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Synthesis and characterization of lipophilic cationic Ga(III) complexes based on the H_2*CHX*dedpa and H_2dedpa ligands and their $^{67/68}$Ga radiolabeling studies†

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^{68}Ga is an attractive isotope for incorporation into a positron-emission tomography (PET) imaging agent, and is finding use as an alternative generator-produced isotope to ^{99m}Tc particularly in imaging of myocardial perfusion. We have synthesized six new chelating ligands based on our previously reported H_2dedpa and H_2*CHX*dedpa scaffolds (*CHX* = cyclohexyl, H_2dedpa = 1,2-[[carboxypyridin-2-yl] methylamino]ethane). These ligands are designed to incorporate several lipophilic appendages at the secondary nitrogens, and upon coordination to ^{68}Ga(III) will form lipophilic, cationic complexes designed to mimic the properties of other clinically relevant myocardial perfusion imaging agents. The non-radioactive Ga(III) complexes were prepared and characterized by NMR spectroscopy; each ligand retained its predicted hexadentate N_4O_2 binding to Ga(III). The radiolabeling properties of the six ligands were assessed using the longer-lived ^{68}Ga surrogate, ^{67}Ga. The absence of 'free' uncomplexed ^{67}Ga in the HPLC radio-chromatograms indicated >99% radiochemical yields (10 minutes at ambient temperature, ligand concentrations of 10^{-4} M). However, the *N*,*N*′-benzyl functionalized derivatives displayed multiple peaks corresponding to the presence of additional ^{67}Ga-complexes which complicated further study. Selected ^{67}Ga-*CHX*dedpa complexes were tested for *in vitro* stability against the metal-binding protein apo-transferrin, and were found to be sufficiently stable (>80%) in a 2 h challenge assay, suggesting that alternative *N*,*N*′-alkylated derivatives which introduce more lipophilic character will be of interest in future studies.

Introduction

Myocardial perfusion imaging (MPI) is a common and powerful technique used to illustrate the function of the heart muscle. Its main application is in diagnosis of coronary artery disease (CAD) and it is of great importance for early detection of heart disease, one of the leading causes of death in the western world.[1] The aim of MPI is to visualise the perfusion of the heart muscle; this blood flow analysis relies on the ensuing accumulation of a radiopharmaceutical in the heart. Areas with good blood circulation will show increased radionuclide uptake compared to areas with poor flow or damaged tissue, thus defining the extent and severity of disease.[1–4]

MPI is dominated by the use of ^{99m}Tc labelled agents for single-photon emission computed tomography (SPECT) imaging. The FDA approved radiopharmaceuticals ^{99m}Tc-sestamibi (Cardiolite), ^{99m}Tc-tetrofosmin (Myoview), and ^{99m}Tc-teboroxime (Fig. 1) are among the most popular agents used clinically. It is believed that the elevated myocardial accumulation of these ^{99m}Tc tracers is rooted in the physical properties of the complexes: these are highly lipophilic and monocationic or neutral charged complexes which contain at least two ether-like linkages.[4,5] Limitations such as low first-pass extraction and high liver uptake of such agents have sustained interest in developing new radiopharmaceuticals for MPI. The ideal perfusion radiotracer should have high heart uptake and retention, with minimal liver and lung uptake to create diagnostically useful images.[4]

The advantageous properties of the β^+ emitter ^{68}Ga ($t_{1/2}$ = 68 min) make it an ideal candidate for incorporation into an MPI agent for positron-emission tomography (PET). A ^{68}Ga-based perfusion imaging agent would possess the specific advantages of spatial resolution obtainable by PET compared to SPECT, whilst relying on the convenience of a generator-

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† Electronic supplementary information (ESI) available: ^{1}H, ^{13}C, ^{1}H-^{1}H COSY and variable temperature NMR spectra of selected precursors, ligands, and complexes. See DOI: 10.1039/c6ra24070d

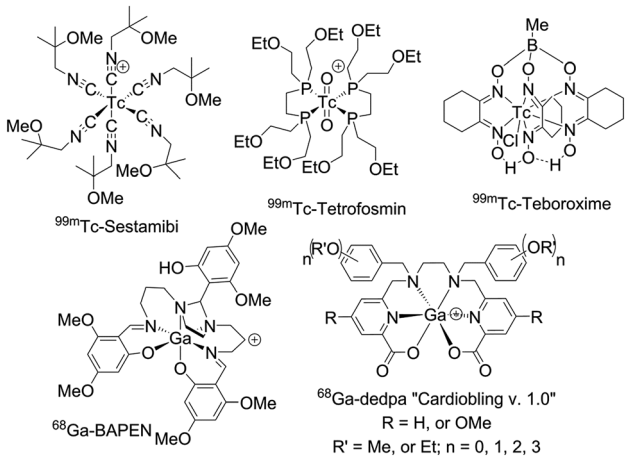


Fig. 1 Commercially available ^{99m}Tc SPECT agents (top) and recently investigated ^{68}Ga PET agents (bottom) for myocardial perfusion imaging.

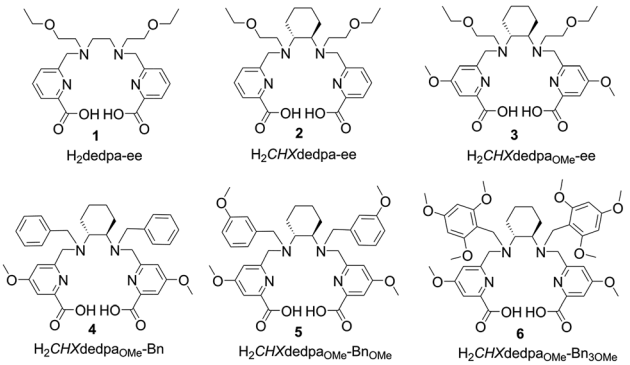


Fig. 2 Structures of novel lipophilic dedpa^{2-} and *CHX*dedpa^{2-} analogues prepared herein (**1–6**).

produced isotope.[6–8] Consequently, much effort has been made towards development of new lipophilic, cationic Ga-complexes with myocardial retention and low liver uptake.[9–12] Specifically, bis- and tris(salicylaldimine) chelators such as tris(4,6-dimethoxysalicylaldimin-*N*,*N*′-bis(3-aminopropyl)-*N*,*N*′-ethylenediamine) (BAPEN)[9,13] and its derivatives[10,11,14] (Fig. 1) showed some promising properties for myocardial perfusion imaging with ^{68}Ga, but non-trivial labelling procedures have limited the progression of these ligands towards the clinic.

Our recent reports of the promising acyclic hexadentate $^{67/68}$Ga chelator H_2dedpa which forms a monocationic complex with Ga(III) sparked interest in developing derivatives which could potentially lead to compounds of increased and prolonged heart uptake.[12,15] A small library of H_2dedpa analogues of varying lipophilicity which possess benzyl residues and a variety of methoxy or ethoxy substituents was evaluated *in vivo*.[12] Despite modest biodistribution results showing initial high uptake and slow clearance from the liver, two complexes showed persistent heart uptake over the course of 2 h.[12] These conclusions have motivated us to develop a second class of dedpa^{2-} analogues which possess varying degrees of lipophilicity.

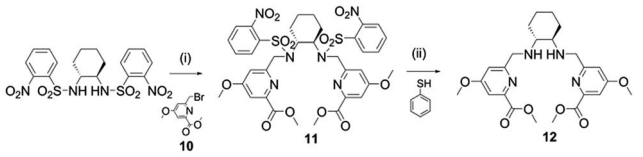
Herein, we report five new derivatives of the chiral acyclic chelator H_2*CHX*dedpa,[16] and one new derivative of H_2dedpa, that have been functionalized to include varying lipophilic appendages. Much like H_2dedpa, the chiral chelator H_2-*CHX*dedpa forms $^{68/67}$Ga complexes of high thermodynamic stability (log $K_{ML} = 27.61(8)$), shows promising kinetic inertness *in vitro*, and exhibits fast and mild $^{67/68}$Ga complexation kinetics (radiochemical yields >99% in 10 minutes at RT at ligand concentrations of 10^{-5} M), making H_2*CHX*dedpa an ideal candidate for further elaboration into a Ga(III) radiopharmaceutical.[16,17] The six novel lipophilic analogues prepared here contain either diethyl ether-like or benzyl linkages with varying number of methoxy groups added onto the benzyl or 4-position of the picolinate moieties (Fig. 2). Moreover, the cyclohexanediamine backbone of *CHX*dedpa^{2-} inherently introduces a degree of added lipophilicity. The addition of ether groups was found to reduce radiotracer liver uptake of cationic ^{99m}Tc complexes resulting in improved target/background ratios.[4] We hope to take advantage of this theory with some of our derivatives by incorporating such ether linkages in our ligands. Indeed, many of the structural components that have been added to this new class of compounds (**1–6**) have been strategically chosen to mimic other ^{99m}Tc and ^{68}Ga MPI agents under investigation. The gallium coordination potential, labelling properties, *in vitro* stability, and log *P* of the six chelating ligands will be explored to assess their potential as myocardial perfusion imaging agents.

