Synthesis of functionalised HP-DO3A chelating agents for conjugation to biomolecules†

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Two novel bifunctional chelating agents (BFCAs) based on the structure of 10-(2-hydroxypropyl)-1,4,7-tetraazacyclododecane-1,4,7-triacetic acid (HP-DO3A) have been designed and synthesized. BFCAs are able to strongly coordinate metals (*e.g.* Gd(III)) and radiometals (*e.g.* ^{90}Y(III), ^{177}Lu(III) and ^{111}In(III)), and find applications in the synthesis of products for the imaging and treatment of several cancer types. Indeed, these two BFCAs have been employed in solid-phase peptide synthesis (SPPS) and coupled to well-known peptides such as bombesin and RGD analogues in order to target tumor cells. We also report here the conjugation of one of them to Lys^8-oxytocin and radiolabeling with ^{111}In(III) to obtain a new radiopharmaceutical product with potential applications in the diagnosis of tumors over-expressing oxytocin receptors.

Introduction

Bifunctional chelating agents (BFCAs) are versatile building blocks for the synthesis of compounds that are used in diagnostic and therapeutic medical applications.[1–4] A BFCA is composed of: (a) an acyclic or macrocyclic ligand that chelates the desired metal ion and (b) a suitable reactive moiety that can bind covalently to a specific targeting vector (TV). This kind of coupling is of broad interest as it finds numerous applications. For example, by conjugating a metallic radionuclide to a TV, such as a monoclonal antibody[5] or a peptide[6–8], radiopharmaceuticals have been prepared and successfully employed in oncology. Moreover, the specificity of contrast agents (CA) used for magnetic resonance imaging (MRI) can be enhanced by conjugating it to a carrier that targets a specific epitope.[9,10] Because trivalent lanthanide ions display a variety of magnetic, optical and catalytic properties,[11] the design and development of synthetic BFCAs for connecting TVs to lanthanide complexes, as well as any improvements in related conjugation and chelation procedures, are matters of great importance to molecular medicine.

The high affinity shown by trivalent metal ions such as Ln(III), Ga(III) and In(III) towards some polyaminocarboxylic acids, such as 1,4,7,10-tetraazacyclododecane-*N*,*N*,*N*,*N*-tetraacetic acid (DOTA) and its derivatives, has been exploited to prepare very stable complexes for various applications.[12,13] For example, these polyaminocarboxylic ligands are very useful for the complexation of β^--emitting radionuclides, such as ^{90}Y ($T_{\frac{1}{2}} = 2.67$ d, $E_{total} = 939.1$ keV) and ^{177}Lu ($T_{\frac{1}{2}} = 6.71$ d, $E_{total} = 146.7$ keV), which are widely employed in nuclear medicine protocols.[5–8]

Almost all macrocyclic BFCAs reported in the literature are based on DOTA or DOTA monoamide (DOTAMA) derivatives bearing a variety of functional groups available for conjugation, *viz.* carboxyls,[14,15] amines,[16] aldehydes or ketones,[16,17] isothiocyanates,[18–19] maleimides,[16,20] alkynes[17] and vinylsulfone.[21] Surprisingly, no reports have appeared so far concerning derivatives of the octadentate macrocyclic ligand HP-DO3A [10-(2-hydroxypropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid]. Gd-HPDO3A (Prohance,® Bracco Imaging), a non-specific CA for MRI, is commercially available and widely employed in clinical practice.[22] A neutral, highly hydrophilic gadolinium chelate, it is endowed with a very high thermodynamic stability (log $K = 23.8$) and kinetic inertness that ensures against any significant release of Gd^{3+} ions under *in vivo* conditions.[12,23] Its relaxivity, r_{1p} (being the relaxation enhancement of solvent water protons at a 1 mM concentration of the paramagnetic chelate), is 3.7 mM^{-1} s^{-1} in water at 40 °C, in line with other macrocyclic Gd complexes.[24]

From a radiolabeling standpoint, the formation kinetics of DOTA chelates leave much to be desired, as either lengthy radiolabeling protocols and/or the use of elevated temperatures are required to achieve acceptable yields and specific activities.[2,4] The use of the HP-DO3A coordinating cage may bring some improvement in the radiolabeling of bioconjugates.

In an optimal BFCA, a spacer is inserted between the macrocyclic coordination cage and the TV to modulate the pharmacokinetic properties and biodistribution of the conjugate by changing its overall charge and hydrophilicity.[4,25] For this purpose, hydrocarbon, PEG or polypeptide spacers are usually employed. For instance, the macrocyclic chelate, often being bulkier than the vector itself, may strongly affect both the conjugation reaction and the bioconjugate interaction with the target; it is therefore advisable to distance it from the vector moiety.[26]

Herein, we report the synthesis and characterization of two new BFCAs based on the HP-DO3A chelating unit, featuring

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† Electronic supplementary information (ESI) available: HPLC and FPLC methods; ^{13}C, DEPT and ^{1}H NMR spectra. See DOI: 10.1039/b905369g

