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14. ABSTRACT:

   We propose to develop novel molecular imaging agents to detect enzyme biomarkers of metastatic breast cancer. These imaging agents are detected using magnetic resonance imaging (MRI) only if they encounter enzymes that are present in metastatic breast tumors. Unlike standard MRI agents, these novel agents are detected by a unique mechanism that allows for evaluations of multiple enzymes during the same MRI experiment. The ability to simultaneously detect multiple enzyme biomarkers within a single MRI scan session will reduce scan times and patient discomfort. The unique detection mechanism employed by these MRI agents will take full advantage of new high-field clinical MRI scanners; this is in contrast to standard MRI agents, which show only limited improvements at high magnetic fields. We will generate these novel agents using standard chemistry protocols. We'll optimize our MRI methods to detect enzyme biomarkers with these agents, and we will use well-established fluorescence imaging methods to validate our results. Finally, we'll apply these agents to detect the enzyme biomarkers within metatstatic breast tumor cells grown in mice. As a long-term goal, these novel molecular imaging agents will be further developed for clinical assessments of metastatic breast cancer.

15. SUBJECT TERMS
Performance of research to accomplish goals listed in the abstract.

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Table of Contents

Introduction

We have developed novel molecular imaging agents that detect matrix metaloprotenase enzyme MMP-2 and MMP-9 that are confirmed biomarkers of metastatic breast cancer. These "smart" imaging agents are activated by MMP-2 or MMP-9 enzymatic activity for detection by magnetic resonance imaging (MRI). Unlike standard MRI agents, these novel molecular imaging agents have unique MR frequencies, and we have shown that multiple agents can be simultaneously used to assess multiple biomarkers. We have also shown that these new agents detect only the active forms of these biomarkers, which is critical for proper evaluation of metastatic breast cancer. We have developed new methodologies to facilitate the synthesis of these agents using standard solution-phase and Fmoc solid-phase peptide synthesis techniques. Our work during the 1-year funded research project has focused on the evaluation of biochemical solutions of our molecular imaging agents. In addition, we have extended our work to develop a similar molecular imaging agent that detects caspase-3, which is an impotant enzyme biomarker for apoptotic therapies against breast cancers and many other disease pathologies. The developments of these new synthesis methods and demonstrations of the detections of many types of enzymes shows that our "smart" PARACEST MRI contrast agents constitute a fundamentally new technology platform to detect many enzymes involved in metastatic breast cancers and other disease pathologies.

BODY

Task 1. To synthesize novel molecular imaging agents that are activated by biomarkers of metastatic breast cancer (Months 1-3).

This task has been successfully accomplished by developing new methods to incorporate our molecular imaging probe, DOTA, at any location along a peptide using industry-standard Fmoc peptide synthesis methods, which greatly faciliatates the development of new peptide-based imaging agents. Details of these methods have been captured in two manuscripts that are under review for publication in the Journal of the American Chemical Society and Tetrahedron Letters. These manuscripts are included in the appendix.

Task 2. To evaluate the abilities of MRI CEST agents to assess biomarkers of metastatic breast cancer (Months 4-6).

This task has been successfully accomplished by testing our peptide-DOTA imaging agents in biochemical solutions of MMP-2 and MMP-9. Details of these studies have been captured in the appendix.

In addition, we have symthesized and evaluated the abilities of MRI CEST agents to assess biomarkers of apoptotic therapies directed against breast cancers and other disease pathologies. Details of our accomplishments have been captured in a manuscript that is under review for publication in the Journal of the American Chemical Society. This manuscript is included in the appendix.

Task 3. To demonstrate the abilities of MRI CEST agents to assess biomarkers of metastatic breast cancer in vivo (Months 7-12).

This task has not been accomplished to date. Problems with detection sensitivity must first be overcome, as described in Task 2.

KEY RESEARCH ACCOMPLISHMENTS

- We have developed new methods to incorporate our molecular imaging probe, DOTA, at any location along a peptide using industry-standard Fmoc peptide synthesis methods, which greatly facilitates the development of new peptide-based imaging agents

- We have developed a MRI contrast agent that detects MMP-2 and MMP-9 enzymes in biochemical solutions. Detection sensitivity is problematic, and our on-going work focuses on polymerization of our agents to improve sensitivity.
- We have extended our methodology to detect caspase-3 with good sensitivity, which is an important biomarker of tumor apoptosis and apoptosis-inducing breast tumor therapies. We have identified that 243 additional protease targets can be detected using our methodologies.

REPORTABLE OUTCOMES

Manuscripts:
1. Yoo B, Pagel MD. A facile synthesis of α-amino-DOTA as a versatile molecular imaging probe. *Submitted to Tetrahedron Lett.*
2. Yoo B, Pagel MD. "Peptidyl Molecular Imaging Contrast Agents Using a New Solid Phase Peptide Synthesis Approach", *Submitted to the Journal fo the American Chemical Society.*
3. Yoo B, Pagel MD. A "Smart" PARACEST MRI Contrast Agent to Detect Enzyme Activity. *Submitted to the Journal fo the American Chemical Society.*

Abstracts and Presentations:
1. *Novel Molecular Imaging Agents to Detect Biomarkers of Metastatic Breast Cancer,* Yoo B, Raam M, Pagel MD. Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting, Philadelphia, PA, 6/8-11/05.
2. *Flexible Synthesis of DOTA-Peptides for Molecular Imaging*, Yoo B, Pagel MD. Society for Molecular Imaging 2005 Meeting, Colgne Germany, 9/7-10/05.

Patents:
1. "Imaging agents for flexibly labeling peptides during peptide synthesis"; Byunghee Yoo and Mark Pagel; Invention Disclosure filed 8/31/05; Non-Confidential Disclosure statement filed 3/11/06 with the Technology Transfer Office of Case Western Reserve University.
2. "Activatable CEST MRI Agent"; Mark Pagel, Byunghee Yoo, Guanshu Liu, and Rachel Rosenblum; Invention Disclosure filed 3/11/06; provisional patent application is under development by the Technology Transfer Office of Case Western Reserve University.

Additional Funding based on this award:
National Cancer Institute, NIH CA-110943-01 $4,911,829 8/1/04-7/31/09
Small Animal Imaging Resource Program (PI: Jeff Duerk)
Development and Management of the Contrast Agent Core Facility of the program ($431,435)
Role: Co-I

Ohio BRTT Partnership Award (PI: P Davis) $4,000,000 7/1/05-6/30/08
Targeted Nanoparticles for Imaging and Therapeutics
Development of novel imaging contrast agents to simultantously track polymeric drug delivery carrier systems and drug release from polymeric carriers within in vivo animal models. ($337,917)
Role: Co-I

Case Western Reserve University $80,000   4/1/06-3/31/08
Cystic Fibrosis Core Center
Assessing MMP-9 activity in Cystic Fibrosis mouse models using MRI
Role: PI

CONCLUSION
To summarize, we have synthesized and characterized our molecular imaging agent to demonstrate that we can detect MMP-2 and MMP-9 in biochemical solutions. Detection sensitivity remains problematic. To address this problem, we are polymerizing our agents by developing a bio-orthogonal synthesis strategy to "click together" our DOTA peptide agent to a biocompatible polymer. Because the CEST effect is linear with concentration, this approach is expected to increase our detection sensitivity by 1000-fold and also improve the localication of the agents through the well-known Enhanced Permeability and Retention (EPR) effect in solid breast tumor tissues.

In addition, we have developed new methods for synthesizing peptides with our DOTA imaging agent. We've demonstrated that our approach provides great flexibility for synthesizing peptide-DOTA agents in solution and in the solid phase, and uses industry-standard Fmoc peptide synthesis methodologies to facilitate the acceptance of our approach. This accomplishment has resulted in two manuscripts, invention disclosure, and Non-Confidential Disclosure for dissemination to industry. This accomplishment provides new opportunities to create peptide-DOTA imaging agents to molecular imaging applications of breast cancer and many other diseases.

Finally, we have demonstrated that our concept of a molecular imaging agent that can detect enzymes can be extended to detect enzymes other than MMP-2 and MMP-9. In particular, we have demonstrated that a peptide-DOTA imaging agent can detect caspase-3, which is an important biomarker of apoptotic therapies applied to breast cancers and many other diease pathologies. This accomplishment has resulted in one manuscript and one pending provisional patent. This demonstration validates that our concept fo a "smart" PARACEST MRI agent is a technology platform that may be used to detect many types of enzymes within breast cancer and other disease pathologies.

REFERENCES
See references in manuscripts in the appendices.

APPENDICES
Three manuscripts are included that provide detail regarding this research:
1. Yoo B, Pagel MD. A facile synthesis of α-amino-DOTA as a versatile molecular imaging probe.
2. Yoo B, Pagel MD. "Peptidyl Molecular Imaging Contrast Agents Using a New Solid Phase Peptide Synthesis Approach".
3. Yoo B, Pagel MD. A "Smart" PARACEST MRI Agent to Detect Matrix Metalloproteinase Enzymes.
4. Yoo B, Pagel MD. A "Smart" PARACEST MRI Contrast Agent to Detect Enzyme Activity.

A facile synthesis of α-amino-DOTA as a versatile molecular imaging probe

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ABSTRACT

DOTA (1,4,7,10-tetraazacyclododecane-N',N'',N'''N''''-tetraacetic acid) has been used as a contrast agent in many molecular imaging studies. However, the carboxylate ligands of DOTA provide limited chemical functionality for derivitization, including the limited coupling of DOTA only to peptidyl amines. To overcome this limitation, an amino group has been introduced into one ligand of DOTA that may couple to peptidyl carboxylates. α-amino-DOTA was synthesized by coupling α-brominated glycine to DO3A-^{t}Bu (1,4,7,10-tetraazacyclododecane-1,4,7-tris(acetic acid, t-butylester)). α-amino-DOTA was coupled to the carboxylate backbone terminus of a peptide to demonstrate the utility for derivitization. T_1 relaxivity of an Amino-Gd^{3+}-DOTA chelate and its para-CEST properties of Tm complex were measured according to pH, Temp, and concentration to verify that the final product can serve as a molecular imaging contrast agent. These results expand peptide-α-amino-DOTA synthesis strategies by conjugating α-amino-DOTA to peptidyl carboxylates, which provides new opportunities to develop novel contrast agents for molecular imaging.

Introduction

Macrocyclic metal chelates using DOTA are often administered to *in vivo* patients and animal models to create or enhance contrast in biomedical molecular imaging studies. Examples include paramagnetic Gd^{3+}-DOTA for magnetic resonance imaging (MRI),[1] radioactive Tc- DOTA for SPECT,[2] and fluorescent Eu(III)-DOTA for fluorescence imaging.[3,4] More recently, metal-DOTA chelates have been conjugated to peptides to affect the pharmacokinetics of the metal-DOTA imaging agent within in vivo systems, which can be used to gain additional information about biological processes at the molecular level. A variety of peptidyl ligands have been employed for these molecular imaging studies, including peptides that bind to specific cell surface receptors,[5-7] peptides that penetrate cell membranes,[8-12] peptides that nonspecifically interact with the extracellular matrix,[13] and very large peptide homopolymers that drastically alter renal clearance rates.[14]

To synthesize these peptidyl imaging agents, the carboxylates of DOTA have been conjugated to the amines of peptides, including the N-terminus, the side chain of lysine, and unnatural amino acid derivatives such as p-NH_2-phenylalanine.[15] Other DOTA derivatives have been devised for conjugation to peptide amino groups, such as succinimide DOTA derivatives[16] and isothiocyanato DOTA derivatives.[17] However, coupling DOTA only to peptide amines can limit synthesis methodologies.[18,19] Also, modification of a peptide N-terminus or side chain amino group can compromise the utility of the peptide for molecular imaging applications, since peptide-biomolecule interactions critically depend on the (native) peptide amino acid sequence.

Experimental

To address these limitations, a specific methodology is required for conjugating DOTA to the C-terminus of a peptide backbone. We have developed new α-amino-DOTA derivatives that conjugate directly to the C-terminus of a peptide. We have developed two different BOC and CBZ protected glycine templates to accommodate future applications via orthogonal protection strategies. Two different synthetic pathways for the α-bromination of glycine templates are shown in Scheme 1.

To brominate α-carbon of BOC-Gly-^{t}Bu, N-bromosuccinimide (1.78g, 10mmol) was added to a solution of BOC-Gly-^{t}Bu (2.31g, 10mmol) dissolved in dry carbon tetrachloride (50mL). The mixture was irradiated with 254nm filtered UV lamp at 25°C for 1.5hrs. The product was obtained in quantitative yield (3.00g, 97%) and used without further purification. As previously reported in other methods,[20-23] the reaction time, temperature, the intensity of UV light and the species of brominating agents were the key factors for this reaction.

The other glycine template, CBZ-Gly(Br)-OMe (N-(Benzyloxycarbonyl)-α-bromoglycine methylester), was acquired with the total yield of 70% according to the previous reported synthetic pathway.[24]

To introduce an amine group to one of the four side ligands, BOC-α-amino-DOTA was synthesized from BOC-Gly(Br)-^{t}Bu (0.62g, 2.0mmol) in dry acetonitrile (100mL) with DO3A(^{t}Bu) (1.1g, 2.1mmol) in the presence of K_2CO_3 (1.66g, 12mmol, 6eq.). The reaction mixture was heated to 70°C for 6hrs. After removal of the undissolved solids by filtration, the solution was concentrated in vacuo, yielding product as a powdery solid (95%). To continue further experiments, the protecting groups of BOC-α-amino-DOTA were completely removed by a cleavage cocktail (95% TFA/2.5% water/2.5% thioanisol) for 30 minutes. The solution was concentrated in vacuo and precipitated with ice cooled diethylether, yielding white solid (90%).

To prepare CBZ-α-amino-DOTA, CBZ-Gly(Br)-OMe (0.6g, 2mmol) was coupled to DO3A(^{t}Bu) (1.1g, 2.1mmol) using the same alkylation conditions, yielding product > 95%. To remove the CBZ group by hydrogenolysis, CBZ-α-amino-DOTA (0.74g, 1mmol) was dissolved in 10mL of absolute ethanol and added 1,4-hexadiene (0.94mL, 10mmol) and 10% Pd/C (0.55g, 10mmol). The catalyst was removed by filtration after 40min of reaction, which is traced by TLC (chloroform/methanol/ammonia=10/10/0.5). The filtrate was concentrated and dried in vacuo. The remaining methyl and *tert*-butyl groups were removed by hydrolysis using 1N-NaOH solution. The aqueous solution was lyophilized after dialysis using a 100 MWCO nitrocellulose dialysis membrane, yielding slightly yellowish solid of α-amino-DOTA (85%).

The BOC and CBZ protecting groups were confirmed to be stable after the coupling reaction using NMR spectroscopy (600MHz Inova NMR spectrometer, Varian Associates, Inc.). Due to the bulky protecting groups of BOC-Gly(Br)-tBu and CBZ-Gly(Br)-OMe, the reaction yields were expected to be lower than conventional exhaustive alkylation reactions,[18,19] but the reactions showed sufficiently high yield to be used in subsequent synthesis steps without further purification. Also for the brominated glycine templates, the α-carbon was racemized by Br introduction, which is confirmed by NMR measurements. The (R) and (S) types were formed almost 1 to 1 ratio. The obtained α-amino-DOTA was very hygroscopic, and refrigerated immediately after drying.

To demonstrate the coupling of α-amino-DOTA with the C-terminus of peptide backbone, a peptide was synthesized with a Wang resin (Fluka, 0.75mmol/g), HBTU and HOBt coupling agents, and standard Fmoc chemistry protocols using an Applied Biosystems 433A Peptide synthesizer.[25-27] The coupling efficiency was monitored by conductivity tests performed by the peptide synthesizer. The synthesized peptide sequence was selected to be a substrate for the MMP-2 enzyme.[28,29] The synthesized peptide sequence was Ac-Pro-Leu-Gly-Met-Trp-Ser-Gly (Ac-PLG-MWSG) and the interaction of the Peptide-α-amino-DOTA complex (Ac-PLG-MWSG-Amino-Ln^{3+}-DOTA) with MMP-2 enzyme will be the focus of a future report.

