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^{68}Ga-Complex Lipophilicity and the Targeting Property of a Urea-Based PSMA Inhibitor for PET Imaging

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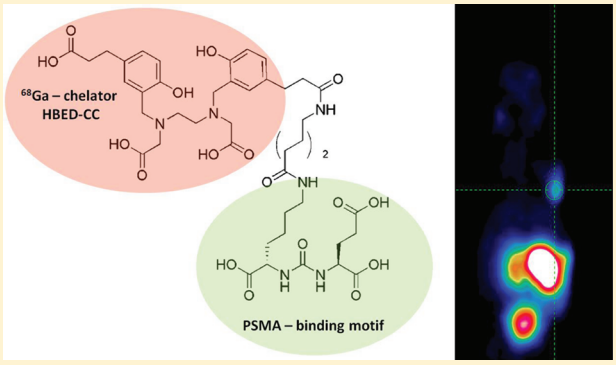
Supporting Information

ABSTRACT: Urea-based inhibitors of the prostate-specific membrane antigen (PSMA) represent low-molecular-weight pepidomimetics showing the ability to image PSMA-expressing prostate tumors. The highly efficient, acyclic Ga(III) chelator *N,N′*-bis [2-hydroxy-5-(carboxyethyl)benzyl] ethylenediamine-*N,N′*- diacetic acid (HBED-CC) was introduced as a lipophilic side chain into the hydrophilic pharmacophore Glu-NH-CO-NH-Lys which was found favorable to interact with the PSMA "active binding site". This report describes the syntheses, in vitro binding analyses, and biodistribution data of the radiogallium labeled PSMA inhibitor Glu-NH-CO-NH-Lys(Ahx)-HBED-CC in comparison to the corresponding DOTA conjugate. The binding properties were analyzed using competitive cell binding and enzyme-based assays followed by internalization experiments. Compared to the DOTA-conjugate, the HBED-CC derivative showed reduced unspecific binding and considerable higher specific internalization in LNCaP cells. The ^{68}Ga complex of the HBED-CC ligand exhibited higher specificity for PSMA expressing tumor cells resulting in improved in vivo properties. ^{68}Ga labeled Glu-NH-CO-NH-Lys(Ahx)-HBED-CC showed fast blood and organ clearances, low liver accumulation, and high specific uptake in PSMA expressing organs and tumor. It could be demonstrated that the PET-imaging property of a urea-based PSMA inhibitor could significantly be improved with HBED-CC.



INTRODUCTION

It is still a challenge to select appropriate treatment options for disseminated prostate cancer because of the lack of sensitive imaging agents for diagnosis and therapy monitoring.[1] Prostate-specific membrane antigen (PSMA) is expressed in nearly all prostate cancers with increased expression in poorly differentiated, metastatic, and hormone-refractory carcinomas.[2] Its expression level is about 1000-fold higher compared to the physiologic levels found in other tissues such as kidney, small intestine, or brain.[3] PSMA is primarily restricted to the prostate, it is abundantly expressed at all stages of disease, it is presented on the cell surface, and it is not shed into the circulation.[4] As a consequence, PSMA can be considered a promising target for specific prostate cancer imaging and therapy.[5−7]

The radiohalogenated inhibitors of PSMA exhibiting Glu-NH-CO-NH-Lys as a pharmacophore showed the ability to image PSMA-expressing prostate tumor xenografts.[8,9] More recently, a corresponding DOTA-conjugate was labeled with ^{68}Ga which represents an attractive generator-based alternative to cyclotron-based PET radiopharmaceuticals.[10,11] The readily available ^{68}Ga decays with 89% probability by positron emission allowing high-resolution PET images with the option of accurate quantification. *N,N′*-Bis[2-hydroxy-5-(carboxyethyl)-benzyl]ethylenediamine-*N,N′*- diacetic acid (HBED-CC) was recently proposed as an efficient ^{68}Ga chelator with fast complexing kinetics and a high in vitro as well as in vivo complex stability.[12,13]

