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NSPs: chromogenic linkers for fast, selective, and irreversible cysteine modification†

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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–C sp²-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 $\geq 2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. 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 in human plasma, fast reaction rates (k_{app} up to $4.4 \text{ M}^{-1} \text{ s}^{-1}$ at 20 °C), high selectivity for cysteine, favorable solubility of the electrophilic moiety and the bathochromic properties of the Click & Lock reaction provide an appealing alternative to existing methods for cysteine conjugation.

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Protein and peptide modification by chemical means serves the study and tracking of biomolecules,^{1,2} the exploration of the proteome,^{3–6} target-selective drug delivery,^{7–9} the improvement of the pharmacokinetic properties of protein drugs,^{10,11} and the immobilization of enzymes,¹² to name a few.^{13–23} The increasing market share of biologicals acts as an additional incentive in this industrious area of research.^{16,17,22,24,25}

Residues targeted for protein modification are most commonly cysteines and lysines. Cysteines are particularly attractive due to their relatively low abundance, the high nucleophilicity of surface-exposed sulfhydryl groups at moderate pH values and the diverse chemistry that can be realized.^{13–15} Reactive cysteines can be introduced recombinantly²⁶ or made accessible by cleavage of intramolecular disulfide bonds under mild reductive conditions. Thiols also serve as attractive nucleophiles in materials and polymer science.^{27–32}

Traditional approaches for thiol conjugation utilize maleimides and haloacetamides as electrophilic moieties and their widespread application continues.³³ For example, the majority of FDA-approved antibody-drug-conjugates are loaded with their drug cargo through a maleimide linker.^{34,35}

Recent examples include new designs for disulfide rebridging electrophiles,^{36–38} tunable equilibrium constants for conjugation,²⁸ and electrophiles that show particularly high reaction rates.^{39,40} Other conjugates can be cleaved⁴¹ or locked on demand,^{36,42} or provide additional functionality for further elaboration.^{43–47} High stability⁴⁸ and excellent pharmacokinetic profiles⁴⁹ have been demonstrated. The introduction of TM-mediated cross-coupling methods has diversified the motifs that can be directly coupled to sulfur further.^{22,50–52} Despite these advances, the search for valuable conjugation techniques continues since a variety of complementary properties is desired.^{53–56}

Inspired by vinyl-pyridinium electrophiles⁵⁷ and the possibility to extend the aromatic system of *o*-nitroarylethane derivatives and related compounds⁵⁸ by intramolecular condensation,^{59,60} 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 can subsequently form the *N*-hydroxyindole product under elimination of water. The reaction enables direct UV-vis spectroscopic monitoring of the conjugation progress due to the accompanying bathochromic shift. This feature can serve to conveniently estimate reaction rates, determine conjugation yields and estimate the availability of free thiols without the need for additional chromophores in the attached cargo or chromatographic analysis. In contrast to Ellman's

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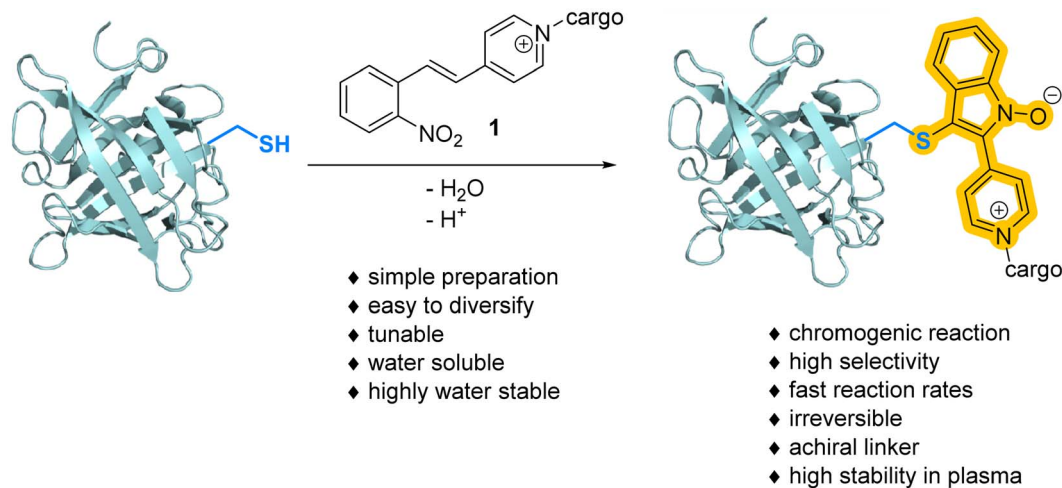
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Scheme 1 The reaction of a cysteine sulphydryl group with a nitrostyryl electrophile results in an irreversible bionconjugation that is accompanied by a large bathochromic shift.

reagent,⁶¹ the chromogenic unit doubles as the functional linker and is directly attached to the protein of interest.

