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Synthesis of chiral, amphiphilic, and water-soluble macrocycles via urea formation

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Abstract—A simple, efficient, and flexible procedure for the synthesis of chiral, amphiphilic, and water-soluble macrocycles is reported. Acylation of *p*-xylylenediamine with N^{α}-Fmoc-protected glycine, L-aspartic acid, L-glutamic acid, and L-arginine, followed by removal of Fmoc-groups, gave amino acid:*p*-xylylene conjugate diamines, which were converted to ten macrocycles via stepwise urea formation using *p*-nitrophenyl chloroformate. L-Aspartic acid-containing macrocyles proved to be soluble in aqueous buffers and a macrocycle containing four aspartate residues was found to recognize arginine and arginine esters with moderate affinity.

1. Introduction

Synthesis of macrocycles has been a rather successful approach towards artificial receptor molecules ever since the pioneering work on crown ethers and elegant and efficient macrocyclic host molecules are continuously being discovered.[1–8] A majority of macrocyclic host molecules rely on polar interactions (charge–charge, charge–dipole, hydrogen bonding etc.) for the recognition of guest molecules and most hosts consequently function in non-polar solvents. However, biologically active molecules (e.g. amino acids, peptides, carbohydrates, glycoconjugates etc.) constitute important targets for artificial receptor molecules and such receptors obviously have to be water-soluble, still providing structural motifs capable of forming molecular interactions in water. Typically, hydrophobic interactions are, in addition to hydrogen bonds, polar and charged interactions, important in a highly competitive aqueous solution[9] and re-organisation of high-energy water molecules in a protein binding site upon displacement by a ligand has been suggested to be a major driving force for a ligand:protein complex formation.[10] Thus, receptor molecules displaying efficacy in polar, i.e. aqueous, environment have to be amphiphilic[10] in order to provide for polar and hydrophobic interactions and for a beneficial re-organisation of water molecules surrounding the receptor molecules, as well as to ensure water-solubility.

A synthetic strategy towards water-soluble amphiphilic macrocycles as potential biomimetic receptors should preferably allow for easy structural diversification, i.e. combinatorial chemistry, and for the incorporation of chirality. We envisioned that acylation of diamines with amino acids would provide molecules amenable for macrocyclisation with bifunctional amine-reactive cross-linking reagents, as well as for incorporation of chirality and water-solubility through the amino acids. Furthermore, such a strategy allows for diversification by variation of both the diamine and amino acid structures.

We herein present a simple system following this strategy; synthesis of *p*-xylylenediamine- and amino acid-based chiral amphiphilic macrocycles obtained via urea formation with *p*-nitrophenyl chloroformate as a bifunctional amine-reactive reagent,[11] as well as their conformational analysis and recognition of basic amino acids. Synthesis of achiral,[12–19] as well as chiral,[20] macrocyclic ureas as host molecules has been reported.

2. Results and discussion

2.1. Synthesis

p-Xylylenediamine was chosen as the diamine component in our synthetic strategy, because it has reactive primary amino groups, it is commercially available, and the aromatic ring provides a site suitable for hydrophobic interactions, cation-π interaction etc. with putative ligands. The charged amino acids L-aspartic acid, L-glutamic acid, and L-arginine were selected as the amino acid components, because they are readily available, they confer water-solubility, and their side-chains are suited for polar (charged) interactions with putative ligands. Furthermore, the amide and urea moieties

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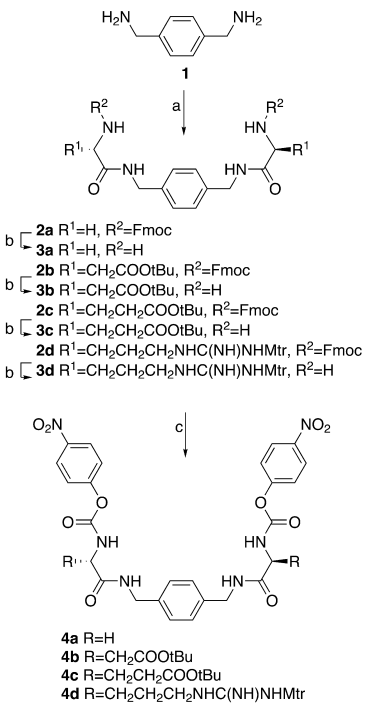
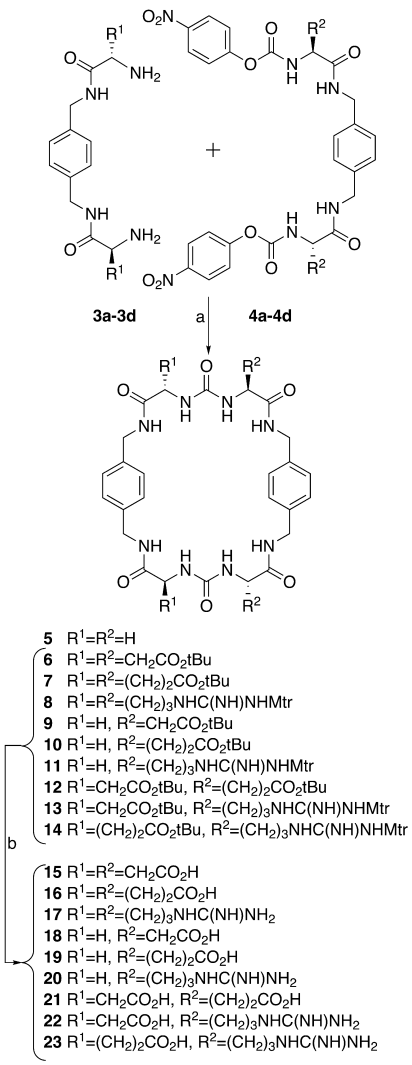
can, in addition to providing sites for hydrogen bonding, be expected to induce rigidity in the final macrocycles.

Diisopropylcarbodiimide (DIC) and 1-hydroxybenzotriazole (HOBt)-mediated acylation of *p*-xylylenediamine **1** with N^{α}-Fmoc-protected glycine, L-(O*t*Bu)aspartic acid, L-(O*t*Bu)glutamic acid, and L-(Mtr)arginine proceeded smoothly to give compounds **2a**–**d**, which were treated with piperidine to afford the free amines **3a**–**d** (Scheme 1). Compounds **3a**–**d** were activated with the bifunctional amine-reactive cross-linking reagent *p*-nitrophenyl chloroformate to give the unstable carbamates **4a**–**d** in 50–67% yields after column chromatography. The moderate yields were due to the instability of the carbamates **4a**–**d** on silica columns. Nevertheless, pure carbamates **4a**–**d** were obtained in acceptable yields and immediately used in macrocyclisation reactions with the diamines **3a**–**d** under high dilution conditions in DMF in the presence of 4-dimethylaminopyridine (DMAP), which resulted in precipitation of the pure macrocycles **5**–**14** in 28–77% (Scheme 2). The protected macrocycles **5**–**14** were all rather insoluble in most solvents, which complicated their analysis by FAB-HRMS. However, MALDI-TOF MS confirmed their cyclic structure and solubility in dimethylsulfoxide was sufficient to allow ^{1}H NMR spectra to be recorded. Fortunately, trifluoroacetic acid-mediated removal of *t*Bu and Mtr protecting groups from **6**–**14** to furnish **15**–**23**, conferred solubility of macrocycles **15**–**16**, **18**–**19**, and **21** in PBS buffer (pH 7.2). Compound **5** was insoluble in aqueous buffers, while L-arginine-based molecules **17**, **20**, **22**–**23** were soluble only under strongly acidic conditions, thus failing the essential criteria for biomimicry, i.e. water-solubility near neutral pH.