Results and discussion

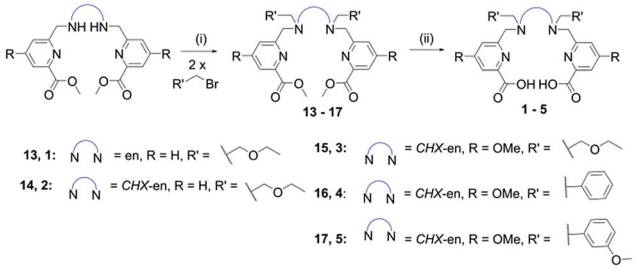
Ligand synthesis and characterization

The synthesis of pro-ligands **3–6** required the use of methyl 6-(bromomethyl)-4-methoxypicolinate (**10**) which was prepared using a previously reported synthesis.[12] Using the methoxy-modified bromopicolinate (**10**), the precursor **12** was prepared using nosyl protection/deprotection chemistry (Scheme 1) analogous to that used in the preparation of previous H_2dedpa and H_2*CHX*dedpa analogues.[16,18] The precursor **12** was nicknamed 'Me_2*CHX*dedpa$_{OMe}$' because it is similar to the previously synthesized analogue Me_2*CHX*dedpa[16,19] in which there are no methoxy (OMe) groups appended to the 4-position of the picolinate moieties.

A similar synthetic pathway was used to prepare five ligands (**1–5**) (Scheme 2). Preparation began with *N*,*N*′-alkylation of Me_2dedpa,[15,18] Me_2*CHX*dedpa,[16] or Me_2*CHX*dedpa$_{OMe}$ (**12**) with a slight excess of the appropriate bromo-alkylating agent under basic condition (K_2CO_3) to yield the methyl ester protected precursors **13–17**. For alkylation reactions with 1-bromo-2-ethoxyethane (Br-ee), 2-ethoxyethane (ee) also replaced the methyl protecting group of one or both of the carboxylic acid(s) which resulted in a mixture of products. For precursors **14** and **15**, separation and isolation of the over-alkylated products *via* column chromatography was not possible, consequently ^{1}H and ^{13}C NMR spectra of pure compound were not collected. Nonetheless, hydrolysis of all ester protected ligands was accomplished in the final deprotection step using lithium hydroxide



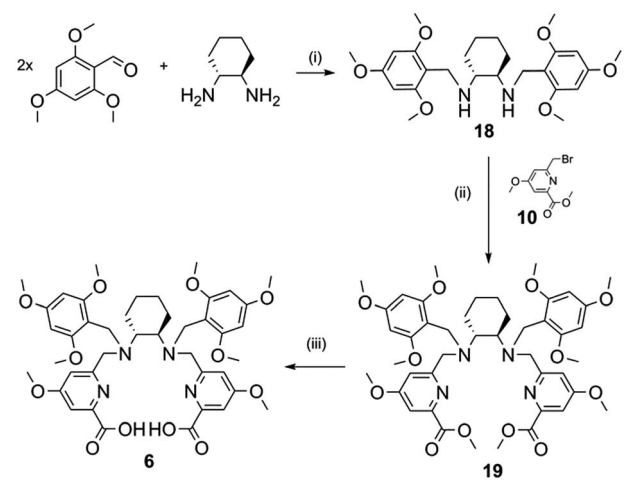
Scheme 1 Synthesis of precursor **12**, Me_2*CHX*$dedpa_{OMe}$. Reagents and conditions: (i) methyl-6-(bromomethyl)-4-methoxypicolinate (2 equiv.), K_2CO_3, CH_3CN, 65 °C, 3 d; (ii) thiophenol (2.1 equiv.), K_2CO_3, THF, RT, 3 d.



Scheme 2 Synthesis of pro-ligands H_2dedpa-ee (**1**), H_2*CHX*dedpa-ee (**2**), H_2*CHX*$dedpa_{OMe}$-ee (**3**), H_2*CHX*$dedpa_{OMe}$-Bn (**4**), and H_2-*CHX*$dedpa_{OMe}$-Bn_{OMe} (**5**). Reagents and conditions: (i) Na_2CO_3, CH_3CN, 75 °C, 2 d; (ii) LiOH (5 equiv.), THF/H_2O (3 : 1), RT, 30 min.

in order to yield all five ligands **1–5** as pure compounds after RP-HPLC purification.

The commercial availability of an aldehyde starting material (2,4,6-trimethoxybenzaldehyde) allowed ligand **6** to be prepared in three synthetic steps, and avoided the need for nosyl protection and deprotection chemistry which was utilized for the preparation of ligands **1–5** through intermediate **12**. Pro-ligand **6** was synthesized in three steps (Scheme 3) through imine formation followed by reductive amination of 2,4,6-trimethoxybenzaldehyde with (1*R*,2*R*)-(−)-cyclohexanediamine to yield **18** which was subsequently used in an *N*,*N*′-alkylation reaction with methyl 6-(bromomethyl)-4-methoxypicolinate (**10**). Finally, the methyl-ester protected ligand **19** was deprotected using lithium hydroxide as was done for the previous ligands to give H_2*CHX*$dedpa_{OMe}$-Bn_{3OMe} (**6**).



Scheme 3 Synthesis of pro-ligand H_2*CHX*$dedpa_{OMe}$-Bn_{3OMe} (**6**). Reagents and conditions: (i) (a) ethanol, 0 °C to RT, 18 h; (b) $NaBH_4$, 0 °C, 1.5 h; (ii) methyl-6-(bromomethyl)-4-methoxypicolinate (2.1 equiv.), Na_2CO_3, CH_3CN, reflux to RT, 2 d. (iii) LiOH (5 equiv.), THF/H_2O (3 : 1), RT, 30 min.

^{1}H NMR spectra of several pro-ligands at 25 °C revealed very broad and unresolvable peaks which precluded ^{13}C NMR data collection. It was hypothesized that intramolecular hydrogen-bonding of the acidified ligand was the cause of such peak broadening; however, NMR samples in deuterated water adjusted to pD of >7.5 using NaOD resulted in only a slight sharpening of peaks. Consequently, variable-temperature (VT) NMR was used to collect ^{1}H NMR spectra of the ligands at temperatures 25 to 75 °C, and resulted in an apparent sharpening of peaks with increasing temperatures (Fig. 3 and S1†); ^{13}C NMR spectra were also obtained at the highest temperature.

Ga(III) complexation

After successful characterization of all six pro-ligands, preparation of metal complexes was attempted with non-radioactive Ga(III). The $dedpa^{2-}$ and *CHX*$dedpa^{2-}$ based ligands **1–6** reported herein were all able to complex Ga(III). In most cases, upon addition of appropriate equivalency of $Ga(ClO_4)_3$ salt solution to a solution of pro-ligand, precipitate formation was observed; the precipitate was isolated and its identity as the Ga-complex was confirmed by HR ESI-MS, and NMR spectroscopy. Stacked ^{1}H NMR spectra of two ligands and their corresponding Ga-complexes are represented in Fig. 4 and S2.†

The diagnostic diastereotopic splitting of hydrogens upon metal-complexation is seen in the NMR spectra of the $dedpa^{2-}$ based ligand **1** (Fig. 4). The two singlets (labelled A and B), assigned to the methylene hydrogens alpha to the pyridine ring and the hydrogens on the ethylenediamine bridge, respectively, of the ligand **1**, become diastereotopic upon gallium complexation and each singlet splits into two doublets (labelled α,α′ and β,β′).

Diastereotopic splitting is visible in pro-ligands **2–6** due to the inherently chiral nature of the *CHX*$dedpa^{2-}$ ligands. Nonetheless, shifts in the ^{1}H resonances of the solution NMR data (Fig. S2†) can be used to confirm successful Ga(III) complexation.

$^{67/68}$Ga radiochemistry and log *P* determination

With a half-life of 3.26 days, ^{67}Ga is used as a longer-lived surrogate for ^{68}Ga complexes, and can be used in studies requiring longer time points by monitoring its γ-emission. ^{68}Ga was also used in the radiolabeling studies herein, when ^{67}Ga was not available. In all cases, the concentration of chelator used for radiolabeling is in great excess relative to the radioisotope. Initial radiolabeling reactions were performed with ligand concentrations of 10^{-4} M and 1 mCi of ^{67}Ga in sodium acetate buffer at pH 4.5, the reaction mixture was left for 10 min at room temperature prior to injection into the HPLC.

The $dedpa^{2-}$ and both *CHX*$dedpa^{2-}$ ligands that possess 2-ethoxyethane functionalities off the secondary amines (**1**, and **2–3**, respectively) displayed one major sharp peak in the radio-