Besides the efficient Ga(III) complexing characteristics, HBED-CC was selected because of its lipophilic nature. It was found that the "active binding site" of PSMA is composed of two structural motifs, one representing a lipophilic pocket and the other interacting with urea-based inhibitors.[14] A study comparing a series of linkers located between the urea-based motif and a ^{99m}Tc chelator revealed a clear dependency of binding properties in favor of the more lipophilic compounds.[14] Further studies described the binding site as a pocket interacting with the carboxylic groups and the zinc complexing urea on one hand and with hydrophobic, mostly aromatic groups on the other hand.[15]

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Table 1. Analytical and PSMA-Binding Data

Ga-peptide complexes	m/z[a]	m/z calculated as $[M+H]^+$	analytical HPLC retention[b]	affinity related IC_{50} [nM] determined in enzyme-based assay[c]	affinity related K_i [nM] determined in cell-based assay[c]
[Ga]**7**	947.4257	947.4250	3.1 min	7.5 ± 2.2	12.0 ± 2.8
[Ga]**8**	819.4104	819.4100	2.4 min	19.4 ± 7.1	37.6 ± 14.3
[Ga]**12**	1284.6368	1284.6364	3.7 min	8.7 ± 3.9	11.1 ± 1.8

[a]High-resolution mass spectrometry data of the free ligands ($[M+H]^+$). [b]Compounds were labeled with ^{68}Ga; runs were performed using a linear A−B gradient (0% B to 100% B in 6 min) at a flow rate of 4 mL/min. Solvent A was 0.1% aqueous TFA and solvent B was MeOH. [c]Compounds were complexed with ^{nat}Ga.

This report describes the syntheses of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (**7**) and Glu-NH-CO-NH-Lys(Ahx)-DOTA (**8**) together with a reference PSMA inhibitor previously published by Banerjee et al.[10] After ^{68}Ga complexation, these compounds were evaluated in vitro and in vivo to study the influence of these two chelators and side chain variations.

MATERIALS AND METHODS

Reagents. All commercially available chemicals were of analytical grade and were used without further purification. ^{68}Ga was obtained from a $^{68}Ge/^{68}Ga$ generator based on pyrogallol resin support.[12,16] Protected amino acids were obtained from Novabiochem (Merck, Darmstadt, Germany) or IRIS Biotech (Marktredwitz, Germany).

The preparations were analyzed using reversed-phase high-performance liquid chromatography (RP-HPLC; Chromolith RP-18e, 100 × 4.6 mm; Merck, Darmstadt, Germany). Runs were performed using a linear A−B gradient (0% B to 100% B in 6 min) at a flow rate of 4 mL/min. Solvent A consisted of 0.1% aqueous TFA and solvent B was 0.1% TFA in CH_3CN or MeOH (in case of determination of radiochemical yield (RCY) or analysis of serum stability).

For all preparative purifications, a Chromolith RP-18e column (100 × 10 mm; Merck, Darmstadt, Germany) was used with a 6 min gradient starting at 0% raised to 50% and followed by a 1 min increase to 100% B. The flow rate was 6 mL/min. The HPLC system (L6200 A; Merck-Hitachi, Darmstadt, Germany) was equipped with a variable UV and a gamma detector (Bioscan; Washington, USA). UV absorbances were measured at 214 and 254 nm. Mass spectrometry was performed with a MALDI-MS Daltonics Microflex (Bruker Daltonics, Bremen, Germany) system. High-resolution mass spectrometry was performed using a system equipped with a mass spectrometer supporting Orbitrap technology (Exactive, Thermo Fisher Scientific). Full-scan single mass spectra were obtained by scanning m/z = 200−4000. NMR data were obtained with Bruker Avance NMR Spectrometers, AV(I)-600 (600 MHz) and AV(III)-400 (400 MHz). The chemical shifts are referenced to solvent signals (DMSO-d_5 = 2.50/39.757 ppm, DMSO-d_6 = 39.50).

