Bioorganic & Medicinal Chemistry 20 (2012) 978–984

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Facile synthesis and evaluation of *C*-functionalized benzyl-1-oxa-4,7,10-triazacyclododecane-*N*,*N*′,*N*″-triacetic acid as chelating agent for ^{111}In-labeled polypeptides

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ARTICLE INFO

Article history:
Received 11 October 2011
Revised 19 November 2011
Accepted 21 November 2011
Available online 1 December 2011

Keywords:
C-ODTA
C-DOTA
Cyclization
Polyazamacrocycle
^{111}In

ABSTRACT

A 12-membered polyazamacrocycle, 1-oxa-4,7,10-triazacyclododecane-*N*,*N*′,*N*″-triacetic acid (ODTA), has been reported to provide an indium chelate of net neutral charge with thermodynamic stability higher than 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA). However, neither synthetic procedure for a *C*-functionalized ODTA (*C*-ODTA) nor its chelating ability with a trace amount of radioactive indium-111 (^{111}In) has been elucidated. We herein present a facile synthetic procedure for *C*-ODTA, and estimated its ability as a chelating agent for radiolabeling peptides and proteins with ^{111}In. The synthetic procedure involves the synthesis of a linear precursor using a *para*-substituted phenylalanine derivative as a starting material. The following intramolecular cyclization reaction was best performed (>73% yield) when Boc-protected linear compound and the condensation reagent, HATU, were simultaneously added to the reaction vessel at the same flow rate. The cyclic compound was then reduced with BH_3 and alkylated with *tert*-butyl bromoacetate. The synthetic procedure was straightforward and some optimization would be required. However, most of the intermediate compounds were obtained easily in good yields, suggesting that the present synthetic procedure would be useful to synthesize *C*-ODTA derivatives. The intramolecular cyclization reaction might also be applicable to synthesize polyazamacrocycles of different ring sizes and cyclic peptides. In ^{111}In radiolabeling reactions, *C*-ODTA provided ^{111}In chelates in higher radiochemical yields at low ligand concentrations when compared with *C*-DOTA. The ^{111}In-labeled *C*-ODTA remained unchanged in the presence of apo-transferrin. The biodistribution studies also showed that the ^{111}In-labeled compound was mainly excreted into urine as intact. These findings indicate that *C*-ODTA would be useful to prepare ^{111}In-labeled peptides of high specific activities in high radiochemical yields.

1. Introduction

Monoclonal antibodies and peptides have been employed as a targeting molecule for the delivery of radionuclides to tumor cells in molecular imaging and targeted radionuclide therapy. Since antibodies and peptides do not form stable chelates with metallic radionuclides, a variety of bifunctional chelating agents have been developed to apply metallic radionuclides of appropriate nuclear properties to the purposes. Among them, a 12-membered polyazamacrocycle, 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid (DOTA; Fig. 1) has been used as a chelating agent for labeling polypeptides with indium-111 (^{111}In) for SPECT imaging,[1] yttrium-90 or lutetium-177 for targeted radionuclide therapy,[2,3] and europium for fluorescent imaging.[4,5] Two major DOTA derivatives have been developed to introduce DOTA molecules to polypeptides. One is a *N*-functionalized DOTA where one of the four carboxylic acids

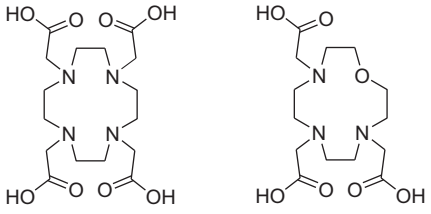

Figure 1. Structures of DOTA (left) and ODTA (right).

in DOTA is converted to an active ester.[6–8] The other involves *C*-functionalized DOTA (*C*-DOTA) where a polypeptide binding site is introduced at a benzyl group extended from the backbone carbon of DOTA.[9–11] However, both DOTA derivatives require high concentration of DOTA-conjugated polypeptides, elevated temperature or long reaction time to provide ^{111}In-labeled polypeptides in high radiochemical yields, due to the extremely slow complex formation rates of the chelating agent. Efforts have been

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doi:10.1016/j.bmc.2011.11.046

made to develop a chelating agent that provides kinetically inert radiometal chelates in high radiochemical yields.[11–14] Although DTPA derivatives provide ^{111}In chelate of high in vivo stability in higher radiochemical yields than DOTA derivatives, ^{111}In-labeled polypeptides exhibit long residence time of radioactivity in the liver (for intact antibodies) or kidney (for polypeptides smaller than albumin) when they are used as bifunctional chelating agents.[15,16]

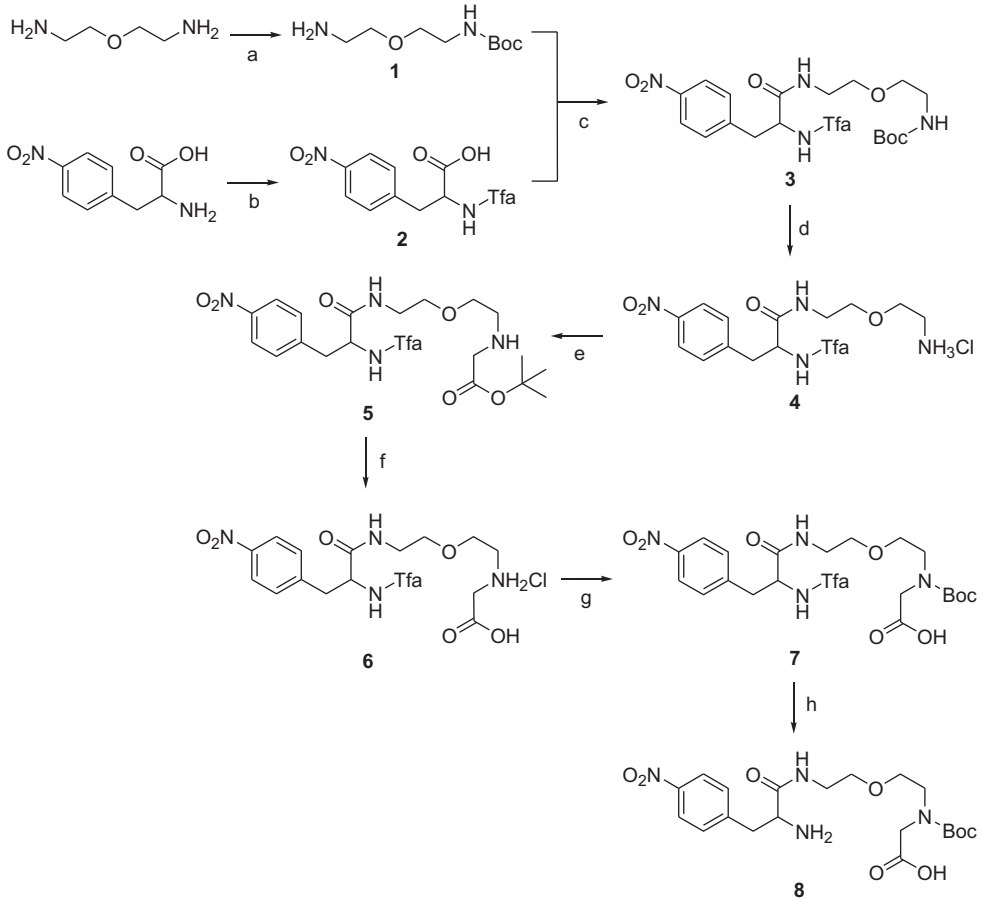
Contrary to DOTA and DTPA, a 12-membered polyazamacrocycle, 1-oxa-4,7,10-triazacyclododecane-*N*,*N*′,*N*″-triacetic acid (ODTA; Fig. 1), provides an indium chelate of net neutral charge similar to a ^{67}Ga chelate of *C*-functionalized 1,4,7-triazacyclononane-*N*,*N*′,*N*″-triacetic acid (NOTA) with thermodynamic stability higher than that of In-DOTA.[17] Such characteristics of ODTA were considered useful to reduce the non-target radioactivity levels when recalling short residence time of ^{67}Ga-NOTA-conjugated methionine from the kidney.[9] However, neither the synthetic procedure for *C*-functionalized ODTA (*C*-ODTA) nor its complexation ability with a trace amount of ^{111}In has been elucidated, which stimulated us to investigate the synthetic procedure of *C*-ODTA and its complexation ability with ^{111}In.