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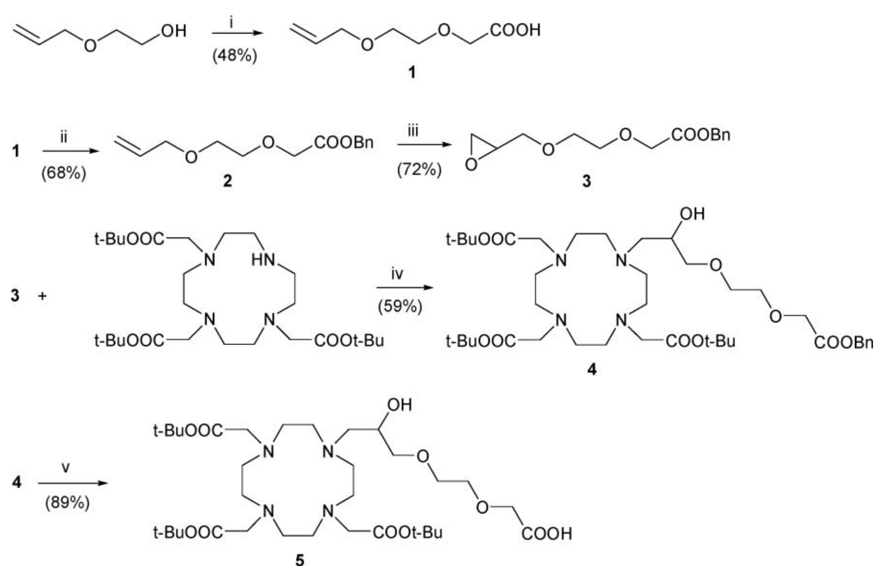
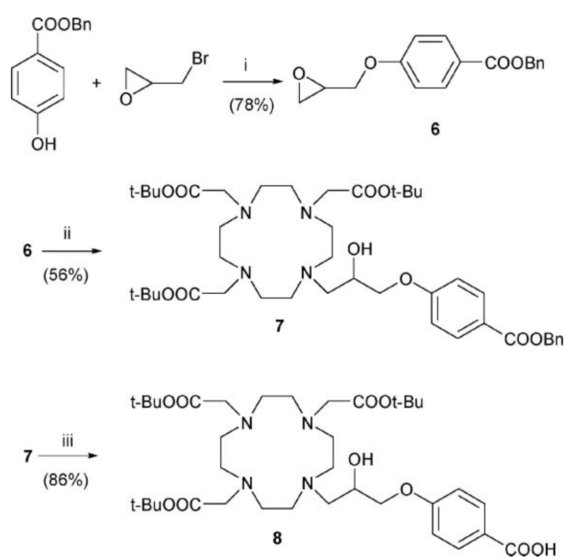


Scheme 1 The synthesis of bifunctional chelating agent **5**. Reagents and conditions: (i) Na, THF, $BrCH_2COONa$, room temperature for 16 h then reflux for 3 h; (ii) BnBr, DBU, toluene, room temperature, 2 h; (iii) 3-chloroperbenzoic acid, $CHCl_3$, room temperature, 48 h; (iv) Et_3N, CH_3CN, 50 °C, 32 h; (v) H_2, 10% Pd/C, MeOH, room temperature, 2 h. Yields are indicated in parentheses.

a free carboxyl group for conjugation to the amino groups of biomolecules. These BFCAs differ in the spacer interposed between the coordinating cage and the carboxyl group; the spacer is a diethylene glycol unit for compound **5** (Scheme 1) and an aromatic moiety for compound **8** (Scheme 2). BFCAs **5** and **8** have been successfully employed in solid-phase peptide synthesis (SPPS),[27] and conjugated to well-known peptides (*e.g.* bombesin[28] and cyclic RGD[29]) that specifically target cells over-expressing their corresponding receptors.[6–8] Moreover, we also exemplify here the conjugation of **8** to Lys[8]-oxytocin to obtain, after labelling with a suitable radionuclide, a product with potential applications in the diagnosis and therapy of tumors over-expressing oxytocin receptors.



Scheme 2 The synthesis of bifunctional chelating agent **8**. Reagents and conditions: (i) K_2CO_3, DMF, 70 °C, 5 h; (ii) DO3A tris-*tert*-butyl ester, CH_3CN, reflux, 16 h; (iii) H_2, 5% Pd/C, MeOH, room temperature, 8 h. Yields are indicated in parentheses.

Results and discussion

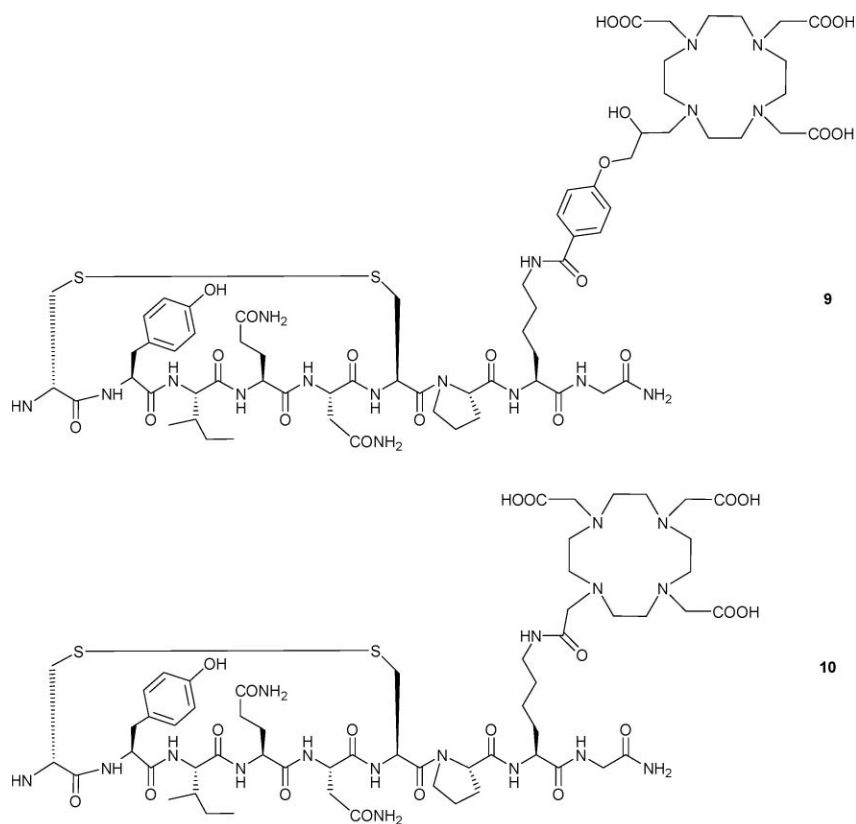
Ligand synthesis

To synthesize ligand **5**, containing the hydrophilic diethylene glycol spacer, we began by preparing sodium 2-allyloxyethanolate by deprotonation of 2-allyloxyethanol with sodium metal in THF, followed by a Wilkinson reaction with bromoacetic acid. The carboxyl group was then protected as a benzyl ester by a reaction with benzyl bromide in toluene with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as the base. At this point, the terminal alkene was oxidized in good yield to the correspondent epoxide with 3-chloroperbenzoic acid in $CHCl_3$. To open the epoxide, we used the secondary amino group of DO3A(tBu)$_3$ as the nucleophile and triethylamine as the base in warm CH_3CN. The alkylated macrocycle was isolated in 59% yield after purification by flash chromatography. Finally, **5** was obtained by catalytic hydrogenolysis of the benzyl ester under a hydrogen atmosphere at atmospheric pressure using Pd/C as the heterogeneous catalyst and MeOH as the solvent. The five-step synthesis had an overall 12% yield.

