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<th>Journal:</th>
<th>Canadian Journal of Chemistry</th>
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<tr>
<td>Manuscript ID</td>
<td>cjc-2016-0179.R1</td>
</tr>
<tr>
<td>Manuscript Type</td>
<td>Article</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>31-May-2016</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Suchy, Mojmir; Western University, Chemistry</td>
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<td>Li, Alex; University of Western Ontario, Robarts Research Institute</td>
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<td>Bartha, Robert; University of Western Ontario, Robarts Research Institute</td>
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<td>Hudson, Robert; University of Western Ontario</td>
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<td>Keyword:</td>
<td>Nitric Oxide Synthase, DOTAM, PARACEST, MRI, Chelate</td>
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Preliminary evaluation of PARACEST MRI agents for the detection of nitric oxide synthase (NOS)

Mojmír Suchý, a,b,1 Alex X. Li, b Yin Liu, c,2 Qingping Feng, c Robert Bartha, b and Robert H. E. Hudson *a,d

a Department of Chemistry and d The Centre for Advanced Materials and Biomaterials Research, The University of Western Ontario, London, Ontario, Canada, N6A 5B7
b Robarts Research Institute, The University of Western Ontario, London, Ontario, Canada, N6A 5K8
c Department of Physiology and Pharmacology, The University of Western Ontario, London, Ontario, Canada, N6A 5C1
1 Current Address: Department of Chemistry and Biomolecular Sciences, University of Ottawa, Ottawa, ON, Canada, K1N 6N5
2 Current Address: Department of Pharmacology, Sichuan University, Chengdu, Sichuan 610041, PR China

E-mail: rhhudson@uwo.ca Tel: 519-661-2111 ext. 86349 Fax: 519-661-3022
Abstract

Several paramagnetic chemical exchange saturation transfer magnetic resonance imaging (PARACEST MRI) agents for the potential detection of nitric oxide synthase (NOS) have been synthesized and evaluated. These agents are based on an amino acid or dipeptide decorated DOTAM (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid amide) chelator possessing either Tm$^{3+}$ or Dy$^{3+}$. The amino acid and dipeptide decorated DOTAMs were designed such that the terminal amino acid pendant group was L-arginine which may be converted to L-citrulline by NOS. Preliminary evaluation has revealed that some of the L-arginine-decorated complexes are recognized and metabolized by the NOS. Differences in the CEST properties between Dy$^{3+}$-metallated L-arginine- and L-citrulline-modified complexes suggest that these might be suitable for imaging of the NOS enzymatic activity.

Keywords: Nitric Oxide Synthase, PARACEST, MRI, DOTAM, thulium, dysprosium, citrulline, arginine, cyclen.
Graphical Abstract

[Chemical diagrams and images related to nitric oxide synthase]
Introduction

The use of magnetic resonance imaging (MRI) for medical purposes has become widely practiced because it offers excellent spatial resolution and deep tissue penetration while being devoid of harmful ionizing radiation. For MR imaging that requires the use of an externally administered contrast agent, those based on Gd$^{3+}$ chelates are often used.\(^1\) In recent years there has been interest in developing chelates of non-gadolinium lanthanide ions as contrast agents. In particular, lanthanide(III) chelates that demonstrate slow exchange of water (bound to bulk) at the metal centre\(^2\) or possess exchangeable protons in the ligand (e.g. N-H, O-H, C(O)NH) can operate by a chemical exchange saturation transfer (CEST) mechanism as originally described by Balaban and coworkers for diamagnetic molecules.\(^3\) When a paramagnetic metal chelate is used, they are termed paramagnetic CEST (PARACEST) MRI agents.\(^4\)

The exchange processes that produce the PARACEST phenomenon are influenced by the microenvironment of the agent, thus it becomes possible to measure physiological of interest conditions such as temperature and pH.\(^5\) Beyond this, if the ligand is transformed chemically, such a change may be manifested in the CEST properties of the chelate.\(^6\) This concept has been exploited for the detection of various enzymatic activities including the proteases caspase-3\(^7\) and cathepsin D,\(^8\) pig liver esterase,\(^9\) urokinase plasminogen activator,\(^10\) \(\beta\)-galactosidase,\(^11\) and transglutaminase.\(^12\)