2. Results and discussion

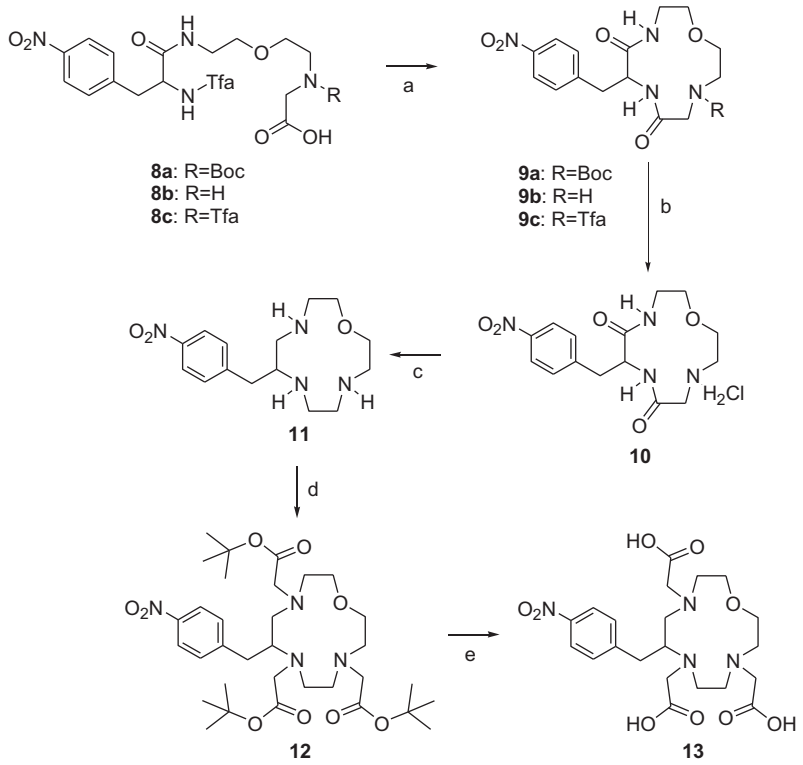
2.1. Synthesis of *C*-ODTA

Among a variety of cyclization methods so far reported,[10,18–20] we employed an intramolecular cyclization procedure, as shown in Schemes 1 and 2. The condensation reaction between *N*-Boc-2,2′-oxybis(ethylamine) **1** and *N*-Tfa-*p*-nitrophenylalanine **2** provided **3** in high yields. The intermediate compound **5** was obtained by deprotection of the Boc group of **3** and subsequent alkylation of the resulting **4** with *tert*-butyl bromoacetate, since our attempts to prepare **5** with *tert*-butyl bromomalonate resulted in lower yields. In the cyclization reaction, we observed two key parameters that significantly affected the synthetic yields. When the cyclization reaction was conducted by an addition of a DMF solution of HATU to a mixed solution of **8a**, DIEA and HOAt in DMF, many side-products impaired the yields. However, when a DMF solution of **8a** and a DMF solution of HATU were simultaneously added to a mixed solution of DIEA and HOAt in DMF, the reduction of side-products gave improved yields in the cyclization reaction (>73%). This would be attributable to rapid reaction rates of HATU-activated **8a** and rapid degradation rates of HATU in the presence of excess base.[21,22] The protecting group of the secondary amine **8** also affected the reaction yields. The best yield was achieved when the amine was protected with Boc group. The linear compound without a protecting group **8b** or with trifluoroacetyl protecting group **8c** resulted in lower cyclization yields (data not shown). These results suggest that the presence of a protecting group with an appropriate molecular size would render the two reaction groups closer each other.

Following deprotection and subsequent reduction of the two amide bonds in **9a** with BH_3-THF, **11** was obtained in 26% yield. When the reduction products were treated with aqueous HCl, the ester bond was readily cleaved, which was contrary to the *C*-DOTA derivatives so far reported. The use of methanol–HCl solution provided **11** at an expense of low synthetic yields. The *C*-ODTA derivative **12** was then obtained in 43% yield by the reaction of **11** with *tert*-butyl bromoacetate. The present synthetic procedure was



Scheme 1. Synthetic procedure for the linear precursor of *C*-ODTA. Reagents and conditions: (a) $(Boc)_2O$, methanol, THF, 0 °C for 1 h, rt for 24 h, 89%; (b) $(Tfa)_2O$, TFA, rt, 15 h, 82%; (c) IBCF, NMM, THF, −15 °C for 30 min, rt for 1 h, 81%; (d) 4 M HCl/ethyl acetate, rt, 1 h, 98%; (e) *tert*-butyl bromoacetate, TEA, DMF, 0 °C to rt, 24 h, 53%; (f) 4 M HCl/ethyl acetate, rt, 3 h, 93%; (g) $(Boc)_2O$, TEA, DMF, 0 °C to rt, 3 h, 79%; (h) 25% NH_3 aq, rt, 6 h.



