Convergent and Sequential Synthesis of Dendritic, Multivalent Complexing Agents

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Abstract: Two series of macrocyclic polyamine derivatives with various length of linkers were synthesized as dendritic ligands containing two, three, four, five or six terminal nitrilotriacetic acid (NTA) groups through convergent and sequential pathways. Tetrafluorophenyl esters were employed as activating reagents in the coupling step of assembling NTA groups to the cyclic polyamine head. A key step of the sequential synthesis is the use of the coupling reagent, 2-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) for the formation of amide bonds between the cyclic polyamine and pendant groups. Finally, two representative compounds were used to demonstrate the formation of stoichiometric protein assemblies.

Key words: complexing agents, polyamines, coupling, nitrilotriacetic acid

Immobilized metal affinity chromatography (IMAC) has become a widely used technique in molecular biology and protein engineering since it was first reported by Porath in 1975.[1] In particular, it has been used for the isolation of proteins, peptides, and nucleic acids. During IMAC, a metal ion, such as Ni^{2+}, Zn^{2+}, or Cu^{2+}, is complexed and immobilized by a chelator, which is covalently linked to a solid matrix. Our interest is based on the use of nitrilotriacetic acid (NTA) which has been employed as a chelator to purify proteins since 1987.[2] NTA chelates a Ni(II) ion through four of its potential six coordination sites, the remaining sites bind two histidine residues forming an octahedral complex. The formation of this type of complex relies on incorporation of 2–6 histidines at one terminus of a protein. The histidine-tagged protein is bound by the metal immobilized on the NTA-derivatized stationary phase, while other proteins are washed off the column. The binding of Ni(II) by NTA is reversible,[3] and the reversibility can be achieved by the use of ethylenediamine tetraacetic acid (EDTA) which has a higher binding constant for Ni(II) ions or imidazole which competes with the histidine binding.

In the course of our study on biomembrane dynamics, we reported a series of macrocyclic polyamine amphiphiles both with and without fluorescent nitrobenzoxadiazole (NBD) labels.[4] These compounds have varied, but defined sizes and shapes and have been useful for investigation of diffusion in biological membranes. The diffusion coefficients of these molecules are size dependent.[5] It is well known that aggregation of membrane proteins plays a key role in signal transduction,[6] so it is of interest to prepare membrane protein aggregates with defined sizes and shapes. To generate aggregates of proteins, two series of complexing agents containing multiple NTA functional groups were designed as shown in Figure 1. These compounds have dendritic arms, which can chelate to histidine-tagged proteins through nickel ions. Changing the size of the central ring will give a corresponding change in the number of dendritic arms. Each dendritic chelating arm can bind to a protein so that different sizes of protein clusters can be created with the various reagents. It may also be possible to control the tightness of the clusters formed by varying the length of the linker chain. In this paper, we report two synthetic strategies for making den-

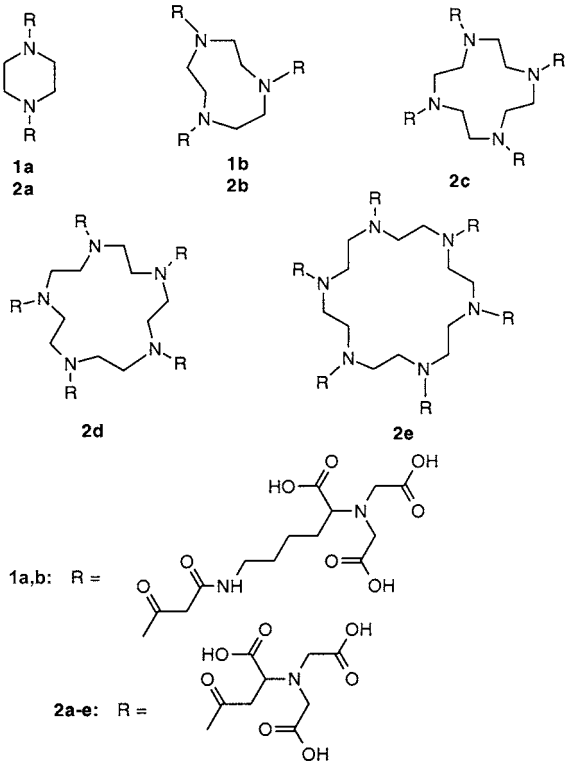

Figure 1 Complexing agents containing multiple NTA functional groups

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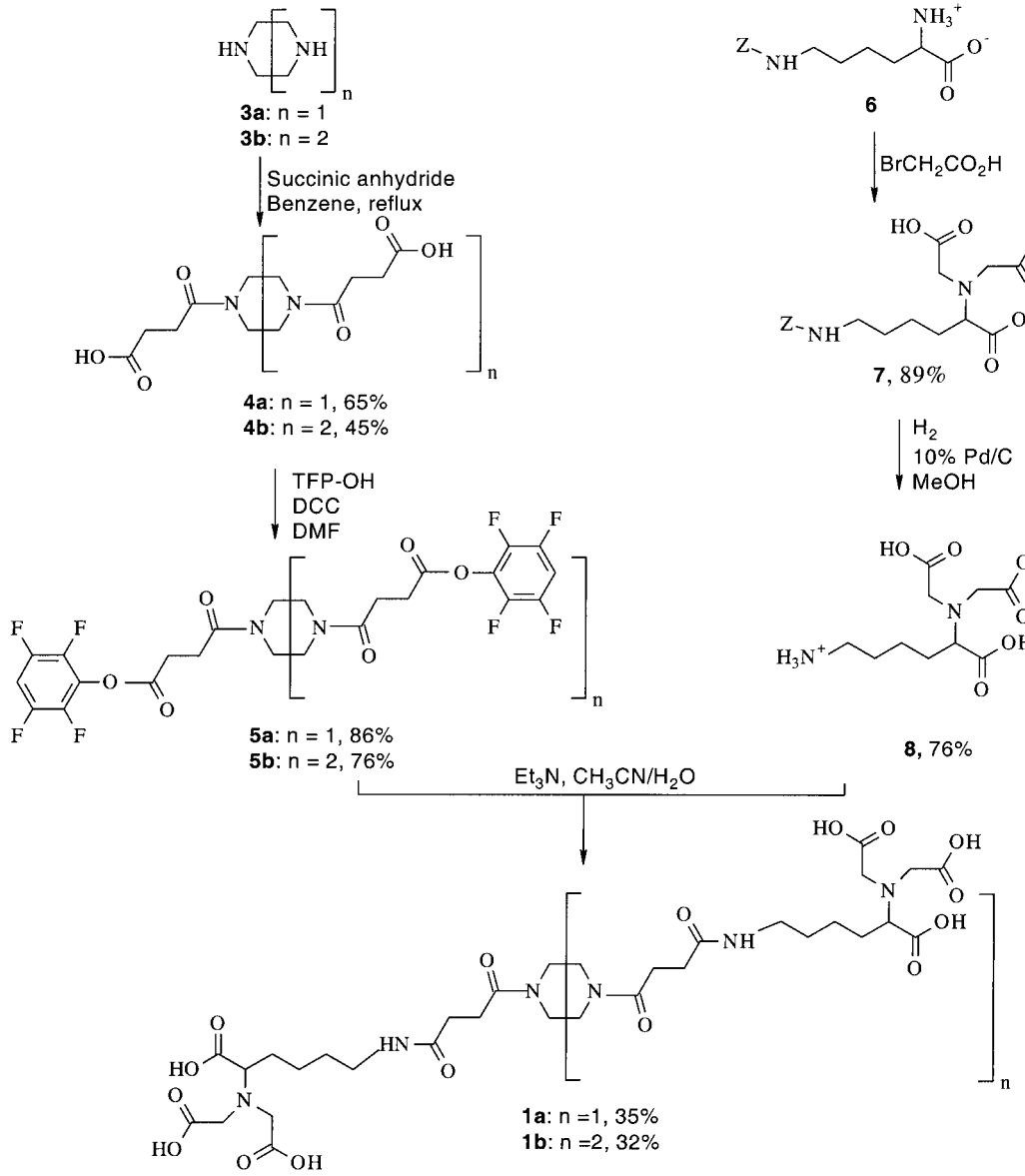
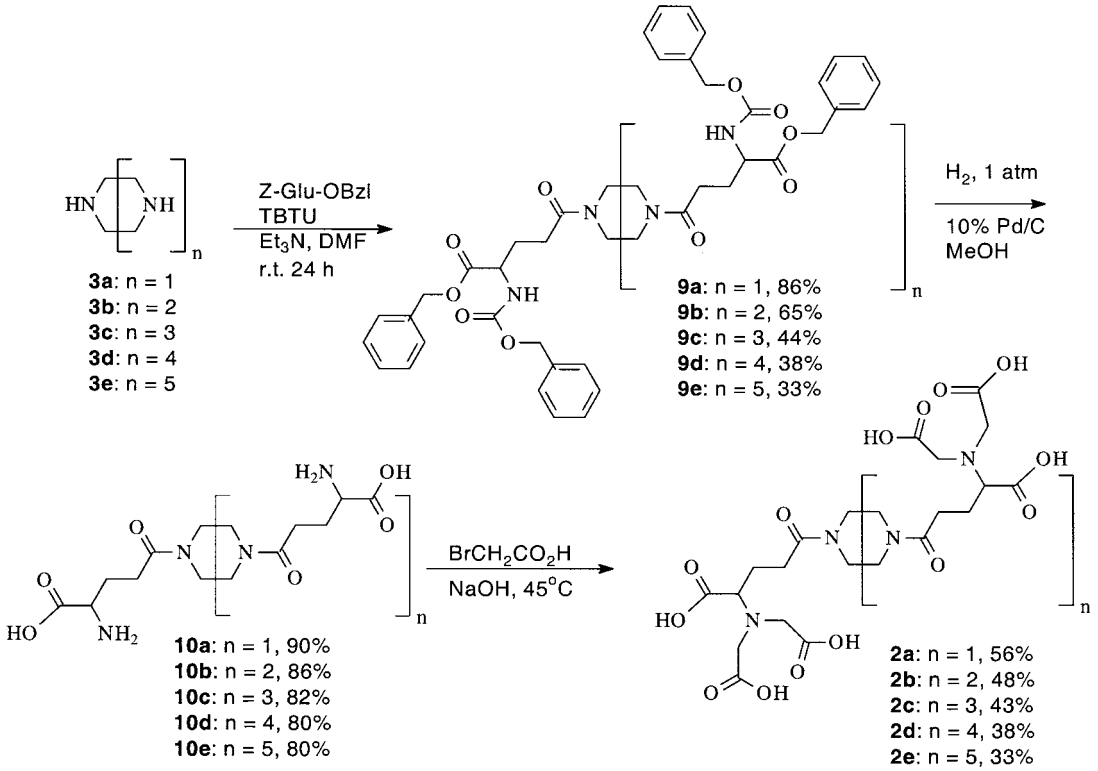
dritic ligands with a macrocyclic polyamine head group and multiple nitrilotriacetic acid pendant groups. The use of these ligands to assemble dimers, trimers, tetramers, pentamers, or hexamers of histidine-tagged proteins will be reported separately, but we illustrate the principle here with making dimers and trimers.

