HCA-310. DOTASA, an Asymmetrical Derivative of DOTA Substituted at one Acetate pendant Arm: ^{1}H NMR and Potentiometric Studies of the Ligand and its Lanthanide(III) Complexes

by **João P. André***[a]), **Ernö Brücher** [b]), **Robert Kiraly** [b]), **Rui A. Carvalho** [c]), **Helmut Mäcke** [d]), and **Carlos F. G. C. Geraldes*** [c])

[a]) Centro de Química, Campus de Gualtar, Universidade do Minho, 4710-057 Braga, Portugal
(e-mail: jandre@quimica.uminho.pt)

[b]) Department of Inorganic and Analytical Chemistry, University of Debrecen, 4010, Hungary

[c]) Department of Biochemistry; NMR Center and Center of Neurosciences and Cell Biology, University of Coimbra, 3001-401 Coimbra, Portugal
(phone +35 123 985 36 08; e-mail: geraldes@ci.uc.pt)

[d] Division of Radiological Chemistry, Institute of Nuclear Medicine, University Hospital, Petersgraben 4, 4031 Basel, Switzerland

Dedicated to Professor *André E. Merbach* on the occasion of his 65th birthday.

*Correspondence to Carlos F. G. C. Geraldes, [c] Department of Biochemistry, Faculty of Science and Technology, University of Coimbra, P.O. Box 3126, 3001-401 Coimbra, Portugal; phone +351239853608, e-mail: geraldes@ci.uc.pt, or to João P. André,[a] Centro de Química, Campus de Gualtar, Universidade do Minho, 4710-057 Braga, Portugal, e-mail: jandre@quimica.uminho.pt

Abstract

DOTASA is a DOTA-like macrocyclic ligand showing a carboxymethyl -CH_2COOH substituent moiety at a C_α carbon of one of the four acetate pendant arms, present as a racemic mixture of *R* and *S* configurations. The protonation constants of the ligand were determined by potentiometry, giving values close to DOTA except for the extra pK_3 value of 5.35 assigned to protonation of the extra carboxylate group in the succinyl arm. The ^1H NMR spectra of DOTASA at different pH values are too complex to allow the determination of its microscopic protonation scheme, due to the presence of multiple isomeric structures in solution. The thermodynamic stability constant of its Gd^{3+} chelate was determined by a potentiometric method, and the value obtained, log K_{ML} = 27.2 (0.2), is higher than for the $[Gd(DOTA)(H_2O)]^-$ complex. The solution structure of the asymmetrical Ln^{3+} chelates of DOTASA was studied by ^1H NMR spectroscopy, indicating the presence of four isomers, corresponding to the combination of the antiprismatic (M) and twisted antiprismatic (m) helicities of the pendant arms and to the *R* and *S* configurations of the substituted pendant arm C_α atom. The m/M isomer ratio decreases along the lanthanide series, with the m isomer decreasing from 90% at La to about 50% from Eu-Lu. This shows that the expected m isomer population of the Gd^{3+} complex with DOTASA is higher than for the unsubstituted Gd^{3+}-DOTA (~15%) but lower than for a Gd^{3+} chelate of a *RRRR* tetrasubstituted DOTA (~70%). Thus the stabilisation of the m isomer by C_α monosubstitution at the DOTA acetate pendant arms in $[Gd(DOTASA)(H_2O)]^{2-}$ is responsible for its increased water exchange rate and higher relaxivity.

1. Introduction. - Due to their paramagnetic, luminescent and radioactive properties, the trivalent lanthanide ions, Ln^{3+}, have found a variety of applications in biomedical sciences [1-5]. The high toxicity of the free ion form can be circumvented by *in vivo* application in the form of stable chelates.

Most magnetic resonance imaging (MRI) contrast agents are gadolinium(III) chelates of polyaminopolycarboxylate-like ligands. In particular, the tetraazacyclododecane polycarboxylic acid ligands proved to be suitable chelating agents in regard of the thermodynamic and kinetic stability of their Gd^{3+} chelates [6,7]. DOTA (1, 4, 7, 10 - tetraazacyclododecane - 1, 4, 7, 10 -tetraacetic acid) became a popular ligand in the last two decades due to its strong capability to encapsulate the Gd^{3+} ion, while allowing the cation to keep one water molecule in the inner coordination sphere [8]. This water molecule conveys the paramagnetic effects of the metal ion to the bulk water. Many attempts have been made to improve the paramagnetic T_1 relaxation effect of the Gd^{3+} ion on the protons of bulk water, by optimising the molecular parameters which determine the relaxivity of those protons, while preserving a DOTA-like coordination pattern within the chelate [7]. In a previous work [9] we reported the synthesis and physicochemical characterisation of the ligand DOTASA (1, 4, 7, 10 – tetraazacyclodo-decane -1-(*R*,*S*)-succinic acid - 4, 7, 10 -triacetic acid) (Fig. 1), which has a succinate pendant arm, resulting from the attachment of a carboxymethyl -CH_2COOH substituent moiety to the C_α carbon of one of the four acetate pendant arms of DOTA, giving a racemic mixture of *R* and *S* configurations at C_α. This extra carboxylate of DOTASA relative to DOTA, which remains free upon coordination to the Gd^{3+} ion in the $[Gd(DOTASA)(H_2O)]^{2-}$ chelate, was shown to be responsible for an increase in the water exchange rate and in the size of the tumbling species in solution with a concomitant overall improvement of circa 30% in its relaxivity in relation to $[Gd(DOTA)(H_2O)]^-$. Additionally, the extra carboxylate group showed to be convenient to couple the chelate to other molecules [10], which is of extreme importance regarding the targeting of organs.

The Ln^{3+} complexes of DOTA-like chelating agents exhibit a variety of conformational and coordination isomers which may display dynamic behaviour on the NMR time scale [6, 7, 11]. The isomers of their Gd^{3+} chelates have been found to have different relaxivity

properties [12]. Thus, in the present work we studied the acid-base properties of DOTASA, as well as the thermodynamic stability and structure of some of its Ln(III) chelates in aqueous solution, both by ^{1}H NMR and potentiometry.

