

A novel tetraazamacrocycle bearing a thiol pendant arm for labeling biomolecules with radiolanthanides†

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The novel tetraazamacrocycle 10-(2-sulfanylethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid (H₄DO3ASH) was synthesized and characterized by multinuclear NMR spectroscopy, 2D NMR techniques and mass spectrometry. The protonation constants of H₄DO3ASH were determined by potentiometry at 25 °C in 0.1 M KCl ionic strength, and the protonation sequence was assigned based on ¹H- and ¹³C-NMR titrations. The stability constants of the DO3ASH complexes with Ce³⁺, Sm³⁺ and Ho³⁺ have been determined by potentiometry and UV-Vis spectroscopy. They are very similar, comprising a narrow range (logK_{ML} = 21.0–22.0). UV-Vis spectrophotometric data on Ce³⁺–DO3ASH and relaxivity measurements on the Gd³⁺–DO3ASH complex suggest that the thiol group does not coordinate to the metal, even in its deprotonated form. For labeling with radioactive lanthanides(III), various conditions were tested and both complexes, ¹⁵³Sm/¹⁶⁶Ho–DO3ASH, were obtained in quantitative yield (> 98%) at pH = 6. At room temperature, formation kinetics were faster for the ¹⁵³Sm than for the ¹⁶⁶Ho complex (5 vs. 60 min, respectively, needed for complete labeling). The stability of these hydrophilic complexes (¹⁵³Sm, logD = –2.1; ¹⁶⁶Ho, logD = –1.6) has been studied in different buffers, in human serum and in the presence of excess of cysteine and glutathione. ¹⁵³Sm–DO3ASH has shown a high stability under these conditions and a relatively low protein binding (2.1%), while ¹⁶⁶Ho–DO3ASH was less stable, including in the presence of cysteine and glutathione, and had a slightly higher protein binding (6.7%). *In vivo* studies have been performed only for the more stable ¹⁵³Sm–DO3ASH complex and its biological profile and *in vivo* stability has been compared to that of ¹⁵³Sm–DO3A in the same animal model. The biodistribution profile presents a similar trend with rapid total excretion from the whole animal body, mainly *via* the urinary pathway. The most striking difference found is related to a slightly slower clearance of ¹⁵³Sm–DO3ASH from organs like blood, bone and muscle as compared to ¹⁵³Sm–DO3A. Additionally, the fraction of ¹⁵³Sm–DO3ASH taken by the hepatobiliary tract is also modestly higher than that of ¹⁵³Sm–DO3A.

Introduction

Radionuclide therapy using specific radiopharmaceuticals is an important emerging area in oncology.^{1–3} The strategy of this therapeutic approach is the delivery of high radiation doses to specific disease sites in target organs or tissues, while sparing healthy cells. The design of an efficient, target-specific radiotherapeutic agent involves the selection of a suitable radionuclide and the use of a carrier molecule to deliver the radionuclide to the desired tumor cells. The choice of radionuclide is determined by their physical, chemical and biological properties, availability and price.^{3,4} Radiolanthanides are largely explored for therapeutic applications as they emit β-particles with a wide range of energy, and they have different half-lives which easily match with different biological vectors and/or medical applications. Among

lanthanides(III), ¹⁵³Sm, ¹⁶⁶Ho and ¹⁷⁷Lu are the most explored for medical applications in nuclear medicine.^{4–6}

Tetraazamacrocycles, and DOTA derivatives in particular, are widely investigated as bifunctional chelating agents (BFCAs) for labelling different biomolecules with radiolanthanides in targeted radiotherapy. Due to their cyclic and preorganized nature, tetraazamacrocycles can encapsulate various metal ions to yield complexes of enhanced thermodynamic stability and kinetic inertness as compared to the analogous acyclic ligands.^{7,8} These properties are very important issues for applications in nuclear medicine, as radiotherapeutic agents must be thermodynamically stable and kinetically inert *in vivo*, avoiding transchelation and/or transmetallation.⁹ Structural factors, such as the rigidity and the cavity size of the macrocycle, the number of pendant arms and the nature of the donor atoms all play a significant role on the stability and, consequently, the chemical and biological behaviour of the complexes.^{10–12} BFCAs derived from tetraazamacrocycles have been applied to label various biomolecules, such as monoclonal antibodies or peptides, with ⁹⁰Y, ¹⁵³Sm, ¹⁶⁶Ho and ¹⁷⁷Lu; the most promising of them currently undergoing clinical evaluation.^{13–29}

Different strategies have been used to conjugate a BFCA to a biomolecule. Usually, it involves a covalent bond between a

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conjugation group of the BFCA and a functional group which is either naturally present in the biomolecule or is introduced synthetically. In peptides, the naturally occurring functional groups include terminal or side-chain amino and carboxyl groups, thiol functions from cysteine and *p*-hydroxyphenyl from tyrosine. Conjugation groups in the BFCAs can also be of a different nature, namely active esters, isothiocyanates, maleimides, hydrazides and *R*-haloamides. Although in proteins the lysine side chain amino groups are the most commonly labelled, conjugation of DOTA through the thiol groups of cysteine residues, using DOTA maleimide derivatives under relatively mild conditions (pH = 7), has also been reported.^{2,30,31} DOTA-like macrocyclic chelators conjugated to peptides through S–S bonds have also shown application as BFCAs for site-specific labelling of proteins,^{32,33} conjugation to antibodies^{34–37} and redox-sensitive or blood-pool MRI contrast agents.^{38–40}

Taking into account these features, the high stability reported for lanthanide DO3A chelates⁴¹ and our interest in finding radiolanthanide complexes suitable for conjugation to biomolecules, we have synthesized the novel tetraaza-macrocyclic 10-(2-sulfanylethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid ($H_4DO3ASH$) to evaluate its coordination capability towards Ln(III) ions and its potential as a bifunctional chelator for labelling biomolecules with radiolanthanides.

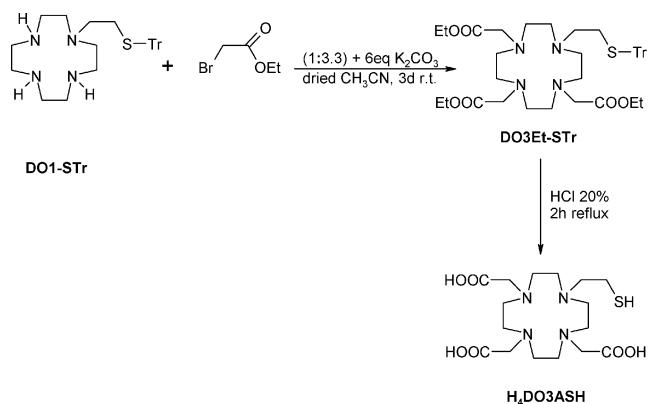
Herein, we report the synthesis, characterization and complexation properties of the novel 12-membered tetraaza-macrocyclic 10-(2-sulfanylethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid ($H_4DO3ASH$). We have studied the labelling of $H_4DO3ASH$ with $^{153}Sm/^{166}Ho$ and the *in vitro* stability of the species formed. In addition, we report the *in vivo* biological profile of the stable ^{153}Sm -DO3ASH complex.

