Solid-Phase Synthesis of DOTA–Peptides

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Abstract: A general synthetic route to two DOTA-linked *N*-Fmoc amino acids (DOTA-F and DOTA-K) is described that allows insertion of DOTA at any *endo*-position within a peptide sequence. Three model pentapeptides were prepared to test the general utility of these derivatives in solid-phase peptide synthesis. Both DOTA derivatives reacted smoothly by means of standard HBTU activation chemistry to the point of insertion of the DOTA amino acid, but extension of the peptide chain beyond the DOTA-amino acid insertion required the use of preactivated *C*-pentafluorophenyl ester *N*-α-Fmoc amino acids. Three Gal-80 binding peptides (12-mers) were then prepared by using this methodology with DOTA positioned either at the N terminus or at one of two different internal positions;the binding of the resulting GdDOTA-12-mers to Gal-80 were compared. The methodology described here allows versatile, controlled introduction of DOTA into any location within a peptide sequence. This provides a potential method for the screening of libraries of DOTA-linked peptides for optimal targeting properties.

Keywords: gadolinium complexes · macrocyclic ligands · peptides · solid-phase synthesis

Introduction

A number of low-molecular-weight peptides have been identified for targeting to specific cell receptors or macromolecules. Early applications of such systems have focused on peptide radiopharmaceuticals for cancer diagnosis and therapy,[1–7] but reports of peptide-based targeting vectors are beginning to appear in the molecular imaging literature, particularly those applications involving optical and magnetic resonance (MRI) imaging.[8–11] Peptide-based pharmaceuticals offer some advantages over typical organic-targeting analogues in that they can be prepared in reasonable quantities by using well-developed solid-phase peptide synthesis (SPPS) methodologies.[12,13] Established combinatorial techniques can then be used to create libraries of peptides for screening purposes.[14] Phage display techniques are particularly useful for screening peptide libraries for targets of specific cell types, tissues, or even individual macromolecules.[15,16] The rapid development of such procedures has made metal-ion-conjugated peptides ideal agents for diagnostic and therapeutic biomedicine. Several types of ligands including DOTA, DTPA, NOTA and TETA,[7] have been attached to peptides. DOTA is of particular interest, since this macrocyclic ligand forms complexes with a variety of metal ions with exceptionally high binding affinities and kinetic stabilities.[17] DOTA–peptides have been prepared by conjugating unprotected DOTA[18–20] or, more conveniently, DOTA-tris(*t*Bu) esters[8,11,21–24] to the N terminus or a Lys–ε–NH_2 residue of resin bound peptides. In a different approach, DOTA–peptides were obtained by synthesizing the DOTA moiety in a stepwise manner on the N terminus of peptides bound to the solid support.[25] A recent communication reported a DOTA–peptide nucleic acid conjugate that was synthesized by using a DOTA-Lys derivative that allows the DOTA to be incorporated into any sequence position.[26]

Recently, we demonstrated[11] that MRI can detect the binding event of a Gd^{3+}–DOTA-labeled peptide to its target protein (Gal-80 is a protein involved in regulation of galactose metabolism[27]). DOTA was added to the N-terminal position of a twelve residue peptide (TFDDLFWKEGHR)[28,29] by treating the resin-bound peptide with DOTA-tris(*t*Bu) ester. Although the resulting DOTA–peptide displayed a ~5-fold lower affinity for Gal-80, the binding event could still be detected at 25 μM, substantially lower than the detection limits of typical Gd^{3+}–

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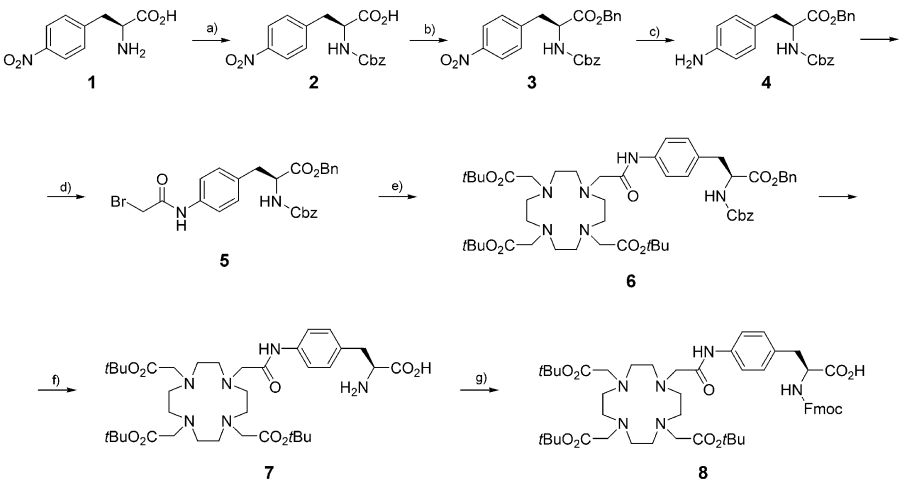
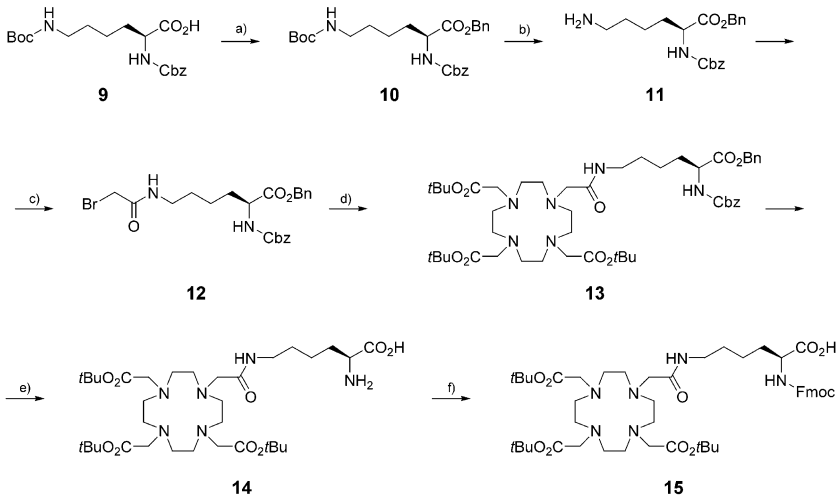
based contrast agents. Given a recent report of antibody recognition of simple LnDOTA chelates,[30] it became evident that one might be able to lower the MRI detection limit of a Gd–peptide/protein recognition process even further if GdDOTA was located in an *endo*-peptide position forming part of the protein binding site. To make the method as versatile as possible, we prepared tris(*t*Bu) ester DOTA derivatives of *N*-α-Fmoc amino acids that could be substituted for standard *N*-α-Fmoc amino acids in SPPS, making it possible to position DOTA anywhere in a peptide sequence. The methods reported here should be useful for generating Ln^{3+}-DOTA–peptide libraries for screening purposes.



