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Design and synthesis of sulfur-35 agents and their applications for protein labeling

Sumei Ren,* Paul McNamara, David Koharski, David Hesk, and Scott Borges

Two new ^{35}S reagents were developed to radiolabel proteins. The first reagent, *N*-succinimidyl-4-(methane [^{35}S]sulfonylamino-methyl)-benzoate (SMSB), acylates the ε-amino group of lysine residues in proteins. The second reagent, 4-(methane [^{35}S]sulfonylamino-methyl)-phenylpropylaldehyde (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. More than ten monoclonal antibodies (mAbs) have been labeled with these ^{35}S labeling reagents, the biological activity of the mAbs was unchanged. Part of this work was presented in the Ninth International Symposium on the Synthesis and Applications of Isotopically Labelled Compounds, Edinburgh, 16–20 July 2006.

Keywords: sulfur-35; radiolabeled proteins; monoclonal antibodies

Introduction

Radiolabeled proteins are valuable tools for biochemical and biomedical research.[1] 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.[2] 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 (BH) reagent,[3–7] 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 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, *N*-succinimidyl-4-(methane [^{35}S]sulfonylamino-methyl)-benzoate (SMSB) and 4-(methane [^{35}S]sulfonylamino-methyl)-phenylpropylaldehyde (MSAPPA), as shown in Figure 1. Both reagents were used to label monoclonal antibodies (mAbs) in good to excellent yields. Preliminary *in vitro* experiments have shown good label stability.[8]

Results and discussion

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

N-hydroxysuccinimide (NHS) esters, such as the BH 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.[4] ^{35}S is most readily introduced into organic molecules by sulfonylation of an amine with methane [^{35}S]sulfonyl chloride.[9] 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 1.

The amine group of benzoic acid **1** was protected with the Cbz group to give **2** in 74.2% yield. Carboxylic acid **2** was converted to *t*-butyl ester **3** in 74.5% yield. The Cbz protecting group is removed by hydrogenolysis to give amine **4** in 93.4% yield. Synthesis of [^{35}S]SMSB from **4** and protein labeling with [^{35}S]SMSB are shown in Scheme 2.

Commercially available methane [^{35}S]sulfonate (5–25 mCi, specific activity: 1400 Ci/mmol) was converted to methane [^{35}S]sulfonyl chloride **5** using a literature procedure[9] and then reacted with **4** to give sulfonamide **6** in 59% overall yield. Acidolysis of the *t*-butyl ester gave carboxylic acid **7** in 70% yield. Treatment of the carboxylic acid **7** with NHS and *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDCI) gave [^{35}S]SMSB in 38% yield after Normal-Phase High-Performance Liquid Chromatography (NP-HPLC) purification. [^{35}S]SMSB was reacted with mAb in pH 9.0 sodium borate buffer for 30 min and then quenched with glycine. The reactant 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.

Schering-Plough Research Institute, 2015 Galloping Hill Road, K-15-4545, Kenilworth, NJ 07033, USA

**Correspondence to: Sumei Ren, Schering-Plough Research Institute, 2015 Galloping Hill Road, K-15-4545, Kenilworth, NJ 07033, USA.*
E-mail: sumei.ren@spcorp.com

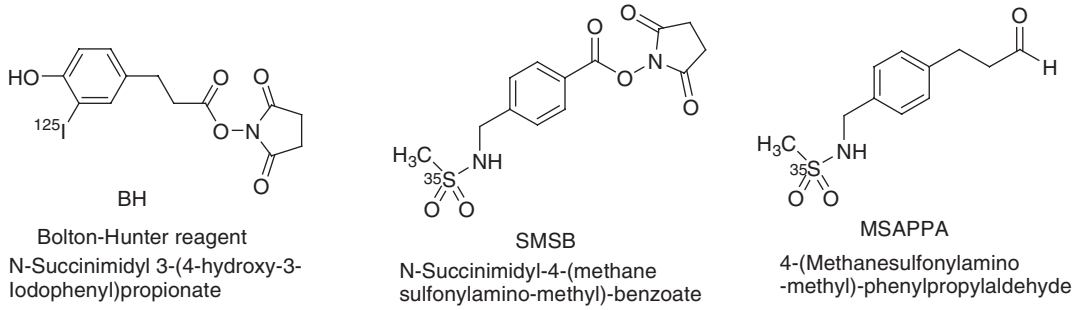
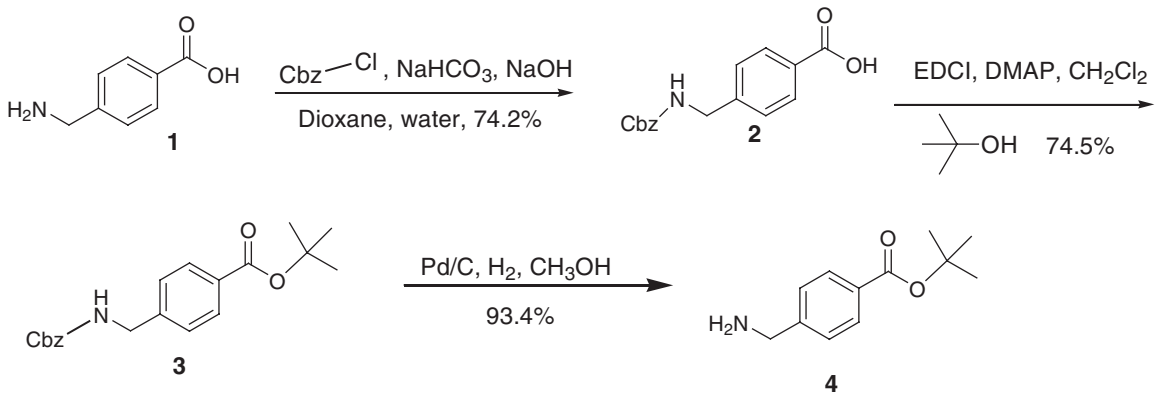
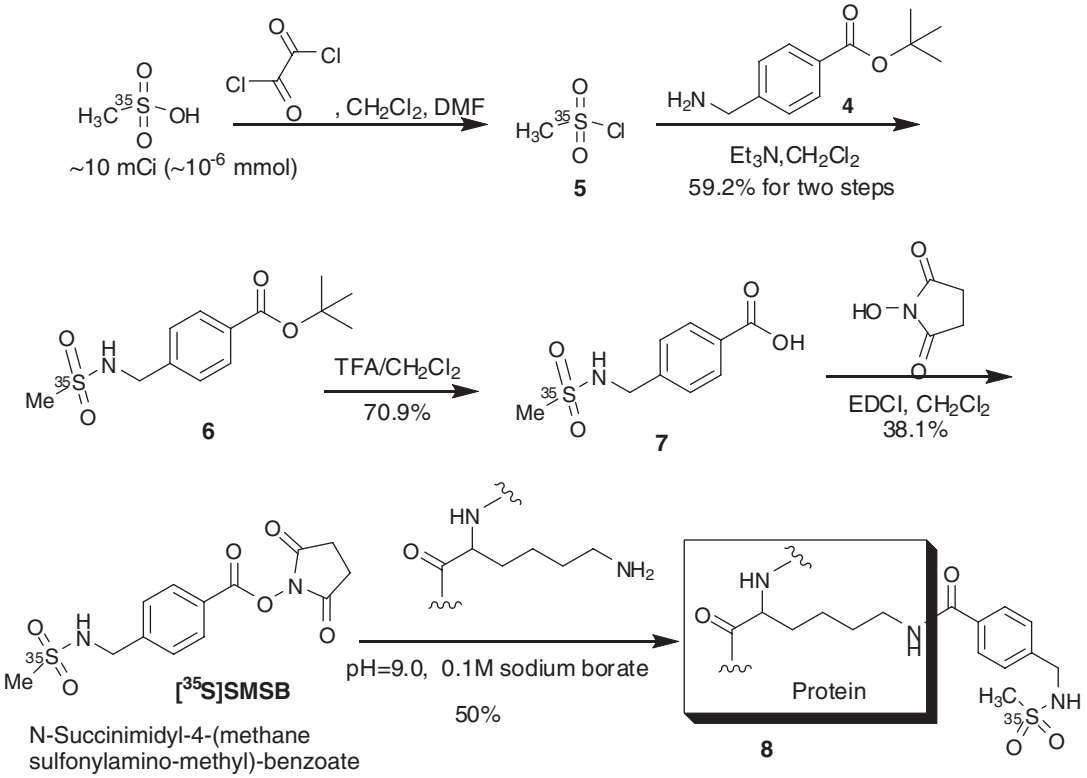


