**Supplementary Information**

# Synthesis and evaluation of a deltic guanidinium analogue of a cyclic RGD peptide

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**General**

All chemical reagents and solvents were purchased commercially from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan), Merck (Darmstadt, Germany), Nacalai Tesque, Inc. (Kyoto, Japan), Fujifilm Wako Pure Chemical Corporation (Osaka, Japan), Kanto Chemical, Co., Inc. (Tokyo, Japan), Kokusan Chemical Co., Ltd. (Tokyo, Japan), and Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). [$^{125}$I]Sodium iodide (644 GBq/mg) was purchased from Perkin Elmer (Waltham, MA, USA). [$^{67}$Ga]$GaCl_3$ was supplied by Nihon Medi-Physics Co., Ltd. (Tokyo, Japan). U-87 MG glioblastoma cells were purchased from DS Pharma Biomedical (Osaka, Japan). The radioactivity was measured by an Auto Gamma System ARC-7010B (Hitachi, Ltd., Tokyo, Japan).

Nuclear magnetic resonance (NMR) spectroscopy was obtained on JEOL JNM-ECS 400 (JEOL Ltd, Tokyo, Japan). Data for $^1$H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), coupling constant (Hz), and integration. For $^1$H NMR in $D_2O$, chemical shifts were reported as δ values relative to HOD (4.79 ppm). Mass spectrometry was performed on JEOL JMS-T100TD (ESI). Analytical high-performance liquid chromatography (HPLC) system was obtained on the Shimadzu SPD-20A system (Shimadzu Corp., Kyoto, Japan).

**Synthesis**

**Synthesis of cyclic[Orn-Gly- Asp(tBu)-Tyr(tBu)-Lys(Boc)] (5)**

H-Asp(tBu)-Tyr(tBu)-Lys(Boc)-Orn(Alloc)-Gly-OH was synthesized manually by a standard Fmoc-based solid-phase methodology using 2-chlorotrityl chloride resin (0.1 mmol). The protected peptide was cleaved from the resin by HFIP/DCM (1.2 mL/1.4 mL) and concentrated *in vacuo*. The crude material was dissolved in DMF (20 mL). To the solution, DPPA (65 µL, 0.3 mmol) and $NaHCO_3$ (42 mg, 0.5 mmol) were added and the solution was stirred at room temperature for 24 h. The mixture was filtered and concentrated *in vacuo*. The crude material was dissolved in DCM (2.0 mL). To the solution, $Pd(PPh_3)_4$ (23 mg, 0.02 mmol) and morpholine (89 µL, 1.0 mmol) were added and the solution was stirred at room temperature. After 3 h, $Pd(PPh_3)_4$ (11.5 mg, 0.01 mmol) and morpholine (45 µL, 0.5 mmol) were added to the mixture. After 4 h, the mixture was concentrated and the residue was purified by RP-HPLC equipped with $5C_{18}$-AR-II (20 × 250 mm) with recycling system at a flow rate of 10 mL/min with an isocratic mobile phase of 75% methanol in water with 0.1% TFA. The solvent was removed by lyophilizationto afford **5** as a colorless oil (54.2 mg, 60%). LRMS(ESI+) calcd for $C_{39}H_{64}N_7O_{10}$ ($[M + H]^+$): 790.5, found: 790.2.

**Synthesis of cyclic[deltic Arg-Gly-Asp-Tyr-Lys] (3)**

To a stirred solution of **5** (54.2 mg, 0.060 mmol) and DIPEA (95.3 µL, 0.17 mmol) in DMF (1.0 mL), dichlorocyclopropene **6** (50.7 mg, 0.068 mmol) was added at room temperature. After 4 h, the mixture was concentrated *in vacuo*. To the residue, 1.0 mL of TFA/water/triisopropylsilane (38/1/1) was added, and the mixture was stirred for 2 h. The solvent was removed by nitrogen gassing, and the residue was purified by RP-HPLC with recycling system equipped with 5$C_{18}$-AR-II (20 × 250 mm) at a flow rate of 10 mL/min with an isocratic mobile phase of 25% methanol in water with 0.1% TFA. The solvent was removed by lyophilization to afford **3** as a pale yellow solid (18.9 mg, 36%).

$^1$H NMR (400 MHz, $D_2O$) δ 7.10 (d, $J$ = 8.7 Hz, 2H), 6.82 (d, $J$ = 8.7 Hz, 2H), 4.51 (dd, $J$ = 11.0, 6.0 Hz, 1H), 4.35 (dd, $J$ = 8.7, 6.4 Hz, 1H), 4.16 (ABq, $J_{AB}$ = 15.1 Hz, 1H), 3.80 (dd, $J$ = 11.5, 4.1 Hz, 1H), 3.45 (ABq, $J_{AB}$ = 14.7 Hz, 1H), 3.26 (t, $J$ = 6.6 Hz, 2H), 3.04-2.68 (m, 6H), 1.90-1.36 (m, 8H), 1.00-0.80 (m, 2H). *One of the α protons of the peptide is not observed presumably because it is obscured by the HOD signal around 4.90-4.70 ppm.

HRMS (ESI+) calcd for $C_{29}H_{42}N_9O_8$ ($[M + H]^+$): 644.3156, found: 644.3150.

**Synthesis of cyclic[deltic Arg-Gly-Asp-Tyr(3-I)-Lys] (4)**

$NaNO_2$ (1.2 mg, 17 μmol) and $I_2$ (4.5 mg, 17 μmol) were dissolved in 1.0 mL of water/MeOH (1/1) and the solution was stirred at room temperature for 1 h. To the solution, **3** (10 mg, 11.5 μmol) was added. After 1 h, the reaction was quenched by $Na_2S_2O_3$, and the resulting mixture was purified by RP-HPLC equipped with $5C_{18}$-AR-II (20 × 250 mm) at a flow rate of 10 mL/min with an isocratic mobile phase of 30% methanol in water with 0.1% TFA. The solvent was removed by lyophilization to afford **4** as a colorless solid (1.8 mg, 16%).

$^1$H NMR (400 MHz, $D_2O$) δ 7.63 (d, $J$ = 1.8 Hz, 1H), 7.10 (dd, $J$ = 8.2, 1.8 Hz, 1H), 6.88 (d, $J$ = 8.2 Hz, 1H), 4.48 (dd, $J$ = 11.4, 5.5 Hz, 1H), 4.35 (dd, $J$ = 8.7, 6.4 Hz, 1H), 4.16 (ABq, $J_{AB}$ = 14.7 Hz, 1H), 3.76 (dd, $J$ = 11.4, 3.6 Hz, 1H), 3.45 (ABq, $J_{AB}$ = 14.7 Hz, 1H), 3.26 (t, $J$ = 6.6 Hz, 2H), 3.04-2.68 (m, 6H), 1.90-1.36 (m, 8H), 1.00-0.80 (m, 2H).
*One of the α protons of the peptide is not observed presumably because it is obscured by the HOD signal around 4.90-4.70 ppm.
HRMS (ESI+) calcd for $C_{29}H_{41}IN_9O_8$ ($[M + H]^+$): 770.2123, found: 770.2137.

