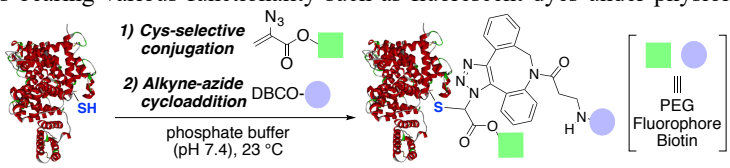
Site-specific Dual Functionalization of Cysteine Residue in Peptides and Proteins with 2-Azidoacrylates

Shinya Ariyasu,[†+] Hirohito Hayashi,[†+] Bengang Xing,*[†] and Shunsuke Chiba*[†]

[†] Division of Chemistry and Biological Chemistry, School of Physical and Mathematical Sciences, Nanyang Technological University, Singapore 637371

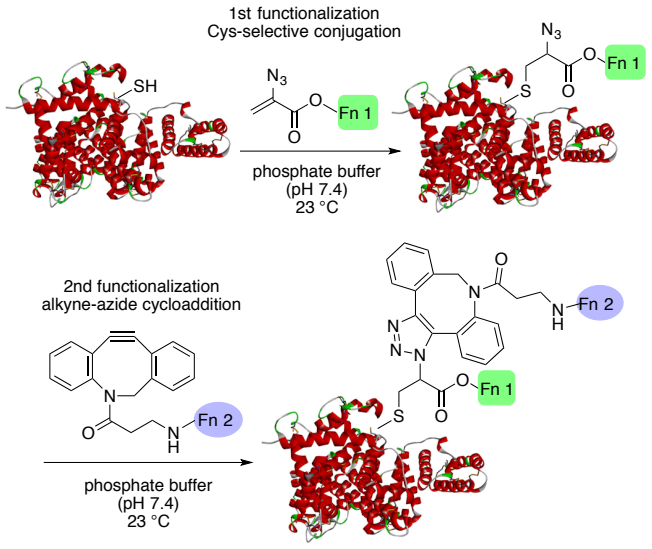
ABSTRACT: Herein, we report use of 2-azidoacrylates to perform site-specific dual functionalization of the cysteine residue of peptides and bovine serum albumin (BSA), a native protein containing one free cysteine residue. The sulfhydryl group of the cysteine residue could be conjugated with 2-azidoacrylates bearing various functionality such as fluorescent dyes under physiological aqueous buffer conditions to afford peptide/protein conjugates anchoring an azide moiety. Successive azide-alkyne cycloaddition enables installation of the 2nd functionality, thus affording dual functionalized peptide/protein based materials.