Results and discussion

Synthesis and characterization of 10-(2-sulfanylethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid ($H_4DO3ASH$)

Different procedures have been described for the synthesis of DO3A derivatives, namely monoalkylation of the acid or ester form of DO3A, tri-alkylation of the corresponding monosubstituted tetraazamacrocyclic or selective modification of one DOTA carboxylic pendant arm.^{2,31} After trying several of these methodologies, we found that the best strategy for the synthesis of the novel tetraazamacrocyclic 10-(2-sulfanylethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid ($H_4DO3ASH$) was the alkylation of the previously described mono-substituted cyclen derivative 10-[2-(tritylsulfanyl)ethyl]-1,4,7,10-tetraazacyclododecane (DO1–STr)⁴² with ethyl bromoacetate, in the presence of K_2CO_3 , using dry acetonitrile as solvent (Scheme 1). After 3 days at room temperature, the compound obtained was purified by silica gel column chromatography, yielding compound DO3Et–STr in a relatively high yield (70%). After refluxing in HCl, the protected thiol/carboxylate compound DO3Et–STr led to the novel mixed cyclen derivative $H_4DO3ASH$ in 85% yield (Scheme 1).

Compound $H_4DO3ASH$ is soluble in water and stable under aerobic conditions at pH ≤ 10 . Above this pH, the ligand suffers oxidation of the thiol group. The characterization of $H_4DO3ASH$ was mainly based on mass spectrometry and multinuclear NMR



Scheme 1 Synthesis of $H_4DO3ASH$.

spectroscopy (1H ; ^{13}C ; 1H , 1H COSY; 1H , ^{13}C HSQC) under acidic conditions. In the ESI-MS positive mass spectrum, three main peaks were found at $m/z = 407$, 429 and 451 corresponding to $[L + H]^+$, $[L + Na]^+$ and $[L - H + 2Na]^+$, respectively.

The 1H and ^{13}C NMR spectra of $H_4DO3ASH$ were recorded at different pD values. At pD 1.20, the 1H NMR spectrum presents five resonances with different multiplicities at 2.76, 2.95, 3.30–3.37, 3.44 and 4.04 ppm in an intensity ratio of 2 : 8 : 10 : 4 : 2. The ^{13}C NMR spectrum recorded at the same pD exhibits ten resonances, a pattern consistent with the two-fold symmetry expected for $H_4DO3ASH$: four resonances were assigned to the carbon atoms of the macrocyclic backbone, two to the carbon atoms of the carboxylate pendant arms, two to the carbon atoms of the thiol pendant arm and the other two to the carbonyl carbon atoms. From 2D NMR studies (g-COSY 1H , 1H and g-HSQC 1H , ^{13}C correlations) performed at pD 1.20 (Fig. 1) we could conclude that the triplet centered at 2.76 ppm (2H) correlates with the carbon atom at 19.71 ppm. Based on the carbon and proton NMR data of the free arms and on our previous results,⁴² the resonance at 19.71 ppm was assigned to the α carbon atom of the thiol pendant arm, C1 (see Fig. 1 for numbering). The triplet at 2.76 ppm also correlates with the multiplet at 3.30–3.37 ppm (10H) and this correlates with the carbon atom at 58.04 ppm, assigned to C2. 1H , 1H g-COSY indicates that the singlets found at 3.44 ppm (4H) and 4.04 ppm (2H) do not correlate with any other resonances, thus they can only correspond to the methylene protons of the acetate pendant arms. Based on the 1H , ^{13}C g-HSQC, these singlets correlate with the carbons at 56.68 and 55.07 ppm which were assigned as C3 and C4, respectively. The resonances at 53.69 and 51.94 ppm were assigned to the C5 and C6 atoms, as they correlate with the multiplet appearing in the range 3.30–3.37 ppm. The multiplet at 2.95 ppm (8H) correlates with the carbon resonance at 50.47 ppm which also correlates with the multiplet at 3.30–3.37 ppm. Based on these data the resonance at 50.47 ppm was assigned to C7 and C8, and the multiplet at 2.95 ppm (8H) was assigned to the protons bound to these carbon atoms.

Acid–base behaviour. The acid–base behaviour of $H_4DO3ASH$ was investigated by pH-potentiometry in the pH range 1.7–11.5 at 0.1 M KCl ionic strength, as well as by 1H - and ^{13}C -NMR spectroscopy in the pD range 1.20–10.40 ($0.80 < pH < 10.00$), without control of the ionic strength. Five protonation constants have been determined by potentiometry

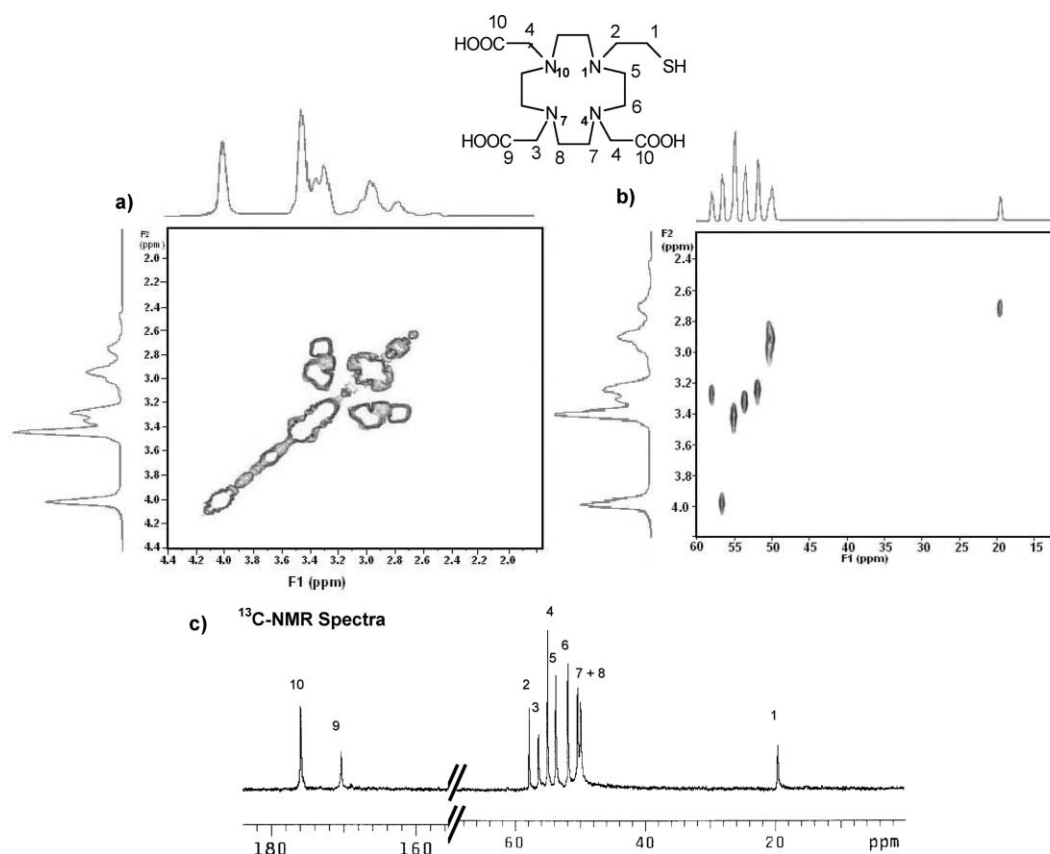


Fig. 1 ^1H , ^1H g-COSY (a) and ^1H , ^{13}C g-HSQC (b) 2D correlations; (c) final attribution of the ^{13}C -NMR spectrum of $\text{H}_4\text{DO3ASH}$, $\text{pD} = 1.20$.