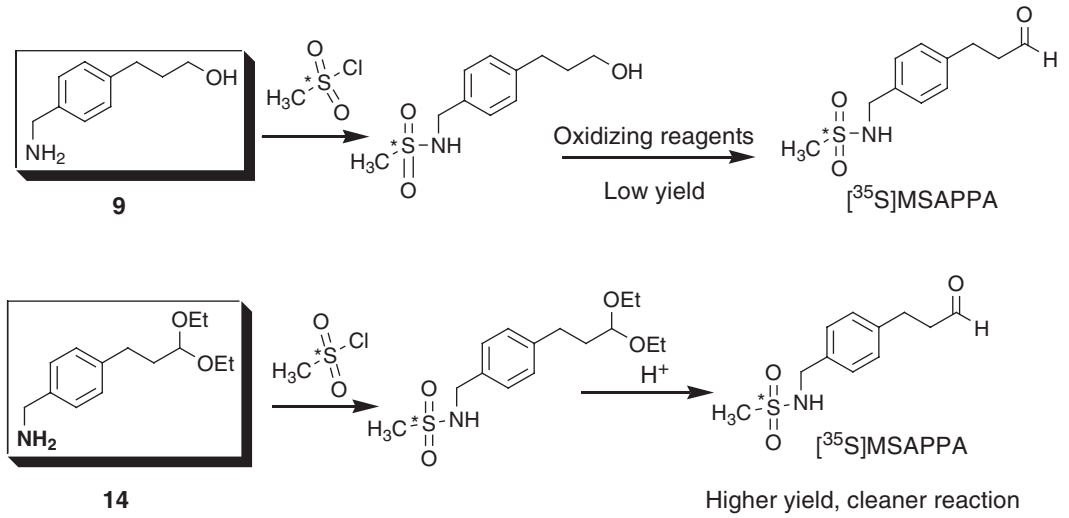
Figure 1. Radioactive reagents for protein labeling.



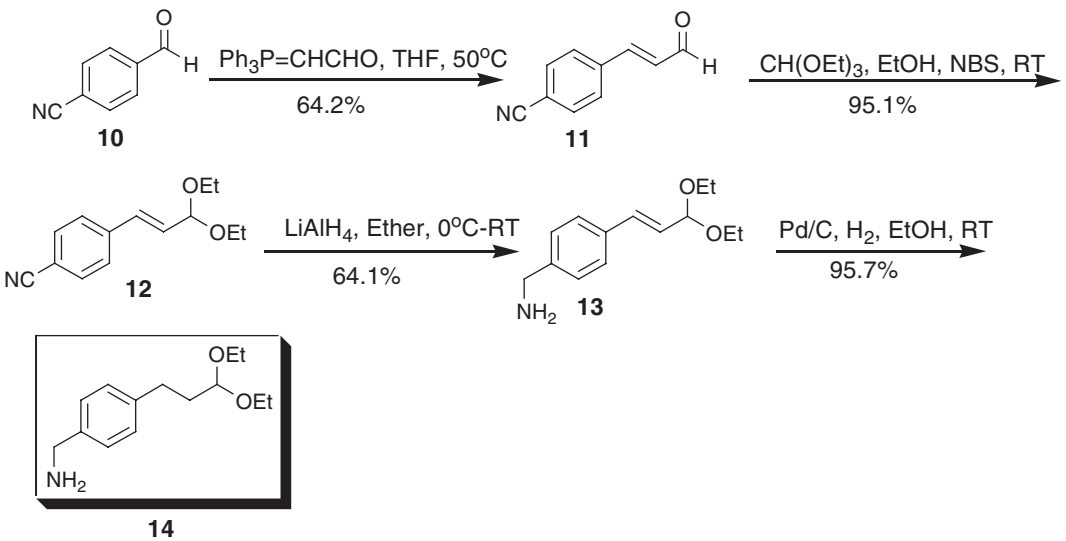
Scheme 1. Synthesis of 4-aminomethyl-benzoic acid *tert*-butyl ester **4**.



Scheme 2. Synthesis of [^{35}S]SMSB and its application for protein labeling.



Scheme 3. Synthetic design of [^{35}S]MSAPPA.



Scheme 4. Synthesis of 4-(3, 3-diethoxy-propyl)-benzylamine.

