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14. ABSTRACT The lacZ gene encoding E. coli beta-gal has already been recognized as the most commonly used reporter system in cancer gene therapy. Moreover, prostate-specific membrane antigen (PSMA) has been identified as an ideal antigenic target in prostate cancer. We propose to develop a novel class of Gd(III)-based MRI contrast agents for in vivo detection of beta-gal or PSMA activity. This new concept of the GD(III)-based MRI contrast agents is composed of three moieties: (A) a signal enhancement group, such as Gd-DOTA or Gd-PCTA; (B) an Fe(III) chelating group; (C) beta-D-galactose or glutamate. Following cleavage by lacZ transgene or PSMA in prostate cancer cells, the released, activated aglycone Fe(III)-ligand will spontaneously trap endogenous Fe(III) at the site of enzyme activity forming a highly stable complex, to restrict motion of the GD(III) chelates enhancing relaxivity and providing local contrast accumulation. We plan to synthesize 8 novel MRI contrast agents for imaging beta-gal or PSMA activity in prostate cancer cell culture, explore the feasibility of applying the most promising analogies to cells grown in vivo in mice and rats.					
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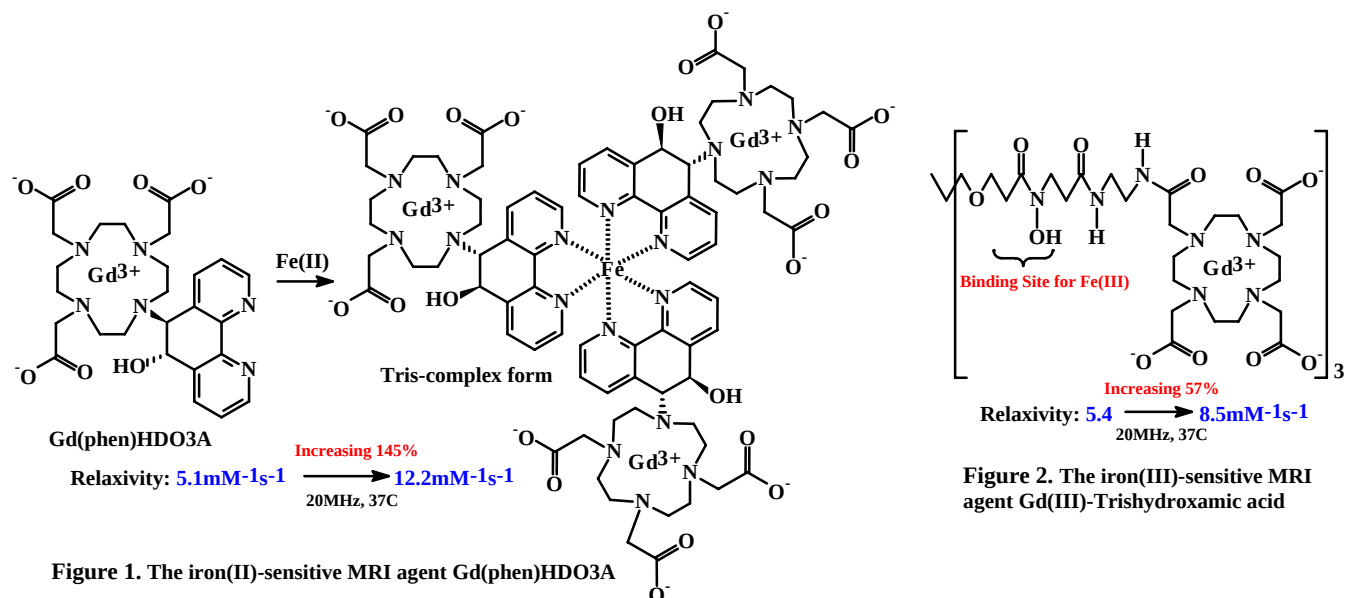
INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and the leading cause of cancer death in men in the United States.[1,2] Gene therapy has emerged as a potentially promising strategy for treatment of prostate cancer.[3-15] The prostate is particularly amenable to gene therapy.[11-16] However, there are major issues in terms of assessing the delivery to target tissue, assessing the uniformity (versus heterogeneity) of biodistribution and determining whether the genes are expressed.[15-33] A viral construct is often readministered on successive occasions, but this should optimally be timed to coincide with loss of expression. Inevitably gene therapy has associated risks, and thus non-invasive *in vivo* determining the duration of gene expression in an individual tumor could greatly enhance the viability of the approach.

Gene expression now is commonly monitored by *in situ* hybridization techniques or by introducing a marker gene to follow the regulation of a gene of interest. Since β -galactosidase (β -gal) activity is readily assessed by histology or in culture, in hosts as evolutionarily diverse as bacteria, yeast, and mammals, its introduction has become a standard means of assaying clonal insertion, transcriptional activation, protein expression, and protein interaction, *lacZ* gene encoding *E. coli* β -gal has already been recognized as the most commonly used reporter system.[34] However, the well-established chromogenic or fluorogenic substrates, relying on the hydrolysis by β -gal to release colorful compounds are limited to histology or *in vitro* assays.[35-39] Non-invasive *in vivo* detecting of transgene expression would be of considerable value in many ongoing and future clinical gene therapy trials.