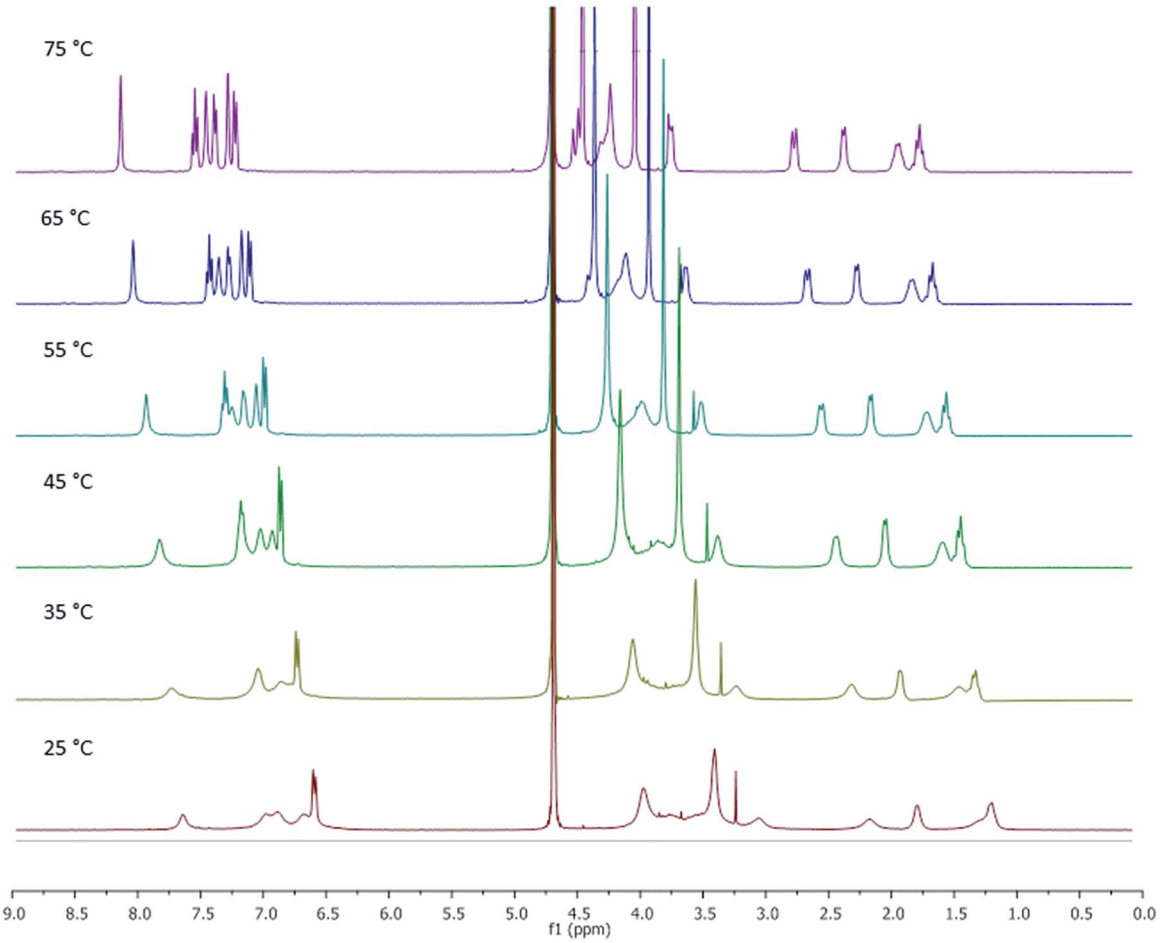


Fig. 3 VT ^{1}H NMR spectra of H_2*CHX*dedpa$_{OMe}$-*N*,*N*′-Bn$_{OMe}$ (**5**) (400 MHz, D_2O, 25–75 °C).

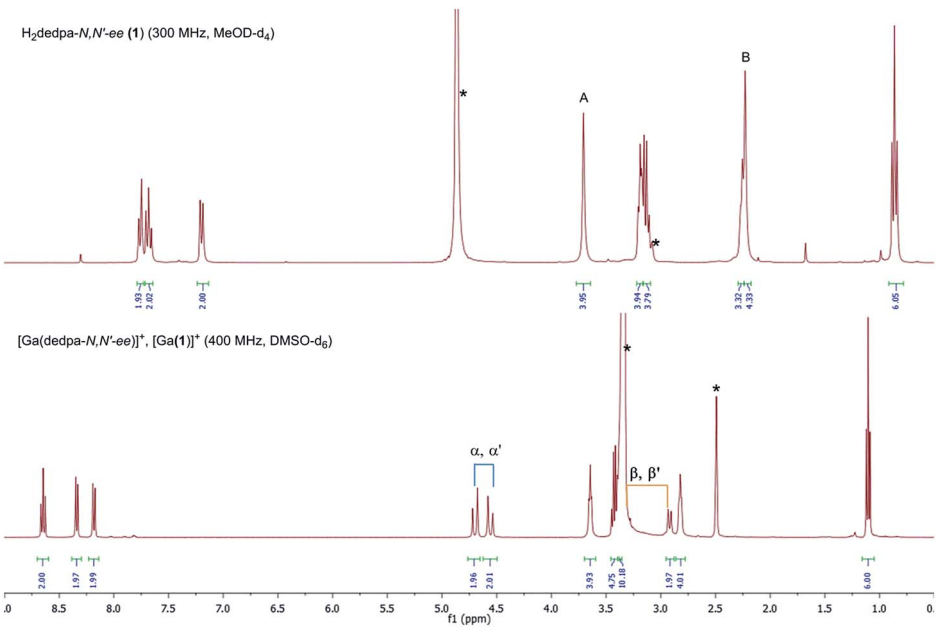


Fig. 4 ^{1}H NMR spectra at 25 °C of (top) H_2dedpa-*N*,*N*′-ee (**1**, 300 MHz, MeOD-d_4) and (bottom) [Ga(**1**)]$^+$ (400 MHz, DMSO-d_6), highlighting diastereotopic splitting of hydrogen resonances upon Ga-complexation. Peak A (top spectrum) splits into peaks α and α′ (bottom spectrum), and peak B (top spectrum) splits into peaks β and β′ (bottom spectrum). *Residual solvent peak.

chromatogram (99, 95, 99% RCY, respectively) assigned to the ^{67}Ga-labelled complex. The remaining three *CHX*dedpa$_{OMe}^{2-}$ ligands which possess benzyl functionalities attached to the secondary amines with none, one, or three methoxy groups per benzene ring (**4**, **5**, and **6**, respectively) display atypical ^{67}Ga labelling radio-traces. Despite the lack of free radioisotope present in the HPLC radio-chromatogram (evinced by absence of a peak at $t_R = 2$–3 min), three sharp peaks of varying intensity are present over a span of about 3 min (Table 1 and Fig. 5). It is possible that small impurities in the ligand solution are able to complex with ^{67}Ga more efficiently that the desired ligand, resulting in multiple peaks in the radio-chromatogram. Ligand impurities, even in small concentrations relative to the pure ligand, can greatly affect labelling efficiency/yield since radiolabeling conditions dictate that the concentration of the chelator is in great excess compared to that of the radioisotope. The peak with the latest retention time in the RP-HPLC radio-trace is believed to be the desired ^{67}Ga-complex, since the chelating ligand will likely be the most lipophilic molecule in the reaction mixture which will subsequently form the most lipophilic ^{67}Ga-complex. Further confirmation to correctly identify the desired ^{67}Ga-complex was performed by injecting an aliquot of the pre-formed non-radioactive Ga-complex ([Ga(**6**)]$^+$) into the HPLC under the same conditions as the ^{67}Ga-complex and revealed one major peak in the UV-Vis absorbance trace (t_R = 14.38 min, Fig. S28†) which matches the latest peak in the radio-trace (t_R = 14.57 min, Fig. 5). Given the shorter retention times of the two additional radio-peaks, the impurities are believed to be the mono-alkylated and non-alkylated by-products. The chelator purity was deemed sufficiently pure post-purification by RP-HPLC by re-injection of a small aliquot of chelator which resulted in one sharp peak and by NMR spectroscopy, so it seemed surprising that the ^{67}Ga radio-traces of the above chelating ligands exhibited three peaks of similar intensity. A second hypothesis is also plausible, one in which multiple radiolabeling peaks arise from ligand radiolysis. If ligand radiolysis is indeed occurring during the course of the radiolabeling reaction, these ligands would be deemed sufficiently unstable and unfit for further testing *in vitro* or *in vivo*. The steric bulk imposed by the benzyl groups on pro-ligands **4**, **5**, and **6** may also contribute to the instability of the chelators, resulting in ease of radiolysis or decomposition

Table 1 Radiolabeling yields, corresponding HPLC radio-chromatogram retention times, and log *P* values for ^{67}Ga-labelled dedpa^{2-} and *CHX*dedpa^{2-} chelators **1**–**6**

Complex	t_R/min (RCY/%)	log *P*
[^{67}Ga(**1**)]$^+$	8.85 (99)	−2.67
[^{67}Ga(**2**)]$^+$	11.50 (99)	−1.76
[^{67}Ga(**3**)]$^+$	12.68 (93)	−1.53
[^{67}Ga(**4**)]$^+$	11.15[a] (20), 11.40[a] (27), 13.42 (32)	ND[b]
[^{67}Ga(**5**)]$^+$	10.91[a] (23), 11.76[a] (53), 13.97 (24)	ND[b]
[^{67}Ga(**6**)]$^+$	11.76[a] (23), 12.34[a] (54), 14.57 (21)	ND[b]

[a] Retention time of ^{67}Ga-labelled by-products produced from ligand impurities or ligand radiolysis. [b] Not determined.

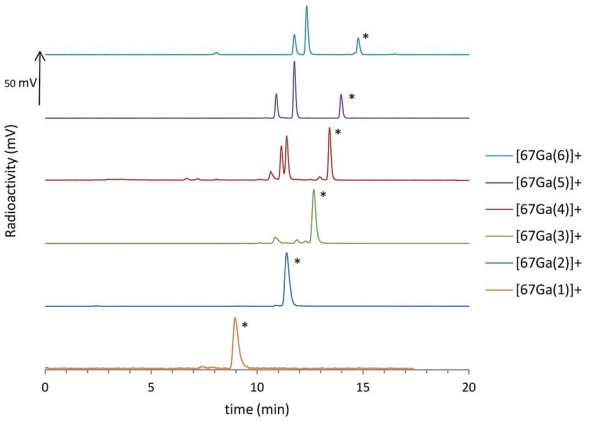


Fig. 5 HPLC radio-chromatograms of ^{67}Ga labelling reactions with lipophilic dedpa^{2-} or *CHX*dedpa^{2-} chelators **1**–**6**. * = desired radio-labelled product.

overtime. In an effort to distinguish whether or not the extra ^{67}Ga-labelled products arise from ligand impurities or radiolysis products, ligand **5** was radiolabelled with 1 mCi of ^{67}Ga and analysed by radio-HPLC at 10 minutes and again at 1 hour. It was hypothesized that if the extra ^{67}Ga-products at 10.91 and 11.76 min in the radio-trace were formed *via* ligand radiolysis, the areas of these peaks would increase over time while the assumed product peak at 13.97 min would decrease as the radiative emissions of ^{67}Ga induce more damage to the radio-complex. However, the relative peak height and areas did not change overtime (% RCYs were within ±2% of each other, Fig. S29†). Furthermore, ligand **6** was radiolabelled with 0.1 mCi of ^{68}Ga at constant ligand concentration (10^{-4} M) and analysed *via* RP-HPLC after 10 minutes at ambient temperature. Again, the ratio (*via* % area) of the three ^{68}Ga-labelled peaks in the radio-trace did not change significantly (23, 60, 16% compared to 23, 54, and 21% for $^{67/68}$Ga-products from shortest to longest retention time, Fig. S30†). We predicted that if indeed the extra peaks at shorter retention were due to ligand radiolysis the relative areas of impurity products would change compared to the desired product peak between the [^{67}Ga(**6**)]$^+$ and [^{68}Ga(**6**)]$^+$ radiolabeling reaction, since radiolysis is dependent on factors such as the time, amount, and type of radiation exposure; ^{67}Ga, a gamma and Auger electron emitter, would inflict a different destructive dose to the ligand compared to the primarily positron emitter ^{68}Ga. The observation of little to no change in the radio-traces of both the ^{67}Ga- and ^{68}Ga-labelled products of ligand **6** further supports the hypothesis that ligand impurities in the benzyl-alkylated ligands **4**–**6** have contributed to the presence of multiple peaks in the radiolabeling reactions. The steric bulk imposed by the benzene rings' close proximity to the cyclohexane ring may be contributing to the instability of pro-ligands **4**–**6**, causing ligand decomposition *via* de-alkylation of one or both of the secondary amines.