Our previous studies have been directed toward the development of synthetic methodologies to access peptide decorated DOTAMs\(^13,14\) and their application as potential PARACEST MRI
contrast agents.\textsuperscript{15,16} These agents generally rely on a CEST effect generated by exchange of a metal bound water, for example Eu\textsuperscript{3+} DOTAM-Gly-Phe-OH (1, Figure 1), or exchange of the amide proton proximal to the cyclen ring, for example Tm\textsuperscript{3+} DOTAM-Gly-Lys-OH (2, Figure 1). The synthetic methodology leading to CAs 1 and 2 was found to be robust, versatile and scalable\textsuperscript{13} and we were therefore interested in expanding its scope. Notably absent in the initially reported method were arginine-decorated DOTAMs. Thus, the method was extended\textsuperscript{17} because we were interested to investigate if arginine-decorated DOTAMs might possess similar or improved properties as compared to Tm\textsuperscript{3+} DOTAM-Gly-Lys-OH (2).\textsuperscript{18}

\textbf{Figure 1} A) Generalized structure of an amino acid decorated DOTAM showing the proximal amide protons responsible for the CEST effect, in bold, and the metal bound water as indicated
by the arrows (R = amino acid sidechain). B) Specific structural examples of dipeptide-decorated DOTAM PARACEST CAs. C) Chemical structures of arginine-decorated CAs 3, 4 (R = amino acid or dipeptide, as indicated).

We have focussed on lanthanide complexes of metals that are able to induce large hyperfine shifts of the proximal amide protons, namely Dy$^{3+}$ and Tm$^{3+}$ in this work, in order to generate a CEST signal well separated from the bulk water signal. With some arginine-decorated DOTAM chelators in hand, we choose to investigate the possibility of using them to detect nitric oxide synthase activity. Our previous studies,$^{13,14}$ and those of others,$^{19}$ indicated that the identity of the amino acid pendant arms influenced the observed CEST signals presumably by modulation of the exchange rates and subtle effects on the ligand conformation. Thus, it was hypothesized that conversion of the charged amino acid arginine to the uncharged citrulline would affect the CEST signals produced by the lanthanide(III) complexes.

Among the various types of MRI CAs, PARACEST agents$^{20}$ are well suited for the potential detection of NOS enzymatic activity. As noted, these CAs are sensitive to their microenvironment and have been previously used for the detection of various enzymatic activities, vide supra. Nitric oxide (NO)$^{21}$ is a key signaling molecule associated with various physiological processes, e.g. vasodilatation, immune response to infection or wound healing. Altered levels of NO can serve as a useful biomarker of certain pathologies, e.g. cardiovascular diseases or breast and prostate cancers. Production of NO within the body is orchestrated by the enzyme nitric oxide synthase (NOS), which utilizes an essential amino acid L-arginine as a substrate.$^{22}$ Enzymatically, L-arginine (L-Arg-OH) is first oxidized to L-N-hydroxyarginine, and
in a second step, further oxidized to L-citrulline (L-Cit-OH) along with the simultaneous production of NO (Scheme 1).  

Scheme 1 Metabolism of L-arginine to L-citrulline accompanied by release of nitric oxide.

Several agents for the detection of NO have been developed over the course of last two decades.  These involve probes which can be tracked by means of optical (fluorescence) imaging or other spectroscopic techniques such as surface-enhanced Raman scattering (SERS).  The detection of NOS enzymatic activity by radiolabeled imaging agents (\(^{11}C, ^{99m}Tc\)) compatible with positron emission tomography (PET) or single photon emission computer tomography (SPECT) has also been reported (Scheme 2).  The sole example of a MRI CA related to the detection of NOS enzymatic activity was described by Pagel and coworkers in 2007 and relies on the detection of nitric oxide (produced by NOS).  Their contrast agent operates in the PARACEST mode and is based on dimerization \(\sigma\)-phenylenediamine-decorated DOTA-derived Yb\(^{3+}\) complex in the presence of NO and \(O_2\) (Scheme 2).  The dimerization of 6 caused an irreversible loss of both CEST effects: the anticipated loss of the CEST effect due to the aromatic
NH$_2$ (at +8 ppm) and the unexpected loss of the CEST effect due to the amide proton (at -11 ppm). In general, for analytical purposes, the loss of a signal could be a result of factors other than the dimerization reaction (e.g. dilution); thus, it is desirable to investigate other agents that provide either a ‘turn on’ of signal or that demonstrate some other observable change such as a change in the chemical shift of the CEST effect.

**Scheme 2** Molecular probes for the detection of NO. A) Radiometal-based imaging agents, 5.

B) PARACEST MRI CA 6 that undergoes NO mediated dimerization.

