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**Development of Novel Oligomer-based Intravascular
Contrast Agents for MRI using Gadolinium Chelates**

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**A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy**

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Krisada Kittigowittana

ABSTRACT

Contrast agents with high relaxivity are needed for increasing the sensitivity of magnetic resonance imaging (MRI) for novel clinical and research applications. Therefore, we are developing a new class of oligomer-based contrast agents by incorporating multiple-gadolinium complexes into peptides of controlled length and structure. This allows synthesis of a library of Gd-peptides with different sequences, peptide length and number of Gd-chelates. Screening this library will enable selection of oligomers with desired properties.

Initially, we have synthesized and characterized two MRI contrast agents (CAs) which are Gd-DO3A conjugated amino acid building blocks derived from glutamate (**CA1**) and lysine (**CA2**). Their longitudinal relaxivities (r_1) measured at 9.4 T, are 6.4 and 5.4 mM⁻¹s⁻¹ in H₂O at 25 °C for **CA1** and **CA2**, respectively. Both are higher than the clinically used MRI contrast agents, Gd-DTPA (Magnevist, Bayer Schering, Germany) and Gd-DOTA (Dotarem, Guerbet, France). *In vivo* imaging in Wistar rats demonstrated considerable signal enhancement (~50%) in the brain artery for **CA2** while lower signal enhancement (~30%) for **CA1**. Comparison with the signal enhancement of Dotarem showed similar signal enhancement to **CA2**, but the enhancement of **CA2** was still high (~30%) even at 52 mins after injection. It was demonstrated that **CA2** has much longer blood half-life (68.1 mins) than the Dotarem and could be potentially advantageous for angiography and tissue targeting.

Our attempts to develop the GdDO3A-dipeptide are unsuccessful in the conjugation of the GdDO3A scaffold to the dipeptides using the standard coupling reagents. Hence, we turned to “Click chemistry”, the Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction, for connecting the cyclen core to the peptides. With the click

chemistry, the GdDO3A-lysine triazole (CA3) has been synthesized and characterized. The longitudinal relaxivity of CA3 is $22.8 \text{ mM}^{-1}\text{s}^{-1}$ in H_2O at 25°C which is much more higher than those of CA1 and CA2. Optimization of the click reaction conditions for GdDO3A-peptides synthesis is in progress.

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LIST OF ABBREVIATIONS

Ac	acetyl
Ar	aryl
app	appeared
Bn	benzyl
br	broad
conc or c	concentration
d	doublet
dd	doublet of doublets
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	dicyclohexylcarbodiimide
dt	doublet of triplets
DIPEA	<i>N,N</i> -diisopropylethylamine
DMAP	<i>N,N</i> -dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DO3A	1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DTPA	Diethylenetriamine pentaacetic acid
ESI	electron ionization
equiv	equivalent
Et	ethyl
h	hour (s)
HOBt	<i>N</i> -hydroxybenzotriazole

LIST OF ABBREVIATIONS (Cont.)

HRMS	high resolution mass spectrometry
IBC	isobutyl chloroformate
IR	infrared spectrum
J	coupling constant
Lit	literature
m	multiplet
min	minute (s)
m/z	a value of mass divided by charge
M ⁺	molecular ion
mp	melting point
Me	methyl
MS	mass spectrum
NMR	nuclear magnetic resonance
NMM	<i>N</i> -methylmorpholine
ppm	part per million
Ph	phenyl
q	quartet
ref.	reference
rt	room temperature
s	singlet
t	triplet
T	temperature

LIST OF ABBREVIATIONS (Cont.)

TFA	trifluoroacetic acid
THF	tetrahydrofuran
TMS	tetramethylsilane
TLC	thin layer chromatography
TOF	time of flight
WSC	Water Soluble Carbodiimide or 1-(3-Dimethylaminopropyl)- 3-ethylcarbodiimide HCl
Z	carbobenzyloxy
δ	chemical shift
$\nu_{\max}$	maximum absorption frequencies

CHAPTER I

INTRODUCTION

Molecular imaging is one of the most interesting and rapidly growing areas in science. Biological processes can be studied in living animals, model systems and humans at the cellular and molecular level using remote imaging detectors. Over the last decade, Magnetic Resonance Imaging (MRI) has evolved into one of the most powerful techniques in diagnostic clinical medicine and biomedical research. The use of MRI eliminates the need for invasive diagnostic procedures and harmful ionizing radiation such as that used in X-ray and CT scanning and, furthermore, it has been shown to provide high quality three-dimensional images for physiological information earlier in clinical investigation. MRI is primarily used to produce anatomical images, it also gives information on the physico-chemical state of tissues, flow diffusion and motion.¹ MRI relies on detecting the NMR signals from hydrogen atoms in water molecules (which make up about 60% of the human body). Signal intensity in MRI is related to the relaxation rate of *in vivo* water protons and can be enhanced by the administration of a contrast agent prior to scanning. These agents utilize paramagnetic metal ions and are evaluated on the basis of their ability to increase the relaxation rate of nearby water proton spins in dependence on the concentration of agent administered.

Nowadays, around 30% of all MRI investigations use a contrast medium, the number of which will certainly further increase with the development of new agents and applications. The most commonly used contrast agents are thermodynamic and kinetically stable low molecular weight gadolinium compounds. Gadolinium(III) has a

large number of unpaired electrons ($S = 7/2$) with its high magnetic moment and moreover its magnetic properties are isotropic. It has a relatively long electron spin relaxation time, $\sim 10^{-9}$ s, which makes it more suitable than other paramagnetic ions such as Dy^{3+} , Eu^{3+} , and Yb^{3+} ($\sim 10^{-13}$ s). These factors are very favorable for nuclear spin relaxation. Gd^{3+} is therefore an ideal candidate for such a proton relaxation agent and is the most widely used metal center for such purposes.^{2,3} Free Gd(III) is very toxic ($\text{LD}_{50} = 0.2 \text{ mmol kg}^{-1}$ in mice)⁴ and has to therefore be administered in the form of stable chelate complexes that will prevent the release of the metal ion *in vivo*. For these reasons, the development of ligands that are suitable for production of high-relaxivity agents with favorable properties for imaging applications remains an important goal.

1. What is MRI

Magnetic Resonance Imaging (MRI) is a non-invasive procedure relying on the magnetic fields of protons within the body, producing three-dimensional views of internal organs or tissues. MRI is based on the principles of nuclear magnetic resonance (NMR), a spectroscopic technique used by scientists to obtain chemical and physical information about molecules. The technique was called magnetic resonance imaging rather than nuclear magnetic resonance imaging (NMRI) because of the negative connotations associated with the word “nuclear” in the late 1970’s.

Biological tissues consist predominantly of water, which contain protons (hydrogen nuclei). The total proton concentration of biological tissues is approximately 100 mM. Protons have an inherent magnetic dipole moment that results from their electrical charge and spin. When a human body is placed in a strong, steady magnetic field, these protons begin to precess around the axis of the external magnetic field, B_0

(Figure 1a) The Larmor frequency, ω_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 magnetization is best described as a vector. A radio frequency (RF) pulse at the Larmor frequency may be used to add energy to the spins, moving spins from the low energy state to the high energy state (Figure 1c). Consequently, the longitudinal component of the 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.

Figure 1 (a) Precession of protons in an external magnetic field (b) Proton spin energy in a magnetic field (c) Change in proton spin energy with increasing RF energy at the Larmor frequency⁵

The relaxation time has two components, longitudinal and transverse relaxation times, known as T_1 and T_2 respectively.⁶ The intensity of a magnetic resonance image

depends primarily on three factors; the water proton density, T_1 and T_2 relaxation times. The visual contrast is determined by the variation in these parameters among tissues. MR images are usually weighted 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).

2. MRI contrast agents

MRI contrast agents are chemical compounds that are used to improve the visibility of internal body structures in MRI by markedly altering the relaxation times of water protons in tissues where they are distributed, rendering a typical MRI brighter (a positive agent) or darker (a negative agent).⁷ This in turn leads to remarkable improvements in medical diagnoses, as they facilitate higher sensitivity, specificity and better tissue characterization.⁸

Contrast agents can be divided into two groups depending on whether they cause changes in either T_1 (longitudinal relaxation time-in simple terms, the time taken for the protons to realign with the external magnetic field) or T_2 (transverse relaxation time-in simple terms, the time taken for the protons to exchange energy with other nuclei) relaxation rates of the water protons, these being commonly described as positive agents, T_1 -agents, or negative agents, T_2 -agents respectively. The ability of an agent to affect T_1 and T_2 is characterized by the concentration-normalized relaxivities, r_1 and r_2 , respectively. These parameters refer to the amount of increase in $1/T_1$ and $1/T_2$, respectively, per millimole of agent, and are normally quoted as a rate ($\text{mM}^{-1}\text{s}^{-1}$).⁴

The values are used to determine the efficiency of a contrast agent, and they consist of contributions from both inner sphere and outer sphere relaxation mechanisms.⁹ It is useful to classify the relevant contributions for water proton relaxivity with respect to three distinct types of interactions, as indicated in Figure 2.

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 theory for solvent relaxation in this case is summarized in the next section. The term “inner-sphere relaxation” is often applied to this type of relaxation mechanism. It should be mentioned that this same theory applies in the case B interaction, i.e., hydrogen-bond waters in the second coordination sphere, if the lifetime of this interaction is long compared with the time required for the water molecule and the chelate to diffuse past each other. Nevertheless, due to the lack of understanding of second coordination sphere interaction, 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 to “outer-sphere relaxation”. The total relaxivity is therefore generally given by equation 1.

$$(1/T_i)_p = (1/T_i)_{inner} + (1/T_i)_{outer} \quad i = 1, 2 \quad (1)$$

The signal observed in MRI tends to increase with an increase in $1/T_1$ and decrease with an increase in $1/T_2$, and it is usual for contrast agents to affect both $1/T_1$ and $1/T_2$ to varying degree.² Positive contrast agents are commonly made up of paramagnetic materials, mainly those based on metal ions with large numbers of unpaired electrons, such as Mn^{2+} (5 unpaired electrons), and Gd^{3+} (7 unpaired electrons). With positive contrast agents, a similar effect is seen on both T_1 and T_2 , but because T_1 is larger than T_2 , shortening of T_1 is observed. This results in the image being brighter within areas where the agents are taken up, due to brightness being a function of T_1 . Hence species with high T_1 values lend themselves to darker images. Negative contrast agents influence the signal intensity by shortening transverse relaxation time (T_2), thereby producing darker image as high T_2 results in increased contrast in an images. Negative contrast agents are commonly formed of superparamagnetic materials such as iron oxide nanoparticles.¹⁰

2.1 Classification of MRI contrast agents

The use of contrast-enhancing agents has become an integral part of MR imaging for many applications. Currently available MRI contrast agents can be categorized according to their magnetic behavior, biodistribution and effect on the image.

2.1.1 Paramagnetic agents

Paramagnetic species are species which have unpaired electrons. It can be a simple substance (i.e. molecular oxygen), organic free radical (i.e. nitroxide radical) or metal ions (i.e. many transition metal ions). Radicals generally cause damage to the living tissues, therefore they are not suitable candidates for medical MRI purposes. The

paramagnetic effect of oxygen, although it is demonstrable, seems to be too weak for practical application.

Metal ions with one or more unpaired electrons are paramagnetic and they therefore have a permanent magnetic moment. In aqueous solution, there is a dipolar magnetic interaction between the electronic magnetic moment of the paramagnetic atom and the much smaller magnetic moments of the protons of the nearby water molecules. Random fluctuations in this dipolar magnetic interaction, mainly a result of molecular motions, reduce both the longitudinal (T_1) and transverse (T_2) relaxation times of the water protons. Gadolinium (Gd^{3+}) and manganese (Mn^{2+}) are examples of paramagnetic ions which are used in MRI contrast agents. Paramagnetic metals can not be used as contrast agents in their ionic form due to an undesirable biodistribution and the relatively high toxicities of the metal ions. By binding the metal to a ligand, a more desirable biodistribution and safety profile can be achieved. The resulting metal-ion complex is called a chelate.^{11,12,13}

2.1.2 Superparamagnetic agents

Iron oxide particles (typically 5-200 nm in diameter) are made up of several thousand magnetic ions and are said to have superparamagnetic properties if the magnetic ions are mutually aligned. Iron oxides are divided into two classes according to the overall size of the crystal: if the diameter (d) is more than 50 nm, they are called superparamagnetic iron oxide (SPIO), while if d is less than 50 nm, they are known as ultra-small superparamagnetic iron oxide (USPIO) particles.

The magnetic moments of the individual iron ions that make up the SPIO and USPIO particles do not cancel out, thus the particles have a permanent magnetic

moment which is very large when exposed to a magnetic field, much larger than that of a single molecule of a Gd chelate. As a consequence of their large size and magnetic moment, SPIOs were initially developed as T_2 agents.¹⁴ However, a new generation of USPIOs with crystal sizes less than 10 nm have also been reported to have excellent T_1 -enhancing properties.^{15,16}

2.1.3 Extracellular Fluid (ECF) agents

Contrast agents which are distributed to the extracellular fluid (ECF) are often referred to as ECF agents. Such agents leak rapidly from the blood into the interstitium with a distribution half-life of about 5 minutes and are cleared by the kidneys with an elimination half-life of about 80 minutes.¹⁷ The presence of the blood brain barrier (BBB) prevents extravascular leakage of ECF agents into the brain in areas with an intact BBB. Since many neuro-pathological conditions are associated with an altered capillary permeability, such conditions can often be better visualized after contrast agent administration due to the selective accumulation of contrast agent in these regions. ECF agents have also been extensively used in extra-cranial applications. Notably, the use of gadolinium-based ECF agents has shown great utility in MR angiography (MRA). By selectively reducing the T_1 relaxation time of blood, high-quality MR angiograms can be obtained after administration of Gd-ECF agents. Since these agents clear rapidly from the blood, the images are typically acquired in the early phase following a bolus injection, when used in conjunction with MRA.

2.1.4 Vascular agents (Blood-Pool agents)

Blood-pool agents (BPAs, or intravascular agents) belong to a new class of MRI contrast agents. These compounds preferentially localize in the vasculature, causing an

increase in $1/T_1$ in the blood that facilitates the acquisition of magnetic resonance angiography (MRA) data. They are significantly larger in size than ECF agents, therefore preventing leakage into the interstitium. Two main approaches are currently pursued with MRI blood-pool agents. One approach is to use ultra-small iron oxide particles with a low r_2/r_1 ratio.^{15,16} The second approach is to use gadolinium-based water-soluble macromolecules with a vascular residence time and relaxivity which is higher than those of ECF agents.¹⁸ The macromolecule can either be a biological entity such as albumin¹⁹ or a chemical entity such as a polymer.²⁰ These agents have been primarily developed for use in MR angiography, where the aim is to have an agent with a relatively long vascular half-life and the highest possible r_1 -relaxivity. At the same time, the r_2 -relaxivity must be low enough to avoid excessive signal loss due to T_2 relaxation. By selectively reducing the T_1 relaxation time of blood, high-quality MR angiograms can be generated. The potential advantage of using a blood-pool agent in MRA is the prolonged imaging window. Since a longer image acquisition time translates into higher image resolution and/or signal-to-noise ratio, higher-quality angiograms can potentially be attained compared to when gadolinium-based ECF agents are used. The disadvantage of this 'steady-state' approach is that both arteries and veins are equally enhanced in the image, thus making their differentiation more challenging.

2.1.5 Organ-specific agents

An organ-specific contrast agent can be defined as a contrast agent which is selectively taken up by a particular type of cell such as Kupffer cells or hepatocytes and thereby only enhances organs where these cells are present (e.g. the liver, the spleen and the lymph nodes). All contrast agents are organ-specific to some extent in that they are

excreted either by the liver or by the kidneys. Iron oxide particle-based agents (SPIOs and USPIOs) accumulate in the reticuloendothelial system in the liver. They can therefore be described as liver specific agents even if they have a long vascular half-life prior to liver accumulation.

Hepatocyte-selective MRI contrast agents were the first examples of organ-specific agents to enter clinical development. Selectivity for hepatocyte uptake caused small focal lesions (e.g., tumours) to appear dark on the brighter background of a T_1 -weighted liver image. Selective delivery of small-molecule MRI contrast media to normal liver tissue was achieved using aryl-modified anionic gadolinium complexes, as exemplified by two compounds in clinical development, Gd-EOB-DTPA²¹ and Gd-BOPTA²² as shown in Figure 3.

Figure 3

Other contrast agents are specifically designed to accumulate in the liver, e.g. Mn-DPDP²³ or Gd-Cy2DOTA²⁴ (Figure 4). Another type of organ-specific agents are the oral contrast agents which are distributed in the gastrointestinal tract to improve delineation of the bowel from surrounding tissues.²⁵

Figure 4

Currently, more than 30% of all MRI scans use a contrast agent, and approximately 35% of those are paramagnetic contrast agents. More than 30 tons of gadolinium contrast agents have been administered since 1988.^{2,26} Gadolinium(III) reagents are commonly focused on due to coupling of the large magnetic moment (spin-only $\mu_{eff} = 7.94$ BM from seven half-filled f orbital electrons) with a long electron spin relaxation time of 10^{-9} s (from the symmetric S electronic state) at the magnetic field strengths used in MRI techniques.⁷ Consideration needs to take into account the toxicity of this heavy metal, as its radial size is approximately equal to that of calcium(II), and as a result it can disrupt calcium(II) mediated signaling, forming strong complexes that can accumulate within the body.² The correct choice of ligand can prevent this *in vivo* transmetallation occurring, and this has enabled gadolinium complexes to be developed for MRI applications.

3. Factors controlling relaxivity

The theory of relaxation in the presence of paramagnetic ions has been described by Solomon, Bloembergen, and Morgan.^{10,27} A contrast agent will increase the longitudinal and transverse relaxation rates, $1/T_1$ and $1/T_2$, respectively, of solvent nuclei. Diamagnetic and paramagnetic relaxation rates are additive and given by

equation 2 where $(1/T_i)_{obs}$ is the observed solvent relaxation rate and the subscripts “d” and “p” refer to diamagnetic and paramagnetic, respectively. The diamagnetic term arises from the relaxation rate of proton nuclei in the absence of a paramagnetic ion and the paramagnetic term can be expressed as the relaxation rate enhancement induced by the paramagnetic species.

$$(1/T_i)_{obs} = (1/T_i)_d + (1/T_i)_p \quad i = 1, 2 \quad (2)$$

Contrast agents are evaluated on the basis of their relaxivity, or how much the relaxation rates of water protons are increased in the presence of the agent at a given concentration. The observed relaxation rate of solvent protons, in this case water protons, is comprised of both diamagnetic and paramagnetic contributions. The paramagnetic contribution is linearly related to the concentration of paramagnetic species present. Relaxivity (r_i , $i = 1, 2$) is then defined as the concentration-dependent increase in relaxation rate of the paramagnetic agent, or the slope of a plot of $(1/T_i)_{obs}$ vs. concentration (equation 3).^{2,5}

$$(1/T_i)_{obs} = (1/T_i)_d + r_i[Gd] \quad (3)$$

Relaxivity of solvent water (bulk water) by a gadolinium complex is a multifaceted phenomenon. Water in close proximity to the ion is relaxed and then rapidly exchanges with the bulk. T_1 relaxation of water hydrogen by Gd(III) occurs *via* a dipolar mechanism. Relaxation will depend on the number of water molecules, their distance to the Gd(III), their rate of exchange with bulk solvent, and some correlation time, τ_c .

Fluctuating magnetic dipoles can induce spin transitions and cause spin relaxation. A correlation time is a time constant for characterizing these fluctuations;

its reciprocal $1/\tau_c$ is the average rate constant for these fluctuating dipoles. There are several processes that lead to fluctuating magnetic dipoles. Electronic relaxation ($1/T_{1e}$) at the Gd(III) ion creates a fluctuating field. Rotation diffusion ($1/\tau_R$) of the complex creates a fluctuating field. Water exchange in and out of the first ($1/\tau_m$) or 2nd ($1/\tau_m'$) coordination sphere also creates a fluctuating field for the hydrogen nucleus. It is the fastest rate (shortest time constant) that determines the extent of relaxation. For water in the second sphere, the relevant correlation time may be the lifetime of this water which may be on the order of 10's of picoseconds. Water in the inner-sphere typically has a much longer residency time (1-10,000 ns), so the relevant correlation time is usually rotational diffusion or electronic relaxation.

As can be seen in Figure 5, the hydrogen nuclei are small magnetic dipoles denoted by the small vectors. There are q water molecules in the inner-sphere with a Gd-H distance, r and a residency time, τ_m and q' waters in the second-sphere at a Gd-H distance, r' and a residency time, τ_m' . The gadolinium ion is a much larger magnetic dipole denoted by the large vector and characterized by spin, S ; it undergoes relaxation that is described by T_{1e} and T_{2e} . Assuming the complex tumbles isotropically, rotational motion is described by rotational correlation time, τ_R . Water in the outer-sphere is described by a translational diffusion correlation time, τ_D and a distance of closest approach, a . This gives 11 parameters to describe water relaxation at a given applied field, B_0 . Moreover, electronic relaxation itself is magnetic field dependent. It is obvious that the relative influence of all these parameters cannot be ascertained by a simple measurement of relaxivity. However, some of these parameters can be determined independently and the effect of others can be stimulated. A better understanding of the molecular basis of relaxivity has led to contrast agents with higher relaxivity.

Figure 5 Molecular parameters that influence inner- and 2nd-sphere relaxivity

3.1 Inner-sphere relaxivity

Paramagnetic relaxation enhancement includes both an inner-sphere component from the proton relaxation of a solvent molecule directly coordinated to the Gd(III) ion and an outer-sphere component from solvent in the second coordination sphere and the bulk solvent. Current agent design focuses mainly on attaining higher inner-sphere, longitudinal relaxivity, r_{1p} from protons of water molecules in the first coordination sphere of the metal. Relaxivity arising from the inner-sphere waters is given by equation 4, which incorporates 2-site exchange where T_{1m} is the T_1 of the water hydrogen in the inner-sphere and $[H_2O]$ is the water concentration in mM. In order to increase relaxivity, either increase hydration number, q or decrease T_{1m} or τ_m .

$$r_1^{IS} = \frac{q/[H_2O]}{(T_{1m} + \tau_m)} \quad (4)$$

3.1.1 Hydration number, q

Hydration numbers are typically estimated using a surrogate ion approach. Horrocks and Sudnick showed that the inner sphere hydration was directly proportional to the difference of fluorescent decay rates measured in H_2O and D_2O because the O-H (but

not O-D) oscillator from bound water is a good quencher of Eu(III) fluorescence.²⁸ It is assumed that a Gd(III) complex has the same hydration number as its Eu(III) analogue. From equation 3, it is clear that inner-sphere proton relaxivity is linearly proportional to the number of water molecules directly coordinated to the Gd(III) ion. In the Gd^{3+} aqua ion, there are eight inner-sphere water molecules which would result in a proportionally high relaxation enhancement. However, the Gd^{3+} aqua ion itself is toxic, since under physiological conditions, it has a strong tendency to precipitate in the form of gadolinium-hydroxide or to form stable complexes with endogenously available anions. The co-ligand, such as DTPA, should form a sufficiently stable complex that Gd(III) ion is not released into the human body. This safety concern limits the ability to increase q to increase relaxivity. Typically as more coordination sites are opened up for water ligation, the thermodynamic stability of the complex decreases and the Gd(III) usually becomes more labile.

A second trade-off with increasing the hydration number is that it often allows coordination of other ligands, such as endogenous phosphate or bicarbonate, which then displace the coordinated water molecules and reduce relaxivity. For example removing an acetate arm from $[\text{Gd}(\text{DOTA})(\text{H}_2\text{O})]^-$ to give $[\text{Gd}(\text{DO3A})(\text{H}_2\text{O})_2]$ (Figure 6) doubles the hydration number but still results in a stable complex.²⁹ However, the water molecules can be displaced by anion binding.³⁰ The hydroxypyridinone (HOPO) (Figure 7) class of compounds described by Raymond and co-workers³¹ appear to be stable $q = 2$ complexes that are resistant to anion coordination.

Figure 6

Figure 7

3.1.2 Inner-sphere water relaxation

The denominator of equation 3 indicates that the relaxation time of the bound water, T_{1m} , should be as short as possible and likewise the water residency time should be short. For the first generation of MRI contrast agents, $T_{1m} > \tau_m$ and it is T_{1m} that limits the relaxivity of these agents. Dipolar relaxation arising from electron-nuclear spin coupling is given by equations 5 and 6.

$$\frac{1}{T_{1m}} = \frac{2}{15} \frac{\gamma_H^2 g_e^2 \mu_B^2 S(S+1)}{r_{GdH}^6} \left[\frac{7\tau_{c2}}{1 + \omega_S^2 \tau_{c2}^2} + \frac{3\tau_{c1}}{1 + \omega_H^2 \tau_{c1}^2} \right] \quad (5)$$

$$\frac{1}{\tau_{ci}} = \frac{1}{\tau_m} + \frac{1}{\tau_R} + \frac{1}{T_{ie}}; i = 1, 2 \quad (6)$$

Relaxation depends on the Gd-H distance, r_{GdH} , the proton Larmor frequency, ω_H (in rad/s), the electron Larmor frequency, ω_S ($\omega_S = 658\omega_H$), and correlation times, τ_{c1} and τ_{c2} . The two terms in square brackets are sometimes referred to as dispersive terms because once $\omega^2\tau_c^2 > 1$, then the relaxation rate becomes slower and disperses with increasing frequency (field). Because the Larmor frequency of the electron is 658 times that of the proton, the first term in square brackets disperses at a lower field than the second. If the correlation time is not dependent on magnetic field, then the relaxation rate will depend on proton Larmor frequency.

The correlation time that defines dipole-dipole relaxation is τ_c and is determined by equation 5 where T_{1e} is the electron spin relaxation time and τ_R is the rotational correlation time. Relaxivity is maximized when τ_c equals the inverse of the Larmor frequency.⁵ Optimization of the parameters shows that τ_m should be decreased but not such that it begins to limit T_{1m} . The above equations demonstrate that proton relaxivity is influenced by a number of parameters and is dependent on magnetic field strength and temperature. Most contrast agents used in clinical applications have relaxivity values approximately 4-10 mM⁻¹s⁻¹, far below the theoretical maximum of ~100 mM⁻¹s⁻¹.⁵ Due to the low relaxivity values of commercial contrast agents, their effective range requires concentrations greater than 0.1 mM.³² In order to increase the relaxivity of Gd(III)-based contrast agents, researchers focus primarily on optimization of three parameters: q , τ_m , and τ_R (Figure 3).

3.1.3 Gadolinium-water distance

The term outside the square brackets in equation 4 contains some physical constants, the spin quantum number of Gd(III), $S = 7/2$, and Gd-H distance, r_{GdH} . Gd(III) is preferred as a relaxation agents due to its high spin number (high number of unpaired electrons) and relatively slow electronic relaxation. If the Gd-H distance could somehow be reduced, this would lead to significant increases in relaxivity. There are many values reported in the literature that the range between 2.7 and 3.3 Å, but these numbers were obtained indirectly, typically from fitting NMRD data. Direct determination of the Gd-H ion-nuclear distance can be done using neutron diffraction on single crystal or using isotopic exchange methods in very concentrated solution.³³

3.1.4 Rotation diffusion

The correlation time is dominated by the shortest correlation time among rotation, electronic relaxation, or chemical exchange and is given by equation 5. By far, the most common field strength used for clinical imaging is 1.5 tesla (64 MHz proton frequency). At this field strength the dominant correlation time is almost always rotational diffusion. For small-molecular-weight Gd(III) chelates, it is the rotational correlation time, τ_R in equation 5, the fast rotation is the limiting factor for proton relaxivity at magnetic field relevant to MRI.

Figure 8 which shows the nuclear magnetic resonance dispersion (NMRD) profile of the low-molecular-weight, and thus fast-rotating, $[Gd(DTPA)(H_2O)]^{2-}$ species ($\tau_{R1} = 58$ ps) and a curve calculated with the same parameters except for the rotational correlation time ($\tau_{R2} = 1000 \times \tau_{R1} = 58$ ns) which is of the order of rotational correlation times measured, e.g. for serum albumins, is well demonstrated. The fact

that the relaxivity of the small Gd(III) chelates is limited by rapid tumbling was recognized at an early stage of contrast agent development and has led to a wide variety of approaches to slow down rotation by increasing the molecular weight of the complex.⁵

Figure 8 An experimental NMRD profile of [Gd(DTPA)(H₂O)]²⁻ (25 °C; bottom curve) and a calculated NMRD profile (upper curve) obtained by using the same parameters as obtained for this species, except for the rotational correlation time, τ_R , which was taken at 1000 times its value.⁵

3.1.5 Electronic relaxation

Electronic relaxation for Gd(III) is a complex, magnetic field dependent phenomenon. At low field ($B_0 < 0.1$ tesla), electronic relaxation is very fast and becomes the dominant correlation time, equation 7. However electronic relaxation decreases with increasing field strength and at some point becomes slower than rotational motion,

equation 8. The result is that the correlation time for nuclear relaxation changes as a function of field.

$$1/T_{1e}, 1/T_{2e} \gg 1/\tau_R; T_{1e} \approx \tau_{c1}, T_{2e} \approx \tau_{c2} \quad (7)$$

At low field ($B_0 < 0.1$ T)

$$1/T_{1e}, 1/T_{2e} \ll 1/\tau_R; T_R \approx \tau_{c1} \approx \tau_{c2} \quad (8)$$

At low field ($B_0 > 1.5$ T)

3.1.6 Water exchange

Water exchange is of obvious importance for relaxivity. The coordinated water must be in rapid exchange with bulk solvent in order to transmit the relaxation effect to the solvent. There is a large body of work on water exchange in Gd(III) complexes. This is a readily accessible parameter via variable temperature ^{17}O NMR transverse relaxation rate measurements at high field. Some useful empirical relationships have emerged. Replacing an acetate oxygen donor with an amido oxygen donor will result in a slower water exchange rate. For complexes undergoing dissociative water exchange (the majority studied), increasing the steric bulk will increase the water exchange rate. For example replacing an acetate oxygen donor with the larger phosphonato oxygen donor increases the exchange rate.³⁴

The water residency time, τ_m ($k_{ex} = 1/\tau_m$) enters into the equation for relaxivity directly and also through the relaxation time of the bound water, T_{1m} . If water exchange is very fast, then the short τ_m can be the correlation time that governs T_{1m} . Relaxivity can be limited if water exchange is too slow because the relaxation effect is

poorly transmitted to the bulk. Relaxivity can also be limited if water exchange is too fast because the water is not coordinated to the Gd(III) long enough to be relaxed.

3.2 2nd-sphere and outer-sphere relaxivity

Contrast agents can display relaxivity even when $q = 0$. Thus, as there is no water in the inner coordination sphere the relaxivity must come from outer-sphere contributions.³⁵ These can take two forms; (i) second-sphere relaxation where water molecules hydrogen bonded to the carboxylate oxygen atoms are relaxed *via* dipolar mechanism and (ii) outer-sphere relaxation which arises due to diffusion of water molecules in the bulk near to the Gd(III) complex.^{2,36} The parameters focused on are the electronic relaxation time of the metal, the distance of the closest approach of solvent and solute and the sum of their diffusion coefficients.⁸ The outer-sphere relaxivity is usually estimated by equations proposed by Freed.¹² It is important to note that this model is only an approximation for the polyaminocarboxylate ligands used in contrast agents because it does not take into account interactions of water with the complex, which for these ligands are important.⁷ On the whole it seems that outer-sphere contributions to relaxivity are not very well understood, and this phenomenon is overlooked when contrast agents are being developed.

4. Clinically approved gadolinium chelates

Of the six clinically approved contrast agents used worldwide for intravenous administration, four of them are gadolinium-based with a polyaminocarboxylate ligand forming highly stable complexes (the other two are mangafodipir trisodium,³⁷ also known as TelescanTM, and ferumoxides^{38,39} which are superparamagnetic iron oxide nanoparticles often coated with dextrans). The anionic complexes of Gd(DTPA)²⁻

(MagnevistTM) and $\text{Gd}(\text{DOTA})^-$ (DotaremTM) were the first complexes used in clinical practice, and $\text{Gd}(\text{DTPA-BMA})$ (OmniscanTM) and $\text{Gd}(\text{HPDO3A})$ (ProhanceTM) are neutral compounds based on the structures of the anionic complexes (Figure 9).

