residue. Treatment of **1** with 1 equiv of *N*-methylbromomaleimide at 0 °C (sodium phosphate buffer, pH 8) for 1 h

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(1) Lundblad, R. L. *Chemical Reagents for Protein Modification*, 3rd ed.; CRC Press: Boca Raton, FL, 2005.
 (2) Chalker, J. M.; Bernardes, G. J. L.; Lin, Y. A.; Davis, B. G. *Chem. Asian J.* **2009**, *4*, 630–640.
 (3) Fodje, M. N.; Al-Karadaghi, S. *Protein Eng.* **2002**, *15*, 353–358.
 (4) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: London, 1996.

(5) Schelte, P.; Boeckler, C.; Frisch, B.; Schuber, F. *Bioconjugate Chem.* **2000**, *11*, 118–123.
 (6) Vanderhooft, J. L.; Mann, B. K.; Prestwich, G. D. *Biomacromolecules* **2007**, *8*, 2883–2889.
 (7) Gregory, J. D. *J. Am. Chem. Soc.* **1955**, *77*, 3922–3923.
 (8) Bednar, R. A. *Biochemistry* **1990**, *29*, 3684–3690.
 (9) Saito, G.; Swanson, J. A.; Lee, K. D. *Adv. Drug Deliver. Rev.* **2003**, *55*, 199–215.
 (10) Baker, J. R.; Tedaldi, L. M.; Smith, M. E. B.; Nathani, R. *Chem. Comm.* **2009**, 6583–6585.

Scheme 1. Modification of the Grb2 SH2 Domain (L111C) with *N*-Methylbromomaleimide**Scheme 2.** Conjugate Addition of Cysteine to Bromomaleimide Generates the Vicinal Bis-cysteine Adduct

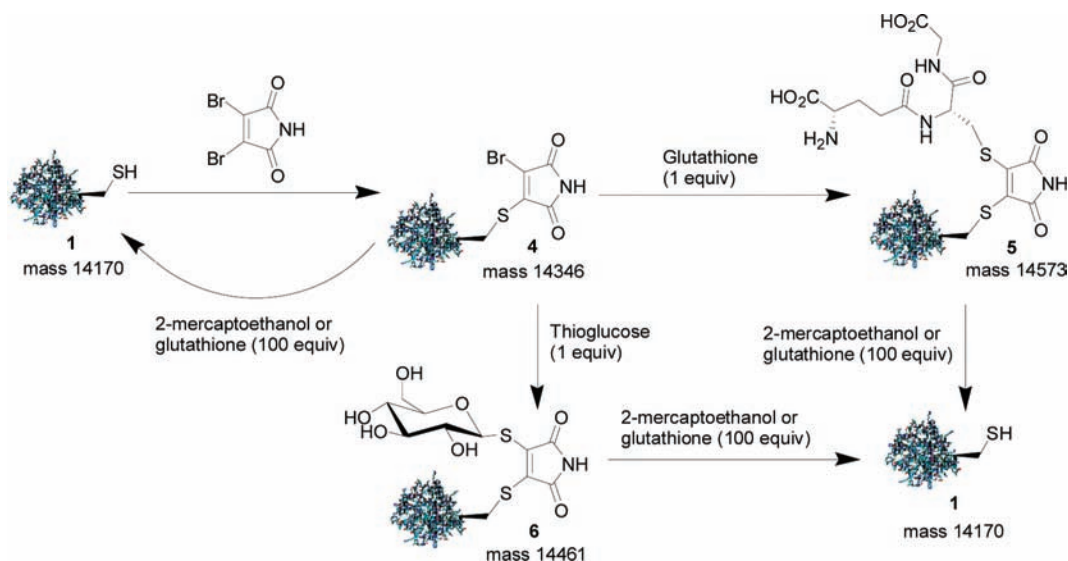
gave complete conversion to the conjugate **2**, as evident by LCMS. Addition of Ellman's reagent to **2** led to no reaction, revealing that the bromomaleimide had reacted exclusively on the cysteine residue. It should be noted that there are eight lysine residues present on this protein, and thus, in this case the reagent is highly selective for thiols over amine nucleophiles. Treatment of the adduct **2** with 100 equiv of TCEP [tris(2-carboxyethyl)-phosphine] resulted in 85% conversion back to protein **1**, demonstrating the potential of bromomaleimides for the reversible covalent modification of cysteine residues in proteins. Further experiments demonstrated that the protein–maleimide adduct **2** could undergo a second thiol conjugate addition. Thus, treatment of **2** with glutathione (1 equiv) led to the protein–peptide conjugate **3** in 95% conversion (Scheme 1). In a similar fashion, treatment of **2** with 2-mercaptoethanol (1 equiv) gave the analogous bis-thioether adduct.

