

Stability Estimation of Gallium Complexes of DOTA Derivatives for Radiotheranostic Applications

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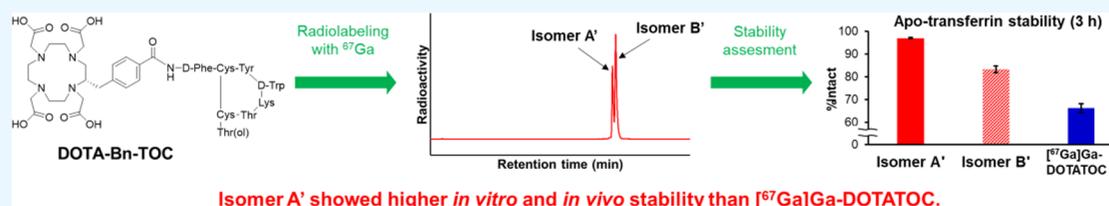
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ABSTRACT: 1,4,7,10-Tetraazacyclododecane-1,4,7-triacetic acid (DO3A) has been used to prepare ⁶⁸Ga-labeled probes for the diagnostic counterpart of radiotheranostic applications. While DO3A provides stable complexes with therapeutic radionuclides such as ⁹⁰Y, ¹⁷⁷Lu, and ²²⁵Ac, further improvement of the *in vivo* stability of the Ga-DO3A complex is required. Considering the high stability of an intact Ga-DOTA complex, the stability of Ga complexes of DOTA and DO3A derivatives, including benzyl-DOTA (Bn-DOTA), was evaluated to gain fundamental knowledge for developing the next-generation radiotheranostic probes using ⁶⁸Ga as a diagnostic counterpart. Following the complexation reaction to prepare ⁶⁷Ga-labeled DOTA and DO3A derivatives, the stability of the resulting ⁶⁷Ga-labeled compounds was evaluated in murine plasma and apo-transferrin challenge. [⁶⁷Ga]Ga-Bn-DOTA produced two isomers, and one of the isomers exhibited the highest stability among the tested complexes. The X-ray crystallography showed that the less stable isomer of Ga-Bn-DOTA suggested an N₃O₃ coordination geometry, while Ga-DOTA and Ga-Bn-DO3A show N₄O₂ coordination. To further evaluate the stability, a synthetic somatostatin analogue, [Tyr³]octreotide (TOC), was used as a model peptide, and *p*-COOH-Bn-DOTA and DO3A were conjugated with TOC to prepare DOTA-Bn-TOC and DOTATOC. [⁶⁷Ga]Ga-DOTA-Bn-TOC also yielded two isomers with varying stability, and one isomer exhibited significantly higher stability than [⁶⁷Ga]Ga-DOTATOC both *in vitro* and *in vivo*. These findings indicate that *para*-substituted Bn-DOTA would constitute a suitable chelating agent for developing next-generation radiotheranostic probes, although high-performance liquid chromatography purification is needed. Thus, further chemical modification on the Bn-DOTA molecule is also needed to avoid the formation of a Ga complex with the N₃O₃ configuration.

INTRODUCTION

1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) forms kinetically inert complexes with various radiometals, such as ^{67/68}Ga, ¹¹¹In, ⁹⁰Y, ¹⁷⁷Lu, and ²²⁵Ac.^{1–5} Thus, various radiolabeled probes have been developed by conjugating bifunctional DOTA derivatives with biomolecules^{6–8} for radiotheranostic applications, which are conducted with the same biomolecules using a pair of radionuclides suitable for imaging and radiotherapy.⁹ The radiotheranostic platform is expected to help accurately assess patient selection and therapeutic efficacy while facilitating personalized medicine.

Positron emission tomography (PET) is a valuable imaging modality for radiotheranostics. ⁶⁸Ga is an attractive radiometal for PET because of its availability from a long-lived ⁶⁸Ge/⁶⁸Ga generator system, allowing for the use of ⁶⁸Ga-labeled probes far from cyclotron facilities.^{10,11} Peptides (including peptidomimetics) accumulate quickly in the target tissue and are rapidly eliminated from the blood, making them suitable for

the short half-life ($T_{1/2} = 68$ min) of ⁶⁸Ga. Thus, various [⁶⁸Ga]Ga-DOTA-based peptide probes have been developed and used for preclinical and clinical applications.^{12–15} Several [⁶⁸Ga]Ga-DOTA-based peptide probes have also been used for radiotheranostics in conjunction with therapeutic radiometals, such as ¹⁷⁷Lu and ²²⁵Ac.^{16–19} 1,4,7,10-Tetraazacyclododecane-1,4,7-triacetic acid (DO3A) derivatives that use one of the carboxylates of DOTA for peptide conjugation have been used in most of these cases. DO3A-conjugated biomolecules labeled with therapeutic radiometals, such as ⁹⁰Y, ¹⁷⁷Lu, and ²²⁵Ac, exhibit high *in vivo* stability.⁵ However, improved resistance of [⁶⁸Ga]Ga-DO3A-conjugated biomolecules to apo-transferrin

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(apoTf) has been needed.^{20,21} Thus, a DOTA derivative providing a more stable Ga complex would contribute to more accurate diagnostics because the loss of Ga ions from the chelators causes the accumulation in the normal tissues, such as the bone and the spleen.^{22–24}

Compared to the Ga-DO3A derivatives such as DO3AM^{Bu} (Figure 1), Ga-DOTA exhibits much higher kinetic inertness,²⁵

