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Methylsulfonyl Benzothiazole (MSBT): A Selective Protein Thiol Blocking Reagent

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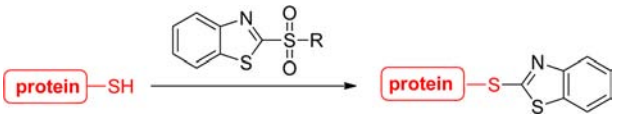
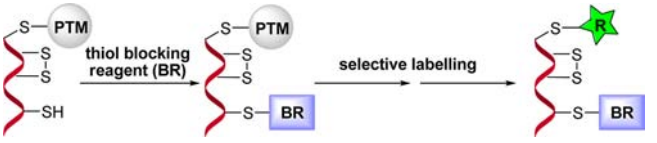
ABSTRACT



A new thiol blocking reagent, methylsulfonyl benzothiazole, was discovered. This reagent showed good selectivity and high reactivity for protein thiols.

Protein cysteine residues are targets of numerous post-translational modifications (PTM) that are essential to maintain cell redox homeostasis as well as signaling.[1] Modifications at cysteine residues are caused by their interaction with reactive oxygen/nitrogen species (ROS/RNS) in response to cellular oxidative damage.[1a,2] Most of these modifications, which comprise the thiol proteome, are reversible and encompass a range of functional groups with very distinctive chemistry including mixed disulfides (RS-SR′, SR′ low molecular weight thiols), nitrosothiols (RS-NO), sulfenic acids (RSOH), sulfinic acids (SO_2H), sulfonic acids (SO_3H), S-lipidation (palmytoylation, RS-COR), and perthiols (RS-SH). This diversity of functionality has possessed some difficulty in selectively determining each modification.[3] Nevertheless many advances have been made in this field, in particular employing chemical methods to detect specific thiol modifications.[3,4] In these methods, a common step involves selective blocking of unmodified thiols (reduced thiols) (Scheme 1).

Scheme 1. Chemical Approach to Study Protein Posttranslational Modifications (PTM) at Cys



To a great extent, the efficiency of these assays relies on the efficiency of the thiol blocking step. Many research efforts have been made to identify reagents that enable blocking or labeling of protein thiols with high selectivity and conversion yields.[5] Among those, thiol-alkylation reagents such as iodoacetamides (IAM) and N-substituted maleimides (NSM) are by far the most commonly used and

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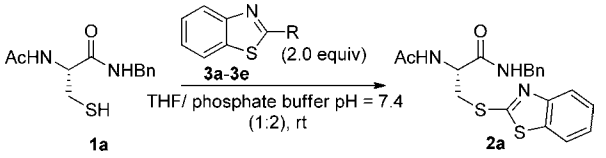
their reactivity profiles have been extensively studied.[6] It is known that, under certain conditions, IAM and NSM can modify other reactive amino acids (e.g., Lys and His).[7] As a consequence, it has been suggested that the selection of the thiol blocking reagent should not be arbitrary. Due to the disparate reactivity of various thiols influenced by their localization within the protein and physiological environment, one must consider the unique property of target protein and necessary experimental conditions to select proper thiol blocking agents.[8]

On the basis of all the above-mentioned, the development of new thiol blocking reagents that possess distinctive reactivity profiles from currently known compounds is needed. Our consideration in this subject was to explore molecules that could react with free thiols via nucleophilic aromatic substitution (NAS). We developed this idea from previous work in our laboratory that studied the reactions of 2-mercaptobenzothiazole (2-SHBT) toward sulfenamides and alkyl disulfides.[9] The results revealed that 2-SHBT was inert to disulfide exchange reactions but showed significant reactivity against more reactive electrophiles, i.e. sulfenamides. In contrast, other aromatic thiols such as thiophenol, 2-mercaptopyridine, and 2-mercaptopyrimidine were found reactive to both alkyl disulfides and sulfenamides. It should be noted that the disulfide exchange is a dynamic equilibrium and thus the progress of the reaction is controlled by both the electrophilicity/nucleophilicity of the starting disulfide/thiol pair as well as those generated. These results suggest that the electron withdrawing effect of the benzothiazole ring decreases the reactivity of the corresponding thiol and inhibits disulfide formation. We envisioned that by placing a leaving group at the C-2 position, benzothiazole might be vulnerable for nucleophilic attack by thiols via the NAS mechanism. With this idea in mind, we designed a series of experiments to examine whether the benzothiazole moiety could be employed as an electrophilic trap for thiols and Cys residues. Here we report our results.

