Bioconjugate Chemistry

Technical Note

pubs.acs.org/bc

Renal Brush Border Enzyme-Cleavable Linkages for Low Renal Radioactivity Levels of Radiolabeled Antibody Fragments

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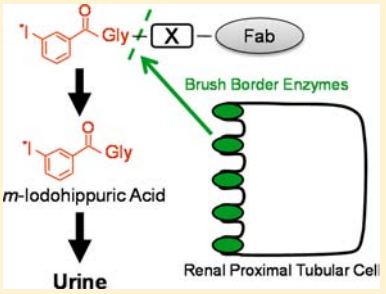
Supporting Information

ABSTRACT: We previously demonstrated that Fab fragments labeled with 3′-[^{131}I]iodohippuryl N^{ε}-maleoyl-L-lysine ([^{131}I]HML) showed low renal radioactivity from early postinjection time, due to a liberation of *m*-[^{131}I]iodohippuric acid by the action of renal brush border enzymes. Since there are lots of enzymes on renal brush border membrane, peptide linkages other than the glycyl-L-lysine were evaluated as the cleavable linkages to explore the chemical design. In this study, we evaluated four peptide linkages with a general formula of *m*-iodobenzoyl-glycyl-X (X: L-tyosine *O*-methyl, L-asparagine, L-glutamine, and N^{ε}-Boc-L-lysine). In vitro studies using renal brush border membrane vesicles (BBMVs) demonstrated that 3′-[^{125}I]iodohippuryl *O*-methyl-L-tyrosine (**2c**) liberated the highest amount of *m*-[^{125}I]iodohippuric acid among the four substrates and the change in the linkage structure altered enzyme species responsible for the hydrolysis reaction. To further assess the applicability of the linkage, a radioiodination reagent containing a glycyl-tyrosine linkage, 3′-[^{125}I]iodohippuryl *O*-((2-maleimidoethyl)carbamoyl)methyl-L-tyrosine (HMT, **12c**), was designed, synthesized, and subsequently conjugated to an Fab fragment. [^{125}I]HMT-Fab exhibited renal radioactivity levels similar to and significantly lower than [^{125}I]HML-Fab and directly radioiodinated Fab, while the blood clearance rates of the three were similar. The analyses of urine for 24 h postinjection of [^{125}I]HMT-Fab showed that *m*-[^{125}I]iodohippuric acid was excreted as the major radiometabolite. The findings indicated that glycyl-tyrosine linkage is also available to reduce renal radioactivity levels of radioiodinated Fab fragments, due to liberation of *m*-iodohippuric acid by the action of enzymes present on renal brush border membrane. These findings suggest that an appropriate selection of peptide linkages would allow the liberation of a designed radiolabeled compound from covalently conjugated polypeptides to prepare radiolabeled polypeptides of low renal radioactivity levels. For the selection of the most appropriate peptide linkage, the in vitro system using BBMVs would be useful to narrow the candidates to just a few.



INTRODUCTION

The antibody fragments/constructs and peptides (abbreviated as polypeptides) constitute useful vectors to deliver radioactivity to target tissues, such as the tumor for molecular imaging and targeted radionuclide therapy, due to rapid elimination rates from the circulation and even distribution in tumor tissue in a size-related manner.[1] However, radiolabeled polypeptides exhibit high and persistent localization in the kidney, which hinders diagnostic accuracy and limits therapeutic application.[2,3] Efforts have been made to reduce the undesirable renal radioactivity levels without impairing the radioactivity levels in the tumors delivered by the vectors.

It is well realized that the renal toxicity of radiolabeled polypeptides is caused by the long residence time of radiometabolites generated after lysosomal proteolysis, following glomerular filtration and subsequent reabsorption into renal cells.[4] The blockage or reduction of tubular reabsorption of radiolabeled polypeptides should constitute a rational approach to reduce renal accumulation of radiolabeled polypeptides. These include the reduction of renal reabsorption by modifying the net charges of peptides,[5,6] and the blockage of renal reabsorption of polypeptides by basic amino acids and other reagents such as Gelofusine.[7−9]

The liberation of a radiometabolite of urinary excretion at the site of renal reabsorption of radiolabeled polypeptides also constitutes an alternative strategy to reduce renal radioactivity levels. Our earlier studies showed that 3′-[^{131}I]iodohippuryl N^{ε}-maleoyl-L-lysine (HML)-conjugated antibody Fab fragment (Figure 1) demonstrated significantly low renal radioactivity levels from early postinjection time, due to the cleavage of the glycyl-lysine linkage by renal brush border enzymes and subsequent excretion of the resulting *m*-[^{131}I]iodohippuric acid into urine.[10−13] Our followup study using an organometallic rhenium compound in place of *m*-iodohippuric acid showed that a change in the chemical structure of a radiolabel attached to the glycyl-lysine linkage significantly affected enzyme recognition.[14] There are many kinds of enzymes that cleave a variety of peptide linkages on the renal brush border

Received: August 2, 2012
Revised: January 14, 2013
Published: January 21, 2013

dx.doi.org/10.1021/bc300428b | *Bioconjugate Chem.* 2013, 24, 291−299

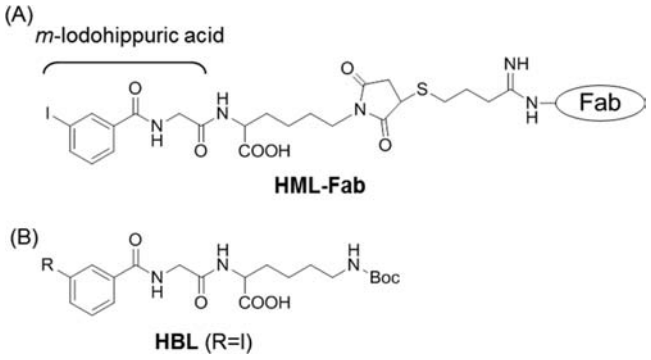


Figure 1. Chemical structures of HML-Fab (A) and HBL (B).

membrane. However, the glycyl-lysine is the only peptide linkage that has been evaluated so far for the purpose.[10−15] The question arises whether the glycy-lysine is the only linkage available for liberating *m*-iodohippuric acid from Fab fragments. The elucidation of the problem would provide a good basis for further application of the chemical design to radionuclides of interest for molecular imaging and targeted radionuclide therapy.

In the present study, three peptide linkages, glycyl-L-tyrosine, glycyl-L-aspartic acid, and glycyl-L-glutamic acid, were selected, due to the presence of side chains convertible to polypeptide binding sites. Low molecular weight model substrates with a general formula of *m*-iodobenzoyl-glycyl-X (X: L-tyrosine-*O*-methyl, L-asparagine, and L-glutamine) were synthesized. The liberation of *m*-[^{125}I]iodohippuric acid from the substrates was compared with 3′-[^{125}I]iodohippuryl-N^{ε}-*tert*-butoxycarbonyl-L-lysine (HBL) in the presence of brush border membrane vesicles (BBMVs).[10] The glycyl-L-tyrosine linkage was further evaluated as the cleavable linkage of choice, and the phenolic hydroxyl group of glycyl-L-tyrosine was converted to a maleimide derivative for polypeptide conjugation. The biodistribution of radioactivity was compared in mice after injection of [^{125}I]iodobenzoic acid-conjugated Fab fragment through the glycyl-L-lysine or glycyl-L-tyrosine linkage. The applicability of peptide linkages other than glycyl-L-lysine will be discussed for designing radiolabeled polypeptides of low renal radioactivity levels.

