An ex situ gaseous reagent for multicomponent amine bioconjugation

Yuxuan Ding, Simon S. Pedersen, Yixian Wang, Han Xiao and Zachary T. Ball*

Department of Chemistry, Rice University, Houston, Texas 77005, United States. E-mail: zb1@rice. edu

Bioconjugation is a large field with many diverse goals, needs, and challenges, that requires a broad toolbox of fundamentally different synthetic approaches. As nucleophilic groups are prevalent in biomolecules, the ability to crosslink two nucleophilic sites offers an attractive approach to construct useful bioconjugates. New technologies for crosslinking with gaseous reagents and with minimal perturbation of natural structure could provide new ways to think about bioconjugation in complex environments. We report a minimalist gaseous sulfonyl chloride-derived reagent for multicomponent bioconjugation with amine, phenol, or aniline reagents to afford urea or carbamate products. In utilizing a gas-phase reagent for a reaction mediated by metal ions, a variety of biologically relevant molecules such as saccharide, PEG, fluorophore, and affinity tag can be efficiently crosslinked to the *N*-terminus or lysine side chain amines on natural polypeptides or proteins. The application of this method to the production of functional, modified proteins was demonstrated by fluorescence imaging of a cancer cell line and by the facile preparation of a peptide–protein conjugate.

Introduction

Chemical protein functionalization has become an indispensable tool for the alteration of protein structure and function.[1] Modified proteins are used in diverse applications, such as hybrid biologic therapeutics,[2] biomaterials synthesis,[3] and biological probe development,[4] that require diverse attributes and capabilities in an ideal bioconjugation reagent. As a result, the last decade has witnessed an explosion of bioconjugation methodologies.[5] Although a plethora of modern bioconjugation technologies have been reported,[6] including redox-based chemistry, cross-coupling, and proximity-driven chemistry,[7] electrophilic reagents that target nucleophilic sites on proteins are still dominant.[5]

Crosslinking two nucleophilic sites is an attractive approach to the preparation of bioconjugates.[8–12] Two-step elaboration of an existing residue to append an electrophile, followed by treatment with a second nucleophilic site is one common approach, but requires careful reagent design.[13–16] A bifunctional bis-electrophile reagent can be employed in a one-step process, with a suitable linker between reactive groups.[17–22] Avoiding multistep manipulations of complex biopolymers is a significant advantage. However, crosslinking selectivity remains an important issue, and thus, this type of method is dominated by the crosslinking of a cysteine thiol and a lysine or *N*-terminal amine.[8,15,17,20,22–26] Meanwhile, bifunctional linkers require significant chemical synthesis and may introduce changes to the biomacromolecule, such as increased hydrophobicity.

Crosslinking of two amines is an attractive alternative that allows reactivity at a common side chain (Figure 1). Amine–amine conjugation remains relatively rare due to the hetero-crosslinking issues.[19,27–29] Squaric acid diester was used as a linchpin reagent for stepwise coupling of two amino groups (Figure 1, a),[30,31] and *ortho*-phthalaldehyde allows a one-pot clamping of two different amines for bioconjugation (Figure 1, b).[32] However, new methods of amine–amine crosslinking would expand the bioconjugation toolbox and may provide new opportunities.

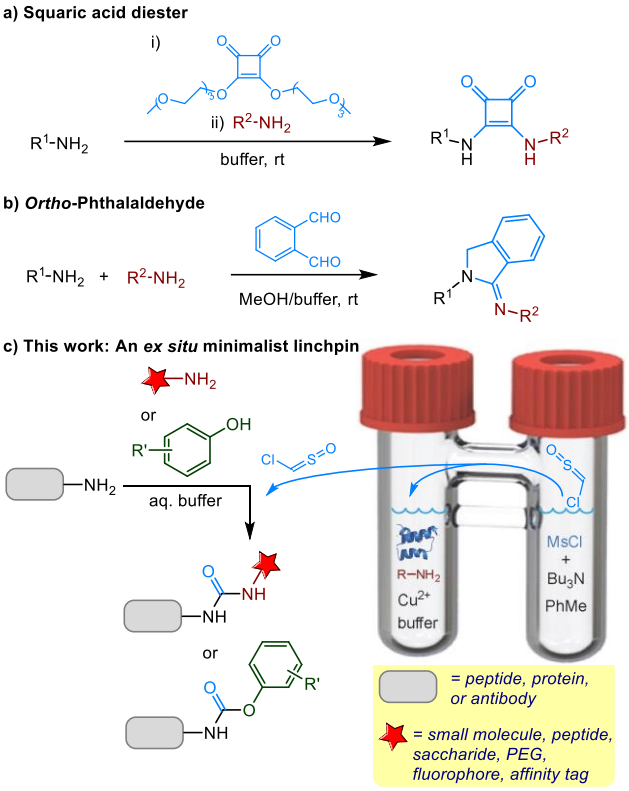

Figure 1. Linchpin reagents for amine–amine conjugation.

