Bifunctional ligands based on the DOTA-monoamide cage

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Efficient routes to DOTA-monoamide ligands bearing amino, hydroxyl, aldehyde and maleimido groups are described. These functional groups, which can be spaced at will from the coordination cage, will readily react with suitable groups of targeting moieties. Bioconjugates obtained in this way can be used for diagnostic imaging and therapeutic applications.

Introduction

In the last few decades many acyclic and macrocyclic compounds have found important applications in diagnostic and therapeutic medicine in the form of metal complexes. These chelates are used both as contrast agents for magnetic resonance imaging (MRI)[1] and as radiopharmaceuticals for the diagnosis and therapy of tumours.[2] A very important goal of current research is to achieve a precise delivery of these compounds to specific cellular targets. The general strategy is to insert them into bioconjugates that will bind to specific receptors that are overexpressed in certain tissues or act as markers to visualize a cell product that is the "signature" of a specific disease. In such conjugates the moiety encapsulating the metal ion is a bifunctional chelating agent (BFCA) that contains a suitable function through which it can be covalently bound to the specific biological carrier. In this regard, the design and development of synthetic BFCAs for connecting these targeting vehicles to metal complexes, and improvements in related conjugation and chelation procedures are topics of outstanding importance in molecular medicine.

In the field of polyaminopolycarboxylic ligands a straightforward route to bifunctional chelating agents is the reaction of DTPA anhydride with amines to form DTPA mono- and bis-amide derivatives.[3,4] The latter chelating moiety can readily coordinate a number of metal ions (transition, group III or lanthanide) of biomedical interest. Relevant examples are found in the use of In or Ga for SPECT studies and Y and Lu for therapeutic applications.[3] However, most recent work on DTPA-amide-containing agents has dealt with the use of Gd complexes for MRI investigations.[4] Evidence has emerged from *in vitro* studies that Gd complexes of DTPA-amide may not have a sufficient thermodynamic/kinetic stability for the intended purposes.[5] This concern may weigh even more when DTPA-amide BFCAs are used in molecular imaging applications for which long contact times with biological structures are required.

It is therefore important to develop BFCAs whose chelating moiety will coordinate metal ions more tightly than DTPA-amide. In this regard, DTPA,[6] DOTA[7–10] and DOTA-monoamide[11–15] systems have been considered as good alternatives. The synthesis of functionalised DOTA derivatives as BFCAs was a hot topic at the end of the '80s and beginning of the '90s. Several patents and papers were published by the groups of Meares, Parker, Gansow and Sherry.[16,17] More recent applications to magnetic resonance molecular imaging and nuclear medicine required the use of new BFCAs, which entailed the development of more efficient synthetic strategies and the optimization of previous protocols. Thus, the commercially available triprotected DOTA-tris(*tert*-butyl) ester,[11] which forms DOTA-monoamide derivatives when it reacts with amines, is one of the most frequently used bifunctional agents.[12] Conjugation to biological vectors has also been exploited with the benzyl-protected analogue DOTA-tris(benzyl) ester,[13] the isothiocyanate-functionalized *p*-NCS-Bz-DOTA both on the macrocyclic backbone and on the pendant arm (C-DOTA and PA-DOTA, respectively),[7] DOTAGA(t-BuO)$_4$,[8] which contains an additional unprotected carboxylic group, 4-acetylphenyl-, ethynylphenyl-[9] and vinyl sulfone-cysteineamido,[14] maleimido-cysteinamido[15] DOTA derivatives containing a methyl ketone, an alkyne, a vinyl sulfone and a maleimido group, respectively.

Herein we report the syntheses of BFCAs based on DOTA-monoamide that display amino, alcohol, aldehyde or maleimido functions to be eventually used for conjugation to biological vectors of interest. Each of these functions may be spaced at will from the chelating moiety by choosing the number of interposed methylene carbons (Scheme 1).

Results

It is well known that DOTA-monoamide derivatives are not the best ligands for Gd(III) for use in MRI because of their long water coordinated exchange time. However, this turns out not to be a real limitation, especially in cell labeling procedures, because it has been reported[18] that when a Gd(III) complex is internalized into the cell by endocytosis its relaxivity is dramatically decreased (and its contrast-enhancing power consequently decreased), both for fast- and slow-water-exchanging complexes. Thus DOTA-monoamide based contrast agents can be successfully used in molecular imaging applications.

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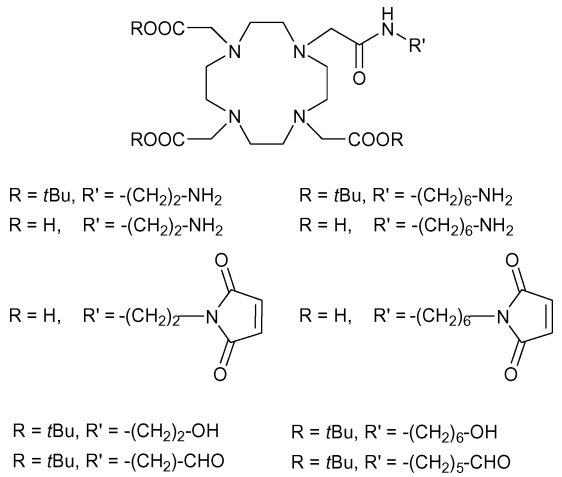


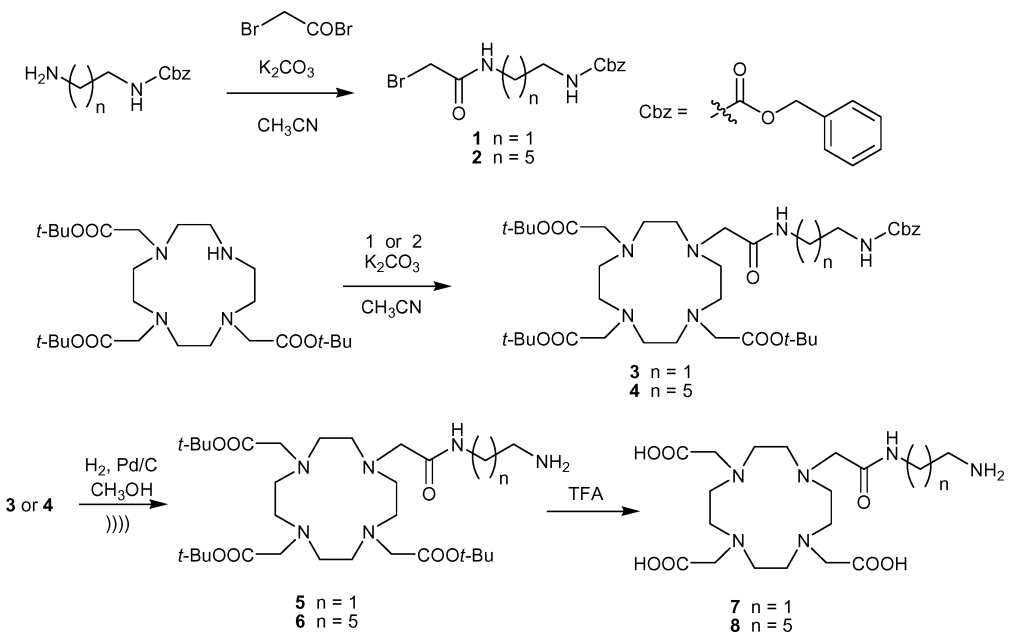
Scheme 1 DOTA-monoamide derivatives bearing different functional groups.

