Design, synthesis and characterization of smart MR contrast agents sensitive to pH / calcium changes during neural activity

Diploma Thesis

Submitted
in partial fulfillment of the requirements
for the

Diploma in Organic Chemistry

by
Anurag Mishra

University of Tübingen
Tübingen, July 2004
This diploma thesis was prepared at the Max-Planck Institute for Biological Cybernetics (chemistry lab of the Department Physiology of Cognitive Processes, Prof. N. K. Logothetis) in collaboration with the Institute for Organic Chemistry, Eberhard Karls University, Tübingen under the guidance of Dr. Anil K. Mishra, Dr. J. Pfeuffer, and Prof. Dr. Klaus Albert during the period from January 2004 to July 2004.

Hereby I declare the fact that I am writing this work and no different than the indicated aids have been used.

Tübingen, 23rd July, 2004
Knowledge in itself is a continuous process. At this moment of my substantial enhancement, I have found this rare opportunity to evince a word of thanks to all those who have played a key role in the successful completion of my project.

My heartfelt thanks to Prof. Dr. Klaus Albert, Institute for Organic Chemistry, Tübingen University, for providing his unconditional guidance and optimal resources allocation that helped me in the successful completion of my diploma thesis project.

I wish to express my deepest reverence to Prof. Dr. Nikos K. Logothetis, Director, Department Physiology of Cognitive Processes, MPI for Biological Cybernetics, for his support, suggestions, valuable guidance and providing me the facilities during the course of the investigation.

I owe a sincere thanks to my esteemed advisors Dr. Josef Pfeuffer, Head of MR Imaging and Spectroscopy, MPI for Biological Cybernetics, and Dr. Anil Mishra, INMAS, Delhi, India for their sincere guidance, co-operation, constant encouragement, patience and belief in me.

I express my cordial gratitude to Michael Beyerlein, Ilgar Mamedov and Ritu Mishra for their help and timely suggestions.

Finally, most of all, I thank my family members for their unconditional love, encouragement and support to complete my diploma thesis project.

I also thank all those who could not find a separate name but have helped directly and indirectly.

ANURAG MISHRA
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1. Introduction

Molecular imaging using Magnetic Resonance Imaging (MRI) techniques is a rapidly growing field in basic neuroscience and diagnostic medicine. The high spatial resolution and an undisputed capacity for differentiating soft tissues have highly contributed to the widespread use of this imaging technique. MRI offers the potential of true three dimensional imaging of biological structures, where the image signal is based upon the resonance from the protons of water or other nuclei. The contrast of MR images in-vivo depends on a couple of parameters like the longitudinal and transverse relaxation times \( T_1 \) and \( T_2 \) and density of (water) protons. Soft tissues are easily identified by MRI, but it has been more difficult to identify damaged tissues, for example in stroke or cancer patients. For neuroimaging, a major technique is the functional MR imagings based on the ‘Blood-oxygenation-level-dependent’ (BOLD) contrast, which is based on the endogenous ‘smart’ contrast agent oxy/deoxyhemoglobin in the blood. MR properties like water relaxivity change dynamically with different oxygenation state in the vasculature during neuronal activation and rest.

In the proposed project, we want to develop novel exogeneous smart contrast agents, which reflect-like hemoglobin in blood–changes of neuronal activity, but which are located in the extra-cellular space, independent of the hemodynamics and may reflect more directly changes in neuronal activity.

The central challenge in developing an MR imaging approach for detecting neuronal activity is to find a way to translate activity into changes in MR image contrast. A modulation of water relaxivity by contrast agents can be achieved by specific physiological or biochemical triggers that can be changes in pH, calcium ion, neurotransmitter concentration, enzymatic activity, or the binding of an intracellular messenger. The purpose of this project is to design and synthesize a new class of Gadolinium-based calcium ion, pH, and enzyme sensitive contrast agents that are capable of reporting on the brain activity status by contrast change in MR images.

The long range goal is to develop effective novel classes of contrast agents that serve as reliable markers for neuronal activity by modulating relaxivity of MR images based on changes in physiological environmental.
The objective of the proposed project is to design and synthesize a couple of pH, calcium ion, enzyme based smart contrast agents that are capable of reporting on the metabolic status of cells as observed by MR images. The development and evaluation of the new smart contrast agents encompasses the following steps

- Synthesis of a variety of cyclen based and C-substituted cyclen based chelating agents and characterization of those by NMR, mass spectrometry, elemental analysis, crystallography and HPLC.
- Complexation of the ligand with the paramagnetic metal ions particularly gadolinium.
- In-vitro MR relaxation measurements simulating different physiological conditions like different pH, calcium ion concentration and enzymatic activity, dependent on the type of contrast agents.
- In-vivo MR measurements in the rat and monkey to evaluate the exogenous contrast agents and its change upon functional activation.

The central hypothesis of the proposed research is that agents can be designed which have switchable protection groups to either provide or prevent access of water to the paramagnetic center of Lanthanide metal containing contrast agents. Changes of the physiological environment in vivo can be translated to changes in the MR signals.

The specific aim is to design and develop smart contrast agents that are extracellular and generate a signal that depends on some variable in their immediate environment. Smart contrast agents have been built on Gd (III) system because their relaxivity can be modulated by their environment. The number of water molecules in the first coordination sphere, the water exchange rate and the rotational correlation time have a strong effect on the relaxivity of the compounds and, exemplified below can be influenced by many factors. Keeping in view the above factors and the central hypothesis three aims are proposed to accomplish the overall objective:

**Specific aim-1**— Design and synthesis of pH-activated contrast agents

**Specific aim-2**— Design and synthesis of calcium-activated contrast agents

**Specific aim-3**— Design and synthesis of enzyme-activated contrast agents
2. Background and Significance

Magnetic Resonance Imaging (MRI) is a non-invasive technique using radio frequency (RF) pulses and strong magnetic fields to create images of internal organs and structures. Computer reconstruction of the data allows high-resolution arbitrary image planes to be generated from a single image imaging procedure. MRI has found applications in almost all areas of medicine; it is used to aid in the diagnosis of cancer, joint and musculoskeletal disorders, and neurodegenerative and cardiovascular diseases.

Magnetic resonance imaging is a valuable and versatile technique for visualizing internal structures technique first described in 1978 [1]. MRI is an extension of nuclear magnetic resonance spectroscopy used in chemistry. Certain atoms have an inherent magnetic dipole moment that results from their electrical charge and spin. When placed in a strong, steady magnetic field, these atoms begin to process around the axis of the external magnetic field, $B_0$ (Figure 1a). The Larmor frequency, $\omega_L$, is the frequency of precession of the atom. The orientation along the longitudinal axis, either with or against $B_0$, determines the energy level of the spin (Figure 1b). The difference in energy levels causes a net magnetization of the sample along the longitudinal axis because of a small excess of spins in the low energy states vs. the high energy state, this net magnetization is best described as a vector. An RF pulse at the Larmor frequency may be used to add energy to the spins, moving spins from the low energy state to a higher-energy state (Figure 1c). Consequently, the longitudinal component of the net magnetization vector diminishes and eventually becomes negative (against $B_0$) as more RF energy is added to the spins. The relaxation time is the time it takes nuclear spins to return to their equilibrium state. The relaxation time has two components, longitudinal and transverse relaxation times, known as $T_1$ and $T_2$ respectively.

The intensity of a magnetic resonance (MR) image depends primarily on three factors-the density of water protons, $T_1$ and $T_2$. The visual contrast is determined by the variation in these parameters among tissues. MR images are usually weighed to highlight differences in either $T_1$ or $T_2$; otherwise images would be fairly featureless because the density of water does not vary significantly.

Although satisfactory images are generated using $T_1$ or $T_2$ weighting, it is sometimes desirable to add additional contrast to an MR image in order to highlight regions of interest.
This is accomplished by the use of small molecules called contrast agents (CA). Contrast agents dramatically shorten the $T_1$ and $T_2$ of water and their presence is easily detected in MRI images at levels as low as 0.1 mM.