Figure 9

$[\text{Gd}(\text{DTPA})(\text{H}_2\text{O})]^{2-}$ forms a stable octacoordinated chelate, similarly to $[\text{Gd}(\text{DOTA})(\text{H}_2\text{O})]^-$, but their formation is very different. DTPA is commercially available, but DOTA requires the preparation of 1,4,7,10-tetraazacyclododecane, which is time consuming.⁴⁰ The DTPA ligand forms stable chelates based on a distorted tricapped trigonal prism with the three nitrogens, five carboxylic oxygens, and the necessary water molecule coordinating to the gadolinium center. DOTA forms very stable lanthanide chelates because the tetra-aza cycle is able to adopt its most stable conformation. The solid state X-ray structure of $\text{Gd}(\text{DOTA})^-$ indicated that Gd^{3+} is situated in the centre of a capped square antiprismatic cage, with the water molecule in an axial position.⁴¹ When in solution, it exists as a pair of interchangeable isomers,

the major square antiprismatic and the minor twisted square antiprismatic (Figure 10).⁴² The neutral complexes of $[\text{Gd}(\text{DTPA-BMA})(\text{H}_2\text{O})]$ and $[\text{Gd}(\text{HPDO3A})(\text{H}_2\text{O})]$ share a similar stability in thermodynamic terms.⁴³ HPDO3A complexes are kinetically stronger because the rigid ring structure of the macrocycle prevents release of the metal, as at least five coordination sites would have to break simultaneously, whereas in DTPA-BMA, it is possible to see sequential breaking of the coordination sites.⁴⁴ The potential for release of Gd^{3+} from the DTPA-BMA ligand is minimized by formulating the complex with 5% excess calcium ligand to scavenge competing biometals.⁴⁵

Figure 10

These first generation contrast agents are very useful, and have accounted for the rise in MRI scans being used in diagnosis. They distribute mainly into the intravascular and interstitial space, and although they are deemed to be non-specific, they can accumulate within the kidneys due to glomerular filtration.⁴⁶ The next generation of contrast agents are designed to be more specific and more effective, with an unusually high relaxivity,⁴⁷ greater thermodynamic stability and a more favourable rate of excretion. This can be achieved by attaching the contrast agent to larger structures such as dendrimers⁴⁸ or micelles.⁴⁹ In efficiency terms, the new reagents are

usually compared to the DOTA and DTPA complexes due to their prior clinical approval.⁵⁰

5. What is known about molecular design of contrast agent and optimization of relaxivity

The substantial clinical and physicochemical experience with MRI contrast agents has yielded important molecular architectural principles for designing new classes of contrast agents. New MRI contrast agents must exhibit substantial gains in diagnostic performance (sensitivity, specificity, cost) in order to penetrate a highly competitive market for current anatomic imaging. However, this is of little concern for more specialized imaging and molecular imaging applications.

Ideal molecular, pharmacological, and radiological⁵¹ properties include:

- 1) Non-ionic to minimize osmolality and the concomitant adverse reactions and pain upon administration (neutral Gd(III) chelates have higher LD₅₀, i.e., lower toxicity; non-ionic chelates such as Gd(HPDO3A) and Gd(DTPA-BMA) are 35-70% lower in osmolality and viscosity than ionic chelates)
- 2) Good water solubility
- 3) High relaxivity per molecular volume for low effective dose
- 4) Shelf stability, heat stability, and sterilizability by autoclaving
- 5) Low toxicity, high patient tolerance, non-immunogenic, and favourable pharmacology
- 6) < 40 – 80 Å for rapid renal excretability and passage through blood capillaries
- 7) High thermodynamic and *in vivo* stability

- 8) Kinetic stability (i.e., slow transmetallation kinetics) in the presence of endogeneous metal ions, with high selectivity of ligand for Gd(III) over Ca(II) (physiological concentration of Ca(II) is 5 mM), Zn(II), and Cu(II), as well as common anions such as phosphate and carbonate
- 9) Presence of at least one inner-sphere water ligand
- 10) Non-spherical with hydrophilic ligands for water interaction in order to increase rational correlation time
- 11) Steric crowding by the ligand at the metal center in order to increase the rate of inner-sphere water exchange, if *via* dissociative mechanism
- 12) Specific *in vivo* distribution
- 13) High degree of imaging signal enhancement per unit of toxicity and per unit cost

Recent strategies in contrast-agent design

The primary developmental focus of next-generation MRI contrast agents has been the synthesis of derivatives of the aminocarboxylate systems used in the clinic. Features of compounds based on ligands such as DTPA and DOTA include inexpensive streamlined syntheses as well as adequate solubility and toxicological parameters.^{1,45,52}

The following examples illustrate several approaches toward optimizing the aminocarboxylate system en route to more efficient relaxation agents.

Research efforts in new contrast-agent design are generally directed towards the optimization of one or more of the aforementioned relaxation parameters through ligand structural modification. For example, Merbach and co-workers have reported numerous studies that probe the factors influencing the water exchange rate of aminocarboxylate Gd(III) complexes.^{53,54,55} The main explanation for increased water exchange rates is steric crowding at the water binding sites, a property that favors the

release of the coordinated water molecule in a dissociative exchange process. Derivatives of DTPA as shown in Figure 11, have been synthesized with varying numbers of carbon atoms in the ligand scaffold.

	τ_m	pGd
<p>DTPA</p>	303 ns	19.1
<p>EPTA</p>	3 ns	14.7
<p>DTTA-Nprop</p>	32 ns	16.9

Figure 11

Water exchange is accelerated in the resultant Gd(III) complexes, with values approaching the optimal range for high relaxivity at higher magnetic fields (60-100 MHz) as shown by the water residency time, τ_m . This rate enhancement is achieved,

however, at the cost of thermodynamic stability, as measured by the relatively low pGd values (equation 9). Typically, stabilities of MRI contrast agents are reported as their pGd values, thus providing a convenient way to compare stabilities of chelates with differing protonation behavior.^{2,56}

$$pGd = -\log[Gd]_{free}; [Gd]_{total} = 1 \mu M, [L]_{total} = 10 \mu M$$

$$(pH 7.4, 25^\circ C, 0.1 M KCl) \quad (9)$$

This phenomenon of decreased stability upon increasing water exchange rates is common for aminocarboxylate ligands and must be addressed when considering these complexes as high-relaxivity agents, particularly at high magnetic field strengths of future clinical scanners.

Increased water exchange rates for macrocyclic complexes based on $[Gd(DOTA)(H_2O)]^-$ have also been reported (Figure 12). The pyridine-*N*-oxide of DOTA resulted in a gadolinium complex with a significantly faster water exchange rate than the parent complex ($\tau_m = 39$ vs. 244 ns^{57}). As with the linear aminocarboxylates mentioned above, the rate acceleration was attributed to an increase in steric crowding. A monophosphinic acid derivative was found to possess an even faster exchange rate, with $\tau_m = 16 \text{ ns}^{58}$. Steric crowding owing to the bulky phosphinate group is given as a rationale for the increased rate, as well as a possible favorable arrangement of water molecules in a second coordination sphere. The relaxivity of this complex is $6 \text{ mM}^{-1}\text{s}^{-1}$ (20 MHz, $26^\circ C$), an improvement over commercial agents.

Figure 12

Complexes with $q > 1$ have also been reported for aminocarboxylate systems in efforts to achieve higher relaxivity. Relaxivity is highly dependent on the hydration number (q) and relaxivity values will always be limited for complexes that possess only one coordinated water molecule ($q = 1$). Two examples of $q = 2$ complexes are depicted in Figure 12. In each case, DTPA complexes are tethered to a central core to produce dinuclear Gd(III) complexes with increased hydration numbers ($q = 2$). The relaxivity values of $[\text{Gd}_2\{p\text{X}(\text{DTTA})_2\}(\text{H}_2\text{O})_4]^{2-}$ and $[\text{Gd}_2\{m\text{X}(\text{DTTA})_2\}(\text{H}_2\text{O})_4]^{2-}$ are 12.8 and 11.6 $\text{mM}^{-1}\text{s}^{-1}$ (20 MHz, 37 °C), respectively, and represent significant increases over that of the parent DTPA complex ($r_{1p} = 4.3 \text{ mM}^{-1}\text{s}^{-1}$).² These values are influenced by the higher q value and (owing to increased molecular weight) by an increase in the rotational correlation time, τ_R .⁵⁹ A supramolecular approach was used to generate another dinuclear $q = 2$ complex *via* iron terpyridine complexes derivatized with DTPA (Figure 13). The high relaxivity of $[\text{Fe}(\text{tpy-DTTA})_2\text{Gd}_2(\text{H}_2\text{O})_4]$ (15.7 $\text{mM}^{-1}\text{s}^{-1}$)

$^1\text{s}^{-1}$, 20 MHz, 37 °C) is attributed to an increase in q as well as a long τ_R value resulting from the higher molecular weight and rigidity of the complex.⁶⁰

While the relaxometric properties of such $q = 2$ compounds are improved, the thermodynamic chelate stability suffers greatly in both cases. The increase in q (Figure 13) from one to two is made possible by removing one carboxylate arm of the parent DTPA to open up a metal coordination site. As is observed in accelerating water exchange in the aminocarboxylate class of compounds, attaining a higher number of coordinated water molecules by decreasing ligand denticity is accompanied by a dramatic decrease in thermodynamic stability. The pGd values for the *para*- and *meta*-substituted xylene-based complexes (16.2 and 15.1, respectively) are significantly lower than the value of 19.1 for the parent DTPA complex.⁴⁵ Even less stable is the iron terpyridine complex, its pGd value of 10.6 represents a decrease by more than five orders of magnitude in stability relative to the DTPA-BMA complex ($pGd = 15.8$, Figure 9), the ligand with the lowest pGd value of all clinically approved agents. This effect of decreased stability resulting from increased q or water exchange rate raises a key concern in contrast-agent design. The optimization of one parameter will often hamper that of another, making the goal of high-relaxivity, practical agents a major challenge. To achieve a practical high-relaxivity agent, the optimal combination of all relevant parameters must be accomplished while maintaining solubility and chelate stability.

Figure 13

As an example of a gadolinium-based contrast agent not focused on traditional aminocarboxylate ligand scaffolds, Wilson and co-workers have reported several studies of Gd(III) encapsulated inside fullerene cages.^{61,62,63} Metallofullerenes encapsulate metal atoms into their interior space, which has potential for medicinal applications because the fullerene cage protects the metal ion from release into the body, potentially allowing longer residence times *in vivo*. The peripheries of these cages are decorated with solubilizing groups to allow for application of C₆₀ in aqueous media. Relaxivities ranging from about 10 to as high as 38 mM⁻¹s⁻¹ (30 MHz, 26 °C) are due entirely to second and outer-sphere relaxation, as there are no inner-sphere water molecules directly coordinated to the gadolinium ion. In solution, these

“gadofullerenes” aggregate, resulting in large assemblies with long rotational correlation times and consequent high relaxivities.⁶³ However, practical concerns such as *in vivo* toxicity and deaggregation in the presence of various salts (thereby limiting the effect of long τ_R values on relaxivity)⁶⁴ may preclude considering such systems for contrast-agent applications.

6. Further developments in contrast agents

6.1 Smart contrast agents

One of the most currently exciting areas in MRI contrast agent research is the development of responsive or ‘smart’ contrast agents. The relaxivity and the corresponding change in signal intensity of these agents respond to changes in physiological environment such as pH, metal ion concentration, partial pressure of oxygen, enzyme activity or temperature.^{9,65}

Unlike X-ray contrast agents (nuclear agents), MRI contrast agents are not directly imaged, they enhance the contrast indirectly by affecting the nuclear magnetic relaxation times of the water protons in surrounding tissues. Through a careful choice of the paramagnetic ion-ligand complex, the interactions between contrast agents and the water protons in surrounding tissues can be modulated by changes in the physiological environment. Such responsive contrast agents should generate a relaxivity or signal that depends on some variable in their immediate environment, enabling them to act as reporters for monitoring physiological change. In this way, MRI contrast agents may have more potential than X-ray contrast agents. However, because of the natural insensitivity of MR technique, robust cellular amplification strategies are needed to confer adequate sensitivity. The rotational correlation time

(τ_R), q and τ_m have a strong effect on the relaxivity of contrast agents. In a responsive contrast agent, one or more of these parameters are affected by changes in the environment. Both the percent change in relaxivity and absolute relaxivity are important factors in generating a better contrast image under different conditions.

6.2 pH Responsive contrast agents

The drive behind the development of a pH responsive contrast agent is based on the fact that healthy tissue has a pH of 7.4, whereas tumor tissue is more acidic (6.8). This could be used to generate contrast agents that can be used to map tumors.

Sherry and co-workers reported the synthesis of a DOTA-tetraamide (DOTAMP) adorned with pendant phosphonates that shows pH responsive behavior in the physiological pH range due to protonation of the phosphonates (Figure 14).⁶⁶ This suggests that the hydrogen-bonding network created by protonation of the phosphonates provides a catalytic pathway for exchange of the bound water protons with protons of bulk water. The results showed that the 2.6 fold increase in water relaxivity over the pH range of 9 to 6 can be largely attributed to protonation of the extended phosphonate groups.⁶⁷

Figure 14

Wilson and co-workers also reported a pH responsive contrast agent based on fullerenes.⁶³ They fully characterized the water soluble gadofullerene derivatives $\text{Gd@C}_{60}(\text{OH})_x$ and $\text{Gd@C}_{60}[\text{C}(\text{COOH})_2]_{10}$.⁶⁸ The relaxivity measured for $\text{Gd@C}_{60}(\text{OH})_x$ were approximately 10 times those of the commercially available contrast agent, with their high field maxima at 40 MHz. The relaxivities at 60 MHz were also measured as a function of pH, and it was observed that the relaxivities for both compounds increased considerably (a factor of 2.6 for $\text{Gd@C}_{60}(\text{OH})_x$ and 3.8 for $\text{Gd@C}_{60}[\text{C}(\text{COOH})_2]_{10}$) with decreasing pH. The explanation offered for this is centred on the pH influencing the proton exchange rate or the molecular rotation rate, but it is unlikely to be solely limited by proton exchange as there is a temperature dependence seen. Static and dynamic light scattering (SLS and DLS) studies were carried out, which indicated aggregation; this is known to affect rotational correlation time and hence proton relaxivity. Therefore gadofullerene compounds of this type are ideal candidates for pH responsive MRI contrast agents, especially as they are able to cross cell membranes for intercellular MRI applications.

Aime and co-worker developed a pH-responsive contrast agent based on reversible intramolecular anion binding (Figure 15).⁶⁹ Intramolecular ligation of β -arylsulfonamide nitrogen was rendered pH-dependent, giving rise to changes in the hydration state, q , at the Gd center. In complexes based on DO3A (GdSaDO3A), variation of the para-substituent in the arylsulfonamide moiety determines the apparent protonation constant $\log K_{\text{MLH}}$ with values of 5.7, 6.4, and 6.7 for the $-\text{CF}_3$, $-\text{CH}_3$, and $-\text{OCH}_3$ substituents, respectively. Introduction of three β -carboxyalkyl substituents, α to three ring nitrogens, inhibits displacement of the bound water by added protein and also suppresses intermolecular binding by endogenous anions (lactate, HCO_3^-). The relaxivity of GdSaDO3A increased from $2.7 \text{ mM}^{-1}\text{s}^{-1}$ at pH 9 to $10.9 \text{ mM}^{-1}\text{s}^{-1}$ at pH 5

(20 MHz, 25 °C). Such a relaxivity value suggested that a $q = 2$ complex predominates in solution below pH5. It also shows that amplification of the relaxivity changes in the pH range of 8 to 5 occurred on protein binding, and a 48% change in relaxivity was reported over the pH range 7.4 to 6.8 in 50% human serum solution.

Figure 15 The pH-dependence of GdSaDO3A

6.3 Metal ion responsive contrast agents

Metals perform a wide spectrum of specific functions associated with biological processes. Several metal ions are recognized as being indispensable to some form of

life while others are known to be very toxic. It is interesting to note that almost half of all enzymes in the human organism are dependent on the presence of one or more metal ions. It is highly desirable to have MRI contrast agents that respond to different *in vivo* concentrations of certain ions.

Iron-activated contrast agents were first developed by Desreux *et al.*⁷⁰ Gd-PhenHDO3A (Scheme 1) formed the supramolecular with iron(II) by self assembly to obtain an increase of relaxivity from 5.1 to 12.5 mM⁻¹s⁻¹ (20 MHz, 298 K) due to the increase of molecular weight and rotational correlation time, τ_R .

Scheme 1 Self-assembly of Gd(phen)HDO3A around Fe(II) resulting in an increased relaxivity

Recently Chang and co-workers designed a smart MRI contrast agent for selective copper sensing.⁷¹ This smart contrast agent is GdDO3A with a pendent iminodiacetate site for binding to Cu^{2+} . In the absence of Cu^{2+} , the pendent receptor will block inner-sphere water to access to Gd^{3+} and thus lower relaxivity ($3.76 \text{ mM}^{-1}\text{s}^{-1}$, 400 MHz, 298 K) whereas the presence of Cu^{2+} triggers about 40% relaxivity enhancement to $5.29 \text{ mM}^{-1}\text{s}^{-1}$ because the binding of Cu^{2+} to the pendent receptor unblocked the site for water access to the inner-sphere and subsequently increased the proton relaxivity (Figure 16).

Figure 16 Proposed action for sensing Cu^{2+}

6.4 Enzyme responsive contrast agents

Because aberrant enzyme catalysis has played an important role in a variety of human diseases, it is important to detect the activity and localization of enzyme associated with a disease state. Some different approaches have been reported in developing enzyme responsive MRI contrast agents.

Meade and co-workers described the first new family of MRI contrast agents⁷² that reports on specific enzymatic activity. (4,7,10-Triacetic acid-1-(2- β -galactopyranosylethoxy)-1,4,7,10-tetraazacyclododecane)gadolinium (EGad), positions a galactopyranose residue at the ninth coordination site of the Gd^{3+} ion.

Exposure of EGad to the common marker enzyme β -galactosidase (β -gal) removes the galactopyranose that sterically blocks the remaining inner-sphere site and replaces this by a water molecule, which causes an irreversible transition from a weak to a strong relaxivity state (Figure 17).

Figure 17 The transition of EGad from weak to strong relaxivity

McMurry and co-workers reported a new strategy in which a specific enzyme activity was coupled to the receptor-induced magnetization enhancement (RIME) mechanism to enhance the MRI signal. The approach relied upon enzymatic transformation of a pro-drug Gd(III) complex with poor HSA affinity and concomitant low relaxivity (a pro-RIME agent) to a species with improved HSA affinity and enhanced relaxivity. The conversion involved enzymatic removal from the Gd(III) complex of a masking group that inhibits HSA binding to expose a binding group with high HSA affinity. A pro-RIME contrast agent as shown in Figure 18, was designed to be cleaved by human carboxypeptidase B, the thrombin-activatable fibrinolysis inhibitor (TAFI).⁷³ TAFI inhibits clot degradation by cleaving C-terminal lysine residues exposed on fibrin binding sites that are recognized by fibrinolytic proteases such as tPA and plasminogen. This enzyme function at the crossroads of coagulation and fibrinolysis and has been implicated in thrombotic disease. The tri-lysine masking group was selected because charged groups have generally poor HSA affinity and the

C-terminal lysine residues were expected to be good substrates for cleavage by TAFI. The residue was expected to bind well to HSA and enhance the relaxivity when unmasked by TAFI turnover.

Figure 18 Enzymatic activation by TAFI

6.5 Site-specific contrast agents

Commonly used blood-pool contrast agents are not specific to a type of tissue. They are not distributed evenly throughout the body, preferring to be distributed within the blood stream due to their hydrophilicity.⁶⁵ Recently, the development of MRI contrast agents that specifically target different types of tissue is a rapidly growing area, some recent examples will be given to demonstrate the applicability of these types of contrast agents.

In addition to the DOTA analogues extensively used in MRI, other derivatives of this basic cyclen macrocycle have been studied including phosphonate esters,⁷⁴ and methylene phosphonates.^{75,76,77} Lukes *et al.* proposed that a bisphosphonate monoamide analogue of DOTA could be used as a potential bone imaging contrast agent.⁷⁸ The presence of phosphorus acid moieties on cyclen macrocycles has been shown to lead to faster water exchange, and enhanced relaxivities for the gadolinium(III) chelates, obviously an attractive property of the ligands.^{58,79} Previous attempts at the creation of a contrast agent featuring bisphosphonates results in a strong affinity between the contrast agent and the bone surface,⁸⁰ but the stability of these complexes was found not to be suitable for *in vivo* applications.⁸¹ Lukes *et al.* reported the properties and synthesis of trivalent lanthanide complexes of BPAMD (Figure 19) in an attempt to apply this complex to bone imaging. The relaxivity was found to be higher than expected ($5.34 \text{ mM}^{-1}\text{s}^{-1}$), higher than that of Gd-DPTA, which was considered to be surprising as the residence time of the water protons was longer than average. This is thought to be overcome by a larger rotational correlation time, giving the larger relaxivity. The potential for bone targeting was measured using a sorption experiment with hydroxyapatite used as the model for bone, and a ^{160}Tb -BPAMD analogue. The uptake was found to be 95% within an hour and reversible within about three days. In addition, after binding to the apatite, the rotational correlation time lengthens further, thus increasing the relaxivity. All the evidence points to this complex as a suitable ligand for MRI of bone tissue.

Figure 19

Another example of specific contrast agents recently reported features a steroid conjugated contrast agent developed to selectively bind to a receptor that causes a gene expression pathway.⁸² Meade *et al.* covalently attached the steroid RU-486 using an aminoxy-functionalized linker to a Gd-DOTA analogue (Figure 20). This was found to bind selectively to the progesterone receptor causing transcription of a gene, which can be used to track the cell signaling pathway. The relaxivity of this compound was $8.5 \text{ mM}^{-1}\text{s}^{-1}$, higher than commercial Gd(III) analogues and assumed to be due to the hydrophobic nature of the steroid which creates a compound amphiphilic enough to aggregate, thus increasing the rotational correlation time and hence relaxivity. This study is currently under further development to optimize the construction of the linker and chelate to provide a useful MRI contrast agent that can specifically track a cell line through the body.

Figure 20 The structure of Gd-DOTA conjugated to RU-486 as demonstrated by Meade and co-workers.⁸²

The Objective

Small Gd(III) complexes such as gadolinium chelates gadopentetate dimeglumine [Gd-DTPA], gadoterate [Gd-DOTA], Gadotericol [Gd-HP-DO3A] and gadodiamide [Gd-DTPA-BMA]^{2,7} are very stable and non-toxic. However, the use of these agents in most cases is still considered inefficient. A promising approach to increase the efficiency of a contrast agent is to increase the molecular weight or the number of Gd atoms. Recently, a number of investigations have aimed to conjugate Gd-DTPA or Gd-DOTA to polymers or macromolecules such as protein or poly-L-glutamic acid.^{83,84} Using polymers as carriers of gadolinium chelates not only influences the complex rotation rate, caused the longer rotation correlation time, τ_R for increasing the relaxivity, but also increases plasma persistence which is critical for clinical applications of contrast agents. The clinical purpose of such diagnostic media, which are designed for magnetic resonance angiography (MRA) protocols, is to visualize blood vessels for detecting abnormalities in the vascular system. For this reason, a BPA needs to be characterized by longer plasmatic half-life than the typical extracellular agents, and its distribution in the interstitial space of healthy tissues has to be minimized. Since the diffusion across the vascular endothelium depends on the size

of a given compound, the simplest approach for developing BPAs has been the synthesis of large-sized paramagnetic compounds.⁸⁵

However, most of these agents have proved to be ineffective. The relaxivities raised by simply increasing the molecular size were less than expected, the clinical applications of many current macromolecular contrast agents are limited by slow excretion from the body, and the potential toxicity of free Gd(III) ions released by metabolism of the contrast agents. Nevertheless, it has been pointed out that “aggregation of Gd(III) centers in a multimeric environment is unlikely to limit ¹H relaxivity”,² rendering the novel oligomeric Gd binding species a fertile challenging area for research which can result in new clinical agents.

In order to develop a novel contrast agent, our objective is to prepare a series of homologous contrast agents containing numbers of Gd atoms. A simple approach to such an agent would be an oligomer bearing an appropriate functional group at regular intervals along the chain. Gd binding units could be then attached at each of these functionalities. By specifically preparing oligomers with known lengths, it would then be possible to prepare agents with defined numbers of Gd atoms.

Peptides represent a series of oligomers that can be constructed in defined lengths and can carry functionality on the amino acid side chains. The technology for peptide synthesis is very well developed and uses standard protecting and coupling methodology. Besides the synthetic aspects, there are several other advantages for using peptide oligomers. First is they are both biodegradable and non-toxic. Ideally, an oligomeric MRI contrast agent should reside in the blood long enough to allow thorough MRI examinations before being degraded and cleared from the body. Peptides can be degraded by enzymes to their basic components, amino acids which

are non-toxic natural compounds. Second, the peptide oligomer can form hydrogen-bonds with water molecules, which may provide a useful route for increasing the hydration state to improve the efficacy of a contrast agent. A typical example is represented by the $[\text{GdDOTP}]^{-5}$ complex.⁸⁶ The relaxivity of this complex which does not possess any water molecules bound to the metal, is equal to the value measured for the tetracarboxylic analog $[\text{GdDOTA}(\text{H}_2\text{O})]^{-}$, where $q = 1$. This suggests that the presence of four phosphonate groups of $[\text{GdDOTP}]^{-5}$ complex promoted the formation of strong hydrogen bonds with water molecules. Third, peptide oligomers are very hydrophilic, thus the final reagents will be water soluble.

The market for MRI contrast reagents is one of the most profitable in the bioimaging industry, with companies like Gerbet, Amersham, Pfizer and Schering accumulating billions of dollars on a yearly basis. Furthermore, the increasingly important role of “molecular imaging” in all other scientific disciplines such as molecular biology, biochemistry, genetics, pharmaceutical chemistry, and bioinformatics will necessitate a shift from non-specific Gd(III)-based contrast reagents to dedicated CR demonstrating high specificity and relaxivity for the precise delineation of the loci of expression of particular genes or proteins. Therefore, the new generation of MRI contrast reagents are likely to promote and enhance a wide range of new applications going from new discoveries in basic science to dedicated and targeted therapy in patients. The potential medical, scientific economic impact of new, efficient and selective reagents is very substantial.

Glutamate and lysine possess side chains with an additional carboxylic acid group and amino group, respectively which would be ideally position for attachment of a Gd(III) binding unit. In addition, naturally occurring amino acids are commercially available as single enantiomers resulting in single diastereoisomers of oligomers. This

avoids the problematic issues of analysis and characterization of stereoisomeric mixtures. Thus, the oligomers of glutamate and lysine would offer the ideal back bone for the Gd agents. The N-terminus could be capped using succinate to provide a final attachment site for a binding unit, or using a fatty acid, such as myristic, and to promote crossing of the blood-brain barrier.⁸⁷ While the C-terminus could be either left exposed or used to attach to a biological probe for the targeting of the agent. A succinate capped glutamate trimer (Succ-(Glu)₃), which is capable of carrying four Gd binding units (R' = carboxylate protecting group) (Figure 21).

Figure 21

Gd binding agents typically employ multi-dentate species with a combination of carboxylate and amino donors. Macrocyclic agents are preferred over acyclic agents due to their greater kinetic stability *in vivo*,² and this proposal will be restricted in this way. In each case, the presence of either an additional hydroxyl or carboxylic group will allow attachment to the carboxylate side chains of the oligomer. For instance, a variety of units may be prepared starting from the tetraazamacrocyclic system, cyclen (Figure 22) in which three of the nitrogen atoms carry carboxylates, while the fourth nitrogen can be functionalized for conjugating to the oligomer.

Figure 22

The binding units can then be conjugated to the various oligomers, as shown in Scheme 2 where the binding unit is conjugated to the succinate capped glutamate trimer.

Scheme 2

Successful coupling allows for a series of oligomers to be obtained with different lengths and variable numbers of binding units, which can then be complexed with the gadolinium. The candidate contrast agents will be assayed for the quantity of

Gd uptake, and assessed by a combination of mass spectrometry and NMR spectroscopy, when it is expected that the complex will experience substantial changes in chemical shift, compared to the uncomplexed moiety.

The described approach allows us to develop strategies for the new oligomer based intravascular MRI contrast reagents by synthesizing large libraries of Gd-peptides with different sequences, peptide length, and number of Gd-chelates, and then to screen or select for oligomers with the desired properties.

Initially, we planned to synthesize the GdDO3A amino acid building block by conjugating the cyclen to amino acid. In the case of glutamate (Scheme 3), the alkynyl linker was attached to the cyclen ring then we can couple the cyclen to the glutamate by esterification.

Scheme 3 Synthetic strategy for GdDO3A glutamate complex

For lysine (Scheme 4), we introduced a different linker to the cyclen which is the propionic acid by the Michael reaction with methyl acrylate. After that the cyclen can be joined to the lysine via amidation. After complexation with Gd metal we would get the GdDO3A amino acid building blocks derived from glutamic acid and lysine.

Scheme 4 Synthetic strategy for GdDO3A lysine complex

From the successful coupling of cyclen to amino acid, we proposed the the cyclen could be attached to a longer peptide with these established strategies to get the Gd-chelate peptide.

CHAPTER II

RESULTS AND DISCUSSION

As a part of our ongoing efforts to develop a novel class of oligomer-based MRI contrast agents, we here report the synthesis of three new MRI contrast agents which are GdDO3A conjugated amino acid building blocks derived from glutamic acid (CA1) and lysine (CA2 and CA3) using alkyne and propionate liners, respectively and also their bioactivities.

Preparation of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (5)

Since the advent of Gd(III) chelates as MRI contrast agents, there has been a continuous search for improving their efficacy. As Gd(III) is characterized by a high toxicity, it is of the utmost importance to use Gd(III) complexes which are both thermodynamically stable and kinetically inert. These two properties are crucial but kinetic inertness is essential, the chelate should not decompose while they are in the body of the patient. For our Gd-binding unit, macrocyclic 1,4,7,10-tetraazacyclododecane-1,4,7-trisacetic acid (DO3A) was selected due to their great kinetic stability (Figure 23). Three of the nitrogen atoms of DO3A carry carboxylates while the fourth nitrogen atom can be functionalized for attaching to the oligomer.

Figure 23 1,4,7,10-Tetraazacyclododecane-1,4,7-trisacetic acid (DO3A)

1,4,7,10-tetraazacyclododecane is by far the most important building block for the preparation of macrocyclic lanthanide (III) complexing agents for using in MRI. However, access to the macrocycle structure remains very difficult as indicated by the high cost of the different tetraazamacrocycles commercially available. Thus, the development of a cost-effective, easy and efficient synthesis of this macrocyclic tetraamine is of the utmost important.

1,4,7,10-tetraazacyclododecane hydrochloride salt (**4**) was prepared from a commercially available triethylenetetraamine (**1**) as previously reported by Handel and co-workers⁹⁰ (Scheme 5). Triethylenetetraamine was initially rigidified by condensation with butanedione in acetonitrile at -10 °C to give the most stable product, tricyclic bis-aminal (**2**) characterized by a maximum number of six membered fused rings with the methyl groups in a *cis*-configuration in high yield (90%). In the literature⁹⁰, the bis-aminal (**2**) was purified by recrystallization from hexanes to provide the pure product in 90% yield. Unfortunately, after we tried to recrystallize the compound from hexanes, it was very difficult to get the pure bis-aminal (**2**) because of a yellow oil impurity. We modified the purification by extracting the crude product with hexanes three times to get rid of the yellow oil impurity before recrystallization from hexanes to obtain tricyclic bis-aminal (**2**) as air and heat sensitive colourless crystals.

Kolinski reported that the glyoxal-based bis-aminal species could be obtained from the condensation between glyoxal and tetraamine as a mixture of the four possible stereoisomers (*gem-cis*, *gem-trans*, *vic-cis* and *vic-trans*, see Figure 24).⁹¹ The relative proportions of these change with time and temperature and this isomerization is catalyzed by water. The *vic-trans* compound rearranges into the *gem-cis* isomer and

the *vic-cis* compound into the *gem-trans* isomer, thus leading to products characterized by the maximum number of six-membered rings. However, it is not certain that all these stereoisomers can be successfully condensed with a bis-electrophile derivative to give the macrocyclic intermediates. Moreover it is well known that the macrocyclic bis-aminals, easily obtained from the reaction between glyoxal and macrocyclic, are very stable and resistant to acid hydrolysis conditions as well as to various reducing agents^{92,93} which causes difficulties in deprotection to release the macrocyclic after cyclization step. Taking into account these various points, butanedione appeared to be an interesting alternative to glyoxal. Handel and co-workers⁹⁰ also reported that the bis-aminal derived from butanedione, is isolated as the sole *gem-cis* isomer (bis-aminal **2**).

Figure 24 The four possible stereoisomers of the bis-aminal intermediate

The *gem*-insertion of butanedione appeared to be favorable to (1+1) cycloaddition, following the literature by Handel and co-workers⁹⁰, the reaction of bis-aminal (**2**) with 1,2-dibromoethane in acetonitrile at 60 °C gave *N*-protected 1,4,7,10-tetraazacyclododecane (**3**) in moderate 52% yield with retention of the *cis*-configuration confirmed by ¹H NMR. The ¹H NMR spectrum showed the signal of protons of the two methyl groups as a singlet at 1.13 ppm. The ¹³C NMR spectrum showed that two aminal-type carbons are equivalent. These results indicated that no isomerization occurred during the cyclization step.

