Reassessment of Diethylenetriaminepentaacetic Acid (DTPA) as a Chelating Agent for Indium-111 Labeling of Polypeptides Using a Newly Synthesized Monoreactive DTPA Derivative

Yasushi Arano,*,† Takashi Uezono,† Hiromichi Akizawa,† Masahiro Ono,† Kouji Wakisaka,† Morio Nakayama,‡ Harumi Sakahara,§ Junji Konishi,§ and Akira Yokoyama†

Department of Radiopharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, and Department of Nuclear Medicine, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan, and Department of Radiopharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Kumamoto University, Kumamoto 862, Japan

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Previous studies on indium-111 (^{111}In) labeling of polypeptides and peptides using cyclic diethylenetriaminepentaacetic dianhydride (cDTPA) as a bifunctional chelating agent (BCA) have indicated that DTPA might be a useful BCA for ^{111}In labeling of polypeptides at high specific activities when DTPA can be incorporated without inducing intra- or intermolecular cross-linking. To investigate this hypothesis, a monoreactive DTPA derivative with a maleimide group as the peptide binding site (MDTPA) was designed and synthesized. A monoclonal antibody (OST7, IgG_1) was used as a model polypeptide, and conjugation of MDTPA with OST7, ^{111}In radiolabeling of MDTPA−OST7, and the stability of ^{111}In−MDTPA−OST7 were investigated using cDTPA and benzyl-EDTA derivatives as references. SDS−PAGE analysis demonstrated that while cDTPA induced intramolecular cross-linking, no such undesirable side reactions were observed with MDTPA. MDTPA generated ^{111}In-labeled OST7 with high radiochemical yields at higher specific activities than those produced using cDTPA and benzyl-EDTA derivatives as the BCAs. Incubation of each ^{111}In-labeled OST7 in human serum indicated that MDTPA generated ^{111}In-labeled OST7 of much higher and a little lower stability than those derived from cDTPA and benzyl-EDTA derivatives, respectively. These findings indicated that the low *in vivo* stability of cDTPA-conjugated antibody reported previously is not attributable to low stability of ^{111}In−DTPA but to formation of intramolecular cross-linking during cDTPA conjugation reactions. The present study also indicated that MDTPA and its precursor, the tetra-*tert*-butyl derivative of DTPA, would be useful BCAs for ^{111}In radiolabeling of polypeptides that have rapid blood clearance with high specific activities.

Introduction

Low molecular weight oligopeptides such as somatostatin analogs and chemotactic peptides have attracted attention as new vehicles for delivery of radioactivity to target tissues in diagnostic nuclear medicine.[1−3] Since the mechanisms of localization of many peptides to the target tissues involve specific binding to receptors expressed on the target cells, radiolabeled peptides of high specific activities are required for diagnostic application.[3] The somatostatin analog, octreotide, was successfully radiolabeled with indium-111 (^{111}In) at high specific activity and high radiochemical yields with cyclic diethylenetriaminepentaacetic dianhydride (cDTPA; Figure 1, compound **3**) used as a bifunctional chelating agent (BCA). In addition, attachment of an ^{111}In−DTPA moiety to the octreotide molecule altered the excretion pathway from hepatobiliary to urinary excretion while maintaining the target localization levels, which reduced upper abdominal radioactivity levels as was observed with a radioiodinated octreotide analog.[4] As a result, clear visualization of somatostatin-positive tumors has been reported in numerous clinical studies.[5] These results suggest that cDTPA would be a suitable BCA for ^{111}In radiolabeling of other oligopeptides and polypeptides such as vasoactive intestinal peptide for receptor scintigraphy.[6,7]

cDTPA has long been used as a BCA for ^{111}In labeling of various polypeptides, especially monoclonal antibodies (mAbs), due to its simple conjugation reaction with polypeptides and its ready availability from commercial sources. Extensive studies of ^{111}In-labeled mAbs using cDTPA as the BCA, however, have demonstrated low *in vivo* stability of the radiolabel. In animal studies, use of cDTPA caused higher hepatic radioactivity levels of ^{111}In-labeled mAbs when compared to ^{111}In-labeled mAbs using benzyl-DTPA and benzyl-EDTA derivatives as the BCAs.[8] The lower kinetic stability of ^{111}In−cDTPA−mAbs in plasma was also demonstrated.[9]

In contrast to these observations, recent metabolic studies of ^{111}In-labeled neoglycoalbumins have indicated that ^{111}In remains stable as intact DTPA chelates *in vivo*.[10,11] Further analyses of radiometabolites after administration of ^{111}In−cDTPA−neoglycoalbumins in mice have confirmed that ^{111}In is present as ^{111}In−DTPA−lysine and ^{111}In−DTPA *in vivo*.[12] Since neoglycoalbumins are prepared by modifying large amounts of accessible amine residues in the albumin backbone with sugar derivatives,[11,13,14] only limited numbers of amine residues are available for cDTPA conjugation. Occupation of amine residues in the albumin molecule with sugar derivatives may also induce steric interfer-

* Address all correspondence to Yasushi Arano, Ph.D., Department of Radiopharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Yoshida-Shimoadachi-cho, 606-01 Kyoto, Japan. TEL: 81-75-753-4567. FAX: 81-75-753-4568. E-mail: arano@pharm.kyoto-u.ac.jp.

† Department of Radiopharmaceutical Chemistry, Kyoto University.

‡ Department of Radiopharmaceutical Chemistry, Kumamoto University.

§ Department of Nuclear Medicine, Kyoto University.

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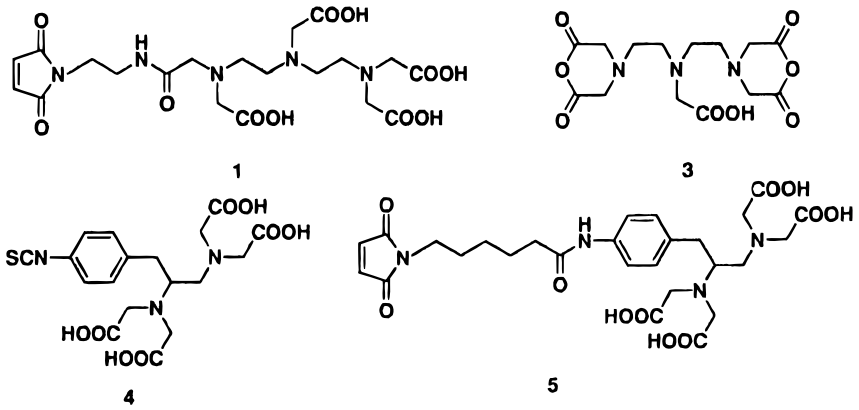


Figure 1. Chemical structures of monoreactive DTPA derivative, 3,6-bis(carboxymethyl)-9-(((2-maleimidoethyl)carbamoyl)methyl)-3,6,9-triazaundecanedioic acid (MDTPA, **1**) and of diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic dianhydride (cDTPA, **3**), 1-(4-isothiocyanatobenzyl)ethylenediaminetetraacetic acid (SCN-Bz-EDTA, **4**), and 1-[4-[(5-maleimidopentyl)amino]benzyl]ethylenediaminetetraacetic acid (EMCS-Bz-EDTA, **5**).

ence against the formation of intramolecular cross-linking during the conjugation reaction with cDTPA. On the other hand, formation of intra- and intermolecular cross-linking has been observed in the conjugation reaction of cDTPA with mAbs.[15] Studies of the reaction of cDTPA with insulin have also demonstrated the unavoidable formation of such unfavorable side products.[16] Thus, it was thought that the difference in stability of ^{111}In between cDTPA-conjugated mAbs and neoglycoalbumins might be attributable to the presence or absence of intramolecular cross-linking. In other words, DTPA might provide ^{111}In-labeled peptides and polypeptides of sufficient stability for diagnostic applications in nuclear medicine if it can be incorporated into polypeptides without inducing unfavorable side reactions, although X-ray structural characterization indicated that the octadentate ligand structure of DTPA is superior to the heptadentate structure of an amide bound DTPA for the thermodynamic and kinetic stabilization of indium.[17] If DTPA actually forms a ^{111}In chelate with sufficient *in vivo* stability for application in nuclear medicine, DTPA would become an attractive BCA, due to its ability to produce ^{111}In-labeled polypeptides of high specific activities with high radiochemical yields. This was supported by much faster ^{111}In complexation with DTPA-conjugated mAb than benzyl-DTPA-conjugated mAb.[15] However, this hypothesis can be confirmed only when DTPA-conjugated polypeptides can be prepared free from unfavorable side products.

