Elution Behavior and Structural Characterization of *N*- and *C*-functionalized DOTA Complexes for the Labelling of Biomolecules

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Two types of lanthanide complexes of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) for the labelling of biomolecules were investigated by HPLC, MS and NMR spectroscopy. The elution behavior of lanthanide complexes of *N*-functionalized DOTA [1,4,7,10-tetraazacyclododecane-1,4,7-triacetic acid-10-maleimidoethylacetamide (nDOTA-Mal) and 1-{2-[4-(maleimido-*N*-propylacetamidobutyl)amino]-2-oxoethyl}-1,4,7,10-tetraazacyclododecane-4,7,10-triacetic acid (nDOTA-Bu-Mal)] and *C*-functionalized DOTA [2-{4-(maleimido-*N*-propylacetamido)benzyl}-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (cDOTA-Bnz-Mal) and 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (cDOTA-Bnz-NCS)] was compared. *N*-functionalized lanthanide DOTA complexes coelute as required for their use as ICAT-analogous reagents. The complexation of the *C*-functionalized DOTA with lanthanides results in two fractions separable by HPLC. Coelution is observed for the main fractions of the lanthanide complexes. The retention times of the minor fractions show a dependence on the ionic radii of the metal ions. MALDI spectra of lanthanide-DOTA-peptide conjugates including different monoisotopic lanthanides demonstrate the advantage of the mass variations for extensive peptide and protein investigations.

Key words: Macrocycles, Lanthanide DOTA Complexes, HPLC, NMR, ICAT, MeCAT

Introduction

The marking of congeneric samples from different origin with the intention of verifying, identifying, comparing, and quantifying the components plays a major role in biochemistry, medicine and also in environmental analysis. An example is the development of approaches for the measurement of the relative expression of peptides and proteins between different samples, which is an essential aspect of proteomics.

Several stable isotope tagging methods are available that include isotope-coded affinity tags (ICAT), isotope tags for relative and absolute quantification (iTRAQ), peptide labelling with [18O]water during proteolytic digestion or [2H]-acylation (GIST), and isotope-differentiated binding energy shift tags (IDBEST) [1 – 6].

Alternative approaches to isotope tagging are element- or metal-coded affinity tags (MeCAT) [7 – 10]. MeCAT is based on tags containing chemically very similar metals. Preferentially monoisotopic lanthanides are used for those methods.

Particularly suitable ligands for the complexation of lanthanides in MeCATs are macrocyclic polyamino polycarboxylates, which are already used in numerous other biochemical and medical applications [11, 12]. DOTA and selectively functionalized DOTA derivatives form very stable lanthanide complexes that are polar and water-soluble. Although there are many publications dealing with the structure, dynamics and isomerization processes of lanthanide DOTA complexes [13, 14], it remains unclear what influence different isomers have on the elution behavior of the complexes. In this paper we compare two types of DOTA-based MeCATs and their *N*- and *C*-functionalized derivatives (Fig. 1), describe details of the complexation with lanthanide ions, compare their HPLC elution behavior, and assess their overall suitability as MeCATs.

nDOTA-Bu-Mal

N-functionalized,
cysteine-specific

nDOTA-Mal

N-functionalized,
cysteine-specific

cDOTA-Bnz-Mal

C-functionalized,
cysteine-specific

cDOTA-Bnz-NCS
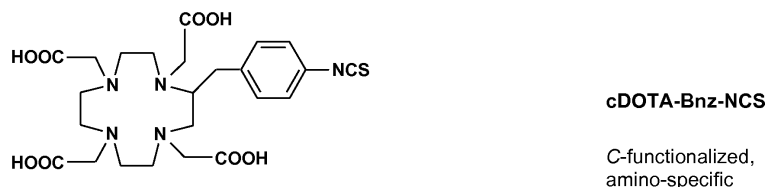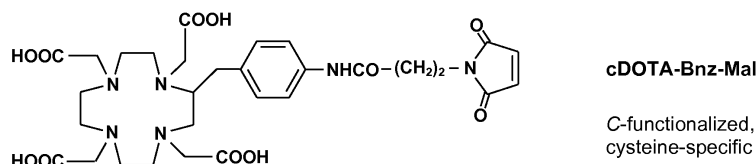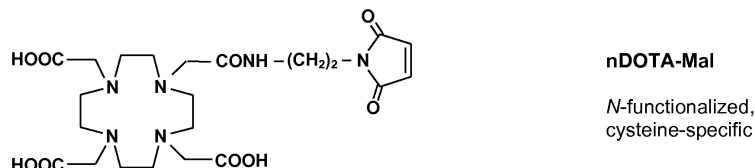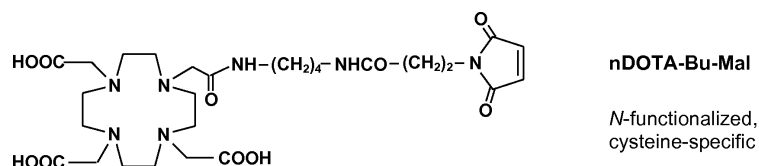
C-functionalized,
amino-specific

Fig. 1. *N*- and *C*-functionalized DOTA ligands with cysteine-specific maleimide and amino-specific isothiocyanate groups.