a) Synthesis of NH_2-functionalized DOTA-monoamide bifunctional agents.

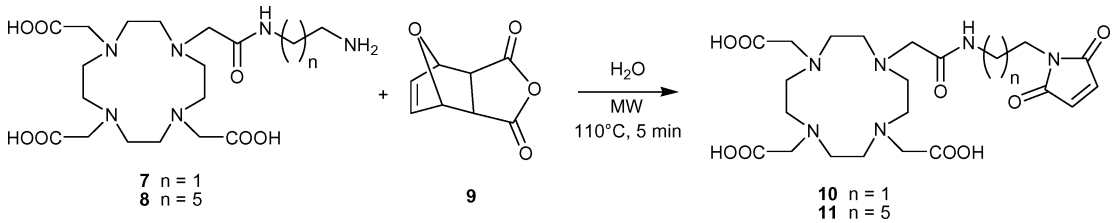
In the present work mono-Cbz alkyldiamino derivatives were converted to bromoacetamido derivatives **1** and **2** by reaction with bromoacetyl bromide. Compounds **1** and **2** were then used for the *N*-alkylation of DO3A(*t*-BuO)$_3$ (1,4,7-tris-*tert*-butoxycarbonylmethyl-1,4,7,10-tetraazacyclododecane) which was prepared as the hydrobromide according to a reported procedure.[19] The secondary ammonium salt of DO3A(*t*-BuO)$_3$ is not nucleophilic enough to react with a primary halide; indeed the reaction gave negligible or poor yields even in the presence of K_2CO_3. This hurdle was overcome by treating DO3A(*t*-BuO)$_3$ hydrobromide with the strongly basic ion-exchange resin Amberlite® IRA 410 in its OH form, that easily deprotonated the ammonium salt to give DO3A(*t*-BuO)$_3$ as free amine. The latter, being more nucleophilic than the hydrobromide salt, reacted better with primary halides in the presence of K_2CO_3. Alternatively, Amberlite® IRA 410 was used as base instead of K_2CO_3 in the *N*-alkylation, thus dispensing with the preliminary treatment of DO3A(*t*-BuO)$_3$ hydrobromide with the ion exchange resin. In this procedure the resin, being added directly to the reaction mixture, played a double role, both deprotonating the ammonium salt and acting as a proton scavenger. As the two protocol variants approximately resulted in the same reaction time and yield, the one-pot reaction was preferred. After the alkylation, the Cbz protecting group was removed by hydrogenation at room pressure (Pd/C in MeOH). When carried out under magnetic stirring, this cleavage was unsatisfactory (still incomplete after 24 h); it went however to completion in 5 hours when a Venturi-effect stirrer was used. Reaction rates and yields were further improved working under power ultrasound[20] (up to 95% in about 2 h). To the best of our knowledge this is the first report of Cbz cleavage under sonochemical conditions. The free NH_2 group of bifunctional chelates **5** and **6** can be exploited while the *tert*-butyl ester protecting groups are maintained on the carboxyl groups; alternatively the latter can be deprotected with TFA to yield ligands **7** and **8**. Another synthetic strategy for the preparation of BCFA **5** has been described by André and coworkers.[21] They started from the monoalkylation of 'cyclen' with ethyl bromoacetate, then *N*-alkylated the remaining amino groups with *tert*-butyl bromoacetate and ended with the aminolysis of the ethyl ester with ethylendiamine; the overall yield was 45%. Although our procedure is one step longer, it was optimized starting from DO3A(*t*-BuO)$_3$ and had a higher overall yield (~ 54%) (Scheme 2).

b) Synthesis of maleimido-functionalized DOTA-monoamide bifunctional agents.

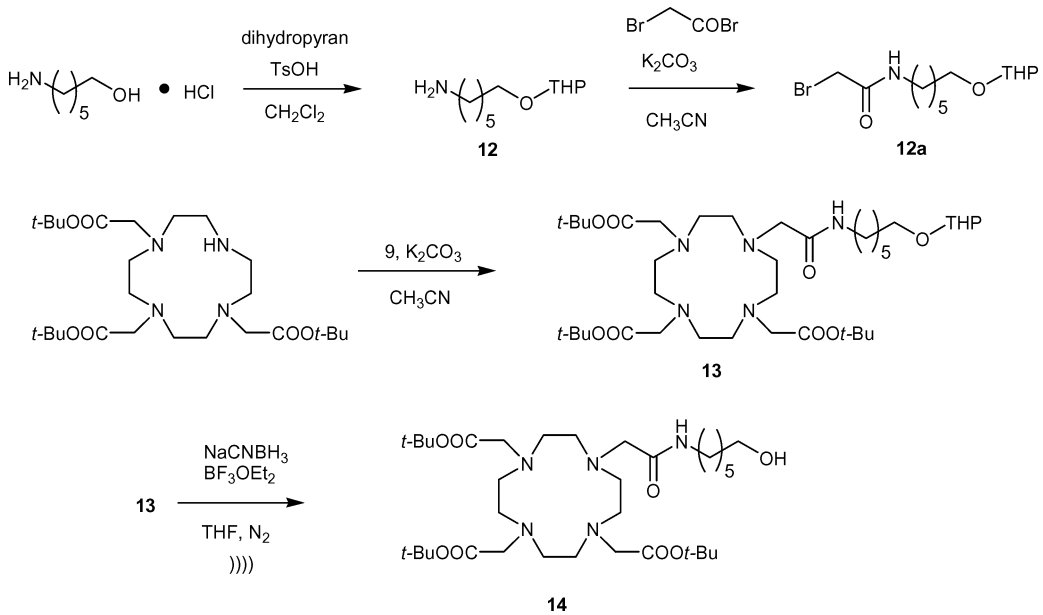
Following the procedure reported by Ondrus *et al.*,[22] which entails the use of a 7-*exo*-Diels–Alder adduct of furan and maleic anhydride (7-*exo*-oxohimic anhydride **9**) and working under microwave irradiation, we converted in good yields ligands **7** and **8** to the maleimido derivatives **10** and **11** (Scheme 3).