Determination of partition coefficients (log *P*) for myocardial perfusion imaging agents is of particular importance because it is believed that differences in heart uptake and liver clearance are caused by the compound lipophilicity which affects the

ability to cross the plasma and mitochondrial membranes (the accepted mechanism of heart uptake).[4] It has been hypothesized that cationic ^{99m}Tc radiotracers should exhibit log *P* values ranging from 0.5–1.2 in order to achieve optimal myocardium retention and fast liver clearance concurrently;[4] therefore, we sought to find a ^{68}Ga-*CHX*dedpa complex which exhibited a partition coefficient within this range.

Due to the presence of multiple species in the ^{67}Ga-labeling reactions of benzyl functionalized ligands **4–6**, (*vide supra*) log *P* values were not determined, since the value obtained through experimental procedures would not accurately depict the lipophilicity of the desired radiometal–ligand complex but instead would be influenced by the mixture of the relatively hydrophilic ^{67}Ga by-products present in the reaction mixture. log *P* of the three ^{67}Ga-ligands functionalized with ee groups (**1–3**) were determined to be −2.67, −1.76, and −1.53, respectively. The structure of ligands **1** and **2** differ only by the presence on the cyclohexane ring on **2**, consequently by comparing directly the log *P* values of $[^{67}Ga(\mathbf{1})]^+$ and $[^{67}Ga(\mathbf{2})]^+$ one can discern the lipophilic character caused by the '*CHX*' backbone. The addition of the cyclohexane ring to the ethylenediamine backbone resulted in a shift of 0.91 log *P* units between $[^{67}Ga(\mathbf{1})]^+$ and $[^{67}Ga(\mathbf{2})]^+$. Moreover, the added lipophilic character of $[^{67}Ga(\mathbf{3})]^+$ due to the addition of two extra methoxy groups off the 4-position of the picolinate moiety are represented by a shift of 0.23 log *P* units compared to $[^{67}Ga(\mathbf{2})]^+$ (in which there is no additional methoxy groups). The comparatively hydrophilic nature of the three evaluated complexes renders their log *P* values well out of the optimal range predicted by Liu.[4] Nonetheless, valuable lessons on ligand design of lipophilic H_2dedpa and H_2*CHX*dedpa analogues have been drawn from the findings of the structures (**1–6**) studied herein.

Human apo-transferrin stability studies

Selected ^{67}Ga-complexes were investigated for their stability in a human apo-transferrin challenge. Compared to unaltered $[^{67}Ga(dedpa)]^+$ which remained 99% intact after 2 hours,[15] the new *CHX*dedpa^{2-} derivatives with *N*,*N*-functionalization displayed moderately reduced stabilities of 81, 86, and 80% for $[^{67}Ga(\mathbf{2})]^+$, $[^{67}Ga(\mathbf{3})]^+$, and $[^{67}Ga(\mathbf{5})]^+$ after 2 hours, respectively (Table 2). It should be noted that the $[^{67}Ga(\mathbf{5})]^+$ labelling reaction used for the stability assay contained a mixture of labelled products; as such the results of the transferrin challenge are representative of the bulk and not the desired single ^{67}Ga-chelate complex. The *in vitro* stability of the pure ^{67}Ga-complex ($[^{67}Ga(CHXdedpa_{OMe}\text{-}Bn_{OMe})]^+$) may differ from the stability of the other side products in the mixture, such as the mono-alkylated ligand.

Table 2 *In vitro* stability of selected ^{67}Ga-labelled *CHX*dedpa^{2-} complexes (**2**, **3**, **5**) against human apo-transferrin (37 °C, 2 h), with stability shown as the percentage of intact ^{67}Ga complex

Complex	15 min (%)	1 h (%)	2 h (%)
$[^{67}Ga(\mathbf{2})]^+$	86.3	84.8	81.4
$[^{67}Ga(\mathbf{3})]^+$	85.0	85.9	85.6
$[^{67}Ga(\mathbf{5})]^{+a}$	97.5	82.9	80.0

[a] Multiple ^{67}Ga products present in reaction mixture.

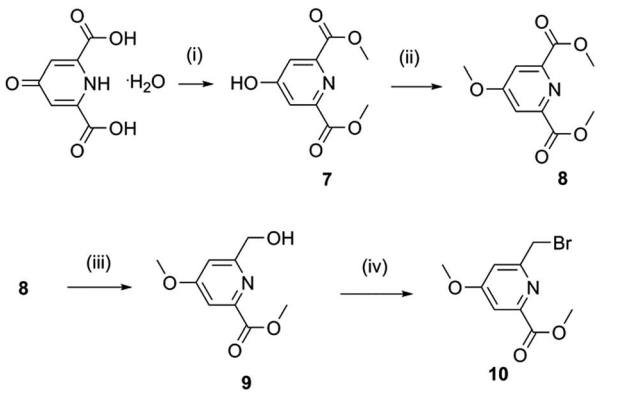
Experimental

Materials and methods

All solvents and reagents were purchased from commercial suppliers (Sigma Aldrich, TCI America, Fisher Scientific) and were used as received. Human apo-transferrin was purchased from Sigma Aldrich. The analytical thin-layer chromatography (TLC) plates were aluminum-backed ultrapure silica gel 60 Å, 250 μm thickness; the flash column silica gel (standard grade, 60 Å, 40–63 μm) was provided by Silicycle. ^{1}H and ^{13}C NMR spectra were recorded at 25 °C unless otherwise noted on Bruker AV300, AV400, or AV600 instruments; NMR spectra are expressed on the δ scale and referenced to residual solvent peaks. Low-resolution mass spectrometry was performed using a Waters ZG spectrometer with an ESCI electrospray/chemical-ionization source, and high-resolution electrospray-ionization mass spectrometry (HR-ESI-MS) was performed on a Micromass LCT time-of-flight instrument at the Department of Chemistry, University of British Columbia. ^{67}Ga-(chelate) apo-transferrin stability experiments were analyzed using GE Healthcare Life Sciences PD-10 desalting columns (size exclusion for MW < 5000 Da) and counted with a Capintec CRC 15R well counter. The HPLC system used for analysis and purification of nonradioactive compounds consisted of a Waters 600 controller, Waters 2487 dual wavelength absorbance detector, and a Waters delta 600 pump. Phenomenex Synergi Hydro-RP 80 Å columns (250 mm × 4.6 mm analytical or 250 mm × 21.2 mm semipreparative) were used for purification of several of the deprotected ligands. Analysis of radiolabelled complexes was carried out using a Phenomenex Synergi 4 μ Hydro-RP 80A analytical column (250 × 4.60 mm 4 μm) using either a Waters Alliance HT 2795 separation module equipped with a Raytest Gabi Star NaI (Tl) detector and a Waters 996 photodiode array (PDA) or an Agilent HPLC system equipped with a model 1200 quaternary pump, a model 1200 UV absorbance detector (set at 250 nm), and a Raytest Gabi Star NaI(Tl) detector. $^{67}GaCl_3$ was cyclotron-produced and provided by Nordion as ~0.1 M HCl solution. ^{68}Ga was obtained from an Eckert & Ziegler (Berlin, Germany) IGG100 ^{68}Ge/^{68}Ga generator and was purified according to previously published procedures[20] using a DGA resin column.

Precursors **7–10** (Scheme 4) were prepared using a modified synthesis according to literature protocol of a similar analogue.[21]

Dimethyl-4-hydroxypyridine-2,6-dicarboxylate (7). Chelidamic acid (4.00 g, 19.9 mmol) was suspended in methanol (40 mL) at −10 °C. To this murky solution, thionyl chloride (11.4 mL, 157 mmol, ~8 equiv.) was added slowly. The solution, which turned clear upon addition, was stirred and allowed to warm to room temperature overnight. The reaction mixture was then refluxed for 2 hours, subsequently cooled to room temperature and concentrated *in vacuo*. The resulting solids



Scheme 4 Synthesis of precursors **7–10**. Reagents and conditions: (i) $SOCl_2$, CH_3OH; (ii) methyl iodide, K_2CO_3, CH_3CN, reflux; (iii) $NaBH_4$, CH_3OH/CH_2Cl_2 (2 : 1), 0 °C, 2 h; (iv) (1) PBr_3, $CHCl_3$, 0 °C–RT, 1.5 h, (2) 0 °C, aq K_2CO_3.

were redissolved in hot ethanol, and cooled in freezer to recrystallize. The recrystallized product was collected by vacuum filtration, and the filtrate was concentrated to half-volume, and the recrystallization procedure was repeated to recover more solid product (2.597 g, 62%). ^{1}H NMR (400 MHz, MeOD) δ 7.88 (s, 2H), 5.01 (s, 3H). ^{13}C NMR (101 MHz, MeOD) δ 174.8, 161.5, 145.4, 117.7, 54.8. MS (ES^+) m/z = 212.3 $[M + H]^+$.