Scheme 1. (a) N^{α}-Fmoc amino acid, DIC, HOBt, THF (70–95%). (b) Piperidine, DMF (80–95%). (c) *p*-Nitrophenyl chloroformate, pyridine, DMF (50–67%).



Scheme 2. (a) Pyridine, DMAP, DMF (28–77%). (b) TFA, H_2O (71–88%).

2.2. Binding studies and conformational analysis

With the water-soluble amphiphilic macrocycles **15**–**16**, **18**–**19**, and **21** at hand, screening for recognition of a panel of commercially available *p*-nitrophenyl glycosides (α-D-gal, β-D-gal, α-D-glc, β-D-glc, α-D-man, β-D-man, α-L-fuc, β-L-fuc, β-lac), amino acids, and amino acid esters was discouraging. However, the addition of esters of L-arginine and L-lysine clearly induced small, but significant, chemical shift changes in the ^{1}H NMR spectra of **15** and, to a lesser extent, of **21**, while no ligand-induced chemical shifts were observed in the ^{1}H NMR spectra of the symmetrical glutamate-based macrocycle **16**, nor of the unsymmetrical glycine containing macrocycles **18** and **19**. The most notable effect on the ^{1}H NMR spectra of **15** and **21** was a shift of the β-protons of aspartic acid side-chains. The induced shift changes were very small, but nonetheless significant and reproducible. Small chemical shift changes were also observed for aromatic, benzylic, and α-protons. The presence of small shift changes suggested weak interactions. This was confirmed by subsequent titration experiments of the symmetrical aspartic acid-based macrocycle **15** and the unsymmetrical macrocycle **21** with

L-arginine and the methyl ester of L-arginine, which provided binding curves fitting well to a 1:1 binding isotherm, suggesting the formation of 1:1 complexes (Fig. 1). Dilution experiments showed that the macrocycles **15** and **21** did not self-aggregate under conditions used.

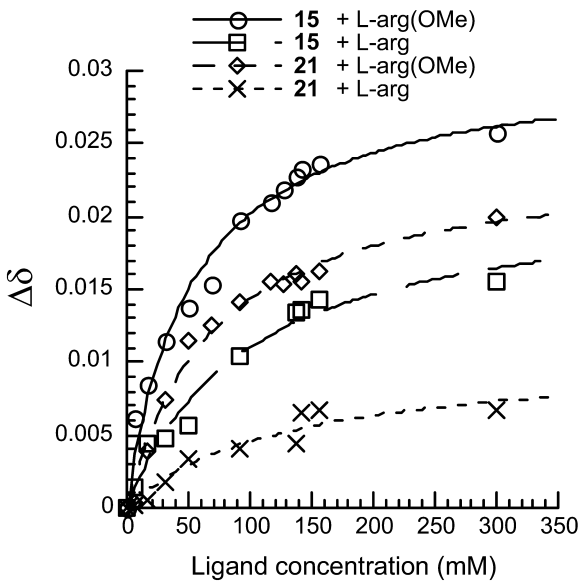
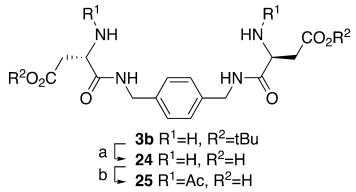


Figure 1. ^{1}H NMR-titration experiments of **15** and **21** with L-arginine and L-arginine methyl ester.

The K_a values of **15** for L-arginine methyl ester and L-arginine were calculated to be 19 and 10 M^{-1}, respectively. The unsymmetrical macrocycle **21**, carrying two aspartate and two glutamate side-chains, appeared a somewhat less efficient receptor for L-arginine methyl ester and L-arginine (K_a 15 and 8 M^{-1}, respectively). Compounds **15**, **16**, **18**, **19**, and **21** all present sites for cation recognition, i.e. carboxylates capable of forming salt-bridges and aromatic rings capable of forming cation-π interactions with ammonium and guanidinium cations. However, only the spectra of **15** and **21** were affected by the presence of L-arginine and the methyl ester of L-arginine, suggesting the involvement of salt-bridges with the side-chain carboxylates. Guanidinium ions are well-known to interact with carboxylates and receptors for guanidium ions, including arginine, typically rely on interaction with carboxylates[21] or sulfonates.[22,23] Furthermore, the receptor carrying four carboxylates close to the macrocycle core, the symmetrical aspartic acid-based macrocycle **15**, displayed the highest affinity for L-arginine and the methyl ester of L-arginine, clearly suggesting that the positions of the side-chain carboxylates are important.

The open structure **25** corresponding to 'half' **15**, prepared by ester hydrolysis of **3b** to give **24**, followed by N-acetylation (Scheme 3), was included in the experiments as a control compound. The flexible and less organised open-chain **25** did not show any ^{1}H NMR chemically induced shifts in the presence of L-arginine or the methyl ester of L-arginine (Fig. 2), further indicating the importance of the organisation of aspartate side-chain carboxylates on the rim of the less flexible macrocyclic core. The enhanced affinity for the methyl ester of L-arginine as compared to L-arginine can be explained by the fact that the receptors **15** and **21** carry four negative charges each, thus forming repulsive interactions with the carboxylate of L-arginine. The recognition of arginine esters was not enantioselective, because **15** showed identical K_a-values for the methyl esters of D-arginine and L-arginine.



Scheme 3. (a) TFA (80%). (b) Ac_2O, MeOH (75%).

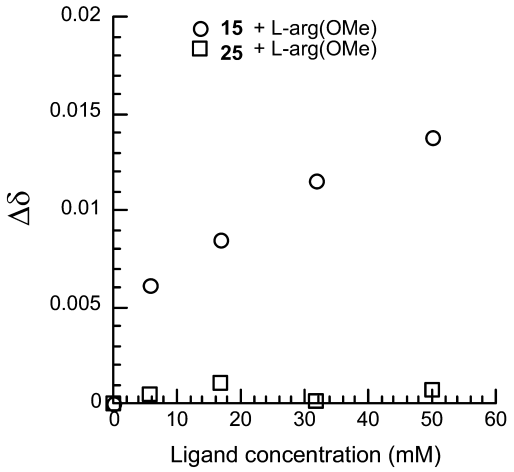


Figure 2. ^{1}H NMR-titration experiments of **15** and **25** with and L-arginine methyl ester.