As part of a program to develop non-traditional bioconjugation methods,[13,33–36] we have recently reported a peptide macrocyclization induced by a chlorosulfine gas, produced *ex situ* from base-induced elimination-disproportionation of methanesulfonyl chloride.[37] Gaseous reagents for bioconjugation are little studied, despite potential advantages, including diffusions/penetration into reaction in complex tissues and porous materials. Herein, we present a copper-mediated linchpin bioconjugation reaction with gaseous chlorosulfine, generated *ex situ* from an elimination/disproportionation process of methanesulfonyl chloride. The reaction acts as a minimalist linchpin reagent that achieves multicomponent coupling[19,38–41] of external

nucleophiles with amine side chains of peptides and proteins (Figure 1, c). While the formation and reactivity of some sulfene or sulfine intermediates have been studied,[42] the application of these species to reaction in complex polyfunctional contexts, including bioconjugation has largely been unexplored.[37]

Results and discussion

We discovered[37] this unique reactivity when investigating peptide modification via carbonylative coupling with carbon monoxide,[35] which was generated in a two-chamber reactor approach for efficient and safe *ex situ* production of CO.[43] When CO was produced from formic acid and methanesulfonyl chloride with triethylamine,[44] bioconjugation reactions of amine reagents persisted even in negative control experiments without formic acid.[37] A brief optimization (Table S2) led to conditions with MsCl and tributylamine in one (releasing) chamber and an aqueous-phase in the second (reaction) chamber of bradykinin **1** and propargylamine **2a** in the presence of $Cu(OAc)_2$,which gave the *N*-terminal urea product **3a** (Figure 1c and Figure 2). The structure of **3a** was confirmed by LC-MS/MS fragmentation and NMR analysis of purified product (Figure S54-S59).

We next sought to examine the scope and efficiency of chlorosulfine-mediated multicomponent coupling for intermolecular reactivity. Using bradykinin **1** as a model, we screened a series of amines (Figure 2). A variety of primary amines (**2a**-**2d**) gave corresponding urea products (**3a**-**3d**) in moderate to high yields. The crosslinking of bradykinin with biologically relevant amines, such as saccharide **2e**, PEG **2f**-**2h**, alkyne **2g**, azide **2h**, fluorophore **2i**, and desthiobiotin tag **2j**-containing amines, were also successful. Most secondary amines (**2k**, **2l**, **2n**) were significantly less efficient. To our surprise, a variety of anilines (**2o**-**2t**) were successfully employed in this reaction, resulting in the corresponding urea products (**3o**-**3t**), despite their dramatically lower nucleophilicity. Anilines with strong electron-withdrawing groups (**2u**-**2v**) provided little to no products. Phenol reagents were also compatible, affording carbamate products (**2w**-**2ab**). No products were observed with benzyl alcohol **2ac** or thiophenol **2ad**.