In the present study, a monoreactive DTPA derivative was designed and synthesized (MDTPA; Figure 1, compound **1**) to investigate this possibility. A mAb against osteogenic sarcoma (IgG_1, OST7) was used as a model polypeptide because there have been numerous studies of ^{111}In radiolabeling of mAbs using cDTPA and because of the ease of detection of interchain cross-linking of antibodies by SDS−PAGE under reducing conditions. The conjugation reaction of MDTPA with OST7, radiochemical yields of the conjugate with ^{111}In, and the stability of the resulting ^{111}In-labeled OST7 in serum were investigated using cDTPA as a reference. In addition, 1-(4-isothiocyanatobenzyl)ethylenediaminetetraacetic acid (SCN-Bz-EDTA; Figure 1, compound **4**) and 1-[4-(5-maleimidopentyl)aminobenzyl]-ethylenediaminetetraacetic acid (EMCS-Bz-EDTA; Figure 1, compound **5**) were used as references to estimate the applicability of MDTPA. Although both SCN-Bz-EDTA and EMCS-Bz-EDTA include an EDTA chelating group and no DTPA group, comparable stability of ^{111}In−Bz-EDTA to ^{111}In−Bz-DTPA has been documented when conjugated to mAbs.[9] Thus, both Bz-EDTA chelating agents were used as representative BCAs to generate ^{111}In-labeled mAbs with *in vivo* stability sufficient for diagnostic applications. Reassessment of DTPA as BCA for ^{111}In labeling of proteins and peptides is discussed.

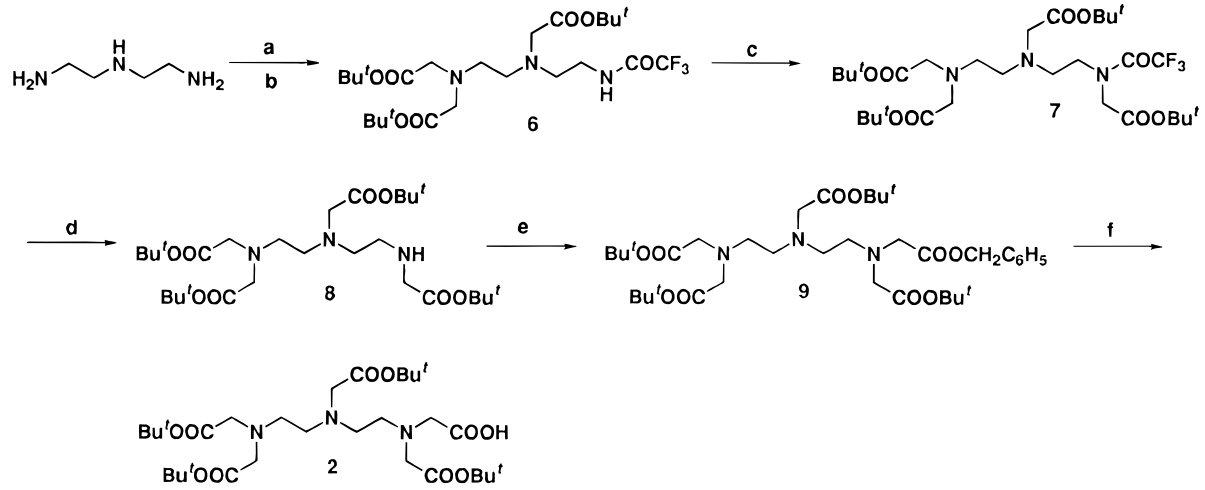
Chemistry

Synthesis of Monoreactive DTPA Derivative. The monomaleimide derivative of DTPA (MDTPA, compound **1** in Figure 1) was synthesized according to the procedure outlined in Schemes 1 and 2. The first isolated synthetic intermediate, compound **6** was prepared by trifluoroacetylation of one terminal amine of diethylenetriamine using ethyl trifluoroacetate, followed by alkylation of unprotected amines with *tert*-butyl bromoacetate with a yield of 66%. The amide nitrogen was then alkylated with *tert*-butyl bromoacetate in the presence of sodium hydride to produce compound **7** in relatively high yield (71%). The trifluoroacetyl protecting group was removed with anhydrous hydrazine in dry *tert*-butyl alcohol, and alkylation of the resulting secondary amine was followed by treatment with benzyl bromoacetate to prepare compound **9** at a yield of 52%. Catalytic hydrogenation with Pd/C in ethyl acetate provided compound **2** in almost quantitative yield (98%).

The amine derivative of maleimide, compound **12**, was synthesized by the reaction of *N*-methoxycarbonylmaleimide with *N*-(*tert*-butoxycarbonyl)ethylenediamine in a saturated aqueous solution of $NaHCO_3$, followed by deprotection of the Boc group of the intermediate with trifluoroacetic acid.

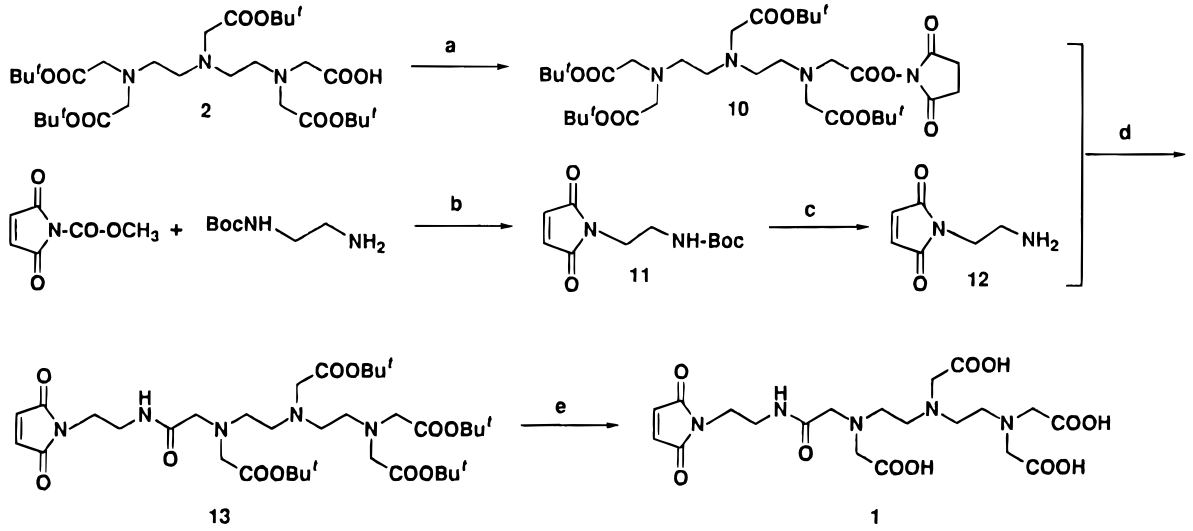
The coupling of compound **2** with compound **12** was achieved by formation of an active ester. The carboxylic acid of compound **2** was converted to *N*-hydroxysuccinimide ester in the presence of *N*,*N*′-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide. The active ester was then reacted with the amine derivative of maleimide without further isolation. After purification of the reaction mixture by silica gel chromatography, the monomaleimide-derived tetra-*tert*-butyl ester of DTPA (compound **13**) was obtained in a yield of 45%. The monomaleimide derivative of DTPA (MDTPA; compound **1**) was then obtained after deprotection of the

Scheme 1[a]



[a] Reagents: (a) $CF_3COOC_2H_5$; (b) $BrCH_2COOBu^t$, $^iPr_2NEt$; (c) $BrCH_2COOBu^t$, NaH; (d) NH_2NH_2, Bu^tOH; (e) $BrCH_2COOCH_2C_6H_5$, $^iPr_2NEt$; (f) Pd/C, H_2.