2. Results and Discussion – 2.1. Ligand protonation studies: potentiometry and NMR. Table 1 summarises the protonation constants of DOTASA, obtained in this study from potentiometric titrations at 25 ^{o}C in the presence of different support electrolytes. These are compared with the corresponding literature values for DOTA [13-15]. The protonation constants of DOTASA are found not to be much different from those of DOTA, indicating that the presence of an extra carboxylic acid group, that is not directly attached to a nitrogen atom from the ring, has very little effect on the protonation constants of the remaining ionisable moieties of the ligand.

Although potentiometry cannot provide information on the microscopic sequence of protonation of the ligand basic sites, a simple comparison with DOTA leads to the assumption that the third protonation in DOTASA occurs mainly in the extra carboxylate and only at the fourth protonation the ligand attains again a DOTA-like protonation pattern [16].This hypothesis could be in principle analysed by studying the microscopic protonation sequence of DOTASA, based on a ^{1}H NMR pH titration of the ligand using K^+ as the counterion, as this cation does not bind significantly to macrocyclic ligands of this type [16]. It is well known that changes in ^{1}H chemical shifts can indicate successive protonation sites of the various basic groups of the ligand because the protonation of donor atoms generally results in a deshielding of the non-labile hydrogen atoms nearby. Under conditions of fast proton exchange among the various protonated species, H_nL, the observed averaged chemical shift of nucleus i is given by $\delta_{obs}{}^{i} = \Sigma_n \delta_n{}^{i} X_{H_nL,}$ where $\delta_n{}^{i}$ are the intrinsic chemical shifts of the H_nL species and X_{H_nL} is the mole fraction of each species. Using the protonation constants obtained by potentiometry, the $\delta_n{}^{i}$ values can be calculated using a multiple linear regression program, which minimises the sums of the squares of the deviation between the observed and calculated $\delta_{obs}{}^{i}$ values [17].Then, these intrinsic shifts can be used to calculate the percentage protonations at the nitrogen (f_N) and oxygen (f_O) sites at each pH, by a well known empirical procedure based on the additivity of the protonation shifts at the various basic sites. According to this procedure,

the observed shifts of the non-exchangeable ligand protons δ_{obs}^{i}, are a function of intrinsic shifts of the fully deprotonated form of the ligand, δ_0^{i}, the fractions of protonation f_N and f_O, and the shielding constants C_O (shift due to α-carboxylate protonation), C_N and $C_{N'}$ (shift due to protonation of a N atom in the α- and β-position, respectively), all relative to the CH_2 group under study. This procedure has been applied with success to many linear and macrocyclic polyamino carboxylate and phosphonate ligands [16-22], including DOTA [16, 20]. All the values of f_N and f_O values calculated for this ligand clearly show that two nitrogens of DOTA are protonated first (at n = 2, f_N ~ 0.50, f_O = 0), and only then the four carboxylate groups are increasingly protonated in the same amounts (at n = 6, f_N ~ 0.50, f_O = 1.00), as expected for the ligand symmetry [16, 20].

This symmetry is broken for DOTASA, due to the presence of the extra carboxymethyl group in one of the pendant arms. Thus, the ^{1}H NMR spectra of DOTASA are very complex at all pH values studied (pH 12.0-0.6) at 25ºC and 70 ºC. Even the COSY spectra obtained at various pH values and both temperatures did not allow a full assignment of the resonances, which made it impossible to obtain proton NMR pH titration curves for DOTASA, and thus prevented a full study of its protonation scheme. The COSY spectra were obtained at 25ºC and 70 ºC, at pH values corresponding to predominance of increasingly protonated states of the ligand, namely pH 12.0 (L), 10.64 (HL), 8.95 (H_2L), 4.27 (H_3L), 3.50 (H_4L), 2.10 (H_5L) and 0.60 (H_6L). They allowed unequivocal assignment of the resonances from the carboxymethyl substituent -CH-CH_2- protons of the DOTASA ring, which should give a well resolved ABX pattern. In fact, a large number of ABX patterns were obtained from this moiety, between 6 and 8 for the forms L up to H_4L, and 3 for H_5L and H_6L at 25 ºC. At 70 ºC, this number decreased to 3 for L, HL, H_3L and H_5L, to 4 for H_4L and did not decrease for H_2L. The complexity of the spectra prevented any further assignments, except for the location of some AB patterns of the methylenic protons of the unsubstituted acetate arms.

The shift of a given resonance of the carboxymethyl substituent CH and CH_2 protons of DOTASA was found to be pH independent at pH 12-8.95, and only started to increase for lower pH values, indicating that the first two protonations are distributed among the three nitrogens N2-N4 of the DOTASA ring bound to the acetate arms, thus

excluding N1 (Fig. 1), and only at lower pH values the carboxylate groups, including the C_α substituent, are protonated.

The complexity of the proton NMR spectra of the unprotonated and protonated forms of DOTASA, reflected not only in the large number of resonances of non-equivalent protons of the unsymmetrical ligand, but specially in the presence of 3 to 8 resonances for each type of proton, is an indication of the presence of a large number of ligand topomers in solution, with exchange processes between them accounting for the partial simplification of the spectra at high temperature. These topomers should have high internal rigidity due to the presence of internal hydrogen bonds between protonated nitrogens and carboxylates, and differ in the relative orientation of the carboxylate arms relative to the tetraaza ring and the *R,S* configuration of the substituted C_α in the pendant arm. However, a detailed study of their structure and dynamics was not undertaken. In the solid state, diprotonated DOTA adopts a [3.3.3.3] square conformation of fourfold symmetry with all the carboxylate arms located on the same side of the tetraaza ring [8]. However, even for this symmetrical ligand the proton NMR spectrum of the H_6L form in strong acidic media becomes quite broad at low temperature (278 K), reflecting the slowing down of its intramolecular dynamic processes [16]. The relatively complex but well-defined proton spectra obtained for the methyl-substituted ligands M4DOTA (with four methyl groups at the tetraaza macrocyclic ring), DOTMA (four methyl substituents at the acetate arms) and M4DOTMA (both types of substitutions) in acidic media have been interpreted through the presence of two slowly exchanging elongated (less symmetric than [3.3.3.3]) conformations for the H_4L and H_6L forms of these ligands [23].

