An ex situ gaseous reagent for multicomponent amine bioconjugation

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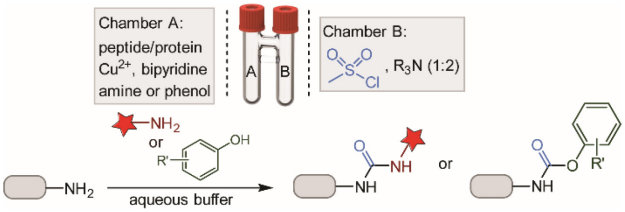

ABSTRACT: We report a minimalist gaseous sulfonyl chloride-derived reagent for multicomponent bioconjugation with amine, phenol, or aniline reagents to afford urea or carbamate products. In utilizing a gas-phase reagent for a reaction mediated by metal ions, a variety of biologically relevant molecules 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.

Chemical protein functionalization has become an indispensable tool for altering protein structure and function.[1] Modified proteins useful in diverse fields, such as biologic therapeutics,[2] biomaterials,[3] and biological probes.[4] 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 targeting nucleophilic side chains remain dominant.[5]

Crosslinking two nucleophilic sites is an attractive approach to the preparation of bioconjugates.[8–10] Two-step elaboration of an existing residue to append an electrophile, followed by treatment with a second nucleophilic reagent is a common approach, but requires careful reagent design.[11–13] A bifunctional bis-electrophile reagent can be employed in a one-step process, with a suitable linker between reactive groups.[14–17] Avoiding multistep manipulations of complex biopolymers is a significant advantage. However, crosslinking selectivity challenges remain, and, this approach is dominated by the crosslinking of a cysteine thiol and a lysine or *N*-terminal amine.[8,13,14,17]

Crosslinking 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.[16] Among reported examples, a squaric acid diester was used as a linchpin reagent for stepwise coupling of two amino groups (Figure 1, a),[18] and *ortho*-phthalaldehyde allows one-pot clamping of two amines (Figure 1, b).[19] However, new methods of amine–amine crosslinking would expand the bioconjugation toolbox and provide new opportunities for the construction of complex bioconjugates.

As part of a program to develop non-traditional bioconjugation methods,[11,20–23] we recently reported a peptide macrocyclization induced by a chlorosulfine gas, produced ex situ

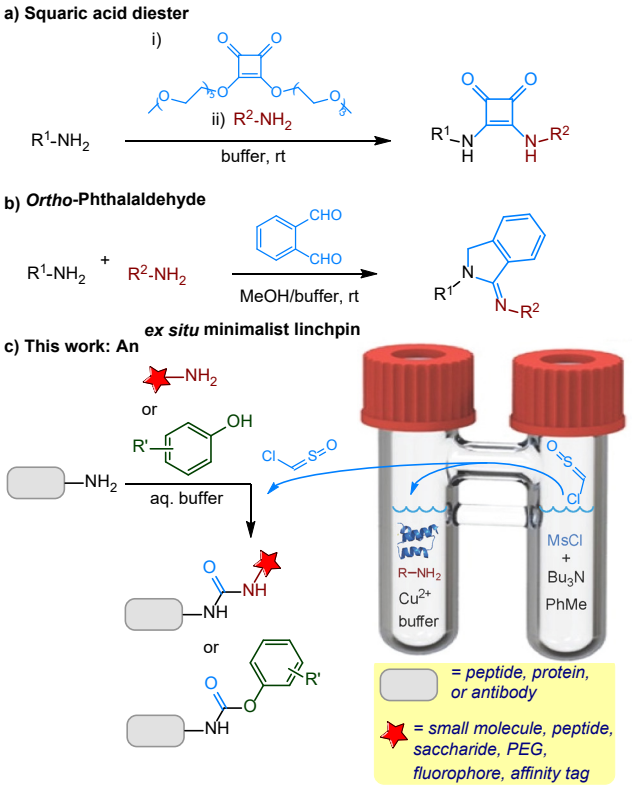

Figure 1. Linchpin reagents for amine–amine conjugation.

from base-induced elimination-disproportionation of methanesulfonyl chloride.[24] 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[16,25,26] of external nucleophiles with amine side chains of peptides and proteins (Figure 1, c). While the reactivity of some sulfene or sulfine intermediates have been studied,[27] the application of these species to reaction in complex polyfunctional contexts, including bioconjugation, is largely unexplored.[24]

We discovered[24,28] this reactivity while investigating carbonylative coupling,[22] in which CO was generated in a two-chambered reactor for safe ex situ production of CO.[29] When CO was produced from formic acid and methanesulfonyl chloride with triethylamine,[30] bioconjugation reactions of amine reagents persisted even in negative control experiments without formic acid.[24] 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 **3a** (Figure 1c and Figure 2). The structure of **3a** was confirmed by 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**). Taken together, these results indicate tolerance of a variety of nucleophile reagents, although some uncharacterized byproducts were sometimes observed, especially in less efficient reactions.