The 14 lowest energy conformations obtained from Monte–Carlo search of **15** were within 10 kJ/mol, which suggests that **15** is relatively flexible. Analysis of the fourteen low energy conformations showed that they could be divided into two groups based on similarity. Furthermore, the two groups of conformers share the common features of a collapsed macrocycle with the two aromatic rings stacked in an edge-face fashion. These collapsed conformations suggest that the interactions with arginine and arginine methyl ester are of the 'nesting' type, which is also supported by the small shift changes observed upon complexation.[24] Two aspartic acid side-chain carboxylates separated by a urea bond and a xylylene unit are placed 10–12 Å apart on the rim of a shallow hydrophobic pocket made up by edge-face stacked xylylene moieties (Fig. 3). A distance of 10–12 Å between the carboxylates allow for salt-bridge formations involving both the ammonium and guanidinium functionalities of L-arginine methyl ester, while the methyl group could fit into the shallow hydrophobic pocket (Fig. 3). Finally, it cannot be ruled out that cation-π interactions are involved in the binding of L-arginine and L-arginine methyl ester by **15** and **21**, nor that the close proximity of one or both xylylene moieties to the one or more side-chain carboxylates is critical in the sense that this results in enhanced strength of salt-bridges. It has been demonstrated in other systems, and hypothesized to be important in protein-ligand interactions, that the positioning of a carboxylate close to an aromatic ring strengthens the

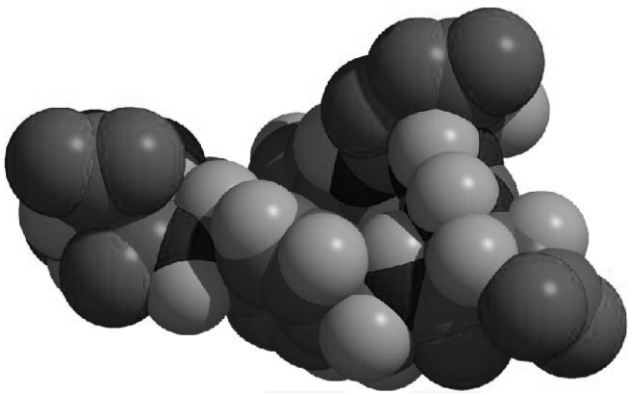


Figure 3. A representative collapsed low-energy minimum conformation of **15**.

salt-bridges it forms,[25] presumably due to a lower cost of desolvation of the carboxylate.

3. Conclusions

In conclusion, we have developed a short and high yielding synthetic procedure towards chiral, amphiphilic, and water-soluble macrocycles, based on the acylation of an aromatic diamine, *p*-xylylenediamine, with amino acids, followed by stepwise macrocyclic urea fomation with *p*-nitrophenyl chloroformate. Despite the short synthesis and structural simplicity, a symmetrical macrocycle **15**, containing four aspartic acid residues, and an unsymmetrical macrocycle **21**, containing two aspartic acid and two glutamic acid residues, were demonstrated to interact with arginine and arginine methyl ester. Although the interactions were weak, they are still noteworthy because of the synthetic and structural simplicity of the macrocycles and of the highly competitive media (PBS buffer, pH 7.2). Furthermore, the binding of L-arginine methyl ester was sensitive to macrocycle structure, because an open-chain analog (**25**) of **15** did not bind, nor did the symmetrical glutamate-based macrocycle **16** and the unsymmetrical glycine containing macrocycles **18** and **19**, suggesting that a salt-bridge with the ammonium and/or guanidinium groups alone is not responsible for the interaction. Instead, the relative positioning of the carboxylates close to the macrocycle core and the xylylene moieties seem to be of importance.

The synthesis is highly flexible in terms of structural modifications. Hence, further structural diversification via the aromatic diamine component and/or the amino acid components may very well lead to improved recognition of biomolecules in aqueous environment. In particular, modification of the diamine component allow for diversification of macrocycle size and of conformational flexibility.

4. Experimental

4.1. General

^{1}H NMR spectra were obtained on Bruker DRX 300, 400, and 500 instruments; chemical shifts are reported in ppm downfield from TMS using residual solvent peaks as internal standards ($CHCl_3$, 7.26 ppm; CHD_2OD, 3.31 ppm, d_5-DMSO, 2.50 ppm; HDO, 4.70 ppm) at 295 K. ^{1}H NMR spectral assignments were made by COSY experiments. PBS (0.15 M, pH 7.2) was prepared by dissolving NaCl (24 g), KCl (0.6 g), $Na_2HPO_4H_2O$ (3.46 g), and KH_2PO_4 (0.6 g) in water (3 L) and adjusting pH to 7.2. Deuterated PBS (*d*-PBS) was prepared by lyophilization of 100 mL PBS, followed by repeated lyophilization from D_2O, and finally desolvation of the deuterated salt residue in D_2O (100 mL). MALDI-TOF-MS spectra were recorded on a Bruker Biflex III instrument and FAB-HRMS on a JEOL MS-SX 102 instrument. Optical rotations were recorded on a Perkin–Elmer 341 polarimeter. Concentrations were made using rotary evaporation with a bath temperature at or below 40°C. TLC were performed on silica gel 60 F_{254} (Merck). Flash chromatography employed silica gel 60 Å (35–70 μm) with distilled solvents. Solid-phase extractions were performed on reversed phase SepPak C_{18} (Waters) and on ion exchange PRS (IST) cartridges. Monte Carlo simulations were performed with the MMFF/water force field implemented in MacroModel. 5000 Conformers were collected within 50 kJ/mol.

4.2. General procedure for the acylation of *p*-xylylene diamine with Fmoc-protected amino acids (→2a–d)

To a solution of 1-hydroxybenzotriazole (292 mg, 2.16 mmol) and Fmoc protected amino acid (2.21 mmol) in dry THF (20 mL), was added diisopropylcarbodiimide (294 mg, 2.16 mmol). The solution was stirred for 45 min. The *N*,*N*′-diisopropyl urea precipitate was removed by filtration and the filtrate was transferred to a solution of *p*-xylylenediamine (100 mg, 0.73 mmol) in THF at room temperature. After 2 h, the product precipitated (except **2b**, which precipitated after concentration) as a white solid, which was filtered off and washed with cold THF:CH_2Cl_2 mixture (60:40). The products were sufficiently pure (>95%) to be used directly in the next step.

4.2.1. Bis-*N*,*N*′-(Fmoc-glycyl)-*p*-xylylenediamine (2a). Fmoc-Gly-OH (0.62 g, 2.21 mmol) gave **2a** (0.484 g, 95%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.30 (t, 2H, J=5.8 Hz, $ArCH_2NH$), 7.86 (d, 4H, J=7.3 Hz, ArH), 7.69 (d, 4H, J=7.2 Hz, ArH), 7.53 (t, 2H, J=6.0 Hz, $COCH_2NH$), 7.39 (t, 4H, J=7.4 Hz, ArH), 7.30 (t, 4H, J=7.2 Hz, ArH), 7.16 (s, 4H, ArH), 4.21–4.26 (m, 10H, OCH_2, CCH, $ArCH_2N$), 3.62 (d, 4H, J=5.9 Hz, CH_2CO). FAB-HRMS *m*/*z* calcd for $C_{42}H_{39}N_4O_6$ (M+H): 695.2870, found: 695.2891.