Table 1 Protonation constants determined for $\text{H}_4\text{DO3ASH}$, compared to those reported for similar macrocyclic ligands

	DO3ASH ^a	DO3A ^b	DOTA ^c
$\log K_{\text{H1}}$	12.22(0.02)	11.55	12.6
$\log K_{\text{H2}}$	10.73(0.01)	9.15	9.70
$\log K_{\text{H3}}$	8.81(0.02)	4.48	4.50
$\log K_{\text{H4}}$	4.31(0.02)	—	4.14
$\log K_{\text{H5}}$	2.55(0.02)	—	2.32
$\log K_{\text{H6}}$	1.8 ^d	—	—

Charges were ignored.^a This work, 25.0 °C, $I = 0.1$ M KCl. ^b 25.0 °C, $I = 0.1$ M KCl, ref. 43 ° 25.0 °C, $I = 0.1$ M NMe_4Cl , ref. 44^a. ^d Determined by $^1\text{H}/^{13}\text{C}$ NMR spectroscopy, without control of the ionic strength.

and an additional sixth constant has been estimated in the acidic region by NMR spectroscopy. The protonation constants are defined as:

$$K_{\text{Hi}} = \frac{[\text{H}_i\text{L}]}{[\text{H}_{i-1}\text{L}][\text{H}^+]},$$

where $i = 1, 2, 3, \dots, n$.

Table 1 summarizes the protonation constants determined for $\text{H}_4\text{DO3ASH}$, compared to those reported in the literature for the related H_4DOTA and $\text{H}_3\text{DO3A}$ ligands.

NMR titrations (Fig. 2) were also used to assess the protonation sequence of the ligand. For a better comparison with the potentiometric data, Fig. 2 shows the chemical shifts as a function of pH ($\text{pH} = \text{pD} - 0.4$). It is generally assumed that when protonation

of a given site occurs, the β -carbons are shifted upfield, while the adjacent protons undergo a downfield shift.⁴⁵ In accordance with the $\log K_{\text{H}}$ values determined by potentiometry, four pH regions can be distinguished in the NMR titration curves: two including two protonation constants each ($14.0 \geq \text{pH} \geq 10.0$ and $\text{pH} \leq 4.0$) and two comprising one protonation constant each ($10.0 \geq \text{pH} \geq 7.5$ and $5.0 \geq \text{pH} \geq 4.0$). The $14.0 \geq \text{pH} \geq 10.0$ region was not studied by NMR, due to the sensitivity of the thiol group at basic pH and under aerobic conditions. However, based on potentiometric studies and according to data published for other related macrocycles ($\text{H}_3\text{DO3A}$ and H_4DOTA), the two highest protonation constants are easily ascribed to the protonation of two nitrogen atoms of the tetraazamacrocycle. Due to electrostatic repulsion, these protonations take place on opposite nitrogen atoms inside the cavity, most probably at N_1 and at N_7 . In the region $10.0 \geq \text{pH} \geq 7.5$, the resonances C1 and C9 are first shifted upfield (until $\text{pH} = 9.4$) and then shifted downfield, while C10 shifts upfield from $\text{pH} \sim 9.50$. In this range, the H1, H3 and H4 protons also undergo a downfield shift. Therefore, the third protonation constant ($\log K_{\text{H3}} = 8.81$) can be ascribed to the thiol group. This value agrees with other $\log K_{\text{H}}$ values reported in the literature for thiols, although in some cases the protonation constants for thiol groups might be highly dependent on the ligand backbone structure, or on the number and nature of the adjacent donor atoms.^{42,46} The attribution of $\log K_{\text{H3}} = 8.81$ to the thiol group was further confirmed by measuring the protonation constant of the Ca-DO3ASH complex. The thiol group is not expected to coordinate to the metal and its protonation should not be significantly influenced by the coordination of Ca^{2+} to

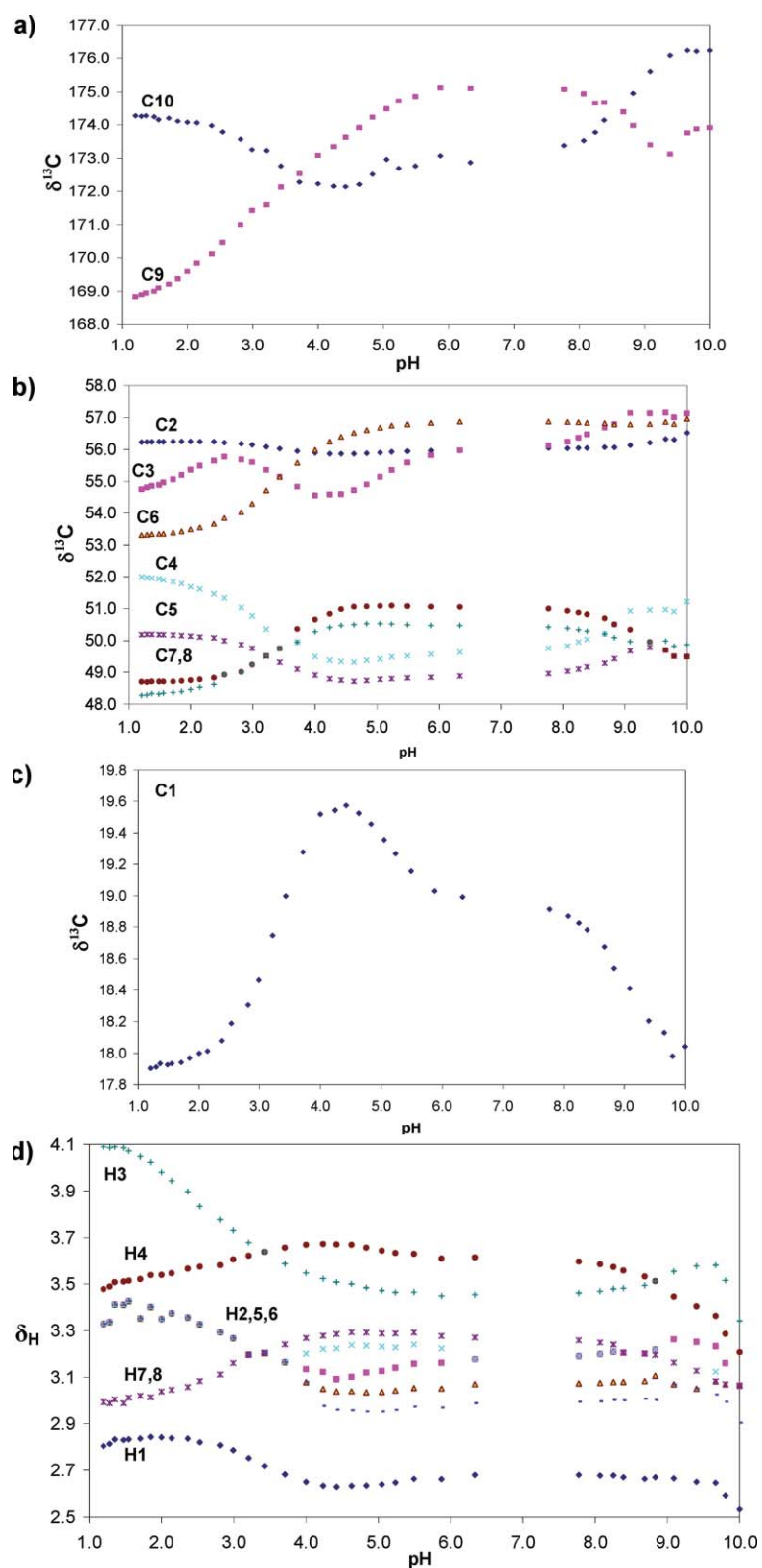


Fig. 2 ^{13}C - and ^1H -NMR titrations of $\text{H}_4\text{DO}_3\text{ASH}$: (a) ^{13}C -NMR titration of carbonyl resonances; (b) ^{13}C -NMR titration of other ^{13}C resonances; (c) ^{13}C -NMR titration of C1; (d) ^1H -NMR titration, all resonances.

the macrocycle. Indeed, from pH-potentiometric titrations we obtained $\log K_{\text{HcaL}} = 8.5 \pm 0.1$ for the protonation constant of the Ca^{2+} complex, a value close to the $\log K_{\text{H3}} = 8.81$ determined for the ligand itself.