We first examined the reactivity of various benzothiazole substrates containing different leaving groups at the C-2 position (Table 1). A cysteine derivative **1a** was used as a thiol model. In a typical experimental setting, to a solution of **1a** in 1:2 THF/phosphate buffer (200 mM, pH = 7.4) were added 2 equiv of benzothiazole substrate respectively. The reaction was monitored by TLC. We tested several commercially available 2-halogenated benzothiazoles (**3a**–**3c**). These compounds are known to react with thiol at high temperature and under strong basic conditions.[10] However, under mild and biologically mimic conditions, these substrates displayed very poor reactivity and only a trace amount of the desired product **2a** was formed. The reaction using 2-diazo substrate **3d** resulted in a complicated mixture of products, and only a small amount of **2a** was produced (judging by TLC and crude NMR). Interestingly, 2-methylsulfonyl benzothiazole (MSBT) showed very high reactivity toward **1a**, with almost quantitative formation of **2a** within 20 min. To the best of our knowledge, this was the first example illustrating the excellent reactivity of MSBT toward alkylthiols in aqueous solutions.

Table 1. Reactions of Benzothiazole Substrates with Model Substrate **1a**



entry	R	time	**2a** % yield
1	—Cl **3a**	4 h	trace
2	—Br **3b**	4 h	trace
3	—I **3c**	4 h	trace
4	—N≡N $^{\oplus}$ $^{\ominus}$ BF_4 **3d**	20 min	< 20
5	—S(=O)(=O)— **3e**	20 min	95

We next investigated whether the pronounced reactivity of MSBT **3e** toward thiols could also occur with other potential nucleophilic species found in proteins. A series of amino acid derivatives (**4a**–**4f**) were then tested under the same conditions. As shown in Scheme 2, side chain functionalities of serine, tyrosine, tryptophan, and methionine are inert to MSBT (5 equiv). In addition, lysine and histidine substrates (**4e** and **4f**) did not react with MSBT to form any product (monitored by TLC), even after 4 h. These outcomes were expected, as known reactions of MSBT with amines and alcohols require high temperature and/or strong basic media.[11] Nevertheless, these results suggested that MSBT is a thiol-selective blocking reagent.

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Scheme 2. Control Experiments

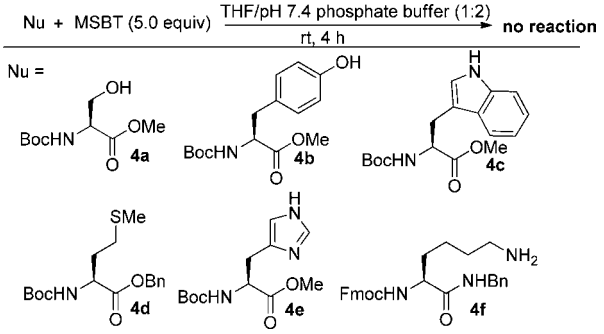
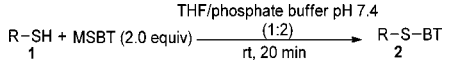
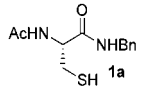
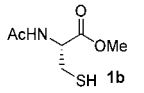
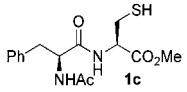
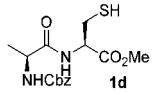
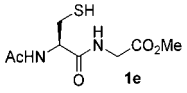
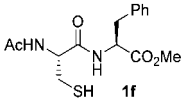
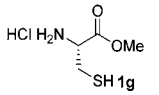


Table 2. Reactions of MSBT with RSH Substrates

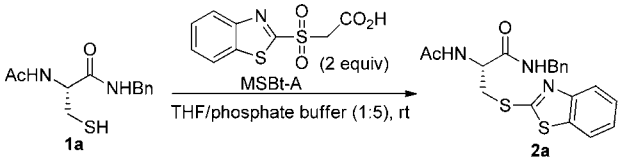


entry	R-SH	% yield
1	 1a	>95
2	 1b	>95
3	 1c	>95
4	 1d	>95
5	 1e	85
6	 1f	86
7	 1g	97

In order to explore the generality of an MSBT mediated thiol-blocking reaction, a series of cysteine derivatives (**1a**−**1g**) were tested. As shown in Table 2, in all the cases the reaction went smoothly and the desired products were obtained in high yields. We did not observe any byproducts in these reactions.

