

Synthesis of *N*-succinimidyl 4-guanidinomethyl-3-[*I]iodobenzoate: a radio-iodination agent for labeling internalizing proteins and peptides

Ganesan Vaidyanathan & Michael R Zalutsky

Department of Radiology, Duke University Medical Center, Durham, North Carolina, USA. Correspondence should be addressed to G.V. (ganesan.v@duke.edu) or M.R.Z. (zalut001@mc.duke.edu).

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This protocol describes a detailed procedure for the synthesis of *N*-succinimidyl 4-guanidinomethyl-3-[*I]iodobenzoate ([*I]SGMIB), an agent useful in the radio-iodination of proteins, including monoclonal Abs, and peptides that undergo internalization after receptor or antigen binding. In this procedure, the tin precursor *N*-succinimidyl 4-[*I]iodobenzoate ([*I]Boc-SGMIB, 3) was first radio-iodinated to [*I]Boc-SGMIB, a derivative of [*I]SGMIB with the guanidine function protected with Boc groups. Treatment of [*I]Boc-SGMIB with trifluoroacetic acid delivered the final product. The total time for the synthesis and purification of [*I]Boc-SGMIB and its subsequent de-protection is approximately 140 min.

INTRODUCTION

The selection of an appropriate labeling method for a protein or peptide requires careful consideration of the fate of the molecule after its interaction with the biological milieu. For radio-iodinated proteins and peptides, circumventing the action of the deiodinases normally involved in thyroid hormone metabolism is an important general concern. This led to the development of reagents such as *N*-succinimidyl 3-[*I]iodobenzoate (SIB), which yield proteins that do not undergo appreciable deiodination *in vivo* based on their Tyr-dissimilar structure^{1,2}. However, when a labeled protein or peptide undergoes cellular internalization after binding to a cell surface receptor or antigen, then, depending on its intercellular routing, considerable loss of label from the targeted cell can occur^{3–5}. Although this discussion is focused on mAbs for simplicity of presentation, this problem can affect other proteins and peptides also. For example, loss of label after cellular internalization of conventionally radio-iodinated epidermal growth factor receptor (EGFR) was first demonstrated 30 years ago by Carpenter and Cohen⁶.

The internalization process can subject mAbs to extensive catabolism, primarily reflecting the action of proteases present in the lysosomal compartment of the targeted cells. Labeling of mAbs with radiometals is generally easy, and for mAbs labeled with radiometals such as ⁹⁰Y and ¹⁷⁷Lu, low-molecular-weight labeled catabolites egress from a tumor to only a small degree⁷. Compared with radio-iodinated mAbs, this results in higher accumulation of radioactivity in the tumor; however, high renal uptake and low tumor/normal tissue ratios are often drawbacks. When mAbs that undergo internalization are labeled with radio-iodine by direct electrophilic substitution on their Tyr residues, in general, the radioactivity is rapidly lost from the tumor cells, primarily in the form of monoiodotyrosine⁸. No significant advantage with respect to tumor retention of radioactivity was seen even when such mAbs were labeled with SIB, confirming that a process other than deiodination was responsible for loss of label after mAb internalization⁹.

Newer radio-iodination methods are needed that will result in higher retention of radioactivity in tumor cells after the internalization

of labeled mAbs. A number of such 'residualizing labels' have been developed. With these methods, the portion of the molecule bearing the label is inert to lysosomal degradation and becomes trapped inside the cell after mAb proteolysis. The first set of reagents for this purpose (e.g., tyramine-cellobiose (TCB) and inulin-tyramine) contained a non-metabolizable carbohydrate moiety in their structure^{10–14}. Use of these methods to label internalizing mAbs generally resulted in improved tumor retention of radioactivity compared with their directly iodinated counterparts. However, there are a number of disadvantages associated with this labeling approach, including low protein-labeling yields, low immunoreactivity of the labeled mAbs, protein cross-linking and (as a result of the latter) higher liver uptake^{10,14–16}. A more successful method for radio-iodination of internalizing mAbs involves the use of oligopeptides composed of D-amino acids^{3,17}. The unnatural D-amino acids are not substrates for proteases; peptides consisting of more than three D-amino acids are expected to be trapped within the cells because passive transport of peptides of that size is poor. A variation of this tactic that has been described previously is to attach very hydrophilic molecules such as DTPA (diethylenetriamine pentaacetic) to the D-amino acid peptide^{4,18}. This is based on the rationale that when mAbs are labeled with radiometals using chelating agents such as DTPA, the radioactivity is retained in the cells in the form of radiometal-DTPA-Lys.