Dimethyl-4-methoxypyridine-2,6-dicarboxylate (8). Compound **7** (1.941 g, 9.2 mmol) and potassium carbonate (3.9 g, 28.2 mmol, ~3 equiv.) were suspended in acetonitrile (50 mL). To this solution, methyl iodide (1.15 mL, 18.4 mmol, 2 equiv.) was added, and the murky solution was stirred at reflux overnight. The resultant mixture was cooled to room temperature, filtered by vacuum filtration to remove excess salts and the filtrate was concentrated *in vacuo*. The crude solid was purified by column chromatography (CombiFlash Rf automated column system; 80 g HP silica; A: hexanes, B: ethyl acetate, 100% A to 100% B gradient) to yield the product as a white solid (1.384 g, 67%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (d, J = 4.9 Hz, 2H), 3.84 (s, 6H), 3.83 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 164.4, 149.2, 113.4, 55.5, 52.5.

Methyl-6-(hydroxymethyl)-4-methoxypicolinate (9). Compound **8** (1.384 g, 6.15 mmol) was dissolved in methanol/dichloromethane (2 : 1, 90 mL) and cooled to 0 °C. Sodium borohydride (0.233 g, 6.15 mmol) was added in small portions, and the reaction mixture was stirred at 0 °C for 2.5 hours. The reaction mixture was quenched with water (50 mL), and the organics were decanted and collected. The aqueous phase was extracted with dichloromethane (8 × 50 mL), organics were collected, dried over $MgSO_4$ and all the organics were concentrated *in vacuo*. The crude product was purified by column chromatography (CombiFlash Rf automated column system; 40 g HP silica; A: hexanes, B: ethyl acetate, 100% A to 100% B gradient) to yield the product as a white solid (0.871 g, 72%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (d, J = 2.3 Hz, 1H), 7.03 (d, J = 2.2 Hz, 1H), 4.70 (s, 2H), 3.84 (s, 3H), 3.79 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.2, 165.5, 163.0, 148.3, 110.4, 109.0, 64.7, 55.5, 52.8.

Methyl-6-(bromomethyl)-4-methoxypicolinate (10). To a solution of **9** (0.74 g, 3.75 mmol) in chloroform (50 mL) at 0 °C, PBr_3 (0.42 mL, 4.50 mmol, 1.2 equiv.) was added. The reaction mixture was allowed to warm to room temperature and stirred for 1.5 hours, while monitoring the reaction progress by TLC (R_f = 0.63, EtOAc). The mixture was subsequently quenched with cold saturated K_2CO_3 (40 mL), transferred to a separatory funnel and the organic layer was collected. The aqueous layer was then washed with dichloromethane (3 × 50 mL). The organic fractions were collected and dried over $MgSO_4$. The organic solution was concentrated *in vacuo* to yield the product as a pink solid (0.70 g, 71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.60 (d, J = 2.3 Hz, 1H), 7.17 (d, J = 2.3 Hz, 1H), 4.59 (s, 2H), 4.01 (s, 3H), 3.93 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.6, 164.6, 158.1, 148.5, 112.0, 110.3, 55.2, 52.4, 32.8.

Dimethyl-6,6′-((((1*R*,2*R*)-cyclohexane-1,2-diyl)bis(((2-nitrophenyl)sulfonyl)azanediyl))bis-(methylene))bis(4-methoxypicolinate) (11). To a stirred solution of *N*,*N*′-((1*R*,2*R*)-cyclohexane-1,2-diyl)bis(2-nitrobenzene-sulfonamide)[16] (0.28 g, 0.58 mmol, 1 equiv.) and K_2CO_3 (0.48 g, 3.6 mmol, 6 equiv.) in CH_3CN (10 mL), methyl-6-(bromomethyl)-4-methoxypicolinate (**10**) (0.31 g, 1.16 mmol, 2 equiv.) in CH_3CN (5 mL) was added, and the reaction mixture was stirred at reflux for 20 hours. The excess inorganic salts were subsequently removed by centrifugation (4000 rpm, 10 min) and filtration, and the filtrate was concentrated *in vacuo*. The crude product was purified by column chromatography (CombiFlash Rf automated column system; 24 g HP silica; A: hexanes, B: ethyl acetate, 70% A to 100% B gradient) to yield the product as a yellow solid (0.46 g, 94%). (TLC R_f (product) = 0.67, 100% EtOAc). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.92 (d, J = 7.7 Hz, 2H), 7.54 (m, 4H), 7.42 (m, 4H), 7.23 (m, 2H), 5.05 (d, J = 16.7 Hz, 2H), 4.61 (d, J = 16.7 Hz, 2H), 4.34 (s, 2H), 3.90 (s, 6H), 3.86 (s, 6H), 2.21 (d, J = 7.0 Hz, 2H), 1.71 (m, 2H), 1.42 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.8, 165.4, 158.5, 148.2, 147.7, 133.7, 133.0, 131.9, 131.3, 123.5, 111.5, 59.7, 55.8, 52.6, 50.2, 32.2, 25.2. MS (ES^+) m/z = 881.4 $[M + K]^+$.

Dimethyl-6,6′-((((1*R*,2*R*)-cyclohexane-1,2-diyl)bis(azanediyl))bis(methylene))bis(4-methoxypicolinate) (12). To a solution of dimethyl **11** (0.46 g, 0.54 mmol, 1 equiv.) and K_2CO_3 (0.90 g, 6.52 mmol, 12 equiv.) in tetrahydrofuran (20 mL), thiophenol (116 μL, 1.14 mmol, 2.1 equiv.) was added and the mixture was stirred at room temperature for 70 hours. The inorganic salts were subsequently filtered out after centrifugation (4000 rpm, 10 min) and the filtrate was concentrated *in vacuo*. The crude product was purified by column chromatography (CombiFlash Rf automated column system; 12 g HP silica; A: dichloromethane, B: 2% triethylamine in methanol, 100% A to 10% B gradient) to yield the product as a yellow oil (0.25 g, 99%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 (d, J = 2.1 Hz, 2H), 7.10 (d, J = 2.1 Hz, 2H), 3.99 (d, J = 15.0 Hz, 2H), 3.83 (d, J = 15.0 Hz, 2H), 3.81 (s, 6H), 3.72 (s, 6H), 3.01 (s, 2H), 2.23 (d, J = 8.7 Hz, 2H), 2.01 (m, 2H), 1.58 (d, J = 7.5 Hz, 2H), 1.07 (dd, J = 17.8, 8.9 Hz, 2H), 0.96–0.86 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.9, 165.7, 163.0, 148.6, 110.9, 110.0, 61.2, 55.4, 52.7, 52.0, 31.4, 24.7. MS (ES^+) m/z = 473.4 $[M + H]^+$.

Me_2dedpa-*N*,*N*′-ee (13). To a solution of Me_2dedpa[15,18] (0.151 g, 0.42 mmol, 1 equiv.) and K_2CO_3 (0.349 g, 2.52 mmol, 6

equiv.) in CH_3CN (5 mL), 2-bromoethyl ethyl ether (119 μL, 1.05 mmol, 2.5 equiv.) was added. The mixture was stirred for 24 h at room temperature. Inorganic salts were filtered out by centrifugation and filtration; the organic solution was concentrated *in vacuo*. The product was purified by column chromatography (CombiFlash Rf automated column system; 12 g HP silica; A: dichloromethane, B: methanol, 100% A to 5% B gradient) to yield pure product (0.159 g, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.94–7.86 (m, 2H), 7.75–7.65 (m, 4H), 3.92 (s, 4H), 3.90 (s, 6H), 3.42 (t, $J = 5.3$ Hz, 4H), 3.32 (q, $J = 7.0$ Hz, 4H), 2.74 (d, $J = 11.7$ Hz, 8H), 1.05 (t, $J = 7.0$ Hz, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 165.8, 147.0, 137.4, 126.3, 123.5, 68.5, 66.4, 60.8, 54.3, 53.0, 52.8, 15.1. MS (ES^+) $m/z = 503.3$ $[M + H]^+$, 525.3 $[M + Na]^+$.

Me$_2$*CHX*dedpa-*N*,*N*′-ee (14). To a solution of Me$_2$*CHX*dedpa[16] (0.231 g, 0.56 mmol, 1 equiv.) dissolved in CH_3CN (5 mL), 2-bromoethyl ethyl ether (133 μL, 1.18 mmol, 2.1 equiv.) and Na_2CO_3 (0.356 g, 3.36 mmol, 6 equiv.) were added. The reaction mixture was stirred at 75 °C for 48 h, subsequently cooled to RT and the salts were removed by vacuum filtration; the filtrate was concentrated *in vacuo*. The crude product was purified by column chromatography (CombiFlash Rf automated column system; 24 g HP silica; A: dichloromethane, B: methanol, 100% A to 10% B gradient) to yield the product as a fluffy off-white solid (0.185 g) in a mixture also containing over-alkylated products where 2-ethoxyethane is attached to the ester function(s). The mixture could not be further separated. MS (ES^+) $m/z = 557.4$ $[M + H]^+$.

