Chapter 2

Modulation of Water Access:

A Gadolinium(III) Chelate for Detection of β-Glucuronidase

Adapted from:

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Introduction

Cancer is a leading cause of death in developed nations prompting extensive research into the myriad aspects of this heterogeneous disease. The ability to non-invasively detect a small number of cells at the onset of metastatic growth presents a major challenge not only to clinicians but to the experimental community. Potential exists for cancer detection through the in vivo, non-invasive visualization of cellular and biochemical events that has been gaining rapid momentum in the burgeoning field of molecular imaging.[1-5] The design of imaging constructs that function within the prodrug paradigm being developed for the chemotherapeutic treatment of cancer is particularly attractive given the extensive research in the latter field.[6-10]

The proven clinical capacity of magnetic resonance imaging (MRI) combined with a lack of ionizing radiation affords a valuable tool for in vivo, whole organism molecular imaging. Since the intrinsic contrast of MRI results from the physical properties and local environment of endogenous water, this technique has predominantly focused on anatomical structure. The quest for higher resolution and shorter acquisition times has spurred the development of agents that increase the observed contrast in MR images.[11, 12] Much work has focused on increasing the effectiveness of small molecule contrast agents based on Gd(III) chelates with particularly promising results derived from interactions with macromolecules.[13-15] While these compounds delineate anatomical structure, new agents that are biochemical and physiological reporters are being developed.[16-21] Barriers to the development of new generations of biologically responsive agents include the lack of adequate in vitro assays to determine efficacy, enzyme kinetics, delivery and inherent contrast.

The similarity in size between the Gd(III) ion and the Ca(II) ion necessitates the confinement of the lanthanide in a sequestering ligand in order to prevent the disruption of Ca(II) dependent biochemical pathways. Ca(II) is an important second messenger, and changes in its concentration can have drastically detrimental effects upon the organism under study. Due to coulombic interactions, gadolinium(III), a trivalent metal ion, can outcompete the similarly sized Ca(II), disrupting the organism's delicate calcium balance. Since gadolinium, like all rare earths, lacks strong bonding orbitals and is coordinatively labile, it must be sequestered in a large chelate structure that can prevent its uptake by the organism being examined. This requirement reduces the number of coordination sites accessible to water molecules thus reducing the effectiveness of the relaxation enhancement. Nonetheless, significant contrast enhancement is still realized and several contrast agents based upon highly kinetically and thermodynamically stable cyclic and acyclic amine-carboxylate chelates have been approved for clinical usage.[12]

Drawing on our previous galactose-based EGad contrast agent (see Chapter 1), the similarity between the mechanisms of action of β-galactosidase and β-glucuronidase,[22] and the prodrug research based on glucuronyl conjugates,[6-10] the development of a Gd(III) MR contrast agent whose effect on water proton T_1 relaxation is modulated by hydrolysis of β-glucuronic acid was undertaken (Scheme 2-1).

The application of β-glucuronide prodrugs in prodrug monotherapy (PMT) has yielded mixed results.[23, 24] The prodrug contains the chemotherapeutic compound but its toxicity is masked by conjugation to β-glucuronic acid. In PMT, the drug is liberated from the prodrug via endogenous extracellular β-glucuronidase. There are two major drawbacks to this approach: high enzyme levels are found only near necrotic tumor

masses that have low perfusion and hence receive less prodrug, and enzyme concentration is variable between individuals.[6, 7] Further complicating matters is the short half-life of glucuronide conjugated prodrugs, necessitating fast enzymatic conversion of the prodrug to its active form.[24] Antibody directed enzyme prodrug therapy (ADEPT; a two-step approach) introduces exogenous enzyme via an antibody targeting moiety and, in principle, should overcome the problems associated with PMT. ADEPT progress, initially curtailed by host immune response to the antibody-enzyme conjugate, has shown promise with the advent of antibodies engineered via phage display[25] and human-based fusion proteins.[26] Another potential avenue involves gene-directed enzyme prodrug therapy (GDEPT). Here, the diseased cells are transfected with DNA coding for the enzyme that is then produced by the cell and effects the prodrug cleavage. The cell surface display of β-glucuronidase has recently been reported as a candidate for GDEPT.[27]

With the necessity of fast cleavage kinetics in mind, compound **1** was designed with incorporation of the nitrophenyl self-immolative linker similar to that seen in the glucuronide conjugated anthracycline prodrug HMR 1826.[28] The kinetics of our previous galactosidase sensitive agent were slow and unsuitable for the current application. The efficacy of compound **1** would be modulated by changing *q*, the number of inner-sphere, Gd(III) coordinated water molecules. The use of a linker longer than the hydroxyethyl structure used in EGad may preclude efficient water blockage by the sugar. However, it is known that seven-coordinate DO3A analogs have reduced relaxivities due to coordination of endogenous bidentate anions such as carbonate.[29-32] The seven-coordinate chelate structure of **1** would allow such anion binding to occur. Upon

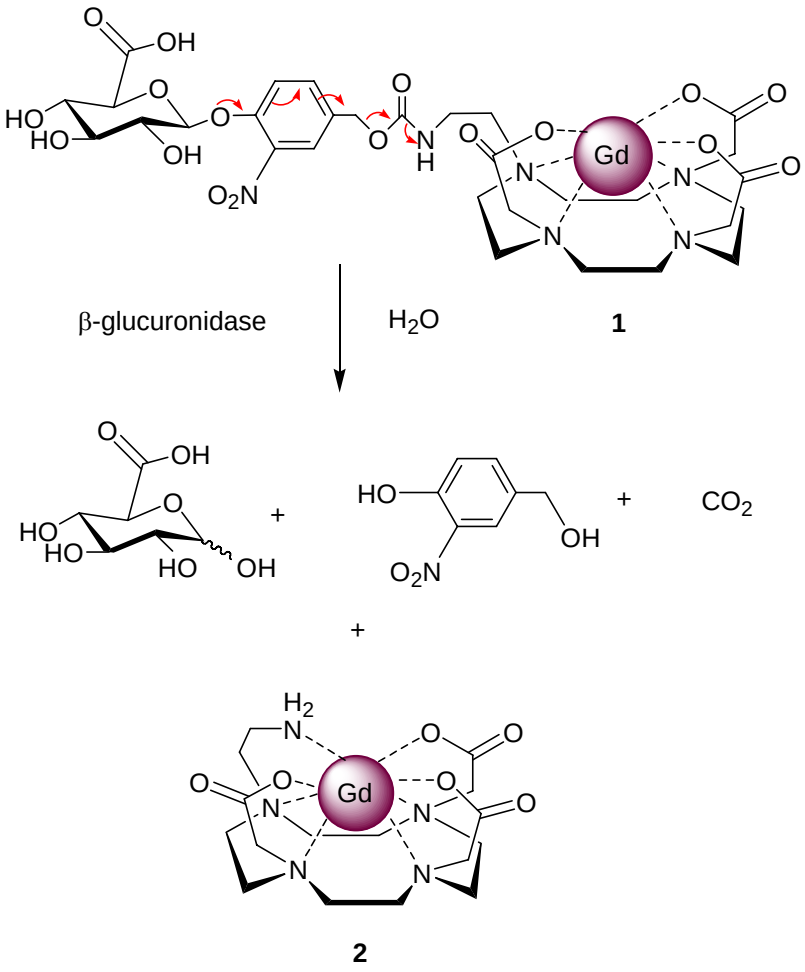
enzymatic cleavage, the aminoethyl arm will bind the metal center, expelling the anion and generating an octadentate center with $q = 1$ thus creating compound **2**. Octadentate complexes such as **2** are known to bind anions with a much lower affinity[32, 33] (3 orders of magnitude for carbonate) and hence water access should approach that of a $q = 1$ complex. The complex **2** would therefore have a higher relaxivity than the nominally $q = 0$ compound **1** in the presence of endogenous anions.

This chapter details work on a new class of MR contrast agents, exemplified by **1**, that uses a self-immolative mechanism for activation in the presence of an enzyme. This agent's effect on water proton T_1 relaxation is modulated by hydrolysis of β-glucuronic acid (Scheme 2-1). Previous studies using β-galactosidase-sensitive EGad and EGadMe (see Chapter 1) revealed that the in vitro behavior of these agents does not reflect their in vivo activity.[16, 17] Therefore, this chapter includes new in vitro assays that have been developed and applied to compounds **1** and **2** to gain insight into factors affecting in vivo activity.

Results and Discussion

Synthesis

Our original investigations with the β-galactosidase sensitive agents, EGad and EGadMe, used an "aqueous" synthetic route to obtain the desired complexes.[16, 17] In that route, the sugar/cyclen conjugate was deprotected in aqueous methanol and the acetate arms were added in alkaline water. The complex was then isolated from this mixture using anion exchange. The large quantities of ammonium acetate used in the ion exchange proved difficult to remove completely and hindered the subsequent metallation

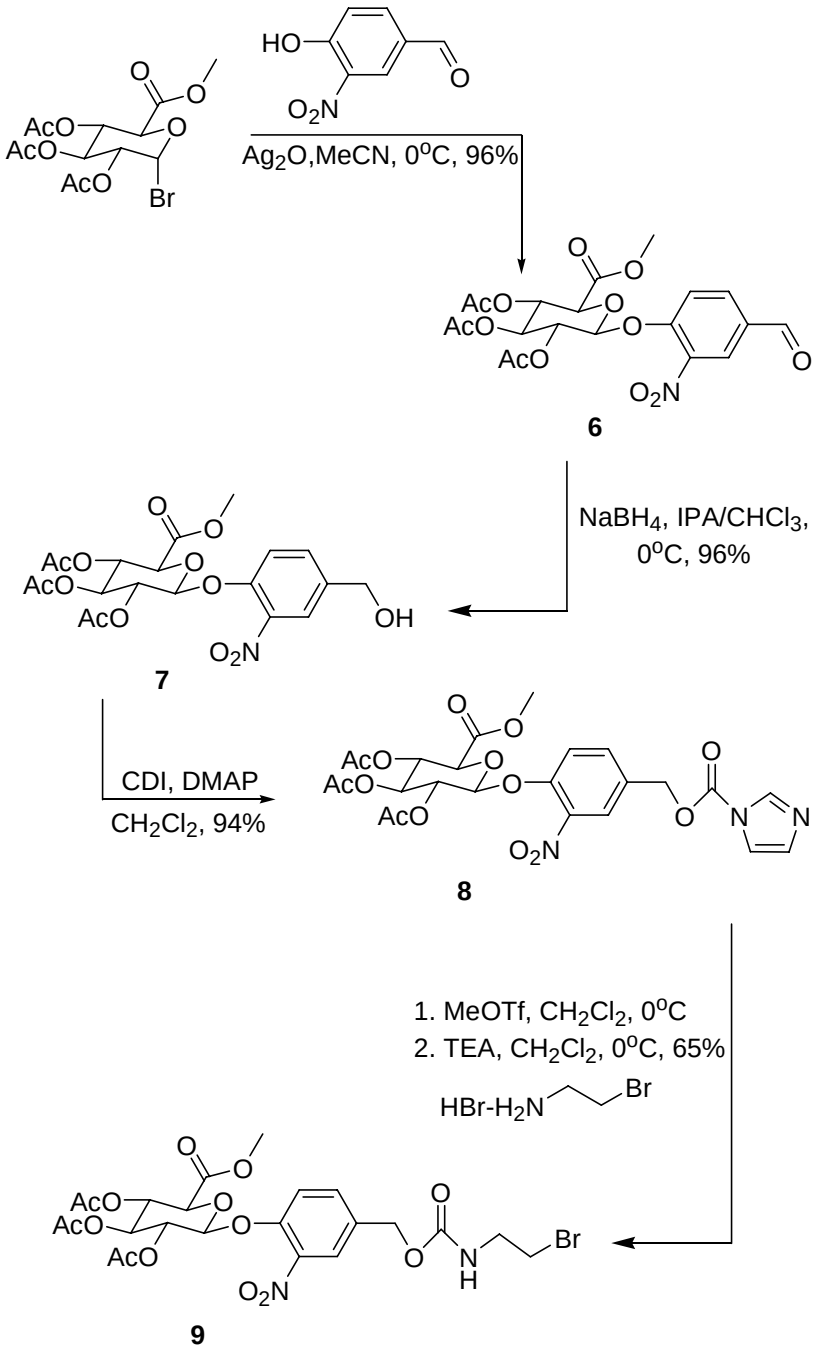


Scheme 2-1: Enzyme-catalyzed hydrolysis of MRI contrast agent, **1**.

