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Cyclic peptides containing a δ-sugar amino acid—synthesis and evaluation as artificial receptors

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Abstract—An Fmoc-protected δ-sugar amino acid, prepared by oxidation of a glucosamine derivative, was coupled to three different tripeptide tert-butyl esters (H-Tyr-Tyr-Tyr-OtBu, H-Tyr-Glu(Obzl)-Tyr-OtBu and H-Tyr-Arg(Mtr)-Tyr-OtBu) and the resulting sugar amino acid/amino acid hybrids were transformed into dimers that were subsequently cyclized to give three C2-symmetric macrocycles. The macrocycles were deprotected and their binding properties towards p-nitrophenyl glycosides, nucleotides, and purines were examined. Of the ligands screened, only some of the purines showed weak, but significant, binding.

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1. Introduction

Interactions of small ligands, such as carbohydrates, metabolites or hormones, with binding sites in proteins are vital to life processes and the synthesis of artificial receptors that mimic such interactions has been an ongoing goal in many research groups for a long time.1 A basic design for biomimetic artificial receptors involves amphiphilic molecules, often macrocycles, with both polar and non-polar regions, thus enabling interactions with both polar and non-polar regions of a ligand.

Sugar amino acids2–5 are carbohydrates that contain at least one amino and one carboxylic acid functionality, which allows for the use of peptide coupling chemistry in order to combine them with amino acids or other building blocks. Sugar amino acids have been used to prepare cyclic homooligomers6–8 and cyclic sugar amino acid/amino acid hybrids,9–15 that have been used in various studies. It has been proposed that such molecules could be interesting artificial receptors, and in one case it has been shown that a cyclodextrin-like cyclic hexamer could bind to benzoic acid and p-nitrophenol in water, although no binding constants were given.6 We decided to explore the use of sugar amino acids as polar structural elements combined with non-polar aromatic amino acids for the construction of amphiphilic molecules as biomimetic receptors.

Keywords: Sugar amino acids; Macrocycles; Cyclic peptides; Artificial receptors; Molecular recognition.

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Herein, we report the synthesis of polyamphiphilic watersoluble macrocyclic sugar amino acid/amino acid hybrid molecules 1a–c (see Fig. 1) and an investigation of their binding properties against biomolecules. We chose to use the δ-sugar amino acid obtained by oxidation of a partially protected methyl β-glycoside of glucosamine together with the aromatic amino acid tyrosine as building blocks for our macrocycles. The δ-sugar amino acid was chosen because of its extended geometry, which prevents turn formation and presumably thus gives rise to more accessible cores of the macrocycles. In addition, we introduced amino acids with charged side chains to enhance solubility and potentially

1a: R = tyrosine side chain
1b: R = CH3CH2COOH
1c: R = CH3CH2CH2NHC(NH)NH2

Figure 1. Synthesized macrocycles.
also binding. Two monosaccharides and six amino acids were used in each macrocyclic ring in order to obtain macrocycles large enough to form a central pocket where ligands might bind.

2. Results and discussion

2.1. Synthesis

The synthetic strategy towards the macrocycles involved two building blocks for each macrocycle, a C-protected tripeptide and an amino sugar precursor, which upon oxidation gives the N-protected sugar amino acid (SAA). The tripeptide and the sugar amino acid were coupled together to give a linear sugar amino acid/amino acid hybrid, which was then transformed into a dimer that was subsequently cyclized to give the desired macrocyclic product, while acidic transesterfication cleanly produced the required C-protected tripeptide. Peptide couplings were prepared in solution in good yields (Scheme 2) to serve as building blocks for each macrocycle, a C-protected tripeptide and an amino sugar precursor, which upon oxidation gives the N-protected sugar amino acid (SAA). 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gave the macrocycle 19a as a major product according to MALDI-TOF analysis of the reaction mixtures.

The HATU and HAPyU reagents have been shown to give less epimerization than TBTU in the cyclization of pentapeptides and these reagents were thus investigated further. Furthermore, it has been shown that DIPEA gives less epimerization than 2,4,6-collidine for the cyclization of pentapeptides by HAPyU, while for segment condensations the opposite is true. Hence, both DIPEA and 2,4,6-collidine were evaluated as bases. In the cyclization of 18a to 19a, DIPEA gave a higher yield and less epimerization.

Compounds 18b–c could also be cyclized to 19b–c using this method (see Table 1). In the cyclization of 18b to 19b, HAPyU gave a better yield than HATU.

The 1H NMR spectra of the protected macrocycles only gave poorly resolved spectra with broad peaks at room temperature, presumably due to slow conformational exchange. Resolved spectra of 19a and 19b could be obtained at 150 and 120 °C, respectively, but compound 19c only gave poorly resolved spectra even at these temperatures.

Macrocycle 19a was deprotected using 10 mM NaOMe in
MeOH to afford 1a in 61% yield. The deprotection of macrocycle 19b started with the cleavage of the benzyl esters using catalytic transfer hydrogenation with palladium black and formic acid as the hydrogen source, followed by treatment of the crude product with NaOMe/MeOH to give 1b in 81% yield. In the case of the macrocycle 19c, the Mtr groups were first cleaved using neat TFA with thioanisole as a scavenger29 and the crude product was then treated with NaOMe/MeOH to afford 1c in 59% yield after HPLC purification.

2.2. Conformational analysis

The deprotected macrocycles 1a–c all gave well resolved NMR spectra at room temperature. Macrocycle 1a in MeOH-d4 and macrocycles 1b–c in D2O all gave the expected 1H NMR spectra for symmetrical compounds. In addition to the major peaks, macrocycle 1c in also gave smaller peaks at 2.81 and 2.56 ppm, as well as some overlapping smaller peaks at 3.74 ppm and in the aromatic region. Heating of 1c in DMSO-d6 brought the 1H NMR signals to coalescence, which shows that the multiple peaks of 1c at ambient temperature were due to slow conformational exchange (Fig. 2).