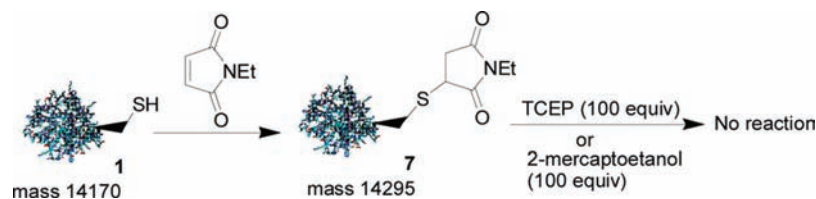
We are proposing that the product **3** is the vicinal and not geminal bis-thioether by analogy with a related experiment

carried out on the single amino acid model system *N*-Boc-Cys-OMe. The addition of an excess of *N*-Boc-Cys-OMe to bromomaleimide in the buffer (sodium phosphate buffer, pH 8) afforded the vicinal bis-adduct as a mixture of diastereomers (Scheme 2), as confirmed by NMR spectroscopy.

We have found that dibromomaleimide can also be employed for protein modification at cysteine. Thus, treatment of the Grb2 SH2 domain **1** with dibromomaleimide led to exclusive formation of the monobromo adduct **4**. Treatment of this adduct with Ellman's reagent resulted in no reaction, confirming once again that the maleimide had added exclusively to the cysteine residue. Addition of 1 equiv of a second nucleophilic thiol, in the form of either glutathione or thioglucose, gave the protein–peptide and protein–sugar conjugates **5** and **6**, respectively (Scheme 3). We envisaged that the maleimide-substituted adducts would still be susceptible to further conjugate addition with nucleophiles resulting in cleavage. However, in this instance we found that TCEP was unsuitable, leading to only a small amount of cleavage and other unidentifiable products. Instead, we found that treatment of these adducts with an excess of a thiol (2-mercaptoethanol or glutathione, 100 equiv) led to clean conversion to the free unmodified protein **1**.

We are proposing that the mechanism involved in the cleavage reactions is a conjugate addition–elimination sequence. In order to provide evidence for our assumption that the analogous conjugate addition using maleimide is irreversible, we prepared the succinimide **7** by treatment of **1** with *N*-

Scheme 3. Reversible Modification of the Grb2 SH2 Domain (L111C) **1** with Dibromomaleimide

Scheme 4. Irreversible Modification of the Grb2 SH2 Domain (L111C) **1** with NEM

ethylmaleimide (NEM). Treatment of **7** with 100 equiv of TCEP or mercaptoethanol led to no reaction, even at 37 °C for 4 h, confirming that NEM irreversibly labels the cysteine residue (Scheme 4).

To gain an appreciation for the relative reactivity of bromomaleimides with cysteines, we decided to compare the reaction times of protein **1** with some commonly employed protein-modifying reagents. Thus, we treated protein **1** with *N*-methylbromomaleimide, dibromomaleimide, *N*-ethylmaleimide, and iodoacetamide, respectively, and monitored the reaction between 5 and 30 min using LCMS (Figure 1). We found that the order of reactivity was *N*-methylbromomaleimide \approx *N*-ethylmaleimide > dibromomaleimide \gg iodoacetamide. Iodoacetamide is not shown in Figure 1, as <5% reaction had taken place after 30 min. The bromomaleimides thus react with the cysteine residue on a similar time scale to NEM, which is notably far faster than that of iodoacetamide.

The observed cleavage of the maleimide conjugates by thiols using excess glutathione suggested the enticing possibility that thiomaleimides could be used to develop conjugates designed to cleave in the cytoplasm of cells.⁹ To test this hypothesis, we subjected the protein–sugar conjugate **6** to conditions that would approximate the conditions of the cytoplasm (20 mM HEPES, 100 mM KCl, 1 mM MgCl₂, 1 mM EDTA, pH 7.4, 1 mM

glutathione,¹¹ 37 °C). We observed after 4 h complete cleavage to afford the protein **1** (Scheme 5). This result suggests that such conjugates will cleave in cells and offers many exciting possibilities which we will be actively pursuing in due course.

