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Femtomolar Ln(III) Affinity in Peptide-Based Ligands Containing Unnatural Chelating Amino Acids

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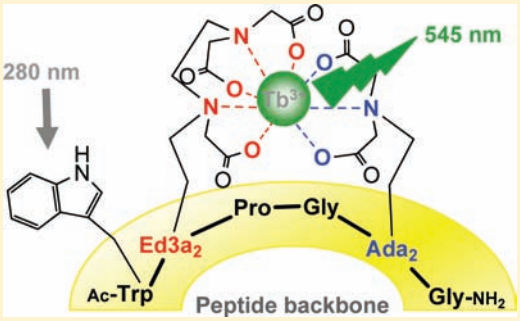
Supporting Information

ABSTRACT: The incorporation of unnatural chelating amino acids in short peptide sequences leads to lanthanide-binding peptides with a higher stability than sequences built exclusively from natural residues. In particular, the hexadentate peptide $\mathbf{P^{22}}$, which incorporates two unnatural amino acids Ada_2 with aminodiacetate chelating arms, showed picomolar affinity for Tb^{3+}. To design peptides with higher denticity, expected to show higher affinity for Ln^{3+}, we synthesized the novel unnatural amino acid $Ed3a_2$ which carries an ethylenediamine triacetate side-chain and affords a pentadentate coordination site. The synthesis of the derivative Fmoc-$Ed3a_2(t\mathrm{Bu})_3$-OH, with appropriate protecting groups for direct use in the solid phase peptide synthesis (Fmoc strategy), is described. The two high denticity peptides $\mathbf{P^{HD2}}$ (Ac-Trp-$Ed3a_2$-Pro-Gly-Ada_2-Gly-NH_2) and $\mathbf{P^{HD5}}$ (Ac-Trp-Ada_2-Pro-Gly-$Ed3a_2$-Gly-NH_2) led to octadentate Tb^{3+} complexes with femtomolar stability in water. The position of the high denticity amino acid $Ed3a_2$ in the hexapeptide sequence appears to be critical for the control of the metal complex speciation. Whereas $\mathbf{P^{HD5}}$ promotes the formation of polymetallic species in excess of Ln^{3+}, $\mathbf{P^{HD2}}$ forms exclusively the mononuclear complex. The octadentate coordination of Tb^{3+} by both $\mathbf{P^{HD}}$ leads to total dehydration of the metal ion in the mononuclear complexes with long luminescence lifetimes (>2 ms). Hence, we demonstrated that unnatural amino acids carrying polyaminocarboxylate side-chains are interesting building blocks to design high affinity Ln-binding peptides. In particular the novel peptide $\mathbf{P^{HD2}}$ forms a unique octadentate Tb^{3+} complex with femtomolar stability in water and an improvement of the luminescence properties with respect to the trisaquo Tb$\mathbf{P^{22}}$ complex by a factor of 4.



INTRODUCTION

Lanthanide-binding peptides are tremendously powerful tools to investigate biologically relevant species such as proteins or DNA.[1] Indeed, they combine the unique spectroscopic and magnetic properties of lanthanide trivalent cations with the characteristics of the peptide scaffold, such as high solubility in water, versatile insertion in proteins by standard peptide synthesis or molecular biology techniques, and most importantly selective recognition of biological partners. The lanthanide series offers a variety of metal ions with diverse physical properties: for instance Eu^{3+} and Tb^{3+} complexes find applications as luminescent sensors because of their long-lived luminescence[2−5] and Gd^{3+} complexes are commonly used as magnetic resonance imaging contrast agents, thanks to the high spin value and long electronic relaxation time of the Gd^{3+} ion.[6−8]

Ln^{3+} cations bind to proteins in Ca^{2+}-binding sites,[9,10] therefore native protein sequences such as the Ca-binding EF-hand motifs were first selected to generate Ln-binding peptides.[11−16] These motifs were subsequently optimized by screening methods to select those combining both stability and efficient luminescence sensitization in the Tb-peptide complexes.[17−19] These optimized sequences have been appended to proteins to investigate their structure, function and dynamics.[1,20,21] Other native sequences may be modified to generate Ln-binding peptides. For instance a "lanthanide-finger" was recently designed by replacing the cysteine and histidine residues involved in the metal coordination of a zinc finger (Cys_2His_2) by aspartic and glutamic acids to generate a tetracarboxylate peptide ligand (Asp_2Glu_2).[22] Another strategy consists in preorganizing the side-chains of coordinating amino acids in de novo designed peptides to afford well-defined Ln^{3+} coordination sites. This methodology was applied to the design of a cyclodecapeptide with a β-sheet structure which allows the preorientation of four amino acid side-chains of glutamic or aspartic acids to coordinate the Ln^{3+} ion. Interestingly, the corresponding Gd^{3+} complex combines the relaxation properties of Gd^{3+} with the high hydrophilicity of the peptide scaffold to provide large second-sphere contributions to water relaxation.[23,24] Natural amino acids with carboxylates, phenolates, or amides as metal-binding groups, show only a moderate affinity for Ln^{3+} ions. Therefore, peptide sequences built exclusively from these natural residues exhibit an affinity for Ln^{3+} in the micromolar range[16,22,24,25] or for the best Ln-binding tags, in the low nanomolar range.[19] For either in vitro