In the range $10.0 \geq \text{pH} \geq 7.5$, the NMR data also indicate that the two protons attached to N_1 and N_7 undergo a redistribution to N_4 and N_{10} . Between pH 7.5 and 5.0, no significant changes are observed, though the smooth upfield shift of C9 may indicate the partial protonation of a carboxylate group (corresponding to $\log K_{\text{H4}} = 4.31$ determined by potentiometry). At $5.0 \geq \text{pH} \geq 4.0$, C9, C3 and C4 are shifted upfield and H3 is shifted downfield therefore, in this region, the carboxylate group opposite to the thiol pendant arm seems to be protonated. Finally, below pH 4.0 the C9 and C1 carbon atoms, as well as H4, H7 and H8 are shifted upfield, while C10, C5, C6, H1, H3, H2, H5 and H6 are shifted downfield. The C3 atom is first shifted downfield and then upfield. Such behaviour indicates a redistribution of the nitrogen protons from $\text{N}_4, \text{N}_{10}$ to N_1, N_7 and a protonation of the carboxylate attached to one C4 atom and a proton transfer from the carboxylate attached to C3 to the other carboxylate arm attached to C4. Finally, another protonation occurs at the carboxylate attached to C3 with a simultaneous redistribution of protons from N_1, N_7 to $\text{N}_4, \text{N}_{10}$. This protonation sequence is similar to the reported sequences proposed for the DO3A and DOTA ligands, for which the first two protonations correspond to amines in *trans* positions, $\log K_{\text{H3}}$ and $\log K_{\text{H4}}$ occur in COOH groups in the position opposite the protonated NH^+ , and the remaining protonations occur in the other COOH groups.^{41d,44b} For DO3ASH, protonation of the last two nitrogen atoms is not detected even by NMR, as it must occur under very acidic conditions.

Stability constants. The stability constant of the Ce–DO3ASH complex was determined by UV-Vis spectrophotometry at 25.0 °C and 0.1 M KCl ionic strength (Fig. 3). In order to get qualitative information on the formation kinetics of the lanthanide(III) complexes, we recorded UV-Vis spectra of the Ce^{3+} –DO3ASH system (245–350 nm) in buffered solutions at pH 4.7, 6.0 and 7.6 ($c_{\text{Ce}} = c_{\text{L}} = 10^{-3}$ M). Under these conditions, the complex was completely formed after 2 h, 15 min and 3 min, respectively. Given the slow formation in the pH range where the complexation is less than 100% (below pH ~ 4), a batch method was used to determine the stability constants. The UV-Vis spectra of the Ce^{3+} complex show only negligible changes upon the deprotonation

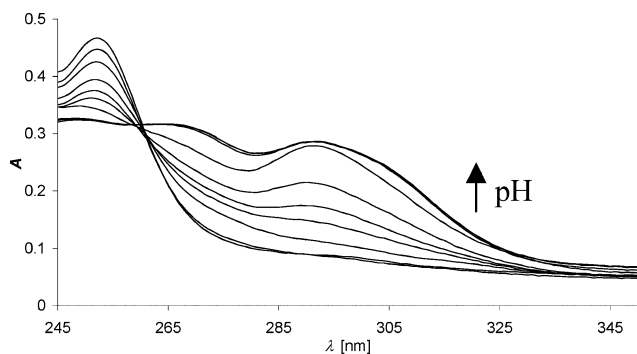


Fig. 3 UV-Vis titration of the Ce^{3+} –DO3ASH system. $c_{\text{L}} = c_{\text{M}} = 0.5$ mM at 25 °C, $I = 0.1$ M (KCl) and variable pH (3.01; 3.27; 3.82; 4.16; 4.41; 4.35; 5.66; 6.84; 7.63; 8.08).

of the –SH group, therefore only the overall stability constant of the Ce–DO3ASH complex protonated on the thiol group could be determined by UV-Vis spectrophotometry ($\beta_{\text{HML}} = [\text{HML}]/([\text{M}][\text{H}][\text{L}])$). Then, the value of the protonation constant, $K_{\text{HML}} = [\text{HML}]/([\text{ML}][\text{H}])$, was obtained from pH-potentiometric titrations of the complex prepared prior to the titration. The stability constant was calculated as $K_{\text{ML}} = \beta_{\text{HML}}/K_{\text{HML}}$. The invariance of the UV-Vis spectrum of the Ce^{3+} complex on deprotonation of the SH group shows that the deprotonated thiol does not coordinate to the lanthanide(III) ion. Even in its deprotonated form, the thiol is not able to replace a water molecule in the inner coordination sphere of the lanthanide. This has been further confirmed by relaxivity measurements on the corresponding Gd^{3+} complex. The relaxivity values were $7.6 \text{ mM}^{-1} \text{ s}^{-1}$ and $6.7 \text{ mM}^{-1} \text{ s}^{-1}$ at pH 4.9 and 7.4, respectively.

Upon coordination of the deprotonated thiol to the Gd^{3+} ion, one would expect the elimination of one inner sphere water molecule from the complex and, consequently, a more important decrease of the proton relaxivity. These findings prove that the thiol is not participating in the coordination of the lanthanide(III), therefore it can be used later for linking the chelate to targeting moieties.^{32,33} It is clear that the protonation constant is considerably lower in the Ln complexes than in the free ligand or in the Ca(II) complex. The higher charge of the lanthanide can be one factor responsible, even without coordination of the deprotonated S function. We also cannot exclude an eventual hydration equilibrium, which could be slightly modified by the pH. We should note that a hydration equilibrium can exist independently of the coordination of S. Luminescence lifetime measurements could bring information on the hydration number, however, the error associated with the q values determined in this way is at least ± 0.3 , which cannot confirm or refute the presence of a hydration equilibrium. An unambiguous answer could be given by the UV-Vis absorption measurements on the Eu complex, however, these experiments require a very large amount of the complex which was, unfortunately, not available.

The stability constants of the Sm^{3+} and Ho^{3+} complexes were determined by UV-Vis spectrophotometry in batch samples *via* competition with the Ce^{3+} ion. Similarly to the Ce^{3+} analogue, protonation constants of both Sm^{3+} and Ho^{3+} complexes were obtained by potentiometry. The complex stability and protonation constants are listed and compared to those of related macrocycles in Table 2.