Our laboratory has been investigating an alternative strategy for labeling internalizing mAbs that is based on the hypothesis that exocytosis of labeled catabolites across cells and lysosomal membranes can be minimized if they are charged species. As the pH of the lysosomal compartment is approximately 5, species containing basic functions are expected to remain positively charged within the lysosomes. To test the feasibility of this approach, studies were performed using *N*-succinimidyl 5-[*I]iodopyridine-3-carboxylate ([*I]SIPC), a reagent containing a positively charged pyridine ring, to label an internalizing mAb, L8A4, reactive with EGFRvIII, a mutant form of the EGFR⁹. Up to 65% higher intracellular retention of radioactivity was observed *in vitro* for L8A4 labeled

using [^{*}I]SIPC compared with L8A4 mAb labeled directly or using [^{*}I]SIB; however, this advantage was transient. On the other hand, excellent tumor/tissue ratios were seen with mAb labeled using [^{*}I]SIPC compared with other methods, primarily because of lower normal organ radio-iodine levels.

The reagent described here, *N*-succinimidyl 4-guanidinomethyl-3-[^{*}I]iodobenzoate ([^{*}I]SGMIB), is an extension of the cationic labeled catabolite concept. Guanidine has a pK_a of approximately 13 and is therefore expected to remain exclusively in the protonated form within the lysosome. We have exploited this property to develop a conjugation labeling agent useful in the radio-iodination of internalizing mAbs¹⁹. [^{*}I]SGMIB was derived simply by attaching a guanidinomethyl function to SIB (Fig. 1). An unlabeled standard of SGMIB (4), its Boc-protected derivative (Boc-SGMIB, 2) and the tin precursor *N*-succinimidyl 4-[^N¹, ^N²-bis(*tert*-butyloxycarbonyl)guanidinomethyl]-3-(trimethylstannyl)benzoate (Boc-SGMTB, 3) were obtained from 3-iodo-4-methylbenzoic acid (1) in multiple steps¹⁹. An SGMIB analog labeled with an α-particle-emitting heavy halogen, ²¹¹At, *N*-succinimidyl 3-[²¹¹At]astato-guanidinomethylbenzoate ([²¹¹At]SAGMB), could be synthesized from the same tin precursor 3 (ref. 20). A considerably higher advantage (twofold to fourfold) in the delivery and retention of radioactivity in tumor was obtained both *in vitro* and *in vivo* when internalizing anti-EGFRvIII mAb L8A4 was radiolabeled with [^{*}I]SGMIB or [²¹¹At]SAGMB compared with the same mAb radio-iodinated using other methods^{19–21}. Proteins are conjugated with labeled SGMIB by incubating [^{*}I]SGMIB with a solution of the protein in pH 8.5 borate buffer for 15–20 min at room temperature (20 °C). The labeled protein is purified using gravity gel filtration chromatography. Typical yields for the conjugation are 60–65% for protein concentrations of 3 mg ml⁻¹ or higher.

With this protocol, it may not be feasible to synthesize [^{*}I]SGMIB at radioactivity levels that are needed for clinical

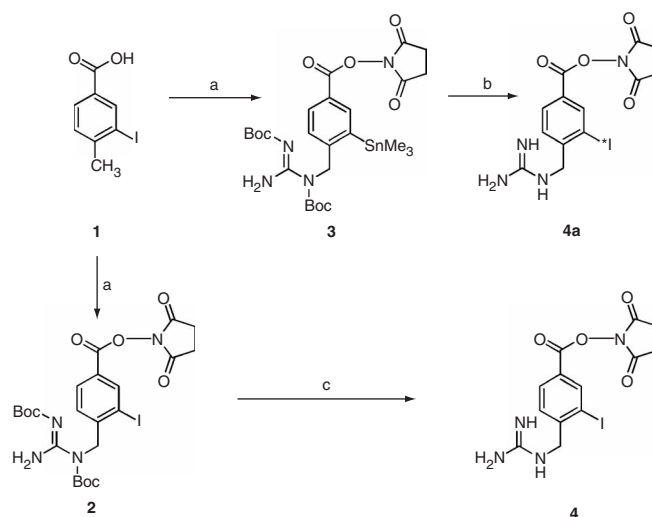


Figure 1 | Scheme for the synthesis of Boc-SGMIB (2), tin precursor (3) and [^{*}I]SGMIB (4a). a. Multiple steps; b. i) ^{*}I, TBHP, HOAc, CHCl₃ ii) TFA; c. TFA.

