Bioconjugation Hot Paper

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

*Narihiro Toda, Shigehiro Asano, and Carlos F. Barbas III**

Proteins modified with fluorescent or biologically active agents are powerful reagents in chemistry, biology, and medicine.[1] Selectivity for certain of the natural 20 amino acids in the presence of many unprotected amino acid residues is necessary for the selective modification of proteins.[2] The development of bioconjugation reactions 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 pair in native proteins, can be readily achieved as a consequence of the relatively low pK_a value 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, alkylation reagents for thiols (such as α-halocarbonyl derivatives) or Michael acceptors (such as maleimide derivatives) are the most commonly used, and their reactivity profiles have been studied extensively. 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 position of endogenous albumin in the blood.[6] Although maleimide chemistry is a powerful tool for the 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 cysteine and glutathione residues 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, methylsulfonylbenzothiazole (**1**, MSBT; Figure 1) was reported as a thiol-blocking reagent.[9] The stability of MSBT-blocked thiols, however, and the broad versatility of methylsulfonyl-functionalized heteroaromatic compounds in thiol conjugation chemistry remains unexplored. Herein, we designed and optimized methylsulfonyl-functionalized heteroaromatic derivatives for rapid protein/peptide conjugation and demonstrate that these conjugates can be significantly more stable than maleimide–cysteine conjugates in human plasma, thereby providing a promising and versatile new approach to protein and peptide conjugates for chemistry, biology, and medicine.

Figure 1. Methylsulfonylbenzothiazole (**1**), a selective protein-thiol blocking reagent.

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] Furthermore, Kocieński and co-workers 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 through a Smiles rearrangement on the heteroaromatic ring (see Figure S1A in the Supporting Information). Furthermore, benzimidazole-derived proton-pump inhibitors are also subject to the Smiles rearrangement under acidic conditions (see Figure S1B in the Supporting Information).[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 conjugation of peptides and proteins.

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–Kocieński reagents. A generalized synthesis is shown in Scheme 1. Methylation of the thiol group of heteroaromatic derivatives **2** gave the corresponding methyl thioether compounds. Methyl thioether derivatives **3** were converted into 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 (Scheme 2). The methyl thioethers **5** (see

[*] Dr. N. Toda,[+] Dr. S. Asano,[+] Prof. Dr. C. F. Barbas III
The Skaggs Institute for Chemical Biology and the Departments of Chemistry and Molecular and Cell Biology
The Scripps Research Institute
10550 North Torrey Pines Road, La Jolla, CA 92037 (USA)
E-mail: carlos@scripps.edu

[+] These authors contributed equally to this work.

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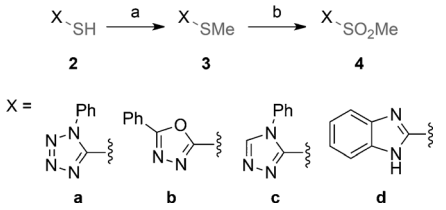
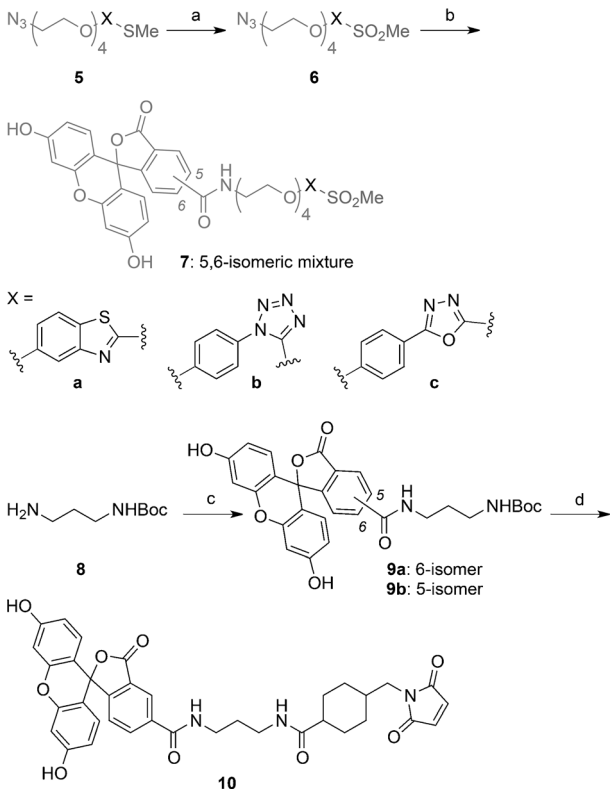
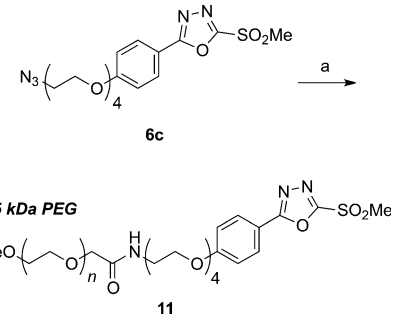