Bifunctional chelating ligand **8**, containing an aromatic spacer, was synthesized in a three-step sequence starting from epibromohydrin and benzyl 4-hydroxybenzoate. The resulting epoxide was reacted with DO3A(tBu)$_3$ to afford the protected precursor of **8** in good yield after purification by flash chromatography. Pd-catalyzed hydrogenolysis of the benzyl ester at room temperature and atmospheric pressure gave the final product in an overall 38% yield.

Conjugation to peptides and labelling

Since most peptides are prepared by SPPS, it is convenient to perform the conjugation step on the same solid support. This approach offers the well-known advantages of the SPPS technique, *i.e.* the attachment of the BFCA to a precise position on the peptide, purification of the conjugate by simple filtration and washing of the solid resin, simultaneous deprotection



Scheme 3 The structure of oxytocin conjugates **9** and **10**.

of the *tert*-butyl esters of the BFCA, removal of all the protecting groups on the peptide and cleavage from the resin. Therefore, **5** and **8** were designed in order to be used in SPPS with *in situ*-activation of the free carboxyl group with tetramethyluronium-type coupling agents such as HATU (*N*,*N*,*N′*,*N′*-tetramethyl-*O*-(7-azabenzotriazol-1-yl)uronium hexafluorophosphate) or HBTU (*N*,*N*,*N′*,*N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate).

Recently, we reported the coupling of **8** to the terminal NH_2 group of BBN[7-14]NH_2, a truncated sequence of bombesin (BBN).[28] BBN, a 14-amino acid peptide isolated from frog skin, is an analogue of the human gastrin-releasing peptide (GRP) that binds to GRP receptors with a high specificity and affinity. BBN has been much studied because it is known that several types of human cancer cells have over-expressed or uniquely expressed GRP receptors.[30,31]

Herein, we report the synthesis of **9**, a conjugate of **8** and Lys^8-oxytocin, as well as its labelling with ^{111}In(III) (Scheme 3). Oxytocin, a cyclic nonapeptide hormone secreted by the neurohypophysis, acts on target cells by binding to specific G-protein-coupled receptors.[32] Oxytocin receptors are normally expressed on endometrial cells, while are over-expressed by several tumor types affecting organs such as breasts, brain and endometrium.[33] Our synthesis of the conjugate was accomplished by Fmoc-based SPPS,[34] incorporating into the peptide sequence Fmoc-Lys(ivDde)OH. This orthogonally-protected lysine has the α-nitrogen protected as 9-fluorenylmethoxycarbonyl (Fmoc) and the ε-nitrogen protected with a 1-(4,4-dimethyl-2,6-dioxo-cyclohexylidene)-3-methylbutyl group (ivDde).[35] The ivDde group is stable under Fmoc deprotection conditions but can be easily cleaved with a hydrazine solution before coupling to **8**. Cleavage from the resin with TFA also removed all the protecting groups; then, the intramolecular disulfide bond was formed by air oxidation in water/DMSO.[36] In order to assess the labelling ability of conjugate **9** with ^{111}In(III), a conjugate containing a DOTAMA chelating unit[10] instead of HP-DO3A was synthesised in a similar manner. Both conjugates **9** and **10** were then purified by preparative HPLC.

A labelling test with $^{111}InCl_3$ was performed on **9** and **10** in order to compare the chelating units HPDO3A and DOTA monoamide for their coordinating ability to ^{111}In(III) (Table 1). Interestingly, labelling was more efficient with **9** (reaching 94%) than **10** (83%). Since the spacers usually do not affect the metal complexation, this difference in labelling efficiency could only be ascribed to the faster In(III) complexation kinetics for HP-DO3A with respect to DOTAMA ligands. However, the results from fast protein liquid chromatography (FPLC) were slightly lower than those obtained by the Sep-Pak method, although the labelling efficiency was still higher for **9** than **10** (83 *vs.* 69%, respectively). Indeed, the Sep-Pak method is a rapid but rough separation method, and it can

Table 1 Labelling percentages obtained after incubation with $^{111}InCl_3$ in 0.1 M sodium acetate buffer at 90 °C for 30 min, followed by purification by Sep-Pak or FPLC

Product	Sep-Pak	FPLC
HPDO3A-Lys^8-oxytocin (**9**)	94	83
DOTAMA-Lys^8-oxytocin (**10**)	83	69

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only provide a first rapid estimation of the labelling efficiency. FPLC provides a more accurate value of the amount of labelled conjugate.

Since the linker between the chelating unit and the carboxyl group in **8** is quite hydrophobic and rigid, **5** was designed to have a more hydrophilic, as well as more flexible, spacer. Because angiogenesis has a crucial role in tumor growth, metastasis and inflammatory diseases, the coupling efficiency of **5** to an $\alpha_v\beta3$ integrin-binding "RGD type" peptide has been the object of a recent patent.[29] In fact, $\alpha_v\beta3$ integrin, a specific endothelial cell surface marker, is up-regulated in angiogenesis, whereas it is almost absent in quiescent blood vessels. For this reason it has been targeted with different imaging probes for molecular imaging applications.[37,38]

Conclusions

New bifunctional chelators **5** and **8**, based on the HP-DO3A coordinating cage, have been synthesized, and the different flexibility and hydrophilicity of their spacers was exploited to tune the characteristics of the final conjugates. Indeed, these two BFCAs have been successfully employed in SPPS, and coupled to a bombesin derivative[28] and a cyclic RGD peptide.[29] Here, in particular, we have reported the coupling of **8** to an oxytocin derivative. Labelling with In(III) proved to be more efficient for HP-DO3A conjugate **9** than the corresponding DOTAMA conjugate **10**. Considering that HP-DO3A gives metal complexes with a high thermodynamic stability (*e.g.* $\log K_{GdHP\text{-}DO3A} = 23.8$ and $\log K_{YHP\text{-}DO3A} = 22.2$),[41] bifunctional chelators **5** and **8** will find applications in the complexation of other metals (*e.g.* Gd(III)) or radiometals (*e.g.* ^{90}Y(III), ^{177}Lu(III)) to achieve new contrast agents for MRI and nuclear medicine.