2.2.Complexation studies by potentiometry. Some spectrophotometric experiments were carried out in the system Ce^{3+}-DOTASA and found that the absorption bands of the Ce^{3+} ion and of the $[Ce(DOTASA)(H_2O)]^{2-}$ complex are overlapping. Thus, they are not suitable for complex stability calculations, but this system is a useful detector of the complex formation. The formation rate of $[Ce(DOTASA)(H_2O)]^{2-}$ is expectably lower than that of $[Gd(DOTASA)(H_2O)]^{2-}$ [24]. This means that, at the time after which the UV-Vis spectrum of the system Ce^{3+}-DOTASA is unchanged, the system Gd^{3+}-DOTASA is also in equilibrium. This helped design a potentiometric determination of the stability constant for the complex $[Gd(DOTASA)(H_2O)]^{2-}$ using an "out of cell" method. The

value obtained, log K_{ML} = 27.2 (0.2), is higher than the one of $[Gd(DOTA)(H_2O)]^-$ (logK_{ML} = 25.3 [14]). This finding is probably due to the extra negative charge of the completely deprotonated ligand.

2.3. NMR Studies of the Ln^{3+}-DOTASA complexes. ^1H NMR is quite useful in the solution study of the isomers of the Ln^{3+} complexes of DOTA and its derivatives [6,7]. The high symmetry of DOTA leads to the well known presence of only two isomers of the $[Ln(DOTA)(H_2O)]^-$ chelates in solution, with square antiprismatic (M) and twisted antiprismatic (m) geometries [8,25-28]. These two isomers have the same [3.3.3.3] square conformation with fourfold symmetry of the tetraazacyclododecane ring, where all its ethylenic groups adopt a δ or λ conformation, thus leading to conformations of clockwise or counterclockwise helicity, λλλλ or δδδδ. They only differ in the layout of the four acetate pendant arms, resulting from rotations around the N-CH_2-COO bonds, with either a clockwise (Λ) or counterclockwise (Δ) helicity. These lead to the two diastereoisomers referred to above, with separate NMR resonances, each of which is an enantiomeric pair: the square antiprismatic (M) geometry results from the opposite helicity of the tetraaza ring and the acetate arms (Δ(λλλλ) or Λ(δδδδ)) while the twisted antiprismatic (m) geometry has the same ring and acetate helicity (Λ(λλλλ) or Δ(δδδδ)). Thus, M and m differ in the value and sign of the twist angle α between the diagonals of the parallel squares formed by the four nitrogens and the four carboxylate oxygens in the coordination polyhedron of the DOTA chelates, with typical values of about α = +35º and α = -15º found from crystallographic structures [7]. The isomer M shows a wider paramagnetic shift range than m throughout the lanthanide series. The isomer m is dominant relative to M for the early Ln^{3+} chelates (Ln = La – Pr), but M becomes dominant for the smaller ions (Ln = Eu-Lu) [28].

The DOTASA ligand used in this study is an asymmetrical derivative of DOTA, with a proton at one of the four acetate C_α atoms substituted with a carboxymethyl group. This introduces a chiral center in the ligand and a site of asymmetry in the complexes which doubles the number of possible isomeric species in solution. As in our study the ligand DOTASA was obtained as a racemic mixture of the enantiomers with *R* and *S* configurations at C_α [9], the complete identification of all the possible steroisomers requires indication of the configuration (*R* or *S*) of the chiral carbon atom of the ligand

together with the four arrangements of the of the ligand itself in the complex described above for DOTA complexes. Thus, in the solutions of the the Ln-DOTASA complexes there can be up to eight stereoisomers of the type W-X (W = *R*, *S*; X = M ($\Delta(\lambda\lambda\lambda\lambda)$ or $\Lambda(\delta\delta\delta\delta)$), m $\Lambda(\lambda\lambda\lambda\lambda)$ or $\Delta(\delta\delta\delta\delta)$), consisting of four enantiomeric pairs: *R*-M, *R*-m, *S*-M and *S*-m. Thus up to four sets of proton NMR signals are to be expected from these enantiomeric pais. The lack of C_4 symmetry removes the signal degeneracy found in the NMR spectra of $[Ln(DOTA)(H_2O)]^-$ complexes and leads to a large number of resonances for each isomer in the proton (25) spectra.

The spectral properties of the paramagnetic Ce^{3+}, Pr^{3+}, Nd^{3+}, Sm^{3+}, Eu^{3+} and Yb^{3+} chelates of DOTASA, as well as of the diamagnetic La^{3+}, Y^{3+} and Lu^{3+}, were investigated using unidimensional and 2D COSY ^{1}H NMR. The chelate of the Y^{3+} ion was included in the present study, as its properties are quite similar to those of the later lanthanides Er^{3+} and Ho^{3+} [29].

The proton NMR spectra of the paramagnetic chelates studied at 25 ºC include a large number of partially overlapping resonances covering paramagnetic shift ranges in accordance with those observed for the corresponding DOTA chelates [25,27]. The spectra of the Eu^{3+} and Yb^{3+} complexes are the best resolved, where about fifty different resonances could be counted, as expected from the presence of two different isomers of the DOTASA chelates. For the earlier lanthanide chelates about half of the resonances observed have much lower intensities that the others.

Previous ^{1}H NMR studies with the symmetrical $[Ln(DOTA)(H_2O)]^-$ chelates, and with a variety of lanthanide derivatives of DOTA [7], have demonstrated that the two diastereoisomers m and M are present in solution, with a relative proportion that is a function of the lanthanide ion, temperature, solvent and also the steric crowding of the chelate [7, 28]. They are characterised by different dipolar shifts, with complexes of the M form often possessing the larger paramagnetic shifts, for a given ligand resonance.

