DOI: 10.1002/cbic.201200231

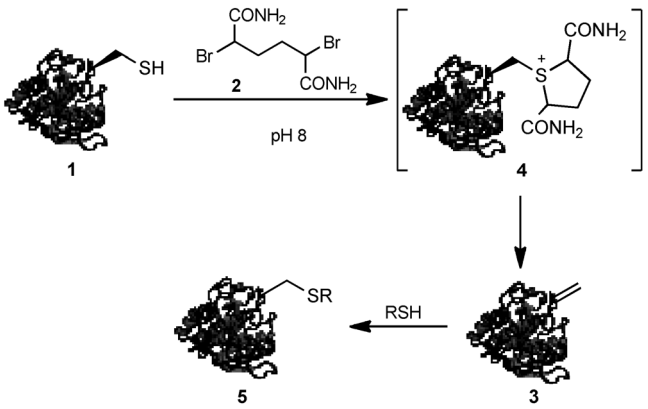
Bioconjugation of Green Fluorescent Protein via an Unexpectedly Stable Cyclic Sulfonium Intermediate

Ramiz Nathani, Paul Moody, Mark E. B. Smith, Richard J. Fitzmaurice, and Stephen Caddick*[a]

The development of methods for the modification of peptides and proteins under benign aqueous conditions has received a great deal of attention from both industrial and academic laboratories.[1] The ability to introduce a range of functional post-translational modifications, either natural or synthetic, to recombinantly expressed proteins, such as sugars, lipids, fluorophores, affinity tags or radionuclides, has been exploited to gain further insight into a plethora of biological process.[2] A number of strategies for protein modification through manipulation of the amino or carboxy terminus have been developed.[3] However, the vast majority of approaches rely on the reactivity of naturally occurring amino acid side chains such as lysine, tyrosine or cysteine.[4]

One bioconjugation approach exploits the elimination of cysteine to dehydroalanine, which can then be modified by adding suitable nucleophiles. A number of reagents have been developed to convert both peptide- and protein-based cysteines to dehydroalanine, for example *O*-mesitylenesulfonylhydroxylamine and hexamethylphosphorous triamide.[4b,5] However, the majority of currently used reagents require harsh conditions that limit their application. Recently, Davis has described the elegant use of 1,4-bishaloalkanes for the β-elimination of cysteine to effect protein modification under mild conditions.[6] An illustrative example is the treatment of a single cysteine mutant of subtilisin from *Bacillus lentus* SBL(S156C) (**1**) with 2,5-dibromohexanediamide (**2**) at pH 8, which afforded intermediate dehydroalanine **3**, presumably via the transient formation of sulfonium **4** (Scheme 1). Dehydroalanine **3** could be subsequently functionalised with a nucleophile to give modified protein **5**.

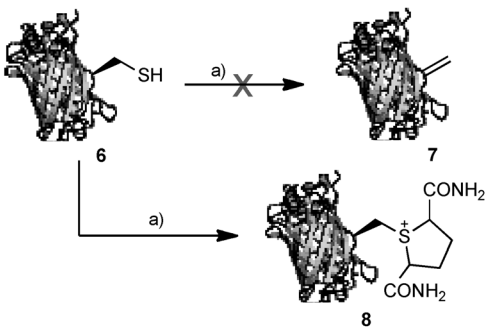
Intrigued by the potential application of the Davis double-alkylation methodology, we explored its applicability to a range of biological systems. Herein, we report preliminary studies on the modification of a cysteine mutant (S147C) of "superfolder" green fluorescent protein (GFP) bearing a single surface-accessible cysteine. We were able to synthesise unusually stable cyclic sulfonium species and to employ these in a novel bioconjugation that is complementary to current dehydroalanine-based technologies for substrates for which dehydroalanine formation is not facile. Although the presence of sulfonium



Scheme 1. Modification of SBL(S156C) by cysteine β-elimination.

ions in peptides and proteins derived from the alkylation of methionine is well established,[7] to our knowledge this represents the first evidence for the formation of a stable cysteine-sulfonium on a protein.

