ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 11086–11088

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COMMUNICATION

Multi-molecule reaction of serum albumin can occur through thiol-yne† coupling‡

Mauro Lo Conte,[a] Samuele Staderini,[a] Alberto Marra,[a] Macarena Sanchez-Navarro,[b] Benjamin G. Davis*[b] and Alessandro Dondoni*[a]

Received 20th July 2011, Accepted 10th August 2011
DOI: 10.1039/c1cc14402b

The free-radical hydrothiolation of alkynes (thiol-yne† coupling, TYC) unites two thiol fragments across the carbon-carbon triple bond to give a dithioether derivative with exclusive 1,2-addition; this reaction can be used for modification of peptides and proteins allowing glycoconjugation and fluorescent labeling. These results have implications not only as a flexible strategy for attaching two modifications at a single site in proteins but also for unanticipated side-reactions of reagents (such as cycloalkynes) used in other protein coupling reactions.

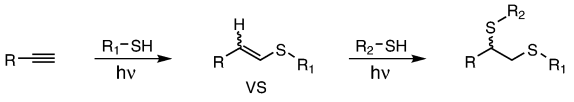
Protein modification and strategies for achieving such modifications with precision have seen tremendous development in the last decade.[1–4] Several reactions have been developed as part of strategies for allowing positional and molecular control and have even been developed to allow not only complex protein multisite protein alteration[5] but even cell surface[6] and *in vivo* conjugations.[7]

Part of the utility of such methods is in the study of natural protein alterations such as post-translational modification, a process that occurs after protein biosynthesis and folding and that incorporates a wide range of chemical moieties including phosphate, sugars, lipids, alkyl and acyl groups.[1,8] Glycosylation is by far the most common and complex of these modifications and it is known to affect both protein structure and function.[9] This is manifested in a variety of biological recognition events such as cell-cell communication, cell growth and differentiation, as well as viral infection. The microheterogeneity of native glycoproteins due to the presence of various glycoforms complicates their characterization and functional determination.

Methods allowing access to either labelled proteins and proteins that contain such post-translational modifications therefore remain in high demand.[10] Among the various chemical and enzymatic glycoprotein synthetic approaches,[11] those entailing the introduction of a functional tag into a protein by site-directed mutagenesis and then treatment with a suitably functionalized glycosyl reagent appears to be quite attractive.[12] Examples include the synthesis of disulfide-linked glycoproteins from proteins containing a cysteine residue as a thiol tag.[13] Desulfurization of these readily available disulfides can also afford thioether-linked glycoproteins.[14] While other examples of this "tag-and-modify" approach have been duly reviewed,[15] one of our groups reported also the free-radical coupling of ene-tagged proteins with glycosyl thiols to give *S*-linked protein glycoconjugates.[16] In this context, another of our groups reported a complementary approach in which *unmodified* native protein bovine serum albumin (BSA) displaying a single cysteine residue was coupled with allyl α-D-*C*-galactoside *via* a photoinduced thiol-ene free-radical reaction.[17] These and other important examples[18,19] have highlighted the selectivity and reactivity of the thiyl radical in protein modification approaches. In some examples of the thiol-ene reaction, however, multiple modifications have been observed that have been attributed to photocleavage of the disulfide bridge of cystine; these suggest that multi-site-selective protein modification can be induced in this way.

The thiol-yne† coupling (TYC), *i.e.* the free-radical addition of two thiol residues to a terminal alkyne,[20] has not yet been explored as a possible tool in protein modification. We have demonstrated[21] for small molecules in organic solvent that the photoinduced hydrothiolation of the triple bond can be carried out by the sequential addition of two different thiols. Thus, under suitable reaction conditions the vinyl sulfide (VS) intermediate formed by addition of a first thiol can be trapped by a second and different thiol *via* a thiol-ene†[22] type coupling process (Scheme 1).

To demonstrate the viability of this approach to more complex and biologically relevant molecules we first examined

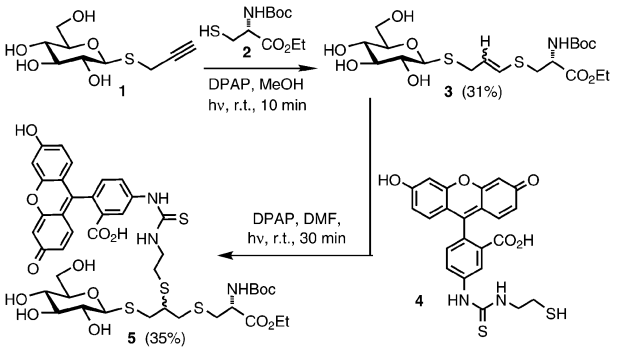


Scheme 1

[a] *Dipartimento di Chimica, Laboratorio di Chimica Organica, Universitá di Ferrara, via L. Borsari 46, 44100 Ferrara, Italy. E-mail: adn@unife.it; Fax: +39-0532-240709; Tel: +39-0532-455176*
[b] *Department of Chemistry, University of Oxford, Chemistry Research Laboratory, Mansfield Road, Oxford, OX1 3TA, UK. E-mail: Ben.Davis@chem.ox.ac.uk; Fax: +44 1865 285002*

† The naming of reaction-type chosen in this paper is dictated by overall stoichiometry as opposed to being indicative of mechanism. Although, we suggest that this reaction is likely to proceed *via* a thiyl radical intermediate and might therefore be more appropriately described as a 'thiyl-yne', this is yet to be determined. The use of the term 'thiol-yne' is also inkeeping with more prevalent current usage of related reactions.