radio-immunotherapy. To accomplish this, the procedure will need to be modified so that it can be performed using a remote-controlled automated synthesizer.

Experimental design

This protocol describes the synthesis of radio-iodinated SGMIB from a protected tin precursor (Boc-SGMTB). Neither Boc-SGMTB nor the corresponding iodo derivative Boc-SGMIB is commercially available, but they can be synthesized following literature protocols¹⁹. For the most part, 0.2 mg Boc-SGMTB is used for a radio-iodination reaction. Methods for synthesis and small quantities of these compounds are available on request.

MATERIALS

REAGENTS

- Boc-SGMIB, 2 (synthesize according to the literature protocol¹⁹)
- Boc-SGMTB, 3 (synthesize according to the literature protocol¹⁹)
- **! CAUTION** As an active ester, SGMIB can undergo slow hydrolysis; however, when it is stored under argon at -20 °C, no detectable decomposition has been observed for months, if not years.
- *tert*-butyl hydroperoxide (TBHP, Sigma-Aldrich, cat. no. B2633)
- **! CAUTION** Strong oxidizer, potential explosion hazard (see material safety data sheet at <http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIAL/B2633>); however, we have had no untoward incidents and have used TBHP from the same bottle for more than 15 years
- ¹³¹I (Perkin-Elmer, cat. no. NEZ-035H)
- Trifluoroacetic acid (TFA; Sigma-Aldrich, cat. no. 302031)
- TFA for HPLC (Pierce, cat. no. 28902)
- Anhydrous sodium sulfate
- Acetic acid (HOAc)
- Chloroform
- Ethyl acetate
- Hexanes
- Acetonitrile (HPLC grade)

EQUIPMENT

- Reacti vial, 1 ml (Pierce, cat. no. 13221)
- Caps for different vials
- Screw-cap glass vials (1/2 dram)

- Calibrated pipette device (Pipetman or similar) and tips
- Disposable test tubes, glass (10–15 ml capacity)
- HPLC equipped with a UV and gamma detector in series (see EQUIPMENT SETUP)
- Normal-phase HPLC column (Alltech Partisil Silica 10 μm, 4.6 × 250 mm, cat. no. 8271)
- Reversed-phase HPLC column (Waters XTerra 5 μm, 4.6 × 250 mm, cat. no. 186000496)
- HPLC syringe (Hamilton Microliter 700 Series syringe: Model 725, 250 μl, 22 gauge, point style 3, cat. no. 80765)

EQUIPMENT SETUP

HPLC setup Turn the UV detector on and equilibrate the normal-phase HPLC column by allowing at least 30 ml 75:25:0.2 (vol/vol/vol) hexanes/ethyl acetate/HOAc to flow through it. Confirm that the gamma detector is functioning and that the flow and back pressure are appropriate. Inject a standard of Boc-SGMIB¹⁹ (unlabeled) to determine the retention time (*t_R*). The quality of de-protected [^{*}I]SGMIB can be checked using reversed-phase HPLC. For this, inject an aliquot of a solution in acetonitrile into a reversed-phase column eluted with a gradient consisting of 0.1% (wt/vol) TFA/water (solvent A) and 0.1% (wt/vol) TFA/acetonitrile (solvent B) at 1 ml min⁻¹. The content of solvent B is increased linearly from 10 to 40% over a period of 30 min and then to 100% in the next 5 min. **! CAUTION** Provide enough lead shielding around HPLC parts such as the injector, column, detector and the container in which the effluent is collected.