We treated GFP(S147C) (**6**) with **2** under Davis' conditions (1500 equiv, 37 °C, 2 h, pH 8; Scheme 2).[6] Unexpectedly the product was not the corresponding GFP dehydroalanine **7**, rather electrospray (ES) MS indicated quantitative conversion to a species with a molecular weight equivalent to that of sulfonium **8** (29486 calcd, 29488 obs.). Indeed, throughout this study, we observed no evidence for the formation of dehydroalanine **7**, or products derived therefrom, even upon incubation of sulfonium **8** for prolonged periods (vide infra). The isolation of this sulfonium species as a stable adduct and its reluctance to undergo elimination to give the corresponding dehydroalanine product **7** was noteworthy and we embarked upon further studies to understand and exploit this unexpected mode of reactivity.



Scheme 2. Reaction of GFP(S-147C) (100 μL, 1 mg mL^{-1} in H_2O, pH 8, 100 mM phosphate) with dibromodiamide (**2**). a) **2** (1500 equiv; 10 μL, 340 mM in DMF), 37 °C, 2 h.

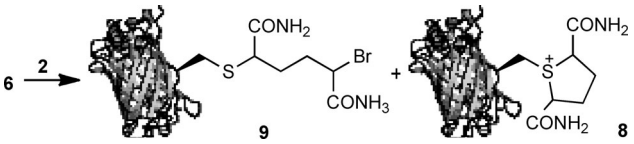
[a] *R. Nathani, P. Moody, Dr. M. E. B. Smith, Dr. R. J. Fitzmaurice, Prof. S. Caddick*
Department of Chemistry, University College London
London, WC1H 0AJ (UK)
E-mail: VPEnterprise@ucl.ac.uk

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201200231.

In order to gain further evidence for the site of reaction between GFP(S147C) and 2,5-dibromohexanediamide, sulfonium **8** was treated with *N*-methylbromomaleimide (1 equiv).[4d] No reaction was observed with **8**, whereas **6** reacted cleanly, thus suggesting that **2** had reacted exclusively on cysteine to generate **8** (see the Supporting Information).

Optimisation of the formation of **8** was achieved by varying a number of parameters including the number of equivalents of **2**, reaction temperature and reaction time (Table 1). Treatment of GFP(S147C) with 10 equivalents of **2** afforded no sulfonium **8** after 5 h; nevertheless, clear evidence for the formation of thioether **9** (29566 calcd, 29563 obs.) was apparent by LCMS (Table 1, entry 2). However, a prolonged incubation time, 20 h, afforded **8** as the only identifiable product in 53% conversion (Table 1, entry 3). Increasing the concentration of 2,5-dibromohexanediamide afforded, as expected, more rapid formation of **8**, with complete conversion of **6** at 21 °C with 25 or 50 equivalents of **2** within 20 h (Table 1, entries 4–9). At 4 °C, no sulfonium formation is apparent after 20 h even using 25 equivalents of 2,5-dibromohexanediamide (Table 1, entries 10 and 11). However, formation of sulfonium **8** at 4 °C is observed at higher concentrations of **2** (Table 1, entry 12). At 37 °C with 50 equivalents of **2** complete conversion of **6** to **8** could be rapidly achieved (Table 1, entry 13).