**Synthesis of c(RGDyK) (1)**

c(RGDyK) (**1**) was synthesized as we reported previously.[1] H-Asp(tBu)-Tyr(tBu)-Lys(Boc)-Arg(Pbf)-Gly-OH was synthesized manually by a standard Fmoc-based solid-phase methodology using 2-chlorotrityl chloride resin (0.1 mmol). The protected peptide was cleaved from the resin by HFIP/DCM (1.2 mL/2.0 mL) and concentrated *in vacuo*. The crude material was dissolved in DMF (15 mL). To the solution, DPPA (65 μL, 0.3 mmol) and $NaHCO_3$ (42 mg, 0.5 mmol) were added and the solution was stirred at room temperature for 24 h. The mixture was filtered and concentrated *in vacuo*. To the residue, 2.0 mL of TFA/water/triisopropylsilane (38/1/1) was added, and the mixture was stirred for 2 h. The solvent was removed by a nitrogen gassing, and the residue was purified with RP-HPLC with recycling HPLC system equipped with 5$C_{18}$-AR-II (20 × 250 mm) at a flow rate of 10 mL/min with an isocratic mobile phase of 30% methanol in water with 0.1% TFA. The solvent was removed by lyophilization to afford **1** as a colorless solid (31.4 mg, 37%). LRMS (ESI+) calcd for $C_{27}H_{42}N_9O_8$ ($[M + H]^+$): 620.3, found: 620.3.

[1] K. Ogawa, T. Takeda, M. Yokokawa, J. A. Makino, Y. Kiyono, K. Shiba, S. Kinuya, A. Odani. *Chem. Pharm. Bull.* **2018,** 66, 651-659.

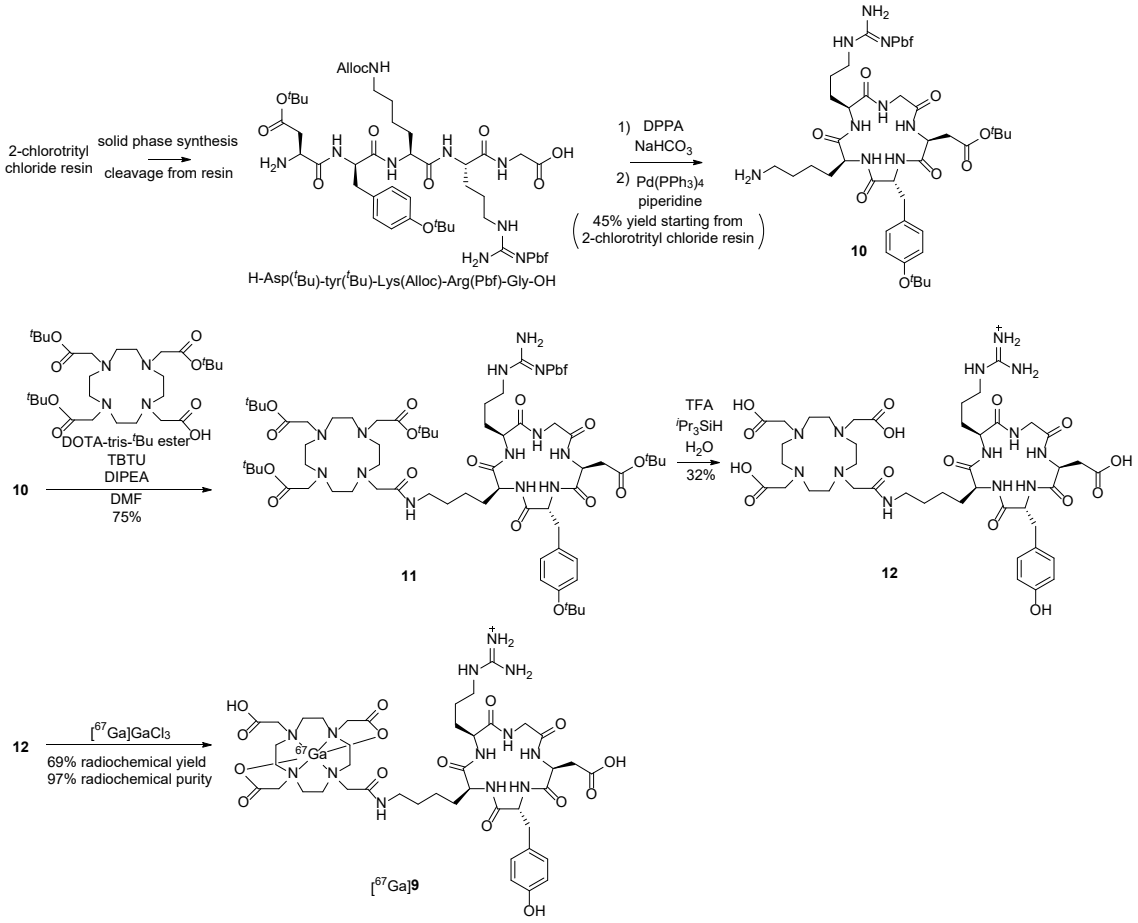
**Synthesis of c(KGDyK) (8)**

H-Asp(tBu)-Tyr(tBu)-Lys(Boc)-Lys(Boc)-Gly-OH was synthesized manually by a standard Fmoc-based solid-phase methodology using 2-chlorotrityl chloride resin (0.1 mmol). The protected peptide was cleaved from the resin by HFIP/DCM (1.2 mL/2.0 mL) and concentrated *in vacuo*. The crude material was dissolved in DMF (15 mL). To the solution, DPPA (65 μL, 0.3 mmol) and $NaHCO_3$ (42 mg, 0.5 mmol) were added and the solution was stirred at room temperature for 24 h. The mixture was filtered and concentrated in vacuo. To the residue, 2.0 mL of TFA/water/triisopropylsilane (38/1/1) was added, and the mixture was stirred for 2 h. The solvent was removed by a nitrogen gassing, and the residue was purified with RP-HPLC with recycling HPLC system equipped with $5C_{18}$-AR-II (20 × 250 mm) at a flow rate of 10 mL/min with an isocratic mobile phase of 30% methanol in water with 0.1% TFA. The solvent was removed by lyophilization to afford **8** as a colorless solid (11.1 mg, 14%). LRMS (ESI+) calcd for $C_{27}H_{42}N_7O_8$ ($[M + H]^+$): 592.3, found: 592.2.

**Synthetic scheme of [$^{67}$Ga]9**

[$^{67}$Ga]**9** was synthesized as described in **Scheme S1**. A cyclic RGD peptide **10** was synthesized via conventional Fmoc solid phase methodology and deprotection of Alloc

group of the ε-amino group in the lysine residue. The peptide **10** was conjugated with DOTA tris(*tert*-butyl ester) to afford **11**. After deprotection under acidic conditions, a peptide **12** was obtained. [$^{67}$Ga]$Ga^{3+}$ was introduced to **12** to afford [$^{67}$Ga]Ga-DOTA-c(RGDyK) ([$^{67}$Ga]**9**).