The full assignment of the complex proton spectra of the paramagnetic lanthanide chelates of DOTASA and use of the paramagnetic shifts of the complexes, in particular of Yb^{3+}, in a structural analysis using the experimental dipolar shifts, is beyond the scope of the present study. The COSY spectra obtained were quite complex and not all the assignments could be made. However, a direct information that can be easily obtained

from these spectra is the M/m isomer ratio. A particularly useful resonance to observe, for this purpose, is the most shifted axial ring proton, ax_1, which is well separated from the others [7,8,25-28]. For example, this resonance is observed at ~ + 30-50 ppm and at ~ + 150-160 ppm for square antiprismatic (M) Eu^{3+} and Yb^{3+} complexes of tetracarboxymethyl-1,4,7,10-tetraaza-cyclododecane derivatives at room temperature, while the corresponding twisted antiprismatic (m) isomers, the same axial ring proton, ax_1, has resonances at lower frequencies, at ~ + 10-30 ppm and ~ + 90-100 ppm for Eu^{3+} and Yb^{3+} complexes, respectively [7,30-32]. In our study, the ax_1 protons gave two sets of four resonances, well separated from all the others, in all cases except Sm^{3+} for the M isomer, corresponding to the asymmetric M and m isomers, with paramagnetic shifts within the expected ranges (Fig. 2 and Table 2). Integration of those resonances gave the isomer ratios M/m also shown in Table 2. Isomer m is dominant for the early lanthanide complexes Ce^{3+}-Nd^{3+}, while for Eu^{3+} up to Yb^{3+} the M/m ratio stays close to one. A comparison of the observed dependence of the M/m isomer ratio on the ionic radius of the lanthanide ion for the DOTASA and DOTA series of complexes (Fig. 4) [28] shows that the C_α carboxymethyl substituent at one of the acetate arms of DOTA significantly stabilises the m isomer relative to M after Nd^{3+}, in particular for the smaller lanthanide ions. An approximate value of the isomer ratio M/m ~ 6:1 can be calculated for $[Gd(DOTA)(H_2O)]^-$ by interpolation of the ratios for the Eu^{3+} and Tb^{3+} chelates, while for $[Gd(DOTASA)(H_2O)]^{2-}$ a M/m ~ 1:1 can be obtained from those of the Eu^{3+} and Yb^{3+} chelates. It is well known that the m isomers of lanthanide macrocyclic DOTA type chelates have about fifty times faster water exchange rates (k_{ex}) than their M isomers [33-36]. Thus, the much larger abundance of the m isomer for $[Gd(DOTASA)(H_2O)]^{2-}$ could explain the 50% increase of water exchange rate (k_{ex}^{298}) observed for $[Gd(DOTASA)(H_2O)]^{2-}$ relative to $[Gd(DOTA)(H_2O)]^-$ [9], which was attributed before to the extra negative charge in the first complex, resulting in higher proton relaxivity.

The possible presence of the *R* and *S* configurations of the substituted pendant arm in the m and M isomers of of the DOTASA complexes was not reflected in any further splitting of the ax_1 and the other proton resonances of the ligand in the complex, including those from the carboxymethyl substituted acetate arm, as seen in the COSY spectra. This has been observed for Ln^{3+} complexes of DOTA-pNP, a DOTA-like ligand

with one acetate C_α proton substituted by a *p*-nitrophenyl group [32], leading to the conclusion that only two of the four possible isomeric species (enantiomeric pairs) were present, where the bulky aromatic group was pointing outward from the coordination cage. However, in the case of DOTASA, the much less bulky carboxymethyl substituent might adopt also the other configuration, and the presence of the four isomeric species might be masked in the paramagnetic spectra by the very similar geometric factors of the protons in the *R* and *S* forms, which determine the large Ln^{3+} induced pseudo-contact shifts. This possibility led us to analyse the spectra of the diamagnetic DOTASA complexes.

The proton NMR spectra of the diamagnetic La^{3+}, Y^{3+} and Lu^{3+} complexes of DOTASA (data not shown), although very crowded in the 5.0-2.6 ppm region, reveal the presence of the stereoisomeric *R* and *S* forms of the substituted pendant arm. The isomer ratio obtained for the Ce^{3+} complex indicates that the spectrum of $[La(DOTASA)(H_2O)]^{2-}$ should be dominated by the resonances of the m isomer. Its COSY spectrum (Fig 3a) shows many cross-peaks, but those involving the CH(d) and CH_2 (e) protons of the carboxymethyl substituted acetate pendant arm are easily assigned. Two strong ABX spin systems (CH - 4.50 ppm, CH_2 - 2.84, 2.68 ppm; and CH – 4.12 ppm, CH_2 - 3.48, 3.40 ppm) are assigned to the major *R*-m and *S*-m isomers. The three sets of CH_2 protons of the non-substituted acetate pendant arms originate a cluster of nearly-overlapping cross-peaks, corresponding to six different AX systems (with shifts of 4.2-3.8 ppm and 3.3-3.1 ppm), reflecting the *R* and *S* configurations of the C_α atom at the substituted arm of the m isomer. Other much less intense cross-peaks of the substituted (ABX) (eg. at 4.12, 2.90, 2.54 ppm) and unsubstituted (AX) acetate arms (eg. at 3.80-3.52 ppm and 3.65-3.20 ppm) and are also present, corresponding to the minor *R*-M and *S*-M isomers. The ring protons give a large number of partially overlapping cross-peaks that were not assigned. The Y^{3+} and Lu^{3+} DOTASA complexes originate much more complex proton 1D and COSY spectra (Fig. 3b). The presence of a much larger number of cross-peaks in the COSY spectra reflects the presence of four (*R*-m, *S*-m, *R*-M, *S*-M) isomers with comparable populations, as expected from the isomer ratios obtained for the paramagnetic complexes. This is clearly illustrated by the presence of cross-peaks from four ABX systems corresponding to the -CH-CH_2- protons of the succinyl arm, with CH

resonances at 4.42, 4.21, 4.02 and 3.85 ppm correlated with signals at 2.65-2.75 ppm, and the large number of heavily overlapping cross-peaks of the CH_2 protons at the other three acetate arms.

Exchange between the m and M isomers of the DOTASA chelates is demonstrated by the broadening and collapse of the resonances of both isomers observed at 70 ºC, both for the diamagnetic and paramagnetic complexes, but the kinetics of the process was not studied further [26].

It is worth comparing the present conclusions on the number and type of isomers for the $[Ln(DOTASA)(H_2O)]^{2-}$ complexes with previous NMR studies of lanthanide chelates of symmetrical and non-symmetrical derivatives of DOTA, modified at the acetate C_α and/or the tetraaza ring [7]. A very similar case to the present study with DOTASA is the introduction of only one chiral center in DOTA by derivatizing one acetate C_α with a *p*-nitrophenyl group, resulting in a non-symmetrical DOTA derivative (*R*,*S*-DOTA-pNP) [32]. The proton NMR spectra of its Ho^{3+} and Yb^{3+} complexes show the presence of only two of the four possible isomers, with M/m ratios (88:12 and 3:1, respectively) indicating that the population of the m isomer is only slightly increased relative to the corresponding $[Ln(DOTA)(H_2O)]^-$ chelates (Fig. 4), indicating that a bulky substituent at an acetate arm (such as in DOTA-pNP) has a much lower stabilizing effect on the m isomer that a flexible, less bulky one (such as in DOTASA).