The macrocyclic polyamine derivatives with multiple NTA functional groups shown in Figure 1 were synthesized as outlined in Scheme 1 and Scheme 2. Their structures were confirmed by mass spectrometry (MS), NMR spectroscopy and FTIR spectroscopy. The synthesis was achieved by both a convergent and a sequential pathway to provide versatility. All of the syntheses started from cyclic polyamines, some of which are not commercially available as free bases. For example, 1,4,7,10,13-pentaazacyclopentadecane (**3d**) and 1,4,7,10,13-hexaazacyclooctadecane (**3e**) can be isolated as such easily from 1,4,7,10,13-pentaazacyclopentadecane pentahydrochloride and hexacyclen trisulfate, respectively. To avoid the formation of byproducts with incomplete carboxymethylation during the preparation of **1a**,**b**, we used lysine-NTA[2a,b] (**8**), to couple with tetrafluorophenyl activated esters of **4a**,**b** as shown in Scheme 1. However, carboxymethylation of the polyamino acids (**10a**–**e)** as shown in the sequential pathway (Scheme 2) yielded, under conditions of controlled pH the fully substituted species, **2a**–**e**, as the major products. The synthesis of polyamides rather than polyamines is chosen to control the shape of the central ring. The rotation about the amide bond is restricted at room temperature decreasing the conformational mobility of the ring. This has been observed previously[4] and will be evident from the NMR data reported here as well. Also, a polyamine ring may acts as a chelator as was demonstrated previously in the use as a radionuclide carrier attached to monoclonal antibodies (mAbs) for cancer chemotherapy.[7] In the case of the macrocyclic system, introduction of



Scheme 1 Convergent pathway to target molecules **1a**,**b**



Scheme 2 The pathway to target molecules **2a–e**
TBTU: 2-(1*H*-benzotriazole-1,1,3,3,-tetramethyluronium tetrafluoroborate
Z-Glu-OBzl: L-glutamic acid *N*-(benzyloxycarbonyl)-1-benzyl ester

the side chain was achieved efficiently and in good yields by direct amidation with succinic anhydride or L-glutamic acid *N*-benzyloxycarbonyl-1-benzyl ester (Z-Glu-OBzl) using coupling agents.

Compounds **4a** and **4b** could be obtained quantitatively by reacting **3a** or **3b** with succinic anhydride using the method of Kung and Izatt,[8] and the products were easily purified (Scheme 1) in 45–65% yields. However, only two polysuccinoyl amines were successfully obtained. This route failed for the larger cyclic polyamines presumably due to steric factors and/or decreased reactivity. The requisite activated macrocyclic polysuccinoyl amine derivatives **5a** and **5b** were prepared as the tetrafluorophenyl (TFP) esters in 76–86% yields by the procedure described by Wilbur et al.[9] They are preferred in our work since they are stable to hydrolysis and give good aminolysis yields for products **1a**,**b** while other activated esters, such as *N*-hydroxysuccinicimide (NHS) esters, are more sensitive to ambient moisture. The benzyloxycarbonyl protecting group of compound **7** was removed by hydrogenolysis under basic conditions. Purification of product **8** was achieved by recrystallization. For the last step of the synthesis of **1a** and **1b**, an acetonitrile–water mixture was used as a solvent to address the problem of the differential solubility of TFP ester and lysine-NTA. Triethylamine was found to be the best base for this reaction, even through it was difficult to remove afterwards.

The key step in Scheme 2 is the coupling of the polyamine with the commercially available derivative of glutamic acid, which was protected by a benzyloxycarbonyl (*Z*) group at the N-terminus and benzyl at the C-terminus [L-glutamic acid *N*-benzyloxycarbonyl-1-benzyl ester or *N*-α-Cbz-L-glutamic acid α-benzyl ester]. The coupling agent, 2-(1*H*-benzotriazol-1-yl)1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), was first used in peptide synthesis by Knorr in 1989,[10] and was reported to be very effective for couplings that involve a proline nitrogen, which is similar to the secondary, cyclic polyamines used in this study. The subsequent cleavage of protecting groups (Z and benzyl) in compounds **9a–e** by hydrogenolysis (10 bar H_2, room temperature, Pd/C) afforded polyamino acids. The crude polyamino acids **10a–e** were obtained by filtration of the catalyst and evaporation of the solvent. The pure compounds **10a–e** were collected in 80–90% yields by recrystallization from ethanol. The last step of the sequential pathway was the carboxymethylation of the polyamino acids **10a–e** to provide dendritic ligands **2a–e** by a standard procedure in good yields.

The final compounds **1a**,**b** and **2a–e** are extremely polar and isolation by standard chromatographic techniques such as silica gel column chromatography was difficult. Anion ion exchange chromatography was used for the purification of **2a**,**b** and **1a**,**b**. It was noted that as the number of NTA groups increases, the solubility decreases. So compounds **2c–e** precipitate out from the mixture of etha-

nol and water, and their purification can be performed by simple filtration. The crude products **2c**–**e** were further purified by recrystallization from methanol and collected in 32–56% yields. Being concerned about the polarity of the second set of dendritic compounds **2a**–**e**, we initially tried another route to obtain **2e** as shown in Scheme 3. We aimed to make a representative methyl ester, i.e. **10e**, followed by carboxymethylation with methyl bromoacetate to give its complete methyl ester **11e**. Subsequent hydrolysis of all the methyl groups under basic conditions would give the desired compound **2e**. However, it was difficult to limit the reaction to tertiary alkylation of the amine **11e** since the quaternary ammonium product formed easily in organic solvents. In contrast, direct alkylation of the amino acid was successfully carried out using bromoacetic acid in aqueous solution under basic conditions (pH 11–12) for a few days and followed by acidification with 6 M hydrochloric acid to pH 1.