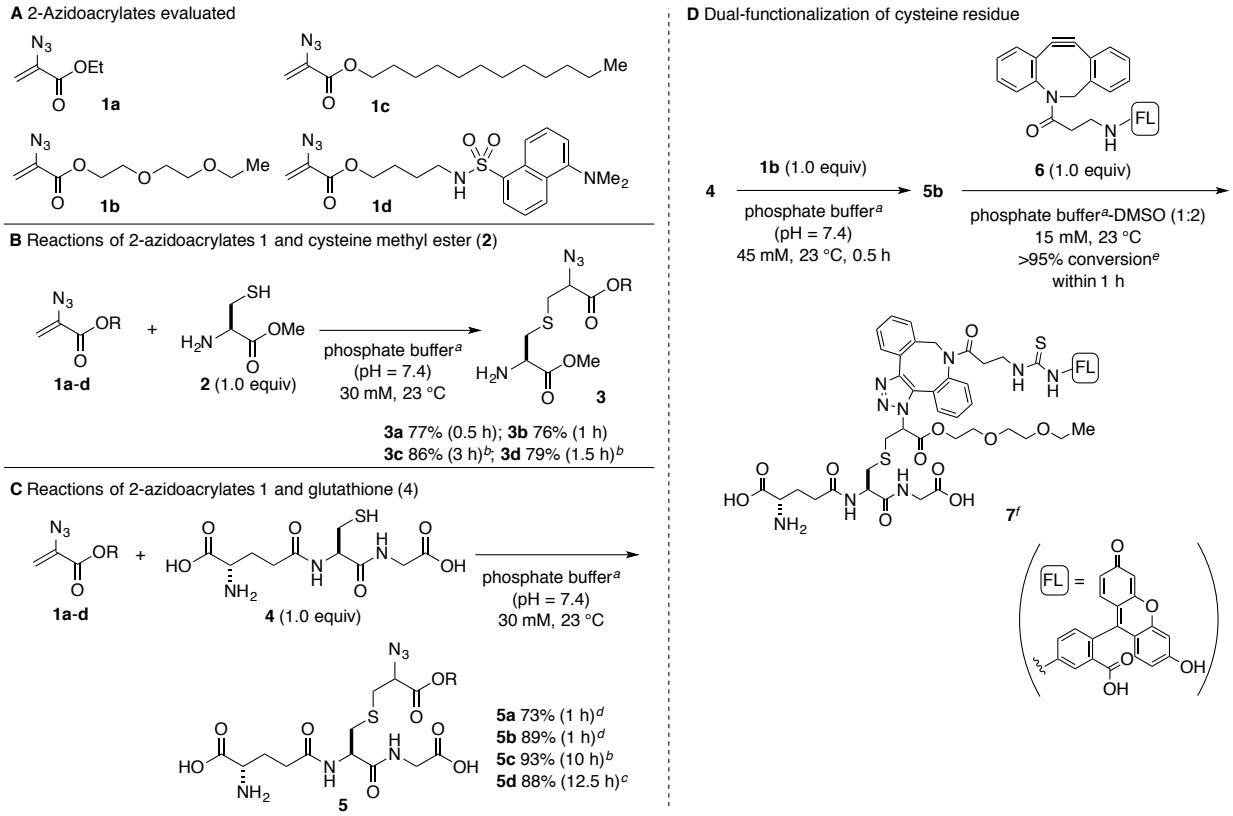
Chemical conjugation of proteins is paramount for various kinds of biological applications such as probing of protein dynamics *in vitro* or *in vivo*,[1-3] investigation of protein structures and their functions,[4,5] enhancement of protein stability in biological systems and resulting therapeutic efficacy,[6-9] development of artificial enzymes or protein-based materials,[10] and construction of protein-drug conjugates.[11-14] As proteins consist of various amino acid residues having different functional groups, protein modification should be carried out selectively at the defined site and should be able to be performed under physiologically relevant conditions. Among about 20 different amino acids involved in the protein composition, cysteine (Cys) is one of the most convenient targets for bioconjugation because of higher nucleophilicity of the sulfhydryl group.[15] In addition, lower natural abundance of the cysteine residue might be useful to avoid undesired multi-site modification. Therefore, various methods for the Cys-modification have been developed, such as alkylation through nucleophilic substitution with α-halo carbonyl reagents[16-18] and 1-(arylsulfonyl)bicyclo[1.1.0]butanes (strain-release reagents),[19] disulfide exchange,[20-22] nucleophilic aromatic substitution (S_NAr) of perfluorobenzene derivatives,[23,24] thiol-yne or -ene reactions,[25-30] and conjugate addition onto the maleimide derivatives,[31-34] vinyl sulfone[35,36] or allenamide derivatives.[37] Among them, the maleimide derivatives are the most commonly used chemical linker because of their excellent kinetic property[38] in spite of instability of the resulting conjugates due to the retro-Michael reaction.

Ability in multi-functionalization for chemical modification of proteins has been highly demanded since advanced peptide probes and protein-based therapeutics frequently need multiple functions on the basis of the conjugation through photo fluorescent dyes or targeting moieties such as biotin, and drug molecules.[39] However, current methods for the multi-functionalization of proteins mainly depend on genetic engineering to prepare artificial proteins having different bioorthogonal handles and subsequent multi-step bioorthogonal reactions under the precisely controlled conditions.[40-42] On the other hand, development of multi-functionalization of native proteins is still amateur due to lacking of the versatile linkers.[43-46] In order to develop the linker capable of multi-functionalizing native proteins, we became interested in design and use of 2-azidoacrylate derivatives as the Michael acceptor for cysteine-selective conjugation, that can simultaneously install an azido group at the same position of the target proteins for the continuous second functionalization by alkyne-azide cycloadditions[47-49] (Scheme 1). The design, development, and preliminary applications of this protocol are described herein.

Scheme 1. Site-specific dual functionalization of proteins with 2-azidoacrylates



Scheme 2. Reactions of 2-azidoacrylates 1 with cysteine (2) or glutathione (4).



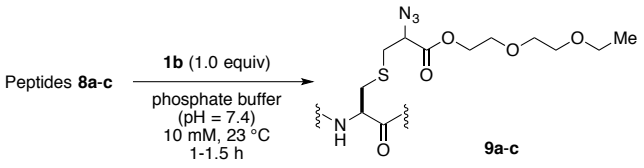
[a] 50 mM phosphate buffer (pH = 7.4) was used. [b] The reaction was conducted in buffer-THF (1:1). [c] The reaction was conducted in buffer-THF (2:1). [d] ^{1}H NMR yields based on *tert*-butanol as the internal standard. [e] Chemical conversion was determined by HPLC. [f] One of the regioisomers is shown.