Monte–Carlo conformational searches were performed on 1a–c using MacroModel 8.5 (MMFFs force field with water as solvent, 20,000 steps, all backbone torsions were selected for random variation) to give for each macrocycle 110–240 conformers within 5 kcal/mol of the global minimum. When these conformers were studied, a coherent picture emerged. The dominant low-energy conformers for macrocycles 1a–c were twisted, oblong structures with extended sugar amino acids and turns formed by the tripeptides (Fig. 3). In the conformers with lowest energy, the axial hydrogen atoms in the two sugar amino acids were facing each other, but conformers where one of the sugar amino acids had rotated to place the hydroxyl groups in position to form hydrogen bonds to the tripeptide, or to the other sugar amino acid, were also found. Hydrogen bonds were occasionally found within the tripeptides, but no pattern could be discerned. The 3JHH coupling constants for all three macrocycles indicate that the sugar amino acids are in the 4C1 conformation. This was the case for the calculated conformers of 1a–b, but many conformers of macrocycle 1c deviated from the expected 4C1 conformation of the pyranose rings. As there is no support for this in the coupling constants, we conclude that it is an artefact in the calculations possibly induced by the strong hydrogen bond formed between the arginine and tyrosine side chains.

2.3. Molecular recognition properties

Compound 1a was not soluble in water and its binding properties were not examined. Macrocycles 1b–c were screened using NMR titrations against a number of putative ligands: p-nitrophenyl glycosides, nucleotides, aromatic
amino acids, aromatic amines and purines. Of all the ligands
screened, only 1b and caffeine and 1c and the purine
electrodes 2'-deoxyadenosine 5'-monophosphate (dAMP)
and 2'-deoxyxyanosine 5'-monophosphate (dGMP) showed
weak, but significant, interactions (Kd ≈ 10 M⁻¹). For
comparison, reference peptide Ac-Tyr-Arg-Tyr-OMe⁵ was
tаilted with dAMP and dGMP and was found to bind
more weakly (Kd ≈ 5 M⁻¹). The binding is most likely due
tо hydrophobic interaction between the purine and tyrosine
rings, and the small increase in affinity for 1c is thus due
tо its dimeric cyclic structure and/or the presence of the sugar
amino acid moieties. However, the weak affinities preclude
conclusions regarding detailed structure-affinity
relationships.

3. Conclusions

We have described the synthesis of three 3-sugar amino
acid/tripeptide dimeric macrocycles and evaluated their
binding properties. Macrocycles 1b-c were found to bind
some purine derivatives with weak, but significant, binding
constants. Apparently, the structures have to be modified
in order to present binding sites pre-organized for higher
affinity binding of biomolecules. However, although the
binding is weak, this shows that sugar amino acid containing
peptides can act as artificial receptors and serves as a
starting point for further research.

4. Experimental

4.1. General methods

THF and CH₂Cl₂ were dried over 4 Å molecular sieves
before use and MeOH was dried over 3 Å molecular sieves
before use. Other solvents were not dried unless specified.
Matrex 35–70 mµ 60 Å silica (Millipore) was used for flash
chromatography. Sephadex LH-20 in CH₂Cl₂/MeOH 1:1
was used for size-exclusion chromatography. Sep-Pak Plus
C₁₈ cartridges (Waters) were used for solid-phase extraction.
Chemical shifts are reported relative to Me₄Si and were
screened, only 1b
before use. Other solvents were not dried unless specified.

4.2. Preparation of sugar amino acid precursor 7

4.2.1. Tri-O-acetyl-2-amino-2-deoxy-α-D-glucopyranosyl
bromide hydrobromide (2).⁶,¹⁷ Acetyl bromide (70 mL, 0.94 mol) was added to D-glucosamine hydrochloride
(21.9 g, 101.6 mmol, dried 24 h in vacuo at 70 °C)
and the mixture was stirred for 3 days at room
temperature. Residual acetyl bromide was removed in vacuo
(21.9 g, 101.6 mmol, dried 24 h in vacuo at 70
0.94 mol) was added to D-glucosamine hydrochloride
(21.9 g, 101.6 mmol, dried 24 h in vacuo at 70
°C). 3-Mercaptobutan-2-one (11.2 g, 35.1 mmol) was added and the solution was stirred
(21.9 g, 101.6 mmol, dried 24 h in vacuo at 70
°C). The product was suspended in water (100 mL) and
chloroform. The crude product was recrystallized in
MeOH/EtOAc to give 7 (37.6 g, 82%) as tiny white
needle. Mp 146–148 °C; lit.¹⁶ 151–152 °C.

4.2.2. Methyl tri-O-acetyl-2-amino-2-deoxy-β-D-glucopyranoside (3). Hydrobromide 2 (37.6 g, 84 mmol) was dissolved in MeOH (800 mL) and pyridine (8 mL, distilled from CaH₂) was added. After 1 h, toluene (150 mL) was added and the mixture was concentrated. The residue was dissolved in chloroform (750 mL), washed with Na₂CO₃ (aq) (5%, 2 × 100 mL) and water (100 mL), dried over Na₂SO₄, and concentrated. The crude product was recrystallized in chloroform/heptane to give 3 (20.3 g, 76%) as small white needles. Mp 146–148 °C.

4.2.3. Methyl 2-amino-2-deoxy-β-D-glucopyranoside hydrochloride (4). Acetyl chloride (67 mL, 0.95 mol) was slowly added to MeOH (320 mL) at 0 °C. Compound 3 (11.2 g, 35.1 mmol) was added and the solution was stirred for 24 h at room temperature. The solution was concentrated and the crude product was recrystallized in MeOH/EtOH/acetic acid to give 4 (7.64 g in two crops, 95%) as small white needles. Mp 192–193 °C; lit.¹⁸ 190 °C; [α]²² D ~ 25 (c 1.0, water), lit.²² [α]²² D = −23.4 (c 1, water); [α]²² D = −24.7 (c 10, water); [α]²² D = 20.3 (c 1, MeOH); [α]²² D = 17.9 (c 1, MeOH); ¹H NMR spectrum is in agreement with published data; HRMS (FAB) calcd for C₁₃H₁₅NO₅Na (M+Na): 342.1165, found 342.1158.