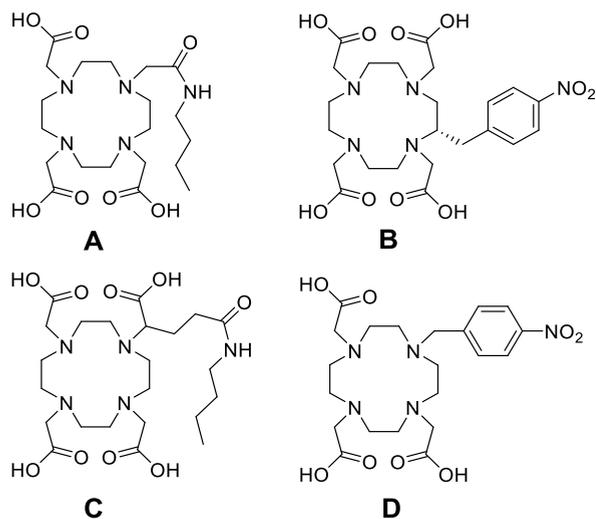


Figure 1. Chemical structures of model chelators used for the stability experiments; DO3AM^{Bu} (A), *p*-NO₂-Bn-DOTA (B), DOTAGAM^{Bu} (C), and *p*-NO₂-Bn-DO3A (D).

indicating that a bifunctional DOTA derivative holding an intact DOTA structure would provide stable Ga complexes.²⁶ One approach for preserving the intact DOTA structure involves the use of 1,4,7,10-tetraazacyclododecane,1-(glutaric acid)-4,7,10-triacetic acid (DOTAGA), which has the 5th carboxylic acid branched from one of the acetate arms of DOTA, allowing for the conjugation with biomolecules to occur. Although a previous study suggested that the *in vivo* stability of the [⁶⁸Ga]Ga-DOTAGA-conjugated peptide was lower than that of the ⁶⁸Ga-labeled peptide prepared with DO3A,²⁷ the details were not investigated. Another approach is to use benzyl-DOTA (Bn-DOTA) derivatives that possess a biomolecule-conjugating moiety, such as amino and isothiocyanate groups, at the *para* position of the benzyl group elongated from the carbon backbone of DOTA. In addition, steric crowding enhanced the stability of the metal-polyaza polycarboxylate complex when introducing a benzyl group.²⁸ Thus, Bn-DOTA may constitute a promising candidate as a bifunctional DOTA to form a more stable Ga complex than DO3A.

This study aimed to assess the stability of ⁶⁷Ga-labeled DOTA derivatives for developing the next-generation radiotheranostic probes that solve the stability problem. We selected Bn-DOTA (*p*-NO₂-Bn-DOTA), DO3A (DO3AM^{Bu}), and DOTAGA (DOTAGAM^{Bu}), as shown in Figure 1, and an intact DOTA as the DOTA derivatives. We also prepared another DO3A derivative, Bn-DO3A, where one of the carboxylate arms of DOTA was replaced with a *para*-substituted benzyl group (*p*-NO₂-Bn-DO3A, Figure 1D) to investigate the role played by the benzyl group on the stability of Ga complex. These studies were conducted with ⁶⁷Ga instead of ⁶⁸Ga because the longer half-life of ⁶⁷Ga (3.3 d) is more suitable for the studies. First, the *in vitro* stability of each

⁶⁷Ga complex was assessed by incubation in murine plasma and apoTf challenge. Then, the stability of [⁶⁷Ga]Ga-Bn-DOTA was further assessed after conjugation to a peptide using a newly developed bifunctional Bn-DOTA, *p*-COOH-Bn-DOTA(^tBu)₄. Its applicability to the solid-phase peptide synthesis was confirmed by synthesizing the *p*-COOH-Bn-DOTA-conjugated [Tyr³]octreotide (TOC) analogue, DOTA-Bn-TOC (Figure 2A).²⁹ The stability of [⁶⁷Ga]Ga-DOTA-Bn-

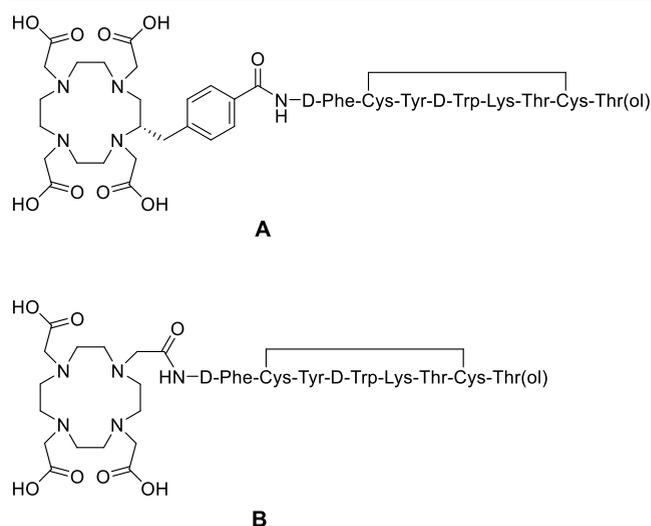


Figure 2. Chemical structures of octreotide analogues; DOTA-Bn-TOC (A) and DOTATOC (B).

TOC (Figure 2A) was compared with [⁶⁷Ga]Ga-DOTATOC (Figure 2B). Future perspectives and problems of bifunctional DOTA derivatives providing highly stable ⁶⁸Ga-labeled probes will be discussed.

RESULTS

Syntheses of ⁶⁷Ga-Labeled Model Chelators. The synthetic procedures for DOTAGAM^{Bu}, DO3AM^{Bu}, and *p*-NO₂-Bn-DO3A are described in Schemes S1–S3. All chelators showed a single peak by high-performance liquid chromatography (HPLC) analysis (Figures 3 and S1–S4). After complexation with ⁶⁷Ga, [⁶⁷Ga]Ga-DOTAGAM^{Bu}, [⁶⁷Ga]Ga-DO3AM^{Bu}, [⁶⁷Ga]Ga-*p*-NO₂-Bn-DO3A, and [⁶⁷Ga]Ga-DOTA showed a single radioactivity peak by HPLC analysis (Figures S1–S4). However, two radioactivity peaks were observed for [⁶⁷Ga]Ga-*p*-NO₂-Bn-DOTA (Figure 3). When the radioactivity peaks were isolated and reanalyzed by HPLC, both complexes of [⁶⁷Ga]Ga-*p*-NO₂-Bn-DOTA remained unchanged. Two peaks were also observed for nonradioactive Ga complexes of *p*-NO₂-Bn-DOTA at similar retention times. Following MS analysis, the isolated peaks showed identical MS spectra. The results indicated that they were isomers. Each isomer was referred to as isomer A and isomer B hereafter (17.6 and 22.1 min of retention time on HPLC system A, respectively, Figure 3).