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

- SMSB reagent reacts with proteins at pH 8.5 and above; some proteins may not stable to this pH range.
- Acylated lysine residues are no longer charged at physiological pH. This could change the activity of the protein.
- Overall yield from methane [^{35}S]sulfonate was low ($\sim$10%).
- The reaction conditions led to some protein aggregation.

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

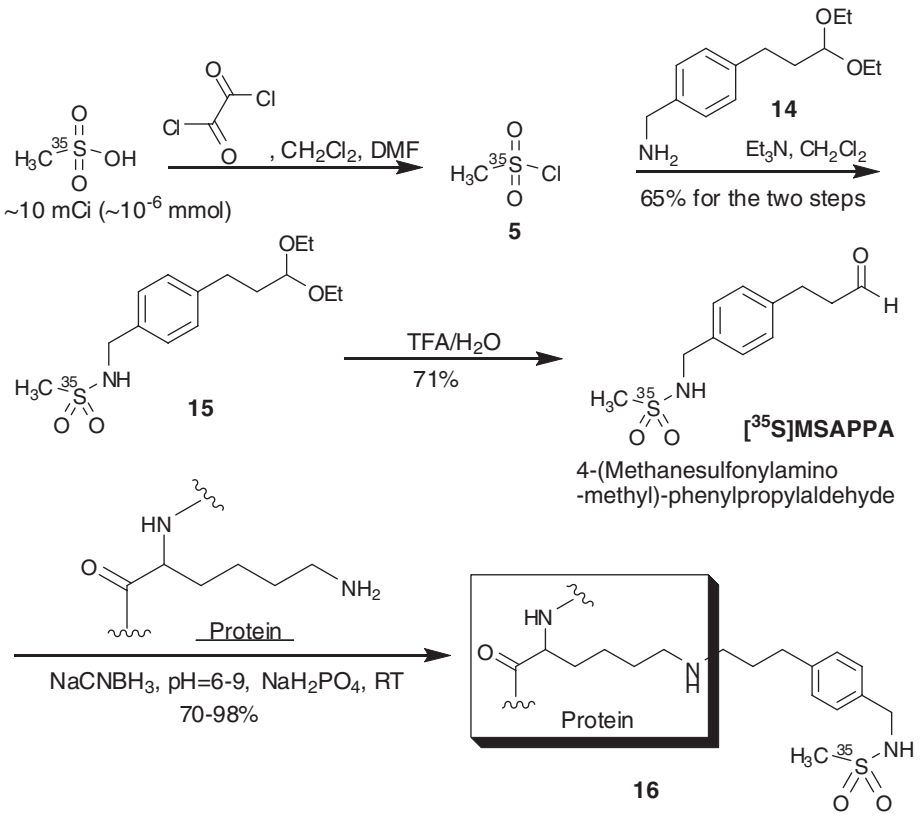
Proteins have been labeled by reductive alkylation using [^{14}C]formaldehyde, sodium [^{3}H]borohydride,[10] and [^{125}I]aldehyde.[11] This chemistry has seen little use, even though it has some useful features in contrast with the BH-type reagent, such as (1) the reductive alkylation of the lysine ε-amino group occurs over a broad pH range (pH 6–9); (2) the modified lysine residues are still charged at physiological pH with small pK_a changes. A ^{35}S 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, analogous to the SMSB reagent. Aliphatic aldehydes alkylate amines in aqueous media with higher yields than aryl aldehydes.[11] [^{35}S]MSAPPA incorporates these structural features. Two possible ways to prepare [^{35}S]MSAPPA were explored, and the route from protected aldehyde **14** gave higher yield and cleaner reactions than the route from alcohol substrate **9** as shown in Scheme 3.

The synthesis of a key intermediate, **14**, is shown in Scheme 4.

4-Cyanobenzaldehyde **10** was reacted with a Wittig-type reagent to yield 64% of α, β-unsaturated aldehyde **11**. Aldehyde **11** was converted to diethyl acetal **12** in 95% yield. Reduction of the cyano group gave amine **13** in 64% yield. Catalytic hydrogenation of **13** afforded **14** in 95% yield. Intermediate **14** was purified by Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) before reaction with methane [^{35}S]sulfonyl chloride.

Synthesis of [^{35}S]MSAPPA from **14** and protein labeling with [^{35}S]MSAPPA are shown in Scheme 5.



Scheme 5. Synthesis of [^{35}S]MSAPPA and its application for protein labeling.

Methane [^{35}S]sulfonate (5–20 mCi, specific activity: 1400 Ci/mmol) was converted to methane [^{35}S]sulfonyl chloride **5**[9] and then reacted with **14** to give sulfonamide **15** in 65% overall yield. Hydrolysis of the acetal **15** gave [^{35}S]MSAPPA in 71% yield after RP-HPLC purification. [^{35}S]MSAPPA was reacted with mAbs and $NaCNBH_3$ in pH 6–9 sodium hydrogen phosphate buffer overnight at room temperature (RT). The reaction pH was chosen based on the stability of the mAbs at the pH and the isoelectric point (pI) of the mAbs, usually 1 pH unit away from pI of the mAbs to avoid precipitation during reaction. The labeled mAbs was purified by passing through a gel-filtration column (Bio-Gel, P-6). Usually, about 70–98% of [^{35}S]MSAPPA was covalently bound to the protein after purification. Overall yield was ~30% from methane [^{35}S]sulfonate.

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

- Reaction is conducted at a broad pH range.
- No aggregated proteins formed, and reactions gave cleaner products.
- Higher overall radiochemical yield was obtained.
- Modified lysine residues are still charged at physiological pH only with small pK_a changes.

Experimental section

General

Chemicals

Chemicals and solvents were purchased from standard commercial sources (Aldrich, Fluka, Fisher, Acros) and were used without further purification. Methane [^{35}S]sulfonate was purchased from PerkinElmer. All mAbs were from Schering-Plough Research Institute.

Analytical methods

Mass spectra were acquired on a JEOL MStation double-focusing magnetic sector mass spectrometer at a Fast-Atom Bombardment (FAB) ionization mode.

^{1}H-NMR and ^{13}C-NMR (400 or 600 MHz) spectra were obtained on a Varian spectrometer.

LC-MS: Waters Micromass with Waters 2695 Separation Module operating in the ES^+ ionization mode. Supelcosil LC-CN, 150 mm × 4.6 mm, 215 nm, isocratic, 0.1% HCO_2H in H_2O:0.1% HCO_2H in CH_3CN (65:35), 1.0 ml/min.