Experimental

Materials and instrumentation

All chemicals were purchased from Sigma-Aldrich Srl (Milan, Italy) or Lancaster Synthesis GmbH (Mühlheim am Main, Germany) and were used without purification. Rink amide NovaGel™ resin and Fmoc amino acids were purchased from Novabiochem® (Merck KGaA, Darmstadt, Germany). 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid tris(1,1-dimethylethyl) ester (DOTA tris-*tert*-butyl ester)[14] and 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid tris(1,1-dimethylethyl) ester (DO3A tris-*tert*-butyl ester)[39] were prepared by following reported procedures. "Reagent B" is a mixture of TFA/phenol/water/triisopropylsilane = 88 : 5 : 5 : 2 (v/v).[40] Sep-Pak C18 cartridges were purchased from Waters (Vimodrone, Italy). NMR spectra were recorded on a Bruker Avance 400 instrument (operating at 9.4 T). Mass spectra were recorded with a ThermoFinnigan TSQ700 triple-quadrupole instrument equipped with an electrospray ionization source. HPLC was performed on a Merck-Hitachi L6200 and L6000 system equipped with a L4500 UV detector. FPLC was carried out on a Pharmacia Biotech LCC-501 system equipped with a UV detector (LKB-UV-MII, Pharmacia Biotech) and a radiodetector (Flow scintillation analyzer, Radiomatic 150 TR, Packard, Meriden, CT). All chromatographic methods are listed in the ESI.†

Preparation of 2-[2-(2-propenyloxy)ethoxy]acetic acid (1). 2-Allyloxyethanol (56.1 g, 0.55 mol) was dissolved in anhydrous THF (200 mL); then, metallic sodium (11.5 g, 0.50 mol) was added in small portions. The mixture was stirred at reflux for 3 h until complete dissolution of the sodium. At the same time, a solution of bromoacetic acid (69.9 g, 0.50 mol) in water (150 mL) was cooled in an ice bath and its pH was adjusted to 7 with 2 N NaOH (250 mL, 0.5 mol). The solution was then lyophilized and the solid suspended in anhydrous THF (200 mL). The suspension was added to the sodium alkoxide solution and the mixture stirred overnight at room temperature and then refluxed for 3 h. The solvent was removed, the residue dissolved in water (250 mL) and the solution extracted first with Et_2O (4 × 50 mL) then with CH_2Cl_2 (4 × 50 mL). The aqueous solution was brought to pH 1 by the addition of 37% HCl, then extracted with CH_2Cl_2 (4 × 50 mL). The organic phase was separated, dried (Na_2SO_4) and evaporated to give **1** (38.6 g, 48%) as a white solid. TLC (silica gel 60 F_{254}, 90 : 10 $CHCl_3$/MeOH, detection: 1% $KMnO_4$ in 1 M NaOH): $R_f = 0.6$; HPLC (system A): $R_t = 9.05$ min, purity 88%; ^{1}H NMR ($CDCl_3$): δ 9.21 (b, 1H), 5.92 (m, 1H), 5.28 (dd, $^3J = 17.2$ Hz, $^2J = 3.2$ Hz, 1H), 5.20 (dd, $^3J = 9.9$ Hz, $^2J = 3.2$ Hz, 1H), 4.18 (s, 2H), 4.05 (m, 2H), 3.77 (t, $^3J = 4.1$ Hz, 2H) and 3.64 (t, $J = 4.1$ Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 174.42, 134.45, 118.15, 72.68, 71.55, 69.51 and 68.91. MS (ESI+): calc. for $[C_7H_{12}O_4+H]^+$ 161.16, found 161.1; calc. for $[C_7H_{12}O_4+Na]^+$ 183.06, found 182.9.

2-[2-(2-Propenyloxy)ethoxy]acetic acid benzyl ester (2). A solution of benzyl bromide (6.57 g, 38.0 mmol) in toluene (30 mL) was added dropwise to a solution of compound **1** (5.13 g, 32.0 mmol) and DBU (4.87 g, 32.0 mmol) in toluene (70 mL). The mixture was stirred for 2 h, then filtered and evaporated under vacuum. The residue was dissolved in $CHCl_3$ (50 mL) and the solution washed with water (3 × 50 mL). The organic layer was separated, dried (Na_2SO_4) and evaporated under vacuum. The residue was purified by flash chromatography (silica gel column, eluent: 8 : 2 petroleum ether/EtOAc) to yield compound **2** (5.40 g, 68%) as a yellow oil. TLC (silica gel 60 F_{254}, 80 : 20 n-hexane/EtOAc, detection: 1% $KMnO_4$ in 1 M NaOH): $R_f = 0.16$; HPLC (system B): $R_t = 11.33$ min, purity 98.7%; ^{1}H NMR ($CDCl_3$): δ 7.37 (s, 5H), 5.92 (m, 1H), 5.28 (dd, $^3J = 17.9$ Hz, $^2J = 3.3$ Hz, 1H), 5.21 (s, 2H), 5.19 (dd, $^3J = 10.0$ Hz, $^2J = 3.3$ Hz, 1H), 4.23 (s, 2H), 4.03 (m, 2H), 3.71 (t, $^3J = 4.3$ Hz, 2H) and 3.65 (t, $^3J = 4.3$ Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 170.74, 135.88, 135.03, 128.96, 117.58, 77.47, 71.34, 69.89, 69.14 and 66.90. MS (ESI+): calc. for $[C_{14}H_{18}O_4+H]^+$ 273.11, found 273.0.