Table 2 Stability ($\log K_{\text{ML}}$) and protonation ($\log K_{\text{HML}}$) constants determined for Ln(III) complexes^a

Ion	DO3ASH ^b		DO3A	DOTA
	$\log K_{\text{ML}}$	$\log K_{\text{HML}}$	$\log K_{\text{ML}}$	$\log K_{\text{ML}}$
Ce^{3+}	21.24(0.03)	5.97(0.03)	19.7 ^c	23.0 ^c
Sm^{3+}	22.0(0.1)	6.0(0.1)	—	23.0 ^c
Eu^{3+}	—	—	20.69 ^d	23.7 ^f
Gd^{3+}	—	—	21.0 ^c	24.6 ^c
Ho^{3+}	21.0(0.1)	6.4(0.1)	—	24.8 ^c
Lu^{3+}	—	—	23.0 ^c	25.5 ^c

^a $K_{\text{ML}} = [\text{ML}]/([\text{M}][\text{L}])$; $K_{\text{HML}} = [\text{HML}]/([\text{ML}][\text{H}])$. ^b This work, 25.0 °C, $I = 0.1$ mM KCl. ^c 25.0 °C, $I = 0.1$ M NMe_4Cl , ref. 41a. ^d Det. by laser-excited Eu^{3+} luminescence excitation spectroscopy, 25.0 °C, ref. 41b. ^e 25.0 °C, $I = 1$ M in NaCl, ref. 47a. ^f 37 °C, ref. 47b.

Table 3 pM Values calculated for Ln(III) complexes

pM	DO3ASH	DO3A	DOTA
Ce ³⁺	8.84	9.28	10.75
Sm ³⁺	9.22	—	10.75
Eu ³⁺	—	9.77	11.35
Gd ³⁺	—	9.93	11.55
Ho ³⁺	8.72	—	11.65
Lu ³⁺	—	10.93	12.00

pM = $-\log[\text{Ln}]_{\text{free}}$; $c_{\text{L}} = c_{\text{M}} = 10^{-6}$ M; pH 7.4.

The three different lanthanides, roughly representing the beginning, the middle and the last part of the lanthanide series, have very similar stability constants without any specific trend. They are two to three orders of magnitude lower than those found for the corresponding Ln–DOTA complexes, however, they are somewhat higher than the constants reported for Ln–DO3A chelates. The protonation constants of the complexes correspond to protonation of the thiol group. In contrast to the Ca–DO3ASH complex, the presence of a lanthanide ion bearing three positive charges considerably lowers the protonation constants of the thiol in the Ln(III) complexes with respect to the free ligand.

Table 3 shows the pM values ($\text{pM} = -\log[\text{M}]_{\text{free}}$) calculated for the Ln(III) complexes of DO3ASH, DOTA and DO3A, at pH 7.4. This parameter allows a better comparison of the affinity of the different ligands towards the same metal ion, as it also takes into account the basicity of the ligands. The values calculated for the DO3ASH complexes are about two log units lower than those for the DOTA analogues. They are also slightly lower than the corresponding pM values calculated for DO3A. This difference mainly reflects the higher protonation constants of DO3ASH, in particular the high values of $\log K_{\text{H}_2}$ and $\log K_{\text{H}_3}$.

Radiochemistry

Synthesis of ¹⁵³Sm and ¹⁶⁶Ho complexes

The complexes ¹⁵³Sm/¹⁶⁶Ho–DO3ASH were prepared by reacting ¹⁵³Sm/¹⁶⁶Ho(NO₃)₃ with the corresponding ligand in water, using a 1 : 2 metal : ligand molar ratio, at different pHs and temperatures. In order to evaluate the effect of the thiol pendant arm, the ligand H₃DO3A was also synthesised and labelled with ¹⁵³Sm. The yield of the reactions was followed by ITLC-SG, using an eluent system in which the ¹⁵³Sm/¹⁶⁶Ho(NO₃)₃ stays at the origin ($R_{\text{f}} = 0$) and the ¹⁵³Sm/¹⁶⁶Ho–DO3ASH or ¹⁵³Sm–DO3A complexes migrate. The experimental conditions were optimized to obtain radiolanthanide complexes with high radiochemical purity (see ESI†). Table 4 presents the experimental conditions used for quantitatively labelling H₄DO3ASH with ¹⁵³Sm/¹⁶⁶Ho

and H₃DO3A with ¹⁵³Sm, together with those previously used for H₄DOTA.⁴⁸ The formation kinetics of ¹⁵³Sm–DO3ASH and ¹⁵³Sm–DO3A compare with that found for ¹⁵³Sm/¹⁶⁶Ho–DOTA, while ¹⁶⁶Ho–DO3ASH forms at a slower rate.

The relatively mild conditions needed for quantitatively labelling H₄DO3ASH encouraged the *in vitro* evaluation of ¹⁵³Sm/¹⁶⁶Ho–DO3ASH complexes. Lipophilicity, plasmatic protein binding and *in vitro* stability studies in physiological media and in fresh human serum were performed. Partition coefficients at physiological pH were determined using the shake–flash method between octanol and PBS (pH = 7.4) and the logD values found were -2.11 and -1.63 for ¹⁵³Sm and ¹⁶⁶Ho, respectively. The protein binding of these hydrophilic complexes was determined by a trichloroacetic acid (TCA) precipitation method and relatively low protein binding was observed (2.1 and 6.7% for samarium and holmium complexes, respectively). The ¹⁵³Sm–DO3ASH complex is stable, at least up to five days, at 37 °C in saline, phosphate buffer (pH 7.4) and human serum. The ¹⁶⁶Ho analogue is only stable up to 3 days in saline, 2 days in human serum and 1 day in the presence of PBS. These results indicate that, under these conditions, ¹⁵³Sm–DO3ASH is more stable than ¹⁶⁶Ho–DO3ASH. While the thermodynamic stability constants and pM values of Sm–DOTA ($\log K_{\text{ML}} = 23$; pM = 10.75) and Sm–DO3ASH ($\log K_{\text{ML}} = 22$; pM = 9.22) are somewhat different, they are equally stable in saline, phosphate buffer and serum at least up to 5 days.

Due to the high level of circulating biomolecules containing thiol groups in healthy human plasma, namely cysteine (201 μM) and glutathione (GSH, 3.60 μM),⁴⁹ it is very important to evaluate the kinetic inertness of the radiocomplexes in the presence of those substrates. Thus, ¹⁵³Sm/¹⁶⁶Ho–DO3ASH (1.6 μmol) were incubated with cysteine solutions in PBS (pH 7.4), and aliquots of these mixtures analysed by ITLC chromatography at different time points. The ¹⁵³Sm complex was stable up to 24 h in 0.5 and 2 mM cysteine solutions. For ¹⁶⁶Ho–DO3ASH, we found that after 24 h incubation with 0.5 and 2 mM cysteine solutions, only 58 and 49% of intact compound was still present, respectively. Due to the instability of ¹⁶⁶Ho–DO3ASH, we only proceeded with the evaluation of ¹⁵³Sm–DO3ASH in 1 mM GSH solution. This complex is resistant to reaction with GSH up to 24 h. Owing to the high stability of ¹⁵³Sm–DO3ASH, compared with ¹⁶⁶Ho–DO3ASH, the *in vivo* studies were only performed for the samarium complex and to have an idea of the effect of the thiol pendant arm, the biological behaviour of ¹⁵³Sm–DO3A was also studied.

Biological studies. Biodistribution studies of ¹⁵³Sm–DO3ASH complex were performed in healthy female CD-1 mice, after intravenous (i.v.) injection through the tail vein at 30 min and 2 h. For comparison, the ¹⁵³Sm–DO3A complex was also studied

Table 4 Labelling conditions for H₄DO3ASH, H₃DO3A and H₄DOTA with ¹⁵³Sm and ¹⁶⁶Ho

Ligand	¹⁵³ Sm–macrocylic complexes		¹⁶⁶ Ho–macrocylic complexes	
	Labelling conditions	Labelling efficiency	Labelling conditions	Labelling efficiency
H ₃ DO3ASH ^a	5 min, rt, pH 6	> 98%	60 min, rt, pH 6	> 98%
H ₃ DO3A ^a	5 min, rt, pH 6–8	> 98%	—	—
H ₄ DOTA ^b	5 min, rt, pH 6–9	> 98%	5 min, rt, pH 6–9	> 98%

^a This work. ^b Ref. 48.