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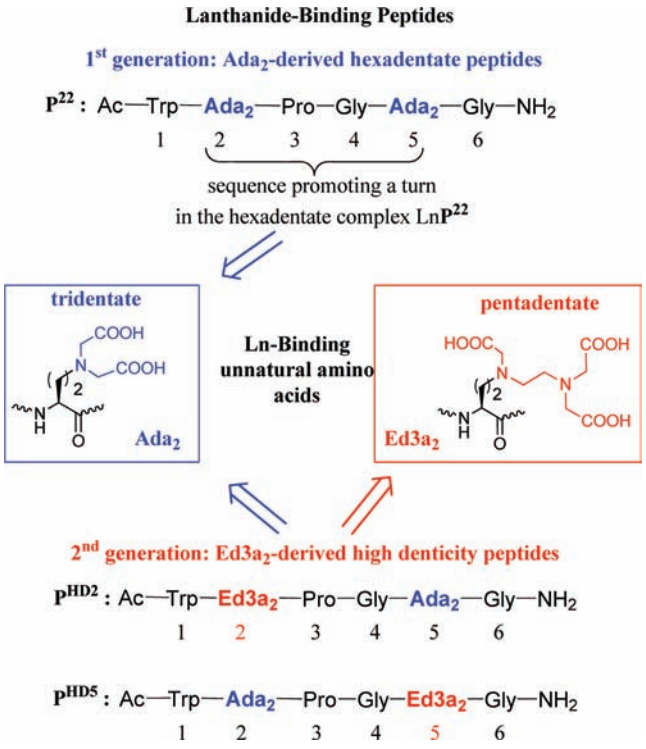
or in vivo applications, these short Ln-binding sequences are inserted in more complex architectures for targeting or detection, and therefore the affinity for the lanthanide ion must be sufficient to avoid leaching of the cation and its coordination in other metal binding-sites such as Ca^{2+}-binding EF motifs.

Therefore, the design of high affinity Ln-binding peptides is a challenging question, which we have chosen to address by using unnatural amino acids carrying high affinity chelating groups. Recently, we developed short peptide sequences containing two unnatural amino acids bearing tridentate aminodiacetate chelating groups (Ada_n). For instance the peptide $\mathbf{P^{22}}$ (see Scheme 1) formed stable and well-defined lanthanide

Scheme 1. Design of Lanthanide-Binding Peptides Incorporating Non Natural Chelating Amino Acids



complexes with stabilizing peptide secondary structure interactions: the stability of $\mathbf{TbP^{22}}$ was found in the picomolar range.[26−28] In this compound, the sequence between the two tridentate chelating aminoacids was chosen to favor the formation of a β-turn, which leads to the hexadentate $\mathbf{TbP^{22}}$ complex with 3 Tb-bound water molecules ($q_{Tb} = 3$) in water.[27]

In this paper, we report the design of peptides forming totally dehydrated Tb^{3+} complexes with a femtomolar stability in water. The two high denticity hexapeptides $\mathbf{P^{HD2}}$ and $\mathbf{P^{HD5}}$ were obtained by replacing one tridentate Ada_2 residue in $\mathbf{P^{22}}$ with the new pentadentate unnatural amino acid $Ed3a_2$, which carries an ethylenediamine triacetate side-chain (Scheme 1). The synthesis of the novel amino acid derivative Fmoc-$Ed3a_2(t\mathrm{Bu})_3$-OH with appropriate protecting groups for direct use during the solid phase peptide synthesis is described, as well as the properties of the Tb^{3+} complexes in water.

EXPERIMENTAL SECTION

Abbreviations. AcOEt (ethyl acetate), $AcONH_4$ (ammonium acetate), Ar (aromatic), Bn (benzyl), Bu (butyl); Fmoc (9-fluorenylmethyloxycarbonyl), HEDTA (2-[2-[bis(carboxymethyl)amino]ethyl-(2-hydroxyethyl)amino] acetic acid), *t*Bu (*tert*-butyl), TFA (trifluoroacetic acid), TLC (Thin Layer Chromatography).

General Remarks. Reagents and solvents were purchased from Aldrich and Novabiochem and used without further purification unless specified. Organic products were characterized by NMR using a Varian Mercury 400 MHz spectrometer at 298 K. Peptides $\mathbf{P^{HD2}}$ and $\mathbf{P^{HD5}}$ were characterized in light water containing 10% D_2O by NMR using a Bruker Avance 500 MHz. Analytical and preparative peptide RP-HPLC were performed with LaChrom and LaPrep systems.

The 3 mM aqueous solutions of lanthanides were prepared by dissolving corresponding salts ($TbCl_3{\cdot}6H_2O$, $Tb(OTf)_3$, $EuCl_3{\cdot}6H_2O$) in deionized water. The precise concentrations were determined by colorimetric titration with 5 mM EDTA (Fisher Chemicals) in the presence of xylenol orange indicator. Peptide solutions in HEPES buffer (10 mM, 0.1 M KCl, pH = 7.0) were prepared directly before use. The precise concentrations were established by UV absorption at 280 nm using the known extinction coefficient of tryptophan (ε_{280} = 5690 L mol^{-1} cm^{-1}). HEPES buffer (10 mM, 0.1 M KCl, pH = 7.0) was prepared from solid 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (Fluka) and potassium chloride in deionized H_2O (or D_2O). The pH (or pD) was adjusted to 7.0 with KOH (or NaOD). A 5 mM HEDTA solution to be used in competition experiments was prepared in pure water from solid HEDTA (Riedel-de-Haën), and its precise concentration was determined pH-metrically.