As a preliminary demonstration of the utility of these dendritic reagents for formation of stoichiometric protein assemblies, we used compounds **1a**,**b** to aggregate His-tagged thioredoxin in solution. Thioredoxin was chosen as a model monomeric protein since it is water-soluble, heat-stable, of low molecular weight and locally available (Dr. Eric Ball, Department of Biochemistry, The University of Western Ontario). Figure 2 shows the native polyacrylamide gel electrophersis analysis of solutions containing mixtures of **1a** and **1b** with His-tagged thioredoxin (lanes 5 and 2, respectively) compared to a solution of protein alone (lanes 1 and 4). It is evident that the majority of the protein in the mixtures move more slowly and that the complex with **1b** moves most slowly. The relative rate of migration is consitent with the majority of the protein moving as monomers in lanes 1 and 4, as dimers in lane 5 and as trimers in lane 2. Lanes 3 and 6 confirm that formation of the stoichiometric assemblies is mediated by the NTA-Ni-His complex since addition of excess EDTA causes the proteins to move as monomers. It should be noted that the mixtures in lanes 3 and 6 were prepared by adding EDTA to solutions after the formation of the protein assemblies but prior to running the gels showing that

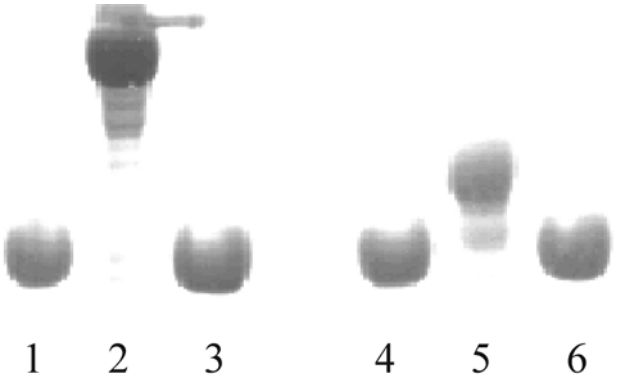
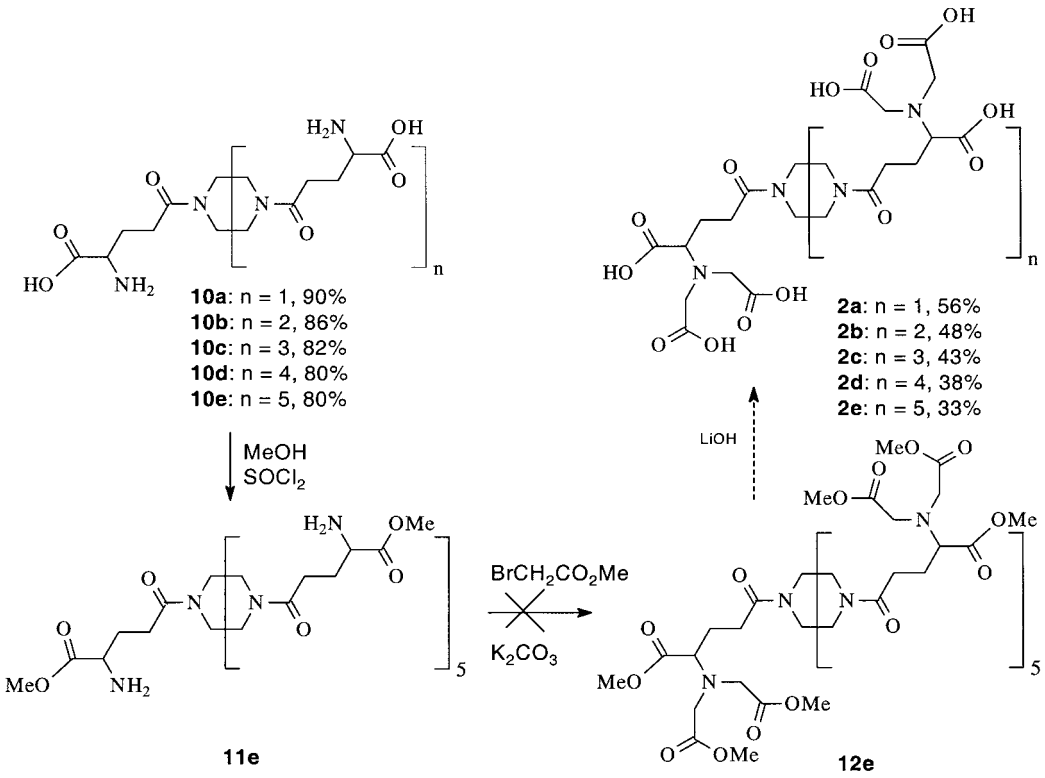


Figure 2 Native PAGE illustration of the aggregation of His-tagged thioredoxin by compounds **1b** (lane 2) and **1a** (lane 5) and the subsequent dissociation by EDTA (lanes 3 and 6 respectively). Lanes 1 and 4 show monomeric protein in the absence of complexing agents. The image of the original gel has been contrast enhanced for clarity.



Scheme 3

complex of *E. coli* His-tagged thioredoxin is observed predominantly as a single oligomeric structure as long as appropriate ratios of ligand:Ni^{2+}:protein were used in solutions. This is consistent with the expectation that the dendritic ligands bind to the His-tagged protein with a high affinity. Although the molecular weight (MW) of the complexes were not determined directly, the gels provide direct evidence that the size of the His-tagged thioredoxin oligomers can be controlled in a reversible fashion by changing the number of NTA groups on the complexing reagents.

In this work, only intermediates **4a**,**7**, and **8** have previously been reported in the literature. We have detailed straightforward methods for the synthesis of two sets of polyfunctional chelators **1a**,**b** and **2a**–**e**. We have tested these compounds to show their ability to form stoichiometric complexes. These and other applications will be reported separately. Access to appropriate ligands with multiple binding features may be a useful step in many aspects of molecular biology or cell biology and protein engineering. The formation constants for 1:1 trivalent metal complexes of NTA (typically 10^8–10^{12}) may be inadequate for in vivo applications, but the ability to form protein-NTA-metal-NTA-protein linkages may provide a non-denaturing and readily reversible means for cross-linking two proteins, such as antibodies and enzymes, for *in vitro* use. The bifunctional NTA ligands may also prove useful for tethering ternary metal complexes to proteins, as was done by Kline.[11] The ability to introduce a protein-reactive substituent into polyamino polycarboxylate chelator by the two pathways described here, should facilitate the identification of additional areas where an interface between coordination chemistry and protein biochemistry may bear fruit.

Piperazine, 1,4,7-triazacyclononane, 1,4,7,10-tetraazacyclododecane, hexacylen trisulfate, 2,3,5,6-tetrafluorophenol (TFP-OH), dicyclohexylcarbodiimide (DCC), bromoacetic acid, and succinic anhydride were obtained from Aldrich Chemical Co. 1,4,7,10,13-Pentaazacyclopentadecane pentahydrochloride was purchased from Parish Chemical Co. TBTU and Z-Glu-OBzl were from Novabiochem. *N*(γ)-Z-L-Lysine was obtained from Fluka. Acrylamide, *N*,*N*′-methylene-bis(acrylamide), Coomassie brilliant blue R250, tris(hydroxymethylaminomethane) hydrogen chloride (Tris-HCl) and glycine were purchased from Life Technologies Inc. Ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED), bromophenol blue, were bought from Sigma Chemical Co. $CaCl_2$, NaCl, sodium dodecyl sulfate (SDS), KCl and ethylenediamine tetraacetic acid (EDTA) were bought from BDH Chemicals. Bio-Rad dye reagent and Mini-Protein II sets were obtained from Bio-Rad laboratories. Native gel electrophoresis was run with a Bio-Rad Model 200/2.0 power supply. All solvents are reagent grade and were used without purification. Column chromatography was performed with 230–400 mesh silica gel. Preparative TLC silica gel F_{254} plates were obtained from EM Separation Technology. Melting points (uncorrected) were determined using a Thomas-Hoover model capillary melting points apparatus. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-300 spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C). IR spectra were recorded as KBr pellets on a Perkin-Elmer System 2000 FTIR spectrometer. High resolution mass spectra were obtained on a Finigan MAT 8320 mass spectrometer. Reactions were monitored by TLC (precoated plates 0.25 mm silica gel 60 F_{254}) and visualized by UV (Z and benzyl groups), ninhydrin (amino group), bromocresol blue (acids and bases), or iodine vapour.