**Scheme S1.** Synthetic scheme of [$^{67}$Ga]**9**.

**Synthesis of c[R(Pbf)GD(OtBu)y(tBu)K] (10)**

H-Asp(tBu)-Tyr(tBu)-Lys(Alloc)-Arg(Pbf)-Gly-OH was synthesized manually by a standard Fmoc-based solid-phase methodology using 2-chlorotrityl chloride resin (0.1 mmol). The protected peptide was cleaved from the resin by HFIP/DCM (1.2 mL/2.0 mL) and concentrated *in vacuo*. The crude material was dissolved in DMF (20 mL). To the solution, DPPA (65 μL, 0.3 mmol) and $NaHCO_3$ (42 mg, 0.5 mmol) were added and the solution was stirred at room temperature for 24 h. The mixture was filtered and concentrated *in vacuo*. The crude material was dissolved in DCM (2.0 mL). To the solution, $Pd(PPh_3)_4$ (23 mg, 0.02 mmol) and morpholine (89 μL, 1.0 mmol) were added and the solution was stirred at room temperature. After 5 h, the mixture was concentrated and the residue was purified by RP-HPLC with recycling system equipped with 5$C_{18}$-AR-II (20 × 250 mm) at a flow rate of 10 mL/min with an isocratic mobile phase of 30% methanol in water with 0.1% TFA. The solvent was removed by lyophilization to afford **10** as a colorless solid (49.0 mg, 45%). LRMS (ESI+) calcd for $C_{48}H_{74}N_9O_{11}S$ ($[M + H]^+$): 984.5, found: 984.2.

**Synthesis of DOTA(OtBu)$_3$-c[R(Pbf)GD(OtBu)y(tBu)K] (11)**

To a stirred solution of tri-*tert*-butyl 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetate (86.2 mg, 0.155 mmol) in DMF (1.0 mL), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) (45.3 mg, 0.141 mmol) and DIPEA (41.3 μL, 0.242 mmol) were added and the solution was stirred at room temperature. After 1 h, **10** (49.0 mg, 0.045 mmol) was added to the solution. After further stirring for 1 h, the mixture was purified by RP-HPLC equipped with 5C$_{18}$-AR-II (20 × 250 mm) at a flow rate of 10 mL/min with an isocratic mobile phase of 20% methanol in water with 0.1% TFA. The solvent was removed by lyophilization to afford **11** as a colorless solid (51.8 mg, 75%). LRMS (ESI+) calcd for $C_{76}H_{124}N_{13}O_{18}S$ ($[M + H]^+$): 1538.9, found: 1539.3.

**Synthesis of DOTA-c(RGDyK) (12)**

Compound **11** (51.8 mg, 0.034 mmol) was dissolved 1.0 mL of TFA/water/triisopropylsilane (38/1/1), and the mixture was stirred for 1 h. The solvent was removed by a nitrogen gassing, and the residue was purified by RP-HPLC with recycling system equipped with 5C$_{18}$-AR-II (20 × 250 mm) at a flow rate of 10 mL/min with an isocratic mobile phase of 20% methanol in water with 0.1% TFA. The solvent

was removed by lyophilization to afford **12** as a colorless solid (12.0 mg, 32%). LRMS (ESI+) calcd for $C_{43}H_{68}N_{13}O_{15}$ ($[M + H]^+$): 1006.5, found: 1006.1.

## Radiolabeling

### Synthesis of [$^{125}$I]c[RGDy(3-I)K] ([$^{125}$I]2)

[$^{125}$I]**2** was synthesized as we reported previously.[1] To a solution of [$^{125}$I]NaI (1.3 MBq) and **1** (50 μg, 0.057 μmol) in 0.1 M pH 7.4 phosphate buffer (100 μL) and 22 mM chloramine-T solution in water (10 μL, 0.22 μmol) were added at room temperature. After 5 min, the mixture was quenched with 1.0 mg/mL $NaHSO_3$ solution (10 μL) and purified with RP-HPLC equipped with Cosmosil 5$C_{18}$-AR-II (4.6 × 150 mm) at a flow rate of 1 mL/min with a gradient mobile phase of 20% of methanol in water with 0.1% TFA to 40% methanol in water with 0.1% TFA for 20 min. The column temperature was maintained at 40 °C. [$^{125}$I]**2** was obtained in 69% radiochemical yield and 97% radiochemical purity.

**Synthesis of [$^{125}$I]c[deltic RGDy(3-I)K] ([$^{125}$I]4)**

To a solution of **3** (50 μg, 0.057 μmol) in 0.1 M pH 7.4 phosphate buffer (50 μL), [$^{125}$I]NaI (1.1 MBq) and 22 mM chloramine-T solution in water (10 μL, 0.22 μmol) were added at room temperature. After 5 min, the mixture was purified with RP-HPLC equipped with Cosmosil 5$C_{18}$-AR-II (4.6 × 150 mm) at a flow rate of 1 mL/min with a gradient mobile phase of 20% of methanol in water with 0.1% TFA to 40% methanol in water with 0.1% TFA for 20 min. The column temperature was maintained at 40 °C. [$^{125}$I]**4** was obtained in 69% radiochemical yield and 97% Radiochemical purity. The identity of [$^{125}$I]**4** was confirmed by comparing the retention times of the radioiodinated compound and non-radioactive **4** in the HPLC analysis. These peaks were shown at the same retention time in the chromatograms.

**Synthesis of [$^{67}$Ga]Ga-DOTA-c(RGDyK) ([$^{67}$Ga]9)**

[$^{67}$Ga]**9** was synthesized according to a previous report.[2] A solution of **12** (50 μg, 0.89 μmol) in 1.0 M pH 5.0 ammonium acetate buffer (100 μL) was added into a solution of a solution of [$^{67}$Ga]$GaCl_3$ (1.5 MBq, 80 μL) and stirred at 80 °C for 20 min. After

[2] U. C. Shin, K. H. Jung, J. W. Lee, K. C. Lee, Y. J. Lee, J. Y. Park, J. Y. Kim, J. H. Kang, G. An, Y. H. Ryu, J. Y. Choi, K. M. Kim, *J. Radiopharm. Mol. Probes,* **2016,** 2, 118-122.

completion of the reaction, the mixture was purified by RP-HPLC equipped with Cosmosil $5C_{18}$-AR-II (4.6 × 150 mm) at a flow rate of 1 mL/min with a gradient mobile phase of 11% of methanol in water with 0.1% TFA to 20% methanol in water with 0.1% TFA for 20 min. The column temperature was maintained at 40 °C. [$^{67}$Ga]**9** was obtained in 69% radiochemical yield and 98% radiochemical purity.

**HPLC chromatograms of 1, [$^{125}$I]2, 3, 4, [$^{125}$I]4, 8, 12, and [$^{67}$Ga]9**

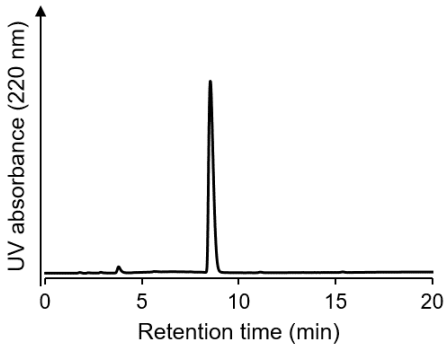



**Figure S1.** HPLC chromatogram of **1**. Condition: a flow rate was 1 mL/min with a gradient mobile phase of 10% methanol in water with 0.1% TFA to 30% methanol in water with 0.1% TFA for 20 min.

