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Thermodynamics of Self-Assembly of Copper(II) 15-Metallacrown-5 of Eu(III) or Gd(III) with (*S*)-α-Alaninehydroxamic Acid in Aqueous Solution

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The equilibria of self-assembly of 15-metallacrown-5 (15-MC-5) complexes of Cu^{2+} and (*S*)-α-alaninehydroxamic acid (α-Alaha, HL) with the lanthanide (Ln) ions Eu^{3+} or Gd^{3+} in aqueous solution are described. The binary Ln^{3+}/α-Alaha systems were first studied by potentiometric and calorimetric in-cell titrations; the latter technique allowed us to define the most suitable speciation model. On the contrary, because the kinetics of formation of the Ln^{3+} 15-MC-5 complexes is slow, their stability constants were determined by out-of-cell (batch) potentiometric titrations. Two 15-MC-5 complexes are formed with both Eu^{3+} and Gd^{3+}, namely, $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ and $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$, with the latter being the hydroxo species of the former. The acidity of the former to give the hydroxo species is remarkably high (log K = 4.40−4.69). Moreover, our potentiometric and spectrophotometric investigations clearly indicate that the hydroxide ion is coordinated to the central Ln ion, as was reported for several 15-MC-5 in the solid state. The formation of $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ starts at ca. pH 3.5, which converts at ca. pH 4.5 into the $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$ species, which predominates up to pH 7, where a purple precipitate occurs. The coexistence of both 15-MC-5 species and the copper(II) 12-MC-4 species of α-Alaha ($[Cu_5L_4H_{-4}]^{2+}$) was observed under appropriate experimental conditions (pH and ligand and metal concentrations). A complete ESI-MS investigation of the Ln^{3+}/Cu^{2+}/α-Alaha system at different pH's confirmed the formation of the two 15-MC-5 species. The 15-MC-5 stability constants were employed to quantitatively evaluate the solution behavior of Ln^{III} MCs regarding their integrity, ligand substitution, and transmetalation processes. In particular, EDTA or DOTA, added in equimolar amounts, should not appreciably interfere with the MC integrity, as found in previous experimental investigations, although it is expected that at higher amounts of EDTA, the MC should be disrupted. Our results also demonstrate that an excess of α-aminohydroxamate does not interfere with the integrity of the MC, and the disappearance of the CD spectra upon addition of the *R* enantiomer to 15-MC-5 containing the *S* enantiomer is due to a very rapid ligand exchange with formation of all possible isomers with no selectivity. The stability of the 15-MC-5 complexes in the presence of transferrin, serum albumin, or an excess of Zn^{2+} is also discussed. With regards to the latter metal ion, we found that the MCs are stable toward Gd^{3+}/Zn^{2+} transmetalation. although the presence of a phosphate buffer promotes the disruption of the MC scaffold by formation of stable Gd^{3+}/phosphate species.

Introduction

Metallacrowns (MCs) are the inorganic analogues of crown ethers, from which they can be ideally obtained by the replacement of C−C bonds with metal ion−nitrogen bonds.[1,2] Topological and structural analogues of the organic 12-crown-4 and 15-crown-5 ligands can be obtained, for instance, by replacing the two methylene groups of each macrocycle arm with −O−M−N− coordination units, where M^{z+} is the *ring metal ion*. In this way, the repetition of the $-(O-M-N)_n-$ unit [n = 4 and 5 for 12-metallacrown-4 (12-MC-4) and 15-metallacrown-5 (15-MC-5), respectively] leads to the formation of metallamacrocycles, which possess a central oxygen cavity capable of encapsulating a metal ion (M'^{n+}, *core metal ion*; Scheme 1).

Through the years, a large number of MCs containing a wide series of ligands and metals have been reported and were extensively reviewed in 2007 by Pecoraro and co-workers.[3] In particular, MCs obtained with aminohydroxamic acids and copper(II) as the ring metal ion have been the most studied and, to date, probably the most interesting, at least with

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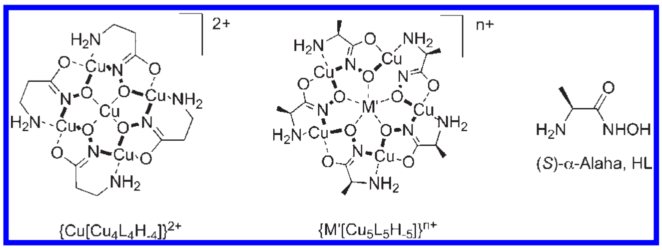
Scheme 1. Schematic Drawing of the Copper(II) (Ring Metal Ion) 12-MC-4 Complex of β-Alaha (Left) and of the 15-MC-5 Complexes (Center) of (*S*)-α-Alaha (Right)[a]



[a] The MC backbones are highlighted. M'^{n+} = Eu^{3+} or Gd^{3+} in this study.

regards to the large variability of structures obtained in the solid state.[3] The main features of aminohydroxamates are their capability of bridging between the metal ions and of possessing a proper number of donor atoms to complete the coordination geometry around the ring metal ions. In this way, both the MC ring closure and completion of the coordination position on the ring metals (with subsequent stabilization of the scaffold) are achieved at the same time.

On the basis of the number of structures obtained for these copper(II) metallacrowns, Pecoraro and co-workers have established a close relationship between the dimension and geometry of the MCs and those of the aminohydroxamates employed in their synthesis. The β-aminohydroxamic acids possess a suitable geometry for the assembly of 12-MC-4 in which both the ring and core metal ions are Cu^{2+} (Scheme 1, left). On the other hand, the shorter spacer between the amino and hydroxamic groups in α-aminohydroxamates forces the MC scaffold into a more expanded structure. This allows use of these ligands to prepare 15-MC-5 complexes in the presence of core cations that can accept an in-plane oxygen five-coordination (Scheme 1, center). To date, a number of 15-MC-5 complexes with Ca^{2+}, Ln^{3+}, UO_2^{2+}, Na^+, Ag^+, Pb^{2+}, Hg^{2+}, Ag^+, and Y^{3+} as core metals have been isolated and characterized in solution or in the solid state.[3-9] This capability of β- and α-aminohydroxamates to form almost planar 12-MC-4 and 15-MC-5, respectively, is called the "Metallacrown Structural Paradigm", and it has been extensively verified in the solid state.

Our research group has recently published a series of systematic studies on the formation in solution of Cu^{II}-12-MC-4 with aminohydroxamates and has clarified the thermodynamic aspects of their self-assembly.[10,11] These studies shed light on the relationship between the type of aminohydroxamate (α, β, or γ and its chirality) and the stability of its Cu^{II}-12-MC-4. Moreover, we also demonstrated that α-aminohydroxamates can form Cu^{2+}-12-MC-4 although of lower stability than those of β-aminohydroxamates. The 12-MC-4 complexes can be converted into 15-MC-5 species by the addition of suitable core metal ions.[8,9] However, the latter MCs cannot form in the absence of such core metals, and in this respect, Cu^{2+} MCs seem to represent structural but not functional analogues of crown ethers.

With regards to 15-MC-5 complexes, by virtue of their easy synthesis, they have been studied as recognition agents for metals and anions,[1,2] as building blocks to prepare mesoporous solids,[12] as single-molecule magnets,[13] as potential MRI contrast agents,[14] and as hosts for the selective binding of carboxylates in water.[15,16] To gain information on their integrity in solution, several studies have been presented in the literature concerning the substitution of the core metal ion, the ligand exchange, and the relative stability of MCs containing different Ln^{3+} ions.[8,9,14,17] However, despite the number of investigations performed to assess the integrity of 15-MC-5 in solution, no quantitative results regarding the overall stability of 15-MC-5 with Ln^{3+} have been published to date. This lack of information allowed only qualitative and, in some cases, even questionable interpretations of the solution equilibria involving 15-MC-5.

In this paper, we present a study in aqueous solution of the formation equilibria of the Eu^{3+} or Gd^{3+}/Cu^{2+} 15-MC-5 of (*S*)-α-alaninehydroxamate (α-Alaha (HL); Scheme 1), with the aim of clarifying the thermodynamic aspects related to their self-assembly. The formation of a hydroxo-15-MC-5, $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$, previously reported in the solid state and studied as a building block for the preparation of MC-based polymorphs, was also studied in detail.[18] The speciation models have been corroborated by electrospray ionization mass spectometry (ESI-MS) data. A quantitative correlation between the mass spectrometric data and the speciation in solution for these MCs was also attempted.

A quantitative interpretation of the results of previously reported experiments on the assembly and integrity of the 15-MC-5 species in the presence of competing ligands [such as ethylenediaminetetraacetic acid (EDTA), transferrin, or albumin] and metal ions (Zn^{2+}) in solution has also been performed, taking advantage of the thermodynamic data obtained here.

Experimental Section

General Information. All chemicals were reagent-grade and were used as received from Sigma Aldrich without further purification. KOH, HCl, and HNO_3 solutions (ca. 0.2 mol dm^{-3}) were prepared with the usual procedures of this laboratory, using Merck Titrisol high-purity, concentrated solutions.[19] All

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solutions for potentiometric, spectrophotometric, and calorimetric investigations were prepared in freshly boiled, bidistilled water. The purity of the ligand was checked by potentiometric titrations and NMR. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance 300 and 400 MHz spectrometers. Samples were dissolved in high purity $(CD_3)_2SO$ and D_2O, and the chemical shifts were referenced to solvent peaks. The deuterated solvents were obtained by Sigma Aldrich. Proton T_1 relaxation rates were measured by means of a standard inversion−recovery sequence. Elemental analyses were carried out by a Carlo Erba model EA1108 microanalyzer. IR spectra were recorded on a Nicolet 5PC FT-IR spectrometer in KBr pellets (4000−400 cm^{-1}). Thermogravimetric analyses (TGA) were carried out using a Perkin-Elmer TGA 7 thermogravimetric analyzer.

Synthesis of (*S*)-*N*-Carboxybenzyl-α-alanine (1). (*S*)-α-Alanine (4.00 g, 44.89 mmol) was dissolved in a 50 mL NaOH solution (3.59 g, 89.78 mmol) in water. A solution of benzyl chloroformate (8.3 mL, 9.92 g, 58.14 mmol) in toluene (30 mL) was added to the solution at 0 °C under stirring. The biphasic mixture was stirred overnight at room temperature. The organic layer was removed, and the aqueous layer was washed with diethyl ether (2 × 50 mL) to remove unreacted benzyl chloroformate. The aqueous solution was acidified with a 0.1 M HCl solution until ca. pH 3 was reached, obtaining a white suspension. The product was extracted in diethyl ether (3 × 50 mL). The organic phase was dried over anhydrous Na_2SO_4 and the solvent removed in vacuo, obtaining a white solid (yield 48%).