EXPERIMENTAL PROCEDURES

DL-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA), phosphoramidon, and tienam were purchased from Calbiochem Co. (San Diego, CA, USA), Peptide Institute (Osaka, Japan), and Banyu Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. The Fab fragments of nonspecific bovine IgG (SIGMA, St. Louis, MO, USA) were prepared using Immobilized Ficin (Pierce, Rockford, IL, USA) under the instructions from the manufacturer. Other reagents were of reagent grade and were used without further purification. Na[^{125}I]I was purchased from MP Biomedicals (Costa Mesa, CA, USA) and was diluted with 0.01 N NaOH when necessary. Analytical reversed-phase HPLC (RP-HPLC) was performed with Cosmosil 5C18-AR-300 column (4.6 × 150 mm; Nacalai Tesque, Kyoto, Japan) at a flow rate of 1 mL/min with a gradient mobile phase starting from 100% water to 100% acetonitrile in 30 min (system 1) or from 100% water containing 0.1% TFA to 100% acetonitrile containing 0.1% TFA in 30 min (system 2). Preparative RP-HPLC was performed with Cosmosil 5C18-AR-300 column (20 × 150 mm; Nacalai Tesque) at a flow rate of 5 mL/min with a gradient mobile phase starting from 100% water containing 0.1% TFA to 100% acetonitrile containing 0.1% TFA in 60 min. Size-exclusion HPLC (SE-HPLC) was performed with Cosmosil 5Diol-300 II (7.5 × 600 mm, Nacalai Tesque) eluted with 0.1 M phosphate buffer (pH 6.8) at a flow rate of 1.0 mL/min. TLC analyses were performed with silica gel 60 (Merck Art 5553). ^{1}H NMR spectra were obtained on a JEOL JNM-ALPHA 400 spectrometer (JEOL Ltd., Tokyo) and chemical shifts are recorded in ppm from tetramethylsilane or chloroform as an internal standard. Mass spectra were obtained on a JEOL JMS-AX500 spectrometer (JEOL Ltd.). 3′-Iodohippuryl-N^{ε}-maleoyl-L-lysine (HML), 3′-(tri-*n*-butylstannyl)hippuryl N^{ε}-maleoyl-L-lysine (Sn-HML), and *N*-succinimidyl 3-(tri-*n*-butylstannyl)hippurate (**1b**) were synthesized according to the procedure of Wakisaka et al.[16] 3′-Iodohippuryl-N^{ε}-*tert*-butoxycarbonyl-L-lysine (HBL) and its stannyl derivative, (3′-(tri-*n*-butylstannyl)hippuryl N^{ε}-*tert*-butoxycarbonyl-L-lysine, Sn-HBL), were synthesized according to the procedure of Fujioka et al.[10] *N*-Succinimidyl 3-iodohippurate (**1a**) was synthesized according to the procedure described previously.[17] Cilastatin and renal BBMVs were prepared as described previously.[10]

3′-Iodohippuryl *O*-Methyl-L-tyrosine (HMeT) (2a). To a suspension of *O*-methyl-L-tyrosine (58.7 mg, 0.30 mmol) in acetonitrile (1.5 mL) was added *N*,*N*-diisopropylethylamine (DIPEA; 52 μL, 0.30 mmol) and a solution of **1a** (110 mg, 0.27 mmol) in acetonitrile (1.5 mL). After stirring at room temperature overnight, 12 mL of H_2O was added to the reaction mixture, then a small amount (up to 1 mL) of 1 N HCl was added to dissolve insoluble matter. The solution was subjected to HPLC purification to afford 63.2 mg (48.5%) of **2a** as a white powder. ^{1}H NMR ($CDCl_3$): δ 2.94−3.11 (2H, m, $CHCH_2$), 3.66 (3H, s, OCH_3), 3.97−4.17 (2H, d, $NHCH_2$), 4.71−4.73 (1H, q, NHC*H*), 6.68−6.70 (2H, d, *aromatic*), 7.01−7.03 (2H, d, *aromatic*), 7.56 (1H, t, *aromatic*), 7.70−7.72 (1H, d, *aromatic*), 7.79−7.81 (1H, d, *aromatic*), 8.13 (1H, s, *aromatic*). FAB-MS calcd for $C_{19}H_{20}IN_2O_5$ (MH^+): *m*/*z* 483. Found: 483. Anal. ($C_{19}H_{19}IN_2O_5 \cdot 1/2CF_3COOH$) C, H, N. mp 81−83 °C.

3′-Iodohippuryl L-Asparagine (HA, 3a). To a cold (0 °C) solution of L-asparagine (302 mg, 2.29 mmol) in a mixture of saturated $NaHCO_3$ (3 mL) and acetonitrile (0.5 mL) was added dropwise a solution of **1a** (230 mg, 0.57 mmol) in acetonitrile (3 mL). The reaction mixture was allowed to warm to room temperature and stirred for 20 h, and was adjusted to pH 3 with 4 N HCl. The solution was subjected to HPLC purification, and fractions containing **3a** were collected and lyophilized to afford 22.2 mg (9.3%) of **3a** as a white powder. ^{1}H NMR (CD_3OD): δ 2.61 (2H, d, $CHCH_2$), 4.08 (2H, d, $NHCH_2$), 4.77 (1H, q, NHC*H*), 7.26 (1H, t, *aromatic*), 7.86 (1H, d, *aromatic*), 7.90 (1H, d, *aromatic*), 8.25 (1H, d, *aromatic*). FAB-MS calcd for $C_{13}H_{15}IN_3O_5$ (MH^+): *m*/*z* 420. Found: 420. Anal. ($C_{13}H_{14}IN_3O_5 \cdot 1/2CF_3COOH$) C, H, N. mp 160−162 °C.

3′-Iodohippuryl L-Glutamine (HG, 4a). This compound was synthesized following the procedure of **3a** using **1a** (230 mg, 0.57 mmol) and L-glutamine (334 mg, 2.29 mmol). **4a** (135 mg) was obtained in 54.7% yield. ^{1}H NMR (CD_3OD): δ 2.31 (2H, m, $NHCHCH_2$), 2.65 (2H, t, CH_2CONH_2), 4.00 (2H, d, $NHCH_2$), 4.44 (1H, q, NHC*H*), 7.22 (1H, t, *aromatic*), 7.85 (1H, d, *aromatic*), 7.88 (1H, d, *aromatic*), 8.24 (1H, t, *aromatic*). FAB-MS calcd for $C_{14}H_{17}IN_3O_5$ (MH^+): *m*/*z* 434.

Found: 434. Anal. ($C_{14}H_{16}IN_3O_5 \cdot 1/2CF_3COOH \cdot 1/2H_2O$) C, H, N. mp 90−92 °C.

3′-(Tri-*n*-butylstannyl)hippuryl *O*-Methyl-L-tyrosine (Sn-HMeT, 2b). This compound was synthesized by the reaction of **1b** (150 mg, 0.27 mmol) with *O*-methyl-L-tyrosine (57 mg, 0.29 mmol) according to the procedures of **2a** to provide 45 mg (25.8%) of **2b**. ^{1}H NMR ($CDCl_3$): δ 0.83−1.54 (27H, m, Sn*Bu*$_3$), 2.93−3.12 (2H, m, CHC*H*$_2$), 3.63 (3H, s, OC*H*$_3$), 3.99−4.20 (2H, d, NHC*H*$_2$), 4.75−4.80 (1H, q, NHC*H*), 6.66−6.68 (2H, d, *aromatic*), 6.78 (1H, s, *amide*), 7.00−7.02 (2H, d, *aromatic*), 7.26 (1H, t, *amide*), 7.33−7.35 (1H, t, *aromatic*), 7.58−7.59 (1H, d, *aromatic*), 7.65−7.66 (1H, d, *aromatic*), 7.90 (1H, s, *aromatic*). FAB-MS calcd for $C_{31}H_{47}N_2O_5Sn$ (MH^+): *m*/*z* 647. Found: 647.

3′-(Tri-*n*-butylstannyl)hippuryl L-Asparagine (Sn-HA, 3b). This compound was synthesized by the reaction of **1b** (250 mg, 0.44 mmol) with L-asparagine (234 mg, 1.77 mmol) according to the procedures of **3a** to provide 40.2 mg (15.6%) of **3b**. ^{1}H NMR ($CDCl_3$): δ 0.84−1.53 (27H, m, Sn*Bu*$_3$), 2.76−2.96 (2H, d, CHC*H*$_2$), 4.10−4.14 (2H, d, NHC*H*$_2$), 4.44−4.45 (1H, d, NHC*H*), 7.29−7.33 (1H, t, *aromatic*), 7.45−7.46 (1H, t, *amide*), 7.56−7.58 (1H, d, *aromatic*), 7.68−7.70 (1H, d, *aromatic*), 7.90 (1H, s, *aromatic*), 9.37−9.38 (1H, d, *amide*). FAB-MS calcd for $C_{25}H_{42}N_3O_5Sn$ (MH^+): *m*/*z* 584. Found: 584.

