Click, Lock & Dye: a chromogenic handle for selective cysteine modification

Yong Hua,[a,b] Zhi Zou,[a,b] Alessandro Prescimone,[a,b] Thomas R. Ward,[a,b,c] Marcel Mayor,[a,b,d,e] Valentin Köhler[a,b]

[a] Department of Chemistry, University of Basel, St. Johannsring 19, CH-4056 Basel, Switzerland, valentin.koehler@unibas.ch

[b] Department of Chemistry, University of Basel, Mattenstrasse 24a, CH-4058 Basel, Switzerland

[c] National Center of Competence in Research (NCCR) "Molecular Systems Engineering", 4058 Basel, Switzerland

[d] Institute for Nanotechnology (INT) and Karlsruhe Nano Micro Facility (KNMFi) Karlsruhe Institute of Technology (KIT) P.O. Box 3640, DE-76021 Karlsruhe Eggenstein-Leopoldshafen, Germany

[e] Lehn Institute of Functional Materials (LIFM), School of Chemistry, Sun Yat-Sen University (SYSU), XinGangXi Road 135, 510275 Guangzhou, P. R. China

Abstract: The addition of a sulfhydryl group to water-soluble N-alkyl(*o*-nitrostyryl)pyridinium ions (NSPs) followed by fast and irreversible cyclization and aromatization results in a stable S-Csp^2-bond. The reaction sequence, termed Click & Lock, engages accessible cysteine residues under the formation of N-hydroxy indole pyridinium ions. The accompanying red-shift of > 70 nm to around 385 nm enables convenient monitoring of the labeling yield by UV-VIS spectroscopy at extinction coefficients of ≥ 2 × 10^4 $M^{-1}cm^{-1}$. The versatility of the linker is demonstrated in the stapling of peptides and the derivatization of proteins, including the modification of reduced Trastuzumab with Val-Cit-PAB-MMAE. The high stability of the linker, fast reaction rates (k_{app} up to 4.4 $M^{-1}s^{-1}$ at 20°C), high selectivity, and the bathochromic properties of the Click & Lock reaction provide an appealing alternative to existing methods for cysteine conjugation.

Keywords: posttranslational modification, click chemistry, bioconjugation, chromogenic reagents

Protein and peptide modification by chemical means serves the study and tracking of biomolecules,[1] target-selective drug delivery,[2] improving the pharmacokinetic properties of protein drugs,[3] and the immobilization of enzymes,[4] to name a few.[5] The increasing market share of biologicals acts as an additional incentive in this industrious area of research.[5a, 6]

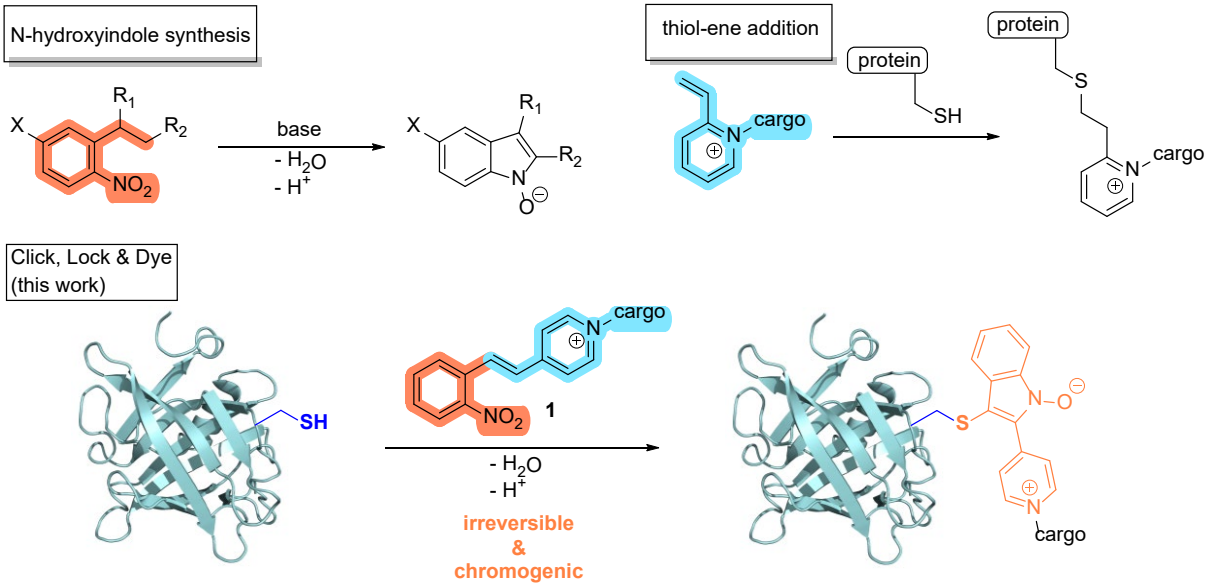
Residues targeted for protein modification are most commonly cysteines and lysines. Cysteines are particularly attractive due to their relatively low abundance and the high nucleophilicity of surface-exposed sulfhydryl groups at moderate pH values. Reactive cysteines can be introduced recombinantly or made accessible by cleavage of intramolecular disulfide bonds under mild reductive conditions.

