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Rapid, Stable, Chemoselective Labeling of Thiols with Julia-Kociński Like Reagents: A Serum-Stable Alternative to Maleimide-Based Protein Conjugation**

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Abstract

Cysteine-maleimide chemistry is widely used for peptide and protein modification. However, the formed succinimide linkage is readily hydrolyzed and is susceptible to an exchange reaction in vivo. We demonstrate that methylsulfonyl phenyloxadiazole compounds react specifically with cysteine under various buffer conditions and found that the resulting protein conjugates had superior stability to cysteine-maleimide conjugates in human plasma. This Thiol-Click chemistry promises a new approach to stable protein conjugates and pegylated proteins.

Keywords

chemoselective; cysteine; drug conjugate; phenyloxadiazole; protein conjugation

Proteins modified with fluorescent or biologically active agents are powerful reagents in chemistry, biology, and medicine.^[1] For the selective modification of proteins, selectivity for certain of the natural 20 amino acids in the presence of many unprotected amino acid residues is necessary.^[2] Bioconjugation reaction development has largely focused on the modification of lysine and cysteine side chains. Conjugation to cysteine, which is a rare amino acid and often exists as a disulfide pairs in native proteins, can be readily achieved due to the relatively low pKa and potent nucleophilicity of the thiolate anion.^[3] Significant research efforts have been made to identify reagents that enable blocking or labeling of protein thiols with high selectivity and conversion yields.^[4] Among those, thiol-alkylation reagents (such as α -halocarbonyl derivatives) or Michael acceptors (such as maleimide derivatives) are the most commonly used and their reactivity profiles have been extensively studied. Indeed, antibody-drug conjugates made through the maleimide-cysteine conjugation method are approved drugs.^[5] Additionally, maleimide-cysteine conjugation is used to make albumin-binding prodrugs, which are rapidly and selectively bound to the cysteine-34

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position of endogenous albumin in the blood.^[6] Although maleimide-chemistry is a powerful tool for selective modification of proteins, limitations have been reported.^[7] The succinimide linkage of the maleimide addition product is susceptible to hydrolysis, and the thioether moiety undergoes exchange reactions with reactive thiols (such as those of albumin) and with free cysteines and glutathiones through the retro-Michael reaction. As a result, heterogeneous mixtures of the conjugates can be produced in the blood that have different pharmacokinetics, *in vivo* efficacy, and toxicity.^[8] Therefore, a cysteine-selective conjugation method that results in a stable linkage is desired. Recently, methylsulfonyl benzothiazole **1** (MSBT, Figure 1) was reported as a thiol blocking reagent.^[9] The stability of MSBT blocked thiols, however, and the broad chemistry of methylsulfonyl hetero aromatics in thiol conjugation chemistry remains unexplored. Herein, we designed and optimized methylsulfonyl hetero aromatic derivatives for rapid protein/peptide conjugation and demonstrate that these conjugates can be significantly more stable in human plasma than maleimide-cysteine conjugates providing a promising and versatile new approach to protein and peptide conjugates for chemistry, biology, and medicine.

The recent disclosure of MSBT (**1**, Figure 1) as a selective protein thiol blocking reagent^[9] stimulated our thinking concerning the reactivity of molecules in this structural class. The compound 2-(alkylsulfonyl)benzothiazole is known as a substrate for a modified one-pot Julia-Lythgoe olefination.^[10] Further, Kociński and coworkers reported the use of related phenyltetrazole derivatives in a stereoselective synthesis of *trans*-1,2-disubstituted alkenes.^[11] One-pot Julia-Lythgoe olefination by these substrates is believed to proceed via a Smiles rearrangement on the heteroaromatic ring (Figure S1A in SI). Furthermore, benzimidazole derived proton pump inhibitors are also subject to the Smiles rearrangement under acidic conditions (Figure S1B in SI).^[12] The reactivity of MSBT and this class of molecules led us to hypothesize that a broader class of heteroaromatic methylsulfones might be exploited to develop a new class of thiol reactive molecules for thiol-selective peptide and protein conjugation.