3′-(Tri-*n*-butylstannyl)hippuryl L-Glutamine (Sn-HG, 4b). This compound was also synthesized by the reaction of **1b** (360 mg, 0.64 mmol) with L-glutamine (372 mg, 2.55 mmol) according to the procedures of **4a** to provide 187 mg (49.0%) of **4b**. ^{1}H NMR ($CDCl_3$): δ 0.77−1.57 (27H, m, Sn*Bu*$_3$), 1.79−2.43 (4H, m, CH(C*H*$_2$)$_2$), 4.20−4.22 (2H, d, NHC*H*$_2$), 4.46 (1H, d, NHC*H*), 5.52−54 (1H, d, *amide*), 6.68 (1H, t, *amide*), 7.29−7.33 (1H, t, *aromatic*), 7.53−7.55 (1H, d, *aromatic*), 7.60−7.64 (1H, d, *aromatic*), 7.83 (1H, s, *aromatic*), 9.37−9.38 (1H, d, *amide*). FAB-MS calcd for $C_{26}H_{44}N_3O_5Sn$ (MH^+): *m*/*z* 598. Found: 598.

***N*$^\alpha$-*tert*-Butoxycarbonyl-*O*-methoxycarbonylmethyl-L-tyrosine *tert*-Butyl Ester (5).** To a solution of L-tyrosine *t*-butyl ester (1.00 g, 4.22 mmol) in a mixture of tetrahydrofuran (THF) and water (1:1, 5 mL), an aqueous solution of K_2CO_3 (1.16 g, 8.44 mmol; 5 mL) was added dropwise, followed by a dropwise addition of a solution of $(Boc)_2O$ (1.01 g, 4.64 mmol) in THF (5 mL). The reaction mixture was warmed to room temperature and stirred for 2 h. After the solution was extracted with ethyl acetate (5 mL × 3), and the combined organic layers were dried over anhydrous $MgSO_4$. After removing the solvent, a white solid of N^α-*tert*-butoxycarbonyl-L-tyrosine *tert*-butyl ester was obtained (1.36 g, 95.7%). ^{1}H NMR ($CDCl_3$): δ 1.36−1.39 (18H, s, *Boc*,*tBu*), 2.94−2.96 (2H, t, C*H*$_2$C$_6$H$_4$), 4.35−4.38 (1H, q, NHC*H*), 4.96−4.98 (1H, d, Boc-N*H*), 6.70−7.01 (4H, *aromatic*). FAB-MS calcd for $C_{18}H_{28}NO_5$ (MH^+): *m*/*z* 338, found 338.

To a suspension of sodium hydride (170 mg, 3.26 mmol) in acetonitrile (5 mL), a solution of N^α-*tert*-butoxycarbonyl-L-tyrosine *tert*-butyl ester (1.00g, 2.96 mmol) in acetonitrile (5 mL) was added dropwise below 0 °C. After stirring for 1 h at room temperature, the mixture was cooled below 0 °C again, and methyl bromoacetate (499 mg, 3.26 mmol) was added dropwise. After stirring the reaction mixture overnight at room temperature, H_2O (10 mL) was added to the reaction mixture, and was extracted with ethyl acetate (10 mL × 3). The combined organic layers were dried over anhydrous $MgSO_4$ and the solvent was removed *in vacuo* to provide **5** (1.1 g, 90.0%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.26−1.39 (18H, s, *Boc*,*tBu*), 2.96−2.98 (2H, t, C*H*$_2$C$_6$H$_4$), 3.77−3.78 (3H, s, COOC*H*$_3$), 4.35−4.38 (1H, q, NHC*H*), 4.59 (2H, s, OC*H*$_2$CO), 4.96−4.98 (1H, d, Boc-N*H*), 6.70−7.01 (4H, *aromatic*). FAB-MS calcd for $C_{21}H_{32}NO_7$ (MH^+): *m*/*z* 410, found 410.

***N*$^\alpha$-*tert*-Butoxycarbonyl-*O*-carboxymethyl-L-tyrosine *tert*-Butyl Ester (6).** To a solution of **5** (1.1 g, 2.69 mmol) in *tert*-butyl alcohol (10 mL), 1 M aqueous solution of NaOH (10 mL) was added dropwise, and the reaction mixture was stirred for 2 h at room temperature. After removal of *tert*-butyl alcohol *in vacuo*, the pH of the solution was brought to 3 with 2 M HCl, and the solution was extracted with ethyl acetate (10 mL × 3). The combined organic layers were dried over anhydrous $MgSO_4$, and the solvent was removed *in vacuo* to provide **6** (1.03 g, 92.0%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.26−1.39 (18H, s, *Boc*,*tBu*), 2.97−2.98 (2H, t, C*H*$_2$C$_6$H$_4$), 4.38−4.40 (1H, q, NHC*H*), 4.62 (2H, s, OC*H*$_2$CO), 4.99−5.01 (1H, d, Boc-N*H*), 6.81−7.10 (4H, *aromatic*). FAB-MS calcd for $C_{20}H_{30}NO_7$ (MH^+): *m*/*z* 396, found 396.

***N*-*tert*-Butoxycarbonyl-ethylenediamine (7).** To a cold (0 °C) solution of ethylenediamine (1.00 g, 16.6 mmol) in chloroform (25 mL), a solution of $(Boc)_2O$ (1.82 g, 8.32 mmol) in chloroform (25 mL) was added dropwise. After stirring at the same temperature for 1 h, precipitates were removed by filtration. The filtrate was concentrated, and the residue was purified by silica gel chromatography (chloroform:methanol:aqueous ammonium = 80:16:3) to provide **7** (1.21 g, 45.5%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.41 (9H, s, *Boc*), 2.74−2.78 (2H, t, C*H*$_2$NH$_2$), 3.11−3.15 (2H, q, NHC*H*$_2$), 4.88 (1H, s, N*H*CH$_2$). FAB-MS calcd for $C_7H_{17}N_2O_2$ (MH^+): *m*/*z* 161, found 161.

***N*-(2-((*tert*-Butoxycarbonyl)amino)ethyl)maleimide (8).** To a cold (0 °C) solution of **7** (630 mg, 3.93 mmol) in saturated $NaHCO_3$ (6 mL), *N*-(methoxycarbonyl)maleimide (NMCM; 610 mg, 3.93 mmol), prepared by the method by Keller et al.,[13] was added. After stirring for 40 min at the same temperature, the reaction mixture was stirred for additional 50 min at room temperature. The reaction mixture was cooled to below 0 °C, and the reaction pH was brought to 3 by a dropwise addition of concentrated sulfuric acid. After the solution was extracted with ethyl acetate (5 mL × 3), the combined organic layers were dried over anhydrous $MgSO_4$. The solvent was removed *in vacuo* to provide **8** (870 mg, 92.1%) as a white solid. ^{1}H NMR ($CDCl_3$): δ 1.38 (9H, s, *Boc*), 3.29−3.33 (2H, q, NHC*H*$_2$), 3.62−3.65 (2H, t, C*H*$_2$N), 4.71 (1H, s, N*H*CH$_2$), 6.69 (2H, s, *maleimide*). FAB-MS calcd for $C_{11}H_{17}N_2O_4$ (MH^+): *m*/*z* 241, found 241.

***N*-(2-Aminoethyl)maleimide Hydrochloride (9).** Compound **8** (1.00 g, 4.16 mmol) was dissolved in 4 M HCl in ethyl acetate (15 mL) for 30 min at room temperature. The addition of diethyl ether at 0 °C provided **9** as a white precipitate (711 mg, 96.7%). ^{1}H NMR (D_2O): δ 3.09−3.12 (2H, t, NH$_2$C*H*$_2$), 3.70−3.73 (2H, t, C*H*$_2$N), 6.78 (2H, s, *maleimide*). FAB-MS calcd for $C_6H_9N_2O_2$ (MH^+): *m*/*z* 141, found 141.

***N*$^\alpha$-*tert*-Butoxycarbonyl-*O*-((2-maleimidoethyl)-carbamoyl)methyl-L-tyrosine *tert*-Butyl Ester (10).** To a cold (0 °C) solution of **9** (203 mg, 1.15 mmol) was added a mixed solution of **6** (500 mg, 1.26 mmol) and *N*-hydroxysuccinimide (146 mg, 1.26 mmol) in THF (6 mL) and triethylamine (160 μL, 1.15 mmol) successively. Then a solution of dicyclohexylcarbodiimide (261 mg, 1.26 mmol) in THF (2 mL) was added dropwise at the same temperature.

dx.doi.org/10.1021/bc300428b | *Bioconjugate Chem.* 2013, 24, 291−299

After stirring at room temperature overnight, the precipitates were removed by filtration. The filtrate was concentrated and the residue was purified by silica gel chromatography (ethyl acetate:hexane = 2:1) to provide **10** (387 mg, 65.0%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 1.40 (18H, s, *Boc,tBu*), 2.97−3.01 (2H, t, $CH_2C_6H_4$), 3.50−3.55 (2H, m, $NHCH_2$), 3.72−3.75 (2H, m, CH_2N), 4.37 (1H, s, NHC*H*), 4.40 (2H, s, OCH_2CO), 4.95−4.97 (1H, d, Boc-N*H*), 6.70 (2H, s, *maleimide*), 6.81−7.10 (4H, *aromatic*). FAB-MS calcd for $C_{26}H_{36}N_3O_8$ (MH^+): *m*/*z* 518, found 518.