To further expand our understanding of the reactivity profile of MSBT, we studied the effect of pH on the reaction. This was driven by the fact that pK_a values of protein thiols are variable within distinctive protein domains and pH fluctuates in different cell compartments.[12] Because the reactivity of many electrophiles toward SH depends on the concentration of thiolate, disruption in the pH will affect the equilibrium between thiol (RSH) and thiolate (RS^-) and therefore change the thiol blocking efficiency.[13] To study this problem, we prepared a water-soluble MSBT reagent (MSBT-A). This compound allowed us to study pH effects in high aqueous buffer containing systems. The results are summarized in Table 3. In acidic media (pH = 6.2), MSBT-A reacted with **1a** slowly and only a small amount of product was obtained after 20 min. At pH 7.0 or 7.4, the reaction went well and afforded the blocking product in good yield in 20 min. When the pH was 9.0, the reactivity of **1a** was greatly enhanced and the reaction was completed in a few minutes. Interestingly, when this reaction was performed in pure organic solvents such as pure THF, we did not observe the formation of **2a** even after 1 h. With these results we concluded that the reactions between MSBT substrates and RSH largely rely on the thiolate concentration. Similar reactivity profiles were observed with both IAM and NSM derivatives.[13,14]

Table 3. pH Dependence of MSBT Reaction



pH	time	% yield
6.2	20 min	34
7.0	20 min	87
7.4	20 min	95
9.0	5 min	95

We also tested the stability of the thiol-blocking adducts (RS-Bt) under common conditions used in protein labeling experiments. For example, tris(2-carboxyethyl)phosphine (TCEP) or dithiothreitol (DTT) are often used for protein thiol reduction or quenching an excess of thiol-blocking reagent. We found that RS-Bt adducts did not show any reaction or decomposition in the presence of an excess of TCEP or DTT (see Supporting Information for details).

Finally we tested the capability of MSBT and MSBT-A in blocking protein thiol residues. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), whose biological function has been shown to be mediated by its cysteine thiol modifications,[15] was used as the model. Briefly (Figure 1A), reduced GADPH was treated with vehicle, MSBT, MSBT-A, or a common thiol blocking reagent methyl

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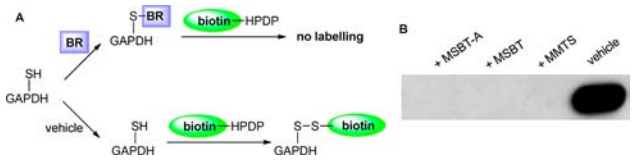

Figure 1. Thiol blocking capability of MSBT and MSBT-A on GAPDH compared to MMTS. (A) Schematic representation of the assay using blocking reagents (MSBT, MSBT-A, MMTS) and vehicle. (B) Western blot results.

methanethiosulfonate (MMTS)[16] and then excess reagents were removed by desalting. The protein sample was then exposed to a thiol labeling reagent *N*-[6-(biotinamido)-hexyl]-3′-(2′-pyridyldithio)propionamide (biotin-HPDP). Biotin labeled GAPDH was detected by nonreducing SDS-PAGE followed by Western blot using the antibiotin antibody. As shown in Figure 1B, MSBT and MSBT-A compared to MMTS exhibited excellent thiol blocking activity. This result confirmed the efficiency of MSBT substrates in thiol specific blocking.

In summary, we have discovered a new reagent, MSBT, capable of blocking protein thiols selectively and effectively. As observed in other thiol blocking reagents, the reactivity profile of MSBT as a function of pH suggests that the rate of reaction depends on thiolate concentration. We expect MSBT substrates will find applications in protein chemistry.

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Supporting Information Available. Spectroscopic and analytical data and selected experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.