At the outset of the project, we prepared four types of 2-azidoacrylates having an ethyl group (for **1a**), a hydrophilic short-PEG moiety (for **1b**) and hydrophobic parts (dodecyl **1c** and fluorescent dansylamide **1d**) (Scheme 2A). The optimization of the reaction conditions of the 1st conjugation using ethyl 2-azidoacrylate (**1a**) and cysteine methyl ester (**2**) revealed that the conjugation proceeds smoothly and selectively in sodium phosphate buffer (50 mM) at pH 7.4, affording α-azidoester-cysteine conjugate **3a** in 77% isolated yield within 0.5 h. Similarly, the reaction with short-PEG derivative **1b** proceeded well to give **3b** in 76% isolated yield. On the other hand, those with hydrophobic **1c** and **1d** should be conducted

in the co-solvent system of aqueous buffer and THF to afford conjugates **3c** and **3d**, respectively in good yields albeit in longer reaction times. The reactions of tripeptide, glutathione (**4**) with 2-azidoacrylates **1a-d** could also show chemoselectivity with the sulfhydryl group to deliver the corresponding conjugates **5a-d**, respectively, in good yields (Scheme 2C). Furthermore, the installed azide group in **5b** smoothly reacted with fluorescein-linked dibenzocyclooctyne (DBCO) **6** through strain-promoted alkyne-azide cycloaddition (SPAAC) to afford dual-functionalized glutathione **7** (Scheme 2D).

In order to obtain the insight into the kinetic property of the present conjugation with 2-azidoacrylate **1b**, the consumption of **1b** in the reaction with glutathione (**4**) under dilute reaction conditions ([**1b**] = 1.0 mM) were monitored at different time points by using high-performance liquid chromatography (HPLC) (see the Supporting Information). The reaction was reached to plateau at about 60% conversion within 2 h, and no side-product was observed in the HPLC analysis. The consumption of **1b** was well fitted second-order manner, and its rate constant of **1b** against **4** was estimated to 0.509 ± 0.03 $M^{-1}s^{-1}$, which is the similar range to that of SPAAC commonly used as the bioorthogonal reactions.[14,50]

Scheme 3. Modification of Cys-containing peptides 8a-c with 1b



8a: H-**Cys**-Gly-Lys-Ser-Arg-Phe-OH

8b: H-Lys-Ser-**Cys**-Gly-Arg-Phe-OH

8c: H-Gly-Lys-Ser-Arg-Phe-**Cys**-OH

Chemoselectivity of the present conjugation with 2-azidoacrylates **1** toward the sulfhydryl group of the cysteine residue could be further proved by the reactions of **1b** with three Cys-containing peptides at *N*-terminus (for **8a**), internal peptide chain (for **8b**) or *C*-terminus (for **8c**). Conjugation of peptide **8a-c** with 1 equivalent of 2-azidoacrylate **1b** were completed within 1.5 h (where [**8a-c**] = 10 mM) selectively at the sulfhydryl group (Scheme 3), which was confirmed by MS/MS analyses of the resulting conjugates **9a-c** (see the Supporting Information). In addition, any multi-labeled peptides were not observed even when 10 equivalents of **1b** were mixed with **8a-c** for 24 h. These studies clearly indicated that surroundings around the sulfhydryl group of the Cys residue in terms of the charge, hydrophilic, and hydrophobic properties do not significantly affect efficiency of the conjugation.