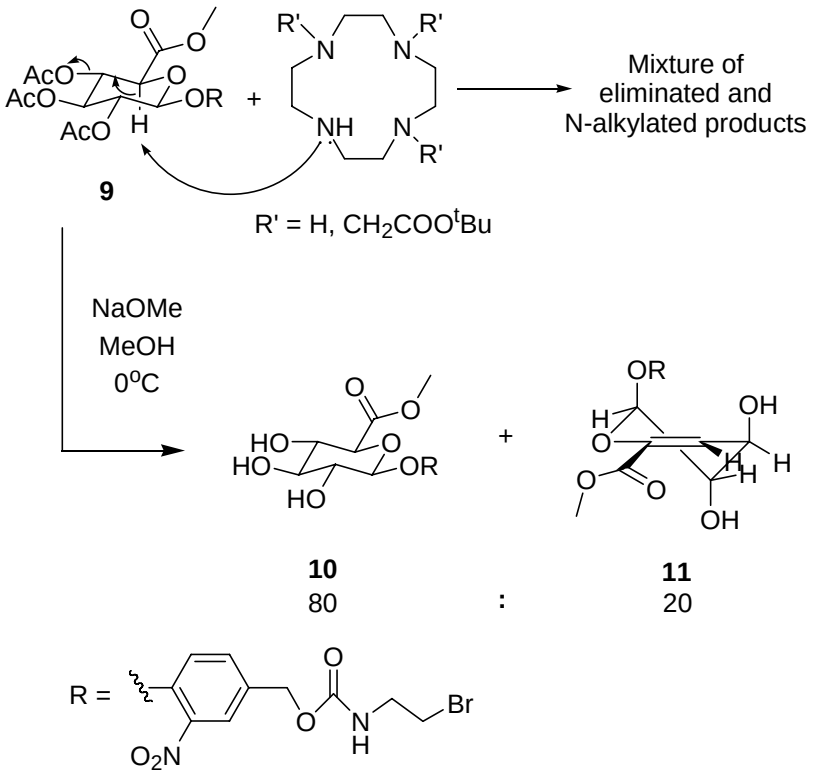
reaction. Furthermore, the one-pot approach did not permit comprehensive characterization of intermediates. For these reasons, an "organic" route to the more complex compound **1** was employed here. This involved the use of protected moieties and permitted more facile characterization with increased reproducibility.

The synthesis of **1** begins with methyl 1-bromo-2,3,4-tri-*O*-acetyl-α-D-glucopyronuronate[34] (Scheme 2-2). Coupling of 4-hydroxy-3-nitrobenzaldehyde to the pyranose via a Koenigs-Knorr reaction followed by sodium borohydride reduction produced **7** in 92% yield without recourse to chromatography. Initial attempts at formation of the carbamate using *p*-nitrophenyl chloroformate[28, 35] and triphosgene[36, 37] gave intermediates that showed insufficient reactivity towards 2-bromoethylamine and 2-hydroxyethylamine. The reaction occurred smoothly however when carbonyl-diimidazole (CDI) was used as the carbonyl equivalent. Methylation of the mono-imidazolyl intermediate gave an increased yield through precipitation of the cationic intermediate.

Alkylation reactions involving **9** with either cyclen or DO3A(tris-^{t}butyl ester)[38] generated large amounts of α,β-unsaturated byproducts due to an acid-base reaction between cyclen and the acidic proton alpha to the methyl ester (Scheme 2-3). It is known that this type of elimination can happen with acetyl protected glucuronic acids,[39, 40] but it is rarely mentioned in the literature. Synthesis of methyl 1-ethoxy-2,3,4-tri-*O*-acetyl-β-D-glucopyronuronate and subsequent monitoring of the reaction with cyclen by ^{1}H NMR confirmed the elimination.[41] The formation of the α,β-unsaturated byproducts was circumvented by selective removal, in good yield, of the acetyl groups from **9** using sodium methoxide, with **11** as the byproduct (Scheme 2-3). Introduction of the



Scheme 2-2: Sugar-linker electrophile synthesis.



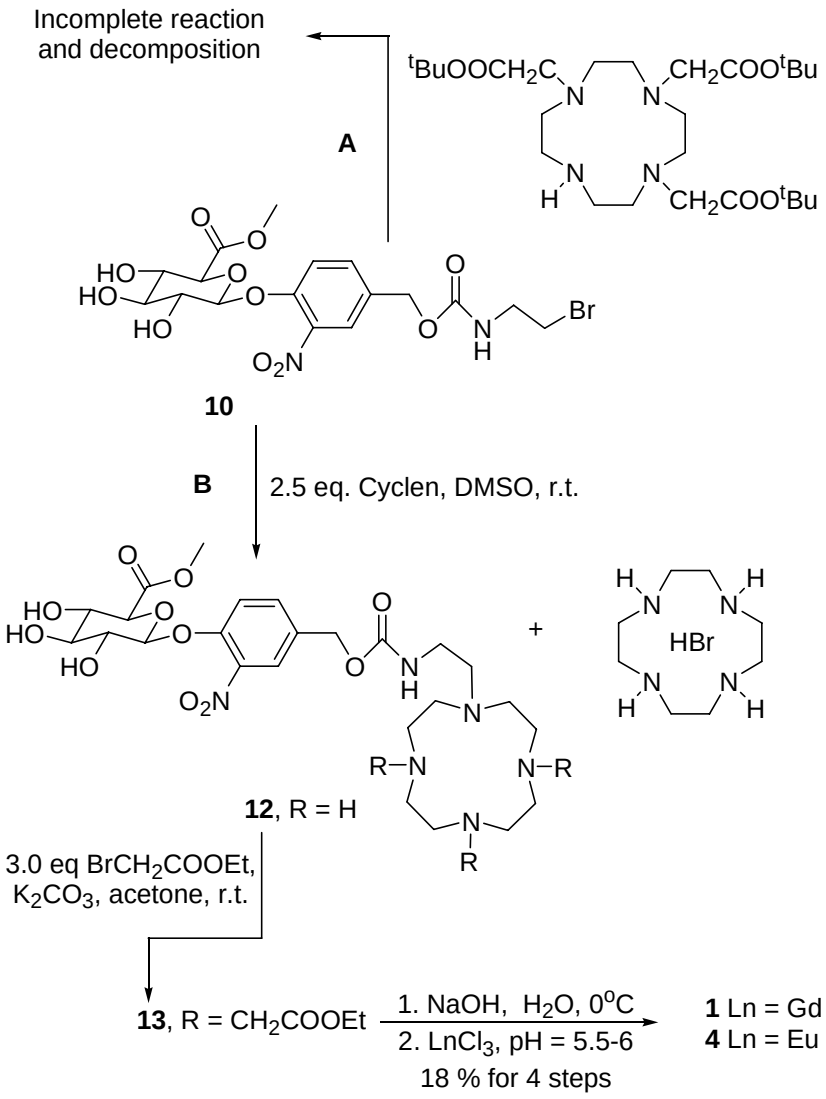
Scheme 2-3: Partial deprotection of sugar-linker electrophile, **9**.

macrocycle was then attempted through two approaches.

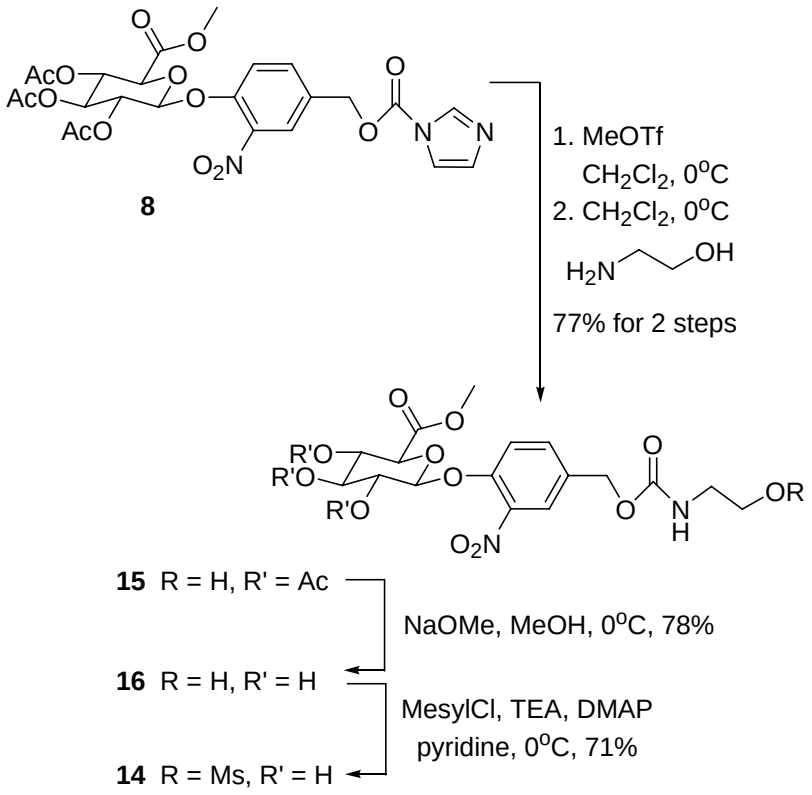
In the first approach, DO3A(tris-^{t}butyl ester) was reacted with **10** (Scheme 2-4, A). After several days at room temperature, the reaction was not complete and byproducts had begun to develop. Attempts involving heating of the reaction induced sugar decomposition. The cyclen route in which the sugar containing arm is added prior to the acetate arms (Scheme 2-4, B) was successful, presumably because the macrocycle free base is more nucleophilic towards the unactivated alkyl bromide electrophile than the DO3A compound. Increasing the electrophilicity of **9** (Scheme 2-2) by making the mesylated compound, **14**, from **8** (Scheme 2-5) was not successful due to the propensity of **14** to cyclize via elimination of the mesyl group in the presence of cyclen.

Purification of **12** proved difficult, so the compound was alkylated with ethyl bromoacetate. Excess cyclen, as the perethyl DOTA ester, could then be removed by chromatography on silica. The protected ligand, **13**, was deprotected using basic hydrolysis due to the presence of the acid-labile glycosidic bond. Final deprotection and metallation were performed in a one-pot procedure. Compound **1** was obtained analytically pure, showing one peak by LC-MS with the appropriate isotope pattern after purification by preparative HPLC. The overall yield for the nine-step procedure was 8.0%. Compound **4** was obtained in 8.2% overall yield by substituting $EuCl_3$ for $GdCl_3$.