Synthesis of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (7). In a first step, the isocyanate **2** of the glutamyl moiety was generated in situ by adding a mixture of 3 mmol of bis(*tert*-butyl) L-glutamate hydrochloride (Bachem, Switzerland) (**1**) and 1.5 mL of *N*-ethyldiisopropylamine (DIPEA) in 200 mL of dry CH_2Cl_2 to a solution of 1 mmol triphosgene in 10 mL of dry CH_2Cl_2 at 0 °C over 4 h. After agitation of the reaction mixture for one further hour at 25 °C, 0.5 mmol of a resin-immobilized (2-chloro-tritylresin, Merck, Darmstadt) ε-allyloxycarbonyl protected lysine was added in one portion (in 4 mL DCM) and reacted for 16 h with gentle agitation leading to compound **3**. The resin was filtered off and the allyloxy-protecting group was removed using 100 mg tetrakis-(triphenyl)palladium(0) (Sigma-Aldrich, Germany) and 400 μL morpholine in 4 mL CH_2Cl_2 for 3 h resulting in compound **4**. The following coupling of the aminohexanoic moiety was performed using 2 mmol of the Fmoc-protected 6-amino-hexanoic acid (Sigma-Aldrich, Germany), 1.96 mmol of HBTU (Merck, Darmstadt, Germany), and 2 mmol of *N*-ethyl-diisopropylamine in a final volume of 4 mL DMF. The product **5** was cleaved from the resin by reacting with 4 mL of a 30% 1,1,1,3,3,3-hexafluoroisopropanole (HFIP) in CH_2Cl_2 for two hours at ambient temperature resulting in the *tert*-butyl protected crude product **6** which was purified via RP-HPLC.

To conjugate HBED-CC, the purified product **6** was reacted with an equimolar amount of HBED-CC-TFP-ester synthesized as previously described[12] in the presence of 2 equiv of DIPEA in *N*,*N*-dimethylformamide (DMF; final volume of 1 mL). After HPLC purification, the remaining *tert*-butyl groups were cleaved at room temperature for one hour using 2 mL trifluoroacetic acid to obtain **7** in ∼35% yield after purification by HPLC. High-resolution mass spectrometry was used to confirm the identity (Table 1), and purity was analyzed via analytical HPLC at λ = 206 nm (Supporting Information). Complete and unequivocal NMR ^1H and ^{13}C signal assignments were obtained (Supporting Information).

Synthesis of Glu-NH-CO-NH-Lys(Ahx)-DOTA (8). Precursor **5** was synthesized as mentioned before. After activation with 3.95 equiv of HBTU and DIPEA for 2 h, 4 equiv of tris(*t*-bu)-DOTA (Chematech, Dijon, France) relative to the resin loading were reacted with **5** after removal of the Fmoc-protecting group in a final volume of 3 mL DMF. The product was cleaved from the resin in a 2 mL mixture consisting of trifluoroacetic acid, triisopropylsilane, and water (95:2.5:2.5). The product was purified via semipreparative RP-HPLC resulting in compound **8** (∼30% yield). High-resolution mass spectrometry was used to confirm the identity (Table 1) and purity was analyzed via analytical HPLC at λ = 206 nm (Supporting Information). Complete and unequivocal ^1H and ^{13}C NMR signal assignments were obtained (Supporting Information).

Synthesis of Reference Compound (12). Synthesis of the DOTA conjugate previously published by Banerjee et al.[10] was performed using a modified solid-phase method. Five equivalents of suberic-acid-bis-(*N*-hydroxysuccinimideester) (Sigma-Aldich, Germany) was conjugated to the side chain of the lysine moiety of compound **4** in the presence of 5 equiv of triethylamine in 3 mL DMF. After 2 h, the resin was washed with DMF. In the presence of five equiv of triethylamine, five equiv of Fmoc-Lys-oAll was coupled to the immobilized NHS-ester of intermediate **9** in a final volume of 3 mL DMF for 16 h resulting in **10**. The remaining conjugations of two phenyl-alanine building blocks and tris(*t*-bu)-DOTA were performed

according to standard Fmoc-protocols resulting in **11**. Cleavage from resin was performed using a 3 mL mixture of trifluoroacetic acid, triisopropylsilane, and water (95:2.5:2.5). The product was purified via RP-HPLC to obtain **12** in ~8% yield. High-resolution mass spectrometry was used to confirm the identity (Table 1), and purity was analyzed via analytical HPLC at λ = 206 nm (Supporting Information).