^{1}H NMR [$(CD_3)_2SO$, 300 MHz]: δ 12.5 (br, 1H, COOH), 7.60 (d, J = 8 Hz, 1H, NH), 7.34 (m, 5H, Ph), 5.01 (s, 2H, CH_2Ph), 4.00 (dq, J_1 = 8 Hz, J_2 = 7 Hz, 1H, C$_\alpha$H), 1.25 (d, J = 7 Hz, 3H, CH_3). IR (KBr, cm^{-1}): 3334 br (OH), 1699 s (C=O(OH)), 1684 s (C=O(NH)), 690 w (Ph). TLC (CH_2Cl_2:MeOH = 9:1): R_f = 0.31. Mp: 84.2−84.4 °C. Anal. Calcd for $C_{11}H_{13}NO_4$: C, 59.49; H, 5.87; N, 6.27. Found: C, 59.4; H, 5.9; N, 6.2.

Synthesis of (*S*)-*N*-Carboxybenzyl-*O*-benzyl-α-alaninehydroxamate (2). The product **1** (4.76 g, 21.34 mmol) was dissolved in 30 mL of dry CH_2Cl_2, and *N*-methylmorpholine (2.16 g, 2.35 mL, 21.35 mmol) and ethyl chloroformate (2.31 g, 2.03 mL, 21.31 mmol) were added at 0 °C under a nitrogen atmosphere, obtaining a pale suspension, which was stirred for 15 min (solution 1). In a separate flask, benzylhydroxylamine hydrochloride (3.74 g, 23.43 mmol) was suspended in 50 mL of dry CH_2Cl_2, and *N*-methylmorpholine (2.36 g, 2.57 mL, 23.38 mmol) was added at 0 °C under a nitrogen atmosphere. After filtration, the supernatant was added to the solution of **1**. The whole suspension was stirred at room temperature for 4 h and then washed with a 1 M HCl aqueous solution (2 × 40 mL), a saturated $NaHCO_3$ aqueous solution (2 × 40 mL), and brine (2 × 40 mL). The organic layer was dried over anhydrous Na_2SO_4, the solvent was removed in vacuo, and the product was collected as a white microcrystalline powder (yield 64%).

^{1}H NMR [$(CD_3)_2SO$, 300 MHz]: δ 11.2 (br, 1H, N*H*OBz), 7.51 (d, J = 7 Hz, 1H, BzOCON*H*), 7.35 (m, 10H, 2Ph), 4.97 (s, 2H, PhCH_2OCO), 4.74 (s, 2H, NHOCH_2Ph), 3.94 (qd, J_1 = J_2 = 7 Hz, 1H, C$_\alpha$H), 1.17 (d, J = 7 Hz, 3H, CH_3). IR (KBr, cm^{-1}): 3294 br (NH), 3183 br (NH), 1688 s (C=O(NHOBz)), 1657 s (C=O(OBz)), 698 w (Ph). Anal. Calcd for $C_{18}H_{20}N_2O_4$: C, 65.84; H, 6.14 N, 8.53. Found: C, 65.8; H, 6.2; N, 8.5.

Synthesis of (*S*)-α-Alaninehydroxamic Acid (3; α-Alaha, HL). The product **2** (2.00 g, 6.09 mmol) was dissolved into a suspension of Pd/C (10%, 300 mg) in 60 mL of dry methanol under a nitrogen atmosphere. Hydrogenation was performed overnight with a Parr apparatus with $p(H_2)$ = 1.5 atm. The catalyst was filtered off, and the solvent was removed in vacuo. The solid product was washed with 20 mL of absolute ethanol and the solvent removed by filtration. The product was obtained as a white powder (yield 97%). The purity of the ligand was determined by potentiometry against a standard KOH solution (purity >98%).

^{1}H NMR (D_2O, 300 MHz): δ 3.94 (q, J = 7 Hz, 1H, C$_\alpha$H), 1.07 (d, J = 7 Hz, 3H, CH_3). ^{13}C NMR (D_2O, 400 MHz): δ 170.6 (C=O), 47.9 (C$_\alpha$), 18.4 (C$_\beta$). IR (KBr, cm^{-1}): 3193 br (NH + OH + NH_2), 1620 s (C=O(NHOH)). Mp: 161.0−161.4 °C. Anal. Calcd for $C_3H_8N_2O_2$: C, 34.61; H, 7.75; N, 26.91. Found: C, 34.7; H, 7.7; N, 26.9.

Synthesis of the MC Complexes: General Method. The 15-MC-5 complexes of both Eu^{3+} and Gd^{3+} with α-Alaha were synthesized using the method described hereafter. A suspension of $Ln(NO_3)_3 \cdot xH_2O$ (x = 5 or 6, 1.09 mmol) in 2 mL of a methanol/water mixture (3:1, v/v) was added to a suspension of α-Alaha (1.09 mmol, 0.113 g) in 20 mL of methanol. In two separate flasks, solutions of $Cu(NO_3)_2 \cdot 3H_2O$ (1.32 g, 5.45 mmol) and KOH (0.614 g, 10.94 mmol) were prepared in 3 mL of a methanol/water mixture (3:1, v/v) and in 5 mL of methanol, respectively. The Ln^{3+}/α-Alaha solution was mixed with 1.5 mL of the copper solution and with 2.5 mL of the KOH solution. The solution was stirred for 3 min. The remaining volumes of copper and KOH solutions were added to the reaction mixtures, and the solutions were left to stir for 30 min. A pale-green precipitate appeared and was filtered on a glass septum. The solvent was removed in vacuo, obtaining a deep-blue material. The crude product was dissolved in dry methanol, leaving white insoluble impurities, which were removed by filtration on a 0.45 μm nylon filter. The solvent was removed in vacuo, and the collected solid was suspended in dry ethanol. The insoluble blue product was collected by filtration as a blue powder and washed with diethyl ether.

Synthesis of $\{Eu[Cu_5L_5H_{-5}]\}(NO_3)_3 \cdot 6H_2O$ (4). This MC was synthesized as reported above, using $Eu(NO_3)_3 \cdot 5H_2O$ (0.47 g, 1.09 mmol).

^{1}H NMR (D_2O, 300 MHz): δ 36.5 [br, 1H, 5C$_\alpha$H, T_1 = 2.8(1) ms], 9.5 [br, 3H, 5CH_3, T_1 = 6.7(2) ms]. IR (KBr, cm^{-1}): 3250 br (NH_2), 1586 s (C=O), 1351 m (NO_3^-). TGA: −9% (205 °C) corresponding to 6H_2O. Anal. Calcd for $EuCu_5C_{15}H_{42}N_{13}O_{25}$: C, 14.14; H, 3.32; N, 14.29. Found: C, 14.1; H, 3.3; N, 14.2.

Synthesis of $\{Gd[Cu_5L_5H_{-5}]\}(NO_3)_3 \cdot 5H_2O$ (5). This MC was synthesized as reported above, using $Gd(NO_3)_3 \cdot 6H_2O$ (0.49 g, 1.09 mmol).

^{1}H NMR (D_2O, 300 MHz): δ 38.0 [br, 1H, 5C$_\alpha$H, T_1 = 0.11(1) ms], 10.1 [br, 3H, 5CH_3, T_1 = 0.39(1) ms]. IR (KBr, cm^{-1}): 3250 br (NH_2), 1589 s (C=O), 1350 m (NO_3^-). TGA: −7% (195 °C) corresponding to 5H_2O. Anal. Calcd for $GdCu_5C_{15}H_{40}N_{13}O_{24}$: C, 14.28; H, 3.20; N, 14.43. Found: C, 14.3; H, 3.4; N, 14.3.

Potentiometry. All potentiometric titrations were carried out in aqueous solution at T = 25.0 ± 0.1 °C and I = 0.1 M (KCl) under a N_2 stream. The potentiometric apparatus consisted of a Metrohm Dosimat 665 dispenser and an Orion 720A potentiometer equipped with a Hamilton combined glass electrode (P/N 238000, for in-cell titrations with automated data acquisition) or a Hamilton microelectrode (P/N 238100, for batch titrations). Both the dispenser and the potentiometer were connected to a PC and controlled by a dedicated software, as previously described.[19] The electrodes were calibrated in terms of [H$^+$] by titrating HCl solutions with KOH (hereafter pH = −log [H$^+$]). The obtained pK_w value was 13.77(1). A standard Cu^{II} solution was obtained by dissolving high-purity $CuCl_2 \cdot 2H_2O$ in bidistilled water (C_{Cu} ca. 0.016 M) and titrated against a standard EDTA solution using Sulphon Black as an indicator. Standard Ln^{3+} solutions were obtained by dissolving high-purity $Eu(NO_3)_3 \cdot 5H_2O$ or $Gd(NO_3)_3 \cdot 6H_2O$ in bidistilled water (C_{Ln} ca. 0.008 M). The titrations against standard EDTA of the Ln^{3+} solutions were performed in 10 mL of an acetate buffer solution (pH 4.5) at 50 °C, using Xylenol Orange as an indicator.

The protonation and Cu^{II} complexation constants of α-Alaha were already published.[11] For the Eu^{III} and Gd^{III} complexation equilibria with α-Alaha, 4−5 in-cell titrations were carried out under a N_2 stream, using 25 mL samples [Ln:L = 1:4−8, C_{Ln} = (0.9−1.1) × 10^{-3} M, approximately pH 3−11].