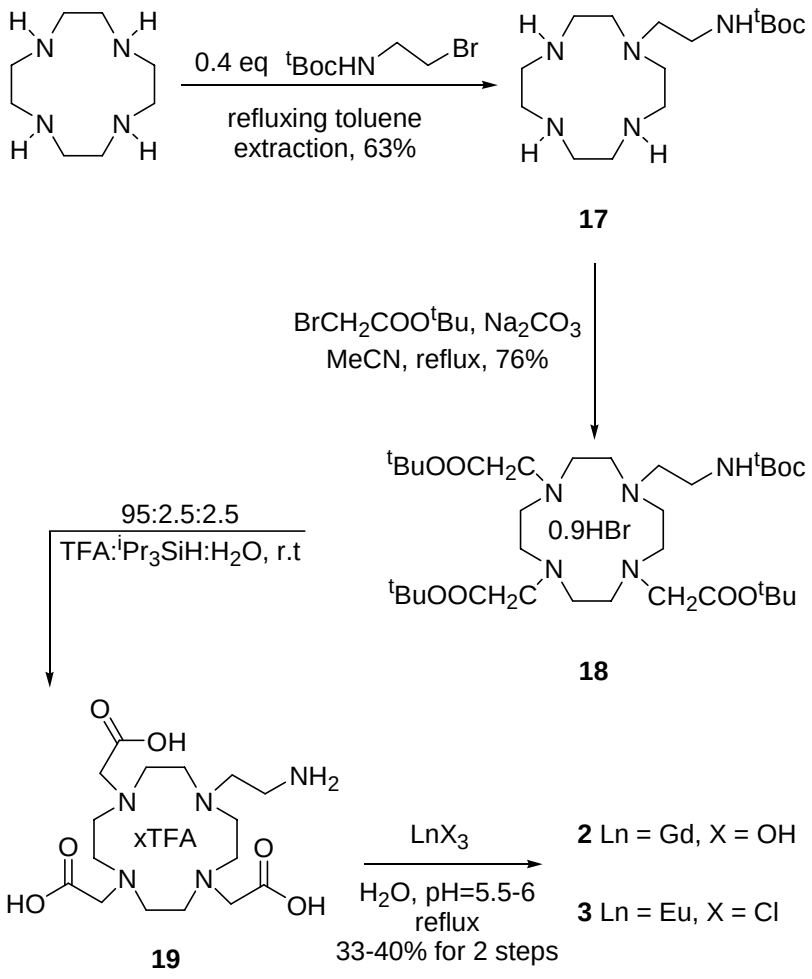
The synthesis of compounds **2** and **3** proved to be more straightforward (Scheme 2-6). Utilizing the mono-alklyation of excess cyclen, intermediate **17** was obtained analytically pure following extraction and an ether wash. Subsequent alkylation with three equivalents of ^{t}butyl bromoacetate gave **18** as a 9:1 mixture of hydrobromide salt to free base. Deprotection was achieved through the use of trifluoroacetic acid. Metallation



Scheme 2-4: Coupling of macrocycle to **10**, via DO3A route (**A**) or cyclen route (**B**).



Scheme 2-5: Synthesis of mesylated sugar-linker conjugate, **14**.



Scheme 2-6: Independent synthesis of the processed agents, **2** and **3**.

of the free ligand, **19**, with $Gd(OH)_3$ gave crude **2**. Purification of **2** via HPLC was difficult for two reasons: the lack of a chromophore on **2** limited detection to fluorescence, and the presence of the primary amine ligand gave a peak with a long tail. These difficulties lowered the overall yield to 19% for four steps. The Eu(III) compound **3** was obtained in 16% overall yield by substituting $EuCl_3$ for $Gd(OH)_3$. Both compounds were determined to be pure by elemental analysis.

Relaxivity

The defining parameter of contrast agent efficacy is relaxivity. In this context, relaxivity, r_1, is a measure of the extent to which the agent, per unit, catalyzes the shortening of the longitudinal relaxation time, T_1, of protons on the hydrogen atoms in bulk water. The presence of other species in solution, be they salts, proteins or small molecules, can have a marked effect on an agent's relaxivity. Relaxivity measurements made in solutions of varying composition not only describe how the agent responds to that composition, but also provide insight into the microscopic processes occurring at or near the Gd(III) center. Attributing relaxivity effects to solution composition can only be made when the contrast agent under study is of a known purity. The use of analytically pure contrast agents allows for facile and accurate determination of agent concentration through the use of Gd(III) ICP-MS. This, in tandem with measurements made in duplicate, reduces the systematic error in the relaxivity measurements.

Relaxivity related to buffer composition

The measurements shown in Figure 2-1 reveal trends in relaxivity related to buffer composition. To provide a reference, the relaxivity of the known $q = 1$ contrast agent, Gd(III)-2-hydroxypropyl-DO3A (GdHP-DO3A), was measured in pH = 7.4 MOPS buffer and gave an $r_1 = 2.99 \pm 0.44$ $mM^{-1}sec^{-1}$ under these conditions. It has been reported that MOPS does not coordinate to the metal center: Bretonniere and coworkers[42, 43] perform experiments in MOPS without interference with carbonate binding to Eu(III) trisamide cyclen complexes, while Bruce et al.[29] indicate that experiments with related compounds may be run in the structurally similar HEPES. Initial relaxivity measurements of **1** and **2** made in the same MOPS buffer showed a higher value for **1** and a value comparable to GdHP-DO3A for **2** (Figure 2-1, MOPS columns). The magnitude of r_1 for **1** is somewhat low for a $q = 2$ complex, however,[44] indicating intramolecular coordination of the sugar-containing arm to the metal center. Determination of q at room temperature via Eu(III) fluorescence[32, 45] for **4** (the Eu(III) analog of **1**) in the absence of buffer salts gave a $q = 1$, supporting the intramolecular coordination postulate. Presumably this coordination occurs through the carbamate carbonyl oxygen. A similar seven-membered intramolecular ring has been invoked to explain low q values for EuDO3A-type complexes containing tethered carboxylates.[31, 46] The donating ability of a carbamate carbonyl oxygen is expected to be lower than a carboxylate due to increased electron density delocalization and charge neutrality. This does not, however, preclude weak binding of the carbamate moiety to the metal center in **1** giving a species that has one inner-sphere water molecule. Measurement of q at 37 °C supports this postulate. Here the q is slightly higher with a value of 1.1-1.2, a result that

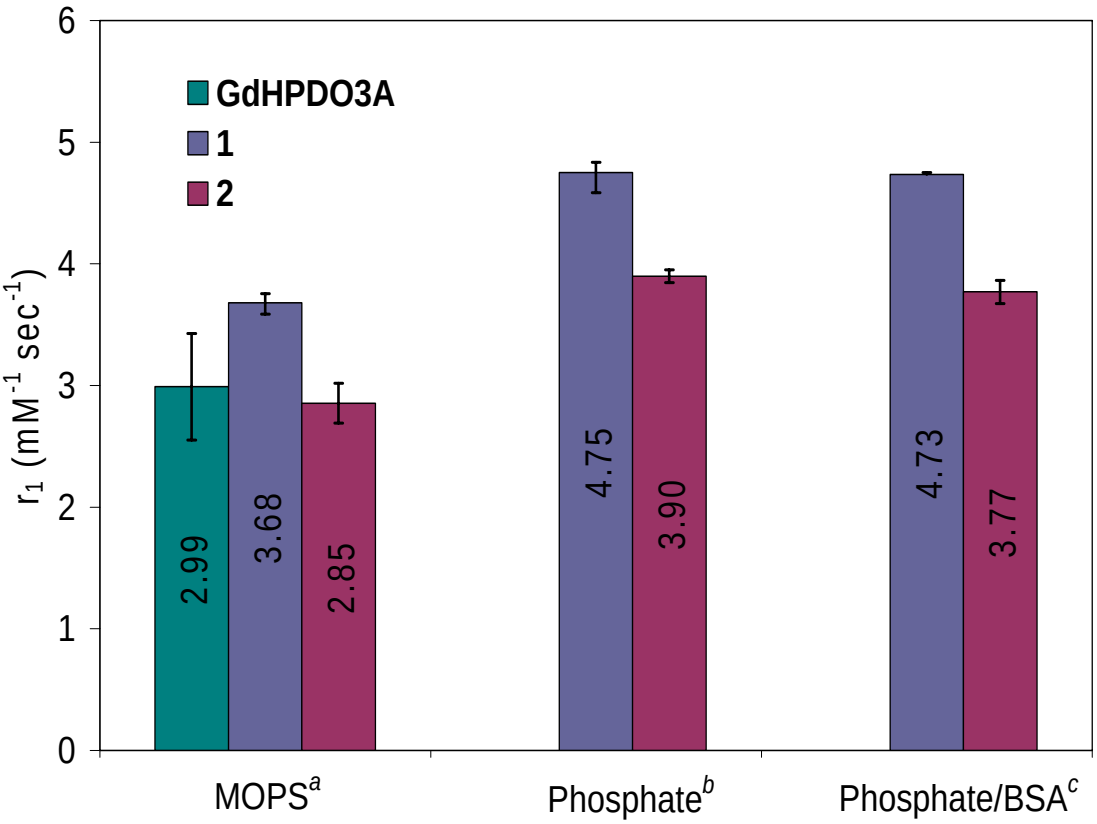


Figure 2-1: T_1 Relaxivity of GdHPDO3A, **1**, and **2** at 60 MHz, 37 °C, pH = 7.4. Error is ± 1 S.D. of duplicate measurements. [a]10 mM MOPS, 100 mM NaCl. [b]100 mM sodium phosphate. [c]100 mM sodium phosphate, 0.01% (w/v) bovine serum albumin (BSA).

indicates a weakening of the interaction between carbamate and metal center.[47]

Due to enzyme instability in MOPS buffer, the relaxivities of **1** and **2** were determined in the enzyme kinetics buffer composed of phosphate and BSA (Figure 2-1). Here, the relaxivities are higher (4.73 vs. 3.68 $mM^{-1}sec^{-1}$) but show the same trend as observed in MOPS buffer, namely a 20% drop in relaxivity upon going from **1** to **2**. In this case, it is unclear if the species in solution is an intramolecularly coordinated **1** or a ternary adduct in which phosphate has displaced carbamate binding. Phosphate is known to coordinate to DO3A analogs in a monodentate fashion[29, 30, 32, 48, 49] and could therefore compete with intramolecular coordination, especially at the relatively high concentrations employed here.

The native lysosomal environment of β-glucuronidase is acidic with maximum activity observed between pH = 4-5.[50] The relaxivity of compounds **1** and **2** were thus measured at pH = 5.0. The results from two different buffers at the same pH are dramatically different. In 10 mM pyridine, 100 mM sodium chloride buffer, compound **1** has a relaxivity of 3.89 ± 0.05 $mM^{-1}sec^{-1}$ while **2** displays an r_1 of 4.16 ± 0.09 $mM^{-1}sec^{-1}$. In 100 mM acetate buffer, those values are 2.53 ± 0.01 $mM^{-1}sec^{-1}$ and 2.16 ± 0.04 $mM^{-1}sec^{-1}$, respectively. The difference between buffers may be due to the coordinating ability of the buffer constituents. Pyridine is expected to be a poor ligand for the oxophilic Gd(III) in water[51] and thus does not restrict water access to the metal center as much as acetate. At pH = 7, propionate binding to GdDO3A has been shown to be monodentate,[52] and TbDO3A in the presence of a large excess of acetate gives a q value of one.[29] To our knowledge, the coordination of acetate or pyridine to LnDO3A and its analogs has not been examined at pH = 5.0.

Relaxivity relevant to physiological conditions

Our studies on EGad and EGadMe demonstrate that in vitro measurements do not correlate with in vivo efficacy. While an MR contrast agent may display efficacy in vitro, the addition of the components found in blood plasma could result in a significant change in the properties of the agent. To investigate these effects and the interplay of coordinating bidentate anions on the present β-glucuronidase sensitive agent, the relaxivity of **1** and **2** was determined in buffers of increasing compositional complexity. These results are depicted in Figure 2-2. For the MOPS and phosphate data, the effects are superimposed upon the overall relaxivity differences observed between the two buffers (Figure 2-1). Addition of physiologically relevant carbonate concentrations (24 mM)[53] to the buffers displayed in Figure 2-1 gave similar results independent of buffer; namely the relaxivity of **1** decreased by 20-30% while that of **2** remained the same within error. This data supports our initial hypothesis of stronger binding of carbonate to **1** versus **2** and would indicate that carbonate binding can displace the seven-membered carbamate chelate. Results with the anion extracellular mimic[29, 53] continue the trend begun in the carbonate-containing buffers. Here, the data exhibit the desired dark to bright (low to higher relaxivity) change. In this case it is a 17% increase. Once again, the decrease is more dramatic for **1** (22%) than **2** (6%), providing more evidence for the increased chelating anion affinity of **1** compared to **2**.