Results and Discussion

HPLC elution behavior of lanthanide(III) complexes of C- and N-functionalized DOTA derivatives

An important prerequisite that MeCAT reagents must fulfill for quantification is the coelution of complexes of different metals in HPLC. The original ICAT reagent, which is available in an octadeuterio- and an octaprotio form, leads to tagged protein fragments that do not exactly coelute [15]. This point is not trivial for MeCATs as it is known from NMR and XRD investigations of other DOTA complexes that they undergo isomerization in solution [16, 17]. The influences of these equilibria on the elution behavior are unknown. Here, the chromatographic behavior of lanthanide complexes of two *N*-functionalized cysteine-specific ligands (nDOTA-Mal and nDOTA-Bu-Mal), a *C*-functionalized amine-specific ligand (cDOTA-Bnz-NCS) and a *C*-functionalized, cysteine-specific ligand (cDOTA-Bnz-Mal) are investigated.

The most important point regarding the elution behavior is the structural variety of the lanthanide complexes of DOTA (Fig. 2). There are many studies dealing with the solid-state and solution structure of DOTA and other polyazapolycarboxylate complexes [18 – 20]. From the solid-state structures of *Ln*(III)-DOTA complexes it is known that the ligand provides eight coordination sites arranged in a square-antiprismatic (SA) (for *Ln* = Pr, Nd, Eu, Gd, Dy, Ho, Lu, Y) or in a twisted square-antiprismatic (TSA) geometry (for *Ln* = La, Ce) around the lanthanide ion [18, 21]. The lanthanide coordination sphere is completed by a water molecule in a capping position above the plane formed by the four carboxylate groups. The TSA structure differs from the SA structure in the twist angle between the two square planes of the antiprism, defining also a different helicity of the isomers. The helicity is determined by two sources: (i) the four five-membered rings formed by the bond between acetate arms to the metal ion with the absolute config-
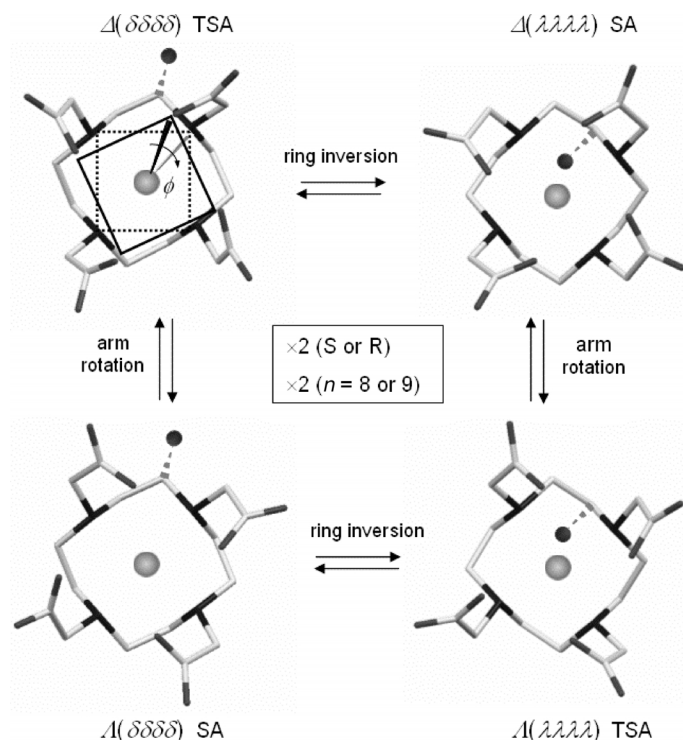
Fig. 2. Structural and isomerizational processes of the diastereoisomers of DOTA-typed complexes. One substituent is shown for the *C*-functionalized ligands cDOTA-Bnz-Mal and cDOTA-Bnz-NCS. The substituent is hydrogen for the *N*-functionalized ligands nDOTA-Bu-Mal and nDOTA-Mal. Symbols Δ and Λ refer to the helicity of the acetate arms, λ and δ to the configuration of the macrocycle. The equilibrium of isomers is additionally complicated by the absence of water in the inner coordination sphere and the occurrence of racemization.

uration Δ or Λ, and (ii) the four five-membered rings formed by the binding of the cyclen with the absolute configuration $\delta\delta\delta\delta$ or $\lambda\lambda\lambda\lambda$ [22]. However, the acetate arm bearing the peptide-specific group of the *N*-functionalized DOTA derivatives is only weakly involved in the complexation of the ion. It has been shown that the SA/TSA isomers may interconvert on the NMR time scale either by rotation of the acetate arms or by conformational interconversion of the ethylenic groups of the macrocycle, resulting in two enantiomeric pairs of four diastereoisomers. The retention times of the enantiomers are expected to be equal on an achiral HPLC column.

