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Short Research Article

Synthesis of sulfur-35 reagents for protein labeling†

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Abstract: Two ^{35}S reagents were developed to radiolabel proteins. The first reagent, a *N*-hydroxysuccinimide (NHS) ester (SMSB), acylates the ε-amino group of lysine residues in proteins. The second reagent, an aldehyde (MSAPPA), labels lysine residues via reductive alkylation. Comparing the two methods, the reductive alkylation method labeled proteins over a broader pH range with higher overall radiochemical yield. The biological activity of the proteins did not change after labeling with these ^{35}S reagents. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: sulfur-35; radiolabeled proteins; monoclonal antibodies

Introduction

Radiolabeled proteins are valuable tools for biochemical and biomedical research. The most common labeling methods use the γ-emitting radionuclide ^{125}I. Direct labeling and conjugation are the most widely used methods to radiolabel proteins.[1] Direct labeling uses sodium [^{125}I]iodide and an oxidant to label tyrosine and histidine residues in proteins. This method exposes proteins to harsh oxidation conditions and the label is partially lost *in vivo*. In the conjugation method, a small, radioiodinated reagent, such as the Bolton–Hunter reagent (BH),[2–6] reacts with the ε-amino group of lysine residues. There are also some limitations for BH type reagents: *in vivo* de-iodination is reduced but still occurs and acylation of lysine residues in proteins is carried out at pH 8.5 or above where some proteins are not stable. In both methods, extra safety procedures are needed when handling a γ-emitting isotope such as ^{125}I. ^{35}S has a high specific activity (1485 Ci/mmol, $t_{1/2}$=87 days) and is a viable alternative to ^{125}I (2169 Ci/mmol, $t_{1/2}$=60 days). Since it is a weak β-emitter, radiation protection procedures are simpler. We designed two ^{35}S reagents, SMSB and MSAPPA, as shown in Scheme 1. Both reagents were used to label monoclonal antibodies (Mabs) in good to excellent yields. Biological activity of the labeled Mabs was unchanged.

Results and discussion

Labeling proteins with activated (^{35}S)NHS ester (SMSB)

N-hydroxysuccinimide esters, such as the Bolton–Hunter reagent, are the most commonly used reagents for labeling proteins. We designed a [^{35}S]NHS ester as follows. Esters of aryl carboxylic acids acylate amines in aqueous media with higher yields than esters of aliphatic carboxylic acids.[3] ^{35}S is most readily introduced into organic molecules by sulfonylation of an amine with [^{35}S]methanesulfonyl chloride.[7] Sulfonylation of alkyl amines proceeds in higher yields than aryl amines. The simplest molecule incorporating these structural features is [^{35}S]SMSB. The synthesis of a key intermediate, **4**, is shown in Scheme 2.

The amine group of benzoic acid **1** was protected with the Cbz group to give **2** in 56.1% yield. Carboxylic acid **2** was converted to the *t*-butyl ester **3** in 74.5% yield. The Cbz protecting group is removed by hydrogenolysis in 93.4% yield to give amine **4**. The synthesis of [^{35}S]SMSB from **4** and protein labeling with [^{35}S]SMSB are shown in Scheme 3.

Commercially available [^{35}S]methane sulfonate (5–10 mCi, specific activity: 1400 Ci/mmmol) was

