A new synthesis of cystamine modified Eu^{3+} DOTAM-Gly-Phe-OH: a conjugation ready temperature sensitive MRI contrast agent†

Mojmír Suchý,[a,b] Alex X. Li,[b] Robert Bartha[b] and Robert H. E. Hudson*[a]

Received 16th May 2008, Accepted 9th June 2008
First published as an Advance Article on the web 11th July 2008
DOI: 10.1039/b808282k

Several approaches towards asymmetrically derivatized peptide-decorated cyclens that yield lanthanide metal chelators, in which three of the nitrogen atoms of cyclen share a common substituent and the fourth nitrogen atom is differentially substituted, have been evaluated. The most effective route consisted of selective monoalkylation followed by peralkylation with a second different electrophile. The unique substituent also possessed a masked sulfanyl group that was suitable for subsequent chemoselective conjugation chemistry.

Introduction

Over the last two decades there have been dramatic developments in the use of lanthanide(III) complexes as responsive MRI contrast agents.[1] A wide variety of MRI contrast agents have been designed and used for the detection of various primary metabolites (*e.g.* proteins, nucleic acids), enzymatic activities (*e.g.* caspase-3, peroxidase, glucuronidase), and metal ions as well as other important physiological parameters such as pH and temperature.[1]

Paramagnetic lanthanide(III) ions are known to induce a large hyperfine shift of coordinated water protons and other exchangeable protons present in the ligand framework.[2] If the exchange rate of these spin systems with bulk water is appropriately slow, a separate MR signal may be observed for each state. Selective saturation of these spins results in a method for the generation of MRI contrast.[2] A new class of MRI contrast agents employing this technique is referred to as paramagnetic chemical exchange saturation transfer (PARACEST). The structures of these agents are often derived from DOTA (**1**) or DOTAM (**2**), Fig. 1.

We have recently designed, synthesized and evaluated the PARACEST properties of a variety of DOTAM-based dipeptide decorated lanthanide(III) complexes.[3] Among them, Eu^{3+} DOTAM-Gly-Phe-OH (**3**, Fig. 1) was found to be an MRI contrast agent suitable for the molecular imaging of temperature.[4] Tissue temperature is an important physiological parameter, which often varies between healthy and disease affected tissues.[1] We are, in particular, interested in using the agent **3** as a molecular probe for the molecular imaging of brain tumors. A major obstacle associated with the intended use of Eu^{3+} DOTAM-Gly-Phe-OH (**3**) is its inability to cross various biological barriers (*e.g.* the blood brain barrier). To overcome this problem, we decided to conjugate Eu^{3+} DOTAM-Gly-Phe-OH (**3**) to a cell penetrating peptide[5] *via* disulfide bond formation. It is expected that the disulfide bridge will be cleaved within the reductive intracellular environment thus leaving the MRI contrast agent trapped inside. To achieve this goal, we required a derivative of **3** bearing a primary sulfanyl (SH) group. Cystamine-modified Eu^{3+} complexes **4a**,**b** (Fig. 1) have been identified as suitable synthetic targets and are derived from the corresponding free ligands **5a**,**b** (Fig. 1). Selective introduction of the cystamine modified side chain present in the structure of **4** and **5** represents the major synthetic challenge associated with the synthesis of desired ligands.

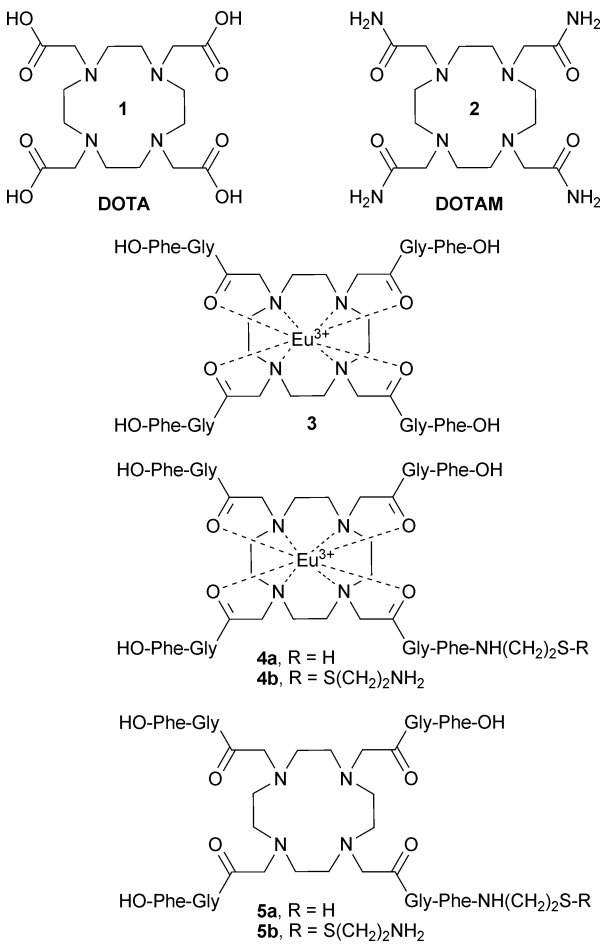


Fig. 1 Structures of DOTA (**1**), DOTAM (**2**), Eu^{3+} DOTAM-Gly-Phe-OH (**3**) and cystamine modified derivatives **4** and **5**.

[a]*Department of Chemistry, The University of Western Ontario, London, ON, Canada, N6A 5B7. E-mail: robert.hudson@uwo.ca*
[b]*Centre for Functional and Metabolic Mapping, Robarts Research Institute, The University of Western Ontario, London, ON, Canada, N6A 5K8*
† Electronic supplementary information (ESI) available: Selected ^{1}H and ^{13}C NMR spectra. See DOI: 10.1039/b808282k

The synthesis of asymmetrically substituted ligands **5a**,**b** and their Eu^{3+} complexes **4a**,**b** has been achieved and moreover, the PARACEST properties associated with complex **4b** have been determined and compared with those of Eu^{3+} DOTAM-Gly-Phe-OH (**3**). In this paper we describe a detailed synthetic approach leading to the synthesis of the sophisticated ligands **5a**,**b** and the Eu^{3+} complexes **4a**,**b** and the evaluation of the PARACEST properties of **4b**.

Results and discussion

Retrosynthetic analysis

To assemble the desired ligands **5** and their Eu^{3+} complexes **4**, synthetic approaches based on the selective alkylation of commercially available cyclen or alkylation of some simple, easily accessible derivatives of cyclen have been evaluated. The various retrosynthetic disconnections that we explored are depicted in Scheme 1.

The first approach we attempted was thought to be the most straightforward at the outset and was based on the selective trialkylation of cyclen (**6**) with *N*-iodoacetyl-Gly-Phe-OEt (**7**)[3] followed by the alkylation of the remaining secondary amine with *N*-iodoacetylGly-Phe-cystamine(Boc) (**8**), Scheme 1. However, we were not able to achieve satisfactory results, which necessitated considering alternative routes. The next possibility investigated was the assembly of ligand **5** *via* *N*-formylcyclen (**9**), which can be obtained from cyclen (**6**) in two steps.[6] Subsequent sequential alkylation of **9** with *N*-iodoacetyl-Gly-Phe-OEt (**7**); removal of the formyl group; and alkylation with *N*-iodoacetyl-Gly-Phe-cystamine(Boc) (**8**, Scheme 1) would yield the desired ligand. Unfortunately, this route had to be abandoned after the *N*-formyl group was found to resist removal.

Another easily accessible cyclen-derived precursor investigated for the synthesis of ligand **5** was *N*-cyclenacetic acid (**10**).[7] Our approach was to trialkylate **10** (Scheme 1) with *N*-iodoacetyl-Gly-Phe-OEt (**7**), followed by coupling with Fmoc-Phe-cystamine(Boc) (**11**, Scheme 1). As described later, the trialkylation of **10** failed to produce the desired result and we turned our attention to the final synthetic approach, which eventually led us to the desired target **5**. The key step in the retrosynthesis of **5** then became the selective monoalkylation of cyclen (**6**) with *N*-haloacetyl-Gly-Phe-O*t*Bu (**12**, Scheme 1). This was followed by peralkylation with *N*-iodoacetyl-Gly-Phe-OEt (**7**), selective removal of the *t*Bu ester group and coupling with mono-Boc-cystamine (**16**), prepared according to a literature procedure.[8]