Table 1. Optimisation of formation of sulfonium **8**.[a]



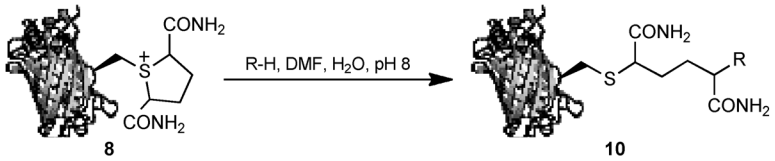
	2 [equiv]	T [°C]	t [h]	6 [%][b]	9 [%][b]	8 [%][b]
1	10	21	2	>95	0	0
2			5	91	9	0
3			20	47	0	53
4	25	21	2	81	19	0
5			5	50	17	33
6			20	0	0	>95
7	50	21	2	56	23	21
8			5	27	25	48
9			20	0	0	>95
10	10	4	20	>95	0	0
11	25		20	80	20	0
12	50		20	56	24	20
13	50	37	2	0	0	>95

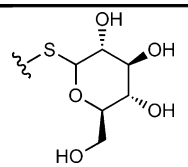
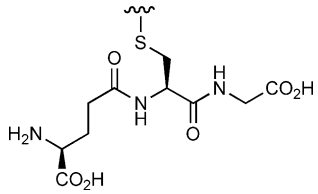
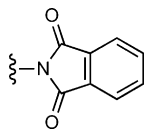
[a] Conditions: **6** (100 μL, 1 mg mL^{-1} in H_2O) at pH 8 (100 mM phosphate), **2** (10 μL in DMF). [b] Determined by ratio of peak heights in deconvoluted mass spectrum.

We then sought to establish the stability and reactivity profile of sulfonium **8**. After preparation of sulfonium **8** under our optimised conditions (50 equiv **2**, 37 °C, 2 h) excess **2** could be readily removed by repeated diafiltration into fresh buffer. Sulfonium **8** was found to be stable in the absence of nucleophiles at 21 °C for 24 h and at 4 °C for 1 month; although, significant decomposition was observed at 37 °C after 4 h (vide supra). Treatment of **8** with β-mercaptoethanol (1000 equiv, 37 °C, 2.5 h) afforded bisthioether **10a** (29564 calcd, 29566 obs.) in complete conversion, presumably through ring opening of the cyclic sulfonium (Scheme 3).

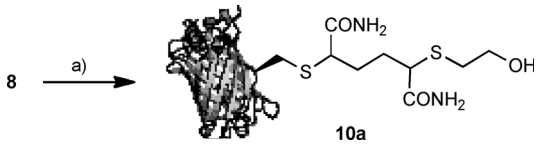
We then evaluated the reactivity of **8** with a range of simple nucleophiles. Treatment of **8** with the sodium salt of 1-thio-β-D-glucose (10 equiv) afforded the expected GFP–thioglucose conjugate **10b** as the sole product, with complete conversion after 5 h (Table 2, entry 1). Similarly, treatment of **8** with glutathione was also successful at both 21 and at 37 °C, generating the corresponding GFP–glutathione adduct **10c** in excellent conversion (entries 3–5). We also confirmed that these reactions could be carried out effectively at room temperature (21 °C) in 5 h, or more rapidly at 37 °C or with increased concentrations of the nucleophile (entries 2, 4 and 5). Treatment of sulfonium **8** with phenyl selenol

Table 2. Conjugation of nucleophiles with GFP sulfonium **8**.[a]



	R–H	Equiv	T [°C]	t [h]	Conv. 8 [%][b]	Product
1		10	37	5	>95	**10b**
2		100	21	5	>95	
3		10	37	5	>95	
4		100	21	5	>95	**10c**
5		200	37	2	>95	
6		100	37	5	>95	**10d**
7		1000	37	2.5	40	**10e**
8		1000	37	2.5	>95	**10f**

[a] Conditions: **8** (100 μL, 1 mg mL^{-1} in H_2O) at pH 8 (100 mM phosphate), NuH (see the Supporting Information). [b] Determined by ratio of peak heights in deconvoluted mass spectrum.



Scheme 3. Reactivity of GFP(S147C) sulfonium **8** (100 μL, 0.9 mg mL^{-1} in H_2O/DMF 10:1, pH 8, 100 mM phosphate) with β-mercaptoethanol (5 μL, 680 mM in H_2O). a) $HSCH_2CH_2OH$ (1000 equiv), 37 °C, 2.5 h, DMF, H_2O, pH 8 (phosphate), >95% conversion.