***O*-((2-Maleimidoethyl)carbamoyl)methyl-L-tyrosine (11).** To a chilled (0 °C) suspension of **10** (387 mg, 0.75 mmol) in anisole (50 μL) was added dropwise TFA (950 μL). After stirring at room temperature for 1 h, TFA was evaporated under a stream of N_2. The residue was treated with diethyl ether at 0 °C, providing **11** as a white precipitate (198 mg, 55.7%). ^{1}H NMR (CD_3OD): δ 3.10−3.12 (2H, t, $CH_2C_6H_4$), 3.29−3.31 (2H, m, $NHCH_2$), 3.43−3.45 (2H, m, CH_2N), 4.19−4.21 (1H, t, NHC*H*), 4.43 (2H, s, OCH_2CO), 6.79 (2H, s, *maleimide*), 6.94−7.27 (4H, *aromatic*). FAB-MS calcd for $C_{17}H_{20}N_3O_6$ (MH^+): *m*/*z* 362, found 362.

3′-Iodohippuryl *O*-((2-maleimidoethyl)carbamoyl)-methyl-L-tyrosine (HMT, 12a). To a chilled (0 °C) suspension of **11** (60 mg, 0.13 mmol) in *N*,*N*-dimethylformamide (DMF, 0.5 mL) was added DIPEA (22 μL, 0.13 mmol) before a dropwise addition of a solution of **1a** (56 mg, 0.14 mmol) in DMF (0.5 mL). After stirring the reaction mixture at room temperature overnight, the solvent was evaporated *in vacuo*. The residue was dissolved in H_2O (1 mL) and the pH was adjusted to 1 using 1 M HCl to provide a white precipitate. The precipitate was dissolved in a mixture of DMF and H_2O (1:1) and the solution was subjected to HPLC purification. Fractions containing the desired product were collected and lyophilized to afford 8.63 mg (10.2%) of **12a** as a white powder. ^{1}H NMR ($CDCl_3$): δ 2.83−2.96 (2H, m, $CH_2C_6H_4$), 3.26−3.27 (2H, t, $NHCH_2$), 3.34−3.51 (2H, t, CH_2N), 3.82−3.87 (2H, d, $NHCH_2CO$), 4.31 (2H, s, OCH_2CO), 4.34 (1H, d, $CHCH_2C_6H_4$), 4.37−4.39 (1H, d, *amide*), 6.77−6.79 (2H, d, *aromatic*), 6.99 (2H, s, *maleimide*), 7.11−7.14 (2H, d, *aromatic*), 7.26−7.29 (1H, d, *aromatic*), 7.84−7.90 (2H, d, *aromatic*), 8.18−8.20 (1H, s, *aromatic*). FAB-MS calcd for $C_{26}H_{25}N_4O_8Na$ (MH^+): *m*/*z* 671, found 671. Anal. ($C_{26}H_{24}IN_4O_8Na{\cdot}2CF_3COOH{\cdot}5H_2O$) C, H, N. mp 102−104 °C.

3′-(Tri-*n*-butylstannyl)hippuryl *O*-((2-maleimidoethyl)carbamoyl)methyl-L-tyrosine (Sn-HMT, 12b). To a chilled (0 °C) suspension of **11** (226 mg, 0.48 mmol) in DMF (4 mL) was added DIPEA (83 μL, 0.48 mmol) before a dropwise addition of **1b** (296 mg, 0.52 mmol) in DMF (1 mL). After stirring at room temperature overnight, the solvent was removed *in vacuo*. The residue was dissolved in H_2O (3 mL) and the pH was brought to 4.0 using 0.1 M HCl. The solution was extracted with ethyl acetate (5 mL × 3) and the combined organic layers were dried over $MgSO_4$. After removing the solvent *in vacuo*, the residue was subjected to purification on silica gel using a mixture of chloroform and methanol (9:1) as an eluent to provide **12b** (102 mg, 26.4%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 0.83−1.54 (27H, m, $SnBu_3$), 3.01−3.17 (2H, m, $CH_2C_6H_4$), 3.49−3.50 (2H, t, $NHCH_2$), 3.67−3.70 (2H, t, CH_2N), 4.08−4.09 (2H, d, $NHCH_2CO$), 4.22−4.24 (1H, d, $CHCH_2C_6H_4$), 4.29 (2H, s, OCH_2CO), 4.75−4.82 [1H, d, *amide*], 6.67 (2H, s, *maleimide*), 6.69−6.71 (2H, d, *aromatic*), 7.03−7.05 (2H, d, *aromatic*), 7.34−7.36 (1H, d, *aromatic*), 7.57−7.60 (1H, d, *aromatic*), 7.65−7.75 (1H, d, *aromatic*), 7.90 (1H, s, *aromatic*). FAB-MS calcd for $C_{38}H_{52}N_4O_8SnNa$ (MH^+): *m*/*z* 835, found 835.

Synthesis of [^{125}I]HBL, [^{125}I]HMeT (2c), [^{125}I]HA (3c), and [^{125}I]HG (4c). Sn-HBL was radioiodinated in the presence of *N*-chlorosuccinimide (NCS) as an oxidant as described previously.[10] In brief, Sn-HBL was dissolved in dry methanol containing 1% acetic acid (0.68 mg/mL), and 80 μL of this solution was mixed with 20 μL of NCS in methanol (0.5 mg/mL) in a sealed vial, followed by the addition of Na[^{125}I]I (1 μL). After incubation for 30 min at room temperature, [^{125}I]HBL was purified with RP-HPLC (system 1). [^{125}I]HMeT (**2c**), [^{125}I]HA (**3c**), and [^{125}I]HG (**4c**) were prepared (55.1%, 43.3%, and 32.5%) under similar conditions using Sn-HMeT (**2b**), Sn-HA (**3b**), and Sn-HG (**4b**), respectively.

Preparation of Radioiodinated Fab Fragments. To a solution of Sn-HMT (**12b**, 1 mM, 60 μL) in methanol containing 1% acetic acid, a solution of *N*-chlorosuccinimide in methanol (0.5 mg/mL, 20 μL) and Na[^{125}I]I (1 to 2 μL) were added successively. After incubation at room temperature for 30 min, [^{125}I]HMT (**12c**) was purified by RP-HPLC (system 1). The solvent was removed *in vacuo* before the conjugation reaction with Fab fragments.

Conjugation of [^{125}I]HMT (**12c**) with the Fab fragment was performed by treating the Fab fragment with 2-iminothiolane, followed by the conjugation reaction between the thiol groups of Fab fragment and the maleimide groups of [^{125}I]HMT (**12c**), as described previously.[12] Briefly, a solution of Fab (200 μL, 1 mg/mL) in well-degassed 0.16 M borate buffer (pH 8.0) containing 2 mM EDTA was allowed to react with 6 μL of 2-iminothiolane solution (2 mg/mL) prepared in the same buffer. After gentle agitation of the reaction mixture for 30 min at room temperature, excess 2-iminothiolane was removed by a centrifuged column procedure,[18] using Sephadex G-50 fine (GE Healthcare, Buckinghamshire, England) equilibrated and eluted with 0.1 M phosphate buffer (pH 6.5) containing 2 mM EDTA. Aliquots of this mixture were sampled for estimation of the number of thiol groups with 2,2′-dipyridyl disulfide.[19] The filtrate (100 μL) was then added to a reaction vial containing [^{125}I]HMT (**12c**). After gentle agitation of the reaction mixture for 1.5 h at room temperature, 14.8 μL of iodoacetoamide (10 mg/mL) in 0.1 M phosphate buffer (pH 6.0) were added. The reaction mixture was further incubated for 30 min to alkylate the unreacted thiol groups. [^{125}I]HMT-Fab was subsequently purified by the centrifuged column procedure using Sephadex G-50 fine equilibrated and eluted with 0.1 M acetate buffer (pH 6.0).