In Vitro Stability Assessments of ⁶⁷Ga-Labeled Model Chelators. The *in vitro* stability experiments were conducted in the presence of plasma or an excess amount of apoTf, either for a short time (3 h) or a long incubation time (24 h), to evaluate the stability of ⁶⁷Ga complexes clearly. All ⁶⁷Ga complexes were purified by HPLC to remove free chelators, and the isomers of [⁶⁷Ga]Ga-*p*-NO₂-Bn-DOTA were simulta-

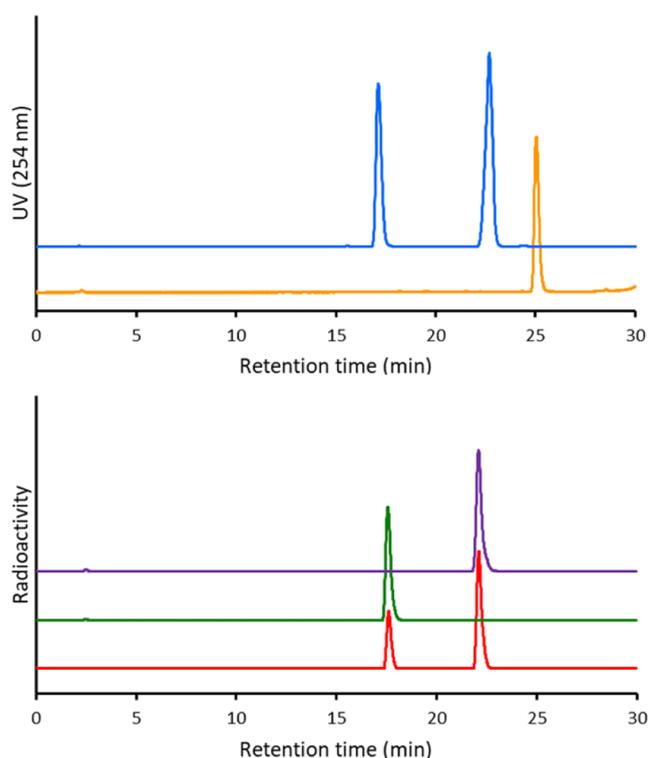


Figure 3. HPLC chromatograms of *p*-NO₂-Bn-DOTA (yellow) and nonradioactive Ga-*p*-NO₂-Bn-DOTA (blue) and HPLC radiochromatograms of [⁶⁷Ga]Ga-*p*-NO₂-Bn-DOTA (red), isomer A (green), and isomer B (purple) on system A. [⁶⁷Ga]Ga-*p*-NO₂-Bn-DOTA exhibited two peaks at retention times of 17.6 min (isomer A) and 22.1 min (isomer B). Each isomer was separated by HPLC and reinjected to HPLC to exhibit a single peak.

neously isolated. The differences in stability were clearly confirmed for apoTf challenge studies (isomer A \approx [⁶⁷Ga]Ga-DOTA > [⁶⁷Ga]Ga-*p*-NO₂-Bn-DO3A > isomer B > [⁶⁷Ga]Ga-DO3AM^{Bu} > [⁶⁷Ga]Ga-DOTAGAM^{Bu}) (Table 1). In the plasma stability study, small but significant differences were partially observed at 3 h incubation, and a similar tendency to an apoTf challenge study was observed at 24 h incubation.

X-ray Structure Analysis of Ga-*p*-NO₂-Bn-DOTA and Ga-*p*-NO₂-Bn-DO3A. To investigate the differences in stability between isomers A and B, we performed X-ray crystallographic analyses of Ga-*p*-NO₂-Bn-DOTA complexes. A single crystal appropriate for X-ray crystallographic analysis

was obtained for isomer B. However, we failed to obtain a single crystal of isomer A after several attempts. In the structure of isomer B, a distorted N₃O₃ octahedral geometry (Figure 4A) was confirmed. Three nitrogen atoms, except an atom next to the carbon atom with the benzyl group, were involved with coordination to the Ga ion, and the carboxylate oxygen atom elongated from the uncoordinated nitrogen atom coordinated to the Ga ion in addition to two oxygen atoms “trans” carboxylates. The coordination bond lengths between the three nitrogen atoms and the Ga ion (2.19–2.24 Å) in isomer B were longer than those in Ga-DOTA (2.09–2.15 Å).^{25,30} In another peculiarity, isomer B located four nitrogen atoms of the macrocyclic ring in the same plane. On the other hand, Ga-*p*-NO₂-Bn-DO3A showed an N₄O₂ coordination environment (Figure 4B).

Radiolabeling of TOC Analogues with ⁶⁷Ga. The applicability of Bn-DOTA for radiotheranostics using ⁶⁸Ga as a diagnostic counterpart was evaluated using a model peptide, the *p*-COOH-Bn-DOTA-conjugated TOC analogue (DOTA-Bn-TOC, Figure 2A). A representative TOC analogue, DO3A-conjugated TOC (DOTATOC, Figure 2B), was used as a reference. [⁶⁷Ga]Ga-DOTATOC showed a single HPLC peak (Figure 5) at a retention time similar to that of the nonradioactive Ga complex identified with MS (Figure S6). On the other hand, two peaks were observed in the radiochromatogram of [⁶⁷Ga]Ga-DOTA-Bn-TOC (Figure 5). Two peaks were also observed for nonradioactive Ga complexes of DOTA-Bn-TOC (Figure S5). Each component was isolated under different elution conditions (system C, Figure S5) in high purities (Figure 5). The MS analyses showed identical spectra, indicating that both are isomers (isomer A': 28.5 min and isomer B': 29.0 min on HPLC system B) (Figure 5).

