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2*H*-Azirines as Potential Bifunctional Chemical Linkers of Cysteine Residues in Bioconjugate Technology

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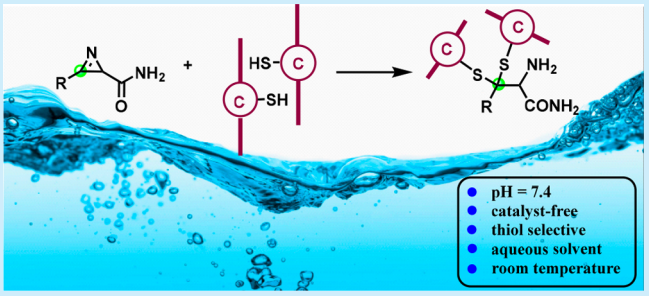
ABSTRACT: 2*H*-Azirine-2-caboxamides have been designed to perform as a new type of bifunctional thiol linker under very mild reaction conditions. The cleavage of a C−N double bond of 2*H*-azirine furnishes an amino amide functional group *in situ* through a thiol addition and ring-opening process. It works with a broad scope of thiols and 2*H*-azirines in the absence of any catalysts at room temperature. Cysteine-containing peptides have also been demonstrated to work efficiently in a completely water solution.



Antibody drug conjugates (ADCs), known as peptides and proteins conjugated through chemical linkers to drugs for use in targeted therapy, have become an increasingly growing research area and could potentially make a fundamental change in cancer chemotherapy.[1] Among them, cysteine-based bioconjugation methods have evolved into a powerful biotechnological tool, allowing varied chemical linkers to have been designed via alkylation,[2] reduction,[3] oxidation,[4] and conjugate addition reactions.[5] Maleimide is recognized as one of the most popular chemical linkers. It has also been applied in FDA-approved drugs such as Adcetris and Kadcyla (Figure 1, 1.a).[6] Allenamide was developed by our group in 2014 as a cysteine linker having excellent chemoselectivity under mild conditions (Figure 1, 1.b).[7] The Buchwald group has also demonstrated the use of an organometallic palladium complex as a cysteine arylation reagent (Figure 1, 1.c).[8]

In general, free thiols do not exist in antibodies, and all cysteine residues are bound in disulfide bonds. To deal with this issue, several new chemical linkers have been designed to target dithiols instead.[9] This approach would optimize the drug-to-antibody ratio (DAR) value and decrease the risk of off-target toxicity of drugs from antibodies.[10] Along these lines, dibromomaleimide-based reagents can be attacked by two reduced cysteines generated from interchain disulfide bonds with the elimination of bromide residues (Figure 1, 2.a).[11] Recently, Griebenow's group developed a photomediated disulfide rebridging method based on a Thiol−yne coupling reaction (Figure 1, 2.b).[12] Pentelute and coworkers have evaluated perfluoaromatic molecules as effective arylation reagents under advanced mild reaction conditions (Figure 1, 2.c).[13] Despite these advances, exploring new synthetic methods to bind two thiols into a single linker is still a developing field, especially in terms of atom economy, high site selectivity, and mild reaction conditions.

2*H*-Azirines are the smallest unsaturated aza-heterocycles and are useful in the preparation of important N-containing compounds through opening the small ring due to their unique ring strain.[14] Most of the research has focused on the cleavage of the 2*H*-azirine C−N single bond to generate 1,3-dipolar intermediates.[15] Similarly, C−C single-bond cleavage of 2*H*-azirines has also been explored in limited examples.[16] However, to the best of our knowledge, there is no example of cleaving the 2*H*-azirine's C−N double bond in a single step. Compared with previous reported disulfide bridging linkers, 2*H*-azirines performed as a new type of bifunctional thiol linker with the advantages of being metal-free and atom-economical, having high site-selectivity, and requiring mild reaction conditions. In our continuing interest in 2*H*-azirine chemistry,[17] herein we describe a bioconjugation method making use of the thiol-2*H*-azirine coupling reaction through a thiol addition followed by a ring-opening process cleavage the C−N double bond.