Scheme 2 Synthesis of DOTAMA(*t*-BuO)$_3$en (**5**), DOTAMA(*t*-BuO)$_3$C$_6$NH$_2$ (**6**), DOTAMAen (**7**), DOTAMAC$_6$NH$_2$ (**8**)



Scheme 3 Microwave-assisted synthesis of DOTA-monoamide maleimido derivatives.



Scheme 4 Synthesis of bifunctional agent **14**.

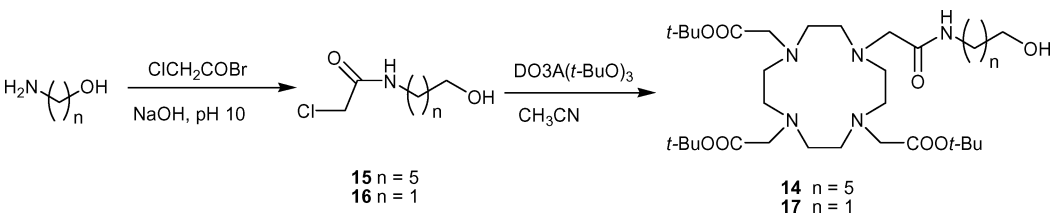
c) Synthesis of OH-functionalized DOTA-monoamide bifunctional agents.

Derivative **14**, bearing a free alcohol function, was prepared with a 42% overall yield from 6-aminohexanol; this was protected with tetrahydropyran and converted to the bromoacetamido derivative **12**. DO3A(t-BuO)$_3$ alkylation with **12** and subsequent removal of the THP group with sodium cyanoborohydride and BF_3·etherate in dry THF under power ultrasound gave the final product (Scheme 4).

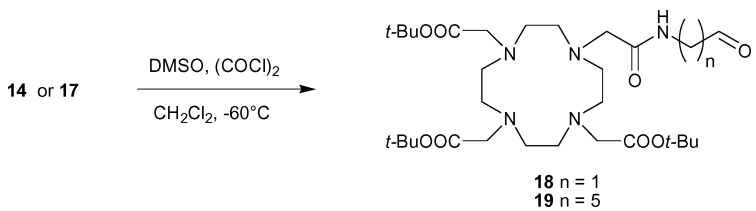
In a more straightforward way, the amino alcohols were reacted with chloroacetyl bromide in water at pH 10 to prevent ester formation. Further alkylation of DO3A(t-BuO)$_3$ gave the derivatives containing a free OH with an overall yield of 47% and 55% for **14** and **17**, respectively (Scheme 5).

d) Synthesis of CHO-functionalized DOTA-monoamide bifunctional agents.

Swern oxidation[23] of **14** and **17** afforded the corresponding aldehydes in good yields (Scheme 6).



Scheme 5 Synthesis of bifunctional agents **14** and **17** without OH protection.



Scheme 6 Swern oxidation of **14** and **17** to yield aldehyde-containing DOTA-monoamide bifunctional agents **18** and **19**.

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Oxidation of **14** and **17** with pyridinium dichromate (PDC), pyridinium chlorochromate (PCC) or Dess–Martin periodinane gave very poor results. Moderate yields were only achieved using PDC under power ultrasound.

Discussion

Thanks to their free -OH, -NH_2, -CHO or maleimido groups, all our novel DOTA-monoamide derivatives can undergo coupling reactions with a wide range of biological vectors.

As already reported for many bifunctional agents, especially DOTA-tris(*tert*-butyl) ester,[12] they can be used in solid-phase Fmoc peptide synthesis (SPPS) by exploiting the orthogonal protecting group strategy and thus be attached to peptides. For example, 4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-protected lysine (lysine-ivDde) can be joined to the DOTA-tris(*tert*-butyl) ester BFCA after removal of the 'ivDde' group with 2% hydrazine in DMF (solid phase reaction). Likewise, amino derivatives **5** and **6** can be coupled by a similar procedure with carboxylic groups on the side chains of glutamic and aspartic acids. In fact, Glu and Asp, orthogonally protected *e.g.* with Dmab (2-{1-[4-(hydroxymethyl)-phenylamino]-3-methylbutylidene}-5,5-dimethyl-1,3-cyclohexan-edione) ester, could be selectively deprotected with 2% hydrazine in DMF and subsequently coupled with **5** and **6**. Aldehydes can also be introduced in solid-phase peptide synthesis, as they will form secondary amines by reductive amination with primary amino groups present in the peptide. A reductive amination protocol for solid-phase peptide synthesis has already been published[24] and works well with our BFCAs **18** and **19** (data not shown). It should be noted that reductive amination must be the last step before cleavage, otherwise Boc protection of the secondary amine would be required. *tert*-Butyl ester protecting groups are easily removed, together with side-chain protecting groups, during peptide cleavage with the usual reagents.

The maleimido group of **10** and **11** is one of the most useful linkers in bioconjugate chemistry because the Michael addition of a thiol to the maleimide ring takes place under very mild conditions and is highly specific.[25] BFCAs **10** and **11** can work either in Fmoc SPPS or in aqueous solution, the maleimido group becoming attached to a cysteine thiol. As in **10** and **11** the carboxyl groups of the chelating moieties are unprotected, their coupling should be the last step of the SPPS before cleavage is carried out. Maleimide is particularly useful in aqueous solution since at neutral or slightly acidic pH it reacts about 1000-fold faster with thiols than with amines, which are mostly protonated.[26] These conditions are most appropriate when the vector is unstable in an organic solvent or under the acidic conditions that are required for *tert*-butyl ester cleavage, a common occurrence with many proteins, monoclonal antibodies and some peptides. It is also possible to start the assembly right away with the metal complex rather than the free ligand; this will avoid problems arising from complex formation by peptides or proteins.

We reported the use of the Gd(III) complex of **7**, converted to the emisquarate derivative (4-alkylamino-3-ethoxy-3-cyclobuten-1,2-dione), for conjugation with a polyornithine.[27] Similarly, **7** and **8** can easily be converted to the emisquarate derivatives for conjugation with any NH_2-containing vector. As in the example reported, **7** and **8** can be first complexed with the appropriate metal ion, then conjugated to a protein or specific antibody.