The superb spatial resolution and the outstanding capacity of differentiating soft tissues have determined the widespread success of magnetic resonance imaging (MRI) in clinical diagnosis.[40] The contrast in an MR image is the result of a complex interplay of numerous factors, including the relative T_1 and T_2 relaxation times, proton density of the imaged tissues and instrumental parameters. It was shown that contrast agent causes a dramatic variation of the water proton relaxation rates, thus providing physiological information beyond the impressive anatomical resolution commonly obtained in the uncontrasted images. Contrast agents are widely used clinically to assess organ perfusion, disruption of the blood–brain barrier, occurrence of abnormalities in kidney clearance, and circulation issues.[40-44] The responsive MRI contrast agents holds great promise in the gene therapy arena.[45,46] The abilities of these contrast agents to relax water protons is triggered or enhanced greatly by recognition of a particular biomolecule opening up the possibility of developing MRI tests specific for biomarkers

indicative of particular disease states and aiding in the early detection and diagnosis of tumors. Desreux *et al* [42,47] demonstrated that, by chelating $\text{Gd}(\text{phen})\text{HDO3A}$ with $\text{Fe}(\text{II})$ to form a highly stable tris-complex, as shown in **Figure 1**, the relaxivity increased 145% at 20MHz and 37°C from $5.1\text{mM}^{-1}\text{s}^{-1}$ per $\text{Gd}(\text{III})$ in $\text{Gd}(\text{phen})\text{HDO3A}$ form to $12.2\text{mM}^{-1}\text{s}^{-1}$ in the tris-complex. Desreux *et al* [42,47] also synthesized another iron-sensitive MRI contrast agent with a tris-hydroxamate (**Figure 2**). After the tris-hydroxamate groups formed a chelate with $\text{Fe}(\text{III})$, free rotation at the $\text{Gd}(\text{III})$ centers was restricted, thereby increasing relaxivity by 57% from 5.4 to $8.5\text{mM}^{-1}\text{s}^{-1}$ at 20 MHz.



Iron is a critically important metal ion for a wide variety of cellular events.[48] Tumor cells, as compared with their normal counterparts, frequently exhibit increased uptake and utilization of iron, as evidenced by an increase in transferrin receptors at the cell surface.[49-51] Additionally, cancer cells are sensitive to the effects of iron chelators because of the critical requirement for iron in proteins that play essential roles in DNA synthesis and energy production.[52,53] Such studies have led to iron chelation therapy to clinically treat some tumors.[54-58]

Based on the MRI contrast agents findings and the biologic features of tumor, we have proposed in this project a novel class of enzyme activated Gd^{3+} -based MRI contrast agent for *in vivo* detection of β -gal activity, in which we try to combine all means of reaching the highest possible relaxivities (**Figure 3**).[42,47]

Additionally, prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein with enzymatic activities: N-acetylated α -linked L-amino dipeptidase (NAALADase) and γ -glutamyl

carboxypeptidase (folate hydrolase).[59-61] Studies with the monoclonal antibodies have demonstrated that PSMA is the most well-established, highly restricted prostate cancer cell surface antigen, it is expressed at high density on the cell membrane of all prostate cancers.[62-64] The high prostate tissue specificity of PSMA has been identified as an ideal therapeutic and diagnostic target for prostate cancer, this potential was exemplified by the recent FDA approval of an ^{111}In -labeled PSMA monoclonal antibody (Prostascint[®]) for diagnostic imaging of prostate cancer.[65-67] Furthermore, phase I and II trials have begun using immunotherapy directed against PSMA.[68-70] By introducing γ -glutamate residue instead of D-galactose in our proposed above new mechanism diagram, we intend to develop a novel class of Gd(III)-based MRI contrast agents for *in vivo* imaging prostate tumor through PSMA activated *in situ* Fe^{3+} -trapped MRI contrast agent formation (Figure 4).