Scheme 2. Synthetic procedure for *C*-ODTA. Reagents and conditions: (a) HATU, HOAt, DIEA, DMF, rt, 20 h, **9a**, 74%, **9b**, N.D., **9c**, 48%; (b) 4 M HCl/ethyl acetate, rt, 3 h, 85%; (c) (1) 1 M BH_3-THF, 0 °C for 1 h, reflux for 24 h, (2) HCl/methanol, reflux, 6 h, 26%; (d) *tert*-butyl bromoacetate, Na_2CO_3, acetonitrile, 0 °C to rt, 24 h, 43%; (e) 10% anisole/TFA, 6 h, rt, 42%.

straightforward and some optimization would be required. However, most of the intermediates were obtained in good yields, suggesting that this procedure would be useful to prepare *C*-ODTA derivatives. The cyclization reaction might be applicable to a synthesis of *C*-functionalized polyazamacrocycles of different ring sizes or cyclic peptides.

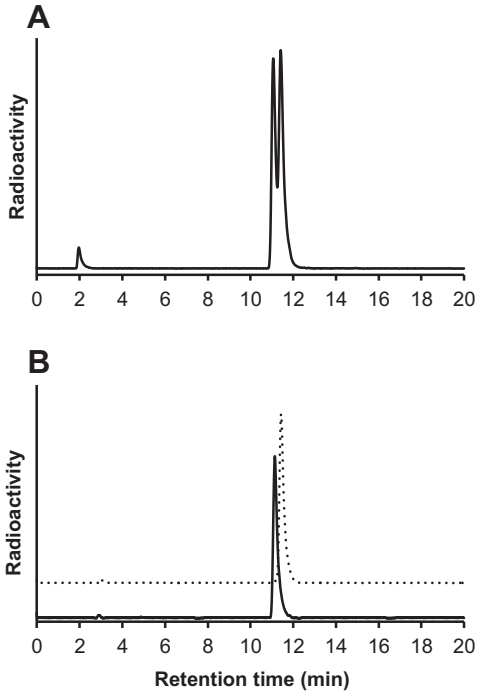


Figure 2. HPLC profiles of ^{111}In-*C*-ODTA (A) and urine samples collected for 6 h postinjection of ^{111}In-*C*-ODTA (B). ^{111}In-*C*-ODTA exhibited two radioactivity peaks at retention times of 11.1 min (complex A) and 11.4 min (complex B). When each complex was separated by HPLC and injected to mice, both complex A and complex B were excreted in the urine intact.

2.2. Radiolabeling of *C*-ODTA and *C*-DOTA with ^{111}In

C-ODTA provided two radioactive ^{111}In peaks at retention times of 11.1 (complex A) and 11.4 min (complex B), as shown in Figure 2. Both complexes remained unchanged when the radioactive peaks were isolated and re-analyzed by HPLC (data not shown). Similar results were observed by the reaction of *C*-ODTA with a stoichiometrically equivalent amount of non-radioactive indium. Both complexes exhibited similar mass spectra, suggesting that both are stereoisomers as also observed with Y^{3+} and Cu^{2+} chelates of *C*-DOTA.[23,24]

Table 1 shows the radiochemical yields of ^{111}In-*C*-ODTA and ^{111}In-*C*-DOTA when the reaction was performed in 1 or 10 μM solution of *C*-DOTA or *C*-ODTA with $^{111}InCl_3$ at 37 or 100 °C. The radiochemical yields of ^{111}In-*C*-ODTA were determined by combining the two complexes. As expected from higher stability constant of ODTA than DOTA for indium,[17] *C*-ODTA provided ^{111}In complexes in much higher radiochemical yield (>95%) than did *C*-DOTA

Table 1
Radiochemical yields of *C*-ODTA and *C*-DOTA

	Radiochemical yields (%)			
	37 °C		100 °C	
	C-ODTA	*C*-DOTA	*C*-ODTA	*C*-DOTA
1 μM	N.D.	N.D.	96.4 ± 1.60	22.8 ± 7.99
10 μM	57.5 ± 0.73	75.9 ± 2.48	N.D.	96.1 ± 0.46

N.D.: not determined.
Results are expressed as means (SD) of five experiments each point.

Table 2
Stability of ^{111}In-*C*-ODTA and ^{111}In-*C*-DOTA against apo-transferrin (aTf)

Time (h)	Percent of intact radiolabeled complex			
	aTf (0.1 mM)		aTf (1.0 mM)	
	C-ODTA	*C*-DOTA	*C*-ODTA	*C*-DOTA
1	98.3 ± 0.70	98.4 ± 0.59	98.3 ± 0.24	98.5 ± 0.22
6	98.5 ± 0.49	98.2 ± 0.32	98.7 ± 0.29	98.0 ± 0.19
24	98.4 ± 0.19	98.1 ± 0.14	98.1 ± 0.42	98.0 ± 0.25
72	98.0 ± 0.25	98.0 ± 0.21	98.8 ± 0.36	97.3 ± 0.19
120	97.4 ± 1.05	97.8 ± 0.20	97.8 ± 0.43	95.7 ± 0.81

Results are expressed as means (SD) of five experiments each point.

(<25%) when the reaction was conducted in 1 μM at 100 °C. However, both *C*-DOTA and *C*-ODTA showed poor radiochemical yields under milder (37 °C) reaction conditions, suggesting that while *C*-ODTA would possess complexation rate with ^{111}In superior to *C*-DOTA, *C*-ODTA still requires harsh conditions not applicable to heat-sensitive proteins.

2.3. Stability of ^{111}In-*C*-ODTA and ^{111}In-*C*-DOTA in apo-transferrin solution

The stability of ^{111}In-*C*-ODTA and ^{111}In-*C*-DOTA were compared in the presence of apo-transferrin (aTf) after removing free *C*-ODTA or *C*-DOTA from the ^{111}In complexes. As shown in Table 2, both ^{111}In-*C*-ODTA and ^{111}In-*C*-DOTA remained intact (>95%) during the experimental intervals, indicating that *C*-ODTA provides ^{111}In chelate stable enough for in vivo applications.