4.2.2. Bis-*N*,*N*″-[Fmoc-L-aspartyl(O*t*Bu)]-*p*-xylylenediamine (2b). Fmoc-Asp(O*t*Bu)-OH (0.91 g, 2.21 mmol) gave **2b** (0.64 g, 93%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 7.77 (dd, 4H, J=3.0, 7.6 Hz, ArH), 7.57 (d, 4H, J=7.1 Hz, ArH), 7.40 (m,4H ArCH), 7.3 (m, 4H, ArH), 7.19 (s, 4H, ArH), 6.78 (bs, 2H, CH_2NH), 5.97 (d, 2H, J=7.9 Hz, CHN*H*), 4.55 (bs, 4H, COCH), 4.43 (m, 8H, NCH_2, OCH_2), 4.20 (t, 2H, J=6.7 Hz, CCH), 2.97 (d, 2H, J=13.7 Hz, $CHCH_2$), 2.62 (d, 2H, J=10.7 Hz, $CHCH_2$), 1.45 (s, 18H, CH_3). FAB-HRMS *m*/*z* calcd for $C_{54}H_{59}N_4O_{10}$ (M+H): 923.4231, found: 923.4218.

4.2.3. Bis-*N*,*N*′-[Fmoc-L-glutamyl(O*t*Bu)]-*p*-xylylenediamine (2c). Fmoc-Glu(O*t*Bu)-OH (0.92 g, 2.21 mmol) gave **2c** (0.60 g, 85%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.35 (t, 2H J=4.6 Hz, CH_2NH), 7.87 (d, 4H, J=7.4 Hz, ArH), 7.86 (d, 2H, J=7.4 Hz, CHN*H*), 7.70 (dd, 4H, J=3.7, 6.7 Hz, ArH), 7.50 (d, 2H, J=8.0 Hz, ArH), 7.44 (t, 4H, J=7.6 Hz, ArH), 7.30 (t, 4H, J=7.4 Hz, ArH), 7.15 (s, 4H, ArH), 4.20–4.28 (m, 10H, NCH_2, OCH_2, CCH), 4.00 (m, 2H, COCH), 2.20 (t, 4H, J=8.1 Hz, $COCH_2$), 1.87, 1.74 (2m, 4H, $CHCH_2$), 1.36 (s, 18H, CH_3). FAB-HRMS *m*/*z*, calcd for $C_{56}H_{63}O_{10}N_4$ (M+H): 951.4544, found: 951.4556.

4.2.4. Bis-*N*,*N*′-[Fmoc-L-arginyl(Mtr)]-*p*-xylylenediamine (2d). Fmoc-Arg(Mtr)-OH (1.31 g, 2.21 mmol) gave **2d** (0.67 g, 70%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 7.87 (d, 4H, J=7.5 Hz, ArH), 7.69 (d, 4H, J=7.4 Hz, ArH), 7.61 (d, 2H, J=8.0 Hz, CHN*H*), 7.39 (t, 4H, J=7.3 Hz, ArH), 7.30 (t, 4 h, J=7.5 Hz, ArH), 7.27 (s, 4H, ArH), 6.65 (bs, 2H, ArH), 4.2–4.26 (m, 10H, NCH_2, OCH_2, CCH), 3.87 (m, 2H COCH), 3.75 (s, 6H, CH_3), 3.00 (d, 4H, J=5.5 Hz, CH_2NH), 2.68 (s, 6H, CH_3), 2.60 (s, 6H, CH_3), 2.0 (s, 6H, CH_3). FAB-HRMS *m*/*z* calcd for $C_{70}H_{81}N_{10}O_{12}S_2$ (M+H): 1317.5477, found: 1317.5486.

4.3. General procedure for the removal Fmoc (2a–d→3a–d)

A solution of Fmoc protected amino acid derivative (**2a–d**) in piperidine/DMF (20% in 20 mL) was stirred for 2 h, then concentrated. The residue was dissolved in water and applied onto a SepPak C18 cartridge. The cartridge was washed with water, then eluted with MeOH/water (60:40). Concentration gave the pure compounds (**3a–d**).

4.3.1. Bis-*N*,*N*′-glycyl-*p*-xylylenediamine (3a). Compound **2a** (1.0 g, 1.44 mmol) gave **3a** (0.35 g, 95%). ^{1}H NMR (300 MHz, CD_3OD) δ 7.27 (s, 4H, ArH), 4.40 (bs, 4H, $ArCH_2$), (d, 4H, J=1.6 Hz, CH_2CO). MALDI-TOF-MS *m*/*z* calcd for $C_{12}H_{18}N_4NaO_2$ (M+Na): 273.1, found: 274.0. FAB-HRMS *m*/*z* calcd for $C_{12}H_{19}N_4O_2$ (M+H): 251.1508, found: 251.1459.

4.3.2. Bis-*N*,*N*′-[L-aspartyl(O*t*Bu)]-*p*-xylylenediamine (3b). Compound **2b** (0.50 g, 0.54 mmol) gave **3b** (0.24 g, 90%). $[\alpha]_D^{25}=-3.1°$ (*c* 1.5, MeOH). ^{1}H NMR (300 MHz, CD_3OD) δ 7.26 (s, 4H, ArH), 4.37 (d, 4H, J=4.4 Hz, $ArCH_2$), 3.64 (t, 2H, J=6.0 Hz, CHCO), 2.67 (dd, 2H, J=5.7, 16.3 Hz, CH_2CO), 2.55 (dd, 2H, J=6.8, 16.3 Hz, CH_2CO), 1.45 (s, 18H, CH_3). MALDI-TOF-MS *m*/*z* calcd for $C_{24}H_{38}N_4NaO_6$ (M+Na): 501.3, found: 501.7. FAB-HRMS *m*/*z* calcd for $C_{24}H_{39}N_4O_6$ (M+H): 479.2870, found: 479.2874.

4.3.3. Bis-*N*,*N*′-[L-glutamyl(O*t*Bu)]-*p*-xylylenediamine (3c). Compound **2c** (0.40 g, 0.42 mmol) gave **3c** (0.20 g, 92%). $[\alpha]_D^{25}=+8.3°$ (*c* 0.4, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ 7.32 (s, ArH, 4H), 4.42 (dd, 4H, J=7.4, 14.4 Hz, $ArCH_2$), 3.96 (t, 2H, J=6.2 Hz, COCH), 2.15 (m, 4H, CH_2CO), 1.71–1.81 (2m, 4H, $CHCH_2$), 1.47 (s, 18H, CH_3). FAB-HRMS *m*/*z* calcd for $C_{26}H_{43}N_4O_6$ (M+H): 507.3183, found: 507.3181.

