SUPPORTING INFORMATION

Reduction of renal radioactivity of ^{111}In-DOTA-labeled antibody fragments with a linkage cleaved by the renal brush border membrane enzymes

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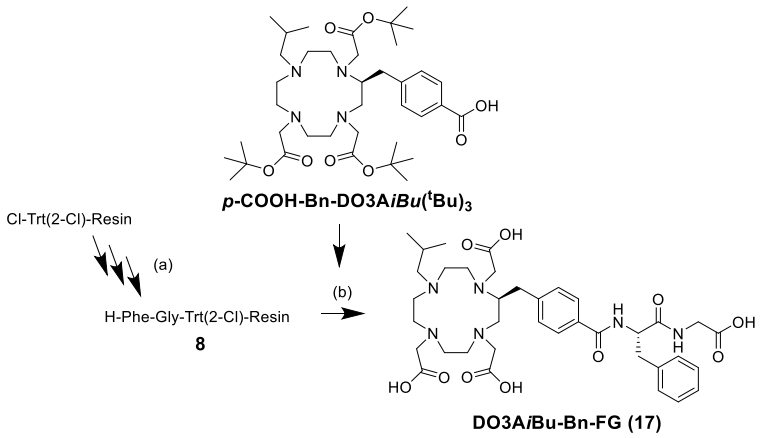
Methods

Synthesis of DO3A*i*Bu-Bn-FG



Scheme S1. Synthetic scheme of DO3A*i*Bu-Bn-FG. Reagents and conditions: (a) 1) Fmoc-amino acids, DIC, HOBt, DMF, rt for 2 h, 2) 20 % piperidine/DMF, rt for 20 min; (b) 1) DIC, HOAt, DMF, rt for 15 h, 2) TFA : triisopropylsilane : water (95/2.5/2.5), rt for 4 h, 19.4%.

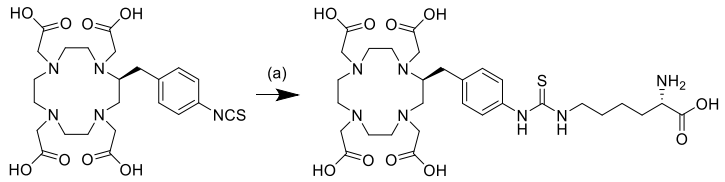
FG-Trt(2-Cl)-Resin (16)

The compound **16** (0.951 mmol/g) was constructed by a conventional Fmoc solid-phase peptide synthesis on Trt(2-Cl)-Resin using *N,N'*-diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole·monohydrate in dry DMF.[1]

DO3A*i*Bu-Bn-FG (17)

DO3A*i*Bu-Bn-FG was prepared according to the same procedure as DO3A*i*Bu-Bn-FGK using 8 (3.48 µmol/3.7 mg) instead of 7. Purification by analytical RP-HPLC (system A, 21.3 min of retention time) provided DO3A*i*Bu-Bn-FG as a white solid (0.5 mg, 0.675 µmol, 19.4%). HR-MS(ESI) calcd. for $C_{37}H_{51}N_6O_{10}$ $[M-H]^-$: m/z 739.36667, found: 739.36597. HPLC purity 99.0%, retention time 21.3 min (system A).

Synthesis of DOTA-Bn-SCN-K



Scheme S2. Synthetic scheme of DOTA-Bn-SCN-K. Reagents and conditions: (a) 1) Boc-Lys-OH, TEA, DMF, rt for 24 h; 2) 10% anisole/TFA, rt for 3 h, 88.7%.

DOTA-Bn-SCN-K (18)

To a solution of *p*-SCN-Bn-DOTA (5.0 mg, 7.27 μmol) (Macrocyclics) in DMF (500 μL) was added TEA and Boc-Lys-OH (1.8 mg, 1 equiv.), and stirred for 24 h at the same temperature. After the solvent was removed *in vacuo*, the residue was dissolved in 10%TFA/anisole (2.0 ml), and stirred for 3 h at room temperature. After the solvent was removed *in vacuo*, diethyl ether (2.0 mL) was added to the residue to give the precipitate. The precipitate was purified by preparative RP-HPLC (system E, 17.5 min of retention time) to provide DOTA-Bn-SCN-K as a white solid (4.5 mg, 6.45 μmol, 88.7%). HR-MS(ESI) calcd. for $C_{30}H_{46}N_7O_{10}S$ $[M-H]^-$: m/z 696.30269, found: 696.29821. HPLC purity >99%, retention time 11.2 min (system A).

Synthesis of non-radioactive In-labeled compounds

A solution of $InCl_3 \cdot 4H_2O$ in 0.25 M acetate buffer (0.1 μmol, 25 μL) was added to a solution of each ligand in 0.25 M acetate buffer (0.01 μmol, 25 μL), and stood for 1 h at room temperature. After the reaction, a solution of 50 mM DTPA in 0.25 M acetate buffer (20 μL) was added to the mixture, and then analyzed by RP-HPLC.

In-DO3A*i*Bu-Bn-F

Retention time: 20.7 and 21.6 min (system A), HR-MS(ESI) calcd. for $C_{35}H_{45}InN_5O_9$ $[M-H]^-$: m/z 794.22561, found: 794.22151 and 794.22304.

In-DOTA-Bn-F

Retention time: 18.2 and 18.9 min (system A), HR-MS(ESI) calcd. for $C_{33}H_{39}InN_5O_{11}$ $[M]^-$: m/z 796.16849, found: 796.17065 and 796.16536.