The complexation of Ln^{III} with Cu^{II} and α-Alaha is kinetically slow, as shown by the marked drift in the emf readings observed for in-cell titrations within 15 min for each titration point. A series of out-of-cell (batch) titrations were then performed (3−4 titrations for each $Ln^{3+}/Cu^{2+}/L$ system). To obtain batch samples, a 100 mL aqueous mother solution was prepared by adding to a ligand solution proper amounts of standard Ln^{III} and Cu^{II} solutions to obtain the molar ratios and concentrations here reported: $Eu^{3+}:Cu^{2+}:L = 1:4.7−9.8:8.9−35.7$, $C_{Eu} = (1.4−4.8) \times 10^{-4}$ M; $Gd^{3+}:Cu^{2+}:L = 1:4.8−10.3:6.1−24.2$, $C_{Gd} = (1.3−4.8) \times 10^{-4}$ M; $I = 0.1$ M (KCl). This solution was divided into 18−20 samples (Teflon capped vials, 5 mL each), and increasing amounts of a standard KOH solution were added to each sample. The vials were kept at 25 °C in a thermostatted bath, and the pH was measured after 2, 6, 12, 24, 48, and 96 h. A drift in the emf values was observed only within 12 h after sample preparation, while the readings were constant after 24 h. The emf values measured 48 h after sample preparation were used for the calculations. For all $Ln^{3+}:Cu^{2+}:L$ ratios, precipitation of a violet solid was observed in the range of pH 6.5−9.8 (C_{Ln} ca. 4×10^{-4} M; C_{Cu} ca. 2.3×10^{-3} M) or 7.8−9.5 (C_{Ln} ca. 1×10^{-4} M; C_{Cu} ca. 1.4×10^{-3} M), which dissolved at higher pH. Only experimental data up to ca. pH 6.5 (absence of precipitation) were taken into account in the calculations.

Absorption and Circular Dichroism (CD) Studies. Visible absorption spectra (400−800 nm) were collected on a Perkin-Elmer Lambda 25 spectrophotometer using matched quartz cells of 1 cm path length and a 0.1 M KCl solution as a reference. $Ln^{3+}/Cu^{2+}/L$ complexation equilibria were studied by collecting the spectra of 15−20 batch samples at appropriate pH between ca. 3 and 6.5 [$Eu^{3+}:Cu^{2+}:L = 1:5.5:8.9$, $C_{Eu} = 4.33 \times 10^{-4}$ M; $Gd^{3+}:Cu^{2+}:L = 1:10.3:24.2$, $C_{Gd} = 1.35 \times 10^{-4}$ M; $Gd^{3+}:Cu^{2+}:L = 1:5.0:9.7$, $C_{Gd} = 4.62 \times 10^{-4}$ M; $I = 0.1$ M (KCl)]. The batch samples were prepared as described above for potentiometric batch titrations. The stability constants of binary and ternary species obtained by potentiometry and the calculated spectra of the Cu^{2+}/L species (previously published)[11] were taken into account as fixed parameters for the spectral data treatment. UV and visible CD spectra of the 15-MC-5 complexes were collected in the spectral range 210−800 nm on a Jasco J715 spectropolarimeter, using 1 and 0.1 cm path length quartz cells for the visible (400−800 nm) and UV (210−400 nm) spectral ranges, respectively. The samples were prepared by dissolving the solid MCs (compounds **4** and **5**) in a 0.1 M (KCl) aqueous solution [$C_{Ln} = (5.0−5.7) \times 10^{-4}$ M].

ESI-MS. ESI-MS spectra were recorded on a single quadrupole ZMD mass spectrometer (Micromass, Manchester, U.K.) fitted with a pneumatically assisted electrospray probe. Data were processed by using the spectrometer software (MassLynx NT, version 3.4). The measurements were performed on Ln^{3+}/Cu^{2+}/α-Alaha solutions at appropriate pH values between 4.1 and 10.4 for Gd^{3+} or 7.75 for Eu^{3+} ($Gd^{3+}:Cu^{2+}:L = 1:4.15:8$, $C_{Gd} = 4.0 \times 10^{-4}$ M; $Eu^{3+}:Cu^{2+}:L = 1:5.5:8.9$, $C_{Eu} = 4.3 \times 10^{-4}$ M). The samples were prepared in a manner similar to that described for potentiometric studies without the addition of KCl in order to avoid the formation of KCl clusters during the ionization process. The solutions were analyzed by direct infusion at 10 μL/min in the positive-ion mode. The conditions were as follows: ES capillary 3.0 kV; cone 30−110 V; extractor 4 V; source block temperature 80 °C; desolvation temperature 150 °C; cone and desolvation gas (N_2) 1.6 and 8 L/min, respectively. Scanning was performed at m/z 100−1800.

Calorimetry. Enthalpy values for binary complexation of Eu^{3+} and Gd^{3+} with α-Alaha were obtained with calorimetric titrations performed with a Tronac 450 isoperibolic calorimeter equipped with a 3 cm^3 Dewar vessel, at $T = 298.15 \pm 0.02$ K and $I = 0.1$ M (KCl). The sample solutions had compositions similar to those employed in potentiometry. The titrant (standard HNO_3) was added using a 1 mL Hamilton syringe also immersed in the thermostatic bath. The pH range explored was approximately 3.5−10.5. A warming resistance (ca. 100 Ω) and a thermistor placed in the solution respectively allowed the instrument to be electrically calibrated and temperature variations to be detected. The accuracy was checked daily by titrating a 2-amino-2-(hydroxymethyl)-1,3-propanediol solution with nitric acid. The completely automated system was managed by a PC via a Tronac 900 interface. The potential difference at thermistor ends was measured by a FLUKE 8840A digital multimeter. Home-made programs for management of the calorimetric system, data acquisition, and gross heat correction for nonchemical contributions[20] and dilution heats (computed from literature data)[21] were all in BASIC language.

Calculations. Stability constants were calculated from potentiometric data using the *Hyperquad 2006* program.[22] Estimated errors of both emf (0.2 mV) and titrant volume (0.008 mL) were considered in the weighing of the potentiometric data. Least-squares treatment was performed by minimization of the sample standard deviation $\sigma = [\sum_i w_i(E_i^\circ - E_i^c)^2/(n - m)]^{1/2}$, where E_i° and E_i^c are the experimental and calculated emf values, n is the number of observations, and m is the number of parameters refined. The statistical weights w_i were set equal to $1/\sigma_i^2$, where σ_i is the expected error on each experimental emf value (E_i°). The potentiometric data treatment for the $Ln^{3+}/Cu^{2+}/L$ systems was performed, keeping the ligand protonation and binary complexation constants, together with the formation constants of Cu^{2+} and Ln^{3+} hydrolytic species, fixed.[23] For each system, the data of different titrations were treated together. ΔH° values were computed from the calorimetric data by means of the least-squares computer program *HypDH*,[24] which minimizes the function $U = \sum w_i(Q_i^\circ - Q_i^c)^2$, where w_i values are statistical weights and Q_i° and Q_i^c are the experimental and calculated heats, respectively, over n observations. A literature ΔH_w° value of 56.4 kJ mol^{-1} was used in the calculations.[25] The spectrophotometric data were treated by means of the *SPECFIT32* program,[26] using unit weights. NMR data were processed by the *MestReC 4.9.9.9*[27] and *SPSS*[28] programs. The speciation diagrams were plotted with the *Hyss 2006* program.[29]

Results and Discussion

Synthesis of the Ligand (*S*)-α-Alaninehydroxamic Acid. The ligand (*S*)-α-alaninehydroxamic acid (α-Alaha) was synthesized by a synthetic route different from that commonly reported in the literature for α-aminohydroxamates.[30] While the literature route allows the synthesis of α-aminohydroxamic acids with only one synthetic step starting from amino acid methyl esters, we have evidenced that the final material does not always fulfill the high-purity requirements for potentiometric studies.

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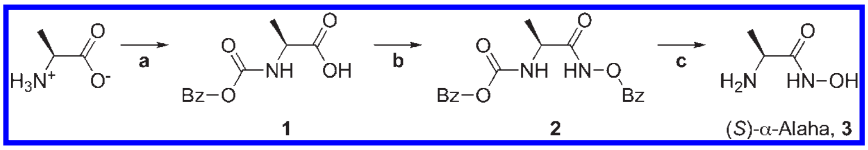
Scheme 2. Scheme of the Synthesis of (*S*)-α-Alaha[a]



1 2 (*S*)-α-Alaha, 3

[a] (a) 1: Benzyl chloroformate, NaOH, water/toluene, room temperature, overnight. 2: HCl. (b) Hydroxylamine hydrochloride, ethyl chloroformate, *N*-methylmorpholine, CH_2Cl_2, 0 °C, 4 h. (c) H_2 (1.5 atm), Pd/C (10%), methanol, overnight.

Table 1. Overall Thermodynamic Parameters for Protonation and Eu^{3+} and Gd^{3+} Complex Formation of α-Alaha and Acha in Aqueous Solution[a]

species	α-Alaha, log β	$-\Delta G°$, kJ mol^{-1}	$-\Delta H°$, kJ mol^{-1}	$\Delta S°$, J K^{-1} mol^{-1}	Acha, log β
LH	9.15[b]	58.3[b]	27.3[b]	84[b]	9.35[b]
LH_2^+	16.48[b]	108.0[b]	62.8[b]	105[b]	
$[EuLH]^{3+}$	13.56(4)	77.3(2)	63(1)	48(4)	
$[EuL]^{2+}$					6.66(3)
$[Eu(LH)_2]^{3+}$	25.88(8)	147.6(5)	121(8)	90(30)	
$[EuL_2]^+$					12.06(3)
$[Eu(LH)_3]^{3+}$	38.59(5)	220.1(3)	151(3)	230(10)	
$[Eu(LH)_2L]^{2+}$	31.16(3)	177.7(2)	109(3)	230(10)	
$[Eu(LH)L_2]^+$	23.50(3)	134.0(2)	73(3)	205(8)	
$[EuL_3]$	15.03(3)	85.7(2)	24(3)	206(8)	16.62(4)
$[EuL_3H_{-1}]^-$	5.58(3)	31.8(2)	−10(3)	140(10)	6.88(5)
$[EuL_3H_{-2}]^{2-}$	−5.55(3)	−31.7(2)			
σ	1.87		2.7×10^{-3}		2.26
n	152		216		297
$[GdLH]^{3+}$	13.49(3)	76.9(2)	56(1)	70(3)	
$[GdL]^{2+}$					6.40(2)
$[Gd(LH)_2]^{3+}$	25.95(4)	148.0(2)	93(3)	184(8)	
$[GdL_2]^+$					11.71(2)
$[Gd(LH)_3]^{3+}$	38.44(4)	219.3(2)	148(2)	239(7)	
$[Gd(LH)_2L]^{2+}$	31.14(2)	177.6(1)	95(2)	277(7)	
$[Gd(LH)L_2]^+$	23.51(2)	134.1(1)	54(2)	267(6)	
$[GdL_3]$	15.10(2)	86.1(1)	5(1)	273(4)	15.89(3)
$[GdL_3H_{-1}]^-$	5.51(2)	31.4(1)	−30(2)	205(6)	5.36(7)
$[GdL_3H_{-2}]^{2-}$	−5.87(2)	−33.5(1)			
σ	2.22		3.2×10^{-4}		2.56
n	463		340		176

[a] T = 25 °C; I = 0.1 M (KCl). Standard deviations on the last figure are given in parentheses. [b] See ref 10.