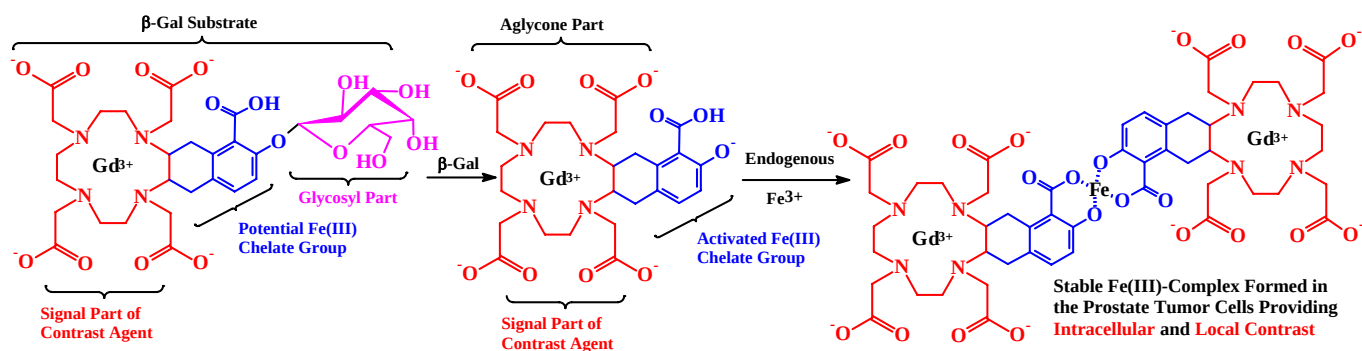


Figure 3. Mechanism of proposed new platform for *in vivo* detection of *lacZ* gene expression through β -gal activated *in situ* Fe^{3+} -trapped MRI contrast agent formation.

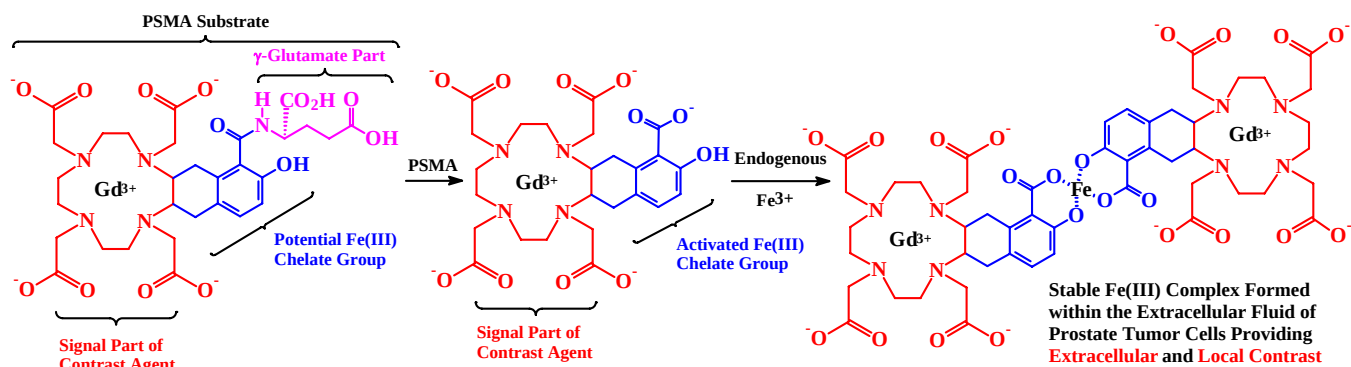


Figure 4. Proposed new mechanism for *in vivo* imaging prostate tumor through PSMA activated *in situ* Fe^{3+} -trapped MRI contrast agent formation.

Especially, PSMA has a large extracellular domain,[70] so the expression of PSMA tethered to the surface of the prostate cancer cells makes that the novel peptide-based MRI contrast agents can be targeted for activation within the extracellular fluid of prostate cancers [71] and overcomes the need for a peptide-based MRI contrast agent to penetrate the tumor cell membrane, thus, providing *in vivo* prostate

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cancer imaging through an **extracellular** MRI approach. The concern of permeability is one of the greatest challenges in the development of *in vivo* MRI contrast agents.[72]

Accordingly, depending upon the enzyme sources either being the *lacZ* transgene or the PSMA from prostate tumors, this new platform could provide *in vivo lacZ* gene expression assay or *in vivo* prostate cancer imaging (in particular, through **extracellular** contrast agents), with combining all the approaches of reaching the highest possible relaxivities.[42,47,72] Furthermore, this new class of responsive MRI contrast agent is composed of three functional moieties, in which the signal enhancing and Fe^{3+} chelating parts are flexible allowing modification in a search for ideal Fe^{3+} -trapped MRI contrast agents. Importantly, the combination of three functional moieties is based on the clinically applied strategies on cancer therapy. These facts strongly suggest the potential of the proposal to future clinical application.