Table 5 Total excretion (% initial dose, ID)

Complex	Time/h	
	0.5	2
$^{153}\text{Sm-DO3ASH}$	49.2 ± 8.9	79.7 ± 3.9
$^{153}\text{Sm-DO3A}$	76.8 ± 7.6	90.3 ± 2.2
$^{153}\text{Sm-DOTA}^a$	60.2 ± 9.9	97.2 ± 1.0

^a Ref. 48.

in the same animal model. Tissue distribution data for the most relevant organs, as a function of time, are depicted in Fig. 4, and total radioactivity excretion is shown in Table 5.

These data point out a comparable pattern for both complexes with no significant accumulation of radioactivity in any major organs, except for the kidneys. A relatively rapid washout from blood stream and most important organs and a high rate of total excretion was found (>79% of the radioactivity is excreted at 2 h post injection, p.i.), a behaviour which correlates well with the high hydrophilic character of the radioactive complexes. In spite of the biodistribution similarities, $^{153}\text{Sm-DO3ASH}$ complex presents a slightly slower clearance from blood and most organs than $^{153}\text{Sm-DO3A}$. Moreover, there was a small difference in the percentage of injected dose taken by the hepatobiliary tract. Actually, the total radioactivity was quite efficiently excreted mainly *via* the urinary excretory route for both complexes but there was also some hepatobiliary uptake that is slightly higher for $^{153}\text{Sm-DO3ASH}$ ($0.9 \pm 0.1\%$ ID g^{-1} organ, 2 h p.i.) than for $^{153}\text{Sm-DO3A}$ ($0.3 \pm 0.1\%$ ID g^{-1} organ, 2h p.i.).

Chromatographic analysis of urine samples collected at different sacrifice time indicated a high *in vivo* stability for both complexes, as no metabolites were detected and/or no free metal was found.

This *in vivo* behaviour with rapid washout from main organs, high rate of whole body excretion and high stability is very similar to that found for $^{153}\text{Sm-DOTA}$ in the same animal model.⁴⁸

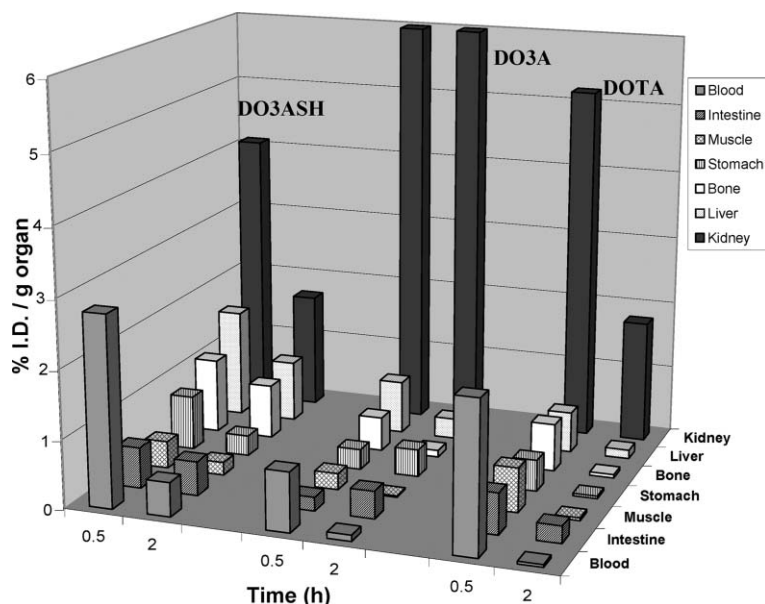
The favourable biodistribution profile of the ^{153}Sm -complex indicates that $^{153}\text{Sm-DO3ASH}$ is a promising candidate for further studies towards biological applications. Moreover, its kinetic inertness remains sufficiently high to avoid *in vivo* transmetallation or transchelation. Our results seem to indicate that the conjugation of the complex *via* the thiol sulfur atom to a biomolecule would not compromise the stability and kinetic inertness of the ^{153}Sm -labelled conjugate.

Concluding remarks

A novel DOTA-like ligand bearing a thiol function, 10-(2-sulfanylethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid ($\text{H}_4\text{DO3ASH}$), was synthesised and fully characterized by the usual analytical techniques. The protonation constants were obtained by potentiometry and a protonation sequence was proposed based on $^1\text{H}/^{13}\text{C}$ -NMR spectroscopic titrations. The stability constants of the complexes formed with Ce^{3+} , Sm^{3+} and Ho^{3+} were determined by UV-Vis spectrophotometry and potentiometry. These lanthanides, roughly representing the beginning, the middle and the last part of the Ln series, have similar stability constants. The values found are lower than those for the corresponding Ln-DOTA and higher than Ln-DO3A. UV-Vis spectroscopy and relaxivity measurements on Gd-DO3ASH have suggested that the thiol group is not coordinated to the metal, even in its deprotonated form.

Compound DO3ASH was successfully labelled with ^{153}Sm and ^{166}Ho in high yields (>98%) and radiochemical purity, using relatively mild conditions. The ligand $\text{H}_3\text{DO3A}$ was also synthesised and labelled with ^{153}Sm . The kinetics of formation of $^{166}\text{Ho-DO3ASH}$ seem to be slower than those of $^{153}\text{Sm-DO3ASH}$ and $^{153}\text{Sm-DO3A}$. The *in vitro* stability studies in physiological media, including in human serum, indicated that $^{153}\text{Sm-DO3ASH}$ is more stable than the corresponding ^{166}Ho complex.

Complexes $^{153}\text{Sm-DO3ASH}$ and $^{153}\text{Sm-DO3A}$ present a similar biodistribution trend, with fast clearance from most organs and a

**Fig. 4** Biodistribution of DO3ASH, DO3A and DOTA labeled with ^{153}Sm .

rapid total excretion at 2 h post injection. The main difference is that ^{153}Sm -DO3A presents a slightly slower clearance than ^{153}Sm -DO3ASH. The *in vivo* stability and favourable biological profile of ^{153}Sm -DO3ASH, together with the non participation of the thiol group in the stabilization of the lanthanide complexes, makes this novel tetraazamacrocyclic suitable for functionalization of biomolecules *via* the thiol pendant arm without compromising the stability and kinetic inertness of the ^{153}Sm -labelled conjugate.

Experimental

Reagents

Chemicals and solvents were of reagent grade and were used without further purification, unless stated otherwise. Acetonitrile, ethyl bromoacetate, potassium carbonate, methanol, dichloromethane, *n*-octanol, sodium chloride, hydrochloric acid, deuterated water and chloroform and sodium hydroxide were obtained from Aldrich Chemical Co. Silica gel 60 (70–230 mesh, pH range = 6.5–7.5) was obtained from Merck. 10-[2-(tritylsulfanyl)ethyl]-1,4,7,10-tetraazacyclododecane (DO1-STr) and 1,4,7-tris(carboethoxymethyl)-1,4,7,10-tetraazacyclododecane (DO3Et) were prepared as previously reported.^{42,50} Before each use, deuterated potassium hydroxide was freshly prepared by reacting KOH with D_2O . Enriched Sm_2O_3 (98.4% ^{152}Sm) was obtained from Campro Scientific and natural Ho_2O_3 (99.9%) from Strem Chemicals. All materials used for radiochemical and biological evaluation of radioactive compounds were reagent grade unless otherwise specified.