Results and discussion

In the initial set of experiments we employed glutathione as the nucleophile and a simple methyl group 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 [NH₄][HCO₃] (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; while electron-withdrawing substituents in the 4-position (**1b–1f**), accelerated the reaction, it was slowed down by electron-donating substituents (**1g**, **1j** and **1l**, 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. Possibly, limited solubility and mass transfer play a role for the lower reaction rate observed with **3m**.

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 NH₄HCO₃ buffer (pH 8.0) was recorded in the wavelength range from 270–470 nm every 3 seconds at 20 °C (Fig. 1). The decreasing absorption of **1a** and the increasing absorption of **3a** in the course of the reaction resulted in a well-defined

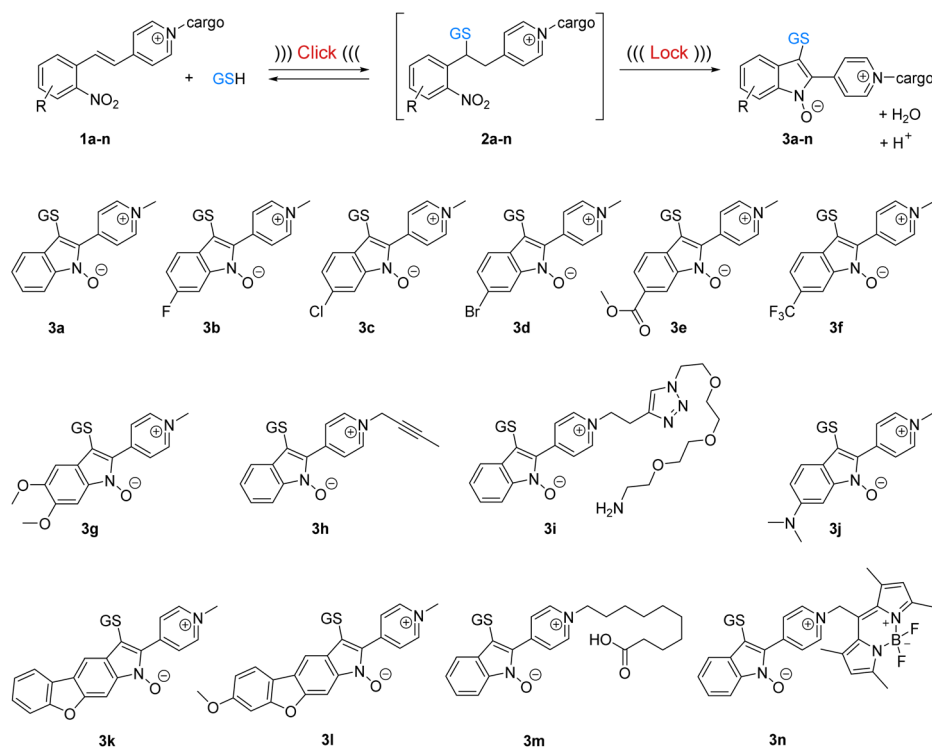
isosbestic point at 355 nm. Its sharp definition indicates that the cyclization (the 'lock') is indeed very fast since the presence of underlying intermediates in significant concentrations can be excluded. The lower limits for the apparent second order rate constants obtained by the method of initial rates range from 1.1 M⁻¹ s⁻¹ for **3m** to 4.4 M⁻¹ s⁻¹ for **3i** (Table 1 and Fig. 1).

No sharp isosbestic point was observed for the reactions of electrophiles **1g–1l** to products **3g–3l**, 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 (Fig. 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*-hydroxy group, hinting at a potential acidity (Fig. 2).^{62,63} 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 (pK_a = 6.71) could be conveniently determined by UV-vis-titration (see ESI†). In the reaction buffer (pH 8.0) 95% of the formed hydroxyindole **6g** is deprotonated.

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** (Fig. 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**, **6f**, 12 and 16 amino acids respectively). Importantly, only singly modified



Table 1 Click & Lock reaction between glutathione (GSH) and various 4-(2-nitrostyryl)pyridinium ions **1a–1n**^a

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} ^d (M ⁻¹ s ⁻¹)
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% (4 h) ^{ef}	312	422	2.2	—
3h	>99%	316	391	2.0	4.4
3i	>99%	324	395	2.3	4.1
3j	Trace (24 h)	—	—	—	—
3k	>99 ^e %	320	405	2.3	—
3l	90% (4 h) ^{ef}	332	413	2.1	—
3m	>99 ^e %	309	387	2.3	1.1
3n	53% (4 h) ^g	525	524	6.1	—

^a 0.40 mM (**1a–1n**), 1.2 equiv. glutathione, 0.1 M NH₄HCO₃ buffer (pH 8.0)/DMF = 9 : 1, 1 h, 37 °C. ^b Determined by LC-MS and LC-UV. Consistent with UV-vis conversions. ^c In 0.1 M NH₄HCO₃ buffer (pH 8.0). ^d k_{app} was determined at 40 μ M **1a–1i**, **1k–1m** in the presence of 4.0 mM glutathione at 20 °C in 0.1 M NH₄HCO₃ buffer (pH 8.0) containing 1% DMF. ^e 0.20 mM **1g**, **1l**, **1m**, 2.4 equiv. glutathione. ^f Conversion determined by UV-vis. ^g Isolated yield; DMF was replaced by MeOH, bodipy is the dominant chromophore. Isolated yields for all compounds (except **3j**) are reported in the ESI. Preparative reactions were carried at room temperature, at higher concentrations (2–4 mM) in buffer/DMF = 5 : 1 (except **3n**, see ESI for further details).

products were observed, confirming that a potential cross-reactivity with lysine is of no concern under the conditions employed. We further scrutinized this conclusion by an attempted reaction of **3a** with lysine itself where no reaction was observed (see ESI†).