According to the same literature by Handel and co-workers⁹⁰, the deprotection was easily achieved under mildly acidic conditions, 10% aqueous hydrochloric acid in ethanol, leading to the corresponding 1,4,7,10-tetraazacyclododecane (cyclen, **4**) as a hydrochloride salt which was dissolved in 2 M sodium hydroxide and then extracted with dichloromethane to obtain the free cyclen **4**. Subsequently, it was found that isolation of the free amine was unnecessary, the cyclen hydrochloride salt can be used in the next step without neutralization. A direct synthesis of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (**5**) was reported by Parker and co-workers,⁹⁴ who showed that selective tri-alkylation of three of four ring nitrogens of cyclen **4** could be achieved, the fourth nitrogen being sluggish towards alkylation under mild conditions. This avoids the original route to DO3^tBu **5**, which requires prior protection of the fourth nitrogen.⁹⁵ The reaction was carried out using 3 equivalents of *tert*-butyl bromoacetate and sodium hydrogen carbonate in acetonitrile to provide 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (**5**) in a good yield of 69% after recrystallization from toluene (Scheme 5).

Scheme 5

Preparation of DO3A conjugated amino acid building blocks

1. DO3A-glutamate (20)

Glutamic acid was chosen as an amino acid building block for development of new MRI contrast agents due to its additional carboxylic side chain which is an ideal position for attaching the Gd binding unit. Therefore, we needed to prepare a protected glutamic acid with a free side-chain carboxylic acid. *N*-benzoyl α -methyl glutamate (10) was synthesized from L-glutamic acid as shown in Scheme 6. Two carboxylic groups of glutamic acid were protected by esterification using benzyl alcohol with *p*-toluenesulfonic acid in toluene to provide L-glutamic acid dibenzyl ester (6) in 83% yield. *N*-Acylation of glutamate (6) using benzoyl chloride and triethylamine in dichloromethane afforded *N*-benzoyl dibenzyl L-glutamate (7) in 94% yield after column chromatography.

Coutsogeorgopoulos and Zervas reported that the γ -benzyl ester of *N*-trityl dibenzyl L-glutamate (11) could be selectively saponified by using lithium hydroxide in acetone to give the trityl α -benzyl glutamate (12) as shown in Scheme 7.⁹⁶ Therefore, we applied this conditions to our *N*-benzoyl dibenzyl L-glutamate (7) and expected that the γ -benzyl ester would be hydrolysed to yield *N*-benzoyl α -benzyl L-glutamate.

Scheme 6

Scheme 7

Interestingly, we found that the α -benzyl ester was removed instead of the γ -benzyl ester to give *N*-benzoyl γ -benzyl L-glutamate (**8**). It was presumed that the chelation of the lithium ion with the carbonyls of the amide and the α -benzyl ester renders the α -benzyl ester more reactive than the γ -benzyl ester (Figure 25) which did not happen in Zervas's case, because the *N*-substituent of their glutamate is a trityl

group which is very hindered, therefore the hydroxyl prefers to attack the carbonyl of the γ -benzyl ester (more positive, less sterically hindered). In this case, the NMR data could not distinguish between cleavage of the α -benzyl ester or the γ -benzyl ester. Therefore, the structure of *N*-benzoyl γ -benzyl L-glutamate (**8**) was elucidated from its X-ray crystal structure analysis as shown in Figure 26.

Figure 25

Figure 26 X-rays crystal structure analysis of *N*-benzoyl γ -benzyl L-glutamate (**8**)

Esterification of *N*-benzoyl γ -benzyl L-glutamate (**8**) using methyl iodide and potassium hydrogen carbonate in DMF afforded *N*-benzoyl α -methyl γ -benzyl L-glutamate (**9**) in high yield (97%) after column chromatography. Deprotection of the γ -benzyl ester by hydrogenolysis using hydrogen gas in the presence of a small amount

of palladium on carbon in ethyl acetate gave *N*-benzoyl α -methyl L-glutamate (**10**) in quantitative yield.

Due to the low yield of the α -selective deprotection of dibenzyl ester (**7**), a shorter alternative pathway was developed for preparation of *N*-benzoyl γ -benzyl L-glutamate (**8**), starting from a commercially available L-glutamic acid (Scheme 8). The γ -carboxylic group was first selectively protected as benzyl ester using benzyl alcohol in 60% aqueous sulfuric acid, the previously reported method developed by Asano *et al.*,⁹⁷ to obtain the L-glutamic acid γ -benzyl ester (**13**) in 48% yield and recovered starting material. The structure of glutamate **13** was confirmed by comparison of its ¹H NMR with the literature data.⁹⁸

N-Acylation of ester (**13**) with benzoyl chloride and sodium hydrogen carbonate in dry dichloromethane gave *N*-benzoyl γ -benzyl L-glutamate (**8**) in good yield (74%). Methylation and subsequent removal of the benzyl group by hydrogenolysis yielded *N*-benzoyl α -methyl L-glutamate (**10**) which was identical with the previously prepared glutamate **10**.

Scheme 8

Having in hand the 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (**5**), we then sought a suitably functionalized linker to attach to

the Gd-DO3A that would allow conjugation to the glutamate. The alkyne **16**, previously developed in our group, was chosen as that required linker because it was anticipated that it would be easy to attach it to the free NH of the cyclen ring *via* nucleophilic substitution. After appending this linker to macrocyclic core and following removal of the TBS protecting group, the DO3'Bu-butynyl alcohol would be formed which would be readily reactive toward most electrophiles especially activated carboxylic acid. The alkyne **16** was synthesized from commercially available butyne-1,4-diol (**14**) as shown in Scheme 9. The selective mono-protection of butyne-1,4-diol was carried out by using an excess amount of butyne-1,4-diol (5 equivalents) with *tert*-butyldimethylsilyl chloride (TBSCl) in the presence of imidazole in THF. After removal of excess diol by washing with H₂O, the TBS-monoprotected butyne-1,4-diol (**15**) was obtained in excellent yield (93%) based on TBSCl. Mesylation of alkyne **15** with methanesulfonyl chloride (MsCl) and triethylamine in dry CH₂Cl₂ gave propargyl mesylate **16** in good yield which was used without further purification in the following step.

Scheme 9

The propargyl mesylate (**16**) was attached to the cyclen (**5**) using potassium carbonate in acetonitrile at 60 °C to give the cyclen (**17**) in 80% yield after purification

by column chromatography. The cyclen (**17**) was reacted with tetrabutyl ammonium fluoride to remove the TBS group, providing the cyclen (**18**) which was subsequently coupled to the *N*-benzoyl α -methyl L-glutamate (**10**) by dicyclohexylcarbodiimide (DCC) with a small amount of 4-dimethylaminopyridine (DMAP) in dry dichloromethane to give the cyclen-glutamate (**19**) in 74% yield after column chromatography over silica gel (Scheme 10). The structure of cyclen-glutamate (**19**) was confirmed by analysis of its spectroscopic data (^1H , ^{13}C NMR, IR and mass spectrometry). The ^1H NMR (300 MHz) spectrum of DO3^tBu-glutamate **19** showed the signals of methylene protons next to the triple bond, $\text{NHCH}_2\text{C}\equiv\text{C}$ and $\text{C}\equiv\text{CCH}_2\text{CO}_2$, at 3.38 and 4.63 ppm, respectively and also of methyl ester protons at 3.77 ppm. The characteristic ^{13}C NMR (100 MHz) signals of the alkynyl carbons, were apparent at 81.4 and 79.6 ppm, as well as the quaternary carbons of *tert*-butyl groups at 82.8 and 82.6 ppm together with the aromatic carbons peaks at 133.6, 131.7, 128.5 and 127.5 ppm. Moreover, the mass spectrum showed the molecular ion (M^+) and the molecular ion with sodium ($[\text{M}+\text{Na}]^+$) signals at 830 and 853, respectively.

Scheme 10

Before adding Gd(III) to the ligand **19**, The *tert*-butyl protecting groups were removed using trifluoroacetic acid (TFA) in dichloromethane to afford DO3A-glutamate (**20**) which was characterized by ^1H , ^{13}C NMR spectroscopy and mass spectrometry (ESI-TOF) (Scheme 11). The ^1H NMR (300 MHz) spectrum of DO3A-glutamate (**20**) showed that the ^1H signals and quaternary carbon signals of *tert*-butyl groups disappeared with acid. The mass spectrum showed the molecular ion signal (M^+) at 661 and molecular weight plus proton signal ($[M+H]^+$) at 662 and also the ^{13}C NMR spectrum showed the absence of quaternary carbon of CF_3 . These indicated that DO3A-glutamate (**20**) was in the free acid form, not as its trifluoroacetate salt. The DO3A-glutamate (**20**) was then used for the next step without purification.

Scheme 11**2. DO3A-lysine (27)**

The protected lysine with a free side-chain amine was required for coupling with the cyclen ring. Lysine derivative **23** was synthesized from commercially available N_ϵ -carbobenzyloxy-L-lysine, H-Lys(Z)-OH, as shown in Scheme 12. The reaction of H-Lys(Z)-OH with benzoyl chloride in 2 M aqueous sodium hydroxide and dichloromethane gave the amide **21** in high yield (85%). Esterification of amide **21** using methyl iodide and potassium hydrogen carbonate in DMF yielded ester **22** in excellent yield (91%). Deprotection of the ϵ -*N*-carbobenzyloxy group was first tried by hydrogenolysis using balloon hydrogen gas in the presence of a small amount of palladium on carbon. Unfortunately, the reaction proved to be inefficient. After that we tried the reaction again by balloon hydrogen gas with the long needle dip into the reaction mixture. The reaction proceeded smoothly to provide lysine **23** in quantitative yield, which was used in the next step without further purification. It should be noted that the deprotection of the ϵ -*N*-carbobenzyloxy group of lysine **22** by using formic

acid as a proton source instead of hydrogen gas also proceeded very well to give lysine **23** in quantitative yield.

Scheme 12

The aza-Michael addition: preparation of DO3'Bu-methyl ester (**24**)

In order to conjugate the DO3'Bu **5** to lysine derivative **23**, we planned to employ a hetero-Michael addition to introduce an electrophilic functional group to the cyclen ring. The aza-Michael addition of DO3'Bu with methyl acrylate, following by hydrolysis of the methyl ester would give a carboxylic acid function which would be available to react with the side-chain amine of protected lysine.

The cyclen ester **24** was synthesized from cyclen **5** and methyl acrylate using hetero-Michael addition. Optimization of the reaction conditions is shown in Table 1.

Table 1 Aza-Michael addition reaction of cyclen **5** and methyl acrylate

Entry	Conditions	Yield (%) ^a
1	EtOH, rt, 16 h	0 ^b
2	EtOH, reflux, 20 h	0 ^b
3	CH ₃ CN, rt, 16 h	0 ^b
4	CH ₃ CN, DBU, rt, 20 h	59 ^b
5	CH ₃ CN, DBU, 50 °C, 20 h	89 ^b

^aIsolated yield after purification by column chromatography^bStarting material was recovered

Firstly, the reaction was carried out in ethanol at room temperature overnight (Table 1, entry 1). Unfortunately, the conjugate addition failed to yield the corresponding adduct **24**. We then increased the reaction temperature by heating at reflux in ethanol (Table 1, entry 2). Again, the reaction proved to be ineffective, the adduct **24** was not observed by ¹H NMR or mass spectrometry. Changing solvent to acetonitrile (Table 1, entry 3) was also not successful.

In 2007, Kim and co-workers developed a convenient and versatile method for aza-Michael addition using a substoichiometric amount of 1,8-diaza-bicyclo[5.4.0]undec-7-ene (DBU).⁹⁹ Consequently, we attempted the Michael addition reaction in the presence of 0.5 equivalent of DBU in acetonitrile at room temperature.

As expected, the reaction proceeded quite well to give the adduct **24** in moderate yield (59%) with recovered starting material (Table 1, entry 4). To improve the yield of the product the reaction temperature was increased to 50 °C, and we obtained adduct **24** in a satisfying yield of 89% after purification by flash column chromatography over silica gel (Table 1, entry 5).

With readily available DO3^tBu methyl ester **24** in hand, hydrolysis of the methyl ester was achieved in good yield using lithium hydroxide, revealing the carboxylic acid **25** after quenching with H₂O, and following by extraction with dichloromethane, which is ready for coupling with the lysine derivative **23** (Scheme 13).

Scheme 13

Afterwards, DCC mediated coupling reaction between DO3^tBu carboxylic acid **25** and lysine **23** was carried out in the presence of HOBt in dry dichloromethane at 0 °C overnight to give the DO3^tBu-lysine **26** in 52% yield after purification by flash column chromatography over silica gel. The structure of DO3^tBu-lysine **26** was evident from the ¹³C NMR, IR spectroscopy and mass spectrometry (ESI-TOF). The ¹³C NMR spectrum showed the presence of five different kinds of carbonyl carbon at 173.3, 173.0, 172.9, 172.1, and 167.7 ppm. The IR signal of O-H stretching of carboxylic acid at 3379 cm⁻¹ disappeared after coupling and the characteristic IR signals of C=O and N-H stretching were shown at 1660 and 3297 cm⁻¹, respectively.

The mass spectrum presented signals at m/z 833 and 834 corresponding to the M^+ and $[M+H]^+$ species, respectively. Cleavage of the *tert*-butyl esters was achieved in quantitative yield using trifluoroacetic acid, TFA, revealing the DO3A carboxylic acid **27** which is ready for incorporation of gadolinium (Scheme 14).

Scheme 14

Incorporation of gadolinium ion

In an initial model investigation for incorporation of gadolinium, the DO3A methyl ester **28** was chosen as a ligand in order to find the reaction conditions. The DO3A methyl ester **28** was obtained by deprotection of *tert*-butyl esters with trifluoroacetic acid from the previously synthesized DO3^{*t*}Bu methyl ester **24** and used in the next step without purification (Scheme 15).

Scheme 15

As can be seen in Table 2, the DO3A derivative **28** was subjected to metallation using gadolinium nitrate ($\text{Gd}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$) with sodium hydroxide in H_2O . After proceeding overnight at room temperature, no product could be detected by mass spectrometry (ESI-TOF), but the signal of a starting material was present (Table 2, entry 1). From the unsatisfactory metallation using gadolinium nitrate ($\text{Gd}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$), we then attempted to use gadolinium oxide (Gd_2O_3) instead by refluxing in water. Again, the metallation proved to be ineffective. Gd complex **29** was not observed by mass spectrometry (ESI-TOF) (Table 2, entry 2). Fortunately, metallation using either gadolinium chloride (GdCl_3) or gadolinium perchlorate ($\text{Gd}(\text{ClO}_4)_3$) in methanol and H_2O with buffer, 0.5 M aqueous ammonium acetate to adjust and maintain the pH of the reaction at 5.8, proceeded smoothly by heating at 70°C for 2 hours. After purification by reverse-phase HPLC, the desired complex **29** was obtained in 40 and 33% yield, respectively (Table 2, entry 3, 4). The analytical HPLC trace of Gd-complex **29** is shown in Annex 1. It should be noted that an attempt to purify by flash column chromatography over silica gel gave an unsatisfactory yield (~10%) of the desired product.

Table 2 Metallation of DO3A methyl ester **28**

Entry	Conditions	Yield (%) ^a
1	Gd(NO ₃) ₃ ·6H ₂ O, NaOH, rt, overnight	0 ^b
2	Gd ₂ O ₃ , H ₂ O, reflux	0 ^b
3	GdCl ₃ , NH ₄ OAc, MeOH, H ₂ O, 70 °C, 2 h	40
4	Gd(ClO ₄) ₃ , NH ₄ OAc, MeOH, H ₂ O, 70 °C, 2 h	33

^aIsolated yield after purification by reverse-phase HPLC^bStarting material was recovered

The electrospray ionization-time of flight mass spectrum (ESI-TOF-MS) showed that complexation had occurred, as seen by the characteristic gadolinium isotope distribution pattern from naturally occurring gadolinium (Gd), composed of 6 stable isotopes, ¹⁵⁴Gd (2.18%), ¹⁵⁵Gd (14.80%), ¹⁵⁶Gd (20.47%), ¹⁵⁷Gd (15.65%), ¹⁵⁸Gd (24.84%) and ¹⁶⁰Gd (21.86%), and 1 radioisotope, ¹⁵²Gd (0.20%), for [complex **29** + H]⁺ and [complex **29** + Na]⁺ (see Annex 2).

GdDO3A-glutamate (CA1)

The DO3A-glutamate ligand (**20**) was subjected to complexation using gadolinium chloride (GdCl₃) and ammonium acetate, to adjust the pH to 5.8, in methanol and H₂O at 70 °C for 2 hours to afford the GdDO3A-glutamate **CA1** in 32% yield after purification by reverse phase high pressure liquid chromatography (Scheme

16). The analytical HPLC trace of complex **CA1** is shown in Annex 3. As in the case of Gd-complex **29**, the correct gadolinium isotope distribution pattern was observed in the ESI-TOF-MS for $[M+H]^+$ GdDO3A-glutamate **CA1** (see Annex 4).

Scheme 16

GdDO3A-lysine (CA2)

With the DO3A-lysine chelate ligand in hand, the complexation with Gadolinium ions was carried out with gadolinium trichloride (GdCl₃) using ammonium acetate, to maintain the pH of the reaction at 5.8, in methanol and H₂O at 70 °C for 2 hours. After purification by reverse-phase HPLC, the desired complex **CA2** was obtained in 31% yield (Scheme 17). The analytical HPLC trace of complex **CA2** is shown in Annex 5. The ESI-TOF-MS characterization of complex **CA2** confirmed the formation of the product with the gadolinium isotope distribution pattern (see Annex 6).

Scheme 17

Determination of bioactivities of GdDO3A-glutamate (CA1) and GdDO3A-lysine (CA2)

Relaxivity measurement

Relaxivity measurements were conducted on a Varian 9.4T MRI system (Palo Alto, CA, USA). The longitudinal relaxation time (T_1) of water proton was measured in aqueous phantoms with concentrations of 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.00625 mM Gd^{3+} , using the inversion recovery spin echo sequence. Longitudinal relaxivity (r_1 in $\text{mM}^{-1}\text{s}^{-1}$) were obtained from linear least-squares determination of the slopes of $1/T_1$ vs $[\text{Gd}^{3+}]$ plots (Figure 27, 28). The correlation of relaxivity with Gd^{3+} concentration was linear with an R^2 value of 0.9877, 0.9976 and 0.9975 for Gd methyl ester **29**, **CA1** and **CA2**, respectively. The study of their relaxivity showed the longitudinal relaxivity (r_1) of three CAs (4.2, 6.4 and $5.4 \text{ mM}^{-1}\text{s}^{-1}$ in H_2O at 25°C for Gd methyl ester **29**, **CA1** and **CA2**, respectively) are higher than those of the clinically used MRI contrast agents [3.9 and $4.1 \text{ mM}^{-1}\text{s}^{-1}$ in H_2O at 25°C for Gd-DOTA (Magnevist, Bayer Schering, Germany) and Gd-DTPA (Dotarem, Guerbet, France), respectively]. The higher longitudinal relaxivities of **CA1** and **CA2** than that of the

GdDO3A methyl ester **29** presumably due to higher molecular weight of **CA1** and **CA2** compared to the GdDO3A methyl ester **29**, rendering the rotation of the molecule was slow down. The slower rotation of the molecule leads to higher relaxivity. Compared to **CA2**, **CA1** had higher longitudinal relaxivity, r_1 possibly because the triple bond in **CA1** making the molecule more rigid. The higher rigidity of the molecular system leads to higher proton relaxivity.^{100,101}

Figure 27 Longitudinal relaxivity (r_1) of GdDO3A methyl ester **29**

Figure 28 Longitudinal relaxivity (r_1) of GdDO3A-glutamate (CA1) and GdDO3A-lysine (CA2)

In vivo imaging study

An *in vivo* study was conducted on Wistar rats (male, weight 320-340 g) under 2% isoflurane anesthesia. CAs were injected (dosage: 0.04 mmol Gd/kg body weight) through the tail vein. T_1 -weighted images were acquired every 6.4 s for 52 min with T_1 -weighted gradient echo sequence (TR/TE = 50/3 ms, flip-angle = 20 degree, resolution = 230 μ m, thickness = 2 mm). *In vivo* imaging studies of the two CAs in Wistar rats demonstrated that signal enhancement in the brain artery occurred immediately after tail vein injection (Figure 29). Time courses of MRI signal enhancement (up to 52 min post-injection) in the brain artery showed considerable signal enhancement (about 50%) for CA2 while lower signal enhancement for CA1 (about 30%). Compared with Dotarem (Guerbet, France), which showed a similar peak enhancement as CA2, the enhancement by CA2 remained high (about 30%) even at 52 mins post-injection. The signal in the brain artery decayed mono-exponentially

with blood half-lives 24.5, 68.1 and 19.8 for **CA1**, **CA2** and Dotarem, respectively (Figure 30).

Although **CA1** had higher relaxivity in H_2O than **CA2**, the *in vivo* study showed that **CA2** had much longer blood half-life. This indicates that **CA2** may bind to serum albumin in plasma¹⁰²⁻¹⁰⁵ or be collagen-targeted^{106,107} in the blood vessel. Protein binding in human plasma will be evaluated. Therefore, **CA2** could be advantageous over the Gd(III) chelates, namely an extended lifetime in the blood pool which is necessary for magnetic resonance angiography applications, to image the detail of blood vessels and tissue targeting.

Figure 29 MR images of the brain artery in Wistar male rat before and after **CA2** compound injection. A. pre-injection, B. post-injection.

Figure 30 Time course of MRI signal enhancement in the brain artery during with CA1, CA2 and Dotarem (dose 0.04 mmol Gd /kg of body weight)

Preparation of peptide oligomers

1. Glu-Glu dipeptide (34)

After the successful synthesis of GdDO3A conjugated amino acid contrast agents, our next target was to synthesize dipeptide Gd-chelates contrast agent. Initially, Glu-Glu dipeptide (34) was synthesized from the previously synthesized *N*-benzoyl γ -benzyl L-glutamate (8) and α -methyl γ -benzyl L-glutamate (32), which was obtained from glutamate 13 with a yield of 84% over three steps, by reaction with di-*tert*-butyldicarbonate (Boc₂O) to yield the glutamate 30. Methylation with methyl iodide gave glutamate 31 and the corresponding α -methyl γ -benzyl L-glutamate (32) was obtained by cleaving the Boc group by treatment with trifluoroacetic acid in dichloromethane (Scheme 18), using standard protecting and coupling methodology. An attempt to optimize the coupling reaction conditions is shown in Table 3.

Scheme 18

Table 3 Coupling reaction

Entry	Conditions	Yield (%) ^a
1	DCC, DIPEA, CH ₂ Cl ₂	8 ^b
2	DCC, HOBT, DIPEA, CH ₂ Cl ₂	12 ^b
3	IBC, NMM, THF/DMF	0 ^b
4	HBTU, HOBT, DIPEA, THF	40 ^b
5	T3P, EtOAc	22 ^b
6	T3P, NEt ₃ , CH ₂ Cl ₂	27 ^b

^aIsolated yields after purification by chromatography^bUnidentified side-products formed and recovered starting material

The coupling reaction of glutamate **8** and **32** was initially attempted by using a standard coupling agent, DCC with *N,N*-diisopropylethylamine, DIPEA to give the corresponding dipeptide **33** in very low yield with recovered starting materials and unidentified side-products after purification using column chromatography over silica gel (Table 3, entry1). We then tried DCC-mediated coupling reaction again with *N*-hydroxybenzotriazole, HOBT, but the coupling reaction proved to be ineffective. The dipeptide **33** was obtained in a little higher yield, but still considered to be very low (Table 3, entry 2). From the unsatisfactory coupling reaction using DCC, we then attempted to use isobutyl chloroformate, IBC, with *N*-methylmorpholine, NMM.

Unfortunately, the reaction failed to yield the corresponding dipeptide **33** (Table 3, entry 3). The HBTU-mediated coupling with HOBt and DIPEA in THF eventually led to the desired dipeptide in an useable yield of 40% (Table 3, entry 4). Modest yields of 22% and 27% of dipeptide **33** were obtained, when the coupling reactions were carried out using T3P without base in ethyl acetate (Table 3, entry 5), and T3P with triethylamine in dichloromethane (Table 3, entry 6), respectively. The benzyl ester groups of dipeptide **33** were removed by treatment with hydrogen gas in the presence of 10% palladium on carbon leading to dipeptide **34** in quantitative yield which is ready to be conjugated with the cyclen chelate ligand **18** (Scheme 19).

Scheme 19

In the mean time, we also tried the coupling reaction of glutamate **30** with glutamate **32** using T3P with *N,N*-diisopropylethylamine in dichloromethane. The reaction proceeded smoothly, no observable epimerization from crude NMR spectrum and the dipeptide **35** was isolated in higher yield of 68% (Scheme 20).

Scheme 20

Afterwards, the Boc group of dipeptide **35** was removed by treatment with trifluoroacetic acid in dichloromethane to give dipeptide **36**. *N*-Acylation of dipeptide **36** was carried out using acetic anhydride with DMAP and triethylamine to give dipeptide **37** in 85% yield. Removal of the benzyl ester groups with H_2 in the presence of a small amount of palladium on carbon in ethyl acetate gave dipeptide **38** in quantitative yield which was ready to conjugate to the cyclen chelate ligand **18** (Scheme 21).

Scheme 21

2. Ac-Lys-Lys-OtBu (**42**)

Preparation of Lys-Lys dipeptide **41** was initially attempted by synthesis of Ac-Lys(Z)-OH (**39**) and H-Lys(Z)-O^tBu (**40**) as shown in Scheme 22. Ac-Lys(Z)-OH (**39**) was synthesized from a commercially available H-Lys(Z)-OH according to the previously reported procedure by Kohn and co-workers¹⁰⁸ using acetic anhydride, triethylamine and sodium hydroxide to provide the corresponding lysine derivative **39** in good yield. By esterification with isobutylene in the presence of concentrated sulfuric acid in dioxane in a sealed tube, lysine derivative **40** was obtained in 88% yield.

Scheme 22

Both of these lysines could be coupled together to obtain the corresponding dipeptide **41** in an excellent yield of 97%, by treatment with T3P with DIPEA and DMAP. Deprotection of the carbobenzyloxy (Z) groups by treatment with H₂ in the presence of palladium on carbon yielded dipeptide **42** in quantitative yield without purification, the dipeptide was ready for coupling with cyclen chelate ligand (Scheme 23).

Scheme 23

Attempts to couple dipeptide with cyclen chelate ligand

The key step for the synthesis of GdDO3A-dipeptide complex was the coupling of dipeptide with cyclen chelate ligand. With PhOC-Glu-Glu-OMe (**34**) in hand, we then tried to conjugate to cyclen **18**, which was used in our previous synthesis of DO3'Bu-glutamate **19**, by the same reaction conditions, treatment with DCC and DMAP in dichloromethane. Unfortunately, after the reaction was stirred at -5 °C overnight, no desired product was detected by ¹H NMR or mass spectrometry. Our attempt to improve the reaction conditions by changing the solvent to acetonitrile instead of dichloromethane, again, failed to yield the corresponding product (Scheme 24). Similarly, the coupling reaction of dipeptide **38** with cyclen **18** using an alternative coupling agent, PyBOP in the presence NMM and DMAP, proved to be ineffective. Dipeptide **44** was not observed in the ¹H NMR spectrum or by mass spectrometry (Scheme 25).

Scheme 24

Scheme 25

From the unsatisfactory coupling of Glu-Glu dipeptide and having in hand the Lys-Lys dipeptide **42**, we now turned our attention to the conjugation of Lys-Lys dipeptide **42** with cyclen **25**, which was previously used to conjugate with lysine monomer **26**. As illustrated in Table 4, Our first attempt with HBTU in the presence of HOBT and NMM in a mixture of dichloromethane and *N,N*-dimethylformamide failed to provide the conjugated product **45**. An unidentified complex mixture was obtained

with recovered starting material (Table 4, entry 1). The conjugation using T3P with DIPEA and DMAP in dichloromethane proved to be unsuccessful, the desired conjugated product again was not detected by ^1H NMR as well as mass spectrometry (Table 4, entry 2). An attempt to couple Lys-Lys dipeptide **42** with DO3'Bu carboxylic acid **25** by treatment with PyBOP in the presence of HOBt and NMM in a combination of dichloromethane and dimethylformamide, also failed to obtain the corresponding conjugated product (Table 4, entry 3). The WSC-mediated coupling was our last attempt for conjugation Lys-Lys dipeptide **42** with DO3'Bu carboxylic acid **25**. Again, the conjugation proved to be ineffective. The desired product was not observed by ^1H NMR and mass spectrometry (Table 4, entry 4).

Table 4 Coupling reaction

Entry	Conditions	Yield
1	HBTU, HOBt, NMM, CH ₂ Cl ₂ /DMF	0 ^a
2	T3P, DIPEA, DMAP, CH ₂ Cl ₂	0 ^a
3	PyBOP, HOBt, NMM, CH ₂ Cl ₂ /DMF	0 ^a
4	WSC, HOBt, DMF	0 ^a

^aUnidentified side-products formed and recovered starting material

The study of spacer effect

From several unsuccessful attempts to conjugate dipeptide with cyclen chelate ligand, it was suspected that the two side chains of the dipeptide were too close to each other creating a hindered environment when the conjugation was attempted. Therefore, a spacer was incorporated into the Lys-Lys dipeptide, glycine, **48** to separate the two side chains and give more space for conjugation. The glycine derivative **48** was prepared from commercially available glycine. According to the previous reported procedure by Senet and co-workers,¹⁰⁹ protection of the amino group of glycine with benzyl chloride in 1 M sodium hydroxide provided *N*-protected glycine **46** in an excellent yield of 95%. A first attempt at esterification of glycine **46** with isobutylene in the presence of concentrated sulfuric acid in dioxane did not give a satisfactory result. The protected glycine **47** was obtained in very low yield (10%) due to the low solubility of starting material. Alternatively, the protected glycine **47** was prepared in higher yield of 41% by treatment with *tert*-butanol and 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide, EDCI in the presence of DMAP in dichloromethane. Removal of the benzyloxycarbonyl group with hydrogen gas in the presence of a small amount of palladium on carbon in THF gave glycine **48** in quantitative yield which was used in the next step without further purification (Scheme 26).

Scheme 26

The incorporation of the spacer glycine **48** into Lys-Lys dipeptide **42** started from the coupling of lysine **39** with glycine **48** using T3P with DIPEA in the presence of a small amount of DMAP in dichloromethane and *N,N*-dimethylformamide to obtain dipeptide **49** in 87% yield. The acid labile protecting group of dipeptide **49** was cleaved with trifluoroacetic acid to give carboxylic acid **50** in quantitative yield and it was used in the next step without purification. The next step involved coupling of carboxylic acid **50** with lysine **40** by treatment with T3P, DIPEA and DMAP in a mixture of dichloromethane and *N,N*-dimethylformamide led to Lys-Gly-Lys tripeptide **51** in moderate yield (68%). Hydrogenolysis of **51** using hydrogen gas in the presence of a small amount of palladium on carbon yielded the Lys-Gly-Lys tripeptide **52** in quantitative yield (Scheme 27).

Scheme 27

With our designed molecule **52** that consisted of a glycine spacer separating two lysine residues in hand, the study of spacer effect of conjugation between tripeptide **52** and DO³tBu carboxylic acid **25** was carried out by treatment with T3P in the presence of DIPEA and DMAP in a combination of dichloromethane and dimethylformamide. Unexpectedly, the reaction proved to be unsuccessful. The desired conjugated product was not observed by ¹H NMR and mass spectrometry (Scheme 28).

Scheme 28

In 2006, Bhujwalla *et al.*¹¹⁰ reported the conjugation of DOTA ester with poly-L-lysine by using *N*-hydroxysuccinimide, NHS ester of DOTA, which was prepared from DO3tBu carboxylic **54** acid with *N*-hydroxysuccinimide by treatment with HBTU in acetonitrile, followed by removal of *tert*-butyl groups using trifluoroacetic acid as shown in Scheme 29. Therefore, we applied their method to our conjugation, starting from esterification of cyclen **25** with *N*-hydroxysuccinimide using HBTU in acetonitrile gave DO3tBu-NHS ester **57** (Scheme 30) which was used to couple with tripeptide **52** without purification. The coupling again was proved to be ineffective as monitored by ¹H NMR and mass spectrometry (Scheme 31).