The peptide was cleaved from the resin with a 95% TFA/ 2.5% water/ 2.5% thioanisol cocktail for 30 minutes.[30] The MMP-2 targeting peptide was obtained as white powder (90%) and characterized with MALDI mass spectrometer (m/z: 811.9[M+Na]$^+$ (calcd. 788.91)[M+H]$^+$.

To show the versatile application of α-amino-DOTA for the coupling to the C-terminus of peptide, the synthesized peptide (120mg, 0.15mmol) was dissolved in NMP (5mL) with 63mg of HBTU and 26mg of HOBt and stirred for 40 min. to activate the carboxyl group of the peptide. The α-amino-DOTA (80mg, 0.15mmol) and TEA (110μl, 0.75mmol) in 1 mL of NMP was added slowly at room temperature. The reaction mixture was stirred for 1hr. After removal of half of the solvents under reduced pressure, the product was precipitated by addition of diethylether (100mL). The obtained crude peptide was purified with a silica column, yielding product as a white solid (71%) and characterized with Maldi-Mass spectrometer (m/z : 1190.3 [M+H]$^+$)

To verify that α-amino-DOTA can serve as a molecular imaging contrast agent, the T_1 relaxivity of α-amino-DOTA-Gd^{3+} was measured to assess the efficiency of the chelate to alter T_1-weighted MR image contrast. A Gd^{3+} chelate was prepared to confirm that the DOTA derivative could tightly bind to lanthanide ions. α-amino-DOTA (25.2mg, 0.06mmol) was dissolved in water at pH 6 and 60°C, and GdCl$_3$ (150ul of 0.4mM solution) was added to this solution and stirred for 1 hr. The pH was adjusted to 8 using 1N NaOH and stirred 30 min. The chelation was monitored using a standard Arsenazo III color test.[31] T_1 measurements were conducted at 18ºC using an inversion-recovery experiment with a 600 MHz NMR spectrometer. As shown in Fig.1, the measured relaxivity of 4.83 mM^{-1} sec^{-1} compared very favorably to the 3.83 mM^{-1} sec^{-1} relaxivity of Gd^{3+}-DOTA that is used in clinical MRI studies.[32]

Also, Tm^{3+} complex was prepared and its para-CEST characteristics were measured according to the change of pH, temperature and concentrations. The results were shown in Fig. 2. The CEST effect was generated from α-amine on the side arm and water, and it was shown at +7ppm, which is very close to the water peak. Because the amine group is located outer space from the core lanthanide (Tm^{3+}) ion and by the neighboring secondary amine and carbonyl groups, the protons could be exchanged very easily with water.

Discussions

This report has demonstrated the preparation and application of α-amino-DOTA, peptide-α-amino-DOTA, and α-amino-DOTA lanthanide chelates. α-amino-DOTA-Gd^{3+} shows T_1 relaxivity (4.83 mM^{-1} sec^{-1}) and it can be applied to the clinical MRI studies. From the para-CEST experiments, the amine group shows very fast water change rate and the CEST effect has increased with the conditions as the decrease of pH, the increase of temperature and the increase of concentrations.

The incorporation of amino group facilitated the conjugation of α-amino-DOTA to the C-terminus of peptide backbone with high efficiency and relatively high yield. Coupling α-amino-DOTA to peptide carboxylates greatly expands peptide-DOTA synthesis strategies by complimenting standard methods that couple DOTA to peptide amines. Further more, the number of amino groups incorporated into DOTA can be controlled from 1 to 4 by the selection of the macrocyclic starting material, such as cyclen (1,4,7,10-tetraazacyclododecane), DO1A-^{t}Bu, DO2A-^{t}Bu or DO3A-^{t}Bu.

Optimization of a combined method for automated peptide-DOTA-peptide syntheses is currently in progress. Because metal DOTA chelates can be detected by multiple biomedical imaging modalities, the combined method for automated peptide-DOTA-peptide syntheses has great commercial value for the molecular imaging diagnostics market.

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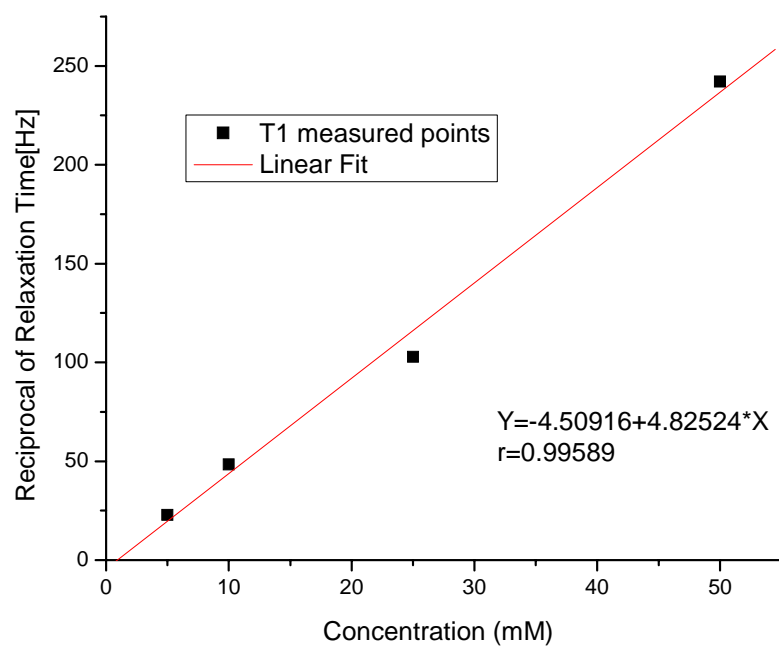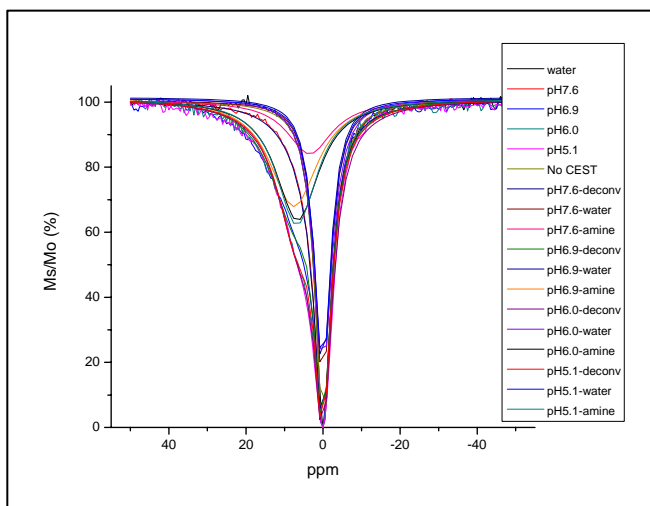
Figure 1. T_1 Relaxivity of AminoDOTA-Gd^{3+}
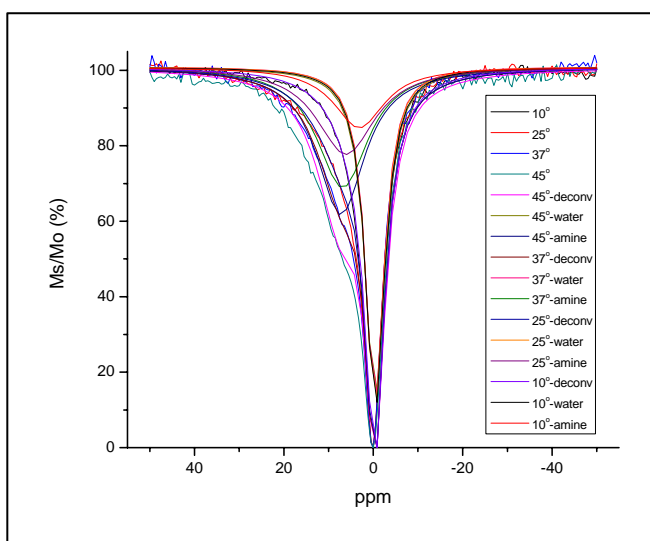

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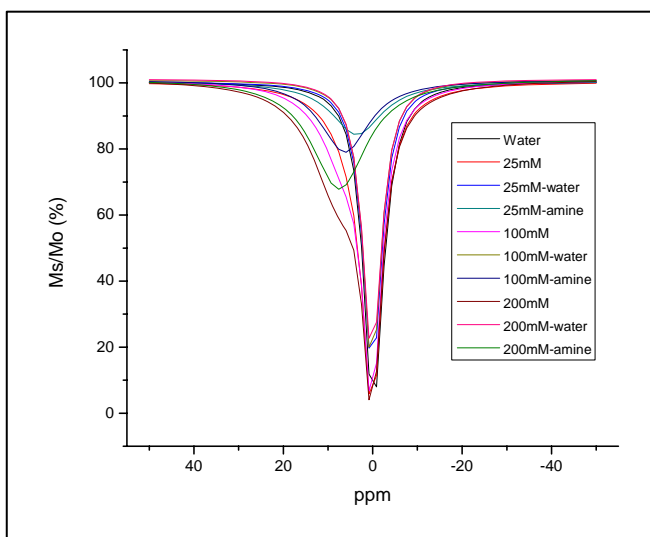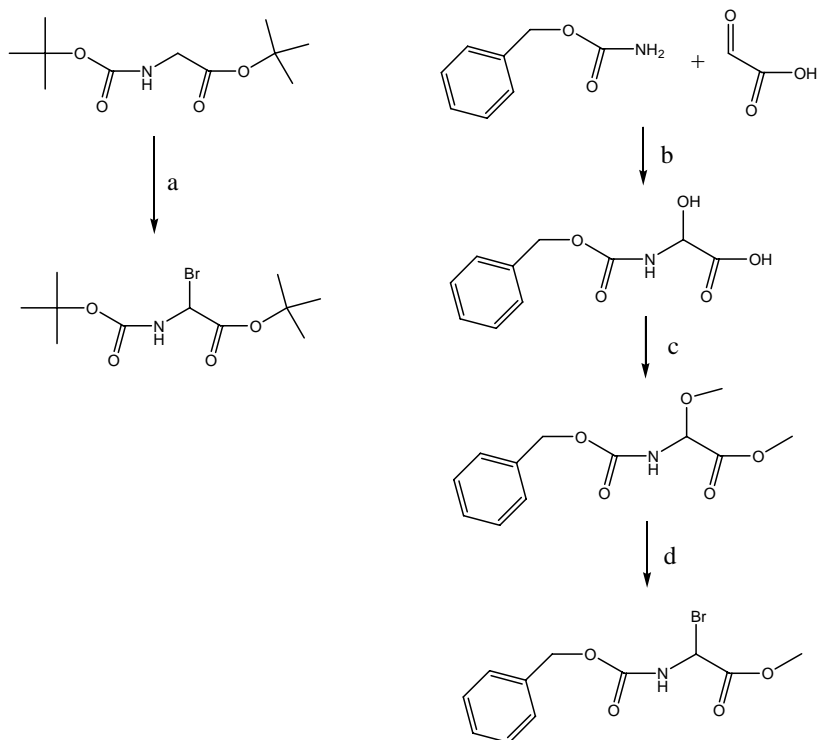r=0.99589
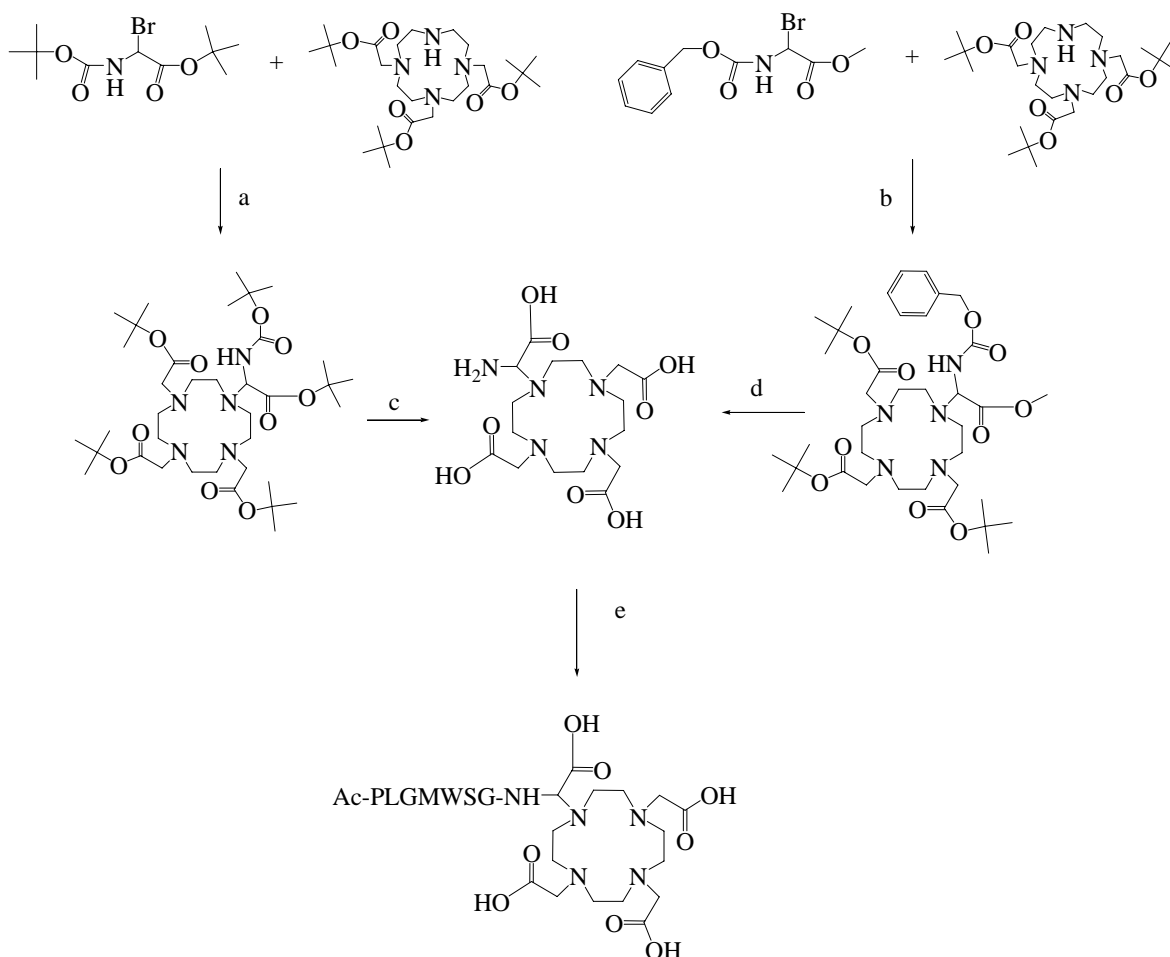
Figure 2. para CEST measurements
a) pH

b) Tempereature

c) Concentration

Scheme 1. Synthesis of α-brominated glycine templates: (a) N-bromosuccinimide, UV (254nm, filtered), CCl4, 25°C, 1.5hrs, 97%; (b) diethylether, rt, 6days, 96%; (c) MeOH, H_2SO_4, rt, 2days, 95%; (d) PBr_3, CCl4, rt, 7days, 75%

Sheme 2. Synthesis of α-amino-DOTA derivatives and coupling to a peptide backbone carboxylate group.

(a) K2CO3 (6eq.), acetonitrile, 70°C, 6hrs, 95%; (b) K2CO3 (6eq.), acetonitrile, 70°C, 6hrs, 90%; (c) 95%TFA/2.5%water/2.5%thioanisol, 30min, 95%; (d) (i)1,4-cyclohexadiene/10% Pd-C, EtOH; (ii) 1N-NaOH; (iii) dialysis (MWCO-100); total yield 85%; (e) (i) Ac-PLGMWSG-OH (1eq.), HBTU (1.1eq), HOBt (1.1eq.), TEA (5eq.), NMP, rt, 1hr; (ii) silica column; 71%

Peptidyl Molecular Imaging Contrast Agents Using a New Solid Phase Peptide Synthesis Approach

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Macrocyclic metal chelates using DOTA (1,4,7,10-tetraazacyclododecane-N-N'-N''-N'''-tetraacetic acid) are often administered to in vivo patients and animal models to create or enhance contrast in biomedical molecular imaging studies.[1-3] More recently, metal-DOTA chelates have been conjugated to peptides to affect the pharmacokinetics of the metal-DOTA imaging agent within in vivo systems, for example, by targeting specific cell surface receptors,[4] penetrating cell membranes peptides,[5] nonspecific interacting peptide with the extracellular matrix,[6] and very large peptide homopolymers that drastically alter renal clearance rates,[7] which can be used to gain detail information about biological processes at the molecular level.