2.4. Biodistribution and metabolic studies

The two ^{111}In-*C*-ODTA complexes were separated by HPLC, and each complex was injected to normal mice. Both ^{111}In-*C*-ODTA complex A (retention time of 11.1 min) and B (retention time of 11.4 min) exhibited rapid elimination rates from the blood with low radioactivity levels in any organs (Table 3). The majority of the radioactivity was excreted in the urine (Table 3), indicating their rapid urinary excretion. However, a small difference was observed in the liver uptake; ^{111}In-*C*-ODTA complex B registered a slightly higher initial radioactivity levels in the liver than those of ^{111}In-*C*-ODTA complex A. This would be attributable to the different stereostructures of the two ^{111}In-*C*-ODTA, as suggested from prior studies[23,24] and from their retention times on HPLC.

Table 3
Biodistribution of radioactivity after intravenous injection of ^{111}In-*C*-ODTA[a]

	Time after injection		
	1 h	6 h	24 h
		Complex A	
Blood	0.12 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
Liver	0.22 ± 0.03	0.09 ± 0.02	0.04 ± 0.01
Spleen	0.06 ± 0.01	0.02 ± 0.01	0.02 ± 0.02
Kidney	3.01 ± 1.36	0.80 ± 0.09	0.30 ± 0.04
Pancreas	0.05 ± 0.02	0.01 ± 0.01	0.01 ± 0.01
Heart	0.07 ± 0.02	0.01 ± 0.01	0.01 ± 0.01
Lung	0.17 ± 0.02	0.02 ± 0.01	0.01 ± 0.01
Bone	0.12 ± 0.09	0.03 ± 0.02	0.02 ± 0.02
Stomach[b]	0.03 ± 0.01	0.13 ± 0.12	0.29 ± 0.24
Intestine[b]	1.07 ± 0.09	0.90 ± 0.57	2.09 ± 1.43
Urine[b]			86.4 ± 2.29
Feces[b]			1.71 ± 1.00
		Complex B	
Blood	0.15 ± 0.10	0.00 ± 0.00	0.00 ± 0.00
Liver	1.42 ± 0.23	0.60 ± 0.12	0.28 ± 0.08
Spleen	0.08 ± 0.03	0.07 ± 0.02	0.02 ± 0.02
Kidney	4.11 ± 1.60	1.33 ± 0.22	0.32 ± 0.15
Pancreas	0.05 ± 0.03	0.02 ± 0.01	0.01 ± 0.01
Heart	0.07 ± 0.03	0.01 ± 0.01	0.01 ± 0.01
Lung	0.21 ± 0.08	0.03 ± 0.01	0.02 ± 0.01
Bone	0.15 ± 0.07	0.02 ± 0.01	0.01 ± 0.01
Stomach[b]	0.09 ± 0.05	0.29 ± 0.35	0.42 ± 0.26
Intestine[b]	2.40 ± 0.54	2.76 ± 1.53	1.59 ± 0.64
Urine[b]			83.0 ± 6.25
Feces[b]			3.76 ± 1.93

Results are expressed as means (SD) of five animals each point.
[a] Tissue radioactivity is expressed as percent of injected dose per gram of wet tissue.
[b] Expressed as percent of injected dose per tissue.

The analysis of urine samples collected for 6 h postinjection of each ^{111}In-*C*-ODTA exhibited a single HPLC profile at a retention time identical to each ^{111}In-*C*-ODTA (Fig. 2B). This reinforced high in vivo stability of ^{111}In-*C*-ODTA under physiological environment.

3. Conclusion

A facile synthetic procedure for *C*-ODTA has been established. Since the majority of synthetic intermediates were obtained in high yields and purities, the present procedure may be useful to a large-scale synthesis of *C*-ODTA. The cyclization reaction may also be applicable to prepare polyazamacrocycles of other ring sizes and cyclic peptides. *C*-ODTA provided two net neutral ^{111}In complexes in higher radiochemical yields than *C*-DOTA at an elevated temperature. The stability of ^{111}In-*C*-ODTA was comparable to that of ^{111}In-*C*-DOTA. The findings in this study indicate that *C*-ODTA possesses an advantage over both DO3A and *C*-DOTA in preparing ^{111}In-labeled peptides of high specific activities without post-labeling purification for molecular imaging. The net neutral charge of ^{111}In-*C*-ODTA may also be useful to decrease the radioactivity levels in non-target tissues.

4. Experimental

4.1. General

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-ALPHA 400 spectrometer (JEOL Ltd, Tokyo, Japan). Fast atom bombardment mass spectra (FAB-MS) were taken on a JEOL JMS-AX500 mass spectrometer (JEOL Ltd, Tokyo). Electrospray ionization mass spectrometry (ESI-MS) was carried out using an Agilent 6130 Series Quadrupole LC/MS electrospray system equipped with diode array detector (Agilent technologies, Tokyo). Elemental analyses were performed by PE-2400 (Perkin-Elmer Japan, Tokyo). The melting points were measured using a Thomas Hoover capillary melting point apparatus and were reported uncorrected. HPLC was performed with a Unison US-C18 column (4.6 × 150 mm, Imtakt, Kyoto, Japan) at flow rate of 1 mL/min with a gradient mobile phase starting from 100% A (0.1% aqueous trifluoroacetic acid (TFA)) and 0% B (methanol with 0.1% TFA) to 70% A and 30% B at 20 min. The radiochemical yields were also determined by TLC (Merck Silica gel 60) developed with a mixture of methanol and water (3:7). $^{111}InCl_3$ (74 MBq/mL) was purchased by Nihon Medi-Physics Co. Ltd (Tokyo). Chemicals purchased from commercial sources were of reagent grade or higher and used without further purification.

4.2. *N*-(*tert*-Butyloxycarbonyl)-2,2′-oxybis(ethylamine) (1)

This compound was synthesized according to the procedure described previously with slight modifications.[25] To a cooled (0 °C) solution of 2,2′-oxybis(ethylamine) (10.0 mL, 94.1 mmol) in methanol (1000 mL) was added dropwise a solution of di-*tert*-butyl dicarbonate ($(Boc)_2O$; 10.3 g, 47.0 mmol) in THF (300 mL), and

the mixture was stirred for 1 h at the same temperature and additional 24 h at room temperature. After removing the solvent in vacuo, the residue was dissolved in 1 N NaOH (200 mL), and extracted with chloroform (300 mL × 3). The organic phases were combined, dried over anhydrous $MgSO_4$. The solvent was removed in vacuo to provide **1** as a colorless oil (6.88 g, 71.5%). ^{1}H NMR ($CDCl_3$): δ 1.39 (9H, s, Boc), 2.79–2.82 (2H, t, CH_2), 3.24–3.28 (2H, dd, CH_2), 3.41–3.43 (2H, t, CH_2), 3.44–3.47 (2H, t, CH_2), 5.00 (1H, d, NH). ESI-MS $(M+H)^+$: *m*/*z* 205, found: 205.