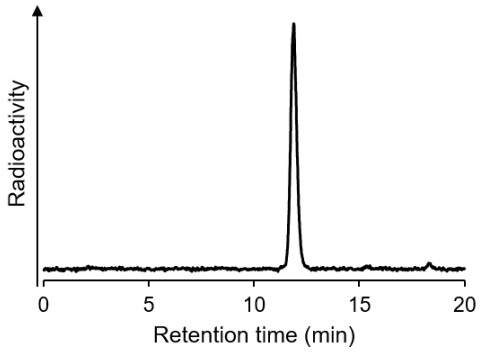



**Figure S2.** HPLC chromatogram of [$^{125}$I]**2**. Condition: a flow rate was 1 mL/min with a gradient mobile phase of 20% methanol in water with 0.1% TFA to 40% methanol in water with 0.1% TFA for 20 min.

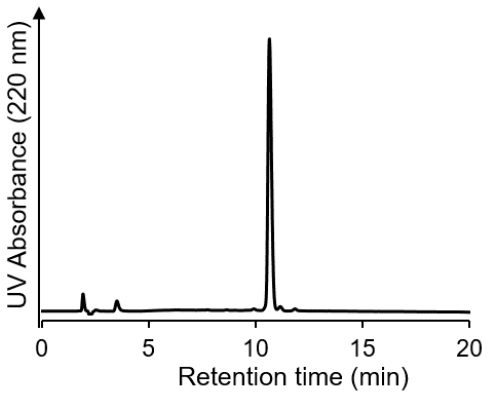



**Figure S3.** HPLC chromatogram of **3**. Condition: a flow rate was 1 mL/min with a gradient mobile phase of 10% methanol in water with 0.1% TFA to 30% methanol in water with 0.1% TFA for 20 min.

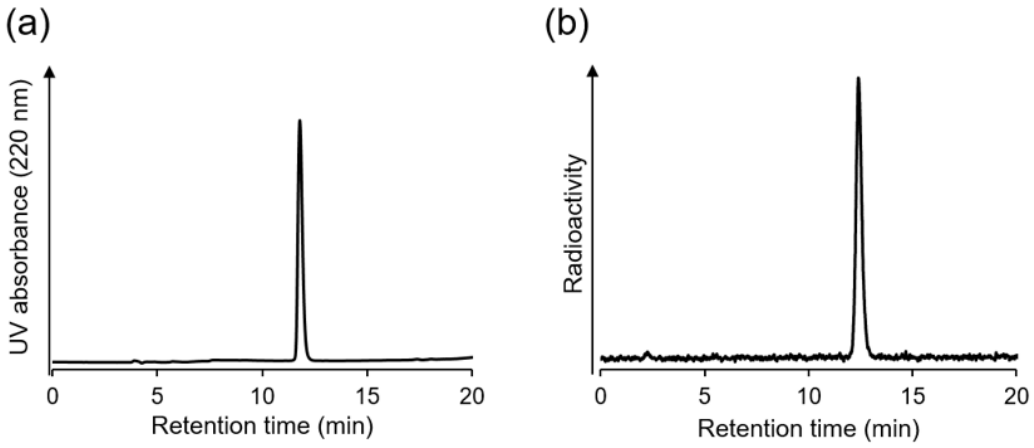



**Figure S4.** HPLC chromatograms of (a) **4** and (b) [$^{125}$I]**4**. Condition: a flow rate was 1 mL/min with a gradient mobile phase of 20% methanol in water with 0.1% TFA to 40% methanol in water with 0.1% TFA for 20 min.

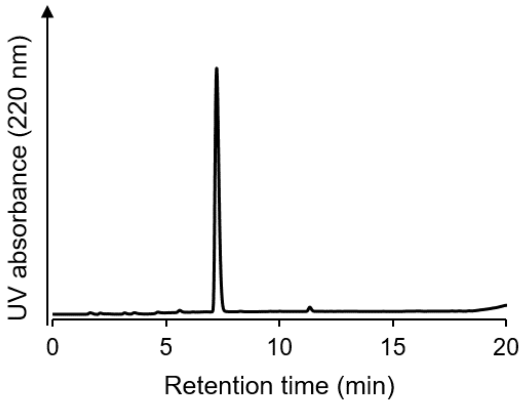



**Figure S5.** HPLC chromatogram of **8**. Condition: a flow rate was 1 mL/min with a gradient mobile phase of 10% methanol in water with 0.1% TFA to 50% methanol in water with 0.1% TFA for 20 min.

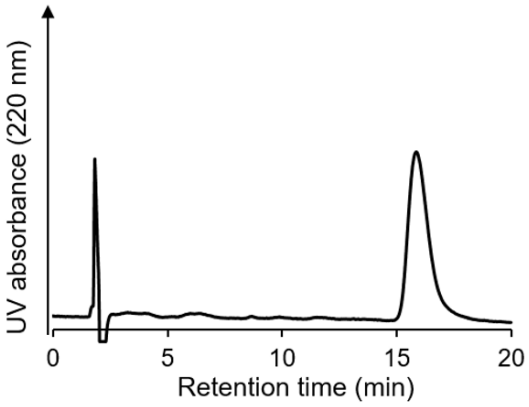



**Figure S6.** HPLC chromatogram of **12**. Condition: a flow rate was 1 mL/min with an isocratic mobile phase of 11% methanol in water with 0.1% TFA.

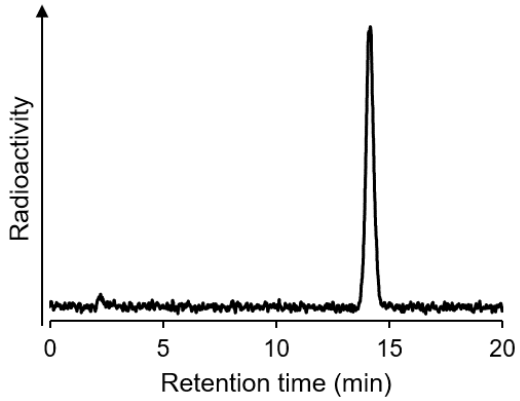



**Figure S7.** HPLC chromatogram of [$^{67}$Ga]**9**. Condition: a flow rate was 1 mL/min with an isocratic mobile phase of 11% methanol in water with 0.1% TFA.

**Determination of distribution coefficient**

The distribution coefficients (log $D_{O/W}$) of [$^{125}$I]c[RGDy(3-I)K] ([$^{125}$I]**2**) and [$^{125}$I]c[deltic RGDy(3-I)K] ([$^{125}$I]**4**) were measured as reported previously with a slight modification. Briefly, [$^{125}$I]**2** or [$^{125}$I]**4** was added to the mixture of each 3 mL of *n*-octanol and 0.1 M PBS (pH 7.4) in a test tube. The test tube was vortexed for 1 min, left at room temperature for 10 min, and centrifuged at 3000g, 4 °C for 5 min. The radioactivity of each 1 mL layer, *n*-octanol, and phosphate buffer, was counted using an auto well gamma counter (n = 4). The log $D_{O/W}$ values were calculated by the logarithm of the ratio of radioactivity per milliliter (cpm/mL) in n-octanol to that in PBS.