A radiolabeling reaction with a final ligand concentration of 10 μM for 5 min at 95 °C yielded [⁶⁷Ga]Ga-DOTA-Bn-TOC and [⁶⁷Ga]Ga-DOTATOC in 97.4 ± 0.5% (isomer A' + isomer B') and 95.9 ± 1.5% radiochemical conversions (determined by RP-TLC), respectively. When the ligand concentration was lowered to 3 μM, radiochemical conversions were comparably decreased for both ⁶⁷Ga-labeled peptides ([⁶⁷Ga]Ga-DOTA-Bn-TOC in 85.2 ± 18.7% and [⁶⁷Ga]Ga-DOTATOC in 84.6 ± 15.1%).

The stability of ⁶⁷Ga-labeled TOC analogues was evaluated after incubation with murine plasma or apoTf, and the results are summarized in Table 2. The same tendency as the results

Table 1. Stability of ⁶⁷Ga-Labeled Model Chelators^a

	murine plasma		ApoTf (0.1 mM)	
	3 h	24 h	3 h	24 h
[⁶⁷ Ga]Ga- <i>p</i> -NO ₂ -Bn-DOTA (A)	97.6 ± 0.3 ^{cdef}	95.9 ± 0.1 ^{cdef}	95.1 ± 1.2 ^{def}	90.3 ± 0.7 ^{cdef}
[⁶⁷ Ga]Ga- <i>p</i> -NO ₂ -Bn-DOTA (B)	96.2 ± 0.4 ^b	85.9 ± 0.4 ^{bcd}	89.5 ± 0.7 ^{bcd}	56.0 ± 0.5 ^{bcd}
[⁶⁷ Ga]Ga-DOTAGAM ^{Bu}	96.1 ± 0.4 ^b	82.2 ± 0.2 ^{bcd}	80.0 ± 1.3 ^{bcd}	27.5 ± 4.6 ^{bcd}
[⁶⁷ Ga]Ga-DO3AM ^{Bu}	96.1 ± 0.6 ^b	83.9 ± 0.5 ^{bc}	87.4 ± 0.5 ^{bc}	43.1 ± 1.1 ^{bc}
[⁶⁷ Ga]Ga- <i>p</i> -NO ₂ -Bn-DO3A	96.7 ± 0.5	87.6 ± 0.3 ^b	93.8 ± 0.6 ^b	67.5 ± 0.6 ^b
[⁶⁷ Ga]Ga-DOTA	97.5 ± 0.5	95.5 ± 0.4	96.4 ± 0.2	89.6 ± 0.5

^aResults are expressed as mean ± SD (*n* = 5). ^bSignificances determined by Tukey's test for the one-way ANOVA; *p* < 0.05 compared with [⁶⁷Ga]Ga-DOTA. ^cSignificances determined by Tukey's test for the one-way ANOVA; *p* < 0.05 compared with [⁶⁷Ga]Ga-*p*-NO₂-Bn-DO3A. ^dSignificances determined by Tukey's test for the one-way ANOVA; *p* < 0.05 compared with [⁶⁷Ga]Ga-DO3AM^{Bu}. ^eSignificances determined by Tukey's test for the one-way ANOVA; *p* < 0.05 compared with [⁶⁷Ga]Ga-DOTAGAM^{Bu}. ^fSignificances determined by Tukey's test for the one-way ANOVA; *p* < 0.05 compared with [⁶⁷Ga]Ga-*p*-NO₂-Bn-DOTA (B).

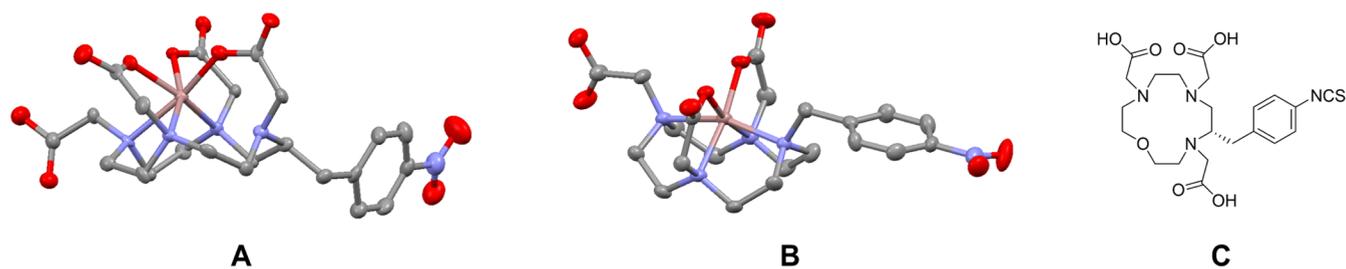


Figure 4. X-ray crystal structures of isomer B (A) and Ga-*p*-NO₂-Bn-DO3A (B) and the chemical structure of *p*-SCN-Bn-oxo-DO3A (C). Hydrogen atoms and solvent molecules are omitted (A,B). Thermal ellipsoids are shown at a 50% probability (A,B).

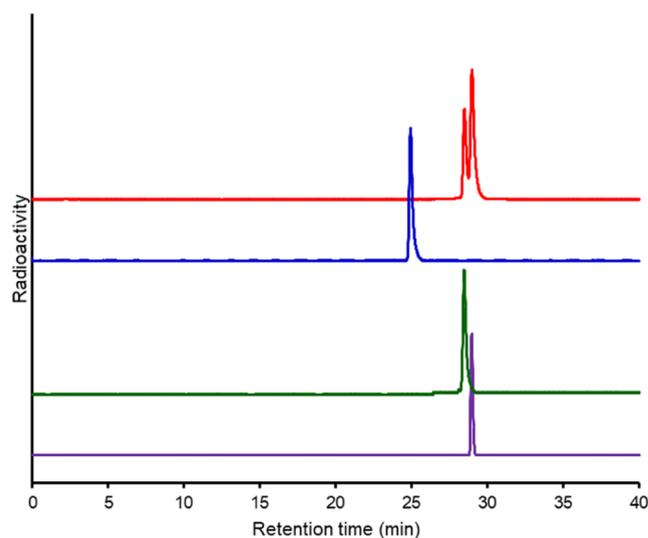


Figure 5. HPLC radiochromatograms of [⁶⁷Ga]Ga-DOTA-Bn-TOC (red), [⁶⁷Ga]Ga-DOTATOC (blue), and the isolated isomer A' (green) and isomer B' (purple) on system B. [⁶⁷Ga]Ga-DOTA-Bn-TOC exhibited two peaks at retention times of 28.5 min (isomer A') and 29.0 min (isomer B'). Each isomer was separated by HPLC and reinjected to exhibit a single peak. *In vitro* stability assessment of ⁶⁷Ga-labeled TOC analogues.

of ⁶⁷Ga-labeled model chelators was observed (isomer A' > isomer B' > [⁶⁷Ga]Ga-DOTATOC, Table 2).

