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ORIGINAL ARTICLE

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Radiosynthesis of ^{125}I-labeled 2-cyanobenzothiazole: A new prosthetic group for efficient radioiodination reaction

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ABSTRACT

Herein we report an efficient radiolabeling method based on a rapid condensation reaction between N-terminal cysteine and 2-cyanobenzothiazole (CBT). Radioiodination of 2-cyano-6-hydroxybenzothiazole 2 was carried out using chloramine-T to give ^{125}I-labeled CBT ([^{125}I]1) with a high radiochemical yield (90±6% isolated yield, n=3) and radiochemical purity (>99%). To evaluate the radiolabeling efficiency of ^{125}I-labeled CBT, model compounds, L-cysteine and N-terminal cysteine conjugated cRGD peptide were reacted with [^{125}I]1 under mild conditions. The radiolabeling reactions rapidly provided the ^{125}I-labeled products [^{125}I]5 and [^{125}I]6 with excellent radiochemical yields and radiochemical purity. Therefore, we demonstrate that [^{125}I]1 will be a useful prosthetic group for radioactive iodine labeling of N-terminal cysteine bearing biomolecules.

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Key Word: 2-Cyanobenzothiazole, Radioiodination, Bioorthogonal reaction, Biomolecules, Radiolabeling

Introduction

In the last few decades, radioactive iodines have been extensively used for radiolabeling of biologically active small molecules and macromolecules (1-4). The biomedical research using several commercially available radioactive iodines include positron emission tomography (PET, ^{124}I) (5-7), single photon emission computed tomography (SPECT, 123I) (8, 9), cancer therapy (^{131}I), pharmacokinetics and radioimmunoassay (^{125}I) (10-13). One of the most frequently used method for the radioiodination of biomolecules is the electrophilic substitution reaction of tyrosine residue in the target molecules (14-19). However such direct radioiodination of biomolecules has some disadvantages, including the fact that radioactive iodine labeled with a tyrosine residue is normally unstable in a living subject and the liberated radioactivity was rapidly accumulate in the thyroid and stomach, which often caused false-positive signals (20, 21). Additionally a strong oxidant is necessary in the electrophilic radiolabeling reaction that frequently involved severe side reaction such as oxidation of methionine side chain and other biologically active sites. Therefore, such labeling procedure often resulted

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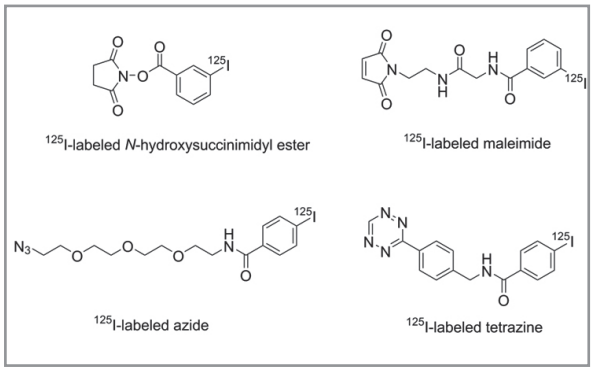


Figure 1. Previously reported prosthetic groups for radioiodination reaction

in loss or reduction of biological activity in some cases (22, 23). To solve these problems, several prosthetic groups have been reported (Figure 1). Well-established radioactive iodine-labeled prosthetic groups contain an activated ester (24) and maleimide (25) for efficient reaction with the amino and thiol group respectively. Recently we have reported radioactive iodine labeled azide (26) and tetrazine analogs (27) for rapid and site-specific radiolabeling of biomolecules and nanomaterial. These prosthetic groups not only offered highly efficient radiochemical results but also provided useful radiotracers for biodistribution and in vivo imaging studies. To apply these methods, however, the target molecules need to be modified to have other artificial functional groups which can be readily reacted with the radiolabeled prosthetic group. For example, a trans-cyclooctene analog has to be conjugated with the biomolecules to react with a radiolabeled tetrazine. These modifications required additional synthetic and purification steps and randomly conjugated functional groups may cause decreased bioactivity of the target molecules. These observations led us to develop a new prosthetic group which can be rapidly and specifically reacted with a natural amino acid. Previously we have reported the radiosynthesis of 18F-labeled 2-cyanobenzothiazole (CBT) (28). This structure showed an excellent specificity and rapid reaction rate toward N-terminal cysteine. An ^{18}F-labeled CBT was applied to the efficient radiolabeling of peptide and protein. Based on these results, we expected that 2-cyano-6-hydroxybenzothiazole 2, which contains a phenolic structure, can be used as a useful prosthetic group for radioiodination of biomolecules. Herein, we report the synthesis of radioactive iodine labeled CBT and its radiolabeling efficiency using N-terminal cysteine bearing cancer targeting peptide. In this study, ^{125}I was used, because it offered a few advantages such as low cost, easy commercial availability, and relatively long half-life (59.4 days) which is suitable for repetitive experiments to optimize radiochemical reactions.

