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Protocol

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Synthesis of ^{18}F−labeled 2-cyanobenzothiazole derivative for efficient radiolabeling of *N*−terminal cysteine−bearing biomolecules

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ABSTRACT

This article provides an efficient ^{18}F−labeling protocol based on a rapid condensation reaction between 2−cyanobenzothiazole (CBT) and *N*−terminal cysteine−containing biomolecules. The ^{18}F−labeled CBT (^{18}F−**1**) was prepared by radiofluorination of the tosylated precursor **4** with 18−crown−6/K+/[^{18}F]F$^-$ complex. Using the purified ^{18}F−**1**, ^{18}F−labeled peptide (^{18}F−**7**) and protein (^{18}F−**8**) could be synthesized efficiently under mild conditions. This strategy would provide a convenient approach for rapid and site−specific ^{18}F−labeling of various peptides and proteins for *in vivo* imaging and biomedical applications.

Key Word: Radiolabeling, Bioorthogonal reaction, Cancer targeting peptide, Protein, PET imaging

Introduction.

Bioorthogonal conjugation can be defined as a ligation reaction in which two reactants are joined selectively in aqueous or biological media to provide the desired product in high chemical yield and short time.(1 - 4) In recent years, these conjugation reactions have been applied to the synthesis of molecular probes for nuclear imaging applications using positron emission tomography (PET) and single-photon emission computed tomography (SPECT) as well as for therapeutic purposes.(5 - 9) Particularly, several important diagnostic radioisotopes have short half-lives, and therefore the radiolabeling procedures require fast and efficient reactions which can provide high radiochemical yield (RCY), radiochemical purity, and minimal formation of undesired side-products. In this regard, the catalyst-free bioorthogonal reaction can be a highly useful tool for radiolabeling of complex biomolecules, which are not stable under harsh reaction conditions such as elevated temperature and extreme pH.

This study focuses on the high reactivity of 2-cyanobenzothizole (CBT) toward an 1,2-amino thiol group.(10 - 13)

In our previous study, we have reported that the F-labeled 2-cyanobenzothiazole (^{18}F-CBT)can be reacted with *N*-termnal cysteine with a second-order reaction rate of ca. 9.1 M^{-1} s^{-1}.(14,15) The rapid condensation reaction using ^{18}F−CBT can be utilized in the rapid radiolabeling of *N*−terminal cysteine containing biomolecules, and the radiolabeled products have been applied to the molecular imaging studies.(16) We herein report a detail protocol for preparation of ^{18}F-CBT and its application to the

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efficient radiolabeling of cancer targeting peptide and bioluminescent protein.

Protocol

1. General methods

All chemicals were purchased from commercial sources and used without purification step. Analytical TLC was performed with silica gel 60F plates with a fluorescent indicator (254 nm).

Plates were visualized by ultraviolet light. ^{1}H and ^{13}C NMR spectra were obtained by using a Bruker 400 MHz magnetic resonance spectrometer. ^{1}H NMR spectra are reported as follows: chemical shifts are reported as δ in units of parts per million (ppm) relative to chloroform−d (δ 7.26, s) or methanol $-d_4$ (δ 3.31); multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), or br (broadened);coupling constants are reported as a J value in Hertz (Hz); the number of protons (n) for a given resonance is indicated nH, and based on the spectral integration values. All mass analyses were performed on a JMS−700 (JEOL, LTD., Tokyo Japan) in Seoul National University (Seoul, Republic of Korea).

2. Synthesis of 2−cyano−6−hydroxy benzo thiazole (3)

2.1) Pyridine hydrochloride (9.0 g, 78 mmol) was added to a round−bottom flask and heated to 200 °C until it was melted completely.

2.2) 2-Cyano-6-methoxybenzothiazole **2** (500 mg, 2.63 mmol) was added to a dissolved pyridine hydrochloride and the reaction mixture was heated for 2 h at 200 °C.

2.3) After the reaction mixture was cooled to 0 °C, saturated aqueous $NaHCO_3$ solution (50 mL) was added slowly to the crude product.

2.4) The precipitated product was collected by filtration, and then it was washed with pure water several times.

2.5) The solid product was dried under reduced pressure to give 2-cyano-6-hydroxybenzothiazole **3** (348 mg, 1.97 mmol, 75%), which was used in the next step without further purification.