Scheme 1. Synthesis of DOTA–phenylalanine derivative **(8)**. Reagents and conditions: a) benzyl chloroformate, Na_2CO_3, dioxane, water, 0 °C; b) benzylbromide, DIEA; c) Zn, EtOH, AcOH, 60 °C; d) bromoacetylbromide, DIEA, CH_2Cl_2; e) DO3A-(tBu)$_3$, K_2CO_3, CH_3CN; f) H_2, Pd on C, *i*PrOH; g) Fmoc-chloride, Na_2CO_3, dioxane, water.



Scheme 2. Synthesis of DOTA-lysine derivative **15**. Reagents and conditions: a) benzyl bromide, DIEA, MeCN; b) TFA, CH_2Cl_2; c) bromoacetyl bromide, DIEA, CH_2Cl_2; d) DO3A-(tBu)$_3$, K_2CO_3, MeCN; e) H_2, Pd on C, *i*PrOH; f) Fmoc-Cl, Na_2CO_3, dioxane, water.

Results and Discussion

Preparation of DOTA-conjugated amino acids: Using the amino acid sequence of the Gal-80 binding peptide identified previously by phage display (TFDDLFWKEGHR), we first chose to prepare three DOTA–peptides differing only in the location of DOTA within this sequence. Conjugation of DOTA to the N terminus of the resin-bound peptide was easily accomplished using DOTA-tris(*t*Bu) ester and standard Fmoc chemistry.[11] To introduce DOTA into any *endo*-position within the sequence requires DOTA amino acid derivatives fully compatible with SPPS conditions. This includes *N*-α-Fmoc protection, a free carboxyl group for coupling, and acid labile protection of the remaining groups on DOTA. Two model amino acid derivatives (DOTA-F and DOTA-K) were chosen to illustrate the method. Lysine and phenylalanine were selected to test the feasibility of applying the proposed chemistry to a charged/aliphatic versus an aromatic sidechain. For both derivatives, the α-amino and carboxylic groups of the parent amino acids were protected with the carboxybenzyl (Cbz) and benzyl (Bn) protecting groups, respectively. These two protecting groups can be removed later by catalytic hydrogenation (Schemes 1 and 2). The fluorenylmethyoxycarbonyl (Fmoc) protecting group cannot be introduced at this point due to its susceptibility to hydrogenation.[31] For DOTA-F (Scheme 1), reduction of the nitro group of p-NO_2-phenylalanine to the amine followed by coupling with bromoacetyl bromide provided intermediate **5.** This was then coupled to DO3A-tris(*t*Bu) ester to give intermediate **6**. Removal of the Cbz and Bn groups by catalytic hydrogenation and introduction of the Fmoc protecting group yielded product **8** in approximately 70% overall yield. Synthesis of DOTA-K was accomplished by using a similar strategy starting with *N*-ε-Boc-protected lysine (Scheme 2). The resulting DOTA tris(*t*Bu) esteramino acid derivatives were then used to create DOTA–peptides by using SPPS methods.

Solid-phase peptide synthesis: To test the general reactivity of DOTA-F, three different peptide sequences, each containing five residues but with a variable DOTA-F position, were selected for synthesis. The reference peptides (GAADF, GAFDG, FAADG) were prepared first, giving an average yield of 65% after purification. Synthesis of (NH_2-F(DO-

TA)AADG-$CONH_2$) using standard Fmoc-protected amino acid chemistry proceeded smoothly but in those sequences where F-DOTA was located at the C-terminus or near the center of the sequence, the peptide sequence could not be extended beyond the F-DOTA position. Furthermore, attempts to add subsequent amino acids beyond F-DOTA using either HATU[32] or TFFH[33] as coupling agents were not successful. These coupling agents are reported[32,33] to have superior coupling capabilities over HBTU for synthesis of "difficult" peptide sequences.

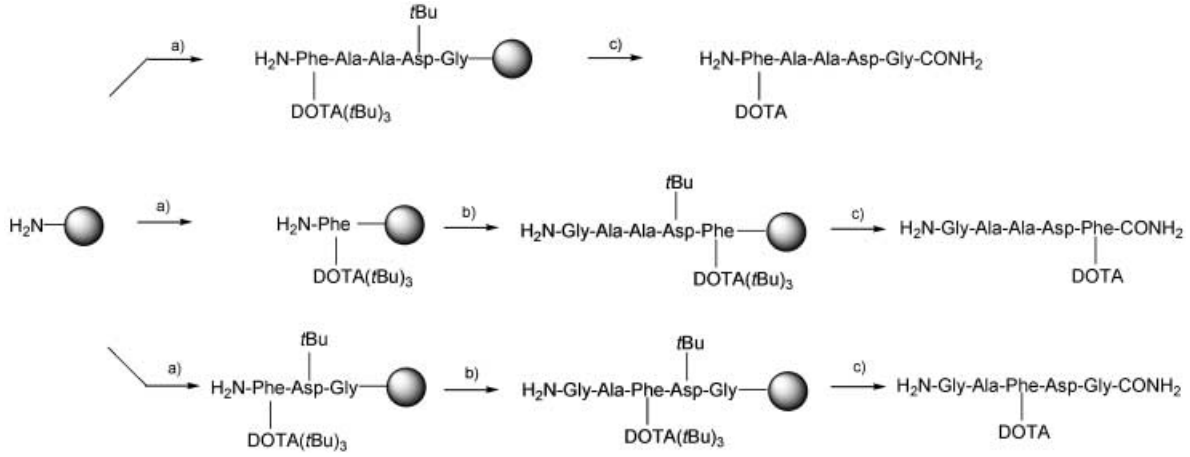
These unexpected results suggest that the bulky DOTA tris(*t*Bu) ester moiety on the peptide alters the coupling efficiency when using HBTU-type activating agents. The fact that *N*-acetyl (capped) derivatives (Ac-F(DOTA)-$CONH_2$ and Ac-F(DOTA)DG-$CONH_2$) were obtained as major products demonstrated that the Fmoc group of DOTA-F had been removed and that the N-terminal amino groups of these derivatives were available for reaction with anhydride. This suggested to us that a more reactive, pre-activated amino acid might be necessary to couple additional amino acids beyond the F-DOTA residue. Indeed, when *N*-α-Fmoc pentafluorophenyl ester (OPfp) derivatives were used, the remaining amino acids of the *endo*-DOTA–peptides were added with an average overall yield of 40% (Scheme 3). Similar chemistry was then used to prepare an analogous K-DOTA sequence (NH_2-FWK(DOTA)LG-$CONH_2$).