Next, bi- and trifunctional linkers (**4a**, **4b**) 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 ($\geq 60\%$). Bicyclic structures were prepared with 6,7- and 7,8-membered rings respectively in $\geq 25\%$ yield.

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



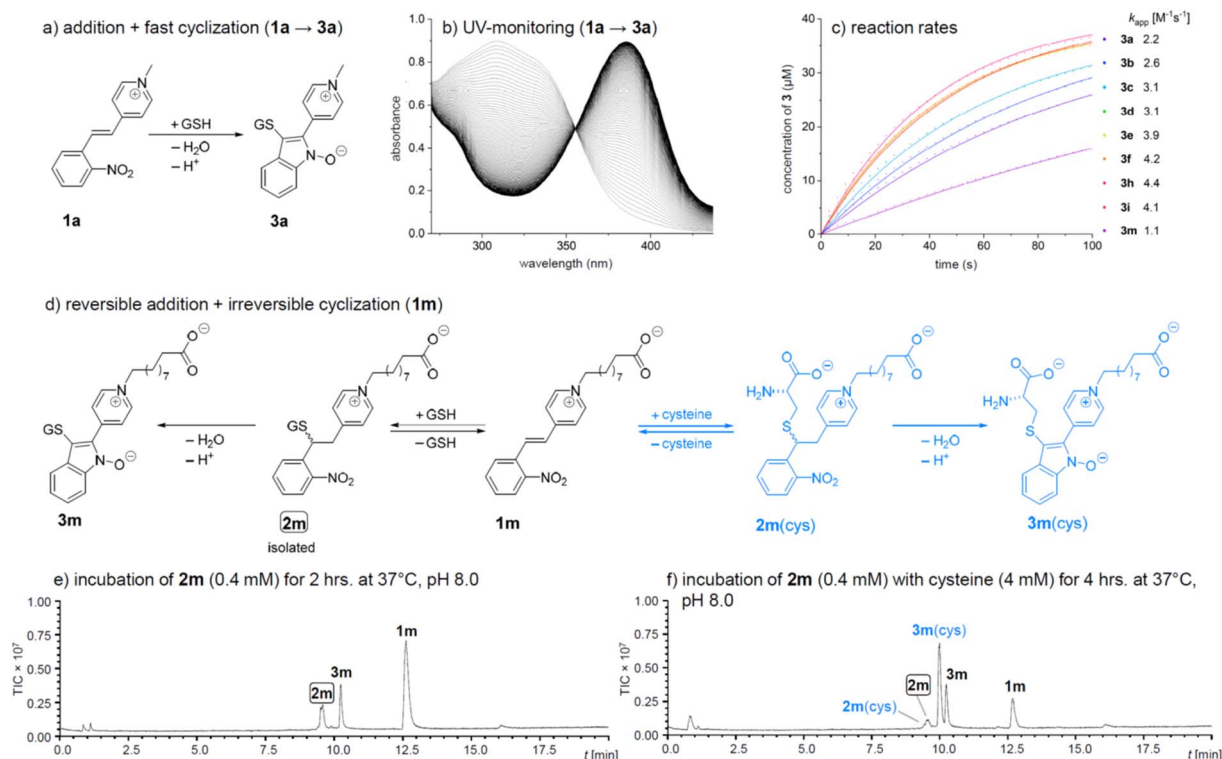


Fig. 1 (a) Electrophiles of type **1** react with thiol nucleophiles under fast addition and even faster cyclization (b) time-resolved UV-vis of the reaction mixture: **1a** (40 μM) and glutathione (4.0 mM), 0.1 M NH₄HCO₃ buffer (pH 8.0), scan from 270 nm to 470 nm every 3 s; (c) apparent second order rate constants for transformation of **1a–1f**, **1h**, **1i**, **1m** (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-Michael addition for slow reacting electrophile **1m**; **2m** was isolated and re-exposed to the reaction buffer with or without cysteine (e) LC-MS ($m/z = 100–1000$) of **2m** (0.40 mM) in NH₄HCO₃ buffer (100 mM, pH 8.0), 37 °C, 2 h incubation; (f) LC-MS ($m/z = 100–1000$) of a mixture of **2m** (0.40 mM) and cysteine (4.0 mM) in NH₄HCO₃ buffer (100 mM, pH 8.0), 37 °C, 4 h incubation.