To complicate matters further, this conformational equilibrium is connected to a coordination equilibrium caused by the loss of the inner-sphere water molecule. Species with the coordination number 8 have been labelled as SA' and TSA', observed in the X-ray solid-state crystal structure of Sc-DOTA and Tm-DOTA, respectively [18]. However, 9-fold coordination is commonly observed, while 8-fold coordination is found for smaller lanthanide ions in the solid state by XRD and also in solution by NMR [17].

The equilibria described for *N*-functionalized DOTA complexes also apply to *C*-functionalized com-

plexes. The complexity is increased by the chiral center within the DOTA macrocycle. Even though the *C*-functionalized ligand has a high optical purity, there is a conformational equilibrium in solution due to ring inversion and nitrogen inversion which gives rise to diastereoisomeric compounds after the complexation of the ligand. Thus non-simultaneous elution of the metal complexes can be caused by a multitude of different phenomena.

C-Functionalized DOTA complexes: Ln-cDOTA-Bnz-NCS and Ln-cDOTA-Bnz-Mal

Chromatograms of lanthanide complexes of the *C*-functionalized ligand cDOTA-Bnz-NCS in hydrogen-carbonate buffer show two peaks in a ratio of about $80:20$, independent of the metal ion. Both peaks exhibit identical MALDI spectra indicating the formation of two isomers in the complexation of cDOTA-Bnz-NCS. Also the fragmentation patterns in the ESI-MS spectra are nearly identical. The MS spectra of both fractions are characterized by the stepwise loss of CO_2 (Fig. 3) which is well observable for all four acetate arms of the later eluting peak. These data demonstrate the presence of the desired complex in both

Ln^{3+}	Ionic radius (pm)	cDOTA-Bnz-NCS minor	cDOTA-Bnz-NCS major	cDOTA-Bnz-Mal minor	cDOTA-Bnz-Mal major	nDOTA-Mal	nDOTA-Bu-Mal
–		17.92		11.80		5.38	9.88
Y	106	16.85	15.54				
Eu	98	17.10	15.54				
Tb	93	16.90	15.53	9.86	10.05	4.78	9.06
Ho	89	16.88	15.55	9.70	10.04	4.79	9.05
Tm	87	16.60	15.54	9.62	10.05	4.78	9.05
Lu	85	16.48	15.54	9.51	10.03	4.78	9.05

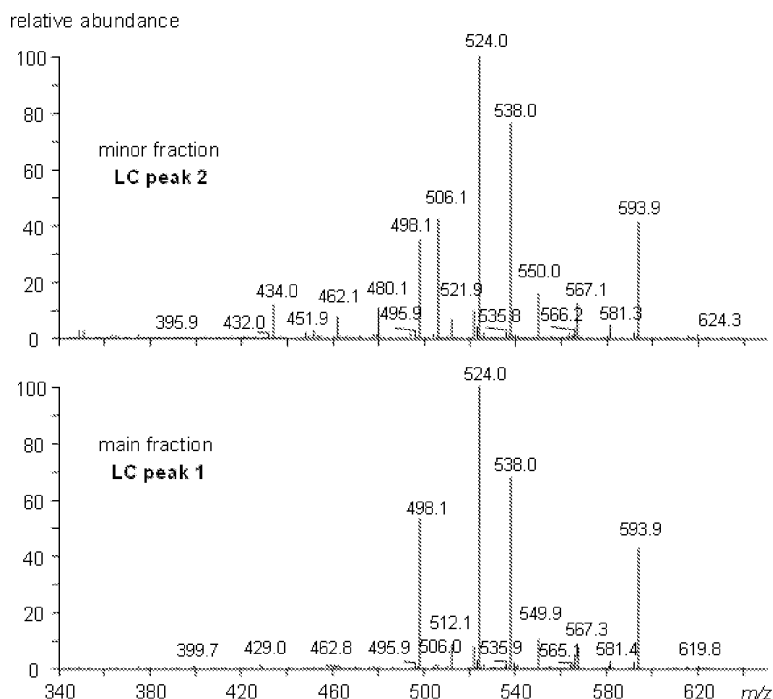Table 1. Elution times of *C*- and *N*-functionalized MeCAT complexes.

Fig. 3. Comparison of MS/MS spectra of the singly charged precursor ion $m/z = 638.0$ obtained from the major and minor HPLC fractions of Lu-cDOTA-Bnz-NCS. Peaks at $m/z = 594, 550, 506,$ and 462 are caused by the stepwise loss of CO_2.

peak components. Different protonation states or incomplete complexation can be excluded.

