Protein Conjugation by Electrophilic Alkynylation using 5-(Alkynyl)dibenzothiophenium Triflates

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ABSTRACT: 5-(Alkynyl)dibenzothiophenium triflates are introduced as new reagents to prepare different protein conjugates through site-selective cysteine alkynylation. The protocol developed allows a highly efficient label of free cysteine-containing proteins with relevant biological roles, such as ubiquitin, the C2A domain of Synaptotagmin-I, or HER2 targeting nanobodies. An electrophilic bis-alkynylating reagent was also designed. The second alkynylating handle thus introduced in the desired protein enables access to protein-thiol, protein-peptide and protein-protein conjugates, and even diubiquitin dimers can be prepared through this approach. The low excess of reagent needed, mild reaction conditions used, short reaction times and stability of the S–C(alkyne) bonds at physiological conditions make this approach an interesting addition to the toolbox of classical, site-selective cysteine-conjugation methods.

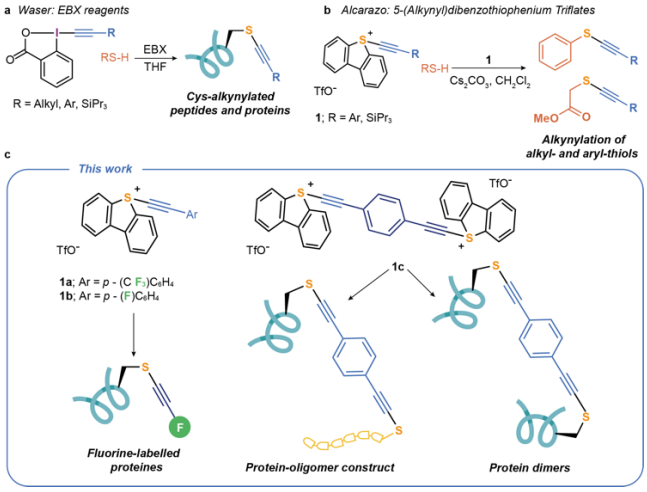
Despite recent efforts to target different amino acids for chemoselective protein modification,[1–7] cysteine bioconjugation remains the most developed approach to achieve homogeneous site-selective protein-conjugate synthesis.[8] This is due to the combination of the inherent nucleophilic reactivity of the thiol moiety at physiological conditions, which is higher than other functionalities found in proteins, such as the hydroxyl group of serine/threonine, or the amine of lysine, and its relative low natural abundance. Over the past few years, a broad variety of new methods for the chemoselective targeting of cysteine residues have been reported.[1] Cysteine alkylation methods were described first, yet, despite the stability of the conjugates formed, their limited reaction kinetics and the later development of other more reactive conjugation reagents, such as Michael acceptors,[9] has restricted their application to the area of proteomics.[10]

Maleimides are the most popular reagents among the Michael acceptors used in cysteine modification because of their fast kinetics.[11] Maleimides are still used extensively to conjugate proteins to fluorescent dyes, affinity labels, and drugs, despite new generations of Michael acceptors with improved stability in plasma being developed.[9] Limitations associated with these approaches; insufficient reactivity, lack of selectivity or low adduct stability are common, which has boosted the design of alternative cysteine modification methods; specifically, arylation protocols developed by adapting typical *S*-arylation reactions to bioconjugation processes.[12] Electron poor aryl halides,[13,14] chlorotetrazines[15] or heteroaryl sulfones[16] are examples of reagents that efficiently promote the arylation of free cysteines through a typical S_NAr mechanism.

Direct alkylation and Michael addition reactions yield S–$C_{(sp3)}$ bonds between the protein and the conjugate, whereas arylation strategies give S–$C_{(sp2)}$ bonds. A third type of possible S–C bond is an S–$C_{(sp)}$ one. The linear, non-sterically demanding, and apolar nature of the acetylene moiety makes it an attractive tether for bioconjugation because it does not add appreciable structural alterations once attached to biomolecules. Methods that provide such a linkage, referred as electrophilic alkynylation reactions, have been described, but are underexplored relative to other methods already mentioned, probably due to the limited number of reagents available to promote this transformation.[17,18] Recently, Waser, Adibekian and collaborators reported the use of hypervalent