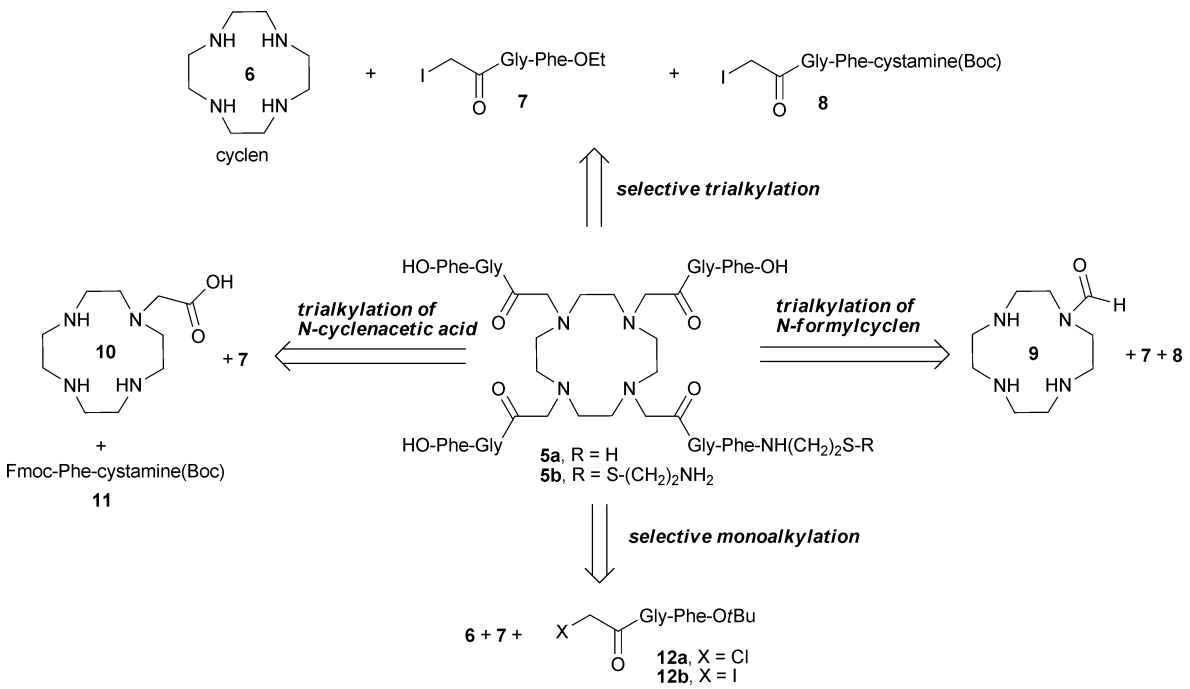
Synthesis of alkylation agents 7, 8 and 12

N-Iodoacetyl-Gly-Phe-OEt (**7**) was prepared from *N*-chloroacetylglycine (**13**) and H-Phe-OEt·HCl according to a recently established literature procedure.[3a] A similar approach was used to prepare electrophiles **12** *via* the reaction of **13** with H-Phe-O*t*Bu·HCl (**14**, Scheme 2). *N*-Chloroacetyl-Gly-Phe-O*t*Bu (**12a**) was obtained in excellent yield (98%), followed by the Finkelstein reaction (NaI, acetone) to furnish *N*-iodoacetyl-Gly-Phe-O*t*Bu (**12b**) in 56% yield (Scheme 2).

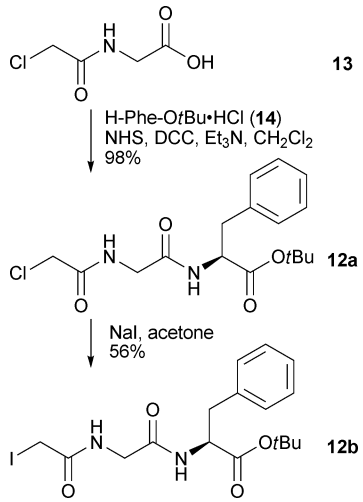
N-Chloroacetylglycine (**13**) was subjected to NHS–DCC-mediated coupling with H-Phe-OH (**15**) followed by coupling with mono-Boc-cystamine[8] (**16**, Scheme 3). The crude *N*-chloroacetyl-Gly-Phe-cystamine(Boc) was obtained, containing *ca.* 20% of *N,N*-dicyclohexylurea (as determined by ^{1}H NMR). This material was treated with NaI in acetone to afford *N*-iodoacetyl-Gly-Phe-cystamine(Boc) (**8**, Scheme 3) in an acceptable yield of 33% (over three steps, based on **13**).

Synthesis of Fmoc-Phe-cystamine(Boc) (11)

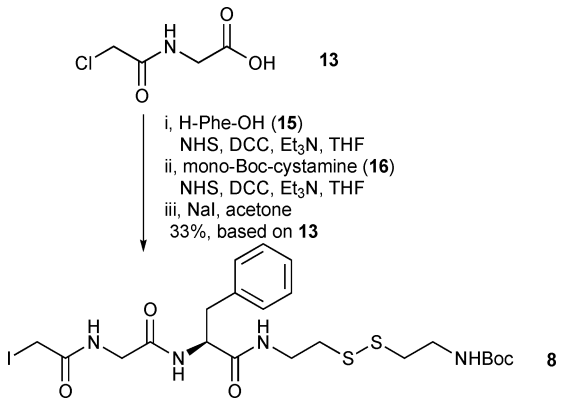
The EDC·HCl–NHS mediated coupling of Fmoc-Phe-OH (**17**) with mono-Boc-cystamine[8] (**16**) was used to prepare



Scheme 1 Comprehensive retrosynthetic analysis of cystamine modified ligands **5**.

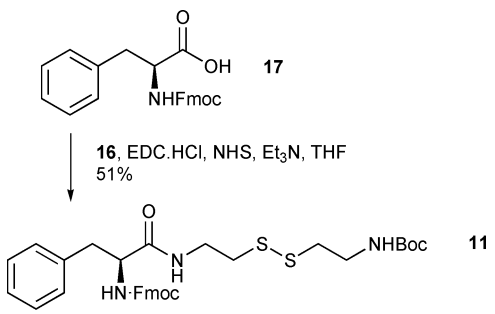


Scheme 2 Synthesis of *N*-haloacetyl-Gly-Phe-O*t*Bu (**12**).



Scheme 3 Synthesis of *N*-iodoacetyl-Gly-Phe-cystamine(Boc) (**8**).

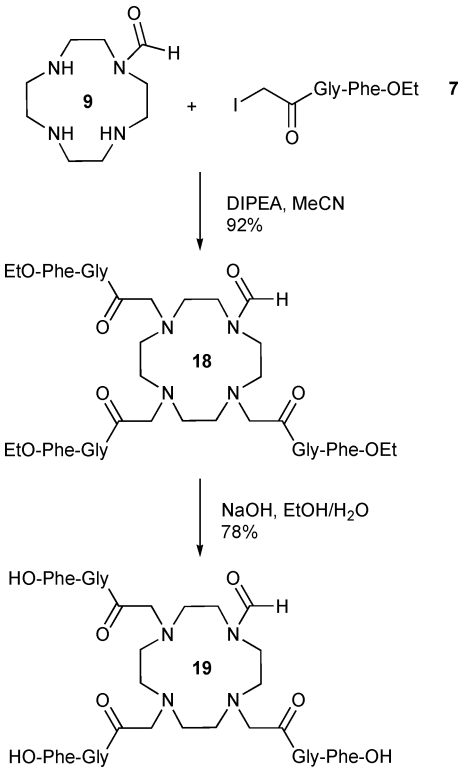
Fmoc-Phe-cystamine(Boc) (**11**) dipeptide in moderate yield (51%), Scheme 4.



Scheme 4 Synthesis of Fmoc-Phe-cystamine(Boc) (**11**).

Alkylation of *N*-formylcyclen (9) and *N*-cyclenacetic acid (10)

N-Formylcyclen (**9**) can be obtained from cyclen (**6**) first by reacting **6** with dimethylformamide dimethylacetal, followed by hydrolysis of unstable tricyclic amine.[6] With *N*-formylcyclen in hand, we turned our attention to the peralkylation of **9** with *N*-iodoacetyl-Gly-Phe-OEt (**7**). The reaction proceeded smoothly and peralkylated cyclen **18** was isolated in 92% yield (Scheme 5). The intermediate triester **18** was expected to undergo the removal



Scheme 5 Alkylation of *N*-formylcyclen (**9**) with *N*-iodoacetyl-Gly-Phe-OEt (**7**) and subsequent saponification of **18** to yield **19**.

of the formyl group, followed by alkylation with *N*-iodoacetyl-Gly-Phe-cystamine(Boc) (**8**, Scheme 1).

The formyl group is a well known nitrogen atom protective group and several methods for its removal have appeared in the literature. For this work, we attempted to remove the formyl group from intermediate **18** employing oxidative,[9] reductive,[10] acidic,[11] or basic[12] conditions. All of these conditions have proved to be unsuitable for the reasons given below.