To synthesize these peptidyl molecular imaging contrast agents, the carboxylates of DOTA have been conjugated to the amines of peptides, including the N-terminus of the backbone and the side chain of lysine.[8] Other DOTA derivatives have been devised for conjugation to peptide amino groups, such as succinimide DOTA derivatives[9] and isothiocyanato DOTA derivatives.[10]

Solid phase peptide synthesis (SPPS) methodologies have been applied to introduce molecular imaging contrast agents on the backbone N-terminus of peptides using PEGA Rink amide resin or unnatural amino acid derivatives such as p-NH$_2$-phenylalanine.[11,12] However, coupling DOTA to the backbone N-terminus or amino side chains of a peptide can limit synthesis methodologies[13] and can compromise the utility of the peptidyl contrast agent for molecular imaging applications, since peptide-biomolecule interactions critically depend on the (native) peptide amino acid sequence.

In this study, we investigated a new and versatile SPPS approach with a resin that is pre-loaded with a molecular imaging contrast agent (Scheme 1). DO3A-tBu was synthesized from cyclen and t-butyl bromoacetate following a previously reported method.[14] To protect the secondary amine of DO3A-^{t}Bu, Fmoc-DO3A-tBu **1** was prepared from Fmoc-Cl and DO3A-^{t}Bu with activated Zn dust.[15] Consequently, Fmoc-DO3A-^{t}Bu was treated with 75% TFA in MC for 40 minutes to remove ^{t}Bu esters, yielding Fmoc-DO3A with free carboxylic acids **2**. This product was coupled to a glycine-preloaded Wang resin **3** (amine contents 0.77mmol/g), and the coupling reaction was monitored by Kaiser's test.[16] Although the Wang resin was pre-loaded with only one glycine amino acid residue in this study, comparable results are anticipated if the Wang resin was pre-loaded with a longer peptide, so that subsequent synthesis steps described below would couple the imaging agent to the backbone N-terminis of this peptide. At the end of the coupling reaction, an excess amount of *t*-BuOH was added to convert the remaining activated

Scheme 1. Solid phase synthesis of peptidyl MR contrast agents.
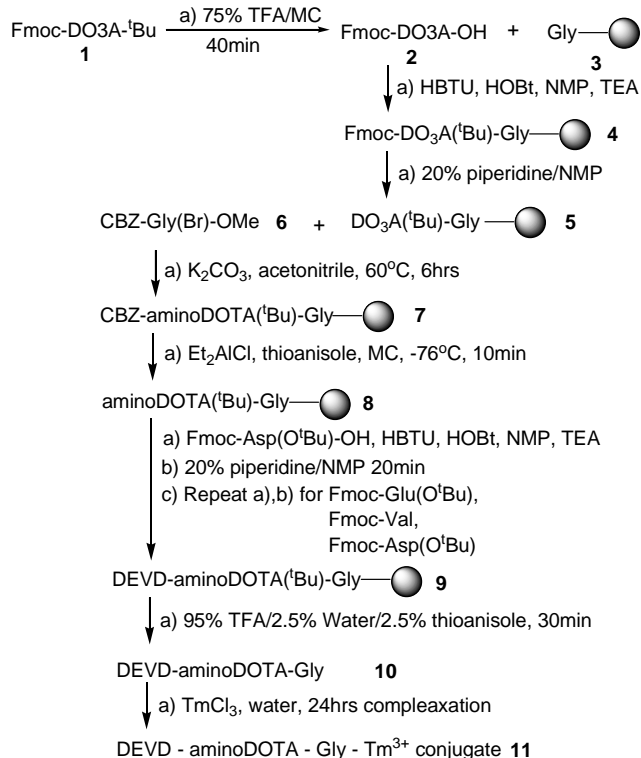
carboxylates to *t*-Bu esters. After washing the resin with NMP, the resin was treated with acetic anhydride to cap the remaining functional reactive sites. To analyze the loading efficiency of Fmoc-DO3A on the resin **4**, the Fmoc group was quantitatively titrated with a UV spectrometer (301nm).[17] The loading efficiency was quantitative during each of 3 trials, and the average amine content on the resin was 0.77($\pm$ 0.05) mmole/g. After the Fmoc group of **4** was removed by 20% piperidine, α-brominated CBZ-Gly-OMe[18] **6** was coupled on the resin, and the reaction was monitored by Kaiser's test. Racemization of the α-carbon of glycine occurred during the first condensation step of benzylcarbamate and glyoxylic acid and continued in the consequent steps. The racemization ratio was measured with NMR spectroscopy to be approximately 50:50. The CBZ group is commonly used to orthogonally protect amine groups during Fmoc SPPS. Cleavage of CBZ groups is typically performed with H$_2$/Pd-C or 1,4-cyclohexadiene/Pd-C in EtOH. However, these cleavage conditions with SPPS resin supports lead to difficult problems with separation and purification.
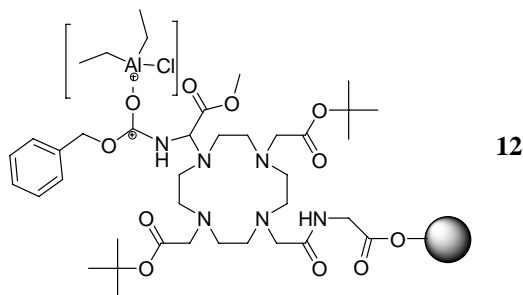
Figure 1. The expected transition state of Et₂AlCl and CBZ-aminoDOTA Wang resin

Figure 2. The CEST spectrum of DEVD-aminoDOTA-Tm^{3+} complex

reactants **7** were varied, and the amine contents on the resin were quantitatively analyzed using picric acid titration and a UV/Vis spectrometer (358nm) to follow the reaction for up to 60 minutes.[20] To eliminate the background measurement of tertiary amines in the cyclen ring, the results of picric acid titration of **7** that was not subjected to Et₂AlCl/thioanisol was subtracted from the measurements of the cleavage reaction. Optimized conditions were determined to consist of a reaction time of 10 minutes at -78ºC, and 1eq. of Et₂AlCl and 2 eq. of thioanisol relative to the CBZ concentration on the resin. These conditions cleaved the CBZ group at quantative yield. These conditions also cleaved an estimated 30% of the benzyl ether group of the Wang linker, assuming pseudo-homomolecular second order kinetics. Although further improvements to reduce cleavage of the Wang linker are under investigation, these conditions provided an acceptable yield for subsequent steps.[19]

Conventional Fmoc SPPS methods were then employed to perform step-wise coupling of amino acids to the aminoDOTA Wang resin.[21] An Asp-Glu-Val-Asp (DEVD) peptide sequence was chosen for this demonstration, because this peptide sequence is preferentially cleaved by the caspase-3 enzyme that is a focus of our molecular imaging research.[22] The total yield of step-wise SPPS of four amino acids to amino-DOTA was 93%, and therefore the coupling efficiency for coupling the first amino acid to amino-DOTA is no lower than 93%. The DEVD-aminoDOTA conjugate was cleaved from the resin **9** with a 95% TFA / 2.5% water / 2.5% thioanisol cocktail for 30 minutes. The obtained DEVD-aminoDOTA **10** was characterized with MALDI-MASS (m/z 949.40[M+H]). To generate a molecular imaging contrast agent, DEVD-aminoDOTA-Tm^{3+} **11** was generated using standard conjugation methods and an Arsenazo III color test. The obtained final product **11** was characterized with MALDI-MASS (m/z 1140.92[M+Na]$^+$).

To demonstrate the application of the final product for molecular imaging, the DEVD-aminoDOTA-Tm^{3+} complex **11** was tested for the PARAmagnetic Chemical Exchange Saturation Transfer (PARACEST) effect (Fig 2).[23] Selective radio frequency irradiation at -51ppm caused a saturation of the amide proton proximal to Tm^{3+} in **11**, and subsequent chemical exchange of this proton with water transferred the saturation to the water, which decreased the MR water signal. This PARACEST effect of **11** occurs at the same chemical shift frequency reported for the PARACEST effect of a similar compound, DOTAMGly-Tm^{3+}.[24] In addition, the PARACEST effect of 11 shows good sensitivity at physiological pH and temperature, indicating that this contrast agent can be used for in vivo molecular imaging.

To summarize, a new SPPS approach has been developed to synthesize peptidyl contrast agents for molecular imaging. Also, the selective cleavage of CBZ protecting groups in SPPS was investigated and optimized for the macrocyclic ligand loaded resin. This scheme provides a convenient and robust method for
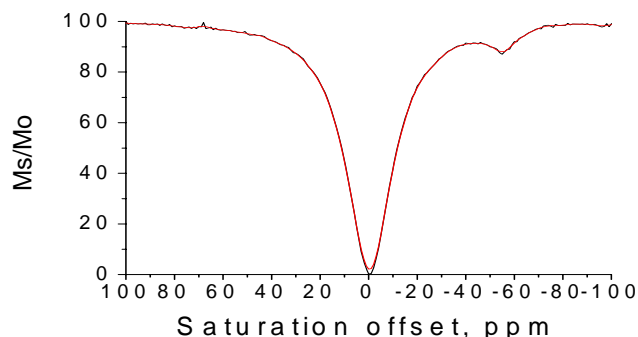
synthesizing peptidyl molecular imaging agents, which can be used to develop individual molecular imaging agents at high yield and/or libraries of molecular imaging agents. Because DOTA macrocyclic rings can strongly chelate a variety of ions, this peptide-aminoDOTA product can be used to develop molecular imaging contrast agents for PARACEST MRI, relaxivity-based MRI (using Gd^{3+} or Dy^{3+}), for nuclear imaging (using ^{99}Tc, ^{111}In), or fluorescence imaging (using Eu^{3+}), which provides a platform technology for molecular imaging.

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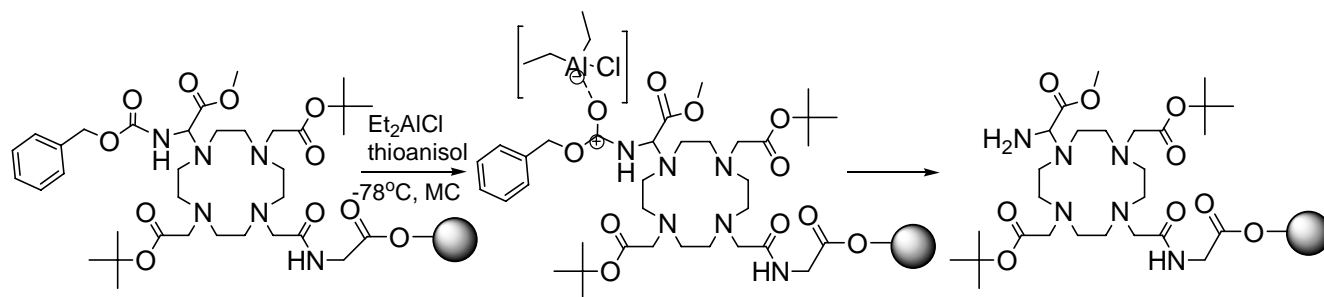
A versatile method is disclosed for solid phase peptide synthesis (SPPS) of molecular imaging contrast agents. A DO3A moiety was derivatized to introduce a CBZ-protected amino group and then coupled to a polymeric support. CBZ cleavage with Et$_2$AlCl/thioanisol was optimized for SPPS. Amino acids were then coupled to the aminoDOTA-resin using conventional step-wise Fmoc SPPS. The peptide-amino-DOTA-Tm^{3+} product showed a PARACEST effect, which demonstrated the utility of this contrast agent for molecular imaging.

Supporting Information

**Peptidyl Molecular Imaging Contrast Agents
Using a New Solid Phase Peptide Synthesis Approach**

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Index

1. General Methods

All the reactions were carried out under argon atmosphere. Dichloromethane and carbon tetrachloride were freshly distilled over P_2O_5. Acetonitrile was distilled over BaO. Fmoc-protected amino acids, acetic anhydride, HBTU (O-Benzotriazol-1-yl-N,N,N'N'-tetramethyluronium hexafluorophosphate), HOBt (1-Hydroxybenzotri azole hydrate), piperidine, DIEA (N,N-diisopropylethylamine) and solvents for peptide synthesis were purchased from Applied Biosystems Co. The DO3A-tBu was purchased from Macrocyclics Co., and other reagents were purchased from Aldrich and Fisher Scientific. Peptides were synthesized using Wang resin (Fluka) and followed Fmoc-chemistry with HBTU and HOBt as coupling agents. The coupling efficiency of each amino acid residues were checked by Kaiser's ninhydrin test. The starting resin has 1.0mmol/g of OH groups and calculated the scale of peptide synthesis based on the substitution ratio of the resin. The loading efficiency of Fmoc-coumpounds and the amine contents of the resin were analyzed quantitatively by UV/Vis/Fluorescence spectrometer (Molecular Devices SpectraMax M2). To analyze the functional groups on the resin, IR (ABB BOMEM Inc., MB-104) was used. To analyze the peptides and Peptidyl-aminoDOTA, final product, HPLC (PerkinElmer Series 200 Pump and UV detector) and was used with C-18 OD-300 reverse phase analytical column. The lanthanide complexation reaction between peptidyl aminoDOTA and $TmCl_3$ was evaluated with Arsenazo III solution color test. ^1H and ^{13}C and paraCEST spectra were measured on a Varian Gemini 300MHz NMR and 600MHz NMR using $CDCl_3$ and DMSO-d_6 as solvents depending on the solubility. Analytical thin layer chromatography was performed on Merck silica gel 60 F254 plates. Compounds were visualized using UV lamp(254nm), iodine chamber and ninhydrin solution. High resolution mass spectral analyses were performed with Mass Spectrometer (Bruker Daltonics Esquire HCT) and MALDI-MASS spectrometer (Bruker BIFLEX III MALDI-TOF).