Synthesis. The aldehyde **2** and *N,N,N′*-tris-(tertbutyloxycarbonylmethyl)ethylenediamine **1** were synthesized following published procedures.[27,29]

Compound (3). The aldehyde **2** (0.496 mmol, 1 equiv, 220 mg) and *N,N,N′*-tris(*tert*butyloxycarbonylmethyl)ethylenediamine (0.893 mmol, 1.8 equiv, 359.5 mg) were dissolved in 1 mL of 1,2-dichloroethane under Ar atmosphere. After addition of $NaBH(OAc)_3$ (183.3 mg, 0.893 mmol, 1.8 equiv), the resulting suspension was vigorously stirred. The progress of coupling was monitored by TLC analysis (CH_2Cl_2/AcOEt *v/v* = 9/1, R_f = 0.2), which indicated complete conversion after 4 h. Dichloromethane (20 mL) and saturated aqueous $NaHCO_3$ (20 mL) were added, and the organic phase was decanted. The aqueous phase was further extracted with CH_2Cl_2 (2 × 20 mL). The combined organic phases were dried over Na_2SO_4 and evaporated. The resulting crude product was purified by silica-gel column chromatography (elution gradient: CH_2Cl_2/AcOEt, v/v = 19/1 to 9/1) to yield **Fmoc-$Ed3a_2(t\mathrm{Bu})_3$-OBn** as a colorless oil (353 mg, 87% yield). $[\alpha]_D^{20}$ = +10.8 (*c* = 1.13 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ(ppm) = 7.75 (2H, d, ^3J(H,H) = 7.5 Hz, Ar); 7.63 (2H, t, ^3J(H,H) = 7.6 Hz, Ar); 7.39−7.35 (4H, m, Ar_{Fmoc}); 7.29 (5H, m, Ar_{Bn}); 7.03 (1H, d, ^3J(H,H) = 8.0 Hz, NH(Fmoc)); 5.17, 5.14 (2H, AB system, J_{AB} = 12.4 Hz, CH_2(Bn)); [4.47 (2H, m), 4.30−4.21 (2H, m), Hα, CH_2 (Fmoc) and CH(Fmoc)]; 3.43, 3.31 (4H, AB system, J_{AB} = 17.5 Hz, $N_\eta CH_2COOt\mathrm{Bu}$); 3.26, 3.18 (2H, AB system, J_{AB} = 17.0 Hz, $N_\delta CH_2COOt\mathrm{Bu}$); 2.87−2.71 (4H, m, H$\varepsilon$, H$\zeta$)); 2.76−2.54 (2H, m, H$\gamma$)); 2.05−1.97 (2H, m, H$\beta$); 1.45 (9H, s, $N_\delta CH_2COOt\mathrm{Bu}$); 1.43 (18H, s, $N_\eta CH_2COOt\mathrm{Bu}$). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ(ppm) = 172.5 (COOBn); 170.9 (1C), 170.8 (2C) (COO*t*Bu); 156.8 (CO(Fmoc)); 144.3 (2C), 141.7 (2C) (C_q (Fmoc)); 136.0 (C_q(Bn)); 128.7 (2C), 128.5 (2C), 128.4 (C_{Ar}-H(Bn)); 127.8 (2C), 127.3 (2C), 125.6, 125.4, 120.2 (2C) (C_{Ar}-H(Fmoc)); 81.1 (3C) (C_q(*t*Bu)); 67.1, 66.9 (CH_2(Bn), (CH_2(Fmoc)); 56.2 ($N_\delta CH_2COOt\mathrm{Bu}$); 55.9 (2C) ($N_\eta CH_2COOt\mathrm{Bu}$); 53.1 (C$\gamma$); 52.2, 51.3 (C$\varepsilon$, C$\xi$); 49.9 (C$\alpha$); 47.3 (CH(Fmoc)); 28.6 (Cβ); 28.4 (9C) (*t*Bu). ES^+-MS (intensity %): *m/z* = 816.2 (100) $[M+H]^+$, 838.3 (96) $[M+Na]^+$.

Fmoc-$Ed3a_2(t\mathrm{Bu})_3$-OH. Compound **3** (350 mg, 0.43 mmol) was dissolved in 50 mL of absolute ethanol and hydrogenated under pressure (3 bar, overnight) over 50 mg of 10% Pd/C. 296 mg of resulting **Fmoc-$Ed3a_2(t\mathrm{Bu})_3$-OH** (95% yield) were recovered after filtration of the reaction mixture through a Celite pad and solvent evaporation. $[\alpha]_D^{20}$ = +4.3 (*c* = 1.03 in $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ(ppm) = 7.76 (2H, d, ^3J(H,H) = 7.5 Hz, Ar); 7.63 (2H, t, ^3J(H,H) = 7.5 Hz, Ar); 7.40 (2H, t, ^3J(H,H) = 7.5 Hz, Ar); 7.32 (2H,d, ^3J(H,H) = 7.5 Hz, Ar); 6.53 (1H, d, ^3J(H,H) = 5.7 Hz,