Attempts to remove the formyl group under oxidative conditions (*m*-CPBA in THF, or H_2O_2 in EtOH–H_2O[9]) led only to intractable mixtures while catalytic hydrogenation[10] led to complete recovery of starting material. Hydrolysis of the formyl group was tried under acidic conditions (HCl in EtOH–H_2O, $SOCl_2$ in EtOH or *p*-TSA in EtOH),[11] but we were plagued by partial hydrolysis of the ester groups present in **18**. When **18** was treated with HCl in EtOH–H_2O, a modest amount of impure product was obtained that showed removal of the formyl group; however, this result could neither be reliably reproduced nor scaled up despite many attempts. It was also found that the reaction of this impure material with *N*-iodoacetyl-Gly-Phe-cystamine(Boc) (**8**, Scheme 1) was rather sluggish and for both of these reasons, this approach was abandoned. Finally, basic conditions were investigated as a way to remove the protecting group. Treatment of **18** with K_2CO_3 in aqueous EtOH even at elevated temperatures (up to reflux) resulted in recovery of the starting material while complete decomposition was observed when NaOEt was used as a base. Classical saponification[12] conditions (NaOH in EtOH–H_2O) led to the formation of macrocycle **19** (Scheme 5), in which the hydrolysis of the ester functionalities took place, while the formyl group remained. Attempts to promote the removal of

the formyl group under identical conditions but using elevated temperatures (up to 50 °C) led to massive decomposition (HPLC inspection). The above mentioned experiments clearly indicated that *N*-formylcyclen (**9**) is not a suitable precursor in the synthesis of desired ligands **5a**,**b** and their Eu^{3+} complexes (Fig. 1).

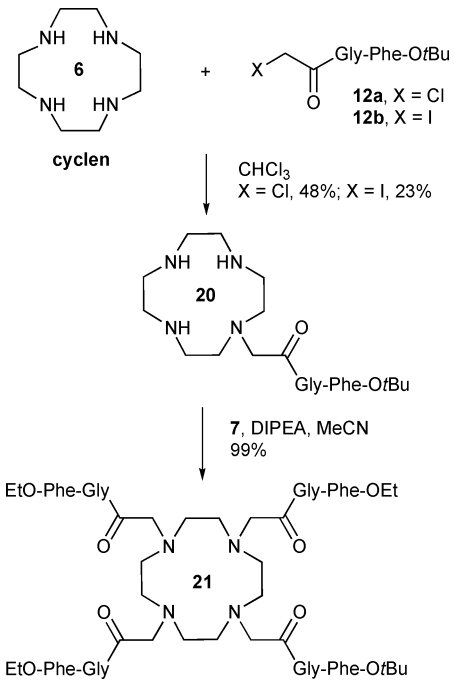
Finding that we were unable to remove the formyl group from **18** we turned our attention to *N*-cyclenacetic acid (**10**) as a starting point. Acid **10** can be obtained by alkylation of cyclen (**6**) with bromoacetic acid.[7] We envisioned the peralkylation of **10** with *N*-iodoacetyl-Gly-Phe-OEt (**7**), followed by coupling with Fmoc-Phe-cystamine(Boc) (**11**, Scheme 1) to furnish the remaining side chain. Somewhat unexpectedly, we observed alkylation of the carboxylic functionality with *N*-iodoacetyl-Gly-Phe-OEt (**7**) leading to the formation of a complex and inseparable mixture of the desired *N*-alkylation product and *O*-alkylated side product,[13] precluding the use of **10** for the synthesis of ligand **5**.

Selective trialkylation and monoalkylation of naked cyclen

Selective trialkylation of unprotected cyclen (**6**) with *N*-iodoacetyl-Gly-Phe-OEt (**7**) followed by the alkylation of the remaining nitrogen atom with *N*-iodoacetyl-Gly-Phe-cystamine(Boc) (**8**, Scheme 1) conceptually was the most straightforward route towards assembling the ligand **5**. In the recent literature,[14] there are several reports describing selective trialkylation of cyclen, followed by the separation of the desired product by chromatography. It is worth mentioning that the alkylation agents used to carry out the above mentioned transformations usually possess simpler structures as compared to *N*-iodoacetyl-Gly-Phe-OEt (**7**). We have found that treatment of cyclen (**6**) with *N*-iodoacetyl-Gly-Phe-OEt (**7**) using Et_3N as a base leads to the formation of a complex mixture. Analysis of the reaction mixture by HPLC (Microsorb-CN column) indicated the presence of the desired trialkylated cyclen (75%) accompanied with the products of peralkylation (20%, this material was compared with an authentic sample[3a] available in our laboratory) and dialkylation (5%). Under these conditions, the products of the reaction were well resolved and we therefore attempted scale up the reaction and to carry out gravity chromatography, using nitrile bonded phase silica gel. Unfortunately, the chromatographic separation could not be reproduced on a larger scale and in fact we observed the desired product to be essentially irreversibly adsorbed onto the stationary phase.

The synthesis of the ligand **5** was eventually accomplished, when selective monoalkylation[15] of cyclen was used as a key step in the synthesis. Cyclen (**6**) was monoalkylated with *N*-haloacetyl-Gly-Phe-O*t*Bu in chloroform (**12**, Scheme 6). The reaction proceeded in moderate yields (**12a**, 48%; **12b**, 23%), affording the monoalkylated cyclen **20** (Scheme 6). The product of monoalkylation was isolated by normal phase column chromatography without difficulty. Peralkylation of **20** with *N*-iodoacetyl-Gly-Phe-OEt (**7**) afforded the orthogonally protected ester **21** in excellent yield (99%, Scheme 6). As described below, compound **21** was later on converted to the desired ligands **5a**,**b**.

Having prepared the orthogonally protected derivative **21** in reasonable quantities and purity, we focused our attention on completing the synthesis of ligands **5**. The *tert*-butyl ester group of **21** was removed by treatment with TFA in dichloromethane and the crude monocarboxylic acid derivative obtained was subjected

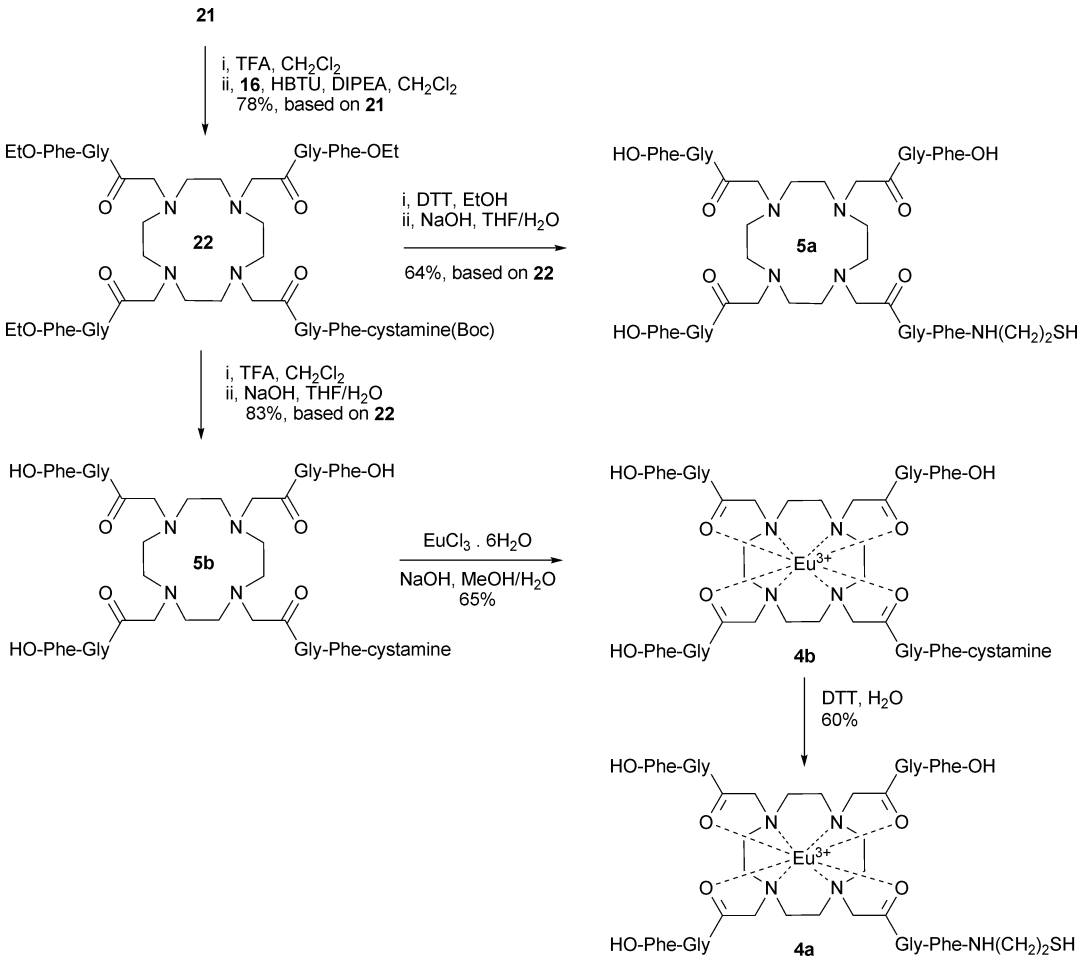


Scheme 6 Preparation of orthogonally protected mono-*t*-butyl ester **21**.

to HBTU-mediated coupling with mono-Boc-cystamine[8] (**16**). Protected ligand **22**, bearing all the necessary substitution was isolated in 78% yield (based on **21**), Scheme 7.