iodine (III) reagents for thiol alkynylation in proteins.[19,20] They achieved the alkynylation of cysteines in different dipeptides and proteins, and performed an array of proteome studies in which EBX derivatives showed superior chemoselectivity but only modest coverage of the cysteonome (**Scheme 1a**).[17] Employing TMS-EBX as alkynylating reagent, the authors also described the selective ethynylation of cysteine under physiological conditions, and the further functionalization of the primarily obtained *S*-alkyne moieties by azide-alkyne click chemistry.[18] Unfortunately, iodine (III) derivatives have some disadvantages. Their synthesis can be demanding and they are often thermally unstable.[21] Furthermore, their applicability is limited by their highly reactive nature; they occasionally act as oxidizing agents, triggering competing reactions, such as cysteine dimerization.[20] Hence, development of alternative approaches to achieve cysteine alkynylation remains desirable. Recently, we reported the use of 5-(alkynyl)dibenzothiophenium triflates (**1**) as thiol alkynylating reagents (**Scheme 1b**).[22] These compounds have slightly reduced kinetics but improved stability and selectivity relative to I(III)-analogues, which shows their potential as protein alkynylation reagents. Here, we evaluate the use of these salts to introduce fluorine labels into proteins through the formation of stable bonds that may be useful for biological studies.

In parallel to cysteine conjugation protocols, efforts have been directed towards the transformation of known protein conjugation methods into chemical cross-linking tools able to deliver protein-peptide and protein-protein constructs.[23,24] The interest in these adducts resides in their potential application in various fields, such as structural analysis,[25] therapeutic agent design,[26] imaging[27] or protein interaction studies.[28] Accessing conjugates through genetic engineering is often tedious, low yielding and limited to *N*-terminal to *C*-terminal bonding;[29] whereas traditional chemoenzymatic[30] or chemical click methods[31] usually yield heterogeneous conjugates of limited stability. The development of new chemical approaches to access these constructs in a stable and homogeneous manner is therefore desirable. In this regard, we report bisalkynylating reagent **1c** that contains a 1,4-diethynylbenzene bridge between the two electrophilic positions, which undergoes stepwise reactions with thiol moieties to give protein-thiol, protein-oligomer and dimeric protein constructs.



Scheme 1. Previous results for thiol alkynylation and the plan to use 5-(alkynyl)dibenzothiophenium salts **1a–c** as alkynylating reagents. THF = tetrahydrofuran.

Initially, to evaluate alkynyldibenzo-thiophenium salts for protein conjugation, compounds **1a** (structure confirmed by X-ray, **Figure S59**) and **1b** were mixed in CH_2Cl_2 with the model thiol N-Boc-cys-OMe at room temperature in the presence of Cs_2CO_3 (1.1 equiv.). The starting material was completely consumed after 1.5 h. The main reaction products were isolated by extraction and purification by column chromatography and determined to be corresponding alkynylated cysteine derivatives **2a** and **2b**, respectively (**Figure 1a**). Driven by this promising reactivity we adapted the reported conditions to study the alkynylation of thiol moieties of free cysteine residues in proteins. Ubiquitin-K63C (**Ub**) was chosen as a model protein because of its small size, established reactivity of the free thiol, and our previous experience of its chemical modification.[32,33] Moreover, ubiquitin conjugates are relevant because ubiquitination is an important post-translational modification of proteins, which regulates important cellular processes, such as protein degradation or DNA repair.[34] Site-specific ubiquitinated proteins are also important research targets,[35,36] but their production is still a challenge.[37]

To test **1a** as a protein alkynylation reagent 5 equiv. were added to **Ub** (20 uM) at pH 8; different co-solvents including dimethyl formamide (DMF), dimethyl sulfoxide and CH_3CN (10%) were added to the reaction mixture to enhance the solubility of **1a**. After 1 h at 25 ºC, no starting material was detected by LC-MS and only the corresponding alkynylation product was observed, which was seen independently of the co-solvent (**Figure 1b**), so DMF was chosen for subsequent studies. Compound **1b**, which has a *p*-fluorine substitution instead of a trifluoromethyl group, showed reduced reactivity under the same conditions, but complete conjugation was obtained with 10 equiv. of **1b** at 37 ºC. These results confirmed the potential of **1a,b** as

Ub alkynylation reagents, so alternative free cysteine containing proteins were tested. We were delighted to see that complete alkynylation of the C2A domain of Synaptotagmin-I (**C2Am**) was achieved under similar conditions with both **1a** and **1b**, which further supports the generality of this method. These results are of interest because C2Am binds to the phosphatidylserine (PS) exposed by apoptotic and necrotic cells, and therefore, it has been developed as an imaging probe for detecting cell death.[38,39]