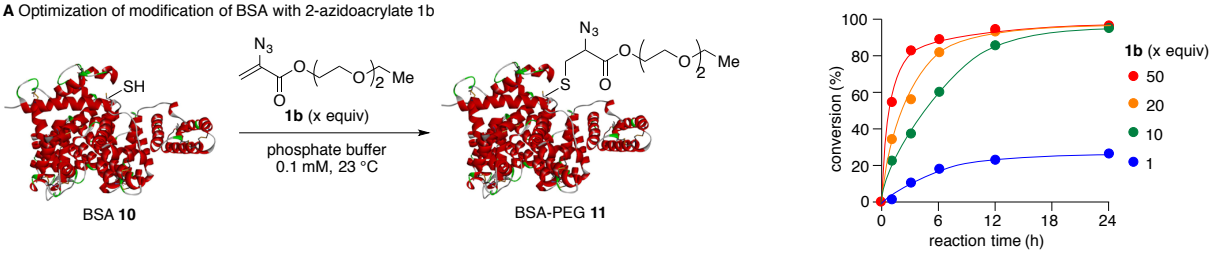
We next investigated capability of 2-azidoacrylate **1b** in modification of the native protein, bovine serum albumin (BSA) (**10**), which contains one free cysteine residue in total 583 amino acid residues (MW = 66.5 kDa) (Scheme 4A). In order to optimize the reaction conditions, remaining quantity of the free sulfhydryl group in BSA (**10**) were traced at different reaction time points by the Ellman test.[51] Differently from the conjugation of short peptides, the conjugation reaction of BSA (**10**) under highly diluted reaction conditions required excess amounts (>10 equivalents) of 2-azidoacrylate **1b** and longer reaction time (>12 h) to achieve sufficient conversion of the conjugation (>85%). We then explored use of the resulting azide moiety in the conjugated-BSA **11** for further functionalization. Namely, successive treatment of **11** with fluorescein-linked DBCO (DBCO-FL) **6** was examined to induce strain-promoted alkyne-azide cycloaddition (SPAAC). This 2nd functionalization took 12 h to attain reasonable conversion (see the Supporting Information) to afford BSA-PEG-FL **12**. In order to confirm the site-specific dual functionalization, the control samples that omitted the incorporation process of either **1b** or **6** were prepared and compared with dual labelled BSA-PEG-FL **12** in SDS-PAGE (Scheme 4B). As shown in Scheme 4B, BSA-PEG-FL **12** in lane 4 showed both protein-staining and fluorescein-emission band, whereas lane 3, which lacked 2-azidoacrylate **1b**, showed very weak emission signal probably originated from the thiol-yne reaction of BSA (**10**) with DBCO-FL **6**.[52] These results indicate that dual functionalization of BSA (**10**) was accomplished. Furthermore, MS spectrum of fragments of BSA-PEG-FL **12** digested by trypsin reveals **1b** and **6** are site-specifically installed at Cys58 in BSA (**10**) (see the Supporting Information).

To demonstrate the versatile capability of the present dual functionalization strategy with 2-azidoacrylates, we further prepared fluorescein-linked 2-azidoacrylate **1e** and use it for conjugation reactions of BSA (**10**) (Scheme 5). By following the procedure of dual functionalization (Scheme 5), BSA (**10**) (in 0.1 M) was treated with 2-azidoacrylate **1e** (10 equiv) to form 1st conjugate **13**, that was followed by SPAAC with cyanine dye-linked dibenzocyclooctyne (DBCO-Cy5) **14** to afford dual color labelled BSA **15**. The SDS-PAGE analysis of **15** in Scheme 5 clearly indicated that BSA was modified with both fluorescein and Cy5 at the single-site. Thus, the present dual color labelling of native proteins would be useful for design of advanced protein-based chemical probes such as ratiometric fluorescent probes and FRET (fluorescence resonance energy transfer) based probes.[1]

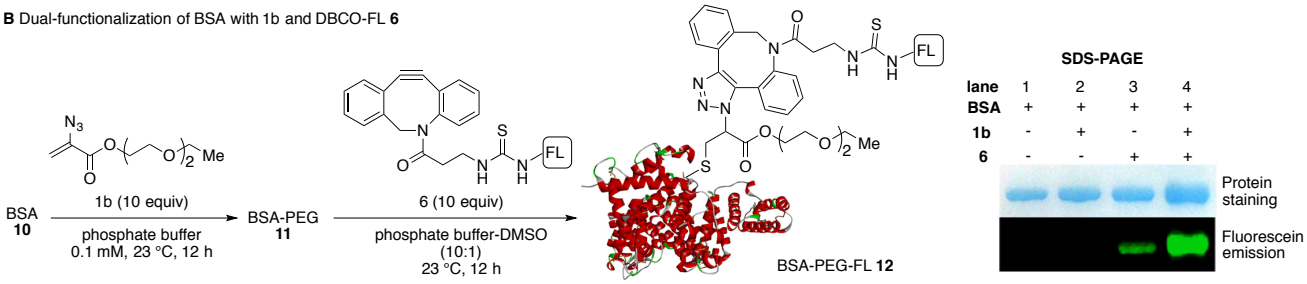
Furthermore, BSA-FL **13** was applied for biotinylation using DBCO-biotin **16** to prepare dual-functionalized BSA-FL-biotin **17** (Scheme 6A). In SDS-PAGE analysis (Scheme 6B), BSA-FL-biotin **17** exhibited green fluorescence originated from fluorescein. To confirm the successful incorporation of biotin, enzyme-linked immunosorbent assay (ELISA) of BSA-FL-biotin **17** and its control samples were conducted by using streptavidin-coated beads, anti-BSA-antibody and horseradish peroxidase (HRP) conjugated secondary antibody (Scheme 6C). Only sample 4 containing BSA-FL-biotin **17** exhibited significant HRP activity, suggesting that biotin moiety is incorporated into BSA through the dual functionalization. Because biotin itself does not bear fluorescence property, concise incorporation of another reporter moiety such as fluorescent dyes into biotinylated proteins would enable tracing the target proteins.