In-DO3A*i*Bu-Bn-FGK(Boc)

Retention time: 25.7 and 26.1 min (system A), 35.0 and 35.9 min (system C), HR-MS(ESI) calcd. for $C_{48}H_{68}InN_8O_{13}$ $[M-H]^-$: m/z 1079.39446, found: 1079.39704 and 1079.39037.

In-DOTA-Bn-FGK(Boc)

Retention time: 23.6 and 24.1 min (system A), 31.2 and 31.9 min (system C), HR-MS(ESI) calcd. for $C_{46}H_{62}InN_8O_{15}$ $[M]^-$: m/z 1081.33734, found: 1081.33592 and 1081.34083.

In-DO3A*i*Bu-Bn-FG

Retention time: 19.5 and 20.4 min (system A), HR-MS(ESI) calcd. for $C_{37}H_{48}InN_6O_{10}$ $[M-H]^-$: m/z 851.24707, found: 851.24821 and 851.24729.

In-DOTA-SCN-K

Retention time: 10.4 and 10.8 min (system A), 18.4 and 19.0 min (system B), HR-MS(ESI) calcd. for $C_{30}H_{43}InN_7O_{10}S$ $[M]^-$: m/z 808.18309, found: 808.18036 and 808.18318.

References

(1) Suzuki, H.; Ichinohe, K.; Araki, M.; Muramatsu, S.; Uehara, T.; Arano, Y. Synthesis and evaluation of a *para*-carboxylated benzyl-DOTA for labeling peptides and polypeptides. *Nucl. Med. Biol.* **2022**, *114-115*, 18-28.

Table S1. The log D values of [^{111}In]In-DO3A*i*Bu-Bn-F and [^{111}In]In-DOTA-Bn-F.[a]

	Log $D_{7.0}$	Log $D_{5.5}$
[^{111}In]In-DO3A*i*Bu-Bn-F	-4.31 ± 0.12	-3.77 ± 0.04
[^{111}In]In-DOTA-Bn-F	-5.19 ± 0.20	-4.74 ± 0.47

[a]Data represent the mean ± SD (n = 3).

Table S2. Plasma stabilities of ^{111}In-labeled Fabs.[a]

Time after incubation (h)	1	3	6	24
[^{111}In]In-DO3A*i*Bu-Bn-FGK-Fab (%)	97.7±0.6	97.4±0.8	97.3±0.3	97.5±0.1
[^{111}In]In-DOTA-Bn-FGK-Fab (%)	97.8±0.7	98.0±1.3	97.9±0.4	97.0±0.4
[^{111}In]In-DOTA-Bn-SCN-Fab (%)	97.3±0.4	97.1±0.1	97.2±0.4	97.0±0.2

[a]Data represent the mean ± SD (n = 3).

Table S3. Biodistribution of radioactivity after intravenous injection of [^{111}In]In-DO3A*i*Bu-Bn-FGK-Fab into normal mice.[a]

	10 min	1 h	3 h	6 h	24 h
Blood	25.29 ± 1.56	11.12 ± 1.29	4.55 ± 0.21	2.23 ± 0.36	0.13 ± 0.04
Liver	4.72 ± 0.40	3.31 ± 0.35	2.24 ± 0.12	1.87 ± 0.36	0.61 ± 0.06
Spleen	3.79 ± 0.42	2.47 ± 0.12	1.41 ± 0.18	0.89 ± 0.17	0.25 ± 0.08
Kidney	18.34 ± 1.70	16.72 ± 3.59[c,d]	13.55 ± 2.15[c,d]	10.21 ± 2.60[c,d]	2.30 ± 0.57[c,d]
Pancreas	1.05 ± 0.11	1.17 ± 0.12	1.40 ± 0.10	1.42 ± 0.18	0.44 ± 0.14
Heart	4.83 ± 0.32	3.66 ± 0.43	2.16 ± 0.23	1.26 ± 0.12	0.27 ± 0.05
Lung	9.07 ± 2.88	5.08 ± 0.99	2.56 ± 0.17	1.57 ± 0.27	0.23 ± 0.08
Stomach[b]	0.38 ± 0.03	0.57 ± 0.12	0.60 ± 0.14	0.45 ± 0.10	0.34 ± 0.22
Intestine[b]	2.33 ± 0.20	4.30 ± 0.17	7.05 ± 0.66	12.75 ± 3.07	2.16 ± 0.56
Muscle	0.76 ± 0.13	1.08 ± 0.16	0.78 ± 0.20	0.44 ± 0.07	0.10 ± 0.03
Bone	2.46 ± 0.81	2.08 ± 1.32	0.90 ± 0.13	0.72 ± 0.14	0.24 ± 0.05
Urine[b]				47.54 ± 11.75[c]	69.31 ± 3.24[c,d]
Feces[b]				0.03 ± 0.03	17.92 ± 8.87

[a]Data represent the mean of %ID/g ± SD (n = 5).

[b]Data represent the mean of %ID ± SD (n = 5).

[c,d]$P < 0.05$ compared with [^{111}In]In-DOTA-Bn-SCN-Fab ([c]) and [^{111}In]In-DOTA-Bn-FGK-Fab ([d]). Significances in the kidney and urine were determined by one-way ANOVA followed by Tukey's test.