Dithiotreitol (DTT) mediated reduction[16] of **22** followed by saponification of the ester functionalities afforded ligand **5a** in 64% overall yield. Studies on the conjugation of compound **5a** as a modified DOTAM-Gly-Phe-OH subunit to cell penetrating peptides and gold nanoparticles are currently in progress and the results of these studies will be described in due course.

We were also interested in preparing the Eu^{3+} complexes of ligands **5** (compounds **4**) in order to compare the PARACEST properties (see below) of these modified ligands with those of parent compound Eu^{3+} DOTAM-Gly-Phe-OH (**3**). It was found that the treatment of ligand **5a** with $EuCl_3 \cdot 6H_2O$ did not afford the desired complex **4a**. The formation of the disulfide bridge between the two molecules of ligand **5a** was found to be an unwanted side reaction and a complex mixture, containing ligand **5a**, the disulfide dimer as well as their Eu^{3+} complexes was obtained. Although we were keenly interested in pursuing this compound, frustratingly, the reaction did not proceed to completion even using a large excess (20 equivalents) of $EuCl_3 \cdot 6H_2O$, thus precluding the isolation of the disulfide dimer and its Eu^{3+} complex. The problem of oxidative disulfide formation was solved by preparing the ligand **5b** *via* Boc-deprotection and subsequent saponification of **22** (Scheme 7). Metalation of **5b** proceeded smoothly affording the complex **4b** (purified by size exclusion chromatography as described previously[3]) in 58% yield (Scheme 7). The absence of free Eu^{3+} was confirmed by negative xylenol orange test.[17] Complex **4b** has been characterized by ^1H NMR and MS (ESI-TOF) spectroscopies. DTT-mediated reduction of **4b** (Scheme 7) was attempted, leading to the formation of Eu^{3+} complex **4a** (confirmed by MS (ESI-TOF) spectroscopy) accompanied with significant amount of Eu^{3+} DOTAM-Gly-Phe-OH (**3**). This unwanted side product apparently results from the hydrolysis of the amide



Scheme 7 Synthesis of target molecules: ligands **5** and their Eu^{3+} complexes **4**.

bond between H-Phe-OH and cystamine. Our experience with molecules described above indicates that this amide bond is quite prone to hydrolysis. Complex **4b** indeed serves as a suitable model compound for a subsequent PARACEST properties study, whereas magnetic studies with impure complex **4a** have not been carried out. Although complexes **4a** and **4b** are shown as eight coordinate (Scheme 7), in aqueous solution the europium centre also coordinates a molecule of water. It is this bound water which, under appropriate conditions of slow exchange, gives rise a signal distinguishable from that of bulk water. These signals are observed in the Z-spectrum in which bulk water appears at $\delta = 0$ ppm. For complexes such as compound **4b**, the bound water signal appears at approximately 40–45 ppm, while the signal for other exchangable protons such as the amide N–H protons is much closer to and often obscured by the bound water signal.[3a]

PARACEST properties of complex 4b

The magnetic resonance PARACEST properties of complex **4b** were investigated at physiological temperature (37 °C) using a previously established experimental protocol.[3a,4] The data obtained were compared (Fig. 2) with the PARACEST properties of Eu^{3+} DOTAM-Gly-Phe-OH (**3**), a new PARACEST MRI contrast agent with the potential ability to perform temperature mapping.[4]

The observed PARACEST effect (35%, *ca.* 45 ppm, at 37 °C) with cystamine modified complex **4b** was found to compare reasonably well with that associated with Eu^{3+} DOTAM-Gly-Phe-OH (**3**, 44%, *ca.* 45 ppm). This finding implies that the cystamine modified complex **4b**, and likely close structural analogues such as **4a** and peptide conjugates, preserve the desirably strong PARACEST properties that are the basis of generating MRI contrast.

Conclusions

Various synthetic approaches have been investigated towards the preparation of asymmetrically peptide-decorated cyclens that possess suitable functionality for chemoselective conjugation to other molecular entities. The challenge was to leave as much of the ligand sphere as possible undisturbed to preserve the PARACEST properties observed for the parent Eu^{3+} DOTAM-Gly-Phe-OH, as previous model studies showed that this was necessary.[3b] The inclusion of a sulfanyl group (−SH) was viewed as a useful functional group that could be addressed chemoselectively. The sulfanyl group adds to the versatility of the molecule as it is reactive towards Michael acceptors, iodoacetyl compounds or can undergo oxidative disulfide formation. It is this latter manifestation of reactivity that we intend to exploit for the formation of peptide–chelator complexes.

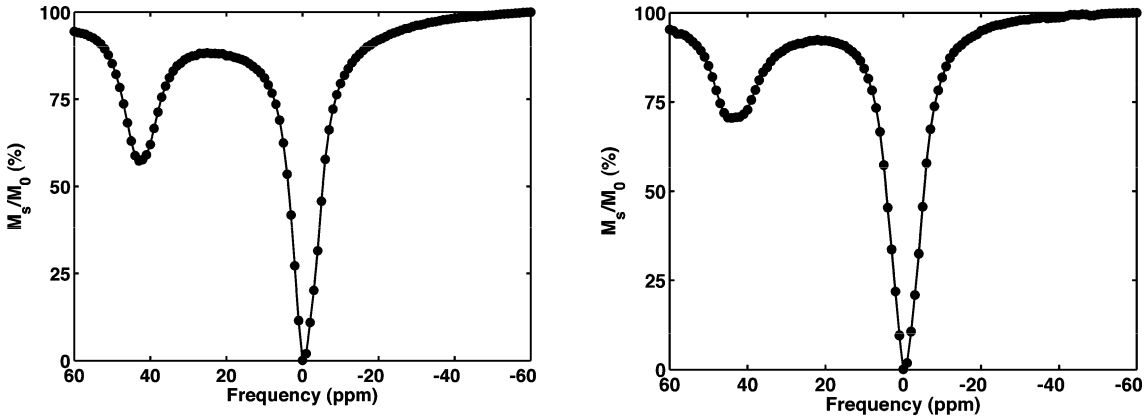


Fig. 2 Z-spectrum of a 10 mM, pH 7.0 solution of Eu^{3+} DOTAM-Gly-Phe-OH (**3**, left) and cystamine modified complex **4b** (right) acquired on a 9.4 T NMR spectrometer using a 10 s, 14 μT saturation pulse at 37 °C.

Synthetic routes based on the peralkylation of easily accessible cyclen derivatives [*N*-formylcyclen (**9**), *N*-cyclenacetic acid (**10**)] permitted us to explore some new chemistry, yet did not lead to a workable synthesis of ligands **5** and their Eu^{3+} complexes. Selective trialkylation of cyclen (**6**) with *N*-iodoacetyl-Gly-Phe-OEt (**7**) was also found to be problematic. Ultimately, the new synthetic route to ligands **5a**,**b** and their Eu^{3+} complexes rested on a selective monoalkylation of cyclen (**6**) with *N*-haloacetyl-Gly-Phe-O*t*Bu (**12**) as a key step. This was followed by peralkylation with **7**, removal of the O*t*Bu group and HBTU-mediated coupling with mono-Boc-cystamine (**16**). After some protecting group manipulations, intermediate **22** was converted into ligands **5a**,**b**. These were successfully loaded with metal by treatment with $EuCl_3 \cdot 6H_2O$ resulting in the formation of complexes **4a**,**b**. Among them, complex **4b** was obtained in pure form and its magnetic properties have been investigated. The PARACEST properties associated with complex **4b** compare favourably with Eu^{3+} DOTAM-Gly-Phe-OH (**3**). It can be envisioned that conjugation of cell penetrating peptide with ligand **5a**, followed by introduction of Eu^{3+} will provide a new advanced PARACEST MRI contrast agent with the potential to cross various biological barriers (*e.g.* the blood brain barrier) while retaining desirable magnetic properties. The development of a cell penetrating peptide conjugate of complex **5a** and its Eu^{3+} complex is underway and will be described later.