Carbonate is known to bind to seven-coordinate cis-divacant LnDO3A analogs more strongly than to eight-coordinate LnDOTA analogs.[29-32] The relaxivities of **1** and **2** (Figure 2-2) follow this trend. Figure 2-3 displays the change in T_1 observed upon titration of these agents with sodium bicarbonate in a physiologically relevant range at

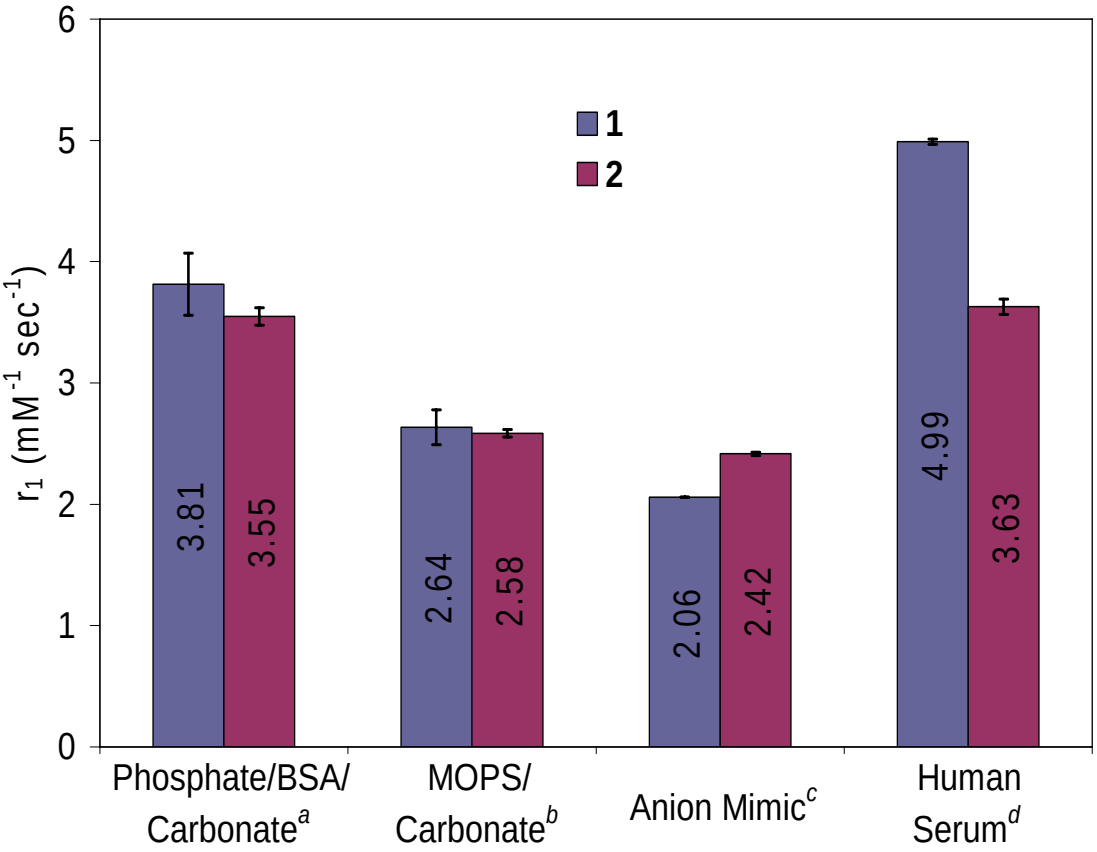


Figure 2-2: T_1 Relaxivity of **1** and **2** at 60 MHz, 37 °C, pH = 7.4. Error is ± 1 S.D. of duplicate measurements. [a]100 mM sodium phosphate, 0.01% (w/v) BSA, 24 mM $NaHCO_3$. [b]10 mM MOPS, 24 mM $NaHCO_3$. [c]100 mM NaCl, 0.9 mM Na_2HPO_4, 30 mM $NaHCO_3$, 0.13 mM sodium citrate, 2.3 mM sodium lactate.[29] [d]Male human blood serum.

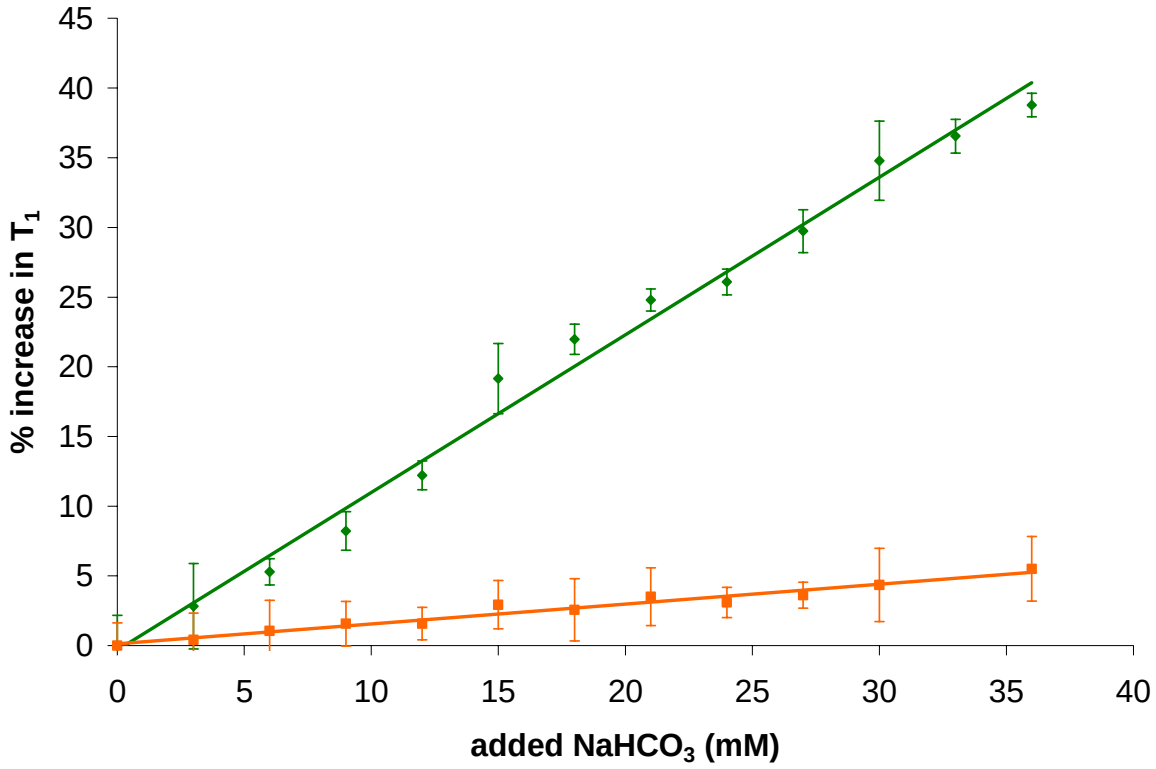


Figure 2-3: Change in T_1 of **1** (♦) and **2** (■) as a function of added sodium bicarbonate. 0.5 mM complex, 100 mM MOPS, 60 MHz, 37 ^{o}C, pH = 7.4. Error is ± 1 S.D. of duplicate measurements. Lines are best linear least-squares fit to data.

pH = 7.4. **2** has a small dependence on added bicarbonate (0.14 % increase in T_1 per mM bicarbonate) in this range while **1** displays a stronger correlation at 1.1 % increase in T_1 per mM bicarbonate[54] indicating displacement of intramolecular carbamate coordination. The results correlate well with the change in relaxivity of the two compounds upon addition of 24 mM bicarbonate (Figures 2-1 and 2-2). The titration of **1** has not begun to saturate at 36 mM added bicarbonate, indicative of a small binding constant for the ternary complex. This result contrasts sharply with the results observed for the tri-positive Ln(III) tris amide cyclen complexes. These complexes display saturation with as little as 2 mM added bicarbonate.[29] The lower affinity for carbonate species of complex **1** compared to the tris amide complexes can be attributed to the lower charge (neutral versus +3) and the binding competition with the intramolecular carbamate chelation.

If the coordination of endogenous anions were the sole intermolecular contributor to the relaxivities observed in these contrast agents then results from the anion extracellular mimic would translate well to results obtained in human blood serum. The present case shows the dramatic differences on going from a competitive extracellular anion mimic to human serum. The data is entirely different as compound **1** shows an increase in relaxivity of 240% while compound **2** displays a 150% increase. Furthermore, the relaxivity differential has switched with **1** being 27% brighter than **2** in serum. For these compounds in serum, the relaxivity indicates a species containing inner-sphere water molecules, in contrast to the $q = 0$ species detected by Aime et al. for DO3A analogs in the presence of albumin.[48] The complex composition of human serum makes it difficult to ascribe the results to any given component, but the higher viscosity

and possible macromolecular interactions could affect τ_R and hence the relaxivity.

Enzyme kinetics

To study the enzymatic processing of **1**, bovine liver β-glucuronidase was used. This commercially available enzyme is more similar to the human variant than the *E. coli* version.[55, 56] The bovine liver enzyme is localized in the lysosome and hence has maximal activity at pH = 5.[50] At neutral interstitial pH, the activity of both the human and bovine liver enzymes is 10% of that at pH = 5 and falls off rapidly in more alkaline solutions.[22, 50] The attenuation of activity is significant since ADEPT and PMT function at interstitial pH. The lower pH around tumor masses is advantageous in this respect as enzyme activity will be higher. Although the bacterial enzyme has much higher activity at extracellular pH,[57] it is obviously not endogenous to humans and its use in ADEPT has resulted in host immune response[25, 27] severely restricting its potential. Furthermore, the 2-nitro-quinone-methide that results from enzymatic processing of **1** is not an irreversible inhibitor of bovine β-glucuronidase.[55]

LC-MS provided a facile means to detect and identify the components of the enzyme catalyzed hydrolysis of **1**. After a 2 h reaction in phosphate buffer, the presence of 4-hydroxy-3-nitrobenzyl alcohol could be easily detected in the LC data at 4.0 min, unreacted **1** was observed at 7.5 min and **2** appeared at 11.8 min. **1** had disappeared after 24 h. The verification of the presence of 4-hydroxy-3- nitrobenzyl alcohol enabled the enzyme kinetics in phosphate/BSA buffer to be quantified using a continuous UV-visible assay.[58] The alcohol has an absorption maximum at 422 nm, while **1** does not. A plot of initial velocity versus substrate concentration fits well to a single-site Michaelis-