Table S4. Biodistribution of radioactivity after intravenous injection of [^{111}In]In-DOTA-Bn-FGK-Fab into normal mice.[a]

	10 min	1 h	3 h	6 h	24 h
Blood	28.52 ± 1.61	13.25 ± 1.15	5.49 ± 0.52	2.55 ± 0.39	0.20 ± 0.03
Liver	5.71 ± 0.96	4.83 ± 0.64	4.88 ± 0.90	4.51 ± 0.89	2.99 ± 0.52
Spleen	4.33 ± 0.29	4.75 ± 1.03	4.35 ± 0.96	3.47 ± 0.74	2.25 ± 0.38
Kidney	16.10 ± 1.57	32.87 ± 4.13[c]	43.61 ± 8.29	41.54 ± 7.53[c]	20.02 ± 3.82[c]
Pancreas	0.92 ± 0.17	1.26 ± 0.22	1.73 ± 0.09	1.88 ± 0.28	0.97 ± 0.08
Heart	5.28 ± 0.42	5.09 ± 0.66	3.75 ± 0.58	3.30 ± 0.55	2.20 ± 0.20
Lung	10.63 ± 1.96	6.10 ± 0.92	3.57 ± 0.65	2.36 ± 0.21	0.84 ± 0.08
Stomach[b]	0.34 ± 0.10	0.61 ± 0.05	0.66 ± 0.08	0.72 ± 0.14	0.36 ± 0.12
Intestine[b]	2.64 ± 0.30	5.08 ± 0.69	6.82 ± 0.83	9.41 ± 0.47	2.98 ± 0.52
Muscle	0.81 ± 0.12	1.21 ± 0.25	1.04 ± 0.18	1.00 ± 0.15	0.55 ± 0.11
Bone	3.20 ± 0.73	2.31 ± 0.31	2.33 ± 0.63	2.22 ± 0.42	1.76± 0.21
Urine[b]				29.64 ± 3.27	51.20 ± 7.25
Feces[b]				0.07 ± 0.02	12.45 ± 3.74

[a]Data represent the mean of %ID/g ± SD (n = 5).

[b]Data represent the mean of %ID ± SD (n = 5).

[c]$P < 0.05$ compared with [^{111}In]In-DOTA-Bn-SCN-Fab. Significances in the kidney and urine were determined by one-way ANOVA followed by Tukey's test.

Table S5. Biodistribution of radioactivity after intravenous injection of [^{111}In]In-DOTA-Bn-SCN-Fab into normal mice.[a]

	10 min	1 h	3 h	6 h	24 h
Blood	25.65 ± 0.95	12.45 ± 0.99	4.14 ± 0.44	1.88 ± 0.20	0.26 ± 0.05
Liver	4.20 ± 0.35	3.80 ± 0.47	3.32 ± 0.59	3.45 ± 0.33	3.35 ± 0.77
Spleen	4.16 ± 0.57	3.72 ± 0.52	3.95 ± 0.75	3.43 ± 0.53	3.05 ± 0.89
Kidney	18.93 ± 1.77	42.23 ± 5.69	53.17 ± 6.89	51.88 ± 4.68	29.08 ± 3.45
Pancreas	0.79 ± 0.10	1.11 ± 0.12	1.62 ± 0.13	1.90 ± 0.19	1.43 ± 0.51
Heart	5.34 ± 0.71	4.81 ± 0.66	3.29 ± 0.09	2.91 ± 0.33	2.56 ± 0.70
Lung	9.43 ± 2.58	5.79 ± 0.71	3.04 ± 0.38	1.95 ± 0.19	0.97 ± 0.16
Stomach[b]	0.38 ± 0.03	0.63 ± 0.07	0.60 ± 0.04	0.59 ± 0.07	0.64 ± 0.19
Intestine[b]	2.43 ± 0.56	4.75 ± 0.49	4.32 ± 0.23	4.50 ± 0.58	6.43 ± 5.02
Muscle	0.72 ± 0.06	1.15 ± 0.09	1.15 ± 0.15	1.00 ± 0.17	0.57 ± 0.16
Bone	2.79 ± 0.63	1.91 ± 0.30	1.79 ± 0.30	1.69 ± 0.16	1.19 ± 0.63
Urine[b]				16.36 ± 2.40	38.27 ± 6.80
Feces[b]				0.05 ± 0.07	1.76 ± 0.43

[a]Data represent the mean of %ID/g ± SD (n = 5).

[b]Data represent the mean of %ID ± SD (n = 5).

Table S6. Biodistribution of radioactivity at 3 h postinjection of [^{111}In]In-DO3A*i*Bu-Bn-FGK-Fab and [^{111}In]In-DOTA-Bn-SCN-Fab into SY tumor-bearing mice.[a]

	[^{111}In]In-DO3A*i*Bu-Bn-FGK-Fab	[^{111}In]In-DOTA-Bn-SCN-Fab
Blood	5.13 ± 0.25	4.14 ± 0.40
Liver	2.91 ± 0.31	7.86 ± 0.73
Spleen	1.61 ± 0.19	4.86 ± 0.67
Kidney	23.92 ± 4.65[c]	113.06 ± 10.68
Pancreas	1.10 ± 0.10	1.45 ± 0.22
Heart	2.01 ± 0.30	2.68 ± 0.09
Lung	3.92 ± 0.24	3.91 ± 0.55
Stomach[b]	0.70 ± 0.39	0.96 ± 0.68
Intestine[b]	9.56 ± 2.62	5.87 ± 1.67
Muscle	0.63 ± 0.06	0.93 ± 0.10
Bone	0.96 ± 0.22	2.11 ± 0.49
Tumor	8.04 ± 1.17	8.26 ± 2.23

[a]Data represent the mean of %ID/g ± SD (n = 6).

[b]Data represent the mean of %ID ± SD (n = 6).

[c]$P < 0.05$ compared with [^{111}In]In-DOTA-Bn-SCN-Fab. Significance in the kidney was determined by Student's t-test.