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 CH_2Cl_2 and THF (dried over Al_2O_3, in a solvent purification system) and water (18.2 MΩ cm millipore water). Organic extracts were dried with Na_2SO_4 and solvents were removed under reduced pressure in a rotary evaporator. Flash column chromatography (FCC) was carried out using silica gel, mesh size 230–400 Å. Size exclusion chromatography was carried out on BIO-GEL P2, 45–90 μm mesh resin (20 g, column size 15 × 2 cm per 0.1 mmol of compound). Ten fractions (10 ml each) were collected. Fractions were identified by UV/I_2 vapours (compounds **4a**, **19**) or by 5% solution of ninhydrin in AcOH/EtOH (compound **4b**). Thin layer chromatography (TLC) was carried out on Al backed silica gel plates, compounds were visualized by UV light or I_2 vapors. Melting points were obtained on Fisher–Johns apparatus and are uncorrected. Specific rotations $[\alpha]_D$ were determined at ambient temperature using a 5 mL, 10 cm path length cell; the units are 10^{-1} deg cm^2 g^{-1} and the concentrations are reported in g 100 mL^{-1}. HPLC analysis was carried out using a high performance liquid chromatograph equipped with an automatic injector, diode array detector (wavelength range 190–600 nm), degasser and a Microsorb-CN column (particle size 5 μm; 4.6 id × 200 mm). Mobile phase: 75% H_2/25% MeCN–25% H_2O/75% MeCN over 35 min, linear gradient, flow rate 1 mL min^{-1}. NMR spectra were recorded on a 400 MHz spectrometer; for ^1H (400 MHz), δ values were referenced as follows $CDCl_3$ (7.26 ppm); DMSO-D_6 (2.49 ppm); D_2O (4.75 ppm) for ^{13}C (100 MHz) $CDCl_3$ (77.0 ppm); DMSO-D_6 (39.5 ppm). Mass spectra (MS) were obtained using electron impact (EI) or electrospray ionization (ESI) techniques.

NHS–DCC mediated coupling of *N*-chloroacetylglycine (13) with H-Phe-O*t*Bu·HCl (14). NHS (575 mg, 5 mmol) and DCC (1.341 g, 6.5 mmol) were added (at 0 °C) to a solution of *N*-chloroacetylglycine (**13**, 758 mg, 5 mmol) in dry CH_2Cl_2 (40 mL). The mixture was stirred for 30 min at 0 °C, H-Phe-O*t*Bu·HCl (**14**, 1.289 g, 5 mmol) and Et_3N (1.4 mL, 10 mmol) added and the stirring continued for 18 h at rt. The mixture was cooled to −20 °C, precipitate was filtered off, the filter was washed with a small amount (*ca.* 15 mL) of cold (−20 °C) dichloromethane, the combined filtrate was washed with 1 M HCl (30 mL) and the aqueous phase was extracted with CH_2Cl_2 (20 mL). The organic phase was dried, concentrated and the residue was subjected to FCC on 50 g SiO_2 (CH_2Cl_2–MeOH, 9 : 1). The residue obtained after concentrating the eluate contained a small amount of *N,N′*-dicyclohexylurea (DCU), which was removed by re-dissolution in a minimal amount of acetone (*ca.* 10 mL) and set aside for 18 h at −20 °C. The precipitate that formed (DCU) was removed by filtration through a cotton plug placed in a Pasteur pipette. The solvent was removed to give *N*-chloroacetyl-Gly-Phe-O*t*Bu (**12a**, 1.734 g, 98%). Slightly yellow oil; $[\alpha]_D^{25}$ +44 (*c* 0.79, CH_2Cl_2). HPLC: t_R 13.7 min; ^1H NMR ($CDCl_3$) δ 7.43 (t, D_2O exch., J = 4.5 Hz, 1H); 7.23 (m, 3H); 7.12 (m, 2H); 6.85 (d, D_2O exch., J = 8 Hz,

1H); 4.73 (m, 1H); 4.00 (d, $J = 2$ Hz, 2H); 3.92 (ddd, $J = 6.5$, 5.5, 1.5 Hz, 2H); 3.07 (dd, $J = 14$, 4.5 Hz, 1H); 3.02 (dd, $J = 14$, 4.5 Hz, 1H); 1.38 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 170.3, 167.6, 166.5, 135.8, 129.3, 128.3, 126.9, 82.5, 53.6, 42.9, 42.2, 37.8, 27.8. HRMS (EI) m/z: found 354.1341 (354.1346 calcd for $C_{17}H_{23}ClN_2O_4$). LRMS (EI) m/z (rel abundance): 354 [M + H]$^+$ (10), 298 (32), 224 (25), 148 (100), 120 (100), 99 (21).

***N*-Iodoacetyl-Gly-Phe-O*t*Bu (12b).** NaI (1.35 g, 9 mmol) was added to a stirred solution of *N*-chloroacetyl Gly-Phe-O*t*Bu (**12a**, 1.065 g, 3 mmol) in acetone (20 mL). The mixture was stirred for 18 hours at rt, was concentrated to *ca.* one-third of its original volume, diluted with EtOAc (50 mL) and washed with 10% Na_2SO_3 solution (50 mL). The aqueous phase was extracted with EtOAc (30 mL), the organic phase was dried, concentrated, and the residue was subjected to FCC on 30 g SiO_2 (hexanes–acetone, 1 : 1) to afford 752 mg (56%) of *N*-iodoacetyl Gly-Phe-O*t*Bu (**12b**). Slightly yellow oil; $[a]_D^{25}$ +29 (*c* 0.69, CH_2Cl_2). HPLC: t_R 13.8 min; ^{1}H NMR ($CDCl_3$) δ 7.26 (m, 3H); 7.18 (m, D_2O exch., 1H); 7.15 (m, 2H); 6.71 (d, D_2O exch., $J = 8$ Hz, 1H); 4.74 (m, 1H); 3.93 (d, $J = 5$ Hz, 2H); 3.71 (s, 2H); 3.10 (dd, $J = 14$, 6.5, Hz, 1H); 3.05 (dd, $J = 12$, 6 Hz, 1H); 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$) δ 170.2, 167.9, 167.8, 135.9, 129.4, 128.4, 127.0, 82.6, 53.8, 43.6, 38.0, 27.9. HRMS (EI) m/z: found 446.0695 (446.0703 calcd for $C_{17}H_{23}IN_2O_4$). LRMS (EI) m/z (rel abundance): 446 [M + H]$^+$ (10), 390 (56), 224 (20), 198 (10), 120 (100), 99 (15).

***N*-Iodoacetyl-Gly-Phe-cystamine(Boc) (8).** DCC (318 mg, 2.2 mmol) was added (at 0 °C) to a stirred suspension of *N*-chloroacetylglycine (**13**, 212 mg, 1.4 mmol) and NHS (161 mg, 1.4 mmol) in dry CH_2Cl_2 (7 mL). The cooling bath was removed and the mixture was stirred for 18 h at rt. The solvent was evaporated, the residue was suspended in dry THF (6 mL), H-Phe-OH (**15**, 231 mg, 1.4 mmol) and Et_3N (390 μL, 2.8 mmol) were added and the mixture was stirred for 2 h at rt. The reaction mixture was then diluted with 1 M HCl (50 mL) and extracted with EtOAc (40 + 3 × 20 mL). The combined organic extracts were dried, concentrated and the residue was dissolved in dry THF (14 mL). The solution obtained was cooled to 0 °C, NHS (161 mg, 1.4 mmol) and DCC (347 mg, 1.7 mmol) were added and the mixture was stirred for 30 min at 0 °C, followed by the addition of mono-Boc-cystamine[8] (**16**, 353 mg, 1.4 mmol) and Et_3N (390 μL, 2.8 mmol). The cooling bath was removed and the mixture was stirred for 18 h at rt. The mixture was concentrated to *ca.* one-third of its original volume, was diluted with brine (50 mL) and was extracted with EtOAc (40 + 2 × 20 mL). The combined organic extracts were dried, concentrated and the residue obtained contained *ca.* 20% of *N*,*N*′-dicyclohexylurea (by ^{1}H NMR). The residue was dissolved in acetone (8 mL) and NaI (341 mg, 2.3 mmol) was added and then the mixture was stirred for 18 h at rt. Subsequently, the reaction mixture was concentrated to *ca.* one-third of its original volume and was diluted with EtOAc (30 mL), washed with 10% Na_2SO_3 solution (20 mL) and the aqueous phase was extracted with EtOAc (20 mL). The organic phase was dried and concentrated to give crude *N*-iodoacetyl compound **8**, which was purified by FCC on 50 g SiO_2 (CH_2Cl_2–MeOH, 19 : 1) and then recrystallized from dichloromethane–hexanes solution.

N-Iodoacetyl-Gly-Phe-cystamine(Boc) [**8**, 288 mg, 33% based on *N*-chloroacetylglycine (**13**)], colorless crystals; mp 130–131 °C; $[a]_D^{25}$ +19 (*c* 0.78, CH_2Cl_2). HPLC: t_R 13.3 min; ^{1}H NMR (DMSO-D_6) δ 8.41 (m, D_2O exch., 1H); 8.18 (m, D_2O exch., 2H); 7.21 (m, 5H); 6.98 (m, D_2O exch., 1H); 4.42 (m, 1H); 3.73 (dd, $J =$ 16.5, 6 Hz, 1H); 3.68 (s, 2H); 3.57 (dd, $J = 16.5$, 6 Hz, 1H); 3.26 (m, 4H); 2.97 (dd, $J = 13$, 5 Hz, 1H); 2.72 (m, 5H); 1.35 (s, 9H); ^{13}C NMR (DMSO-D_6) δ 170.8, 168.1, 167.9, 155.5, 137.7, 129.1, 128.1, 126.3, 77.8, 54.1, 42.4, 39.4, 38.0, 37.8, 37.5, 36.9, 28.2. HRMS (ESI) m/z: found 625.1000 [M + H]$^+$ (625.1015 calcd for $C_{22}H_{34}IN_4O_5S_2$).