4.2.4. Methyl 2-(9-fluorenyl methoxycarbonyl)amino-2-
deoxy-β-D-glucopyranoside (5). Compound 4 (2.78 g, 12.1 mmol) was dissolved in water (22 mL) and NaHCO₃ (1.02 g, 12.1 mmol) was added. After the evolution of gas had ceased, additional NaHCO₃ (1.02 g, 12.1 mmol), acetonitrile (22 mL), and N-(9-fluorenylmethoxycarbonyloxy)succinimide (4.08 g, 12.1 mmol) were added. The reaction mixture was stirred overnight and solidified during the reaction. The product was suspended in water (100 mL) and chloroform (100 mL), filtered off, and washed with water and chloroform. The crude product was recrystallized in methanol to give 5 (3.81 g in three crops, 76%) as tiny white needles. Mp 163–164 °C; [α]²² D = −20 (c 0.5, MeOH); [α]²² D = −20 (c 0.5, MeOH); [α]²² D = −20 (c 0.5, MeOH); [α]²² D = −20 (c 0.5, MeOH); ¹H NMR (400 MHz, MeOH-d₄) δ 7.79 (d, J = 7.5 Hz, 2H, Fmoc), 7.68 (d, J = 6.9 Hz, 2H, Fmoc), 7.38 (t, J = 7.2 Hz, 2H, Fmoc), 7.30 (t, J = 7.5 Hz, 2H, Fmoc), 4.28 (m, 4H, 3 × Fmoc-H + H'), 3.88 (dd, J = 11.9, 2.1 Hz, 1H, H'), 3.68 (dd, J = 11.8, 5.8 Hz, 1H, H'); 3.46 (s, 3 H, OMe), ~3.34

⁵ In agreement with previously published data, but a COSY experiment shows that the signals had been incorrectly assigned.

⁶ Ac-Tyr-Arg(Mtr)-Tyr-OMe was prepared from Ac-Tyr-OH, H-Arg(Mtr)-OtBu and H-Tyr-OMe analogously to the synthesis of 9a–b. The Mtr group was cleaved as in the synthesis of 1c.
poured over ice. The product was extracted with chloroform (300 MHz) δ 7.77 (d, J = 7.5 Hz, 2H, Fmoc), 7.56 (d, J = 7.7 Hz, 2H, Fmoc), 7.37 (t, J = 7.5 Hz, 2H, Fmoc), 7.28 (m, 2H, Fmoc), 7.03 (d, J = 8.5 Hz, 2H, Tyr-H), 7.00 (d, J = 8.4 Hz, 2H, Tyr-H), 6.68 (d, J = 8.5 Hz, 2H, Tyr-H), 6.67 (d, J = 8.5 Hz, 2H, Tyr-H), 4.45 (t, J = 7.0 Hz, 1H, Tyr-H), 4.30 (m, 2H, 1xTyr-H+1xFmoc-H), 4.16 (m, 2H, Fmoc), 3.05–2.85 (m, 3H, 3xTyr-H), 2.71 (dd, J = 13.9, 9.3 Hz, 1H, Tyr-H), 1.37 (s, 9H, O'Bu). HRMS (FAB) calcd for C35H46NO7O7 (M + H): 623.2757; found 623.2748.

4.3.2. Fmoc-Tyr-Tyr-O'Bu (9a). Compound 8a (951 mg, 1.53 mmol) was dissolved in CH2Cl2 (12 mL) and Et3SiH (0.61 mL, 3.8 mmol) and TFA (6 mL) were added. The mixture was stirred for 4 h and coevaporated with toluene. The crude free acid was dissolved in THF (14 mL) and H-Tyr-O'Bu (363 mg, 1.53 mmol), HOBt (206 mg, 1.53 mmol), EDC·HCl (308 mg, 1.60 mmol) and N-methylmorpholine (0.340 mL, 3.06 mmol) were added. The mixture was stirred overnight and then evaporated. The residue was dissolved in MeOH and impregnated on silica. The product was purified with flash chromatography (toluene/MeOH 5:1, Rf = 0.36) to give 9a (1.13 g, 94%) as a white amorphous solid. [α]D22 = −22 (c 0.5, MeOH); 1H NMR (MeOH-d4, 300 MHz) δ 7.77 (d, J = 7.5 Hz, 2H, Fmoc), 7.55 (d, J = 7.7 Hz, 2H, Fmoc), 7.37 (t, J = 7.5 Hz, 2H, Fmoc), 7.26 (m, 2H, Fmoc), 7.00 (d, J = 8.5 Hz, 6H, Tyr-H), 6.66 (m, 6H, Tyr-H), 4.55 (t, J = 6.4 Hz, 1H, Tyr-H), 4.42 (t, J = 6.9 Hz, 1H, Tyr-H), 4.20 (4m, 4H, 1xTyr-H+Fmoc), 3.05–2.75 (m, 5H, 5xTyr-H), 2.66 (dd, J = 14.5, 9.5 Hz, 1H, Tyr-H), 1.35 (s, 9H, O'Bu); HRMS (FAB) calcd for C49H48N6O3 (M + H): 786.3391; found 786.3398.