Scheme 2[a]



[a] Reagents: (a) dicyclohexylcarbodiimide, *N*-hydroxysuccinimide; (b) $NaHCO_3$; (c) CF_3COOH; (d) $^iPr_2NEt$; (e) CF_3COOH.

tert-butyl esters in the presence of trifluoroacetic acid at a yield of 73%.

Conjugation of MDTPA, cDTPA, SCN-Bz-EDTA, and EMCS-Bz-EDTA with OST7. Conjugation of MDTPA or EMCS-Bz-EDTA with the mAb against osteogenic sarcoma (OST7, IgG_1) was performed by maleimide−thiol chemistry using the maleimide group of each reagent and freshly thiolated OST7 by 2-iminothiolane. The number of chelating agents introduced per molecule of OST7 was estimated to be 2.2 for both MDTPA- and EMCS-Bz-EDTA-conjugated OST7s by measuring the thiol groups of OST7 before and after each conjugation reaction. Conjugation of cDTPA or SCN-Bz-EDTA with OST7 was achieved by reaction of acid anhydride or isothiocyanate with primary amine residues of the proteins. ^{111}In labeling of unpurified cDTPA- and SCN-Bz-EDTA-OST7 conjugates by method A (see the Experimental Section) and subsequent analyses by thin layer chromatography estimated the number of each chelating agent attached per molecule of OST7 to be 2.2 for cDTPA−OST7 and 2.8 for SCN-Bz-EDTA−OST7, respectively. When analyzed by SDS−PAGE under reducing conditions, MDTPA-, SCN-Bz-EDTA-, and EMCS-Bz-EDTA-conjugated OST7s indicated two bands at molecular masses of 25 and 50 kDa, corresponding to light and heavy chains, respectively (Figure 2). However, cDTPA−OST7 showed multiple additional bands at higher molecular weight although cDTPA−OST7 was purified by size-exclusion HPLC to remove intermolecular cross-linking.

^{111}In Radiolabeling of Conjugates. ^{111}In labeling of each conjugate was performed by two methods, A and B (see the Experimental Section for details). In method A, ^{111}In labeling was performed in the presence of high acetate concentration to stabilize ^{111}In against hydrolysis. When non-chelate-conjugated OST7 (original OST7) was labeled with ^{111}In by method A, no radioactivity was detected in the protein fractions by cellulose acetate electrophoresis (CAE) analysis. Addition of EDTA solution (100 molar excess over OST7) to the OST7 solution caused migration of more than 93% of the ^{111}In activity to the ^{111}In−EDTA fractions (Figure 3). Figure 4 illustrates the radioactivity profiles of OST7−cDTPA−^{111}In and OST7−MDTPA−^{111}In by method A when analyzed by size-exclusion HPLC (A−C) and CAE (D−F). OST7−cDTPA−^{111}In indicated a small radio-

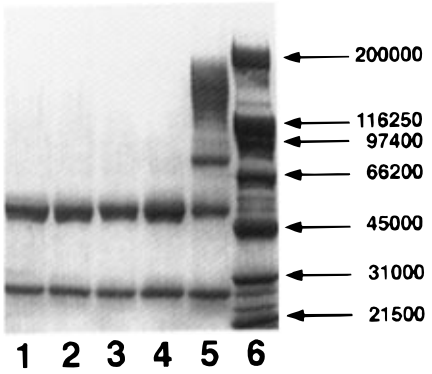


Figure 2. SDS−PAGE profiles of the four conjugates under reducing conditions. Molecular weight standards (lane 6) are myosin (200 kDa), β-galactosidase (116.25 kDa), phospholipase-*b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonoic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa). OST7−SCN-Bz-EDTA (lane 2), OST7−EMCS-Bz-EDTA (lane 3), and OST7−MDTPA (lane 4) indicated two bands corresponding to heavy and light chains, respectively, as also observed with the unmodified OST7 (lane 1). On the other hand, OST7−cDTPA (lane 5) showed multiple bands with molecular weights higher than the other two bands, although intermolecular cross-linking was excluded by size-exclusion HPLC prior to SDS−PAGE analysis. Intramolecular cross-linking was thus demonstrated in the OST7−cDTPA conjugates.

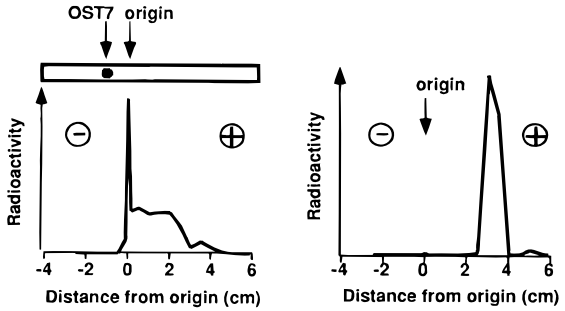


Figure 3. Cellulose acetate electrophoresis profiles of ^{111}In-labeled unmodified OST7 by method A (see the Experimental Section) before (left) and after (right) EDTA addition. No radioactivity was found at the protein fractions, and more than 94% of the radioactivity was detected at the ^{111}In−EDTA fractions after addition of EDTA. Thus, hydrolysis of ^{111}In was minimized under these conditions.

activity peak other than the protein fraction, which was observed at a longer retention time (ca. 26 min) on HPLC and ca. 0−1 cm anode on CAE (Figure 4A,D). While HPLC purification of the OST7−cDTPA−^{111}In increased the radiochemical purity as determined by HPLC analysis (Figure 4B), CAE analysis still indicated the presence of ca. 20% of the non-protein-associated radioactivity at the 0−1 cm anode (Figure 4E). Addition of excess EDTA and subsequent purification by centrifuged gel-filtration column did not improve the radiochemical purities of OST7−cDTPA−^{111}In determined by CAE analysis (data not shown). On the other hand, more than 92% of the radioactivity of OST7−MDTPA−^{111}In was present in the antibody fraction as determined by both HPLC (Figure 4C) and CAE (Figure 4F) analyses without any further purification.

Radiochemical yields of the four ^{111}In-labeled OST7s are summarized in Table 1. Method A was used for preparation of ^{111}In-labeled OST7s at the specific activity of 0.25 mCi (9.25 MBq)/mg, whereas method B was employed for ^{111}In labeling of OST7s at higher specific activities (3 and 6 mCi (111 and 222 MBq)/mg). OST7−MDTPA generated ^{111}In-labeled OST7 in radiochemical yields of more than 92% from low to high specific activity. No significant changes in the radiochemical purities were observed before and after EDTA addition to OST7−MDTPA−^{111}In. OST7−cDTPA showed lower radiochemical yields than OST7−MDTPA with similar radiochemical yields under both radiolabeling conditions. Both OST7−SCN-Bz-EDTA and OST7−EMCS-Bz-EDTA indicated radiochemical yields similar to OST7−MDTPA at low specific activity. However, significant decreases in the radiochemical yields were observed with increases in the specific radioactivity. Although OST7−SCN-Bz-EDTA contained a slightly more chelating agent per molecule of protein than OST7−EMCS-Bz-EDTA, the latter provided higher radiochemical yields.