3. 2−(2−cyanobenzothiazol−6−yloxy) ethyl 4−methylbenzenesul fonate (4)

3.1) To a solution of 2-cyano-6-hydroxybenzothia-zole **3** (260 mg, 1.48 mmol) in DMF (1 mL), ethylene di (p−toluenesulfonate) (1,640 mg, 4.42 mmol) and potassium carbonate (510 mg, 3.69 mmol) were added sequentially (Scheme 1).

3.2) The reaction mixture was stirred for 24 h at room temperature under nitrogen atmosphere, and then the reaction was quenched by adding aq. 1 M HCl (4 mL) at 0 °C

3.3) The mixture was partitioned between water (50 mL) and ethyl acetate (50 mL). The aqueous layer was separated and extracted with ethyl acetate (50 mL × 2).

3.4) The combined organic layer was dried with $MgSO_4$, and then the solvent was removed by rotary evaporation.

3.5) The crude product was purified with SiO_2 column chromatography (dichloromethane: hexanes = 4:1) to give the compound **4** (294 mg, 0.78 mmol, 53%).

4. 2−cyano−6−(2−fluoroethyoxy)benzo-thiazole (1)

4.1) To a solution of 2-cyano-6-hydroxybenzothiazole **3** (260 mg, 1.48 mmol) in DMF (1 mL), 2-fluoroethyl 4-methylbenzenesulfonate (120 mg, 0.55 mmol) and and potassium carbonate (138 mg, 1.0 mmol) were added (Scheme 1).

4.2) The reaction mixture was stirred for 24 h at room temperature under nitrogen atmosphere, and then the reaction was quenched by adding aq. 1 M HCl (1 mL) at 0 °C.

4.3) The mixture was partitioned between water (50 mL) and ethyl acetate (50 mL). The aqueous layer was separated and extracted with ethyl acetate (50 mL × 2). The combined organic ayer was dried with $MgSO_4$, and then the solvent was removed by rotary evaporation.

4.4) The crude product was purified with SiO_2 column chromatography (ethyl acetate: hexanes = 5:1) to give the compound **1** (78 mg, 0.35 mmol, 70%).

5. Synthesis of ^{18}F−labeled 2−cyanobenzothiazole derivative (^{18}F−1)

5.1) Radioactive fluoride ([^{18}F]-fluoride) (1,000 mCi) was prepared by proton bombardment of 2.5 mL [^{18}O] enriched water target via the ^{18}O(p,n)^{18}F nuclear reaction.

5.2) The [^{18}F]-fluoride was then trapped onto Sep-Pak QMA cartridge.

5.3) 18-Crown-6/K_2CO_3 solution (1 mL, 15:1 CH_3CN/H_2O, 16.9 mg of 18-crown-6, 4.4 mg of K_2CO_3) was used to elute the [^{18}F]-fluoride from QMA cartridge into a dried glass reactor.

5.4) The resulting solution was azeotropically dried with sequential CH_3CN evaporations at 90 °C.

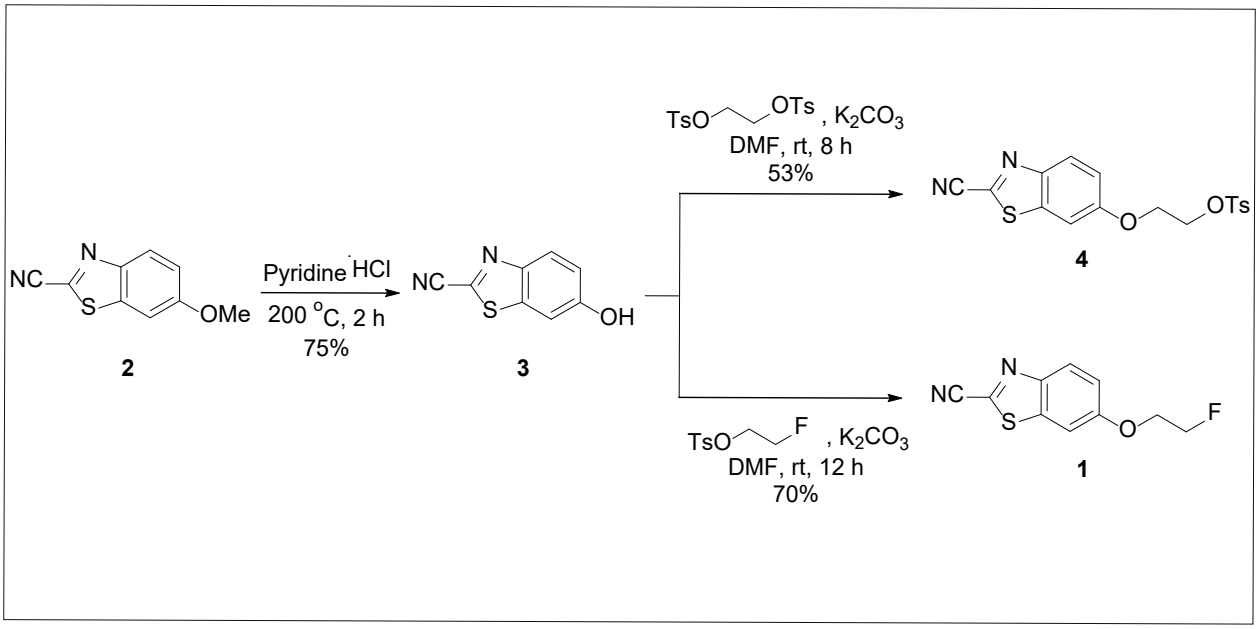
5.5) A solution of compound **4** (2 mg in 1 mL of anhydrous CH_3CN) was added to the reactor and heated at 90 °C for 10 min (Scheme 2A).