Therefore, for the present study, we decided to perform the synthesis of the ligand α-Alaha starting from (*S*)-alanine using the protection-activation strategy reported in Scheme 2. In this way, the last synthetic step was a simple catalytic hydrogenation on a highly pure benzyl-protected ligand, which afforded a >98% pure α-Alaha.

Solution Equilibria of (*S*)-α-Alaha/Eu^{III} or Gd^{III} Binary Systems. The solution equilibria of α-Alaha (HL) with Eu^{III} or Gd^{III} ions were investigated by glass-electrode potentiometry as a necessary step for the speciation of the ternary systems Ln^{3+}/Cu^{2+}/α-Alaha. Moreover, the equilibria of Eu^{3+} and Gd^{3+} with acetohydroxamic acid (Acha, LH) were also studied in order to obtain reference data for the complexing behavior of a simple O,O$^-$ chelating hydroxamate ligand. The results on the system Eu^{3+}/Acha (see Table 1) are in good agreement with a recent spectrophotometric study of Sinkov and co-workers (log β_{ML} = 6.19; log β_{ML_2} = 11.97; β_{ML_3} = 15.85).[31]

The two Ln^{3+} ions behave in a very similar way; therefore, only the results concerning the Eu^{3+} ion are discussed here in detail. For both Ln^{3+}/α-Alaha systems, data processing gave two different speciation models that described equally well the experimental curves on the basis of the statistical fitting parameters. Both models contained the species $[LnLH]^{3+}$, $[LnL_3]$, $[LnL_3H_{-1}]^-$, and $[LnL_3H_{-2}]^{2-}$, in addition to either $[Ln(LH)_2]^{3+}$, $[Ln(LH)_3]^{3+}$, $[Ln(LH)_2L]^{2+}$, and $[Ln(LH)L_2]^+$ (model 1, eight species) or $[LnL]^{2+}$, $[Ln(LH)L]^{2+}$, and $[LnL_2]^+$ (model 2, seven species). A model containing all 11 proposed species was impossible to refine, even though it cannot be excluded that all of the above complexes form in solution, some of them in rather low amounts. The analysis of these binary systems by ESI-MS under different experimental conditions (e.g., pH value, cone voltage, etc.) revealed no appreciable signals other than those of the $[Ln(LH_{-1})]^+$, $[LnL_2]^+$, and $[LnL_3]H^+$ species. This confirmed the absence of polynuclear species in solution, but these data were of no help for the choice between models 1 and 2 in which the species differ from each other only by the number of bound protons, although the sample standard deviation of potentiometric data refined with model 2 is higher than that of model 1 for both Ln ions (see the Supporting Information for model 2). To shed more light on these systems, a complete calorimetric study was thus performed. The calculated enthalpy values were found to be strongly dependent on the hypothesized speciation model, and only those calculated with log β values of model 1 were reasonable. The results obtained with model 1 are reported in Table 1, and a representative

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Table 2. Overall Stability Constants and Visible (Absorption, CD) Spectral Parameters of the 15-MC-5 Complexes of Eu^{3+} and Gd^{3+} of α-Alaha and Cu^{2+} in Aqueous Solution[a]

species	log β	λ_{max} (nm), ε (cm^{-1}, M^{-1})	λ_{max} (nm), $\Delta\varepsilon$ (cm^{-1}, M^{-1})
$\{Eu[Cu_5L_5H_{-5}]\}^{3+}$	54.14(3)	577, 373	609, −0.240; 345, +0.167; 234, +7.27
$\{Eu[Cu_5L_5H_{-5}](OH)\}^{2+}$	49.74(4)	572, 419	-
$\{Gd[Cu_5L_5H_{-5}]\}^{3+}$	53.82(3)	578, 375	616, −0.267; 345, +0.169; 234, +6.64
$\{Gd[Cu_5L_5H_{-5}](OH)\}^{2+}$	49.13(6)	573, 382	-

$\{Ln[Cu_5L_5H_{-5}](OH_2)\}^{3+} = \{Ln[Cu_5L_5H_{-5}](OH)\}^{2+} + H^+$; pK_a = 4.40(5) (Eu^{3+}), 4.69(7) (Gd^{3+}). [a] $T = 25$ °C; $I = 0.1$ M (KCl). Standard deviations are given in parentheses.

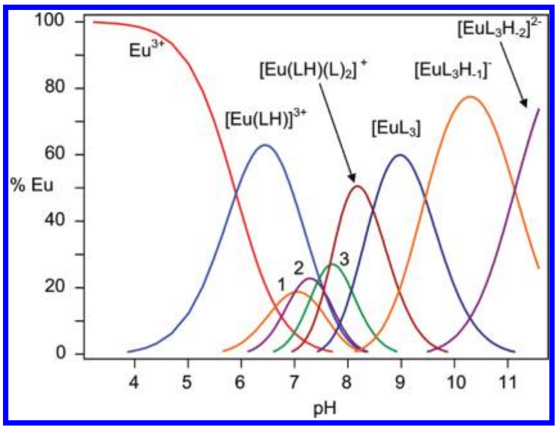

Figure 1. Representative distribution diagram of the system Eu^{3+}/α-Alaha (Eu:L = 1:6, C_{Eu} = 1.0 × 10^{-3} M). 1 = $[Eu(LH)_2]^{3+}$; 2 = $[Eu(LH)_3]^{3+}$; 3 = $[Eu(LH)_2L]^{2+}$.

distribution diagram is presented in Figure 1. The schematic structures proposed for Eu^{3+} complexes of model 1 are reported as Supporting Information, together with the thermodynamic data refined using model 2.

The complexation starts at ca. pH 5 with the successive formation of the species $[Eu(LH)]^{3+}$, $[Eu(LH)_2]^{3+}$, and $[Eu(LH)_3]^{3+}$. In these three complexes, α-Alaha is protonated on its amino group, and most probably it acts as an (O,O^-) chelating ligand, as suggested by the stepwise constants of the equilibria $[Eu(LH)_{n-1}]^{3+} + LH = [Eu(LH)_n]^{3+}$ (log K = 6.23, 4.99, and 5.38 for n = 1−3, respectively), which are comparable with those of the Eu^{3+}/Acha system (6.66, 5.40, and 4.56). With regards to the calorimetric results, the overall formation enthalpy of $Eu(LH)_3^{3+}$ (−151 kJ mol^{-1}; Table 1) corresponds to a mean contribution of −50 ± 10 kJ mol^{-1} for the reaction $Eu^{3+} + L^- + H^+ = [EuLH]^{3+}$. Therefore, a mean value of −14.5 kJ mol^{-1} is obtained for the formation of one O, O^- bond between the Eu^{3+} ion and a zwitterionic (HL) α-Alaha [−50 − (−35.5) = −14.5 kJ mol^{-1}; see Table 1 and the Supporting Information]. It is worth noting that this type of chelation has been found by X-ray analysis in two complexes of zwitterionic DL- and L-Alaha (HL) with Eu^{3+}, where dimeric cations $[Eu(HL)(H_2O)_6]_2^{6+}$ are present.[32]

The $[Eu(LH)_3]^{3+}$ complex has its maximum at neutral pH, and it deprotonates at the three ammonium groups to give the $[EuL_3]$ complex, which predominates at ca. pH 9. The corresponding three pK_a values (7.4, 7.7, and 7.5) are actually pretty close to the pK_a of the ammonium group in the free α-Alaha (7.33). The enthalpy changes for these three deprotonation steps (mean value: +42 ± 6 kJ mol^{-1}) are also similar to that of the ammonium group in the free ligand (+35.5 kJ mol^{-1}; Table 1). With regards to the deprotonation processes of $[EuL_3]$ to form the species $[EuL_3H_{-1}]^-$ and $[EuL_3H_{-2}]^{2-}$, these should involve the hydroxamate NH deprotonation (same as that for $[EuL_3]$ of Acha) rather than the formation of hydroxo species. Actually, the pK_a values for the $[EuL_3] = [EuL_2(LH_{-1})]^- + H^+$ process for α-Alaha and Acha are very similar (9.45 and 9.74, respectively). The absence of signals corresponding to hydroxo species in the ESI-MS spectra and the detection of the species $[Ln(LH_{-1})]^+$ (although not found by potentiometry) further support the hypothesis that $[EuL_3H_{-1}]^-$ and $[EuL_3H_{-2}]^{2-}$ are hydroximate and not hydroxo complexes.

Solution Equilibria of Eu^{3+} or Gd^{3+}/Cu^{2+}/α-Alaha Ternary Systems. The ternary systems Eu^{3+} or Gd^{3+}/Cu^{2+}/α-Alaha were initially studied by in-cell potentiometric titration, but a marked drift in the emf values was observed; thus, batch titrations were performed (see the Experimental Section). This inertness in the Ln^{3+} 15-MC-5 formation in water was also recently put into evidence by Pacco and co-workers for (*S*)-serine- and (*S*)-threoninehydroxamic acids: their spectrophotometric study showed that the formation equilibrium was reached only 30 min after reactant mixing, although we have measured a drift in the emf readings within 12 h.[33] In the present investigation, the titrations were limited to pH 6.5 because at higher pH the formation of an insoluble purple gelatinous material was observed. No direct calorimetric investigation with our isoperibolic instrument was possible because of the slow kinetics of these ternary systems.

The speciation models consist of two ternary complexes: $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ and $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$. Both are 15-MC-5 complexes, with the latter being the hydroxo species of the former because no further acidic protons are available on the ligand. The formation constants for the 15-MC-5 species are reported in Table 2, and representative distribution diagrams for the Gd^{3+} system are shown in Figures 2 and 3. The corresponding diagrams for Eu^{3+} are very similar and are reported as Supporting Information.