^{68}Ga-Labeling. Typically, 0.1−1 nmol of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (**7**, in 0.1 M HEPES buffer pH 7.5) or 1 nmol of the DOTA conjugates (**8**/**12**, in 0.1 M HEPES buffer pH 7.5) were added in a volume of 100 μL to a mixture of 10 μL 2.1 M HEPES solution and 10 μL [^{68}Ga]Ga^{3+} eluate (50−100 MBq). The pH of the labeling solution was adjusted to 4.2. Depending on the chelator, the reaction mixture was incubated either at ambient temperature or at 80 °C for 2 min. The radiochemical yield (RCY) was determined using RP-HPLC.

^{67}Ga-Labeling. ^{67}Ga was purchased from MDS Nordion (Fleurus, Belgium) as [^{67}Ga]$GaCl_3$ in 0.1 N HCl. Typically, 0.1 nmol of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (**7**) was added in a volume of 100 μL to a mixture of 10 μL 2.1 M HEPES solution, 2 μL 1 N HCl, and 10 μL [^{67}Ga]$GaCl_3$ (~100 MBq) in 0.1 N HCl resulting in a solution with a pH of 4.2. The reaction mixture was incubated for 2 min at ambient temperature. The RCY was determined using RP-HPLC.

^{nat}Ga-Complexes. A 10× molar excess of Ga(III)-nitrate (Sigma Aldrich, Germany) in 0.1 N HCl (10 μL) was reacted with the compounds **7**, **8**, or **12** (1 mM in 0.1 M HEPES buffer pH 7.5, 40 μL) in a mixture of 10 μL 2.1 M HEPES solution and 2 μL 1 N HCl for 2 min at 80 °C.

Radiochemical Stability. The radiochemical stability of the ^{68}Ga-labeled compounds **7**, **8**, and **12** was determined by incubating in both PBS and human serum for 2 h at 37 °C. An equal volume of MeCN was added to the samples to precipitate serum proteins. Subsequently, the samples were centrifuged for 5 min at 13 000 rpm. An aliquot of the supernatant and the PBS sample was analyzed by RP-HPLC. In addition, serum samples of compound [^{68}Ga]**7** were run on a Superdex 200 GL 5/150 gel filtration column (GE Healthcare, Munich, Germany) to analyze protein binding. To analyze the complex stability against human transferrin, a 400 μL aliquot of [^{68}Ga]**7** was added to 250 μg apo-transferrin in PBS at pH 7 and incubated at 37 °C (water bath) for 2 h. The complex stability was determined using a Superdex 200 GL 5/150 short column with PBS pH 7 as eluent.

Naaladase Assay. Recombinant human PSMA (rhPSMA, R&D systems, Wiesbaden, Germany) was diluted in assay buffer (50 mM HEPES, 0.1 M NaCl, pH 7.5) to 0.4 μg/mL. The substrate Ac-Asp-Glu (Sigma, Taufkirchen, Germany, 40 μM final concentration) was mixed with [^{nat}Ga]**7**, [^{nat}Ga]**8**, or [^{nat}Ga]**12** at concentrations ranging from 0.05 nM to 1000 nM in a final volume of 125 μL assay buffer. The mixtures were combined with 125 μL of the rhPSMA solution (0.4 μg/mL) and incubated for one hour at 37 °C. The reaction was stopped by heating at 95 °C for 5 min. 250 μL of a 15 mM solution of *ortho*-phthaldialdehyde (Sigma, Taufkirchen, Germany) was added to all vials and incubated for 10 min at ambient temperature. Finally, 200 μL aliquots of the reaction solutions were loaded onto a F16 Black Maxisorp Plate (Nunc, Langenselbold, Germany) and read at excitation and emission wavelengths of 330 and 450 nm, respectively, using a microplate reader (DTX-880, Beckman Coulter, Krefeld, Germany). The data were analyzed by a one-site total binding regression algorithm of *GraphPad Prism* (GraphPad Software, California, USA).