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NH(Fmoc)); [4.38−4.28 (2H, m), 4.22 (1H, m) C*H*(Fmoc), CH_2(Fmoc)]; 4.14, 4.09 (2H, AB system, J_{AB} = 17.3 Hz, $N_\delta CH_2COOtBu$); 4.40 (1H, m, Hα); 3.44 (4H, s, $N_\eta CH_2COOtBu$); 3.42−3.34 (4H, m, Hγ, Hε or Hζ); 3,14 (2H, m, Hε or Hζ); 2.31 (2H, m, Hβ); 1.49 (9H, s, $N_\delta CH_2COOtBu$); 1.45 (18H, s, $N_\eta CH_2COOtBu$). ^{13}C NMR (100 MHz, $CDCl_3$, 298 K): δ(ppm) = 173.5 (COOH); 171.0 (2C), 166.4 (1C) (*COO*tBu); 156.6 (*CO*-(Fmoc)); 144.4, 144.2, 141.7 (2C) (C_q (Fmoc)); 128.1 (2C), 127.6 (2C), 125.8, 125.7, 120.3 (2C) (C_{Ar}-H(Fmoc)); 84.3 (1C), 82.4 (2C) (C_q(*t*Bu)); 67.5, (CH_2(Fmoc)); 56.2 (3C) ($NCH_2COOtBu$); 53.8 (Cα); 54.4, 50.5, 52.5 (Cγ, Cε, Cξ); 47.5 (CH(Fmoc)); 28.5 (6C), 28.4 (4C) (*tBu*, Cβ). ES$^+$-MS (intensity %): m/z = 726.3 (40) [M +H]$^+$, 748.3 (100) [M+Na]$^+$.

The enantiopurity of Fmoc-Ed3a$_2$(*t*Bu)$_2$-OH was checked by analyzing the NMR spectrum of the coupling product with the chiral amine α-methylbenzylamine either in enantiopure (*S*) or in racemic form, as described previously for Ada$_n$ amino acids.[27,28] In the spectra of the adducts, a splitting of the NH amide resonances was detected only for the coupling products with the racemic amine. This indicates that no racemization of Fmoc-Ed3a$_2$(*t*Bu)$_2$-OH occurred during the synthesis (assuming that 2% diastereomeric product would be detected by NMR, *ee* > 96%).

Peptide Synthesis and Purification. These were performed as previously described for Ada$_2$-based peptides.[27]

***P*^{HD2}**. Ac-Trp-Ed3a$_2$-Pro-Gly-Ada$_2$-Gly-NH$_2$, Yield of the on-resin synthesis (UV): 68%, Isolated mass: 49.0 mg (isolated yield assuming that the solid is **P**^{HD2}·3TFA: 50%). ES$^+$-MS ($AcONH_4$, pH = 7.0): m/z = 990.3 [M+H]$^+$, ES$^-$-MS ($AcONH_4$, pH = 7): m/z = 988.3 [M-H]$^-$; RP-HPLC: t_R = 8.4 min, >95% purity (NMR).

***P*^{HD5}**. Ac-Trp-Ada$_2$-Pro-Gly-Ed3a$_2$-Gly-NH$_2$, Yield of the on-resin synthesis (UV): 24%, Isolated mass: 17.2 mg (isolated yield assuming that the solid is **P**^{HD5}·3TFA: 17%). ES$^+$-MS ($AcONH_4$, pH = 7.0): m/z = 990.5 [M+H]$^+$, ES$^-$-MS ($AcONH_4$, pH 7): m/z = 988.5 [M-H]$^-$; RP-HPLC: t_R = 8.4 min, >95% purity (NMR).

Electrospray Mass Spectrometry of Eu Complexes. The mass spectrometry measurements were performed with a LXQ-type Thermo Scientific spectrometer equipped with an electrospray ionization source and a linear trap detector. The samples of Eu**P**^{HD} complexes were prepared by adding $EuCl_3$ (0, 0.5, 1.0, 2.0 equiv) to a peptide solution (60 μM) in $AcONH_4$ buffer (20 mM, pH = 7.0). Resulting mixtures were injected into the spectrometer at a flow rate of 5 μL min^{-1}. 2 kV voltage and 250 °C capillary temperature were applied.

Circular Dichroism Measurements. The CD spectra were collected on an Applied Photophysics Chirascan spectrometer in a 1 cm path length quartz cell at 25 °C. Twenty μM solutions of free peptides and Tb-peptide complexes (0 to 2 equiv. of Tb(OTf)$_3$) were prepared in deionized water, and the pH was adjusted to 7.0 with KOH. Subsequently, the CD measurements were performed in 280−200 nm range with 1 nm interval, 2 s time constant, 1 nm bandwidth, and three scans. The CD signal was reported in molar ellipticity per α-amino acid residue ([θ] in units of deg cm^2 mol^{-1}; [θ]= θ_{obs}/(10*lcn*), where θ_{obs} is the observed ellipticity in m°, *l* is the optical path length of the cell in cm, *c* is the peptide concentration in mol L^{-1}, and *n* is the number of residues in the peptide (*n* = 6 in this case)).

Luminescence Measurements. The luminescence measurements were performed on a LS50B spectrofluorimeter connected to a computer equipped with FLWINLAB 2.0. The spectra were recorded in a 1 cm path length quartz luminescence cell at 25 °C, by exciting the sample at 280 nm and recording the emission with maximum at 350 nm (tryptophan fluorescence) or 545 nm (Tb-centered luminescence). For fluorescence measurements, excitation slit: 3.0 nm and emission slit: 8.0 nm were applied. The terbium and europium emission spectra were recorded after 0.05 ms delay, with gate time/excitation slit/emission slit: 1 ms/10 nm/5 nm and 0.5 ms/10 nm/15 nm for Tb^{3+} and Eu^{3+}, respectively. The 430 nm cutoff filter was used during all tryptophan-sensitized luminescence measurements.