We have also investigated the utilization of dibromomaleimides for the modification of disulfides. Recent work by Brocchini and co-workers has highlighted the potential of modifying disulfides via bridging reagents that retain the structure and function of the protein.^{12–14} We envisaged that dibromomaleimide could serve as an alternative reagent for the selective modification of disulfides without the requirement for introduction of an additional asymmetric carbon and with the resulting adduct incorporating a two-carbon bridge between the two cysteine residues.

The endogenous peptide hormone somatostatin served as an excellent system to test our hypothesis. It is a 14-amino acid peptide containing a disulfide bridge, and stable analogues of somatostatin are clinically employed in the treatment of conditions including acromegaly and gastroenteropancreatic tumors.^{15,16} Treatment of somatostatin at pH 6.2 with 1 equiv of TCEP followed by 1.1 equiv of dibromomaleimide led to complete conversion to the bridged somatostatin **8** (Scheme 6). This reaction, together with the conditions used for the Grb2 SH2 domain (pH = 8), demonstrate the broad pH suitability of these reagents. Treatment of the maleimide bridged peptide **8** with 2-mercaptoethanol led to complete reversion to afford the reduced somatostatin **9** after 1 h at room temperature. This demonstrated that dibromomaleimides offer the first effective reagent for controlled reversible bridging of disulfides.

Fluorescein-5-maleimide is a commercially available reagent used widely in protein labeling.⁴ To demonstrate that bromomaleimides provide three possible points of attachment for protein bioconjugation, we synthesized the fluorescein–dibromomaleimide **10**. The synthesis was carried out by treatment of dibromomaleic anhydride¹⁷ with fluoresceinamine at room temperature, followed by heating at reflux in AcOH to effect cyclization to give the maleimide. We chose to treat somatostatin with this reagent to illustrate the fluorescent labeling of disulfide-containing biomolecules. Thus, treatment of somatostatin with *N*-fluoresceindibromomaleimide **10** afforded the fluorescent bridged construct **11** in quantitative conversion after 10 min. In order to demonstrate the reversible nature of this modification,

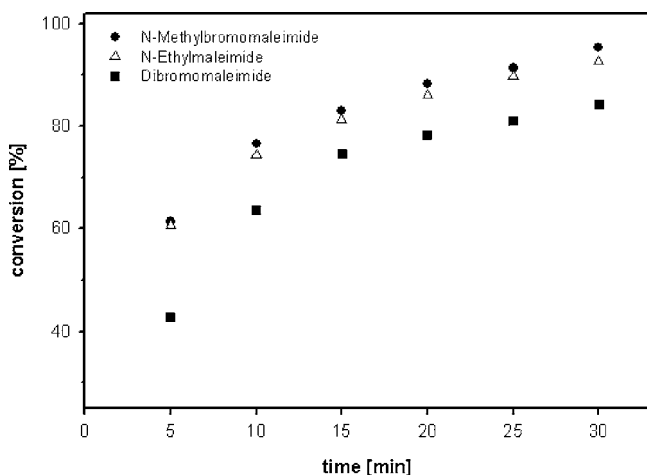
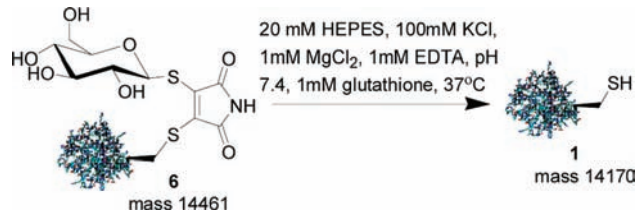
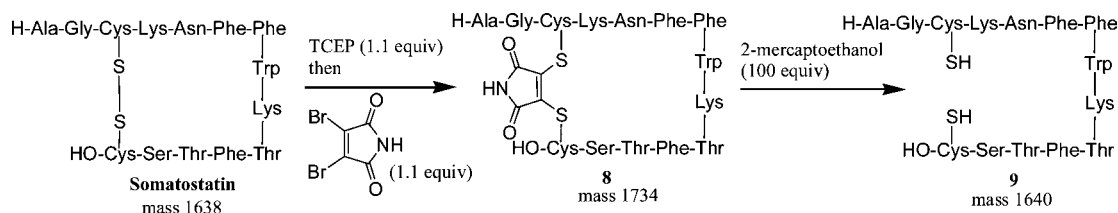
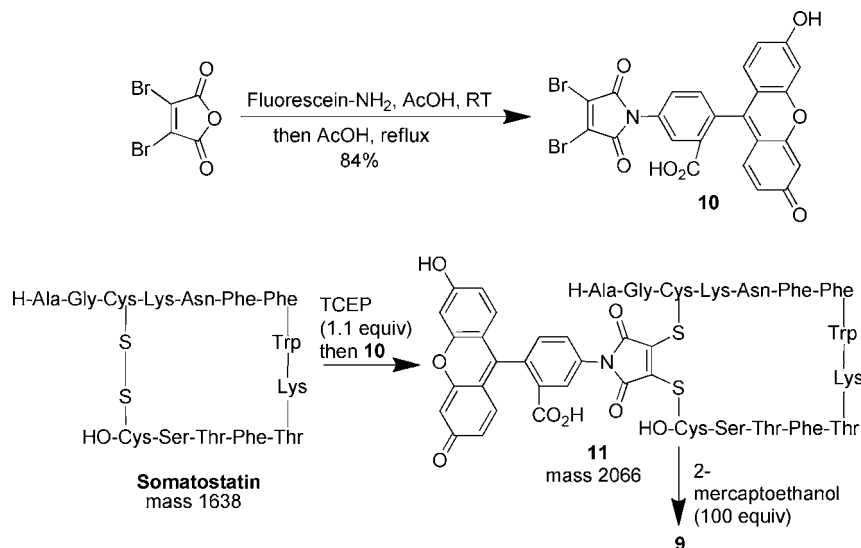