Analytical HPLC

Waters 600 Multisolvent Delivery System with Waters 2487 Absorbance Detector and Radiomatic 525TR Radioflow Detector, Packard Flo-Scint III liquid scintillation cocktail (1:3). HPLC elute:

System 1: Zorbax extend C18, 5 μm, 150 mm × 3.0 mm, 254 nm, 0.05 M TEAA (pH = 9.0):CH_3CN (65:35) for 15 min followed by a step gradient to CH_3CN, 0.5 ml/min.

System 2: Phenomenex Prodigy 5 μm silica, 150 mm × 3.2 mm, 254 nm. EtOAc:hexane:HOAc = 65:35:2, 0.5 ml/min.

System 3: TosoHaas TSK Gel G3000 SWXL column, 300 mm × 7.8 mm, 280 nm, 30°C, 20 mM NaOAc/150 mM NaCl, pH = 5.2, 1.0 ml/min.

System 4: Bio-Rad Bio-Sil 250 5 SEC column, 300 mm × 7.8 mm (or equiv.), 280 nm, 20 mM NaOAc/150 mM NaCl, pH 5.0, 1.0 ml/min.

Semi-preparative HPLC

HPLC purification was conducted on a Waters Delta Prep 4000 with Waters 486 Tunable Absorbance Detector; the following systems were used:

System a: Phenomenex Prodigy 5 μm silica, 250 mm × 10 mm, 254 nm. EtOAc:hexane:HOAc = 55:45:2, 4.0 ml/min.

System b: Zorbax extend C18, 250 mm × 9.4 mm, 254 nm, 0.05 M TEAA (pH = 8.9):CH_3CN (65:35), 4.0 ml/min.

System c: Phenomenex Prodigy 5 μm silica, 250 mm × 10 mm, 254 nm. EtOAc:hexane:HOAc = 70:30:1, 4.0 ml/min.

System d: Zorbax extend C18, 250 mm × 9.4 mm, 254 nm, 0.05 M TEAA (pH = 9.0):CH_3CN (60:40), 4.0 ml/min.

Radioactivity was measured on a Packard 2200CA liquid scintillation analyzer using Scintiverse BD liquid scintillation cocktail. TLC plates were scanned on a Bioscan 1000 linear analyzer.

Purification of protein

Bio-Spin columns with Bio-Gel P-6 were purchased from Bio-Rad Laboratories. The column was used to initially clean up the labeled proteins by using a swing bucket centrifuge. The centrifuge (IEC Centra, CL2) was purchased from International Equipment Company, and operated at 3.3 × 1000 RPM for 2 min per each run.

The labeled proteins from SMSB route may be further purified by the size-exclusion HPLC system e. The HPLC conducted was on a Bio-Rad BioLogic Duo-flow protein purification system with BioLogic QuadTec UV–Vis Detector. Superdex 200 column, 300 mm × 10 mm, 280 nm. Isocratic elution with 0.150 M NaCl, 0.020 M NaOAc, pH = 5.50 buffer, 0.5 ml/min.

Synthesis of unlabeled corresponding compounds as authentic standards

All corresponding unlabeled compounds were synthesized and fully analyzed. The reaction conditions were different from the labeled reactions due to the difference of the concentration and the stoichiometry of the reagents in the reactions.

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

*Synthesis of 4-(benzyloxycarbonylamino-methyl)-benzoic acid (**2**)*

A solution of 4-(aminomethyl)benzoic acid (3.0 g, 20.0 mmol) in dioxane (30 ml) and 10% $NaHCO_3$ (60 ml) was cooled to 5°C, and benzyl chloroformate (2.86 ml, 20.0 mmol) was added. The reaction pH was adjusted to 8.0 with NaOH (1.0 N). Additional NaOH may be added to keep the pH constant during the reaction. The reactant was warmed to RT and stirred overnight. The solvent was removed and H_2O (100 ml) was added. The solution was washed with Et_2O (2 × 25 ml) and acidified to pH = 1 with 2 N HCl. The resulting solid was filtered, washed with H_2O (2 × 5 ml), and dried under vacuum to give 4.2 g (74.2%) of **2** as a white solid. ^{1}H-NMR (DMSO-d_6, 400 MHz, δ): 7.86–7.89 (m, 3H), 7.4 (m, 7H), 5.03 (s, 2H), 4.25 (d, J = 6.4 Hz, 2H). ^{13}C-NMR (DMSO-d_6, δ): 167.43 (1C), 156.70 (1C), 145.22 (1C), 137.42 (1C), 129.75 (2C), 129.68 (1C), 128.74 (2C), 128.20 (1C), 128.15 (2C), 127.34 (2C), 66.00 (1C), 44.16 (1C). LC-MS *m/z*: 286 $(M+H)^+$, 571 $(2M+H)^+$. HRMS-FAB (*m/z*): $[M+H]^+$calcd. for $C_{16}H_{16}NO_4$, 286.1079; found 286.1068.

*Synthesis of 4-(benzyloxycarbonylamino-methyl)-benzoic acid tert-butyl ester (**3**)*

To a solution of 4-(benzyloxycarbonylamino-methyl)-benzoic acid (**2**, 2.0 g, 7.0 mmol), DMAP (0.856 g, 7.0 mmol), and *tert*-butanol (6.0 ml, 63.9 mmol) in anhydrous CH_2Cl_2 (6.0 ml) at 5°C, EDCI (3.35 g, 17.5 mmol) was added. The reactant was warmed up to RT and stirred under N_2 overnight. The reactant was washed with H_2O (2 × 50 ml), brine (50 ml), dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash chromatography on silica gel (elution with 1–20% EtOAc/hexanes with 1% Et_3N) to give 1.79 g (74.5%) of compound **3** as a colorless oil. ^{1}H-NMR (DMSO-d_6, 400 MHz, δ): 7.92 (t, 1H, NH), 7.83 (d, J = 8.0 Hz, 2H), 7.34 (m, 7H), 5.03 (s, 2H), 4.25 (d, J = 6.4 Hz, 2H), 1.52 (s, 9H). ^{13}C-NMR (DMSO-d_6, δ): 164.56 (1C), 156.16 (1C), 144.71 (1C), 136.88 (1C), 129.70 (1C), 128.93 (2C), 128.22 (2C), 127.68 (1C), 127.62 (2C), 126.81 (2C), 80.52 (1C), 66.45 (1C), 43.61 (1C), 27.86 (3C). MS *m/z*: 286 $(M-(t\text{-Bu})+2H)^+$, 342 $(M+H)^+$, 364 $(M+Na)^+$. HRMS-FAB (*m/z*): $[M+H]^+$ calcd. for $C_{20}H_{24}NO_4$, 342.1705; found 342.1703.