Lifetime measurements were performed on peptide samples containing 0.9 equiv of $TbCl_3$ to ensure exclusive contribution from the mononuclear complexes. The emission intensities of the most intense Tb^{3+} emission band were recorded after excitation at 280 nm with a first delay of 0.05 ms, a delay increment of 0.05 ms, and the number of measurements adjusted to have a final delay >4τ.

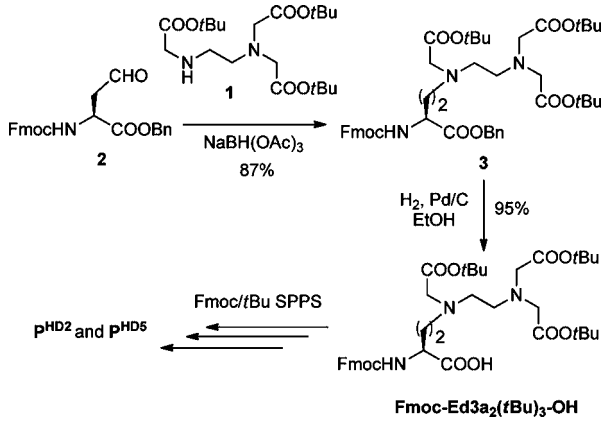
The quantum yield measurements were performed according to Chauvin et al.[30,31] in a 1 cm path length luminescence cell at 25 °C on aqueous solutions of Tb**P**^{HD} (50 μM, A_{280} = 0.3), [Tb(dpa)$_3$]$^{3-}$ (0.107 mM, A_{280} = 0.295) in HEPES buffer (10 mM, 0.1 M KCl, pH = 7.0). The standard solutions of [Tb(dpa)$_3$]$^{3-}$ was prepared by mixing Tb^{3+} with dipicolinic acid (dpa) in 1/3 equivalent ratio in HEPES buffer (10 mM, 0.1 M KCl, pH = 7.0) and stirring the resulting mixture during 5 min. The quantum yields Φ have been calculated using the equation $\Phi x/\Phi_r = (E_x/E_r) \times (A_r(\lambda_r)/A_x(\lambda_x))$, where *x* refers to the sample and *r* to the reference, *E* is the integrated luminescence intensity, and *A* is the absorbance of the solution at the excitation wavelength λ. The value of the quantum yields $\Phi_{Tb(dpa)3}$ = 18.4 ± 2.5% (0.107 mM, A_{280} = 0.295) was applied to the calculations assuming that the change of a buffer from Tris (0.1 M, pH = 7.45) to HEPES (10 mM, 0.1 M KCl, pH = 7.0) has affected the reference values in the error range.[30,31]

The conditional stability constants of Tb**P**^{HD2} and Tb**P**^{HD5} were measured by recording the Tb-centered luminescence of these complexes (16 μM) in HEPES buffer (10 mM, 1 M KCl, pH = 7.0) upon addition of aliquots of a HEDTA solution. Forty minutes waiting between two successive points ensured reaching the thermodynamic equilibrium. The data obtained from these competitions were fitted with a fixed value of the conditional stability constant of the reference Tb(HEDTA), log $\beta_{11}^{pH\ 7}$= 12.6(5), determined by taking into account the known equilibrium constants of HEDTA and global stability constant of Tb(HEDTA).[32]

RESULTS

Synthesis of the Octadentate Hexapeptides. The novel chelating unnatural amino acid Ed3a$_2$ was synthesized with protections compatible with Fmoc/*t*Bu strategy in solid phase peptide synthesis, that is, in the form Fmoc-Ed3a$_2$(*t*Bu)$_3$-OH (Scheme 2). The synthesis of Fmoc-Ed3a$_2$(*t*Bu)$_3$-OH consists

Scheme 2. Synthesis of the High Denticity Peptides P^{HD2} and P^{HD5}



in two steps from the aldehyde **2**, which we previously described.[27] Compound **1** was first prepared according to Micklitsch et al.[29] and then coupled to aldehyde **2** to afford the benzylated amino acid **3**, which was deprotected by catalytic hydrogenation. High denticity peptides were then manually assembled by solid phase peptide synthesis starting from Fmoc-Ada$_2$(*t*Bu)$_2$-OH, Fmoc-Ed3a$_2$(*t*Bu)$_3$-OH and commercial Fmoc derivatives of natural amino acids, on Rink amide MBHA resin using standard conditions as performed previously for **P**^{22}.[27] No significant byproducts were detected by ^{1}H NMR, showing that the unnatural amino acid Fmoc-Ed3a$_2$(*t*Bu)$_3$-OH displayed normal reactivity in the on-resin synthetic steps. The pure

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peptides were obtained after preparative HPLC purification. Their identity and purity were confirmed by electrospray ionization mass spectrometry and ^{1}H NMR (Supporting Information, Table S1 and S2).