The OH group present on bifunctional agents **14** and **17** could be transformed into a sulfonyl derivative (*e.g.* mesyl, tosyl, nosyl), to be subsequently reacted with a variety of nucleophilic species. Still more interestingly, it can form a carboxylic ester. This type of ester could be easily cleaved even *in vivo* by hydrolysis with esterase; this could be an advantage in terms of detoxification and catabolism. After target recognition has taken place, it could be advantageous for the imaging probe to be cleaved off from its vector. In this way each of the two moieties would follow its metabolic pathway independently from the other, thus reducing overall toxicity.

Aldehyde derivatives **18** and **19** can easily react by reductive amination with free amino groups present on the vector surface. This reaction would be especially useful when the vector is a small molecule, whose surface modification could dramatically affect interaction with the target site. Reductive amination preserves the overall charge of the vector, which is not the case when conjugation is achieved through an amide bond.

Finally, it should be pointed out that for each functional group two different BFCAs were synthesized, with spacers consisting of either two or six methylene groups. The possibility of choosing between different spacers affords a control of the complex–vector distance. The distance between the DOTA coordination cage and the vector may strongly affect both the conjugation reaction and bioconjugate interaction with the target. In fact, the DOTA-monoamide cage, being often bulkier than the vector itself, may hinder the interaction very considerably. As shown in our previous work on the glutamine vector,[28] such hindrance can be minimized by increasing the complex–vector distance.

Conclusions

In the present paper we have reported the synthesis of a series of bi-functional building blocks to be exploited for various applications in diagnostic and therapeutic medicine. Their chelating moieties are based on the DOTA-monoamide cage which forms highly stable coordination complexes with both the Gd(III) ion (used in MRI) and several radioactive metal ions used in nuclear medicine (PET, SPECT). They offer a wide range of reactive groups and the possibility of choosing between two spacer lengths; these features are useful toward optimizing BFCA conjugation to the vector and eventually synthesizing a large library of specific molecular probes. While most of the published BFCAs are normally prepared on a small scale, the present protocols are well suited for multigram preparations. Finally, conjugation of these BFCAs to targeting vectors can be performed with different procedures: in organic solvents with protecting groups still present on the chelating moieties, followed by deprotection and complexation with the desired metal ion; or alternatively in aqueous media, using the preformed complex if this proves more convenient.

Experimental

All chemicals were purchased either from Sigma-Aldrich Co. or Lancaster Synthesis GmbH and were used without purification unless otherwise stated. NMR spectra were recorded on a Bruker Avance 300 (operating at 7 Tesla) and on a JEOL Eclipse Plus 400 (operating at 9.4 Tesla). ESI mass spectra were recorded on a Waters Micromass ZQ and elemental analyses on a Flash EA-1112 CHNS-O Elemental Analyzer (Thermoquest Inc.).

MW-promoted reactions were carried out in a MicroSYNTH oven (Milestone, Bergamo). Sonochemical apparatus used in the present work was developed in the authors' laboratory in collaboration with Danacamerini sas (Torino).[29] DO3A(t-BuO)$_3$ was prepared following a reported procedure.[16]

General procedure for bromo-acetamidation of monoprotected alkanediamines

In a round-bottom flask mono-Cbz-alkanediamine (10 mmol) (Cbz = benzyloxycarbonyl) and K_2CO_3 (20 mmol) were mixed in 20 mL of CH_3CN. The mixture was cooled down to 0 °C, then a solution of bromoacetyl bromide (1.1 mol eq. in 10 mL CH_3CN) was slowly added over 1 hour. After 4 hours stirring at room temperature, the solid was removed by filtration and the organic phase evaporated under reduced pressure. The residue was dissolved in CH_2Cl_2 (50 mL) and washed with 5% Na_2CO_3 (2 × 20 mL), H_2O (20 mL), 0.01 N HCl (2 × 20 mL), and finally again with H_2O (20 mL). The organic phase was dried with Na_2SO_4, filtered and evaporated under reduced pressure. The residue was then dried under vacuum to yield the product as a white solid.

General procedure for chloroacetamidation of unprotected amino alcohols

To a solution of amino alcohol (0.20 mol) in water (50 mL), cooled to 5–7 °C, chloroacetyl chloride (16.3 mL; 0.20 mol) was slowly added while pH 10 was being maintained by adding 10 N NaOH. The solution was stirred for 30 min, then neutralized with HCl and concentrated under vacuum; inorganic salt was eliminated by adding methanol. After evaporation of the solvent, the product was recovered as a colourless oil.

General procedure for the attachment of a bromoacetamide pendant arm to DO3A(t-BuO)$_3$ using K_2CO_3 as base (a)

DO3A(t-BuO)$_3$ free base was obtained by dripping DO3A(t-BuO)$_3$ HBr over Amberlite® IRA 410 in its basic form and eluting it with water/ethanol 1:1. The bromoacetamide derivative (10 mmol) was dissolved in CH_3CN (30 mL) and slowly dripped, over 1 h, into a mixture of DO3A(t-BuO)$_3$ free base (10 mmol) and solid K_2CO_3 (15 mmol) suspended in CH_3CN (50 mL). The mixture was stirred at room temperature for 4 h. After filtration, the solvent was removed by evaporation under reduced pressure to yield the product as a white solid.

General procedure for the attachment of a bromoacetamide pendant arm to DO3A(t-BuO)$_3$ using Amberlite® IRA 410 as base (b)

Bromoacetamide (8 mmol) and DO3A(t-BuO)$_3$ HBr (8 mmol) were dissolved in CH_3CN (150 mL); then 16 mL of Amberlite® IRA 410, previously activated with 10% NaOH, washed with H_2O to neutral pH and finally conditioned with CH_3CN, were added to the solution. The mixture was stirred at room temperature for 4 h. After filtration, the solvent was removed by evaporation under reduced pressure to yield the product as a colourless oil.

General procedure for the attachment of a chloroacetamide pendant arm to DO3A(t-BuO)$_3$

DO3A(t-BuO)$_3$ free base (0.1 mol) was dissolved in acetonitrile (970 mL) and the chloroacetamido derivative (0.1 mol) was added. After 1 hour stirring at room temperature, DIPEA (0.1 mol) was added and the mixture was stirred for another 24 hours. The solvent was removed under vacuum, the residue solubilized in MeOH and purified over a XAD 1600 chromatographic column by gradient elution (water–MeOH). After solvent evaporation a white solid was obtained.