Having established the protocol for conjugate formation, we moved to study the residue selectivity of **1a** and **1b** and how the conjugation affects the tertiary structure and function of the original protein. MS/MS studies after trypsin digestion of conjugate **3a** showed that alkynylation occurs selectively on cysteine residues to leave other potentially nucleophilic positions, such as lysines or serines, untouched (**Figure 1c**). No conjugation was observed when a protein which did not contain any free cysteine such as lysozyme (**Figure S42**) was incubated in the presence of **1a** under the reaction conditions, which further confirmed the selectivity of the method for free cysteine moieties. Circular Dichroism (CD) measurements of **Ub** and conjugate **3a** were analysed; no significant differences were noted, which suggests that the tertiary structure of the protein was not perturbed by conjugation to **1a** (**Figure 1d**). Additional support for this conclusion has been found from the comparison of the affinities of **C2Am** conjugate **4a** and the naked protein for PS (**Figure 1e**). Despite its conjugation, the affinity of both species proved to be analogous. Of note, these binding studies were performed by using a microfluidic diffusional sizing method, which enables measurements to be taken entirely in solution, thus eliminating the need for surface functionalization and also minimizes non-specific interactions in surfaces.[40,41] The binding constant was calculated by keeping the protein concentration constant (10 nM) and exploring different PC:PS lipid concentrations while measuring the hydrodynamic radius (**Figure 1e**). A small difference in the binding potency of **C2Am** and **4a** was detected, but both have similar binding properties as reflected by the K_d (2.4 x 10^{-4} versus 1.6 x 10^{-4}). Once the methodology for site-selective alkynylation of free cysteine residues was established we conducted several conjugate stability tests. Interestingly, the alkynylated proteins were stable overnight in human plasma and in the presence glutathione at pH 8 for over 66 h (**Figures S47–S50**).

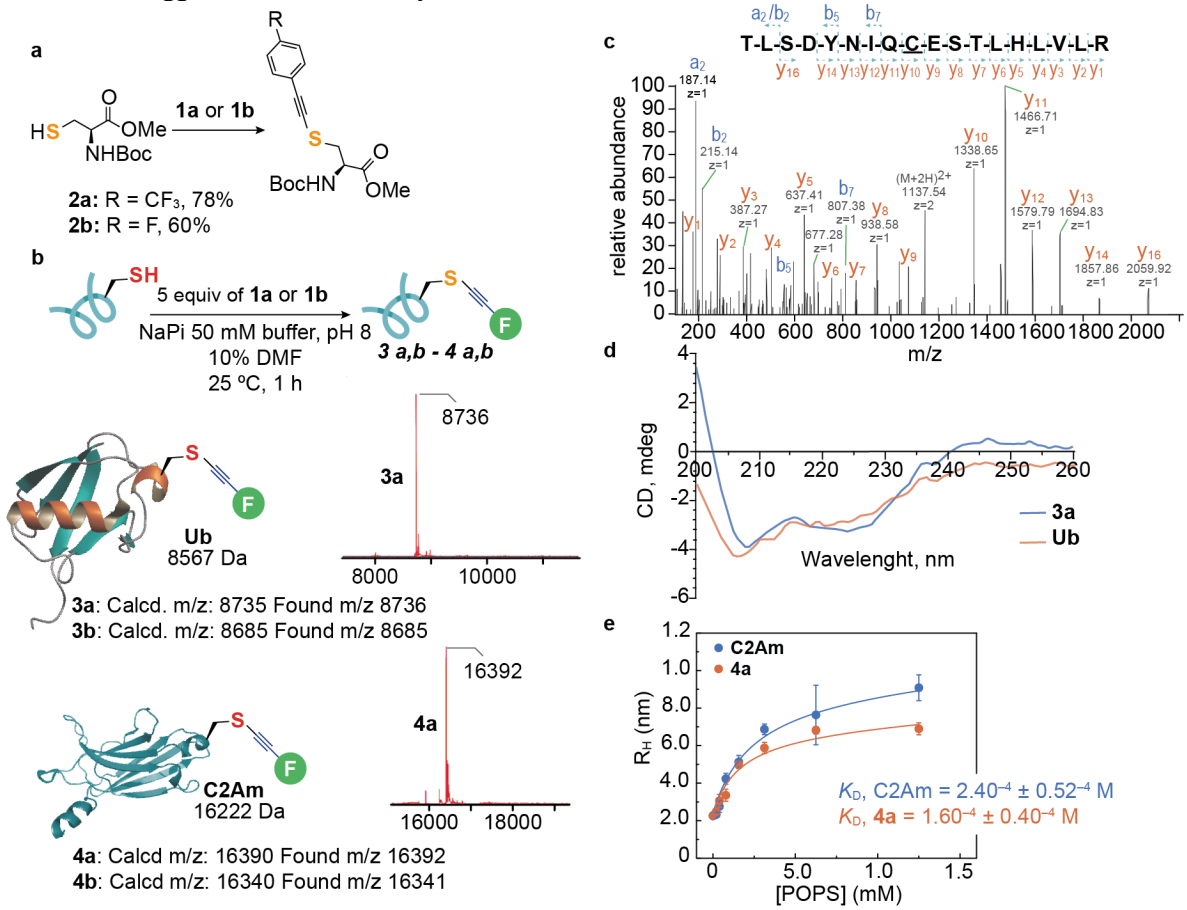
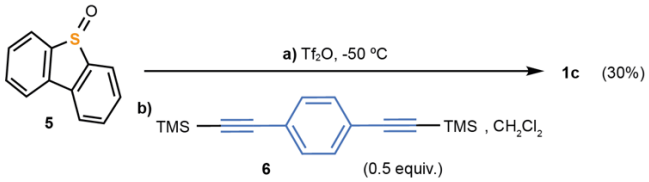