We commenced our study by exploring the proposed double addition of thiophenol **1a** to the 2*H*-azirine **2a** in an aqueous solvent system under mild reaction conditions (Table 1). To our delight, the dithiol product **3aa** was formed when an organic cosolvent (Table 1, entries 1−4) was used with the aqueous phosphate buffer saline (PBS) solution. EtOH turned out to be an optimal cosolvent. The role of the cosolvent is likely to improve the solubility of thiophenol **1a**. Meanwhile,

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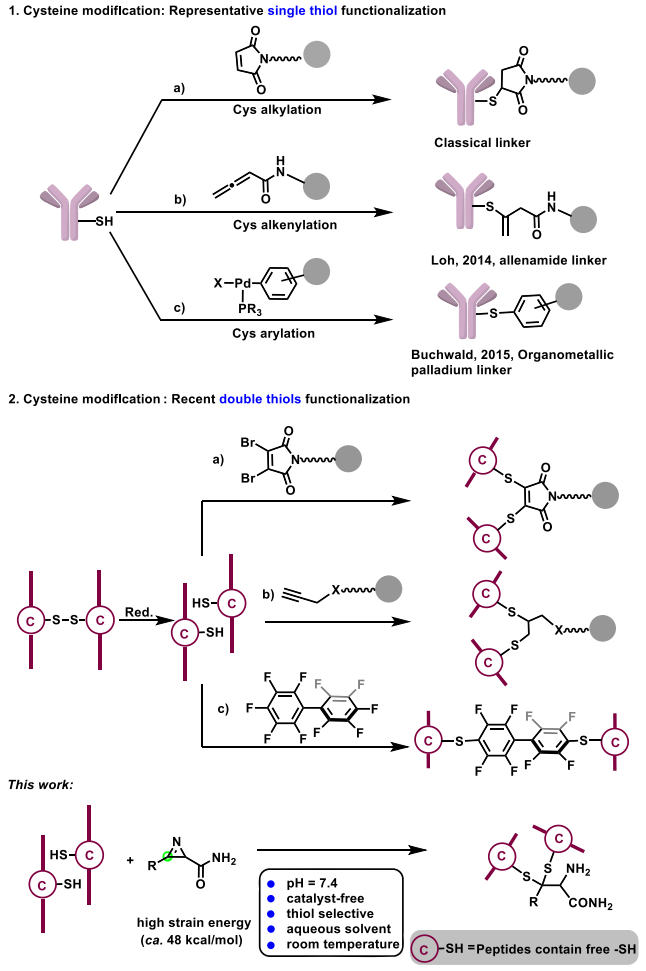
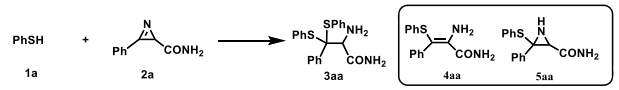


Figure 1. Modification of cysteines in bioconjugation.

Table 1. Optimization of Reaction Conditions[a]



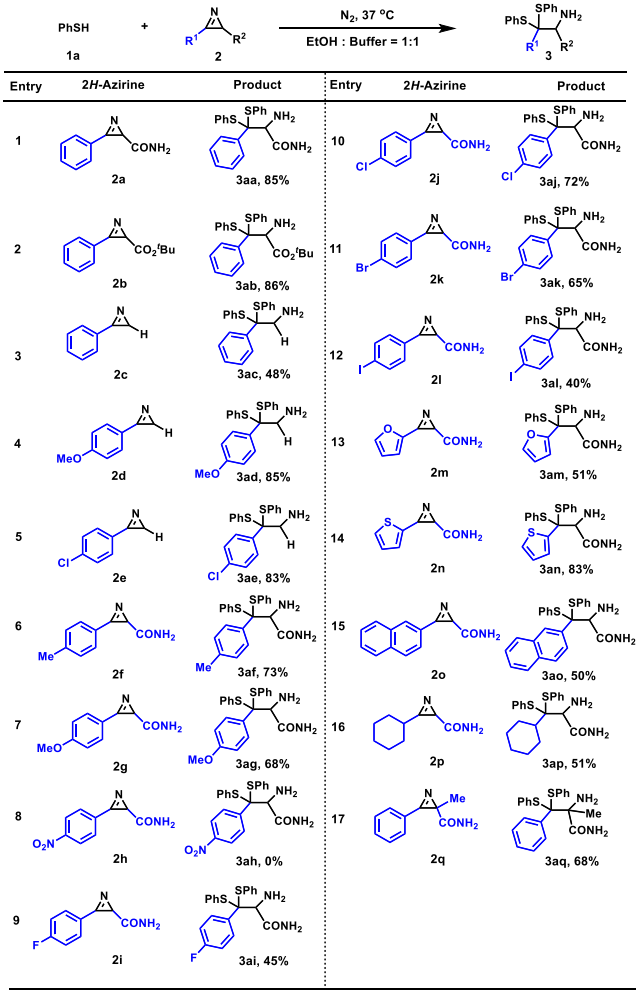
entry	temp. (°C)	pH	1a:2a	solvent	3aa yield (%)[b]
1	37	7.0	6:1	CH_3CN	40
2	37	7.0	6:1	THF	56
3	37	7.0	6:1	H_2O	22
4	37	7.0	6:1	EtOH	60
5	37	6.0	6:1	EtOH	45
6	37	7.4	6:1	EtOH	70
7	37	7.4	4:1	EtOH	50
8	**37**	7.4	8:1	EtOH	**85**
9	37	7.4	10:1	EtOH	79
10	20	7.4	8:1	EtOH	78
11	50	7.4	8:1	EtOH	65
12	**37**	7.4	8:1	EtOH	**78**[c]