Scheme 4. Reactions of 2-azidoacrylates 1 with cysteine (2) or glutathione (4)

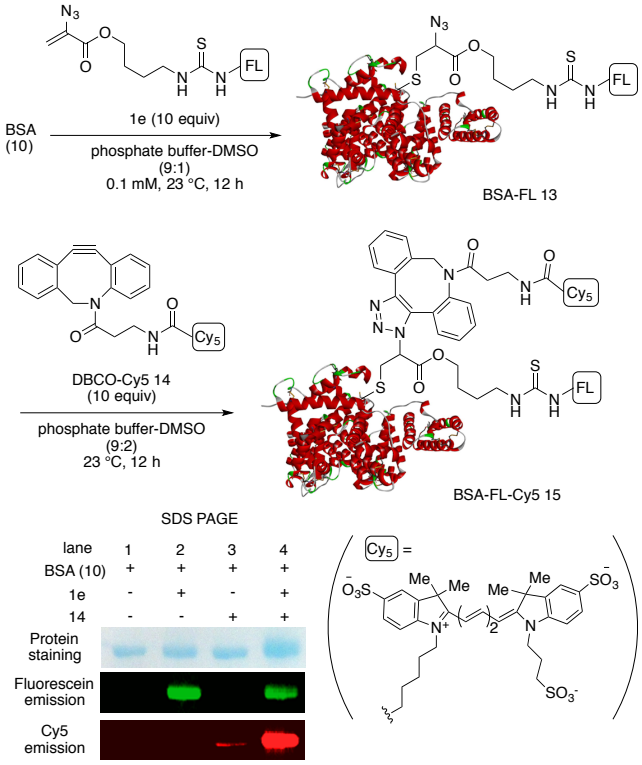
A Optimization of modification of BSA with 2-azidoacrylate 1b



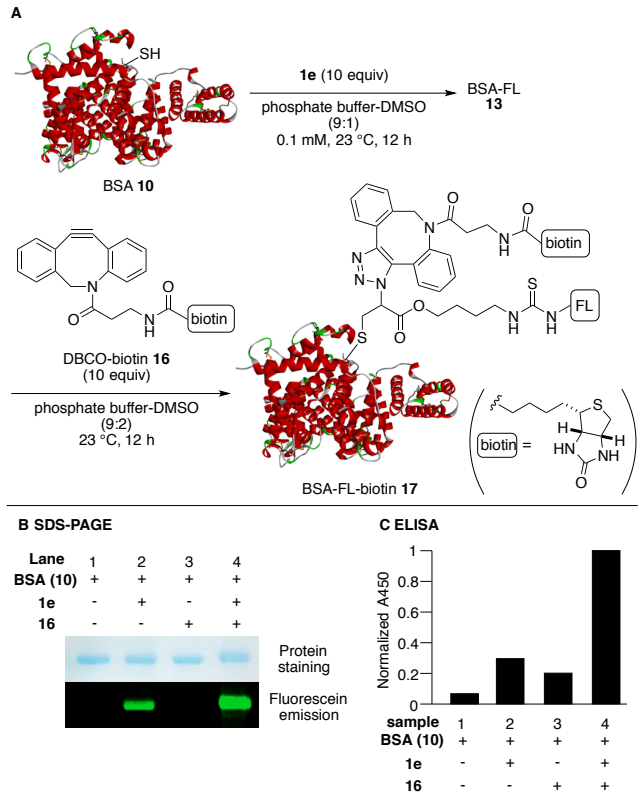
B Dual-functionalization of BSA with 1b and DBCO-FL **6**



Scheme 5. Dual color labeling of BSA (10) with 1e and DBCO-Cy5 14.



Scheme 6. Dual-functionalization of BSA (10) with 1e and DBCO-biotin 16.



In summary, we have developed site-specific dual-functionalization of the cysteine residue of peptides and bovine serum albumin (BSA), a native protein containing one free cysteine residue with 2-azidoacrylates linked with various hydrophilic and hydrophobic functionalities. 2-Azidoacrylates could react with the sulfhydryl group of the cysteine residues in a chemoselective manner over other amino acid residues to give the α-azidoester conjugate, which was further functionalized through alkyne-azide cycloaddition, thus enabling facile dual-functionalization at the single cite of the cysteine residue. We anticipate that the concept for concise dual-functionalization of proteins will be useful to develop advanced protein-based chemical probes or therapeutics. The future work will be directed toward development of the site-specific conjugation of the targeted Cys residue(s) in various native proteins having multiple Cys residues using functionalized 2-azidoacrylates of rational design.

ASSOCIATED CONTENT

Supporting Information

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

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

AUTHOR INFORMATION

Corresponding Author

S.C. e-mail: shunsuke@ntu.edu.sg

B.X. e-mail: bengang@ntu.edu.sg

Author Contributions

[+]S.A. and H.H. contributed equally to this work.