**$\alpha_v\beta_3$ Integrin-Binding Assay**

Binding affinities of synthesized peptides, c(RGDyK) (**1**), c(deltic RGDyK) (**3**), and c(KGDyK) (**9**), for $\alpha_v\beta_3$ integrin were evaluated by competitive inhibition between the peptides and [$^{125}$I]c[RGDy(3-I)V], which was prepared by $^{125}$I labeling of c(RGDyV) synthesized via Fmoc solid-phase synthesis, according to a previously reported procedure.[1] The half-maximal inhibitory concentration ($IC_{50}$) values of the peptides were calculated by curve fitting with nonlinear regression using GraphPad Prism 5.04

(GraphPad Software Inc., San Diego, CA). Each data point is the average of four determinations, and $IC_{50}$ values were expressed as mean ± standard deviation (SD) from three independent experiments.

**Animals**

Male ddY mice and female BALB/c nu/nu mice were purchased from Japan SLC Inc. (Hamamatsu, Japan). The mice were housed in a cage with free access to food and water at constant temperature (23–25 °C) with a 12 h light/dark cycle. For preparing tumor-bearing mice, U-87 MG cells ($5 \times 10^6$ cells) were grown and injected subcutaneously into 4-week-old female BALB/c nude mice as previously reported.[2] The tumor reached palpable size after approximately 2-weeks post-inoculation. Animal experiments were conducted in accordance with the Care and Use of Animals Laboratory Guidelines of Kanazawa University. The animal handling protocol was approved by the Kanazawa University Animal Care Committee.

***In vitro* stability experiment**

The *in vitro* stability of [$^{125}$I]**4** was studied in murine plasma. The plasma was prepared by centrifugation of murine blood at 4 °C for 20 min at 1200 g. The reaction was initiated by the addition of [$^{125}$I]**4** (44 kBq, 10 μL) in the plasma (100 μL). The mixture was incubated in a shaker 300 rpm at 37 °C. After 24 h, to the mixture, 120 μL acetonitrile was added for deproteinization. The samples were left in the fridge for 15 min and then centrifugation at 4 °C for 15 min at 1,700 g. The supernatant was filtered by 0.45 μm pore size then analyzed by RP-HPLC equipped with Cosmosil 5$C_{18}$-AR-II (4.6 × 150 mm) at a flow rate of 1 mL/min with a gradient mobile phase of 40% of methanol in water with 0.1% TFA to 60% methanol in water with 0.1% TFA for 20 min. The column temperature was maintained at 40 °C. The radioactivity was determined by an auto well gamma counter.

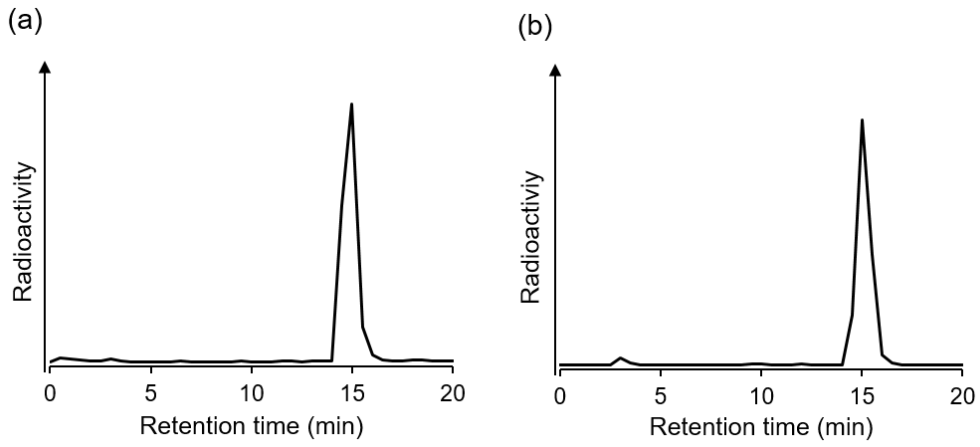



**Figure S8.** (a) HPLC chromatogram of [$^{125}$I]**4** before the incubation in murine plasma. (b) Representative HPLC chromatogram of [$^{125}$I]**4** after 24 h incubation in murine plasma. HPLC conditions: flow rate was 1 mL/min with a gradient mobile phase of 20% methanol in water with 0.1% TFA to 40% methanol in water with 0.1% TFA for 20 min. The sample from HPLC was fractionated every 0.5 min, and the radioactivity of each fraction was measured by γ-counter. (Retention time is slightly different from the chromatogram in Figure S4 due to the difference of the HPLC systems).

***In vivo* metabolic stability analysis**

The metabolites of [$^{125}$I]**2** and [$^{125}$I]**4** in urine were evaluated. [$^{125}$I]**2** (100 kBq) or [$^{125}$I]**4** (200 kBq) was injected into male ddY mice via the lateral tail vein and the mice were individually housed in metabolic cages (Metabolica, Sugiyama-Gen, Tokyo, Japan),

and urine and feces were collected over 24 h. The urine was filtered by 0.45 μm pore size then analyzed by RP-HPLC with mobile phase gradient system water (A) and methanol (B), B: 20-40%, for 20 min using a Cosmosil $5C_{18}$ AR-II (4.6 × 150 mm) column at flow rate 1 mL/min. The column temperature was maintained at 40 °C. The radiochemical purity of the recovered intact material was determined by an auto well gamma counter.

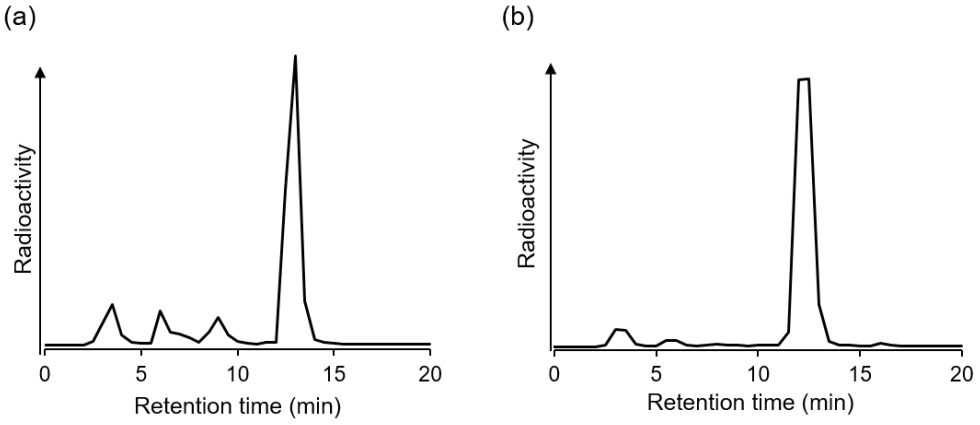



**Figure S9.** Representative RP-HPLC analysis of a urine sample collected from a mouse. HPLC condition: flow rate was 1 mL/min with a gradient mobile phase of 20% methanol in water with 0.1% TFA to 40% methanol in water with 0.1% TFA for 20 min. The sample from HPLC was fractionated every 0.5 min, and the radioactivity of each fraction was measured by γ-detector. (a) HPLC chromatogram after administration with [$^{125}$I]**2**. (b) HPLC chromatogram after administration with [$^{125}$I]**4**. For the measurement of the radioactivity, the sample from HPLC was fractionated every 0.5 min, and the radioactivity of each fraction was measured by γ-counter.