Me$_2$*CHX*dedpa$_{OMe}$-*N*,*N*′-ee (15). To a solution of Me$_2$-*CHX*dedpa$_{OMe}$ (**12**) (79 mg, 0.17 mmol, 1 equiv.) in CH_3CN (2 mL), 1-bromo-2-ethoxyethane (66 μL, 0.58 μmol, 3.5 equiv.) and Na_2CO_3 (106 mg, 1.00 mmol, 6 equiv.) were added. The reaction mixture was stirred at 80 °C for 96 h. Excess salts were removed by centrifugation (4000 rpm for 10 min) and the filtrate was concentrated *in vacuo*. The crude product was purified by column chromatography (CombiFlash Rf automated column system; 24 g HP silica; A: DCM, B: methanol, 100% A to 20% B gradient) and the product (yellowish oil (122 mg)) was obtained as a mixture of product 6.7 and over-alkylated products where 2-ethoxyethane is attached to the ester function(s). The mixture could not be further separated. MS (ES^+) $m/z = 639.6$ $[M + Na]^+$.

Me$_2$*CHX*dedpa$_{OMe}$-*N*,*N*′-Bn (16). To a solution of Me$_2$-*CHX*dedpa$_{OMe}$ (**12**) (110 mg, 0.220 mmol) in CH_3CN (2 mL), bromobenzene (68 μL, 0.576 mmol, 2.5 equiv.) and Na_2CO_3 (0.15 g, 1.38 mmol, 6 equiv.) were added. The reaction mixture was stirred at 60 °C for 36 hours. Excess salts were removed by centrifugation (4000 rpm for 10 min) and filtration; the filtrate was concentrated *in vacuo*. The crude product was purified by column chromatography (CombiFlash Rf automated column system; 24 g HP silica; A: DCM, B: methanol, 100% A to 30% B gradient) to afford the product as a brown oil (0.135 g, 80%). ^{1}H NMR signals were broad and could not be integrated. ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.9, 165.2, 163.5, 148.8, 139.6, 129.1, 128.0, 126.9, 111.8, 109.5, 59.3, 55.4, 53.5, 53.6, 46.4, 25.5, 24.5. MS (ES^+) $m/z = 653.6$ $[M + H]^+$.

Me$_2$*CHX*dedpa$_{OMe}$-*N*,*N*′-Bn$_{OMe}$ (17). To a stirred solution of Me$_2$*CHX*dedpa$_{OMe}$ (**12**) (0.39 g, 0.80 mmol) in CH_3CN (8 mL) were added Na_2CO_3 (0.53 g, 5.00 mmol, ~6 equiv.) and 1-bromomethyl-3-methoxybenzene (243 μL, 1.7 mmol, 2 equiv.). The mixture was stirred at 60 °C overnight, after which excess salts were removed by centrifugation (4000 rpm for 10 min) and filtration; the filtrate concentrated *in vacuo*. The crude product was purified by column chromatography (CombiFlash Rf automated column system; 24 g HP silica; A: dichloromethane, B: methanol, 100% A to 20% B gradient) to give the product as a yellow oil (0.48 g, 85%). HR-ESI-MS m/z for $C_{40}H_{49}N_4O_8$ $(M + H)^+$ calcd (found): 713.3550 (713.3548) (−0.3 ppm).

(1*R*,2*R*)-*N*^{1},*N*^{2}-Bis(2,4,6-trimethoxybenzyl)cyclohexane-1,2-diamine (18). To a solution of (1*R*,2*R*)-(−)-cyclohexanediamine (0.162 g, 1.42 mmol, 1 equiv.) in ethanol (8 mL) at 0 °C, a solution of 2,4,6-trimethoxybenzaldehyde (0.557 g, 2.84 mmol, 2 equiv.) in ethanol/methanol (1 : 1, 20 mL) was added. The reaction mixture was stirred overnight and allowed to warm to RT. Bis-imine formation was confirmed by ES-MS ($m/z = 471.4$ $[M + H]^+$), and the solution was again cooled to 0 °C on ice. Sodium borohydride (0.17 g, approx. 3 equiv.) was added to the reaction mixture in small portions, and the mixture was stirred for 1.5 hours. The mixture was then quenched with saturated NH_4Cl (20 mL), and extracted with dichloromethane (3 × 40 mL). The organics were collected, dried over $MgSO_4$ and concentrated *in vacuo* to yield the product as a faint yellow solid (0.675 g, >99%) which was used in the subsequent step without further purification. ^{1}H NMR (400 MHz, MeOD) δ 6.22 (s, 4H), 4.10 (d, $J = 12.5$ Hz, 2H), 3.82 (s, 6H), 3.79 (d, $J = 12.5$ Hz, 2H), 3.71 (s, 12H), 2.48 (s, 2H), 2.24 (d, $J = 7.3$ Hz, 2H), 1.81 (s, 2H), 1.30 (d, $J = 6.3$ Hz, 4H). ^{13}C NMR (75 MHz, MeOD) δ 163.4, 160.7, 105.0, 91.6, 61.1, 56.2, 56.0, 39.2, 29.9, 25.5. MS (ES^+) $m/z = 475.4$ $[M + H]^+$.

Me$_2$*CHX*dedpa$_{OMe}$-*N*,*N*′-Bn$_{3OMe}$ (19). To a solution **15** (0.276 g, 0.58 mmol, 1 equiv.) and methyl-6-(bromomethyl)-4-methoxypicolinate (**10**) (0.322, 1.23 mmol, 2.1 equiv.) in CH_3CN (30 mL), Na_2CO_3 (0.355 g, 3.35 mmol, 5.6 equiv.) was added, and the reaction mixture was stirred at reflux for 6 hours and subsequently at RT for 2 days. The excess salts were removed by vacuum filtration, and the filtrate was concentrated *in vacuo*. The crude product was purified by column chromatography (CombiFlash Rf automated column system; 24 g HP silica; A: dichloromethane, B: methanol, 100% A to 10% B gradient) to yield the product as a fluffy white solid (0.382 g, 79%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (s, 2H), 7.59 (s, 2H), 5.87–5.75 (m, 4H), 4.67 (d, $J = 14.2$ Hz, 2H), 4.30 (d, $J = 13.8$ Hz, 2H), 4.10 (d, $J = 13.7$ Hz, 2H), 4.00 (s, 6H), 3.87 (s, 6H), 3.76 (d, $J = 15.3$ Hz, 4H), 3.68 (s, 6H), 3.46 (s, 12H), 3.30 (d, $J = 9.1$ Hz, 2H), 2.16 (d, $J = 11.9$ Hz, 2H), 1.82 (d, $J = 8.1$ Hz, 2H), 1.44–1.34 (m, 2H), 1.12–1.03 (m, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.0, 165.4, 161.8, 159.8, 157.5, 149.1, 113.6, 109.6, 102.1, 90.1, 61.2, 56.1, 55.4, 55.2, 53.5, 52.5, 52.2, 44.6, 26.0, 25.0. MS (ES^+) $m/z = 833.7$ $[M + H]^+$.

General procedure for methyl ester deprotection

To a stirred solution of methyl ester-protected ligand (**13–17**, **19**) (approx. 60 mg) in THF/water (3 : 1, 4 mL), LiOH (12 mg, approx. 5 equiv.) was added. The reaction mixture was stirred at

ambient temperature for 1.5 h or until reaction progress was complete (confirmed by absence of starting material peak in MS, and disappearance of starting material spot on TLC (R_f (product) < R_f (starting materials)) in 10% MeOH/dichloromethane). The reaction mixture was concentrated *in vacuo* and used without further purification, or purified by semi-preparative RP-HPLC (A: CH_3CN, B: 0.1% trifluoroacetic acid (TFA) in water, 5–100% A linear gradient, 25 min, 10 mL min^{-1}). Product fractions were lyophilized to yield the ligand as a white solid (80–99% yield).

H_2dedpa-*N,N′*-ee (1). ^{1}H NMR (300 MHz, MeOD) δ 7.76 (d, J = 7.5 Hz, 2H), 7.68 (t, J = 7.6 Hz, 2H), 7.20 (d, J = 7.4 Hz, 2H), 3.71 (s, 4H), 3.24–3.16 (m, 4H), 3.18–3.06 (m, 4H), 2.29–2.24 (m, 4H), 2.23 (s, 4H), 0.86 (t, J = 7.0 Hz, 6H). ^{13}C NMR (75 MHz, MeOD) δ 172.2, 159.5, 154.5, 139.6, 125.5, 123.0, 68.1, 67.3, 61.3, 53.9, 51.6, 15.5. MS (ES^+) m/z = 475.3 $[M + H]^+$, 481.3 $[M + Li]^+$, 497.3 $[M + Na]^+$.

H_2*CHX*dedpa-*N,N′*-ee (2). ^{1}H NMR (400 MHz, MeOD) δ 8.10 (d, J = 7.4 Hz, 2H), 8.01 (t, J = 7.5 Hz, 2H), 7.80 (d, J = 7.2 Hz, 2H), 4.48 (d, J = 14.0 Hz, 2H), 4.06 (d, J = 14.1 Hz, 2H), 3.79–3.66 (m, 4H), 3.56–3.48 (m, 2H), 3.41–3.33 (m, 2H), 3.27–3.16 (m, 4H), 3.16–3.05 (m, 2H), 2.38 (d, J = 10.8 Hz, 2H), 1.99 (d, J = 6.4 Hz, 2H), 1.94–1.76 (m, 2H), 1.70–1.56 (m, 2H), 1.51–1.32 (m, 4H), 1.12 (s, 4H), 0.96 (t, J = 6.5 Hz, 6H). MS (ES^+) m/z = 529.5 $[M + H]^+$, 535.5 $[M + Li]^+$.