Fmoc-Phe-cystamine(Boc) (11). NHS (115 mg, 1 mmol) and EDC·HCl (383 mg, 2 mmol) were added to a solution of Fmoc-Phe-OH (**17**, 387 mg, 1 mmol) in dry THF (5 mL). The mixture was stirred for 18 h at rt, was concentrated, the residue was dissolved in water (40 mL) and was extracted with EtOAc (40 + 2 × 20 mL). The organic extract was dried and the solvent was evaporated to leave the corresponding NHS-ester. This material was dissolved in dry THF (5 mL) followed by a dropwise addition (over a period of 10 min, at 0 °C) of a solution of mono-Boc-cystamine (**16**, 252 mg, 1 mmol) and Et_3N (210 μL, 1.5 mmol) in dry THF (5 mL). The stirring continued for 3 h at 0 °C, the mixture was diluted with brine (60 mL), and was extracted with EtOAc (40 + 20 mL). The organic extract was dried and then concentrated to leave crude compound **11**, which was purified by FCC on 50 g SiO_2 (CH_2Cl_2–MeOH, 19 : 1) followed by crystallization from dichloromethane–hexanes solution.

Fmoc-Phe-cystamine(Boc) (**11**, 318 mg, 51%), colorless crystals; mp 154–155 °C; $[a]_D^{25}$ +13 (*c* 0.80, DMSO). HPLC: t_R 11.0 min; ^{1}H NMR (DMSO-D_6) δ 8.21 (m, D_2O exch., 1H); 7.87 (m, 2H); 7.63 (m, 2H); 7.42 (m, 2H); 7.23 (m, 7H); 6.98 (m, D_2O exch., 1H); 4.15 (m, 3H); 3.33 (m, 3H); 3.19 (m, 2H); 2.96 (dd, $J = 13.5$, 4.5 Hz, 1H); 2.75 (m, 5H); 1.35 (s, 9H); ^{13}C NMR (DMSO-D_6) δ 171.5, 155.8, 155.5, 143.8, 143.7, 142.6, 140.7, 139.4, 138.1, 137.4, 129.3, 129.2, 128.9, 128.1, 128.0, 127.6, 127.3, 127.0, 126.2, 125.4, 125.3, 121.4, 120.0, 109.7, 77.8, 65.6, 56.2, 46.6, 39.4, 38.0, 37.7, 37.6, 37.1, 28.2. HRMS (EI) m/z: found 621.2296 (621.2331 calcd for $C_{33}H_{39}N_3O_5S_2$). LRMS (EI) m/z (rel abundance): 621 [M + H]$^+$ (10), 308 (10), 223 (16), 178 (79), 120 (100), 91 (10).

Alkylation of *N*-formylcyclen (9) with *N*-iodoacetyl-Gly-Phe-OEt (7). DIPEA (260 μL, 1.5 mmol) and *N*-iodoacetyl-Gly-Phe-OEt[3] (**7**, 628 mg, 1.5 mmol) were added to a solution of *N*-formylcyclen[6] (**9**, 100 mg, 0.5 mmol) in MeCN (10 mL). The mixture was stirred for 18 h at 50 °C, cooled to rt and then diluted with EtOAc (40 mL). The organic solution was washed with water (40 mL) and the aqueous phase was extracted with EtOAc (20 mL). The organic extract was dried and was concentrated and the resulting oil was triturated (twice) with a small amount (*ca.* 15 mL) of hexane to leave *N*-formyl-*N*-triacetyl-Gly-Phe-OEt cyclen (**18**, 494 mg, 92%). Colorless solid; $[a]_D^{25}$ +55 (*c* 0.55, CH_2Cl_2). HPLC: t_R 21.8 min; ^{1}H NMR (DMSO-D_6) δ 8.26 (m, D_2O exch., 2H); 7.87 (m, D_2O exch., 2H); 7.09 (m, 15H); 4.35 (m, 3H); 3.89 (m, 6H); 3.62 (m, 6H); 3.26–2.38 (br m, 28H); 0.96 (m, 9H); ^{13}C NMR (DMSO-D_6) δ 171.9, 171.4, 170.9, 170.7, 170.5, 170.4, 169.6, 169.1, 168.9, 168.3, 163.3, 137.0, 136.9, 129.1, 128.3, 126.6, 60.6, 53.7, 52.5, 41.8, 41.7, 37.0, 36.9, 13.9. HRMS (ESI) m/z: found 1071.5514 [M + H]$^+$ (1071.5515 calcd for $C_{54}H_{75}N_{10}O_{13}$).

Saponification of *N*-formyl-*N*-triacetyl-Gly-Phe-OEt cyclen (18). NaOH (1 M, 980 μL) was added to a solution of

N-formyl-*N*-triacetyl-Gly-Phe-OEt cyclen (**18**, 107 mg, 0.1 mmol) in EtOH (3 mL). The mixture was stirred for 18 h at rt, EtOH was evaporated, the aqueous residue was cooled to 0 °C and the pH was adjusted to 6 (1 M HCl). The obtained solution was subjected to size exclusion chromatography as described in the general experimental procedures; fractions containing the product were combined and were lyophilized to leave *N*-formyl-*N*-triacetyl-Gly-Phe-OH cyclen (**19**, 76 mg, 78%). Colorless solid; $[\alpha]_D^{25}$ −7 (*c* 0.72, MeOH). HPLC: t_R 9.4 min; ^{1}H NMR (D_2O) δ 7.20 (m, 16H); 4.36 (m, 3H); 3.75 (m, 6H); 3.45–2.84 (br m, 28H); ^{13}C NMR (DMSO-D_6) δ 178.1, 173.4, 170.5, 170.3, 166.5, 138.2, 138.0, 129.7, 128.9, 127.1, 56.5, 54.0, 53.3, 51.8, 50.5, 42.5, 42.0, 39.6, 37.8. HRMS (ESI) *m/z*: found 987.4575 $[M + H]^+$ (987.4576 calcd for $C_{48}H_{63}N_{10}O_{13}$).

Selective monoalkylation of cyclen (6) with *N*-haloacetyl-Gly-Phe-O*t*Bu (12) to give *N*-monoacetyl-Gly-Phe-O*t*Bu cyclen (20). Separate solutions of *N*-chloroacetyl-Gly-Phe-O*t*Bu (**12a**, 562 mg, 1.57 mmol) or *N*-iodoacetyl-Gly-Phe-O*t*Bu (**12b**, 705 mg, 1.57 mmol) in $CHCl_3$ (2 mL) were added dropwise (over a 10 min period) to a stirred solution (at 0 °C) of cyclen (**6**, 541 mg, 3.14 mmol) in $CHCl_3$ (4 mL). The slow addition of electrophile and careful control of the temperature is needed to have reproducible results. The mixtures were stirred for 2 h at 0 °C and 2 h at rt; a clear solution was obtained with *N*-chloroacetyl-Gly-Phe-O*t*Bu (**12a**); it was concentrated and the residue was subjected to FCC on 15 g SiO_2 (CH_2Cl_2–MeOH–NH_4OH, 80 : 20 : 1). Evaporation of the eluate afforded *N*-monoacetyl-Gly-Phe-O*t*Bu cyclen (**20**, 368 mg, 48%). A white precipitate formed when *N*-iodoacetyl-Gly-Phe-O*t*Bu (**12b**) was used, it was filtered off, the filtrate was concentrated and the residue was subjected to FCC as described above, leaving *N*-monoacetyl-Gly-Phe-O*t*Bu cyclen (**20**, 176 mg, 23%). Colorless oil; $[\alpha]_D^{25}$ +19 (*c* 0.52, MeOH). HPLC: t_R 7.5 min; ^{1}H NMR (DMSO-D_6) δ 8.48 (m, D_2O exch., 1H); 7.23 (m, 5H); 4.34 (m, 1H); 3.76 (s, 2H); 3.48 (br m, D_2O exch., 1H); 3.22 (s, 2H), 2.92 (m, 2H); 2.67 (m, 16H); 1.29 (s, 9H); ^{13}C NMR (DMSO-D_6) δ 171.1, 170.5, 169.2, 137.2, 129.2, 128.2, 126.5, 80.7, 58.0, 54.3, 51.4, 48.6, 46.7, 45.4, 44.8, 41.3, 37.0, 30.7, 27.5. HRMS (ESI) *m/z*: found 491.3349 $[M + H]^+$ (491.3346 calcd for $C_{25}H_{43}N_6O_4$).