‡ Electronic supplementary information (ESI) available: Syntheses and characterization data of all new compounds. See DOI: 10.1039/c1cc14402b

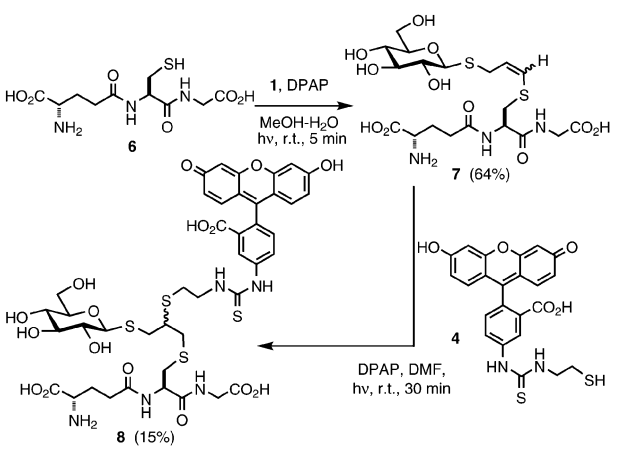


Scheme 2

a model system that used a representative L-cysteine derivative (**2**) and carbohydrate **1** (Scheme 2). Conditions were sought that would allow one equivalent only of cysteine derivative **2** to add to the alkynyl sugar **1**. Thus, coupling of **2** with a significant excess of **1** (4 equiv) was carried out at room temperature in a protic solvent (MeOH) by irradiation with a UV-lamp (λ_{max} 365 nm) using 2,2-dimethoxy-2-phenylacetophenone (DPAP, 10%) as a radical initiator. The reaction was carried out in a glass vial and no care was taken to exclude either air or moisture. After 10 min irradiation, the NMR spectrum of the reaction mixture revealed the presence of olefinic proton signals in the 5–6.5 ppm region. The excess of sugar alkyne **1** was recovered almost quantitatively (3 equiv.) by chromatography with pure vinyl sulfide **3** being isolated in fair yield (31%) as a 1 : 1 mixture of *E* and *Z* isomers. This key initial experiment confirmed the feasibility of step one of our intended two-step process.

Next, a solution of this intermediate **3**, fluorescein thiol **4** (4 equiv.) and DPAP in DMF was irradiated at λ_{max} 365 nm for 30 min. The ^{1}H NMR spectrum of the reaction mixture showed the complete conversion of alkene (as judged by ^{1}H NMR of **3**); chromatography over Sephadex LH-20 allowed the isolation of conjugated cysteine derivative **5**.

Excited by these results with a model amino acid, the same strategy was next explored in tripeptide glutathione GSH **6** (Scheme 3). Again photoinduced reaction of **1** with **6** under the same conditions afforded an alkene intermediate **7** (64% isolated yield) which was successfully reacted with fluorescein thiol **4** to give a corresponding doubly-conjugated product **8** as