Scheme 29

Scheme 30

Scheme 31

To confirm that the spacer effect caused the unsuccessful conjugation of Lys-Lys dipeptide with the cyclen chelate ligand, we also studied the coupling of Lys-Gly dipeptide **60** which was prepared from a commercially available glycine following the previous literature by Poojary and co-workers¹¹¹ by treatment with acetic anhydride in H_2O to yield the glycine derivative **58** in good yield (74%), further coupling to the lysine **40** using T3P with DIPEA and DMAP in dichloromethane and *N,N*-dimethylformamide gave the Lys-Gly **59** in 87% yield, following by hydrogenolysis of **59** using hydrogen gas in the presence of palladium on carbon obtained the Lys-Gly dipeptide **60** in quantitative yield, with DO_3^tBu carboxylic acid **25** using T3P with DIPEA and DMAP in a mixture of dichloromethane and *N,N*-dimethylformamide. The reaction failed to yield the corresponding product **61** (Scheme 32). It was concluded that the unsuccessful conjugation of Lys-Lys dipeptide **42** with the cyclen **25** is not due to the steric hindrance between two side chains of Lys-Lys dipeptide.

Scheme 32

“Click” chemistry: to link dipeptide to a macrocyclic

After many unsuccessful attempts at the conjugation of dipeptides to cyclen chelate ligands with standard coupling methods attempts, we now turned our attention to “click” chemistry for coupling. “Click” chemistry is a modular approach that uses only the most practical and reliable chemical transformations. Its applications are increasingly found in all aspects of drug discovery, ranging from lead finding through

combinatorial chemistry and target-templated *in situ* chemistry, to proteomics and DNA research, using bioconjugation reactions. The click reaction, copper(I)-catalyzed 1,2,3-triazole formation from azide and terminal acetylenes is a particularly powerful linking reaction (Scheme 33), due to its high degree of dependability, complete specificity, and the bio-compatibility of the reactants. The triazole products are more than just passive linkers, they readily associate with biological targets, through hydrogen bonding and dipole interactions. This triazole moiety may also increase the relaxivity of contrast agent from second-sphere relaxation where water molecules hydrogen bonded to the triazole nitrogen atoms are relaxed.

Scheme 33

A number of copper(I) sources can be used directly in the click cycloaddition. Sharpless and co-workers¹¹² reported that the Cu(I) catalyst is better prepared *in situ* by reduction of copper(II) salts, which are less costly and often purer than copper(I) salts (copper(II) sulfate pentahydrate serves well). As the reductant to reduce copper(II) to copper(I), ascorbic acid and/or sodium ascorbate has proved to be excellent.

The mechanism as shown in Scheme 34 was proposed by Sharpless.¹¹² It starts unexceptionally with formation of the copper(I) acetylide (**I**)¹¹³, extensive density functional theory calculations offer compelling evidence which strongly disfavors the concerted [2+3] cycloaddition (B-direct) and points to a stepwise, annealing sequence

(B1→B2→B3, hence the term “ligation”), which proceeds via the six-membered copper-containing intermediate **III**.¹¹⁴

Scheme 34 Proposed catalytic cycle for the Cu(I)-catalyzed ligation; [Cu] represents either mononuclear or dinuclear copper(I) complex fragment

In 2005, Sharpless and Fokin reported the computational studies which revealed a stepwise mechanism (B1→B3, Scheme 34) involving unprecedented metallacycle intermediates.¹¹⁵ It appears to be common for a variety of dipoles other than organic azide. The proposed mechanism accounts for the key experimental observations. First, the dramatic rate increase observed in the copper-catalyzed synthesis of 1,2,3-triazoles is in excellent agreement with the computed activation barriers, which are as much as 11 kcal/mol lower than in the corresponding concerted cycloadditions. Second, the exclusive regioselectivity of the copper(I)-catalyzed processes is both predicted computationally and observed experimentally. Third, the proposed mechanism suggests that dipoles other than organic azide should engage in the analogous stepwise sequence with copper(I) acetylides.

A stepwise pathway with copper(I) acetylide catalysys and a copper(I) triazolide intermediate is generally assumed (B1→B3, Scheme 33). In 2005, Fokin and Finn suggested that the alkyne-azide click reaction presumably involves dinuclear copper(I) complexes in the rate-determining step by indirect kinetic measurement (Scheme 35).¹¹⁶

Scheme 35 Outline of species involved in the copper catalytic cycle^x

The isolation of a molecular copper(I) triazolide complex was reported by Straub and co-workers.¹¹⁷ It is a piece of direct evidence for the existence of such structures, which to date have only been assumed as intermediates in the alkyne-azide click reaction. Mechanistic studies can go beyond indirect kinetic measurements of reactions towards the direct investigation of elementary steps.

A model study of the click reaction was first tried with the Lys-Gly azide **63** and propargyl alcohol. The Lys-Gly azide **63** was prepared from the previously synthesized dipeptide **60** by reaction with chloroacetic anhydride in the presence of potassium carbonate and DMAP in dichloromethane to give chloride **62** in good yield

(73%), following by treatment with sodium azide to give Lys-Gly azide **63** in moderate yield (Scheme 36). The IR spectrum of azide **63** showed the characteristic azide peak at 2104 cm^{-1} . The azide-alkyne cycloaddition of Lys-Gly azide **66** and propargyl alcohol using 2 mol% of copper sulfate (CuSO_4) and 5 mol% of sodium ascorbate in a 1:1:1 mixture of water, *tert*-butanol and dichloromethane as expected, proceeded smoothly to give 87% of the corresponding triazole **64** which was characterized by ^1H NMR and mass spectrometry (Scheme 37). The ^1H NMR spectrum showed a new peak at 7.82 ppm corresponding to the triazole ring proton and the disappearance of the peak at 2.51 ppm typical for the terminal alkyne proton found in propargyl alcohol.

Scheme 36

Scheme 37

Our next target was the synthesis of the GdDO3A lysine building block using the click reaction. To design the appropriate DO3A derivative for attachment to peptides via Cu(I)-catalyzed azide-alkyne cycloaddition, we decided to introduce the alkyne functionality to the DO3A derivative. As shown in Scheme 38, the synthesis of cyclen alkyne **65** was started from previously prepared cyclen carboxylic acid **30**. Esterification of carboxylic acid **30** with propargyl alcohol by treatment with T3P in the presence of triethylamine and DMAP in dichloromethane gave propargyl ester **65** in good yield (84%).

Scheme 38

Alternatively, cyclen propargyl ester **65** was prepared by avoiding two steps, from cyclen **5** with propargyl acrylate **66** using the Michael addition reaction. The propargyl acrylate **66** was obtained in 64% yield by alkylation of propargyl alcohol

with acryloyl chloride and DIPEA in dichloromethane. The reaction of cyclen **5** with propargyl acrylate **66** in the presence of DBU in acetonitrile gave cyclen propargyl ester **65** in 66% yield as illustrated in Scheme 39.

Scheme 39

Azido functionalized lysine **70** was readily accessible, by *N*-acylation of lysine **45** using acetic anhydride and triethylamine and aqueous solution of sodium hydroxide and dichloromethane, following by hydrogenolysis by treatment with H_2 in the presence of palladium on carbon to give lysine **68**. Subsequently, lysine **68** was reacted with chloroacetic anhydride in the presence of potassium carbonate and DMAP in dichloromethane to obtain, in the excellent yield, the chloride **69** which was treated with sodium azide in *N,N*-dimethylformamide to give azide **70** in 93% yield (Scheme 40). After our positive result with the model study (azide-alkyne cycloaddition of azide **63** and propargyl alcohol) we attempted to couple the cyclen alkyne **65** with azido functionalized lysine **70** using the click reaction (Table 5).

Scheme 40

Table 5 Click reaction

Entry	Conditions	Yield ^a
1	2 mol% CuSO ₄ , 5 mol% Na ascorbate, H ₂ O/ ^t BuOH/CH ₂ Cl ₂	0 ^b
2	2 mol% CuSO ₄ , 5 mol% Na ascorbate, H ₂ O/ ^t BuOH/CH ₂ Cl ₂ , microwave	trace amount ^b
3	10 mol% CuSO ₄ , 10 mol% Na ascorbate, H ₂ O/ ^t BuOH	0 ^b
4	5 mol% CuSO ₄ , 10 mol% Na ascorbate, H ₂ O/THF	0 ^b

^aDetected by ¹H NMR and mass spectrometry^bRecovery of starting material

As illustrated in Table 5, the azide-alkyne cycloaddition of azide **70** and cyclen alkyne **65** was first attempted by treatment with 2 mol% of copper sulfate and 5 mol% of sodium ascorbate in a 1:1:1 mixture of water, *tert*-butanol and dichloromethane. The reaction failed to yield the desired triazole product **71**, and only recovered starting material was observed by ¹H NMR and mass spectrometry (Table 5, entry 1). An

attempt to promote the azide-alkyne cycloaddition under identical conditions but using microwave irradiation (80 °C, 120 W) proved to be unsuccessful, only a trace amount of triazole **71** was observed by mass spectrometry (Table 5, entry 2). When the amount of copper sulfate and sodium ascorbate were increased to 10 mol% in a 1:1 mixture of water and *tert*-butanol (Table 5, entry 3), the desired triazole **71** still was not observed by ¹H NMR and mass spectrometry. When the cycloaddition reaction was carried out by changing the solvent to water and THF using 5 mol% copper sulfate and 10 mol% sodium ascorbate, the reaction also failed to give the desired product (Table 5, entry 4).

Many attempts to couple the azide **70** with cyclen alkyne **65** via the Cu(I)-catalyzed cycloaddition reaction using a small amount of copper sulfate failed with only trace amounts of product detectable (Table 5, entry 2). The cause of the low Cu(I)-catalyzed azide-alkyne cycloaddition efficiency was attributed to the strong coordination of copper ions to the macrocyclic core, making it unavailable for further catalysis. However, there are three ways to overcome this obstacle, first is coupling the lysine azide **70** with propargyl acrylate **66** using click reaction before the conjugation to cyclen **5**. The second is an increase in the copper concentration. The last is adding the gadolinium ion to the cyclen ring of DO'Bu alkyne **65** before coupling to the azide **70** via click reaction. Initially, we tried to solve the problem by coupling the lysine azide **70** and propargyl acrylate **66** by treatment with 2 mol% of copper sulfate and 5 mol% of sodium ascorbate in 1:1:1 mixture of water, *tert*-butanol and dichloromethane. The reaction proceeded smoothly to give the corresponding triazole **72** in good yield (87%) as shown in Scheme 41.

Scheme 41

Unfortunately, the conjugation of triazole **72** and cyclen **5** with the Michael reaction using DBU in acetonitrile at 50 °C overnight fail to yield the desired product **71** (Scheme 42).

Scheme 42

An attempt to overcome the problem of copper sequestration by increasing the copper concentration to a molar ratio of 1.3 to 1 of copper to cyclen **65** with 1.8 equivalents of sodium ascorbate in a 1:1:1 mixture of water, *tert*-butanol and dichloromethane was found to generate the desired triazole **71** in modest yield (10%) after being stirred overnight. The structure of triazole product **71** was confirmed by ¹H NMR and mass spectrometry. The ¹H NMR spectrum included the characteristic peak at 7.75 ppm of triazole ring as well as the disappearance of a peak at 2.47 typical for the primary alkyne proton found in cyclen **65**. The yield of the triazole product **71** was improved to 32% under identical conditions but using microwave irradiation at 80 °C, 120 W for 1 hour. Some improvement (about 8%) was shown when the reaction was allowed to proceed for a longer time (3 days) at room temperature as shown in Table 6.

Table 6 Click reaction

Entry	Conditions	Yield (%) ^a
1	1.3 eq. CuSO_4 , 1.8 eq. Na ascorbate, $\text{H}_2\text{O}/t\text{BuOH}/\text{CH}_2\text{Cl}_2$, rt, overnight	10
2	1.3 eq. CuSO_4 , 1.8 eq. Na ascorbate, $\text{H}_2\text{O}/t\text{BuOH}/\text{CH}_2\text{Cl}_2$, microwave, 80 °C, 120 W, 1 h	32
3	1.3 eq. CuSO_4 , 1.8 eq. Na ascorbate, $\text{H}_2\text{O}/t\text{BuOH}/\text{CH}_2\text{Cl}_2$, rt, 3 days	40

^aIsolated yields after purification by chromatography

The completion of the click reaction between the cyclen alkyne **65** and azide **70** was monitored with ESI-TOF-MS which showed that the alkyne **65** was completely consumed, followed by removal of the copper catalyst by extraction from the organic phase to the aqueous 1 wt% sodium EDTA solution. The organic phase was extracted

until the blue colouration due to Cu^{2+} disappeared. After that the crude product was purified by flash column chromatography. It should be stated that the removal of the copper was conveniently monitored using ESI-TOF-MS technique.

Cleavage of the *tert*-butyl esters by treatment with trifluoroacetic acid in dichloromethane gave the DO3A-lysine triazole **73** in quantitative yield which was ready for incorporation with gadolinium ion (Scheme 43). The structure of triazole **73** was confirmed by ^1H NMR (400 MHz) where the signals corresponding to the *tert*-butyl protons disappeared. The mass spectrum (ESI-TOF-MS) showed the molecular ion signal (M^+) at 728, molecular weight with proton ($[\text{M}+\text{H}]^+$) at 729, and molecular weight with sodium ($[\text{M}+\text{Na}]^+$) at 729.

Scheme 43

Incorporation of gadolinium ions

A molar ratio of 1:1 between the DO3A-lysine triazole **73** and gadolinium(III) chloride, GdCl_3 were stirred in methanol and H_2O at 70°C for 2 hours using 0.5 M aqueous ammonium acetate to adjust pH to ~ 5.8 to obtain the GdDO3A -lysine triazole

CA3 in 22% yield (Scheme 44) after purification by using reverse-phase HPLC, the analytical HPLC trace of Gd-complex **CA3** is shown in Annex 7. The ESI-TOF-MS technique was used to monitor the incorporation of Gd^{3+} . The mass spectrum showed the gadolinium isotope distribution pattern signals of the $[\text{CA3} + \text{H}]^+$ and $[\text{CA3} + \text{Na}]^+$ (see Annex 8), it implies that a Gd complex was formed.

Scheme 44

Due to the unsatisfactorily low yield of the click cycloaddition between DO3^tBu alkyne **65** and lysine azide **70**, we moved to the third strategy, the addition of the gadolinium ion to the cyclen ring of DO3^tBu alkyne **65** before click reaction. As can be seen in Scheme 45, the *tert*-butyl esters of DO3^tBu alkyne **65** were removed by trifluoroacetic acid in dichloromethane to provide DO3A alkyne **74** in quantitative yield. The incorporation of Gd^{3+} to the alkyne **74** was allowed to proceed by treatment with gadolinium(III) chloride in methanol and H_2O at room temperature for 1.5 hours, and using 0.5 M aqueous ammonium acetate buffer to adjust the pH to 5.8. The crude

product was washed with absolute ethanol to remove the impurity to give the Gd complex **75** as a white powder in 42% yield. In the work up step, we first removed the solvent by evaporation with heat and it was found that the product decomposed, therefore it is necessary to use a freeze dryer to remove the solvent (methanol and water) after reaction. The ESI-TOF-MS data showed that the Gd^{3+} ion was incorporated in the macrocycle (see Annex 9).

Scheme 45

Attempts to couple the azide **70** and the alkynyl GdDO3A **75** via click cycloaddition using are summarized in Table 7.

Table 7 click reaction of azide **70** and GdDO3A **75**

Entry	Condition	Yield ^a
1	2 mol% CuSO ₄ , 5 mol% Na ascorbate, H ₂ O/ ^t BuOH/CH ₂ Cl ₂ , overnight	0 ^b
2	2 mol% CuSO ₄ , 5 mol% Na ascorbate, H ₂ O/ ^t BuOH/CH ₂ Cl ₂ , microwave, 80 °C, 120 W, 30 mins	0 ^c
3	2 mol% CuSO ₄ , 5 mol% Na ascorbate, H ₂ O/ ^t BuOH, 2 days	trace amount ^b

^aDetected by mass spectrometry^bRecovery of starting material^cDecomposition of starting material

We firstly tried the reaction by treatment with 2 mol% of copper(II) sulfate and 5 mol% sodium ascorbate in a 1:1:1 mixture of H₂O, *tert*-butanol, and dichloromethane at room temperature overnight. Unfortunately, the cycloaddition proved to be inefficient (Table 7, entry 1). The click reaction under the identical

conditions using microwave at 80 °C, 120 W for 30 minutes (Table 7, entry 2) also failed to give the conjugated complex **76**. By changing the solvent to a 1:1 mixture of H₂O and *tert*-butanol using the same molar ratio of copper(II) sulfate and sodium ascorbate, the reaction proved to be unsuccessful with only a trace amount of Gd complex **76** being observed by mass spectrometry (Table 7, entry 3).

Currently, optimization of the click cycloaddition of the lysine azide **70** and the alkynyl GdDO3A **76** is still in progress for further synthesis of Gd-dipeptide complex. In order to synthesize the Gd-dipeptide, the bis-azido Lys-Lys derivative **78** was prepared from the previously synthesized Lys-Lys dipeptide **42**. The reaction of Lys-Lys **42** with chloroacetic anhydride in the presence of potassium carbonate and DMAP in dichloromethane yielded the bis-chloro Lys-Lys **77** in good yield (73%) which was treated with sodium azide in *N,N*-dimethylformamide to obtain the bis-azido Lys-Lys derivative **78** in 89% yield which is ready for click reaction (Scheme 46).

The click reaction model study of the bis-azido Lys-Lys derivative **78** was carried out with propargyl alcohol using 2 mol% of copper(II) sulfate and 5 mol% sodium ascorbate per azide in a 1:1:1 mixture of H₂O, *tert*-butanol, and dichloromethane at room temperature overnight. The reaction proceeded smoothly to provide bis-triazole **79** in 85% yield after purification by flash column chromatography (Scheme 46). From the successful model study of click reaction, our next target is to synthesize the GdDO3A bis-triazole lysine dipeptide by click reaction of lysine dipeptide and cyclen propargyl ester, which is in progress (Scheme 47). After incorporating the Gd metal, we would get the GdDO3A bis-triazole lysine dipeptide. After that this method could be applied for further preparation of a number of Gd-chelate peptides.

Scheme 46

Scheme 47

Relaxivity measurement of CA3

Longitudinal relaxivity (r_1 in $\text{mM}^{-1}\text{s}^{-1}$) were also obtained from linear least-squares determination of the slopes of $1/T_1$ vs $[\text{Gd}^{3+}]$ plots same as those of **CA1** and **CA2** (Figure 31). The correlation of relaxivity with Gd^{3+} concentration was linear with an R^2 value of 0.9926. The relaxivity measurement of **CA3** showed a much greater value ($22.9 \text{ mM}^{-1}\text{s}^{-1}$ in H_2O at 25°C) than those of the clinically used MRI contrast agents [3.9 and $4.1 \text{ mM}^{-1}\text{s}^{-1}$ in H_2O at 25°C for Gd-DOTA (Magnevist, Bayer Schering, Germany) and Gd-DTPA (Dotarem, Guerbet, France), respectively] and

previously synthesized **CA1** and **CA2** (6.4 and $5.4 \text{ mM}^{-1}\text{s}^{-1}$ in H_2O at 25°C , respectively). It may be suggested that the high relaxivity of **CA3** probably comes from the triazole moiety which makes the molecule more rigid. The rigid nature of the triazole linker hinders the local rotation of the complex which leads to higher proton relaxivity.^{100,101} Another presumption is that the high relaxivity may come from the second-sphere relaxation where water molecules hydrogen bonded to the triazole nitrogen atoms are relaxed *via* dipolar mechanism. The paramagnetic relaxation of the water protons originates from the dipole-dipole interactions between the nuclear spins and the fluctuating local magnetic field caused by the unpaired electron spins. This magnetic field around the paramagnetic center vanishes rapidly with distance. Therefore, specific chemical interactions that bring the water protons into the immediate proximity of the metal ion play an important role in transmitting the paramagnetic effect towards the bulk solvent. At this point, it is highly interesting to further investigate the MRI imaging of this complex and its analogs.

Figure 31 Longitudinal relaxivity (r_1) of **CA3**

CHAPTER III

CONCLUSIONS

In conclusion, to develop a new novel Gd-chelate oligomer-based intravascular MRI contrast agent, two MRI contrast agents (CAs), Gd-DO3A conjugated amino acid building blocks derived from glutamate (CA1) and lysine (CA2) have been synthesized using the standard coupling reagent to couple the GdDO3A to an amino acid. These two MRI contrast agents exhibited longitudinal relaxivities (r_1) higher than those of the commercial MRI contrast agents, Magnevist and Dotarem. The *in vivo* imaging experiment results demonstrated that CA2 provide high contrast enhancement similar to the clinically used Gd-DTPA (Dotarem), but has much longer blood half-life.

Unfortunately, conjugation of GdDO3A scaffold to dipeptides by the standard coupling reagents, failed to obtain the corresponding conjugated product. Therefore, we have developed the conjugation of cyclen core to lysine by the Cu(I)-catalyzed 1,3-dipolar cycloaddition, “click” reaction leading to the successful synthesis of GdDO3A-lysine triazole **76**. This conjugation method will be applied for further preparation of Gd-chelate dipeptide.

CHAPTER IV

EXPERIMENTAL SECTION

General Methods

When required, reactions were carried out under an inert atmosphere of nitrogen in oven-dried glassware. Dichloromethane and acetonitrile were dried from CaH_2 immediately prior to use. All other solvents and reagents were used as received or purified if required, using standard methods.¹¹⁸ IR spectra were recorded on a Bio-Rad FTS 165 spectrometer or Bruker ALPHA-E, either neat or as Nujol mulls using NaCl plates. ^1H NMR spectra were recorded on a Bruker AV300 (300 MHz), or AV400 (400 MHz), or AV500 (500 MHz), or Jeol ECA400 (400 MHz), or Jeol ECA400SL (400 MHz) with residual protic solvent as the reference. ^{13}C spectra were recorded at the corresponding frequencies on the same instruments. NMR data are reported as follows: ^1H NMR chemical shifts are in parts per million (ppm) and coupling constants, J , are in Hertz (Hz). Multiplicities are reported as singlet (s), broad singlet (br s), triplet (t), quartet (q), and multiplet (m). ^{13}C spectra were recorded in ppm with residual non-deuterated solvent peak as the internal standard. Mass spectra were recorded on a Finnigan LCQ DECA XP MAX with ESI mode. High-resolution mass spectra were recorded on a Finnigan MAT95XP instrument, also using EI mode. Optical rotations were measured at the sodium D line (589 nm), with a Jasco P-7030 polarimeter using a 10 mm path length cell and are given in units of $10^{-1}\text{degcm}^2\text{g}^{-1}$. HPLC was performed on a Shimadzu system using reverse-phase column with detection by UV. Analytical reverse-phase HPLC was performed on Alltima C18 column (250×3.0 mm, 5 μm 100 °A) at flow rate of 1.0 mL/min and semi-preparative HPLC was performed on Kromacil C18 column (250×10.0 mm, 5 μm 100 °A) at flow rate of 2.5 mL/min. The eluent was a linear gradient from water (0.1% TFA) to acetonitrile (0.1% TFA) over 30 minutes. The retention time (t_R) of HPLC is given in minutes with the gradient in percentage of acetonitrile.

Bis-aminal (2).⁹⁰

A solution of butanedione (7 mL, 80 mmol) in dry acetonitrile (150 mL) was added dropwise to a cooled (-10 °C) and stirred solution of triethylenetetramine (19.9 mL, 80 mmol) in dry acetonitrile (150 mL). The reaction mixture was stirred at -10 °C for 2 hours. The solvent was removed under reduced pressure and the residue was extracted with hexanes (3×100 mL). The combined extracts were concentrated and kept in a refrigerator overnight. The precipitate was filtered to give the product as colourless crystals (14.12 g, 71.9 mmol, 90% yield).

mp 60°C dec (Lit⁹⁰ dec with heat), R_f =0.36 (15% MeOH/CH₂Cl₂)

$\nu_{\max}/\text{cm}^{-1}$ 3294, 2721, 2507, 1489, 1319, 1290, 1258, 1242, 1190, 1140, 1125, 1109, 1076, 1016, 937, 875, 850;

δ_{H} (400 MHz; CDCl₃) 3.10–2.54 (m, 12H, 6×CH₂), 1.87 (br s, 2H, 2×NH), 1.37 (s, 3H, CH₃), 1.17 (s, 3H, CH₃);

δ_{C} (100 MHz; CDCl₃) 77.1, 68.2, 53.3, 48.3, 41.0, 24.2, 12.6;

m/z , 197 ([M+H]⁺).

Butanedione-protected 1,4,7,10-tetraazacyclododecane (3).⁹⁰

1,2-Dibromoethane (10.3 mL, 120 mmol) was added to a suspension of bis-aminal (2) (16.81 g, 85.6 mmol) and potassium carbonate (59.18 g, 428 mmol) in dry acetonitrile (700 mL). The mixture was stirred at 60 °C under N₂ for 2 days. The reaction mixture was then allowed to cool to room temperature, before being filtered through celite and the filtrate was evaporated to dryness. The crude product was

purified by flash column chromatography (Al_2O_3 , 2% MeOH in CH_2Cl_2) to give the product as a orange oil (9.87 g, 44.4 mmol, 52% yield).

$R_f=0.50$ (15% MeOH/ CH_2Cl_2)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3385, 2983, 2900, 2818, 1491, 1441, 1369, 1323, 1275, 1244, 1213, 1194, 1179, 1132, 1082, 1038, 1009, 949, 847, 748;

δ_{H} (400 MHz; CDCl_3) 3.04-2.91 (m, 4H, $2\times\text{CH}_2$), 2.91-2.78 (m, 8H, $4\times\text{CH}_2$), 2.76-2.64 (m, 4H, $2\times\text{CH}_2$), 1.13 (s, 6H, $2\times\text{CH}_3$);

δ_{C} (100 MHz; CDCl_3) 78.3, 48.4, 47.7, 13.5;

m/z , 223 ($[\text{M}+\text{H}]^+$).

1,4,7,10-Tetraazacyclododecane (4).⁹⁰

10% aqueous hydrochloric acid (400 mL) was added to a stirred solution of butanedione-protected 1,4,7,10-tetraazacyclododecane (3) (7.32 g, 32.9 mmol) in ethanol (200 mL). The reaction mixture was stirred at 60 °C for 2 days. The reaction mixture was concentrated to about 100 mL and ethanol (100 mL) was added. The mixture was kept in a refrigerator overnight. The precipitate was filtered and washed with ethanol to give the cyclen hydrochloride salt which was then taken up in saturated aqueous sodium hydroxide. The mixture was extracted with dichloromethane and evaporated to give cyclen 4 as a colourless solid (4.57 g, 26.3 mmol, 80% yield).

mp 109-111 °C, $R_f=0.30$ (15% MeOH/ CH_2Cl_2)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3275, 1740, 1605, 1296, 1261, 1246, 1150, 1111, 1049, 941, 841, 810, 745;

δ_{H} (400 MHz; CDCl_3) 2.67 (s, 16H, $8\times\text{CH}_2$), 2.12 (s, 4H, $4\times\text{NH}$);

δ_{C} (100 MHz; CDCl_3) 46.1;

m/z , 173 ($[\text{M}+\text{H}]^+$).

1,4,7-Tris(*tert*-butoxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (5).¹¹⁹

tert-Butyl bromoacetate (3.1 mL, 20.8 mmol) was added dropwise over 10 minutes to a suspension of 1,4,7,10-tetraazacyclododecane (**4**) (1.09 g, 6.3 mmol) and sodium hydrogen carbonate in freshly distilled acetonitrile (40 mL) under N₂ at 0 °C. After the addition, the reaction mixture was allowed to reach ambient temperature and stirred for a further 48 hours. The mixture was filtered through celite and the filtrate was evaporated under reduced pressure to leave a beige solid. Recrystallization from toluene afforded the title ester **5** as a colourless solid (2.24 g, 4.35 mmol, 69%)

mp 181-183 °C (Lit¹¹⁹ 178-180 °C), *R*_f=0.60 (15% MeOH/CH₂Cl₂)

$\nu_{\text{max}}/\text{cm}^{-1}$ 2734, 1728, 1367, 1162, 1147, 849;

δ_{H} (300 MHz; CDCl₃) 3.39 (s, 4H, 2×CH₂CO₂), 3.31 (s, 2H, CH₂CO₂), 3.18-3.06 (m, 4H, 2×CH₂NH), 3.03-2.81 (m, 12H, 6×CH₂N), 1.48 (s, 27H, *t*-Bu);

δ_{C} (100 MHz; CDCl₃) 170.5, 169.6, 162.3, 81.7, 58.2, 51.4, 49.2, 47.5, 28.2;

m/z, 515 (M⁺);

4-(*tert*-Butyldimethylsilyloxy)but-2-yn-1-ol (15).

A solution of TBSCl (3.50 g, 23.2 mmol) in dry THF (13 mL) was added dropwise to stirred solution of butyne-1,4-diol (10 g, 116.2 mmol) and imidazole (1.74 g, 25.3 mmol) in dry THF (26 mL). The reaction mixture was stirred for 3 hours. Saturated aqueous ammonium chloride (20 mL) and H₂O (10 mL) were then added, and the mixture was extracted with ethyl acetate (3×30 mL). The organic layer was washed with H₂O (3×30 mL) and brine before drying over anhydrous magnesium

sulfate. The solvent was removed and the crude product was purified by distillation (80 °C, 4 mmHg) to give silyl ether **15** as a yellow oil (4.33 g, 21.6 mmol, 93% yield).

$R_f=0.51$ (50% EtOAc/hexanes)

$\nu_{\max}/\text{cm}^{-1}$ 3368, 2955, 2930, 2859, 1472, 1462, 1362, 1256, 1132, 1082, 1015, 837, 777;

δ_{H} (400 MHz; CDCl_3) 4.32 (s, 2H, CH_2OSi), 4.26 (s, 2H, CH_2OH), 0.89 (s, 9H, *t*-Bu), 0.10 (s, 6H, $2\times\text{CH}_3$);

δ_{C} (100 MHz; CDCl_3) 84.4, 83.1, 51.8, 51.2, 25.9, 18.4, -5.1.

4-(*tert*-Butyldimethylsilyloxy)but-2-ynyl methanesulfonate (**16**).

Methanesulfonyl chloride (1.83 mL, 23.60 mmol) was added to a stirred solution of 4-(*tert*-butyldimethylsilyloxy)but-2-yn-1-ol (**15**) (4.30 g, 21.46 mmol) and triethylamine (4.20 mL, 30.05 mmol) in dry dichloromethane (70 mL) at 0 °C. The reaction mixture was stirred for 2 hours, then the reaction mixture was quenched with H_2O (30 mL) and extracted with diethyl ether (3×30 mL). The combined organic layers were washed with H_2O , brine and dried over anhydrous magnesium sulfate. The solvent was removed to give sulfonate **16** a yellow oil (4.90 g, 17.6 mmol, 82% yield).

$R_f=0.56$ (50% EtOAc/hexanes)

$\nu_{\max}/\text{cm}^{-1}$ 3426, 2955, 2930, 2859, 1472, 1464, 1362, 1256, 1177, 1144, 1086, 976, 945, 837, 806, 779;

δ_{H} (400 MHz; CDCl_3) 4.81 (s, 2H, CH_2OSi), 4.30 (s, 2H, CH_2OSO_2), 3.07 (s, 3H, CH_3SO_2), 0.86 (s, 9H, *t*-Bu), 0.05 (s, 6H, $2\times\text{CH}_3$);

δ_{C} (100 MHz; CDCl_3) 88.7, 57.9, 51.6, 39.1, 25.8, 18.3, -5.2.

***tert*-Butyl 2,2',2''-(10-(4-(*tert*-butyldimethylsilyloxy)but-2-ynyl)-1,4,7,10-tetraaza-cyclododecane-1,4,7-triyl)triacetate (17).**

A solution of 4-(*tert*-butyldimethylsilyloxy)but-2-ynyl methanesulfonate (**16**) (1.40 g, 5.04 mmol) in dry acetonitrile (32 mL) was added to a stirred solution of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7-tetraazacyclododecane (**5**) (2 g, 3.88 mmol) and potassium carbonate (1.34 g, 9.70 mmol) in dry acetonitrile (64 mL). The reaction mixture was stirred at 60 °C for 24 hours. The reaction mixture was then allowed to cool to room temperature, before being filtered through celite and the filtrate was evaporated to dryness. The crude product was purified by flash column chromatography (SiO₂, 10% MeOH in CH₂Cl₂) to give cyclen **17** as a pale yellow solid (2.52 g, 3.62 mmol, 93% yield).

mp 61-63°C, *R_f*=0.45 (15% MeOH/CH₂Cl₂)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3325, 2924, 2851, 2673, 2492, 1728, 1713, 1454, 1369, 1307, 1258, 1227, 1157, 1107, 1076, 1011, 895, 837, 775;

δ_{H} (400 MHz; CDCl₃) 4.23 (s, 2H, NCH₂C≡C), 3.34 (s, 2H, CH₂OSi), 3.24-2.18 (m, 22H, 11×CH₂N), 1.44 (s, 18H, 2×*t*-Bu), 1.39 (s, 9H, *t*-Bu), 0.85 (s, 9H, *t*-Bu), 0.06 (s, 6H, 2×CH₃);

δ_{C} (100 MHz; CDCl₃) 173.6, 172.8, 84.1, 82.8, 82.5, 79.2, 56.8, 55.9, 51.5, 51.1, 50.4, 46.4, 43.3, 27.9, 27.8, 25.8, 18.2, -5.2;

m/z, 719 ([M+Na]⁺), 697 (M⁺);

HRMS Found 719.4750 ([M+Na]⁺, C₃₆H₆₈N₄O₇SiNa requires 719.4755).