The portfolio of cysteine-reactive electrophiles is dominated by maleimide derivatives that exhibit very fast reaction kinetics and high selectivities.[7] For example, the majority of FDA-approved antibody-drug-conjugates are loaded with their drug cargo through the reaction with a maleimide reagent.[8] A frequently encountered challenge is the reversibility of the thiol-ene reaction that leads, in the case of antibody-drug conjugates, to increased systemic toxicity and reduced target activity through the untimely release of the cargo.[2a, 7, 9] Despite the convenience and resulting popularity of maleimide cysteine conjugation, alternative methods with complementary profiles are frequently investigated.[10] The relevance of linker moieties with diverse structural features is readily illustrated in the cyclization of peptides with cysteine-reactive multifunctional entities: Here, the nature of the linker directly affects, which conformations of the constrained peptide can be adapted, and how functional motifs are displayed.[11]

The thiol-ene reaction, e.g., the addition of reactive cysteines to maleimides, leads to the formation of an S-Csp^3-bond. If subsequent elimination of an alternative leaving group[12] could proceed under aromatization,[13] one might gain the increased stability of an S-Csp^2 bond without forming a potent electrophile.

N-alkylated *o*-vinyl-pyridinium groups, developed by Bernardes et al,[10d] react under the formation of an ethyl-pyridinium thioether and combine fast reaction kinetics with high water solubility. The alkylation of nitrogen not only dramatically enhances reactivity and solubility but also doubles as a convenient handle to load the reactive linker with cargo. Certain *o*-nitroarylethanes on the other hand can be easily converted into 1-hydroxyindoles in the presence of a suitable base as reported by Makosza and coworkers.[14]

Inspired by vinyl-pyridinium electrophiles and the possibility to extend the aromatic system of *o*-nitroarylethanes by cyclization and elimination, we designed N-alkyl(*o*-nitrostyryl)pyridinium ions **1** (Scheme 1). Here the nucleophilic attack of a thiol at an electron-deficient alkene results in an *o*-nitroarylethane intermediate that subsequently cyclizes to form the N-hydroxyindole product.



Scheme 1. Merging the thiol-ene reaction of vinyl-pyridinium ions[10d] with N-hydroxyindole synthesis[14] results in an irreversible cysteine bionconjugation that is accompanied by a large bathochromic shift.

The determination of the reaction yield of bioconjugations typically requires a chromatographic analysis. The Click & Lock reaction described here enables the direct monitoring of the reaction progress without the need for chromatography due to the large bathochromic shift that accompanies the transformation of reagent to coupling product. This feature can serve to conveniently estimate reaction rates and determine conjugation yield in the absence of additional chromophores in the attached cargo, i.e., for the attachment of Val-Cit-PAB-MMAE to Trastuzumab.

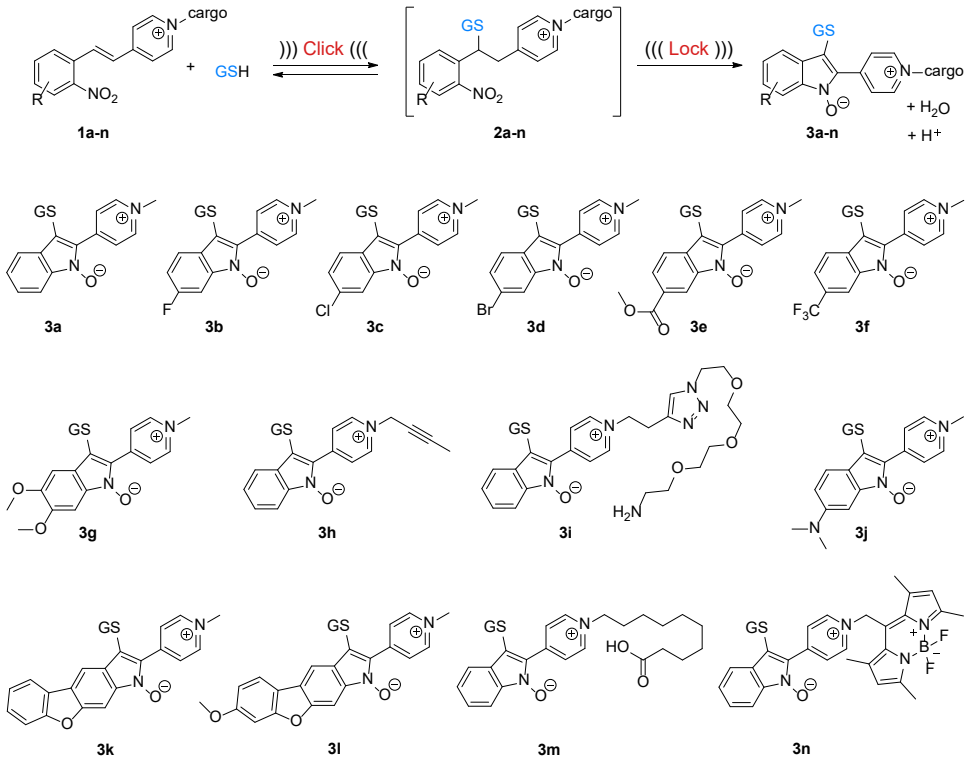
Results and Discussion:

In the initial set of experiments we employed glutathione as the nucleophile and a simple methylgroup as the 'cargo' in electrophile **1a**. Glutathione conjugates were formed quantitatively within 1 hour as revealed by LC-MS (2 mM **1a**, 1.2 eq. of GSH, 37 °C, 100 mM $[NH_4][HCO_3]$ (pH 8.0), buffer:DMF = 9:1. The reaction proceeded cleanly and was accompanied by a red-shift of 75 nm. A range of substituents at the nitroaryl moiety were tolerated, and even accelerated by electron-withdrawing substituents in the 4-position (**1b**-**f**) while electron-donating substituents slowed the reaction: 3,4-dimethoxy substituted **1g** required already 4 hours to reach high conversion, and the 4-dimethylamino-substituted compound **1j** yielded only traces of product, even after prolonged reaction times (Table 1). The products showed in all cases a strong red shift (>70 nm) relative to the N-alkyl(*o*-nitrostyryl)pyridinium ions allowing product formation to be detected by eye as a yellow hue of the solution.

Cargo can be attached to the pyridine ring directly by alkylation as shown for the bodipy chromophore in **3n** or via additional linker moieties that have alternative functional groups, such as an alkyne in **3h** or a primary amine displayed by a hydrophilic PEG-linker in **3i**. Intriguingly, the nature of the linker can also

affect reaction rates: I.e., the reaction of **1i** to **3i** carrying a triazole-PEG-amine cargo was significantly faster than the reaction of **1m** to **3m** having a lipophilic hydrocarbon chain and a terminal carboxylate.

Table 1[a]: Click & Lock reaction between glutathione (GSH) and various 4-(2-nitrostyryl)pyridinium ions **1a-n**.



ID	conversion[b]	λ_{max} (nm) substrate (**1**)[c]	λ_{max} (nm) product (**3**)[c]	ε ($\times 10^4$ M^{-1} cm^{-1}) product (**3**)[c]	k_{app} ($M^{-1}s^{-1}$)[d]
3a	>99%	310	385	2.2	2.2
3b	>99%	309	385	2.2	2.6
3c	>99%	311	387	2.2	3.1
3d	>99%	313	387	2.2	3.1
3e	>99%	308	379	2.1	3.9
3f	>99%	299	374	2.0	4.2
3g	90% (4h)[e, f]	312	422	2.2	-
3h	>99%	316	391	2.0	4.4
3i	>99%	324	395	2.3	4.1
3j	trace (24h)	-	-	-	-
3k	>99%[e]	320	405	2.3	-
3l	90% (4h)[e, f]	332	413	2.1	-
3m	>99%[e]	309	387	2.3	1.1
3n	53%(4h)[g]	525	524	6.1	-

a) 0.40 mM **1**(**a**-**n**), 1.2 equiv glutathione, 0.1 M NH_4HCO_3 buffer (pH 8.0) / DMF = 9:1, 1 hr, 37°C. b) determined by LC-MS and LC-UV. Consistent with UV-VIS conversions. c) in 0.1 M NH_4HCO_3 buffer (pH 8.0). d) k_{app} was determined at 40 µM **1a**-**i**,**k**-**m** in the presence of 4.0 mM glutathione at 20 °C. e) 0.20 mM **1m**, 2.4 equiv. glutathione. f) Conversion determined by UV-VIS. g) Isolated yield; DMF was replaced with MeOH; bodipy is the dominant chromophore. Isolated yields for all compounds (except **3j**) are reported in the SI.