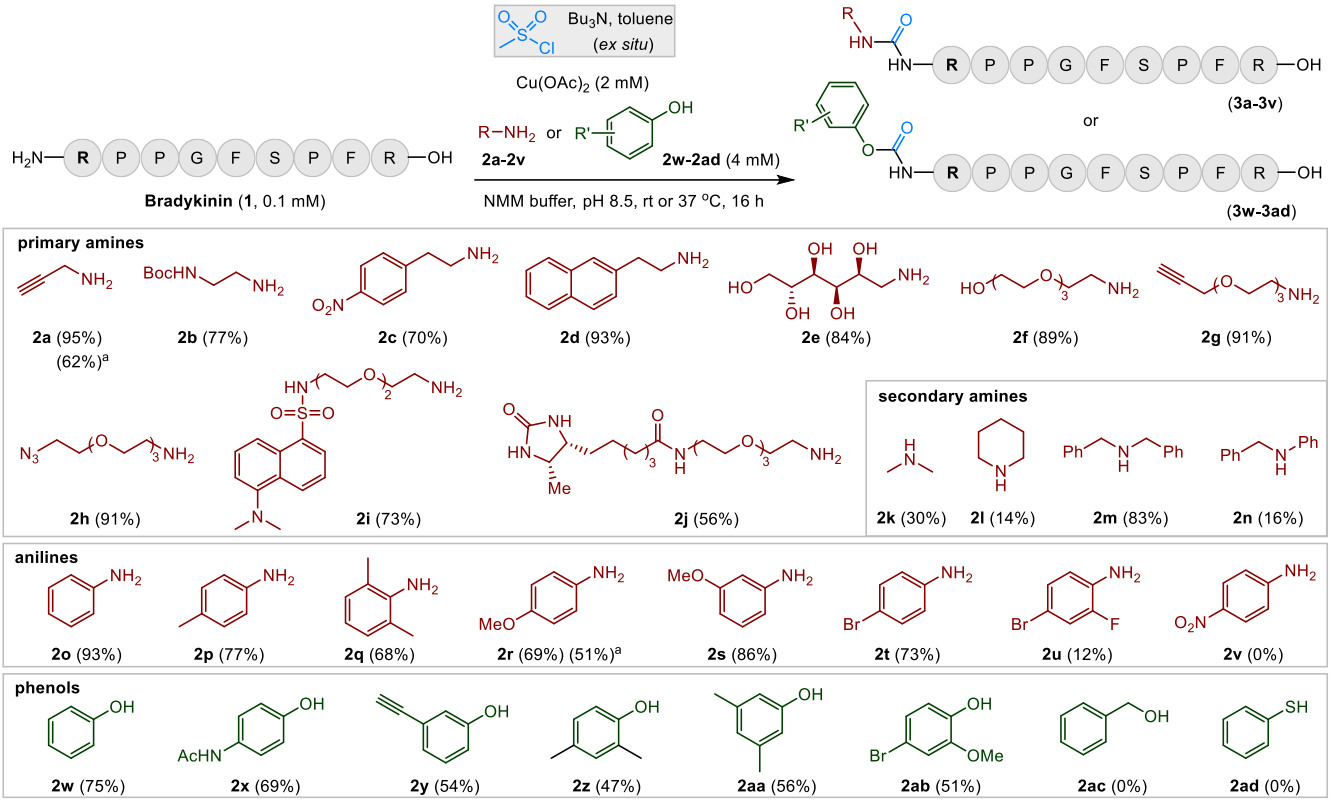

Figure 2. Scope of amines and phenols. Conditions: releasing chamber: MsCl (0.181 mmol) and tributylamine (0.362 mmol) in toluene (0.85 mL) at rt or 37 °C for 16 h; reaction chamber: **1** (0.1 mM), $Cu(OAc)_2$ (2 mM), and **2a**-**2ad** (4 mM) in NMM buffer (50 mM, pH 8.5) at rt or 37 °C for 16 h. Yields were determined by LC-MS. [a]Isolated yields.

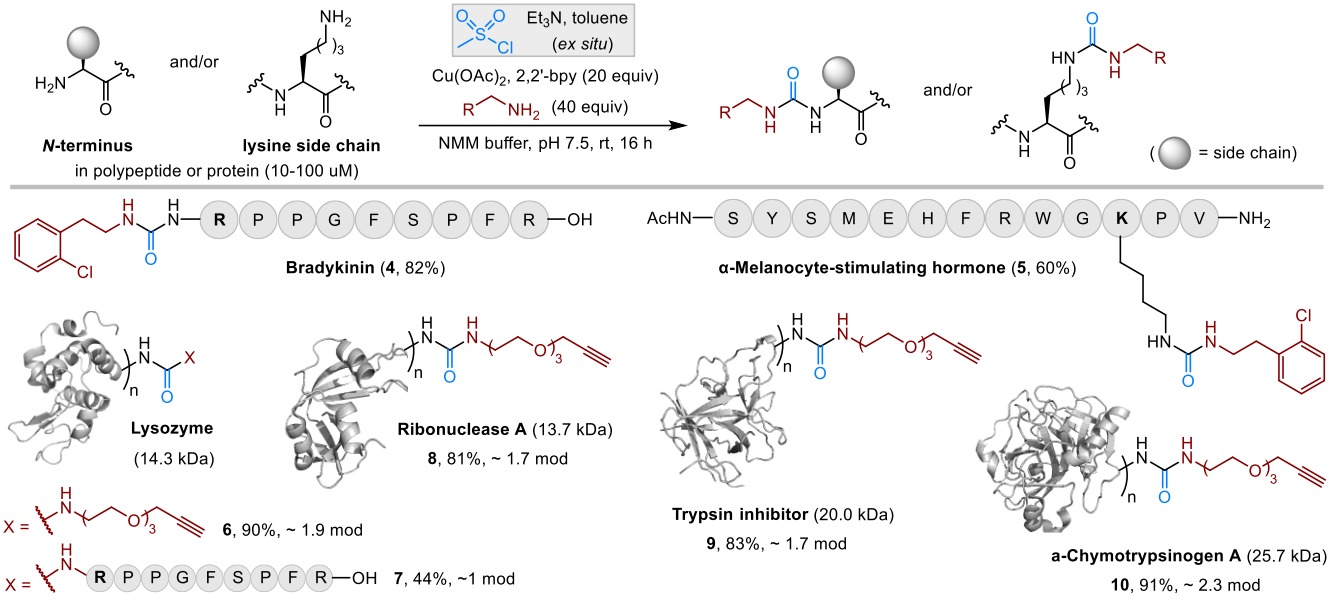

Figure 3. Scope of polypeptides and proteins. Conditions: releasing chamber: MsCl (0.181 mmol) and triethylamine (0.362 mmol) in toluene (0.85 mL) at rt for 16 h; reaction chamber: peptide (100 µM) or protein (10 µM), $Cu(OAc)_2$ (0.2-2 mM), 2,2′-bipyridine (0.2-2 mM) and 2-(2-chlorophenyl)ethyl-amine (4 mM) or propargyl-PEG3-amine (0.4 mM) in NMM buffer (50 mM, pH 7.5) at rt for 16 h. Yields and average modification numbers were determined by LC-MS.