Scheme 1. Synthesis of methylsulfone derivatives. Reagents and conditions: a) CH_3I, TEA, THF, RT, 1–2 h, **3a** 78%, **3b** 94%, **3c** 84%; b) $(NH_4)_6[Mo_7O_{24}]\cdot 4H_2O$, 30% H_2O_2, C_2H_5OH, RT, 2-6 h, **4a** 76%, **4b** 54%, **4c** 84%, **4d** 89%. TEA = triethylamine, THF = tetrahydrofuran.

Scheme S2 in the Supporting Information) were oxidized with *m*CPBA to afford the corresponding methylsulfone compounds **6**. The azide groups of compounds **6** were reduced by hydrogenation or through 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 Boc-protected 1,3-propanediamine (**8**) with NHS-5(6)-fluorescein. Deprotection gave the primary amines, and a subsequent 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 ME-050AS from azide **6c** in two steps (Scheme 3).



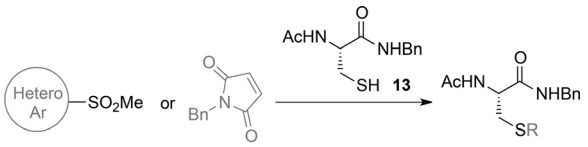
Scheme 2. Synthesis of fluorescein derivatives. Reagents and conditions: a) *m*CPBA, CH_2Cl_2, **6a** 51%, **6b** 63%, **6c** 37%; b) 1. 10% Pd/C, H_2, THF or PPh_3, H_2O, THF; 2. NHS-5(6)-fluorescein, TEA, DMSO, **7a** 12%, **7b** 28%, **7c** 14%; c) NHS-5(6)-fluorescein, TEA, DMSO, **9a** 38%, **9b** 54%; d) 1. TFA, CH_2Cl_2; 2. Sulfo-SMCC, TEA, DMF, 51%. Boc = *tert*-butoxycarbonyl, *m*CPBA = *m*-chloroperbenzoic acid, NHS-5(6)-fluorescein = [fluorescein-5(6)-carboxamido]hexanoic acid *N*-hydroxysuccinimide ester, DMSO = dimethylsulfoxide, TFA = trifluoroacetic acid, DMF = *N*,*N*-dimethylformamide.

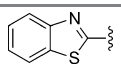
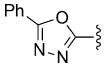
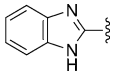
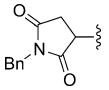


Scheme 3. Synthesis of PEGylated derivative. Reagents and conditions: a) 1. PPh_3, H_2O, THF; 2. SUNBRIGHT ME-050AS, CH_3CN, 14%.