***In Vivo* Stability Assessment of ⁶⁷Ga-Labeled TOC Analogues.** Figure 6 shows the biodistribution of radioactivity in the blood, spleen, and bone after intravenous injection of the two ⁶⁷Ga-labeled TOC analogues into mice, and the details are shown in Table S1. The isomer A' showed significantly lower radioactivity levels in normal tissues, such as the spleen ($p < 0.05$ at 1 and 3 h postinjection) and the bone ($p < 0.05$ at 1 h postinjection) compared with [⁶⁷Ga]Ga-DOTATOC. However, no significant differences were

observed in the biodistribution between the isomer B' and [⁶⁷Ga]Ga-DOTATOC.

DISCUSSION

The size of the DOTA cavity is suitable for binding large cations such as ¹¹¹In and ⁹⁰Y, and small Ga prefers NOTA.^{31–33} However, a DOTA derivative that provides a stable Ga complex for *in vivo* applications is preferable to radiotheranostic applications. Since intact [⁶⁷Ga]Ga-DOTA was stable in plasma and against the apoTf challenge, we evaluated Bn-DOTA derivatives for developing next-generation radiotheranostic probes using ⁶⁸Ga-PET as diagnostic probes.

We first evaluated the gallium complexation reactions with DOTA and DO3A derivatives (Figure 1). DOTA, DO3AM^{Bu}, DOTAGAM^{Bu}, and *p*-NO₂-Bn-DO3A produced a single Ga complex. However, *p*-NO₂-Bn-DOTA provided two Ga complexes with the same molecular masses (Figures 3 and S8, S9), referring to isomer A and isomer B. The prolonged reaction times to prepare [⁶⁷Ga]Ga-*p*-NO₂-Bn-DOTA did not change the formation ratios of the two isomers (data not shown). The isolated isomer B did not produce isomer A after heating in the presence of excess *p*-NO₂-Bn-DOTA (data not shown). Thus, isomer B is not a precursor for isomer A, and isomer B remained stable in the reaction solution once formed. X-ray crystallographic analysis of isomer B showed a distorted N₃O₃ octahedral geometry (Figure 4A). However, Ga complexes of intact DOTA and DO3A derivatives exhibited an N₄O₂ coordination geometry.^{25,30,34} Ga-*p*-NO₂-Bn-DO3A also showed an N₄O₂ coordination geometry (Figure 4B). A prior study suggested that oxo-DO3A (Figure 4C) generated a Ga complex with N₃O₃ configuration and was unstable in human serum (>80% was decomposed for 1.5 h).³⁵ Taken together, isomer A would possess an N₄O₂ coordination geometry, although we could not obtain a crystal for X-ray crystallography for isomer A. The small ionic radius of Ga would react with a Bn-DOTA specie preferring N₃O₃ configuration upon complexation, and the benzene ring would stabilize the resulting complex.

Table 2. Stability of [⁶⁷Ga]Ga-DOTA-Bn-TOC and [⁶⁷Ga]Ga-DOTATOC^a

	murine plasma		ApoTf (0.1 mM)	
	3 h	24 h	3 h	24 h
[⁶⁷ Ga]Ga-DOTA-Bn-TOC (A')	97.4 ± 0.4 ^{bc}	96.6 ± 1.0 ^{bc}	97.1 ± 0.3 ^{bc}	91.9 ± 1.2 ^{bc}
[⁶⁷ Ga]Ga-DOTA-Bn-TOC (B')	95.2 ± 0.5	86.5 ± 1.5 ^b	83.3 ± 1.5 ^b	20.5 ± 0.8 ^b
[⁶⁷ Ga]Ga-DOTATOC	94.4 ± 0.2	82.8 ± 0.9	66.2 ± 2.2	2.9 ± 0.2

^aResults are expressed as mean ± SD ($n = 5$). ^bSignificances determined by Tukey's test for the one-way ANOVA; $p < 0.05$ compared with [⁶⁷Ga]Ga-DOTATOC. ^cSignificances determined by Tukey's test for the one-way ANOVA; $p < 0.05$ compared with [⁶⁷Ga]Ga-DOTA-Bn-TOC (B').

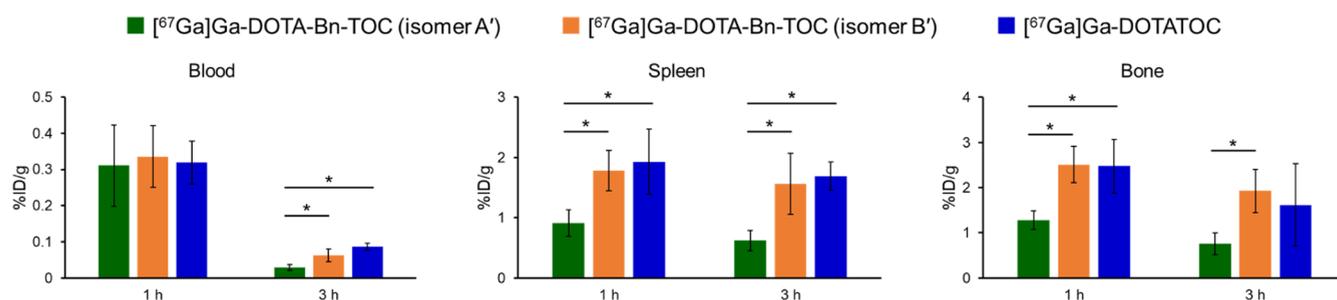


Figure 6. Radioactivity in the blood, spleen, and bone after injection of ^{67}Ga -labeled TOC analogues in ICR mice at 1 h and 3 h postinjection. Significances were determined by Tukey's test for the one-way ANOVA (* $p < 0.05$).