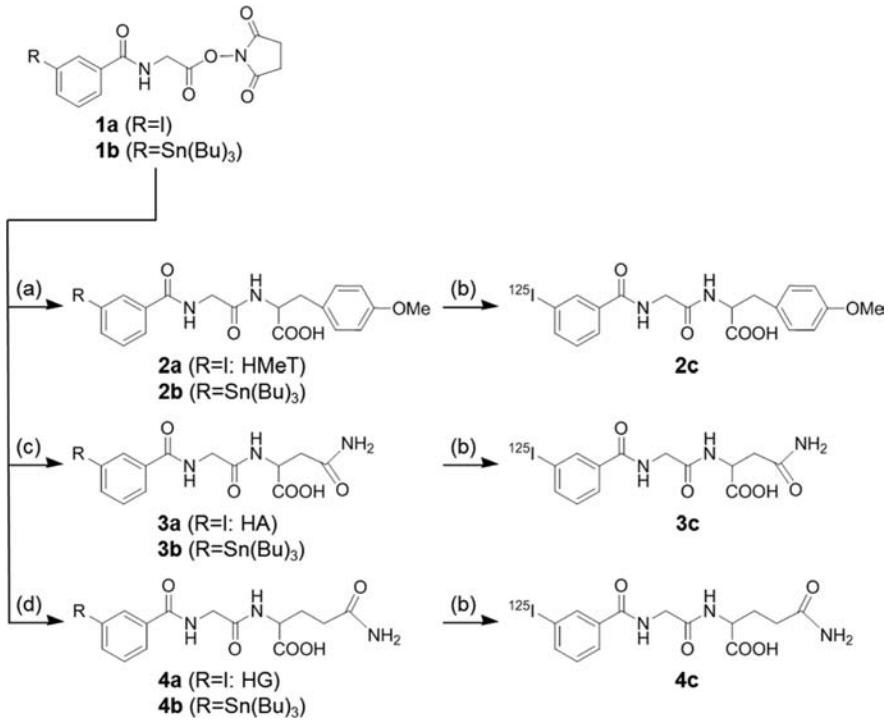
[^{125}I]HML-Fab and directly radioiodinated Fab ([^{125}I]Fab) were prepared according to the procedure as described previously.[12]

Radiochemical purities of the radioiodinated Fab fragments were determined by SE-HPLC and TLC developed with a mixture of methanol and water (4:1).

In Vitro Studies of Model Substrates. Synthetic substrates were incubated with BBMVs as follows:[10] a solution of BBMVs (20 μL) was preincubated for 10 min at 37 °C, followed by the addition of each substrate solution (20 μL) in 0.1 M phosphate buffer (pH 7.0). After incubation for 15 min and 3 h at 37 °C, aliquots of samples were taken from the solution and analyzed immediately by TLC (chloroform:methanol:water = 15:8:1). Each sample was also analyzed by RP-HPLC (system 2) after ultrafiltration with a 10 kDa cutoff membrane (Microcon-10, Millipore, Tokyo, Japan). A solution

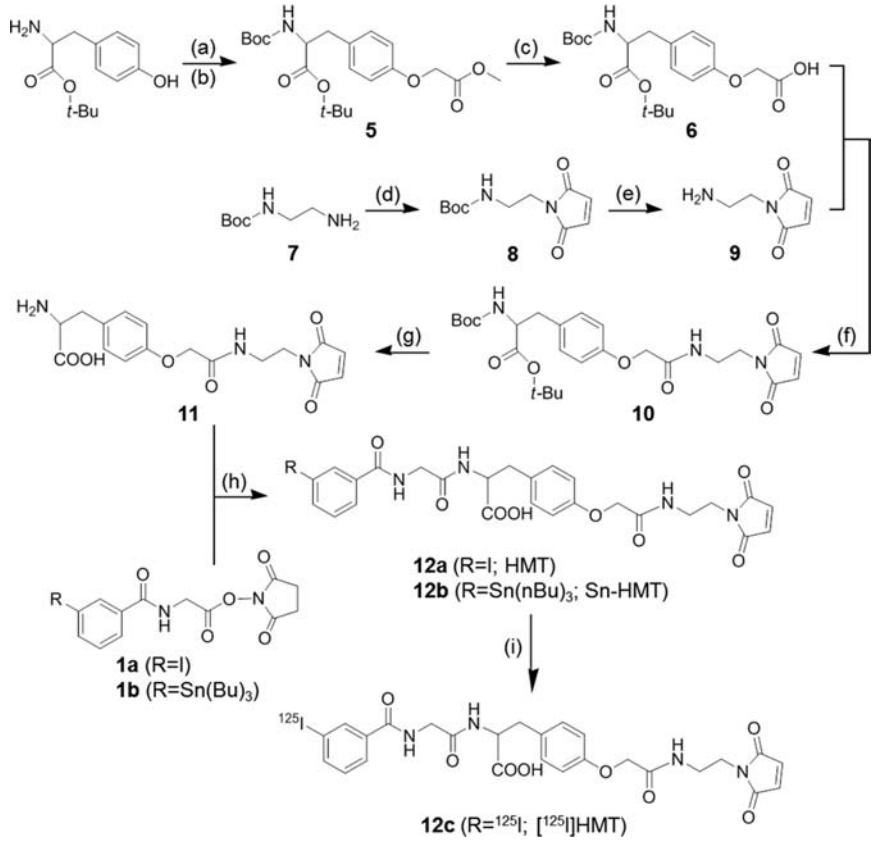
dx.doi.org/10.1021/bc300428b | *Bioconjugate Chem.* 2013, 24, 291−299

Scheme 1. Synthesis of HMeT, HA, and HG[a]



[a]Reagents: (a) *O*-methyl-L-tyrosine, DIPEA; (b) *N*-chlorosuccinimide, Na[^{125}I]I; (c) L-asparagine, sat. $NaHCO_3$; (d) L-glutamine, sat. $NaHCO_3$.

Scheme 2. Synthesis of HMT[a]



[a]Reagents: (a) $(Boc)_2O$; (b) NaH, methyl bromoacetate; (c) 1 M NaOH; (d) *N*-methoxycarbonyl maleimide, sat. $NaHCO_3$; (e) 4 M HCl/ethyl acetate; (f) *N*-hydroxysuccinimide, dicyclohexylcarbodiimide, NEt_3; (g) anisole, TFA; (h) DIPEA; (i) **12b**, *N*-chlorosuccinimide, Na[^{125}I]I.

dx.doi.org/10.1021/bc300428b | *Bioconjugate Chem.* 2013, 24, 291−299

of MGTA, phosphoramidon, or cilastatin in 0.1 M phosphate buffer was added to preincubating solution with a final concentration of 1 mM, and the release of *m*-[^{125}I]iodohippuric acid from each substrate was estimated by TLC and RP-HPLC as described above.

Stability of Model Substrates in Mouse Plasma. A 20 μL solution of each radiolabeled model substrates (**2c**, **3c**, and **4c**) was added to 230 μL of freshly prepared mouse plasma. After incubation for 1, 3, 6, and 24 h at 37 °C, aliquots of samples were taken from the solutions, and the percentages of intact model substrates were determined by TLC.

In Vivo Studies. Biodistribution of radioactivity in normal mice were determined at 10 and 30 min and 1, 3, 6, and 24 h post intravenous injection of [^{125}I]HMT-Fab, [^{125}I]HML-Fab, or [^{125}I]Fab to 6-week-old male ddY normal mice weighing 28−30 g.[20] Groups of three mice, each receiving 20 μg of Fab fragments, were used for the experiments. Organs of interest were removed and weighed, and the radioactivity was determined with a well counter (ARC 380M; Aloka, Tokyo). To determine the amounts and routes of radioactivity excretion from the body, mice were housed in metabolic cages for 24 h after administration of the respective preparation. Urine and feces were collected for 24 h postinjection, and the radioactivity counts were determined.

To elucidate the radiolabeled species excreted in the urine, the urine samples collected for 24 h postinjection of [^{125}I]HMT-Fab (37 kBq) were immediately analyzed by TLC developed with a mixture of chloroform, methanol, and water (15:8:1). Under this condition, *m*-iodohippuric acid was observed with an R_f value of 0.3. The urine samples were also analyzed by SE-HPLC and RP-HPLC after filtration through a polycarbonate membrane with a pore diameter of 0.45 μm (Cosmonice Filter, Nacalai Tesque) for SE-HPLC and a 10 kDa cutoff ultrafiltration membrane (Microcon-10) for RP-HPLC. On RP-HPLC analysis, *m*-iodohippuric acid had retention time of 15 min.