Figure 1. Relative reactivity of Grb2 SH2 domain (L111C) **1** with *N*-methylbromomaleimide, dibromomaleimide, and *N*-ethylmaleimide.

Scheme 5. Cleavage of Grb2 SH2 Domain–Thioglucose Conjugate **6** under Intracellular-like Conditions

- (11) Schafer, F. Q.; Buettner, G. R. *Free Radic. Biol. Med.* **2001**, *30*, 1191–1212.
- (12) Balan, S.; Choi, J. W.; Godwin, A.; Teo, I.; Laborde, C. M.; Heidelberger, S.; Zloh, M.; Shaunak, S.; Brocchini, S. *Bioconjugate Chem.* **2007**, *18*, 61–76.
- (13) Brocchini, S.; Godwin, A.; Balan, S.; Choi, J. W.; Zloh, M.; Shaunak, S. *Adv. Drug Delivery Rev.* **2008**, *60*, 3–12.
- (14) Shaunak, S.; Godwin, A.; Choi, J. W.; Balan, S.; Pedone, E.; Vijayarangam, D.; Heidelberger, S.; Teo, I.; Zloh, M.; Brocchini, S. *Nat. Chem. Biol.* **2006**, *2*, 312–313.
- (15) Freda, P. U. *J. Clin. Endocrinol. Metab.* **2002**, *87*, 3013–3018.
- (16) Oberg, K. *Expert Rev. Anticancer Ther.* **2009**, *9*, 557–566.
- (17) Dubernet, M.; Caubert, V.; Guillard, J.; Viaud-Massuard, M. C. *Tetrahedron* **2005**, *61*, 4585–4593.

Scheme 6. Reversible Modification of the Disulfide Bond of Somatostatin by Dibromomaleimide**Scheme 7.** Reversible Modification of the Disulfide Bond of Somatostatin by a Fluorescent Dibromomaleimide Reagent

we treated conjugate **11** with 2-mercaptoethanol (100 equiv), which led after 1 h to complete cleavage of the fluorescent maleimide to regenerate the reduced somatostatin (Scheme 7).

The fluorescent properties of fluoresceindibromomaleimide **10** and somatostatin conjugate **11** were confirmed (Figure 2). Fluoresceinamine is known to be a poor fluorophore until conjugated (e.g., to a maleimide)¹⁸ and this was observed.

Summary and Implications

In summary, we have shown that mono- and dibromomaleimides can be used for the selective modification of a cysteine residue in a protein and that the modification can be reversed. Thus, bromomaleimides can be employed in temporary cysteine

modification.¹⁹ In addition to the potential benefits afforded by this temporary or reversible modification, these reagents also provide opportunities for further attachment of functional groups. Thus, a second thiol can also be added to the protein–maleimide adducts to form maleimide or succinimide conjugates. As with other maleimide based reagents,⁴ a functional moiety can also be attached to the nitrogen, thus offering three points of attachment for protein bioconjugation and we have illustrated this by synthesizing a fluorescently labeled somatostatin. We envisage numerous potential applications for bromomaleimides incorporating functional groups, including biotin or solid supports for protein purification and immobilization; fluorophores, radiolabels, and quantum dots for imaging; polymers, e.g., PEG, for protein stability and others. In the present work, we have also shown that the dithiomaleimide constructs cleave under conditions which approximate those encountered in the cytoplasm, and this introduces the possibility that these maleimide reagents could serve as a new motif for the design of prodrugs. We will report on further developments with this powerful new class of reagent, in due course.