To explore the reactivity of this class of molecules and the relative stability of the thiol conjugates they might form, we synthesized a family of these molecules guided by the known reactivity of Julia-Kociński reagents. A generalized synthesis is shown in Scheme 1. Methylation of the thiol group of hetero aromatic derivatives **2** gave the corresponding methylthioether compounds. Methylthioether derivatives **3** were converted to methylsulfone compounds **4** by oxidation with hydrogen peroxide in the presence of ammonium molybdate as a catalyst.^[13]

Next, in order to study protein conjugation, fluorescein derivatives of the sulfone and maleimide compounds **7** and **10** were synthesized as shown in Scheme 2. The methylthioethers **5**, which were synthesized as shown Scheme S2 (see SI), were oxidized with *m*CPBA to afford the corresponding methylsulfone compounds **6**. Azide groups of compounds **6** were reduced by hydrogenation or with the Staudinger reaction, and these primary amines were coupled with commercially available NHS-5(6)-fluorescein[®] to yield the desired fluorescein compounds **7**. The maleimide derivative was prepared by coupling of Boc-protected 1,3-propane diamine **8** with NHS-5(6)-fluorescein[®]. Deprotection gave the primary amines followed by coupling with Sulfo-SMCC[®] yielded the desired maleimide **10** as a single isomer at the 5-position. PEGylated derivative **11** was synthesized with commercially available NHS-PEG derivative, SUNBRIGHT AS-050[®], from azide **6c** in two steps (Scheme 3).

We first examined the relative reactivity of sulfones (**1**, **4a–d**) and commercially available maleimide (**12**) with (*R*)-2-acetamido-*N*-benzyl-3-mercapto-propanamide **13** (Table 1 and Figure S2a–d in SI). To a solution of 1.2 equivalents of protected cysteine **13** in THF/200

mM PBS, compounds **1**, **4a–d**, or **12** were added, and the reactions were monitored by HPLC (220 nm). The conversion of phenyltetrazole compound **4a**, which is known as a key structure for Julia-Kocienski olefination, was faster than that of benzothiazole **1**, while triazole compound **4c** did not react with **13**. We were pleased to discover that phenyloxadiazole compound **4b** reacted most rapidly and provided desired conjugate **14c** in quantitative yield after only 5 min (Table 1, entry 3). In contrast, benzimidazole compound **4d**, chosen based on the core structure of known proton pump inhibitors, was unreactive. With **4d**, activation of the benzimidazole ring by strong acid might be necessary for reactivity.

Given the promising reactivity of phenyloxadiazole compound **4b**, we studied its reactivity with other potential nucleophilic species in proteins. Reactivities with amino acid derivatives **13** and **15a–e** were evaluated (Scheme S4 in SI). Only the protected cysteine-phenyloxadiazole adduct was observed. The other amino acid derivatives (**15a–e**) did not react with **4b**. Thus, phenyloxadiazole derivative **4b** was selective for the free thiol of cysteine and substantially more reactive than **1** (Table 1).

In order to further investigate the reactivity of **4b**, we studied the effects of buffer concentration and pH dependence on the reaction of **4b** with **13** (Table S1 in SI). When this reaction was performed in pure organic solvents (e.g., THF, entry 1), the desired adduct **14c** was not observed. In the case of one to one mixture of THF and water, **14c** was detected but conversion was low (entry 2). For reactions at pH 7.4, reaction conversion increased with increasing buffer concentration (entries 3–7). In HEPES or Tris buffer at pH 7.4 with THF (1:1, v/v), % conversion was similar to that in PBS (entries 6, 11, and 12). At pH 7.0 and pH 8.0, conversion was complete within 30 min, but % conversion at pH 5.8 was moderate (entry 8–10).

We also studied the substrate scope of the thiol modifying reaction (Table 2). **4b** reacted with cysteine derivatives **16b** and **16c** with free amino and carboxyl groups, respectively (entries 2 and 3). These studies suggest that the sulfone compound should react with the thiol group of cysteines in peptides or proteins regardless of location.