Stability Estimation of ^{111}In-Labeled OST7s. OST7s labeled with ^{111}In using MDTPA, cDTPA, and SCN-Bz-EDTA as the BCAs were purified by size-exclusion HPLC and incubated at 37 °C in freshly prepared human sera. Figure 5 illustrates typical radioactivity profiles of size-exclusion HPLC of OST7−cDTPA−^{111}In before incubation (Figure 5A) and after 48 h of incubation (Figure 5B). The size-exclusion HPLC profile of the radioactivity of ^{111}In-labeled apotransferrin is also shown in Figure 5C. At 48 h incubation, approximately 15% of the initial radioactivity of OST7−cDTPA−^{111}In was present in the same fractions containing transferrin. These results are summarized in Figure 6, where the initial radiochemical purities of each ^{111}In-labeled OST7 are normalized to 100%. OST7−cDTPA−^{111}In released the radioactivity after incubation, and 26% of the initial radioactivity was detected at the transferrin fractions at 120 h postincubation. On the other hand, only a slight migration of the radioactivity (less than 5%) to the transferrin fractions was observed even at 120 h postincubation of OST7−SCN-Bz-EDTA−^{111}In. OST7−MDTPA−^{111}In significantly reduced the migration of radioactivity from antibody to transferrin fractions when compared with OST7−cDTPA−^{111}In. At 48 and 120 h postincubation, OST7−MDTPA−^{111}In liberated approximately 6 and 10% of the initial radioactivity, respectively.

Discussion

A monoreactive DTPA with a coordination geometry similar to cDTPA can be prepared by protecting one carboxylate attached to a terminal amine with a reagent that can be removed under conditions different from the protecting group of the other four carboxylates in the DTPA skeleton. We selected the benzyl-protecting group for one carboxylate and the *tert*-butyl protecting group for the rest of the four carboxylates (compound **9**). Since compound **9** has an asymmetrical structure, the symmetrical diethylenetriamine was first modified to an asymmetrical structure by acylation of one of the two terminal amines with ethyl trifluoroacetate, followed by alkylation of the unprotected primary and secondary amines with *tert*-butyl bromoacetate to generate compound **6**. Alkylation of the amide nitrogen of this molecule was performed with another *tert*-butyl bromoacetate in the presence of sodium hydride to produce compound **7**. In the deprotection reaction of the trifluoroacetyl group of compound **7**, the standard procedure using aqueous $NaHCO_3$ solution failed to

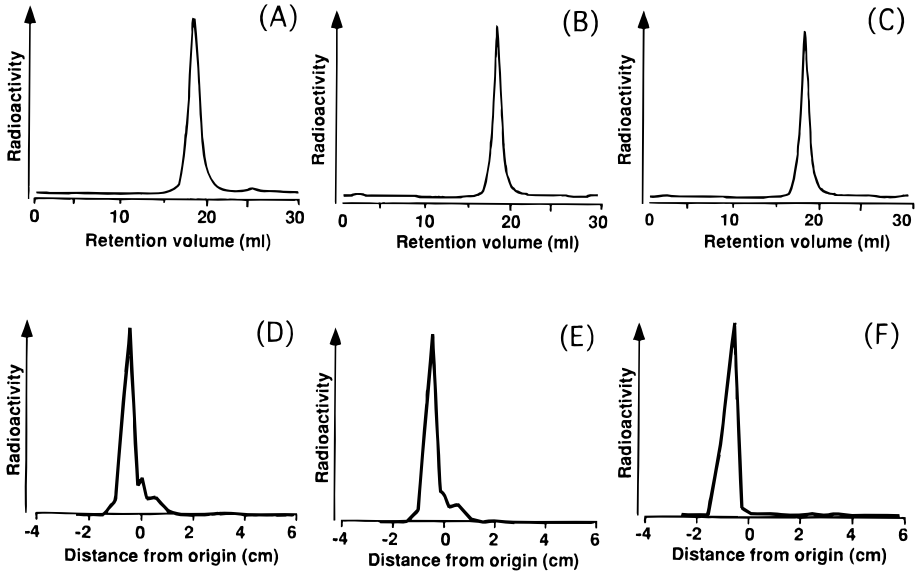


Figure 4. Size-exclusion HPLC (A−C) and cellulose acetate electrophoresis (D−F) profiles of OST7−cDTPA−^{111}In and OST7−MDTPA−^{111}In by method A. Panels A and D are of OST7−cDTPA−^{111}In; panels B and E are of OST7−cDTPA−^{111}In after purification by size-exclusion HPLC; panels C and F are of OST7−MDTPA−^{111}In. OST7−cDTPA−^{111}In showed a radioactivity peak in fractions other than those of protein (A and D). Although size-exclusion HPLC purification was shown to increase radiochemical purity of OST7−cDTPA−^{111}In by repeated size-exclusion HPLC analysis (B), CAE analysis indicated the presence of such radioactive species (E). OST7−MDTPA−^{111}In showed much higher radiochemical yields by both analytical methods (C and F).

Table 1. Radiochemical Yields[a] of ^{111}In-Labeled OST7s

specific activity (mCi/mg)	OST7−MDTPA−^{111}In	(+)-EDTA[b]	OST7−cDTPA−^{111}In	(+)-EDTA[b]	OST7−SCN-Bz-EDTA−^{111}In	(+)-EDTA[b]	OST7−EMCS-Bz-EDTA−^{111}In	(+)-EDTA[b]
0.25	92.5 (1.0)	93.3 (1.1)	70.2 (3.5)	65.5 (5.8)	92.9 (1.5)	93.7 (0.7)	92.3 (1.4)	91.5 (2.1)
3.0	94.5 (1.5)	93.4 (0.5)	nd[c]	nd	24.5 (8.3)	12.8 (2.6)	76.1 (9.0)	72.1 (13.1)
6.0	93.3 (1.6)	92.4 (1.6)	64.5 (5.4)	64.2 (8.2)	5.3 (2.6)	3.8 (1.8)	30.4 (5.3)	22.7 (1.3)

[a] Radiochemical yields determined by CAE. Each value represents the mean (1 sd) for three experiments. [b] To estimate nonspecific binding of ^{111}In in each preparation, EDTA was added to each ^{111}In-labeled OST7 (EDTA to OST7 molar ratio of 100:1). After incubation for 1 h at 23 °C, the antibody-associated radioactivity was reanalyzed by CAE. [c] nd = not determined.

cleave the protecting group. Deprotection under stronger alkaline conditions accompanied solvolysis reactions of the *tert*-butyl ester groups. Among the various reagents tested, anhydrous hydrazine in dry *tert*-butyl alcohol provided a high degree of selective deprotection. The resulting secondary amine of this key intermediate was then alkylated with benzyl bromoacetate to produce compound **9**. The precursor of the monoreactive DTPA, compound **2**, was obtained in high yield by the catalytic hydrogenation of the benzyl ester.

This compound is a monocarboxylic acid derivative of DTPA with the rest of the four carboxylates being protected with acid-removable *tert*-butyl esters, which are soluble in organic solvents such as acetonitrile and dimethylformamide (DMF). Since low reaction yields of cDTPA with oligopeptides were caused by intermolecular cross-linking, even when the reactions were performed with oligopeptides possessing only one amine residue per molecule or under solid-phase synthesis conditions,[1,2,18] compound **2** would serve as a good reagent to introduce a DTPA skeleton to oligopeptides with high reaction yields. The high solubility of compound **2** in organic solvent would render the reagent versatile for incorporating DTPA groups into oligopeptides by either liquid- or solid-phase synthesis.

The monoreactive DTPA derivative was prepared as illustrated in Scheme 2. When considering the structural similarity to cDTPA, a monoactive ester of DTPA would be desirable. We attempted to convert the free carboxylic acid of compound **2** to *N*-hydroxysuccinimide ester and subsequently deprotected the *tert*-butyl esters. However, compound **10** underwent decomposition during purification by silica gel column chromatography. Use of other alcohols such as tetrafluorophenol in place of *N*-hydroxysuccinimide did not allow isolation of the active ester precursor. Therefore, we selected a maleimide group as the protein binding site, and freshly prepared unpurified compound **10** was reacted with an amine derivative of maleimide, compound **12**. After column chromatography on silica gel, compound **13** was isolated in pure form. The final product, MDTPA (compound **1**), was then obtained by cleavage of the *tert*-butyl ester in the presence of trifluoroacetic acid.