4.3. *N*-Trifluoroacetyl-4-nitrophenylalanine (2)

To a solution of 4-nitro-L-phenylalanine (5.26 g, 25.0 mmol) in TFA (50 mL) was added dropwise a mixture of trifluoroacetic anhydride ($(Tfa)_2O$; 8.9 mL, 63.0 mmol) and TFA (20 mL) for 2 h with stirring at 60 °C. The mixture was stirred for additional 15 h at room temperature. The solvent was removed in vacuo, and the residue was azeotroped with toluene in vacuo. The residue was dissolved in 5% $NaHCO_3$ (100 mL), and washed with chloroform (50 mL × 3). The aqueous phase was then acidified with 10% citric acid (100 mL), and extracted with ethyl acetate (300 mL × 3). The organic phases were combined, dried over anhydrous $MgSO_4$. After removing the solvent in vacuo, the resulting pale yellow solid was collected, washed with hexane and dried in vacuo to provide **2** (6.27 g, 81.7%) melting at 139–140 °C. ^{1}H NMR ($CDCl_3$): δ 3.27–3.31, 3.42–3.48 (2H, dq, C*H*$_2$), 4.93–4.96 (1H, dd, C*H*), 6.77–6.78 (1H, d, N*H*), 7.30–7.33 (2H, d, *aromatic*), 8.17–8.19 (2H, d, *aromatic*). ESI-MS $(M-H)^-$: *m*/*z* 305, found: 305. Anal. Calcd for $C_{11}H_9N_2O_5F_3{\cdot}0.2H_2O$: C, 42.65; H, 3.06; N, 9.04. Found: C, 42.57; H, 2.89; N, 8.80.

4.4. 1-(*tert*-Butyloxycarbonyl)-10-trifluoroacetyl-9-(4-nitrobenzyl)-8-oxo-1,7,10-triaza-4-oxadecane (3)

To a chilled solution (−15 °C) of **2** (1.41 g, 4.61 mmol) in THF (20 mL) was added *N*-methylmorpholine (NMM; 0.51 mL, 4.61 mmol) and subsequently isobutylchloroformate (IBCF; 0.61 mL, 4.61 mmol) under N_2 atmosphere. After 5 min, a solution of **1** (0.857 g, 4.19 mmol) in THF (10 mL) was added dropwise at the same temperature, and the mixture was stirred for 30 min at the same temperature and then at room temperature for 1 h. After removing the solvent in vacuo, the residue was dissolved in ethyl acetate (50 mL). The organic phase was washed with 5% $NaHCO_3$ (30 mL × 2), 5% citric acid (30 mL × 3), saturated NaCl solution (30 mL × 1), successively and dried over anhydrous $MgSO_4$. After removal of the solvent in vacuo, the residue was subjected to flash chromatography on silica gel using a mixture of chloroform/methanol (100:1) as an eluent to provide **3** as a pale yellow solid (1.67 g, 80.6%) melting at 128–129 °C. ^{1}H NMR ($CDCl_3$): δ 1.44 (9H, s, *Boc*), 3.17–3.51 (10H, overlapped, C*H*$_2$), 4.76–4.78 (1H, dd, C*H*), 6.89 (1H, d, N*H*), 7.35–7.37 (2H, d, *aromatic*), 8.14–8.16 (2H, d, *aromatic*). ESI-MS $(M+Na)^+$: *m*/*z* 515, found: 515. Anal. Calcd for $C_{20}H_{27}N_4O_7F_3$: C, 48.78; H, 5.53; N, 11.38. Found: C, 48.46; H, 5.30; N, 11.21.

4.5. 10-Trifluoroacetyl-9-(4-nitrobenzyl)-8-oxo-1,7,10-triaza-4-oxadecane hydrochloride (4)

Compound **3** (1.52 g, 3.09 mmol) was dissolved in 4 M HCl/ethyl acetate (15 mL), and the mixture was stirred for 1 h at room temperature. After removing the solvent in vacuo, the resulting white solid was collected and washed with diethyl ether, and then dried in vacuo to provide **4** as a white solid (1.30 g, 98.4%) melting at 211–212 °C. ^{1}H NMR (D_2O): δ 2.96–2.99 (2H, t, C*H*$_2$), 3.07–3.36 (6H, overlapped, C*H*$_2$), 3.47–3.49 (2H, t, C*H*$_2$), 4.51–4.55 (1H, t, C*H*), 7.31–7.34 (2H, d, *aromatic*), 8.04–8.06 (2H, d, *aromatic*). ESI-MS $(M+H)^+$: *m*/*z* 393, found: 393. Anal. Calcd for $C_{15}H_{20}N_4O_5F_3{\cdot}1.4HCl$: C, 40.63; H, 4.64; N, 12.64. Found: C, 40.80; H, 4.52; N, 12.62.

4.6. *tert*-Butyl 12-Trifluoroacetyl-11-(4-nitrobenzyl)-10-oxo-3,9,12-triaza-6-oxadodecanate (5)

To a chilled (0 °C) suspension of **4** (5.63 g, 13.1 mmol) and triethylamine (TEA; 2.75 mL, 19.7 mmol) in DMF (30 mL) was added dropwise *tert*-butyl bromoacetate (1.93 mL, 13.1 mmol). After the addition, the mixture was stirred for 24 h at room temperature. The solvent was removed in vacuo, and the residue was dissolved in ethyl acetate (50 mL). The organic phase was washed with 5% $NaHCO_3$ (20 mL) and saturated NaCl solution (5 mL) successively, and dried over anhydrous Na_2SO_4. After removing the solvent in vacuo, the residue was subjected to column chromatography on silica gel using a mixture of chloroform/methanol (60:1) as an eluent to provide **5** as a pale yellow solid (3.53 g, 53.1%) melting at 55–58 °C. ^{1}H NMR ($CDCl_3$): δ 1.42 (9H, s, ^{t}*Bu*), 2.85–2.88 (2H, m, C*H*$_2$), 3.13–3.18, 3.30–3.36 (2H, dq, C*H*$_2$), 3.41–3.58 (8H, overlapped, C*H*$_2$), 4.85–4.87 (1H, dd, C*H*), 7.38–7.40 (2H, d, *aromatic*), 7.55 (1H, s, N*H*), 8.12–8.14 (2H, d, *aromatic*). ESI-MS $(M+H)^+$: *m*/*z* 507, found: 507. Anal. Calcd for $C_{21}H_{29}N_4O_7F_3{\cdot}H_2O$: C, 48.09; H, 5.96; N, 10.68. Found: C, 48.06; H, 5.74; N, 10.44.