RESULTS

Chemistry. The low molecular weight substrates, HMeT (**2a**), HA (**3a**), and HG (**4a**), were synthesized by reacting *N*-succinimidyl 3-iodohippurate (**1a**) with L-tyrosine *O*-methyl ether, L-asparagine, and L-glutamine, respectively, as shown in Scheme 1. The stannyl precursors of HMeT (**2b**), HA (**3b**), and HG (**4b**) were also prepared by similar procedures using *N*-succinimidyl 3-(tri-*n*-butylstannyl)hippurate (**1b**) (Scheme 1). Each stannyl precursor was radioiodinated in the presence of *N*-chlorosuccinimide as an oxidant. After purification by RP-HPLC, [^{125}I]HMeT (**2c**), [^{125}I]HA (**3c**), and [^{125}I]HG (**4c**) were obtained with over 95% of radiochemical purity.

Scheme 2 illustrates the synthetic procedures for 3′-iodohippuryl *O*-((2-maleimidoethyl)carbamoyl)methyl-L-tyrosine (HMT, **12a**) and its stannyl derivative, Sn-HMT (**12b**). The amine group of L-tyrosine *tert*-butyl ester was protected with Boc group, and the phenolic hydroxyl group was alkylated with methyl bromoacetate with the aid of sodium hydride to prepare **5**. Following hydrolysis of the methyl ester, the resulting carboxylic acid **6** was condensed with an amine group of *N*-aminoethyl maleimide **9** prepared by the method of Keller et al.[21] in the presence of dicyclohexylcarbodiimide and *N*-hydroxysuccinimide to prepare **10**. Following the simultaneous cleavage of the Boc and *tert*-butyl protecting groups with TFA, the resulting **11** was reacted with *N*-succinimidyl 3-iodohippurate (**1a**) or *N*-succinimidyl 3-(tri*n*-butylstannyl)-hippurate (**1b**) to provide HMT (**12a**) or its stannyl precursor, Sn-HMT (**12b**). [^{125}I]HMT (**12c**) was prepared by the reaction of Sn-HMT (**12b**) with Na[^{125}I]I in the presence of *N*-chlorosuccinimide as an oxidant as described above. After RP-HPLC purification, [^{125}I]HMT was obtained with radiochemical yield of 50.2%.

The Fab fragment was modified with 2-iminothiolane according to the procedure as described previously[12] to introduce an average of 2.2 thiol groups per molecule of Fab, as determined by 2,2′-dipyridyl disulfide.[19] [^{125}I]HMT-Fab was prepared by the thiol-maleimide chemistry using the maleimide group of [^{125}I]HMT and the thiolated Fab fragment. After the conjugation reaction, [^{125}I]HMT-Fab was purified by the centrifuged column procedure to provide [^{125}I]HMT-Fab with a radiochemical yield and purity of ca. 24% and over 94.5%.

In Vitro Studies. All the low molecular weight model substrates remained stable after incubation in murine plasma (Table 1). The glycyl-tyrosine linkage also remained stable after conjugation to Fab fragment (Table 1). Figure 2 summarizes the percentage of radioactivity in *m*-[^{125}I]iodohippuric acid fractions after 15 min and 3 h incubation of the low molecular weight model substrates in the presence of BBMVs at 37 °C, as determined by TLC. [^{125}I]HMeT liberated the highest amount of *m*-[^{125}I]iodohippuric acid, followed by [^{125}I]HBL and [^{125}I]HG.

Table 1. Stability of [^{125}I]HBL, [^{125}I]HMeT, [^{125}I]HA, [^{125}I]HG, and [^{125}I]HMT-Fab after Incubation in Murine Plasma at 37 °C[a]

	% intact structure		
	incubation time		
reagent	1 h	3 h	24 h
[^{125}I]HBL	98.07 (0.93)	98.03 (0.45)	96.93 (0.81)
[^{125}I]HMeT	96.69 (0.87)	99.00 (0.56)	97.00 (1.02)
[^{125}I]HA	95.07 (1.13)	96.61 (0.94)	97.00 (1.02)
[^{125}I]HG	95.93 (0.72)	96.77 (0.94)	97.43 (0.64)
[^{125}I]HMT-Fab	96.95 (1.56)	95.30 (1.88)	95.21 (1.56)

[a]Each value represents the mean (SD) for three experiments.

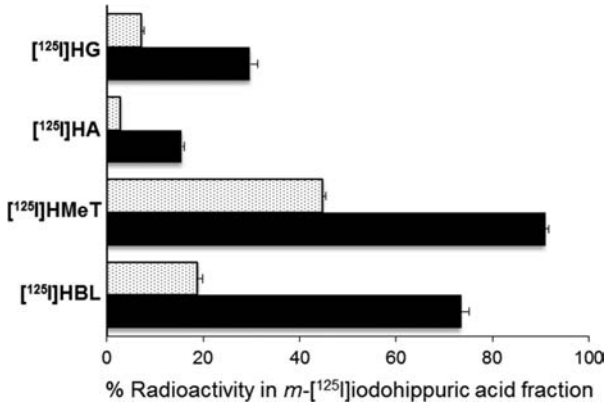


Figure 2. Liberation of *m*-[^{125}I]iodohippuric acid from low molecular weight substrates with different peptide linkages after incubation in BBMVs at 37 °C for 15 min (hatch) and 3 h (solid). Columns and bar represent the mean and SD of three experiments.

dx.doi.org/10.1021/bc300428b | *Bioconjugate Chem.* 2013, 24, 291−299

The effect of representative inhibitors for the BBMVs enzymes on the liberation of *m*-[^{125}I]iodohippuric acid from [^{125}I]HMeT is shown in Figure 3. All the inhibitors partially inhibited the release of *m*-[^{125}I]iodohippuric acid from [^{125}I]HMeT.

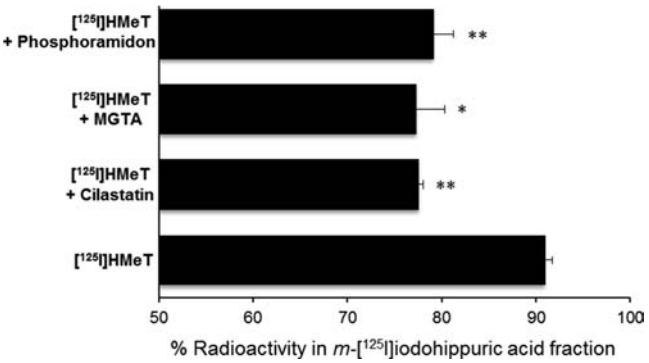


Figure 3. Liberation of *m*-[^{125}I]iodohippuric acid from [^{125}I]HMeT after incubation in BBMVs at 37 °C for 3 h in the presence of an inhibitor of neutral endopeptidase (phosphoramidon), carboxypeptidase (MGTA), or dipeptidase (cilastatin). Columns and bar represent the mean and SD of three experiments. Significance was determined by unpaired *t*-test [(*) $p < 0.05$ and (**) $p < 0.01$].

In Vivo Studies. Figure 4 and Table S1 show the biodistribution of radioactivity after injection of [^{125}I]HMT-

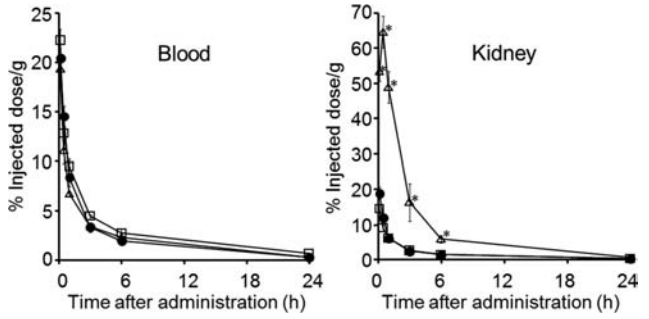


Figure 4. Biodistribution of radioactivity after injection of [^{125}I]HML-Fab (open square), [^{125}I]HMT-Fab (solid circle) and directly radioiodinated [^{125}I]Fab (open triangle) to normal mice. Significance was determined by unpaired *t*-test [(*) $p < 0.01$].