Cell Binding Studies. Cell binding studies were performed using PSMA$^+$ LNCaP cells (metastatic lesion of human prostatic adenocarcinoma, ATCC CRL-1740) and PSMA$^-$ PC-3 cells (bone metastasis of a grade IV prostatic adenocarcinoma, ATCC CRL-1435) cultured in DMEM medium supplemented with 10% fetal calf serum and 2 mmol/L L-glutamine (all from Invitrogen). During cell culture, cells were grown at 37 °C in an incubator with humidified air, equilibrated with 5% CO_2. The cells were harvested using trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA; 0.25% trypsin, 0.02% EDTA, all from Invitrogen).

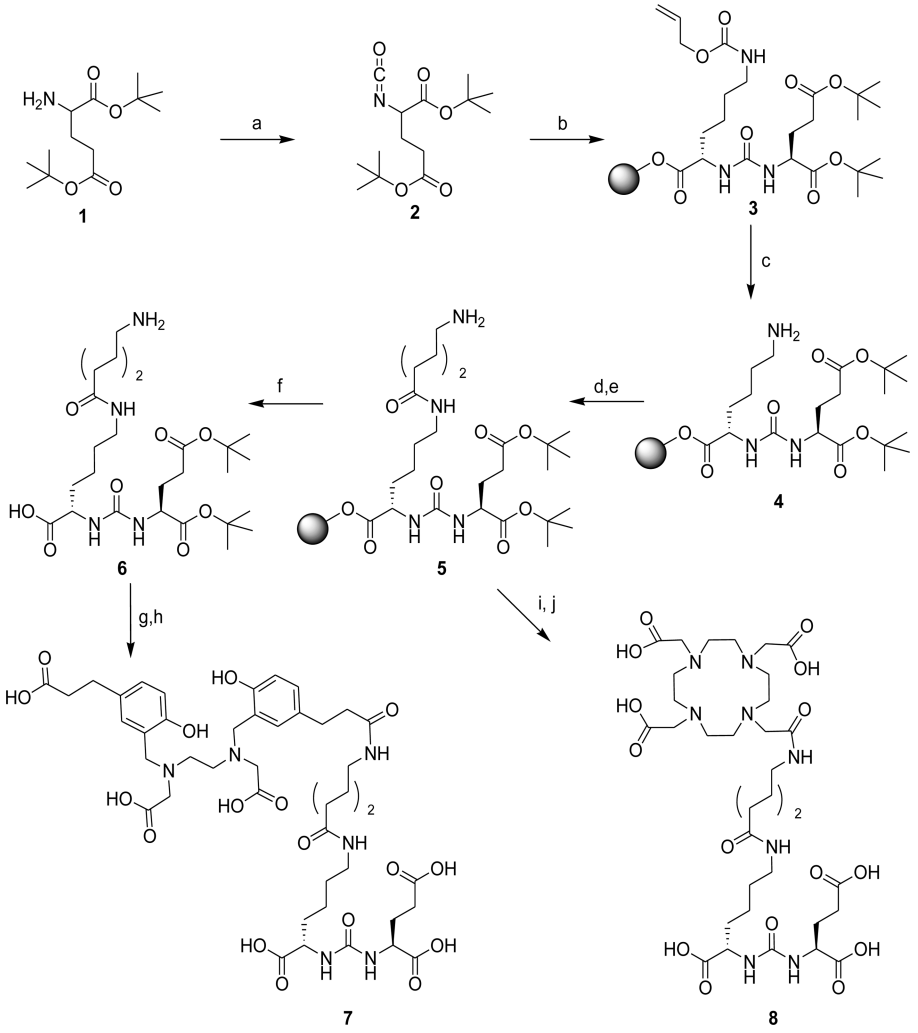
In order to determine the binding affinity, a competitive cell binding assay was performed. LNCaP cells (10^5 per well) were incubated with a 0.2 nM solution of [^{67}Ga]**7** in the presence of 12 different concentrations of [^{nat}Ga]**7**, [^{nat}Ga]**8**, or [^{nat}Ga]**12** (0−5000 nM, 200 μL/well). After incubation at ambient temperature for 1 h with gentle agitation, the binding buffer was removed using a multiscreen vacuum manifold (Millipore, Billerica, MA). After washing twice with 100 μL and once with 200 μL ice-cold binding buffer, the cell-bound radioactivity was measured with a gamma counter (Packard Cobra II, GMI, Minnesota, USA). The 50% inhibitory concentration (IC_{50}) values were calculated by fitting the data using a nonlinear regression algorithm (GraphPad Software). Experiments were performed at least three times including quadruplicate sample measurements.