4.7. 12-Trifluoroacetyl-11-(4-nitrobenzyl)-10-oxo-3,9,12-triaza-6-oxadodecanoic acid hydrochloride (6)

Compound **5** (3.43 g, 6.77 mmol) was dissolved in 4 M HCl/ethyl acetate (30 mL), and the mixture was stirred for 3 h. After removing the solvent in vacuo, the resulting white solid was collected, and washed with diethyl ether, and then dried in vacuo to provide **6** as a white solid (3.07 g, 93.0%) melting at 188–189 °C. ^{1}H NMR (D_2O): δ 3.03–3.36 (10H, overlapped, C*H*$_2$), 3.51–3.55 (2H, m, C*H*$_2$), 4.51–4.55 (1H, t, C*H*), 7.31–7.33 (2H, d, *aromatic*), 8.03–8.06 (2H, d, *aromatic*). ESI-MS $(M-H)^-$: *m*/*z* 449, found: 449. Anal. Calcd for $C_{17}H_{21}N_4O_7F_3{\cdot}HCl$: C, 41.94; H, 4.55; N, 11.51. Found: C, 41.88; H, 4.58; N, 11.28.

4.8. 3-(*tert*-Butyloxycarbonyl)-12-trifluoroacetyl-11-(4-nitrobenzyl)-10-oxo-3,9,12-triaza-6-oxadodecanoic acid (7)

To a chilled (0 °C) solution of **6** (3.07 g, 6.31 mmol) and TEA (1.32 mL, 9.46 mmol) in DMF (20 mL) was added dropwise a solution of $(Boc)_2O$ (1.65 g, 7.56 mmol) in DMF (10 mL). After the addition, the mixture was stirred for 3 h at room temperature. The solvent was removed in vacuo, and the residue was dissolved in ethyl acetate (40 mL). The organic phase was washed with 5% citric acid (20 mL × 3), and dried over anhydrous $MgSO_4$. After removing the solvent in vacuo, the residue was subjected to column chromatography on silica gel using a mixture of chloroform/methanol (20:1) as an eluent to provide **7** as a pale yellow solid (2.73 g, 78.6%) melting at 70–71 °C. ^{1}H NMR ($CDCl_3$): δ 1.45 (9H, s, *Boc*), 3.11–3.73 (10H, overlapped, C*H*$_2$), 3.81–3.87, 4.09–4.16 (2H, dq, C*H*$_2$), 5.06–5.18 (1H, m, C*H*), 7.39–7.42 (2H, d, *aromatic*), 7.69–7.71, 7.91–7.93 (1H, dd, N*H*), 8.13–8.15 (2H, d, *aromatic*). ESI-MS $(M+Na)^+$: *m*/*z* 573, found: 573. Anal. Calcd for $C_{22}H_{29}N_4O_9F_3{\cdot}0.2H_2O$: C, 47.69; H, 5.35; N, 10.11. Found: C, 47.79; H, 5.43; N, 9.74.

4.9. 10-(*tert*-Butyloxycarbonyl)-5,8-dioxo-6-(4-nitrobenzyl)-1-oxa-4,7,10-triaza-cyclododecane (9a)

Compound **7** (2.12 g, 3.85 mmol) was dissolved in 25% NH_3 aqueous solution (20 mL). After the mixture was stirred for 6 h at room temperature, the solvent was removed in vacuo. The residue (**8a**) was dissolved in DMF (35 mL) to prepare solution 'A'. *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU; 2.20 g, 5.78 mmol) was dissolved in DMF (35 mL) to prepare solution 'B'. Both the solution 'A' and 'B' were loaded into gas-tight syringes, and the two syringes were locked

onto syringe pump. To a mixed solution of *N*,*N*-diisopropylethylamine (DIEA; 2.68 mL, 15.4 mmol) and HOAt (786 mg, 5.78 mmol) in DMF (1.5 L) was added each solution 'A' and 'B' simultaneously at a flow rate of 1.2 mL/h with stirring. After the addition, the mixture was stirred for another 20 h at room temperature. After removing the solution in vacuo, the residue was dissolved in ethyl acetate (100 mL) and washed three times with a mixture of 5% $NaHCO_3$ (40 mL) and saturated NaCl solution (10 mL), and dried over anhydrous $MgSO_4$. The solvent was removed in vacuo, and the residue was subjected to column chromatography on silica gel using a mixture of chloroform/methanol (20:1) as an eluent to provide **9a** as a white solid (1.24 g, 73.5%) melting at 102–103 °C. ^{1}H NMR ($CDCl_3$): δ 1.41 (9H, s, *Boc*), 2.98–3.65 (10H, overlapped, C*H*$_2$), 3.89–3.94 (1H, m, C*H*$_2$), 4.39–4.43 (1H, d, C*H*$_2$), 4.57–4.63 (1H, dd, C*H*), 6.61 (1H, m, N*H*), 7.41–7.43 (2H, d, *aromatic*), 7.73–7.75 (1H, d, N*H*), 8.14–8.16 (2H, d, *aromatic*). ESI-MS $(M+Na)^+$: *m*/*z* 459, found: 459. Anal. Calcd for $C_{20}H_{28}N_4O$: C, 55.04; H, 6.47; N, 12.84. Found: C, 54.71; H, 6.56; N, 12.50.

4.10. 5,8-Dioxo-6-(4-nitrobenzyl)-1-oxa-4,7,10-triazacyclododecane (10)

Compound **9a** (1.23 g, 2.83 mmol) was dissolved in 4 M HCl/ethyl acetate (10 mL), and the solution was stirred for 3 h at room temperature before the solvent was removed in vacuo. The resulting white solid was dissolved in water (10 mL), and 1 N NaOH was added to bring the pH of the solution to 11. The aqueous solution was extracted with ethyl acetate (80 mL × 3), and the combined organic phases were dried over anhydrous $MgSO_4$. After removing the solvent in vacuo, the resulting white solid was collected and washed with diethyl ether, and then dried in vacuo to provide **10** (899 mg, 85.2%) melting at 231–232 °C. ^{1}H NMR (DMSO): δ 2.58 (2H, s, *CH*$_2$), 2.69–2.73 (1H, d, *CH*$_2$), 2.95–3.01 (1H, dd, *CH*$_2$), 3.07–3.31 (6H, overlapped, C*H*$_2$), 3.33–3.53 (2H, m, C*H*$_2$), 4.35–4.40 (1H, m, C*H*), 7.50–7.52 (2H, d, *aromatic*), 7.65–7.68 (1H, dd, N*H*), 8.13–8.15 (2H, d, *aromatic*), 8.32–8.34 (1H, d, N*H*). ESI-MS $(M+H)^+$: *m*/*z* 337, found: 337. Anal. Calcd for $C_{15}H_{20}N_4O_5F_3{\cdot}0.25H_2O$: C, 52.86; H, 6.06; N, 16.44. Found: C, 53.25; H, 6.08; N, 16.05.