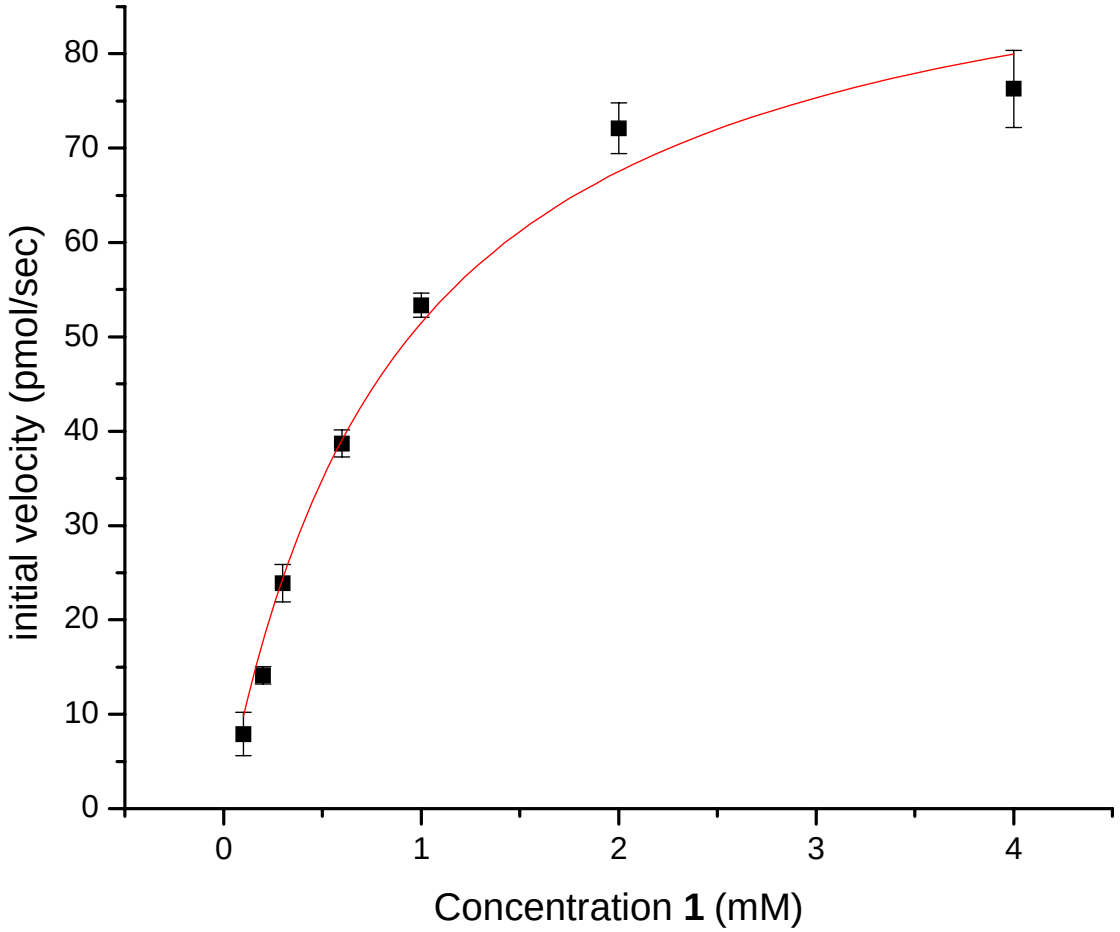


Figure 2-4: Kinetics of hydrolysis of **1** catalyzed by bovine liver β-glucuronidase. Each point is the average of three runs ± one S.D. Line represents best fit to Michaelis-Menten model. Conditions are 1.0 mg/mL enzyme in 100 mM sodium phosphate, 0.01% (w/v) bovine serum albumin (BSA), pH = 7.4 at 37 °C.

Menten model (Figure 2-4). The kinetic parameters from the non-linear fit are tabulated in Table 2-1. For comparison, k_{cat}/K_m was determined for the standard assay substrate, *p*-nitrophenyl-β-D-glucuronide (PNG). The data obtained show that **1** is a better substrate by an order of magnitude for bovine-liver β-glucuronidase than PNG (Table 2-1). These kinetics demonstrate the marked improvement afforded by incorporation of the self-immolative linker. In contrast, the non-immolative β-galactosidase sensitive agent EGadMe displayed enzyme kinetics that were two to three orders of magnitude worse than the standard β-galactosidase substrate 2-nitrophenyl-β-D-galactopyranoside.[17]

The kinetics can also be monitored via relaxivity using magnetic resonance. The use of MR allowed observation of kinetics in human serum, something that could not be done by visible light spectroscopy due to light scatter by suspended particles. Figure 2-5 shows the normalized change in T_1 as a function of enzyme incubation time. The results show excellent correlation with the relaxivities of the substrate, **1**, and the product, **2**, measured in the absence of enzyme (Figures 2-1 and 2-2). The largest change detected for the pH = 7.4 buffers comes from the data in human serum. Here, a 14% increase is observed over the course of one hour and the curve has not saturated at this time. The control without enzyme maintains a constant T_1 over this period. These serum experiments demonstrate that the contrast agent functions well in the complex biological milieu represented by human serum. Enzyme instability precluded determination at long reaction times, but the shapes of the curves match those obtained from the UV-visible assays, indicating that compound **2** forms on a time scale similar to the change in absorbance of the aromatic linker. In particular, the longitudinal relaxation time

Table 2-1: Enzyme kinetic parameters for **1** and PNG.

substrate	pH[a]	K_m (mM)	V_{max} (pmol s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
1[b]	7.4	0.906 ±0.133	98 ± 5.5	74.0 ± 10.9
PNG[b]	7.4	18.5 ± 5.6	184 ± 30	6.76 ± 2.05
PNG[c]	5	0.33	--	--
PNG[d]	7.2	1.40 ± 0.42	--	47

[a]37 °C. [b]1.0 mg/mL bovine liver β-glucuronidase (type B-1), 100 mM sodium phosphate, 0.01% (w/v) bovine serum albumin (BSA). Data average of 3 runs ± 1 S.D. [c]Bovine liver β-glucuronidase[50]. [d]Human recombinant β-glucuronidase.[59]

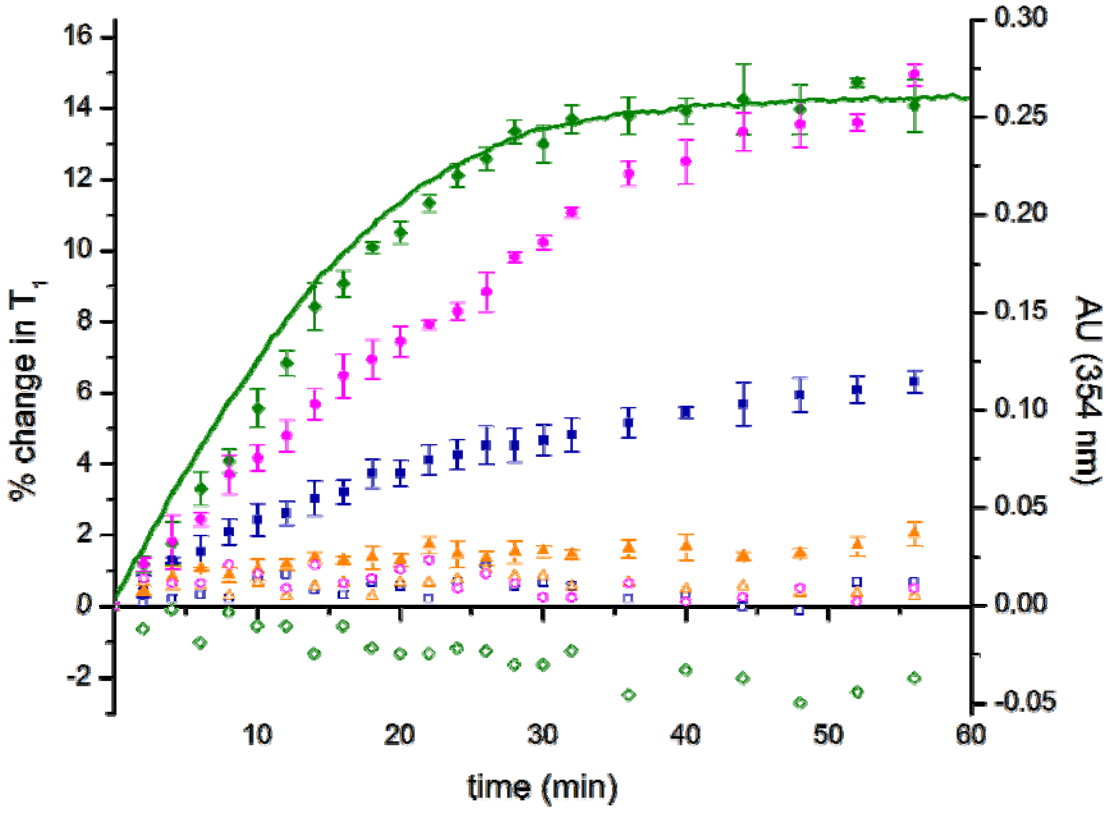


Figure 2-5: Kinetics of enzyme catalyzed hydrolysis of **1** monitored by bulk water T_1 relaxation (60 MHz, 37 °C). Error bars on data (filled symbols) are ± 1 S.D. of 3 independent measurements. Open symbols are control runs without enzyme. ■: 0.2 mM **1**, 1.0 mg/mL β-glucuronidase, 100 mM sodium phosphate, 0.01% (w/v) bovine serum albumin (BSA), pH = 7.4. ▲: 0.2 mM **1**, 1.0 mg/mL β-glucuronidase, 100 mM sodium phosphate, 0.01% (w/v) bovine serum albumin (BSA), 24 mM $NaHCO_3$, pH = 7.4. Green line is hydrolysis of **1** monitored by UV-visible at 354 nm, conditions as for ♦: 0.2 mM **1**, 0.1 mg/mL β-glucuronidase, 100 mM sodium acetate, pH = 5.0. ●: 0.2 mM **1**, 1.0 mg/mL β-glucuronidase, male human blood serum.

observed in acetate buffer levels off at +15% around 35 min, corroborating well with both the complete conversion detected by absorption spectroscopy (solid line in Figure 2-5) and the 15% decrease in relaxivity observed between the pure compounds in acetate buffer.

Conclusion and Future Work

The successful synthesis of an MRI contrast agent sensitive to the oncologically relevant enzyme, β-glucuronidase, expands the repertoire of agents responsive to biochemical reactions into the realm of clinically important processes. This agent shows considerably improved enzyme kinetics compared to EGad and EGadMe, kinetics which are necessary due to the short in vivo retention time of glucuronide conjugates. The relaxivity and kinetic assays developed in the present work elucidate the marked dependence that buffer composition can have upon the efficacy of an MR agent. While the mechanism of relaxivity modulation is not fully understood, it is apparent that binding of bidentate anions plays an important role in determining the relaxivity of the agents.

Affinity for anions such as carbonate may be improved by increasing the electrostatic charge at the metal center through the replacement of anionic carboxylate ligands with neutral amide coordinating groups. The use of amides should also generate compounds with long τ_m values. Europium(III) derivatives of these amide-containing compounds could be useful as ParaCEST (paramagnetic chemical exchange saturation transfer) agents.[60, 61]

Experimental

General Methods:

All reagents were used as purchased. 1,4,7,10-tetraazacyclododecane (cyclen) was obtained from Strem. Prohance was purified from the clinically available sample from Bracco Inc. using HPLC. Bovine liver β-glucuronidase [EC 3.2.1.31] Sigma cat G 0251 (Type B-1), BSA fraction V Sigma cat A 3059, *p*-nitrophenyl-β-D-glucuronide, and male human blood serum Sigma cat H 1388 were procured from Sigma. Dry solvents where indicated were obtained from Aldrich as anhydrous Sure-Seal bottles. Water was purified using a Millipore Milli-Q Synthesis purifier. Sugar-containing compounds were visualized on silica TLC plates with CAM stain (1g $(NH_4)_4Ce(SO_4)_4$, 2.5 g $(NH_4)_4Mo_2O_7$, 6 mL conc. H_2SO_4, 94 mL water), while compounds containing unmetallated cyclen could be easily detected using a platinum stain (150 mg K_2PtCl_6, 10 mL 1 N HCl, 90 mL water, 3g KI). NMR spectra were recorded on either a Varian Mercury 400 MHz or Varian Inova 500 MHz instrument. Peaks were referenced to an internal TMS standard. Infrared spectra were measured using a KBr plate on a Biorad FTS-60 FTIR spectrometer. Electrospray mass spectra were obtained via direct infusion of a methanolic solution of the compound of interest on a Varian 1200L single quadrupole mass spectrometer. Elemental analysis was performed by Desert Analytics (Tucson, AZ). ICP-MS were recorded on a VG Elemental PQ Excell spectrometer standardized with eight concentrations spanning the range 0-50 ppb Gd(III). One ppb In(III) was used as the internal standard for all runs. UV-visible spectroscopy was performed on a HP 8452A diode array spectrometer thermostated to 37 °C.