Experimental Section

Lyophilized somatostatin, 3,4-dibromomaleimide, and fluoresceinamine isomer 1 were purchased from Sigma-Aldrich and used without further purification. Bromomaleimide and *N*-methylbromomaleimide were synthesized as described previously.¹⁰ LCMS

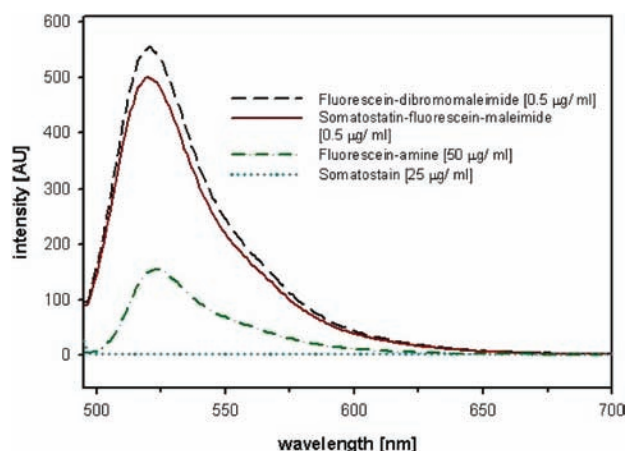


Figure 2. Fluorescence measurements of samples excited at 488 nm.

(18) Munkholm, C.; Parkinson, D. R.; Walt, D. R. *J. Am. Chem. Soc.* **1990**, *112*, 2608–2612.

(19) Baker, J. R.; Caddick, S.; Smith, M. E. B. Patent applications 0913965.0 (reversible covalent linkage of functional moieties), 0913967.6 (functionalization of solid substrates), and 0914321.5 (thiol protecting group), August 2009.

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 \times 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 were 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 preinstalled on MassLynx software.

Modification of Grb2-SH2 (L111C) 1 with Ellman's Reagent.

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 $^{\circ}\text{C}$ was added Ellman's reagent (5 μL , 282 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 10 min, after which the mixture was analyzed by LCMS. Analysis showed a single product with a mass of 14 370, corresponding to the mixed disulfide, confirming that C111 was available for functionalization.

Modification of Grb2-SH2 (L111C) 1 with *N*-Methylbromomaleimide.

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 $^{\circ}\text{C}$ was added *N*-methylbromomaleimide (5 μL , 2.82 mM solution in DMF). The mixture was vortexed for 1 s and then maintained at 0 $^{\circ}\text{C}$ for 1 h. Analysis using LCMS showed that the conjugate 2 had been formed in quantitative conversion (mass 14 280). Identical solutions of 2 formed in this manner were then subjected to the following experiments.

Ellman's Test.

The solution of 2 was treated with Ellman's reagent (5 μL , 282 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 10 min, after which the mixture was analyzed by LCMS. Analysis showed that no reaction with Ellman's reagent was evident, highlighting that *N*-methylbromomaleimide functionalization had occurred at C111.

TCEP-Mediated Cleavage of 2 To Regenerate Protein 1.

The solution of 2 was treated with TCEP·HCl (5 μL , 282 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 3 h after which the mixture was analyzed by LCMS. Analysis showed that the protein–*N*-methylbromomaleimide adduct 2 had been cleaved, yielding Grb2-SH2 (L111C) 1 (mass = 14 170) in 85% conversion.

Formation of the Protein–Glutathione Bioconjugate 3.

The solution of 2 was treated with glutathione (5 μL , 2.82 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 2 h, after which the mixture was analyzed by LCMS. Analysis showed that diconjugate 3 had been formed (mass = 14 588) in 95% conversion. The remaining material was Grb2-SH2 (L111C) 1.

Formation of the Protein–Mercaptoethanol Bioconjugate.

The solution of 2 was treated with 2-mercaptoethanol (5 μL , 2.82 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 2 h, after which the mixture was analyzed by LCMS. Analysis showed that the Grb2-SH2 domain L111C–*N*-methylbromomaleimide–2-mercaptoethanol adduct (mass = 14359) had been formed in 90% conversion. The remaining material was Grb2-SH2 domain L111C.