Materials and Methods

1.1 General Methods

All chemicals including 2-cyano-6-hydroxybenzothiazole (CBT), L-cysteine hydrochloride, Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), and N,N-Diisopropylethyamine (DIPEA) were purchased from Sigma Aldrich. The cysteine conjugated cRGD peptide (cRGD-Cys) 4 was purchased from Peptide International, Inc. All the chemicals were used without further purification steps. ^{1}H NMR and ^{13}C NMR spectra were measured with JEOL 500 MHz spectrometer and dimethyl sulfoxide (DMSO-d_6) and chloroform-d ($CDCl_3$) were used as solvent. Chemical shifts are reported as δ in the units of parts per million (ppm) relative to an internal standard (tetramethylsilane, 0.0 ppm); multiplicities are reported as follows: s (singlet), d (doublet), dd (doublet of doubles), or m (multiplet). HPLC experiments were carried out using Agilent HPLC system: 0.1% formic acid containing water (solvent A), 0.1% formic acid containing acetonitrile (solvent B) were used as eluents. Preparative HPLC was equipped with Eclipse XDB-C18 column (7 μm, 21.2 x 150 mm) and analytical HPLC system was equipped with Eclipse XDB-C18 column (5 μm, 4.6 x 250 mm). MALDI-Tof

mass was measured by using a 4800 MALDI TOF/TOF Analyzer (ABSciex).

1.2. Synthesis of 6-hydroxy-7-iodobenzo[d]thiazole-2-carbonitrile (1)

To a solution of 2-cyano-6-hydroxybenzothiazole 2 (1.0 g, 5.7 mmol) in DMSO (10 mL), iodine monochloride (0.927 g, 5.7 mmol) was added at room temperature. The reaction mixture was allowed to stir at room temperature for 60 min. An aqueous solution of sodium metabisulfite (2.0 M, 5.7 mL) was added to quench the reaction. Crude product was diluted with ethyl acetate (100 mL) and it was washed with brine (100 mL x 2). The combined organic phase was dried over MgSO4, and concentrated under reduced pressure. Silica gel chromatography (Hexanes : EtOAc = 4:1) was used to provide the product 1 as yellow solid (1.3 g, 84%). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 8.03 (d, 2H, J = 8.8 Hz), 7.21 (d, 2H, J = 8.8 Hz); ^{13}C NMR (DMSO-d_6, 500 MHz) δ 159.49, 145.86, 143.20, 130.81, 125.94, 117.50, 114.13, 72.54; HRMS ($[M+H]^+$) calculated for $C_8H_5IN_2OS^+$:302.8041; found 302.8024.

1.3. Synthesis of 2-(6-hydroxy-7-iodobenzo[d]thiazol-2-yl)-4,5-dihydrothiazole-4-carboxylic acid (5)

To a solution of the compound 1 (1.0 g, 3.3 mmol) in DMF (10 mL), L-Cysteine hydrochloride 3 (0.521 g, 3.3 mmol), TCEP (1.8 g, 6.6 mmol) and DIPEA (5.1 g, 39 mmol) were sequentially added. The reaction mixture was allowed to stir for 30 min at room temperature and then the reaction was quenched by adding aqueous acetic acid (10%) to adjust the reaction mixture weak acidic (pH = 3-4). Crude product was diluted with ethyl acetate (100 mL) and washed with brine. The combined organic phase was dried over $MgSO_4$, and concentrated under reduced pressure. Silica gel chromatography (Hexanes : EtOAc = 1:9) was used to provide the product 5 as light yellow solid (1.05 g, 79%). ^{1}H NMR (DMSO-d_6, 500 MHz) δ 13.14 (s, 1H), 11.4 (s, 1H), 7.95 (d, 2H, J = 9.0 Hz), 7.11 (d, 2H, J = 9.0 Hz), 5.34 (t, 2H, J = 8.2 Hz), 3.72 (dd, 1H, J = 8.2, 9.8 Hz), 3.64 (dd, 1H, J = 8.5, 9.8 Hz); ^{13}C NMR (DMSO-d_6, 500 MHz) δ 171.63, 164.95, 157.85, 155.76, 145.36, 144.21, 125.30, 116.12, 78.63, 73.43, 35.39; HRMS ($[M+H]^+$) calculated for $C_{11}H_8IN_2O_3S_2^+$: 406.9093; found 406.9081.