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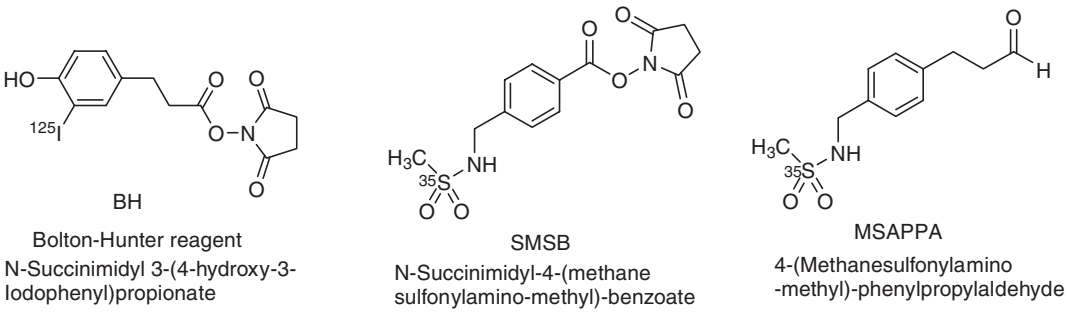
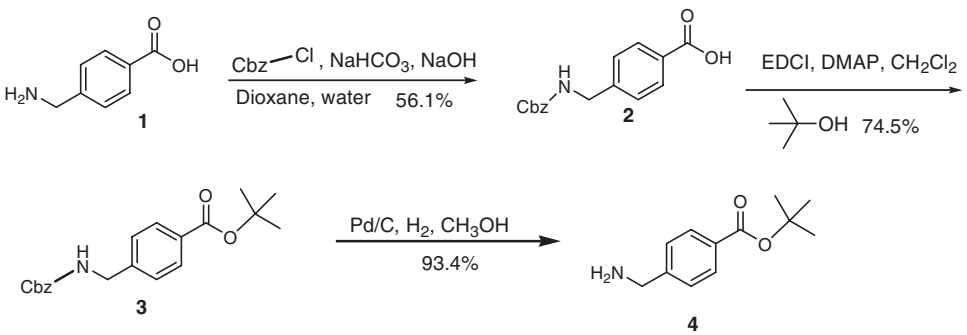
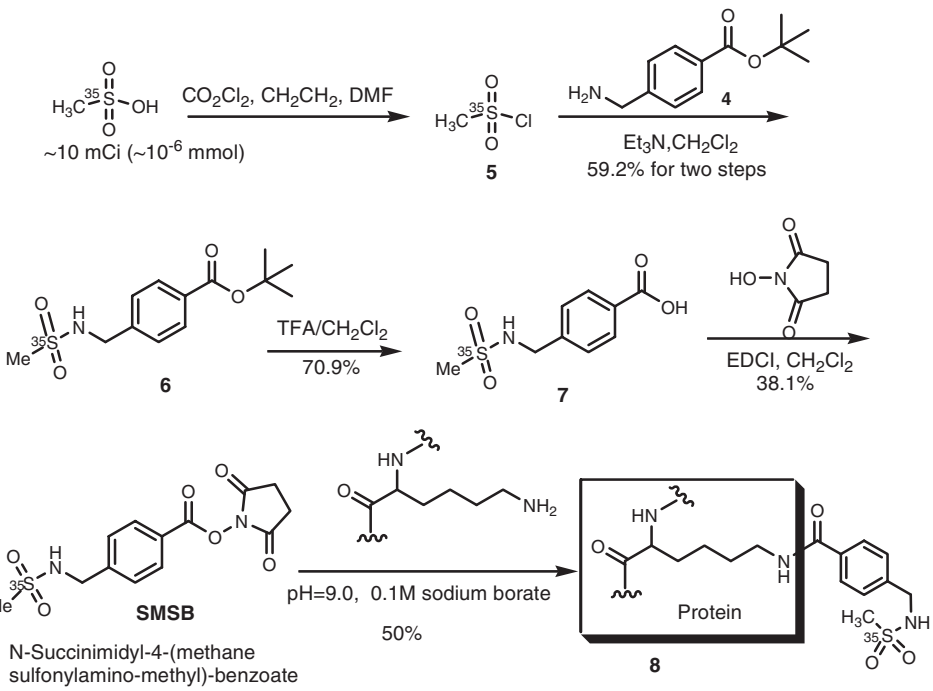