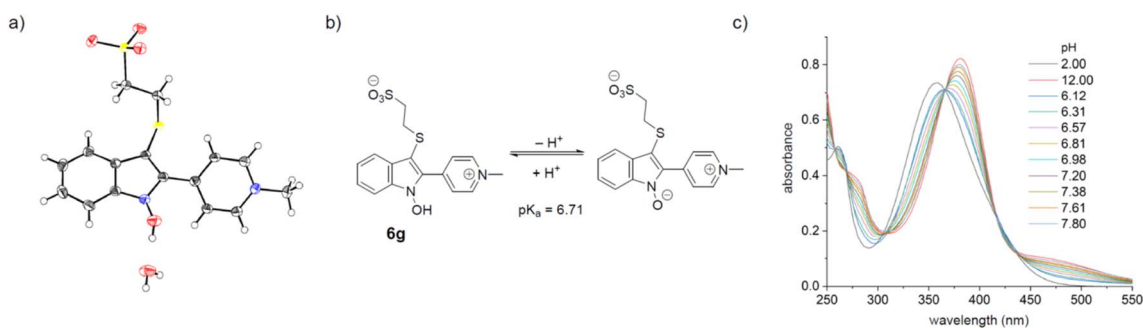


Fig. 2 (a) The X-ray structure of conjugate **6g** reveals an associated water molecule; CCDC ID 2278235 (b) protonation equilibrium of conjugate **6g** (c) pH-dependence of the absorption spectrum of conjugate **6g**.

polypeptide (see ESI[†]). 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 h (Fig. 4).

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** ($k_{app} = 1.9 \text{ M}^{-1} \text{ s}^{-1}$) 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 (Fig. 5b). This contrasts



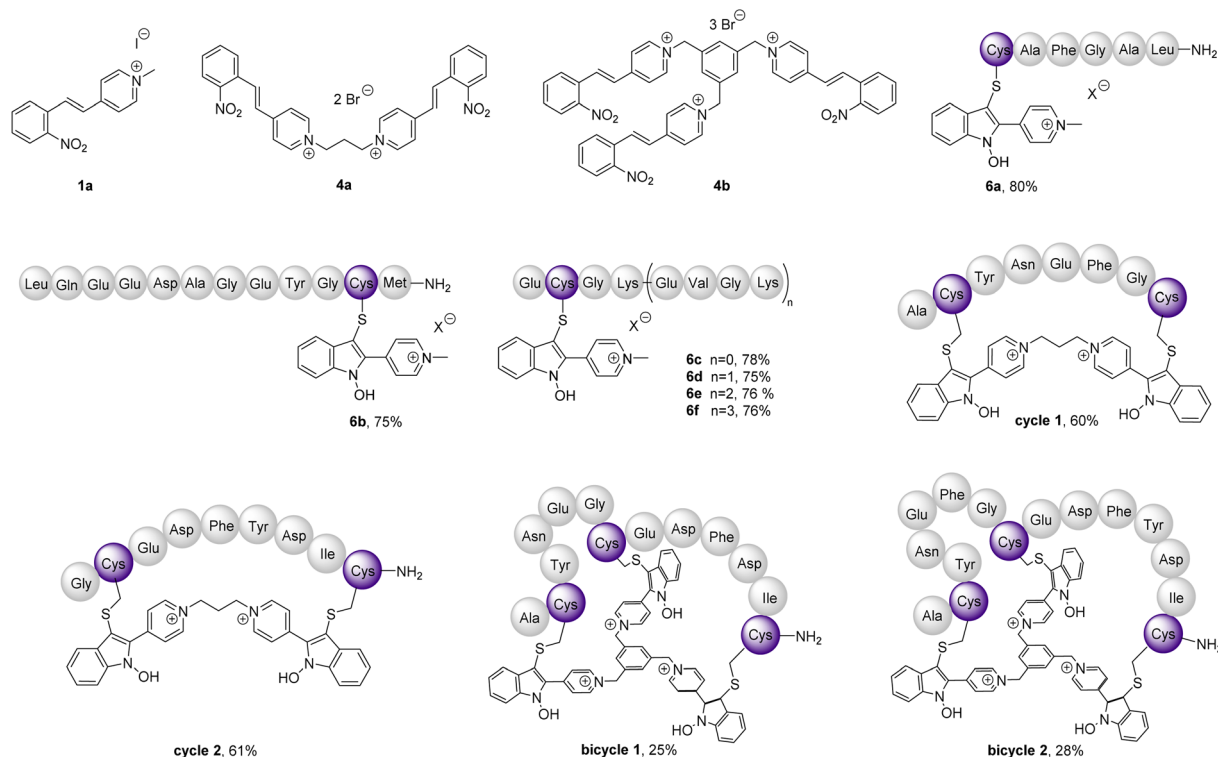


Fig. 3 Selective modification of one, two or three sulfhydryl groups in peptides with mono-, bi- and trifunctional 4-(2-nitrophenyl)pyridinium linkers. Conditions for the coupling reactions: peptides with a single cysteine residue: 2.0 mM **1a**, 1.5 equiv. peptide **5a–5f**, 100 mM NH_4HCO_3 buffer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