The component eluting first (LC peak 1) shows approximately the same retention time for all lanthanides, whereas that of component 2 (LC peak 2) depends strongly on the lanthanide. The different elution times of LC peak 2 correlate well with the ionic radii of the lanthanide ions (Table 1, Fig. 4). The relative amounts of LC peak 1 and LC peak 2 do not change significantly at room temperature over 24 h. Even if the complexation is carried out at 60 °C the ratio is unchanged overnight. However, the extreme reaction conditions are accompanied by decomposition and formation of additional peaks in the chromatogram, in particular of the thiourea by-product.

Both components can be separated chromatographically and remain unchanged for several months provided they are stored below 0 °C.

The ratio of the peaks is expected to change depending on the metal ion present if they are due to isomers of different helicity. For example, the ratio of the diastereoisomers $\Delta(\delta\delta\delta\delta) : \Delta(\lambda\lambda\lambda\lambda)$ of the enantiomeric pair for LaDOTA is 50 : 1, but 1 : 80 for Lu-DOTA [16, 21, 23]. In our system there was no observable change in peak ratios within the *Ln*-cDOTA-Bnz-NCS series, and peak 1 was always largest independent of the lanthanide ion used. Therefore, isomerization resulting from rotation of the acetate arms or motions within the macrocycle is unlikely to be the cause for the phenomena.

The fraction of LC peak 1 from the Eu-cDOTA-Bnz-NCS complex shows the expected number of signals in the ^{1}H and ^{13}C NMR spectra. Similar signals were found for the second component present in < 5 %. Most signals can be assigned by analogy with the NMR spectra of Eu-DOTA to axial and equatorial acetate
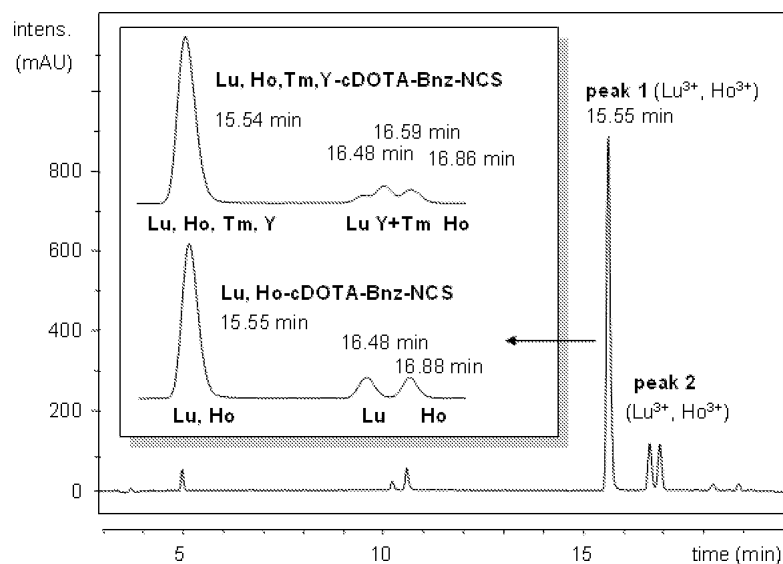
Fig. 4. The reversed-phase HPLC-UV spectrum of a mixture of cDOTA-Bnz-NCS with Lu, Ho, Tm, and Y ions shows the coelution for LC peak 1 and the effect of the ionic radii dependence of LC peak 2. Also the main isomer of cDOTA-Bnz-NCS with Y(III) shows coelution and is therefore suitable as a MeCAT.
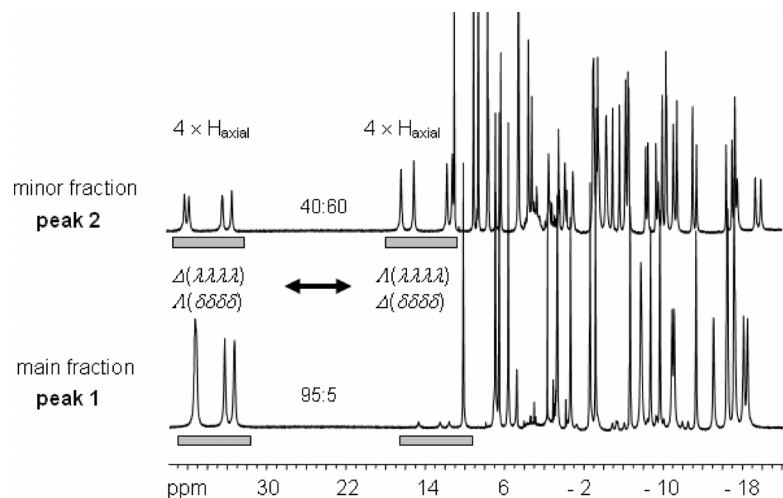

Fig. 5. ^{1}H NMR spectra of the separated HPLC fractions of Eu-cDOTA-Bnz-NCS in D_2O at 278 K. Both spectra show two sets of resonance signals with similar chemical shifts but different contents of isomers.