***tert*-Butyl 2,2',2''-(10-(4-hydroxybut-2-ynyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (18).**

Tetra-*n*-butylammonium fluoride (1.71 g, 5.42 mmol) was added to a stirred solution of cyclen **17** (1.89 g, 2.71 mmol) in THF (18 mL). The reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was diluted with dichloromethane (10 mL) and washed with H₂O (3×15 mL), brine and dried over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 8% MeOH in CH₂Cl₂) to give alcohol **18** as a waxy brown solid (1.06 g, 1.82 mmol, 67% yield).

mp 72–74°C, *R*_f=0.39 (15% MeOH/CH₂Cl₂)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3395, 2978, 2936, 2839, 1724, 1639, 1454, 1369, 1312, 1227, 1161, 1107, 1015, 849, 756;

δ_{H} (400 MHz; CDCl₃) 4.27 (s, 2H, CH₂OH), 3.37 (s, 2H, NCH₂C≡C), 3.18 (s, 4H, 2×CH₂CO₂), 3.06 (s, 2H, CH₂CO₂), 2.98–2.20 (m, 16H, 8×CH₂N), 1.49 (s, 18H, 2×*t*-Bu), 1.43 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl₃) 173.6, 172.6, 85.4, 82.8, 82.5, 78.6, 56.9, 55.9, 50.1, 43.5, 28.0, 27.9;

m/z, 606 ([M+Na]⁺), 583 (M⁺);

HRMS Found 583.4059 (M⁺, C₃₀H₅₄N₄O₇ requires 583.4071).

1-Methyl5-(4-(4,7,10-tris(2-*tert*-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)but-2-ynyl)2-benzamidopentanedioate (19).

A solution of cyclen **18** (1 g, 1.72 mmol) in dry dichloromethane (6 mL) was added to a stirred solution of *N,N'*-dicyclohexylcarbodiimide (0.39 g, 1.89 mmol) and a small amount of *N,N*-dimethyl aminopyridine in dry dichloromethane (12 mL) at -5 °C. After being stirred for 10 minutes, a solution of 4-benzamido-5-methoxy-5-oxopentanoic acid (0.46 g, 1.72 mmol) in dry dichloromethane (6 mL) was added dropwise. The reaction mixture was stirred at -5 °C for 18 hours. The reaction mixture was filtered through celite and the filtrate was evaporated to dryness. The crude product was purified by flash column chromatography (SiO₂, 10% MeOH in CH₂Cl₂) to give ester **19** as a glass-like solid (1.14 g, 1.37 mmol, 80% yield).

mp 67-68°C, *R_f*=0.48 (15% MeOH/CH₂Cl₂)

[α]_D²² = 12.7 (c = 0.70, MeOH)

*ν*_{max}/cm⁻¹ 3392, 2979, 2836, 1722, 1651, 1537, 1454, 1383, 1369, 1310, 1227, 1158, 1104;

δ_H (300 MHz; CDCl₃) 7.94 (d, *J*=6.8 Hz, 2H, Ar-H), 7.66 (br d, *J*=7.2 Hz, 1H, NH), 7.53-7.38 (m, 3H, Ar-H), 4.80-4.72 (m, 1H, CH), 4.62 (s, 2H, C≡CCH₂CO₂), 3.75 (s, 3H, CH₃), 3.38 (s, 2H, NCH₂C≡C), 3.13 (s, 4H, 2×CH₂CO₂), 3.04 (s, 2H, CH₂CO₂), 2.98-2.17 (m, 20H, 8×CH₂N and 2×CH₂ of glutamate), 1.46 (s, 18H, 2×*t*-Bu), 1.41 (s, 9H, *t*-Bu);

δ_C (100 MHz; CDCl₃) 173.7, 173.6, 172.9, 172.4, 167.3, 133.6, 131.7, 128.5, 127.5, 82.8, 82.6, 81.4, 79.6, 56.9, 55.9, 53.5, 52.5, 52.3, 46.1, 43.2, 30.4, 27.9, 27.8, 26.9;

m/z, 853 ([M+Na]⁺), 830 (M⁺);

HRMS Found 830.4896 (M⁺, C₄₃H₆₈N₄O₁₁ requires 830.4915).

2,2',2''-(10-(4-(4-Benzamido-5-methoxy-5-oxopentanoyloxy)but-2-ynyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (20).

Trifluoroacetic acid (5 mL) was added to a stirred solution of cyclen **19** (1 g, 1.20 mmol) in dry dichloromethane (7 mL). The reaction mixture was stirred at room temperature for 16 hours. After removal of solvent under reduced pressure, acid was removed by addition and evaporation of successive portion of dichloromethane (2×10 mL), methanol (2×10 mL) and diethyl ether (2×10 mL), respectively to give cyclen **20** as a pale yellow solid which was used in the next step without further purification.

mp 81-83°C, $R_f=0.13$ (15% MeOH/ CH_2Cl_2)

$[\alpha]^{22}_{\text{D}} = 5.4$ ($c = 1.24$, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3400, 1720, 1646, 1540, 1353, 1178, 1128, 1086, 799, 719;

δ_{H} (300 MHz; CD_3OD) 7.87 (d, $J=7.2$ Hz, 2H, Ar-H), 7.63-7.44 (m, 3H, Ar-H), 4.78 (s, 2H, CH_2CO_2), 4.68 (dd, $J=9.3, 5.4$ Hz, 1H, CH), 4.11 (s, 2H, CH_2N), 3.95-3.70 (m, 6H, CH_2CO_2), 3.77 (s, 3H, CH_3), 3.51-3.11 (m, 16H, CH_2), 2.57 (t, $J=6.6$ Hz, 2H, CH_2) 2.34 (dt, 1H, CH_2), 2.20-2.04 (m, 1H, CH_2);

δ_{C} (125 MHz; CD_3OD) 172.3, 172.2, 169.1, 167.0, 133.6, 131.7, 128.3, 127.2, 81.6, 77.0, 53.5, 52.1, 51.6, 51.5, 49.7, 48.8, 48.5, 46.5, 29.6, 25.8;

m/z , 662 ($[\text{M}+\text{H}]^+$), 661(M^+);

HRMS Found 662.3038 ($[\text{M}+\text{H}]^+$, $\text{C}_{31}\text{H}_{43}\text{N}_5\text{O}_{11}$ requires 662.3037)

GdDO3A-glutamate (CA1).

A solution of GdCl_3 (0.4 g, 1.51 mmol) in H_2O (3 mL) was added to a stirred solution of cyclen **20** (1 g, 1.51 mmol) in methanol (10 mL). The pH was adjusted and maintained at 5.8 using 0.5 M aqueous ammonium acetate. The reaction mixture was stirred at 70 °C for 2 hours. After removal of solvent under reduced pressure, the crude product was purified by semipreparative reverse phase HPLC (2→60% CH_3CN , t_{R} =12.9 min) to give complex **CA1** as a pale yellow solid (0.34 g, 0.42 mmol, 37% yield).

mp 101-102°C

$[\alpha]_{\text{D}}^{21} = -10$ ($c = 0.02$, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3412, 2873, 2427, 1681, 1602, 1437, 1365, 1203, 1134, 1084, 944, 836, 801, 723;

m/z , 811, 813, 814, 815, 816, 817, 819 ($[\text{M}+\text{H}]^+$), 833, 835, 836, 837, 838, 839, 841 ($[\text{M}+\text{Na}]^+$);

HRMS Found 817.1994 ($[\text{M}+\text{H}]^+$, $\text{C}_{31}\text{H}_{41}\text{N}_5\text{O}_{11}^{158}\text{Gd}$ requires 817.2044) with Gd isotope distribution pattern.

***tert*-Butyl 2,2',2''-(10-(3-methoxy-3-oxopropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (24).**

1,8-Diazabicyclo[5.4.0]undec-7-ene, DBU (54 μ L, 0.36 mmol) was added to a stirred suspension of 1,4,7-tris(tert-butoxycarbonylmethyl)-1,4,7-tetraazacyclododecane (**5**) (0.37 g, 0.72 mmol) and methyl acrylate (0.1 mL, 1.08 mmol) in acetonitrile (3 mL). After being stirred at 50 $^{\circ}$ C for 20 hours, The solvent was removed and the residue was taken up in dichloromethane (10 mL), washed with saturated aqueous ammonium chloride, H₂O, and brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 10% MeOH in CH₂Cl₂) to give cyclen **24** as a white foam (0.39 g, 0.65 mmol, 89% yield).

mp 71-72 $^{\circ}$ C, R_f =0.55 (15% MeOH/CH₂Cl₂)

$\nu_{\max}/\text{cm}^{-1}$ 3402, 2978, 2847, 1728, 1667, 1454, 1369, 1312, 1227, 1161, 1107, 849, 756;

δ_{H} (300 MHz; CDCl₃) 3.66 (s, 3H, CH₃), 3.30-1.90 (m, 26H, CH₂), 1.47 (s, 18H, 2 $\times$ *t*-Bu), 1.44 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl₃) 173.2, 173.0, 172.9, 82.4, 82.2, 56.5, 55.8, 51.9, 50.5, 50.3, 30.8, 28.0;

m/z , 623 ([M+Na]⁺), 601 ([M+H]⁺);

HRMS Found 601.4177 ([M+H]⁺, C₃₀H₅₆N₄O₈ requires 601.4176).

3-(4,7,10-Tris(2-*tert*-butoxy-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)-propanoic acid (25).

Aqueous lithium hydroxide (3 mL of a 1 M solution) was added to a stirred solution of cyclen **24** (0.25 g, 0.42 mmol) in MeOH (9 mL) at 0 °C. The reaction mixture was stirred at room temperature for 4 hours. The solvent was removed and the residue was taken up in dichloromethane (10 mL), washed with H₂O. The solvent was removed under reduced pressure to give cyclen **25** as a white solid (0.17 g, 0.29 mmol, 69% yield).

mp 89-90°C, *R_f*=0.41 (15% MeOH/CH₂Cl₂)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3379, 2978, 2847, 1728, 1589, 1454, 1392, 1369, 1312, 1227, 1161, 1107, 1007, 976, 903, 849, 756;

δ_{H} (400 MHz; CDCl₃) 3.45 (s, 2H, NCH₂CO₂), 3.42 (s, 4H, NCH₂CO₂), 2.98-2.35 (m, 20H, CH₂), 1.46 (s, 9H, *t*-Bu), 1.44 (s, 18H, 2×*t*-Bu);

δ_{C} (100 MHz; CDCl₃) 177.5, 172.9, 169.8, 169.7, 81.9, 81.8, 81.7, 55.6, 54.4, 54.0, 50.3, 49.9, 49.8, 49.3, 34.8, 27.9;

m/z, 587 ([M+H]⁺);

HRMS Found 587.4003 ([M+H]⁺, C₂₉H₅₅N₄O₈ requires 587.4020).

***tert*-Butyl 2,2',2''-(10-(3-(5-benzamido-6-methoxy-6-oxohexylamino)-3-oxopropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (26).**

A solution of cyclen **25** (0.7 g, 1.18 mmol) in dry CH_2Cl_2 (4 mL) was added to a stirred solution of *N,N'*-dicyclohexylcarbodiimide (0.27 g, 1.30 mmol) and *N*-hydroxybenzotriazole (0.16 g, 1.18 mmol) in dry CH_2Cl_2 (4 mL) at 0 °C was. After being stirred for 10 minutes, a solution of methyl 6-amino-2-benzamidohexanoate (0.31 g, 1.18 mmol) in dry dichloromethane (4 mL) was added dropwise. The reaction mixture was stirred at 0 °C for 18 hours. The reaction mixture was filtered and the filtrate was evaporated to dryness. The crude product was purified by flash column chromatography (SiO_2 , 10% MeOH in CH_2Cl_2) to give cyclen **26** as a glass-like solid (0.51 g, 0.61 mmol, 52% yield).

mp 75-76°C, $R_f=0.43$ (15% MeOH/ CH_2Cl_2)

$[\alpha]_D^{22} = -21.2$ (c = 0.62, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3297, 3093, 2950, 1734, 1660, 1165, 767, 688;

δ_{H} (400 MHz; CDCl_3) 8.18 (d, $J=1.8$ Hz, 2H, Ar-H), 7.51-7.35 (m, 3H, Ar-H), 4.69-4.60 (m, 1H, CH), 3.72 (s, 3H, CH_3CO_2), 3.42-1.57 (m, 34H, CH_2), 1.46 (s, 27H, CH_3CO_2);

δ_{C} (100 MHz; CDCl_3) 173.3, 173.0, 172.9, 172.1, 167.7, 133.8, 131.2, 128.2, 128.1, 82.3, 82.2, 82.0, 77.7, 60.4, 56.4, 55.7, 52.2, 51.6, 38.2, 32.3, 30.6, 28.0, 27.9;

m/z , 834 ($[\text{M}+\text{H}]^+$), 833 (M^+);

HRMS Found 833.5390 ($[\text{M}+\text{H}]^+$, $\text{C}_{43}\text{H}_{73}\text{N}_6\text{O}_{10}$ requires 833.5388).

2,2',2''-(10-(3-(5-benzamido-6-methoxy-6-oxohexylamino)-3-oxopropyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (27).

Trifluoroacetic acid (2.6 mL) was added to a stirred solution of cyclen **26** (0.56 g, 0.68 mmol) in dry dichloromethane (5 mL). The reaction mixture was stirred at room temperature for 16 hours. After removal of solvent under reduced pressure, acid was removed by addition and evaporation of successive portion of dichloromethane

(2×10 mL), methanol (2×10 mL) and diethyl ether (2×10 mL) to give cyclen **27** as a pale yellow solid which was used in the next step without further purification.

mp 79-81 °C, $R_f=0.10$ (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{22} = -7.3$ ($c = 1.07$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3294, 3073, 2950, 1637, 1578, 1538, 1385, 1179, 1129, 833, 799, 717;

δ_{H} (400 MHz; CD₃OD) 7.87 (d, $J=7.2$ Hz, 2H, Ar-H), 7.62-7.45 (m, 3H, Ar-H), 4.64 (dd, $J=9.2, 5.0$ Hz, 1H, CH), 3.76 (s, 3H, CH₃CO₂), 3.71-2.95 (m, 26H, CH₂), 2.77 (br s, 2H, CH₂NH), 2.05-1.81 (m, 2H, CH₂), 1.69-1.43 (m, 4H, CH₂);

δ_{C} (100 MHz; CD₃OD) 173.0, 172.8, 169.1, 167.1, 133.8, 131.6, 128.2, 127.2, 54.6, 52.1, 51.4, 48.5, 38.8, 30.6, 28.3, 23.1;

m/z , 665 ($[\text{M}+\text{H}]^+$);

HRMS Found 665.3505 ($[\text{M}+\text{H}]^+$, C₃₁H₄₉N₆O₁₀ requires 665.3510).

GdDO3A-lysine (CA2).

A solution of GdCl₃ (0.28 g, 1.05 mmol) in H₂O (2 mL) was added to a stirred solution of cyclen **27** (0.7 g, 1.05 mmol) in methanol (7 mL). The pH was adjusted and maintained at 5.8 using 0.5 M aqueous ammonium acetate. The reaction mixture was stirred at 70 °C for 2 hours. After removal of solvent under reduced pressure, the crude product was purified by semi-preparative reverse phase HPLC (2→60% CH₃CN, $t_R=11.8$ min) to give complex **CA2** as a pale yellow solid (0.26 g, 0.32 mmol, 31% yield). –

mp 99-100°C

$[\alpha]_D^{21} = 4.8$ ($c = 0.77$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3273, 3100, 2866, 1680, 1601, 1439, 1395, 1361, 1321, 1181, 1130, 1084, 834, 745, 717;

m/z , 814, 816, 817, 818, 819, 820, 822 ($[M+H]^+$);

HRMS Found 820.2444 ($[M+H]^+$, $C_{31}H_{44}N_6O_{10}$ ^{158}Gd requires 820.2390) with Gd isotope distribution pattern.

2-Amino-5-(benzyloxy)-5-oxopentanoic acid (13).⁹⁷

L-Glutamic acid (15 g, 100.95 mmol), 60% sulfuric acid (9 mL) and benzyl alcohol (10.6 mL, 100.95 mmol) were mixed at 70 °C. The reaction mixture was stirred at the same temperature for 1 hour. The mixture was concentrated under reduced pressure and the residue was taken up in 1 M aqueous sodium hydrogen carbonate and the pH was adjusted to 7. The precipitate was filtered and washed with diethyl ether to give amino acid **13** as a colourless solid (11.69 g, 49.27 mmol, 48% yield).

mp 187-188°C (Lit⁹⁸ 188-189°C), $R_f=0.07$ (15% MeOH/ CH_2Cl_2)

$[\alpha]_D^{22} = 9.4$ ($c = 1.00$, AcOH), Lit⁹⁷ $[\alpha]_D^{20} = 19.1$ ($c = 1.400$, AcOH)

$\nu_{\max}/\text{cm}^{-1}$ 2936, 1722, 1578, 1516, 1452, 1410, 1387, 1370, 1351, 1280, 1170, 1155, 1000, 856, 730;

δ_H (400 MHz; D_2O) 7.43-7.15 (m, 5H, Ar-H), 5.03 (s, 2H, CH_2Ph), 3.58 (t, $J=3.0$ Hz, 1H, CH), 2.60-2.35 (m, 2H, CH_2), 2.13-1.88 (m, 2H, CH_2);

δ_C (100 MHz; D_2O) 177.0, 176.4, 138.0, 131.4, 130.9, 69.8, 56.5, 32.4, 28.0;

m/z , 260 ($[M+\text{Na}]^+$), 238 ($[M+H]^+$).

2-Benzamide-5-(benzyloxy)-5-oxopentanoic acid (8).

To a stirred suspension of L-glutamic acid γ -benzyl ester **13** (1 g, 4.21 mmol) in saturated aqueous sodium hydrogen carbonate (25 mL) and CH_2Cl_2 (25 mL), was added benzoyl chloride (0.54 mL, 4.64 mmol) dropwise. After being stirred at room temperature for 24 hours, the aqueous layer was separated and acidified with 2 M aqueous hydrochloric acid, extracted with ethyl acetate (2 $\times$ 20 mL). The combined organic extracts were dried over anhydrous magnesium sulfate and evaporated to dryness to give amide **8** as a colourless solid (1.05 g, 3.08 mmol, 74% yield).

mp 102-104°C, R_f =0.29 (15% MeOH/ CH_2Cl_2)

$[\alpha]_D^{22} = 30.5$ ($c = 0.11$, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 2920, 1728, 1579, 1448, 1415, 1322, 1296, 1214, 1171, 1151, 1067, 754;

δ_{H} (400 MHz; CDCl_3) 8.10 (d, $J=8.0$ Hz, 1H, NH), 7.84 (d, $J=7.2$ Hz, 2H, Ar-H), 7.58–7.40 (m, 3H, Ar-H), 7.39–7.29 (m, 5H, Ar-H), 5.12 (s, 2H, CH_2Ph), 4.91–4.79 (m, 1H, CH), 2.74–2.50 (m, 2H, CH_2), 2.46–2.34 (m, 1H, CH_2), 2.32–2.20 (m, 1H, CH_2);

δ_{C} (100MHz; CDCl_3) 175.2, 174.0, 168.3, 135.5, 133.1, 132.1, 128.7, 128.4, 128.3, 127.4, 66.9, 52.9, 30.7, 26.5;

m/z , 364 ($[\text{M}+\text{Na}]^+$), 342 ($[\text{M}+\text{H}]^+$), 341 (M^+).

5-Benzyl 1-methyl 2-benzamidopentanedioate (9).

To a stirred suspension of 2-benzamide-5-(benzyloxy)-5-oxopentanoic acid **8** (1.4 g, 4.10 mmol) and potassium hydrogen carbonate (0.82 g, 8.20 mmol) in dry DMF (14 mL) at room temperature was added methyl iodide (0.46 mL, 7.40 mmol). After being stirred for 2 hours, the reaction mixture was partitioned between ethyl acetate

and H₂O. The organic layer was separated and washed successively with H₂O (3×30 mL), brine, dried over anhydrous magnesium sulfate and evaporated to dryness. The crude product was purified by flash column chromatography (SiO₂, 25-40% ethyl acetate in hexane) to give ester **9** as a colourless solid (1.12 g, 3.15 mmol, 76% yield).

mp 77-79°C, R_f =0.44 (50% EtOAc/hexanes)

$[\alpha]_D^{22} = 17$ (c = 0.50, CHCl₃)

$\nu_{\max}/\text{cm}^{-1}$ 3323, 2957, 1746, 1722, 1658, 1520, 1434, 1392, 1353, 1302, 1202, 1169, 1153, 1102, 956, 752, 693;

δ_H (400 MHz; CDCl₃) 7.83 (d, J =7.2 Hz, 2H, Ar-H), 7.58-7.42 (m, 3H, Ar-H), 7.41-7.30 (m, 5H, Ar-H), 7.03 (br d, J =8.0 Hz, 1H, NH), 5.12 (s, 2H, CH₂CO₂), 4.91-4.81 (m, 1H, CH), 3.79 (s, 3H, CH₃CO₂), 2.67-2.46 (m, 2H, CH₂), 2.43-2.31 (m, 1H, CH₂), 2.26-2.13 (m, 1H, CH₂);

δ_C (100MHz; CDCl₃) 173.1, 172.5, 167.2, 135.7, 133.6, 131.9, 128.7, 128.4, 128.3, 127.2, 66.8, 52.7, 52.4, 30.5, 27.2;

m/z , 356 ([M+H]⁺).

4-Benzamido-5-methoxy-5-oxopentanoic acid (**10**).

A solution of ester **9** (1 g, 2.81 mmol) in ethyl acetate (18 mL) containing 10% palladium on carbon (0.1 g) was stirred under H₂ for 18 hours. The reaction mixture was filtered through celite and the filtrate was evaporated to give acid **10** as a colourless solid which was used in the next step without further purification.

mp 80-81°C (Lit¹²⁰ 79-81°C), R_f =0.06 (50% EtOAc/hexanes)

$[\alpha]_D^{21} = -16.7$ (c = 1.00, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 2959, 1745, 1717, 1626, 1574, 1543, 1494, 1445, 1406, 1362, 1257, 1214, 1158, 1101, 1069, 996, 856, 712, 711, 629;

δ_{H} (400 MHz; CDCl_3) 7.82 (d, $J=7.6$ Hz, 2H, Ar-H), 7.59-7.39 (m, 3H, Ar-H), 7.14 (br d, $J=7.6$ Hz, 1H, NH) 4.96-4.79 (m, 1H, CH), 3.79 (s, 3H, CH_3CO_2), 2.65-2.42 (m, 2H, CH_2), 2.41-2.27 (m, 1H, CH_2), 2.23-2.05 (m, 1H, CH_2);

δ_{C} (100MHz; CDCl_3) 177.5, 172.5, 167.6, 133.4, 132.1, 128.7, 127.2, 52.9, 52.2, 30.2, 27.4;

m/z , 266 ($[\text{M}+\text{H}]^+$), 265 (M^+).

2-Benzamido-6-(benzyloxycarbonylamino)hexanoic acid (**21**).¹²¹

Benzoyl chloride (83 μL , 0.71 mmol) was added dropwise to a stirred suspension of H-Lys(Z)-OH (0.2 g, 0.71 mmol) in 2 M aqueous sodium hydroxide (5 mL) and dichloromethane (5 mL). After being stirred at room temperature for 24 hours, the aqueous layer was separated and acidified with 2 M aqueous hydrochloric acid, extracted with diethyl ether (2×10 mL), dried over anhydrous magnesium sulfate and evaporated to give amide **21** as a colourless solid (0.23 g, 0.61 mmol, 85% yield).

mp 171-173°C, $R_f=0.23$ (15% MeOH/ CH_2Cl_2)

$[\alpha]_{\text{D}}^{22} = 12.4$ ($c = 1.11$, MeOH),

$\nu_{\text{max}}/\text{cm}^{-1}$ 3320, 2938, 2861, 1685, 1526, 1489, 1455, 1258, 1243, 1134, 1117, 1010, 691;

δ_{H} (300 MHz; CD_3OD) 7.87 (d, $J=7.2$ Hz, 2H, Ar-H), 7.61-7.42 (m, 3H, Ar-H), 7.39-7.24 (m, 5H, Ar-H), 5.03 (s, 2H, CH_2CO_2), 4.55 (dd, $J=8.7, 4.8$ Hz, 1H, CH), 3.14 (t, $J=6.9$ Hz, 2H, CH_2NH), 2.08-1.76 (m, 2H, CH_2), 1.67-1.38 (m, 4H, CH_2);

δ_{C} (100 MHz; CD_3OD) 175.7, 168.7, 157.6, 137.1, 134.2, 131.4, 128.2, 128.1, 127.6, 127.4, 127.1, 66.0, 53.8, 40.2, 31.3, 29.2, 22.9;

m/z , 407 ($[\text{M}+\text{Na}]^+$), 385 ($[\text{M}+\text{H}]^+$), 384 (M^+).

Methyl 2-benzamido-6-(benzyloxycarbonylamino)hexanoate (22).

Methyl iodide (0.29 mL, 4.59 mmol) was added to a stirred suspension of amide **21** (0.98 g, 2.55 mmol) and potassium hydrogen carbonate (0.51 g, 5.10 mmol) in dry DMF (9 mL) at room temperature. After being stirred for 2 hours, the reaction mixture was partitioned between ethyl acetate and H₂O. The organic layer was separated and washed successively with H₂O (3×20 mL) and brine, dried over anhydrous magnesium sulfate and evaporated to dryness. The crude product was purified by flash column chromatography (SiO₂, 50% ethyl acetate in hexane) to give ester **22** as a pale yellow solid (0.93 g, 2.33 mmol, 91% yield).

mp 191–192°C, *R_f*=0.69 (15% MeOH/CH₂Cl₂)

[α]_D²² = -16.6 (*c* = 1.99, MeOH)

*ν*_{max}/cm⁻¹ 3308, 2952, 1743, 1686, 1634, 1490, 1436, 1210, 1172, 1158, 1130, 719;

δ_H (300 MHz; CDCl₃) 7.85 (d, *J*=7.2 Hz, 2H, Ar-H), 7.57–7.40 (m, 3H, Ar-H), 7.40–7.29 (m, 5H, Ar-H), 6.81 (br d, *J*=7.5 Hz, 1H, NH), 5.06 (dd, *J*=17.4, 12.2 Hz, 2H, CH₂Ph), 4.94–4.77 (m, 2H, NH, CH), 3.80 (s, 3H, CH₃CO₂), 3.30–3.14 (m, 2H, CH₂NH), 2.04–1.75 (m, 2H, CH₂), 1.65–1.34 (m, 4H, CH₂);

δ_C (100 MHz; CDCl₃) 173.2, 167.4, 156.7, 136.6, 133.9, 131.9, 128.7, 128.6, 128.2, 128.1, 127.2, 66.7, 52.6, 52.5, 40.5, 32.1, 29.5, 22.4;

m/z, 399 ([M+H]⁺).

Methyl 6-amino-2-benzamidohexanoate (23).

A solution of ester **22** (0.92 g, 2.39 mmol) in ethanol (10 mL) containing 10% palladium on carbon (0.09 g) and formic acid (0.9 mL) was stirred under N₂ for 2 hours. The reaction mixture was filtered through celite and the filtrate was evaporated

to give amine **23** as a yellow oil which was used in the next step without further purification.

$R_f=0.10$ (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{22} = -4.6$ ($c = 0.51$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3272, 3062, 2952, 2869, 1739, 1637, 1601, 1577, 1533, 1489, 1438, 1344, 1210, 1164, 1001, 713, 694;

δ_H (400 MHz; CDCl₃) 7.83 (d, $J=7.6$ Hz, 2H, Ar-H), 7.59-7.39 (m, 3H, Ar-H), 6.84 (br d, $J=7.6$ Hz, 1H, NH), 4.93-4.79 (m, 1H, CH), 3.80 (s, 3H, CH₃), 2.71 (t, $J=6.6$ Hz, 2H, CH₂NH), 2.10–1.75 (m, 2H, CH₂), 1.69-1.35 (m, 4H, CH₂);

δ_C (100 MHz; CDCl₃) 173.2, 168.2, 133.5, 132.0, 128.6, 127.5, 53.0, 52.6, 39.4, 31.1, 28.7, 22.8;

m/z , 265 ($[M+H]^+$).

5-(Benzyloxy)-2-(*tert*-butoxycarbonyl)-5-oxopentanoic acid (**30**).

A solution of di-*tert*-butyldicarbonate (5.38 g, 24.7 mmol) in THF (30 mL) was added to a stirred suspension of L-glutamic acid γ -benzyl ester (4.5 g, 19 mmol) in saturated aqueous sodium hydrogen carbonate (110 mL) at 0 °C. The reaction mixture was stirred at room temperature 16 hours. THF was removed and the residue was acidified 10% aqueous citric acid. The mixture was extracted with ethyl acetate (3×30 mL). The organic layer was washed with brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 10% MeOH in CH₂Cl₂) to give glutamate **30** as a colourless oil (5.73 g, 17 mmol, 90% yield).

$R_f=0.41$ (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{22} = 12.9$ ($c = 1.08$, CHCl₃), Lit¹²² $[\alpha]_D^{20} = 6.0$ ($c = 1.20$, CHCl₃)

$\nu_{\max}/\text{cm}^{-1}$ 3364, 2979, 1711, 1500, 1454, 1392, 1367, 1248, 1212, 1154, 1052, 1027,

910, 852, 779, 732, 696;

δ_{H} (300 MHz; CDCl_3) 7.44-7.30 (m, 5H, Ar-H), 5.21 (br d, $J=6.0$ Hz, 1H, NH), 5.15 (s, 2H, CH_2Ph), 4.43-4.32 (m, 1H, CH), 2.64-2.44 (m, 2H, CH_2), 2.34-2.21 (m, 1H, CH_2), 2.11-1.98 (m, 1H, CH_2), 1.46 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl_3) 175.9, 173.0, 155.8, 135.8, 128.7, 128.3, 80.4, 66.7, 52.9, 30.4, 28.4, 27.6;

m/z , 360 ($[\text{M}+\text{Na}]^+$).

5-Benzyl 1-methyl 2-(*tert*-butoxycarbonyl)pentanedioate (31).

Methyl iodide was added to a suspension of glutamate **30** (4 g, 11.9 mmol), and potassium hydrogen carbonate (2.37 g, 23.7 mmol) in dry dimethylformamide (38 mL). After being stirred at room temperature for 2 hours, the reaction mixture was partitioned between ethyl acetate and H_2O . The organic phase was separated and washed with H_2O (3×30 mL), and brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO_2 , 25% EtOAc in hexanes) to give glutamate **31** as a white solid (3.88 g, 11 mmol, 93% yield).

mp 41-42°C (Lit¹²³ 38-41°C), $R_f=0.58$ (50% EtOAc/hexanes)

$[\alpha]_{\text{D}}^{21} = -25.5$ ($c = 1.00$, MeOH), Lit¹²³ $[\alpha]_{\text{D}}^{20} = -17.0$ ($c = 1.00$, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3366, 2978, 1711, 1500, 1453, 1391, 1366, 1249, 1211, 1154, 1049, 1027, 780, 739, 698;

δ_{H} (400 MHz; CDCl_3) 7.37 (m, 5H, Ar-H), 5.14 (s, 2H, CH_2Ph), 4.42-4.31 (m, 1H, CH), 3.75 (s, 3H, CH_3CO_2), 2.57-2.39 (m, 2H, CH_2), 2.29-2.15 (m, 1H, CH_2), 2.06-1.92 (m, 1H, CH_2), 1.46 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl_3) 172.8, 172.6, 155.5, 135.8, 128.6, 128.3, 80.1, 66.6, 52.9, 52.5, 30.4, 28.4, 27.7;

m/z , 375 ($[\text{M}+\text{Na}+\text{H}]^+$), 374 ($[\text{M}+\text{Na}]^+$).