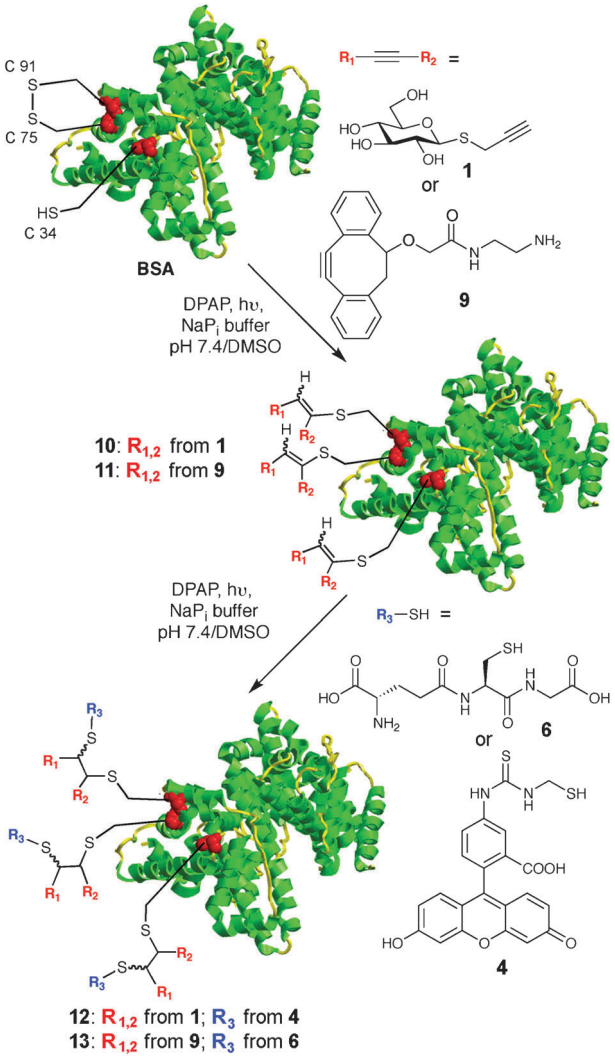


Scheme 3

a mixture of diastereomers with good conversion (>95% by NMR); isolated yield was more modest (15%). In all of the above cases, conversions were higher than isolated yields due to difficulties in purification associated with the amphiphilicity of these compounds. These key proof-of-principle experiments illustrated that a strategy could be developed for the dual modification at the same site of representative cysteinyl derivatives with both a biologically-relevant biomolecule (carbohydrate) and a label that is contingent on this first modification (here fluorescein).

Guided by the above preliminary experiments on small molecules, the same dual glycoconjugation and fluorolabeling of a representative thiol-containing protein, bovine serum albumin (BSA), was examined. Experimentation established an optimized procedure: a mixture of BSA, excess alkynyl sugar **1** (33 equiv) and photoinitiator DPAP (3 equiv) were dissolved in DMSO/phosphate buffer at pH 7.4 (5% v/v) and irradiated at λ_{max} 365 nm for 5 min. Again the experiment was conducted at room temperature without any caution to exclude air. The crude reaction mixture was purified by size-exclusion centrifugation to remove small molecule reagent sugar alkyne **1** and then the resulting solution containing the protein intermediate **10** was mixed with phosphate buffer (pH = 7.4) and an excess of fluorescein thiol **4** (160 equiv.) and DPAP (16 equiv.) dissolved in DMSO. The resulting solution was again irradiated at λ_{max} 365 nm for 10 min. MS (MALDI-TOF) analysis of the resulting synthetic conjugate **12** (found 68546 Da; calculated 68565 Da Fig. S3 ESI) indicated overall incorporation of three molecules of **1** and three molecules of **4** consistent with sequential dual modification at three cysteinyl sites (Scheme 4): the free cysteine at 34 and two thiyls 75 and 91 created by the photoinduced opening of the corresponding 75–91 cystine, as observed previously.[17] Consistent with these observations the fluorescence spectrum (Fig. S1 ESI) confirmed the incorporation of fluorescein residues. It should be noted that if disulfides provide structural integrity critical to function then this type of cystine cleavage and modification may therefore clearly prove detrimental to protein activity.

These key experiments revealed that a representative mammalian serum albumin (here BSA) reacts by undergoing representative dual modifications (*e.g.*, glycosylation and fluorolabeling) at up to three different positions *via* a TYC-based strategy. Not only does this valuably allow the dual conjugation of proteins at single sites in proteins containing cysteine residues it highlighted the possibility that other reagents that contain alkynes may react analogously. Foremost amongst these are the cycloalkynes used in so-called 'Cu-free CLICK' reactions that take place between azides and strained cycloalkynes to yield triazole diastereomeric products.[23–26] To test this possibility cycloalkyne **9** (commercially available as a Click-iT® reagent from Invitrogen), which is the core structure of several so-called DIBO-alkynes that have been used in strain-promoted reactions with azides, was reacted (Scheme 4) with BSA. The resulting intermediate **11** (found 67451 Da; calculated 67425 Da) was reacted with glutathione **6** as a representative thiol that can be present in significant levels *in vivo*. Ready conversion to the corresponding dually-modified conjugate **13** was observed (found 68359 Da; calculated 68346 Da). Furthermore, when cycloalkyne **9** was reacted alone in the



Scheme 4

absence of glutathione **6**, light and initiator the formation of a conjugate **14** (see ESI‡) that is the product of the addition of a single copy of **9** was observed (found 66 786 Da; calculated 66 783 Da). Tryptic digest followed by MS/MS analysis of the resultant peptides suggested reaction at the free cysteine that is present in serum albumin at position 34 (see ESI).

Although conditions for *in vitro* experiments can never adequately reproduce those *in vivo* our results confirm that alternative reactive pathways exist for such strained alkyne reagents. Indeed, our results are consistent with the 'dark' reactions of simple aliphatic thiols with cyclooctyne[27] and Bertozzi *et al.* have recently noted[25] that such alkynes show unusual high affinity for murine serum albumin, possibly consistent with the formation of a covalent linkage that is not due to reaction with an azide. Taken together with our results we suggest that thiols in such albumins may act as potential unwanted reaction partners during such experiments in the manner we disclose here. It should be noted that other reactions that involve the use of excessive double-bond containing reagents (such as so-called photoclick variants[28]) may also suffer from similar limitations. Further utility of our dual site conjugation methods using the TYC are under exploration.

We thank Dr A. Chambery (II Universitá di Napoli, Italy) for HRMS analyses, Dr S. Caramori (University of Ferrara) for recording fluorescence emission spectra and Dr J. S. O. McCullagh for MS analyses. MSN thanks Fundación Ramón Areces for funding, BGD is a Royal Society Wolfson Research Merit Award recipient.

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