We next probed the bathochromic properties of the reaction for the estimation of reaction rate constants under pseudo-first-order conditions. The UV-spectrum of a solution containing initial concentrations of **1a** (40 µM) and GSH (4.0 mM) in 100 mM NH_4HCO_3 buffer (pH 8.0) was recorded in the wavelength range from 270 - 470 nm every 3 seconds at 20 °C (Figure 1). The decreasing absorption of **1a** and the increasing absorption of **3a** in the course of the reaction resulted in a well-defined isosbestic point at 355 nm. Its sharp definition indicates that the cyclization (the 'lock') is indeed very fast since the presence of underlying intermediates in significant concentrations can be excluded. The lower limits for the apparent second order rate constants obtained by the method of initial rates range from 1.1 $M^{-1}s^{-1}$ for **3m** to 4.4 $M^{-1}s^{-1}$ for **3i** (Table 1 and Figure 1).

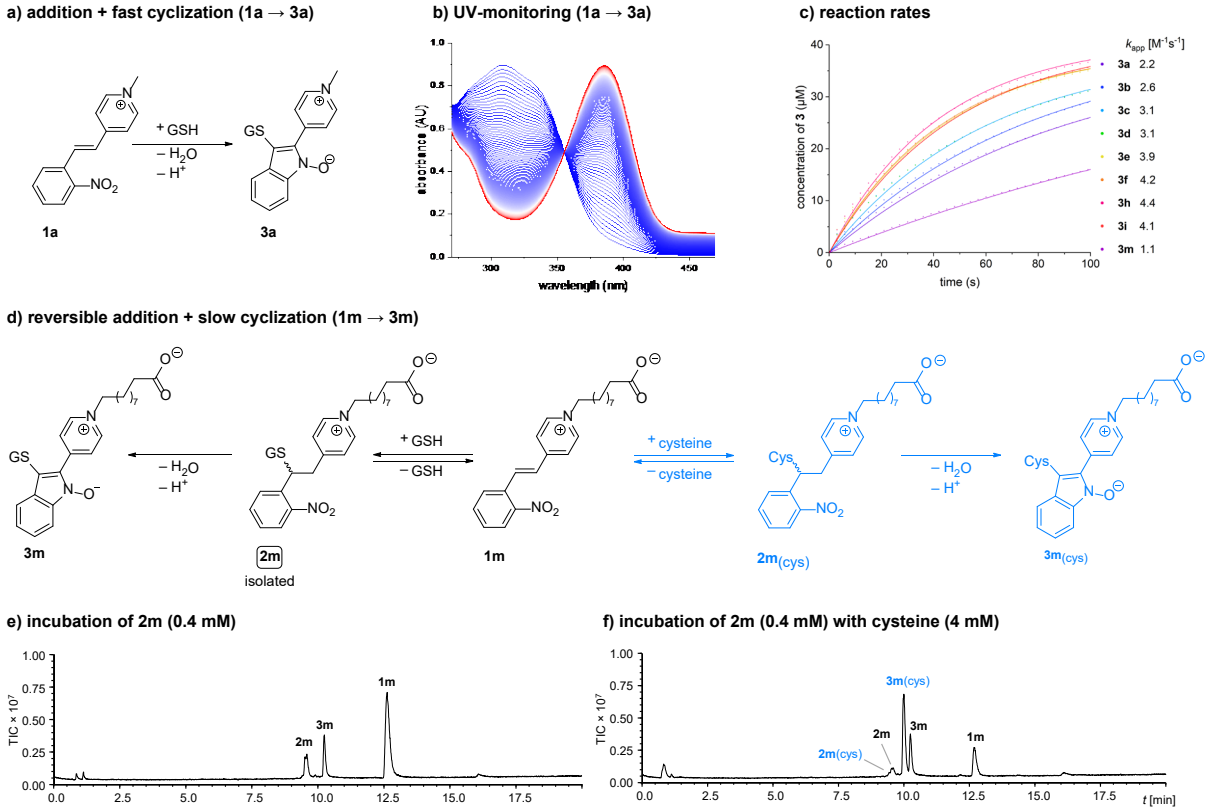


Figure 1. (a): Electrophiles **1a**-**1m** react with thiol nucleophiles under fast addition and even faster cyclization (b) time-resolved UV-VIS of the reaction mixture: **1a** (40 µM) and glutathione (4.0 mM), 0.1 M NH_4HCO_3 buffer (pH 8.0), scan from 270 nm to 470 nm every 3 s; (c): apparent second order rate constants for transformation of **1a – f**,**h**,**i**,**m** (40 µM) with glutathione (4.0 mM), 20 °C obtained by a linear fit of the early data-points of the UV-VIS data under pseudo-first order conditions; the lines show a first order fit to guide the eye. (d): Reversibility of thiol-ene addition for slow reacting electrophile **1m**; **2m** was isolated and re-exposed to the reaction buffer with or without cysteine (d) LC-MS (m/z = 100-1000) of **2m** (0.40 mM) in NH_4HCO_3 buffer (100 mM, pH 8.0), 37°C, 2h incubation; (e) LC-MS (m/z = 100-1000) of a mixture of **2m** (0.40 mM) and cysteine (4.0 mM) in NH_4HCO_3 buffer (100 mM, pH 8.0), 37°C, 4h incubation.