1.4 Synthesis of the peptide 6

To a solution of iodo CBT 1 (5.0 mg, 16.6 μmol) in DMF (1 mL), cRGD-Cys peptide 4 (5.0 mg, 7.0 μmol), TCEP (4.0 mg, 14 μmol) and DIPEA (13.5 mg, 105 μmol) were added. The reaction mixture was allowed to stir for 30 min at room temperature. The reaction was quenched by adding aqueous acetic acid to adjust pH of the reaction mixture weakly acidic. Purification of the crude product was carried out by using a preparative HPLC (flow rate: 10 mL/min, eluent gradient: 95% solvent A in 5% solvent B for 0-2 min; a linear gradient to 35% solvent A in 65% solvent B for 2-30 min, retention time: 17.5 min). The solvent was removed under reduced pressure followed by lyophilization to give the desired product 6 (5.0 mg, 71%). MALDI-TOF ($[M+H]^+$) calculated for $C_{38}H_{47}IN_{11}O_9S_2^+$: 992.27; found 992.28.

2. Radiochemistry

2.1 Synthesis of ^{125}I-labeled 2-cyano-6-hydroxybenzothiazole ([^{125}I]1)

To a solution of CBT 2 (1 mg) in 100 μL of DMSO, 3 μL of acetic acid and 10 μL of aqueous chloramine-T solution (0.1 mg) were added and 130 MBq of [^{125}I] NaI solution (0.1 M NaOH) was then added at room temperature. The reaction was carried out for 10 min at room temperature and then it was quenched by addition of aqueous sodium metabisulfite solution (1.0 M, 20

μL). The crude product was purified with a preparative HPLC (flow rate: 10 mL/min, eluent gradient: 80% solvent A in 20% solvent B for 0-2 min; 20% solvent A in 80% solvent B for 2-22 min, retention time: 18.2 min) and 120 MBq of 125I-labeled product ([^{125}I]1) were obtained (92% of radiochemical yield). Radiochemical purity was >99% as determined by analytical HPLC (flow rate: 1 mL/min, eluent gradient: 80% solvent A in 20% solvent B for 0-2 min; a linear gradient to 20% solvent A in 80% solvent B for 2-22 min, retention time: 16.7 min). After purification of the radiolabeled product using a preparative HPLC, the fraction containing the desire product ([^{125}I]1) was diluted with 45 mL of deionized water. The diluted solution was loaded with a SepPak C18 cartridge (Waters) which was preconditioned with 5 mL of ethanol and 10 mL of deionized water. The product trapped in the SepPak C18 cartridge was eluted with 1.5 mL of DMSO (or absolute ethanol) for further experiment.

2.2 Radiosynthesis of [^{125}I]5

To a mixture of L-Cysteine hydrochloride 3 (1 μL in DMF), TCEP (2 μL, 2 equiv.), and DIPEA (15 μL,15 equiv.), 2.0 MBq of ^{125}I-labeled CBT ([^{125}I]1, 32 μL in DMF) was added. Total reaction volume was 50 μL and the final concentration of 3 was 0.1 mM, 0.5 mM or 1.0 mM. The labeling reaction was conducted at room temperature or 40 oC. Radiochemical yields were determined by integration of analytical radio-HPLC chromatogram (flow rate: 1 mL/min, eluent gradient: 20% solvent B in solvent A for 0-2 min; 20-80% solvent B in solvent A for 2-22 min; 100% solvent B for 22-28 min, retention time of [^{125}I]5: 11.8 min). The observed conversion yields were summarized in the table 1.

2.3. Radiosynthesis of [^{125}I]6

To a mixture of cRGD-Cys 4 (1 μL in DMF), TCEP (2 μL, 2 equiv.), and DIPEA (15 μL,15 equiv.), 125I-labeled CBT ([^{125}I]1, 2.0 MBq, 32 μL in DMF) was added. Total reaction volume was 50 μL and the final concentration of 4 was 0.1 mM, 0.5 mM or 1.0 mM. The labeling reaction was conducted at room temperature or 40°C. Radiochemical yields were determine by integration of analytical radio-HPLC chromatogram (flow rate: 1 mL/min, eluent gradient: 5% solvent B in solvent A for 0-2 min; 5-65% solvent B in solvent A for 2-32 min; retention time of [^{125}I]5: 15.7 min). The observed conversion yields were summarized in the table 2.