While both ^{67}Ga - $p\text{-NO}_2\text{-Bn-DO3A}$ and ^{67}Ga - DO3AM^{Bu} possess N_4O_2 configuration, the former showed higher stability than the latter, indicating the role played by the benzene ring in stabilizing the metal complexes through steric crowding as has been observed with the other radiometal chelates.²⁸ The higher stability of isomer A than ^{67}Ga - $p\text{-NO}_2\text{-Bn-DO3A}$ also suggests the importance of four carboxylic acids in DOTA to stabilize the Ga complex. On the other hand, ^{67}Ga - $\text{DOTAGAM}^{\text{Bu}}$ was the least stable among the 6 complexes (Table 1), as observed in an *in vivo* study.²⁷ Thus, the conjugation to a butylamine through the sidearm carboxylate impaired the inherent ability of DOTA to complex with the metal ion that possesses a small ionic radius for the DOTA cavity.

TOC was then selected as a model peptide to evaluate the stability of Bn-DOTA-derived ^{67}Ga -labeled probes. After conjugation of $p\text{-COOH-Bn-DOTA}$ with TOC, ^{67}Ga - Ga-DOTA-Bn-TOC was synthesized by reacting ^{67}Ga - GaCl_3 . Two isomers were again observed in HPLC analysis of ^{67}Ga - Ga-DOTA-Bn-TOC (Figure 5). The stability estimation showed that isomer A' possessed the highest stability, followed by isomer B' and ^{67}Ga - DOTATOC (Table 2). The stability of isomer B' and ^{67}Ga - DOTATOC drastically decreased in the apoTf challenge (Table 2) compared to murine plasma. The presence of plasma proteins may have protected the attack of apoTf on the radiometal chelates. The drastic decrease after TOC conjugation (Figure S10) suggests that the TOC molecule may have facilitated the access of apoTf to the chelates through some interactions. Under the conditions, isomer A' remained stable, which confirms the high stability of isomer A'. In biodistribution studies in mice, isomer A' exhibited significantly lower radioactivity levels in the spleen and the bone where free radiogallium accumulates (Figure 6).^{22–24} The lowest radioactivity levels in the blood at 3 h postinjection also support the high stability of isomer A'. When considering clinical studies, radiolabeled peptides would be diluted over 2,000 times upon intravenous injection compared to mice. Thus, the small but significant differences in the *in vivo* stability observed in mice would be enhanced in the clinical studies. These findings indicate that Bn-DOTA would constitute an appropriate chelating moiety for radiotheranostic applications using ^{68}Ga as a diagnostic counterpart. However, the formation of two Ga isomers with Bn-DOTA presented a new problem to be solved for developing the next-generation radiotheranostic probes. A bifunctional Bn-DOTA providing a single Ga complex with high *in vivo* stability is needed. The structural modifications of the Bn-DOTA skeleton avoiding the N_3O_3 configuration upon Ga complexation may constitute a way.³⁶

CONCLUSIONS

We evaluated the stabilities of Ga chelates of DOTA derivatives to develop the next-generation of radiotheranostic probes using ^{68}Ga as a diagnostic counterpart. The present study indicates that one isomer of Bn-DOTA provided ^{67}Ga chelate with the highest stability among the tested DOTA and DO3A derivatives shown in Figure 1. Bn-DOTA can be conjugated to peptides of interest using a commercially available $p\text{-NH}_2\text{-Bn-DOTA}$ directly or after converting to a carboxylic acid with succinic anhydride or glutamic anhydride or with $p\text{-COOH-Bn-DOTA}$ conducted in this study. However, Bn-DOTA generated two isomers with different stability, which needed HPLC purification after the radiolabeling reaction and decreased the radiochemical yields. The X-ray crystallography indicated that the less stable isomer possesses an N_3O_3 geometry. Thus, further studies are needed to develop bifunctional Bn-DOTA derivatives that avoid the formation of a Ga complex with an N_3O_3 configuration.

METHODS

General. ^1H NMR spectra were recorded on a JEOL JNM-ECS 400 spectrometer (JEOL, Tokyo, Japan). Mass spectrometry was performed using an AccuTOF LC-plus (JMS-T100LP, JEOL, Tokyo, Japan). The analytical methods for reversed-phase HPLC (RP-HPLC) are described in detail in the Supporting Information. Radiochemical conversions and *in vitro* stability were determined by reversed-phase (RP)-TLC (Silica gel 60 RP-18 F_{254S}, Merck, Tokyo, Japan) developed with a mixture of 0.1% aqueous TFA and MeCN with 0.1% TFA (2:3, v/v). ^{67}Ga - GaCl_3 was purchased from FUJIFILM RI Pharma (Tokyo, Japan). $p\text{-NO}_2\text{-Bn-DOTA}$ and DOTA were purchased from Macrocyclics (Texas, USA) and Tokyo Chemical Industry (Tokyo, Japan), respectively. DOTA-Bn-TOC and DOTATOC were prepared from the same procedure described previously.²⁹ The syntheses of DOTA-GAM^{Bu}, DO3AM^{Bu}, and $p\text{-NO}_2\text{-Bn-DO3A}$ are described in the Supporting Information. The other chemicals obtained from the commercial sources were of reagent grade or higher and were used without further purification. Data for single crystal X-ray diffraction analyses were collected on a Rigaku R-AXIS RAPID diffractometer using a graphite monochromator with Cu $K\alpha$ radiation ($\lambda = 1.54187 \text{ \AA}$). Data collection and reduction were performed using RAPID AUTO. The crystallography structures were solved by direct methods using SHELXL97 and refined with SHELXL97.