Figure 1. a, Preliminary test to use a protected cysteine as substrate; **b**, Alkynylation method for proteins; **c**, MS/MS spectrum of the tryptic peptide containing the modification at the cysteine residue of **3a**. The generated

C-terminal y fragment ions are consistent with the mass of the modification; **d**, CD of tagged and untagged protein; **e**, Binding curve between **C2Am**, **4a** and PS (see ESI). Measurements we performed using a Fluidity One-W instrument from Fluidic Analytics.

The stability of the cysteine alkynylated conjugates obtained suggests that this approach might be suitable for the synthesis of more complex species, such as protein-oligomer or protein-protein constructs. This would provide an alternative to existing methods using dimethyl sulfone or iodoacetic acid, which usually need high pH and excess of reagents.[42] To build this type of conjugates we designed bisalkynylation reagent **1c**, in which the two electrophilic positions are connected by a rigid 1,4-diethynylbenzene bridge. To obtain salt **1c**, dibenzothiophene-*S*-oxide **5** was treated with triflic acid anhydride (1 equiv.) at –50 ºC. Then, 1,4-bis((trimethylsilyl)ethynyl)benzene (**6**, 0.5 equiv.) was added at the same temperature to form slowly **1c** as a light yellow solid, which was filtered (**Scheme 2**). While **1a,b** were fully stable, we observed decomposition of the bisalkynylation reagent **1c** upon long-term storage (SI).

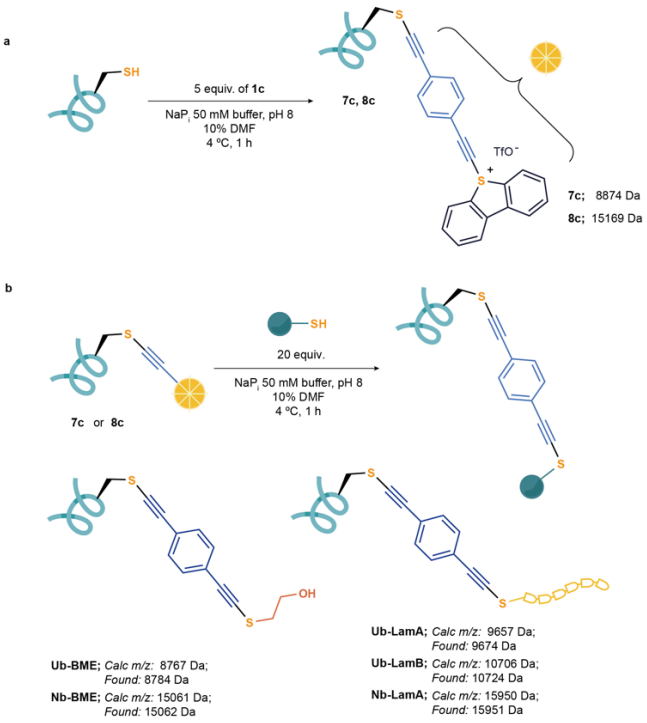


Scheme 2. Synthesis of reagent **1c**.