*Synthesis of 4-aminomethyl-benzoic acid tert-butyl ester (**4**)*

To a solution of 4-(benzyloxycarbonylamino-methyl)-benzoic acid *tert*-butyl ester **3** (1.77 g, 5.18 mmol) in CH_3OH, Pd/C (10% wt on dry, wet, 200 mg) was added. The flask was degassed, and the reactant was stirred under an H_2 balloon at RT for 1 h. The mixture was filtered through a celite pad and concentrated to dryness. The crude product was purified by flash chromatography on silica gel (elution with 1–5% CH_3OH/CH_2Cl_2 with 1% Et_3N) to give 1.00 g (93.4%) of compound **4** as a colorless oil. ^{1}H-NMR (DMSO-d_6, 400 MHz, δ): 7.81 (d, J = 8.0 Hz, 2H), 7.42 (d, J = 8.4 Hz, 2H), 3.75 (s, 2H), 1.97 (br, 2H, NH_2), 1.52 (s, 9H). ^{13}C-NMR (DMSO-d_6, δ): 164.70 (1C), 149.10 (1C), 129.07 (1C), 128.70 (2C), 126.83 (2C), 80.30 (1C), 45.27 (1C), 27.87 (3C). LC-MS *m/z*: 191 $(M-NH_3+H)^+$, 208 $(M+H)^+$, 415 $(2M+H)^+$. HRMS-FAB (*m/z*): $[M+H]^+$ calcd. for $C_{12}H_{18}NO_2$, 208.1338; found 208.1332.

*Synthesis of 4-(methane sulfonylamino-methyl)-benzoic acid tert-butyl ester (**6**)*

To a solution of 4-aminomethyl-benzoic acid *tert*-butyl ester (**4**, 100 mg, 0.483 mmol) in anhydrous CH_2Cl_2 (5.0 ml), methane sulfonyl chloride (38 μl, 0.489 mmol) and Et_3N (100 μl, 0.754 mmol) were added. The reactant was stirred at RT under N_2 for 1 h, diluted with CH_2Cl_2 (20 ml), and washed with saturated $NaHCO_3$ (2 × 15 ml). The organic phase was dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash chromatography on silica gel (elution with 0–0.5% CH_3OH/CH_2Cl_2) to give 115 mg (83.9%) of compound **6** as a white solid. ^{1}H-NMR (DMSO-d_6, 400MHz, δ): 7.87 (d, J = 8.4 Hz, 2H), 7.66 (t, 1H, NH), 7.44 (d, J = 8.0 Hz, 2H), 4.21 (d, J = 6.4 Hz, 2H), 2.86 (s, 3H), 1.53 (s, 9H). ^{13}C-NMR (DMSO-d_6, δ): 165.04 (1C), 143.87 (1C), 130.56 (1C), 129.50 (2C), 127.96 (2C), 81.13(1C), 46.19 (1C), 40.51(1C), 28.40 (1C). LC-MS *m/z*: 230 (acid $+H)^+$, 459 $(2acid+H)^+$, 571$(2M+H)^+$, 593 $(2M+Na)^+$. HRMS-FAB (*m/z*): $[M-t\text{-Bu}+2H]^+$ calcd. for $C_9H_{12}NO_4S$, 230.0487; found 230.0495.

*Synthesis of 4-(methane sulfonylamino-methyl)-benzoic acid (**7**)*

To the solution of 4-(methane sulfonylamino-methyl)-benzoic acid *tert*-butyl ester (**6**, 852 mg, 3.0 mmol) in CH_2Cl_2 (30 ml), TFA

(4.0 ml) was added. The reactant was stirred at RT under N_2 for 6 h, and the solvent was removed under vacuum. The crude product was dried under vacuum to give 680 mg (99.4%) of compound **7** as a white solid. The compound was directly used in the next step without further purification. ^{1}H-NMR (DMSO-d_6, 400 MHz, δ): 7.90 (d, J = 8.0 Hz, 2H), 7.66 (t, 1H, NH), 7.45 (d, J = 8.0 Hz, 2H), 4.21 (d, J = 6.4 Hz, 2H), 2.87 (s, 3H). ^{13}C-NMR (DMSO-d_6, δ): 167.41 (1C), 143.85 (1C), 129.98 (1C), 129.78 (2C), 127.96 (2C), 46.21 (1C), 40.50 (1C). LC-MS *m/z*: 212 $(M-H_2O+H)^+$, 230 $(M+H)^+$. HRMS-FAB (*m/z*): $[M+H]^+$ calcd. for $C_9H_{12}NO_4S$, 230.0487; found 230.0482.

*Synthesis of N-succinimidyl-4-(methane sulfonylamino-methyl)-benzoate (**SMSB, unlabeled**)*

A solution of 4-(methane sulfonylamino-methyl)-benzoic acid **7** (100 mg, 0.436 mmol), NHS (251 mg, 2.18 mmol), and EDCI (416 mg, 2.18 mmol) in anhydrous CH_2Cl_2 (5.0 ml) was stirred at RT under N_2 overnight. The reactant was diluted with CH_2Cl_2 (10 ml), washed with H_2O (2 × 10 ml), dried over Na_2SO_4, filtered, and concentrated. The crude product was first purified by flash chromatography on silica gel (elution with 0–40% EtOAc/hexanes in 1% HOAc), and then purified by NP-HPLC (HPLC system a) to give 130 mg (91.5%) of **SMSB** as a white solid. ^{1}H-NMR (DMSO-d_6, 400 MHz, δ): 8.07 (d, J = 8.4 Hz, 2H), 7.74 (t, 1H, NH), 7.60 (d, J = 8.4 Hz, 2H), 4.29 (d, J = 6.0 Hz, 2H), 2.91 (s, 3H), 2.87 (s, 4H). ^{13}C-NMR (DMSO-d_6, δ): 170.61 (2C), 161.89 (1C), 147.11 (1C), 130.54 (2C), 128.73 (2C), 123.58 (1C), 46.11 (1C), 40.49 (1C), 26.16 (2C). LC-MS *m/z*: 212 (acid$-H_2O+H)^+$, 327 $(M+H)^+$, 349 $(M+Na)^+$, 653 $(2M+H)^+$. HRMS-FAB (*m/z*): $[M+H]^+$ calcd. for $C_{13}H_{15}N_2O_6S$, 327.0651; found 327.0635.