2. Synthesis & Analysis

N-(Benzyloxycarbonyl)-α-hydroxyglycine (CBZ-Gly(OH)-OH)[1]

Benzylcarbamate (15.1g, 0.1mol) and glyoxylic acid monohydrate (9.2g, 0.1mol) were stirred together for 6 days in dry diethylether (250ml). At first it looks like suspension, within 6 hs, the solution become clear and after 2 days the product was precipitated as white solid. The product was filtered and used crude for further reactions. White solid (21.5g, quantitative yield) : ^1H NMR (300MHz, $CDCl_3$) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H) ; ^{13}C (125MHz, DMSO-d_6) δ 172.77, 156.17, 137.49, 129.03, 128.54, 128.50, 87.42, 66.17; MS-ESI m/z : 223.93(calcd. 225.06) [M-H]$^-$

Methyl N-(Benzyloxycarbonyl)-α-methoxyglycinate (CBZ-Gly(OMe)-OMe)

To an ice-cooled solution of N-(Benzyloxycarbonyl)-α-hydroxyglycine (6.0g, 26.6mmol) in anhydrous methanol (100ml) was added concentrated sulfuric acid (1.0ml). The reaction was allowed to warm to room temperature and stirred for 48hrs. At this point the reaction was quenched by being poured into ice-saturated $NaHCO_3$ (saturated aqueous, 200ml). The product was extracted into EtOAc (4 X 100ml) and the organic phase was dried over anhydrous $MgSO_4$, filtered and concentrated in vacuo, yielding product as a powdery white solid (yield 95%). The product was obtained with quantatative yield and used without further purification : ^1H NMR (300MHz, $CDCl_3$) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H) ; ^{13}C (125MHz, DMSO-d_6) δ 168.60, 156.55, 137.23, 129.07, 128.64, 128.55, 81.03, 66.56, 55.37, 52.85; MS-ESI m/z : 256.21(calcd. 253.10) [M+H]$^+$

Methyl N-(Benzyloxycarbonyl)-α-bromoglycinate (CBZ-Gly(Br)-OMe)

To a suspension of methyl N-(Benzyloycarbonyl)-α-methoxyglycinate (2.51g, 10mmol) in carbon tetrachloride (100ml), under an argon atmosphere, was added phosphorous tribromide (8.2g, 30mmol, 3eq.). The reaction mixture was stirred at room temperature for 7 days. At this point the reaction solution was concentrated in vacuo and triturated with dry n-hexane (100ml) for 24 hrs. The reaction mixture was then filtered, yielding a white solid (yield > 75%) : ^{1}H NMR (300MHz, CDCl$_3$) δ 1.45(s, 9H), 1.50(s, 9H), 5.90(d, 1H), 6.20(d, 1H) ; ^{13}C (125MHz, DMSO-d$_6$) δ 170.71, 156.07, 137.37, 129.03, 128.57, 128.53, 73.78, 66.25; MS-ESI *m/z* : 304.21 (calcd. 302.12) [M+H]$^+$

Fmoc-DO3A-tBu[2)]

To a solution of DO3A-tBu(2.57g, 5mmol) in 10mL of acetonitrile, activated zinc dust was added in small portions until the reaction mixture attained neutral pH. The solution of Fmoc-Cl (1.35g, 5mmol, 1eq.) in 5mL of acetonitrile and zinc dust (325mg, 5mmol, 1eq.) was added to the reaction mixture in one portion and the reaction mixture was stirred at room temperature for about 20 min. The progress of reaction was monitored by TLC(CHCl$_3$/MeOH=1.1, Rf=0.75). The reaction mixture was filtered and dried in vacuo, yielding 3.32g of white solid (yield 90%) : ^{1}H NMR (600MHz, DMSO-d$_6$) δ 1.47(s, 27H), 2.50(s, 8H, overlapped with solvent), 2.71(t, 4H), 2.79(t, 4H), 3.39(s, 6H), 5.15(t, 1H), 6.29(d, 2H), 7.42(t, 2H), 7.47(t, 2H), 7.63(d,2H), 7.84(d, 2H) ; ^{13}C (125MHz, DMSO-d$_6$) δ 170.61, 153.83, 135.59, 135.36, 129.47, 127.23, 123.88, 121.36, 119.99, 81.22, 65.88, 55.14, 53.53, 50.48, 48.44, 47.10, 27.75; MALDI-Mass *m/z* : 738.96 (calcd. 736.94) [M+H]$^+$

Fmoc-DO3A

2.94g (4mmol)of Fmoc-DO$_3$A-tBu was dissolved in 5 ml of 75% TFA in MC and treated for 40 min. The reaction was traced by TLC(CHCl$_3$/MeOH=1.1, Rf=0.22). The solution was dried under reduced pressure. the remained solid was re-dissolved in MC and precipitated with diethyl ether. The precipited solid was filtered and dried in vacuo, yielding 1.70g (3.0mmol, yield 75%), and it was TFA salt complex form because the measured chemical shift was shown up field then expected. : ^{1}H NMR (600MHz, DMSO-d$_6$) δ 3.05(s, 4H), 3.20(s, 4H), 3.45(t, 4H), 3.62(t, 4H), 3.60(s, 6H), 4.25(t, 1H), 4.55(d, 2H), 7.35(t, 2H), 7.42(t, 2H), 7.65(d,2H), 7.94(d, 2H) ; ^{13}C (125MHz, DMSO-d$_6$) δ 174.21, 155.44, 143.85, 140.63, 127.08, 125.07, 120.01, 67.24, 54.30, 51.88, 50.65, 48.68, 45.79; MALDI-Mass *m/z* : 570.64 (calcd. 568.62) [M+H]$^+$

Coupling of Fmoc-DO3A on the resin

1.3g of Fmoc-Gly preloaded Wang resin was treated with 20% piperidine in NMP for 20 min and washed with NMP, acetone, MC and NMP sequentially and immediately after the washing, the resin was used for peptide synthesis (amine contents 1mmol). Fmoc-DO3A (1.14g, 2mmol), HBTU (2.5g, 6.6mmol, 3.3eq.) and HOBt (1.0g, 6.6mmol, 3.3eq.) were dissolved for 40 min to activate carboxylates of Fmoc-DO3A and the solution was added to the peptide reaction vessel containing the resin. TEA (1.82mL, 13eq.) was added and the reaction continued for 4hrs. During the reaction, a small amount of resin was sampled for the Kaiser's test. After filter the resin, the resin was dispersed in the solution of tBuOH (1.48g, 20mmol, 20eq.) in 20ml of NMP and continued the reaction for 1hrs. The resin was washed and dried in vacuo, yielding 1.63g of resin (yield 95% by weight)

Loading Efficiency (Fmoc titration) [3]

Fmoc concentration on the resin was measured according to the previous reported method.

Fmoc amino acyl resins (4~8mg) were shaken or stirred in piperidine-DMF (3:7) (0.5mL) for 30 min, following which MeOH (6.5mL) is added and the resin is allowed to settle. The resultant fulvene-piperidine adduct has UV absorption maxima at 267nm ($\varepsilon=17,500M^{-1}cm^{-1}$), 290nm ($\varepsilon=5800M^{-1}cm^{-1}$), and 301nm ($\varepsilon=7800M^{-1}cm^{-1}$). For reference, a piperidine-DMF-MeOH solution (0.3:0.7:39) is prepared. Spectrophotometric analysis is carried out at 301nm, with comparison to a free Fmoc amino acid (Fmoc-Ala) of known concentration treated under identical conditions.

Sub. Level = Abs. $\times 10^6 \mu$mol/mol $\times 0.007$L $\times 7800 \times 1$cm $\times$mg of resin

The measured Fmoc concentration was 0.47mmol/g of resin (calcd. 0.62mmol/g)

Coupling of CBZ-Gly(Br)-OMe on the resin

Fmoc-DO3A loaded resin (1g, Sub. Level 0.47mmol/g) was treated with 20% piperidine for 30 min and washed with NMP, MC, acetone, MC and acetonitrile sequentially. The resin was transferred to the flask with CBZ-Gly(Br)-OMe (0.6g, 2mmol, 4.3eq.) and K_2CO_3 (1.66g, 12mmol, 25.5eq.) in dry acetonitrile (100ml). The solution was stirred and heated 70°C for 6hrs. After filter out the solution, the resin was washed with MeOH/Water, water, MeOH, MC sequentially and dried in vacuo, yielding 0.97g (97% by weight)

Cleavage of CBZ on the resin [4]

In 3 flasks, 0.20g (0.1mmol scale) of resin was dispersed and swelled in MC (5ml) for 1hr and cooled down to -78°C. Et_2AlCl was transferred by 1, 2 and 5 eq. with air-tight syringe and stayed for 15min. 2, 4, and 10eq. of thioanisol (the molar ratio of Et_2AlCl/thioanisol (1/2) was fixed) was added in each flasks and sample the resin according to pre-scheduled time intervals (5, 15, 30 and 60 min). The sampled resin was washed with MC and prepared for the picric acid titration. The picric acid titration was carried out according to the previous reported method.[5] To prevent the influence of cyclen secondary amines, the CBZ protected resin was treated and compared as a reference.

	1eq.	2eq.	5eq.
5	0.60	0.55	0.32
15	0.46	0.26	0.07
30	0.32	0.09	-
60	0.11	-	-

* The amine contents on the resin were measured slightly larger than the Sub.Level of Fmoc groups.

IR spectra

CBZ cleaved aminoDOTA-Wang resin was analyzed with FT-IR to confirm the introduction of functional groups. 3440cm^{-1} (cyclen ring N-H, str), 3014cm^{-1} (aromatic C-H, str), 2917cm^{-1} (alkyl C-H, str), 1743cm^{-1} (tBu ester C=O, str), 1677cm^{-1} (amide C=O, str), 1601cm^{-1} (aromatic C=C, str), 1450cm^{-1} (C-H, sci, ben), 1306cm^{-1} (cyclen ring C-N, str), 1176cm^{-1} (C-O, str).

Peptide synthesis and HPLC analysis[6]

0.3g of resin (0.14mmol scale) was used for the solid phase peptide synthesis, the peptide sequence was Asp-Glu-Val-Asp (DEVD) and Fmoc-Asp(OtBu)-OH (0.2g, 0.5mmol), Fmoc-Val-OH (0.17g, 0.5mmol) and Fmoc-Glu(OtBu)-OH (0.23g, 0.5mmol) were used as building amino acids and HBTU (0.19g, 0.5mmol) and HOBt (73mg, 0.5mmol) were used for the coupling agents. Freshly distilled TEA (140μL, 1mmol) was used as a base and the solvent was NMP. Each amino acid was reacted twice and 20% piperidine in NMP was used to cleave Fmoc group after one amino acid coupling. Each amino acids were coupled twice at each step.

After the peptide synthesis, Fmoc goup of peptide was removed and the peptide-aminoDOTA was cleaved form the resin with a 95% TFA/2.5% water/2.5% thioanisol cocktail for 30 minutes. After removing solvents, it was washed with diethylether and dried in vauo, yielding 115mg (0.12mmol, yield 93%).

The obtained product was characterized by MALDI-MASS (*m/z* : 949.40 (calcd. 948.93) [M+H]$^+$) and HPLC (Column : OD300 RP-18 column, Detector : 222nm, Flow rate : 1.0mL/min, Eluent : 0.1% TFA in water / Acetonitrile (gradient from 85%/15% to 5%/95% for 30 min, Retention time : 2.9min).

Complexation of Tm^{3+}

The obtained peptide-aminoDOTA (100mg, 0.11mmol) was dissolved in water (3mL) at pH 8, 60°C and TmCl$_3$ (27mg, 0.1mmol) in water (0.5mL) was added drop by drop for 1 hr and adjusted pH to 8 with 0.5N NaOH solution. The solution stirred for 18 hrs at 60°C and adjusted pH 8 when pH drops. The complete complexation was evaluated with Arsenazo III color test. When the test showed negative, the reaction mixture was cooled down to room temperature. pH was adjusted to 9 and filter white precipitates. The solution was freeze dried, yielding quantitative product : MALDI-Mass *m/z* : 1140.92 (calcd. 1117.86) [M+Na]$^+$

CEST Spectrum (Z-spectrum)

Conditions : Concentration 150mM, pH=8.0, Saturation power = 37, Saturation delay = 4, Spectral width=200ppm (+100 ~ -100ppm), temperature 37°C,
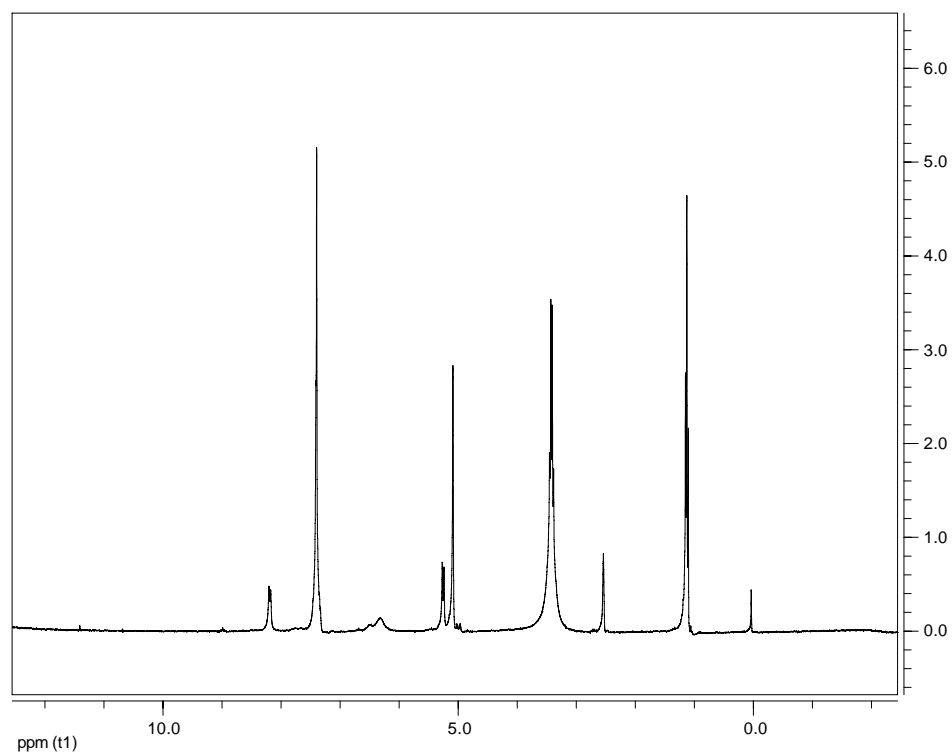
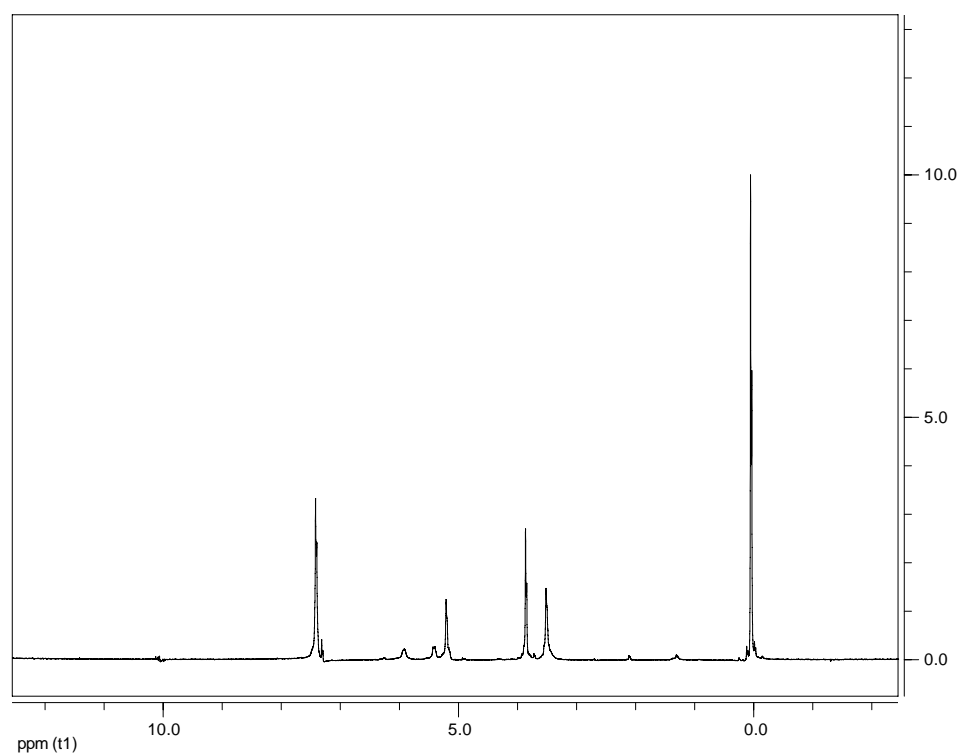**Figure 1**. ^{1}H-NMR Spectrum of Cbz-Gly(OH)-OH
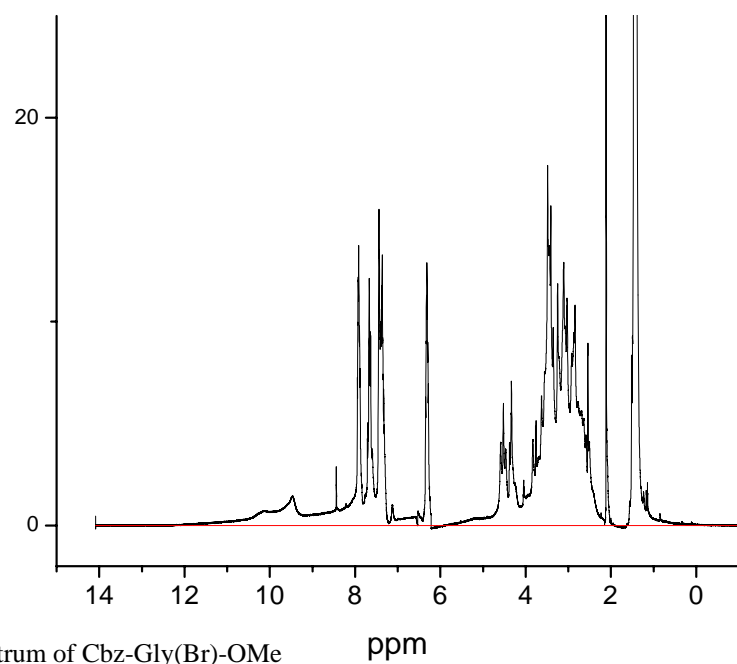