The aim of using a contrast agent in MRI is to accelerate the relaxation of water protons in the surrounding tissue [2-4]. Paramagnetic ions as a result of their unpaired electrons are able to influence the relaxation time of water protons [3]. Previous research shows that ferric nitrate and Mn(II) salts could be used as paramagnetic substances, but they were weak chelates and easily dissociated in in-vivo studies. Today, Gd(III) complexes are by far the most widely used contrast agents in clinical practice [1-5, 15].

The choice of Gd (III) complexes is explained by the following properties which show its relevance as a good MR contrast agent:

- It has seven unpaired electrons which make it the most stable paramagnetic metal ion.
- It has symmetric electronic ground state, (S$^8$).
- It has relatively long electronic relaxation time.
- It has very high magnetic moment [4].

The unpaired electrons of gadolinium shorten the relaxation time of nearby water protons, making voxels appear ‘brighter’ in $T_1$-weighted images. Through-space dipole-dipole interactions between nearby water protons and the unpaired electrons cause this enhancement. Unfortunately, gadolinium ions also interfere with calcium-dependant processes and are toxic.

![Figure 1](image-url)
2.1. Relaxation theory

The general theory of solvent nuclear relaxation in the presence of paramagnetic substance was developed by Solomon, Bloembergen and others [5-7]. The observed relaxation rate, $1/T_{i, \text{obs}}$, is the sum of a diamagnetic term $1/T_{i,d}$, corresponding to the relaxation rate of the solvent nuclei without the paramagnetic solute, and a paramagnetic term $1/T_{i,p}$, which is the relaxation rate enhancement caused by the paramagnetic substances:

$$\frac{1}{T_{i, \text{obs}}} = \frac{1}{T_{i,d}} + \frac{1}{T_{i,p}} \quad i = 1, 2 \quad (1)$$

In the absence of solute-solute interactions, the solvent relaxation rates are linearly dependent on the concentration of the paramagnetic species, $[\text{Gd}]$; relaxivity, $R_i$, is defined as the slope of this dependence in units of M$^{-1}$s$^{-1}$ or, more commonly, mM$^{-1}$s$^{-1}$:

$$\frac{1}{T_{i, \text{obs}}} + \frac{1}{T_{i,d}} + R_i[\text{Gd}] \quad i = 1, 2 \quad (2)$$

The large and fluctuating local magnetic field in the vicinity of a paramagnetic center provides this additional relaxation pathway for solvent nuclei. Since these fields fall off rapidly with distance, random translational diffusion of solvent molecules and the complex as well as specific chemical interactions that bring the solvent molecules near the metal ion are important in transmitting the paramagnetic effect. Each type of chemical interaction can yield different relaxation efficiencies as governed by the distance and time scale of the interaction; the sum of these contributions and that due to translational diffusion gives the total relaxivity of the paramagnetic species. For better understand of these, three distinct type of interactions showing below (Figure-2):

- A: $\text{M} - \text{OH}_2$
- B: $\text{M} - \text{X} - \text{H} - \text{O}, \text{M} - \text{X} - \text{H} - \text{H} - \text{O}$
- C: $\text{M} - \text{H}_2\text{O}$

**Figure-2**
In case A, a water molecule binds in the primary coordination sphere of the metal ion and exchanges with the bulk solvent. The term “inner-sphere relaxation” is often applied loosely to this type of relaxation mechanism. It should be mentioned, however, that this same theory applies in the case B interaction, i.e., hydrogen-bounded waters in the second coordination sphere. Nevertheless, due to the lack of understanding of second coordination sphere interactions investigators often do not distinguish between this relaxation mechanism (case B) and that due to translational diffusion of the water molecule past the chelate (case C), referring simply to “outer-sphere relaxation”. The total relaxivity of a paramagnetic agent is therefore generally given by the below equation:

\[
\frac{1}{T_1} p = \frac{1}{T_1} IS + \frac{1}{T_1} SS + \frac{1}{T_1} OS = r_i^{IS} + r_i^{SS} + r_i^{OS}
\]

Where IS, SS and OS refers for inner sphere, secondary sphere and outer sphere.

Today, monomeric Gd (III)-based contrast agents, the outer-sphere and inner-sphere relaxation mechanisms contribute approximately to the same extent to the observed proton relaxivity at the imaging fields. It is the inner-sphere term that can be considerably augmented, whereas the outer-sphere contribution can hardly be modified. Consequently, for the new generation agents of higher relaxivity, the inner-sphere term becomes relatively much more important and represents the major contribution to the overall proton relaxation rate. Here the inner-sphere relaxation, second sphere relaxation and outer-sphere relaxation mechanism (Figure-4) is briefly described:

### 2.1.1 Inner Sphere proton relaxation

The inner sphere contribution to proton relaxivity results from the chemical exchange of the coordinated water protons with the bulk. The longitudinal and transverse inner sphere relaxation rates, $1/T_1$ and $1/T_2$, of the bulk solvent nuclei (the only observable NMR signal) are given by below equations:
\[
\left(\frac{1}{T_i}\right)_{IS} = \left(\frac{cq}{55.5}\right) \left(\frac{1}{T_{1m} + \omega_M}\right) = P_m \left(\frac{1}{T_{1m} + \omega_M}\right)
\] (4)

\[
\left(\frac{1}{T^2}\right)_{IS} = \left(\frac{P_m}{tM}\right) \left(\frac{T_{2m}^{-2} + \tau_{M}^{-1}T_{2m}^{-1} + \Delta \sigma_m^2}{T_{2m}^{-2} + \tau_{M}^{-1}}\right) + \Delta \sigma_m^2
\] (5)

where \(c\) is the molar concentration, \(q\) is the number of bound water nuclei per Gd (hydration number), \(P_m\) is the mole fraction of the bound water nuclei, \(\tau_M\) is the lifetime of a solvent (water) molecule in the inner sphere of the complex (equal to the reciprocal water exchange rate, \(1/k_{ex}\)), \(1/T_m\) and \(1/T_{2m}\) are the longitudinal and transverse proton relaxation rates in the bound water, and \(\Delta \omega_m\) is the chemical shift difference between bound and bulk water.

Enhancement of relaxation is also dependent on the effective correlation time \(\tau_c\) which depends on the molecular motion, on the rate of relaxation of the electron spins and on the exchange rate between free and bound water. \(\tau_c\) is given by the equation:

\[
\frac{1}{\tau_c} = \frac{1}{\tau_M} + \frac{1}{\tau_R} + \frac{1}{\tau_S}
\] (6)

Where

- \(\tau_M\)----residence time of the water molecule in the first hydration sphere of the metal
- \(\tau_S\)----the electron correlation time.
- \(\tau_R\)----rotation correlation time. (Figure-3)
Figure-3: Schematic representation of a Gd(III) chelate with one inner-sphere water molecule, surrounded by bulk water, $k_{ex}$ refers to the water/proton exchange rate and $1/T_{1,2e}$ to the relaxation rate of the Gd(III) electron spin

Only $\tau_R$ appears easily modifiable. Slowing the rotation time by increasing the molecular weight of the complex or by chelating the metal ion with bulky ligands will result in an optimized rotational correlation time that could elicit relaxivities of up to 50 l/mmol$^{-1}$/s$^{-1}$.