Syntheses of Non-Radioactive Gallium-Labeled Model Chelators. A solution of 0.1 M GaCl_3 in 0.2 M AcOH-AcONa buffer (pH 5.0) (equivalent molar to each chelator) was added to a solution of chelators (6 mg) in 0.5

mL of 0.2 M AcOH–AcONa buffer (pH 5.0). The mixture was reacted for 25 min at 95 °C. After cooling to room temperature, the mixture was purified by preparative or analytical HPLC. Fractions containing the product were lyophilized to provide nonradioactive Ga complexes.

Ga-*p*-NO₂-Bn-DOTA. *p*-NO₂-Bn-DOTA (8.75 μmol) was used as the chelator to provide isomer A (1.9 mg, 3.13 μmol, 35.8%) and isomer B (3.4 mg, 5.61 μmol, 64.1%).

Isomer A: 14.5 min of retention time of preparative HPLC on system F and 17.6 min of retention time of analytical HPLC on system A. ¹H NMR (D₂O): δ 2.95–3.82 [24H, m, DOTA, CH₂], 4.16 [1H, d, J = 18.8 Hz, DOTA], 7.46 [2H, d, J = 8.0 Hz, CH], 8.12 [2H, d, J = 8.8 Hz, CH]. HR-MS(ESI) calcd. for C₂₃H₂₉N₅O₁₀Ga [M – H][–]: *m/z*, 604.11702; found, 604.11551 and *m/z*, 606.11615; found, 606.11638.

Isomer B: 23.2 min of retention time of preparative HPLC on system F and 22.7 min of retention time of analytical HPLC on system A. ¹H NMR (D₂O): δ 2.69–3.78 [25H, m, DOTA, CH₂], 7.40 [2H, d, J = 8.8 Hz, CH], 8.10 [2H, d, J = 8.8 Hz, CH]. HR-MS(ESI) calcd. for C₂₃H₂₉N₅O₁₀Ga [M – H][–]: *m/z*, 604.11702; found, 604.11497 and *m/z*, 606.11615; found, 606.11397.

Ga-DOTAGAM^{Bu}. DOTAGAM^{Bu} (6.07 μmol) was used as the chelator to provide Ga-DOTAGAM^{Bu} (2.8 mg, 4.68 μmol, 77.0%). 26.5 min of retention time of preparative HPLC on system F and 21.6 min of retention time of analytical HPLC on system A. ¹H NMR (D₂O): δ 0.74 (3H, t, J = 7.4 Hz, CH₃), 1.09–1.19 (2H, m, CH₂), 1.26–1.33 (2H, m, CH₂), 1.89–1.98 (2H, m, CH₂), 2.23–2.80 (2H, m, CH₂), 3.03 (2H, t, J = 7.0 Hz, CH₂), 3.12–3.80 (23H, m, DOTA, CH₂). HR-MS(ESI) calcd. for C₂₃H₃₇N₅O₉Ga [M – H][–]: *m/z*, 596.18471; found, 596.18385 and *m/z*, 598.18383; found, 598.18545.

Ga-DO3AM^{Bu}. DO3AM^{Bu} (6.55 μmol) was used as the chelator to provide Ga-DO3AM^{Bu} (1.8 mg, 3.42 μmol, 52.2%). 24.3 min of retention time of preparative HPLC on system F and 19.9 min of retention time of analytical HPLC on system A. ¹H NMR (D₂O): δ 0.73 (3H, t, J = 7.4 Hz, CH₃), 1.12–1.22 (2H, m, CH₂), 1.31–1.38 (2H, m, CH₂), 3.07 (2H, t, J = 7.2 Hz, CH₂), 3.24–3.30 (8H, m, DOTA), 3.38–3.44 (4H, m, DOTA), 3.53 (2H, s, DOTA), 3.62 (2H, s, DOTA), 3.77–3.79 (6H, m, DOTA), 3.88–3.94 (2H, m, DOTA). ESI-MS [M – H][–]: *m/z*, 524; found, 524 and *m/z*, 526; found, 526.

Ga-*p*-NO₂-Bn-DO3A. *p*-NO₂-Bn-DO3A (6.40 μmol) was used as the chelator to provide Ga-*p*-NO₂-Bn-DO3A (3.5 mg, 6.38 μmol, 99.8%). 14.5 min of retention time of preparative HPLC on system F and 16.7 min of retention time of analytical HPLC on system A. ¹H NMR (D₂O): δ 3.03 [2H, d, J = 14.4 Hz, DOTA], 3.20–3.32 [6H, m, DOTA], 3.38–3.46 [6H, m, DOTA], 3.63 [2H, s, DOTA], 3.78–3.95 [6H, m, DOTA], 4.07 [2H, s, CH₂], 7.62 [2H, d, J = 7.2 Hz, aromatic], 8.18 [2H, d, J = 7.6 Hz, aromatic]. HR-MS(ESI) calcd. for C₂₁H₂₉N₅O₈Ga [M + H]⁺: *m/z*, 548.12719; found, 548.12654 and *m/z*, 550.12632; found, 550.12933.

Ga-DOTA. DOTA (14.8 μmol) was used as the chelator to provide Ga-DOTA (6.9 mg, 14.6 μmol, 98.7%). 8.6 min of retention time of analytical HPLC on system E. ¹H NMR (D₂O): δ 3.25 (8H, br, CH₂), 3.42 (4H, br, CH₂), 3.64 (4H, s, CH₂), 3.75 (4H, s, CH₂), 3.89 (4H, br, CH₂). ESI-MS [M – H][–]: *m/z*, 469; found, 469 and *m/z*, 471; found, 471.