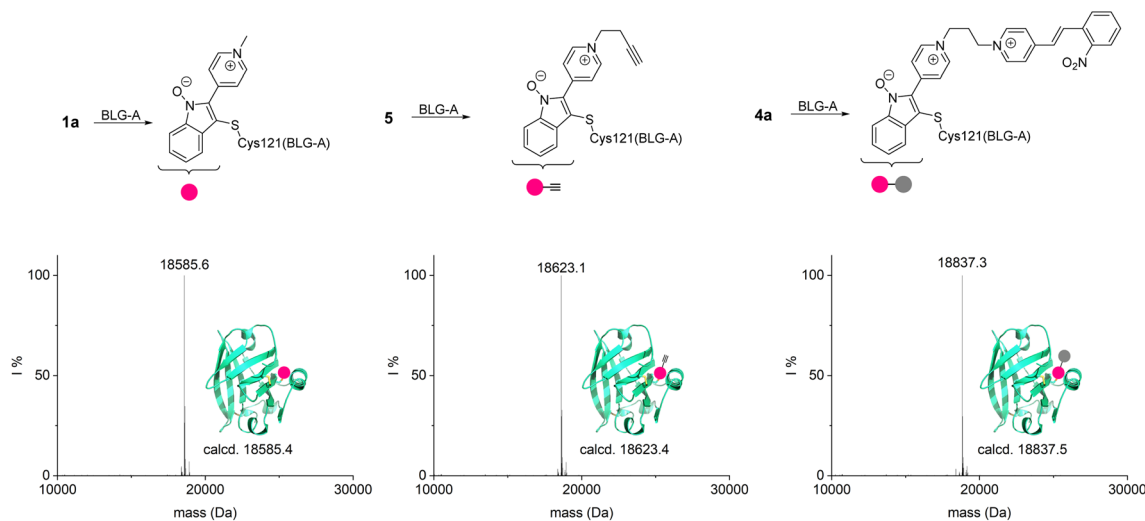


Fig. 4 Modification of BLG-A with different NSPs (20 μM BLG-A, 10 eq. **1a** and **5**, 5 eq. **4a**, respectively). Deconvoluted mass spectra are shown.

with observations made with the commercial maleimide drug-linker MC-Val-Cit-PAB-MMAE (see ESI[†]).⁶⁴

We further tested the stability of the glutathione conjugates **3a**, **3f**, **3g** in human plasma (pooled human plasma (blood-derived) K2 EDTA, Loxo GmbH). Within 48 hours more than half of the species with an intact conjugated-*N*-hydroxy indole pyridinium unit remained. Identified decomposition products

stemmed from cleavage of the glutamate–cysteine amide bond in the glutathione moiety (49% for **3a**, 56% for **3f**, and 12% for **3g**, see ESI[†]).

The high stability of the conjugated *N*-hydroxy indole pyridinium unit against thiol exchange and in human plasma illustrates the superior applicability of the conjugation method.