Figure 2. ^{1}H-NMR Spectrum of Cbz-Gly(OMe)-OMe

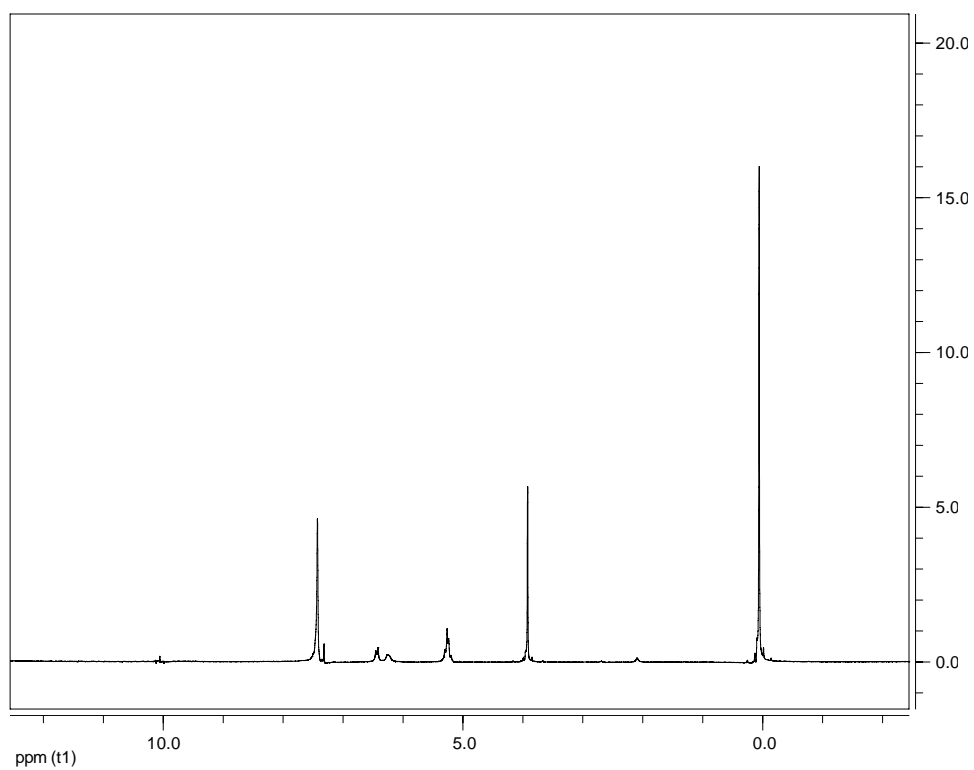**Figure 3.** ^{1}H-NMR Spectrum of Cbz-Gly(Br)-OMe

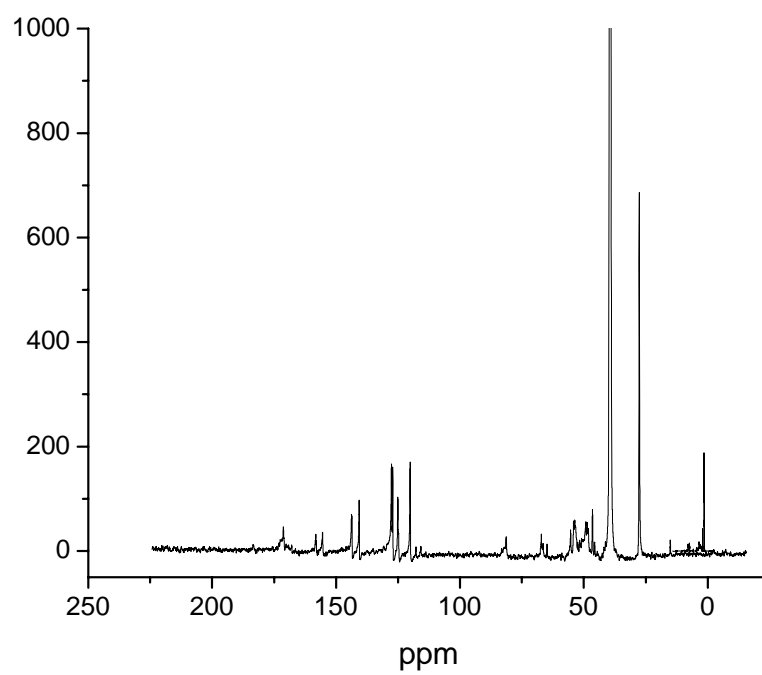**Figure 4.** ^{1}H-NMR Spectrum of Fmoc-DO3A-tBu

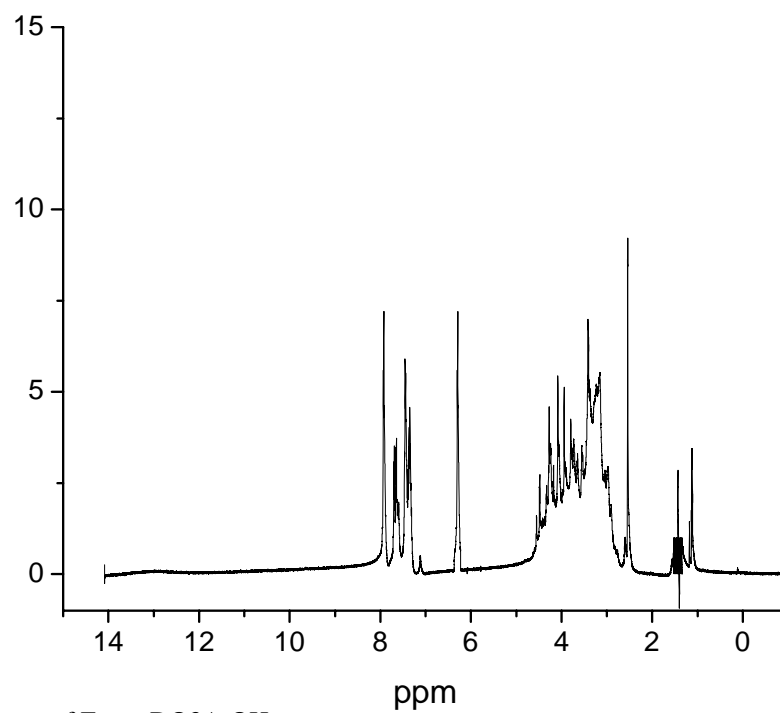***Figure 5***. ^{13}C-NMR Spectrum of Fmoc-DO3A-tBu
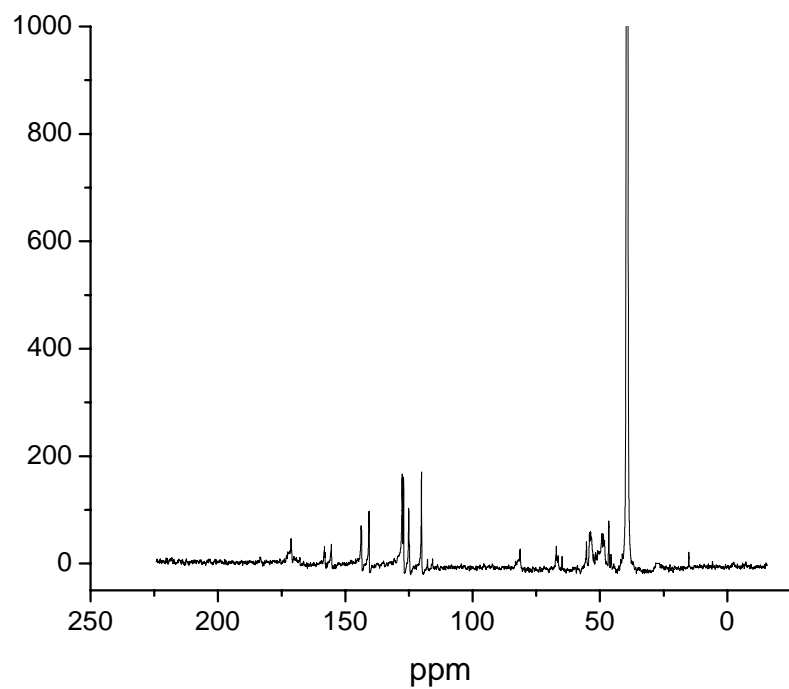

Figure 6. ^{1}H- NMR Spectrum of Fmoc-DO3A-OH

Figure 7. ^{13}C-NMR Spectrum of Fmoc-DO3A-OH
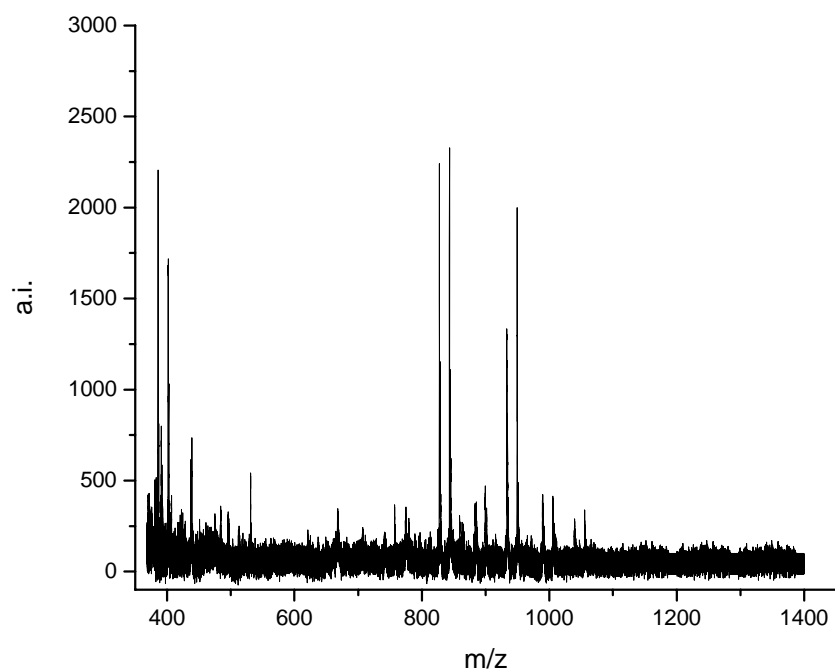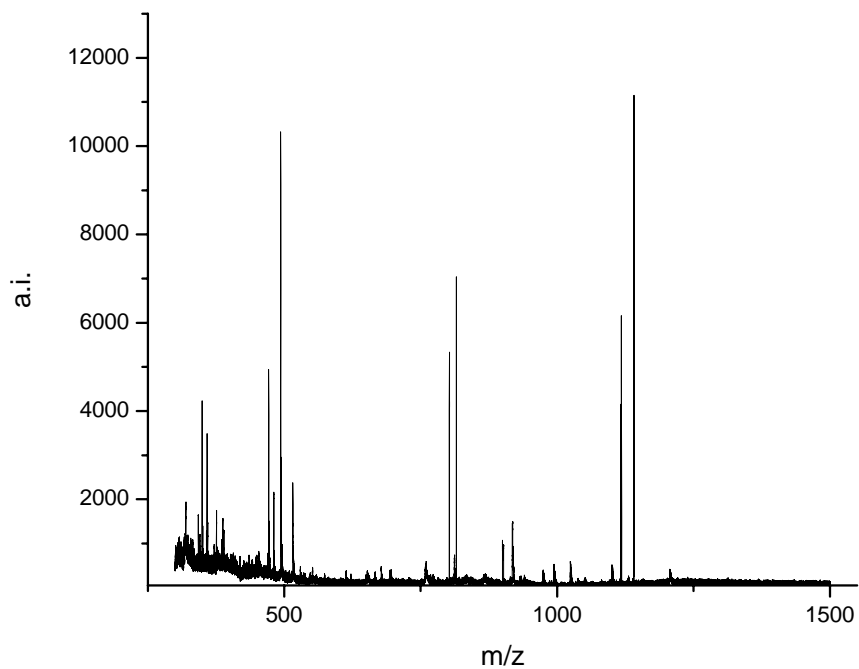

Figure 8. MALDI-MASS Spectrum of DEVD-aminoDOTA.
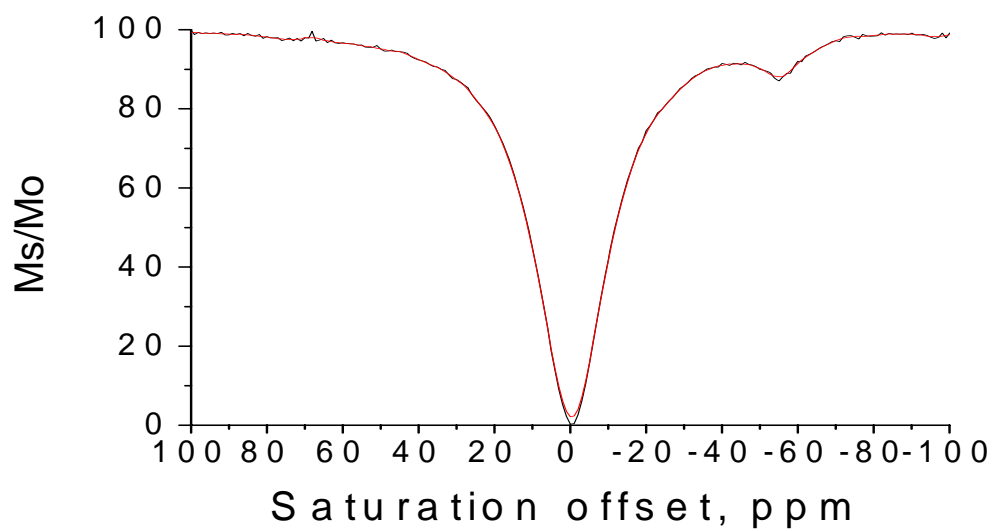
Figure 9. MALDI-MASS Spectrum of DEVD-aminoDOTA-Tm^{3+} complex.