4.3.3. H-Tyr-Tyr-Tyr-O'Bu (10a). Compound 9a (2.27 g, 2.89 mmol) was suspended in CH2Cl2 (80 mL) and piperidine (14.3 mL) was added. After stirring for 30 min, toluene (50 mL) was added and the mixture was evaporated. The residue was dissolved in CH2Cl2 and purified with flash chromatography (toluene/MeOH 3:1, Rf = 0.13) to give 10a (1.42 g, 87%) as a white foam. [α]D22 = −14 (c 0.5, MeOH); 1H NMR (MeOH-d4, 300 MHz) δ 6.97 (m, 6H, Tyr-H), 6.67 (m, 6H, Tyr-H), 4.56 (dd, J = 7.9, 6.0 Hz, 1H, Tyr-H), 4.44 (t, J = 7.1 Hz, 1H, Tyr-H), 3.44 (dd, J = 8.0, 5.0 Hz, 1H, Tyr-H), 3.00–2.75 (m, 5H, 5xTyr-H), 2.52 (dd, J = 13.8, 8.0 Hz, 1H, Tyr-H), 1.38 (s, 9H, O'Bu); HRMS calcd for C41H38N6O7 (M + H): 564.2701; found 564.2710.

4.3.4. Fmoc-Tyr-Glu(Obzl)-O'Bu (8b). The title compound was prepared from Fmoc-Tyr-OH (1.26 g, 3.12 mmol) and H-Glu(Obzl)-O'Bu·HCl (1.03 g, 3.12 mmol) using the method described in the synthesis of 8a, but using an additional equivalent of base to neutralize the hydrochloride salt. The product was purified with flash chromatography (toluene/EtOAc 2:1, Rf = 0.19) to give 8b (1.82 g, 86%) as a white foam. [α]D22 = −16 (c 0.5, MeOH); 1H NMR (MeOH-d4, 300 MHz) δ 7.77 (d, J = 7.5 Hz, 2H, Fmoc), 7.55 (d, J = 6.6 Hz, 2H, Fmoc), 7.37 (t, J = 7.4 Hz, 2H, Fmoc), 7.27 (m, 7H, Fmoc + Bzl), 7.06 (d, J = 8.4 Hz, 2H, Tyr-H), 6.68 (d, J = 8.3 Hz, 2H, Tyr-H), 5.06 (m, 2H, Bzl), 4.30 (m, 3H, Tyr-H+Glu-H+1xFmoc-H), 4.16 (m, 2H, Fmoc), 3.02 (dd, J = 13.9 Hz, J = 5.2 Hz, 1H, Tyr-H), 2.77 (dd, J = 14.0, 9.4 Hz, 1H, Tyr-H), 2.43 (t,

4.3.5. Fmoc-Tyr-Glu(OBzl)-Tyr-O’Bu (9b). The title compound was prepared from 8b (1.73 g, 2.55 mmol) and H-Tyr-O’Bu (605 mg, 2.55 mmol) using the method described in the synthesis of 9a. The product was purified with flash chromatography (toluene/EtOAc 1:3, 2% signal), 3.12 (m, 2H, Tyr-H), 2.07 (d, 1J = 8.4 Hz, 2H, Tyr-H), 2.07 (m, 1H, Glu-H), 1.89 (m, 1H, Glu-H), 1.35 (s, 9H, O’Bu); HRMS (FAB) calced for C29H44N3O10 (M+H): 606.2916; found 606.2958.

4.3.6. H-Tyr-Glu(OBzl)-Tyr-O’Bu (10b). The Fmoc group of 9b (1.74 g, 2.06 mmol) was removed using the same method as in the synthesis of 10a. The product was purified using flash chromatography (EtOAc/MeOH 40:1, Rf = 0.21) to give 10b (1.05 g, 88%) as a white foam. [α]D22^22 = −10 (c 0.5, MeOH); 1H NMR (300 MHz, MeOH-d4) δ 7.72 (d, J = 7.5 Hz, 2H, Fmoc), 7.55 (dd, J = 7.3, 3.0 Hz, 2H, Fmoc), 7.37 (t, J = 7.4 Hz, 2H, Fmoc), 7.28 (m, 2H, Fmoc + Bzl), 7.04 (d, J = 8.2 Hz, 2H, Tyr-H), 7.02 (d, J = 8.2 Hz, 2H, Tyr-H), 6.69 (d, J = 8.5 Hz, 2H, Tyr-H), 6.67 (d, J = 8.4 Hz, 2H, Tyr-H), 5.05 (m, 2H, Bzl), 4.41 (m, 2H, Tyr-H + Glu-H), 4.28 (m, 2H, 1X-Tyr-H + 1X-Fmoc), 4.25 (m, 2H, Fmoc), 2.92 (m, 3H, Tyr-H), 2.75 (dd, J = 13.8, 9.5 Hz, 1H, Tyr-H), 2.40 (t, J = 7.7 Hz, 2H, Tyr-H), 2.07 (m, 1H, Glu-H), 1.89 (m, 1H, Glu-H), 1.35 (s, 9H, O’Bu); HRMS (FAB) calced for C38H52N3O10 (M+H): 842.3653; found 842.3646.

4.3.7. Fmoc-Arg(Mtr)-Tyr-O’Bu (11). The title compound was prepared from Fmoc-Arg(Mtr)-OH (854 mg, 2.12 mmol) and 12 (1.28 g, 2.12 mmol) using the method described in the synthesis of 8a. The product was purified with flash chromatography (toluene/EtOAc 1:4, Rf = 0.16) to give 13 (1.74 g, 83%) as a white foam. [α]D22^22 = −8 (c 0.5, MeOH); 1H NMR (300 MHz, MeOH-d4) δ 7.76 (d, J = 7.6 Hz, 2H, Fmoc), 7.55 (d, J = 7.1 Hz, 2H, Fmoc), 7.36 (t, J = 7.4 Hz, 2H, Fmoc), 7.26 (t, J = 7.2 Hz, 2H, Fmoc), 7.04 (d, J = 7.8 Hz, 2H, Tyr-H), 7.01 (d, J = 8.2 Hz, 2H, Tyr-H), 6.68 (d, J = 8.0 Hz, 2H, Tyr-H), 6.67 (d, J = 8.1 Hz, 2H, Tyr-H), 6.61 (s, 1H, Mtr-ArH), 4.42 (t, J = 7.2 Hz, 1H, Tyr-H), 4.35 (dd, J = 8.3, 5.1 Hz, 1H, Arg-H), 4.31 (m, 2H, 1X-Tyr-H + 1X-Fmoc), 4.16 (m, 2H, Fmoc), 3.78 (s, 3H, Mtr-OMe), 3.12 (br s, 2H, Arg-H), 2.94 (m, 3H, Tyr-H), 2.75 (d, J = 13.8, 9.9 Hz, 1H, Tyr-H), 2.65 (s, 3H, Mtr-Me), 2.59 (s, 3H, Mtr-Me), 2.08 (s, 3H, Mtr-Me), 1.74 (m, 1H, Arg-H), 1.58 (m, 1H, Arg-H), 1.48 (m, 2H, Arg-H), 1.35 (s, 9H, O’Bu); HRMS (FAB) calced for C35H50N4O14S (M+H): 991.4276; found 991.4250.