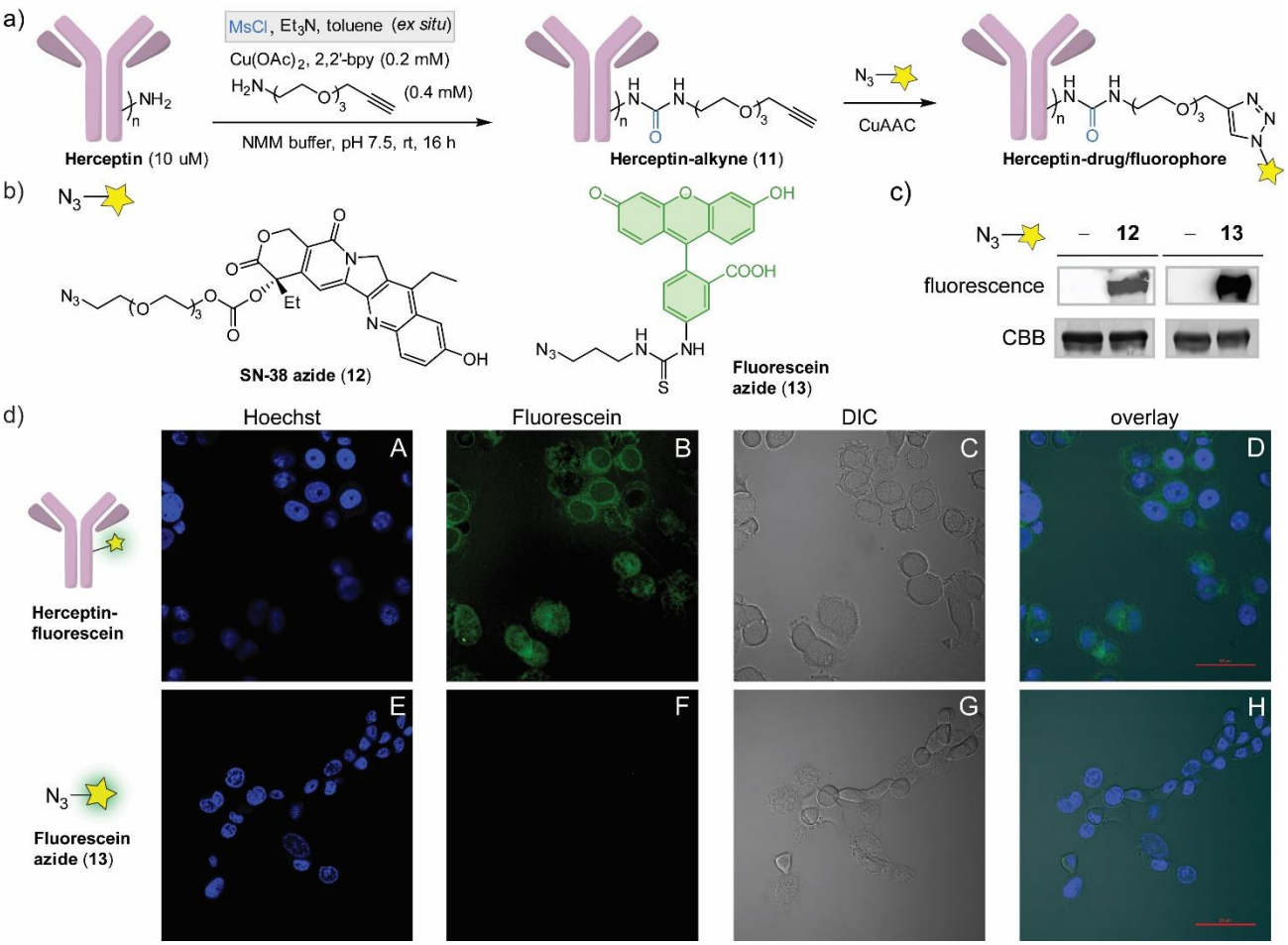

Figure 4. Antibody functionalization and fluorescence cell imaging. a) Herceptin modification with propargyl-PEG3-amine and azide–alkyne cycloaddition (CuAAC) reaction of Herceptin-alkyne **11** with SN-38 azide **12** or FITC azide **13**. b) Structures of SN-38 azide **12** and FITC azide **13**. c) Coomassie brilliant blue (CBB)-stained gel and fluorescence blot imaging of Herceptin modification and CuAAC reactions. SN-38: 365 nm excitation with 515 nm long-pass filter; FITC: 460 nm excitation with 515 nm long-pass filter. d) Fluorescence microscopy image of SK-BR-3 cells treated with the Herceptin–FITC conjugate (A–D) or FITC azide **13** (E–H). Scale bar: 50 µm. For detailed reaction and incubation conditions, please see the SI.