We first examined the relative reactivity of sulfones (**1**, **4a**–**d**) and commercially available maleimide (**12**) with (*R*)-2-acetamido-*N*-benzyl-3-mercaptopropanamide (**13**; Table 1 and see Figure S2a–d in the Supporting Information). Compounds **1**, **4a**–**d**, or **12** were added to a solution of 1.2 equivalents of protected cysteine **13** in THF/200 mM PBS, and the reactions monitored by HPLC (220 nm). The conversion of phenyltetrazole compound **4a**, which is known as a key

Table 1: Reaction 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 equiv), 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] Diastereomeric mixture. PBS = phosphate buffered saline.

structure for Julia–Kocieński 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 the 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. In the case of **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. Thus, the reactivity of **4b** with amino acid derivates **13** and **15a–e** was evaluated (see Scheme S4 in the Supporting Information). 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).

To further investigate of the reactivity of **4b** we studied the effects of buffer concentration and pH dependence on the reaction of **4b** with **13** (see Table S1 in the Supporting Information). When this reaction was performed in pure organic solvents (e.g. THF; Table S1, entry 1), the desired adduct **14c** was not observed. In the case of a 1:1 mixture of THF and water, **14c** was detected but conversion was low (Table S1, entry 2). For reactions at pH 7.4, reaction conversion increased with increasing buffer concentration (Table S1, entries 3–7). The conversion in HEPES (2-[4-(2-hydroxethyl)-1-piperazinyl]ethanesulfonic acid) or Tris (tris(hydroxymethyl)aminomethane) buffer at pH 7.4 with THF (1:1, v/v) was similar to that in PBS (Table S1, entries 6, 11, and 12). Conversion was complete within 30 min at pH 7.0 and pH 8.0, but was moderate at pH 5.8 (Table S1, entries 8–10).

We also studied the substrate scope of the thiol-modifying reaction (Table 2). **4b** reacted with cysteine derivatives **16b** and **16c**, which have free amino and carboxy groups, respectively (entries 2 and 3). These studies suggest that the sulfone compound should react with the thiol group of cysteine residues in peptides or proteins regardless of their 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 the maltose-binding protein, MBP-C-HA, which has a single cysteine residue in the linker between the protein and the HA peptide tag. Compound **6c** (10 equiv) was added to a solution of recombinant HSA in PBS 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 see Figure S3 in the Supporting Information),

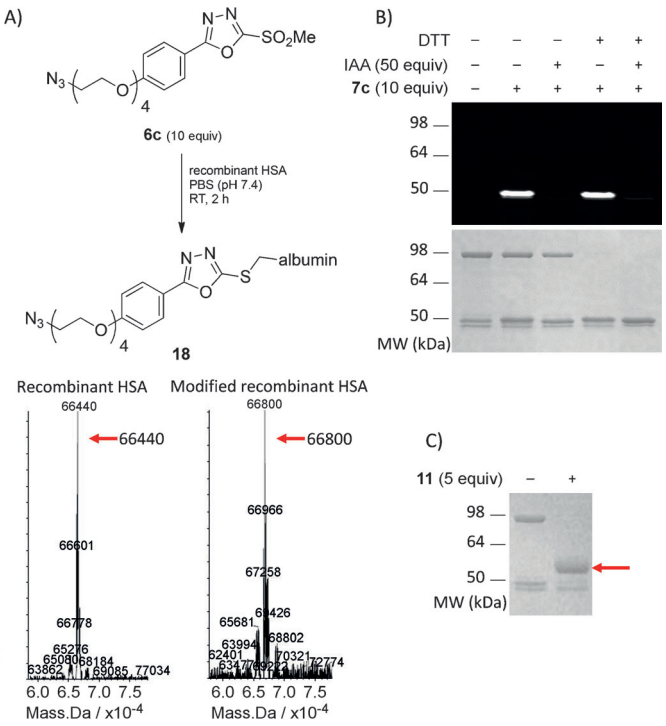
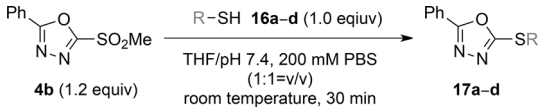


Figure 2. Thiol labeling of recombinant HSA and MBP-C-HA using 1,3,4-oxadiazole derivatives **6c**, **7c**, and **11**. A) Conjugation reaction of oxadiazole compound (**6c**) with recombinant HSA, and the ESI-MS spectra of the conjugate. B) Gel image of the conjugation reaction of fluorescein-attached compound (**7c**) in the presence or absence of iodoadetamide. C) Gel image of the conjugation reaction of PEGylated oxadiazole compound (**11**) with reduced MBP-C-HA protein.