5.6) After the reaction mixture was cooled to 25 °C, 0.05 M HCl (2.5 mL) was added to quench the reaction and prevented basic hydrolysis of the product.

5.7) The crude mixture was then purified with a semipreparative HPLC [Phenomenex Gemini column: 10 × 250 mm, 5 μm, 3 mL/min, and eluent gradient: 0 - 3 min 40% (0.1% TFA containing CH_3CN in 0.1% TFA containing H_2O); 3 - 35 min 40 - 100% (0.1% TFA containing CH_3CN in 0.1% TFA containing H_2O), Retention time (R_t) of the product (^{18}F-**1**) = 21.0 min.]

5.8) The collected product (^{18}F-**1**) was diluted with pure water (20 mL) and passed through a C18 cartridge. The cartridge was further washed with pure water (20 mL).

5.9) The trapped ^{18}F-**1** was eluted out with Et_2O (2.5 mL), and then Et_2O was removed by helium stream and used for next reaction. The isolated radiochemical yield of ^{18}F-**1** was ca. 20% (140 - 150 mCi, decay-corrected to end of bombardment).



Scheme 1. Synthesis of the tosylated CBT **4** and fluorinated product **1** (^{19}F analog).

6. Radiolabeling of dimeric cRGD using ^{18}F

6.1) Dimeric cRGD **5** (1.2 mg) was dissolved in DMF (200 μL) containing TCEP·HCl (2 equiv.) and DIPEA (15 equiv.).

6.2) The resulting solution was added to ^{18}F-**1** (40 mCi) in DMF (200 μL) at room temperature (25 °C) (Scheme 2B). At different time points (1, 5, 10, and 20 min), the sample was taken from the crude mixture and the reaction was quenched with 10% AcOH aqueous solution. The conversion yields were monitored by using radio−TLC.

6.3) After 20 min, the reaction was quenched by adding 10% AcOH aqueous solution and then the crude product was purified by a semipreparative HPLC to give ^{18}F-**7** with 80% RCY (decay−corrected to the end of synthesis). [Phenomenex Gemini column: 10 × 250 mm, 5 μm, 5 mL/min, and eluent gradient: 0 - 50 min 10 - 50% (0.1% TFA containing CH_3CN in 0.1% TFA containing H_2O), R_t of the product (^{18}F - **7**) = 34.4 min.] The molar activity of ^{18}F-**7** was 48 ± 5.6 GBq/μmol.

7. Radiolabeling of Cys−RLuc8 using ^{18}F−1

7.1) ^{18}F-**1** (10.7 mCi, 7.5 μL) in DMSO solution was added to a solution of *N*-terminal bearing luciferase (Cys-RLuc) **6** (5 nmol) in PBS buffer (150 μL, pH = 7.5 with 200 μM of TCEP), and stirred at 37 °C for 30 min (Scheme 2B).

7.2) After the reaction, the crude mixture was diluted with PBS buffer until total volume was up to 1mL. The crude mixture was directly loaded onto a NAP-10 column, which was pre-conditioned with elution buffer (PBS, pH = 7.4).

7.3) The crude solution (1 mL) was allowed to enter the column completely, and then, 1.5 mL of elution buffer (PBS, pH = 7.4) was added into the column to collect the product ^{18}F-**8** (12 ± 0.7%, n = 3, decay-corrected to end of synthesis, isolated yield). The molar activity of ^{18}F-**8** was 9.7 GBq/μmol.