In addition to the *N*-terminus modification of bradykinin (**4**), the reaction of α-melanocyte-stimulating hormone with 2-(2-chlorophenyl)ethylamine provided the lysine side-chain modification product **5** (Figure 3). 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[31] 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,[32] we screened potential ligands and observed improved reaction efficiency with 2,2'-bipyridine or 4,4'-dimethyl-2,2'-bipyridine (Figure S2).

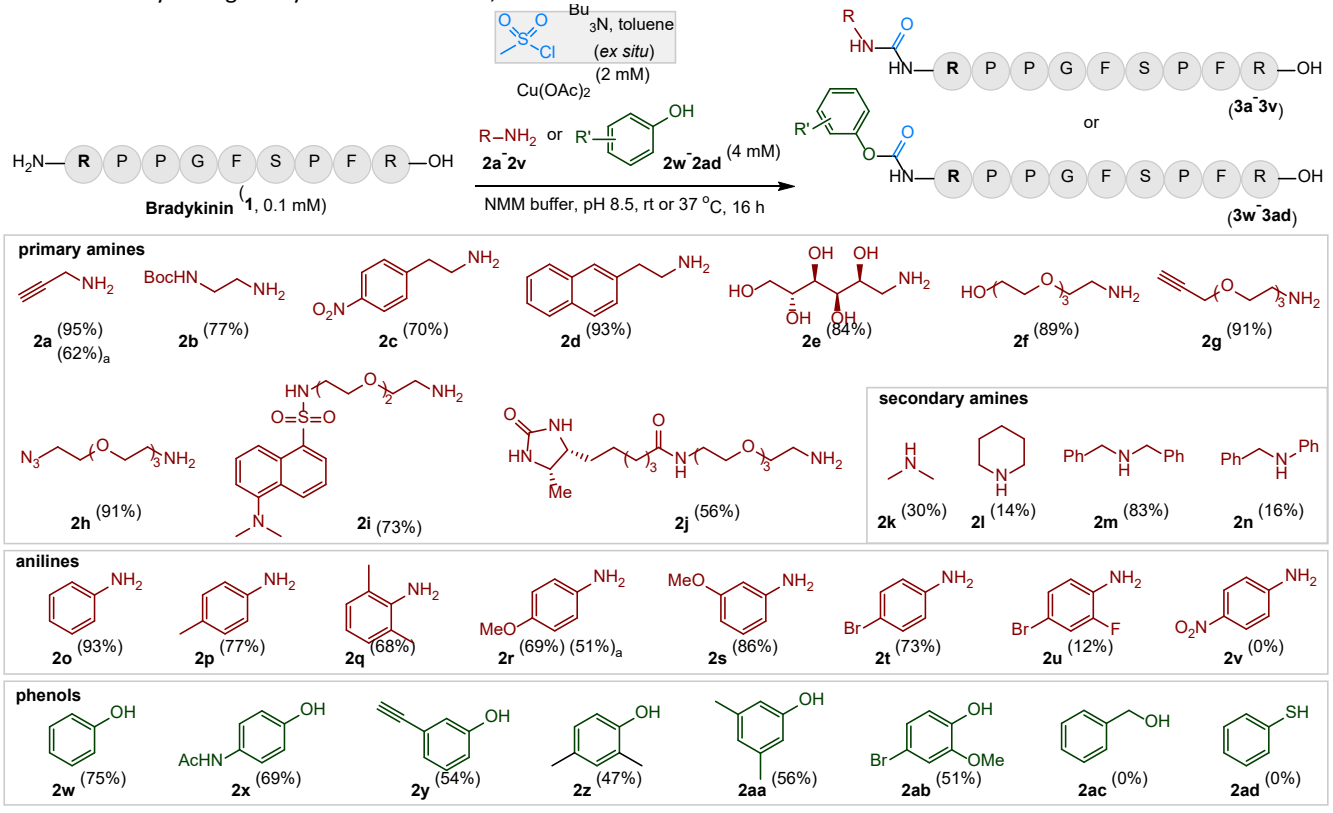

Figure 2. Scope of amines and phenols. Condns: releasing chamber: MsCl (0.18 mmol), Bu_3N (0.36 mmol) in toluene (0.85 mL); reaction chamber: **1** (0.1 mM), $Cu(OAc)_2$ (2 mM), **2** (4 mM) in aq *N*-methylmorpholine (50 mM, pH 8.5). Yields determined by LC-MS. [a]Isolated yield.