protons [16]. However, an exact assignment is not possible due to missing cross peaks in the HMBC and NOESY spectra of the paramagnetic Eu-cDOTA-Bnz-NCS. 1D NOE difference measurements of the component of LC peak 1 have shown that the four signals between 32 and 38 ppm exchange with the four signals between 10 and 15 ppm. The observed equilibrium within peak 1 is caused by the equilibrium between the $\Delta(\lambda\lambda\lambda\lambda)/\Lambda(\delta\delta\delta\delta)$ and $\Lambda(\lambda\lambda\lambda\lambda)/\Delta(\delta\delta\delta\delta)$ isomers shown in Fig. 5. Also the NMR spectra of the fraction of LC peak 2 show the expected number of proton signals and resemble the spectra of LC peak 1, but here the $\Lambda(\lambda\lambda\lambda\lambda)/\Delta(\delta\delta\delta\delta)$ isomers are favored (60 %). Ring inversion of the macrocycle and rotation of the acetate

arms occur in both samples, but they are not the cause of the two peaks in the HPLC.

The NMR spectra of the two fractions isolated from Lu-cDOTA-Bnz-NCS show results similar to those of Eu-cDOTA-Bnz-NCS. For LC peak 1 the equilibrium strongly favors the $\Lambda(\delta\delta\delta\delta)$ or enantiomeric $\Delta(\lambda\lambda\lambda\lambda)$ conformation (99 %). There are again two distinct sets of signals in the NMR spectra of the fraction of LC peak 2. In the NOESY spectrum, each proton of one species shows an exchange peak with a proton of the second species, and both species are equally populated. Chemical shifts of both species are different to those found for the compound of LC peak 1.

It should be noted that transformations between 8- and 9-fold coordinated species, which differ in the co-ordination of one water molecule, appear to be fast on the proton NMR time scale. Thus the species are not distinguishable by this technique, as the dissociation and association processes have rate constants in the range $10^5 – 10^8$ s^{-1} [17].

A further interesting observation is related to the solubility of both components in water. The major component of Lu-cDota-Bnz-NCS is easily, the minor one is noticeably less soluble in water. The observations for cDOTA-Bnz-NCS apply also to the Tb^{3+}, Ho^{3+}, Tm^{3+}, and Lu^{3+} complexes of cDOTA-Bnz-Mal. These lanthanide complexes can also be separated into two components by HPLC in a ratio of *ca.* 20 : 80, which changes to 25 : 75 in HEPES buffer. Here the minor component elutes earlier and shows a remarkable dependence of the retention time on the Ln^{3+} ionic radius. The latter peaks show the desired coelution (Table 1). The isomeric ratio remains constant over several days at room temperature. There was no interconversion of the purified components at 60 ℃ for 24 h, although at this temperature conversion of the reactive maleimide moiety into the unreactive maleoyl group and decomposition take place.

The peak ratios do not depend on the metal ion present. Hence, we initially concluded that the isomerism is caused by the ligand side chain and assumed that an enantiomeric pair exists. After the complexation both enantiomers form a pair of diastereomers, which can be separated by chromatography and distinguished by NMR spectroscopy. However, measurements of the ligand Cyclen-Bnz-NO$_2$, a precursor of cDOTA-Bnz-NH$_2$, on a chiral HPLC column showed no separation into *R*- and *S*-isomers. Also NMR measurements of Cyclen-Bnz-NO$_2$ with a chiral additive ((*R*)-(+)-α-methoxy-α-(trifluoromethyl)phenyl-acetic acid) in solution did not result in a signal doubling. This is in accordance with the manufacturers statement (Macrocyclics, Dallas, Texas), excluding the presence of a racemic ligand.

N-Functionalized DOTA complexes: Ln-nDOTA-Bu-Mal and Ln-nDOTA-Mal

The formation of complexes with the *N*-functionalized ligands nDOTA-Bu-Mal and nDOTA-Mal was performed analogously to those of the *C*-functionalized ligands at pH = 7 and at room temperature. The progress of the reaction was monitored by HPLC. Chromatograms of the reaction mixtures of nDOTA-Bu-Mal with lanthanide chlorides showed two peaks after several hours in addition to those of the unco-ordinated ligand. During further reaction the ligand peak and one of the two additional peaks disappeared, and after 12 h only one peak remained. For the reaction of nDOTA-Mal with lanthanide ions a decrease of the ligand peak and an increase of the peak of one reaction product was observed. After complete coordination only one defined product was obtained for *N*-functionalized DOTA ligands. The lanthanide complexes of nDOTA-Bu-Mal coeluted as well as those of nDOTA-Mal, hence both are suitable candidates as MeCAT reagents. The transient peaks found during nDOTA-Bu-Mal coordination also coeluted suggesting that these peaks do not correspond to the minor fraction of the *C*-functionalized complexes.