In addition to the *N*-terminus modification of bradykinin (**4**), the reaction of α-MSH (α-melanocyte-stimulating hormone) with 2-(2-chlorophenyl)ethylamine provided the lysine side-chain modification product **5** (Figure 3). LC–MS/MS fragmentation definitively established lysine as the modified site (Figure S40). To move forward with protein substrates, lysozyme was first tested. Propargyl-PEG3-amine **2g** was used to visualize the modified proteins on a blot membrane by chemical blotting[45] with a fluorogenic azide. Modification of lysozyme under conditions developed for peptides was rather sluggish. Having seen useful beneficial effects from a ligand additive in other copper-catalyzed bioconjugation reactions,[46] we screened potential ligands and observed significantly improved reaction efficiency with 2,2'-bipyridine or 4,4'-dimethyl-2,2'-bipyridine (Figure S2). Several proteins, including lysozyme, ribonuclease A, trypsin inhibitor, and α-chymotrypsinogen A were modified smoothly with propargyl-PEG3-amine **2g**, as determined by MS and chemical blotting (Figure 3 and S41-S45). The bradykinin peptide could itself be used as the reagent in protein modification, affording a peptide conjugate **7** directly, demonstrating the potential applicability of this method in crosslinking different biomolecules. The formation of peptide–protein conjugate **7** was confirmed by ESI-MS (Figure S46), and SDS-PAGE analysis shows an appropriate mass shift and no significant change in soluble protein levels after the reaction (Figure S46).

We next sought to assess the function of modified protein by exploring imaging applications of a modified antibody (Figure 4). Herceptin, an antibody that targets HER2 receptors, was labeled with propargyl-PEG3-amine **2g**. The resulting Herceptin-alkyne conjugate **11** was then conjugated with SN-38 azide **12** or FITC azide **13** to afford an antibody-drug conjugate or antibody-fluorophore conjugate, respectively (Figure 4, a-b). Fluorescence band visualization confirmed the incorporation of SN-38 and FITC (Figure 4, c). Next, a HER2-overexpressing breast cancer cell line, SK-BR-3, was treated with the Herceptin-FITC conjugate, and confocal microscopy indicated localization of fluorescence at cellular membranes, absent in control experiments (Figure 4, d), demonstrating the modified antibody retains antigen-binding properties.

The efficient incorporation of aniline reaction partners prompted us to explore kinetic selectivity questions. Consistent with expectation based on nucleophilicity, amine reagents react preferentially in the presence of phenol groups. Quite surprisingly, however, reactions conducted in the presence of a mixture of aniline and amine reagents showed significant selectivity in favor of aniline bioconjugation (Figure 5, a), a finding significantly at odds with expectation based on nucleophilicity.

To shed further light on the bioconjugation reaction, we measured the kinetic course of the reaction of peptide **1** with amine **2a** while varying the concentration of reagents in the aqueous phase (Figure 5, c-e). As expected for a two-chamber reaction, we observed an induction period of ~30 minutes, but otherwise found quite clean and reproducible kinetics and reaction efficiency. The maximum rate of product formation displayed a first-order dependence on peptide **1** concentration