*Synthesis of methane [^{35}S]sulfonyl chloride (**5**)*

An aliquot of methane [^{35}S]sulfonate (25 mCi, 0.32 ml, specific activity: 1400 Ci/mmol) was transferred to a 10 ml reaction vial by a syringe, the syringe was washed with EtOH (6 × 50 µl), and the washing solution was added to the vial. The mixture was stirred for 5 min, and the solvent was removed under N_2. The residue was coevaporated with EtOH (2 × 0.8 ml), CH_2Cl_2 (2 × 0.8 ml) under N_2, and dissolved in anhydrous CH_2Cl_2 (1.5 ml). To the solution, oxalyl chloride (0.20 ml, 2.3 mmol) was added. After stirring at RT under N_2 for 50 min, DMF (100 µl of 10% DMF in CH_2Cl_2) was added slowly and the reactant was stirred at the same condition overnight. After dilution with CH_2Cl_2 (1.5 ml), the reactant was cooled to 5°C, and carefully washed with $NaHCO_3$ (1% solution, 3 × 2.0 ml) and $NaHSO_3$ (2% solution, 2 × 2 ml). The organic phase was dried over Na_2SO_4, filtered, and concentrated by distillation at 60°C under N_2 (1 atm). The crude product **5** was used immediately in the next step without further purification.

*Synthesis of 4-(methane [^{35}S]sulfonylamino-methyl)-benzoic acid tert-butyl ester (**^{35}S-6**)*

To the crude product of methane [^{35}S]sulfonyl chloride **5**, a solution of 4-aminomethyl-benzoic acid *tert*-butyl ester (**4**, 15 mg, 0.072 mmol) and Et_3N (20 µl, 0.151 mmol) in anhydrous CH_2Cl_2 (150 µl) was added. The reactant was stirred at RT in a sealed vial for 1 h, and the solvent was removed under N_2. The crude product was purified by the RP-HPLC system b to give 14.8 mCi (59.2% for the first two steps) of **^{35}S-6**. The radiochemical purity was 99.7% by the HPLC system 1. **^{35}S-6** was co-eluted with an authentic standard **6** by the HPLC system 1 (t_R = 11.0 min).

*Synthesis of 4-(methane [^{35}S]sulfonylamino-methyl)-benzoic acid (**^{35}S-7**)*

To the solution of 4-(methane [^{35}S]sulfonylamino-methyl)-benzoic acid *tert*-butyl ester (**^{35}S-6**, 14.8 mCi) in CH_2Cl_2 (300 µl), TFA (50 µl) was added. The reactant was stirred at RT in a sealed vial overnight, and the solvent was removed under N_2. The crude product was purified by the NP-HPLC system c to give 10.5 mCi (70.9%) of **^{35}S-7**. The radiochemical purity was 96.6% by the HPLC system 1. **^{35}S-7** was co-eluted with an authentic standard **7** by the HPLC system 1 (t_R = 1.8 min).

*Synthesis of N-succinimidyl-4-(methane sulfonylamino-methyl)-benzoate (**^{35}S-SMSB**)*

A solution of 4-(methane [^{35}S]sulfonylamino-methyl)-benzoic acid **^{35}S-7** (10.5 mCi), NHS (15 mg, 0.13 mmol), and EDCI (20 mg, 0.10 mmol) in anhydrous CH_2Cl_2 (300 µl) was stirred at RT in a sealed vial overnight. The solvent was removed under N_2, and the crude product was purified by the NP-HPLC system c to give 4.0 mCi (38.1%) of **^{35}S-SMSB**. The radiochemical purity was 97.2% by the HPLC system 2. **^{35}S-SMSB** was co-eluted with an authentic standard **SMSB** by the HPLC system 2 (t_R = 5.2 min).

*General procedure for labeling protein (mAb) (**^{35}S-16**) using **^{35}S-SMSB***

^{35}S-SMSB (4.0 mCi) in CH_2Cl_2 (2.0 ml) was transferred to a 2 ml plastic vial, and the solvent was removed under N_2. To the residue, DMF (10 µl) was added and the vial was shaken for 5 min, and then a solution of mAbs (4.0 mg) in borate buffer (300 µl, pH = 9.0, 0.1 M) was added. The reactant was incubated at RT for 20 min and quenched by the addition of glycine (20 µl, 0.2 M). The labeled mAbs was initially purified by passing through a gel-filtration column (Bio-Gel, P-6) to give 2.49 mCi (62.3%) of **^{35}S-16** with the radiochemical purity of 80.1% by the HPLC system 3. The product was further purified by the size-exclusion HPLC system e to give 1.1 mCi of **^{35}S-16** with the radiochemical purity of 96.7% by the HPLC system 3.

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

*Synthesis of 4-cyano-cinnamaldehyde (**11**)*

To a solution of 4-cyanobenzaldehyde (1.02 g, 7.78 mmol) in THF (40 ml), (triphenyl phosphoramylidene)acetaldehyde (2.20 g, 6.94 mmol) was added. The suspension was stirred at 50°C under N_2 for 8 h and concentrated to dryness under vacuum. The crude product was purified by flash chromatography on silica gel (elution with 0–40% EtOAc/hexanes) to give 0.70 g (64.2%) of compound **11** as a white solid. ^{1}H-NMR (DMSO-d_6, 400 MHz, δ): 9.70 (d, J = 7.6 Hz, 1H, COH), 7.93 (s, 4H), 7.80 (d, J = 16.0 Hz, 1H), 7.01 (dd, J = 7.6 Hz, 16.0 Hz, 1H). HRMS-FAB (*m/z*): $[M+H]^+$ calcd. for $C_{10}H_8NO$, 158.0606; found 158.0605.