No sharp isosbestic point was observed for the reactions of electrophiles **1g, k, l** to products **3g, k, l**, respectively, possibly due to a reduced electrophilicity of the nitro group. When the reactions for slow reacting substrates **1g** and **1m** with GSH (1.2 eq.) were performed at higher substrate concentrations (4.0 mM) for preparative purposes, the intermediates **2g** and **2m**, respectively, could be detected by LC-MS. A larger scale reaction under these conditions allowed the isolation of intermediate **2m** by preparative HPLC in the presence of TFA. When **2m** was exposed to the reaction buffer, a mixture of **1m**,

2m and **3m** was observed by LC-MS after 2 hours (Fig. 1e). The addition of cysteine to **2m** led to competitive formation of the cysteine addition and cyclization product, **2m**(cys) and **3m**(cys), respectively (Figure 1f), confirming that the aromatization is indeed necessary to render the reaction irreversible.

The X-ray structure of the cyclized conjugate **6g** revealed the presence of a water molecule associated with the N-hydoxy group, hinting at a potential acidity (Fig 2).[15] Since the OH-group is part of the chromophore, the absorption spectrum of the cyclized conjugates was expected to be pH-dependent. A red-shift of 24 nm was observed for **6g** between pH 2 and 12 and the pK_a-value (pKa = 6.71) could be conveniently determined by UV-VIS-titration (see SI). In the reaction buffer (pH 8.0) 95% of the formed hydroxyindole **6g** is deprotonated.

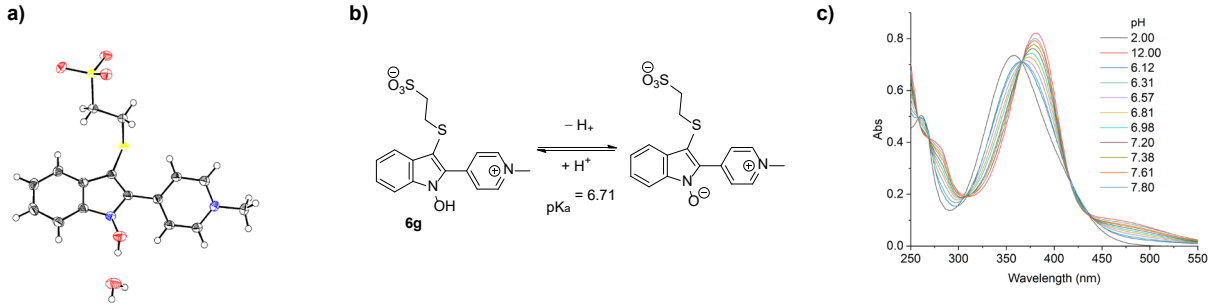


Figure 2. a) the X-ray structure of conjugate **6g** reveals an associated water molecule; CCDC ID 2278235 b) protonation equilibrium of conjugate **6g** c) pH-dependence of the absorption spectrum of conjugate **6g**.

To test the applicability of the NSP-motif, first a small set of peptides (4-16 AA) with a single cysteine residue were reacted with electrophile **1a** (Figure 3). The conjugates were isolated in good yields after preparative HPLC (75-80%) and had formed quantitatively after 30 minutes (**6c**, 4 amino acids; **6d**, 8 amino acids) or after 2 hours for longer peptides (**6e**,**f,** 12 and 16 amino acids respectively). Importantly, only singly modified products were observed, confirming that a potential cross-reactivity with lysines is of no concern under the conditions employed.

Next, bi- and trifunctional linkers (**4a**,**b**) for peptide stapling were prepared by reacting dibromopropane or 1,3,5-tri(bromomethyl)benzene with (*E*)-4-(2-nitrostyryl)pyridine, respectively. Peptides with two or three cysteine residues could be conveniently constrained into cyclic or bicyclic structures under moderately dilute conditions. The monocyclic constructs with ring sizes of 7 and 8 amino acids were isolated in good yields (⩾60 %). Bicyclic structures were prepared with 6,7- and 7,8-membered rings respectively in ⩾25% yield.