Liu *et al.* also observed only one isomer for the *N*-functionalized DOTA complex Y-nDOTA-MBA (MBA = methyl-benzylamine) by HPLC [11]. By contrast, reversed-phase HPLC has indicated that the *N*-functionalized Y-labeled nDTPA-monoamide complex exists as a mixture of two detectable isomers which interconvert even at room temperature [24]. Thus the isomer equilibrium proposed for DTPA complexes does not apply to the DOTA complexes, because of the rigidity of the latter.

DOTA and its *C*- and *N*-functionalized derivatives are very flexible. Nitrogen inversion occurs as well as motion of the acetate arms and ethylenic groups. These processes induce conformational equilibria although the number of stable conformers is restricted. The most stable conformers are the strainless conformers with diamond lattice-derived forms [25]. Structures contain as few *gauche* bonds as possible, and additional dipole-dipole interactions and hydrogen bonds are important. Various conformations of the cycloalkanes (boat, chair and twist) may be favored, and after complexation freedom of movement is strongly restricted [26]. In aqueous solution, lanthanide DOTA complexes exist as a mixture of two stereoisomers (four enantiomers) with four equivalent ethylenic and acetate groups (Fig. 2). In the NMR spectra only a quarter of each molecule is represented. One peak is observed in the HPLC, but two sets of NMR signals. Both sets of signals are due to the C_4 symmetry of the complex, and the ratio of isomers is dependent on the central atom. Modification of one acetate arm in *Ln*-DOTA, *e. g.* in *Ln*-nDOTA-Bu-Mal or *Ln*-nDOTA-Mal, removes the C_4 symmetry. These complexes again show only one peak in the HPLC, but the NMR spectra show two complete sets
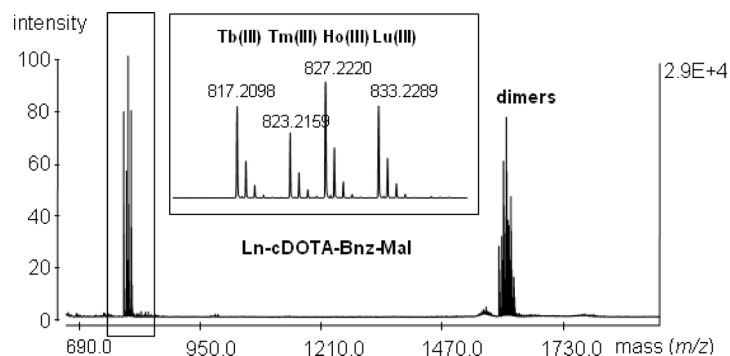
Fig. 6. MALDI spectrum of *Ln*-cDOTA-Bnz-Mal with Tb(III), Tm(III), Ho(III) and Lu(III) lanthanide ions.

of resonance signals. There is also an isomeric equilibrium dependent on the radius of the central atom.

In the case of *C*-functionalized DOTA, two preferred conformers in a ratio of 80 : 20 result from coordination. The potential energy of the two conformers is determined by the different positions of the substituents, and the ratio is independent of the central atom, as this is determined by the conformer ratio in the uncoordinated state. Whereas both conformers of the uncoordinated ligand exist in a fast equilibrium, there is no interchange after coordination. Two separate chromatographic peaks are observed for components which have NMR spectra similar to those of the *N*-functionalized complexes. Both show two signal sets arising from mobility of the acetate arms and ring inversion. The isomeric ratio depends on the central atom, but is very different for LC peak 1 and LC peak 2 despite the same central atom (Fig. 5).

MALDI-TOF investigations of MeCAT and MeCAT-peptide conjugates

It could be shown by HPLC that coelution occurs for the complexes of *C*- (main component) and *N*-functionalized DOTA with different lanthanides. Additionally, the complexes were studied by mass spectroscopy. MALDI-TOF and MALDI-TOF-TOF measurements of the pure complexes and the conjugates of the complexes with model peptides were carried out.

Fig. 6 shows the MALDI spectrum of the purified mixture of *Ln*-cDOTA-Bnz-Mal containing the lanthanide ions [159]Tb(III), [165]Ho(III), [169]Tm(III), and [175]Lu(III). This MeCAT mixture allows for the parallel detection and quantification of the peptide and protein content of four samples. The resolution of the spectra in the reflector mode is very high. The different metal complexes show the expected mass differences due to the different lanthanide ions. The largest mass differ-

ence is 16 Da, arising from [159]Tb-cDOTA-Bnz-Mal and [175]Lu-cDOTA-Bnz-Mal. In addition, the expected ^{12}C/^{13}C isotope pattern is observable for each metal complex. A further prominent group of peaks results from the formation of dimers. As there are both homo- and heterometallic dimers, more complex isotope patterns become visible.

The fact that a multitude of mass differences can be reproduced is demonstrated by the example *Ln*-cDOTA-Bnz-NCS. Besides [165]Ho(III), [169]Tm(III) and [175]Lu(III), the monoisotopic ion [89]Y(III) was used for complexation [27]. The latter leads to an MS peak well separated from the others. Such a mixture may prove useful in the case of complicated samples, where the signal assignment is not unambiguous or where peak overlappings prevent the exact quantification.