4.3.4. Bis-*N*,*N*′-[L-arginyl(Mtr)]-*p*-xylylenediamine (3d). Compound **2d** (0.20 g, 0.22 mmol) gave **3d** (0.1 g, 80%). $[\alpha]_D^{25}=+4.1°$ (*c* 1.4, MeOH). ^{1}H NMR (400 MHz, CD_3OD) δ 7.25 (s, 4H, ArH), 6.65 (s, 2H, ArH), 4.37 (d, 2H, J=14.9 Hz, $ArCH_2$), 4.29 (d, 2H, J=14.9 Hz, $ArCH_2$), 3.3 (m, 2H, COCH), 3.14 (bs, CH_2NH), 2.66 (s, 6H, CH_3), 2.60 (s, 6H, CH_3), 2.11 (s, 6H, CH_3), 1.64 (m, 4H, $CHCH_2$), 1.54 (m, 4H, $CH_2CH_2CH_2$). FAB-HRMS *m*/*z* calcd for $C_{40}H_{61}N_{10}O_8S_2$ for (M+H): 873.4115, found: 873.4110.

4.4. General procedure for the synthesis of *p*-nitrophenyl carbamates (3a–d→4a–d)

p-Nitrophenyl chloroformate (6 equiv.) was added to **3a–d** and pyridine (6 equiv.) in dry DMF at 0°C, and the solution was stirred for 1 h. The solution was then brought to room temperature and allowed for stirring for further 10 h. Concentration of the reaction mixture and flash chromatography (SiO_2, CH_2Cl_2/MeOH 50:1) gave **4a–d**. The compounds were unstable and used immediately in the next step. Satisfactory FAB-HRMS nor elemental analysis data could be recorded due to the instability of the compounds.

4.4.1. Bis-*N*,*N*′-(*p*-nitrophenoxycarbonyl-glycyl)-*p*-xylylenediamine (4a). Compound **3a** (0.17 g, 0.68 mmol) gave **4a** (0.20 g, 50%). ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.55 (t, 2H, J=5.6 Hz, $ArCH_2NH$), 8.27 (d, 4H, J=9.0 Hz, ArH), 7.42 (d, 4H, J=9.0 Hz, ArH), 7.22 (s, 4H, ArH), 4.27 (d, 4H, J=5.5 Hz, $ArCH_2$), 3.75 (d, 4H, J=5.8 Hz, CH_2CO).

4.4.2. Bis-*N*,*N*′-[*p*-nitrophenoxycarbonyl-L-aspartyl (O*t*Bu)]-*p*-xylylenediamine (4b). Compound **3b** (0.10 g, 0.20 mmol) gave **4b** (0.11, g, 67%). ^{1}H NMR (400 MHz, CD_3OD) δ 8.65 (t, 2H, J=5.4 Hz, CH_2NH), 8.26 (d, 4H, J=9.2 Hz, ArH), 7.37 (d, 4H, J=9.2 Hz, ArH), 7.26 (s, 4H, ArH), 4.57 (m, 2H, CHCO), 4.40 (m, 4H, $ArCH_2$), 2.87 (dd, 2H, J=5.6, 16.2 Hz, CH_2CO), 2.68 (dd, 2H, J=8.1, 16.2 Hz, CH_2CO), 1.46 (s, 18H, CH_3).

4.4.3. Bis-*N*,*N*′-[*p*-nitrophenoxycarbonyl-L-glutamyl (O*t*Bu)]-*p*-xylylenediamine (4c). Compound **3c** (0.10 g, 0.197 mmol) gave **4c** (90 mg, 55%). ^{1}H NMR (300 MHz, CD_3OD) δ 8.63 (t, 2H, J=5.0 Hz, CH_2NH), 8.25 (d, 4H, J=8.4 Hz, ArH), 7.40 (d, 4H, J=9.0 Hz, ArH), 7.26 (s, 4H, ArH), 4.40 (d, 4H, J=5.4 Hz, $ArCH_2$), 4.20 (dd, 2H, J=5.2, 9.0 Hz, COCH), 2.40 (t, 4H, J=7.4 Hz, $CHCH_2$), 1.97–2.15 (2m, 4H, CH_2CO), 1.46 (s, 18H, CH_3).

4.4.4. Bis-*N*,*N*′-[*p*-nitrophenoxycarbonyl-L-arginyl(Mtr)]-*p*-xylylenediamine (4d). Compound **3d** (75 mg, 86 μmol) gave **4d** (52 mg, 51%). ^{1}H NMR (300 MHz, d_6-DMSO) δ 8.15 (d, 4H, J=9.2 Hz, ArH), 7.30 (d, 4H, J=9.2 Hz, ArH), 7.20 (s, 4H, ArH), 6.60 (s, 2H, ArH), 4.32 (d, 4H, J=6.4 Hz, $ArCH_2$), 4.12 (dd, 2H, J=5.5, 8.9 Hz, COCH), 3.75 (s, 6H, OCH_3), 3.20 (bs, 4H, CH_2NH), 2.63 (s, 6H CH_3), 2.55 (s, 6H, CH_3), 2.05 (s, 6H, CH_3), 1.55–1.75 (2m, 8H, $CHCH_2CH_2$).

4.5. General procedure for macrocyclization (3+4→5–14)

A solution of **4** (20–210 μmol) in DMF (20–70 mL) was

added to a vigorously stirred solution of **3** (20–210 μmol, 1 equiv.), pyridine (2–16 μl), and DMAP (0.5–2 mg) in DMF (20–40 mL). After 12 h, the white precipitate formed was isolated by centrifugation (400 rpm, 5 min), washed with DMF and MeOH and centrifuged repeatedly to give **5**–**14**.

4.5.1. Macrocycle 5. Compounds **3a** (27.5 mg, 110 μmol) and **4a** (63.8 mg, 110 μmol) gave **5** (23 mg, 38%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.49 and 8.42 (2 s, 2H each, $ArCH_2NH$), 7.29 (s, 8H, ArH), 6.60 (bs, 4H, $COCH_2NH$), 4.35 (bs, 8H, $ArCH_2$), 3.77 (d, 8H, J=15.8 Hz, CH_2CO). MALDI-TOF-MS m/z calcd for $C_{26}H_{32}N_8NaO_6$ (M+Na): 575.2, found: 575.5.

4.5.2. Macrocycle 6. Compounds **3b** (53 mg, 111 μmol) and **4b** (90 mg, 111 μmol) gave **6** (63 mg, 56%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.15 (bs, 4H, $ArCH_2NH$), 7.12 (bs, 8H, ArH), 6.54 (bs, 4H, CHNH), 4.24 (bs, 8H, $ArCH_2$), 4.10 (bs, 4H, J=5.9 Hz, COCH), 1.35 (s, 36H, CH_3). MALDI-TOF-MS m/z calcd for $C_{50}H_{73}N_8O_{14}$ (M+H): 1009.5, found: 1009.4.

4.5.3. Macrocycle 7. Compounds **3c** (60 mg, 120 μmol) and **4c** (100 mg, 120 μmol) gave **7** (50 mg, 40%). ^{1}H NMR (400 MHz, d_6-DMSO), δ 8.12 (bs, 4H, $ArCH_2NH$), 7.17–7.19 (2 s, 8H, ArH), 6.26 (t, 4H, J=7.1 Hz, CHNH), 4.24 (d, 8H, J=5.3 Hz, $ArCH_2$), 4.15 (t, 4H, J=5.9 Hz, COCH), 2.27 (t, 4H, J=7.6 Hz, CH_2CO), 2.19 (t, 4H, J=7.2 Hz, CH_2CO), 1.92–1.72 (2m, 8H, $CHCH_2$), 1.37–1.40 (2 s, 18H, CH_3). MALDI-TOF-HRMS m/z calcd for $C_{54}H_{80}N_8NaO_{14}$ (M+Na): 1087.6, found: 1086.3.