Fab, [^{125}I]HML-Fab, and [^{125}I]Fab to normal mice. The three radioiodinated Fab fragments showed similar blood clearances, and similar amounts of the radioactivity were excreted in the urine for 24 h postinjection (Figure 5). Both [^{125}I]HMT-Fab and [^{125}I]HML-Fab exhibited renal radioactivity levels significantly lower than those of [^{125}I]Fab. The kidney to blood ratios of radioactivity of both [^{125}I]HMT-Fab and [^{125}I]HML-Fab were less than 1 from 10 min postinjection.

Figure 6 shows the radiochromatograms of urine samples obtained for 24 h postinjection of [^{125}I]HMT-Fab when analyzed by SE-HPLC (A), TLC (B), and RP-HPLC (C). On SE-HPLC, two major peaks of radioactivity were observed in the earlier (Fab) and the later fractions. The radioactivity ratio between the two fractions was 1 to 2.4. Similar ratios of radioactivity were observed in TLC analyses where two major radioactivity peaks were detected at the origin and an R_f value identical to that of *m*-iodohippuric acid (R_f = 0.3). On RP-HPLC analyses, the majority of the radioactivity was eluted at a

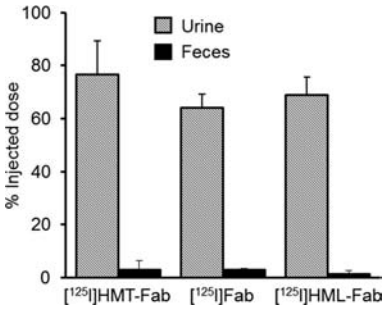


Figure 5. Radioactivity levels in the urine (hatch) and feces (solid) for 24 h postinjection of [^{125}I]HML-Fab, [^{125}I]HMT-Fab, and [^{125}I]Fab to normal mice. Columns and bar represent the mean and SD of three experiments.

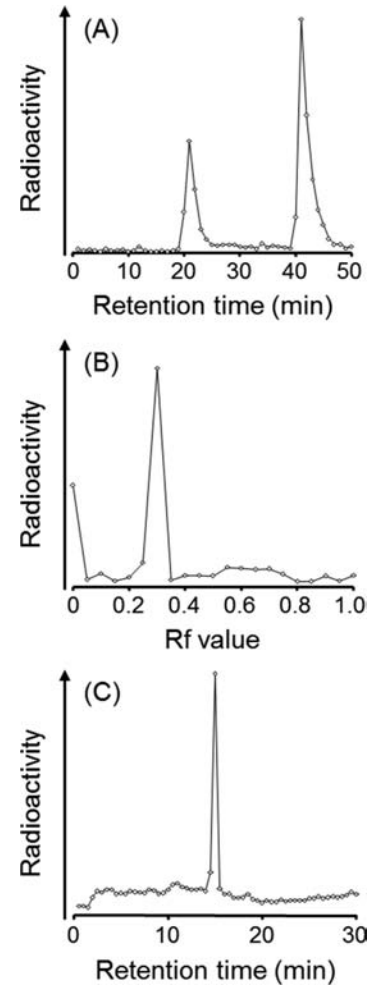


Figure 6. Radiochromatograms of urine samples collected for 24 h postinjection of [^{125}I]HMT-Fab by (A) SE-HPLC after filtration through a 0.45 μm polycarbonate membrane, (B) TLC, and (C) RP-HPLC after filtration through a 10 kDa cutoff membrane.

retention time identical to that of *m*-iodohippuric acid (15 min).

DISCUSSION

In the design of radiolabeling agents for polypeptides with cleavable linkages by renal brush border enzymes, the linkages should be stable against enzymes in plasma so as not to impair the radioactivity levels in the target tissue delivered by the

dx.doi.org/10.1021/bc300428b | Bioconjugate Chem. 2013, 24, 291−299

vectors. The four linkages with a general formula of *m*-iodobenzoyl-glycyl-X (X: N^{ε}-Boc-L-lysine, *O*-methyl-L-tyrosine, L-glutamine, and L-asparagine) were found to satisfy the criterion (Table 1). On the other hand, huge differences were observed in BBMV-mediated release of *m*-[^{125}I]iodohippuric acid from the substrates. Despite the difference in only one carbon length of the side chain, [^{125}I]HG released much higher amounts of *m*-[^{125}I]iodohippuric acid than did [^{125}I]HA. The highest release of *m*-[^{125}I]iodohippuric acid from [^{125}I]HMeT suggested that the glycyl-L-tyrosine linkage would constitute an alternative for the glycyl-L-lysine linkage as the cleavable linkage to liberate *m*-[^{125}I]iodohippuric acid from covalently conjugated polypeptides by renal brush border enzymes (Figure 2). These results also suggested that the in vitro system using BBMVs would be useful to narrow a variety of candidates down to just a few. Further characterization of HMeT showed that more than two enzymes including renal dipeptidase would be involved in the cleavage of the glycyl-L-tyrosine sequence (Figure 3). Under similar conditions, the glycyl-L-lysine sequence of HBL was not recognized by the enzyme.[10]

To further evaluate the glycyl-L-tyrosine sequence as the cleavable linkage to liberate *m*-iodohippuric acid, the hydroxyl group of *m*-iodobenzoyl-glycyl-L-tyrosine was derivatized to a maleimide group to prepare HMT (Scheme 2), and the in vivo cleavage of the linkage was determined after conjugation with Fab fragments.

When injected to mice, [^{125}I]HMT-Fab exhibited renal radioactivity levels similar to and significantly lower than those of [^{125}I]HML-Fab and [^{125}I]Fab, respectively, from an early postinjection time, while all the three radioiodinated Fab fragments registered similar blood clearance rates (Figure 4). The analyses of urine samples obtained for 24 h postinjection of [^{125}I]HMT-Fab showed the liberation of *m*-[^{125}I]-iodohippuric acid (Figure 6). These findings along with those in previous studies using [^{125}I]HML-Fab[10−14] suggested that the low renal radioactivity levels achieved by [^{125}I]HMT-Fab would be attributable to the liberation of *m*-[^{125}I]iodohippuric acid by the action of renal brush border enzymes at lumen of renal tubules. Thus, HMT would constitute an alternative for HML to liberate *m*-iodohippuric acid from covalently conjugated polypeptides.

The similar pharmacokinetics between [^{125}I]HMT-Fab and [^{125}I]HML-Fab showed that both glycyl-L-tyrosine and glycyl-L-lysine linkages liberated *m*-[^{125}I]iodohippuric acid at similar rates when conjugated with Fab fragments. The discrepancies between in vitro (HMeT) and in vivo (HMT) studies would be attributable to the restricted access of the substrates to enzymes responsible for cleaving the peptide bond by covalent conjugation with bulky Fab molecule. Under similar experimental conditions, the cleavage rates of the peptide bond between [^{186}Re]CpTR-glycine and L-lysine were similar to those of *m*-[^{125}I]iodohippuric acid and L-aspartic acid. Despite the low cleavage rates, [^{186}Re]CpTR-GK-conjugated Fab exhibited slightly higher renal radioactivity levels than those of [^{125}I]HML-Fab.[14] Our preliminary studies of Fab radioiodinated with a *m*-[^{125}I]iodohippuryl-aspartic acid linkage exhibited similar biodistribution profiles to those of [^{186}Re]-CpTR-GK-Fab (data not shown). These studies suggest that in vitro studies using BBMVs would be useful to predict in vivo behavior and select an appropriate linkage for further derivatization to radiolabeling agents for polypeptides. These studies also suggest that in vitro cleavage rates of [^{125}I]HBL would be sufficient to reduce renal radioactivity levels when conjugated to polypeptides. In this study, we focused our efforts to select potential peptide linkage to liberate *m*-[^{125}I]-iodohippuric acid. Since a change in chemical structure of a radiolabel attached to a cleavable linkage significantly altered enzyme species responsible for the hydrolysis reaction,[14] the peptide linkages other than glycyl-lysine might be useful to liberate a designed radiolabeled compound other than *m*-iodohippuric acid from covalently conjugated Fab fragments. The interposition of a spacer molecule of appropriate length and hydrophilicity between the cleavable linkage, and the antibody molecule may facilitate in vivo cleavage of the linkage, as observed previously.[11,15]

In conclusion, this study was undertaken to estimate cleavable linkages other than glycyl-L-lysine to liberate *m*-iodohippuric acid from covalently conjugated Fab fragment by the action of renal brush border enzymes, and found that glycyl-L-tyrosine sequence would constitute a potential alternative for glycyl-L-lysine linkage to liberate *m*-[^{125}I]-iodohippuric acid. This study also indicates that the brush border enzymes responsible for cleavaging the glycyl-L-tyrosine linkage were different from those for the glycyl-L-lysine sequence. Since there are many kinds of enzymes on the renal brush border membrane, an appropriate selection of peptide linkages would allow the liberation of a designed radiolabeled compound from covalently conjugated polypeptides. For the selection of the most appropriate peptide linkage, the in vitro system using BBMVs would be useful to narrow the candidates to just a few.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Biodistribution data of [^{125}I]HML-Fab, [^{125}I]HMT-Fab and [^{125}I]Fab in normal mice. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

HML, 3′-iodohippuryl N^{ε}-maleoyl-L-lysine; BBMVs, brush border membrane vesicles; HMT, 3′-iodohippuryl *O*-((2-maleimidoethyl)carbamoyl)methyl-L-tyrosine; MGTA, DL-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid; HBL, 3′-Iodohippuryl-N^{ε}-*tert*-butoxycarbonyl-L-lysine; HMeT, 3′-Iodohippuryl *O*-methyl-L-tyrosine; HA, 3′-Iodohippuryl L-asparagine; HG, 3′-Iodohippuryl L-glutamine.