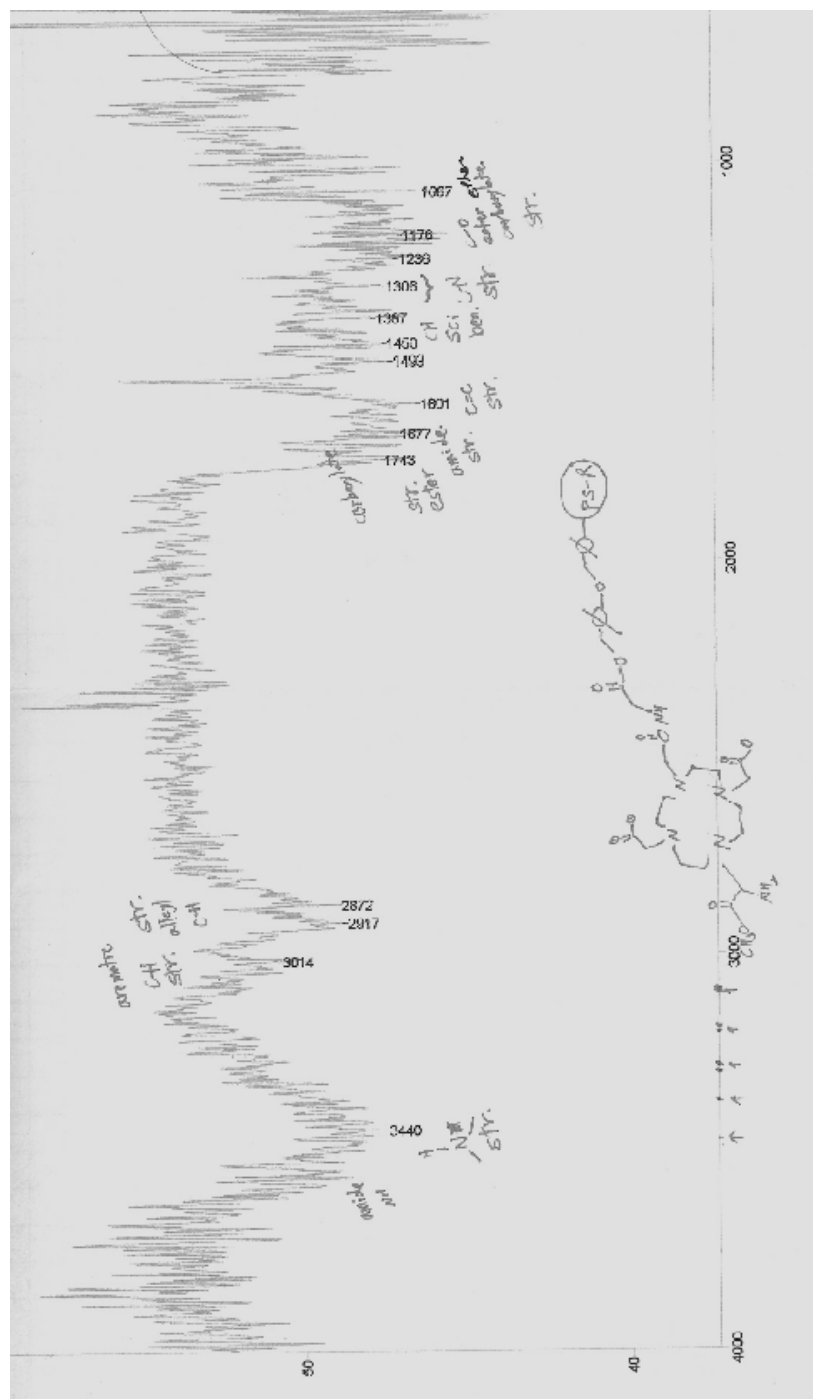**Figure 10.** The CEST spectra of DEVD-aminoDOTA-Tm^{3+} complex

Figure 11. IR spectrum of aminoDOTA-Wang Resin

29

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A "Smart" PARACEST MRI Agent to Detect Matrix Metalloproteinase Enzymes

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Introduction

Matrix metalloproteinases MMP-2 and MMP-9 are biomarkers of metastatic breast cancer.[1,2] We have developed fundamentally new types of molecular imaging agents that detect MMP-2 and MMP-9 through the mechanism of PARAmagnetic Chemical Exchange Saturation Transfer (PARACEST). These "smart" imaging agents incorporate peptides that are specifically cleaved by MMP-2 and MMP-9 to produce a peptide with fewer amide functional groups that are detected by MRI. Unlike standard MRI agents that are indistinguishable, the unique MR frequencies of these amino groups are exploited to detect the presence of each specific MMP enzyme within the same scan session, which can be useful for assessing the stage of breast cancer metastasis. Furthermore, these "smart" agents can only detect the active form of MMPs, which is critical for properly assessing metastatic breast cancer tumors. The detection sensitivity of the agents improves with increasing MRI magnetic field strength, so that these "smart" agents are poised to take advantage of high-field MRI scanners used for small animal research and clinical applications. Still, the detection sensitivity of PARACEST agents is modest, in the 10-50 mM range for these agents, and therefore additional improvements are needed before translation to in vivo studies. Yet these results demonstrate the feasibility of using these novel molecular imaging agents for the non-invasive assessment of MMPs and other breast cancer biomarkers.

Chemical Synthesis

CEST-MMP-2 and CEST-MMP-9 consist of a peptide coupled to DO3A (Figure 1). Steps 1-3 synthesize protected Bromo-α-amino-Glycine with quantitative yield. Step 4 adds the protected Glycine to DO3A (Macrocyclics, Inc., Dallas TX) with 85% yield. Peptides are coupled to the Glycine amine at >75% yield in solution using two couplings at a 4:1 ratio of peptide to DO3A-α-amino-Glycine. CEST-MMP-2 consists of DO3A coupled to the peptide sequence VPLS-LYSG, and CEST-MMP-9 couples DO3A to VPLS-LTMG (cleavage site indicated by the dash).

PARAmagnetic Chemical Exchange Saturation Transfer

MRI contrast agents based on the PARAmagnetic Chemical Exchange Saturation Transfer effect (PARACEST) form a new class of diagnostic agents.[3,4] PARACEST agents contain exchangeable hydrogens with shifted magnetic resonance frequencies due to their proximity to a lanthanide ion (Figure 2A). MR saturation methods can be applied to "scramble" the coherent magnetic signal of these hydrogens. Rapid exchange between the agent's hydrogens and water hydrogens effectively transfers the saturation to the water, causing a decrease in the water MR signal (Figure 2B). Continuous saturation and exchange enhances the sensitivity of the CEST effect relative to MR spectroscopy.

We have developed "smart" or activatable PARACEST agents that show a PARACEST effect from amide functional groups before an enzyme cleaves the agent's peptidyl ligand (Figure 3). Fewer amide functional groups remain after cleavage, which causes a decrease in the PARACEST effect. However, the number of amide functional groups decreases from 8 to 4, which only produces a modest change in the PARACEST effect: 10-50 mM of contrast agent is required to develop a 5% change in the PARACEST effect, depending on temperature and pH.

Molecular Modeling indicates that the 4-residue peptidyl ligand remaining after cleavage has the conformational flexibility to position at least some amide groups near the lanthanide ion (Figure 4). The PARACEST spectrum also indicates that the PARACEST effects from the amide

groups of two different agents can be distinguished, so that the reactants of two cleavage reactions by two different proteases can be monitored during a single MR experiment.

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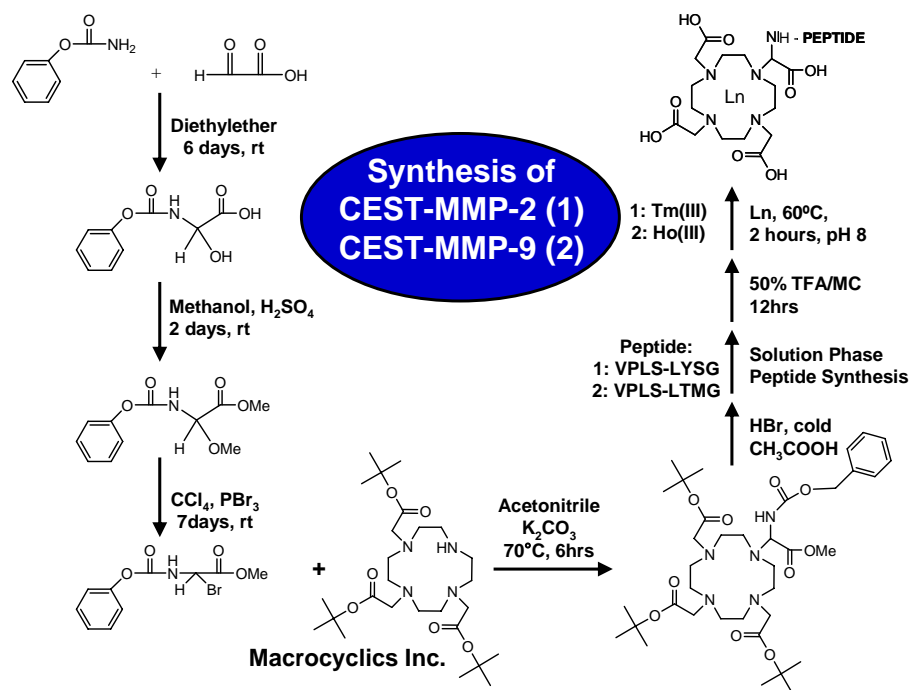9. Coussens LM, Fingleton B, Matrisian LM, Science, 2002, 295:2387-2392.

Figure 1: **Synthesis of CEST-MMP-2** (**1**) CEST-MMP-9 (**2**). The first 4 steps synthesize protected DO3A-α-amino-Glycine. Peptides were synthesized using solid-phase synthesis techniques, and were coupled to the t-Boc-deprotected DO3A-α-amino-Glycine in solution.
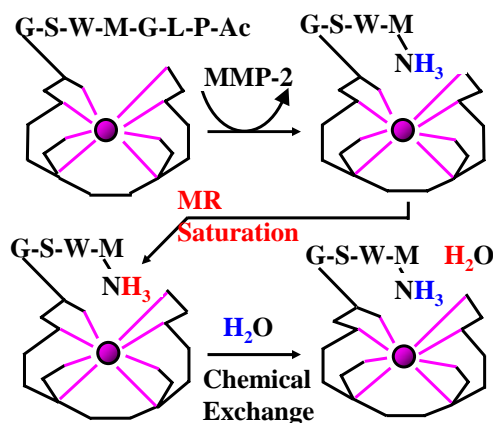

Figure 2: Mechanism of "Smart" CEST agents. Steps 2 & 3 constitute the standard CEST mechanism. Step 1 generates a new amino peptidyl terminus that can be selectively detected via CEST. This amino peptide terminus is created only after an enzyme cleaves the peptidyl ligand, so that the CEST effect from the amino group is detected only if the active enzyme is present.
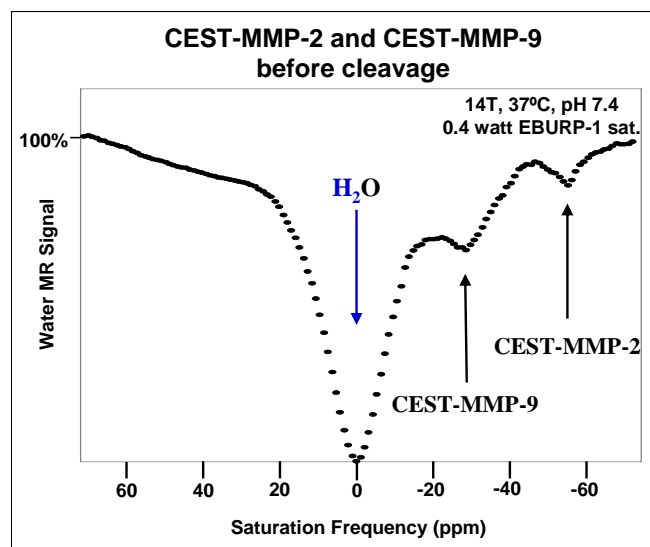
Figure 3: CEST Spectrum of CEST-MMP-2 and CEST-MMP-9 before cleavage by MMP-2 and MP-9. Initial concentrations of CEST-MMP-2 and CEST-MMP-9 were 10 mM in 50 mM HEPES, 10 mM CaCl2, pH 7.5. 1.0 Unit of each enzyme was added to cleave the peptidyl ligands. After establishing equilibrium, the spectrum was acquired at 14T, 37ºC, with a 0.4 watt EBURP-1 saturation pulse. CEST peaks were identified through comparison with CEST spectra of each pre-cleaved agent, and each separately cleaved agent.
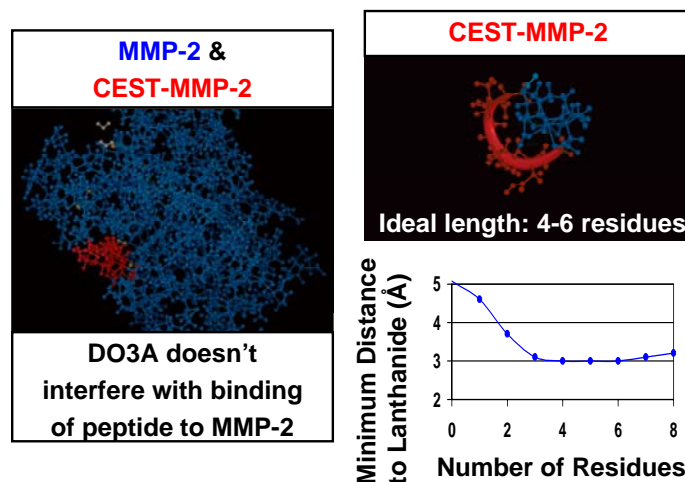

Figure 4: Molecular Modeling of CEST-MMP-2 with Discover / InsightII (Molecular Simulations, Inc). Simular results were obtained with CEST-MMP-9 and the PDB structure file for MMP-9. A) The model of CEST-MMP-2 and MMP-2 was generated by overlaying the peptidyl ligand onto the peptide substrate included in the PDB structure file for MMP-2, removing the peptide substrate, and then minimizing the energy of CEST-MMP-2 and MMP-2 residues within 10 Å of CEST-MMP-2. The final conformation has no steric interactions between CEST-MMP-2 and MMP-2. B) The model of CEST-MPP-2 was generated through 1000 iterations of simulated annealing. The average conformation was determined using the lower quartile of energy-ranked structures. C) Graph of the average minimum distance between the amino terminus and lanthanide ion for the lower quartile of energy ranked structures. The size of each dot approximates the standard deviations.

34

A "Smart" PARACEST MRI Contrast Agent to Detect Enzyme Activity

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Many recent reports have demonstrated the advantages of PARAmagnetic Chemical Exchange Saturation Transfer (PARACEST)[1] for providing molecular-scale information using Magnetic Resonance Imaging (MRI). Measurements of tissue pH,[2] temperature,[3] glucose concentrations[4] and metabolite levels[5] have been accomplished by detecting the PARACEST effect of exogenous agents that chelate lanthanide ions. However, the modest sensitivity of PARACEST agents, often requiring a minimum concentration of 1-5 mM for adequate detection, has limited the applicability of this approach to detect endogenous molecular targets that only exist at high concentrations within tissues.[6]

To address this limitation, we hypothesized that enzymatic catalysis may be exploited to change the chemical structure of a high concentration of PARACEST agents and cause a detectable change in the PARACEST effect, in order to indirectly detect a relatively low concentration of the enzyme. For example, enzymatic conversion of an amide to an amine will accelerate the chemical exchange rate between the agent and water from ~300 sec^{-1} to ~3000 sec^{-1}.[7] More importantly, the MR chemical shift frequency of the amide and amine will be significantly different, especially if these functional groups are proximal to a paramagnetic lanthanide ion. This chemical shift change may be especially advantageous for detection, because MR methods are very sensitive to changes in MR frequencies. This type of chemical agent is often termed as "smart", because the chemical agent alertly responds to its molecular environment.[8]

To demonstrate our approach, we have designed a "smart" PARACEST MRI contrast agent that can detect caspase-3. This enzyme is known as an "executioner" in the metabolic death cascade during cell apoptosis, and therefore serves as a critical early biomarker for evaluating apoptosis-promoting anti-tumor therapies.[9] Among the identified substrates of caspase-3, DEVD (Asp-Glu-Val-Asp) is efficiently and selectively cleaved by caspase-3, and has been incorporated in fluorescence dyes for detecting caspase-3 such as DEVD-AMC.[10,11] Our "smart" PARACEST MRI agent uses a similar motif by replacing AMC with DOTA (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid).

To synthesize DEVD-DOTA amide **4**, a polymer support pre-loaded with a DOTA derivative **2** was developed by surface modification of Wang resin **1**. Standard Fmoc solid phase peptide synthesis methods were then used to "grow" the DEVD peptide chain onto the amino group of **2**. Following the synthesis, the acquired compound **4** was characterized with MALDI-MASS (m/z 885.80[M+H]). Thullium was chelated with **4** to prepare the final compound **5** (m/z 1088.74[M+Na]$^+$).