1,4-Bis(3′-carboxypropanoyl)diazacyclohexane (4a); Typical Procedure

Piperazine (**3a**; 0.87 g, 10 mmol) was dissolved in benzene (10 mL) and heated to 60–70 °C. Succinic anhydride (2.10 g, 20 mmol) in benzene (20 mL) was added at this temperature and then the solution was refluxed for 48 h. After this period, the benzene was removed under vacuum. The product **4a** (1.85 g, 6.5 mmol) was purified by crystallization from EtOH; yield: 65%; mp 156.6–158.5 °C.

IR (KBr): 3030 (OH stretch), 1720 (C=O stretch for carboxylic acid), 1600 (C=O stretch for tertiary amide), 1490 cm^{-1} (CH_2 deformation).

^{1}H NMR (D_2O): δ = 2.56 (deformed t, *J* = 8 Hz, 4 H, $CH_2CH_2CO_2H$), 2.66 (deformed t, *J* = 8 Hz, 4 H, $NCOCH_2CH_2$), 3.62–3.48 (t, at r.t.; br s, at 55 °C, due to the existence of rotamers, 8 H, $OCNCH_2CH_2NCO$).

^{13}C NMR (DMSO-d_6): δ = 27.7, 29.3, 44.5, 52.4, 170.3, 174.3.

HRMS (FAB, oxalic acid/glycerol): *m*/*z* calcd for $C_{12}H_{18}N_2O_6$: 287.1243 $[M + H]^+$; found: 287.1236 $[M + H]^+$.

1,4,7-Tris(3′-carboxypropanoyl)triazacyclononane (4b)

Compound **4b** (0.21 g, 0.49 mmol) was prepared from 1,4,7-triazacyclononane (**3b**; 0.14 g, 1.08 mmol) and succinic anhydride (0.33 g, 3.24 mmol) according to the procedure used for **4a** in 45% yield as a white solid; mp 126–130 °C.

IR (KBr): 3010 (OH stretch), 1718 (C=O stretch for carboxylic acid), 1602 (C=O stretch for tertiary amide), 1486 cm^{-1} (CH_2 deformation).

^{1}H NMR (DMSO-d_6): δ = 2.46 (t, *J* = 6 Hz, 6 H, $OCCH_2CH_2CO_2H$), 2.58 (t, *J* = 8 Hz, 6 H, $NOCCH_2CH_2$), 3.68–3.38 (2 br, 12 H, $OCNCH_2CH_2NCO$, conformational isomer).

^{13}C NMR (DMSO-d_6): δ = 27.6, 29.2, 40.4, 52.1, 170.2, 174.1.

HRMS (FAB, oxalic acid/glycerol): *m*/*z* calcd for $C_{18}H_{27}N_3O_9$: 430.1825 $[M + H]^+$; found: 430.1848 $[M + H]^+$.

1,4-Bis[3′-(2,3,5,6-tetrafluorophenoxycarbonyl)propanoyl]diazacyclohexane (5a); Typical Procedure

To a stirred solution of compound **4a** (0.28 g, 0.98 mmol) in DMF (10 mL) under argon was added 2,3,5,6-tetrafluorophenol (TFP-OH) (0.41 g, 2.50 mmol) at 50 °C. A solution of dicyclohexylcarbodiimide (DCC, 0.45 g, 2.04 mmol) in DMF (10 mL) was added dropwise at this temperature. The reaction mixture was stirred overnight, and the solution was cooled to r.t. The white precipitate of dicyclohexylurea was filtered out, and the filtrate was evaporated under vacuum. The residue of reaction was triturated with MeCN–EtOAc (1:1, 15 mL). A white solid (0.49 g) was collected by vacuum filtration; yield: 86%; mp140–142 °C.

IR (KBr): 3422, 3086 (CH stretch for aromatic compound), 2970, 2930, (CH stretch), 1778 (C=O stretch for ester), 1696 (C=C stretch), 1651 (C=O stretch for tertiary amide), 1485, 1451 (CH_2 deformation), 1216 (C–F stretch), 851 cm^{-1} (CH out of plane deformation).

^{1}H NMR ($CDCl_3$): δ = 2.80 (t, *J* = 6 Hz, 4 H, $NCOCH_2CH_2COO$), 3.08 (t, *J* = 6 Hz, 4 H, $NCOCH_2CH_2COO$), 3.76–3.52 (2 m, *J* = 6 Hz, 8 H, $CONCH_2CH_2NCO$, conformational isomer), 6.98 (m, *J* = 5 Hz, 2 H, 2 C_6HF_4).

^{13}C NMR ($CDCl_3$): δ = 26.4, 30.3, 41.1, 48.3, 128.5, 135.2, 172.0, 173.2.

HRMS (EI, 70 V): *m*/*z* calcd for $C_{24}H_{18}F_8N_2O_6$: 582.1037 $[M]^+$; found: 582.1040 $[M]^+$.

1,4,7-Tris[3′-(2,3,5,6-tetrafluorophenoxycarbonyl)propanoyl]-triazacyclononane (5b)

Compound **5b** (0.15 g, 0.17 mmol) was obtained from **4b** (0.11 g, 0.25 mmol), TFP-OH (0.15 g, 0.88 mmol) and DCC (0.18 g, 0.82 mmol) according to the procedure used for **5a** as a white solid in 67% yield; mp 120–122 °C.

IR (KBr): 3412, 3085 (CH stretch for aromatic compound), 2950 (CH stretch), 1776 (C=O stretch for ester), 1686 (C=C stretch), 1650 (C=O stretch for tertiary amide), 1480, 1450 (CH_2 deformation), 1216 (C–F stretch), 850 cm^{-1} (CH out of plane deformation).

^{1}H NMR ($CDCl_3$): δ = 2.82 (t, *J* = 8 Hz, 6 H, NOCC*H*$_2$CH$_2$COO), 3.07 (t, *J* = 8 Hz, 6 H, NOCCH$_2$C*H*$_2$COO), 3.72–3.53 (2 m, *J* = 7 Hz, 12 H, CONC*H*$_2$C*H*$_2$NCO, conformational isomer), 6.97 (m, *J* = 5 Hz, 3 H, 3 C$_6$*H*F$_4$).

^{13}C NMR ($CDCl_3$): δ = 27.3, 30.8, 41.5, 45.5, 128.0, 135.3, 172.3, 174.2.

HRMS (EI, 70 V): *m*/*z* calcd for $C_{36}H_{27}F_{12}N_3O_9$: 873.1556 $[M]^+$; found: 873.1558 $[M]^+$.

***N*-(5-Benzyloxycarbonylamino-1-carboxypentyl)iminodiacetic Acid [*N*(γ)-Z-Lys-NTA, 7]**

Bromoacetic acid (4.17 g, 30 mmol) was dissolved in NaOH solution (15 mL, 2 M) and cooled to 0 °C. *N*(γ)-Benzyloxycarbonyl-L-lysine (**6**, 4.20 g, 15 mmol) in aq 2 M NaOH solution (22.5 mL) was added dropwise at 0 °C with stirring. After 2 h, the ice bath was removed and the mixture was stirred overnight at r.t. The temperature was then raised to 50 °C for 2 h and aq 1 M HCl solution (45 mL) was added. After the mixture had been cooled, the crystals that formed were filtered off, and redissolved again in aq 1 M NaOH solution. The product precipitated out by the dropwise addition of aq 1 M HCl solution and was collected by filtration. Compound **7** (5.28 g) was obtained as a white solid in 89% yield; mp 172–174 °C.

IR (KBr): 3206 (OH stretch), 1720 (C=O stretch for carboxylic acid), 1652 [C=O stretch for O(C=O)NH], 1640 (C=O stretch for tertiary amide), 1594 (C=C stretch), 1464 (CH_2 deformation), 1431, 1287 cm^{-1} (C–N stretch).

^{1}H NMR (DMSO-d_6): δ = 1.76–1.36 (br, 6 H, NHCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH), 2.02 (q, *J* = 6 Hz, 2 H, NHC*H*$_2$CH$_2$), 3.55 (br s, 5 H, NC*H*$_2$CO$_2$H and NCHCOO), 4.98 (s, 2 H, C$_6$H$_5$C*H*$_2$), 7.33 (br s, 5 H, C$_6$H$_5$).

^{13}C NMR (DMSO-d_6): δ = 23.3, 27.8, 28.6, 46.4, 54.6, 59.9, 64.2, 65.2, 117.7, 118.3, 128.5, 158.5, 173.2, 174.8.