Speciation of the Ln Complexes of P^{HD2} and P^{HD5}. Several analytical techniques were used to investigate the speciation of metal complexes formed with the two octadentate peptides $\mathbf{P}^{HD2}$ and $\mathbf{P}^{HD5}$. Electrospray ionization mass spectrometry (ES-MS) gives a qualitative insight into the speciation of metal-peptide complexes in solution. Circular dichroism (CD) is sensitive to peptide conformational changes upon complexation. The tryptophan (Trp) residue in position 1 is a Tb^{3+} sensitizer, and we demonstrated that the fluorescence evolution of the Trp indole moiety upon Tb^{3+}complexation was dominated in our systems by the modification of the indole environment.[27,28] These three techniques point to different behaviors of the two peptides. Compound $\mathbf{P}^{HD2}$ forms exclusively a monometallic complex $Ln\mathbf{P}^{HD2}$, which is the only species detected in the ES-MS spectra of the Eu^{3+} complexes either as a monocation ($[Eu\mathbf{P}^{HD2}\text{-}2H]^+$, m/z = 1140.3) or as a dication ($[Eu\mathbf{P}^{HD2}\text{-}H]^{2+}$, m/z = 570.8) (see Supporting Information, Table S3). The formation of the Tb^{3+} complex induces a decrease of Trp fluorescence which stabilizes after one metal equivalent (Figure 1) as the evolution of the CD signal (Supporting Information,

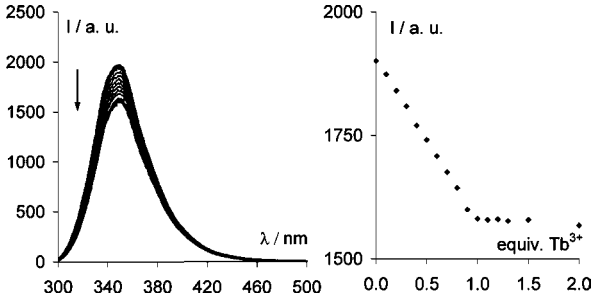

Figure 1. Fluorescence of $\mathbf{P}^{HD2}$ (45 μM) in HEPES buffer (10 mM, KCl 0.1 M, pH = 7.0) upon excitation at 280 nm, with addition of $TbCl_3$ (0 to 2 equiv).

Figure S1). Whereas all these data demonstrate the formation of a unique $Ln\mathbf{P}^{HD2}$ monometallic complex, the same experiments performed with $\mathbf{P}^{HD5}$ show the transformation of the monometallic species $Tb\mathbf{P}^{HD5}$ into polymetallic adducts in excess of metal. Indeed Trp fluorescence (Supporting Information, Figure S2) and CD spectra (Supporting Information, Figure S3) clearly evolve in excess of metal ion, and polymetallic complexes are also detected in the ES-MS spectra in particular the bimetallic adduct ($[Eu_2\mathbf{P}^{HD5}\text{-}4H]^{2+}$, m/z = 644.6) (Supporting Information, Figure S4). This indicates that $\mathbf{P}^{HD5}$ sequence allows an independent coordination by the two chelating amino acids, which leads to polymetallic species, at the expense of the mononuclear complex formation. Interestingly, the sequence of $\mathbf{P}^{HD2}$, which differs only from $\mathbf{P}^{HD5}$ by the positions of the two chelating amino acids with respect to the Pro-Gly sequence, promotes exclusively an octadentate monometallic complex which does not evolve to polymetallic species in excess of Ln^{3+} to give bimetallic complexes.

Luminescence Properties of the Tb Complexes. Owing to the antenna effect, that is, Tb^{3+} luminescence sensitization by energy transfer through Trp indole absorption at 280 nm, the Tb-centered $^5D_4 \rightarrow {}^7F_J$ emission bands (J = 3−6) build up upon addition of Tb^{3+} to the free peptide in water at pH 7 as seen in Supporting Information, Figure S5. The luminescence spectra are not sensitive to the presence of water as seen in Figure 2,

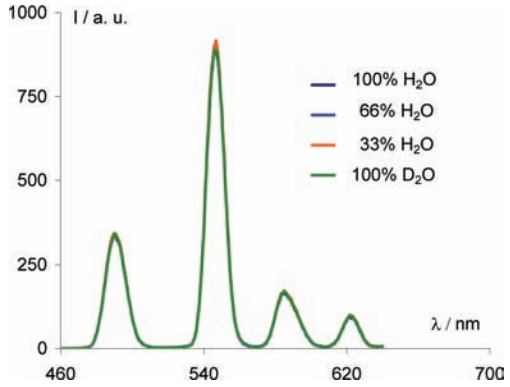

Figure 2. Tb-centered luminescence of 30 μM $Tb\mathbf{P}^{HD2}$ in D_2O/H_2O mixtures HEPES buffer (10 mM, pH = 7.0).

which shows the perfect superimposition of spectra acquired in light or heavy water, in which there is no deactivation by the potentially coordinated O−D vibrators

The luminescence lifetimes of Tb^{3+} in the mononuclear complexes $Tb\mathbf{P}^{HD}$ in water at pH 7 have very similar values (τ_{H2O} = 2.3 ms). These values compare well with luminescence lifetimes measured for totally dehydrated octadentate Tb^{3+} complexes, for instance Barbara Imperiali's Tb-binding peptides (τ_{H2O} = 2.6 ms)[19] or octadentate cages of Tb^{3+} 2-hydroxyisophthalamides (2.45 ms < τ_{H2O} < 2.6 ms).[33]

The corresponding values in D_2O were determined by the extrapolated limit of the luminescence decay rates in solutions of increasing D_2O molar fractions tending to a H_2O-free solution (Supporting Information, Figure S6). The hydration numbers were calculated from the lifetimes in heavy and light water according to Parker's equation (Table 1).[34] They are very close to 0 and confirm the total dehydration of the $Tb\mathbf{P}^{HD}$ complexes and therefore the absence of deactivation because of inner-sphere water molecules. The octadentate nature of the peptide ligands in the complexes is confirmed by these measurements.