4.3.8. H-Arg(Mtr)-Tyr-O’Bu (12). The Fmoc group of 11 (2.01 g, 2.42 mmol) was removed using the same method as in the synthesis of 10a. The product was purified using flash chromatography (EtOAc/MeOH 10:1, Rf = 0.31) to give 12 (1.33 g, 90%) as a white foam. [α]D22^22 = +7 (c 0.5, MeOH); 1H NMR (300 MHz, MeOH-d4) δ 7.01 (d, J = 8.5 Hz, 2H, Tyr-H), 6.69 (d, J = 8.5 Hz, 2H, Tyr-H), 6.65 (s, 1H, Mtr-ArH), 4.48 (dd, J = 8.1, 6.5 Hz, 1H, Tyr-H), 3.82 (s, 3H, Mtr-OMe), ~3.28 (Arg-H), partially obscured by solvent signal), 3.12 (br s, 2H, Arg-H), 2.98 (dd, J = 13.9, 6.4 Hz, 1H, Tyr-H), 2.86 (dd, J = 13.9, 8.2 Hz, 1H, Tyr-H), 2.66 (s, 3H, Mtr-Me), 2.60 (s, 3H, Mtr-Me), 2.12 (s, 3H, Mtr-Me), 1.58 (m, 1H, Arg-H), 1.45 (m, 3H, 1X-Ar-H + 2X-Arg-H), 1.38 (s, 9H, O’Bu); HRMS (FAB) calced for C29H44N3O7S (M+H): 606.2916; found 606.2958.

4.4. Synthesis of the macrocycles 1a–c

4.4.1. Fmoc-SAA(di-O-Bz)-Tyr-O’Bu (14a). Compound 7 (1.60 g, 2.56 mmol) was dissolved in acetonitrile (380 mL) and the mixture was cooled to 0 ºC. Jones’s reagent (4 M, 25.6 mL, prepared by dissolving 12.0 g CrO3 and 6.9 mL concd H2SO4 in 23.1 mL water) was added. The solution was stirred at room temperature for 1.5 h and then quenched by addition of MeOH (100 mL). The mixture was carefully evaporated (caution: bumping) and the residue was dissolved in water (200 mL) and EtOAc (200 mL). The phases were separated and the aqueous phase was extracted with EtOAc (2×200 mL). The organic phases were...
combined and washed with water (2 x 200 mL), dried over Na2SO4 and evaporated. The crude oxidation product was dissolved in THF (45 mL) and H-Tyr-Tyr-Tyr-O'Bu 10a (1.44 g, 2.56 mmol), HOBT (0.346 g, 2.56 mmol), EDC·HCl (0.515 g, 2.69 mmol), and N-methylmorpholine (0.56 mL, 5.12 mmol) were added. After 16 h, the mixture was concentrated, dissolved in MeOH and impregnated on silica. The product was purified with flash chromatography (Toluene/EtOAc 2:3, Rf = 0.10) to give 14a (1.37 g, 45%). As a white amorphous solid [α]D22 = −5 (c 0.5, MeOH); 1H NMR (DMSO-d6, 400 MHz) δ 9.22 (s, 1H, Tyr-OH), 9.13 (s, 1H, Tyr-OH), 9.12 (s, 1H, Tyr-OH), 8.29 (d, J = 7.3 Hz, 1H, NH), 8.12 (d, J = 8.2 Hz, 1H, NH), 7.97 (d, J = 8.2 Hz, 1H, NH), 7.83 (d, J = 6.7 Hz, 2H, Fmoc), 7.79 (d, J = 7.9 Hz, 2H, Bz-o), 7.75 (d, J = 7.2 Hz, 2H, Bz-o), 6.72 (m, 3H, Bz-p+NH), 7.51 (d, J = 7.6 Hz, 1H, Fmoc), 7.42 (t, J = 7.7 Hz, 4H, Bz-m), 7.38 (d, J = 8.8 Hz, 1H, Fmoc), 7.34 (td, J = 7.5, 3.4 Hz, 2H, Fmoc), 7.15 (m, 2H, Fmoc), 7.01 (d, J = 8.4 Hz, 2H, Tyr-H3), 6.95 (d, J = 8.5 Hz, 2H, Tyr-H3), 6.90 (d, J = 8.4 Hz, 2H, Tyr-H3), 6.67 (d, J = 8.5 Hz, 2H, Tyr-H3), 6.59 (d, J = 8.4 Hz, 2H, Tyr-H3), 6.55 (d, J = 8.4 Hz, 2H, Tyr-H3), 5.50 (t, J = 9.9 Hz, 1H, SAA-H4), 5.33 (t, J = 9.6 Hz, 1H, SAA-H4), 4.68 (t, J = 8.3 Hz, 1H, SAA-H4), 4.43 (m, 2H, 2 × Tyr-H2), 4.27 (m, 3H, 1 × Tyr-H2 + SAA-H2 + 1 × Fmoc-H), 4.15 (dd, J = 10.6, 6.9, 1H, Fmoc), 4.02 (t, J = 6.5 Hz, 1H, Fmoc), 3.73 (q, J = 9.2 Hz, SAA-H3), 3.42 (s, 3H, OMe), 2.78 (m, 5H, 5 × Tyr-H2), 2.58 (dd, J = 14.7, 8.1 Hz, 1H, Tyr-H3), 1.31 (s, 9H, O'Bu); HRMS (FAB) calcd for C76H86N30O18Na (M + Na): 1205.4372; found 1205.4388.