**Biodistribution study in normal mice**

Male ddY mice (6 weeks) were injected intravenously with a solution of [$^{125}$I]**4** (30 kBq) and [$^{67}$Ga]**9** (60 kBq) in saline (100 μL) via the lateral tail vein. The ddY mice were sacrificed at 10 min, 1 h, 4 h, 6 h, and 24 h postinjection. The selected tissues were collected, weighed, and the radioactivity of each tissue was counted using an auto well gamma counter. A window from 16 to 71 keV was used for counting $^{125}$I and a window from 70 to 400 keV was used for counting $^{67}$Ga. More than one month after the experiments, the radioactivity counts of $^{125}$I were measured after attenuation of $^{67}$Ga. The biodistribution data are expressed as percent injected dose per gram tissue (%ID/g) along with the SD. Biodistribution of $^{125}$I and $^{67}$Ga were compared using paired Students' *t* test. The level of statistical significance was set to a $p < 0.05$.

**Biodistribution study in tumor bearing mice**

The tumor bearing mice were injected intravenously with a solution of [$^{125}$I]**2** (60 kBq) or [$^{125}$I]**4** (60 kBq) and [$^{67}$Ga]**9** (60 kBq) in saline (100 μL) via the lateral tail vein. The mice were sacrificed at 24 h. The selected tissues and organs were collected, weighed, and the radioactivity of each tissue was counted using an auto well gamma counter. In blocking experiments, mice were intravenously administered a mixed solution of [$^{125}$I]**2** (60 kBq) or [$^{125}$I]**4** (60 kBq), [$^{67}$Ga]**9** (60 kBq), and c(RGDyK) (200 μg/mouse). The mice were sacrificed at 1 h postinjection, and biodistribution analysis were conducted as described in the biodistribution study in normal mice. Biodistribution of $^{125}$I and $^{67}$Ga were compared using paired Students' *t* test. The level of statistical significance was set to a $p < 0.05$.

## Detailed biodistribution data

**Table S1.** Biodistribution of radioactivity after concomitant intravenous injection of [$^{125}$I]**4** and [$^{67}$Ga]**9** in normal mice

| Tissue | Time after injection | | | |
|---|---|---|---|---|
| | 10 min | 1 h | 4 h | 24 h |
| [$^{125}$I]**4** | | | | |
| Blood | 2.83 (0.12) | 0.35 (0.06) | 0.02 (0.01) | 0.01 (0.01) |
| Liver | 2.66 (0.14) | 2.97 (0.30) | 1.00 (0.24) | 0.03 (0.00) |
| Kidney | 17.22 (2.61) | 12.71 (3.81) | 5.77 (2.06) | 0.11 (0.01) |
| Small-Intestine | 1.13 (0.13) | 1.77 (0.24) | 0.66 (0.20) | 0.06 (0.04) |
| Large-Intestine | 0.67 (0.08) | 0.23 (0.04) | 3.41 (0.55) | 0.25 (0.23) |
| Spleen | 0.95 (0.11) | 0.34 (0.13) | 0.22 (0.04) | 0.07 (0.03) |
| Pancreas | 1.16 (0.43) | 0.24 (0.05) | 0.24 (0.09) | 0.02 (0.03) |
| Lung | 2.79 (0.17) | 0.48 (0.08) | 0.17 (0.03) | 0.05 (0.00) |
| Heart | 1.52 (0.33) | 0.26 (0.05) | 0.07 (0.02) | 0.01 (0.01) |
| †Stomach | 0.56 (0.09) | 0.37 (0.04) | 0.22 (0.10) | 0.05 (0.02) |
| Bone | 2.78 (0.43) | 0.54 (0.10) | 0.90 (0.16) | 0.14 (0.22) |
| Muscle | 1.05 (0.09) | 0.20 (0.08) | 0.11 (0.11) | 0.04 (0.01) |
| Brain | 0.15 (0.02) | 0.06 (0.02) | 0.04 (0.02) | 0.01 (0.00) |
| †Neck | 2.92 (0.19) | 0.39 (0.11) | 0.17 (0.03) | 0.03 (0.01) |
| †Urine | - | - | - | 60.48 (14.48) |
| †Feces | - | - | - | 10.55 (2.72) |

| [$^{67}$Ga]**9** | | | | |
|---|---|---|---|---|
| Blood | 2.50 (0.21) | 0.23 (0.05) | 0.02 (0.00) | 0.01 (0.00) |
| Liver | 1.26 (0.12) | 0.90 (0.14) | 0.74 (0.07) | 0.37 (0.07) |
| Kidney | 12.32 (2.92) | 6.00 (2.87) | 2.71 (1.28) | 0.87 (0.07) |
| Small-Intestine | 1.32 (0.11) | 1.27 (0.23) | 0.70 (0.21) | 0.28 (0.03) |
| Large-Intestine | 0.89 (0.08) | 0.42 (0.03) | 1.75 (0.39) | 0.39 (0.29) |
| Spleen | 1.17 (0.11) | 0.58 (0.16) | 0.45 (0.09) | 0.43 (0.04) |
| Pancreas | 1.19 (0.38) | 0.35 (0.16) | 0.22 (0.04) | 0.14 (0.03) |
| Lung | 2.70 (0.06) | 0.58 (0.06) | 0.31 (0.06) | 0.17 (0.01) |
| Heart | 1.31 (0.13) | 0.28 (0.05) | 0.16 (0.02) | 0.09 (0.01) |
| [†]Stomach | 0.66 (0.06) | 0.40 (0.06) | 0.24 (0.04) | 0.13 (0.02) |
| Bone | 2.49 (0.53) | 0.62 (0.13) | 0.90 (0.16) | 0.32 (0.03) |
| Muscle | 0.93 (0.12) | 0.25 (0.02) | 0.20 (0.10) | 0.10 (0.01) |
| Brain | 0.14 (0.02) | 0.04 (0.01) | 0.02 (0.00) | 0.02 (0.00) |
| [†]Neck | 2.62 (0.14) | 0.53 (0.11) | 0.35 (0.08) | 0.25 (0.01) |
| [†]Urene | - | - | - | 56.03 (13.62) |
| [†]Feces | - | - | - | 7.25 (3.18) |

Expressed as % injected dose per gram. Each value represents the mean (SD) for three or four animals. [†]Expressed as % injected dose.