It is recognized that NOS is a promiscuous enzyme with a wide substrate scope.$^{22}$ As shown by recent work from Correia and colleagues, Re(III) complexes of the general structure 5 (Scheme 2) were found to be recognized by NOS and served as competitive inhibitors of its enzymatic activity. Moreover, these complexes were found to cross cellular membranes and their $^{99m}$Tc counterparts have been used as SPECT probes for the detection of NOS enzymatic activity.$^{28}$ Armed with this knowledge we have investigated the use of a series of L-arginine-decorated PARACEST MRI CAs (structures 3 and 4, Figure 1 and Scheme 3) that were recently prepared
in our laboratory\textsuperscript{18} for their ability to be metabolized by NOS. The metabolism of 3 or 4 by NOS would produce the corresponding \textit{L}-citrulline-decorated DOTAMs (Scheme 3) and release NO. We have independently synthesized the \textit{L}-citrulline-derived CAs 7 and 8, evaluated their CEST properties and compared them with those associated with 3 and 4. The metabolism of complexes 3 or 4 was determined by enzymatic mediated NO production using commercially available bovine endothelial NOS (eNOS). The production of NO was detected electrochemically and compared to the free amino acid \textit{L}-arginine, the natural substrate of NOS. The results of these studies are described below and indicate that CA 4a shows promise for the PARACEST MRI-based detection of NOS enzymatic activity.

\textbf{Scheme 3} The proposed NOS-mediated metabolism of \textit{L}-Arg modified CAs 3 and 4 to form the \textit{L}-Cit modified CAs 7 and 8
Results and discussion

Chemistry

The synthesis of CAs 7 and 8a,b was accomplished by the “cyclen tetraalkylation” approach that has been previously employed for the preparation of L-Arg-decorated CAs 3 and 4. Briefly, esterification (SOCl₂/MeOH) of commercially available L-Citulline (L-Cit-OH, Scheme 4) afforded the corresponding methyl ester 9 (L-Cit-OMe·HCl) in quantitative yield. A dicyclohexylcarbodiimide (DCC)/N-hydroxysuccinimide (NHS)-mediated coupling between 9 and chloroacetic acid or N-chloroacetylglutamine provided electrophiles 10a (81%) or 10b (30%), respectively, Scheme 4. The full spectral characterization of 10a and 10b can be found in Experimental and in Supplemental Information. An aqueous extractive workup, previously used for the preparation of related electrophiles, was not used, as we anticipated poor partitioning of 10a and 10b into organic solvents commonly used. Instead the reaction mixtures were adsorbed on SiO₂ and then subjected to flash column chromatography (FCC) purification. The relatively low yield of the more polar electrophile 10b is attributed to an increased retention on the silica gel.

Scheme 4 Preparation of electrophiles 10a,b
With the electrophiles 10a and 10b the peralkylation of cyclen (11) was performed (Scheme 5). The free ligands, DOTAM-Cit-OCH₃ (10a) and DOTAM-Gly-Cit-OCH₃ (10b) were purified by semi-preparative high pressure liquid chromatography (HPLC) and were obtained as the trifluoroacetate salts in moderate yields (Scheme 5, see Experimental and Supplemental Information for details). Metallation of the ligands was performed by treatment with corresponding lanthanide(III) chlorides as shown in Scheme 5. The pH of the reaction was initially maintained at ca. 10 to ensure complete hydrolysis of ester groups. Once the esters hydrolyzed, the pH was lowered to ca. 5-6 to achieve the complete metellation. Resulting reaction mixtures were purified by size exclusion chromatography (SEC) affording L-citrulline-modified CAs 7 and 8 (Scheme 5) in good yields (66-73%). The identities and purities of CAs 7 and 8 were confirmed by high resolution mass spectrometry (HRMS) as described in the Experimental and depicted in the Supplemental Information.
CEST spectra of CAs 3, 4, 7 and 8

With the desired CAs 3, 4, 7 and 8 in hand their CEST spectra were collected (10 mM solutions in a phosphate buffer, pH 7.0, 37 °C). Further details related to CEST spectra acquisition can be found in the Experimental. As depicted in Scheme 3, we hypothesized that L-arginine-modified CAs 3 and 4 would be recognized and metabolized by NOS with the formation of corresponding L-citrulline-decorated CAs 7 and 8. We were interested in comparing the differences (e.g. chemical shift, signal intensity) observed in the CEST spectra associated with CAs 3, 4, 7, and 8. These results are shown in Figure 2; for the sake of clarity the CEST spectra associated with L-arginine-modified CAs 3 and 4 are depicted in red while the
CEST spectra associated with L-citrulline-modified CAs 7 and 8 are depicted in green.