MDTPA was conjugated to OST7 by maleimide−thiol chemistry using 2-IT.[19,20] A similar method was employed for EMCS-Bz-EDTA conjugation of OST7 while cDTPA and SCN-Bz-EDTA were conjugated by reaction of amine residues of the mAb.[8,9,11,21−23] When analyzed by SDS−PAGE under reducing conditions, MDTPA-, EMCS-Bz-EDTA-, and SCN-Bz-EDTA-conjugated OST7s demonstrated two bands, corresponding to the light and heavy chains (Figure 2), which indicated the absence of intermolecular and intramolecular (interchain) cross-linking of the three conjugates. However, although OST7−cDTPA was purified from intermolecularly cross-linked forms by size-exclusion HPLC prior to analysis, SDS−PAGE of cDTPA−OST7 showed multiple bands at a molecular mass higher than 66 kDa as well as the

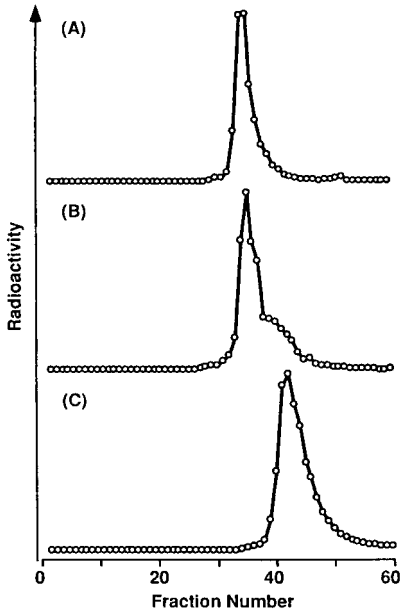


Figure 5. Size-exclusion HPLC profile of OST7−cDTPA−^{111}In before (A) and after 48 h (B) incubation in human serum at 37 °C and size-exclusion HPLC profile of ^{111}In-labeled apotransferrin (C). Approximately 15% of the radioactivity was transferred from the antibody to the fractions containing transferrin during 48 h incubation.

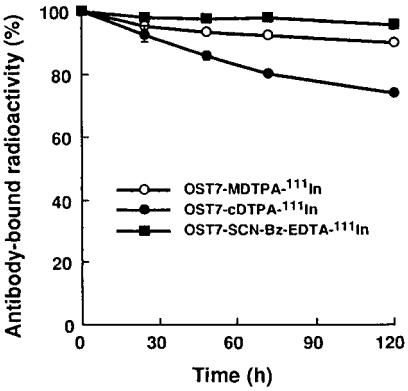


Figure 6. Percent radioactivity in the antibody fractions after incubation of ^{111}In−MDTPA−OST7, ^{111}In−cDTPA−OST7, and ^{111}In−SCN-Bz-EDTA−OST7 in freshly prepared human serum at 37 °C. Each value was calculated by dividing the antibody-bound radioactivity at various incubation times by the radiochemical purities of respective ^{111}In-labeled antibody immediate after size-exclusion HPLC purification. OST7−MDTPA−^{111}In liberated radioactivity slightly higher and much lower than OST7−SCN-Bz-EDTA−^{111}In and OST7−cDTPA−^{111}In, respectively. The radioactivity was transferred from the antibody to serum components such as transferrin.

two bands corresponding to heavy and light chains (Figure 2). Although cDTPA conjugation reactions were performed at various cDTPA-to-OST7 molar ratios, similar SDS−PAGE profiles were observed. Thus, intramolecular (interchain) cross-linking was unavoidable when cDTPA was used as a BCA with polypeptides, as has been documented previously.[15,16] The effects of such side reactions on complexation with ^{111}In and on the stability of the resulting ^{111}In-labeled protein were then examined.

Since the radiolabeling of ^{111}In with chelate-conjugated proteins is a competitive reaction between complexation and hydrolysis of ^{111}In, radiolabeling of each conjugate was performed at high acetate concentration to minimize the hydrolysis of ^{111}In (method A). Slow hydrolysis kinetics of ^{111}In under these reaction conditions was confirmed by the high-yield production (>94%) of ^{111}In−EDTA chelate after addition of EDTA to a mixed solution of ^{111}In acetate and unmodified OST7 (Figure 3). Under these conditions, MDTPA-, SCN-Bz-EDTA-, and EMCS-Bz-EDTA-conjugated OST7s showed radiochemical yields of more than 92% as determined by CAE analyses. However, OST7−cDTPA−^{111}In indicated significantly lower radiochemical yields (Table 1). Furthermore, although size-exclusion HPLC purification of OST7−cDTPA−^{111}In increased the radiochemical purity (>95%) when reanalyzed by HPLC, CAE analysis demonstrated that ca. 20% of the radioactivity was still present in the non-protein fractions (Figure 4E). The addition of a large amount of EDTA to the OST7−cDTPA−^{111}In did not alter the antibody-associated radioactivity when analyzed by CAE as shown in Table 1. These results indicated that OST7−cDTPA−^{111}In contained ^{111}In species that could not be removed by size-exclusion HPLC purification or EDTA chase but were dissociated from the conjugate during CAE analysis. Similar results were observed with ^{111}In radiolabeling of OST7−MDTPA and OST7−cDTPA at higher specific activities by method B. While OST7−MDTPA afforded radiochemical yields of over 92%, significantly lower yields were observed with OST7−cDTPA (ca. 65%) (Table 1). Since MDTPA possesses coordination geometry similar to cDTPA when cDTPA is introduced without any side reactions, the differences in radiochemical yield between MDTPA- and cDTPA-conjugated OST7 would be attributable to the presence of intramolecular (interchain) cross-linking in OST7−cDTPA, as demonstrated by SDS−PAGE (Figure 2). DTPA molecules that formed intramolecular cross-links may have acted as another chelating site for ^{111}In and hindered ^{111}In complexation with the DTPA moiety in OST7−cDTPA. Since such ^{111}In species were not removed by size-exclusion HPLC, which is commonly used for the purification of ^{111}In−cDTPA-labeled polypeptides, contamination by such ^{111}In species in ^{111}In−cDTPA-labeled polypeptides would constitute one reason for the observed instability of the radiolabel.

To further characterize the ^{111}In complexation reaction with respect to ligand structure, radiochemical yields of MDTPA−OST7 were compared with the two Bz-EDTA-conjugated OST7s. While MDTPA−OST7 provided radiochemical yields of over 92% under the three conditions tested, both Bz-EDTA-conjugated OST7s showed decreased radiochemical yields with increases in their specific activities. These results may have been due to the rigid ligand structure of Bz-EDTA chelating agents, which confers high kinetic stability on the resulting ^{111}In chelate but which may also hinder the complexation reaction. These findings indicated an important advantage of monoreactive DTPA for the preparation of ^{111}In-labeled polypeptides at high specific activities. The higher radiochemical yields of EMCS-Bz-EDTA−OST7 than SCN-Bz-EDTA−OST7 suggested the importance of the linkage structure between protein binding site and chelating site to increase the number of chelating groups available for complexation with metal ions.[24,25] Although a similar explanation may account for the radiochemical yields of MDTPA− and cDTPA−OST7s, the similar radiochemical yields of OST7−cDTPA−^{111}In under the two radiolabeling condi-

tions (methods A and B) suggested that this hypothesis is unlikely.