Determination of Binding Specificity and Internalization. Internalization experiments were performed as previously described.[17] Briefly, 10^5 LNCaP or PC-3 cells were seeded in poly(L-lysine)-coated 24-well cell culture plates 24 h before incubation. After washing with PBS, the cells were incubated with the radiolabeled compounds [^{68}Ga]**7**, [^{68}Ga]**8**, and [^{68}Ga]**12** (25 nM final concentration) for 45 min at 37 °C and at 4 °C, respectively. To determine specific cell uptake, cells were blocked with 2-(phosphonomethyl)-pentanedioic acid (PMPA, Axxora, Loerrach, Germany) to a final concentration of 100 μM. Cellular uptake was terminated by washing 4 times with 1 mL of ice-cold PBS. To remove surface-bound radioactivity, cells were incubated twice with 0.5 mL glycine-HCl in PBS (50 mM, pH 2.8) for 5 min at room temperature. The cells were washed with 1 mL of ice-cold PBS and lysed using 0.5 mL of 0.3 M NaOH. The surface-bound and internalized fractions were measured in a gamma counter.

Specificity of binding was additionally analyzed in a concentration-dependent cell uptake experiment. Solutions of [^{68}Ga]**7**, [^{68}Ga]**8**, or [^{68}Ga]**12** at final concentrations of 2.5, 25, and 250 nM were added to 7 × 10^5 cells suspended in 50 μL OPTIMEM medium (Gibco, Auckland, New Zealand). After 45 min at 37 °C, the samples were briefly vortexed and a 10 μL aliquot was transferred to a 400 μL microcentrifuge tube (Roth, Germany) containing 350 μL of a 75:25 mixture of silicon oil, density 1.05 (Sigma Aldrich, Germany), and mineral oil, density 0.872 (Acros, Nidderau, Germany). Separation of cells from the medium was performed by centrifugation at 12 000 rpm for 2 min. After freezing the tubes using liquid nitrogen, the bottom tips containing the cell pellet were cut off. The cell pellets and the supernatants were separately counted in a gamma counter.

Biodistribution and PET Imaging. Five ×10^6 cells of either LNCaP or PC-3 in 50% Matrigel (Becton Dickinson, Heidelberg, Germany) were subcutaneously inoculated into the right trunk of male 7- to 8-week-old BALB/c nu/nu mice

Scheme 1. Syntheses of Glu-NH-CO-NH-Lys(Ahx)-HBED-CC (7) and Glu-NH-CO-NH-Lys(Ahx)-DOTA (8)



(a) Triphosgene, DIPEA, CH_2Cl_2, 0 °C; (b) H-Lys(Alloc)-2CT-Resin, CH_2Cl_2; (c) $Pd[P(C_6H_5)_3]_4$, morpholine, CH_2Cl_2; (d) Fmoc-6-Ahx-OH, HBTU, DIPEA, DMF; (e) 20% piperidine, DMF; (f) hexafluoroisopropanol/CH_2Cl_2; (g) HBED-CC-TFP ester, DIPEA, DMF; (h) TFA; (i) tris(*t*-bu)DOTA, HBTU, DIPEA; (j) TFA.

(Charles River Laboratories). The tumors were allowed to grow for 3 to 4 weeks until approximately 1 cm^3 in size.

The ^{68}Ga-radiolabeled compounds of **7**, **8**, and **12** were injected via tail vein (1−2 MBq per mouse; 0.1−0.2 nmol). At 1 h after injection, the animals were sacrificed. Organs of interest were dissected, blotted dry, and weighed. The radioactivity was measured with a gamma counter and calculated as % ID/g.