(100 equiv) afforded the mixed thio/seleno bisether **10d** in excellent conversion (entry 6). Although the conversion from reaction between **8** and phthalimide to give **10e** was only modest (entry 7), we were delighted to observe clean reaction in high conversion upon treatment of **8** with sodium azide (1000 equiv) to generate azide-labelled GFP **10f** (entry 8).

In conclusion, we have shown that treatment of superfolder GFP with bis-alkylating agents can be used to prepare highly stable, yet synthetically useful cyclic protein sulfonium adducts. These species undergo clean addition reactions to allow the introduction of a variety of useful chemical motifs. We envisage that the stability of the generated cysteine-derived sulfonium is presumably strongly correlated to the chemical environment of the alkylated cysteine. This new bioconjugation technique could provide a useful entry into multiply functionalised proteins and further work will be directed toward extending these findings to other systems.

Acknowledgements

We gratefully acknowledge EPSRC (EP/E000754/1), UCL, the Wellcome Trust (090145/Z/09/Z), UCLB and GSK for support of our programme.

Keywords: alkylation · bioconjugation · cysteine · protein modifications · sulfonium

[1] a) J. M. Antos, M. B. Francis, *Curr. Opin. Chem. Biol.* **2006**, *10*, 253–262; b) L. M. Artner, L. Merkel, N. Bohlke, F. Beceren-Braun, C. Weise, J. Dernedde, N. Budisa, C. P. R. Hackenberger, *Chem. Commun.* **2012**, *48*, 522–524; c) C. R. Behrens, J. M. Hooker, A. C. Obermeyer, D. W. Romanini, E. M. Katz, M. B. Francis, *J. Am. Chem. Soc.* **2011**, *133*, 16398–16401; d) L. Cohen, *Annu. Rev. Biochem.* **1968**, *37*, 695–726; e) A. N. Glazer, *Annu. Rev. Biochem.* **1970**, *39*, 101–130; f) T. J. Holmes, Jr., R. G. Lawton, *J. Am. Chem. Soc.* **1977**, *99*, 1984–1986; g) C. T. Walsh, S. Garneau-Tsodikova, G. J. Gatto, *Angew. Chem.* **2005**, *117*, 7508–7539; *Angew. Chem. Int. Ed.* **2005**, *44*, 7342–7372.

[2] For example: a) W. Song, Y. Wang, J. Qu, Q. Lin, *J. Am. Chem. Soc.* **2008**, *130*, 9654–9655; b) J. Wang, W. Zhang, W. Song, Y. Wang, Z. Yu, J. Li, M. Wu, L. Wang, J. Zang, Q. Lin, *J. Am. Chem. Soc.* **2010**, *132*, 14812–14818; c) Q.-F. Li, Y. Yang, A. Maleckis, G. Otting, X.-C. Su, *Chem. Commun.* **2012**, *48*, 2704–2706; d) P. Moody, M. E. B. Smith, C. P. Ryan, V. Chudasama, J. R. Baker, J. Molloy, S. Caddick, *ChemBioChem* **2012**, *13*, 39–41; e) D. F. Qi, C. M. Tann, D. Haring, M. D. Distefano, *Chem. Rev.* **2001**, *101*, 3081–3111; f) M. P. O. Richter, K. Holland-Nell, A. G. Beck-Sickinger, *Tetrahedron* **2004**, *60*, 7507–7513; g) T. Stafforst, *ChemBioChem* **2012**, *13*, 505–507.