Analytical methods

^1H (300 MHz) and ^{13}C (75.5 MHz) NMR spectra were recorded in a Varian Unit Inova-300 spectrometer at 293 K. The spectra were performed in CDCl_3 (δ/ppm : ^1H : 7.24; ^{13}C : 77.00) or D_2O versus an external reference of 1,4-dioxane (δ/ppm : ^1H : 3.75; ^{13}C : 69.20). The proton relaxivity measurements have been carried out on a Stelar Smartracer Fast Field Cycling relaxometer at 1 MHz and 25 °C. Positive-ion mass spectra were obtained in an ESI/QITMS Bruker HCT. pH Measurements were performed in an ORION SA 720 potentiometer and the pH of the solutions was measured directly in the NMR tube with a Mettler-Toledo U402-M3-S7/200 microelectrode. HPLC analyses were performed on a Perkin Elmer LC pump 200 coupled to a Shimadzu SPD 10AV UV-Vis detector, using an analytic Macherey–Nagel C18 reversed-phase column (Nucleosil 100–10, 250 × 4 mm) with a flow rate of 1.0 $\text{cm}^3 \text{min}^{-1}$. Purification of DO3A was achieved on a semi-preparative Waters μ Bondapak C18 (150 × 19 mm) with a flow rate of 5.0 $\text{cm}^3 \text{min}^{-1}$. UV detection: 220 nm. Eluent system: 0–20 min; CH_3CN (10%)– H_2O (90%).

Production of ^{153}Sm and ^{166}Ho

^{153}Sm ($t_{1/2}$ 46.8 h; β_{max} 0.67 MeV, 34%; 0.71 MeV, 44%; 0.81 MeV, 21%; γ 0.103 MeV, 38%) and ^{166}Ho ($t_{1/2}$ 26.8 h; β_{max} 1.85 MeV, 51%; 1.77 MeV, 48%; γ 80.6 keV, 7.5%; 1.38 MeV, 0.90%) were produced in the ITN Portuguese Research Reactor (RPI) by thermal neutron bombardment of isotopically enriched $^{152}\text{Sm}(\text{NO}_3)_3$ or natural $\text{Ho}(\text{NO}_3)_3$, respectively, as previously described.⁴⁸ The specific activities of the radionuclides, after 3 h irradiation and at EOB,

were 110–150 MBq mg^{-1} for ^{153}Sm and 220–260 MBq mg^{-1} for ^{166}Ho . The radionuclidic purity of the ^{153}Sm and ^{166}Ho solutions was assessed by γ -ray spectrometry using a Ge (Li) detector coupled to an Accuspec B Canberra multichannel analyzer.⁴⁸ The spectra were processed, following efficiency calibration with a ^{152}Eu source. The ^{153}Sm and ^{166}Ho activities produced after irradiation were measured in a dose calibrator (Aloka Curimeter IGC-3).

Synthesis of triethyl 10-[2-(tritylsulfanyl)ethyl]-1,4,7,10-tetraazacyclododecane-1,4,7-triacetate (DO3Et-STr)

To a suspension of DO1-STr⁴² (300 mg, 0.65 mmol) and K_2CO_3 (540 mg; 3.9 mmol) in dried acetonitrile (50 cm^3), ethylbromoacetate (360 mg; 2.1 mmol) was added. The reaction mixture was stirred for 3 days at room temperature. The reaction mixture was filtered, the supernatant evaporated to dryness and the resulting oil was purified by silica-gel chromatography using a mixture of CH_2Cl_2 –MeOH as eluent (gradient 100:0 → 100:20). TLC: R_f = 0.2 in 90:10 CH_2Cl_2 –MeOH, yielding compound DO3Et-STr (334 mg, 70%); δ_{H} (300 MHz, CDCl_3 ; ppm): 7.37 (6H, d, aromatic protons), 7.27–7.05 (9H, m, aromatic protons), 4.09–3.97 (6H, m), 2.99–3.03 (6H, d), 2.70 (8H, m), 2.13 (8H, m), 1.16–1.08 (9H, m). δ_{C} (75.5 MHz, CDCl_3 ; ppm): 173.58 (C=O), 173.13 (C=O), 144.12 (Ph), 129.07 (Ph), 127.54 (Ph), 126.36 (Ph), 67.77 (SCPh₃), 66.46 ($\text{CO}_2\text{CH}_2\text{CH}_3$), 61.36 ($\text{CH}_2\text{CO}_2\text{Et}$), 61.11 ($\text{CH}_2\text{CO}_2\text{Et}$), 60.66 ($\text{CH}_2\text{CH}_2\text{SCPh}_3$), 55.49 (C_{ring}), 54.93 (C_{ring}), 53.27 (C_{ring}), 52.73 (C_{ring}), 51.59 (C_{ring}), 50.49 (C_{ring}), 49.17 (C_{ring}), 27.03 ($\text{CH}_2\text{CH}_2\text{SCPh}_3$), 13.75 ($\text{CO}_2\text{CH}_2\text{CH}_3$), 13.69 ($\text{CO}_2\text{CH}_2\text{CH}_3$).

Synthesis of 10-(2-sulfanylethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid ($\text{H}_3\text{DO3ASH}$)

Compound DO3Et-STr was refluxed in 20% HCl, for 18 h. After cooling to room temperature, the resulting solution was washed with dichloromethane, the aqueous extracts separated and vacuum dried yielding $\text{H}_3\text{DO3ASH}$ (158 mg, 85%); δ_{H} (300 MHz, D_2O , pD = 1.05; ppm): 4.04 (2H, s, C(3)H); 3.44 (4H, s, C(6)H); 3.30–3.37 (10H, m, C(4)H + C(5)H); 2.95 (8 H, m, C(2)H); 2.76 (2H, t, C(1)H). δ_{C} (75.5 MHz, D_2O –1,4-dioxane, pD = 1.20; ppm): 176.05 (C10); 170.87 (C9); 58.04 (C2); 56.68 (C3); 55.07 (C4); 53.69 (C6); 51.94 (C5); 50.47 (d, C7 + C8); 19.71 (C1). *m/z* (ESI-MS): calc. for $[\text{C}_{16}^1\text{H}_{30}^{14}\text{N}_4^{16}\text{O}_6^{32}\text{S}]^+$: 406.50, $[\text{C}_{16}^1\text{H}_{29}^{14}\text{N}_4^{16}\text{O}_6^{32}\text{S}^{23}\text{Na}]^+$: 428.49, $[\text{C}_{16}^1\text{H}_{28}^{14}\text{N}_4^{16}\text{O}_6^{32}\text{S}^{23}\text{Na}_2]^+$: 450.46; found: $[\text{L} + \text{H}]^+$: 407, $[\text{L} + \text{Na}]^+$: 429 and $[\text{L} - \text{H} + 2\text{Na}]^+$: 451. Anal. calc. for $\text{C}_{16}\text{H}_{30}\text{N}_4\text{O}_6\text{S}\cdot 4.5\text{H}_2\text{O}\cdot 4\text{HCl}$: C, 30.24; H, 6.65; N, 8.50; S, 4.30. Found: C, 30.34; H, 6.84; N, 8.55; S, 5.06.