Finally, stability of the label, ^{111}In, derived from cDTPA-, MDTPA-, and Bz-EDTA-conjugated antibodies was estimated in freshly prepared human serum. OST7−cDTPA−^{111}In released significantly more radioactivity than OST7−MDTPA−^{111}In and OST7−SCN-Bz-EDTA−^{111}In (Figure 6). Most of the radioactivity released from OST7−cDTPA−^{111}In was observed in the same fractions containing transferrin (Figure 5), reinforcing the involvement of transchelation of ^{111}In from cDTPA-conjugated antibodies to transferrin in plasma.[15,26−29] Since OST7−cDTPA−^{111}In contained ca. 20% of the non-chelate-associated radioactivity even after size-exclusion HPLC purification, and the difference in radioactivity release between OST7−cDTPA−^{111}In and OST7−MDTPA−^{111}In was close to the value (ca. 15%) at 120 h postincubation (Figure 6), the presence of nonchelated ^{111}In species in OST7−cDTPA−^{111}In appears to account for the higher radioactivity release from the conjugate. These studies also indicated that Bz-EDTA forms an ^{111}In chelate with a little higher kinetic stability than DTPA even when DTPA was conjugated to mAb without inducing intra- or intermolecular cross-linking.

In conclusion, these findings suggested that low *in vivo* stability of ^{111}In-labeled polypeptides derived from cDTPA may be caused primarily by a formation of intramolecular cross-linking during conjugation reactions and may not be due to inherent stability of ^{111}In−DTPA chelate. Use of monoreactive DTPA in place of cDTPA as the BCA increased the radiochemical yield and the stability in serum of ^{111}In-labeled polypeptide. Furthermore, MDTPA facilitated generation of ^{111}In-labeled OST7 in high radiochemical yields even when radiolabeling was performed at high specific activities. Thus, MDTPA and its precursor appear to be appropriate BCAs for ^{111}In radiolabeling of polypeptides at high specific activities. However, their applications would be limited to polypeptides with rapid blood clearance due to the slightly lower stability of ^{111}In−MDTPA than Bz-EDTA−^{111}In. The discrepancies in the stability between ^{111}In−cDTPA-labeled mAbs and neoglycoalbumins could be attributed to the higher level of intramolecular cross-linking in the former and much faster blood clearance rate of the latter.

Experimental Section

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on an AC 300 (300 MHz) spectrometer (Bruker), and the chemical shifts are reported in ppm downfield from an internal tetramethylsilane standard. Fast atom bombardment mass spectra (FABMS) were obtained with a JMS-HX/HX110 A model (JEOL Ltd., Tokyo, Japan). Size-exclusion HPLC was performed with Cosmosil 5Diol-300 (7.5 × 600 mm) eluted with 0.1 M phosphate buffer (pH 6.8) at a flow rate of 1 mL/min.

Monoclonal Antibody. The monoclonal antibody against osteogenic sarcoma (OST7, IgG_1) was produced by standard hybridoma technology, as described previously.[30]

Synthesis of Di-*tert*-butyl 3-((*tert*-Butoxycarbonyl)-methyl)-6-(2-((trifluoroacetyl)amino)ethyl)-3,6-diazaoctanedioate (6). Diethylenetriamine (1.06 g, 10.3 mmol) was dissolved in dry dichloromethane (8 mL), and ethyl trifluoroacetate (1.46 g, 10.3 mmol) in dry dichloromethane (12 mL) was added dropwise to keep the reaction temperature below 0 °C. After 2 h of stirring below 0 °C, the reaction mixture was stirred for an additional 1.5 h at room temperature. The solvent and unreacted ethyl trifluoroacetate were removed *in vacuo*, and the oily residue was dissolved in dry acetonitrile (40 mL). The solution was cooled below 0 °C, and *N*,*N*-diisopropylethylamine (4.65 g, 36 mmol) was added to the solution, followed by *tert*-butyl bromoacetate (7.02 g, 36 mmol) while the reaction temperature was kept below 0 °C. After the reaction mixture was stirred at room temperature overnight, the solution was concentrated *in vacuo*. Ethyl acetate (50 mL) was added to the residue, and the organic phase was washed three times with a saturated aqueous solution of sodium bicarbonate. After the organic layer was dried over anhydrous calcium sulfate, ethyl acetate was removed *in vacuo*. The oily residue was chromatographed on silica gel using dichloromethane−methanol (70:1) as an eluent to produce compound **6** (3.68 g, 66%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$): δ 1.45 (27H, s, *t*-Bu), 2.75−2.79, 2.83−2.88 (6H, m, $NCH_2CH_2NCH_2$), 3.29 (2H, s, NCH_2COO-*t*-Bu), 3.33−3.38 (2H, m, CH_2NHCO), 3.44 (4H, s, NCH_2COO-*t*-Bu), 8.59 (1H, br, amide). FABMAS calcd for $C_{24}H_{43}F_3N_3O_7$ (MH^+): *m*/*z* 542, found 542. Anal. Calcd for ($C_{24}H_{42}F_3N_3O_7$) C, H, N.

Synthesis of Di-*tert*-butyl 3,6-Bis((*tert*-butoxycarbonyl)methyl)-9-(trifluoroacetyl)-3,6,9-triazaundecanedioate (7). To a suspension of 60% sodium hydride (0.122 g, 3.05 mmol) in dry DMF (13 mL) was added compound **6** (1.38 g, 2.54 mmol) in dry DMF (2 mL) to maintain the reaction temperature below −15 °C. The reaction temperature was then brought to room temperature, and stirring was continued until evolution of hydrogen ceased. The reaction solution was chilled to below 0 °C again, and *tert*-butyl bromoacetate (0.74 g, 3.81 mmol) was added dropwise while the reaction temperature was kept below 0 °C. The solution was warmed to room temperature again, and the stirring was continued overnight. Ethyl acetate (50 mL) was added to the reaction solution, and the organic phase was washed three times with a saturated aqueous solution of sodium bicarbonate. The organic phase was dried over anhydrous calcium sulfate, and the solvent was removed *in vacuo*. The oily residue was chromatographed on silica gel using ethyl acetate−hexane (1:6) as an eluent to produce compound **7** as a pale yellow oil (1.18 g, 71%). ^{1}H-NMR ($CDCl_3$): δ 1.45−1.47 (36H, s, *t*-Bu), 2.76−2.79, 2.85−2.92 (6H, m, $NCH_2CH_2NCH_2$), 3.31 (2H, s, NCH_2COO-*t*-Bu), 3.42 (4H, s, NCH_2COO-*t*-Bu), 3.55 (2H, m, CH_2NCOCF_3), 4.16 (1H, s, NCH_2COO-*t*-Bu), 4.29 (1H, d, NCH_2COO-*t*-Bu). FABMAS calcd for $C_{30}H_{53}F_3N_3O_9$ (MH^+): *m*/*z* 656, found 656. Anal. Calcd for ($C_{30}H_{52}F_3N_3O_9$) C, H, N.

Synthesis of 1-Benzyl *tert*-Butyl 3,6,9-Tris((*tert*-butoxycarbonyl)methyl)-3,6,9-triazaundecanedioate (9). Compound **7** (2.81 g, 4.28 mmol) was dissolved in *tert*-butyl alcohol (28 mL), and anhydrous hydrazine (13.5 mL, 428 mmol) was added below 0 °C. After 3.5 h of stirring at the same temperature, dichloromethane (150 mL) was added to the reaction mixture, and the organic phase was washed with a saturated aqueous solution of sodium bicarbonate. The organic layer was separated and dried over anhydrous calcium sulfate, and the solvent was removed *in vacuo*. The residue was then dissolved in dry DMF (22 mL), and *N*,*N*-diisopropylethylamine (0.78 g, 6.03 mmol) was added. After the reaction solution was chilled to below 0 °C, benzyl bromoacetate (1.38 g, 6.03 mmol) was added dropwise while the temperature was maintained below 0 °C. The solution was warmed to room temperature, and stirring was continued overnight. Ethyl acetate (150 mL) was added to the solution, and the organic phase was washed with a saturated aqueous solution of sodium bicarbonate. After the organic layer was dried over anhydrous calcium sulfate, the solvent was removed *in vacuo*. The residue was chromatographed on silica gel using ethyl acetate−hexane (1:3) as an eluent to produce compound **9** (1.59 g, 52%) as a pale yellow oil. ^{1}H-NMR ($CDCl_3$): δ 1.44 (36H, s, *t*-Bu), 2.74−2.86 (8H, m, $NCH_2CH_2NCH_2CH_2N$), 3.33 (2H, s, NCH_2-COO-*t*-Bu), 3.43 (4H, s, NCH_2COO-*t*-Bu), 3.46 (2H, s, NCH_2-COO-*t*-Bu), 3.64 (2H, s, NCH_2COOBz), 5.14 (2H, s, CH_2Ph), 7.33−7.36 (5H, m, aromatic). FABMAS calcd for $C_{37}H_{62}N_3O_{10}$ (MH^+): *m*/*z* 708, found 708. Anal. Calcd for ($C_{37}H_{61}N_3O_{10}$) C, H, N.