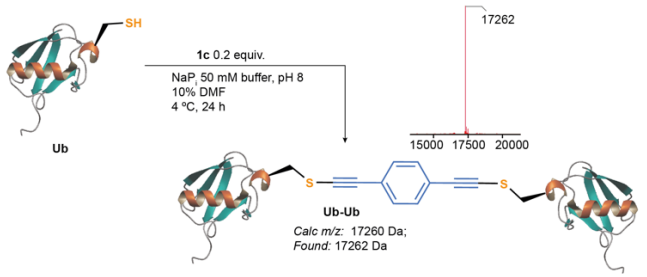
The reactivity of **1c** was then explored. To achieve mono-alkynylation of **Ub** with **1c** it is necessary to work at 4 ºC; otherwise, adjacent nucleophilic (e.g. the N-terminus amine of Ub) residues react with the second alkynylation site to create a double bonded conjugate, which quenches the second reactive site. Optimal conditions for single modification to selectively occur are **1c** (5 equiv.), 4 ºC and 1 h with 20 μM of protein. Under these reaction conditions we were able to obtain alkynylated **Ub** (**7c**) and conjugate HER 2 targeting nanobody (**Nb**-U4604)[43] **8c** (**Scheme 3**). The selective reaction of only one of the sulfonium functionalities in **1c**, while the other remains untouched but conjugated to the protein, transforms cysteine residues into new electrophilic anchoring positions. Hence, these reactive sites can then be sequentially reacted with other thiols or free cysteine containing peptides and proteins.

Mercaptoethanol (BME) was initially used as a model thiol to study the kinetics and optimize the conditions for the second addition. Thus, addition of BME (20 equiv.) at 4 ºC in NaP_i buffer at pH 8 resulted in complete incorporation of the thiol into the conjugate for both **7c** and **8c**, to give **Ub-BME** (calcd. m/z = 8767 Da found: 8784 Da) and **Nb-BME** (calcd. m/z = 15061 Da found: 15062 Da), respectively (**Scheme 3**). Please note that the mass obtained for **Ub-BME** is 16 Da higher than predicted, which is probably due to oxidation of the sulfur atom to sulfoxide analogs. The stability of the construct did not seem to be affected by this oxidation and seemed to be related to the cysteine environment because it is only observed for **Ub** constructs; no trace of oxidation is observed for **8c** derivatives. Additionally, cysteine-containing peptides can also be used for the second coupling by using analogous conditions to the ones described for BME. Specifically, two *N*-terminal cysteine peptides, CNPGYIGSR (**LamA**) that contains 9 residues, and a more elaborate one that contains 19 residues, CSRARKQAASIKVAVSADR (**LamB**), were used to afford desired protein-peptide conjugates **Ub-LamA**, **Ub-LamB** and **Nb-LamA**, respectively (**Scheme 3**).

After having established a chemical approach to building protein-oligomer constructs, we tested its scope on the formation of protein-dimers. Pleasingly, when **Ub** and **1c** were mixed at 4 ºC, for 24 h, at pH 8 in a 5:1 ratio, selective formation of a **Ub** dimer **Ub-Ub** was detected by LCMS (**Scheme 4**). The final construct was purified by AMICON size-exclusion filters and its presence was confirmed by SDS-PAGE of the crude reaction mixture (**Figure S41**). Only unlabelled Ub (used in excess) and dimer were observed, by LCMS and SDS-PAGE, while no traces of **7c**, which means complete consumption of **1c** towards forming Ub-Ub is achieved. This data demonstrates the utility of the bisalkynylating reagent **1c** to build protein-protein constructs.



Scheme 3. Chemical approach to the synthesis of protein-oligomer constructs.



Scheme 4. Chemical approach to the synthesis of protein-protein protein constructs.

A new method for site-selective cysteine-oriented protein alkynylation has been developed by using *S*-alkynyl sulfonium reagents. This approach has exquisite site selectivity for cysteine residues to form stable S–C$_{(sp)}$ bonds in physiological conditions. The conjugates were shown to maintain the tertiary structure and binding ability of the respective unconjugated native protein. Moreover, through the preparation of a new bisalkynylating reagent the protocol could be extended to the synthesis of protein-oligomer and protein-protein constructs. Ubiquitin-ubiquitin homodimeric constructs were synthesized to showcase the potential application of the method to chemically bond two free cysteine-containing proteins.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website.

Materials, methods, data including NMR and mass spectrometry (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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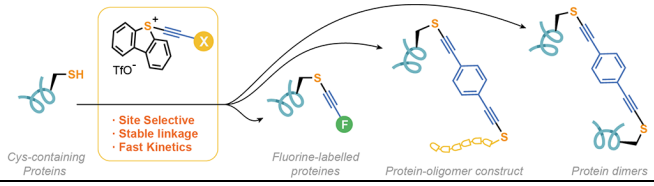
SH
TfO-
X
· Site Selective
· Stable linkage
· Fast Kinetics
F
Cys-containing Proteins
Fluorine-labelled proteines
Protein-oligomer construct
Protein dimers