Scheme 1



Scheme 2



Scheme 3

converted to methane [^{35}S]sulfonyl chloride **5** [7] and then reacted with **4** to give sulfonamide **6** in about 59% yield. Acidolysis of the t-butyl ester gave carboxylic acid **7** in 70% yield. Treatment of the carboxylic acid **7** with *N*-hydroxysuccinimide and EDCI, gave [^{35}S]SMSB in 38% yield after NP-HPLC purification. [^{35}S]SMSB was

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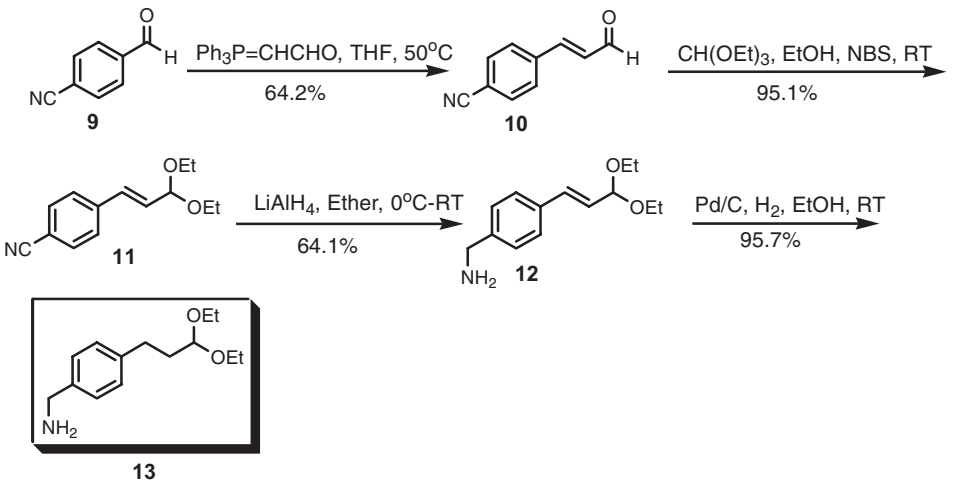
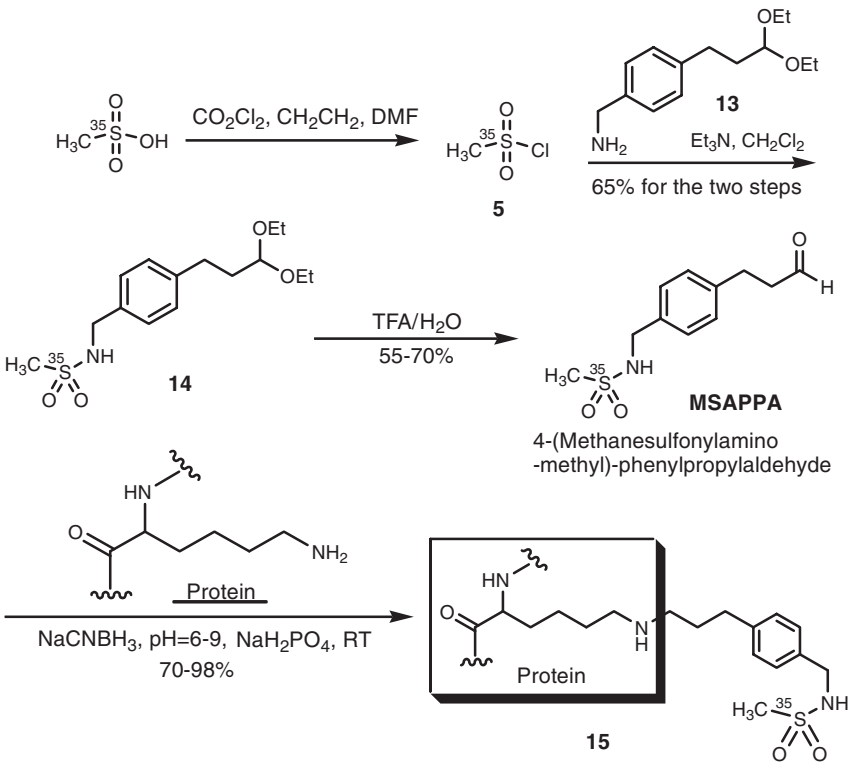
reacted with Mab in pH 9.0 sodium borate buffer for 30 min and then quenched with glycine. The reaction was passed through a short gel-filtration column. Usually, about 50% of SMSB was covalently bound to the protein after initial purification. About 20% of the labeled Mab was larger MW aggregated protein. The aggregated protein was removed by size-exclusion HPLC.