2.1.2. Second and outer sphere proton relaxation:

Water molecules not directly coordinated to the metal ion also experience relaxation enhancement in the presence of the CA. These water molecules may be organized into a second- and outer-coordination sphere (Figure-4). Solomon-Bloembergen-Morgan theory may also be applied second-sphere water molecules. The deviating terms are noted with a prime, e.g., $q'$, $r'$ and $\tau_M'$, to differentiate them from the unvarying terms, $w_H$ and $S$. Outer-sphere relaxation enhancement may be modeled using theories developed by Hwang and Freeman [8,9]. $T_1^{OS}$ is determined by using Equations 7-10. Essentially $T_1^{OS}$ is determined by $T_{1e}$ (vide supra), $a$ – the minimum distance of approach between the metal complex and the outer-sphere water molecules, and $D$ – the sum of the diffusion constants of outer-sphere water molecules and the CA. The remaining terms are $N_A$ - Avogadro’s number and $M$ – concentration of CA.

$$\frac{1}{T_1^{OS}} = C[3j(w_H) + 7j(w_S)]$$ (7)
\[ C = \left( \frac{32\pi}{405} \right) \gamma^2 n g^2 \mu_B^2 (S + 1) \frac{NaM}{1000aD} \]  

(8)

\[ f(w_i) = \text{Re} \left[ \frac{1 + \frac{1}{4} z_i}{1 + z_i + \frac{4}{9} z_i^2 + \frac{1}{9} z_i^3} \right] \]

\[ i = H, s \]  

(9)

\[ z_j = \sqrt{iw_j \frac{a^2}{D} + \frac{a^2}{DT_{le}}} \]

\[ i = \sqrt{-1}, j = H, s \]  

(10)

Separating the inner-, second- and outer-sphere components of (T1) p is problematic. An approach often used to approximate T1 IS of a CA is to simply deduct the (T1)p of a second, related CA that has q = 0. In the case of the second CA, T1 IS is zero (because q = 0) and the observed relaxation enhancement is attributed solely to T1 SS and T1 OS. This provides a reasonable estimate of T1 SS and T1 OS for the first CA because these parameters are not expected differ significantly among structurally related Contrast agents.

\[ \text{Figure-4: Three coordination spheres, the inner sphere molecules are directly bound to the metal; the second sphere consists of water molecules coordinated to other parts of the CA while remaining water molecules comprise in the outer sphere} \]
Several factors necessary for enhancement of relaxation rate of contrast agent are briefly given below [2, 3].

- Increase in the hydration number of the metal ion (q).
- Shortening of the metal-water proton distance (r), r is usually 2.5Å for Gd(III).
- The rate of exchange of water bound to the paramagnetic metal ion with bulk water should be fast enough that bulk water experiences relaxation enhancement (τ_M).
- The water exchange rate is highly dependent on the ligands in the complex.
- Slow molecular rotation can increase the relaxivity of complex, called as rotational correlation time (τ_R).

2.2. Contrast agent

The chemistry and design of new contrast agents is presently a very active domain of research. The design of more powerful contrast agents requires not only the understanding of nuclear relaxation principles in the presence of unpaired electrons but also the exploration of the relationship between molecular structure and each of the factors which determine relaxivity. Certainly, contrast agent development is not possible without efficient synthetic methods. Two of the contributions deal with ligand synthesis, representing the two main streams (acyclic and macrocyclic ligands) in the chelation of Gd (III).

Amongst the acyclic ligands; first and most widely used chelators for the complexation of Gd(III) is Diethylenetriaminepentaacetate (DTPA) (Figure-5a). DTPA is a readily available octadentate ligand, accumulates nonspecifically and is excreted rapidly, constitutes the first generation of acyclic contrast agents. DTPA chelates are easily derivatized. Various complexes have been designed and evaluated using thermodynamic stability, rates of excretion, toxicity, lipophilicity, and biodistribution, percent change in MR signal intensity as criteria keeping Gd DTPA as the standard. The complexes are anionic, and therefore easily soluble in water. They are marked by unselective biodistribution over the extra cellular fluid and the resultant contrast is dependent on their tissue permeability versus clearance rate. Stability of the majority of macrocyclic Gd chelates is higher than that of acyclic ligands.
Macrocyclic ligands containing paramagnetic metals are particularly well suited as they are both thermodynamically stable and kinetically inert. Both these properties are crucial but kinetic inertness is essential and the chelates should not decompose while in the body of the patient. Gd (III), the paramagnetic metal which is being used in the proposed research is highly toxic when it is free. Chelate of DOTA or 1, 4, 7, 10-tetra (carboxymethyl-1, 4, 7, 10-tetraazacyclododecane, (Figure-6a), is currently used as an MRI contrast agent under the brand name Dotarem (Guerbet, France) [10]. DOTA became the parent molecule of an ever-growing family of macrocyclic lanthanide (III) chelating agents. This ligand forms lanthanide complexes that are exceedingly stable and inert at physiological pH and in blood serum. This stability and inertness has been ascribed to the macrocyclic structure of the ligands.

Most of the molecules being developed are from 1, 4, 7, 10-tetraazacyclododecane (cyclen), which is a tetraazamacrocycle with 12 atoms. Out of these four nitrogen atoms have their lone pair of electron pointing towards the metal ions and rest methylene groups are fully staggered. This arrangement hinders the entry of protons and slows down the dissociation of the chelates [10, 11]. (Figure -5b)

As reported in Silvio et al [13], gadolinium complexes containing ionisable ligands which are derived from 1, 4, 7, 10-tetraazacyclododecane with three acetate oxygens and one amide oxygen to ensure the stability of Gd complexes (Figure-6b). One or two ionisable groups (carboxylate or phosphonate) have been introduced on position fourth nitrogen (to the
10th position of the ring) to forms ion pairs with the polyaminoacids like polyornithine or polyargin which makes it sensitive to pH. In fact it has been reported [13-18] that DO3A [1,4,7 tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane] with three acetate groups form stable complexes with Gd/ Lanthanide and fourth amide oxygen is not needed to stabilize the Gd complex. Amide linkages are not very stable under physiological conditions and get digested by enzymes thus forming DOTA like complexes. All chelating agents derived from DO3A, play an important role to hold the metal ion strongly as well as to behave as responsive group in the presence of polyaminoacids/ pH/ calcium ions.

![Figure-6a](image1.png) ![Figure-6b](image2.png)

The design and preparation of a new generation of MR contrast agents that are – apart from being brighter, selectively targeted and remaining longer in vivo[19] – also capable of reporting on the physiological status and metabolic activity of cells or organisms have been prepared. These ‘Procontrast’ agents have been designed to exploit three fundamental physical properties of paramagnetic complexes that function as the switch or trigger to make them detectable by MRI. These properties are: first, \( q \), the number of water molecules coordinated to the paramagnetic ion; second, \( \tau_M \), the life time of a water molecule bound to the paramagnetic ion; and third, \( \tau_R \), the rotational correlation time of the complex. Varying \( q \), \( \tau_M \) or \( \tau_R \) can increase or decrease the observed signal intensity, and therefore manipulation of these parameters in the design of an agent produces what we call ‘activatable’ MR agents. Here briefly examples of these recently prepared agents are reviewed, organized by the mechanism (\( q \), \( \tau_M \) or \( \tau_R \)) and their identity as T1, T2 contrast agents [2, 3].
2.2.1. *pH-activated contrast agents*

pH is one of the most important factors for designing smart contrast agents. We all know that, the external medium is slightly basic but the opposite situation is observed in tumor tissues [20]. The intracellular pH, on the other hand is almost the same for both kinds of cells due to homeostatic mechanism. Therefore triggering MRI contrast agents by pH variation seems like a promising method for highlighting tumors. Several pH sensitive MRI contrast agents have already been developed.

Increased glycolytic activity may cause a significant pH decrease in the extracellular region of certain tumors relative to surrounding healthy tissue. Because pH is an important physiological indicator, many research groups have designed pH-sensitive contrast agents. **Mikawa and coworkers** [21] developed a MRI contrast agent that is responsive to pH (Figure-7a). The agent is based on a micro environmental responsive polyion complex in the form of a mixture of two polymers. The complex exhibits a fifty percent increase in relaxivity upon decreasing pH from 7.0 to 5.0. The mechanism of how the complex works is unknown; however, it is detectable in the presence of tumors in mice but is not in the absence of tumors.