HPLC:

LC-MS:

Analytical LC-MS was performed on a computer controlled Varian Prostar system consisting of a 410 autosampler equipped with a 100 µL sample loop, two 210 pumps with 5 mL/min heads, a 363 fluorescence detector, a 330 photodiode array (PDA) detector and a 1200L single quadrupole ESI-MS. All runs were executed with a 0.8 mL/min flow rate using a ThermoElectron 4.6 x 150 mm 5 µm Aquasil C18 column with a 3:1 split directing one part to the MS and 3 parts to the series-connected light detectors. Mobile phase consisted of water (solvent A) and HPLC-grade acetonitrile (solvent B) except where noted. All injections were full-loop.

Preparative LC:

The preparative system is a Varian Prostar. Two 210 pumps with 25 mL/min heads fed a 5 mL manual inject sample loop. Detection was performed after a 20:1 split by a two channel 325 UV-visible detector and, on the low-flow leg, a HP 1046A fluorescence detector. The mobile phases were the same as in the LC-MS instrument. Preparative runs were typically 50-100 mg dissolved in water and run at 15 mL/min on a ThermoElectron 20 x 250 mm 5 µm Aquasil C18 column.

Methyl 1-(4-formyl-2-nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D**-glucopyronuronate: (6)**[28]

Methyl 1-bromo-2,3,4-tri-*O*-acetyl-α-D-glucopyronuronate[34] (10.75 g, 27.1 mmol) was dissolved in 250 ml anhydrous MeCN. 4-hydroxy-3-nitrobenzaldehyde (7.64 g, 45.7 mmol) was then added followed by 28.5 g (123 mmol) of Ag_2O. The resulting slurry was stirred in the dark under N_2 for 4 h. The solution was filtered through Celite to remove solids and the filtrate concentrated *in vacuo*. The residue was brought up in

EtOAc (400 ml) and washed with saturated $NaHCO_3$ (6 x 200 ml), water and brine. The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The beige solid was triturated with hexanes yielding **6** (12.52 g, 96%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.01, 2.02, 2.03 (3s, 3 x 3H, OAc), 3.64 (s, 3H, $COOCH_3$), 4.80 (d, 1H, H-5, J = 10 Hz), 5.15 (m, 2H, H-2, H-4), 5.74 (m, 1H, H-3), 5.94 (d, 1H, H-1 J = 8 Hz), 7.64 (d, 1H, ArH, J = 9 Hz), 8.22 (dd, 1H, ArH, J = 9 Hz, J' = 1.5 Hz), 8.44 (d, 1H, ArH, J = 1.5 Hz), 9.98 (s, 1H, CHO); ^{13}C NMR (126 MHz, DMSO-d_6) δ 20.17, 20.22, 20.26, 52.65, 68.49, 69.67, 70.43, 71.18, 97.25, 117.58, 126.23, 131.07, 134.74, 140.25, 152.10, 166.85, 168.72, 169.34, 169.49, 190.48; IR (KBr plate) ν 2956, 1756, 1700, 1612, 1538, 1368, 1235, 1074, 1039 cm^{-1}; ESI-MS *m/z* $(M + Na)^+$ 506.1; Anal. Calcd for $C_{20}H_{21}NO_{13}$: C 49.69, H 4.38, N 2.90; Found C 49.92, H 4.55, N 2.80.

Methyl 1-(4-hydroxymethyl-2-nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D-glucopyronuronate: (7)[28, 35]

1.41 g (37.3 mmol) $NaBH_4$ were added to a stirring solution of 12.03 g (24.9 mmol) **6** and 5 g silica gel at 0 °C in 300 mL 1:5 IPA:$CHCl_3$. After 45 min, the solution was poured into 300 mL of ice water and filtered through Celite. The layers were separated and the organic fraction washed with brine, dried ($MgSO_4$), concentrated *in vacuo* and triturated with Et_2O, yielding **7** as a white solid (11.65 g, 96 %). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.99 (s, 3H, OAc), 2.02 (s, 6H, OAc), 3.33 (br OH), 3.64 (s, 3H, $COOCH_3$), 4.51 (d, 2H, CH_2OH, J = 5.5 Hz), 4.73 (d, 1H, H-5, J = 10 Hz), 5.10 (m, 2H, H-2, H-4), 5.44 (m, 1H, H-3), 5.71 (d, 1H, H-1 J = 8 Hz), 7.38 (d, 1H, ArH, J = 8 Hz), 7.62 (d, 1H, ArH, J = 8 Hz), 7.80 (s, 1H, ArH); ^{13}C NMR (126 MHz, DMSO-d_6) δ 20.19, 20.22, 20.28, 52.64, 61.32, 68.73, 69.94, 70.75, 71.02, 98.06, 117.70, 122.30, 131.97,

138.54, 140.18, 146.92, 166.92, 168.74, 169.32, 169.51; IR (KBr plate) ν 3527, 1756, 1535, 1367, 1232, 1077, 1039 cm^{-1}; ESI-MS *m/z* $(M + Na)^+$ 508.3; Anal. Calcd for $C_{20}H_{23}NO_{13}$: C 49.49, H 4.78, N 2.89; Found C 49.50, H 4.90, N 3.12.

Methyl 1-(4-(2-bromo-ethylcarbamoyloxymethyl)-2-nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D**-glucopyronuronate: (9)**

3.75 g (7.73 mmol) sugar **7** and 189 mg (1.55 mmol) DMAP in 50 mL dry CH_2Cl_2 under N_2 were subjected to 2.51 g (15.5 mmol) 1,1'-carbonyl-diimidazole. When the reaction was complete by TLC (silica, 5% MeOH/CH_2Cl_2) (2.25 h), the solution was washed with water, 5% NaH_2PO_4, sat. $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$) and concentrated *in vacuo* to yield 4.19 g of the imidazolyl intermediate **8**. **8** was dissolved in 65 mL anhydrous CH_2Cl_2 under N_2 and cooled to 0 °C. 0.90 mL (7.95 mmol) MeOTf was added over 5 min. After 30 min, the reaction mixture was diluted with 30 mL Et_2O and cooled to -20 °C to allow all methylated product to precipitate. The white solid was collected by filtration, washed with Et_2O and dried *in vacuo*. The activated compound was suspended in 50 mL anhydrous CH_2Cl_2 under N_2 and 2.22 g (10.85 mmol) 2-bromoethylamine hydrobromide were added. The slurry was brought to 0 °C and 1.51 mL (10.85 mmol) TEA added in one portion. The reaction stirred for 2 h and was then washed with water and brine. The organic layer was dried ($MgSO_4$), concentrated *in vacuo* and purified by chromatography (silica, 0-55% EtOAc in hexanes) to give **9** (2.99 g, 65 %) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.00 (s, 3H, OAc), 2.02 (s, 6H, OAc), 3.39 (t, 2H, J = 6 Hz), 3.47 (t, 2H, J = 6 Hz), 3.64 (s, 3H, $COOCH_3$), 4.73 (d, 1H, H-5, J = 10 Hz), 5.06-5.14 (m, 4H, benzylic CH_2, H-2, H-4), 5.46 (m, 1H, H-3), 5.74 (d, 1H, H-1 J = 8 Hz), 7.43 (d, 1H, ArH, J = 9 Hz), 7.67 (m, 2H,

ArH, N*H*), 7.89 (s, 1H, ArH); ^{13}C NMR (101 MHz, DMSO-d_6) δ 20.22, 20.26, 20.31, 32.44, 42.34, 52.63, 63.81, 68.65, 69.83, 70.66, 71.01, 97.79, 117.72, 123.86, 132.77, 133.51, 139.99, 147.58, 155.70, 166.78, 168.62, 169.21, 169.38; ESI-MS *m/z* $(M + Na)^+$ 657.0, 659.0 (Br isotope pattern); Anal. Calcd for $C_{23}H_{27}BrN_2O_{14}$: C 43.48, H 4.28, N 4.41; Found C 43.74, H 4.20, N 4.34.

Methyl 1-(4-(2-bromo-ethylcarbamoyloxymethyl)-2-nitrophenyl)-β-D-**glucopyronuronate: (10)**

2.84 g (4.47 mmol) **9** were suspended in 90 mL dry MeOH under N_2 at 0 °C. 650 μL 30% w/v NaOMe in MeOH were added and the solution stirred for 100 min. The reaction was quenched with 197 μL acetic acid. Removal of solvent was followed by purification on silica (10% MeOH/CH_2Cl_2). The resulting solid was dissolved in acetone and filtered through a 0.2 μm PTFE filter to remove any silica. The solution was concentrated *in vacuo* and triturated (Et_2O) to give 1.80 g (79%) **10**. ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.26-3.42 (m, 5H), 3.47 (t, 2H, *J* = 6 Hz), 3.65 (s, 3H, $COOCH_3$), 4.13 (d, 1H, H-5, *J* = 10 Hz), 5.04 (s, 2H, benzylic C*H*$_2$), 5.31 (m, 2H, H-1, O*H*), 5.49 (d, 1H, O*H* *J* = 6 Hz), 5.54 (d, 1H, O*H* *J* = 5 Hz), 7.44 (d, 1H, ArH, *J* = 9 Hz), 7.63 (m, 2H, ArH, N*H*), 7.86 (s, 1H, ArH); ^{13}C NMR (126 MHz, DMSO-d_6) δ 32.44, 42.35, 52.02, 63.94, 71.19, 72.71, 75.17, 75.61, 99.76, 116.86, 124.09, 131.27, 133.54, 139.86, 148.58, 155.88, 169.06; ESI-MS *m/z* $(M + Na)^+$ 530.9, 532.9 (Br isotope pattern); Anal. Calcd for $C_{17}H_{21}BrN_2O_{11}$: C 40.09, H 4.16, N 5.50; Found C 40.32, H 4.46, N 5.39. The major byproduct of this reaction was α,β-unsaturated compound **11**, resulting from base-catalyzed β-acetate elimination. ^{1}H NMR (500 MHz, CD_3OD) δ 3.23 (t, 2H, *J* = 5 Hz), 3.45 (t, 2H, *J* = 5 Hz), 3.77 (s, 3H, $COOCH_3$), 4.09 (m, 2H), 5.09 (s, 2H, benzylic C*H*$_2$),

5.90 (br. s, 1H, H-4), 6.28 (d, 1H, H-1 J = 2.5 Hz), 7.57 (d, 1H, ArH, J = 9 Hz), 7.66 (d, 1H, ArH, J = 9 Hz), 7.92 (s, 1H, ArH); ^{13}C NMR (126 MHz, CD_3OD) δ 33.67, 44.41, 53.12, 65.83, 66.77, 70.58, 100.08, 114.14, 120.19, 126.04, 134.37, 135.11, 141.05, 142.02, 150.24, 158.73, 163.96.