Labeling proteins with the [^{35}S]SMSB has the following limitations:

- The SMSB reagent reacts with proteins at pH 8.5 and above; some proteins may not be stable at this pH range.
- Acylated lysine residues are no longer charged at physiological pH. This could change the activity of the protein.



Scheme 4



Scheme 5

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- The overall yield from [^{35}S]methane sulfonate was low (~10%).
- The reaction conditions led to some protein aggregation.

Labeling proteins with an (^{35}S)aldehyde (MSAPPA) by reductive alkylation

Proteins have been labeled by reductive alkylation using [^{14}C]formaldehyde, [^{3}H]sodium borohydride[8] and a [^{125}I]aldehyde[9]. This chemistry has seen little use, even though it has some useful features. Reductive alkylation of the lysine ε-amino group occurs over a broad pH range (pH 6–9). In contrast with Bolton-Hunter type reagent, the modified lysine residues are still charged at physiological pH with small pKa changes. A ^{35}S-labeled aldehyde labeling reagent will broaden the scope of ^{35}S labeling chemistry.

^{35}S labeled aldehyde was designed as follows. The benzyl amine moiety was used to introduce ^{35}S, in analogous fashion to SMSB reagent. Aliphatic aldehydes alkylate amines in aqueous media with higher yields than aryl aldehydes.[9] [^{35}S]MSAPPA incorporates these structural features.

The synthesis of a key intermediate, **13**, is shown in Scheme 4. 4-Cyanobenzaldehyde **9** was reacted with a Wittig type reagent to yield 64% of α, β-unsaturated aldehyde **10**. Aldehyde **10** was converted to diethyl acetal **11** in 95% yield. Reduction of the cyano group gave amine **12** in 64% yield. Catalytic hydrogenation of **12** afforded **13** in 95% yield. Intermediate **13** was purified by RP-HPLC before reaction with [^{35}S]methanesulfonyl chloride.

Synthesis of [^{35}S]MSAPPA from **13** and protein labeling with [^{35}S]MSAPPA is shown in Scheme 5.

[^{35}S]methane sulfonate (5–10 mCi, specific activity: 1400 Ci/mmmol) was converted to methane [^{35}S]sulfonyl chloride **5**[7] and then reacted with **13** to give sulfonamide **14** in 65% yield. Hydrolysis of the acetal **14** gave [^{35}S]MSAPPA in 55–70% yield after RP-HPLC purification. [^{35}S] MSAPPA was reacted with Mab and $NaCNBH_3$ in pH 6–9 sodium hydrogenphosphate buffer overnight at room temperature. The reaction was passed through a short gel-filtration column. Usually, about 70–98% of [^{35}S]MSAPPA was covalently bound to the protein after initial purification. The labeled protein was further purified by size-exclusion HPLC. Overall yield was ~30% from [^{35}S]methane sulfonate.

Advantages of labeling proteins with [^{35}S] aldehyde (MSAPPA) by reductive alkylation are as follows:

- The reaction may be conducted over a broad pH range;
- No aggregated proteins were formed and the reactions gave cleaner products;
- Higher overall radiochemical yield was obtained;
- Modified lysine residues are still charged at physiological pH only with small pKa changes.

Conclusion

Two ^{35}S reagents were developed to radiolabel proteins in good to excellent yield. Five monoclonal antibodies (Mabs) have been labeled with these ^{35}S-reagents and their bioactivity was unchanged. Reductive alkylation (MSAPPA reagent) approach is our preferred method.

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