![Figure-7a: Contrast agent sensitive to pH based on a polyion complex composed of a mixture of two polymers](image.png)
Aime and coworkers [22] have developed a pH-sensitive contrast agent with thirty gadolinium (III) chelates and 114 ornithine residues (Figure-7b). The chelates are conjugated to the amino acid chain via squaric esters, which readily react with amines. At low pH, the amines are protonated and do not interact with the squaric ester residues. When the pH rises, the amine side chains become deprotonated and interact with the squaric ester linkers. This interaction rigidifies the polymer creating an increase in $\tau_R$, which results in an increase in relaxivity. This agent is sensitive in the physiological range from pH 4.5 to 8.5.

Hovland and coworkers [23] have developed a pH-sensitive contrast agent which is a DO3A derivative with a tertiary amine-containing side arm (Figure-7c). The side arm amine contains two long alkyl chains. When the amine is protonated (pH 3-6) the relaxivity is low. Upon deprotonation (pH 8-10), the agents form colloidal aggregates due to the higher lipophilicity of the deprotonated complex. The aggregation causes an increase in $\tau_R$ and a subsequent increase in relaxivity of 142%.

Figure-7b: CA sensitive to pH based on a squaric ester containing polymer.

Figure-7c: CA sensitive to pH based on Ampiphilic molecules.
The Gd(III) complex of a DOTA tetramide derivative has been prepared by Zhang et al.[24] who observed an interesting behavior of the agent with change of pH. The relaxivity of the pH-sensitive contrast agent increases when pH increases from 4 to 6, but decreases between pH 6 to 8.5 and is constant between 8.5 to 10.5 and then increases again. According to analysis of these type of DOTA based contrast agents shows important parameters for designing pH sensitive contrast agents. The unusual pH dependence of the relaxivity could be accounted for by the presence of the ligand molecule of uncoordinated phosphonate groups. The proton of these ionisable groups between pH 9 and 6 can catalyze the exchange of protons between the bound water molecule and the bulk by providing an efficient hydrogen bond network. This network could be disrupted at lower pH by further protonation. (Figure-7d).

Aimes and coworkers (1996) have introduced hydrogen carbonate and pH sensitive contrast agent (Figure-7e). [25]. It has been a ternary complex between a Gd(III) chelate and carbonate ions. The coordination sphere of a Gd(III) complexed by a heptadentate ligand is incomplete. The relaxivity of this complex can therefore be affected by the saturation of its coordination sphere either by two water molecules or a bidentate ligand such as hydrogenocarbonate. This was readily observed by measuring the relaxivity of the complex as a function of pH in a saturated aqueous solution of sodium hydrogenocarbonate. The relaxivity of the complex changes from about 7.5mM⁻¹s⁻¹ at low pH to 1.9mM⁻¹s⁻¹ at high pH, reflecting the replacement of two water molecules in the first coordination sphere by a carbonate ion.
Finally, Aime and coworkers [26] have created a CEST agent which is responsive to changes in pH (Figure 7f). The agent is an ytterbium (III) complex of the tetruglycineamide derivative of DOTA. Saturation of the amide protons that are in close proximity to the paramagnetic center results in the best CEST agents to date. The observed effect is pH-dependent since $k_{ex}$ for amide protons is pH-modulated. By irradiating the amide N-H resonances, saturation transfer to the water resonance occurs (increasing with pH from pH 5.5 to 8.1) leading to a decrease in the water signal intensity which determines the contrast in MRI.

**Figure-7f: CA sensitive to pH based on CEST effects**
### 2.2.2. Metal ion concentration activated contrast agents

Several metals ions are essential or beneficial to life while others, such as Calcium, Magnesium, iron, copper etc. Its deficiency and excess may cause several deceases. Copper deficiency causes Anemia while excess copper can lead to willson’s diseases (Liver cirrhosis) In vivo determination of metal ion distribution is thus highly desirable and progresses have been towards the design of MRI contrast agents sensitive to the concentration of metal ions.

Calcium ion is probably one of the most important metal ions for life. Its roles are important both in healthy and damaged tissues. Intracellular calcium ion plays an important role in muscular contraction, neural transduction, and hormonal secretion. The development of selective reporter molecule has helped in the understanding of calcium multiple roles.

An important intracellular secondary messenger of signal transduction is calcium (II). Changes in the cytosolic concentration of calcium (II) trigger changes in cellular metabolism and are responsible for cell signaling and regulation. Li and coworkers [27, 31] have developed a contrast agent that can specifically detect calcium ions (Figure 8a). This agent is a gadolinium-based and possesses two limiting conformational states with regard to calcium(II) concentration. In the absence of calcium (II), the aromatic aminoacetates of the ligand interact with the two gadolinium (III) ions. In the presence of calcium (II), the aromatic iminoacetates rearrange to bind calcium (II) thereby allowing water to bind directly to gadolinium (III). This increase in $q$ yields an increase in relaxivity.
Iron is a key element in biology and in many enzymes. An iron-sensitive contrast agent was synthesized by Aime and coworkers [32] by functionalizing DTPA with salicylate moieties as shown in (Figure 8b). Upon addition of iron (III), the gadolinium (III) DTPA-salicylate complexes bind to the iron ions via the salicylate functional groups. This binding yields an increase in $T_1$ and relaxivity.
Figure-8b: Contrast agents sensitive to the presence of Iron Ions

On the above same approach, another iron-sensitive contrast agent was designed. [33] This time, DOTA was conjugated to the ligand 5, 6-dihydrophenanthroline. Self-assembly around iron (II) ions leads to an increase in $\tau_R$ and a subsequent increase in relaxivity from 5.1 to 12.5 mM$^{-1}$s$^{-1}$ (Figure 8c).

Figure-8c: Contrast agents sensitive to the presence of Iron Ions
2.2.3. **Enzyme-activated contrast agents**

The elaboration of enzyme responsive MRI contrast agents could provide a means of measuring enzyme activity and enzyme localization. The sensitivity of MRI contrast agents to specific enzymes depends on the mechanism of their interaction. Provided the interactions between a contrast agent and an enzyme are sufficiently strong, a large increase in relaxivity will be observed due to the increased rotational correlation time of the adduct [34].

The first enzyme activated macrocyclic MR contrast agents was reported by *Meade co-workers*, and was developed in response to the need to correlate developmental biological events with gene expression during an imaging experiment [35, 36]. The mechanism of the inner sphere $T_1$ relaxation phenomena ($q$) suggested a means to create a contrast agent with two distinct relaxation states, weak and strong (Figure-9a). By blocking the one remaining open coordination site, water protons are excluded from the inner sphere and the effect of the Gd$^{3+}$ ion on the $T_1$ of water is diminished. The agent ‘Egad’ was designed to be activated by the enzyme $\beta$-galactosidase. The enzyme substrate (sugar) occupies all nine coordination sites, inhibiting water access to the paramagnetic ion. The contrast agent irreversibly turned ‘on’ when $\beta$-galactosidase cleaves the sugar and water becomes accessible to the ion, thus modulating $q$. These agents have been successfully used *in vivo* to monitor gene expression in Xenopus laevis [35].
**Figure-9a:** MRI contrast agent activated by β-galactosidase.

Anelli and coworkers [37] have synthesized a DTPA derivative which can detect carbonic anhydrase (Figure-9b). The gadolinium complex contains a sulfonamide group in place of one of the carboxylic acid arms of the DTPA, helping it to selectively target the enzyme carbonic anhydrase. Upon binding to the enzyme, the relaxivity Increases significantly (approximately five fold at 40 MHz). This increase in relaxivity is due to an increase in \( \tau_R \) caused by binding to the large enzyme.

![Figure-9a](image)

**Figure-9b:** DTPA based carbonic anhydrase sensitive contrast agent

Nivorozhkin and coworkers [38] prepared an agent that is sensitive to the presence of human carboxypeptidase B (a thrombin-activatable fibrinolysis inhibitor (TAFI)), which
has been implicated in thrombotic disease (Figure 9c). TAFI cleaves a trilysine masking group attached to the agent exposing an aromatic functional group. This aromatic group has a high binding affinity for human serum albumin (HSA). The contrast agent binds HSA leading to an increase in $\tau_R$ resulting in an increase in relaxivity. This event is known as a receptor-induced magnetization enhancement (RIME). The trilysine chain makes this agent a pro-RIME agent because the trilysine chain inhibits interaction with HSA.