2-[2-(2-Oxiranylmethoxy)ethoxy]acetic acid phenylmethyl ester (3). A solution of 3-chloroperbenzoic acid (3.96 g, 23.0 mmol) in $CHCl_3$ (70 mL) was added dropwise to a solution of compound **2** (5.20 g, 21.0 mmol) in $CHCl_3$ (50 mL). The mixture was stirred for 48 h, then washed with 10% aq. Na_2SO_3 (3 × 150 mL) and water (3 × 150 mL). The organic layer was separated, dried (Na_2SO_4) and evaporated under vacuum. The residue was purified by flash chromatography (silica gel column, eluent: 1 : 1 CH_2Cl_2/Et_2O) to yield compound **3** (4.05 g, 72%) as a yellow oil. TLC (silica gel 60 F_{254}, 80 : 20 n-hexane/EtOAc detection: 1% $KMnO_4$ in 1 M NaOH): $R_f = 0.2$; HPLC (system B): $R_t = 9.69$ min, purity 90%; ^{1}H NMR ($CDCl_3$): δ 7.34 (s, 5H), 5.17 (s, 2H), 4.19 (s, 2H), 3.73 (m, 5H), 3.50 (m, 1H), 3.13 (m, 1H), 2.75 (m, 1H) and 2.58 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 170.65, 135.87, 128.99, 128.81, 128.79,

72.36,71.33, 71.13, 69.07, 66.87, 51.13 and 44.56. MS (ESI+): calc. for $[C_{14}H_{18}O_5+Na]^+$ 289.10, found 289.0.

10-[2-Hydroxy-3-[2-[2-oxo-2-(phenylmethoxy)ethoxy]ethoxy]propyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid 1,4,7-tris(1,1-dimethylethyl) ester (4). A solution of compound **3** (2.44 g, 9.20 mmol) in CH_3CN (30 mL) was added dropwise to a solution of DO3A tris-*tert*-butyl ester (3.19 g, 6.20 mmol) and Et_3N (1.27 mL, 9.20 mmol) in CH_3CN (30 mL). The mixture was stirred at 50 °C for 32 h, then cooled down to room temperature and evaporated under vacuum. The residue was dissolved in $CHCl_3$ (50 mL), and the solution washed with water (50 mL) and brine (50 mL). The organic layer was separated, dried (Na_2SO_4) and evaporated under vacuum. The residue was purified by flash chromatography (silica gel column, eluent: 90 : 10 : 1 $CHCl_3$/MeOH/NH_4OH) to yield compound **4** (2.87 g, 59%) as a yellow oil. TLC (silica gel 60 F_{254}, 90 : 10 : 1 $CHCl_3$/MeOH/NH_3, detection: 1% $KMnO_4$ in 1 M NaOH): $R_f = 0.33$; HPLC (system B): $R_t = 9.59$ min, purity 81%; ^{1}H NMR ($CDCl_3$): δ 7.34 (s, 5H), 5.16 (s, 2H), 4.13 (s, 2H), 3.68 (m, 9H), 3.53 (m, 4H), 3.34 (m, H) and 3.23 (d, $J = 17.1$ Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 173.20, 172.77, 172.52, 170.54, 129.01,127.41, 82.71, 82.42, 74.52, 71.22, 70.94, 68.98, 66.91, 56.87, 56.36, 55.91, 52.90, 52.60, 50.98, 49.67, 48.91, 28.53 and 28.30. MS (ESI+): calc. for $[C_{40}H_{68}N_4O_{11}+Na]^+$ 803.48, found 803.5.

10-[3-[2-(Carboxymethoxy)ethoxy]-2-hydroxypropyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid 1,4,7-tris(1,1-dimethylethyl) ester (5). 10% Pd/C (386 mg) was added to a solution of compound **4** (1.93 g, 2.47 mmol) in MeOH (50 mL). The reaction mixture was stirred under a hydrogen atmosphere for 2 h, then filtered through a Millipore® apparatus (FH 0.5 μm) and evaporated to yield **5** (1.53 g; 89%) as a colorless oil.

TLC (silica gel 60 F_{254}, 80 : 20 $CHCl_3$/MeOH, detection: 1% $KMnO_4$ in 1 M NaOH): $R_f = 0.42$; HPLC (system B): $R_t = 3.52$ min, purity 90%; ^{1}H NMR ($CDCl_3$): δ 4.34 (br, 2H), 4.13 (m, 3H), 3.76 (m, 2H), 3.69 (br, 10H), 3.51 (br, 2H), 3.47 (s, 4H), 3.43 (br, 2H), 3.37 (br, 2H), 3.09 (br, 4H), 2.86 (br, 4H), 1.47 (s, 9H) and 1.45 (s, 18H). ^{13}C NMR ($CDCl_3$): δ 172.77, 170.93, 82.49, 73.33, 71.51, 70.84, 69.52, 64.75, 56.56, 55.27, 53.00, 51.00, 28.53 and 28.49. MS (ESI+): calc. for $[C_{33}H_{62}N_4O_{11}+H]^+$ 691.45, found 691.5; calc. for $[C_{33}H_{62}N_4O_{11}+Na]^+$ 713.43, found 713.5.

4-(Oxyranylmethoxy)benzoic acid phenylmethyl ester (6). Anhydrous K_2CO_3 (11.8 g, 85.4 mmol) was added to a solution of benzyl 4-hydroxybenzoate (12.9 g, 56.7 mmol) and 1-bromo-2,3-epoxypropane (10.5 g, 76.7 mmol) in *N*,*N*-dimethylformamide (DMF) (60 mL). The mixture was stirred for 5 h at 70 °C and 12 h at room temperature. The solvent was then evaporated under vacuum and the crude product purified by flash chromatography (silica gel, petroleum ether/EtOAc 6 : 4, then petroleum ether/EtOAc 1 : 1). A second purification was required (silica gel, petroleum ether/EtOAc 6 : 4) to afford **6** (12.6 g, 78%) as a white solid. TLC (silica gel 60 F_{254}, petroleum ether/EtOAc 1 : 1, detection: UV 254 nm): $R_f = 0.45$; HPLC (system C): $R_t = 25.92$ min, purity 100%; ^{1}H NMR ($CDCl_3$): δ 8.06 (d, $J = 8.3$ Hz, 2H), 7.40 (m, 5H), 6.96 (d, $J = 8.3$ Hz, 2H), 5.36 (s, 2H), 4.30 (dd, $J = 11.2$ Hz, $J = 3.0$ Hz, 1H), 3.97 (dd, $J = 11.2$ Hz, $J = 3.0$ Hz, 1H), 3.37 (m, 1H), 2.92 (dd, $J = 4.9$ Hz, $J = 2.6$ Hz, 1H) and 2.77 (dd, $J = 4.9$ Hz, $J = 2.6$ Hz, 1H). ^{13}C NMR ($CDCl_3$): δ 166, 162, 137, 132, 129, 128, 123, 115, 69, 67, 50 and 45. MS (ESI+): calc. for $[C_{17}H_{16}O_4+Na]^+$ 307.09, found 307.0.