Table 1. Radiolabeling results of L-cysteine 3 using [^{125}I]1[a]

Entry	Conc. of 3 (mM)	Reaction Conditions	RCY(%)[b]
1	1	DMF, 40ºC	>99
2	0.5	DMF, 40ºC	93
3	0.1	DMF, 40ºC	85
4	0.1	DMF, RT	82
5	0.1	DMF/H_2O 1:1, 40 ºC	80
6	0.1	DMF/H_2O 1:1, RT	70

[a] Reaction time:5 min
[b] Radiochemical yield was determined by radio-HPLC.

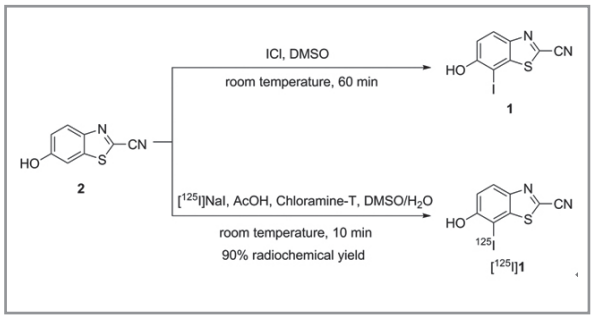
Table 2. Radiolabeling results of cysteine bearing cRGD peptide 4 using [^{125}I]1[a]

Entry	Conc. of 4 (mM)	Reaction Conditions	RCY(%)[b]
1	1	DMF, 40ºC	>99
2	0.5	DMF, 40ºC	90
3	0.1	DMF, 40ºC	80
4	0.1	DMF/H_2O 1:1, 40 ºC	75
5	0.1	DMF, RT	63
6	0.1	DMF/H_2O 1:1, RT	56

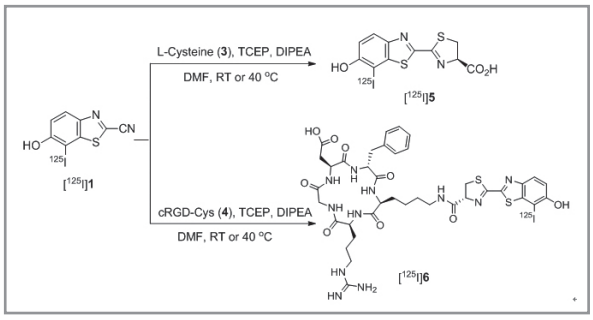
[a] Reaction time: 30 min
[b] Radiochemical yield was determined by radio-HPLC.

Results and Discussion

A non-radioactive analogue, iodinated CBT 1, was synthesized using iodochloride (ICl) as a reference



Scheme 1. Synthesis of [^{125}I]1 and the standard compound 1



Scheme 1. Synthesis of [^{125}I]1 and the standard compound 1

for HPLC characterization of [^{125}I]1 (Scheme 1). Radioiodination of 2 was carried out using [^{125}I]NaI and chloramine-T as an oxidizing agent. Because the cyano group in the compound 2 can be easily hydrolyzed to amide or carboxylic acid under the basic condition, the pH of reaction mixture was adjusted to less than 8 to obtain high radiochemical results. The iodination reaction was quenched by adding aqueous sodium metabisulfite. After HPLC purification of the crude product, [^{125}I]1 was obtained with a high radiochemical yield (90±6%, n=3). HPLC chromatogram of the crude mixture clearly showed the major product at 16.7 min of retention time (Figure 2a) and the radiochemical purity of the purified [^{125}I]1 was more than 99% as determined by analytical HPLC (Figure 2b). Radiolabeling reaction was performed by using varying amount of radioactivity (10-130 MBq) however, the radiochemical results were consistent. The radiolabeling efficiency of ^{125}I-labeled

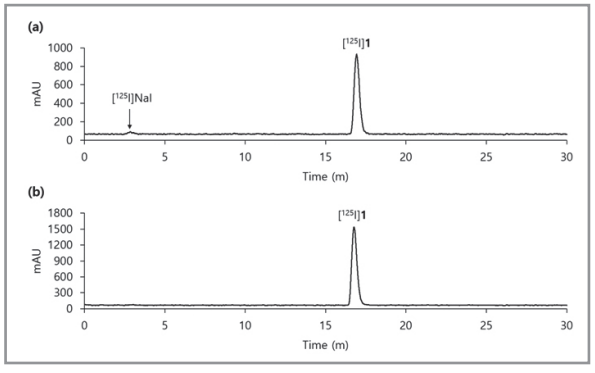


Figure 2. Radio-HPLC chromatogram (a) crude product and (b) purified [^{125}I]1

CBT was evaluated by using L-cysteine 3 and cysteine bearing cyclic RGD peptide (cRGD) 4 (Scheme 2). The standard compounds 5 and 6 were synthesized by using the iodinated CBT 1 for HPLC characterization of the radiolabeled products [^{125}I]5 and [^{125}I]6.