Figure-9c: Contrast agent sensitive to TAFI.

Bogdanov and coworkers [39] prepared a peroxidase activatable agent. This agent consists of a gadolinium (III) chelate linked to benzene-1, 2-diol that acts as a monomer.
In the presence of peroxide, the monomers are oligomerized yielding a threefold increase in relaxivity due to an increase in $\tau_R$. This MRI signal amplification (MRamp) can detect peroxidase concentration in vitro and has been used to detect E-selectin expression on human endothelial cells in cell culture by imaging the high local enzymatic activity of antibody-bound peroxidase associated with the plasma membrane of these cells.

**Perez and coworkers** [40] have utilized the difference in relaxivity between solitary CLIO particles and those in close proximity to other CLIO particles to detect DNA-cleaving agents (Figure 9d). Two strands of complementary DNA are each conjugated to a CLIO particle. When the complementary strands bind, the CLIO particles from each strand come into close proximity to each other. Upon cleavage of the double strand by a DNA-cleaving agent, the two CLIO particles become separated leading to a detectable change in relaxivity.

**Figure-9d: Contrast agent that detects DNA cleaving agents**

Utilizing a similar mechanism, **Zhao and coworkers** [41] have developed a protease sensitive MRI contrast agent (Figure 9e). With this agent, the strong interaction between biotin and avidin is exploited. A molecule of biotin is conjugated to each side of a peptide that is cleaved by proteases. CLIO particles coated with avidin are exposed to the biotinylated peptides. In the presence of protease specific for the peptide, the CLIO particles will not aggregate; however, in the absence of protease, aggregation of the CLIO particles...
will occur resulting in an increase in relaxivity. Currently this agent has only been used in vitro.

*Figure-9e: CLIO-based contrast agent that is sensitive to the presence of protease*
3. Aims

The aim was to design and synthesized two novel chelates from a new synthetic route, DO3A-EP and DO3A-EA-P5P, which have the potential to act as smart biochemical markers of physiological changes. DO3A-EP and DO3A-EA-P5P are similar in their way of synthesis path, but react differently in the different physiological environment. Our aims were

3.1. Synthesis of Cyclen (Intermediate molecule)

3.2. Synthesis of DO3A-EP (pH sensitive contrast agent)

3.3. Synthesis of DO3A-EA-P5P (Enzyme sensitive contrast agent)

The design and synthesis starts for the most of the macrocycle molecules are from cyclen, which is a 1, 4, 7, 10- tetraazacyclododecane. Cyclen is by far the most important building block for the preparation of macrocyclic complexing agents for use in MRI.

The first pH sensitive contrast agent, DO3A-EP, was synthesized in four steps. In each step we found good yield and each step was reproducible. It contains -tris-carboxymethyl and -phosphono-ethyl moieties on tetraazacyclododecane. Phosphonate derivatives are known to have very high affinity towards calcium and can function as reporters of pH or calcium ions. After loading with paramagnetic metal ion such as Gd(III), this Gd-DO3A-EP therefore can be used as an extracellular contrast agents reacting to pH/calcium-related changes on cellular activity and neurotransmission.

The second contrast agent DO3A-EA-P5P is enzyme sensitive, and contains pyridoxal 5-phosphate which can be cleaved at imine/phosphate ester by GABA-aminotransferase or PLP enzymes [35]. After cleavage, the water molecule can enter the inner coordination sphere, which enhances MR relaxivity. In addition, Gd-DO3A-EA-P5P can report on changes in pH.
4. Methods

4.1. General Procedures:

All the experiments were performed under nitrogen. TLC was run on plastic backed silica gel plates with 0.2 mm thick silica gel 60 F254 (E. Merck, Germany) using the MeOH/DCM (1:9) and 10 w/v aqueous ammonium acetate/CH₃OH (1:1 v/v) mobile phase. TLCs are scanned in 254nm UV lamp, I2 Chamber and Ninhydrin solution. Analytical HPLC was used on a system equipped with titanium piston washing pump heads (Beckman, 32 Karat software). Solvents were mixed using a Dynamax dual-chamber dynamic mixer (Titanium). UV absorbance was measured using an absorbance/fluorescence monitor (ISCO model UA-5) at 254 or 354 nm. Reversed-phase HPLC was performed at room temperature with a Dynamax 21.4x50 nm e18 column, generally using CH₃OH or CH₃CN gradients and 0.1M ammonium acetate (pH 6) or 0.1% TFA with a flow rate of 1mL/min. All the solvents for HPLC and reaction mixtures were filtered through a nylon 66 Millipore filter (0.45µm) prior to use.

4.2. Analytical Characterization:

NMR data were recorded on Bruker 250 MHz or 400 MHz systems (Department of Organic Chemistry, University of Tübingen) using standard ¹H, ¹³C and ³¹P protocols. Mass spectra (MS or further fragmentations) were obtained in-house from an ion trap SL 1100 system (Agilent, Waldbronn, Germany) using electrospray ESI-MSⁿ in positive and negative ion mode.

4.3. Materials Used:

Reagents used in different synthetic schemes were diethanolamine, diethyltriamine, Toluene-4-sulphonyl chloride, sulphuric acid, cesium carbonate, tert-butylbromoacetate, ethyl-2-bromoacetate, diethyl-2-bromoethyl phosphonate, pyridoxal 5’ phosphate monohydrate, 2-bromoethylamine hydrochloride, triethylamine, lithium hydroxide, potassium carbonate, sodium hydroxide, trifluoroacetic acid, 38% HBr/HCOOH, gadolinium trichloride hexahydrate, di-tert-butyldicarbonate, and other solvents (chloroform, dichloromethane, methanol, ethanol, diethyl ether, hexane) were used as received from Sigma Aldrich, Germany.
5. Synthesis

5.1. Synthesis of Cyclen (Intermediate Molecule) (Figure-10)

Cyclen (1, 4, 7, 10-teraazacyclododecane) was synthesized by the reactions of toluene-4-sulphonyl chloride with diethyltriamine and diethanolamine to get tritossylated diethyltriamine and tritossylated diethanolamine. Both were put together in DMF and cesium carbonate at 120 °C for 20-24h to get tetratossylated cyclen. Tetratossylated cyclen was appended in sulphuric acid at 120-130 °C for 72h to get protonated cyclen.

![Diagram of Cyclen synthesis](Figure-10)
**Synthesis of 1, 4, 7-N-tris (toluene-p-sulfonyl) diethyltriamine 1**

Add 1 equivalent of diethyltriamine (20g) in 200mL of water and aqueous solution of 3 equivalent of sodium hydroxide (23.76g in 100mL) at 10 °C in to round bottom flask with vigorous stirring. Then added p-toluene sulfonylchloride (113.16g) in 500mL diethylether. After completion of addition stirring was continued for an additional 14-16h. Obtained gummy product was separated from the mother solution and washed with water, ether and finally ethanol. All the solvents were carefully decanted from the product and treated with methanol was evaporated and dried under reduced pressure for 3-4h. Finally white solid powder formed and the yield was 74% (66.8g).

**ESI-MS (+):** calculated for C\textsubscript{25}H\textsubscript{31}N\textsubscript{3}O\textsubscript{6}S\textsubscript{3}: m/z 565.0; found: 566.9[M+1] \textsuperscript{+}, 587.9 [M+Na], 603.9 [M+K]. **ESI-MS-MS of 566.9 (supporting data):** 395.2 [M-168] \textsuperscript{+}, 227.1 [M-169\times2]. **\textsuperscript{13}C NMR (DMSO, 62.9MHz) \(\delta\) (ppm):** 143.7, 143.1, 137.7, 135.6, 130.2, 130.0, 129.93, 127.2, 126.9, 48.7, 41.9, 40.4-39.1, 21.3. **\textsuperscript{1}H NMR (DMSO), 250MHz) \(\delta\) (ppm):** 7.8-7.4 (12H of phenyl), 4.2-4.1 (2H of NH), 3.4-2.8 (8H of CH\textsubscript{2}), 2.4 (9H of CH\textsubscript{3}).