The quantum yields were measured as previously described[28] following the procedure of Bünzli et al., which uses the Tb *tris*(dipicolinate) complex as a secondary standard, which excitation band perfectly overlaps with the excitation band of tryptophan in our peptides.[30,31] The quantum yields are 1% for both $Tb\mathbf{P}^{HD}$ complexes which are significantly enhanced with respect to the $Tb\mathbf{P}^{22}$ complex (Φ = 0.24%). Of course these values are still low because the energy transfer from Trp to Tb^{3+} is not the most efficient. Anyway, Tb^{3+} is sufficiently sensitized upon binding to the peptides as previously described in other Ln-binding peptides or proteins to allow detection in the nM range.[10,17−19,35] The second-generation peptides $\mathbf{P}^{HD}$ provide a gain in luminescence efficiency of a factor 4 with respect to the first generation of Ada_2-based peptides since they promote total dehydration of the Tb ion.

Stability Constant Determination. The pK_a's of $\mathbf{P}^{HD5}$ were measured by potentiometry (see Supporting Information, Figure S7). Six acidity constants could be determined in our experimental conditions (2.5 < pH < 11). The three higher values (pK_a = 9.1, 8.4, and 5.6) correspond to the

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Table 1. Comparison between P^{HD2} and P^{HD5} Properties

complex	τ_{H2O} (ms)[a]	τ_{D2O} (ms)[a]	q[b]	log $\beta_{11}^{pH=7}$[c]	log β_{110}[d]	polymetallic Ln complexes
TbP^{HD2}	2.35(2)	2.86(2)	0.08(3)	12.7(5)	16.2(5)	no
TbP^{HD5}	2.30(2)	2.81(2)	0.09(3)	12.7(5)	16.2(5)	yes

[a] τ_{H2O} and τ_{D2O} are the luminescence lifetimes of Tb^{3+} in the complexes in HEPES buffer (10 mM, KCl 0.1 M, pH = 7.0) in 100% H_2O and extrapolated to 100% D_2O, respectively. [b] q is the hydration number calculated from the lifetimes with Parker's equation.[34] [c] $\beta_{11}^{pH=7}$ is the conditional stability constant of TbP^{HD} at pH 7, 298 K in 0.1 M KCl. [d] β_{110} is the global stability constant of TbP^{HD} calculated from $\beta_{11}^{pH=7}$ and the peptide pK_a's.

deprotonation of the 3 ammonium functions of the chelating amino acids and the three lower values to the deprotonation of three carboxylic acid moieties. Potentiometry requires large amounts of material (mM concentration experiments), especially for reliable determination of metal-complex stability constants.[36,37] Therefore the affinity of the two peptides for Tb^{3+} was investigated by luminescence (16 μM concentration) using competition experiments.

The two octadentate peptides demonstrate significantly improved affinities for Tb^{3+} in comparison to the hexadentate peptide P^{22}, as shown by competition experiments with HEDTA. As seen in Figure 3, the Tb-centered luminescence

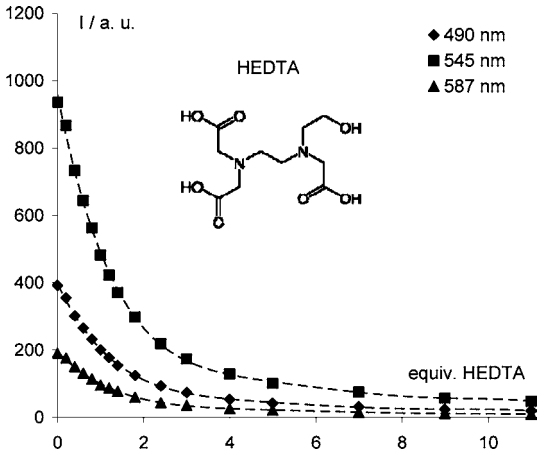

Figure 3. Tb-centered emission at selected wavelengths during the titration of 16 μM TbP^{HD2} with HEDTA in HEPES buffer (10 mM, 0.1 M KCl, pH = 7.0). Dashed lines represent calculated data.

of TbP^{HD2} decreases by nearly 50% upon addition of 1 equiv of HEDTA, which points to similar stabilities for TbP^{HD2} and Tb(HEDTA) at pH 7. The conditional stability constant of Tb(HEDTA) at pH 7 was calculated from published data:[32] log $\beta_{11}^{pH\ 7}$ = 12.6, and the fit of the competition experiments led to conditional stability constants log $\beta_{11}^{pH\ 7}$ = 12.7 for the two octadentate peptide P^{HD}.

The global stability constants β_{110}, independent of the basicity of the ligand and the pH, were calculated by taking into account the three protonation constants, which significantly contribute at pH 7, that is, pK_a = 9.1, 8.4, and 5.6. The calculation leads to logβ_{110} = 16.0(5). The octadentate peptides P^{HD2} and P^{HD5} provide thus totally dehydrated Tb^{3+} complexes with a femtomolar stability in water.