[a]Unless otherwise noted, the reactions were carried out using PhSH **1a** and 2*H*-azirines **2a** (0.2 mmol) in PBS (1 mL) and organic solvent (1 mL) at 37 °C under a nitrogen atmosphere (1 atm). [b]Yields were determined by NMR versus CH_2Br_2 as internal standard. [c]10 mL of PBS and 10 mL of EtOH were used.

the monothiol adducts could also be detected and characterized as enamine **4aa** and 2*H*-aziridine **5aa**. To improve the selectivity of the thioacetals, we then screened the reaction pH and found that the yield of **3aa** improved to 70% at pH 7.4 (Table 1, entries 4−6). Further study found that the thiol loading had an important role in the reaction. When an eight-fold excess of thiophenol **1a** was used, the yield of **3aa** further improved to 85%. Finally, temperature had only a minor influence on the reaction in the range from 20 to 50 °C (Table 1, entries 9−11). Thus the optimal reaction conditions were established by using PhSH **1a** (1.6 mmol, 8.0 equiv) with 2*H*-azirines **2a** (0.2 mmol, 1.0 equiv) in EtOH and PBS (1:1 v/v) as a cosolvent system (3 mL) at 37 °C under a nitrogen atmosphere (1 atm) until **2a** was completely consumed (~20 h).

With the optimized reaction conditions in hand, we then screened a series of 2*H*-azirines to establish a range of potential ADC linker candidates (Scheme 1). Initially, different 2-

Scheme 1. Substrate Scope of 2*H*-Azirines[a,b]



[a]Unless otherwise noted, reactions were carried out using PhSH **1a** (1.60 mmol, 8.0 equiv) and 2*H*-azirines **2** (0.2 mmol, 1.0 equiv) in EtOH (1 mL) and PBS (1 mL) solution at 37 °C for 20 h under a nitrogen atmosphere (1 atm). [b]Isolated yields.

substituted 2*H*-azirines, including carboxylic acid, esters, and amides, were investigated. In all cases, the 2*H*-azirines gave the corresponding functionalized thioacetals in moderate to excellent yield (**3aa**−**ae**). Of particular interest to bioorthogonal chemistry are the 2-amide-substituted 2*H*-azirines, which would be expected to be soluble in aqueous media. Thus we screened a variety of different 2-amide-2*H*-azirines. The aryls

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with electron-donating substituents (**3af**–**ag**) worked efficiently, whereas electron-withdrawing (**3ah**) groups failed to react. The halogen substituents (F, Cl, Br, and I) on the parasite of the aryl ring reacted smoothly and afforded the desired products in 40 to 72% yield (**3ai**–**al**). The halogen-functionalized thioacetal adducts are useful because they can be applies in further cross-coupling reactions.[18] Heterocyclic- (**3am**–**an**) and naphthalenyl-substituted (**3ao**) substrates also proceeded smoothly, albeit in fluctuant yield ranging from 50 to 83%. Cyclohexyl-substituted 2*H*-azirine also performed well and gave the corresponding adduct in 51% yield (**3ap**). Finally, a 2*H*-azirine bearing a quaternary carbon also reacted efficiently and gave the thioacetal product in 68% yield (**3aq**).