4-[2-Hydroxy-3-[4,7,10-tris[2-(1,1-dimethylethoxy)-2-oxoethyl]-1,4,7,10-tetrazacyclododec-1-yl]propoxy]benzoic acid phenylmethyl ester (7). A solution of **6** (1.1 g, 3.7 mmol) and DO3A tris-*tert*-butyl ester (1.6 g, 3.1 mmol) in CH_3CN (25 mL) was stirred for 16 h at reflux. The solvent was evaporated, the oily residue dissolved in $CHCl_3$ (25 mL), washed with H_2O (3 × 10 mL) and brine (2 × 10 mL), dried (Na_2SO_4) and evaporated under vacuum. The crude was purified by flash chromatography (silica gel, $CHCl_3$/MeOH 9 : 1) to afford **7** (1.3 g, 56%) as a pale yellow solid. TLC (silica gel 60 F_{254}, $CHCl_3$/MeOH/NH_4OH 6 : 4 : 2, detection: UV 254 nm, 1% $KMnO_4$ in 1 M NaOH): $R_f = 0.35$; HPLC (system C): $R_t = 13.32$ min, purity 98.3%; ^{1}H NMR ($CDCl_3$): δ 7.96 (d, $J = 8.52$ Hz, 2H), 7.35 (m, 5H), 6.92 (d, $J = 8.52$ Hz, 2H), 5.30 (s, 2H), 5.15 (d, $J = 7.57$ Hz, 1H), 4.35 (m, 1H), 4.30 (br, 1H), 4.01 (m, 1H), 3.75 (d, $J = 16.9$ Hz, 1H), 3.34 (d, $J = 17.9$ Hz, 1H), 3.22 (d, $J = 17.8$ Hz, 1H), 3.10–2.80 (enveloped signals, 10H), 2.52 (t, $J = 12.4$ Hz, 1H), 2.41 (t, $J = 12.6$ Hz, 1H), 2.36–2.21 (enveloped signals, 4H), 2.20–2.01 (enveloped signals, 5H), 1.46 (s, 18H) and 1.42 (s, 9H). ^{13}C NMR ($CDCl_3$): δ 173, 172, 156, 153, 137, 132, 129, 128, 123, 83, 82, 72, 67, 65, 57, 56, 55, 52, 50, 49 and 48. MS (ESI+): calc. for $[C_{43}H_{66}N_4O_{10}+H]^+$ 799.48, found 799.6; calc. for $[C_{43}H_{66}N_4NaO_{10}+Na]^+$ 821.47, found 821.5.

4-[2-Hydroxy-3-[4,7,10-tris[2-(1,1-dimethylethoxy)-2-oxoethyl]-1,4,7,10-tetrazacyclododec-1-yl]propoxy] benzoic acid (8). 5% Pd/C (310 mg) was added to a solution of **7** (2.2 g, 2.8 mmol) in MeOH (50 mL) and the mixture was stirred under a hydrogen atmosphere at room temperature for 8 h. Then, more 5% Pd/C (310 mg) was added and the mixture was stirred for a further 8 h. The catalyst was then filtered off (paper filter, then Millipore FH 0.5 μm filters) and the solvent evaporated to afford **8** (1.7 g, 86%) as a white solid. TLC (silica gel 60 F_{254}, $CHCl_3$/MeOH/NH_4OH 9 : 1 : 0.1, detection: 1% $KMnO_4$ in 1 M NaOH): $R_f = 0.15$; HPLC (system C): $R_t = 10.12$ min, purity 99%; ^{1}H NMR ($CDCl_3$): δ 7.76 (br, 2H), 6.70 (br, 2H), 4.67 (br, 2H), 4.42–4.16 (enveloped signals, 2H), 4.10 (br, 2H), 4.03–3.49 (enveloped signals, 8H), 3.36 (br, 3H), 3.10 (br, 4H), 2.78 (br, 6H) and 1.40 (s, 27H). ^{13}C NMR ($CDCl_3$): δ 171, 162, 132, 123, 114, 82, 70, 58, 57, 55, 53, 52, 50, 48 and 29. MS (ESI+): calc. for $[C_{36}H_{60}N_4O_{10}+H]^+$ 709.44, found 709.5; calc. for $[C_{36}H_{60}N_4O_{10}+Na]^+$ 731.42, found 731.6.

Resin-anchored oxytocin peptide

In a vessel suitable for SPPS, Fmoc amino acid (2.4 mmol), *N*-hydroxybenzotriazole (HOBt) (0.37 g, 2.4 mmol) and *N*,*N*′-diisopropylcarbodiimide (DIC) (0.38 mL, 2.4 mmol) were added to a suspension of Rink amide NovaGel™ resin (1.0 g, 0.6 mmol) in *N*,*N*-dimethylacetamide (DMAC) (15 mL). The mixture was shaken for 3 h at room temperature, the liquid flushed off and the resin washed with DMAC (5 × 45 mL). The resin was then shaken with 50% morpholine in DMAC (15 mL) for 15 min, the liquid flushed off and fresh 50% morpholine in DMAC (15 mL) added. The suspension was shaken for 20 min more, the liquid flushed off and the resin washed with DMAC (5 × 15 mL). This procedure was applied sequentially using the following amino acids: *N*-α-Fmoc-glycine, *N*-α-Fmoc-*N*-ε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl-L-lysine,

N-α-Fmoc-L-proline, *N*-α-Fmoc-*S*-trityl-L-cysteine, *N*-α-Fmoc-*N*-β-trityl-L-asparagine, *N*-α-Fmoc-*N*-γ-trityl-L-glutamine, *N*-α-Fmoc-L-isoleucine and *N*-α-Fmoc-*O*-*tert*-butyl-L-tyrosine. In the last coupling reaction, *N*-α-Boc-*S*-trityl-L-cysteine (1.12 g; 2.4 mmol), HOBt (0.37 g, 2.4 mmol) and DIC (0.38 mL, 2.4 mmol) were added to the resin in DMAC (15 mL). The mixture was then shaken for 3 h at room temperature, the liquid flushed off and the resin washed with DMAC (5 × 15 mL) then CH_2Cl_2 (5 × 15 mL) and finally vacuum-dried. The resin-anchored oxytocin peptide was then used in subsequent steps without further purification.