**Synthesis of N, O, O'-tris (toluene-p-sulfonyl) bis (2-hydroxy-ethyl) amine 2**

Add 1 equivalent of diethanolamine (20g) in 3 equivalent of triethylamine (19.23g, density-0.723 = 26.60mL) at 10 °C in to round bottom flask with vigorous stirring. Then added p-toluene sulfonylchloride (108.86g) in 500mL diethylether. After completion of addition stirring was continued for a 1h. During addition white precipitate appeared which was allowed to stir overnight at room temperature (21 °C). Added excess of water to dissolve the inorganic materials, filtered, dried and recrystallized in minimum quantity of ethanol. Filtered, washed and dried under reduced pressure for 3 h. Finally white solid powder formed and the yield was 62g (56%).

**ESI-MS (+):** calculated for C\textsubscript{25}H\textsubscript{29}NO\textsubscript{8}S\textsubscript{3}: m/z 567.0; found: 567.9 [M+1] \textsuperscript{+}, 589.9 [M+Na], 605.9 [M+K]; **ESI-MS-MS of 567.9 (supporting data):** 396.2 [M-171]. **\textsuperscript{13}C NMR (DMSO, 62.9 MHz) \(\delta\) (ppm):** 145.5, 144.1, 137.7, 135.6, 130.5, 130.2, 128.4, 27.3, 125.8, 68.5, 47.7, 45.9, 40.5-39.1, 21.3. **\textsuperscript{1}H NMR (DMSO), 250MHz) \(\delta\) (ppm):** 7.8-7.4 (12H of phenyl), 4.1-3.3 (8H of CH\textsubscript{2}), 2.4-2.4 (9H of CH\textsubscript{3}).

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Synthesis of 1, 4, 7, 10-tetraazacyclododecane 3

Took 1 equivalent of compound 1 (50g) in 300mL DMF and stirred at 120-125°C, at this temperature added 2 equivalent of K₂CO₃ (24.34g) and allowed stirring for 1 h. Followed by the addition of 1 equivalent of compound 2 (49.55g in 200mL DMF) with in 1 h. After complete addition reaction mixture (RM) were allowed to stir for an additional 24 h. Cooled the RM and filtered to remove the inorganic salts, reduced the volume solution to 300mL of the original volume, cooled at ice temperature and added 2 L of water slowly in vigorous stirring. Obtained yellow precipitate, filtered, washed with water and dried under reduced pressure at 30 °C for 6-7 h. Finally yellow solid powder obtained and the yield was 86% (60g).

ESI-MS (+): calculated for C₃₆H₄₄N₄O₈S₄: m/z 788.0; found: 789.0 [M+1]⁺, 811.0 [M+Na]. ¹³C NMR (CDCl₃, 62.9 MHz) δ (ppm): 144.3, 130.2, 130.1, 128.1, 127.5, 68.5, 47.7, 45.8, 40.4-39.1, 21.9. ¹H NMR (CDCl₃, 250MHz) δ (ppm): 7.7-7.3 (12H of phenyl), 3.4 (16H of CH₂), 2.4(9H of CH₃).

Synthesis of 1, 4, 7, 10-tetraazacyclododecane (Cyclen) 4.

Tetratossylated cyclen 3 (60g) treated in 96% sulfuric acid at 110-120°C for 72h. The resulting solutions cooled at 0-5 °C and add dropwise 1:1, ethanol and diethylether (1L). During addition exothermic reaction occur, so the reaction mixture should be cooled at 0-5 °C. After complete addition brown precipitate formed, which was filtered, washed with ethanol and ether simultaneously and dried under reduced pressure for 4-5 h, dissolved in 150 mL of water and extracted several times with chloroform by pH 12. Organic phase evaporated to give cyclen. Whit solid product obtained and the yield was 43% (6.4 g).

ESI-MS (+): calculated for C₈H₂₀N₄: m/z 172.3; found: 173.2 [M+H]⁺; ¹³C NMR (D₂O, 62.9MHz) δ (ppm): 46.5; ¹H NMR (D₂O, 250MHz) δ (ppm): (S, 2.5)
5.2. Synthesis of DO3A-EP (pH sensitive contrast agent) (Figure-11)

DO3A-EP was synthesized from 1,4,7,10-tetraazacyclododecane (5 equivalent cyclen) by the reaction of diethyl-2-bromoethyl phosphonate to get mono-substituted cyclen. It was appended by reaction with ethyl-2-bromoacetate. The corresponding carboxylate derivative was obtained by cleaving the ethyl group with 48% HBr / acetic acid and heating at 80°C. All the derivatives were characterized by spectral analysis (1H, 13C, 31P NMR and ESI-MS), see (Figure-11).
**Synthesis of 1-[N-2(diethyl-phosphano-ethyl)]-4, 7, 10-tetraazacyclododecane 2**

1 equivalent of diethyl-2-bromoethyl phosphonate (0.427g) were added drop wise (in ethanol= 20 mL) to a solution (20 mL ethanol and 5 mL water) of 5 equivalent tetraazacyclododecane (1.5g) and 1 equivalent of LiOH(0.042g) at 4°C. After complete addition the solution was stirred at 80°C for 6-7 h. This produced a mixture of the two products (cyclen and monosubstituted cyclen). The free protonated cyclen was then removed by chloroform in basic condition, cyclen extract by chloroform and the resulting monosubstituted product was dissolved in water. Water was evaporated under reduced pressure and purify by classical chromatography and yielding 40%, 0.783g of mono-N-substituted tetraazacyclododecane 2, in this step cyclen can be recycle.

**ESI-MS (+):** calculated C_{14}H_{33}N_{4}O_{3}P: m/z 336.2; found 337.3 [M+H]^+. **ESI-MS-MS** of 337.3 (Supporting data): 286.0, 253.0. $^{13}$C NMR (D$_2$O, 62.9MHz) δ (ppm): 63.9, 63.6, 63.5, 47.7, 46.8, 46.2, 45.2, 44.8, 42.4, 41.6, 37.2, 36.4, 35.8, 15.9, 15.8; $^{1}$H NMR (D$_2$O, 250MHz) δ (ppm): 4.1-4.0 (4H of 2 –OCH$_2$-), 3.2-2.7 (16H, -CH$_2$ of cyclen), 2.1-2.0 (2H of –NCH$_2$), 1.8 (2H of –CH$_2$-P-), 1.2-1.2 (6H of –CH$_3$). $^{31}$P NMR (D$_2$O) δ (ppm): 34.9.

**Synthesis of 1,4,7-tris(carboethoxymethyl)10-[N-2(diethyl-phosphano-ethyl)]-1,4,7,10-tetraazacyclododecane 3:**

6 equivalents of 2-bromo ethylacetate in (10 mL) acetonitrile(ACN) were added to a solution of 2 (0.780g) using 10 equivalents of powdered anhydrous Na$_2$CO$_3$ (2.45g) in ACN. The reaction mixture was stirred at 90-100°C for 20-24 h and the reaction was monitored by TLC (MeOH: DCM:: 1:9) and ESI-MS. After completion, the reaction mixture was brought to room temperature, filtered by G-4 sintered funnel, and evaporated under reduced pressure. The product was dissolved in chloroform and washed several times with 100mL of water and the organic phase was dried over anhydrous Na$_2$SO$_4$, filtered and evaporated to dryness, got 1.129g, yielding 82% of 3.
ESI-MS (+): calculated C\textsubscript{26}H\textsubscript{51}N\textsubscript{4}O\textsubscript{9}P: m/z 594.3; found 595.2 [M+H]\+ 617.3 [M+Na]. ESI-MS-MS of 617.3 (Supporting data): 515.2, 487.1, 451.3. \(^{13}\)C NMR (CDCl\textsubscript{3}), 62.9MHz) δ (ppm) 175.0, 169.0, 61.4, 55.9, 53.9, 51.6, 50.6, 49.8, 49.3, 45.7, 44.0, 16.8, 14.5. \(^1\)H NMR (CDCl\textsubscript{3}), 250MHz) δ (ppm): 4.2-3.9 (10H of –OCH\textsubscript{2}–), 3.3-2.9 (16H, -CH\textsubscript{2} of cyclen), 2.2-2.1 (8H of –NCH\textsubscript{2}), 1.9 (2H of –CH\textsubscript{2}-P-), 1.4-1.2 (15H of –CH\textsubscript{3}).