In the case of symmetrical C_α-substitution, the introduction of four identical chiral centres of *R* or *S* configuration in the acetate arms of DOTA may originate twelve stereoisomers (six enantiomeric pairs), resulting from the combinations of the C_α configurations (*RRRR*, *RRRS*, *RRSS*, *RSRS*, *RSSS* and *SSSS* with a 1:4:4:2:4:1 statistical relative intensity) and the helicity (Δ/Λ) of the pendant arms. However, the number of isomers actually observed in the DOTA tetraderivatives is sometimes much lower. In the case of the ligand (*RRRR*)-DOTMA, where the four C_α atoms are substituted with four methyl groups of *R* configuration, the ^1H NMR spectrum of $[Yb(DOTMA)(H_2O)]^-$ shows only two isomers, m and M, resulting from the helicity (Δ/Λ) of the pendant arms, with *RRRR* configuration [37]. The presence of the four C_α substituents again increases the rigidity of the chelate and makes the m isomer dominant relative to M in $[Yb(RRRR)\text{-}(DOTMA)(H_2O)]^-$ (m:M ~ 15:1, compare with the other chelates in Fig. 4) [37]. The

(*RRRR*), (*RRRS*), (*RRSS*) and (*RSRS*) stereoisomers of the $[Eu(TCE\text{-}DOTA)(H_2O)]^{5-}$ complex, where TCE-DOTA is the symmetrically substituted tetra(carboxyethyl) C_α derivative of DOTA, were individually analysed by proton NMR in solution, in order to study their isomeric composition [30,31]. The M/m ratios obtained were (*RRSS*)- 1:0 > (*RSRS*)- 4:1 > (*RRRS*)- 2:1 (*RRRR*)- 1:4, showing that in the first two cases the absolute configuration of the chiral C_α centres does not significantly affect the stereoisomeric M/m ratio found for $[Eu(DOTA)(H_2O)]^-$, but for the less symmetric (*RRRS*)- and specially (*RRRR*)- the C_α centres determine the left-handed helicity of the complexes, favouring the m isomer [30,31]. For the (*RRRR*)- isomer, the m form predominates along the lanthanide series, but changes differently from the $[Ln(DOTA)(H_2O)]^-$ or $[Ln(DOTASA)(H_2O)]^{2-}$ series, with a minimum at Tb^{3+} (Fig. 4).

If DOTA derivatisation at the acetate arms affects the isomer ratio in ways that depend on the relative configuration of the substituents, its derivativatisation at the tetraaza ring has a dominant effect on the isomers formed, as shown also by proton NMR [38]. The Yb^{3+} complex of the four-fold symmetrical (*RRRR*)-M4DOTA ligand, with four methyl substituents at the tetraaza ring, shows three isomers M, m and m' in the ratio 1: 0.095:0.013. The orientation of the methyl groups has a large influence on the stability of the square antiprismatic (M) and twisted antiprismatic (m, m') structures, as their "equatorial up" orientation (present in the M and m isomers) is much more stable than the "equatorial down" orientation (m' isomer) [38]. Here, the dominance of the M isomer in $[Yb(DOTA)(H_2O)]^-$ is maintained in $[Yb(RRRR)\text{-}(M4DOTA)(H_2O)]^-$. The Yb^{3+} complex of the non-symmetrical racemic (*R*/*S*)-MDOTA ligand, monomethylated at the tetraaza ring, has four isomers, with relative intensities M (M-eq up) > m' (m-eq down) >> M' (M-eq down) > m (m-eq up) [38]. Here the orientation of the ring methyl group is more important than the helicity of the acetate arms in determining the stability of the isomers. Finally, the Yb^{3+} complex of the symmetrical M4DOTMA ligand, with four methyl substituents at the tetraaza ring and on the acetate arms in the *SSSS* and *RRRR* configurations, respectively, features a proton spectrum with only one isomer, M (M-eq up) [38]. Again, the most stable orientation of the ring methyl groups dominates, inverting the helicity of the most stable isomer (m) in $[Yb(RRRR)\text{-}(DOTA)(H_2O)]^-$.

3. Conclusion. The present studies using the ligand DOTASA, where one C_α proton of an acetate arm is substituted by a carboxymethyl group (*R*/*S* racemic mixture), further illustrate the multiple effects that derivatisation of the DOTA ligand has on the properties of the corresponding Gd^{3+} chelate, with consequences on its potential application as an MRI contrast agent. While the protonation constants of DOTASA are not much changed relative to DOTA except for an extra p*K* value due to protonation of the extra carboxylate group in the succinyl arm, the stability constant of its Gd^{3+} chelate is significantly increased relative to $[Gd(DOTA)(H_2O)]^-$. The number of isomers in solution of the Ln^{3+} chelates of DOTASA also doubles, combining the M and S structures of the framework of the complexes with the *R* and *S* configurations of the substituted pendant arm C_α atom. More importantly, the m isomer population of the Gd^{3+} complex with DOTASA is 3-4 times increased relative to Gd^{3+}-DOTA. This is in contrast with the C_α effect of the more sterically bulky para-nitrophenyl group, like in the Gd^{3+}-DOTA-pNP chelate [32], where the % m increase is much smaller. The effect of the flexible carboxymethyl group in Gd^{3+}-DOTASA on the % m increase is almost as high as that resulting from *RRRR* tetrasubstitution of DOTA [31], and can thus be considered a good strategy of connecting a Gd^{3+}-tetrazamacrocycle to a carrier while increasing its water exchange rate, and thus its relaxivity.

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Experimental Part

Chemicals and Materials. The ligand DOTASA was prepared, isolated and purified as a racemic mixture of the enantiomers with *R* and *S* configurations at the substituted acetate C_α atom, as described elsewhere [9]. It was used in the form $C_{18}H_{30}N_4O_{10}x2.30HClx1.73H_2OxNaBr$, as obtained by elemental analysis [9]. The

lanthanide chlorides, other salts and deuterated solvents were obtained form Sigma. The Ln^{3+} and Y^{3+} complexes (Ln=La, Lu, Pr, Nd, Sm, Eu, Yb) for NMR studies were prepared by adding a D_2O solution containing 40 mmol (1 eq.) of the ligand to the same volume of an equimolar solution of $LnCl_3$ in D_2O. The pH of the solutions was slowly adjusted to 7-8 with aq. KOD.