Table S7. Biodistribution of radioactivity after intravenous injection of [^{111}In]In-DO3A*i*Bu-Bn-F into normal mice.[a]

	10 min	30 min	1 h	3 h	6 h
Blood	1.16 ± 0.12	0.43 ± 0.14	0.06 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
Liver	2.31 ± 0.04	1.74 ± 0.22	0.57 ± 0.14	0.55 ± 0.35	0.58 ± 0.16
Spleen	0.30 ± 0.05	0.24 ± 0.13	0.46 ± 0.52	0.01 ± 0.01	0.00 ± 0.00
Kidney	6.26 ± 1.97	6.47 ± 1.98	3.34 ± 2.33	0.43 ± 0.09	0.11 ± 0.04
Pancreas	0.37 ± 0.05	0.29 ± 0.15	0.24 ± 0.29	0.02 ± 0.02	0.00 ± 0.00
Heart	0.57 ± 0.23	0.48 ± 0.42	0.10 ± 0.05	0.00 ± 0.01	0.00 ± 0.00
Lung	1.06 ± 0.14	0.50 ± 0.18	0.12 ± 0.03	0.05 ± 0.05	0.01 ± 0.00
Stomach[b]	1.04 ± 0.19	0.29 ± 0.19	1.27 ± 0.65	0.87 ± 1.40	0.51 ± 0.89
Intestine[b]	12.91 ± 3.00	16.72 ± 2.31	22.94 ± 2.71	24.05 ± 3.41	26.59 ± 3.31
Muscle	0.33 ± 0.12	0.16 ± 0.08	0.06 ± 0.03	0.06 ± 0.08	0.00 ± 0.00
Bone	0.39 ± 0.05	0.24 ± 0.10	0.10 ± 0.02	0.03 ± 0.03	0.01 ± 0.02
Urine[b]					56.66 ± 3.79
Feces[b]					0.44 ± 0.23

[a]Data represent the mean of %ID/g ± SD (n = 4).

[b]Data represent the mean of %ID ± SD (n = 4).

Table S8. Biodistribution of radioactivity after intravenous injection of [^{111}In]In-DOTA-Bn-F into normal mice.[a]

	10 min	30 min	1 h	3 h	6 h
Blood	2.55 ± 0.14	0.61 ± 0.14	0.13 ± 0.12	0.01 ± 0.01	0.00 ± 0.00
Liver	4.22 ± 0.47	1.88 ± 0.14	0.81 ± 0.40	0.19 ± 0.10	0.23 ± 0.28
Spleen	0.56 ± 0.05	0.20 ± 0.07	0.07 ± 0.03	0.02 ± 0.02	0.01 ± 0.01
Kidney	16.12 ± 5.23	4.85 ± 2.86	5.30 ± 1.95	0.80 ± 0.23	0.46 ± 0.08
Pancreas	0.71 ± 0.15	0.23 ± 0.06	0.35 ± 0.23	0.03 ± 0.02	0.00 ± 0.00
Heart	1.13 ± 0.31	0.25 ± 0.06	0.19 ± 0.10	0.03 ± 0.02	0.01 ± 0.01
Lung	1.99 ± 0.17	0.52 ± 0.16	0.19 ± 0.09	0.04 ± 0.02	0.01 ± 0.01
Stomach[b]	0.49 ± 0.20	0.39 ± 0.13	0.35 ± 0.15	0.28 ± 0.06	0.02 ± 0.01
Intestine[b]	5.29 ± 0.99	10.81 ± 1.66	15.77 ± 4.11	17.65 ± 6.09	16.22 ± 3.65
Muscle	0.74 ± 0.07	0.22 ± 0.04	0.10 ± 0.07	0.01 ± 0.02	0.00 ± 0.00
Bone	0.68 ± 0.08	0.17 ± 0.04	0.07 ± 0.04	0.03 ± 0.03	0.01± 0.01
Urine[b]					61.36 ± 12.42
Feces[b]					0.75 ± 0.59

[a]Data represent the mean of %ID/g ± SD (n = 4).

[b]Data represent the mean of %ID ± SD (n = 4).