Methyl 1-(4-(2-hydroxy-ethylcarbamoyloxymethyl)-2-nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D**-glucopyronuronate: (15)**

1.05 mL (9.25 mmol) MeOTf were added over 5 min to a solution of 4.87 g (8.41 mmol) **8** in 60 mL anhydrous CH_2Cl_2 under N_2 at 0 °C. After 30 min, the reaction was diluted with 30 mL Et_2O and cooled to -20 °C to allow all methylated product to precipitate. The white solid was collected by filtration, washed with Et_2O and dried *in vacuo*. The activated compound was suspended in 60 mL anhydrous CH_2Cl_2 under N_2 and brought to 0 °C. 761 μL (12.6 mmol) 2-hydroxyethylamine were added and the solution was allowed to warm to room temperature over 2 h before it was washed with water, 5% NaH_2PO_4, sat. bicarbonate and brine. The organic layer was dried ($MgSO_4$), concentrated *in vacuo* and purified by chromatography (silica, 0-5% MeOH in CH_2Cl_2) to give 3.68 g (77 %) **15** as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.99 (s, 3H, OAc), 2.02 (s, 6H, OAc), 3.05 (q, 2H, J = 6 Hz), 3.33 (s, 1H, O*H*), 3.38 (q, 2H, J = 6 Hz), 3.64 (s, 3H, $COOCH_3$), 4.64 (t, 0.5H, N*H*, J = 6 Hz), 4.74 (d, 1H, H-5, J = 10 Hz), 5.02 (s, 2H, benzylic CH_2), 5.10 (m, 2H), 5.46 (t, 1H, J = 10 Hz), 5.74 (d, 1H, H-1, J = 8 Hz), 7.27 (t, 0.5H, N*H*, J = 6 Hz), 7.43 (d, 1H, ArH, J = 9 Hz), 7.67 (d, 1H, ArH, J = 9Hz), 7.88 (s, 1H, ArH); ^{13}C NMR (126 MHz, DMSO-d_6) δ 20.20, 20.23, 20.28, 43.09, 52.64, 59.86, 63.59, 68.69, 69.87, 70.71, 71.05, 97.89, 117.80, 123.89, 133.12, 133.55, 140.11, 147.63, 155.93, 166.90, 168.74, 169.33, 169.51; IR (KBr plate) ν 3394, 2962, 1756,

1708, 1535, 1366, 1235, 1073, 1039 cm^{-1}; ESI-MS *m/z* $(M + Na)^+$ 595.3; Anal. Calcd for $C_{23}H_{28}N_2O_{15}$: C 48.25, H 4.93, N 4.89; Found C 47.96, H 5.14, N 4.91.

Methyl 1-(4-(2-hydroxy-ethylcarbamoyloxymethyl)-2-nitrophenyl)-β-D-**glucopyronuronate: (16)**

3.49 g (6.10 mmol) **15** in 100 mL anhydrous MeOH were cooled to 0 °C under N_2. 776 µL (4.27 mmol) 30% NaOMe in MeOH were added and the solution was allowed to stir for 1 h. 210 µL acetic acid were added and the volatiles were removed *in vacuo*. The resulting solid was purified by chromatography (silica, 10-15% MeOH in CH_2Cl_2) and excess silica was removed by filtration of an acetone solution through a 0.2 µm PTFE filter. Trituration of the solid with Et_2O yielded 2.15 g (79%) **16** as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.05 (q, 2H, J = 6 Hz), 3.25-3.44 (m, 6H), 3.66 (s, 3H, $COOCH_3$), 4.12 (d, 1H, H-5, J = 10 Hz), 4.63 (t, 0.5H, N*H*, J = 5.5 Hz), 5.00 (s, 2H, benzylic CH_2), 5.31 (d, 2H, H-1, OH), 5.49 (d, 1H, OH, J = 5.5 Hz), 5.54 (d, 1H, OH, J = 4.5 Hz), 7.25 (t, 0.5H, N*H*, J = 6 Hz), 7.44 (d, 1H, ArH, J = 9 Hz), 7.63 (d, 1H, ArH, J = 9Hz), 7.85 (s, 1H, ArH); ^{13}C NMR (126 MHz, DMSO-d_6) δ 43.10, 52.03, 59.88, 63.70, 71.20, 72.72, 75.17, 75.61, 99.80, 116.86, 124.05, 133.51, 133.51, 139.87, 148.53, 155.99, 169.07; IR (KBr plate) ν 3352, 2954, 1737, 1705, 1533, 1354, 1252, 1083, 1060, 1019 cm^{-1}; ESI-MS *m/z* $(M + Na)^+$ 469.2; Anal. Calcd for $C_{17}H_{22}N_2O_{12}$: C 45.74, H 4.97, N 6.28; Found C 47.92, H 5.06, N 5.98.

Methyl 1-(4-(2-methanesulfonyloxy-ethylcarbamoyloxymethyl)-2-nitrophenyl)-β-D-**glucopyronuronate: (14)**

950 µL (6.81 mmol) triethylamine, 100mg (0.85 mmol) DMAP and 1.90 g (4.26 mmol) **16** were dissolved in 50 mL anhydrous pyridine and cooled to 0 °C. 529 µL (6.81

mmol) methanesulfonyl chloride were added and the reaction was checked by TLC (10% MeOH/CH_2Cl_2). After 1 h an additional 0.5 eq (2.13 mmol) each of triethylamine and methanesulfonyl chloride were added and the solution was allowed to stir for 1 h more. The volatiles were then removed *in vacuo* and the resulting oil was purified by chromatography (silica, 10% MeOH/CH_2Cl_2). The solid was dissolved in acetone, filtered as in **16**, concentrated and triturated with hexanes to give 1.60 g (72%) **14**. The compound decomposes upon prolonged storage at ambient temperature even desiccated under N_2. ^{1}H NMR (500 MHz, CD_3OD) δ 3.03 (s, 3H), 3.44 (m, 2H), 3.48-3.66 (m, 3H, H2-4), 3.76 (s, 3H, $COOCH_3$), 4.09 (d, 1H, H-5, *J* = 10 Hz), 4.25 (m, 2H), 5.09 (s, 2H, benzylic C*H*$_2$), 5.19 (d, 1H, H-1), 7.37 (d, 1H, ArH, *J* = 8 Hz), 7.59 (d, 1H, ArH, *J* = 8Hz), 7.83 (s, 1H, ArH); ^{13}C NMR (126 MHz, CD_3OD) δ 37.32, 41.37, 53.11, 66.06, 69.95, 72.77, 74.48, 76.89, 77.25, 102.44, 118.94, 125.64, 133.44, 134.52, 142.24, 150.61, 158.62, 170.81; Anal. Calcd for $C_{18}H_{24}N_2O_{14}S$: C 41.22, H 4.61, N 5.34; Found C 41.52, H 4.55, N 5.24.

Methyl 1-(4-(2-(1-(1,4,7,10-tetrazaacyclododecyl))-ethylcarbamoyloxymethyl)-2-nitrophenyl)-β-D**-glucopyronuronate: (12)**

Cyclen (541 mg, 3.14 mmol) and **10** (640 mg, 1.26 mmol) were combined in 19 mL DMSO and the reaction was allowed to stir overnight. TLC analysis at this time (10% MeOH/CH_2Cl_2) indicated no unreacted sugar. The solvent was removed *in vacuo* yielding a viscous yellow oil. The oil was dissolved in 7 mL MeOH and a pale yellow solid precipitated upon addition of 50 mL Et_2O. Upon storage at -20 °C for 1 h, the hygroscopic solid was collected on a glass frit, washed with Et_2O (3 x 3 mL) and dried under vacuum yielding 908 mg of solid. TLC (silica; 1:9:90 sat KNO_3

(aq.):water:MeCN; Pt stain visualization) and ESI-MS showed very low-intensity di- and tri-substituted side product peaks. The precipitation procedure removed excess free base cyclen. However, MS and ^{1}H NMR showed that the desired product was contaminated with cyclen hydrobromide salt. This mixture was used in the subsequent reaction without further purification.

Methyl 1-(4-(2-(1-(4,7,10-tris-ethylcarboxymethyl-(1,4,7,10-tetrazaacyclododecyl)))-ethylcarbamoyloxymethyl)-2-nitrophenyl)-β-D**-glucopyronuronate: (13)**

887 mg of the mixture containing **12** and 1.23 g K_2CO_3 were suspended in 30 mL acetone. 820 μL α-bromo-ethylacetate were added and the solution was allowed to stir at room temperature overnight. An additional 164 μL α-bromo-ethylacetate and 210 mg K_2CO_3 were added after 24h. At 48 h, the reaction mixture was filtered to remove solids and purified by flash chromatography (silica, 0-13.3% MeOH in CH_2Cl_2). The resulting solid was dissolved in acetone and filtered through a 0.2 μm PTFE filter to remove excess silica. This yielded 510 mg of **13**. Elemental bromine analysis indicated the presence of a mixture of free base and hydrobromide salt. ESI-MS and ^{1}H NMR showed no evidence of lactamization. ^{1}H NMR (500 MHz, CD_3OD) δ 1.25 (m, 9H, $COOCH_2CH_3$), 2.0-3.4 (br, 24H, cyclen H's, 2H-sugar, acetate CH_2), 3.52 (m, 4H), 3.64 (m, 1H), 3.76 (s, 3H, $COOCH_3$), 4.10 (d, 1H, J = 10 Hz), 4.12-4.24 (m, 6H, $COOCH_2CH_3$), 5.07 (s, 2H, benzylic CH_2), 5.21 (d, 1H, H-1, J = 7 Hz), 7.39 (d, ArH, J = 8 Hz), 7.60 (d, 1H, ArH, J = 8 Hz), 7.83 (s, 1H, ArH); ^{13}C NMR (126 MHz, CD_3OD) δ 14.64, 14.67, 38.73, 53.10, 55.99, 56.44, 56.88 (br), 62.61, 62.83, 66.01, 72.79, 74.46, 76.90, 77.25, 102.30, 118.81, 125.33, 133.50, 134.31, 142.19, 150.54, 159.14, 170.76, 175.57, 175.66 (br). ESI-MS *m/z* $(M + H)^+$ 859.2 (40%), $(M + Na)^+$ 881.2 (100%); Anal. Calcd for

$C_{37}H_{58}N_6O_{17}$·acetone·2.5H_2O·0.75HBr: C 46.98, H 6.87, N 8.22, Br 5.86; Found C 47.05, H 6.55, N 8.23, Br 6.07.

Gadolinium(III) 1-(4-(2-(1-(4,7,10-tris-carboxymethyl-(1,4,7,10-tetrazaacyclododecyl)))-ethylcarbamoyloxymethyl)-2-nitrophenyl)-β-D-**glucopyronuronate: (1)**

A portion of 455 mg **13** in 10 mL water was cooled to 0 °C. 2.12 mL 1 N NaOH were added over one min and the solution was allowed to stir for 75 min. The pH was brought to 6.5 with 0.1 N HCl and 216 mg $GdCl_3$ (dissolved in 5 mL water and brought to pH = 6.5 with NaOH) were added dropwise. The pH was kept above 5.5 with 1 N NaOH during metal addition. The solution was allowed to warm to room temperature while stirring and the pH adjusted periodically to keep it between 6-6.5. After 3 days at room temperature, the pH showed no change and the reaction was considered complete. The pH was brought to 8.2 and the solution centrifuged to remove excess gadolinium as $Gd(OH)_3$. Trace solids were removed by filtration through a 0.2 μm nylon filter and the solution lyophilized. The solid was brought up in 3 mL water and purified on preparative HPLC using the following method: 0-10% B over 10 min, hold for 15 min at 10% B, then wash to 98% B before returning to 0% B. Two runs using this method were sufficient to give material that was pure by microanalysis. Yield: 185 mg **1** (17.7% from **10**). The compound was stored at -20 °C. Analysis of this material by analytic LC-MS (using the same method as in the preparative runs) gave a single peak in the PDA at 12.9 min with *m/*z = 914.4 ($M - H^+$ ESI-MS) of appropriate isotope pattern. Calcd for $C_{30}H_{40}N_6O_{17}Gd$·2.5H_2O (87% Na^+ salt): C 37.85, H 4.46, N 8.83, Gd 16.52, Na 2.10; Found C 37.69, H 4.28, N 9.12, Gd 16.88, Na 2.10.

Europium(III) 1-(4-(2-(1-(4,7,10-tris-carboxymethyl-(1,4,7,10-tetrazaacyclododecyl)))-ethylcarbamoyloxymethyl)-2-nitrophenyl)-β-D-**glucopyronuronate: (4)**

This compound was synthesized and purified in the same manner as **1** using 168 mg **13** and substituting $EuCl_3$ for $GdCl_3$. Yield: 70 mg **4** (18.1% from **10**). The compound was stored at -20 °C. Analysis of this material by analytic LC-MS (using the same method as in the preparative runs) gave a single peak in the PDA at 12.9 min with *m/z* = 907.2 ($M - H^+$ ESI-MS) of appropriate isotope pattern.