Most recently, Merbach *et al* [73-76] observed the remarkably high T_1 relaxivity gain by the heterometallic, self-assembled metallostear formation with six efficiently relaxing Gd^{III} centers from $(\text{tpy-DTTA})\text{Gd}(\text{H}_2\text{O})$ with $7.3\text{mM}^{-1}\text{s}^{-1}$ to $\{\text{Fe}^{\text{II}}[\text{Gd}^{\text{III}}_2(\text{tpy-DTTA})_2(\text{H}_2\text{O})_4]_3\}^{4-}$ with $15.7\text{mM}^{-1}\text{s}^{-1}$ at 20MHz and 37°C (**Figures 5**), significantly, their detailed studies on structure and dynamics of the trinuclear complex $\{\text{Fe}^{\text{II}}[\text{Gd}^{\text{III}}_2(\text{tpy-DTTA})_2(\text{H}_2\text{O})_4]_3\}^{4-}$ indicate that the heterometallic self-assemblies attain high T_1 relaxivities by influencing three factors: water exchange, rotation, and electron relaxation, which are fully consistent with the expecting results shown as above in **Figures 3** and **4**, the effectiveness of contrast agents can be increased by restricting the motion of $\text{Gd}(\text{III})$ chelates by linking them rigidly to macromolecules through covalent or non-covalent bonds, by an improvement of their intrinsic relaxivity or by attaching several paramagnetic entities to biological or synthetic oligomers. Obviously, these most recently comprehensive investigations as relevant evidences strongly support for our current proposal.

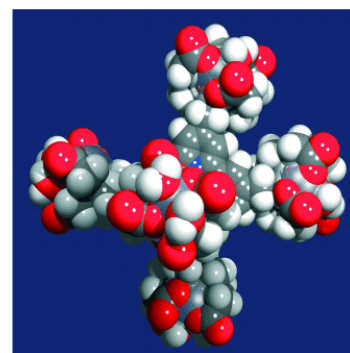


Figure 5. $[\text{Fe}\{\text{Gd}_2\text{L}(\text{H}_2\text{O})_4\}_3]^{4-}$

STATEMENT OF WORK

Specific Aim 1 Design and synthesize model “smart” MRI contrast agents to report β -gal or PSMA activities with the ability of trapping Fe^{3+} ion.

Task 1 Design and optimization of synthetic strategies for reporter molecules. (**Months 1-18**)

Task 2 Structural characterizations of the synthesized molecules. (**Months 4-20**)

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Specific Aim 2 Test the properties of molecules in solution and *in vitro* with cultured prostate cancer cells.

Task 3 Evaluation the basic properties of the agents **in solution**. (Months 20-22)

Task 4 Evaluation of the properties of the optimal molecules *in vitro* **with cultured prostate cancer cells**. (Months 23-25)

Specific Aim 3 Scale up synthesis of the most promising MRI contrast agent(s) and apply to animal investigations.

Task 5 Scale up synthesis of the most promising ^1H MRI contrast agent(s). (Months 26-28)

Task 6 Apply the most promising ^1H MRI contrast agent(s) to assess β -gal transfection efficiency, *lacZ* gene expression (spatial and temporal) in prostate tumors *in vivo* (48 mice + 48 rats). (Months 29-35)

Task 7 Test dosing protocols, timing, MR detection protocols (48 mice) (Months 29-35)

Task 8 Prepare manuscripts and final report (Month 36)

BODY

In this second supported year, our work continued followed the research plan of the approved proposal W81XWH-05-1-0593 on: **Task 1** Design and optimization of synthetic strategies for reporter molecules; **Task 2** Structural characterizations of the synthesized molecules; **Task 3** Evaluation the basic properties of the agents in solution; and **Task 4** Evaluation of the properties of the optimal molecules *in vitro* with cultured prostate cancer cells.

For the designed molecules **M**₁ and **M**₂, in the first supported year we have successfully built the key structure (*see the red structure*) of Gd^{3+} and Fe^{3+} chelators according to the approaches as shown in **Figure 6**. After alkylation by reacting with ethyl bromoacetate, much attempts to selectively cleave benzyl ether (*see the blue Bn-*) in the presence of benzyl ester (*see the magenta Bn-*) by Pd/C hydrogenolysis were not fruitful under various reaction conditions: **Solvents**: MeOH, EtOH, THF, DMF, pyridine, MeOH-dioxane, MeOH-DMF, MeOH-pyridine, EtOH-pyridine; **Catalysts**: Pd/C 1%, 3%, 5%, 10% wt; **H₂ Pressures**: 5, 10, 15, 20 parr; **Temperatures**: room temperature, 50, 75°C, only di-benzyl deprotection product **A** (R=H), not the desired product (R=Bn), was isolated in moderate yield. The literature survey indicates that it is a high desire so far to develop selective removal methods for a range of benzyl-type protecting groups with different reactivities. Direct galactosylation using 2, 3, 4, 6-tetra-O-acetyl- α -D-galactopyranosyl bromide with **A** accomplished the di-galactosyl compound **B**, in which

the galactosyl ester was hoped to be removed within the cleavage of ethyl groups from tetraacetyl esters. However, the conditions being able to cleave ethyl groups from tetraacetyl esters either by hydrolysis or esterolysis are always too strong to destroy the desirable galactosyl ether moiety, yielding compound **C**, not the expected compound **D**, very close analogue to the target molecule **M₁**.