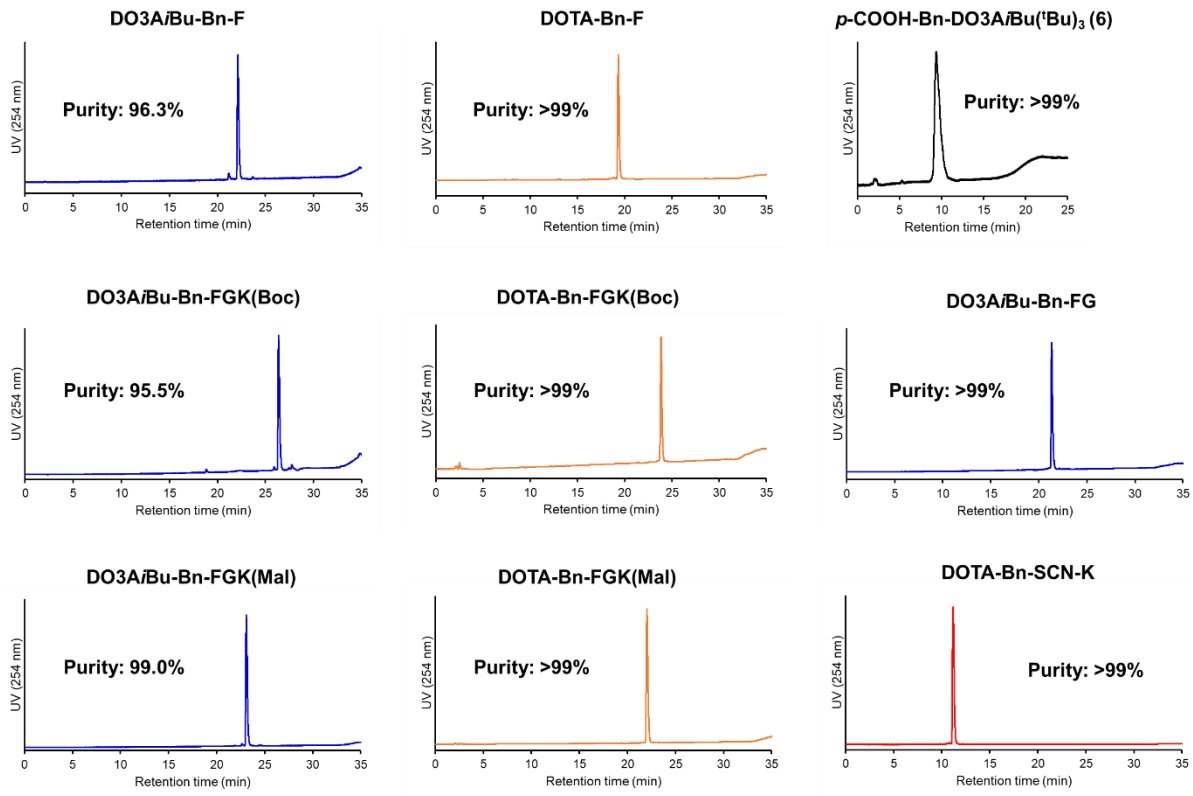


Figure S1. RP-HPLC analyses for purity check of key intermediate (**6**) and the precursors used for radiolabeling reactions. Chemical purities of all compounds were greater than 95%.

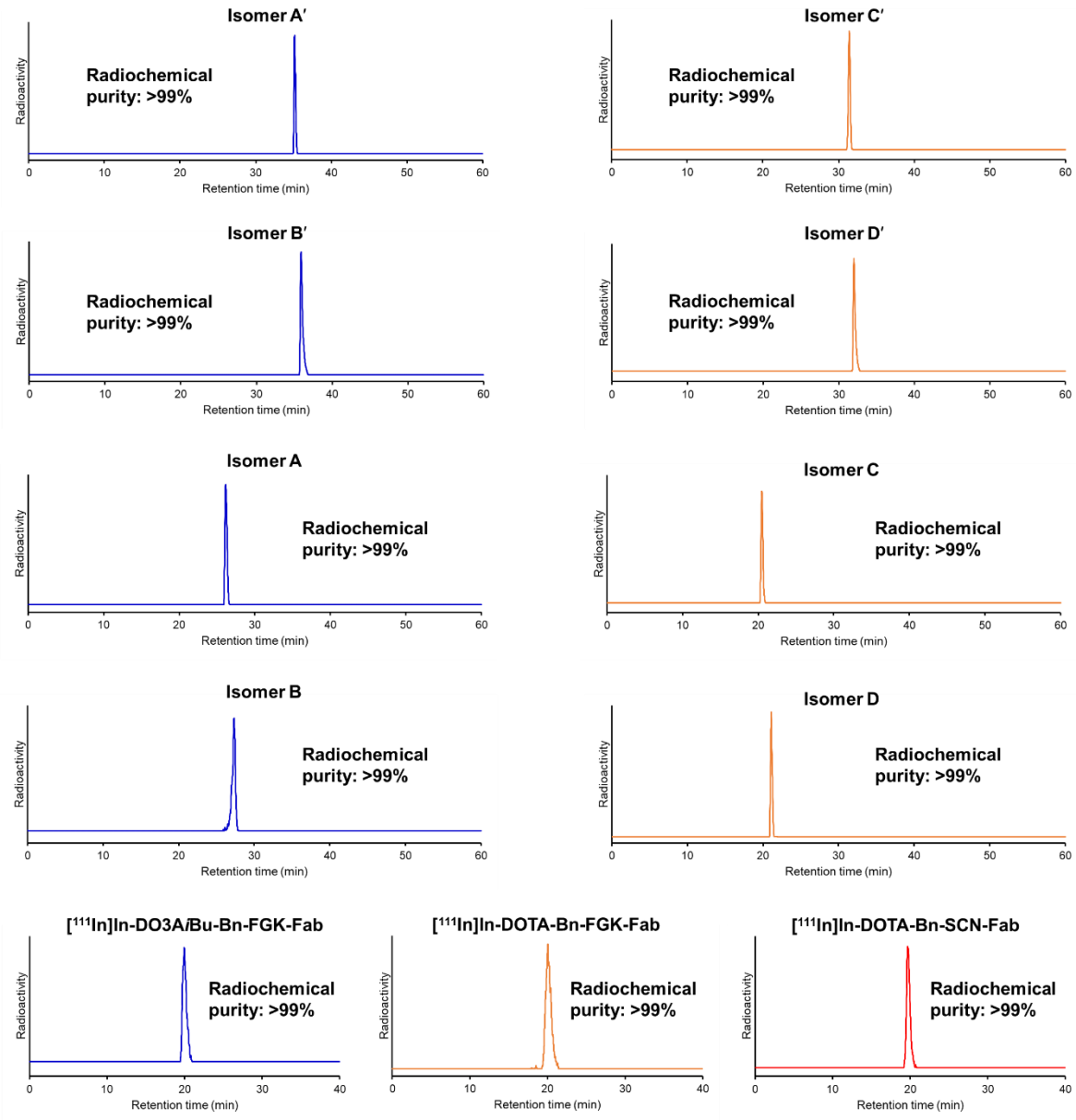


Figure S2. RP-HPLC and SE-HPLC analyses for purity check of ^{111}In-labeled compounds. Radiochemical purities of all tested compounds determined by RP-HPLC or SE-HPLC were greater than 99%.