Results and Discussion

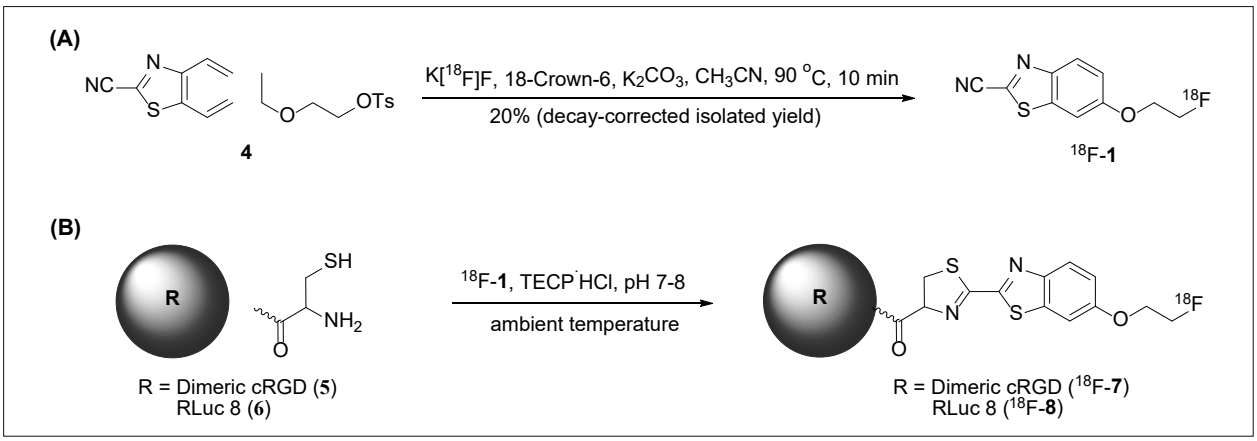
1. Spectroscopic data of the organic compounds

2−cyano−6−hydroxybenzothiazole (3)

^{1}H−NMR(CD_3 OD 400 MHz): 7.99 (d, 1H, *J* = 8.8), 7.39 (d, 1H, *J* = 2.4), 7.15 (dd, 1H, *J* = 8.8, 2.4).

2−(2−cyanobenzothiazol−6−yloxy)ethyl 4−methylbezenesulfonate (4)

^{1}H-NMR ($CDCl_3$, 400 MHz): 8.05 (d, 1H, *J* = 9.2), 7.82 (d, 2H, *J* = 8.0), 7.35 (d, 2H, *J* = 8.0), 7.28 (d, 1H, *J* = 2.4), 7.12 (dd, 1H, *J* = 9.2, 2.4), 4.44 (m, 2H), 4.27 (m, 2H), 2.45 (s, 3H); ^{13}C-NMR ($CDCl_3$, 100 S3 MHz): 158.6, 147.1, 145.1, 137.1, 133.7, 132.5, 129.8, 127.9,



Scheme 2. (A) ^{18}F-Fluorination reaction of tosylated CBT **4**,(B) Radiolabeling reaction of *N*-terminal cysteine bearing biomolecules using ^{18}F-**1**.

158.6, 147.1, 145.1, 137.1, 133.7, 132.5, 129.8, 127.9, 125.8, 118.5, 113.0, 104.0, 67.7, 66.1, 21.6. MS ESI (m/z) calculated for $C_{17}H_{15}N_2O_4S_2$ $[M + H]^+$ 375.05, observed 375.02.

2-cyano-6-(2-fluoroethyoxy)benzothiazole (1)

^{1}H-NMR ($CDCl_3$, 400 MHz): 8.10 (d, 1H, J = 9.2), 7.39 (d, 1H, J = 2.4), 7.29 (dd, 1H, J = 9.2, 2.4), 4.88 (m, 1H), 4.77 (m, 1H), 4.36 (m, 1H), 4.29 (m, 1H); ^{13}C-NMR ($CDCl_3$, 100 MHz): 159.4, 147.4, 137.5, 134.0, 126.2, 119.0, 113.4, 104.2, 82.7, 81.0, 68.2, 68.0. MS ESI (m/z) calculated for $C_{10}H_8FN_2OS$ $[M + H]^+$ 223.03, observed 223.04.