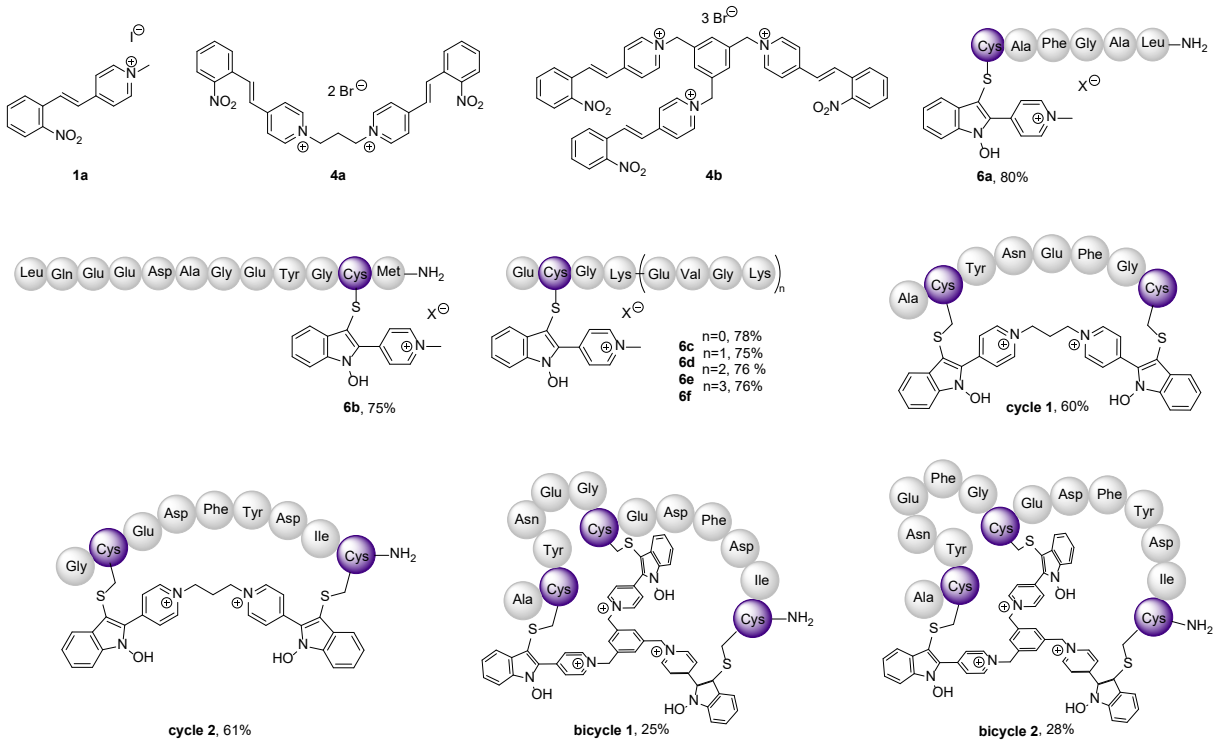


Figure 3: Selective modification of one, two or three sulfhydryl group in peptides with mono-, bi- and trifunctional 4-(2-nitrostyryl)pyridinium linkers. Conditions for the coupling reactions: peptides with a single cysteine residue: 2.0 mM **1a**, 1.5 equiv. peptide **5a-f**, 100 mM NH_4HCO_3 buffer (pH 8.0), 37°C, 0.5 - 2hrs; peptides with two cysteine residues: 0.50 mM **4a**, 1.0 equiv. peptide **5g**, **h**, 100 mM NH_4HCO_3 buffer (pH 8.0), rt, 1 hour; peptides with three cysteine residues: 0.20 mM **4b**, 1.0 equiv. peptide **5i**, **j**, 100 mM NH_4HCO_3 buffer (pH 8.0), 0°C→rt, 1 hour. Isolated yields; products purified by prep. HPLC.

We further tested the modification of bovine aprotinin (58 AA) where the three disulfide bonds had been reduced by addition of 6 eq. of TCEP. Direct addition of 20 eq. of **1a** to the reduced mixture without workup resulted in the hexa-modified polypeptide (see SI). The excess of **1a** served to compensate for competing addition of TCEP to **1a**.

Next, to probe NSPs for the selective modification of an unpaired cysteine on a protein, β-lactoglobulin A (BLG-A, 162 AA) that contains two disulfide bridges and an unpaired buried cysteine was selected. Despite being buried this single cysteine can be modified under mild conditions. Reagent **1a**, alkynyl-functionalized **5** and bifunctional **4a** were tested for modification at 37 °C and at pH 8.0: all reactions resulted in singly modified BLG-A within 2 hrs (Figure 4).