Similarly, the syntheses of analogues **M₃** - **M₆** have encountered the same situations. We are now stressing to explore the various methods to simultaneously remove the ethyl and galactosyl in ester moieties, while preserving the galactosyl ether part stable, for it seems that only one step to success.

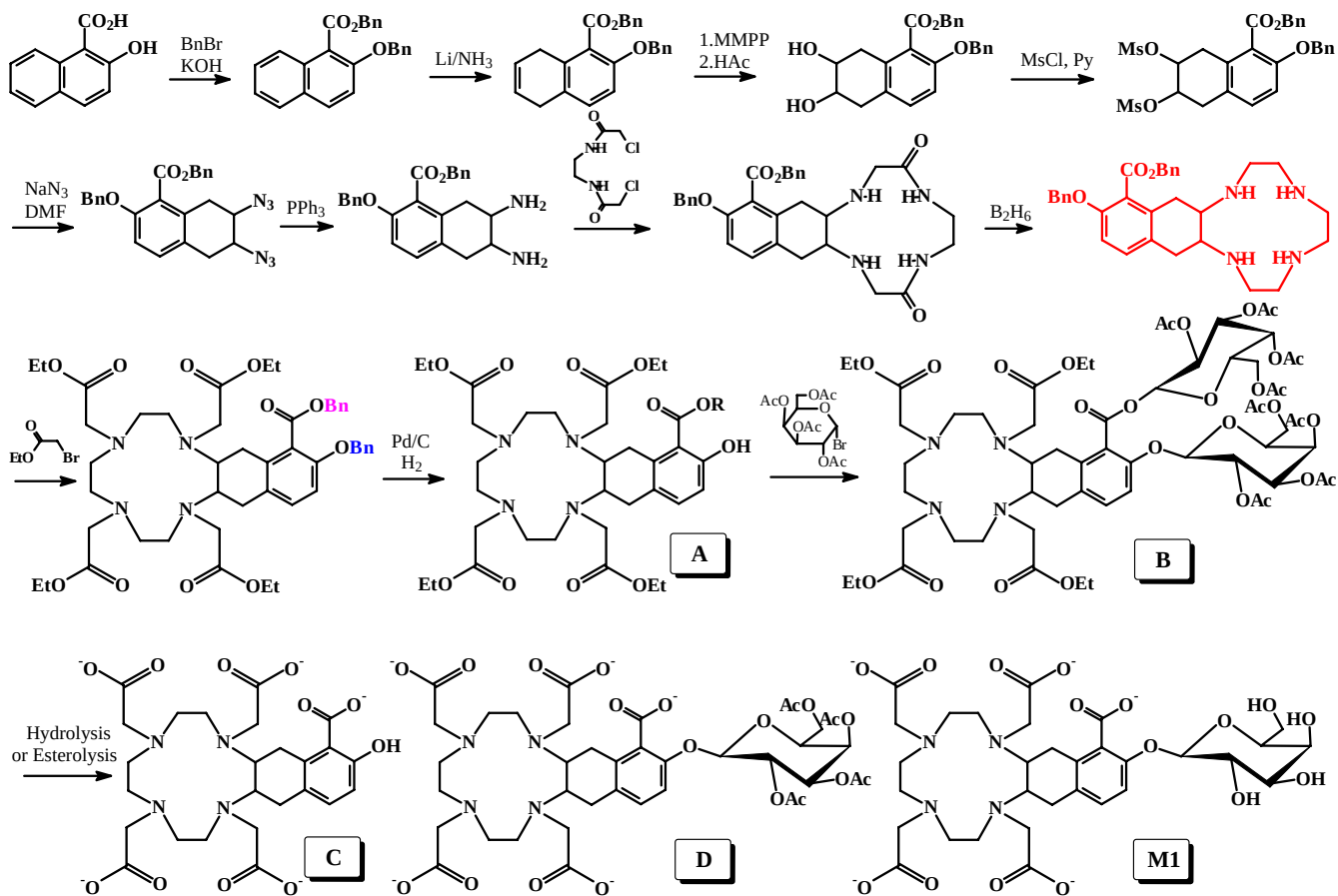


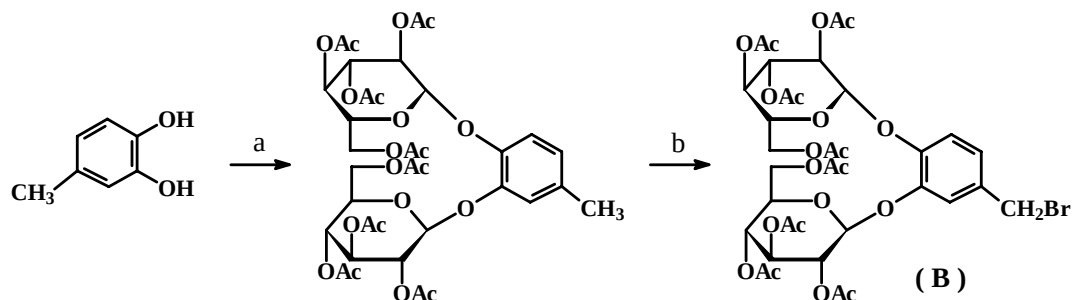
Figure 6

On the other hand, we MODIFIED the synthetic strategies based on the encountered difficulties, for example in **Figure 7**: tert-Butyl (instead of Ethyl) and benzyl groups were chosen, since they can be readily and selectively removed. Actually, the modified synthetic strategies have been already applied successfully in synthesis of other target molecules, such as **M₇**.