2. Radiosynthesis of ^{18}F-labeled 2-cyanobenzothiazole (CBT) derivative (^{18}F-1)

The labeling of tosylated **4** with ^{18}F was first conducted by using a traditional phase transfer catalyst (PTC) Kryptofix 222 (K_{222}) and potassium carbonate. However, undesired side product was mainly observed on analytical radio-HPLC instead of the expected product (^{18}F-**1**). The tertiary amine group in K_{222} could facilitate facile hydrolysis of the cyanogroup on CBT structure under this reaction condition. To investigate a suitable radiofluorination condition, we utilized 18-crown-6 as a PTC. Alternatively, the substitution reaction of **4** using anhydrous 18-crown-6/K^+/[^{18}F]F^- complex at 90 °C suppressed the formation of the byproduct and provided ^{18}F-labeled CBT as a major product (Figure 1A).

Automated syntheses have been accomplished with up to 1 Ci of radioactivity, and the desired ^{18}F-**1** was obtained at a 20% RCY (decay-corrected to end of bombardment, isolated yield). Analytical radio-HPLC showed that the radiochemical purity of the purified product (^{18}F-**1**) was more than 99% (Figure 1B).

3. Radiosynthesis of ^{18}F-labeled dimeric cRGD using ^{18}F-1

N-terminal cysteine conjugated cRGD peptide **5** was synthesized by following the procedure in our previous study. The dimeric cRGD derivative was reacted with ^{18}F-**1** in anhydrous DMF in the presence of tris(2-carboxyethyl) phosphine (TCEP) to minimize the undesired oxidation of free thiol group during the radiolabeling procedure. After incubating 20 min with the ^{18}F-labeled CBT (^{18}F-**1**) at room temperature, the conversion yield reached 92% as determined by analytical radio-TLC (Figure 1C).

The same reaction could be conducted in a mixed

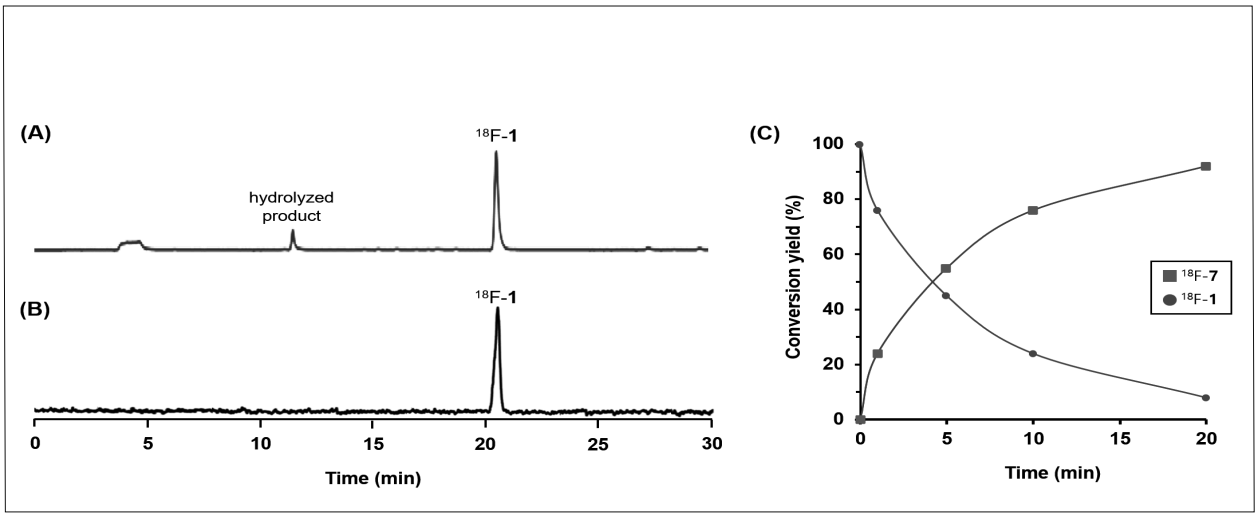


Figure 1. (A) Radiochromatogram of the crude product (^{18}F-**1**) using 18-crown-6, (B) Radiochromatogram of the purified product (^{18}F-**1**), (C) Conversion yield of ^{18}F-labeled dimeric cRGD peptide (^{18}F-**7**).