Next, we investigated whether our sulfone derivatives were able to selectively modify cysteine in the context of a protein. We evaluated reactions with recombinant human serum albumin (HSA), in which Cys34 is reactive, and a fusion protein of our design based on maltose binding protein, MBP-C-HA, which has a single cysteine in the linker between the protein and the HA peptide tag. To a solution of recombinant HSA in PBS was added compound **6c** (10 eq) at room temperature, and the reaction was incubated for 2 h at room temperature. ESI-MS analysis indicated that HSA was modified with a single label (Figure 2A and Figure S3 in SI). The result suggests that Cys34 was labeled with compound **6c**. To obtain further evidence for specific cysteine labeling, we reduced the disulfide bond of the homodimer MBP-C-HA by treatment with DTT or TCEP. The reduced MBP-C-HA was incubated in the presence or absence of iodoacetamide (IAA, 50 eq) at room temperature. After 1 h, fluorescein-linked phenyloxadiazole **7c** (10 eq) was added to the reaction solution and then the solution was incubated for 1 h. Analysis by SDS-PAGE showed strong fluorescence of the MBP-C-HA conjugate was observed without IAA blocking (lane 2 and 4 in Figure 2B) even though pretreatment of reduced MBP-C-HA with IAA suppressed the labeling for compound **7c** (lane 3 and 5 in Figure 2B). Unreduced MBP-C-HA migrated as a homodimer of around 100 kDa and did not fluoresce. Additionally, the fluorescein-attached MBP-C-HA conjugate was detected by ESI-MS (Figure S4b in SI). These results suggest that compound **7c** modified only the single cysteine in MBP-C-HA and is exquisitely chemoselective since no labelling of the disulfide linked homodimer was observed. The introduction of poly(ethylene) glycol chains (PEGylation) is widely used to modify the

pharmacokinetic properties of proteins, typically through maleimide labelling chemistry.^[14] To study the potential of this sulfone approach in protein PEGylation we studied the reaction of compound **11**, which has a linear 5-k Da PEG chain, with MBP-C-HA. As demonstrated in Figure 2C and Figure S5 in SI, **11** selectively pegylated MBP-C-HA at a single site suggesting the broad potential of this chemistry in the preparation of protein therapeutics.

Finally, we investigated the stability of our conjugates under several conditions. Significantly, under basic conditions, all heteroaryl compounds (**14a–c**) were more stable than the maleimide-cysteine conjugate (**14d**). For example, no degradation of the cysteine-benzothiazole conjugate **14a** was observed under these basic conditions. The succinimide ring of the maleimide-cysteine conjugate, however, was opened under these basic conditions, and hydrolyzed products were observed (Figure S6d in SI). Under acidic conditions, no degradation of heteroaryl conjugates (**14a–c**) was observed after 3 days. In the presence of glutathione at neutral pH for 5 days, **14a–c** were stable, but an exchange reaction occurred from *N*-benzyl cysteine to glutathione in maleimide-cysteine conjugate **14d** (Figure S8d in SI). These results indicate that these heteroaromatic- cysteine conjugates have superior stability relative to the maleimide-cysteine conjugate in a variety of conditions.