DISCUSSION

Recent work in our laboratory has been focused on the development of Ln-binding peptides, which incorporate unnatural chelating amino acids to enhance the affinity for Ln^{3+} cations. We demonstrated that the introduction of two chelating Ada_n residues, with tridentate aminodiacetate groups, was very powerful to design high affinity Ln-binding peptides.[26−28] Indeed, peptides built exclusively from natural amino acids, even in very well-defined structures, show at most a nanomolar affinity for Ln^{3+} ions, which need high coordination numbers.[1,19] The incorporation of phosphorylated amino acids such as phosphotyrosine or phosphoserine, with more efficient coordinating side-chains than other natural amino acids, also lead to μM affinity for Tb^{3+}, in α-synuclein peptide fragments.[38,39] On the contrary, hexapeptides built with two Ada_n residues show up to picomolar affinity for TbP^{22} in water.[27] To further enhance the denticity of these peptides, we used the shorter side-chain analogue Ada_1, which may act as a tetradentate donor thanks to the coordination of its backbone carbonyl in a six-membered chelate ring. This attempt was successful in decreasing the hydration state of the Ln^{3+} ion (q_{Tb} = 0−1)[26,28] but did not lead to greater stability of the Ln^{3+} complexes. Even though the carbonyl function of Ada_1 was involved in the metal coordination by the two peptides P^{12} and P^{11},[26,28] no significant stabilization was observed because of this additional donor. Indeed, supplementary carbonyl binding groups are neutral donors, which do not provide significant stabilization of Ln^{3+} complexes in water, as evidenced before with neutral tripodal *N,O* ligands.[40]

On the contrary, the novel chelating amino acid $Ed3a_2$ developed in the present study bears a pentadentate polyaminocarboxylate side-chain and therefore an extra amino-acetate group which has a significant affinity for Ln^{3+} and thus contributes to both dehydration and stability. The protected derivative Fmoc-$Ed3a_2(tBu)_3$-OH could be introduced in short peptide sequences with standard solid phase peptide synthesis protocols. $Ed3a_2$ was inserted in place of one of the two tridentate residues in P^{22}, to obtain two second-generation hexapeptides P^{HD2} and P^{HD5}, differing in the position of the pentadentate residue. The two latter high denticity peptides are able to bind Ln^{3+} in an octadentate coordination mode and the mononuclear Tb^{3+} complexes demonstrate femtomolar stability in water, that is, a gain of 4 orders of magnitude with respect to the first generation hexapeptide P^{22}.

The octadentate coordination of Tb^{3+} by P^{HD} leads to total dehydration of the metal ion in the mononuclear complexes with long luminescence lifetimes (>2 ms). This results in an improvement of the luminescence properties with respect to the trisaquo TbP^{22} complex of a factor 4. The quantum yield values remain quite low (~1%) because the energy transfer from the indole group of tryptophan to Tb^{3+} is not very efficient. Nevertheless, as previously described by other groups working with Ln-binding peptides or proteins, Tb^{3+} is sufficiently sensitized by Trp upon binding to the peptides to allow detection in the nanomolar range.[10,17−19,35] In the future, the replacement of Trp in the peptide sequence by more efficient Ln^{3+} sensitizers could be performed to enhance the energy transfer to the Ln^{3+} ion. For instance, non natural amino acids bearing carbostyril 124 or acridone fluorophores, which are excited at lower energy than tryptophan, have been inserted

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in lanthanide binding tags without modifying the coordination properties of the peptide scaffold.[41] The chromophore 1,8-naphtalimide has also been appended at the N-terminus of a Ca-binding loop from paravalbumin to obtain luminescent Eu^{3+} peptide complexes.[42,43] These strategies could thus be applied to our hexapeptides to improve the luminescence properties.

The position of the high denticity amino acid $Ed3a_2$ in the hexapeptide sequence appears to be critical for the control of the metal complex speciation. The two peptides $\mathbf{P^{HD2}}$ and $\mathbf{P^{HD5}}$ only differ by the positions of the two chelating amino acids with respect to the Pro-Gly spacer and show different behaviors. Whereas $\mathbf{P^{HD5}}$ promotes the formation of polymetallic species in excess of Ln^{3+}, $\mathbf{P^{HD2}}$ forms exclusively the mononuclear complex. Incidentally, the introduction of the tetradentate aminoacid Ada_1 leads to similar observation: Ada_1 acts as a tetradentate donor only in position 2 and induces the formation of polymetallic species if in position 5.[26,28] It appears then that positioning the high denticity amino acid, either Ada_1 or $Ed3a_2$ in position 5 after the Pro-Gly spacer favors an independent coordination of the two chelating moieties. Hence, to control speciation and stabilize the octadentate coordination mode that involves the two chelating arms of the hexapeptides, the higher denticity amino acid has to be introduced in position 2, prior to the cyclic proline residue, which probably constrains more efficiently the peptide backbone in a β-turn structure.

To conclude, the incorporation of two unnatural amino acids with polyaminocarboxylate side-chains in short peptide sequences leads to high stability Ln^{3+} complexes. In particular, the design of the novel pentadentate chelating amino acid $Ed3a_2$ and its insertion in $\mathbf{P^{22}}$ sequence in place of one Ada_2 was demonstrated to afford octadentate Tb^{3+} complexes with femtomolar stability in water. The octadentate coordination provides totally dehydrated complexes with better luminescence properties than the previously described $\mathbf{TbP^{22}}$ complex. The pentadentate amino acid position is demonstrated to be critical: it has to be inserted in position 2, to control the speciation and avoid the formation of polymetallic complexes. Overall, $\mathbf{P^{HD2}}$ is a short Ln-binding peptide which gives a unique octadentate Tb^{3+} complex with femtomolar stability, which could be inserted in more complex peptide or protein sequences to investigate biologically relevant functions. In particular, the greater stability of these Ln-peptide complexes will prevent the decoordination of the Ln^{3+} ion from the probe when interacting with biological molecules, which bear potential Ln^{3+}-binding sites such as proteins with metal-binding EF hands or DNA sequences.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

pH-metric titrations, ES-MS data, supplementary luminescence data, circular dichroism titrations, NMR tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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