ACKNOWLEDGMENT

This work was supported by Nanyang Technological University and Singapore Ministry of Education (Academic Research Fund Tier 2: MOE2013-T2-1-060 to SC and Academic Research Fund Tier 1: RG2/15 to SC as well as RG11/13 & RG35/15 to B.X.).

ABBREVIATIONS

Cys, cysteine; S_NAr, nucleophilic aromatic substitution; THF, tetrahydrofuran; DMSO, dimethyl sulfoxide; PEG, polyethylene glycol; DBCO, dibenzocyclooctyne; SPAAC, strain-promoted alkyne-azide cycloaddition; HPLC, high-performance liquid chromatography; Gly, glycine; Lys, lysine; Ser, serine; Arg, arginine; Phe, phenylalanine; MS/MS, tandem mass spectrometry; BSA, bovine serum albumin; FL, fluorescein; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase

REFERENCES

(1) Fernandez-Suarez, M., and Ting, A. Y. (2008) Fluorescent probes for super-resolution imaging in living cells. *Nat. Rev. Mol. Cell. Biol. 9*, 929−943.

(2) Jing, C., and Cornish, V. W. (2011) Chemical tags for labeling proteins inside living cells. *Acc. Chem. Res. 44*, 784−792.

(3) Tamura, T., and Hamachi, I. (2014) Recent progress in design of protein-based fluorescent biosensors and their cellular applications. *ACS Chem. Biol. 9*, 2708−2717.

(4) Royer, C. A. (2006) Probing protein folding and conformational transitions with fluorescence. *Chem. Rev. 106*, 1769−1784.

(5) Spasser, L., and Brik, A. (2012) Chemistry and biology of the ubiquitin signal. *Angew. Chem. Int. Ed. 51*, 6840−6862.

(6) Harris, J. M., and Chess, R. B. (2003) Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug. Discov. 2*, 214−221.

(7) Pelegri-O'Day, E. M., Lin, E. W., and Maynard, H. D. (2014) Therapeutic protein-polymer conjugates: advancing beyond PEGylation. *J. Am. Chem. Soc. 136*, 14323−14332.

(8) Nishcan, N., and Hackenberger, C. P. (2014) Site-specific PEGylation of proteins: recent developments. *J. Org. Chem. 79*, 10727−10733.

(9) Hu, Q. Y., Berti, F., and Adamo, R. (2016) Towards the next generation of biomedicines by site-selective conjugation. *Chem. Soc. Rev. 45*, 1691-1719.

(10) Ueno, T., Tabe, H., and Tanaka, Y. (2013) Artificial metalloenzymes constructed from hierarchically-assembled proteins. *Chem. Asian J. 8*, 1646−1660.

(11) Gordon, M. R., Canakci, M., Li L., Zhuang, J., Osborne, B., and Thayumanavan, S. (2015) Field Guide to Challenges and Opportunities in Antibody-Drug Conjugates for Chemists. *Bioconjugate Chem. 26*, 2198−2215.

(12) Agarwal, P., and Bertozzi, C. R. (2015) Site-specific antibody-drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. *Bioconjugate Chem. 26*, 176−192.

(13) Chudasama, V., Maruani, A., and Caddick, S. (2016) Recent advances in the construction of antibody-drug conjugates. *Nat. Chem. 8*, 114−119.

(14) Krall, N., da Cruz, F. P., Boutureira, O., and Bernardes, G. J. (2016) Site-selective protein-modification chemistry for basic biology and drug development. *Nat. Chem. 8*, 103−113.

(15) Gunnoo, S. B., and Madder, A. (2016) Chemical protein modification through cysteine. *ChemBioChem 17*, 529−553.

(16) Goddard, R. D., and Leonor, M. (1935) Derivative of keratin *J. Biol. Chem. 112*, 361− 371.

(17) Nielsen, M. L., Vermeulen, M., Bonaldi, T., Cox, J., Moroder, L., and Mann, M. (2008) Iodoacetamide-induced artifact mimics ubiquitination in mass spectrometry. *Nat. Methods 5*, 459−460.

(18) Weerapana, E., Wang, C., Simon, G. M., Richter, F., Khare, S., Dillon, M. B., Bachovchin, D. A., Mowen, K., Baker, D., and Cravatt, B. F. (2010) Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature 468*, 790−795.

(19) Gianatassio, R., Lopchuk, J. M., Wang, J., Pan, C. -M., Malins, L. R., Prieto, L., Brandt, T. A., Collins, M. R., Gallego, G. M., Sach, N. W., et al. (2016) Organic chemistry. Strain-release amination. *Science 351*, 241−246.