Hydrogenation of the Cbz group (benzyloxycarbonyl) on a mmol scale (general)

The Cbz-protected DOTA-monoamide derivative (2 mmol) was dissolved in MeOH (40 mL) and 10% its weight of Pd/C-10% was added. The reaction was carried out under H_2 at atmospheric pressure and room temperature for 2 hours, using a high-power ultrasound bath operating at 21 kHz and 80 W. The mixture was then filtered and the solvent evaporated. The residue was washed with diethyl ether (2 × 20 mL) and dried under vacuum to yield a white solid.

Hydrogenation of the Cbz group (benzyloxycarbonyl) on a mol scale (general)

The Cbz-protected DOTA-monoamide derivative (0.1 mol) was dissolved in MeOH (50 mL) and 10% its weight of Pd/C-10% was added. Hydrogenation was carried out for 5 h at room pressure and temperature, under stirring with a Venturi-effect stirrer (MRK1 Buddenberg) spinning at 2000 rpm. Then the mixture was filtered, the solvent evaporated and the residue dissolved in 30 mL of 0.5 N HCl (this concentration did not affect the *tert*-butyl esters, while eliminating carbamic acid residues). After stirring under vacuum for 10 min until the evolution of CO_2 had ceased, the pH was raised to about 10 by adding NaOH 1 N at 0 °C and the product was extracted with CH_2Cl_2 (4 × 50 mL). The organic phase was dried with Na_2SO_4, filtered and evaporated under reduced pressure. The residue was dried under vacuum to yield the product as a white solid.

General procedure for alcohol oxidation (Swern)

A mixture of dichloromethane (4 mL) and oxalyl chloride (2.08 mmol, 178 µl) was placed in a 10 mL two-necked round-bottom flask and cooled down to −60 °C. DMSO (4.16 mmol, 295 µl) was then added and the mixture was stirred for 10 min. Then the alcohol, dissolved in DMSO–CH_2Cl_2 (0.74 mmol, 500 µl–500 µl), was added over 5 min and the mixture stirred for 45 min. Triethylamine (1.20 mL, 8.6 mmol) was added and the mixture was stirred for 2 h at room temperature. It was then washed with water (5 mL) and the aqueous layer extracted with dichloromethane (3 × 3 mL). The organic layers were combined, washed first with saturated NH_4Cl, then with brine and dried over anhydrous sodium sulfate. The aldehyde was collected after solvent evaporation as a pale yellow oil.

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General procedure for *tert*-butyl ester deprotection

The compound protected with *tert*-butyl ester groups (8 mmol) was dissolved in dichloromethane (50 mL) and the mixture cooled down to 0–5 °C. Trifluoroacetic acid (TFA, 48 mmol) was then slowly added under magnetic stirring and the temperature slowly increased to RT. The mixture was concentrated, 3% triisopropylsilane in TFA (18 mL) was added and the solution stirred for 48 h. A white solid was collected after addition of diethyl ether (50 mL), washed three times with diethyl ether (10 mL) and dried under vacuum.

Synthesis of *N*-(6-benzyloxycarbonylaminohexyl)bromoacetamide (2)

6-Benzyloxycarbonylaminohexanamine (3.0 g, 10.45 mmol), K_2CO_3 (2.89 g, 20.9 mmol) and bromoacetyl bromide (2.34 g, 11.5 mmol) were reacted according to the above-reported general procedure for bromoacetamidation of monoprotected alkanediamines, giving 3.52 g (9.48 mmol, 90.7% yield) of product **2**. ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 7.35$ (s, 5H), 6.51 (b, 1H), 5.08 (s, 2H), 4.75 (b, 1H), 3.87 (s, 2H), 3.26 (m, 2H), 3.18 (m, 2H), 1.50 (m, 4H), 1.34 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 165.6$, 156.5, 136.8, 128.7, 128.3, 128.2, 66.8, 40.1, 29.9, 29.3, 29.1, 26.3, 26.1. ESI-MS (*m*/*z*): 371.30 (M + H$^+$) (calc. 371.09).

Synthesis of 1-(3,10-diaza-2,11-dioxo-11-phenyloxyundecyl)-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (4)

DO3A(*t*-BuO)$_3$ free base (4.71 g, 9.15 mmol), K_2CO_3 (1.57 g, 11.4 mmol) and **2** (3.52 g, 9.48 mmol) were reacted according to the general procedure for the attachment of a bromoacetamide pendant arm to DO3A(*t*-BuO)$_3$ using K_2CO_3 as base, and gave 7.08 g (8.8 mmol, 95% yield) of **4**. The alternative method: using Amberlite® IRA 410 as base instead of K_2CO_3 gave **4** in 90% yield. ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 7.35$ (s, 5H), 7.29 (b, 1H), 5.07 (s, 2H), 5.02 (b, 1H), 4.37–2.75 (b, 28H), 1.55 (s, 9H), 1.49 (s, 9H), 1.44 (s, 9H), 1.73 (m, 2H), 1.41 (m, 2H), 1.25 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 171.9$, 171.7, 171.5, 156.5, 136.9, 128.4, 127.9, 66.3, 58.2, 56.8, 56.5, 53–48 (b), 40.9, 39.1, 29.2, 28.2, 26.3, 26.1, 28.1, 28.0. ESI-MS (*m*/*z*): 805.7 (M + H$^+$), (calc. 805.5).

Synthesis of 1-(9-amino-3-aza-2-oxononyl)-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane—DOTAMA(*t*-BuO)$_3$-C$_6$-NH$_2$ (6)

Product **4** (0.88 g, 1.09 mmol) was deprotected by hydrogenation at room pressure, accordingly to the above-reported procedure (mmol scale), yielding 0.68 g (1.01 mmol, yield 93%) of product **6**. Gram-scale deprotection of **4**, carried out using a Venturi-effect stirrer, gave **6** in 90% yield. ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta =$ 8.54 (b, 1H), 8.37 (b, 2H), 4.44 (s, 2H), 4.25 (s, 2H), 3.71 (s, 4H), 3.60–2.70 (b, 20H), 1.89 (t, 2H, $J = 7.3$ Hz), 1.60 (m, 2H), 1.50 (m, 2H), 1.44 (s, 27H), 1.40 (m, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 171.8$, 171.6, 170.2, 81.9, 58.2, 56.7, 56.2, 53–48 (b), 39.8, 39.1, 28.4, 26.9, 25.8, 25.7, 28.2, 28.1, 28.0. IR (ν_{max}/(cm^{-1}), liquid film): 3451, 3266, 1732, 1654, 1537, 1456, 1368, 1155.