A recent report showed that *N*-α-Fmoc-DOTA-ε-lysine could be introduced into a peptide sequence by using standard HBTU-type activating agents when using a XAL-PEG-PS resin.[26] PEG-PS solid supports are prepared by grafting readily soluble polar PEG chains onto microporous polystyrene-*co*-divinylbenzene and have been shown to be superior to conventional resins for the synthesis of hydrophobic peptides.[35] This suggests that the DOTA tris(*t*Bu) estermoiety attached to peptides on the Rink amide resin causes the growing peptide to aggregate and that this aggregation may be prevented using a PEG-PS based resin.

DOTA conjugates of a Gal-80 binding peptide: This same methodology was then used to synthesize the three DOTA analogues of the Gal-80 peptide binding sequence, TFDDLFWKEGHR, shown in Table 1. After cleavage of these peptides from Rink resin using trifluoroacetic acid and purification by HPLC, the Gd^{3+} complexes were formed and the resulting GdDOTA–peptides were purified once again by HPLC. The association constants of each GdDOTA–peptide with Gal-80 were determined by competitive binding against fluorescein-TFDDLFWKEGHR-$CONH_2$ using fluorescence polarization.[11] A saturation experiment was first performed to determine the K_D for the fluorescein-BP/Gal-80 interaction. A fit of these data to a model that assumes 1:1 binding stoichiometry gave a K_D of 173 ± 13 nM,which is similar to the value obtained previously from a different protein preparation.[11] Competition binding experiments were then performed by starting with 2 nM fluorescein-BP and 130 nM Gal-80 and titrating in aliquots of either *N*-acetyl-BP, *exo*-GdDOTA-BP, *endo*-GdDOTA(F)-BP, or *endo*-GdDOTA(K)-BP. Competition binding curves for the first three systems are presented in Figure 1. Addition of *endo*-GdDOTA(K)-BP gave similar results as that shown for *endo*-GdDOTA(F)-BP. It was clear from these data that both *exo*-peptides, *N*-acetyl-BP and *exo*-GdDOTA-BP, compete effectively with fluorescein-BP, while the two *endo*-GdDOTA–peptides do not. The competitive binding constants, K_D', for the two *exo*-peptides were determined using Equation (1),[34] in which IC50 corresponds to the concentration of competing peptide required to decrease the fluorescence polarization by 50%. This fit-

Table 1. Derivatives of the Gal-80 binding peptide (BP).

Abbreviation	Peptide Sequence
Fluorescein-BP	Fluorescein-TFDDLFWKEGHR-$CONH_2$
N-acetyl-BP	Ac-TFDDLFWKEGHR-$CONH_2$
Exo-DOTA-BP	DOTA-TFDDLFWKEGHR-$CONH_2$
Endo-DOTA(K)-BP	Ac-TFDDLFW(K-DOTA)EGHR-$CONH_2$
Endo-DOTA(F)-BP	Ac-TFDDL(F-DOTA)WKEGHR-$CONH_2$



Scheme 3. Solid-phase peptide synthesis of three pentapetides with variable DOTA position. Reagents and conditions: a) Rink amide resin, Fmoc-amino acids, HBTU, HOBT, DIEA; b) Fmoc-amino acid pentafluorophenyl esters, HOBT; c) CF_3CO_2H, thioanisole, 1,2-ethanedithiol, anisole.

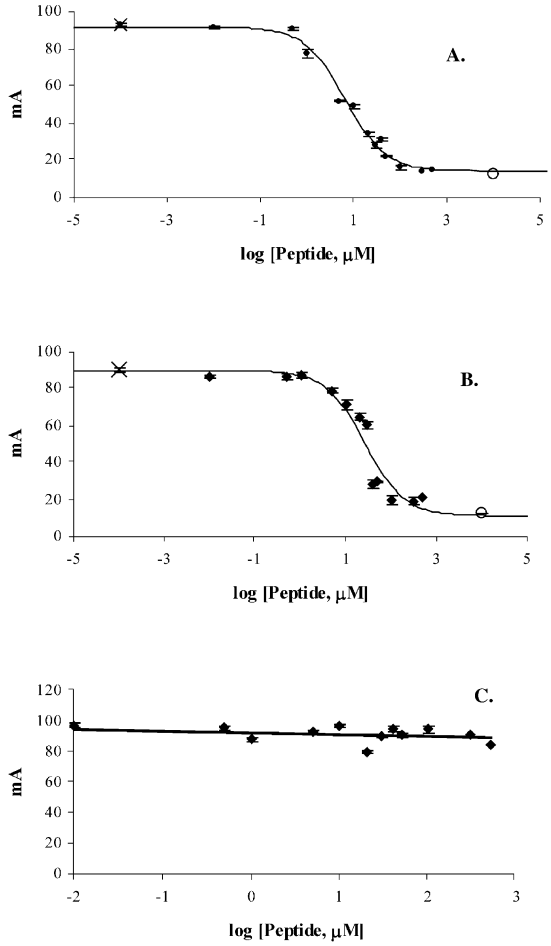


Figure 1. Changes in fluorescence polarization (measured in mA) upon incremental addition of either *N*-acetyl-BP (**A**), *exo*-GdDOTA-BP (**B**), or *endo*-GdDOTA(F)-BP (**C**) to a solution of 2 nM fluorescein-BP plus 130 nM Gal-80. [represents a data point where no competing peptide has been added, and ○ represents a data point where a large excess of competing peptide has been added and all fluorescein-BP is assumed to be free.

ting procedure gave a K_D' of $6.4 \pm 0.3\,\mu M$ for *N*-acetyl-BP and $25.9 \pm 0.9\,\mu M$ for *exo*-GdDOTA-BP. (This K_D value is ~10-fold higher than the value reported in reference 11 due to an error in the previous calculation.)

$$K_{D'(\text{competing peptide})} = \frac{IC_{50}}{1 + \left\{ [\textit{flourescein}\text{-BP}]/K_D(177\,nM) \right\}} \quad (1)$$