To show the practical application of **5** to detect the activity of caspase-3, the PARACEST spectrum of **5** was recorded by applying selective saturation in 1 ppm increments from +100 ppm to -100

Scheme 1. Synthesis of DEVD-(Tm-DOTA) using Fmoc chemistry.
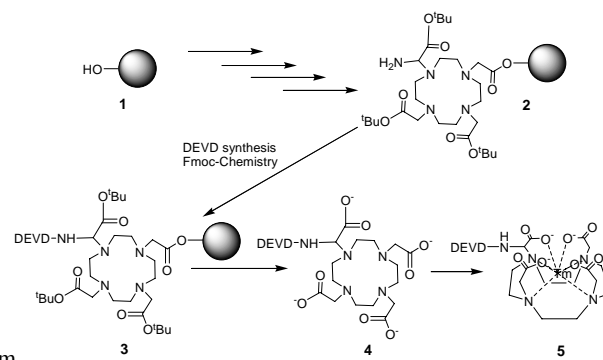
ppm (Figure 1A). A PARACEST effect was detected at -51ppm, which was assigned to the amide most proximal to the lanthanide ion in **5**, based on identical results obtained from a similar compound, Tm^{3+}-DOTAMGly.[12] After 48 nM of caspase-3 was added and the mixture was incubated at 37°C and pH 7.4 for 1 hour, the PARACEST effect at -51 ppm was dramatically decreased and an asymmetrical shape in the PARACEST spectrum was observed near water (0ppm). This asymmetry was analyzed by spectral deconvolution to show a PARACEST effect at +8ppm. Considering that the PARACEST spectrum of **2** also shows an identical PARACEST peak at +8ppm (data not shown), this effect further confirms that caspase-3 has converted the DOTA-amide of **5** to the DOTA-amine of **2**.
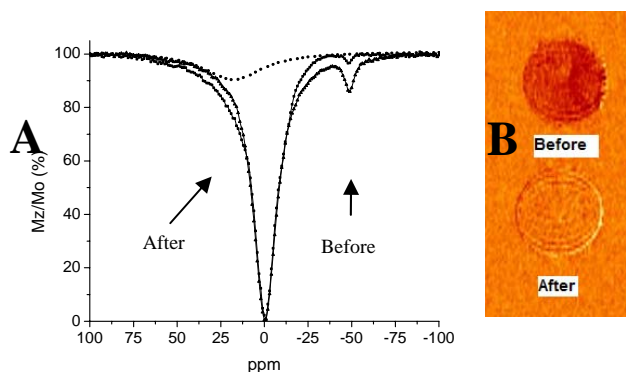

Figure 1. PARACEST spectra (A) and MR parametric map (B) of DEVD-(Tm-DOTA) amide before and after adding caspase-3. PARACEST spectra were acquired with a concentration of 25 mM at 37° C and pH 7.4 with a continuous wave saturation pulse applied at 31uT for 4 seconds. The deconvoluted PARACEST spectrum of the product after reaction, showing a PARACEST effect at +8ppm, is also shown. MR images were acquired at 37 °C and pH 7.4 with a Bruker Biopsin 9.4 T MR scanner. A MSME T1 method was used with TR/TE = 1623/10.9 ms and a train of Gaussian-shaped saturation pulses applied at 50 µT for 1.106 s, and with saturation offsets at -51 ppm and +51 ppm. The parametric map was obtained by subtracting the MR image with a saturation offset at -51 ppm from the MR image with saturation offset at +51 ppm. The magnitude of the scale of the original MR images was used as the scale for this parametric map, to properly represent the difference in MRI contrast obtained with different saturation offsets.

To further demonstrate the utility of **5**, a MR image with selective saturation at -51ppm was acquired with **5** before and after reaction with caspase-3 (Figure 1B). A MR image with selective saturation at +51 was also acquired as a control to account for direct saturation of water. The difference between these images showed a 14.5% decrease in water MR signal before the enzymatic reaction due to the PARACEST effect, and no significant change in water MR signal after reaction.

To determine the sensitivity of detecting **5** under physiological conditions, the PARACEST effect of the agent was correlated with concentrations using modified Bloch equations.[13] After validating a linear relationship between concentration and T_1 relaxation under selective saturation conditions, and confirming that the selective saturation pulse was sufficiently long to achieve steady-state conditions, this theory was further modified to obtain a linear relationship that correlates concentration to the PARACEST effect (Figure 2). These results indicate that 0.90 mM of the agent can be detected by saturating the amide MR frequency to generate a 1% change in water MR signal.
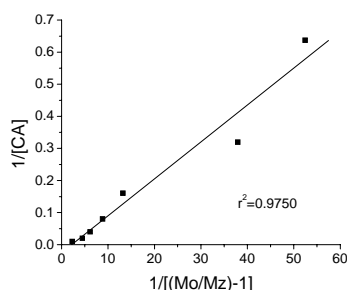

Figure 2. The correlation of concentration and PARACEST of DEVD-(Tm-DOTA) amide using modified Bloch equations. PARACEST was measured at 37 ^{o}C and pH 7.4, using a continuous wave saturation pulse applied at -51ppm and +51ppm at 31μT for 4 seconds. (Concentrations: 100, 50, 25, 12.5, 6.25, 3.13 and 1.57mM).

Temperature can influence chemical exchange rates and therefore can modulate the PARACEST effect.[11] However, changes in temperature caused only a moderate change to the PARACEST effect of **5** when the temperature was changed from 25 ^{o}C to 45 ^{o}C (Figure 3). This behavior can be explained by complicated effect of temperature on water T_1 and $\Delta\omega$, in addition to the proton chemical exchange rate. More importantly, the effect of temperature is negligible throughout the relatively invariant range of temperatures under physiological conditions.

pH can also influence the PARACEST effect, as proton chemical exchange between water and amides is catalyzed by hydroxide ions.[13] The amide proton showed increasingly greater PARACEST with increasing pH, reaching the greatest effect at near pH 8 (Figure 3). The proton chemical exchange rate between an amide and water is relatively slow on the MR time scale, which is characterized by the chemical shift difference between the amide and water. Therefore, an increasing hydroxide ion concentration accelerates this rate to improve the PARACEST effect.

To summarize, DEVD-(Tm-DOTA) amide **5** shows PARACEST with good sensitivity at physiological pH and temperature, indicating that this MRI contrast agent can be used for in vivo molecular imaging. The detection of catalytic activity of caspase-3, rather than the presence of caspase-3, has many important advantages for molecular imaging. A relatively low concentration of enzymes with rapid activity can quickly convert a high concentration of MRI contrast agents for detection using PARACEST MR methods. Caspase-3 is constitutively expressed as an inactive proenzyme, so that detecting enzyme activity avoids detection of the inactive form. Specificities for different substrates

are relatively good for different members of the caspase enzyme family, so that detecting enzyme activity can exploit substrate specificity. Finally, a variety of important enzyme biomarkers can catalyze the conversion of amines, amides, and other functional groups that exchange protons with water. Therefore, this concept of a "smart" PARACEST MRI contrast agent constitutes a fundamentally new type of molecular imaging agent that may have broad applicability for assessing enzyme biomarkers in many biological processes and disease pathologies.
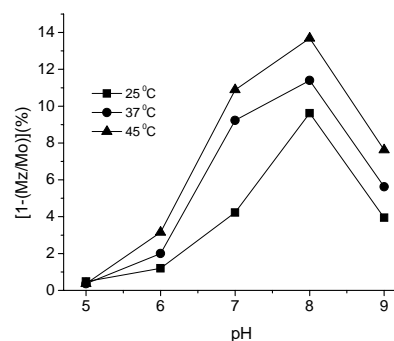

Figure 3. The effect of pH and temperature on PARACEST of 25 mM DEVD-(Tm-DOTA) amide. PARACEST was measured using a continuous wave saturation pulse applied at -51ppm and +51ppm at 31μT for 4 seconds.

SUPPORTING INFORMATION AVAILABLE: Detailed experimental procedures are available free of charge via the Internet at http://pubs.acs.org.

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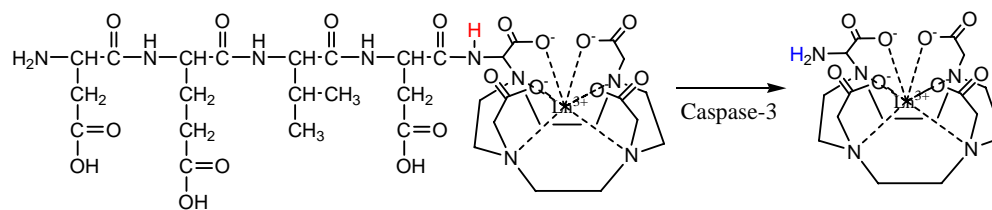
A "smart" PARACEST MRI contrast agent was synthesized to detect caspase-3, which is an important biomarker in apoptosis. The well-known caspase-3 substrate, DEVD (Asp-Glu-Val-Asp), was elongated using the amino group on one side arm of lanthanide ligand anchored on the polymer support. The amide of DEVD-(Tm-DOTA) showed a PARACEST effect with MR saturation at -51ppm. DEVD-(Tm-DOTA) amide was successfully cleaved by caspase-3 exposing the free amine group, which showed PARACEST with saturation at +8ppm. The enzymatic activity of caspase-3 can be detected by the change in PARACEST effect caused by this biotransformation.

A "Smart" PARACEST MRI Contrast Agent to Detect Enzyme Activity

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Theory

A modified Bloch equation for two proton pools undergoing exchange was used to describe the relationship of the PARACEST effect and concentration of **1** (equation 1).[1,2]

$$\frac{M_s}{M_0} = \frac{1}{1 + \dfrac{n_{CA}\,[CA]\,T_{1sat}}{n_{H2O}\,[H2O]\,\tau_M}} \tag{1}$$

M_s: MR signal of water proton pool during selective saturation of the contrast agent proton pool

M_0: MR signal of water proton pool without selective saturation

n_{CA}: number of exchangeable protons of the contrast agent proton pool

n_{H2O}: number of exchangeable protons of the water proton pool (2)

[CA]: concentration of contrast agent

[H_2O]: concentration of water (~55 M)

T_{1sat}: T_1 relaxation time constant of the water proton pool during selective saturation of the contrast agent proton pool

τ_M: average lifetime of the proton on the contrast agent

$1/T_{1sat}$ was found to be linearly related to contrast agent concentration by using a T_1 inversion recovery method with selective saturation at the amide or amine chemical shifts. By substituting T_{1sat} with a linear relationship based on [CA], the modified Bloch equation can be further simplified (equation 2), where m and b represent the slope and intercept of the linear relationship between T_1 and [CA]. This equation was exploited to determine the sensitivity of detecting contrast agent **1** in this paper.

$$\frac{1}{[CA]} = \frac{1}{\left(\dfrac{M_z}{M_0} - 1\right)}\left[\frac{n_{CA}}{b\,n_{H2O}\,\tau_M}\right] + \frac{m}{b} \tag{2}$$

Experiments

1. General Methods

All the reactions were carried out under argon atmosphere. Dichloromethane and carbon tetrachloride were freshly distilled over P_2O_5. Acetonitrile was distilled over BaO. Fmoc-protected amino acids, acetic anhydride, HBTU (O-Benzotriazol-1-yl-N,N,N'N'-tetramethyluronium hexafluorophosphate), HOBt (1-Hydroxybenzotri azole hydrate), piperidine, DIEA (N,N-diisopropylethylamine) and solvents for peptide synthesis were purchased from Applied Biosystems Co. The DO3A-tBu was purchased from Macrocyclics Inc., and other reagents were purchased from Aldrich and Fisher Scientific. Peptides were synthesized using Wang resin (Fluka) and followed Fmoc-chemistry with HBTU and HOBt as coupling agents. The coupling efficiency of each amino acid residues were checked by Kaiser's ninhydrin test. The starting resin had 1.0mmol/g of OH groups and the scale of peptide synthesis was

calculated based on the substitution ratio of the resin. The loading efficiency of Fmoc compounds and the amine contents of the resin were analyzed quantitatively with a UV/Vis/Fluorescence spectrometer (Molecular Devices SpectraMax M2 spectrometer). IR was used to analyze the functional groups on the resin (ABB BOMEM Inc., MB-104). To analyze the peptides and Peptidyl-aminoDOTA final product, HPLC (PerkinElmer Series 200 Pump and UV detector) was used with a C-18 OD-300 reverse phase analytical column. The lanthanide complexation reaction between peptidyl aminoDOTA and $TmCl_3$ was evaluated with Arsenazo III solution color test. ^1H and ^{13}C and PARACEST spectra were measured with a Varian Gemini 300MHz NMR spectrometer and Varian Inova 600MHz NMR spectrometer using $CDCl_3$ and DMSO-d_6 as solvents depending on the solubility. Analytical thin layer chromatography was performed with Merck silica gel 60 F254 plates. Compounds were visualized using a UV lamp(254nm), iodine chamber and ninhydrin solution. High resolution mass spectral analyses were performed with a Mass Spectrometer (Bruker Daltonics Esquire HCT spectrometer) and a MALDI-MASS spectrometer (Bruker BIFLEX III MALDI-TOF spectrometer).

2. Synthesis

Fmoc-DO3A

2.94g (4mmol) of Fmoc-DO$_3$A-tBu was dissolved in 5 ml of 75% TFA in MC and treated for 40 min. The reaction was traced by TLC (CHCl$_3$/MeOH=1.1, Rf=0.22). The solution was dried under reduced pressure. The remaining solid was re-dissolved in MC and precipitated with diethyl ether. The precipitated solid was filtered and dried in vacuo, yielding 1.70g (3.0mmol, yield 75%). This complex was determined to be a TFA salt complex based on the NMR chemical shifts that were upfield relative to expected values: ^1H NMR (600MHz, DMSO-d_6) δ 3.05(s, 4H), 3.20(s, 4H), 3.45(t, 4H), 3.62(t, 4H), 3.60(s, 6H), 4.25(t, 1H), 4.55(d, 2H), 7.35(t, 2H), 7.42(t, 2H), 7.65(d,2H), 7.94(d, 2H) ; ^{13}C (125MHz, DMSO-d_6) δ 174.21, 155.44, 143.85, 140.63, 127.08, 125.07, 120.01, 67.24, 54.30, 51.88, 50.65, 48.68, 45.79. In addition, the product was confirmed with MALDI-MASS spectrometry (Bruker BIFLEX III MALDI-TOF spectrometer): MALDI-Mass *m/z* : 570.64 (calcd. 568.62) [M+H]$^+$.

Coupling of Fmoc-DO3A on the resin

1.0g of Wang resin was used for peptide synthesis (amine contents 1mmol). Fmoc-DO3A (1.14g, 2mmol), HBTU (2.5g, 6.6mmol, 3.3eq.) and HOBt (1.0g, 6.6mmol, 3.3eq.) were dissolved for 40 min to activate carboxylates of Fmoc-DO3A and the solution was added to the peptide reaction vessel containing the resin. TEA (1.82mL, 13eq.) was added and the reaction continued for 4hrs. During the reaction, a small amount of resin was sampled for the Kaiser's test. After filtering, the resin was dispersed in a solution of ^{t}BuOH (1.48g, 20mmol, 20eq.) in 20ml of NMP and the reaction was continued for 1 hour. The resin was washed and dried in vacuo, yielding 1.63g of resin (yield 95% by weight).

Loading Efficiency (Fmoc titration)

The Fmoc concentration on the resin was measured according to a previously reported method.[3]

Fmoc amino acyl resins (4~8mg) were shaken or stirred in piperidine-DMF (3:7) (0.5mL) for 30 min. MeOH (6.5mL) was added and the resin was allowed to settle. The resultant fulvene-piperidine adduct had UV absorption maxima at 267nm (ε=17,500$M^{-1}cm^{-1}$), 290nm (ε=5800$M^{-1}cm^{-1}$), and 301nm (ε=7800$M^{-1}cm^{-1}$). For reference, a piperidine-DMF-MeOH solution (0.3:0.7:39) was prepared. Spectrophotometric analysis was carried out at 301nm, with comparison to a free Fmoc amino acid (Fmoc-Ala) of known concentration treated under identical conditions. The following algorithm was used to quantify the Fmoc concentration :

Sub. Level = Abs. $\times 10^6 \mu$mol/mol $\times$ 0.007L $\times$ 7800 $\times$ 1cm $\times$mg of resin

The measured Fmoc concentration was 0.47mmol/g of resin (calcd. 0.62mmol/g).

Coupling of CBZ-Gly(Br)-OMe on the resin

Fmoc-DO3A loaded resin (1g, Sub. Level 0.47mmol/g) was treated with 20% piperidine for 30 min and sequentially washed with NMP, MC, acetone and acetonitrile. The resin was transferred to a flask with CBZ-Gly(Br)-OMe (0.6g, 2mmol, 4.3eq.) and K_2CO_3 (1.66g, 12mmol, 25.5eq.) in dry acetonitrile (100ml). The solution was stirred and heated 70°C for 6hrs. After filtering the solution, the resin was sequentially washed with MeOH/Water, water, MeOH, MC and dried in vacuo, yielding 0.97g (97% by weight).