■ REFERENCES

(1) Yokota, T., Milenic, D. E., Whitlow, M., and Schlom, J. (1992) Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. *Cancer Res. 52*, 3402−3408.

(2) Behr, T. M., Becker, W. S., Bair, H. J., Klein, M. W., Stuhler, C. M., Cidlinsky, K. P., Wittekind, C. W., Scheele, J. R., and Wolf, F. G. (1995) Comparison of complete versus fragmented technetium-99m-labeled anti-CEA monoclonal antibodies for immunoscintigraphy in colorectal cancer. *J. Nucl. Med. 36*, 430−441.

(3) Choi, C. W., Lang, L., Lee, J. T., Webber, K. O., Yoo, T. M., Chang, H. K., Le, N., Jagoda, E., Paik, C. H., Pastan, I., Eckelman, W. C., and Carrasquillo, J. A. (1995) Biodistribution of ^{18}F- and ^{125}I-

labeled anti-Tac disulfide-stabilized Fv fragments in nude mice with interleukin 2 alpha receptor-positive tumor xenografts. *Cancer Res. 55*, 5323−5329.
(4) Akizawa, H., Uehara, T., and Arano, Y. (2008) Renal uptake and metabolism of radiopharmaceuticals derived from peptides and proteins. *Adv. Drug Delivery Rev. 60*, 1319−1328.
(5) Akizawa, H., Arano, Y., Mifune, M., Iwado, A., Saito, Y., Mukai, T., Uehara, T., Ono, M., Fujioka, Y., Ogawa, K., Kiso, Y., and Saji, H. (2001) Effect of molecular charges on renal uptake of ^{111}In-DTPA-conjugated peptides. *Nucl. Med. Biol. 28*, 761−768.
(6) Kobayashi, H., Le, N., Kim, I. S., Kim, M. K., Pie, J. E., Drumm, D., Paik, D. S., Waldmann, T. A., Paik, C. H., and Carrasquillo, J. A. (1999) The pharmacokinetic characteristics of glycolated humanized anti-Tac Fabs are determined by their isoelectric points. *Cancer Res. 59*, 422−430.
(7) Gainkam, L. O., Caveliers, V., Devoogdt, N., Vanhove, C., Xavier, C., Boerman, O., Muyldermans, S., Bossuyt, A., and Lahoutte, T. (2011) Localization, mechanism and reduction of renal retention of technetium-99m labeled epidermal growth factor receptor-specific nanobody in mice. *Contrast Media Mol. Imaging 6*, 85−92.
(8) Wild, D., Wicki, A., Mansi, R., Behe, M., Keil, B., Bernhardt, P., Christofori, G., Ell, P. J., and Macke, H. R. (2010) Exendin-4-based radiopharmaceuticals for glucagonlike peptide-1 receptor PET/CT and SPECT/CT. *J. Nucl. Med. 51*, 1059−1067.
(9) van Eerd, J. E., Vegt, E., Wetzels, J. F., Russel, F. G., Masereeuw, R., Corstens, F. H., Oyen, W. J., and Boerman, O. C. (2006) Gelatin-based plasma expander effectively reduces renal uptake of ^{111}In-octreotide in mice and rats. *J. Nucl. Med. 47*, 528−533.
(10) Fujioka, Y., Satake, S., Uehara, T., Mukai, T., Akizawa, H., Ogawa, K., Saji, H., Endo, K., and Arano, Y. (2005) In vitro system to estimate renal brush border enzyme-mediated cleavage of Peptide linkages for designing radiolabeled antibody fragments of low renal radioactivity levels. *Bioconjugate Chem. 16*, 1610−1616.
(11) Fujioka, Y., Arano, Y., Ono, M., Uehara, T., Ogawa, K., Namba, S., Saga, T., Nakamoto, Y., Mukai, T., Konishi, J., and Saji, H. (2001) Renal metabolism of 3′-iodohippuryl N(epsilon)-maleoyl-L-lysine (HML)-conjugated Fab fragments. *Bioconjugate Chem. 12*, 178−185.
(12) Arano, Y., Fujioka, Y., Akizawa, H., Ono, M., Uehara, T., Wakisaka, K., Nakayama, M., Sakahara, H., Konishi, J., and Saji, H. (1999) Chemical design of radiolabeled antibody fragments for low renal radioactivity levels. *Cancer Res. 59*, 128−134.
(13) Nakamoto, Y., Sakahara, H., Saga, T., Sato, N., Zhao, S., Arano, Y., Fujioka, Y., Saji, H., and Konishi, J. (1999) A novel immunoscintigraphy technique using metabolizable linker with angiotensin II treatment. *Br. J. Cancer 79*, 1794−1799.
(14) Uehara, T., Koike, M., Nakata, H., Hanaoka, H., Iida, Y., Hashimoto, K., Akizawa, H., Endo, K., and Arano, Y. (2007) Design, synthesis, and evaluation of [188Re]organorhenium-labeled antibody fragments with renal enzyme-cleavable linkage for low renal radioactivity levels. *Bioconjugate Chem. 18*, 190−198.
(15) Li, L., Olafsen, T., Anderson, A.-L., Wu, A., Raubitschek, A. A., and Shively, J. E. (2002) Reduction of kidney uptake in radiometal labeled peptide linkers conjugated to recombinant antibody fragments. Site-specific conjugation of DOTA-peptides to a Cys-diabody. *Bioconjugate Chem. 13*, 985−995.
(16) Wakisaka, K., Arano, Y., Uezono, T., Akizawa, H., Ono, M., Kawai, K., Ohomomo, Y., Nakayama, M., and Saji, H. (1997) A novel radioiodination reagent for protein radiopharmaceuticals with L-lysine as a plasma-stable metabolizable linkage to liberate m-iodohippuric acid after lysosomal proteolysis. *J. Med. Chem. 40*, 2643−2652.
(17) Arano, Y., Wakisaka, K., Ohmomo, Y., Uezono, T., Mukai, T., Motonari, H., Shiono, H., Sakahara, H., Konishi, J., Tanaka, C., et al. (1994) Maleimidoethyl 3-(tri-n-butylstannyl)hippurate: a useful radioiodination reagent for protein radiopharmaceuticals to enhance target selective radioactivity localization. *J. Med. Chem. 37*, 2609−2618.
(18) Meares, C. F., McCall, M. J., Reardan, D. T., Goodwin, D. A., Diamanti, C. I., and McTigue, M. (1984) Conjugation of antibodies with bifunctional chelating agents: isothiocyanate and bromoacetamide reagents, methods of analysis, and subsequent addition of metal ions. *Anal. Biochem. 142*, 68−78.
(19) Grassetti, D. R., and Murray, J. F., Jr. (1967) Determination of sulfhydryl groups with 2,2′- or 4,4′-dithiodipyridine. *Arch. Biochem. Biophys. 119*, 41−49.
(20) Imai, S., Morimoto, J., Tsubura, T., Esaki, K., Michalides, R., Holms, R. S., Deimling, O., and Higlgers, J. (1986) Genetic marker patterns and endogenous mammary tumor virus genes in inbred mouse strains in Japan. *Exp. Anim. 35*, 263−276.
(21) Keller, O., and Rudinger, J. (1975) Preparation and some properties of maleimide acids and maleoyl derivatives of peptides. *Hel. Chim. Acta. 58*, 531−540.