\textit{Synthesis of 1,4,7-tris(carboxymethyl)10-[N-2(phosphono-ethyl)]-1,4,7,10-tetraazacyclododecane (DO\textsubscript{3}A-EP): 4}

The ethyl groups of compound 4 were de-protected by treatment with 48% HBr in acetic acid at 80°C for 20-24 h. Reaction was monitored by ESI-MS. After completion of reaction, reaction mixture was concentrated under reduced pressure at 50°C for 3-4 h. Compound was purified by reversed phase HPLC and got 0.721g of compound yield was 93%.

ESI-MS (+): calculated for C\textsubscript{16}H\textsubscript{31}N\textsubscript{4}O\textsubscript{9}P: m/z 454.2; found 455.0 [M+H]\+ 477.1 [M+Na]. ESI-MS-MS of 454.2 (Supporting data): 437.0, 407.0, 397.1, 373.0, 354.0, 336.0, 286.0, 253.0, 234.0. \(^{13}\)C NMR (MeOD, 62.9MHz) δ (ppm) 175.0, 168.9, 56.0, 53.9, 51.6, 50.6, 49.8, 49.3, 45.7, 44.0. \(^1\)H NMR (D\textsubscript{2}O), 250MHz, δ (ppm): 4.2-3.4 (16H, -CH\textsubscript{2} of cyclen), 3.2-2.8 (8H of –NCH\textsubscript{2}), 2.2-2.1 (2H of –CH\textsubscript{2}-P-). \(^{31}\)P NMR (D\textsubscript{2}O) δ(ppm) 18.4.
5.3. Synthesis of DO3A-EP-P5P (Figure-12)

DO3A-EP-P5P was synthesized from 1,4,7,10 tetraazacyclododecane (cyclen) by the reaction of N-BOC protected 2-bromoethylamine to get the mono-substituted product. It was further reacted with tert-butylbromoacetate to get 1,4,7-(tris(carboxymethyl)-10-(BOC-aminoethyl)-1,4,7,10 tetraazacyclododecane. The corresponding carboxylate derivative [DO3A-EA, 1-(2-Aminoethyl)-4, 7, 10-tris (carboxymethyl)-cyclen] was obtained by cleaving the tert-butyl groups by the treatment of TFA at room temperature. After getting this precursor (DO3A-EA), it will be further reacted with pyridoxal 5’-phosphate monohydrate [31] to obtain DO3A-EP-P5P. Initial analytical data are given in (Figure-12).

![Chemical structure of DO3A-EP-P5P](image-url)
Synthesis of N-Boc-2-aminoethyl bromide 1.

1N solution of sodium hydroxide (80 mL) was added dropwise over 15 minutes to a vigorously stirring biphasic system of 2-bromoethylaminehydrobromide (9.02 g, 1 equivalent) in water (50 mL) and di-t-butyl-dicarbonate (4.75 g, 1.2 equivalents) in dichloromethane (50 mL). After 2 hours, the layers were separated and the organic layer was washed with 1N HCl (25 mL) twice and a saturated sodium chloride (25 mL) once. The organic layer was then dried over sodium sulfate and concentrated in vacuum to colorless oil. The oil was dissolved in hexanes (5mL) and placed at −80°C overnight. 1.1 g of a white solid that crystallized was filtered and washed with cold hexanes. The combined filtrates were again placed at −80°C for 1 h to precipitate a second batch of crystals weighing 2.4 g. Total yield, 70% yield (3.5 g).

ESI-MS (+): calculated for C7H14NO2Br: m/z 223.4; found 245.9, 247.9 (M+Na).
ESI-MS-MS of 247.9 (Supporting data): 189.8, 191.8. 13C NMR (CDCl3, 62.9MHz) δ (ppm): 156.0, 80.0, 42.7, 40.9, 32.9, 28.7. 1H NMR (CDCl3), 250MHz, δ (ppm): 5.1 (1H of NH), 3.5-3.4 (4H of –CH2), 1.4 (9H of –CH3)

Synthesis of (N-Boc-2-aminoethyl) cyclen 2.

1 equivalent of compound 1 were added dropwise (in ethanol= 20 ml) to a solution (10 ml ethanol and 10 ml water) of 5 equivalent tetraazacyclododecane and 1 equivalent of LiOH. After complete addition the solution was stirred at 80°C for 6-7 h. This produced a mixture of the two products (cyclen and monosubstituted cyclen). The free protonated cyclen was then removed by chloroform in basic condition, cyclen extract by chloroform and the resulting monosubstituted product was dissolved in water. Water was removed evaporated under reduced pressure and purify classical chromatography and yielding 40% of mono-N-substituted tetraazacyclododecane 2, which was used for the next step. In this step cyclen was recycled.

ESI-MS (+): calculated C15H33N5O2: m/z 315.26; found 316.2 [M+H] +, 322.2 [M+Li]. ESI-MS-MS of 316.2 (Supporting data): 216. 13C NMR (D2O, 62.9MHz) δ
(ppm): 158.2, 82.7, 62.5, 60.1, 52.7, 49.3, 48.5, 44.7, 42.5, 42.0, 37.2, 28.1. $^1$H NMR (D$_2$O), 250MHz, $\delta$ (ppm): 3.2 (2H of –CH$_2$-N), 3.1-2.6 (18H of cyclen and –N–CH$_2$), 1.3 (9H of –CH$_3$).

**Synthesis of (N-Boc-2-aminoethyl)-4, 7, 10-tri(t-butoxycarbonylmethyl)-cyclen 3.**

A solution of 2 (1g, 1equivalent), sodium carbonate (2.6 g, 10 equivalent) and t–butyl bromoacetate (2.4 mL, 5equivalent) in acetonitrile (100 mL) was stirred at 70°C for 20-24 h. Reaction was monitored by TLC and ESI-MS, after completion of reaction, the reaction mixture was filtered and washed with DCM (20 ml, 2x). The filtrate was then concentrated in vacuum to obtain pale yellow oil. Compound was purified by classical chromatography using stationary phase as silica gel 60 F$_{254}$ and mobile phase DCM: EtOH. The white solid obtained after complete evaporation. Total yield, 1.79 g (85% yield).

ESI-MS (+): calculated C$_{33}$H$_{63}$N$_5$O$_8$: m/z 657.5; found 658.5 $[\text{M+H}]^+$, 680.4 $[\text{M+Na}]^+$. ESI-MS-MS of 658.5 (Supporting data): 602.4, 546.4, 490.4, 446.4, 390.3. $^{13}$C NMR (CDCl$_3$, 62.9 MHz) $\delta$ (ppm) 172.9, 170.4, 166.6, 61.2, 56.1 55.0, 53.0, 52.4, 49.9, 49.0, 45.8, 28.4. $^1$H NMR (CDCl$_3$), 250MHz, $\delta$ (ppm): 5.5 (1H of –NH-), 3.4-2.2 (16H of cyclen, 8H of –N–CH$_2$-, 2H of –CH$_2$-N), 1.6-1.4 (36H of –CH$_3$).

**Synthesis of 1-aminoethyl-4, 7, 10-tri(carboxymethyl)-cyclen (DO3A-EA) 4.**

A solution of 3 (1.6 g, 1 equivalent) in trifluoroacetic acid (50 mL) was stirred at room temperature for 20-24h. Reaction was monitored by ESI-MS. The remained solution was added dropwise to cold diethylether. The pale orange solid that formed immediately was filtered, and then re-suspended in a minimal amount of water (~3 mL). The final product was triturated and filtered off by the addition of a large excess of acetone (~100 mL). Total yield was 0.630 g (67.5% yield).