5-Aminopentylnitrilotriacetic Acid or N^{α},N^{α}-Bis[carboxymethyl]-l-lysine (8)

N(γ)-Z-Lys-NTA (**7**; 3.02 g, 7.50 mmol) was dissolved in aq 1 M NaOH (50 mL). After the addition of a spatula tip of Pd/C (10%), the mixture was hydrogenated at r.t. and normal pressure. The reaction was complete after 6 h as monitored by TLC (MeCN–H_2O, 4:1) and visualized with UV, I_2, and ninhydrin. The catalyst was filtered off, and solvent was removed in vacuo. The resulting residue was redissolved in EtOH (25 mL), the product crystalized at 0 °C within 2 d. The crystals (1.49 g) were collected and dried in vacuum; yield: 76%; mp 202–204 °C (dec.).

IR (KBr): 3016 (OH stretch), 1724 (C=O stretch for carboxylic acid), 1710 (COO^- antisym stretch), 1464 (CH_2 deformation), 1381, 1287 cm^{-1} (C–N stretch).

^{1}H NMR (D_2O): δ = 1.91–1.52 (br, 6 H, NHCH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH), 2.88 (t, *J* = 7 Hz, 2 H, NHC*H*$_2$CH$_2$), 3.84–3.50 (br s, 5 H, NC*H*$_2$CO$_2$H and NCHCOO).

^{13}C NMR (D_2O): δ = 26.3, 28.9, 32.6, 42.9, 59.1, 70.8, 174.7, 175.8.

1,4-Bis[5′-*N*,*N*-di(carboxymethyl)amino-5′-carboxypentylamino-3-oxopropanoyl]diazacyclohexane (1a); Typical Procedure

Lysine-NTA (**8**; 0.26 g, 0.99 mmol) was dissolved in H_2O (4 mL). To this solution was added Et_3N (0.3 mL), followed by a solution of **5a** (0.29 g, 0.49 mmol) in warm MeCN (5 mL). The reaction solution turned cloudy immediately and was stirred at r.t. for 12 h. The reaction was monitored by TLC, the developed component was visualized by ninhydrin spray. When the reaction was complete, it was quenched by the addition of aq 1 M HCl to pH 1. After extraction with $CHCl_3$, the aqueous layer was evaporated to dryness under vacuum and the resulting product redissolved in H_2O. The resulting residue was purified twice on by anion exchange column chromatography and collected to give **1a** (0.13 g) in 35% yield; mp 170 °C (dec.).

IR (KBr): 3330 (OH stretch), 2948, 2854 (CH stretch), 1730 (C=O stretch for carboxylic acid), 1680 (C=O stretch for tertiary amide), 1436 (CH_2 deformation), 1380 cm^{-1} (C–N stretch).

^{1}H NMR (D_2O): δ = 1.85–1.37 (br, 12 H, CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH), 2.38 (t, *J* = 7 Hz, 4 H, CH$_2$C*H*$_2$CO), 2.58 (t, *J* = 7 Hz, 4 H, NCOC*H*$_2$CH$_2$), 3.10 (t, *J* = 6 Hz, 4H, NHC*H*$_2$CH$_2$), 3.56–3.46 (two b, 8 H, C*H*$_2$C*H*$_2$NCO), 3.89–3.80 (br s, 10 H, NC*H*$_2$CO$_2$H and CH$_2$C*H*CO$_2$H).

^{13}C NMR (D_2O): δ = 23.0, 26.7, 28.2, 30.8, 38.8, 46.8, 54.4, 63.9, 66.8, 168.1, 170.5, 171.6, 172.5.

HRMS (FAB, oxalic acid/glycerol): *m*/*z* calcd for $C_{32}H_{50}N_6O_{16}$: 775.3362 $[M + H]^+$; found 775.3386 $[M + H]^+$.

1,4,7-Tris[5′-*N*,*N*-di(carboxymethyl)amino-5′-carboxypentylamino-3′-oxopropanoyl]triazacyclononane (1b)

Compound **1b** was obtained from **5b** (0.25 g, 0.28 mmol) and lysine-NTA (**8**; 0.28 g, 1.17 mmol) according to the method given for **1a** as a white solid (0.1 g, 32%); mp 200 °C (dec.).

IR (KBr): 3331 (OH stretch), 2945, 2857 (CH stretch), 1732 (C=O stretch for carboxylic acid), 1685 (C=O stretch for tertiary amide), 1434 (CH_2 deformation), 1381 cm^{-1} (C–N stretch).

^{1}H NMR (D_2O): δ = 1.85–1.37 (br, 18 H, CH$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH), 2.44 (t, *J* = 6 Hz, 6 H, CH$_2$C*H*$_2$CONH), 2.80 (t, *J* = 6 Hz, 6 H, NCOC*H*$_2$CH$_2$), 3.18 (t, *J* = 6 Hz, 6 H, NHC*H*$_2$CH$_2$), 3.55–3.45 (br s, 12 H, OCNC*H*$_2$C*H*$_2$NCO), 3.89 (br, 15 H, NC*H*$_2$CO$_2$H and CH$_2$C*H*CO$_2$H).

^{13}C NMR (D_2O): δ = 22.9, 23.0, 27.0, 28.2, 30.8, 38.1, 46.8, 54.5, 64.8, 66.9, 168.6, 170.0, 171.4, 172.3.

HRMS (FAB, oxalic acid/glycerol): *m*/*z* calcd for $C_{48}H_{75}N_9O_{24}$: 1162.5003 $[M + 1]^+$; found 1162.4982 $[M + 1]^+$.

1,4-Bis[4′-(*N*-benzyloxycarbonyl)amino-4′-benzyloxycarbonylbutanoyl]diazacyclohexane (9a); Typical Procedure

Z-Glu-OBzL (2.5 g, 6.0 mmol) and TBTU (1.926 g, 6.0 mmol) were dissolved in DMF (4 mL), followed by the addition of Et_3N (0.83 mL, 6.0 mmol). To this solution, was added a warm solution of piperazine (0.206 g, 2 mmol) in DMF (2 mL). After stirring overnight, the reaction mixture was evaporated to dryness. The viscous residue was dissolved in $CHCl_3$ (80 mL), the $CHCl_3$ solution was washed twice with aq 5% $NaHCO_3$, brine, and H_2O, sequentially. The organic layer was dried (Na_2SO_4), and the solvent was removed by rotary evaporation. The residue was subjected to silica gel column chromatography, and the desired product was isolated by preparative TLC; the fourth band was collected (R_f 0.32, developed by a 5:2:5:1 mixture of EtOAc, $CHCl_3$, hexane, and MeOH). The product (1.36 g) was collected as a white solid; yield: 86%; mp 39–42 °C.

IR (KBr): 3300 (NH stretch), 3033 (CH stretch for aromatic compound), 2947 (CH stretch for alkane), 1721 (C=O stretch for ester), 1636 (C=O stretch for tertiary amide), 1530 (C=C stretch), 1455 (CH_2 deformation), 1215 (C–O–C stretch), 699 cm^{-1} (CH out of plane deformation).

^1H NMR ($CDCl_3$): δ = 2.05 (br, 4 H, CH_2CH_2CH), 2.26 (t, *J* = 7 Hz, 4 H, $COCH_2CH_2$), 3.20 (br s, 4 H, $OCNCH_2CH_2NCO$, isomer), 3.48 (br s, 4 H, $OCNCH_2CH_2NCO$), 4.45 (t, *J* = 7 Hz, 2 H, CH_2CHCOO), 5.06 (s, 4 H, $C_6H_5CH_2OCO$), 5.16 (q, *J* = 3 Hz, 4 H, $NCO_2CH_2C_6H_5$), 5.70 (d, *J* = 8 Hz, 2 H, CHN*H*CO), 7.34–7.25 (br s, 20 H, 4 C_6H_5).

^{13}C NMR ($CDCl_3$): δ = 27.6, 29.1, 41.4, 45.0, 53.7, 67.1, 128.2, 128.5, 128.7, 135.3, 136.3, 156.8, 170.4, 171.7.

HRMS (EI, 70 V): *m*/*z* calcd for $C_{44}H_{48}N_4O_{10}$: 792.3370 $[M]^+$; found: 792.3305 $[M]^+$.