The radiolabeling reaction was performed by mixing the cysteine substrates (0.1, 0.5, and 1 mM) with 2.0 MBq of [^{125}I]1 at room temperature or 40 °C. TCEP was added to the reaction mixture for preventing undesired oxidation of the thiol group and pH was adjusted to 7.0−7.5 by using DIPEA. Two solvents system, anhydrous DMF and DMF/H2O (1:1 mixture), were used in these reactions. The ligation between CBT and cysteine was carried out in a small volume (50 μL) for optimization of the radiochemical reactions. The radiochemical yields in the Table 1 and 2 were measured by the integration of radio-HPLC. For accurate determination of the radiolabeling yields and kinetics, each reaction was quenched by adding acidic aqueous solution (0.1 M HCl) at each time points. As shown in the Table 1, rapid condensation reactions were observed between [^{125}I]1 and 3. The radiochemical yields of [^{125}I]5 were dependent on the concentration of cysteine substrate. More than 99% and 85% of [^{125}I]5 were obtained by using 1.0 mM and 0.1 mM of L-cysteine respectively in 5 min at 40 °C (entry 1 and 3). In addition, more than 99% of [^{125}I]1 was converted to [^{125}I]5 by using 0.1 mM of cysteine in 30 min. Interestingly, the radiochemical yield of

[^{125}I]5 was slightly decreased in the presence of water (entry 3 vs. entry 5) at 40 oC and similar results were also observed in the Table 2 (entry 3 vs. entry 4). These results probably due to the fact that water solvent could form a hydrogen bond with the amino group of the cysteine that resulted in hindering nucleophilic addition reaction with the carbon in the cyano group.

Next, we carried out the radiolabeling of cRGD-Cys 4 using TCEP and DIPEA (Scheme 2). A longer reaction time (30 min) were necessary to achieve satisfactory radiolabeling efficiency of [^{125}I]6, because the size of peptide substrate is bigger than a single amino acid, L-cysteine. As shown in the Table 2, the observed radiochemical yield of [^{125}I]6 were also depended on the amount of cysteine conjugated cRGD and reaction temperature. Using 0.1 mM of the peptide, as high as 80% and 63% of [^{125}I]1 was converted to the desired product [^{125}I]6 at 40 oC and room temperature respectively (entry 3 and 5). The reaction condition utilizing a higher concentration of 4 provided more than 90% of radiochemical yield in 30 min. Anhydrous reaction condition provided better radiochemical yields than those of aqueous solvent system (entry 3 vs. entry 4) which were similar results with those observed in the Table 1. The presented results clearly suggested that [^{125}I]1 can be used as an efficient prosthetic group for radioactive iodine labeling of cysteine bearing peptides.

Previously reported prosthetic groups such as a radioactive iodine labeled N-hydroxyl succinimidyl ester and maleimide could not produce a site-specifically labeled product when a peptide substrate contains large number of lysine or cysteine groups. On the other hand, CBT structure can only react rapidly with N-terminal cysteine (1, 2-amino thiol moiety) to provide a condensation product and thus [^{125}I]1 can allow a site-specific radiolabeling result. In general, N-terminal cysteine can be easily incorporated into a peptide sequence by using a protected cysteine as the last amino acid in the procedure of solid phase peptide synthesis. Moreover, the preparation of [^{125}I]1 required a single reaction step from a commercially available precursor 2 and the radiolabeling yield of [^{125}I]1 was higher than those of our previous prosthetic groups,^{125}I-labeled tetrazine (65%) [27] and ^{125}I-labeled azide (75-85%) (29). Therefore, it is expected that the radiolabeling reaction using [^{125}I]1 will be used as a quite useful platform methodology to synthesize radioactive iodine labeled products for molecular imaging (SPECT) and other biomedical applications.

Conclusion

In conclusion, we demonstrated a simply and highly efficient ^{125}I-labeling method based on a rapid condensation reaction between CBT and N-terminal cysteine. We anticipated that other kinds of radioisotopes such as ^{124}I (PET imaging), ^{131}I and ^{211}At (therapy of disease) labeled CBT can also be synthesized by using the same protocol. Radiolabeling of cancer targeted large peptide and affibody using the present method will be our next study.

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