[3] a) L. Yi, H. Sun, Y.-W. Wu, G. Triola, H. Waldmann, R. S. Goody, *Angew. Chem.* **2010**, *122*, 9607–9611; *Angew. Chem. Int. Ed.* **2010**, *49*, 9417–9421; b) M. Lewinska, C. Seitz, A. Skerra, F. P. Schmidtchen, *Bioconjugate Chem.* **2004**, *15*, 231–234; c) G. Volkmann, X.-Q. Liu, *PLoS ONE* **2009**, *4*, e8381; d) J. M. Gilmore, R. A. Scheck, A. P. Esser-Kahn, N. S. Joshi, M. B. Francis, *Angew. Chem.* **2006**, *118*, 5433–5437; *Angew. Chem. Int. Ed.* **2006**, *45*, 5307–5311; e) J. Alam, T. H. Keller, T.-P. Loh, *Chem. Commun.* **2011**, *47*, 9066–9068; f) X. Zhang, F. Li, X.-W. Lu, C.-F. Liu, *Bioconjugate Chem.* **2009**, *20*, 197–200.

[4] For example: a) J. M. Antos, M. B. Francis, *J. Am. Chem. Soc.* **2004**, *126*, 10256–10257; b) G. J. L. Bernardes, J. M. Chalker, J. C. Errey, B. G. Davis, *J. Am. Chem. Soc.* **2008**, *130*, 5052–5053; c) J. M. Chalker, L. Lercher, N. R. Rose, C. J. Schofield, B. G. Davis, *Angew. Chem.* **2012**, *124*, 1871–1875; *Angew. Chem. Int. Ed.* **2012**, *51*, 1835–1839; d) M. E. B. Smith, F. F. Schumacher, C. P. Ryan, L. M. Tedaldi, D. Papaioannou, G. Waksman, S. Caddick, J. R. Baker, *J. Am. Chem. Soc.* **2010**, *132*, 1960–1965; e) Y. Zhu, W. A. van der Donk, *Org. Lett.* **2001**, *3*, 1189–1192.

[5] a) G. J. L. Bernardes, E. J. Grayson, S. Thompson, J. M. Chalker, J. C. Errey, F. El Oualid, T. D. W. Claridge, B. G. Davis, *Angew. Chem.* **2008**, *120*, 2276–2279; *Angew. Chem. Int. Ed.* **2008**, *47*, 2244–2247; b) Z. Miao, J. P. Tam, *Org. Lett.* **2000**, *2*, 3711–3713.

[6] J. M. Chalker, S. B. Gunnoo, O. Boutureira, S. C. Gerstberger, M. Fernández-González, G. J. L. Bernardes, L. Griffin, H. Hailu, C. J. Schofield, B. G. Davis, *Chem. Sci.* **2011**, *2*, 1666–1676.

[7] a) M. Amunugama, K. D. Roberts, G. E. Reid, *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1631–1642; b) G. Battistuzzi, J. Stampler, M. Bellei, J. Vlasits, M. Soudi, P. G. Furtmüller, C. Obinger, *Biochemistry* **2011**, *50*, 7987–7994; c) S. Lang, D. E. Spratt, J. G. Guillemette, M. Palmer, *Anal. Biochem.* **2006**, *359*, 253–258; d) C. L. Metcalfe, M. Ott, N. Patel, K. Singh, S. C. Mistry, H. M. Goff, E. L. Raven, *J. Am. Chem. Soc.* **2004**, *126*, 16242–16248; e) J. E. Meza, G. K. Scott, C. C. Benz, M. A. Baldwin, *Anal. Biochem.* **2003**, *320*, 21–31; f) G. E. Reid, K. D. Roberts, R. J. Simpson, R. A. J. O'Hair, *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 1131–1150; g) G. A. Rogers, N. Shaltiel, P. D. Boyer, *J. Biol. Chem.* **1976**, *251*, 5711–5717; h) K. L. Taylor, J. Pohl, J. M. Kinkade, *J. Biol. Chem.* **1992**, *267*, 25282–25288.

Received: March 1, 2012
Published online on May 25, 2012