1,4,7-Tris[4′-*N*-benzyloxycarbonylamino-4′-benzyloxycarbonylbutanoyl]triazacyclononane (9b)

Compound **9b** (0.95 g, 0.52 mmol) was obtained as a white solid from 1,4,7-triazacyclononane (**3b**; 0.10 g, 0.80 mmol), Z-Glu-OBzl (1.35 g, 3.60 mmol) and TBTU (1.15 g, 3.60 mmol) according to the procedure used for **9a** in 65% yield; mp 40–43 °C.

IR (KBr): 3305 (NH stretch), 3031 (CH stretch for aromatic compound), 2948 (CH stretch for alkane), 1725 (C=O stretch for ester), 1634 (C=O stretch for tertiary amide), 1528 (C=C stretch), 1458 (CH_2 deformation), 1215 (C–O–C stretch), 700 cm^{-1} (CH out of plane deformation).

^1H NMR ($CDCl_3$): δ = 1.95 (br s, 6 H, $COCH_2CH_2CH$), 2.25 (br s, 6 H, $COCH_2CH_2$), 3.30 (br s, 9 H, $OCNCH_2CH_2NCO$, due to isomer), 3.64 (br s, 3 H, $OCNCH_2CH_2NCO$), 4.38 (t, *J* = 8 Hz, 3 H, CH_2CHCOO), 5.05 (s, 6 H, $C_6H_5CH_2OCO$), 5.15 (m, *J* = 2 Hz, 6 H, $C_6H_5CH_2OCON$), 6.01 (d, *J* = 8 Hz, 3 H, CHN*H*CO), 7.33–7.25 (br s, 30 H, 6 C_6H_5).

^{13}C NMR ($CDCl_3$): δ = 26.5, 30.0, 46.0, 50.9, 54.0, 67.1, 67.3, 128.3, 128.6, 128.8, 136.3, 158.1, 172.2, 173.4.

HRMS (EI, 70 V): *m*/*z* calcd for $C_{66}H_{72}N_6O_{15}$: 1188.5056 $[M]^+$; found 1188.5067 $[M]^+$.

1,4,7,10-Tetrakis[4′-(*N*-benzyloxycarbonyl)amino-4′-benzyloxycarbonylbutanoyl]tetraazacyclododecane (9c)

Compound **9c** (0.17 g, 0.11 mmol) was obtained from cyclen or 1,4,7,10-tetraazacyclododecane (**3c**; 0.043 g, 0.24 mmol), Z-Glu-OBzl (0.54 g, 1.44 mmol) and TBTU (0.46 g, 1.44 mmol) according to the procedure used for **9a** as a white solid in 44% yield; mp 60–62 °C.

IR (KBr): 3310 (NH stretch), 3023 (CH stretch for aromatic compound), 2946 (CH stretch for alkane), 1720 (C=O stretch for ester), 1638 (C=O stretch for tertiary amide), 1533 (C=C stretch), 1456 (CH_2 deformation), 1205 (C–O–C stretch), 698 cm^{-1} (CH out of plane deformation).

^1H NMR ($CDCl_3$): δ = 1.95 (br, 8 H, $COCH_2CH_2CH$), 2.50 (br, 8 H, $COCH_2CH_2$), 3.71–3.13 (2 br s, 16 H, $OCNCH_2CH_2NCO$), 4.43 (br, 4 H, CH_2CHCOO), 5.05 (s, 8 H, $C_6H_5CH_2OCO$), 5.15 (m, *J* = 2 Hz, 8 H, $C_6H_5CH_2OCON$), 6.04 (br s, 4 H, CHN*H*CO), 7.33–7.25 (br s, 40 H, 8 C_6H_5).

^{13}C NMR ($CDCl_3$): δ = 26.9, 29.8, 38.7, 49.7, 53.5, 67.0, 67.4, 128.2, 128.9, 136.2, 156.5, 171.9, 172.5.

HRMS (EI, 70 V): *m*/*z* calcd for $C_{88}H_{96}N_8O_{20}$: 1584.6741 $[M]^+$; found 1584.6712 $[M]^+$.

1,4,7,10,13-Pentaazacyclopentadecane (3d)

Pentaaza-15-crown-5 pentahydrochloride (0.17 g, 0.42 mmol) was dissolved in aq 0.1 M HCl (1 mL), to which a large excess of aq 6 M NaOH was added. The solution was extracted with chloroform (4 × 40 mL) and dried (Na_2SO_4). Removal of the solvent gave **3d** (0.07 g) in 75% yield.

1,4,7,10,13-Pentakis[4′-(*N*-benzyloxycarbonyl)amino-4′-benzyloxycarbonylbutanoyl]pentaazacyclopentadecane (9d)

Compound **9d** (0.21 g, 0.11 mmol) was prepared as a white solid from **3d** (0.06 g, 0.28 mmol), Z-Glu-OBzl (0.77 g, 2.06 mmol) and TBTU (0.66 g, 2.06 mmol) according to the procedure used for **9a** as a white solid in 38% yield; mp 56–58 °C.

IR (KBr): 3305 (NH stretch), 3030 (CH stretch for aromatic compound), 2948 (CH stretch for alkane), 1719 (C=O stretch for ester), 1630 (C=O stretch for tertiary amide), 1528 (C=C stretch), 1456 (CH_2 deformation), 1210 (C–O–C stretch), 697 cm^{-1} (CH out of plane deformation).

^1H NMR ($CDCl_3$): δ = 2.05 (br, 10 H, CH_2CH_2CH), 2.45 (br, 10 H, $COCH_2CH_2$), 3.55–3.23 (2 br s, 20 H, $OCNCH_2CH_2NCO$), 4.43 (br, 5 H, CH_2CHCOO), 5.04 (s, 10 H, $C_6H_5CH_2COO$), 5.15 (br, 10 H, $C_6H_5CH_2O$), 5.96 (d, *J* = 8 Hz, 5 H, CHN*H*CO), 7.35–7.25 (br s, 50 H, 10 C_6H_5).

^{13}C NMR ($CDCl_3$): δ = 27.0, 29.8, 38.7, 49.9, 53.5, 66.8, 67.3, 128.2, 128.9, 136.1, 157.5, 171.8, 172.0.

HRMS (EI, 70 V): *m*/*z* calcd for $C_{110}H_{120}N_{10}O_{25}$: 1980.8246 $[M]^+$; found 1980.8265 $[M]^+$.

1,4,7,10,13-Hexaazacycloocadecane or Hexacyclen (3e)

Hexacyclen trisulfate (0.46 g, 0.83 mmol) was dissolved in aq 6 M NaOH (40 mL). The solution was extracted with $CHCl_3$(4 × 40 mL) and dried (Na_2SO_4). Removal of the solvent gave **3e** (0.14 g) in 65% yield.

1,4,7,10,13,16-Hexakis[4′-(*N*-benzyloxycarbonyl)amino-4′-benzyloxycarbonylbutanoyl]hexaazacycloocadecane (9e)

Compound **9e** (0.07 g, 0.03 mmol) was obtained as a white solid from hexacyclen (**3e**) (0.024 g, 0.093 mmol), Z-Glu-OBzl (0.35 g, 0.93 mmol) and TBTU (0.30 g, 0.93 mmol) according to the procedure used for **9a** as a white solid in 33% yield; mp 56–58 °C.

IR (KBr): 3304 (NH stretch), 3030 (CH stretch for aromatic compound), 2947 (CH stretch for alkane), 1720 (C=O stretch for ester), 1628 (C=O stretch for tertiary amide), 1521 (C=C stretch), 1452 (CH_2 deformation), 1207 (C–O–C stretch), 699 cm^{-1} (CH out of plane deformation).

^1H NMR ($CDCl_3$): δ = 2.05 (br, 12 H, CH_2CH_2CH), 2.45 (br, 12 H, $COCH_2CH_2$), 3.54–3.27 (2 br s, 24 H, $OCNCH_2CH_2NCO$), 4.40 (br, 6 H, CH_2CHCOO), 5.02 (s, 12 H, $C_6H_5CH_2COO$), 5.12 (br, 12 H, $C_6H_5CH_2OCON$), 6.05 (br, 6 H, CHN*H*CO), 7.35–7.18 (br s, 60 H, 12 C_6H_5).

^{13}C NMR ($CDCl_3$): δ = 27.5, 29.0, 47.5, 53.9, 67.5, 67.7, 127.9, 128.8, 128.9, 136.9, 156.8, 172.5, 173.6.

HRMS (EI, 70 V): *m*/*z* calcd for $C_{132}H_{144}N_{12}O_{30}$: 2377.0111 $[M]^+$; found 2377.0178 $[M]^+$.