The Tm$^{3+}$ complexes 4b (Arg) and 8b (Cit) exhibited a CEST effect (4b, 28%; 8b, 19%) due to the presence of exchangeable amide protons at -45 ppm (Figure 2, panels A and B). Unfortunately, we did not observe a difference in the chemical shift between L-arginine- and L-citrulline-modified CAs, implying that the CA 8b would not be suitable for the detection of NOS enzymatic activity by PARACEST MRI.

The Dy$^{3+}$ complexes 3, 4a, 7 and 8a appeared more promising in terms of their future use for the detection of NOS enzymatic activity. Dy$^{3+}$ complexes 4a (Arg) and 8a (Cit) featuring the Gly linker exhibited a CEST effect (4a, 13%; 8a, 7%) due to the presence of exchangeable amide protons at 71 ppm (4a) and 69 ppm (8a, Figure 2, panels C and D). Although the difference in the chemical shift between the CEST effects associated with L-arginine-modified (4a) and L-citrulline-modified (8a) CAs is rather small (Δδ 2 ppm), further use of CAs 4a and 8a for the imaging of NOS enzymatic activity in cell culture studies or in vivo maybe be feasible since similar small differences in chemical shifts are oftentimes exploited when using endogenous macromolecules (e. g. proteins, saccharides) as diamagnetic CEST (DIACEST) CAs for in vivo imaging.31

The absence of the Gly linker in the complexes 3 (Arg) and 7 (Cit) resulted in a more dramatic difference in chemical shift between the CEST effects associated with 3 (71 ppm, 10%) and 7 (76 ppm, 5%, Figure 2, panels E and F, Δδ 5 ppm). The signal intensity decrease of ca. 50% in all three instances, (Figure 2) may be attributed to the different proton exchange rates in L-arginine- (3 and 4) and L-citrulline-modified (7 and 8) complexes. The L-citrulline-modified CAs in this case would possess less favorable proton exchange rates resulting in a CEST signal intensity decrease. It is also interesting to point out, that the CAs 3 and 7 bearing no Gly linker...
possess weaker CEST signal (Figure 2) as opposed to CAs 4 and 8 bearing the Gly linker. Similar results have been previously observed by our group, when comparing the CEST spectra associated with CAs 1 and 2 (Figure 1) with their counterparts lacking a Gly linker within their structure.
Figure 2 Comparison of the CEST spectra associated with CAs 4b and 8b (panels A, B); 4a and 8a (panels C, D); 3 and 7 (panels E, F). The CEST spectra associated with L-arginine modified agents are depicted in red; the CEST spectra associated with L-citrulline modified agents are depicted in green. Full view of the CEST spectra (-100 to 100 ppm) is shown on the left (panels A, C, E); expanded view of the region of interest is shown on the right (panels B, D, F). CEST spectra were collected on 10 mM solutions of chelator at pH 7.0 and 37 °C using a 9.4 Tesla MRI scanner with 14 μT saturation pulse, 3.95 second in duration.

Metabolism of 3, 4a, 7 and 8a with eNOS

Having selected two pairs of L-arginine- and L-citrulline-modified PARACEST CAs (3 and 7, 4a and 8a) showing promise in detecting NOS enzymatic activity by CEST MRI, we determined whether or not NOS can recognize and metabolize the L-arginine-modified CAs 3 and 4a. The NOS enzymatic activity assay was performed with bovine eNOS and relied on the sensitive detection of NO production by electrochemical means, the details are found in the Experimental and the results are summarized in Table 1. The rates of metabolism were qualitatively judged by comparison of the amount of NO produced relative to the natural substrate L-arginine.

Only the L-arginine-modified CA 4a, featuring a Gly linker was found to be metabolized by eNOS with a rate of metabolism that was similar to the natural substrate L-arginine (Scheme 1). The L-arginine-modified CA 3, lacking the Gly linker was, on the other hand, not metabolized by eNOS. We hypothesize that the linker in 3 between the core of the chelator and the pendant arginine residue is too short to permit proper interaction with enzyme. Our observation is
consistent with the results described by Correia and coworkers, wherein the complexes of general structure 5 (Scheme 2) possessing longer side chain linkers (5, Scheme 2, \( n = 4 \)) exhibited increased competitive inhibitory activity toward NOS when compared to the inhibitory activity of those possessing a shorter linker (5, Scheme 2, \( n = 1 \)).\(^{28}\) As expected, the \( L \)-citrulline-modified CAs 7 and 8a did result in the production of NO in the presence of eNOS.