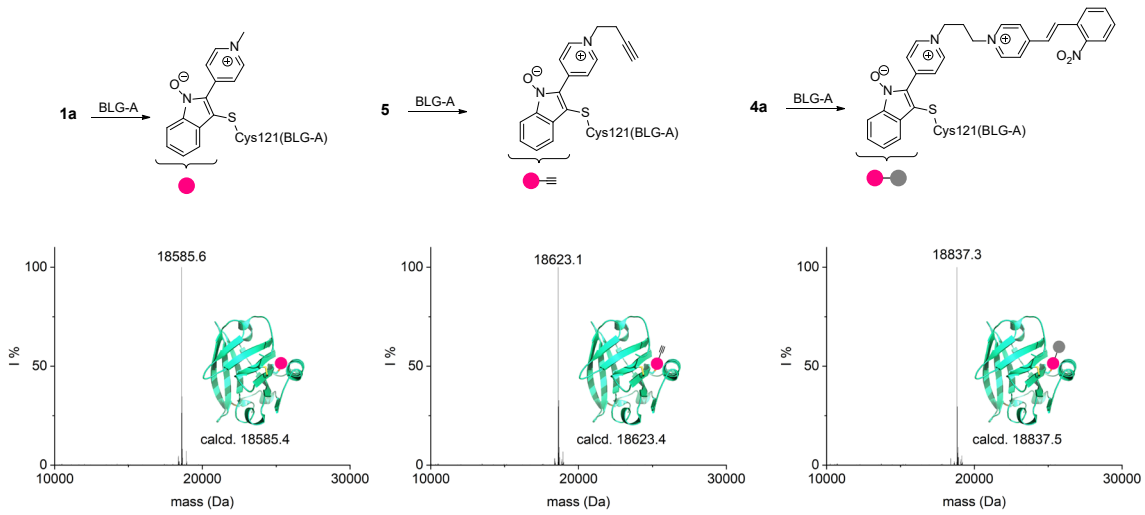


Figure 4. Modification of BLG-A with different NSPs (20 µM BLG-A, 10 eq. **1a** and **5**, 5 eq. **4a**, respectively). Deconvoluted mass spectra are shown.

Toward a pharmacologically relevant application, we equipped the cytotoxic Val-Cit-PAB-MMAE with the electrophilic NSP moiety. The resulting reactive drug-linker NSP-Val-Cit-PAB-MMAE was first tested in the reaction with glutathione. A similar reaction rate to **1a** (k_{app} = 1.9 $M^{-1}s^{-1}$) indicates that the larger payload does not impede the reaction rate (Val-Cit-PAB-MMAE vs. methyl in **1a**). Importantly the GSH conjugate (0.20 mM) showed only minimal decomposition after 7 days of incubation at 37 °C and pH 8.0 in the presence of 2.0 mM cysteine according to LC-MS and LC-UV analysis. This contrasts with observations made with the commercial maleimide drug-linker MC-Val-Cit-PAB-MMAE (Fig. 5a,b), where we observed ~ 60% exchange with cysteine and ~ 40% hydrolysis of the GSH-conjugate after 3 days under identical conditions (see SI). No hydrolysis product of the formed cysteine-conjugate of MC-Val-Cit-PAB-MMAE could be detected, indicating that not only the cargo but also the conjugated peptide can affect the rate of hydrolysis.[9b]

Encouraged by these results, we set out to modify the widely applied anti-HER2 antibody trastuzumab. Interchain disulfide bonds were reduced with TCEP and the mixture subsequently treated with 2.5 eq. of NSP-MMAE per free thiol (4 µM trastuzumab, 40 µM TCEP, 37°, 90 min, followed by 80 µM NSP-MMAE, 3 hrs; 3 days dialysis at room temperature). HRMS showed, as expected, the singly modified light chain and the triply modified heavy chain. Additional signals of the heavy chain are due to heterogeneous glycosylation commonly observed in commercial trastuzumab.[16]

The red-shifted absorption spectrum of the conjugate allows for a simple estimation of the drug to antibody ratio by comparing the absorption at 280 nm and 392 nm resulting in a value of DAR = 7.3 under not further optimized conditions. A small signal for the doubly modified half-antibody indicates that the DAR < 8.0 might be caused by incomplete TCEP reduction (Fig. 5).