**Table S2.** (A) Biodistribution of radioactivity after intravenous administration of [$^{125}$I]**2** and [$^{67}$Ga]**9** at 1 h and 4 h in U-87 MG tumor bearing mice. (B) Biodistribution of radioactivity after intravenous administration of [$^{125}$I]**2**, [$^{67}$Ga]**9**, and c(RGDyK) (0.2 mg/mouse) at 1 h in U-87 MG tumor bearing mice

(A)

| | [$^{125}$I]c[RGDy(3-I)K] ([$^{125}$I]**2**) | | [$^{67}$Ga]Ga-DOTA-c(RGDyK) [$^{67}$Ga]**9** | |
|---|---|---|---|---|
| | Time after injection | | | |
| Tissue | 1 h | 4 h | 1 h | 4 h |
| Blood | 0.29 (0.06) | 0.03 (0.02) | 0.17 (0.02) | 0.03 (0.00) |
| Liver | 3.70 (0.10) | 1.16 (0.11) | 1.38 (0.08) | 0.93 (0.09) |
| Kidney | 12.80 (1.42) | 5.51 (0.33) | 2.73 (0.37) | 1.64 (0.14) |
| Small-Intestine | 3.24 (0.33) | 1.36 (0.28) | 2.17 (0.64) | 1.04 (0.26) |
| Large-Intestine | 2.11 (0.38) | 3.47 (0.45) | 1.25 (0.44) | 2.89 (0.44) |
| Spleen | 2.47 (0.03) | 1.40 (0.18) | 1.26 (0.07) | 0.90 (0.11) |
| Pancreas | 0.66 (0.06) | 0.35 (0.03) | 0.28 (0.03) | 0.17 (0.01) |
| Lung | 1.75 (0.20) | 0.86 (0.18) | 0.77 (0.09) | 0.35 (0.04) |
| Heart | 0.64 (0.08) | 0.25 (0.01) | 0.32 (0.03) | 0.17 (0.02) |
| †Stomach | 0.66 (0.11) | 0.24 (0.03) | 0.32 (0.17) | 0.13 (0.02) |
| Bone | 1.03 (0.19) | 0.44 (0.22) | 0.90 (0.11) | 0.40 (0.06) |
| Muscle | 0.58 (0.08) | 0.30 (0.04) | 0.25 (0.04) | 0.12 (0.00) |
| Brain | 0.08 (0.01) | 0.04 (0.01) | 0.04 (0.00) | 0.03 (0.00) |
| †Neck | 1.47 (0.34) | 0.60 (0.17) | 0.66 (0.17) | 0.31 (0.08) |
| U87MG | 5.88 (0.32) | 2.59 (0.58) | 2.83 (0.18) | 1.76 (0.34) |

Expressed as % injected dose per gram. Each value represents the mean (SD) for three or four animals. †Expressed as % injected dose.

(B)

| Tissue | [$^{125}$I]c[RGDy(3-I)K] ([$^{125}$I]**2**) | | [$^{67}$Ga]Ga-DOTA-c(RGDyK) ([$^{67}$Ga]**9**) | |
|---|---|---|---|---|
| | Control | Blocking | Control | Blocking |
| Blood | 0.29 (0.06) | 0.18 (0.07) | 0.17 (0.02) | 0.09 (0.02)** |
| Liver | 3.70 (0.10) | 1.57 (0.22)*** | 1.38 (0.08) | 0.17 (0.05)*** |
| Kidney | 12.80 (1.42) | 8.44 (1.30)** | 2.73 (0.37) | 1.61 (0.39)* |
| Small-Intestine | 3.24 (0.33) | 1.72 (0.45)** | 2.17 (0.64) | 1.42 (0.51) |
| Large-Intestine | 2.11 (0.38) | 0.44 (0.22)*** | 1.25 (0.44) | 0.28 (0.16)* |
| Spleen | 2.47 (0.03) | 0.26 (0.13)*** | 1.26 (0.07) | 0.19 (0.03)*** |
| Pancreas | 0.66 (0.06) | 0.51 (0.28) | 0.28 (0.03) | 0.31 (0.23) |
| Lung | 1.75 (0.20) | 0.40 (0.07)*** | 0.77 (0.09) | 0.17 (0.03)*** |
| Heart | 0.64 (0.08) | 0.07 (0.03)*** | 0.32 (0.03) | 0.06 (0.01)*** |
| †Stomach | 0.66 (0.11) | 0.55 (0.23) | 0.32 (0.17) | 0.42 (0.23) |
| Bone | 1.03 (0.19) | 0.20 (0.07)*** | 0.90 (0.11) | 0.11 (0.04)*** |
| Muscle | 0.58 (0.08) | 0.26 (0.11)** | 0.25 (0.04) | 0.14 (0.16) |
| Brain | 0.08 (0.01) | 0.03 (0.02)** | 0.04 (0.00) | 0.01 (0.00)*** |
| †Neck | 1.47 (0.34) | 0.25 (0.08)** | 0.66 (0.17) | 0.11 (0.03)** |
| U87MG | 5.88 (0.32) | 0.50 (0.22)*** | 2.83 (0.18) | 0.37 (0.13)*** |

Expressed as % injected dose per gram. Each value represents the mean (SD) for three or four animals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. †Expressed as % injected dose.

**Table S3.** (A) Biodistribution of radioactivity after intravenous administration of [$^{125}$I]**4** and [$^{67}$Ga]**9** at 1 h and 4 h in U-87 MG tumor bearing mice. (B) Biodistribution of radioactivity after intravenous administration of [$^{125}$I]**4**, [$^{67}$Ga]**9**, and c(RGDyK) (0.2 mg/mouse) at 1 h in U-87 MG tumor bearing mice

(A)

| | [$^{125}$I]c[deltic RGDy(3-I)K] ([$^{125}$I]**4**) | | [$^{67}$Ga]Ga-DOTA-c(RGDyK) ([$^{67}$Ga]**9**) | |
|---|---|---|---|---|
| | Time after injection | | | |
| Tissue | 1 h | 4 h | 1 h | 4 h |
| Blood | 0.37 (0.19) | 0.27 (0.16) | 0.29 (0.09) | 0.18 (0.04) |
| Liver | 4.43 (0.39) | 2.13 (0.21) | 1.83 (0.15) | 1.83 (0.15) |
| Kidney | 11.74 (0.74) | 6.70 (0.63) | 3.70 (0.05) | 2.83 (0.20) |
| Small-Intestine | 4.76 (0.51) | 2.60 (1.54) | 3.49 (0.58) | 2.41 (1.17) |
| Large-Intestine | 0.52 (0.10) | 17.00 (2.49) | 0.96 (0.11) | 12.91 (2.93) |
| Spleen | 0.66 (0.05) | 0.53 (0.05) | 1.32 (0.07) | 1.13 (0.11) |
| Pancreas | 0.46 (0.13) | 0.27 (0.03) | 0.53 (0.09) | 0.32 (0.04) |
| Lung | 0.65 (0.07) | 0.28 (0.05) | 0.91 (0.09) | 0.49 (0.09) |
| Heart | 0.30 (0.02) | 0.17 (0.03) | 0.46 (0.02) | 0.31 (0.01) |
| [†]Stomach | 0.46 (0.09) | 0.22 (0.08) | 0.38 (0.04) | 0.19 (0.03) |
| Bone | 0.65 (0.18) | 0.51 (0.15) | 0.90 (0.16) | 0.73 (0.12) |
| Muscle | 0.38 (0.28) | 0.16 (0.04) | 0.43 (0.23) | 0.22 (0.03) |
| Brain | 0.05 (0.01) | 0.04 (0.00) | 0.04 (0.00) | 0.03 (0.00) |
| [†]Neck | 0.48 (0.06) | 0.36 (0.05) | 0.76 (0.08) | 0.74 (0.10) |
| U87MG | 0.98 (0.07) | 0.67 (0.03) | 2.85 (0.35) | 2.51 (0.11) |