This illustrates that the chemical properties of the N-terminal group (fluorescein versus acetyl versus GdDOTA) has a dramatic effect on the resulting peptide/Gal-80 binding interaction either as a result of altered peptide conformation or by additional repulsive forces between the N-terminal moiety and the protein. This binding order suggests that hydrophobic interactions at the N-terminal site are more favorable (fluorescein-BP) than hydrophilic interactions (*exo*-GdDOTA-BP). Neither *endo*-GdDOTA(K)-BP nor *endo*-GdDOTA(F)-BP could significantly displace fluorescein-BP from Gal-80, even at very highly competitive concentrations. This indicates that the phenylalanine and lysine residues in the native peptide are especially critical to the protein–peptide interaction and that introduction of the GdDOTA moiety disrupts these crucial binding interactions. Even though the *exo*-GdDOTA-BP/Gal-80 binding constant is only ~26 μM, this interaction has been shown to be strong enough to allow detection of binding by MRI.[11]

Conclusion

The Gal-80 binding peptide, TFDDLFWKEGHR, was modified by introducing a DOTA-chelating group at three different residues (Thr1, Phe6, or Lys8). After addition of Gd^{3+} to each peptide–DOTA conjugate, competition binding experiments showed that the peptide labeled with GdDOTA at the N-terminal (T) had a reasonable affinity for Gal-80 ($K_a = 3.9 \times 10^4\,M^{-1}$), while those peptides labeled with GdDOTA at either Phe6 or Lys8 had no detectable binding affinity for Gal-80. This demonstrates that location of the GdDOTA moiety greatly affects the binding specificity of a peptide–protein system in which structure–activity relationships have not been previously well defined, and illustrates that screening libraries of DOTA-linked peptides may be useful in identifying targeted metal-ion-based systems for new MRI diagnostic applications.

Experimental Section

General procedures: 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro phosphate (HBTU), *N*-hydroxybenzotriazole (HOBT), Fmoc-L-amino acids, Fmoc-L-amino acid pentafluorophenyl esters (OPfp) and Rink amide resin were obtained from NovaBiochem (San Diego, CA). Fmoc-L-threonine (O*t*Bu)-OPfp was obtained by esterification of Fmoc-L-threonine(O*t*Bu) with pentafluorophenol (Lancaster) by using DCC (Sigma). 2-(1*H*-Azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and tetramethylfluoroformamidinium hexafluorophosphate (TFFH) were purchased from Applied Biosystems (Foster City, CA). 1,4,7-Tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane (DO3A-tris(*t*Bu)) ester was supplied by Macrocyclics (Dallas, TX). Silica gel (200–400 mesh, 60 Å) for column chromatography was purchased from Aldrich. TLC was conducted on Whatman precoated silica gel on polyester plates. All other chemicals and solvents were purchased from commercial sources and were used without further purification. ^1H and ^{13}C NMR spectra were taken using a JEOL Eclipse 270 MHz spectrometer with tetramethylsilane as the standard. Chemical shifts are reported as parts per million (ppm), with splitting patterns designated as singlet (s), doublet (d), triplet (t), multiplet (m), or doublet of doublets (dd). Coupling constants are reported in Hertz (Hz). No special experiments were performed to obtain definitive ^1H and ^{13}C peak assignments. Melting points were determined by using a Fisher Johns Melting Point Apparatus and are uncorrected. HPLC analysis was performed over a C18 reverse-phase column on an HP1100 HPLC instrument equipped with an automated injector and 1100 Series diode array UV/Vis detector. Peptide purification was performed on a semi-preparative HPLC (Waters 600) using a C18 reverse-phase column. Products were eluted at flow rates of $1\,mL\,min^{-1}$ (HP1100) or $10\,mL\,min^{-1}$ (Waters) using a linear gradient of H_2O/TFA(0.1%) and acetonitrile/H_2O/TFA (90/10/0.1).

***N*-α-Carbobenzyloxy-*p*-nitro-L-phenylalanine (2)**: *p*-Nitro-L-phenylalanine (**1**) (9.56 g, 45.5 mmol) was dissolved in a mixture of dioxane (80 mL) and Na_2CO_3 solution (16.5 g, 3 equiv, in 145 mL water) and cooled in an ice bath. Benzyl chloroformate (9.56 g, 95%, 1.05 equiv) in dioxane (100 mL) was added dropwise while stirring. The reaction mixture was allowed to stand at RT overnight. The solvent was removed by rotary evaporation, and the residue was dissolved in ethyl acetate

(100 mL), washed with water (50 mL), brine (2×50 mL) and water (2× 50 mL). The solvent was evaporated to give a clear oil that crystallized from pentane/CH_2Cl_2. The white solid was filtered off and dried under vacuum overnight to yield 15.02 g (95.6%) of product. M.p.: 126–127°C; ^{1}H NMR (270 MHz, $CDCl_3$, 25°C, TMS):: δ=8.07 (d, 2H), 7.33–7.29 (m, 7H), 5.38 (d, 1H), 5.06 (m, 2H), 4.71 (t, 1H), 3.29 ppm (m, 2H); ^{13}C NMR (67.5 MHz, $CDCl_3$, 25°C, TMS): δ=174.8, 155.9, 147.2, 143.6, 135.8, 130.4, 128.7–128.3, 123.8, 67.5, 54.4, 37.8 ppm. Elemental analysis calcd. (%) for $C_{17}H_{18}N_2O_6$: C 59.30, H 4.68, N 8.14. Found: C 59.01, H 4.60, N 8.11; MS-ESI: m/z: 367 (calcd.367) $[M+Na]^+$, 343 (calcd.343) $[M-H]^-$.