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



Entry	R-SH	Yield [%]
1	BocHN, CO_2Me, HS **16a**	99 (**17a**)
2	H_2N, CO_2Me, HS, HCl **16b**	78 (**17b**)
3	AcHN, CO_2H, HS **16c**	83 (**17c**)
4	n-$C_{12}H_{25}SH$ **16d**	90 (**17d**)

which suggest 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 1,4-dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The reduced MBP-C-HA was incubated in the presence or absence of iodoacetamide (IAA, 50 equiv) at room temperature. Fluorescein-linked phenyloxadiazole **7c** (10 equiv) was added to the reaction solution after 1 h, and then the solution was incubated for 1 h. Analysis by SDS-PAGE (sodium dodecyl sulfate polyacrylamide electrophoresis) showed strong fluorescence of the MBP-C-HA conjugate without IAA blocking (lanes 2 and 4 in Figure 2B) even though pretreatment of reduced MBP-C-HA with IAA suppressed the labeling for compound **7c** (lanes 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 the Supporting Information). These results suggest that compound **7c** modified only the single cysteine residue in MBP-C-HA and is exquisitely chemoselective since no labeling 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-labeling reactions.[14] To study the potential of this sulfone approach in protein PEGylation we studied the reaction of compound **11**, which has a linear 5 kDa PEG chain, with MBP-C-HA. Compound **11** selectively pegylated MBP-C-HA at a single site (Figure 2C and Figure S5 in the Supporting Information), thus suggesting the broad potential of this approach for the preparation of protein therapeutics.

Finally, we investigated the stability of our conjugates under several conditions. Significantly, under basic conditions, all the heteroaryl compounds (**14a–c**) were more stable than the maleimide–cysteine conjugate (**14d**, Table 3). 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 (see Figure S6d in the Supporting Information). No degradation of the heteroaryl conjugates (**14a–c**) was observed after 3 days in acidic conditions. **14a–c** were stable in the presence of glutathione at a neutral pH value for 5 days, but an exchange reaction occurred from *N*-benzylcysteine to glutathione in maleimide–cysteine conjugate **14d** (see Figure S8d in the Supporting Information). 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. Solutions of the cysteine conjugates (**14a–d**, 50 μL) in DMSO were added to human plasma (950 μL), and the solutions were incubated for 72 h at 37 °C. After precipitation of protein with CH_3CN, the supernatants were analyzed by HPLC, and the amount of conjugate remaining was calculated based on an internal standard. Cysteine-heteroaryl conjugates **14a–c** were more stable than cysteine–maleimide conjugate **14d** (green line, $t_{1/2} = 4.3$ h; Figure 3A); benzothiazole derivative **14a** had the longest half-life (blue line, $t_{1/2} = 191$ h). After 72 h, the solution of **14c** was analyzed by HPLC with compound **2b**, which was expected as a by-product (see Figure S9d in the Supporting Information) and then by LC/MS to confirm two unknown peaks (see Figure S9e in the Supporting Information). The result showed that the peak at 9.79 min in the HPLC trace was compound **2b**, and both the *exo*-methylene β-eliminated product and compound **2b** were detected by LC/MS. To study the stability of the labeled MBP-C-HA protein monomers, the disulfides were reduced with TCEP and the protein incubated with fluorescein-modified reactants (**7a–c** and **10**). After removal

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) K_2CO_3 (4 equiv), THF/H_2O (1:1), room temperature, 20 h; B) THF/0.1 M HClaq (1:1), room temperature, 3 days; C) THF/pH 4.0 200 mM PBS (1:1), room temperature, 3 days; D) glutathione (3 equiv), THF/pH 7.4 200 mM PBS (2:3), 37 °C, 5 days. [b] HPLC (254 nm).