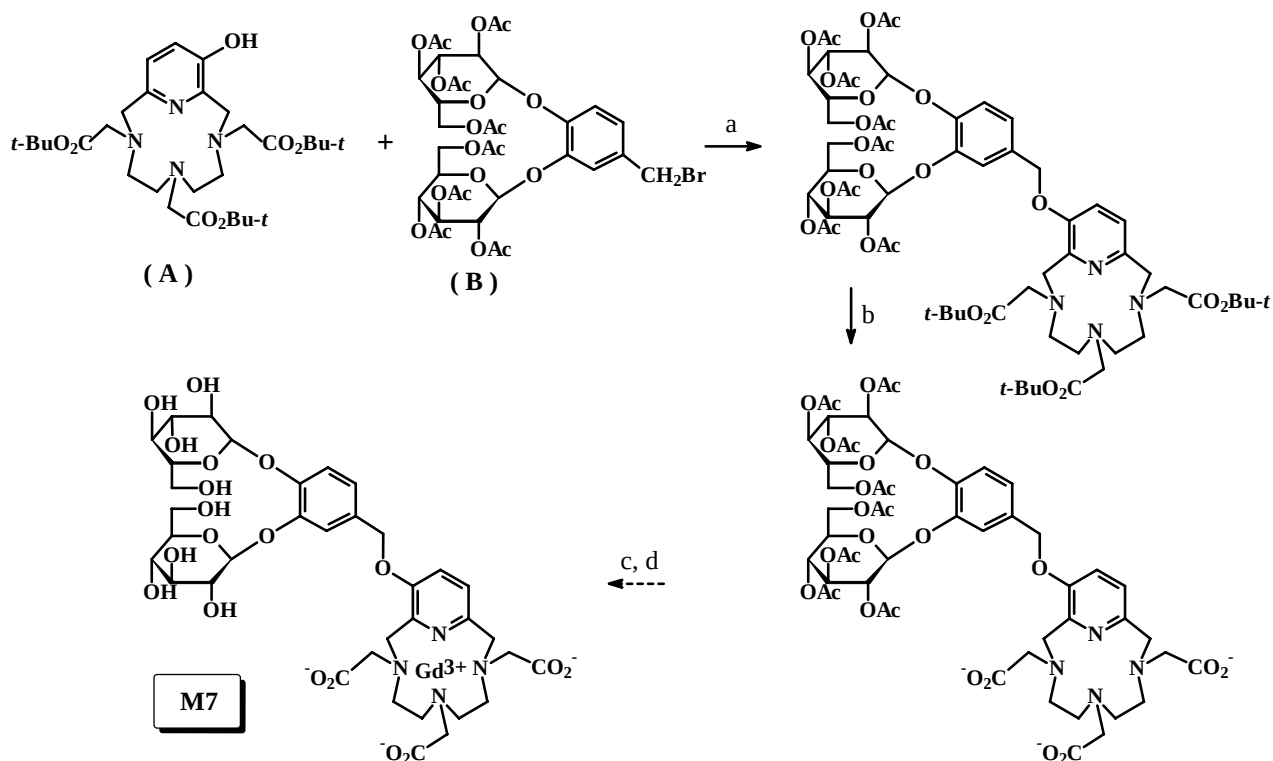


Scheme 1 illustrates the synthesis of macrocyclic ligand (A). The process begins with 2,6-dihydroxynicotinic acid, which is converted to 2,6-dihydroxy-1-methyl-4-(benzyloxy)pyridine (intermediate 1) via step (a). Intermediate 1 is then chloromethylated to form 2,6-dihydroxy-1-methyl-4-(benzyloxy)-2,6-dichloromethylpyridine (intermediate 2) in step (b). In step (c), intermediate 2 reacts with Ns-NH-CH₂-CH₂-NH-Ns to form a macrocyclic intermediate with three Ns-protected amine groups. Step (d) involves deprotection with H₂ to yield a macrocycle with three primary amine groups. Step (e) shows the reaction of this macrocycle with t-BuO₂C-CH₂-CH₂-NH₂ to form a macrocycle with three amine-terminated side chains. Finally, step (f) is the cyclization to form the macrocyclic ligand (A), which is a cage-like structure with three amine-terminated side chains and a central pyridine ring.