***N*-α-Carbobenzyloxy-*p*-nitro-L-phenylalanine benzyl ester (3)**: Compound (**2**) (14.90 g, 43.2 mmol) was suspended in acetonitrile (300 mL), then diisopropyl ethyl amine (6.42 g, 1.1 equiv) was added to give a yellow homogeneous solution. Benzyl bromide (8.30 g, 1.05 equiv) in acetonitrile (100 mL) was added and the flask was purged with argon and sealed. The reaction mixture was stirred at RT for 2 days. Solid formation was observed during the reaction. When the reaction was complete as monitored by TLC, the solvent was evaporated and the oily residue was dissolved in dichloromethane (500 mL) and washed with water (3× 400 mL). The organic phase was collected, and dried over Na_2SO_4, and the solvent was evaporated to yield a white solid. It was dried under vacuum overnight to give 17.9 g (95.1%) of product. R_f=0.68 (SiO_2 TLC; hexanes/ethyl acetate, 60:40); m.p. 84–85°C; ^{1}H NMR (270 MHz, $CDCl_3$ 25°C, TMS):: δ=7.96 (d, 2H), 7.35–7.32 (m, 10H), 7.08 (d, 2H,) 5.34 (d, 1H), 5.18 (m, 2H) 5.06 (m, 2H), 4.71 (t, 1H), 3.17 ppm (m, 2H); ^{13}C NMR (67.5 MHz, $CDCl_3$, 25°C, TMS):: δ=170.7, 155.5, 147.1, 143.5, 136.1, 134.8, 130.6, 128.9–128.3, 123.6, 67.7, 67.2, 54.5, 38.2 ppm. Elemental analysis calcd. (%) for $C_{24}H_{22}N_2O_6$: C 66.35, H 5.10, N 8.45. Found: C 66.00, H 4.98, N 8.48; MS-ESI: m/z: 457 (calcd.457) $[M+Na]^+$.

***N*-α-Carbobenzyloxy-*p*-amino-L-phenylalanine benzyl ester (4)**: Compound (**3**) (5.56 g, 12.8 mmol) and zinc dust (<10 micron) were suspended in absolute ethanol (60 mL). Glacial acetic acid (60 mL) was added and the reaction mixture was heated to 65°C with an oil bath and stirred for 2 h. The reaction mixture was cooled in an ice bath and crushed ice (20 mL) was added. The pH of the solution was adjusted to 9.0 by slow addition of a 9M NaOH solution. The resulting white thick slurry was diluted with water, and ethyl acetate (400 mL) was added. The solids were filtered out from the mixture and washed with ethyl acetate (~200 mL). The organic phase was washed with saturated $NaHCO_3$ solution (2× 200 mL), and brine (2×200 mL), and dried over K_2CO_3. Removal of the solvent gave a light yellow solid that was dried overnight under vacuum, which yielded 4.7 g (90.9%) of product. R_f=0.38 (SiO_2 TLC; hexanes/ethyl acetate, 60:40); m.p. 87–89°C; ^{1}H NMR (270 MHz, $CDCl_3$, 25°C, TMS): δ=7.29–7.27 (m, 10H), 6.75 (d, J=8.16 Hz, 2H), 6.46 (d, 2H), 5.53 (d, 1H), 5.07 (m, 4H), 4.60 (t, 1H), 3.65 ppm (brs, 2H), 2.94 (m, 2H); ^{13}C NMR (67.5 MHz, $CDCl_3$, 25°C, TMS): δ=171.7, 155.9, 145.8, 136.5, 135.4, 130.2, 128.6–128.1, 125.0, 115.3, 67.1, 66.9, 55.3, 37.3. Elemental analysis calcd. (%) for $C_{24}H_{24}N_2O_4$: C, 71.27; H, 5.98; N, 6.93. Found: C 69.28, H 5.90, N 6.91; MS-ESI: m/z: 405 (calcd.405) $[M+H]^+$.

***N*-α-carbobenzyloxy-*p*-bromoacetylamide-L-phenylalanine benzyl ester (5)**: Compound(**4**) (14.4 g, 35.6 mmol) was dissolved in CH_2Cl_2 (250 mL) and diisopropylethylamine (4.83 g, 1.05 equiv) was added. The reaction flask was purged with argon and cooled to −97°C (the freezing point of CH_2Cl_2) in liquid N_2. Bromoacetyl bromide (7.70 g, 1.05 equiv) was added when the mixture started to melt. The reaction mixture was allowed to warm to RT and stirring continued overnight. The mixture was washed with water (2×100 mL), 0.1M HCl (2×100 mL) and water (1× 100 mL), dried over K_2CO_3, and evaporated to yield 18.1 g (96.7%) of a white solid. R_f=0.50 (SiO_2 TLC; hexanes/ethyl acetate, 60:40). M.p. 134–135°C; ^{1}H NMR (270 MHz, $CDCl_3$, 25°C, TMS): δ=8.13 (s, 1H), 7.36–7.26 (m, 12H), 6.95 (d, J=7.92 Hz, 2H) 5.27 (d, 1H), 5.11 (m, 4H), 4.67 (t, 1H), 3.97 (s, 2H), 3.06 ppm (m, 2H); ^{13}C NMR (67.5 MHz, $CDCl_3$, 25°C, TMS): δ=171.3, 163.4, 155.7, 136.2, 136.1, 135.1, 132.5, 130.1, 128.7–128.2, 120.1, 67.4, 67.1, 54.9, 37.7, 29.5 ppm. Elemental analysis calcd. (%) for $C_{26}H_{25}BrN_2O_5$: C 59.44, H 4.80, N 5.33. Found: C 59.65, H 4.93, N 5.51; MS-ESI: m/z: 524 (calcd.524) $[M-H]^-$.

1,4,7-Tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane-10-*N*-α-carbobenzyloxy-*p*-acetylamide-L-phenylalanine benzyl ester (6): 1,4,7-tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane (2.62 g, 5.1 mmol) was dissolved in acetonitrile (100 mL) and solid K_2CO_3 was added (1.78 g, 12.9 mmol). The temperature was increased to 60°C using an oil bath, and the resultant slurry was stirred for 15 minutes. Compound (**5**) (2.68 g, 5.1 mmol) in acetonitrile (50 mL) was added dropwise over 20 minutes and the solution was stirred at 60°C for three days. After filtering out residual solids the solvent was evaporated to give a quantitative yield of yellow oil. ^{1}H NMR (270 MHz, $CDCl_3$, 25°C, TMS): δ=10.40 (s, 1H) 7.64 (d, J=7.18 Hz, 2H), 7.31 (m, 10H), 6.98 (d, J=7.18 Hz, 2H), 5.58 (d, 1H), 5.14–5.08 (m, 4H), 4.66 (m, 1H), 3.32–2.65 (overlapping multiplets, 26H), 1.39 ppm (s, 27H, –C(CH); ^{13}C NMR (67.5 MHz, $CDCl_3$, 25°C, TMS): δ=170.4, 169.9–169.5, 154.8, 136.8, 135.4, 134.3, 129.9, 128.8, 127.6–127.1, 118.8, 79.9, 66.1, 65.9, 59.3, 58.5, 55.9, 54.9, 54.2, 53.9, 51.7, 51.3, 50.9, 36.6, 27.3. Elemental analysis calcd. (%) for $C_{52}H_{74}N_6O_{11}$: C, 65.11; H, 7.78; N, 8.76. Found: C 65.13, H 7.71, N 8.79; MS-ESI: m/z: 960 (calcd.960) $[M+H]^+$.