4.4.2. H-SAA(di-o-Bz)-Tyr3-O'Bu (15a). Compound 14a (400 mg, 0.338 mmol) was dissolved in THF (10 mL) and N-(2-mercaptoethyl)aminomethyl polystyrene (2.0 mmol/g, 1.69 g) and DBU (76 µL, 0.507 mmol) were added. After stirring the mixture for 6 h, the solid phase was filtered off and washed with THF (2 × 8 mL) and MeOH (2 × 8 mL). The filtrate and washes were combined and evaporated. The residue was dissolved in CH2Cl2/MeOH 9:1 and filtered through silica. Evaporation of the filtrate gave 15a (306 mg, 94%) as a yellowish amorphous solid. [α]D22 = −13 (c 0.5, MeOH); 1H NMR (MeOH-d4, 300 MHz) δ 7.86 (m, 8H, Bz-o), 7.49 (m, 4H, Bz-p), 7.35 (m, 8H, Bz-m), 6.97 (m, 6H, Tyr-H2), 6.90 (d, J = 8.7 Hz, 2H, Tyr-H3), 6.81 (d, J = 8.8 Hz, 2H, Tyr-H3), 6.79 (d, J = 8.9 Hz, 2H, Tyr-H3), 6.69 (d, J = 8.3 Hz, 6H, Tyr-H6), 6.61 (d, J = 8.5 Hz, 4H, Tyr-H4), 6.57 (d, J = 8.5 Hz, 2H, Tyr-H5), 5.73 (t, J = 10.0 Hz, SAA-H4), 5.42 (m, 3H, SAA-H2 + 2 × SAA-H4), 4.77 (d, J = 8.3 Hz, 1H, SAA-H3), 4.50–4.30 ppm (m, 7H, SAA-H3 + 6 × Tyr-H3), 4.26 (d, J = 10.0 Hz, 1H, SAA-H3), 4.15 (dd, J = 10.6, 8.4 Hz, 1H, SAA-H3), 4.12 (d, J = 9.9 Hz, 1H, SAA-H3), 3.56 (s, 3H, OMe), 3.48 (s, 3H, OMe), 3.04 (dd, J = 10.2, 8.0 Hz, 1H, SAA-H3), 2.95–2.40 (m, 12H, Tyr-H3), 1.34 (s, 9H, O'Bu); HRMS (FAB) calcd for C104H106N30O27Na (M + Na): 1869.7152; found 1869.7136.

4.4.5. Cyclo[SAA(di-o-Bz)-Tyr3-SAA(di-o-Bz)-Tyr3]-O'Bu (19a). Compound 18a (37.6 mg, 20.3 µmol) was dissolved in CH2Cl2 (1.6 mL) and Et3SiH (8.1 µL, 51 µmol) and TFA (0.8 mL) were added. The mixture was stirred for 4 h and coevaporated with toluene. The crude product was dissolved in THF (20 mL) and DIPEA (10 µL, 61 µmol) and HAPyU (10.6 mg, 24.4 µmol) were added. The mixture was stirred for 1 h at room temperature and then evaporated. The product was purified by flash chromatography (CH2Cl2/MeOH 6:1, Rf = 0.29) followed by size-exclusion chromatography to give 19a (13.3 mg, 37%) as a white amorphous solid. [α]D22 = −6 (c 0.5, MeOH); 1H NMR (DMSO-d6, 400 MHz, 150 °C) δ 8.47 (s, 2H, Tyr-Oh), 8.41 (s, 2H, Tyr-Oh), 8.34 (s, 2H, Tyr-Oh), 7.86 (d, J = 8.4 Hz, 2H, NH), 7.80 (t, J = 7.2 Hz, 8H, Bz-o), 7.47 (m, 4H, Bz-p), 7.41 (d, J = 8.0 Hz, 2H, NH), 7.34 (m, 10H, Bz-m + 2 × NH), 7.05 (d, J = 8.3 Hz, 2H, NH), 6.89 (d, J = 8.5 Hz, 4H, Tyr-H3), 6.83 (d, J = 8.4 Hz, 4H, Tyr-H3), 6.79 (d, J = 8.3 Hz, 4H,
Ty

4.4.6. Cyclo(SAAS-Tyr3-SAA3-Tyr1) (1a). Compound 19a (89.1 mg, 0.50 mmol) was dissolved in MeOH (18 mL) and stored for 18 h, then neutralised with AcOH and evaporated. The mixture was stirred for 18 h, then purified by size-exclusion chromatography on a short column to afford 1a (41.7 mg, 61%) as a white amorphous solid. [α]D 20 = -15 (c 0.5, MeOH); 1H NMR (MeOH-d 4, 300 MHz) δ 6.97 (m, 12H, Tyr-H), 6.70 (m, 12H, Tyr-H), 4.75 (d, J = 8.5 Hz, 2H, SAA-H 5), 4.52 (dd, J = 9.4, 5.1 Hz, 2H, Tyr-H), 4.41 (dd, J = 8.5, 4.7 Hz, 2H, Tyr-H), 4.29 (t, J = 6.8 Hz, 2H, Tyr-H), 3.82 (d, J = 9.7 Hz, 2H, SAA-H 1), 3.76 (t, J = 9.4 Hz, 2H, SAA-H 3), 3.41 (t, J = 9.4 Hz, 2H, SAA-H 5), 3.33 (s, 6H, OMe), 3.3 (SAA-H 2, obscured by solvent signal), 3.05-2.80 (m, 10H, Tyr-H), 2.54 (d, J = 14.1, 9.4 Hz, 2H, Tyr-H); HRMS (FAB) calced for C 59H 38N 4O 17Na (M + Na) : 1795.6020; found 1795.6010.