Figure 2 shows that $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ and $\{Gd[Cu_5L_5H_{-5}](OH)\}^{2+}$ are the only Gd^{3+} species formed in solution. The former reaches ca. 80% total Gd^{3+} at pH 4, while the latter amounts to more than 90% at pH > 5.7. It is worth noting that the refined stability constants for the 15-MC-5 species are independent of the model of the Ln^{3+}/α-Alaha system employed in the calculations (i.e., models 1 or 2; see above).

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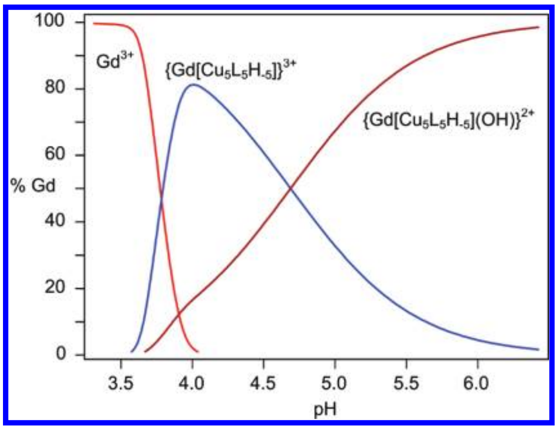

Figure 2. Representative Gd^{3+} distribution diagram for the $Gd^{3+}/Cu^{2+}/\alpha$-Alaha system ($Gd^{3+}:Cu^{2+}:L = 1:10.3:24.2$, $C_{Gd} = 1.36 \times 10^{-4}$ M).

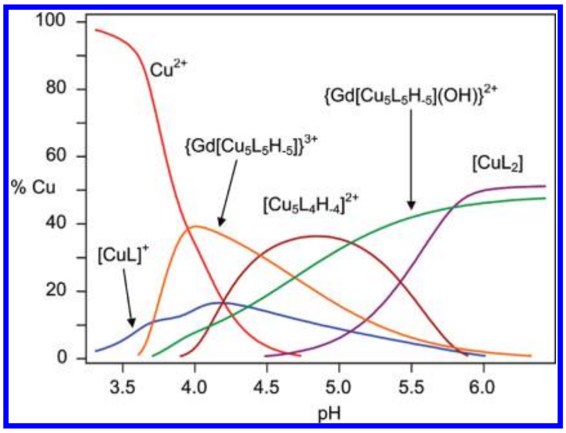

Figure 3. Representative Cu^{2+} distribution diagram for the $Gd^{3+}/Cu^{2+}/\alpha$-Alaha system ($Gd^{3+}:Cu^{2+}:L = 1:10.3:24.2$, $C_{Gd} = 1.36 \times 10^{-4}$ M; same conditions as those in Figure 2).

The potentiometric batch titrations of both Ln^{3+} ions were performed with an excess of both Cu^{2+} and the ligand with respect to the theoretical Ln:Cu:L = 1:5:5 ratio needed for the 15-MC-5 assembly (see the Experimental Section). These conditions were optimized in order to attain the equilibrium conditions within a few hours after sample preparation and to investigate the possible coexistence of binary Cu^{2+}/α-Alaha and 15-MC-5 species in the same pH range. In particular, the coexistence of the 12-MC-4 $[Cu_5L_4H_{-4}]^{2+}$ and the two 15-MC-5 species can be attained in the range of pH 4−5.8 for $Gd^{3+}:Cu^{2+}:L = 1:10.3:24.2$ (Figure 3). However, it is worth noting that the Cu^{II} distribution diagram drawn for a strictly stoichiometric Gd:Cu:L = 1:5:5 system (see the Supporting Information) does not show the formation of the 12-MC-4 species, suggesting that the 15-MC-5 complexes are thermodynamically stable in solution above pH 4.

Within our experimental conditions, $[CuL]^+$ and $[CuL_2]$ are also formed, in addition to the MC species. The $[CuL]^+$ species reaches 20% total copper at ca. pH 4, as observed in the Cu^{2+}/α-Alaha system,[11] while $[CuL_2]$ is formed at pH > 5, reaching 50% total copper at pH 5.5−6. Actually, the $[CuL_2]$ and $[CuL_2H_{-1}]^-$ complexes of α-aminohydroxamates are very stable because the ligand is coordinated to Cu^{2+} through 5-membered NH_2,N^- chelate rings, and the formation of $[CuL_2]$ is responsible for the disappearance of the 12-MC-4 species in the binary Cu^{2+}/α-Alaha system.[10,11,34] Their stability is, however, not high enough to compete with that of the 15-MC-5 species: the $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$ complexes reach their maximum concentration (100% total Ln^{3+}) at pH > 5.5 even in the presence of a high excess of ligand (up to $Ln^{3+}:L = 1:36$).

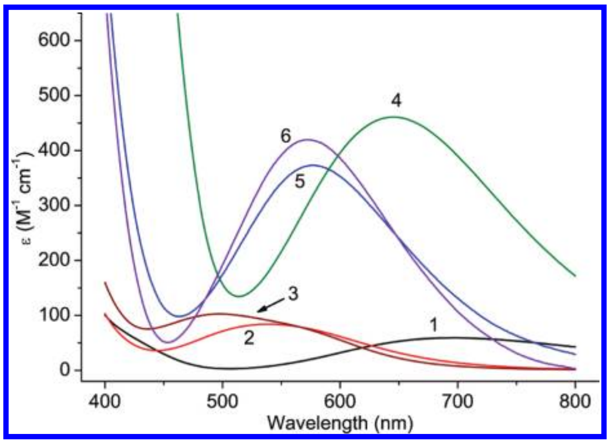

Figure 4. Calculated molar absorbances for the complexes in the $Eu^{3+}/Cu^{2+}/\alpha$-Alaha system. Spectra of the binary Cu^{2+}/α-Alaha species are those reported in ref 11. 1 = $[CuL]^+$; 2 = $[CuL_2]$; 3 = $[CuL_2H_{-1}]^-$; 4 = $[Cu_5L_4H_{-4}]^{2+}$; 5 = $\{Eu[Cu_5L_5H_{-5}]\}^{3+}$; 6 = $\{Eu[Cu_5L_5H_{-5}](OH)\}^{2+}$.

The pK_a of the deprotonation of a water molecule in $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ to give $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$ is 4.40 for Eu^{3+} and 4.69 for Gd^{3+}. In order to establish whether OH^- is coordinated either on the Ln^{3+} ion or on a peripheral Cu^{2+} ion, visible spectrophotometric titrations of the two $Ln^{3+}/Cu^{2+}/\alpha$-Alaha systems were performed in the range of pH 3−6.5. These spectra show that λ_{max} of $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$ presents a blue shift of 5 nm with respect to that of the $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ species for both Ln^{3+} ions (Table 2). The calculated molar absorbances for all of the species in the $Eu^{3+}/Cu^{2+}/\alpha$-Alaha system are reported in Figure 4 (see also the Supporting Information for further experimental data on both Eu^{3+} and Gd^{3+}).

In the literature, the X-ray structures of a number of Ln^{3+}/Cu^{2+} 15-MC-5 have been reported since 1994, some of them containing OH^- as one of the counterions of the $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ cation.[3] However, the precise localization of the hydroxide ion could, in general, not be achieved because the crystals normally contain several cocrystallized water molecules. Since the earliest works by Pecoraro and co-workers, the question was raised whether OH^- is coordinated to Cu^{2+} or to Ln^{3+},[3,35] although in a very recent paper the structure of the La^{III} 15-MC-5 with Pheha is described where one OH^- ion is bound to the La^{3+} ion.[18]

The position of the deprotonated water molecule can be inferred by considering the hydrolytic tendencies of both Cu^{2+} and Ln^{3+} ions. It is known that the pK_a for the hydrolytic process of the Cu^{2+} aquaion ($[Cu(OH_2)_x]^{2+} = [Cu(OH_2)_{x-1}(OH)]^+ + H^+$) is ca. 7.5,[23] while for Cu^{II} complexes, the acidity of water is lower and it depends on its coordination position. In particular, the axial water

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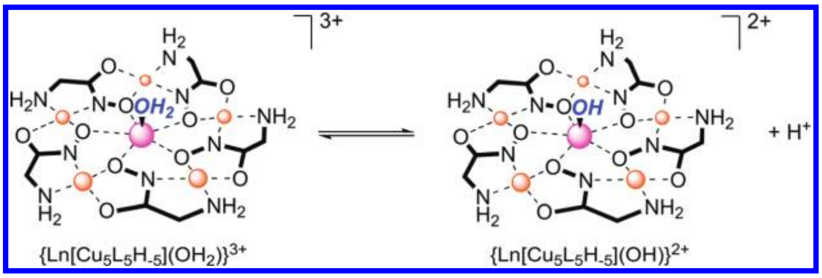
Scheme 3. Schematic Representation of the Proton Dissociation of the Axial Water Molecule in $\{Ln[Cu_5L_5H_{-5}](OH_2)\}^{3+}$ (Orange = Cu^{2+}; Magenta = Ln^{3+})



molecule of the (4N) equatorially coordinated Cu^{2+} in $[Cu(trien)(H_2O)]^{2+}$ occurs with a pK_a of 10.7,[23] while an even higher value (pK_a = 11.82) was observed for the $[CuL_2H_{-2}(H_2O)]$ complex of (*S*)-alanineamide (L).[36] It is of note that for the latter complex the deprotonation was accompanied by a small λ_{max} red shift of 5 nm.