PROTOCOL

PROCEDURE

[¹³¹I]SGMIB synthesis

1| Prepare an approximately 30% (wt/vol) solution of TBHP in chloroform by mixing 370 μ l 90% TBHP (475 μ l 70% TBHP) and 670 μ l chloroform (same volume of chloroform for both). Add a few crystals of anhydrous sodium sulfate to this mixture to remove water present in the TBHP solution. Use the supernatant for the reaction. Prepare solutions of HOAc (3% vol/vol) and Boc-SGMTB (20 mM, 1 mg per 75 μ l) in chloroform.

! CAUTION In our laboratory, we use a 90% solution of TBHP that was procured several years ago. However, probably owing to its potential explosion hazard, it is now sold as a 70% solution.

2| Using a Pipetman with a micro tip attached, dispense the required amount of radio-iodine in 0.1 N NaOH into a 1 ml Reacti vial.

! CAUTION Radio-iodine is volatile (particularly in the oxidized form) and needs to be handled in a well-ventilated charcoal-filtered hood with adequate lead shielding. If you are not familiar with dealing with radioactivity, it is recommended that you consult your institutional Radiation Safety Office to determine appropriate protective measures and radioactivity monitoring devices. If the volume of radio-iodine solution is more than 3 μ l, it should be concentrated to a volume of 3 μ l or less using a gentle stream of argon.

▲ CRITICAL STEP It is best to use fresh batches of radio-iodine. With time, byproducts of higher oxidation states such as iodate and periodate may be formed, which can compromise the efficiency of the radio-iodination reaction. Although radiochemical yields are generally considerably higher, it is recommended that you conservatively assume a yield of 50% to calculate the initial amount of radio-iodine added to the reaction vessel.

3| Add 3 μ l solution of HOAc in chloroform (3%, vol/vol). If radio-iodine volumes higher than 3 μ l must be used, adjust the concentration of the HOAc solution so that the volume will still be 3 μ l. For example, if 6 μ l radio-iodine is used, then use 3 μ l 6% (vol/vol) HOAc.

4| Add 5 μ l TBHP solution prepared in Step 1.

5| Add a solution of Boc-SGMTB in chloroform (15 μ l 20 mM, 0.2 mg).

6| Add a triangular stir bar, cap the vial and stir the mixture at room temperature (20 °C) for 30 min.

7| Withdraw the entire reaction mixture into a 250 μ l HPLC syringe. Rinse the reaction vial with 50 μ l chloroform and withdraw the rinse into the same HPLC syringe. Inject the syringe contents onto a normal-phase HPLC column eluted isocratically with 25:75 ethyl acetate/hexanes containing 0.2% (vol/vol) HOAc at a flow rate of 1 ml min⁻¹.

▲ CRITICAL STEP Approximately 5% of the radioactivity frequently sticks to the reaction vial. Additional chloroform (50 μ l) rinses can extract some of this radioactivity; however, larger volumes of chloroform in the injectant will compromise chromatographic resolution.

? TROUBLESHOOTING

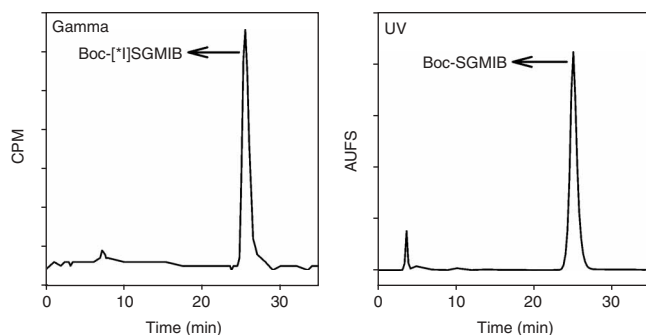


Figure 3 | HPLC profiles of Boc-[¹³¹I]SGMIB isolated from the reaction mixture by HPLC and co-injected with a sample of unlabeled standard.

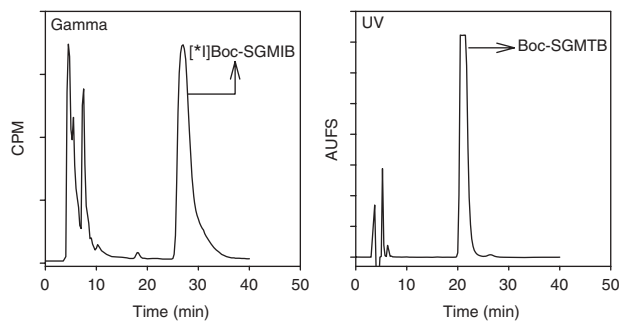


Figure 2 | HPLC profiles of the crude reaction mixture from Boc-[¹³¹I]SGMIB synthesis by the radio-iodination of Boc-SGMTB.