1,4-Bis(4′-amino-4′-carboxybutanoyl)diazacyclohexane (10a); Typical Procedure

Compound **9a** (0.65 g, 0.82 mmol) was dissolved in MeOH (10 mL). A small amount of Pd/C (10%) was added to this solution. After stirring the suspension for 2 min, H_2 gas was introduced into the flask at r.t. under normal pressure. The reaction progress was monitored by TLC, visualized by illumination with UV light. The catalyst was filtered off, washed with H_2O, and the filtrate was dried in vacuo. The resulting precipitate was redissolved in hot EtOH, and allowed to crystallize at 0 °C. The crystals were filtered off and dried in vacuo; yield: 90% (0.25 g); mp 168–170 °C.

IR (KBr): 3435 (NH and OH stretch), 2934 (CH stretch for alkane), 1716 (C=O stretch for carboxylic acid), 1630 (C=O stretch for tertiary amide), 1530 (NH_3^+ deformation), 1443 (CH_2 deformation), 1427 cm^{-1} (COO^- sym stretch).

^1H NMR (D_2O): δ = 1.99 (q, *J* = 6 Hz, 4 H, CH_2CH_2CH), 2.48 (t, *J* = 7 Hz, 4 H, $COCH_2CH_2$), 3.60–3.46 (2 br s, 8 H, CH_2CH_2NCO), 3.74 (t, *J* = 8 Hz, 2 H, CH_2CHCOO).

^{13}C NMR (D_2O): δ = 25.2, 28.4, 41.7, 45.1, 173.0, 174.1.

HRMS (FAB, oxalic acid/glycerol): *m*/*z* calcd for $C_{14}H_{24}N_4O_6$: 345.1774 $[M + H]^+$; found 345.1761 $[M + H]^+$.

1,4,7-Tris(4′-amino-4′-carboxybutanoyl)triazacyclononane (10b)

Compound **10b** (0.11 g, 0.22 mmol) was obtained from **9b** (0.30 g, 0.25 mmol) according to the procedure used for **10a** as a white solid, which was deliquescent, but could be recrystallized from hot EtOH; yield: 86%; mp 210 °C (dec.).

IR (KBr): 3436 (NH and OH stretch), 2936 (CH stretch for alkane), 1723 (C=O stretch for carboxylic acid), 1638 (C=O stretch for tertiary amide), 1445 (NH_3^+ deformation), 1429 cm^{-1} (COO^- sym stretch).

^1H NMR (D_2O): δ = 2.02 (br , 6 H, CH_2CH_2CH), 2.42 (br , 6 H, $COCH_2CH_2$), 3.67–3.37 (2 br s, 12 H, $OCNCH_2CH_2NCO$), 3.69 (t, *J* = 8 Hz, 3 H, CH_2CHCOO).

^{13}C NMR (D_2O): δ = 25.7, 29.5, 44.1, 48.6, 50.5, 174.5, 175.3.

HRMS (FAB, glycerol/oxalic acid): *m*/*z* calcd for $C_{21}H_{36}N_6O_9$: 517.2622 $[M + H]^+$; found 517.2690 $[M + H]^+$.

1,4,7,10-Tetrakis(4′-amino-4′-carboxybutanoyl)tetraazacyclododecane (10c)

Compound **10c** (0.04 g, 0.06 mmol) was obtained from **9c** (0.12 g, 0.07 mmol) according to the procedure used for **10a** as a white solid in 82% yield; mp 210 °C (dec.).

IR (KBr): 3432 (NH and OH stretch), 2933 (CH stretch for alkane), 1720 (C=O stretch for carboxylic acid), 1634 (C=O stretch for tertiary amide), 1442 (NH_3^+ deformation), 1430 cm^{-1} (COO^- sym stretch).

^1H NMR (D_2O): δ = 2.05 (q, *J* = 6 Hz, 8 H, CH_2CH_2CH), 2.44 (t, *J* = 7 Hz, 8 H, $COCH_2CH_2$), 3.63–3.43 (2 br, 16 H, $OCNCH_2CH_2NCO$), 3.67 (t, *J* = 7 Hz, 4 H, CH_2CHCOO).

^{13}C NMR (D_2O): δ = 25.6, 29.5, 45.7, 48.6, 51.4, 174.2, 175.0.

HRMS (FAB, glycerol/oxalic acid): *m*/*z* calcd for $C_{28}H_{48}N_8O_{12}$: 689.3469 $[M + H]^+$; found 689.3445 $[M + H]^+$.

1,4,7,10,13-Pentakis(4′-amino-4′-carboxybutanoyl)pentaazacyclopentadecane (10d)

Compound **10d** (0.10 g, 0.12 mmol) was obtained from **9d** (0.30 g, 0.15 mmol) according to the procedure of **10a** as white solid in 80% yield; mp 200 °C (dec.).

IR (KBr): 3430 (NH and OH stretch), 2931 (CH stretch for alkane), 1716 (C=O stretch for carboxylic acid), 1640 (C=O stretch for tertiary amide), 1448 (NH_3^+ deformation), 1430 cm^{-1} (COO^- sym stretch).

^1H NMR (D_2O): δ = 2.01 (br, 10 H, CH_2CH_2CH), 2.42 (br , 10 H, $COCH_2CH_2$), 3.63–3.43 (2 br, 20 H, $OCNCH_2CH_2NCO$), 3.67 (br, 5 H, CH_2CHCOO).

^{13}C NMR (D_2O): δ = 25.2, 29.2, 45.6, 48.0, 50.9, 174.2, 175.1.

HRMS (FAB, glycerol/oxalic acid): *m*/*z* calcd for $C_{35}H_{60}N_{10}O_{15}$: 861.4317 $[M + H]^+$; found 861.4325 $[M + H]^+$.

1,4,7,10,13,16-Hexakis(4′-amino-4′-carboxybutanoyl)hexaazacycloocadecane (10e)

Compound **10e** (0.04 g, 0.04 mmol) was obtained from **9e** (0.11 g, 0.05 mmol) according to the procedure used for **10a** as a white solid in 80% yield; mp 200 °C (dec.).

IR (KBr): 3436 (NH and OH stretch), 2933 (CH stretch for alkane), 1717 (C=O stretch for carboxylic acid), 1635 (C=O stretch for tertiary amide), 1447 (NH_3^+ deformation), 1426 cm^{-1} (CH_2 deformation).

^1H NMR (D_2O): δ = 2.02 (br, 12 H, CH_2CH_2CH), 2.64 (br, 12 H, $COCH_2CH_2$), 3.56–3.46 (2 br, 24 H, $OCNCH_2CH_2NCO$), 3.78 (t, *J* = 8 Hz, 6 H, CH_2CHCOO).

^{13}C NMR (D_2O): δ = 25.5, 28.5, 46.1, 51.0, 173.9, 174.7.

HRMS (FAB, glycerol/oxalic acid): *m*/*z* calcd for $C_{42}H_{72}N_{12}O_{18}$: 1033.5165 $[M + H]^+$; found 1033.5158 $[M + H]^+$.

1,4-Bis[4′-*N*,*N*-di(carboxymethyl)amino-4′-carboxylbutanoyl)]diazacyclohexane (2a)

A solution of bromoacetic acid (0.24 g, 1.72 mmol) in aq 1.5 M NaOH (2 mL) was cooled to 0 °C and was added dropwise to a solution of **10a** (0.13 g, 0.38 mmol) in NaOH (2 mL, 2 M). The pH of the resulting solution was adjusted to 12. Then the reaction mixture was stirred at 45 °C for 7 d. The pH was maintained in the range of 11–12 by periodic addition of aq 2 M NaOH and the reaction progress was monitored by TLC. After cooling to r.t., the mixture was evaporated to dryness under vacuum to give a white solid. The solid was redissolved in H_2O, pH adjusted to10, and applied to a Bio-Rad AG1-X4 anion-exchange column (formate ion). The column was eluted successively with H_2O, aq 4 M formic acid and aq 5 M formic acid. The fraction containing the desired product determined by TLC and was applied to a second Bio-Rad AG1-X4 anion-exchange column that was eluted with H_2O, aq 5 M formic acid, and aq 6 M formic acid. The desired product eluted slowly in the aq 5 M formic acid fractions and these were combined, evaporated to dryness under vacuum, and redissolved in aq 4 M HCl. The resulting solution was again evaporated to dryness, and the residue was repeatedly redissolved in H_2O. The solution was dried to give a white solid (0.12 g, 56%); mp 210 °C (dec.).