H_2*CHX*dedpa$_{OMe}$-*N,N′*-ee (3). ^{1}H NMR (400 MHz, D_2O, 55 °C) δ 7.67 (s, 2H), 7.25 (s, 2H), 4.46 (d, J = 13.0 Hz, 2H), 4.26 (d, J = 12.0 Hz, 2H), 4.16 (s, 6H), 4.09–3.97 (m, 4H), 3.89–3.79 (m, 4H), 3.76 (d, J = 7.8 Hz, 2H), 3.49–3.34 (m, 4H), 2.53 (d, J = 11.5 Hz, 2H), 2.21 (d, J = 8.2 Hz, 2H), 1.88–1.74 (m, 2H), 1.66 (t, J = 9.9 Hz, 2H), 1.44 (t, J = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, D_2O, 55 °C) δ 172.1, 167.8, 156.6, 155.9, 118.4, 115.5, 111.9, 109.3, 67.0, 62.4, 56.1, 24.6, 24.4, 14.5. HR-ESI-MS m/z for $C_{30}H_{45}N_4O_8$ $(M + H)^+$ calcd (found): 589.3237 (589.3237).

H_2*CHX*dedpa$_{OMe}$-*N,N′*-Bn (4). ^{1}H NMR (400 MHz, D_2O, 55 °C) δ 7.72 (dd, J = 13.9, 6.2 Hz, 8H), 7.61–7.53 (m, 6H), 4.20 (s, 6H), 4.09 (d, J = 15.4 Hz, 2H), 4.03–3.92 (m, 6H), 3.20 (d, J = 6.7 Hz, 2H), 2.60 (d, J = 11.0 Hz, 2H), 2.16 (d, J = 6.9 Hz, 2H), 1.62–1.50 (m, 4H). ^{13}C NMR (101 MHz, D_2O, 55 °C) 129.8, 128.5, 127.5, 110.7, 108.2, 100.1, 60.0, 55.9, 54.6, 54.3, 25.9, 23.9. HR-ESI-MS m/z for $C_{36}H_{41}N_4O_6$ $(M + H)^+$ calcd (found): 625.3026 (625.3020).

H_2*CHX*dedpa$_{OMe}$-*N,N′*-Bn$_{OMe}$ (5). ^{1}H NMR (400 MHz, D_2O, 75 °C) δ 8.22 (s, 2H), 7.63 (t, J = 7.8 Hz, 2H), 7.54 (s, 2H), 7.47 (d, J = 7.3 Hz, 2H), 7.37 (s, 2H), 7.31 (d, J = 8.3 Hz, 2H), 4.60 (d, J = 16.3 Hz, 2H), 4.55 (s, 6H), 4.38 (d, J = 18.2 Hz, 2H), 4.33 (s, 4H), 4.13 (s, 6H), 3.84 (d, J = 8.3 Hz, 2H), 2.86 (d, J = 12.0 Hz, 2H), 2.47 (d, J = 8.8 Hz, 2H), 2.10–1.97 (m, 2H), 1.93–1.80 (m, 2H). ^{13}C NMR (101 MHz, D_2O, 75 °C) δ 160.16, 153.42, 149.07, 131.06, 123.55, 116.97, 114.87, 113.66, 111.83, 100.12, 60.57, 57.75, 56.08, 55.36, 51.87, 24.52, 23.98. HR-ESI-MS m/z for $C_{38}H_{45}N_4O_8$ $(M + H)^+$ calcd (found): 685.3237 (685.3231).

H_2*CHX*dedpa$_{OMe}$-*N,N′*-Bn$_{3OMe}$ (6). ^{1}H NMR (400 MHz, MeOD) δ 7.65–7.43 (m, 4H), 5.88 (s, 4H), 3.91 (s, 6H), 3.68 (d, J = 26.7 Hz, 2H), 3.63 (s, 6H), 3.55 (d, 2H), 3.48 (s, 12H), 3.32 (d, J = 14.2 Hz, 4H), 2.55 (br s, 2H), 2.24–1.98 (m, 2H), 1.73–1.54 (m, 2H), 1.09–0.90 (m, 4H). HR-ESI-MS m/z for $C_{42}H_{52}N_4O_{12}$ $(M + H)^+$ calcd (found): 805.3660 (805.3658) (−0.2 ppm).

General procedure for Ga(III) complexation of pro-ligands (1–6)

A solution of pro-ligand (**1–6**) (0.02 mmol) in water/methanol (1 : 1, 2 mL) was adjusted to pH 2–3 using 0.1 M HCl (aq). A solution of $Ga(ClO_4)_3 \cdot 6H_2O$ (0.03 mmol, 1.5 equiv.) in water (500 μL) was added to this solution, and the pH was then adjusted to 4–4.5 with 0.1 M NaOH (aq). The reaction mixture was stirred at room temperature overnight, over which time a white precipitate formed. The solid was subsequently isolated by centrifugation (4000 rpm, 10 min) and dried further under vacuum to yield the Ga-complex as a white solid (55–89% yield).

[Ga(dedpa-*N,N′*-ee)][ClO_4], [Ga(1)][ClO_4]. ^{1}H NMR (400 MHz, DMSO) δ 8.66 (t, J = 7.8 Hz, 2H), 8.35 (d, J = 7.6 Hz, 2H), 8.19 (d, J = 7.9 Hz, 2H), 4.71 (d, J = 17.6 Hz, 2H), 4.57 (d, J = 17.5 Hz, 2H), 3.65 (t, J = 4.8 Hz, 4H), 3.43 (q, J = 14.1, 7.1 Hz, 5H), 3.37 (d, J = 11.1 Hz, 2H), 2.93 (d, J = 11.1 Hz, 2H), 2.83 (t, J = 4.6 Hz, 4H), 1.11 (t, J = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 150.9, 146.4, 143.6, 128.2, 123.3, 65.8, 64.5, 56.3, 50.9, 48.5, 15.0. HR ESI-MS m/z for $C_{24}H_{32}{}^{69}GaN_4O_6$ (M^+) calcd (found): 541.1584 (541.1578).

[Ga(*CHX*dedpa-*N,N′*-ee)][ClO_4], [Ga(2)][ClO_4]. ^{1}H NMR (400 MHz, DMSO) δ 8.82 (t, J = 7.8 Hz, 2H), 8.50 (d, J = 7.6 Hz, 2H), 8.35 (d, J = 7.9 Hz, 2H), 5.14 (d, J = 18.2 Hz, 2H), 4.60 (d, J = 18.2 Hz, 2H), 3.64–3.37 (m, 8H), 3.19 (d, J = 8.2 Hz, 2H), 3.09–2.98 (m, 4H), 2.37 (d, J = 11.3 Hz, 2H), 1.89 (d, J = 7.0 Hz, 2H), 1.62–1.48 (m, 2H), 1.45–1.31 (m, 2H), 1.21 (t, J = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO) δ 162.0, 151.9, 147.0, 144.0, 126.8, 123.6, 66.1, 65.9, 63.8, 57.6, 51.3, 48.8, 27.338, 23.9, 14.9. HR-ESI-MS m/z for $C_{28}H_{38}{}^{69}GaN_4O_6$ $(M)^+$ calcd (found): 595.2047 (595.2048) (0.2 ppm).

[Ga(*CHX*dedpa$_{OMe}$-*N,N′*-ee)][ClO_4], [Ga(3)][ClO_4]. ^{1}H NMR (300 MHz, DMSO) δ 7.76 (s, 2H), 7.75–7.70 (m, 2H), 4.87 (d, J = 17.7 Hz, 2H), 4.27 (d, J = 17.8 Hz, 1H), 4.11 (s, 6H), 3.39–3.16 (m, 8H), 2.99 (d, J = 3.9 Hz, 2H), 2.88–2.75 (m, 2H), 2.16 (d, J = 9.7 Hz, 2H), 1.77–1.64 (m, 2H), 1.44–1.29 (m, 2H), 1.26–1.15 (m, 2H), 1.07 (t, J = 6.9 Hz, 6H). ^{13}C NMR (resonances obtained from HSQC) (75 MHz, DMSO) δ 111.5, 110.1, 66.3, 63.9, 58.3, 51.5, 51.1, 49.0, 27.7, 24.2, 15.3. HR-ESI-MS m/z for $C_{30}H_{42}{}^{69}GaN_4O_8$ $(M)^+$ calcd (found): 655.2258 (655.2253) (−0.8 ppm).

[Ga(*CHX*dedpa$_{OMe}$-*N,N′*-Bn)][ClO_4], [Ga(4)][ClO_4]. ^{1}H NMR (300 MHz, DMSO) δ 7.87–7.83 (m, 2H), 7.79 (d, J = 1.6 Hz, 2H), 7.65 (d, J = 6.8 Hz, 4H), 7.45–7.32 (m, 6H), 4.71 (br s, 4H), 4.17 (d, J = 13.7 Hz, 2H), 4.14 (s, 6H), 3.46 (d, J = 13.7 Hz, 2H), 3.25 (2, J = 6.5 Hz, 4H), 1.32–1.21 (m, 2H), 1.20–1.06 (m, 2H), 1.03–0.90 (m, 2H). ^{13}C NMR (resonances obtained from HSQC) (75 MHz, DMSO) δ 132.1, 128.7, 112.1, 110.2, 62.5, 62.1, 58.2, 49.3, 40.1. HR-ESI-MS m/z for $C_{36}H_{38}{}^{69}GaN_4O_6$ $(M)^+$ calcd (found): 691.2061 (691.2074) (2.0 ppm).