Primary amine contents on the resin[4]

In 3 flasks, 0.20g (0.1mmol scale) of resin was dispersed and swelled in MC (5ml) for 1hr and cooled down to -78°C. Et_2AlCl was transferred in 1, 2 and 5 eq. with an air-tight syringe and stayed for 15min. 2, 4, and 10 eq. of thioanisol was added to each flask (the molar ratio of Et_2AlCl/thioanisol (1/2) was fixed) and the resin was sampled according to pre-scheduled time intervals (5, 15, 30 and 60 min). The sampled resin was washed with MC and a picric acid titration was carried out according to a previously reported method.[5] To prevent the influence of cyclen secondary amines, the CBZ protected resin was treated and compared as a reference.

Table 1. The amine contents on the resin were measured to be slightly greater than the Sub.Level of Fmoc groups.

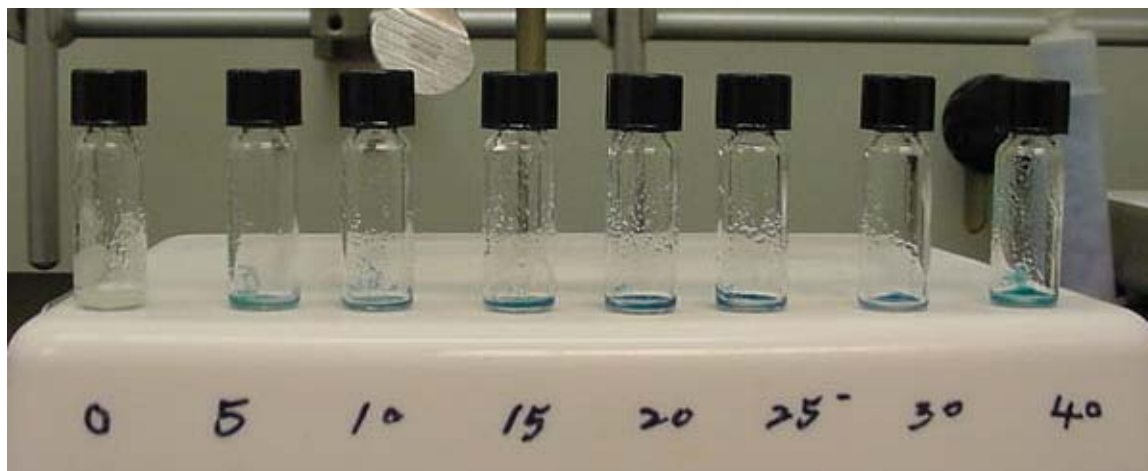|	1eq.	2eq.	5eq.
5	0.60	0.55	0.32
15	0.46	0.26	0.07
30	0.32	0.09	-
60	0.11	-	-

Figure S1. A ninhydrin color test was performed to qualitatively analyze the amine content on the resin. After 25 minutes of reaction time, the resin showed darkest color and after 30 minutes of reaction time, the color of resin became weaker. The reaction time was fixed at 25 minutes during subsequent studies.

IR spectra

CBZ cleaved aminoDOTA-Wang resin was analyzed with FT-IR to confirm the introduction of functional groups. $3440cm^{-1}$ (cyclen ring N-H, str), $3014cm^{-1}$ (aromatic C-H, str), $2917cm^{-1}$ (alkyl C-H, str), $1743cm^{-1}$ (tBu ester C=O, str), $1677cm^{-1}$ (amide C=O, str), $1601cm^{-1}$ (aromatic C=C, str), $1450cm^{-1}$ (C-H, sci, ben), $1306cm^{-1}$ (cyclen ring C-N, str), $1176cm^{-1}$ (C-O, str).

Peptide synthesis and HPLC analysis[6]

0.3g of resin (0.14mmol scale) was used for the solid phase peptide synthesis of a Asp-Glu-Val-Asp (DEVD) peptide sequence. Fmoc-Asp(OtBu)-OH (0.2g, 0.5mmol), Fmoc-Val-OH (0.17g, 0.5mmol) and Fmoc-Glu(OtBu)-OH (0.23g, 0.5mmol) were used as building amino acids and HBTU (0.19g, 0.5mmol) and HOBt (73mg, 0.5mmol) were used for the coupling agents. Freshly distilled TEA (140μL, 1mmol) was used as a base and the solvent was NMP. A double coupling scheme was used for each amino acid and 20% piperidine in NMP was used to cleave the Fmoc group after one amino acid coupling.

After peptide synthesis, the Fmoc goup of the peptide was removed and the peptide-aminoDOTA was cleaved from the resin with a 95% TFA/2.5% water/2.5% thioanisol cocktail for 30 minutes. After removing solvents, it was washed with diethylether and dried in vauo, yielding 115mg (0.12mmol, yield 93%).

The obtained product was characterized by MALDI-MASS (*m/z* : 885.80 (calcd. 884.80) [M+H]+) and HPLC (Column : OD300 RP-18 column, Detector : 222nm, Flow rate : 1.0mL/min, Eluent : 0.1% TFA in water / Acetonitrile (gradient from 85%/15% to 5%/95% for 30 min, Retention time : 2.9min).

Complexation of Tm^{3+}

The obtained peptide-aminoDOTA (100mg, 0.11mmol) was dissolved in water (3mL) at pH 8 and 60°C, and $TmCl_3$ (27mg, 0.1mmol) in water (0.5mL) was added dropwise for 1 hour and adjusted pH to 8 with 0.5N NaOH solution. The solution was stirred for 18 hrs at 50°C and adjusted to pH 8 when the pH dropped. The complete complexation was evaluated with an Arsenazo III color test. When the test showed negative results for free lanthanide ions, the reaction mixture was cooled to room temperature. The pH was adjusted to 9 and white precipitates were filtered. The solution was freeze dried, yielding a quantitative product : MALDI-Mass m/z : 1088.74 (calcd. 1053.73) $[M+Na]^+$

CEST Spectrum (Z-spectrum)

Conditions: Concentration 25 mM, pH=7.4, Saturation power = 31µT, Saturation delay = 4, Spectral width=200ppm (+100 ~ -100ppm with 1ppm increment).
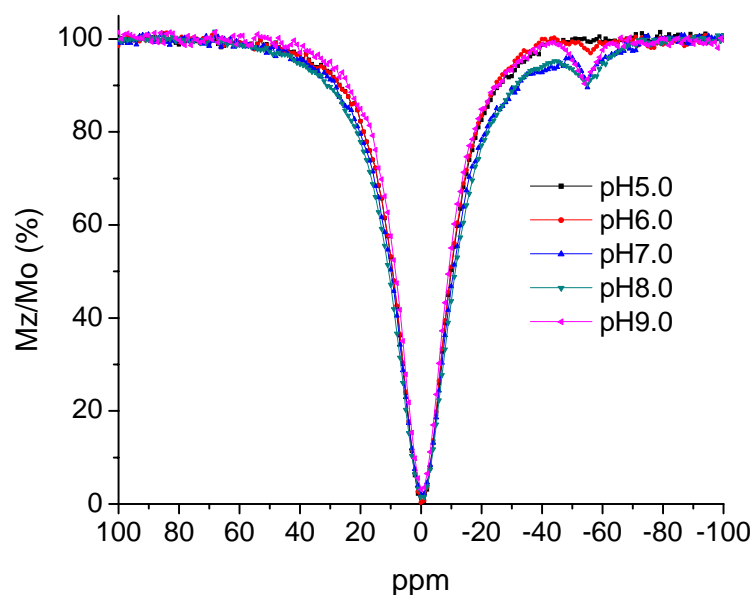

Figure S2. PARACEST spectra at different pH values. PARACEST spectra were collected with a modified presaturation pulse sequence with a continuous wave saturation pulse, saturation pulse power of 31µT, saturation delay of 4 seconds and in 1 ppm increments from 100 ppm to -100 ppm at 25° C.
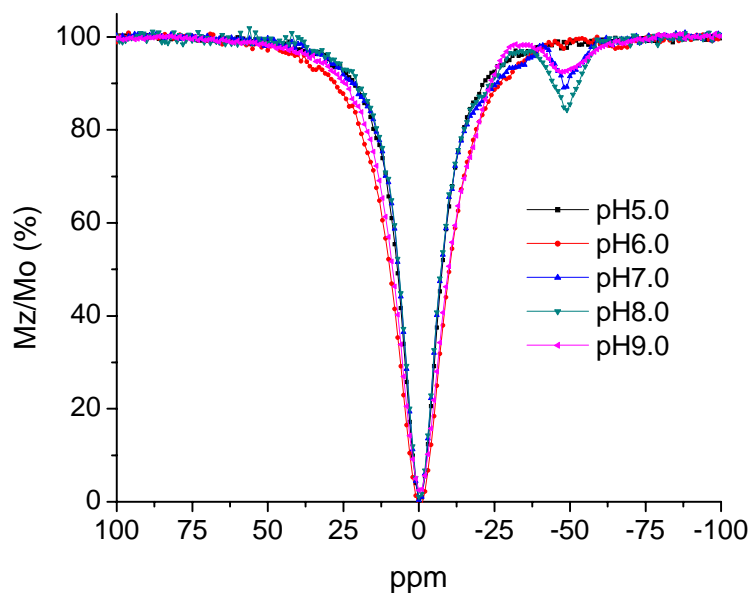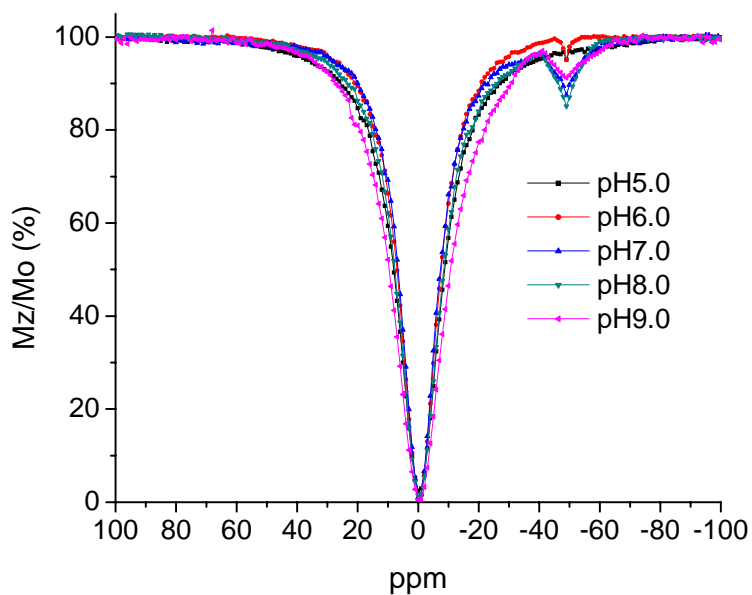
Figure S3. PARACEST spectra at different pH values. PARACEST spectra were collected with a modified presaturation pulse sequence with a continuous wave saturation pulse, saturation pulse power of 31μT, saturation delay of 4 seconds and in 1 ppm increments from 100 ppm to -100 ppm at 37° C.

Figure S4. PARACEST spectra at different pH values. PARACEST spectra were collected with a modified presaturation pulse sequence with a continuous wave saturation pulse, saturation pulse power of 31μT, saturation delay of 4 seconds and in 1 ppm increments from 100 ppm to -100 ppm at 45° C.
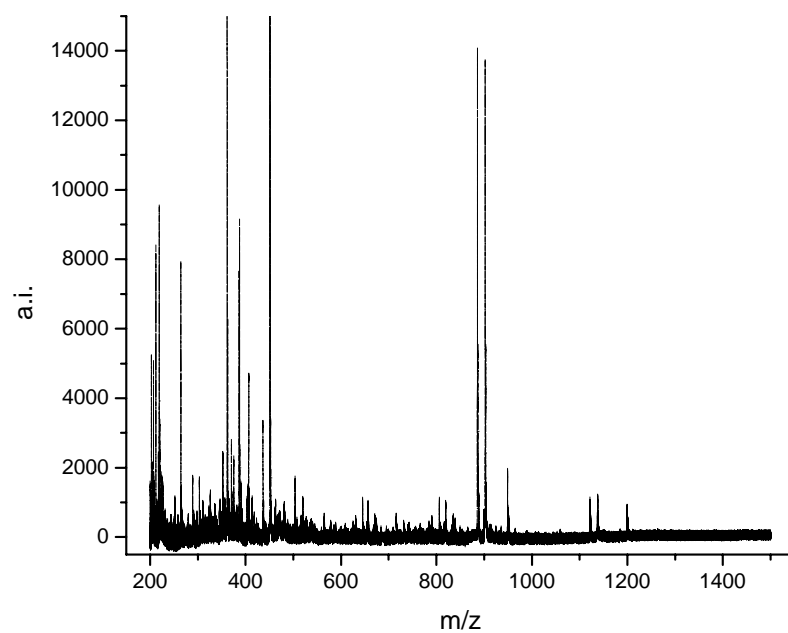
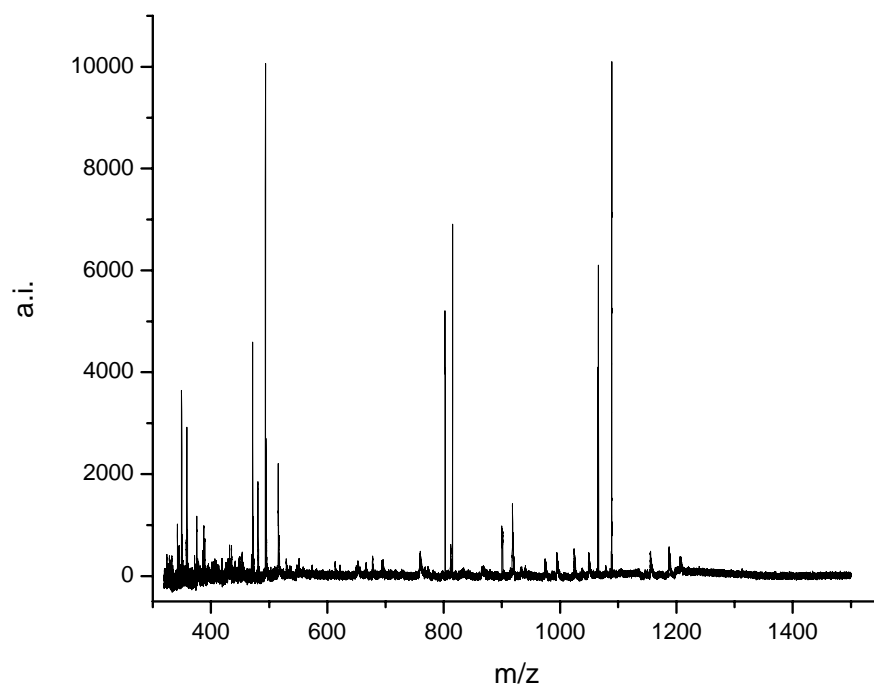**Figure S5**. MALDI-MASS Spectrum of DEVD-DOTA amide.

Figure S6. MALDI-MASS Spectrum of DEVD-(Tm-DOTA) amide complex.

3. DEVD-(Tm-DOTA) amide reaction with Caspase-3 (PARACEST measurements)

To measure the enzymatic reaction of Caspase-3 with DEVD-(Tm-DOTA) amide, samples were prepared in the concentration of 25mM DEVD-(Tm-DOTA) amide using 1X reaction buffer containing 5% D_2O. 500Unit of Caspse-3 (enzyme concentration in reaction solution was 48nM) was reacted with DEVD-(Tm-DOTA) amide at 37°C, pH 7.4 for 1hour. The reaction was traced with the % CEST change from PARACEST spectra. The result of enzymatic reaction of caspase-3 with DEVD-(Tm-DOTA) amide complex was shown in Figure 1.

To verify the activity of caspase-3, a Caspase-3 Assay kit #1(DEVD-AMC, fluorescence, Invitrogen Inc.) was used, following the protocol provided by Invitrogen Inc.

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