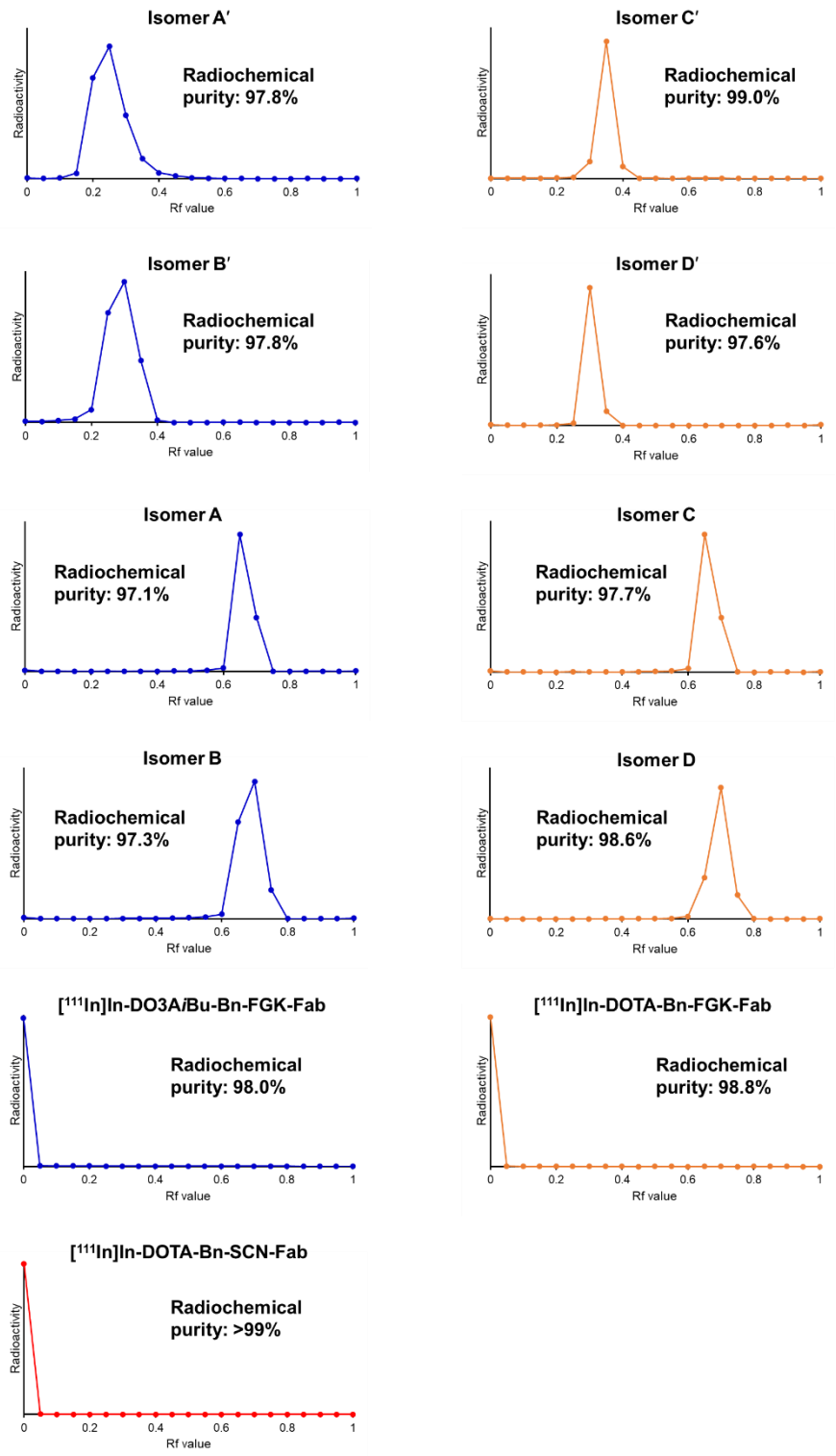


Figure S3. RP-TLC analyses for purity check of ^{111}In-labeled compounds. Radiochemical purities of all tested compounds determined by RP-TLC were greater than 97%.