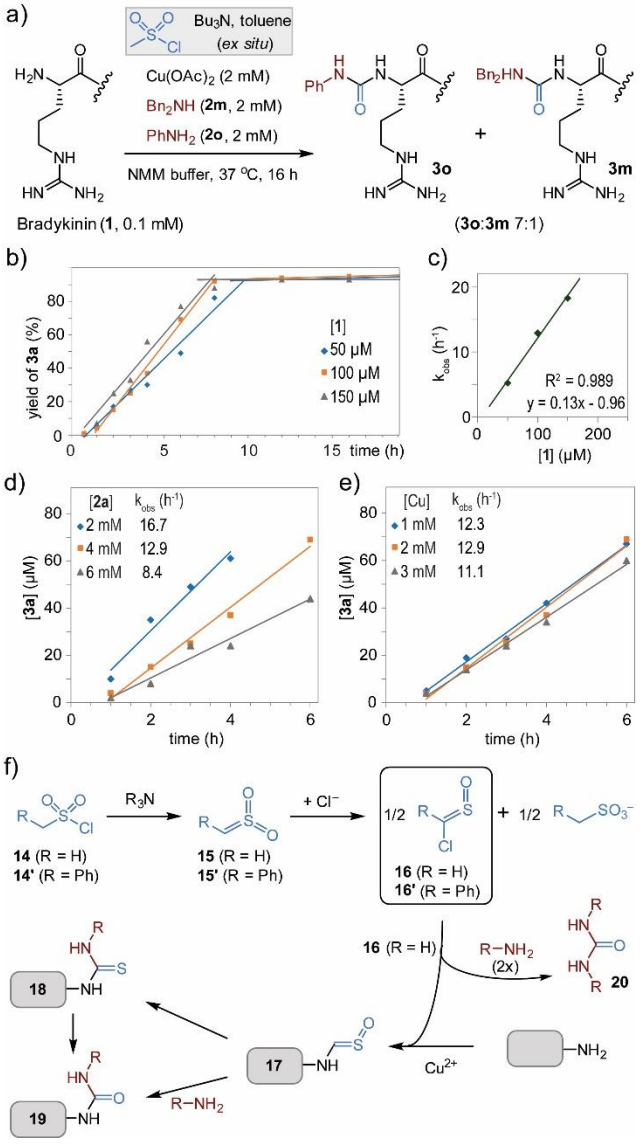

Figure 5. a) Competition reaction between an aniline and an amine. b) Kinetic analysis of product formation for reaction of **1** with **2a.** c) Plot of k_{obs} vs [**1**]. d,e) Kinetic analysis measuring k_{obs} with varying concentrations of **2a** (d) and copper (e). f) Proposed mechanistic pathway.

(Figure 5, c). However, reaction rate (i.e. the slope of [prod] vs. time) is constant for the entire course of the reaction, indicating that the rate of product formation within a given reaction is independent of changing peptide concentrations over time (Figure 5, b). Reaction rates are inhibited by increasing concentrations of small-molecule amine **2a** (Figure 5, d). Taken together, these data are

consistent with a reaction rate dependent on diffusion of a gaseous sulfonyl-derived reagent into the aqueous phase, where its reactivity partitions between reaction with peptide **1** or small molecule amine **2a**. At relevant concentration ranges, the reaction rate is unaffected by copper salt concentration (Figure 5, e).

These kinetics data are consistent with a mechanistic pathway which we proposed previously (Figure 5, f),[37] involving diffusion of chlorosulfine **16** (a species we observe in head space analysis by GC-MS[37]) into the aqueous reaction chamber. Substitution of the chlorine leaving group with an amine[42,47–49] would afford an amino-sulfine **17**, and reaction of the sulfine species **17** with amine nucleophile is postulated to undergo an internal redox reaction, affording a thiourea **18**, akin to a reported transformation for which mechanisms have been postulated.[50] Product formation would then require desulfurization in water to afford urea **19**. Indeed, we observed conversion of a model thiourea into a urea under the copper/sulfine reaction conditions,[37] although other pathways to product **19** without the intermediacy of a thiourea **18** are possible. We previously ruled out some other potential 1-ccarbon electrophiles, including thiophosgene, OCS, CO_2, and CS_2. The specific role for copper in this reaction remains uncertain. However, several different metal salts are similarly effective at mediating this transformation, including redox-inactive metals (e.g. Ca^{2+}, Mg^{2+}), which led us to postulate a role for $Cu(OAc)_2$ as a Lewis acid thiophile for activation/sequestration of sulfur.[37] Finally, the addition of radical traps in the aqueous chamber (BHT, TEMPO) did not affect reaction efficiency, which generally provides evidence against a radical pathway.[37]

Conclusions

We report an operationally simple gas-phase reagent for one-carbon linchpin bioconjugation that achieves amine–amine and amine–phenol conjugation to afford urea or carbamate products. A variety of biologically relevant molecules such as PEG, saccharide, fluorophore, and affinity tag can be efficiently crosslinked to the *N*-terminus or lysine side chain amines on natural polypeptides, proteins, and antibodies. Neither the aqueous chemistry nor the metal-mediated reactivity of chlorosulfine or related structures are well understood, and represents a new class of biocompatible electrophile, and the reaction is mediated by several bio-available metals—e.g. Fe^{3+}, Ca^{2+}, Mg^{2+}, Zn^{2+}—in addition to copper.[37] The remarkable selectivity favoring aniline conjugation over a dialkylamine is evidence of novel mechanistic and selectivity concepts at play. This work also provides a cautionary tale for use of the MsCl/triethylamine/formic acid system for CO generation,[44] given the appearance of chlorosulfine-derived byproducts under those conditions.

Acknowledgements

We acknowledge support from the Robert A. Welch Foundation Research Grant C-1680 and C-1970, the National Science Foundation under grant number CHE-2203948, and the NIGMS (R35-GM133706) grant. We are highly appreciative of financial support from the Danish National Research Foundation (grant no. DNRF118), NordForsk (grant no. 85378), Independent Research Fund Denmark/Technology and Production Sciences, Innovation Fund Denmark, and Aarhus University. We thank Prof. László Kürti for useful discussions. We also thank Prof. Jun Ohata at NCSU and Genentech, Inc. (San Francisco, CA) for their generous gifts of the SN-38 azide molecule (**12**) and the Herceptin antibody, respectively.