5-Benzyl 1-methyl 2-aminopentanedioate (32).

Trifluoroacetic acid (5 mL) was added to a stirred solution of glutamate **31** (0.48 g, 1.38 mmol) in dry dichloromethane (5 mL). The reaction mixture was stirred at room temperature for 18 hours. After removal of solvent under reduced pressure, acid was removed by addition and evaporation of successive portion of dichloromethane (2×10 mL), methanol (2×10 mL) and diethyl ether (2×10 mL), respectively to give amine **32** as a white solid which was used in the next step without further purification.

mp 131-132°C (Lit¹²⁴ 129°C), R_f =0.07 (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{21}$ = 2.6 (c = 1.00, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3033, 2948, 1731, 1668, 1509, 1454, 1422, 1354, 1261, 1185, 1137, 1082, 1004, 947, 910, 838, 799, 722, 696;

δ_H (400 MHz; CDCl₃) 7.45-7.23 (m, 5H, ArH), 5.12 (s, 2H, CH₂Ph), 4.25-4.10 (m, 1H, CH), 3.75 (s, 3H, CH₃CO₂), 2.73-2.55 (m, 2H, CH₂), 2.41-2.20 (m, 1H, CH₂);

δ_C (100 MHz; CDCl₃) 173.5, 172.8, 140.7, 128.7, 127.8, 127.2, 65.3, 56.1, 52.2, 25.2, 24.6;

m/z, 252 ([M+H]⁺).

5-Benzyl 1-methyl 2-(2-benzamido-5-(benzyloxy)-5-oxopentanamido)pentanedioate (33).

A solution of carboxylic acid **8** (0.25 g, 0.73 mmol) in THF (1.8 mL) and *N,N*-diisopropylethylamine (0.13 mL, 0.73 mmol) were added to a stirred solution of (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (0.31 g, 0.81 mmol) and *N*-hydroxybenzotriazole (0.11 g, 0.81 mmol) in THF (1.8 mL) at -5 °C. After being stirred for 5 minutes, a solution of amine **32** (0.2 g, 0.81 mmol) and *N,N*-diisopropylethylamine (0.26 mL, 1.46 mmol) in THF (1.8 mL) was added dropwise. The reaction mixture was stirred at -5 °C for 3 hours and at room temperature for 16 hours. The solvent was removed and the residue was taken up in ethyl acetate (5 mL), washed with 10% aqueous citric acid, 4% aqueous sodium hydrogen carbonate, and brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 30→40% EtOAc in hexanes) to give dipeptide **33** as a pale yellow solid (0.17 g, 0.29 mmol, 40% yield).

mp 87-88°C, *R_f*=0.26 (50% EtOAc/hexanes)

$[\alpha]_D^{21} = 9.6$ (*c* = 0.79, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3303, 3066, 3034, 2954, 1733, 1638, 1578, 1531, 1489, 1453, 1388, 1261, 1211, 1165, 1125, 1082, 973, 737, 697;

δ_{H} (400 MHz; CDCl₃) 7.79 (d, 2H, Ar-H), 7.53-7.37 (m, 3H, Ar-H), 7.37-7.26 (m, 5H, Ar-H), 5.10 (s, 2H, CH₂Ph), 5.06 (s, 2H, CH₂Ph), 4.77-4.69 (m, 1H, CH), 4.63-4.52 (m, 1H, CH), 3.71 (s, 3H, CO₂CH₃), 2.73-1.94 (m, 8H, 4×CH₂);

δ_{C} (100 MHz; CDCl₃) 173.8, 172.7, 171.9, 171.4, 167.5, 135.7, 133.5, 132.0, 131.9, 128.7, 128.4, 127.3, 127.2, 66.7, 52.9, 52.7, 52.0, 30.6, 30.3, 27.9, 26.9;

m/z, 575 ([M+H]⁺);

HRMS Found 575.2387 ([M+H]⁺, C₃₂H₃₅N₂O₈ requires 575.2393).

Bz-Glu-Glu-OMe (34).

A solution of dipeptide **33** (0.16 g, 0.27 mmol) in ethyl acetate (2.5 mL) containing 10% palladium on carbon (30 mg) was stirred under H₂ for 18 hours. The reaction mixture was filtered through celite and the filtrate was evaporated to give diacid **34** as a colourless solid which was used in the next step without further purification.

$R_f = 0.05$ (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{21} = 10.1$ (c = 1.00, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3309, 2960, 2927, 1733, 1640, 1576, 1534, 1490, 1438, 1406, 1216, 1180, 712, 694;

δ_H (300 MHz; MeOD) 7.94-7.31 (m, 2H, Ar-H), 7.62-7.40 (m, 3H, Ar-H), 4.70-4.57 (m, 1H, CH), 4.56-4.45 (m, 1H, CH), 3.72 (s, 3H, CH₃CO₂), 2.62-2.37 (m, 4H, 2×CH₂), 2.36-1.88 (m, 4H, 2×CH₂);

δ_C (100 MHz; MeOD) 173.0, 172.2, 169.0, 133.8, 131.5, 128.2, 127.2, 53.7, 52.1, 51.4, 30.9, 30.3, 27.1, 26.4;

m/z , 418 ([M+Na+H]⁺), 417 ([M+Na]⁺), 396 ([M+2H]⁺), 395 ([M+H]⁺);

HRMS Found 395.1453 ([M+H]⁺, C₁₈H₂₃N₂O₈ requires 395.1454).

5-Benzyl 1-methyl 2-(5-(benzyloxy)-2-(tert-butoxycarbonyl)-5-oxopentanamido) pentanedioate (35).

50% Solution of T3P in DMF (0.16 mL, 0.28 mmol) was added to a stirred solution of acid **30** (68 mg, 0.2 mmol), amine **32** (55 mg, 0.22 mmol) and *N,N*-diisopropylethylamine (90 μ L, 0.5 mmol) in dry dichloromethane (2 mL) at 0 °C. The reaction mixture was allowed to reach ambient temperature and stirred for 18 hours. The reaction was quenched with 1 M aqueous sodium hydroxide. The organic layer was separated and washed with H₂O (3 $\times$ 5 mL), and brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 30% EtOAc in hexanes) to give dipeptide **35** as a colourless oil (78 mg, 0.14 mmol, 68% yield).

R_f =0.52 (50% EtOAc/hexanes)

$[\alpha]_D^{21} = 11.3$ ($c = 0.69$, CHCl₃), Lit¹²⁵ $[\alpha]_D^{25} = 7.1$ ($c = 1.00$, CHCl₃)

$\nu_{\max}/\text{cm}^{-1}$ 3369, 2977, 1732, 1512, 1497, 1454, 1391, 1366, 1250, 1210, 1157, 1049, 1028, 733, 697;

δ_H (300 MHz; CDCl₃) 7.42-7.31 (m, 10H, Ar-H), 6.90 (br d, $J=7.5$ Hz, 1H, NH), 5.23 (br d, $J=7.5$ Hz, 1H, NH), 5.14 (d, $J=5.1$ Hz, 2H, CH₂Ph), 4.62 (dt, $J=8.0$, 5.1 Hz, 1H, CH), 4.27-4.14 (m, 1H, CH), 3.74 (s, 3H, CH₃CO₂), 2.65-2.35 (m, 4H, CH₂), 2.33-2.10 (m, 2H, CH₂), 2.10-1.87 (m, 2H, CH₂), 1.44 (s, 9H, *t*-Bu);

δ_C (100 MHz; CDCl₃) 173.3, 172.6, 171.7, 171.3, 155.6, 135.8, 128.7, 128.4, 80.3, 66.6, 52.4, 51.7, 30.4, 30.2, 28.4, 27.8, 27.1;

m/z , 594 ([M+Na+H]⁺), 593 ([M+Na]⁺), 572 ([M+2H]⁺), 571 ([M+H]⁺).

5-Benzyl 1-methyl 2-(2-amino-5-(benzyloxy)-5-oxopentanamido)pentanedioate (36).

Trifluoroacetic acid (1 mL) was added to a stirred solution of dipeptide **35** (0.14 g, 0.24 mmol) in dry dichloromethane (2 mL). The reaction mixture was stirred at room temperature for 16 hours. After removal of solvent under reduced pressure, acid was removed by addition and evaporation of successive portion of dichloromethane (2×5 mL), methanol (2×5 mL) and diethyl ether (2×5 mL) to give amine **36** as a yellow oil which was used in the next step without further purification.

$R_f=0.06$ (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{21} = 4.1$ ($c = 0.74$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3249, 3075, 2956, 1733, 1673, 1542, 1454, 1392, 1261, 1202, 1176, 1134, 973, 835, 800, 737, 722, 698;

δ_H (300 MHz; CDCl₃) 8.00 (br s, 1H, NH), 7.45-7.20 (m, 10H, Ar-H), 5.09 (d, 4H, CH₂Ph), 4.67-4.47 (m, 1H, CH), 4.42-4.22 (m, 1H, CH), 3.66 (s, 3H, CH₃CO₂), 2.80-2.57 (m, 2H, CH₂), 2.57-2.34 (m, 2H, CH₂), 2.34-2.10 (m, 2H, CH₂), 2.10-1.92 (m, 2H, CH₂);

δ_C (100 MHz; CDCl₃) 173.6, 173.1, 172.4, 171.6, 135.6, 128.7, 128.3, 66.8, 52.7, 52.2, 51.9, 30.5, 29.5, 29.3, 26.4;

m/z , 471 ([M+H]⁺).

5-benzyl 1-methyl 2-(2-acetamido-5-(benzyloxy)-5-oxopentanamido)pentanedioate (37).

A small amount of *N,N*-dimethylaminopyridine and triethylamine (0.24 mL, 1.7 mmol) were added to a stirred solution of dipeptide **36** (0.4 g, 0.85 mmol) in acetic anhydride (5.6 mL). The reaction mixture was stirred at room temperature for 4 hours. After removal of solvent under reduced pressure, the residue was taken up in brine and extracted with ethyl acetate (3×10 mL). The combined ethyl acetate extracts were washed with 0.2 M aqueous hydrochloric acid, 5% aqueous sodium hydrogen carbonate and brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 4% MeOH in CH₂Cl₂) to give dipeptide **37** as a white solid (0.37 g, 0.72 mmol, 85% yield).

mp 91–92°C, *R_f*=0.34 (5% MeOH/CH₂Cl₂)

[α]_D²¹ = 7.1 (c = 0.19, MeOH)

*ν*_{max}/cm^{−1} 3283, 3068, 2953, 1734, 1647, 1542, 1499, 1454, 1387, 1262, 1211, 1166, 986, 741, 699;

δ_H (300 MHz; CDCl₃) 7.42–7.29 (m, 10H, Ar-H), 7.13 (d, *J*=4.8 Hz, 1H, NH), 6.54 (d, *J*=4.5 Hz, 1H, NH), 5.17–5.08 (m, 4H, CH₂Ph), 4.60–4.51 (m, 2H, CH), 3.72 (s, 3H, CH₃CO₂), 2.62–2.36 (m, 4H, 2×CH₂), 2.27–2.08 (m, 2H, CH₂), 2.06–1.92 (m, 2H, CH₂), 1.95 (s, 3H, CH₃);

δ_C (100 MHz; CDCl₃) 173.5, 172.7, 171.8, 171.3, 170.4, 135.7, 128.7, 128.4, 66.8, 66.7, 52.7, 52.4, 51.9, 30.5, 30.3, 28.0, 26.9, 23.2;

m/z, 535 ([M+Na]⁺), 513 ([M+H]⁺);

HRMS Found 513.2233 ([M+H]⁺, C₂₇H₃₃N₂O₈ requires 513.2237).

Ac-Glu-Glu-OMe (38).

A solution of dipeptide **37** (0.3 g, 0.59 mmol) in methanol (4.2 mL) containing 10% palladium on carbon (60 mg) was stirred under H₂ for 18 hours. The reaction mixture was filtered through celite and the filtrate was evaporated to give diacid **38** as a colourless solid which was used in the next step without further purification.

R_f=0.04 (15% MeOH/CH₂Cl₂)

[α]_D²¹ = 5.4 (c = 0.53, MeOH)

ν_{max}/cm⁻¹ 2959, 2927, 2860, 1728, 1648, 1461, 1380, 1272, 1123, 1072, 1040, 723;

δ_H (300 MHz; MeOD) 4.47 (dd, *J*=9.0, 5.1 Hz, 1H, CH), 4.39 (dd, *J*=7.7, 6.5 Hz, 1H, CH), 3.74 (s, 3H, CH₃CO₂), 2.44 (t, *J*=7.1 Hz, 4H, CH₂), 2.27-2.05 (m, 2H, CH₂), 2.05-1.65 (m, 2H, CH₂);

δ_C (100 MHz; MeOD) 175.3, 175.2, 173.5, 172.5, 172.0, 55.1, 51.9, 51.7, 29.4, 27.0, 21.0;

m/z, 356 ([M+Na+H]⁺), 355 ([M+Na]⁺), 334 ([M+2H]⁺), 333 ([M+H]⁺);

HRMS Found 355.1107 ([M+Na]⁺, C₁₃H₂₀N₂O₈Na requires 355.1117).

2-Acetamido-6-(benzyloxycarbonyl)hexanoic acid (39).¹⁰⁸

Acetic anhydride (1.4 mL, 15 mmol) and triethylamine (4.2 mL, 30 mmol) were added in two portions to a suspension of H-Lys(Z)-OH (3 g, 10.7 mmol) in 0.5 M aqueous sodium hydroxide (25 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 hour and another 1 hour at room temperature. The mixture was washed with

diethyl ether (15 mL), adjusted pH to 2 using 3 M aqueous hydrochloric acid. The mixture was extracted with ethyl acetate (3×30 mL). The organic layer was washed with brine before drying over anhydrous magnesium sulfate. The solvent was removed to give lysine **39** as a colourless solid (2.9 g, 9.0 mmol, 84% yield).

mp 116-117°C (Lit¹⁰⁸ 119-120°C), $R_f=0.37$ (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{22} = 0.71$ (c = 0.51, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3325, 2942, 1702, 1655, 1527, 1455, 1374, 1245, 1137, 1026, 907, 725, 696, 646;

δ_H (400 MHz; CDCl₃) 7.48-7.30 (m, 5H, Ar-H), 6.73 (br d, 1H, NH), 5.21-5.02 (m, 3H, CH₂Ph and NH), 4.63-4.01 (m, 1H, CH), 3.31-3.08 (m, 2H, CH₂), 2.04 (s, 3H, CH₃), 2.00-1.71 (m, 2H, CH₂), 1.63-1.32 (m, 4H, 2× CH₂);

δ_C (100 MHz; CDCl₃) 175.0, 171.9, 157.1, 136.5, 128.6, 128.2, 128.1, 66.7, 52.5, 40.5, 31.3, 29.4, 22.7, 22.4;

m/z, 346 ([M+Na+H]⁺), 345 ([M+Na]⁺), 324 ([M+2H]⁺), 323 ([M+H]⁺).

***tert*-Butyl 2-amino-6-(benzyloxycarbonyl)hexanoate (40).**

A suspension of H-Lys(Z)-OH (3 g, 10.7 mmol), concentrate sulfuric acid (3 mL) and isobutylene (30 mL) in dioxane (30 mL) was stirred in sealed flask for 18 hours. The reaction mixture was poured onto 2 M aqueous sodium hydroxide (50 mL) at 0 °C. The aqueous phase was extracted with diethyl ether (3×30 mL). The combined diethyl ether extracts were washed with brine before drying over anhydrous magnesium sulfate. The solvent was removed to give lysine **40** as a yellow oil (3.15 g, 9.36 mmol, 88% yield).

$R_f=0.48$ (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{21} = 5.7$ (c = 1.30, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3337, 2977, 2938, 1703, 1529, 1455, 1367, 1246, 1151, 1026, 846, 736, 696;

δ_{H} (400 MHz; CDCl_3) 7.37-7.26 (m, 5H, Ar-H), 5.07 (s, 2H, CH_2Ph), 4.87 (app br s, 1H, NH), 3.34-3.27 (m, 1H, CH), 3.23-3.12 (m, 2H, CH_2), 1.99 (br s, 2H, NH_2), 1.75-1.35 (m, 6H, $3\times\text{CH}_2$), 1.44 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl_3) 175.1, 156.6, 128.5, 128.1, 81.2, 67.1, 40.8, 34.3, 29.7, 28.1, 22.8;

m/z , 338 ($[\text{M}+2\text{H}]^+$), 337 ($[\text{M}+2\text{H}]^+$).

Ac-Lys(Z)-Lys(Z)-O^tBu (41).

A small amount of 4-dimethyl aminopyridine was added to a solution of carboxylic acid **39** (0.5 g, 1.55 mmol) and amine **40** (0.52 g, 1.55 mmol) in dry dichloromethane (16 mL). The resulting mixture was stirred under N_2 at room temperature for 10 minutes. After the addition of *N,N*-diisopropylethylamine (0.68 mL, 3.9 mmol), the reaction mixture was cooled to 0 °C whereupon a 50% solution of T3P in DMF (1.3 mL, 2.2 mmol) was slowly added. The reaction mixture was stirred at 0 °C for 30 minutes and then for 18 hours at room temperature. The reaction was quenched with 1 M sodium hydroxide, the organic phase was separated and washed with H_2O (3×10 mL), brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO_2 , 10% MeOH in CH_2Cl_2) to give dipeptide **41** as a colourless oil (0.9 g, 1.5 mmol, 97% yield).

$R_f=0.54$ (15% MeOH/ CH_2Cl_2)

$[\alpha]_{\text{D}}^{22} = -20.4$ ($c = 1.03$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3326, 2976, 2937, 2865, 1702, 1531, 1455, 1393, 1368, 1246, 1151, 1025, 913, 846, 775, 736, 697;

δ_{H} (400 MHz; CDCl_3) 7.41-7.19 (m, 10H, Ar-H), 6.83 (br d, 1H, NH), 6.46 (br d, 1H, NH), 6.83 (br d, 1H, NH), 5.32 (app br s, 1H, NH), 5.19-4.98 (m, 5H, CH_2Ph and NH), 4.52-4.30 (m, 2H, $2\times\text{CH}$), 3.25-3.03 (m, 4H, $2\times\text{CH}_2$), 1.92 (s, 3H, CH_3), 1.87-1.20 (m, 12H, $6\times\text{CH}_2$), 1.42 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl_3) 171.9, 171.3, 170.7, 156.8, 136.7, 128.6, 128.2, 82.2, 66.7, 66.6, 53.0, 52.8, 40.4, 32.0, 31.5, 29.3, 23.0, 22.3;

m/z , 664 ($[\text{M}+\text{Na}+\text{H}]^+$), 663 ($[\text{M}+\text{Na}]^+$), 642 ($[\text{M}+2\text{H}]^+$), 641 ($[\text{M}+\text{H}]^+$);

HRMS Found 641.3541 ($[\text{M}+\text{H}]^+$, $\text{C}_{34}\text{H}_{49}\text{N}_4\text{O}_8$ requires 641.3550).

Ac-Lys-Lys-O^tBu (42).

A solution of dipeptide **41** (0.5 g, 0.84 mmol) in ethanol (6 mL) containing 10% palladium on carbon (0.1 g) was stirred under H_2 for 18 hours. The reaction mixture was filtered through celite and the filtrate was evaporated to give diamine **42** as a yellow oil which was used in the next step without further purification.

$R_f=0.04$ (15% MeOH/ CH_2Cl_2)

$[\alpha]_{\text{D}}^{21} = -12.5$ ($c = 1.00$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3264, 3071, 2942, 1724, 1639, 1576, 1460, 1371, 1347, 1253, 1153, 1041, 959, 843;

δ_{H} (400 MHz; CDCl_3) 4.42-4.20 (m, 2H, CH), 2.99-2.71 (m, 4H, CH_2N), 1.97 (s, 3H, CH_3), 1.89-1.51 (m, 8H, $4\times\text{CH}_2$), 1.51-1.30 (m, 4H, $2\times\text{CH}_2$), 1.40 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl_3) 173.2, 172.1, 171.3, 81.7, 53.4, 52.5, 39.1, 31.0, 30.5, 26.9, 26.5, 22.5, 22.4, 21.1;

m/z , 373 (M^+);

HRMS Found 373.2808 (M^+ , $\text{C}_{18}\text{H}_{37}\text{N}_4\text{O}_4$ requires 373.2815).

2-(*tert*-Butoxycarbonyl)acetic acid (46).¹⁰⁹

Benzyl chloroformate (16 mL, 100 mmol) was added to an ice-cold stirred solution of glycine in 1 M sodium hydroxide (100 mL) dropwise. The pH of the reaction was maintained at about 10 by addition of 1 M sodium hydroxide. The reaction mixture was stirred for 2 hours and then washed with diethyl ether. The aqueous layer was acidified to a pH 1, the precipitate was filtered and washed with water before drying under reduced pressure to give amide **46** as a yellow oil (19.7 g, 94 mmol, 95% yield).

mp 121-122°C (Lit¹⁰⁹ 118-119°C), R_f =0.06 (50% EtOAc/hexanes)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3325, 1721, 1689, 1530, 1467, 1351, 1318, 1240, 1167, 1052, 976;

δ_{H} (300 MHz; MeOD) 7.44-7.25 (m, 5H, Ar-H), 5.12 (s, 2H, CH_2Ph), 3.85 (s, 2H, CH_2);

δ_{C} (100 MHz; CDCl_3) 174.5, 156.6, 128.7, 128.4, 128.2, 67.4, 42.7;

m/z , 210 ($[\text{M}+\text{H}]^+$), 209 (M^+).

***tert*-Butyl 2-(*tert*-butoxycarbonyl)acetate (47).**

Procedure A

A mixture of glycine **46** (1 g, 4.78 mmol), concentrate sulfuric acid (15 mL) and isobutylene (15 mL) in dioxane (15 mL) was stirred in sealed flask for 18 hours. The reaction mixture was poured into an ice-cold 2 M sodium hydroxide (25 mL). The

aqueous layer was extracted with diethyl ether (3×30 mL). The combined diethyl ether extracts were washed with brine before drying over anhydrous magnesium sulfate. The solvent was removed to give ester **47** as a yellow oil (0.12 g, 0.47 mmol, 10% yield).

Procedure B

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide, EDCI was added to an ice-cold stirred solution of glycine **46** (2 g, 9.56 mmol), 4-dimethyl aminopyridine (0.58 g, 4.78 mmol) and *tert*-butanol (0.81 g, 10.9 mmol) in dry dichloromethane. The reaction mixture was stirred at 0 °C for 2 hours and then for 18 hours at room temperature. After removal of solvent under reduced pressure, the residue was taken up in ethyl acetate (30 mL) and H₂O (30 mL). The organic layer was separated, washed with saturated aqueous sodium hydrogen carbonate (2×10 mL) and H₂O (2×10 mL) before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 30% EtOAc in Hexanes) to give ester **47** as a yellow oil (1.03 g, 3.9 mmol, 41% yield).

R_f =0.60 (30% EtOAc/hexanes)

$\nu_{\max}/\text{cm}^{-1}$ 3353, 2979, 2934, 1708, 1520, 1500, 1455, 1392, 1367, 1349, 1219, 1150, 1052, 992, 848, 736, 697;

δ_{H} (500 MHz; CDCl₃) 7.43-7.30 (m, 5H, Ar-H), 5.24 (br s, 1H, NH), 5.12 (s, 2H, CH₂Ph), 3.90 (d, J =5.5 Hz, 2H, CH₂), 1.49 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl₃) 169.2, 156.4, 136.4, 128.4, 128.2, 82.2, 67.0, 43.5, 28.1;

m/z , 289 ([M+Na+H]⁺), 288 ([M+Na]⁺).

tert-Butyl 2-aminoacetate (**48**).

A solution of glycine **47** (0.9 g, 3.39 mmol) in THF (6.8 mL) containing 10% palladium on carbon (90 mg) was stirred under H₂ for 18 hours. The reaction mixture was filtered through celite and the filtrate was evaporated to give amine **48** as a yellow oil which was used in the next step without further purification.

$R_f=0.46$ (15% MeOH/CH₂Cl₂)

$\nu_{\max}/\text{cm}^{-1}$ 2923, 1738, 1662, 1538, 1457, 1394, 1369, 1229, 1156, 1034, 945, 846, 711;

δ_{H} (500 MHz; CDCl₃) 3.33 (s, 2H, CH₂), 1.49 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl₃) 166.5, 83.4, 41.2, 27.8;

m/z , 131 ([M+H]⁺).

***tert*-Butyl 2-(2-acetamido-6-(*tert*-butoxycarbonyl)hexanamido)acetate (49).**

A small amount of 4-dimethyl aminopyridine was added to a solution of carboxylic acid **39** (0.19 g, 0.59 mmol) and amine **48** (77 mg, 0.59 mmol) in dry dichloromethane (6 mL). The resulting mixture was stirred under N₂ at room temperature for 10 minutes. After the addition of *N,N*-diisopropylethylamine (0.26 mL, 1.47 mmol), the reaction mixture was cooled to 0 °C whereupon a 50% solution of T3P in DMF (0.24 mL, 0.82 mmol) was slowly added. The reaction mixture was stirred at 0 °C for 30 minutes and then for 18 hours at room temperature. The reaction was quenched with 1 M sodium hydroxide, the organic phase was separated and washed with H₂O (3×5 mL), brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 15% MeOH in CH₂Cl₂) to give dipeptide **49** as a colourless oil (0.17 g, 0.39 mmol, 66% yield).

$R_f=0.53$ (15% MeOH/CH₂Cl₂)

$[\alpha]_{\text{D}}^{22} = -11.3$ ($c = 1.24$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3305, 2978, 2937, 1703, 1650, 1529, 1455, 1393, 1368, 1225, 1152, 1028, 846, 736, 698;

δ_{H} (400 MHz; CDCl₃) 7.38-7.25 (m, 5H, Ar-H), 6.79 (app br s, 1H, NH), 6.47 (app br s, 1H, NH), 5.17-5.01 (m, 1H, NH), 5.07 (s, 2H, CH₂Ph), 4.49-4.40 (m, 1H, CH), 3.98-

3.80 (m, 2H, $\text{NHCH}_2\text{CO}_2^t\text{Bu}$), 3.23-3.13 (m, 2H, CH_2), 1.99 (s, 3H, CH_3), 2.06-1.31 (m, 6H, $3\times\text{CH}_2$), 1.44 (s, 9H, $t\text{-Bu}$);

δ_{C} (100 MHz; CDCl_3) 172.0, 170.5, 168.8, 156.8, 136.6, 128.6, 128.2, 128.1, 82.5, 66.7, 52.9, 40.0, 40.2, 31.7, 29.5, 28.1, 23.2, 22.1;

m/z , 459 ($[\text{M}+\text{Na}+\text{H}]^+$), 458 ($[\text{M}+\text{Na}]^+$), 436 ($[\text{M}+\text{H}]^+$);

HRMS Found 458.2264 ($[\text{M}+\text{Na}]^+$, $\text{C}_{22}\text{H}_{33}\text{N}_3\text{O}_6\text{Na}$ requires 458.2267).

2-(2-Acetamido-6-(*tert*-butoxycarbonyl)hexanamido)acetic acid (**50**).

Trifluoroacetic acid (1 mL) was added to a stirred solution of dipeptide **49** (0.14 g, 0.24 mmol) in dry dichloromethane (0.5 mL). The reaction mixture was stirred at room temperature for 1 hour. After removal of solvent under reduced pressure, acid was removed by addition and evaporation of successive portion of dichloromethane (2×5 mL), methanol (2×5 mL) and diethyl ether (2×5 mL) to give acid **50** as a colourless oil which was used in the next step without further purification.

$R_f=0.40$ (15% $\text{MeOH}/\text{CH}_2\text{Cl}_2$)

$[\alpha]_{\text{D}}^{22} = 2.7$ ($c = 0.18$, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3294, 2935, 2855, 1745, 1698, 1653, 1540, 1455, 1439, 1375, 1255, 1206, 1182, 1135, 1028, 739, 722, 699;

δ_{H} (400 MHz; CDCl_3) 7.38-7.21 (m, 5H, Ar-H), 5.04 (s, 2H, CH_2Ph), 4.32-4.23 (m, 1H, CH), 3.95 (dd, AB, $J=17.9$ Hz, 1H), 3.85 (dd, AB, $J=17.9$ Hz, 1H), 3.15-3.04 (m, 2H, CH_2), 1.96 (s, 3H, CH_3), 1.89-1.18 (m, 6H, $3\times\text{CH}_2$);

δ_{C} (100 MHz; CDCl_3) 173.7, 172.0, 170.3, 157.6, 137.0, 128.1, 127.6, 127.4, 65.9, 53.2, 51.2, 40.4, 40.1, 31.3, 29.2, 22.6, 21.1;

m/z , 380 ($[\text{M}+\text{H}]^+$), 379 (M^+);

HRMS Found 402.1647 ($[\text{M}+\text{Na}]^+$, $\text{C}_{22}\text{H}_{33}\text{N}_3\text{O}_6\text{Na}$ requires 402.1641).

***tert*-Butyl 2-(2-(2-acetamido-6-(*tert*-butoxycarbonyl)hexanamido)acetamido)-6-(*tert*-butoxycarbonyl)hexanoate (51).**

A small amount of 4-dimethyl aminopyridine was added to a solution of carboxylic acid **50** (0.13 g, 0.33 mmol) and amine **40** (0.11 g, 0.33 mmol) in dry dichloromethane (4 mL). The resulting mixture was stirred under N₂ at room temperature for 10 minutes. After the addition of *N,N*-diisopropylethylamine (0.26 mL, 1.47 mmol), the reaction mixture was cooled to 0 °C whereupon a 50% solution of T3P in DMF (0.14 mL, 0.46 mmol) was slowly added. The reaction mixture was stirred at 0 °C for 30 minutes and then for 18 hours at room temperature. The reaction was quenched with 1 M sodium hydroxide, the organic phase was separated and washed with H₂O (3×5 mL), brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 10% MeOH in CH₂Cl₂) to give tripeptide **51** as a colourless oil (0.16 g, 0.23 mmol, 68% yield).

$R_f=0.56$ (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{22} = -5.9$ (c = 0.27, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3311, 2935, 1700, 1655, 1535, 1455, 1369, 1251, 1154, 1027, 846, 776, 736, 698;

δ_H (500 MHz; CDCl₃) 7.44-7.28 (m, 10, Ar-H), 7.02 (app br s, 1H, NH), 6.85 (br d, $J=7.5$ Hz 1H, NH), 6.47 (app br s, 1H, NH), 5.39 (app br s, 1H, NH), 5.19-5.00 (m, 5H, CH₂Ph and NH), 4.55-4.41 (m, 1H, CH), 4.32-4.22 (m, 1H, CH), 4.06-3.81 (m, 2H, NCH₂CO), 3.35-3.10 (m, 4H, 2×CH₂), 1.69 (s, 3H, CH₃), 1.99-1.23 (m, 12H, 6×CH₂), 1.47 (s, 9H, *t*-Bu);

δ_C (100 MHz; CDCl₃) 172.7, 171.2, 168.5, 156.7, 136.7, 128.6, 128.3, 128.2, 128.1, 82.2, 66.7, 53.7, 52.5, 43.3, 40.6, 40.1, 31.5, 30.8, 29.5, 29.4, 28.1, 23.0, 22.0;

m/z , 721 ($[M+Na+H]^+$), 720 ($[M+H]^+$), 699 ($[M+2H]^+$), 698 ($[M+H]^+$);

HRMS Found 698.3749 ($[M+H]^+$, $C_{36}H_{52}N_5O_9$ requires 698.3765).

***tert*-Butyl 2-(2-(2-acetamido-6-aminohexanamido)acetamido)-6-aminohexanoate (52).**

A solution of tripeptide **51** (0.14 g, 0.2 mmol) in ethanol (1.5 mL) containing 10% palladium on carbon (28 mg) and formic acid (75 μ L) was stirred under N_2 for 2 hours. The reaction mixture was filtered through celite and the filtrate was evaporated to give diamine **52** as a colourless oil which was used in the next step without further purification.

R_f =0.04 (15% MeOH/ CH_2Cl_2)

$[\alpha]_D^{21} = -12.8$ ($c = 0.33$, MeOH)

ν_{max}/cm^{-1} 2952, 2922, 2854, 1732, 1654, 1542, 1456, 1373, 1249, 1202, 1155, 1087, 1038, 838, 800, 721;

δ_H (500 MHz; MeOD) 4.35-4.21 (m, 1H, CH), 3.73 (d, AB, $J=19.8$ Hz, 1H, NCH_2CO), 3.66 (d, AB, $J=19.8$ Hz, 1H, NCH_2CO), 3.57-3.48 (m, 1H, CH), 2.85-2.68 (m, 2H, CH_2), 1.88 (s, 3H, CH_3), 1.81-1.14 (m, 12H, $2\times CH_2$), 1.33 (s, 9H, *t*-Bu);

δ_C (100 MHz; MeOD) 173.7, 172.4, 171.2, 170.2, 81.7, 53.9, 52.7, 42.1, 39.1, 39.0, 30.6, 30.5, 26.9, 26.8, 26.6, 22.4, 21.2;

m/z , 452 ($[M+Na]^+$), 430 ($[M+H]^+$);

HRMS Found 430.3027 ($[M+H]^+$, $C_{20}H_{40}N_5O_5$ requires 430.3029).