*Synthesis of 4-cyano-cinnamaldehyde diethyl acetal (**12**)*

To a solution of 4-cyano-cinnamaldehyde (**11**, 695 mg, 4.42 mmol) in EtOH (16 ml), triethyl orthoformate (1.15 ml,

6.91 mmol) and *N*-bromosuccinimide (14 mg, 0.079 mmol) were added. The reactant was stirred at RT under N_2 for 4 h, quenched with NaOH solution (10%, 30 ml), and extracted with Et_2O (3 × 30 ml). The organic phase was washed with brine (20 ml), dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash chromatography on silica gel (elution with 0–15% EtOAc/hexanes) to give 970 mg (95.1%) of compound **12** as a white solid. ^{1}H-NMR (DMSO-d_6, 400 MHz, δ): 7.78 (d, J = 8.4 Hz, 2H), 7.69 (d, J = 8.4 Hz, 2H), 6.73 (d, J = 16.4 Hz, 1H), 6.46 (dd, J = 5.2, 16 Hz, 1H), 5.06 (dd, J = 5.2, 0.8 Hz, 1H), 3.67–3.51 (m, 2H), 3.55–3.45 (m, 2H), 1.13 (t, J = 7.2 Hz, 6H). HRMS-FAB (*m/z*): $[M+H]^+$ calcd. for $C_{14}H_{18}NO_2$, 232.1338; found 232.1327.

*Synthesis of 4-aminomethyl-cinnamaldehyde diethyl acetal (**13**)*

To a solution of 4-cyano-cinnamaldehyde diethyl acetal **12** (950 mg, 4.11 mmol) in Et_2O (10 ml) at 0°C, $LiAlH_4$ (1.0 M, 25 ml, 25 mmol) was added dropwise for 30 min under N_2. The reactant was stirred at 0°C for 30 min, warmed up to RT, and stirred for 2 h. The reactant was diluted with Et_2O (50 ml), cooled to 0°C, and quenched slowly with H_2O (30 ml). The organic phase was washed with NaOH (10%, 30 ml), brine (30 ml), dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash chromatography on silica gel (elution with 0.5–4% CH_3OH (7 N NH_3)/CH_2Cl_2) to give 620 mg (64.1%) of compound **13** as a colorless oil. ^{1}H-NMR (DMSO-d_6, 400 MHz, δ): 7.39 (d, J = 7.2 Hz, 2H), 7.27 (d, J = 7.6 Hz, 2H), 6.62 (d, J = 16.0 Hz, 1H), 6.19 (dd, J = 16.0, 4.8 Hz, 1H), 5.06 (d, J = 5.2 Hz, 1H), 3.67 (s, 2H), 3.60–3.56 (m, 2H), 3.48–3.44 (m, 2H), 1.13 (t, J = 7.2 Hz, 6H). HRMS-FAB (*m/z*): $[M+H]^+$ calcd. for $C_{14}H_{22}NO_2$, 236.1651; found 236.1642.

*Synthesis of 4-(3, 3-diethoxy-propyl)-benzylamine (**14**)*

To a solution of 4-aminomethyl-cinnamaldehyde diethyl acetal (**13**, 590 mg, 2.51 mmol) in EtOH (25 ml), Pd/C (10% wt on dry, wet, 240 mg) was added. The flask was degassed, and the reactant was stirred under H_2 balloon at RT for 5 h. The mixture was filtered through a celite pad and concentrated to dryness to give crude 4-(3, 3-diethoxy-propyl)-benzylamine **14** (570 mg, 95.8% yield) as a colorless oil. The product may be purified by HPLC d before reacting with methane [^{35}S]sulfonyl chloride **5.** ^{1}H-NMR (DMSO-d_6, 600 MHz, δ): 7.20 (d, J = 7.6 Hz, 2H), 7.10 (d, J = 7.6 Hz, 2H), 4.14 (t, J = 5.6 Hz, 1H), 3.65 (s, 2H, CH_2NH_2), 3.57–3.53 (m, 2H), 3.42–3.38 (m, 2H), 3.31 (br, 2H, NH_2), 2.54–2.51 (t, J = 7.6 Hz, 2H), 1.77–1.75 (m, 2H), 1.10 (t, J = 7.2 Hz, 6H). ^{13}C-NMR (DMSO-d_6, δ): 141.51 (1C), 139.22 (1C), 127.82 (2C), 126.91 (2C), 101.43 (1C), 60.40 (2C), 45.26 (1C), 34.98 (1C), 29.93 (1C), 15.24 (2C). HRMS-FAB (*m/z*): $[M+H]^+$ calcd. for $C_{14}H_{24}NO_2$, 238.1807; found 238.1805.

*Synthesis of N-[4-(3, 3-diethoxy-propyl)]-methane sulfonamide (**15**)*

To a solution of 4-(3, 3-diethoxy-propyl)-benzylamine (**14**, 288 mg, 1.22 mmol), Et_3N (0.87 ml, 6.10 mmol), and five seeds of 5 Å molecular sieve in anhydrous CH_2Cl_2 (2.0 ml), methane sulfonyl chloride **5** (0.14 ml in 0.6 ml of CH_2Cl_2, 1.46 mmol) was added slowly. The reactant was stirred at RT in a sealed vial for 3 h, and then diluted with CH_2Cl_2 (20 ml). The reactant was washed with saturated $NaHCO_3$ (10 ml), brine (10 ml), dried over Na_2SO_4, filtered, and concentrated. The crude product was purified by flash chromatography on silica gel (elution with 0–20% EtOAc/hexanes) to give 200 mg (52.3%) of compound **15** as a colorless oil. ^{1}H-NMR (DMSO-d_6, 600 MHz, δ): 7.48 (t, 1H, NH), 7.23 (d, J = 8.0 Hz, 2H), 7.16 (d, J = 8.0 Hz, 2H), 4.41 (t, J = 5.6 Hz, 1H), 4.09 (d, J = 6.4 Hz, 2H), 3.57–3.53 (m, 2H), 3.43–3.38 (m, 2H), 2.81 (s, 3H), 2.57 (t, J = 8.0 Hz, 2H), 1.79–1.76 (m, 2H), 1.10 (t, J = 6.4 Hz, 6H). ^{13}C-NMR (DMSO-d_6, δ): 140.89 (1C), 135.98 (1C), 128.60 (2C), 128.09 (2C), 101.92 (1C), 61.06 (2C), 46.39 (1C), 39.67 (1C), 35.56 (1C), 30.66 (1C), 16.00 (2C). HRMS-FAB (*m/z*): $[M+Na]^+$ calcd. for $C_{15}H_{25}NO_4SNa$, 338.1402; found 338.1396.