ESI-HRMS (*m*/*z*): 671.5065 (M + H$^+$), (calcd. 671.5071). $C_{34}H_{66}N_6O_7$ (670.92) calcd. C, 60.87; H, 9.92; N, 12.53; found C, 61.18; H, 10.50; N, 12.90%.

Synthesis of 1-(9-amino-3-aza-2-oxononyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane—DOTAMA-C$_6$-NH$_2$ (8)

Compound **6** was fully deprotected with trifluoroacetic acid (TFA) according to the above-reported procedure. DOTAMA-C$_6$-NH$_2$ (**8**) was obtained as trifluoroacetate salt in quantitative yield. By acidimetric titration, and CHN analysis, 2.5 TFA molecules were found for each DOTAMA-C$_6$-NH$_2$ molecule. ^{1}H NMR (DMSO-d$_6$, 300 MHz): $\delta = 8.33$ (b, 1H), 7.92 (b, 2H), 4.40 (s, 2H), 4.22 (s, 2H), 3.69 (s, 4H), 3.60–2.70 (b, 20H), 1.85 (t, 2H, $J = 7.3$ Hz), 1.60 (m, 2H), 1.50 (m, 2H), 1.40 (m, 2H). ^{13}C NMR (DMSO-d$_6$, 75 MHz): $\delta = 171.6$, 171.1, 168.8, 57.9, 56.3, 55.8, 53–48 (b), 39.4, 38.7, 28.0, 26.4, 25.3, 25.1. IR (ν_{max}/(cm^{-1}), liquid film): 3443, 3250, 1729, 1654, 1463, 1425, 1202, 1138

ESI-HRMS (*m*/*z*): 503.3208 (M + H$^+$) (calcd. 503.3193). $C_{22}H_{42}N_6O_7 \cdot 2.5\ C_2HO_2F_3$ (791.46) calcd. C, 40.97; H, 5.67; N, 10.62; found C, 40.72; H, 5.38; N, 10.30%.

Synthesis of 1-(9-maleimido-3-aza-2-oxononyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane—DOTAMA-C$_6$-Nmal (11)

Compound **8** (87 mg, 0.11 mmol) was dissolved in water (20 mL) and 7-*exo*-oxohimic anhydride (**9**) (19 mg, 0.11 mmol) was added. The mixture was placed in a two-necked round-bottom flask which was inserted in the cavity of the professional microwave oven. The flask was equipped with a fiber-optic thermometer and a condenser. The mixture was irradiated with microwave for 5 min (30 s at 600 W, 4.5 min at 350 W) the reaction temperature remaining constant at 110 °C. The solvent was then evaporated under vacuum and the residue triturated with diethyl ether to give a white solid. The crude material was dissolved in water, loaded over an Amberlite XAD 1600 column and eluted with a water–methanol gradient (100 : 0 to 0 : 100). After solvent evaporation product **11** was collected as a white solid (yield 60%).^{1}H NMR (DMSO-d$_6$, 300 MHz): $\delta = 8.33$ (b, 1H), 6.90 (s, 2H), 4.40 (s, 2H), 4.22 (s, 2H), 3.69 (s, 4H), 3.60–2.70 (b, 20H), 1.85 (t, 2H, $J = 7.3$ Hz), 1.60 (m, 2H), 1.50 (m, 2H), 1.40 (m, 2H). ^{13}C NMR (DMSO-d$_6$, 75 MHz): $\delta = 171.6$, 171.1, 170.7, 168.8, 134.2, 57.9, 56.3, 55.8, 53–48 (b), 38.7, 32.7, 28.0, 26.4, 25.3, 25.1. ESI-HRMS (*m*/*z*): 583.3077 (M + H$^+$) (calc. 583.3092). $C_{26}H_{42}N_6O_9$ (582.64) calcd. C, 53.60; H, 7.27; N, 14.42; found C, 53.27; H, 6.90; N, 14.13.

Synthesis of *N*-(2-benzyloxycarbonylaminoethyl)bromoacetamide (1)

2-(Benzyloxycarbonylamino)ethanamine (24.7 g, 0.127 mol), K_2CO_3 (0.150 mol, 20.7 g) and bromoacetyl bromide (0.127 mol, 25.6 g, 11.0 mL) were reacted according to the above-reported general procedure for bromoacetamidation of monoprotected alkanediamines, giving product **4** (29.4 g, 0.093 mol) in 73.4% yield. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 7.34 = (s, 5H), 6.55 (b, 1H), 5.10 (s, 2H), 4.80 (b, 1H), 3.85 (s, 2H), 3.31 (m, 2H), 3.22 (m, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 166.2$, 158.0, 136.9, 128.8,

128.4, 128.3, 67.0, 40.1, 38.0, 29.9. ESI-MS (m/z): 315.27 (M + H^+) (calcd. 315.03).

Synthesis of 1-(3,6-diaza-2,7-dioxo-7-phenyloxyheptyl)-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (3)

14.5 g (0.028 mol) of DO3A(*t*-BuO), 4.64 g (0.034 mol) of K_2CO_3 and 8.82 g (0.028 mol) of **1** were reacted according to the above-reported general procedure for the attachment of a bromoacetamide pendant arm to DO3A(*t*-BuO)$_3$ using K_2CO_3 as base, giving product **3** in 85% yield. The alternative method using Amberlite® IRA 410 as base instead of K_2CO_3 gave **3** in 80% yield. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 7.30 (s, 5H), 6.80 (b, 2H), 5.05 (s, 2H), 3.50–2.55 (b, 28H), 1.45 (s, 9H), 1.41 (s, 9H), 1.38 (s, 9H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 172.1, 171.8, 171.5, 157.0, 137.9, 128.4, 127.9, 82.9, 67.3, 59.2, 57.1, 56.5, 53–48 (b), 40.9, 39.1, 29.2, 29.0. ESI-MS (m/z): 749.3 (M + H^+), (calcd. 749.7).

Synthesis of 1-(5-amino-3-aza-2-oxopentyl)-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane—DOTAMA(*t*-BuO)3-en (5)

22.5 g (0.03 mol) of **3** and 2.0 g of catalyst were subjected to the general procedure for hydrogenating the Cbz group on the molar scale. 17.5 g (0.028 mol) of **5** were obtained, a 95% yield. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.80 (b, 1H), 8.71 (b, 2H), 3.60 (b, 4H), 3.40 (s, 4H), 3.21 (s, 4H), 3.20–2.70 (b, 16H), 1.44 (s, 27H). ^{13}C NMR ($CDCl_3$, 75 MHz): δ = 171.8, 171.6, 170.2, 81.9, 59.2, 57.5, 56.2, 53–48 (b), 40.8, 39.7, 28.8, 28.5, 28.3. IR (ν_{max}/(cm^{-1}), liquid film): 3452, 3259, 1730, 1655, 1540, 1455, 1370. ESI-HRMS (m/z): 615.4425 (M + H^+) (calcd. 615.4445), $C_{30}H_{58}N_6O_7$ (614.81) calcd. C, 58.61; H, 9.51; N, 13.67; found C, 58.26; H, 9.18; N, 13.50%.