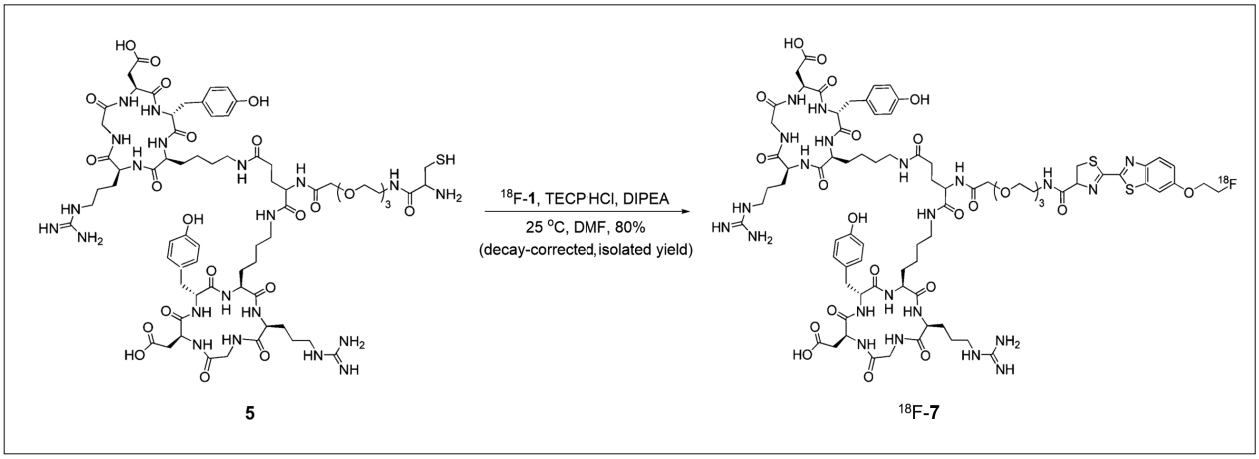
solvent(organic/H_2O). However, the condensation reaction in aqueous media showed unidentified side products, which resulted in decreased RCY. After purification of the crude product by semi-preparative HPLC, the observed RCY of the ^{18}F-labeled product ^{18}F-**7** was 80% (decay-corrected yield). During the HPLC purification, all the reagent, impurity, and starting material have distinct retention time from the product ^{18}F-**7** and only tiny peaks appeared around the product peak on the HPLC chromatograph at both γ and UV detector.(16) The radiochemical purity was more than 99% and molar activity of the ^{18}F-labeled dimeric cRGD was 48 ± 5.6 GBq/μmol as determined by analytical radio-HPLC analysis.

4. Radiosynthesis of ^{18}F−labeled Rluc8 using ^{18}F−1

To investigate the radiolabeling of biomacromolecule using ^{18}F-**1**, we selected the bioluminescent protein Renilla luciferase (RLuc8) as a model protein. *N*-terminal cysteine bearing RLuc8 (Cys-RLuc8) was prepared by the previous report. In the ^{18}F-labeling reaction, a solution of ^{18}F-**1** was added to protein **6** (5 nmol) in phosphate buffered saline (PBS, pH = 7.5 with 200 μM TECP), and then the labeling reaction proceeded at 37 °C for 30 min. The purification was accomplished by using size exclu sion .chromatography (NAP − 10 column) eluted with PBS (pH = 7.4) to provide ^{18}F-labeled RLuc8 (^{18}F-**8**) with 9.7 GBq/μmol of molar activity. The observed RCY was 12 ± 0.7% (n = 3, decay−corrected to end of synthesis, isolated yield) and radiochemical purity was more than 99% as determined by radio-TLC analysis. As a comparison experiment, we have conducted the same condensation reaction with non-modified bioluminescent protein, which did not contain the *N*-terminal cysteine group for the labeling, showed no radiolabeled product. This result indicated that ^{18}F-labeling of **6** with F-**1** was accomplished in a site-specific manner. In addition, the bioluminescent property of RLuc8 remained unchanged after the radiolabeling procedure, suggesting that the condensation reaction does not affect the biological function of the protein.(16)

Conclusion

This protocol provides the synthetic procedure of tosylated CBT derivative **4** and radiofluorination reaction for preparing ^{18}F-**1**. As CBT group showed fast and selective reactivity toward an 1,2-amino thiol moiety under mild conditions, ^{18}F-**1** could be applied to the efficient radiolabeling of *N*-terminal cysteine bearing biomolecules such as cancer targeting peptide (dimeric cRGD) and engineered bioluminescent protein (RLuc8). It is efficient and site-specific abeling Radiosynthesis of ^{18}F-labeled dimeric cRGD using ^{18}F-**1**



Scheme 3. ^{18}F-Labeling of dimeric cRGD peptide **5** using ^{18}F-**1**.

N-terminal cysteine residue.

Acknowledgement

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