For the microPET studies, 10−25 MBq of compounds [^{68}Ga]**7** and [^{68}Ga]**8** in a volume of 0.15 mL (~0.5 nmol) were injected via a lateral tail vein into mice bearing LNCaP tumor xenografts. The anesthetized animals (2% sevoflurane, Abbott, Wiesbaden, Germany) were placed in prone position into the Inveon small animal PET scanner (Siemens, Knoxville, Tenn, USA) to perform a 50 min dynamic microPET scan starting at 1 min post injection followed by a 20 min static scan.

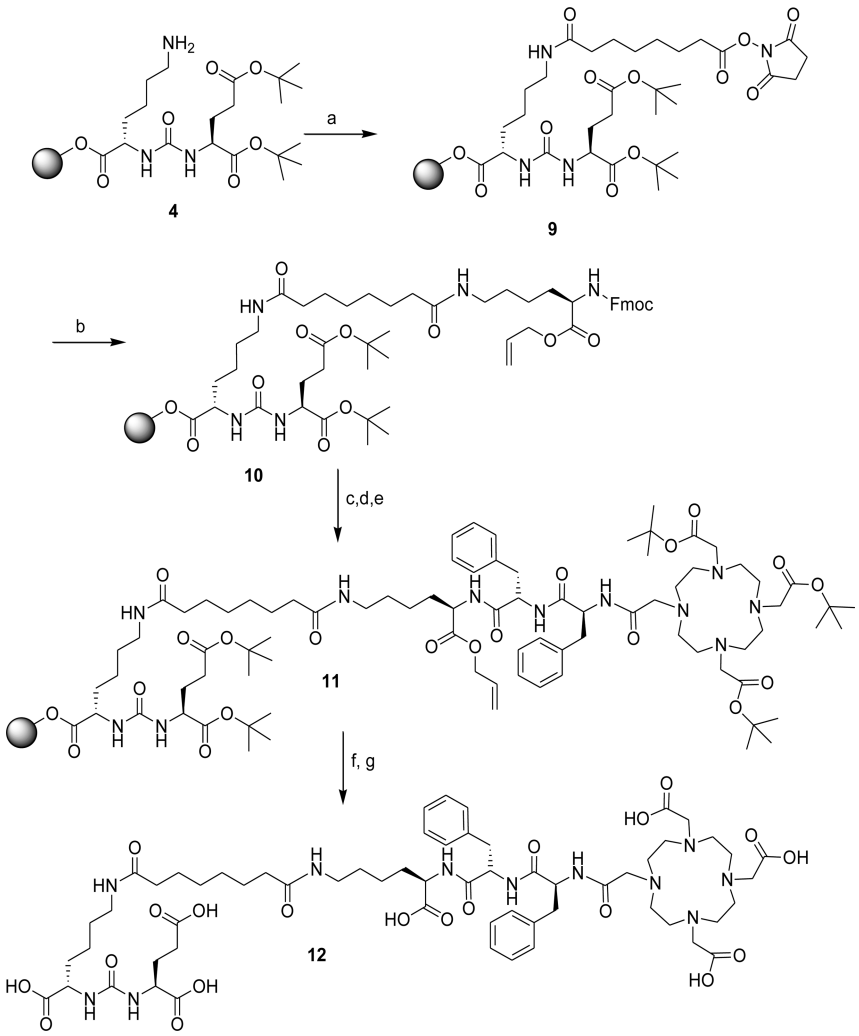
Statistical Aspects. All experiments were performed at least in triplicate. Quantitative data were expressed as mean ± SD. If applicable, means were compared using Student's *t*-test. *P* values of <0.05 were considered statistically significant.

RESULTS

Chemistry. The synthesis of the resin-bound intermediate **5** was performed using solid-phase chemistry as outlined in Scheme 1. To couple the respective chelators, compound **5** was either cleaved from resin and reacted with an equimolar amount of HBED-CC-TFP-ester resulting in **7** or coupled with HBTU activated tris(t-bu)DOTA resulting in the DOTA-conjugate **8**. The preparation of the reference compound **12** previously published by Banerjee et al.[10] was performed using a modified solid-phase method as outlined in Scheme 2. Briefly, disuccinimidyl suberate was conjugated to the ε-amino group of the lysine group of **4**. After Fmoc-Lys-OAll was coupled to the resulting immobilized NHS-ester, the conjugation of two phenylalanine building blocks and tris(*t*-bu)-DOTA were performed according to standard Fmoc-protocols. Analytical data of compounds **7**, **8**, and **12** are summarized in Table 1.