Both amino- and thiol-specific MeCAT mixtures were reacted with model peptides. The conjugation was done by standard procedures [28, 29]. As an example for the thiol-specific MeCATs, a ten-fold excess of the mixture Tb, Ho, Tm, Lu-cDOTA-Bnz-Mal was reacted with the model peptide, CYENPQVIASQQL (monoisotopic mass [M+H]$^+$ 1492.7). The mixture was studied by MS without further purification. Beside the peaks for the pure MeCATs the MALDI spectrum shows signals due to MeCAT-peptide conjugate and additionally the sodium adduct. The conjugate is characterized by the isotope pattern caused by the four lanthanides, *e. g. m/z* = 2308.9 for the Tb-cDOTA-Bnz-Mal conjugate, *m/z* = 2314.9 for the Ho(III) label, *m/z* = 2318.9 for Tm(III) and *m/z* = 2324.9 for Lu(III). Fig. 7 shows the MALDI-TOF-TOF spectrum of the precursor at *m/z* = 2308.9. Seven fragments of the b series and two of the y series can be observed. The fragments of the b series include the metal chelate, yet not only the Tb(III)-containing complex is visible but also the peaks of the other three lanthanide ions are de-
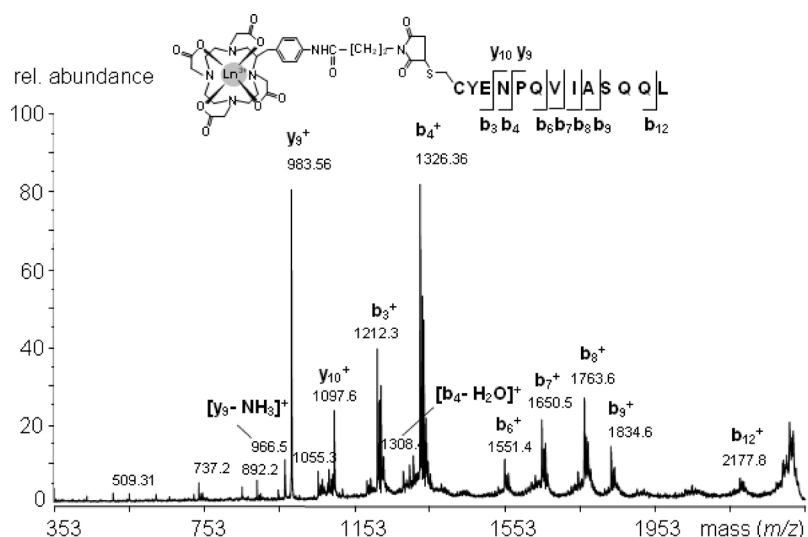
Fig. 7. MALDI-TOF-TOF tandem mass spectrum of an MeCAT-peptide conjugate at *m/z* = 2308.9 (Tb-cDOTA-Bnz-Mal / CYEN-PQVIASQQL). Tb(III), Tm(III), Ho(III) and Lu(III) lanthanide ions were used for the complexation of cDOTA-Bnz-Mal. Some peaks of the b and y series of the precursor are denoted.

tectable. On the other hand, MALDI-TOF-TOF spectra of the [1]H-ICAT-labelled peptides show also fragment peaks of the [2]H-ICAT-labelled ones and *vice versa*.

Quantitative investigations of protein digestions using ICP-MS were also carried out and published separately [9]. Further investigations performed in our Institute concerning the MS fragmentation behavior of MeCAT-labelled model peptides and trypsinized BSA peptides under collision-induced dissociation (CID), electron capture dissociation (ECD) and infrared multiphoton dissociation (IRMPD) conditions were recently published [30].

Conclusion

Both *Ln*-nDOTA derivatives and the major isomers of *Ln*-cDOTA-Bnz-Mal/NCS fulfill the requirement of coelution necessary for their use as MeCAT reagents. The conditions for complexation must be controlled very carefully to reach high yields. As known from the synthesis of the lanthanide DOTA complexes, the formation of the complexes is slow [31, 32]. A pH value of 7 is essential for the entrance of the lanthanide ions into the tetraaza coordination pockets of the ligands, which otherwise would be blocked by protonation of the strongly basic ring nitrogens. Based on our experiences, we would advice against tagging proteins first with the ligand and to complex it thereafter with the lanthanide ions. The influence of lanthanide salts on the peptide has not been investigated systematically as yet. The lanthanide can bind additionally in an unspecific way at amino acid side chains, and obviously pep-

tide degradation processes occur. Thus the following order should be kept:

1. Coordination of the DOTA ligand.
2. Purification or separation of the main product.
3. Attaching the main product to the peptides and proteins under proper experimental conditions.