Alkylation of *N*-monoacetyl-Gly-Phe-O*t*Bu cyclen (20) with *N*-iodoacetyl-Gly-Phe-OEt (7). DIPEA (385 μL, 2.2 mmol) and *N*-iodoacetyl-Gly-Phe-OEt[3] (**7**, 919 mg, 2.2 mmol) were added to a solution of *N*-monoacetyl-Gly-Phe-O*t*Bu cyclen (**20**, 359 mg, 0.73 mmol) in MeCN (8 mL). The mixture was stirred for 18 h at 70 °C, was cooled to rt and was diluted with EtOAc (30 mL). The organic solution was washed with water (30 mL), the aqueous phase was extracted with EtOAc (20 mL) and the organic phase was dried and concentrated. The resulting oil was triturated (twice) in a small amount (*ca.* 15 mL) of hexanes to leave *N*-monoacetyl-Gly-Phe-O*t*Bu-*N*-triacetyl-Gly-Phe-OEt cyclen (**21**, 986 mg, 99%). Colorless solid; $[\alpha]_D^{25}$ +28 (*c* 0.91, CH_2Cl_2). HPLC: t_R 23.0 min; ^{1}H NMR (DMSO-D_6) δ 8.28 (m, D_2O exch., 2H); 8.05 (m, D_2O exch., 2H); 7.22 (m, 20H); 4.41 (m, 4H); 4.00 (m, 6H); 3.71 (m, 8H); 3.01–2.87 (br m, 16H); 2.60–2.53 (br m, 16H); 1.27 (s, 9H); 1.05 (m, 9H); ^{13}C NMR (DMSO-D_6) δ 171.8, 171.3, 170.4, 168.8, 168.3, 166.2, 137.0, 136.8, 129.2, 129.1, 128.2, 128.1, 126.6, 126.5, 80.8, 60.5, 59.7, 54.0, 53.6, 41.5, 37.2, 37.0, 27.5, 13.9. HRMS (ESI) *m/z*: found 1361.7184 $[M + H]^+$ (1361.7186 calcd for $C_{70}H_{97}N_{12}O_{16}$).

Removal of *t*Bu group from 21, followed by HBTU-mediated coupling with mono-Boc-cystamine (16). TFA (1 mL) was added to a solution of *N*-monoacetyl-Gly-Phe-O*t*Bu-*N*-triacetyl-Gly-Phe-OEt cyclen (**21**, 408 mg, 0.3 mmol) in CH_2Cl_2 (2 mL). The mixture was stirred for 18 h at rt, was concentrated, the residue was dissolved in EtOAc (40 mL) and the solution obtained was washed with saturated $NaHCO_3$ solution (20 mL). The aqueous phase was extracted with EtOAc (20 mL), and the organic phase was separated, dried and concentrated to leave *N*-monoacetyl-Gly-Phe-OH-*N*-triacetyl-Gly-Phe-OEt cyclen (342 mg, 87%) of sufficient purity (HPLC inspection) for the next step. This material was dissolved in dry CH_2Cl_2 (6 mL), the solution obtained was cooled to 0 °C followed by the addition of DIPEA (91 μL, 0.52 mmol) and HBTU (99 mg, 0.26 mmol). The mixture was stirred for 15 min at 0 °C, a solution of mono-Boc-cystamine[8] (**16**, 66 mg, 0.26 mmol) in dry CH_2Cl_2 (2 mL) was added dropwise (over the period of 1 min), the cooling bath was removed and the mixture was stirred for 4 h at rt. The solvent was evaporated, the residue was dissolved in EtOAc (50 mL) and the solution obtained was consecutively washed with saturated $NaHCO_3$ solution (30 mL), 1 M HCl (30 mL) and saturated $NaHCO_3$ solution (20 mL). The organic phase was dried and was concentrated to leave a colorless solid, which was purified by trituration (twice) with a small amount (*ca.* 15 mL) of hexane to leave *N*-monoacetyl-Gly-Phe-cystamine(Boc)-*N*-triacetyl-Gly-Phe-OEt cyclen (**22**, 358 mg, 90%). Colorless solid; $[\alpha]_D^{25}$ +30 (*c* 0.67, CH_2Cl_2). HPLC: t_R 23.2 min; ^{1}H NMR (DMSO-D_6) δ 8.44 (m, D_2O exch., 2H); 8.21 (m, D_2O exch., 3H); 7.22 (m, 20H); 4.45 (m, 4H); 3.99 (m, 6H); 3.71 (m, 8H); 3.20–2.31 (br m, 40H); 1.36 (s, 9H); 1.04 (m, 9H); ^{13}C NMR (DMSO-D_6) δ 171.8, 171.7, 171.3, 170.8, 170.3, 168.9, 168.8, 168.5, 168.3, 166.3, 155.5, 137.5, 137.0, 136.8, 129.2, 129.1, 128.2, 128.1, 126.6, 77.8, 60.5, 59.8, 57.0, 53.7, 47.6, 41.4, 38.2, 37.0, 33.4, 32.3, 28.2, 25.4, 25.3, 24.7, 24.5, 14.1, 13.9. HRMS (ESI) *m/z*: found 1539.7303 $[M + H]^+$ (1539.7380 calcd for $C_{75}H_{107}N_{14}O_{17}S_2$).

DTT-mediated reduction and saponification of 22. DTT (358 mg, 2.32 mmol) was added to a solution of *N*-monoacetyl-Gly-Phe-cystamine(Boc)-*N*-triacetyl-Gly-Phe-OEt cyclen (**22**, 358 mg, 0.23 mmol) in EtOH (8 mL). The mixture was stirred for 18 h at rt, was concentrated, the residue was dissolved in EtOAc (40 mL), and the solution obtained was washed with saturated $NaHCO_3$ solution (20 mL). The aqueous phase was extracted with EtOAc (20 mL), the organic phase was dried and then concentrated to leave a solid residue (*N*-monoacetyl-Gly-Phe-NH$(CH_2)_2$SH-*N*-triacetyl-Gly-Phe-OEt cyclen) that was used in the next step without further purification. The solid residue obtained was dissolved in THF (4.5 mL), NaOH solution (1M, 4.5 mL) was added and the mixture was stirred vigorously for 2 h at rt. THF was evaporated, the aqueous solution was cooled to 0 °C, was acidified (1 M HCl, pH 5) and was set aside for 2 h at 0 °C. Water was decanted, the oily product deposited on the flask walls was washed with water, was coevaporated with toluene (ca. 20 mL) and was triturated (twice) in hexanes (*ca.* 15 mL) to leave *N*-monoacetyl-Gly-Phe-NH$(CH_2)_2$SH-*N*-triacetyl-Gly-Phe-OH cyclen (**5a**, 189 mg, 64% based on **22**). Colorless solid; $[\alpha]_D^{25}$ −5 (*c* 0.94, MeOH). HPLC: t_R 11.6 min; ^{1}H NMR (DMSO-D_6) δ 8.27 (m, D_2O exch., 6H); 7.20 (m, 20H); 4.40 (m, 4H); 3.71 (m, 8H); 3.51–2.15 (br m, 36H); ^{13}C NMR (DMSO-D_6) δ 173.1, 169.2, 168.3, 137.7, 137.6, 137.5, 129.3,

129.2, 128.3, 128.2, 126.4, 126.3, 56.0, 55.9, 53.9, 51.1, 50.9, 50.4, 47.6, 45.4, 42.2, 41.7, 41.6, 41.5, 37.8, 36.9, 33.4, 32.3, 28.2, 27.0. HRMS (ESI) *m/z*: found 1280.5720 $[M + H]^+$ (1280.5774 calcd for $C_{62}H_{82}N_{13}O_{15}S$).

Boc-deprotection and saponification of 22. TFA (1.5 mL) was added to a solution of *N*-monoacetyl-Gly-Phe-cystamine(Boc)-*N*-triacetyl-Gly-Phe-OEt cyclen (**22**, 328 mg, 0.21 mmol) in CH_2Cl_2 (3 mL), the mixture was stirred for 30 min at rt and was concentrated to leave *N*-monoacetyl-Gly-Phe-cystamine-*N*-triacetyl-Gly-Phe-OEt cyclen that was used for the next step without further purification. This material was dissolved in THF (1.7 mL) and NaOH solution (2.5 M, 1.7 mL) was added. The mixture was stirred vigorously for 2 h at rt. THF was evaporated, the aqueous solution was cooled to 0 °C, was acidified (1 M HCl, pH 5) and was set aside for 2 h at 0 °C. Water was decanted, an oily product deposited on the flask walls was washed with water, was coevaporated with toluene (*ca.* 20 mL) and was triturated (twice) in hexanes (*ca.* 15 mL) to leave *N*-monoacetyl-Gly-Phe-cystamine-*N*-triacetyl-Gly-Phe-OH cyclen (**5b**, 240 mg, 83% based on **22**). Colorless solid; $[\alpha]_D^{25}$ +9 (*c* 0.59, MeOH). HPLC: t_R 13.0 min; ^{1}H NMR (DMSO-D_6) δ 8.32 (m, D_2O exch., 6H); 7.19 (m, 20H); 4.38 (m, 4H); 3.78–2.77 (br m, 48H); ^{13}C NMR (DMSO-D_6) δ 172.9, 172.8, 171.8, 171.1, 168.8, 168.6, 137.6, 137.4, 129.2, 128.2, 128.1, 126.5, 126.4, 57.0, 53.8, 50.8, 41.6, 38.1, 36.9, 34.0, 33.4, 32.9, 32.4. HRMS (ESI) *m/z*: found 1355.5850 $[M + H]^+$ (1355.5917 calcd for $C_{64}H_{87}N_{14}O_{15}S_2$).