With regards to the hydrolysis of Ln^{III} complexes, the Gd^{3+} aquaion has a pK_a of ca. 8.4,[23] which, as observed for Cu^{2+}, increases in its complexes with multidentate ligands. For Gd^{3+}/DOTA-type complexes, hydrolytic processes have been studied, and the pK_a range for the deprotonation of a coordinated water molecule is ca. 10−11.[37] On the contrary, a greater acidity has been shown by complexes with similar ligands, such as $[Ln(DTMA)(H_2O)]^{3+}$ [DTMA = DOTA tetrakis(methylamide)], which presents two pK_a values of 7.0 (Eu^{3+}) and 8.0 (Gd^{3+}).[38] With regards to in-plane five-coordinated Gd^{3+} complexes, a photophysical study of a Gd^{III} complex with a pentaazadentate porphyrin-like ligand, which has shown a change in the visible absorption spectra between pH 6.1 and 8.9, has been reported recently.[39] Although these studies were performed in methanol and the spectral change has been attributed to possible sandwich-type μ-oxo-bridged complexes, the pH interval 6.1−8.9 indicates a pK_a of ca. 7.5. Among the other Ln^{3+} ions, pK_a values for azacycles similar to those of DOTA with La^{3+}, Sm^{3+}, and Ho^{3+} between 7.03 and 8.02 have been reported.[40]

Although not as acidic as the two present $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ cations, these data show that Ln^{3+} complexes are expected to be, in general, more acidic than Cu^{2+} complexes. Thus, a pK_a of ca. 4.5 for the $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ complex (Table 2) is inconsistent with the deprotonation of a water molecule coordinated to Cu^{2+} in the axial position. The formation of the $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$ species must then involve the deprotonation of a water molecule coordinated to the Ln^{3+} core metal (Scheme 3). The $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ complexes are then the most acidic Eu^{3+} and Gd^{3+} complexes reported until now and, still surprisingly, are more acidic than the corresponding Ln^{3+} aquaions of 4 orders of magnitude. To explain this behavior, we put forward the hypothesis that in 15-MC-5 the hydroximate oxygen atoms of the cavity, which are involved also in Cu^{2+} coordination, most likely act as worse donor atoms toward the core metal than the water oxygen atoms of the Ln^{3+} aquaion. The enhanced hydrolytic behavior is thus a result of a high effective charge on the lanthanide center. The blue shift of the Cu^{2+} d−d absorption band upon water deprotonation is the result of the reduced charge on Ln^{3+}, which makes the hydroximate oxygen atoms more effective in Cu^{2+} coordination with an increase of the ligand field.

Table 3. ESI-MS Ions for the MC Species of the Systems Ln^{3+}/Cu^{2+}/α-Alaha (Ln^{3+} = Gd^{3+}, Eu^{3+})[a]

	Gd^{3+}	Eu^{3+}
$\{MC\}^{3+}$	328.9	327.7
$\{MC\}(H_2O)^{3+}$	334.2	332.9
$\{MC\}(H_2O)_2{}^{3+}$	340.2	338.7
$\{MC\}(OH)^{2+}$	501	498.5
$\{MC\}Cl^{2+}$	511	507.5
$\{MC\}(NO_3)^{2+}$	523.5	
$\{MC\}(L)^{2+}$	544	
$\{K[Cu_5L_5H_{-5}]\}^{+}$	864	
$\{MC\}Cl_2{}^{+}$	1057	1050
$\{MC\}Cl(NO_3)^{+}$	1084	1077
$\{MC\}(NO_3)_2{}^{+}$	1109	1104
$\{MC\}Cl_2(KCl)^{+}$	1131	1126

[a] The *m*/*z* values refer to the most intense peak of the multiplet. $\{MC\}^{3+}$ stands for $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$.

ESI-MS Measurements. Solutions of the system Gd^{3+}/Cu^{2+}/α-Alaha with the same concentrations as those employed in the potentiometric studies, without the addition of KCl, were studied by ESI-MS at different pH values (4.1, 4.3, 6.0, 6.9, 7.8, and 10.4). The samples at pH 6.9 and 7.8 presented a purple precipitate, which was filtered out, and the solution was passed through a 0.45 μm nylon filter before injection. For both Ln ions, ions associated with the $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ and $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$ species were detected as triple-, double-, and single-charged ions, as reported in Table 3. A representative spectrum of the Gd^{3+}/Cu^{2+}/α-Alaha system is reported in Figure 5.

As a general observation, the single-charged ions are 2−10 times less intense than the double-charged ones. It is worth noting that the relative intensities of all signals were highly dependent on the cone voltage conditions: the signals of 2+ species are favored over those of 1+ ions at voltages < 70 V, while at 70 V cone voltage, they are of almost equal intensity. Triple-charged ions have been clearly observed only at 30 V cone voltage. At higher voltages (110 V), only 1+ ions can be detected, but the whole intensity of the spectrum decreases dramatically.

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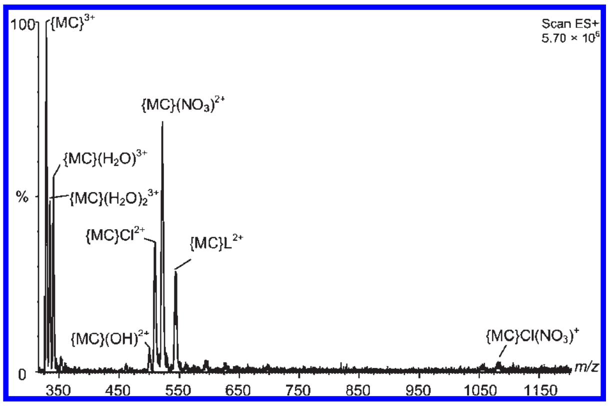

Figure 5. ESI-MS spectrum of a $Gd^{3+}/Cu^{2+}/\alpha$-Alaha system (Gd:Cu:L = 1:4.15:8, $C_{Gd} = 4.0 \times 10^{-4}$ M) in an aqueous solution at pH 4.1 and at 30 V cone voltage. $\{MC\}^{3+}$ stands for $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$.

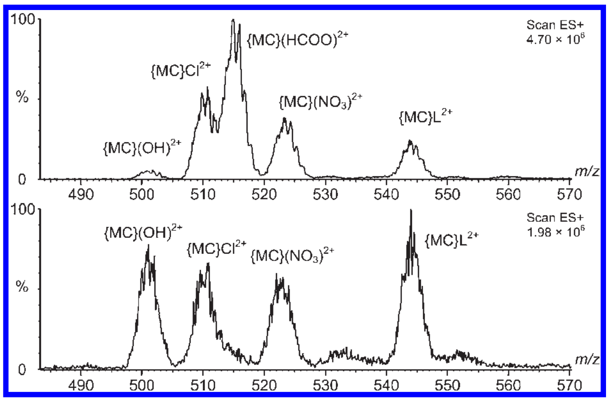

Figure 6. ESI-MS spectra of a $Gd^{3+}/Cu^{2+}/\alpha$-Alaha system (Gd:Cu:L = 1:4.15:8, $C_{Gd} = 4.0 \times 10^{-4}$ M) in an aqueous solution at pH 4.3 (top) and 6.0 (bottom) and at 40 V cone voltage. $\{MC\}^{3+}$ stands for $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$. The formate ion is a residue coming from the washing solutions employed for instrument cleaning.

The $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ ion has been identified as an aggregate with NO_3^-, Cl^-, and L^- anions. At 40 V cone voltage, the intensity of the $\{Gd[Cu_5L_5H_{-5}](OH)\}^{2+}$ ion at pH 4.3 is lower than those of the $\{Gd[Cu_5L_5H_{-5}]\}X^{2+}$ ions ($X^- = Cl^-$, NO_3^-; Figure 6), but it becomes as intense as the other signals at pH 6.0, where the hydroxo species is ca. 95% total Gd^{3+} (see Figure 2). The same agreement between the peak intensities and the distribution diagram was not observed at higher cone voltages. Actually, at pH 4.1 and 6.0 (70 V cone voltage), the signals of $\{Gd[Cu_5L_5H_{-5}](OH)\}^{2+}$ and $\{Gd[Cu_5L_5H_{-5}]\}X^{2+}$ are very similar, and this indicates that at high cone voltages ESI-MS spectra cannot be used for quantitative measurements of these complexes in solution.

The signal intensities of the $\{Gd[Cu_5L_5H_{-5}](OH)\}^{2+}$ ion as a function of the cone voltage reveal a high stability of this complex under the ESI-MS conditions. While the absolute intensities of the $\{Gd[Cu_5L_5H_{-5}]\}X^{2+}$ ions increase in the range of 30−40 V cone voltage and then decrease in the 40−70 V range, the signal of $\{Gd[Cu_5L_5H_{-5}](OH)\}^{2+}$ increases in the 30−40 V range and then it remains constant. The high intensity of the peak of the hydroxo complex at high voltages is consistent with the high acidity of the coordinated water molecules in $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$.

At pH 6.0−7.8 and at low cone voltages (30−40 V), additional peaks attributable to multiple-charged aggregates could be observed that tend to disappear at higher voltages (70 V) in favor of the $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ and $\{Gd[Cu_5L_5H_{-5}](OH)\}^{2+}$ species. In particular, the spectra collected at pH 6.0 presented peaks at $m/z = 627$ and 575, which cannot be attributed to simple MC species (Figure 7). These two peaks are large multiplets, spread over 8 m/z units and characterized by peaks at $\Delta m/z = 0.5$. Because of the Gd:Cu ratio in 15-MC-5 species and because of their isotopic distribution, $\Delta m/z = 0.5$ could be associated with 4+ ions, which should thus have molecular weights of ca. 2508 and 2300 Da for m/z 627 and 575, respectively. These multiplets are present also in the spectra of samples at pH 6.9 and 7.8, where the formation of a purple gel was observed, but only the peak at m/z 627 was observed at 30 V cone voltage. Attempts were performed to analyze separately the purple gel material by mass spectrometry. The solid is insoluble in many solvents of different polarities from methanol to chloroform, with the exception of dimethylformamide/water, where a very pale-purple solution was obtained. Unfortunately, analysis of this solution gave no appreciable signals, most probably because of a very low concentration of the analytes.

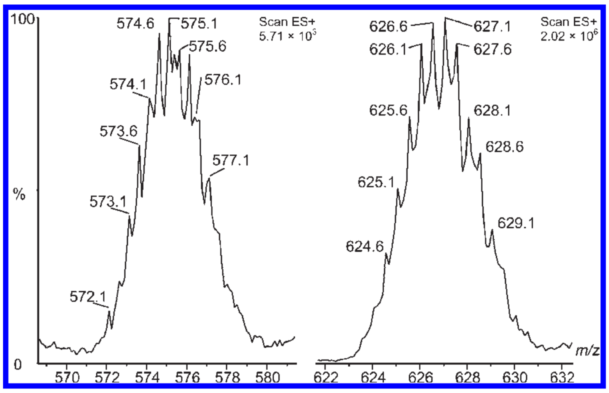

Figure 7. ESI-MS peaks assigned to aggregated MCs in a $Gd^{3+}/Cu^{2+}/\alpha$-Alaha system (Gd:Cu:L = 1:4.15:8, $C_{Gd} = 4.0 \times 10^{-4}$ M) in an aqueous solution at pH 6.0 and at 40 V cone voltage.