Modification of Grb2-SH2 (L111C) 1 with Dibromomaleimide.

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 $^{\circ}\text{C}$ was added dibromomaleimide (5 μL , 2.82 mM solution in DMF). The mixture was vortexed for 1 s then maintained at 0 $^{\circ}\text{C}$ for 2 h. Analysis using LCMS showed that conjugate 4 had been formed in quantitative conversion (mass 14346). Identical solutions of 4 formed in this manner were then subjected to the following experiments.

Ellman's Test.

The solution of 4 was treated with Ellman's reagent (5 μL , 282 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 10 min, after which the mixture was analyzed by LCMS. Analysis showed that no reaction with Ellman's reagent was evident, highlighting that dibromomaleimide functionalization had occurred at C111.

Mercaptoethanol- or Glutathione-Mediated Cleavage of 4 To Regenerate Protein 1.

The solution of 4 was treated with 2-mercaptoethanol or glutathione (5 μL , 282 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 4 h, after which the mixture was analyzed by LCMS. Analysis showed that the protein–dibromomaleimide adduct had been cleanly cleaved, yielding the Grb2-SH2 (L111C) 1 (mass = 14170) in quantitative conversion.

Formation of the Protein–Glutathione Bioconjugate 5.

The solution of 4 was treated with glutathione (5 μL , 2.82 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 2 h, after which the mixture was analyzed by LCMS. Analysis showed that the double conjugate 5 had been formed (mass = 14 573) in near quantitative conversion.

Formation of the Protein–Sugar Bioconjugate 6.

The solution of 4 was treated with β -1-thioglucofuranose, sodium salt (5 μL , 2.82 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 2 h, after which the mixture was analyzed by LCMS. Analysis showed that the double conjugate 6 (mass = 14461) was formed in near quantitative conversion.

Mercaptoethanol- or Glutathione-Mediated Cleavage of 5 To Regenerate Protein 1.

The solution of 5 was treated with 2-mercaptoethanol or glutathione (5 μL , 282 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 4 h, after which the mixture was analyzed by LCMS. Analysis showed that the Grb2-SH2 (L111C) 1 (mass = 14 170) was formed in quantitative conversion.

Mercaptoethanol- or Glutathione-Mediated Cleavage of 6 To Regenerate Protein 1.

The solution of 6 was treated with 2-mercaptoethanol or glutathione (5 μL , 282 mM solution in H_2O) at 0 $^{\circ}\text{C}$. The mixture was vortexed for 1 s and maintained at 0 $^{\circ}\text{C}$ for 4 h, after which the mixture was analyzed by LCMS. Analysis showed that the Grb2-SH2 (L111C) 1 (mass = 14170) was formed in quantitative conversion.

Modification of Grb2-SH2 (L111C) 1 with NEM.

To a solution of Grb2-SH2 (L111C) (100 μL , [protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0 $^{\circ}\text{C}$ was added *N*-ethylmaleimide (5 μL , 2.82 mM solution in DMF). The mixture was vortexed for 1 s and then maintained at 0 $^{\circ}\text{C}$ for 1 h. Analysis using LCMS showed that the desired conjugate 7 had been formed in quantitative conversion (mass 14 295). The solution of 7 was treated with either 2-mercaptoethanol (5 μL , 282 mM solution in H_2O) or TCEP·HCl (5 μL , 282 mM solution in H_2O), vortexed for 1 s, and maintained at 37 $^{\circ}\text{C}$ for 4 h, after which the mixtures were analyzed by LCMS, showing no reaction had occurred in either case.

Reaction Profile Experiments of *N*-Methylbromomaleimide, Dibromomaleimide, *N*-Ethylmaleimide, and Iodoacetamide with Grb2-SH2 (L111C) 1.

To a solution of Grb2-SH2 (L111C) (100 μL , [protein] 2.0 mg/mL, 100 mM sodium phosphate, 150 mM NaCl, pH 8.0) at 0 $^{\circ}\text{C}$ was added the relevant electrophile (5 μL , 2.82 mM solution in DMF). The mixture was vortexed for 1 s and then maintained at 0 $^{\circ}\text{C}$. The reaction was sampled at regular time intervals and reaction progress monitored by MS. Percentage reaction completion was determined using total ion count (TIC) via the formula $\text{TIC adduct}/(\text{TIC adduct} + \text{TIC unreacted Grb2}) \times 100$. Iodoacetamide resulted in <5% reaction after 30 min, and thus no time points are shown for this reaction. Reactions were performed in triplicate, and percentage reaction completion was plotted as the mean value. Errors were determined and are reported both as standard deviations and standard errors in the Supporting Information.