1,4,7-tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane-10-*N*-α-(9-fluorenylmethoxy carbonyl)-*p*-acetylamide-L-phenylalanine (8): 1,4,7-tris-(*tert*-butylacetate)-1,4,7,10-tetraaza cyclododecane-10-*N*-α-carbobenzyloxy-*p*-acetylamide-L-phenylalanine benzyl ester (**6**), (3.01 g, 3.12 mmol) was dissolved in isopropanol (25 mL) and 10% Pd–C catalyst (0.6 g) was added. The reaction mixture was hydrogenated in a Parr hydrogenation apparatus at 40 psi for 3 days. The solution was filtered to remove the catalyst and the solvent was removed by rotary evaporation to yield 2.29 g (99.3%) of 1,4,7-tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane-10-*p*-acetylamide-L-phenylalanine (**7**). This product was used in the next step without further purification. A mixture of **7** (2.29 g, 3.12 mmol) in dioxane (35 mL) and aqueous Na_2CO_3 (0.99 g, 3 equiv, 35 mL) was added over 2 h to a solution of 9-fluorenylmethylchloroformate (0.87 g, 1.05 equiv) in dioxane (10 mL) with cooling in an ice bath. After completion, the solution was allowed to stand overnight at RT. The solvent was evaporated at 30°C under a high vacuum to give a water insoluble gummy residue. It was washed with water (2×10 mL) followed by ether (2×15 mL), yielding 2.61 g (87.3%) of a yellow solid product. ^{1}H NMR (270 MHz, $CDCl_3$, 25°C, TMS): δ=11.37 (s, 1H) 7.69–7.20 (m, 12H), 6.13 (s, 1H), 5.07 (bs, 1H), 4.41 (bs, 2H), 3.90–2.70 (overlapping multiplets, 26H), 1.41 (s, 27H). ^{13}C NMR (67.5 MHz, $CDCl_3$, 25°C, TMS): δ= 174.5, 172.2–170.6, 155.6, 145.4, 144.3, 137.3, 133.5, 129.9, 128.4, 128.2, 126.9.8, 125.5, 119.5, 81.6, 66.2, 56.9–47.3, 37.5, 27.9. Elemental analysis calcd. (%) for $C_{52}H_{72}N_6O_{11}.2H_2O$: C, 62.88; H, 7.71; N, 8.46. Found: C, 62.52; H, 7.87; N, 8.56. MS-ESI: *m/z:* 958 (calcd. 958) $[M+H]^+$.

***N*-ε-(*tert*-butoxycarbonyl)-*N*-α-carbobenzyloxy-L-lysine benzyl ester (10)**: *N*-ε-(*t*butoxycarbonyl)-*N*-α-carbobenzyloxy-L-lysine (**9**) (5.14 g; 13.5 mmol), di-isopropyl ethyl amine (1.83 g; 1.05 equiv) and benzyl bromide (2.48 g; 1.05 equiv) were dissolved in acetonitrile (120 mL). The reaction mixture was stirred under argon at RTfor two days. The solvent was removed by rotary evaporation and the residue was dissolved in dichloromethane (250 mL) and washed with water (4x100 mL). The organic phase was dried over Na_2SO_4, filtered and removal of the solvent gave a clear oil which crystallized from ether/hexanes yielding 6.28 g (98.7%) of a white solid. R_f=0.63 (SiO_2 TLC; hexanes/ethyl acetate, 60/40). M. p. 63–64°C. ^{1}H NMR (270 MHz, $CDCl_3$, 25°C, TMS): δ=7.26 (s, 10H.), 5.94 (d, 1H,) 5.06 (m, 4H), 4.34 (m, 1H), 2.97 (m, 2H), 1.80–1.64 (m, 4H), 1.36 (s, 11H). ^{13}C NMR (67.5 MHz, $CDCl_3$, 25°C, TMS): δ=172.5, 156.3, 136.2, 135.5, 128.6–128.1, 78.9, 67.0, 66.9, 54.0, 40.0, 31.8, 29.5, 28.5, 22.5. Elemental analysis calcd. (%) for $C_{26}H_{34}N_2O_6$: C, 66.36; H, 7.28; N, 5.95. Found: C, 66.16; H, 7.28; N, 5.98. MS-ESI: *m/z:* 493 (calcd. 493) $[M+Na]^+$.

***N*-α-carbobenzyloxy-L-lysine benzyl ester (11)**: N-ε-(*t*butoxycarbonyl)-*N*-α-carbobenzyloxy -L-lysine benzyl ester (**10**) (6.28 g; 13.4 mmol) was dissolved in a mixture of CH_2Cl_2 (50 mL) and TFA (50 mL). The solution was stirred at RT for 5 h. The solvent was evaporated by rotary evaporation and the product was dissolved in water (100 mL) and crushed ice (20 mL). The pH was adjusted to 9.5 by addition of Na_2CO_3. The aqueous layer was extracted with CH_2Cl_2 (2×500 mL). The organic phases were combined, washed with water (300 mL), dried over Na_2SO_4, filtered and removal of the solvent gave 4.68 g (94.6%) of a clear oil. R_f=0.83 (Al_2O_3 TLC; CH_2Cl_2/MeOH, 85/15). ^{1}H NMR (270 MHz, $CDCl_3$, 25°C, TMS): δ=7.26 (s, 10H), 5.79 (s, 1H) 5.11 (m, 4H), 4.37 (m, 1H), 2.54 (m, 2H), 1.80–1.64 (m, 4H), 1.35–1.17 (m, 2H). ^{13}C NMR (67.5 MHz, $CDCl_3$, 25°C, TMS): δ=172.5, 156.2, 136.4, 135.4, 128.6–128.1, 67.0, 66.9, 54.0, 41.8, 33.1, 32.3, 22.5. Elemental analysis calcd. (%) for $C_{21}H_{26}N_2O_4$: C, 68.09; H, 7.07; N, 7.56. Found: C, 68.15; H, 7.00; N, 7.53.