To evaluate our conjugates under therapeutically relevant conditions, we evaluated the stability of the cysteine conjugates (**14a–d**) and MBP-C-HA conjugates (**20a–d**) in human plasma. Cysteine conjugates (**14a–d**, 50 μ L) in DMSO were added to human plasma (950 μ L), and the solutions were incubated for 72 h at 37 °C. After precipitation of protein with CH₃CN, the supernatants were analyzed by HPLC, and % conjugate remaining was calculated based on an internal standard. As shown in Figure 3A, cysteine-heteroaryl conjugates **14a–c** were more stable than cysteine-maleimide conjugate **14d** (green line, $T_{1/2}$ =4.3 h); benzothiazole derivative **14a** had the longest half-life (blue line, $T_{1/2}$ =191 h). After 72 hours, the solution of **14c** was analyzed by HPLC with compound **2b**, which was expected as a by-product (Figure S9d in SI) and injected to LC/MS to confirm two unknown peaks (Figure S9e in SI). The result showed that the peak at 9.79 min of HPLC eluted as compound **2b**, and both the *exo*-methylene β -eliminated product and compound **2b** were detected by LC/MS. To study the stability of the labelled MBP-C-HA protein monomers, disulfides were reduced with TCEP and the protein incubated with fluorescein-modified reactants (**7a–c** and **10**). After the removal of excess small molecules, MBP-C-HA conjugates (**20a–d**) were mixed with human plasma, and the solutions were incubated for 72 h at 37 °C. The derivative **7c** reacted more rapidly than the other fluorescein-modified reactants with MBP-C-HA. The efficiency of reaction of **7b** was similar to that of maleimide compound **10**, known to react with cysteine residues of proteins (Figure 3B and S10 in SI). Benzothiazole derivative **7a** reacted slowly with MBP-C-HA and the protein monomer was re-oxidized to the disulfide dimer (Figure S10 in SI, red arrow). The stabilities of MBP-C-HA conjugates (**20a–d**) in human plasma were analyzed by SDS-PAGE (Figure 3C). No fluorescent bands were observed at the molecular weight expected for the sulfone-HSA conjugates of **7a–c**. Fluorescence bands gradually appeared in the maleimide-MBP-C-HA conjugate lanes (Figure 3C, red arrow) at the molecular weight of HSA. This assay was designed to explore the exchange reaction from labelled MBP-C-HA to Cys34 of HSA, the most abundant protein in human plasma and a troublesome side reaction of therapeutic drug conjugates. In the Coomassie-stained gel of maleimide-protein conjugate **20d**, we observed that the free MBP-C-HA monomer, which was re-produced by exchange reaction with HSA in human plasma, formed a heterodimer with another protein, which was likely HSA (Figure 3C, green arrow). The MBP-C-HA homodimer was not formed under these conditions (Figure 3C, blue arrow). The heterodimer was also observed with the benzothiazole-MBP-C-HA conjugate (**20a**) incubated in plasma. As previously mentioned, the conjugation

reaction with benzothiazole derivative **7a** was slow, therefore the solution included MBP-C-HA homodimer, formed by re-oxidation during the conjugation reaction, and an exchange reaction from the MBP-C-HA homodimer to an S-S exchanged heterodimer occurred (Figure 3C, green arrow). The half-lives of conjugates **20a–d** were 135, 84.4, 117, 59.9 h, respectively. All heteroaromatic-protein conjugates were more stable than the maleimide-protein conjugate in plasma.

In conclusion, we developed a class of sulfone derivatives for applications in protein conjugation chemistry, and we have compared the newly synthesized conjugates to maleimide conjugates. Methylsulfonyl 5-member monocyclic compounds, such as phenyltetrazole or phenyloxadiazole, reacted rapidly and specifically with thiols in small molecules and proteins with exquisite chemoselectivity at biologically relevant pHs, 5.8 to 8.0. Designer heteroaromatic sulfones allowed for the selective introduction of a fluorophore and poly(ethylene) glycol chains (PEGylation), and provided protein conjugates with superior stability in human plasma as compared to maleimide-conjugated proteins. Given the speed, selectivity, and stability of the sulfone-cysteine chemistry described herein, we anticipate that this “Thiol-phenyloxadiazole Click” approach will find broad application in peptide and protein chemistry and for the development of antibody drug conjugates.^{[5][8a][15][16]}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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16. Through an agreement with Sigma-Aldrich, compound **4b** will be commercially available as product L511749. Other products based on this chemistry will be introduced shortly.

Figure 1.
Methylsulfonyl benzothiazole **1**, a selective protein thiol blocking reagent.

Figure 2.

Thiol labeling of recombinant HSA and MBP-C-HA using 1,3,4-oxadiazole derivatives **6c**, **7c**, and **11**. [A] Conjugation reaction of oxadiazole compound (**6c**) with recombinant HSA, and the ESI-MS spectra of the conjugate; [B] Gel image of conjugation reaction of fluorescein-attached compound (**7c**) in the presence or absence of iodoacetamide; [C] Gel image of conjugation reaction of PEGylated oxadiazole compound (**11**) with reduced MBP-C-HA protein.