Potentiometric Titrations. Ligand solutions were titrated in a thermostated cell under a stream of nitrogen with a 0.5 M standard solution of tetramethylammonium hydroxide (standardised with potassium hydrogenophtalate) at an ionic strength adjusted to 0.50 M with tetramethylammonium nitrate in a Metrohm 665 Dosimat with a 1 mL piston burette. Excess nitric acid was added to the ligand solutions (25 mL, 1.5 mM) to determine the lower protonation constants. All the solutions were prepared with deionised water (Millipore) which was previously boiled in order to remove the CO_2. pH measurements were carried out with a Metrohm 654 digital pH-meter equipped with a Metrohm 0.0204.100 glass electrode combined with a calomel (3 M KCl) reference electrode. The glass electrode was calibrated with pH 4.0 and 7.0 buffers at 25 ^{o}C. The pH-meter-electrode system was calibrated by titration of a 3.2 mM TRIS solution with 0.5 M tetramethylammonium hydroxide solution at an ionic strength adjusted to 0.5 M with tetramethylammonium nitrate. The data were handled and calculated with the program TITFIT [39]. Another set of potentiometric measurements was performed using a ionic strength of 0.10 M in TMACl.

For the potentiometric determination of the stability constant of $[Gd(DOTASA)]^{2-}$, 20 samples of 4.00 mL volume were prepared. The concentrations of the Gd^{3+} ion and of the ligand were the same: 0.005 M. The pH values were between 1.8 and 2.4 at equilibrium. These "out of cell" samples were kept at the temperature of 60 ^{o}C during 2 days, then at 40 ^{o}C during 4 days, and finally at 25 ^{o}C during 2 weeks. The fitting parameter related to 20 data pairs is 0.007 mL. The standard deviation is a little bit higher than usually but even the stability constant is high. The pH measurements were carried out with a PHM85 Radiometer pH meter and with a combined electrode 6.0234.100 (Metrohm). The effect of the liquid-junction potential of the electrode was taken into consideration. For obtaining H^{+} ion concentration from the measured pH values, the method suggested by Irving et al. [40] was used.

The value pK_w = 13.89, which was determined in our cell, was also used for calculations. For the computation the software PSEQUAD [41] was used.

A sample of pH 2.07 containing 0.0005 M Ce(III) and DOTASA was also kept together with the studied systems. The changes in this solution were followed spectrophotometrically with a Cary 1E spectrophotometer.

NMR Experiments. ^{1}H NMR spectra were obtained on a Varian Unity 500 spectrometer. Probe temperatures were accurate to ±1 ^{o}C. NMR spectra were recorded in D_2O (versus sodium 3-(trimethylsilyl)propanesulfonate (TSP). Solutions of DOTASA (0.08 M) for NMR pH titrations were prepared in D_2O and the pD was adjusted with DCl or KOD. The final pH was determined with a Hanna 8417 pH meter fitted with a combined Hanna H11310 electrode and calibrated at 20±1 ^{o}C with two standard buffers at pH 4.0 and 7.0 and corrected for the deuterium isotope effect using pH=pD-0.4 [42]. 1D ^{1}H NMR spectra and 2D COSY spectra were recorded as a function of added base.

Solutions of the diamagnetic (La^{3+}, Lu^{3+} and Y^{3+}) and paramagnetic (Ce^{3+}, Pr^{3+}, Nd^{3+}, Sm^{3+}, Eu^{3+}, Yb^{3+}) metal ion complexes of DOTASA for NMR measurements contained 40 mM of ligand and 1 equivalent of the trivalent metal chloride in D_2O adjusted to pH 7-8 with DCl or KOD. Proton 1D and 2D COSY spectra of the complexes were obtained at 25 and 70 ^{o}C on a Varian Unity 500 spectrometer with presaturation (transmitter and decoupler, respectively) to suppress the residual water solvent signal.

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Table 1 - Protonation constants (log K_i) of DOTASA and DOTA at 25 ^{o}C obtained by potentiometry.

log K_i	**DOTASA**		**DOTA**		
	0.1M Me_4NCl	0.5M Me_4NNO_3	0.1M Me_4NCl [15]	0.1M Me_4NCl [14]	0.1M Me_4NNO_3 [13]
logK_1	10.99 (0.02)	11.17	11.74	11.73	12.09
logK_2	9.18 (0.02)	9.38	9.76	9.40	9.68
logK_3	5.35 (0.02)	5.25	4.68	4.50	4.55
logK_4	4.40 (0.02)	4.21	4.11	4.19	4.13
logK_5	3.75 (0.02)	3.28	2.37	-	-
logK_6	2.93 (0.02)	-	-	-	-
logK_7	1.8 (0.04)	-	-	-	-
logK_8	1.2 (0.06)	-	-	-	-

Table 2. NMR shifts (δ – ppm) of the ax_1 protons of the paramagnetic $[Ln(DOTASA)(H_2O)]^{2-}$ chelates in the M and m isomeric forms.

Ln	m isomer	M isomer	M/m
Ce	-8.31, -8.62, -9.58, -11.73	-18.52, -20.48, -20.94, -21.43	1:11.5
Pr	-23.53, -23.99, -27.65, -30.39	-40.35, -46.05, -46.17, -46.30	1:6.7
Nd	-7.80, -8.05, -9.97, -11.67	-21.18, -23.65, -23.99, -24.63	1:3.3
Sm	not assigned	-2.47, -2.96, -2.96, -3.60	-
Eu	17.93, 16.36, 13.76, 13.22	37.28, 36.23, 35.94, 32.85	1:0.9
Yb	101.01, 90.44, 77.56, 71.92	152.22, 145.05, 142.52, 132.49	1:1.2

Legends

Fig. 1. Structure of DOTASA

Fig. 2. ^{1}H NMR resonances of the ax_1 protons of the M and m isomers of paramagnetic $[Ln(DOTASA)(H_2O)]^{2-}$ (D_2O, pH 8.0, 298 K): a) Ln = Ce; b) Ln = Eu; c) Ln = Yb.