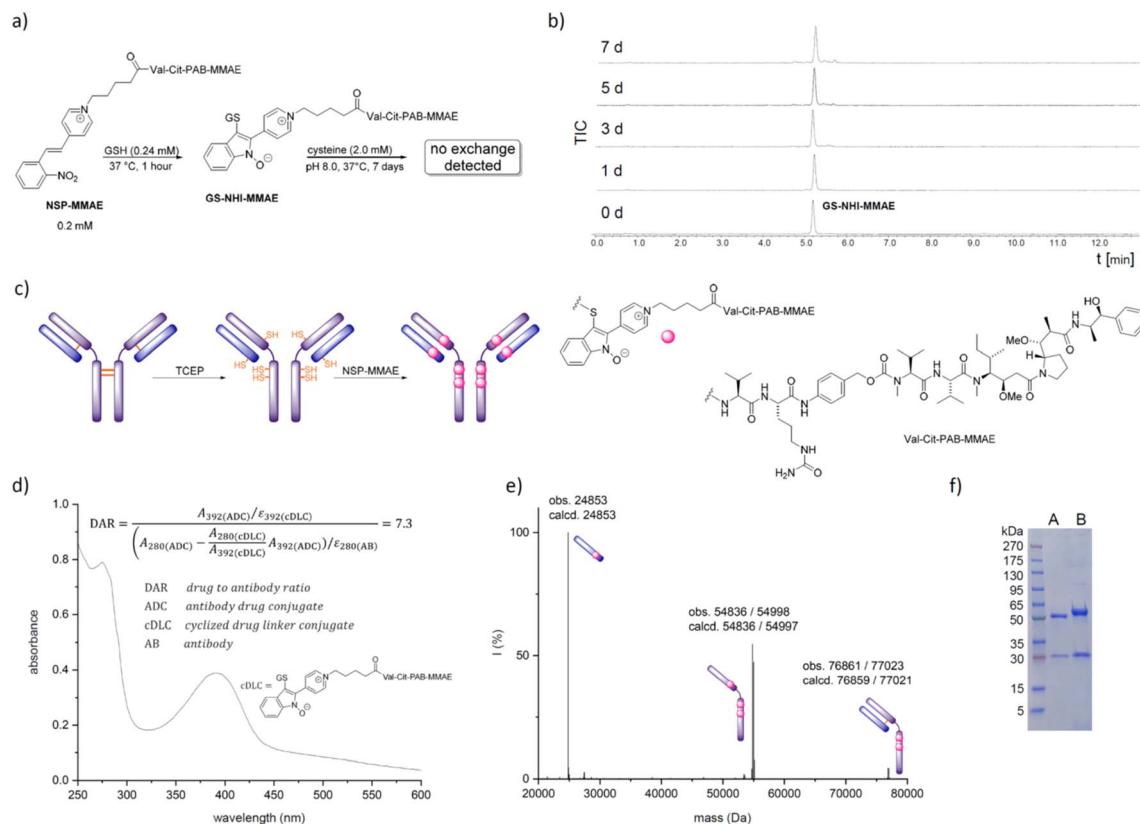


Fig. 5 (a) The GSH conjugate of the NSP-MMAE linker does not exchange with cysteine and shows superior stability. (b) Stability test of incubated GS-NHI-MMAE by LC-MS. (c) Conjugation of NSP-MMAE with trastuzumab. (d) Determination of DAR by UV-vis. (e) Deconvoluted HRMS of the trastuzumab NSP-MMAE conjugates. (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; samples were heated to 95 °C for 10 minutes before they were applied to the gel.

Encouraged by these results, we modified 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 μ M trastuzumab, 40 μ M TCEP, 37°, 90 min, followed by 80 μ M NSP-MMAE, 3 h; 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.⁶⁵

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. 5e).

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 thiol *N*-hydroxy indole

pyridinium unit furthermore showed half-lives of >48 hours in human plasma. The chromogenic reaction proved to be an effective method for the determination of the reaction rate constants, the concentration of free sulfhydryl residues in the reaction mixture 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.

Data availability

The data is included in the ESI.†

Author contributions

Conceptualization: Y. Hua; formal analysis: Y. Hua, V. Köhler; funding acquisition: M. Mayor, V. Köhler, T. R. Ward; investigation: Y. Hua, Z. Zou, A. Prescimone; methodology: Y. Hua; project administration: M. Mayor, V. Köhler; resources: M. Mayor, T. R. Ward, V. Köhler; supervision: V. Köhler, M. Mayor, T. R. Ward; validation: Y. Hua, V. Köhler; visualization: Y.



Hua, V. Köhler, Z. Zou, A. Prescimone; writing – original draft: Y. Hua, V. Köhler; writing – review & editing: Y. Hua, V. Köhler, T. R. Ward, M. Mayor.

Conflicts of interest

The authors declare no competing financial interest.