Cytoplasm Mimicking Cleavage Experiment. A solution of sugar–protein bioconjugate **6** (made as described above) was subjected to a buffer swap (Micro Bio-Spin 6 Chromatography Column, Bio-Rad). To a solution of **6** (95 μ L, [adduct] 0.2 mg/mL, 20 mM HEPES, 100 mM KCl, 1 mM MgCl_2 , 1 mM EDTA, pH 7.4) was added glutathione (5 μ L, 20 mM solution in 20 mM HEPES, 100 mM KCl, 1 mM MgCl_2 , 1 mM EDTA, pH 7.4). The mixture was vortexed for 1 s then maintained at 37 °C for 4 h. Analysis showed that Grb2-SH2 (L111C) **1** was formed (mass = 14 170) in quantitative conversion.

Bridging of Somatostatin with Dibromomaleimide. Lyophilized somatostatin (mass = 1638) was solubilized in buffer (50 mM sodium phosphate, pH 6.2, 40% MeCN, 2.5% DMF) to yield a concentration of 152.6 μ M (0.25 mg/mL) and reduced with 1.1 equiv of TCEP for 1 h at 20 °C. Completeness of the reduction was confirmed by LCMS (mass = 1640); 1.1 equiv of dibromomaleimide was added and the reaction maintained at 20 °C for 1 h. Quantitative insertion of the maleimide into the disulfide bond to give conjugate **8** was confirmed by LCMS (mass = 1734).

Mercaptoethanol-Mediated Cleavage of **8 To Regenerate Reduced Somatostatin **9**.** The solution of **8** was treated with 2-mercaptoethanol (100 equiv) and the reaction maintained at 4 °C for 1 h. Analysis by LCMS showed complete cleavage of the conjugate, yielding reduced somatostatin **9** (mass = 1640).

Bridging of Somatostatin with *N*-Fluoresceindibromomaleimide. Lyophilized somatostatin (mass = 1638) was solubilized in buffer (50 mM sodium phosphate, pH 6.2, 40% MeCN, 2.5% DMF) to yield a concentration of 152.6 μ M (0.25 mg/mL) and reduced with 1.1 equiv of TCEP for 1 h at 20 °C. Completeness of the reduction was confirmed by LCMS (mass = 1640); 1.1 equiv of *N*-fluoresceindibromomaleimide was added and the reaction maintained at 20 °C for 10 min. Quantitative insertion of the maleimide into the disulfide bond to give conjugate **11** was confirmed by LCMS (mass = 2066) [M^{+1} peak of product above spectrometer threshold, M^{+2} (m/z = 1033) and M^{+3} (m/z = 689) clearly visualized].

Mercaptoethanol-Mediated Cleavage of **11 To Regenerate Reduced Somatostatin **9**.** The solution of **11** was treated with 2-mercaptoethanol (100 equiv) and the reaction maintained at 4 °C for 1 h. Analysis by LCMS showed complete cleavage of the conjugate yielding reduced somatostatin **9** (mass = 1640).

2-*tert*-Butoxycarbonylamino-3-[4-(2-*tert*-butoxycarbonylamino-2-methoxycarbonyl-ethylsulfanyl)-2,5-dioxopyrrolidin-3-ylsulfanyl]-propionic Acid Methyl Ester (Inseparable Mixture of Diastereomers). *N*-Boc-Cys-OMe (660 mg, 2.81 mmol) in *N,N*-dimethylformamide (DMF) (0.25 mL) was added to a stirred solution of bromomaleimide (50 mg, 0.281 mmol) in aqueous buffer (100 mM sodium phosphate, 150 mM NaCl, pH 8.0):DMF 95:5 (9.25 mL), and the reaction was stirred at 25 °C for 5 min. The aqueous reaction mixture was extracted with ethyl acetate (3 \times 25 mL). The combined organic layers were washed with saturated lithium chloride solution (aq) (5 \times 25 mL), water (25 mL), and brine (25 mL); dried (MgSO_4); and filtered, and the solvent was removed in vacuo. Purification by column chromatography (gradient elution 10–40% ethyl acetate in petroleum ether 40–60 °C) gave the product as a yellow waxy oil (150 mg, 0.265 mmol, 94% yield),