(20) King, T. P., Li, Y., and Kochoumian, L. (1978) Preparation of protein conjugates via intermolecular disulfide bond formation. *Biochemistry 17*, 1499−1506.

(21) Bernardes, G. J., Grayson, E. J., Thompson, S., Chalker, J. M., Errey, J. C., Oualid, F. El., Claridge, T. D., and Davis, B. G. (2008) From disulfide- to thioether-linked glycoproteins. *Angew. Chem. Int. Ed. 47*, 2244−2247.

(22) Steiner, M., Hartmann, I., Perrino, E., Casi, G., Brighton, S., Jelesarov, I., Bernardes, G. J. L., and Neri, D. (2013) Spacer length shapes drug release and therapeutic efficacy of traceless disulfide-linked ADCs targeting the tumor neovasculature. *Chem. Sci. 4*, 297−302.

(23) Spokoyny, A. M., Zou, Y., Ling, J. J., Yu, H., Lin, Y. S., and Pentelute, B. L. (2013) A perfluoroaryl-cysteine S(N)Ar chemistry approach to unprotected peptide stapling. *J. Am. Chem. Soc. 135*, 5946−5949.

(24) Zhang, C., Welborn, M., Zhu, T., Yang, N. J., Santos, M. S., Van Voorhis, T., Pentelute, B. L. (2016) π-Clamp-mediated cysteine conjugation. *Nat. Chem. 8*, 120−128.

(25) Floyd, N., Vijayakrishnan, B., Koeppe, Koeppe, J. R., and Davis, B. G. (2009) Thiyl glycosylation of olefinic proteins: S-linked glycoconjugate synthesis. *Angew. Chem. Int. Ed. 48*, 7798−7802.

(26) Dondoni, A., Massi, A., Nanni, P., and Roda, A. (2009) A new ligation strategy for peptide and protein glycosylation: photoinduced thiol-ene coupling. *Chem. Eur. J. 15*, 11444−11449.

(27) Lo Conte, M., Pacifico, S., Chambery, A., Marra, A., and Dondoni (2010) Photoinduced addition of glycosyl thiols to alkynyl peptides: use of free-radical thiol-yne coupling for post-translational double-glycosylation of peptides. *J. Org. Chem.75*, 4644−4647.

(28) Bernardim, B., Cal, P. M., Matos, M. J., Oliveira, B. L., Martínez-Sáez, N., Albuquerque, I. S., Perkins, E., Corzana, F., Burtoloso, A. C., Jiménez-Osés, G., et al. (2016) Stoichiometric and irreversible cysteine-selective protein modification using carbonylacrylic reagents. *Nat. Commun. 7*, 13128.

(29) Shiu, H.–Y., Chan, T.–C., Ho, C.–M., Liu, Y., Wong, M.–K. and Che, C.–M. (2009), Electron-Deficient Alkynes as Cleavable Reagents for the Modification of Cysteine-Containing Peptides in Aqueous Medium. *Chem. Eur. J. 15*, 3839 – 3850.

(30) Koniev, O., Leriche, G., Nothisen, M., Remy, J. S., Strub, J. M., Schaeffer-Reiss, C., Van Dorsselaer, A., Baati, R., and Wagner, A. (2014) Selective Irreversible Chemical Tagging of Cysteine with 3-Arylpropiolonitriles. *Bioconjugate Chem. 25*, 202-206.

(31) Fridmann, E. Spectrophotometric investigation of the interaction of glutathione with maleimide and *N*-ethylmaleimide. (1952) *Biochim. Biophys. Acta 9*, 65−75.

(32) Zhang, Y., Bhatt, V. S., Sun, G., Wang, P. G., and Palmer, A. F. (2008) Site-selective glycosylation of hemoglobin on Cys beta93. *Bioconjugate Chem. 19*, 2221−2230.

(33) Tavaré, R., Torres Martin De Rosales, R., Blower, P. J., and Mullen, G. E. (2009) Efficient site-specific radiolabeling of a modified C2A domain of synaptotagmin I with $[^{99m}Tc(CO)_3]^+$: a new radiopharmaceutical for imaging cell death. *Bioconjugate Chem. 20*, 2071−2081.

(34) Junutula, J. R., Flagella, K. M., Graham, R. A., Parsons, K. L., Ha, E., Raab, H., Bhakta, S., Nguyen, T., Dugger, D. L., Li, G., et al. (2010) Engineered thio-trastuzumab-DM1 conjugate with an improved therapeutic index to target human epidermal growth factor receptor 2-positive breast cancer. *Clin. Cancer Res. 16*, 4769−4778.

(35) Morpurgo, M., Veronese, F. M., Kachensky, D., and Harris, J. M. (1996) Preparation of characterization of poly(ethylene glycol) vinyl sulfone. *Bioconjugate Chem. 7*, 363−368.