***N*-ε-bromoacetylamide-*N*-α-carbobenzyloxy-L-lysine benzyl ester (12)**: A solution of *N*-α-carbobenzyloxy-L-lysine benzyl ester (**11**) (4.670 g; 12.6 mmol) and di-isopropyl ethyl amine (1.713 g; 1.05 equiv) in CH_2Cl_2 (280 mL) was added to a solution of bromoacetyl bromide (2.729 g; 1.05 equiv) over one hour while maintaining the temperature at −30 °C. Then the solution was stirred at −30 °C for one hour and then allowed to stand at RT overnight. The organic phase was washed with water (2× 200 mL), 0.1 N HCl (2×200 mL) and water (2×200 mL), dried over Na_2SO_4, filtered and removal of the solvent gave 6.05 g (97.7 %) of a white solid. R_f=0.83 (Al_2O_3 TLC; CH_2Cl_2/MeOH, 85/15). M.p. 70–72 °C. ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS): δ=7.32 (s, 10H), 6.57 (s, 1H), 5.51 (s, 1H), 5.14 (m, 4H), 4.40 (m, 1H), 3.79 (s, 2H), 3.17 (m, 2H), 1.84–1.23 (m, 6H). ^{13}C NMR (67.5 MHz, $CDCl_3$, 25 °C, TMS): δ=172.3, 165.70, 156.1, 136.4, 135.3, 128.7–128.2, 67.3, 67.1, 53.7, 39.8, 32.2, 29.3, 28.7, 22.4. Elemental analysis calcd. (%) for $C_{23}H_{27}BrN_2O_5$: C, 56.22; H, 5.54; N, 5.70. Found: C, 56.09; H, 5.57; N 5.68. MS-ESI: *m/z:* 490 (calcd. 490) $[M-H]^-$.

1,4,7-tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane-10-*N*-ε-acetylamide-*N*-α-carbobenzyloxy-L-lysine benzyl ester (13): 1,4,7-tris(*t*butylacetate)-1,4,7,10-tetraazacyclo dodecane (1.08 g, 2.1 mmol) was dissolved in acetonitrile (50 mL) and K_2CO_3 (0.76 g, 5.53 mmol) was added. The reaction mixture was stirred at 60 °C for 15 minutes. *N*-ε-bromoacetylamide-Nα-carbobenzyloxy-L-lysine benzyl ester (**12**) (1.04 g, 2.1 mmol) was dissolved in acetonitrile (50 mL) and added dropwise over 20 minutes. The solution was stirred at 60 °C for 3 days. The solids were filtered out and the solvent was removed by rotary evaporation. The oily residue was dried to a constant mass to give the product quantitatively. ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS): δ=8.62 (s, 1H), 7.29–7.24 (m, 10H), 5.58 (d, 1H), 5.12 (s, 2H), 5.05 (s, 2H), 4.32 (m, 1H), 3.19, 3.16, 2.97, 2.80, 2.78, 2.64, 2.44 (overlapping multiplets, 26H), 1.84–1.50 (m, 6H), 1.38 (s, 27H). ^{13}C NMR (67.5 MHz, $CDCl_3$, 25 °C, TMS): δ=172.4, 172.2, 170.7, 170.6, 156.1, 136.4, 135.5, 128.6–128.1, 80.9, 67.1, 66.9, 60.4, 58.2 , 56.8, 56.3, 55.0, 54.1, 53.6, 52.6, 52.1, 38.7, 31.9, 29.5, 28.3, 22.8. Elemental analysis calcd. (%) for $C_{49}H_{76}N_6O_{11}$: C, 63.61; H, 8.28; N, 9.06. Found: C, 63.31; H, 8.22; N, 9.10. MS-ESI: *m/z:* 926 (calcd. 926) $[M+H]^+$.

1,4,7-tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane-10-*N*-ε-acetylamide-L-lysine (14): 1,4,7-tris(*t*butylacetate)-1,4,7,10-tetraazacyclododecane-10-*N*-ε-acetylamide-*N*-α-carbobenzyloxy-L-lysine benzyl ester (**13**) (1.02 g, 1.1 mmol) was dissolved in absolute ethanol (25 mL) and 10 % Pd–C catalyst (0.4 g) was added. The reaction mixture was hydrogenated in a Parr hydrogenation apparatus at 50 psi for 2 days. The solution was filtered to remove the catalyst and the solvent was removed by rotary evaporation. The residual oil was dried under vacuum to give 0.77 g (100 %) of a light yellow oil. The product was obtained as a white solid by dissolving the oil in CH_2Cl_2 and extracting it into water and lyophilizing the aqueous phase. R_f=0.21 (Al_2O_3 TLC; CH_2Cl_2/MeOH, 85/15). ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS): δ=9.14 (s, 1H), 4.08 (m, 1H), 3.69, 3.46, 3.27, 3.11, 2.90 (overlapping multiplets, 26H,), 1.97–1.56 (m, 6H), 1.44 (s, 27H). ^{13}C NMR (67.5 MHz, $CDCl_3$, 25 °C, TMS): δ=172.1, 168.8, 168.3, 164.6, 80.2, 79.9, 56.1, 55.1, 54.3, 53.3, 51.6, 50.4, 48.9, 47.4, 37.2, 28.8, 26.7, 20.7.

1,4,7-tris(*tert*-butylacetate)-1,4,7,10-tetraazacyclododecane-10-*N*-ε-acetylamide-*N*-α-(9-fluorenylmethoxycarbonyl)-L-lysine (15): 1,4,7-tris(*t*butylacetate)-1,4,7,10-tetraazacyclo dodecane-10-*N*-ε-acetylamide-L-lysine (**14**) (0.887 g, 1.27 mmol) was dissolved in dioxane (8 mL) and an aqueous solution of Na_2CO_3 (0.403 g, 3 equiv in 8 mL of water) was added. The reaction mixture was immersed in an ice bath and a solution of 9-fluorenylmethylchloroformate (0.328 g, 1.23 mmol) in dioxane (5 mL) was added under argon atmosphere. The solution was allowed to warm to RT and stirred overnight. The solvents were evaporated under high vacuum at 30 (C. The solid residue was washed with water and dried to a constant mass to give 1.01 g (86.4 %) of a white solid. R_f=0.67 (Al_2O_3 TLC; CH_2Cl_2/MeOH, 85/15), ^{1}H NMR (270 MHz, $CDCl_3$, 25 °C, TMS): δ= 7.64–7.11 (m, 8H), 4.17 (m, 1H), 3.20–2.42 (overlapping multiplets, 26H), 1.84–1.50 (m, 6H), 1.32 (s, 27H). ^{13}C NMR (67.5 MHz, $CDCl_3$, 25 °C, TMS): δ=173.8, 170.6, 170.2, 169.3, 154.0, 143.9,139.3, 125.7, 125.3, 123.8, 118.0, 79.9, 64.5, 54.3.0–45.5, 37.1, 30.2, 26.3, 20.3. Elemental analysis calcd. (%) for $C_{49}H_{74}N_6O_{11}$: C, 63.75; H, 8.08; N, 9.10. Found C, 63.51, H, 8.05, N, 9.15. MS-ESI: *m/z:* 922 (calcd. 922) $[M-H]^-$.