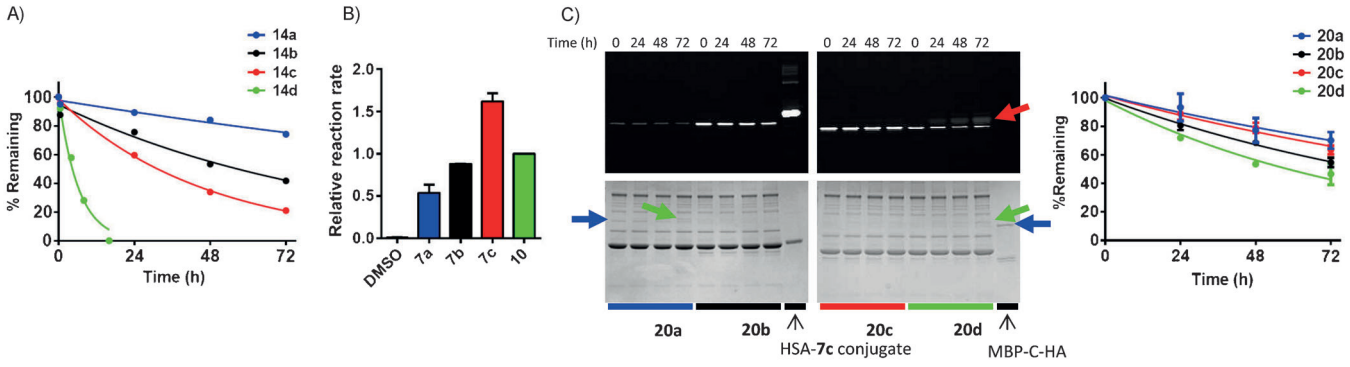


Figure 3. Stability of the cysteine conjugates (**14a–d**) and MBP-C-HA conjugates (**20a–d**) in human plasma, benzothiazole (blue), phenyltetrazole (black), phenyloxadiazole (red), and meleimide (green). A) Time-dependent stability of the cysteine conjugates (**14a–d**) in human plasma. B) Relative reaction rate of the 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 percentage remaining versus time of fluorescein-attached MBP-C-HA conjugates (**20a–d**).

of excess small molecules, the MBP-C-HA conjugates (**20a–d**) were mixed with human plasma, and the solutions were incubated for 72 h at 37 °C. The phenyloxadiazole derivative **7c** reacted more rapidly than the other fluorescein-modified reactants with MBP-C-HA. The efficiency of the reaction of **7b** was similar to that of maleimide compound **10**, which is known to react with cysteine residues of proteins (Figure 3B and see Figure S10 in the Supporting Information). Benzothiazole derivative **7a** reacted slowly with MBP-C-HA and the protein monomer was re-oxidized to the disulfide dimer (see Figure S10 in the Supporting Information, 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**. Fluorescent 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 labeled MBP-C-HA to Cys34 of HSA, the most abundant protein in human plasma and a troublesome side reaction of therapeutic drug conjugates. We observed in the Coomassie-stained gel of maleimide–protein conjugate **20d** that the free MBP-C-HA monomer, which was re-generated by an 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 when the benzothiazole–MBP-C-HA conjugate (**20a**) was 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 reoxidation 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, and 59.9 h, respectively. All the heteroaromatic–protein conjugates were more stable than the maleimide–protein conjugate in plasma.

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

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[16] Through an agreement with Sigma–Aldrich, compounds **4b** and **6c** will be commercially available as products L511757 and L511722. Other products based on this approach will be introduced shortly.