Overall our results indicate that among the series of CAs herein studied, \( \text{Dy}^{3+} \) DOTAM-Gly-Arg-OH (4a, Scheme 3) is the probe of choice for the detection of NOS enzymatic activity. It exhibits different CEST properties (chemical shift, signal intensity) compared to the corresponding \( \text{Dy}^{3+} \) DOTAM-Gly-Cit-OH (8a, Scheme 3) counterpart. Moreover 4a was qualitatively judged to be metabolized by eNOS by comparison to the natural substrate, \( L \)-arginine (Scheme 1), in terms of the rate and quantity of NO liberated as determined by electrochemical detection.\(^{32}\)

**Table 1** Metabolism of \( L \)-arginine (1) and CAs 3, 4a, 7 and 8a by bovine eNOS

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<th>Compound</th>
<th>Observed result</th>
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<td>( L )-arginine</td>
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<tr>
<td>( \text{Dy}^{3+} ) DOTAM-Arg-OH (3)</td>
<td>not metabolized</td>
</tr>
<tr>
<td>( \text{Dy}^{3+} ) DOTAM-Gly-Arg-OH (4a)</td>
<td>metabolized</td>
</tr>
<tr>
<td>( \text{Dy}^{3+} ) DOTAM-Cit-OH (7)</td>
<td>not metabolized</td>
</tr>
<tr>
<td>( \text{Dy}^{3+} ) DOTAM-Gly-Cit-OH (8a)</td>
<td>not metabolized</td>
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**Experimental**

**General experimental procedures**
All amino acids (naturally occurring L isomers) and reagents were commercially available, unless otherwise stated. All solvents were HPLC grade and used as such, except for water (18.2 MΩ·cm⁻¹ Millipore water) and THF (dried over Al₂O₃ in a solvent purification system). Solvents were removed under reduced pressure in a rotary evaporator. Aqueous solutions were lyophilized. Flash column chromatography (FCC) was carried out using silica gel, mesh size 230 – 400 Å. Thin layer chromatography (TLC) was carried out on Al backed silica gel plates, compounds were visualized by anisaldehyde stain, phosphomolybdic acid stain and I₂ vapours. Specific rotations [α]D were determined by polarimeter at ambient temperature using a 1 mL, 10 cm path length cell; the units are 10⁻¹ deg cm² g⁻¹ and the concentrations are reported in g/100 mL. Size exclusion chromatography (SEC) was performed on BIO-GEL P2, 45-90 µm mesh resin (15 g, per 0.03 mmol of compound). Ten fractions (10 mL each) were collected and identified by UV or ninhydrin test, fractions containing complexes 7 and 8 were combined and lyophilized. Absence of free lanthanide(III) ions was verified by a xylenol orange test. HPLC analysis and purification was done using a Delta-Pak C18 300Å column (particle size 15µm; 8 × 100 mm Radial-Pak cartridge). Mobile phase: Method A: 90% H₂O/10% MeCN – 45% H₂O/55% MeCN over 10 min; linear gradient and flow rate 3 mL/min. Ultra performance liquid chromatography (UPLC) was performed using a BEH C18 column (particle size 1.7µm; 1.0 id × 100 mm) and HR-ESI-MS detector. Mobile phase: Method B: 100% H₂O – 100% MeCN over 5 min, linear gradient, flow rate 0.25 mL/min. NMR spectra were recorded on 400 MHz spectrometer; for ¹H (400 MHz), δ values were referenced as follows DMSO-D₆ (2.49 ppm), D₂O (4.75 ppm), for ¹³C (100 MHz) DMSO-D₆ (39.5 ppm). Mass spectra (MS) were obtained using chemical ionization (CI) or electron spray ionization (ESI) Time-of-Flight (TOF) instrument. PARACEST properties of CAs 3, 4, 7, and 8 have been evaluated as follows: NMR
tubes containing solutions of 3, 4, 7, or 8 in phosphate buffer (10 mM, pH 7.0) were imaged on a 9.4 Tesla MRI scanner (Agilent, Santa Clara, CA) at 37 °C. The temperature was monitored and controlled by blowing hot air using a Model 1025 small animal monitoring and gating system (SA Instruments, Inc., Stony Brook, NY). 18 A fast spin echo pulse sequence (FOV: 12.8 × 12.8 mm², matrix: 32 × 32, TR = 4000 ms, 4 echoes, and effective TE = 10 ms) was used, preceded by a frequency selective saturation pulse (B₁ = 14 μT, saturation range = -100 to 100 ppm in steps of 1 ppm, saturation time = 3.95 s). CEST spectra were generated using the total signal intensity from each tube. 18