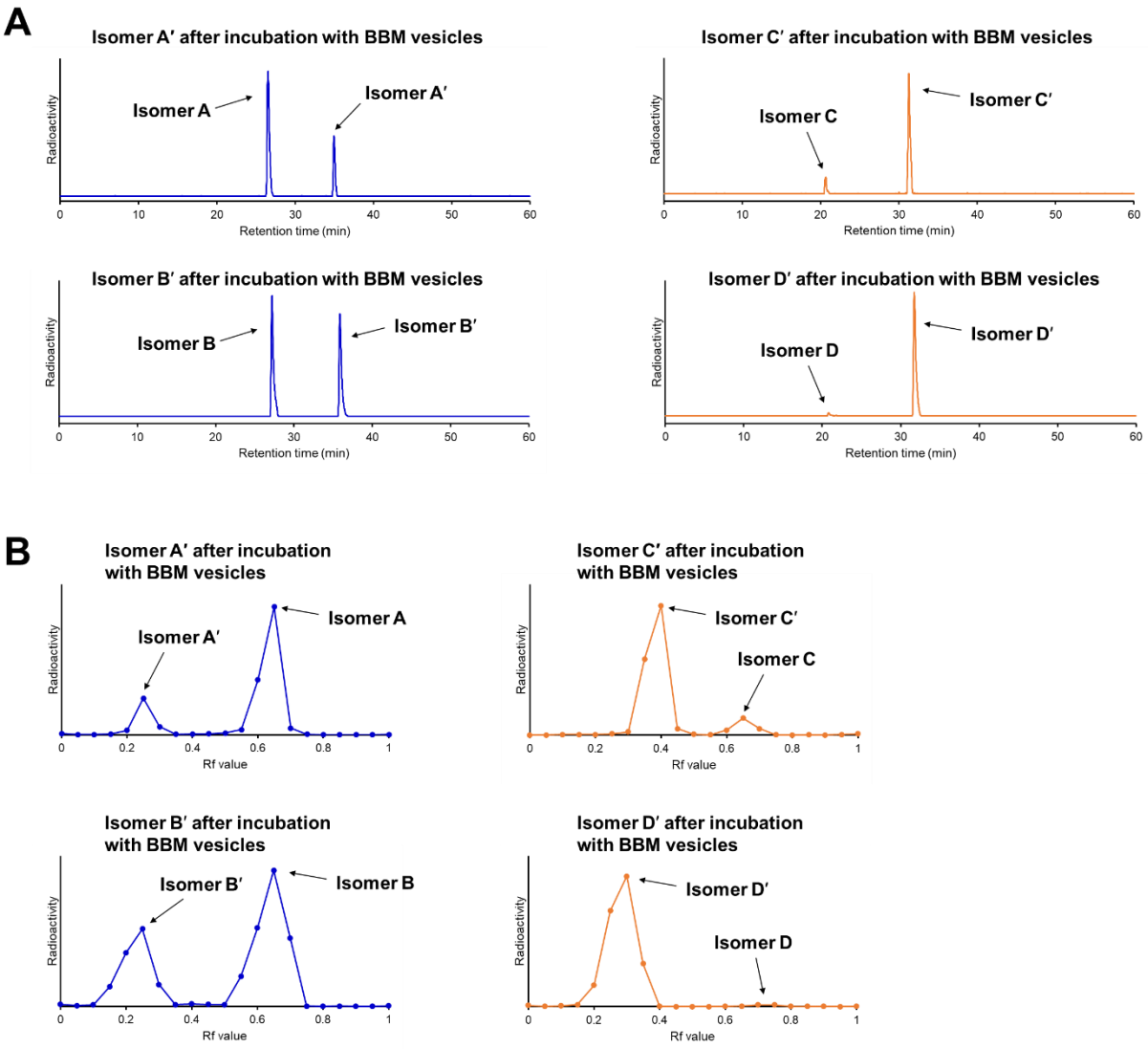


Figure S4. (A) RP-HPLC elution profiles of *in vitro* metabolic studies using ^{111}In-labeled low-molecular-weight (LMW) substrates. (B) RP-TLC analyses of *in vitro* metabolic studies using ^{111}In-labeled LMW substrates.