With regards to the possible nature of these aggregates, it is worth noting that α-norvalinehydroxamate forms with Cu^{2+} a metallahelicate with the formula $[Cu_{28}(LH_{-1})_{20}]^{16+}$, which has been isolated and structurally characterized (chlorides and acetates as counterions). However, analysis of a solution of metallahelicate with ESI-MS evidenced the presence only of ions related to the 12-MC-4 species.[3,41] Moreover, the formation of polymorphic dimers and helices in crystals of 15-MC-5 complexes of Ln^{3+}, Cu^{2+}, and phenylalaninehydroxamayte has recently been reported and discussed.[13,18] The hypothesis we put forward for our system is that a kind of supramolecular aggregate containing Ln^{3+}, Cu^{2+}, and the ligand in the $(LH_{-1})^{2-}$ form gives rise to the purple material, which, in turn, is related to the broad multiplets at ESI-MS. It is then possible that at proper pH values some kind of either MC- or metallahelicate-derived supramolecular aggregates are formed, a hypothesis consistent with the observation that at high cone voltage the aggregates are disrupted to give 15-MC-5 ions. The

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presence of the Ln^{3+} cation in these aggregates is suggested by the purple color, which is, in general, related with a Ln^{3+}-containing system while Cu^{II} 12-MC-4 with aminohydroxamates are green. With the aim to check if these aggregates may originate from a direct rearrangement of the 15-MC-5 complexes in solution, the ESI-MS spectra of solutions of the complex $\{Gd[Cu_5L_5H_{-5}]\}(NO_3)_3 \cdot 5H_2O$ in less solvating solvents (EtOH, 1:5 dimethyl sulfoxide/MeOH, or CH_3CN) were collected. Only in acetonitrile have two peaks of low intensity corresponding to dimeric ($\{Gd[Cu_5L_5H_{-5}]\}_2(NO_3)_5{}^{+}$, *m*/*z* 2281) and trimeric 15-MC-5 ($\{Gd[Cu_5L_5H_{-5}]\}_3(NO_3)_7{}^{2+}$, *m*/*z* 1695) been detected but no broad multiplets (see the Supporting Information). All of these results evidenced that the 15-MC-5 supramolecules are stable, although the change in the pH may promote the formation and precipitation of different species, whose high insolubility unfortunately prevented their precise identification.

Competition Study. Along the literature, the reactivity of Ln^{3+} 15-MC-5 toward several competing ligands and metals was evaluated with different methods (UV/vis and CD titrations, NMR, and proton relaxometry).[3,6,8,9,14,17] Among competing ligands, metal sequestering agents such as $EDTA^{4-}$ or natural metal ion transporters such serum transferrin were tested,[17] while transmetalation equilibria using Ca^{2+}, $UO_2{}^{2+}$, and Zn^{2+} were studied for possible core ion displacement.[4,6,8,14] The stability constants determined for the 15-MC-5 species reported here allow a quantitative (re)evaluation of some of the so far published results. As concerns ligand-exchange equilibria in particular, we performed these calculations assuming that no mixed-ligand species are formed. This is a reasonable approximation because the ligands taken into account are multidentate (like EDTA, transferrin, or albumin, see below). On the other hand, the speciation calculations for systems containing Ln^{3+} ions other than Eu^{3+} and Gd^{3+} (e.g., Nd^{3+}) were performed using the stability constants of Gd^{3+} MCs.

Pecoraro and co-workers reported a CD study concerning the addition of EDTA to a $\{Nd[Cu_5L_5H_{-5}]\}(NO_3)_3$ solution in water (HL = α-Alaha).[17] Upon the addition of 1 equiv of EDTA, only a partial disruption of the MC occurred, with a 15% decrease of the CD intensity, while a complete disappearance of the CD spectrum occurred at Nd^{3+}:EDTA = 1:6.[17] These data have been compared to the Gd^{3+} species distribution of the analogous system containing $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ and EDTA at equimolar concentrations (1×10^{-3} M, HL = α-Alaha) calculated in the range of pH 4−8 (Figure 8; literature formation constants for Gd^{3+} and Cu^{2+} with EDTA were used).[23] The corresponding Cu^{2+} and EDTA distribution diagrams are reported as Supporting Information.

The results of these calculations are in good agreement with the experimental observation of only a partial MC disruption. Actually, $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ and $\{Gd[Cu_5L_5H_{-5}](OH)\}^{2+}$ are the major species in the entire pH range (70−80% total Gd), with the latter prevailing at pH >4.7. Above pH 5, the concentration of both the hydroxometallacrown and $[Gd(EDTA)]^{-}$ increases, with the latter reaching 28% total Gd at pH 8. Above pH 6.5, no free EDTA is present (see the Supporting Information): this ligand is 20−25% involved in Gd^{3+} complexation and 75−80% in the binding to Cu^{2+}, with the latter released in solution together with the Gd^{3+} removed from the MC. Calculations showed that, upon an increase in the EDTA concentration to 4×10^{-3} M (Gd:EDTA = 1:4), 95% total Gd^{3+} is involved in EDTA at pH 7 (see the Supporting Information).

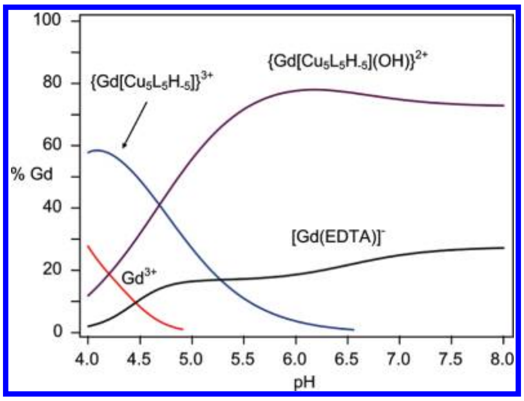

Figure 8. Gd^{3+} distribution diagram for the system containing $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ and $EDTA^{4-}$. 15-MC-5:EDTA = 1:1; $C_{15\text{-MC-5}} = 1 \times 10^{-3}$ M.

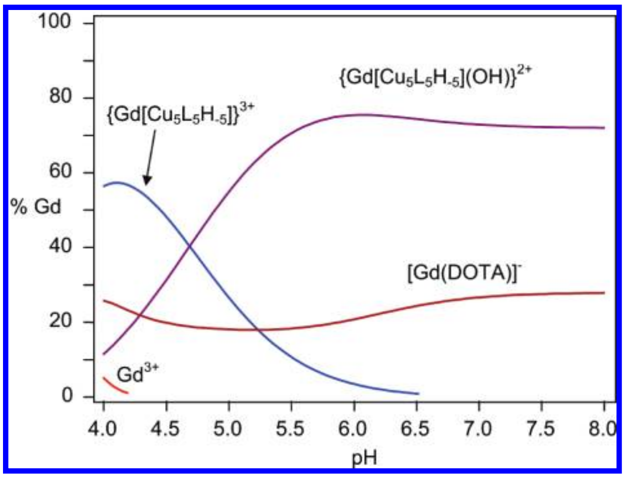

Figure 9. Distribution diagram for the system containing $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ and DOTA. 15-MC-5:DOTA = 1:1; $C_{15\text{-MC-5}} = 1 \times 10^{-3}$ M.

The competition between $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ and the $DOTA^{4-}$ ligand (one of the most relevant MRI contrast agents) was also evaluated.[42] The distribution diagram for the $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$/DOTA system at a 1:1 molar ratio (1×10^{-3} M; Figure 9) was calculated on the basis of the Gd^{3+} and Cu^{2+}/DOTA binary complex constants reported in the literature.[23] The behavior is quite similar to that of EDTA, discussed above.

The possible disruption of the 15-MC-5 complex by an excess of α-aminohydroxamate has also been discussed in the literature.[17] In particular, a decrease in the CD intensity upon the addition of 5 equiv of (*R*)-α-Alaha to a millimolar solution of $\{Nd[Cu_5L_5H_{-5}]\}(NO_3)_3$ with (*S*)-α-Alaha was observed. In the resulting solution, where the *R*/*S* enantiomeric ratio is 1:1, the CD spectrum

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(42) *The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging*; Merbach, A. E., Tóth, É., Eds.; John Wiley & Sons: Chichester, U.K., 2001.

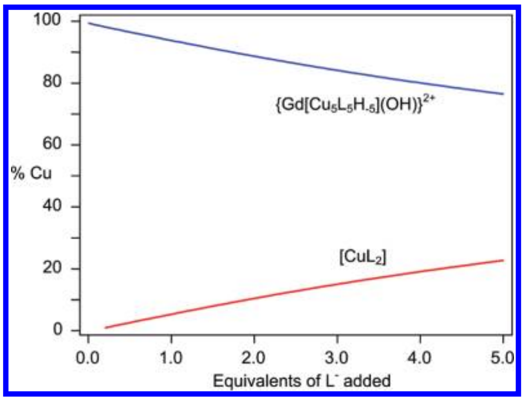

Figure 10. Distribution diagram for the system containing 1×10^{-3} M {Gd[Cu$_5$L$_5$H$_{-5}$]}$^{3+}$ upon the addition of (*R*)-α-Alaha at buffered pH 7.

slowly vanished in 10 h. This behavior was ascribed to the disruption of the MC and the formation of a racemic [CuL$_2$] complex. However, the diagram reported in Figure 10 shows that the addition of 5 equiv of (*R*)-α-Alaha produces a {Gd[Cu$_5$L$_5$H$_{-5}$](OH)} $^{2+}$ decrease of only 25% at pH 7 (buffered): approximately 75% of the initial MC remains intact (for these calculations, the stability constants of both the homochiral and eterochiral [CuL$_2$] complexes of α-Alaha were considered equal).