2-Acetamidoacetic acid (58).¹¹¹

Acetic anhydride (19 mL, 200 mmol) to a stirred solution of glycine (7.5 g, 100 mmol) in H₂O (50 mL). The reaction mixture was stirred vigorously at room temperature for 20 minutes. The mixture was kept overnight in a refrigerator. The precipitate was filtered to obtain the product as white solid (8.7 g, 74.3 mmol, 74% yield).

mp 204-205°C (Lit¹¹¹ 207°C), R_f =0.13 (15% MeOH/CH₂Cl₂)
$$\nu_{\max}/\text{cm}^{-1} \text{ 3272, 2945, 2833, 1717, 1581, 1547, 1439, 1380, 1350, 1274, 1227, 1133, 1022, 902, 681;}$$
 δ_{H} (500 MHz; D_2O) 3.88 (s, 2H, CH_2), 1.95 (s, 3H, CH_3); δ_{C} (100 MHz; D₂O) 174.7, 173.6, 41.1, 21.6;

m/z , 140 ($[M+Na]^+$), 118 ($[M+H]^+$).

***tert*-Butyl 2-(2-acetamidoacetamido)-6-(*tert*-butoxycarbonyl)hexanoate (59).**

A small amount of *N,N*-dimethylaminopyridine was added to a solution of carboxylic acid **58** (0.35 g, 2.97 mmol) and amine **40** (1 g, 2.97 mmol) in dry dichloromethane (30 mL) and dry dimethylformamide (5 mL). The resulting mixture was stirred under N₂ at room temperature for 10 minutes. After the addition of *N,N*-diisopropylethylamine (0.26 mL, 1.47 mmol), the reaction mixture was cooled to 0 °C whereupon a 50% solution of T3P in DMF (2.4 mL, 4.2 mmol) was slowly added. The reaction mixture was stirred at 0 °C for 30 minutes and then for 18 hours at room

temperature. The reaction was quenched with 1 M sodium hydroxide, the organic phase was separated and washed with H₂O (3×20 mL), brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 10% MeOH in CH₂Cl₂) to give dipeptide **59** as a colourless oil (1.13 g, 2.6 mmol, 87% yield).

$R_f=0.53$ (15% MeOH/CH₂Cl₂)

$[\alpha]_D^{22} = -12.5$ ($c = 0.57$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3294, 2935, 1653, 1529, 1455, 1368, 1247, 1149, 1099, 1028 846, 776, 734, 697;

δ_H (500 MHz; CDCl₃) 7.42-7.33 (m, 5H, Ar-H), 6.60 (br d, $J=7.5$ Hz, 1H, NH), 6.33 (app s, 1H, NH), 5.11 (s, 2H, CH₂Ph), 5.04 (app s, 1H, NH), 4.51-4.43 (m, 1H, CH), 3.98 (dd, ABX, $J=17.0, 5.5$ Hz, 1H, CH₂), 3.94 (dd, ABX, $J=17.0, 5.5$ Hz, 1H, CH₂), 3.26-3.17 (m, 2H, CH₂), 2.03 (s, 3H, CH₃), 1.94-1.30 (m, 6H, 3×CH₂), 1.48 (s, 9H, *t*-Bu);

δ_C (100 MHz; CDCl₃) 171.4, 171.1, 169.1, 156.4, 136.7, 128.6, 128.1, 82.3, 66.6, 52.7, 43.2, 40.5, 31.8, 29.3, 28.0, 22.9, 22.2;

m/z , 458 ([M+Na]⁺), 436 ([M+H]⁺);

HRMS Found 436.2447 ([M+H]⁺, C₂₂H₃₄N₃O₆ requires 436.2448).

***tert*-Butyl 2-(2-acetamidoacetamido)-6-aminohexanoate (**60**).**

A solution of dipeptide **59** (1.4 g, 3.23 mmol) in ethanol (15 mL) containing 10% palladium on carbon (0.14 g) and formic acid (1.2 mL) was stirred under N₂ for 2 hours. The reaction mixture was filtered through celite and the filtrate was evaporated to give diamine **60** as a colourless oil which was used in the next step without further purification.

$R_f=0.08$ (15% MeOH/ CH_2Cl_2)

$[\alpha]_D^{21} = -16.2$ ($c = 0.30$, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3275, 3075, 2975, 2936, 1730, 1650, 1563, 1461, 1370, 1346, 1292, 1253, 1154, 1032, 846, 762;

δ_{H} (400 MHz; CDCl_3) 7.90 (app br s, 1H, NH), 7.62 (app br s, 1H, NH), 4.45–4.26 (m, 1H, CH), 4.23–3.73 (m, 2H, CH_2NHCO_2), 3.25 (br s, 2H, NH_2), 3.03–2.77 (m, 2H, CH_2), 2.00 (s, 3H, CH_3), 1.90–1.12 (m, 6H, $3\times\text{CH}_2$), 1.44 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl_3) 171.9, 171.8, 171.5, 82.3, 52.9, 42.9, 39.2, 30.9, 28.1, 27.4, 22.9, 22.2;

m/z , 302 ($[\text{M}+\text{H}]^+$);

HRMS Found 302.2078 ($[\text{M}+\text{H}]^+$, $\text{C}_{14}\text{H}_{28}\text{N}_3\text{O}_4$ requires 302.2080).

***tert*-Butyl 2-(2-acetamidoacetamido)-6-(2-chloroacetamido)hexanoate (62).**

A solution of chloroacetic anhydride (62 mg, 0.37 mmol) in dry dichloromethane (1 mL) was added to a stirred mixture of amine **60** (0.1 g, 0.33 mmol), a small amount of 4-dimethyl aminopyridine and potassium carbonate (91.7 mg, 0.66 mmol) in dry dichloromethane (3 mL). The reaction mixture was stirred at room temperature for 1 hour. The reaction mixture was filtered through celite and the filtrate was evaporated. The crude product was purified by flash column chromatography (SiO_2 , 8% MeOH in CH_2Cl_2) to give chloride **62** as a pale yellow oil (82 mg, 0.22 mmol, 73% yield).

$R_f=0.31$ (10% MeOH/ CH_2Cl_2)

$[\alpha]_D^{22} = -15.4$ ($c = 0.88$, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3291, 2936, 1731, 1650, 1537, 1440, 1368, 1233, 1030, 846, 682;

δ_{H} (300 MHz; CDCl_3) 7.11 (d, 1H, NH), 7.02-6.87 (m, 2H, NH), 4.48-4.36 (m, 1H, CH), 4.03 (s, 2H, CH_2Cl), 3.97 (dd, ABX, $J=17.1, 5.7$ Hz, 1H, CH_2), 3.90 (dd, ABX, $J=17.1, 6.2$ Hz, 1H, CH_2), 3.38–3.13 (m, 2H, CH_2), 2.02 (s, 3H, CH_3), 1.92-1.28 (m, 6H, $3\times\text{CH}_2$), 1.43 (s, 9H, *t*-Bu);

δ_{C} (75 MHz; CDCl_3) 171.2, 171.0, 169.1, 166.4, 82.2, 52.6, 43.2, 42.7, 39.3, 31.7, 28.7, 28.0, 22.9, 22.2;

m/z , 400, 402 ($[\text{M}+\text{Na}]^+$), 377, 380 ($[\text{M}+\text{H}]^+$);

HRMS Found 378.1792 ($[\text{M}+\text{H}]^+$, $\text{C}_{16}\text{H}_{29}\text{N}_3\text{O}_5$ ^{35}Cl requires 378.1796).

***tert*-Butyl 2-(2-acetamidoacetamido)-6-(2-azidoacetamido)hexanoate (63).**

Sodium azide (52 mg, 0.79 mmol) was added to a stirred solution of chloride **62** (0.2 g, 0.53 mmol) in dimethylformamide (10 mL). The reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was quenched with H_2O and extracted with dichloromethane (3×10 mL). The combined dichloromethane extracts were washed with brine before drying over anhydrous magnesium sulfate. The solvent was removed to give azide **63** as a colourless oil (0.14 g, 0.36 mmol, 68% yield).

$R_f=0.28$ (10% MeOH/ CH_2Cl_2)

$[\alpha]_{\text{D}}^{21} = -6.2$ ($c = 1.11$, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3288, 2934, 2104, 1731, 1651, 1540, 1439, 1368, 1252, 1155, 1098, 1032, 1010, 845, 661;

δ_{H} (400 MHz; CDCl_3) 6.90 (br d, $J=7.8$ Hz, 1H, NH), 6.84 (app br s, 1H, NH), 6.74–6.65 (m, 1H, NH), 4.48-4.36 (m, 1H, CH), 4.04 (s, 2H, CH_2Cl), 3.97 (dd, ABX,

$J=16.5, 5.5$ Hz, 1H, CH₂), 3.90 (dd, ABX, $J=16.5, 5.0$ Hz, 1H, CH₂), 3.37–3.18 (m, 2H, CH₂), 2.03 (s, 3H, CH₃), 1.89–1.26 (m, 6H, 3×CH₂), 1.44 (s, 9H, *t*-Bu);
 δ_{C} (100 MHz; CDCl₃) 171.3, 171.0, 168.9, 167.2, 82.4, 52.6, 43.3, 38.9, 31.8, 28.8, 28.0, 23.0, 22.2;
 m/z, 407 ([M+Na]⁺), 408 ([M+Na+H]⁺), 385 ([M+H]⁺), 384 (M⁺);
 HRMS Found 385.2192 ([M+H]⁺, C₁₆H₂₉N₆O₅ requires 385.2199).

Butyl 2-(2-acetamidoacetamido)-6-(2-(4-(hydroxymethyl)-1*H*-1,2,3-triazol-1-yl)acetamido)hexanoate (64).

Aqueous copper sulfate (5.5 μL of a 0.3 M solution) and aqueous sodium ascorbate (8.2 μL of a 0.5 M solution) were added to a stirred solution of azide **63** (32 mg, 0.08 mmol) and propargyl alcohol (7.4 μL , 0.08 mmol) in a 1:1:1 mixture of H₂O, *tert*-butanol and dichloromethane (0.9 mL). The reaction mixture was stirred at room temperature for 24 hours. The reaction mixture was quenched with H₂O and extracted with dichloromethane (3×10 mL). The combined dichloromethane extracts were washed with brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 10% MeOH in CH₂Cl₂) to give triazole **64** as a colourless oil (32 mg, 0.07 mmol, 87% yield).

$R_f=0.32$ (10% MeOH/CH₂Cl₂)

$\nu_{\text{max}}/\text{cm}^{-1}$ 2926, 1731, 1652, 1541, 1457, 1369, 1259, 1231, 1155, 1038, 1013, 915, 844, 801, 728;

δ_{H} (500 MHz; CDCl_3) 7.82 (s, 1H, $\text{NCH}=\text{C}$), 6.91-6.81 (m, 2H, NH), 6.71 (app s, 1H, NH), 5.15 (d, $J=15.5$ Hz, 1H, CH_2OH), 5.04 (d, $J=15.5$ Hz, 1H, CH_2OH), 4.84 (s, 2H, COCH_2N), 4.50-4.42 (m, 1H, CH), 4.06 (dd, ABX, $J=16.5$, 5.8 Hz, 1H, CH_2), 3.92 (dd, ABX, $J=16.5$, 5.5 Hz, 1H, CH_2), 3.40-3.21 (m, 2H, CH_2), 2.11 (s, 3H, CH_3), 1.92-1.20 (m, 6H, $3\times\text{CH}_2$), 1.48 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl_3) 171.3, 171.2, 171.1, 169.2, 141.4, 123.6, 82.6, 56.5, 53.2, 52.3, 43.5, 39.0, 31.8, 28.0, 27.8, 23.0, 21.7;

m/z , 464 ($[\text{M}+\text{Na}+\text{H}]^+$), 463 ($[\text{M}+\text{Na}]^+$), 441 ($[\text{M}+\text{H}]^+$);

HRMS Found 441.2461 ($[\text{M}+\text{H}]^+$), $\text{C}_{19}\text{H}_{33}\text{N}_6\text{O}_6$ requires 441.2462).

Prop-2-ynyl acrylate (66).

N,N-Diisopropylethylamine (8.6 mL, 49.3 mmol) were added to a stirred solution of propargyl alcohol (2.65 mL, 44.9 mmol) in dry dichloromethane (10 mL) at 0 °C. After being stirred for 5 minutes, a solution of acryloyl chloride (4 mL, 49.3 mmol) in dry dichloromethane (8 mL) was added dropwise. The reaction mixture was stirred at 0 °C for 1 hour and at room temperature for 5 hours. The resulting mixture was diluted with dichloromethane (30 mL), washed with saturated aqueous sodium hydrogen carbonate, H_2O , and brine before drying over anhydrous magnesium sulfate. The solvent was removed and the crude product was purified by distillation (80 °C, 4 mmHg) to give acrylate **66** as a colourless liquid (3.16 g, 28.7 mmol, 64% yield).

$R_f=0.59$ (10% MeOH/ CH_2Cl_2)

$\nu_{\text{max}}/\text{cm}^{-1}$ 1812, 1728, 1408, 1296, 1271, 1184, 1052, 984, 809, 670, 639;

δ_{H} (400 MHz; CDCl_3) 6.46 (dd, $J=17.4$, 1.4 Hz, 1H, $\text{CH}_2=\text{CH}$), 6.14 (dd, $J=17.4$, 10.5 Hz, 1H, $\text{CH}_2=\text{CH}$), 5.88 (dd, $J=10.5$, 1.4 Hz, 1H, $\text{CH}_2=\text{CH}$), 4.75 (d, $J=2.3$ Hz, 2H, $\text{CH}_2\text{C}\equiv\text{C}$), 2.48 (t, $J=2.3$ Hz, 1H, $\text{C}\equiv\text{CH}$);

δ_{C} (100 MHz; CDCl_3) 165.3, 132.0, 127.6, 77.6, 75.1, 52.1;

m/z , 112 ($[\text{M}+2\text{H}]^+$), 111 ($[\text{M}+\text{H}]^+$).

{4,10-Bis-*tert*-butoxycarbonylmethyl-7-[2-propynylcarboxylate)-ethyl]1,4,7,10-tetraazacyclododec-1-yl}-acetic acid *tert*-butyl ester (65).

1,8-Diazabicyclo[5.4.0]undec-7-ene, DBU (54 μ L, 0.36 mmol) was added to a stirred suspension of 1,4,7-tris(*tert*-butoxycarbonylmethyl)-1,4,7-tetraazacyclododecane (**5**) (0.35 g, 0.68 mmol) and propargyl acrylate (0.11 g, 1.02 mmol) in acetonitrile (8 mL). After being stirred at 50 $^{\circ}$ C for 20 hours, The solvent was removed and the residue was taken up in dichloromethane (20 mL), washed with saturated aqueous ammonium chloride, H₂O, and brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 8% MeOH in CH₂Cl₂) to give alkyne **65** as a white solid (0.38 g, 0.6 mmol, 89% yield).

mp 75-77 $^{\circ}$ C, R_f =0.28 (10% MeOH/CH₂Cl₂)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3403, 2978, 2832, 2120, 1720, 1671, 1455, 1427, 1368, 1311, 1258, 1226, 1154, 1101, 1031, 988, 937, 848, 787;

δ_{H} (400 MHz; CDCl₃) 4.64 (d, J =2.5 Hz, 2H, CH₂C $\equiv$ C), 3.38-1.86 (m, 26H, CH₂), 2.47 (t, J =2.5 Hz, 1H, CH), 1.46 (s, 18H, 2 $\times$ *t*-Bu), 1.43 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl₃) 173.3, 173.0, 172.0, 82.5, 77.3, 75.4, 56.5, 55.8, 52.3, 50.8, 50.1, 30.8, 28.0, 27.9;

m/z , 626 ([M+H]⁺), 625 (M⁺);

HRMS Found 647.4006 ([M+Na]⁺, C₃₂H₅₆N₄O₈Na requires 647.3996).

***tert*-Butyl 2-acetamido-6-(benzyloxycarbonyl)hexanoate (67).**

Acetic anhydride (0.39 mL, 4.2 mmol) and triethylamine (1.2 mL, 8.3 mmol) were added in two portions to a suspension of lysine **45** (1 g, 2.97 mmol) in 0.5 M aqueous sodium hydroxide (5 mL) and dichloromethane (5 mL) at 0 °C over 30 minutes. The reaction mixture was stirred at 0 °C for 1 hour and another 1 hour at room temperature. The organic layer was separated and washed with H₂O, and brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO₂, 8% MeOH in CH₂Cl₂) to give lysine **67** as a colourless oil (0.9 g, 2.38 mmol, 80% yield).

R_f =0.54 (10% MeOH/CH₂Cl₂)

$[\alpha]_D^{21}$ = -13.2 (c = 1.08, CHCl₃)

$\nu_{\max}/\text{cm}^{-1}$ 3321, 2937, 1704, 1657, 1535, 1455, 1369, 1248, 1147, 1026, 915, 846, 733, 697;

δ_H (400 MHz; CDCl₃) 7.39-7.25 (m, 5H, Ar-H), 6.09 (br d, 1H, NH), 5.08 (s, 2H, CH₂Ph), 4.82 (app br s, 1H, NH), 4.46 (td, J =7.5, 5.3 Hz, 1H, CH), 3.17 (dt, J =6.6, 6.6 Hz, 2H, CH₂), 1.99 (s, 3H, CH₃), 1.86-1.23 (m, 6H, 3×CH₂), 1.44 (s, 9H, *t*-Bu);

δ_C (100 MHz; CDCl₃) 171.9, 169.9, 156.6, 136.6, 128.6, 128.2, 82.3, 66.7, 52.5, 40.7, 32.3, 29.6, 28.1, 23.3, 22.2;

m/z , 402 ([M+Na+H]⁺), 401 ([M+Na]⁺), 380 ([M+2H]⁺), 379 ([M+H]⁺);

HRMS Found 401.2056 ([M+Na]⁺, C₂₀H₃₀N₂O₅Na requires 401.2052).

***tert*-Butyl 2-acetamido-6-aminohexanoate (**68**).**

A solution of lysine **67** (0.8 g, 2.1 mmol) in ethanol (12 mL) containing 10% palladium on carbon (0.08 g) was stirred under H₂ for 4 hours. The reaction mixture was filtered through celite and the filtrate was evaporated to give amine **68** as a yellow oil which was used in the next step without further purification.

R_f =0.05 (10% MeOH/CH₂Cl₂)

$[\alpha]_D^{21} = -17.7$ ($c = 0.98$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3271, 2977, 2935, 2865, 1731, 1653, 1479, 1456, 1368, 1304, 1252, 1231, 1150, 919, 847, 644;

δ_H (400 MHz; CDCl₃) 4.21 (dd, $J=3.2, 5.0$ Hz, 1H, CH), 3.32-3.24 (m, 2H, CH₂), 1.95 (s, 3H, CH₃), 1.83-1.70 (m, 1H, CH₂), 1.70-1.56 (m, 1H, CH₂), 1.54-1.31 (m, 2H, CH₂), 1.44 (s, 9H, *t*-Bu);

δ_C (100 MHz; CDCl₃) 172.0, 170.1, 82.1, 52.7, 41.5, 32.5, 32.4, 28.1, 23.2, 22.4;

m/z , 245 ($[M+H]^+$);

HRMS Found 267.1688 ($[M+Na]^+$, C₁₂H₂₄N₂O₃Na requires 267.1685).

***tert*-Butyl 2-acetamido-6-(2-chloroacetamido)hexanoate (**69**).**

A solution of chloroacetic anhydride (0.38 g, 2.25 mmol) in dry dichloromethane (5 mL) was added to a stirred mixture of amine **68** (0.5 g, 2.05 mmol), a small amount of 4-dimethyl aminopyridine and potassium carbonate (0.57 g, 4.09 mmol) in dry dichloromethane (15 mL). The reaction mixture was stirred at room temperature for 1 hour. The reaction mixture was filtered through celite and the filtrate was evaporated. The crude product was purified by flash column chromatography

(SiO₂, 8% MeOH in CH₂Cl₂) to give chloride **69** as a colourless oil (0.62 g, 1.93 mmol, 94% yield).

R_f =0.45 (10% MeOH/CH₂Cl₂)

$[\alpha]_D^{21} = -17.2$ (c = 1.12, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3294, 2936, 1732, 1650, 1538, 1455, 1440, 1368, 1233, 1146, 1040, 846, 734, 691;

δ_H (400 MHz; CDCl₃) 6.46 (app br s, 1H, NH), 6.12 (d, J =3.2, 5.0 Hz, 1H, NH), 4.47 (ddd, J =7.8, 7.8, 5.0 Hz, 1H, CH), 3.96 (s, 1H, CH₂Cl), 3.28 (td, J =6.7, 6.7 Hz, 2H, CH₂), 2.02 (s, 3H, CH₃), 1.87-1.25 (m, 6H, 3×CH₂), 1.46 (s, 9H, *t*-Bu);

δ_C (100 MHz; CDCl₃) 171.8, 170.1, 166.2, 82.4, 52.4, 42.7, 39.5, 32.3, 28.8, 28.1, 23.3, 22.3;

m/z , 345 ([M+Na]⁺), 343 ([M+Na]⁺), 322 (M⁺), 320 (M⁺);

HRMS Found 321.1585 ([M+H]⁺, C₁₄H₂₆N₂O₄³⁵Cl requires 321.1581).

***tert*-Butyl 2-acetamido-6-(2-azidoacetamido)hexanoate (70).**

Sodium azide (0.17 g, 2.57 mmol) was added to a stirred solution of chloride **69** (0.55 g, 1.71 mmol) in dimethylformamide (10 mL). The reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was quenched with H₂O and extracted with dichloromethane (3×10 mL). The combined dichloromethane extracts were washed with brine before drying over anhydrous magnesium sulfate. The solvent was removed to give azide **70** as a colourless oil (0.52 g, 1.59 mmol, 93% yield).

R_f =0.45 (10% MeOH/CH₂Cl₂)

$[\alpha]_D^{21} = -14.9$ (c = 1.04, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3295, 2933, 2103, 1733, 1652, 1539, 1438, 1387, 1367, 1255, 1155, 1094, 847, 733, 660;

δ_{H} (400 MHz; CDCl_3) 6.45 (br s, 1H, NH), 6.13 (br s, 1H, NH), 4.49-4.41 (m, 1H, CH), 3.94 (s, 2H, CH_2N_3), 3.31-3.22 (m, 2H, CH_2), 2.00 (s, 3H, CH_3), 1.87-1.23 (m, 6H, $3\times\text{CH}_2$), 1.44 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl_3) 171.8, 170.2, 167.0, 82.2, 52.6, 52.4, 39.0, 32.2, 28.8, 28.0, 23.2, 22.4;

m/z , 351 ($[\text{M}+\text{Na}+\text{H}]^+$), 350 ($[\text{M}+\text{Na}]^+$), 329 ($[\text{M}+2\text{H}]^+$), 328 ($[\text{M}+\text{H}]^+$);

HRMS Found 350.1809 ($[\text{M}+\text{Na}]^+$, $\text{C}_{14}\text{H}_{25}\text{N}_5\text{O}_4$ requires 350.1804).

***tert*-Butyl 2-acetamido-6-(2-(4-(acryloyloxymethyl)-1*H*-1,2,3-triazol-1-yl) acetamido)hexanoate (72).**

Aqueous copper sulfate (32 μL of a 0.3 M solution) and aqueous sodium ascorbate (48 μL of a 0.5 M solution) were added to a stirred solution of azide **70** (0.16 g, 0.49 mmol) and propargyl acrylate (80 mg, 0.73 mmol) in a 1:1:1 mixture of H_2O , *tert*-butanol and dichloromethane (4.6 mL). The reaction mixture was stirred at room temperature for 24 hours. The reaction mixture was quenched with H_2O and extracted with dichloromethane (3×10 mL). The combined dichloromethane extracts were washed with brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO_2 , 10% MeOH in CH_2Cl_2) to give triazole **72** as a white solid (0.19 g, 0.43 mmol, 87% yield).

mp 131-132°C, $R_f=0.26$ (10% MeOH/ CH_2Cl_2)

$\nu_{\max}/\text{cm}^{-1}$ 3275, 3075, 2934, 2862, 1731, 1657, 1621, 1495, 1444, 1369, 1260, 1230, 1200, 1156, 1084, 1041, 1014, 912, 845, 803, 726, 644;

δ_{H} (400 MHz; CDCl_3) 6.64 (app s, 1H, NH), 6.43 (dd, $J=10.4$, 1.4 Hz, 1H, $\text{CH}_2=\text{CH}$), 6.12 (dd, $J=17.4$, 10.5 Hz, 1H, $\text{CH}_2=\text{CH}$), 5.85 (dd, $J=17.4$, 1.4 Hz, 1H, $\text{CH}_2=\text{CH}$), 5.31 (s, 2H, CH_2CO_2), 5.07 (d, AB, $J=16.0$ Hz, 1H, COCH_2N), 4.99 (d, AB, $J=16.0$ Hz, 1H, COCH_2N), 4.45 (td, $J=8.2$, 4.6 Hz, 1H, CH), 3.32-3.16 (m, 2H, CH_2), 2.04 (s, 3H, CH_3), 1.82-1.26 (m, 6H, $3\times\text{CH}_2$), 1.45 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl_3) 171.8, 170.4, 166.0, 165.0, 143.3, 131.8, 128.0, 125.6, 82.5, 57.7, 53.0, 52.1, 39.5, 32.7, 28.1, 23.5, 22.3;

m/z , 460 ($[\text{M}+\text{Na}]^+$), 461 ($[\text{M}+\text{Na}+\text{H}]^+$), 438 ($[\text{M}+\text{H}]^+$);

HRMS Found 438.2350 ($[\text{M}+\text{H}]^+$, $\text{C}_{20}\text{H}_{32}\text{N}_5\text{O}_6$ requires 438.2353).

DO3'*t*-Bu-lysine triazole (71).

Copper sulfate (35 mg, 0.14 mmol) and aqueous sodium ascorbate (0.16 mL of a 1 M solution) were added to a stirred solution of azide **70** (35.6 mg, 0.11 mmol) and DO3'*t*-Bu-alkyne **65** (68 mg, 0.11 mmol) in a 1:1:1 mixture of H_2O , *tert*-butanol and dichloromethane (0.6 mL). The reaction mixture was stirred at room temperature for 3 days. The reaction mixture was diluted with dichloromethane (5 mL). EDTA disodium salt was then added and stirred for 30 minutes, before being filtered through celite and the filtrate was evaporated to dryness. The crude product was purified by flash column

chromatography (SiO₂, 10% MeOH in CH₂Cl₂) to give DO3'Bu **71** as a colourless oil (41.7 mg, 0.04 mmol, 40% yield).

R_f =0.18 (10% MeOH/CH₂Cl₂)

$[\alpha]_D^{21} = 4.8$ ($c = 0.27$, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3271, 2980, 2837, 1725, 1636, 1461, 1425, 1393, 1370, 1312, 1233, 1152, 1087, 971, 842;

δ_H (400 MHz; CDCl₃) 7.75 (s, 1H, CH), 6.42 (app br s, 1H, NH), 6.27 (d, $J=7.5$ Hz, 1H, NH), 5.08 (d, $J=7.5$ Hz, 2H, NCH₂CO), 4.85 (s, 2H, CO₂CH₂CN=C), 4.50-4.39 (m, 1H, CH), 3.49-2.21 (m, 28H, 14×CH₂), 2.08 (s, 3H, CH₃), 1.89-1.15 (m, 42H, 3×CH₂ and 4×*t*-Bu);

δ_C (100 MHz; CDCl₃) 172.9, 172.5, 172.0, 171.1, 165.9, 141.8, 125.1, 83.1, 82.8, 81.3, 58.3, 56.5, 55.7, 53.5, 53.1, 50.2, 49.8, 38.6, 30.9, 28.6, 28.3, 28.2, 28.1, 28.0, 23.1, 22.6;

m/z , 976 ([M+Na+H]⁺), 975 ([M+Na]⁺), 954 ([M+2H]⁺), 953 ([M+H]⁺);

HRMS Found 952.6083 ([M+H]⁺, C₄₆H₈₂N₉O₁₂ requires 952.6083).

DO3A-lysine triazole (**73**).

Trifluoroacetic acid (0.1 mL) was added to a stirred solution of DO3'Bu **71** (10 mg, 0.01 mmol) in dry dichloromethane (0.2 mL). The reaction mixture was stirred at

room temperature for 18 hours. After removal of solvent under reduced pressure, acid was removed by addition and evaporation of successive portion of dichloromethane (2×5 mL), methanol (2×5 mL) and diethyl ether (2×5 mL), respectively to give DO3A **73** as a pale yellow solid which was used in the next step without further purification.

$R_f=0.08$ (10% MeOH/CH₂Cl₂)

$[\alpha]_D^{21} = 5.8$ (c = 0.47, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3327, 2961, 1640, 1574, 1546, 1536, 1441, 1402, 1261, 1203, 1141, 801, 693;

δ_H (400 MHz; MeOD) 8.07 (s, 1H, CH), 5.25 (s, 2H, $\bar{N}CH_2CO$), 5.14 (s, 2H, CO₂CH₂CN=C), 4.37-4.28 (m, 1H, CH), 4.22-4.06 (m, 2H, CH₂), 3.74-2.85 (m, 26H, CH₂), 1.96 (s, 3H, CH₃), 1.90-1.31 (m, 12H, CH₂);

δ_C (100 MHz; MeOD) 173.9, 172.0, 170.7, 166.4, 166.2, 141.9, 124.3, 65.5, 57.5, 55.1, 52.3, 52.2, 51.7, 51.3, 50.0, 48.8, 46.5, 39.0, 30.8, 28.4, 28.3, 22.8, 21.0;

m/z, 752 ([M+Na+2H]⁺), 751 ([M+Na+H]⁺), 750 ([M+Na]⁺), 729 ([M+2H]⁺), 728 ([M+H]⁺), 727 (M⁺);

HRMS Found 750.3403 ([M+Na]⁺, C₃₀H₄₉N₉O₁₂Na requires 750.3398).

GdDO3A-lysine triazole (CA3).

A solution of GdCl_3 (3 mg, 0.01 mmol) in H_2O (0.1 mL) was added to a stirred solution of DO3A **73** (7.6 g, 0.01 mmol) in methanol (0.5 mL). The pH was adjusted and maintained at 5.8 using 0.5 M aqueous ammonium acetate. The reaction mixture was stirred at 70 °C for 2 hours. After removal of solvent under reduced pressure, the crude product was purified by semipreparative reverse phase HPLC (2→60% CH_3CN , t_{R} =6.8 min) to give complex **CA3** as a pale yellow solid (3.05 mg, 0.003 mmol, 33% yield).

mp 131-132°C

$[\alpha]_{\text{D}}^{21} = 9.8$ ($c = 0.59$, MeOH)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3137, 3046, 2879, 1669, 1576, 1441, 1405, 1324, 1200, 1135, 1086, 838, 800, 722;

m/z , 880, 881, 882, 883, 884, 885, 886 ($[\text{M}+\text{H}]^+$), 902, 903, 904, 905, 906, 907, 908 ($[\text{M}+\text{Na}]^+$);

HRMS Found 883.2592 ($[\text{M}+\text{H}]^+$, $\text{C}_{30}\text{H}_{47}\text{N}_9\text{O}_{12}$ ^{158}Gd requires 883.2585) with Gd isotope distribution pattern.

***tert*-Butyl 2-(2-acetamido-6-(2-chloroacetamido)hexanamido)-6-(2-chloroacetamido)hexanoate (77).**

A solution of chloroacetic anhydride (0.91 g, 5.32 mmol) in dry dichloromethane (9 mL) was added to a stirred mixture of bis-amine **42** (0.9 g, 2.42 mmol), a small amount of 4-dimethyl aminopyridine and potassium carbonate (1.34 g,

9.66 mmol) in dry dichloromethane (26 mL). The reaction mixture was stirred at room temperature for 1 hour. The reaction mixture was filtered through celite and the filtrate was evaporated. The crude product was purified by flash column chromatography (SiO₂, 8% MeOH in CH₂Cl₂) to give bis-chloride **77** as a colourless oil (0.92 g, 1.75 mmol, 73% yield).