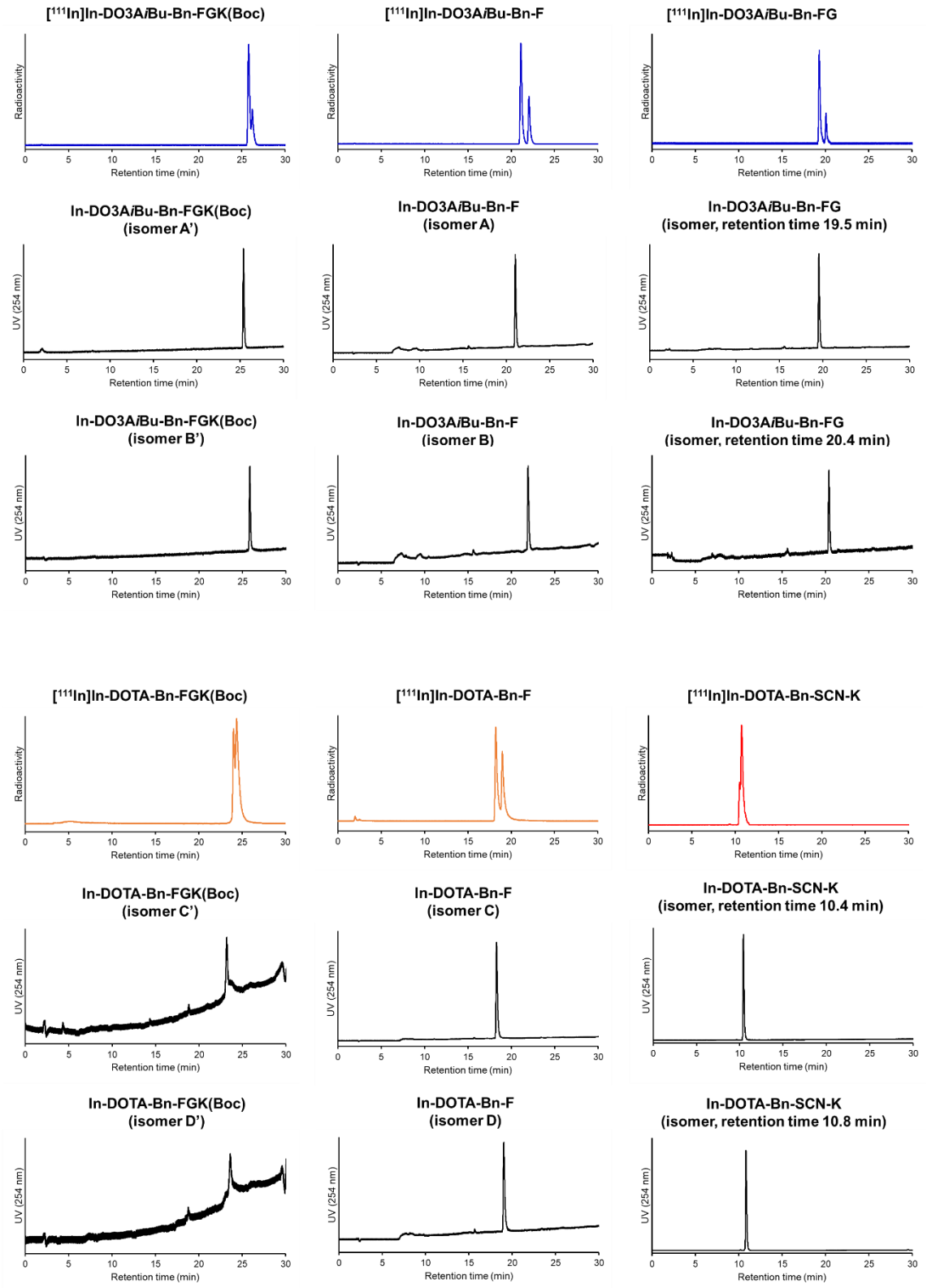


Figure S5. RP-HPLC analyses for characterization of ^{111}In-labeled LMW compounds. All ^{111}In-labeled compounds were characterized by the corresponding non-radioactive In-labeled compounds.

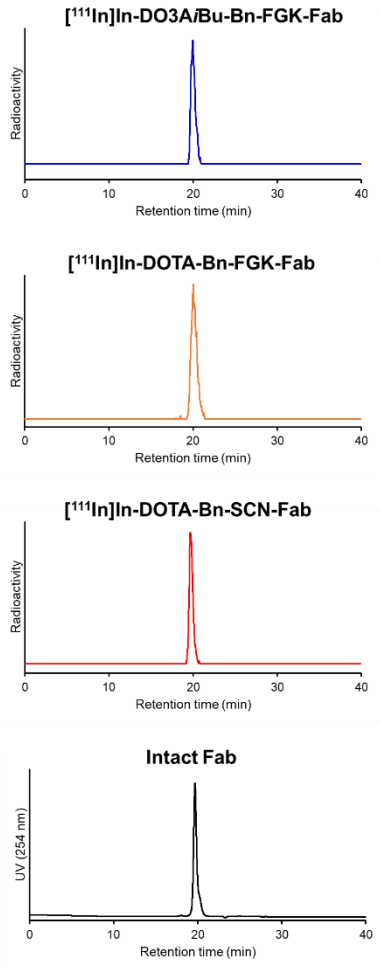


Figure S6. SE-HPLC analyses for characterization of ^{111}In-labeled Fabs. All ^{111}In-labeled compounds were eluted in the fraction of Fab.

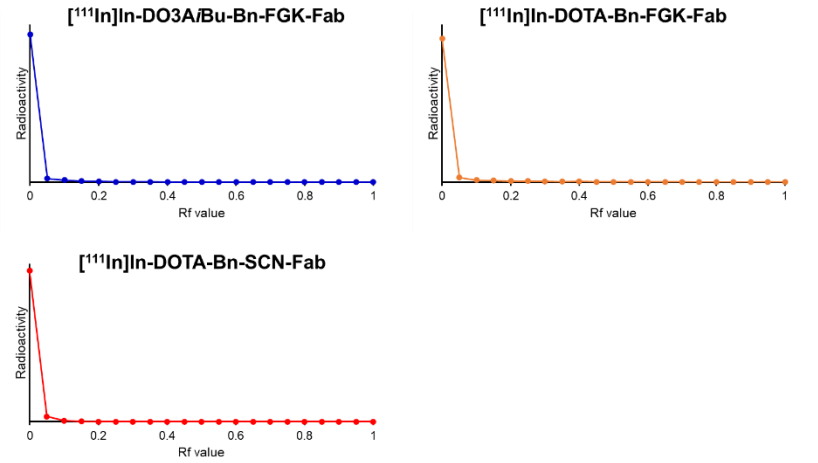
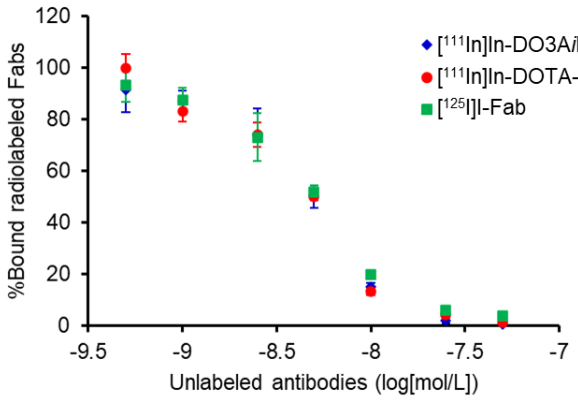


Figure S7. RP-TLC analyses of the *in vivo* plasma samples at 3 h postinjection of ^{111}In-labeled Fabs. All ^{111}In-labeled Fabs remained stable against *in vivo* plasma stability study (>95%). Radioactivity at the LMW fractions of [^{111}In]In-DO3A*i*Bu-Bn-F and [^{111}In]In-DOTA-Bn-F (Rf values: 0.6–0.8, Figure S3) were hardly observed for [^{111}In]In-DO3A*i*Bu-Bn-FGK-Fab and [^{111}In]In-DOTA-Bn-FGK-Fab, respectively (< 1%).



The IC_{50} values of an intact antibody calculated from each radiolabeled antibody

Radiolabeled antibody	IC_{50} (nM)	95% confidential interval (nM)
[111In]In-DO3AiBu-Bn-FGK-Fab	5.33	4.73–6.00
[111In]In-DOTA-Bn-SCN-Fab	4.94	4.26–5.72
[125I]I-Fab	5.25	4.62–5.97

Figure S8. *In vitro* competitive inhibition assay of radiolabeled Fabs. The IC_{50} values of an intact antibody determined from each radiolabeled Fabs were comparable.