Synthesis of 1-*tert*-Butyl Hydrogen 3,6,9-Tris((*tert*-butoxycarbonyl)methyl)-3,6,9-triazaundecanedioic Acid (2). A mixture of compound **9** (1.57 g, 2.22 mmol) and 10%

palladium on carbon (0.1 g) in ethyl acetate (18 mL) was hydrogenated at room temperature for 6 h. The catalyst was removed by filtration, and the filtrate was evaporated *in vacuo* to provide compound **2** as a pale yellow oil (1.36 g, 98%). ^{1}H-NMR ($CDCl_3$): δ 1.45−1.47 (36H, s, *t*-Bu), 2.85−3.00 (8H, m, $NCH_2CH_2NCH_2CH_2N$), 3.44 (4H, s, NCH_2COO-*t*-Bu), 3.47 (2H, s, NCH_2COO-*t*-Bu), 3.48 (2H, s, NCH_2COO-*t*-Bu), 3.49 (2H, s, NCH_2COOH), 5.16 (1H, br, COO*H*). FABMAS calcd for $C_{30}H_{56}N_3O_{10}$ (MH^+): *m*/*z* 618, found 618. Anal. Calcd for ($C_{30}H_{55}N_3O_{10}\cdot{}^1/_2H_2O$) C, H, N.

Synthesis of *N*-(2-((*tert*-Butoxycarbonyl)amino)ethyl)-maleimide (11). To a solution of *N*-(*tert*-butoxycarbonyl)-ethylenediamine[31] (1.28 g, 8 mmol) in a saturated aqueous bicarbonate (50 mL) was added *N*-(methoxycarbonyl)maleimide[32] (1.55 g, 10 mmol) at 0 °C. After 30 min of stirring at the same temperature, the mixture was stirred for an additional 1 h at room temperature. The aqueous phase was extracted with chloroform (50 mL) three times. The combined organic phase was dried over anhydrous calcium sulfate, concentrated *in vacuo*, and chromatographed on silica gel using a mixture of chloroform−acetonitrile (20:1) as an eluent to yield compound **11** (0.9 g, 46.8%). ^{1}H-NMR ($CDCl_3$): δ 1.41 (9H, s, *t*-Bu), 3.30−3.36 (2H, m, CH_2NHBoc), 3.64−3.68 (2H, m, CH_2N-maleimide), 4.76 (1H, br, N*H*-Boc), 6.71 (2H, s, maleimide). FABMAS calcd for $C_{11}H_{17}N_2O_4$ (MH^+): *m*/*z* 241, found 241. Anal. Calcd for ($C_{11}H_{16}N_2O_4$) C, H, N.

Synthesis of *N*-(2-Aminoethyl)maleimide Salt of Trifluoroacetic Acid (12). Compound **11** (0.4 g, 1.66 mmol) was stirred in a mixed solution of trifluoroacetic acid (5 mL) and anisole (200 μL, 1.84 mmol) for 1 h at room temperature. After trifluoroacetic acid was removed *in vacuo*, the residue was treated with dry ether to produce compound **12** as a white crystal (0.33 g, 78%). ^{1}H-NMR (DMSO-d_6): δ 3.00 (2H, t, NH_2CH_2), 3.66 (2H, t, CH_2N-maleimide), 7.06 (2H, s, maleimide), 7.96 (2H, br, NH_2). FABMAS calcd for $C_6H_9N_2O_2$ (MH^+): *m*/*z* 141, found 141. Anal. Calcd for ($C_6H_8N_2O_2\cdot CF_3$-COOH) C, H, N.

Synthesis of Di-*tert*-butyl 3,6-Bis((*tert*-butoxycarbonyl)methyl)-9-(((2-maleimidoethyl)carbamoyl)methyl)-3,6,9-triazaundecanedioate (13). To a solution of compound **2** (0.5 g, 0.798 mmol) in dry acetonitrile (5 mL) was added dropwise a solution of *N*,*N*′-dicyclohexylcarbodiimide (175 mg, 0.85 mmol) in dry acetonitrile (1.5 mL) while the reaction temperature was maintained below 0 °C. After 15 min of stirring, *N*-hydroxysuccinimide (98 mg, 0.85 mmol) in dry acetonitrile (0.4 mL) was added. After 30 min of stirring below 0 °C, the reaction solution was stirred at room temperature for 5.5 h. A mixture of compound **12** (205 mg, 0.809 mmol) and *N*,*N*-diisopropylethylamine (148 μL, 0.85 mmol) in dry acetonitrile (3 mL) was then added to the solution, and the reaction mixture was stirred at room temperature overnight. After filtration of the organic salt, the filtrate was evaporated *in vacuo*, and the residue was chromatographed on silica gel using ethyl acetate as an eluent to produce compound **13** as a pale yellow oil (267 mg, 45%). ^{1}H-NMR ($CDCl_3$): δ 1.45 (36H, s, *t*-Bu), 2.69−2.83 (8H, m, $NCH_2CH_2NCH_2CH_2N$), 3.21 (2H, s, CH_2CONH), 3.30 (2H, s, NCH_2COO-*t*-Bu), 3.34 (2H, s, NCH_2COO-*t*-Bu), 3.44 (4H, s, NCH_2COO-*t*-Bu), 3.46−3.50 (2H, m, maleimide-NCH_2CH_2), 3.65−3.69 (2H, m, maleimide-NCH_2CH_2), 6.69 (2H, s, maleimide), 8.31 (1H, t, amide). FABMAS calcd for $C_{36}H_{62}N_5O_{11}$ (MH^+): *m*/*z* 740, found 740. Anal. Calcd for ($C_{36}H_{61}N_5O_{11}\cdot{}^1/_2H_2O$) C, H, N.

Synthesis of 3,6-Bis(carboxymethyl)-9-(((2-maleimidoethyl)carbamoyl)methyl)-3,6,9-triazaundecanedioic Acid (MDTPA, 1). Compound **13** (120 mg, 0.16 mmol) was stirred in a mixed solution of trifluoroacetic acid (2 mL) and anisole (80 μL, 0.736 mmol) at room temperature for 3 h. After trifluoroacetic acid was removed *in vacuo*, dry tetrahydrofuran was added to the residue. Compound **1** was obtained as a white crystal after addition of hexane to the solution (72.8 mg, 73%). ^{1}H-NMR (DMSO-d_6): δ 2.93−3.00 (4H, m, CH_2CH_2), 3.18−3.24 (8H, m, $NCH_2CH_2NCH_2CH_2N$), 3.40 (2H, s, NCH_2CONH), 3.47−3.49 (6H, m, NCH_2COOH), 3.98 (2H, s, NCH_2COOH), 6.99 (2H, s, maleimide), 8.14 (1H, t, amide). FABMAS calcd for $C_{20}H_{30}N_5O_{11}$ (MH^+): *m*/*z* 516, found 516. Anal. Calcd for ($C_{20}H_{29}N_5O_{11}\cdot{}^1/_2CF_3COOH\cdot{}^1/_2C_4H_8O\cdot H_2O$) C, H, N.