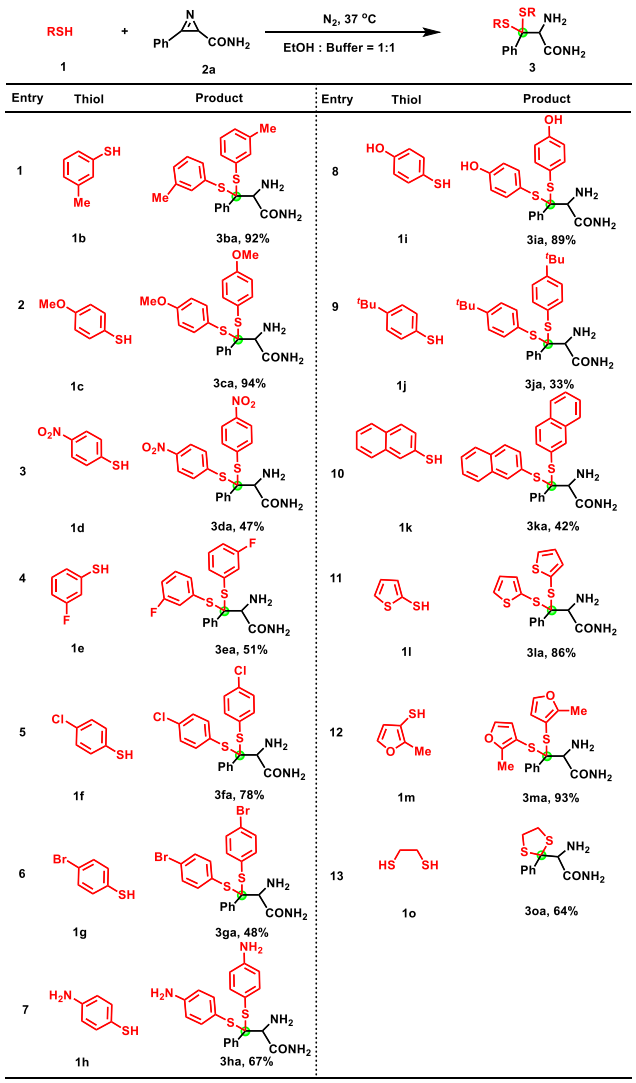
Subsequently, we explored different thiols under the standard conditions (Scheme 2). In general, the reaction tolerated a broad array of thiols carrying useful functionalities including halogen, NO_2, NH_2, and OH groups. Considering the electronic effects, thiophenols with electron-rich groups reacted more efficiently and gave the thioacetal adduct products in 92% to 94% yield (**3ba**–**ca**), whereas electron-deficient groups resulted in moderate to good yield (**3da**–**ga**). Importantly, thiophenols containing active hydrophilic groups such as amino and hydroxyl groups underwent the reaction smoothly and offered the desired products with excellent chemoselectivity (**3ha**–**ia**). No N- or O-addition products were observed. Naphthalenyl- (**3ka**) and heterocyclic-substituted (**3la**–**ma**) thiols also reacted smoothly in excellent yield. Also, alkyl dithiol reacted smoothly, albeit in slightly decreased yield (**3oa**).

To further demonstrate the utility of our method in biological chemistry, we explored the reactivity of cysteine derivatives with 2*H*-azirines. Cysteine **1p** reacted with the simple 2*H*-azirine **2a** under the standard reaction conditions to give the expected thioacetal product in a 42% yield (Scheme 3). We also noted that the ethanol cosolvent was not necessary, and a 65% yield of the cysteine adduct was obtained in just the buffer solution alone. This further underlines the biocompatibility of our proposed linker method. Finally, we examined different 2*H*-azirines with MeO, Cl, and CN functional groups. The 4-methoxyl-2*H*-azirine **2g** reacted with cysteine in excellent yield (95% crude yield and up to 87% by isolation).

Having established the desired linker mode for cysteines, we then explored different peptides containing free thiol moieties to react with the best performing methoxy 2*H*-azirine **2g** (Scheme 4). Peptides with two cysteines resulted in the formation of cyclic thioacetal adduct bridges (Scheme 4, entries 3–6). On the contrary, peptide sequences with single cysteine residues resulted in the linker working in a linchpin mode, forming a dimeric thioacetal (Scheme 4, entries 1 and 2). It should be noted that in both cases, the reaction was carried out in an aqueous solution at room temperature and tolerated several other amino acid residues including NH_2, OH, and COOH groups.