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Reaction Conditions: (a) 2,3,4,6-tetra-*O*-acetyl- α -D-galactopyranosyl bromide, $\text{Hg}(\text{CN})_2$, 4Å M.S., CH_2Cl_2 , 88%; (b) NBS, 78%;



Reaction Conditions: (a) K_2CO_3 , MeCN, 75%; (b) $\text{CF}_3\text{CO}_2\text{H}$, CH_2Cl_2 , 81%; (c) GdCl_3 , Pyridine, 82%; (d) MeOH, MeONa, 89%.

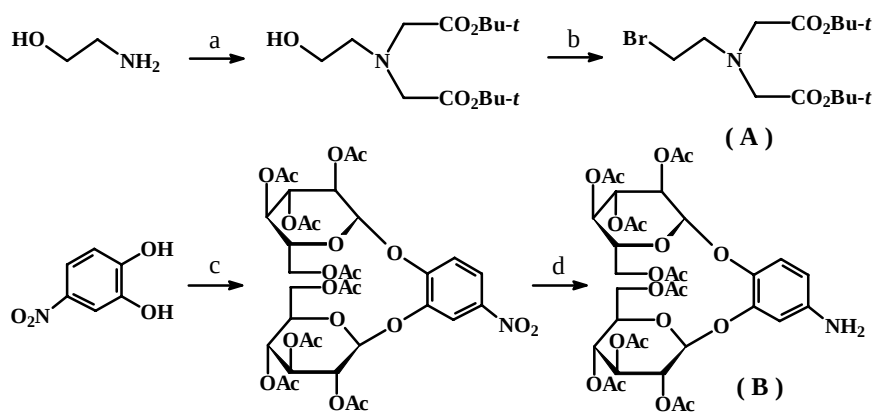
This part work has been reported on the IMPaCT Meeting in Atlanta, Georgia, Sept. 5-8, 2007. We are now evaluating the characterization of this target molecule with β -gal reaction, the Fe-complex formation, and the resulting relaxation changes.

IMPROVEMENTS

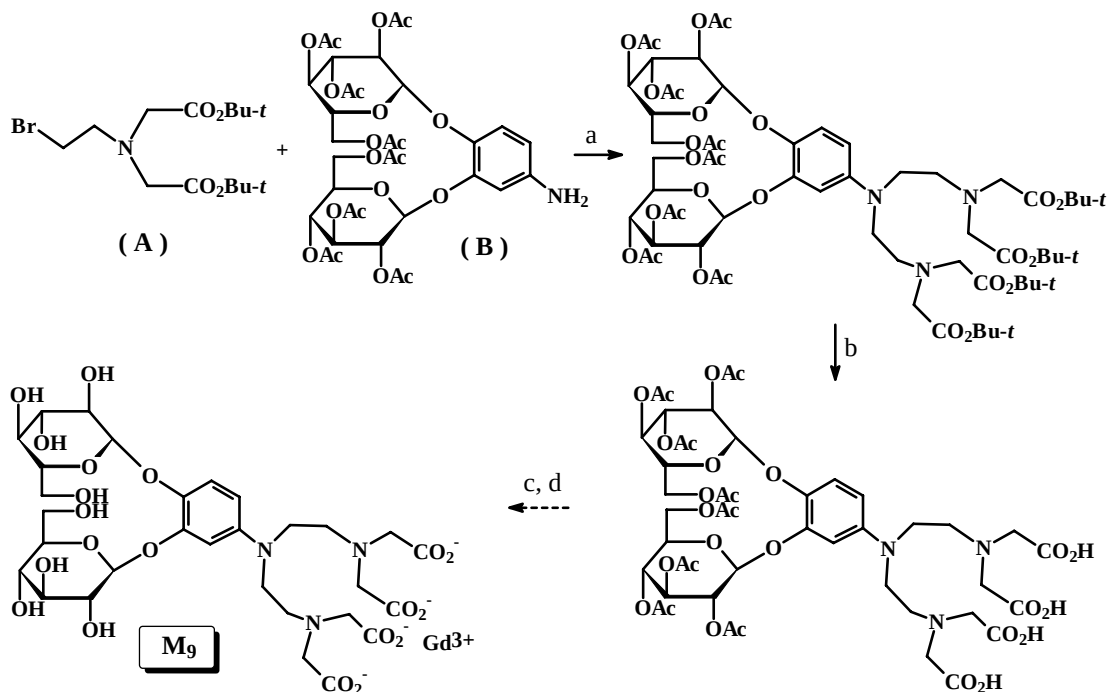
We always keep in mind that our objective is to develop and establish a novel platform for *in vivo* prostate cancer imaging and evaluation of prostate cancer gene therapy as described in the approved proposal W81XWH-05-1-0593, but not exploring the chemical synthesis methodology. Meanwhile on struggling for accomplishing the representative target molecules $\text{M}_1 \sim \text{M}_6$, we also attempted to develop

the alternate candidates based on the novel mechanism proposed in W81XWH-05-1-0593, and combining with the most recent findings about MRI contrast agents.