4.11. 6-(4-Nitrobenzyl)-1-oxa-4,7,10-triazacyclododecane (11)

To a chilled (0 °C) suspension of **10** (870 mg, 2.33 mmol) in THF (10 mL) was added BH_3-THF (1 M, 29.5 mL, 29.5 mmol) under N_2 atmosphere. The mixture was stirred for 1 h at 0 °C, and then heated to reflux for 24 h. The solution was cooled to 0 °C, quenched with methanol (15 mL) for 1 h, and then evaporated to dryness. The residue was dissolved in methanol (30 mL) again, and the solvent was evaporated. The residue was dissolved in methanol saturated with HCl gas (5 mL). The solution was refluxed for 6 h. After the solution was cooled to 0 °C, 1 N NaOH (30 mL) was added, and extracted with chloroform (30 mL × 3). The combined organic phases were dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo before the residue was subjected to flash chromatography on silica gel using a mixture of chloroform/methanol/25% NH_3 aqueous solution (12:4:1) as an eluent to provide **11** as a brown oil (184 mg, 25.5%). ^{1}H NMR ($CDCl_3$): δ 2.39–3.02 (11H, overlapped, C*H*, C*H*$_2$), 3.46–3.52 (2H, m, C*H*$_2$), 3.56–3.63 (2H, overlapped, C*H*$_2$), 7.31–7.33 (2H, d, *aromatic*), 8.11–8.13 (2H, d, *aromatic*).

4.12. Tris(*tert*-butyl) 6-(4-nitrobenzyl)-1-oxa-4,7,10-triazacyclododecane-*N*,*N*′,*N*″-triacetate (12)

To a chilled suspension (0 °C) of **11** (184 mg, 0.595 mmol) and Na_2CO_3 (189 mg, 1.79 mmol) in acetonitrile (5 mL) was added *tert*-butyl bromoacetate (262 μL, 1.79 mmol) under N_2 atmosphere. After the addition, the mixture was stirred for 24 h at room temperature under the same conditions. The reaction mixture was filtered, and the filtrate was evaporated in vacuo. The residue was dissolved in a mixture of saturated $NaHCO_3$ solution (10 mL) and saturated NaCl solution (4 mL), and extracted with chloroform (20 mL × 3). The combined organic phases were dried over anhydrous $MgSO_4$, and the solvent was removed in vacuo. The residue was subjected to flash chromatography on silica gel using a mixture of chloroform/methanol/25% NH_3 aqueous solution (120:10:1) as an eluent to provide **12** as a red-brown oil (165 mg, 42.5%). ^{1}H NMR ($CDCl_3$): δ 1.36 (9H, s, ^{t}*Bu*), 1.40 (9H, s, ^{t}*Bu*), 1.44 (9H, s, ^{t}*Bu*), 2.27–3.59 (23H, overlapped, C*H*, C*H*$_2$), 7.42–7.44 (2H, d, *aromatic*), 8.08–8.10 (2H, d, *aromatic*). ESI-MS $(M+H)^+$: *m*/*z* 651, found: 651. Anal. Calcd for $C_{33}H_{54}N_4O_9$: C, 60.90; H, 8.36; N, 8.61. Found: C, 60.54; H, 8.50; N, 8.22.

4.13. 6-(4-Nitrobenzyl)-1-oxa-4,7,10-triazacyclododecane-*N*,*N*′,*N*″-triacetic acid (13) (*C*-ODTA)

Compound **12** (54.7 mg, 0.253 mmol) was dissolved in a mixture of TFA (1.8 mL) and anisole (0.2 mL), and the solution was stirred for 6 h at room temperature. The solvent was concentrated in vacuo, and diethyl ether (3 mL) was added. The precipitate was collected and dried in vacuo to provide *C*-ODTA as a pale yellow solid (28.9 mg, 41.7%). ^{1}H NMR (D_2O): 2.64–4.07 (23H, overlapped, C*H*, C*H*$_2$), 7.36–7.38 (2H, d, *aromatic*), 8.07–8.09 (2H, d, *aromatic*). ESI-MS $(M+H)^+$: *m*/*z* 483, found: 483. Anal. Calcd for $C_{21}H_{30}N_4O_9{\cdot}1.5H_2O{\cdot}2CF_3COOH$: C, 40.71; H, 4.78; N, 7.60. Found: C, 40.81; H, 4.58; N, 7.66.

4.14. ^{111}In radiolabeling of *C*-ODTA and *C*-DOTA

To a 1 M acetate buffer (pH 4.5, 15 μL) was added $^{111}InCl_3$ (10 μL). After the mixture was allowed to stand for 5 min at room temperature, a solution of *C*-ODTA or *C*-DOTA in 0.1 M acetate buffer (pH 4.5, 25 μL) was added. The final chelate concentrations were 10 μM. The mixture was incubated for 1 h at 37 °C.

^{111}In radiolabeling of *C*-ODTA and *C*-DOTA was also conducted at 100 °C for 10 min under similar reaction conditions as stated above except for using 0.1 or 1 M acetate buffer (pH 3.0) with the final chelate concentration of 1 or 10 μM.

4.15. Stability assessments of ^{111}In-*C*-ODTA and ^{111}In-*C*-DOTA

^{111}In-*C*-ODTA and ^{111}In-*C*-DOTA were prepared as described above with the final chelate concentration of 10 μM. After incubation for 10 min at 100 °C, the ^{111}In-labeled compounds were subjected to HPLC purification to remove free chelates. The radioactive fractions were collected and evaporated to dryness. The radioactive residue dissolved in 0.1 M sodium bicarbonate buffer (pH 7.4, 15 μL) was added to a solution of apo-transferrin (30.0 mg/210 μL or 3.0 mg/210 μL) in 0.1 M sodium bicarbonate buffer (pH 7.4). The mixture was incubated at 37 °C. After incubation for 1, 6, 24, 72, 120 h, a 5–10 μL aliquot of each sample was drawn, and the % of intact complex were determined by TLC.