1-(2-^{t}Boc-aminoethyl)-(1,4,7,10-tetrazaacyclododecane): (17)

1.0 g (4.46 mmol) 2-^{t}Boc-aminoethylbromide[62] were added to a stirring solution of 1.92 g (11.1 mmol) cyclen in 60 mL dry toluene. The solution was refluxed overnight under N_2 and extracted with 3 x 100 mL water. The aqueous layer was extracted with 3 x 75 mL CH_2Cl_2 and the combined CH_2Cl_2 extracts were dried over $MgSO_4$. Removal of solvent gave a white solid that was washed with cold Et_2O and dried *in vacuo*. This yielded 890 mg (63%) **15**. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.44 (s, 9H), 2.59 (br s, 10H), 2.63 (br s, 4H), 2.82 (br s, 4H), 3.22 (br s, 2H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 28.58, 38.65, 46.21, 47.28, 47.92, 52.20, 54.28, 78.96, 156.14; ESI-MS *m/z* $(M + H)^+$ 316.3; Anal. Calcd for $C_{15}H_{33}N_5O_2$: C 57.11, H 10.54, N 22.20; Found C 57.43, H 10.47, N 22.51.

1-(2-^{t}Boc-aminoethyl)-4,7,10-(tris-^{t}butylcarboxymethyl)-(1,4,7,10-tetrazaacyclododecane): (18)

To a solution of 950 mg **17** (3.01 mmol) and 3.29 g (31.0 mmol) Na_2CO_3 in dry MeCN under N_2, was added 2.4 mL (15.1 mmol) α-bromo-^{t}butylacetate. The suspension

was refluxed for 24 h, filtered, washed with 3 x 250 mL hexanes and concentrated *in vacuo* to give a yellow oil. The resulting oil was purified by chromatography (silica, 0-10% MeOH in CH_2Cl_2) to give 1.70 g (76%) of **18** as a white solid. Spectral and analytic data indicate a mixture of free base and HBr salt. The ^{1}H NMR was very broad between 2-3.8 ppm and therefore unassignable; ^{13}C NMR (126 MHz, $CDCl_3$) δ (major product) 27.73, 27.92, 28.03, 28.32, 37.69, 48.01, 49.88, 50.09, 52.50, 53.01, 53.82, 55.60, 56.40(br), 56.91, 79.17, 81.70, 82.74, 156.40, 169.99, 172.51, (minor peaks): 79.30, 81.80, 82.36, 170.33, 173.28(br); ESI-MS *m/z* $(M + H)^+$ 658.4 (60%), $(M + Na)^+$ 680.3 (100%); Anal. Calcd for $C_{33}H_{63}N_5O_8 \cdot 0.9HBr \cdot H_2O$: C 52.94, H 8.87, N 9.35, Br 9.60; Found C 52.81, H 8.99, N 9.02, Br 9.78.

1-(2-aminoethyl)-4,7,10-(tris-carboxymethyl)-(1,4,7,10-tetrazaacyclododecane) TFA salt: (19)

Deprotection of 192 mg **18** was achieved by stirring at room temperature in 4.75 mL trifluoroacetic acid with 125 μL each triisopropylsilane and water. After 17 h the volatiles were removed *in vacuo* and 40 mL Et_2O were added to precipitate the ligand. The suspension was centrifuged and the white pellet washed with 3 x 50 mL Et_2O. The resulting solid was dried under vacuum and yielded 135 mg of the TFA salt, **19.** ^{1}H NMR showed no remaining ^{t}butyl resonances while ^{19}F NMR showed a signal for TFA. ESI-MS *m/z* $(M + H)^+$ 390.2.

Gadolinium(III)1-(2-aminoethyl)-4,7,10-(tris-carboxymethyl)-(1,4,7,10-tetrazaacyclododecane): (2)

128 mg (0.61 mmol) $Gd(OH)_3 \cdot H_2O$ and 239 mg **19** were combined in 10 mL water and the suspension refluxed for 48 h. The solution was brought to pH = 10 with

conc. NH_4OH and centrifuged to remove excess $Gd(OH)_3$. The pellet was washed and the combined washings and supernatant were lyophilized. The resulting solid was dissolved in water and purified by successive runs on preparative HPLC using the following method: 0-20% B over 10 min, hold at 20% B for 15 min, then wash to 98% B before returning to 0% B. Due to lack of chromophores, the compound displays little UV absorption; fluorescence, however, can be detected by exciting at 271 nm and monitoring the emission at 314 nm. Due to peak tailing, fractions were analyzed by analytic LC-MS and those containing **2** were pooled and lyophilized. 101 mg **2** were obtained analytically pure by this approach (40% from **18**). ESI-MS *m/z* $(M + Na)^+$ 567.0 with Gd isotope pattern. Anal. Calcd for $C_{16}H_{28}N_5O_6Gd \cdot H_2O$: C 34.21, H 5.38, N 12.47; Found C 34.16, H 5.31, N 12.08.

Europium(III)1-(2-aminoethyl)-4,7,10-(tris-carboxymethyl)-(1,4,7,10-tetrazaacyclododecane): (3)

128 mg **19** and 132 mg (0.36 mmol) $EuCl_3 \cdot 6H_2O$ were combined in water and the pH adjusted to 6 with 1 N NaOH. The reaction was stirred for 3 days at room temperature, filtered and lyophilized. The freeze-dried solid was purified in the same manner (and exhibited similar peak tailing) as **2** except fluorescence detection used λ_{ex} = 395 nm and λ_{em} = 615 nm. 45 mg **3** were obtained analytically pure in this fashion (33% from **18**). ESI-MS *m/z* $(M + Na)^+$ 559.8, 561.7, Eu isotope pattern. Anal. Calcd for $C_{16}H_{28}N_5O_6Eu \cdot 0.5H_2O$: C 35.11, H 5.34, N 12.79, Eu 27.76; Found C 35.03, H 5.41, N 12.54, Eu 27.89.

Relaxivity:

A 4 mM stock solution of either **1** or **2** in the appropriate buffer was diluted to

give 500 μL each of seven approximate concentrations for each run: 0, 0.05, 0.15, 0.3, 0.5, 1.0 and 2.0 mM. The T_1 of each concentration was determined using an inversion recovery pulse sequence with appropriate recycle delays on a Bruker mq60 Minispec. This instrument has a proton Larmor frequency of 60 MHz and operates at 37 °C. The resulting curves were fit to a monoexponential function to obtain T_1. 10 μL of each sample was digested in concentrated nitric acid, diluted with water and analyzed for exact Gd(III) concentration using ICP-MS. The reciprocal of the longitudinal relaxation time was plotted against the concentration obtained from ICP-MS and fit to a straight line. All lines fit with $R^2 > 0.998$. This was performed for each buffer in duplicate. The buffers were all made to have the appropriate pH at 37 °C and remade if the pH had drifted more than 0.05 pH units upon storage. Anion mimic and carbonate containing buffers were made fresh daily.

Enzyme Kinetics:

Stability:

Bovine liver β-glucuronidase (Type B-1, Sigma G 0251) stability in several buffers was assayed by the Sigma quality control assay for β-glucuronidase from bovine liver. It was determined that MOPS and anion mimic[53] engendered the enzyme with poor stability, while 100 mM phosphate with 0.01% (w/v) bovine serum albumin (BSA), pH = 7.4 at 37 °C gave suitable stability (> 2h at 37 °C without loss of activity) provided the enzyme concentration was greater than 0.5 mg/mL. The enzyme was stable for at least 24 h at 37 °C at its native pH of 5.0 in 100 mM acetate buffer.

LC-MS:

After incubating **1** (0.2 mM) with bovine liver β-glucuronidase (1 mg/mL) in the

above phosphate buffer at 37 °C, the reaction mixture was analyzed by LC-MS and showed the presence of 4-hydroxy-3-nitrobenzyl alcohol at 4.0 min, substrate **1** at 7.5 min and **2** at 11.8 min. The alcohol and **1** are readily distinguished by their absorption spectra (PDA), their appropriate negative mode ESI-MS patterns and through spiking with authentic compound. **2** could be observed using fluorescence. The HPLC method was as follows: 0-10% B over 10 min, hold at 10% B for 15 min, with fluorescence using λ_{ex} = 271 nm, λ_{em} = 314 nm.

UV-visible:

The enzymatic hydrolysis of the pyranose from **1** and subsequent linker decomposition generates 4-hydroxy-3-nitrobenzyl alcohol. The molar absorptivity, ε, of this alcohol at 422 nm was determined in triplicate to be 2840 ± 40 $M^{-1}cm^{-1}$ in 100 mM phosphate, 0.01% (w/v) BSA, pH = 7.4 at 37 °C. At pH = 7.4, the substrate, **1**, does not absorb at this wavelength. The kinetics were sampled on the HP 8452A at 37 °C every 5 s for ten min. The initial velocities for pH = 7.4 buffers were determined through a linear fit of the first 10% change in absorbance. The Michaelis-Menten parameters for **1** were determined by plotting the initial velocity versus substrate concentration for concentrations ranging from 0.1–4.0 mM. The measurements were made in triplicate with 1.0 mg/mL enzyme in a 500 µL sample. Control experiments without enzyme or substrate showed negligible absorption over the time period of the experiment. Fitting of the resulting data was performed using Origin 7.0. Analysis of the reaction mixture by LC-MS using the method detailed in purification of **1** after 10 min confirmed the presence of 4-hydroxy-3-nitrobenzyl alcohol and compound **2**. The ε of *p*-nitrophenol at 422 nm was determined to be 7660 ± 180 $M^{-1}cm^{-1}$ in the same fashion as 4-hydroxy-3-

nitrobenzyl alcohol. The determination of K_m and V_{max} for *p*-nitrophenyl-β-D-glucuronide was performed in the same manner as for **1** except the substrate concentrations were varied from 0.5–30.0 mM.

Magnetic Resonance:

Samples consisting of 0.2 mM **1** and 1.0 mg/mL enzyme in 500 μL phosphate buffer were monitored by UV-visible and magnetic resonance. T_1 was determined using a saturation recovery (90-τ-90) pulse sequence on the Bruker mq60 operating as detailed in the relaxivity section. The runs were performed in triplicate. The substrate-only control was also examined in this manner. The enzyme-only controls showed T_1's that were identical to neat buffer. In all instances with the exception of the magnetic resonance substrate control in acetate buffer, the controls showed very little change over the 1 h duration of each experiment. The substrate in acetate buffer showed a slight (2%) decrease in T_1 over the course of an hour. This trend is in the opposite direction of the kinetics runs that show an increase in T_1.

Determination of *q*:

Europium(III) compound **4** was dissolved in H_2O and D_2O. The emission was monitored at 614 nm with excitation at 394 nm on a Hitachi F4500 fluorometer operating in Phosphorescence Lifetime (short) mode. The shortest lifetime measurable with this instrument is about 0.3 ms. Fifteen scans were averaged and fit to a monoexponential decay to give phosphorescent lifetimes. $\tau(D_2O)$ = 1.274 ms; $\tau(H_2O)$ = 0.527 ms at room temperature. Using the equation of Supkowski and Horrocks,[32] q = 0.89 (correction for a single N-H amide-like oscillator gives q = 0.81) while the equation from Beeby et al.[45] generates q = 1.0 (N-H correction gives q = 0.95). At 37 °C, $\tau(D_2O)$ = 1.197 ms; $\tau(H_2O)$

= 0.458 ms. Supkowski and Horrocks give $q = 1.2$ (correction for a single N-H amide-like oscillator gives $q = 1.1$). Using the equation from Beeby et al. gives $q = 1.3$ (N-H correction gives $q = 1.2$).

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