Very recently, diethylenetriamine-*N, N, N'', N''*-tetraacetate (DTTA) as Gd(III) chelator has been proven to ensure the complex to sufficient thermodynamic stability, a water exchange faster (closer to optimal) than that of commercial agents and two inner sphere water molecules to double the inner sphere contribution to the relaxivity.[40,73] So merging DTTA into our responsive MRI contrast agents instead of DOTA or PCTA as Gd(III) chelating moiety should produce particularly high relaxivity and sensitivity. **Figure 8** depicts the successful syntheses of such kind new target molecule **M₉**.



Reaction Conditions: (a) $\text{BrCH}_2\text{CO}_2\text{Bu-t}$, KHCO_3 , 88%; (b) Ph_3P , NBS, 86%; (c) 2,3,4,6-tetra-O-acetyl- α -D-galactopyranosyl bromide, $\text{Hg}(\text{CN})_2$, 4 Å M.S., CH_2Cl_2 , 92%; (d) H_2 , Pd/C, 100%.



Reaction Conditions: (a) K_2CO_3 , MeCN, 78%; (b) $\text{CF}_3\text{CO}_2\text{H}$, CH_2Cl_2 , 84%; (c) GdCl_3 , Pyridine, 80%; (d) MeOH, MeONa, 86%.

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Comparing with the representative target molecules **M₁**, **M₃** and **M₅**, **M₉** exhibits the following merits: (1) DTTA has been proven to ensure the complex to sufficient thermodynamic stability, a very important factor for *in vivo* safety; (2) DTTA guarantees faster water exchange, closer to optimal; (3) Gd-DTTA has two inner sphere water molecules to double the inner sphere contribution to the relaxivity; (4) Gd-DTTA and Fe-chelator are directly connected *via* a covalent C-N bond to have the shortest and most rigid linker, remarkably restrict the motion of Gd-DTTA, then particularly improving its intrinsic relaxivity; (5) Practically, the syntheses are much straightforward.

This part work has been reported on the AMI/SMI Joint Molecular Imaging Conference in Providence, Rhode Island, Sept. 8-11, 2007. We are now evaluating the characterization of this target molecule with β -gal reaction, the Fe-complex formation, and the resulting relaxation changes.

RESEARCH ACCOMPLISHMENTS

Two target molecules **M₇** and **M₉** have been successfully achieved, the molecule **M₉** shows the following merits: (1) DTTA has been proven to ensure the complex to sufficient thermodynamic stability, a very important factor for *in vivo* safety; (2) DTTA guarantees faster water exchange, closer to optimal; (3) Gd-DTTA has two inner sphere water molecules to double the inner sphere contribution to the relaxivity; (4) Gd-DTTA and Fe-chelator are directly connected *via* a covalent C-N bond to have the shortest and most rigid linker, remarkably restrict the motion of Gd-DTTA, then particularly improving its intrinsic relaxivity; (5) Practically, the syntheses are much straightforward. All structures for syntheses of these two target molecules **M₇**, **M₉** are verified by NMR data.

REPORTABLE OUTCOMES

The design and synthesis of target molecules **M₇**, **M₉** have been reported on the IMPaCT Meeting in Atlanta, Georgia, Sept. 5-8, 2007 and the AMI/SMI Joint Molecular Imaging Conference in Providence, Rhode Island, Sept. 8-11, 2007, respectively.

CONCLUSIONS

Prostate cancer is the most commonly diagnosed cancer and the second most common cause of cancer death in men in the United States. The advent of effective screening measures can sharply decrease the mortality of prostate cancer through detecting this disease at an earlier stage. However, the evidence for mortality benefit from prostate cancer screening has been disappointing to date. Expanding knowledge of prostate cancer biology with combination of imaging technologies would be of considerable value in many ongoing and future clinical prostate cancer diagnosis and gene therapy trials.

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Based on the biologic features of prostate cancer, we proposed in this project a new approach for *in vivo lacZ* gene expression assay or *in vivo* prostate cancer imaging (in particular, through **extracellular** contrast agents). The ultimate objective is to demonstrate the utility and reliability of this new approach to measure β -gal or PSMA activities *in vivo*. We have accomplished two target molecules **M₇**, **M₉**, and verified by NMR data. We are now focusing on evaluating the characterization of these two target molecules with β -gal reaction, the Fe-complex formation, and the resulting relaxation changes, and speeding up the syntheses based on the modified synthetic strategies, anticipating to identify 1-2 as of the most promising MRI contrast agents for the sequence of tests with prostate cancer *in vitro* and *in vivo*.

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APPENDICES

- (1) Poster #P30-19 on the IMPaCT Meeting in Atlanta, Georgia, Sept. 5-8, 2007.
- (2) Poster #0322 on the AMI/SMI Joint Molecular Imaging Conference in Providence, Rhode Island, Sept. 8-11, 2007.