Radiolabeling and Stability. Radiolabeling with ^{68}Ga was performed at pH 4.2 by incubating the conjugates **7**, **8**, or **12** in a mixture consisting of 50−100 MBq [^{68}Ga]Ga^{3+} and HEPES. Radiochemical yields were determined by RP-HPLC analysis of the reaction mixtures. The retention times obtained for each compound are shown in Table 1. An amount of 0.1 nmol of the

Scheme 2. Modified Solid-Phase Synthesis of the DOTA Conjugate Previously Published by Banerjee et al.[10]



(a) Disuccinimidyl suberate (DSS), TEA, DMF; (b) Fmoc-Lys-oAll, TEA, DMF; (c) 20% piperidine, DMF; (d) Fmoc-Phe-OH, HBTU, DIPEA, DMF; (e) tris(*t*-bu)DOTA, HBTU, DMF; (f) $Pd[P(C_6H_5)_3]_4$, morpholine, CH_2Cl_2; (g) TFA, triisopropylsilane, H_2O (95/2.5/2.5).

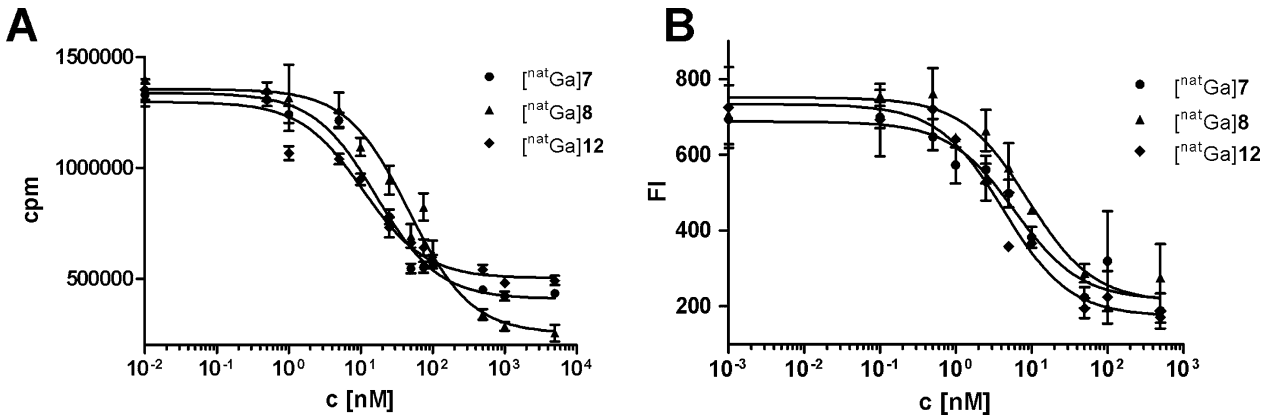


Figure 1. Determination of binding affinity of compounds [^{nat}Ga]**7**, [^{nat}Ga]**8**, and [^{nat}Ga]**12** by competitive titration on LNCaP cells (A) and purified receptor using an enzyme-based assay (B). Data are expressed as mean ± SD (n = 4). cpm = counts per minute; FI = fluorescence intensity.

HBED-CC conjugate **7** in a final concentration of 1.7 μM led to a radiochemical yield of more than 99% in less than 1 min at room temperature. As a consequence, specific activities in the range 500−1000 GBq/μmol were obtained. In order to achieve comparable high radiochemical yields with the DOTA-conjugates **8** and **12**, the compounds were incubated for 2 min at 80 °C using 1 nmol precursor.

Incubation of the ^{68}Ga-labeled compounds **7**, **8**, and **12** in human serum for 2 h at 37 °C resulted in no detectable changes in the radiograms. Stability of the ^{68}Ga-labeled HBED-CC-

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