4.5.4. Macrocycle 8. Compounds **3d** (72 mg, 82 μmol) and **4d** (100 mg, 83 μmol) gave **8** (108 mg, 72%). ^{1}H NMR (400 MHz, d_6-DMSO), δ 8.25 (bs, 4H $ArCH_2NH$), 7.2 (s, 8H, ArH), 6.65 (s, 4H, ArH), 6.16 (d, 4H, J=8.7 Hz, CHNH), 4.45 (m, 4H, $ArCH_2$), 3.93 (m, 4H, $ArCH_2$), 3.85 (bs, 4H, COCH), 3.75 (s, 12H, OCH_3), 3.0 (s, 8H, CH_2NH), 2.67 (s, 12H, CH_3), 2.00 (s, 12H, CH_3), 1.54 (bs, 8H, $CHCH_2$), 1.40 (bs, 8H, $CH_2CH_2CH_2$). MALDI-TOF-MS m/z calcd for $C_{82}H_{117}N_{20}O_{18}S_4$ (M+H): 1797.8, found: 1798.2.

4.5.5. Macrocycle 9. Compounds **3b** (100 mg, 209 μmol) and **4a** (121 mg, 209 μmol) gave **9** (46 mg, 28%). ^{1}H NMR (400 MHz, d_6-DMSO) 8.32 (bs, 4H, $ArCH_2NH$), 7.14 (s, 4H, ArH), 7.12 (s, 4H, ArH), 6.52 (dd, 2H, J=8.2, 14.4 Hz, $NHCOCH$), 6.43 (t, 2H, J=7.0 Hz, $NHCOCH_2$), 4.41 (dd, 2H, J=8.2, 14.4 Hz, CH), 4.16–4.32 (m, 8H, $ArCH_2$), 3.78 (dd, 2H, J=6.9, 16.7 Hz, $COCH_2NH$), 3.44 (dd, 2H, J=2.5, 16.7 Hz, $COCH_2NH$), 2.59–2.63 (m, 4H, $CHCH_2$), 1.36 (s, 18H, CH_3). MALDI-TOF-MS m/z calcd for $C_{30}H_{37}N_8O_{10}$ (M−2Bu+H): 669.3, found: 669.8.

4.5.6. Macrocycle 10. Compounds **4a** (24 mg, 47 μmol) and **3c** (27 mg, 47 μmol) gave **10** (29 mg, 76%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.33, 8.25 (2m, 4H, $ArCH_2NH$), 7.12, 7.13 (2 s, 8H, ArH), 6.43 (d, 2H, J=7.6 Hz, CHNHCO), 6.24 (t, 2H, J=7.1 Hz, CH_2NHCO), 4.40, 3.90 (2m, 8H, $ArCH_2$), 4.30, 4.10 (2m, 4H, COCH), 3.79, 3.4 (2m, 4H, $COCH_2N$), 2.24 (m, 4H, CH_2CH_2CO), 1.89–1.67 (2m, 4H, $CHCH_2$), 1.37 (s, 18H, CH_3). MALDI-TOF-MS m/z calcd for $C_{32}H_{41}N_8O_{10}$ (M−2Bu+H): 697.3, found: 696.7.

4.5.7. Macrocycle 11. Compounds **3d** (20 mg, 34 μmol) and **4a** (30 mg, 34 μmol) gave **11** (30 mg, 75%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.40 (bs, 2H, $ArCH_2NH$), 8.30 (bs, 2H, $ArCH_2NH$), 7.93 (s, 8H, ArH), 6.65 (bs, 2H, ArH), 6.38 (d, 2H, J=7.6 Hz, CHNH), 6.26 (d, 2H, J=5.3 Hz, CH_2NH), 4.50, 3.97 (2m, 4H, $ArCH_2$), 4.22, 4.05 (2m, 4H, $ArCH_2$), 4.10 (m, 2H, COCH), 3.93, 3.60 (2m, 4H, CH_2NH), 3.00 (s, 4H, CH_2NH), 2.65 (s, 6H, CH_3), 2.55 (s, 6H, CH_3), 2.02 (s, 6H, CH_3), 1.55 (s, 4H, $CHCH_2$), 1.36 (s, 4H, $CH_2CH_2CH_2$). MALDI-TOF-MS m/z calcd for $C_{54}H_{75}O_{12}N_{14}S_2$ (M+H): 1175.5, found: 1176.0.

4.5.8. Macrocycle 12. Compounds **3b** (57 mg, 119 μmol) and **4c** (100 mg, 119 μmol) gave **12** (35 mg, 29%). Alternatively, compounds **3c** (32 mg, 62 μmol) and **4b** (50 mg, 62 μmol) gave **12** (27 mg, 42%). ^{1}H NMR (400 MHz, d_6-DMSO), δ 8.25 (t, 2H, J=6.6 Hz, $ArCH_2NH$), 8.17 (t, 2H, J=7.9 Hz, $ArCH_2NH$), 7.17 (s, 4H, ArH), 7.13 (s, 4H, ArH), 6.51 (d, 2H, J=7.1 Hz, CHNH), 6.30 (d, 2H, J=8.1 Hz, CHNH), 4.44, 3.99 (2m, 8H, $ArCH_2$), 4.30–4.10 (2m, 4H, COCH), 2.60 (m, 4H, $CHCH_2CO$), 2.30 (m, 4H, CH_2CH_2CO), 1.70–1.68 (m, 4H, CH_2CH_2CO), 1.40, 1.38 (2 s, 36H, CH_3). MALDI-TOF-MS m/z calcd for $C_{52}H_{76}N_8O_{14}Na$ (M+Na): 1059.5, found: 1060.2.

4.5.9. Macrocycle 13. Compounds **3d** (50 mg, 57 μmol) and **4b** (46 mg, 57 μmol) gave **13** (60 mg, 75%). ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.26 (bs, 2H, $ArCH_2NH$), 8.14 (bs, 2H, $ArCH_2NH$), 7.17 (s, 4H, ArH), 7.11 (s, 4H, ArH), 6.65 (s, 2H, ArH), 6.42 (d, 2H, J=7.1 Hz, CHNH), 6.28 (d, 2H, J=8.8 Hz, CHNH), 4.45, 3.94 (2m, 4H $ArCH_2$), 4.35–4.10 (2m, 4H, $ArCH_2$), 4.20 (m, 2H, COCH), 3.92 (m, 2H, COCH), 3.75 (s, 6H, OCH_3), 3.00 (s, 4H, CH_2NH), 2.65 (s, 6H, CH_3), 2.20 (s, 6H, CH_3), 2.00 (8 s, 6H, CH_3), 1.55 (bs, 4H, $CHCH_2$), 1.40 (bs, 4H, $CH_2CH_2CH_2$), 1.37 (s, 18H, CH_3). MALDI-TOF-MS m/z calcd for $C_{66}H_{94}O_{16}N_{14}S_2$ (M+H): 1403.6, found: 1402.9 and m/z calcd for $C_{66}H_{94}O_{16}N_{14}S_2Na$ (M+Na): 1425.6, found: 1424.8.