4.4.7. Fmoc-SAA(di-OBz)-Tyr-Glu(OBzl)-Tyr-O'Bu (14b). The title compound was prepared from 14b (604 mg, 0.487 mmol) and 15b (496 mg, 0.487 mmol) using the method described in the synthesis of 17a. The product was purified with flash chromatography (CH 2 Cl 2 /MeOH 15:1, R f = 0.18) followed by size-exclusion chromatography to give 17b (767 mg, 72%) as a white amorphous solid. [α]D 20 = +4 (c 0.5, DMSO); 1H NMR (DMSO-d 6, 400 MHz) δ 9.23 (s, 1H, Tyr-OH), 9.16 (s, 1H, Tyr-OH), 9.13 (s, 1H, Tyr-OH), 9.10 (s, 1H, Tyr-OH), 8.36 (d, J = 8.5 Hz, 1H, NH), 8.25 (d, J = 7.1 Hz, 1H, NH), 8.20 (m, 2H, 2 × NH), 8.14 (d, J = 7.7 Hz, 1H, NH), 8.09 (d, J = 7.3 Hz, 1H, NH), 7.85 (m, 3H, 2 × Fmoc-H 2 + NH), 7.77 (m, 4H, Bz-o), 7.70 (m, 4H, Bz-o), 7.55 (m, 6H, 4 × Bz-p + 1 × NH + 1 × Fmoc-H), 7.37 (m, 21H, Bz-m + 3 × Fmoc-H + 2 × Bzl), 7.15 (m, 2H, Fmoc), 7.01 (d, J = 8.5 Hz, 2H, Tyr-H), 6.97 (d, J = 8.5 Hz, 2H, Tyr-H), 6.97 (d, J = 8.6 Hz, 2H, Tyr-H), 6.77 (d, J = 8.4 Hz, 2H, Tyr-H), 6.66 (d, J = 8.5 Hz, 2H, Tyr-H), 6.60 (d, J = 8.4 Hz, 2H, Tyr-H), 6.59 (d, J = 8.1 Hz, 2H, Tyr-H), 6.47 (d, J = 8.4 Hz, 2H, Tyr-H), 5.51 (t, J = 9.8 Hz, 1H, SAA-H 3), 5.47 (t, J = 10.1 Hz, 1H, SAA-H 4), 5.37 (t, J = 9.8 Hz, 1H, SAA-H 5), 5.31 (t, J = 9.7 Hz, 1H, SAA-H 3), 5.06 (s, 2H, Bzl), 5.05 (s, 2H, Bzl), 4.71 (d, J = 8.2 Hz, 1H, Tyr-H), 4.66 (d, J = 8.2 Hz, 1H, SAA-H 3), 4.44 (m, 3H, SAA-H 2 + 2 × Tyr-H), 4.29 (m, 5H, SAA-H 2 + 2 × Tyr-H), 4.13 (b, 2 × Tyr-H), 4.02 (t, J = 6.5 Hz, 1H, Fmoc), 3.71 (q, J = 9.4 Hz, 1H, SAA-H 4), 3.17 (b, 3H, OMe), 3.33 (s, 3H, OMe), 2.75 (m, 7H, 7 × Tyr-H), 2.56 (dd, J = 15.3, 11.2 Hz, 1H, Tyr-H), 2.23 (t, J = 8.0 Hz, 2H, Glu-H), 2.10 (m, 2H, Glu-H), 1.83 (m, 2H, Glu-H), 1.70 (m, 2H, Glu-H), 1.56 (m, 1H, Glu-H), 1.28 (s, 9H, O'Bu); HRMS (FAB) calced for C 212H 298N 13O 31Na (M + Na): 2203.7957; found 2203.7947.

4.4.10. H-SAA(di-OBz)-Tyr-Glu(OBzl)-Tyr-SAA(di-OBz)-Tyr-Glu(OBzl)-Tyr-O'Bu (18b). The title compound was prepared from 17b (676 mg, 0.310 mmol) using the method described in the synthesis of 15a to give 18b (599 mg, 99%) as a yellowish amorphous solid. [α]D 20 = -23 (c 0.5, MeOH); 1H NMR (MeOH-d 4, 400 MHz) δ 7.72 (d, J = 8.5 Hz, 2H, Bz-o), 7.67 (d, J = 8.4 Hz, 2H, Bz-o), 7.63 (d, J = 8.5 Hz, 2H, Bz-o), 7.58 (d, J = 8.4 Hz, 2H, Bz-o), 7.28 (m, 4H, 2 × Bzl-p), 7.12 (m, 18H, Bz-m + Bzl), 6.81 (m, 6H, Tyr-H), 6.63 (d, J = 8.5 Hz, 2H, Tyr-H), 6.48 (m, 6H, Tyr-H), 6.33 (d, J = 8.5 Hz, 2H, Tyr-H), 5.51 (t, J = 10.0 Hz, 1H, SAA-H 3), 5.29 (t, J = 9.8 Hz, 1H, SAA-H 4), 5.24 (t, J = 9.7 Hz, 1H, SAA-H 5), 5.20 (t, J = 9.6 Hz, 1H, SAA-H 3), 4.87 (s, 4H, Bzl), 4.55 (d, J = 8.4 Hz, 1H, SAA-H 3), 4.30 (t, J = 6.5 Hz, 1H, Tyr-H), 4.23 (m, 3H, SAA-H 2 + 2 × Tyr-H), 4.16 (dd, J = 9.1 Hz, J = 5.0 Hz, Tyr-H), 4.06 (m, 3H, 2 × SAA-H + Glu-H), 3.93 (m, 2H, SAA-H 2 + Glu-H), 3.34 (s, 3H, OMe), 3.25 (s, 3H, OMe),
2.83 (dd, J = 10.1 Hz, J = 8.0 Hz, 1H, SAA-H^t), 2.72 (m, 6H, 6\times\text{Tyr-H}^t), 2.55 (dd, J = 14.2, 5.0 Hz, 1H, Tyr-H^t), 2.26 (dd, J = 13.9, 9.4 Hz, 1H, Tyr-H^t), 2.07 (m, 2H, 2\times\text{Glu-H}^t), 1.94 (m, 2H, 2\times\text{Glu-H}^t), 1.72 (m, 1H, Glu-H^t), 1.57 (m, 3H, 3\times\text{Glu-H}^t), 1.14 (s, 9H, O’Bu); HRMS (FAB) calcd for C_{106}H_{110}N_{29}O_{29}Na (M+Na): 1981.7276; found 1981.7268.