*Synthesis of 4-(methane sulfonylamino-methyl)-phenylpropylaldehyde) (**MSAPPA**)*

N-[4-(3, 3-diethoxy-propyl)]-methane sulfonamide (**15**, 50 mg, 0.16 mmol) was dissolved in TFA/water (1:3, 1.2 ml). The reactant was stirred at RT for 1 h, then diluted with CH_2Cl_2 (10 ml), and quenched slowly with $NaHCO_3$ (saturated, 10 ml) for 10 min. The aqueous phase was backextracted with CH_2Cl_2 (10 ml). The combined organic phase was dried over Na_2SO_4, filtered, and concentrated under vacuum. The crude product was purified by flash chromatography on silica gel (elution with 20–60% EtOAc/hexanes) to give 30 mg (78.9%) of the compound **MSAPPA** as a white solid. ^{1}H-NMR (DMSO-d_6, 600 MHz, δ): 9.68 (s, 1H, COH), 7.50 (t, 1H, NH), 7.23 (d, J = 7.2 Hz, 2H), 7.18 (d, J = 7.6 Hz, 2H), 4.09 (d, J = 6.0 Hz, 2H), 2.85–2.82 (m, 5H), 2.75 (t, J = 6.8 Hz, 2H). ^{13}C-NMR (DMSO-d_6, δ): 202.96 (1C, CHO), 140.08 (1C), 136.16 (1C), 128.62 (2C), 128.11 (2C), 46.35 (1C), 44.94 (1C), 40.25 (1C), 27.76 (1C). HRMS-FAB (*m/z*): $[M+Na]^+$ calcd. for $C_{11}H_{15}NO_3SNa$, 264.06703; found 264.07012.

*Synthesis of N-[4-(3, 3-diethoxy-propyl)]-methane [^{35}S]sulfonamide (**^{35}S-15**)*

To the crude product of methane [^{35}S]sulfonyl chloride **5** (18.5 mCi, 1.28×10^{-5} mmol), a solution of 4-(3, 3-diethoxy-propyl)-benzylamine (**14**, 12 mg, 0.050 mmol) and Et_3N (10 μl, 0.072 mmol) in anhydrous CH_2Cl_2 (140 μl) was added. The reactant was stirred at RT in a sealed vial for 1 h 30 min, and the solvent was removed under N_2. The crude product was purified by RP-HPLC system b, and the collected fractions were concentrated to dryness and then dissolved in CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated. The residue was dissolved in CH_3CN (3.0 ml) to give 13.8 mCi (74.6%) of **^{35}S-15**. The radiochemical purity was 99.5% by the HPLC system 1. **^{35}S-15** was co-eluted with an authentic standard **15** by the HPLC system 1 (t_R = 12.5 min).

*Synthesis of 4-(methane [^{35}S]sulfonylamino-methyl)-phenylpropylaldehyde) (**^{35}S-MSAPPA**)*

An aliquot of *N*-[4-(3, 3-diethoxy-propyl)]-methane [^{35}S]sulfonamide (**^{35}S-15**) in CH_3CN (0.43 ml, 2.0 mCi) was transferred to a plastic vial (7 ml), and the solvent was removed under N_2. To the residue, TFA/water (1:3, 200 μl) was added. After stirring at RT for 1 h, the reactant was diluted with CH_2Cl_2 (0.5 ml), cooled to 5°C, and quenched slowly with $NaHCO_3$ (saturated, 0.5 ml) for 10 min. The aqueous phase was backextracted with CH_2Cl_2 (3 × 0.5 ml). The combined organic phase was dried over Na_2SO_4, filtered, and concentrated under N_2 to give 1.35 mCi (67.5%) of **^{35}S-MSAPPA** with the radiochemical purity of 73.3% by the HPLC

system 1. **^{35}S-MSAPPA** was co-eluted with an authentic standard **^{35}S-MSAPPA** by the HPLC system 1 ($t_R = 4.0$ min)**.** The crude product was directly used in the next step without further purification.

General procedure for labeling protein (mAb) using ^{35}S-MSAPPA

To the vial containing **^{35}S-MSAPPA** (1.35 mCi), DMSO (10 μl), and NaH_2PO_4 buffer (50 μl, pH = 7.5, different buffer solutions may be used depending on the protein), a solution of mAbs (2.0 mg) in the reaction buffer (120 μl) and $NaCNBH_3$ (50 μl, 0.1 M) were added. The reactant was incubated at RT for 20 h and purified by passing through a gel-filtration column (Bio-Gel, P-6) to give 0.91 mCi (74%) of **^{35}S-16**. The radiochemical purity of the protein was 95% by the HPLC system 4.

Conclusion

Two ^{35}S reagents were developed to radiolabel proteins in good to excellent yield. More than ten mAbs have been labeled with these ^{35}S reagents and their bioactivity was unchanged. Reductive amination (MSAPPA reagent) approach is our preferred method.

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References

[1] S. Ren, P. McNamara, D. Koharski, D. Hesk, S. Borges, *J. Labelled Compd. Radiopharm.* **2007**, *50*, 395–398.
[2] D. S. Wilbur, *Bioconjugate Chem.* **1992**, *3*, 433–470.
[3] A. E. Bolton, W. M. Hunter, *Biochem. J.* **1973**, *133*, 529–539.
[4] G. Vaidyanathan, M. R. Zalutsky, *Bioconjugate Chem.* **1990**, *1*, 269–273.
[5] D. Beiki, S. Shahhosseini, A. Khalaj, M. Eftekhari, *J. Labelled Compd. Radiopharm.* **2002**, *45*, 927–934.
[6] O. R. Pozzi, E. O. Sajaroff, M. M. Edreira, *Appl. Radiat. Isot.* **2006**, *64*, 668–676.
[7] F. Nader, A. Yaron, A. Ewenson, M. Tallon, C. B. Xue, J. V. Srinivasan, E. Eriotou-Bargiota, J. M. Becker, *Biopolymers* **1990**, *29*, 237–245.
[8] O. Y. So, P. Lapresca, N. Shinsky-Bjorde, Unpublished results, **2007**.
[9] D. C. Dean, R. P. Nargund, S. S. Pong, L. Y. P. Chaung, P. Griffin, D. G. Melillo, R. L. Ellsworth, L. H. T. Van Der Ploeg, A. A. Patchett, R. G. Smith, *J. Med. Chem.* **1996**, *39*, 1767–1770.
[10] G. E. Means, R. E. Feeney, *Anal. Biochem.* **1995**, *224*, 1–16.
[11] J. R. Panuska, C. W. Parker, *Anal. Biochem.* **1987**, *160*, 192–201.