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References

- B. N. G. Giepmans, S. R. Adams, M. H. Ellisman and R. Y. Tsien, *Science*, 2006, **312**, 217–224.
- L. Wang, M. S. Frei, A. Salim and K. Johnsson, *J. Am. Chem. Soc.*, 2019, **141**, 2770–2781.
- E. Weerapana, C. Wang, G. M. Simon, F. Richter, S. Khare, M. B. Dillon, D. A. Bachovchin, K. Mowen, D. Baker and B. F. Cravatt, *Nature*, 2010, **468**, 790–795.
- K. M. Backus, B. E. Correia, K. M. Lum, S. Forli, B. D. Horning, G. E. González-Páez, S. Chatterjee, B. R. Lanning, J. R. Teijaro, A. J. Olson, D. W. Wolan and B. F. Cravatt, *Nature*, 2016, **534**, 570–574.
- Y. Liao, S. Chin Chan, E. A. Welsh, B. Fang, L. Sun, E. Schonbrunn, J. M. Koomen, D. R. Duckett, E. B. Haura, A. Monastyrskyi and U. Rix, *ACS Chem. Biol.*, 2023, **18**, 251–264.
- K. C. Tang, S. M. Maddox, K. M. Backus and M. Raj, *Chem. Sci.*, 2022, **13**, 763–774.
- A. Beck, L. Goetsch, C. Dumontet and N. Corvaia, *Nat. Rev. Drug Discovery*, 2017, **16**, 315–337.
- M. Fani, H. R. Maecke and S. M. Okarvi, *Theranostics*, 2012, **2**, 481–501.
- S. J. Walsh, J. D. Bargh, F. M. Dannheim, A. R. Hanby, H. Seki, A. J. Counsell, X. Ou, E. Fowler, N. Ashman, Y. Takada, A. Isidro-Llobet, J. S. Parker, J. S. Carroll and D. R. Spring, *Chem. Soc. Rev.*, 2021, **50**, 1305–1353.
- J. M. Horn and A. C. Obermeyer, *Biomacromolecules*, 2021, **22**, 4883–4904.
- P. Bailon and C.-Y. Won, *Expert Opin. Drug Delivery*, 2009, **6**, 1–16.
- C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2007, **40**, 1451–1463.
- J. M. Chalker, G. J. Bernardes, Y. A. Lin and B. G. Davis, *Chem.-Asian J.*, 2009, **4**, 630–640.
- S. B. Gunnoo and A. Madder, *ChemBioChem*, 2016, **17**, 529–553.
- P. Ochtrop and C. P. R. Hackenberger, *Curr. Opin. Chem. Biol.*, 2020, **58**, 28–36.
- O. Boutureira and G. J. L. Bernardes, *Chem. Rev.*, 2015, **115**, 2174–2195.
- N. Krall, F. P. da Cruz, O. Boutureira and G. J. L. Bernardes, *Nat. Chem.*, 2016, **8**, 103–113.
- N. Stephanopoulos and M. B. Francis, *Nat. Chem. Biol.*, 2011, **7**, 876–884.
- L. Taiariol, C. Chaix, C. Farre and E. Moreau, *Chem. Rev.*, 2022, **122**, 340–384.
- E. D. Holz, M. Zou, D. B. Tesar and W. Shatz-Binder, *Pharmaceutics*, 2023, **15**, 1–54.
- P. G. Isenegger and B. G. Davis, *J. Am. Chem. Soc.*, 2019, **141**, 8005–8013.
- J. Ohata, S. C. Martin and Z. T. Ball, *Angew. Chem., Int. Ed.*, 2019, **58**, 6176–6199.
- C. S. Wu and L. Cheng, *ChemBioChem*, 2023, **24**, e202200468.
- N. L. Kjaersgaard, T. B. Nielsen and K. V. Gothelf, *ChemBioChem*, 2022, **23**, e202200245.
- C. Sornay, V. Vaur, A. Wagner and G. Chaubet, *R. Soc. Open Sci.*, 2022, **9**, 211563.
- R. Ohri, S. Bhakta, A. Fourie-O'Donohue, J. dela Cruz-Chuh, S. P. Tsai, R. Cook, B. Wei, C. Ng, A. W. Wong, A. B. Bos, F. Farahi, J. Bhakta, T. H. Pillow, H. Raab, R. Vandlen, P. Polakis, Y. Liu, H. Erickson, J. R. Junutula and K. R. Kozak, *Bioconjugate Chem.*, 2018, **29**, 473–485.
- C. Bednarek, U. Schepers, F. Thomas and S. Bräse, *Adv. Funct. Mater.*, 2024, **34**, 2303613.
- A. E. Crolais, N. D. Dolinski, N. R. Boynton, J. M. Radhakrishnan, S. A. Snyder and S. J. Rowan, *J. Am. Chem. Soc.*, 2023, **145**, 14427–14434.
- A. B. Lowe, *Polym. Chem.*, 2010, **1**, 17–36.
- M. H. Stenzel, *ACS Macro Lett.*, 2013, **2**, 14–18.
- D. P. Nair, M. Podgórski, S. Chatani, T. Gong, W. Xi, C. R. Fenoli and C. N. Bowman, *Chem. Mater.*, 2014, **26**, 724–744.
- C. Bahou, R. J. Spears, A. M. Ramírez Rosales, L. N. C. Rochet, L. J. Barber, K. S. Stankevich, J. F. Miranda, T. C. Butcher, A. M. Kerrigan, V. K. Lazarov, W. Grey, V. Chudasama and C. D. Spicer, *Biomacromolecules*, 2023, **24**, 4646–4652.
- G. T. Hermanson, in *Bioconjugate Techniques*, ed. G. T. Hermanson, Academic Press, Boston, 3rd edn, 2013, pp. 229–258.
- C. S. B. Chia, *ChemistryOpen*, 2022, **17**, e202200032.
- J. You, J. Zhang, J. Wang and M. Jin, *Bioconjugate Chem.*, 2021, **32**, 1525–1534.
- A. M. Tallon, Y. Xu, G. M. West, C. W. am Ende and J. M. Fox, *J. Am. Chem. Soc.*, 2023, **145**, 16069–16080.
- F. M. Dannheim, S. J. Walsh, C. T. Orozco, A. H. Hansen, J. D. Bargh, S. E. Jackson, N. J. Bond, J. S. Parker, J. S. Carroll and D. R. Spring, *Chem. Sci.*, 2022, **13**, 8781–8790.
- L. Xu, M. J. S. A. Silva, P. M. P. Gois, S. L. Kuan and T. Weil, *Chem. Sci.*, 2021, **12**, 13321–13330.