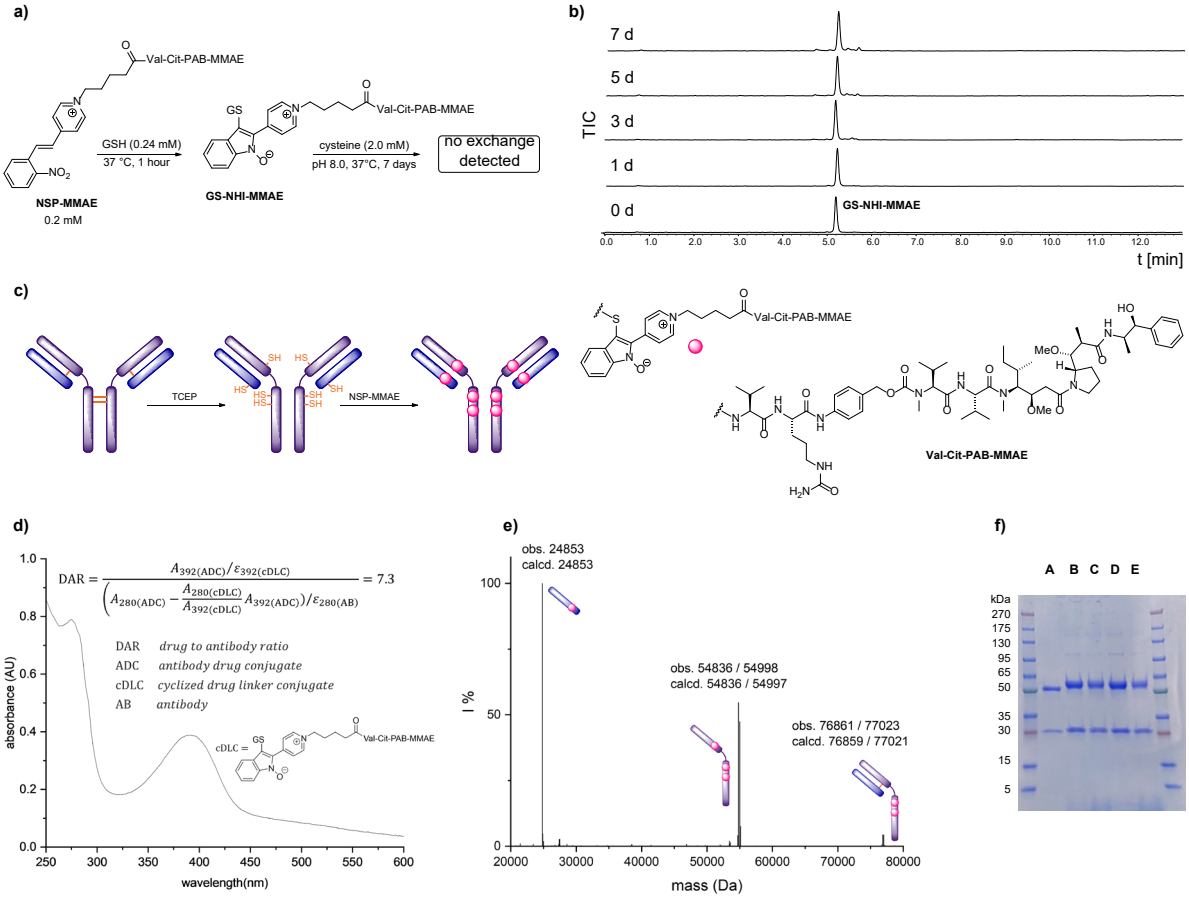


Figure 5. a) The GSH conjugate of the NSP-MMAE linker does not exchange with cysteine and shows superior stability. b) Stability test of incubated GS-NHI-MMAE by LC-MS. c) Conjugation of NSP-MMAE with trastuzumab. d) Determination of DAR by UV-VIS. e) Deconvoluted HRMS of the trastuzumab NSP-MMAE conjugates. f) SDS-PAGE of reduced trastuzumab after reaction with NSP-MMAE, A: no NSP-MMAE; B: 20 eq. NSP-MMAE; 3 h incubation; 3 days of dialysis at room temperature; C: 20 eq., 12 h incubation; D: 40 eq., 3 h incubation; E: 40 equiv, 12 h incubation; samples were heated to 95°C for 10 minutes before they were applied to the gel.

Conclusion:

In summary we present a versatile and readily accessible electrophilic moiety for aqueous cysteine conjugation that allows facile monitoring of the reaction progress by virtue of its bathochromic properties and whose conjugates show superior stability to thiol exchange. The chromogenic reaction proved to be an effective method for the determination of the reaction rate constants and the conjugation yield as exemplified in the drug-to-antibody ratio (DAR) of an antibody-drug conjugate (ADC). Moreover, a synthesized drug conjugate (GS-NHI-MMAE) displayed excellent stability, even when confronted with an excess of free thiol groups. These features collectively make Click & Lock an appealing alternative to existing methods for cysteine conjugation and open new possibilities in the field of protein modification and bioconjugation strategies.

Acknowledgements: This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreements #860713. We acknowledge funding by the Gordon & Betty Moore foundation under grant agreement #10771. We thank Michael Pfeffer for the HRMS analysis and Daniel Häussinger for advice on NMR spectroscopy.

Notes: The authors declare no competing financial interest.

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