L-Citulline methyl ester hydrochloride, L-Cit-OMe•HCl, 9

SOCl₂ (190 µL, 2.6 mmol) was added dropwise (over ca. 1 min period) to MeOH (1.5 mL) cooled to 0 °C. L-Citrulline (350 mg, 2 mmol) was then added, the cooling bath was removed and the resulting mixture was stirred for 48 h at room temperature (RT). The solvent was evaporated and the residue was dried in high vacuum overnight to afford L-citrulline methyl ester hydrochloride (450 mg, quantitative) as colorless oil of sufficient purity to be used in the next step. [α]D +15.6 (c 0.7, H₂O). 1H NMR (D₂O) δ 3.99 (t, J = 6.5 Hz, 1H); 3.65 (s, 3H); 2.98 (t, J = 6.5 Hz, 2H); 1.78 (m, 2H); 1.44 (m, 2H). 13C NMR (D₂O) δ 170.4, 161.1, 53.6, 52.5, 39.1, 26.9, 24.5. HRMS (CI) m/z: found 190.1198 [M + H]⁺ (calcd 190.1192 for C₇H₁₅N₃O₃).

Preparation of electrophiles 10a and 10b

N-hydroxysuccinimide (219 mg, 1.9 mmol) was added to separate solutions of chloroacetic acid (10a, 180 mg, 1.9 mmol) or N-chloroacetylglycine (10b, 288 mg, 1.9 mmol) in dry THF (10 mL). The mixtures were cooled to 0 °C, followed by the addition of DCC (510 mg, 2.5 mmol)
and subsequent stirring for 1 h at 0 °C. Separate solutions of L-Cit-OMe•HCl (9, 450 mg, 2 mmol) in MeOH (2 mL)/THF (7 mL) were added, followed by the addition of Et₃N (1.06 mL, 7.6 mmol). The cooling bath was removed and the mixture was stirred for 18 h at RT. The solvents were evaporated; the residues were resuspended in acetone (ca. 20-30 mL), SiO₂ was added and the volatiles were removed. Resulting slurries were subjected to FCC on 40 g of SiO₂, CH₂Cl₂/MeOH/NH₄OH (90:9:1). Evaporation of the eluates afforded desired products containing small amount of Et₃N•HCl. The impurity was removed by repeated FCC on 40 g of SiO₂, CH₂Cl₂/MeOH/NH₄OH (80:19:1, 10a) or 20 g of SiO₂, CH₂Cl₂/MeOH/NH₄OH (90:9:1, 10b). Evaporation of eluates afforded the desired products as colorless oils.

N–Chloroacetyl-Cit-OMe (10a, 409 mg, 81%); [α]D -13.4 (c 1, MeOH). ¹H NMR (DMSO-D₆) δ 8.68 (d, D₂O exch., J = 7.5 Hz, 1H); 5.95 (t, D₂O exch., J = 5.5 Hz, 1H); 5.39 (s, D₂O exch., 1H); 4.25 (m, 1H); 4.11 (dd, J = 14.5, 13.0 Hz, 2H); 3.63 (s, 3H); 2.94 (dd, J = 13.0, 6.5 Hz, 2H); 1.72 (m, 1H); 1.59 (m, 1H); 1.36 (m, 2H). ¹³C NMR (DMSO-D₆) δ 172.1, 166.1, 158.7, 52.2, 52.0, 42.2, 38.6, 28.3, 26.5. HRMS (ESI) m/z: found 266.0895 [M + H]⁺ (calcd 266.0908 for C₉H₁₇ClN₃O₄).

N–Chloroacetyl-Gly-Cit-OMe (10b, 182 mg, 30%); [α]D -15.3 (c 1, MeOH). ¹H NMR (DMSO-D₆) δ 8.41 (m, D₂O exch., 2H); 5.94 (t, D₂O exch., J = 5.5 Hz, 1H); 5.39 (s, D₂O exch., 1H); 4.24 (m, 1H); 4.13 (s, 2H); 3.79 (m, 2H); 3.62 (s, 3H); 2.94 (dd, J = 13.0, 6.5 Hz, 2H); 1.65 (m, 1H); 1.58 (m, 1H); 1.37 (m, 2H). ¹³C NMR (DMSO-D₆) δ 172.5, 168.5, 166.2, 158.8, 51.9, 45.6, 42.5, 41.8, 38.6, 28.4, 26.6. HRMS (ESI) m/z: found 323.1126 [M + H]⁺ (calcd 323.1122 for C₁₁H₂₀ClN₄O₅).