References

1 M. M. Müller, *Biochemistry*, 2018, **57**, 177–185.
2 B. Leader, Q. J. Baca and D. E. Golan, *Nat. Rev. Drug Discov.*, 2008, **7**, 21–39.
3 M. A. Wronska, I. B. O'Connor, M. A. Tilbury, A. Srivastava and J. G. Wall, *Adv. Mater.*, 2016, **28**, 5485–5508.
4 E. Baslé, N. Joubert and M. Pucheault, *Chem. Biol.*, 2010, **17**, 213–227.
5 O. Boutureira and G. J. L. Bernardes, *Chem. Rev.*, 2015, **115**, 2174–2195.
6 J. Ohata, S. C. Martin and Z. T. Ball, *Angew. Chem. Int. Ed.*, 2019, **58**, 6176–6199.
7 L. Cheng, Y. Wang, Y. Guo, S. S. Zhang and H. Xiao, *Cell Chem. Biol.*, 2024, **31**, 428–445.
8 C. H. P. Cheung, T. H. Chong, T. Wei, H. Liu and X. Li, *Angew. Chem. Int. Ed.*, 2023, **62**, e202217150.
9 B. Li, H. Tang, A. Turlik, Z. Wan, X. Xue, L. Li, X. Yang, J. Li, G. He, K. N. Houk and G. Chen, *Angew. Chem. Int. Ed.*, 2021, **60**, 6646–6652.
10 J. Iegre, J. S. Gaynord, N. S. Robertson, H. F. Sore, M. Hyvönen and D. R. Spring, *Adv. Ther.*, 2018, **1**, 1800052.
11 Y. H. Lau, P. de Andrade, Y. Wu and D. R. Spring, *Chem. Soc. Rev.*, 2014, **44**, 91–102.
12 Y. Zhang, Q. Zhang, C. T. T. Wong and X. Li, *J. Am. Chem. Soc.*, 2019, **141**, 12274–12279.
13 M. K. Miller, M. J. Swierczynski, Y. Ding and Z. T. Ball, *Org. Lett.*, 2021, **23**, 5334–5338.
14 A. J. Rojas, J. M. Wolfe, H. H. Dhanjee, I. Buslov, N. L. Truex, R. Y. Liu, W. Massefski, B. L. Pentelute and S. L. Buchwald, *Chem. Sci.*, 2022, **13**, 11891–11895.
15 J. Konč, L. Brown, D. R. Whiten, Y. Zuo, P. Ravn, D. Klenerman and G. J. L. Bernardes, *Angew. Chem. Int. Ed.*, 2021, **60**, 25905–25913.
16 H. H. Dhanjee, A. Saebi, I. Buslov, A. R. Loftis, S. L. Buchwald and B. L. Pentelute, *J. Am. Chem. Soc.*, 2020, **142**, 9124–9129.
17 J. Ceballos, E. Grinhagena, G. Sangouard, C. Heinis and J. Waser, *Angew. Chem. Int. Ed.*, 2021, **60**, 9022–9031.
18 R. Morewood and C. Nitsche, *Chem. Sci.*, 2021, **12**, 669–674.