Metallation of *N*-monoacetyl-Gly-Phe-cystamine-*N*-triacetyl-Gly-Phe-OH cyclen (5b). $EuCl_3 \cdot 6H_2O$ (32 mg, 0.089 mmol) was added to a suspension of *N*-monoacetyl-Gly-Phe-cystamine-*N*-triacetyl-Gly-Phe-OH cyclen (**5b**, 120 mg, 0.089 mmol) in MeOH (2 mL) and water (4 mL). The pH of the reaction mixture was adjusted to *ca.* 9 (2.5 M NaOH solution), the mixture was stirred for 18 h at rt and was subjected to size exclusion chromatography as described in the general experimental procedures; fractions containing the product (the absence of free Eu^{3+} was confirmed by negative xylenol orange test[17]) were combined and were lyophilized to leave Eu^{3+} *N*-monoacetyl-Gly-Phe-cystamine-*N*-triacetyl-Gly-Phe-OH cyclen (**4b**, 87 mg, 65%). Colorless solid; ^{1}H NMR (D_2O) δ 24.57 (s); 23.87 (s); 8.45 (s); 7.17–6.84 (m); 6.59 (s); 4.28–4.21 (m); 4.04 (s); 3.74–3.43 (m); 3.20–2.05 (m); 1.47 (s); −3.08 (s); −3.49 to −3.83 (m); −5.09 (s); −8.92 to −9.19 (m); −9.98 (s); −12.24 (s); −13.00 to −13.10 (m). HRMS (ESI) *m/z*: found 1503.4946 $[M - 2H]^+$ (1503.4881 calcd for $C_{64}H_{84}N_{14}O_{15}S_2Eu$).

DTT-mediated reduction of 4b. DTT (43 mg, 0.28 mmol) was added to a solution of Eu^{3+} *N*-monoacetyl-Gly-Phe-cystamine-*N*-triacetyl-Gly-Phe-OH cyclen (**4b**, 42 mg, 0.028 mmol) in H_2O (2 mL). The mixture was stirred for 18 h at rt and was subjected to size exclusion chromatography as described in the general experimental procedures; fractions containing the product were combined and were lyophilized to leave impure Eu^{3+} *N*-monoacetyl-Gly-Phe-NH$(CH_2)_2$SH-*N*-triacetyl-Gly-Phe-OH cyclen (**4a**, 24 mg, 60%) containing a significant amount of Eu^{3+} DOTAM-Gly-Phe-OH (**3**). The absence of free Eu^{3+} was confirmed by negative xylenol orange test.[17] Colorless solid; HRMS (ESI) *m/z*: found 1428.4727 $[M - 2H]^+$ (1428.4738 calcd for $C_{62}H_{79}N_{13}O_{15}SEu$).

Acknowledgements

We thank the Ontario Institute for Cancer Research (OICR), for providing the operating funds and equipment needed to perform this work.

References and notes

1 B. Yoo and M. D. Pagel, *Front. Biosci.*, 2008, **13**, 1733–1752, and references cited therein.
2 S. Zhang, M. Merritt, D. E. Woessner, R. E. Lenkinski and A. D. Sherry, *Acc. Chem. Res.*, 2003, **36**, 783–790.
3 (*a*) F. Wojciechowski, M. Suchý, A. X. Li, H. A. Azab, R. Bartha and R. H. E. Hudson, *Bioconjugate Chem.*, 2007, **18**, 1625–1636; (*b*) M. Suchý, A. X. Li, R. Bartha and R. H. E. Hudson, *Bioorg. Med. Chem.*, 2008, **16**, 6156–6166.
4 A. X. Li, F. Wojciechowski, M. Suchý, C. K. Jones, R. H. E. Hudson, R. Menon and R. Bartha, *Magn. Reson. Med.*, 2008, **59**, 374–381.
5 For the recent reviews on the use of cell penetrating peptides in drug delivery see: (*a*) W. M. Pardridge, *Mol. Intervention*, 2003, **3**, 90–105; (*b*) M. Zorko and Ü. Langel, *Adv. Drug Delivery Rev.*, 2005, **57**, 529–545; (*c*) W. M. Pardridge, *Curr. Opin. Pharmacol.*, 2006, **6**, 494–500; (*d*) K. M Stewart, K. L. Horton and S. O. Kelly, *Org. Biomol. Chem.*, 2008, **6**, 2242–2255.
6 (*a*) D. D. Dischino, E. J. Delaney, J. E. Emswiler, G. T. Gaughan, J. S. Prasad, S. K. Srivastava and M. F. Tweedle, *Inorg. Chem.*, 1991, **30**, 1265–1269; (*b*) J. Platzek, P. Blaszkiewicz, H. Gries, P. Luger, G. Michl, A. Müller-Fahrnow, B. Radüchel and D. Sülzle, *Inorg. Chem.*, 1997, **36**, 6086–6093.
7 I. Meunier, A. K. Mishra, B. Hanquet, P. Cocolios and R. Guilard, *Can. J. Chem.*, 1995, **73**, 685–695.
8 K. A. Jacobson, B. Fischer and X. Ji, *Bioconjugate Chem.*, 1995, **6**, 255–263.
9 (*a*) G. Losse and W. Zönnchen, *Liebigs Ann. Chem.*, 1960, **636**, 140–143; (*b*) J. O. Thomas, *Tetrahedron Lett.*, 1967, **8**, 335–336.
10 G. Losse and D. Nadolski, *J. Prakt. Chem.*, 1964, **24**, 118–124.
11 (*a*) J. Yoo, D. E. Reichert and M. J. Welch, *J. Med. Chem.*, 2004, **47**, 6625–6637; (*b*) A. C. Ferrand, D. Imbert, A. S. Chauvin, C. D. B. Vandevyver and J. C. G. Bünzli, *Chem.–Eur. J.*, 2007, **13**, 8678–8687.
12 V. Boldrini, G. B. Giovenzana, R. Pagliarin, G. Palmisano and M. Sisti, *Tetrahedron Lett.*, 2000, **41**, 6527–6530.
13 Products of peralkylation at nitrogen including some *O*-alkylations have not been isolated but their structures were inferred from the MS (ESI-TOF) analysis.
14 For selected examples see: (*a*) A. Roigant, I. Gardinier, H. Bernard, J. J. Yaounac and H. J. Handel, *J. Chem. Soc., Chem. Commun.*, 1995, 1233–1234; (*b*) J. I. Bruce, R. S. Dickins, L. J. Govenlock, T. Gunnlaugsson, S. Lopinski, M. P. Lowe, D. Parker, R. D. Peacock, J. J. B. Perry, S. Aime and M. Botta, *J. Am. Chem. Soc.*, 2000, **122**, 9674–9684; (*c*) T. Gunnlaugsson, J. P. Leonard, S. Mulready and M. Nieuwenhuyzen, *Tetrahedron*, 2004, **60**, 105–113; (*d*) C. Li and W. T. Wong, *Tetrahedron*, 2004, **60**, 5595–5601; (*e*) L. S. Karfeld, S. R. Bull, N. E. Davis, T. J. Meade and A. E. Barron, *Bioconjugate Chem.*, 2007, **18**, 1697–1700.
15 For selected examples see: (*a*) W. J. Kruper, P. R. Rudolf and C. A. Langhoff, *J. Org. Chem.*, 1993, **58**, 3869–3876; (*b*) A. Heppler, S. Froidevaux, H. R. Mäcke, E. Jermann, M. Béhé, P. Powell and M. Hennig, *Chem.–Eur. J.*, 1999, **5**, 1974–1981; (*c*) M. Woods, G. E. Kiefer, S. Bott, A. Castillo-Muzquiz, C. Eshelbrenner, L. Michaudet, K. McMillan, S. D. K. Mudigunda, D. Ogrin, G. Tircsó, S. Zhang, P. Zhao and A. D. Sherry, *J. Am. Chem. Soc.*, 2004, **126**, 9248–9256; (*d*) N. J. Wardle, A. H. Herlihy, P. W. So, J. D. Bell and S. W. A. Bligh, *Bioorg. Med. Chem.*, 2007, **15**, 4714–4721; (*e*) J. Massue, S. E. Plush, C. S. Bonnet, D. A. Moore and T. Gunnlaugsson, *Tetrahedron Lett.*, 2007, **48**, 8052–8055.
16 Reduction of **22** with $NaBH_4$ and $NaBH_3CN$ was also attempted but led to a complex mixture of products due to reduction of the ester functionalities.
17 A. Barge, G. Cravotto, E. Gianolio and F. Fedeli, *Contrast Media Mol. Imaging*, 2006, **1**, 184–188.