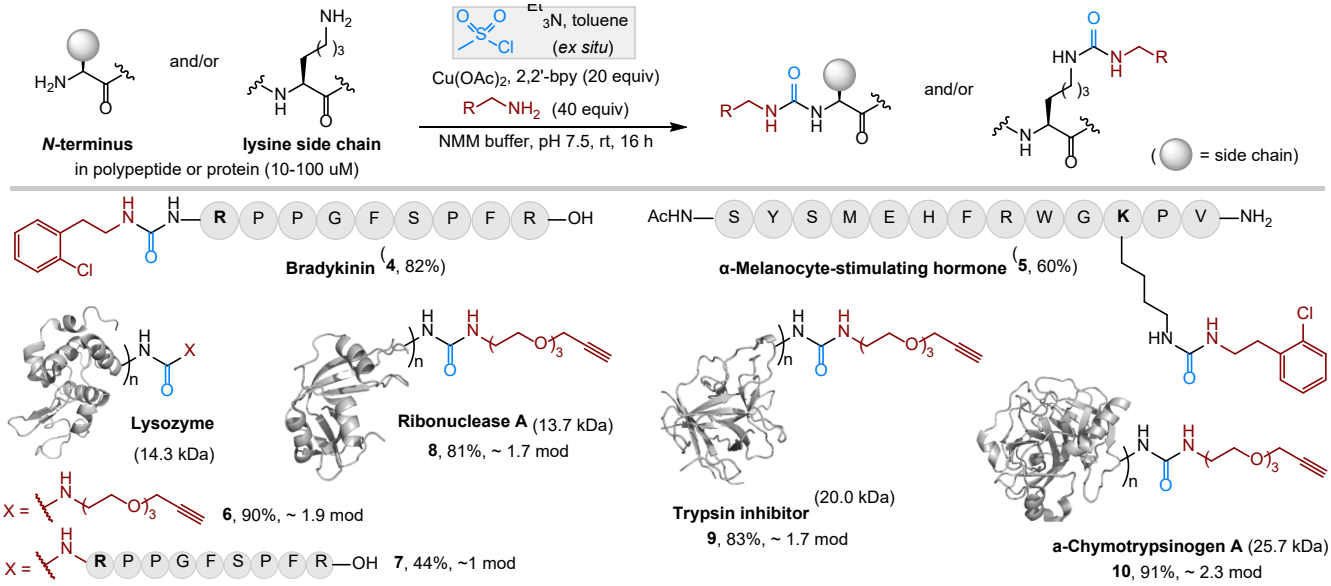

Figure 3. Scope of peptides and proteins. Conditions: releasing chamber: MsCl (0.181 mmol), Et_3N (0.362 mmol) in toluene (0.85 mL); reaction chamber: peptide (100 μM) or protein (10 μM), $Cu(OAc)_2$ (0.2–2 mM), 2,2'-bipyridine (0.2–2 mM) and amine (0.4 mM) in *N*-methylmorpholine buffer (50 mM, pH 7.5). Yields and average modification numbers were determined by LC-MS.

Several proteins, including lysozyme, ribonuclease A, trypsin inhibitor, and α-chymotrypsinogen A were modified with **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 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). The MS analysis of reactions with protein substrates did indicate some instances of minor byproducts ($[M+26]^+$) indicative of intramolecular reactions (Figures S41-S44), which may be a limitation of this chemistry.

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, reactions 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

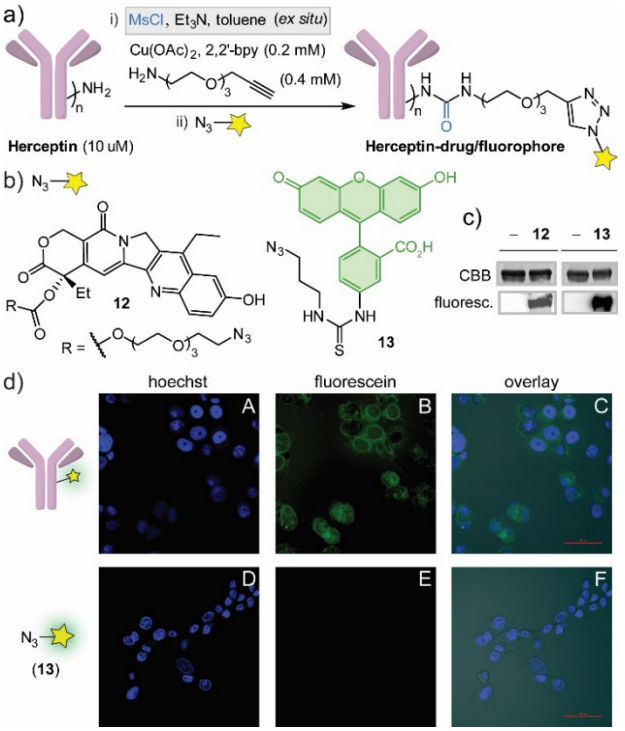

Figure 4. Antibody functionalization. a) Herceptin modification with propargyl-PEG3-amine and subsequent CuAAC with **12** or **13**. b) Structures of **12**, **13**. c) CBB-stained gel and fluorescence blot imaging of labeled Herceptin. SN-38: 365-nm ex. with 515-nm long-pass filter; FITC: 460-nm ex. with 515 nm long-pass filter. d) Fluorescence microscopy image of SK-BR-3 cells treated with the Herceptin–FITC conjugate (A–C) or **13** (D–F). Scale bar: 50 μm.