19 M. G. Ricardo, D. Llanes, L. A. Wessjohann and D. G. Rivera, *Angew. Chem. Int. Ed.*, 2019, **58**, 2700–2704.
20 K. Kubota, P. Dai, B. L. Pentelute and S. L. Buchwald, *J. Am. Chem. Soc.*, 2018, **140**, 3128–3133.
21 A. J. Rojas, C. Zhang, E. V. Vinogradova, N. H. Buchwald, J. Reilly, B. L. Pentelute and S. L. Buchwald, *Chem. Sci.*, 2017, **8**, 4257–4263.
22 M. Todorovic, K. D. Schwab, J. Zeisler, C. Zhang, F. Bénard and D. M. Perrin, *Angew. Chem. Int. Ed.*, 2019, **58**, 14120–14124.
23 M. Jbara, J. Rodriguez, H. H. Dhanjee, A. Loas, S. L. Buchwald and B. L. Pentelute, *Angew. Chem. Int. Ed.*, 2021, **60**, 12109–12115.
24 H. H. Dhanjee, I. Buslov, I. W. Windsor, R. T. Raines, B. L. Pentelute and S. L. Buchwald, *J. Am. Chem. Soc.*, 2020, **142**, 21237–21242.
25 B. Yang, H. Wu, P. D. Schnier, Y. Liu, J. Liu, N. Wang, W. F. DeGrado and L. Wang, *Proc. Natl. Acad. Sci.*, 2018, **115**, 11162–11167.
26 S. Kolodych, O. Koniev, Z. Baatarkhuu, J.-Y. Bonnefoy, F. Debaene, S. Cianférani, A. Van Dorsselaer and A. Wagner, *Bioconjug. Chem.*, 2015, **26**, 197–200.
27 B. Li, L. Wang, X. Chen, X. Chu, H. Tang, J. Zhang, G. He, L. Li and G. Chen, *Nat. Commun.*, 2022, **13**, 311.
28 M. J. Matos, B. L. Oliveira, N. Martínez-Sáez, A. Guerreiro, P. M. S. D. Cal, J. Bertoldo, M. Maneiro, E. Perkins, J. Howard, M. J. Deery, J. M. Chalker, F. Corzana, G. Jiménez-Osés and G. J. L. Bernardes, *J. Am. Chem. Soc.*, 2018, **140**, 4004–4017.
29 O. Nwajiobi, A. K. Verma and M. Raj, *J. Am. Chem. Soc.*, 2022, **144**, 4633–4641.
30 F. Wurm, T. Steinbach and H.-A. Klok, *Chem. Commun.*, 2013, **49**, 7815.
31 K. I. Taylor, J. S. Ho, H. O. Trial, A. W. Carter and L. L. Kiessling, *J. Am. Chem. Soc.*, 2023, **145**, 25056–25060.
32 X. Chu, B. Li, H. Liu, X. Sun, X. Yang, G. He, C. Zhou, W. Xuan, S. Liu and G. Chen, *Angew. Chem. Int. Ed.*, 2023, **62**, e202212199.
33 R. D. Vargas, Y. Ding, H. O. Trial, R. Qian and Z. T. Ball, *Chem. Commun.*, 2023, **59**, 13030–13033.
34 M. J. Swierczynski, Y. Ding and Z. T. Ball, *Bioconjug. Chem.*, 2022, **33**, 2307–2313.
35 Y. Ding, S. S. Pedersen, A. Lin, R. Qian and Z. T. Ball, *Chem. Sci.*, 2022, **13**, 14101–14105.
36 Z. T. Ball, *Acc. Chem. Res.*, 2019, **52**, 566–575.
37 Y. Ding, S. S. Pedersen, H. Wang, B. Xiang, Y. Wang, Z. Yang, Y. Gao, E. Morosan, M. R. Jones, H. Xiao and Z. T. Ball, *Angew. Chem. Int. Ed.*, 2024, e202405344.
38 M. G. Ricardo, Y. Vázquéz-Mena, Y. Iglesias-Morales, L. A. Wessjohann and D. G. Rivera, *Bioorganic Chem.*, 2021, **113**, 104987.
39 M. G. Ricardo, A. M. Ali, J. Plewka, E. Surmiak, B. Labuzek, C. G. Neochoritis, J. Atmaj, L. Skalniak, R. Zhang, T. A. Holak, M. Groves, D. G. Rivera and A. Dömling, *Angew. Chem. Int. Ed.*, 2020, **59**, 5235–5241.
40 L. Reguera and D. G. Rivera, *Chem. Rev.*, 2019, **119**, 9836–9860.
41 L. Reguera, Y. Méndez, A. R. Humpierre, O. Valdés and D. G. Rivera, *Acc. Chem. Res.*, 2018, **51**, 1475–1486.
42 P. G. McCaw, N. M. Buckley, S. G. Collins and A. R. Maguire, *Eur. J. Org. Chem.*, 2016, **2016**, 1630–1650.
43 S. D. Friis, A. T. Lindhardt and T. Skrydstrup, *Acc. Chem. Res.*, 2016, **49**, 594–605.
44 C. Veryser, S. Van Mileghem, B. Egle, P. Gilles and W. M. De Borggraeve, *React. Chem. Eng.*, 2016, **1**, 142–146.
45 J. Ohata, F. Vohidov and Z. T. Ball, *Mol. Biosyst.*, 2015, **11**, 2846–2849.
46 K. Hanaya, J. Ohata, M. K. Miller, A. E. Mangubat-Medina, M. J. Swierczynski, D. C. Yang, R. M. Rosenthal, B. V. Popp and Z. T. Ball, *Chem. Commun.*, 2019, **55**, 2841–2844.
47 B. Zwanenburg, L. Thijs and J. Strating, *Recl. Trav. Chim. Pays-Bas*, 1970, **89**, 687–695.
48 F. Cerreta, C. Leriverend and P. Metzner, *Tetrahedron Lett.*, 1993, **34**, 6741–6742.
49 S. A. Siry, V. M. Timoshenko and J.-P. Bouillon, *J. Fluor. Chem.*, 2012, **137**, 6–21.
50 M. Baltas, K. Raouf-Benchekroun, A. De Blic, L. Cazaux, P. Tisnès, L. Gorrichon, K. Hussein and J.-C. Barthelat, *Tetrahedron*, 1996, **52**, 14865–14876.