8-L-[*N*6-[4-[2-Hydroxy-3-[4,7,10-tris(carboxymethyl)-1,4,7,10-tetrazacyclododec-1-yl]propoxy]benzoyl]lysine]oxytocin (9). The resin-anchored oxytocin peptide (0.5 g, 0.3 mmol) was shaken with 10% hydrazine in DMF (7 mL) for 15 min, the liquid flushed off and fresh 10% hydrazine in DMF (7 mL) added. The suspension was stirred for another 20 min, the liquid was then flushed off and the resin washed with DMF (5 × 7 mL). Bifunctional chelate **8** (0.9 g, 1.2 mmol), HOBt (0.2 g, 1.2 mmol), DIC (0.2 mL, 1.2 mmol), *N*-ethyldiisopropylamine (0.4 mL, 2.4 mmol) and DMAC (7 mL) were added to the resin. The mixture was shaken for 40 h at room temperature, the liquid flushed off and the resin washed with DMAC (5 × 7 mL), CH_2Cl_2 (5 × 7 mL) and vacuum dried. The resin was shaken in a flask with "reagent B" (25 mL) for 4.5 h; the mixture was then filtered and the filtrate evaporated under reduced pressure. The resulting oily crude product was treated with Et_2O (5 mL) to give a precipitate that was collected by centrifugation, washed with Et_2O (4 × 20 mL) and vacuum dried. The crude product was dissolved in H_2O (2 L) and DMSO (45 mL), the pH adjusted to 8.5 by the addition of 25% aq. NH_4OH and the solution stirred for 48 h at room temperature; it was then concentrated and purified by preparative HPLC (system F). The fractions containing the product were collected and lyophilised to give **9** as a white fluffy solid (66 mg, 7%; purity according to HPLC system D was >99%; R_t = 6.67 min). MS (ESI+): calc. for $[C_{67}H_{101}N_{17}O_{21}S_2+H]^+$ 1544.69, found 1544.8.

8-L-[*N*6-[[4,7,10-Tris(carboxymethyl)-1,4,7,10-tetrazacyclododec-1-yl]acetyl]lysine]-oxytocin (10). The resin-anchored oxytocin peptide, obtained as described above (1.0 g, 0.6 mmol), was shaken with 10% hydrazine in DMF (15 mL) for 15 min, the liquid flushed off and fresh 10% hydrazine in DMF (15 mL) added. The suspension was stirred for another 20 min, the liquid was then flushed off and the resin washed with DMF (5 × 15 mL). DOTA tris-*tert*-butyl ester (1.56 g, 2.4 mmol), HOBt (0.37 g, 2.4 mmol), DIC (0.38 mL, 2.4 mmol), *N*-ethyldiisopropylamine (0.82 mL, 4.8 mmol) and DMAC (15 mL) were added to the resin. The mixture was shaken for 24 h at room temperature, the liquid flushed off and the resin washed with DMAC (2 × 15 mL), CH_2Cl_2 (5 × 15 mL) and vacuum dried. The resin was shaken in a flask with "reagent B" (25 mL) for 4 h; it was then filtered and the filtrate evaporated under reduced pressure. The resulting oily crude product was treated with Et_2O (5 mL) to give a precipitate that was collected by centrifugation, washed with Et_2O (5 × 5 mL) and vacuum-dried. The crude product was dissolved in H_2O (8 L) and DMSO (420 mL), the pH adjusted to 8.5 by the addition of 25% aq. NH_4OH and the solution stirred for 48 h at room temperature; it was then concentrated and purified by preparative HPLC (system G). The fractions containing the product were collected and lyophilised to give **10** as a white fluffy solid (59 mg, 6.9%; purity according to HPLC system E was 98%, R_t = 6.07 min). MS (ESI+): calc. for $[C_{59}H_{93}N_{17}O_{19}S_2+H]^+$ 1408.63, found 1409; calc. for $[C_{59}H_{93}N_{17}O_{19}S_2+Na]^+$ 1430.62, found 1431.0.

Labelling test

The labelling test with $^{111}InCl_3$ was performed by incubating 5 μg of conjugate with 500 μCi of ^{111}In(III) at 90 °C for 30 min in 0.1 M sodium acetate buffer (pH 5) in the presence of gentisic acid (0.26 M) as a radical scavenger. The labelling percentage was determined both by the Sep-Pak method and by FPLC. A small amount of EDTA solution was added to the incubation medium in order to complex excess ^{111}In(III). The sample was loaded onto Sep-Pak and eluted first with 0.1 M sodium acetate to collect a fraction containing the complex $[^{111}InEDTA]^-$ (F1), then with MeOH to collect a second fraction containing the labelled oxytocin conjugate (F2). The labelling percentage was calculated as follows:

$$Labelling\ percentage = \frac{F_2\,activity}{(F_1 + F_2)\,activities}$$

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References

1 M. Woods and A. D. Sherry, *Chim. Oggi*, 2005, **23**, 31.
2 S. Liu, *Chem. Soc. Rev.*, 2004, **33**, 445.
3 J. Fichna and A. Janecka, *Bioconjugate Chem.*, 2003, **14**, 3.
4 S. Liu and D. S. Edwards, *Bioconjugate Chem.*, 2001, **12**, 7.
5 D. E. Milenic, E. D. Brady and M. W. Brechbiel, *Nat. Rev. Drug Discovery*, 2004, 488.
6 R. E. Weiner and M. L. Thakur, *Appl. Radiat. Isot.*, 2002, **57**, 749.
7 M. Langer and A. G. Beck-Sickinger, *Curr. Med. Chem.: Anti-Cancer Agents*, 2001, **1**, 71.
8 A. Heppeler, S. Froidevaux, A. N. Eberle and H. R. Maecke, *Curr. Med. Chem.*, 2000, **7**, 971.
9 Z. Zhang, S. A. Nair and T. J. McMurry, *Curr. Med. Chem.*, 2005, **12**, 751.
10 M. Woods, Z. Kovacs and A. D. Sherry, *J. Supramol. Chem.*, 2002, **2**, 1.
11 The Lanthanides and Their Interrelations with Biosystems, *Metal Ions in Biological Systems*, ed. A. Sigel and H. Sigel, CRC Press, Boca Raton, FL, 2003, vol. 40.
12 A. E. Martell and R. M. Smith, *Critical Stability Constants*, Plenum Press, New York, 1974, vol. 4.
13 M. L. De León Rodriguez and Z. Kovacs, *Bioconjugate Chem.*, 2008, **19**, 391.
14 A. Heppeler, S. Froidevaux, H. R. Maecke, E. Jermann, M. Behe, P. Powell and M. Hennig, *Chem.–Eur. J.*, 1999, **5**, 1974.
15 K. P. Eisenwiener, P. Powell and H. R. Maecke, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 2133.
16 A. Barge, L. Tei, D. Upadhyaya, F. Fedeli, L. Beltrami, R. Stefanìa, S. Aime and G. Cravotto, *Org. Biomol. Chem.*, 2008, **6**, 1176.
17 S. Knor, A. Modlinger, T. Poethko, M. Schottelius, H.-J. Wester and H. Kessler, *Chem.–Eur. J.*, 2007, **13**, 6082.
18 S. J. Kline, D. A. Betebenner and D. K. Johnson, *Bioconjugate Chem.*, 1991, **2**, 26.
19 T. J. McMurry, M. W. Brechbiel, K. Kumar and O. A. Gansow, *Bioconjugate Chem.*, 1992, **3**, 108.
20 M. R. Lewis and J. E. Shively, *Bioconjugate Chem.*, 1998, **9**, 72.
21 L. Li, S.-W. Tsai, A.-L. Anderson, D. A. Keire, A. A. Raubitschek and J. E. Shively, *Bioconjugate Chem.*, 2002, **13**, 110.
22 M. F. Tweedle, *Eur. Radiol.*, 1997, **7**((Suppl. 5)), S225.

23 K. Kumar, C. A. Chang, L. C. Francesconi, D. D. Dischino, M. F. Malley, J. Z. Gougoutas and M. F. Tweedle, *Inorg. Chem.*, 1994, **33**, 3567.
24 P. Caravan, J. J. Ellison, T. J. McMurry and R. B. Lauffer, *Chem. Rev.*, 1999, **99**, 2293.
25 S. M. Quadri and H. M. Vriesendorp, *Q. J. Nucl. Med.*, 1998, **42**, 250.
26 S. Geninatti Crich, C. Cabella, A. Barge, S. Belfiore, C. Ghirelli, L. Lattuada, S. Lanzardo, A. Mortillaro, L. Tei, M. Visigalli, G. Forni and S. Aime, *J. Med. Chem.*, 2006, **49**, 4926.
27 *Solid-Phase Synthesis: A Practical Guide*, ed. S. A. Kates and F. Albericio, CRC Press, Boca Raton, FL, 2000.
28 E. Cappelletti, L. Lattuada, K. Linder, E. Marinelli, P. Nanjappan, N. Raju, R. Swenson and M. Tweedle, *Int. Pat. Appl.*, WO2004065407, 2004.
29 L. Lattuada, P. Morosini, F. Uggeri, S. Aime, E. Terreno, D. Delli Castelli, C. Scolastico, L. Manzoni and D. Arosio, *Int. Pat. Appl.*, WO2006095234, 2006.
30 C. J. Smith, W. A. Volkert and T. J. Hoffman, *Nucl. Med. Biol.*, 2003, **30**, 861.
31 L. E. Lantry, E. Cappelletti, M. E. Maddalena, J. S. Fox, W. Feng, J. Chen, R. Thomas, S. M. Eaton, N. J. Bogdan, T. Arunachalam, J. C. Reubi, N. Raju, E. C. Metcalfe, L. Lattuada, K. E. Linder, R. E. Swenson, M. F. Tweedle and A. D. Nunn, *J. Nucl. Med.*, 2006, **47**, 1144.
32 G. Gimpl and F. Fahrenholz, *Physiol. Rev.*, 2001, **81**, 629.
33 P. Cassoni, E. Fulcheri, M. L. Carcangiu, A. Stella, S. Deraglio and G. Bussolati, *J. Pathol.*, 2000, **190**, 470.
34 *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*, ed. W. C. Chan and P. D. White, Oxford University Press, Oxford, 2000.
35 S. R. Chhabra, B. Hothi, D. J. Evans, P. D. White, B. W. Bycroft and W. C. Chan, *Tetrahedron Lett.*, 1998, **39**, 1603.
36 J. P. Tam, C.-R. Wu, W. Liu and J.-W. Zhang, *J. Am. Chem. Soc.*, 1991, **113**, 6657.
37 M. Neeman, A. A. Gilad, H. Dafni and B. Cohen, *J. Magn. Reson. Imaging*, 2007, **25**, 1.
38 E. Garanger, D. Boturyn and P. Dumy, *Anticancer Agents Med. Chem.*, 2007, **7**, 552.
39 R. Ranganathan, E. Marinelli, R. Pillai and M. Tweedle, *Int. Pat. Appl.*, WO 9527705, 1995.
40 N. A. Sole and G. Barany, *J. Org. Chem.*, 1992, **57**, 5399.
41 K. Kumar, C. A. Chang, L. C. Francesconi, D. D. Dischino, M. F. Malley, J. Z. Gougoutas and M. F. Tweedle, *Inorg. Chem.*, 1994, **33**, 3567.

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