8| Collect radioactivity fractions corresponding to labeled [¹³¹I]Boc-SGMIB (t_R under these HPLC conditions is 28–29 min) in glass test tubes, and, using a Pasteur pipette with a rubber bulb attached, combine these fractions into one test tube. Evaporate solvents from pooled fractions to approximately 0.1 ml with a gentle stream of argon.

▲ CRITICAL STEP During the initial evaporation of HPLC fractions, do not let the fractions dry completely.

? TROUBLESHOOTING

9| Transfer the residual radioactive solution to a 1/2-dram vial and dry it further with a gentle stream of argon. Rinse the original test tube that contained the radioactivity with 0.1 ml

ethyl acetate, transfer the rinse to the 1/2-dram vial and evaporate the ethyl acetate. Repeat the rinse-transfer-evaporation process one time.

10| Add 0.1 ml TFA to the radioactivity in the 1/2-dram vial, gently vortex the vial and allow the de-protection reaction to proceed at room temperature (20 °C) for 15 min.

11| Evaporate the TFA with a stream of argon. To ensure complete removal of TFA, add 3 × 0.1 ml ethyl acetate and evaporate it each time.

● **TIMING**

Step 1: 10 min; Step 2: 5 min (if no evaporation required)
Steps 3–5: 5 min; Step 6: 32 min; Steps 7 and 8: 45–50 min
Step 9: 10 min, Step 10: 17 min; Step 11: 10 min

? **TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
7,8	Radioactivity from HPLC fractions sticks to test tube	Evaporation to dryness	Do not let the solution dry. Evaporate to a small volume (approximately 0.1 ml), transfer to a vial in which mAb labeling will be performed and then dry
8	Poor radiochemical yields	Poor quality of SGMTB, reagents and/or radio-iodine	Purify SGMTB using chromatography, if necessary ¹⁹ . Use fresh radio-iodine and reagents
8	Shorter t_R and split peak for [¹²⁵ I]SGMIB	Higher volumes of chloroform in the injectant	Try to keep the volume of injectant 50 µl or less
8	t_R of Boc-[¹²⁵ I]SGMIB is different from that given in text	Different batch of HPLC columns; slight changes in mobile phase composition, flow rate and/or temperature	Always inject a standard beforehand to ascertain the t_R

ANTICIPATED RESULTS

Typical yields

Typical isolated yields of [¹³¹I]Boc-SGMIB will be 65%. The de-protection of [¹³¹I]Boc-SGMIB to form the desired [¹³¹I]SGMIB proceeds nearly quantitatively.

HPLC data

Typical HPLC profiles of the Boc-[¹³¹I]SGMIB preparation reaction mixture and of the isolated product are shown in **Figures 2** and **3**, respectively. The t_R of Boc-[¹³¹I]SGMIB under our HPLC conditions is typically approximately 28–29 min. Reversed-phase HPLC profiles of de-protected [¹²⁵I]SGMIB and co-injected standard are shown in **Figure 4**. The t_R of the de-protected SGMIB under the reversed-phase HPLC conditions used is approximately 17 min.

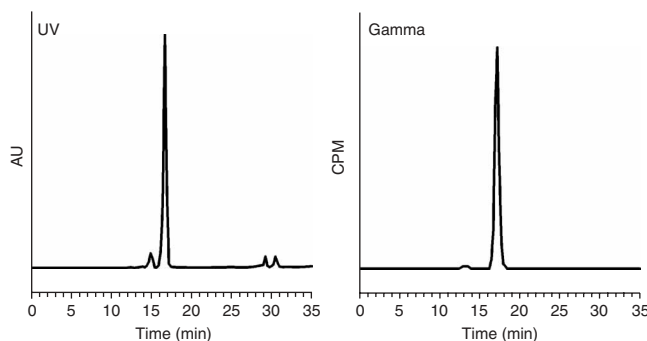


Figure 4 | Reversed-phase HPLC profile of [¹²⁵I]SGMIB co-injected with a sample of unlabeled SGMIB.

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