(36) Lopez-Jaramillo, F. J., Ortega-Muñoz, M., Megia-Fernandez, A., Hernandez-Mateo, F., and Santoyo-Gonzalez, F. (2012) Vinyl sulfone functionalization: a feasible approach for the study of the lectin-carbohydrate interactions. *Bioconjugate Chem. 23*, 846−855.

(37) Abbas, A., Xing, B., and Loh, T. P. (2014) Allenamides as orthogonal handles for selective modification of cysteine in peptides and proteins. *Angew. Chem. Int. Ed. 53*, 7491−7494.

(38) Saito, F., Noda, H., and Bode, J. W. (2015) Critical evaluation and rate constants of chemoselective ligation reactions for stoichiometric conjugations in water. *ACS Chem. Biol. 10*, 1026−1033.

(39) Maruani, A., Richards, D. A., and Chusasama, V. (2016) Dual modification of biomolecules. *Org. Biomol. Chem. 14*, 6165−6178.

(40) Haney, C. M., Wissner, R. F., and Petersson, E. J. (2015) Multiply labeling proteins for studies of folding and stability. *Curr. Opin. Chem. Biol. 28*, 981-984.

(41) Sachdeva, A., Wang, K., Elliott, T., and Chin, J. W. (2014) Concerted, rapid, quantitative, and site-specific dual labeling of proteins. *J. Am. Chem. Soc. 136*, 7785−7788.

(42) Lee, T. C., Kang, M., Kim, C. H., Schultz, P. G., and Chapman, E., and Daniz, A. A. (2016) Dual Unnatural Amino Acid Incorporation and Click-Chemistry Labeling to Enable Single-Molecule FRET Studies of p97 Folding. *ChemBioChem 17*, 981−984.

(43) Morales-Sanfrutos, J., Lopez-Jaramillo, F. J., Hernandez-Mateo F., and Santoyo-Gonzalez, F. (2010) Vinyl sulfone bifunctional tag reagents for single-point modification of proteins. *J. Org. Chem. 75*, 4039−4047.

(44) Viault, G., Dautrey, S., Maindron, N., Hardouin, J., Renard, P. Y., and Romieu, A. (2013) The first "ready-to-use" benzene-based heterotrifunctional cross-linker for multiple bioconjugation. *Org. Biomol. Chem. 11*, 2693−2705.

(45) Vaněk, V., Pícha, J., Fabre, B., Buděšínský, M., Lepšík, M., and Jiráček, J. (2015) The Development of a Versatile Trifunctional Scaffold for Biological Applications. *Euro. J. Org. Chem. 2015,* 3689−3701.

(46) Maruani, A., Smith, M. E., Miranda, E., Chester, K. A., Chudasama, V., and Caddick, S. (2015) A plug-and-play approach to antibody-based therapeutics via a chemoselective dual click strategy. *Nat. Commun. 6*, 6645.

(47) Yang, M., Li, J., and Chen, P. R. (2014) Transition metal-mediated bioorthogonal protein chemistry in living cells. *Chem. Soc. Rev. 43*, 6511−6526.

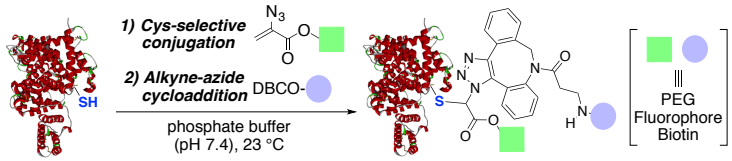
(48) Zhang, G., Zheng, S., Liu, H., and Chen, P. R. (2015) Illuminating biological processes through site-specific protein labeling. *Chem. Soc. Rev. 44*, 3405−3417.

(49) Chen, X., and Wu, Y. W. (2016) Selective chemical labeling of proteins. *Org. Biomol. Chem. 14*, 5417−5439.

(50) Gordon, C. G., Mackey, J. L., Jewett, J. C., Sletten, E. M., Houk, K. N. and Bertozzi, C. R. (2012) Reactivity of biarylazacyclooctynones in copper-free click chemistry. *J. Am. Chem. Soc. 134*, 9199−9208.

(51) Ellman, G. L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys. 82*, 70−77.

(52) van Geel, R., Pruijn, G. J., van Delft, F. L., and Boelens, W. C. (2012) Preventing thiol-yne addition improves the specificity of strain-promoted azide-alkyne cycloaddition. *Bioconjugate Chem. 23*, 392−398.


SH
1) Cys-selective conjugation
2) Alkyne-azide cycloaddition
DBCO-
phosphate buffer
(pH 7.4), 23 °C
S
PEG
Fluorophore
Biotin