4.4.11. Cyclo[SAA(di-OBz)-Tyr-Glu(OBzl)-Tyr-SAA(di-OBz)-Tyr-Glu(OBzl)-Tyr] (19b). The title compound was prepared from 18b (314 mg, 0.166 mmol) using the method described in the synthesis of compound 19a. The product was purified with flash chromatography (CH_{3}Cl/MeOH 12:1, R_f = 0.25) followed by size-exclusion chromatography to give 19b (166 mg, 53%) as a white foam.

\[ \text{[1298 mg, 1.30 mmol] using the method described in the synthesis of 14a. The product was purified with flash chromatography (toluene/MeOH 7:1, R_f = 0.21) followed by size-exclusion chromatography to give 14c (837 mg, 1.39 g, 46%) as a white foam.} \]

4.4.12. Cyclo[SAA-Tyr-Glu-Tyr-SAA-Tyr-Glu-Tyr] (1b). Palladium black (75 mg) was suspended in MeOH containing 5% formic acid (3 mL). Compound 19b (149 mg, 78.8 \mu mol) was dissolved in MeOH containing 5% formic acid (9 mL) and added to the suspension. After 20 min, the catalyst was filtered off (caution: catalyst may catch fire when filtered to dryness), toluene (5 mL) was added, and the mixture was evaporated. The residue was dissolved in MeOH (30 mL) and NaOMe/MeOH (1 M, 450 \mu L) was added. The solution was stirred for 22 h, then neutralised with AcOH, and evaporated. The residue was dissolved in water and applied to a C_{18} cartridge. The column was washed with water and the compound was eluted with 30% MeOH in water to afford 1b (82.3 mg, 81%) as a fluffy white powder after lyophilization. [\[1298 mg, 1.30 mmol] using the method described in the synthesis of 14a. The product was purified with flash chromatography (toluene/MeOH 7:1, R_f = 0.21) followed by size-exclusion chromatography to give 14c (837 mg, 1.39 g, 46%) as a white foam.} \]

4.4.15. Fmoc-SAA(di-OBz)-Tyr-Arg(Mtr)-Tyr-O’Bu (15c). The title compound was prepared from 14c (315 mg, 0.227 mmol) using the method described in the synthesis of 15a to give 15c (334 mg, 99%) as a yellowish foam. [\[1298 mg, 1.30 mmol] using the method described in the synthesis of 14a. The product was purified with flash chromatography (toluene/MeOH 7:1, R_f = 0.21) followed by size-exclusion chromatography to give 14c (837 mg, 1.39 g, 46%) as a white foam.} \]
was prepared from J 19c. Compound 19c (25.5 mg, 11.7 μmol) was dissolved in TFA containing 5% thioanisole (2.5 mL). After 24 h, toluene (2.5 mL) was added and the mixture was evaporated. The residue was dissolved in MeOH (5 mL) and NaOMe/MeOH (1 M, 75 μL) was added. After 24 h, the mixture was acidified with AcOH and evaporated. The product was purified using preparative HPLC (C18 column, 15 → 20% B in A over 60 min; A: H2O + 0.1% TFA, B: CH3CN + 0.1% TFA, τg = 10 min) to afford 1c (9.3 mg, 59%) as a fluffy white powder after lyophilization. [α]D 20 = −15 (c 0.2, MeOH); 1H NMR (D2O, 400 MHz) δ 7.03 (d, J = 8.4 Hz, 4H, Tyr-H3), 7.00 (d, J = 8.2 Hz, 4H, Tyr-H3), 6.77 (d, J = 8.3 Hz, 4H, Tyr-H3), 6.68 (d, J = 7.8 Hz, 4H, Tyr-H3), 4.57 (m, 4H, 4 × Tyr-H4, partially obscured by solvent signal), 4.43 (d, J = 8.6 Hz, 2H, SAA-H3), 3.81 (t, J = 7.0 Hz, 2H, Arg-H2), 3.66 (m, 4H, SAA-H3 + SAA-H4), 3.40 (t, J = 9.8 Hz, 2H, SAA-H3), 3.32 (s, 6H, OMe), 3.18 (t, J = 9.5 Hz, SAA-H1), 3.11 (dd, J = 15.1, 7.4 Hz, 2H, Tyr-H4), 2.96 (m, 8H, 4 × Tyr-H4 + 4 × Arg-H2), 2.32 (dd, J = 12.3, 11.6 Hz, 2H, Tyr-H4), 1.66 (m, 2H, Arg-H2), 1.52 (m, 2H, Arg-H3), 1.27 (m, 2H, Arg-H4), 1.16 (m, 2H, Arg-H7); HRMS (FAB) calcd for C62H82N14O20Na (M + Na): 1365.5728; found 1365.5729.

4.5. NMR titrations

All experiments were performed at 400 MHz in a deuterated phosphate buffer (100 mM phosphate, pH 7.2). In the titration experiments, a stock solution with 0.5 mM receptor concentration was prepared. The ligand to be titrated was dissolved in a portion of the stock solution to give a solution with 0.5 mM receptor and 100 mM ligand. These two solutions were mixed in different proportions to give a series of solutions with 0.5 mM receptor and ligand concentrations up to 80 mM. 1H NMR experiments were performed on the solutions and the chemical shifts of receptor signals were fitted to a 1:1 binding isotherm using non-linear regression.31 Acidic and basic ligands were used as sodium salts and hydrochloride salts, respectively.

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References and notes