4.5.10. Macrocycle 14. Compounds **3c** (10 mg, 20 μmol), and **4d** (25 mg, 20 μmol) gave **14** (16 mg, 56%) ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.38 (bs, 2H, $ArCH_2NH$), 8.25 (bs, 2H, $ArCH_2NH$), 7.16 (s, 4H, ArH), 7.14 (s, 4H, ArH), 6.65 (s, 2H, ArH), 6.20 (d, 2H, J=7.9 Hz, CHNH), 6.07 (d, 2H, J=6.8 Hz, CHNH), 4.46, 4.00 (2m, 4H, $ArCH_2$), 4.21, 4.05 (2m, 4H, $ArCH_2$), 4.10 (m, 2H, COCH), 3.95, (m, 2H, COCH), 3.75 (s, 6H, OCH_3), 3.00 (s, 4H, CH_2NH), 2.65 (s, 6H, CH_3), 2.55 (s, 6H, CH_3), 2.40 (m, 4H, CH_2CH_2), 2.00 (s, 6H, CH_3), 1.85, 1.70 (2brs, 4H, CH_2CH_2), 1.65 (s, 4H, $CHCH_2$), 1.54 (s, 4H, $CH_2CH_2CH_2$), 1.38 (s, 18H, CH_3). MALDI-TOF-MS m/z calcd for $C_{68}H_{98}O_{16}N_{14}S_2$ (M+H): 1431.7, found 1431.3, and m/z calcd for $C_{68}H_{98}O_{16}N_{14}S_2Na$ (M+Na): 1453.7, found: 1453.3.

4.6. General procedure for deprotection of compounds 6–7, 9, 10, and 12 (→15–16, 18–19, and 21)

Compounds **6**–**7**, **9**–**10**, and **12** were stirred with TFA (5 mL) at rt for 12 h. The solutions were concentrated and

co-concentrated with toluene. The resulting TFA salts were dissolved in PBS (pH 7.2), followed by precipitation upon acidification to pH 1, filtration and thorough washing with water followed by methanol. The residues were dissolved in 0.1 M HCl (2 mL) applied onto an ion exchange cartridge. Washing with water (2×5 mL), methanol (2×5 mL) and water (2×2 mL), elution with 4 M HCl, concentration, and co-concentration with methanol afforded pure macrocycles **15**–**16**, **18**–**19**, and **21**. The macrocycles did not provide satisfactory FAB-HRMS or analysis results, presumably due to their low solubility and/or hygroscopic nature. NMR and MALDI-TOF MS-analysis gave satisfactory results in all cases.

4.7. General procedure for deprotection of compounds 8, 11, 13–14 (→17, 20, and 22–23)

Compounds **8**, **11**, and **13**–**14** were stirred with TFA (5 mL) at rt for 12 h. The solutions were concentrated and co-concentrated with toluene. The resulting TFA salts were dissolved in 0.1 M HCl (2 mL) applied onto an ion exchange cartridge. Washing with water (2×5 mL), methanol (2×5 mL) and water (2×2 mL), elution with 4 M HCl, concentration, and co-concentration with methanol afforded pure macrocycles **17**, **20**, and **22**–**23**. The macrocycles did not provide satisfactory FAB-HRMS or analysis results, presumably due to their low solubility and/or hygroscopic nature. NMR and MALDI-TOF MS-analysis gave satisfactory results in all cases.

4.7.1. Macrocycle 15. Compound **6** (20 mg, 19 μmol) gave **15** (12 mg, 78%). ^{1}H NMR (400 MHz, *d*-PBS, pD 7.2) δ 7.17 (s, 8H, ArH), 4.27 (ABq, 8H, J=15.9, 22.2 Hz, ArCH_2), 4.24 (dd, 4H, J=4.6, 8.3 Hz, CHCH$_2$), 2.56 (dd, 4H, J=4.6, 16.0 Hz, CHCH_2), 2.47 (dd, 4H, J=8.3, 16.0 Hz, CHCH_2). MALDI-TOF-MS m/z calcd for $C_{34}H_{41}N_8O_{14}$ (M+H): 785.3, found: 784.7.

4.7.2. Macrocycle 16. Compound **7** (8.0 mg, 7.5 μmol) gave **16** (4.0 mg, 78%). ^{1}H NMR (400 MHz, *d*-PBS, pD 7.2) δ 7.15 (s, 8H, ArH), 4.25 (m, 8H, ArCH_2), 3.95 (m, 4H, CHCO), 2.14 (t, 8H, J=8.5 Hz, CH_2COOH), 1.97 and 1.80 (2m, 4H each, CHCH_2). MALDI-TOF-MS calcd for $C_{38}H_{49}O_{14}N_8$ (M+H): 841.3, found: 841.8.

4.7.3. Macrocycle 17. Compound **8** (20 mg, 11 μmol) gave **17** (10 mg, 80%). ^{1}H NMR (400 MHz, *d*-PBS, pD 7.2) δ 7.12 (s, 8H, ArH), 4.22 (bs, 8H, ArCH_2), 3.90 (dd, 4H, J=5.0, 8.9 Hz, CHCO), 3.02 (t, 8H, J=7.0 Hz, CH_2NH), 1.60 (m, 8H, CHCH_2), 1.50 (m, 8H, CHCH_2CH$_2$). MALDI-TOF-MS m/z calcd for $C_{42}H_{69}O_6N_{20}$ (M+H): 949.6, found: 949.9.

4.7.4. Macrocycle 18. Compound **9** (25 mg, 32 μmol) gave **18** (17 mg, 80%). ^{1}H NMR (400 MHz, *d*-PBS, pD 7.2) δ 7.05 (s, 4H, ArH), 7.01 (s, 4H, ArH), 4.35 (dd, 2H, J=4.6, 8.6 Hz, CHCH$_2$), 4.27 (d, 2H, J=15.7 Hz, ArCH_2), 4.17 (d, 2H, J=15.5 Hz, ArCH_2), 4.15 (d, 2H, J=15.7 Hz, ArCH_2), 4.10 (d, 2H, J=15.5 Hz, ArCH_2), 3.90 (d, 2H, J=17.5 Hz, CH$_2$CON), 3.62 (d, 2H, J=17.5 Hz, CH$_2$CON), 2.58 (dd, 2H, J=4.6, 15.7 Hz, CHCH_2), 2.46 (dd, 2H, J=8.6, 15.7 Hz, CHCH_2). MALDI-TOF-MS m/z calcd for $C_{30}H_{37}N_8O_{10}$ (M+H): 669.3, found: 669.6. FAB-HRMS m/z calcd for $C_{30}H_{37}N_8O_{10}$ (M+H): 669.2633, found: 669.2647.