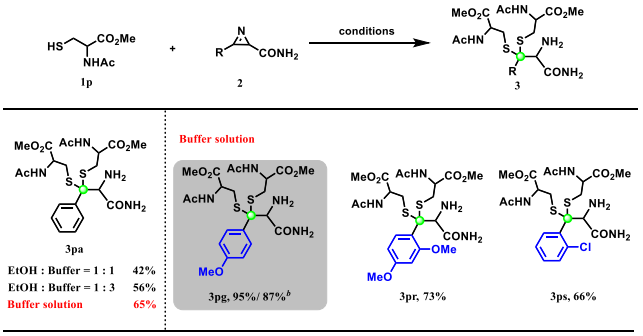
With the obtained results in hand, we propose a possible reaction pathway for the 2*H*-azirine thioacetalization (Scheme 5a). Upon thiol **1a**'s initial attack of 2*H*-azirine **2a**, two possible products (enamide **4aa** or aziridine **5aa**) can be envisioned. To test our hypothesis, we prepared compounds **4aa** and **5aa** independently and allowed them to react with **1a** under standard reaction conditions (Scheme 5b). In this control

Scheme 2. Substrate Scope of Thiols[a,b]



[a]Unless otherwise noted, the reactions were carried out using thiol compounds **1** (1.6 mmol, 8.0 equiv) and 2*H*-azirines **2a** (0.2 mmol, 1.0 equiv) in EtOH (1 mL) and PBS (1 mL) solution at 37 °C for 20 h under a nitrogen atmosphere (1 atm). [b]Isolated yield.

Scheme 3. Optimization of Reaction Conditions for Cysteine[a]



[a]Unless otherwise noted, the reactions were carried out using cysteine **1p** (1.6 mmol, 8.0 equiv) and 2*H*-azirine **2** (0.2 mmol, 1.0 equiv) in PBS (1 mL) at 37 °C for 20 h under a nitrogen atmosphere (1 atm), and the yields were determined by NMR. [b]Isolated yields.

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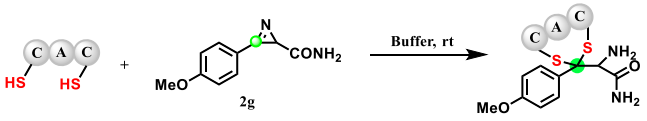
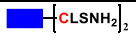
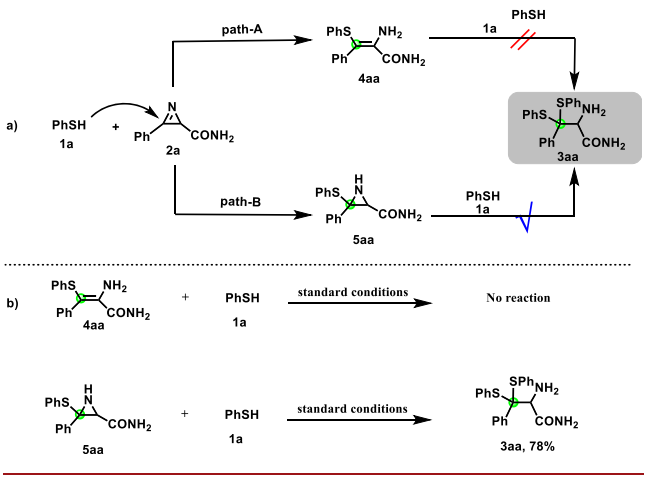
Scheme 4. Reactions of Peptide Sequences*



Entry	Peptide sequence	Conv. (%) [a]
1	CLS-NH_2	
2	CTWSL-NH_2	
3	RCLiTCK-NH_2	
4	CKARGGC-NH_2	
5	RACKGGCG	
6	CPCRAGKG-NH_2	

*Unless otherwise noted, the reactions were carried out using peptide (1.6 mmol, 8.0 equiv) and 2*H*-azirine **2g** (0.2 mmol, 1.0 equiv) in PBS (1 mL) at 37 °C for 20 h under a nitrogen atmosphere (1 atm). [a]Conversion was quantitative in each case (determined by LC−MS).

Scheme 5. Mechanism Study



experiment, only aziridine **5aa** reacted completely to the desired product thioacetal **3aa** in 78% yield. This result supports the **path-B** pathway involving an aziridine intermediate **5aa**.

In summary, we have demonstrated the use of 2*H*-azirine-2-caboxamides as a new type of biocompatible bifunctional thiol linker through a thiol addition and ring-opening cascade process. The linking reaction works with a broad substrate scope under very mild reaction conditions without any catalysts with excellent chemoselectivity. The method also tolerates the presence of other nucleophilic functional groups, such as NH_2 and OH. Cysteine-containing peptides have also been demonstrated to work efficiently in aqueous solutions. The developed method has potential for use in bifunctional chemical linkers for constructing antibody−drug conjugates.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c00415.

Experimental procedures and characterization data for new compounds (PDF)

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Notes

The authors declare no competing financial interest.

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