an inseparable 1:1 mix of two symmetrical diastereomers: ^1H NMR (400 MHz, CDCl_3) δ 8.62 (s, 1H, NH from one diastereomer), 8.66 (s, 1H, NH from one diastereomer), 5.62 (d, 2H, J = 8.4, 2 \times NH from one diastereomer), 5.51 (d, 2H, J = 8.0, 2 \times NH from one diastereomer), 4.72–4.58 (m, 4 \times α -CH from both diastereomers), 3.80 (s, 6H, 2 \times CH_3 from one diastereomer), 3.79 (s, 6H, 2 \times CH_3 from one diastereomer), 3.68 (s, 2H, 2 \times succinimide CH from one diastereomer), 3.64 (s, 2H, 2 \times succinimide CH from one diastereomer), 3.46 (dd, 2H, J = 4.8 and 12.0 Hz, 2 \times *CHH* from one diastereomer), 3.37 (dd, 2H, J = 6.0 and 14.4, 2 \times *CHH* from one diastereomer), 3.21 (dd, 2H, J = 4.8 and 14.0 Hz, 2 \times *CHH* from one diastereomer), 3.11 (dd, 2H, J = 6.4 and 14.0 Hz, 2 \times *CHH* from one diastereomer), 1.463 (s, 18H, 6 \times CH_3 from one diastereomer), 1.460 (s, 18H, 6 \times CH_3 from one diastereomer); ^{13}C NMR (125 MHz, CDCl_3) (three signals missing due to overlap of diastereomers) δ 174.32 (C=O), 171.25 (C=O), 155.33 (C=O), 80.61 (C), 80.58 (C), 53.51 (CH), 53.18 (CH), 52.91 (CH_3), 52.90 (CH_3), 48.45 (CH), 47.89 (CH), 34.66 (CH_2), 34.59 (CH_2), 28.37 (CH_3), 28.36 (CH_3); IR (thin film, neat) 3348, 2978, 1719 cm^{-1} ; LRMS (EI) 566 (20), 564 (100 [$\text{M} - \text{H}$] $^-$); HRMS (EI) calcd for $\text{C}_{22}\text{H}_{34}\text{N}_3\text{O}_{10}\text{S}_2$ [$\text{M} - \text{H}$] $^-$ 564.1669; observed 564.1686.

***N*-Fluoresceindibromomaleimide **10**.** Dibromomaleic anhydride¹⁷ (77.0 mg, 0.30 mmol) was added in one portion to a solution of fluoresceinamine isomer **1** (105 mg, 0.30 mmol) in AcOH (10 mL) and the reaction mixture was stirred for 6 h at room temperature. The solid was then filtered off, washed with EtOAc, and resuspended in AcOH (10 mL). The reaction mixture was then heated to reflux for 3 h. Upon cooling to room temperature, toluene (10 mL) was added and the solvent removed in vacuo. This procedure was repeated twice more to azeotropically remove the AcOH, affording **10** as an orange solid (148 mg, 0.25 mmol, 84% yield): ^1H NMR (400 MHz, CD_3OD) δ 8.07 (d, J = 1.5 Hz, 1H, CH), 7.81 (dd, J = 1.5, 2.0 Hz, 1H, CH), 7.34 (d, J = 8.5 Hz, 1H, CH), 6.71 (d, J = 2.5 Hz, 2H, CH), 6.66 (d, J = 8.5 Hz, 2H, CH), 6.58 (dd, J = 1.5, 2.5 Hz, 2H, CH); ^{13}C NMR (150 MHz, CD_3OD) δ 170.23 (C), 164.34 (C), 161.63 (C), 154.18 (C), 152.93 (C), 134.59 (C), 134.19 (CH), 131.01 (C), 130.35 (CH), 129.25 (C), 126.25 (CH), 123.63 (CH), 113.84 (CH), 111.02 (C), 103.55 (CH); IR (MeOH) 3063, 2924, 1725, 1588 cm^{-1} ; HRMS (ES $^+$) calcd for $\text{C}_{24}\text{H}_{12}\text{NO}_7\text{Br}_2$ [$\text{M} + \text{H}$] $^+$ 583.8980, observed 583.8964; mp >220 °C (dec).

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Supporting Information Available: Full experimental details on the cloning and expression of Grb2-SH2 L111C mutant **1**, MS spectra on the protein modification experiments, and NMR spectra on the small molecule experiments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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