Fig. 3. COSY spectra of $[Ln(DOTASA)(H_2O)]^{2-}$ (D_2O, pH 8.0, 298 K): a) Ln = La; b) Ln = Lu.

Fig. 4. Molar fractions of the isomer m for Ln-tetraazamacrocycles in aqueous solution, pH 7, 298 K, as a function of the complexes metal ion obtained from ^{1}H NMR: (■) $[Ln(DOTASA)]^{2-}$ (this work); (●) $[Ln(DOTA)(H_2O)]^{-}$ [11,25]; (▲) [Ln(*RRRR*)-(TCE-DOTA)$(H_2O)]^{2-}$ [31]; (▼) $[Ln(DOTA\text{-}pNP)(H_2O)]^{-}$ [32].

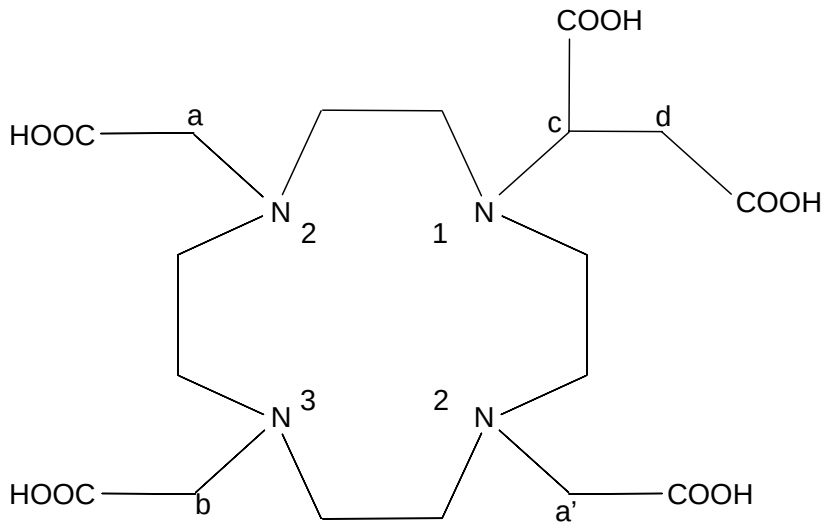

Fig. 1

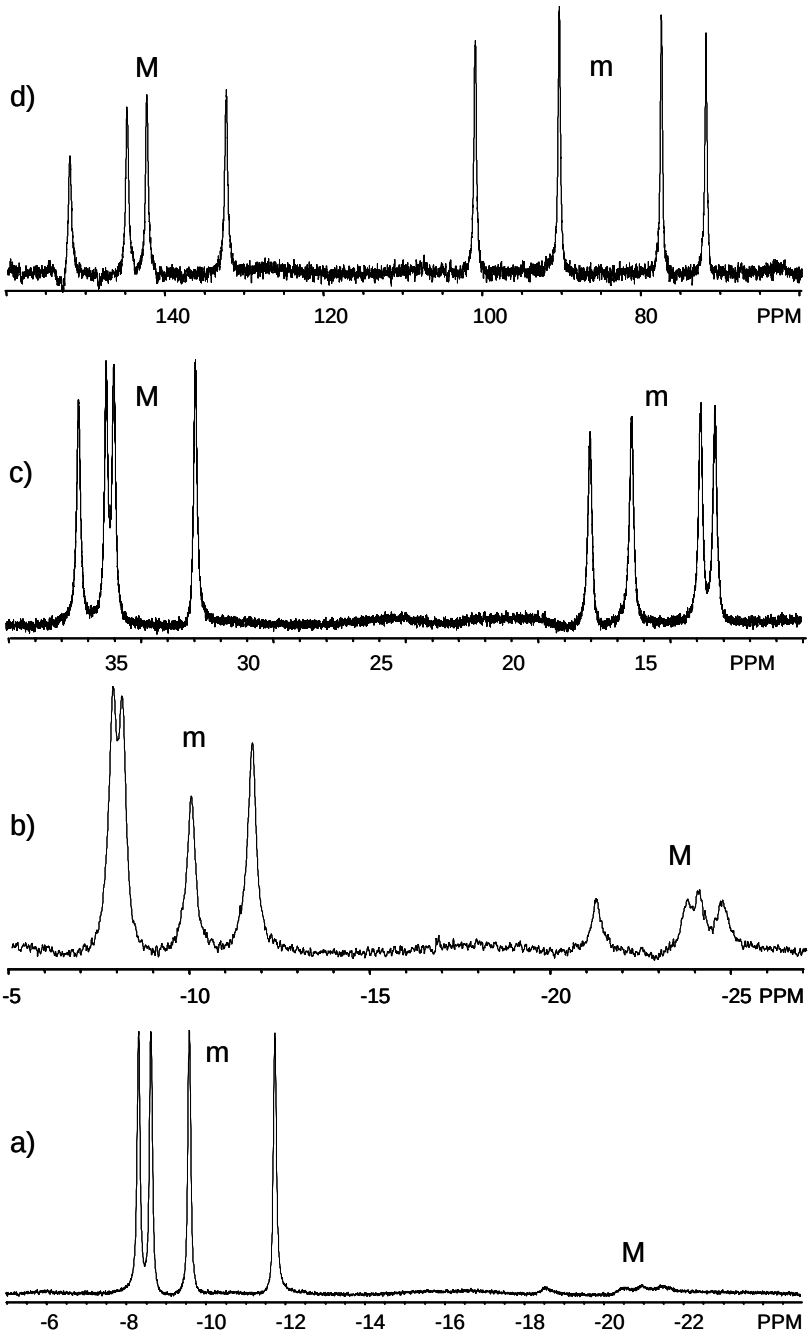
d)
M
m
140
120
100
80
PPM
c)
M
m
35
30
25
20
15
PPM
b)
m
M
-5
-10
-15
-20
-25 PPM
a)
m
M
-6
-8
-10
-12
-14
-16
-18
-20
-22
PPM