Figure 3.

Stability of the cysteine conjugates (**14a–d**) and MBP-C-HA conjugates (**20a–d**) in human plasma, benzothiazole (blue), phenyltetrazole (black), phenyloxadiazole (red), and meleimide (green). [A] Time-dependent stability of the cysteine conjugates (**14a–d**) in human plasma; [B] Relative reaction rate of conjugation reaction of fluorescein-attached compounds (**7a–c** and **10**) with MBP-C-HA protein in PBS (pH 7.4); [C] Gel image and plot of % remaining vs time of fluorescein-attached MBP-C-HA conjugates (**20a–d**).

Scheme 1.

Synthesis of methylsulfone derivatives. Reagents and conditions: a) CH_3I , TEA, THF, rt, 1–2 h, **3a** 78%, **3b** 94%, **3c** 84%; b) $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$, 30% H_2O_2 , $\text{C}_2\text{H}_5\text{OH}$, rt, 2–6 h, **4a** 76%, **4b** 54%, **4c** 84%, **4d** 89%. TEA = triethylamine, THF = tetrahydrofuran.

Scheme 2.

Synthesis of fluorescein derivatives. Reagents and conditions: a) *m*CPBA, CH₂Cl₂, **6a** 51%, **6b** 63%, **6c** 37%; b) 1) 10% Pd/C, H₂, THF or PPh₃, H₂O, THF; 2) NHS-5(6)-fluorescein[®], TEA, DMSO, **7a** 12%, **7b** 28%, **7c** 14%; c) NHS-5(6)-fluorescein[®], TEA, DMSO, **9a** 38%, **9b** 54%; d) 1) TFA, CH₂Cl₂; 2) Sulfo-SMCC[®], TEA, DMF, 51%. *m*CPBA = *m*-chloroperbenzoic acid, DMSO = dimethylsulfoxide, TFA = trifluoroacetic acid, DMF = *N,N*-dimethylformamide, TEA = triethylamine.

Scheme 3.

Synthesis of PEGylated derivative. Reagents and conditions: a) 1) PPh_3 , H_2O , THF; 2) SUNBRIGHT AS-050[®], CH_3CN , 14%.

Table 1Reaction of methylsulfonyl or maleimide derivatives **1**, **4a–d**, and **12** with protected cysteine **13**^[a]

entry	Substrate	R=	Conversion (%) [5 min] ^[b]
1	1		45 ^[c] (14a)
2	4a		93 (14b)
3	4b		>99 (14c)
4	4c		No reaction
5	4d		No reaction
6	12		>99 ^[d] (14d)

^[a] Reagents and Conditions: (*R*)-2-Acetamido-*N*-benzyl-3-mercaptopropanamide **13** (1.2 eq.), THF/pH 7.4, 200 mM PBS (1/1=v/v), room temperature;

^[b] Conversion was measured by HPLC (220 nm),

^[c] 86% [60 min];

^[d] diastereo mixture. PBS = phosphate buffered saline.

Table 2Reactions of compound **4b** with thiol substrates

entry	R-SH	Isolated yield (%)
1	 16a	99 (17a)
2	 16b	78 (17b)
3	 16c	83 (17c)
4	<i>n</i> -C ₁₂ H ₂₅ SH 16d	90 (17d)

Table 3

Stability evaluation

Conjugate	Conditions ^[a]	% Remaining ^[b]
14a	A	> 99
	B	> 99
	C	> 99
	D	94
14b	A	88
	B	98
	C	> 99
	D	> 99
14c	A	82
	B	> 99
	C	> 99
	D	99
14d	A	2.7
	B	71
	C	69
	D	34

^[a] (A) K₂CO₃ (4 eq.), THF/H₂O (1/1), room temperature, 20 hours; (B) THF/0.1 M HCl_{aq} (1/1), room temperature, 3 days; (C) THF/pH 4.0 200 mM PBS (1/1), room temperature, 3 days; (D) glutathione (3 eq), THF/pH 7.4 200 mM PBS (2/3), 37 °C, 5 days;

^[b] HPLC (254 nm).