Peralkylation of cyclen (11) with electrophiles 10a and 10b
Separate solutions of *N*-chloroacetyl-Cit-OMe (10a, 114 mg, 0.43 mmol) or *N*-chloroacetyl-Gly-Cit-OMe (10b, 140 mg, 0.43 mmol), cyclen (11, 19 mg, 0.11 mmol) and DIPEA (76 µL) in MeCN (1.8 mL)/DMF (0.2 mL) were stirred under reflux for 48 h. Solvents were evaporated; the residues were dissolved in MeOH/H₂O (1.5 mL each) and were subjected to semipreparative HPLC purification as described in General experimental procedures. Concentration of fractions containing the desired products as afforded colorless semi-solid residues.

**DOTAM-Cit-OMe·4CF₃COO⁻** (78 mg, 46%); *t*ᵣ 5.4 min (Method A); ¹H NMR (DMSO-D₆) δ 8.75 (m, D₂O exch., 4H); 7.87 (m, D₂O exch., 4H); 4.28 (m, 4H); 3.69 (br m, 8H); 3.64 (s, 12H); 3.26 (br m, 16H); 2.97 (m, 8H); 1.72 (m, 4H); 1.60 (m, 4H); 1.41 (m, 8H). HRMS (ESI) *m/z*: found 1089.5997 [M + H]⁺ (calcd 1089.6016 for C₄₄H₸₁N₁₆O₁₆).

**DOTAM-Gly-Cit-OMe·4CF₃COO⁻** (62 mg, 32%); *t*ᵣ 4.3 min (Method A); ¹H NMR (DMSO-D₆) δ 8.53 (m, D₂O exch., 8H); 7.77 (m, D₂O exch., 4H); 6.12 (m, D₂O exch., 4H); 4.25 (m, 4H); 3.84 (m, 8H); 3.70 (br m, 8H); 3.62 (s, 12H); 3.26 (br m, 16H); 2.95 (m, 8H); 1.68 (m, 4H); 1.57 (m, 4H); 1.39 (m, 8H). HRMS (ESI) *m/z*: found 1317.6915 [M + H]⁺ (calcd 1317.6875 for C₅₂H₹₃N₂₀O₂₀).

**Metallation of the ligands DOTAM-Cit-OMe and DOTAM-Gly-Cit-OMe**

Separate solutions of DOTAM-Cit-OMe·4CF₃COO⁻ (45 mg, 2.9 × 10⁻⁵ mol) and DOTAM-Gly-Cit-OMe·4CF₃COO⁻ (51 mg, 2.9 × 10⁻⁵ mol) in water (2 mL) were treated with DyCl₃·6H₂O (11 mg, 2.9 × 10⁻⁵ mol, both ligands) or TmCl₃·H₂O (8 mg, 2.9 × 10⁻⁵ mol, DOTAM-Gly-Cit-OMe·4CF₃COO⁻). The pH was adjusted to ca. 9-10 (1 M NaOH) and the mixtures were stirred for 24 h at RT, complete saponification took place, along with incomplete metallation.

**DOTAM-Cit-OH**; HRMS (ESI) *m/z*: found 1033.5345 [M + H]⁺ (calcd 1033.5390 for
C\textsubscript{40}H\textsubscript{73}N\textsubscript{16}O\textsubscript{16}).

DOTAM-Gly-Cit-OH; HRMS (ESI) \( m/z \): found 1261.6304 [M + H]\(^+\) (calcd 1261.6249 for C\textsubscript{48}H\textsubscript{85}N\textsubscript{20}O\textsubscript{20}).

The pH was then adjusted to ca. 5-6 (1 M HCl) and the mixtures were stirred for 24 h at RT, followed by SEC as described in General experimental procedures. Fractions containing the products were concentrated to leave the desired complexes as colorless solids.

Dy\(^{3+}\) DOTAM-Cit-OH (7, 25 mg, 73%); HRMS (ESI) \( m/z \): found 1192.4462 [M - 2H]\(^+\) (calcd 1192.4424 for C\textsubscript{40}H\textsubscript{70}N\textsubscript{16}O\textsubscript{16}Dy).

Dy\(^{3+}\) DOTAM-Gly-Cit-OH (8a, 28 mg, 69%); HRMS (ESI) \( m/z \): found 1421.5372 [M - 2H]\(^+\) (calcd 1421.5302 for C\textsubscript{48}H\textsubscript{82}N\textsubscript{20}O\textsubscript{20}Dy).