Fig. 2

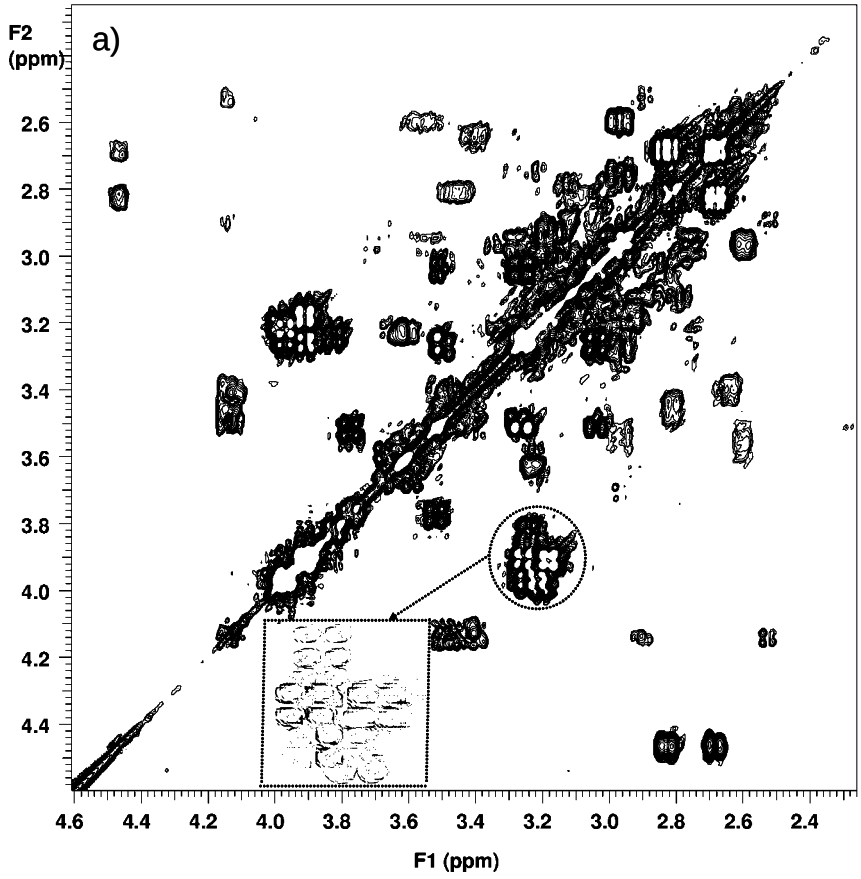
a)
F2
(ppm)
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
F1 (ppm)

Fig. 3a

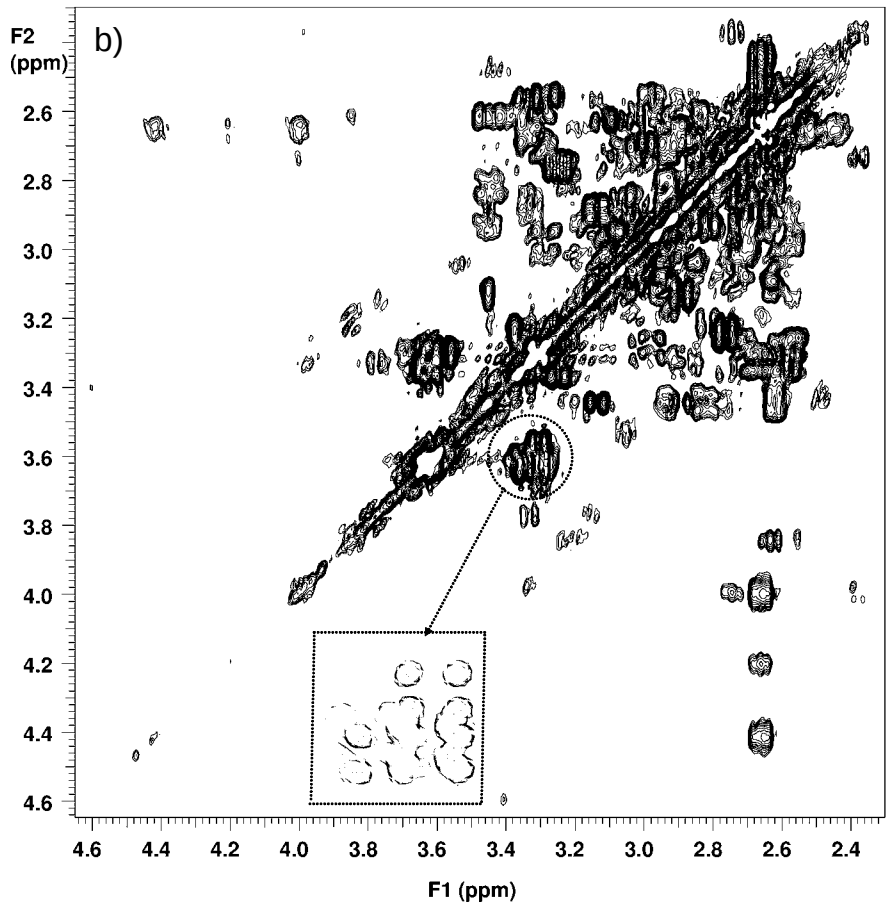
b)
F2 (ppm)
2.6
2.8
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0 2.8 2.6 2.4
F1 (ppm)

Fig. 3b

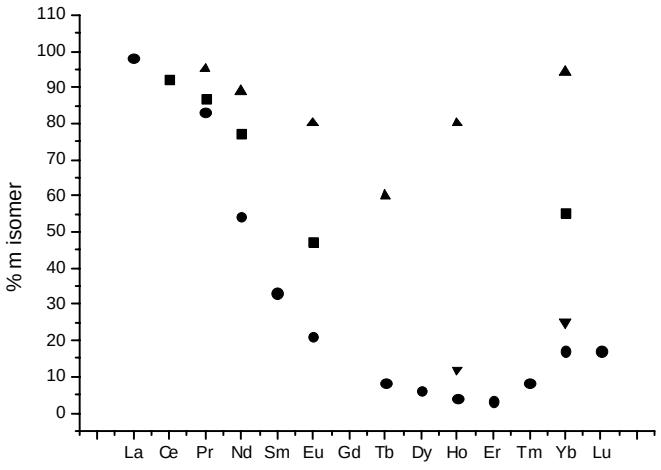
% m isomer
110
100
90
80
70
60
50
40
30
20
10
0
La
Ce
Pr
Nd
Sm
Eu
Gd
Tb
Dy
Ho
Er
Tm
Yb
Lu

Fig. 4