Synthesis of 1-(5-amino-3-aza-2-oxopentyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraazacyclododecane—DOTAMA-en (7)

Compound **3** was fully deprotected with trifluoroacetic acid according to the above-reported procedure. DOTAMA-en (**7**) was obtained as trifluoroacetate salt in quantitative yield. By acidimetric titration, and CHN analysis, 2.5 TFA molecules were found for each DOTAMA-en molecule.^{1}H NMR (DMSO-d$_6$, 300 MHz): δ = 8.30 (b, 1H), 7.90 (b, 2H), 3.63 (b, 4H), 3.38 (s, 4H), 3.24 (s, 4H), 3.10–2.60 (b, 16H). ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ = 171.6, 171.0, 168.5, 57.2, 55.5, 55.2, 53–48 (b), 38.8, 37.7. IR (ν_{max}/(cm^{-1}), liquid film): 3443, 3248, 1728, 1650, 1459, 1427, 1208, 1140. ESI-HRMS (m/z): 447.2585 (M + H^+) (calcd. 447.2567). $C_{18}H_{34}N_6O_7$ 2.5 $C_2HO_2F_3$ (731.23) calcd. C, 37.76; H, 5.03; N, 11.49; found C, 38.53; H, 5.39; N, 12.07%.

Synthesis of 1-(5-maleimido-3-aza-2-oxopentyl)-4,7,10-tris(carboxymethyl)-1,4,7,10-tetraaza cyclododecane—DOTAMA-en-mal (10)

Compound **7** (80 mg, 0.11 mmol) was dissolved in water (20 mL) and 7-*exo*-oxohimic anhydride (**9**) (19 mg, 0.11 mmol) was added. By the procedure described for product **11**, product **10** was obtained as a white solid in 65% yield.^{1}H NMR (DMSO-d$_6$, 300 MHz): δ = 8.30 (b, 1H), 6.90 (s, 2H), 3.63 (b, 4H), 3.38 (s, 4H), 3.24 (s, 4H), 3.10–2.60 (b, 16H). ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ = 171.6, 171.0, 10.7, 168.5, 134.2, 57.2, 55.5, 55.2, 53–48 (b), 38.0, 32.7. ESI-HRMS (m/z): 527.2484 (M + H^+) (calcd. 527.2466). $C_{22}H_{34}N_6O_9$ (526.54) calcd. C, 50.18; H, 6.51; N, 15.96; found C, 50.45; H, 6.87; N, 16.32%.

Synthesis of 2-(6-aminohexyloxy)tetrahydropyran (12)

Dihydropyran (DHP, 25 mmol) was added to a solution of 6-amino-1-hexanol hydrochloride (8.5 mmol) in CH_2Cl_2 (50 mL). The liquid was cooled down to 0 °C, *p*-toluenesulfonic acid (9.3 mmol) was added, and the mixture stirred at 0 °C for 1 h. After another 8 h at room temperature the reaction was complete. Ice was then added and the phases were separated. The organic phase was washed with saturated $NaHCO_3$ solution (2 × 10 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to give the protected alcohol **12** in quantitative yield as a colourless oil. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 4.48 (t, J = 4.4 Hz, 1H), 3.83 (m, 1H), 3.69 (m, 1H), 3.45 (m, 1H), 3.33 (m, 1H), 2.65 (t, J = 8 Hz, 2H), 1.32–1.69 (m, 14H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.0, 99.2, 67.1, 62.5, 40.2, 19.8, 30.8, 29.4, 29.3, 26.7, 25.9, 25.5. ESI-MS (m/z): 202.10 (M + Na^+), (calcd. 202.17).

Synthesis of bromoacetamidohexyl tetrahydropyran-2-yl ether (12a)

2-(6-Aminohexyloxy)tetrahydropyran (**12**) (14.9 mmol), K_2CO_3 (20.9 mmol) and bromoacetyl bromide (1.36 mL, 15.6 mmol) were reacted under the above-reported general conditions for bromoacetamidation of monoprotected alkanediamines. By silica-gel column chromatography (CH_2Cl_2 : CH_3OH 98 : 2, R_f 0.32) 10.44 mmol of product were obtained (70% yield).^{1}H NMR ($CDCl_3$, 300 MHz): δ = 6.49 (s, 1H), 4.53 (t, 1H, J = 4.0 Hz), 3.85 (s, 2H), 3.83 (m, 1H), 3.71 (m, 1H), 3.28 (m, 1H), 3.24 (m, 1H), 1.34–1.57 (m-overlapped, 14H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.0, 99.2, 67.1, 62.5, 40.2, 19.8, 30.8, 29.7, 29.4, 29.3, 26.7, 25.9, 25.5. ESI-MS (m/z): 322.4 (M + Na^+), (calcd. 322.1).

Synthesis of 1-(tetrahydropyran-2-yloxyhexylcarbamoylmethyl)-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (13)

DO3A-tris-*tert*-butyl ester free base (3.7 mmol), K_2CO_3 (14.8 mmol) and bromoacetamidohexyl tetrahydropyran-2-yl ether (**12**) (1.19 g, 3.7 mmol) were reacted under the above-reported general conditions (a) for substitution of DO3A(*t*-BuO)$_3$, giving **13** in 76% yield. ^{1}H NMR ($CDCl_3$, 300 MHz): δ = 8.5 (s, 1H), 4.56 (t, 1H, J = 4.0 Hz), 3.86 (m, 1H), 3.73 (m, 1H), 3.50 (m, 1H), 3.38 (m, 1H), 3.28 (s, 2H), 3.23 (s, 2H), 3.22 (s, 4H), 3.20 (m, 2H), 3.00 (s, 2H), 2.89–2.51 (b, 16H), 1.71–1.38 (m-overlapped, 14H), 1.45 (s, 27H). ^{13}C NMR ($CDCl_3$, 400 MHz): δ = 171, 98.91, 81.03, 81.00, 67.6, 62.4, 58.0, 56.7, 56.2, 54.9, 53.4, 52.7, 52,0, 39.4, 30.8, 30.0, 29.8, 28.3, 27.2, 26.2, 25.5, 19.7. ESI-MS (m/z): 778.50 (M + Na^+) (calcd. 778.53).