[Ga(*CHX*dedpa$_{OMe}$-*N,N′*-Bn$_{OMe}$)][ClO_4], [Ga(5)][ClO_4]. ^{1}H NMR (600 MHz, DMSO) δ 7.83 (d, J = 6.0 Hz, 4H), 7.29 (t, J = 7.9 Hz, 2H), 7.25 (s, 2H), 7.19 (d, J = 7.4 Hz, 2H), 6.99 (dd, J = 8.4, 1.7 Hz, 2H), 4.71 (d, J = 17.8 Hz, 2H), 4.65 (d, J = 17.8 Hz, 2H), 4.15 (s, 6H), 4.12 (d, 2H), 3.83 (d, 2H), 3.74 (s, 6H), 3.23–3.17 (m, 2H), 1.29–1.20 (m, 4H), 1.16–1.07 (m, 4H). HR-ESI-MS m/z for $C_{38}H_{42}{}^{69}GaN_4O_8$ $(M)^+$ calcd (found): 751.2258 (751.2266) (1.1 ppm).

[Ga(*CHX*dedpa$_{OMe}$-*N*,*N*′-Bn$_{3OMe}$)][ClO$_4$], [Ga(6)][ClO$_4$]. ^{1}H NMR (600 MHz, DMSO) δ 7.82 (s, 2H), 7.80 (s, 2H), 6.19 (s, 4H), 4.43 (dd, J = 17.8 Hz, 4H), 4.14 (s, 6H), 4.09 (d, J = 14.0 Hz, 2H), 3.76 (s, 12H), 3.69 (s, 6H), 3.29 (d, J = 14.5 Hz, 2H), 2.90 (d, J = 6.2 Hz, 2H), 1.49–1.38 (m, 4H), 1.16–1.05 (m, 2H), 0.88–0.78 (m, 2H).^{13}C NMR (151 MHz, DMSO) δ 173.4, 162.3, 162.0, 159.7, 153.1, 146.5, 111.8, 109.6, 101.4, 90.8, 65.4, 58.0, 56.0, 55.5, 50.9, 49.7, 29.5, 24.5. HR ESI-MS m/z for $C_{42}H_{50}{}^{69}GaN_4O_{12}$ (M^+) calcd (found): 871.2681 (871.2684) (0.3 ppm).

$^{67/68}$Ga radiolabeling studies

The ^{67}Ga radiolabeling protocol of all pro-ligands followed those previously described.[16,17] Briefly, for initial labeling studies and human apo-transferrin challenge assays, all chelators were made up as stock solutions (1 mg mL^{-1}, $\sim$10^{-3} M) in deionized water. A 100 μL aliquot of each chelator stock solution was transferred to screw-cap mass spectrometry vials and diluted with pH 4 NaOAc (10 mM) buffer such that the final volume was 1 mL after the addition of $^{67}GaCl_3$, to a final chelator concentration of $\sim$10^{-4} M for each sample. An aliquot of $^{67}GaCl_3$ ($\sim$1 mCi for labeling studies and log P determination, and $\sim$3–6 mCi for apo-transferrin competitions) was added to the vials containing the chelator and allowed to radiolabel at ambient temperature for 10 min, then analyzed by RP-HPLC to confirm radiolabeling and calculate yields. Areas under the peaks observed in the HPLC radiotrace were integrated to determine radiolabeling yields. Elution conditions used for RP-HPLC analysis were gradient: A: 10 mM NaOAc buffer, pH 4.5, B: CH_3CN; 0 to 100% B linear gradient 20 min.

For ^{68}Ga radiolabeling studies, selected pro-ligands were prepared as above (1 mg mL^{-1}, $\sim$10^{-3} M) in deionized water. A 100 μL aliquot of each chelator stock solution was transferred to screw-cap mass spectrometry vials and diluted with pH 5.5 NaOAc (100 mM) buffer such that the final volume was 1 mL after the addition of $^{68}GaCl_3$ (50–100 μL), to a final chelator concentration of 10^{-4} M and reaction pH of 4.5. Reaction vials were left to sit at ambient temperature for 10 minutes, and analysed by RP-HPLC as above for ^{67}Ga reactions. Elution conditions used for RP-HPLC analysis of ^{68}Ga reactions were gradient: A: 0.1% TFA (trifluoroacetic acid), B: CH_3CN; 0 to 100% B linear gradient 20 min.

log *P* determination

The log P of ^{67}Ga-complexes were measured experimentally following procedures outlined previously.[17] Briefly, ^{67}Ga-labelled complex (30 μCi/6 μL) was added to 1.5 mL centrifuge tubes containing phosphate buffered saline (PBS) (494 μL) and 1-octanol (500 μL), mixed vigorously for 1 minute, and subsequently centrifuged to separate phases (3000 rpm, 5 min). Equal aliquots (20–100 μL) of the organic and aqueous phases were diluted in a standard volume (20 mL) of water for measurement in an N-type Co-axial HPGe gamma spectrometer from Canberra fitted with a 0.5 mm beryllium window and calibrated (energy and efficiency) with a 20 mL ^{152}Eu source. The samples were counted for 10 minutes, with a dead time of less than 5%. Measurements were carried out in triplicate.

Human apo-transferrin stability studies

The *in vitro* kinetic inertness assays of ^{67}Ga-complexes against human apo-transferrin were performed according to a previously published method.[15,17,18] The radiolabelled complexes [^{67}Ga(**2**, **3**, or **5**)]$^+$ were prepared with the radiolabeling protocol as described above. In duplicate for each ^{67}Ga-complex above, solutions were prepared in vials with 500 μL of freshly prepared 1 mg mL^{-1} apo-transferrin in $NaHCO_3$ (10 mM, pH 7) solution, 300 μL of ^{67}Ga-complex, and 200 μL of phosphate buffered saline (PBS, pH 7.4), and incubated at 37 °C in a water bath. At time points 15 min, 1 and 2 hours, 300 μL of the apo-transferrin competition mixture was removed from each vial, diluted to a total volume of 2.5 mL with PBS, and then counted in a Capintec CRC 15R well counter to obtain a value for the total activity to be loaded on the PD-10 column (F). The 2.5 mL of diluted apo-transferrin mixture was loaded onto a PD-10 column that had previously been conditioned *via* elution with 20 mL of PBS, and the empty vial was counted in a well counter to determine the residual activity left in the vial (R). The 2.5 mL of loading volume was allowed to elute into a waste container, and then the PD-10 column was eluted with 3.5 mL of PBS and collected into a separate vial. The eluent which contained ^{67}Ga bound/associated with apo-transferrin (size exclusion for MW < 5000 Da) was counted in a well counter (E) and then compared to the total amount of activity that was loaded on the PD-10 column to obtain the percentage of ^{67}Ga that was bound to apo-transferrin and therefore no longer chelate-bound using the equation:

$$\left(1 - \frac{E}{(F - R)}\right) \times 100.$$

Conclusions

The synthesis and characterization of six new lipophilic derivatives of H_2dedpa or H_2*CHX*dedpa for the intended purpose of myocardial perfusion imaging were described. All six derivatives were screened for their ability to bind Ga(III) and retained hexadentate N_4O_2 binding. Initial radiolabeling experiments with ^{67}Ga resulted in abnormal radiolabeling HPLC chromatograms in which three ^{67}Ga-labelled products were formed for the *N*,*N*′-benzyl functionalized ligand derivatives (**4–6**), while ligands (**1–3**), with *N*,*N*′-ethyl ether moieties, exhibited single peaks in the radio-chromatogram as expected. In an attempt to further identify the additional by-product peaks in the radiolabeling reactions of [$^{67/68}$Ga(**4–6**)]$^+$ as either ligand impurities or ligand radiolysis products, reaction mixture [^{67}Ga(**5**)]$^+$ was analysed over the course of one hour, and pro-ligand **6** was radiolabelled with ^{68}Ga as well as ^{67}Ga. The relative peak intensities of the three radiolabelled products remained constant despite the changing reaction parameters, suggesting ligand impurities present in even low concentrations are able to complex Ga(III) more efficiently than the desired pro-ligand. Even though the ligands were deemed sufficiently pure post purification *via* HPLC and NMR characterization, the relative steric-bulk imposed by benzyl groups next to the cyclohexane backbone may be conceivably inducing ligand decomposition overtime.

Therefore, ligands **4–6** are not suitable candidates as radiopharmaceuticals, as a major requirement of such pharmaceuticals is that they be chemically stable and have a suitable shelf-life.

Partition coefficients of *CHX*dedpa^{2-} *N*,*N*-ethyl ether functionalized ligands (**2** and **3**) were determined to be in the range of −1.5 to −1.8, and are not in the predicted suitable log *P* range for myocardial perfusion imaging agents. The ^{67}Ga-dedpa complex ([Ga(**1**)]$^{+}$, [Ga(dedpa-ee)]$^{+}$) exhibited a log *P* nearly one full log unit lower than the analogous ^{67}Ga-*CHX*dedpa complex ([Ga(**2**)]$^{+}$, [Ga(*CHX*dedpa-ee)]$^{+}$), −2.7 and −1.8, respectively. This finding suggests that H$_2$*CHX*dedpa presents a potentially useful framework to design lipophilic ligands in future studies. Moreover, selected ^{67}Ga-*CHX*dedpa complexes were sufficiently stable (>80%) in a 2 h apo-transferrin challenge assay.

Though *N*,*N*′-alkylation of the ligands with benzyl-like attachments was an unfortunate choice of appendage which led to non-trivial radiolabeling and potentially unstable ligands, important lessons on ligand design and alteration of the H$_2$-*CHX*dedpa scaffold have been learned. The results obtained from this set of ligands may be used to design a new library of H$_2$*CHX*dedpa ligands which offer more lipophilic features, but do not incorporate benzyl groups off the 2° nitrogens in order to prepare Ga(III) complexes within the optimal log *P* range (0.5–1.2). The findings in this study are an important first step towards defining the design requirements for the next generation of H$_2$*CHX*dedpa lipophilic tracers that may have potential use as myocardial perfusion imaging agents.

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