Solid-phase Peptide Synthesis: Solid-phase peptide synthesis was carried out either manually or in an Applied Biosystem ABI 433A Automatuzed Peptide Synthesizer following standard Fmoc protocols using Rink amide resin. Peptides were manually synthesized on a 0.1 mmol scale using single step couplings of two equivalent Fmoc-amino acids (or Fmoc-amino acid pentafluorophenyl esters), two equivalents coupling agent (HBTU/HOBT or HOBT with the pentafluorophenyl esters) and 6 equivalents di-isopropyl ethyl amine in DMF at RT. Free unreacted amino groups were capped with a 4.75 % v/v acetic anhydride solution in NMP. During manual synthesis, coupling and capping completion was monitored by the ninhydride test. A 10-fold excess of reagents was used for the automated synthesizer. Following linear peptide assembly, cleavage from the resin and final deprotection was carried out with TFA: thioanisole: 1,2-ethanedithiol: anisole (9:0.5:0.3:0.2) at 25 °C for 2 h. The resin was filtered off and the filtrate concentrated with a gentle nitrogen flow. Peptides were precipitated with cold diethyl ether. The precipitate was filtered, washed with cold ether, dried and redissolved in 10 % acetic acid. The crude peptides were purified by reverse phase HPLC, lyophilized and characterized by MS-ESI.

Peptide Characterization

H_2N-GAADF-$CONH_2$, HPLC: R_t=16.7 min, MS-ESI: *m/z:* 479 (calcd. 479) $[M+H]^+$.

H_2N-GAFDG-$CONH_2$, HPLC: R_t=16.0 min, MS-ESI: *m/z:* 465 (calcd. 465) $[M+H]^+$.

H_2N-FAADG-$CONH_2$, HPLC: R_t=14.6 min, MS-ESI: *m/z:* 479 (calcd. 479) $[M+H]^+$.

H_2N-(F-DOTA)DG-$CONH_2$, HPLC: R_t=9.3 min, MS-ESI: *m/z:* 738 (calcd. 738) $[M+H]^+$, 736 (calcd. 736) $[M-H]^-$.

CH_3CONH-(F-DOTA)-$CONH_2$, HPLC R_t=11.0 min, MS-ESI: *m/z:* 607 (calcd. 607) $[M+H]^+$.

H_2N-GAAD(F-DOTA)-$CONH_2$, HPLC: R_t=10.5 min, MS-ESI: *m/z:* 880 (calcd. 880) $[M+H]^+$, 878 (calcd. 878) $[M-H]^-$.

H_2N-GA(F-DOTA)DG-$CONH_2$, HPLC: R_t=11.1 min, MS-ESI: *m/z:* 866 (calcd. 866) $[M+H]^+$, 864 (calcd. 864) $[M-H]^-$.

H_2N-(F-DOTA)AADG-$CONH_2$, HPLC: R_t=13.6 min, MS-ESI: *m/z:* 880 (calcd. 880) $[M+H]^+$, 878 (calcd. 878) $[M-H]^-$.

H_2N-FW(K-DOTA)EG-$CONH_2$, HPLC: R_t=22.5 min, MS-ESI: *m/z:* 1052 (calcd. 1052) $[M+H]^+$, 1050 (calcd. 1050) $[M-H]^-$.

(DOTA-T)FDDLFWKEGHR-$CONH_2$, HPLC: R_t=33.1 min, MS-ESI: *m/z:* 1936 (calcd. 1937) $[M+H]^+$, 1934 (calcd. 1935) $[M-H]^-$.

CH_3CONH-TFDDLFWKEGHR-$CONH_2$, HPLC: R_t=36.9 min, MS-ESI: *m/z:* 1593 (calcd. 1592) $[M+H]^+$, 1590 (calcd. 1590) $[M-H]^-$.

CH_3CONH-TFDDLFW(K-DOTA)EGHR-$CONH_2$, HPLC: R_t=36.6 min, MS-ESI: *m/z:* 1979 (calcd. 1979) $[M+H]^+$, 1976 (calcd. 1977) $[M-H]^-$.

CH_3CONH-TFDDL(F-DOTA)WKEGHR-$CONH_2$, HPLC: R_t=30.1 min, MS-ESI: *m/z:* 1995 (calcd. 1995) $[M+H]^+$, 1993 (calcd. 1993) $[M-H]^-$.

Gadolinium Complex Formation: Complexes were prepared by adding a $GdCl_3$ stock solution to a DOTA–peptide ligand solution, pH 7.4 (HEPES buffer) in stoichiometric amounts (1:1). The solution was stirred at RTfor 24 h and then centrifuged to remove any precipitated $Gd(OH)_3$. The presence of free Gd^{3+} was evaluated by colorimetry using xylenol orange as an indicator. In a solution containing the complex GDDOTA–peptide at pH 5.2, a deep purple color indicates free Gd^{3+}. Resultant peptide complexes were further purified by HPLC using the previously described procedure. The purified complex solution was lyophilized to give the gadolinium complex as a powder solid.

Protein Binding Studies: To measure the K_D of the Gal-80-fluorescein N-labeled peptide (provided by Dr. Kodadek, University of Texas Southwestern Medical Center, Dallas, Texas), the indicated amounts of His_6-Gal-80 and 2 nM of labeled peptide were mixed in 200 μL of buffer (PBS with 0.2 mg mL^{-1} bovine serum albumin). Each solution was equilibrated at RT for 20 minutes prior to measuring the fluorescence polarization of each sample. Competition binding studies were done by titrating a competing peptide into a sample containing 2 nM of Fluorescein-labeled peptide with 130 nM Gal-80 (75 % of K_D). The dissociation of the fluorescein-peptide from Gal-80 was monitored by fluorescence polarization spectroscopy.

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