IR (KBr): 3436 (OH stretch), 1733 (C=O stretch for carboxylic acid), 1631 (C=O stretch for tertiary amide), 1365 cm^{-1} (N–C stretch).

^1H NMR (D_2O): δ = 2.06 (br s, 4 H, CH_2CH_2CH), 2.54 (br s, 4 H, $NCOCH_2CH_2$), 3.65–3.43 (2 br s, 8 H, $OCNCH_2CH_2NCO$), 3.89 (br s, 10 H, NCH_2CO_2H and CH_2CHCO_2H).

^{13}C NMR (D_2O): δ = 22.5, 30.3, 40.6, 49.2, 54.1, 64.5, 170.1, 171.2, 175.3, 176.6.

HRMS (FAB, glycerol/oxalic acid): *m*/*z* calcd for $C_{22}H_{32}N_4O_{14}$: 577.1993 $[M + H]^+$; found 577.1942 $[M + H]^+$.

1,4,7-Tris[4′-*N*,*N*-di(carboxymethyl)amino-4′-carboxybutanoyl]triazacyclononane (2b)

Compound **2b** (0.017 g, 0.02 mmol) was obtained from **10b** (0.02 g, 0.04 mmol) and bromoacetic acid (0.034 g, 0.25 mmol) according to the procedure used for **2a** as a white solid in 48% yield; mp 210 °C (dec.).

IR (KBr): 3435 (OH stretch), 1735 (C=O stretch for carboxylic acid), 1630 (C=O stretch for tertiary amide), 1367 cm^{-1} (N–C stretch).

^1H NMR (D_2O): δ = 2.05 (br, 6 H, CH_2CH_2CH), 2.54 (br, 6 H, $NCOCH_2CH_2$), 3.58–3.35 (2 br, 12 H, $OCNCH_2CH_2NCO$), 3.92–3.74 (2 br, 15 H, NCH_2CO_2H and CH_2CHCO_2H).

^{13}C NMR (D_2O): δ = 24.5, 30.4, 48.7, 54.3, 66.6, 169.8, 175.0, 175.3.

HRMS (FAB, glycerol/oxalic acid): *m*/*z* calcd for $C_{33}H_{48}N_6O_{21}$: 865.2951 $[M + H]^+$; found 865.2921 $[M + H]^+$.

1,4,7,10-Tetrakis[4′-*N*,*N*-di(carboxymethyl)amino-4′-carboxybutanoyl]-tetraazacyclododecane (2c)

Compound **2c** (0.04 g, 0.035 mmol) was obtained from **10c** (0.056 g, 0.081 mmol) and bromoacetic acid (0.10 g, 0.73 mmol) according to the procedure used for **2a** as white solid in 43% yield; mp 220 °C (dec.).

IR (KBr): 3430 (OH stretch), 1723 (C=O stretch for carboxylic acid), 1634 (C=O stretch for tertiary amide), 1387 cm^{-1} (N–C stretch).

^1H NMR (D_2O): δ = 2.01 (br, 8 H, CH_2CH_2CH), 2.54 (br, 8 H, $NCOCH_2CH_2$), 3.85–3.45 (2 br, 36 H, NCH_2CO_2H, CH_2CHCO_2H and $OCNCH_2CH_2NCO$).

^{13}C NMR (D_2O): δ = 24.7, 29.6, 47.4, 55.0, 66.8, 169.3, 171.0, 174.3, 175.4.

HRMS (FAB, oxalic acid): *m*/*z* calcd for $C_{44}H_{64}N_8O_{28}$: 1153.3908 $[M + H]^+$; found 1153.3938 $[M + H]^+$.

1,4,7,10,13-Pentakis[4′-*N*,*N*-di(carboxymethyl)amino-4′-carboxybutanoyl]pentaazacyclopentadecane (2d)

Compound **2d** (0.044 g, 0.03 mmol) was obtained from **10d** (0.07 g, 0.08 mmol) and bromoacetic acid (0.12 g, 0.88 mmol) according to the procedure used for **2a** as a white solid in 38% yield; mp 220 °C (dec.).

IR (KBr): 3433 (OH stretch), 1730 (C=O stretch for carboxylic acid), 1637 (C=O stretch for tertiary amide), 1364 cm^{-1} (N–C stretch).

^1H NMR (D_2O): δ = 2.01 (br, 10 H, CH_2CH_2CH), 2.70 (br, 10 H, $NCOCH_2CH_2$), 3.86–3.45 (2 br, 45 H, NCH_2CO_2H, CH_2CHCO_2H and $OCNCH_2CH_2NCO$).

^{13}C NMR (D_2O): δ = 26.5, 30.4, 48.6, 54.7, 64.8, 170.0, 174.8, 175.2.

HRMS (FAB, glycerol/oxalic acid): *m*/*z* calcd for $C_{55}H_{80}N_{10}O_{35}$: 1441.4865 $[M + H]^+$; found 1441.4856 $[M + H]^+$.

1,4,7,10,13,16-Hexakis[4′-*N*,*N*-di(carboxymethyl)amino-4′-carboxybutanoyl]hexaazacyclooctadecane (2e)

Compound **2e** (0.13 g, 0.007 mmol) was obtained from **10e** (0.023 g, 0.022 mmol) and bromoacetic acid (0.04 g, 0.30 mmol) according to the procedure used for **2a** as a white solid in 33% yield; mp 230 °C (dec.).

IR (KBr): 3430 (OH stretch), 1729 (C=O stretch for carboxylic acid), 1680 (C=O stretch for tertiary amide), 1360 cm^{-1} (N–C stretch).

^1H NMR (D_2O): δ = 2.01 (br, 12 H, CH_2CH_2CH), 2.72 (br, 12 H, $NCOCH_2CH_2$), 3.86–3.46 (2 br, 54 H, NCH_2CO_2H, CH_2CHCO_2H and $OCNCH_2CH_2NCO$).

^{13}C NMR (D_2O): δ = 27.5, 31.4, 48.7, 54.2, 57.4, 65.8, 67.8, 169.6, 171.0, 174.8, 175.2.

HRMS (FAB, glycerol/oxalic acid): *m*/*z* calcd for $C_{66}H_{96}N_{12}O_{42}$: 1729.5823 $[M + H]^+$; found 1729.5801 $[M + H]^+$.

Native Polyacrylamide Gel Electrophoresis (PAGE)

A solution of His-tagged thioredoxin obtained from Dr. E. Ball was further dialysed against 20 mM HEPES buffer (20 mM Tris-HCl, 100 mM NaCL, and 2 mM $CaCl_2$), pH 8.0 using a Spectra/por1 wet tubing MWCO 1000. The resulting solution was concentrated with lyophilizer (ETS System, Inc.), and the size and purity was determined by SDS-PAGE. The protein concentration was determined from the absorbance at 595 nm using the Bradford method.[12] The conditions for native PAGE were adapted from Weber and Osborn.[13] The apparatus routinely used generates an 8.5 cm (height) × 7.5 cm separating gel, 1 mm thickness. The electrode buffer contained 25 mM Tris base, 0.19 M glycine, and 20 mM KCl, pH 8.0. Samples for native PAGE were prepared by mixing one part of the protein sample with five parts of native PAGE sample buffer (15.5 mL of 1 M Tris-HCl pH 8.0, 2.5 mL of a 1% solution of bromophenol blue, 7 mL of H_2O and 25 mL of glycerol). The protein samples as described below were electrophoresized toward the anode (+) for 30 minutes at 40 V, and followed by 210 mins at 70 V until the dye front reached the bottom of the gel. The gel was then placed in a staining solution containing 0.25% Coomassie Blue G-250 (0.25 g Coomassie Blue G-250, 125 mL MeOH, 25 mL glacial AcOH, and 100 mL H_2O) overnight. Then the gel was destained in a solution containing 10% MeOH and 10% AcOH for 4 h. The resulting gel was dried and scanned.

The model protein was loaded to lane 1 and lane 4. Lanes 2 and 5 contain the complex formed by a combination of ligand **1a** and **1b**, $NiCl_2$ and protein with molar ratio of 1:2.5:2.5 and 1:3.5:3.5, respectively. Lanes 3 and 6 represent the complex (lanes 2 and 5) being dissociated by the addition of EDTA with molar ratios of 1/4 (Dimeric complex/EDTA) or 1/8 (trimeric complex/EDTA). The same amount of protein (15 μL of a 0.06 mg/mL solution) was loaded in each lane. The gel was formed at 17% polyacrylamide.

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