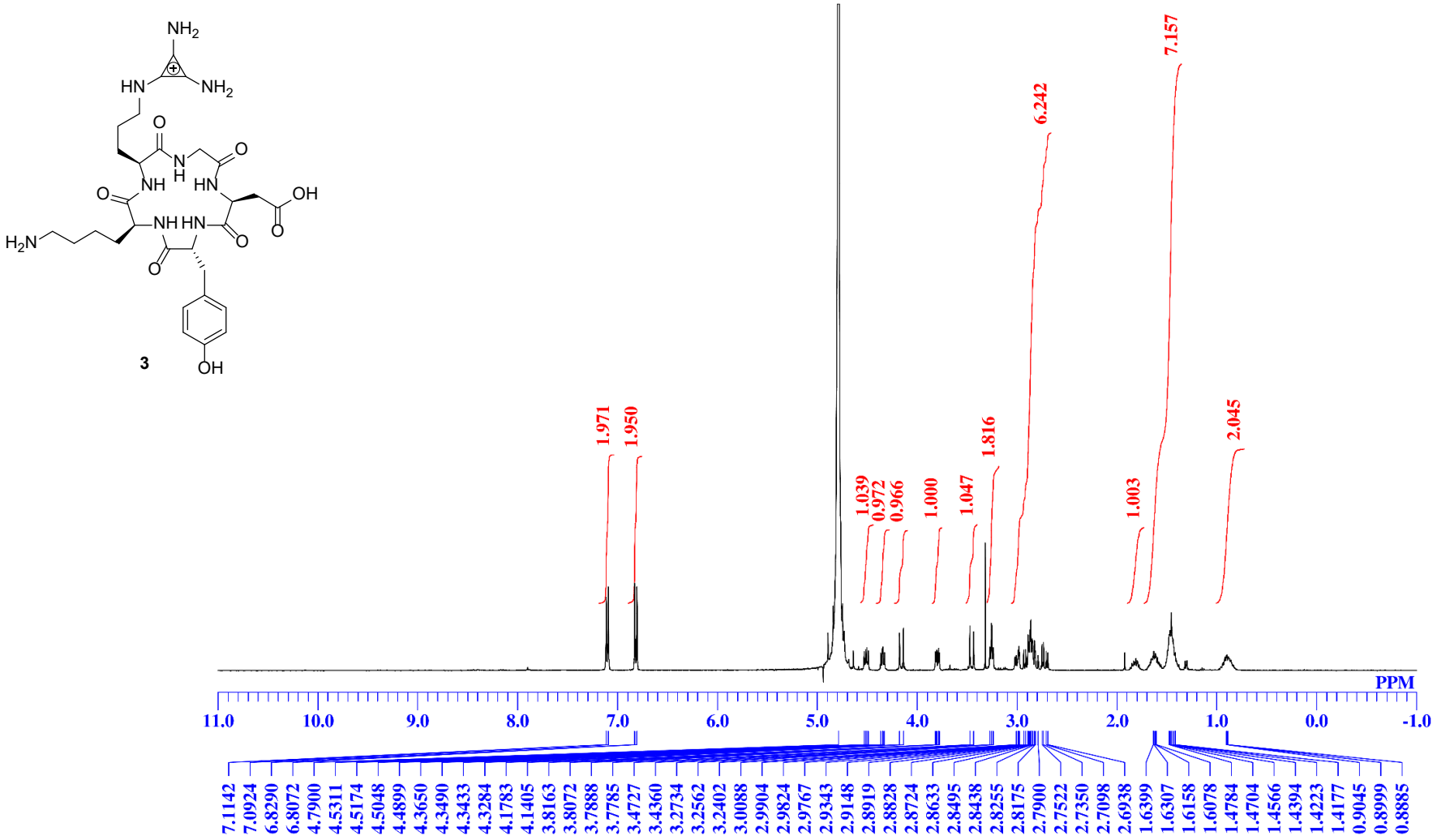
Expressed as % injected dose per gram. Each value represents the mean (SD) for three or four animals. †Expressed as % injected dose.

(B)

| | [$^{125}$I]c[deltic RGDy(3-I)K] ([$^{125}$I]**4**) | | [$^{67}$Ga]Ga-DOTA-c(RGDyK) [$^{67}$Ga]**9** | |
|---|---|---|---|---|
| Tissue | Control | Blocking | Control | Blocking |
| Blood | 0.37 (0.19) | 0.68 (0.27) | 0.29 (0.09) | 0.52 (0.10)* |
| Liver | 4.43 (0.39) | 3.69 (0.48) | 1.83 (0.15) | 1.45 (0.76) |
| Kidney | 11.74 (0.74) | 9.89 (1.13) | 3.70 (0.05) | 4.82 (1.21) |
| Small-Intestine | 4.76 (0.51) | 3.62 (0.85) | 3.49 (0.58) | 3.26 (0.48) |
| Large-Intestine | 0.52 (0.10) | 0.39 (0.12) | 0.96 (0.11) | 0.34 (0.10)*** |
| Spleen | 0.66 (0.05) | 0.29 (0.10)** | 1.32 (0.07) | 0.20 (0.17)*** |
| Pancreas | 0.46 (0.13) | 0.22 (0.16) | 0.53 (0.09) | 0.27 (0.15)* |
| Lung | 0.65 (0.07) | 0.64 (0.08) | 0.91 (0.09) | 0.48 (0.05)*** |
| Heart | 0.30 (0.02) | 0.19 (0.03)** | 0.46 (0.02) | 0.19 (0.06)*** |
| †Stomach | 0.46 (0.09) | 0.74 (0.37) | 0.38 (0.04) | 0.84 (0.46) |
| Bone | 0.65 (0.18) | 0.48 (0.14) | 0.90 (0.16) | 0.49 (0.23)* |
| Muscle | 0.38 (0.28) | 0.22 (0.13) | 0.43 (0.23) | 0.23 (0.18) |
| Brain | 0.05 (0.01) | 0.06 (0.02) | 0.04 (0.00) | 0.03 (0.02) |
| †Neck | 0.48 (0.06) | 0.40 (0.04) | 0.76 (0.08) | 0.28 (0.07)*** |
| U87MG | 0.98 (0.07) | 0.57 (0.10)** | 2.85 (0.35) | 0.69 (0.13)*** |

Expressed as % injected dose per gram. Each value represents the mean (SD) for three or four animals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. †Expressed as % injected dose.

$^{1}$H NMR spectrum of **3** (400 MHz, $D_2O$)



$^{1}$H NMR spectrum of **4** (400 MHz, $D_2O$)