4.16. In vivo studies

Animal studies were conducted in accordance with our institutional guidelines and were approved by Chiba University Animal Care Committee. ^{111}In-*C*-ODTA was prepared as described above, and subjected to HPLC purification to separate the two peaks. Biodistribution studies were performed by intravenous administration of a 0.1 mL of phosphate-buffered saline (1 mM, pH 7.4) solution of each radioactive fraction (0.3 μCi/100 μL) to 6-week-old male ddY mice (Japan SLC Inc., Shizuoka, Japan). Groups of five

mice were used for the experiments. Organs of interest were removed, weighed, and the radioactivity counts were determined with an auto-well γ counter at 1, 6, and 24 h post-injection. To determine the amounts and routes of excretion from the body, mice were housed in metabolic cages after injection of ^{111}In-*C*-ODTA (0.3 μCi/100 μL). Urine and feces were collected for 24 h postinjection, and the radioactivity counts were determined. Mice were also housed in metabolic cages after injection of ^{111}In-*C*-ODTA (5 μCi/100 μL). Urine samples were collected for 6 h postinjection, and were analyzed by HPLC after filtration through a 10 kDa cutoff ultrafiltration membrane (Microcon-10, Millipore).

Acknowledgments

This work was supported in part by a Grant-in-Aid for Technology Development Program for Advanced Measurement and Analysis from Japan Science and Technology Agency and Special Funds for Education and Research (Development of SPECT Probes for Pharmaceutical Innovation) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This work was dedicated to our distinguished colleague, the late Mr. Yuichiro Taira.

References and notes

1. Miao, Y.; Gallazzi, F.; Guo, H.; Quinn, T. P. *Bioconjugate Chem.* **2008**, *19*, 539.
2. Forrer, F.; Uusijärvi, H.; Storch, D.; Maecke, H. R.; Mueller-Brand, J. *J. Nucl. Med.* **2005**, *46*, 1310.
3. Brouwers, A. H.; Van Eerd, J. E. M.; Frielink, C.; Oosterwijk, E.; Oyen, W. J. G.; Corstens, F. H. M.; Boerman, O. C. *J. Nucl. Med.* **2004**, *45*, 327.
4. Beeby, A.; Botchway, S. W.; Clarkson, I. M.; Faulkner, S.; Parker, A. W.; Parker, D.; Williams, J. A. G. *J. Photochem. Photobiol., B* **2000**, *57*, 83.
5. Thibon, A.; Pierre, V. C. *Anal. Bioanal. Chem.* **2009**, *394*, 107.
6. Mier, W.; Hoffend, J.; Krämer, S.; Schuhmacher, J.; Hull, W. E.; Eisenhut, M.; Haberkorn, U. *Bioconjugate Chem.* **2005**, *16*, 237.
7. Mukai, T.; Namba, S.; Arano, Y.; Ono, M.; Fujioka, Y.; Uehara, T.; Ogawa, K.; Konishi, J.; Saji, H. *J. Pharm. Pharmacol.* **2002**, *54*, 1073.
8. Anelli, P. L.; Lattuada, L.; Gabellini, M.; Recanati, P. *Bioconjugate Chem.* **2001**, *12*, 1081.
9. Wu, C.; Jagoda, E.; Brechbiel, M.; Webber, K. O.; Pastan, I.; Gansow, O.; Eckelman, W. C. *Bioconjugate Chem.* **1997**, *8*, 365.
10. Renn, O.; Meares, C. F. *Bioconjugate Chem.* **1992**, *3*, 563.
11. Chappell, L. L.; Ma, D.; Milenic, D. E.; Garmestani, K.; Venditto, V.; Beitzel, M. P.; Brechbiel, M. W. *Nucl. Med. Biol.* **2003**, *30*, 581.
12. Chong, H. S.; Garmestani, K.; Ma, D.; Milenic, D. E.; Overstreet, T.; Brechbiel, M. W. *J. Med. Chem.* **2002**, *45*, 3458.
13. Chong, H. S.; Milenic, D. E.; Garmestani, K.; Brady, E. D.; Arora, H.; Pfiester, C.; Brechbiel, M. W. *Nucl. Med. Biol.* **2006**, *33*, 459.
14. Försterová, M.; Petřík, M.; Lázničková, A.; Lázníček, M.; Hermann, P.; Lukeš, I.; Melichar, F. *Appl. Radiat. Isot.* **2009**, *67*, 21.
15. Tsai, S. W.; Li, L.; Williams, L. E.; Anderson, A. L.; Raubitschek, A. A.; Shively, J. E. *Bioconjugate Chem.* **2001**, *12*, 264.
16. Camera, L.; Kinuya, S.; Garmestani, K.; Brechbiel, M. W.; Wu, C.; Pai, L. H.; McMurry, T. J.; Gansow, O. A.; Pastan, I.; Paik, C. H., et al *Eur. J. Nucl. Med.* **1994**, *21*, 640.
17. Delgado, R.; Quintino, S.; Teixeira, M.; Zhang, A. *J. Chem. Soc., Dalton Trans.* **1997**, 55.
18. Richman, J. E.; Atkins, T. J. *J. Am. Chem. Soc.* **1974**, *96*, 2268.
19. McMurry, T. J.; Brechbiel, M.; Kumar, K.; Gansow, O. A. *Bioconjugate Chem.* **1992**, *3*, 108.
20. Mishra, A. K.; Gestin, J. F.; Benoist, E.; Faivre-Chauvet, A.; Chatal, J. F. *New J. Chem.* **1996**, *20*, 585.
21. Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *63*, 9678.
22. Jeremic, T.; Linden, A.; Moehle, K.; Heimgartner, H. *Tetrahedron* **2005**, *61*, 1871.
23. Ferreira, C. L.; Yapp, D. T.; Lamsa, E.; Gleave, M.; Bensimon, C.; Jurek, P.; Kiefer, G. E. *Nucl. Med. Biol.* **2008**, *35*, 875.
24. Schlesinger, J.; Koezle, I.; Bergmann, R.; Tamburini, S.; Bolzati, C.; Tisato, F.; Noll, B.; Klussmann, S.; Vonhoff, S.; Wuest, F.; Pictzsch, H. J.; Steinbach, J. *Bioconjugate Chem.* **2008**, *19*, 928.
25. Herges, R.; Dikmans, A.; Jana, U.; Köhler, F.; Jones, P. G.; Dix, I.; Fricke, T.; König, B. *Eur. J. Org. Chem.* **2002**, 3004.