Preparation of Conjugates: MDTPA−OST7. Conjugation of MDTPA to OST7 was performed by maleimide−thiol chemistry using 2-iminothiolane (2-IT), as described previously.[19] To a solution of OST7 (10 mg/mL) in well-degassed 0.16 M borate buffer (pH 8.0) containing 2 mM ethylenediaminetetraacetic acid (EDTA) was added 45 μL of 2-IT (2 mg/mL) in the same buffer. The reaction mixture was gently agitated at room temperature for 1 h. After the unreacted reagent was removed by centrifuged gel-filtration column[22] using Sephadex G-50 (Pharmacia Biotech, Tokyo, Japan) equilibrated with 0.1 M phosphate-buffered saline (PBS, pH 6.0) containing 2 mM of EDTA, the protein concentration was adjusted to 5 mg/mL with the same buffer. MDTPA solution (92.5 μL; 8.4 mg/mL) in the same buffer was then added to the freshly thiolated solution of OST7 (1.85 mL), and the reaction mixture was stirred gently at room temperature for 2 h. The solution was further agitated gently for 30 min after addition of iodoacetamide (190 μL; 10 mg/mL) in the same buffer. The number of MDTPA groups introduced per molecule of OST7 was estimated by measuring the number of thiol groups before and after MDTPA addition using 2,2′-dithiopyridine,[33] as previously described.[19] The OST7−MDTPA conjugate was separated from unreacted small molecules using Sephadex G-50 column chromatography (1.8 × 40 cm) equilibrated and eluted with 20 mM of 2-(*N*-morpholino)ethanesulfonic acid (MES)-buffered saline (pH 6.0). The conjugate fractions collected were subsequently adjusted to 1 mg/mL with the same buffer.

EMCS-Bz-EDTA−OST7. EMCS-Bz-EDTA−OST7 conjugate was prepared according to the procedure described above using EMCS-Bz-EDTA (Dojindo Labs., Kumamoto, Japan) in place of MDTPA. The number of EMCS-Bz-EDTA groups attached per molecule of OST7 was also estimated using 2,2′-dithiopyridine, as described above.

cDTPA−OST7. Conjugation of cDTPA to OST7 was performed according to the procedure of Paik et al.[23] with slight modifications. To a solution of OST7 (400 μL; 6 mg/mL) in 0.05 M borate-buffered saline (pH 8.5) was added 20 μL of cDTPA (Dojindo Labs.) in dry dimethyl sulfoxide (5.7 mg/mL). After 30 min of gentle stirring at room temperature, a small aliquot (10 μl) was removed for ^{111}In radiolabeling to estimate the number of DTPA molecules conjugated to each OST7 molecule, according to the procedure described previously[34,35] with slight modifications. Briefly, 10 μL of acetate buffer (0.1 M, pH 4.5) was added to the reaction mixture, and 5 μL of ^{111}In acetate, prepared by method A (see ^{111}In radiolabeling section), was then added. After being left to stand at room temperature for 1 h, protein-bound radioactivity was determined by thin layer chromatography (Merck Art 5553) developed with a mixture of 10% aqueous ammonium formate−methanol−0.2 M aqueous citric acid (2:2:1). The remainder of the reaction mixture was purified by size-exclusion HPLC, followed by Sephadex G-50 column chromatography (1.8 × 40 cm) equilibrated with 20 mM of MES-buffered saline (pH 6.0). The protein concentration was adjusted to 1 mg/mL with the same buffer.

SCN-Bz-EDTA−OST7. 1-(4-Isothiocyanatobenzyl)ethylenediaminetetraacetic acid (SCN-Bz-EDTA; Dojindo Labs.) was conjugated to OST7 according to the previously described procedure.[13] Briefly, a 20 μL solution of SCN-Bz-EDTA in dry DMF (15 mg/mL) was added to a 1 mL solution of OST7 (10 mg/mL) in borate-buffered saline (0.05 M, pH 8.5) at 37 °C for 20 h. OST7−SCN-Bz-EDTA was then purified by gel permeation chromatography using Sephadex G-50 (1.8 × 40 cm), as described above. The number of SCN-Bz-EDTA molecules attached per molecule of OST7 was measured by a procedure similar to that used for OST7−cDTPA.

SDS−PAGE. Each conjugate was characterized by SDS−PAGE (Mini PROTEAN II, BioRad Co. Ltd., Richmond, CA) under reducing conditions using 4−20% gradient gels (BioRad Co. Ltd.). Each conjugate (approximately 1 μg) was denatured by dilution in an equal volume of sample buffer containing 65 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% 2-mercaptoethanol. All the samples were heated in boiling water for 3 min before being loaded onto the gel. Molecular weight marker proteins (BioRad Co. Ltd.),

which included myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase-*b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybeen trypsin inhibitor (22 kDa), were also treated in the same way.

^{111}In Radiolabeling of Conjugates. Method A. ^{111}In radiolabeling of each conjugate was performed according to the procedure of Brechbiel et al.[8] with slight modifications as described previously.[13] Briefly, 5 μL of $^{111}InCl_3$ (2 mCi (74 MBq)/mL) in 0.02 M HCl was added to a mixed solution (5 μL) of 1 M sodium acetate and 1.75 M hydrochloric acid (4:1). After 3 min, a 40 μL aliquot of conjugate or unmodified OST7 (1 mg/mL) was added, and the reaction mixture was kept at 23 °C for 1 h.

Method B. Each OST7 conjugate was also labeled with ^{111}In at higher specific activity. A solution of sodium acetate (1M, 2.25 μL) was mixed with a 30 μL solution of $^{111}InCl_3$ (2 mCi/mL) in 0.02 N HCl. After the mixture was allowed to stand for 3 min, a 10 or 20 μL aliquot of conjugate (1 mg/mL) was added, and the reaction mixture was incubated at 23 °C for 30 min.

Radiochemical yields of each ^{111}In-labeled OST7 were determined by size-exclusion HPLC and cellulose acetate electrophoresis (CAE). CAE was run at an electrostatic field of 0.8 mA/cm for 45 min in a veronal buffer (I = 0.06, pH 8.6).

To estimate nonspecific binding of ^{111}In in each ^{111}In-labeled OST7, 2 mM ethylenediaminetetraacetic acid (EDTA) in acetate buffer (0.1 M, pH 5.0) was added to each ^{111}In-labeled OST7 to a 100 M excess over each protein molecule. After incubation for 1 h at 23 °C, the reaction mixture was analyzed by CAE.

^{111}In radiolabeling of bovine apotransferrin (Nacalai Tesque, Kyoto) was performed as follows: A 40 μL solution of $^{111}InCl_3$ in 0.02 M HCl was mixed with a 40 μL solution of sodium acetate (1 M), and the resulting ^{111}In acetate solution (50 μL) was added to a protein solution (150 μL; 5 mg/mL) in PBS (0.1 M, pH 7.4). After a 10 min incubation at 37 °C, a 20 μL solution of EDTA (2 mM in PBS) was added. After a 30 min incubation at 23 °C, ^{111}In-labeled transferrin was purified by centrifuged gel-filtration column.

Serum Stability of ^{111}In-Labeled OST7s. MDTPA-, cDTPA-, and SCN-Bz-EDTA-conjugated OST7s were labeled with ^{111}In by method B and purified by size-exclusion HPLC. Each ^{111}In-labeled OST7 was added to 500 μL of freshly prepared human serum containing 0.1% of sodium azide to reach a final OST7 concentration of 20 μg/mL, and the solutions were incubated at 37 °C. After 1, 2, 3, and 5 days of incubation, a 5−10 μL aliquot of each sample was drawn, and the radioactivity was analyzed by size-exclusion HPLC. Fractions were subsequently collected, and the radioactivity was determined using a well counter (ARC 2000, Aloka, Tokyo, Japan). Each value was calculated by dividing the antibody-bound radioactivity at various intervals by the radiochemical purities of freshly purified respective preparations. Data are expressed as means and sd of three experiments.

Statistical Analysis. Data are expressed as means ± standard deviation where appropriate. Results were analyzed for statistical significant using Student's unpaired *t*-test. Differences were considered statistically significant when the *p* value was less than 0.05.

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