4.7.5. Macrocycle 19. Compound **10** (8.0 mg, 1.0 μmol) gave **19** (4.0 mg, 88%). ^{1}H NMR (400 MHz, *d*-PBS, pD 7.2) δ 7.05 (s, 4H, ArH), 7.00 (s, 4H, ArH), 4.31 (d, 2H, J=19.9 Hz, ArCH_2), 4.17 (ABq, 4H, J=15.0 Hz, ArCH_2), 4.06 (d, 2H, J=19.9 Hz, ArCH_2), 4.03 (dd, 2H, J=5.0, 8.4 Hz, CHCO), 3.91 (d, 2H, J=17.2 Hz, CH$_2$CO), 3.62 (d, 2H, J=17.2 Hz, CH$_2$CO), 2.19 (bt, 4H, J=7.4 Hz, CH_2-COOH), 1.95 and 1.82 (2m, 2H each, CHCH_2). MALDI-TOF-MS m/z calcd for $C_{32}H_{40}O_{10}NaN_8$ (M+Na): 719.3, found: 719.0.

4.7.6. Macrocycle 20. Compound **11** (15 mg, 12 μmol) gave **11** (7.0 mg, 80%). ^{1}H NMR (500 MHz, *d*-PBS, pD 7.2) δ 7.10 (s, 4H, ArH), 7.05 (s, 4H, ArH), 4.23 (ABq, 4H, J=12.2 Hz, ArCH_2), 4.13 (ABq, 4H, J=11.6 Hz, ArCH_2), 4.05 (dd, 2H, J=4.6, 6.3 Hz, COCH), 3.90 (d, 2H, J=13.8 Hz, CH$_2$CO), 3.61 (d, 2H, J=13.8 Hz, CH$_2$CO), 3.10 (t, 4H, J=4.5 Hz, CH$_2$CH_2NH), 1.75 (m, 2H, CHCH_2-CH$_2$), 1.60 (m, 6H, CHCH_2CH$_2$, CHCH$_2$CH_2). MALDI-TOF-MS m/z calcd for $C_{34}H_{51}O_6N_{14}$ (M+H): 751.4; found: 751.4.

4.7.7. Macrocycle 21. Compound **12** (24 mg, 29 μmol) gave **21** (15 mg, 81%). ^{1}H NMR (400 MHz, *d*-PBS, pD 7.2) δ 7.17 (s, 4H, ArH), 7.14 (s, 4H, ArH), 4.25 (m, 8H, ArCH_2), 4.21 (m, 2H, COCH), 3.94 (dd, 2H, J=8.6, 5.0 Hz, COCH), 2.55 (dd, 2H, J=16.1, 5.4, Hz, CHCH_2CO), 2.47 (dd, 2H, J=8.12, 15.62 Hz, CHCH_2CO), 2.13 (t, 4H, J=7.58, Hz, CH$_2$CH_2CO), 1.94, 1.80 (2m, 4H, CHCH_2-CH$_2$). MALDI-TOF-MS m/z calcd for $C_{36}H_{45}O_{14}N_8$ (M+H): 813.3, found: 815.2.

4.7.8. Macrocycle 22. Compound **13** (30 mg, 21.3 μmol), gave **22** (15 mg, 80%). ^{1}H NMR (500 MHz, *d*-PBS, pD 7.2) δ 7.20, (2 s, 4H each, ArH), 7.19 (s, 4H, ArH), 4.25 (m, 8H, ArCH_2), 4.18 (m, 2H, CHCO), 4.00 (m, 2H, CHCO), 3.00 (m, 4H, CH_2NH), 2.58 (dd, 2H, J=9.17, 16.25 Hz, CHCH_2), 2.48 (dd, 2H, J=6.52, 16 Hz, CHCH_2), 1.75 (m, 4H, CHCH_2CH$_2$) 1.55 (m, 4H, CHCH$_2$CH_2). MALDI-TOF-MS m/z calcd for $C_{38}H_{55}O_{10}N_{14}$ (M+H): 867.4, found: 867.4.

4.7.9. Macrocycle 23. Compound **14** (12 mg, 8.0 μmol), gave **23** (5.0 mg, 71%). ^{1}H NMR (400 MHz, *d*-PBS, pD 7.2) δ 7.17 (bs, 8H, ArH), 4.33–4.20 (m, 8H, ArCH_2), 4.10–3.90 (m, 4H, COCH), 3.10 (s, 4H, CH_2NH), 2.40 (bs, 4H, CH$_2$CO), 2.00–1.50 (m, 12H, CHCH$_2$CH$_2$). MALDI-TOF-MS m/z calcd for $C_{40}H_{59}O_{10}N_{14}$ (M+H): 895.5, found: 895.0.

4.7.10. Bis-L-aspartyl-*p*-xylyldiamide 24. Compound **3b** (130 mg, 0.27 mmol) was stirred with TFA (2.5 mL) at rt for 6 h. The solution was concentrated and TFA was removed by co-evaporation with toluene. The residue was applied onto a C18 cartridge and elution with water methanol (2:1) afforded **24** (80 mg, 81%). $[\alpha]_D^{25}=-24°$ (c 0.0026, MeOH). ^{1}H NMR (400 MHz, D_2O) δ 7.19 (s, 4H, ArH), 4.30 (d, J=15.3 Hz, ArCH_2), 4.26 (d, 2H, J=15.1 Hz, ArCH_2), 4.14 (t, 2H, J=5.5 Hz, CHCO), 2.72 (dd, 2H, J=17.2, 4.7 Hz, CHCH_2), 2.62 (dd, 2H, J=18.9, 9.1 Hz, CHCH_2).

FAB-HRMS *m/z* calcd for $C_{16}H_{22}N_4NaO_6$ (M+Na): 389.1437, found: 389.1462.

4.7.11. Bis-L-(*N*-acetyl)-aspartyl-*p*-xylyldiamide 25. Compound **24** (4.0 mg, 11 μmol) was stirred with methanol (0.6 mL) and Ac_2O (1.5 mL) for 12 h, then concentrated. The residue was dissolved in water and applied onto a C18 cartridge. Elution (water/methanol 2:1) afforded **25** (3.8 mg, 77%). $[\alpha]_D^{25}=-9.0°$ (*c* 0.0013, MeOH). ^{1}H NMR (400 MHz, D_2O) δ 7.30 (s, 4H, ArH), 4.57 (s, $ArCH_2$), 4.4 (m, 2H, CHCO), 2.55 (dd, 4H, *J*=14.9, 7.4 Hz, $CHCH_2$). FAB-HRMS *m/z* calcd for $C_{20}H_{26}N_4NaO_8$ (M+Na): 473.1648, found: 473.1631.

4.8. NMR-titrations

NMR titration experiments[26] were performed at 298K by recording the ^{1}H NMR spectra of a macrocyclic compound (2 mM) in the presence of varying concentration of a ligand in *d*-PBS. The change of proton chemical shifts were plotted against the ligand concentration and the data points were fitted to a standard 1:1 binding isoterm using the program Kaleidagraph (Abelbeck Software Inc.), which provided the association constants.

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