It looks thus reasonable to imagine that the {Gd[Cu$_5$L$_5$H$_{-5}$](OH)}$^{2+}$ species slowly undergoes ligand exchange with distribution of the chiral ligands over all possible configurations, leading to a racemic 15-MC-5 mixture and the corresponding disappearance of the CD spectrum. This behavior is consistent with the ligand exchange and mixing observed for Nd^{3+} 15-MC-5 of (*S*)-tyrosine- and (*S*)-phenylalaninehydroxamate, which also occurred in the hours time scale.[9] Moreover, the synthesis of Nd^{3+}/Cu^{2+} 15-MC-5 with racemic mandelohydroxamate in a 1:1 ratio led to a product that gave no CD signal, as was expected for a material containing all possible isomers.[7] Finally, the tendency of (*R*)- and (*S*)-α-aminohydroxamates to mix over all possible configurations is consistent with that recently found for Cu^{II} 12-MC-4 of α-aminohydroxamates.[10]

With regards to the stability of MCs in human serum, it is worth noting that many bioligands, from proteins to inorganic anions, may affect the integrity of the MC by removing Gd^{3+} and/or Cu^{2+}. An earlier experimental investigation showed that Nd^{3+} 15-MC-5 of α-Alaha is stable in the presence of human serum transferrin:[17] the species distributions for systems containing Gd^{3+} 15-MC-5 and transferrin at different concentrations are shown in Figures 11 and 12.

Despite the fact that human transferrin has two very high stepwise binding constants for Cu^{2+} at pH 7.4 (log $K_1 = 12.26$; log $K_2 = 11.08$; 0.1 M KCl)[43] and quite high stability constants for Gd^{3+} (log $K_1 = 7.96$; log $K_2 = 5.94$; 0.01 M HEPES),[44] the Gd^{3+} 15-MC-5 was found to be stable in the presence if this protein.[17] This behavior was interpreted on the basis of the inertness of 15-MC-5 assembly and deassembly,[17] but our speciation calculations clearly prove that the MC integrity resides also in their high thermodynamic stability. Actually, in systems containing {Gd[Cu$_5$L$_5$H$_{-5}$]}$^{3+}$ and transferrin at millimolar concentrations and at pH 7.0, which in some way reproduce the experiment cited above,[17] the amount of {Gd[Cu$_5$L$_5$H$_{-5}$](OH)}$^{2+}$ is ca. 80% total Gd^{3+}, while only the remaining 20% is bound to transferrin (Figure 11). With regards to the protein distribution, 75% serum transferrin (Tf) is bound to either Cu^{2+} or Gd^{3+}, while ca. 25% total transferrin is in the free form at pH 7 (Figure 12). Remarkably, for concentrations closer to those expected in vivo ($C_{\mathrm{Tf}} = 3.1 \times 10^{-5}$ M, $C_{\text{15-MC-5}} = 1.5 \times 10^{-4}$ M, pH 7.4), the {Gd[Cu$_5$L$_5$H$_{-5}$](OH)}$^{2+}$ complex should involve more than 95% total Gd.

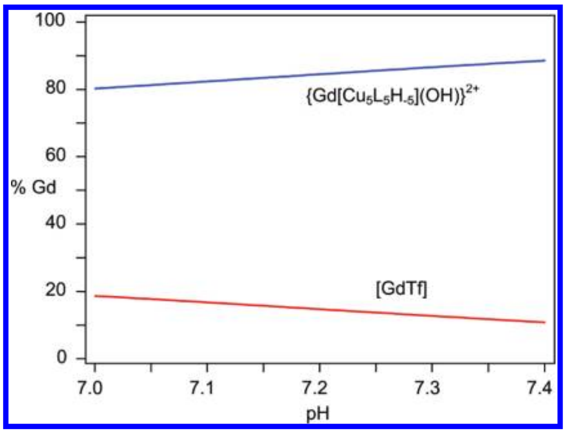

Figure 11. Gd^{3+} distribution diagram of the {Gd[Cu$_5$L$_5$H$_{-5}$]}$^{3+}$/transferrin (Tf) system (15-MC-5:Tf = 1:1, $C_{\text{15-MC-5}} = 1 \times 10^{-3}$ M).

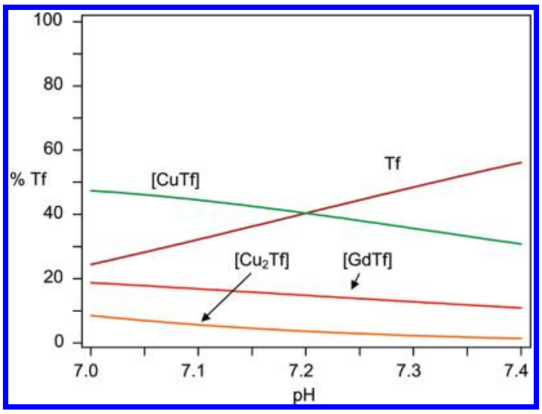

Figure 12. Transferrin distribution diagram of the {Gd[Cu$_5$L$_5$H$_{-5}$]}$^{3+}$/transferrin (Tf) system (15-MC-5:Tf = 1:1, $C_{\text{15-MC-5}} = 1 \times 10^{-3}$ M).

Similar calculations were performed by taking into account the capability of serum albumin (SA) to bind metal ions.[45−48] In the presence of this protein, Gd^{3+} 15-MC-5 (both at millimolar concentrations) is expected to remain intact. However, when Gd^{3+} 15-MC-5 and albumin are at 0.1 and 1 mM concentrations, respectively, the excess of albumin is expected to disassemble the 70% of the MC at pH 7.4. The distribution diagrams for the Gd^{3+}

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15-MC-5/SA systems are reported as Supporting Information (C_{SA} = 1 × 10^{-3} M, $C_{15\text{-}MC\text{-}5}$ = 1 × 10^{-3} or 1 × 10^{-4} M). These diagrams were calculated by taking into account the SA binding constants toward Cu^{2+} (log K = 12.0 at pH 7.4)[47] or Gd^{3+} (log K = 6.16),[48] together with the pK_a of the Gd^{3+} binding site (log K = 6.63).[48]

In a recent paper, it has also been suggested that 15-MC-5 possesses a poor stability in vivo because of transmetalation processes operated by Zn^{2+} that can substitute the core Gd^{3+}.[14] These studies were performed in a phosphate buffer and showed a change in the water proton relaxivities with loss of 80% of the proton longitudinal paramagnetic relaxation rate within 50 h. Although it does not have any tendency to assume planar five-coordination, Zn^{2+} forms, however, complexes with α-aminohydroxamic acids and, in principle, it could compete with Gd^{3+} and Cu^{2+} in coordinating α-Alaha.[49] We have thus calculated the effect of the Zn^{2+} addition to $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ at the experimental concentrations reported in the literature ($C_{15\text{-}MC\text{-}5}$ = C_{Zn} = 2.5 × 10^{-3} M).[14] Under these conditions, and even in a 1000-fold excess of Zn^{2+}, the $\{Gd[Cu_5L_5H_{-5}]\}^{3+}$ species remains >99% intact, suggesting that Zn^{2+} cannot be itself responsible of the 15-MC-5 disruption because of the very low stability of Zn^{2+}/α-Alaha complexes.

Although gadolinium(III) carbonate and phosphate are highly insoluble, it has been reported that Gd^{3+} complexes currently used as MRI contrast agents do not decompose with the formation of precipitates in either carbonate or phosphate buffers.[42] For these complexes, it was demonstrated that the anions may coordinate to available axial positions on the Gd^{3+} ion, but the removal of Gd^{3+} from the macrocycle is not observed for kinetic reasons.[42] We thus calculated a distribution diagram for a Gd^{3+} 15-MC-5 in the presence of a phosphate buffer at pH 7−7.4 under the experimental conditions of the transmetalation experiments and including the solubility product of $GdPO_4$ and $Cu_3(PO_4)_2$ (pK_{sp} = 25.6, solubility = $10^{-12.8}$ M, and 36.9, solubility = $10^{-7.8}$ M, respectively).[14,23,50] The result is that >99% total Gd^{3+} is expected to form $GdPO_{4(s)}$, while Cu^{2+} remains as $[Cu_5L_4H_{-4}]^{2+}$ and $[CuL_2]$ (80% and 20% total copper, respectively). The same calculations performed assuming a 15-MC-5 concentration of 1.5 × 10^{-4} M in the presence of a 1.3 × 10^{-3} M phosphate buffer, which is approximately the physiological serum concentration, gave as a result that >95% total Gd^{3+} is present as $GdPO_{4(s)}$ in the overall range of pH 7−7.4. A similar effect is not expected to be produced by carbonates because $Gd_2(CO_3)_3$ (pK_{sp} = 32.2, solubility = $10^{-6.9}$ M) is more soluble than $GdPO_4$.[23] Parac-Vogt and co-workers have not described clearly whether or not a precipitate appeared during the transmetalation experiments, but our results strongly suggest that phosphate has a role in removing Gd^{3+} and disassembling the 15-MC-5 complex.[14]

Conclusion

The equilibria of the ternary systems Cu^{II}/α-Alaha/Eu^{III} or Gd^{III} were studied in aqueous solution, and the stability constants of two 15-MC-5, namely, $\{Ln[Cu_5L_5H_{-5}]\}^{3+}$ and $\{Ln[Cu_5L_5H_{-5}](OH)\}^{2+}$, were determined. The Ln^{III} distribution diagrams evidenced that the MCs are the only solution species containing the Ln^{3+} ions, suggesting that the metallamacrocyclic scaffold obtained by the direct self-assembly of the ligand and the metal ions is stable in solution. Moreover, the speciation of these systems demonstrated the possibility for the 15-MC-5 and the 12-MC-4 complexes to coexist in solution as a function of the Ln:Cu:L molar ratios employed and that the amount of both types of MC complexes can be tuned with the pH.

ESI-MS experiments performed as a function of the pH and of various solvents allowed one to find the most suitable conditions for the analysis of the MC in solution and to establish a possible correlation with the speciation diagrams.

Finally, simulations based on the stability constants obtained for the 15-MC-5 complexes allowed us to critically reevaluate on a quantitative ground a number of experimental observations concerning ligand- and metal-exchange processes for 15-MC-5 reported in the past decade. As a major point, it has been established that Ln^{3+} MCs are stable in the presence of competing ligands (either α-Alaha itself or EDTA and DOTA). With regards to competing bioligands, the 15-MC-5 integrity is preserved in the presence of transferrin but not in the presence of an excess of SA. Transmetalation processes involving Zn^{II} to replace the encapsulated Ln^{3+} ions could be excluded, although the presence in solution of other components able to form very insoluble salts (such as phosphate) also may affect the MC integrity.

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Supporting Information Available: Schematic drawings of the Eu^{3+} binary complexes with α-Alaha (HL) and Acha (LH), a detailed discussion of the proton dissociation equilibria, representative distribution diagrams for the ternary systems, experimental and calculated visible spectra, CD spectra of 15-MC-5, and ESI-MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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