Synthesis of 1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid ($\text{H}_3\text{DO3A}$)

Tris(ethyl)ester of DO3A⁵⁰ was refluxed in 20% HCl for 24 h. After cooling to room temperature, the resulting solution was washed with dichloromethane, the aqueous extracts separated and vacuum dried. The resulting oil was purified by HPLC yielding $\text{H}_3\text{DO3A}$ (t_{R} = 1.52 min); δ_{H} (300 MHz, D_2O , pD = 1.05; ppm): 3.70 (s, 2H); 3.32 (t, 8H); 3.11–2.87 (m, 12H). δ_{C} (75.5 MHz, D_2O –1,4-dioxane, pD = 1.05; ppm): 176.76; 170.25; 56.10; 55.36; 52.77; 51.81; 49.53; 48.54; 42.75.

Potentiometry

Titration curves were carried out in a cell thermostated at 25 °C, at an ionic strength $I(\text{KCl}) = 0.1 \text{ M}$ using a combined glass electrode (LL Biotrode, Metrohm) connected to a Metrohm Titrino 702 automatic burette. The initial volume was 3 ml. An inert atmosphere was ensured by constant passage of N_2 through the solution. For the determination of the ligand protonation constants, the concentration of the ligand was 4 mM and the titration was carried in the $-\log[\text{H}^+]$ range 1.7–11.5. The potentiometric titrations have been carried out from acidic to basic pH and then the reverse and the forward titration curves were identical which is an unambiguous proof of the non-degradation of the ligand under the conditions of the potentiometry. For the determination of the complex protonation constants, the concentration of the complex was 1 mM. The complex was prepared as follows prior to the titration: the ligand and the metal solutions were mixed in the titration cell, KOH solution was added stepwise to reach pH ~ 9 and the mixture was stirred. After 2 h, HCl was added to reach pH ~ 5. The protonation constant of the Ca^{2+} complex has been determined by titrating a solution containing Ca^{2+} and the ligand in equimolar ratio from pH 7 to 11.

The constants (with standard deviations) were calculated with the program OPIUM.⁵¹ The program minimizes the criterion of the generalized least-squares method using the calibration function $E = E_0 + S \log[\text{H}^+] + j_A [\text{H}^+] + j_B [\text{OH}^-]$, where the additive term E_0 contains the standard potentials of the electrodes used and contributions of inert ions to the liquid-junction potential, S corresponds to the Nernstian slope and $j_A [\text{H}^+]$ and $j_B [\text{OH}^-]$ terms are contributions of the H^+ and OH^- ions to the liquid-junction potential. It is clear that j_A and j_B cause deviation from a linear dependence between E and $-\log[\text{H}^+]$ only in strong acid and strong alkaline solutions. The calibration parameters were determined from titration of standard HCl with standard KOH before any titration of the ligand to give a pair of calibration titrations used for calculations of the constants.

UV-Vis spectrophotometry

Measurements were carried out on a PERKIN ELMER Lambda 19 spectrometer in the region 200–350 nm with data steps of 1 nm. A constant temperature of 25 °C was maintained by using thermostated cells with a 1 cm optical length. The stability constants were determined at an ionic strength of $I(\text{KCl}) = 0.1 \text{ M}$, in the pH range of 3–5. The ligand and the metal concentrations were 0.5 mM. In the competition experiments, the two metals had identical concentrations (0.5 mM). Each point was prepared in a separate Eppendorf tube and equilibrated for 14 d at room temperature. After another 7 days, the measurement was repeated to confirm the thermodynamic equilibrium. The values of the stability constant were calculated with program OPIUM⁵¹ by simultaneous treatment of the data in the range 245–305 nm.

Synthesis of ^{153}Sm and ^{166}Ho complexes

Radiolabelling of the tetraazamacrocycles ($\text{H}_4\text{DO3ASH}$ and $\text{H}_3\text{DO3A}$) was performed by dissolving 5 mg of the ligand in 0.4 mL double-distilled water followed by the addition of an adequate amount of ^{153}Sm or ^{166}Ho solution to achieve a 2 : 1 ligand : metal molar ratio. The pH was adjusted to the desired

value with freshly prepared 1.0 M NaOH solution. Final ligand concentration was 8 μmol 500 μL^{-1} . The labelling was optimized by changing the pH (from 5 to 9), the temperature and the reaction time. Labelling efficiency, chelation kinetics and stability of the radiolanthanide complexes were followed by ascending silica gel ITLC (polygram, Macherey–Nagel) developed with the mobile phase $\text{MeOH} : \text{H}_2\text{O} : \text{NH}_4\text{OH} (2 : 4 : 0.2)$. In this system, the $^{153}\text{Sm}/^{166}\text{Ho}-\text{DO3ASH}$ migrate with $R_f = 1.0$ and $^{153}\text{Sm}-\text{DO3A}$ with $R_f = 0.80$, while $^{153}\text{Sm}(\text{NO}_3)_3$ and $^{166}\text{Ho}(\text{NO}_3)_3$ remain at the origin. The colloidal radioactive forms, if present, also remain at the origin. These species can be assessed by ascending instant thin layer chromatography using silica gel ITLC-SG strips developed with saline. In this system both, the radiolanthanide complexes and $^{153}\text{Sm}/^{166}\text{Ho}(\text{NO}_3)_3$ migrate with $R_f = 1.0$.

In vitro studies

In vitro stability studies

The *in vitro* stability of the complexes was evaluated in saline, 0.1 M phosphate buffer (PBS) (pH 7.4) and fresh human serum, at 37 °C at various time points (up to five days). Typically, 50 μL of each ^{153}Sm - or ^{166}Ho -complex (0.8 μmol) were added to 100 μL of the different solutions and stored at 37 °C. Daily, an aliquot of each mixture was taken and evaluated by ITLC analysis, as described above. The percentage of radiochemical impurities was then calculated.

Complex lipophilicity and protein binding. Lipophilicity was assessed by determination of the partition coefficient (P) *n*-octanol/saline and expressed as $\log D$, according to the previously described method⁴⁸.

Plasmatic protein binding was determined by a trichloroacetic acid (TCA) precipitation method, after 1 h incubation of the radiolanthanide complex (100 μL) with 1 mL of fresh human blood plasma.

Challenge studies in the presence of cysteine and glutathione. The radiocomplex (1.6 mM) was incubated at 37 °C, in the presence of 0.5 and 2 mM solutions of cysteine and 1 mM glutathione. Aliquots of these solutions were taken after 0.3, 2 and 24 h and their stability was analysed by ITLC.

In vivo studies

Biodistribution studies. The *in vivo* behaviour of the radioactive complexes was evaluated in groups of 4–5 female CD-1 mice (randomly bred, from Charles River Laboratories, Spain) weighing approximately 20–22 g. Animals were intravenously (i.v.) injected through the tail vein with 100 μL (10–15 MBq 100 μL^{-1}) of the radiolanthanide complex, were maintained on normal diet *ad libitum* and were sacrificed by cervical dislocation at 30 min and 2 h post injection, according to a previously described method.⁴⁸ Results were expressed as percentage of injected dose per gram of organ (% ID g^{-1} organ \pm SD). Whole body excretion of the radioactivity was assumed to be the difference between the measured radioactivity in the injected and sacrificed animal and was expressed as percentage of injected dose (% ID). The *in vivo* stability of the complexes was assessed by urine ITLC analysis, using the above referred experimental conditions for the radiochemical purity evaluation.

The experiments were conducted in compliance with the National Law and with the EU guidelines for Animal Care and Ethics for Animal Experiments.

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