Thermodynamic stability and kinetic inertness of the investigated *C*- and *N*-functionalized *Ln*-DOTA complexes are comparable with those of both *Ln*-DOTA and *Ln*-DO3A complexes and are higher than for *Ln*-DTPA complexes [13]. Also on that score, both are suitable candidates for the use as MeCAT reagents. *N*-functionalized ligands are easier and less costly to synthesize in comparison to the *C*-functionalized DOTA [11, 33, 34]. Also the yield of the MeCAT reagent after complexation with lanthanide ions is higher for *Ln*-nDOTA derivatives.

The MALDI spectra presented here and in related work have shown that MeCAT is a very promising method for peptide and protein labeling, identification and quantification. MeCAT is applicable as a stand-alone or a complementary method to ICAT, ITRAQ and 2D-GE.

Experimental Section

The ligands cDOTA-Bnz-NH$_2$, cDOTA-Bnz-NCS and nDOTA-Mal were purchased from Macrocyclics (Richardson, TX). The modification of cDOTA-Bnz-NH$_2$ and the synthesis of nDOTA-Bu-Mal were performed according to published procedures [27, 35]. All reagents used for the synthesis of the ligands were purchased from commercial sources.

Metallation of chelates

Using a 0.1 M Na$_2$CO$_3$/HCl, pH = 7.5 buffer, 0.1 mmol of lanthanide chlorides were dissolved and combined with 0.075 mmol of the MeCAT ligand. The pH was adjusted to pH = 7.0, and the samples were kept at room temperature over night. Analytical HPLC was carried out to verify the coordination to the *Ln*(III) ions. The complexes were purified on an Agilent-Prep-C18 column (solvent A: 0.1 % TFA in water; solvent B: 0.1 % TFA, 90 % aq. CH$_3$CN). The analytical HPLC method for the metal-DOTA conjugates used an Agilent 1100 HPLC system and was performed on a Zorbax 300SB-C18 4.6 × 150 mm column (Agilent) with a flow rate of 1 mL min^{-1} and a linear gradient of 100 % solution A to 60 % solution B in 30 min (solvent A: 0.1 % TFA in water; solvent B: 0.1 % TFA, 90 % aq. CH$_3$CN) with spectrophotometric monitoring at $\lambda = 220$ nm.

HPLC

Analytical reversed-phased HPLC (Agilent, 1100 series, Paolo Alto, CA) was performed at r.t. at 1 mL min^{-1} on a Zorbax 300SB-C18 4.6 × 150 mm column (Agilent) with a linear gradient of 100 % solution A to 60 % solution B in 30 min (A: 1000 mL of H$_2$O, 2 mL of TFA; B: 500 mL of CH$_3$CN, 100 mL of H$_2$O, 1 mL of TFA with spectrophotometric monitoring at $\lambda = 220$ nm.

Preparative reversed-phase HPLC (Shimadzu L10) was performed at room temperature at 70 mL min^{-1} using a Vydac C18 protein 40 × 300 mm column (The Seperations Group, Hesperia, CA) with a linear gradient of 80 % solution A to 70 % solution B in 50 min.

Mass Spectrometry

ESI

The lyophilized samples were freshly dissolved in methanol/water/acetic acid (50 : 49 : 1, v/v) and analyzed by MS and MS/MS using an electrospray ion-trap mass spectrometer (LCQ, Thermo-Finnigan, San Jose, CA, USA). It was operated with XCALIBUR software (v1.3). Both samples were injected with a syringe pump at a flow rate of 3 μL min^{-1}. A spray voltage of 4.5 kV was applied. The heated metal capillary was maintained at 200 °C. For MS/MS analysis the normalized collision energy was set at 40 %.

MALDI

One μL of each sample was mixed with 1 μL of α-cyano-4-hydroxycinnamic acid matrix solution consisting of 5 mg matrix dissolved in 1 mL 0.3 % TFA in acetonitrile/water (1 : 1, v/v). From the resulting mixture 1 μL was applied to the sample plate. MALDI-MS and MS/MS measurements were performed using a MALDI-TOF/TOF instrument (4700 Proteomics Analyzer, Applied Biosystems, Framingham, MA, USA) equipped with an Nd:YAG laser (355 nm) operating at a frequency of 200 Hz. MS/MS spectra were obtained by accumulation of up to 10 000 consecutive laser shots. Spectra were processed using the DATA EXPLORER software v4.8.

NMR

Samples for NMR spectroscopy were prepared by dissolving 5 mg of the complex in 0.75 mL of D$_2$O in a 5 mm sample tube (Wilmad). NMR spectra were acquired at 600 MHz on a Bruker AV600 spectrometer. 2D spectra (DQF-COSY, ROESY, TOCSY, HSQC and HMBC) were recorded at 278 K. The signal due to residual HDO was suppressed by continuous low-power irradiation. Data matrices typically consisted of 2048 complex points in F2 for each of the 512 F1 increments, the data being zero-filled to a 4096 × 1024 complex matrix before transformation. 2D spectra were processed using the program TOPSPIN on a HP workstation.

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