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Synthesis of *N*-(6-hydroxyhexyl)chloroacetamide (15)

Product **15** was obtained in 85% yield from chloroacetyl bromide and 6-aminohexanol following the general procedure for chloroacetamidation. ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 9.95$ (b, 1H), 4.50 (b, 1H), 4.05 (s, 2H), 3.50 (t, 2H, $J = 7.0$ Hz), 3.19 (t, 2H, $J = 7.2$ Hz), 1.50 (m, 4H), 1.30 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.0, 62,0, 52.3, 40.3, 30.3, 32.1, 26.5, 25.6. ESI-MS (m/z): 194.15 (M + Na^+), (calcd. 194.09).

Synthesis of 1-(6-hydroxyhexylcarbamoylmethyl)-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (14)

Sodium borocyanohydride (5.7 mmol) was added to a solution of the THP ether (compound **13**, 2.9 mmol) and $BF_3{\cdot}OEt_2$ (14.5 mmol) in dry THF (10 mL). The mixture was irradiated with ultrasound (21 kHz, 50 W) for 2 hours, then filtered to give **14** as a white solid in 79% yield. Alternatively, **14** was obtained in 55% yield by direct *N*-alkylation of DO3A(t-BuO)$_3$ with *N*-(6-hydroxyhexyl)chloroacetamide (**15**) following the general procedure for the attachment of a chloroacetamide pendant arm. ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 8.5$ (s, 1H), 4.27 (b, 4H), 4.13 (b, 2H), 4.0 (b, 2H), 3.61 (m, 2H), 3.67–3.40 (b, 16H), 3.21 (m, 2H), 1.55–1.27 (m-overlapped, 35H). ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta =$ 169.0, 166.0, 164.0, 84.8, 82.4, 68.2, 62.3, 55.4, 55.0, 54.2, 51.6, 48.9, 48.6, 39.5, 32.3, 28.9, 28.0, 27.9, 26.5, 25.2. IR (ν_{max}/(cm^{-1}), liquid film): 3453, 3252, 1728, 1652, 1543, 1451, 1154, 1223. ESI-HRMS (m/z): found 672.4895 (M + H^+) (calcd. 672.4911). $C_{34}H_{65}N_5O_8$ (671.90) calcd. C, 60.78; H, 9.75; N, 10.42; found C, 60.37; H, 9.32; N, 10.21%.

Synthesis of 1-(6-oxohexylcarbamoylmethyl)-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (19)

By the general procedure for alcohol oxidation, compound **14** was converted to **19** in 90% yield. ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 9.56$ (s, 1H), 8.7 (s, 1H), 3.54–1.96 (b, 28H), 1.4–1.18 (m-overlapped, 35H). ^{13}C NMR ($CDCl_3$, 75 MHz): $\delta = 203.0$, 173.1, 172.2, 171.0, 81.6, 81.3, 72.9, 62.0, 56.1, 55.7, 55.6, 52.0, 46.1, 43.8, 38.8, 29.1, 28.1, 27.9, 26.4, 21.7. IR (ν_{max}/(cm^{-1}), liquid film): 3275, 3250, 1730, 1669, 1452, 1360, 1225, 1153, 1122. ESI-HRMS (m/z): found 670.4770 (M + H^+) (calcd. 670.4755). $C_{34}H_{63}N_5O_8$ (669.89) calcd. C, 60.96; H, 9.48; N, 10.45; found C, 60.71; H, 8.91; N, 10.14%.

Synthesis of *N*-(2-hydroxyethyl)chloroacetamide (16)

16 was obtained in 85% yield from chloroacetyl bromide and ethanolamine following the general procedure for chloroacetamidation of amino alcohols.

^{1}H NMR ($CDCl_3$, 400 MHz): $\delta = 9.95$ (b, 1H), 4.26 (b, 1H), 3.90 (s, 2H), 3.66 (t, 2H, $J = 7.0$ Hz), 3.26 (t, 2H, $J = 7.3$ Hz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 165.0, 60.8, 52.0, 47.5. ESI-MS (m/z): 138.35 (M + H^+) (calcd. 138.02).

Synthesis of 1-(2-hydroxyethylcarbamoylmethyl)-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (17)

Product **17** was obtained in 65% yield by direct *N*-alkylation of DO3A(t-BuO)$_3$ free base with *N*-(2-hydroxyethyl)chloroacetamide following the general procedure for the attachment of a chloroacetamide pendant arm. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta =$ 4.07 (s, 1H), 3.74 (m, 2H), 3.46 (m, 2H), 3.35–3.27 (b, 8H), 3.0–2.8 (b, 16H), 1.45 (s, 27H).^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 173.1$, 170.6, 169.7, 167.1, 81.8, 81.6, 60.8, 58.0, 55.6, 51.3, 49.3, 47.4, 28.2. IR (ν_{max}/(cm^{-1}), KBr): 5480, 1741, 1722, 1469, 1452, 1361, 1255, 1151, 1168. ESI-HRMS (m/z): 616.4300 (M + H^+) (calcd. 616.4285). $C_{30}H_{57}N_5O_8$ (615.80) calcd. C, 58.51; H, 9.33; N, 11.37; found C, 58.83; H, 9.84; N, 11.62%.

Synthesis of 1-(2-oxoethylcarbamoylmethyl)-4,7,10-tris(*tert*-butyloxycarbonylmethyl)-1,4,7,10-tetraazacyclododecane (18)

By the general procedure for alcohol oxidation, compound **14** was converted to **19** in 90% yield. ^{1}H NMR ($CDCl_3$, 400 MHz): $\delta =$ 4.07 (1H), 3.81 (m, 2H), 3.32–3.20 (b, 8H), 2.7–2.5 (b, 16H), 1.36 (s, 27H). ^{13}C NMR ($CDCl_3$, 400 MHz): $\delta = 201.0$, 173.1, 171.1, 170.2, 81.0, 82.1, 55.7, 52.8, 49.3, 46.1, 28.2. IR (ν_{max}/(cm^{-1}), liquid film): 3277, 3249, 1738, 1675,1553, 1452, 1390, 1371, 1223, 1150. ESI-HRMS (m/z): found 614.4145 (M + H^+) (calcd. 614.4129). $C_{30}H_{55}N_5O_8$ (613.79) calcd. C, 58.70; H, 9.03; N, 11.41; found C, 59.11; H, 9.47; N, 11.81%.

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