Tm\(^{3+}\) DOTAM-Gly-Cit-OH (8b, 27 mg, 66%); HRMS (ESI) \( m/z \): found 1427.5377 [M - 2H]\(^+\) (calcd 1427.5357 for C\textsubscript{48}H\textsubscript{82}N\textsubscript{20}O\textsubscript{20}Tm).

**L-Arginine-modified CAs 3, 8a and 8b**

These CAs and the synthetic intermediates leading to them were prepared as described previously.\(^\text{18}\)

**NOS enzyme assay with 3, 4, 7, 8\(^\text{32}\)**

The NOS assay was carried out using a NO sensor with the “inNO” Nitric Oxide Measuring System (Innovative Instruments Inc., USA) and used bovine eNOS (Sigma) and ancillary reagents from a Cayman Chemical NOS activity assay kit. The assays were performed according to manufacturer’s instructions using 100 µL of 100 mM solutions of L-arginine-modified agents 3 and 4a. L-Citrulline-modified agents 7 and 8a were used as a negative
control; *L*-arginine was used as a positive control. The rate of metabolism was judged by the time to return to baseline reading in the amperogram and the amount was judged by the maximum peak height.

**Conclusions**

In this study, we have described PARACEST MRI CAs capable of detecting NOS enzymatic activity directly. From the chemical structure point of view, the agents are ligands composed of amino acid decorated-cyclens which have been metallated with Dy$^{3+}$ and Tm$^{3+}$. The complexes feature terminal mono- or dipeptide arms incorporating *L*-arginine as a substrate for NOS or *L*-citrulline as a product of enzymatic transformation mediated by NOS. The structure of CAs described in this work resembles other dipeptidic PARACEST MRI CAs$^{13,30}$ developed in our laboratory and utilized for *in vivo* temperature and pH mapping.$^{15,16}$

A detailed evaluation of CEST spectra associated with the CAs revealed that Tm$^{3+}$ complexes are not suitable for the detection of NOS enzymatic activity as there is no difference in chemical shift of the CEST effect (amide protons), when the *L*-arginine-modified agents and *L*-citrulline modified counterparts are compared. The Dy$^{3+}$-metallated complexes are more favourable as NOS enzymatic activity sensors because they produced a 2 ppm (8a, Gly linker present) or 5 ppm (7, Gly linker absent) difference of the chemical shift for the proximal amide protons in their CEST spectra of the *L*-arginine- vs. *L*-citrulline-containing agents, respectively. The small change in the frequency and intensity of the CEST signal is attributed to modest changes the conformation of the ligand ($\delta$ shift) and amide proton exchange rates (intensity change) due to the structural change of the pendant arms and concomittant changes in their solvation.
The selected agents which showed promise for the detection of NOS enzymatic activity by PARACEST MRI (i.e. 3, 4a) were subjected to a qualitative NOS enzymatic activity assay. This assay revealed only agent 4a (Gly linker) was metabolized by NOS. This observation is consistent with the results observed by other researchers\textsuperscript{28} and provides a useful insight for the design of other oligopeptide-based CAs/enzyme inhibitors capable of detecting/inhibiting NOS enzymatic activity.

When compared to the previously reported “turn off” NO PARACEST MRI based sensor 6,\textsuperscript{29} wherein the presence of NO is detected by the disappearance of the CEST effects associated with 6, CA 4a has two notable differences. Firstly, NOS enzymatic activity is detected by a change in signal, i.e. the difference of the amide proton chemical shift in the CEST spectra associated with the L-arginine- vs. L-citrulline-modified agents. Secondly, rather than detect the short lived, diffusible NO molecule, as in the case of 6,\textsuperscript{29} 4a is directly metabolized by the enzyme and potentially may respond to the level of expression of NOS. Based on analogy with previously described cell wall permeable complexes of general structure 5 (Scheme 2),\textsuperscript{28} the L-arginine-decorated CA 8a is also anticipated to be internalized within the cell compartment. Taken altogether, Dy\textsuperscript{3+} DOTAM-Gly-Arg-OH (8a) may find use for imaging NOS enzymatic activity in cell culture and animal models overexpressing NOS. However, before this can be done, more quantitative evaluation including the rate and extent of the metabolism of the agent by the various isoforms of NOS is required.

Acknowledgements

We thank The University of Western Ontario (The Major Academic Development Fund Grant
for RHEH) and The Ontario Institute for Cancer Research (OICR) and The Natural Sciences and Engineering Research Council of Canada (NSERC) for the financial support for this work. We also gratefully acknowledge Prof. J. M. Chong of the University of Waterloo for assistance with optical rotation measurements.