Preparation of the Crystal for X-ray Crystallographic Analysis. *Isomer B.* A solution of isomer B (1 mg) in water (120 μL) was prepared in a 0.3 mL tube and stored in a 50 mL vial including THF (ca. 5 mL). Standing of the solution for 1

week provided a colorless crystal. A selected crystal was used for the analysis. The crystallographic information file for isomer B can be obtained from Cambridge structure data base with the CCDC no. 2204293.

Ga-*p*-NO₂-Bn-DO3A. A solution of Ga-*p*-NO₂-Bn-DO3A (2 mg) in water (120 μL) was prepared in a 0.3 mL tube and slowly evaporated for 1 week to provide a colorless needle crystal. A selected crystal was used for the analysis. The crystallographic information file for Ga-*p*-NO₂-Bn-DO3A can be obtained from Cambridge structure data base with the CCDC no. 2204295.

Syntheses of Non-Radioactive Gallium-Labeled TOC Analogues. **Ga-DOTA-Bn-TOC.** GaCl₃ was dissolved in a 1 M AcOH–AcONa buffer (pH 3.5) (5 mg/mL) and stood for 5 min at room temperature. A solution of GaCl₃ (100 μL) was added to a solution of DOTA-Bn-TOC (50 μL, equivalent molar to Ga). The mixture was reacted for 30 min at 100 °C. After cooling to room temperature, the mixture was analyzed by analytical HPLC. Retention time of analytical HPLC: 28.4 (isomer A', 50.2%) and 28.9 min (isomer B', 45.0%) on system B, ESI-MS [M + H + Na]²⁺: *m/z*, 823.3; found, 823.2 and [M + H + K]²⁺: *m/z*, 831.3; found, 831.2 for both isomers.

Ga-DOTATOC. Ga-DOTATOC was prepared according to the same procedure as Ga-DOTA-Bn-TOC using DOTATOC. 24.4 min of retention time (86.7%) of analytical HPLC on system B. ESI-MS [M + H]⁺: *m/z*, 1489.5; found, 1489.6.

Radiolabeling with ⁶⁷Ga. To a 1 M AcOH–AcONa buffer (pH 4.0) was added the same volume of [⁶⁷Ga]GaCl₃. The mixture was allowed to stand for 5 min at room temperature to provide [⁶⁷Ga]Ga-acetate solution. The same volume of a solution of chelators (20 μM) or TOC analogues (6 or 20 μM) in 0.1 M AcOH–AcONa buffer (pH 4.0) was added to the solution. A radiolabeling reaction was conducted for 5 min at 95 °C, and the radiochemical conversion was determined by RP-TLC. Then, ⁶⁷Ga-labeled compounds were subjected to analytical HPLC purification to remove free chelators: 17.6 and 22.1 min of retention time for [⁶⁷Ga]Ga-*p*-NO₂-Bn-DOTA on system A, 22.4 min of retention time for [⁶⁷Ga]Ga-DOTAGAM^{Bu} on system A, 20.2 min of retention time for [⁶⁷Ga]Ga-DO3AM^{Bu} on system A, 16.9 min of retention time for [⁶⁷Ga]Ga-*p*-NO₂-Bn-DO3A on system A, 8.8 min of retention time for [⁶⁷Ga]Ga-DOTA on system D, 23.5 and 25.1 min of retention time for [⁶⁷Ga]Ga-DOTA-Bn-TOC on system C, and 24.6 min of retention time for [⁶⁷Ga]Ga-DOTATOC on system B. The collected radioactive fractions were applied to Sep-Pak Plus C18 Cartridge (Nihon waters K.K., Tokyo), washed with water (3 mL), and then eluted with methanol (3 mL) for solvent change. The solution was evaporated to dryness, and the radioactive residue was redissolved in D-PBS(–) (Wako Pure Chemical Industries, Ltd., Osaka). The final solution of each sample was analyzed by analytical HPLC.

Stability Assessment in Murine Plasma. ⁶⁷Ga-labeled chelators or TOC analogues (74 kBq/20 μL) were added to freshly prepared murine plasma (180 μL). The mixture was incubated at 37 °C and 5% CO₂. After incubation for 3 and 24 h, a 5 μL aliquot of each sample was drawn, and analyzed by RP-TLC.

Stability Assessment in apoTf Solution. ⁶⁷Ga-labeled chelators or TOC analogues (74 kBq/100 μL) were added to the same volume of 0.2 mM apoTf in 200 mM sodium bicarbonate buffer (pH 7.4). The mixture was incubated at 37

°C and 5% CO₂. After incubation for 3 and 24 h, a 5 μL aliquot of each sample was drawn and analyzed by RP-TLC.

Biodistribution. Animal studies were conducted following our institutional guidelines and were approved by the Chiba University Animal Care Committee. Biodistribution studies were performed by intravenous administration of 0.1 mL of D-PBS(−) containing 0.1% BSA solution of each radioactive fraction (11 kBq/100 μL/mouse) to a 5 week old male ICR mice (Japan SLC Inc., Shizuoka, Japan). Groups of five mice were used for the experiments. Organs of interest were removed and weighed, and the radioactivity counts were determined with an auto-well γ counter at 1 and 3 h postinjection.

Statistical Analyses. Data are expressed as the means ± SD where appropriate. The results were statistically analyzed using Tukey's test for the one-way ANOVA.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.2c06814>.

Synthesis of DOTAGAM^{Bu}, DO3AM^{Bu}, and *p*-NO₂-Bn-DO3A; biodistribution of ⁶⁷Ga-labeled TOC analogues; RP-HPLC chromatograms of ⁶⁷Ga complexes, non-radioactive complexes, and ligands of DOTAGAM^{Bu}, DO3AM^{Bu}, *p*-NO₂-Bn-DO3A, DOTA, DOTA-Bn-TOC, and DOTATOC; RP-TLC radiochromatograms of ⁶⁷Ga-labeled model chelators and TOC analogues; MS spectra of isomer A and isomer B; and comparison of *in vitro* stability (PDF)

Crystallographic information of isomer B (CIF)

Crystallographic information of Ga-*p*-NO₂-Bn-DO3A (CIF)

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Notes

The authors declare no competing financial interest.

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