R_f =0.37 (10% MeOH/CH₂Cl₂)

$[\alpha]_D^{22}$ = -10.9 (c = 1.07, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3274, 3068, 2937, 2867, 1730, 1650, 1539, 1442, 1369, 1224, 1155, 617, 846, 726;

δ_{H} (300 MHz; CDCl₃) 7.21 (d, 1H, NH), 7.06 (t, 1H, NH), 6.96 (t, 1H, NH), 7.21 (d, 1H, NH), 4.44 (ddd, J =5.8, 7.7, 7.7 Hz, 1H, CH), 4.33 (ddd, J =4.7, 7.8, 7.8 Hz, 1H, CH), 4.08 (d, AB, J =14.7 Hz, 1H, CH₂Cl), 4.02 (d, AB, J =14.7 Hz, 1H, CH₂Cl), 4.02 (s, 2H, CH₂Cl), 3.40-3.14 (m, 4H, 2×CH₂), 1.99 (s, 3H, CH₃), 1.89-1.25 (m, 12H, 6×CH₂), 1.44 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl₃) 171.9, 171.2, 170.7, 166.6, 166.4, 82.1, 52.9, 52.8, 42.7, 39.3, 39.1, 31.8, 31.2, 28.7, 28.0, 23.0, 22.4, 22.3;

m/z , 549 ([M+Na]⁺), 547 ([M+Na]⁺), 527 ([M+H]⁺), 525 ([M+H]⁺);

HRMS Found 525.2250 ([M+H]⁺, C₂₂H₃₉N₄O₆³⁵Cl₁₂ requires 525.2247).

***tert*-Butyl 2-(2-acetamido-6-(2-azidoacetamido)hexanamido)-6-(2-azidoacetamido)hexanoate (78).**

Sodium azide (0.18 g, 2.74 mmol) was added to a stirred solution of bis-chloride **77** (0.48 g, 0.91 mmol) in *N,N*-dimethylformamide (10 mL). The reaction mixture was stirred at room temperature for 18 hours. The reaction mixture was quenched with H₂O and extracted with dichloromethane (3×10 mL). The combined dichloromethane extracts were washed with brine before drying over anhydrous magnesium sulfate. The solvent was removed to give bis-azide **78** as a pale yellow solid (0.44 g, 0.81 mmol, 89% yield).

$R_f=0.37$ (10% MeOH/CH₂Cl₂)

$[\alpha]_D^{21} = -31.3$ (c = 0.35, MeOH)

$\nu_{\max}/\text{cm}^{-1}$ 3286, 3080, 2937, 2867, 2103, 1732, 1649, 1541, 1439, 1369, 1257, 1156, 911, 846, 729;

δ_{H} (300 MHz; CDCl₃) 6.92 (d, 1H, NH), 6.76 (t, $J=5.7$ Hz, 1H, NH), 6.62 (t, $J=5.7$ Hz, 1H, NH), 4.48-4.32 (m, 1H, CH), 4.04 (d, AB, $J=16.2$ Hz, 1H, CH₂Cl), 3.97 (d, AB, $J=16.2$ Hz, 1H, CH₂Cl), 3.97 (s, 2H, CH₂Cl), 3.47-3.12 (m, 4H, 2×CH₂), 2.02 (s, 3H, CH₃), 1.94-1.24 (m, 12H, 6×CH₂), 1.47 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; CDCl₃) 171.9, 171.3, 170.7, 167.5, 167.2, 82.2, 53.0, 52.8, 52.6, 38.9, 38.7, 31.9, 31.2, 28.8, 28.1, 23.1, 22.4, 22.2;

m/z , 562 ([M+Na+H]⁺), 561 ([M+Na]⁺), 540 ([M+2H]⁺), 539 ([M+H]⁺);

HRMS Found 539.3057 ([M+H]⁺, C₂₂H₃₉N₁₀O₆ requires 539.3054).

***tert*-Butyl 2-(2-acetamido-6-(2-(4-(hydroxymethyl)-1*H*-1,2,3-triazol-1-yl)acetamido)hexanamido)-6-(2-(4-(hydroxymethyl)-1*H*-1,2,3-triazol-1-yl)acetamido)hexanoate (79).**

Aqueous copper sulfate (6.8 μL of a 0.3 M solution) and aqueous sodium ascorbate (10 μL of a 0.5 M solution) were added to a stirred solution of bis-azide **78** (27.5 mg, 0.05 mmol) and propargyl alcohol (8.6 mg, 0.15 mmol) in a 1:1:1 mixture of H_2O , *tert*-butanol and dichloromethane (1.8 mL). The reaction mixture was stirred at room temperature for 24 hours. The reaction mixture was quenched with H_2O and extracted with dichloromethane (3 $\times$ 5 mL). The combined dichloromethane extracts were washed with brine before drying over anhydrous magnesium sulfate. After removal of solvent under reduced pressure, the crude product was purified by flash column chromatography (SiO_2 , 10% MeOH in CH_2Cl_2) to give bis-triazole **79** as a colourless oil (28.2 mg, 0.04 mmol, 85% yield).

R_f =0.20 (15% MeOH/ CH_2Cl_2)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3274, 3088, 2933, 2865, 1727, 1656, 1551, 1458, 1370, 1259, 1231, 1156, 1041, 1012, 843, 802;

δ_{H} (400 MHz; MeOD) 7.91 (s, 2H, NCH=C), 5.11 (s, 4H, CH_2OH), 4.67 (s, 4H, COCH₂N), 4.30-4.18 (m, 1H, CH), 3.30-3.12 (m, 4H, 2 $\times$ CH₂), 1.95 (s, 3H, CH₃), 1.88-1.29 (m, 12H, 6 $\times$ CH₂), 1.43 (s, 9H, *t*-Bu);

δ_{C} (100 MHz; MeOD) 171.9, 171.4, 166.5, 147.7, 124.3, 81.5, 55.2, 53.3, 52.9, 51.8, 39.0, 31.3, 30.6, 28.5, 28.3, 26.9, 22.7, 22.6, 21.1;

m/z , 674 ($[M+Na+H]^+$), 673 ($[M+Na]^+$), 652 ($[M+2H]^+$), 651 ($[M+H]^+$);

HRMS Found 673.3402 ($[M+Na]^+$, $C_{28}H_{46}N_{10}O_8Na$ requires 673.3398).

DO3A-alkyne (74).

Trifluoroacetic acid (1.7 mL) was added to a stirred solution of DO3'Bu **65** (0.16 g, 0.26 mmol) in dry dichloromethane (1.7 mL). The reaction mixture was stirred at room temperature for 18 hours. After removal of solvent under reduced pressure, acid was removed by addition and evaporation of successive portion of dichloromethane (2×10 mL), methanol (2×10 mL) and diethyl ether (2×10 mL), respectively to give DO3A-alkyne **74** as a pale yellow solid which was used in the next step without further purification.

$R_f=0.09$ (15% MeOH/ CH_2Cl_2)

$\nu_{\text{max}}/\text{cm}^{-1}$ 3429, 3090, 2859, 1718, 1671, 1509, 1460, 1388, 1354, 1182, 1131, 1089, 988, 832, 799, 721;

δ_{H} (400 MHz; MeOD) 4.74 (d, $J=2.6$ Hz, 2H, $CH_2C\equiv C$), 4.11 (app s, 2H, CH_2), 3.76-2.86 (m, 24H, $12 \times CH_2$), 2.95 (t, $J=2.6$ Hz, 1H, $C\equiv CH$);

δ_{C} (100 MHz; MeOD) 173.4, 173.0, 169.9, 76.9, 75.5, 54.3, 52.4, 52.3, 51.2, 49.9, 49.8, 48.9, 48.5, 48.4, 28.0;

m/z , 479 ($[M+Na]^+$), 458 ($[M+2H]^+$), 457 ($[M+H]^+$);

HRMS Found 457.2296 ($[M+H]^+$, $C_{20}H_{33}N_4O_8$ requires 457.2298).

GdDO3A-alkyne (75).

A solution of GdCl_3 (40 mg, 0.15 mmol) in H_2O (0.3 mL) was added to a stirred solution of DO3A-alkyne **74** (0.069 g, 0.15 mmol) in methanol (0.2 mL) and H_2O (0.2 mL). The pH was adjusted and maintained at 5.8 using 0.5 M aqueous ammonium acetate. The reaction mixture was stirred at room temperature for 1.5 hours. After removal of solvent under reduced pressure, the crude product was washed with absolute ethanol to give complex **75** as a white powder (39 mg, 0.064 mmol, 42% yield).

mp 120-122°C

$\nu_{\text{max}}/\text{cm}^{-1}$ 3188, 3034, 2874, 1666, 1581, 1546, 1408, 1323, 1200, 1182, 1132, 1086, 1018, 941, 838, 801, 722, 675;

m/z , 608, 609, 610, 611, 612, 614, $([\text{M}+\text{H}]^+)$, 630, 631, 632, 633, 634, 636 $([\text{M}+\text{Na}]^+)$;

HRMS Found 612.1316 $([\text{M}+\text{H}]^+)$, $\text{C}_{20}\text{H}_{30}\text{N}_4\text{O}_8^{158}\text{Gd}$ requires 612.1305) with Gd isotope distribution pattern.

Relaxivity measurements

The relaxivity was determined on a Varian 9.4T MRI system (Palo Alto, CA, USA). The longitudinal relaxation time (T_1) of protons in water was measured in aqueous phantoms with concentrations 0.4, 0.2, 0.1, 0.05, 0.025, 0.0125 and 0.00625 mM Gd^{3+} , using inversion-recovery (IR) spin echo sequence with $\text{TR} = 7500$ ms, $\text{TE} = 6.28$ ms for inversion time, $\text{TI} = 5, 200, 500, 700, 900, 1000, 1100, 1300, 1500, 2000, 5000, 7480$ ms. The T_1 was determined in triplicate and the average T_1 measurement was calculated.

In vivo MR imaging

In vivo contrast enhanced MR imaging study was conducted on Wistar rats (male, weight 320-340 g). The animals were cared for under an approved protocol (IACUC Number: 060188) and the guidelines of the Responsible Care & Use of Laboratory Animals Committee, Biological Resource Center, A*star (Agency for Science, Technology and Research). CAs were injected (dosage: 0.04 mmol Gd/kg body weight) through tail vein under 2% isoflurane anesthesia. T_1 -weighted images were acquired every 6.4 s for 52 min with T_1 -weighted gradient echo sequence (TR/TE = 50/3 ms, flip-angle = 20 degree, resolution = 230 μ m, thickness = 2 mm) on a Varian 9.4T scanner. Dotarem and CA1 were investigated in a group of three rats while CA2 was investigated in a group of five rats.

REFERENCES

1. Lauffer, R. B. *Chem. Rev.* **1987**, *87*, 901-927.
2. Caravan, P.; Ellison, J. J.; McMurry, T. J.; Lauffer, R. B. *Chem. Rev.* **1999**, *99*, 2293-2352.
3. Tóth, É.; Helm, L.; Merbach, A. E. *Top. Curr. Chem.* **2002**, *221*, 61-101.
4. Ranganathan, R. S.; Raju, N.; Fan, H.; Zhang, X.; Tweedle, M. F.; Desreux, J. F.; Jacques, V. *Inorg. Chem.* **2002**, *41*, 6856-6866.
5. Merbach, A. E.; Tóth, É. *The Chemistry of Contrast Agents in Medical Magnetic Resonance Imaging*. John Wiley & Sons, Ltd., Chichester, **2001**.
6. Hinshaw, W. S.; Andrew, E. R.; Bottomley, P. A.; Holland, G. N.; Moore, W. S.; Worthington, B. S. *Neuroradiology* **1978**, *16*, 607-609.
7. Aime, S.; Botta, M.; Terreno, E. *Adv. Inorg. Chem.* **2005**, *57*, 173-237.
8. Aime, S.; Botta, M.; Fasano, M.; Terreno, E. *Chem. Soc. Rev.* **1998**, *27*, 19-29.
9. Lowe, M. P. *Aust. J. Chem.* **2002**, *55*, 551-556.
10. Weissleder, R.; Papisov, M. *Rev. Magn. Reson. Med.* **1992**, *4*, 1-20.
11. Solomon, I. *Phys. Rev.* **1955**, *99*, 559-565.
12. Freed, J.H.; *Chem. Phys.* **1978**, *68*, 4034-4037.
13. Koenig, S. H.; Brown, R. D. *Prog. Nucl. Magn. Reson. Spectrosc.* **1991**, *22*, 487-567.
14. Vogl, T. J.; Hammerstingl, R.; Schwarz, W.; Mack, M. G.; Muller, P. K.; Pegios, W.; Keck, H.; Eibl-Eibesfeldt, A.; Hoelzl, J.; Woessmer, B.; Bergman, C.; Felix, R. *Radiology* **1996**, *198*, 881-887.
15. Koenig, S. H.; Kellar, K. E. *Magn. Reson. Med.* **1995**, *34*, 227-233.

16. Kellar, K. E.; Fujii, D. K.; Gunther, W. H. H.; Briely-sæbø, K.; Bjørnesud, A.; Spiller, M.; Koenig, S. H. *Magn. Reson. Imaging* **2000**, *11*, 488-494.
17. Van Wagoner, M.; Worah, D. *Invest. Radiol.* **1993**, *28*, S44.
18. Koenig, S. H.; Kellar, K. E. *Acad. Radiol.* **1998**, *5*, S200-S205.
19. Lauffer, R. B.; Parmelee, D. J.; Dunham, S. U.; Ouellet, H. S.; Dolan, R. P.; Witte, S.; McMurry, T. J.; Walovitch, R. C. *Radiology* **1998**, *207*, 529-538.
20. Dong, Q.; Hurst, D. R.; Weinmann, H. J.; Chenevert, T. L.; Londy, F. J.; Prince, M. R. *Invest. Radiol.* **1998**, *33*, 699-708.
21. Reimer, P.; Rummeny, E. J.; Daldrup, H. E.; Hesse, T.; Balzer, T.; Tombach, B.; Peters, P. E. *Eur. Radiol.* **1997**, *7*, 275-280.
22. Vittadini, G.; Felder, E.; Musu, C.; Tirone, P. *Invest. Radiol.* **1990**, *25*, S59-S60.
23. Wang, C.; Ahlström, H.; Ekholm, S.; Fagertun, H.; Helleström, M.; Hemmingsson, A. *Acta. Radiol.* **1997**, *38*, 643-649.
24. Runge, V. M.; Wells, J. W.; Williams, N. M. *Invest. Radiol.* **1995**, *30*, 123-130.
25. Jacobson, T. F.; Laniado, M.; Van Beers, B. E.; Dupas, B.; Boudghene, F. P.; Rummeny, E.; Falke, T. H.; Rinck, P. A.; MacVicar, D.; Lundby, B. *Acad. Radiol.* **1996**, *3*, 571-580.
26. Aime, S.; Barge, A.; Cabella, C.; Crich, S. G.; Gianolio, E. *Curr. Pharm. Biotechnol.* **2004**, *5*, 509-518.
27. Bloembergen, N.; Morgan, L. O. *J. Chem. Phys.* **1961**, *34*, 842-850.
28. Horrocks, W. D. Jr.; Sudnick, D. R. *J. Am. Chem. Soc.* **1979**, *101*: 334-340.
29. Kumar, K.; Chang, C. A.; Francesconi, L. C.; Dischino, D. D.; Malley, M. F.; Gougoutas, J. Z.; Tweedle, M. F. *Inorg. Chem.* **1994**, *33*, 3567-3575.
30. Supkowski, R. M.; Horrocks, W. D. Jr. *Inorg. Chem.* **1999**, *38*, 5616-5619.

31. Raymond, K. N.; Pierre, V. C. *Bioconjugate Chem.* **2005**, *16*, 3-8.
32. Caravan, P. *Chem. Soc. Rev.* **2006**, *36*, 512-523.
33. Cossy, C.; Helm, L.; Powell, D. H.; Merbach, A. E. *New J. Chem.* **1995**, *19*, 27-35.
34. Kotek, J.; Lebduskova, P.; Hermann, P.; Van der Elst, L.; Muller, R. N.; Geraldès, C. F.; Maschmeyer, T.; Lukes, I.; Peters, J. A. *Chem. Eur. J.* **2003**, *9*, 5899-5915.
35. Aime, S.; Batsanov, A. S.; Botta, M.; Howard, J. A. K.; Parker, D.; Senanayake, K.; Williams, G. *Inorg. Chem.* **1994**, *33*, 4696-4707.
36. Botta, M. *Eur. J. Inorg. Chem.* **2000**, 399-407.
37. Zuo, C. S.; Seoane, P. R.; Hu, J.; Harnish, P. P.; Rofsky, N. M. *Radiology* **2004**, *232*, 160-163.
38. Gamarra, L. F.; Brito, G. E. S.; Pontuschka, W. M.; Amaro, E.; Parma, A. H. C.; Goya, G. F. *J. Magn. Mater.* **2005**, *289*, 439-441.
39. Kim, D. K.; Mikhaylova, M.; Wang, F. H.; Kehr, J.; Bjelke, B.; Zhang, Y.; Tsakalakos, T.; Muhammed, M. *Chem. Mater.* **2003**, *15*, 4343-4351.
40. Weisman, G. R.; Reed, D. P. *J. Org. Chem.* **1996**, *61*, 5186-5187.
41. Chang, C. A.; Francesconi, L. C.; Malley, M. F.; Kumar, K.; Gougoutas, J. Z.; Tweedle, M. F.; Lee, D. W.; Wilson, L. J. *Inorg. Chem.* **1993**, *32*, 3501-3508.
42. Bombieri, G.; Artali, R. *J. Alloys. Comp.* **2002**, *344*, 9-16.
43. Tweedle, M. F.; Wedeking, P.; Kumar, K. *Invest. Radiol.* **1995**, *30*, 372-380.
44. Gibby, W. A.; Gibby, K. A. *Invest. Radiol.* **2004**, *39*, 138-142.
45. Cacherris, W. P.; Quay, S. C.; Rocklage, S. M. *Magn. Reson. Imaging.* **1990**, *8*, 467-481.

46. Weinmann, H. J.; Ebert, W.; Misselwitz, B.; Schmitt-Willich, H. *Eur. J. Radiol.* **2003**, *46*, 33-44.
47. Fatin-Rouge, N.; Tóth, E.; Meuli, R.; Bunzli, J. C. G. *J. Alloys Compd.* **2004**, *374*, 298-302.
48. Nicolle, G. M.; Tóth, E.; Schmitt-Willich, H.; Raduchel, B.; Merbach, A. E. *Chem. Eur. J.* **2002**, *8*, 1040-1048.
49. Nicolle, G. M.; Tóth, E.; Eisenwiener, K. P.; Macke, H. R.; Merbach, A. E. *J. Biol. Inorg. Chem.* **2002**, *7*, 757-769.
50. Feng, J.; Sun, G.; Pei, F.; Liu, M. *Bioinorg. Med. Chem.* **2003**, *11*, 3359-3366.
51. Mathur-Devre, R.; Lemort, M. *Brit. J. Radiol.* **1995**, *68*, 225-247.
52. Desreux, J. F.; Barthelemy, P. P. *Nucl. Med. Biol.* **1988**, *15*, 9-15.
53. Helm, H.; Merbach, A. E. *Chem. Rev.* **2005**, *105*, 1923-1959.
54. Jászberényi, Z.; Sour, A.; Tóth, E.; Benmelouka, M.; Merbach, A. E. *Dalton Trans.* **2005**, 2713-2719.
55. Laus, S.; Ruloff, R.; Tóth, E.; Merbach, A. E. *Chem. Eur. J.* **2003**, *9*, 3555-3566.
56. Harris, W. R.; Raymond, K. N.; Weitz, F. L. *J. Am. Chem. Soc.* **1981**, *103*, 2667-2675.
57. Polášek, M.; Rudovský, J.; Hermann, P.; Lukeš, I.; Van der Elst, L.; Muller, R. N. *Chem. Commun.* **2004**, *22*, 2602-2603.
58. Rudovský, J.; Kotek, J.; Hermann, P.; Lukeš, I.; Mainero, V.; Aime, S. *Org. Biomol. Chem.* **2005**, *3*, 112-117.
59. Costa, J.; Tóth, E.; Helm, L.; Merbach, A. E. *Inorg. Chem.* **2005**, *44*, 4747-4755.

60. Costa, J.; Ruloff, R.; Burai, L.; Helm, L.; Merbach, A. E. *J. Am. Chem. Soc.* **2005**, *127*, 5147-5157.
61. Bolskar, R. D.; Benedetto, A. F.; Husebo, L. O.; Price, R. E.; Jackson, E. F.; Wallace, S.; Wilson, L. J.; Alford, J. M. *J. Am. Chem. Soc.* **2003**, *125*, 5471-5478.
62. Sitharaman, B.; Bolskar, R. D.; Rusakova, I.; Wilson, L. J. *Nano. Lett.* **2004**, *4*, 2373-2378.
63. Tóth, E.; Bolskar, R. D.; Borel, A.; Gonzalez, G.; Helm, L.; Merbach, A. E.; Sitharaman, B.; Wilson, L. J. *J. Am. Chem. Soc.* **2005**, *127*, 799-805.
64. Laus, S.; Sitharaman, B.; Tóth, E.; Borel, A.; Helm, L.; Asokan, S.; Wong, M. S.; Wilson, L. J.; Merbach, A. E. *J. Am. Chem. Soc.* **2005**, *127*, 9368-9369.
65. Jacques, V.; Desreux, JF. *Top. Curr. Chem.* **2002**, *221*, 123-164.
66. Zhang, S.; Wu, K.; Sherry, A. D. *Angew. Chem. Int. Ed.* **1999**, *38*, 3192-3194.
67. Raghunand, N.; Howison, C.; Sherry, A. D.; Zhang, S.; Gillies, R. *Magn. Reson. Med.* **2003**, *49*, 249-257.
68. Shinohara, H. *Rep. Prog. Phys.* **2000**, *63*, 843-892.
69. Lowe, M. P.; Parker, D.; Reany, O.; Aime, S.; Botta, M.; Castellano, G.; Gianolio, E.; Pagliarin, R. *J. Am. Chem. Soc.* **2001**, *123*, 7601-7609.
70. Comblin, V.; Gilsoul, V.; Hermann, M.; Humblet, V.; Jacques, V.; Mesbahi, M.; Sauvage, C.; Desreux, J. F. *Coord. Chem. Rev.* **1999**, *185*, 451-470.
71. Que, E. L.; Chang, C. J. *J. Am. Chem. Soc.* **2006**, *128*, 15942-15943.
72. Moats, R. A.; Fraser, S. E.; Meade, T. J. *Angew. Chem. Int. Ed.* **1997**, *36*, 726-728.
73. Nivorozhkin, A. L.; Kolodziej, A. F.; Caravan, P.; Greenfield, M. T.; Lauffer, R. B.; McMurry, T. J. *Angew. Chem. Int. Ed.* **2001**, *40*, 2903-2906.

74. Lazar, I.; Sherry, A. D.; Ramasamy, R.; Brucher, E.; Kiraly, R. *Inorg. Chem.* **1991**, *30*, 5016-5023.
75. Sherry, A. D.; Geraldès, C. F. G. C.; Cacheris, W. P. *Inorg. Chim. Acta.* **1987**, *139*, 137-139.
76. Geraldès, C. F. G. C.; Sherry, A. D.; Cacheris, W. P. *Inorg. Chem.* **1989**, *28*, 3336-3341.
77. Sherry, A. D. *J. Alloys Compd.* **1997**, *249*, 153-157.
78. Kubicek, V.; Rudovsky, J.; Kotek, J.; Hermann, P.; Van der Elst, L.; Muller, R. N.; Kolar, Z. I.; Wolterbeek, H. T.; Peters, J. A.; Lukes, I. *J. Am. Chem. Soc.* **2005**, *127*, 16477-16485.
79. Rudovský, J.; Cigler, P.; Kotek, J.; Hermann, P.; Vojtisek, P.; Lukeš, I.; Peters, J. A.; Van der Elst, L.; Muller, R. N. *Chem. Eur. J.* **2005**, *11*, 2373-2384.
80. Adzamli, I. K.; Johnson, D.; Blau, M. *Invest. Radiol.* **1991**, *26*, 143-148.
81. Adzamli, I. K.; Blau, M. *Magn. Reson. Med.* **1991**, *17*, 141-148.
82. Lee, J.; Zylka, M. J.; Anderson, D. J.; Burdette, J. E.; Woodruff, T. K.; Meade, T. J. *J. Am. Chem. Soc.* **2005**, *127*, 13164-13166.
83. Wen, X.; Jackson, E. F.; Price, R. E.; Kim, E.; Wu, Q.; Wallace, S.; Charnsangavej, C.; Gelovani, J. G.; Li, C. *Bioconjugate Chem.* **2004**, *15*, 1408-1415.
84. Yan, G. P.; Liu, M. L.; Li, L. Y. *Bioconjugate Chem.* **2005**, *16*, 967-971.
85. Port, M.; Meyer, D.; Bonnemain, B.; Corot, C.; Schaefer, M.; Rousseaux, O.; Simonot, C.; Bourrinet, P.; Benderbous, S.; Dencausse, A.; Devoldere, L. *MAGMA* **1999**, *8*, 172-176.
86. Aime, S.; Botta, M.; Crich, S. G.; Giovenzana, G. B.; Pagliarin, R.; Piccinini, M.; Sisti, M.; Terreno, E. *J. Biol. Inorg. Chem.* **1997**, *2*, 470-479.

87. Pham, W.; Zhao, B.-Q.; Lo, E. H.; Medarova, Z.; Rozen, B.; Moore, A. *Neuroimage* **2005**, *28*, 287-292.
88. Desreux, J. F. *Inorg. Chem.* **1980**, *19*, 1319-1324.
89. Wang, X. Y.; Jin, T. Z.; Comblin, V.; Lopez-Mut, A.; Merciny, E.; Desreux, J. F. *Inorg. Chem.* **1992**, *31*, 1095-1099.
90. Hervé, G.; Bernard, H.; Le Bris, N.; Yaouanc, J. J.; Handel, H.; Toupet, L. *Tetrahedron Lett.* **1998**, *39*, 6861-6864.
91. Jazwinski, J.; Kolinski, R. A. *Tetrahedron Lett.* **1981**, *22*, 1711-1714.
92. Caulkett, P. W. R.; Greatbanks, D.; Turner, R. W.; Jarvis, J. A. J. *J. Chem. Soc. Chem. Commun.* **1977**, 150-151.
93. Weisman, G. R.; Ho, S. C. H.; Jonhson, V. *Tetrahedron Lett.* **1980**, *21*, 335-338.
94. Aime, S.; Barge, A.; Botta, M.; Howard, J. A. K.; Katakya, R.; Lowe, M. P.; Moloney, J. M.; Parker, D.; de Sousa, A. S. *Chem. Commun.* **1999**, 1047-1048.
95. Dischino, D. D.; Delaney, E. J.; Emswiler, J. E.; Gaughan, G. T.; Prasad, J. S.; Srivanasta, S. K.; Tweedle, M. F. *Inorg. Chem.* **1991**, *30*, 1265-1269.
96. Coutsogeorgopoulos, C.; Zervas, L. *J. Am. Chem. Soc.* **1961**, *83*, 1885-1888.
97. Asano, Y.; Umezaki, M.; Li, Y. F.; Tsubota, S.; Lübbehüsen, T. L. *J. Molec. Catal. B: Enzymatic* **2001**, *12*, 53-59.
98. Albert, R.; Danklmaier, J.; Hönig, H.; Kandolf, H. *Synthesis* **1987**, 635-637.
99. Yeom, C. E.; Kim, M. J.; Kim, B. M. *Tetrahedron* **2007**, *63*, 904-909.
100. Livramento, J. B.; Sour, A.; Borel, A.; Merbach, A. E.; Tóth, É. *Chem. Eur. J.* **2006**, *12*, 898-1003.
101. Caravan, P. *Chem. Soc. Rev.* **2006**, *35*, 512-523.

102. Lauffer, R. B.; Parmelee, D. J.; Dunham, S. U.; Ouellet, H. S.; Dolan, R. P.; Witte, S.; McMurry, T. J.; Walovitch, R. C. *Radiology* **1998**, *207*, 529-538.
103. Fink, C.; Goyen, M.; Lotz, J. *Eur. Radiol.* **2007**, *Suppl 2*, B38-44.
104. Preda, A.; Novikov, V.; Möglich, M.; Turetschek, K.; Shames, D. M.; Brasch, R. C.; Cavagna, F. M.; Roberts, T. P. *Invest. Radiol.* **2006**, *41*, 279-291.
105. Faccioli, N.; Marzola, P.; Boschi, F.; Sbarbati, A.; D'Onofrio, M.; Mucelli, R. *P. Radiol. Med.* **2007**, *112*, 319-328.
106. Caravan, P.; Das, B.; Deng, Q.; Dumas, S.; Jacques, V.; Koerner, S. K.; Kolodziej, A.; Looby, R. J.; Sun, W. C.; Zhang, Z. *Chem. Commun.* **2009**, 430-432.
107. Caravan, P.; Das, B.; Dumas, S.; Epstein, F. H.; Helm, P. A.; Jacques, V.; Koerner, S. K.; Kolodziej, A.; Shen, L.; Sun, W. C.; Zhang, Z. *Angew. Chem. Int. Ed.* **2007**, *46*, 8171-8173.
108. LeTiran, A.; Stables, J. P.; Kohn, H. *J. Med. Chem.* **2002**, *45*, 4762-4773.
109. Sennyey, G.; Barcelo, G.; Senet, J. P. *Tetrahedron Lett.* **1986**, *27*, 5375-5376.
110. Li, C.; Winnard, Jr. P. T.; Takagi, T.; Artemov, D.; Bhujwalla, Z. M. *J. Am. Chem. Soc.* **2006**, *128*, 15072-15073.
111. Holla, B. S.; Malini, K. V.; Sarojini, B. K.; Poojary, B. *Synthetic Commun.* **2005**, *35*, 333-340.
112. Rostovtsev, V. V.; Green, L. G.; Fokin, V. V.; Sharpless, K. B. *Angew. Chem. Int. Ed.* **2002**, *41*, 2596-2599.
113. Koten, G.; Noltes, J. G. *Comprehensive organometallic chemistry* (Ed: Wilkinson G.), Pergamon, Oxford **1983**, *2*, 720.
114. Doyle, M. P.; McKervy Ye, T. *Modern catalytic methods for organic synthesis with diazo compounds*, Wiley, New York **1997**, 163-248.

115. Himo, F.; Lovell, T.; Hilgraf, R.; Rostovtsev, V. V.; Noodleman, L.; Sharpless, K. B.; Fokin, V. V. *J. Am. Chem. Soc.* **2005**, *127*, 210-216.
116. Rodionov, V. O.; Fokin, V. V.; Finn, M. G. *Angew. Chem. Int. Ed.* **2005**, *44*, 2210-2215.
117. Nolte, C.; Mayer, P.; Straub, B. F. *Angew. Chem. Int. Ed.* **2007**, *46*, 2101-2103.
118. Perrind, D. D.; Armarego, W. L. F. *Purification of laboratory chemicals*, 3rd Ed., Peramon press, Oxford **1988**.
119. Dadabhoy, A.; Faulkner, S.; Sammes, P. G. *J. Chem. Soc. Perkin Trans. 2* **2002**, 348-357.
120. Slama, J. T.; Satsangi, R. K.; Simmons, A.; Lynch, V.; Bolger, R. E.; Suttie, J. *J. Med. Chem.* **1990**, *33*, 824-832.
121. Chen, F. M. F.; Benoiton, N. L. *Can. J. Chem.* **1987**, *65*, 1224-1227.
122. Fotakopoulou, I.; Barbayianni, E.; Conatantinou-Kokotou, V.; Bornscheuer, U. T.; Kokotos, G.; *J. Org. Chem.* **2007**, *72*, 782-786.
123. Biron, E.; Voyer, N. *Chem. Commun.* **2005**, *37*, 4652-4654.
124. Boudreault, P. L.; Voyer, N. *Org. Biomol. Chem.* **2007**, *5*, 1459-1465.
125. Pettit, G. R.; Nelson, P. S. *J. Org. Chem.* **1986**, *51*, 1282-1286.

– **APPENDIX**

Annex 1 Reverse-phase HPLC trace (UV 220 nm) of Gd-complex 29 (t_R : 3.6 min)

Annex 2 ESI-TOF-MS spectrum of Gd-complex 29

Annex 3 Reverse-phase HPLC trace (UV 220 nm) of Gd-complex CA1 (t_R : 12.9 min)

Annex 4 ESI-TOF-MS spectrum of Gd-complex CA1

Annex 5 Reverse-phase HPLC trace (UV 220 nm) of Gd-complex CA2 (t_R : 11.8 min)

Annex 6 ESI-TOF-MS spectrum of Gd-complex CA2

Annex 7 Reverse-phase HPLC trace (UV 220 nm) of Gd-complex **CA3** (t_R : 6.8 min)

Annex 8 ESI-TOF-MS spectrum of Gd-complex **CA3**

Annex 9 ESI-TOF-MS spectrum of Gd-complex 75