otherwise found clean and reproducible kinetics and reaction efficiency. The maximum rate of product formation displayed a first-order dependence on peptide **1** concentration (Figure 5c). However, reaction rate (i.e. the slope of [prod] vs. time) is constant throughout 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 rate dependent on diffusion of gaseous chlorosulfene into the aqueous phase, where its reactivity partitions between reaction with peptide **1** or small molecule amine **2a**. At relevant concentration ranges, the rate is unaffected by copper salt concentration (Figure 5e).

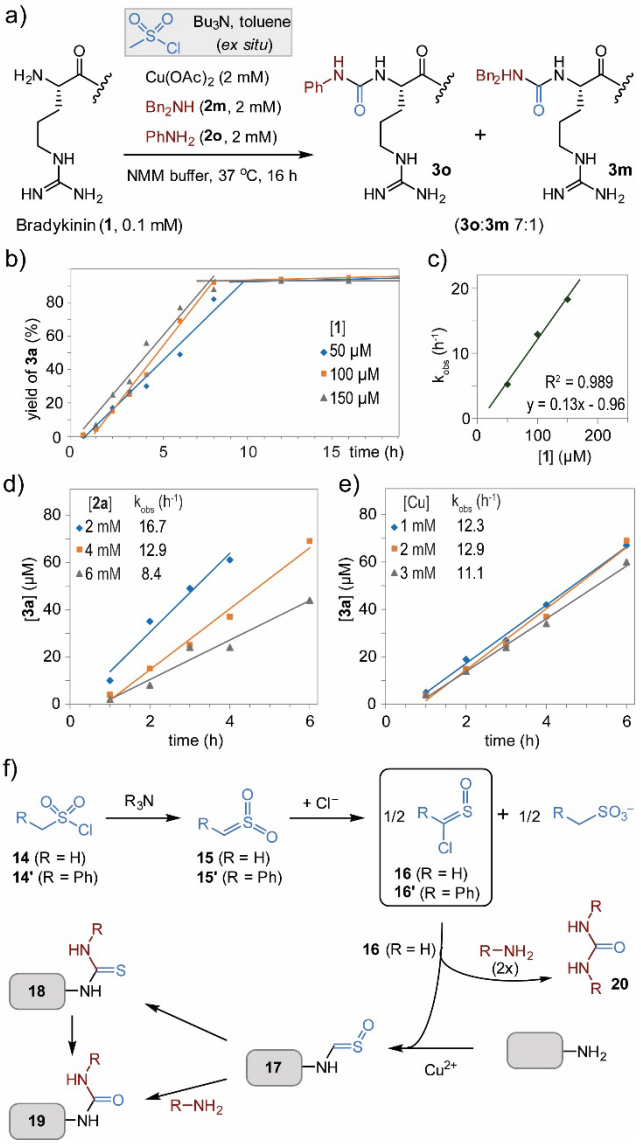

Figure 5. a) Competition reaction between an aniline and an amine. b) Kinetic analysis of product formation for reaction of **1** with **2a.** c) Plot of k_{obs} vs [**1**]. d,e) Kinetic analysis measuring k_{obs} with varying concentrations of **2a** (d) and copper (e). f) Proposed mechanistic pathway.

These kinetics data are consistent with a mechanistic pathway which we proposed previously (Figure 5, f),[24] involving diffusion of chlorosulfine **16** (a species we observe in head space analysis by GC-MS[24]) into the aqueous reaction chamber. Substitution of the chlorine leaving group with an amine[27,33–35] 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.[36] Product formation would then require desulfurization in water to afford urea **19**. Indeed, we previously observed conversion of a model thiourea into a urea in the presence of the combination of copper and chlorosulfine (but crucially not as effectively with either reagent individually), leading us to propose a role for copper as a thiophile in this desulfurization step.[24] Other pathways to product **19** without the intermediacy of a thiourea **18** are also 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 leads us to postulate a role for $Cu(OAc)_2$ as a Lewis acid thiophile for activation/sequestration of sulfur.[24] 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.[24]

In conclusion, 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 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 Cu^{2+}.[24] The 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/Et_3N/formic acid system for CO generation,[30] given the appearance of chlorosulfine-derived byproducts under those conditions.

ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.
Experimental procedures, LC-MS and NMR spectra.

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