- 39 B. M. Lipka, D. S. Honeycutt, G. M. Bassett, T. N. Kowal, M. Adameczyk, Z. C. Cartnick, V. M. Betti, J. M. Goldberg and F. Wang, *J. Am. Chem. Soc.*, 2023, **145**, 23427–23432.
- 40 F. J. Chen and J. Gao, *Chem.–Eur. J.*, 2022, **28**, e202201843.
- 41 S. Maity, C. Bingham, W. Sheng, N. Ehyaei, D. Chakraborty, S. Tahmasebi-Nick, T. E. Kimmel, C. Vasileiou, J. H. Geiger and B. Borhan, *Analyst*, 2023, **148**, 1085–1092.
- 42 K. Gavriel, D. C. A. van Doeselaar, D. W. T. Geers and K. Neumann, *RSC Chem. Biol.*, 2023, **4**, 685–691.
- 43 I. Koutsopetras, A. K. Mishra, R. Benazza, O. Hernandez-Alba, S. Cianferani, G. Chaubet, S. Nicolai and J. Waser, *Chem.–Eur. J.*, 2023, **29**, e202302689.
- 44 M. Ahangarpour, I. Kavianinia, P. A. Hume, P. W. R. Harris and M. A. Brimble, *J. Am. Chem. Soc.*, 2022, **144**, 13652–13662.
- 45 C. F. Afonso, M. C. Marques, J. P. M. Antonio, C. Cordeiro, P. M. P. Gois, P. M. S. D. Cal and G. J. L. Bernardes, *Angew. Chem., Int. Ed.*, 2022, **61**, e202208543.
- 46 L. Xu, S. L. Kuan and T. Weil, *Angew. Chem., Int. Ed.*, 2021, **60**, 13757–13777.
- 47 S. Teng, Z. Zhang, B. Li, L. Li, M. C. L. Tan, Z. Jia and T.-P. Loh, *Angew. Chem., Int. Ed.*, 2023, **62**, e202311906.
- 48 I. N. Gober, R. Sharan and M. Villain, *J. Pept. Sci.*, 2023, **29**, e3495.
- 49 P. Ochtrop, J. Jahzerah, P. Machui, I. Mai, D. Schumacher, J. Helma, M.-A. Kasper and C. P. R. Hackenberger, *Chem. Sci.*, 2023, **14**, 2259–2266.
- 50 A. J. Rojas, J. M. Wolfe, H. H. Dhanjee, I. Buslov, N. L. Truex, R. Y. Liu, W. Masefski, B. L. Pentelute and S. L. Buchwald, *Chem. Sci.*, 2022, **13**, 11891–11895.
- 51 H. R. Montgomery, M. S. Messina, E. A. Doud, A. M. Spokoiny and H. D. Maynard, *Bioconjugate Chem.*, 2022, **33**, 1536–1542.
- 52 J. Rodriguez, H. H. Dhanjee, B. L. Pentelute and S. L. Buchwald, *J. Am. Chem. Soc.*, 2022, **144**, 11706–11712.
- 53 Z. Zhang, L. Li, H. Xu, C. K. Lee, Z. Jia and T. P. Loh, *J. Am. Chem. Soc.*, 2024, **146**, 1776–1782.
- 54 R. J. Spears and V. Chudasama, *Curr. Opin. Chem. Biol.*, 2023, **75**, 102306.
- 55 W.-Y. O, J.-F. Cui, Q. Yu, K. K.-Y. Kung, S.-F. Chung, Y.-C. Leung and M.-K. Wong, *Angew. Chem., Int. Ed.*, 2023, **62**, e202218038.
- 56 H. Seki, S. J. Walsh, J. D. Bargh, J. S. Parker, J. Carroll and D. R. Spring, *Chem. Sci.*, 2021, **12**, 9060–9068.
- 57 M. J. Matos, C. D. Navo, T. Hakala, X. Ferhati, A. Guerreiro, D. Hartmann, B. Bernardim, K. L. Saar, I. Compañón, F. Corzana, T. P. J. Knowles, G. Jiménez-Osés and G. J. L. Bernardes, *Angew. Chem., Int. Ed.*, 2019, **58**, 6640–6644.
- 58 J. Hanusek and V. Machacek, *Collect. Czech. Chem. Commun.*, 2009, **74**, 811–833.
- 59 Z. Wróbel and M. Mąkosza, *Synlett*, 1993, 597–598.
- 60 Z. Wrobel and M. Makosza, *Tetrahedron*, 1997, **53**, 5501–5514.
- 61 G. L. Ellman, *Arch. Biochem. Biophys.*, 1959, **82**, 70–77.
- 62 M. Somei, in *Advances in Heterocyclic Chemistry*, Academic Press, 2002, vol. 82, pp. 101–155.
- 63 E. E. Bykov, S. N. Lavrenov and M. N. Preobrazhenskaya, *Chem. Heterocycl. Compd.*, 2006, **42**, 42–44.
- 64 R. P. Lyon, J. R. Setter, T. D. Bovee, S. O. Doronina, J. H. Hunter, M. E. Anderson, C. L. Balasubramanian, S. M. Duniho, C. I. Leiske, F. Li and P. D. Senter, *Nat. Biotechnol.*, 2014, **32**, 1059–1062.
- 65 T. Li, X. Tong, Q. Yang, J. P. Giddens and L.-X. Wang, *J. Biol. Chem.*, 2016, **291**, 16508–16518.