ESI-MS (+): calculated C$_{16}$H$_{31}$N$_5$O$_6$: m/z 389.23; found 390.2 $[\text{M+H}]^+$, 412.2
[M+Na]. ESI-MS-MS of 390.2 (Supporting data): 373,359. $^{13}$C NMR (D$_2$O, 62.9MHz), $\delta$ (ppm) 176.2, 171.5, 168.1, 59.6, 55.0, 53.0, 52.4, 49.9, 48.9, 45.8. $^1$H NMR (D$_2$O), 250MHz, $\delta$ (ppm): 3.8-2.6 (16H of cyclen, 8H of –N-CH$_2$-, 2H of –CH$_2$-N),

Synthesis of 1,4,7 tris(carboxymethyl)-10-{2-[(3-hydroxy-2-methyl-5-phosphonoxyrnylpyridin-4-ylmethylene)-amino]-ethyl}-1,4,7,10-tetraazacyclododecane (DO3A-EA-P5P) 5

To a solution of pyridoxal 5-phosphate (0.100g) and dried 3Å molecular sieves (0.100g w/w) a methanolic solution of 1-aminoethyl-4, 7, 10-tri(carboxymethyl)-cyclen 4 (0.147g in 5mL) was added slowly at 4°C. The reaction mixture was stirred at ambient temperature (25°C) for 4-6 h and the reaction was monitored by mass spectrometry.

Note: - No product was formed in this duration and hence the reaction was continued up to 24h. Even then the product was not obtained and so the reaction was tried by four different methods as given below:

- With reference to Raymond's [42], the reaction was put taking 1 equivalent of pyridoxal 5-phosphate, 1 equivalent of DO3A-EA and 2 equivalent of NaOH in Methanol and water solution [2:1] and stir at RT for 24 h. Reaction was monitored by TLC and ESI-MS. They indicated only the presence of starting material. Thus the reaction was repeated trying different concentration of NaOH increasing from 2-5 equivalents.

- Took 1 equivalent of pyridoxal 5-phosphate, 1 equivalent of DO3A-EA and 1 equivalent of K$_2$CO$_3$ in ethanol and stir at RT for 24 h. Reaction was monitored by TLC and ESI-MS. The reaction did not proceed by this method.

- Took 1 equivalent of pyridoxal 5-phosphate, 1 equivalent of DO3A-EA and 1 equivalent Na-Acetate buffer in ethanol and stir at RT for 24 h. Reaction was monitored by TLC and ESI-MS. No indication of product formation was observed.

- Took 1 equivalent of pyridoxal 5-phosphate, 1 equivalent of DO3A-EA in ethanol and refluxed for 24 h. Reaction was monitored by TLC and ESI-MS. The reaction did not proceed.
- Took 1 equivalent of pyridoxal 5-phosphate, 1 equivalent of DO3A-EA and 4-6 equivalents anhydrous sodium sulphate in dry THF and stir at RT for 24 h. Reaction was monitored by TLC and ESI-MS. No product formation.

The overall conclusion of these reactions was that possibly the presence of three carboxylate groups created a problem in imine formation or leads to instability of the final product. Thus the synthetic scheme for DO3A-EA-P5P was modified as follows.
Synthesis of 1-Aminoethyl-4, 7, 10-tri (methoxycarbonylmethyl) cyclen (MP-DO3A-EA) \(5'\)

Took 1 equivalent of pyridoxal 5-phosphate, 1 equivalent of DO3A-EA in methanol and passed HCl gas for 3h and put it on stirring for overnight. Reaction was monitored by TLC and ESI-MS. Methyl-protected DO3A-EA was formed. This product was used further without purification.

**ESI-MS** (+): calculated C\(_{19}\)H\(_{37}\)N\(_5\)O\(_6\): m/z 431.2; found 432.4 \([\text{M+H}]^+\), 454.2 \([\text{M+Na}]\). ESI-MS-MS of 432.4: 415, 382.4, 300.3, 231.3, 185.3.

Synthesis of 1,4,7 tri(methoxycarbonylmethyl)-10-{2-[(3-hydroxy-2-methyl-5-phosphonooxymethyl-pyridin-4-ylmethylene) -amino]-ethyl}-1,4,7,10-tetraazacyclododecene (MP-DO3A-EA-P5P) \(6\)

Took 1 equivalent of pyridoxal 5-phosphate, 1 equivalent of DO3A-EA and 2 equivalent of NaOH in Methanol and water solution [2:1] and stir at RT for 3-4 h. Reaction was monitored by TLC and ESI-MS. Product was formed and concentrated under reduced pressure.

**ESI-MS** (+): calculated C\(_{27}\)H\(_{45}\)N\(_6\)O\(_{11}\)P: m/z 660.2; found 661.4 \([\text{M+H}]^+\), 683.4 \([\text{M+Na}]\), 705.4 \([\text{M-1+2Na}]\). ESI-MS-MS of 705.4: 603.4, 585.4, 454.4; ESI-MS-MS of 683.4: 603.4, 585.4, 454.4.

**Note:** - Deprotection of methyl groups of MP-DO3A-EA-P5P for the formation of final compound DO3A-EA-P5P is under investigation.
5.4. Complexation

The gadolinium complexes of DO3A-EP and DO3A-EA-P5P were prepared by solutions of 1 equivalent of both compound and solution of GdCl$_3$.6H$_2$O 1.1 equivalent was stirred at 80° C for 20-24h. The pH was periodically checked and adjusted to 6.0~7.0 using a solution of sodium hydroxide (1M) as needed. After 20-24 h, sodium hydroxide was added to bring the pH to ~12 and reaction was syringed through a nylon 0.2 µM filter. The filtrate was then lyophilized to a white powder that was re-suspended in a minimal amount of methanol (~1 mL). The final product was triturated from this solution and filtered off by the addition of a large excess of acetone (~40 mL). This solution was purified over an ion-exchange resin (Bio-Rad Chelex), dialyzed (100 MWCO) and lyophilized to an off-white powder.

Free Gd(III) was identified by a Xylenol test. In this test small quantity of the complex was taken in 1mL solution of 0.1N sodium acetate and 0.1N acetic acid (2:1). First 20µL pyridine and finally 60µL of Xylenol was added. Appearance of purple color indicated the presence of free Gd(III) while yellow color showed no free Gd(III) existed.

*Figure 13: Structures of complexation of DO3A-EP-Gd, DO3A-EA-P5P-Gd*
6. Summary

Two novel gadolinium-based magnetic resonance (MR) contrast agents, DO3A-EP and DO3A-EA-P5P for molecular imaging were designed and synthesized. These agents are based on the tetraazacyclododecane (cyclen) ring. The macrocycle contains three carboxalate groups, which are essential to form the stable complex with Gd. The fourth nitrogen was appended with a responsive group, which participated in the coordination with a lower affinity than carboxylate.

DO3A-EP was synthesized by a novel synthetic scheme that are standardized and reproducible. DO3A-EP was synthesized in 3 steps and gave a good yield at each step.

The final step of synthesis of the other enzyme based contrast agent DO3A-EA-P5P is under investigation.

Both of these compounds offer wide range of chemical and biological applications for MRI contrast agents. These contrast agents will enhance relaxivity of healthy or damaged tissues, and has the potential to be an invaluable tool for molecular imaging of experimental animals. With improved targeted delivery, these agents could become more sensitive, accurate, and ultimately simplified.

Such activatable agents that respond to biological phenomena by altering the intensity of signal enhancement in a conditional fashion are steps toward unraveling the complex connectivity of biological systems. Further, these agents may represent the prelude to complete, noninvasive, medical examinations that are safe, fast, and accurate.
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Figure 13: Structures of complexation of DO3A-EP-Gd, DO3A-EA-P5P-Gd
8. References


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